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Report Title:
Development of Advanced Raman Spectroscopy Methods and Databases for the Evaluation of Trace Evidence and the Examination of Questioned Documents - Phase II.

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Abstract:

Researchers from the City University of New York, West Virginia University, the New York Police Department Crime Lab, and The Metropolitan Museum of Art have formed a multidisciplinary collaboration to further the application of Raman spectroscopy and non-destructive surface-enhanced Raman scattering (SERS) analysis techniques as applied to the evaluation of trace evidence and the examination of questioned documents. Through the sharing of instrumentation, reference materials, and their respective research expertise the team has assembled a reference database of spectra for forensic applications and optimized new techniques for non-destructive SERS analysis.

Raman spectroscopy is an established and increasingly utilized technique for the rapid and non-destructive analysis of paints, inks, fibers, mineral residues, pharmaceuticals, and controlled substances; however, the development of underlining reference databases for identifications has not kept pace. In particular, high quality, comprehensive databases of pigments, dyes, and inks are not available commercially. Moreover, while the technique is applicable to a variety of substances, analytes of forensic interest such as some natural and synthetic dyes found in textiles, inks, and paints display an excessive fluorescent background limiting Raman efficiency in most situations, leading to poor analytical results. Recent work in SERS has demonstrated that organic colorants present in inks, paints, and textile fibers can be easily identified from microscopic samples by treatment of the sample with silver nanoparticles prior to analysis.

The work in Phase I of the project successfully demonstrated that SERS can be applied to the identification of organic colorants present in inks, paints, and textile fibers. The techniques we developed are especially suited for handling microscopic samples: textile dyes were successfully identified from samples as small as a one-millimeter section of a single silk fibril of fifty-micrometer diameter and even from textiles severely degraded by burial. Analytical procedures for SERS of a number of representative dyes were developed, the core

of a high quality spectral database was assembled as a proof of concept experiment, and innovative non-destructive approaches were investigated.

The research conducted in Phase II aimed to further explore the application of Raman spectroscopy to the evaluation of trace evidence and the examination of questioned documents, with the aim of solving the problems that have so far limited the applicability of this technique to the identification of organic colorants and other materials in trace amounts.

First of all, we optimized a highly reproducible method of production of silver nanoparticles based on a microwave-assisted reduction of silver sulfate with glucose in the presence of sodium citrate as a capping agent. We continued developing the SERS database, to which spectra of several additional natural and synthetic dyes, as well as controlled substances, were added. In particular, initial studies on problematic tattoo inks systems were performed with the aim of creating a combined normal Raman / SERS approach to their characterization. In addition, we investigated the coupling of SERS with separation techniques such as thin layer chromatography (TLC), as well as emerging methods for completely non-destructive SERS analysis of documents and textile fibers. We refined a non-destructive sampling approach based on the contact transfer of dyes to a gel support to improve its applicability to non-destructive analysis of gel inks. Finally, we conducted validation studies on the methods developed.

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Executive Summary

Raman spectroscopy is a well established technique in the forensic laboratory. It is an ideal tool for its ability to rapidly identify organic and inorganic compounds in small samples without any need for cumbersome preparations. Raman spectroscopy is characterized by very high spectral resolution, leading to effective discrimination among various species, and it has the added advantage of allowing non-destructive, *in-situ* detection. It is also preferable to infrared (IR) spectroscopy in that Raman spectra may readily be obtained in aqueous solution, while the large IR absorption of water precludes use of IR for many practical applications. The possibility of using fiber optics for remote detection is another advantage of Raman spectroscopic techniques. However, the low intensity of normal Raman scattering has prevented its wide application as a sensitive spectroscopic probe. Furthermore, interference from fluorescence often obscures the much weaker Raman signal. Surface-enhanced Raman scattering (SERS) has been found useful to overcome these restrictions. The SERS effect is characterized by an enormous increase in the Raman intensity (by factors of $>10^8$) for species adsorbed on rough metal surfaces compared to that obtained from the same number of molecules in solution or the gas phase. At the same time, proximity to the surface provides a non-radiative pathway for relaxation from the excited state, which successfully quenches fluorescence.

We have investigated the application of SERS to the evaluation of trace evidence and questioned documents. Although the technique has been in wide use for several decades, recent advances in specialized handling techniques provide an opportunity to apply this in a practical and reliable way to forensic science. SERS provides a technique for the rapid and non-destructive analysis of paints, inks, fibers, mineral residues, pharmaceuticals, and controlled substances. While advances in commercial instrumentation have rendered the technique routinely accessible and reliable to use, development of reference databases has not kept pace with its increased use. There is a special need for high quality, and

comprehensive databases of pigments, dyes, and inks, which are not available commercially. Moreover, while the technique is applicable to a variety of substances, some analytes of forensic interest, such as some natural and synthetic dyes found in textiles, inks, and paints, produce a large fluorescent background obscuring the Raman signal, often leading to poor analytical results.

The SERS enhancement requires nanoscale surface features in order to support a plasmon resonance. This may be achieved by several techniques, including mechanical roughening, chemical etching, vapor deposition of silver islands, electrochemical oxidation-reduction cycles, and use of metallic colloids in aqueous solution. The first three techniques are especially useful for the construction of portable probes for utilization in fieldwork, but are difficult to produce and often not uniform in reproducibility. Colloids, on the other hand, are quite easy to make, and provide a large surface area, for convenient Raman studies in the laboratory. Under certain circumstances they may also be useful for portable probes or *in-situ* studies. In addition the effects of pH and electrolyte can be precisely measured.

During Phase II of the project, we optimized a fast and reproducible method of production of Ag nanoparticles based on a microwave-assisted reduction of silver sulfate in the presence of glucose (reducing agent) and sodium citrate (capping agent). This method could also be reproduced using regular home microwave ovens with Teflon, polyethylene and polycarbonate vessels with slightly degraded results. Secondly, we investigated the coupling of thin layer chromatography (TLC) and SERS for the separation and identification of dyes in mixtures, and developed a gel transfer method which enables dye extraction and SERS identification of an unknown colorant without removing a sample from the work of art and preserving its physical integrity and appearance. Moreover, we have collected Raman and SERS spectra from several tattoo inks and controlled substances, which were incorporated into our comprehensive spectral database. We optimized a fast and reliable library search method based on spectral treatments such as baseline correction and cutting, smoothing, normalization and second derivative computation, combined with the correlation coefficient (CC)

algorithm; this method has allowed us to identify correctly 20 query spectra of dyes from works of art and museum objects, and could find easy application in the forensic field as well. Finally, we have performed initial validation studies to ensure that the analytical procedures developed could be reproduced and applied in different laboratories.

Synthesis of Ag nanoparticles by microwave-assisted reduction

The most commonly used procedure to prepare Ag nanoparticles is the Lee and Meisel recipe, in which Ag nanoparticles are synthesized by heating a solution of $AgNO_3$ with citrate as a reducing agent. This method has the advantage of simplicity, and for many applications it is adequate. However, the absorption band of such particles tends to be broad, suggesting a broad nanoparticle size range, and the samples are not stable over long periods. As a forensic tool, it is of considerable importance to have a reliable standardized method for production of such particles, which can then be stored for later field use, and will provide reproducible SERS spectra from laboratory to laboratory. With these considerations in mind, we optimized nanoparticle production using a microwave digestion system (CEM Ultimate Digestion Vessel UDV 10, CEM Corporation). We found that with addition of glucose as well as citrate to an Ag_2SO_4 solution, narrow band samples of Ag nanoparticles could be prepared in just 90 seconds. These were stable over a long period and gave reproducible SERS spectra under a variety of conditions. However, if needed, those results could be reproduced using home microwave ovens with Teflon, polyethylene and polycarbonate vessels, although the nanoparticle size distribution was not as narrow, most likely due to less uniform temperature distribution in the oven. It is our understanding that high efficiency microwave ovens are generally found in forensic laboratories, due to their use in the preparation of samples for atomic absorption and inductively coupled plasma emission spectroscopy.

TLC-SERS for the separation and identification of dyes in mixtures

Although increasingly accepted as an analytical tool, SERS is not a separation technique and, therefore, it is not suitable for distinguishing different components in a mixture. We have recently investigated coupling of thin layer chromatography (TLC) and SERS as a tool for the separation and identification of four alkaloids, namely harmalol, harmaline, harmane and harmine, from the seed extract of Syrian rue (*Peganum harmala*). The alkaloids contained in this plant were historically used as a dye and for medicinal purposes, and have recently drawn attention due to their antitumor activity. The use of TLC over high-performance liquid chromatography (HPLC) is a convenient way to reduce the amount of material, sophisticated equipment and time needed for the analysis, and coupling of TLC with SERS allows us to overcome the poor limit of detection of TLC as such. Complementary HPLC analyses were also carried out to ascertain the actual composition of Syrian rue extract and validate the results obtained from TLC-SERS investigations. In addition, a general characterization of Syrian rue extract and its alkaloid components commercially purchased was performed by FT-Raman, Raman and SERS spectroscopies, in order to provide valuable reference data to be used for identification purposes. It is interesting to notice that harmalol, harmaline, harmane and harmine have analogous molecular structures and therefore, in most cases, provided very similar spectra. However, we have identified discriminant bands which can be used to differentiate among them, which is one of the main issues in forensic applications.

Gel transfer SERS for non-destructive analysis of dyes and inks

We have developed a gel transfer SERS method which involves a matrix transfer of the free dye to a suitable gel substrate followed by SERS analysis. The procedure relies on the application of a bead of a polymer hydrogel loaded with a solution containing water, an organic solvent and a chelating agent, to the substrate containing the insoluble colorant. The dye-mordant-fabric bond is broken down by the combined action of the chelating agent and the organic solvent, and the uncomplexed dye is transferred to the gel. The gel is then removed from the substrate, transferred to a microscope slide, covered with a drop

of Ag colloid and analyzed by SERS. Transfer of the dye from the substrate to the gel eliminates the need to obtain a sample from the object under examination, thus preserving its physical integrity; the amount of dye removed is so small that no appreciable fading is detected by the eye, and the size of the polymer bead can be reduced to a fraction of a millimeter in order to minimize any impact on the work of art, without detriment on the effectiveness of the method. Spectrophotometric measurements confirmed that no appreciable color loss resulted from the use of hydrogel transfer.

Raman and SERS characterization of tattoo inks

Besides organic colorants, we have initiated extensive research on tattoo inks. In the forensic field, the recognition and identification of both inorganic and organic pigments in human tissue can aid in the identification of charred, decomposed, mummified or otherwise unidentifiable remains in criminal investigations and mass disasters (natural, accidental and as a result of terrorism). In the field of art conservation and cultural heritage, the characterization and archiving of organic pigments in traditional tattoo inks can aid in future anthropological and archaeological studies of human culture and history. The criminal justice field has long studied the culture and impact of tattooing, especially in criminal behavior and incarcerated individuals. A more detailed knowledge of the composition of tattoo inks can assist in understanding criminal behavior and cultural practices of individuals in prison settings and among social groups.

Several tattoo inks have been thus characterized by documenting their physical properties both macroscopically and microscopically and by identifying their optical and chemical properties spectroscopically. This was done in an effort to qualitatively identify tattoo inks, resulting in the ability to discriminate between different colors, within similar colors and between different brands of tattoo inks. The lack of an established method of analysis of tattoo inks for identification and comparison was an additional catalyst for this research. The primary means of characterization was based upon molecular structural determination using normal

micro-Raman spectroscopy and SERS. This method was supplemented by several other techniques commonly employed in forensic science and art conservation laboratories, such as FT-Raman, FT-IR, X-ray fluorescence (XRF) and UV/Vis spectroscopy.

Raman and SERS characterization of controlled substances

We have extended our previous studies on controlled substances on morphine derivatives by evaluating the normal Raman and the SERS of five sympathomimetic amines: phenethylamine, amphetamine, methamphetamine, ephedrine and 3,4-methylenedioxymethamphetamine (MDMA, Ecstasy). Quantum mechanical calculations – geometry optimization and calculations of the harmonic vibrational frequencies – were also carried out using the Density Functional Theory (DFT) approach, and vibrational assignments were performed by comparing the experimental and calculated spectra. Both normal Raman and SERS provided excellent spectra for the drugs tested. We found that by using Ag substrates produced by microwave reduction the SERS spectra obtained were reproducible over long periods of time (measured in months), and that the technique showed very high sensitivity at 514.5 nm. Certain conditions, such as response to various laser wavelengths and background fluorescence of the analyte were easily managed using SERS techniques. We also identified a set of discriminant bands, useful for distinguishing the five compounds, despite their structural similarities.

Searchable databases for the identification of dyes using SERS

Over the last few years, the development of several micro-invasive analytical approaches has considerably increased the chances of success in the identification of minute amounts of dyes by SERS. However, the need for searchable databases which could provide a reliable match between spectra from unknowns and reference materials is still to be fulfilled. Recently, we have assembled the core of a comprehensive library which contains 100 Raman and SERS reference spectra of natural and synthetic organic colorants. Experiments to

classify 20 query SERS spectra of dyes from museum objects against the library
were conducted using the correlation coefficient algorithm (CC) and principal
component analysis (PCA). The effect of spectral transformations such as
baseline correction, cutting, normalization, smoothing and second derivative on
the search process was systematically evaluated. Despite the remarkable spectral
variations of reference data collected from the same dye depending on the
experimental conditions, the results of our tests allowed us to optimize an
identification protocol which resulted in 100% of success in the classification of
the query spectra. Such method relies on the application of a baseline correction
and cutting between 420 and 1675 cm^{-1}, smoothing on 5 points repeated 20 times,
scaling between 0 and 100, and second derivative computation, combined with
the CC algorithm. Our work shows that, even when combined with the most
elementary statistical techniques and library search algorithms, SERS can provide
fast and reliable identification of a number of colorants from different substrates.

Validation of the analytical procedures developed

We have performed validation studies in order to ensure that the methods
and analytical procedures developed during Phases I and II of this project can be
used on a regular basis and that the results obtained so far can be easily
reproduced. In fact, SERS has recently been at the center of a powerful
renaissance, with over 2,600 articles published on this topic. However, careful
validation is needed for SERS to be of use in forensic science on a regular basis.

The SERS methods and analytical procedures that we have developed
were incorporated into a step-by-step protocol which will be carefully tested
outside our research group by forensic practitioners from several other
laboratories.

The validation process has been articulated into several steps:

1) Synthesis of Ag colloids using the microwave digestion system
available at the Metropolitan Museum, and preparation of the other chemicals
needed for SERS experiments (e.g. 0.5 M KNO$_3$ aqueous solution to induce

nanoparticle aggregation). The procedure has been repeated multiple times and the resulting nanoparticles tested by UV/Vis, in order to ensure reproducibility.

2) Synthesis of Ag colloids using a home microwave system (Haier), in order to evaluate the feasibility of the reduction method when a laboratory microwave digester is not available. Experiments have been conducted using Teflon, polycarbonate as well as polyethylene vessels to check whether or not it is possible to produce stable nanoparticles using any container normally available in a laboratory. UV/Vis spectra have been collected for each stock of Ag colloids, and the nanoparticles thus produced will be tested soon to evaluate their SERS performances.

3) We have previously reported that hydrolysis with HF vapor can be effective in obtaining good spectra from various textiles and paintings (see Phase I technical report). Evaluation of the HF hydrolysis procedure when applied to different sets of samples, i.e. two commercial lake pigments (carmine and madder lakes purchased from Kremer Pigments), dyes on different mordanted fabrics (madder applied on silk and wool using alum/Fe/Sn mordants), and gel pen inks on different paper supports (PaperMate pen ink on print paper, notebook paper and Whatman filter paper). Such samples have been analyzed by SERS upon HF hydrolysis inducing the nanoparticle aggregation with 0.5 M KNO_3, after HF hydrolysis without KNO_3, and without hydrolysis, and the results obtained were thus compared.

Future steps of the validation process will include library search experiments designed to search the SERS spectra obtained from pure known dyes, reference dyes from different substrates, and dyes from real forensic samples against the database, in order to verify the effectiveness of the identification method and the correctness of matching criteria.

Impact on the forensic community

In addition to over 37 publications in journals such as Journal of Forensic Sciences, Journal of Analytical Chemistry, Journal of Raman Spectroscopy, Proceedings of the National Academy of Sciences, and the Journal of Physical

Chemistry, we have presented talks at conferences such as American Academy of Forensic Sciences, Gordon Conference on Noble Metal Nanoparticles, International Conference on Raman Spectroscopy, Eastern Analytical Symposium. Trace Evidence Symposium, Meeting of the International Association of Forensic Sciences, American Academy of Forensic Sciences and several American Chemical Society meetings. Our research has been featured in an article in Laser Focus ("When photonics meets forensics, crime really doesn't pay", February, 2012, by Gail Overton).

Due to close collaboration with Professor Thom Kubic at John Jay College of Criminal Justice in New York City, we have directed the research for several Master's and Ph.D. level students some of whom have gone on to careers in Forensic Sciences. Among them are (along with their current status):

Masters Level

Irina Geiman (United States Secret Service), Vinesh Rana (Hamilton Robotics), Peter DeCuzzi (Current), Francis Taplin (Corepharma), Amanda Gallagher (Current)

Ph.D.

Michelle Miranda (Assistant Professor, Criminal Justice Department, Farmingdale State College, SUNY)

Post-Doctoral Fellow

Deanna O'Donnell (Assistant Professor, Forensic Sciences, Hamline University)

Federica Pozzi (current)

I. Introduction

1. *Statement of the Problem*

1.1 Surface Enhanced Raman Spectroscopy (SERS)

Raman spectroscopy provides a technique for the rapid and non-destructive analysis of paints, inks, fibers, mineral residues, pharmaceuticals, and controlled substances[1]. While the technique is applicable to a variety of substances, some analytes of forensic interest, such as some natural and synthetic dyes found in textiles, inks, and paints produce a large fluorescent background obscuring the Raman signal, often leading to poor analytical results. We propose here to investigate the application of an ultra-sensitive technique, surface enhanced Raman scattering (SERS), to the evaluation of trace evidence and questioned documents, and to develop high quality, and comprehensive databases of pigments, dyes, and inks, which are not available commercially.

Raman spectroscopy is characterized by very high spectral resolution, leading to effective discrimination among various species, and it has the added advantage of allowing non-destructive, *in-situ* detection. It is also preferable to infrared (IR) spectroscopy in that Raman spectra may readily be obtained in aqueous solution, while the large IR absorption of water precludes use of IR for many practical applications. However, the low intensity of normal Raman scattering has prevented its wide application as a sensitive spectroscopic probe. Furthermore, interference from fluorescence often obscures the much weaker Raman signal. Surface enhanced Raman scattering (SERS) has been found useful to overcome these restrictions. The SERS effect is characterized by an enormous increase in the Raman intensity by many orders of magnitude for species adsorbed on rough metal (usually silver) surfaces compared to that obtained from the same number of molecules in solution or the gas phase[2,3,4,5]. At the same time, proximity to the surface provides a non-radiative pathway for relaxation from the excited state, which successfully quenches fluorescence.

Organic molecules that perform most strongly under SERS conditions are generally characterized by extensive aromatic systems carrying nitrogen and oxygen substituents with lone electron pairs[6]. This is often the case for several molecules of forensic interest such as alkaloids (drugs of abuse) and dyes (textile dyes and inks). It is now generally accepted that there are two major contributions to SERS. One is due to enrichment of the electric field (EM enhancement) caused by surface plasmon resonances induced by the laser light in nano-sized metal clusters on the surface[7]. The second factor is associated with chemisorption, and involves either molecule-metal or metal-molecule charge transfer[8].

Both electromagnetic and chemical SERS theories predict that the SERS enhancement requires nanoscale surface roughness. This may be achieved by several techniques, including mechanical roughening, chemical etching, vapor-deposition of silver islands, electrochemical roughening and use of metallic colloids in aqueous solution. The first three techniques are especially useful for the construction of portable probes for utilization in fieldwork. Colloids, on the other hand, are quite easy to make, and provide a large surface area, for convenient Raman studies in the laboratory. Under certain circumstances they may also be useful for portable probes or *in-situ* studies. Electrochemical studies in SERS are more difficult for practical applications due to the bulk and electronic equipment needed, but since the electrochemical potential can be controlled they are quite useful for basic scientific studies, such as determining the effect of oxidation state on the Raman spectrum. In addition the effects of pH and electrolyte can be precisely measured. Electrochemical studies are especially sensitive to the charge-transfer contributions to SERS, and since this is associated with chemisorbed molecules, they can elucidate the mode of adsorption on the surface as well as the molecular orientation with respect to the surface plane.

1.2 SERS and Raman Spectroscopy in Forensic Science

The potential of SERS in forensic science, and in particular in the trace evidence, controlled substances, and questioned documents fields has been recently highlighted in a review of analytical tools for forensic science[9].

Research so far conducted mostly in Europe has shown that SERS can be effectively applied to problems such as the discrimination of jet printer inks *in situ*[10], the fast identification of synthetic dyes on fiber samples[11,12], and the sensitive determination of narcotics[13,14]. Further work has involved SERS as applied to inks[15,16], as well as in an in-situ analysis of lipstick stains[17]. SERS was needed in this latter study due to strong fluorescence of the sample. Recent work conducted at the Department of Scientific Research of the Metropolitan Museum of Art, in collaboration with CUNY-CCNY and ORNL on the somewhat germane topic of dye and ink identification on ancient and modern works of art shows great promise for forensic applications.

As for forensic scientists, preserving the integrity of the object, to analyze, is paramount when working in art authenticity testing. SERS has been successfully applied to the study of ancient textiles and documents at the Metropolitan Museum of Art, and analytical protocols and references databases have been developed for organic dyes and inks. Moreover, a non-destructive technique for examining textile fibers and documents has been developed. An example of the potential of the work so far conducted is given by the successful identification of the dye contained in a silk textile unearthed in an archaeological expedition: the textile, a silk fragment measuring less than one cm^2 was severely degraded after ten centuries of burial. The color of the textile was dark brown and the silk was extremely brittle. UV-Vis Spectroscopy or ordinary Raman spectroscopy could not detect any colored compound. Analysis of a fragment of the textile measuring less than 2 mm in length following the SERS protocol developed at the museum proved that the textile had originally been dyed with the natural dye madder[18]. A further development of the SERS protocol in use at the museum allows preservation of the integrity of the object examined, by means of non-sampling analysis. Dyes and inks present in a textile sample or a questioned document can be extracted from the object using a specially designed hydroxygel polymer, which is then subsequently analyzed by SERS. The matrix transfer procedure does not require that a fragment be detached from the object, nor does it affect the appearance or integrity of the object. The amount of dye transferred to

the gel is so small that no fading or adverse colorimetric effect can be perceived; yet due to the extreme sensitivity of SERS, dye identification is still possible[19].

Work conducted in collaboration with ORNL shows that the sensitivity of SERS for the analysis of dyes is in the femtogram range, and that excellent reproducibility can be obtained with the proper nanosupports[20]. The possibility of using SERS with a variety of textile dyes and organic colorants has been likewise demonstrated[21].

The analyses and detection of explosives is of utmost importance in today's world. Even more important is a positive association of a suspect to the explosive. In 1995, Cheng, *et al.*[22] reported the *in-situ* detection of the high explosive SEMTEX-H. Using Raman instrumentation, they were able to identify both components of SEMTEX-H (RDX and PETN) from latent fingerprints produced by individuals who had handled SEMTEX-H. Remote analysis of the high explosive SEMTEX-H has been demonstrated. RDX and PETN, the two components of SEMTEX-H, were identified in fingerprints. The analysis was conducted using a fiber optic probe at a range of four meters from the instrument[23].

In 1999, Ryder[24] reported the identification and characterization of solid mixtures of the controlled substances cocaine, heroin, and MDMA using Near-IR and Raman spectroscopy. Quantitative results were obtained from these narcotics mixed with various amounts of diluents.

2. *Statement of Hypothesis or Rationale for the Research*

2.1 Creation of a SERS searchable database

Dye identification, which is usually performed by comparison of query spectra from art or forensic samples with reference spectra from a given spectral library, is a tricky task to be accomplished. In fact, a certain fraction of search tests in the matching process may result in false positive or false negative identification, mostly due to random spectral variations related to the experimental conditions used, to the incompleteness of the reference database as well as to deficiencies of the search algorithm employed. In this context, the

availability of extensive spectral databases as well as the accessibility of library search methods designed to match a query spectrum to the closest library references are crucial to allow for certain identification. Both things play a key role in decreasing false positive / false negative probability and increasing the spectral recognition rate, and should be thus pursued to make the identification process considerably easier and more reliable.

With these considerations in mind, we focused our efforts on the creation of a comprehensive SERS spectral database of dyes, inks and controlled substances, which we plan to update by constant addition of new compounds. In order to achieve fast identification of spectra, the database was made searchable by the use of statistical techniques and library search algorithms.

2.2 Non-destructive analysis with GT-SERS

One of the main challenges in the scientific analysis of culturage heritage materials and forensic evidence is represented by the fact that sampling of art objects and forensic samples is always limited to microscopic fragments, when at all allowed. During Phase II of this project, significant effort has been put into the development of a non-destructive SERS method for the non-invasive identification of dyes. We have expanded our previous research on this topic by exploring the use of hydrogels saturated with various solvents. Our research on the gel transfer (GT) SERS technique was motivated by the necessity of obtaining a spectroscopic fingerprint of an unknown compound without compromising the appearance and physical integrity of the object itself.

2.3 Separation and identification of mixtures using TLC-SERS

The recent introduction of SERS in the forensic field has been crucial to accomplish the ultrasensitive identification of a number of analytes, including organic colorants and controlled substances. Nevertheless, SERS is not a separation technique and therefore it does not always allow for reliable differentiation of several components in a mixture. This is certainly one of the main limitations of this technique. Drugs, as well as many other forensic samples, are usually complex mixtures of chemical compounds. Likewise,

organic colorants were historically derived from natural sources such as plants and insects, and the resulting extracts contained several molecules; moreover, particular hues could be only produced by mixing different dyes. In these cases, SERS may detect just a few, or even only one of the chemical compounds contained in the mixture, i.e. those that have the strongest affinity for the metal substrate or the largest Raman cross-section. The use of hyphenated techniques such as high performance liquid chromatography (HPLC) - SERS or thin layer chromatography (TLC) - SERS presents a means toward effective separation of different components in a mixture and provides valuable vibrational information for each constituent, to be used for identification purposes. In particular, compared to HPLC-SERS, TLC-SERS would allow to reduce the amount of material, equipment and time needed for the analysis.

These considerations prompted us to undertake a study to explore the applicability of TLC-SERS to the separation and identification of substances in mixture. As an example, we report in the following the analysis of the main alkaloid compounds from the extract of *Peganum harmala* seeds.

2.4 Sensitivity Enhancement with Immobilized Colloids

Preliminary experiments in our laboratory have demonstrated that an increment in sensitivity of up to 3 orders of magnitude can be obtained by immobilizing, on the tip of a glass fiber, or on a glass microscope slide, a small amount of colloid (a dot about 0.5 mm in diameter). The active surface thus obtained is left in contact with a large amount of diluted dye solution (10^{-9} M concentration) for a relatively long time. The silver nanoparticles act as an extraction and concentration device, removing from the solution the dye molecules. The advantage of using a small amount of colloid concentrated in a small space is that colloid saturation can be achieved even at very low analyte concentrations, leading to a situation in which a large number of silver nanoparticles carrying adsorbed dye molecules are concentrated within the instrument's excitation beam. We plan to develop this procedure so that it can be

used for the analysis of microsamples extracted from specimens on ink writing, or other evidence.

2.5 DFT Calculations to Assign Spectral Lines

In order to compare spectra obtained from classes of similar molecules, it is necessary to identify discriminant bands, i.e. those which enable us to examine an unknown sample which might contain a mixture of related molecules and determine which are actually present in that sample. Although discriminant bands can be identified by taking spectra of individual compounds separately and in various chosen mixtures, it may also be of value to identify the exact nature of the vibrations involved in the discriminant bands observed. To this end we employ Density Functional Theory (DFT) to calculate the Raman spectra, and compare with the observed spectra. This allows detailed assignment of the lines, and DFT has been shown to be a valuable and reliable tool for these assignments. We think that with enough experience in this area, we will be able to predict the likely discriminant bands from knowledge of the molecular structure alone. Although not a substitute for experiments, this approach can guide us in designing experiments, and explain the cause of our observation, possibly enabling generalizations as to the nature of the effects we observe.

II. Methods

1.   *Preparation of Ag Colloids*

Lee-Meisel method. Ag colloids were prepared following the method of Lee and Meisel, i.e. by reduction of silver nitrate (Aldrich 209139 Silver Nitrate 99.9%) with sodium citrate (Aldrich W302600 Sodium Citrate Dihydrate). The colloid thus prepared shows an absorption maximum at 406 nm and FWHM of 126 nm, as measured with a Cary 50 UV-Vis Spectrophotometer (after a 1:4 dilution with ultrapure water to keep maximum absorbance within the instrumental range). To further concentrate the colloid for use, a volume of 10 ml of the original colloid was centrifuged at 5000 rpm for 2 minutes. The supernatant was discarded and the settled portion was resuspended in 1 ml of ultrapure water. All glassware was cleaned with Pierce PC54 cleaning solution, rinsed with ultrapure water and finally in acetone and methanol. This method proved to be as effective as the use of aggressive cleaning agents such as aqua regia or piranha solution, and was preferred for health and safety reasons. Only ultrapure water was used for the preparation of the various solutions. SERS measurement were made simply by adding 1 μl of dye solution to a 2 μl drop of colloid deposited on a gold coated microscope slide, followed by addition of 2 μl of a 0.2 M KNO_3 solution. Raman measurements were taken directly from the drop using a 50 or 100x microscope objective and focusing on the microscope slide surface. SERS spectra could be obtained two or three minutes after addition of the KNO_3 and remained constant in quality until evaporation of the liquid.

Microwave-assisted reduction. Silver colloids were also synthesized by microwave-supported glucose reduction of silver sulfate in the presence of sodium citrate as a capping agent. In detail, 100 mg of $AgNO_3$ were dissolved in 5 mL of cold ultrapure water and 10% H_2SO_4 was added dropwise to precipitate Ag_2SO_4. The precipitate was washed twice with ultrapure water, allowed to dry on a piece of filter paper and then dissolved in ultrapure water to give a 5 x 10^{-4} M

solution. 25 mL of the silver sulfate solution were added to a pressure resistant Teflon microwave vessel (CEM Ultimate Digestion Vessel UDV 10, CEM Corporation), together with 2 mL of a 1%w solution of glucose and 1 mL of a 1%w solution of sodium citrate. The resulting mixture was shaken vigorously for a few seconds to mix the reagents, and heated to 120°C for a total of 60 s using a CEM MDS-2100 microwave digestion system with temperature and pressure monitoring. The stock colloid was wrapped in an aluminium foil and kept refrigerated. To reduce the amount of citrate in competition with the analyte for adsorption on the nanoparticles and prepare this colloid for use, 1 mL was centrifuged for 5 minutes at 16,060 x g RCF (relative centrifugal force) with a Fisher Scientific Accuspin 400 centrifuge, and 900 μL of the supernatant were then removed and replaced with the same amount of 18 MΩ ultrapure water (Millipore Simplicity 185 water purification system). These nanoparticles were found to have several advantages towards the traditional Lee-Meisel colloids. First of all, the use of microwave radiation can alleviate heat transfer and reagents mixing issues, as the solution is heated at a fast rate without temperature gradients, and this is crucial to achieve a better control of the reaction. As a consequence, the resulting colloid is characterized by a narrower absorption band and a considerably lower particle size dispersion in comparison to the traditional citrate-reduced nanoparticles obtained according to the Lee-Meisel procedure. In detail, our experiments gave rise to the following results: FWHM (full width at half maximum) of microwave colloid ~ 50 nm versus FWHM of Lee-Meisel colloid > 120 nm; size distribution of microwave colloid = 3-10 nm versus size distribution of Lee-Meisel colloid = 3-50 nm. Moreover, sequential UV-vis measurements of the colloid absorption over time and SERS analyses of probe molecules such as 4-methylpyridine showed that microwave nanoparticles, unlike Lee-Meisel colloids, are stable and efficient over several months, thus leading to more reproducible SERS performances.

2.   *HF hydrolysis of mordant dyes and lake pigments*

While SERS investigations can generally be carried out directly without any pretreatment steps on a range of materials, we demonstrated that an increase in sensitivity can be obtained by acid treating the sample prior to analysis. Most natural dyes are in fact mordant dyes, fixed to the fabric by bridging metal atoms (mordants) bound to charged groups in the dye molecule and in the textile fiber. When used in painting, the dyes are similarly complexed to metal ions to form insoluble lake pigments. In high-performance liquid chromatography (HPLC) - the preferred, albeit sample intensive method for dye analysis - dye extraction is usually performed by treatment with HCl and methanol. This method results in quantitative dye removal but causes extreme degradation of the substrate itself. As milder alternative, a non-extractive gas-solid hydrolysis procedure performed by exposing the sample to HF vapor in a closed microchamber was developed specifically for SERS analysis at the Metropolitan Museum of Art in collaboration with CCNY. The reaction is carried out in a microchamber fashioned out of a BEEM size 00 polyethylene vial (8 mm ID x 20 mm H) and affixed to a glass slide. A 10 μL drop of HF is placed in the vial: the drop is confined to the pyramidal shaped bottom of the vial due to surface tension. A capsule thus prepared can be used for over a week without refilling with HF. The sample holder is obtained by removing the cap from a BEEM size 3 vial (5.6 mm ID). Due to its smaller size when compared to the 00 vial, the cap fits snugly within the microchamber. The sample is placed in the sample holder, introduced in the microchamber, which is then closed by snapping the lid shut, and exposed to the HF saturated atmosphere for 5 minutes. Following removal from the chamber, the sample holder is deposited on a glass microscope slide and left to stand in air under a fume hood for 5 minutes to allow any HF absorbed on the sample to evaporate. This step is a precaution against damage to the microscope optics rather than a health and safety measure, as the amount of HF absorbed by the microscopic samples under analysis is deemed to be too small to pose any health hazard. The use of glass slides as a base for the HF microchamber and to support the sample holder after retrieval from the chamber provides sensitive and

convenient method to detect HF leaks. The colloid is then deposited directly on the sample in the sample holder, which is finally transferred to the Raman microscope for SERS analysis.

3.   *Raman and SERS Spectra*

The normal Raman spectra of the pure powders were recorded in the dispersive mode using a Bruker Senterra Raman spectrometer equipped with an Olympus 50x long working distance microscope objective and a charge-coupled device (CCD) detector. A Spectra Physics Cyan solid state laser, a Melles Griot He-Ne laser and a continuous wave diode laser, emitting at 488 nm, 633 nm and 785 nm respectively, were used as the excitation sources. Two holographic gratings (1800 and 1200 rulings/mm) provided a spectral resolution of 3-5 cm^{-1}. Raman analyses were carried out by collecting a single integration of 30 s, using an output laser power of 0.25-2.5 mW for 488 nm excitation, 2-5 mW for 633 nm excitation, and 1-10 mW for 785 nm excitation.

Since the fluorescence of the dyes in some cases prevented the acquisition of a Raman spectrum, FT-Raman spectra were also acquired with a Bruker RamII Vertex 70 spectrometer equipped with a liquid nitrogen-cooled Ge detector. The 1064 nm line provided by a Nd:YAG laser was used for excitation, with a resolution of 4 cm^{-1} and a 180° geometry. The measurements were taken by collecting 64/128 scans, using a 25-100 mW output laser power.

Additional SERS work on Ag colloids is carried out using the Bruker Senterra Raman microscope using 488/633/785 nm excitation, and power at the sample ranging from 2.5 to 25 mW. All the SERS spectra were obtained by adding to the sample (or to the reference solution) in sequence 0.8 μL of Ag colloid and 0.1 μL of a 0.5 M KNO_3 solution (to induce aggregation of the colloid). In most cases, the spectra could be observed immediately after addition of the colloid and KNO_3 solution, but generally improved in quality as the aggregation proceeded, before deteriorating when the liquid was fully evaporated.

4.   Immobilized Colloids

In a typical application, a microscopic fragment, such as a paper fiber with ink traces, or a textile fiber is pre-treated with HF vapor to facilitate the solubilization of the dye, and then deposited on a microscope slide (or other appropriate surface) and covered with a drop of water (or other appropriate development solvent). The immobilized silver colloid (on the tip of a glass fiber or on a microscope coverslip) is then placed in contact with the solvent and left there for a suitable time. We eventually plan to develop this technique into a microfluidic system able to handle microsamples pretreated with HF vapor.

5.   Gel Transfer SERS

Using a gel as a medium for the solvent mixture confines its action only to the areas of the substrate covered by the gel bead. The extraction is carried out for 30 seconds - 8 hours at room temperature. The gel bead is then removed, transferred to a microscope slide, covered with a drop of Ag colloid, and examined with the Raman microscope. Transfer of the dye from the substrate to the gel does not require removing a fragment from the object; the amount of dye removed is so small that no appreciable fading is detected by the eye. The size of the polymer bead can be reduced to a fraction of a millimeter in order to minimize any impact on the substrate, without detriment on the effectiveness of the method. The one-step procedure combining extraction and hydrolysis is extremely efficient and time saving. Additionally, problems previously encountered in obtaining SERS spectra from alizarin[25] and due to its difficult adsorption on silver nanoparticles do not appear to affect its analysis by GT-SERS. In an alternative procedure, instead of using a silver colloid to obtain the SERS effect, the gel fragment is coated with silver nanoislands by thermal evaporation of silver in a high vacuum evaporator (Edwards E306A). The method has been found to yield the same results as the use of a colloid.

Different hydrogels can be used for the GT procedure. We have so far worked with either a 1:1 random copolymer of 2,3-dihydroxypropyl methacrylate

with 2-hydroxyethyl methacrylate (Benz 5X, also known as GMA) or 2-hydroxyethyl methacrylate (known as p(2-HEMA) or Benz 38 or HEMA). The gel is prepared for use by soaking for 10 minutes in a solution of dimethylformamide (DMF) and water with EDTA (1% w/w), with water and DMF in a 1:1 ratio. This solution is known as a solvent for extraction of dyes from fibers. Different solvents however could be used: the possibility of using pure DMF for the extraction of vat dyes has been demonstrated in our tests.

The procedure described above is the first example of the use of a solvent gel to hydrolyze/extract insoluble dyes from an object for the purpose of non-invasive identification by SERS techniques. The current standard procedure for dye extraction when HPLC is used is a destructive procedure based on the removal of a sample and its treatment with an appropriate reagent capable of dissolving the dye-aluminum complex and removing the dye from the textile fiber or other support. This reagent can be an acidic solution, or the same H_2O/EDTA/DMF mixture used here, with the difference that the procedure of reference is carried out at boiling.

Gel supports for SERS analysis have been described before[26] and are commercially available[27]. The bulk of the published literature however deals with gels containing silver particles, and having the specific functions of stabilizing the silver particles to increase their efficiency for SERS analysis. The innovative aspect of the GT-SERS procedure is that it is the first method ever proven effective for the non-invasive analysis of insoluble dyes contained in textiles, paintings, documents, etc. We expect this technique to be especially useful in the analysis of writing and printing inks containing lake pigments and pigment dyes, because of the unique ability of the solvent gel system to hydrolyze and solubilize such materials.

6.   *TLC-SERS of dye mixtures*

TLC-SERS experiments were typically conducted as explained in the following. First of all, the extract obtained from the plant material (e.g. in the case

of *Peganum harmala*) as well as the commercial references (~ 2 μL) were deposited onto a silica gel TLC plate by using a glass capillary and eluted in a glass developing chamber using an appropriate solvent mixture. The TLC spots were visualized and marked under ultraviolet light. SERS analyses were subsequently performed directly on the TLC plate, upon deposition of 0.8 μL of Ag colloid and 0.1 μL of 0.5 M KNO$_3$ aqueous solution on top of each spot. Good quality SERS spectra could be obtained while the spots were still wet.

7. *Searchable database for the identification of dyes*

Series of subsequent tests led us to assess a library search protocol which has proven to be extremely successful, in that it enabled the correct classification of all the query spectra selected for our experiments. We have added over 80 new compounds to the database since the start of phase II, including 4 drugs, 4 β-carboline alkaloids and several additional dyes. We currently have over 135 compounds, for which spectra were taken at 4 different excitation wavelengths and following various sample treatments.

All the spectra were baseline corrected (using a ten point curve subtraction) and the region between 420 and 1675 cm^{-1}, where the main Raman and SERS signals are located. This was aimed to remove possible inconsistencies due to fluorescence effects, baseline distortions and artifacts produced by the stray light coming from the Raman spectrometers. The spectra were also normalized by scaling their values between 0 and 100. After that, the 5 noisiest and the 5 least noisy spectra of the database were selected in order to evaluate the optimal degree of smoothing which could significantly reduce the level of noise without compromising the spectrum profile. A 5-point smoothing procedure repeated 20 times was found to be ideal to remove as much spectral noise as possible, avoiding at the same time peak shifts higher in wavenumber than the spectral resolution (4 cm^{-1}). As a final step, the second derivative of the smoothed spectra has been calculated in order to achieve an improved accuracy in the classification

tests. By comparison with other smoothing techniques, we found that this technique was the simplest and most reliable of the possibilities.

An experimental comparison of the spectral transformations applied has been performed by using two of the most popular classification methods: the correlation coefficient algorithm (CC) and principal component analysis (PCA) with Euclidean distance as the dissimilarity measure.

Baseline correction has been performed by means of the OPUS software, while spectral cutting, normalization, smoothing, second derivative computation, as well as all the classification tests, have been carried out by using MATLAB.

The best results, i.e. 100% of success in the classification tests performed on 20 query SERS spectra of colorants from works of art and museum objects, were achieved when the spectral transformations described above were combined with the use of the CC algorithm.

8.   *Density Functional Calculations*

Density Functional Theory (DFT) calculations are performed with Gaussian 03 at the B3LYP level of theory and employing the 6-31+G* basis set. This technique automatically optimizes the geometry and predicts the normal mode frequencies, and their symmetries. In general vibrational normal mode assignments are based on the best-fit comparison of the calculated Raman spectrum with the observed normal Raman spectrum. Where needed slight scaling of the calculated spectrum will be utilized (usually 0.96-0.99). In instances where there is spectral congestion, such as in the carbonyl stretch region (near 1600 cm^{-1}), the relative intensities of the calculated spectra are matched to those of the observed spectra, so that the most intense calculated lines were assigned to the most intense observed lines. In this way we obtain reliable spectral assignments. Using the program "Gaussview" we can obtain visual depictions of the normal modes involved, and therefore confirm the exact nature of the lines observed.

III. Results

1. Statement of Results

1.1 SYNTHESIS OF AG COLLOIDS BY MICROWAVE-ASSISTED REDUCTION

The most commonly used procedure to prepare Ag nanoparticles is the Lee and Meisel recipe, in which Ag nanoparticles are synthesized by heating a solution of $AgNO_3$ with citrate as a reducing agent. This method has the advantage of simplicity, and for many applications it is adequate. However, the absorption band of such particles tends to be broad, suggesting a broad nanoparticle size range, and the samples are not stable over long periods. As a forensic tool, it is of considerable importance to have a reliable standardized method for production of such particles, which can then be stored for later field use, and will provide reproducible SERS spectra from laboratory to laboratory. With these considerations in mind, we optimized nanoparticle production using a microwave digestion system (CEM Ultimate Digestion Vessel UDV 10, CEM Corporation) (Fig. 1). We found that with addition of glucose as well as citrate to an Ag_2SO_4 solution, Ag nanoparticles could be prepared in just 90 seconds (Fig. 2). These had a narrow particle size range (Fig. 3), were stable over a long period of time (Fig. 4), and gave reproducible SERS spectra under a variety of conditions (Fig. 5). However, if needed, those results could be reproduced using home microwave ovens with Teflon, polyethylene and polycarbonate vessels, although the nanoparticle size distribution was not as narrow, most likely due to less uniform temperature distribution in the oven. It is our understanding that high efficiency microwave ovens are generally found in forensic laboratories, due to their use in the preparation of samples for atomic absorption and inductively coupled plasma emission spectroscopy.

1.2 TLC-SERS FOR THE IDENTIFICATION OF DYES IN MIXTURE

Although increasingly accepted as an analytical tool, SERS is not a separation technique and, therefore, it is not suitable for distinguishing different components in a mixture. We have recently investigated coupling of thin layer chromatography (TLC) and SERS (Fig. 6) as a tool for the separation and identification of four alkaloids, namely harmalol, harmaline, harmane and harmine (Fig. 7; UV-Vis spectra in Fig. 8), from the seed extract of Syrian rue (*Peganum harmala*). The alkaloids contained in this plant were historically used as a dye and for medicinal purposes, and have recently drawn attention due to their antitumor activity. The use of TLC over high-performance liquid chromatography (HPLC) is a convenient way to reduce the amount of material, sophisticated equipment and time needed for the analysis, and coupling of TLC with SERS allows us to overcome the poor limit of detection of TLC as such. The results of TLC as such, as well as those obtained from the SERS analysis of the TLC spots, are shown in Figs. 9-13. Complementary HPLC analyses were also carried out to ascertain the actual composition of Syrian rue extract and validate the results obtained from TLC-SERS investigations (Figs. 14-15). In addition, a general characterization of Syrian rue extract and its alkaloid components commercially purchased was performed by FT-Raman, Raman and SERS spectroscopies (Figs. 16-20), in order to provide valuable reference data to be used for identification purposes. It is interesting to notice that harmalol, harmaline, harmane and harmine have analogous molecular structures and therefore, in most cases, provided very similar spectra. However, we have identified discriminant bands which can be used to differentiate among them, which is one of the main issues in forensic applications.

1.3 GEL TRANSFER SERS FOR NON-DESTRUCTIVE ANALYSIS

We have developed a gel transfer SERS method which involves a matrix transfer of the free dye to a suitable gel substrate followed by SERS analysis. The procedure relies on the application of a bead of a polymer hydrogel loaded with a

solution containing water, an organic solvent and a chelating agent, to the substrate containing the insoluble colorant. The dye-mordant-fabric bond is broken down by the combined action of the chelating agent and the organic solvent, and the uncomplexed dye is transferred to the gel. The gel is then removed from the substrate, transferred to a microscope slide, covered with a drop of Ag colloid and analyzed by SERS. Transfer of the dye from the substrate to the gel eliminates the need to obtain a sample from the object under examination, thus preserving its physical integrity; the amount of dye removed is so small that no appreciable fading is detected by the eye (Fig. 21), and the size of the polymer bead can be reduced to a fraction of a millimeter in order to minimize any impact on the work of art, without detriment on the effectiveness of the method. This technique was successfully applied to the identification of alizarin in a tapestry (Figs. 22-24) and crystal violet in a Japanese print (Figs. 25-26). Spectrophotometric measurements confirmed that no appreciable color loss resulted from the use of hydrogel transfer (Figs. 27-28).

1.4 RAMAN AND SERS ANALYSIS OF TATTOO INKS

Besides organic colorants, we have initiated extensive research on tattoo inks. In the forensic field, the recognition and identification of both inorganic and organic pigments in human tissue can aid in the identification of charred, decomposed, mummified or otherwise unidentifiable remains in criminal investigations and mass disasters (natural, accidental and as a result of terrorism). In the field of art conservation and cultural heritage, the characterization and archiving of organic pigments in traditional tattoo inks can aid in future anthropological and archaeological studies of human culture and history. The criminal justice field has long studied the culture and impact of tattooing, especially in criminal behavior and incarcerated individuals. A more detailed knowledge of the composition of tattoo inks can assist in understanding criminal behavior and cultural practices of individuals in prison settings and among social groups.

Several tattoo inks have been thus characterized by documenting their physical properties both macroscopically and microscopically and by identifying their optical and chemical properties spectroscopically. This was done in an effort to qualitatively identify tattoo inks, resulting in the ability to discriminate between different colors, within similar colors and between different brands of tattoo inks. The lack of an established method of analysis of tattoo inks for identification and comparison was an additional catalyst for this research. The primary means of characterization was based upon molecular structural determination using normal micro-Raman spectroscopy and SERS. This method was supplemented by several other techniques commonly employed in forensic science and art conservation laboratories, such as FT-Raman, FT-IR, X-ray fluorescence (XRF) and UV/Vis spectroscopy.

1.5 RAMAN AND SERS ANALYSIS OF SYMPATHOMIMETIC AMINES

We have extended our previous studies on controlled substances on morphine derivatives by evaluating the normal Raman and the SERS of five sympathomimetic amines: phenethylamine, amphetamine, methamphetamine, ephedrine and 3,4-methylenedioxymethamphetamine (MDMA, Ecstasy). Quantum mechanical calculations – geometry optimization and calculations of the harmonic vibrational frequencies – were also carried out using the Density Functional Theory (DFT) approach, and vibrational assignments were performed by comparing the experimental and calculated spectra. Both normal Raman and SERS (Fig. 29) provided excellent spectra for the drugs tested. We found that by using Ag substrates produced by microwave reduction the SERS spectra obtained were reproducible over long periods of time (measured in months), and that the technique showed very high sensitivity at 514.5 nm. Certain conditions, such as response to various laser wavelengths and background fluorescence of the analyte were easily managed using SERS techniques. We also identified a set of discriminant bands, useful for distinguishing the five compounds, despite their structural similarities.

1.6 SEARCHABLE DATABASES FOR THE IDENTIFICATION OF DYES

Over the last few years, the development of several micro-invasive analytical approaches has considerably increased the chances of success in the identification of minute amounts of dyes by SERS. However, the need for searchable databases which could provide a reliable match between spectra from unknowns and reference materials is still to be fulfilled. Recently, we have assembled the core of a comprehensive library which contains 100 Raman and SERS reference spectra of natural and synthetic organic colorants (examples in Figs. 30-47). Experiments to study the structure of the database (Table 1) and classify 20 query SERS spectra of dyes from museum objects against the library (Tables 2-3) were conducted using the correlation coefficient algorithm (CC) and principal component analysis (PCA). The effect of spectral transformations such as baseline correction, cutting, normalization, smoothing and second derivative on the search process was systematically evaluated. In detail, a baseline correction has been applied to the spectra using an interactive mode: for each spectrum, 20 baseline points connected by straight lines were manually set to eliminate possible inconsistencies due to fluorescence, baseline distortions and stray light artifacts. After that, We carried out extensive smoothing tests using both the OPUS software (Savitzky-Golay algorithm) and our home-made MATLAB software (in which the smoothing algorithm replaces each group of 5 points with their average value), in order to evaluate the optimal degree of smoothing which could significantly reduce the level of noise without compromising the spectrum profile. The 5 noisiest and the 5 least noisy spectra of the database were selected for this purpose, and smoothing tests were performed using the two algorithms on different number of points and repeated a different number of times. A 5x20 smoothing procedure applied with the MATLAB algorithm was found to be ideal to remove as much spectral noise as possible, avoiding at the same time peak shifts higher in wavenumber that the spectral resolution (4 cm^{-1}). Despite the remarkable spectral variations of reference data collected from the same dye depending on the experimental conditions (Figs. 48-49), the results of our tests allowed us to optimize an identification protocol

which resulted in 100% of success in the classification of the query spectra (Table 3). Such method relies on the application of a baseline correction and cutting between 420 and 1675 cm^{-1}, smoothing on 5 points repeated 20 times, scaling between 0 and 100, and second derivative computation, combined with the CC algorithm. As shown in Table 3, the smoothing procedure allowed us to improve the classification of query spectra against the database: the percentage of correct matches changes in fact from 40% to 50% with PCA and, more significantly, from 80% to 100% for second derivative data. Our work shows that, even when combined with the most elementary statistical techniques and library search algorithms, SERS can provide fast and reliable identification of a number of colorants from different substrates.

1.7 VALIDATION OF THE ANALYTICAL PROCEDURES DEVELOPED

We have performed validation studies in order to ensure that the methods and analytical procedures developed during Phases I and II of this project can be used on a regular basis and that the results obtained so far can be easily reproduced. In fact, SERS has been recently at the center of a powerful renaissance, with over 2,600 articles published on this topic. However, careful validation is needed for SERS to be of use in forensic science on a regular basis.

The SERS methods and analytical procedures that we have developed were incorporated into a step-by-step protocol which will be carefully tested outside our research group by forensic practitioners from several other laboratories.

The validation process has been articulated into several steps:

1) Synthesis of Ag colloids using the microwave digestion system available at the Metropolitan Museum, and preparation of the other chemicals needed for SERS experiments (e.g. 0.5 M KNO_3 aqueous solution to induce nanoparticle aggregation). The procedure has been repeated multiple times and the resulting nanoparticles tested by UV/Vis, in order to ensure reproducibility.

2) Evaluation of the HF hydrolysis procedure when applied to different sets of samples, i.e. two commercial lake pigments (carmine and madder lakes purchased from Kremer Pigments) (Figs. 50-51), dyes on different mordanted fabrics (madder applied on silk and wool using alum/Fe/Sn mordants) (Figs. 52-54), and gel pen inks on different paper supports (PaperMate pen ink on print paper, notebook paper and Whatman filter paper) (Figs. 55-57). Such samples have been analyzed by SERS upon HF hydrolysis inducing the nanoparticle aggregation with 0.5 M KNO_3, after HF hydrolysis without KNO_3, and without hydrolysis, and the results obtained were thus compared.

3) Synthesis of Ag colloids using a home microwave system (Haier), in order to evaluate the feasibility of the reduction method when a laboratory microwave digester is not available. Experiments have been conducted using Teflon, polycarbonate as well as polyethylene vessels to check whether or not it is possible to produce stable nanoparticles using any container normally available in a laboratory (Figs. 58-60). UV/Vis spectra have been collected for each stock of Ag colloids, and the nanoparticles thus produced will be tested soon to evaluate their SERS performances.

Future steps of the validation process will include more extensive library search experiments designed to search the SERS spectra obtained from pure known dyes, reference dyes from different substrates, and dyes from real forensic samples against the database, in order to verify the effectiveness of the identification method and the correctness of matching criteria.

2. *Tables and Figures*

Synthesis of Ag colloids by microwave-assisted reduction
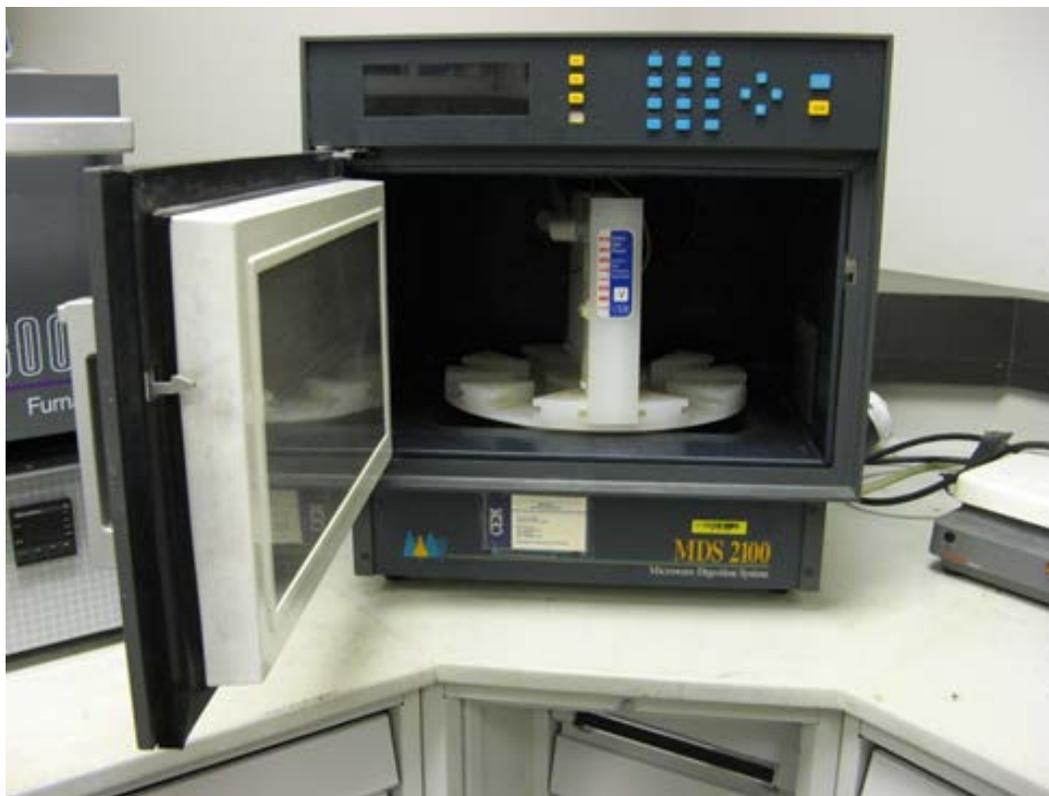
Figure 1. The CEM MDS-2100 microwave digestion system with a pressure resistant Teflon microwave vessel where the nanoparticle synthesis is carried out.

Figure 2. Ag colloid synthesized by microwave reduction.
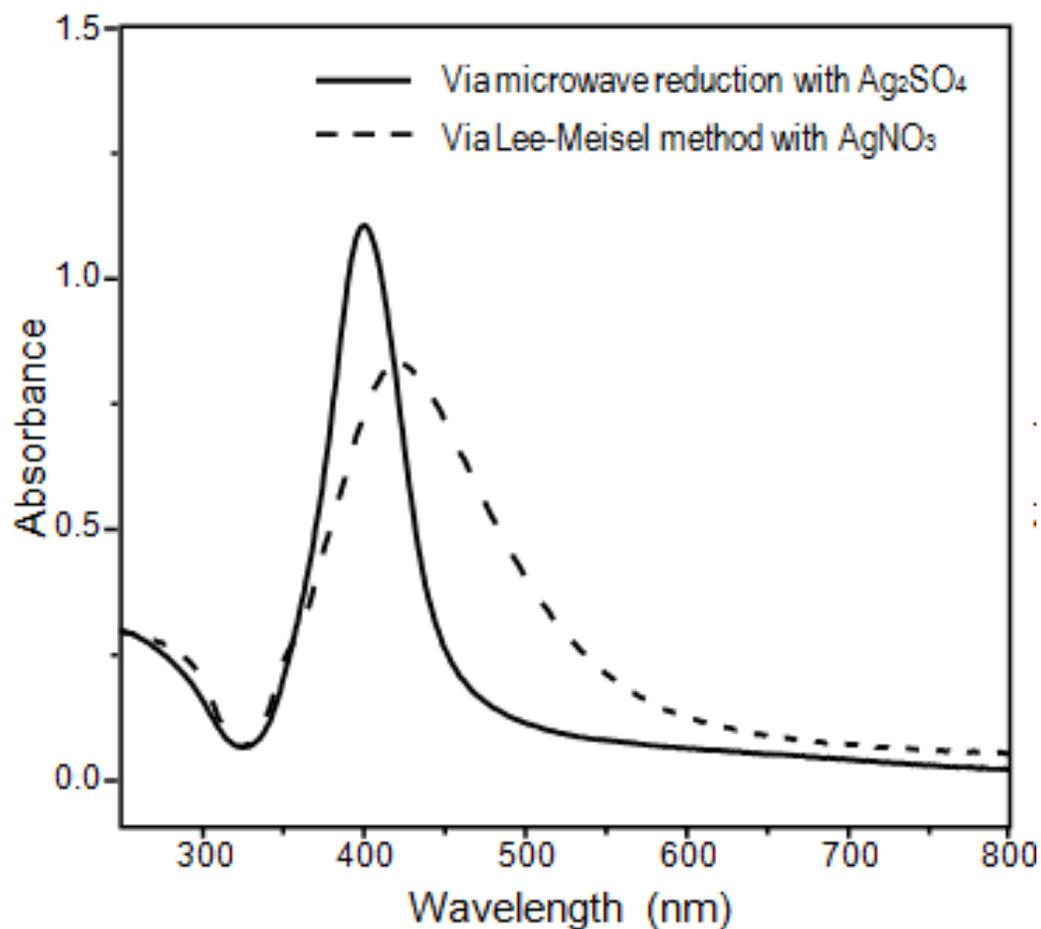
Figure 3. comparison between UV-vis spectra of Ag nanoparticles synthesized by microwave-assisted reduction of silver sulfate and according to the traditional Lee-Meisel procedure: a narrower absorption band, corresponding to a lower particle size distribution, is obtained when the first method is used.
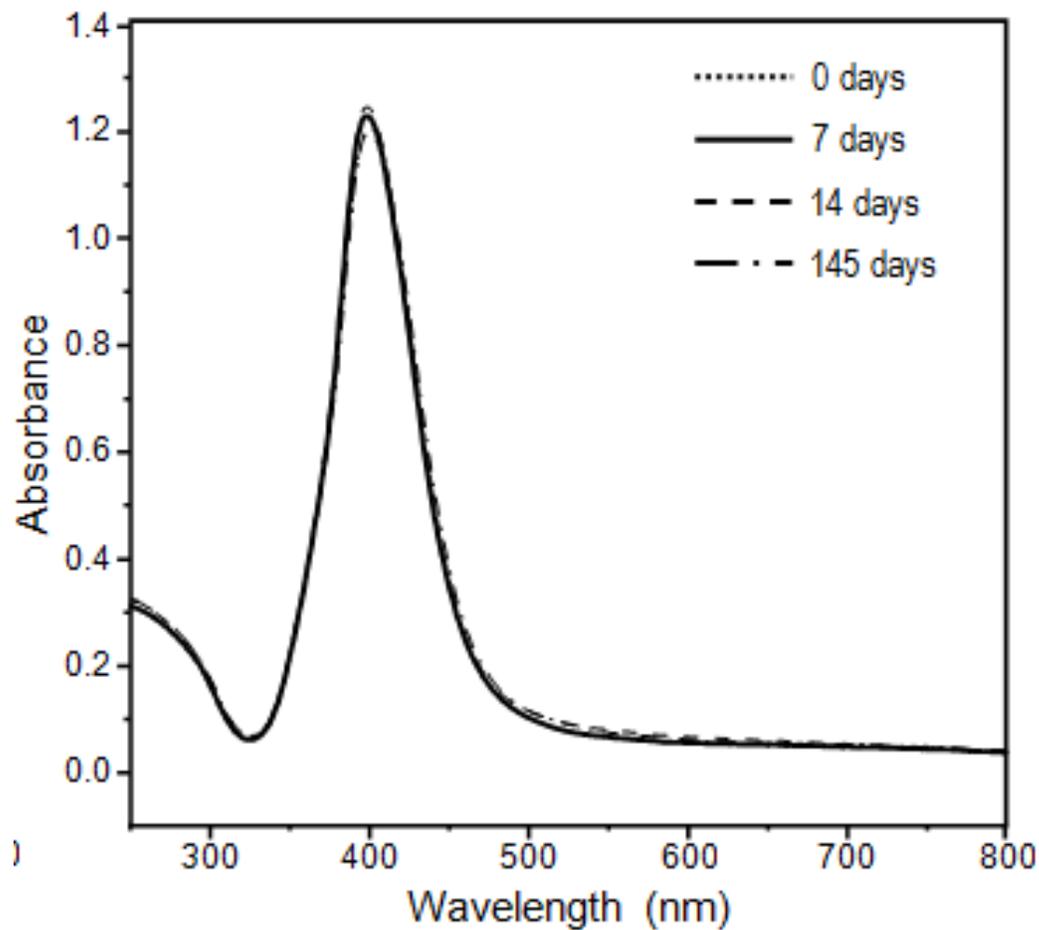
Figure 4. UV-vis measurements of Ag microwave nanoparticles taken 0, 7, 14 and 145 days after their synthesis, showing a very high stability for this colloid over the time.
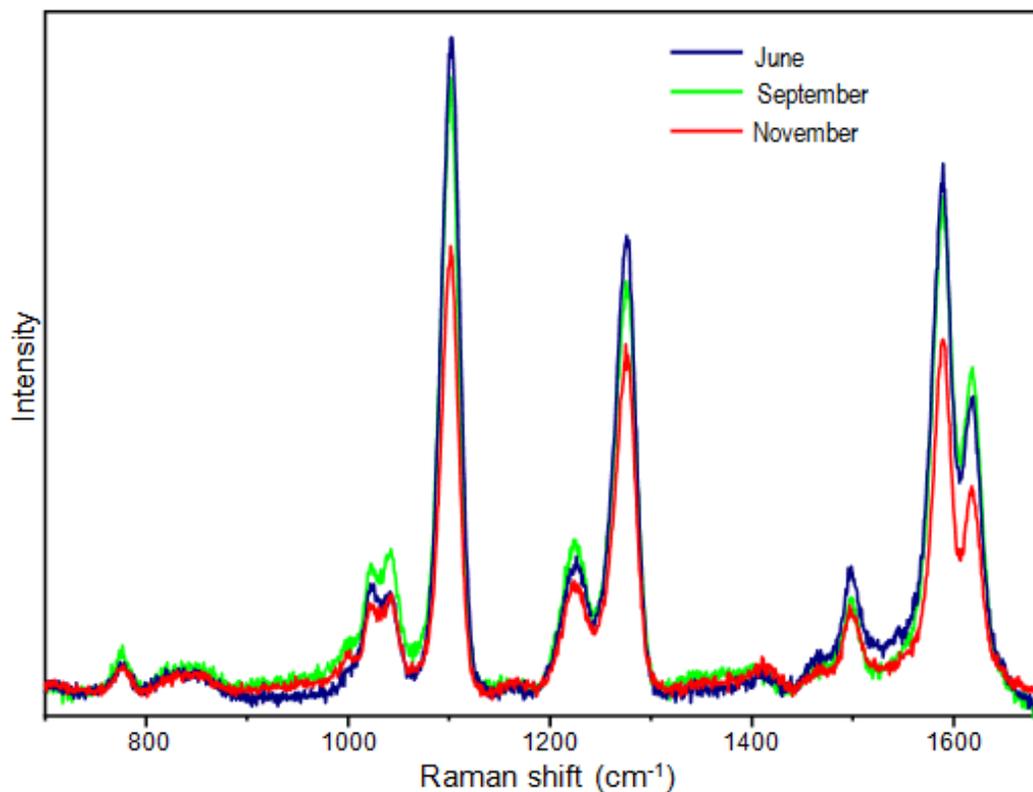
Figure 5. SERS spectra of 4-methylpyridine on Ag nanoparticles synthesized by microwave-assisted reduction of silver sulfate taken in June, September and November, using the same stock colloid prepared in June, right before the first set of measurements was taken. Spectra obtained from sequential measurements display comparable intensities, showing that a high stability and prolonged efficiency over time is associated with such nanoparticles.

TLC-SERS for the identification of dyes in mixture
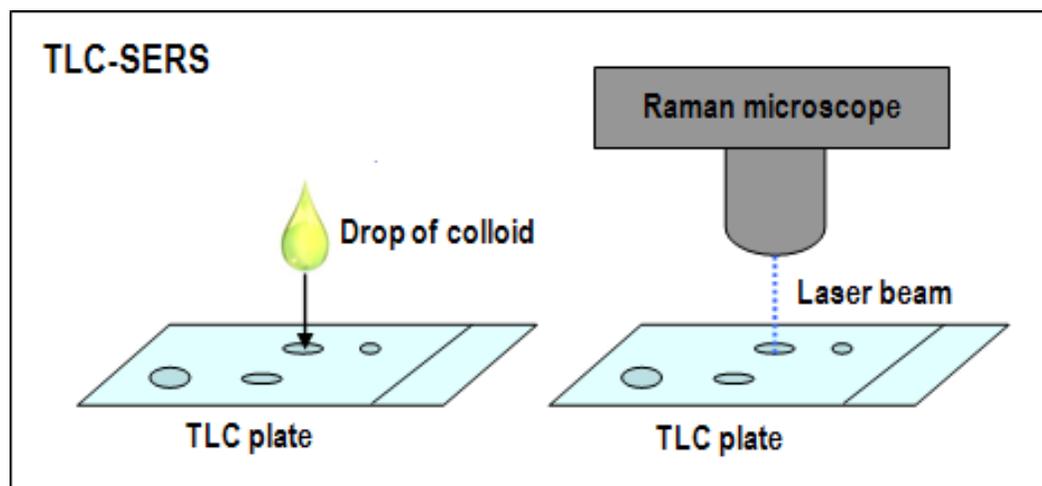
Figure 6.TLC-SERS analytical procedure. Syrian rue seed extract, commercial harmalol, harmaline, harmane and harmine were deposited onto a silica gel TLC plate and eluted. SERS analyses were performed directly on the TLC plate, upon deposition of the Ag colloid and KNO_3.
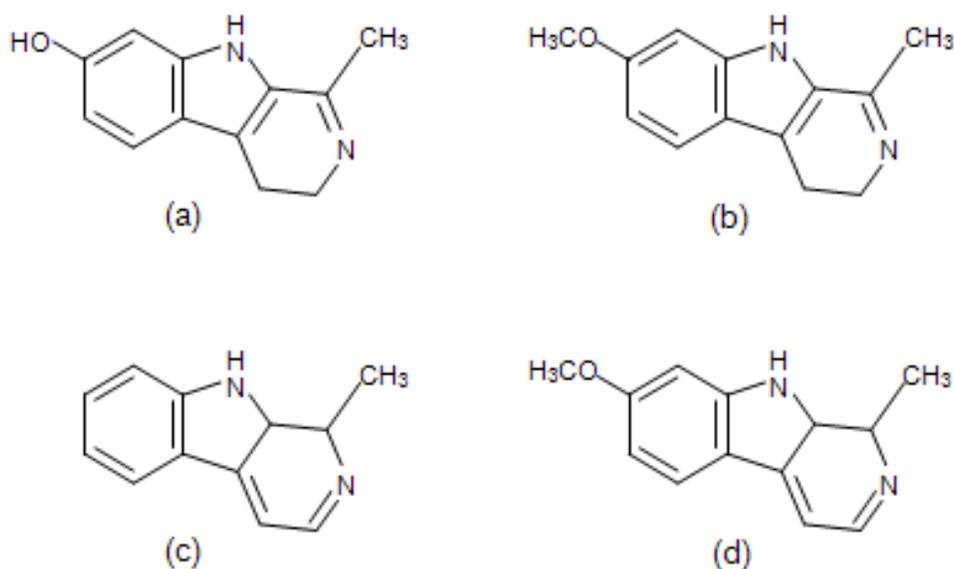
Figure 7. Molecular structures of the main alkaloid components of Syrian rue (Peganum harmala), i.e. (a) harmalol, (b) harmaline, (c) harmane and (d) harmine.
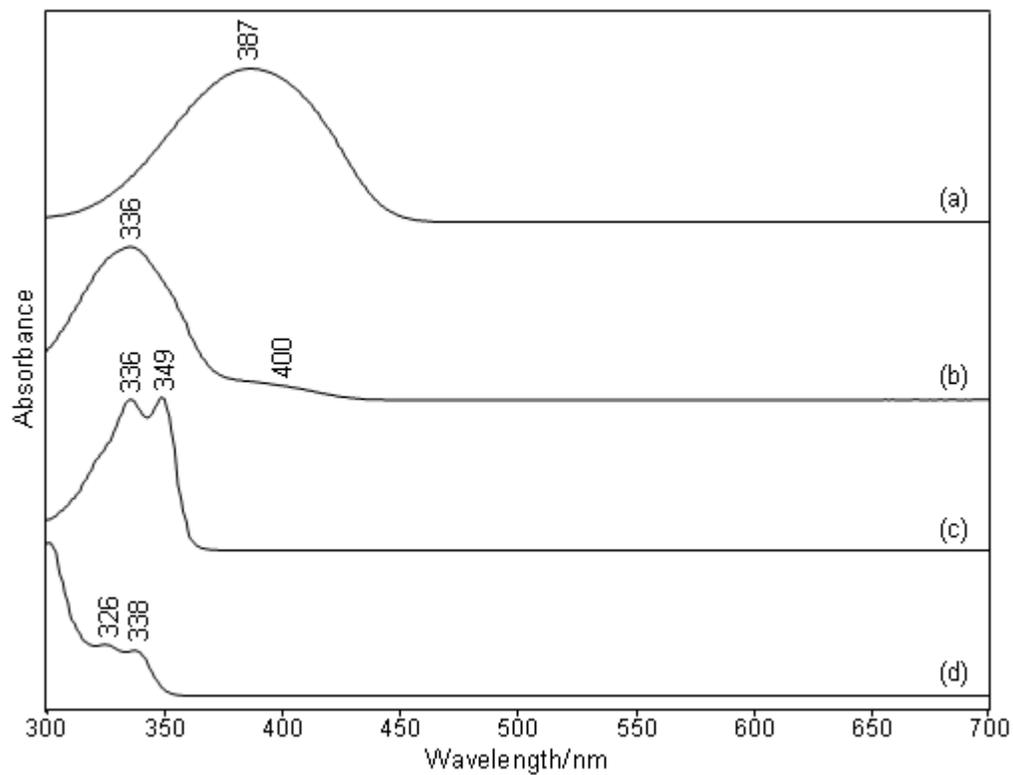
Figure 8. UV/Vis spectra of (a) harmalol, (b) harmaline, (c) harmane and (d) harmine in ethanol.
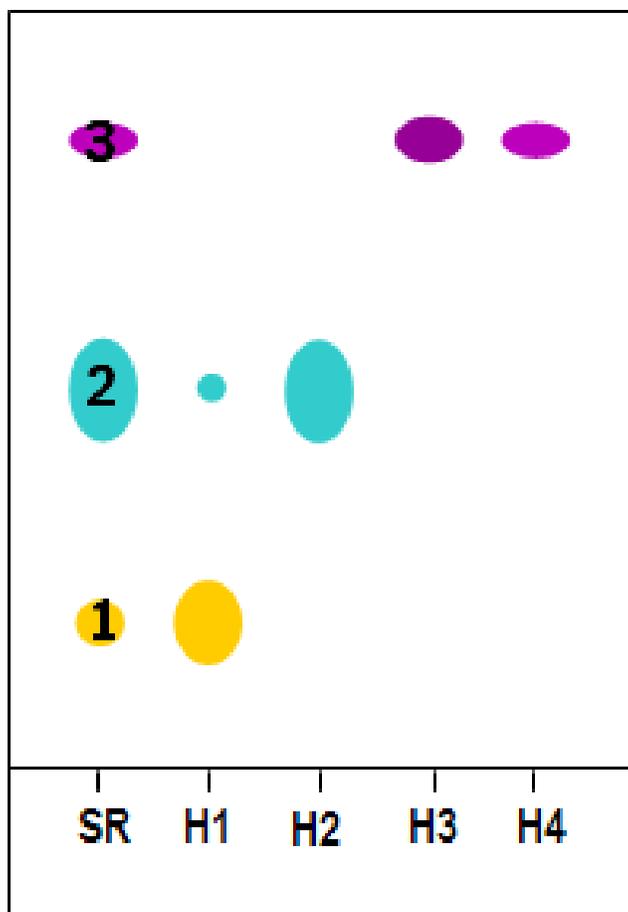
Figure 9. Results of TLC analysis of Syrian rue seed extract (SR), eluted along
with its main alkaloid components purchased from Sigma-Aldrich, i.e. harmalol
(H1), harmaline (H2), harmane (H3) and harmine (H4).
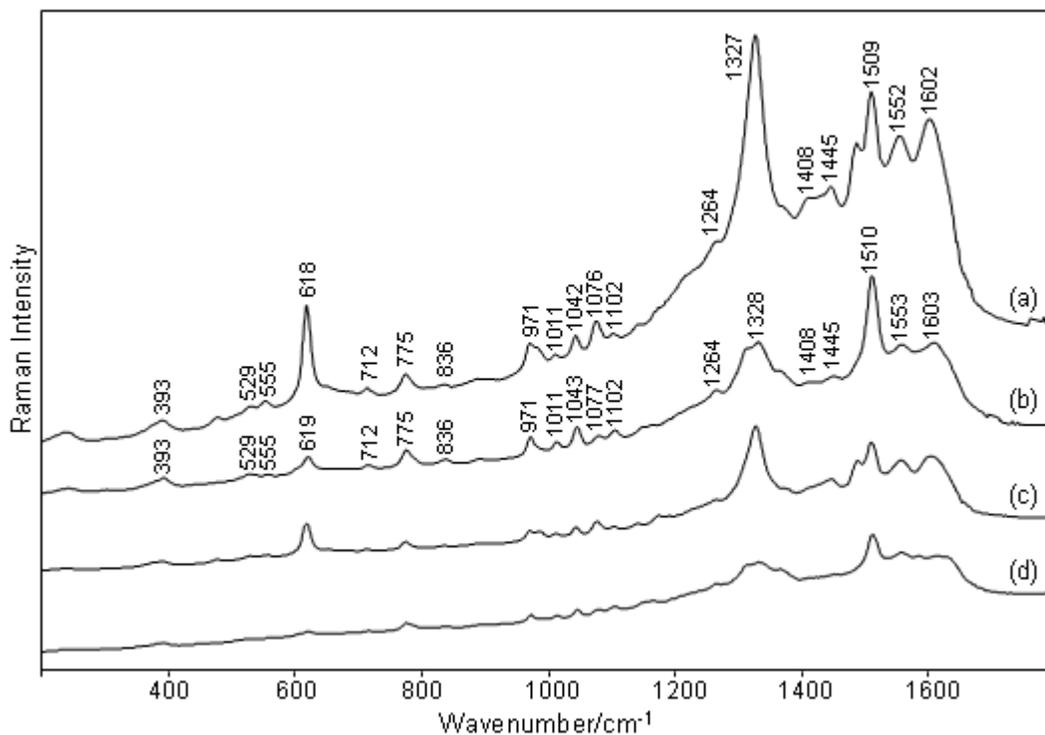
Figure 10. SERS spectra of (a) harmalol and (b) component 1 from Syrian rue seed extract eluted on TLC plate on concentrated nanoparticles, and (c) harmalol and (d) component 1 from Syrian rue seed extract eluted on TLC plate on regular nanoparticles (λ_{exc} = 488 nm). Spectra were collected using a 2.5 mW output laser power, with a single integration of 30 s.
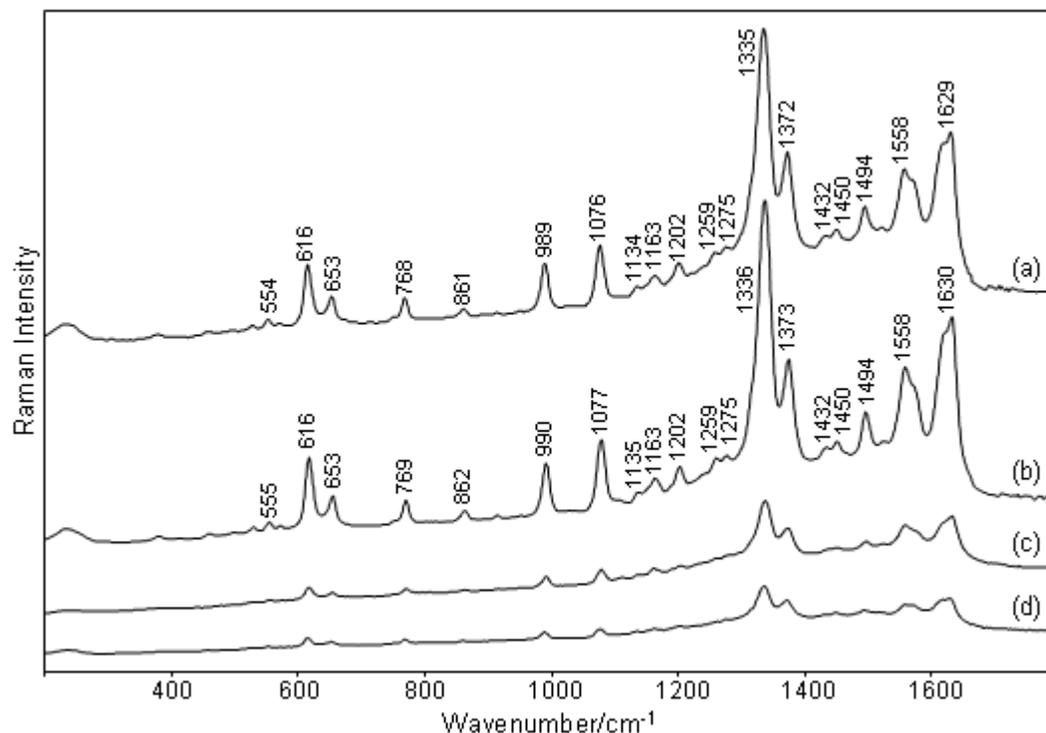
Figure 11. SERS spectra of (a) component 2 from Syrian rue seed extract and (b) harmaline eluted on TLC plate on concentrated nanoparticles, and (c) component 2 from Syrian rue seed extract and (d) harmaline eluted on TLC plate on regular nanoparticles (λ_{exc} = 488 nm). Spectra were collected using a 2.5 mW output laser power, with a single integration of 30 s.
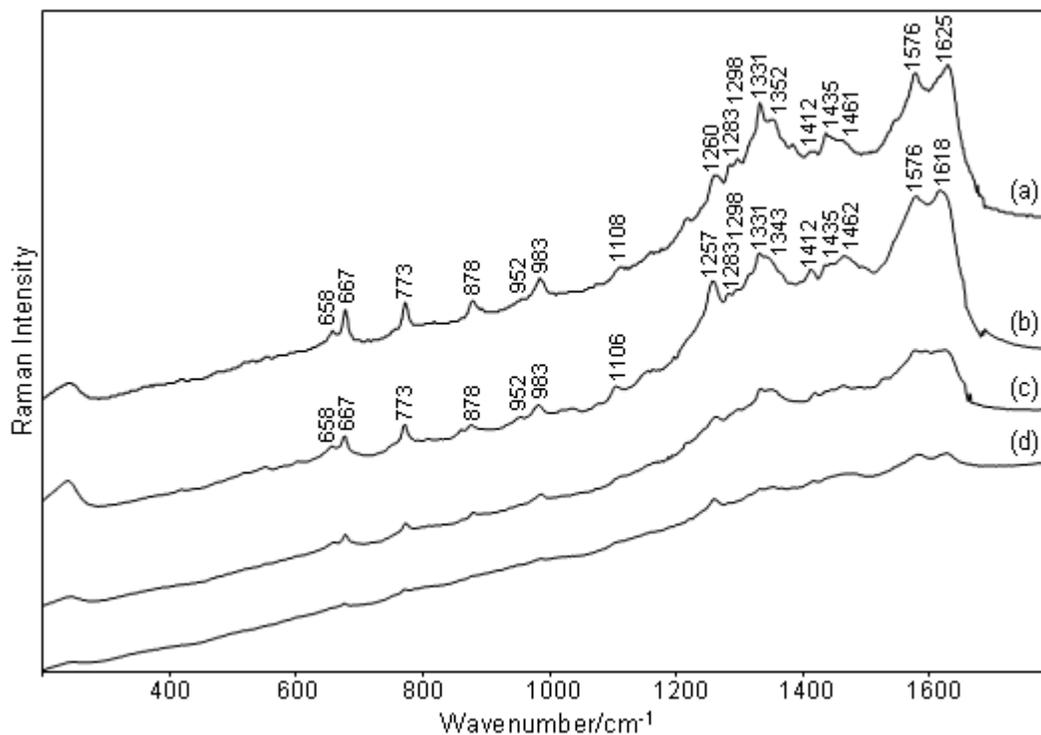
Figure 12. SERS spectra of (a) component 3 from Syrian rue seed extract and (b) harmine eluted on TLC plate on concentrated nanoparticles, and (c) component 3 from Syrian rue seed extract and (d) harmine eluted on TLC plate on regular nanoparticles (λ_{exc} = 488 nm). Spectra were collected using a 2.5 mW output laser power, with a single integration of 30 s.
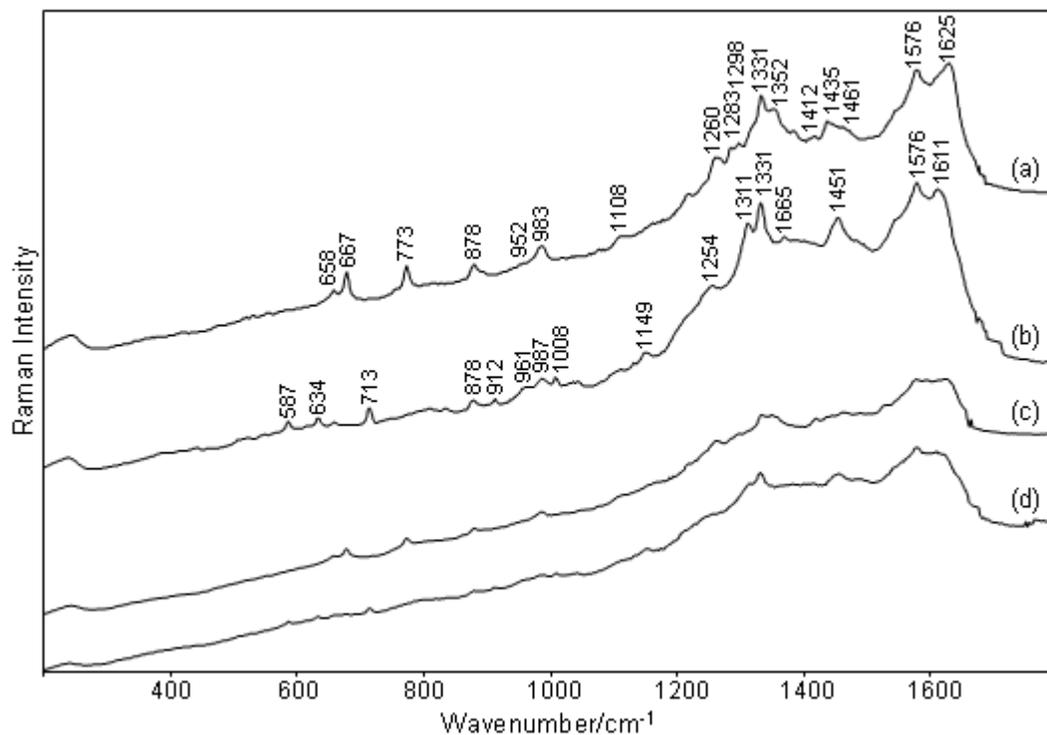
Figure 13. SERS spectra of (a) component 3 from Syrian rue seed extract and (b) harmane eluted on TLC plate on concentrated nanoparticles, and (c) component 3 from Syrian rue seed extract and (d) harmane eluted on TLC plate on regular nanoparticles (λ_{exc} = 488 nm). Spectra were collected using a 2.5 mW output laser power, with a single integration of 30 s.
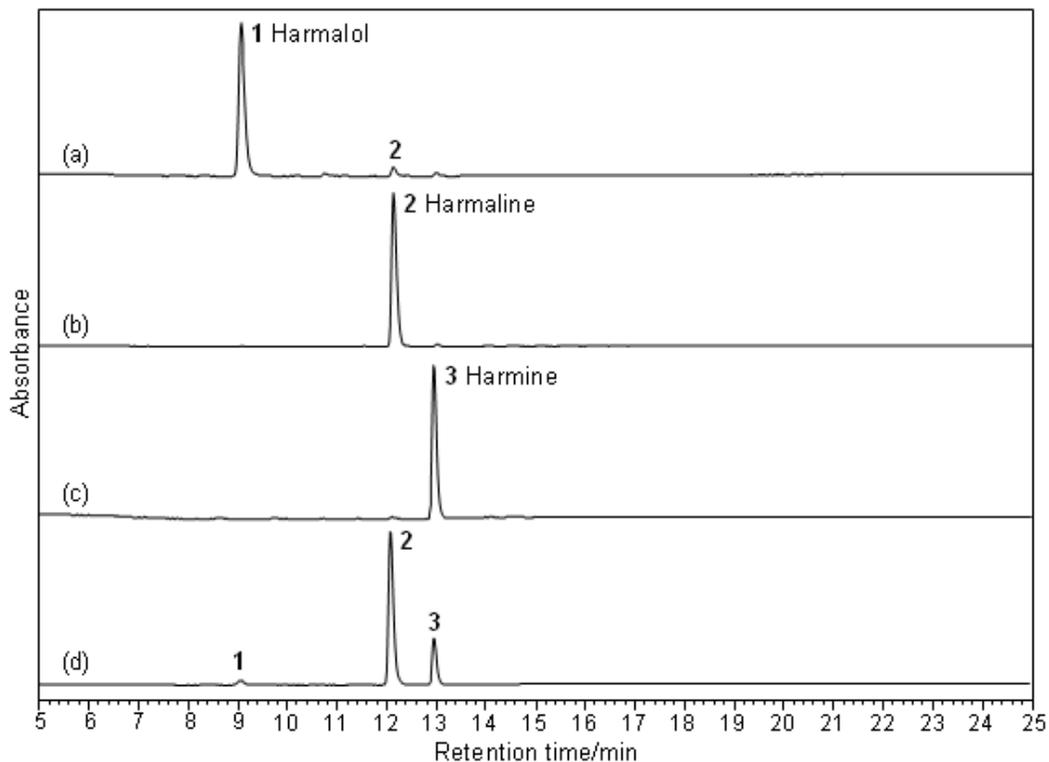
Figure 14. HPLC chromatograms of commercial (a) harmalol, (b) harmaline and (c) harmine compared to (d) that of Syrian rue seed extract. Retention times (t_R) are: t_R (Harmalol) = 9.1 min; t_R (Harmaline) = 12.1 min; t_R (Harmine) = 13.0 min. Based on retention times and visible spectra of the components, Syrian rue seed extract was found to contain (1) harmalol, (2) harmaline and (3) harmine. Chromatograms were recorded at 350 nm.
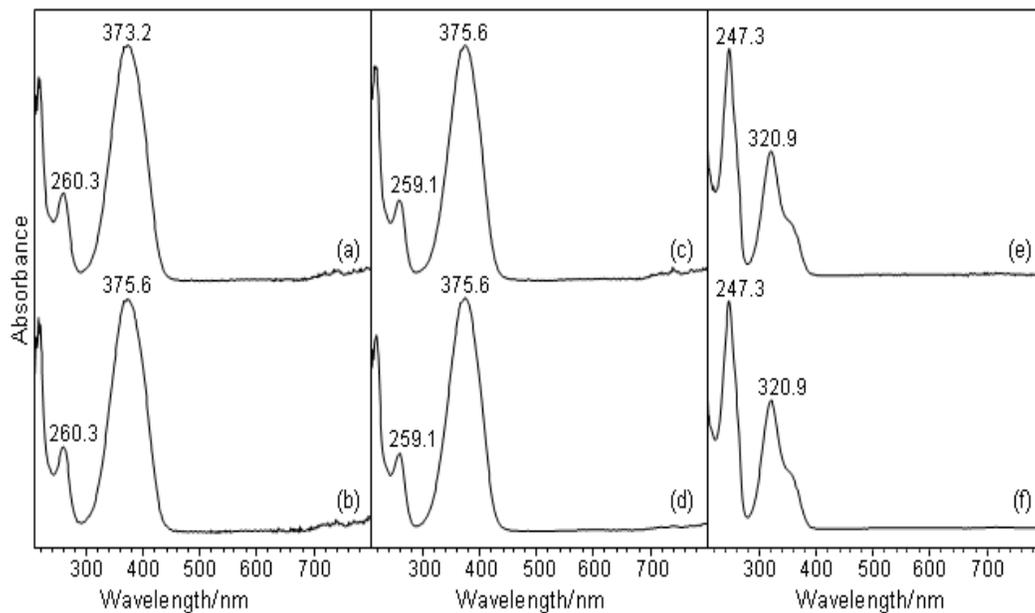
Figure 15. UV/Vis spectra of (a) commercial harmalol compared to (b) compound 1 in Syrian rue seed extract, (c) commercial harmaline compared to (d) compound 2 in Syrian rue seed extract, and (e) commercial harmine compared to (f) compound 3 in Syrian rue seed extract.
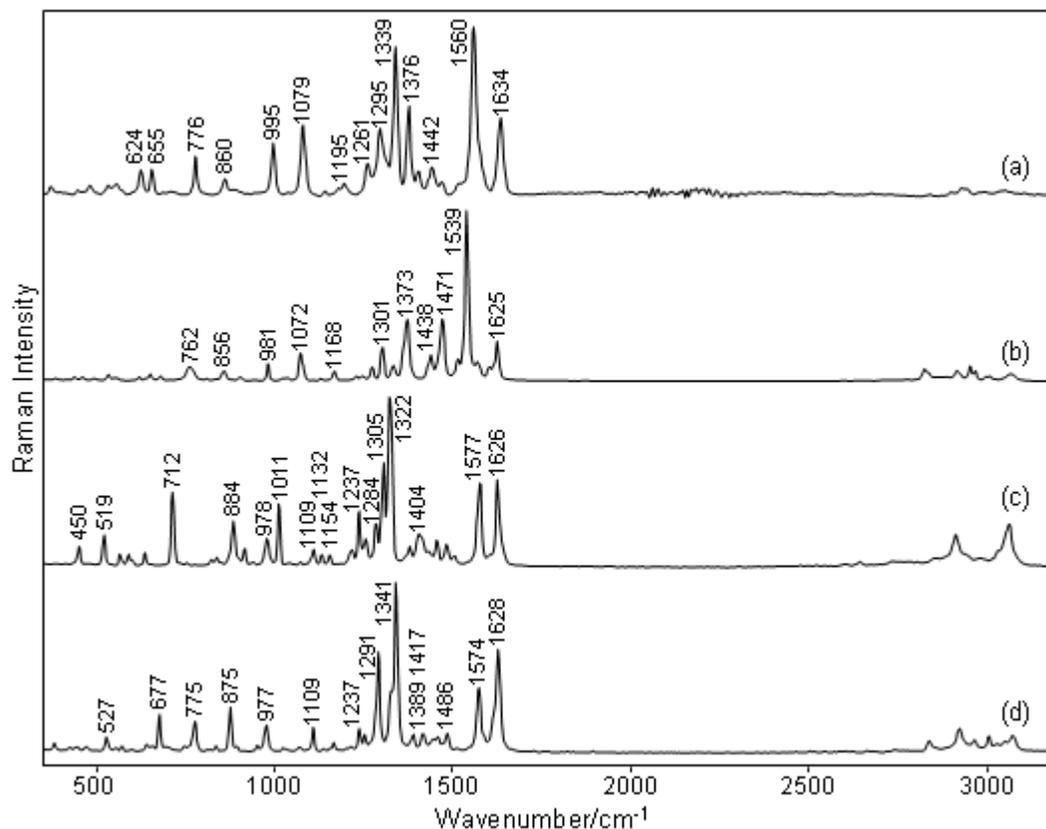
Figure 16. FT-Raman spectra of (a) harmalol, (b) harmaline, (c) harmane and (d) harmine (λ_{exc} = 1064 nm). A baseline correction was applied to spectrum (a). Spectra were recorded using a 25 mW output laser power, by collecting 128 scans.
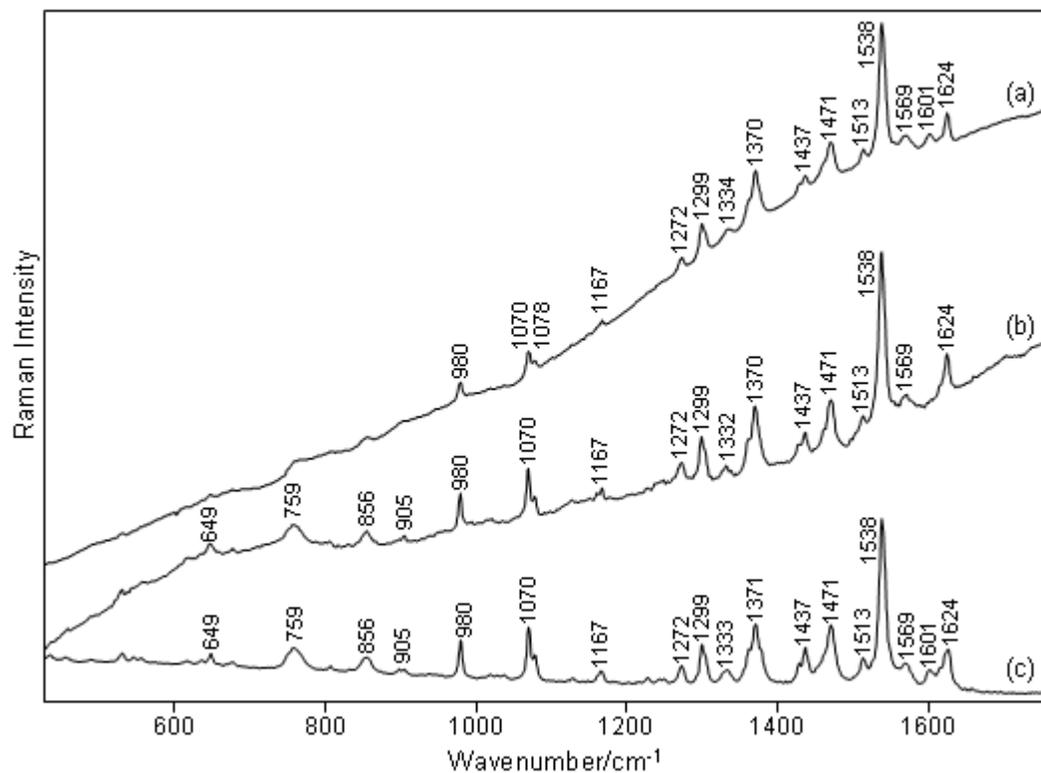
Figure 17. Raman spectra of harmaline at excitation (a) 488 nm, (b) 633 nm and (c) 785 nm. Spectra were collected using a 2.5, 5 and 10 mW output laser power, respectively, with a single integration of 30 s.
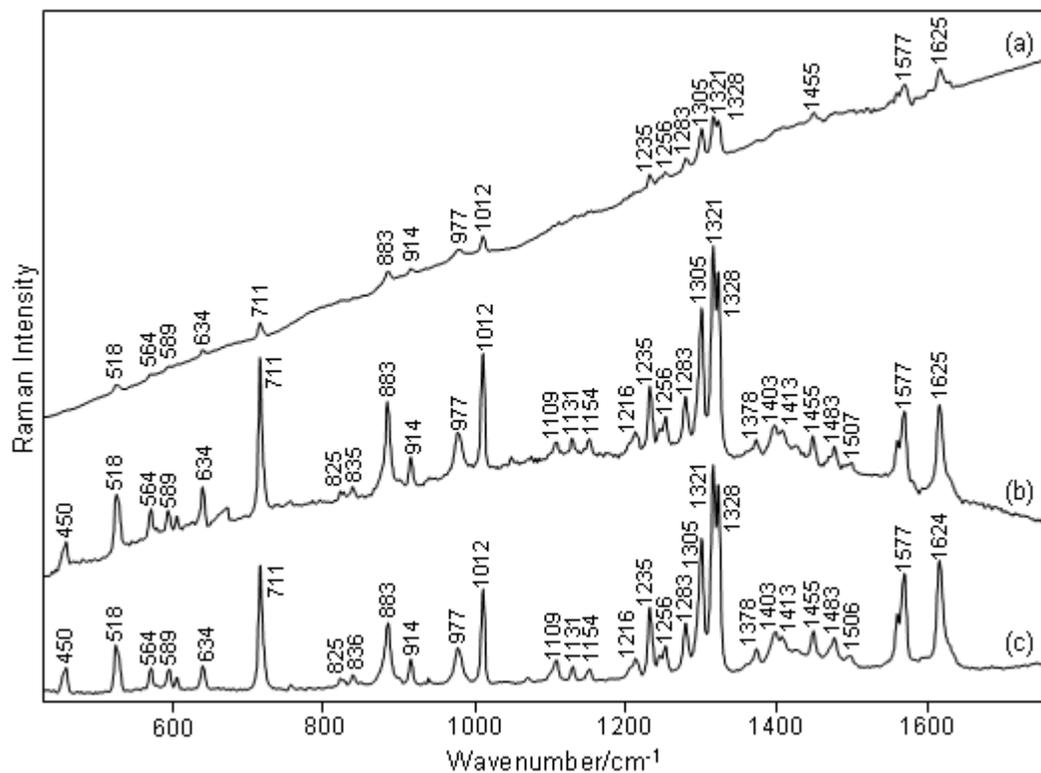
Figure 18. Raman spectra of harmane at excitation (a) 488 nm, (b) 633 nm and (c) 785 nm. Spectra were collected using a 2.5, 5 and 10 mW output laser power, respectively, with a single integration of 30 s.

Figure 19. Raman spectra of harmine at excitation (a) 488 nm, (b) 633 nm and (c) 785 nm. Spectra were collected using a 2.5, 5 and 10 mW output laser power, respectively, with a single integration of 30 s.
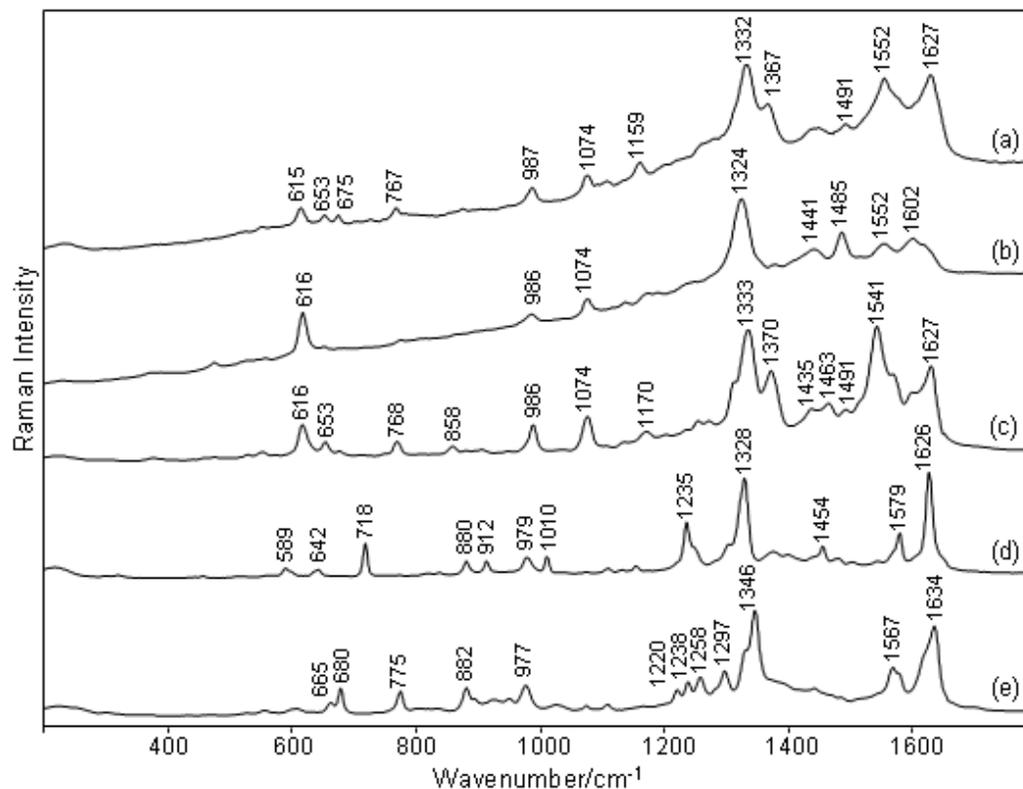
Figure 20. (a) SERS spectrum of Syrian rue extract compared to those of (b)
harmalol, (c) harmaline, (d) harmane and (e) harmine (λ_{exc} = 488 nm). Spectra
were collected using a 2.5 mW output laser power, with a single integration of
30 s.

Gel transfer SERS for non-destructive analysis
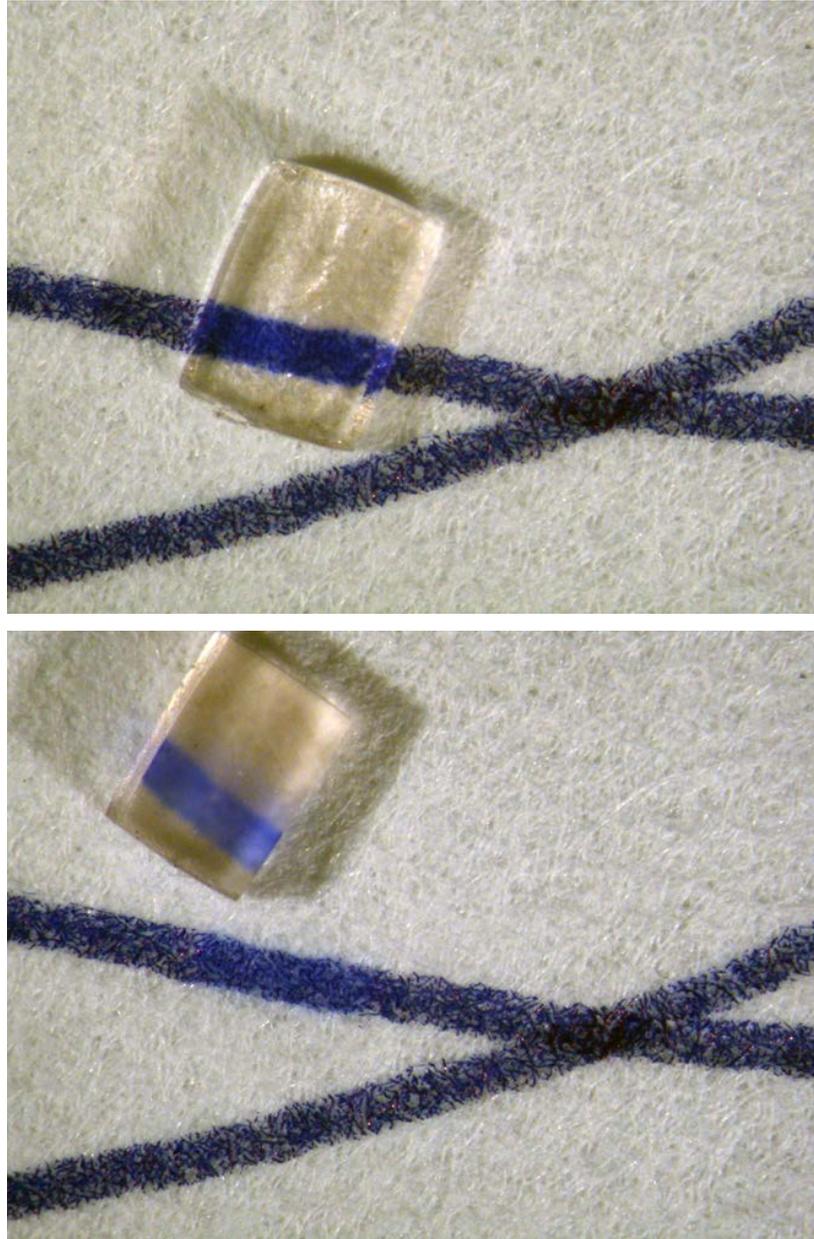
Figure 21. Pen ink on paper, during and after gel extraction.

Figure 22. "The maiden's companion signals to the hunters" tapestry, from "The hunt of the unicorn" series (South Netherlandish, ca. 1495-1505. The Metropolitan Museum of Art, 38.51,1.2; gift of John D. Rockefeller Jr., 1937).

**Figure 23.** Extraction of a red dye of the tapestry using the gel transfer technique.
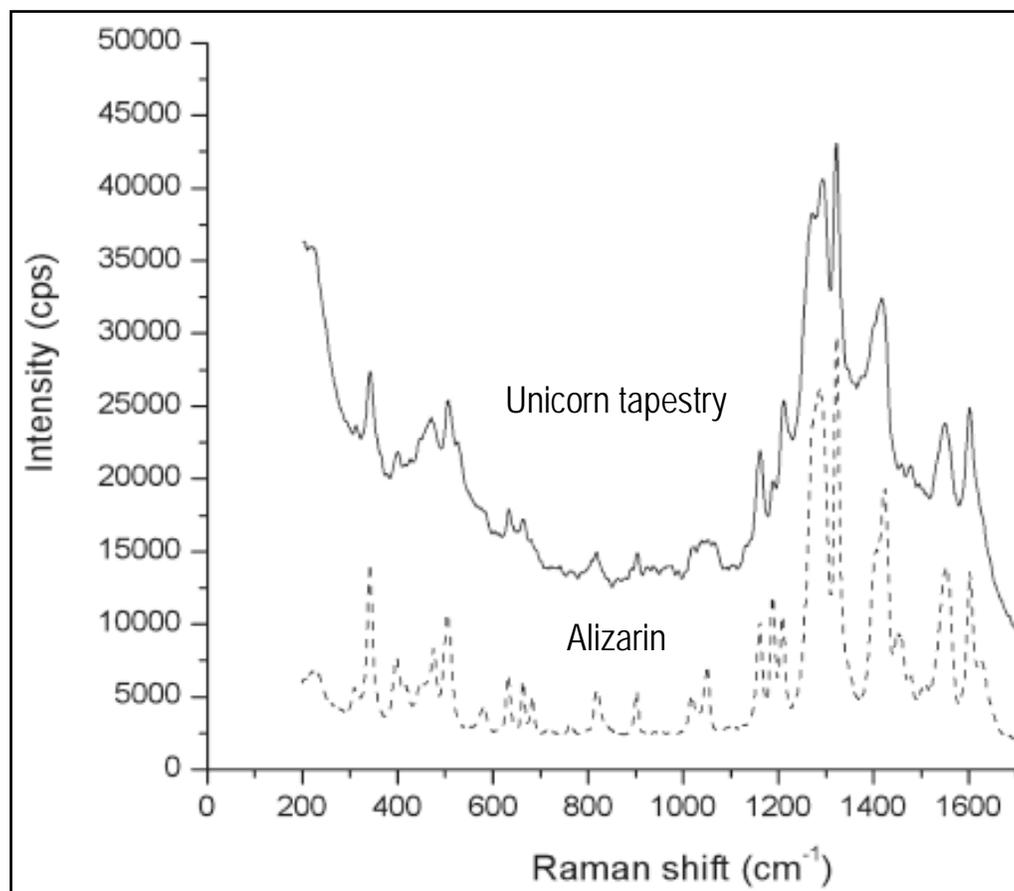
Figure 24. SERS spectra from a gel strip applied to a red thread in the Unicorn tapestry and from an alizarin reference solution.

Figure 25. "Sekigahara Homare no Gaika" ("A poem about the battle of Sekigahara") by Kunichika, 1892. Woodblock print on paper, triptych, each sheet originally *oban* size (27 x 39 cm), slightly trimmed. Private collection.
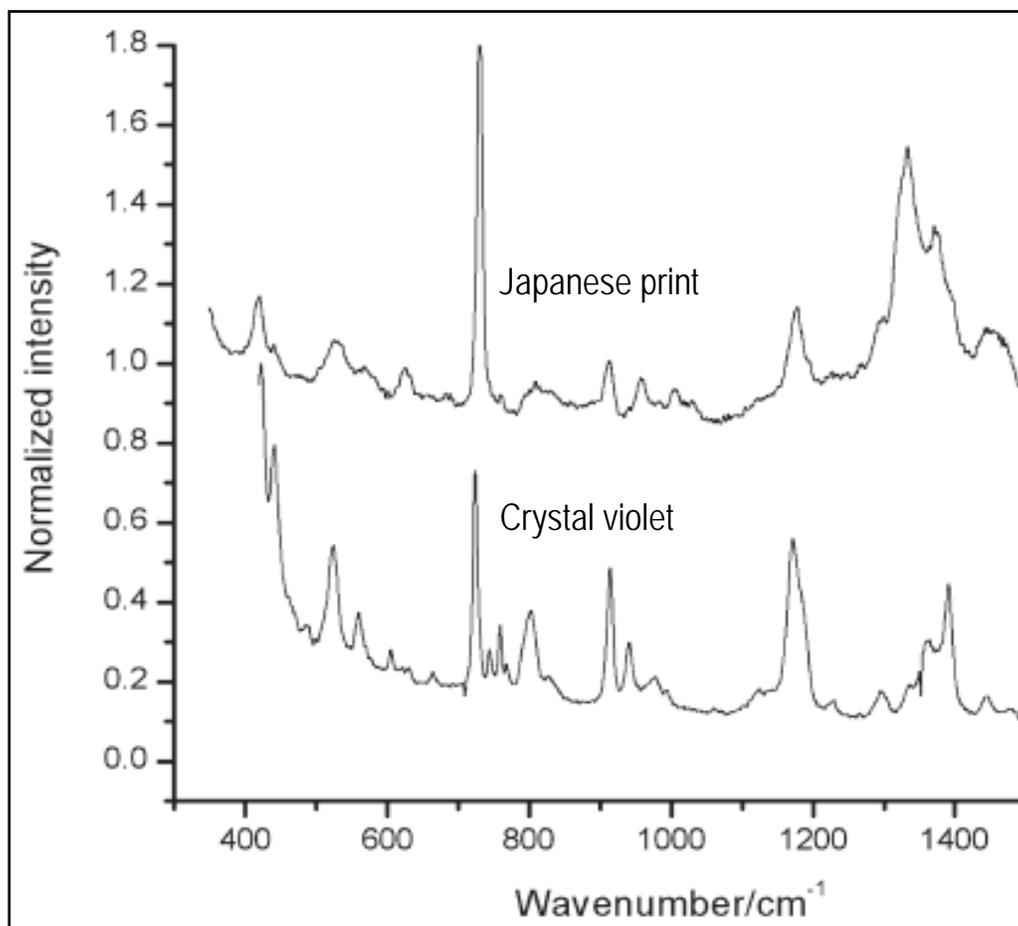
Figure 26. SERS spectra from a gel fragment removed from a violet area on the print "Sekigahara Homare no Gaika" and from a reference solution of crystal violet. Spectra were normalized for ease of comparison.

Figure 27. Reflectance measurements on a purple area of the Japanese woodblock print to evaluate the color change before and after gel extraction.
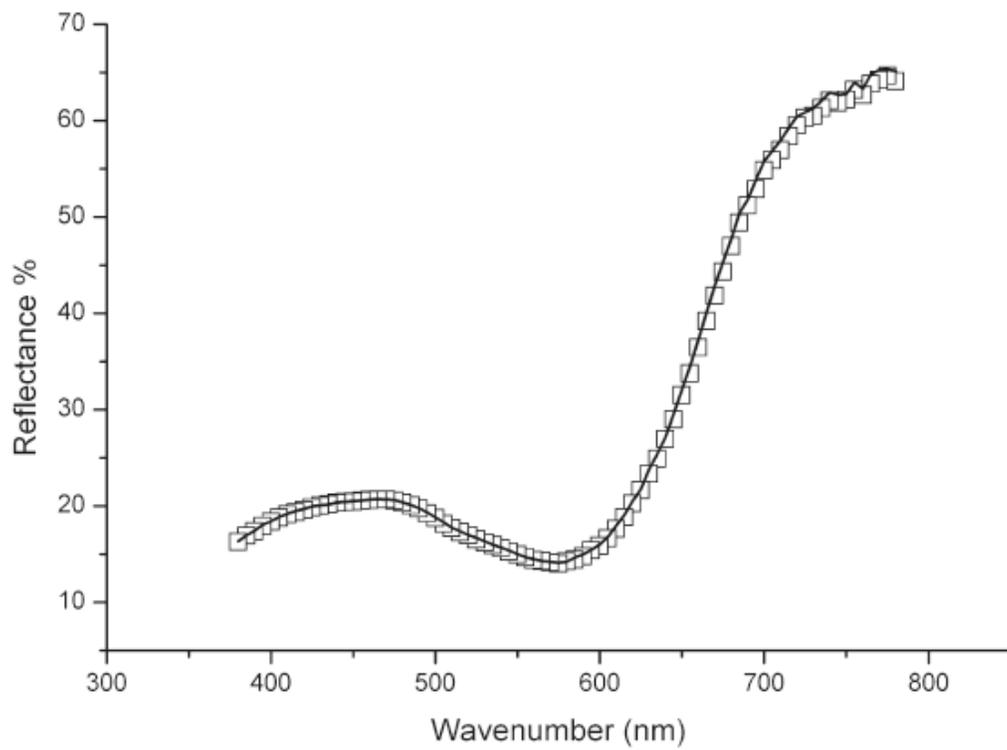
Figure 28. Means of ten measurements each of the same location, before extraction (squares) and after extraction (solid line).

## Raman and SERS analysis of sympathomimetic amines
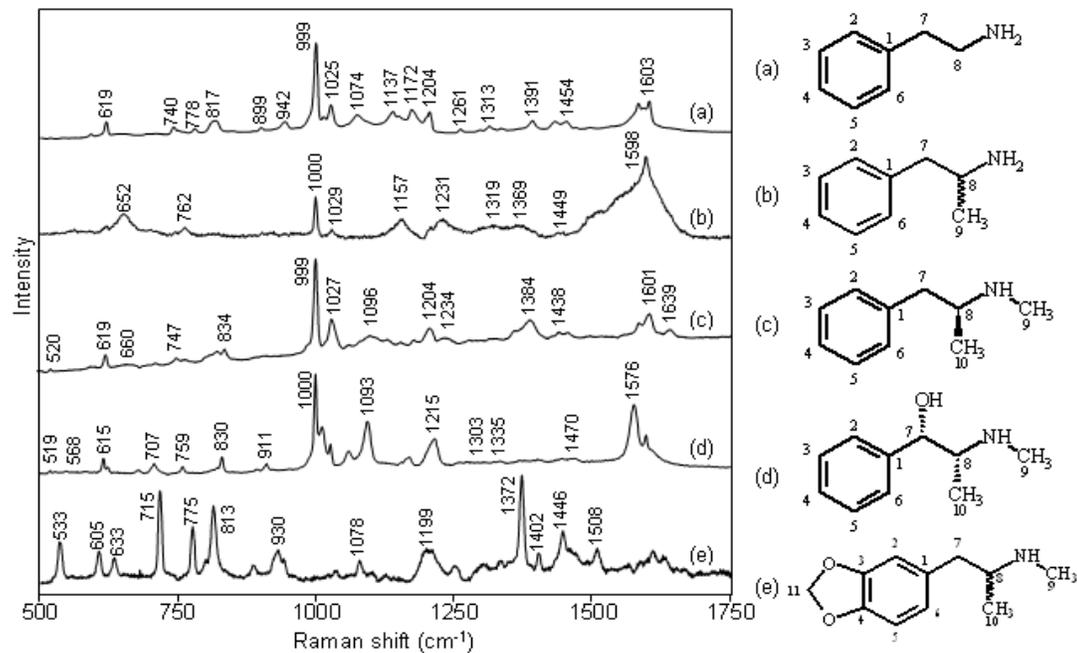
Figure 29. SERS spectra of (a) phenethylamine, (b) amphetamine, (c) methamphetamine, (d) ephedrine and (e) MDMA taken at excitation 514 nm. The molecular structures of these substances are also shown.

Raman and SERS analysis of synthetic dyes
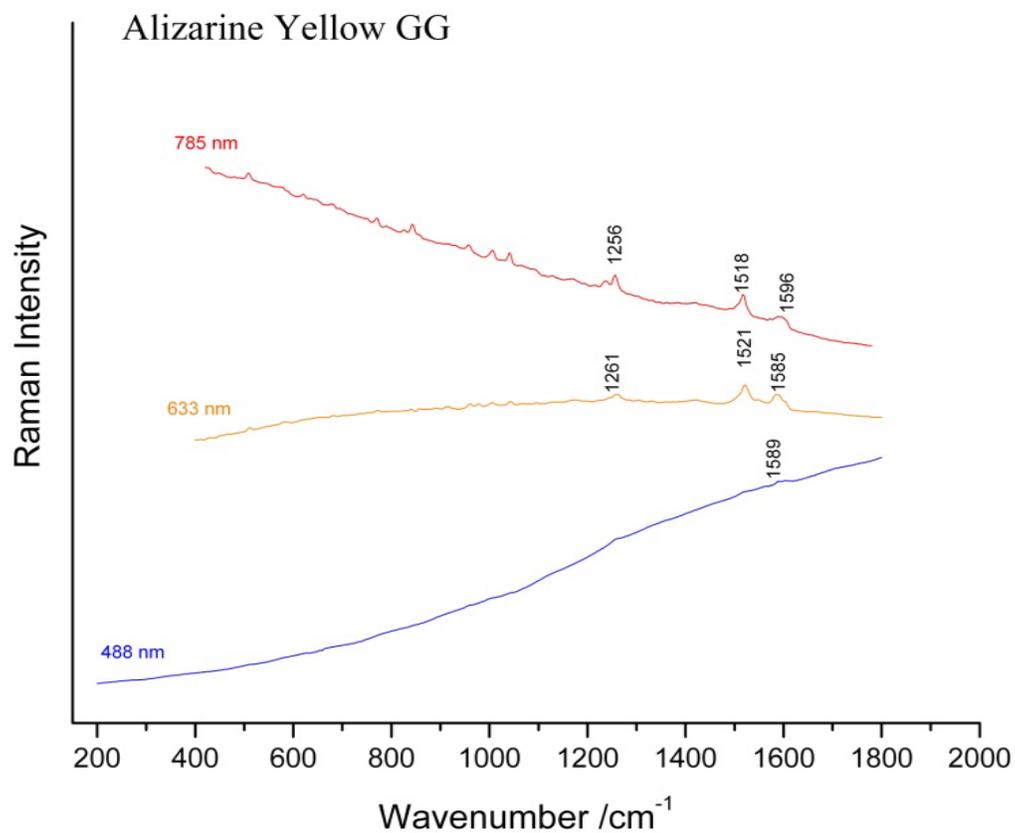
**Figure 30.** Raman spectra of alizarine yellow GG at 488, 633 and 785 nm.
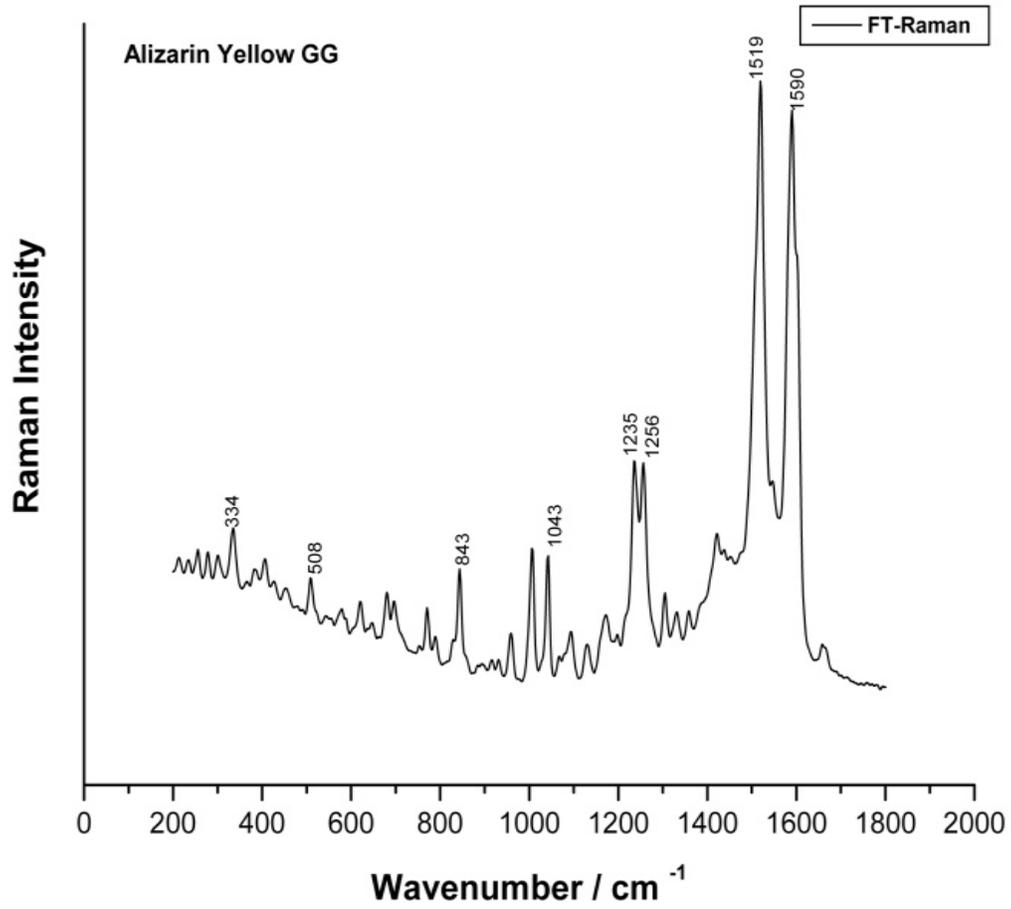
Figure 31. FT-Raman spectrum of alizarine yellow GG.
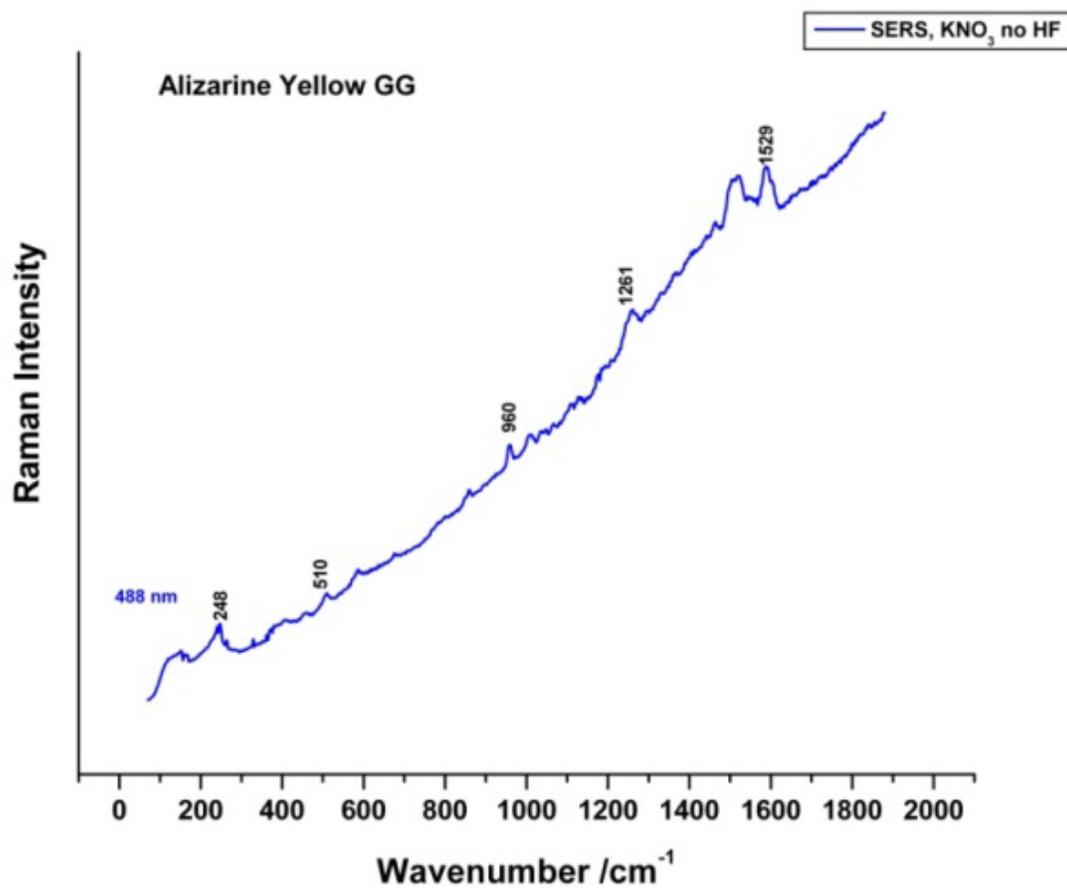
Figure 32. SERS spectrum of alizarine yellow GG at 488 nm.
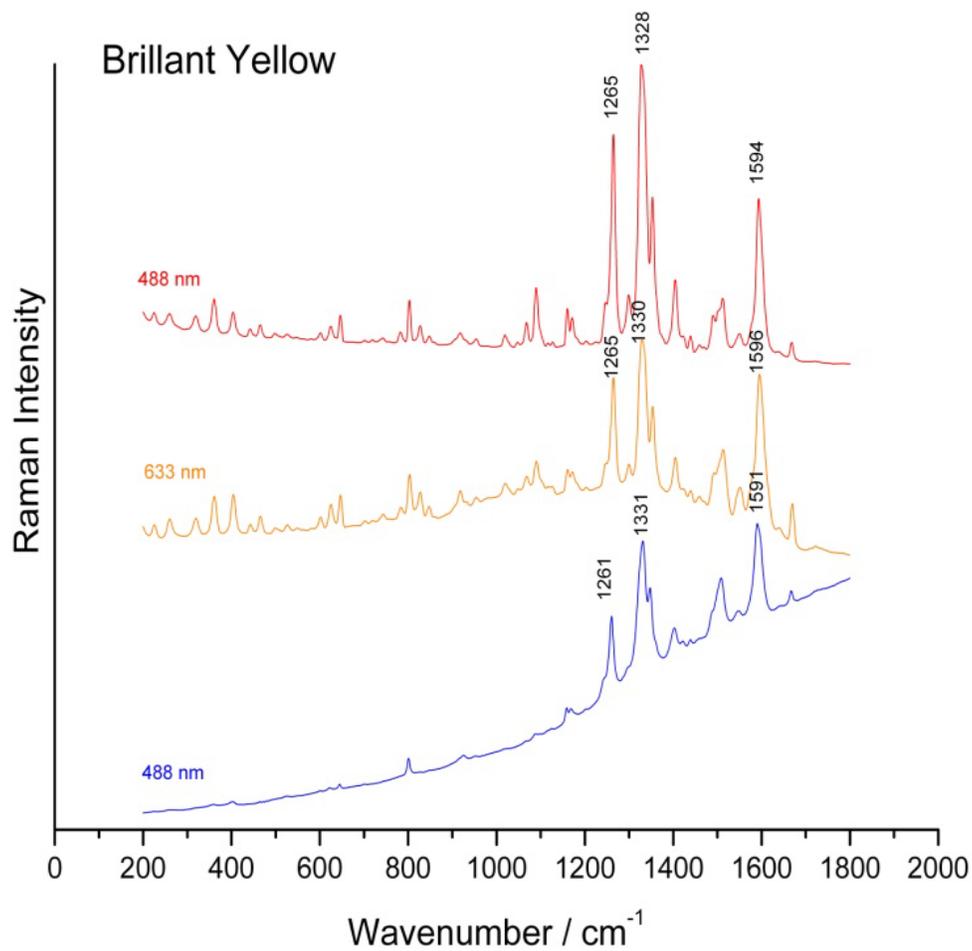
Figure 33. Raman spectra of brilliant yellow at 488, 633 and 785 nm.
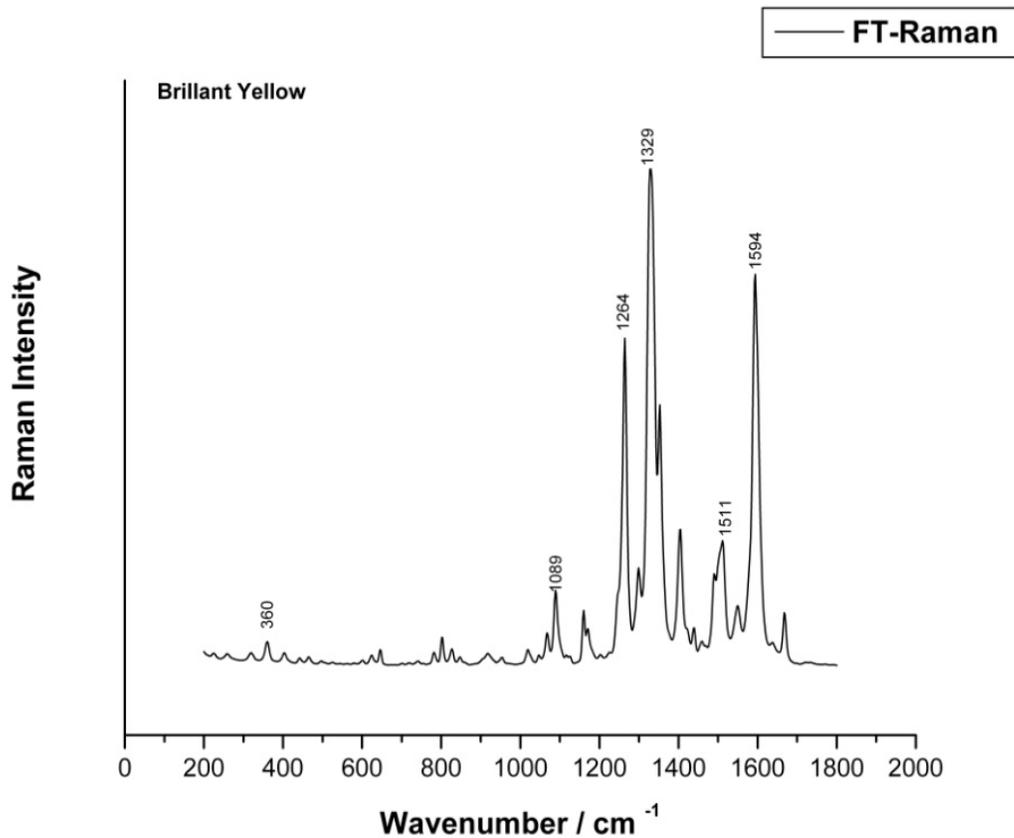
Figure 34. FT-Raman spectrum of brilliant yellow.
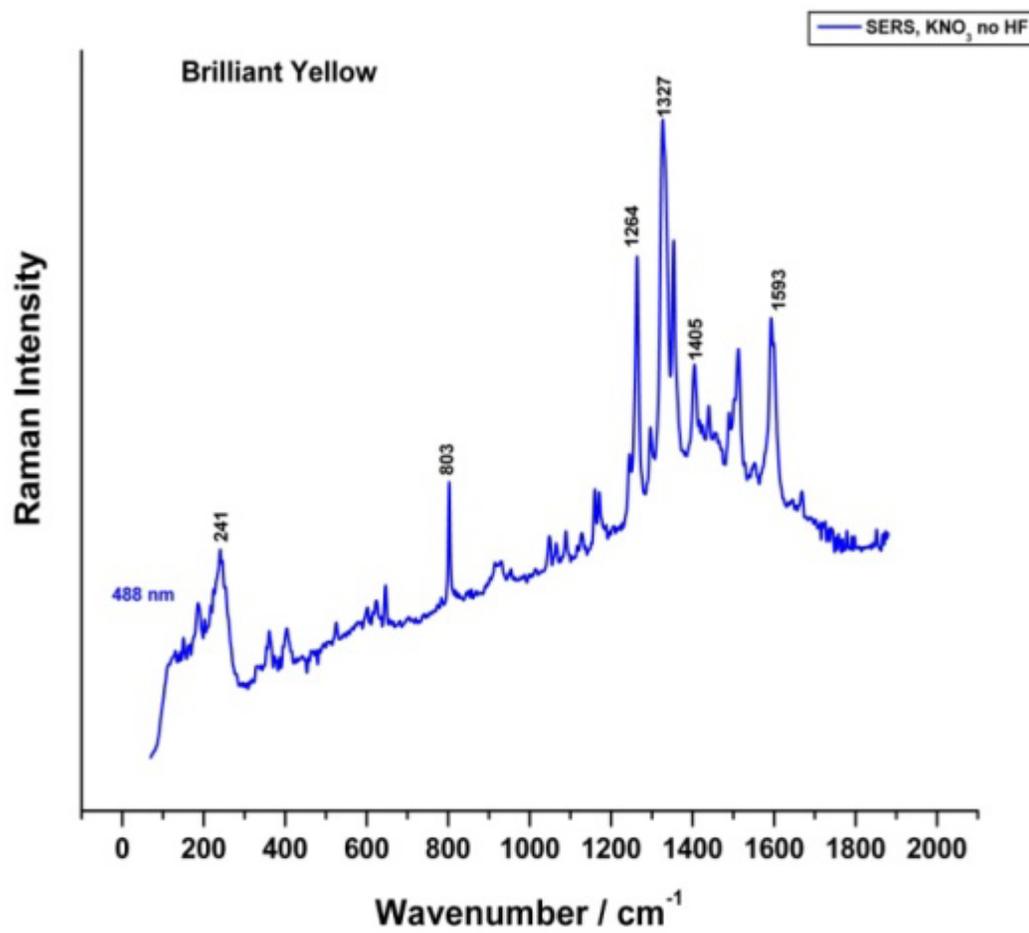
Figure 35. SERS spectrum of brilliant yellow.
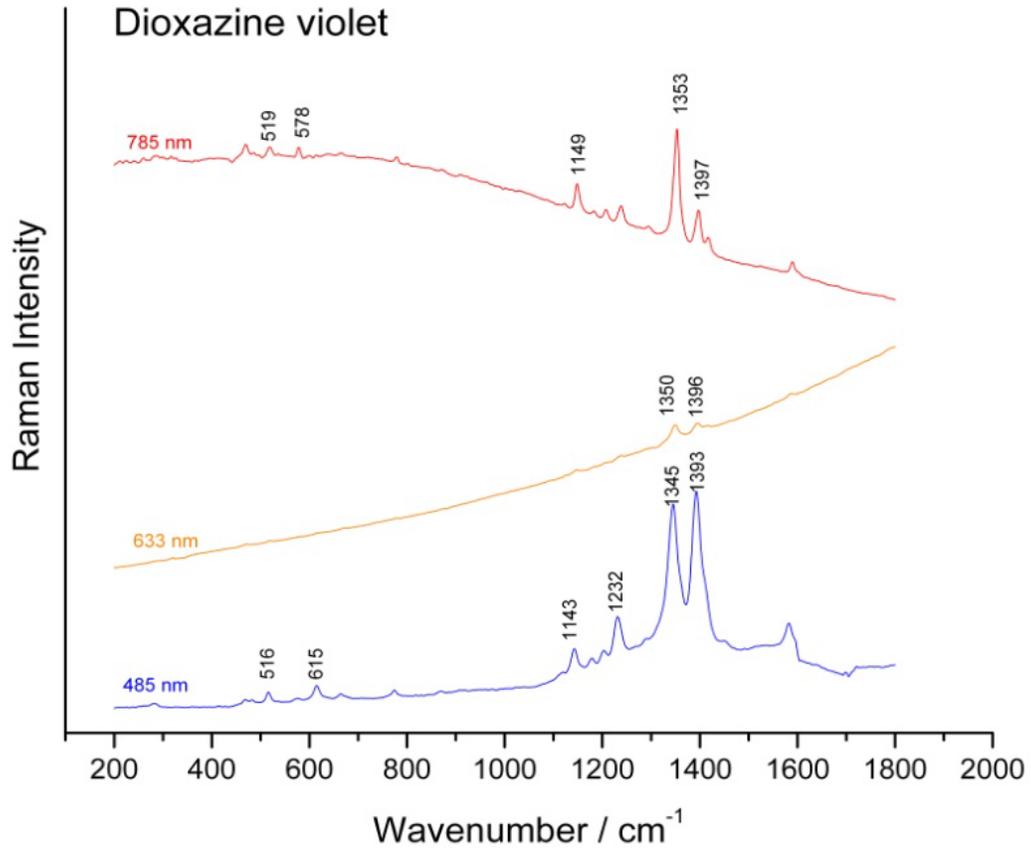
Figure 36. Raman spectra of dioxazine violet at 488, 633 and 785 nm.
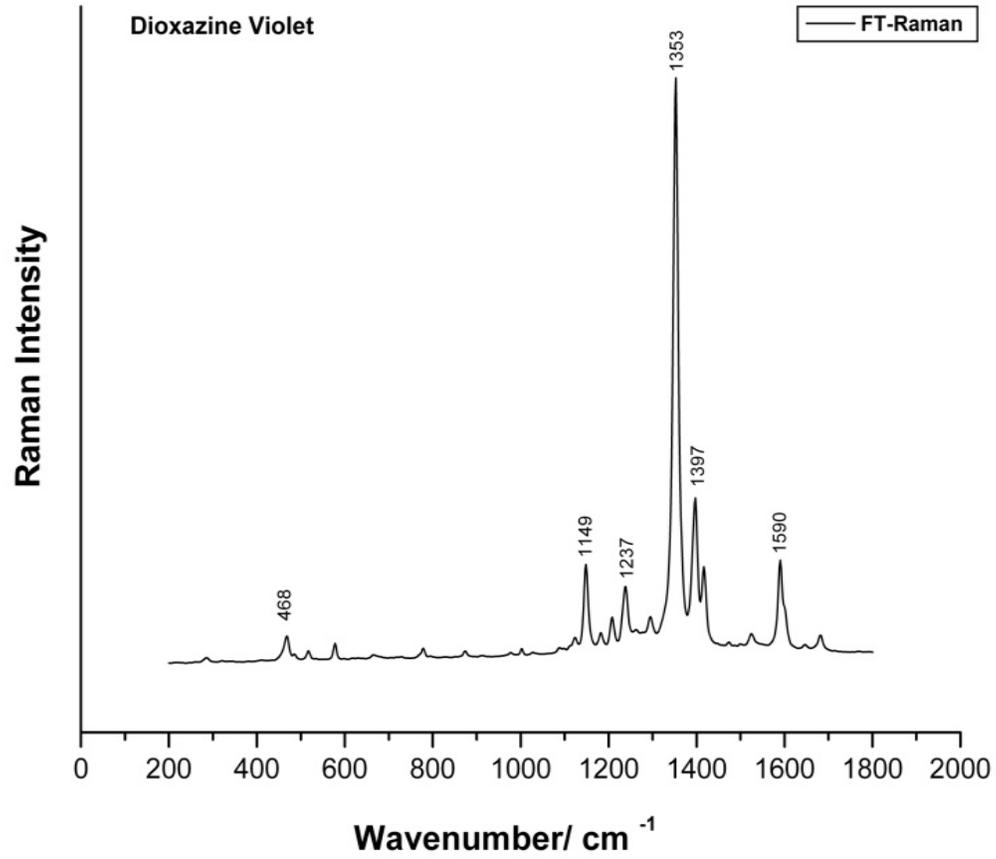
Figure 37. FT-Raman spectrum of dioxazine violet.
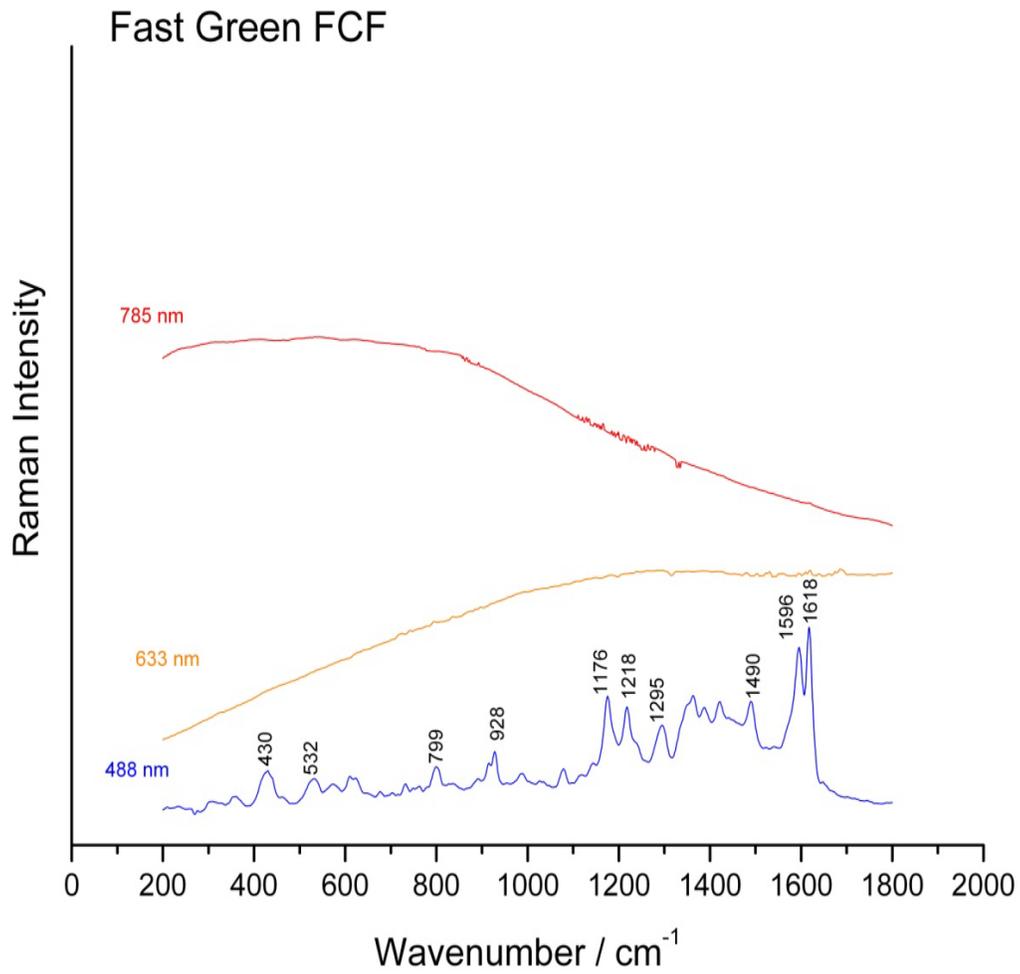
Figure 38. Raman spectra of fast green FCF at 488, 633 and 785 nm.
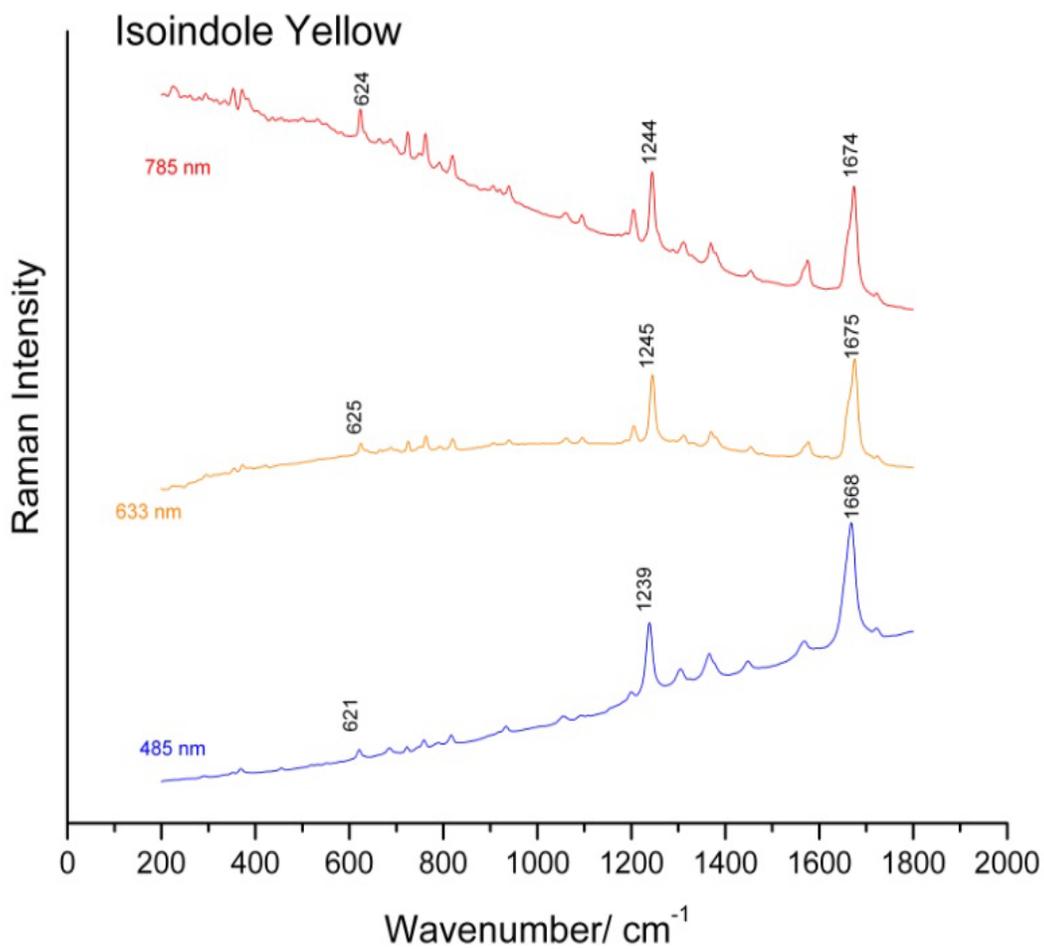
Figure 39. Raman spectra of isoindole yellow at 488, 633 and 785 nm.
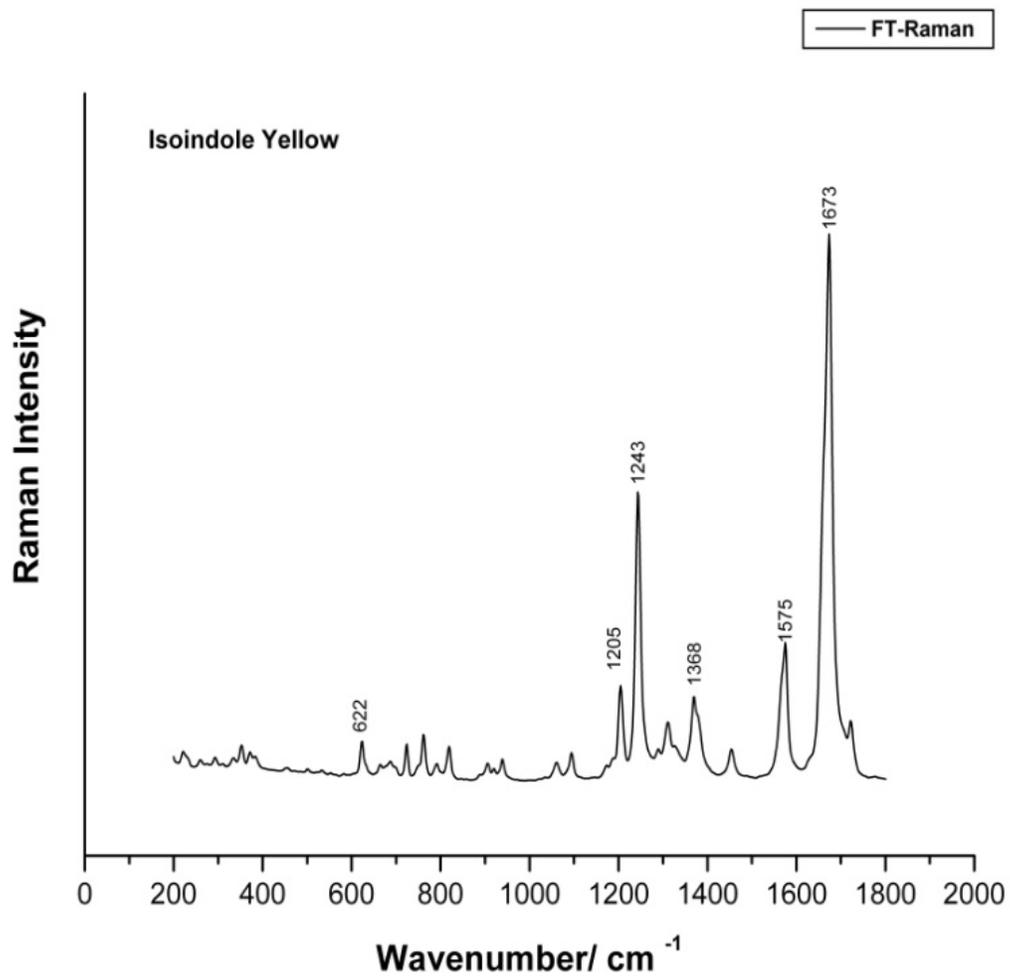
Figure 40. FT-Raman spectrum of isoindole yellow.
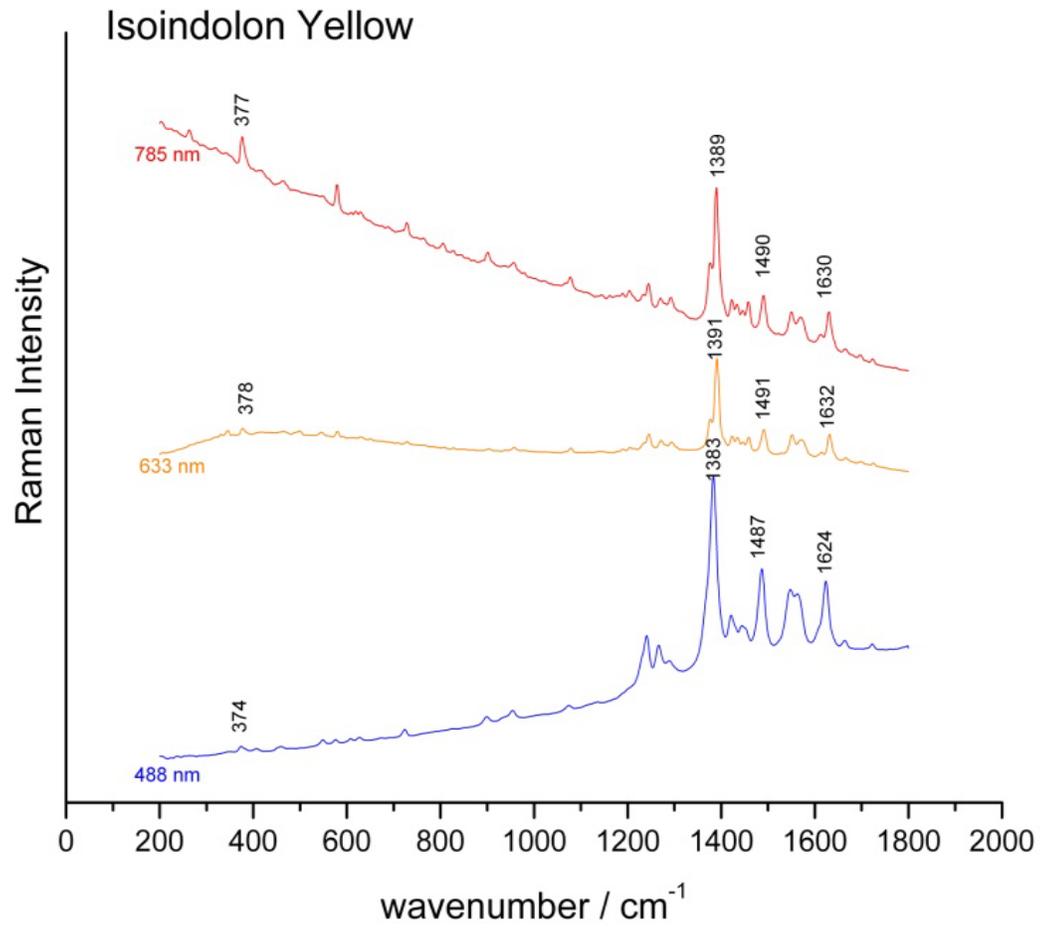
Figure 41. Raman spectra of isoindolon yellow at 488, 633 and 785 nm.
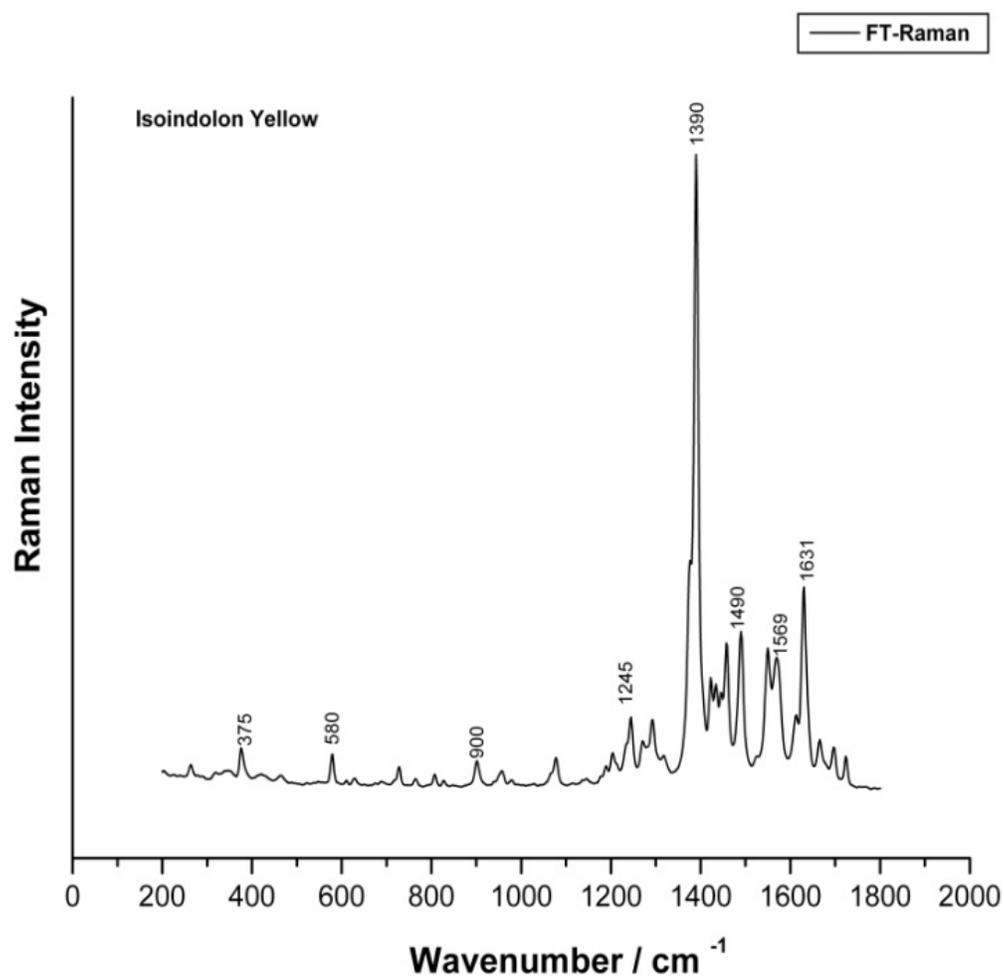
Figure 42. FT-Raman spectrum of isoindolon yellow.
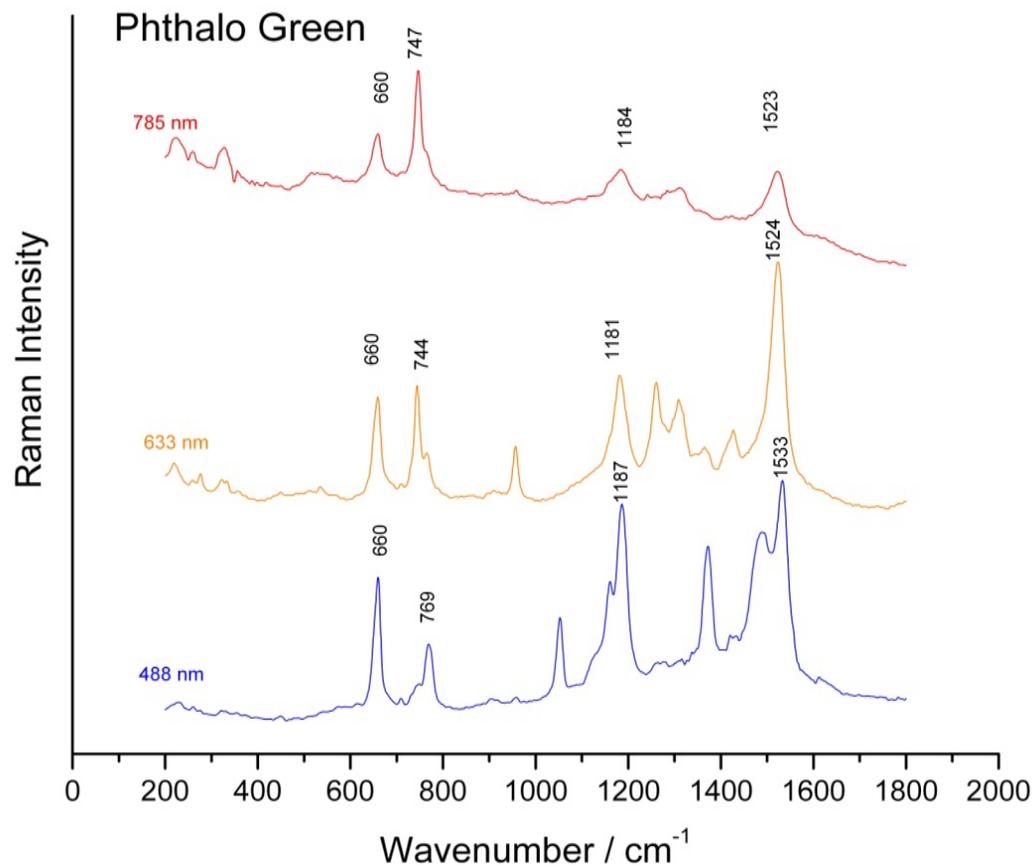
Figure 43. Raman spectra of phthalo green at 488, 633 and 785 nm.
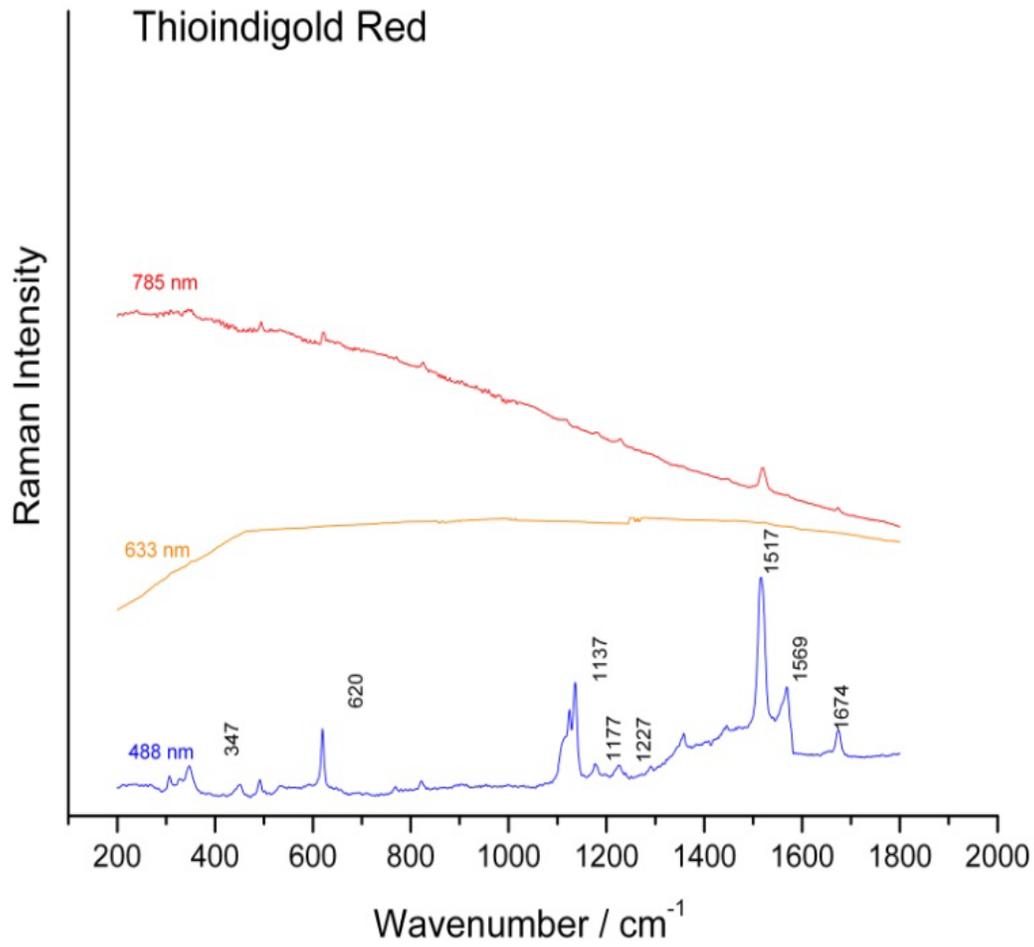
Figure 44. Raman spectra of thioindigoid red at 488, 633 and 785 nm.
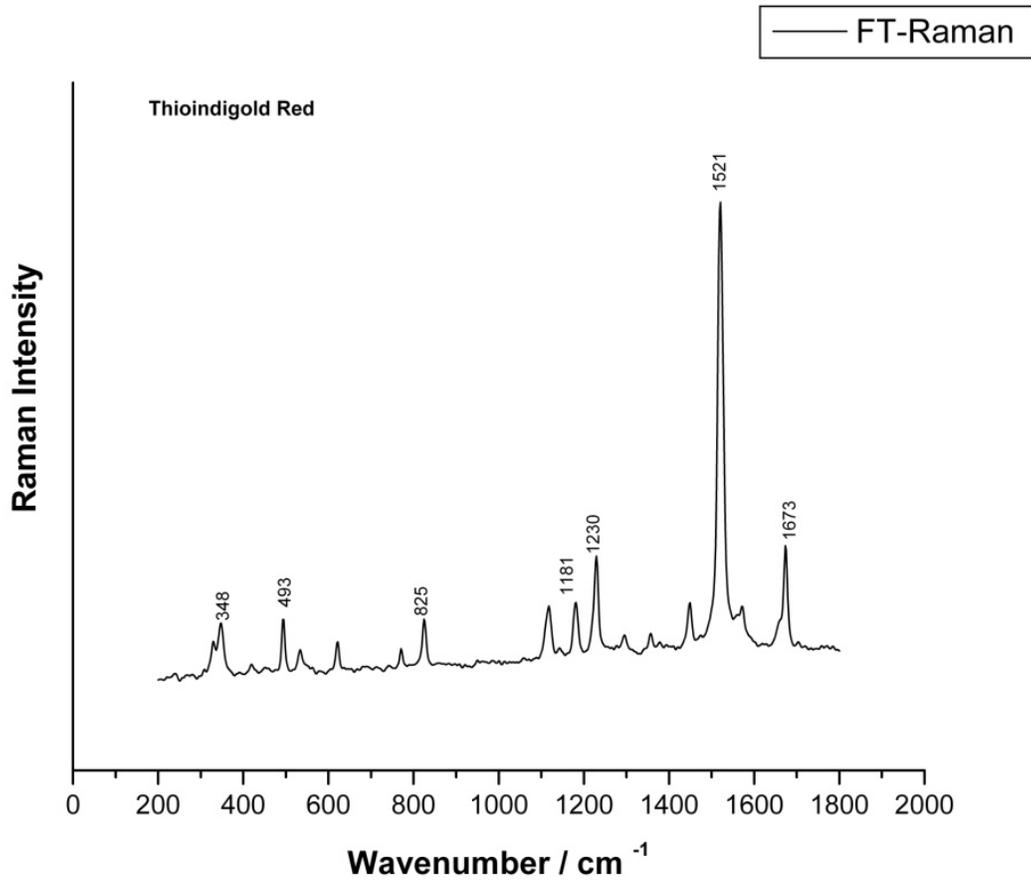
Figure 45. FT-Raman spectrum of thioindigoid red
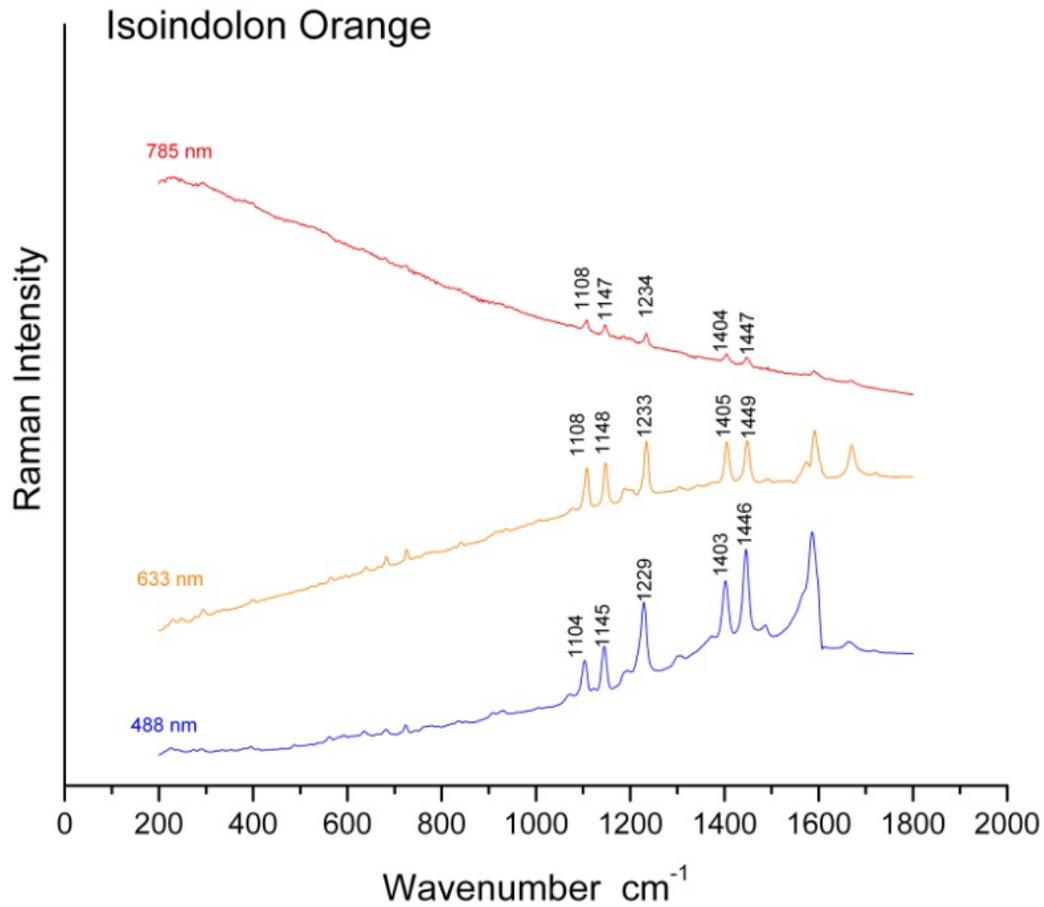
Figure 46. Raman spectra of isoindolon orange at 488, 633 and 785 nm.
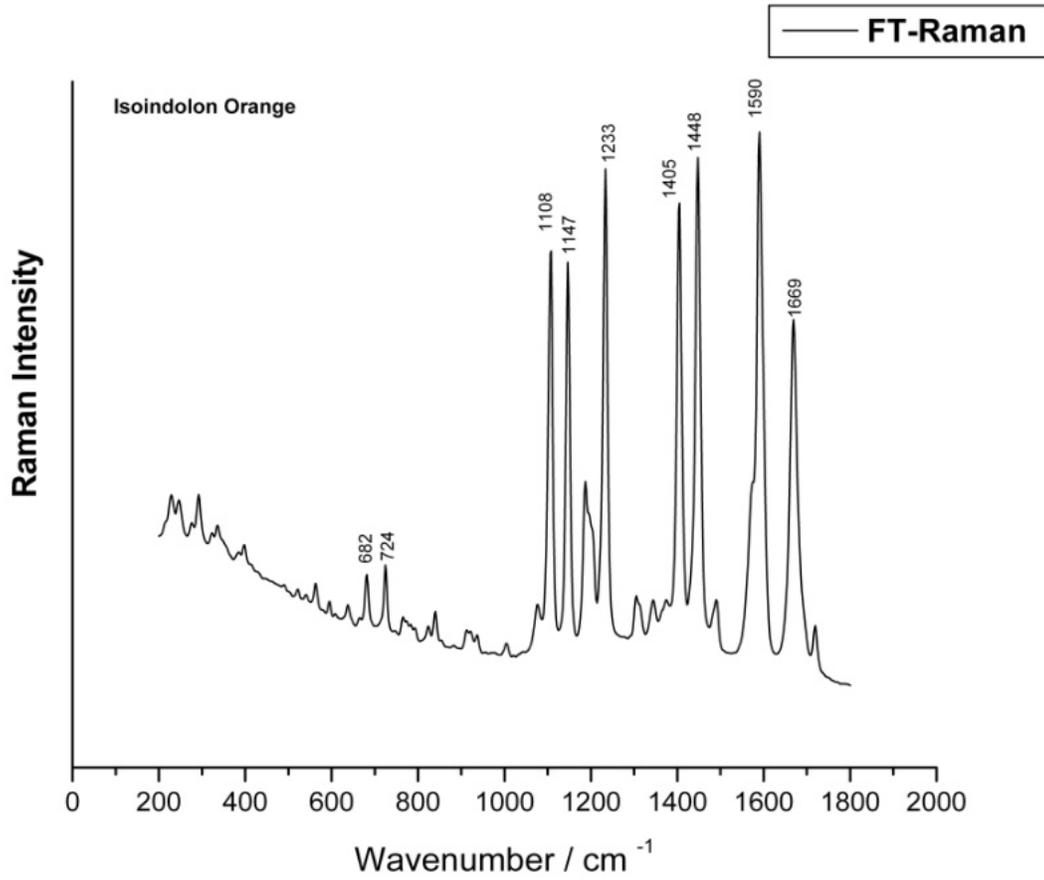
Figure 47. FT-Raman spectrum of isoindolon orange.

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Searchable databases for the identification of dyes

Table 1. Results of classification tests of the reference spectra of the database with PCA and CC algorithm using a cross validation method, when applying subsequent degrees of spectral transformation (expressed in percentages).

Database	PCA (number of PCs)	CC
Baseline Correction and Cutting (BCC)	36.0% (23)	55.9%
BCC/Scaling 0-100	53.0% (35)	55.9%
BCC/Smoothing 5 points x20	35.0% (24)	54.0%
BCC/Smoothing 5 points x20/Scaling 0-100	55.0% (36)	55.9%
BCC/Scaling 0-100/II Derivative	51.0% (22)	71.0%
BCC/Smoothing 5 points x20/Scaling 0-100/II Derivative	76.0% (15)	78.5%

Table 2. Works of art and museum objects from which a sample was taken and analyzed by SERS are reported along with the correct matches for the corresponding spectra searched.

Sample	Colorant
Lake pigment from Corinth	Madder
Alizarin crimson (Winsor & Newton)	Alizarin
Carmine (Winsor & Newton)	Carminic acid
Mauve (Winsor & Newton)	Crystal violet
Feathered bag from Peru	Madder
Cap with feathers from Chile	Madder
Tunic from Peru	Madder
Tasseled tunic from Peru	Carminic acid
Crucifix from Spain	Laccaic acid
Bust of Saint Barbara	Madder
Statue of Caligula	Madder
Mandolin by Vinaccia	Carminic acid
Nur al-Din reproduction panel, red	Carminic acid
Nur al-Din reproduction panel, violet	Laccaic acid
Painted cloth from India	Laccaic acid
Silver ball, barge, and trees by Dove	Madder
The card players by Lievens, red 1	Madder
The card players by Lievens, red 2	Madder
The card players by Lievens, red 3	Madder
The card players by Cézanne	Madder

Table 3. Results of classification tests of query spectra from works of art with PCA and CC algorithm, when applying subsequent degrees of spectral transformation (expressed as number of correct matches / total number of query spectra).

Database	PCA (number of PCs)	CC
Baseline Correction and Cutting (BCC)	9/20 - 45.0% (23)	18/20 - 90.0%
BCC/Scaling 0-100	16/20 - 80.0% (35)	18/20 - 90.0%
BCC/Smoothing 5 points x20	9/20 - 45.0% (24)	18/20 - 90.0%
BCC/Smoothing 5 points x20/Scaling 0-100	15/20 - 75% (36)	18/20 - 90.0%
BCC/Scaling 0-100/II Derivative	8/20 - 40.0% (22)	16/20 - 80.0%
BCC/Smoothing 5 points x20/Scaling 0-100/II Derivative	10/20 - 50.0% (15)	20/20 - 100.0%
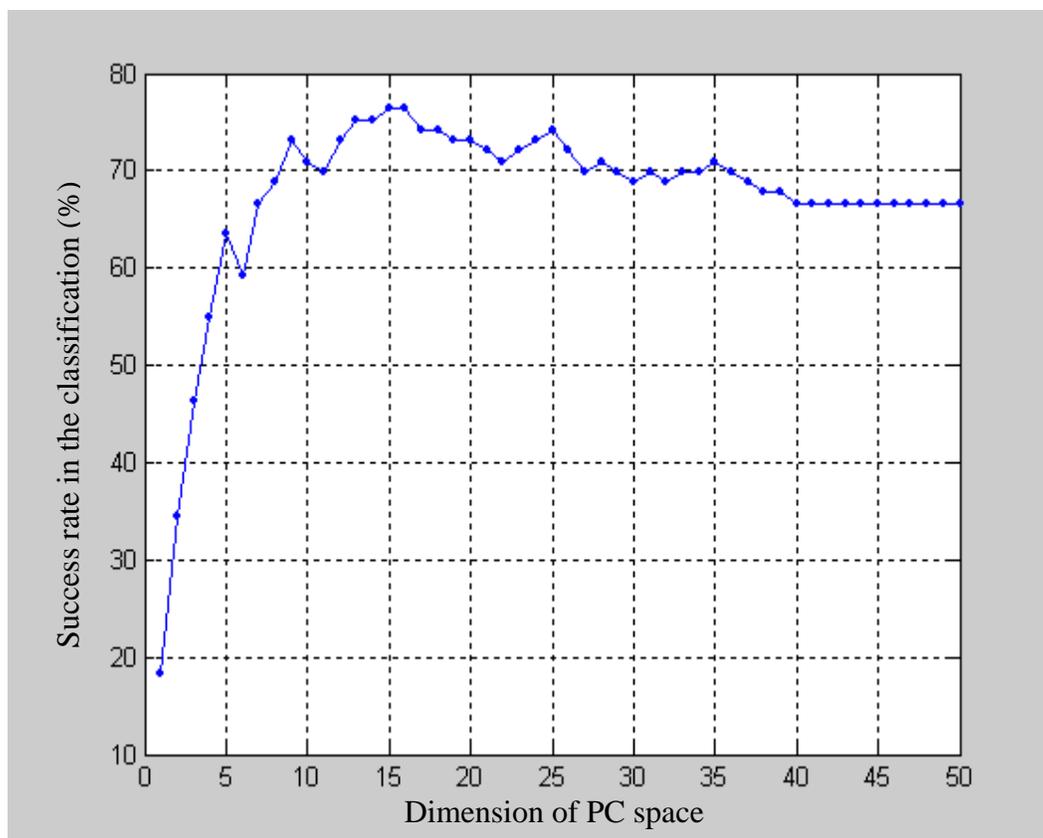
Figure 48. Results obtained from the re-assignment of the reference spectra of the database to each dye group when applying baseline correction/cutting/smoothing/scaling/second derivative. The percentage of correct assignments is plotted against the dimension of PC space. The highest success rate is achieved when working with the first 15 PCs.
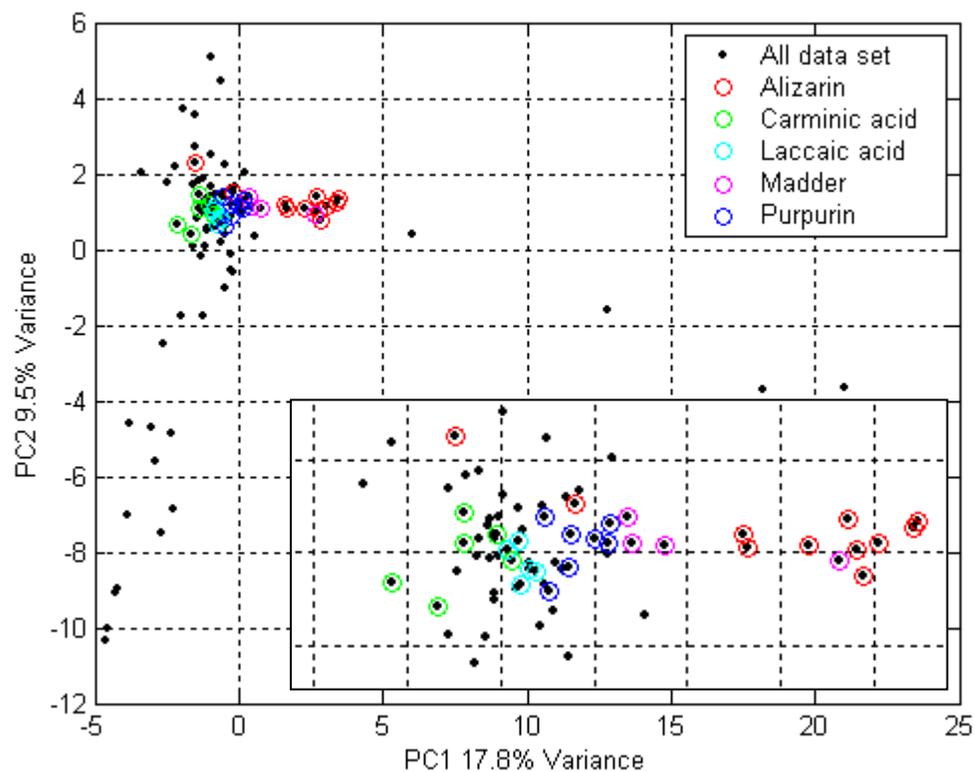
Figure 49. PCA score plot of the first two PCs of the reference data set.

Validation of the analytical procedures developed
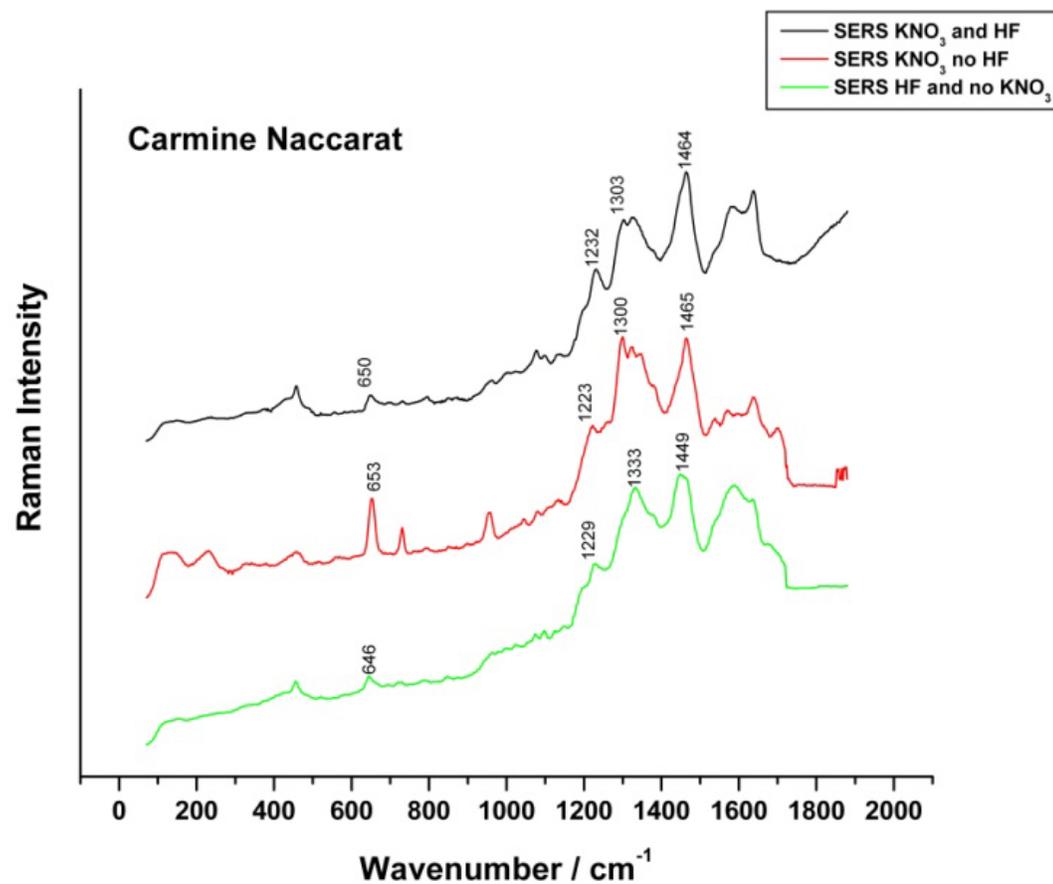
Figure 50. SERS spectra of carmine naccarat purchased from Kremer Pigments in different experimental conditions.
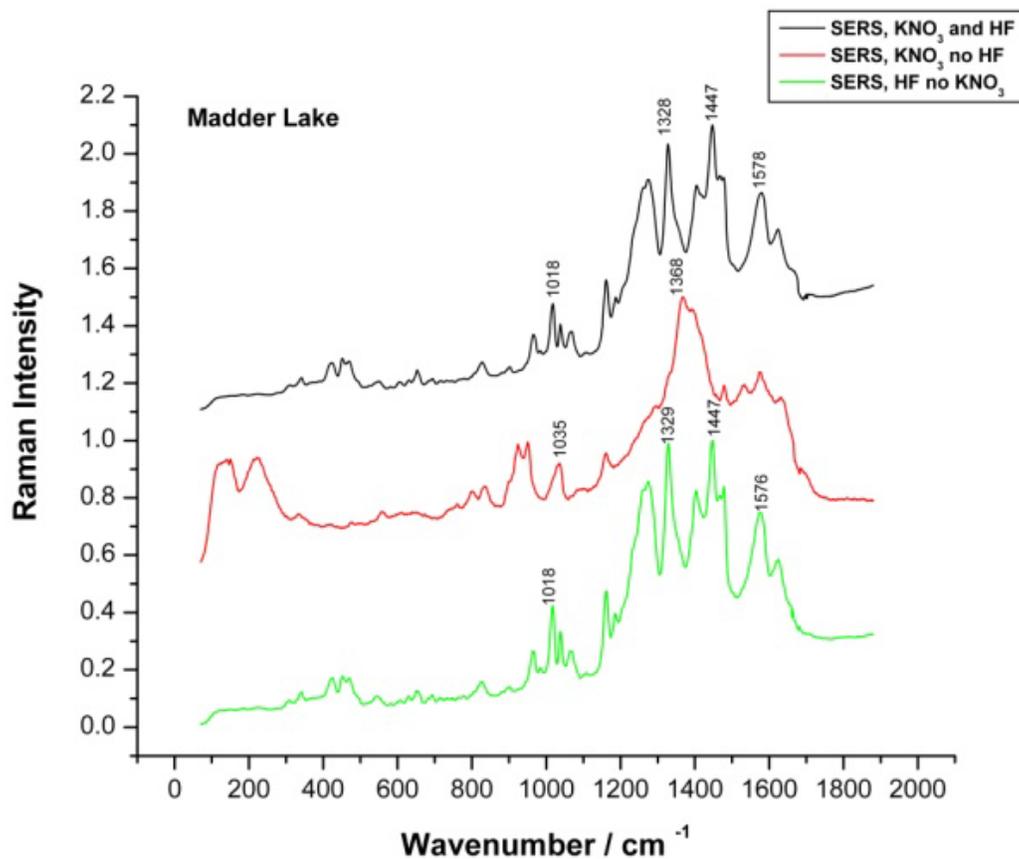
Figure 51. SERS spectra of madder lake purchased from Kremer Pigments in different experimental conditions.
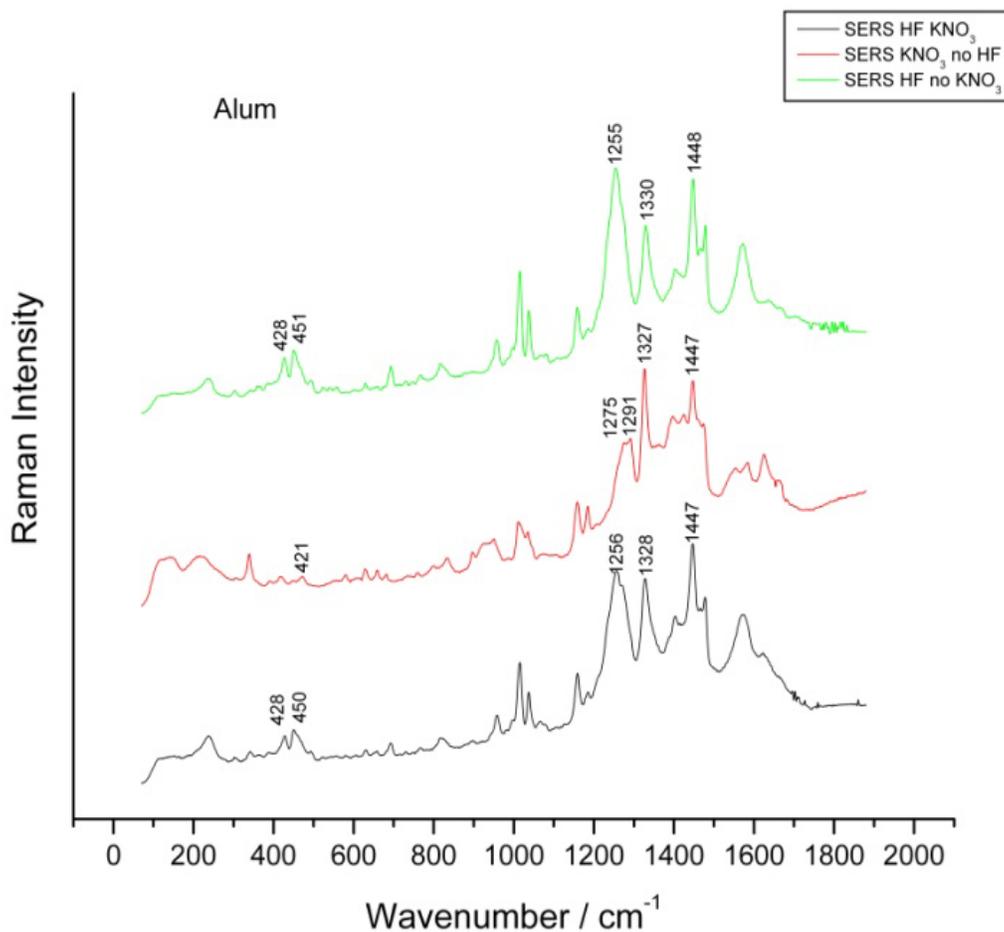
Figure 52. SERS spectra of madder on alum-mordanted wool in different experimental conditions.
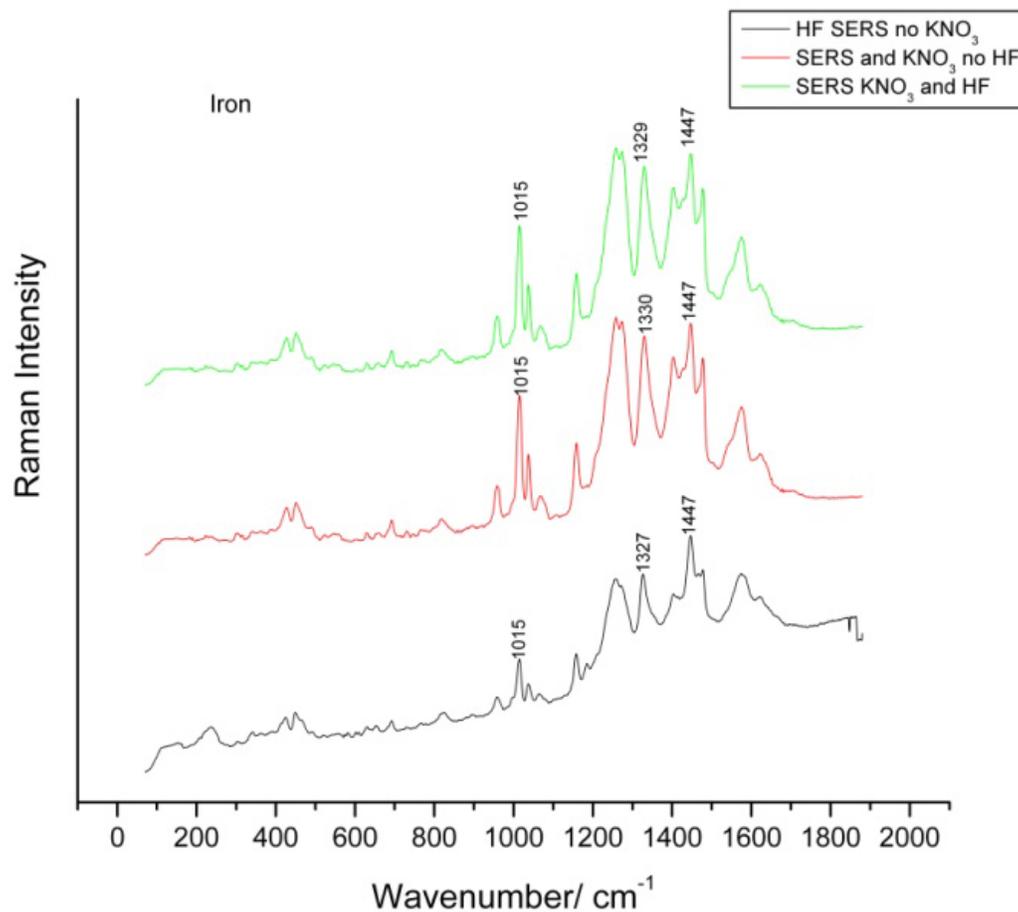
Figure 53. SERS spectra of madder on iron-mordanted wool in different experimental conditions.
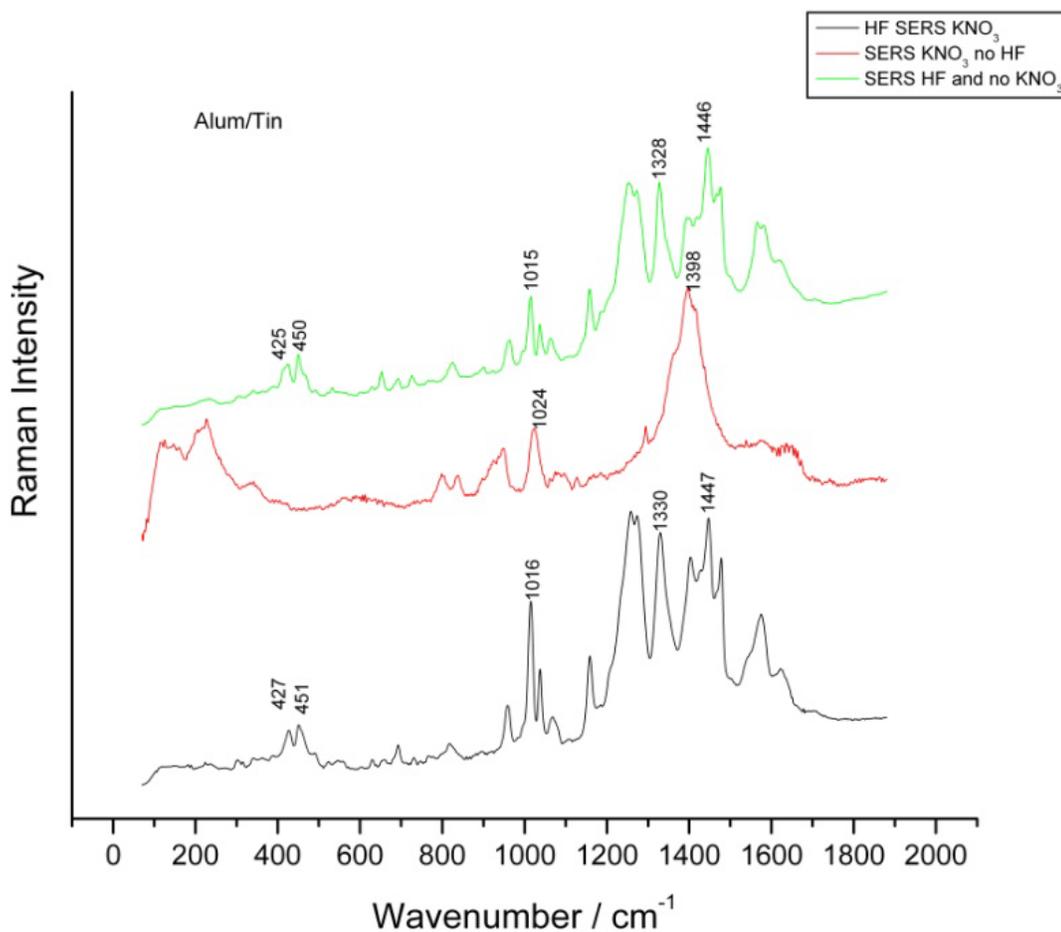
Figure 54. SERS spectra of madder on alum/tin-mordanted wool in different experimental conditions.
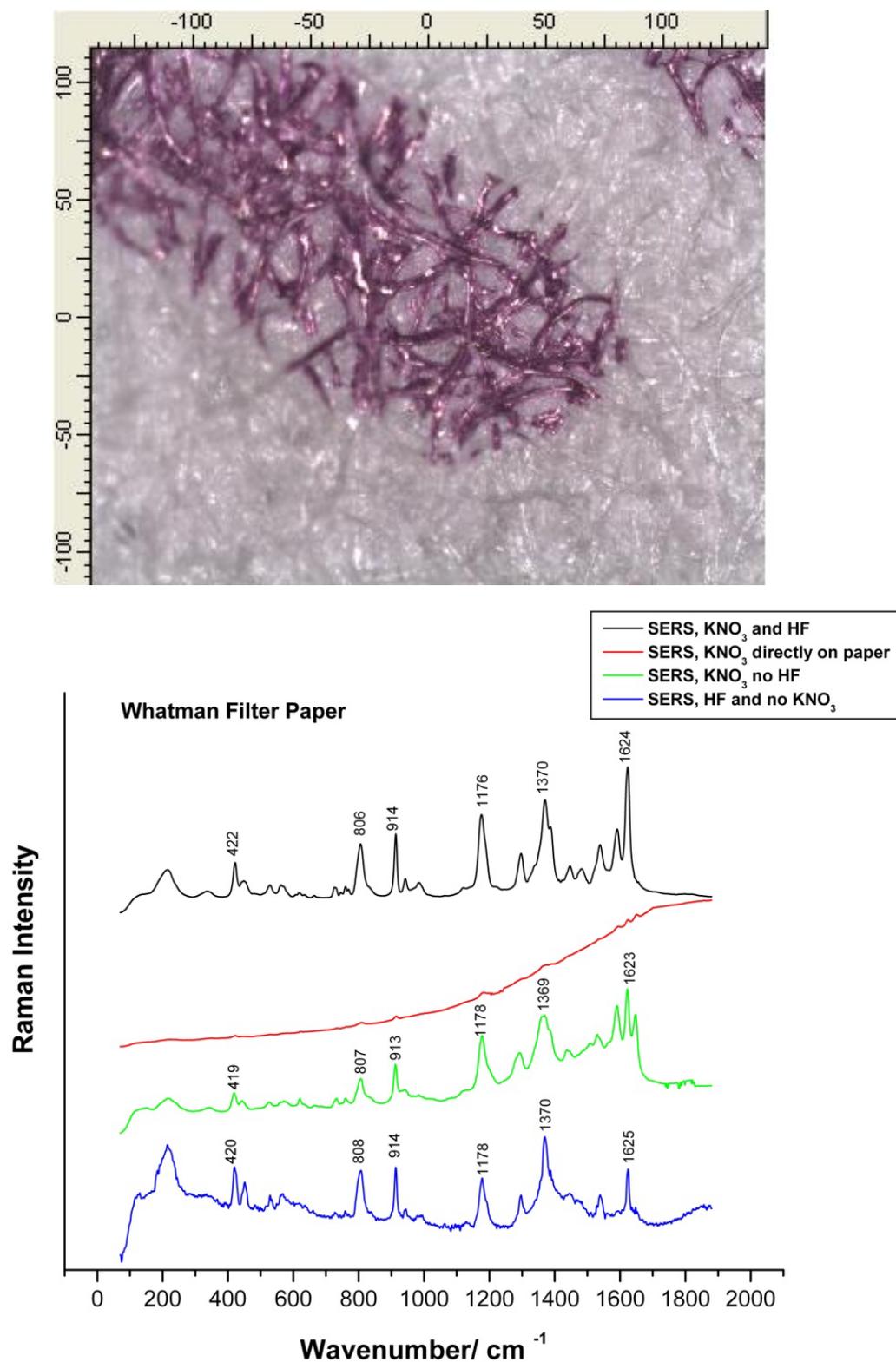
Figure 55. Gel pen ink on Whatman filter paper (top). SERS spectra of the gel pen ink acquired in different experimental conditions (bottom).
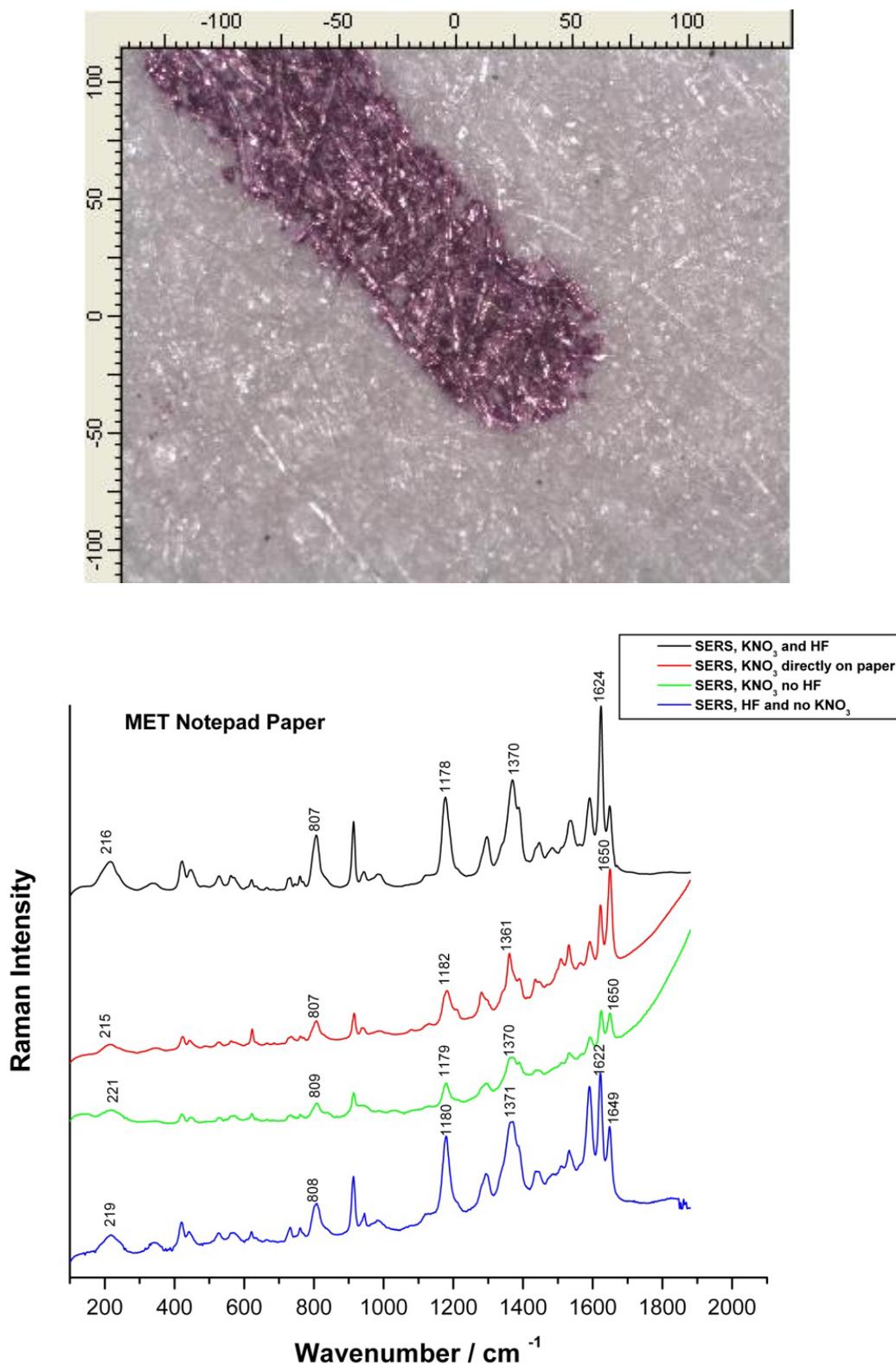
Figure 56. Gel pen ink on notebook paper (top). SERS spectra of the gel pen ink acquired in different experimental conditions (bottom).
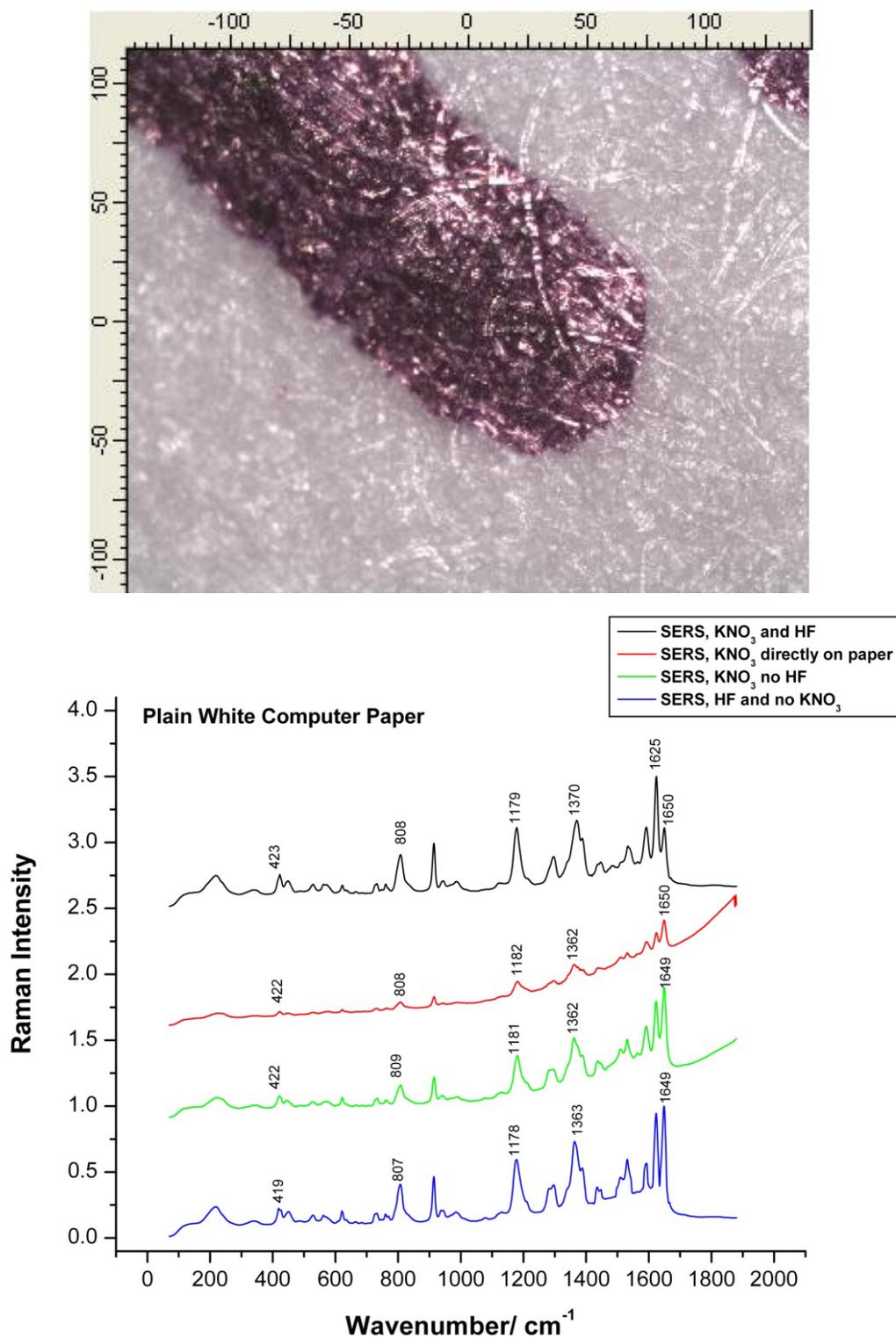
Figure 57. Gel pen ink on print paper (top). SERS spectra of the gel pen ink acquired in different experimental conditions (bottom).
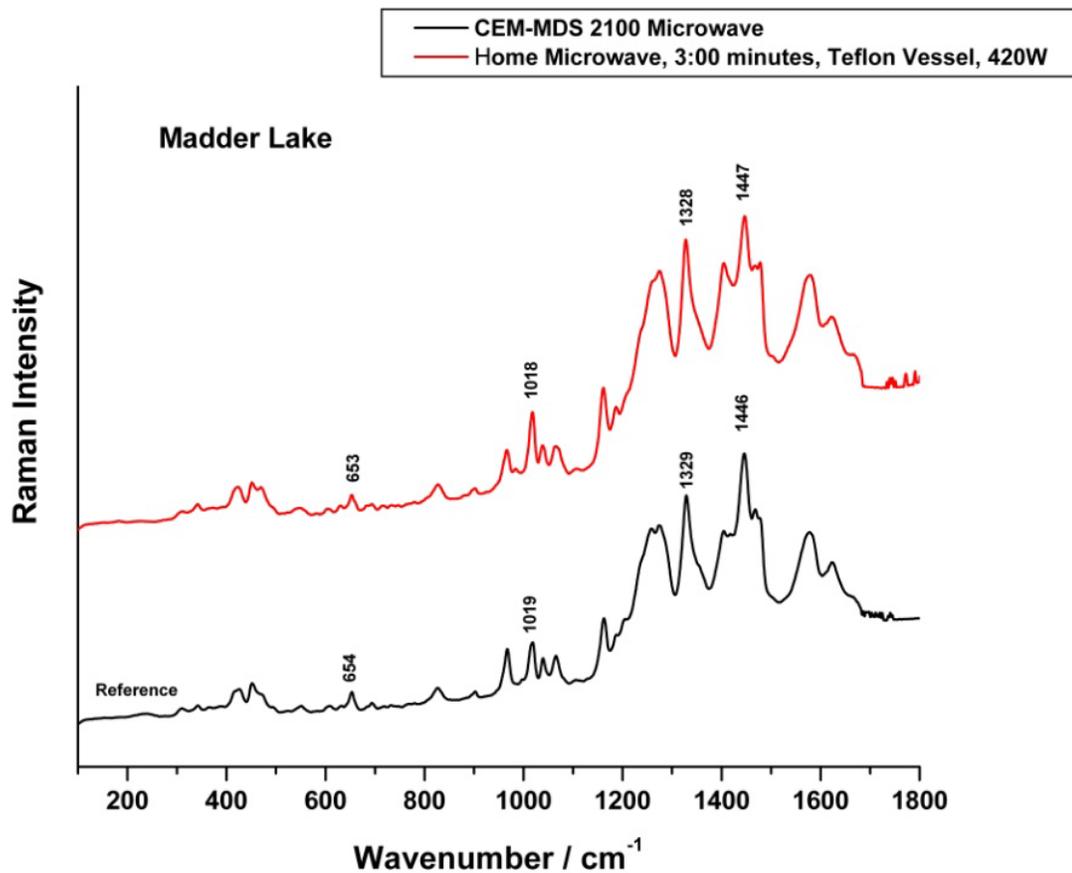
Figure 58. SERS spectra of commercial madder lake on Ag nanoparticles produced in the microwave digestion system and in a conventional home microwave oven.
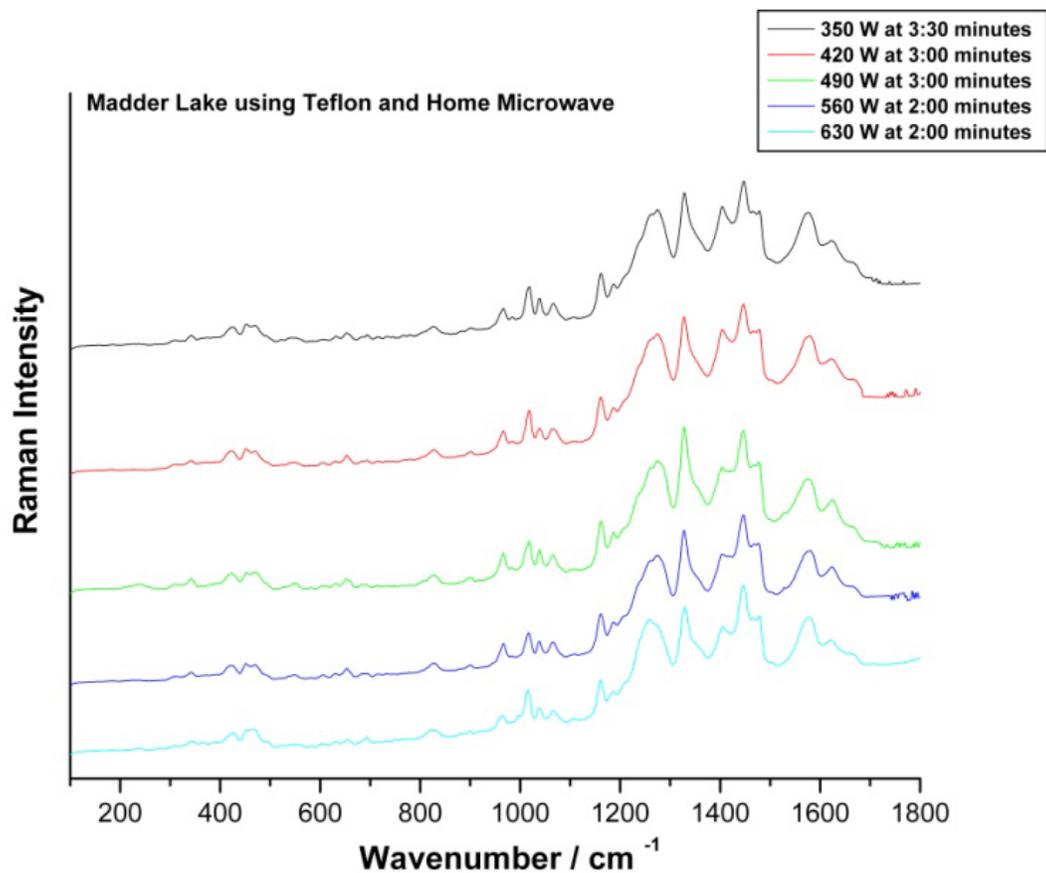
Figure 59. SERS spectra of commercial madder lake on Ag nanoparticles produced in a conventional home microwave oven at different powers and times, using a Teflon vessel.
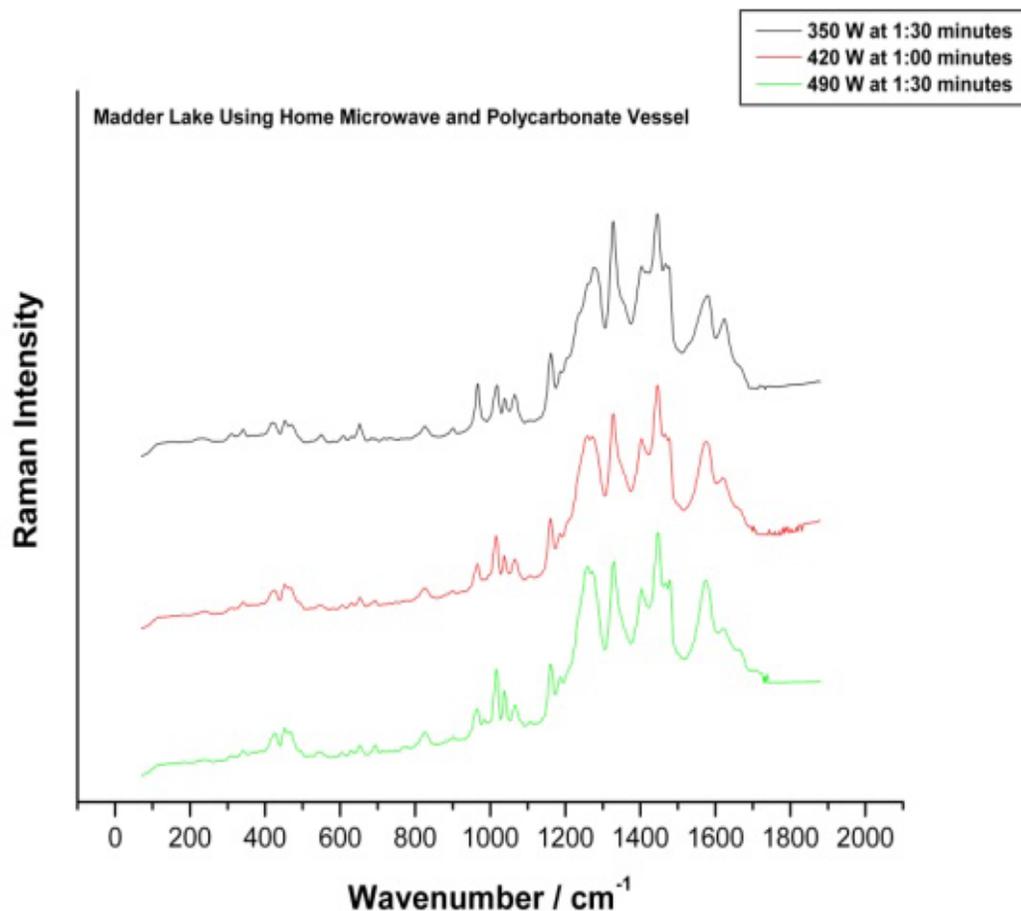
Figure 60. SERS spectra of commercial madder lake on Ag nanoparticles produced in a conventional home microwave oven at different powers and times, using a polycarbonate vessel.

IV. Conclusion

1. Discussions of Findings

The SERS spectral library has been expanded by the addition of new natural and synthetic dyes. Each substance was characterized by Raman and SERS using Ag colloids. For all the materials analyzed normal Raman results were compared with those obtained from SERS, and the conditions in which each technique may be of value were investigated. As an example, we would like to mention the FT-Raman, normal dispersive Raman and SERS characterization of 10 new synthetic organic colorants (Alizarine violet, Alizarine yellow GG, Brilliant yellow, Dioxazine violet, Fast green FCF, Isoindole yellow, Isoindolinon yellow, Isoindolon orange, Phthalo green yellowish, Thioindigoid red), all purchased from Kremer Pigments, that we recently undertook using the four excitation wavelengths (488, 633, 785, 1064 nm) currently available at the Metropolitan Museum.

One of the main issues in forensic applications is the ability to distinguish between two or more compounds of similar molecular structure, in order to determine with certainty which derivative of a compound is actually present in an unknown sample. In our work, special attention was dedicated to this problem. In this regard, we reported as an example our recent investigation of the main β-carboline alkaloids from the seeds of *Peganum harmala*, i.e. harmalol, harmaline, harmane and harmine. The extract obtained from *Peganum harmala* seeds was traditionally used in Western Asia for ritual purposes and as a dye for carpets and wool fabrics. Also, β-carbolines from Peganum harmala are of interest as a drug and have recently drawn attention for their significant antitumor activity. In addition, they are relevant for their antibacterial, anti-radialization, anti-trypanosome and neural activities, as well as mutagenic and co-mutagenic properties. We have thus performed a general FT-Raman, normal dispersive Raman and SERS characterization of harmalol, harmaline, harmane and harmine, in order to provide valuable data which could be used as a reference in identification studies. Raman spectra of these compounds were collected at the

museum by the use of four different excitation wavelengths, namely 1064, 785, 633 and 488 nm. FT-Raman spectra acquired at 1064 nm display excellent signal intensity and signal-to-noise ratio in all cases, and exhibit distinctive bands which could be used as a fingerprint for the identification of these four alkaloids. Harmalol was found to be characterized by a significant fluorescence emission when analyzed in the dispersive mode, regardless of the laser line employed for excitation. Instead, good quality Raman spectra were obtained for harmaline, harmane and harmine especially when using the 785 nm laser. When moving to lower excitation wavelengths, namely 633 and 488 nm, an increasing fluorescence emission was encountered for these three molecules as well. Excellent results were obtained by SERS at 488 nm, which provided remarkable signal enhancements and fluorescence suppression, allowing us to collect a high quality spectrum even for harmalol. It is interesting to point out that these four compounds have analogous molecular structures and thus provided SERS spectra which, in some cases, looked very similar to one another. However, besides those bands which are common to all the alkaloids examined, a few intense and isolated signals were observed exclusively for some of them. We have identified these signals and suggested they could be used as discriminant bands for specific identification of the corresponding substances in mixtures.

Particularly relevant was our work on phenethylamine (PEA) and substituted phenethylamines, a broad class of compounds that affect the central nervous system. The drugs act as stimulants (amphetamine, methamphetamine), psychedelics (mescaline), empathogens (MDMA, Ecstasy), or decongestants (pseudoephedrine) depending on the substitution on the aromatic ring and along the aliphatic chain. Three endogenous phenethylamines are the neurotransmitters dopamine and norepinephrine and the hormone adrenaline. The neurotransmitters are associated with the reward pathways of the brain and play a part in the addictive qualities of sympathomimetics while adrenaline is associated with the "fight or flight" response in animals.

The wide and varied supply of phenethylamines makes their investigation necessary. While methamphetamine and Ecstasy are still the most popular of

these types of compounds, recent events show that there will most certainly be new varieties encountered by law enforcement. Therefore quick detection and identification of this class of molecules is of on-going importance to hospitals and law enforcement agencies. The detailed results of our study are discussed in the following.

Phenethylamine

Normal Raman and DFT spectra were compared without applying a scaling factor to the DFT calculated spectrum. A scaling factor is some number (usually 0.9-1) that is applied to the wavenumber scale of the calculated spectrum so that the calculated spectrum can then be better correlated with the experimental spectrum. Intensity profiles and Raman shifts were consistent, providing unambiguous assignments without the necessity of a scaling factor. Predictably the DFT calculated bands appear at higher frequencies than the corresponding bands in the experimental spectrum. These differences are attributed to the DFT calculation that treats the molecule as a harmonic oscillator in a vacuum rather than the real-world state of the molecule as an anharmonic oscillator in solid form.

In the normal Raman spectrum of phenethylamine (PEA) distinct bands are seen at 593, 621, 743, 820, 1002, 1029, 1207, 1316, 1455, 1586 and 1605 cm^{-1}. The corresponding bands in the DFT spectrum appear at 606, 634, 743, 835, 1015, 1052, 1222, 1358, 1488, 1630, and 1652 cm^{-1}. All the intense bands of the molecule involve motion associated with in-plane and out-of-plane vibrations of the aromatic ring except for the band at 1455 cm^{-1} which corresponds to vibrations limited to the alkyl side-chain. The most intense band at 1002 cm^{-1} can be assigned to the ring breathing mode $\upsilon12$ with a noticeable contribution from C7-C8 stretching. Bands at 1029 and 1207 cm^{-1} are both assigned to $\upsilon8a$ and have a C1-C7 stretching contribution with the band at 1207 cm^{-1} having the higher displacement between C1-C7 along with a pronounced C7H2 wag that is not seen in the band at 1029 cm^{-1}. The band at 1316 cm^{-1} has a non-symmetrical C-H bend of the ring ($\upsilon19b$) with an in-phase twist from C8H2 and NH2. The

bands between 1100 and 1500 cm^{-1} are primarily due to deformations along the alkyl chain.

The SERS analysis of PEA was performed using 514, 633, and 1064 nm laser light. A comparison was made using the NR of PEA at 514 nm and the SERS spectra acquired at 514, 633, and 1064 nm. The SERS generated spectra appeared similar to the normal Raman spectra with some slight differences in intensities of some of the bands. The most intense band at 1002 cm^{-1} remains the most intense but the relative intensities of some of the other bands appear changed and/or shifted in location. Additional bands appear in the SERS spectra which are not observed in the NR spectrum. The NR bands at 743 and 820 cm^{-1} are now at 743 and 821 cm^{-1} in the SERS spectrum with a corresponding increase in relative intensities. These bands are associated with out-of-plane υ11 and in-plane υ6a vibrational modes along with strong C1-C7 stretching and NH2 wagging. The band at 782 cm^{-1} in the NR spectrum has its corresponding SERS band at 781 cm^{-1} but at slightly lower intensity. Bands appear at 1077 and 1095 cm^{-1} in the SERS spectrum which are not apparent in the NR spectrum. These bands appear in the region of the spectrum associated with stretching of the alkyl chain along with out-of-phase twisting of C7H2, C8H2, and NH2. The bands at 1263, 1394, and 1455 cm^{-1} in the NR spectrum appear at 1264, 1394, and 1457 cm^{-1} in the SERS spectrum but with increased relative intensity. The NR band at 1586 cm^{-1} now appears at 1583 cm^{-1} in the SERS spectrum and with slightly greater intensity. There appears some band broadening of the 1603 cm^{-1} NR band with the 1593 cm^{-1} NR band being undetectable in the SERS spectrum.

The increase in relative intensities of the part of the spectrum associated with vibrations restricted to the alkyl chain is a good indication that the molecule is attached to the Ag colloid through some portion of the aromatic ring rather than the chain. Phenethylamine responded well to 514, 633, and 1064 nm lasers although acquisition times were significantly shorter with the 514 nm laser.

Ephedrine

While this drug is more complex than phenethylamine a comparison of the two quickly reveals 10 easily assignable bands. The NR spectrum has distinct bands at 619, 762, 833, 1000, 1014, 1028, 1165, 1210, 1585, and 1601 cm^{-1}. These bands are assignable to bands 629, 745, 831, 1014, 1043, 1051, 1156, 1214, 1615, and 1652 cm^{-1} in the calculated spectrum.

In the NR spectrum (514nm) of ephedrine there is a band at 619 cm^{-1} which has a small shoulder at 630 cm^{-1}. These overlapping bands correspond to the overlapping bands seen at 629 cm^{-1} and 633 cm^{-1} of the DFT spectrum. There is extensive rocking of the entire side chain in both bands due to in-plane υ6b (619 cm^{-1}) and υ6a (630 cm^{-1}) modes of the aromatic ring. The band at 833 cm^{-1} also shows a small shoulder at 825 cm^{-1}. Though the intensities are reversed in the DFT spectrum, the bands can be assigned to 831 cm^{-1} and 860 cm^{-1} respectively. The band at 825 cm^{-1} has υ10b mode and rocking of all the atoms in the side chain. On the other hand, the band at 833 cm^{-1} has a weak υ6a mode but has the same strong rocking motions associated with the chain.

The SERS analysis of ephedrine was performed using 514, 633, and 1064 nm laser light. It was discovered that SERS spectra of ephedrine gave better results when using the 514 nm laser. It can be seen that the relative intensities of the bands change from the normal Raman to the SERS spectrum due to the interaction of the drug with the Ag nanoparticles. For example the bands at 619, 762, and 833 cm^{-1} all see a decrease in intensity when compared to the NR spectrum. These bands are also shifted slightly to lower frequency. The corresponding SERS bands appear at 615, 759, and 830 cm^{-1} respectively. These bands all involve both in-plane and out-of-plane deformations of the aromatic ring. The shift to lower wavenumbers along with the decrease in intensity suggests that the molecule interacts with the Ag through the aromatic ring. The region of the molecule associated with side chain vibrations sees a relative increase in intensity. The bands at 1056, 1170, and 1215 cm^{-1} all see intensity increases as does the band at 1014 cm^{-1}. All of these bands are associated with movement in the alkyl chain. The band at 1056 cm^{-1} has C7-O and C8-C10 stretching. The 1170 cm^{-1} band has stretching of the bonds throughout the chain

(C7-C8-N-C9 and C7-O). Stretching of the C1-C7 and C8-N are responsible for the band at 1210 cm^{-1} while the 1014 cm^{-1} band has C8-C10 and C9-N stretching.

3,4-methylenedioxymethamphetamine (MDMA)

Normal Raman (633 nm) and DFT calculated spectra of MDMA were compared without scaling and the spectrum generated with the 633 nm laser was used as the reduced fluorescence produced better results. In the NR spectrum distinct bands are seen at 528, 605, 624, 811, 919, 939, 1010, 1251, 1608, and 1631 cm^{-1}. The corresponding bands in the calculated spectrum appear at 563, 611, 646, 827, 928, 946, 1015, 1281, 1654, and 1671 cm^{-1}. All of the intense bands of the drug involve motion associated with in-plane and out-of-plane vibrations of the aromatic ring except for the band at 1010 cm^{-1} which corresponds to vibrations limited to the side chain. The most intense band at 811 cm^{-1} has mode υ1 along with in-plane breathing of the 5-membered ring. The second most intense band at 1251 cm^{-1} has in-plane ring breathing mode υ1 in addition to symmetrical stretching of C3-O and C4-O. Bands at 771 cm^{-1} and 1010 cm^{-1} are of equal intensity but are the result of vibrations in separate parts of the molecule. The 771 cm^{-1} band results predominantly from out-of-plane υ11 with some minor bending of the alky chain while the 1010 cm^{-1} band is exclusively due to stretching of C7-C8-C10 and C9-N. It is important to note that the Raman spectrum of MDMA does not contain the intense band at around 1000 cm^{-1} like the drugs previously described. That is because the methylenedioxy ring structure constains the υ12 mode.

The SERS analysis of MDMA was performed using 514, 633, and 1064 nm laser light. A comparison was made using the NR of MDMA at 633 nm and the SERS spectra acquired at 514, 633, and 1064 nm. The SERS experiment gave better results using the 514 or 633 nm laser. There are changes in intensity in several of the bands. For instance the band at 811 cm^{-1} is no longer the most intense. The bands between 1000-1500 cm^{-1} see great enhancement of their signals. For example the bands at 1074, 1193, 1352, 1383, and 1501 cm^{-1} are greatly enhanced by the Ag surface. The corresponding SERS bands now appear

at 1071, 1185, 1358, 1388, and 1500 cm^{-1}. The band at 1074 cm^{-1} is assigned C7_C8_C10 stretching, C9-N stretching, and C7H2 twisting. Stretching, rocking, and twisting vibrations are assigned to the broad band at 1193 cm^{-1} and scissoring vibrations are assigned to 1500 cm^{-1}. The band at 1383 cm^{-1} has C11H2 wagging. All of these bands involve movement of some portion of the side chain with the exception of the 1352 cm^{-1} band. The band at 714 cm^{-1} also sees an increase in intensity. This band is assigned to breathing of the 5-membered ring along with NH wagging. Excitation with the 633 nm laser appeared to be ideal for analyzing MDMA. Although excitation at 514 nm was satisfactory there was some fluorescence interference in the NR spectrum of MDMA which the use of Ag colloid eliminated.

SERS Comparison

By examining the SERS spectra, it can be seen that the three drug compounds can be easily distinguished from each other using the discriminant bands. The presence of a very intense peak at around 1000 cm^{-1} due to the symmetric breathing mode of the benzene ring is a good marker of a monosubstituted benzene like phenethylamine and ephedrine. The molecule that lacks this intense peak is MDMA due to the addition of the methylenedioxy ring that constrains breathing of the benzene ring. Phenethylamine has weak and medium bands at 590, 899, 942, and 1137 cm^{-1}. Some of these bands (815, 1132, and 1438 cm^{-1}) overlap with bands in MDMA but the presence of the intense peak at 1000 cm^{-1} distinguishes methamphetamine from MDMA. Finally, MDMA has many distinct bands as it is not a monosubstituted benzene. It has other intense bands that do not appear in any of the other spectra. Bands at 533, 605, 715, 775, 813, 930, 1372, and 1508 cm^{-1} can be used to distinguish MDMA from other compounds in this group. Therefore, looking first at the region between 1000 and 500 cm^{-1} then considering the rest of the spectrum, it is possible to make a positive identification of all three compounds.

2. *Implications for Policy and Practice*

With over 2,600 articles published on SERS since its discovery in 1974, it is clear that SERS is a generally accepted scientific technique. Thus, we believe that the basic criteria of Court Admissibility as put forth in *Frye*[28] are met by this technique. However, as indicated by the number of cases in which attempts at questioning techniques with a much longer track record than SERS were made[29], careful validation will be needed for the technique to be of use in forensic science.

Recently we have performed initial validation studies in order to ensure that the analytical procedures developed can be easily applied and reproduced by practitioners following step-by-step instructions. This study will be furthered and completed in the next future. The validation of SERS should be based upon the data obtained by CCNY, ORNL, NYPD, and MMA. Validation should include documentation to demonstrate that the application of SERS to forensic samples is a reliable, reproducible, and powerful technique. Also useful is the methodology delineated in the American Society of Crime Laboratory Directors Manual[30] and the ISO (the International Organization for Standardization) and IEC (the International Electrotechnical Commission) Manual[31]. A series of round robin samples should further support the use of SERS in the analysis of forensic samples. The initial data obtained showed that the methodology provides quality analytical data. Validation of the database will also be required. This research will result in providing greater discrimination between questioned and known forensic samples. The ability to provide greater discrimination between similar materials will result in a reduction of false inclusions, and an analysis that shows consistency between samples will result in a stronger association. The result of this research will provide the qualification and validation necessary for Raman spectroscopy to meet the standards delineated in *Frye* and *Daubert*. Calibration of the instruments should be performed prior to use by following individual instrument manufacturers recommendations and by following the American Society of Testing and Materials (ASTM) standard guide for calibrating Raman spectrometers, ASTM E1840 (1996)[32]. Quality Raman spectra depend on optimum instrumental parameters. Two Raman spectrometers have been used in

this study, a Renishaw Dispesive Micro-Raman spectrometer (750nm laser, CCD detector) and a Nicolet FT-Raman system (1064nm laser, InGaS and Ge detectors). Both spectrometers are be adjusted to obtain high quality spectra. The parameters for the Renishaw Raman instrument that can be adjusted include: the use of neutral density filters, the number of scans, exposure times, changing the laser spot size, and using different microscope objectives. The parameters for the Nicolet Raman instrument that can be adjusted include: the number of scans, the use of a neutral density filter, and the laser power. A reliable database will facilitate the identification of individual components present in complex matrices. The qualification and validation SERS and the data base(s) developed will be modeled to meet the standards delineated in *Frye* and *Daubert*[33].

This study provides analytical data on forensic samples that does not appear in the literature: in fact, no systematic library of SERS spectra is currently available. This research will result in providing greater discrimination between questioned and known samples. The ability to provide greater discrimination between similar samples will result in a reduction of false inclusions, and an analysis that shows consistency between samples will result in a stronger association.

3. Implications for Further Research

The work carried out in Phase II of the research project demonstrated that SERS is a valuable technique for the identification of organic colorants used in inks and for the dyeing of textile fibers, as well as for trace analysis of pharmaceuticals and of drugs of abuse. Analytical procedures for SERS of a number of representative dyes were developed, the core of a high quality spectral database was assembled as a proof of concept experiment, and innovative non-destructive approaches were investigated. To date our colorants database includes approximately 100 spectra of natural and synthetic dyes, and it is, to our knowledge, the first SERS spectral database ever assembled. The database is both chemically inclusive (as dyes belonging to the principal classes are represented) and spectrally comprehensive, since both SERS spectra obtained at different

wavelengths (488, 633, 785 nm) and normal dispersive Raman (488, 633, 785 nm), and FT-Raman (1064 nm) spectra are also included.

For SERS to become a routine technique, the database must be further expanded by the addition of spectra from dyes, pigments, inks, and controlled substances. In particular, as far as controlled substances are concerned, to the materials characterized so far we plan to add several as yet uninvestigated compounds such as heroin, cocaine, fentanyl, methadone, oxycodone, and natural as well as synthetic cannabinoids. Most cannabinoids involve an indole ring coupled by a keto link to naphthalene. In addition to natural cannabinoids, many synthetic versions are currently coming to market and are commercially available for research purposes through Cayman Chemical (http://www.caymanchem.com/app/template/Product.vm/catalog/). Such compounds are ideal for SERS study, since they possess two possible points at which they can be adsorbed onto a Ag nanoparticle, either the indole N or the keto C=O. We plan to select several compounds with the more common features for initial study to determine the spectrum for inclusion in our controlled substance database, as well as to define limits of detection and to enumerate the spectral features which can be used to distinguish these compounds. Also, our SERS experiments on opiates, cannabinoids and other controlled substances will be related and compared to "real-world" situations through the use of this technique on actual samples received by the NYPD Police Laboratory.

Beside controlled substances, the database will be expanded by systematic inclusion of new spectra from other materials of forensic interest, such as pen and printer inks, as well as pigments of several additional classes. Our initial work on tattoo inks will be furthered and finalized. Experiments with artificially aged written and printed samples will also be conducted using an Atlas Fade-o-meter, which is available at The Metropolitan Museum of Art for accelerated aging experiments.

The new spectra will be included in our searchable library, where they will be stored in specific reserved subsets. The final database will be structured into sub-libraries containing spectra from materials belonging to specific classes. This

has the advantage of more rapid and certain identification of trace substances which are suspected to be those contained in a certain sub-library. Spectra will be presented in our database together with information on the source, state (neat, extracted, type of support, etc.), on the SERS methodology used for spectra acquisition and extraction conditions.

As already pointed out, the possibility to distinguish between two or more compounds of similar molecular structure is of utmost importance in forensic science, as it can allow for certain identification of particular derivatives of a compound which are present in a sample. In our previous work on the β-carboline and protoberberine alkaloids, on the flavonoid, and on several controlled substances (morphine, codeine and hydrocodone, MDMA and analogs), we have been sensitive to this issue, and we have thus identified and clearly delineated discriminant bands in the SERS spectra. In our future work on the library we plan to continue this detailed investigation of chemical compounds of similar structure, in order to ease the ability of practitioners in the field to analyze spectra more quickly and with greater confidence in identification.

As far as library search approaches based on statistic techniques are concerned, to date we have evaluated their use only for the identification of colorants in a limited series of preliminary tests, as described above. Once a standardized library search procedure has been optimized, the same procedure will be also tested for the identification of different classes of compounds included in the database, i.e. inks, drugs and controlled substances.

Sample handling techniques must also be improved, with the goal of matching the non-destructive approach of conventional Raman spectroscopy. Two promising methods have been developed, gel transfer SERS (GT-SERS) and inkjet nanoparticle delivery SERS (IND-SERS), and their use in the analysis of written and printed documents should be carefully explored. As far as GT-SERS is concerned, we plan to expand on the range of gel systems studied, as the hydroxyacrylic gels – commercially available in bulk quantities as blanks for the production of soft contact lenses – are relatively expensive. In particular, we will evaluate methylcellulose and agar, which have been recently used in this context.

Finally, further attention should be given to the analysis of problematic ink systems, such as gel pen inks and black inks. We have received a set of 45 black gel pen ink samples, which are currently under investigation in the laboratories of the NYPD. We plan to develop extraction techniques for gel inks suitable for SERS analysis, and in particular we will refine the hydroxygel transfer method to improve its applicability to non-destructive sampling of gel inks.

In addition, given the promising results obtained from our initial TLC-SERS tests, we plan to carry out an extensive work for the application of the TLC-SERS protocol developed to the separation and identification of compounds of similar structure from different classes of mixtures relevant to forensics, e.g. controlled substances and drugs, in order to expand the study conducted thus far and ensure that the method developed could be massively exploited in the field. Experimental work will include tests with different TLC substrates and various solvent mixtures aiming to determine the best combination of stationary and mobile phase which can be used to achieve the most efficient separation of the different compounds from each forensic mixture. Furthermore, we plan to perform experiments on mixtures of reference materials at different concentrations to determine the limit of detection of the TLC-SERS technique for each substance of interest.

In the future, we will work to place SERS in an analytic flow of evidentiary chain, and to extend the Raman library, at least to include all commonly used compounds, and ensure that as new spectra are obtained the can readily be added to the database. We also plan to test the validation techniques and the library database. Future steps of the validation process will include further library search experiments designed to search the SERS spectra obtained from pure known dyes, reference dyes from different substrates, and dyes from real forensic samples against the database, in order to verify the effectiveness of the identification method and the correctness of matching criteria. We will also examine real samples obtained from crime scenes provided by Philip Antoci at the NYPD, these methods and techniques will be applied, when feasible, to the examination of related casework samples submitted to the NYPD crime

laboratory. We will carry out further validation studies using 45 black gel pen ink

samples kindly supplied to us by the Secret Service.

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VI. Dissemination of Research Findings

1. Presentations and Information Exchange

a) Forensic Applications of Raman Spectroscopy

One-day workshop at the 62nd Annual Scientific Meeting of the American Academy of Forensic Sciences, February 22, 2010, Seattle, WA.

Part 1: Scientific Presentations
Welcome & Introduction (**Patrick Buzzini**)
Raman spectroscopy & Criminalistics (**Patrick Buzzini**)
Introduction to SERS techniques with an emphasis on the analysis of trace evidence and questioned documents (**John Lombardi**).

b) Gordon Research Conferences - Noble Metal Nanoparticles Preparation, Modeling and Applications

Mount Holyoke College, June 20-25, 2010, South Hadley, MA
Chair: Teri W. Odom**;** Vice Chair: Michael J. Natan

Presentation Title - "Unifying the Disparate Strands of SERS" (**John Lombardi** , City College New York)

c) XXII International Conference on Raman Spectroscopy

Boston Park Plaza Hotel, August 8 -13, 2010, Boston, MA

Presentation Title - "A Unified Theory of Surface Enhanced Raman Scattering" (**John Lombardi**, City College New York)

Presentation Title - "Microanalytical and Non-Destructive Approaches for the Investigation of Works of Art by Surface-Enhanced Raman Spectroscopy (SERS)" (**Marco Leona**, the Metropolitan Museum of Art and **John Lombardi**, City College New York)

d) Invited Talk – PITTCON 2011 Conference

Georgia World Conference Center, March 13-18, 2011, Atlanta, Georgia

Presentation Title – "Applications of Surface-Enhanced Raman Spectroscopy to Trace Analysis of Crime Scene Evidence" (**John Lombardi**, City College New York)

e) **Invited Talk- State Key Laboratory for Supramolecular Nanostructures**

Jilin University, July 5, 2011, Changchun, Jilin, People's Republic of China

Presentation Title – "Spectral Blinking in single molecule Surface Enhanced Raman Scattering" (**John Lombardi**, City College New York)

f) **Invited Talk – Changchun University of Science and Technology Science Division Colloquium**

Changchun University of Science and Technology, July 6, 2011, Changchun, Jilin, People's Republic of China

Presentation Title – "Surface Enhanced Raman Spectroscopy: Theory and Applications" (**John Lombardi**, City College New York)

g) **241st ACS National Meeting**

Anaheim Convention Center, March 27-31, 2011, Anaheim, CA

Presentation Title – "Open Air and simple method to synthesize narrowly distributed and size tunable PbSe nanocrystals" (**Yi Pan** & **John Lombardi**, City College of New York) and

Presentation Title - "New, simple method to synthesize lead telluride (PbTe) nanocrystals" (**Yi Pan** & **John Lombardi**, City College of New York)

h) **TechConnect World Conference and Trade Show**

Hynes Convention Center, June 13-16, 2011, Boston, MA

Presentation Title – "A Simple, Open Air Method to Synthesize Monodisperse and Size Selective PbSe Quantum Dots" (**Yi Pan**, City College of New York)

i) **6th International Conference on Advanced Vibrational Spectroscopy**

June 12-17, 2011, Sonoma County, CA

Presentation Title - "Searchable databases for the identification of organic colorants using surface-enhanced Raman scattering (SERS): critical evaluation and possible approaches" (**Federica Pozzi**, the Metropolitan Museum of Art and the University of Milan)

j) **Invited Talk – State Key Laboratory for Supramolecular Nanostructures Jilin University**

Changchun, Jilin, People's Republic of China, July 4, 2011

Presentation Title - "Introduction to Surface-Enhanced Raman Spectroscopy" (**John Lombardi**, City College of New York)

k) **Invited Talk – State Key Laboratory for Supramolecular Nanostructures Jilin University**

Changchun, Jilin, People's Republic of China, July 5, 2011

Presentation Title - "Recent Results from City College" (**John Lombardi**, City College of New York)

l) **Trace Evidence Symposium 2011**

August, 2011, Kansas City, Missouri

Presentation Title - "Surfaced Enhanced Raman Spectroscopy and Databases for the Characterization of Dyes" (**Deanna O'Donnell**, City College of New York)

m) **6th International Congress on the Application of Raman Spectroscopy in Art and Archaeology**

September 5-8, 2011, Parma, Italy

Presentation Title - "Surface-enhanced Raman scattering (SERS) and Raman study of red and violet water-colour pigments from an original Winsor & Newton handbook dating to 1887" (**Federica Pozzi**, the Metropolitan Museum of Art and the University of Milan)

Presentation Title - "Comparative study of surface-enhanced Raman scattering (SERS) methodologies for the detection of organic colorants in cultural heritage investigations" (**Federica Pozzi**, the Metropolitan Museum of Art and the University of Milan)

n) **National Institute of Justice Conference**

June 18-20, 2012, Arlington, VA

Presentation Title - "Development of surface-enhanced Raman scattering (SERS) methods and databases for the identification of substances of forensic interest" (**Federica Pozzi**, the City College of New York)

o) **SERS in Art and Forensics Workshop** for the combined NYU (New York University) and CCNY Summer REU (research experience for undergraduates) programs, July 20, 2012, held at CCNY.

Presentation Title - "Introduction to surface-enhanced Raman spectroscopy" (**John R. Lombardi**, Professor, The City College of New York)

Presentation Title - "Surface-enhanced Raman spectroscopy and quantum chemistry calculations" (**Ronald Birke**, Professor, The City College of New York)

Presentation Title - "Surface-enhanced Raman spectroscopy in art and archaeology: the identification of organic colorants" (**Federica Pozzi**, Post-doctoral research associate, The City College of New York and the Metropolitan Museum of Art)

Presentation Title - "The chemical analysis of modern tattoo inks" (**Michelle Miranda**, Professor, The Farmingdale State College)

p) **Gordon Conference - Scientific Methods in Cultural Heritage Research**

Mount Snow Resort, West Dover, Vermont (**John Lombardi, Marco Leona, Federica Pozzi, and Pablo Londero**), July 29 - August 3, 2012. Chair: Marco Leona.

Presentation Title - "A compact OPO Raman microscope for wavelength-tunable multi-analytic microanalysis" (**Pablo Londero**, the Metropolitan Museum of Art)

Presentation Title - "Development of surface-enhanced Raman scattering (SERS) techniques and databases for the ultra-sensitive identification of organic colorants in works of art" (**Federica Pozzi**, the City College of New York)

q) **Society of Applied Spectroscopy -** (**Yi Pan, Syed Islam**)

December 5th, 2012, Rutgers, New York

Presentation Title - "Synthesis of semiconductor nanoparticles" (**Yi Pan**, the City College of New York)

Presentation Title - "SERS on semiconductor nanoparticles" (**Syed Islam**, the City College of New York)

2.   *Publications Resulting from this Award*

Published or Accepted for Publication:

1. "Size and Wavelength Dependence of the Charge-Transfer Contributions to SERS in Ag/PATP/ZnO Junctions", A.P. Richter, J.R. Lombardi, B. Zhao, *J. Phys. Chem.C,* 2010, 114, 1610.

2. "Improved Surface-Enhanced Raman Scattering Properties of TiO$_2$ Nanoparticles by Zn dopant", L. Yang, Y. Zhang, W. Ruan, B. Zhao, W. Xu, J.R. Lombardi, *J. Raman Spectrosc.*, 2010, 41, 721.

3. "Evaluation of Raman and SERS analytical protocols in the analysis of Cape Jasmine dye (*Gardenia augusta* L.)", M.V. Cañamares, M. Leona, M. Bouchard, C.M. Grzywacz, J. Wouters , K. Trentelman, *J. Raman Spectrosc.*, 2010, 41(4), 391.

4. "Identification of Organic Colorants in Fibers, Paints, Glazes and other media by Surface Enhanced Raman Scattering", Francesca Casadio, Marco Leona, John R. Lombardi, Richard P. Van Duyne, *Accts. Chem. Res.*, 2010, 43, 782.

5. "Excitation Profile of Surface-Enhanced Raman Scattering in Graphene-Metal Nanoparticle based Derivatives", X. Fu, F. Bei, X. Wang, S. O'Brien, J.R. Lombardi, *Nanoscale*, 2010, 2, 1461.

6. "Excitation profiles and the continuum in SERS: Identification of Fano lineshapes", J.R. Lombardi and R.L. Birke, *J. Phys. Chem. C,* 2010, 7812.

7. "A Charge-Transfer surface enhanced Raman scattering model from time-dependent density functional theory calculations", R.L. Birke, V. Znamenskiy, J.R. Lombardi, *J. Chem. Phys.*, 2010, 132, 214707.

8. "Study of Surface Enhanced Raman Scattering for Molecules Adsorbed on Cu-Doped TiO$_2$ Nanoparticles", X. Xue, W. Ji, Z. Mao, W. Ruan, B. Zhao, S. Ma, J.R. Lombardi, *Scientia Sinica Chimica*, in press, (2010).

9. "Surface Enhanced Raman Spectroscopy of Pyridine on CdSe/ZnBeSe Quantum Dots Grown by Molecular Beam Epitaxy", R. Livingstone, X. Zhou, M.C. Tamargo, J.R. Lombardi, *J. Phys. Chem. C*, 2010, 114, 17460.

10. "Scanned Chemical Enhancement of Surface-Enhanced Raman Scattering Using a Charge-Transfer Complex", W. Ji, X. Xue, W. Ruan, C. Wang, N. Ji, L. Chen, Z. Li, W. Song, B. Zhao, J.R. Lombardi, *Chem. Comm.*, in press (2010), DOI:10.1039/b000000x.

11. "The auxiliary determination of the binding site of berberine binding to human serum albumin by surface-enhanced Raman scattering", W. Zhang, Y. Zhao, X. Bai, G. Hui, J.R. Lombardi, D. Zhao, B. Zhao, *Vibr. Spectrosc.*, 2011, 55, 65.

12. "Surface-Enhanced Raman Spectroscopy for Trace Identification of Controlled Substances: Morphine, Codeine, and Hydrocodone", V. Rana, M.V. Cañamares, T. Kubic, M. Leona, J.R. Lombardi, *Journal of Forensic Sciences*, 2011, 56, 200.

13. "Quantum Confinement Effects on Charge-Transfer Between PbS Quantum Dots and 4-Mercaptopyridine", X. Fu, Y. Pan, X. Wang, J.R. Lombardi, *J. Chem. Phys.*, 2011, 134, 024707.

14. "Single Molecule SERS Spectral Blinking and Vibronic Coupling", J.R. Lombardi, R.L. Birke, G. Haran, *J. Chem. Phys. C.*, 2011, 115, 4540.

15. "Enhanced Raman Spectroscopy of Nanostructured Semiconductor Phonon Modes", S. Ma, R. Livingstone, B. Zhao, J.R. Lombardi, *J. Chem. Phys. Letters*, 2011, 2, 671.

16. "Surface-enhanced Raman spectroscopy of 4-tert-butylpyridine on a silver electrode", R. Livingstone, J.R. Lombardi, *J. Raman Spectrosc.*, 2011, 42, 1945.

17. "Potential Induced Changes in Neuromedin B Adsorption on Ag, Au, and Cu Electrodes Monitored by SERS", I. Ignatjev, E. Podstawke-Proniewicz, N. Gediminas, J.R. Lombardi, L. Proniewicz, *J. Phys. Chem. B*, 2011, 115, 10525.

18. "Metal-Semiconductor Contacts Induced Charge-Transfer Mechanism of Surface-Enhanced Raman Scattering", Z. Mao, W. Song, L. Chen, W. Ji, X. Xue, W. Ruan, Z. Li, H. Mao, S. Ma, J.R. Lombardi, B. Zhao, *J. Phys. Chem. C*, 2011, 115, 18378.

19. "Non-destructive Identification of Natural and Synthetic Organic Colorants in Works of Art by Surface Enhanced Raman Scattering", M. Leona, P. Decuzzi, T.A. Kubic, G. Gates and J.R. Lombardi, *Anal. Chem.*, 2011, 83 (11), 3990.

20. "Raman spectrum of monobromoindigo", H. Ajiki, F. Pozzi, L. Huang, L. Massa, M. Leona, J.R. Lombardi, J. Raman Spectrosc., 2012, 43, 520.

21. "Sample treatment considerations in the analysis of organic colorants by surface-enhanced Raman scattering", F. Pozzi, J.R. Lombardi, S. Bruni, M. Leona, Anal. Chem., 2012, 84, 3751.

22. "TLC-SERS study of Syrian rue (Peganum harmala) and its main alkaloid constituents", F. Pozzi, N. Shibayama, M. Leona, J. R. Lombardi, *J. Raman Spectrosc.*, (2012), in press.

23. "OPO Raman microscope for wavelength-tunable multi-analytic microanalysis", P. Londero, J. R. Lombardi, M. Leona, *J. Raman Spectrosc.*, (2012), in press.

24. "Singular thermochromic effects in dyeings with indigo, 6-bromoindigo, and 6,6'-dibromoindigo", O. Lavinda, I. Mironova, S. Karimi, F. Pozzi, J. Samson, H. Ajiki, L. Massa, K. Ramig, *Dyes and Pigments*, (2012), in press, DOI: doi.org/10.1016/j.dyepig.2012.07.014.

In Preparation:

"Winsor & Newton original handbooks: a surface-enhanced Raman scattering (SERS) and Raman spectral database of dyes from modern watercolor pigments", F. Pozzi, M. Leona, J. R. Lombardi, to be submitted to Studies in Conservation.

"Development of searchable databases for the identification of natural and synthetic dyes in works of art using surface-enhanced Raman spectroscopy (SERS)", F. Pozzi, S. Porcinai, M. Leona, J. R. Lombardi, to be submitted to Analytical Chemistry.

"Microwave-assisted synthesis of Ag nanoparticles for SERS applications", D. O'Donnell, F. Pozzi, D. Kirby, M. Leona, J.R. Lombardi, to be submitted to Analytical Chemistry.