PhD Course in Science for the Conservation of Cultural Heritage

Cycle XXVI

PhD Coordinator
Prof. Piero Baglioni

"Synthesis and Application of Ag-agar gel SERS substrates for the non-destructive detection of organic dyes in works of art"

CHIM/02

Tutor
Prof. Emilio M. Castellucci

PhD Candidate
Elena Platania

PhD Coordinator
Prof. Piero Baglioni

Years 2011/2013

To Diego,
My love, joy and strength.

To my family and my family-in-law,
For all their endless support

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Abbreviations

HPLC High Performance Liquid Chromatography
SERS Surface Enhanced Raman Scattering
MT-SERS Matrix Transfer Surface Enhanced Raman Spectroscopy
TLC Thin Liquid Chromatography
TLC-SERS Thin Liquid Chromatography Surface Enhanced Raman Spectroscopy
FT-IR Fourier Transform Infrared Spectroscopy
ATR-FTIR Attenuated Total Reflectance - Fourier Transform Infrared Spectroscopy
FORS Fiber Optic Reflectance Spectroscopy
UV-Vis Ultra Violet-Visible Spectroscopy
NIR Near Infrared Spectroscopy
LSPRs Localized Surface Plasmon Resonances
LSP Localized Surface Plasmon

Acknowledgements

I would like to acknowledge first of all my PhD tutor, Prof. Castellucci, for his important help and supervision during these three years, Dr. Cristiana Lofrumento for her dedication and her remarkable support and friendship, Dr. Marilena Ricci for her important advices and suggestions, Dr. Maurizio Becucci for his constant help and ideas for the progress of the project.

I would like also to acknowledge Dr. John R. Lombardi and Dr. Marco Leona for all their unequal support and dedication during the year spent in New York. Thank you to all the "Met" and City College friends and colleagues for their special friendship and constant support.

I would like to acknowledge my fiancé Diego for his endless love, patience and support, my mom Patrizia, my dad Angelo and my sisters, Alessia and Federica for their unconditional love and irreplaceable help. A warm thanks to my mother and father-in-law, Janice and Gaetano, for their support, my sister and brother-in-laws Lynds and Rodrigo for their endless helpfulness and friendship during my stay in USA, and Felipe and Roberta for their kindness.

This research has been founded by the Italian Ministery of Instruction, University and Research (MIUR) through the PRIN 2008 project (prot. 2008ZRSHHB), by the Department of Justice Office of Justice Programs, National Institute of Justice Cooperative Agreement 2009-DN-BX-K185 and partially by the Center for Exploitation of Nanostructures in Sensor and Energy Systems (CENSES) under NSF Cooperative Agreement Award Number 0833180.

Introduction

In the last few years a high attention has been paid by conservation scientists to the analysis and detection of organic dyes in works of art. These compounds, representing an essential part of the world's ecological and cultural heritage, have been playing a remarkable role throughout history, being one of the most important sources involved in economic and cultural exchanges among countries and continents. Organic dyes were extracted from natural sources of animal and vegetal nature, such as plants, shellfish, fungi, lichens and insects, widely employed for the dyeing of clothes, paintings, hair, skin or simply used as food colorants. Different procedures and methodologies were developed by our ancestors, for the treatment and the extraction of these compounds, whose use was in some cases a noteworthy indicator of the social status of the people using them.

From an analytical point of view, the detection of organic dyes in artworks is a quite challenging issue. As a matter of fact, these coloring substances, because of their high tinting power, were used in artifacts at very low concentration. Moreover, dyes were often used in mixtures with other dye compounds, and because of their organic nature, they are extremely susceptible to degradation processes. Among the most commonly consolidated techniques for the recognition of organic dyes on artworks, high performance liquid chromatography is one of the most powerful tools for the detection of these compounds. Moreover, currently non-destructive techniques such as UV-Vis reflectance spectroscopy and fluorescence spectroscopy have been widely employed as well for the detection of organic dyes on artworks.

In the last few years, the introduction of surface enhanced Raman spectroscopy (SERS), has allowed for the non-destructive and ultra-sensitive detection of organic dyes in works of art, due to the possibility of overcoming several disadvantages usually encountered when working by means of conventional dispersive Raman Spectroscopy, such as the fluorescence background, and providing promising and successful insights on the application of this technique in the field of art conservation.

In this work, a cutting-edge methodology has been developed, based on matrix transfer SERS methodologies (MT-SERS), dealing with the synthesis and application of a

nanostructured matrix made of agar gel coupled with silver nanoparticles, for the minimal invasive and non-destructive extraction of organic dyes on works of art. The presence of silver nanoparticles within the agar gel medium, has allowed for the ultra-detection of the analyte extracted, because of the SERS effect provided by surface plasmon resonances supported by the metallic nanoparticles. The methodology has been found to be extremely efficient for the recognition of organic dyes in textiles and panel paintings, without any detriment of the analyzed object. No fading or discoloring effects have been noticed on the investigated area after the micro-extraction process, confirming the high safety of this technique. In particular, the system has provided remarkable results when applied to sample of unknown chemical composition, revealing the identity of the analyte used for the dyeing process. The use of a natural polysaccharide such as agar-agar, makes the synthesis of the Ag-agar gel extremely safe for the operator and in agreement with the new eco-sustainability requirements for the accomplishment of green chemistry probes based on eco-friendly materials. For all the aforementioned advantages, Ag agar gel has been confirmed to be a highly safe and powerful analytical tool for the detection and the non-destructive micro-extraction of organic dyes in works of art.

Chapter 1

Organic dyes

1.1 Natural and synthetic dyes

The use of natural dyes in art is one of the oldest cultural activity of mankind. Natural coloring substances extracted from insects, plants, fungi, and animals (Fig.1.1) were widely employed since ancient times for the dyeing of textiles and in form of lake pigments for pictorial purposes. Before the introduction of synthetic dyes in the second half of the XIXth century, when Perkin synthesized serendipitously mauveine for the first time, natural dyes were the primary source of coloring compounds in our civilization, representing for this reason an essential part of the world's ecological and cultural heritage.[1-2]

Fig.1.1 On the left: Indian collecting cochineal with a deer tail by Josè Antonio de Alzate y Ramirez (1777); on the right: Illustration of cochineal insects from the Florentine Codex Book X (Biblioteca Medicea Laurenziana).

Dyes are organic compounds soluble in water and/or an organic solvent, in contrast with pigments, which are inorganic compounds insoluble in the paint medium (water, oil, etc.) and dispersed in the matrix. In particular, a lake pigment is a pigment formed by precipitation, usually by complexation with a metal ion, forming a dye on the surface of an inorganic substance[3].

The chemical structure of dyes is characterized by the presence of two important groups called **chromophores** and **auxochromes,** who influence the chromatic properties of the molecule. Chromophores are functional groups responsible for the dye's color. The most important chromophores are the azo (-N=N-), carbonyl (C=O), methine (-CH=) and nitro (NO_2) groups. An auxochrome, instead, is a functional group of atoms attached to the chromophore, which modifies the ability of the chromophore to absorb light. In particular, it increases the wavelength at which the light is adsorbed and intensifies the dye's absorption. Commonly encountered auxochrome groups include hydroxyl (OH) and amino (NH_2) groups.[4] According to the process involving the attachment of the dye to the fiber, we can distinguish the following classes of dyes.[5]

Acid dyes. This class of dyes is characterized by water-soluble anionic dyes with a good affinity for protein fibers, such as wool and silk. These dyes are sodium salts of a sulphuric, carboxylic or phenol organic acid usually applied to the fiber by means of an acid or a neutral dyebath. Acid dyes affix to fibers by hydrogen bonding, van der Waals forces and ionic bonding, and the strength of the bond is related to the tendency of the dye to remain dissolved in water over fixation to the fiber.

Basic dyes. These are cationic dyes containing basic groups such as $–NH_2$, -NHR, $-NR_2$ and their salts (mostly in the form of hydrochloride salts). The dyes in this category include malachite green, magenta, and para-rosaniline.

Direct or substantive dyes. Direct dyes are a long and well-established class of dyes deriving their name by the fact that they could be applied directly to the fiber, without any need of a fixation mordant process. In this case, the chemical bonds involved in the attachment process are essentially hydrogen bonds.[4]

Mordant dyes. This class of dyes requires the presence of a polyvalent metal ion (mordant), to help the dye being attached to the fiber. As a matter of fact many colorants do not show a good affinity for the textile fiber, which requires to be treated with a mordant

solution before being dyed. The use of mordants was crucial not only in terms of the attachment of the dye to the fiber, but they helped as well to improve the fastness properties of the dye and to provide a higher brightness and intensity of the color produced. The mordanting treatment can be performed in three ways; before the dyeing process (Pre-mordanting or onchrome), after the dyeing process (Post-mordanting or afterchrome) or added in the dye-bath itself (Meta-mordanting or metachrome). The attachment of the dye to the fiber occurs by chelation-covalent bond formation (Fig. 1.2). The chelate complex formed from a mordant dye and a metal is called lake. The chemical reaction between the mordant and the dye involves the formation of a covalent bond with a hydroxyl oxygen, (or a carboxyl oxygen) and a coordinate bond with another oxygen. In particular, mordants are able to increase the fastness of the dyes since the dye molecule is bonded to the fiber. Aluminum and iron salts were the most common traditional mordants, while copper, tin and chrome came into use much later. In rural areas where these metals were not easily available, plants were also used as mordants, such as the ones of the *Lycopodiaceae* family. Common dye mordants were also tannic acid, cream of tartar, urine, chrome alum, sodium chloride and certain salts of aluminium, chromium, copper, iron, iodine, potassium, sodium, and tin.

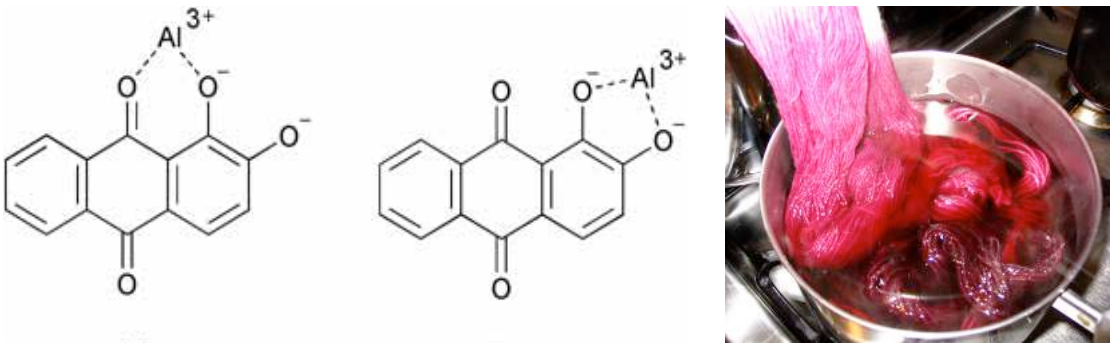

Fig.1.2 On the left: An example of mordanted complex, two possible coordination sites of the aluminum complexed alizarine; on the right: Dyeing process of a mordanted piece of textile.

Vat dyes. The term vat is referred to the large vessel (vat) where the dyeing process is carried out. These dyes are applied directly on the fiber, without any mordanting process, and they are generally insoluble in water. Vat dyes can be used only on cotton and not on silk and on wool. Being insoluble, these dyes are first of all converted into a water-soluble form by reduction in alkaline medium. The fabric to be dyed is dipped first in the solution of the reduced dye, then it is dried in air where oxidation takes place and colored fabric is obtained. Indigo is a dye belonging to this class.

Azo dyes. This class of dyes is characterized by compounds containing azo-groups (-N=N-) linked to methane or aromatic sp^2- hybridized C-atoms.

Sulphur dyes. These dyes are water-insoluble compounds characterized by disulfide (S-S) or oligosulfide ([S-S_n]) bonds between aromatic residues. In the classical dyeing process, dispersions of sulfur dyes are reduced with Na_2S at their S-S bonds. The soluble monomeric dye anions are then adsorbed and re-oxidized on the fiber.[6]

The introduction of synthetic dyes in the middle of the XIX^{th} century was of remarkable importance for the textile market. The new compounds, whose production and optimization were especially centered in France, Britain and Germany, were successfully introduced, providing very rapidly a wide range of new dyes. Synthetic dyes were found to be brighter, easier to apply, cheaper and purer with respect to the highly fugitive natural compounds, whose extraction procedure and treatments were extremely long, difficult and expensive. After the discovery of mauveine, a wide range of new dyes was introduced, such as fuchsine in 1859, a rich red dye, also known as magenta. Then followed synthetic indigo, discovered by Adolf von Baeyer in 1883, and synthetic alizarin in 1868, whose introduction made pink colors available at lower cost[7].

Even today, organic and synthetic dyes are still widely employed for many purposes, as food colorants, hair coloring, textiles, inks etc.; the analysis and detection of these materials is extremely remarkable since it provides important pieces of information about the cultural heirloom of humanity throughout history.

1.2 Molecular classes of natural organic dyes

As discussed before, natural dyes were the first source of colorant compounds used for artistic purposes by our civilization since the prehistoric period. These materials were extracted from a huge number of organic materials of animal and vegetal origin such as leaves, roots, berries, bark, woods, fungi, lichens and invertebrates. Dyes were treated and processed in several ways in order to obtain a high quality final product that could provide a good capability of being fixed on the support and a high fastness. In this paragraph is reported a brief outline concerning the history and chemical properties of molecular classes of the main natural dyes used in art and archaeology throughout history.

1.2.1 Anthraquinones

This class of dyes is one of the most common found in nature and widely used since ancient times. The main chromophores belonging to this molecular class are alizarin, purpurin, carminic acid, laccaic acid, kermesic acid and aloin. Alizarin and purpurin are the most abundant components of Madder, even if other components such as munjistin, pseudopurpurin, ibericin and lucidin are present at lower concentration. The dye, native to Europe, the Mediterranean and Western Asia, was obtained by depositing an extract of the roots from various plant of the *Rubiaceae* species (mainly *Rubia tinctorum*)(Fig.1.3), onto an insoluble base.[8] Mentioned by Pliny the Elder, Vitruvius, and other notorious Greek and Roman historians, the use of madder is reported since 1500 B.C. This dye is particularly substantive for cotton even if it was used for the dyeing of silk and wool as well. Madder produces a wide range of colors from yellow through orange to red, depending on the modality of extraction and the mordant used for the complexation of the dye to the substrate.

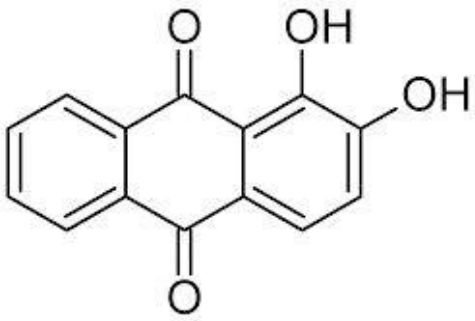

Fig.1.3 On the left: Roots of the plant species *Rubia Tinctorum* from where madder dye is extracted; on the right: chemical structure of alizarin, the main chromophore of madder.

Carminic acid is the main chromophore of the known bright purple dye Cochineal. Cochineal was extracted grinding the dried bodies of the female insects of the species *Dactylopius Coccus*, who grow on the Cactus plants in Central and South America. It was widely used by Pre-Columbian people for the dyeing of textiles of remarkable religious importance, as part of rituals and ceremonials. Before the introduction of the "american" quality of cochineal by Spaniards, Polish Cochineal was the unique source of madder used by Europeans, extracted from the insect species *Porphyrophora Polonica*. After the discovery of America the use of Polish cochineal ceased rapidly, displaced by the quality of cochineal imported from the American colonies.[9.10]

Kermesic acid was extracted from the dried bodies of the female insects of the genus Kermes that was extensively traded throughout the classical world, first of all by Phoenicians. Pliny the Elder pointed out that the use of this dye was reserved for the military costumes of generals. Lac dye also known as *Kerria Lacca*, whose main chromophore is laccaic acid, was particularly used in India and Southeast Asia. Another dye belonging to the molecular class of anthraquinones is aloe. This dye, extracted from the evaporated juice of the cut leaves of the botanical *genus* "Aloe" of the *Liliaceae* family, is featured by yellow-brown shades; it was used as glaze by XVIth century artists, in paintings and decorative arts, moreover it was widely employed as a textile dye during the XXth century.[11] Aloe is characterized by the presence of the anthrone C-glycosides aloin and aloe emodin, which are the major constituents of this colorant.

1.2.2 Flavonoids

Flavonoids are one of the major groups of yellow natural dyes and are secondary metabolites found in plants and characterized by the C_6-C_3-C_6 carbon framework. This group of natural dyes is divided into three classes depending on the position of the linkage of the benzopyrano moiety to the aromatic ring. We can distinguish flavonoids (2-phenylbenzopyrans), isoflavonoids (3-benzopyrans) and neoflavonoids (4-benzopyrans).[12] Subgroups of flavonoid class are: flavonols (morin, rhamnazin, emodin, xanthorhamnin, fisetin, quercetin), flavones (luteolin, apigenin, genistein), chalcones (carthamin, rottlerin, hydroxysafflor yellow A), neoflavones (catechin, 7,4'dihydroxyflavilium, dracoflavylium), biflavonoids (santalin A, santalin B) and neoflavonoids (brasilein, hematein). These dyes, being direct dyes, were applied directly to the fabric without any use of mordants.[13]

The two flavones luteolin and apigenin are the main chromophores of weld dye, even known as Dyer's rocket, an ancient yellow dye extracted from the roots of the plant of the species *Reseda luteola,* which is one of the oldest Mediterranean yellow dye plant used since the first millennium B.C.[14] Weld dye was commonly combined with indigo in order to produce the famous medieval green colors, Saxon Green and Lincoln Green. Morin, together with small amounts of kaempferol, is the main chromophore of old fustic dye. Imported to Europe around the XVIth century, it was extracted from Argentinian and Mexican trees of the species *Maclura tinctoria* and *Chlorophora tinctoria,* and from the leaves of *Psidium guajava*. Fisetin and quercetin are flavonols found in fruits, vegetables, leaves and grains. The flavonols rhamnazin, emodin and xanthoramnin, are the main chromophores of Stil de grain dye, also known as sap green. This dye, extracted from both ripe and unripe buckthorn berries, is characterized by a poor lightfastness. Safflower, extracted[14] from *Carthamus tinctoria* native of south-west and central Asia. Archaeological evidences show the use of this dye since the Early Bronze Age. The well-known dye Dragon's blood is a natural resin obtained from various trees of Liliaceae family.[3,8] The main chromophores of this dye are 7,4'dihydroxyflavilium, dracoflavylium. These dyes found many applications throughout history, such as dye in clothing, varnish for furniture, compounds for religious and magical rituals. Another neoflavone dye is Cate-

chu, an extract of acacia plants and related indian species, used in the past for dyeing cotton and calicos printing.

Sandalwood, whose constituents are the bioflavonoids Santalin A, Santalin B and Deoxysantalin, is extracted from the heartwood of Indian Red sanders tree (*Pterocarpus santalinus*). It was used since ancient times as dye for textiles, but also as medicine, hair coloring and wood polish. Brazilwood is a dye derived from species of the *genus Caesalpinia* and the tropical hardwood of the family *Leguminosae*. Its main chromophore is the neoflavonoid brasilein. Coming from Asia, it was traded in powder form and used as a red dye in the manufacture of luxury textiles. After the discovery of America, brazilwood was found to be extremely abundant in Brazil, allowing Portugueses to start a rich commerce of the dye holding the monopoly of brazilwood for three centuries. Another neoflavonoid dye is logwood, (whose chromophore is hematoxylin), derived from the flowering tree *Haematoxylum campechianum.* Logwood was considered a versatile dye and it was widely used for textiles and paper.

1.2.3 Naphtoquinones

Naphtoquinones are dark yellow pigments with a high range of pharmacological properties. Belonging to this class are the three main dyes henna, alkanet and walnut. These dyes are direct dyes since they don't need any mordant to be attached to the fiber. Henna is one of the[3] oldest mordent dyes used by our civilization for thousands of years. It is derived from the plant *Lawsonia inermis* that contains the naphtoquinone lawsone as a glycoside. Although its use was mainly ascribed to the dyeing of hair and body, it could have been employed as textile as well. Alkanet, whose main chromophore is alkannin, was extracted from the roots of *Alkanna tinctoria*.[15] It was used mainly to dye textiles, food products and cosmetics. Walnut dye was derived from green walnut shells and leaves of *Juglans regia* and it was traditionally used as a natural dye for clothing and fabrics, particularly ink and as ink, but even as coloring agent for cosmetics, food and hair.

1.2.4 Orchil dyes

This class of dyes comprehends compounds extracted from lichens whose major source comes from the archil lichen *Roccella tinctoria*. The extracted compound is orcinol, which is converted into orcein by means of ammonia and air. The bright purple dye, mentioned by Theophrastus and Pliny the Elder as being brighter than Tyrian purple - even if unlike the mollusk dye it was extremely fugitive - was widely employed by Phoenicians, Greeks and Romans. Orchil dyes were introduced in the Western Europe around the XVth century by a Florentine noble family who started the manufacture of "oricello" in Florence; for this reason the family took the name of Oricellari, later corrupted to Ruccellai.[16,17] Lichen dyes have a particular affinity for animal fibres, but they were used as colouring compounds for wood, leather, marble, wine and food as well.

1.2.5 Carotenoid

Carotenoids are lipid soluble organic dyes found in the photosynthetic apparatus of plants. They can be divided in carotenes, containing only carbon and hydrogen and xanthophylls, made up of carbon, hydrogen and oxygen.[18] This class of dyes is one of the most complex, with around 750 different structures identified.[19] Among the most important carotenoids used throughout history as dyes for textiles saffron and annatto are the most well-known. Saffron is extracted from the stigma of *Crocus sativus*, and its main chromophore is crocetin. It was an important and expensive dye in ancient Greece, reserved for people of high social *status*; used by Romans to color the marriage robe it was even used as medicine, food and perfume.[20] Annatto is the carotenoid based dye extracted from the seeds of the tropical tree *Bixa orellana*, whose main chromophore is bixin. It was widely employed by Aztecs, who used it as a textile, body dye and as food colorant. Annatto was introduced in Europe after the 16th century, but it never became commercially important because of its low light fastness.[21] Recent studies have shown the antioxidant properties of this carotenoid dye.[22, 23]

1.2.6 Curcuminoids

These compounds are natural phenols extracted from plants of the ginger family and characterized by a bright yellow color. The most important dye belonging to this class is turmeric, extracted from the rhizomes of the plant *Curcuma longa*. Turmeric, described by Marco Polo as a "vegetable with the properties of sapphron", has been extensively used as a dye, medicine and flavoring spice since 600 B.C.[24] This dye presents a poor light fastness, as a matter of fact it is extremely fugitive, moreover in presence of alakalis it turns his color into red-brown.[25]

1.2.7 Indigoid dyes

Indigo, used by our civilization for over 4000 years, is one of the most stable organic colorant. It was extracted from species of the tropical *Indigofera* plant, while in medieval Europe it was extracted from the woad plant, *Isatis tinctoria*. The leaves of the plant were soaked in water and fermented in order to convert the glycoside present into the blue dye indigotin.[26] Tyrian purple, whose main chromophore is 6,6'-dibromoindigotin, was known since Phoenicians as early as 1570 B.C. In the Mediterranean, before the advent of Christianity, a whole dyeing industry arose around Tyrian Purple, a natural dye extracted from the mucous glands of some species of *Purpura* and *Murex* shellfish. The shells were crushed to extract this fluid, which only turns purple once it has been applied to the fiber and exposed to light and oxidation with the air. This dye was one of the most expensive since a huge number of shellfish was required for the obtainment of few grams of dye.[27]

1.3 Analytical approaches to the study of organic dyes in artworks

The analysis and detection of dyes is of great importance for a better understanding of several matters related to these compounds, such as craftsmanship methodologies, trade routes, cultural and religious issues that accompanied people in all over the world throughout history. Tracing the use of these compounds has provided remarkable pieces of information helping conservators and restorers in detecting forgeries and falsifications. Unfortunately, the study of these substances is an extremely challenging issue. First of all, due to their high tinting power, dyes were used at very low concentration in artworks, issue that implies the application of extraction methodologies for their detection. Moreover, dyes were often used in mixtures, incorporated in complex matrixes such as paint layers or attached to fibers through a mordant. Analytical techniques such as High Performance Liquid Chromatography (HPLC) have been used as a powerful tool for the discrimination of the single components in a mixture. Despite the high specificity and sensitivity of this analytical tool, in the specific case of artworks analysis, HPLC requires big samples for the analyses (1-2mm of a fabric thread), issue that makes the technique not always applicable because of the lack of samples available to be analyzed via a destructive way. An additional problem related to the chemical characterization of these compounds concerns the extreme susceptibility to degradation of organic dyes, due to their organic nature. Deterioration processes play an important role in terms of dye's detection since they can completely change the original appearance and the molecular structure of the primary organic compound with respect to the final degradation products, making the detection of the original coloring species extremely difficult. Among the most common degradation processes affecting natural colorants, the photo-oxidation is one of the most frequently observed; examples reported in literature regard the debromination of indigoid dyes[28] and the production of 2,4-dihydroxybenzoic acid and 2,4,6-trihydroxybenzoic acid deriving from flavonoid degradation.[29] It is worth to be mentioned that degradation products can sometimes be used as molecular markers for the identification of the original dye source.[29] Non-destructive techniques, such as UV-Vis reflectance spectroscopy have allowed for the characterization of many pictorial materials, such as the pre-Columbian pigment Maya blue[30] and several natural dyes[31,32] in silk[33] and Japanese prints[34]. In par-

ticular, the application of Fiber Optic Reflectance Spectroscopy (FORS), has been proven to be useful for the non-invasive study of natural dyes in Japanese paintings[35] and pigments in frescoes[36] glass[37] and renaissance Italian paintings.[38] Despite the non-destructiveness of the aforementioned techniques, electronic methods are anyway strongly affected by matrix interference, show poor specificity, weak wavelength resolution and weak fingerprinting ability in comparison with vibrational techniques such as Fourier Transform Infrared Spectroscopy (FTIR) or Raman spectroscopy. Vibrational techniques, characterized by a high level of specificity and molecular fingerprinting, have been widely employed in the last decades for the characterization of organic and inorganic materials in artworks. Infrared spectroscopies have allowed for a wide characterization of binding media used in artworks, such as albumin, egg yolk, linseed oils, etc., and of natural organic dyes, such as madder, present in silk, cotton wool and linen. In particular, FTIR, involving a more easily preparation of the sample in conjunction with a smaller amount of sample required, has been preferred with respect to traditional IR spectroscopy, whose sample manipulation after the removal from the artifacts, is quite laborious.[39]

Normal dispersive Raman, although successfully applied for the characterization of inorganic pigments in illuminated manuscripts,[40] paintings,[41] statues,[42] enamels[43] and archaeological objects[44], is not as suitable for the analyses of organic dyes due to the strong fluorescent background obscuring the weak Raman signal, which can not be overridden even when using a laser line at a higher wavelength. In the last few years the introduction of Surface Enhanced Raman Spectroscopy (SERS), has proven to be a suitable and powerful tool for the characterization of almost all the molecular classes of dyes. As a matter of fact, this technique, based on the enhancement of the Raman scattering when the analyte is adsorbed on a nanostructured metal surface, is extremely sensitive, able to provide spectra of the single molecule. Moreover, it offers the possibility of investigating quite small samples, of the order of the micrometer. For this reason, it is a quite promising analytical tool for the ultra-detection of analytes present in trace amounts in the objects to be studied. In the next chapter, a brief outline related to the state of the art in the application of surface enhanced Raman spectroscopy will be illustrated.

Chapter 2

Surface Enhanced Raman Spectroscopy

2.1 Discovery and Principles of Surface Enhanced Raman Spectroscopy

Surface enhanced Raman spectroscopy (SERS), is a technique based on the amplification of Raman scattering by several orders of magnitude, when a molecule is adsorbed on a nanostructured metal surface. The discovery of the SERS effect was achieved in the early ’70, when Fleischmann showed how the pyridine Raman spectrum might be enhanced by several orders of magnitude by putting the molecule in contact with a roughened silver electrode.[45]

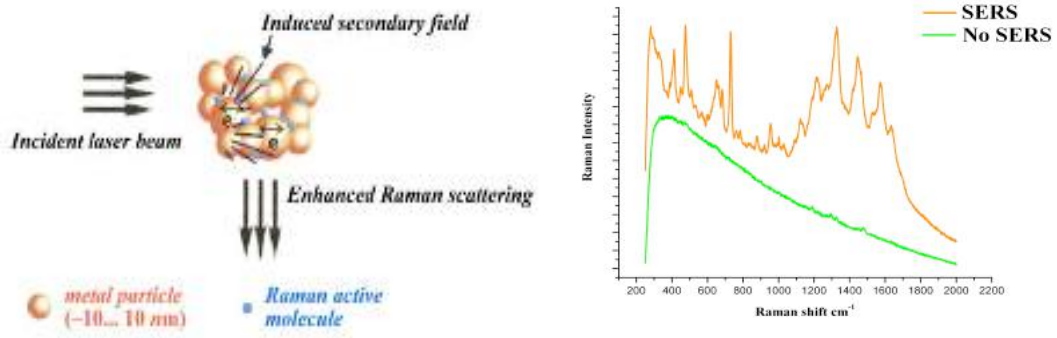

Fig.2.1 On the left graphical illustration of the SERS effect; on the right: comparison between the SERS spectrum of carminic acid in absence (green line) and in presence (orange line) of SERS enhancement. The SERS enhancement dramatically quenches the fluorescence background.

In 1977 Jean Marie and Van Duyne proposed for the first time a theoretical approach able to explain the observed enhancement phenomenon, introducing the concept of electro-magnetic effect, while Albrecht and Creighton, introduced subsequently the concept of

charge transfer SERS, whose theories are still matter of debate in literature. Starting from that moment several analytical improvements were triggered in the field of SERS, providing a plethora of applications in many fields of research, such as forensics, biochemistry, and recently in the field of conservation of cultural heritage. This analytical tool presents many advantages with respect to normal dispersive Raman, such as the suppression of the fluorescence background, the higher spectral specificity and the higher sensitivity that make this technique particularly suitable for the ultra-detection of molecular species found at very low concentration. Evidences of the possibility of performing single molecule SERS (SM-SERS) detection, whose enhancement factor (EF), is around 10^7-10^8 orders of magnitude, [46, 47] have been widely reported in literature[48, 49] revealing the great potential of this powerful application.

The SERS effect is based essentially on two main contributions. The first contribution, known commonly as electromagnetic contribution (EM), [50, 51, 52] is related to the existence of surface plasmon resonances (SPR) due to the collective oscillation of the electrons in the conduction band of the metallic substrate. The EM enhancement is a consequence of electromagnetic excitation of the SPR of nanostructured metals.[53] In particular the incident electromagnetic field excites a collective oscillation of the conduction electrons of a noble metal (silver, gold), producing enhanced fields close to the surface. The Raman scattering intensity is proportional to the square of the local electromagnetic field $|E_0|^2$ which is enhanced by the presence of the metallic surface. For a small metallic sphere the electromagnetic field intensity at the surface of the nanoparticle is given by:

$$|E_{out}|^2 = 2E_0^2/|(\varepsilon_{in} - \varepsilon_{out})/(\varepsilon_{in} + 2\varepsilon_{out})|^2$$

where ε_{in} and ε_{out} are the dielectric constants of the metal nanoparticle and external environment, respectively. The equation shows that the maximum enhancement occurs when $\varepsilon_{in} \approx -2\varepsilon_{out}$, a resonant condition satisfied in the visible range for Au and Ag.[54]

The other contribution, identified as the chemical contribution [54, 55], is based on the charge transfer (CT) resonance, which is a particular resonance Raman scattering (RRS)

process. When the sample is irradiated with an exciting radiation whose energy corresponds to that of the electronic transition of the molecule, we are in presence of resonance Raman scattering (RRS); under resonance conditions, the intensity of some Raman bands may be selectively enhanced by several orders of magnitude.[56] In particular, the CT phenomena, who takes place only if the molecule is chemisorbed on the metal substrate, involves the transfer of one electron from the Fermi level of the metal to an unoccupied molecular orbital (LUMO) of the adsorbate and viceversa.[50]

A third contribution, playing an important role in terms of SERS enhancement, has been introduced and described by Lombardi et al.,[57] concerning the resonances within the adsorbed molecule itself. This contribution is particularly important since it is related to the observation of the single molecule effect.

A unified expression that takes into account the aforementioned three contributions, has been recently published by the same authors.[57] This equation, which describes the SERS contributions involved in the case of a single spherical particle, is reported below:

$$R_{IFK}(\omega) = \frac{\mu_{KI}\mu_{FK}h_{IF}\langle i|Q_k|f\rangle}{((\varepsilon_1(\omega)+2\varepsilon_0)^2+\varepsilon_2^2)(\omega_{FK}^2-\omega^2+\gamma_{FK}^2)(\omega_{IK}^2-\omega^2+\gamma_{IK}^2)}$$

The surface-enhanced Raman intensity is proportional to the square of the polarizability and therefore, for a single dominant term, to $|R_{IFK}(\omega)|^2$. In this expression I, F, and K refer to the ground state, a charge-transfer state, and an excited molecular state of the molecule-metal system, respectively. The first denominator $(\varepsilon_1(\omega)+2\varepsilon_0)^2+\varepsilon_2^2)$ is due to the plasmon resonance at $\varepsilon_1(\omega) = -2\varepsilon_0$, where ε_1 and ε_2 are the real and imaginary parts of the Ag dielectric constant and ε_0 is the real part of the dielectric constant of the surrounding medium. The second resonance, which may be potential (Fermi energy)-dependent and represents charge-transfer resonance $(\omega_{FK}^2-\omega^2+\gamma_{FK}^2)$ occurs at $\omega = \omega_{FK}$, and the third $(\omega_{IK}^2-\omega^2+\gamma_{IK}^2)$ represents a molecular resonance at $\omega = \omega_{IK}$.

The geometry of the molecule attached to the metal nanostructured surface is of remarkable importance when analyzing a SERS spectrum (Fig.2.2). As a matter of fact, when

molecules are adsorbed onto a surface, their symmetry can strongly change leading to differences in mode selection.[56,175,176] The loss of a center of symmetry eliminates the requirements of the mutual exclusion rule, which states that modes can only be either Raman or Infrared actives. Thus, modes that would normally appear only in the infrared spectrum of the free molecule can appear as well in the SERS spectrum. In order to better understand the mechanism of analyte adsorption on the metallic substrate, *ab initio* computational methods, such as density functional theory (DFT) calculations have been widely exploited for the understanding of the geometry of the molecule attached to the nanocomposite metal substrate, and for the assignment of the normal modes of SERS spectra for a better characterization of the SERS data whose interpretation can be sometimes highly challenging.

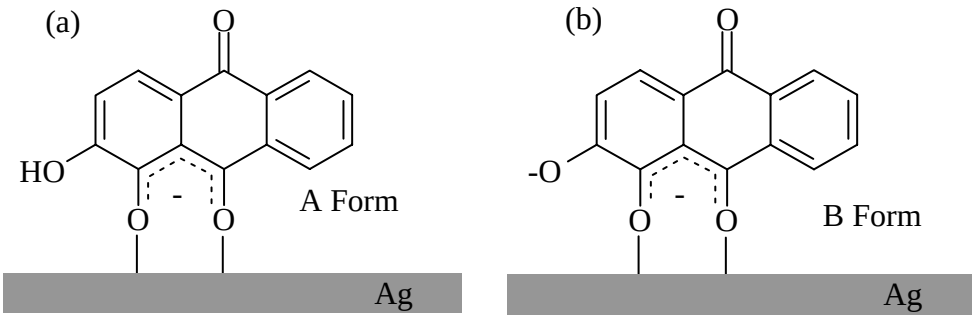

Fig.2.2 Adsorption mechanisms A and B deduced for alizarin on silver surfaces. (From M.V. Cañamares, J.V. Garcia-Ramos, C. Domingo, S. Sanchez-Cortes, Journal of Raman Spectroscopy, 2004; 35, 921-927).

2.2 SERS substrates

As aforementioned, SERS theories predict that the SERS enhancement requires nanoscale surface roughness substrates to occur, and the magnitude of the SERS enhancement is strongly dependent upon the SERS substrate adopted for the analyses. This condition may be achieved by the use of specific substrates synthesized *ad hoc* for SERS purposes by means of several methodologies, such as mechanical roughening, vapor deposition of silver islands, electrochemical roughening, chemical etching and by the use of metallic colloids in aqueous solution. Good and efficient SERS substrates present high enhancement factor, high reproducibility of the results, large surface area and favor the attachment of the molecule to the substrate. The most commonly employed SERS substrates are constituted by metallic nanoparticles. As a matter of fact these substrates present several advantages that make nanoparticles extremely versatile as being used for the accomplishment of SERS analyses. First of all nanoparticles are characterized by a larger surface area which is responsible for the increase of the potential number of molecules that might produce SERS effect. Moreover, nanoparticles can be synthesized extremely easily, via chemical or physical methods, without any need of expensive laboratory equipment such as vacuum evaporation chambers. In addition, compared to electrochemical substrates such as electrodes, the structure of the analyte is not altered by oxidation-reduction processes.[58] Nanoparticles, whose size usually ranges from 10 to 100nm, have specific surface plasmon properties, compared to that of their bulk materials, that make them ideal SERS substrates because of the high enhancement of the Raman scattering observed on or near their surface. In particular, among the most commonly employed substrates, colloidal dispersions of metallic nanoparticles of various size, shape and compositions are the most synthesized and applied substrates for SERS purposes. Several procedures have appeared in the last few years dealing with the synthesis of nanoparticles of several metals. In particular, gold and silver, because of their LSPRs (localized surface plasmon resonances) that cover most of the visible and near infrared wavelength range (Fig.2.3) where most Raman measurements occur, are the most efficient noble metals used for SERS applications. The use of other metals, such as copper, for the synthesis of nanoparticles, has been exploited as well.[59,60]

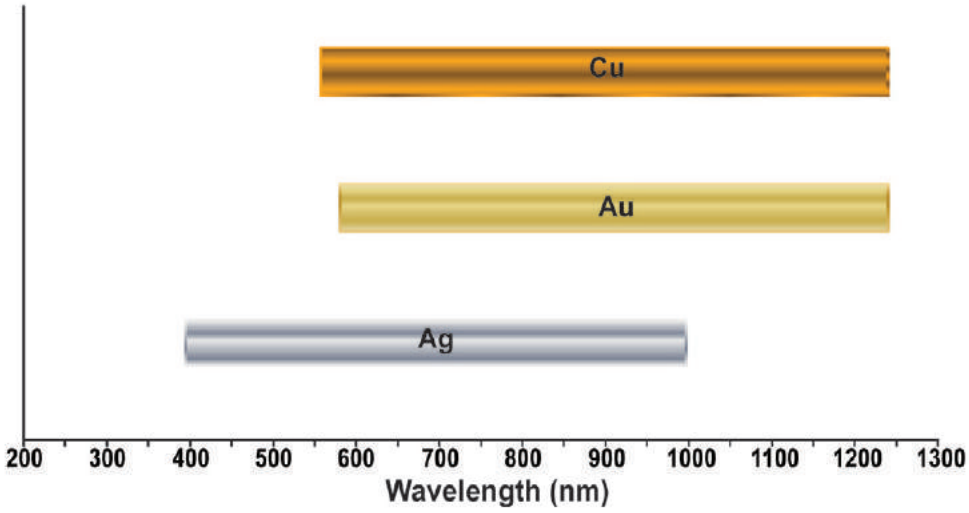

Fig.2.3 Approximate wavelength ranges where Ag, Au and Cu have been well characterized and are established to support SERS. (Picture from B. Sharma, R.R. Frontiera, A.I. Henry, E. Ringe, R.P. Van Duyne, Materials Today, 2012; 15, 16-25)

With regards to colloidal dispersions obtained via chemical reaction, the first reported SERS application of spherical nanoparticles in a colloidal solution was observed in 1979 by Creighton, who used ice-cold $NaBH_4$ solution to reduce $AgNO_3$ and $K[AuCl_4]$ for the synthesis of spherical silver and gold nanoparticles, adopting pyridine as probe molecule for the study of the SERS activity of the colloids.[61] Creighton evidenced moreover the strong dependence between the SERS signal and the excitation wavelength adopted. In 1982 was published the Lee-Meisel protocol regarding the synthesis of silver spherical nanoparticles,[62] whose procedure requires the reduction of $AgNO_3$ by means of sodium citrate. This colloid is still one of the most employed for carrying on SERS measurements but it is not particularly suitable when a certain monodispersity of the nanoparticles is required for analyses, due to the high polydispersity of this colloid. Another well-consolidated synthesis procedure concerns the reduction of the silver salt by means of hydroxylamine hydrochloride.[63,64] Recently, several chemical procedures have been carried out, providing a wide choice of sizes and shapes of nanoparticles (Fig.2.4), such as cubes,[65] prisms,[66] rods,[67] stars[68] and flowers.[69] The nanoparticles SERS activity is strong-

ly dependent on the nanoparticle size, moreover, it has been shown that in order to get the best SERS signal, is necessary to choose the preferred size and the corresponding excitation wavelength to excite surface plasmon resonance on the nanoparticles.[70,71]

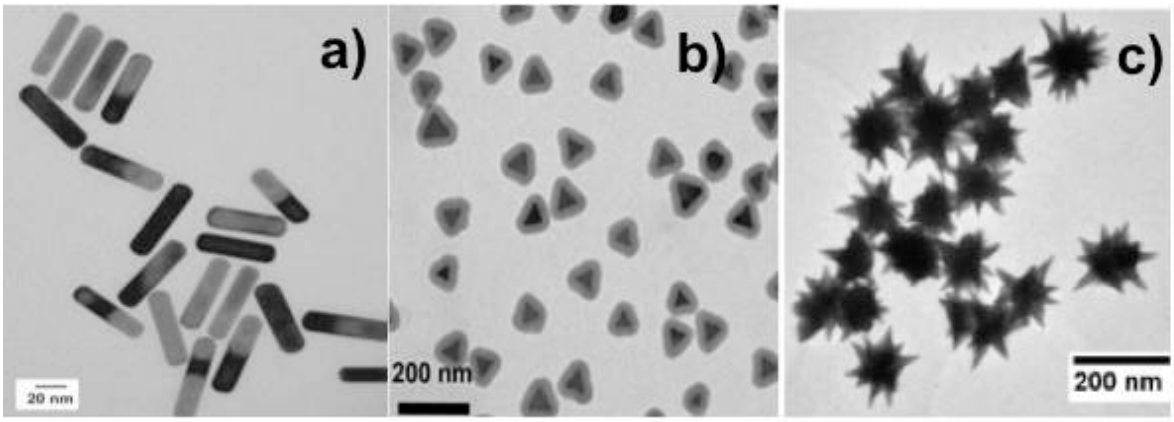

Fig.2.4 Examples of nanoparticle shapes a) nanorods; b) nanoprisms c) nanostars

Another important issue in terms of SERS activity of metal nanoparticles regards their aggregation, condition achieved by adding an inorganic salt to the colloidal dispersion. As a matter of fact, the much stronger electromagnetic field is induced inside narrow gaps between nanoparticles, the so-called "hot-spots", where the enhancement factor can be of the order of 10^6-10^{11}.[72] In the last few years a high number of papers concerning the synthesis of colloids according to "green chemistry" requirements have been published, where it is introduced the use of natural sources, such as plants[73,74] carbohydrates[75] vitamins[76], etc., as reducing agents for the metallic salts. These colloids are usually characterized by quite small nanoparticles (10-20nm) and they have been found extremely suitable for SERS applications in several fields of research. Another procedure for the synthesis of metal nanoparticles is done by means of laser ablation[77-78] and photoreduction.[79-81] The advantage of these procedures, is related to the purity of the final product; as a matter of fact the metal is reduced upon laser (usually a 1064nm pulse Nd:Yag laser) without any need of reducing agents, whose residual ions often interfere with the SERS spectrum of the investigated probe. In the case of photoreduction instead, gamma or UV laser irradiation is adopted for the reduction of the metal salt.[58]

Nanotechnology, which has attracted the attention of a high number of researchers, is constantly in progress. The development of new methodologies, focused mainly on eco-compatible efforts, is the cornerstone of several fields of research.

2.3 Application of SERS for the detection of organic dyes in artworks

As previously anticipated in Chapter 1, the analysis of organic dyes in artworks is a quite challenging issue. The major problems related to the detection of these compounds concern first of all the low concentration in which these compounds are found. As a matter of fact, due to their high tinting power, tiny amounts of dye were sufficient for the dyeing process. Secondly, being organic compounds, they are extremely susceptible to degradation, whose processes can often held to the change of the molecular structure of the original compound, making the detection and the identification of these species particularly difficult. Another issue, that makes the analysis of dyes particularly tough, is related to the fact that they were often used in mixtures, incorporated in complex matrixes - such as paint layers - or attached to fibers through a mordant. Since it is not always possible to perform destructive HPLC analyses on artwork because of the lack of sample available, the discrimination of the single components of the mixture is sometimes impossible. Although currently available non-invasive methods, such as UV-visible absorption or fluorescence spectroscopy help allay concerns about maintaining the integrity of the artifact, they, unfortunately, are of limited use due to their poor specificity.

In the last few years, the introduction of SERS in the field of science applied for the conservation and the preservation of cultural heritage has provided successful results because of its ability of detecting compounds at very low concentration, in conjunction with the suppression of the fluorescence background usually observed when working upon normal dispersive Raman conditions.

The pioneers in the application of SERS for the analysis of organic dyes were Guichard and Guineau[82] who understood the high potential of this technique for the accomplishment of non-destructive analyses of organic dyes used in paintings and illuminated manuscripts. The successful application of SERS for the analysis of art objects triggered a

plethora of scientific works that greatly enriched the pieces of info available about organic dyes. In particular, rich databases of almost all the components of the most important molecular classes of dyes have been provided, anthraquinones,[83-89] flavonoids,[83,88,90,91] naphthoquinones,[83,92] tannins,[83,93] orchil dyes,[83,94] redwoods,[83,87,92] carotenoids,[88,93] indigoids,[83,94,95] curcuminoids,[93] and xanthones,[95] whose attempt of implementation in a searchable database has been presented by Pozzi et al. in a recent publication, where the authors have assembled the core of a comprehensive library composed of 100 Raman and SERS reference spectra of natural and synthetic organic colorants, as a reliable tool for finding the right match of experimental data.[96] The importance of applying SERS for the detection of organic dyes in works of art concerns first of all the advantage of handling samples of the micrometer scale, which are sufficient to provide excellent spectra of the dyeing agent present. Several methodologies have been developed in the last few years aimed to easy and minimal invasive approaches for the characterization of textiles, prints, paintings and sculptures. Leona et al. have optimized a non-extractive hydrolysis method that involves the pre-treatment of the sample, prior to SERS analysis, with the vapors of fluorhydric acid (HF). This process allows for the decomplexation of the dye from the metal, making easier the SERS detection of the compound whose analysis is performed by simply putting a drop of a colloidal dispersion aggregated with KNO_3 on the top of the sample.[97]

In particular, in the last few years, a new interesting analytical tendency has regarded the application of natural and synthetic polymers as medium for SERS analyses. Leona et al.[98] have developed a methacrylate hydroxygel system, saturated with water, dimethylformamide (DMF) and the chelating agent ethylenediaminetetraacetic acid (EDTA) as extraction vehicle, for the solid phase micro-extraction (MT-SERS) of dyes on artworks. The method, which is completely non-destructive and minimal invasive, has shown to be quite suitable for the micro-extraction of madder on a Renaissance tapestry and of the synthetic dye crystal violet on a Japanese print. Other works, focused on the use of polymers for the SERS detection of dyes, have been carried out as well by other authors. Cañamares et al. have synthesized a zircon organic modified silicate complex (Zr-Ormosil) specifically tailored for the selective incorporation and identification of alizarin in mixtures,[99] while Doherty et al. have developed a matrix of methylcellulose loaded with a

dispersion of silver nanoparticles, to be used as active film for the SERS detection of lakes in paintings.[100] Recently, Brosseau et al., and Casadio et al.[101-103] have proposed the use of a high concentrated silver colloidal paste, obtained after several cycles of centrifugation of the colloid, for the direct, extractionless non-hydrolysis detection of highly fluorescing colorants from historical samples such as fibers, pastels and watercolors. Another promising application in the field of surface enhanced Raman spectroscopy applied to art objects, regards the use of hyphenated techniques such as Thin Liquid Chromatography (TLC) in conjunction with SERS for the ultra sensitive detection and separation of different components in a mixture.[102,104]. As a matter of fact, SERS, despite its high sensitivity, is not a separation technique and it does not always allow for reliable differentiation of several components in a mixture. The possibility of associating SERS to a separation technique provides remarkable insights on the study of complex mixtures of compounds. Interesting studies have furthermore evidenced how artworks themselves can work as SERS substrates. Centeno et al. have showed how an enhancement of the Raman bands due to deterioration products on daguerrotypes silver surfaces, was observed when these compounds were analyzed *in situ*.[105]

The field of SERS analyses in conservation science is still dramatically increasing. Application and implementation of SERS with others techniques, such as the atomic force microscope for the accomplishment of tip enhanced Raman spectroscopy (TERS) analyses is one of the new goals in conservation science and other fields of research.

Chapter 3

Agar gel in art conservation

3.1 Chemistry and properties of agar-agar

Agar-agar is a Phycocolloid, a gelling product extracted from the cell walls of some species of red algae, primarily from the *genera Gelidium* and *Gracilaria*. It was discovered in the XVIIth century by the Japanese Minoya Tarozaemon who accidentally noticed the thermoreversibility of this gel. Agar was subsequently introduced by Europeans who learned from the eastern world how to use the biopolymer for the preparation of fruit jellies and subsequently imported it to Europe. In 1882 Robert Koch, the founder of modern bacteriology, introduced the use of agar-agar as a culture medium in his famous experiments on tuberculosis bacteria.

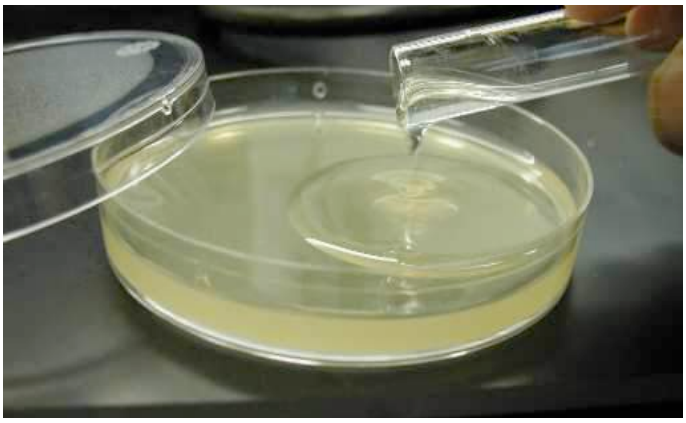

Fig.3.1 Agar-agar gel.

Agar-agar consists of a mixture of a linear, neutral, sulphate free polysaccharide, agarose, and of a heterogeneous mixture of smaller ionic molecules called agaropectins, which are mixed in variable proportions depending on the original raw material and on the manu-

facturing process employed[106]. Agarose is a disaccharide consisting of alternating D-galactose and 3,6-anhydro-L-galactopiranose linked by a $\alpha-(1\rightarrow 3)$ and $\beta-(1\rightarrow 4)$ glycosidic bonds (Fig.4.1). This basic agarobiose repeat unit forms long chains with an average molecular mass of 120,000 daltons, representing about 400 agarobiose units.[107]

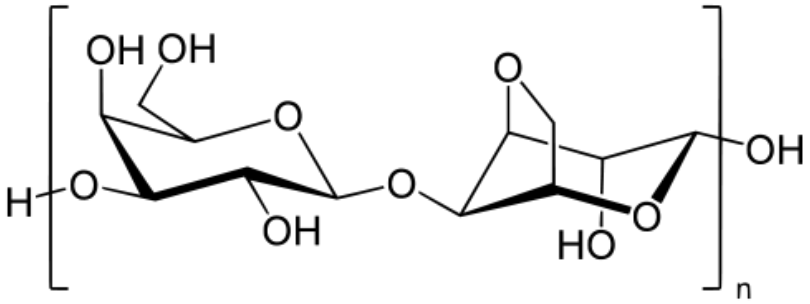

Fig.3.2 Basic repeating disaccharide unit of the polysaccharide component agarose.

Agarose is the polysaccharide fraction responsible for the gelation of agar-agar since agaropectin has an extremely weak gelling ability. The mechanism of gelation of agar-agar, (Fig.3.3) is a process involving more steps. Basically, at temperatures above the melting point of the gel (>85°C), due to thermal agitation that overcomes the tendency to form helices, the polymer exists in solution as a random coil (Fig.3.3). Upon cooling (35-40°C), two gelation steps can be observed. A first step (Gel I) involves the formation of anti-symmetric double helices, that work as junction points of the polymer chains, and a second step (Gel II) concerns the building up of the three-dimensional network, where the double helices connect to each others by means of hydrogen bonds to form the hydrogel macro-reticulum.[106] The gelation process of agar agar is not a polymerization but a simple electrostatic attraction, that makes the gel highly thermoreversible.[106]

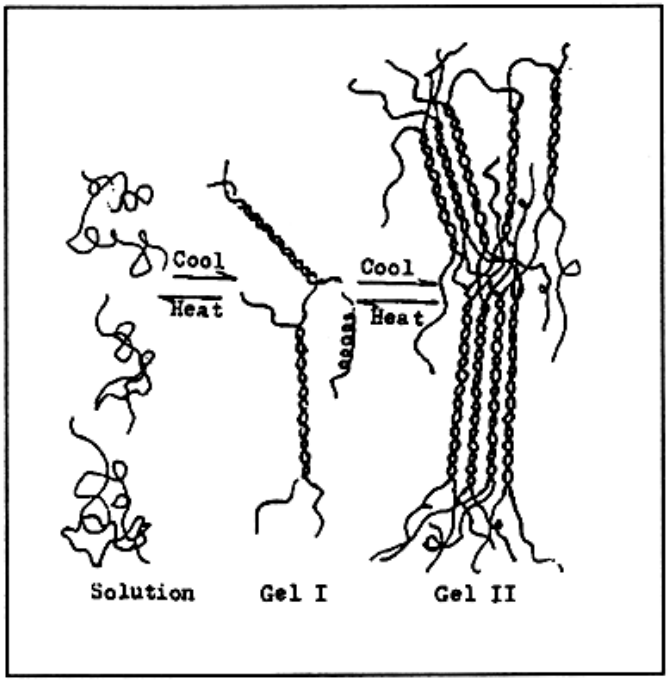

Fig.3.3 Gelling mechanism of agar gel

Agar gel melts under heating (~85°C) but it gelates again upon cooling (~35°C). This process can be repeated endlessly in the absence of aggressive substances that could hydrolize the agarose molecules or destroy them by oxidation. The gelation process of agar-agar does not require any additional substance to gel, as in the case of alginates that need the presence of divalent cations for the gelation process to occur. Due to the peculiar gelling mechanism of agar gels, they hold within their network a big amount of water, which can move freely along the macro-reticulum. Agar gel is even characterized by a high gelling hysteresis, parameter defined as the temperature difference between its gelling and melting temperatures.[108] The gel strength of agar-agar depends on the content of agarose which is influenced by the species of red algae from where the polysaccharide is extracted. It can reach 500 g/cm^2 or more after alkali modification. The gel viscosity, that rarely exceeds 10-15 cp at 1% concentration, is usually lower as the gel strength is greater.[106]
Agar-agar is still widely employed for several uses and in several fields, such as food additive in food industry, bakery, confectionery and meat packing; in pharmaceuticals as medium for microbiological cultures and dental impression materials, and in biomedicine and biotechnology as medium in electrophoresis, chromatography, immunology and as a

inert carrier for enzymes and cells. The use of this biopolymer has been successfully extended even to the field of art conservation. Interesting methodologies have been developed in the last few years for the cleaning of paper, stone and painting artworks. A brief discussion about these applications is presented in the next paragraph.

3.2 Applications of agar gel in conservation science

In the last few years polysaccharide based rigid gels have been successfully employed in the field of conservation science as media for the cleaning of artwork surfaces. The use of hydrophilic polymers allows for the increase of the viscosity of water, which is released on the surface in a quite small amount, limiting processes of diffusion and capillarity that can strongly affect the integrity of the object, as for example the internal layers of a painting. The use of rigid agar gels for cleaning purposes in art conservation was introduced firstly by Richard Wolbers in the mid-1980s who demonstrated the potentiality of agar as being applied for the cleaning of painted surfaces (Fig.4.3).[109,110]

Fig.3.4 Application of a rigid agar-gel to a tempera-painted wooden cross to remove a discolored proteinaceous coating. (From: P. Cremonesi, Smithsonian contributions to museum conservation, 2010; 3, 179-183).

The application of the natural polymer was then broadened to other artifacts such as wooden objects, plaster sculptures and mural paintings.[111] The "Istituto per il Restauro e la Conservazione del Patrimonio Archivistico e Librario" (ICRCPAL) in Rome, has been dealing in the last few years with projects concerning the use of polysaccharide based rigid gels for paper cleaning treatments. This technique has been found extremely suitable since it removes degradation products from prints and drawings without any detriment of the analyzed substrate. As a matter of fact, the use of gels allows for the gradual and controlled release of water molecules on paper that in conjunction with the capability of agar of adsorbing water-soluble degradation products, the ease of application and removal and the viscoelasticity of the system, helps allay concern to maintain the integrity of the artwork surface.[112] Moreover, the application of a gel for cleaning purposes confines the action only in correspondence of the area covered by the hydroxygel system, making the cleaning procedure quite localized on a specific point of application. As well as solvents, gels can carry enzymes,[113] buffers, chelates, surfactants, salts and thickeners, in order to tailor the system to act in a specific way upon the substrate. In particular is extremely suitable as carrier for enzymes[114] and it is compatible with buffers and chelates. Wolbers has suggested the use of small agarose gel discs to be placed in temporary contact with paper, as a pH probe able to provide an indication of surface pH of the paper avoiding any kind of unpleasant tidelines on it. Another great advantage of using agar gel in conservation science regards, furthermore, the eco-compatibility of the material, which can be safely handled by the operator and easily synthesized without requiring sophisticated tools or devices. For all the reasons mentioned above, agar gel is a quite safe and promising material in the world of art objects, whose applications, as evidenced by the high numbers of articles in the scientific literature dealing with this subject, are increasing exponentially year by year.

3.3 Development of Ag-agar gel SERS substrate for the micro-extraction of organic dyes on artworks

As already introduced, agar-agar is an extremely versatile natural polysaccharide employed in many scientific applications, such as electrophoresis, microbiology and pharmaceuticals. Moreover, as explained in the previous paragraph, it has been successfully adopted in the field of art conservation as medium for the cleaning of paintings, stone and paper artworks. In the last few years, several works have appeared in literature concerning the synthesis of nanostructred hybrid organic-inorganic matrices, made of natural polymers, such as agar-agar, alginates, chitosan, starch and methylcellulose coupled with metal nanoparticles, used for many purposes, such as carriers of drug delivery[115] eco-friendly bactericides,[116] scaffolds for tissue engineering and other biomedical applications.[117] The advantage of using natural polymers for the synthesis of hydrogels is related firstly to their high biocompatibility, biodegradability and their low cost. Moreover, because of the presence of metal nanoparticles within their structure, these systems have been found to possess a high antimicrobial activity that makes the nanocomposite matrices extremely stable in time. As a matter of fact, trapping silver nanoparticles inside a rigid structure, as the one of a hydrogel, has the advantage of showing a better stability with respect to the colloid by itself.[118] The application of these polymeric systems has recently attracted the field of Surface enhanced Raman spectroscopy, because of the metal nanoparticles supporting localized surface plasmon resonances (LSPRs), providing interesting insights on the use of polymeric nanocomposite matrices for the ultra-detection of molecules found at very low concentration.

In this work, a SERS substrate made of agar-agar gel coupled with silver nanoparticles has been developed for the non-destructive micro-extraction of organic dyes present in works of art. In particular, the proposed gel formula has been synthesized coupling the biopolymer with a colloidal dispersion prepared according to Lee-Meisel protocol. The system has been found to be extremely suitable for the accomplishment of non-destructive micro-extractions of organic dyes in works of art and as SERS substrate for the detection of the analyte extracted because of the nanoparticles present within the hydrogel structure, able to quench the strong fluorescence background usually encountered

when working in dispersive Raman conditions. The technique has been successfully applied for the detection of mordanted dyes on textiles (wool, silk, cotton and printed cotton) and lakes present on a mock-up panel painting. No detriment of the investigated surfaces has been detected after the extraction, moreover the Ag-agar gel system has been found to be extremely sensitive and minimal invasive for the detection of the molecular species recognized by means of SERS analyses. The extracting vehicle for the accomplishments of the micro-extraction is the free water present inside the network of the biopolymer even if the addition of further chemicals has been explored in order to improve the extractive capabilities of the nanocomposite compound. A detailed description of properties and applications of Ag-agar gel will be provided in the following chapters.

Chapter 4

SERS detection of red organic dyes by Ag-agar gel

Abstract

Micro-Raman spectroscopy has been widely employed in the last few years for the study of artworks, allowing for the characterization of a high class of pictorial materials. However, the detection of organic dyes by means of dispersive conventional Raman spectroscopy is extremely challenging, due to the high fluorescence background provided by these compounds. Recently, outstanding improvements have been achieved by the introduction of surface enhanced Raman spectroscopy (SERS) for the analysis of organic dyes in artworks. A SERS probe made of agar-agar coupled with silver nanoparticles, has been developed for the accomplishment of non-destructive and minimally invasive micro-extractions of dyes from textiles. Ag-agar gel has been applied firstly on textile mock-ups dyed with three anthraquinone dyes, alizarin, purpurin and carminic acid. SERS measurements have been performed adopting laser light excitations at 514.5 and 785nm of a micro-Raman setup. Highly structured SERS band intensities have been obtained. Moreover, after having verified the safety of the method by colorimetric, X-ray fluorescence and attenuated total reflectance Fourier transform infrared techniques, a real case, a pre-Columbian piece of textile, have been investigated by Ag-agar gel. This cutting-edge method offers new possibilities for a sensitive and non-destructive analysis of fluorescent materials.

Introduction

Since the 1980s, micro-Raman spectroscopy has been a very reliable technique for the characterization of artists' pigments and pictorial materials. However, the analysis of organic dyes by means of conventional dispersive Raman spectroscopy is a very challenging issue. The difficulties encountered in the characterization of this class of materials stem mainly from their high fluorescence emission upon laser excitation, which covers the weak Raman signal. Moreover, due to their high tinting power, these compounds are present at very low concentrations in artifacts. Among the most commonly used techniques for the identification of dyes, high performance liquid chromatography (HPLC) has been widely employed for the study of this class of materials in archaeological artworks and historical textiles, due to its ability to resolve complex mixtures of compounds.[119,120] Despite its high sensitivity, HPLC requires large samples (1 or 2 mm of a fabric thread) for analysis, raising concerns about the preservation of the physical integrity of the art object. Although currently available non-invasive methods, such as UV–visible absorption or fluorescence spectroscopy, help allay concerns about maintaining the integrity of the artifact, unfortunately, they are of limited use due to their poor specificity. In the last few years, surface enhanced Raman spectroscopy (SERS) has become a powerful analytical technique for the study of fluorescent organic materials of artistic interest. The ultrasensitive detection of organic dyes in artworks, exploiting the high sensitivity of SERS, has been particularly incisive in tracing the use of these materials, following trade routes, identifying relationships among archaeological objects, detecting forgeries and attributing works of art.[121-123] SERS analyses of dyes have enabled the majority of their molecular classes to be characterized, providing rich and detailed reference databases of anthraquinones,[83-89] flavonoids,[83,88,90,91] naphthoquinones,[83,92] tannins,[83,93] orchil dyes,[83,94] redwoods,[83,87,92] carotenoids,[88,93] indigoids,[83,94,95] curcuminoids,[93] and xanthones.[95] The dyeing agents were studied by adsorption on nanoscale metallic particles dispersed in a colloidal solution,[122,88,89,90] embedded in self-assembled films,[121-123, 83,84] or prepared as very thin active films.[121,122,83,84,87,90] SERS is a process whereby the Raman scattering signal is enhanced when the dye molecule is spatially confined within the electromagnetic field of the localized surface plasmon resonance (LSPR) of a nanostructured

metal surface.[124-125] The SERS effect can give rise to enhancements of the normal Raman effect of greater than 8 orders of magnitude. The enhancement of the Raman signal and the quenching of the underlying fluorescence allow for the overcoming of the above-mentioned problems associated with the application of the normal Raman effect to dye materials to be largely overcome. In order to improve the quality of the enhancement of the Raman scattering, and the reproducibility of the spectra, a wide class of nanostructured substrates such as rough electrodes,[126] colloids,[127-129] nanoisland films,[130,131] nanostars,[132,133] nanorods,[134,135] and other nanocomposite technological supports have been studied. Recently, interesting methods, adopting polymers and sol-gel matrices, have been developed for the identification of molecules at extremely low concentrations.[136-138] This aspect has attracted the field of dye analysis in artworks, providing cutting-edge methodologies for the study of this class of materials such as polymeric beads of methacrylate[98] for a non-destructive extraction of dyes from ancient textiles and drawings; organic modified silicate matrices, combined with zirconium, tailored for a selective identification of alizarin;[99] methylcellulose active films for the detection of painting lakes.[100] In particular agar-agar, successfully applied in the field of stone works,[139-140] paintings[141] and paper cleaning,[142,112] and combined with silver nanoparticles for the production of antibacterial organic-inorganic systems,[143-145,116] has been selected as an ideal gelling material for our research. In this work a nanocomposite Ag-agar hydrogel has been developed for non-destructive extraction of dyes from artworks. Agar-agar is a polysaccharide consisting of a mixture of agarose and agaropectin. It was the first phycocolloid (gelling product extracted from marine algae endowed with colloidal properties) extensively used as food additive in our civilization since 300 years ago.[143] Chemically inert and non-toxic, it has a good solubility at high temperature (>50°C) in water and can form easily a rigid and thermoreversible hydrogel, due to its high crosslinking properties.[144] It is cheap and environmentally friendly. All the aforementioned attributes make this material particularly suitable for the development of nanocomposite matrices, which are highly stable and easily stored for long periods, as suggested by studies published over the last few years.[145-147, 116] Moreover, the shrinkage of the gel upon drying makes it an excellent mechanical molecular trap for the silver nanoparticles, which approach each other as the network volume decreases. This process generates high plasmonic electromagnetic fields

that engender the Raman signal amplification.[147]

Ag-agar gels have enabled the accomplishment of two important goals. As a matter of fact, the nanocomposite matrix acts not only as an absorbent probe for the micro-extraction of dye molecules from textiles, but also as efficient enhancer of Raman scattering, due to the silver nanoparticles trapped in its structure. The system has been found to be extremely stable, easy to use and to produce, minimally invasive, easy to store and able to be analyzed even after long time intervals, maintaining unaltered its enhancement properties without any detriment of the extracted compound. Ag-agar gel has been tested on three anthraquinone dyes: alizarin (1,2-dihydroxyanthraquinone), purpurin (1,2,4 trihydroxyanthraquinone), and carminic acid. These molecules are the main chromophores of two red dyes: madder (alizarin and purpurin) and cochineal (carminic acid). Madder, also called red madder, is an extract obtained by boiling the roots of the madder plant (Rubia tinctorum), while cochineal is extracted from the dried bodies of the females of cochineal insects (Dactylopius coccus) living on the Nopal cactuses of Central and South America.[148] Since this class of dyes has been widely studied in the past, it offers a solid basis for the comparison of several techniques related to the preparation of SERS active substrates. The efficiency of dye extraction by nanocomposite Ag-agar gel has been tested on textile mock-ups, dyed with alizarin, purpurin, and carminic acid, prior to being applied to ancient artworks. Further analyses, performed by means of complementary techniques, have allowed the feasibility and the safety of the method to be assessed. The Ag-agar gel has been proven to be extremely safe applied to artworks, since it does not release any residual and it does not show any discoloring effects. In addition, it is not gluey, thus it can be safely applied and removed without any risk of damage to the artwork. The procedure has been applied to a real case study, an ancient pre-Columbian piece of textile. SERS analyses, performed after the micro-extraction step by means of Ag-agar gel, have revealed the presence of alizarin, the main chromophore of madder dye.

4.1 The basic of Plasmon Activation of SERS

Metals, like Ag and Au, possess negative real and small positive imaginary dielectric constant components. This physical property enables the condition for SPR to be established in these metals. Surface plasmons are coherent oscillations of the conduction electrons on the surface of metals, driven by the electromagnetic radiation. The controlled production and manipulation of metallic structures on the nanoscale have allowed useful applications, taking advantage of the localized surface plasmon resonances (LSPR).[149,150] The LSPR of Ag and Au nanoparticles gives rise to light absorption in the UV–Vis range of the electromagnetic spectrum. The localized plasmon resonance furnishes a means to enhance electromagnetic energy in proximity of the surface of the metallic nanoparticles, such as light scattering phenomena of species adsorbed on the metallic surface. In this manner, such scattering phenomena on the metallic surface can benefit from a substantial increase in the incoming and outgoing (diffused) electromagnetic fields (SERS). It has been shown[151] that the maximum enhancement occurs when the laser excitation wavelength is close to the plasmon resonance. In fact, large enhancement factors can be obtained when the LSPR falls within a 100 nm range that encompasses both the excitation and the Raman shifted wavelengths. However, the effect is sensitive to even larger differences in wavelengths. Surface enhanced [resonance] Raman scattering (SE[R]RS) is known to produce further enhancement of the Raman signal. In SERRS, the laser excitation has sufficient energy to promote an electronic transition in the molecule, which is promoted usually to the first excited electronic state. When the LSPR of the enhancing substrate is also in the proper energy region, the SERRS enhancement factor is roughly the product of the enhancement factor for non-resonant SERS and the resonance Raman spectrum intensification factor of the molecule.

4.2 Experimental

4.2.1 Textile dyeing process

Three pieces of cotton fabric have been dyed, according to traditional dyeing methodologies, with three anthraquinone dyes: purpurin (Sigma-Aldrich), alizarin (Sigma-Aldrich) and carminic acid (Sigma-Aldrich) (Figg. 4.1- 4.2). The textiles have been treated prior to the dyeing step with an alum (Zecchi, Firenze) mordant solution in distilled water (J. T. Baker HPLC Gradient Grade). After the dyeing step, the cotton fabrics have been thoroughly washed with distilled water several times in order to remove unmordanted dye molecules to the surface, and left to dry.

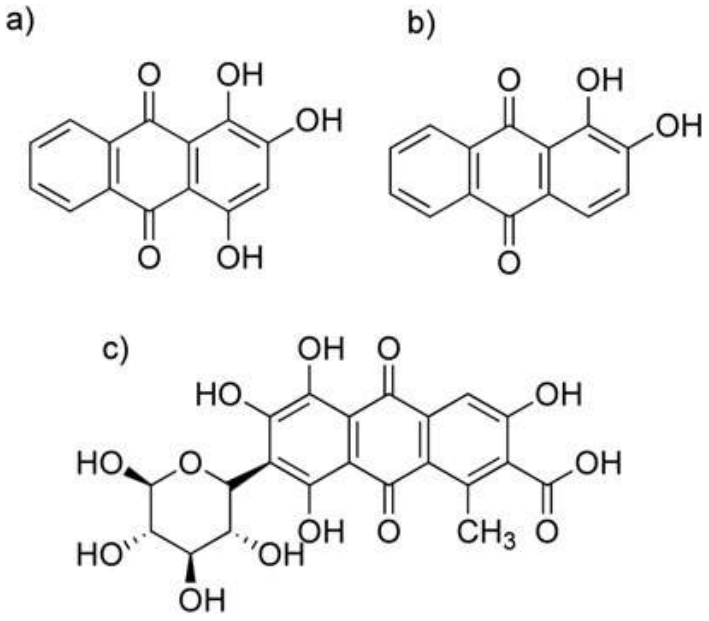

Fig.4.1 Molecular structure of the three anthraquinon dyes a) purpurin b) alizarin and b) carminic acid.

Fig.4.2 On the left: Pieces of cotton fabrics dyed with alizarin, carminic acid and purpurin, the three main chromophores of madder lake and cochineal. On the right: potash alum used for the mordanting of the pieces of textile.

4.2.2 Synthesis of Ag-agar gel and micro-extraction procedure

Common grade agar-agar in flakes was washed repeatedly with distilled water, in order to remove any traces of chloride ions. It was then dried and ground. The silver colloidal solution, used for the preparation of the nanocomposite matrix, was synthesized according to the Lee–Meisel procedure.[127] In brief, 0.008495g of $AgNO_3$ are dissolved in 50ml of HPLC grade tridistilled water. The solution is heated on a hotplate and brought to boil. Once boiling, 1ml of a sodium citrate solution (0.05 g in 5ml) are added dropwise to the silver salt solution. The solution is then left boiling under reflux for one hour, time necessary for the reduction of silver. In particular, after a reduction time of 60 minutes, the flask containing the colloidal solution was placed into an ice-bath to cool. As a matter of fact it has been noticed how the prompt cooling process of the colloidal dispersion provides a wider number of nanoparticles characterized by an average diameter of 40 nm.[152] Prior to being used for the gel synthesis, no aggregation salts, such as KNO_3, NaI or NaCl have been added to the colloid. For the preparation of Ag-agar gel, 0.2 g of agar-agar have been mixed in a beaker with 10 ml of the silver colloidal dispersion. The sol–gel mixture was heated in a microwave oven at 300 W for a couple of seconds. The Ag-agar

viscous solution was then poured into a Petri dish and left to cool down to room temperature (Fig.4.3). Ag-agar gel is stored at room temperature and conserved inside a glass container hermetically sealed. This storage condition avoids the dehydration of the gel, that otherwise, would not be suitable for the accomplishment of the micro-extraction when dried.

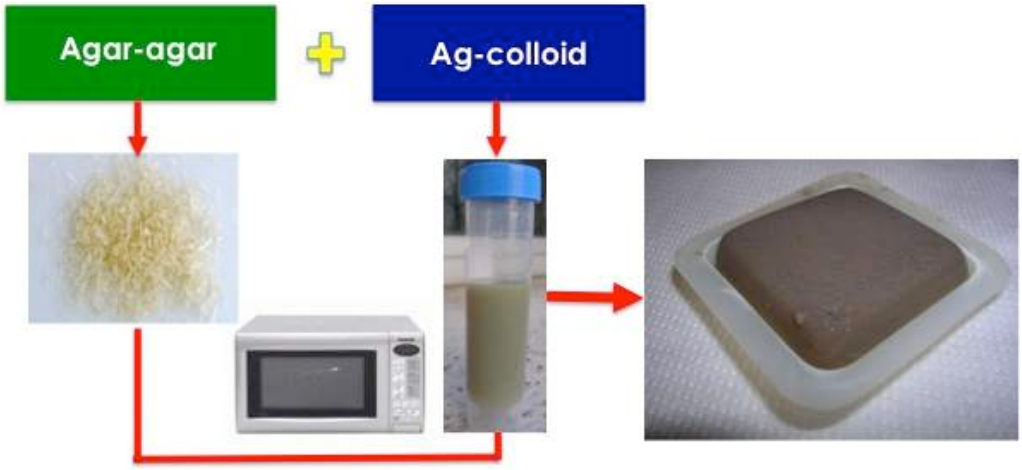

Fig.4.3 Synthesis of Ag-agar gel

With regards to the dye extraction process, it is performed by first cutting small cubes of about 4x4x4 mm^3 from the Ag-agar gel matrix. The face of the cube to be placed in contact with the textile surface is then wet with a drop of ethyl alcohol and subsequently positioned at the point of interest on the textile. The extraction time observed for the cotton textiles dyed in laboratory was of 30 minutes. After the extraction, the cube of gel charged with silver nanoparticles and dye molecules was placed on a microscope glass slide and left to dry. Once dried, SERS measurements were performed by means of a conventional micro-Raman instrumental setup (Fig.4.4).

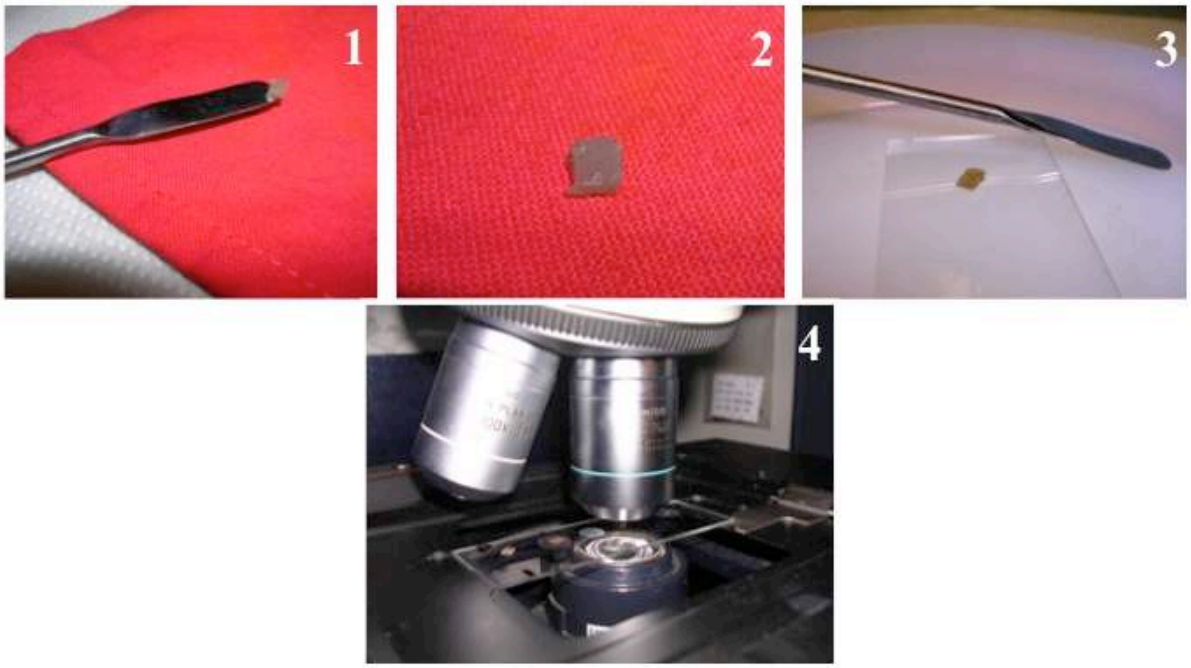

Fig.4.4 Micro-extraction steps by means of Ag-agar gel. 1) A bead of few millimiters extracted from the nanocomposite matrix and placed on the point of of interest; 2) The bead is left on the surface of the object to analyze for a time necessary for the extraction; 3) After the accomplishment of the micro-extraction, the bead is placed on a microscope glass slide and left dry; 4) The dried bead is ready to be analyzed by means of a micro-Raman instrumental set-up.

4.2.3 Instrumentation

The colorimetric analyses have been performed using a CM-2600d Konica-Minolta portable spectrophotometer equipped with the integrative sphere inside the apparatus and a Xenon lamp to pulse the light on the sample surface. The measurement aperture is 3 mm, and light is reflected from surface at an angle of 8°. Color coordinates are based on CIEL*a*b* system using an illuminant D65 with an observer angle of 10°. Electronic absorption spectra have been recorded with a Varian Cary 5 Spectrophotometer, with a spectral interval ranging from 350 to 550 nm at the scan rate of 200 nm/min and an integration time of 10s. Spectra were baseline corrected. SERS spectra have been acquired with a micro-Raman Renishaw RM2000 spectrometer (Fig.4.5) coupled with an argon ion laser excitation at 514.5nm and a solid state diode laser at 785nm. The light collected

in a back-scattering geometry (180°) by a 50x objective of a Leica microscope is filtered by a holographic notch filter to remove the elastic scattering. The Raman photons are then dispersed by a diffraction grating (1200 lines/mm) and detected by a charge coupled device (CCD), maintained at -20°C by means of Peltier effect. The whole system is characterized by a spectral resolution of 3cm^{-1} and by a spatial resolution of 2 µm. Using a 50x objective the laser was focused on different points of the sample with a spot size of approximately 5x5 $µm^2$.

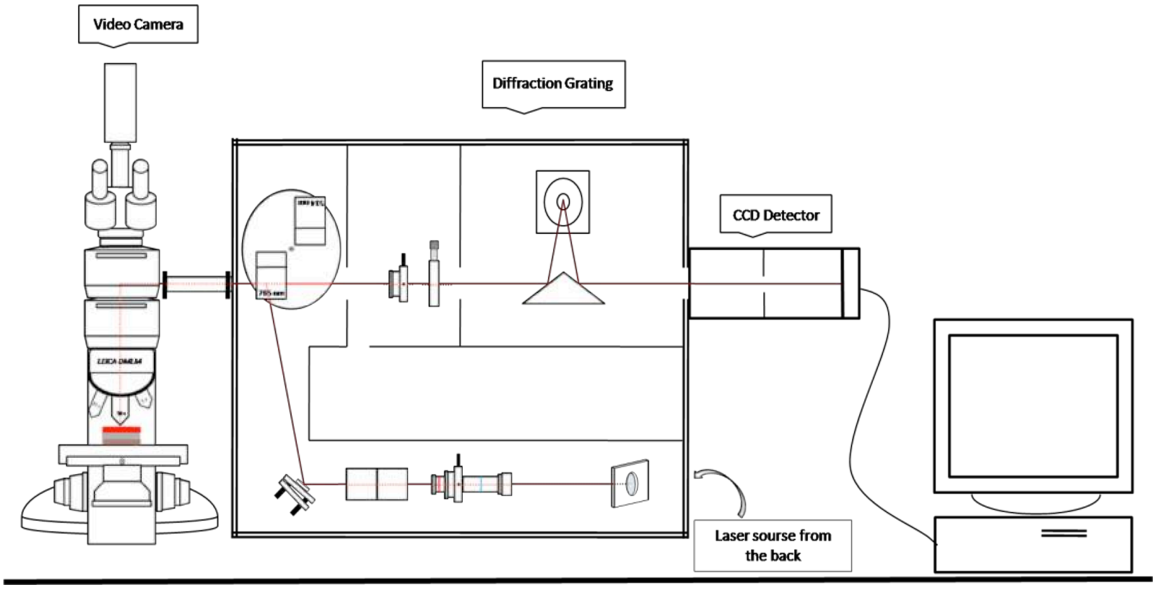

Figure 4.5 Scheme of the Raman apparatus in the Chemistry Department of Florence University.

The 50X objective has been used for both wavelengths and signal collection. Laser power at the surface of the agar-agar gel cube was estimated to be ~20 µW in the case of the argon (514.5 nm) and ~600 µW in the case of the diode laser (785 nm) emission excitations. The time constant for signal accumulation has been typically 10s and the laser spot diameter ~2 µm. The study of the shape and the size of the nanoparticles was performed by means of a Philips CM12 transmission electron microscope (TEM), equipped with a high definition digital camera.

4.3 Results and discussion

4.3.1 Ag-agar matrix characterization

Due to the dependence of nanoparticles size on the reduction time of Ag^{+},[152] both the UV–Vis absorption spectra and the TEM images of the Ag-colloid were acquired (Fig.4.6 a–b) to evaluate the size of the nanoparticles obtained. The measured LSPR absorption maximum of the colloid occurs at ~420 nm, in agreement with the value expected for Lee–Meisel colloids.[127] The TEM micrograph of the silver colloidal dispersion (Fig. 4.6b) shows aggregates of almost spherical nanoparticles, with an average diameter of 40 nm. In order to evaluate the distribution of the silver nanoparticles inside the dry Ag-agar gel, the UV–VIS absorption spectrum and the TEM micrographs have also been recorded (Fig.4.6c–d). The TEM pictures show the same shape and size of the nanoparticles observed in the colloidal dispersion.

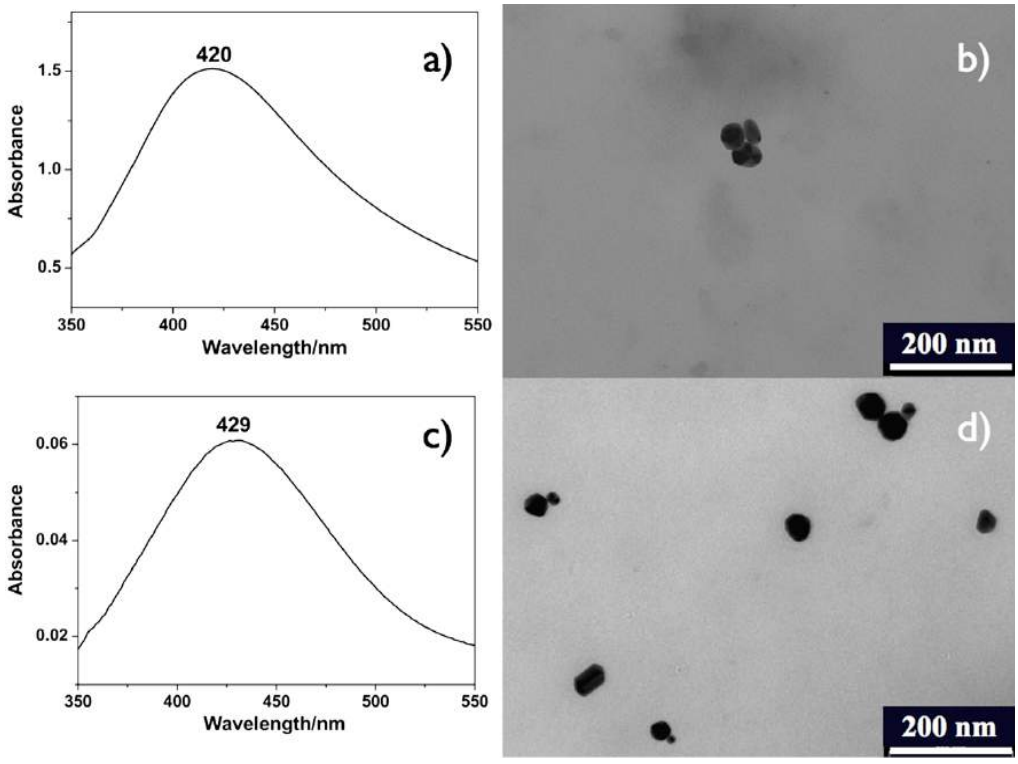

Fig.4.6 a) UV–VIS absorption spectrum of the Ag colloidal suspension; b)TEM image of the colloid; c) UV–Vis spectrum of Ag-agar gel; d) TEM image of Ag-agar gel.

In addition, the increase of the silver nanoparticle concentration after the Ag-agar gel shrinkage upon drying is confirmed. This process facilitates the generation of the electromagnetic coupling between two or more metallic nanoparticles for ultra-detection.[146] The UV–Vis spectrum of the Ag-agar gel (Fig.4.6c), dissolved in tri-distilled water in a volume of 3.5 ml by microwave oven heating, has revealed a red shift in the SPR band (430 nm) compared to that of the colloid alone (420 nm).

This behavior could be due to two main causes: particle nucleation under microwave heating,[147] and/or high concentration of silver nanoparticles inside the sol–gel structure, which is responsible for the multipole interactions among the nanoparticles, leading to the red shift of the observed SPR band.[153]

Further UV–Vis analyses have been performed in order to evaluate the amount of dye extracted by Ag-agar gel. Since the silver LSPR band could overlap the dye absorption band, UV–Vis spectra of agar-gel only, without silver nanoparticles embedded in it, have been acquired after extraction. These measurements were performed in order to better evaluate the amount of the extracted dye. In addition, absorption spectra of solutions of alizarin, purpurin and carminic acid in ethanol, at concentrations varying from 10^{-4} to 10^{-7} M, have been collected in order to estimate the minimal concentration detectable for each dye (Fig.4.7).

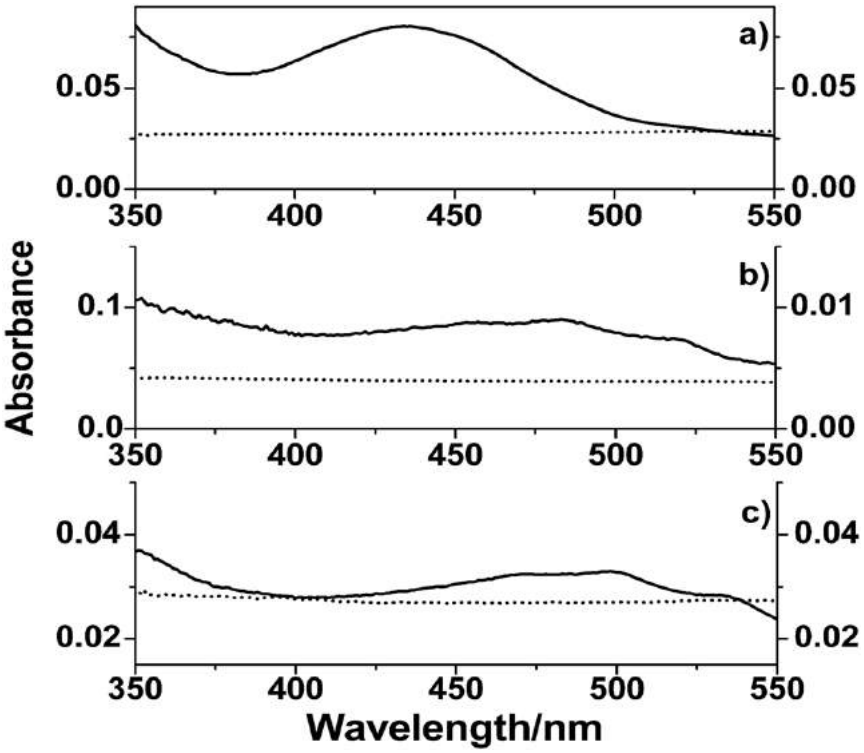

Fig.4.7 UV–VIS spectra of solutions of alizarin 10^{-6} M (a), purpurin 10^{-7} M (b) and carminic acid 10^{-6} M (c) (solid line, right Y axis) and UV–VIS spectra of agar gel after the extraction of the same dyes from the textile mock-ups (dot line, left Y axis).

By comparing the UV–Vis spectra of the solutions and the UV–Vis spectra of the agar gel cube after the extraction, the amount of extracted dye is estimated to be lower than 10^{-6} M for alizarin and carminic acid, and 10^{-7} M for purpurin, thus confirming the minimal invasiveness of the method. The limit of detection of the absorbance spectrum has allowed for the calculation of the number of molecules extracted from the textile for each dye. It has been estimated lower than 10^{10}. The degree of invasiveness of the method has been also estimated by colorimetric, attenuated total reflectance Fourier transform infrared (ATR-FTIR) and X-ray fluorescence (XRF) measurements. While the colorimetric coordinates have demonstrated that the surface did not show any color alteration (Fig.4.8) after the extraction, ATR-FTIR (Fig. 4.8) and XRF analyses have confirmed the absence of gel and silver residuals on the textile. These results confirm the high level of safety of the method.

	L* before	a* before	b* before	L* after	a* after	b* after	ΔE
Alizarin	44.39	45.73	21.02	44.45	45.69	20.99	0.078
Purpurin	38.04	44.71	13.06	38.1	44.7	13.04	0.064
Carminic acid	45.32	27.37	-12.13	45.4	27.35	-12.11	0.084

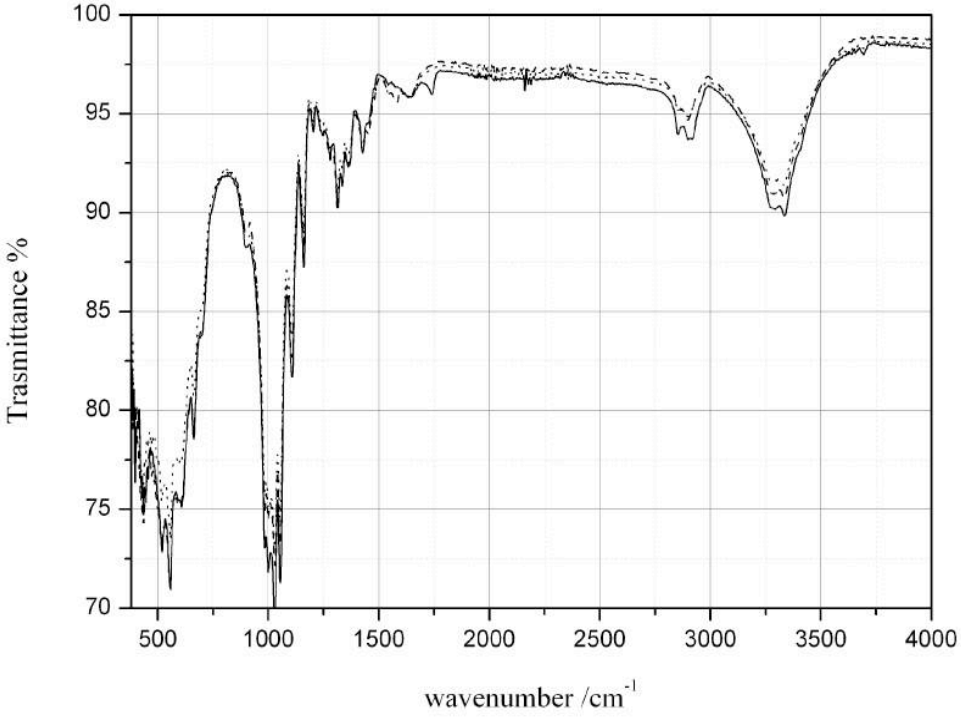

Fig.4.8 On the top: Colorimetric CIEL*a*b* coordinates before and after the micro-extraction of Ag-agar gel on the mock-up textiles dyed with alizarin, purpurin and carminic acid. Results clearly shows the absence of any color detriment on the surfae after the micro-extraction. On the bottom: ATR-FTIR spectra of a piece of cotton fabric (solid line), and of a piece of dyed textile before (dot line) and after (dashed line) Ag-agar gel extraction. No gel residuals have been detected.

4.3.2 SERS measurements

SERS measurements were performed first on dried Ag-agar cubes, wetted on one face with a few drops of 10^{-4} M solutions of alizarin, purpurin and carminic acid in ethanol, in order to obtain reference spectra of the dyes in Ag-agar gel. The spectra were recorded using the 514.5nm laser line excitation, with an integration time of 10s. These results have been compared with SERS spectra of the same reference dyes in ethanol, combined with drops of Lee–Meisel colloidal dispersion (Fig.4.9). Nanoparticles of the colloidal solution were aggregated before use by addition of 1M NaCl. Band wavenumbers of the dyes are reported in Table 4.1.

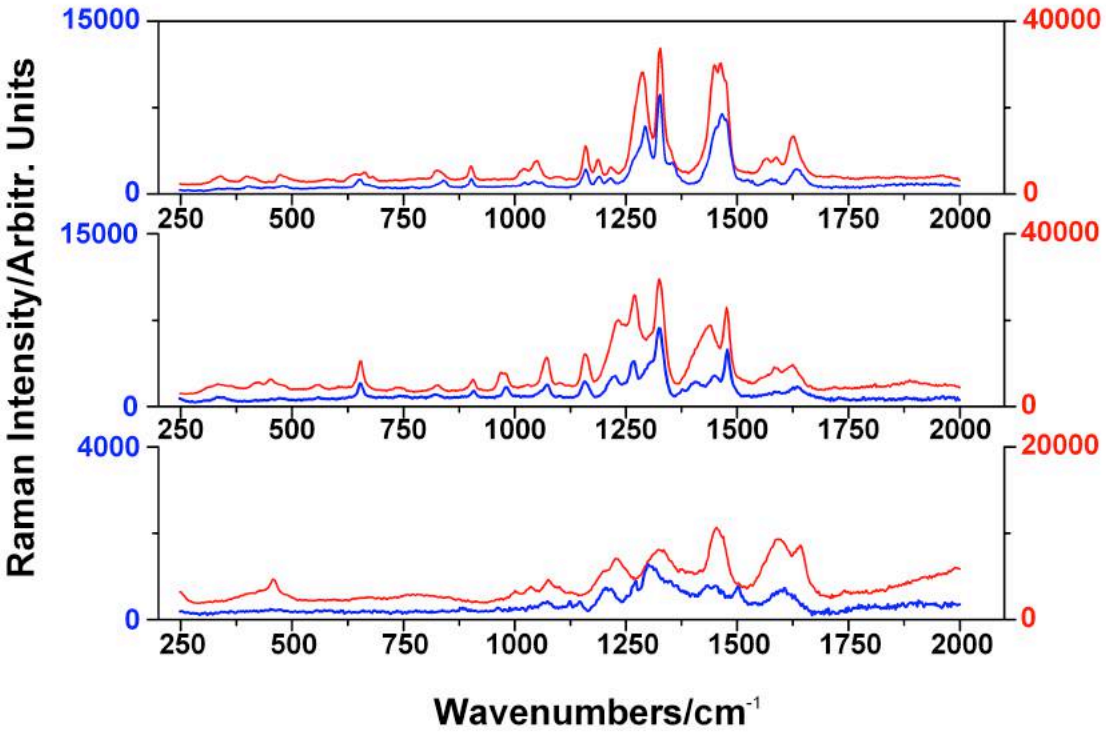

Fig.4.9 Comparison among SERS spectra of 10^{-4}M solutions of a) alizarin; b) purpurin; c) and carminic acid in ethanol combined with drops of NaCl (1M) aggregated silver colloid (blue line, left Y axis) and SERS spectra of the same solutions in Ag-agar gel (red line, right Y axis). Spectra have been collected with 514.5nm laser wavelength excitation.

Alizarin		**Purpurin**		**Carminic acid**	
ϋ (cm^{-1})	Intensity	ϋ (cm^{-1})	Intensity	ϋ (cm^{-1})	Intensity
338	vw	337	vw	401	w
401	w	364	w	424	w
479	w	421	w	458	w
581	vw	452	w	489	w
642	vw	476	sh	662	w
662	vw	556	w	765	w
681	sh	608	vw	970	sh
721	vw	653	m	1001	w
824	w	734	w	1036	w
833	sh	751	sh	1075	m
902	w	825	w	1099	vw
1020	w	906	w	1002	vw
1045	w	970	w	1202	sh
1092	vw	1028	w	1076	w
1159	m	1070	m	1139	sh
1187	w	1101	vw	1229	s
1215	w	1124	vw	1331	s
1287	s	1160	m	1253	sh
1325	s	1235	sh	1452	m
1447	s	1271	s	1468	sh
1461	s	1302	vw	1459	s
1472	sh	1326	s	1588	m
1561	w	1411	sh	1643	m
1585	w	1436	s	-	-
1624	m	1477	s	-	-
-	-	1558	vw	-	-
-	-	1585	m	-	-
-	-	1615	m	-	-

Table.4.1 Main Raman wavenumbers (cm^{-1}) of alizarin, purpurin and carminic acid.

Dyes with chromophores having an anthraquinone structure exhibit quite similar SERS spectra,[121-133,136,154] In particular, the following main common features have been observed

for alizarin, purpurin and carminic acid:

- series of bands around 1600 cm^{-1}, assigned to the C=O stretching modes of the anthraquinone rings;
- strong bands at around 1450 cm^{-1}, which can be attributed to ring CC and d C-OH modes;
- strong bands around 1300 cm^{-1}, which can be assigned d C-H in plane modes;
- series of weak/medium intensity bands between 400cm^{-1} and 1000 cm^{-1} attributed mainly to ring modes.

The effects of the change of the Ag-agar polymeric framework on the SERS signal intensity, as a consequence of the different water content, have been previously reported.[146] The volume reduction of the agar matrix, which occurs when the gel collapses upon dehydration, pushes the embedded colloidal silver nanoparticles closer to each other. This process is responsible for the increase of the particle density in the sample and the electromagnetic fields around the dye molecules. For this reason, a strong enhancement of the Raman scattering is obtained. In particular, the improved enhancement provided by the shrinkage of the gel polymeric framework could be a consequence of the generation of particular sites in which the local plasmonic amplification is considerably efficient.[146] Thereafter, in order to evaluate the efficiency of the proposed methodology, spectra recorded on dried beads of Ag-agar gel after the dye extraction on the pieces of cotton dyed in laboratory with alizarin, purpurin and carminic acid have been acquired (Fig.4.10).

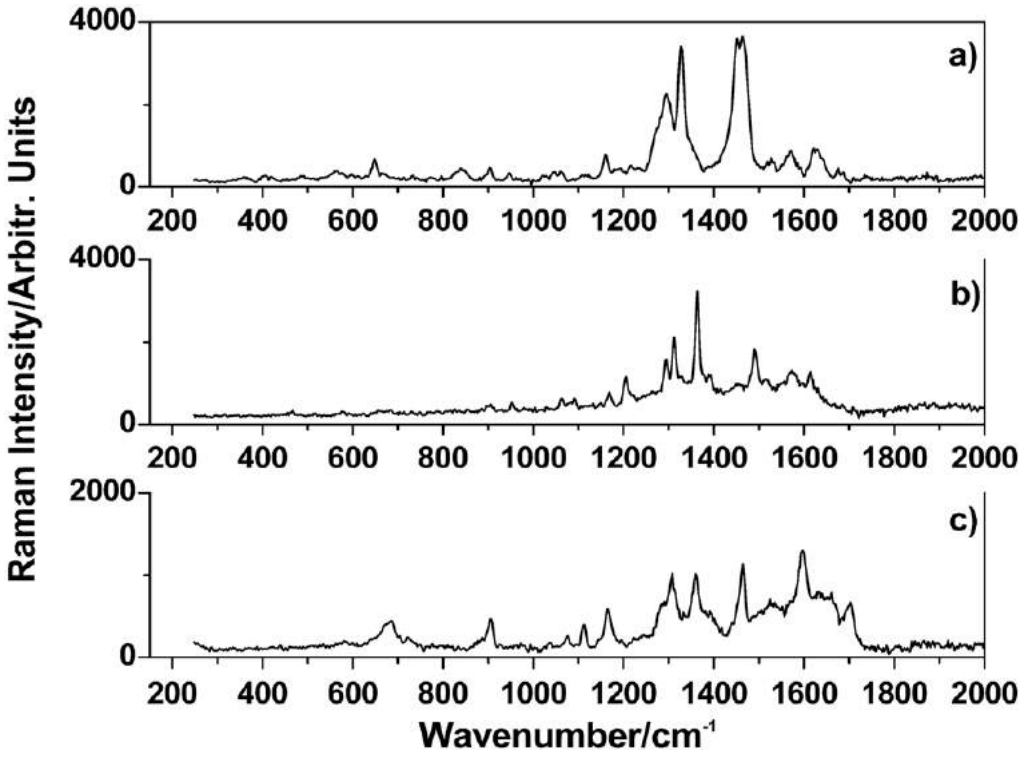

Fig.4.10 SERS spectra of a) alizarin; b) purpurin; c) and carminic acid extracted from the textiles mock-ups by means of Ag-agar gel. Excitation at 514.5nm.

This preliminary assessment was also necessary before application of the procedure to textile samples having artistic/historical value to prevent the risk of damaging the samples. The spectrum of alizarin, extracted from the textile, shows higher band resolution with respect to the spectra of purpurin and carminic acid. Other minor extra features appear in the spectrum of the extracted dye, due probably to the matrix background. To further ascertain the feasibility of the method, a cube of Ag-agar gel has been applied to a real case. The micro-extraction has been performed on a pre-Columbian piece of textile (private collection) that has revealed the presence of alizarin, the main chromophore of madder lake. A good quality SERS spectrum of alizarin (Fig. 4.11) has been obtained, confirming the sensitivity of the method for the study of dyes in historical textiles of artistic interest.

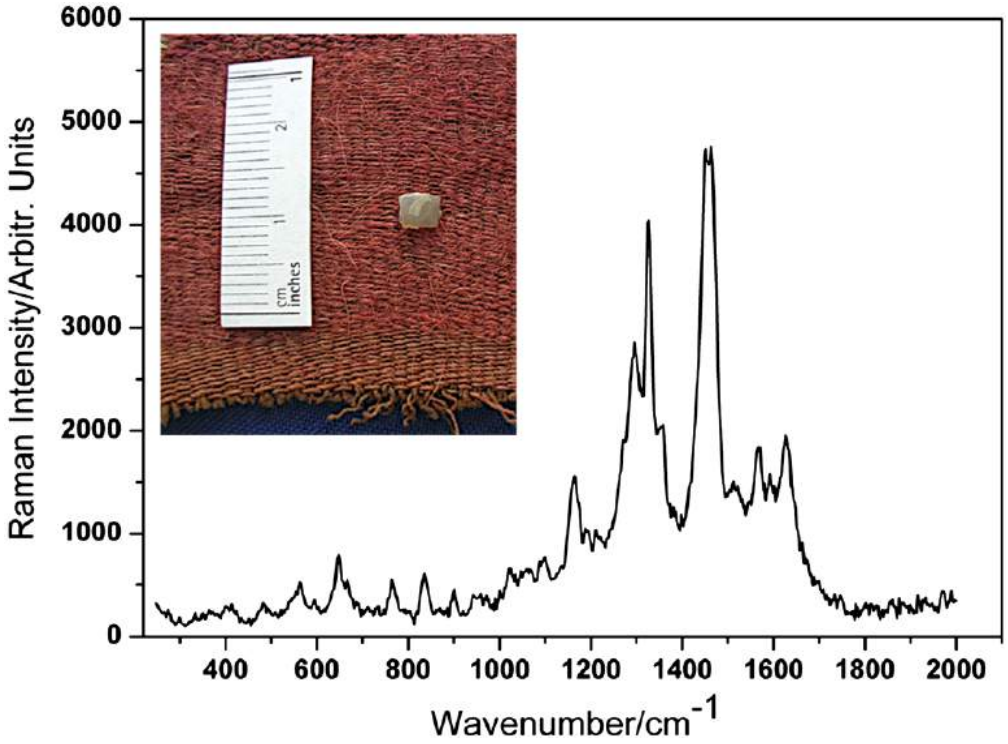

Fig.4.11 SERS spectra of alizarin acquired on the Ag-agar gel dried bead after the micro-extraction on a pre-Columbian piece of textile. Excitation wavelength at 514.5nm.

4.3.3 Excitation wavelength dependence

Alizarin, purpurin and carminic acid all absorb in the visible region of the electromagnetic spectrum, with a broad maximum in the blue region. The use of green light at 514.5nm should give rise to resonance Raman scattering and, due to the proximity of the LSPR absorption maximum of the Ag-agar matrix at ~ 430 nm, it should provide a stronger amplification of the SERS signal with respect to the 785nm diode laser wavelength excitation.[146] In order to evaluate the relative importance of the laser excitation wavelength, and therefore to assess the most effective conditions for enhancing the weak Raman signal of the investigated red dyestuffs, the two different laser excitation wavelengths (514.5 and 785 nm) were used. The spectra obtained for the different excitation wavelengths are very similar, showing peaks at the same frequencies. However, whereas those obtained with the 514.5 nm excitation wavelength show higher relative intensities at higher relative

wavenumbers (Fig.4.12 d–e–f), those acquired at 785 nm excitation exhibit higher relative intensities at lower relative wavenumbers (Fig. 4.12 a–b–c).

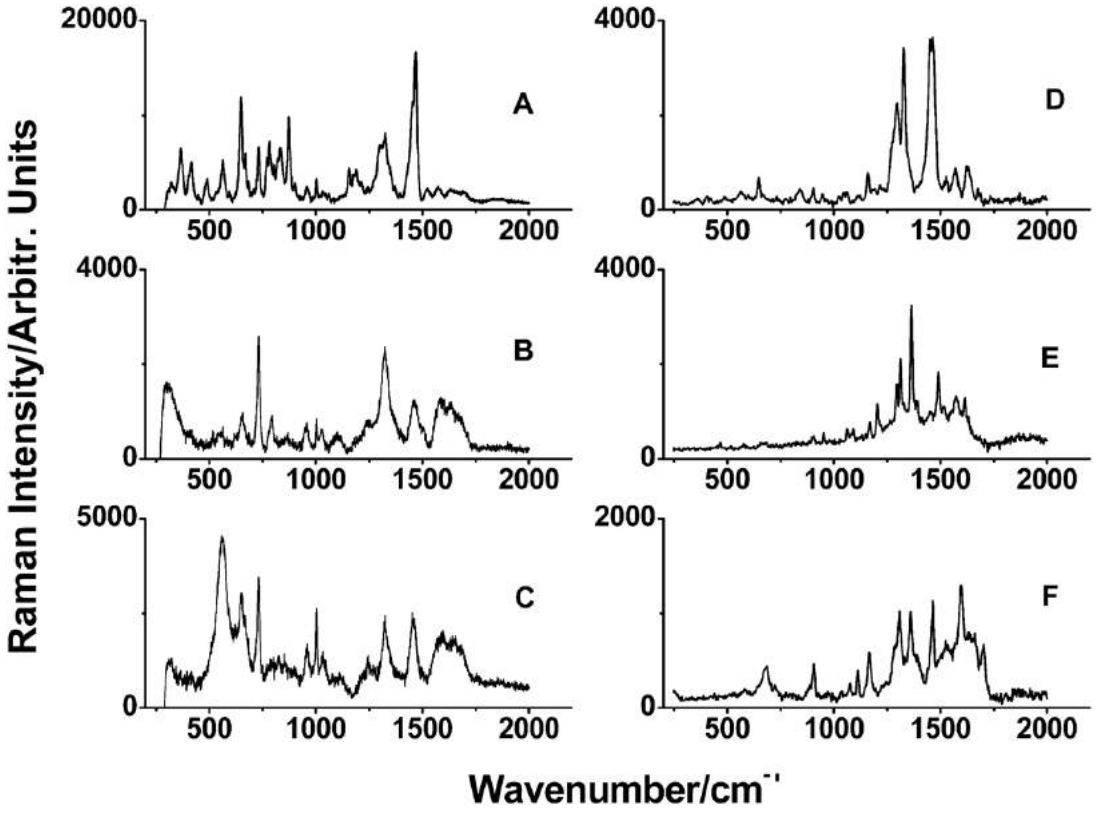

Fig.4.12 Comparison among SERS spectra alizarin (4.12 a,d), purpurin (4.12 b,e), and carminic acid (4.12 c,f) extracted from the textile mock-ups by means of Ag-agar gel. Spectra have been collected by means of a solid state diode laser at 785nm (4.12 a,b,c) and an argon ion laser excitation at 514.5nm (4.12 d,e,f).

This phenomenon could be due to several factors. As suggested by Cañamares et al.,[86] as the analyte is adsorbed on the metal surface in different forms, the relative intensities of the bands in a SERS spectrum, using different laser excitations, depend on the degree of coupling of the vibrational transitions with the electronic transitions. When both transitions coincide, high resonant enhancement is observed, obtaining a SERRS spectrum. Moreover, the deprotonation order of the analyte, when it is adsorbed on a metallic sur-

face, could be responsible for the red shift of the absorption band, thus explaining the different relative intensities observed depending on the excitation wavelength used. In the study presented by Baran et al.,[155] the variability of the band intensities observed, using different laser wavelengths is due to the degree of attachment of the chelate ring, formed after the interaction between the analyte and the metal surface. For Aldeanueva et al.,[146] the LPSR red shift, due to the electromagnetic field between two or more metallic nanoparticles, induces an overlap with the 785nm laser line, thus allowing the increase of the lower wavenumbers range of the corresponding spectra. The closer the laser excitation wavelength is to LSPR absorption maximum, the higher should be the enhancement. This is clear since in the Raman resonance mechanism it is possible to induce different molecular vibrations with different excitation wavelengths. In this case, it can be assumed that the resonance effect is induced with the shorter wavelength Argon laser excitation, while the red shift of the LSPR maximum for the Ag-agar matrix justifies the intense features in the lower relative wavenumbers range of the SERS spectra acquired upon 785nm excitation.[146]

Conclusions

The paper outlines the development of a SERS substrate-probe, synthesized by mixing agar-agar with a colloidal dispersion of silver nanoparticles, and used for a minimal invasive extraction of dye molecules from textiles. The intent was to provide a simple, efficient and safe method to extract dye molecules from textiles fibers of historical and artistic interest, exploiting the high sensitivity of SERS spectroscopy. The method was found to be very efficient and have advantages with respect to other possible methodologies. The amount of extracted dye molecules is extremely low, confirming the minimal invasiveness of Ag-agar gel extraction. The simplicity of the procedure to perform the SERS measurements, the stability of the dry silver charged gel, the strong enhancement of the observed SERS signal, are factors that assure the prompt recognition of the dyestuff. Furthermore, they demonstrate the potential of this approach as an easy to use non-destructive sampling technique in the field of conservation. The high level of enhance-

ment achieved is probably due to the shrinkage of the Ag-agar gel structure upon drying, which could favor the interaction of the silver nanoparticles by creating high plasmon density sites. Ag-agar gel has been tested on a pre-Columbian piece of textile, revealing the presence of alizarin, the main chromophore of madder dye. Relevant spectra on dyed mock-ups were obtained with both Argon (514.5 nm) and diode (785 nm) laser excitations. In particular, the spectra registered with diode laser excitation showed some differences with respect to those obtained with Argon laser one. SERS spectra collected by means of 785-nm laser excitation wavelength showed higher intensity modes at lower wavenumbers, while SERS spectra collected by means of 514.5 nm laser excitation wavelength showed higher intensity modes at high wavenumbers. The differences observed in the spectra obtained with the two excitation wavelengths should depend on three factors: (1) the 514.5-nm excitation is closer to the local plasmon absorption, such that a higher SERS enhancement is in order; (2) the 514.5 nm excitation is closer to the absorption wavelengths of the dye molecules, such that an enhancement due to a resonance effect can be envisaged (SERRS); (3) the LPSR red shift induces an overlapping with the 785-nm laser line, thus allowing the signal increase of the lower wavenumbers range of the corresponding spectra.

Chapter 5

Suitability of Ag-agar gel for the micro-extraction of organic dyes on different substrates: the case study of wool, silk, printed cotton and panel painting mock-up

Abstract

A SERS substrate made of agar gel coupled with silver nanoparticles has been applied for the micro-extraction and the ultra-sensitive detection of dyes on different type of fabrics (silk, wool, printed cotton) and on a mock-up panel painting. In particular, the Ag-agar gel formula previously developed has been improved by the addition of the chelating agent EDTA, which has been found to play an important role as a stabilizer of the nano-composite matrix and for the improvement of the micro-extractive performances of Ag-agar gel. The system has been confirmed to be non-destructive and minimally invasive for the analyzed samples, showing a better capability of trapping the dye molecules inside its structure. Ag-agar gel-EDTA has been an invaluable and powerful tool for the characterization of the dye present in a printed cotton sample of unknown chemical composition. SERS analyses performed on the Ag-agar gel-EDTA matrix after the micro-extraction on the piece of cotton, have evidenced the presence of madder. These results have been corroborated by high performance liquid chromatography (HPLC) analyses, which have confirmed the presence of the analyte detected. The possibility of easily detecting compounds at very low concentration coupled with the high sensitivity of SERS, makes this technique a valid and versatile device for the non-destructive recognition of molecules in works of art.

Introduction

The detection of dye molecules at very low concentration by means of minimal invasive and non-destructive techniques is a relevant issue in the field of forensics and art conservation, where the quantity of sample available for the analyses is usually extremely small, making the investigation of these compounds particularly challenging. Among the most consolidated analytical techniques for the recognition of organic dyes, such as high performance liquid chromatography (HPLC), fluorescence spectroscopy and UV-Vis absorbance spectroscopy, in the last few years, surface enhanced Raman spectroscopy (SERS), has been successfully applied for the non-destructive detection of fluorescent organic dyes in works of art [83-93,156] and forensics.[91,157,158] Exploiting the strong enhancement in the Raman intensity when a molecule is in the vicinity of a metal nanoparticle, coupled with the suppression of fluorescence, this technique is an invaluable, high-resolution method for the detection of trace quantities of target molecules.[57,159]

Recently, cutting-edge methodologies have been developed, involving the application of SERS in conjunction with solid phase micro-extraction techniques that use synthetic and natural polymeric materials as media for the extraction of organic dyes from textiles and drawings[98] or simply as SERS substrates for the recognition of dyes[99] and painting lakes[100]. In the previous chapter, the synthesis of the polymeric matrix made of agar gel loaded with silver nanoparticles has been described, synthesized to act both as medium for the non-destructive micro-extraction of organic dyes on textiles, and as a SERS substrate for the ultra-detection of the extracted analyte because of the silver nanoparticles trapped in its structure. Agar is a strongly gelling hydrocolloid extracted from marine algae. It is a physical gel where the polymer molecules are linked solely by hydrogen bonds; this simple electrostatic attraction is responsible for the thermoreversibility of the gel and its high cross-linking properties.[143,144] The application of this environmentally friendly polysaccharide has been recently introduced in the field of artwork cleaning,[111,139,140] and several studies have appeared in the last few years concerning the synthesis of a stable and sensitive agar SERS substrate able to detect molecules at very low concentration.[118,136,145-147]

As a weak Raman scatterer, agarose gel avoids fluorescence background problems in SERS measurements, making it particularly suitable for the synthesis of SERS substrates.[160] Ag-agar gel has been found to be an excellent gel medium, able to provide safe micro-extractive performances without any detriment of the analyzed surface (the concentration of dye extracted has been estimated to be lower than 10^{-8} M). Moreover, the use of a gel system confines its action only to the investigated area avoiding any kind of sampling essay of the object under study. The high capability of the gel to incorporate in its structure silver nanoparticles makes it a stable and efficient SERS substrate, confirming this methodology as extremely useful for the study of fluorescent molecules at very low concentration. In this work, an improvement of the previous gel formula of Ag-agar gel has been accomplished by adding a chelating agent (EDTA) to the nanocomposite matrix structure. It has been noticed that the presence of EDTA has drastically improved both the micro-extraction performances and the stability of the gel. Ag-agar-gel-EDTA has been successfully applied for the micro-extraction and SERS detection of painting lakes on a mock-up panel painting and for the dye characterization of pieces of textile samples of wool, silk and printed cotton. In particular, the application of this technique has been shown useful for the chemical characterization of the dyeing agent of a piece of printed cotton of unknown chemical composition. SERS measurements have revealed the presence of alizarin, the main chromophore of madder lake. Cross reference analyses, performed by means of high performance liquid chromatography (HPLC), have corroborated the SERS results, confirming the safety of Ag-agar-gel-EDTA as medium for the non-destructive micro-extraction and detection of organic dyes in artworks and forensics.

5.1 Experimental

5.1.1 Textile samples and practice board painting panel

Samples of wool, silk and printed cotton, have been provided by the Department of Textile Conservation of the Metropolitan Museum of Art (Fig.5.2). The printed cotton samples were of unknown composition. The mordant agents of the silk and wool samples are displayed in Table 5.1.

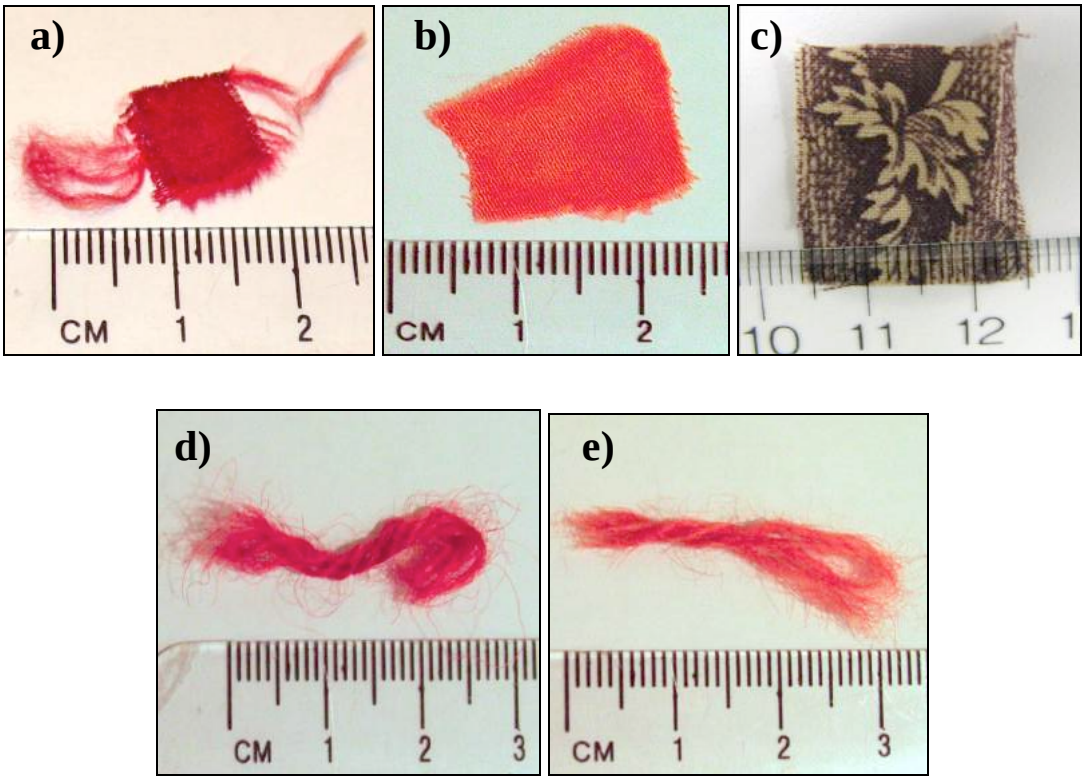

Fig.5.2 Pieces of textiles provided by the Department of Textile Conservation for analyses. a) silk dyed with cochineal; b) silk dyed with madder; c) printed cotton sample of unknown chemical composition; d) wool dyed with lac dye; e) wool dyed with madder.

	Lac dye	Madder lake	Cochineal
Wool	Potassium alum 15%, potassium bitartrate.	Potassium alum 15%, potassium bitartrate.	-
Silk	Potassium alum 15%, potassium bitartrate.	Potassium alum 15%, potassium bitartrate.	Tin, acetic acid

Table.5.1 Table displaying the mordant agents for each textile sample of silk and wool analyzed.

The panel painting used for the analyses (Fig.5.3) is a laboratory reproduction of the Nur-Al-Din panel from the Ottoman reception room (Damascus, Syria, 1280-1924) provided by the Department of Scientific Research of the Metropolitan Museum of Art.

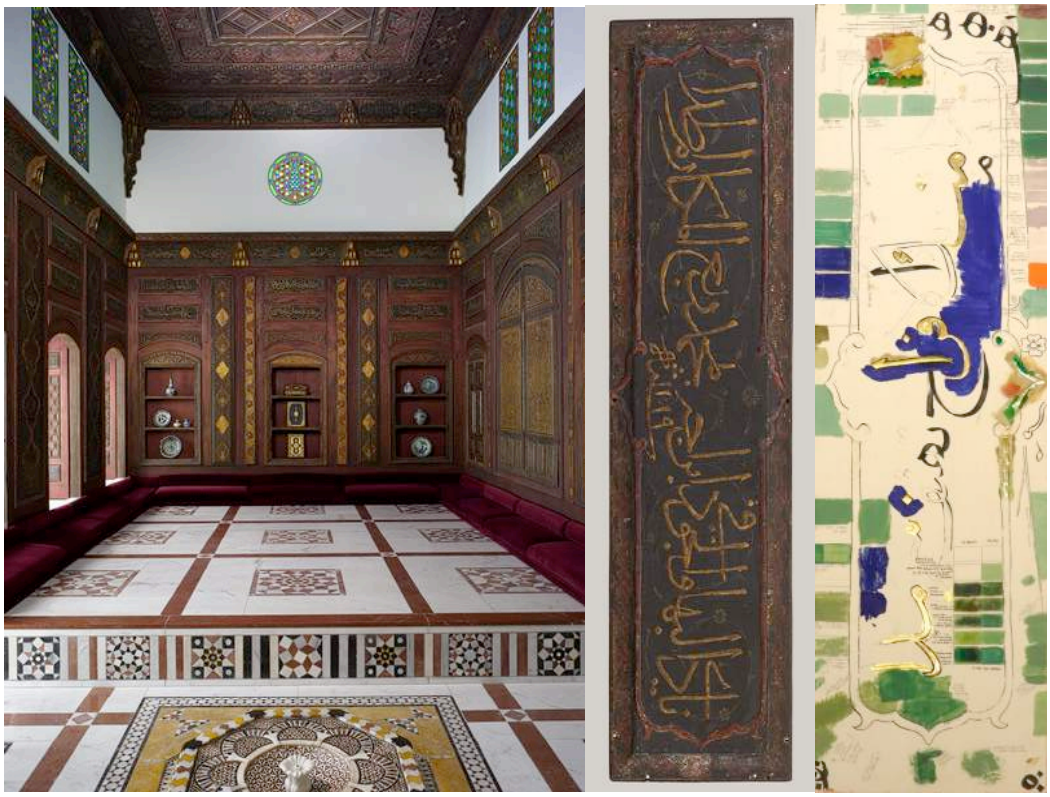

Fig.5.3 On the right: the laboratory reproduction of one of the Nur-al-din panel paintings conserved in the Syrian section of the Metropolitan Museum of Art.

5.1.2 Ag-agar gel-EDTA synthesis

Ag-agar gel has been synthesized according to the procedure described in the previous chapters. In brief, 0.2g of agar-agar in flakes (analytical grade) have been mixed in a beaker with 0.1g of EDTA (Fisher Scientific) and 10ml of silver colloid. The mixture has been heated in a common microwave for a few seconds at the lowest heating power and then poured inside a petri dish (Fig.5.4). Once cooled, the petri dish containing the nano-composite matrix is stored in the dark inside a sealed glass container. In these storage conditions the gel has been found to be stable for up to 6 months. The colloidal solution used for the synthesis of Ag-agar gel was prepared according to the Lee-Meisel procedure[127]. In particular, after a reduction time of 60 minutes, the flask containing the colloid has been put in an ice-bath to cool down, instead of being cooled at room temperature. It has been observed that the prompt cooling process of the silver colloid provides a wider number of nanoparticles with a diameter of about 40nm[152], which have been found extremely suitable in terms of SERS enhancement when combined with the agar gel matrix.

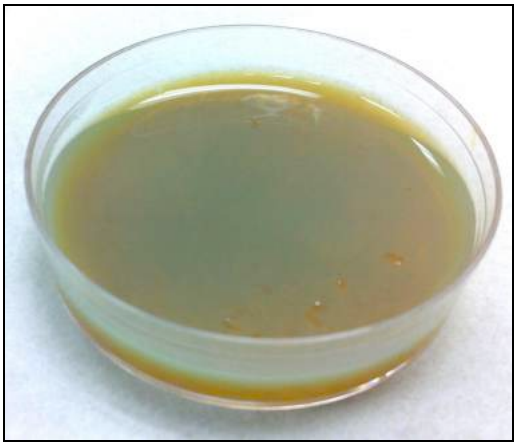

Fig.5.4 Ag-agar gel with the further addition of the chelating agent EDTA inside the matrix's structure.

5.1.3 Micro-extraction process by means of Ag-agar gel-EDTA

For the micro-extraction, a small cube of about 4x4x4mm^3 is cut off from the nanocomposite agar gel by means of a spatula and placed on the point of interest. The extraction time ranges among few seconds (ink on paper) to 30-50 minutes, depending on the object to be analyzed. After the time required for extraction, the gel bead is removed from the artwork surface, placed on a microscope glass slide and let dry. Once dried, SERS measurements are performed on it. The drying process is crucial in terms of SERS enhancement since it decreases the distance between the nanoparticles producing aggregation. This process creates a high density distribution of the nanoparticles inside the dried nanocomposite matrix, that helps the improvement of the gel's enhancement efficiency due to the strong electromagnetic local field produced.

5.2 Instrumentation

SERS spectra have been collected using a Bruker Senterra Raman apparatus with a charge-coupled device detector and a holographic grating with a spectral resolution of 3-5cm^{-1} (1800 rulings/mm for the 488nm laser). A Spectra Physics Model 2020 BeamLock Ar^+, at 488nm, has been adopted as laser wavelength for the spectral acquisition. Measurements have been performed with the following experimental conditions: integration time 30s, 1 scan, output laser power at 0.4mW (0.03mW on the sample), 20X long working distance Olympus microscope objective. The UV-Vis spectra have been collected with a Varian Cary 50 spectrophotometer using a quartz cell with a path length of 1mm. Scanning electron microscope pictures have been collected by means of a Zeiss Supra55VP field emission SEM, maximum resolution of 1nm, secondary and backscattered electron imaging in high vacuum or in variable pressure mode, low voltage scanning transmission electron microscopy (STEM), electron beam lithography, energy dispersive x-ray spectrometry, and electron backscatter diffraction (EBSD). Images of the nanocomposite gel have been taken in backscattered conditions. Microscope pictures have been taken in transmitted light conditions, by means of a Zeiss Axioplan 2, objective 5X.

HPLC analyses have been performed by means of a 1525µ binary HPLC pump, 2996 PDA detector, 1500 series column heater, in-line degasser (Waters Corporation), and a Rheodyne 7725i manual injector with 20 µl loop. A Waters Xterra RP18 (3.5µm, 2.1 mm I.D. x 150.0mm) reverse-phase column was used with a guard column (Xterra RP18 3.5µm, 2.0 mm I.D. x10.0 mm) with a flow rate of 0.2 ml/min. The column prefilter (Upchurch ultra-low Volume pre- column filter with 0.5µm stainless steel frit (Sigma-Aldrich, St. Lois, MO) was attached in front of the guard column. Column temperature was 40°C. The mobile phase was eluted in a gradient mode of 0.88% formic acid in de-ionized water (v/v) (pH 2.3) (A) and methanol (B). The samples were extracted using 100µl of a mixture of 1N hydrochloric acid (HCl) in deionized water (v/v): methanol (6:4) at 95°C for 15 minutes and the extract was dried in a desiccator with sodium hydroxide at the bottom. The residue was dissolved in 20 µl of a mixture of 0.88% formic acid in de-ionized water (v/v): methanol (8:2) and the solution was centrifuged for 10 minutes at 7000 RPM/g; the supernatant was injected into the HPLC system.

5.3 Discussion and results

5.3.1 UV-Vis and SEM investigations

Preliminary UV-Vis, and SEM analyses (Fig.5.5a-b) have been performed in order to study the effect of EDTA on the size and the shape of the silver nanoparticles. The UV-Vis spectrum of Ag-agar gel EDTA (Fig.5.5a) has shown an absorption maximum at 415nm, the same observed for the colloid by itself. With respect to the previously discussed Ag-agar gel formula, no red shift of the absorption spectrum has been noticed after the addition of EDTA and after the heating of the gel/nanoparticles system by microwave. This experimental result could be explained by the fact that the chelating agent EDTA, acting as derivatizing agent, stabilizes the silver nanoparticles so that they do not further nucleate after the heating process.[127,147,152,160,173,174]

In particular, EDTA, coating the silver nanoparticles (AgNp), is chemisorbed onto the surface by means of carboxylate groups. The two oxygen atoms of the carboxylate groups are coordinated symmetrically to the Ag atoms. The surface of AgNps remains negatively charged and in presence of counterions acquires an electrostatic double layer, which, providing a repulsive force, enables silver colloid to be stable.[161-163]

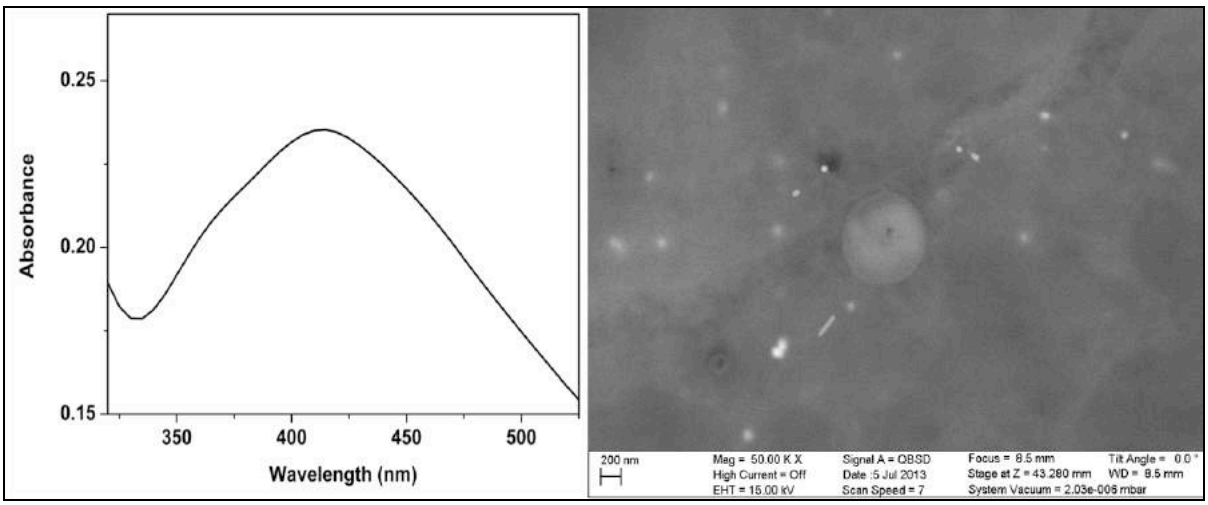

Fig.5.5 UV-Vis absorption spectrum (a) and SEM image (b) of Ag-agar gel-EDTA. Absorbance maximum at 418nm.

The SEM image, (Fig.5.5b) clearly shows the spherical shape of the silver nanoparticles. Rod shaped silver nanoparticles have been observed as well. In particular, the nanoparticles show a larger size (60-100nm), probably due by the layer of EDTA surrounding their surface. Particle aggregates have been observed in the dried Ag-agar-EDTA gel matrix.

5.3.2 SERS measurements

Ag-agar gel-EDTA has been applied for the micro-extraction of dyes on pieces of textile fabric of silk, wool, printed cotton and on a mock-up panel painting. Different extraction times have been observed for each sample, respectively 15 minutes for silk and panel painting and 50 minutes for the wool and the printed cotton samples. Before the dye ex-

traction, no pre-treatment of the surface to be analyzed, such as hydrofluoric acid (HF) vapor exposition[164] has been required for the hydrolysis of the organic dye from the substrate. SERS measurements have been performed on the beads of agar gel completely dried. As a matter of fact, this has been found to be requisite for the collection of good SERS spectra, since the shrinkage of the gel upon drying, pushing the silver nanoparticles close to each others, allows for the generation of high plasmonic electromagnetic fields, responsible for the high enhancement of the Raman scattering.[146] In addition, the presence of chloride ions in the chemical composition of agar gel, could be responsible for the enhancement of the Raman bands when the electrostatic interaction between Cl^- ions and the functional groups of the dye molecules takes place.[165] In Fig.5.2 are reported the SERS spectra of carmine lake and lac dye extracted from the Nur-al-Din panel painting mock-up provided by the Department of Scientific Research of The Metropolitan Museum of Art. Good spectra of laccaic acid and carminic acid have been collected, showing the typical band frequencies observed for these dyes (Tab.5.2).

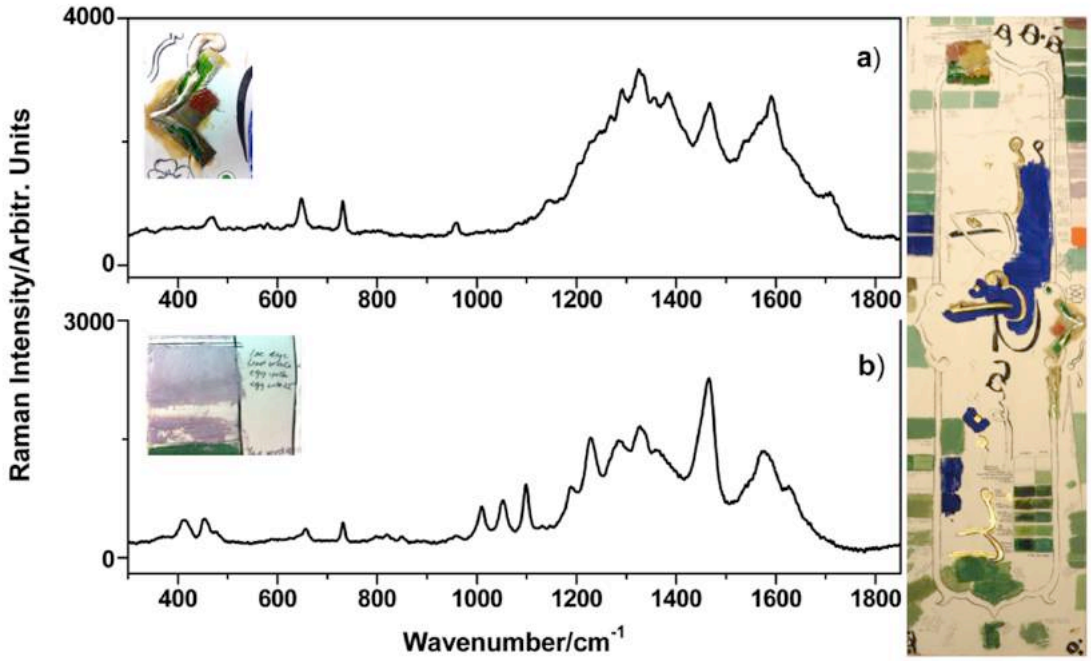

Fig.5.5 SERS spectra of carminic acid (a) and laccaic acid (b) collected from Ag-agar gel-EDTA dried beads, after the micro-extraction on the "Nur-Al-Din" mock-up panel painting, provided by the Metropolitan Museum of Art. Spectra collected on dried Ag-agar-gel-EDTA beads. Excitation at 488nm.

Alizarin		Purpurin		Carminic		Laccaic	
ṽ (cm^{-1})	*Intensity*	*ṽ (cm^{-1})*	*Intensity*	*ṽ (cm^{-1})*	*Intensity*	*ṽ (cm^{-1})*	*Intensity*
338	vw	**337**	vw	**401**	w	**411**	w
401	w	**364**	w	**424**	w	**455**	vw
479	w	**421**	w	**458**	w	**654**	vw
581	vw	**452**	w	**489**	w	**1010**	w
642	vw	**476**	sh	**662**	w	**1059**	w
662	vw	**556**	w	**765**	w	**1100**	w
681	sh	**608**	vw	**970**	sh	**1130**	w
721	vw	**653**	m	**1001**	w	**1191**	w
824	w	**734**	w	**1036**	w	**1230**	m
833	sh	**751**	sh	**1075**	m	**1291**	s
902	w	**825**	w	**1099**	vw	**1332**	w
1020	w	**906**	w	**1002**	vw	**1345**	vs
1045	w	**970**	w	**1202**	sh	**1365**	w
1092	vw	**1028**	w	**1076**	w	**1454**	s
1159	m	**1070**	m	**1139**	sh	**1532**	m
1187	w	**1101**	vw	**1229**	s	**1569**	m
1215	w	**1124**	vw	**1331**	s	**1580**	m
1287	s	**1160**	m	**1253**	sh	**1610**	m
1325	s	**1235**	sh	**1452**	m	**1630**	m
1447	s	**1271**	s	**1468**	sh	**1657**	sh
1461	s	**1302**	vw	**1459**	s	-	
1472	sh	**1326**	s	**1588**	m	-	
1561	w	**1411**	sh	**1643**	m	-	
1585	w	**1436**	s	-	-	-	
1624	m	**1477**	s	-	-	-	
-	-	**1558**	vw	-	-	-	
-	-	**1585**	m	-	-	-	
-	-	**1615**	m	-	-	-	

Tab.5.2 Main SERS frequencies (cm^{-1}) of alizarin, purpurin, carminic acid and laccaic acid.

Ag-agar gel-EDTA has been also applied for the micro-extraction of organic dyes on silk, wool and printed cotton samples provided by the Department of Textiles of the Metropolitan Museum of Art, respectively dyed, with lac dye, cochineal and madder, using cream of tartar and alum as mordants. The SERS frequencies occurring in the SERS spectra correspond to the ones usually observed for these anthraquinone molecules (Tab.1). In particular, the band at 730cm^{-1}, characteristic only of carminic acid, may be both attributed to the citrate ions adsorbed on the Ag colloid[83] and to the C-O-C bending mode of the two glycosidic linkages (α 1,3 and β 1,4) of agar gel, which is the frequency often observed for agar gel when performing SERS measurements.[166] The micro-extraction of silk

by means of Ag-agar gel EDTA (Fig.5.6), has provided very good results, not only in terms of spectral response, but also in terms of time's extraction, since excellent spectra have been recorded after placing the gel bead on the textile's surface for only 15 minutes and without performing any sample pre-treatment for the hydrolysis of the mordanted dye. Lac dye has been found to be particularly hard to be detected for both silk and wool.

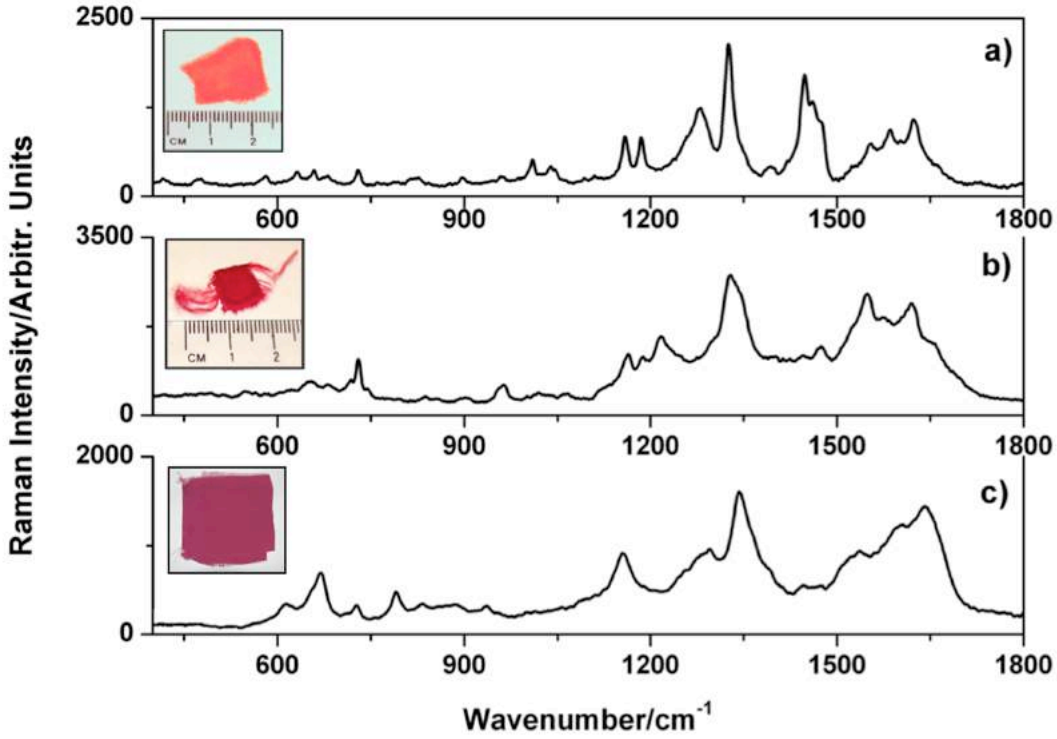

Fig.5.6 SERS spectra of madder (a), cochineal (b) and lac dye (c), performed on the Ag-agar gel-EDTA dried beads after the extraction on silk samples. Excitation at 488nm.

The SERS spectra shown in Fig.5.7 have been collected after the micro-extraction on wool samples dyed with the two anthraquinone dyes, lac dye and madder. In this case, the time requested for the extraction was of 50 minutes.

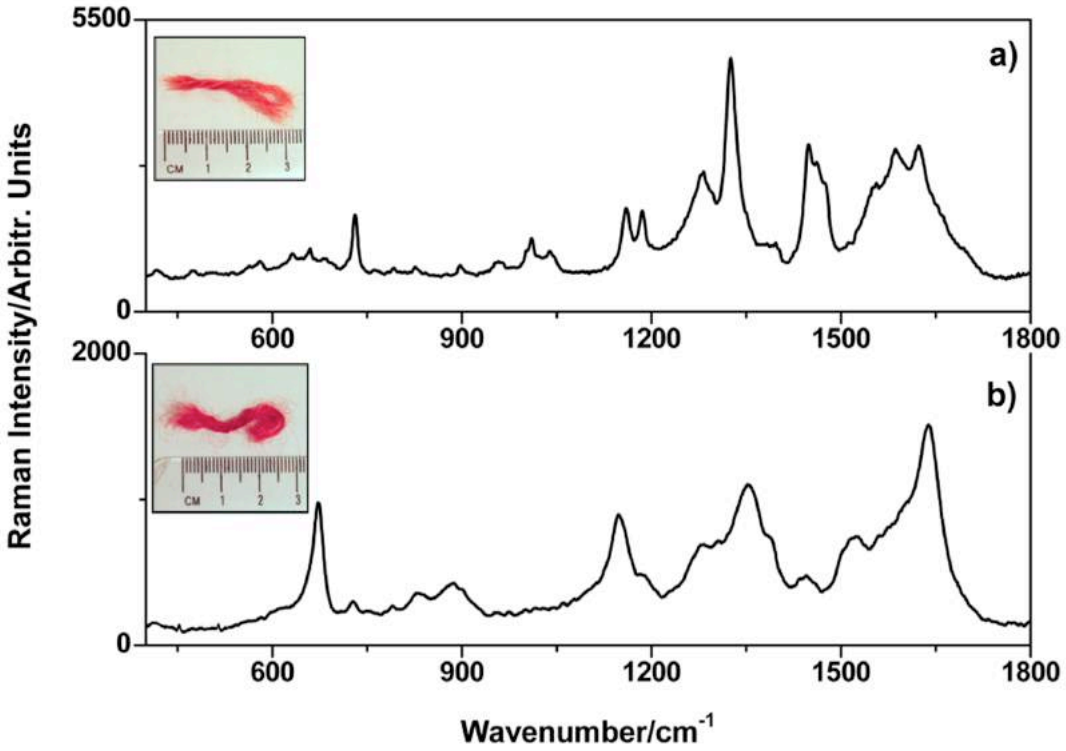

Fig.5.7 SERS spectra of madder (a) and lac-dye (b) performed on the Ag-agar gel-EDTA dried beads after the extraction on wool samples. Excitation at 488nm.

The micro-extractive technique has been found to be extremely helpful for the detection of the dye molecules of a purple printed cotton sample of unknown chemical composition. Due to the purple color of the piece of textile, it was at first supposed to be carminic acid. SERS measurements performed on the dried beads of Ag-agar gel-EDTA after the micro-extraction, have revealed instead the presence of alizarin (Fig.5.8) the main chromophore of madder.

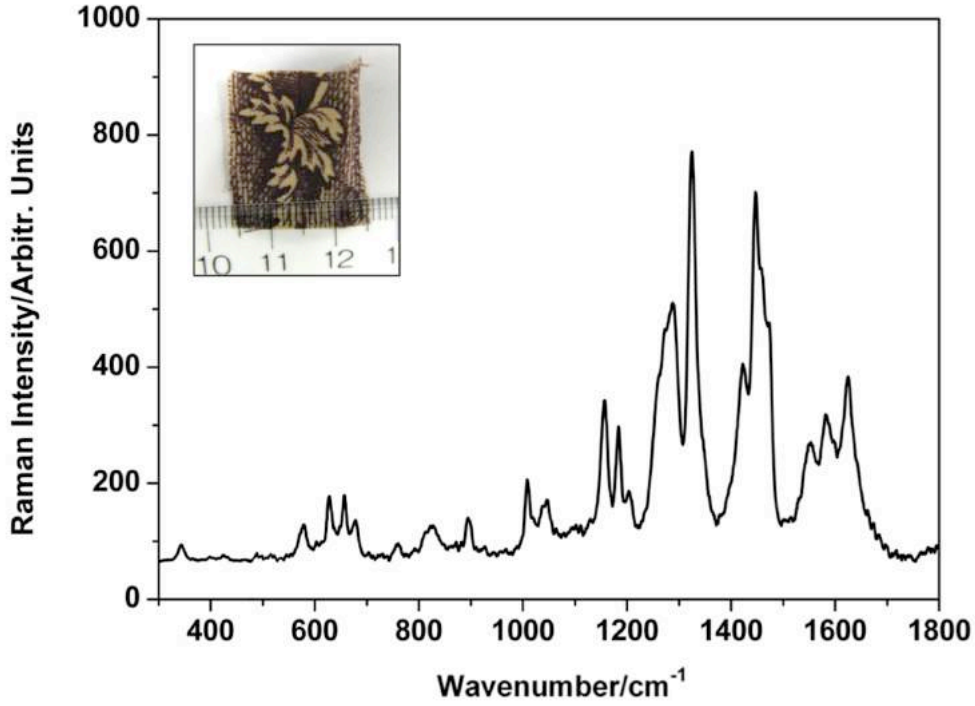

Fig.5.8 SERS spectrum of alizarin, from a piece of printed cotton of unknown chemical composition. Excitation at 488nm.

In order to evaluate the safety of the micro-extraction by means of Ag-agar gel with the add of EDTA within its structure, pictures of the analyzed area of the printed cotton sample, before and after the micro-extraction, have been taken. Microscope pictures have clearly showed the extreme safety of the method (Fig.5.9), since no blurring or shading have been noticed after the extraction, confirming the suitability of this technique for the detection of organic dyes on artworks.

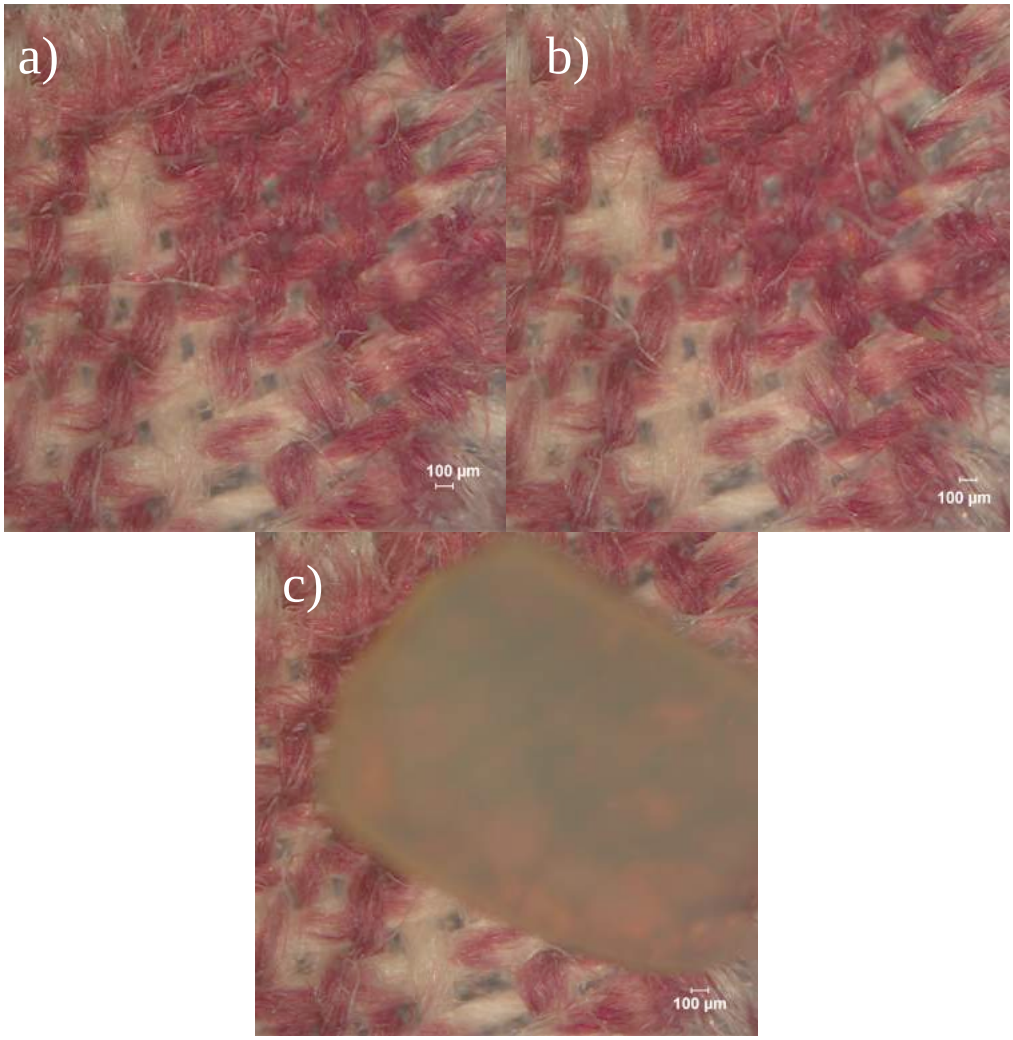

Fig.5.9 Pictures taken under microscope of the printed cotton area before (a) and after (b) the micro-extraction by means of Ag-agar gel. On the bottom (c) microscope picture of Ag-agar gel EDTA taken during the micro-extraction on the printed cotton sample of unknown chemical composition.

In order to corroborate the SERS results, HPLC analyses have been performed on the printed cotton sample. The chromatogram (Fig.5.10) has confirmed the presence of madder as dyeing agent. Unlike HPLC results of reference samples dyed with madder, where alizarin is usually found to be abundant together with purpurin, munjistin and other anthraquinones as minor colorants, in this case, only alizarin was detected as a major colorant, and a very low contribution of purpurin and munjistin has been noticed on the sam-

ple. These results could be ascribed to the use of synthetic madder as dyeing agent or, the natural madder used, might have been processed for printing purposes.

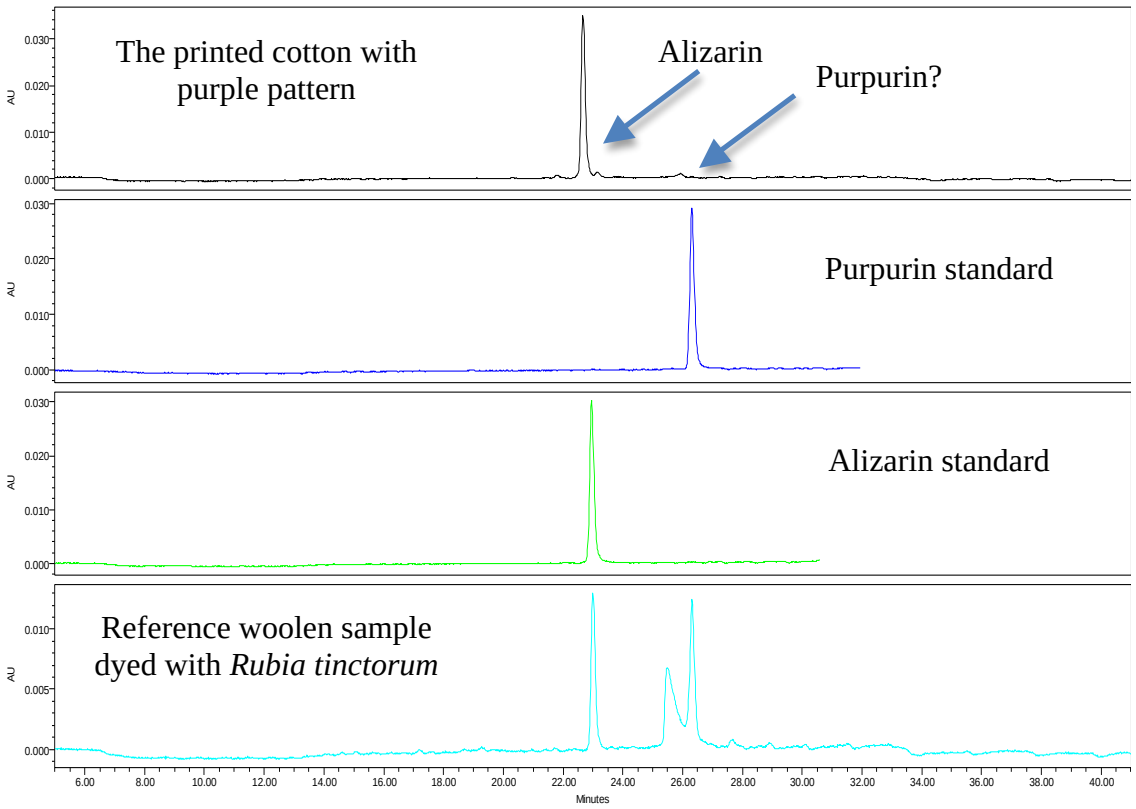

Fig.5.10 Chromatograms at 450nm of HCl extract from the cotton purple sample and a reference woolen sample dyed with madder, and alizarin and purpurin standard.

The HCl extract of the purple yarn sample from the printed cotton showed a main peak at 227 minutes. The UV-spectrum of the peak and the retention time matched those of alizarin (Figg. 5.10, 5.11-5.16). A minute peak at 25.7 minute was detected in the chromatogram in Fig.5.13. Since the peak is extremely weak, it is hard to identify it, however, the peak may be purpurin because the UV-spectra is similar to that of purpurin and eluted at the same retention time. Other minute peaks were observed at 21.8 and 23.1 minutes and they may be anthraquinones, however they are not commonly detected from samples dyed with madder, *Rubia tinctorum*. It appeared that the extract of the purple yarn sample of printed cotton contained alizarin as a major colorant. Further study is necessary to in-

vestigate the reason why only alizarin was detected: Synthetic alizarin may be used, or the natural madder dye may be processed.

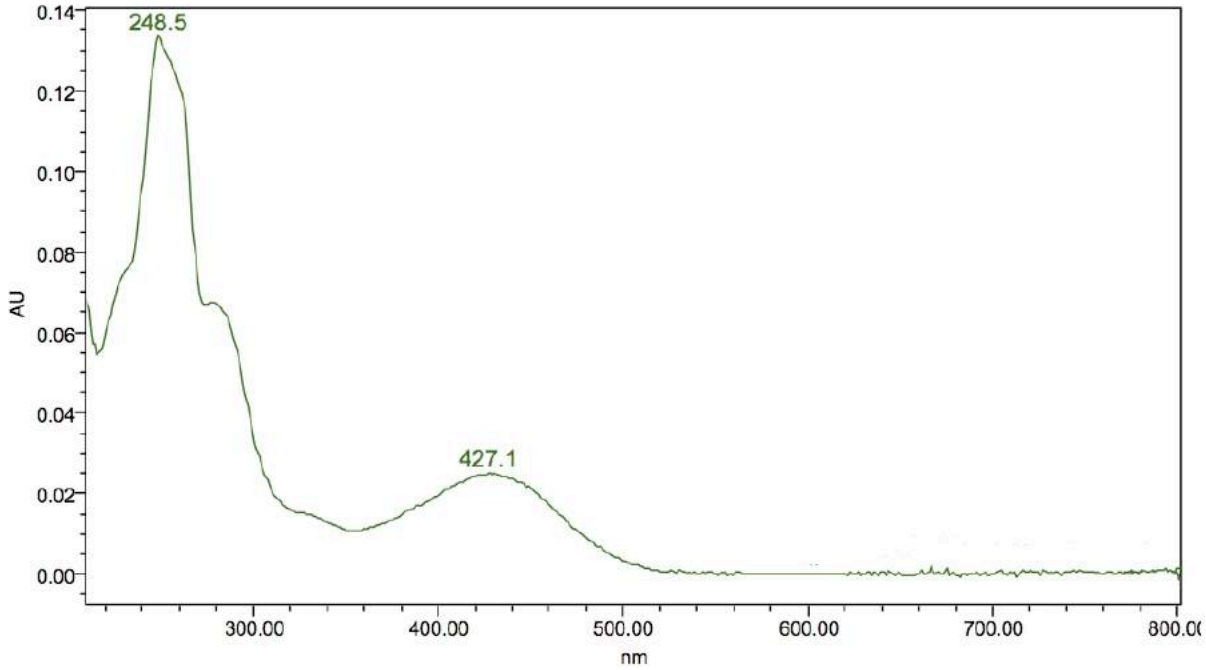

Fig.5.11 UV-Vis transmittance spectrum of alizarin standard.

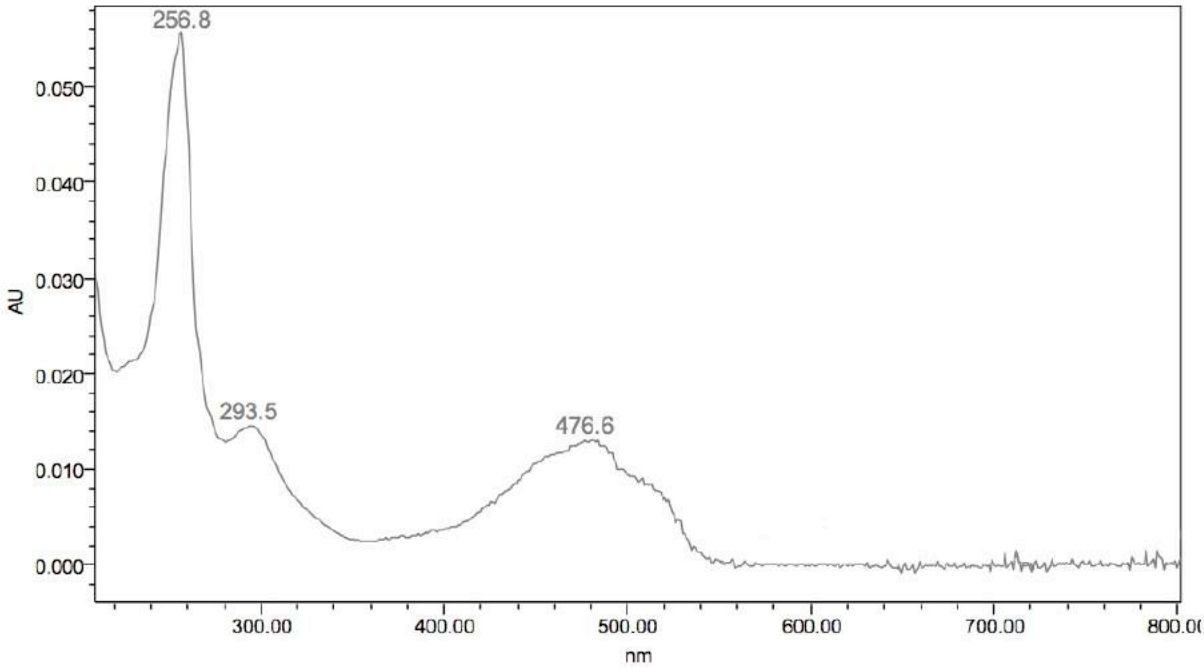

Fig.5.12 UV-Vis transmittance spectrum of purpurin standard.

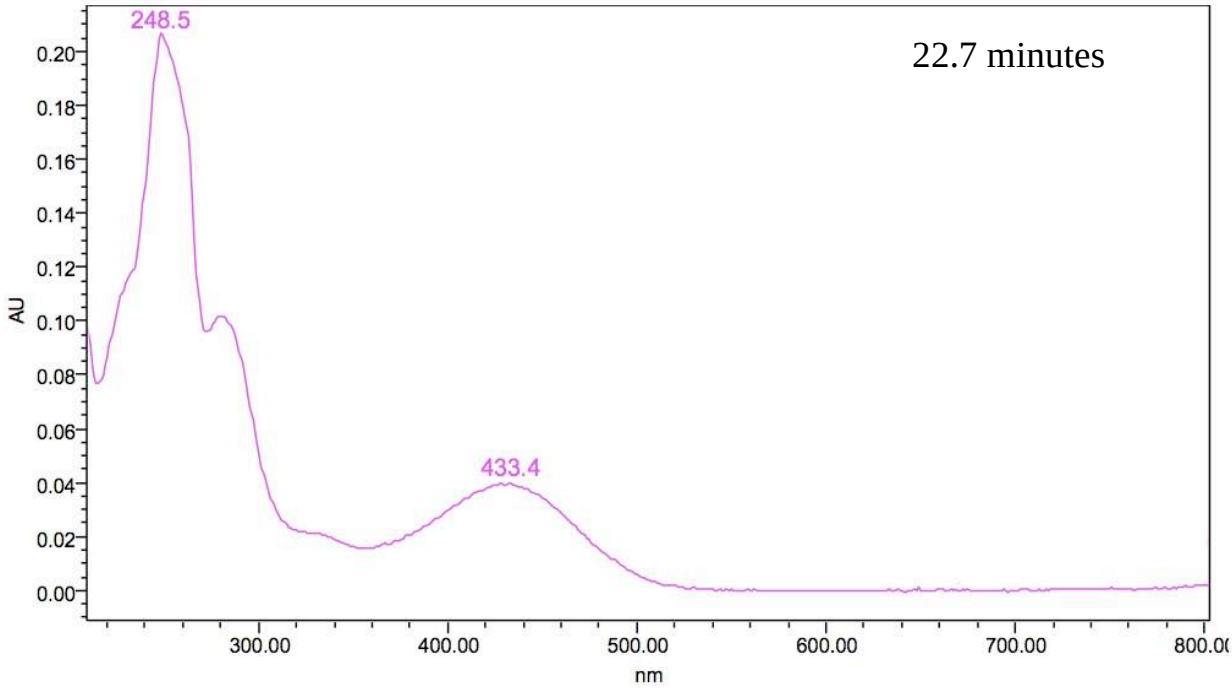

Fig.5.13 UV-Vis transmittance spectra of the peaks at 22.7 minutes of the HCl extract from the printed cotton.

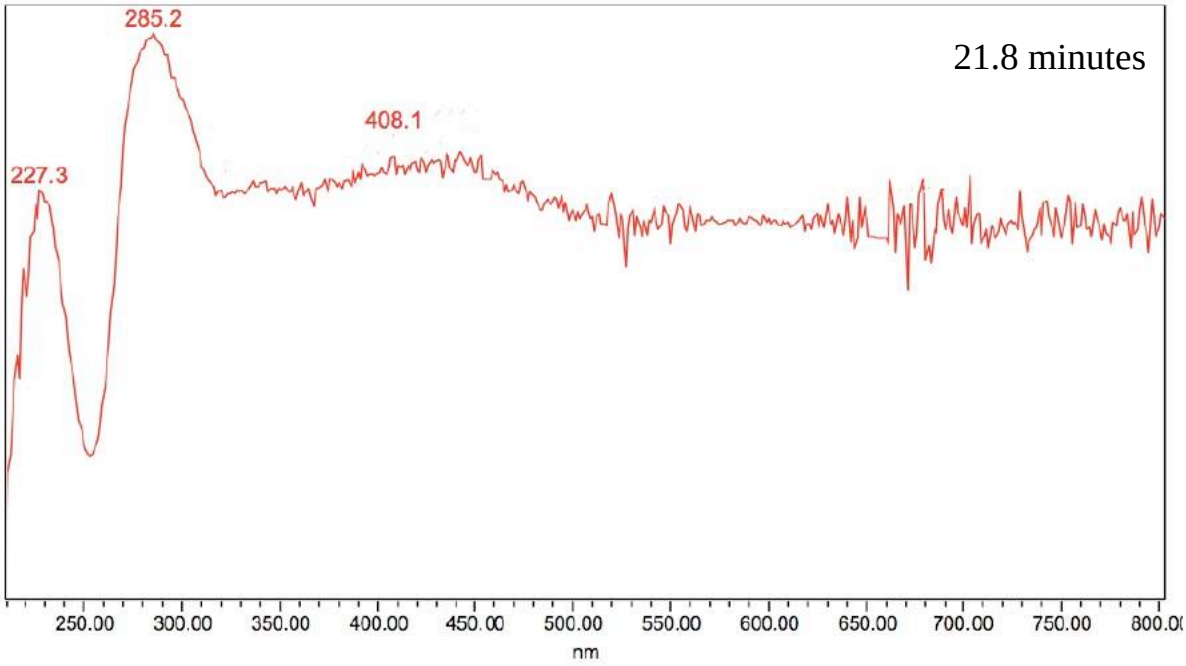

Fig.5.14 UV-Vis transmittance spectra of the peaks at 25.9 minutes of the HCl extract from the printed cotton.

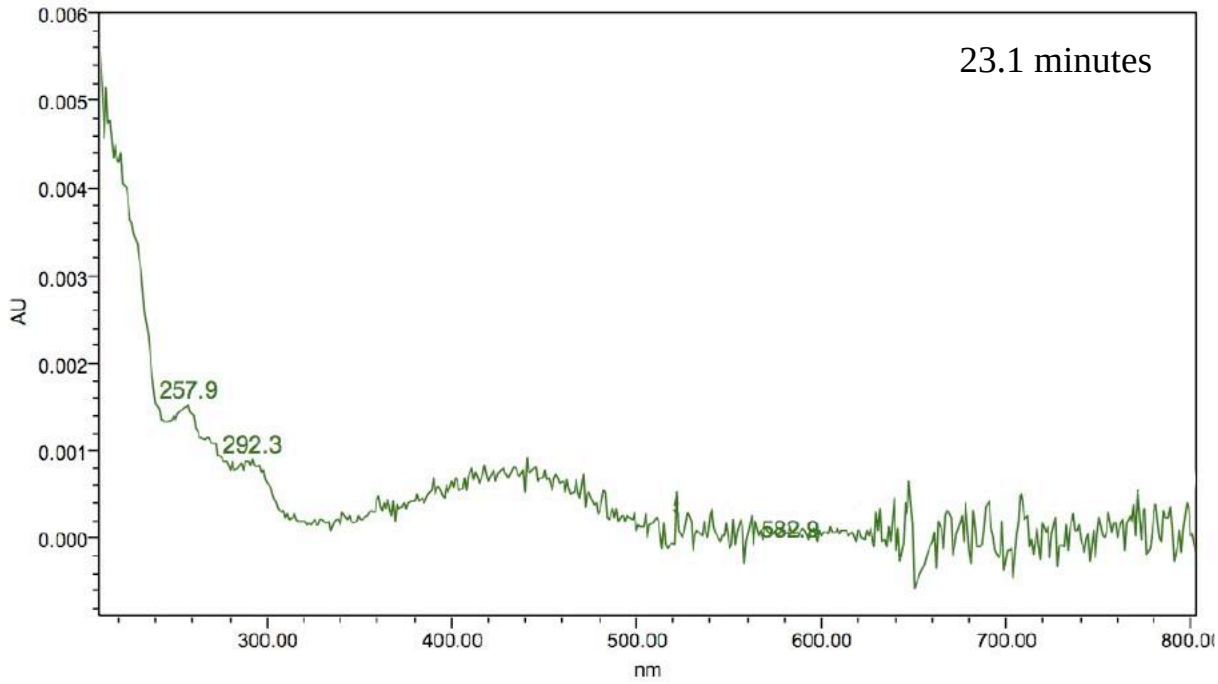

Fig.5.15 UV-Vis transmittance spectra of the peaks at 23.1 minutes of the HCl extract from the printed cotton.

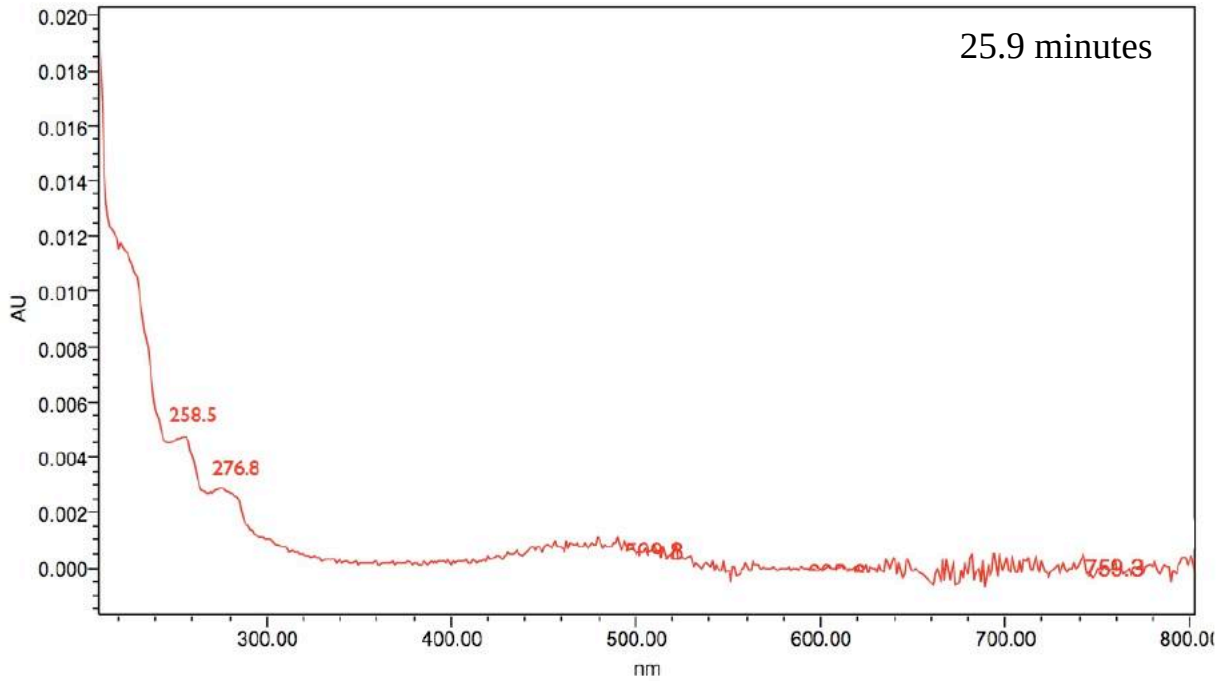

Fig.5.16 UV-Vis transmittance spectra of the peaks at 25.9 minutes of the HCl extract from the printed cotton.

Conclusions

In this work, Ag-agar gel formula ha been improved for the micro-extraction of mordanted dyes on textile samples of silk, wool, printed cotton and for the micro-extraction of lakes on a mock-up panel painting. The addition of a further chemical to the Ag-agar gel formula, the chelating agent EDTA, has dramatically increased the gel capability of extracting the analyte from the substrate. The medium has been found to be extremely safe for the analyzed substrates, confirming its high potential in being applied in the field of artworks conservation for the detection of molecules at very low concentration. Different extraction times have been noticed for each substrate, showing that the micro-extraction efficiency strongly depends on the nature of the analyzed substrate. No hydrolysis treatments, such as the exposition to hydrofluoric acid vapors have been required before performing the extraction of the dye compound. The methodology has been particularly suitable for the understanding of the chemical composition of a purple printed cotton sample provided by the Department of Textile Conservation of the Metropolitan Museum of art. SERS measurements performed on the dried Ag-agar gel EDTA dried bead, after the micro-extraction on the piece of textile have revealed the presence of alizarin. These results have been corroborated by means of high performance liquid chromatography analyses, which have confirmed the suitability of Ag-agar gel substrates for the ultra-detection of organic dyes in works of art. The possibility of adding further solvents and components within the gel structure for the improvement of the extractive performance of the nanocomposite matrix, offers the possibility of tailoring the gel for the extraction of specific molecules and for the potential discrimination and recognition of single components of a mixture.

Chapter 6

Comparison between Lee-Meisel and Microwave colloid in Ag-agar gel

Abstract

Ag-agar gel, synthesized coupling agar-agar gel with the silver colloidal dispersion prepared according to Lee-Meisel procedure, has been compared with a Ag-agar gel formula synthesized according to the "microwave colloid" preparation, whose protocol has been carried out by the Department of Scientific Research of the Metropolitan Museum of Art. The advantages provided by the microwave colloid, with respect to Lee Meisel colloid are related to a higher stability and a higher monodispersity. Spectroscopic results have shown that the Lee-Meisel colloid present in the agar gel nanocomposite matrix, is more suitable for the enhancement of the analytes englobed within its structure with respect to the Ag agar gel formula prepared with the "microwave colloid".

Introduction

Colloidal dispersions trapped inside hydrogel networks made of natural or synthetic polymers, have been found to be extremely suitable for the SERS detection of molecules at very low concentration. Nanoparticles can be coupled to the polymer by means of several procedures. By previously synthesizing the colloid via chemical or physical pathways, or simply by using the polymer as reducing and capping agent for the metal salt, accomplishing an *in situ* reduction of the nanoparticles inside the nanocomposite matrix macroreticulum. As a matter of fact, due to the large free space offered by hydrogels between the crosslinked networks in the swollen state, they can act as nanoreactors for the nucleation and growth of the nanoparticles.[167] Each procedure provides systems characterized

by a wide range of nanoparticles size, shape and distribution, depending on the purpose and use of the gel to be followed. Trapping nanoparticles inside a polymer should favor a higher stability and a greater uniformity of the silver nanoparticles distribution. In particular, natural occurring biopolymers such as agarose, because of their oxygen rich functionalities and their high affinity towards metals, are extremely suitable for the stabilization of metal nanoparticles.[168,169] Moreover, these nanocomposite systems, have been found to have a strong anti-microbial activity, whose most common mechanisms proposed concern the gradual release of free silver ions followed by disruption of ATP production and DNA replication, direct damage of the cell membranes produced by silver nanoparticles and the development of reactive oxygen species by silver nanoparticles and silver ions.[170-172] With regards to the analysis of organic dyes in artworks, whose concentration is extremely low due to the high tinting power of these materials, the possibility of applying non-destructive and ultra-sensitive methodologies for their detection, is one of the most discussed issues in the world of art conservation. The use of Ag-agar gel formula, discussed in this work have appeared to be a quite successful and cutting-edge technique for the ultra-detection of organic dyes in artworks without any detriment of the analyzed object. The entrapment of the dye molecules inside the nanocomposite network of the hydrogel, followed by their recognition by means of SERS analyses, is an extremely suitable technique for the accomplishment of non-destructive ultra-detection of organic dyes. Until now, the use of the silver colloidal dispersion synthesized according to Lee-Meisel protocol[127] has been widely exploited, providing excellent SERS results. In this chapter the comparison among Ag-agar gel coupled with silver nanoparticles synthesized according to Lee-Meisel procedure and the Ag-agar gel formula prepared using silver nanoparticles synthesized by means of a microwave digestion system, is presented.

6.1 Experimental

6.1.1 Synthesis of Ag-agar gel with Lee-Meisel and "microwave colloid"

Two colloidal solutions have been prepared according to Lee-Meisel procedure and to the "microwave" procedure. Silver colloid synthesized by microwave-supported glucose reduction of silver sulfate in the presence of sodium citrate as a capping agent has been prepared according to a previously published protocol by Leona et al.[97] In detail, 100 mg of $AgNO_3$ are dissolved in 5 mL of cold ultrapure water and 10% H_2SO_4 is added dropwise to precipitate Ag_2SO_4. The precipitate is washed twice with ultrapure water and then left to dry on a piece of filter paper. It is then dissolved in ultrapure water to give a 5 x 10^{-4} M solution. For the synthesis in the microwave digestion system, 25 mL of the silver sulfate solution were added to a pressure resistant Teflon microwave vessel (CEM Ultimate Digestion Vessel UDV 10, CEM Corporation)(Fig.6.1), together with 2 mL of a 1% w/w solution of glucose and 1 mL of a 1% w/w solution of sodium citrate.

Fig.6.1 On the left: CEM-MDS 2100 microwave digestion system used for the synthesis of the silver sulphate colloid; On the right: pressure resistant Teflon microwave vessel where the nanoparticle synthesis is carried out.

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 (Fig.6.1). 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 the colloid for use, it was centrifuged for 5 minutes at 16,060 x g RCF (relative centrifugal force) with a Fisher Scientific Accuspin 400 centrifuge, and the supernatant was then removed and replaced with the same amount of 18 MΩ ultrapure water (Millipore Simplicity 185 water purification system). Silver nanoparticles synthesized by microwave-supported reduction present several advantages with respect to the Lee-Meisel colloid. 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 (Fig.6.2).

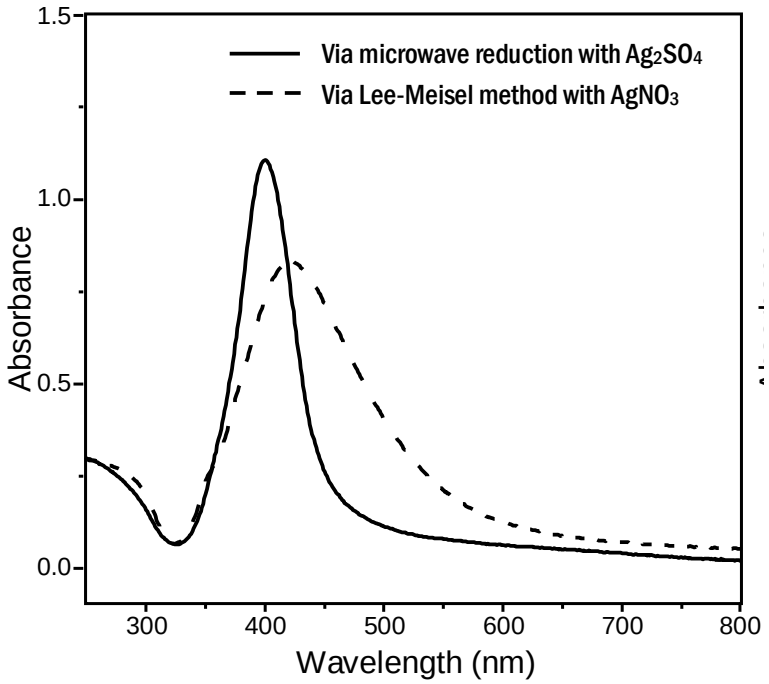

Fig.6.2 Comparison between UV-Vis spectra of silver nanoparticles synthesized according to microwave assisted reduction (solid line) of silver sulfate and according to the Lee-Meisel protocol (dot line).

In detail, previous 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 = 20-100 nm. Sequential UV-Vis measurements of the colloid absorption over time (Fig.6.3) shows that microwave nanoparticles, are stable and efficient over several months, thus leading to more reproducible SERS performances.

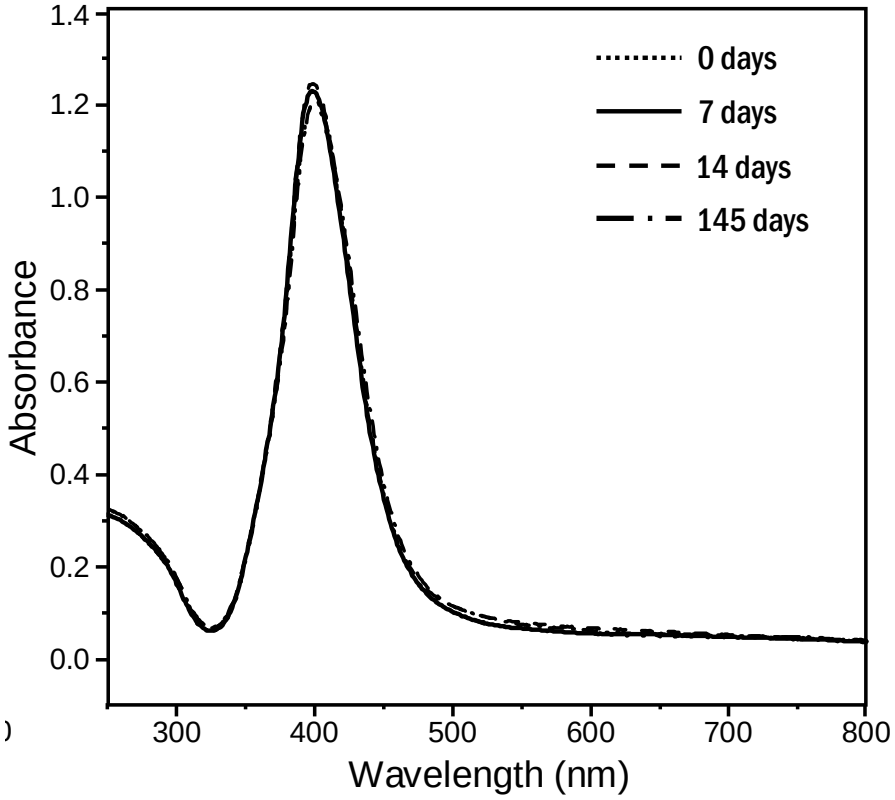

Fig.6.3 UV-Vis measurements of the microwave assisted reduction colloid taken at several time intervals, showing the high stability of the colloid in time.

The other colloidal solution used for the synthesis of Ag-agar gel has been prepared according to the Lee-Meisel protocol[34]. In particular, 0.008495g of $AgNO_3$ have been dissolved in 50ml of 18 MΩ ultrapure water (Millipore Simplicity 185 water purification system). Another solution has been prepared, dissolving 0.05g of sodium citrate in 5ml of 18 MΩ ultrapure water. The silver salt solution has been brought to boil and once boiling,

1ml of the sodium citrate solution has been added dropwise. The solution has been left boiling under reflux for one hour. After the time required for the reduction of the Ag ions, the flask containing the colloid has been put in an ice-bath to cool down, instead of being cooled at room temperature. As a matter of fact, it has been observed that the prompt cooling process of the silver colloid provides a wider number of nanoparticles with a diameter of about 40nm[35], which have been found extremely suitable in terms of SERS enhancement when combined with the agar gel matrix.

For the synthesis of Ag-agar gel, 0.2g of agar-agar in flakes (analytical grade) have been mixed in a beaker with 10ml of the Lee-Meisel and the microwave colloidal dispersions (Fig.6.2). The mixture has been heated in a common microwave for a few seconds at the lowest heating power and then poured inside a petri dish. Once cooled, the petri dish containing the nanocomposite matrix is stored in the dark inside a sealed glass container. In these storage conditions the gels have been found to be stable for up to 6 months.

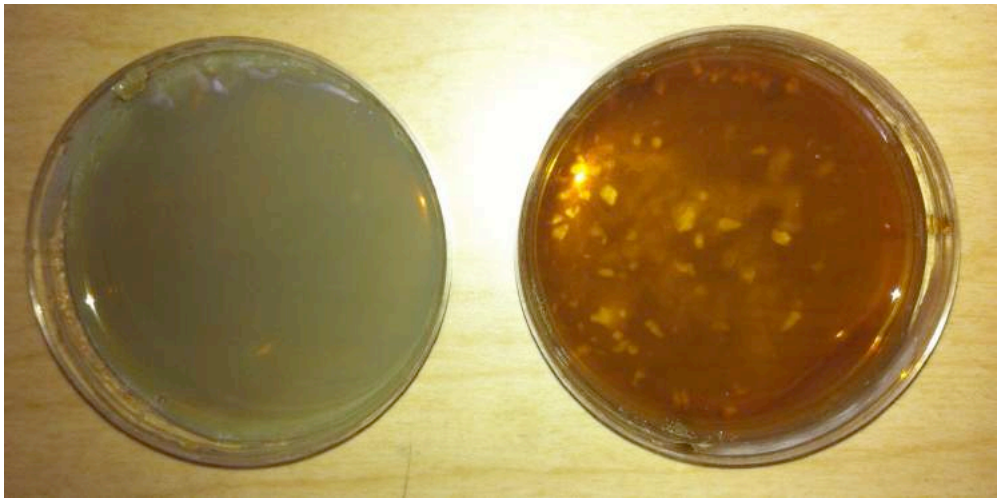

Fig.6.2 Ag-agar gel coupled with Lee-Meisel colloid (left) and microwave colloid (right).

6.1.2 Preparation of Ag-agar gel for SERS measurements

Two solutions of alizarin (Sigma-Aldrich) and purpurin (Sigma-Aldrich), in ethanol at a concentration of 10^{-4} M have been prepared as reference analyte solutions for the accomplishment of the SERS analyses. Alizarin and purpurin have been chosen because of their wide characterization for SERS purposes. From the Ag-agar gel matrices prepared using Lee-Meisel and microwave colloids, four beads of few millimiters, have been cut out by means of a spatula. On each bead has been pipetted one drop of the ethanolic solutions of purpurin and alizarin, which have been left to dry. Once dried, SERS measurements have been performed by means of a micro-Raman instrumental set-up.

6.1.3 Instrumentation

SERS spectra have been collected using a Bruker Senterra Raman apparatus with a charge-coupled device detector and a holographic grating with a spectral resolution of 3-5cm^{-1} (1800 rulings/mm for the 488nm laser). A Spectra Physics Model 2020 BeamLock Ar^{+}, at 488nm, has been adopted as laser wavelength for the spectral acquisition. Measurements have been performed with the following experimental conditions: integration time 30s, 1 scan, output laser power at 0.4mW (0.03mW on the sample), 20X long working distance Olympus microscope objective. The UV-Vis spectra of the colloidal dispersions have been collected with a Varian Cary 50 spectrophotometer using a quartz cell with a path length of 1mm.

6.2 Results and discussion

SERS measurements have been performed both on the dried beads of Ag-agar gel coupled with Lee-Meisel colloid and Ag-agar gel coupled with microwave colloid, where drops of ethanolic solutions of alizarin and purpurin at 10^{-4}M were pipetted before the drying of the gel.

These dye molecules, have been chosen as probes for the analyses, because of their high well-known SERS activity. Excellent spectra have been acquired in terms of signal to noise ratio in the case of both dyes, whose drops of the ethanolic solutions at 10^{-4}M were pipetted on the still wet beads of Ag-agar gel prepared by adding the silver colloidal dispersion synthesized according to Lee-Meisel protocol (Figg.6.3-6.4). The spectral acquisition has been carried out by means of a 488nm argon laser source, 50X objective and an integration time of 10 seconds.

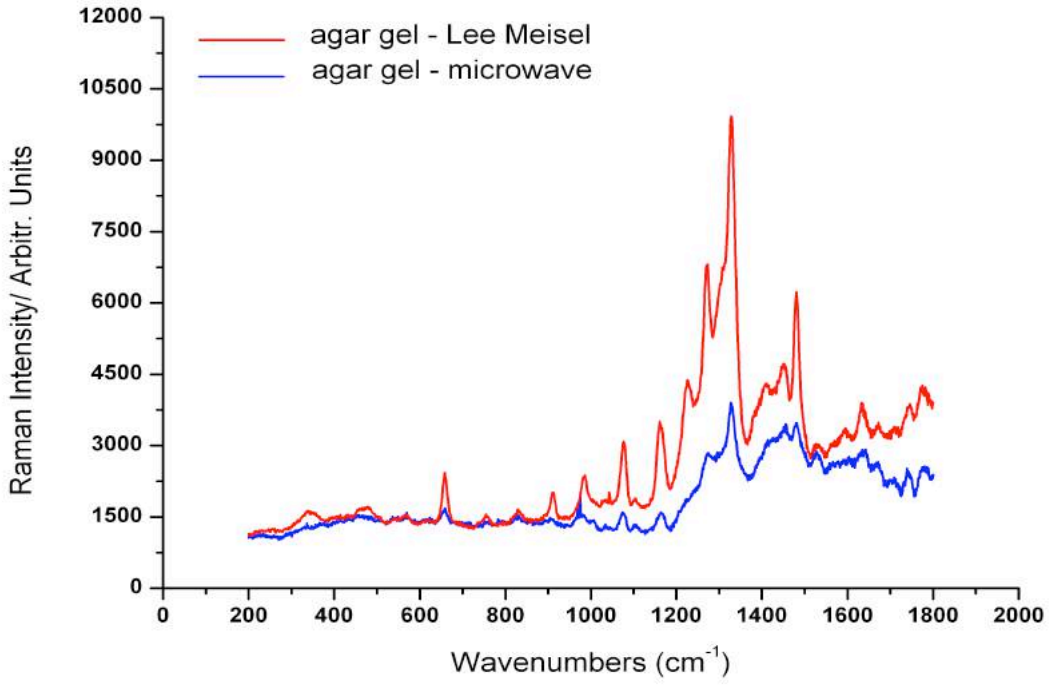

Fig.6.3 SERS spectra of the ethanolic solution of purpurin at a concentration of 10^{-4}M, on Lee-Meisel Ag-agar gel and microwave Ag-agar gel. 488nm excitation laser line.

Good results have been obtained as well in the case of the detection of the two anthraquinone dyes on Ag-agar gel coupled with the microwave colloid, even if the relative intensities noticed are quite weaker with respect to the spectra of alizarin and purpurin registered on the Lee-Meisel nanocomposite matrix. Relevant differences can be noticed among the spectra of alizarin and purpurin detected on the nanocomposite matrix of Ag-agar gel prepared according to the two different synthesis protocols. Although the same Raman frequencies have been observed for both compounds, the spectral profiles of alizarin and purpurin enhanced by Lee-Meisel colloid in Ag-agar gel, are quite different with respect to the spectral profiles of the same probes enhanced by the microwave colloid entrapped inside the gel macro-reticulum. In particular, the spectrum of alizarin absorbed on the microwave Ag-agar gel colloid, the frequencies at 1451 cm^{-1}, attributed to a CC stretching, and a COH and CH in-plane bendings, and at 1420 cm^{-1}, are clearly divided into two single bands, while in the SERS spectrum of alizarin absorbed on the Lee-Meisel Ag-agar gel the two frequencies are unified in a single band.

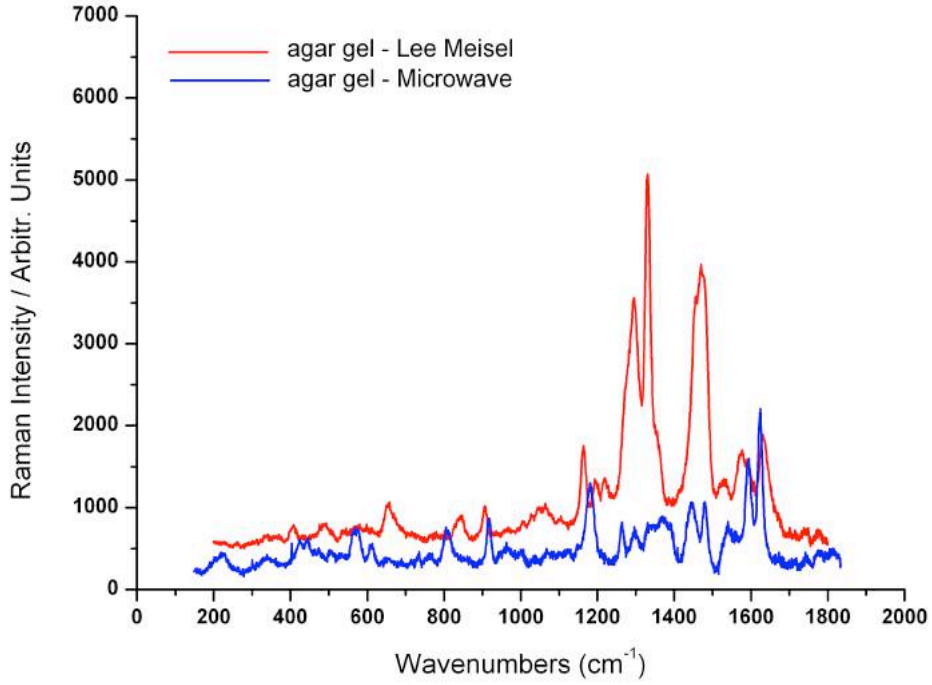

Fig.6.4 SERS spectra of the ethanolic solution of alizarin at a concentration of 10^{-4}M, on Lee-Meisel Ag-agar gel and microwave Ag-agar gel. 488nm excitation laser line.

Ag-agar gel matrix coupled with Lee-Meisel colloid has provided the most intense enhancement, as evidenced by Figg.6.2 and 6.3. In particular, a three times higher relative intensity can be observed in the spectrum of both alizarin and purpurin on Lee-Meisel Ag-agar gel with respect to the spectrum of the same probes on the microwave colloid. This behavior could be related to the size and distribution of the nanoparticles trapped inside the hydrogel network, which differs dramatically between the two colloids. As a matter of fact, as anticipated in the previous paragraphs, the Lee-Meisel colloid, characterized by a wide absorption band, with a FWHM > 120nm, presents a wide nanoparticle size distribution that ranges from few nanometers to 100nm. Instead, the microwave colloid, because of the narrower absorption band, is characterized by a high monodispersity of the metal nanoparticles with respect to the polydispersity of the Lee-Meidel colloid. In particular the particle size range is very small (3-20nm) if compared with the one of the Lee-Meisel colloid. Another explanation could be related to the geometry of the molecule of alizarin when attached to the nanostructured surface, justifying the differences of the bands observed in the spectrum of anthraquinone dye between the Lee-Meisel Ag-agar gel and the microwave Ag-agar gel.

Conclusions

Ag-agar gel has been synthesized by adopting two different colloidal dispersions. Lee-Meisel colloid and microwave colloid. Lee-Meisel colloid, synthesized by reducing $AgNO_3$ by means of sodium citrate, is a high polydisperse colloid characterized by a broad absorption band and by a wide size distribution of nanoparticles. The microwave colloid, is synthesized instead from Ag_2SO_4 which is reduced by means of sodium citrate and glucose. It has a narrow absorption band, indicator of a high monodispersity of the colloid whose nanoparticle size ranges between 3 to 20nm.

Two nanocomposite agar matrices have been prepared by coupling the biopolymer with the Lee-Meisel colloid and with the microwave colloid. In order to check the suitability of both systems, few drops of ethanolic solutions of alizarin and purpurin at a concentra-

tion of 10^{-4} M, have been pipetted on the beads of Lee-Meisel Ag-agar gel and microwave Ag-agar gel, chosen as probes for the assessment of the suitability of the nanostructured substrates for the ultra-detection of organic dyes at low concentration. SERS spectra acquired on the dried beads of Ag-agar gel for each colloidal dispersion, have shown several differences in terms of signal to noise ratio, spectral profile and relative intensity. In particular, the gel coupled with Lee-Meisel colloid has been found to provide the best SERS spectra of alizarin and purpurin. Ag-agar gel synthesized with microwave colloid have provided a quite different spectral profile of alizarin, that could be related to the small size of the nanoparticles inside the microwave Ag-agar gel or to the geometry of the attachment of the alizarin molecule on the nanostructured surface.

General conclusions

In this work a nanocomposite hydrogel made of agar-agar coupled with silver nanoparticles has been developed for a non-destructive micro-extraction in solid phase of organic dyes in works of art. Agar-agar has been chosen as suitable gelling material for this purpose because of its wide application in the field of art conservation as medium for the cleaning of stone, paper and panel paintings. This biopolymer due to its high crosslinking properties, thermoreversibility, biocompatibility and biodegradability is an extremely advantageous medium for the field of conservation of artistic objects whose application agrees with the development of respectful methodologies able to preserve the integrity of the object to be analyzed, without any detriment of it.

Because of the high impact of surface enhanced Raman spectroscopy (SERS) for the recognition of organic dyes in works of art - whose detection was until a decade ago extremely difficult when working in dispersive Raman conditions because of the strong fluorescence background that obscured the weak Raman signal of this compounds - the hydrogel developed for the micro-extraction in solid phase of organic dyes has been coupled with silver nanoparticles. The hybrid organic-inorganic nanostructured surface has been adopted not only as extraction vehicle of organic dyes on works of art but even as a SERS substrate able to enhance the Raman signal of the analyte absorbed. For the synthesis of the nanostructured surface, agar-agar has been coupled with a colloidal dispersion of silver nanoparticles prepared according to the Lee-Meisel protocol, where the metal salt $AgNO_3$ is reduced by means of the capping agent sodium citrate. This colloid has been found particularly suitable in terms of SERS enhancement of the nanocomposite substrate moreover, the presence of metal particles of nanometer size, because of their high surface area, provides the gel with antimicrobial activity.

In order to check the capability of the nanostructured system of actually micro-extracting organic dyes from artistic substrates, it has been applied on cotton mock-up textiles dyed in laboratory using potash alum as mordant agent of the three anthraquinone dyes, alizarin, purpurin and carminic acid, widely employed in the past and found in the majority of the artworks analyzed. In particular alizarin and purpurin are the main chromophores of madder lake, a dye extracted from the roots of the plant species *Rubia tinctorum*, while

carminic acid is the main chromophore of cochineal, extracted from the dried bodies of the females of the insect *Dactylopius coccus* growing on the cactus of central and south America.

SERS measurements performed on the dried beads of Ag-agar gel after the micro-extraction have provided excellent spectra of the extracted chromophores, showing the high potentiality of this analytical tool for the detection of organic dyes on artworks.

Before applying the nanostructured hydrogel on a real case, complementary techniques have been adopted for the assessment of the safety and the sensitivity of the method for the ultra-detection of dyes at very low concentration. While FTIR-ATR analyses performed on the analyzed textiles before and after the extraction have revealed that no residuals of the nanostructured biopolymer are released on the surface, XRF and colorimetric analyses have allowed to assess the absence of silver on the analyzed area and the absence of discoloring, fading and blurring effects, confirming the technique an extremely safe method for the non-destructive micro-extraction of organic dyes on art object. Once estimated the safety of the method, a real case has been analyzed, a pre-Columbian piece of textile of a private collection, whose composition was not clear. SERS measurements of the dried Ag-agar gel bead after the micro-extraction have shown the presence of alizarin, the main chromophore of madder lake.

In the second part of the work, Ag-agar gel has been applied on a new class of substrates in order to assess its suitability on silk, wool, printed cotton and on a mock-up panel painting, whose samples have been provided by the Department of Textile Conservation and Scientific Research of the Metropolitan Museum of Art. In order to improve the micro-extractive performances of Ag-agar gel on these substrates, a further agent has been added to the nanostructured matrix, the chelating agent EDTA, widely used among the extractive vehicles as water and DMF for the accomplishment of solid phase micro-extraction applications. Ag-agar gel EDTA formula has been found to be extremely suitable for the detection of organic dyes of the aforementioned samples. In particular, the technique has been crucial for the recognition of the dyeing agent presents in the printed cotton sample of unknown chemical composition. SERS spectra have revealed the presence of alizarin, main chromophore of madder lake. SERS results have been corroborated by HPLC analyses, which have confirmed the suitability of Ag-agar gel EDTA for the de-

tection of the coloring compound. Ag-agar gel synthesized by coupling the biopolymer with Lee-Meisel colloid, has been moreover compared with another formula of gel coupled with a colloidal dispersion synthesized in a digestion microwave system. SERS measurements on both gels have revealed a better enhancement provided by the Lee-Meisel colloidal dispersion.

Thus, Ag-agar gel has been proven to be an efficient and suitable medium for the microextraction and SERS detection of organic dyes in works of art. Further improvements may be accomplished in order to make the nanocomposite gel able to selectively extract organic dyes from artworks.

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