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DOCTORAL THESIS

Exploiting the potentiality of surface-enhanced Raman scattering (SERS) for the identification of natural dyes in ancient textiles: from hyphenated techniques to *in-situ* analysis

CHIM/01 - CHIM/12

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Autobiography in Five Chapters

Chapter 1

I walk down the street.
There is a deep hole in the sidewalk.
I fall in.
I am lost… I am hopeless.
It isn't my fault.
It takes forever to find a way out.

Chapter 2

I walk down the same street.
There is a deep hole in the sidewalk.
I pretend I don't see it.
I fall in again.
I can't believe I am in the same place.
But it isn't my fault.
It still takes a long time to get out.

Chapter 3

I walk down the same street.
There is a deep hole in the sidewalk.
I see it is there.
I still fall in… it's a habit.
My eyes are open.
I know where I am.
It is my fault.
I get out immediately.

Chapter 4

I walk down the same street.
There is a deep hole in the sidewalk.
I walk around it.

Chapter 5

I walk down another street

(Portia Nelson)

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INTRODUCTION

Surface-enhanced Raman scattering (SERS) is nowadays a well-assessed spectroscopic tool for the identification of historical natural dyes in the scientific community of analytical chemists, even if not yet entirely established in the common practice of laboratories devoted to the chemical characterization of works of art.

This technique is based on the enhancement of Raman bands that takes place when a molecule is placed in the proximity of, or possibly adsorbed on, a roughened metal surface, provided by a pre-treated electrode, a metal colloid or a metal thin film. Silver is the most frequently used metal, thanks to the relevant enhancement it provides with visible excitation wavelengths, and colloids are the preferred substrates for analytical applications, given their easiness of preparation. Researchers first composed databases of SERS spectra of natural dyes in solution obtained with different excitation wavelengths to provide suitable references for the identification of the same in ancient textiles and paintings. Subsequently, new protocols of analysis have been developed to improve the identification of dyes in complex matrices, such as micro-samples coming from valuable artistic and historical objects.

In the present Ph.D. project, surface-enhanced Raman scattering is studied in order to overcome some limitations both inherent to the technique and to ancient colored samples and artifacts. The methodologies developed evolved from the knowledge of a very recent literature, with continuous cutting-edge progresses.

In more detail, as suggested above, to complete the transfer of the SERS technique from the research laboratory to the specialized analytical laboratory, a further enlargement of the database is still required. Indeed, the range of natural dyes used historically worldwide in craftsmanship and art is really huge and, even if some of them were employed in different geographical areas, many others were typical of a given zone being obtained from local vegetable (or, less frequently, animal) sources.

In order to contribute to accomplish this aim, a SERS study of anthocyanins by means of SERS was carried out for the first time and reported in the present doctoral thesis. The work regarded both SERS analyses of anthocyanins as pure molecules and in mixture with other compounds in plants and textiles. Moreover, the hypothesis of an anthocyanin content of the so-called *folium* dye, whose main chromophores are still unknown, was assessed.

It is worth remembering that, even if SERS shows, as usual for vibrational spectroscopy, a remarkable specificity in the identification of an analyte, its use can be partially hindered when the sample is more properly a mixture of compounds, as frequently happens for natural dyes that contain many colored and uncolored substances and that were often used in mixtures to yield a given tint. Indeed, this is the reason why a separative technique, namely high-performance liquid chromatography (HPLC), is still the most widely employed analytical tool for this sort of studies, usually coupled with UV-visible diode-array detection (HPLC-PDA). In spite of its sensitivity, however, this kind of detection can sometimes lack in specificity. Therefore, SERS proposes itself as an alternative, or parallel, detection method for HPLC analysis of natural dyes. Few studies have been reported in the literature about the development of equipments combining HPLC and SERS techniques, mainly applied to the analysis of drugs. A part of the present project was therefore devoted to the optimization of a laboratory-made HPLC-PDA-SERS hyphenated system to be used specifically for the identification of natural organic dyes.

Of course, being conceived for solutions, the application of SERS as well as of HPLC to the analysis of colorants in ancient objects requires as first step the extraction of the dyes from a sample drawn from the object itself. Dyestuffs were indeed fixed on textiles or incorporated in painting as metal complexes. Extractions are of course time-consuming, as well as obviously destructive with regard to the sample. For this reason, non-extractive protocols based on SERS analysis performed directly on the

sample itself have been recently suggested in the literature.[1] "Extractionless" analyses can be performed on textile fragments as such, or on samples previously hydrolyzed by acid vapors. In this latter case, usually a hydrolysis pre-treatment of micro-samples is executed, in order to break the dye-mordant complexes without removing the dye from textiles, followed by direct SERS analysis onto the sample. A further aim of the present project was indeed to contribute to the development of an "extractionless" protocol to be applied to the widest range of dyes and to the least amount of sample, possibly reducing problems such as the interference of the textile substrate and the fluorescence background typical of ancient samples. The use of a less-energetic source of radiation, as in the Fourier-transform Raman spectroscopy (1064 nm), was therefore investigated to match these aims. Finally, it should be noted that even extractionless methods are not completely respectful of the integrity of the artefact being examined, requiring a sample even if as small as possible. Being this an issue of the outmost importance for conservators, it is evident that an entirely non-destructive application of SERS technique would be highly desirable. Some attempts in this regard were also carried out, both at Duke University (USA) in collaboration with the research group of Prof. Tuan Vo Dinh and at the Department of Chemistry of Università degli Studi di Milano. Different solid nanoplatforms were experimented and tested for the enhancement of Raman signals of dyes directly on textiles, exploiting solid-solid interactions between the dyes and the nanomaterials with the final aim to allow the identification of these molecules without need of displacing the artefacts to which they belong from their site of storage.

Finally, it is worth underlining that, as SERS protocols need to be tested not only on reference dyes, but also on ancient samples to verify their performance in real analytical problems, the present project investigated also the applicability of the SERS techniques developed for recognizing dyes in ancient textile samples from artistic collections.

[1] F. Pozzi, J. R. Lombardi, S. Bruni, M. Leona, Analytical Chemistry, 84, 3751-3757 (2012).

CHAPTER 1

The chemical characterization of natural organic dyes: state of art

Abstract

This introductory Chapter is intended to provide a short overview of natural organic dyes and their use both in textile dyeing and applied to objects of historical and artistic value. The chemical aspect of traditional processes involved in the dyeing of artifacts will be described, as well as the analytical methods employed by researchers aiming to the identification of dyes. Among the different analytical techniques, special emphasis is placed on the application of surface-enhanced Raman scattering, a rather newly developed analytical tool that has recently attracted increasing attention in this context thanks to its great potential in detecting minuscule amounts of colorants with very high sensitivity.

Organic colorants in art and history

"Ogni erbaccia fa tinta", literally "any weed gives a dye", was the expression that Florentine dyers of the early 15th century were used to say when they want to joke.[1] Indeed, prior to the introduction of synthetic dyes in the 19th century, all colorants were derived from the large palette offered by natural species. Coloring matters were extracted from both vegetal sources, such as roots, berries, wood, bark and leaves and living organisms, such as lichens, insects and sea snails. Therefore, it is easy to understand that the range of natural dyes used historically worldwide in craftsmanship and art is really huge. However, on the other hand, ancient dyers acquired such a relevant knowledge concerning dye-plants (or animals) from which extract the coloring matter that they started to select plant families and animal species that were particularly rich in certain colorants and to use them as raw material to dye textiles. Moreover, they experimented a wide variety of recipes to improve the fixing of the color to the fibers: in the dye-baths, besides the dyeing matter, other substances, both mineral (salts, mud, metal oxides) and organic (urine, excrement, fats etc.) were added, as that appeared to improve the fixing of dyes on

textiles. It should be considered a form of “mordanting”, word coming from the Latin *mordere*, “to bite”. Indeed it was thought that metals could attached themselves to the fibers by “biting” textiles.[1] In this regard, natural organic colorants used in textile dyeing can be divided into three different categories, depending on the procedure according to which they were applied to fabrics:[1-4]

- **Vat dyes:** The name of these dyes origins from the dye-baths used in the process of dyeing textiles, the so-called “vats”. Colorants belonging to this class are water-insoluble in their colored form and the necessity to render them soluble put dryers to test. They rapidly discovered that under reducing conditions these dyes can be converted into a “leuco” form, soluble in alkali that, in ancient times, were derived from either wood ash, plant ash, lime or stale urine. Immersion of textiles into the dye solution allows the dissolved molecules of colorant to penetrate the fibers. After that, upon removal of wet textiles from the bath and exposure to atmospheric oxygen, these substances were oxidized back to their colored forms. The best-known blue dyes, indigo and woad, as well as Tyrian purple, belong to this category.

- **Mordant dyes:** The majority of the dyeing molecules of natural origin does not have a strong chemical affinity for textile fibers and therefore require to be treated prior to the dyeing stage. On the other hand, they can combine with various metallic salts, the so-called “mordants”. In the past, a solution of a mordant was used to impregnate the fibers, then the colorant was added to the pre-treated textile. In chemical terms, mordant dyestuffs exhibit appropriate functional groups, i.e. hydroxyl, amino or carboxyl groups, that are able to form a complex

Figure 1. A step of the dyeing process of textiles

with metal ions. Also textile fibers are natural materials rich in functional groups suitable to bond complexed dyestuffs, in particular wool and silk display carboxyl and amino groups. The metal ion therefore seems to mediate the interaction between the dye molecule and the fiber itself. Examples of mordants are aluminum, iron, tin, chromium and copper ions; one of the substances most commonly employed in ancient times for this purpose was potassium alum, ($KAl(SO_4)_2 \cdot 12H_2O$), a compound that naturally occurs in various minerals such as kalinite, alunite and leucite. Commonly employed in association with mordants were dye-assistants such as cream of tartar or oxalic acid that were thought to brighten the colors, protect the fibers and help the absorption of the mordants.

- **Direct dyes:** Also called substantive dyes, they were applied directly to textiles without requiring any special treatment, but usually they resulted less wash- and lightfast than vat or mordant dyes. Direct dyes are bound to the fibers by secondary bonds (van der Waals forces, dipole association and hydrogen bonding). Chemically, they are often large linear, flat, water-soluble molecules, whose flat shapes promote an easy diffusion into the fibers.[2] A typical example of direct dyes is the carotenoid crocetin in saffron.

Nowadays the main chemical compounds responsible for the colors observed are well known and characterized. It is worth remembering that materials appear colored as they selectively absorb light in the visible region of the electromagnetic spectrum. These molecules usually exhibit ketone groups, conjugated double bond systems or aromatic systems, being the latter chemically more stable than long chain conjugated compounds.[4] In more detail, a *chromophore* is that part of a molecule which led to absorption in the visible region of the electromagnetic spectrum, thus it is responsible for the observed color. It usually consists of functional groups belonging to systems of conjugated double bonds, such as the vinylene group (-C=C-), the

carbonyl group (-C=O) and the nitroso group (-N=O).[2, 4] On the other hand, *auxochromes* are chemical moieties that do not give rise to color themselves, but caused a shift of absorption of an already colored compound. Hydroxyl groups (-OH), carboxyl groups (-COOH) and the amino groups ($-NH_2$) could be considered auxochromes. If the absorption is shifted to longer wavelength the effect is called *bathochromic*, while the reverse is named *hypsochromic* effect.

On the bases of the chemical structures of the main compounds contained in natural dyes, it is possible to classify them. Generally, chromophores of red dyes are mostly anthraquinones, while the yellow tints were given by flavonoids and the blue ones by indigoids (Figure 2), even if other compounds belonging to different chemical classes, such as carotenoids, naphtoquinones and tannins were encountered as well.

Figure 2. (a) Madder roots, from which an antraquinone-based dye is extracted, (b) flowers of dyer's broom from which a flavonoid dye is extracted and (c) lengths of indigo-dyed cotton hanging in a courtyard. (Ph. J.Balfour)

Moreover, several other hues could be achieved by the combined use of different dyestuffs; green shades, for instance, were traditionally obtained as mixtures of a blue and a yellow dye. In the following, some historical as well as chemical information about the most common natural dyes used in the past are provided, according to their different chemical classes.

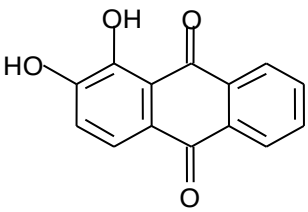
- Anthraquinone dyes

From a chemical point of view, the most important red dyes are based on molecules possessing an anthraquinone skeleton. These compounds, as detailed in the following, were extracted from very different natural sources, both vegetal and animal: the plant family of *Rubiaceae* (from the Latin *ruber*: red) and scale insects.

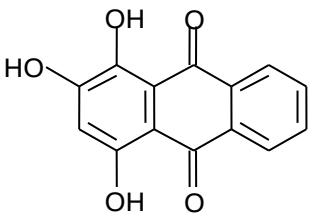
Madder

The knowledge of madder as dyeing plant was very diffused since ancient times, as several ancient historians of the Greek and Roman period mentioned madder to dye textiles.[5, 6] Madder plant originated in India, but soon it became widely cultivated in the Middle East and in Europe.[3] The red dye was extracted from the roots of plants belonging to the plant family of *Rubiaceae.* More properly, the precise name of the red dye depends on the botanic source from which it was extracted: "madder", the most common one, if the plant source is *Rubia tinctorum L.*, "wild madder" if the source is *Rubia peregrina L.*, "munjeet" if it is *Rubia cordifolia L.*. The main components of madder are the anthraquinones alizarin, purpurin and pseudopurpurin, whose chemistry was intensively studied by Thomson in 1960.[7]

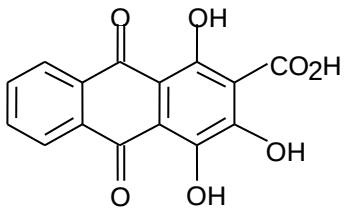
The first chemical investigations on madder date back to the nineteen century: the identification of alizarin as the main chromophore of this colorant and its synthetic routes discovered in 1870 led to the industrial production of synthetic alizarin, which largely displaced the natural dye in commercial use.[3, 4, 8]



Alizarin



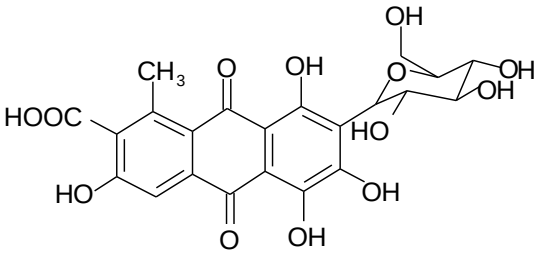
Purpurin



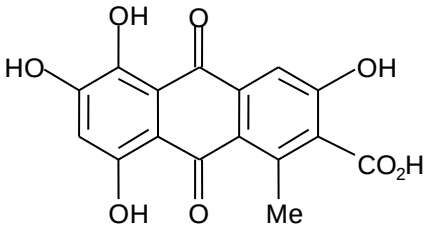
Pseudopurpurin

Carmine

Carmine is the generic name currently accepted to refer both to the dyes cochineal and kermes, which however have a different origin and a different chemical composition. Cochineal is one of the main red dyes of animal origin. It was obtained from the scale insects *Dactylopius coccus* and other parasite species living on the *Opuntia* cactus family, indigenous to the New World and in particular of Mexico, Central and South America, where Aztecs employed this pigment for dyeing and painting. Only in the 16th century, following the Spanish conquest, cochineal was brought to Europe. The major constituent of all cochineals is the anthraquinone carminic acid, but the various species contain different amounts of other anthraquinone minor compounds which can be of help in distinguishing the insect of origin in historical samples. Kermes instead was extracted from the female scale insect *Kermes vermilio*, which lives on Mediterranean oaks *Quercus coccifera* and *Quercus ilex*.[3, 5] Therefore kermes was the principal insect dye in Europe before the discovery of America and, thus, of cochineal itself. Kermes mainly owes its color to kermesic acid, another anthraquinone compound.



Carminic acid

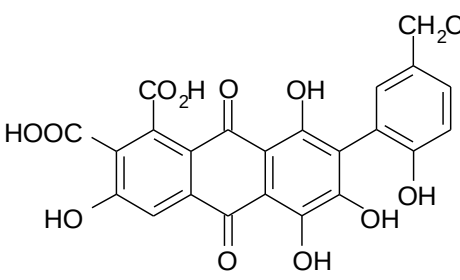


Kermesic acid

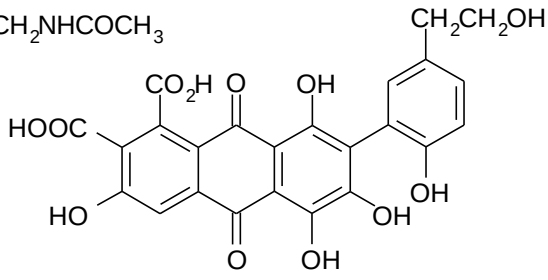
Lac dye

Another anthraquinone dye of animal origin is lac dye, whose source is the insect *Laccifer lacca*, also known as *Coccus laccae*. The dye was extracted from a resin produced by the insect on host trees situated in a region extending from Northern India to Indochina.[9] Due to its origins, it was known and used in India and Japan

since antiquity to dye silk, while it was not widely mentioned as a textile dye during the same period in Europe, where it was fully introduced only in the late 18th century.[4, 8, 9] In spite of this, lac dye was employed as an artists' pigment from the 13th century, with an extensive use in 15th century Italy and onwards.[8] The main coloring components of this dye are laccaic acids A and B.



Laccaic acid A



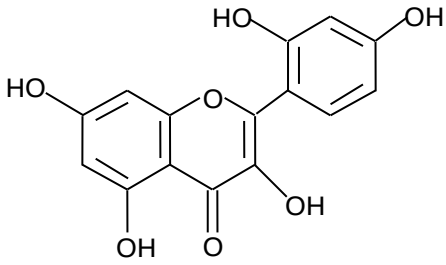
Laccaic acid B

- Flavonoid dyes

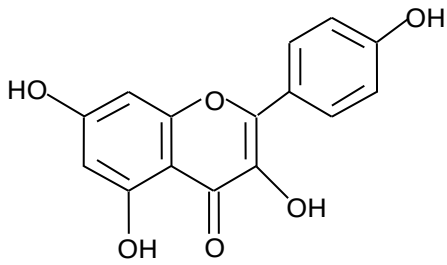
Most yellow colorants owe their color to chemical compounds called flavonoids. They occur in a wide variety of botanical sources, mainly as glycosides. Since a large range of plants rich in flavonoids can be easily found all over the world, no individual source of yellow dye became predominant over the centuries – as opposite to what happened with the red dyes madder and cochineal – and, in the past, the use of the plant source obviously depended on the species that were locally available. Under the name "flavonoids" (from the Latin *flavus*: yellow), several compounds are taken into consideration. In addition, not all the flavonoids give a yellow hue: for example, as pointed out in Chapter 2, anthocyanins are an important subgroup of flavonoids that are responsible for the red, purple and blue colors of a wide variety of flowers, fruit and cereal grains. In the following, the most important flavonoid-based yellow dyes are presented, while examples of other important compounds used to obtain different tints but always belonging to the flavonoid family will be described separately.

Old fustic

Old fustic was extracted from the wood of the dyer's mulberry *Chlorophora tinctoria* Gard., typical of America, and particularly present in Cuba, Jamaica, Puerto Rico and other West Indian islands. This dye was imported to Europe during the 16th century, becoming soon an important source for a yellow mordant dye. The colors obtained from it were not very lightfast, but its use became widespread because of its low cost. In association with the main flavonoid compound morin, smaller amounts of kaempferol were recognized in the extract of old fustic.[3]



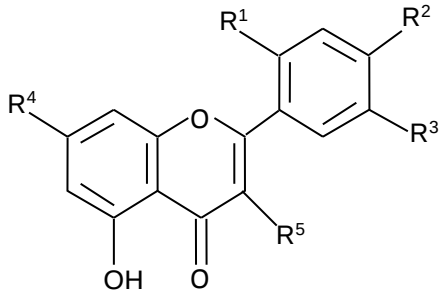
Morin



Kaempferol

Stil de grain

Stil de grain was obtained by extraction from the berries of *Rhamnus cathartica*, a wild plant diffused in the Northern hemisphere, Brazil and Southern Africa. This gold-yellow dye was also called Rhamno lake, Spincervino lake or buckthorn lake. It was known since ancient times and largely used from Middle Age to the 19th century for dyeing textiles, but it was also employed in artistic techniques such as miniature, tempera and oil during the same period.[10] Different glycosides of the flavonols quercetin, rhamnetin, rhamnazin and kaempferol are costituents shared in common with all Rhamnus

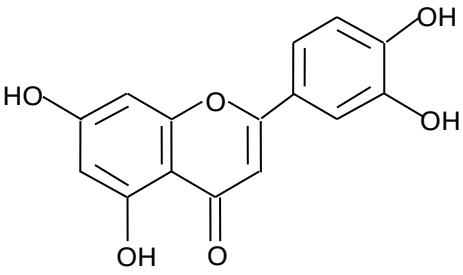


Rhamnetin if R1=H, R2=R3=R5=OH and R4=OCH3, Rhamnazin if R1=H, R2=R5=OH and R3=R4=OCH3, Quercetin if R1=H and R2=R3=R4=R5=OH.

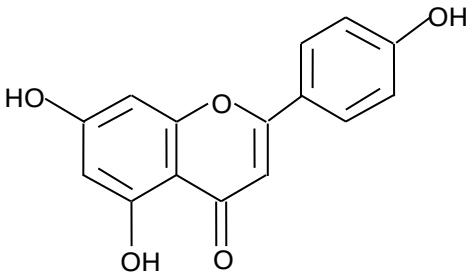
species, as well as the anthraquinone emodin. It was used to produce orange and scarlet shades in mixture with cochineal, rather than for making pure yellows, because the color resulted very fugitive.[11]

Weld

Weld was extracted from *Reseda luteola*, a plant that grew wild in most Europe but was also cultivated. Its use as textile dye was well attested in ancient recipes for dyeing fabrics and indeed it was found in European textiles and early Anatolian carpets. Following the discovery of America it was partially replaced by fustic and, after the 18th century, by quercitron as well.[4] Weld provided a lightfast yellow color and was used as mordanted dye. The main constituents of weld are the flavones luteolin and apigenin which allowed to obtain bright yellow colors.[12] Its employment in combination with woad or indigo to produce green dyes was also reported.[3]



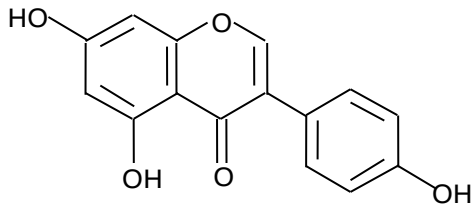
Luteolin



Apigenin

Dyer's broom

Dyer's broom was obtained from *Genista tinctoria* plants that are commonly found throughout Central and Southern Europe, Russia and Asia. It was native to England, where it was the only yellow dye before importation of others in the Middle Ages.[4] It owes its color to the flavone luteolin and the isoflavone genistein.



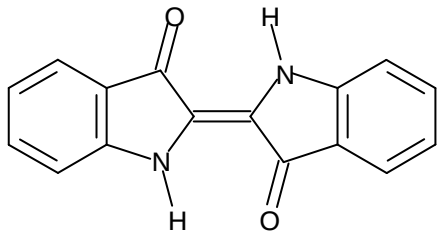
Genistein

- Indigoid dyes

Indigo and Tyrian purple belong to the oldest and most appreciated blue and purple dyes in the world. The natural sources of these dyes are vegetal for indigo and animal for Tyrian purple. Their main coloring agents, i.e. indigotin and 6,6'-dibromoindigotin, are classified as vat dyes. Therefore, in order to dye textiles, the two molecules were reduced to water-soluble compounds which can bind to the fiber and were then re-oxidized into their original insoluble blue/purple form.

Indigo

Employed since 2000 B.C. in Egypt, indigo is a very stable organic dye, fact that also explains its wide use in oil paintings by the greatest masters of the 17th and 18th centuries.[13] Two were the main natural sources of indigo: *Indigofera tinctoria* plant, native of tropical Asia and *Isatis tinctoria*, available in Southern and Central Europe, North Africa and West Asia, from which woad can be extracted.[3] In the Middle Ages woad was extensively cultivated in Europe and only small amounts of indigo from *Indigofera* were imported from India. Over the years, the overland trade of indigo acquired increasing importance, especially after the circumnavigation of the Cape of Good Hope by Vasco de Gama in 1498, which opened a new trade route.[3] As previously mentioned, indigotin is the main chromophore of indigo. Natural indigotin was displaced by the synthetic pigment first prepared by Von Baeyer in 1878, bringing a halt to the cultivation of the natural indigo plant.[14] Indigo was used, apart to provide blue shades, in mixture with red, yellow and brown dyes to produce purple, green and black colors.[3]



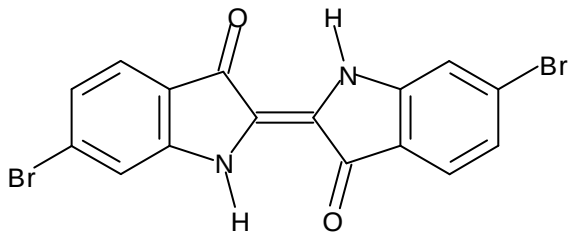
Indigotin

Tyrian purple

Tyrian purple is the most precious dye among those used in antiquity. Since the Phoenician period, a shellfish purple dye industry was well attested and evidences in this regard have also been provided by archaeological findings, that allowed to date back its use to the 14th century B.C..

As in the case of indigo plant sources, the dye is not present in the live mollusc. It had to be generated by the enzymatic hydrolysis of precursors found in the animals' hypobranchial glands, followed by photochemical conversion to the purple pigment. The process was described in details by Pliny the Elder: it consisted in the capture of the molluscs, the removal of the colorless glands and their treatment with salt and water and a ten-day heating process in urine and water.[3] Only very small amounts of dye can be obtained from each mollusc, making these dyes very expensive commodities, indicating membership of the highest ranks of society. The Roman emperor, Nero, and the Byzantine emperor, Theodosius, issued edicts prohibiting on pain of dead the wearing of some types of purple cloth by anyone other than themselves.[1]

The three main species of molluscs used in the Mediterranean region were spiny dye-murex (*Bolinus brandaris* L. or *Murex brandaris*), rock-shell (*Thais haemastoma* L. or *Purpura haemastoma*), and banded dye-murex (*Hexaplus trunculus* L. or *Murex trunculus*). Depending on the species, it is possible to obtain a purple dye with different amount of 6,6'-dibromoindigotin, 6-bromoindigotin, indigotin, indirubin and their derivatives.[15]



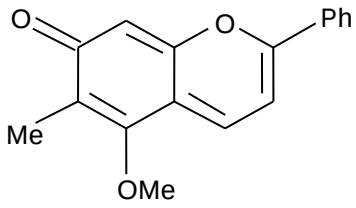
6,6'-dibromoindigotin

- Other dyes

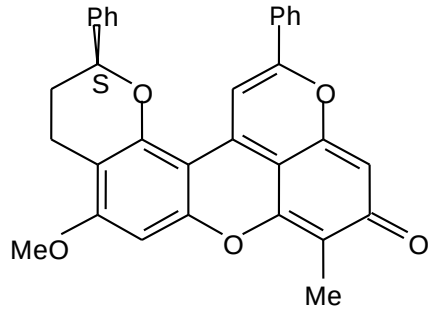
In this section, other important dyestuffs belonging to different molecular classes were briefly described. Again, it is worth remembering that the range of natural dyes used historically worldwide in craftsmanship and art is really huge and, even if some of them were employed in different geographical areas, many others were typical of a given zone being obtained from local vegetable (or, less frequently, animal) sources. Therefore only the most used are here reported.

The neoflavone dye dragon's blood

Dragon's blood dye is actually a natural resin displaying a red color, that was historically employed in the Mediterranean area for several aims, such as to dye wool or to glue and decorate pottery, but in India it was also used for medical purposes and for magic rituals and ceremonies. Moreover, its use in painting and for enhancing the color of precious stones and staining glass, marble and wood of 16th - 18th century Italian violins was attested. The source for dragon's blood first used in Europe in ancient times was the plant *Dracaena cinnabari* from the island of Socotra. By the Medieval and Renaissance periods, alternative genera started to be used, such as *Dracaena draco,* coming from Canary Islands and Madeira, *Daemonorops* species from Southeast Asia and *Croton* species from tropical areas.[16, 17] The main components responsible for the color of the resin are the neoflavone dracorhodin and dracorubin. The overall chemical composition however



Dracorhodin



Dracorubin

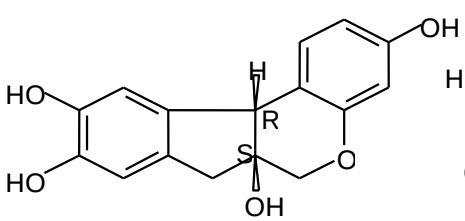
changes according to the botanical sources employed. *D. draco* contains 50% of abietic acid, while *D. cinnabari* is mainly composed of biflavonoids and dihydrochalcones.[16, 17] In recent times, two compounds called dracoflavylium and 7,4'- dihydroxyflavylium were isolated from *D. draco* and *D. cinnabari*.[18] Most of the dragon's blood which is traded today is classified as *Daemonorops*.

Soluble redwoods: neoflavonoid dyes

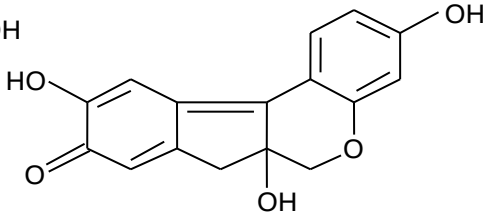
Dyes called "soluble redwoods" are constituted by principal coloring components belonging to the family of neoflavonoids and are, as the name suggests, readly soluble in water and used as mordanted dyes.

Brazilwood

These kind of soluble redwood colorants were obtained from various species of the plant genus *Caesalpinia*. The most used varieties were brazilwood, peachwood, sappanwood, limawood and pernabuco wood, though they were collectively known as brazilwood. They were native of Brazil, Nicaragua, Mexico, Jamaica (brazilwood, peachwood, pernabuco wood) and of Southeast Asia (sappanwood).[3, 4] Cheaper than other red dyes, brazilwood appears to have been used less frequently in paintings probably because its poor lightfastness,[8] documented also by researchers who identified the degradation products of this dye.[19-23] Named "type B and C", these products were used as markers for the identification of soluble redwood dyes in historical samples. The main coloring compound of brazilwood is brazilein, which is formed by oxidation of its precursor, brazilin, upon exposure to air and light.



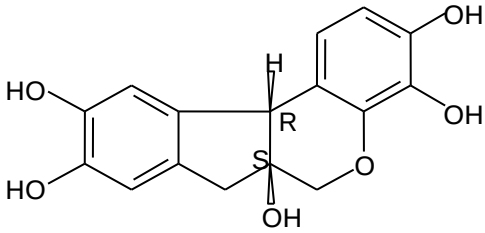
Brazilin



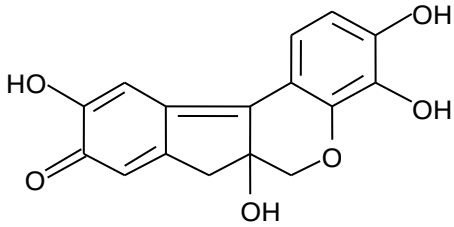
Brazilein

Logwood

Logwood is obtained from the plant species *Haematoxylon campechianum*, native to Central America, and has been known in Europe since the 18th century.[14] The dye itself is red, but, in combination with different mordants, it produces a wide range of colors, including black (with copper, iron or chromium-based mordants) gray (with iron salts), blue and purple (with alum and tin mordants).[3] The compound responsible for the color of logwood is hematein, which originates from the oxidation of hematoxylin, present in the fresh wood, upon exposure to air.



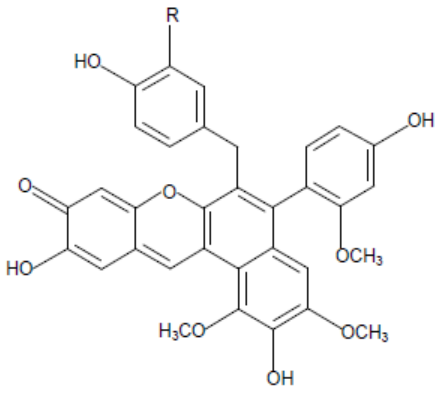
Hematoxylin



Hematein

Insoluble redwood dye: the biflavonoid sandalwood

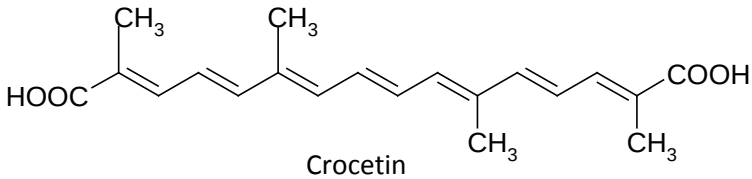
Insoluble redwood dyes are based on chromophores belonging to bioflavonoids compounds and, as the name suggests, they are very sparingly soluble in water. Representative of this class of dyes is sandalwood, extracted from *Pterocarpus santalinus* and related species, typical of tropical Asia. The mordanted dye was widely used since antiquity in India for dyeing textiles, while in Europe it is known from the middle of the 16th century.[2] In combination with other pigments, it provided dark red and brown colors. The main coloring agents are molecules called santalins.[3]



Santalin A if R = OH;
Santalin B if R = OCH3

The carotenoid dye saffron

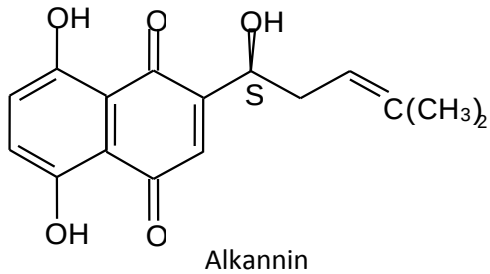
Known from antiquity, evidences of its use date back to Egyptian times, but it was very popular in Persia in classical times as well. Due its high cost, saffron was employed as a textile dye reserved for the noble classes, particularly in China and India. It was obtained from the stigmas of the flowers of *Crocus sativus* and could be used both as a direct dye, providing a orange-yellow color, and in association with alum mordants. In the long run, it is an unstable colorant and the imparted vibrant hue quickly fades to a pale and creamy yellow. It was later replaced by cheaper yellow dyes like weld, with better fastness properties.[3] *Cis* and *trans*-crocins, which are glucosides of the carotenoid crocetin are the molecules that confer the characteristic golden yellow hue to dishes and textiles.



Crocetin

The naphtoquinone dye alkanet

From the root of *Alkanna tinctoria* Lamm., species found in Southeastern Europe and in Asia Minor, a red dye called alkanet is obtained.[4] It was known from ancient times, at least in the later periods of Egyptian history,[2] and mentioned by the Greek historian Theophrastus and the Roman Pliny, who describes the blood-red dyestuff obtainable from its roots.[6] The main coloring component of alkanna is the naphthoquinone alkannin, which displays a red color in acids and becomes blue or green in alkalis.



Alkannin

With the discovery of synthetic dyes in the middle of the 19th century, the decline of traditional natural dyes started. In 1856 William Henry Perkin, at the age of 18, accidentally discovered the purple dye "mauve" when trying to synthesize quinine, the only known remedy for malaria. Perkin's synthesis of mauve and the establishment of a factory in 1862 to produce it commercially marked the beginnings of the modern dye industry.[24] In 1883 Adolf von Baeyer discovered the chemical structure of indigo, and by the end of the 19th century a commercially feasible manufacturing process had been developed.[2, 4] In relatively short time, several improved coloring substances, offering a vast range of new colors at a lower price, became available. Consequently, artists were strongly influenced by the introduction of new industrial materials into the art market, which ranged from traditional pigments to commercial paint and new synthetic pigments and colorants. In addition, the new formulations contained other compounds used as stabilizers, extenders and fillers. Thanks to their availability and low cost, artists mixed and experimented with these new paints, even if the long-term stability of new colors were far from being completely understood.[25]

Nowadays natural and synthetic organic dyes are used all over the world for a multitude of purposes. They are constituents of inks and paints and are used as food colorants, cosmetic and textile dyes. Some of the most popular natural dyes employed in ancient times, as for example saffron and cochineal, have retained an economic importance over the centuries as coloring agents in food, drinks, drugs and cosmetics. In spite of this, synthetic dyes, often cheaper than the natural ones, increased the range of their commercial applications over the years. It is worth remembering, however, that the recent discovery of potential health issues associated with some synthetic food color additives induced researchers to search safer alternatives among the range of natural dyes, giving them new a new life and possibilities in the today market.

The chemical characterization of organic dyes

The art of dyeing has been developed from old times, in all civilizations: ancient Egyptian, Chinese, Jewish and Persian people mainly colored wood and textiles, but, with the evolution of humankind, colored substances were applied to an infinite range of objects and surfaces. During the centuries, some of these precious objects entered into museums' collection and are still visible nowadays. Colors were indeed employed in the realization of paintings, but they were used also in a multitude of other objects, whose colored nature seems to us quite obvious. For example, a wide variety of colored textiles is present in museums and collections: they can be sacred vestments, costumes, every-day clothes, but also tapestries, carpets and wallpapers. The use of inks on paper and of several dyes to decor almost every object produced by the mankind are just cited, as the list of colored artifacts could be endless.

The knowledge of the materials from which these historic objects have been made can contribute to the understanding and appreciation of the artefacts as well as providing an insight into their past.[2] Notably, several aspects are involved in the characterization of dyes: from a mere historical point of view, the analysis of natural dyes can reveal what kind of substances were available in particular historical periods and in defined geographical areas, providing valuable data about the lifestyle, the craftsmanship and the technical knowledge reached by a population in a given historical age, as well as information about the provenance of materials, pigments and dyestuffs. In this regard, notions concerning interactions between different cultures and the trade routes that may have allowed the usage of a particular colorant far from its geographical source could be deduced from the identification of dyes. An art historian could be probably more attracted in the chemical characterization of a work of art by the possibility to have a new insights into the artist's original intention and changes of mind, as well as to discover the techniques used. Again art collectors, art historians and archaeologists' interests could be directed to the possibility of the dating of artefacts, possibly leading to the

uncovering of falsifications and forgeries. For example, the discovery of America brought several changes in the use of dyeing matters, with the introductions of new dyes and the replacing of some natural sources with cheaper ones. Therefore, the use of one of these new dyes in an European artefact dated before the 1492 could open the way to a reconsideration of the dating or of the authenticity of the same. Furthermore, from the point of view of the art conservator, scientific analysis applied to the study of dyes may contribute to assess suitable conservation and restoration procedures to be applied to paint defects and degraded pigments in works of art of any kind; in fact, time and environmental conditions unavoidably cause damage and deterioration to art objects which require careful conservation. Moreover, as restoration was attempted also in a period in which the knowledge of the chemistry of dyes was not so exhaustive, the analysis of restored particulars could allow to distinguish the posterior reshuffles from the original materials.

The identification of historical dyes remains however one of the most challenging tasks in the chemical investigation of art materials. First of all, colorants in works of art are usually included in complex matrixes such as paint layers or cloth fibers, where they are mixed with other substances, such as binding media or mordants. Moreover, due to their high tinting power, they are present in very low concentrations. Besides, sampling of art objects is always limited to microscopic fragments, when at all allowed. Moreover, the unavoidable deterioration of organic materials could led to the formation of specimens with a different molecular structure in comparison to the primary organic dye. However, the scientific literature offers interesting examples of useful identification of dyes in ancient artifacts, in spite of evidences of severe degradation of dyes molecules. For examples, madder and cochineal-based purples and reds were detected, in addition to inorganic compounds, in an important watercolor by Winslow Homer. This identification was essential both to determine how the colorless appearance of the sky was actually the result of a fading process and to hypothesize how the painting most likely looked just

after its completion.[26] Another example is constituted by the investigation of the red lakes from Renoir's painting "Madame Léon Clapisson" (1883) carried out at the Art Institute of Chicago. While the painting showed a significant fading of the red lake across the background, the original color was preserved along the edges where the painting was protected from light by the frame. The sampling from unfaded areas and the subsequent analysis allowed to highlight the use of a cochineal lake.[27] The newly discovered information was further used to generate a digital image with the faded red color restored to the background (Figure 3), intended as a visualization of the painting's original appearance.[28] Finally, the identification of an unstable red dye coming from the plant sappanwood in several Chinese carpets produced in the Ningxia region allowed to solve the apparent inconsistency between the ancient representations of those carpets, depicted as bright red, and the appearance of almost all the surviving exemplars of this type, that show yellow or reddish-yellow shades instead. Indeed the original red dye used for the carpets faded away possibly by accident or according to the dyer's intention. The obtained results could be considered in favor of the hypothesis of a "voluntary" fading of the textiles.[29]

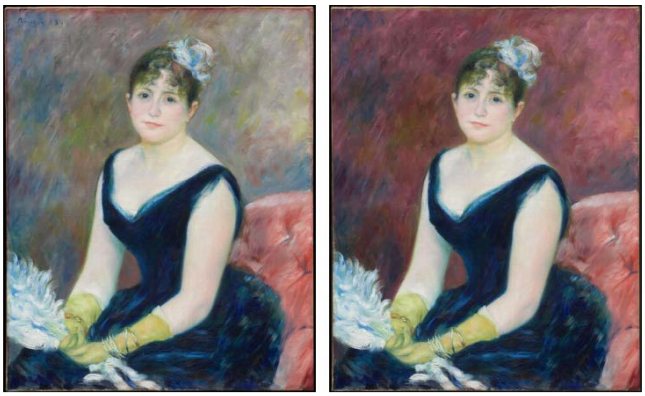


Figure 3. Original (left) and recolorized visualization (right) of "Madame Léon Clapisson" (Renoir, 1883) (The Art Institute of Chicago)

Interestingly, in most of the cases considered above, the identification of dyes was made possible thanks to the application of a rather new spectroscopic technique: surface enhanced Raman scattering (SERS). This analytic tool, on whose use the present doctoral thesis is based, is presented and discussed in detail in the following section.

Introduction to surface-enhanced Raman scattering

Surface enhanced Raman scattering (SERS) is a valuable technique based on the enhancement of the Raman signals of a molecule when it is in the proximity, or even adsorbed on, a roughness metal surface. This effect was first observed in 1974 by Fleishmann et al.,[30] while using Raman scattering to study the electrochemical reactions of pyridine at a roughened silver electrode. Resulting from the adsorption of chemicals on specially roughened metallic surfaces, a Raman scattering signal enhancement factor of up to 10^8 was reported by Jeanmaire and Van Duyne [31] and by Albrecht and Creighton[32] in 1977.

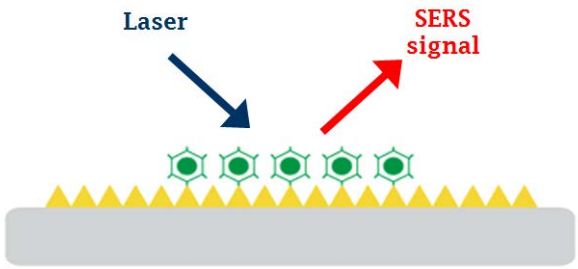


Figure 4. Conceptual illustration of SERS mechanism.

There is a large variety of SERS substrates, including modified electrodes, colloids and metal island films. Notably, wet chemistry provides an inexpensive and versatile approach to metal nanoparticle fabrication. Since the first reported SERS experiment on silver and gold sols in 1979,[33, 34] metal colloids have become the most commonly used nanostructures for SERS. They were produced by chemical reduction of a starting metal salt by a suitable chemical agent. The colloidal suspensions contain nanoparticles with variable sizes and shapes depending on the method of production. Generally, the size regime relevant to SERS experiments is comprised between 10 and 80 nm. Depending on their size, shape and on the dielectric constant of the metal of which they are constituted, these nanoparticles exhibit different plasmon resonances, [35] that for silver and gold fall in the range of the visible region

of the electromagnetic spectrum. Thanks to this property, colloidal suspensions have been often characterized by their absorption spectra.

Indubitably, the reduction of Ag^+ by addition of sodium citrate[34] is the most common method for the fabrication of Ag sols to be used in SERS. The relative stability of freshly prepared metal colloids is mainly due to the counter ions existing on the metal surface coming from the ionic species added to the mixture during the preparation. These ions are normally adsorbed on the metal surface and confer to the nanoparticles enough charge to maintain them suspended in the dispersion medium, generally water, thanks to the inter-particle repulsion forces. However, with the passing of time, the lower electrostatic barrier of colloids makes easier the approaching of nanoparticles to form macroscopic aggregates and flocculate.[35] Interestingly, it was found out that SERS signals increase when nanoparticles are aggregated, forming the so-called "hot-spots".[36, 37] Clearly, we are considering only partial aggregation of the nanoparticles, therefore a different phenomenon with respect to the flocculation cited above. This partial aggregation of the colloidal particles can be induced by adding an aggregation agent, which is typically a salt, such as sodium perchlorate[38] or potassium nitrate.[39]

The exact mechanism of the enhancement effect of SERS is still a matter of debate in the literature. The precise physics of this effect have been in dispute, as experiments indicate there may be more than one enhancement mechanism. Today's consensus is that two effects are operative: the electromagnetic (EM) and the chemical effect (CT). According to the electromagnetic theory, the increase in intensity of the Raman signal for molecules on particular surfaces is due to an enhancement in the electric field provided by the surface itself; this explanation can apply even in those cases where the specimen is only physisorbed to the surface, without chemical interactions. On the other hand, the chemical theory proposes charge-transfer effects between molecular orbitals of the target analyte and the conduction band of

the noble metal substrate; it only applies for species which have formed a chemical bond with the surface.[40]

The chemical structure of the studied adsorbate is also important to ensure a strong SERS signal. In fact, a certain degree of interaction between the adsorbate and the metal surface is required to have intensification of the Raman signal through the mechanisms involved in SERS enhancement. The EM mechanism needs the adsorbate to be close enough to the surface because of the short range of the surface-enhanced electromagnetic field.[40] On the other side, the CT mechanism implies an effective metal–adsorbate interaction for the CT process to take place.[41]

As previously stated, depending on how an adsorbate interacts with nanostructures, it could be chemically or physically adsorbed to the surface. Since the initial layer of adsorbed molecules benefits most from the local-field enhancement, both chemisorbed and physisorbed molecules will experience a maximum enhancement for the first layer ("first-layer effect"). However, there is a substantial difference in the way adsorption, be it physical or chemical, affects the polarizability derivatives of the adsorbate. Chemisorption can lead to a new species at the surface with a strong chemical bond, which belong to a new symmetry point-group, and as such have a different irreducible representation for the internal modes. The adsorption on a surface may therefore lower the symmetry of an analyte, as it can remove a possible symmetry plane from the molecular point group, resulting in the appearance of vibrational bands forbidden for the free molecule in the observed spectra. In other words, a quite different Raman spectrum from the free molecule is expected.[42, 43] Therefore, the presence of a surface on which analytes adsorb needed a suitable theory able to explain the resulting SERS spectra. The modifications of the adsorption, emission and Raman scattering intensities of molecules near metal surfaces were thus studied[42, 44, 45] and they are usually referred as surface selection rules. Moskovits[44] demonstrated that for the composite analyte-metal system, vibrations whose transition dipoles in the molecule are directed tangentially to the

metal surface would have zero composite transition dipoles; whereas, vibrations whose transition dipoles have a component normal to metal surface would have that component reinforced in the composite system. It follows that strong signals are observable for those vibrational modes which are perpendicular to the metallic surface.[44]

Surface-enhanced Raman scattering combines extremely high sensitivity, due to enhanced Raman cross-sections comparable or even better than fluorescence, providing one of the most incisive analytical methods for chemical and biochemical detection and analysis. To further increase the sensitivity of SERS, surface-enhanced resonance Raman scattering (SERRS) strategies were adopted.[46-48] SERRS exploits the resonance Raman (RR) scattering condition that occurs when the excitation frequency of the laser source lies near to the maximum of absorption of a molecular chromophore. In this case (Figure 5c), the excitation source has sufficient energy to promote an electronic transition in the molecule and the coupling of the vibrational transitions with the electronic one increases the induced dipole moment, that, in turn, results in an increased efficiency for Raman scattering.[49] Interestingly, the

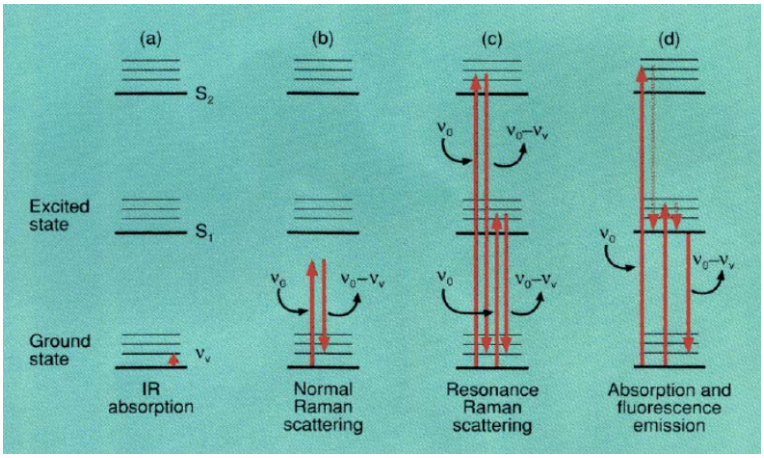


Figure 5. (a) vibrational IR absorption; (b) inelastic scattering of light of normal Raman scattering; (c) excitation occurring within an electronic transition produces resonant Raman scattering. (d) absorption and fluorescence emission in which an excited state is created. (Figure from ref. 49)

selective enhancement of the signals of the species in resonance associated with RR is retained also in SERRS, giving good molecular specificity, free from interference from non-SERRS-active contaminants.[50] In addition, further surface enhancement is achieved if the excitation wavelength coincides with the absorption bands of the surface plasmon of the nanometric material.[51] Thus SERRS developed into an extremely powerful analytical tool that yields information about the molecular structure of the analyte with sensitivity comparable or even better than that achieved using fluorescence spectroscopy. [52, 53] In this context, it is worth remembering the exploitation of SERRS to single-molecule SERS detection (SM-SERS).[48, 54-56] For "single molecule detection" it is intended the possibility of identifying $1/N_A$ (1.66×10^{-24}) mol of a substance. This can mean acquiring the spectroscopic signal arising from a *ca.* 10 picoliter of a 10^{-10} molar solution of the analyte. Detecting the spectroscopic signal from such a small fraction requires an extraordinary sensitivity of the analytical tool employed, feature recognized to SERS and in particular to SERRS.

Another interesting feature of SERS is that while Raman signals are enhanced, fluorescence is quenched as the molecule approaches the metal surface. In more detail, fluorescence and resonance Raman emissions from the adsorbed molecule may benefit from the enhanced absorption processes as well as the amplification of the field of the emitted light (Figure 5); however, on the other hand, the emission in fluorescence is limited by the competition between radiative and non radiative decay from the excited state, the rate of which are modified by different mechanisms in close vicinity to a metal surface. In addition, Raman profits from radiative enhancements in both the incoming (laser) and Stokes-scattered photon, while fluorescence only profits from an absorption enhancement in the first step of the process. As a result, for molecules adsorbed directly on the metal, the Raman signal is typically enhanced by several orders of magnitude more than the fluorescence signal and the fluorescence quantum yield is overall reduced.[57, 58]

The identification of organic dyes by means of SERS

The application of SERS in the field of the diagnostic of cultural heritage is relative recent, although already in 1987 Guineau and Guichard exploited SERS to identify colorants of artistic and archaeological interest.[59] However, because of the objective difficulties in achieving consistent and reproducible results with this technique, only recently, roughly in the last two decades, the enhancement provided by SERS has been extensively exploited by scientists in order to identify several organic dyes used in the past to dye textiles or applied on paintings as lakes.[26, 39, 60, 61]

Several features of SERS reveal that it is the ideal analytical tool for the identification of natural organic dyes: as discussed in the previous excursus on the chemistry of dyestuffs, most chromophores exhibit large π-electron systems or atoms carrying lone electron pairs, factors that favor their adsorption on metal nanoparticles; most of dyestuffs are fluorescent molecules, and hence while their conventional Raman spectra are obscured by very high backgrounds, SERS, quenching the fluorescence, allows the univocal identification of these compounds; finally, being them present in microscopic quantities in samples, an ultrasensitive technique is required.[61] Ruled out Raman spectroscopy, referring to other analytical tools to be possibly applied to the same aim, it is worth remembering that non-destructive UV-visible reflectance techniques can in some cases lead to positive identification or at least to some level of discrimination. In the majority of cases though, UV-visible spectra do not have the necessary degree of detail for a univocal identification of unknown materials. Therefore analytical techniques with greater discriminatory power, but needing samples and therefore destructive, such as high performance liquid chromatography (HPLC), were employed and are still the most used for this aim.[61]

As previously mentioned, dyes are present in works of art as insoluble complexes with aluminum or other metal ions. In this form, dyes can hardly adsorb on metal nanoparticles, making the acquisition of SERS signal challenging. Textile fibers and paint samples have been therefore subjected to hydrolysis procedures in order to

obtain the free dye. This process however introduces other variables in the analysis: hydrolysis and extraction techniques must therefore be accurately evaluated to determine their influence on the SERS effect.[61] These protocols initially employed harsh chemical extraction in strong acids in order to isolate the organic dye from the medium, invariably resulting in degradation of the host material. Interestingly, the recent development of milder extraction procedures,[62] non-extractive hydrolysis methods[39, 61] as well as non-hydrolysis approaches[63-65] has allowed the identification of several organic dyes in tapestries, carpets, paintings and other art objects. In this context, Chapter 4 of the present doctoral thesis describes an original extractionless approach combined with the use of Fourier-transform Raman instrumentation.

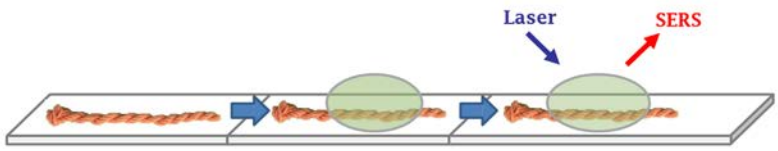


Figure 6. Conceptual illustration of an extractionless approach.

A lot of research carried out thus far in the field of SERS applied to art has been dealing with the characterization of reference substances: the anthraquinones alizarin and purpurin have been the subject of most works available in the scientific literature[8, 9, 61, 66-71] thanks to the easiness of absorption of these molecules on silver nanoparticles and their high SERS response. It is worth remembering that researchers investigated also the behavior of pure molecules belonging to the flavonoid[72-74] and indigoid families.[75, 76] In these studies, reference spectra of pure molecules were recorded and carefully examined. The influence of pH was also taken into consideration, as the different degree of protonation can influence the way on which compounds can bound to the nanometric roughened surface.[9, 67, 71] Therefore other aspects such as the complexation geometry and orientation of the analyzed molecules with respect to the metal substrate were deduced from SERS spectra.

Eventually, recently published databases[38, 61] included a large number of reference spectra both of pure chromophores and of dyes as such, i.e. as extracted from plant or animal sources. Nevertheless, it is worth remember that a further enlargement of the database is still required to cover the large variety of botanical sources exploited in the past to dye and paint.

As every analytical technique, SERS has some limitations that thus far can be ascribed to its non-separative and micro-destructive nature. The former feature entails the obtainment of limited information when the sample analyzed contains more than one coloring agents or when chemotaxonomic data are desired. In such cases complex spectra are obtained or, alternatively, just one component gives a prevailing SERS response, while the others cannot be detected. For this reason, the joining of SERS with chromatographic techniques for the analysis of dyes could be of great interest and was indeed already attempted with thin layer chromatography (TLC)[24, 63, 77] and, in this doctoral thesis (Chapter 3), with high performance liquid chromatography (HPLC).

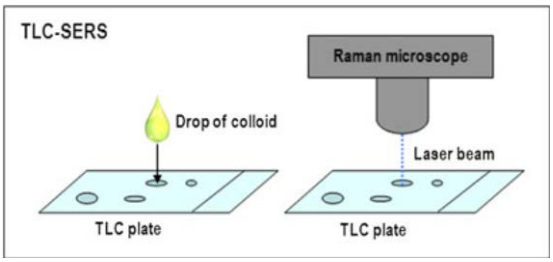


Figure 7. Schematic illustration of TLC-SERS from ref.77

As far as the micro-destructive feature of SERS is concerned, nowadays researchers are trying to convert this valuable but destructive technique into a non-destructive one, in order to apply it to the analysis of artifacts. Several attempts were done in this regard, using different approaches, as for example the development of hydrogels based on solid phase micro-extraction techniques extract trace amounts of dyes[78-81] and the laser ablation followed by SERS analysis of the ablated substances.[82]

Great opportunities are therefore still open in the field of the identification by means of SERS, in particular in the field of the setting up of multifunctional devices or joined systems that combine SERS with other analytical techniques.

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CHAPTER 2

Surface-enhanced Raman scattering of anthocyanins

Abstract

In the present Chapter, anthocyanin-based dyes were investigated by means of SERS. Anthocyanins are an important class of natural compounds, present in several plant sources that were employed in the past as dyeing matters. Anthocyanins belong to the family of flavonoids, but the colors provided are not in the range of yellow shades. Red, purple and blue were the hues that berries, flowers and cereal grains exhibit and the colors that were consequently imparted to textiles.

Anthocyanins are present in nature as glycosylated molecules, while their respective aglycones are named "anthocyanidins". The first part of the present work was thus devoted to the characterization of anthocyanidins by means of SERS at different pH values, exploiting the chemical specificity of the technique and its sensitivity to subtle changes in molecular structures, phenomena that occur extensively in the chemistry of anthocyanins.

The aim of the second part of the project was to provide experimental procedures for the identification of anthocyanin-based dyes used in antiquity. In particular, we identified anthocyanins both in plant extracts and in dyed textiles by means of SERS. Finally, due to the instability of isolated anthocyanins, a study to verify the possibility to identify such molecules also in faded textiles was carried out upon exposure of dyed wool samples to artificial ageing.

To the best of our knowledge, a complete SERS study of such important molecules was carried out in the context of the present doctoral work for the first time and it led to the enrichment of the SERS database of dyes available in the literature.[1, 2]

Introduction

Anthocyanins (from the Greek *anthos*, flower; and *kyanos*, dark blue) are natural compounds responsible for the red, purple and blue colors in a large number of flowers, fruits and leaves. They are sometimes contained in other plant sources such

as roots, tubers, stems, bulbils, cereal grains[3] and in the skin of red grapes, playing a key role in the organoleptic characteristics of wines.[4]

Anthocyanins belong to the family of flavonoids, being glycosylated polyhydroxy- and polymethoxy-derivatives of 2-phenylbenzopyrylium (flavylium) salts (Figure 1).

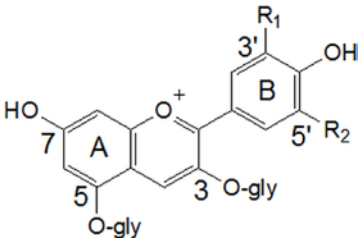

Figure 1. Chemical structure of an anthocyanin from ref 1.

The little differences in their chemical structures are responsible for the colors they produce: for example, by changing the hydroxylation or methoxylation pattern of ring B a shift of some nanometers in the maximum of their visible absorption spectra can be verified.[5] Moreover, glycosidic substitutions can occur at the 3 and/or 5 positions, and the mono-, di- or tri-saccharides may be acylated with aliphatic or cinnamic acids, leading to considerable structural variations, with over 600 anthocyanins having been identified in nature.[6] Despite this fact, 90% of the naturally occurring anthocyanidins are based on only six structures (Figure 2): cyanidin (CYN), delphinidin (DEL), pelargonidin (PEL), peonidin (PEO), malvidin (MAL) and petunidin (PET), usually known as anthocyanidins.[3]

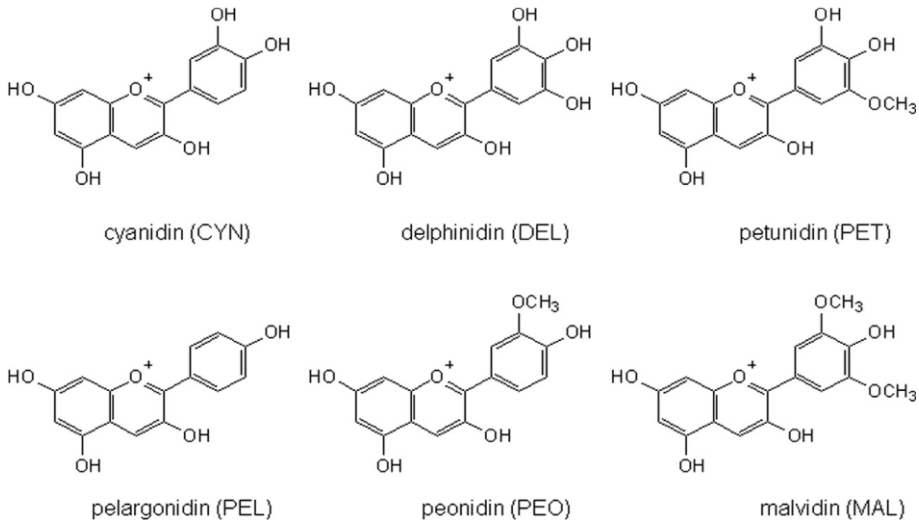

Figure 2. Chemical structures of the six most common anthocyanidins. (from ref. 1)

Several factors such as pH, storage temperature, concentration, light, oxygen, solvents and the presence of enzymes, flavonoids, proteins and metallic ions can affect the stability of isolated anthocyanins.[7] As far as the dependence on pH is concerned, the predominant species at pH 1 is the flavylium cation (AH^+), which shows a red color, but a series of chemical reactions take place if the pH is increased (Figure 3): the hydration of the flavylium cation gives rise to a colorless hemiketal (form B) that can undergo a tautomerisation reaction responsible for ring opening, giving the pale yellow cis-chalcone form (Ccis). The latter can moreover isomerizes to the trans-chalcone (Ctrans).[8, 9] Ultimately, a proton transfer transforms the red flavylium cation into a blue quinoidal base (A).[8, 9] As a general rule, at pH above 4, yellow (chalcone form), blue (quinoidal base) and colorless (hemiketal) products are formed, while the red flavylium cation is predominant at pH 1–2[10] and the blue quinoidal base A is the major component around pH 6–8.[6, 11, 12]

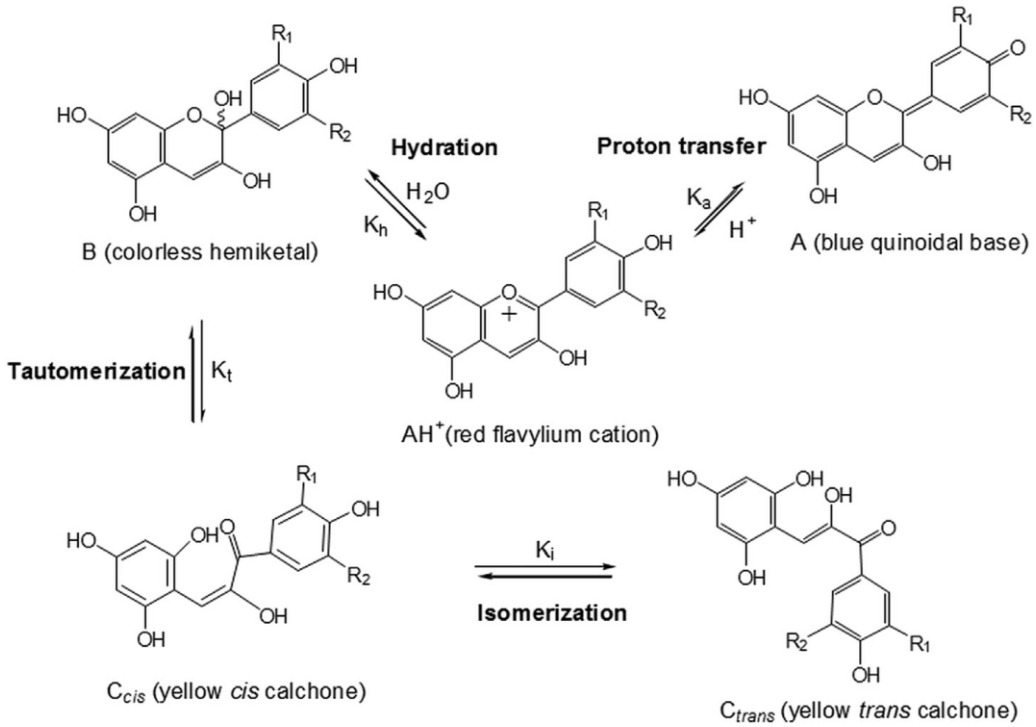

Figure 3. Equilibria in aqueous solution for an anthocyanidin (from ref 1).

Anthocyanins recently attracted the interest of many researchers, as they exhibit antioxidant properties[13, 14] and could be used as food colorants.[15, 16] Indeed, with an increasing awareness of the potential health issues associated with synthetic food color additives, food industry has become particularly interested in replacing them with natural alternatives, including fruit and vegetable extracts containing anthocyanins.[16] In addition, thanks to their antioxidant properties, which give them a potential role in prevention of various diseases associated with oxidative stress,[3] the interest in this class of compounds as potential nutritional supplements for humans recently increased. Thus in addition to their functional roles as colorants, anthocyanin extracts may improve the nutritional quality of food and beverages.[6] Finally, together with other polyphenolics, anthocyanins have been studied to evaluate the ripening stage of grape for chemotaxonomic purposes.[4]

Besides the recently discovered properties of anthocyanins, it is indubitable that the colors of flowers and fruits have fascinated the humankind since ancient times: the use of anthocyanins as dyeing matter is reported in the Roman period by Pliny the Elder[17] and the architect Vitruvius.[9] Moreover, ancient recipes show that early Celtic and Germanic peoples used the strongly colored juices of wild berries to dye their hair and clothes obtaining purple shades much more cheaply than with seashells.[17] Also the pre-Columbian civilizations, such as Maya[18] and Incas,[19] seem to have used the local vegetable sources of anthocyanins for dyeing: for example, the colorant extracted from purple corn (*Zea Mays L.*) was used by the Inca civilization to prepare food and to dye textile fibers.[19] Finally, as well resumed by Melo,[18] the use of vegetable sources of anthocyanins as “watercolors” was frequently described in several treatises or recipe books on illumination such as the Strasbourg manuscript, an early Portuguese work on manuscript illumination and the Italian *De arte illuminandi*. Especially, plants containing anthocyanins could be used to produce clothlets (“pezzuole”) as described by Cennino Cennini in the 15th century.[20] However

the colors obtained were not durable, as they are very light-sensitive and pH-sensitive, giving hues from violet-red in acid dye-baths to blue alkaline ones.[17]

The identification of anthocyanins in plant extracts is nowadays mostly accomplished by HPLC–MS analysis[21, 22] and only limited literature can be found concerning the vibrational studies of these compounds. In this context, it is worth citing the pioneering work of Merlin and co-workers,[23, 24] who in the 90s obtained the resonance Raman spectra of anthocyanidins and some anthocyanins, suggesting an assignment of their vibrational frequencies. These authors also performed some *in vivo* micro analyses of the pigments present in a single cell of the skin of ''Pinot noir'' grape berries.[25] More recently, Raman analyses of anthocyanins as such[26] or complexed with Fe^{3+} and interacting with polysaccharides,[27] or again complexed with Fe^{3+} and Al^{3+}[28] were published. Also an *in-situ* Fourier transform Raman study for the analysis of pigments on the colored petals of pansy cultivars was reported.[29]

The first aim of the present study was to assess the identification of anthocyanidins by means of surface-enhanced Raman scattering (SERS), exploiting the chemical specificity of the technique and its sensitivity to changes in molecular structures, phenomena that, as detailed above, occur extensively in the chemistry of anthocyanins. Moreover, this spectroscopic technique, already applied to the characterization of other flavonoid compounds,[30, 31] can improve the limited number of Raman studies of anthocyanidins, which indeed resulted not fully effective in order to discriminate between one or another compound, especially in solution.

The positive identification obtained allowed us to further characterize anthocyanins in more complex conditions, such as in plant extracts and in dyed textiles, by means of surface-enhanced Raman scattering. Also in these cases, the influence of pH on SERS spectra was evaluated and, in order to verify the possibility of identifying anthocyanins also in extracts not subjected to purification, a study of the SERS response of a mixture of anthocyanidin and flavonoid, both present in the plant sources analyzed and both able to absorb onto silver nanoparticles, was carried out.

Ultimately, being known the instability of anthocyanins, the possibility to identify such molecules also in faded textile samples was verified: wool threads dyed with anthocyanin-rich vegetable sources were exposed to artificial ageing and then analyzed by means of SERS, bearing in mind that the final aim of the study was to develop a protocol of analysis suitable for ancient historical objects.

Natural sources of anthocyanins used in art and history

The use of bilberry, elderberry, sumac, purple corn and hollyhock as natural sources of anthocyanins is reported in detail in the following sections, highlighting their relevance in art and history and listing their most characteristic anthocyanins.[2]

Elder (*Sambucus nigra L.*)

Elderberry was mentioned by Pliny the Elder in the *Naturalis Historia* probably for the first time. In the Roman period, different parts of elder were used to dye textiles, leather and paper. From berries, the part of the plant which contains anthocyanins, blue and purple colors were obtained: the shades could be different depending on the kind of mordant adopted.[32] A recently published Raman study of a 6th century illuminated manuscript allowed to identify the presence of elderberry lake as painting material.[33]

The principal anthocyanins present in the berries of *Sambucus nigra L.* are cyanidin 3-glucoside, cyanidin 3-sambubioside and cyanidin 3-sambubioside-5-glucoside.[34-36]

Bilberry (*Vaccinium myrtillus L.*)

As regards the evidences of use of these berries as dyeing matter in the Roman period, Pliny (*Naturalis Historia* XVI:17) reports that the Gauls used bilberries to obtain a purple dye for clothes that, on account of its poor colorfastness, were worn principally by slaves. The famous architect Vitruvius described instead the “bilberry

purple", obtained by encapsulating the berry juice within a clay, to be used in mural paintings as a substitute for the true purple pigment.[9] A dye obtained from bilberries is mentioned also in the oldest "recipe book" preserved in Western Europe (8th century A.D.), the *Compositiones ad tingenda musiva, pelles et alia* ("Recipe for dyeing mosaics, leathers and other things").[17] Recipes for dyeing with bilberries also appear in a number of medieval manuscripts and printed treatises from Germany and Sweden.[17]

The anthocyanin composition of bilberries is characterized by several anthocyanidin glycosides in which five anthocyanidins (delphinidin, cyanidin, petunidin, peonidin, malvidin) are combined with different sugars.[17, 37] In *Vaccinium myrtillus* species, a large amount of cyanidin was detected.[37]

Sumac (*Rhus coriaria L.*)

Rhus coriaria L. is commonly known as sumac, being the name originated from *sumaga*, which means "red" in Syriac. This plant has been known and widely used since Greek times, as described by Theophrastus (4th–3rd century B.C.). Sumac can still be found in the Central Sahara, where the wild plant has a long and important history of local exploitation.[38, 39]

In 1997, Mavlyanov et al.[40] isolated cyanidin 3-glucoside, delphinidin 3-glucoside and delphinidin from the fruits of sumac; ten years later Kosar et al.,[41] using HPLC-MS, identified the 3-glucosides of peonidin, petunidin and delphinidin, petunidin-3-glucoside coumarate, delphinidin-3-glucoside coumarate, delphinidin coumaroyl glucoside and cyanidin coumaroyl glucoside.

Purple corn (*Zea Mays L.*)

The colorant extracted from purple corn (*Zea Mays L.*) seems to have been used by the Inca civilization both to prepare food and to dye textile fibers[19]. Still nowadays people from the Andean region use the purple extract from this corn together with

cochineal to dye textiles in red and purple colors, in order to save the more expensive cochineal itself.[42]

The principal anthocyanins found in *Zea Mays L.* are cyanidin 3-glucoside, peonidin 3-glucoside and cyanidin 3-(6''-malonylglucoside).[43]

Hollyhock (*Althaea rosea L.* var. *Nigra*)

Hollyhocks were traditionally cultivated in the gardens of oasis towns in central Asia, most famously Bukhara and Samarkand, both for their ornamental qualities and for their purplish-black dyes, used for silk and leathers, coming from their dark purple flowers.[17] In the 19th century, in Germany, especially in Bavaria, hollyhock petals were first used as a colorant for red wines; they were then tried for dyeing and printing cotton cloth and soon used on a commercial scale. Around 1870, the greater part of this dyestuff was supplied by Turkey where about 700 tons of dried flowers were produced each year.[17, 44]

Although the major anthocyanins of black flowers are generally delphinidin glycosides, those of *A. rosea Nigra* comprise also petunidin and malvidin. Thus, it was presumed that black flower color of this cultivar is due to co-occurrence of many anthocyanins. In fact, the absorption maxima of anthocyanins differ slightly from one another and anthocyanin content is high in petals, so that their light absorption spreads in the entire visible range giving rise to the black color.[45] As regards the glycosides, in a first study[46] the pigments were reported to be 3-glucosides and 3,5-diglucosides of delphinidin, petunidin and malvidin. In 2012, another work based on LC-MS and spectroscopic analysis of the black hollyhock flowers[45] identified again, as the major anthocyanidins, those found previously (delphinidin, petunidin and malvidin); however the anthocyanins present in major amount resulted to be delphinidin 3-glucoside, delphinidin 3-rutinoside, petunidin 3-rhamnosylglucoside and malvidin 3-rhamnosylglucoside. Glycosides of cyanidin were also detected, even if in lesser amount.

Experimental

Materials

Silver nitrate ($AgNO_3$) (purity $\geq$ 99.5%), sodium perchlorate monohydrate (assay ≥ 99.0%) and trifluoroacetic acid (assay ≥ 99.5%) were obtained from Fluka; methanol (assay ≥ 99.9%), acetonitrile (assay ≥ 99.9%) and trisodium citrate dihydrate (assay 100.2%), from Sigma–Aldrich; hydrochloric acid (min. 37%) from Riedel-de Haën, ethyl acetate from VWR. C18 cartridges (100 mg sorbent) used for purification of plant extracts were obtained by Supelco. The anthocyanidins cyaniding chloride, delphinidin chloride, malvidin chloride, pelargonidin chloride, peonidin chloride were kindly donated by Dr. L. Santagostini (Department of Chemistry, Università degli Studi di Milano) and Dr. S. Pilu (Department of Agricultural and Environmental Sciences, Università degli Studi di Milano), while petunidin chloride and cyanidin 3-glucoside were purchased from Extrasynthese (France). The purity of all the anthocyanidin samples was checked by HPLC. All the aqueous solutions were prepared using ultrapure water (Millipore MilliQ). 10^{-4} M solutions of anthocyanidins were prepared in methanol acidified with 0.01% HCl.

Blueberries were purchased from the herbalist's shop "ErbeSalute" (Milan, Italy), while elderberries and hollyhock flowers were donated by "A. Minardi & Figli" and purple corn glumes were obtained from a *Morado* like variety named *Reduno*. The term "corn glumes" comes from the Latin *gluma*, meaning "husk of grain". Very briefly, they are the external sheath that wind up the grains of purple corn.

Reference wool threads dyed with sumac berries were washed, treated with alum, $KAl(SO_4)_2 \cdot 12H_2O$, acid potassium tartrate and dyed as described elsewhere.[38] Wool samples dyed with blueberries, elderberries and hollyhock were donated by Dr. Paola Cicuta, while wool samples dyed with purple corn were produced at the Department of Scienze Agrarie e Ambientali - Produzione, Territorio, Agroenergia of Università degli Studi di Milano. In all cases a mordanting process with alum, $KAl(SO_4)_2 \cdot 12H_2O$, was used to fix the dye on the textiles.

Extraction and purification of anthocyanins from plant sources

A total of 3 g of sumac berries, bilberries and hollyhock flowers were finely grounded, suspended in 10 mL MeOH with 1% of HCl and sonicated for 15 minutes. The obtained solutions were then filtered through a 0.45 µm GHP Acrodisc membrane filter. For elderberries, the same procedure was carried out using 6 g of starting material. For what concerns colored maize, 1 g of purple corn glumes was milled and boiled in 100 mL of distilled water for 1 hour. After 7 hours, the solution was filtered through a 0.45 µm GHP Acrodisc membrane filter.

The extracts were then loaded onto Discovery Supelco C18 SPE cartridges previously pre-conditioned by 2 mL of MeOH and 3 ml of MilliQ water + 0.01% HCl. Organic acids and free sugars were washed away with 2 mL of MilliQ water + 0.01% HCl, while polyphenols were removed with 2 mL of ethyl acetate. Finally, the anthocyanin-contained fractions were eluted using 3 mL of MeOH acidified with 0.01% of HCl. The obtained solutions were finally evaporated under a N_2 gentle stream and re-dissolved in 300 µl of MeOH.[47]

Extraction of anthocyanin-based dyes from reference dyed wool samples

About 12 or 6 mg of each wool thread was suspended in 3 ml of MeOH with 100 µL of HCl 37% and placed in a water bath at 70°C for 1 hour. Then the extracts were filtered through a 0.45 µm GHP Acrodisc membrane filter, evaporated under a N_2 gentle stream and re-dissolved in 3 mL of MeOH.[48, 49]

The procedure was carried out also with lower amount of sample, until 0.5 mg, in order to verify the possibility to obtain SERS response even in these conditions.

Ageing by artificial light of reference dyed wool samples

Reference wool threads were exposed to a xenon arc lamp for 4, 12, 24 and 36 hours. The exposure device was a Xenotest 150S+ (Atlas) set as required by UNI EN ISO 105-B02, in normal conditions for temperate zone (black panel temperature: 47 ± 3 °C,

effective humidity: 40%, irradiance: 42 ± 2 W/m^2 in the wavelength range 300 to 400 nm). The light source consists of a xenon arc lamp of color temperature 5500 – 6500 K, with a filter system providing a reasonable simulation of solar radiation filtered by typical window glass.

The transmission of the filter system used shall be at least 90% between 380 and 750 nm, falling to 0 between 310 and 320 nm and attenuating infrared radiation to allow a better control of the sample temperature.

The color fading was evaluated by reflection UV-visible spectroscopy before and after the exposure of the samples.

Silver colloid synthesis

To perform SERS analyses, a citrate-reduced silver colloid obtained according to the Lee and Meisel procedure[50] was chosen as substrate, due to its ease of preparation and use. The protocol for the synthesis was previously described.[51] Briefly, 18 mg of $AgNO_3$ was suspended in a round flask with 100 mL of deionized water previously degassed under a gentle N_2 stream and heated to boiling. Then 2 mL of a 1% solution of trisodium citrate was slowly dropped under vigorous magnetic stirring; the solution was held at boiling point for 60 min with continuous stirring. The measured pH of the colloid was 7.

Samples preparation and analysis

For SERS analyses, 100 μL of analyte, i.e. both 10^{-4} M solutions of anthocyanidins prepared in methanol acidified with 0.01% HCl and extracts (purified or not) from plant sources and textiles, were added to 1 mL of Ag sol under magnetic stirring.

The aggregation of silver nanoparticles was achieved with the addition of 41.5 μL of $NaClO_4$ 1.8 M. With a Pasteur pipette, a drop of the solution was placed on a glass slide and posed under the laser beam.

In order to perform SERS analysis at different pH values, the pH of the colloidal solution was varied adding the proper amount of HNO_3 0.01 M or KOH 0.01 M. The pH of such solution was measured before the addition of the analyte. To check the final pH value experimented by the anthocyanidins, pH measurements of the final sample solutions were performed, confirming the values obtained before the addition of the analytes. SERS spectra were reproducibly obtained for each compound at a given pH value, as was verified by repeating the analysis on at least two preparations of the system analyte-colloid and on at least three drops from each preparation.

Instrumentation

SERS spectra were collected by a compact micro-Raman instrument composed of: a Jasco RMP100 probe with a notch filter and a 50x microscope objective; a Lot Oriel MS25 spectrometer equipped with a 1800 lines/mm grating and a Peltier-cooled charge coupled device (CCD) detector. A frequency-doubled Nd:YAG laser provided the exciting radiation at 532 nm and the laser power at the sample used was around 1.5 mW. All SERS spectra were recorded between 2000 and 300 cm^{-1} by collecting 30 scans with an exposure time of 2 s. A resolution around 8 cm^{-1} was estimated in the considered spectral range.

Fourier-transform (FT) Raman spectra were obtained by means of a Jasco FT/IR-620 spectrometer equipped with a RFT-600 Raman attachment. A Nd-YAG laser source (λ_{exc} = 1064 nm) was used for the excitation, with a power at the sample of about 50–60 mW. The spectra were obtained as average of 300 accumulations.

HPLC analyses were performed by means of a HPLC PU-1580 Jasco pump, equipped with a LG-1580-02 Jasco gradient valve, a Jasco DG-2080-53 solvent degasser and an MD 1510 Jasco diode-array detector in order to obtain spectral information between 200 and 600 nm. The analyses were executed, using a 20 µL injection volume of solution of the sample, on a Supelco Discovery C18 column (25 cm×3 mm, diameter 5 µm) by using (A) H2O with 0.1% trifluoroacetic acid and (B) acetonitrile with 0.1%

trifluoroacetic acid as solvents, with a flow of 0.4 mL/min. Parameters for gradient elution are reported elsewhere.[52]

UV-visible reflectance spectra of samples were acquired in the range 350-880 nm using a Jasco UV/VIS/NIR V-570 spectrophotometer equipped with a $BaSO_4$-coated integrating sphere. From the obtained spectra, CIELab values for dyed wool samples with different exposure times were determined referring to the CIE D65/2° illuminant–observer conditions.

Results and discussion

SERS spectra of anthocyanidins at different pH

In the present section, the SERS analyses of the six most common anthocyanidins recorded at different pH values comprised between 3 and 10 were described.[1] As it was expected for molecules strongly dependent on pH, they showed significantly different spectral patterns varying the pH of the sample solution. However, the obtained SERS spectra at pH 3 showed the same spectral patterns of those recorded at pH 4 and the SERS signals at above pH 7 were similar to those recorded around neutrality. Therefore the SERS spectra reported in the present doctoral thesis (Figure 4 and band wavenumbers listed in Tables 1 and 2) are only those at pH 4 and 7 respectively, i.e. at those pH values in which the differences in terms of spectral pattern are significant.

For the sake of completeness, it should be noted that, the SERS spectra obtained at alkaline pH for most anthocyanidins resemble those recorded at pH 7, but for peonidin and pelargonidin SERS spectra at alkaline pH could not be obtained. As the degradation of flavonoids occurs for those molecules with a catechol moiety,[53] such as CYN, DEL and PET, for which however SERS spectra could be obtained in alkaline conditions, this could led to exclude degradation or polymerization processes. In our case, it seems that those molecules that display only one OH group on the phenyl ring are the most penalized by the performance of the colloid in alkaline conditions.

The final concentration of each anthocyanidin analyzed, taking into account the dilution due to the additions of silver sol and of the $NaClO_4$ solution, is 10^{-6} M.

From the UV–vis spectra of anthocyanins at the pH values of 4 and 7,[26] it clearly appears that the use of the 532 nm exciting radiation entails the occurrence of the resonance conditions, therefore the SERS spectra here reported can be defined, more precisely, as surface-enhanced resonance Raman scattering (SERRS) spectra. Indeed the use of exciting radiation at 785 nm, obviously not fulfilling the resonance condition, did not give an appreciable SERS response. The resonance conditions moreover allowed to selectively record the SERS features of the colored species, the only resonant with the excitation wavelength, even if there are other but colorless species present in the complex multi-equilibria systems at the different pH values examined.

In 1994, Merlin and co-workers[24] showed that the visible chromophore of anthocyanidins is mainly located on the benzopyrylium moiety, rather than on the phenyl ring (ring B). On the other hand, anthocyanidins are differentiated by the substitution patterns on ring B. Indeed, even if some differences are observed in the low-frequency region of resonance Raman (RR) spectra of anthocyanidins, the reported spectral patterns are rather similar, especially for example for malvidin in comparison with petunidin or for peonidin in comparison with cyanidin.[23] On the other hand, the SERS spectra obtained in the present work show greater differences, leading to a further step in the identification of such important compounds by means of vibrational spectroscopy. The work of Merlin et al. concerning resonance Raman (RR) analysis of anthocyanins[23-25] is considered as the main term of comparison for the SERS spectra reported in the present study. It is worth remembering that, while the RR spectra were obtained from aqueous solutions, we dissolved anthocyanidins in MeOH + 0.01% HCl. However, the same authors demonstrated that anthocyanidins in these two solvents showed the same spectral patterns,[23] allowing the comparison of the results.

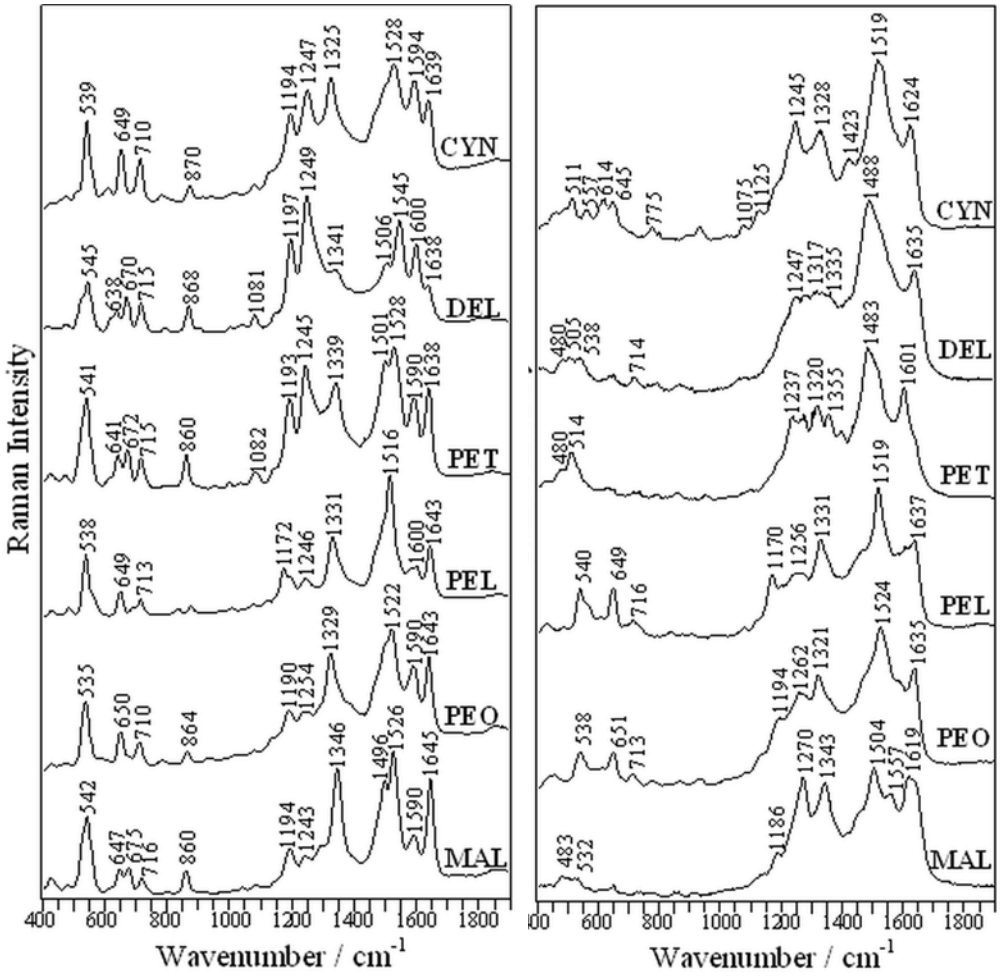

Figure 4. SERS spectra of anthocyanidins 10^{-6} M at pH 4 (left) and pH 7 (right). (Fig. from ref 1)

As shown in Figure 4 and similarly to what suggested for RR spectra,[24] the SERS signals between 1400 and 1650 cm^{-1} of anthocyanidins can be attributed to ring stretching vibrational modes, ν (CC), both of benzopyrylium moiety and phenyl ring. However, upon pH variation, a difference arises: at pH 4 two signals can be found at around 1600 and 1640 cm^{-1}, while at pH 7 the compounds show only one strong signal at around 1630 cm^{-1}. This feature was recognized also in RR spectra of anthocyanidins and the different bands were attributed to the flavylium cationic form and to the quinoidal base, respectively.[25] Indeed, the band at ca. 1630 cm^{-1} at pH 7

can present a contribution from the C=O stretching of the quinoidal form.[26] Moreover, under acidic pH, where the red flavylium cation and the colorless hemiketal usually predominate, it is reasonable that SERRS spectra exhibit the vibrational modes characteristic of the flavylium cation, i.e. of that species whose signals are enhanced by the resonance conditions.[23] An interesting structural feature of anthocyanins, but also of flavonoids in general, is the torsional angle θ that reflects the relative contributions of the π-electronic interaction between the B ring and the remainder of the molecule.[23, 54] The inter-ring bond stretching mode, which falls at ca. 1350 cm^{-1}, would be indeed sensitive to changes in delocalized π-electron density within the rings and therefore, an increase of the delocalization would lead to an increase in force constant and therefore of the wavenumber of the signal.[23] At pH 4, the SERS bands attributed to inter-ring vibration fall at wavenumbers that seem to confirm the change of the bond order reported by Merlin and co-workers as logical consequence of the degree of substitution: delphinidin, petunidin and malvidin, which carry three substituents on the phenyl ring, have their inter-ring bands at higher wavenumbers (1341, 1339 and 1346 cm^{-1}, respectively). However, the inter-ring stretching mode is expected to couple with ring vibrations and can be involved in many normal modes,[23, 24] therefore it is not easy to distinguish between the contributions of different vibrations that fall at the same frequency. The explanation of this signal in the spectra at pH 7, when the main form is no more the flavylium cation but the hemiketal and the quinoidal base,[6, 11, 12] is more difficult as the change in aromaticity modifies the SERS spectral patterns, similarly to what happens in the resonance Raman experiments.[26]

At pH 4, the SERS spectra of cyanidin, petunidin and delphinidin (Figure 4, left) show, in the region between 1100 and 1260 cm^{-1}, strong signals which could be reasonably attributed to the bending modes of the hydroxyl groups and to the stretching of C-OH bond, having these molecules 2 or 3 OH groups on the phenyl ring. The other compounds show instead only medium-weak signals at the same wavenumbers. At

pH 7, most anthocyanidins show only weak SERS signals at these frequencies (Figure 4, right), as the molecules in these conditions are deprotonated. At this pH value, the main signal of the SERS spectra of anthocyanidins is the band that falls at ca. 1480–1520 cm^{-1}, attributed to ring stretching vibrational modes, ν(CC). At lower wavenumbers, the assignment of bands results more difficult, however some of them could be attributed to skeleton out-of-plane bending modes, Γ (CC) (Tables 1 and 2).

Table 1. Band wavenumbers (cm^{-1}) of SERS spectra of anthocyanidins at pH 4 and their tentative assignments

CYN	DEL	PET	PEL	PEO	MAL	Tentative assignment
1639 (s)	1638 (w)	1638 (s)	1643 (m)	1643 (s)	1645 (s)	ν (CC)
1594 (s)	1600 (s)	1590 (s)	1600 (w)	1590 (w)	1590 (w)	ν (CC)
1528 (s)	1545 (s)	1528 (s)	1516 (s)	1522 (s)	1526 (s)	ν (CC)
	1506 (w)	1501 (s)		1502 (sh)	1496 (s)	ν (CC)
1325 (s)	1341 (w)	1339 (s)	1331 (m)	1329 (m)	1346 (s)	$\nu(CC)_i;\delta(CH)$
1247 (s)	1249 (vs)	1245 (s)	1246 (w)	1254 (w)	1243 (vw)	ν (CO)
1194 (s)	1197 (s)	1193 (s)	1194 (w)	1190 (w)	1194 (w)	δ (OH)
			1172(m)			δ (CH)
	1081 (w)	1082 (w)				
870 (m)	868 (m)	860 (m)	875 (vw)	864 (vw)	860 (w)	γ (CH)
710 (s)	715 (m)	715 (m)	713 (w)	710 (w)	716 (w)	
	670 (m)	672 (m)			675 (w)	
649 (s)	638 (w)	641 (m)	649 (w)	650 (w)	647 (w)	
539 (s)	545 (m)	541 (s)	538 (m)	535 (m)	542 (s)	δ (CC)
	474 (vw)	472 (w)	483 (vw)	479 (vw)	477 (vw)	Γ (CC)
		424 (w)	428 (vw)	426 (vw)	427 (vw)	Γ (CC)

sh, shoulder; s, strong; m, medium; w, weak; ν, stretching; δ, in-plane bending; γ, out-of-plane bending; Γ, skeleton out-of-plane bending; i, inter-ring.

Table 2. Band wavenumbers (cm^{-1}) of SERS spectra of anthocyanidins at pH 7 and their tentative assignments

CYN	DEL	PET	PEL	PEO	MAL	Tentative assignment
1624 (s)	1635 (s)	1635 (sh)	1637 (s)	1635 (s)	1619 (s)	ν(CC); ν(CO)
		1601 (s)				ν (CC)
					1557 (sh)	ν (CC)
1519 (vs)	1488 (vs)	1483 (s)	1519 (s)	1524 (vs)	1504 (s)	ν (CC)
1423 (w)						
		1396 (w)				ν (CC)
	1335 (m)	1355 (m)				ν (CC)
1328 (s)	1317 (m)	1320 (m)	1331 (s)	1321 (s)	1343 (s)	ν $(CC)_i$;ν(CC)
	1276 (m)	1274 (m)	1256 (sh)	1262 (m)	1270 (s)	ν (CO)
1245 (s)	1247 (sh)	1237 (m)				ν (CO)
			1199 (vw)	1194 (sh)	1186 (w)	δ (OH)
			1170 (m)			δ (CH)
1075 (w)						
775 (w)				772 (vw)		
	714 (vw)		716 (w)	713 (w)		
645 (w)			649 (m)	651 (w)		
614 (w)						
557 (vw)						
511 (w)	538 (w,br)	514 (w)	540 (m)	538 (w)	532 (vw)	δ (CC)
	505 (w,br)					
	480 (w,br)	480 (vw)			483 (vw)	Γ (CC)
	415 (vw)	424 (vw)	431 (w)	455 (vw)		Γ (CC)

sh, shoulder; s, strong; m, medium; w, weak; ν, stretching; δ, in-plane bending; Γ, skeleton out-of-plane bending; i, inter-ring.

Comparison among SERS spectra of anthocyanidins at the same pH

As already mentioned, the RR analyses of different anthocyanidins gave back essentially the same spectral pattern, since the B-ring substituents are not chromophoric in the investigated spectral region, and therefore no signal directly assignable to their internal modes can be observed. On the contrary, important differences among the SERS spectra of the most common anthocyanidins can be noted, leading to hypothesize that the spectra reflect not only the influence of the benzopyrylium moiety, but also that of the B-ring, whose substitution pattern is different for each anthocyanidin.

As it can be seen in Figure 4, at pH 4 PEL, PEO and MAL present strong signals due to the ring stretching and to the inter-ring bond at ca. 1330 cm^{-1}, while CYN, DEL and PET display stronger bands at ca. 1245 cm^{-1} in correspondence of the C–OH stretching and strong signals at ca. 1190 cm^{-1} due to the in-plane bending of OH. The SERS spectra of CYN, DEL and PET are anyway different in terms of relative intensity of the just mentioned bands, feature that allows the univocal identification of the anthocyanidins.

According to the assignments of RR bands reported in the literature,[23] the signals observed for all examined compounds in the 1500–1650 cm^{-1} interval are mostly due to C–C stretching vibrations of the benzopyrylium moiety, with the exception of the two bands located at 1590–1600 cm^{-1} and at 1496–1501 cm^{-1} (exact wavenumbers depend on the compound and can be checked in Figure 4), attributed to the 8*a* and 19*a* modes (using the Wilson's notation) of the phenyl ring, respectively. It should be noted that, at acidic pH, the SERS spectra of CYN, DEL and PET show an appreciable intensification of bands due to OH groups with respect to those due to the aromatic system, especially in comparison with both RR spectra in solution[23] and FT-Raman spectra obtained on solid chlorides (Figure 5). This is not the case for the SERS spectra obtained at the same pH value for PEL, PEO and MAL.

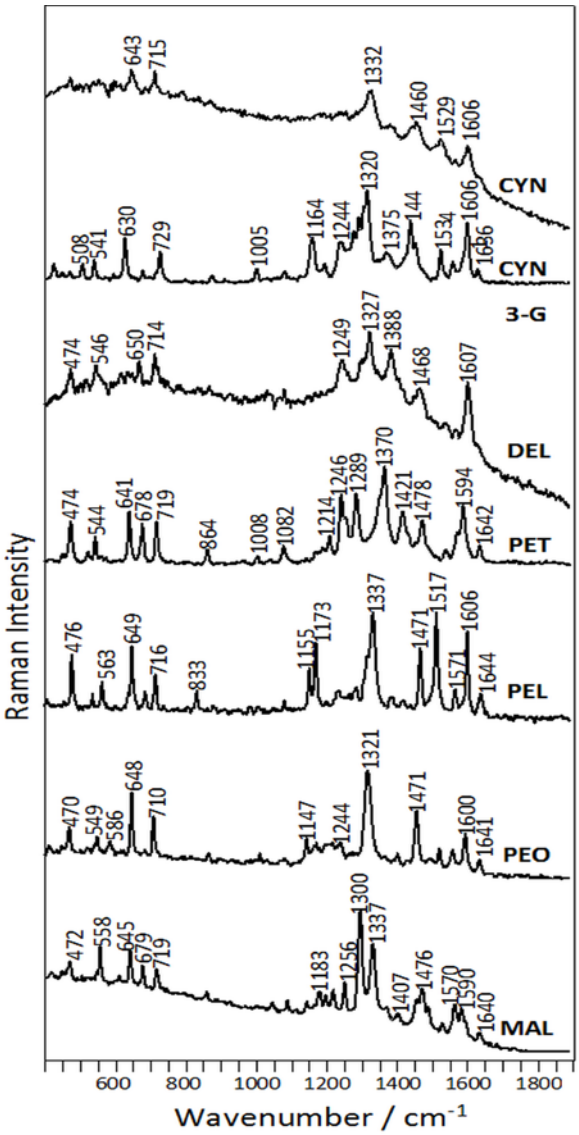

Figure 5. FT-Raman spectra of anthocyanidins in powder (figure from ref. 1)

As far as the comparison among anthocyanidins at pH 7 is concerned, Figure 4 shows that cyanidin, delphinidin and petunidin have similar features, as it happens for pelargonidin, peonidin and malvidin. A similar behavior is therefore linked with the different substitution patterns of the molecules: CYN, DEL and PET exhibit two hydroxyl groups in position 3' and 4', while PEL, PEO and MAL display only one hydroxyl group in 4' (Figure 2). In contrast with the influence of the hydroxyl groups, the presence of a single methoxyl group seems not to affect significantly the SERS spectral pattern, as it can be seen comparing the SERS spectra (Figure 4) of petunidin (CH_3 in position 5') and peonidin (CH_3 in position 3'). Again this observation is in good agreement with the literature.[23] It is worth highlighting that also at this pH value, the spectral features of each analyte allow to discriminate between one or another anthocyanidin.

Interactions between anthocyanidins and Ag nanoparticles

It is well known that metal complexation is possible only for those anthocyanidins that exhibit at least two ortho-hydroxyl groups in the B-ring, i.e. cyanidin, delphinidin and petunidin.[55] If the interaction of anthocyanidins with Ag nanoparticles had been similar to that of chelation, only three molecules could have shown a SERS response. Nevertheless, all six anthocyanidins gave back a SERS spectrum, showing that the kind of interaction with the metallic support can be different. Therefore, even if the evaluation of the interactions between anthocyanidins and Ag nanoparticles from SERS spectra is not an easy task, some hypotheses can be formulated in this respect.[1] As far as the interactions occurring at acidic pH is concerned, CYN, DEL and PET seem to interact with the metal through the two hydroxyl groups in position 5' and 4', as in their SERS spectra the signals related to the C-OH and OH vibrations are particularly intensified (Table 1). On the contrary, for PEL, PEO and MAL the adsorption on metal nanoparticles could occur by means of the aromatic system present in both the flavylium and the hemiketal forms, as suggested by the fact that their strongest signals are attributed to the ring stretching vibrational modes, ν (CC).

However, it should be noted that for all examined molecules SERS spectra show bands due to in-plane ring modes mainly of the benzopyrylium moiety, also in the low-frequency region (bands at about 540, 650 and 670 cm^{-1}, assigned to in-plane CC bending), probably due also to the resonance component of the observed SERRS effect. Even for PEL, PEO and MAL a completely flat-on orientation of the molecules on the Ag surface should be thus ruled out. Therefore, it is more reasonable to hypothesize that CYN, DEL and PET have probably an end-on orientation insisting on the two chelating hydroxyl groups: indeed, at least for the first two compounds, the signal at ca. 1590 cm^{-1} due to an in-plane phenyl ring stretching shows a remarkable intensity, while it is slightly weaker for PET, the orientation of which is probably perturbed by the presence of a methoxyl group on the same ring. As far as PEL, PEO

and MAL are concerned, a tilted orientation involving both hydroxyl groups and π-electron system in the interaction with the metal is deemed probable.

When the pH arises to the neutrality, for CYN, DEL and PET the relative intensity of the bands in the region comprised between 1100 and 1450 cm^{-1} (i.e. the signals related to OH and C–OH vibrations) decreases: this could possibly be explained hypothesizing the occurrence of co-existent interactions between the silver nanoparticles and both the hydroxyl groups remaining after the formation of the quinoidal bases and the π-electron system of the same molecules, as the spectra exhibit an intense signal at ca. 1485 cm^{-1} related to ν (CC) (at 1519 cm^{-1} for cyanidin).

It is worth remembering that, on passing from the flavylium cation to the hemiketal and the quinoidal base, the conjugation of π bonds is less extended though still present.[26, 56] Moreover, the equilibrium reaction between the AH^+ and A species, previously described as a single proton transfer process (Figure 3), is valid only when there is one free hydroxyl group and must be replaced by a much more complicated system when there are four ionizable hydroxyls, as in the cases of anthocyanidins.[56] However, we could hypothesize that for those molecules that probably interact with the silver surface also through their aromatic rings at acidic pH (i.e. PEL, PEO and MAL), in a neutral environment the contribution to the interaction of the less extended π-electron system is reduced in favor of the involvement of the remaining hydroxyl groups, as suggested by the increased magnitude assumed by the signals related to OH groups at pH 7 in comparison with the bands of the ring vibrations (Figure 4).

Study of the effect of the glycosidic moiety on SERS spectra

The effect of glycosylation was investigated by the comparison of the spectral features of cyanidin (CYN) and cyanidin 3-glucoside (CYN 3-G), whose SERS spectra are reported in Figure 6.[1]

At acidic pH, the bands at 1247 and 1594 cm^{-1} of CYN 3-G are less intense than the same signals in the aglycone spectrum, while the signal at 1467 cm^{-1} of the glycosylated compound appears as a shoulder in the spectrum of CYN. In the corresponding RR spectra reported in the literature,[23] the differences between the two compounds are far less marked, even if, similarly to what observed in the SERS spectra, the band at 1246 cm^{-1} is weaker and that at 1480 cm^{-1} is stronger for the glycoside in comparison with the aglycone. The more significant variation promoted by glycosylation in the SERS spectrum of cyanidin is most probably connected with the change of the molecular orientation with respect to the metal surface due to the steric hindrance of the sugar moiety. Observing the variation in the relative band intensities, it can be hypothesized that for the glycoside the chelation through the two hydroxyl groups on the B ring is probably less favored. Interestingly, the SERS spectral pattern observed at acidic pH for CYN 3-G partly resembles that described above for PEL, PEO and MAL, i.e. those anthocyanidins that cannot chelate Ag through two ortho hydroxyl groups.

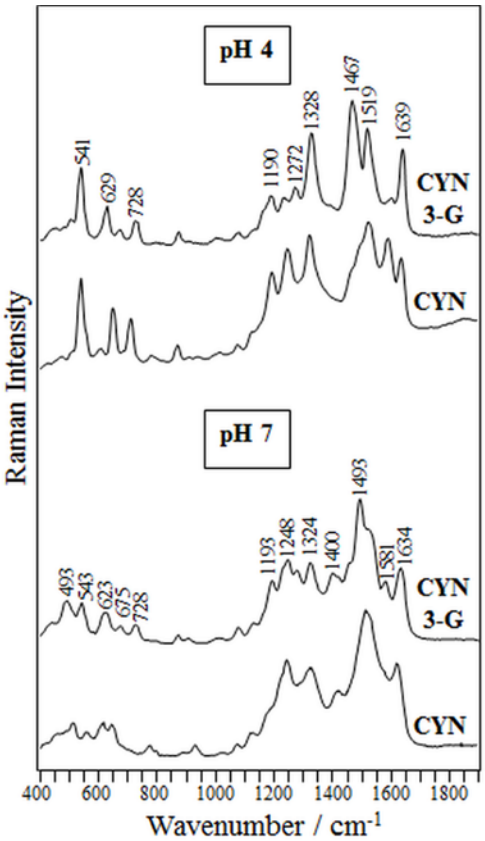

Figure 6. SERS spectra of cyanidin (CYN) and cyanidin 3-glucoside (CYN 3-G) at pH 4 and 7 (figure from ref. 1)

Coherently, at pH 7 the SERS spectra of the aglycone and the corresponding glycoside are more similar, except for the signal at ca. 1190 cm^{-1}, which is only a weak shoulder in the spectral pattern of CYN. Indeed at pH above neutrality, the observed signals are

due to the quinoidal base, for which the involvement of the B ring in the interaction with Ag is less important, as already discussed above for anthocyanidins.

SERS analysis of extracts from plant sources

The SERS spectra obtained from purified vegetable extracts at pH 4 and 7 are shown in Figure 7 and the corresponding wavenumbers are listed in Tables 3 and 4, respectively.[2]

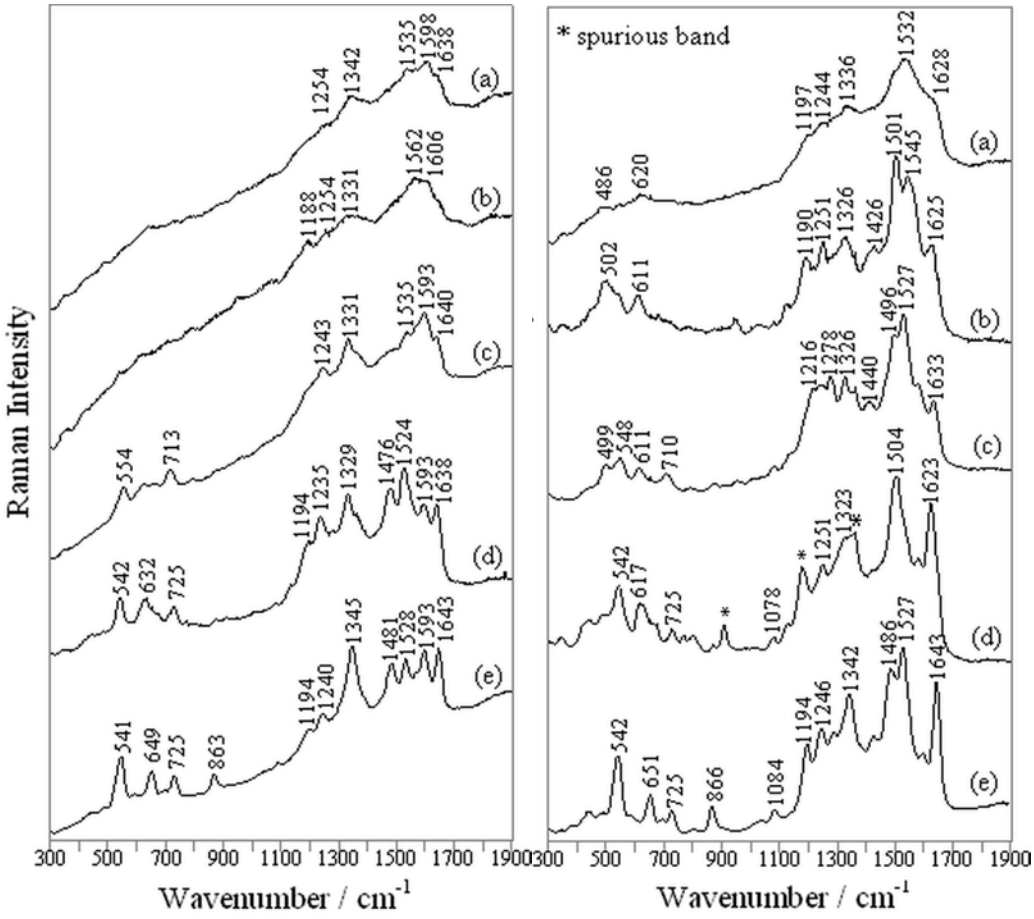

Figure 7. SERS spectra at pH 4 (left) and pH 7 (right) of the extracts from plant sources of: (a) elderberry, (b) bilberry, (c) sumac, (d) purple corn, (e) hollyhock (figure from ref. 2).

Table 3. Wavenumbers (cm^{-1}) of SERS spectra of anthocyanins in plant extracts and dyed wool at pH 4:									
elderberry		bilberry		sumac		purple corn		hollyhock	
berries	dyed wool	berries	dyed wool	berries	dyed wool	cob glumes	dyed wool	flowers	dyed wool
1638 (sh)	1628 (sh)	1606 (sh)	1630 (sh)	1640 (sh)	1624 (sh)	1638 (m)	1638 (m)	1643 (s)	1648 (m)
1598 (m)	1590 (m)	1562 (m)	1598 (s)	1593 (s)	1594 (m)	1593 (m)	1595 (m)	1593 (s)	
1535 (m)	1524 (s)		1529 (s)	1535 (m)	1536 (s)	1524 (s)	1527 (s)	1528 (s)	1524 (vs)
						1476 (s)		1481 (s)	
1342 (m)	1350 (sh)							1345 (vs)	1334 (m,br)
	1326 (m)	1331 (br)	1342 (s)	1331 (s)	1330 (m)	1329 (s)	1334 (m)		
1254 (w)	1240 (m)	1254 (w)	1243 (s)	1243 (m)	1245 (m)	1235 (m)	1240 (m)	1240 (m)	
	1197 (sh)	1188 (w)				1194 (sh)		1194 (sh)	1175 (m)
									1112 (m,br)
								863 (w)	
	707 (w)			713 (m)		725 (m)	722 (w)	725 (w)	
	651 (w, br)		654 (vw)	656 (vw)	650 (w)			649 (w)	
				623 (w)		632 (m)	643 (w)		
	538 (w)		545 (w)	554 (m)		542 (m)	545 (w)	541 (m)	

Table 4. Wavenumbers (cm^{-1}) of SERS spectra of anthocyanins in plant extracts and dyed wool at pH 7:									
elderberry		bilberry		sumac		purple corn		hollyhock	
berries	dyed wool	berries	dyed wool	berries	dyed wool	cob glumes	dyed wool	flowers	dyed wool
								1643 (s)	1650 (m)
1628 (sh)	1627 (sh)	1625 (m)	1631 (m)	1633 (m,sh)	1629 (sh)	1623 (s)	1633 (sh)		
				1578 (m)	1574 (sh)		1527 (s)	1596 (vw)	1565 (sh)
1532 (s)	1504 (s,br)	1545 (s)	1529 (s)	1527 (s)				1527 (s)	1524 (s)
		1501 (s)		1496 (s)	1492 (s)	1504 (s)		1486 (s)	
		1426 (m)	1423 (m)	1410 (m)	1425 (m)			1424 (w)	
				1361 (m)			1353 (m)		
1336 (m)	1335 (m)	1326 (m)	1337 (m)	1326 (m)	1338 (m,br)	1323 (m)	1332 (m)	1342 (s)	1337 (m,br)
	1319 (m)			1278 (m)				1298 (w)	
1244 (m)	1241 (m)	1251 (m)	1238 (m)	1247 (br)	1248 (w)	1251 (m)	1240 (m)	1246 (m)	
				1216 (sh)					
			1186						
1197 (sh)	1192 (sh)	1190 (m)	(wv,sh)		1185 (sh)		1200 (sh)	1194 (m)	
						1127 (sh)	1137 (sh)		
						1078 (vw)		1084 (vw)	1117 (w,br)
								866 (w)	
				710 (w)		725 (w)		725 (w)	
						629 (sh)	645 (w)		
620 (vw)	608 (w)	611 (w)	614 (vw)	611 (w)		617 (m)	615 (w)	651 (w)	
			541 (m)	548 (w)	514 (m)	542 (m)	545 (w)	542 (m)	
486 (vw)	483 (w)	502 (m)	474 (w)	499 (w)					

It is worth remembering that, as detailed in the section “Natural sources of anthocyanins used in art and history”, each extract contains various anthocyanins, constituted by the anthocyanidin skeleton to which several and different glycosides were linked. Therefore the assignment of each SERS band to a single anthocyanin is not possible. Interestingly, however, extracts mainly containing cyanidin-based anthocyanins, i.e. bilberry, sumac, purple corn and elderberry (even if with a lower signal-to-noise ratio) show similar spectra at both pH values and can be distinguished from the spectral pattern of hollyhock, in which glycosides of other anthocyanidins are predominant. In more detail, at pH 4 they show a characteristic spectral pattern with strongest signals at ca. 1240, 1330, 1530, 1590 and 1640 cm^{-1}. All these bands were attributed to ring stretching vibrational modes, with the exception of the signals at ca. 1240 cm^{-1} that are related to the stretching of C-OH bond, as discussed above.

Referring to the study of the influence of the glycosidic moiety on SERS spectra of anthocyanins, the spectral patterns of cyanidin-based extracts can be related to those of cyanidin and its 3-glucoside, even if some differences in the exact wavenumbers of the signals could contribute to the differentiation of the spectra. In the cases of elderberry, bilberry and sumac, the SERS bands at acidic pH are superimposed on large signals at ca. 1300 and 1580 cm^{-1} due to the formation of amorphous carbon from photodissociation of organic molecules at the silver colloid surface. The occurrence of this phenomenon was already observed in SERS experiments[57-60], in particular when the pH of the sol is decreased by means of the addition of strong acids.[61] Therefore, at pH 4, bands possibly related to glycosylated anthocyanins can be better distinguished in the SERS spectra of purple corn and hollyhock. For example, the signal at 1476 cm^{-1} observed for purple corn can be related with cyanidin 3-glucoside. Moreover, a distinctive signal, possibly related to its content in malvidin, can be found at 1345 cm^{-1} in the spectrum of hollyhock at acidic pH (see Figure 7), allowing to distinguish it from the cyanidin-based species.

At neutral pH, the SERS spectra are more detailed and, also in this case, it is possible to identify a common spectral pattern for cyanidin-based extracts. Common features can be found at ca. 1190 cm^{-1}, whose presence can be attributable to a contribution of cyanidin 3-glucoside, and at ca. 1250, 1320-30 and 1630 cm^{-1}. While the latter two signals could be related to ring stretching vibrational modes, the bands at 1630 cm^{-1} present also a contribution from the C=O stretching of anthocyanins in their blue quinoidal form, predominant at this pH value with respect to the flavylium species. Moreover, two signals at ca. 1500 and 1530 cm^{-1} are present in all the spectra, with the exception of purple corn which exhibits only the signal at 1504 cm^{-1}. It should be noted that at pH 7 the SERS spectra are not affected by the signals of amorphous carbon, but spurious bands were detected in the spectrum of purple corn at 1362, 1178 and 908 cm^{-1}, corresponding to the so-called anomalous bands already observed by several authors in the SERS spectra obtained with the Lee and Meisel colloid.[62]

Again, the SERS spectrum of the extract from flowers of hollyhock (spectrum (e) of Figure 7, right) is slightly different with respect to the other SERS spectra obtained from plant sources, coherently with the different kind of anthocyanins contained in this plant. In particular, the SERS spectrum of hollyhock shows diagnostic peaks at 1342 cm^{-1}, possibly related to the SERS spectrum of malvidin, and 1643 cm^{-1}.

More generally, it is worth noting that the strongest SERS signal of the anthocyanin-based samples here examined, located between 1490 and 1550 cm^{-1}, falls at different wavenumbers for the different plant extracts; this could be possibly explained considering this large signal as the superimposition of the contributions of each anthocyanin present in the plant sources examined, weighed for its relative amount. This feature could lead to facilitate the distinction among the different plant sources, even if the major differences in spectral patterns can be noted between the cyanidin-based species and hollyhock.

SERS analyses of extracts from dyed wool

In order to recover the greater amount of colored matter from textiles, the pre-treatment of these kind of samples did not include the purification step. Nevertheless, extracts from dyed wool showed good-quality SERS spectra for all the samples examined, as it can be seen in Figure 8.

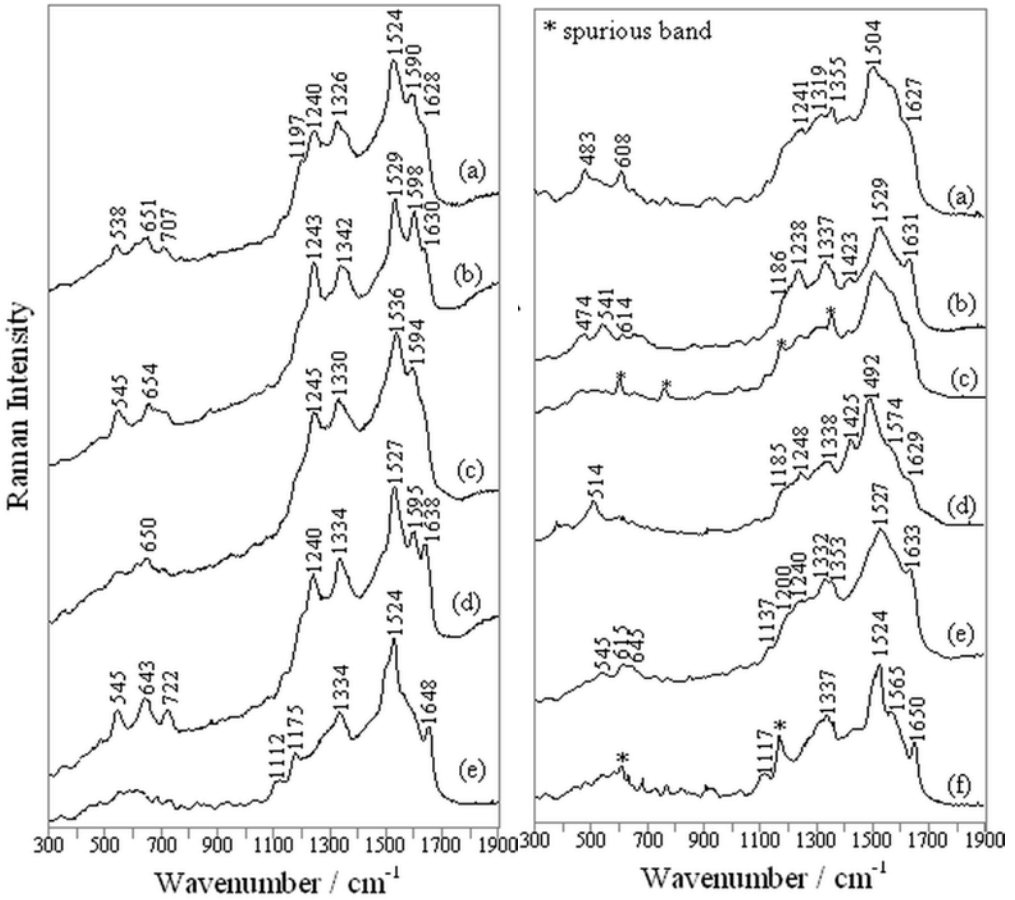

Figure 8. SERS spectra at pH 4 (left) and pH 7 (right) of the extracts from wool samples dyed with: (a) elderberry, (b) bilberry, (c) sumac, (d) purple corn, (e) hollyhock (fig. from ref. 2).

It is well known that plant sources used for dyeing, i.e. berries, flowers and cob glumes, contain not only anthocyanins but also organic acids, sugars, polyphenols and other flavonoids. These latter molecules in particular are able to interact with silver nanoparticles showing characteristic SERS signals.[30] Therefore, in order to verify the

possibility to detect SERS signals of anthocyanins in non-purified extracts of textile samples, a mixture of the main anthocyanin and the main flavonol present in elderberry,[36] respectively cyanidin and quercetin, was prepared and analyzed. The SERS spectra of an equimolar mixture of cyanidin (CYN) and quercetin (QUC) 10^{-4} M are reported in Figure 9. It is easy to see that at acidic pH the SERS spectrum obtained is very similar to that of CYN at the same pH, while the SERS spectrum of the CYN+QUC solution at neutral pH shows spectral features which belong both to the anthocyanin CYN and to the flavonol QUC. In more detail, the signals at 1591, 1400, 1187, 845 and 594 cm^{-1} are attributed to QUC, while the vibration modes of CYN are responsible for the bands at 1519, 1331, 1246, 776 and 555 cm^{-1}. Ultimately, the signals at 648 and 513 cm^{-1} are present in the spectra of both pure compounds, while the band at 1637 cm^{-1} finds a better correspondence with QUC, even if a contribution by CYN cannot be excluded. The detection of the signals of CYN at acidic pH and of those of both QUC and CYN at neutral pH could be explained with the loss of the chelating hydroxyl groups of CYN when the pH arises, feature which could decrease its affinity

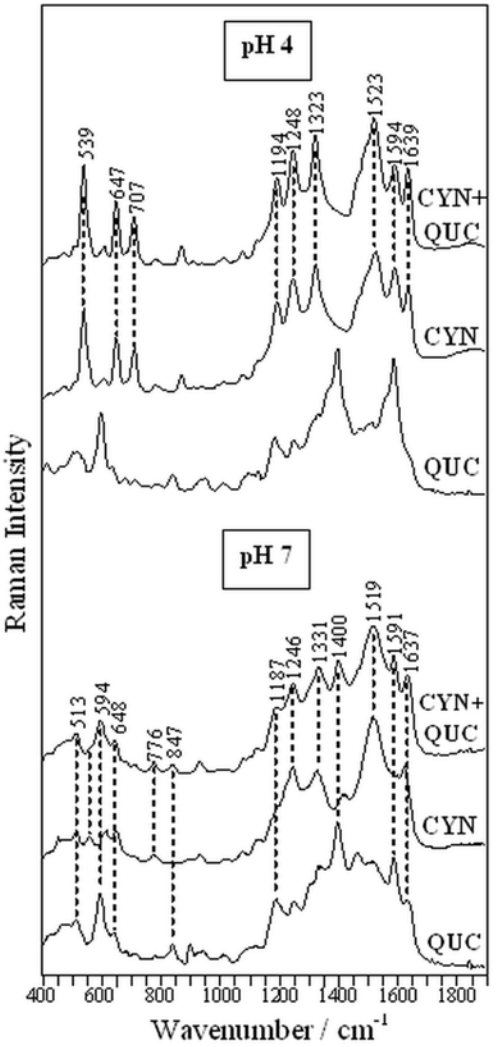

Figure 9. SERS spectra of a mixture of CYN and QUC 10^{-4} M at different pH compared with reference SERS spectra of pure CYN and QUC (fig. from ref.2).

for the Ag nanoparticles, in favor of the flavonol quercetin. Therefore, while at acidic pH no interference of other compounds in the spectra of anthocyanins occurs, in neutral condition we detected a coexistence of signals related to different species. Nevertheless, also in this case the spectral pattern is dominated by anthocyanin signals. Thus, the SERS spectra obtained from wool samples without purification were considered reliable and the observed wavenumbers are listed in Tables 3 and 4.

Again, wool samples dyed with cyanidin-based plant sources gave place to SERS spectra with common features (Figure 8). In this case, however, the spectral patterns show a better correspondence with the SERS spectrum of the aglycone cyanidin than to that of cyanidin 3-glucoside, which is instead recognizable in the spectra obtained from the plant sources (Figure 7). Such hypothesis is formulated mainly looking at the SERS spectra obtained at acidic pH, the condition at which the glucoside and its aglycone show remarkably different spectral patterns, as previously demonstrated. In more detail, at this pH value, the extracts from dyed textiles mainly containing cyanidin show signals at ca. 1240 and 1330 cm^{-1}, the strongest signal at ca. 1530 cm^{-1} and ultimately a band at ca. 1590 cm^{-1}. The strongest signal of the spectrum obtained from wool dyed with sumac falls at 1536 cm^{-1}, therefore at a higher wavenumber with respect to the others. Again, the principal bands observed are related to the ring stretching vibrational modes, with the exception of the signals at ca. 1240 cm^{-1} that are related to the stretching of C-OH bond. It should be remembered that it has been already reported in the literature[63, 64] that extraction procedures using MeOH/HCl to hydrolyze the dye-aluminum complexes formed during the mordanting process can break the glycosidic bonds of dyes. The occurrence of this breaking, which entails the release in solution of the corresponding aglycones, was confirmed in the present study also by means of HPLC analyses. Indeed, for those plant species which contain cyanidin 3-glucoside, the comparison between the chromatograms obtained from plant extracts and those from wool samples allowed to detect, in the former samples, the presence of cyanidin 3-glucoside and, in the latter ones, only cyanidin or a co-

existence of the two molecules, as in the case of wool dyed with elderberry (Figure 10).

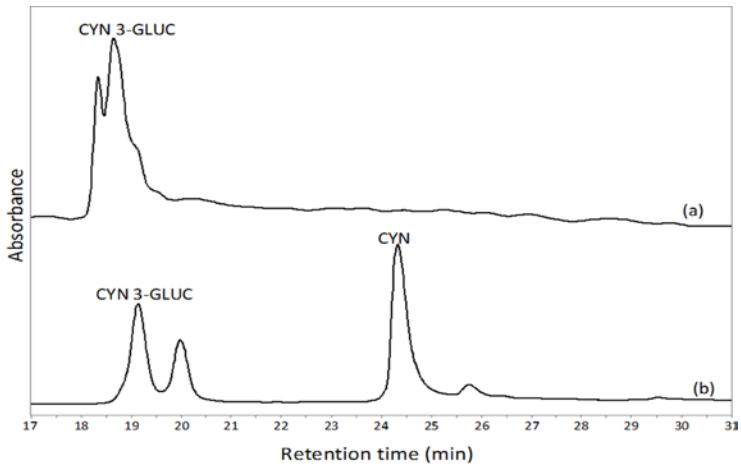

Figure 10. Chromatograms at λ = 520 nm of (a) extract from elderberries, (b) extract from wool dyed with elderberries (fig. from ref. 2).

A trend for cyanidin-based extracts can be detected also at neutral pH, with principal signals falling at ca. 1240, 1330, 1520 and 1630 cm^{-1}. Again the SERS spectrum of sumac shows a difference, displaying the strongest band at 1492 cm^{-1}. As previously reported for the extracts from plant sources, the main signals at ca. 1330, 1520, 1630 cm^{-1} of the spectral patterns observed could be related to ring stretching vibrational modes, and for the bands at 1630 cm^{-1} a contribution from the C=O stretching of anthocyanins in their blue quinoidal form should be considered. As far as the wool dyed with hollyhock is concerned, it shows SERS spectra with features slightly different from the previously described cyanidin-based ones. Diagnostic peaks, falling at higher wavenumbers with respect to the spectra of the other species, can be found at 1648 and 1650 cm^{-1} at acidic and neutral pH values, respectively. Ultimately, the extraction procedure was carried out also on lower amounts of wool dyed with bilberry to test the SERS response obtainable upon reducing the sample size. As shown in Figure 8, we could observe a reliable SERS spectrum even when using just 0.5 mg of wool thread.

SERS analysis of extract from aged dyed wool

As previously mentioned, anthocyanins are unstable to light, fading away more easily than other dyeing compounds. This fact could probably explain their limited use in history for dyeing textiles; however their attested use as coloring matters[17, 18, 20] justifies the need to include them in the SERS database at the disposal of laboratories involved in the identification of dyestuffs. In order to verify the possibility to identify anthocyanins also in aged textile samples, dyed wool samples were subjected to artificial ageing, exposing them to a xenon lamp for 4, 12, 24 and 36 hours, until they lost their original color. The color change was monitored through the colorimetric coordinates obtained by reflection UV-visible analyses (Table 5).

Table 5. CIELab* values of dyed wool samples exposed to xenon lamp

sample	L*	a*	b*	ΔE^*_{ab}
elderberry	32.40	8.75	8.00	0
elderberry 4h	35.79	9.77	11.78	5.18
elderberry 12h	40.29	9.92	14.36	10.20
elderberry 24h	44.69	8.43	15.55	14.42
elderberry 36h	44.28	7.52	15.57	14.14
bilberry	42.63	8.61	4.69	0
bilberry 4h	47.57	7.87	8.69	6.40
bilberry 12h	43.67	6.06	10.47	6.40
bilberry 24h	52.48	6.53	14.63	14.15
bilberry 36h	52.20	5.86	15.34	14.58
sumac	50.58	6.59	19.25	0
sumac 4h	53.94	6.67	20.14	2.99
sumac 12h	52.53	6.76	20.11	2.14
sumac 24h	51.61	6.77	20.54	1.65
sumac 36h	53.11	5.07	19.74	3.48
hollyhock	43.28	12.13	10.56	0
hollyhock 4h	45.51	10.91	12.06	2.95
hollyhock 12h	49.62	9.37	13.15	7.39
hollyhock 24h	53.51	7.37	14.17	11.85
hollyhock 36h	54.07	6.22	14.44	12.91

The light-induced color changes produced during accelerated ageing showed a similar trend for elderberry, bilberry and hollyhock: an increase in lightness (L*) and yellowness (b*) values is detected whereas redness (a*) values decrease. In the case of sumac, this trend is less evident, probably because the natural degradation of the dye already occurred, as this sample was dyed in our laboratory some years ago. However, the achievement of a rather advanced fading of the dyeing matter on wool can be hypothesized as the color difference (ΔE*) values of samples exposed 24 hours are similar to those obtained exposing the samples for 36 hours, probably indicating that a maximum of color fading was reached. The scarceness of wool dyed with purple corn at our disposal did not allow us to obtain reliable colorimetric data for purple corn, nevertheless SERS spectra are available (Figure 11). As these latter analyses is concerned, in each case, the residual dyes extracted from the aged wool samples exhibited reliable SERS signals, obviously characterized by a lower intensity with respect to the unaged wool samples and a lower signal-to noise ratio, as exemplified in Figure 11 for wool dyed with purple corn and bilberry. In the figure, the SERS spectra are aligned on the same Raman intensity scale. These results suggest that the identification of anthocyanins by SERS is possible even if the color faded away, thus the technique could be suitable also for the analysis in historical textiles.

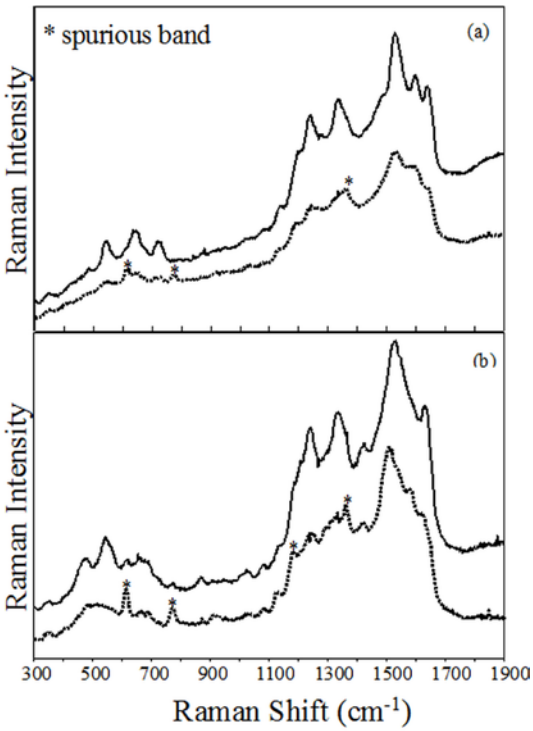

Figure 11. SERS spectra of extract from unaged wool (continuum line) of (a) purple corn at pH 4 and (b) bilberry at pH 7 compared with those of samples of the same wool aged 36 h (dotted line).

Conclusions

With the present work[1, 2] a deeper insight into the spectroscopic characterization of anthocyanins by means of SERS was provided. First of all, reference samples of the most common anthocyanidins were investigated, producing also an original study of the dependence on the pH of SERS spectra of these molecules. The spectra obtained show both features shared in common by all the analytes at the same pH, leading to identify the main species in solution, and diagnostic signals that allow the univocal identification of the anthocyanidins. Moreover, on the basis of the spectra recorded, the influence of the glycosidic moiety of anthocyanins and the interactions between the analytes and silver nanoparticles, used as substrate for SERS analyses, were studied.

A further step in the development of a protocol for the analysis of ancient art objects possibly dyed with anthocyanins was then approached. In particular, experimental procedures for the identification of anthocyanins-based dyes used in antiquity were reported: the extraction and the purification of dyes from plant sources and the extraction from both unaged and artificially aged textiles allowed to obtain dye samples which gave back reliable SERS signals. It was also demonstrated that, even if the colors of textiles dyed with anthocyanins fade away upon ageing, their identification can still be possible by means of SERS.

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APPENDIX TO CHAPTER 2

The *folium* dye: an anthocyanin?

Introduction

The so-called *folium* dye (or *Torna-ad-solem*) has been quoted in the medieval recipe of Heraclius *De coloribus et Artibus Romanorum* and by Theophilus in the recipe *Diversarum artium schaedula*.[1] This dyeing matter is obtained from the fruit and other parts of the plant scientifically called *Chrozophora tinctoria Juss.* (Figure 1), native of the East but also imported in Africa and Italy. It is now known under the name of *Heliotrope minor* or even *Tornasole*, a term that has often created confusion with another type of vegetable dye, also called *Tornasole* or *Laccamuffa* and corresponding to various species of lichens (*Roccella tinctoria*) from which orchil, another purple dye, is obtained.[2] The name *Tornasole* is however full of meaning: if, in fact, the extract of the plant is treated with even weak acids, the color becomes light purple, ranging up to red depending on the pH (Figure 1). In this way, by the same vegetable matter, different colors could be obtained, which the miniaturists in the Middle Ages called "*Folium rubeum*" if the color was red-brown, "*Folium Purpureum*" if a violet or purple was obtained, "*Folium Saphireum*" if the color was blue.[1] The final color seems also to depend on the amount of quicklime added in the preparations.[3]

It seems that f*olium* dye was used as clothlets ("pezzuole" in Italian), as described by Cennino Cennini in the 15th century.[2] Clothlets were small pieces of cloth imbibed with the juices expressed from berries or other parts of dye-plants and worked as carriers for the dye. When required, the illuminator dipped the cloth in a medium that allowed the dye to dissolve in solution and applied it as a watercolor. The name *folium* probably originated from the place in which the *pezzuole* were conserved: "inter cartas librorum" said Cennini,[2] i.e. "among the pages of books", where a sheet of paper is said "folium" in Latin. Interestingly, as already reported in Chapter 2, the use of vegetable sources of anthocyanins as "watercolors" was frequently described in several ancient treatises or recipe books, in which recipes for *pezzuole* were also reported.[2, 4]

Folium dye has been scarcely studied from a chemical point of view, especially it is not known what molecules are responsible for the colors observed. A large contribution in the attempt to clarify the origin of *folium* was given by Guineau,[3] who reported some spectroscopic and mass spectrometry analyses of this mysterious dye, observing the presence of orcinol, a compound also present in lichen dyes. Indeed, also other researchers[5, 6] suggested the similarity between *folium* and orchil, the red dye cited above and obtained from various lichen species upon fermentation in ammonia. Other studies,[6, 7] however, hypothesized that, according to its properties of changing color on varying pH, *folium* could contain anthocyanin compounds.

Very recently, different non-invasive and micro-invasive techniques were used by Aceto and co-workers[8, 9] in order to increase the diagnostic information available on this dye. In particular, they compared the results obtained from *folium* dye with those from orchil. Nevertheless, in spite of the multitude of analytical techniques used, the chemical composition of *folium* seems to be elusive, therefore the authors aimed to identify *folium* on artworks rather than characterize its structure or composition.

At the same time, some analyses on this dye were carried out also in our laboratory. In particular, we assessed the possibility that the chemical origin of *folium* could be that, already formulated but never demonstrated, of an anthocyanin-based dye. In this brief Appendix, preliminary analyses on *folium* dye with different analytical techniques and the application of the developed protocol for the identification of anthocyanins described in Chapter 2 were reported.

Figure 1. *Folium* dye on parchment (left) and berries of *Chrozophora tinctoria* (right).

Experimental

Materials

Folium dye is commercially available as dry extract of *Chrozophora tinctoria* on textile carrier (Kremer Pigmente), thus it needs to be dissolved in polar solvents and then dried under a gentle stream of N_2 in order to obtain a solid sample. The same procedure was conducted onto those samples obtained with an aqueous extraction from berries of *Chrozophora tinctoria*, kindly donated by Oron Peri (Israel).

The dye was extracted from these sources with different methodologies; however, those reported in this Appendix were conducted with aqueous solutions in which the pH value is changed by means of the addition of hydrochloric acid (min. 37%) and sodium hydroxide. The acid was purchased from Riedel-de Haën, while the base from Sigma-Aldrich, as well as methanol (assay ≥ 99.9%) and formic acid (reagent grade ≥ 95%). The neutral extracts were also laid on parchment, as described in the ancient treatises cited above.

Instrumentation

The Raman instrumentation, as well as the HPLC and UV-vis instruments were the same used for the development of the analytical protocol for the identification of anthocyanins and for whose description we refer to Chapter 2.

FT-SERS spectra were recorded between 4000 and 200 cm^{-1} with an RFT-600 Jasco FT-Raman spectrometer with a resolution of 4 cm^{-1}, using for excitation the 1064 nm emission of a Nd:YAG laser.

SEM-EDX analyses were recorded by means of a Hitachi-TM 1000 scanning electron microscope covering a magnification range between ×20 and ×10,000. The instrument was equipped with an energy-dispersive electron microprobe, a pre-centred cartridge filament and a high-sensitive semiconductor backscattered electron detector.

Micro-Fourier transform infrared (μ-FTIR) spectra were obtained with a Jasco IRT-3000 spectrometer, with a spectral range of 4000–600 cm^{-1} and a resolution of 4 cm^{-1}.

Results and discussion

Our preliminary approach was to experiment different extraction methodologies varying the pH of the extracting solutions in order to assess the color changes described in ancient treatises, monitoring these changes through UV-vis analyses of the extracts. However, neither with the commercially available *folium* nor with berries of *Chrozophora tinctoria* relevant changes were observed, excepting a change from violet to purple-pink of the commercially available dye after some hours (Figure 2). The UV-vis spectra obtained form this source show in all the cases two maxima of absorption at 535 and 570 nm, which do not correspond any of known red dyes, being the maxima of absorption of anthraquinones comprised between 428 and 495 nm (in methanol), those of anthocyanins between 518 and 530 nm (in acid solution), those of redwoods at ca. 442-556 nm (in methanolic or aqueous solution, depending on the solubility of dyes), those of alkanet at 489, 518 and 556 nm (in methanol) and those of orchil at 528-555 nm (in methanol). Eventually, UV-visible reflectance spectroscopy analyses were also performed on *folium* dye both on the textile carrier and laid on parchment, obtaining results in good agreement with the spectra reported by Guineau (results not shown).[3]

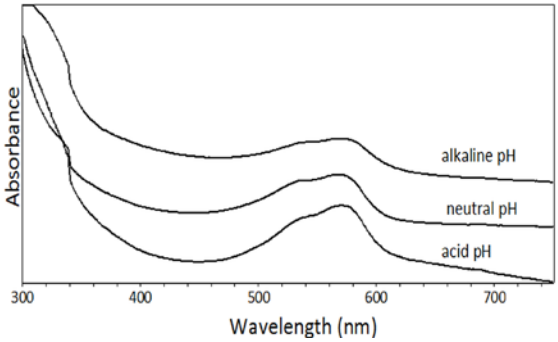

Figure 2. Vis spectra of aqueous solutions from commercially available folium dye upon extractions at different pH values.

As already pointed out in Chapter 1, UV-vis spectroscopy hardly allows specific identification of dyes, as it shows poor specificity. Therefore, other analytical

techniques were employed, such as those based on vibrational spectroscopies, that could achieve a suitable degree of specificity in the recognition of chemical compounds. Unfortunately, most of the spectroscopic analyses conducted onto the different dyeing matters obtained upon extractions revealed that *folium* dye is very fluorescent, thus both from Raman, with different excitation wavelengths, and FT-Raman analyses no vibrational information could be obtained. The micro-FTIR spectra shown in Figure 3 were instead obtained from the extracts of the commercially available *folium* dye at different pH values. At acidic pH the dye gave back a spectral pattern in which the signal at ca. 1700 cm^{-1} could be consistent with a carboxylic acid, species that could be present also in the spectrum at neutral pH as carboxylated salt.

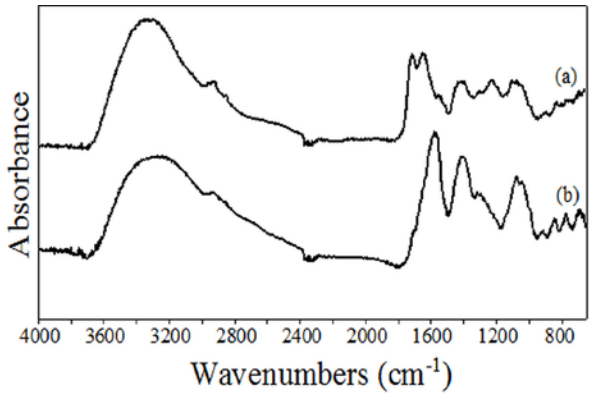

Figure 3. Micro-FTIR of extracts from the commercially available folium dye (a) at acidic pH, (b) at neutral pH.

The spectra obtained could be consistent with those of anthocyanins,[10] even if scarce literature concerning the spectroscopic characterization of anthocyanins by means of FTIR spectroscopy can be found. Interestingly, a SEM-EDX analysis of the textile carrier confirmed the presence of calcium on the textile, supporting the hypothesis, deriving from the FTIR analyses, of the presence of an organic salt on the fibers and the possible treatment of the dye with quicklime (CaO), as described by ancient treatises. However, other analyses were required to confirm this hypothesis. In

particular, we wanted to approach the chromatographic separation of all the components of the dye in order to isolate and identify the molecules responsible of the colors. Therefore, several gradient HPLC-PDA analyses were conducted in order to find the best composition of eluents and elution program to achieve this aim. Unfortunately, as regards the commercially available *folium* on a textile carrier, no satisfactory separation was achieved, while the best separation of the extract from berries was obtained using water acidified with formic acid and methanol, as reported in Table 1 together with the elution program.

Table 1. Elution program

Time (min)	%A	%B
0	93	7
2	93	7
8	85	15
35	45	55
37	20	80
39	20	80
49	93	7

A = H_2O + 5% HCOOH
B = MeOH

Figure 4 shows the chromatogram from the extract of berries obtained in these conditions:

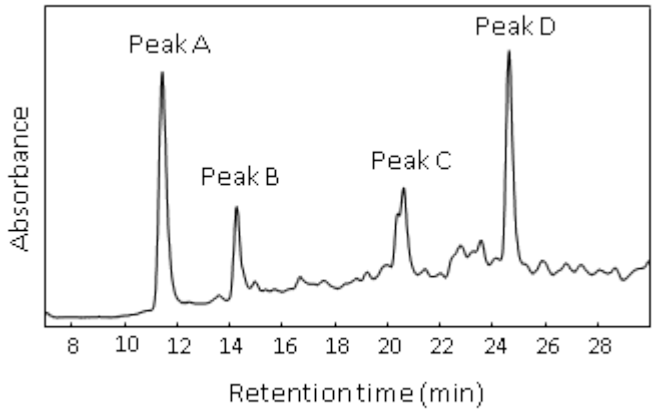

Figure 4. Chromatogram (λ = 560 nm) of the red eluted compounds from the extract of *Chrozophora tinctoria* berries.

Among the eluted species, four compounds with UV-vis spectra (Figure 5) coherent with the red color of the extract were recognized. However, their spectra did not correspond to those of anthocyanins,[11] or of other known red dyes, being the maxima of the recorded spectra at higher wavelengths. Moreover, even if the solution was concentrated till it apparently acquired a very dark red-brown color, the absorbance spectra of the red eluted compounds showed low absorbance values, probably indicating great molar absorption coefficients of the unknown molecules, which allow the extract to be colored even if the chromophoric components are present in a very small amount. The four compounds were tentatively separated, but, for the reasons just cited, the amount of the products collected was really low.

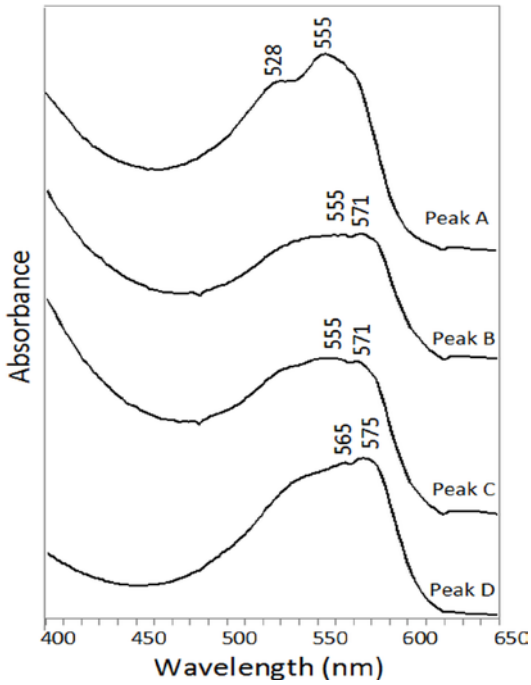

Figure 5. Vis spectra of the four compounds of the extract from berries analyzed by HPLC.

Ultimately, also to try to overcome the problem of fluorescence, the protocol described in Chapter 2 for the identification of anthocyanins by means of SERS was

applied to the extract obtained from berries. Unfortunately, also in this case the signals were completely overlapped by a very fluorescent background, already observed in the Raman analyses. SERS analyses of the single compounds separated by HPLC and collected were tentatively performed, but no SERS signal could be recorded due to the fluorescence. The only reliable SERS spectrum obtained was a Fourier-transform (FT) SERS spectrum of the whole extract from berries (Figure 6). As it will be discussed in Chapter 4, the use of FT-Raman instrumentation can further reduce the fluorescence typical of natural dyes. Interestingly, the FT-SERS spectrum recorded is in accordance with the SERS one recently published on the dye spread on parchment.[8] In ref. [8] the authors claimed that their SERS spectrum of *folium* dye displays spectral features similar to its FT-Raman spectrum, whose interpretation was hindered by the fact that it resulted to be dominated by the spectral features of the parchment on which the folium dye was laid. In contrast with this, our spectrum is not affected by the problem of parchment, being recorded from the extract of *Chrozophora tinctoria* berries. A comparison of the FT-SERS spectrum of folium with that of cyanidin (Figure 6), one of the most common anthocyanidin, confirmed differences in the spectral patterns, therefore adding a proof against the hypothesis of its anthocyanin content.

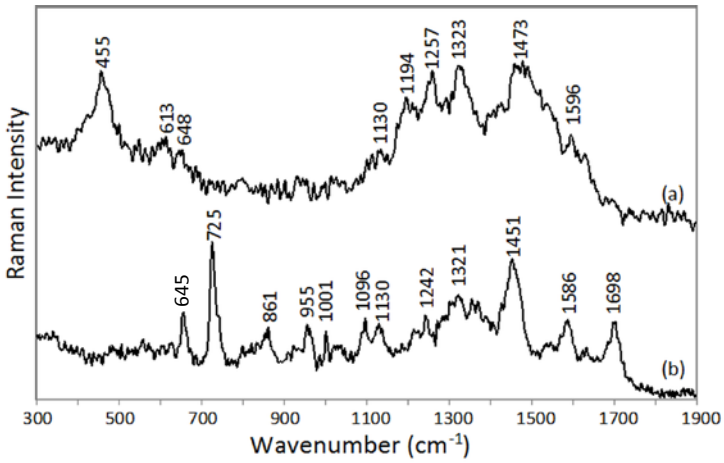

Figure 6. FT-SERS spectra of (a) cyanidin, (b) the aqueous extract from berries of *Chrozophora tinctoria.*

Very briefly, the investigation of this dye was also attempted by means of mass spectrometry analyses, such as ESI-MS and FAB-MS. However, the mass fragments observed (ESI +: 453, 497, 541, 585 m/z) seem to be not coherent with that reported for anthocyanins,[12] and they resulted not useful for the identification of its molecules. Our results found a confirmation in the literature, as this discordance was already documented, even if no experimental data were provided.[6]

Conclusions

In conclusion, HPLC-PDA, MS and SERS analyses seem to rule out an anthocyanin content of *folium* dye. Its chemical content resulted once again elusive, but the renewed attention on this dye led to new spectroscopic and analytical information of the extract of *Chrozophora tinctoria*. As far as the chemical composition of *folium* dye is concerned, we could only form a hypothesis based on our most detailed spectral information obtained: the FT-SERS spectrum. Comparing this spectrum with those reported in the literature, some affinity in the trend of the spectral pattern could be revised with the FT-Raman spectrum of naphtazarin,[13] a benzoquinone compound whose derivatives were extracted from plant pigments of the *Boraginaceae* family as copper complexes (Figure 7).[14] Its UV-vis spectra displays absorption maxima at 488, 514, 575 and 614 nm depending on the pH, changing its color from red to blue.[15] Moreover, its capability of forming chelate polymers with some divalent metal ions is also interesting and it may further support the hypothesis that the colored molecules of *folium* dye could belong either to the benzoquinones class or to some complex of these molecules with metals, as suggested by Krekel.[16] We deemed it probable that some derivatives of this molecule could be responsible for the colors of *folium* dye. But this should be intended just as an hypothesis until verified.

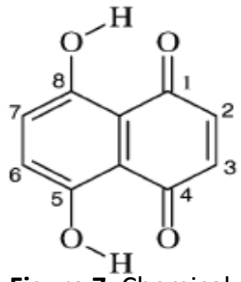

Figure 7. Chemical structure of naphtazarin.

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CHAPTER 3

On-line coupling of HPLC with SERS for the identification of historical dyes

Abstract

High-performance liquid chromatography (HPLC) is still today the technique of choice for the analysis of natural dyes in artistic objects and historic textiles, particularly in association with photodiode-array detection (PDA). In the last two decades surface-enhanced Raman scattering (SERS) gained also increasing importance for these investigations thanks to its sensitivity and limited requirements in terms of sample quantity. In favor of SERS its high specificity in molecular recognition typical of vibrational spectroscopy should be mentioned, whereas this non-separative technique is obviously disadvantaged in the analyses of mixed chromophores, as is the case of many natural dyes and also of tints obtained by the combined use of different colorants. An optimized experimental setup combining the two techniques, HPLC-PDA and SERS, is proposed in the present work,[1] allowing on-line SERS detection of different dyeing compounds eluting from the HPLC column. Examples are presented concerning some of the colorants most widely used in history, such as morin and luteolin for yellow dyes, alizarin, purpurin, laccaic, kermesic and carminic acids for red ones and indigotin for blue tints.

Introduction

Separative techniques obviously imposed themselves for the analysis of dyes, starting at first with thin-layer chromatography (TLC)[2, 3] to continue with high-performance liquid chromatography (HPLC), the prominent position of which was definitely established with the introduction of photodiode array (PDA) detectors.

Only more recently, approximately in the last two decades, surface-enhanced Raman scattering (SERS) has gained increasing importance thanks to its sensitivity for dye molecules and the requirement of small amount of samples, especially important for objects of artistic or historic value. In comparison with UV-visible spectroscopy, SERS also exhibits the relevant specificity for molecular recognition typical of vibrational

spectra. The main limitation of this non-separative technique is obviously encountered in the analysis of mixture of compounds, as is often the case of many natural dyes that contain more than one chromophore, or of objects dyed with combined colorants to obtain a particular hue. In such cases complex spectra are obtained or, alternatively, just one component gives a prevailing SERS response, while the others cannot be detected.[4]

For this reason, the joining of SERS with chromatographic techniques for the analysis of dyes could be of great interest and was indeed already attempted with TLC.[5-7] Even if, compared to TLC-SERS, the sample amount could be greater and the time of analysis longer, an HPLC-PDA-SERS system could provide a very detailed characterization of samples. Indeed, TLC-SERS analyses have some limitations, as for example the co-elution of chemically similar compounds[5, 7] and the interaction of the analytes with the silica gel TLC substrate, which can cause adsorption-induced spectral distortions of SERS spectra and, in some cases, a significant decrease of the signal resolution and of the overall spectral quality.[7] Moreover, TLC-SERS analyses, as SERS analyses themselves, were often followed by complementary HPLC-PDA analyses,[4, 7, 8] therefore the joined HPLC-SERS system can actually reduce the time of analysis.

As far as HPLC is concerned, in the literature different experimental approaches to the hyphenated HPLC-SERS technique were described[9-14] but only few analytes were tested: among them it is worth remembering purine bases[9, 10] and illicit drugs.[11-14] While, as far as the detection of dyes with joined systems is concerned, besides the previously cited works related to the identification of selected natural dyes by means of TLC-SERS, it was performed for few synthetic dyes, such as Basic Red 9 (CI 42500), Acid Orange 7 (CI 15510), and Food Yellow 3 (CI 15985)[15] with a coupled capillary electrophoresis (CE)-SERS system and for crystal violet with a microfluidic system which allowed on-line SERS measurements.[16]

In the present work,[1] the development of an on-line HPLC-PDA-SERS system for the detection of the most important natural dyes used in antiquity is presented. The use of this hyphenated technique can offer, besides an effective separation of dyes in mixture, valuable vibrational information for each constituent, to be used for identification purposes. Moreover, differently from the studies previously reported, the analytes taken into consideration in this work[1] belong to different molecular classes, namely anthraquinones, flavonoids and indigoids. It follows that the development of a suitable HPLC elution program for several dye molecules characterized by different chemical features was essential, in contrast with most on-line HPLC-SERS studies in the literature which adopted isocratic conditions for the chromatographic separation.[9-12]

Experimental

Materials

Silver nitrate ($AgNO_3$) (purity ≥ 99.5%), alizarin, purpurin and luteolin were obtained from Fluka, while lac dye was purchased from Zecchi (Florence, Italy). Methanol (assay ≥ 99.9%), trisodium citrate dihydrate (assay 100.2%), magnesium sulfate heptahydrate, formic acid (reagent grade ≥ 95%), morin hydrate, carminic acid and indigotin were purchased from Sigma-Aldrich. Hydrochloric acid 37% and dimethylformamide (assay ≥ 99.9%) were obtained from Riedel-de Haën, while kermes lice (*Kermes vermilio (Planchon)*) was purchased from Kremer Pigmente (Aichstetten, Germany).

Instrumentation

HPLC analyses were performed by means of the HPLC instrumentation described in Chapter 2. It should be noted that the use of an HPLC column with an internal diameter of 3 mm resulted to be advantageous as it allowed low flow rates and

therefore longer times of permanence of the sample under the laser beam. The optimized elution gradient used for the analyses is reported in Table 1.

Table 1. Elution program

Time (min)	%A	%B
0	85	15
3	55	45
7	40	60
16	25	75
19	10	90
27	5	95
30	85	5

A = H_2O + 1% HCOOH
B = MeOH + 1% HCOOH

SERS spectra were collected by means of a portable micro-Raman equipment with an excitation wavelength of 532 nm as described in Chapter 2. All SERS spectra were recorded between 2000 and 300 cm^{-1} in a kinetic acquisition mode by collecting series of 3 scans, each scan with an exposure time of 2 s.

Description of the coupled HPLC-PDA-SERS system

In our system (Figure 1), the micro-Raman spectrometer is a compact and portable instrument, suitable to be located near the HPLC instrumentation, feature which allows to minimize the tube length between the HPLC column and the flow cell, decreasing the problem of the broadening of chromatographic peaks. In order to be efficiently mixed with the silver colloid, before getting into the cell the eluted solution from the chromatographic column (flow rate 0.3 mL/min) is sent through a 12 cm long Teflon tube (0.3 mm internal diameter) in a PEEK inverted Y shaped valve.

The colloid is addressed to the valve from a reservoir by means of a Fluid Metering (INC) pump with a controlled flux of 0.8 mL/min. The two inlet channels of the valve are arranged with 30° angles with respect to the outlet channel (Figure 2a). Besides

the valve, to obtain a satisfying mixing we adopted, similarly to Cotton and co-workers,[9] a device proposed in the literature for postcolumn derivatization, known as knitted open tubular (KOT) reactor.[17] KOTs are Teflon tubes with small diameters, knitted with different loops around a backbone. The mixture of the eluted solution and the silver colloid coming out from the valve is thus addressed to the KOT, that is connected to the flow cell. The best performing KOT resulted to be a tube with 0.8 mm internal diameter, knitted 12 times around a Tygon tube (7 mm external diam., 4 mm internal diam.) according to the pattern shown in Figure 2b and forming a loop with a diameter of 5.5 cm. The flow cell is a glass capillary 15 cm long (1 mm internal diam., 1.5 mm external diam.).

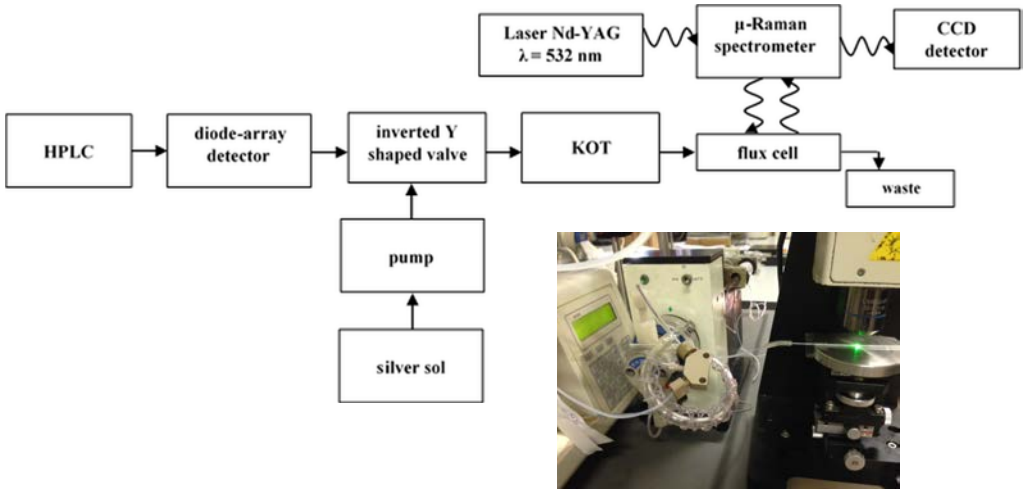

Figure 1. Schematic diagram and photograph of the HPLC-PDA-SERS system (from ref 1).

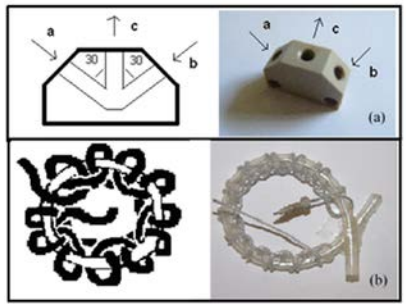

Figure 2. Devices used to interface the HPLC column and the Raman spectrometer: (a) inverted Y shaped valve, (b) KOT (figure from ref 1).

Colloid synthesis and aggregation

Ag colloids were prepared according to the Lee and Meisel procedure,[18] by reduction of silver nitrate with trisodium citrate dihydrate, as described in Chapter 2.[19]

In order to find out the best aggregation state of the colloid, tests were carried out using $3 \cdot 10^{-5}$ M alizarin solution as reference dye. To 3 mL of silver sol, amounts of 50/75/100 µL of $MgSO_4$ 0.026 M were added in three different test tubes under stirring. SERS analyses were then performed several minutes after the addition, in order to verify aggregation during time.

Sample preparation

Solutions of commercially available chromophoric molecules and dyes were prepared in methanol, water or dimethylformamide according to the solubility properties of each dye. Whenever necessary, solutions were filtered through a 0.45 µm GHP Acrodisc membrane filter. The concentration of the compounds in solution was dependent on the molecular class to which they belong and on the related intensity of SERS response: anthraquinones were prepared with a final concentration of $2.5 \cdot 10^{-4}$ M, flavonoids and indigoids were $2 \cdot 10^{-3}$ M.

Extraction of dyes from complex matrices

Solutions of dyes were also obtained upon extraction from different sources, i.e. kermes insect and a pink thread from a Renaissance Italian carpet.

The textile sample was treated with 3 mL of MeOH and 100 µL of HCl 37% at 65°C for 60 min;[20] the obtained solution was filtered, dried under N_2 and re-dissolved in methanol. The extraction from the kermes insect was performed according to a procedure reported elsewhere,[21] then the extract was re-dissolved in 50 µL of ultrapure water and injected in the HPLC-PDA-SERS system.

Results and discussion

The main difficulty in the design of a HPLC-SERS system was that of coupling an instrumentation which works in continuum (HPLC) with another which usually works in a static mode (SERS). Therefore it was necessary to develop a system in which also SERS analyses could be conducted in a continuous mode. Moreover, the system must be suitable for the detection of all the analytes considered, which, in our study, belongs to different molecular classes, as detailed in Chapter 1. To this aim, several aspects were taken in consideration and optimized, as described in the following sections.

Aggregation of the Ag colloid

Working in continuum with SERS entails some difficulties: indeed, while in static condition the laser itself can favor the sol aggregation increasing the Raman signal, under flowing conditions the irradiation time is substantially shorter.[9] It was therefore necessary to find out a method to accomplish the aggregation of the metallic substrate. It is well known that the SERS signals increase when the metallic substrates display nanometric roughness or some nanoparticles aggregations, the so-called "hot-spots".[22, 23] Therefore, the possibility to heat the colloid at 90°C in order to induce the aggregation was considered, as suggested by Cotton and co-workers.[9] However, since a lack of reproducibility in the results was noticed, we preferred to pre-aggregate the silver colloid. A partial aggregation of the colloidal nanoparticles can be induced by controlling volume and concentration of a suitable aggregating agent. In our laboratory, a SERS procedure for the identification of natural dyes by means of silver colloid aggregated with sodium perchlorate was previously developed.[19] However, combining HPLC and SERS, we avoided the use of $NaClO_4$, taking into account that the pre-aggregated colloid has to be conserved during the entire HPLC analysis (30 minutes) and that some residuals could deposit into tubes or inside the pump: even if the oxidation of nanoparticles is thermodynamically

disadvantaged, it is known that the perchlorates of heavy metals, such as silver, are explosive when they are dried. Therefore, electrolytes different from sodium perchlorate were tested. Initially, the possibility to use NaCl was assessed, but good quality SERS spectra could be obtained only for alizarin. As reported in the literature,[24, 25] some analytes cannot be detected by means of SERS in presence of NaCl because of their lower tendency to adsorb onto the metallic surface with respect to Cl^- anion, while the SERS spectra of the same analytes could be obtained in the presence of $MgSO_4$, that has much lower affinity towards active sites on silver particles. Also in the present study the use of $MgSO_4$ as aggregating agent allowed to record satisfying SERS spectra for all the chromophoric molecules analyzed.

Moreover, the aggregated colloid needs to keep its stability throughout the HPLC analysis. An unstable aggregation indeed affects the reproducibility of the results[26] and favors the so-called "memory effect" that occurs when nanoparticles deposit in the Raman cell; this fact could lead to record both the signals of the analyte which is passing through the flow cell and of the analytes previously eluted.[9, 11] To avoid unstable aggregation or metal precipitation during HPLC analysis, the suitable amount of electrolyte resulted to be 0.026 M $MgSO_4$ added to Ag nanoparticles with a volume ratio of 75 µL : 3 mL. However, as it is known that Lee and Meisel silver colloids are scarcely reproducible,[21] the exact quantity of $MgSO_4$ to obtain the best aggregation was verified by an off-line test (see Experimental) every time a new colloid was synthesized.

HPLC mobile phase and elution gradient

Even if most HPLC experiments concerning the separation of natural dyes are performed using water and acetonitrile (AcN) as constituents of the mobile phase (see for example references [27-29]), the use of AcN in an on-line HPLC-SERS system is not suitable, as it competes significantly with the analytes for the adsorption on the

Ag surface.[11, 14, 30] Therefore water and methanol (MeOH) were chosen as eluents for the mobile phase.

It is well known that the use of an acid modifier improves chromatographic resolution for ionizable compounds by sharpening the eluting bands and avoiding possible salt precipitation within the system. However, an alteration in the pH of the eluting solution can produce an effect also on the SERS response of the target molecules, whose structure in our case is indeed rich in carboxylic and hydroxyl groups and therefore significantly dependent on the pH.[31-33] Formic acid was the acid modifier that gave the best results in comparison with acetic and trifluoroacetic acid, both from the point of view of the chromatographic sharpening of bands and as far as the acquisition of SERS signals is concerned, as detailed in the following. Notably, the use of formic acid as modifier of a H_2O/MeOH mobile phase was already attested for some HPLC-MS applications concerning natural dyes.[34, 35]

The optimized elution gradient, already shown in Table 1, allowed a satisfying chromatographic separation of all the substances tested and had a suitable time duration to prevent the precipitation of the silver colloid in the system. As shown in Figure 3, the acidity provided by formic acid in water at the beginning of the elution program allowed to obtain satisfying chromatographic peaks for rather hydrophilic compounds such as carminic acid and laccaic acids C and

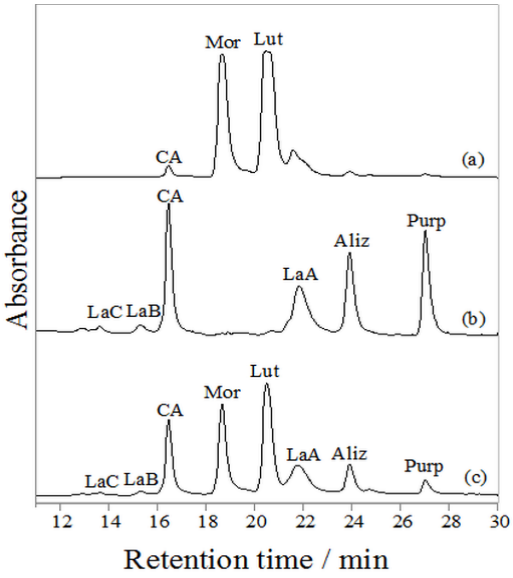

Figure 3. UV-vis chromatograms of mixture of anthraquinones and flavonoids at (a) λ= 350 nm, (b) 450 nm, (c) 280 nm; (figure from ref. 1).
Abbreviation: LaC = laccaic acid C, LaB = laccaic acid B, CA= carminic acid, Mor = morin, Lut = luteolin, LaA= laccaic acid A, Aliz = alizarin, Purp = purpurin.

B, while the reduced acidity provided by both the decrease of the percentage of water and the higher pH value experimented by the molecules when HCOOH is dissolved in methanol allowed the SERS detection of more hydrophobic compounds, such as flavonoids, alizarin and purpurin.

HPLC-PDA-SERS analyses of natural dyes

The chromophoric molecules and natural dyes tested with the combined HPLC-PDA-SERS system are listed in Table 2 together with their initial concentrations. As a proof-of-concept, a mixture of antraquinones (i.e. carminic acid, lac dye, alizarin, purpurin) and flavonoids (morin, luteolin) was prepared for the on-line detection.

Table 2. Chromophores tested with the HPLC-PDA-SERS system

	Chromophores	Molecular class	Concentration	Retention time / min (λ_{max})
1	Laccaic Acid C	anthraquinone	$1.2 \cdot 10^{-5}$ M [a]	13.6 (490 nm)
2	Laccaic Acid B	anthraquinone	$1.6 \cdot 10^{-5}$ M [a]	15.2 (490 nm)
3	Carminic acid	anthraquinone	$2.5 \cdot 10^{-4}$ M	16.4 (495 nm)
4	Morin	flavonoid	$2.0 \cdot 10^{-3}$ M	18.6 (357 nm)
5	Luteolin	flavonoid	$2.0 \cdot 10^{-3}$ M	20.5 (359 nm)
6	Laccaic Acid A	anthraquinone	$2.2 \cdot 10^{-4}$ M [a]	21.8 (490 nm)
7	Alizarin	anthraquinone	$2.5 \cdot 10^{-4}$ M	23.9 (428 nm)
8	Flavokermesic acid	anthraquinone	- [b]	23.5 (430 nm)
9	Kermesic acid	anthraquinone	- [b]	24.0 (491 nm)
10	Indigotin	indigoid	$2.0 \cdot 10^{-3}$ M	24.6 (615 nm)
11	Purpurin	anthraquinone	$2.5 \cdot 10^{-4}$ M	27.0 (479 nm)

[a] concentration estimated from the total concentration of lac dye
[b] initial concentration non available as extracted from kermes insect

The three-dimensional SERS-detected chromatogram of a mixture of chromophores, obtained using the optimized elution program, is shown in Figure 4.

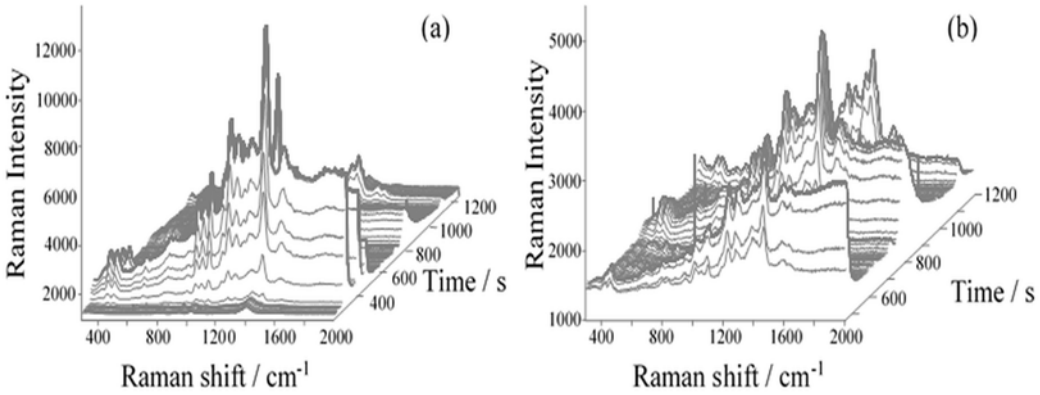

Figure 4. 3D SERS chromatograms of a mixture of anthraquinones and flavonoids. (a) the most intense spectra are those of laccaic acids with lower retention times. Being the baseline of SERS spectra of the laccaic acids higher due to the fluorescence of the molecules, Figure 4b shows the three-dimensional map of the compounds with higher retention times (figure from ref 1).

A two-dimensional SERS chromatogram is shown in Figure 5, plotting the peak intensity at 1470 cm^{-1} as a function of retention time. Interestingly, the laccaic acids present in less amount in lac dye (i.e. laccaic acids B and C)[36] were detected as small peaks in the UV-vis chromatogram (Figure 4), while they showed a high response when analyzed with SERS, allowing an easy detection of these compounds.

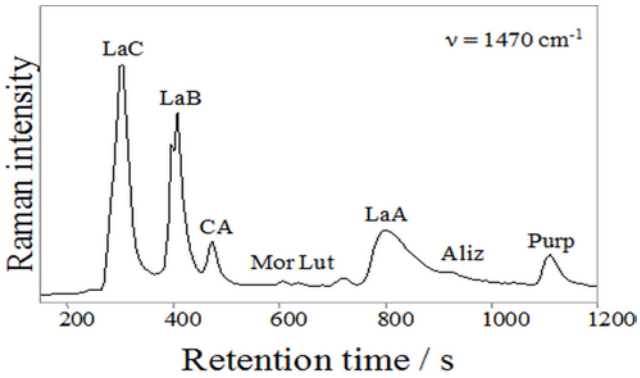

Figure 5. SERS chromatogram at ν=1470 cm^{-1} of a mixture of anthraquinones and flavonoids (fig. from ref 1).

Abbreviation: LaC = laccaic acid C, LaB = laccaic acid B, CA= carminic acid, Mor = morin, Lut = luteolin, LaA= laccaic acid A, Aliz = alizarin, Purp = purpurin.

As regards the SERS signals obtained, the spectra of each anthraquinone compound tested are shown in Figure 6, while those of flavonoids are reported in Figure 7.

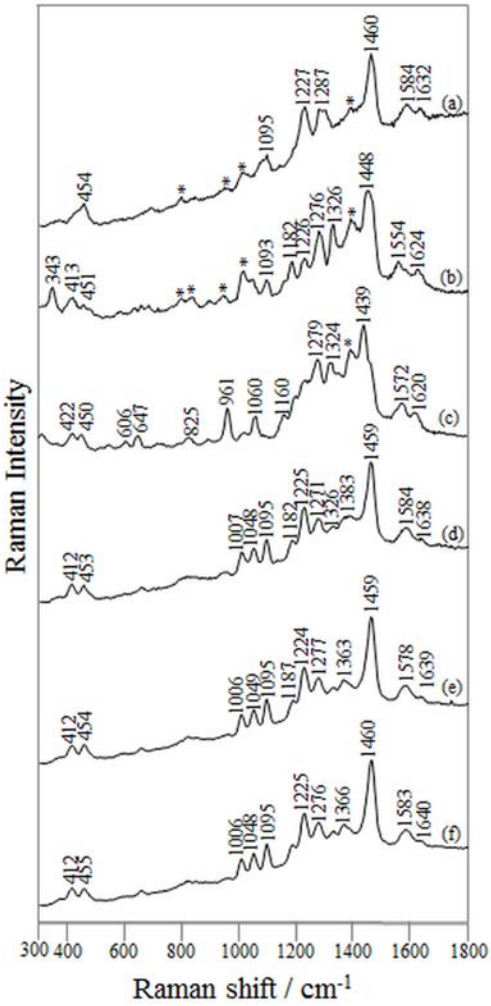

Figure 6. SERS spectra of (a) carminic acid, (b) alizarin, (c) purpurin, (d) laccaic acid A, (e) laccaic acid B, (f) laccaic acid C. Signals due to oxidation products of citrate are indicated with *. (figure from ref 1).

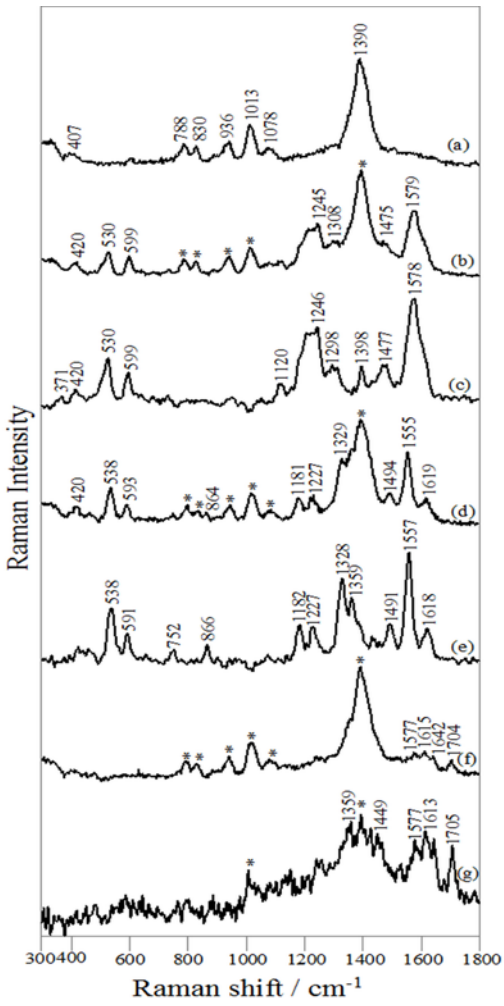

Figure 7. SERS spectra of (a) aggregated Ag colloid mixed with the eluent, (b) luteolin, (c) luteolin with the subtraction of the signals of the colloid, (d) morin, (e) morin with the subtraction of the signals of the colloid, (f) indigotin, (g) indigotin with the subtraction of the signals of the colloid. Bands marked with * in the SERS spectra of dyes are assigned to the colloid (fig. from ref 1).

A list of the Raman shifts of bands in each SERS spectrum is reported in Table 3:

Table 3. Raman shift (cm^{-1}) of the SERS spectra of the chromophores analyzed:	
Carminic acid	1632 (w), 1584 (w), 1460 (vs), 1287 (s), 1227 (m), 1095 (m), 454 (w)
Alizarin	1624 (w), 1554 (w), 1448 (s), 1326 (m), 1276 (m), 1226 (w), 1182 (w), 1093 (w), 1046 (w), 451 (vw), 413 (w), 343 (w)
Purpurin	1620 (w), 1572 (w), 1439 (vs), 1324 (s), 1279 (s), 1235 (sh), 1201 (sh), 1160 (sh), 1060 (m), 961 (m), 825 (w), 647 (w), 606 (vw), 450 (w), 422 (w)
Laccaic acid A	1638 (vw), 1584 (w), 1459 (vs), 1383 (m), 1326 (w), 1271 (m), 1225 (s), 1182 (m),1095 (m), 1048 (m), 1007 (m), 453 (w), 412 (m)
Laccaic acid B	1639 (vw), 1578 (m), 1459 (vs), 1363 (w), 1328 (w), 1277 (m), 1224 (s), 1187 (sh), 1095 (m), 1049 (m), 1006 (m), 454 (w), 412 (w)
Laccaic acid C	1640 (vw), 1583 (m), 1460 (vs), 1366 (m), 1331 (w), 1276 (m), 1225 (s), 1185 (sh), 1095 (m), 1048 (m), 1006 (m), 455 (w), 412 (w)
KvI compound	1637 (m), 1580 (m), 1462 (vs), 1412 (vs), 1299 (s), 1234 (s), 1134 (m), 672 (m), 447 (m)
Flavokermesic acid	1663 (m), 1584 (m), 1450 (s), 1288 (s), 1244 (sh), 1117 (w), 657 (w), 462 (m)
Kermesic acid	1637 (m), 1590 (m), 1454(vs), 1299 (s), 1234 (s), 1184 (sh), 1126 (m), 1059 (w), 975 (w), 949 (w), 884 (w), 663 (m), 450 (m), 418 (w)
Morin (background subtracted)	1618 (m), 1558 (vs), 1491 (m), 1359 (s), 1328 (s), 1227 (m), 1182 (m), 866 (w), 752 (w), 591 (m), 538 (s)
Luteolin (background subtracted)	1578 (vs), 1477 (m), 1398 (m), 1289 (m), 1246 (s), 1120 (w), 599 (m), 530 (m), 420 (w), 371 (vw)
Indigotin (background subtracted)	1705 (s), 1642 (m), 1613 (s), 1577 (s), 1449 (m), 1359 (s)

In more detail, the SERS signal of carminic acid is in good accordance with the SERS spectrum obtained by Cañamares et al.[32] at pH 3, while the SERS spectrum of alizarin matches that reported by the same authors[31] at pH 5. This is not surprising, being the acid percentage of the elution program very different at the retention times of the two chromophores. A spectral pattern similar of that of alizarin can be seen in the SERS spectrum of purpurin, which indeed differs in its molecular structure from alizarin only for the presence of a third hydroxyl group. As far as the laccaic acids analyzed is concerned, they show very similar spectral patterns: it was already reported that lac dye displays similar patterns both at pH 3 and 5,[33] which is indeed the same spectral pattern we recorded for all the laccaic acids tested in our work, i.e. both for laccaic acids B and C that eluted when the acidity of the HPLC eluents is higher and for laccaic acid A, whose retention time is close to that of alizarin.

It is worth remembering that when only eluents and silver sol were mixed, the spectrometer detects the signals due to the oxidation products of citrate at the pH experimented after the mixing in the KOT. This SERS spectrum, shown in Figure 7a, was generally hidden by the SERS signals of dyes adsorbed onto silver nanoparticles, except in the cases of flavonoids and indigotin. The SERS pattern of the colloid was thus subtracted from the SERS spectra of such dyes. The spectrum obtained for luteolin (Figure 7c) is in good accordance with the reference SERS spectrum previously recorded in our laboratory at the pH value of the colloid,[19] while the SERS spectral pattern of morin (Figure 7e) is probably more affected by the pH value of the eluted solution, being more acidic than other flavonoids with a 7-OH group.[37, 38] Eventually, the subtraction of the signals not related to the analytes resulted important during the analysis of the mixture: in this case some signals of anthraquinone dyes with a high SERS response can still be noted in the SERS spectra of those molecules that have less affinity for the silver colloid, i.e. flavonoids, but the subtraction of background signals recorded at a time immediately preceding the elution of each compound allowed to obtain the same spectral patterns observed when analyzing a single

flavonoid dye. It should be noted that the presence of signals of already eluted compounds is not due to the "memory effect", as no deposition of silver nanoparticles occurred during the analysis, but to the high affinity of anthraquinones to the silver substrate, feature that allowed their identification even when they are present at trace level.

As far as indigotin is concerned, the enhancement of signals is highly pH dependent due to the formation of singly protonated and possibly doubly protonated forms of the molecule at acidic pH, being the optimum signal intensification at approximately pH 1.75.[39] However, in our system the final solution sent to the flow cell is at higher pH, therefore the adsorption of indigo molecules on silver nanoparticles can be difficult and it is not surprising that the SERS spectrum of indigotin shows strong bands due to the citrate oxidation products (Figure 7f). Nevertheless, the background subtraction allowed to assign the principal bands of indigo.[40] Changes in the elution program at the retention time of indigotin in order to decrease the pH were tested, but they affected also the obtainment of the SERS spectra of the anthraquinones alizarin and purpurin, whose retention times are close to that of indigotin. Moreover, the pH value at this point of the elution program is limited also by the composition of the mobile phase: in order to obtain a satisfying separation of alizarin, purpurin and indigotin, the small percentage of water must be kept small, therefore the effective acidity of the mobile phase is invariably reduced.

On-line HPLC-PDA-SERS detection of dyes in complex samples

Case a) Chromophores contained in the insect *Kermes vermilio* (Planchon)

Among the natural sources of dyes used in the past, scale insects represented an important group and were used for the production of red dyes for wool and silk.[41] In particular, kermes has been used since antiquity, and one of the earliest mentions of this material can be found in the Old Testament.

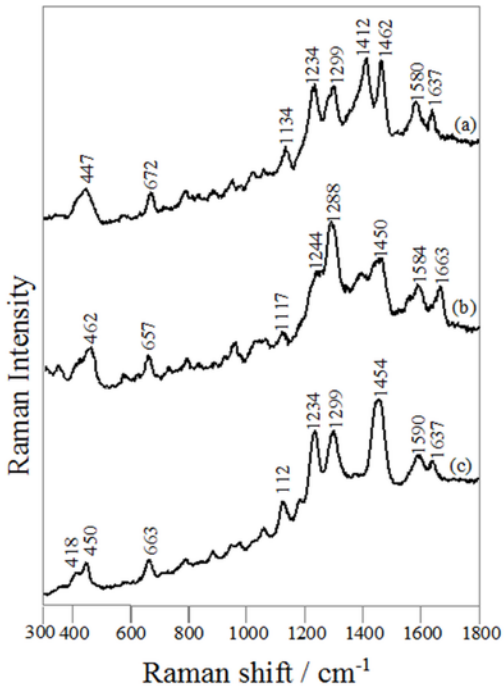

Figure 8. SERS spectra obtained by HPLC-PDA-SERS analysis of the extract from *Kermes vermilio*: (a) kvI compound, (b) flavokermesic acid, (c) kermesic acid (figure from ref 1).

Beside the identification of flavokermesic acid and kermesic acid (Figure 8), the two chromophores of kermes, we detected other colored species present in smaller amount, as already reported.[41] Among them, the compound named "kvI" by Wouters and Verhecken[41] shows an intense chromatographic peak and its SERS spectrum is reported in Figure 8a. Notably, this the first time, according to our knowledge, that the SERS spectra of flavokermesic acid and "kvI" compound are reported.

Case b) Dyeing matter extracted from a pink thread of an ancient Italian carpet

A pink thread from an Italian carpet, dated to Renaissance, was analyzed with the optimized HPLC-PDA-SERS system. The chromatographic data (Figure 9), in excellent accordance with the spectroscopic ones (Figure 10), allowed to reveal in the extract the predominance of the chromophore purpurin, usually found in association with alizarin in the roots of madder (*Rubia tinctorum L.*), a very common source of red coloring matter since antiquity. As alizarin was not detected in this analysis, a different botanical source was hypothesized. The identification of purpurin instead of the most common alizarin in ancient red dyes has been already reported in the literature.[42, 43] Also in this case, the plant source could be a Mediterranean species, such as wild madder (*Rubia peregrina L.*), Lady's bedstraw (*Galium verum L.*) or dyer's

woodruff (*Asperula tinctoria L.*), in which purpurin is predominant with respect to alizarin.[44, 45]

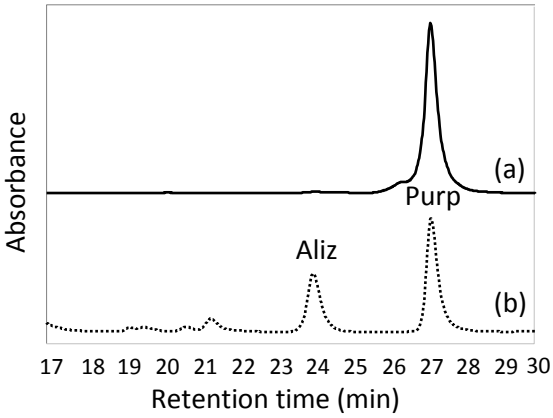

Figure 9. UV-vis chromatograms (λ= 480 nm) of (a) the extract from an ancient pink thread, (b) reference alizarin and purpurin (figure from ref. 1).

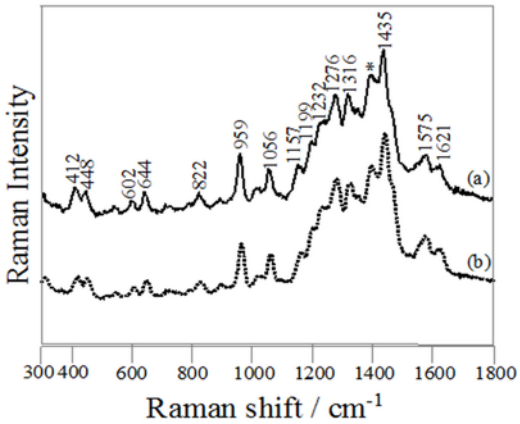

Figure 10. SERS spectra obtained by HPLC-PDA-SERS analysis of (a) the extract from a pink thread of an Italian carpet, (b) purpurin (fig from ref.1).

Conclusions:

The development and optimization of a coupled HPLC-PDA-SERS system for the identification of natural dyes historically used was carried out. The main parameters optimized were the efficiency of the mixing between the eluted analytes and the pre-aggregated silver sol, the construction of the elution gradient and the aggregation of the colloid. The pH of the eluted solution, which plays a key role in the obtainment of the SERS spectra, was successfully modulated by means of both the acid modifier and the elution program. The SERS spectra obtained from reference dyes showed a better affinity between anthraquinones and silver colloid, nevertheless also flavonoids and indigotin could be detected. The method of analysis here described resulted to be successful also on samples of different origin, such as the extract from the animal source of kermes and an ancient dyed thread, demonstrating the effectiveness and the stability of the method. Indeed it allows to obtain a valuable characterization of samples in a single analysis.

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CHAPTER 4

Extractionless FT-SERS analysis of dyed textiles

Abstract

In the present Chapter, an extractionless procedure followed by SERS analyses performed with a Fourier-transform Raman instrumentation for the identification of historical dyes on textiles is optimized and presented.[1] The term "extractionless" refers to a method of SERS analysis applied directly on the fiber, thus avoiding the extraction of dyes from textile samples. It can be performed on samples as such, or previously hydrolyzed by acid vapors. The combination of a low-energy source of radiation, as in the FT-Raman technique, with SERS can bring the important advantage of reducing the fluorescence typical of ancient samples and organic dyes. Upon the acquisition of a FT-SERS spectral database from solutions of pure dyes, extractionless both hydrolysis and non-hydrolysis FT-SERS analyses were performed on ancient textile fibers. The FT-SERS spectrum of an iron-gall dye was acquired without hydrolysis, while, with an HF hydrolysis pre-treatment, madder, lac dye and the very fluorescent dye brazilwood were clearly recognized.

Introduction

In order to perform SERS analysis of historical dyes, it was usually necessary to extract the dye itself from the ancient artefact by more or less aggressive extractive methods. Indeed, most dyes are fixed on textiles by mordanting, i.e. by treating the fibers with metal salts so that the metal ion mediates the interaction between the dye molecule and the fiber itself. In the same way, organic dyes were used in paintings as lakes, i.e. as metal complexes that could be dispersed in the binder. It was thought that, in this form, they could not adsorb on metal nanoparticles and therefore SERS could not take place. Thus textile fibers and paint samples were pre-treated to obtain the free dye. Initially strong acids were employed to isolate the organic dye from the medium,[2] but these protocols invariably entailed the degradation of the host material and the destruction of the sample. In recent years, researchers developed milder extraction

procedures,[3] as well as non-extractive hydrolysis methods[4, 5] in which the dye-mordant complex is broken without removing the dye from the fiber, followed by direct SERS analysis onto the pre-treated sample. Moreover, in 2008, Jurasekova and co-workers[6] proposed for the first time a non-extractive, non-hydrolysis, direct SERS method for the identification of organic colorants on wool and silk textiles, showing that SERS effect takes place also on mordanted dyes and thus making the extraction procedures even less necessary. On the other hand, in spite of these interesting results, some of the above mentioned methods require silver colloids obtained by unconventional synthesis such as microwave reduction of Ag_2SO_4[4, 5] or *in situ* photoreduction of Ag nanoparticles.[6] Only Brosseau et al.[7] and, more recently, Idone et al.[8] reported non-hydrolysis *on the fiber* analyses performed by the use of a concentrated citrate-reduced Ag colloid.

The present work[1] studies the applicability of an extractionless procedure, based on the use of a Ag Lee-Meisel colloid,[9] to obtain Fourier-transform SERS (FT-SERS) spectra directly from fibers. The sol synthesis is simple and does not require any specific instrumentation, thus its application can possibly lead to a diffusion of SERS analysis in the common practice of laboratories specialized in the analysis of ancient textiles or paintings. The possibility to use a different excitation wavelength besides the typically employed visible ones, namely a near infrared radiation (1064 nm) as in the FT-Raman technique, was evaluated in order to reduce fluorescence and diminish photo-induced degradation of samples, as reported by Kneipp[10] and Caudin.[11] Indeed, even if it is well known that SERS effect itself produces an important quenching of fluorescence,[12] in some *on the fiber* analyses, as it will be discussed, the use of visible laser excitation in order to record a SERRS spectrum can be anyway hindered by samples fluorescence. Thus, using a less-energetic source of radiation, as in the FT-Raman technique, the population of the electronic excited states producing fluorescence can be reduced.

As regards the application of non-resonance SERS techniques for the identification of organic colorants, only few references can be found in literature: in 1990's Kneipp and co-workers[10, 13] carried out some near infrared-SERS (NIR-SERS) analyses of synthetic dyes, while, more recently, other researchers exploited the use of the 785 nm excitation wavelength for the identification of natural dyes[5, 14] and the synthetic dye Eosin Y.[15] In the latter reference, the study carried out includes also the FT-SERS analysis of the fluorone dye, showing the potentiality of the near-infrared wavelengths as excitation sources for SERS analyses. However, the use of FT-SERS for the identification of natural dyes has been scarcely studied: few examples are provided by the FT-SERS analyses of carotenoids,[11] curcumin dye,[16] the anthraquinone pigments alizarin and carminic acid[17-19] and the alkaloid dye berberine.[20]

In the present work,[1] FT-SERS spectra of the most used natural dyes were recorded, leading to the construction of a new database, not redundant with respect to the previously published ones [5, 21] as positions and relative intensities of the Raman bands result in many cases dependent on the excitation wavelength. Indeed, as it will be discussed, the use of different excitation wavelengths can enhance different vibrational modes in the resulting SERS spectrum, especially when the SERS-active molecule does not experiment the resonant condition anymore. Then reference spectra were compared to those obtained from both wool threads dyed in our laboratory according to historical recipes and from wool, cotton and silk fibers from art objects, namely Caucasian and Chinese textiles. Special attention was paid to obtain reliable SERS spectra from amounts of sample as small as possible, so that the method can be considered, if not entirely non destructive, at least micro-destructive. Moreover, an evaluation of the opportunity to perform a hydrolysis pre-treatment on the same samples was also carried out.

Experimental

Materials

Cochineal carmine, lac dye, dragon's blood, brazilwood, logwood, sandalwood, old fustic were purchased from Zecchi (Florence, Italy), while catechu from Kremer (Aichstetten, Germany). Silver nitrate ($AgNO_3$) (purity ≥ 99.5%), sodium perchlorate monohydrate (assay ≥ 99.0%), fluoridric acid (47-51%) and the chromophores alizarin, purpurin, quercetin, luteolin and rutin trihydrate, were obtained from Fluka. Methanol (assay ≥ 99.9%), trisodium citrate dihydrate (assay 100.2%), orcein, carminic acid and morin hydrate were purchased from Sigma-Aldrich. All the aqueous solutions were prepared using ultrapure water (Millipore MilliQ).

Silver colloid synthesis

To perform SERS analyses, citrate-reduced silver colloid obtained according to Lee-Meisel procedure[9] was chosen, due to its ease of preparation and use. The protocol for the synthesis of silver nanoparticles is described in Chapter 2 of the present doctoral thesis.

Reference wool samples

In order to prepare reference samples, wool threads were washed, treated with alum, $KAl(SO_4)_2 \cdot 12H_2O$, and with acid potassium tartrate and dyed as described elsewhere.[22] Before analysis, about 1 cm of dyed thread was allowed to stay in 1 mL of pure methanol from 4 hours to one night in order to wash away the not-mordanted dye possibly remaining on the fibers. This step was performed for all the laboratory dyed threads.

Ancient Samples

The analyzed ancient fibers (Figure 1) came from cotton and silk threads from Caucasian Kaitag textiles (17th-18th centuries) and wool threads from Chinese Ningxia textiles (18th-19th centuries) belonging to the Moshe Tabibnia Gallery (Milan, Italy).

Figure 1. Ancient textiles from which samples were taken and analysed (fig. from ref. 1).

As far as the Chinese textile artefacts are concerned, they came from the Ningxia region, whose handcrafted production of traditional carpets was famous and appreciated also by the Chinese emperor Kangxi (1662-1722). From a chair cover with dragons embroidery (Figure 1a, cat. n. 13901, 18th century) a red wool fiber from the background was taken, while an orange wool fiber was obtained from a knot on the reverse side of a Chinese carpet (Figure 1b, cat. n. 6563), produced in the first half of 19th century.

As regards Kaitag textiles, they constitute an example of the artistic textile production of Daghestan (Russia). Today, only a few hundreds of these precious antique specimens can still be found, and surviving examples are mostly from the 17th and 18th centuries, even if the earliest examples of these are dating to the 16th century.[23] These art objects, composed by cotton squares with silk embroideries, were intended for ritual purposes and used by families on special occasions such as the birth, marriage or death of one of their members. In detail, the samples at our disposal were a red thread from the cotton ground of a Kaitag textile dating to the 17th century (Figure 1c, cat. n. 50) and a dark brown thread coming from the silk embroideries of another Kaitag artefact dating to the 18th century (Figure 1d, cat. n. 7).

Instrumentation

FT-SERS spectra were recorded between 4000 and 200 cm^{-1} with an RFT-600 Jasco FT-Raman spectrometer, using for excitation the 1064 nm emission of a Nd:YAG laser and collecting different numbers of scans (between 50 and 200) depending on the sample. When a single fiber was examined, the spectra were acquired in the micro-sampling mode with a 50x objective and with an output laser power varying from 60 to 90 mW. Instead, when analyses of whole threads or of reference dyes in solution were performed, the macro-sampling mode was preferred. In this case the output laser power was kept between 240 and 280 mW, and optimized for each sample in order to obtain the highest intensity of the Raman signals. The resolution was set to 4 cm^{-1}.

SERS spectra with exciting radiation at 532 nm were collected by a the compact micro-Raman instrument described in Chapter 2. All SERS spectra were recorded between 2000 and 200 cm^{-1} by collecting 50 scans with an exposure time of 2 s.

UV-visible absorption spectra of silver sol and of dyes in solution were obtained with a Jasco UV/VIS/NIR V-570 spectrophotometer, using 1 mm optical path cells.

Scanning electron microscope (SEM) observations were performed by a JEOL 5500 LV EDS-equipped instrument (20 kV, 10-11 A, 2 μm beam diameter).

Sample preparation and analysis

As pointed out elsewhere,[21] the optimum aggregation of Lee-Meisel silver colloid is obtained with the addition of an appropriate amount of sodium perchlorate 1.8 M. In comparison with ref. 21, in the present study[1] a greater volume of electrolyte is added to induce aggregation, in order to increase the absorption of the colloid at the wavelength of 1064 nm chosen for excitation.

Figure 2 shows that the aggregation process leads to an intensity decrease of the maximum of the plasmonic band, which appears at 425 nm for the Ag sol as such, and to an overall increase of absorbance in the region of the NIR wavelengths.

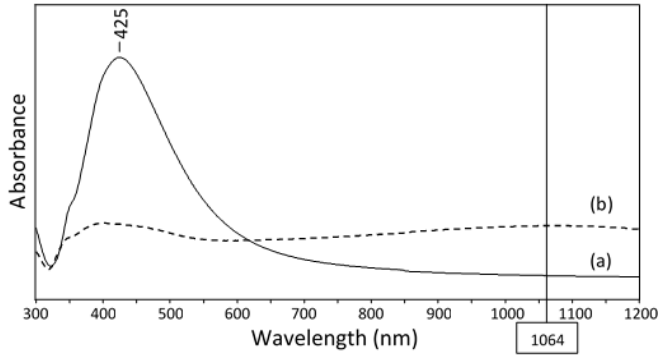

Figure 2. UV-vis absorption spectra of (a) the silver colloid as such, (b) the colloid aggregated with $NaClO_4$ 1.8 M (figure from ref. 1).

The addition of the colloid to the fibers seems not to induce a further aggregation of the nanoparticles, as was observed by SEM (Figure 3).

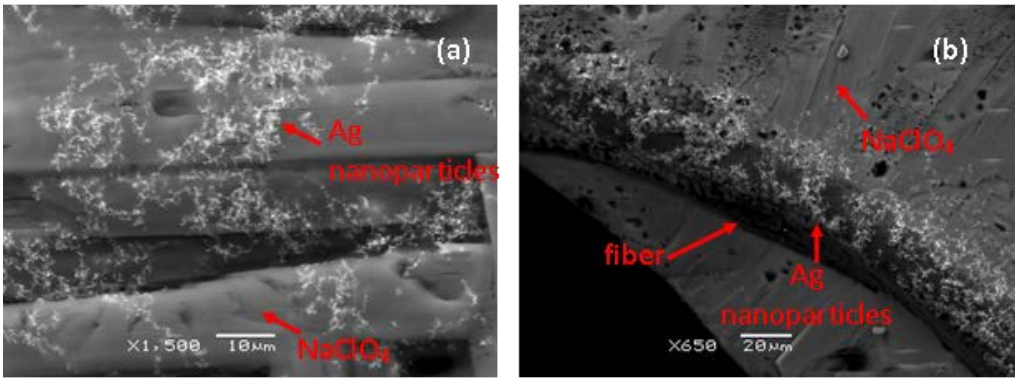

Figure 3. SEM images of (a) the aggregated silver colloid, (b) a fiber covered with the aggregated silver sol (figure from ref. 1).

One of the aims of this study is to reduce, as much as possible, the size of the sample requested for the analysis. This achievement is obviously very important for artistic objects with an historical value. Therefore, we started by analyzing a whole thread 5 mm long and then we decreased the sample dimensions, in most cases, to a single wool fiber. In the following are reported the details of the experimental procedures

adopted for analyses on solutions of the reference dyes as well as on fibers both with and without hydrolysis. For best clarity about the dimensions of samples see Figure 4.

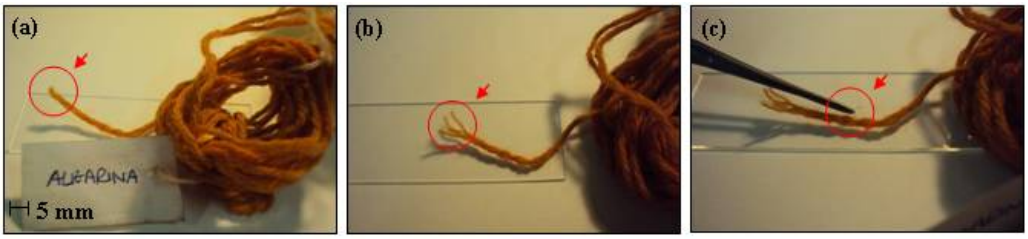

Figure 4. Photographs of (a) the whole thread, (b) a single ply, (c) a single fiber from reference wool (figure from ref. 1).

FT-SERS analysis of reference dyes in solution

Solutions of reference dyes and chromophores were prepared daily at a concentration of 10^{-3} M, in methanol or water according to the solubility properties of each dye. Whenever necessary, solutions were filtered through a 0.45 µm GHP Acrodisc membrane filter. For FT-SERS analyses, first 60 µl of $NaClO_4$ 1.8 M and then 100 µl of 10^{-3} M dye solution were added in a test tube to 1 ml of Ag sol under magnetic stirring. With a Pasteur pipette a drop of the solution was placed on a glass slide and posed under the laser beam.

FT-SERS extractionless analysis of dyes on the fiber

1) Non-hydrolysis procedures:

a - Analysis of a wool thread

The addition of 100 µl of Ag sol in a 5 ml test tube, under magnetic stirring, was followed by the addition of 6 µl of $NaClO_4$ 1.8 M. Then, a 5 mm long thread was placed into the tube, in order to wet the wool with the aggregated sol. With the help of a spatula, the thread was posed on a glass slide and a drop of the sol was put on the wool. Finally, the sample was placed under the laser beam.

b - Analysis of a single ply from a wool thread

In a test tube, 6 µl of $NaClO_4$ 1.8 M were added to 100 µl of Ag sol under magnetic stirring. Then a drop of the solution (about 15-20 µl) was put by a Pasteur pipette on the ply previously posed on a glass slide. The sample was thus ready to be analyzed.

c - Analysis of a single wool fiber

With the help of a stereomicroscope, a wool fiber was taken from a thread and placed on a glass slide. Then 5 µl of the aggregated sol, prepared as above, were added to the fiber and the sample was placed under the laser beam.

2) Hydrolysis procedure:

The hydrolysis method used to break the dye-mordant complex in extractionless analyses (Figure 5) is based on the procedure reported by Leona and co-workers.[4, 5, 14] After putting 10 µL HF in the conical tip of a polyethylene microvial, the textile sample, previously deposed in a cap of a smaller vial, was inserted in this sort of micro-reaction chamber and exposed to the acid vapors for 30 minutes. Then, the sample was left alone outside the vial for 30 minutes more in order to remove the HF vapors completely. Finally, the textile fiber was transferred onto a glass slide, covered with 5 µl of the aggregated sol prepared as above, and placed under the laser beam.

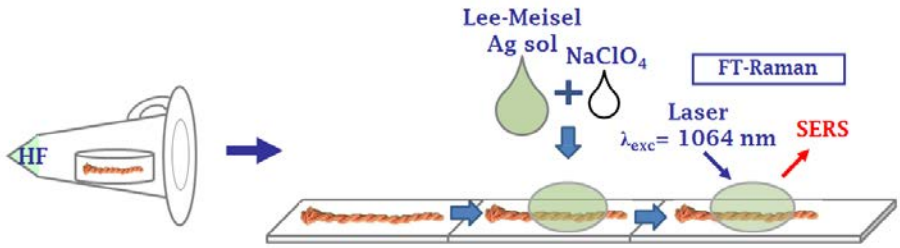

Figure 5. Scheme of the hydrolysis pre-treatment.

All samples, subjected both to extractionless non-hydrolysis and hydrolysis analyses, were tracked over time. SERS signals usually appeared after few minutes, during which the sol further aggregated, leading to the highest enhancement. The variation of the required waiting time in repeated experiments on the same fiber was really negligible and the spectra were reproducible both in pattern and intensity. As far as ancient samples are concerned, it resulted that they needed a longer aggregation time to show quite a good quality spectrum. For these reasons, the analyses carried out on historical samples were conducted for a longer time and, even if initially no SERS signals were observed, the acquisition was repeated till their appearance. More in detail, when the first colloid drop had dried, another 5 µL drop of aggregated sol was added and then the sample was placed again under the laser beam. Only when it was necessary, this operation was carried out up to 3 drops of colloid. The spectrum reported in the present paper for each sample is the best one obtained after one or repeated additions of the colloid.

Results and discussion

FT-SERS spectra of reference dyes in solution

As it will be discussed, a different enhancement of the vibrational bands obviously occurs when the SERS spectrum of a colored compound is obtained under resonance conditions rather then with an excitation wavelength far from the visible region of the electromagnetic spectrum, as in the present work.[1] For this reason, we deemed it useful to record FT-SERS spectra on solutions of reference dyes, belonging to several molecular classes, namely anthraquinones, flavonoids, neoflavonoids, biflavonoids and phenazone derivatives (orcein). In detail, FT-SERS spectra were obtained for 10^{-3} M solutions of the chromophores carminic acid, orcein, morin, luteolin, rutin, quercetin (Figure 6), alizarin (Figure 7) and purpurin (Figure 8) and of the natural organic dyes cochineal carmine, catechu, dragon's blood, logwood, sandalwood, old

fustic (Figure 6), madder (Figure 9), lac dye (Figure 10) and brazilwood (Figure 12). Table 1 reports the complete lists of the corresponding Raman shifts.

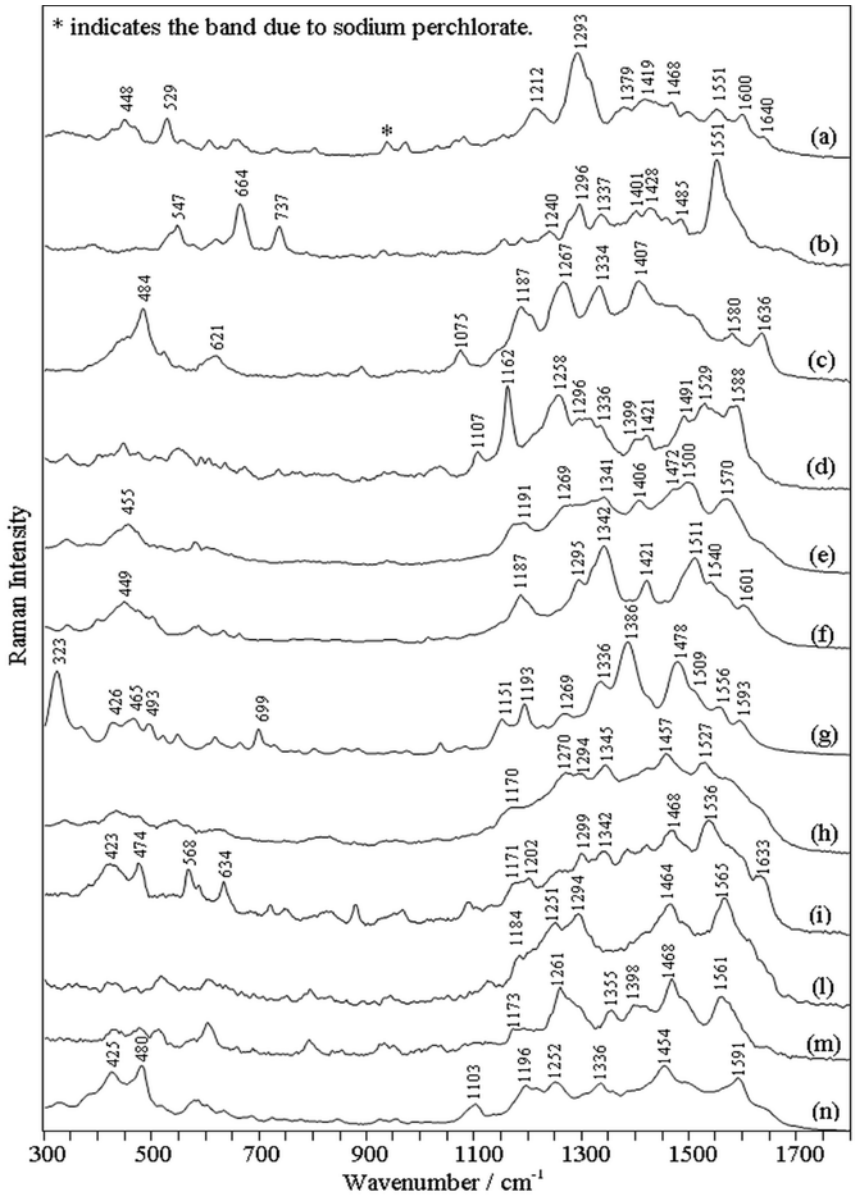

Figure 6. FT-SERS spectra (λ_{exc} = 1064 nm) from 10^{-3} M solution of (a) carminic acid, (b) cochineal carmine, (c) orcein, (d) dragon's blood, (e) catechu, (f) sandalwood, (g) logwood, (h) old fustic, (i) morin, (l) luteolin, (m) rutin, (n) quercetin (figure from ref 1).

Table 1. Raman shift (cm^{-1}) of bands in FT-SERS spectra of reference dyes in solution:	
Alizarin	345 (m), 399 (m), 477 (m), 503 (m), 581 (w), 633 (m), 662 (w), 681 (w), 762 (w), 1017 (m), 1048 (m), 1155 (m), 1186 (m), 1290 (s), 1321 (sh), 1422 (s), 1453 (sh), 1511 (sh), 1549 (s), 1603 (s), 1626 (sh)
Purpurin	386 (m), 431 (w), 462 (m), 528 (s), 610 (m), 650 (m), 974 (m), 1032 (w), 1064 (w), 1156 (m), 1212 (s), 1300 (s), 1318 (sh), 1389 (s), 1472 (s), 1501 (s), 1553 (sh), 1605 (s)
Carminic acid	335 (w), 448 (m), 529 (m), 558 (w), 606 (w), 656 (w), 973 (w), 1032 (vw), 1081 (w), 1212 (s), 1293 (s), 1379 (m), 1419 (s), 1468 (m), 1551 (s), 1600 (m), 1640 (w)
Cochinel	547 (m), 620 (w), 664 (s), 733 (s), 1156 (w), 1188 (w), 1240 (m), 1296 (s), 1337 (s), 1401 (m), 1428 (m), 1485 (m), 1551 (vs)
Lac dye	476 (w), 524 (s), 639 (m), 658 (m), 800 (w), 1013 (w), 1040 (w), 1058 (m), 1098 (m), 1130 (w), 1193 (s), 1282 (s), 1352 (s), 1372 (s), 1447 (s), 1555 (s), 1595 (s)
Sandalwood	342 (w), 339 (sh), 449 (s), 473 (sh), 500 (sh), 579 (w), 586 (w), 633 (w), 662 (w), 1014 (w), 1187 (s), 1295 (sh), 1342 (vs), 1421 (s), 1511 (vs), 1540 (sh), 1601 (sh)
Quercetin	383 (sh), 425 (s), 480 (s), 579 (m), 586 (m), 603 (m), 633 (w), 685 (w), 845 (w), 925 (w), 954 (w), 1103 (m), 1196 (s), 1216 (s), 1252 (s), 1336 (s), 1454 (s), 1494 (sh), 1591 (s)
Morin	423 (m), 474 (m), 568 (m), 587 (sh), 634 (m), 721 (w), 747 (w), 824 (w), 833 (w), 879 (w), 967 (w), 1087 (w), 1171 (sh), 1202 (m), 1258 (sh), 1299 (s), 1342 (s), 1385 (s), 1422 (s), 1468 (s), 1536 (s), 1633 (sh)
Old fustic	337 (w), 433 (s), 473 (m), 545 (m), 625 (w), 831 (w), 952 (w), 1170 (sh), 1270 (s), 1294 (s), 1345 (s), 1424 (sh), 1457 (s), 1527 (s), 1573 (sh)
Rutin	1023 (w), 1038 (w), 1023 (w), 1038 (w), 1173 (sh), 1261 (vs), 1278 (sh), 1296 (sh), 1315 (sh), 1355 (s), 1398 (m), 1402 (m), 1422 (sh), 1468 (vs), 1486 (sh), 1494 (sh), 1561 (s), 1574 (sh)

Luteolin	420 (w), 516 (w), 603 (w), 794 (w), 943 (w), 1126 (w), 1184 (sh), 1211 (sh), 1251 (s), 1280 (sh), 1294 (s), 1415 (sh), 1437 (sh), 1464 (s), 1487 (sh), 1497 (sh), 1552 (sh), 1565 (s), 1613 (sh), 1634 (sh)
Dragon's blood	342 (w), 446 (m), 550 (m), 592 (w), 607 (w), 636 (w), 672 (w), 736 (w), 949 (w), 998 (w), 1036 (w), 1107 (m), 1162 (vs), 1258 (vs), 1296 (m), 1306 (m), 1315 (m), 1336 (sh), 1399 (sh), 1406 (m), 1421 (m), 1491 (s), 1529 (s), 1542 (sh), 1552 (sh), 1579 (sh), 1588 (s)
Catechu	340 (w), 455 (s), 580 (w), 584 (sh), 602 (sh), 1173 (sh), 1191 (s), 1269 (sh), 1279 (sh), 1291 (s), 1310 (sh), 1319 (sh), 1330 (sh), 1341 (s), 1406 (s), 1472 (sh), 1500 (s), 1570 (s)
Brazilwood	334 (s), 369 (sh), 420 (sh), 465 (s), 490 (sh), 530 (w), 550 (m), 622 (w), 673 (w), 727 (w), 812 (w), 1167 (s), 1192 (s), 1218 (s), 1251 (sh), 1274 (sh), 1314 (sh), 1347 (s), 1392 (s), 1428 (sh), 1488 (s), 1495 (sh), 1515 (s), 1557 (vs)
Logwood	323 (s), 369 (sh), 426 (m), 465 (m), 493 (m), 521 (w), 548 (w), 618 (w), 699 (w), 1037 (w), 1151 (m), 1193 (m), 1269 (sh), 1336 (s), 1386 (vs), 1478 (vs), 1509 (sh), 1556 (sh), 1593 (sh)
Orcein	455 (sh), 484 (s), 522 (sh), 602 (sh), 621 (m), 890 (w), 1075 (m), 1187 (s), 1205 (sh), 1267 (s), 1334 (s), 1407 (s), 1449 (sh), 1476 (sh), 1507 (sh), 1580 (m), 1636 (sh)

Some FT-SERS spectra of anthraquinone dyes were already reported in literature, since they are the most studied natural colorants thanks to their very intense SERS response.[17-19] Cañamares and co-workers[18, 19] reported FT-SERS spectra of alizarin and carminic acid which correspond to those reported in this study. As regards purpurin, lac dye and cochineal carmine, the FT-SERS spectra acquired in the present work[1] show some similarities with those previously obtained with visible excitation in our laboratory.[21] In the case of purpurin, however, the signals at 1501 and 528 cm^{-1} are not observed at 532 nm, while bands at 1505 and 537 cm^{-1} are reported for 785 nm excitation wavelength.[5] The commercial cochineal carmine here analyzed is indeed a lake of carminic acid, as verified by FTIR analysis (result not shown),

therefore leading to a different SERS spectrum in comparison with that of the acid itself.

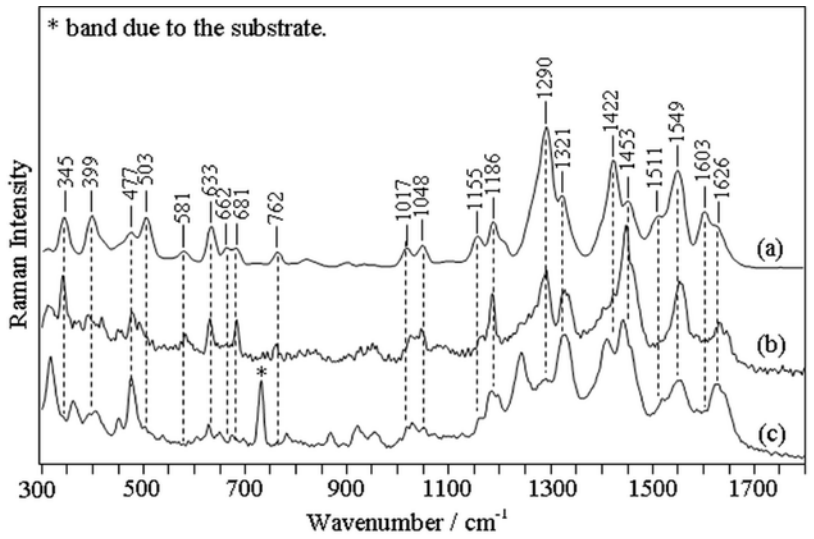

Figure 7. FT-SERS spectra (λ_{exc} = 1064 nm) of (a) 10^{-3} M alizarin methanolic solution, (b) single ply of reference wool dyed with alizarin without HF hydrolysis, (c) single ply of reference wool dyed with alizarin after HF hydrolysis (figure from ref. 1).

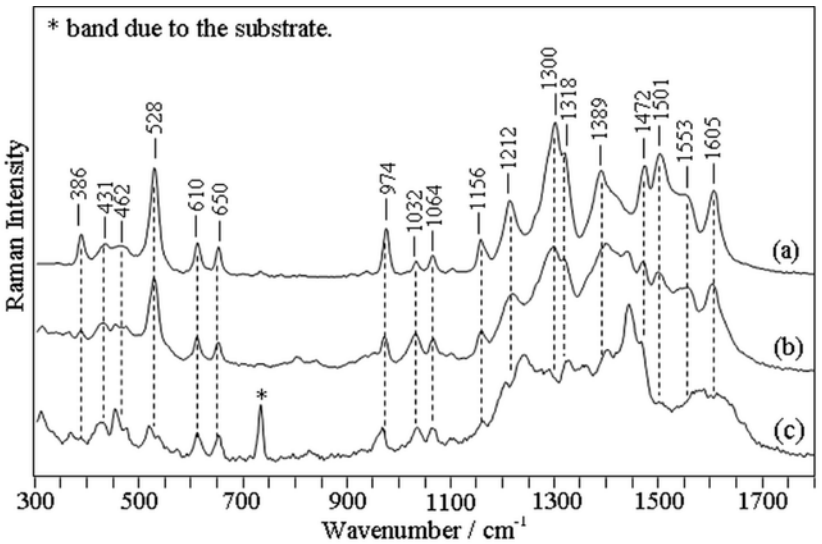

Figure 8. FT-SERS spectra (λ_{exc} = 1064 nm) of (a) 10^{-3} M purpurin methanolic solution, (b) single ply of reference wool dyed with purpurin without HF hydrolysis, (c) single ply of reference wool dyed with purpurin after HF hydrolysis (figure from ref. 1).

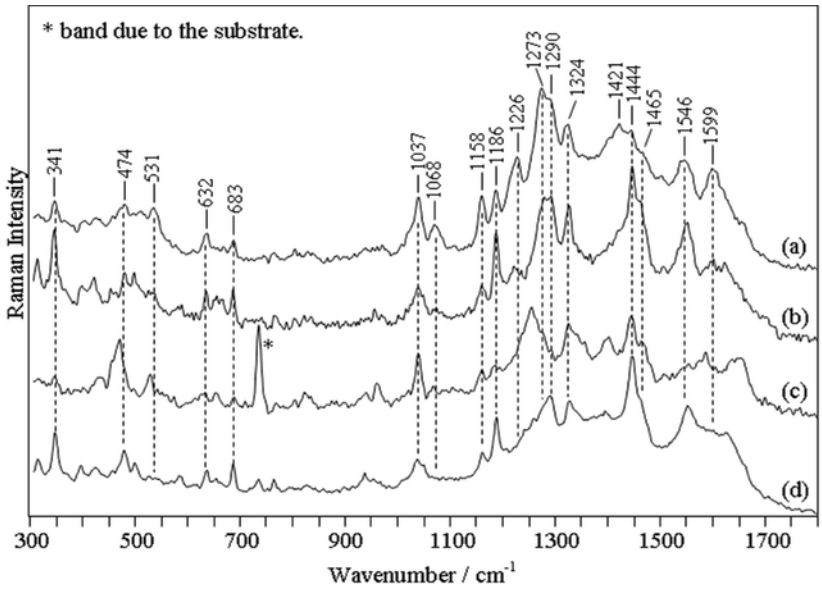

Figure 9. FT-SERS spectra (λ_{exc} = 1064 nm) of (a) single ply of reference wool dyed with madder without HF hydrolysis, (b) whole thread of reference wool dyed with madder without HF hydrolysis, (c) single ply of reference wool dyed with madder after HF hydrolysis, (d) red cotton thread from Kaitag textile (cat. n. 50) after HF hydrolysis (figure from ref. 1).

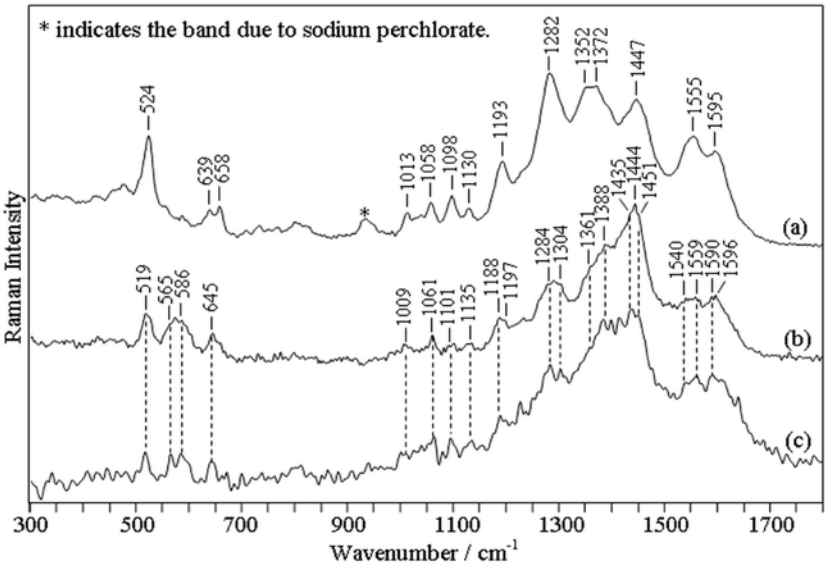

Figure 10. FT-SERS spectra (λ_{exc} = 1064 nm) of (a) 10^{-3} M lac dye methanolic solution, (b) fiber of reference wool dyed with lac dye after HF hydrolysis, (c) red fiber from Chinese cover chair (cat.n. 13901) after HF hydrolysis (figure from ref. 1).

As far as orcein is concerned, its FT-SERS spectrum is similar, more for band positions than for relative intensities, to the one reported in the literature[5] with λ_{exc} = 785 nm, even if the spectral region from 300 to 900 cm^{-1} shows some differences.

Dragon's blood and catechu belong to the molecular class of neoflavone dyes. The FT-SERS spectrum of the former displays several bands in common with the SERS spectrum obtained under visible excitation,[21] even if their relative intensities are significantly different. More specifically, the main signals at 1162 and 1529 cm^{-1} are present also in the spectrum recorded at 532 nm but with different relative intensities, while the band at around 1258 cm^{-1} is significantly enhanced in the FT-SERS spectrum. Ultimately, the FT-SERS spectrum of dragon's blood does not correspond completely to the SERS one.[21] Indeed the UV-Vis spectrum of the dye solution (Figure 11) shows that the 532 nm wavelength lies very close to the absorbance maximum of the dye, thus in the SERS spectrum at this excitation wavelength a resonance component exists, which is obviously absent in the spectrum recorded at 1064 nm. As regards catechu, the FT-SERS spectrum is in good accordance with the previously reported one,[21] in which the band at 1638 cm^{-1} appears as a shoulder. The FT-SERS spectrum of the biflavonoid dye sandalwood resembles that of catechu in the spectral region from 1300 to 1600 cm^{-1}, possibly due, as already suggested,[21] to the catechol moiety of the santalin A chromophore. In comparison with the spectrum obtained upon visible excitation, different relative intensities are observed, especially for the signal at 1511 cm^{-1}, which is significantly intensified in the

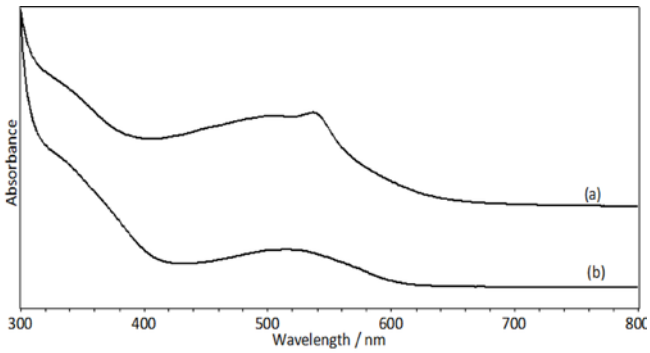

Figure 11. UV-vis spectra of (a) an aqueous solution of brazilwood, (b) a methanolic solution of dragon's blood (figure from ref. 1).

FT-SERS spectrum. As regards the neoflavonoid dyes logwood and brazilwood, evident differences were observed in comparison with the visible-excited spectra.[21] For logwood, spectrum (g) in Figure 6 shows some bands in common with the spectrum at 532 nm,[21] but with very different relative intensities. Moreover upon visible excitation the strong signal at 1386 cm^{-1} is absent, while it is present when a 785 nm excitation source is employed.[5] Indeed we can state that the SERS spectrum of logwood shows a remarkable dependence on the excitation wavelength. As far as brazilwood is concerned, it is worth noting that, while with visible excitation the SERS spectrum shows a high fluorescence background,[21] in the FT-SERS spectrum strong signals are observed (Figure 12).

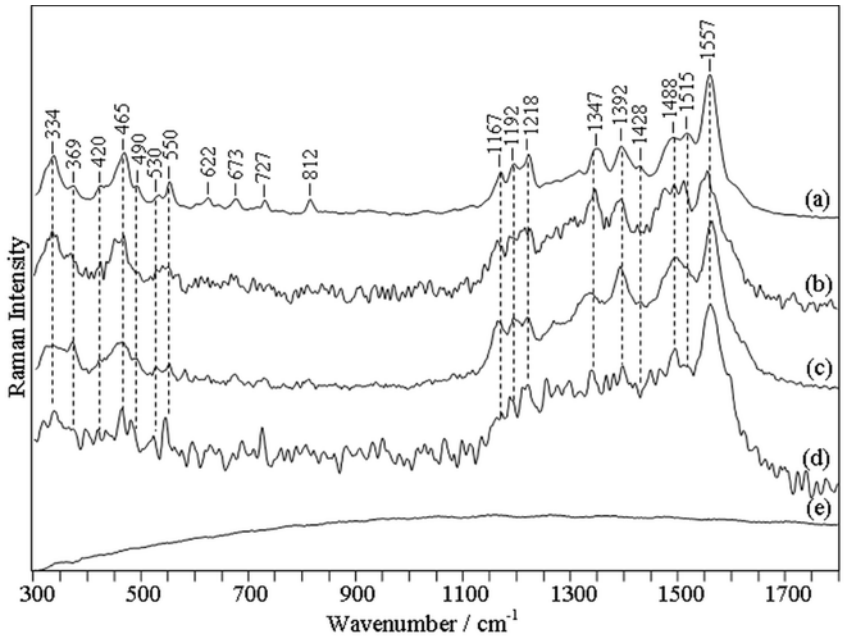

Figure 12. FT-SERS spectra (λ_{exc} = 1064 nm) of (a) 10^{-3} M brazilwood aqueous solution, (b) fiber of reference wool dyed with brazilwood without HF hydrolysis (a smoothing correction has been applied), (c) fiber of reference wool dyed with brazilwood after HF hydrolysis, (d) orange fiber from Chinese Ningxia carpet (cat.n. 6563) after HF hydrolysis (a smoothing correction has been applied), (e) SERS spectrum of the orange fiber from Chinese Ningxia carpet (cat.n. 6563) after HF hydrolysis obtained with 532 nm excitation wavelength (figure from ref.1).

However the FT-SERS spectrum has several bands in common with the SERS one after subtraction of the fluorescence background.[21] The main differences are the signals at 965 e 1043 cm^{-1} observed with 532 nm-excitation. This phenomenon can be partially explained by considering that, as in the case of dragon's blood, also for brazilwood the excitation wavelength of 532 nm lies very close to the absorbance maximum of the visible spectrum in solution (Figure 11), thus adding a resonance contribution to the SERS effect.

Flavonoid dyes are a very important class of molecules that provide a wide range of yellow hues. In the present work[1] the FT-SERS spectra of the main chromophores are reported together with that of old fustic, whose main component is morin. These compounds exhibit a strong dependence of the SERS signals on the excitation wavelength. In particular, the spectra of quercetin, morin, luteolin and old fustic (see spectra (n), (i), (l) and (h) of Figure 6) display remarkable differences with the previously reported ones[21] obtained with visible excitation. The FT-SERS spectrum of rutin, the glycoside of quercetin, shows a band at 1468 cm^{-1} with a stronger intensity compared to the SERS spectrum obtained with visible excitation (Figure 13), but is similar to what obtained by Leona and co-workers[5] with a 785 nm excitation wavelength: the enhancement of this signal can be attributed to the different sources of excitation.

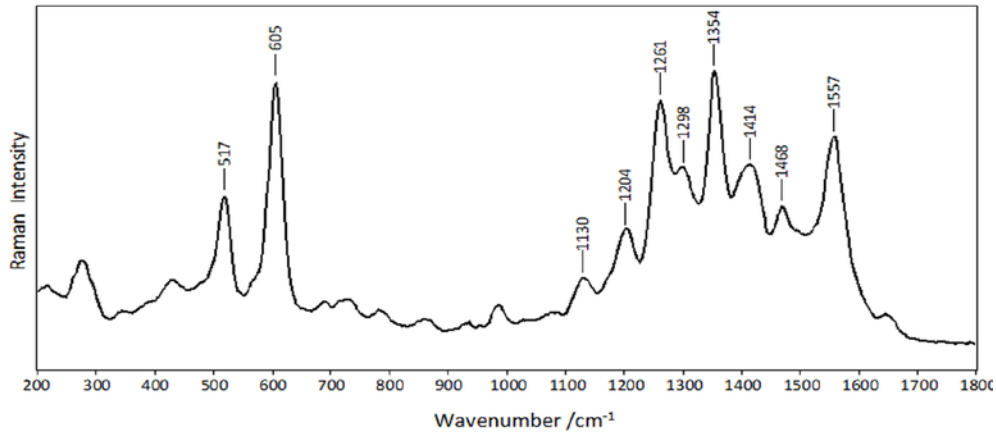

Figure 13. SERS spectrum (λ_{exc} = 532 nm) of rutin in solution (fig. from ref.1).

Extractionless FT-SERS analysis of reference wool samples

The laboratory-dyed wool threads subjected to extractionless analyses were chosen on the basis of the colors of the ancient samples we had at our disposal: they are red (cotton thread from Kaitag background, wool fiber from Chinese chair cover), orange (wool fiber from Chinese carpet) and brown (silk thread from Kaitag embroidery), thus extractionless analyses were performed on wool threads dyed with alizarin, purpurin, madder, lac dye, brazilwood and logwood.

As far as the extractionless non-hydrolysis analyses are concerned, the FT-SERS spectra obtained *on the fiber* are in good agreement with those obtained in solution for brazilwood (Figure 12), alizarin (Figure 7), purpurin (Figure 8) and logwood (result not shown). Moreover, the extractionless non-hydrolysis SERS spectrum of madder is very similar to that of alizarin, obtained under the same conditions (Figure 9). Only in the case of lac dye, it was not possible to obtain a reliable SERS spectrum directly on dyed wool fibers without hydrolysis.

Further, since only one historical sample, the brown silk thread, gave a reliable FT-SERS spectrum without the hydrolysis procedure, it was necessary to perform extractionless hydrolysis FT-SERS analyses not only on ancient samples, but also on reference dyed wool, in order to evaluate accurately the influence of such treatment on the FT-SERS spectra. Figures 7-9 show the extractionless FT-SERS spectra of alizarin, purpurin and madder upon HF hydrolysis compared to those obtained without hydrolysis. As it can be seen, after the hydrolysis treatment the spectra changed, not only in terms of relative intensity, but also of position of the enhanced signals. This fact could be possibly attributed to a different degree of protonation of the molecules, and thus to a different way of adsorption on the Ag colloid, resulting from the different pH conditions experimented. Anthraquinones are indeed rich in hydroxyl and carboxylic groups, which make these molecules subjected to pH conditions.[17, 18, 24, 25] In particular, a band at about 1250-1240 cm^{-1} (the exact wavenumber varies according to the particular dye considered) appears in the spectra

of wool plies dyed with alizarin, purpurin and madder after HF hydrolysis, gaining intensity at expenses of the band situated at about 1300-1275 cm^{-1} (again the precise wavenumber is different for each dye). This observation is in agreement with what reported by Shadi et al.,[25] who describe a similar shift for alizarin and purpurin upon changing the pH from alkaline to acid values. In the case of lac dye, for which an extractionless analysis could not be performed without hydrolysis, a similar trend could be observed in comparison with the spectrum of the dye recorded in solution (Figure 10): HF hydrolysis enhances the signal at 1447 cm^{-1} and decreases the intensity of the strong bands at 1282, 1352 and 1372 cm^{-1}.

As far as brazilwood is concerned, HF hydrolysis does not affect the spectral pattern, as shown in Figure 12. Finally, it is worth mentioning that the aim to reduce, as much as possible, the size of the thread used for the analysis could be achieved: starting from 5 mm of thread, the decrease of the sample size to a single ply or a single fiber of wool led to obtain again good quality SERS spectra, especially when the HF pre-treatment was performed (see Fig. 10, spectrum (b) and Fig. 12, spectrum (c)).

Extractionless FT-SERS analysis of ancient samples

Case study 1. The dark red background of the Chinese wool chair cover: lac dye

The identification of lac dye on the red fiber from the Chinese chair cover was straightforward after performing the HF hydrolysis pre-treatment of the sample. The spectral pattern obtained is indeed in good accordance with the one recorded on the reference wool fiber dyed with lac dye and subjected to HF hydrolysis prior to SERS analysis (Figure 10, spectra (b) and (c)). The complete lists of the Raman shifts of this and other FT-SERS spectra of ancient samples are reported in Table 2. The analysis of a similar sample from the same artefact by high-performance liquid chromatography (HPLC) is reported elsewhere[26] and confirmed the assignment based on the FT-SERS spectrum. Notably, the identification of lac dye in a Chinese Ningxia textile artefact is interesting, as many sources[27, 28] reported that the most common red dyes used

were madder, sandalwood and brazilwood, all dyes of vegetable origins, while the use of carmine and lac dye was very limited.[29, 30]

Case study 2. The orange background of the Chinese wool Ningxia carpet: the "fugitive" dye brazilwood

Also in this case an HF hydrolysis pre-treatment was required, but the main bands of the FT-SERS spectrum of the orange wool fiber (Figure 12, spectrum (d)) at 337, 463, 546, 1187, 1220, 1342, 1398, 1496 and 1561 cm^{-1} are consistent with the reference spectrum recorded for wool dyed with brazilwood. Notably, the same extractionless hydrolysis analysis performed on the same fiber but with an excitation wavelength of 532 nm (spectrum (e) of Fig. 12) led only to observe a fluorescence background, without SERS signals. These results demonstrate that the use of a FT-Raman instrumentation with an excitation wavelength of 1064 nm can be especially advantageous to obtain SERS spectra from strongly fluorescent dyes, such as brazilwood. However, it is worth mentioning that the SERS spectrum of this neoflavonoid dye was also recorded with good resolution by Idone et al.[8] with an excitation wavelength of 633 nm. It should be also remarked that, for geographical reasons, the dye originally used for this carpet was most probably the so-called sappanwood, obtained from *Caesalpinia sappan* L. and chemically analogous to brazilwood deriving instead from *Caesalpinia echinata* Lam..[31]

As far as our sample is concerned, of particular interest is the fact that the fiber exhibited, as remarked above, an orange color, a hue that is common to many Ningxia carpets, as they can be seen nowadays. However, historical representations of such carpets in Chinese paintings usually show they had a bright red background.[26] Moreover it is well known that the ancient Chinese dyers were used to employ on purpose light-fading red dyes, also in mixture with other dyes, to obtain, upon exposure of the textiles to light and air, the desired tint ranging from pink to peach, apricot and salmon colors. These red dyes often belonged to the redwoods family,

such as sappanwood itself.[29, 32, 33] Indeed, the HPLC analysis[26] of the same thread here examined by SERS led to detect not the intact chromophores of the dye, but the so-called "type C compound", recognized as a characteristic degradation product of the dye itself.[34-38] Interestingly, it has been recently suggested[35] that the "type-C compound" can originate from the aging of brazilwood as a result to the effect of light. The above considerations prompted us to hypothesize that the original dye used for the carpet faded away, possibly by accident or according to the dyer's intention. Nevertheless, it is interesting to note that the SERS technique allowed us to identify the redwood dye. The good correspondence with the SERS spectrum of the unaltered colorant could possibly suggest that the molecular moiety responsible for the interaction with the silver particles is maintained in the degraded chromophores.

Case study 3. The red cotton background of Kaitag textile cat. n. 50: madder

Again the HF hydrolysis pre-treatment allowed to obtain the FT-SERS spectrum of madder from the red cotton thread belonging to the background of a Kaitag textile. This spectrum surprisingly resembles much more the spectral pattern obtained from the non-hydrolyzed reference ply dyed with madder or alizarin (see Fig.9, spectra (b) and (d) and Fig.7, spectrum (b)) than the same after HF hydrolysis. However, it is worth remembering that, while our reference threads were made of wool, this sample comes from a cotton fabric. It is well known that the light-fastness of a dye depends greatly both on the kind of fiber to which it is applied and on the mordant used. For example, Padfield and Landi[39] found out that madder is very much more light-resistant on cotton than on wool, revealing a different behavior for the two fabrics. As far as the mordanting procedure is concerned, a SEM-EDX analysis of the red thread showed that, besides Al, also Fe and Ca are present on the fibers (mean values: Al 34 %, Fe 16 %, Ca 14 %, for details about this analysis see reference [40]). In this context, it is worth noting that many recipes for the production of Turkey red, another dye made from madder, insist not only on aluminum, but also on lime, in

order to achieve the red-purplish color and the high fastness properties for which it is famed. The presence of calcium in our sample could possibly suggest the use of a similar mordanting process, resulting in a metal complex of alizarin dianion, namely $CaAl(OH)(Az)_2$, whose structure was reported by Kiel and Heertjes.[41] This kind of chelation could lead to a complex more resistant to hydrolysis, as also Ca^{2+} need to be removed to obtain the free dye. As a consequence, the hydrolysis on a Ca and Al mordanted thread can lead to a situation in which the dye is not completely removed from the complex with metal ions, as it happens for the extractionless non-hydrolysis SERS analyses.

Case study 4. The dark brown corroded silk embroidery of Kaitag textile cat. n. 7: an iron-gall dye

As previously mentioned, FT-SERS extractionless non-hydrolysis analyses of historical samples gave appreciable results only in the case of the brown silk thread from a Kaitag textile. The thread is a part of an embroidery that shows evident signs of corrosion (Figure 1). Figure 14 reports the obtained FT-SERS spectrum in comparison to the FT-Raman spectra of the same sample and of an undyed silk thread: it is easy to

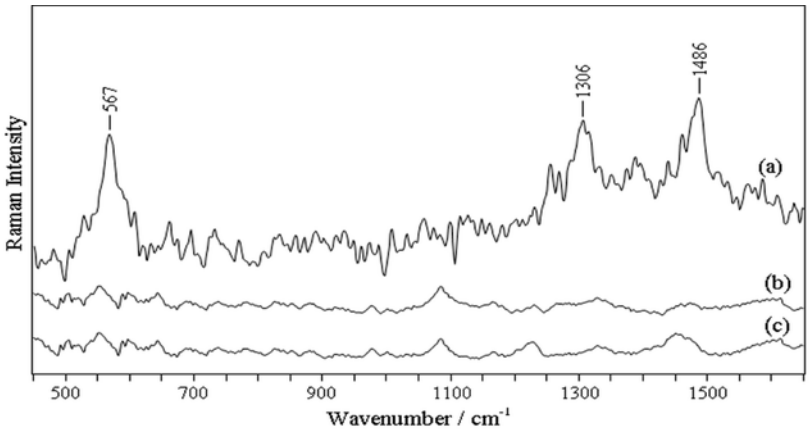

Figure 14. (a) FT-SERS spectrum (λ_{exc} = 1064 nm) from a silk brown thread from Kaitag textile (cat. n. 7) (a smoothing correction has been applied), compared with (b) FT-Raman spectrum of the same thread and (c) FT-Raman spectrum of a undyed silk thread (figure from ref. 1).

see that only the enhancement given by SERS allow to record a spectrum different from that of the fiber and showing a spectral pattern consistent with that of an iron-gall dye.[42] Such dyes were historically obtained from vegetable parts rich in tannins and iron(II) salts. Indeed significant amounts of iron were detected in these dark brown threads by X-ray fluorescence (XRF) and scanning electron microscopy combined with energy dispersive X-ray analysis (SEM-EDX), as reported elsewhere.[40] The use of this dye is in fact responsible for the observed corrosion and is confirmed by the detection of ellagic acid in the high-performance liquid chromatography (HPLC) analysis of a sample from the same embroidery.[40] To the best of our knowledge, the identification of an iron-gall dye by an *on-the-fiber* SERS analysis was reported in this study[1] for the first time.

Table 2 - Raman shift (cm^{-1}) of bands in FT-SERS spectra of ancient samples:	
Brown silk thread from Kaitag textile, extractionless non-hydrolysis analysis	567 (s), 1255 (m), 1306 (s), 1385 (m), 1486 (s)
Red cotton thread from Kaitag textile, extractionless HF hydrolysis analysis	309 (w), 342 (m), 391 (w), 418 (w), 474 (m), 495 (w), 632 (w), 650 (vw), 682 (w), 730 (w), 760 (w), 1035 (m), 1047 (sh), 1159 (m), 1186 (s), 1291 (s, br), 1327 (s), 1339 (sh), 1395 (w), 1446 (vs), 1462 (sh), 1552 (s), 1626 (sh)
Red wool fiber of Chinese chair cover, extractionless HF hydrolysis analysis	517 (w), 565 (w), 585 (w), 642 (w), 1001 (vw), 1012 (vw), 1064 (w), 1095 (w), 1135 (w), 1188 (sh), 1227 (sh), 1284 (m), 1303 (m), 1359 (sh), 1385 (s), 1400 (s), 1414 (s), 1439 (vs), 1540 (sh), 1561 (m), 1592 (m), 1610 (sh)
Orange fiber from Ningxia carpet, extractionless HF hydrolysis analysis	337 (w), 365 (vw), 463 (w), 481 (w), 546 (w), 726 (w), 1163 (sh), 1187 (m), 1220 (m), 1256 (m), 1342 (m), 1398 (m), 1496 (s), 1561 (vs)

Conclusions

With the present work[1] we provided a deeper insight into the potentialities of FT-SERS for the identification of natural dyes in ancient textiles and works of art. We were able to collect the FT-SERS spectra of eight chromophores and eight natural organic dyes belonging to several molecular classes, namely anthraquinones, flavonoids, neoflavonoids, biflavonoids and phenazone derivatives (orcein), and thus we started the construction of a new database of FT-SERS spectra of dyes in solution. Moreover, an extractionless procedure with and without hydrolysis was tested on reference dyed wool and ancient samples, whose dimensions were decreased in most cases to a single fiber. It was thus demonstrated that the excitation at 1064 nm resulted to be especially advantageous for strongly fluorescent dyes, such as brazilwood, that could be identified in an historical sample from a Chinese Ningxia carpet in spite of its fading. Moreover, a dark brown iron-gall dye was recognized for the first time[1] by SERS extractionless analysis on a Kaitag textile.

Acknowledgments

Mr. Moshe Tabibnia is thanked for giving us the opportunity to access his own collection of precious textiles, and Dr. Elisabetta Mero and Dr. Tiziana Marchesi (Moshe Tabibnia Gallery) for providing the samples. Moreover, Dr. Italo Campostrini (Università degli Studi di Milano) is thanked for acquiring the SEM images.

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CHAPTER 5

Dry-state methodologies for direct SERS analysis of dyes on textiles

Abstract

The present Chapter is intended as the description of a preliminary study assessing the use of nanomaterials for direct solid-state SERS analysis of dyed textiles. The final aim is that of developing a simple method to identify natural dyes on textiles by means of a "dry-state SERS" approach, i.e. exploiting the interactions between a solid nanometallic substrate and the molecules of dyes present on textiles, with the intent to avoid any necessity to take samples from the analyzed objects. Different nanosubstrates were taken into account, as well as different excitation sources. The preliminary data obtained can open a new window of opportunities towards the non destructive analysis of dyes on artefacts.

Introduction

As extensively demonstrated, surface-enhanced Raman scattering is a valuable tool for the identification of chemicals at the trace level. Nowadays, researchers dealing with the identification of dyes in works of art by means of SERS are trying to convert this valuable but destructive technique in a non-destructive one, in order to safely apply it to the analysis of artefacts without any risks for the art objects. Several attempts were done in this regard, using different approaches: the development of hydrogels based on solid phase micro-extraction techniques that extract trace amounts of dyes,[1-4] the laser ablation followed by SERS analysis of the ablated substances[5, 6] and the exploitation of *in situ* tip-enhanced Raman spectroscopy (TERS), whose key features include high mass sensitivity, high spatial resolution and precise positioning of the tip.[7]

In the present work, the proof of concept of the possibility to obtain a SERS response starting from a solid nanometric substrate in contact with dry dyes on textiles is verified and tested on laboratory-prepared samples. It is worth underlining that this kind of interactions are obviously more challenging than those occurring between a

solution of the target dye and the same substrate, as in the former case the electromagnetic mechanism should play a key role, while molecules in solution can be also chemisorbed on the nanomaterial. Being a new approach, it needs a preliminary assessment of feasibility by the analysis of reference samples, with the ultimate goal of the developing nanodevices to be used for in-situ non-destructive analyses.

As far as "dry-state SERS" is concerned, in 2004 Vo Dinh and co-workers demonstrated the feasibility of localized, nondestructive SERS-based analysis of dry surfaces using a nanoprobe coated with nanoislands: the probe induced the SERS effect when brought into contact with dried drops of chemicals on a glass slide.[8] In 2006, the possibility to obtain the SERS effect from pure dyes in powder was tested by Van Duyne and co-workers using nanoislands.[9] Silver nanoislands are a SERS-active substrate produced by vapor deposition of a thin film (< 100 Å) of silver onto a suitable surface; the deposited film is constituted by discontinuous island-like particles, thus the name "nanoislands". This substrate has been used several times to obtain SERS spectra of natural dyes: the samples were solutions of dyes extracted from textile samples[10] or dyed fragments which were covered with the layer of silver nanoparticles.[11, 12] Recently, this substrate was chosen also for laser ablation-SERS (LA-SERS) analysis, thanks to its ease to manufacture and its relatively high enhancement factor.[5, 6] In order to accomplish "dry-state SERS" analyses, silver nanoislands could be advantageous, being deposited onto surfaces as thin metallic films, therefore constituting a solid platform for SERS analyses. The high reproducibility is another key feature of this substrate.[9, 13, 14] Indeed, even if the structure of the particles are dependent on several experimental factors, including the substrate material, the temperature of the substrate during and following metal deposition, the thickness of the film and the rate of metal deposition, these conditions are easy to control and reproducible SERS-active island films can be prepared by maintaining a consistent experimental procedure.[13] Therefore, the first

part of the present project, focusing onto the exploitation of Ag nanoislands (AgI) to obtain SERS signals from dyed textiles, was carried out at Duke University (USA).

However, thus far the most used substrates for SERS purposes are silver colloids, whose use entails the wetting of the artefacts, if not the extraction itself of dyes from the samples,[11, 15-17] thus resulting not suitable for a non-destructive analysis. In spite of this, examples of silver films obtained from colloids were reported in the literature (see for example refs. [18-20]). These SERS substrates consist of metallic nanoparticles synthesized by a wet chemistry method and subsequently immobilized onto a solid support, exploiting the self-assembly of metallic nanoparticles onto solid surfaces based on electrostatic attraction with polymers[21-23] and biomolecules.[24, 25] Although the chemical and electrostatic self-assemblies are popular for fabricating SERS substrates, different approaches have also been explored. Interestingly, silver films obtained from colloids and based on the suppression of the so-called "coffee ring effect" were recently developed.[26] The "coffee ring effect" is widely known as a typical evaporation-driven self-assembly and self-organization.[27, 28] When a droplet of solutions containing nonvolatile solutes (e.g. coffee particles) dries on a substrate, it leaves a dense, ring-like deposit of the solutes, i.e. a "coffee ring" along the perimeter. This because, during the drying process, the droplet contact line remains pinned in all suspensions, and fluid (carrying particles) flows outwards from the drop center to replenish the edges. Spherical particles are efficiently transported to the edge, either in the bulk or along the air–water interface, leaving a ring after

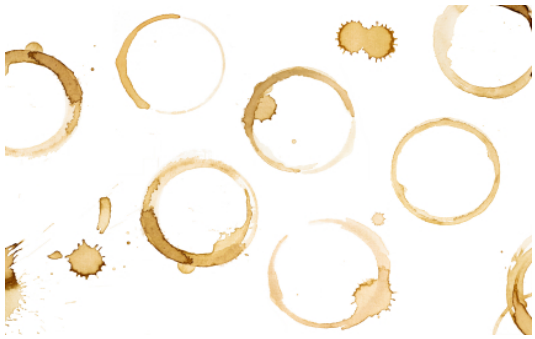

Figure 1. The coffee ring effect

evaporation has been completed. In more detail, the evaporation flux at the edge of the droplet is much higher than that at the center, leading to more solvent loss at the edge of the droplet than at the center. To keep the contact line pinned, the solvent must flow from the droplet center toward the edge to compensate for the solvent loss. Consequently, a flow is generated in the evaporating droplet and more particles are carried to the edge. Translating this phenomenon in the physics of colloidal solutions, it obviously hinders the possibility to obtain homogenous films from colloids by the mere evaporation of the solvent. In ref. [26] the authors managed to avoid this effect by modifying the conditions for films formation. More in detail, they demonstrated that at concentrations of silver nanoparticle solutions ranging from 50 mM to 0.1 M, smooth silver nanoparticle films can be formed on the surface of silicon wafers at temperatures of about 50°C. Indeed, at lower concentrations, almost all of the nanoparticles were deposited on the outer ring and no film was generated inside. Increasing the concentration up to 10 mM, scattering particles were deposited inside the ring. When the concentration was high enough, such as 50 mM or 0.1 M, the silver nanoparticles promptly filled the solid-liquid contact line and thereby formed a smooth film. In the light of these interesting results, at the Università degli Studi di Milano we exploited the possibility to avoid the "coffee ring effect" to create silver films starting from different silver colloids and to use them as "dry-state SERS" probes for the identification of dyes on textiles.

Experimental

Materials

Alizarin, trisodium citrate dihydrate (assay 100.2%), hydroxylamine hydrochloride and sodium hydroxide were purchased by Sigma-Aldrich, while ethanol, sulfuric acid and hydrogen peroxide from VWR. Silver nitrate ($AgNO_3$) was obtained from Fluka. The wool samples are laboratory-dyed textiles, whose fabrication is described elsewhere.[29]

Preparation of substrates:

1) Silver nanoislands

SERS-active silver island film substrates were prepared by depositing a statistically random array of silver islands on microscope slides. Briefly, commercially available 1.2 mm thick glass microscope slides (Baxter, McGaw Park, IL) were sonicated in ethanol or in piranha solution (H_2SO_4: H_2O_2 = 3:1) for 5 minutes and dried with nitrogen. Each slide was then coated with a 6 nm mass thickness of silver in an electron-beam evaporator (CHA Industries Solution E-Beam) at a deposition rate of 5 angstroms per second. The pressure within the evaporator was no higher than 10^{-6} torr during AgI deposition. These substrates were stored at room temperature in the dark and in a nitrogen atmosphere until use.

2) Silver films obtained from colloids

Two different starting colloids were synthesized to be tested for the fabrication of solid nanometallic substrates.

a - Lee and Meisel (LM) silver colloid

The protocol used for the synthesis of the citrate-reduced silver colloid according Lee-Meisel procedure[30] is described in Chapter 2 of the present doctoral thesis.

b - Leopold and Lendl (LL) silver colloid

In 2003, Leopold and Lendl[31] reported a very effective and simple way to produce silver colloids for surface-enhanced Raman scattering (SERS) experiments by reduction of silver nitrate with hydroxylamine hydrochloride (HYA) at alkaline pH and at room temperature. The silver colloid tested in this study was synthesized accordingly: to obtain a rather monodispersed size of the silver particles, 10 mL of a concentrated hydroxylamine hydrochloride/sodium hydroxide solution ($1.5 \cdot 10^{-2}$ M /

$3 \cdot 10^{-2}$ M, respectively) were instantaneously added to 90 mL of silver nitrate solution ($1.11 \cdot 10^{-3}$ M). The mixed solution was kept stirring for 60 minutes.

To obtain silver films from the colloidal solutions synthesized, the sols underwent a centrifugation process in order to increase the concentration of the silver nanoparticle solution.[26] The LM silver colloid was centrifuged for 15 minutes at 7000 rpm, while the LL colloid was centrifuged for 20 minutes at the same speed. The solvent was removed from the centrifuge tubes using a pipette, leaving aqueous-based Ag nanoparticle paste at the bottom. For the latter colloid, a difficulty in the separation of particles from the solvent was noted. This observation is coherent with the different particle size of the two sols prepared: while with the Lee–Meisel method a large variety of 60–200 nm sized Ag nanoparticles with a wide range of shapes is yielded,[32] those obtained from the synthesis by Leopold and Lendl have a mean diameter of 34 nm.[31] The silver pastes at the bottom of the centrifuge tubes were collected and conveniently diluted until the required concentration solution was obtained. Subsequently, glass slides were cut into the required size. The glass surfaces were cleaned with diluted nitric acid, washed with MilliQ water, acetone and dried. The cleaned glass slides were then laid in a glass container before the introduction in the oven, whose temperature was set to 130°C, for the evaporation of the solvent. The concentrated solutions (0.05 or 0.1 M) of silver nanoparticles were deposited onto the glass slides and the evaporation in the oven was carried out. After evaporation of the solvent, self-assembled silver nanoparticle films were obtained.

Instrumentation

SERS measurements using silver nanoislands (AgI) as nanostubstrates were performed by a Renishaw InVia confocal Raman microscope. A HeNe laser (Coherent, model 106−1) emitting a 632.8 nm line was used as the excitation source. The laser power on the sample was 6 mW. The light from the laser was passed through a line filter and

focused on the sample via a 10× microscope objective. The Raman scattered light was collected by the same objective and passed through a holographic notch filter to block the Rayleigh scattered light. An 1800 groove/mm grating was used to disperse the collected light, providing a spectral resolution of 1 cm^{-1}. The Raman scattering was detected using a 1024 × 256 pixel RenCam charge-coupled device (CCD) detector. SERS spectra from dyes in solution were collected after a 2 µL drop of reference dye was put on the nanometric substrate, with a laser power of 100% and with an exposure time of 10 s.

SERS analyses with silver films obtained from colloids were performed by the compact micro-Raman instrument with exciting radiation at 532 nm described in Chapter 2. All SERS spectra were recorded between 2000 and 200 cm^{-1} by collecting 50 scans with an exposure time of 2 s.

In all cases, threads of the wool samples and grains of pure dyes were posed on nanometric substrates and pressed with another glass slide to improve the contact between the molecules of dyes and the nanomaterials, as shown in Figure 2 for the analysis performed by means of silver nanoislands.

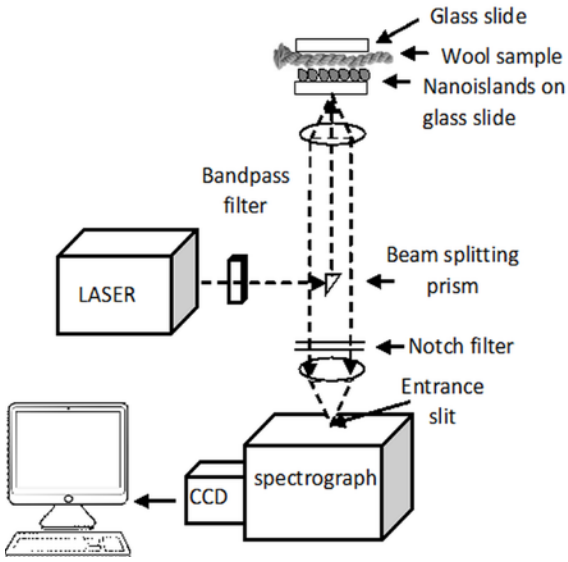

Figure 2. Schematic diagram of the detection system used for "dry-state SERS" analysis.

Being wool threads intrinsically inhomogeneous, for each sample analyzed the exposure time and the laser power were adjusted as necessary to avoid detector saturation.

SEM-EDX analyses were recorded by means of a Hitachi-TM 1000 scanning electron microscope covering a magnification range between ×20 and ×10,000. The instrument was equipped with an energy-dispersive electron microprobe, a pre-centred cartridge filament and a high-sensitive semiconductor backscattered electron detector.

Results and discussion

Dry-state SERS analyses using silver nanoislands

Silver nanoislands are a reproducible substrate, commonly used to obtain SERS spectra.[9-12] Van Duyne and co-workers[9] previously demonstrated that the use of a 633 nm excitation wavelength, i.e. that adopted in the present study, in conjunction with silver island film substrates gives back enhanced and detailed SERS spectra of the common natural red dyes. This occurrence could be explained with the achievement of resonance conditions between the excitation wavelength and the absorption band of the localized surface plasmonic band of AgI.[9] Interestingly, we found out that another parameter to take into account for the obtainment of enhanced Raman signals is the cleaning procedure of glass slides before the deposition of the metal. Figure 3 reports the SERS spectra of a 10^{-3} M methanolic solution of alizarin obtained with nanoislands deposited onto glass slides cleaned with two different methods before undergoing the same deposition process. The glass slides sonicated in ethanol resulted to give a better enhancement (Figure 3(a)), more than twice the signal obtained with the glass slides cleaned with the piranha solution (Figure 3(b)). This is probably due to the lower efficiency of ethanol in the cleaning process, which could lead to the presence of more roughness on glass slides at the moment of the deposition of the silver layer, thus inducing a more enhanced Raman signal.

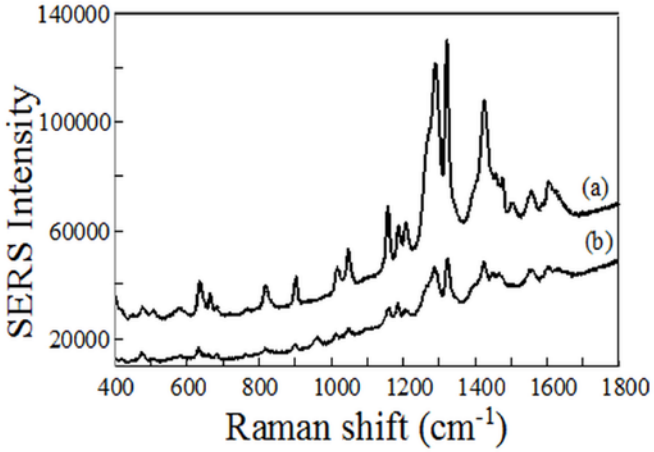

Figure 3. SERS spectra of alizarin ($4.8 \cdot 10^{-4}$ ng/cm^2) spotted on (a) glass slide cleaned with ethanol, (b) glass slide cleaned with piranha solution.

The instrumentation adopted (Figure 2) allowed us to record the Raman and SERS spectra shown in Figures 4 and 5, obtained directly from wool samples. As easily expected, conventional Raman analyses of dyed threads led only to observe a high fluorescence background, while, applying the "dry-state SERS" protocol of analysis, SERS signals of dyes are well recognizable, even if an intense background is present. This is particularly true for purpurin (Figure 4a, right) and for lac dye (Figure 5b), whose spectrum is highly affected by the background signal. The background subtraction, however, allowed to highlight the signals belonging to the dyes.

The SERS spectra obtained are in good accordance with those recorded from solutions of the same dyes deposited on silver nanoislands (see Figure 3 for alizarin, the inset of Figure 4b right for purpurin and the inset of Figure 5b for lac dye). All the SERS spectra are moreover coherent with those previously reported in the literature for the same dyes in solution or powdered.[9, 10]

The results obtained thus clearly demonstrated the possibility to directly acquire SERS spectra from dry dyes on textiles by means of a solid nanometallic substrate in a simple way.

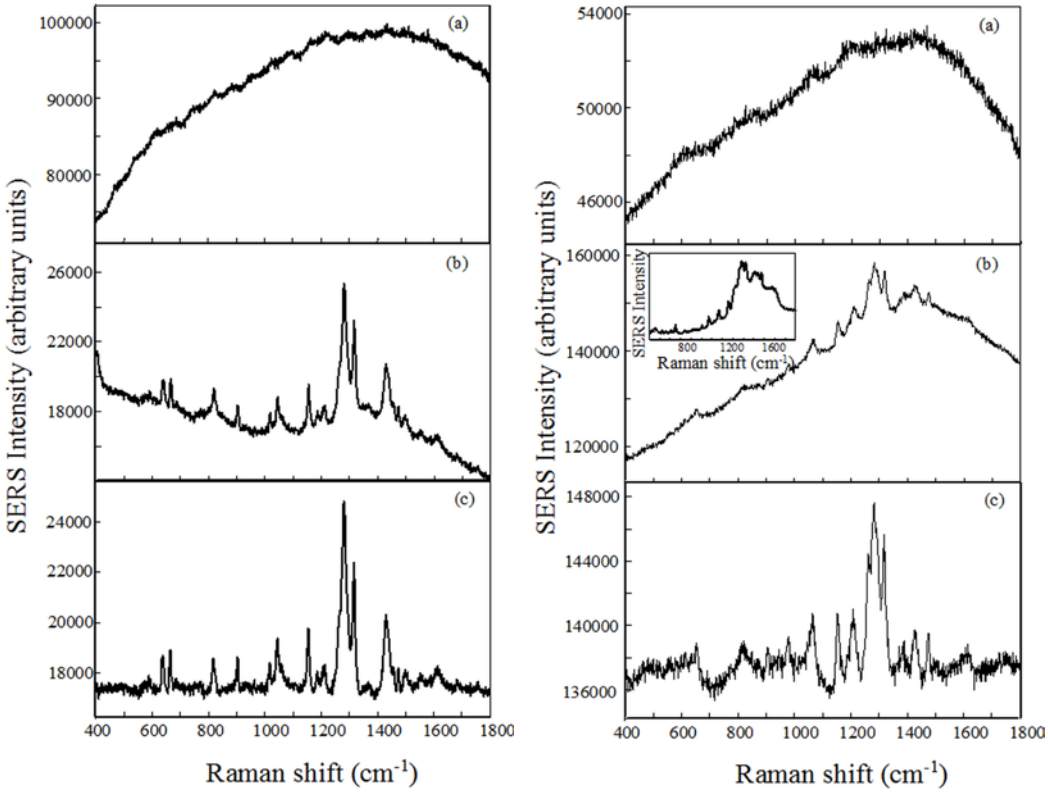

Figure 4. (a) Raman spectra of wool threads dyed with alizarin (left) and purpurin (right); (b) SERS spectra of the same samples on Ag nanoislands without background correction (the right inset shows the reference SERS spectrum of purpurin in solution); (c) on Ag nanoislands with background correction. The laser power was set to 10% of the excitation source, excepting for spectrum (a, right) for which it was set to 1% to avoid the signal saturation. The exposure time was of 20 s, excepting for spectrum (a, left) for which it was set to 10 s to avoid the signal saturation.

It is worth remembering also that the most of natural dyes were anchored to textile fibers with a "mordant", generally alum ($KAl(SO_4)_2 \cdot 12H_2O$). The SERS spectra shown in Figures 4 and 5 were obtained on wool samples that underwent a mordanting process and, after the dyeing process, were washed with distilled water. However, some free dye molecules, not complexed with the metallic salt, could be present on fibers and the obtainment of the SERS spectra could be due mostly to these molecules. The same analysis on mordanted dyed wool washed with methanol gave back some results whose reproducibility was not satisfying (results not shown).

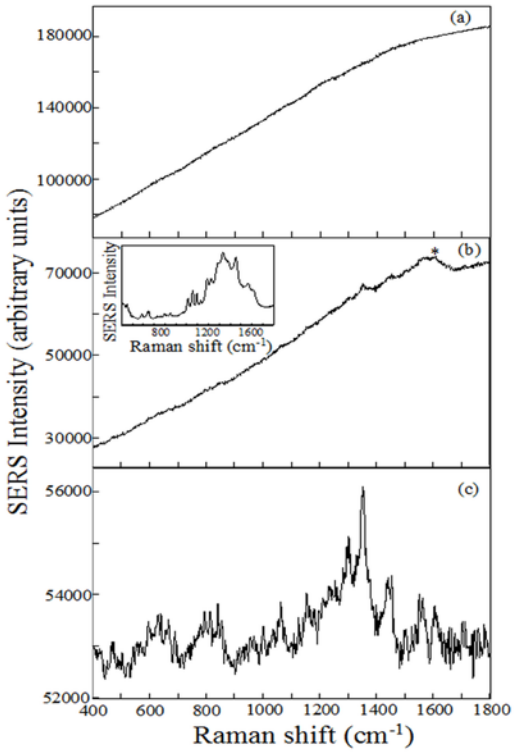

Figure 5. (a) Raman spectrum of a wool thread laboratory-dyed with lac dye; (b) SERS spectrum of the same sample on Ag nanoislands without background correction (the inset shows the reference SERS spectrum of lac dye in solution) and (c) with background correction. The laser power was set to 50% of the excitation source, excepting for spectrum (a) for which it was set to 10% to avoid the signal saturation. The exposure time was set to 10 s.

Eventually, another drawback of using Ag nanoislands is constituted by the fact that AgI can peel off extremely easily, entailing the possibility of transferring silver to the textile upon physical contact between the two. Therefore, as detailed in the following, other substrates were taken into account.

Dry-state SERS analyses using silver films obtained from colloids

As described above, a procedure recently reported in the literature[26] to obtain silver films from colloids was also applied in the present work. However, since our aim was that of recovering the scattered radiation from bulk materials, the geometry of the instrumental set-up was invariably subjected to some constrains. In particular, to reach the detector, the signal must pass through the nanomaterial and its solid support (Figure 2). Moreover, the analysis of a textile artwork should allow to easily identify the point under measure. Therefore, differently from ref. [26] where silicon wafers were used, but similarly to the fabrication of nanoislands, we selected glass slides as solid support for the deposition of films.

As far as the preparation of SERS-active substrates from colloids is concerned, it should be remembered that Shah and co-workers already exploited a concentrated Lee-Meisel silver colloidal substrate, that they named "silver colloidal pastes", to detect dyes directly on fibers.[33] In the present work, however, the concept of pastes is further exploited to obtain silver films onto glass slides. Moreover, two different starting colloids were chosen and tested to this aim, as described in the experimental section. The Lee and Meisel (LM) silver sol[30] is the most used in the field of SERS of natural dyes, while the colloid introduced by Leopold and Lendl in 2003[31] was selected since, contrary to the former sol which exhibits signals in the 1100-700 cm^{-1}

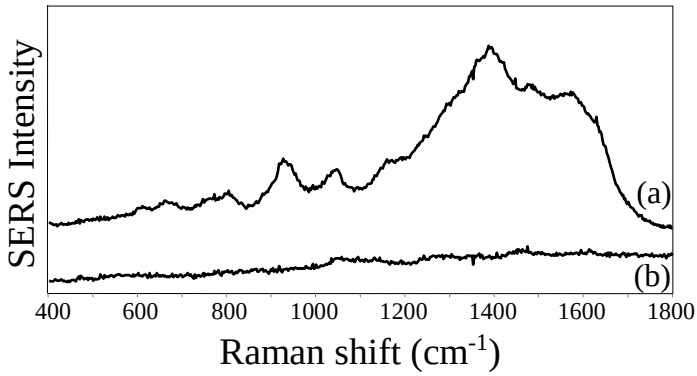

Figure 6. SERS spectra of Ag films obtained from (a) Lee and Meisel sol, (b) Leopold and Lendl sol.

and 1300-1700 cm^{-1} regions due to citrate oxidation products, it displays only a narrow and intense band attributed to the ν(Ag-Cl) vibration at 244 cm^{-1},[34] therefore far from the spectral region where SERS signals of dyes are situated. As shown in Figure 6, the films obtained from the two sols retain the spectral features of their starting colloids.

Another parameter taken into account to evaluate the best condition to obtain a SERS response was the concentration of the re-dissolved nanoparticles solutions after centrifugation. Hence two different concentrations, 0.05 and 0.1 M, were examined.

The two colloids were first tested, before centrifugation, to compare their SERS performances: the analyses were conventionally carried out with a reference analyte, i.e. alizarin, in methanolic solution and by adding a metal salt, i.e. sodium perchlorate, already reported for SERS experiments,[16] to induce the aggregation of nanoparticles. The two colloids provided similar results, with enhanced Raman signals of the same magnitude (results not shown).

Figure 7 shows the image of silver films obtained from LM and LL colloids at the concentration of 0.1 M. It can be seen that the films exhibit a very little "coffee ring effect" and are rather homogeneous, even if the silver layers display a variable degree of thickness.

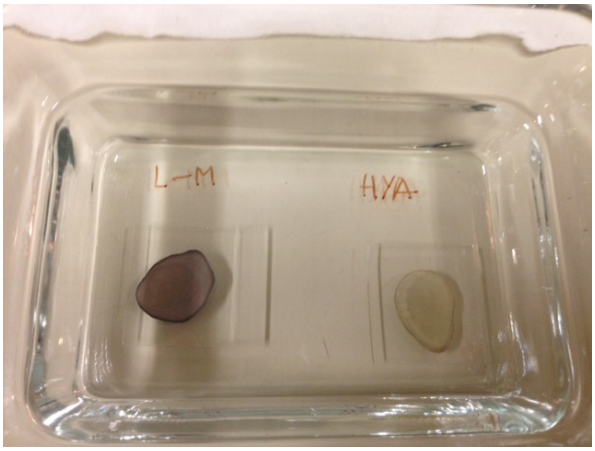

Figure 7. Silver films obtained from 0.1 M colloid of Lee and Meisel (left) and of Leopold and Lendl (right).

The two films, in particular that obtained from the LM sol, resulted to be SERS active both towards alizarin in powder and a wool thread dyed with the same dye (Figure 8). It is easy to see that, even if the quality of SERS spectra was dependent on the area of the film under analysis, the film obtained from the LL silver colloid gave back SERS signals with a fluorescent background, while the film fabricated with the LM colloid allowed to record more detailed SERS spectra. Experimentally, the best SERS spectra were obtained in those areas of films that showed a lower thickness.

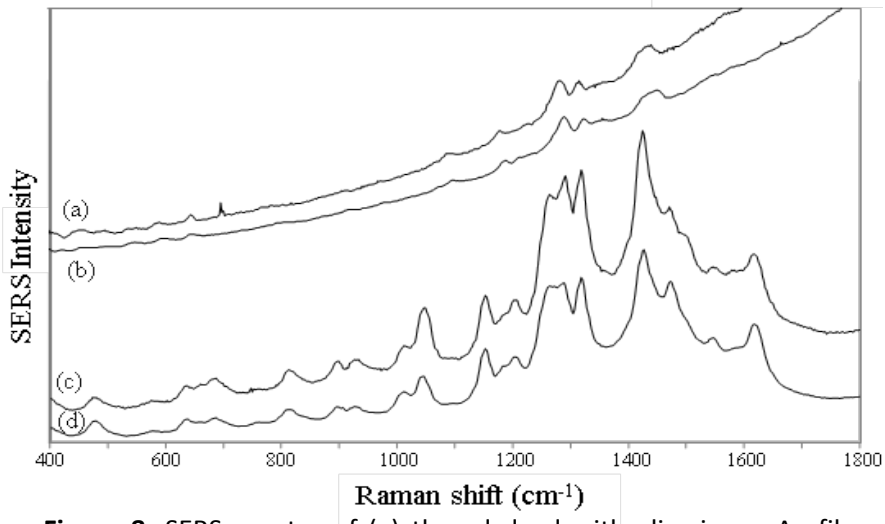

Figure 8. SERS spectra of (a) thread dyed with alizarin on Ag film from LL sol; (b) grain of alizarin in powder on Ag film from LL sol; (c) thread dyed with alizarin on Ag film from LM sol; (d) grain of alizarin in powder on Ag film from LM sol.

One drawback experimented was the difficulty in the placement of the sample under the laser beam, as the films were not completely transparent.

In the light of these results, two colloids with a concentration of $5 \cdot 10^{-2}$ M were prepared to obtain films onto glass slides (Figure 9). The films were thin but less homogenous with respect to the previous ones and exhibit a more pronounced "coffee ring effect". This is particularly true for the silver film obtained from the LL colloid.

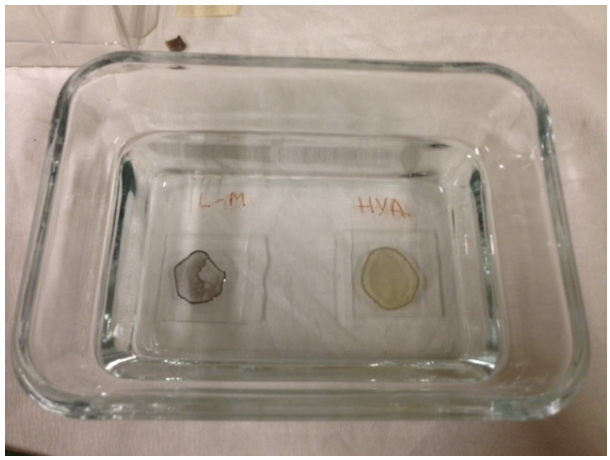

Figure 9. Silver films obtained from 0.05 M colloid of Lee and Meisel (left) and of Leopold and Lendl (right).

At this concentration, the film obtained from the LL colloid gave back no SERS results, whereas good quality SERS spectra of both alizarin in powder and on textile fibers were recorded using the film obtained with the LM colloid (Figure 10).

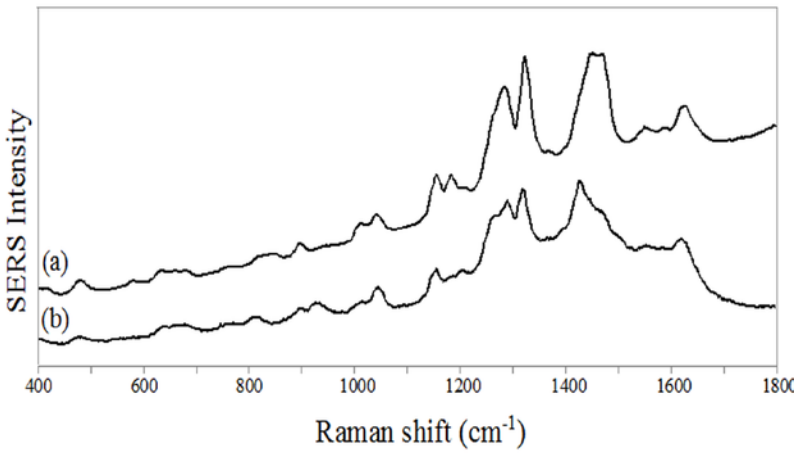

Figure 10. SERS spectra recorded with the Ag film carrying LM nanoparticles and obtained from (a) grain of alizarin in powder; (b) wool thread dyed with alizarin.

The better performances of films obtained from the LM colloid in comparison to the other one were thus confirmed. In comparison with the spectra reported in Figure 8, those of Figure 10 seem less resolved, indicating the silver film obtained from 0.1 M LM colloid as the most performant SERS-active substrate among those here experimented.

The silver films obtained were characterized by UV-vis reflectance analysis (Figure 11). It is evident that the plasmonic band, characteristic of metal colloids as such, is replaced in films by a large band spread along the whole spectrum of the visible light. Interestingly, the different visible reflection spectra of the films can explain why that obtained from the LM silver sol resulted more SERS performant than the other one: the higher absorption of this film at the Raman excitation wavelength (532 nm) favors the resonance condition, allowing to increase the SERS effect, as described in Chapter 1.

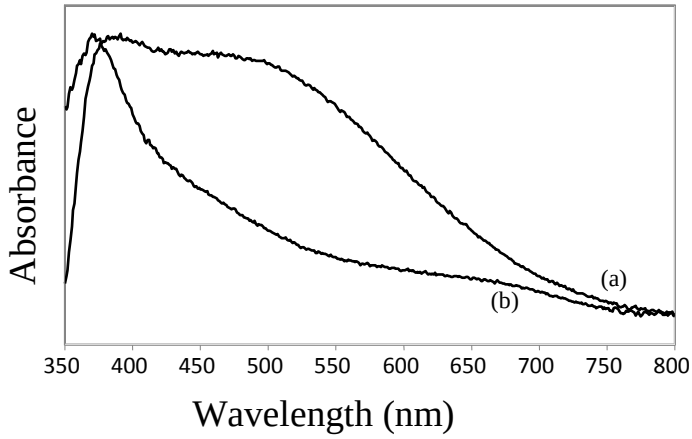

Figure 11. UV-vis reflectance spectra of films obtained from (a) 0.1 M Lee and Meisel sol, (b) 0.1 M Leopold and Lendl sol.

Eventually, it should be noted that an interesting aspect of the silver films obtained from colloids is that they peeled off much less than nanoislands, a desirable feature for application on works of art, even if a careful evaluation of the possible contamination of artefacts with silver should be carried out. To this aim, as a

preliminary study, we analyzed by SEM-EDX those wool threads that were put in contact with the silver films. Analyzing large areas of threads, between 600 x 600 μm and 3 x 3 mm, no silver could be detected by EDX analysis (see for example Figure 12), while sulfur present in wool was obviously identified.

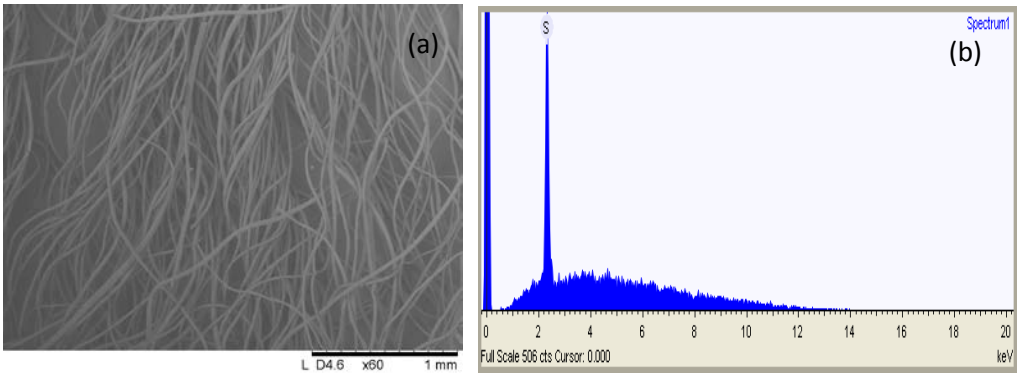

Figure 12. (a) SEM image of an area of wool thread put in contact with silver film, (b) EDX analyses of the same area.

At higher magnifications, however, we recognized on textile fibers few aggregates of silver nanoparticles with a mean diameter comprised between 1 and 3 μm, as exemplified in Figure 13. Even if the dimensions of the particles are very small, the oxidation of silver may originate issues related to the tarnish of the metal, with the result of a change in the color of art objects. The contamination of artifacts therefore

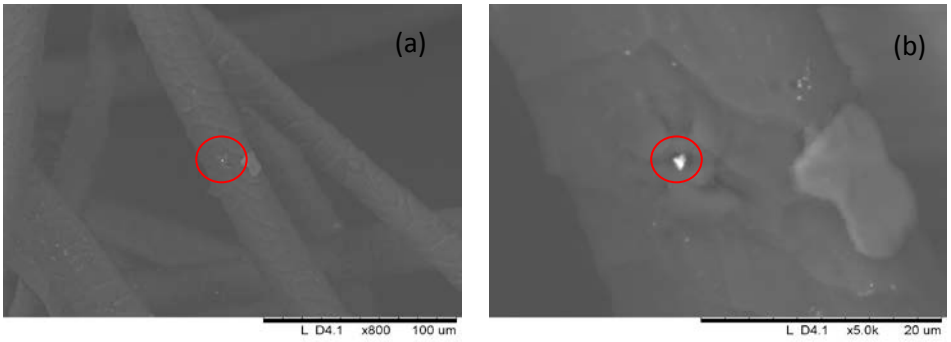

Figure 13. SEM images of a wool thread in contact with silver films fabricated with the Lee-Meisel silver sol: (a) silver particle on the fiber, (b) magnification of the same particle.

is another important parameter to consider in this kind of approach towards non-destructive SERS and, even if passing from nanoislands to films obtained from colloids, the contamination is surely reduced, further studies in order to avoid any deposition of metal on valuable objects need to be performed.

Conclusions and future directions

The possibility to adopt a "dry-state SERS" approach exploiting the interactions between a solid nanometallic substrate and the molecules of dyes present on textiles was positively verified. The study started with the exploitation of the most used solid nanoplatform for SERS analyses, i.e. Ag nanoislands, and approached to the fabrication of new SERS-active nanosubstrates.

Several aspects will be the object of future studies in order to further improve the reproducibility of the fabricated silver films. Indeed, in this context, it is worth highlighting two different aspects: the limited reproducibility of silver colloids obtained with the Lee-Meisel procedure[35] and the variables involved in the fabrication of this kind of nanosubstrates. While the first parameter is not avoidable, the latter ones can be further investigated to achieve a better control of the process. The fabricated nanosubstrates will be also properly characterized.

Moreover, the role of the mordanting agent in the obtainment of "dry-state SERS" from dyed textiles will be studied, as well as the suitability of the protocol of analysis for the identification of dyes belonging to different molecular classes from that of anthraquinones tested in this preliminary study.

Particular attention will be devoted to the development of suitable methods to both assess and avoid the possible contamination of artefacts with the nanomaterials needed for SERS analyses. For example, in order to try to make "dry-state SERS" analysis less harmful for textiles, the use other metals, more tarnish resistant, such as gold, to produce the nanostructured films should be tested. Alternatively, films prepared by attaching metal nanoparticles to glass slides derivatized with

mercaptosilane compounds are reported in the literature (see for example refs. [19, 36, 37]). The application of these latter nanodevices to “dry-state SERS” analyses should be verified, as the presence of organic compounds directly linked to silver nanoparticles could lead to the obtainment of a prevailing SERS response from mercaptosilanes with respect to dyes molecules.

Particularly interesting is the fact that, being the sample area under investigation very small (ca. 10 µm), the dimensions of the support on which the metallic substrate is deposited could be reduced to the diameter of a fiber optic: in this way the non destructive approach could be suitable also for *in situ* analysis, a very important feature for some particular art objects conserved in museums. Ultimately, the “dry-state SERS” approach could open a new window of opportunity towards non destructive SERS.

Acknowledgments

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CONCLUSIONS

The present doctoral thesis work aimed to improve pre-existing analytical methods as well as to develop innovative and effective procedures for the identification of natural organic dyes of artistic and historical interest, based on the exploitation of a rather new spectroscopic tool, such as surface-enhanced Raman scattering (SERS).

In particular, the work carried out allowed to:

- investigate the SERS response of molecules never characterized by means of this technique, such as anthocyanins. In more detail, the SERS characterization of pure anthocyanidins, i.e. anthocyanins without the glycosidic moiety, was intended to provide a contribution to the enlargement of searchable databases of reference materials, so important for the identification of unknown compounds in art objects. The work provided also an original study of the dependence on the pH of SERS spectra of these molecules and the development of experimental procedures for the identification of anthocyanin-based dyes used in antiquity both from plant sources and textiles, as well as the validation of the possible identification of anthocyanins in faded textiles, carried out analyzing artificially aged textiles.
- acquire new spectral data on *folium* dye, obtained with different analytical techniques, providing also new starting points for the analysis of this complex dye.
- overcome some limits of SERS, such as its feature of non-separative technique, with the development of a coupled HPLC-PDA-SERS system for the identification of natural dyes historically used. The optimization of several parameters influencing the hyphenate HPLC-SERS technique including the aggregation of the colloid, the efficiency of the mixing between the eluted analytes and the silver sol, the construction of the elution gradient and the pH of the eluted solution, was carried out. Moreover, the HPLC-PDA-SERS system was validated analyzing both reference dyes and complex samples of different origin, such as the extract from the animal

source of kermes dyestuff and an ancient dyed thread, demonstrating the effectiveness and the stability of the method.

- overcome the need of extraction of dyes from art objects, with the development of an extractionless FT-SERS protocol of analysis for the identification of natural dyes in ancient textile fibers. The samples' dimensions were decreased until a single fiber, making the protocol of analysis micro-destructive. Moreover, a new database of FT-SERS spectra of the most common dyes used in antiquity was acquired. Notably, the in the same project, the issue of fluorescence, commonly related to ancient dyed samples was taken into account, and the work carried out allowed to
- demonstrate that the coupling with SERS of a less-energetic source of radiation with respect to the commonly employed ones, as in the Fourier transform Raman technique, resulted to be especially advantageous for fluorescent dyes, such as brazilwood, that could be identified in an historical sample from a Chinese carpet.
- perform a preliminary assessment of the possibility to carry out SERS using a solid nanometallic substrates in a solid state, in order to start a study possibly leading to the non-destructive application of SERS analysis directly to artefacts. Conventional nanoplatform, such as silver nanoislands, were initially tested and encouraging results were obtained. However, some drawbacks of silver islands led us to
- fabricate new nanometric substrates for solid state SERS analysis based on the modification of the drying process of concentrated silver colloids. Some parameters, such as the type of colloid and its concentration, were taken into account, while new aspects, such as the issue of contamination of art objects, arose.

In conclusion, the present doctoral work introduced solutions to some issues related to the complex samples under investigation, such as ancient dyed art objects with an historical value, and proposed new application of SERS, overcoming its nature of non-separative and destructive technique.

LIST OF PUBLICATIONS

E. De Luca, G. Poldi, M. Redaelli, C. Zaffino, S. Bruni*
"Identification of ancient Chinese dyestuffs used in Ningxia carpets"
Archaeological and Anthropological Sciences, submitted.

C. Zaffino, G. Bedini, G. Mazzola, V. Guglielmi, S. Bruni*
"On-line coupling of high-performance liquid chromatography with surface-enhanced Raman spectroscopy for the identification of historical dyes"
Journal of Raman Spectroscopy, submitted.

L. Bonizzoni, S. Bruni*, G. Fanti, P. Tiberio, C. Zaffino
"Ageing of flax textiles: fingerprints in micro-Raman spectra of single fibres"
Microchemical Journal, submitted.

C. Zaffino*, S. Bruni, B. Russo, R. Pilu, C. Lago, G. M. Colonna
"Identification of anthocyanins in plant sources and textiles by surface-enhanced Raman spectroscopy (SERS)"
Journal of Raman Spectroscopy, in press, DOI: 10.1002/jrs.4786.

C. Zaffino*, B. Russo, S. Bruni
"Surface-enhanced Raman scattering (SERS) study of anthocyanidins"
Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy 149 (2015) 41-47.

C. Zaffino, V. Guglielmi, S. Faraone, A. Vinaccia, S. Bruni*
"Exploiting external reflection FTIR spectroscopy for the in-situ identification of pigments and binders in illuminated manuscripts. Brochantite and posnjakite as a case study"
Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy 136 (2015) 1076-1085.

C. Zaffino, S. Bruni*, V. Guglielmi, E. De Luca
"Fourier-transform surface-enhanced Raman spectroscopy (FT-SERS) applied to the identification of natural dyes in textile fibers: an extractionless approach to the analysis"
Journal of Raman Spectroscopy 45 (2014) 211-218.