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On the identification of *folium* by SERS: from crude extracts to illuminated codices

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The ancient purple dye known as *folium* is still a mystery for both scientists and art historians. Today, it is commonly assumed that *folium* was produced from the fruits of *Chrozophora tinctoria* (L.) A. Juss., a plant belonging to the Euphorbiaceae family, and efforts have been mainly devoted to highlight the analytical features of the dyes extracted from this plant, whereas detection in ancient manuscripts has been mainly based on poorly selective, non-invasive analytical techniques. As a consequence, the possibility that the actual source for the dye could have been so far misunderstood cannot be excluded. Surface-enhanced Raman spectroscopy (SERS), a highly selective and sensitive analytical technique, has been used here to characterize both extracts from *C. tinctoria* and a microsample taken from a medieval manuscript. The behaviour of the dyes as SERS probes has been investigated in order to set up an accurate and selective procedure for the identification of the dye in ancient artworks.

By unambiguously detecting the dye by SERS in the microsample of the medieval manuscript, we also demonstrated that the purple dye mentioned in ancient treatises is definitely linked with the aqueous extract from purple fruits of *C. tinctoria*.

Keywords: SERS; *folium*; *Chrozophora tinctoria*; illuminated manuscripts

Introduction

The identification of the botanical source of the ancient purple dye known as *folium* was achieved by considering the features of the plant in comparison with the descriptions given in the ancient written sources, and it is presently accepted that *folium* was obtained from *Chrozophora tinctoria* (L.) A. Juss., a plant belonging to the Euphorbiaceae family. The plant has been possibly known at least since Roman age, as Pliny the Elder, in his *Naturalis historia*,[1] mentions a plant called *Heliotropium tricoccum* characterized by trilobate fruits, which are typical of *C. tinctoria*. The terminology used for citing the plant or its extract is rather ambiguous and varies from time to time and according to local traditions. The most common terms for the plant have been *heliotrope*, *tournesol*, *morella* or *maurelle* or *maurelle des teinturiers*, *croton* and *ricinoide*.

The dyes are contained in the fruits, specifically in the outermost layer of the pericarp (the epicarp). Neither the seeds nor the layers which directly surround them (endocarp and mesocarp) carry the coloured molecules. Extraction of the dyes, purification and transformation into a painting or a dyeing material are described in some technical treatises at least since Early Middle Ages.[2–6]

Despite this well-known information, the use of *folium* has been identified very rarely in artworks. The scientific literature reports the identification of *folium* by Guineau[7] on some 9th–11th century manuscripts by means of Ultraviolet–Visible diffuse reflectance spectrophotometry and by Roger[8,9] on two purple codices with the same technique, while a tentative identification was proposed by Thomas and Flieder in the analysis of a 6th century purple codex by means of gas chromatography–mass spectrometry.[10]

Some information on the chemical nature of the extract from *C. tinctoria* was provided by Guineau,[7] Wallert[11] and Krekel,[12] although they did not report a definite structure for the purple dyes.

Recently, our team provided further diagnostic information on the purple dyes extracted from *C. tinctoria*, upon preparation of model samples of dyed (or painted) parchment and their analysis with different spectroscopic techniques.[13] The identification of some spectral features on model samples of purple parchment by means of fibre optic Ultraviolet–Visible diffuse reflectance spectrophotometry with optic fibres (FORS) and spectrofluorimetry allowed us to suggest the identification of *folium* in some medieval illuminated manuscripts.[14] Raman spectra of extracts from *C. tinctoria* obtained by means of Fourier-transform (FT) Raman spectroscopy and by surface-enhanced Raman spectroscopy (SERS) with silver colloidal pastes[13] were different from the one obtained with FT Raman spectroscopy by Edwards and Benoy[15] who tentatively attributed to *folium* a blue colorant identified in the *Madonna and Child* tondo painting by de Brécy.

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It is evident that the lack of diagnostic information has prevented the straightforward identification of *folium* in artworks and in ancient manuscripts in particular. For these precious artefacts, in fact, only a microscopic amount of sample is normally (if ever) available.

This issue can be advantageously addressed by SERS, thanks to its high specificity, huge sensitivity and very low consumption of sample. Using different substrates, Raman signals are amplified and luminescence is depressed. As a consequence, microsamples can be investigated under the microscope of a micro-Raman apparatus employing laser sources in the visible spectral range. Several publications recently contributed to the development of an ideal database of SERS spectra of a wide range of dyes detected in paintings and textiles.[16–29] By considering the whole of the published results, it is evident that the spectroscopic features of the target molecules can be (at least partially) influenced by the complex analytical matrix; therefore, the relatively large number of published data also gives an idea of different situations that can be encountered when tackling real samples.

As for *folium*, the available information is presently limited[13]; therefore, in this work, the behaviour of *folium* as a SERS probe has been considered throughout in order to select SER signals that can be unambiguously linked to the presence of dyes from *C. tinctoria* in microsamples detached from ancient manuscripts.

To this aim, extracts from fruits of *C. tinctoria* at different stages of maturation have been obtained; after that, two semi-preparative chromatographic separations (C18 reverse phase column followed by size exclusion chromatography) have been performed, slightly modifying the procedures reported by Krekel,[12] in order to test the homogeneity of the dye composition. Several fractions with different chromatic and size features were obtained after the chromatographic separations, and their SER spectra were collected under 633-nm excitation. Finally, a microsample taken from a medieval manuscript previously investigated by FORS was considered for SERS. The codex (ms F.I.4) is held at the Biblioteca Nazionale Universitaria in Torino (Italy). It is an *antiphonarium* composed in the Bobbio abbey in 14th century. In this manuscript, *folium* appears to be used in the delicate filigrees decorating some capital letters (Fig. 1).

The overall work also allowed us to observe peculiar changes in the colour of the extracts from *C. tinctoria* which are discussed here in the frame of the information known from ancient technical treatises.

Experimental

Ultra-high quality (UHQ) water, employed throughout the experimental work, was obtained by a Millipore (Darmstadt, Germany) Direct-q 3 system. The metal ions for the study of complexes with *folium*, with the exception of Sn(IV) purchased as chloride, were purchased as nitrates from Sigma-Aldrich (Schnelldorf, Germany). Details on other chemicals are reported in each specific paragraph.

Extraction of the dyes

Although the dye is present only in epicarps and not in mesocarps nor in endocarps, we chose to use whole pericarps for practical reasons. Pericarps of *C. tinctoria* (L.) A. Juss. from Sardinia (Italy) were soaked in UHQ water at room temperature for 1 h; the extract (about 100 mL per batch) was filtered, and the solution was allowed to dry overnight at room temperature in an open Petri dish. The recovery of the dye, calculated with respect to the initial weigh of the extracted pericarps, was ~17% w/w. The same extraction method was used for seeds, although the dye is not present in them.

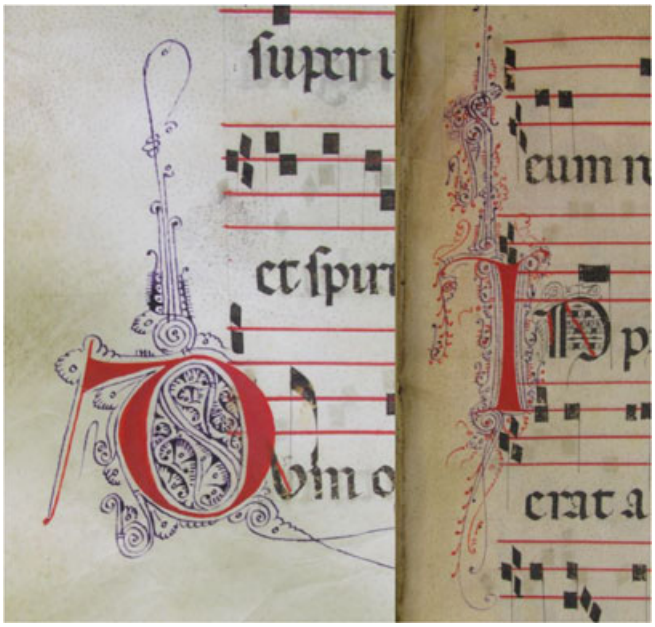

Figure 1. Examples of purple filigrees from ms F.I.4 (Biblioteca Nazionale Universitaria, Torino). [Colour figure can be viewed at wileyonlinelibrary.com]

Chromatographic separation

Solvents for chromatographic separations (e.g. methanol and formic acid) were purchased from Sigma-Aldrich (Schnelldorf, Germany). In order to obtain fractions of the dye with specific features, an aqueous extract was prepared from 20 g of purple pericarps in 200 mL of UHQ water. After concentrating the extract to a little volume (~2 mL), this was loaded onto a semi-preparative C18 column and separated with a water/methanol 40/60 w/w mobile phase with 0.1% formic acid in an isocratic elution. The deep-purple fraction, which was recovered among others during the elution, was then subjected to size exclusion chromatography on an LH-20 column with water/methanol 20/80 w/w mobile phase with 0.1% formic acid in an isocratic elution.

Sampling from the manuscript

A microsample of purple paint was detached by means of a scalpel. Figure 2 shows it under the magnification of an optical microscope, in order to give a clear indication on its actual dimensions.

SERS analysis

All the reagents and solvents (e.g. nitric acid, hydrochloric acid, methanol, silver nitrate and sodium citrate dihydrate) were purchased from Carlo Erba Reagents (Arese, Italy). SERS analysis was performed by means of Ag colloidal pastes, according to the procedure described elsewhere,[30,31] based on the Lee and Meisel reduction of silver nitrate.[32]

Samples considered for SERS were the following:

- dry extracts from unripe blue pericarps;
- dry extracts from ripe purple pericarps;
- dry extracts from seeds;
- dry fractions obtained after elution on the C18 column;

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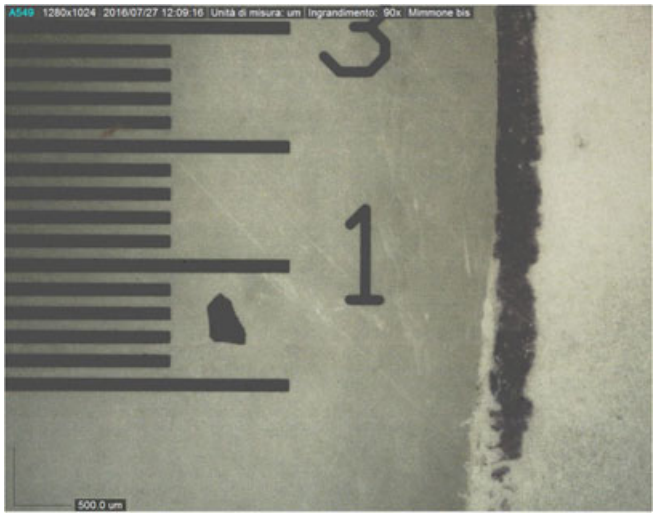

Figure 2. ×90 image of the sample taken from ms F.I.4. [Colour figure can be viewed at wileyonlinelibrary.com]

- dry fractions obtained after elution on the LH-20 column; and
- microsample from a 14th century manuscript.

None of the considered samples gave significant spectra in conventional Raman mode; therefore, SERS was employed in all instances.

Dry extracts and fractions were dissolved in UHQ water prior to the SERS tests. Silver colloidal paste (0.5 μL) was dropped on 0.5 μL of the solution under investigation and mixed with the tip of the pipette; the mixture was allowed to dry before exposing it to the laser beam.

As for the manuscript, SERS analysis was performed directly on the sample, by pouring on it 1 μL of Ag colloidal paste. Also in this case, the mixture was allowed to dry before exposing it to the laser beam.

Surface-enhanced Raman spectra were collected with a high-resolution dispersive Horiba (Villeneuve d'Ascq, France) LabRAM HR model spectrophotometer coupled with a confocal microscope. The instrument was equipped with a 633-nm excitation laser, two (600 and 1800 lines/mm) dispersive gratings, an 800-mm path monochromator, and a Peltier cooled charge coupled device detector; the 600 lines/mm grating was used preferentially. The optical arrangement gave a spectral resolution of about 2 cm^{-1}. Spectra were taken by placing the samples on the microscope stage and by observing them with long working distance (×20, ×50 and ×80) objectives. The sampled area was identified and focused by using either a video camera or the microscope binoculars. Laser power at the sample was kept very low (30 μW) by means of a series of neutral density filters, in order to prevent any thermal degradation of the molecules. Exposure time was set between 1–120 s according to the specific needs of the analytical spot, with three accumulations for each spectrum. The system was managed with LABSPEC 5 software running under Windows XP.

Results and discussion

Folium rubeum, purpureum, and saphireum

In Theophilus' *De diversis artibus*,[2] which is one of the most important bibliographic sources for the history of art techniques, the author reports the possibility of preparing three different colorants from *folium*, according to its behaviour at acid, neutral and basic conditions. He calls them *folium rubeum* (red), *folium purpureum* (purple) and *folium saphireum* (blue) respectively.

In this study, we verified that this information is not correct when extracts from *C. tinctoria* are considered. Actually, blue and purple extracts are obtained from unripe and ripe fruits respectively. No colour variation is observed when purple solutions of ripe fruits are treated with acids or with bases, whereas the blue solution from unripe fruits turns rapidly (and irreversibly) to purple upon addition of a few drops of diluted HCl. Moreover, the blue solution from unripe fruits turns spontaneously to purple after about 48 h of stirring in air (whereas the transformation does not occur if the solution is stirred under a nitrogen flow).

It is therefore apparent that the purple colour represents the most stable form of the dye and possibly the one which can be found on artworks. Moreover, it is evident that the colour change from blue to purple is due to an irreversible chemical transformation - which occurs in oxic conditions and is catalysed by protons - and not to acid/base equilibria.

Interestingly, various colours can be obtained upon addition of some metal ions to a purple extract. Even if no recipe is known about the preparation of a folium lake, i.e. by precipitating the dye on an inorganic support, we chose to explore this aspect by using metals that could have been actually present as contaminants in the preparation of the dye. Fe(III), Al(III), Zn(II), Pb(II), Cu(II) and Sn(IV) ions were mixed with purple extracts. The results were as follows:

- Cu(II) gave a blue solution, possibly due to the colour of the cation itself in an aqueous solution.
- Zn(II) and Sn(IV) produced grey and light purple–red solutions respectively.
- With Pb(II), which provides a dark grey solution, and with Sn(IV), a precipitate started to settle.
- Fe(III) and Al(III) were the most interesting species because their mixtures with the purple extract gave a red colour.

No colour change, on the contrary, occurred upon addition of Ca^{2+} which could resemble the use of lime in the preparation of clothlets. Indeed, the main observed interaction between Ca^{2+} ions and the dye should be provided by electrostatic nature and not by coordination bonds that are related to changes of colour in the solutions treated with the other metal ions cited.

In the end, we can hypothesize that *folium saphireum* and *folium purpureum* are obtained from unripe and ripe fruits respectively; *folium rubeum*, instead, may have occurred either as a result of contamination in the preparation of the *folium* dye with iron tools or, more probably, by the intentional addition of an excess of an iron salt or of alum to the purple extract.

The behaviour of extracts from *C. tinctoria* when mixed with metal ions is definitely not unusual. Natural organic ligands often establish metal complexation via carboxylic groups. However, the highly polar nature of these moieties does not provide in Raman techniques the strong experimental results of FT-IR analysis.[33]

SER examination of extracts from blue and purple pericarps

Besides, it is unlikely that *folium saphireum* from *C. tinctoria* could have been actually used in artworks (blue turns rapidly and irreversibly to purple, and it is very unlikely that medieval artists were not aware of this behaviour), it is interesting to compare SER spectra of blue and purple extracts, trying to identify the differences in peak positions, therefore gaining information potentially useful for the characterization of the molecule.

The spectrum of the blue extract (Fig. 3, top) is characterized by a strong band at 708 cm^{-1} with a shoulder at 685 cm^{-1} and additional spectral features at 369, 439, 539, 831, 1318, 1408, 1459, 1589 and 1653 cm^{-1}. Figure 3 (middle) reports also a spectrum obtained from a blue extract left 2 days in an open beaker, so that it eventually turned to purple. After exposing the blue extract to air, its spectrum loses the components at lower Raman shifts (369, 439 and 539 cm^{-1}) and presents only a very weak signal at 504 cm^{-1}. The spectrum in this region is dominated by a signal at 635 cm^{-1} (with a shoulder at 579 cm^{-1}), which was present in the spectrum of the blue extract only with a very low intensity, while the peak at 708 cm^{-1} is decreased to a small band at 711 cm^{-1}. In the 1700–1300 cm^{-1} interval, the signals at 1644 cm^{-1}, which exhibits a shoulder at lower Raman shift, might correspond to a shift of the 1653 cm^{-1} peak in the blue extract spectrum. The component at 1463 cm^{-1} presumably coincides to the 1459 cm^{-1} band in the blue extract spectrum, while all the other components observed in the spectrum of the blue extract are missing. Finally, the signal at 930 cm^{-1} is imputable to the Ag colloidal paste. The spectrum of the purple extract shows a significant similarity compared with the spectrum of the blue extract after exposure to air. In more detail, the main components in the 1700–1300 cm^{-1} range are the peaks at 1640 and 1462 cm^{-1}, which should correspond to the signals at 1644 and 1463 cm^{-1}, while the less intense band at 1338 cm^{-1} has no noticeable correspondence. In the lower Raman shift region, the small peak at 711 cm^{-1} is not present, the signal at 637 cm^{-1} (635 cm^{-1} in the spectrum of the air exposed blue extract) is even more intense, the shoulder at 579 cm^{-1} is converted in a peak at 580 cm^{-1}, and the small signal at 504 cm^{-1} appears as a strong band at the same Raman shift.

It is interesting to note that the same spectrum is obtained from a blue extract turned to purple after exposure to air and from a blue extract turned to purple after acidification: It seems like the blue dye could change to purple according to both oxidation and acid hydrolysis mechanisms.

A SER spectrum of a *folium* purple extract was already reported by Aceto *et al.* in a previous publication.[13] Comparing the already published spectrum with the spectrum reported in the present paper, a few differences are immediately evident. Indeed, in the present work, the acquisition of a definitely higher number of spectra on different purple extracts of *folium* evidenced the occasional presence of other signals in addition to those illustrated in Fig. 3. On the other hand, the examination of the spectra led us to select here the spectroscopic pattern with the highest reproducibility and statistical significance to be used for diagnostic purposes.

Even if the reported spectra here do not enable an unambiguous interpretation of the molecular structures of the involved species, it is apparent that the molecules corresponding to the blue extract on the one hand and to both the blue extract after air exposure and the purple extract on the other hand must have significant differences. These dissimilarities should be ascribable to chemical transformations that occur when the blue extract is left to air. In the blue extracted dye, the supplementary spectral features may be related indeed with a supplementary assembled aromatic moiety. The strong peak at 708 cm^{-1} can be actually linked to an aromatic γC–H out-of-plane bending mode, and the multiple peaks in the 1650–1300 cm^{-1} range can be vinylic or aromatic νC=C stretching modes and mixed aromatic νC=C stretching and δC–H in-plane bending modes.[34,35] The peak at 1338 cm^{-1} can be tentatively assigned to a C–H bond vibrational mode (a CH symmetric deformation in an alkane) or to a $(C{=}O)O^{-}$ symmetric stretching in a carboxylic acid. The peaks at 637, 580 and 504 cm^{-1} can be attributed to ring deformation modes. Based on this interpretation, an aromatic structure with carboxylic acid and/or ester functionalities might be hypothesized.

Also, it is noteworthy that the SER spectra of both blue and purple extracts differ significantly from those reported by Zaffino *et al.*[36] in their characterization of anthocyanins from plant sources. In the past, some authors hypothesized that the main constituents of *folium* could be anthocyanins,[37] but our results compared with those of Zaffino *et al.* seem to exclude this hypothesis.

SER examination of extracts from seeds

The trilobate fruits of *C. tinctoria* contain three seeds. The aqueous extract from seeds is colourless, so that this part does not

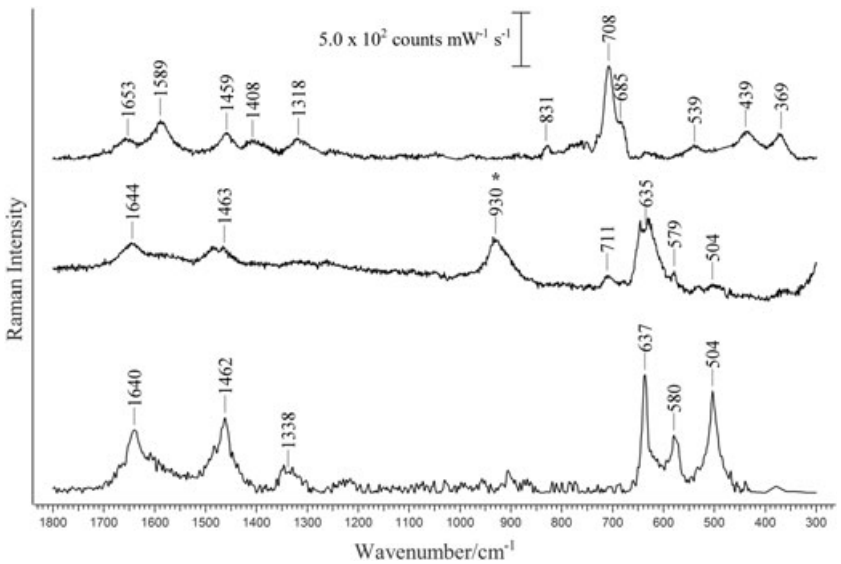

Figure 3. SERS spectra of fresh blue extract (top), of blue extract after oxidation by air (middle) and of purple extract (bottom). Spectra are offset for clarity. The peak at 930 cm^{-1} evidenced with an asterisk in the middle spectrum is ascribable to the Ag colloidal paste.

contribute to the overall hue of *folium*; nevertheless, SER features of the extract were also considered because in the preparation of the dye upon extraction in water of the fruits, the seeds could have been extracted as well (if the extraction is performed on whole fruit rather than on pericarps only). The SERS spectrum of an aqueous extract from seeds (Fig. 4) shows bands occurring at 698, 742, 880, 1336, 1459, 1508 and 1548 cm^{-1}. No signals appear to be present at higher regions. The comparison with the spectra of Fig. 3 highlighted an almost complete lack of correspondence between the spectral pattern obtained from the aqueous extract from the *C. tinctoria* seeds and the spectra registered on the extracts from the blue and the purple pericarps. More in detail, the signals at lower Raman shift do not show any analogy, while in the 1700–1300 cm^{-1} range, the only accidental similarities could be recognized in the 1336 cm^{-1} signal, which can find a correspondence in the 1338 cm^{-1} peak in the spectrum of the purple extract and in the 1459 cm^{-1} band, which is also recognizable in all the three spectra of Fig. 3 (at 1459, 1463 and 1462 cm^{-1}, from top to bottom).

SER examination of chromatographic fractions (from C18 separation)

In order of elution, the following fractions were isolated by the semi-preparative C18 column: a yellow fraction, an orange fraction and eight purple fractions. The SERS spectra corresponding to the yellow and orange fractions are shown in Fig. 5. The spectrum of the yellow fraction definitely shows the most complex pattern, with signals at 459 (sh), 476 (m), 513 (m), 563 (m), 591 (m), 662 (vs), 719 (m), 784 (vw), 835 (w), 890 (m), 964 (sh), 981 (w), 1024 (m), 1069 (w), 1160 (w), 1217 (m), 1277 (m), 1318 (s), 1398 (sh), 1419 (m), 1464 (m),

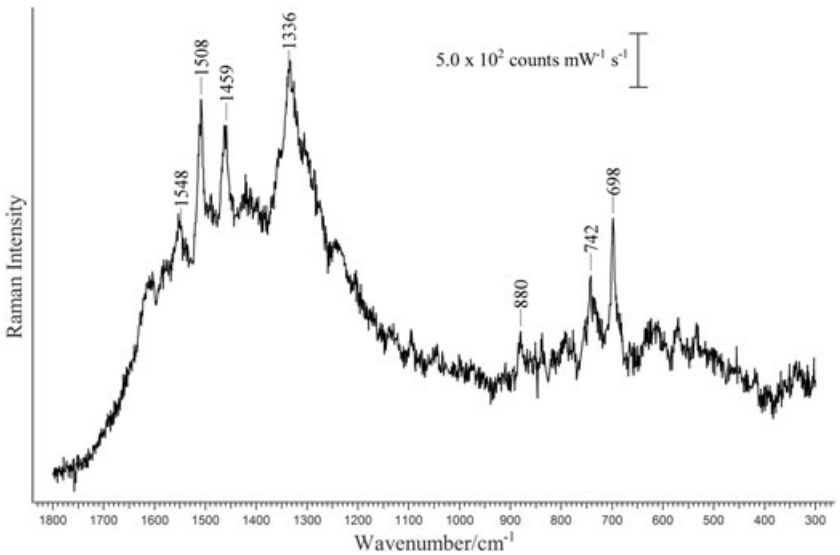

Figure 4. SERS spectrum of an aqueous extract from *C. tinctoria* seeds.

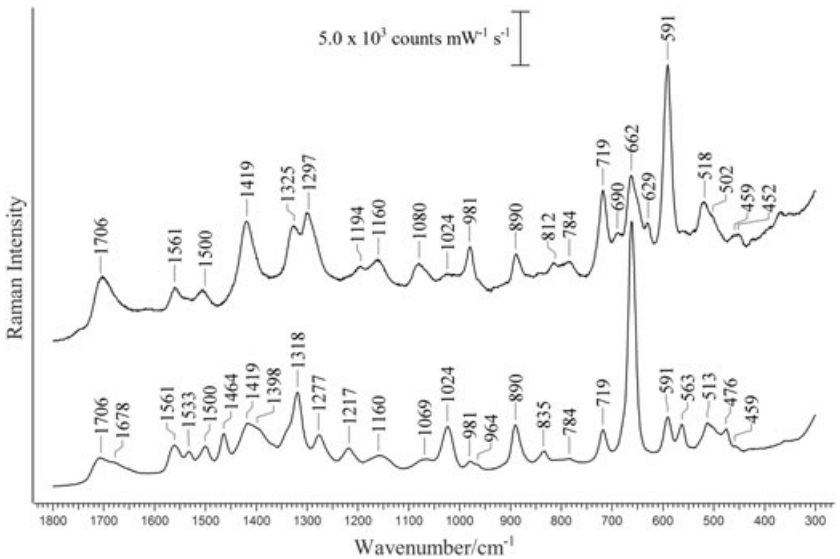

Figure 5. SERS spectra of yellow (bottom) and orange (top) fractions obtained upon separation of a purple extract on a C18 column. Spectra are offset for clarity.

1500 (m), 1533 (w), 1561 (m), 1678 (sh) and 1706 (m) cm^{-1}. The spectrum of the orange fraction is indeed less structured, but it exhibits several similarities with the spectrum of the yellow fraction, namely the peaks at 591, 662, 719, 784, 890, 981, 1024, 1160, 1419, 1500, 1561 and 1706 cm^{-1}. On the other hand, a few bands are not common with the other spectrum, and some of the peaks are shifted with respect to the spectrum of the yellow fraction. More in details, two small components appear at 452 and 459 cm^{-1}, the peak at 513 is shifted to 518 cm^{-1}, two new bands are present around 629 and 690 cm^{-1}, the signal at 1069 is shifted at 1080 cm^{-1}, a new feature emerges at 1194 cm^{-1}, and the pattern in the range of 1300–1400 cm^{-1} appears completely different, with two peaks centred at 1297 and 1325 cm^{-1}. Moreover, the comparison of these two spectra with the spectrum of the purple extract illustrated in Fig. 3 reveals that the patterns of the two fractions are somewhat different, with only a slight correspondence of the signals around 1460 cm^{-1}.

SER examination of chromatographic fractions (from LH-20 separation)

The fraction bearing the most intense purple colour recovered from the C18 column was subjected to a second semi-preparative chromatographic separation on a gel filtration LH-20 resin. Upon elution, eight fractions were obtained. As all the fractions presented the same features, two of them are shown in Fig. 6 to represent the general picture.

It is apparent that the fractions eluted on the LH-20 column, differing mostly for the molecular weight, contain definitely the same spectral features, which in turn are very similar to those of the crude

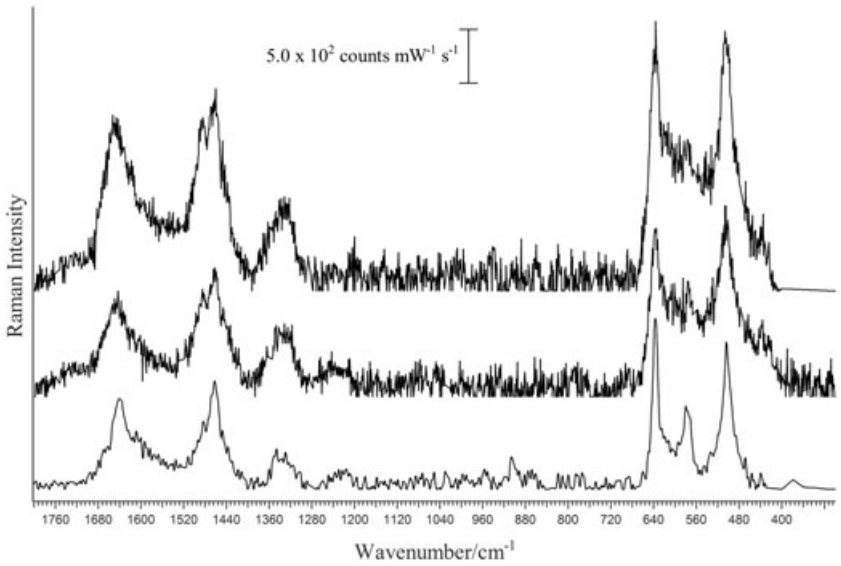

Figure 6. SERS spectra of two fractions obtained upon separation on LH20 column (top and middle) of a purple fraction previously isolated by means of C18 separation. The spectrum of a raw purple extract is shown for comparison (bottom). Spectra are offset for clarity.

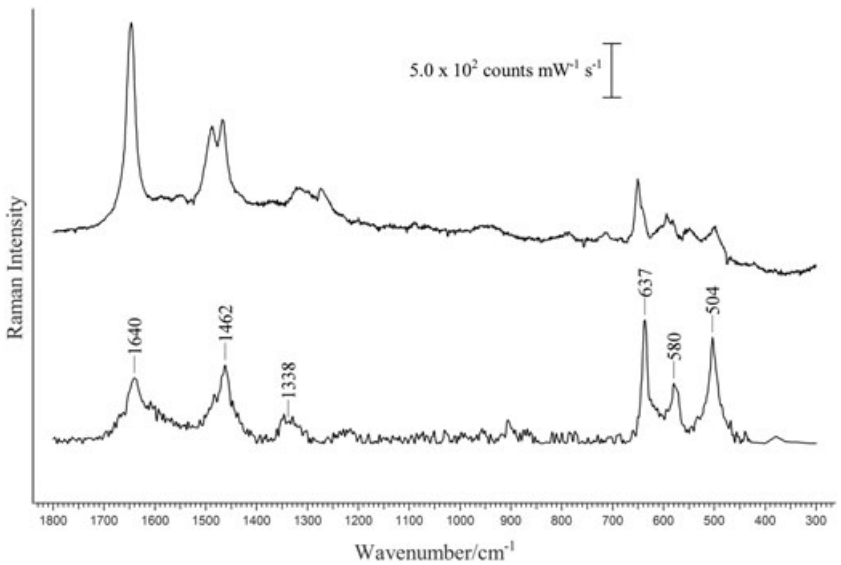

Figure 7. SERS spectra of a purple area from ms F.I.4 (top) and of a raw purple extract from *C. tinctoria* (bottom). Spectra are offset for clarity.

purple extract. The only slight difference is the evident splitting of the peak around 1460 cm^{-1} into two components in the spectra of the two fractions, while the corresponding signal in the pattern of the raw purple extract merely shows a shoulder at higher wavenumbers. This result suggests that the extract from purple fruits of *C. tinctoria* could contain molecules with different molecular weights, characterized by a same core structure, bearing a variable number of substituents, possibly sugars, which is common in plants.

Identification of folium on a 14th century codex

In order to verify the application of SERS in the identification of the *folium* dye on artworks, a survey was made on a large series of illuminated manuscripts, selecting by FORS those possibly containing *folium*.[14] The identification was carried out by using the *apparent absorption* spectral features as described by Aceto *et al.*,[38] according to the bands at approximately 545 and 575 nm.[13] Among the manuscripts analysed, one was selected for sampling according to the criterion of the minimal impact on the decoration of the manuscript itself. These features were found to be compatible with ms F.I.4 (Biblioteca Nazionale Universitaria, Torino), which was sampled and analysed as described in the Experimental section.

A SERS spectrum obtained on the sample is reported in Fig. 7 (top). The spectrum allowed us to definitely confirm the presence of *folium*, according to the main spectral features at 504, 580, 637, 1338, 1462 (split into two peaks as in some of the analysed purple fractions) and 1640 cm^{-1}.

This result definitely links the purple colorant found in the manuscript to *C. tinctoria* and indirectly supports the robustness of the non-invasive detection of this dye in manuscripts.

Conclusions

The systematic investigation of extracts from *C. tinctoria* allowed us to obtain much information on the behaviour of the dye under different pH conditions. In particular, the general belief supported by the information given in the treatise from Theophilus, who claims that *folium* shall behave as an acid–base indicator, was rebutted. The plant can actually produce different colours (namely blue, purple and red). They are related to different maturation stages of the fruits from which the dye is obtained as far as blue and purple are concerned. Red is instead obtained when the purple solution is treated with Fe(III) salts or with the addition of an excess of Al(III) ions.

Surface-enhanced Raman investigation enabled the selection of the most significant signals to be employed for diagnostic purposes for detecting *folium* in microsamples detached from ancient illuminated manuscripts.

The large number of SER tests we made on *folium* indicated that the dye can be considered a 'decent' SERS probe, as the signals are normally weak and signal-to-noise ratio much lower than the one expected for 'good' SERS probes. Nevertheless, *folium* is even a worse Raman scatter, as no signals at all were detected without the silver nanoparticle substrate. Moreover, the compounds are easily decomposed by the laser beam; therefore, the power of the laser shall be kept very low to prevent degradation, and such conditions are detrimental for the intensity of the signals.

Surface-enhanced Raman tests on both crude extracts and purified fractions allowed us to select, among the set of the obtained spectra, the ones showing the signals appearing most frequently, which are therefore more robust for diagnostic purposes. As the dye is not an excellent SER probe, only a few signals are normally detected, although richer spectra can also appear sporadically if many analytical spots are considered. The comparison of the obtained SER spectra here with those obtained from other natural colorants that can produce purple hues such as cochineal,[39] brazilwood,[39] plants bearing anthocyanins,[36] alkanet (unpublished data) and purple dyes from lichens[13] excludes any structural similarity with the dyes from *C. tinctoria*. The structure of the dye-extracted *Chrozophora* is therefore still to be clarified, and presently, we cannot rely on an accurate spectroscopic interpretation of the SER signals to guide the assignment. Nevertheless, the robustness of the selected spectra for detecting *folium* in artworks has been confirmed here by the signals obtained from the 14th century manuscript.

For the first time, we obtained direct evidence through SERS that *C. tinctoria* was actually used to obtain a purple material employed to decorate medieval manuscripts. Moreover, the result obtained by such a selective spectroscopic technique indirectly supports the accuracy of the non-invasive approach by FORS,[13,14] where spectra with a few and broad absorption bands may lack in selectivity.

Author contributions

The manuscript was written through contributions of all authors.

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