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**A diagnostic study on folium and orchil dyes with non-invasive and micro-destructive methods**

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Abstract: Folium and orchil are dyes of vegetal origin. Folium is obtained from the Chrozophora tinctoria plant, whereas orchil is obtained from Roccella, Dendrographa and Lecanora genera of lichens. These dyes were used since long times to impart purple hue to paintings and textiles as substitutes for the more prised Tyrian purple dye, obtained from shellfishes. Despite several citations in ancient technical treatises dating at least to the Greek-Roman age, the identification of these dyes in artworks is rare. In the case of folium, an additional drawback is that its composition is at present unknown.In this work different non-invasive (FT-IR, FT-Raman, Fiber Optic Reflectance Spectrophotometry, spectrofluorimetry, X-Ray Fluorescence spectrometry) and micro-invasive (Surface Enhanced Raman Spectroscopy, Matrix Assisted Laser Desorption Ionization - Time of Flight - Mass Spectrometry, Inductively Coupled Plasma - Mass Spectrometry) techniques were used in order to increase the diagnostic information available on these dyes. Measurements were carried out on the dyes extracted from raw materials and on painted or dyed parchments. The possibility to distinguish among folium and orchil is discussed.

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**To the attention of the Editorial Office of *Spectrochimica Acta Part A***

Dear Editor

Enclosed please find the manuscript "A diagnostic study on folium and orchil dyes with non-invasive and micro-destructive methods" by M. Aceto, A. Arrais, F. Marsano, A. Agostino, G. Fenoglio, A. Idone and M. Gulmini, which is submitted for publication in *Spectrochimica Acta Part A*. In this work, we aim to improve the diagnostic information available for identification of two historical dyes, *folium* and *orchil*, which are known since long times for their use both in painting and in textile art, but whom evidence in artworks is hard to individuate. At present, in fact, the number of their identifications is very low if compared to their frequent citations in old artistic treatises (e.g. *De Arte Illuminandi*, *Stockholm Papyrus* and writings by Theophrastus, Dioskurides and Pliny the Elder among others). These difficulties are most probably connected with the fact that there has been in the past a large amount of ambiguity in identifying the sources of these dyes and frequently they have been mistaken. It is only in the last centuries that folium and orchil have been considered as separate materials and their sources well defined. In the case of folium, a major drawback is the fact that the knowledge of the chemical composition is almost totally unknown; only very few studies have hypothesised its composition but they are largely missing chemical information. Apart from this, the identification of dyes is generally harder than the identification of pigment as far as non-invasive techniques are concerned.

While we are carrying out a thorough study on the composition of the cited dyes, with particular concern to folium, we propose in this preliminary work the application of different analytical approaches for their identification, discussing whether the various techniques used are suitable or not for application *in situ*. The final scope of this study is, in fact, the possibility of identifying these dyes on artworks in the most possible non-invasive way.

Particular concern has been devoted to the historical reconstructions of the studied dyes. This has been achieved following carefully the recipes described in the ancient treatises. Samples of different lichen species (for orchil) and of *Chrozophora tinctoria* (for folium) have been obtained

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from reliable sources, after botanical identification. The obtained dyes have been used to produce samples of parchment painted and dyed with folium and orchil, which constituted the standard references for the spectral investigation.

Among the strong points of our work, we believe the most important be the fact that is presents the first available spectra of folium in most of the spectroscopic techniques used. We hope that these achievements will be useful in further identifications of folium on artworks.

Another relevant point is evidencing the content of bromine in both dyes and their raw materials. Bromine has been considered, up to now, as a key marker for the indirect identification of the highly-prised Tyrian purple dye, due to the molecule 6,6'-dibromoindigotine. Our results highlight that this statement is not valid anymore.

Thank you for your time and consideration.

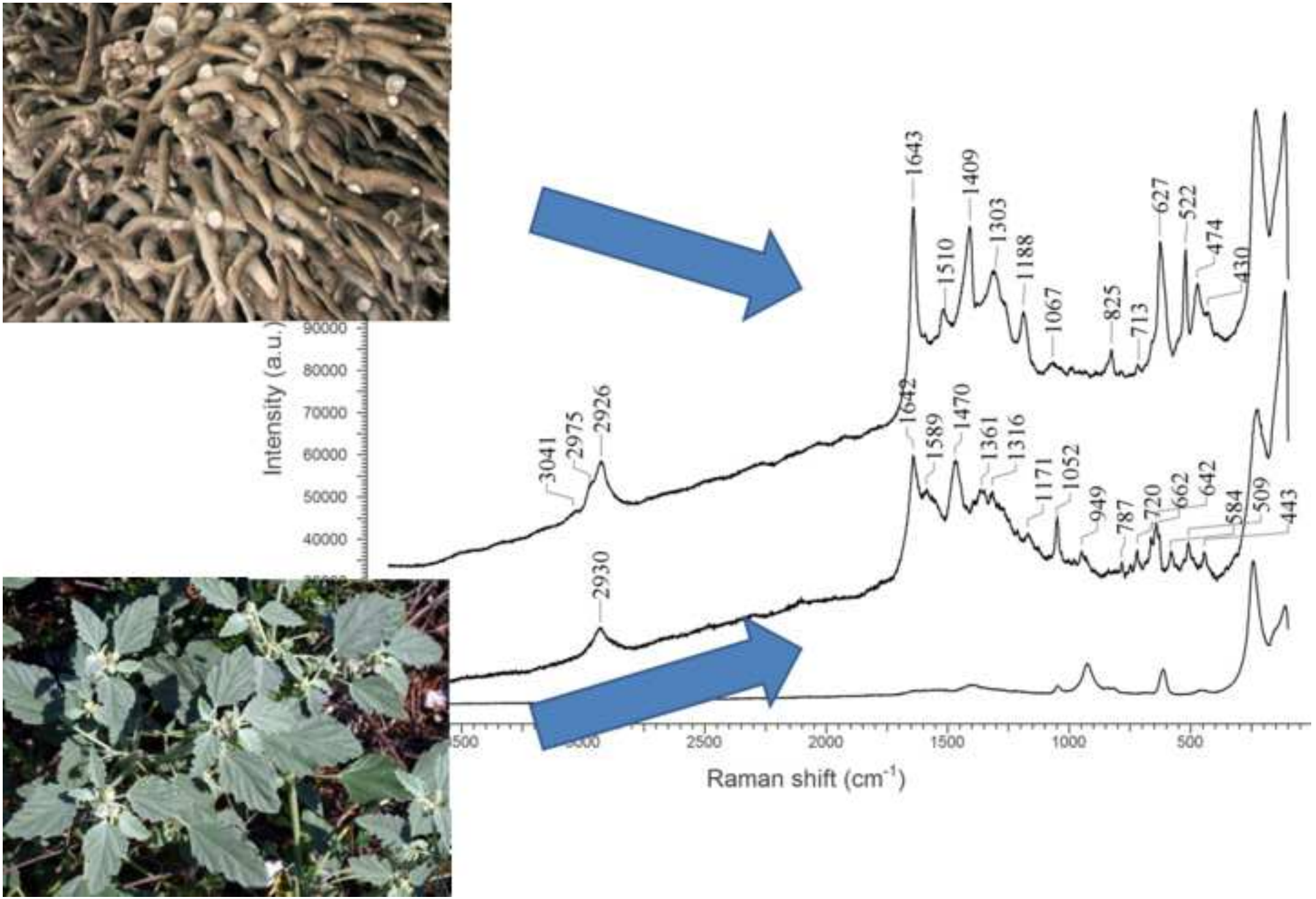
I look forward to your reply.

Yours faithfully,

Dr. Maurizio Aceto and co-authors

Alessandria, 28/11/2014

**Graphical Abstract (for review)**



- Non-invasive and micro-invasive techniques used for folium/orchil identification
- Diagnostic information on these dyes strongly increased
- Historical reconstructions performed in order to have reliable standards
- Evidence that bromine is not a key marker exclusive for Tyrian purple
- Most folium spectral features presented for the first time in a scientific work

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# A diagnostic study on *folium* and *orchil* dyes with non-invasive and micro-destructive methods

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**Abstract**: *Folium* and *orchil* are dyes of vegetal origin. Folium is obtained from the *Chrozophora tinctoria* plant, whereas orchil is obtained from *Roccella*, *Dendrographa* and *Lecanora* genera of lichens. These dyes were used since long times to impart purple hue to paintings and textiles as substitutes for the more prised Tyrian purple dye, obtained from shellfishes. Despite several citations in ancient technical treatises dating at least to the Greek-Roman age, the identification of these dyes in artworks is rare. In the case of folium, an additional drawback is that its composition is at present unknown.

In this work different non-invasive (FT-IR, FT-Raman, Fiber Optic Reflectance Spectrophotometry, spectrofluorimetry, X-Ray Fluorescence spectrometry) and micro-invasive (Surface Enhanced Raman Spectroscopy, Matrix Assisted Laser Desorption Ionization – Time of Flight - Mass Spectrometry, Inductively Coupled Plasma - Mass Spectrometry) techniques were used in order to increase the diagnostic information available on these dyes. Measurements were carried out on the dyes extracted from raw materials and on painted or dyed parchments. The possibility to distinguish among folium and orchil is discussed.

**Keywords**: folium, orchil, solid-state characterisation, Raman, SERS, FORS

## 1 Introduction

The names *folium* and *orchil* are used today to indicate two kinds of dyes of vegetal origin, respectively obtained from the *Chrozophora tinctoria* plant and from *Roccella*, *Dendrographa* and *Lecanora* genera of lichens. These dyes have been in use since long times to impart purple hue to artworks, either dyed or painted; they were in fact mostly used as substitutes for the more priced Tyrian purple, the famous dye obtained from shellfishes. For several centuries, though, folium and orchil were hardly considered as different materials, as the historical terminology used for their description in the technical and artistic literature was confusing. In many medieval manuscripts, similar names were given to lichen dyes and to dyes obtained from *Chrozophora* species. As an example, lichens of *Roccella* species were also known as *tournesol* which is a traditional name used for *Chrozophora tinctoria* plant. It is only in the 19th century that ambiguity was resolved [1,2] and the different origin of these dyes was ascertained.

*Orchil* and its dialectic variants *archil*, *orseille* and *oricello* refer primarily to dyes obtained from *Roccella* species. It was in use at least since Greek-Roman times: literary citations from Theophrastus, Dioskurides and Pliny the Elder are known [3,4] which highlight its role as a substitute of Tyrian purple in dyeing. Pliny the Elder, in particular, suggested that orchil could be used in dyeing of wool textiles as a background where a small amount of Tyrian purple was applied, a procedure known as top-dyeing [5]. Moreover, several recipes in the Greek manuscript known as *Stockholm Papyrus* (3rd century A.D.) recommended the use of dyes obtained from lichens to imitate purple [6]. For what concerns painting, medieval treatises cited orchil as a suitable colourant, such as the manuscript *Ut auro scribatur* [7] where its use is suggested as a paint (not as a dye) to impart purple colour to parchment in purple codices. The composition of orchil, though complex, has been elucidated and reviewed in several studies [8,9]. Lichens contain derivatives of orsellinic acid; after extraction, these compounds are hydrolysed and decarboxylated to colourless orcinol, which is oxidised to orcein upon introduction of ammonia. Orcein is actually made up of a mixtures of phenazone derivatives such as hydroxyorceins, amino-orceins and amino-orceinimines.

Folium is extracted from *Chrozophora tinctoria (L.) A. Juss*, a plant known as *turnsole* or *morella*, native of coastal Mediterranean countries. Interestingly, the German name for turnsole has been for long *lackmuskraut*, a term meaning litmus-herb, where litmus is another dye produced from *Roccella tinctoria* lichens differing from orchil in reason of its polymeric structure. Literary citations concerning the use of *folium* in artworks are found in later manuscripts than those concerning citations of orchil. The first recipes date back to 11th century A.D. but its use in painting can be probably

referred to early Middle Ages [10] since it is cited in the 9th century *Mappae Clavicula* treatise [11]. However, it is highly probable that turnsole was already in use in Roman times. Pliny the Elder [5] in his *Naturalis historia*, book XXII, chapter 29, mentions in fact a vegetal species which he called *Heliotropium tricoccum*. This may refer to three cells in the capsule of the plant, as the characteristic tri-lobed fruits yielding folium dye. Among others, Theophilus in his famous *De diversis artibus* treatise [12] and the anonymous author of the *De arte illuminandi* treatise [13] highlighted the fact that this plant could produce a red, violet or blue dye when berries were extracted respectively with an acid, neutral or alkaline solution: the so-called *folium rubeum*, *folium purpureum* and *folium saphireum*. The name folium, however, is historically referred to the purple-violet phase. The scientific knowledge on the composition of folium is relatively scarce with respect of orchil. Early studies [14] suggested that, according to its properties of changing colour on varying pH, folium could be made of anthocyanin compounds. Other studies [15-17], instead, suggested the similarity between folium and orchil from a compositional point of view. Guineau [10] in his detailed historical and diagnostic study showed results from Time-of-Flight Mass Spectrometry (ToF-MS) analysis which highlighted the presence of orcinol, a compound also present in lichen dyes.

Identifications of folium and orchil on artworks are rare, with particular reference to non-invasive analyses. Orchil was identified by Clementi *et al.* [18,19] by means of fluorescence spectroscopy in some Renaissance tapestries and in purple details of the miniatures of the *Book of Kells* [20], a famous 8th-9th century A.D. manuscript. Using fluorescence spectroscopy and Subtracted Shifted Raman Spectroscopy, the same authors identified orchil on the parchment of the *Bible de Théodulfe* (9th century) [21] for which a similar identification was gained by HPLC by Eveno *et al.* [22]. Aceto *et al.* [23] analysed the parchment of the *Codex Brixianus*, a 6th century A.D. purple manuscript, using UV-visible diffuse reflectance spectrophotometry, spectrofluorimetry and X-ray Fluorescence spectrometry and suggested that both orchil and folium could be present. Recently Bicchieri [24] identified orchil on the parchment of the precious *Codex Rossanensis*, another 6th century A.D. purple manuscript, with UV-visible diffuse reflectance spectrophotometry. Finally, the identification of litmus was carried out by Baraldi *et al.* [25] with Raman spectroscopy on a 17th century painted table. For what concerns folium, the number of identifications is very limited since it can be circumscribed to the pioneering work by Guineau [10] in which the author identified the dye in some 9th-11th century manuscripts by means of UV-visible diffuse reflectance spectrophotometry, to the tentative identification on the *Sinope Gospels* (a 6th century A.D. purple manuscript) by means of GC-MS by Thomas and Flieder [15] and to the tentative attribution to folium of blue areas in the de Brécy *Madonna and Child* tondo painting, analysed with FT-Raman spectroscopy by Edwards and Benoy [26].

From the artistic point of view, the use of folium and orchil in painting is certainly suitable for obtaining a range of hues from red to blue through purple, as described in several medieval artistic treatises. Therefore, despite the very low

number of identifications on artworks, the number of instances in which these dyes could have been used is possibly much larger than the number of actual identifications. Moreover, the overview on the literature reported above highlights that the diagnostic information concerning these dyes is very limited or, as in the case of folium, almost absent. In the present work we aim to increase the diagnostic information available for the detection of folium and orchil by means of those spectroscopic techniques that are normally used in the analysis of painted artworks, with particular concern to illuminated manuscripts; therefore in this study folium and orchil have been subjected to a deep analytical investigation with particular focus on the use of a non-invasive or a micro-invasive approach. In particular, the following non-invasive techniques were considered: Fourier Transform Raman Spectroscopy (FT-Raman), Fourier Transform Infrared Spectrophotometry (FT-IR) both in transmission mode and in Attenuated Total Reflection (ATR) mode, Spectrofluorimetry, UV-Visible Diffuse Reflectance Spectrophotometry with Optical Fibres (FORS) and X-Ray Fluorescence Spectrometry (XRF). In order to assess the accuracy of the non-invasive approach and to gain further information on the dyes, micro samples were analysed by means of Surface Enhanced Raman Spectroscopy (SERS) and Matrix-Assisted Laser Desorption-Ionization Time-of-Flight Mass Spectrometry (MALDI-ToF MS). Finally, additional elemental analysis on lichens and *Chrozophora tinctoria* samples was performed by means of Inductively Coupled Plasma – Mass Spectrometry (ICP-MS). For all the above cited techniques, measurements were carried out both on raw powders and on standard paints and dyes on parchment; the results were compared with non-invasive analyses on some purple and violet painted areas on illuminated manuscripts.

## 2 Materials and methods

*2.1 FT-IR Spectrophotometry*. Solid-state FT-IR Spectra were collected on a FT-IR Bruker (Bruker Optics Inc., Ettlingen, Germany) Equinox 55 spectrophotometer, at 2 $cm^{-1}$ resolution, in anhydrous KBr discs (average of 50-100 scans). Measurements in ATR mode were carried out with a Thermo Scientific Nicolet (Madison, Wisconsin, USA) iN10$^{TM}$ model FT-IR spectrometer equipped with an iZ10 external module bearing a Smart iTR$^{TM}$ diamond ATR Sampling Accessory.

*2.2 FT-Raman Spectroscopy*. FT-Raman measurements were performed with a Bruker (Bruker Optics Inc., Ettlingen, Germany) Vertex 70 spectrometer equipped with a RAM II accessory, a 1064 nm Nd/YAG laser source and a Ge diode detector. Spectral parameters were as follows: 100 mW laser power, 500 scans, and 4 $cm^{-1}$ resolution.

*2.3 Surface Enhanced Raman Spectroscopy (SERS)*. SERS analysis was performed by means of Ag colloidal pastes, according to the procedure described by Idone *et al.* [27]. All the materials employed (e.g. nitric acid, hydrochloric acid, methanol, formic acid, silver nitrate and sodium citrate dihydrate) were purchased from Carlo Erba reagents (Arese,

Italy), while Ultra high quality (UHQ) water was obtained by a Millipore (Darmstadt, Germany) Direct-q 3 system. Citrate-reduced Ag nanoparticles were synthesized by modifying the procedure of Lee and Meisel [28]. Raman measurements were performed with a Renishaw (Stonehouse, Great Britain) inVia micro-Raman spectrometer equipped with a 633 nm laser, 1800 lines/mm grating and a 100x Leica (Wetzlar, Germany) microscope objective to focus the laser beam onto the sample. Power at the samples was kept very low (never exceeding 300 μW) by a series of neutral density filters in order to avoid any thermal damage. Analysis of samples of dyed parchment was performed both directly and upon extraction of the dye. In the first case, 0.5 μL of silver colloidal paste were dropped on the parchment. In the last case, 50 μl of concentrated formic acid were added to a 1 $mm^2$ fragment of parchment and kept at 40°C for three hours; then, 2 μl of extract were mixed with 2 μl of Ag colloidal paste.

*2.4 UV-Visible diffuse reflectance Spectrophotometry with optic fibres (FORS)*. FORS analysis was performed with an Avantes (Apeldoorn, The Netherlands) AvaSpec-ULS2048XL-USB2 model spectrophotometer and an AvaLight-HAL-S-IND tungsten halogen light source; detector and light source were connected with fibre optic cables to an FCR-7UV200-2-1,5x100 probe. In this configuration, light is sent and retrieved with a unique fibre bundle positioned at 45° from the surface normal, in order not to include specular reflectance. The spectral range of the detector was 200-1160 nm; depending on the features of the monochromator (slit width 50 μm, grating of UA type with 300 lines/mm) and of the detector (2048 pixels), the best spectra resolution was 2,4 nm calculated as FWHM (Full Width at Half Maximum). Diffuse reflectance spectra of the samples were referenced against the WS-2 reference tile provided by Avantes and guaranteed to be reflective at 98% or more in the spectral range investigated. Since the correction for blank was not efficient on both extremes of the spectral range, the regions 200-250 and 900-1160 were cut from original spectra in order to show better spectra. The diameter of the investigated area on the sample was 1 mm. In all measurements the distance between probe and sample was kept constant at 1 mm. The instrumental parameters were as follows: 10 ms integration time, 100 scans for a total acquisition time of 1.0 s for each spectrum. The whole system was managed by means of AvaSoft v. 8™ dedicated software, running under Windows 7™.

*2.5 Spectrofluorimetry*. An Ocean Optics (Dunedin, Florida, USA) Jaz model spectrophotometer was employed to measure molecular fluorescence spectra. The instrument is equipped with a 365 nm Jaz-LED internal light source; a QF600-8-VIS/NIR fibre fluorescence probe is used to drive excitation light on the sample and to recover emitted light. The spectrophotometer is working in the range 191-886 nm; according to the features of monochromator (200 μm slit width) and detector (2048 elements), the spectral resolution available is 7.6 nm calculated as FWHM. The investigated area on the sample is 1 mm in diameter. In all measurements the sample-to-probe distance was kept constant to 1 mm (corresponding to focal length) with aid of a small black cylinder inserted on top of the probe, in order also to exclude

contributions from external light. Instrumental parameters were as follows: 2 s integration time, 3 scans for a total acquisition time of 6 s for every spectrum. The system was managed with SpectraSuite™ software under Windows 7™.

*2.6 Matrix-Assisted Laser Desorption-Ionization Time-of-Flight Mass Spectrometry (MALDI-ToF MS).* MALDI-ToF-MS experiments were performed in positive-ion mode on a time of flight (ToF) mass spectrometer Voyager DE-PRO model (Applied Biosystems Italia, Monza, Italy). Desorption/ionization was obtained by using a 337-nm nitrogen laser and the accelerating voltage of +20 kV. To obtain good resolution and signal-to-noise (S/N) ratios, laser power was adjusted to slightly above the threshold and each mass spectrum was generated by averaging 100 laser pulses. The calibration of mass spectra was performed externally using Sequazime Peptide Mass Standard, Calibration mixture 1 (AB Sciex Italia, Brugherio, Italy) and matrix peaks. Sample preparation was carried out as follows:

- Matrix solution: 5 g/L of sinapinic acid solution was obtained with a 1:1 volumetric ratio of acetonitrile to 0.1% trifluoroacetic acid in ultrapure water.
- Sample solution: 5 mg of dye powder were dissolved in matrix solvent to obtain a mother solution.
- Parchment samples: analysis of samples of dyed and painted parchment was performed after extraction of the dye with 50 µl of concentrated formic acid.

The same amounts of matrix and sample mother solution were mixed and then deposited as 1 µl drop on a stainless steel 96-well target and allowed to dry before MALDI-ToF-MS analysis.

All organic solvents (HPLC grade), formic acid and ultrapure water were purchased from VWR, Milan, Italy). Sinapinic acid recrystallized matrix was purchased from LaserBio Labs (Sophia-Antipolis, France).

*2.7 X-Ray Fluorescence spectrometry (XRF).* XRF measurements were performed with an EDXRF Thermo (Waltham, USA) NITON spectrometer XL3T-900 GOLDD model, equipped with Ag tube (max. 50 kV, 100 µA, 2 W), large area SDD detector, energy resolution of about 136 eV at 5.9 keV. Analysed spot had an average diameter of 3 or 8 mm and was focused by a CCD camera, with a working distance of 2 mm. Total time of analysis is 240s. The instrument is held in position with a moving stage allowing micrometric shifts, in order to reach the desired probe-to-sample distance; the stage is laid on a tripod. The obtained spectra have been processed with the commercial software WinAxil, derived by the academic software QXAS from IAEA.

*2.8 Inductively Coupled Plasma – Mass spectrometry (ICP-MS).* ICP-MS was used to determine the amount of bromine and iodine in raw materials, i.e. in scraps of lichens and in parts of *Chrozophora tinctoria* fruits. For this task, 50 mg of sample were subjected to acid digestion with 2 ml of concentrated $HNO_3$ TraceSelect grade (Sigma-Aldrich, Milan, Italy) in a microwave oven. The dissolved sample was diluted to 100 ml with high purity water. The analytical

conditions used for ICP-MS were the same as described in [29]; $^{79}Br$, $^{81}Br$ and $^{129}I$ were the isotopes used for quantification.

*2.9 Extraction of the dyes from raw materials*.

Folium was obtained by extraction of fruits of *Chrozophora tinctoria* from Turkey in cold water at neutral pH for 1 hour; extract was filtered and allowed to dry. Orchil, following the indications by Kok [30], was obtained by extraction of scraps of *Roccella tinctoria* from Canary Islands in 30% v/v ammonia, with frequent stirring to favour introduction of air and oxidation of orcinol to orcein; after 3 weeks the extract was filtered and left at room temperature until dryness.

*2.10 Preparation of painted and dyed parchment*.

Paints and dyes of folium and orchil were prepared following the recipes indicated in ancient treatises and applied on parchment. In particular, a solution with 1 g/ml in gum Arabic and 2 g/ml in sucrose was used as painting medium. Folium and orchil (ca. 0.25 g/ml) were dissolved in the medium and applied on parchment by means of a brush. Parchment was dyed according to the procedures employed for dyeing textiles with mordant dyes. The parchment was soaked for 1 hour in a solution containing the dye and alum (30% by weight with respect to the weight of parchment)

## 3 Results and discussion

The results will be presented and discussed indicating whether they refer to raw powdered dyes or to painted/dyed samples.

*3.1 FT-IR Spectrophotometry analysis*.

The FT-IR spectra of raw orchil (above) and folium (bottom) are reported in Figure 1; for sake of comparison, an offset has been applied along Y axis. The lichen dye is provided with strong hydrophilic features, noticed by three main broad absorptions set at ca. 3400, 1630 and 1000 $cm^{-1}$, which are diagnostic of alcoholic –OH moieties, whilst folium retains a more lipophilic nature, probably determined by an amphiphilic structure, as suggested by the fact that folium is easily extracted in cold water. In both spectral patterns, peaks in the fingerprint regions can be correlated to the normal vibrational modes of aromatic and polycyclic aromatic skeletal frames [31,32]. In particular, the 3050-3000 $cm^{-1}$ region is patterned with the –CH aromatic stretching modes, the 1650-1580 $cm^{-1}$ region with the $sp^2$ C=C aromatic stretching modes, the 1450-1200 $cm^{-1}$ region with the coupled C=C stretching and in-plane CH bending modes and the 900-700 $cm^{-1}$ region with the out-of-plane CH bending modes. In this perspective, reported modes can be observed in both spectral patterns. Noteworthy, the out-of-plane –CH deformation modes, i.e. the $\gamma$(CH) modes, are provided with very

strong infrared intensities (the highest or among the highest of the entire aromatic pattern), and they are by far the most distinctive region of the spectra [31,33,34]. Hence, the pattern of folium, featured with a strong sharp absorption at 872 $cm^{-1}$, can be associated with a main molecular aromatic frame, whilst the pattern of orchil may be correlated to a mixture of different products. In this pattern, $sp^2$ aromatic –CH stretching modes, set at 3000 $cm^{-1}$ or higher wavenumbers, are not observed. However, the experimental result is coherent with the relative intensities of γ(CH) modes, embedded in a spectral profile with the very strong broad absorptions determined by the highly-polar –OH groups. Therein, the peak at 1659 $cm^{-1}$ may be correlated to a conjugated ketonic moiety. The small shoulder set at about 3200 $cm^{-1}$ can be determined by ring-conjugated N-H modes. The –CH stretching mode peaks under 3000 $cm^{-1}$, with the related –CH in-plane deformation modes, in the 1450-1200 $cm^{-1}$ spectral region, are associated to aliphatic ring-substituents. In this context, the overlapping broad profile of peaks between 1450 and 1200 $cm^{-1}$ supports the presence of a mixture of different products. As a whole, the comprehensive spectral pattern matches in appropriate results with orcein-like molecular frames that can be actually extracted and purified from lichen substrates [8]. A similar substance is provided by folium, with less polar groups and with specific hydrophobic features, although a peak at 1746 $cm^{-1}$ is observed which can be determined by ketonic moieties.

The reported spectrum of orchil is substantially in agreement with that shown by Beecken *et al.* [8] while the spectrum of folium, to the authors' knowledge, is the first ever published.

Neither painted nor dyed samples of folium and orchil on parchment yielded a significant FT-IR spectrum in transmittance and ATR modes. Indeed, the corresponding spectra (not reported) were dominated by the spectral features of the parchment and it was not possible to recognise any useful features from the dyes.

*3.2 FT-Raman Spectroscopy analysis.*

FT-Raman spectra of raw orchil (above) and folium (bottom) are reported in Figure 2; for sake of comparison, an offset has been applied along Y axis. Both Raman patterns are generally coherent with results obtained from infrared spectroscopy. In the orchil pattern, different partially overlapping peaks fill the 1450-1200 $cm^{-1}$ region of in-plane –CH deformation modes, which can be associated to a coexisting mixture of different products. As in infrared pattern, in the FT-Raman spectrum of orchil –CH aromatic stretching mode peaks are barely observed. Noteworthy, the strong peaks at 2926 and at 2927 $cm^{-1}$ (with a shoulder at 2864 $cm^{-1}$) for orchil and folium, respectively, supported by the strong signals observed in the in-plane –CH deformation mode region, have to be correlated to aliphatic ring-substituents. In both spectra, sharp peaks (at 1075 and at 1087 $cm^{-1}$) can be associated to –C–O–C- ether groups, whilst weak peaks at 3250 $cm^{-1}$ can be associated to –NH groups.

It is difficult to find in the literature a suitable comparison for the here reported spectra. The spectrum of orchil shows limited resemblance to those reported by Edwards *et al.* [35-37] in their works on the characterisation of substances obtained from lichens (lecanoric acid, parietin, gyrophoric acid, etc.) but these compounds are actually the precursors of orchil and therefore they may not be a correct reference to compare with. A more proper comparison can be carried out with the spectrum of orcein recently published by Zaffino *et al.* [38] which shows similar spectral features. In the case of folium there is no reliable reference to compare with; the spectrum of a blue area, tentatively attributed to folium in a work by Edwards *et al.* [26], largely differs from those obtained here.

Also in this case, the spectra of painted and dyed samples were dominated by the spectral features of parchment, although very few characteristic features of the dyes could be singled out. Peaks occurring at 1271 and 1248 $cm^{-1}$, which can be attributed to in-plane ring stretching and to -CO aromatic ether stretching respectively, appear in folium, orchil and parchment itself, but in the case of orchil the peak at 1271 $cm^{-1}$ is clearly higher. In the spectrum of folium a distinctive peak is the one occurring at 975 $cm^{-1}$, due to ring breathing or to -CH out-of-plane bending; this peak is weak in orchil and it is absent in the spectrum of parchment.

*3.3 SERS analysis*.

The SER spectra obtained from application of Ag colloidal pastes to the raw powdered dyes are shown in Figure 3. They support FT-Raman results with some differences. In the spectrum of orchil the modes at 1643, 1409, 1312, 626 and 522 $cm^{-1}$ are clearly enhanced, while the SER spectrum of folium appears more similar to its FT-Raman spectrum. Noteworthy, the deposition of dye molecules on the heterogeneous surface of colloidal Ag nanoparticles determines broad overlapping peaks and a smoothened vibrational profile [39].

Similar results were obtained by analysing raw dyes and samples of dyed parchment, either as such or upon extraction of the dye with formic acid. Silver colloidal pastes directly spread onto the parchment dyed with orchil were effective in enhancing the signals of the dye, even though their intensity was lower than what observed for the powder sample. In particular, peaks below 800 $cm^{-1}$ were more intense, while weaker signals were found in the 1000-1700 $cm^{-1}$ region. The position of the peaks was in quite good accordance with that of powder orchil: 419 (w), 461 (w), 476 (w), 522 (s), 602 (sh), 619 (s), 630 (sh), 658 (w), 818 (m), 1186 (w), 1410 (m), 1526 (w) and 1645 (m). SER spectra recorded on the parchment dyed with folium presented the stronger signals at 1467, 1483 (sh) and 1641 $cm^{-1}$, with medium peaks at 503, 572 and 640 $cm^{-1}$ and weak peaks at 370, 583 (sh), 595 (sh), 684, 1000, 1033, 1068, 1117, 1289, 1319, 1555 $cm^{-1}$, most of which corresponding to the SER peaks observed for powdered folium. SERS analysis allowed to establish a reliable micro-invasive and micro-destructive procedure for identification of these dyes.

The SER spectrum of orchil is in good agreement with those reported by Leona *et al.* [40] and by Doherty *et al.* [41], while some differences arise upon comparison with the one reported by Rosi *et al.* [21] which was obtained, at any rate, with Subtracted Shifted Raman spectroscopy. On the other hand, the only comparison available in the literature for folium is the above mentioned FT-Raman spectrum obtained by Edwards from the blue areas in de Brécy *Madonna and Child* tondo painting [26]. To the authors' knowledge, this is the very SER spectrum of folium ever published, together with the FT-Raman spectrum reported above.

*3.4 FORS analysis.*

FORS spectra were acquired in reflectance mode and transformed in Log(1/R) in order to obtain *apparent absorbance* coordinates and to better appreciate the absorption spectral features (Figure 4). The spectra from painted and dyed parchment samples were identical, as already evidenced before [23]. FORS spectra of folium and orchil are rather similar and characterised by a large absorption band structured in two sub-bands. The absorption maxima of folium are located at ca. 546 and 577 nm, while those of orchil occur at ca. 544 and 588 nm.

For what concerns folium, the spectral features are in good agreement with those reported by Guineau [10] and by Clarke [42] which are, to the authors' knowledge, the only references available in the literature. Noteworthy, folium extracts with metal ions (e.g. aluminium, copper, iron, lead, tin, zinc) can provide alterations of the absorption profile, reasonably due to metal chelation (Unpublished results); these metals, with particular concern to iron, might well be available during the procedure of extraction and preparation of the dye, considering the tools involved into it. Even in the case of orchil we have scarce references to compare with: Clementi *et al.* [18] published spectra of orchil in acetonitrile, ethanol and aqueous solutions; in the last case they reported a marked red shift of the maxima in alkaline solution, which are hardly comparable to those found in our solid-state spectra.

FORS analysis appears to be the most reliable totally non-invasive method, among those cited in this work, for identification and discrimination of folium and orchil.

*3.5 Spectrofluorimetry analysis.*

The fluorescence spectra were registered using a 365 nm LED source. Emission spectra are shown in Figure 5; the spectrum of the underlying parchment is also reported for comparison. Again, we found no differences in the spectra from painted and dyed parchment samples [23]. The spectrum of folium is dominated by a band at 595 nm with a shoulder at ca. 625 nm. In this case also, we have no previous data to compare with. For what concerns orchil, the spectrum obtained has a neat peak at ca. 625 nm which well compares with spectra reported in the literature, for example in the work by Rosi *et al.* [21] and references therein. According to the spectral features exhibited by the two dyes, spectrofluorimetric analysis with the setup used in this work could be selective enough to allow distinguishing

folium and orchil. 3d techniques, analysis in synchro mode or determination of half-life times could provide more reliable alternatives [43].

*3.6 MALDI-ToF-MS analysis*.

The application of MALDI-ToF-MS analysis enabled the development of another interesting procedure for a micro-invasive, micro-destructive procedure for the identification of folium and orchil. The amount of sample requested was in fact less than 1 $mm^2$ of parchment, which was subjected to hydrolysis with formic acid as described in the Experimental section. The results of MS analysis are shown in Figure 6. Following a sort of *untargeted* approach, once having obtained the mass spectra from the dyes some peaks were identified as markers, setting aside the identification of the exact chemical nature of the compounds involved to further future research. It appears that two peaks, one for folium and one for orchil, can be considered as markers. The mass spectrum of folium (Figure 6, bottom) is dominated by a peak at 266 m/z. The mass spectrum of orchil (Figure 6, top) has its main feature in a peak occurring at 672 m/z. The main coloured chemical species known to be present in orchil according to the literature, that, is amino- and hydroxy-orceins, are barely detectable in the spectrum. The reason of this phenomenon is uncertain; it can be hypothesised that, considering the spectral features of these molecules (see apparent absorbance spectrum in Figure 4), they strongly absorbed laser radiation at 337 nm and resonance effect could led to molecule degradation or at least rearrangement.

*3.7 XRF analysis*. Being an elemental technique, XRF spectrometry was used in order to check whether heavy elements were present in the composition of the dyes. Surprisingly, it was found that both folium and orchil contained bromine. Scraps of *Roccella tinctoria* from Canary Islands, of other coastal lichen samples and a sample of orchil were analysed according to the conditions described in the Experimental section. A semi-quantitative determination of bromine yielded an amount of ca. 100 mg/Kg in lichen samples. Also interesting is the fact that not only bromine is present in the raw material, i.e. lichens, but also that this element, entirely or in part, follows orcinol in the extraction with ammonia, therefore resulting in the final composition of orchil. Bromine in lichens may be due to their exposure to marine aerosol, since *Roccella, Dendrographa* and *Lecanora* lichens grow on coastal lands. The enrichment of some elements in lichens compared to natural crustal composition has been well demonstrated [44] and bromine, together with chlorine and magnesium, is representative of the contribution of sea-spray. In a study on lichens from Azores and Madeira Archipelagos [45], enrichment factors between 10 and 100 were found for bromine. Bromine could come from low molecular weight organobromine compounds which are known to be produced by living organisms [46].

A similar response was obtained by analysing the fruits of *Chrozophora tinctoria* and the folium powder, even if the amount of bromine was found to be lower than in lichens and close to the detection limit of XRF technique. The

presence of bromine in this plant can be connected to sea-spray exposure also, if we consider that it grows mainly in coastal lands of the Mediterranean basin (e.g. Sardinia, Southern France, Turkey, etc.).

*3.8 ICP-MS analysis*. To improve the information obtained by XRF identification of bromine, a more accurate quantitative result was obtained by means of ICP-MS analysis. Samples of *Roccella tinctoria* from Canary Islands, *Lasallia pustulata* from England and *Ocrolechia tartarea* from Dartmoor (Southern England) were considered, along with a sample of raw orchil powder obtained from *Roccella tinctoria*. To evaluate the indication of sea-spray as the origin of bromine, a comparison was carried out among two samples of *Ochrolechia tartarea*, collected respectively near the coast (sample A) and several kilometres far from the seaside (sample B). For what concerns *Chrozophora tinctoria*, analysis was carried out on the external pericarp (the part richest in purple dye), on the internal seed and on the raw folium powder.

The results are shown in Table 1: it is apparent that lichens living on coastal lands (*Roccella tinctoria* and *Ocrolechia tartarea*) have a higher content in bromine and, accordingly, in iodine, than lichens living in internal lands (*Lasallia pustulata*). This fact is confirmed by analysis of the two samples of *Ocrolechia tartarea*: sample A, coming from the coast, has more than two times the amount of bromine of sample B and a higher amount of iodine. In the case of *Chrozophora tinctoria*, it is interesting to note that the concentration of bromine is higher in the pericarp than in the internal seed, according to the hypothesis of the contribution from sea-spray.

From the diagnostic point of view, there is a significant consequence in the results of elemental analysis of orchil and folium: the identification of bromine in the analysis of purple artworks cannot be considered as a reliable clue for the presence of Tyrian purple. Some studies on ancient manuscripts involving XRF analysis [23,47,48] led to the hypothesis that the precious dye obtained from shellfishes had been used, due to the identification of bromine, but the present study actually demonstrates that Tyrian purple, orchil and folium show a similar behaviour.

*3.9 Analysis of purple and violet panted areas on illuminated manuscripts*. To verify the possibility of identifying and distinguish folium and orchil on painted artworks, non-invasive analyses were performed on purple and violet painted areas of several illuminated manuscripts. As an example, Figure 7 reports the FORS spectra obtained from two manuscripts held in Italian libraries. Ms. CIV or *Libri S. Augustini de Trinitate* is a 9$^{th}$ century codex held in the Archivio Capitolare at Vercelli (Piedmont), while ms. J.II.1, also known as *Beatus of Liébana - Turin Codex*, is a 12$^{th}$ century codex held in the Biblioteca Nazionale Universitaria at Torino (Piedmont). According to the spectral features, in the first case there is a very good match against orchil; the second manuscript, instead, appears to be decorated with folium.

**4 Conclusions**

The application of different techniques to the identification of folium and orchil allowed to select the most suitable procedures of analysis. FORS has shown to be the best technique for a totally non-invasive approach. Of course, micro-invasive techniques such as SERS and MALDI-ToF-MS allowed to obtain far better diagnostic information for the identification and discrimination of these ancient dyes which at present can be considered largely unexplored. A wider application of SERS and MALDI-ToF-MS is strongly recommended since they can provide unique information at the expense of a very limited amount of sample. In this work, the very first FT-IR, FT-Raman and SER spectra of folium have been obtained.

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| Sample | Bromine (mg/Kg) | Iodine (mg/Kg) |
|---|---|---|
| *Roccella tinctoria* | 196,0 | 0,902 |
| *Lasallia pustulata* | 10,2 | 0,635 |
| *Ocrolechia tartarea*, sample A | 113,5 | 2,18 |
| *Ocrolechia tartarea*, sample B | 48,9 | 1,42 |
| Orchil from *Roccella tinctoria* | 159,7 | n.d.* |
| *Chrozophora tinctoria* pericarp | 19,4 | 0,127 |
| *Chrozophora tinctoria* seed | 8,14 | 0,045 |
| Folium | 104,2 | n.d.* |

Table 1 – ICP-MS analysis of raw matters and dyes

* not detected (above detection limit)

**Figure captions**

Figure 1. FT-IR spectra in transmittance coordinates of folium (bottom line) and orchil (top line)

Figure 2. FT-Raman spectra of folium (bottom line) and orchil (top line)

Figure 3. SER spectra of folium (middle line) and orchil (top line); the spectrum of Ag colloidal paste is also reported (bottom line)

Figure 4. FORS spectra of folium (bottom line) and orchil (top line)

Figure 5. Fluorescence spectra of folium (medium line) and orchil (top line); the spectrum of parchment is also reported (bottom line)

Figure 6. MALDI-ToF-MS spectra of folium (bottom line) and orchil (top line)

Figure 7. FORS spectra of orchil (solid line), ms. CIV (dashed line), folium (dotted line) and ms. J.II.1 (dotted-dashed line)

**Figure 1**

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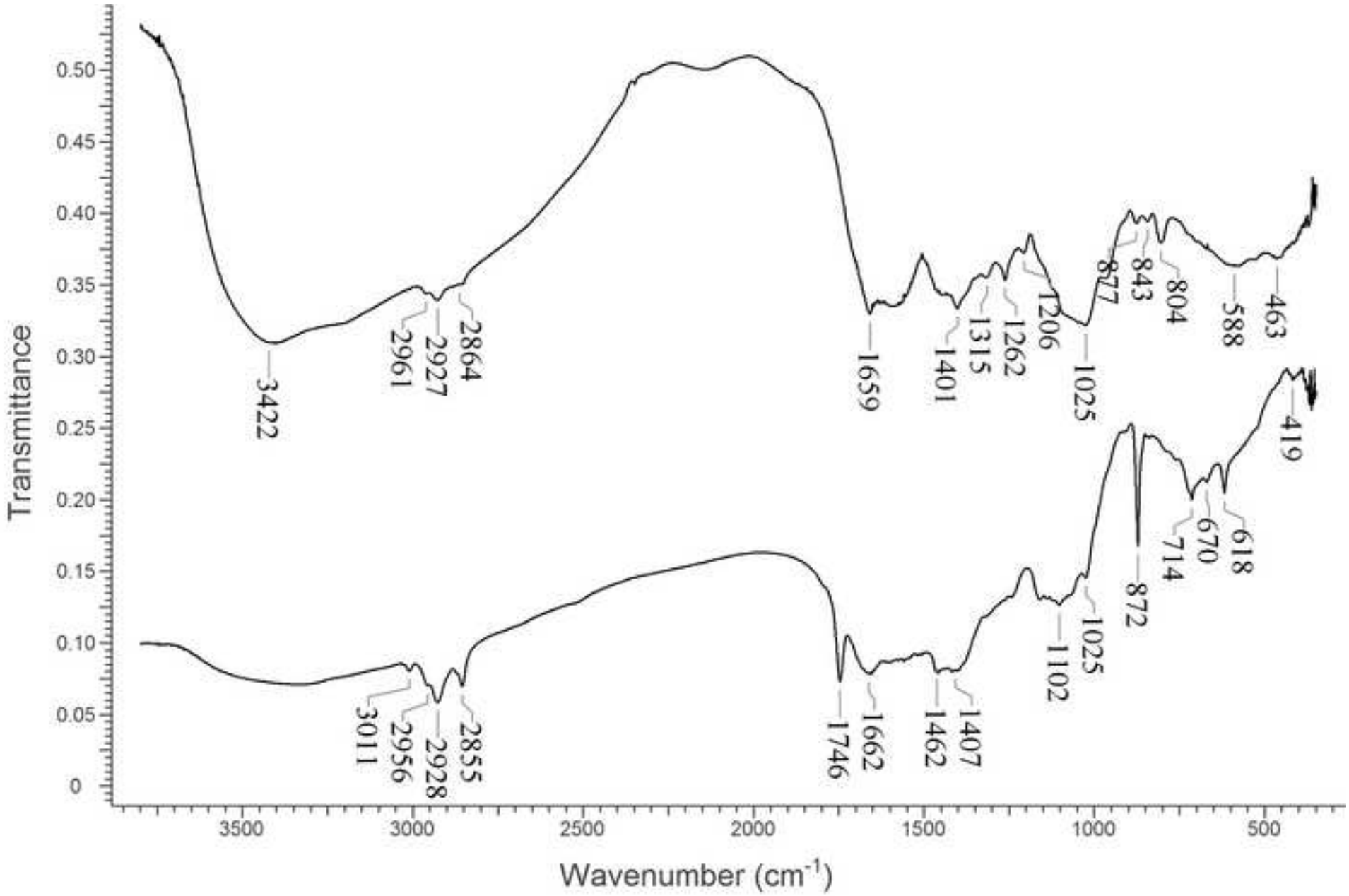

**Figure 2**

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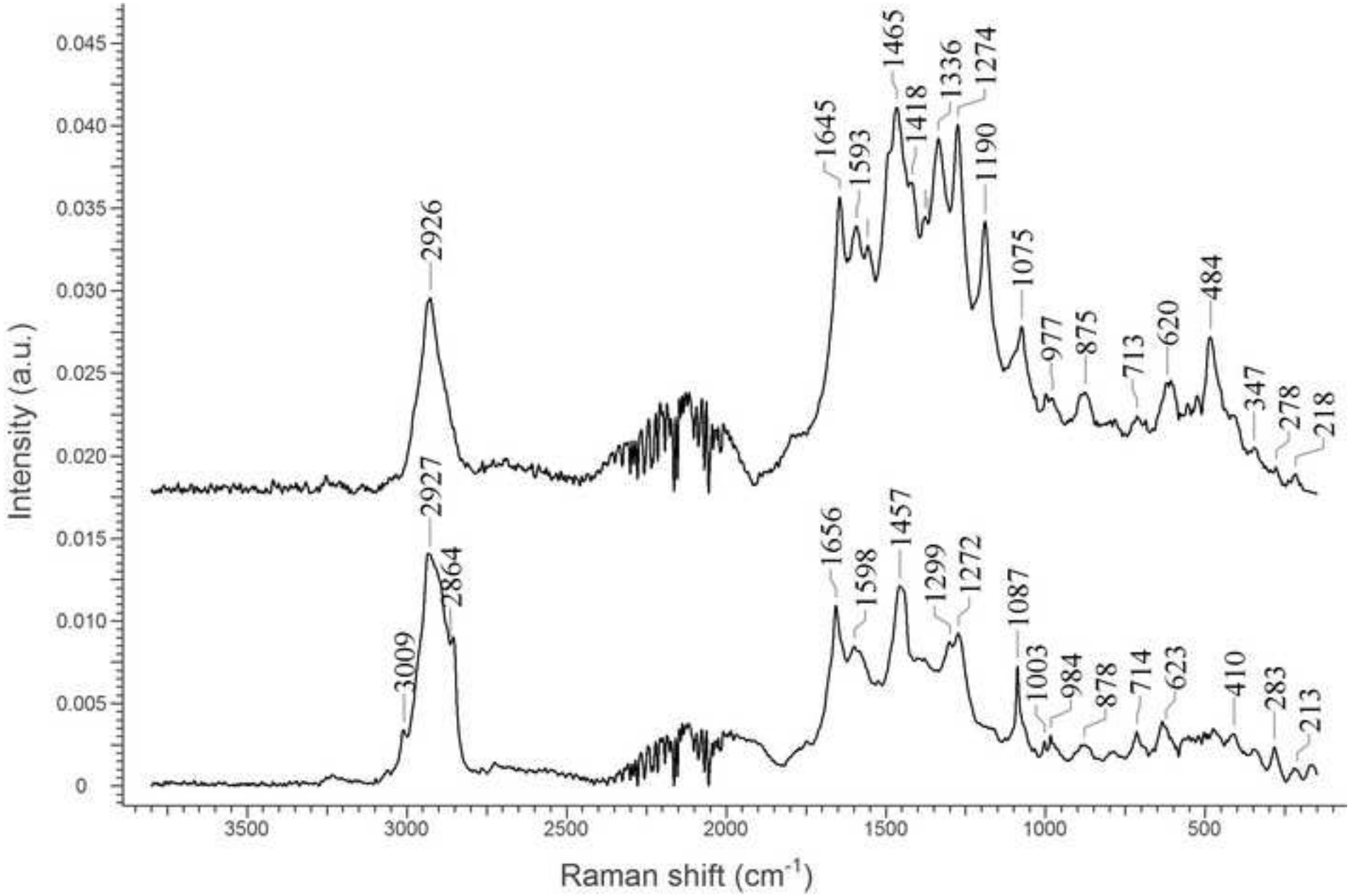

**Figure 3**

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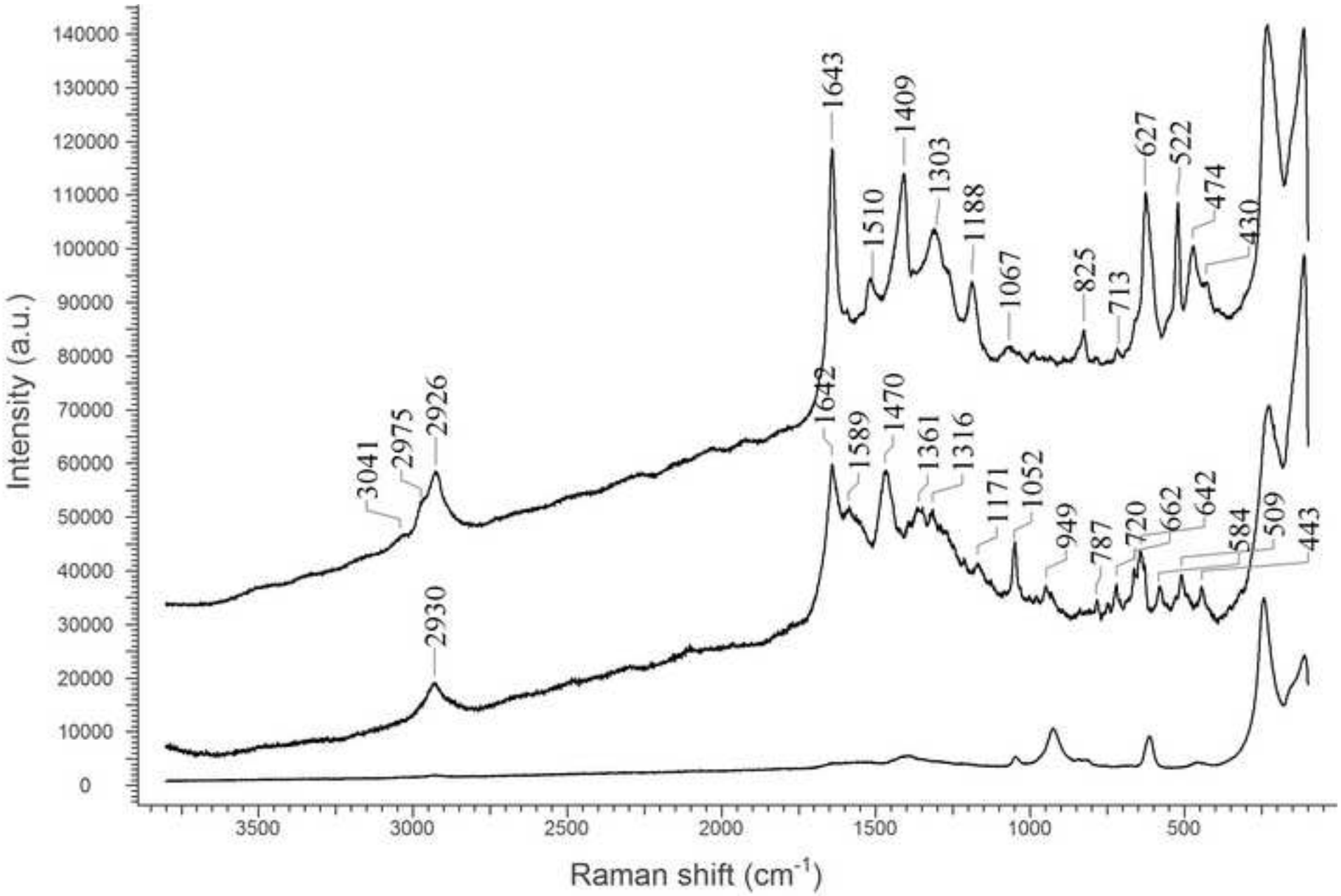

**Figure 4**

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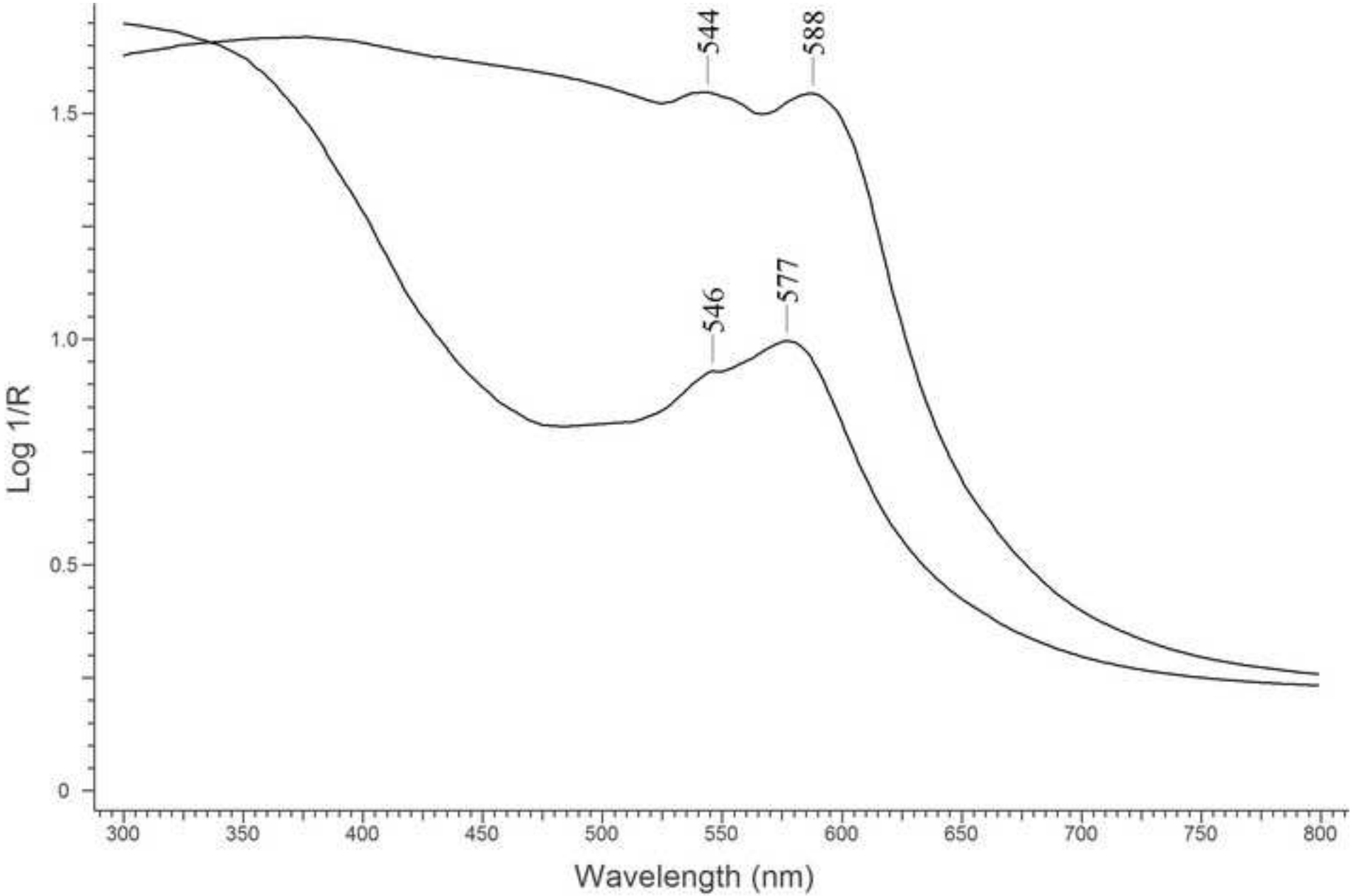

**Figure 5**

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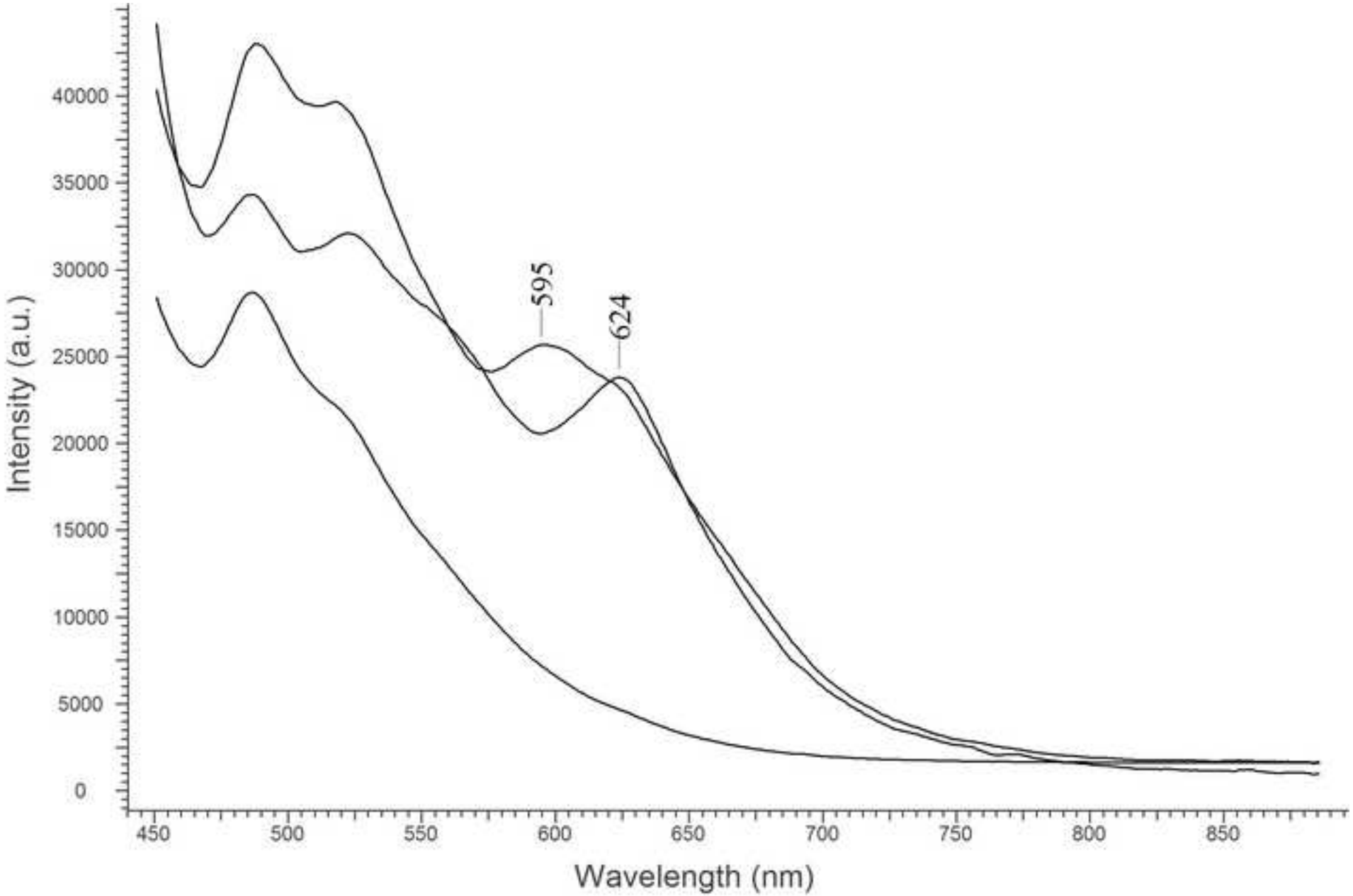

**Figure 6**

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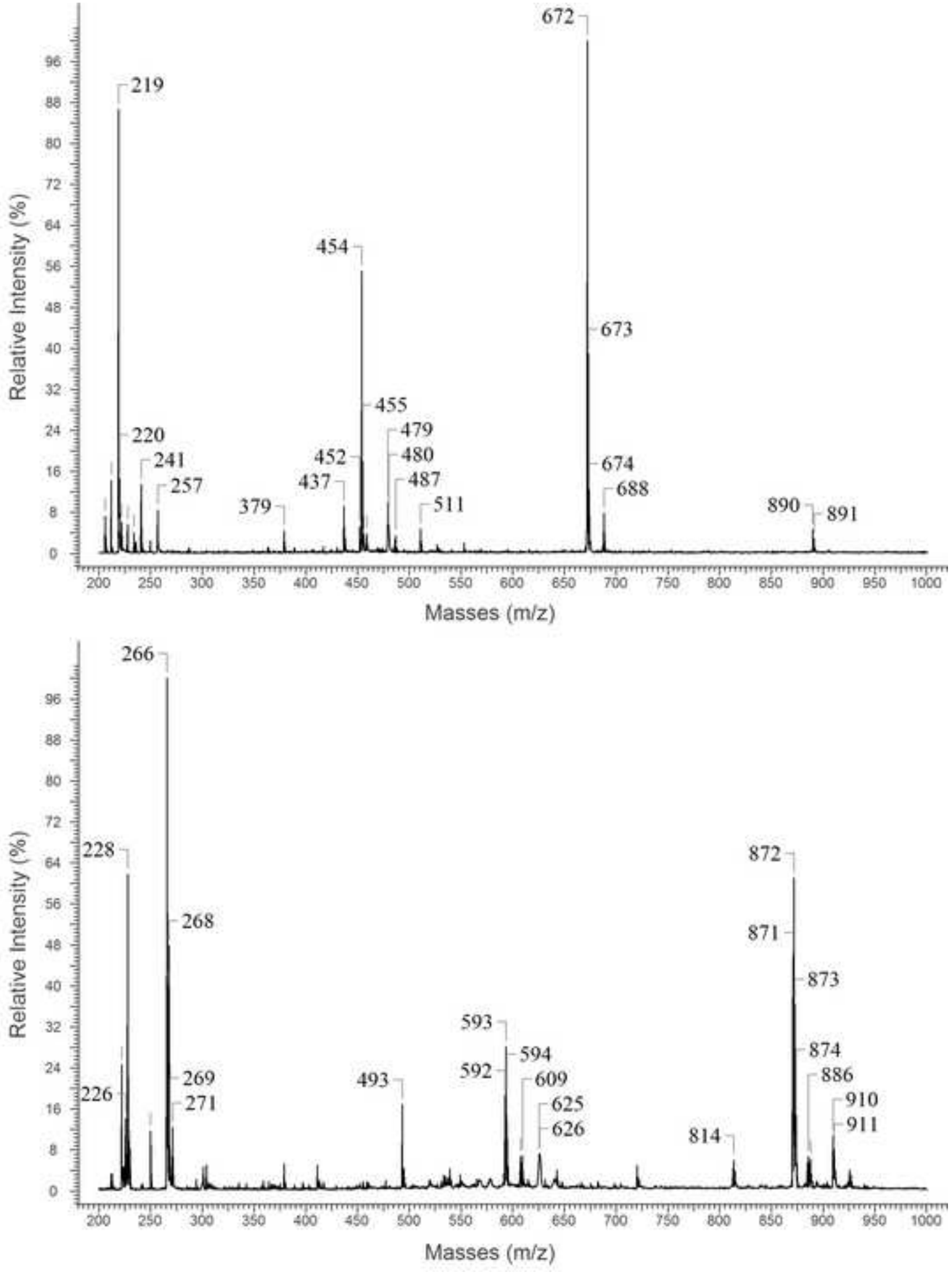
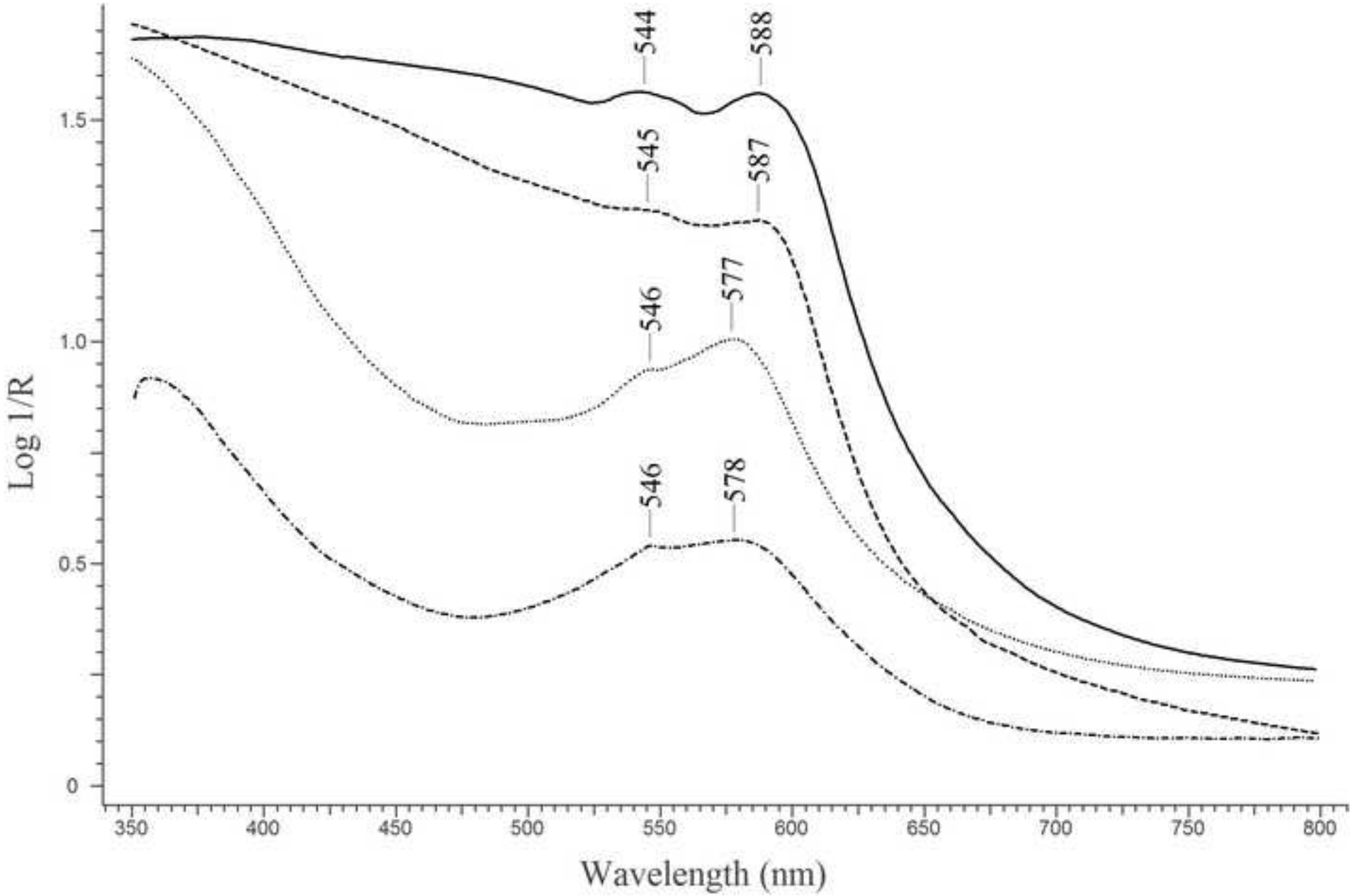


Figure 7

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