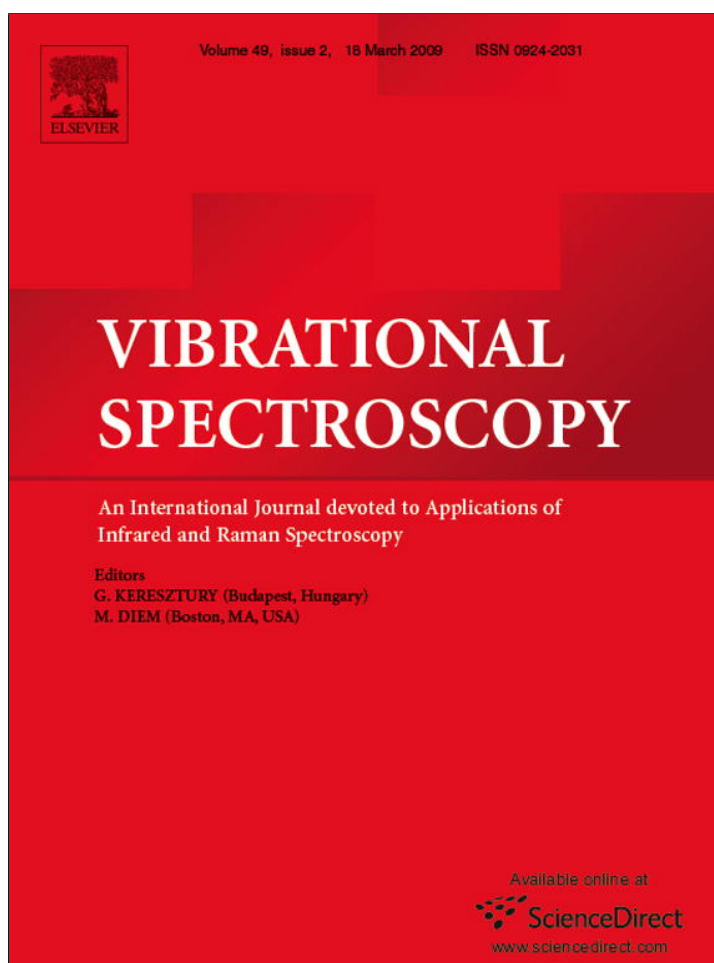


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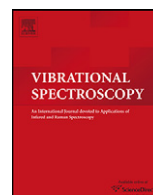
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Raman and surface-enhanced Raman spectra of chrysin, apigenin and luteolin

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ABSTRACT

The FT-Raman and surface-enhanced Raman (SER) spectra of three flavonoids, namely chrysin, apigenin and luteolin, have been obtained. The SERS spectra were obtained on citrate reduced Ag colloids. Assignments of the experimentally obtained normal vibrational modes were aided by density functional theory (DFT) calculations using the B3LYP functional and the 6-31+G* basis set. Excellent fits were obtained for the observed spectra with little or no scaling. The most intense lines in the three flavonoids SERS spectra are those in the C=O stretching region and around 1250 cm⁻¹. The first ones are often weakened by proximity of the metal surface, whereas the latter are not affected by the Ag. On the other hand, the lines at lower wavenumbers, assigned to in-plane ring deformation, are strongly enhanced by the surface, indicating a perpendicular orientation of the flavonoids on the Ag surface. The spectra of the flavonoids are compared, and a case study of application to detect weld, a mixture of apigenin and luteolin, in a textile is presented.

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1. Introduction

Phenolic derivatives of flavone are responsible for the brightly colored pigments of many fruits and vegetables. Polyphenols are found in high concentrations in wine, tea, grapes and in a wide variety of other plants and have been associated with prevention of heart disease and cancer [1]. Due to their polyphenolic nature, flavonoids exhibit strong antioxidant properties and have been widely used as ingredients in pharmaceutical products. They can be subdivided into: flavonols (e.g. quercetin), flavones (e.g. apigenin, luteolin), flavanols (e.g. catechin) and isoflavones (e.g. genistein). Flavones are also the main components of various natural dyes (e.g. weld, fustic, quercitron), mainly yellow but ranging from brown to green and olive-green, used in textile industries since ancient times [2,3]. Present in plants as glycosides, they can be used as mordant dyes by hydrolysis of glycoside bonds and chelating with metals such as aluminum or copper [4].

Fig. 1 shows the structure of chrysin (5,7-dihydroxyflavone), apigenin (5,7,4'-trihydroxyflavone) and luteolin (5,7,3',4'-tetrahydroxyflavone). They differ only in that apigenin and luteolin have a 4' or 3' and 4' OH group on the benzene ring. Among those, chrysin has been the least studied by spectroscopic techniques. Even though it contains two hydroxyl groups, this molecule is relatively insoluble in water, making the preparation of the samples difficult. Chrysin and its complexes with Al³⁺, Ga³⁺ and In³⁺ have been studied by IR, UV-vis and NMR [5]. The IR spectra shows $\nu(\text{OH})$ bands at 3600–2400 cm⁻¹, $\nu(\text{C=O})$ at 1655 cm⁻¹, aromatic $\nu(\text{C=C})$ at 1505 cm⁻¹, other bands at 1460 and 1168 cm⁻¹, $\gamma(\text{CH})$ at 850 cm⁻¹ and 815 cm⁻¹. Only the UV resonance Raman (UVR) spectrum of chrysin has been reported in the study on extractable compounds in Scots pine wood, where the flavonoid was used as a reference [6]. The reported Raman bands are the symmetric aromatic ring stretching at 1605 cm⁻¹, the C=C ring stretching at 1649 cm⁻¹, and the asymmetric ring vibration at ~1500 cm⁻¹.

On the other hand, apigenin and luteolin are the main coloring matters of the natural dyestuff weld. It originates from the leaves and stem of *Reseda luteola* L. found in Central Europe, India and China. Before the discovery of America, weld was the dyestuff most used in Western Europe. In 1775, quercitron became available in large quantities and superseded weld and fustic as the principal yellow dye [7]. Apigenin and luteolin are very abundant in textile

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¹ Note, the first two authors carried out much of the work involved in this research and should be considered equally as principal authors.

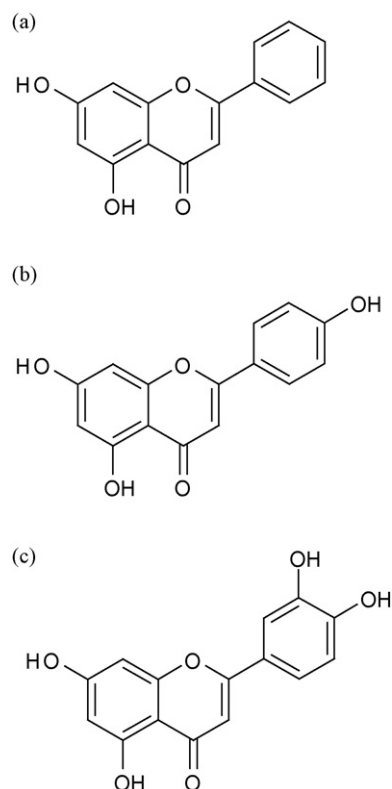


Fig. 1. Structure of chrysin (a), apigenin (b) and luteolin (c).

dyes from various samples and HPLC, GCMS, IR, Raman and SERS spectra on silver colloid data have been reported [8–11]. Their structure differs by only one hydroxyl group, so they exhibit similar structural properties. Luteolin shows $\nu(\text{O-H})$ vibrations around 3220 cm^{-1} and $\nu(\text{C=O})$ at 1655 cm^{-1} in IR. FT-Raman spectra of luteolin and apigenin in solid state show similar trend of the region corresponding to $\nu(\text{C=O})$ and $\nu(\text{C=C})$ vibrations at $1660\text{--}1550\text{ cm}^{-1}$, but differences in the $1400\text{--}1000\text{ cm}^{-1}$ region. This is explained by appearance of different $\delta(\text{O-H})$ and $\nu(\text{C-O})$ motions due to the different number of hydroxyl groups. SERS spectra of luteolin on silver colloid showed a strong downward shift of the aromatic $\nu(\text{C=C})$ vibration at 1608 cm^{-1} in solid to 1577 cm^{-1} in colloid. This was explained by the effect of polymerization of the catechol-like moiety on the Ag nanoparticles. Apigenin was reported to have fewer differences between the Raman spectrum of the solid and the SERS spectrum on the colloid [11].

The limited number of studies on chrysin by Raman and SERS spectroscopy may be due to its poor solubility in water as well as the strong fluorescence upon excitation, which obscures regularly weak Raman spectra. Our recent study on other flavonoid molecules including the pentahydroxyflavone quercetin has shown effectiveness of SERS spectrum in quenching the fluorescence and inducing spectra intensity by the few orders of magnitude [12,13]. This method also allows using very little sampling which is crucially important in art painting and textile conservation [14] as well as in bioresearch and forensic science [15].

In this article we report FT-Raman and SERS spectra on Ag colloid of all three molecules. These studies are aided in their interpretation by DFT calculations. We provide comparative analysis of the spectra of molecules with relation to each other. Our objective is to apply techniques similar to our past studies to obtain spectra of flavonoids and to further study their structures

and possibly seek better methods of analysis suitable for art preservation and other fields.

In the next section we present details of our experimental procedure, as well as DFT calculations. Following that we present the analysis of the spectra of chrysin, apigenin and luteolin, and we compare the results of both the normal Raman and SERS spectra. We will then provide a comparative analysis of several of the normal modes of each molecule, showing the effect of various hydroxy substituents on the observed frequencies. In the final section we present the SERS spectrum analysis of weld, the most universally used dye plant.

2. Experimental

Chrysin, apigenin and luteolin were purchased from Sigma. Stock solutions of the dyes were prepared in ethanol in a concentration 10^{-2} M . Then, a water/ethanol mixture (60/40 v/v) was added to prepare 10^{-4} M solution of the dye.

Ag colloid was prepared following the method of Lee and Meisel [16] by reduction of silver nitrate (Aldrich 209139 Silver Nitrate 99.9%) with sodium citrate (Aldrich W302600 Sodium Citrate Dihydrate). The colloid thus prepared shows an absorption maximum at 406 nm and FWHM of 106 nm , as measured with a Cary 50 UV-Vis Spectrophotometer (after a 1:4 dilution with ultrapure water to keep maximum absorbance within the instrumental range). To further concentrate the colloid for use, a volume of 10 ml of the original colloid was centrifuged at 5000 rpm for 2 min . The supernatant was discarded and the settled portion was resuspended in 1 ml of ultrapure water. All glassware was cleaned with Pierce PC54 cleaning solution, rinsed with ultrapure water and finally in acetone and methanol. This method proved to be as effective as the use of aggressive cleaning agents such as aqua regia or piranha solution, and was preferred for health and safety reasons. Only ultrapure water was used for the preparation of the various solutions. SERS spectrum measurement were made simply by adding $1\text{ }\mu\text{l}$ of dye solution to a $2\text{ }\mu\text{l}$ drop of colloid deposited on a gold coated microscope slide, followed by addition of $2\text{ }\mu\text{l}$ of a 0.2 M KNO_3 solution. Raman measurements were taken directly from the drop using a $50\times$ or $100\times$ microscope objective and focusing on the microscope slide surface. SERS spectrum could be obtained 2 or 3 min after addition of the KNO_3 and remained constant in quality until evaporation of the liquid.

A weld reference sample (Weld NS Al-acetate, Silk, 10.7.05) from the Metropolitan Museum of Art reference collection was prepared by dyeing the mordanted silk yarn with the dried plant *Reseda luteola* (Kremer pigment). Weld was extracted using a hydrolysis procedure consisting of a treatment of the fiber with HF vapor. Then the extracted dye was dissolved in ethanol/water (1/1 v/v).

The NR spectra of solids were obtained in the region of $100\text{--}4000\text{ cm}^{-1}$ directly from pure powder samples. Since the fluorescence of the dyes prevented the acquisition of a Raman spectrum, FT-Raman spectroscopy was carried out using a Bruker Ram II FT-Raman-Vertex 70 FTIR Micro spectrometer. The 1064 nm line of an Nd:YAG laser was used as the excitation line. The resolution was set to 4 cm^{-1} in back scattering mode. A liquid nitrogen cooled Ge detector was used to collect 100 scans for a good Raman spectrum. The laser output was kept at 150 mW for the SERS spectra and 50 mW for the solid samples.

Additionally, SERS spectroscopy on Ag colloids was carried out using a Bruker Senterra Raman microscope using 785 nm excitation, a 1200 rulings/mm holographic grating, a CCD detector and power at the sample ranging from 8 to 80 mW .

Density functional theory (DFT) calculations were performed with Gaussian 03 [17] at the B3LYP level of theory and employing

the 6-31+G* basis set. The geometry optimization resulted in a planar geometry and no imaginary frequencies were observed in the calculated spectrum. This basis set was chosen to be consistent with earlier work, and because the fit obtained was excellent (see below). In general vibrational normal mode assignments were based on the best-fit comparison of the calculated Raman spectrum with the observed normal Raman spectrum. Where needed slight scaling of the calculated spectrum was utilized (usually 0.96–0.99). In instances where there was spectral congestion, such as in the carbonyl stretch region (near 1600 cm^{-1}), the relative intensities of the calculated spectra were matched to those of the observed spectra, so that the most intense calculated lines were assigned to the most intense observed lines.

3. Results and discussion

3.1. Chrysin

The normal Raman spectrum of chrysin powder is shown in Fig. 2. Included also in each figure is a plot of the Raman spectrum predicted by a density functional theory calculation. The DFT wavenumbers have been adjusted by a factor of 0.963 and 0.985 above and below 1150 cm^{-1} , respectively. This was done in order to obtain the best fit of observed data. As can be seen the fit is excellent and we may therefore have great confidence in the assignments. The measured and calculated wavenumbers are tabulated in Table S1 (see supplementary materials), along with the assigned normal modes and a brief description of the major vibrational displacements which contribute to the assigned mode. Note the band at 1000 cm^{-1} (ν_{43}) is the trigonal stretch of ring B. The most intense bands in the normal Raman spectrum are around 1600 cm^{-1} (ν_{69}), 1611 cm^{-1} (ν_{70}), and 1652 cm^{-1} (ν_{71}), which these involve the C=O stretching motion. Other prominent bands involve the C3H in-plane bend (ν_{55}) at 1247 cm^{-1} as well as the ring C–C in-plane deformations (ν_{23}) at 614 cm^{-1} . A broad band is observed at 3069 cm^{-1} which is most likely an overlap of various CH stretching motions, while the lines predicted to be the OH stretches (ν_{80} , ν_{81}) at around 3600 cm^{-1} are not seen in this spectrum, as has been the case for other hydroxyflavones in this study.

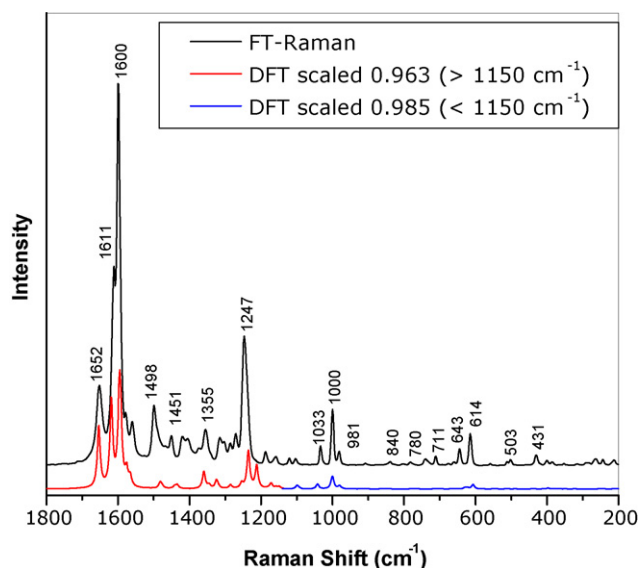


Fig. 2. FT-Raman and DFT calculated spectra of chrysin (powder). (For interpretation of the references to color in the artwork, the reader is referred to the web version of the article.)

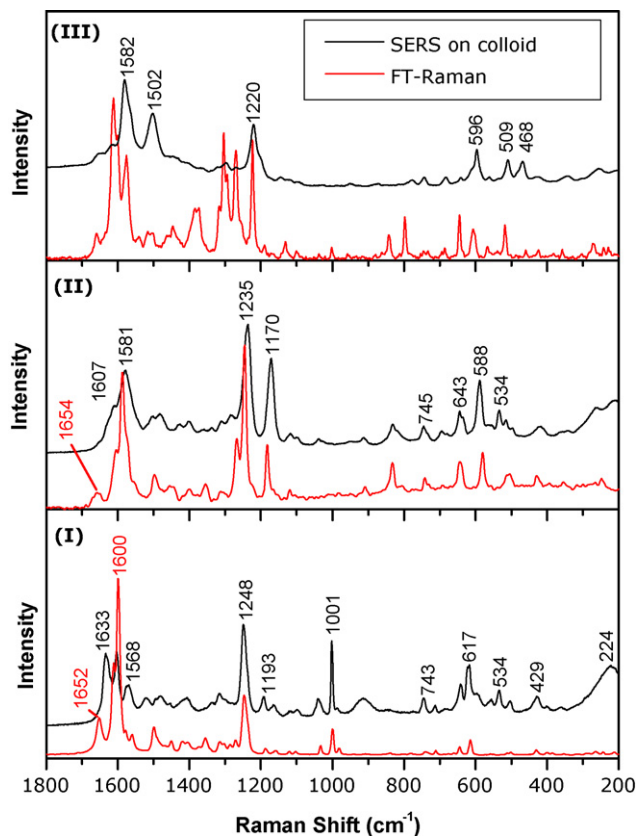


Fig. 3. Comparison of the FT-Raman and the SERS spectra of chrysin (I), apigenin (II) and luteolin (III). (For interpretation of the references to color in the artwork, the reader is referred to the web version of the article.)

The SERS spectrum is shown in Fig. 3I in comparison with the normal Raman spectrum. It can be seen that several of the prominent lines are present in both, including 1600 (ν_{69}), 1247 (ν_{55}), 1000 (ν_{43}) and 614 cm^{-1} (ν_{23}) (giving the normal Raman measurements). However, the relative intensities have been drastically altered. The bands at 1633 (ν_{71} , C=O stretching), 1603 (ν_{70} , C=O, C2=C3 and rings A/C quinoid stretchings) and 1569 cm^{-1} (ν_{66} , B quinoid stretching) are no longer the most intense. On the other hand, the bands at 1248 (ν_{55}) and 1101 cm^{-1} (ν_{43}), assigned to the C3H in-plane bend and to the ring B trigonal stretching, respectively, undergo a great enhancement when chrysin is on the Ag surface. Moreover, the bands in the region $750\text{--}400\text{ cm}^{-1}$ (642 (ν_{26}), 617 (ν_{23}) and 429 cm^{-1} (ν_{17})), assigned to ring CC in-plane deformation modes, are highly enhanced by proximity to the metallic colloid, in comparison to their intensity in the normal Raman spectrum. Other bands, such as the ones at 743 , 596 and 534 cm^{-1} appear in the SERS spectrum and were not shown either in the normal Raman or in the calculated spectrum, likely due to the interaction of chrysin with the Ag surface. This pattern of intensity redistribution on the Ag surface is similar to that found in other flavone derivatives [13]. Thus, the decrease in the intensity of the C=O bands indicate that the molecule may be attached to the surface by the carbonyl group in position 4, possibly chelated with the OH in C5. The fact that the most enhanced bands correspond to in-plane modes in the SERS spectrum indicates that chrysin must be in a perpendicular orientation with respect to the metal surface. A few broad lines namely at 224 , 914 and 1407 cm^{-1} are also observed, and they are attributed to the colloid background [13].

3.2. Apigenin

In Fig. 4 we show the FT-Raman spectrum of solid apigenin along with the DFT calculated spectrum (scaled by a factor of 0.97 above 1000 cm^{-1}). As can be seen, there is an excellent fit in the higher wavenumber region, and we feel that this indicates that our assignments of the normal modes are correct. In the region below 1000 cm^{-1} we found it totally unnecessary to scale the calculations at all. Both the wavenumbers and intensities can be seen to fit quite well. The observed and calculated wavenumbers are listed in Table S2 (see supplementary materials). As in the other flavone derivatives, the region near 1600 cm^{-1} is the most intense and may be assigned to the C=O stretch in combination with either C2=C3 stretches or ring quinoidal stretches. Also prominent between 1400 and 1500 cm^{-1} are various OH in-plane bending modes. Note the strong band at 1245 cm^{-1} attributable to a combination of the 7OH bend and the C3-H bend. The ring B trigonal stretch, often prominent in other flavones (and observed near 1000 cm^{-1}) is shifted to 983 cm^{-1} and observed to be very weak in apigenin. This is presumably due to the 4'OH on ring B, which severely distorts this mode. The lower wavenumber region, between 200 and 1000 cm^{-1} is relatively weak in the solid spectrum and is dominated by CH in-plane bends and CC in-plane deformations of various rings.

On the colloid, shown in Fig. 3II along with the solid Raman spectrum for comparison, we observed that, relatively speaking, the region of around 1600 cm^{-1} is decreased in intensity. These bands, at 1607 (ν_{72}) and 1581 cm^{-1} (ν_{71}), are assigned to the C=O and rings A/B quinoid stretching modes, and to the C5-OH bending and the C=O and rings B/C quinoid stretchings, respectively. Note that the line at 1654 cm^{-1} almost completely disappears (or becomes a very weak shoulder). This line (ν_{74}) also involves the C=O and C quinoid stretching and the bending of the 5OH. In comparison, SERS spectrum bands in the lower wavenumber region (745 (ν_{33}), 643 (ν_{27}), 588 (ν_{24}), 534 and 514 cm^{-1} (ν_{21})), which corresponds to in-plane deformations, are considerably enhanced. The above observations lead us to believe that, as with other flavones, the molecule is attached perpendicular to the Ag surface through a chelated structure of the C=O and OH. Confirming this is the relative lack of any serious effect of the

metal on the 1266 cm^{-1} (ν_{58} , C4'-OH stretch) or the 1245 cm^{-1} (ν_{56} , 7OH bend).

3.3. Luteolin

In Fig. 5 we display a comparison of the FT-Raman spectrum of luteolin powder with the density functional calculation (scaled by a factor of 0.97 above 1550 cm^{-1}). With few exceptions (such as the line at 842 cm^{-1}) the fit is quite good. The assignments are listed in Table S3 (see supplementary materials), along with the measured wavenumbers, and a brief description of the normal mode. In the last columns of Table S3 the surface-enhanced spectra obtained on Ag colloid is listed.

The FT-Raman spectrum of solid luteolin is quite similar to those of the other flavones. In that, the most intense lines are those of the C=O and C2=C3 combined stretches near 1600 cm^{-1} . The lines at 1224 , 1270 and 1304 cm^{-1} are also quite intense. The first of these is due to a combination of C5'H and 4'OH in-plane bend, while the line at 1270 is a C3H in-plane bend and 1304 is the ring B breathing mode. Lines between 500 and 850 cm^{-1} , mainly attributable to ring CC in-plane deformations, are somewhat weaker.

In Fig. 3III we show a comparison of the FT-Raman spectrum of solid luteolin with the SERS spectrum on the colloid. The overall pattern of changes is similar to those observed in other flavones [13]. Note the intense line at 1612 cm^{-1} , attributable to the C=O, C2=C3 stretch (ν_{75}), is considerably weakened on the colloid. By contrast the line at 1576 cm^{-1} (ν_{72}) is shifted to 1582 cm^{-1} and is the strongest line in the colloid spectrum. Furthermore the lines at 1220 cm^{-1} (ν_{57}) and 1502 cm^{-1} (ν_{69}) are strongly enhanced relative to nearby lines. These lines involve the 3'OH and the 4'OH in-plane bend. The three lines at 468 , 509 and 596 cm^{-1} are also considerably enhanced by proximity to the surface.

These results indicate that the molecule is most likely attached to the surface by the C=O, possibly chelated with the 5OH. Absence of out-of-plane modes in the enhanced spectra indicates that the plane of the molecule is perpendicular to the metal surface. The lines involving the OH bends on ring B are strongly enhanced, as is observed on related molecules such as quercetin (3,5,7,3',4'-pentahydroxy flavone).

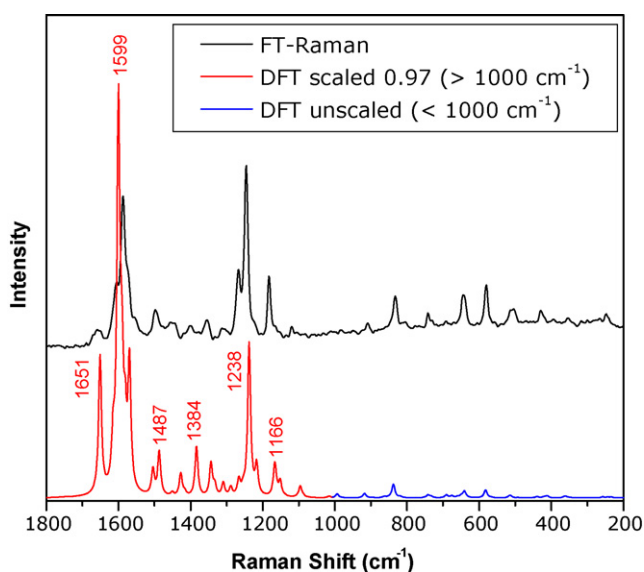


Fig. 4. FT-Raman and DFT calculated spectra of apigenin (powder). (For interpretation of the references to color in the artwork, the reader is referred to the web version of the article.)

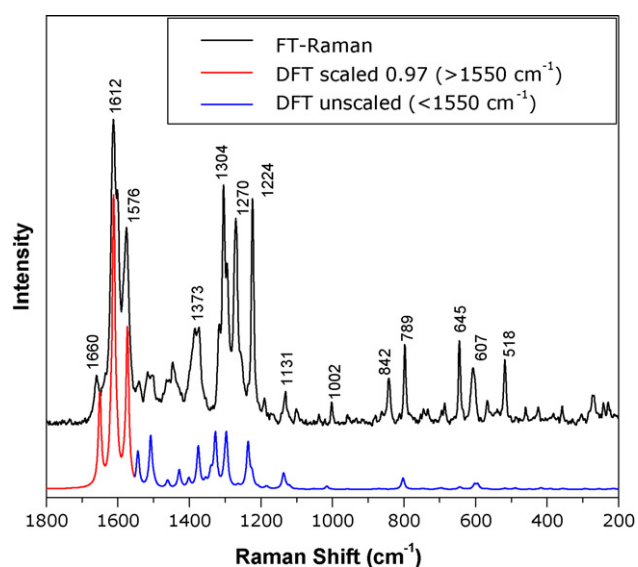


Fig. 5. FT-Raman and DFT calculated spectra of luteolin (powder). (For interpretation of the references to color in the artwork, the reader is referred to the web version of the article.)

3.4. Comparative results

In Fig. 6 we compare the SERS spectrum of 3-HF, 5-HF, 7-HF, chrysin, apigenin, 3',4'-DHF, luteolin and quercetin. The SERS spectra of the first four molecules, that is, the ones without OH group in the B ring, show an intense band at approximately 1000 cm^{-1} . This band is assigned to the ring B trigonal stretching (Table S1). However, when any OH substitution occurs in the B ring, the intensity of that band is negligible.

On the other hand, an enhancement of the low frequency bands occurs, as in our previous paper [13], which is difficult to explain. In the SERS spectra of the flavones with an OH substitution in C3, namely apigenin, 3',4'-DHF, luteolin and quercetin, the most intense bands are the ones at lower wavenumbers. However, in the other molecules only an enhancement of those bands is observed. Thus, the possibility of a photoproduct formed by reaction of the diphenol moiety can be ruled out, and the enhancement of lower wavenumbers bands in luteolin can be attributed only to the adsorption of the dye on the metal surface.

3.5. Case studies/applications

In order to apply SERS spectroscopy to the analysis of dyes on mixtures we have to identify bands that allow an easy discrimination of different dyes of the same family. We compared the SERS spectra of chrysin, apigenin and luteolin (Fig. 7) and discriminant bands of each dye were determined. Their wavenumbers are collected in Table 1. A good discriminant line would be relatively intense and isolated from nearby lines, which could possibly interfere. Such lines can serve as markers to identify the presence of each compound in a dye mixture. In apigenin the bands

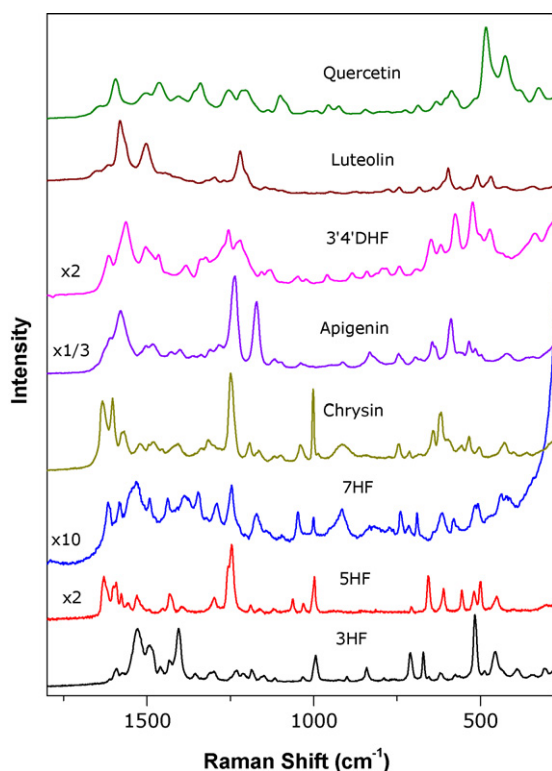


Fig. 6. SERS spectra on Ag colloid of various flavones. Spectra were translated vertically and the intensities were scaled for clarity in comparison. (For interpretation of the references to color in the artwork, the reader is referred to the web version of the article.)

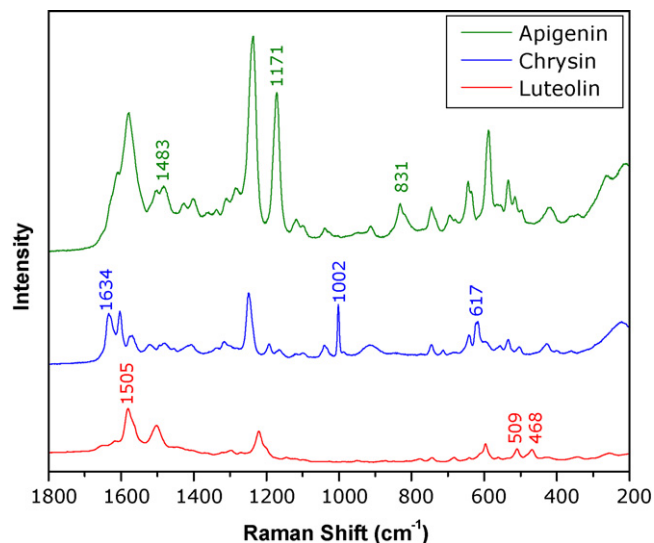


Fig. 7. SERS spectra of chrysin, apigenin and luteolin. Spectra were translated vertically in order to view them clearly. The discriminant bands are labeled with the wavenumber. (For interpretation of the references to color in the artwork, the reader is referred to the web version of the article.)

Table 1
Discriminant bands of chrysin, apigenin and luteolin

Chrysin (cm^{-1})	Apigenin (cm^{-1})	Luteolin (cm^{-1})
		468m
617m		509m
1002s	831w	
	1171s	
	1483w	
1634m		1505m

Abbreviations: s, strong; m, medium; w, weak.

at 831 , 1171 and 1483 cm^{-1} are intense and relatively uncluttered by nearby bands. In chrysin the bands at 1002 and 1634 cm^{-1} serve this purpose, while in luteolin the best discriminants are 468 , 509 and 1505 cm^{-1} . The most intense discriminant bands are located at 1002 , 1171 and 1505 cm^{-1} for chrysin, apigenin and luteolin,

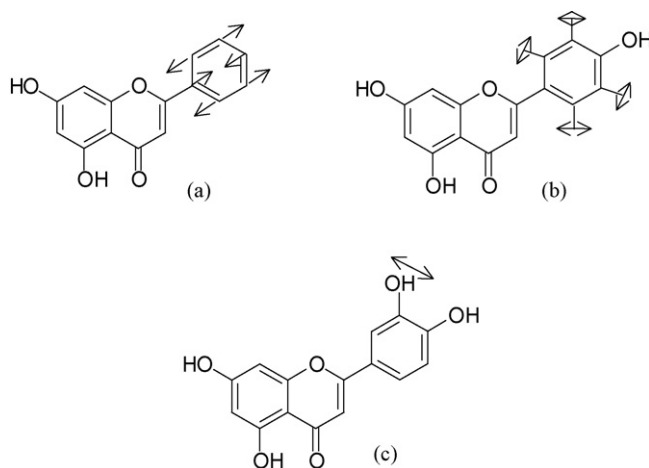


Fig. 8. Vibrations involved in the most intense discriminant bands of chrysin (1002 cm^{-1}) (a), apigenin (1171 cm^{-1}) (b) and luteolin (1505 cm^{-1}) (c).

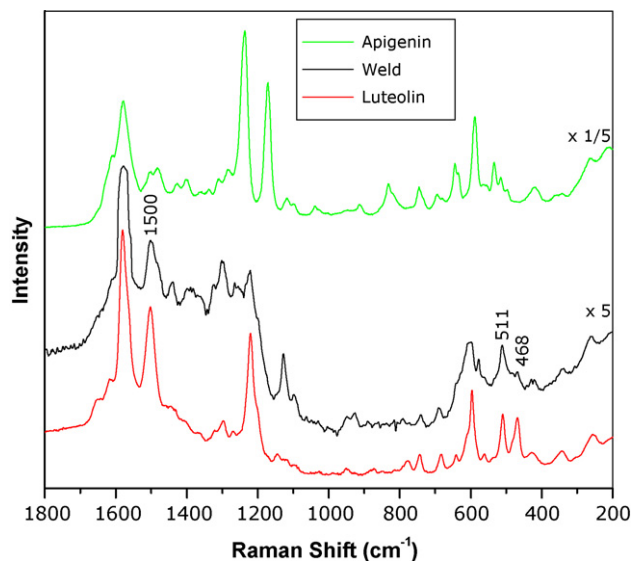


Fig. 9. SERS spectra of apigenin, luteolin and the dye weld. (For interpretation of the references to color in the artwork, the reader is referred to the web version of the article.)

respectively. The motions involved in those vibrations (Fig. 8) show that due to the different position of the OH substituents in the B ring, they are unique for each flavonoid.

These discriminant bands were used to determine by SERS spectroscopy the components of the yellow dye weld, extracted from a dyed silk yarn (Fig. 9). If the SERS spectra of the weld extract, apigenin and luteolin are compared, only the discriminant bands of the latter flavonoid are found. This is due to the much higher proportion of luteolin in the fiber, as was determined by HPLC analysis (luteolin 40.5% and apigenin 6.5%).

4. Conclusions

We have obtained the normal Raman and SERS spectra of three flavonoids, namely chrysin, apigenin and luteolin, which structures differ only on the number of OH substituents in the benzene ring. The SERS spectra technique enabled us to obtain intense spectra from a small quantity of material while simultaneously suppressing fluorescence. We have compared the relative intensities from the NR spectra with colloidal SERS spectra and with DFT calculations. It is concluded that DFT calculations provide a source of accurate normal mode assignments, as well as a basis of comparison of the effects of successive OH substitutions on the normal modes of the parent flavonoid (chrysin in this case). Besides, a general comparison of the enhancement produced by SERS spectra on the spectra of different flavonoid hydroxyl derivatives was provided. The SERS spectra of the molecules without OH groups in the B ring show an intense band at approximately 1000 cm^{-1} . However, when any OH substitution occurs in the B ring, the intensity of that band is negligible. Finally, the SERS spectrum bands that allow an easy discrimination among chrysin, apigenin and luteolin were determined. These bands were used in the SERS spectra identification of the dye weld, a mixture of apigenin and luteolin, previously extracted from a silk fiber.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.vibspec.2008.07.012](https://doi.org/10.1016/j.vibspec.2008.07.012).

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