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Effect of pH on the chemical modification of quercetin and structurally related flavonoids characterized by optical (UV-visible and Raman) spectroscopy

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ABSTRACT

In this work we report the study of the chemical modifications undergone by flavonoids, especially by quercetin (QUC), in alkaline conditions by UV-visible absorption, Raman and surface-enhanced Raman scattering (SERS) spectroscopy, whereas the study was performed in aqueous solution and also on Ag nanoparticles (AgNPs). Several processes are involved in the effect of alkaline pH both in solution and on AgNPs: autoxidation affecting mainly the C-ring of the molecule and giving rise to the molecular fragmentation leading to simpler molecular products, and/or the dimerization and further polymerization leading to species with a higher molecular weight. In addition, there exists a clear structure-instability correlation concerning mainly particular groups in the molecule: the C3-OH group in the C-ring, the catechol moiety in the B-ring and the C2=C3 bond also existing in the C-ring. QUC possesses all these groups and exhibits a high instability in alkaline solution. SERS spectra registered at different pH revealed a change in the dimerization protocol of QUC going from the A- and C-rings-like-condensation to B-ring-like-condensation. Increasing the knowledge of the chemical properties of these compounds and determining the structure-activity relationship under specific environmental factors allow to improve their beneficial properties for the health as well as their preservation in the objects of the Cultural Heritage, for example, by preventing their degradation.

KEYWORDS: Quercetin, Flavonoids, Oxidative polymerization, Raman spectra, SERS, Silver nanoparticles, Catalysis

1. INTRODUCTION

Flavonoids are widely distributed in plants where they develop many functions. For instance, these molecules are the most important ones for flower and fruit coloration. They produce yellow or red/blue pigmentation in petals which are visual cues for pollinator animals.[1] Besides, active color components of yellow organic dyestuffs used in art objects are mostly flavonoid dyes, mainly flavones and flavonols.[2, 3] Flavonoids were also reported to have multiple advantageous properties for the health whereas most of them are attributed to their antioxidant properties. These compounds can behave as antioxidants mainly because of their ability to scavenge free radicals and chelate metal ions.[4-9] The antioxidant activity could also be the reason to display anti-allergic,[10, 11] anti-inflammatory,[12, 13] anti-microbial,[14, 15] anti-cancer,[16-18] anti-diarrheal,[19, 20] cardioprotective,[21, 22] hypoglycemic and/or antidiabetic activities.[23, 24]

The antioxidant-activity of a flavonoid is closely related to its chemical structure. Three structural requirements are important for high antioxidant activity of a flavonoid: (i) the catechol structure in the B-ring; (ii) the 2,3-double bond, in conjugation with the 4-oxo function in the C-ring; and (iii) the 3- and 5-OH groups in the A-ring. The catechol group induces a greater stability of aryloxy radicals produced as a result of flavonoid oxidation, possibly through H-bonding and electron-delocalization. Another function of the catechol moiety in the B-ring is the possible chelation of transition metal ions that may otherwise cause radical oxygen species formation via Fenton-type reaction[25]. The unsaturated bonds localized in the C-ring act enhancing the electron-transfer and radical scavenging actions through electron-delocalization. Finally, the presence of OH groups in the A-ring enables the formation of stable quinonic structures upon flavonoid oxidation. Furthermore, the

substitution of the 3-OH results in increase in torsion angle and loss of coplanarity, and subsequently, reduced antioxidant-activity.[26, 27]

Quercetin (3,3',4',5,7-pentahydroxyflavone, QUC, Figure 1) is of interest both as a dye as well as a biologically active molecule because of its chemical structure and unique properties. The study of QUC modifications can be interesting for controlling the chemical degradation of this compound, and consequently avoiding undesirable effects such as browning of QUC-containing mixtures (this aspect could be important, *i.e.* in food processing storage and art preservation). On the other hand, the antioxidant activity of flavonoids is related to their high tendency to undergo deep chemical changes. Quercetin possesses all the reactive sites that confer instability to flavonoids, then displaying the highest antioxidant capacity.[5, 28, 29]

In a previous work we have already shown by Raman spectroscopy that QUC undergoes significant structural changes at alkaline,[25] whereas the B-ring is involved in ring condensation reactions. However, although the initial step of these condensation processes is the conversion of the catechol moiety in the B-ring to *o*-quinone, the QUC condensation products may differ depending on pH because of the existence of different reaction pathways. This fact is particularly evident in the presence of silver nanoparticles, where the deprotonation state of the OH groups changes the reactive sites of QUC interacting with the surface, favouring one or more reaction pathways. Recently we have followed in more detail the chemical processes undergone by QUC on Ag nanoparticles (Ag NPs).[30] On these systems, QUC and other flavonoids display very complex chemical modifications due to the existence of different labile points in these molecules subjected to follow different processes: condensation, metal complexation, and a possible degradation. From SERS spectra obtained at different excitation wavelengths, concentrations and in the presence of chloride we have deduced that the interaction of QUC with the Ag surface mainly takes place through C5-

OH/C4=O and C4=O/C3-OH groups placed in A- and C- rings. We have also deduced that a chemical change takes place through the formation of C-C and C-O-C bonds between rings.

In the present work we have studied in more detail the influence of the pH on the chemical modification of quercetin and other related flavonoids in order to get more insight on the structural components that may be more important in these processes. This study was performed in aqueous solution and also on Ag nanoparticles to investigate the role of metal surfaces on the possible catalytical transformation of the flavonoid compounds. When Ag NPs are employed, the presence of C(3)-OH groups was important regarding the adsorption and catalytical modification of QUC, while at alkaline pH the presence of the catechol structure in the B-ring was reported to have a key importance in the head-to-tail condensation of the flavonol quercetin.[30] Therefore, in this work we have tried to investigate in more detail this issue and to evaluate the relative importance of these groups by obtaining the Raman and UV-visible spectra of structurally related flavones (luteolin, LUT; apigenin, APG), flavonols (quercetin, QUC; fisetin, FIS; morin, MOR; kaempferol, KMP; galangin, GAL), flavanone (taxifolin, TAX) and flavanol (catechin, CAT). This work was aimed at increasing the knowledge of the chemical properties of these compounds in order to improve their beneficial properties for the health by preventing their degradation by environmental factors, which will be also of interest for the preservation of the Cultural Heritage.

2. MATERIALS AND METHODS

QUC and other flavonoids were purchased in its highest purity from Sigma-Aldrich and used without further purification. For the most part, stock solutions of the dye in ethanol were prepared at a concentration of 10^{-2} M and stored in dark in order to protect them from light[31].

For the same reason, to minimize a possible photodegradation of flavonoid molecules, the examined solutions were protected from light during the out-of-measurement-times. Silver nitrate, trisodium citrate and other reagents were of analytical grade and purchased from Sigma-Aldrich and Fluka. All solutions were freshly prepared with triple distilled water before experiments and used immediately.

UV−visible spectra were obtained by using a UV-visible−NIR Shimadzu 3600 spectrometer equipped with a PMT for light detection in the UV−visible range and an InGaAs detector for the NIR region.

The normal Raman spectra were obtained directly from FT-Raman measurements. The liquid samples (flavonoid solution) were placed in 1-cm-path-length glass cuvettes. The FT-Raman spectra were obtained by using a Bruker RFS 100/S spectrometer. Radiation of 1064 nm from an air-cooled Nd: YAG laser was used for excitation. The resolution was set to 4 cm^{-1} and a 180° geometry was employed. The output laser power was 300 mW, and the laser power at the sample was 30 mW. The Raman spectra displayed in this work are the result of averaging 1000 accumulations.

The SERS spectra were obtained on Ag nanoparticles (Ag NPs) prepared by chemical reduction of silver nitrate with trisodium citrate dihydrate (AgC).[32] The colloid was activated before adding the dye. This activation consisted in a partial aggregation of the colloidal particles and, to accomplish this, an aliquot (usually 50 μL) of 0.5 M potassium nitrate were added to 1 mL of the colloid. Nitric acid and sodium hydroxide were employed to vary the pH value. In the latter cases even no nitrate was added, since the nitric acid and the hydroxide also induced colloid aggregation.

The SERS spectra registered at 785 nm laser line excitation were recorded in a Raman Microscope Renishaw RM 2000 instrument equipped with an electrically refrigerated CCD camera, working under macro conditions. The output laser power at the sample was 2 mW.

The geometry optimization and frequency calculations were performed by applying the DFT method using GAUSSIAN 09 package[33] at the B3LYP level supplemented with the 6-311++G** basis set. The PCM model[34], with water as a solvent, was employed as is implemented in GAUSSIAN 09 by default. The theoretical Raman intensities were derived from the computed Raman scattering activities.[35] Where needed, a wavenumber-linear scale factor on the calculated spectrum was utilized taking into account methods found in the literature for similar basis sets.[36]. This scaling is commonly needed in DFT calculations of Raman spectra in order to correct for correlation effects incompletely accounted for in DFT. All quantum mechanical computations were done on a high computational cluster server *Trueno* performed at the Instituto de Estructura de la Materia, CSIC in Madrid, Spain.

3. RESULTS AND DISCUSSION

UV-visible absorption spectra

Figure 2A shows the absorption spectra of QUC at different pH. As can be seen, the complex variation of the absorption spectra indicates that the species existing at different pH are not the simple result of a deprotonation of the QUC hydroxyl groups. In contrast, the absorption spectrum of LUT, a flavone which does not bear an OH group in the C3 position, at different pH shows a normal behavior regarding the pH (Fig. 2C), resulting from a molecular deprotonation as the pH increases.[37] At acidic pH LUT shows an intense band at 345 nm, which progressively decreases as the pH is raised while a new band at 398 nm appear. This red shift is attributed to the deprotonation of OH groups placed in B-ring.

FIS, another flavonol with similar structure to QUC, undergoes a similar pH behavior (Fig. 2B). In both cases (QUC and FIS) the increase of pH leads first to the appearance of a new peak at higher wavelengths (396 and 402 nm, respectively) at pH=9.2. At pH between 9.2 and 11.1 the latter peak undergoes a red shift, and a new peak at lower wavelengths is seen (311 and 335 nm, respectively). The absorption of this new lower peak grows progressively dominating the spectra at pH 12.4. The appearance of a band at low wavelength in both flavonols suggests that a deep chemical change involving the C-ring occurs, thus leading to a loss of electronic resonance between rings A and B.[25, 38] These results suggest the existence of a correlation between the flavonols molecular structure and the spectral behaviour at different pH.[39] In particular, the presence of the C3-OH group in both flavonols seems to be the responsible for the above behavior.

Raman spectra

Effect of alkaline environment

Figure 3 shows the Raman spectra of QUC, FIS and LUT dissolved in ethanol and in 0.5 M NaOH aqueous solution (pH ~ 12-13). As expected, QUC and FIS molecules present very similar Raman spectra at high pH (Fig. 3b and d) and at the same time very different to the Raman of these compounds in ethanol solution (Fig. 3a and c). On the contrary, the Raman spectrum of LUT at alkaline pH (Fig. 3f) shows no significant changes produced by increasing the pH in relation to the ethanolic solution (Fig. 3e). This is probably related to the lower planarity deduced for the latter molecule[40]. Thus, confirming again that the OH group at position C3 is an important source of instability in flavonoids under alkaline conditions.

The Raman study was also extended to other structurally similar flavonoids at alkaline pH in order to monitor the influence of the C3-OH group on the molecular instability of flavonols. KPF and GAL, two flavonols bearing the C3-OH group in its structure, but lacking

the catechol moiety in the B-ring show a lower reactivity as can be deduced from their Raman spectra (Fig. S1b and d, respectively). The spectral changes observed for the latter molecules can be rather associated to the deprotonation of the molecule. Besides, the Raman spectrum of MOR, a structurally isomeric molecule of QUC which only differs in the substitution pattern of the B-ring, at alkaline pH exhibits a behavior similar to QUC and FIS, *i.e.* with significant differences regarding the Raman in ethanol (Fig. S1f). Moreover, the lack of the double bond C2=C3 in the structure of TAX breaks electronic resonance between the B-ring and the A and C rings giving rise to a Raman spectrum which significantly differs from the flavonols (Fig. S1g and h). Finally, APG, which possess neither C3-OH group nor catechol moiety, demonstrates a higher stability at alkaline conditions, as in the case of LUT (Fig. S1j).[39]

From the above results we can conclude that there are several important structural points which determine the reactivity of the flavonoid molecule at alkaline pH. The OH group at position C-3 plays an important role in the chemical modification of these molecules, but only when catechol or resorcinol moieties are present in the B-ring the chemical modifications are thus much stronger. If the molecule possesses the catechol group, but lacks the C3-OH group, the chemical modifications are minor and they are dominated by deprotonation of the molecule. Finally, the lack of the double bond C2=C3 gives rise to a different behaviour. QUC possesses both the C3-OH group as well as catechol moiety in the B-ring showing a higher reactivity under alkaline conditions. Thus our investigation was focused on this flavonoid.

Oxidation of quercetin provoked by high pH

In order to study in more detail the oxidation process undergone by QUC at high pH, the Raman spectra of QUC in aqueous alkaline solutions at pH values from 8.6 to 13.4 was

studied. Some representative spectra are depicted in Figure 4. Raman spectra of QUC in aqueous alkaline solutions with pH from 6.7 to 9.5 (Fig. 4a-c) only showed a progressive weakening of bands at 1402 and 1547 cm^{-1}. These bands are assigned to the vibrations of δ(C5,7-OH), δ(CH(A)), and δ(C5-OH) coupled with the ν(C=O) and ν(A) vibrations respectively.[30, 40, 41] Other bands which are also associated to the vibrational modes of the A- and C-rings, for example, at 1327 and 1589 cm^{-1}, decrease their relative intensity and shift to lower wavenumbers[40, 41]. The changes observed at these mild alkaline conditions can be related to the deprotonation of QUC, as also suggested by the absorption spectra, which seems to involve the C7-OH group of the A-ring.

A further increase of the pH (up to 11.1) leads to stronger spectral changes in QUC (Fig. 4d-e). Specifically, the band associated with the ν(C=O) did not significantly change its intensity, but it shifted from 1665 to 1635 cm^{-1}. On the other hand, the bands at 1518 and 1364 cm^{-1}, which are attributed to the B- and C-ring, significantly increased. The structural changes involving the C-ring influence also the vibrational modes connected with the A- and B-rings, in particular, the δ(CH) and δ(C-OH) vibrations. Based on the observed changes at these pH values we suggest that QUC undergoes the ionization of the OH group at position C3 (C-ring), probably related to the red shift of the absorption maximum at pH between 9.0 and 11.0.

The most prominent changes observed in the spectrum of QUC on varying the pH occur when increasing the pH above 11.5 (Fig. 4f-g). In particular, the band at 1562 cm^{-1} becomes the most intense of the spectrum. This band is assigned to the stretching vibration of the C=O group coupled to CH and C-OH in-plane deformations in the A-ring[41]. On the other hand, the band at 1636 cm^{-1}, associated with ν(C2=C3)/δ(C3-OH) vibrations, almost disappeared.

Moreover, the band at 1603 cm^{-1} shifts to 1586 cm^{-1} and becomes a shoulder, while the band at 1518 cm^{-1} shifts to 1501 cm^{-1}. The change of the latter band is likely associated to a substantial change of the C2-C1' bond connecting the C- and B-rings. Finally, the 1111/1094 cm^{-1} doublet (Fig. 4g), associated with the B- and C-rings but especially with the C-O-C bond, is significantly intensified. Thus, three different regions can be clearly observed what suggests at least three different processes which take place through the QUC molecule.

In order to study all these changes in a more systematic way, we have analyzed the ratios of representative bands which undergo significant modifications in the three pH regions mentioned above. Thus the I(1547/1372), I(1518)/I(1372), I(1603)/I(1372) ratios were plotted against the pH giving rise to three sigmoid figures that, linked together and subjected to mathematical treatment, give rise to the plot displayed in the inset of Figure 4. From the latter plot two pKa values of QUC were deduced: 9.19±0.11 and 11.06±0.04 corresponding to the two first inflection points. These values are in very good agreement with those deduced from UV-Vis absorption spectra[39] as well as those found in the literature.[42, 43] The third pKa expected above the pH 13 was not able to be determined because of the lack of data owed to the high instability of samples at extreme pH.

All the above results indicate that QUC experiences a significant structural modification at high pH that mainly affects the C-ring, which is modified thanks to the ionization induced on the C3-OH group at alkaline pH. The resulting Raman spectrum (Fig. 4g and that of Fig. 3b) has some similarities with the calculated spectrum of benzofuranone (Fig. S2b), thus we suggest that this is the QUC oxidation product (QUC*) generated at pH above 11.0 (inset in Fig. 3b).

Oxidation of fisetin provoked by high pH

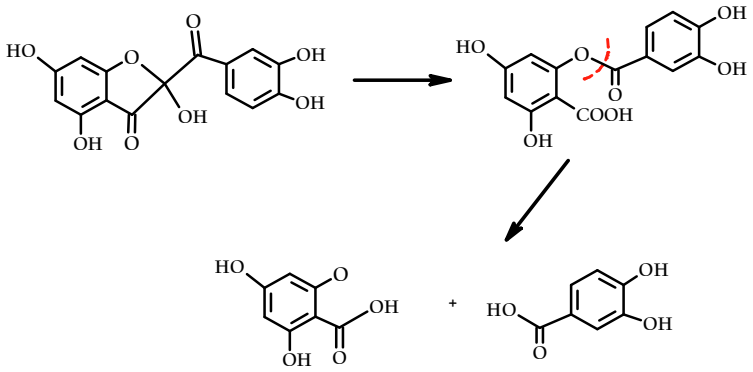
FIS displays a similar trend at alkaline pH as deduced from the Raman spectrum of FIS at high pH (Fig. 3d). The latter spectrum may correspond to an oxidation product of FIS (FIS*) which shows some structural correlation with that of QUC*. The intense band at 1605 cm^{-1} seen in the FIS* spectrum suggests the existence of the C2=C3 double bond in the FIS* which is not observed in the Raman spectrum of QUC*. A band at 1561 cm^{-1} is observed in both spectra, likely associated to the benzoyl group. However, FIS* also shows an intense band at 1397 cm^{-1} attributed to the vibration δ(C7-OH). The band at 1358 cm^{-1}, assigned mainly to δ(C-OH) vibrations and stretching vibrations of A- and B-ring[30], is much more intense in the spectrum of QUC*, thus indicating a lower electronic resonance between the B-ring and the rest of the molecule. All the above features led us to proposed for FIS* the structure indicated in Figure 3d. Therefore, the Raman spectra indicate that the chemical structures of QUC* and FIS* match quite well that of the products proposed by other authors in the literature[44, 45].

DFT calculations of QUC* and FIS* oxidation products were carried out in order to have more insight on the nature of the oxidation products of these flavonols. The calculated spectra are shown in Figure S2 and S3, for QUC* and FIS* respectively, showing a large number of similarities between the experimental and calculated data in what respect the position of the theoretical bands, although the relative intensity is not well reproduced. The presence of a large number of features in the experimental spectra of these products indicates that many different molecular species may be formed at alkaline pH and that the nature of these products may evolve at different reaction times.

Time evolution: quercetin degradation

It is important to highlight that all the spectra shown here at high pH were obtained immediately after sample preparation and in the presence of oxygen. In previous works we have reported the effect of O_2 on the chemical transformation of QUC both in solution and at alkaline pH.[25] In addition, the time is also an important parameter in the QUC degradation, affecting specially the oligomer/polymer formation.[25] To monitor this effect we have obtained Raman spectra at conditions at which the autoxidation of QUC is already induced (*i.e.*, QUC dissolved in 0.5 M NaOH aqueous solution, corresponding to pH ≈ 12-13) as a function of time (from hours to nearly two months) (Figure 5). At first look, the Raman spectra show a gradual decrease of the total intensity which denotes a removal of the QUC products from the surface. In addition, the spectral profile also changed significantly with the time loosing resolution in comparison to the first spectrum. The Raman spectrum registered after 56 days shows bands at 1575 and 1627 cm^{-1} (Fig. 5h) indicating the existence of systems with both C-C double bonds and carbonyl group. On the other hand, the doublet at 1111/1091 cm^{-1} (Fig. 5a) corresponding to ν(C-O-C) shifted and converted to a single band at 1065 cm^{-1} in Fig. 5h. The 1111/1065 cm^{-1} intensity ratio was plotted against the time (Fig. 5, inset). As can be seen, after 2 hours there is a drastic intensity decrease of the latter band indicating that the degradation process starts after a previous activation. These results together with the data on the degradation of flavonols and flavones found in the literature[44, 46-50] suggest that QUC first undergone an oxidation to QUC* and then a fragmentation leading to simpler molecular products, likely hydroxybenzoic acids, as indicated in Scheme 1. This is corroborated by the intensification of the absorbance band at 311 nm in the absorption spectrum (Fig. 2A), which can be attributed to the formation of smaller aromatic compounds in the suspension. A similar fragmentation process has been also reported for other flavonoids by other authors.[46, 51, 52]

Scheme 1



The analysis of the resulting oxidation and degradation products represents indeed a very valuable structural information which allows to determine potential spectral markers of flavonoids used in the original dyeing of historical textiles which are supposed to have undergone similar processes in the textile fibers.[53]

Furthermore, there are a number of other spectral markers which also indicates the possible key role of other processes in the chemical modification of flavonoids with the high pH and the time. In particular, the doublet at 1111/1091 cm^{-1} (Fig. 5a), which indicates that a process of polymerization giving rise to the formation of higher molecular weight products, as also reported in the literature[54], may also take place in the mixture. This polymerization was already observed in previous works carried out by our group for several simpler phenolic compounds, such as catechol, caffeic acid and gallic acid.[55-57] However, in the latter cases the polymerization was due to the presence of nanostructured metal surfaces. The polymerization process is also corroborated by the absorption spectra of UV-Vis QUC registered at alkaline pH over the time,[25, 58, 59] which confirmed the formation of quinonic structures and the subsequent formation of oligomers and/or polymers formation caused by the autoxidation of

QUC and the consecutive condensation reactions thereafter. A similar conclusion was deduced for catechin upon analysis of the UV-visible absorption spectra.[58] The results evidence two possible polymerization mechanisms for QUC: (a) condensation reactions between the oxidation products of QUC created without breaking the C-ring; and (b) condensation reaction between the simplest units coming from the degradation QUC at high pH. These results indicate that autoxidation and polymerization processes play an important role in the chemical transformation experienced by QUC in the long-term scales and that constitutes the basis of the general process of what we called flavonoids degradation.

SERS spectra

Quercetin

The SERS spectra of QUC on Ag NPs measured at different pH, ranging from the acidic to the alkaline regions, are shown in Figure 6. As reported in previous works, QUC undergoes a large chemical modification on metal NPs even at neutral pH mainly involving the dimerization and possible oligomerization of the flavonoid on the surface.[25, 30] When increasing the pH a general weakening of the spectrum intensity is seen. This effect is attributed to the ionization of QUC upon deprotonation at alkaline pH, which induces the appearance of negative charges in QUC, and to the increasing concentration of hydroxyl ions which tend to be strongly attached to the metal surface. Another important effect observed at alkaline pH is the modification of the spectral profile. In particular, we have focused our attention into the modification of the relative intensities of fingerprint bands at 481 and 421 cm^{-1} appearing at acidic pH on going from acidic to alkaline pH. In this regard, the 481/421 cm^{-1} intensity ratio changes with the pH and these bands are shifted to 444 and 525 cm^{-1} at pH

values above 7.0, thus indicating the formation of different condensation products. In previous work we have already proposed that the bands observed at neutral pH at 481 and 421 cm^{-1} can be associated with the condensation processes in which the A- and C-ring play an important role.[30] Thus, the spectral changes occurring in this region of the SERS spectrum on increasing the pH can be attributed to a change in the dimerization protocol with a possible key role of the B-ring.

Once again the comparison with other flavonoids was crucial to understand the polymerization mechanism of QUC. To help in this study we have obtained the SERS spectrum of catechin (CAT; Fig. 6h). CAT is a flavonoid which does have neither the carbonyl group in position C4 nor the C2=C3 double bond, thus the main reactive group in this molecule and a possible anchoring point to the surface is the catechol moiety in the B-ring. The SERS of CAT shows a similar profile in the 400-600 cm^{-1} region in comparison to the SERS of QUC registered at pH 12.5 (Fig. 6g). In addition, the 1100-1700 cm^{-1} region also shows a large number of similarities regarding the CAT spectrum, thus indicating that the polymerization mechanism of QUC at alkaline pH takes place through the B-ring since this is the main reactive point in CAT. In addition, it is known that the oxidation of CAT in solution leads to the formation of oligomers/polymers mainly due to the condensation reactions between the A-ring of one unit and the B-ring of another.[60] Thus, although different condensation pathways are possible for QUC, the prevalence of the A-B reactions seems to be favored under alkaline conditions.[25] In relation to this, the conversion of *o*-phenol to *o*-quinone occurring at high in the B-ring seems to facilitate the interaction of QUC with the surface and its subsequent dimerization as also reported by other authors for CAT in solution.[60-62]

Fisetin

The effect of pH was also checked in the case of FIS (Figure 7). This molecule shows at neutral pH an intense band at 475 cm^{-1} (Fig. 7d), which is intensified at acidic pH (Fig. 7b-c). The latter band is weakened at pH above 7.0, as in the case of QUC, while the band at 396 cm^{-1} shifts to 414 cm^{-1}. Since these usually strong bands present in the low wavenumber region are attributed to skeletal modes of the polymeric species, it is obvious that the process of polymerization undergoes a dramatic change at high pH for FIS molecule. The marked intensity decrease of bands below 600 cm^{-1} suggests that a lower polymerization occurs in this molecule. This is likely due to the lack of the OH group in the C5 position of the A-ring. In addition the bands falling in the 1100-1700 cm^{-1} region also undergo strong changes and a general broadening, but it is difficult to separate the effect of deprotonation to that of a possible polymerization at high pH.

Luteolin

Finally, SERS spectra of LUT on Ag NPs at different pH are displayed in Figure 8. This flavone displays low spectral changes at pH above 7.0, in contrast to what happens to QUC and FIS. It is necessary to increase the pH to 10.0 in order to observe strong changes in the SERS spectra which can be rather attributed to the ionization and interaction with the silver.[63] Thus, we can conclude that the presence of the C3-OH group is important to induce the flavonoid condensation as indicated the absence of bands corresponding to the molecular oligomerization at alkaline pH conditions in the SERS of flavonoids which do not contain this group in their structure.

4. CONCLUSIONS

Flavonoids undergo a clear chemical modification in alkaline aqueous solution consisting in an autoxidation that mainly affects the C-ring. This process occurs at pH above 11.0 and implies the formation of a benzofuranone as a first step which can further undergo a fragmentation leading to simpler molecular products, which can undergo a subsequent polymerization.

The comparison of Raman spectra of different flavonoids indicates that there are several important structural points which determine the reactivity of the flavonoid molecule at alkaline pH: (i) the C3-OH group in the C-ring; (ii) the catechol moiety in the B-ring; and (iii) the C2=C3 bond in C-ring. QUC possesses all these groups and exhibits a high instability in alkaline solution. The reactivity of the C3-OH group is enhanced by the presence of a catechol moiety in the B-ring. If the molecule possesses the catechol group, but lacks the C3-OH group, the chemical modifications are minor and they are dominated by deprotonation of the molecule. The lack of the double bond C2=C3 gives rise to different chemical modifications.

On Ag NPs the SERS spectra of QUC reveal that this molecule undergoes a large chemical modification on metal NPs even at neutral pH mainly involving the dimerization and possible oligomerization of the flavonoid on the surface where the A- and C-rings have high importance. At alkaline pH, the spectral changes revealed a change in the dimerization protocol of QUC with a possible key role of the B-ring. FIS and LUT also undergo chemical changes following different polymerization process at alkaline pH, although in these cases it is difficult to discern the effect of deprotonation to that of polymerization.

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Legends of Figures

Figure 1

Molecular structure and atomic numbering of flavonols quercetin and fisetin, and flavone luteolin.

Figure 2

UV-vis absorption spectra of 5 x 10^{-5} M aqueous solution of quercetin (A), fisetin (B) and luteolin (C) at different pH.

Figure 3

FT-Raman spectra of quercetin, fisetin and luteolin in absolute ethanol (EthOH) and in 0.5 M NaOH aqueous solution (NaOH); 20mg/mL, pH ~ 12-13. Band marked with asterisk corresponds to the EthOH residues. The excitation line was 1064 nm. Inset: Molecular structures of the oxidation products proposed for QUC and FIS.

Figure 4

FT-Raman spectra of QUC in aqueous solution (20mg/mL) at the following pH: (a) 6.7; (b) 8.9; (c) 9.5 M; (d) 10.6; (e) 11.1; (f) 13.0; (g) 13.4. The excitation line was 1064 nm. The spectra have been normalized to the intensity of the band at ≈ 600 cm^{-1}. Inset: Average of three intensity ratios (I(1547/1372); I(1518)/I(1372); I(1603)/I(1372)) plotted against the pH.

Figure 5

FT-Raman spectra of QUC in 0.5 M NaOH aqueous solution (20mg/mL) recorded at different times: (a) 0 h; (b) 0.1 h; (c) 2.1 h; (d) 5.1 h; (g) 24 h; (j) 392 h; (o) 1200 h; (p) 1340 h ≈ 56 days. The excitation line was 1064 nm. The spectra have been normalized to the intensity of the band at ~ 130cm^{-1}. Inset: The 1111/1065 cm^{-1} intensity ratio plotted against the time.

Figure 6

SERS spectra of quercetin (QUC; 1 x 10^{-5} M) in AgC colloid recorded at different pH values: (a) 4; (b) 5; (c) 6; (d) 7; (e) 8; (f) 10.5; and (g) 12.5. (h) SERS spectrum of catechin (CAT; 1 x 10^{-5} M) in AgC colloid recorded at neutral pH. The excitation line was 785 nm. The spectra of QUC have been normalized to the intensity of the band at 1337-1343 cm^{-1}. The spectrum of CAT has been normalized to the intensity of the band at 444 cm^{-1}. Bands marked with asterisk correspond to the citrate present in the AgC colloid.

Figure 7

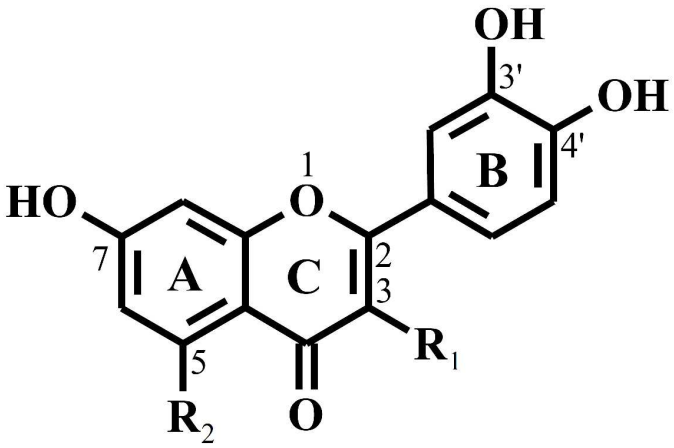
SERS spectra of fisetin (FIS; 1 x 10^{-5} M) in AgC colloid recorded at different pH values: (a) 4; (b) 5; (c) 6; (d) 7; (e) 9; (f) 11.5. The excitation line was 785 nm. The spectra of FIS

have been normalized to the intensity of the band at 1352-1355 cm^{-1}. Bands marked with asterisk correspond to the citrate present in the AgC colloid.

Figure 8

SERS spectra of luteolin (LUT; 1 x 10^{-5} M) in AgC colloid recorded at different pH values: (a) 4; (b) 5; (c) 6; (d) 8; (e) 10.5; (f) 12.5. The excitation line was 785 nm. The spectra of LUT have been normalized to the intensity of the band at 1212-1219 cm^{-1}. Bands marked with asterisk correspond to the citrate present in the AgC colloid.

Figure 1



Quercetin (QUC): R1 = OH, R2 = OH

Fisetin (FIS): R1 = OH, R2 = H

Luteolin (LUT): R1 = H, R2 = OH

Figure 2

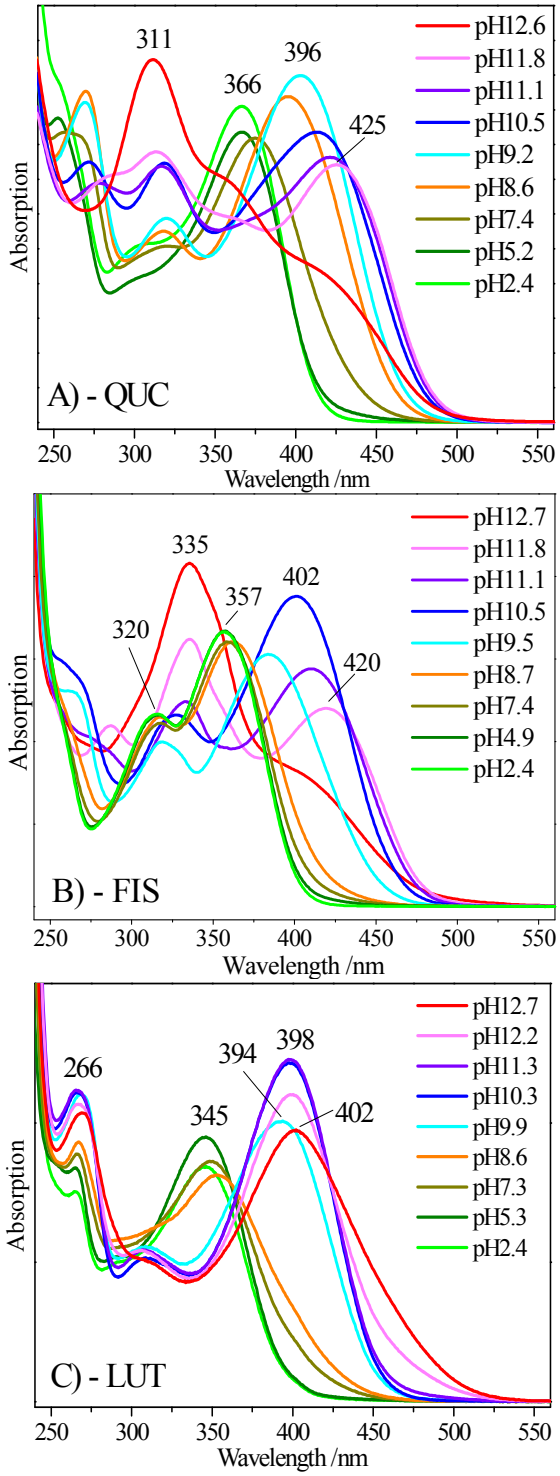


Figure 3

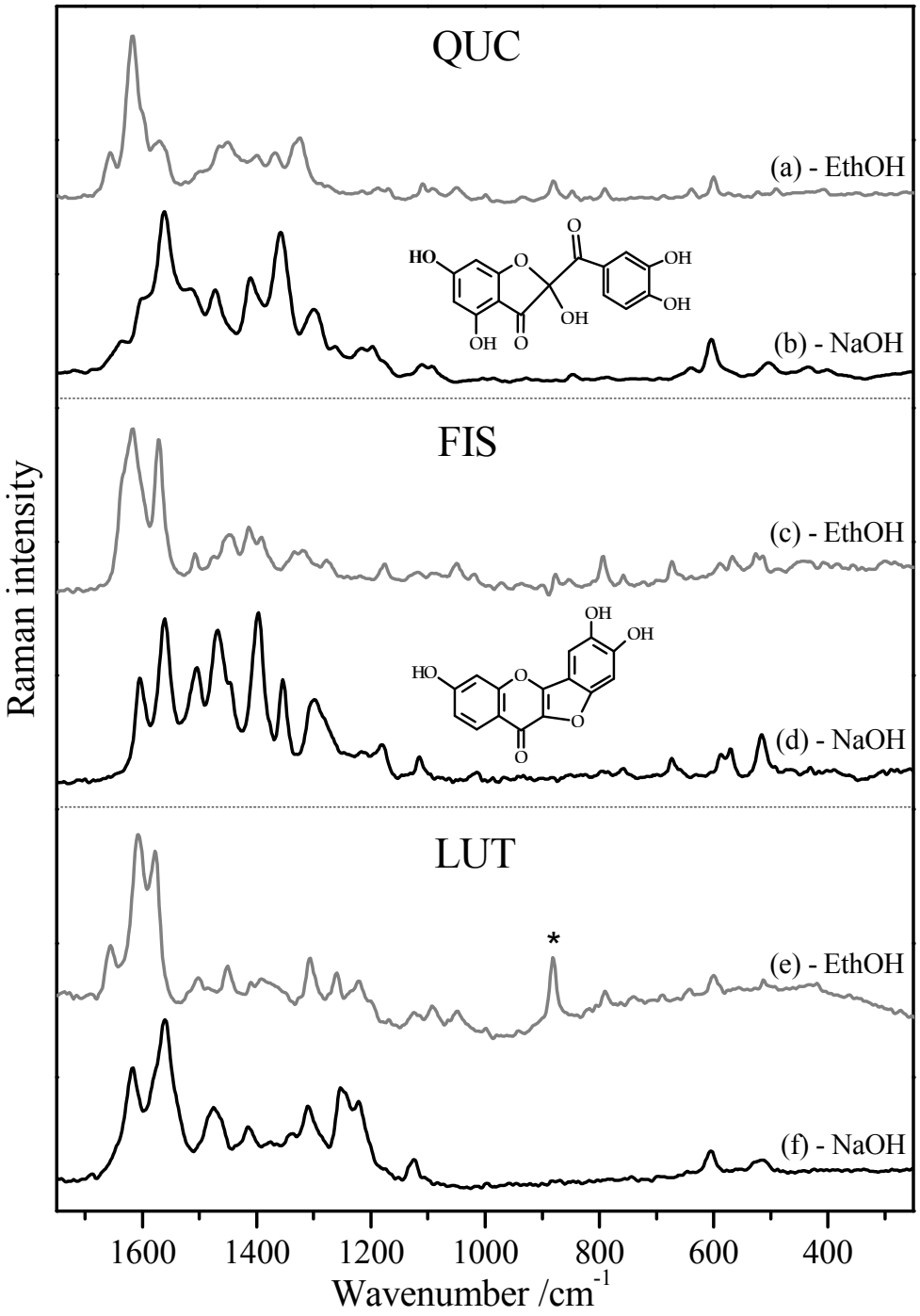


Figure 4

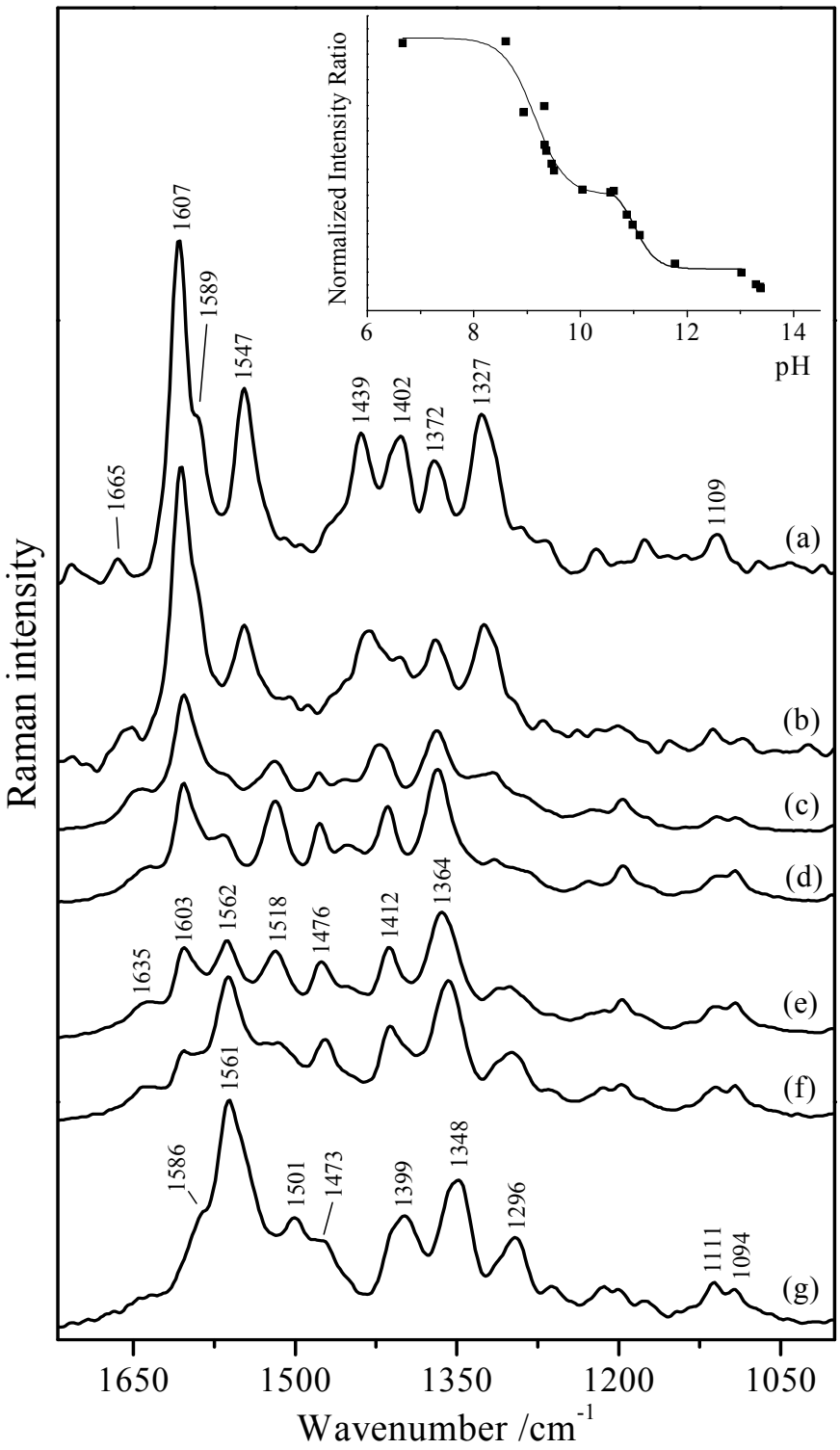


Figure 5

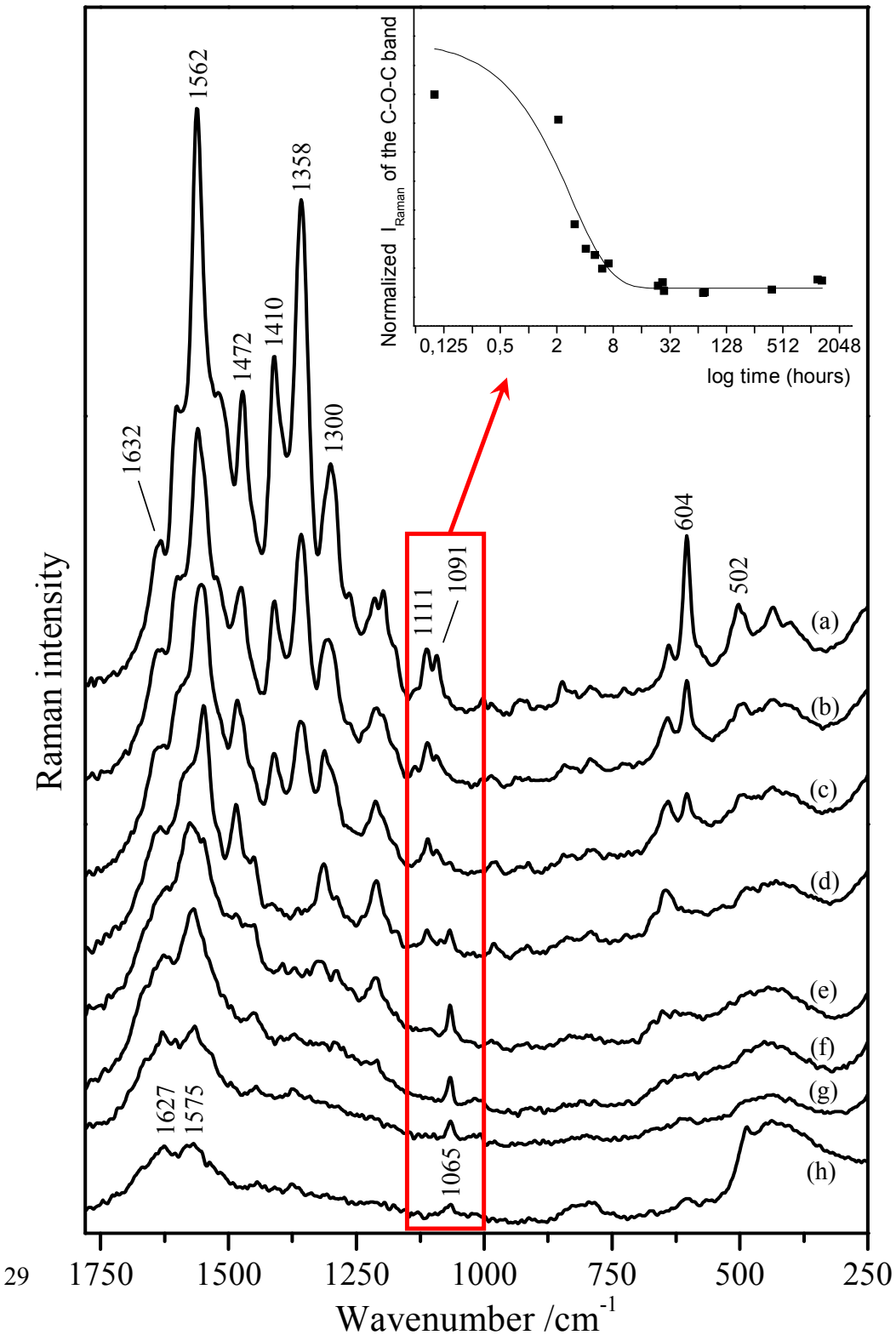


Figure 6

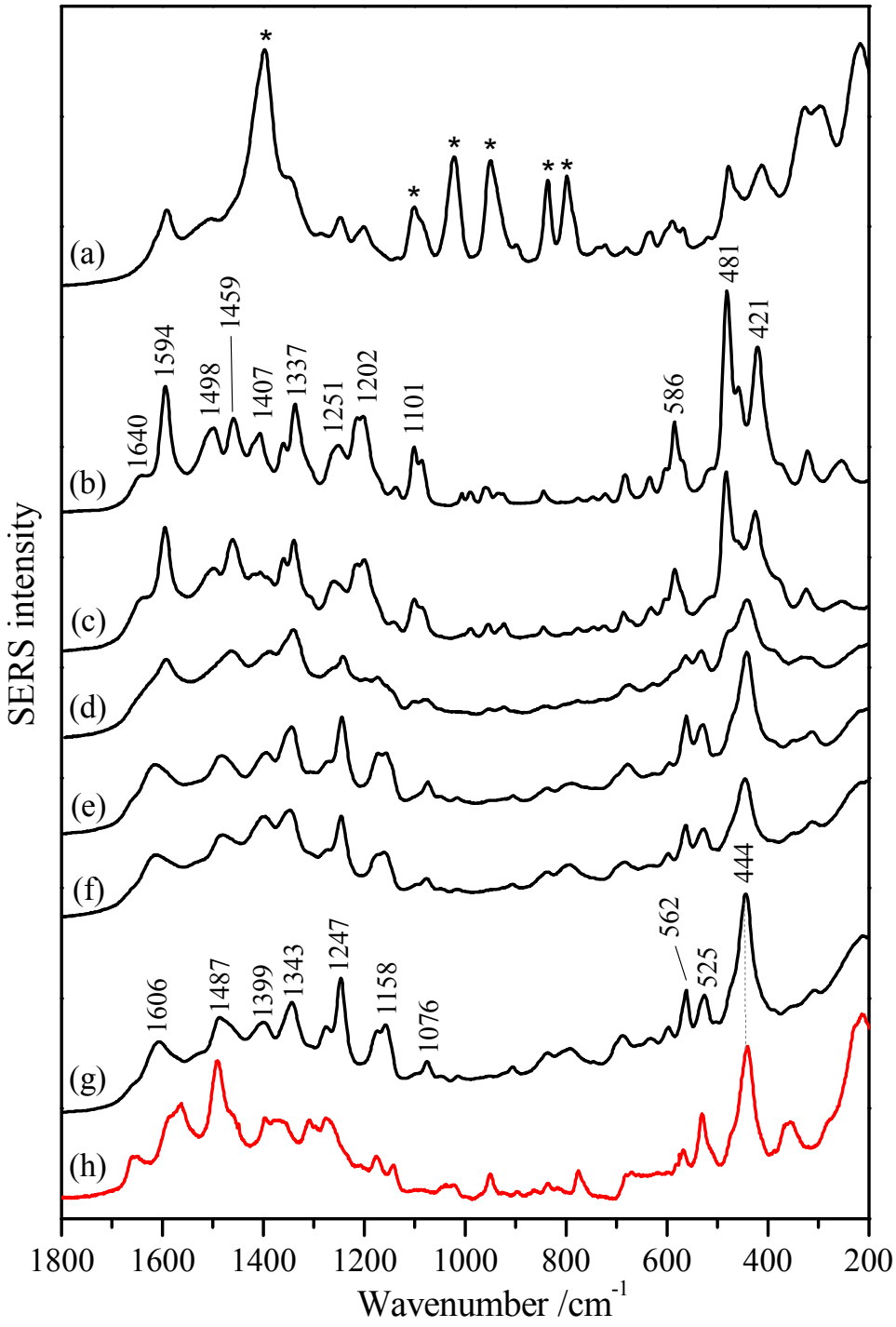


Figure 7

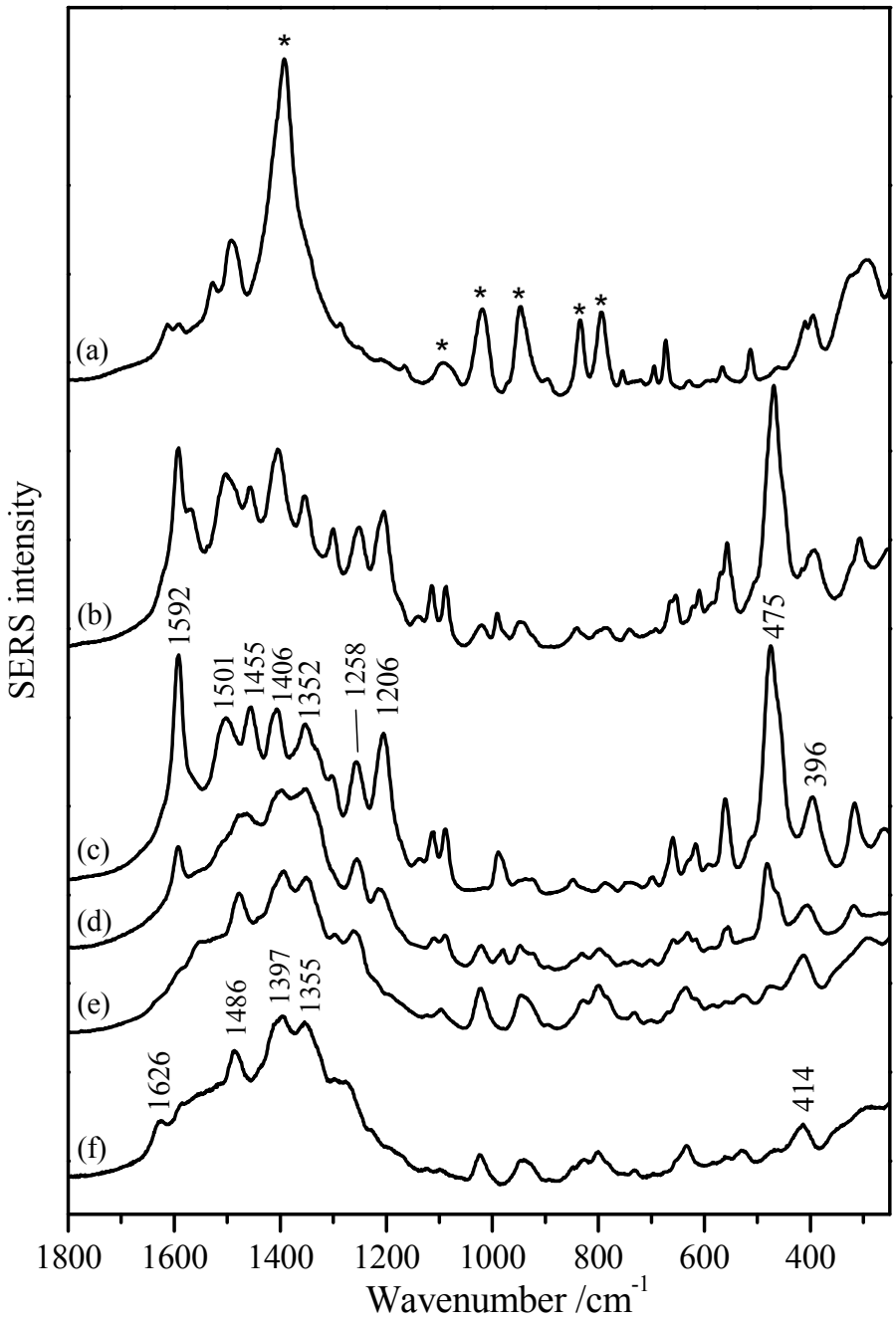
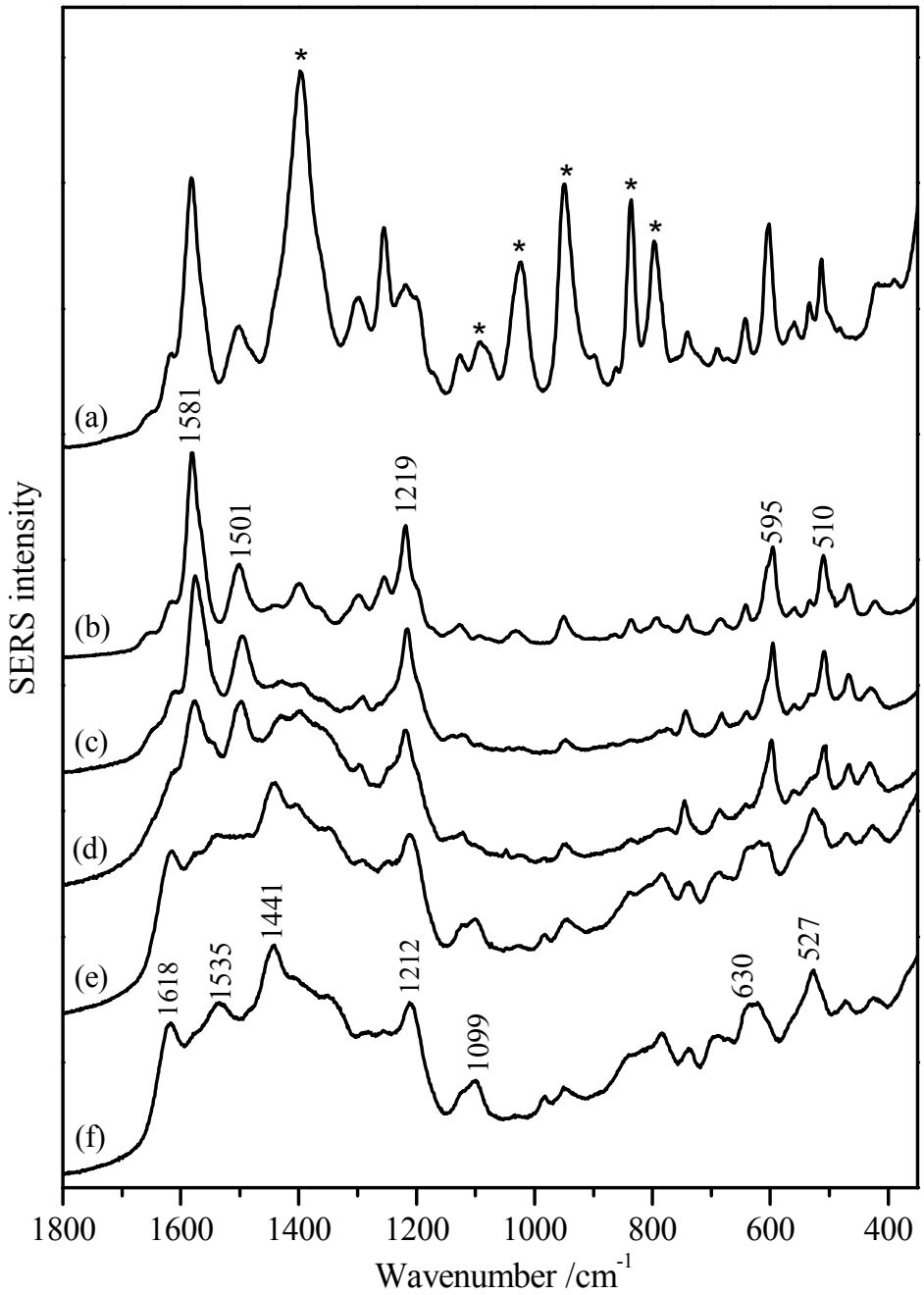


Figure 8



Graphical Abstract