Surface-enhanced Raman and fluorescence joint analysis of soil humic acids

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Abstract
Surface-enhanced Raman scattering (SERS) and surface-enhanced fluorescence (SEF) combined emissions were used in this work to the analysis of humic acids (HA). This study examined HA structure at different pH and HA concentrations and assessed the structural differences taking place in HA as a result of various amendment trials. Raman and fluorescence emissions behave in opposite ways due to the effect of the metal surface on the aromatic groups responsible for these emissions. The information afforded by these techniques can be successfully employed in the structural and dynamic analysis of these important macromolecules. The surface-enhanced emission (SEE) spectra, that is the sum of the Raman and the fluorescence emissions, were acquired by using both macro- and micro-experimental configurations in order to apply imaging and confocal Raman and fluorescence spectroscopy techniques on the analysis of HA.

1. Introduction
Humic substances (HS) are the most important organic components of soil and the most widely distributed organic products of biosynthesis on the face of the earth [1]. In addition to the quantitative importance of HS, these substances act as a sink and source of C, they influence the soil fertility through the release of nutrients and affect the fate of contaminants leading to soil remediation [2,3]. Although HS are widespread in soils and water, during a long time their structure remained unknown, but in the last years the knowledge on these compounds has notably increased [2,4–6]. HS are divided into two main groups: humic acids (HA) and fulvic acids (FA) each displaying a different solubility in acidic media [7].

The inherent complexity of HS has seriously limited the application of more traditional optical spectroscopies, such as Raman and fluorescence spectroscopy, in structural and dynamic studies of HS. Fluorescence spectroscopy has been used so far in the characterisation of HA by several authors [8–11]. However, the high complexity of HS limits the application of this technique to these compounds. Raman spectroscopy is a technique, which has been widely applied, in structural studies of organic molecules and biological compounds [12]. However, normal Raman spectroscopy cannot be employed in the structural characterisation of HS due to the intense fluorescence emission, which overlaps the Raman bands.

Over recent years, several optical spectroscopy techniques (Raman, IR and fluorescence) have undergone a renaissance due to the notable characteristics of metal nanostructures (MNs) where localised surface plasmon (LSP) can occur. LSP leads to a remarkable local electromagnetic field enhancement owing to the high absorption of light in the vicinity of metal nanoparticles and thus induces a huge enhancement of
spectroscopic signals, mainly in the case of emission signals of molecules placed in the proximities of the surface [13–15].

Raman scattering is greatly enhanced as a consequence of LSP. For this reason, the surface-enhanced Raman scattering (SERS) technique can be successfully applied to the study of highly fluorescent molecules in water, due to the fluorescence quenching occurring on the metal surface via a charge-transfer mechanism [16]. This effect enables Raman to be applied to the characterisation of HS [17–20].

SERS spectra can provide important information on the HS structure in function of factors such as the humification degree [21], extraction and purification processes [22], the soil amendment trials [23], HS extraction fractions [17,24] and pH [25]. The growing importance of SERS in the analysis of HS has recently been highlighted in several reviews dealing with techniques applied to the analysis of HS [26–28]. SERS has also been used to examine pesticide–HA interaction [29]. However, SERS is not a quantitative technique because of effects such as the resonance Raman and the adsorption on surfaces [30], although these characteristics can also serve to study structural and dynamic processes undertaken by macromolecules in the presence of surfaces [31].

The surface-enhanced fluorescence (SEF) technique has not been employed as much as SERS because of charge-transfer effects, which take place on the surface and which, in turn, lead to an actual quenching of the fluorescence. SEF effect is also induced by LPR near a metal surface [32,33]. However, the net effect seems to vary depending on the distance between the fluorophore and the surface, as well as on the intrinsic quantum yield of the fluorophore [34]. In general, the fluorescence is not quenched if the molecule is relatively far from the surface. The estimated optimal distance for a good SEF enhancement is achieved above 100 Å [35,36]. At distances further than this value, SERS and SEF signals can be simultaneously emitted and registered for molecular species placed in the vicinity of MNs [36–38]. Since the average size of HA is larger than this value [39], the fluorophores included in the HA structure could be good candidates to give rise to intense SEF + SERS joint emission spectra, without the intrinsic quenching observed on a surface. This is possible due to the fact that the HS cross-sections of either Raman or fluorescence are of the same order on MNs, and thus, a surface-enhanced emission (SEE) is seen for HS adsorbed on these substrates (Fig. 1, bottom). Furthermore, the SEF emission affords information on the HA macromolecules adsorbed onto a surface,

Fig. 1 – Top: experimental set-up for SEE measurements. Bottom left: a SEM image of typical Ag nanoaggregates. Bottom right: scheme displaying the adsorption of HA micelles and the emission of Raman a fluorescence combined process.
2. Materials and methods

The soil samples analysed (horizon Ap, 0–40 cm depth) were taken from plots of a 30 plus year field experiment conducted at the agricultural farm of the University of Bologna’s Agricultural Faculty (Cadriano, Italy). This soil is classified (Soil Survey Staff, USDA SCS 1989) as a Typic Udorthent.

The experimental design included plots amended over a 30-year period with cattle manure (CM) and crop residues (CR) constituted by wheat straw or corn stalks (each biomass followed in succession), with unamended soil as control (C). CM constituted by wheat straw or corn stalks (each biomass followed in succession), with unamended soil as control (C). The combination of surface-enhanced techniques and microscopy is also a powerful methodology which has attracted much attention in the analysis of biological systems such as cells or bacteria \[42,43\] and which could be very helpful in the characterisation of HS.

In this work we analysed SEE spectra of HA. This work represents the first time that SEF technique is used in the characterisation of HA. The HA compounds selected for this study were extracted from a soil amended over a period of 30 years under several conditions and the corresponding control experiments in order to evaluate the structural variations induced by the different treatments. The SEE spectra were acquired by using both macro- and micro-experimental configurations in order to investigate the abilities of the new imaging and confocal Raman and fluorescence spectroscopy techniques on the analysis of these HA.

2.1. SERS spectroscopy

Colloidal silver nanoparticles were prepared by using hydroxylamine hydrochloride as reducing agent. In a recent work, a deep study of Ag MNs prepared by reduction with hydroxylamine (AgHx) was accomplished \[44\]. AgHx was activated by the addition of potassium nitrate, which induced the particle aggregation to form other larger aggregations. This process is necessary when using these systems as a substrate for SERS, since specific morphological requirements are needed to enhance the electromagnetic field on the metal surface. A typical Ag MNs used to record the SEE spectra is shown in Fig. 1 (bottom).

Samples for SEE measurements were made by adding an aliquot of an aqueous HS solution, prepared by solving 1 mg of the corresponding humic fraction in 1 mL of tri-distilled water, to 1 mL of the silver colloid until the desired concentration. The pH of the mixture was adjusted by adding 0.1 M NaOH or HNO₃. All solutions were prepared with tri-distilled water.

The SERS spectra were recorded by means of a Renishaw Raman Microscope System RM2000, using the macro-configuration equipped with a notch filter and an electrically refrigerated CCD camera (575 × pixels) as indicated in Fig. 1. The Raman and fluorescence emission spectrum is recorded by the CCD camera. The SERS spectra were obtained by using a 100× objective (NA = 0.9). Confocal micro-SERS and Raman images were recorded by using a PRIOR motorised stage coupled to the microscope with a 1 μm step. The SERS spectra were baselined to withdraw the contribution of the fluorescence by using the algorithm provided by the Origin 6.0 Program.

3. Results and discussion

3.1. SERS spectra of humic acids

Fig. 2 shows the SERS spectra of HA at pH 10.0, after correction of the fluorescence background. At pH 10.0 the SERS spectra of all HA samples are dominated by two very strong and narrow bands, at 1308 and 1611 cm⁻¹, which can be attributed to the aromatic part of HS and in particular to polycyclic aromatic hydrocarbon (PAH) structures. The nature of these PAH residues is still unknown, but the similarity of the SERS pattern observed at high pH and the Raman spectra of, perylene \[45\] and coronene \[46\] compounds suggests that similar structures could exist in HA, either from anthropogenic...
or natural origins [2,7]. The situation seems to be different in the case of FA, since no PAH bands are observed in these compounds [24]. The presence of PAH in the SERS spectrum recorded at alkaline pH, suggests that PAH residues might be mainly localised inside the HA structure, since a HA structure expansion is expected at high pH due to ionisation of carboxylic and phenolic groups [47]. Although the macromolecular expansion is limited by chemical cross-linkages [48], it seems to be enough to allow a higher exposure of the polyaromatic groups to the metal surface. The bands corresponding to PAH residues are resonantly enhanced as revealed the appearance of the overtone and combination bands at 1948, 2577, 2913 and 3228 cm\(^{-1}\) in the SERS spectra (Fig. 2). However, we cannot deduce that PAH groups are quantitatively predominant in the HA structure, since these groups undergone a selective enhancement by SERS.

The SERS spectra in Fig. 2 were normalised to the water band appearing at 3420 cm\(^{-1}\). As can be seen, the CR and CM samples display a lower SERS intensity in comparison to the control samples C0 and C30. In order to better understand this behaviour we carried out a more detailed SERS study of HA at different pH values using the C30 sample (Fig. 3), since this sample was the one, which displayed the most intense variation.

The bands at 1308 and 1611 cm\(^{-1}\) observed at pH 10.0 (Fig. 3a) decreased markedly at pH 5 (Fig. 3d), as a consequence of: (i) an increase of intermolecular interactions, due to both a decrease of electric repulsions and an increase [47] of H-bonds and (ii) coagulation of smaller HA micelles into larger ones [49]. These facts lead to a macromolecular shrinkage. Below this pH value, a significant enhancement of the SERS intensity was observed (Fig. 3e), which is related to the HA adsorption tendency increase of at low pH as also reported by several authors [50,51]. The HA adsorption increase is related to the higher hydrophobic nature of HA at low pH caused by the negative charge decrease upon protonation of acidic HA groups. The increase of HA molecules adsorbed on the metal is further intensified due to the reduction of the area per molecule induced by the molecular shrinkage occurring at low pH [52].

The SERS spectrum seen at low pH changes with respect to that observed at high pH (Fig. 3a). The analysis of the Raman broad bands seen at low pH is rather difficult owed to the sum of different components and the possible contribution from carbonaceous materials such as charcoal [53,54], which may be incorporated in the humic structure of that can be formed due to photodegradation as indicated by other authors [55]. Nevertheless, the different Raman profile with respect to charcoal Raman spectrum [54], the variation of this spectrum with the pH, as revealed below, and the appearance of bands which are attributable to functional groups actually existing in HA, such as benzoic, polyphenolic and aliphatic ones, discharge the possibility of a massive photodegradation of the adsorbed HA as an effect of light irradiation.

The dependency of SERS intensity on pH is better seen in the inset figure of Fig. 3, which displays a minimum at ca. 5.5. Recently, Terashima et al. [52] have demonstrated that the apparent pK (pK\(_{\text{app}}\)) of HA is approximately 5.5 and, as a consequence, at pH 5.5 drastic changes take place in several physical properties, such as surface tension, surface area occupied by HA molecules when adsorbed onto a surface and HA hydrophobicity. A similar pH value was reported by other authors when studying the aggregation tendency of HA [56,57].

It therefore follows that the lower SERS intensity of CR and CM samples in comparison to the control C0 and C30, can be attributed to the effect of amendments. The introduction of lignin in the HA structure may increase the stability of the macromolecule due to the higher H-bonds formed between OH groups afforded by lignin. In the case of CM, the enrichment in fatty acids, due to the treatment with manure, also lead to a higher stabilisation of the macromolecule due to the formation of hydrophobic interactions between aliphatic chains of fatty acid residues.

### 3.2 Surface-enhanced emission spectra of humic acids

Fig. 4 compares the normal Raman spectrum of the C30 sample (1\(\mu\)g mL\(^{-1}\)) with SEE spectrum of the same sample in the presence of Ag NPs. The emission spectrum of the solution shows the Raman bands of water at 1640 cm\(^{-1}\) (very weak) and 3420 cm\(^{-1}\) (strong) and a broad fluorescence band produced by HA centered at 630 nm, while no Raman band of HA are seen. In contrast, the SEE spectrum of HA was characterised by strong Raman bands in the 1200–1650 cm\(^{-1}\) region, and by a fluorescence emission with a maximum at 650 nm, which is threefold intensified in relation to that seen in aqueous solution. The intensification of Raman bands corresponding to the HA and the shift observed for the fluorescence emission maximum suggests structural changes in the HA upon adsorption on the metal. Thus, the SEE spectrum of the C30 sample provided a combined enhanced Raman and fluorescence information because the cross-sections corresponding

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**Fig. 3** – SERS spectra of C30 sample (10\(\mu\)g mL\(^{-1}\)) after fluorescence correction at the following pH: (a) 10.0, (b) 8.0, (c) 6.0, (d) 5.5 and (e) 3.0. Inset: variation of the 1609 cm\(^{-1}\) SERS band on changing the pH.
Fig. 4 – Surface-enhanced emission (SEE) spectra of C30 sample (1 µg/mL) in absence (a) and presence (b) of Ag nanoparticles.

to both emissions are of the same magnitude order. Although the total emission measured from adsorbates adsorbed on metal NPs is also a combined luminescence signal with contributions from both the molecule and the metal background luminescence [58], we can consider that the latter one is negligible in relation to the luminescence of HA in the SEE spectra.

SEE spectra recorded at three different HA concentrations are shown in Fig. 5. At high concentration, the fluorescence signal predominates over the Raman signal, while at lower concentrations the opposite behaviour is witnessed. This is probably due to the formation of HA multilayers on the Ag NPs, which leads to higher fluorescence intensities as the multilayer width is increased due to a lower quenching effect by the metal. The predominance of the SERS signal over the SEF one (in fact limit of detection of 1–100 ppb can be obtained, depending on the case) points out the powerful analytical possibilities of SERS in comparison to the fluorescence, in spite of the higher sensibility of fluorescence over the Raman effect.

The effect of pH on the Raman and fluorescence of HA samples is shown in Fig. 6. The Raman intensity changes with the pH reaching a minimum at pH 5.5 (Fig. 6b). In contrast, the analysis of the fluorescence emission revealed an opposite behaviour, i.e. a maximum at precisely pH 5.5 (Fig. 6a).

This contrary behaviour of the fluorescence and SERS signals of HA can be explained on the basis of the structural modifications induced on HA as a consequence of the acidic groups ionisation on increasing the pH. Other authors have also reported similar variations with pH of the HS fluorescence emission [8,59]. The HA uncoiling or structural expansion occurring in the pH range between 8 and 10 provoked an opposite response on the fluorescence and Raman intensities. By one hand, it favours the approach of the inner PAH residues of HA to the metal leading to an enhancement of the SERS signal. On the other hand, this expansion induces the fluorescence quenching due to the higher exposure of fluorophores to the aqueous polar medium and the charge-transfer induced by the closer metal surface.

In contrast, at pH 5.5 the HA is compacted, and consequently the PAHs residues remain inside the HA structure, and therefore, far from the metal surface, yielding a weaker SERS intensity. However, the fluorescence signal is enhanced due to the lower fluorescence quenching of fluorophore groups, which are therefore protected from the aqueous medium and the metal surface quenching effects. This behaviour is similar to the fluorescence change in proteins due to folding–unfolding processes observed on changing the pH [60].

At pH below 5.0, the Raman intensity is remarkably intensified due to the higher adsorption tendency of HA, as mentioned above, but the fluorescence quenching observed at lower pH is now attributed to the protonation of its acidic groups ionisation.
groups and the lower quantum efficiency of protonated groups [8].

Although this is a general behaviour of HA, as we have seen for many HA types, they also display differences, which can be attributed to the structural changes induced by amendments, which the soil have received for over 30 years. For instance, the CR and C30 samples show the minimum and maximum variations of fluorescence and Raman signals, respectively. This can be attributed again to the presence of a larger number of phenolic groups in CR sample due to the incorporation of lignin derivatives coming from the residual crops used in the amendment. Several authors have also detected a higher sensitivity to pH in HS bearing a higher content in phenolic hydroxyl groups [8,61,62]. CM sample exhibits an intermediate behaviour between CR and C30 samples due to the presence of a high amount of aliphatic chains introduced by the residual fatty acids of manures, which may contribute to a further stabilisation of the HS structure through hydrophobic interactions, as also corroborated in a previous work [63].

3.3. Micro-SEE spectra of HA

Micro-SEE spectra were obtained from Ag nanoaggregates immobilised on a glass substrate corresponding to the optical image shown in Fig. 7a. One of the distinct advantages of micro-Raman instruments is the possibility to record images and maps, which enable the distribution of a certain species to be followed by recording the intensity of a characteristic band. The intense band at 1611 cm\(^{-1}\) was used as reference to study the SERS intensity obtained from different points in the same metal aggregate onto which the C30 was adsorbed. Fig. 7b and c shows the images obtained by mapping the intensity of the 1611 cm\(^{-1}\) Raman band and the fluorescence at 572 nm (with excitation at 514.5 nm) in 50 \(\mu\)m \(\times\) 50 \(\mu\)m areas of the aggregate shown in the optical image of Fig. 7a. The spectroscopic images indicate that both the Raman and fluorescence signals are greatly enhanced in the vicinity of the metal aggregate, thus highlighting the fact that the emission spectra are actually produced by surface-enhanced Raman and fluorescence effects with a negligible contribution from the bulk.

The maps of Fig. 7b and c indicate that the emission intensity is higher in the lower part of the nanoaggregate. According to the electromagnetic mechanism of LSP-based surface-enhanced spectroscopy [13–15], the enhancement depends on the morphology of metallic clusters. In addition, the existence...
of areas with large SERS enhancements, so-called hot spots, has also been reported on metallic NPs. Suitable hot spots are believed to be formed, for example, at a junction between two metallic nanoparticles [64,65] and are highly localised. Thus it seems that the local morphology of the lower aggregate of Fig. 7a is highly favourable to an enhancement of the electromagnetic field, as deduced from the high SEE signal observed. This is probably due to the existence there of hot spots.

A random Raman mapping was also performed in a region where different Ag nanoaggregates having different sizes and shapes can be found (Fig. 8b). The micro-SERS spectra recorded for the points labelled with letters in Fig. 8b are represented in Fig. 9, while the Raman intensity of the 1611 cm\(^{-1}\) band is plotted in Fig. 8a for all the examined points. The analysis of the overall Raman intensity and relative intensity of the observed features was interesting to gain an insight into the adsorption of a macromolecular heterogeneous system such as HA.

Fig. 8a indicates that the Raman intensity depends on the morphology of the aggregate studied. The "d" nanoaggregate was the most efficient at enhancing the Raman intensity. This can be attributed to the more appropriate morphology for electromagnetic field enhancement on the surface of this aggregate. In contrast, aggregate a yields a very low Raman intensity, even if the size is comparable to that of the “d” aggregate. This study affords interesting information to investigate the influence of the Ag nanoaggregate morphology on the SERS enhancement.

Fig. 9 shows that the spectral pattern undergoes a slight variability from point to point, which was not observed in the corresponding macro-SEE spectra. This effect can be attributed to the intrinsic structural heterogeneity of HA, which can be adsorbed onto the surface through the different chemical groups existing in their structure.

It is worth noting that the different features detected in the micro-SERS spectra obtained at the different points indicated in Fig. 8a precisely matched the bands resulting from the curve-fitting of the macro-SERS spectrum registered in macro-conditions for the same sample, as indicated in the upper spectrum of Fig. 9 (see the arrows). This fact clearly indicates that in the surface-enhanced analysis of macromolecular systems the spectrum obtained in macro is actually the sum of the different spectra, which can be obtained, in micro-conditions.

4. Conclusions

SEE is a promising combined Raman plus fluorescence technique to be applied in the structural and dynamic analysis of HA. SEE signal is very sensitive to the HA shrinkage, which in turn may vary with the pH, the concentration and the structural modifications of HA induced by different soil treatments.

Raman and fluorescence emissions follow an opposite behaviour due to the effect of the metal surface on the groups responsible for these emissions. This fact can be related to the different shrinking state of the HA structure, which may vary with the pH or the amendment trials. At high HA concentration the SEE spectra are dominated by the fluorescence emission. However, at low HA concentrations the SERS signal predominates over the fluorescence and a limit of detection as low as 1 ppb can be achieved.

The sensitivity of SEE is further increased in microspectroscopy. A very low detection limit can be achieved in micro-conditions, with the detection of few HA molecules (probably much lower taking into account that HA molecules placed in hot spots are those contributing to a highest extent to the overall SEE signal). The micro-SERS spectra show a high variability from point to point due to the inherent complexity of HA micelles.

The SEE technique has promising applications in the influence of other factors affecting the HA structure such as: origin, humification rank, and interaction with pollutants. Indeed, this is a work that we are currently doing.
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