Synthesis and properties of model humic substances derived from gallic acid**

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Abstract. A model humic acid (HA) was synthesized from a strong natural antioxidant, 3,4,5-trihydroxybenzoic acid (gallic acid-GA), in a slow oxidative polymerization/condensation reaction catalysed by OH⁻ at pH ca. 8. The resulting dark-brown product (HAG), acidified to pH ca. 2, did not precipitate from the reaction solution and it was isolated and purified by dialysis. Its physicochemical and spectroscopic properties, as determined by means of elemental analysis, high performance liquid chromatography (HPLC), Fourier transform infrared (FTIR), ultraviolet-visible (UV-VIS), fluorescence and electron paramagnetic resonance (EPR) spectroscopy, showed a close resemblance to natural humic substances. The antioxidative activity of HAG was assayed by quenching of chemiluminescence of lucigenin and compared to that of standard antioxidants. The similarity and differences between HAG and natural humic substances and the role of the HAG antioxidative activity are discussed.

Keywords: gallic acid, humic acid, spectroscopic and antioxidizing properties

INTRODUCTION

Gallic acid (GA) is a natural phenolic antioxidant widely found in the plant kingdom, soil and aqueous environments (Fig. 1). Phenolic materials like flavonoids, tannins, lignin and fulvic acids (FAs) provide plants, soil and aquatic microorganisms with protection against free radicals and oxidative damage. In complex interactions taking place during the biological and chemical degradation of organic residues, phenolic compounds and their degradation products can undergo oxidative coupling reactions and became part of natural humic substances (HSs) (Cieślewicz et al., 1997; Frimmel and Christman, 1988; Huang et al., 2002; Labieniec et al., 2003). However, some results show that the phenolic compounds could also act as proxidants (Labieniec et al., 2003). Here the question arises whether GA-derived HSs are efficient antioxidants. Control of the chemical composition and conformational structure of HS may be of importance in regulating their reactivity in the environment. The problem of antioxidative activity of HS is especially important with respect of increasing penetration of UV radiation, ozone and N₂O₅ and increase of global temperature. These physicochemical factors accelerate the oxidative degradation of HS, thus increasing the concentration of atmospheric CO₂ and global warming.

The majority of synthetic model HSs have been obtained from dihydroxybenzenes and quinones (Cataldo, 1998; Czuchajowski and Krzeczek, 1966; Orlov, 1985) and phenols, and by coupling these phenolics with amino acids and proteins (Flaig et al., 1975; Janos, 2003). The resulting products suffered, as models, from the significantly lower content of COOH functional groups in comparison with natural HSs. A relatively good model, particularly of FAs, should contain precursor-derived aromatic rings, OH, COOH groups facilitating intermolecular associations, and exhibit physicochemical properties characteristic of HSs. Till now GA was used as a substrate for humic polymers in studies of the effect of Mn, Fe (II) and Fe (III) oxides on the rate and efficiency of HS formation (Shindo, 1994). However, no research on the mechanism of synthesis and properties of the resulting macromolecular material was carried out. The objective of this study was, therefore, to synthesize a humic acid (HA)-like model from GA and to characterize its physicochemical properties, particularly the antioxidative activity.
MATERIALS AND METHODS

Gallic acid monohydrate (analytical purity) and lucigenin from Sigma Aldrich (Germany) were used. Other chemicals were of analytical grade (POCh, Gliwice, Poland).

Mild conditions for the polymerization/condensation reaction of GA (‘humification’) were used: spontaneous auto-oxidation of 0.1M GA in 0.1 M Na₂CO₃ diluted to keep pH at ca. 8, with reaction time of 37 d. The reaction solution was protected against microbiological contamination. Then, the resulting homogenous dark solution was treated with HCl to a pH of ca. 2 and kept for 2 d at 8°C. No precipitation of HA fraction was observed. The solution was dialysed against distilled water using dialysis benzoylated tubing (Sigma Aldrich). The dialysed solution was dried under reduced pressure at room temperature and dried at 70°C. The yield was 24.5% with respect to the initial amount of GA.

The synthetic HAG was soluble in water and alkali and insoluble in MeOH, EtOH and dimethyl sulfoxide (DMSO). During the polymerization/condensation reaction the pH and absorption spectra were measured.

Dried preparations of HAG were analysed for C and H using Vario ELII analyser, and FTIR spectra were obtained (Bruker IFS 113 V spectrometer) using KBr pellets (3-6 mg HAG/5 mg KBr). UV-VIS absorption spectra were measured with a Top Sensor System (Ocean Optics, The Netherlands) equipped with H source, quartz fibre and a photodiode array detector sensitive in the spectral range of 220-800 nm. Quartz cuvettes, 0.1 and 0.2 cm wide for UV and 1 cm wide for the visible range were used. From the measured numerical values of absorbance (A) at specified λ = 260, 400 and 600 nm, colour coefficients (Q₂₁/₂₃) were calculated.

HPLC-MS analysis of HAG was performed with Waters Micro Mass USA, using HPLC-2690 chromatograph, symmetry column C₁₈ id = 4.6 mm and 150 mm in length, photodiode array detector type 996 Waters and Electro spray MS type ZQ.

Fluorescence excitation and emission spectra were recorded with a Shimadzu RF 5001PC spectro-fluorimeter using quartz cuvettes and water solutions of HAG with absorbance <0.2.

EPR spectra of solid HAG were measured using a 2 mm quartz tube. In order to calculate the spin content of the sample, a spin standard [4-proto-3-carbonyl-2,2,5,5-tetra- perdeuteromethyl-3-pyrroline-1-yloxy, (mHCTPO)] was used. EPR measurements were carried out using a Bruker ESP 300 E spectrometer operating in the X-band and equipped with 100 kHz field modulation.

A chemiluminescent method for the evaluation of antioxidant activity (AOA) of HAs based on quenching of the chemiluminescence (CL) of lucigenin was applied (S³awiñska and Rolewski, 2003). The basic system was 0.5 mM lucigenin, 10 mM H₂O₂ and NH₄OAc buffer pH 8.3. To measure chemiluminescence intensity, a RCA 5566A photomultiplier was used.
RESULTS AND DISCUSSION

Elemental analysis

Oxidation processes involving organic precursors (mainly plant residues) are considered to be a basis for the formation and diagenetic changes of HSs. Such an internal oxidation process may be quantified using the following formula (Dêbska, 1997):

\[ \omega = \frac{2O + 3N}{C} \]

where: C, O, N and H are the atomic percentages of the elements C, O, N and H. Atomic percentages are calculated from the averages of the weight percentages determined from the elemental composition (Table 1). The parameter \( \omega \) (the degree of internal oxidation) is closely related to oxidation and degradation reactions. It is supposed that the degradation process involves the loss of CH3 groups and partial oxidation, thus decreasing the number and length of aliphatic chains in the HA. High positive values of \( \omega \) indicate a high degree of internal oxidation.

As can be seen from Table 2, a high value of \( \omega \) was obtained for HAG formed under mild conditions. However, the evident decrease in the percentage of C indicates a de-carboxylation process.

HAG obtained from GA contains only 56.1% of C in comparison to the average content of C in reference or standard humic acid obtained from peat or river (http://www.ihss.gatech.edu, 2003). The content of oxygen in HAG is 56.4% higher than in reference or standard HA isolated from natural sources (river or peat). The percentage of H is the same in HAG and natural-derived HA.

A high amount of O-containing hydrophilic groups explains the solubility of HAG in waters and weak solubility in organic solvents. This is also supported by strong absorption bands in 3400-3200, 1720-1700, 1640-1610 and 1260-1250 cm\(^{-1}\), reflecting oxygen-containing functional groups. Part of these groups is dissociable (OH, COOH), which causes electrostatic repulsion leading to more loose structure of oligomeric and polymeric subunits of HAG. In this respect the obtained HAG better resembles the properties and structure of supramolecular ensembles of natural HS, stabilized by electrostatic and weak intermolecular forces (Picollo et al., 2003).

Absorption spectra

Humic substances exhibit high absorbance in the UV region because of the presence of aromatic chromophores. Figure 2 shows absorption spectra of the substrate GA (1), reaction mixture (2) and final isolated product HAG (3). At stage (2) a blue-green colour formed and a new long wavelength transient absorption band with a maximum at 640 nm appeared (Fig. 2). The band slowly disappeared and a brown colour of the solution developed and intensified. Such a blue-green intermediate has been observed in the modified Trautz reaction and ascribed to the formation of 2,3,4,6-tetrahydroxy-5H-benzocycloheptan-5-one (purpurogalline) and oxidative polymerization of a metastable semiquinone of the corresponding substrate (polyphenol). A ‘remnant’ of this semiquinone is still reflected in the flat shoulder visible in the absorption spectra of HAG (\( \lambda = 500-650 \) nm). Oxidative

<table>
<thead>
<tr>
<th>Substance</th>
<th>N</th>
<th>C</th>
<th>H</th>
<th>O</th>
</tr>
</thead>
<tbody>
<tr>
<td>GA</td>
<td>-</td>
<td>49.38</td>
<td>3.63</td>
<td>46.99</td>
</tr>
<tr>
<td>HAG</td>
<td>-</td>
<td>29.65</td>
<td>2.555</td>
<td>67.455</td>
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</table>

Table 1. Elemental analysis of humic acid (HAG) from GA

<table>
<thead>
<tr>
<th>Substance</th>
<th>H/C</th>
<th>O/C</th>
<th>( \omega )</th>
</tr>
</thead>
<tbody>
<tr>
<td>GA</td>
<td>0.074</td>
<td>0.952</td>
<td>0.536</td>
</tr>
<tr>
<td>HAG (pH 8)</td>
<td>0.085</td>
<td>2.248</td>
<td>2.326</td>
</tr>
</tbody>
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Table 2. The oxidation parameter (\( \omega \)) and H/C and O/C ratio for HA from GA

Fig. 2. Absorption spectra and pH values of the reaction solution during the oxidative polymerization/condensation of gallic acid: 1) \( 1.6 \times 10^{-4} \) M GA, 2) transient reaction mixture observed after 20 h, 3) \( 5 \times 10^{-5} \) g ml\(^{-1}\) final product HAG.
transformation of orthotrihydroxybenzenes and certain of their derivatives leads to the condensation of two molecules with decarboxylation and formation of hydroxybenzotropolones (Sławińska *et al*., 1979). The trace 500-650 nm absorption band might indicate the presence of 6-7 of such member rings. Moreover, semiquinones of hydroxybenzotropolones stabilized in the 3D structure would contribute to a strong EPR signal observed in the preparation obtained.

HPLC method resolved the HAG solution into three fractions characterized by the absorption spectra in Fig. 3. The absorption spectrum of the first eluting fraction (1.21 min) shows maxima at 210, 235 and 260 nm, characteristic of simple aromatic structures. However, monotonically decreasing absorbance with increasing wavelength extends up to 575 nm. The absorbance at 260 nm is used to characterize the relative content of aromatic structures because the ππ* transition of benzenes and phenols occurs at this wavelength. Absorbance at < 340 nm indicates the presence of C=O functional groups because they give weaker absorption in the UV region. This part of the absorption spectrum probably reflects the presence of monomeric quinoid and semiquinoid structures. The next fraction (1.76 min) exhibits a maximum at 205 nm and barely observable peaks at 260 and as a shoulder at 315 nm, characteristic for ketonic C=O functionality. This spectrum ends at 420 nm. The third fraction shows a broad band with maximum at 260 nm and a bump at 315 nm. This spectrum is similar to the second fraction spectrum except that the peak at 260 nm is much more pronounced. This indicates that HAG contains mainly phenolic, ketonic and quinoid functional groups. Calculated Q_{260/400} and Q_{400/600} values are 14.3 ± 1.1 and 6.7 ± 0.4, respectively. The low absorption in the visible region indicates the rather low degree of condensation in HAG.

The long tail observed in the third fraction suggests a continuous distribution of the different oligomeric structures with increasing molecular weight formed on the basis of phenolic and/or quinoid dimeric units.

The absorption maxima at 266 and 350 nm can be assigned to the 2-OH-p-benzoquinone moiety present in the polymer backbone which is reported to absorb at 254 and 365 nm (Cataldo, 1998). However, if the o-BQ moiety is present in the chain, it should absorb at a similar wavelength. The o-BQ moiety is reported to have maxima at 254 and 368 nm (Tratnyek *et al*., 2001). The broad shoulder at 303 nm is due to a 2-OH-p-hydroquinone moiety present in the backbone because hydroquinones in DMSO absorb at 300 nm. All these data also confirm that HAG can be seen as hydroxylated/carboxylated copolymer of reduced and oxidized HQ and Q units which can interact by weak intermolecular forces like π-π and hydrogen bonds, forming larger associations. Additionally, the solubility of HAG in water and alkaline solution, but not in acetone, DMSO, EtOH and MeOH, suggests that HAG is highly hydrophilic and contains many OH and/or COOH groups. This suggestion agrees with the ionized form of GA at pH < 8.5 and electrostatic potential distribution (Fig. 1).

**Fluorescence spectra**

HSs fluoresce in the 250 to 550 nm range and a number of studies allow definition of three major ranges where fluorescence is likely to occur (Bilenko *et al*., 2003; Senesi and Miano, 1994). The long wavelength and low intensity emission peak indicates the presence of condensed aromatic rings, unsaturated bond systems with a high degree of conjugation, and electron accepting groups such as C=O and COOH groups. The short wavelength and high intensity band is associated with low aromatic content, low molecular weight components, and electron donating groups such as OH. Emission in the range > 400 nm is influenced by polycondensation between carbonyls and phenolic structures. In such a case it is difficult to resolve and identify relevant structures responsible for the observed fluorescence.

![Fig. 3. Absorption spectra of three fractions of HAG obtained from HPLC chromatographic column with retention time: a) 1.2, b) 1.7, and c) 2.16 min.](image)
Fluorescence was used to distinguish different structures in HAG. The excitation spectrum for emission at 510 and 450 nm exhibits one peak with a maximum at 350 nm (Fig. 4). For a simple fluorescent compound it can be expected that the observed excitation spectrum and the corresponding absorption spectrum would be similar. For HAG the fluorescence excitation spectrum and absorption spectrum are different. While the absorption spectrum is almost featureless, with an increasing absorption towards the UV region, a well-resolved band is observed in the fluorescence excitation spectrum. These differences indicate that only a part of the light-absorbing chromophores in HAG are fluorescent.

The fluorescence emission spectra (Fig. 5) exhibit a broad, featureless band with a maximum at 450 nm when excited at 260, 350 and 390 nm. In the range from 260 to 510 nm, the excitation at 330 and 350 nm is the most efficient and the maximum intensity in fluorescence emission is observed between 446-452 nm. The fluorescence efficiency decreases with excitation wavelength in the order 260 < 390 < 350 nm.

**Fig. 4.** Fluorescence excitation spectra for $2 \times 10^{-5}$ g ml$^{-1}$ HAG observed at 510 and 450 nm. Fluorescence intensity given in arbitrary units (a.u.).

**Fig. 5.** Fluorescence emission spectra for $2 \times 10^{-5}$ g ml$^{-1}$ HAG recorded at excitation wavelength 260, 350, and 390 nm. Fluorescence intensity given in arbitrary units (a.u.).
The concentration dependence of the fluorescence intensity at \( \lambda = 450 \text{ nm} \), excited at 350 nm, is linear for concentrations < 50 \( \mu \text{g ml}^{-1} \) (Fig. 6). This indicates low tendency to form associations between HAG sub-units and is in agreement with the proposed negatively charged HAG sub-units.

A general conclusion is that HAG contains a multifluorophore system in which excitation energy transfer occurs. This results in a continuous broad band fluorescence emission in the range of 400-600 nm, with two dominant bands at 450 and 510 nm.

**EPR spectra of solid HAG sample**

EPR spectroscopy was used to measure the free radical content in HAG. The calculated spin density is \( 6 \times 10^{18} \text{ spin g}^{-1} \) and measured width equals 6.45 Gs with \( g = 2.0018 \). The spectrum is broad without any fine structure, shown as an inset in Fig. 7. This stable free radical is most likely a semiquinone type radical and seems to be universal for all types of humic acids and melanins (Rozanowska et al., 1995). It is well known that information on spin-lattice relaxation rates may be obtained (Cataldo, 1998) by performing progressive

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**Fig. 6.** Fluorescence emission spectra of HAG solutions with different concentration. Fluorescence intensity given in arbitrary units (a.u.). Excitation wavelength 350 nm.

**Fig. 7.** Dependence of microwave power saturation versus area in arbitrary units (a.u.). Inset shows EPR spectrum of a solid HAG sample.
saturation experiments with increasing microwave power. The power saturation curve for the double-integrated intensity of the in-phase first harmonic absorption spectra is given in Fig. 7. From such an experiment we can calculate $P_{1/2}$ and, from a fitting procedure to Eq. (1), which is the empirical expression used to fit saturation data (Galli et al., 1996), it is possible to obtain the non-homogeneity parameter $b$.

$$Y' = K P_{1/2} / \left(1 + \left(P / P_{1/2}\right)^{b/2}\right)$$  \hspace{1cm} (1)

where: $Y'$ is the EPR derivative signal amplitude, $K$ is a constant, $P$ is the microwave power, $P_{1/2}$ is the microwave power at half saturation and $b$ is the non-homogeneity parameter.

Applying a least square fitting procedure to the data in Fig. 7 inset gave a value for $b$ of 1, which suggests high dipolar-relaxation enhancement occurring in the HAG solid state phase.

HAG dissolved in water or alkaline solution also exhibits an EPR signal dependent on pH, atmosphere and time (Pawlak et al., 2005).

**FTIR spectra of GA and HAG**

Functional groups of substituted phenols play a crucial role during humic acid formation. In GA carboxylic and phenolic hydroxyl groups are the main source of dissociable protons during HAG formation. IR is considered to provide some information about functional groups in HAs. In our study FTIR spectra were used as a tool for monitoring the changes in absorption bands and therefore in functional groups in HAG. The positions of IR absorption peaks of the humic acid (HAG) obtained from gallic acid (GA) are given in Table 3. Not all possible assignments for specific bands are given because absorptions for humic substances often overlap. Accordingly, only the most characteristic bands are assigned. The peaks for GA at 3492, 3367 and 3285 cm$^{-1}$ correspond to different modes of the OH groups. The bands in the 2850 to 2950 cm$^{-1}$ region are assigned to stretching vibrations of aliphatic CH, CH$_2$ and CH$_3$ side chain groups of aromatic rings. The peaks between 1701 to 1721 cm$^{-1}$ belong to carboxyl as well as to carbonyl groups. Those between 1608-1460 cm$^{-1}$ represent aromatic C and C=O stretching vibrations of esters, ethers and phenols. The band around 1080 cm$^{-1}$ is attributed to aliphatic C-C stretching. That at 750 cm$^{-1}$ is attributed to H-bonded OH stretching in carboxylic groups and that at 525 cm$^{-1}$ to COOH deformation. The fingerprint region (below 1300 cm$^{-1}$) is difficult to assign because of the variety of combination bands and a single functional group may contribute to the absorbance in several regions.

The FTIR spectrum of the HAG humic acid obtained from GA is shown in Fig. 8, where it can be seen that after oxidative condensation most of the bands are broadened and shifted compared to those for pure GA. The spectrum shows some characteristic and strong absorption bands in the 3400-3200, 1700-1720, 1610-1640 and 1250-1260 cm$^{-1}$ regions. The spectra recorded from 500 to 3500 cm$^{-1}$ of the synthesised HAG are not very different from that of the un-polymerised GA. Absorption in the range 3400-3200 cm$^{-1}$ is assigned to O-H vibrations and reflects the increase in these groups during HA formation. In HAG the peaks are merged under a very broad band with a maximum at 3424 cm$^{-1}$. The spectrum of HAG also resembles those of natural humic acids (Tratnyek et al., 2001) but clearly shows more -COOH and carbonyl C=O groups with abundance of -OH groups.
their presence is also confirmed by the strong C-OH bending band at 1205 cm\(^{-1}\). The peak at 2927 cm\(^{-1}\) is assigned to asymmetric and symmetric C-H motions and indicates the low level of aliphatic structures. The band at 1630 cm\(^{-1}\) is due to the 1,2- and 1,4- quinone groups in the ring of the monomeric unit, while the C=O stretching band at 1720 cm\(^{-1}\) indicates the presence of -C=O, lactonic O=C-O and -COOH groups. The basic aromatic nature of HAG is confirmed by the phenyl bands at 1500, 1460 and 835 cm\(^{-1}\) (out-of-plane aromatic C-H bending).

The formation of chelated C=O....OH rings can cause a shift in the quinoid C=O peak from its usual 1680-1650 cm\(^{-1}\) position toward lower wave numbers. The fusion of the C=O absorption band near 1650 cm\(^{-1}\) with the neighbouring 1600 cm\(^{-1}\) peak results in the single broad band typical of the IR spectra of humic acids (Czuchajowski and Krzeczek, 1966).

In HAG the peaks at 1720 and at 1249 cm\(^{-1}\) indicate the presence of carboxylic groups. The peak at 1249 cm\(^{-1}\) reflects C=O stretching or O-H deformation of COOH or OPh groups. The known GA dissociation constants (Fig. 1) suggest that in the mildly alkaline conditions during the oxidative condensation the resulting HAG should also contain quinoid and COO- groups. The broad band with maxima at 1608 and 1383 cm\(^{-1}\) indicates the presence of COO- groups in the HAG structure. The FTIR results confirm the presence of such groups.

**Chemiluminescent assay of antioxidative activity of HAG**

The \(\text{H}_2\text{O}_2-\text{CH}_3\text{COONH}_4\) system generates reactive oxygen species (ROS) and low level chemiluminescence (CL). At the chosen reagent concentrations and pH of 8.3 the reaction rate is very slow and suited for analytical assay. In order to enhance the CL intensity, lucigenin – a widely used chemiluminescent probe – is added. In the presence of substances scavenging ROS, the intensity of CL decreases (Fig. 9). A relative measure of antioxidant activity (AOA) is the \(\beta\) value (Polewski et al., 2002):

\[
\beta = \frac{(I_0 - I)}{I_0} [S]^{-1},
\]

where: \(I_0\) and \(I\) are the intensity of CL without and in the presence of scavenger, respectively, and \([S]\) is the scavenger concentration.

The \(\beta\)-values calculated from the stationary intensities of CL for 5 to 500 µg ml\(^{-1}\) of HAG are in the range of 1.5 \(\times\) 10\(^3\) to 8 \(\times\) 10\(^3\) g\(^{-1}\). Gallic acid – a well-known antioxidant, gives a \(\beta\) value of 0.26 \(\times\) 10\(^4\) g\(^{-1}\) when used at 10\(^{-3}\) M (1.88 \(\times\) 10\(^4\) g ml\(^{-1}\)) concentration. This means that the HAG preparation reveals an AOA one order of magnitude less than that of GA, but still sufficiently high to potentially play a physiologically significant role with respect to, for example, soil microflora and root systems, and to protective effects against oxidative deterioration of soil organic matter.
CONCLUSIONS

1. From the elemental, chromatographic, UV-Vis, fluorescence, EPR, FTIR and chemiluminescence quenching analysis data we can conclude that HAG formed from GA shows high degree of substituted aromatic structure with carboxylic acidity. A carboxyl group has a negative effect on the conversion of GA to humus-like polymers. Electrostatic repulsion between anionic forms of GA1 and GA2, at pH > 8.5 and steric hindrance decrease the rate and degree of polycondensation and leads to relatively low molecular structures soluble in water. A previous study confirmed that hydroxylation at C-2 of hydroquinone and p-benzoquinone takes place due to OH$^-$ attack. Such a reaction seems to be less probable in the case of GA, but the results of elemental analysis show an increase in oxygen content in HAG in comparison with that of the starting GA.

2. EPR studies revealed the interesting fact that HAG possesses a high spin density despite its low polymerization degree. However, its solubility in water resembles that of fulvic acid, which exhibits low spin density. Increasing amount of ROS (O$_3$, NO$_x$, UV radiation) in the environment may lead to a gradual oxidative deterioration of humic substances – a precious component of soil and waters. Therefore a search for a hydrophilic paramagnetic (high spin density) model HS with high AOA may be useful for better understanding of interactions of HS with the environment.

3. A newer aspect of our research regards anti-oxidizing activity of HS. This aspect is related to the protective role of HS against oxidative stress caused by ozone, N$_2$O$_y$, $^{1}O_2$', acid rains and UV radiation in soil and water ecosystems. Antioxidant activity of HS is also important with respect to the contribution of oxidative degradation of HS to the increasing CO$_2$ in the troposphere.

REFERENCES


