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Long-term kinetics study and quantitative characterization of the antioxidant capacities of humic and humic-like substances

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Abstract

Purpose Humic substances (HS) play important functions in the environment by radical scavenging in biogeochemical redox reactions, thus influencing behavior of pollutants and preventing damage to cell membranes; this is due to antioxidant properties of HS. Previous studies focused primarily on assessing endpoint antioxidant capacity (AOC) of HS. Our work aimed to estimate long-term kinetics of the antioxidant capacities of humic and humic-like substances under different pH in relation to their specific structural features.

Materials and methods The 10-h kinetic profiles of four standard HS and two fungi-produced humic-like substances (HLS) were established with Trolox equivalent antioxidant capacity (TEAC) approach using the ABTS decolorization assay. Three pH levels (3.75, 4.25, and 6.80) and a broad range of humic material concentrations (0.5–10 mg L^{-1}) were examined. The data were divided into intervals and fit using exponential functions to evaluate the endpoint AOCs as well as rate constants for the reaction of humic materials with the ABTS radical cation. To further explore the nature of the antioxidant activities of humic materials, the physicochemical features and antioxidant activities of humic compounds were subjected to correlation analysis.

Results and discussion Our results demonstrated that during the first 40 min, the determined AOCs did not exceed 50 % of the endpoint AOCs for studies of humic materials, indicating that short-term measures of the AOCs of humic materials provide artificially low values due to the presence of slow-acting antioxidant compounds. Due to the instability of ABTS$^{\bullet+}$ at neutral and alkaline pH values, only the fast antioxidant moieties of humic materials can be assessed with ABTS decolorization approach under these conditions. Our results show that at acidic pH, the antioxidant activity of HLS is mainly related to the presence of nitrogen-containing groups rather than phenols. However, for HS, both nitrogen-containing compounds and phenolic compounds should be considered.

Conclusions To obtain clearer information concerning the AOC of humic materials, kinetic profiles should first be established, and then endpoint measurements should be taken at a time when the reaction has reached, or at least neared, the endpoint.

Keywords ABTS · Antioxidant capacity · Humic-like substances · Humic substances · Kinetics

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1 Introduction

Humic substances (HS) are ubiquitous in soil and aquatic ecosystems and play a fundamental role in the environment. HS can affect soil fertility, mineral weathering, and water acidity; they are involved in the transport, sequestration, and mitigation of contaminants. As the products of stochastic synthesis processes, HS are polydispersed substances that have elemental compositions that are non-stoichiometric and structures that are irregular and heterogeneous. Thus, it is not

possible to assign exact structures to HS. Instead, they are operationally defined using a model structure predicated on available compositional, structural, functional, and behavioral data: a model structure that contains all the same basic structural units and types of reactive functional groups (Senesi 1993). Recently, the ability of different microorganisms, including fungi (Koroleva et al. 2007) and bacteria (Ortega-Retuerta et al. 2009), to produce humic-like substances (HLS) which resemble HS in their compositional and structural properties has also been demonstrated.

A special characteristic of HS is their antioxidant capacity. Antioxidant moieties in HS are expected to affect the concentrations and lifetimes of reactive oxidants in soils and aquatic systems (Aeschbacher et al. 2012). Due to their antioxidant activity, HS and HLS can also exert protective functions in ecosystems by interrupting radical reactions and preventing damage to cell membranes and biological macromolecules (Kulikova et al. 2005; Tarasova et al. 2015). Phenolic moieties formed from more complex plant precursor molecules, such as lignin and tannins, have been suggested as the major electron-donating moieties of HS and thus are thought to provide their antioxidant activity (Aeschbacher et al. 2012).

Quantitative estimation of antioxidant properties is based upon the kinetic parameters of the reaction between antioxidant molecules and free radicals, and the reaction rate constant for this reaction can be used as a measure of antioxidant activity. As is the case with HS, which are heterogeneous organic macromolecules, the antioxidant capacity (AOC) rather than the antioxidant activity is usually used for such an evaluation. Due to difficulties in measuring the individual antioxidant components of a complex mixture such as HS, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) equivalency is used as a measure for the antioxidant capacity of such a mixture. Trolox equivalent antioxidant capacity (TEAC) is most often measured using the 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) decolorization assay (Re et asl. 1999). The main advantages of the TEAC approach using ABTS include (1) applicability to both lipophilic and hydrophilic antioxidants and (2) direct non-fermentative generation of the ABTS radical monocation with no involvement of an intermediary radical (Re et al. 1999). Therefore, the TEAC assay is a widely accepted method to measure the antioxidant capacity of foods, beverages, and supplements (Huang et al. 2005; Prior et al. 2005) as well as for natural organic matter (Romera-Castillo and Jaffé 2015).

For individual compounds, the characteristic times for their reaction with the cation radical ABTS$^{\bullet+}$ typically do not exceed 1 min (Re et al. 1999; Tan et al. 2003). For doxorubicin, where a two-stage interaction with ABTS$^{\bullet+}$ occurs, two values for the characteristic reaction time were established (< 5 s and >3 min), and the value for AOC measured in the first stage was identical to that measured in the second stage (Reszka and

Britigan 2007). The characteristic reaction time was demonstrated to be greater than 1 h for the hormone melatonin (Tan et al. 2003). Considering that the TEAC approach is an end-point assay, the kinetic parameters for the reactions between the antioxidant and the ABTS$^{\bullet+}$ radical are also of great importance. Besides, in TEAC assay, relative reactivity is based on deprotonation and ionization potential of the reactive functional group, so reactions are pH dependent (Karadag et al. 2009). In general, ionization potential values decrease with increasing pH, reflecting increased electron donating capacity with deprotonation (Prior et al. 2005). The pH values have an important effect on the reducing capacity of antioxidants. At acidic conditions, the reducing capacity may be restrained due to protonation on antioxidant compounds, whereas in basic conditions, proton dissociation of phenolic compounds would increase the sample's reducing capacity (Huang et al. 2005). As HS and HLS are complex mixtures of different molecules, a kinetics study into their antioxidant properties allows for a deeper understanding of the antiradical activities both of the HS and of the possible formation of their coupling products in the environment.

Our work aimed to estimate the main parameters of the antioxidant activity of HS and HLS under different pH in relation to their specific structural features. To this end, AOCs and the reaction rate constants for the reaction with ABTS$^{\bullet+}$ were estimated for a set of standard HS and two HLS produced by fungi. These characteristics of antioxidant strength were then compared to the main physicochemical properties of humic materials to assess the effects of the properties of HS and HLS on their antioxidant activity.

2 Materials and methods

2.1 Humic materials used

Standard samples of HS including Suwannee River humic (SRHA) and fulvic acids (SRFA) and dissolved organic matter (SRDOM) were kindly provided by the coordinator of the Russian Chapter of the International Humic Substances Society (IHSS, USA), Prof. I.V. Perminova. Commercially available coal humic acid (AldHA) was purchased from Sigma-Aldrich (USA). Two samples of HLS, HLS45 and HLS70, were produced in our laboratory by the basidiomycete *Trametes maxima 0275* cultivated on oat straw. HLS45 and HLS70 were isolated after 45 and 70 days of solid phase cultivation, as described earlier (Koroleva et al. 2007).

2.2 Characterization of humic materials

The assays for C, H, and N in humic materials were carried out in a model 1106 (Carlo Erba Strumentazione, Italy) elemental analyzer. The oxygen content was calculated as the differences

between the dry ash-free weights of the samples and the total contents of carbon, hydrogen, and nitrogen. The ash content was determined after incinerating the sample in a muffle furnace at 800 °C.

Size-exclusion chromatography (SEC) analyses of humic materials were performed according to Perminova et al. 2003. Based on the data obtained, number-averaged molecular weight Mn and weight-averaged molecular weight Mw were calculated using GelTreat software (Kudryavtsev et al. 2000).

The acidic contents of the analyzed humic materials were determined by potentiometric titration, as described in Balcke et al. 2002. Titrations were conducted under N_2 atmosphere, pH 2.6 as the starting point and pH 11.0 as the endpoint. The molar amount of NaOH wasted for raising pH of HS solution from 2.6 till 7.5 was normalized to mass quantity of HS present in the solution and treated as its carboxylic acidity, and from pH 7.5 to the endpoint of titration—as the phenolic acidity of HS. Total acidity was calculated as the sum of carboxylic and phenolic acidity.

Quantitative ^{13}C solution-state NMR spectra were recorded on a Bruker Avance 400 spectrometer (Bruker BioSpin, Germany) operating at 100 MHz according to Kovalevskii et al. 2000. To quantify the spectra obtained, the following assignments were made (in ppm): 5–50, aliphatic H- and C-substituted C atoms (C_{Alk}); 50–108, aliphatic O-substituted C atoms ($C_{Alk–O}$); 108–145, aromatic H- and C-substituted atoms (C_{Ar}); 145–165, aromatic O-substituted C-atoms ($C_{Ar–O}$); 165–187, C atoms of carboxylic and esteric groups (C_{COO}); and 187–220, C atoms of quinone and ketone groups ($C_{C=O}$). In addition to the integrals of the given ranges, the ratio $\Sigma C_{Ar}/\Sigma C_{Alk}$ was calculated, where ΣC_{Ar} and ΣC_{Alk} are the sums $C_{Ar} + C_{Ar–O}$ and $C_{Alk} + C_{Alk–O}$, respectively.

The total phenolic (TP) contents of the HS and HLS samples were determined according to a previously published report (Singleton and Rossi 1965).

2.3 Assay of antioxidant capacity

The AOCs of HS and HLS were measured based on a decrease in the absorbance of ABTS$^{•+}$ in the presence of the studied substances at constant temperature 25 °C. The reactions were monitored by measuring the absorbance at 734 nm (A_{734}), as HS do not have significant absorbance at this wavelength. The radicals were generated according to a published report (Re et al. 1999). ABTS (Sigma-Aldrich, USA) and potassium peroxodisulfate were dissolved in water and incubated in the dark at final concentrations of 7 and 2.45 mM, respectively, at ambient temperature for 12–16 h. The ABTS$^{•+}$ stock solution obtained under these conditions was stable and could be used for 48 h. For measurements, ABTS$^{•+}$ stock solution was further diluted with an appropriate buffer (0.1 M sodium citrate buffer (pH 3.75 or 4.25) or 0.1 M

potassium phosphate buffer (pH 6.80)) to obtain final concentrations of 43 μM for the measurements.

Samples of HS and HLS were dissolved in 1 mL of 0.1 M NaOH and then diluted to 100 mL with deionized water to obtain final stock concentrations of 100 mg L^{-1}. Further dilutions of HS and HLS were made in such a manner that the final concentrations for the measurements would be 0.5, 1, 2, 4, 6, 8, and 10 mg L^{-1}. The reaction was initiated by mixing 20 μL of each dilution with 180 μL of ABTS$^{•+}$, followed by measurement of A_{734} for 10 h using a kinetic program on a Synergy 2 96-well plate reader (BioTek, USA). The assay was carried out at 25 °C. All solutions were prepared in 18 MΩ cm water or HPLC-grade ethanol from Panreac Quimica (Spain). All measurements were performed on four replicates.

Trolox is the most often used standard antioxidant in the ABTS decolorization assay, and therefore it was used as a benchmark for the AOC determinations in this study. A standard Trolox (Sigma-Aldrich, USA) stock solution was prepared by dissolving a sample of Trolox of known weight in 1 mL of ethanol and then diluting it with water to 100 mL to obtain a final concentration of 100 μM. To create a calibration curve, decreases in absorbance ΔA_{734} in the presence of Trolox at the concentrations 10, 30, 50, 80, and 100 μM were measured. The data thus obtained were fitted to the equation

$$\Delta A_{734}=r.c.\cdot C(Trolox) \tag{1}$$

where $C(Trolox)$ indicates the Trolox concentration in micromoles and $r.c.$ is the regression calibration coefficient. The calibration coefficients obtained for each studied buffer were used to further transform optical units from the ABTS decolorization assay into Trolox equivalents (TEs).

2.4 Auto-decay of cation-radical ABTS$^{•+}$

An absorbance A_{734} decrease corresponds to the transformation of ABTS$^{•+}$ into the formed ABTS. This process may occur due to both the presence of antioxidants in the system and ABTS$^{•+}$ auto-decay. To consider the latter, the kinetics of A_{734} decrease were monitored in the studied buffers containing ABTS$^{•+}$ alone (without HS introduction). The dependences thus obtained were fitted to the equation

$$A_{734}=A_{734}^{0}+b \times t \tag{2}$$

where A_{734}^{0} is A_{734} at the time = zero point, b is the coefficient of spontaneous auto-decay for the cation-radical ABTS$^{•+}$, and t is time in minutes. Taking into consideration that a decrease in absorbance in the presence of HS results from both ABTS$^{•+}$ auto-decay and its reduction by HS, one can derive the following expression for the decrease in absorbance ΔA_{734} due to only HS activity:

$$\Delta A_{734}=A_{734}^{0}-A_{734}+b \times t \tag{3}$$

where ΔA_{734} corresponds to the change of concentration of ABTS$^{\bullet+}$ formed due to reaction with HS, and the concentration of ABTS formed can be further calculated as

$$C(ABTS) = \frac{\Delta A_{734}}{\varepsilon \times 1} \qquad (4)$$

where ε is the extinction coefficient for ABTS$^{\bullet+}$ (1.5×10^4 mol^{-1} L cm^{-1}) and l is the optical path length (0.59 cm). The optical path length was estimated for the plate reader for a sample volume of 200 μL using the Beer–Lambert–Bouguer law, by reading ΔA_{734} of a solution of ABTS$^{\bullet+}$ of known concentration. Alternatively, optical units can be converted directly to the units of TE using the equation calibration curve (4).

2.5 Interval treatment of kinetic curve and calculation of AOC

Considering that HS is a set of individual hydrogen-donating antioxidants (AHs), a set of chemical reactions occurring in the HS–ABTS$^{\bullet+}$ media can be simplified as follows:

$$ABTS^{\bullet+} + AH_i \xrightarrow{k_i} ABTS + A_i^{\bullet} \qquad (5)$$
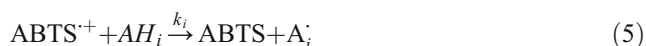
where $i = 1, 2, \dots n$, corresponding to the number of individual antioxidants in HS and k_i is the reaction rate constant for the scavenging of ABTS$^{\bullet+}$ by AH$_i$. Overall scavenging of ABTS$^{\bullet+}$ in the presence of HS results from n bimolecular reactions between the cation radical and each individual antioxidant. Therefore, the resulting time-dependent changes observed in the ABTS$^{\bullet+}$ concentration are rather difficult to formalize. However, the reaction between ABTS$^{\bullet+}$ and each AH$_i$ can be considered to be pseudo-first order, as it is likely that an excess of ABTS$^{\bullet+}$ will exist in the reaction medium. This reaction can thus be described in terms of pseudo-first-order kinetics:

$$AH_i \xrightarrow{\alpha_i} A_i^{\bullet} \qquad (6)$$

where α_i is an elementary constant of the pseudo-first-order reaction rate and

$$\alpha_i = k_i \cdot C(ABTS^{\bullet+})^0 \qquad (7)$$

where $C(ABTS^{\bullet+})^0$ is the initial concentration of ABTS$^{\bullet+}$ (at time = 0) and k_i is the second-order reaction constant.

Assuming that n reactions occur independently and simultaneously, the time-dependent change in the concentration of antioxidants in the reaction media can be expressed as:

$$C\left(A_i^{\bullet}\right) = C(AH_i)^0 \cdot \left(1 - e^{-\alpha_i \times t}\right) \qquad (8)$$

Given that

$$C\left(A_i^{\bullet}\right)(t \rightarrow \infty) = C(AH_i)^0 \qquad (9)$$

The overall *AOC* of HS can be expressed as a sum of the concentrations of the individual antioxidants, i.e.,

$$AOC = \sum_{i=1}^{n} C\left(AH_i\right)^0 \qquad (10)$$

Because the AOC is defined as the ability to reduce the cation-radical ABTS$^{\bullet+}$, and formation of 1 M of $A_i^{\bullet}$ requires one mole of ABTS$^{\bullet+}$, the following expression for the time-dependent concentration of ABTS can be derived:

$$C(ABTS) = \sum_{i=1}^{n} C(AH_i)^0 \cdot \left(1 - e^{-\alpha_i \cdot t}\right) \qquad (11)$$

where *C(ABTS)* can be calculated for each point of time t based on ΔA_{734} according to (4). Using (4), one can re-write (11) in the following way:

$$\Delta A_{734} = \sum_{i=1}^{n} C(AH_i)^0 \times \left(1 - e^{-\alpha_i \cdot t}\right) \times \varepsilon \times l \qquad (12)$$

Therefore, to find the endpoint AOC of HS, the limit of the function (12) should be determined. The reaction rate constant k can be calculated from α using (7). To obtain values of AOC in the units of TE, the experimentally obtained values in optical units can be converted using (1).

ABTS$^{\bullet+}$-based approaches assume that the antioxidants simply reduce the radical back to the parent substrate ABTS. So, for the reactions where an individual antioxidant reacts with ABTS$^{\bullet+}$ irreversibly in one step, time-dependent concentration of ABTS should be described using (11) when $n = 1$. However, the experimental data obtained here did not fit the exponential equation (11) at $n = 1$ well (data not shown). The latter probably resulted from certain chemical reactions of polyphenoles leading to reproduction of phenolic –OH moieties in the polymeric products also donating electrons. So, the unusual scavenging activities of polyphenols may be ascribed to such reproduction of –OH moieties by oxidative polymerization (Hotta et al. 2001). On the other hand, the redox potential in the reaction media did not stay constant over time as ABTS$^{\bullet+}$ was consumed and ABTS formed, and a decrease in ABTS$^{\bullet+}$ to ABTS ratio would also slow down the reactions as the thermodynamic driving force for oxidation decreased. Indeed, the experiments conducted under different initial ABTS$^{\bullet+}$/ABTS ratio varying from 2.5 (ratio used in this study) to 1 demonstrated its strong effect on AOC, especially at concentrations of humic materials exceeding 10 mg L^{-1} (Fig. S1 proposed as Electronic Supplementary Material). However, under selected conditions, final the ABTS$^{\bullet+}$/ABTS ratio reached 1.6 which could decrease measured AOC by no more than 10 % (Fig. S1, Electronic Supplementary Material). Finally, a loss of exponential fit could be a result of the fact that most phenolics display a biphasic kinetic pattern of

ABTS$^{\bullet+}$ scavenging, involving fast and slow steps (Walker and Everette 2009). Therefore, the obtained results were further divided into two intervals, namely, 0–40 min (interval I) and 41–600 min (interval II). Then, AOC$_I$ and k_I were derived for the interval 0–40 min. This was followed by subtracting the data for 0–40 min from the overall dataset, prior to calculation of AOC$_{II}$ and k_{II}. For the latter portion of the derivation of AOC and k, the concentration of ABTS$^{\bullet+}$ at the 40th min was used as C(ABTS$^{\bullet+}$)^0. The total AOC was calculated as a sum of AOC$_I$ and AOC$_{II}$. Considering that both AOC$_I$ and AOC$_{II}$ represent the limits of the function (12), i.e., asymptotic values rather than ones estimated at a certain point of time, their sum can be considered an endpoint value. The latter is of special importance, as the ABTS decolorization assay is thought to be an endpoint-only approach.

2.6 Statistical data treatment

All results were presented as the mean ± SD. To reveal the relationship between humic materials' physicochemical characteristics and their antioxidant properties, a correlation analysis was applied. To visualize clustering, a cluster analysis was performed. All of the statistical data treatments were carried out using Statistica 8.0 Software.

3 Results

3.1 Properties of HS and HLS used

The obtained physicochemical characteristics of the HS and HLS samples used are presented in Tables S1 and S2, Electronic Supplementary Material. The studied HS and HLS differed significantly in their structural-molecular characteristics. The atomic ratio of H/C for HLS was slightly higher compared to HS. In addition, HLS possessed a lower C/N ratio. The aromaticity degree, calculated as the ratio of the carbon content in aromatic, non-substituted ΣC_{Ar} structures to that in non-substituted ΣC_{Alk} structures, was at a minimum in the fungi-produced HLS45 and at a maximum in the coal HA, AldHA. The values of the weight-average molecular masses ranged from 6.9 kD (SRFA) to 22.1 kD (HLS45) and agreed well with data published for similar humic samples (Perminova et al. 2003).

3.2 Stability of the ABTS$^{\bullet+}$ cation radical at different pH values

The stability of ABTS$^{\bullet+}$ in different solutions, estimated as a decrease in A_{734}, clearly demonstrated the instability of the ABTS$^{\bullet+}$ cation radical at pH 6.80 under spectrophotometric assay conditions (Fig. S2, Electronic Supplementary Material). After 10 h of incubation, the A_{734} level dropped to

approximately 32 % of the initial value, whereas 10 h of incubation at pH 3.75 or 4.25 led to an A_{734} value equal to 96 % of the initial value. An excess of the ABTS$^{\bullet+}$ in the reaction mixture at pH 6.80 (a value equal to at least 90 % of the initial concentration) was observed only during the initial 40 min of the reaction. Therefore, the data obtained at pH 6.8 were excluded from further examination. The values calculated for the coefficient b are presented in Table 1.

The calibration coefficient $r.c.$ at pH 3.75 or 4.25 was calculated according to the calibration curve (1) and was 0.014 ± 0.001 a.u. per micromole.

3.3 Kinetic curves for ABTS$^{\bullet+}$ scavenging by HS and HLS

Data on ABTS$^{\bullet+}$ scavenging by different humic materials, expressed as A_{734} versus time, are presented in Figs. S3 and S4 (Electronic Supplementary Material). Based on the data obtained, the amount of ABTS was calculated after 10 h for each studied humic compound at various concentrations in buffers of pH 3.75 and 4.25 (Fig. 1).

As can be seen from Fig. 1, there were increases in the amount of ABTS formed with increasing concentrations of the humic compounds. However, a linear dependence between C(ABTS) and the concentrations of the humic compounds was observed only if their concentration was below 4 mg L^{-1}. A decrease in the Eh due to depletion of ABTS$^{\bullet+}$ over the course of the measurements might explain the non-linear character of the reduction of ABTS$^{\bullet+}$ by the studied humic compounds (Fig. 1). Indeed, average values of Eh calculated according to the Nernst equation decreased from 701.78 to 668.64–646.98 mV depending on the humic material or from 701.78 to 681.31–641.12 mV depending on the humic material at pH 3.75 or 4.25, respectively (Table S3, Electronic Supplementary Material). The latter was indicative for the fact that a failure of the reaction medium to contain an excess of cation radical occurred at HS concentration exceeding 4 mg L^{-1}. So, only data for the experiments with humic compounds' concentrations in the range of 0.5–4 mg L^{-1} were used in further analyses. Data on the AOCs and the reaction rate constants for the scavenging of ABTS$^{\bullet+}$ by humic compounds, calculated as averages of the values obtained with humic compounds in the concentration range of 0.5–4 mg L^{-1}, are presented in Table 2.

Table 1 Coefficient of spontaneous ABTS$^{\bullet+}$ auto-decay b at different pH values

	pH 3.75	pH 4.25	pH 6.80
b^a, 10^{-5} a.u. min^{-1}	−2.2 ± 0.2^b	−2.9 ± 0.3	−42 ± 5
R^2	0.9900	0.9935	0.9232

^{a} The value of the linear approximation (2)

^{b} ± represents SD, $n = 3$

Fig. 1 The reduction of ABTS$^{\bullet+}$ by the studied humic materials at pH 3.75 (*open dots*) or 4.25 (*black dots*) at different concentrations. The reaction time was 10 h. *Lines* represent linear approximation for the data in the range 0–4 mg L^{-1}. *Error bars* represent standard deviation ($n = 4$)
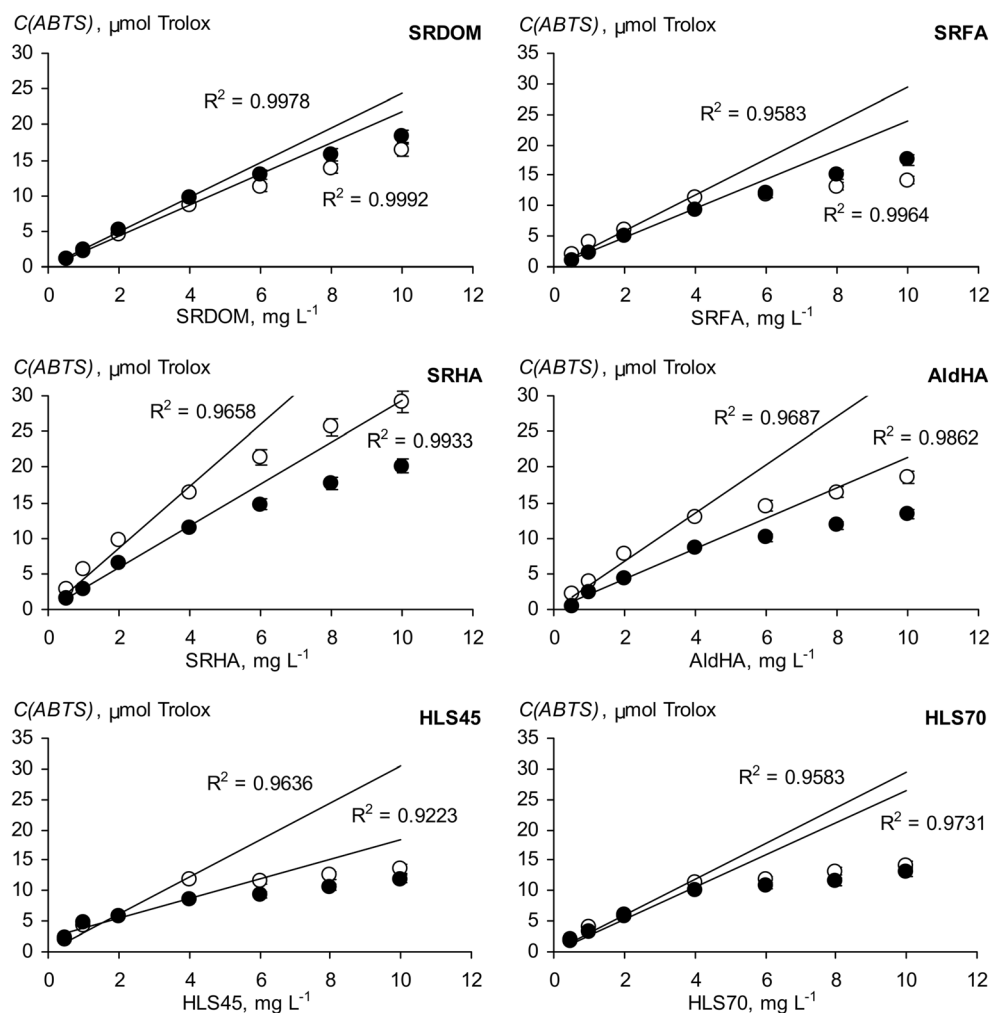

Table 2 AOCs and reaction rate constants for the scavenging of ABTS$^{\bullet+}$ by HS and HLS, determined at pH 3.75 and 4.25

Humic material	Constant rate, $(s \times M)^{-1}$		Antioxidant capacity, mmol ABTS$^{\bullet+}$/g			Antioxidant capacity, mmol TE/g		
	k_I	k_{II}	AOC_I	AOC_{II}	AOC	AOC_I	AOC_{II}	AOC
				pH 3.75				
SRDOM	18 ± 3[a]	1.6 ± 0.5	2.0 ± 0.2	1.6 ± 0.2	3.6 ± 0.4	1.3 ± 0.1	1.0 ± 0.1	2.3 ± 0.2
SRFA	16 ± 3	1.3 ± 0.1	1.8 ± 0.3	1.7 ± 0.2	3.5 ± 0.4	1.2 ± 0.2	1.1 ± 0.1	2.2 ± 0.2
SRHA	20 ± 4	1.3 ± 0.3	2.8 ± 0.7	2.3 ± 0.5	5.1 ± 0.5	1.8 ± 0.5	1.4 ± 0.3	3.2 ± 0.3
AldHA	36 ± 5	1.8 ± 0.6	2.0 ± 0.7	1.7 ± 0.6	3.7 ± 0.4	1.3 ± 0.5	1.1 ± 0.4	2.3 ± 0.2
HLS45	40 ± 7	1.6 ± 0.3	2.3 ± 0.6	3.1 ± 1.0	5.4 ± 0.5	1.5 ± 0.4	1.9 ± 0.7	3.4 ± 0.3
HLS70	47 ± 7	1.6 ± 0.4	2.3 ± 0.5	3.0 ± 0.7	5.3 ± 0.5	1.4 ± 0.3	1.9 ± 0.5	3.3 ± 0.3
				pH 4.25				
SRDOM	24 ± 4	1.3 ± 0.2	1.9 ± 0.2	2.0 ± 0.3	3.9 ± 0.4	1.2 ± 0.1	1.2 ± 0.2	2.5 ± 0.2
SRFA	19 ± 1	1.1 ± 0.2	1.7 ± 0.2	2.1 ± 0.2	3.8 ± 0.3	1.10 ± 0.08	1.3 ± 0.1	2.4 ± 0.2
SRHA	25 ± 5	1.3 ± 0.4	2.4 ± 0.3	2.3 ± 0.3	4.7 ± 0.3	1.5 ± 0.2	1.4 ± 0.2	3.0 ± 0.2
AldHA	27 ± 5	1.3 ± 0.4	2.0 ± 0.7	1.6 ± 0.5	3.6 ± 0.5	1.2 ± 0.5	1.0 ± 0.3	2.2 ± 0.3
HLS45	37 ± 3	1.2 ± 0.1	2.0 ± 0.9	3.2 ± 0.9	5.2 ± 0.2	1.2 ± 0.6	2.0 ± 0.6	3.3 ± 0.1
HLS70	44 ± 6	1.5 ± 0.5	1.9 ± 0.2	2.6 ± 0.5	4.6 ± 0.5	1.2 ± 0.3	1.7 ± 0.3	2.9 ± 0.3

[a] ± represent SD, $n = 4$

Among the humic materials studied, those produced by the Basidiomycetes were characterized by the highest AOCs and reaction rate constants, i.e., HLS45 and HLS70 possessed faster antioxidant moieties or greater amounts of fast antioxidant compounds compared to HS.

During the first 40 min, only approximately 50 % of the total AOC was reached for both HS and HLS, with average values of 51 and 47 % obtained at pH values 3.75 and 4.25, respectively. The latter was indicative of undervaluation of the AOC of humic materials using short-term measuring due to the presence of slow-acting antioxidant compounds.

4 Discussion

4.1 Reliable experimental conditions for AOC determination using the TEAC assay

In this study, we have examined the experimental conditions for the evaluation of the antioxidant properties of HS and HLS using a TEAC approach with the ABTS$^{\bullet+}$. The results obtained confirmed the instability of ABTS$^{\bullet+}$ at pH 6.80 at spectrophotometric assay conditions due to oxygen presence and were indicative of the limited opportunity for use of this radical for long-term experiments. It is apparent that, at pH 6.80, the assay duration should not exceed 40 min.

To describe the antioxidant properties of HS and HLS in terms of the bimolecular reaction between the ABTS$^{\bullet+}$ and the individual antioxidants in humic materials, a range of concentrations with an excess of ABTS$^{\bullet+}$ in the reaction media should be employed. To perform this, curves of C(ABTS) calculated after 10 h of reacting, according to (4), versus HS or HLS concentration were analyzed (Fig. 1). Our results demonstrated a linear dependence between ABTS$^{\bullet+}$ and the concentration of humic materials only in the range of 0.5–4 mg L^{-1} (Fig. 1), indicating that there is a substantial consumption of ABTS$^{\bullet+}$ at higher humic compound concentrations.

The next issue was selection of the reaction time, as the latter is a critical point to obtain reliable and comparable data (Walker and Everette 2009). Scavenging of ABTS$^{\bullet+}$ by humic compounds possesses very slow kinetics, indicating that short-term experiments result in underestimation of AOCs for humic compounds. Therefore, a short-term assay is recommended for the evaluation of fast constituents, rather than for determination of the endpoint AOCs of humic compounds, using the ABTS$^{\bullet+}$ decolorization assay. The findings reported here correspond well to previous data demonstrating the complex kinetic behaviors of phenols and polyphenols due to formation of electron-donating polymeric products (Hotta et al. 2001) or covalent adducts scavenging ABTS radicals (Osman et al. 2006).

To overcome this problem, a novel kinetic matching approach was developed to determine the total (endpoint) AOC of red wines (Magalhaes et al. 2012). This was achieved by using a standard compound with a kinetic profile similar to the food sample, which allows for the assessment of antioxidant values independent of the selected reaction time. Nevertheless, the feasibility and applicability of the methodology to other types of food products containing different profiles of antioxidants were not proven (Magalhaes et al. 2014). Therefore, there is still no adequate approach to determine the endpoint antioxidant capacity for compounds that demonstrate complex kinetic behavior in their reaction with ABTS$^{\bullet+}$. So, in this study, endpoint AOCs for HS and HLS were evaluated after the longest possible time of interaction (10 ; a longer period of time could result in ABTS$^{\bullet+}$ decay). To avoid underestimation of the endpoint AOC, due to the possibility of an insufficient duration for the ABTS$^{\bullet+}$–humic compound interaction, the sum of limits of the function (12) for the time intervals 0–40 min and 41–600 min was calculated. The obtained values were averaged for HS or HLS concentrations in the range of 0.5–4 mg L^{-1} (Table 2).

4.2 Antioxidant activity of the HS and HLS used

The antioxidant activities of the HS and HLS used were characterized with reaction rate constants and endpoint AOCs, as well as partial AOCs related to the 0–40- and 41–600-min time intervals, assigned as AOC_I and AOC_{II}, respectively (Table 2). The results showed a clear-cut distinction in reaction rate constants and AOCs between HS and HLS. For the first interval (0–40 min), reaction rate constants ranged from 16 to 47 (s × M)$^{-1}$ at pH 3.75 and from 19 to 44 (s × M)$^{-1}$ at pH 4.25, reaching their maximum values in both cases for HLS. Moreover, time-resolved changes in A_{734} for the reaction of HLS with ABTS$^{\bullet+}$ (Figs. S3 and S4, Electronic Supplementary Material) demonstrated an apparent gap between the curves at concentrations 2 and 4 mg L^{-1}; however, no such gap was observed for HS samples. Though additional studies should be performed to explain the phenomena discovered here, these findings illustrate strongly pronounced differences in the realization of antioxidant properties of HS and HLS. Most likely, the differences observed could be attributed to their differences in chemical composition and structural features (Tables S1 and S2, Electronic Supplementary Material). HLS are enriched with hydrocarbon compounds, compared to HS (Table S1, Electronic Supplementary Material). In addition, HLS manifested a higher nitrogen content, along with lower oxygen and TP contents. The greater AOCs we observed for humic compounds possessing low phenolic contents were unexpected, as the antioxidant activity of humic materials is usually supposed to be due to the presence of phenolic electron-donating moieties, which may act as antioxidants (Aeschbacher et al.
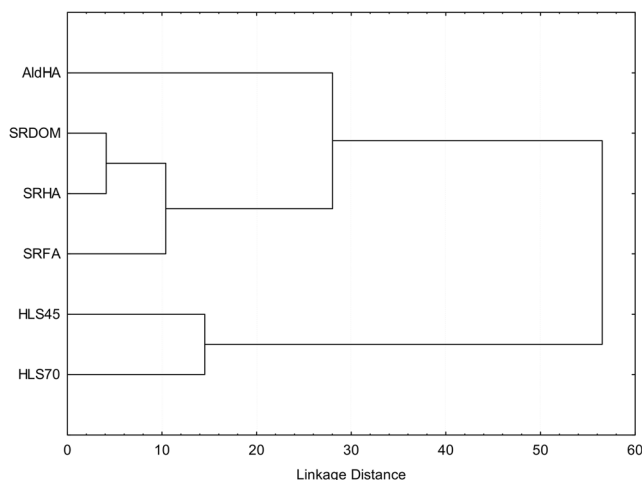
Fig. 2 A tree diagram (complete linkage, city-block (Manhattan) distances) for the HS and HLS studied. Clustering was performed based antioxidant property data (Table 2)

2012). The phenomenon observed could be probably attributed to the relatively low pH values in the reaction media used in this work. Besides, it was recently demonstrated (Rammer and Abbott 2011) that for soil–NaOH extracts (i.e., non-fractionated HS), phenolic compounds only contributed to a very small percentage of the overall AOC. Thus, other antioxidant compounds are expected to be present in humic materials. A correlation between peptide and carbohydrate content in soil fulvic acids and AOCs was demonstrated recently by Schlichting and co-workers (Schlichting et al. 2013), who attributed that phenomenon to microbial activity, which contributes to the stabilization of dissolved organic matter. The difference thus discovered was visualized by cluster analyses (Fig. 2), in which HS and HLS formed two groups.

To obtain insight into the relationship between antioxidant properties and the structural features of humic compounds, a correlation analysis was conducted (Table S4, Electronic Supplementary Material). Data on the elemental composition, SEC, ^{13}C NMR, potentiometric titration, and TP contents were used as descriptors of HS and HLS (Tables S1 and S2, Electronic Supplementary Material). It displayed a significant ($p < 0.1$) relationship between the endpoint AOC and nitrogen

enrichment of humic materials C/N ($r = -0.94$ at pH 3.75 or $r = -0.92$ at pH 4.25). This is most likely due to the leading roles of N-containing structural moieties such as peptides and amino acid residues (Schlichting et al. 2013), aromatic amsines (Aeschbacher et al. 2012), or insole derivatives (Cano et al. 2003) in determining the antioxidant properties of HS and HLS (Fig. 3).

Values related to antioxidant capacity (endpoint AOC, AOC_I, and AOC_{II}) almost always correlated with the C/N ratio (except for AOC_I at pH 4.25, where $r = -0.53$), whereas no relationship was observed for the phenolic contents. A significant ($p < 0.1$) positive linear relationship between AOC and TP in HS both at pH 3.75 ($r = 0.96$) and at pH 4.25 ($r = 0.98$) was revealed only when HLS samples were excluded from the dataset. The antioxidant activity of humic materials under selected conditions therefore cannot be attributed to phenol contents alone at least in case with HLS.

A negative correlation between the reaction rate constants and acidic group contents was discovered (Table S4, Electronic Supplementary Material). According to current opinion in the field, humic materials have a large amount of phenolic groups coupled with carboxylic groups. Being electron accepting groups, the carboxylic groups may decrease antioxidant activity of phenols via withdrawing electron density from the phenol ring. Recently, Al-Sehemi and Irfan 2013 published a theoretical study where geometries of phenol and its derivatives with electron donor or accepting groups including p-hydroxybenzoic acid were optimized by using the density functional theory (DFT) and bond dissociation enthalpy (BDE), and the adiabatic ionization potential (IP) were calculated. BDE and IP were used as indicators of the ease by which these molecules can deactivate free radicals. It was demonstrated that BDE is enhanced by substituting the electron withdrawing group COOH at *para* position which would decrease the antioxidant activity. Unlike carboxylic group COOH, carboxylate COO$^-$ does not possess electron withdrawing properties. So, deprotonation of carboxylic group COOH would enhance antioxidant activity of phenol. Carboxylic groups are mainly dissociated at pH 5 or higher. According to Illés and Tombácz 2006, the amount of

Fig. 3 Relationship between the endpoint AOC and the C/N ratio at pH 3.75 (**a**) and 4.25 (**b**)
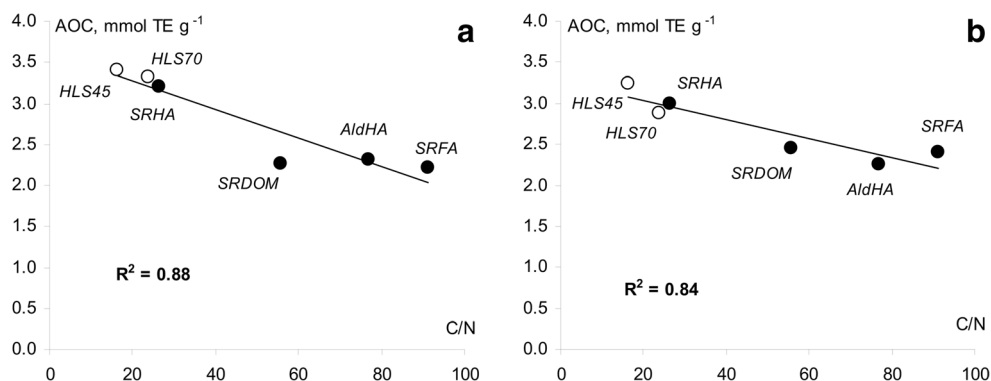
dissociated acidic functional groups, as determined by different potentiometric methods at pH 4.0, was 0.75–0.80 mmol g^{-1} HA, while their overall content was 4 mmol g^{-1}. Therefore, we can expect that, at selected acidic pH ranges, the amounts of dissociated acidic groups do not exceed 20 % of their maximum value. So, carboxylic groups were predominantly protonated under the experimental conditions used in this study (pH 3.75 or 4.25) which could result in low antioxidant activity of phenol moieties and so to negative correlation between AOC and TP in humic materials.

Therefore, under selected conditions, the antioxidant activity of HLS might be attributed mainly to nitrogen-containing moieties, whereas for HS, both nitrogen-containing compounds and phenolic compounds should be considered.

5 Conclusions

In summary, we observed that humic materials studied displayed a complex kinetic pattern for their reaction with ABTS$^{•+}$. All humic compounds tested here did not reach stable endpoints at 40 min. Our findings indicate that, to obtain clearer information concerning the antioxidant capacities of humic materials, kinetic profiles should first be established, and then endpoint measurements should be taken at a time when the reaction has reached, or at least neared, the endpoint. Our study is unique in that it demonstrates that quantitative characterization of the AOCs of HS or HLS using the ABTS decolorization approach can be applied only under acidic pH conditions. Due to the instability of ABTS$^{•+}$ at neutral and alkaline pH values, only the fast antioxidant moieties of humic materials can be assessed with this method. Our results show that at acidic pH values alone with phenolic compounds, nitrogen-containing groups should be also considered when antioxidant activity of humic materials is discussed.

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