

Development and Validation of Antioxidant Capacity Assessment Protocol for Humic and Humic-Like Substances

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Keywords: antioxidant capacity, humic and humic-like substances

1. INTRODUCTION

Humic substances (HS) are considered to be involved in realization of various processes in soil and aquatic ecosystems (1, 2). The main feature of HS is their polyfunctionality as they can act as sorbents, soil conditioners, fertilizers, bioactive compounds etc. Due to their structure and functions HS have been widely explored for practical use. Nevertheless there is still little knowledge about quantitative characteristics of their biological activity. Therefore such parameters are of great importance. HS are known to take part in redox and radical processes and their antioxidant capacity (AOC) can be proposed as one of essential parameter for evaluation of their properties. Thus, AOC should be a quantitative characteristic of HS efficiency as reactive oxygen species scavenger or trap providing comparison of different HS preparations both for fundamental study and their application. The aim of this study was to develop standardized protocol for quantitative estimation of HS and humic-like substances antioxidant capacity.

2. MATERIALS AND METHODS

Standard and commercially available samples of HS including Suwannee River humic (SR-HA), fulvic acids (SR-FA) and dissolved organic matter (SR-DOM) and Aldrich coal humic acid (CHA-ALD) were chosen for development of antioxidant capacity measurement protocol. For verifying of the developed protocol two samples of humic-like substances (HLS) produced by basidiomycetes *Cerrena maxima* 0275 cultivated on oat straw were used. HLS-45 and HLS-70 were isolated from nutritional media after 45 and 70 days of cultivation as described earlier (3).

Antioxidant capacity (AOC) of HS and HLS was measured as a decrease in the absorbance at 734 nm of radical-cation ABTS ((2,2-azyno-bis-(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt) in their presence. A water soluble analogue of vitamin E

trolox (6-hydroxi-2,5,7,8-tetramethylchroman-2-carboxylic acid) was used as a calibration standard. The obtained results were expressed in trolox molar equivalents per mg of sample that offers possibility of their easy comparison to AOC values for other objects (4). Measurements were carried out using Perkin Elmer lambda 25 UV-VIS spectrophotometer (USA). Generation of $ABTS^{•+}$ radical-cation was made according to protocol of Re and co-workers (5).

HS and HLS AOC assessment was performed at three pH levels using 0.1M sodium citrate buffer solution (pH 3.75 and 4.25) and 0.1M potassium phosphate buffer (pH 6.80). The reaction was initiated by adding 100 μ l of sample solution to 900 μ l of $ABTS^{•+}$ dissolved in suitable buffer followed by measurement of change of absorbance at 734 nm after 3 min. AOC for humics was measured over concentration range of 1-10 mg/l.

3. RESULTS AND DISCUSSION

Development of HS antioxidant capacity protocol requires determination of such conditions as pH of the reaction mixture and a range of HS concentrations where its relationship to AOC is linear. Also $ABTS^{•+}$ stability should be determined in chosen matters.

Our findings showed that $ABTS^{•+}$ exhibited maximal stability in deionized water. Increasing of reaction mixture pH led to gradual decrease of $ABTS^{•+}$ concentration (Fig. 1).

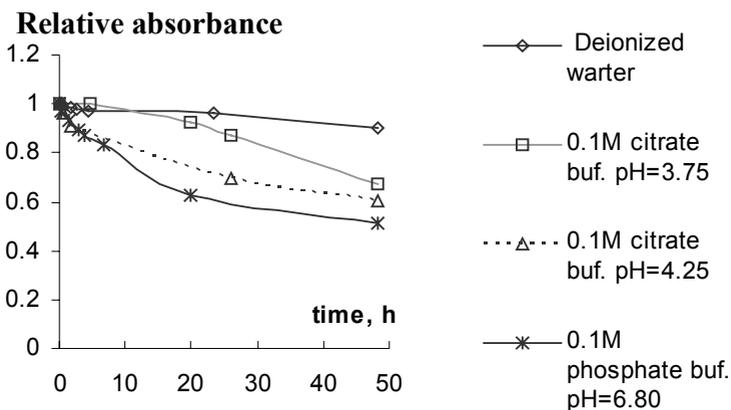


Figure 1. Stability of $ABTS^{•+}$ radical-cation in different reaction mixtures. Relative absorbance is the ratio of optical density monitored in the course of experiment to initial value at 734 nm (D/D_0).

Concentration-dependent AOC over used concentration range was characterized by smooth, non saturating curves for all studied humics (Fig. 2A). At low concentrations of HS

(1-5 mg/l) AOC noticeably decreased along with increase in HS concentration followed by reaching plateau at about 6 mg/l. The exception was AOC measured at pH 6.8 where smooth decrease of that parameter was observed over concentration 1-10 mg/l.

Estimation of AOC at different pH demonstrated its increase with growing pH (Fig. 2B) what probably reflected processes of HS conformational rearrangements and dissociation. Besides mechanism of HS antioxidant action shifts from hydrogen atom transfer to electron transfer in neutral and alkaline pH could be proposed under such conditions (6).

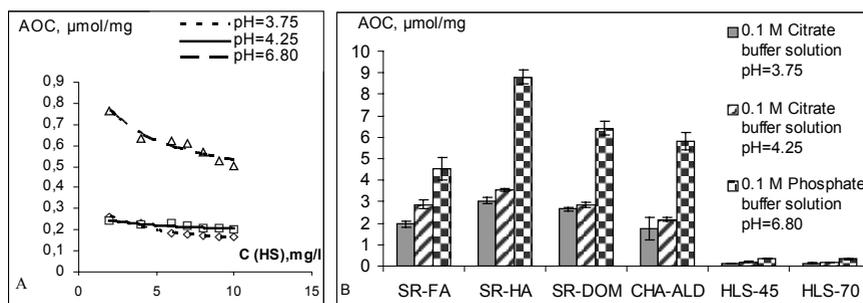


Figure 2. AOC of the studied HS and HLS at different concentrations (A; on the example of CHA-ALD) and pH level (B).

Values of specific AOC at pH 4.25 for HS varied from 2.18 to 3.56 $\mu\text{mol}/\text{mg}$ what did not differ significantly from AOC of well known natural antioxidant dihydroquercetine (2.67 $\mu\text{mol}/\text{mg}$). At pH 6.80 those values increased to 4.54-8.79 $\mu\text{mol}/\text{mg}$ that was corresponded to 6.77-12.29 μmol of trolox per mg of organic carbon (OC). The latter met well (7) where AOC range 4.38-26.57 $\mu\text{mol}/\text{mg}$ of OC were reported for alkaline soil extracts. HLS have antioxidant capacity on the average 17-times lower in comparison to HS (Fig. 1B). This can be hypothesized by different functional groups content.

4. CONCLUSIONS

The trolox equivalent antioxidant capacity protocol has been developed for HS and HLS. The determined AOC was demonstrated to be concentration independent for both HS and HLS in the range of concentrations 4-10 mg/l. The results obtained at different pH levels showed significant increasing in HS and HLS antioxidant capacity with growing pH from 3.75 to 6.80. However, under neutral and particularly alkaline pH ABTS^{•+} was rather unstable, so mild acidic (pH 4.0-5.0) conditions could be recommended for HS and HLS antioxidant capacity determination.

ACKNOWLEDGEMENTS

Authors express their deepest appreciation to Prof. Irina Perminova for the samples of HS. This research was financially supported by the International Science and Technology Center (#KR-993.2)

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