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Influence of pH on the speciation of copper(II) in reactions with the green tea polyphenols, epigallocatechin gallate and gallic acid

Katharina F. Pirker [a], Maria Camilla Baratto [b], Riccardo Basosi [b], Bernard A. Goodman [a,c,*]

[a] *Health and Environment Department, Environmental Resources and Technologies, AIT Austrian Institute of Technology GmbH, Konrad-Lorenz-Straße 24, 3430 Tulln, Austria*
[b] *Department of Chemistry, Via A. de Gasperi 2, University of Siena, 53100 Siena, Italy*
[c] *State Key Laboratory for Conservation and Utilization of Subtropical Agro-Bioresources, Guangxi University, Nanning, 520004 Guangxi, China*

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ABSTRACT

Changes in speciation of copper(II) in reactions with epigallocatechin gallate (EGCG) and gallic acid (GA) as a function of pH have been investigated by multifrequency (X- and S-band) EPR spectroscopy in the fluid and frozen states. The EPR spectra show the formation of three distinct mononuclear species with each of the polyphenols, and these are interpreted in terms of one *mono*- and two *bis*-complexes. However, di- or polymeric complexes dominate the Cu(II) speciation in the pH range 4–8, and it is only at alkaline pH values that these mononuclear complexes make appreciable contributions to the metal speciation. Each mononuclear complex displays linewidth anisotropy in fluid solution as a consequence of incomplete averaging of the spin Hamiltonian parameters through molecular motion. Rotational correlation times for the individual complexes have been estimated by analysing the lineshape anisotropy of the fluid solution spectra using parameters determined by simulation of the rigid limit spectra. These show that the molecular masses increase with increasing pH, indicating either coordination of increasing numbers of polyphenol molecules as ligands to the copper or the increasing involvement of polyphenol dimers as ligands in the copper coordination sphere.

1. Introduction

Interactions between transition metal ions and phenolic compounds are widespread in nature, and can involve complexation of metal ions by the phenols or their oxidation products, polymerisation and redox reactions. Although polymerisation and complexation reactions between Cu(II) and a number of polyphenols have been reported [1,2], it is generally assumed, especially in the biological literature [3–7], that redox is the major reaction process. In redox reactions between Cu(II) and polyphenol molecules, Cu(II) is reduced to Cu(I) and the hydroquinone (H_2Q) is oxidised to the semiquinone (HQ). In a second oxidation step, the semiquinone (HQ) is oxidised to the quinone (Q) also by Cu(II) [8].

$$\mathrm{Cu(II)} + \mathrm{H_2Q} \rightarrow \mathrm{Cu(I)} + \mathrm{HQ} \tag{1}$$

$$\mathrm{Cu(II)} + \mathrm{HQ}\cdot \rightarrow \mathrm{Cu(I)} + \mathrm{Q} \tag{2}$$

We have recently investigated the reaction between Cu(II) and gallic acid (GA) over a wide range of pH values, and found no evidence to support either reactions (1) or (2) [9]. The observed oxidation of GA in the alkaline pH region was the result of autoxidation, which was in fact inhibited by Cu(II). In that work, the EPR spectra, which were recorded in fluid solution only, indicated the formation of two, and possibly three, different complexes whose intensities depended on the pH and the Cu:GA ratio, along with the precipitation of a di- or polymeric EPR silent species in the approximate pH range 4–8.

There is extensive epidemiological evidence for the health benefits of green tea (e.g. [10]), and recently there have been proposals to make use of the metal chelating properties of its major polyphenol, epigallocatechin gallate (EGCG), in the treatment of neurodegenerative disorders (e.g. [11–13]). However, there is also evidence that the prion diseases, such as Creuzfeld-Jacob's disease and Alzheimer's, are associated with copper deficiency [14–16]. Thus it is important that the reaction between Cu and EGCG is understood as fully as possible, especially if the chemistry of EGCG mirrors that of GA where precipitation of copper complexes occurs at physiological pH values.

Although both GA and EGCG belong to the same family of polyphenols, there are important differences in their structures. The structure of GA is simple and consists of a carboxyl group attached to a pyrogallol entity (Fig. 1a). The structure of EGCG is more complex with two pyrogallol groups in the molecular structure (one on ring B and one on ring D (Fig. 1b), and one resorcinol group on ring A, but no free carboxyl group. Therefore the principal objective of the present investigation was to determine the extent to which the

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* Corresponding author at: State Key Laboratory for Conservation and Utilization of Subtropical Agro-Bioresources, Guangxi University, Nanning, 520004 Guangxi, China, Tel.: +86 771 3272475; fax: +86 771 3237873.

E-mail addresses: katharina.pirker@ait.ac.at (K.F. Pirker), baratto@unisi.it (M.C. Baratto), basosi@unisi.it (R. Basosi), bernard_a_goodman@yahoo.com (B.A. Goodman).

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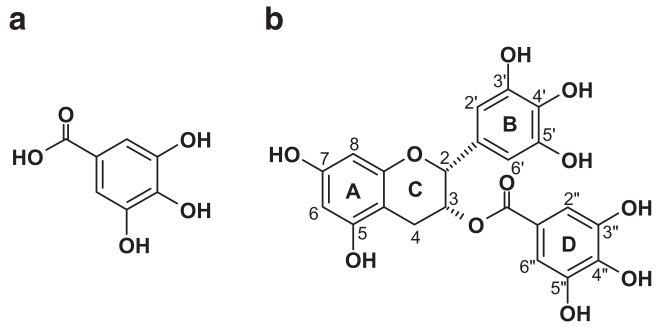

Fig. 1. Molecular structures of (a) gallic acid (GA) and (b) epigallocatechin gallate (EGCG).

reactions of GA and EGCG with transition metal ions such as Cu(II) follow similar or different pathways, and to gain information on the complex formation of these polyphenols with Cu(II). For example, the formation of di- or polymeric species involving Cu(II) and the carboxylate group was proposed by Ferreira Severino et al. [9] for the identity of the "EPR silent" species in the reaction of Cu(II) with GA, but since there is no free carboxyl group in EGCG, a similar reaction would not be expected with that polyphenol.

In the previous report of the reactions between Cu(II) and GA, EPR spectra were only obtained from fluid solutions, since the objective of that investigation was simply to distinguish between the relative importance of redox, complexation and polymerisation reactions at different pH values. No anisotropic (rigid limit) spectral parameters were reported, although these could provide additional information on the Cu coordination environment in the mononuclear complexes. Furthermore, the Cu(II) spectra all showed the presence of linewidth anisotropy as a result of incomplete averaging of the anisotropic spectral parameters through molecular motion, but these were not analysed in detail apart from the derivation of approximate values for the isotropic g-values and hyperfine coupling constants. However, if the anisotropic values from the rigid limit spectra are available, it is possible to analyse the fluid solution spectral lineshapes to produce rotational correlation times that are related to the molecular masses of the complexes.

In the present paper we report the results of a comprehensive EPR spectroscopic investigation of the EGCG/Cu(II) system along with additional measurements on the GA/Cu(II) reaction to extend those reported by Ferreira Severino et al. [9]. Spectra were recorded with fluid and frozen solutions at X-band (~9 GHz) and S-band (~3 GHz) frequencies for samples with a wide range of pH values and Cu: polyphenol ratios. The use of S-band measurements for the EGCG/Cu(II) system is important for understanding the fluid solution results, because of the large linewidth anisotropy in the spectra recorded at X-band frequencies [17–19].

2. Materials and methods

2.1. Sources of materials

Epigallocatechin gallate (EGCG, 95% purity) and gallic acid (GA, ≥98%) were purchased from Sigma–Aldrich Handels GmbH (Vienna, Austria), and copper sulphate anhydrous ($CuSO_4$) was bought from Merck (VWR International GmbH, Vienna, Austria).

2.2. Sample preparation

Solutions of different concentration ratios of Cu:GA (1:0, 1:0.5, 1:1, 1:2, 1:10 for X-band measurements and 1:5 for S-band measurements) and Cu:EGCG (1:0, 1:0.5, 1:1, 1:2, 1:5 for X-band measurements and 1:5 for S-band measurements) were prepared with pH values ranging between 1 and 13 with a constant Cu(II) concentration of 2 mM. EPR spectra were recorded at room temperature and low temperature (77 K or 160 K) at both X- and S-band frequencies in solutions containing 5% glycerol, which was added to aid glass formation for the frozen solution studies.

2.3. EPR measurements

EPR spectra were acquired as first derivatives of the microwave absorption with either a Bruker EMX CW spectrometer, operating at X-band frequencies (9 GHz) or a Bruker 200D SRC operating at S-band frequencies (3 GHz). For X-band measurements, a high sensitivity cavity was used and microwaves were generated by a Gunn diode; the microwave frequency was recorded continuously with an in-line frequency counter. Low temperature spectra were recorded using a quartz "finger dewar" containing liquid nitrogen inserted into the microwave cavity. S-band EPR spectra were obtained using a S-band bridge ($\nu = 2$–4 GHz) SB-1111 Jagmar (Poland), and low temperatures were controlled with a Bruker ER 4111VT variable temperature unit.

The Cu(II) EPR spectra were acquired using 20 mW microwave power (MP) for room temperature and 2 mW MP for low temperature measurements, 100 kHz modulation frequency (MF) and 1 mT modulation amplitude (MA). g-values were determined by reference to the signal of DPPH ($g = 2.0036$), which was used as an external standard.

2.4. Data analysis

Signal intensities of the fluid solution spectra were determined by double integration (DI) using the Bruker WINEPR software. For determination of the Cu(II) intensity, the DI of the whole Cu(II) spectrum was carried out, followed by subtraction of the DI of the intensity of the free radical signal in the measurements at very high pH.

Easyspin [20] was used for spectral simulation and analysis. Parameters were determined for the frozen solution spectra using the fitting function "pepper", and these were then used as the basis for simulation of the fluid solution spectra. The Easyspin software assumes the natural abundance ratio of ^{63}Cu and ^{65}Cu isotopes, but returns hyperfine splittings for the ^{63}Cu isotope only; thus the tabulated results apply only to this nucleus (note: the Cu hyperfine parameters for many spectra reported in the literature give a weighted mean from the two isotopes). The frozen solution spectra were all simulated using a model assuming axial symmetry and co-axial g- and hyperfine matrices. For the latter, the spectra provide information on the absolute magnitudes of the $A_{//}$ and $A_{\perp}$ values, but not on their relative signs. Therefore, simulations to produce the rotational correlation signs were performed initially for situations where these principal values of the hyperfine coupling constant had the same or opposite signs. Fast motion solution spectra (S- and X-band spectra from Complex I, II, and III of GA/Cu and Complex I of EGCG/Cu) were simulated using the "garlic" function, whereas slow motion solution spectra (S- and X-band spectra from Complex II and III of EGCG/Cu) were fitted using the Easyspin function "chili".

3. Results and interpretation

3.1. Dependence of Cu(II) signals in fluid solutions on pH and polyphenol concentration

The Cu(II) spectral intensities at X-band frequencies are presented in Fig. 2 as a function of pH for various Cu(II):polyphenol ratios for the Cu/GA and Cu/EGCG reaction systems. Similar curves are observed for both polyphenols; the total signal intensity, and hence the copper speciation, is dependent on both the pH and the Cu(II):polyphenol ratio. The results for the GA system (Fig. 2a) are similar to those reported previously for the Cu/GA system in 1:1 methanol/water