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Attempting to Understand Health Effects Associated with Tea Consumption by Investigating Complexation Reactions with Copper

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Abstract: Tea consumption is associated with many health benefits including protection against neurodegenerative disorders, heart disease and viral infections, and stimulation of brain activity, it has also been reported to help diabetes prevention, and has been linked with anticancer properties. This presentation addresses the chemical components in teas that might be responsible for the reported health effects. Although many beneficial effects of tea consumption are associated with the high polyphenol contents of the beverages, the chemical forms of the specific polyphenols differ greatly among tea types, primarily as a consequence of their processing conditions. Furthermore, the wide ranges of beneficial health effects associated with tea consumption, makes it unlikely that all are derived from a single family of chemicals. Therefore, special consideration is given to the search for non-phenolic components in teas with potential biological activities.

Keywords: Green tea, oolong tea, copper complex, EPR spectroscopy, HPLC

Introduction

There is extensive literature on the association of tea consumption with health benefits, as summarized in various reviews (e.g.[1-5]). Based on epidemiological, clinical and experimental studies, important specific links have been reported for cancer protection (e.g.[6-12]) and cardiovascular health,[13-17], along with protection against neurodegenerative disorders,[18-21] stimulation of brain activity[22-23] and stroke prevention,[24], protection against viral infections,[25,26] helping to prevent diabetes,[27-29] and metabolic disorders,[30,31] promoting healthy skin,[32-34] and the treatment of obesity.[35-38] However, as indicated by the research of Woodward & Tunstall-Pedoe[39], other lifestyle factors can lead to a negative association in specific communities, and caution should be exercised in isolating tea consumption from other lifestyle factors in developing links to general health. It should also be borne in mind that the modes of action of teas are complex, and synergistic properties of various components in tea extracts may be potentially important for their medicinal benefits. Nevertheless, there seems to be a broad consensus that many of the beneficial effects of tea consumption are derived from their antioxidant properties.[40-43] Since the polyphenol catechins represent the major antioxidants in teas, they are considered to be largely responsible for the positive effects of tea consumption,[44-49] especially since teas are rich in such polyphenols,[50] which are also readily bioavailable.[51]

Although fresh tea leaves may contain up to 30% of their dry weight as catechins,[52] these may be altered appreciably during processing. For example, aerobic oxidation during the formation of black tea results in condensation reactions that generate bisflavanols, theaflavins, epitheaflavic acids, and thearubigens.

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Also, as pointed out by Cooper *et al.*,[53] characteristics unrelated to antioxidant properties of teas may be responsible for anticancer activity and improvements in cardiac health and atherosclerosis. Teas also contain theanine (5-N- ethylglutamine), which has a role in reducing stress, oxidized catechins which may reduce cholesterol levels in blood, caffeine, which has long been recognized as a brain stimulant,[54-56] and small amounts of theobromine and theophylline; they also accumulate the metals aluminum and manganese.

As mentioned above, antioxidant activity (reducing ability) is an important property that is commonly associated with polyphenols; another is metal chelation.[19, 57-59] In recent years, we have investigated reactions with Cu^{2+} as a method to probe these properties with both teas and individual polyphenols using the technique of electron paramagnetic resonance (EPR) spectroscopy to characterize the molecules produced. Interestingly, neither the polyphenols gallic acid[60] nor epigallocatechin gallate[61] show any significant reducing ability towards Cu(II) over a wide range of pH values. Furthermore, these polyphenols produce insoluble Cu(II) species in the pH range 4-6.5, with no complexation occurring at lower pH. In contrast, Cu(II) complexation with both green and black teas commences at pH <2, and an appreciable fraction of the Cu(II) remains in solution in the pH range 4-6.5.[62,63] Indeed it is only under alkaline conditions that complexation with polyphenols dominates the solution chemistry of Cu(II) in teas.

In view of these results and the importance of tea as a common beverage with medicinal properties, we are attempting to identify the components that are able to complex copper at acidic pH values. Some preliminary results were reported by Stolze et al.,[63] and the present paper describes recent progress, and includes results obtained with an oolong tea which was not investigated in our previous measurements.

Methodology

Materials

The teas used in this study were commercial products obtained from China; they consisted of a green tea of Shandong origin, and an oolong tea (known as King's oolong) from Fujian. HPLC solvents were from purchased from VWR Chemicals Vienna, Austria, and all other chemicals from Sigma-Aldrich, Vienna, Austria.

Preparation of tea extracts

Leaves of green tea or oolong tea were extracted with water (1g/50ml) at ~90°C for 10 min to yield samples which we refer to as GTB#1 and Koo#1, respectively. These extracts were cooled to room temperature (~22°C), filtered using MN 620 ¼ from Macherey-Nagel, Graz, Austria, and used immediately for HPLC and EPR measurements.

HPLC measurements

HPLC measurements used a Waters HPLC system equipped with a PDA 996 detector. 10 µl of the aqueous tea extract were injected and run at 25°C and 1ml/min on a Phenomenex Synergi Hydro C18 column 150 x 4.6mm / 5µm particle size using the following gradient (trifluoroacetic acid in water (pH 3)): 0-5min: 0% acetonitrile (ACN); 5-15 min: 0-10% ACN; 15-30min: 10% ACN; 30-50min: 10-15% ACN; 50-70min: 15-20% ACN; 70-90min: 20-25%ACN; 90-110%: 25-50%ACN; 110-120min: 50-100% ACN;120-125min: 100% ACN: 125-140min: 0% ACN.

EPR spectroscopy of copper complexes

Reaction of the various extracts with Cu(II) were investigated over a wide range of acidic-to-neutral pH values (1.7-7.4) in an attempt to identify the different types of complexes that form in these solutions on the basis of their EPR spectral parameters. EPR spectra were acquired using a Bruker ESP300E spectrometer operating at X-band frequencies. The measurement protocol was as follows. Firstly, the pH of 4ml of freshly prepared aqueous tea extract (1g/50ml H_2O, 90°C, 10min) was measured and the first EPR spectrum recorded using 500µl in a TM_{110} flat cell. Then copper(II) sulfate solution was added to give a copper concentration of either 2 mM or 300 µM, and the pH and EPR spectrum measured again. Measurements of the pH and EPR spectra were repeated following addition of successive amounts of H_2SO_4 (1M) until the pH decreased to 1.7. EPR acquisition conditions were: field range 1500 G; microwave frequency 9.797 GHz; microwave power 20 mW; modulation frequency 100 kHz; modulation amplitude 10 G; receiver gain $1x10^4$, time constant 81.9 msec; correlation time 81.9 msec; 1024 data points and 5 accumulations.

Spectral Analysis

The first EPR spectrum (without copper added) was used as the background tea signal, and an additional

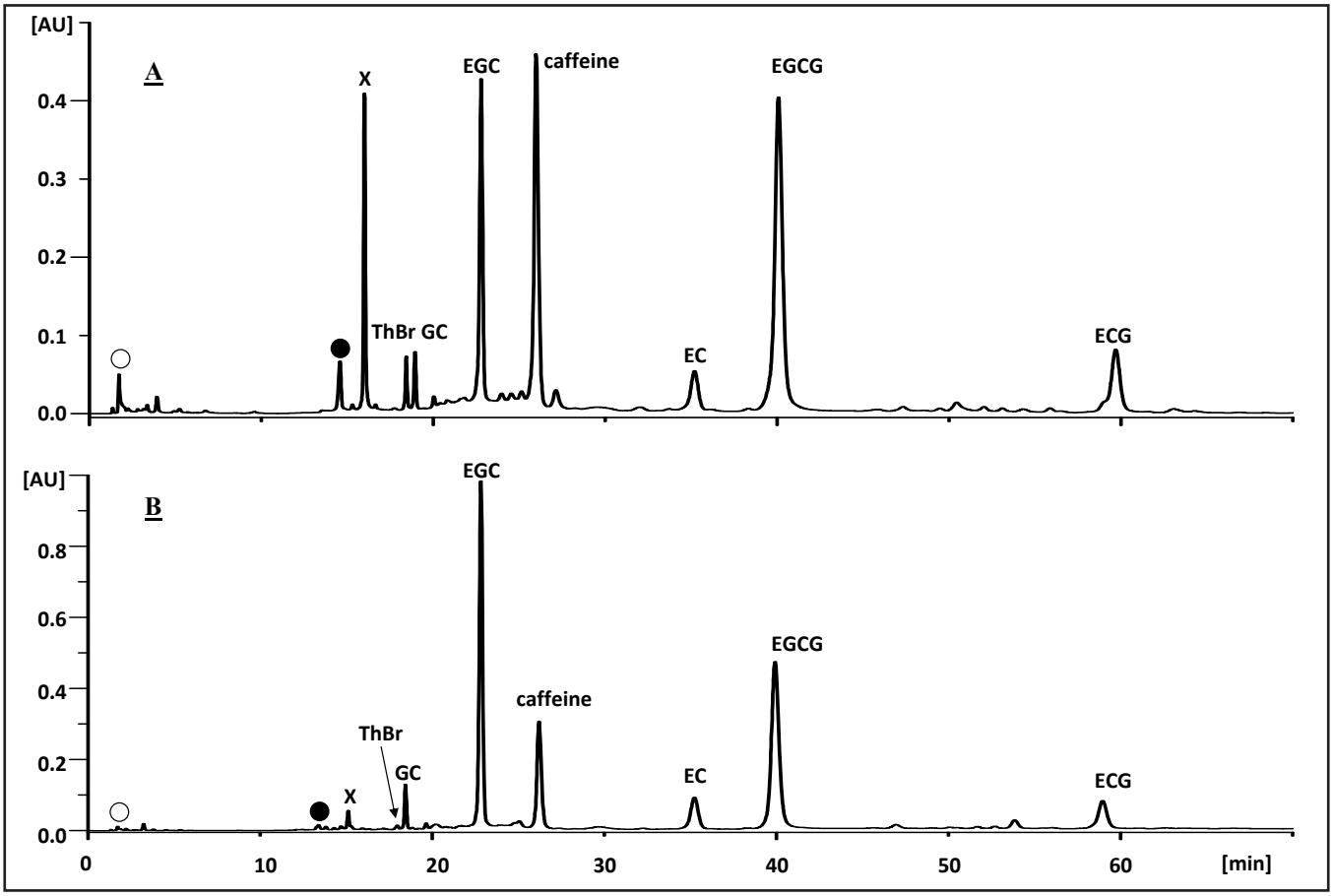
background scan was also made with water in the flat cell to provide a system background signal. Both background signals were subtracted from the EPR spectra of the tea to produce a set of spectra which correspond to the products of reactions of copper with tea components (i.e. free from contributions of Mn^{2+} and the flat cell background). Difference spectra were generated by subtracting spectra obtained at different pH values and these resulting spectra were then analyzed.

Results

HPLC

The HPLC trace for the initial green tea extract is shown in Fig. 1a. Standards were available for gallic acid (GA), theobromine (ThBr), theophyllin, catechin (C), caffeine, epicatechin (EC), and epigallocatechin gallate (EGCG), whereas gallocatechin (GC), epigallocatechin (EGC), and epicatechin gallate (ECG) were identified according to their UV spectra, relative retention time and relative abundance reported in the literature.[64] With the exception of theobromine which was present only as a minor component, these compounds were also found in the oolong tea (Figure 1b), but with slightly different relative concentrations. In addition, three major peaks (marked "○", "●", and "x") in the green tea extract were considerably weaker in the oolong tea

The 1st peak (at 2.0 min) did not show a characteristic UV spectrum, that of the 2nd peak (at 11.9 min), was a superposition of gallic acid (GA) and an unknown substance with an uncharacteristic UV spectrum, whereas the UV spectrum of the 3rd peak (at 16.0 min) was similar, but not identical, to the spectrum of gallic acid. Comparison with data from the literature[64, 65] suggests that this last compound might be an adduct of quinic acid (e.g. 3- or 5-O-galloyl quinic acid).



(A) HPLC chromatogram of green tea extract (1g in 50 ml water for 10min / 90°C). The major peaks are indicated as theobromine (ThBr), gallocatechin (GC), epigallocatechin (EGC), caffeine, epicatechin (EC), epigallocatechin gallate (EGCG) and epicatechin gallate (ECG). The peaks of the unknown compounds are marked as "○", "●", and "x". The compound "●" partially overlaps with gallic acid (GA), The UV spectrum of "x" is similar to gallic acid derivatives.

(Bb) The corresponding HPLC chromatogram of the oolong tea extract. The compounds "○", "●", and "x" are markedly weaker than in the green tea.

EPR spectra

The original EPR spectra of both green and oolong tea extracts were dominated by the sextet signal of free Mn^{2+} ions (not shown), and in addition contained a background (baseline drift and impurities) from the flat cell. These signals were routinely subtracted from the experimental spectra to yield results that corresponded to the Cu^{2+} spectra at each of the pH values.

(i) Green tea extract

The Cu^{2+} EPR spectra obtained for seven different pH values with Cu^{2+} concentrations of either 2 mM or 300 µM are shown in Figure 2. These generally consist of overlapping signals, which we attempted to resolve by performing weighted subtractions of the results from different pHs. These yielded the results shown in Figures 3 and 4.

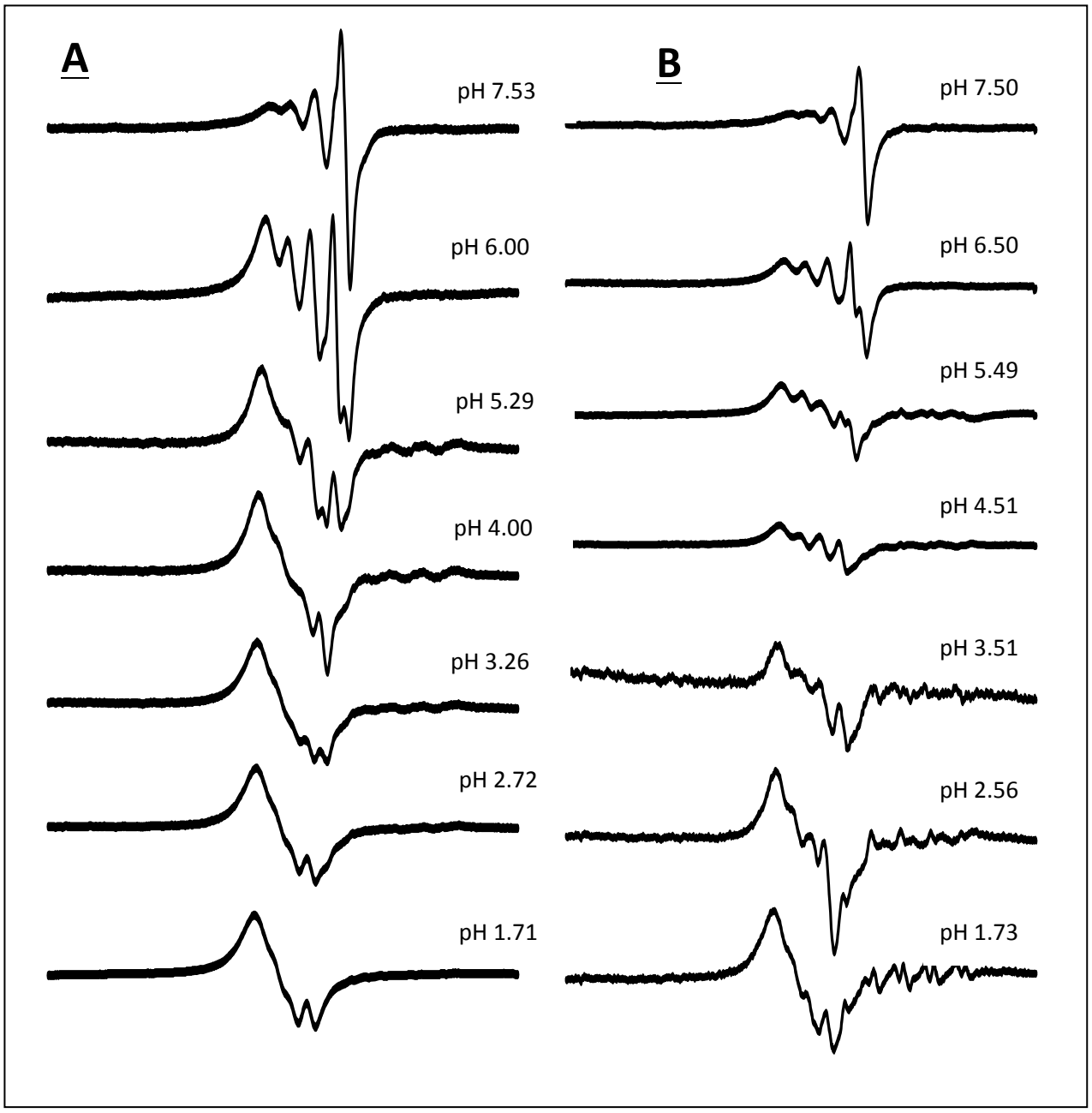
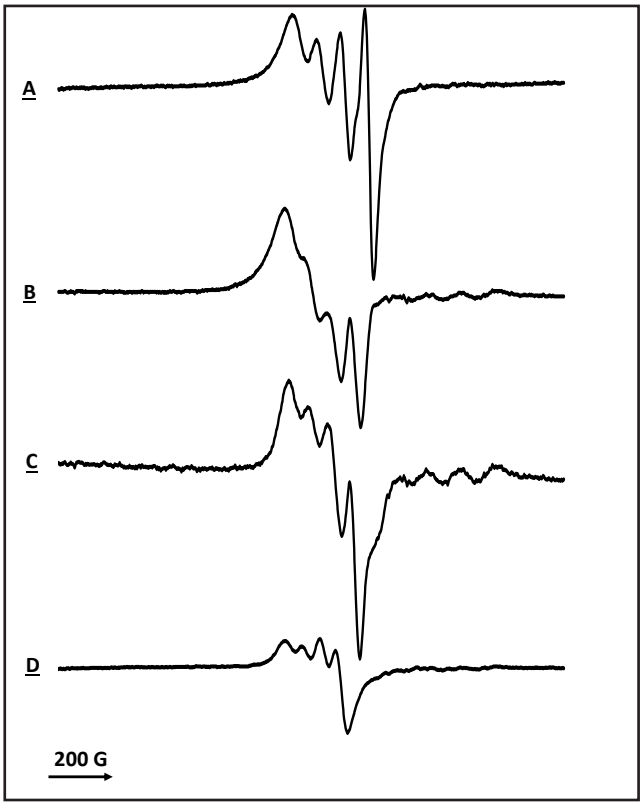
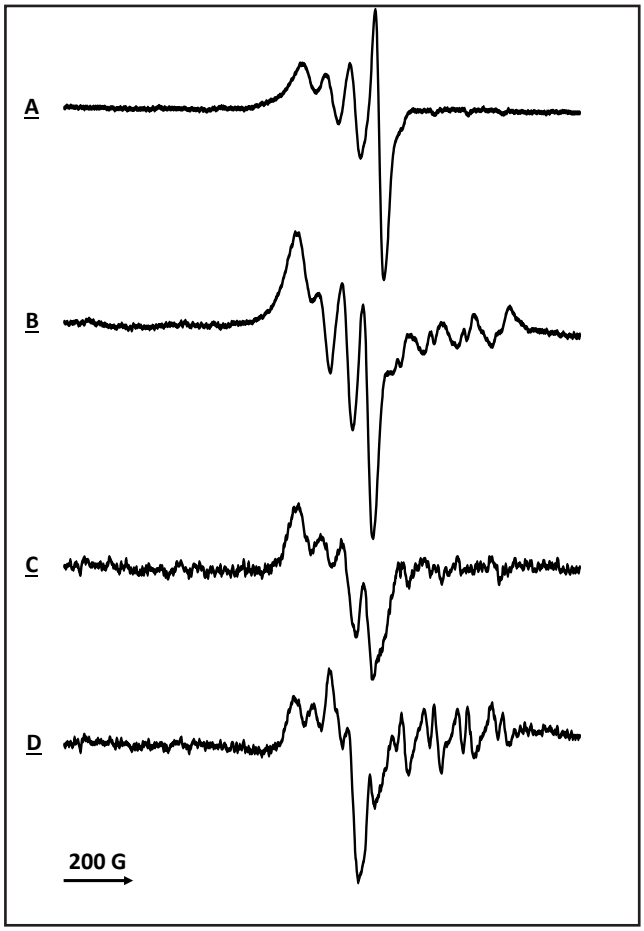


Figure 2 EPR spectra of green tea extract #1 after addition of a) 2mM and b) 300µM Cu^{2+}

EPR spectra from the green tea extract in the presence of (A) 2 mM, and (B) 0.3 mM CuSO4 acquired with different pH values.



EPR difference spectra calculated from individual spectra in Figure 2A (2 mM Cu^{2+}):
(A) EPR (pH 6) – 0.6 * EPR (pH 7.53), (B) EPR (pH 4) – 0.75 * EPR (pH 5.29), (C) EPR (pH 4) – 0.5 * EPR (pH 1.71), and (D) EPR (pH 1.71) – 0.45 * EPR (free Cu^{2+} signal).



EPR difference spectra calculated from individual spectra in Figure 2B (0.3 mM Cu^{2+}):
(A) EPR (pH 6.5) – 0.6 * EPR (pH7.5), (B) EPR (pH 4.51) – 0.4 * EPR (pH 5.49), (C) EPR (pH 3.51) – 0.25 * EPR (pH 1.73), and (D) EPR (pH 1.73) – 0.18 * EPR (free Cu^{2+} signal).

Two major components can be separated from the difference spectra. The spectra in Figures 3a, and 4a are similar to those of bis theanine copper complexes,[62] and although they are not identical this is their most probable assignment considering the identification of theanine in the HPLC traces, and the ability of amino acids to form bis complexes with Cu^{2+} at similar pH values.[66] The spectra for pH values <2 (Figs. 3d,4d) also correspond to a single Cu(II) complex along with free Cu^{2+} ions, although this has not yet been identified. However, the difference spectra calculated for slightly acidic pH values (Figures 3b,4b, and 3c,4c) still seem to correspond to mixtures of complexes.

(ii) Oolong tea extract

The results from the oolong tea extracts at different pH values after correcting for the background signal from the flat cell and the Mn^{2+} component (Fig. 5) were similar to those from the green tea, although the respective signal intensities were lower, especially around pH 1.7, where the signal of free Cu^{2+} ions was predominant. The difference spectra between different pH values (Figures 6 and 7) for the two separate Cu^{2+} concentrations were obtained in a similar way to those from green tea shown above

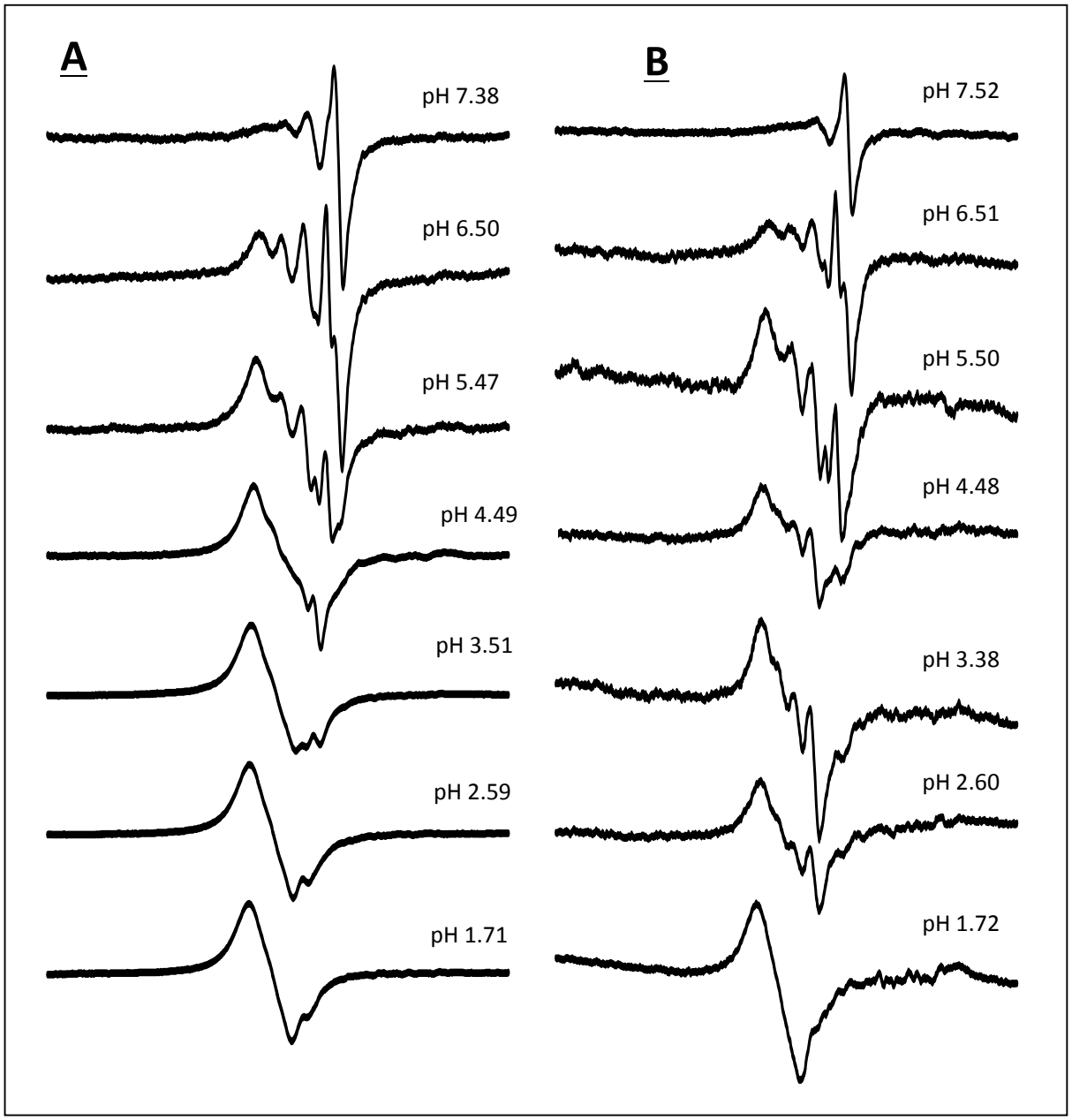
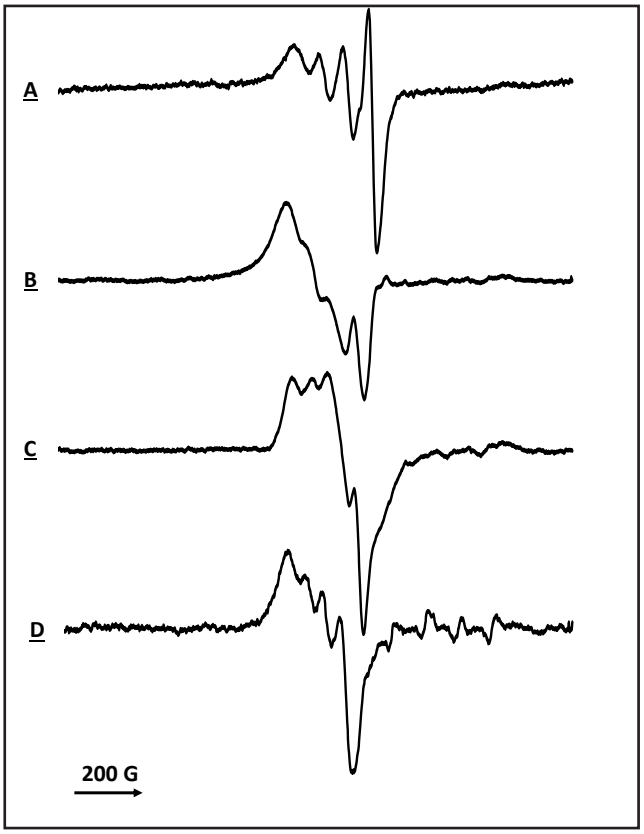
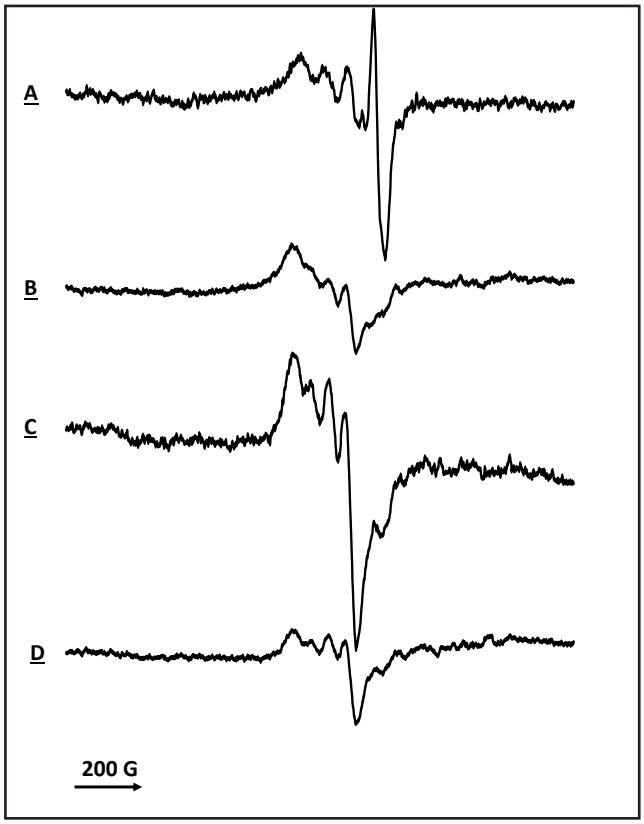


Figure 5 EPR spectra of oolong tea extract #1 after addition of a) 2mM and b) 300µM Cu^{2+}

EPR spectra from the oolong tea extract in the presence of (A) 2 mM, and (B) 0.3 mM $CuSO_4$ acquired with different pH values.



EPR difference spectra calculated from individual spectra in Figure 5A (2 mM Cu^{2+}):
(A) EPR (pH 6.5) – 2.7 * EPR (pH 7.38), (B) EPR (pH 4.49) – 0.8 * EPR (pH 5.47), (C) EPR (pH 4.49) – 0.32 * EPR (pH 1.71), and (D) EPR (pH 1.71) – 0.37 * EPR (free Cu^{2+} signal).



EPR difference spectra calculated from individual spectra in Figure 5B (0.3 mM Cu^{2+}):
(A) EPR (pH 6.51) – 0.43 * EPR (pH 7.52), (B) EPR (pH 4.48) – 0.25 * EPR (pH 5.5), (C) EPR (pH 3.48) – 0.1 * EPR (pH 1.72), and (D) EPR (pH 1.72) – 0.05 * EPR (free Cu^{2+} signal).

As with the green tea (Figures 3a, 4a), the spectra in Figures 6a, and 7a have parameters similar, but not identical to those of bis theanine copper complexes,[62] although there might also be minor contributions from other complexes. Also, at pH values <2 (Figs. 6d,7d) the spectrum consists of a mixture of free Cu^{2+} ions and a complex which is similar to that seen with the green tea, but with the oolong tea the contribution of the complex is much weaker than with the green tea. As with green tea, the difference spectra for weakly acidic pH values (Figures 6b,7b and 6c,7c) seem to be mixtures, possibly involving the mono theanine complex and/or the gallic acid derivative (with the HPLC peak at 14.9 min) (b-series), or the complex stable around pH 1.7 (c-series).

Discussion

The major polyphenols (e.g. EGCG) have already been excluded as being responsible for the Cu(II) complexes in acidic solutions.[63] Furthermore, they can also be easily separated from tea solutions by an SPE column, which removes all compounds eluting later than 15-20 min). Since most of the major peaks in the HPLC traces of the two teas are of comparable intensity (Fig. 1a and b), they can be excluded as being responsible for the Cu^{2+} complex formed at low pH values. The exception is the first four peaks. However, theobromine can also be excluded, since it does not form a stable copper complex under the experimental conditions used. The other three peaks are more difficult to evaluate, because they may consist of more than one component. However, on the basis of a comparison of our HPLC and UV data, peak 3 could be a gallic acid derivative, probably as a quinic acid ester that has been reported previously by other groups.[64, 65] The first two major peaks cannot be identified yet, primarily because they overlap with various other smaller peaks. One of these is gallic acid itself, but the products if its reactions with copper