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Interaction of quercetin with copper ions: complexation, oxidation and reactivity towards radicals

Anna Pękal · Magdalena Biesaga · Krystyna Pyrzynska

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Abstract Quercetin, one of the most common dietary flavonols, was investigated in the presence of Cu(II) ions in methanolic solution in order to obtain some explanation on the mechanism interaction and its action against free radical-mediated damage. The spectroscopic studies (UV–VIS, IR, ESI–MS) were used to assess the extent to which it undergo complex formation through chelation or modification through oxidation. The reaction of quercetin with Cu(II) resulted in the formation of 1:1 metal–ligand complex ($\lambda_{max} = 436$ nm) through the carbonyl oxygen and 3-OH group in C ring. Then quercetin is oxidized to the benzoquinone type products. The addition of EDTA destroyed the complex but did not regenerate the whole original spectrum of quercetin. From the other hand, the presence of EDTA inhibits formation of copper–quercetin complex and quercetin oxidation. The antioxidant activity of the Q + Cu solutions was evaluated by using 2,2-diphenyl-1-picrylhydrazyl radical (DPPH•) radical scavenging method and from an electrochemical point of view. The complex is much more effective as free radical scaveninger than the free flavonoid.

A. Pękal · M. Biesaga · K. Pyrzynska (✉)
Department of Chemistry, University of Warsaw, Warsaw, Poland
e-mail: kryspyrz@chem.uw.edu.pl

Keywords Flavonoid · Quercetin · Copper complexation · Antioxidant properties

Introduction

Flavonoids are polyphenolic compounds that occur ubiquitously in the plant kingdom. A multitude of substitution patterns in the two benzene rings (A and B) of the basic structure occur in nature. Variations in their heterocyclic rings rise to flavonols, flavones, catechins, flavonones, anthocyanidins and isoflavones. The epidemiological studies point out to their possible role in preventing cardiovascular diseases and cancer (Fiorucci et al. 2007; Grazul and Budzisz 2009). The consumption of flavonoid-rich food throughout life may have the potential to limit or even reverse age-dependent deteriorations in memory and cognition (Spencer et al. 2009). Flavonoids behave as antioxidants by a variety of ways including direct trapping of reactive oxygen species, inhibition of enzymes responsible for superoxide anion production, chelation of transition metals involved in processes forming radicals and prevention of the peroxidation process by reducing alkoxyl and peroxyl radicals (Fiorucci et al. 2007; Murota and Terao 2003). Quercetin, 3,3′,4′,5,7-pentahydroxylflavone (Fig. 1) is one of the most abundant flavonoids present in fruits and vegetables. It has attracted the attention of many researchers because of its biological

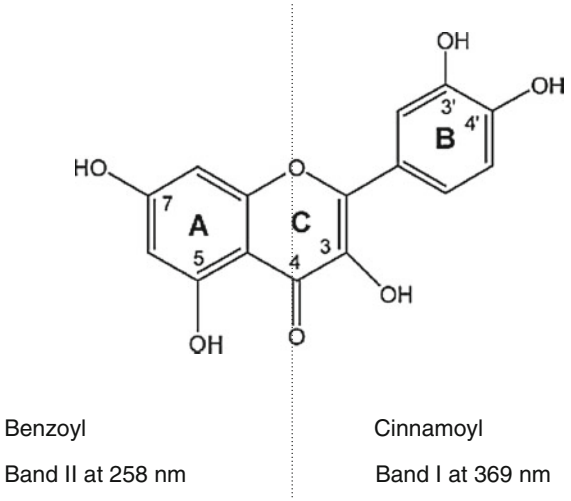

Fig. 1 Chemical structure of quercetin and the division of bands I and II related to UV–VIS absorption bands

and pharmaceutical properties (Boots et al. 2008; Biesaga and Pyrzynska 2009).

Many of the beneficial effects of quercetin are related to its ability to act as free radical acceptor, however, the metal-complexing properties of this molecule may contribute to its total antioxidant activity. Metal ions which possess redox activity as cofactors for various enzymes are also cytotoxic as they generate reactive oxygen species. Generally by chelating metal ions flavonoids prevent metal-catalyzed free radical generation and their subsequent reactions and accordingly protect very important biologically active molecules from oxidative stress (Fiorani et al. 2002). Quercetin possesses three possible metal chelating sites that can interact with metal ions: the 3′,4′-dihydroxy group located on the B ring and the 3- or 5-hydroxy and 4-carbonyl group in the C ring (Fig. 1). Cornard and Merlin (2003) have reported that in acidic conditions, the 3-hydroxy-4-ketone or the 5-hydroxy-4-ketone groups of quercetin are involved in complex formation with the stoichiometry of Me:L = 1:1. In alkaline media, the second implicated site is the 3′,4′-dihydroxy group located on the B ring which allows formation of high stoichiometry complexes (1:2).

Some papers deal with the complex formation of quercetin with metal ions, but data on the composition, structure and conditions of complex formation are sometimes incomplete and contradictory. For steric reasons the complexes usually include no more than two flavonoid molecules, however, four different Al–quercetin stoichiometry have been proposed, e.g. 1:1, 1:2, 1:3 and 2:3 (Gutierrez and Gehlen 2002; De Souza and De Giovani 2005; Cornard and Merlin 2002). It was demonstrated that the degree of complex formation was dependent on the Al–ligand ratio and the solvent system employed; in general methanol favoured formation of the 1:2 dimer, whereas dimeric complex was suppressed in acetonitrile and *i*-propanol (Deng and van Berkel 1998). Iron binding by quercetin has been studied by a number of groups and the formation of 1:1 as well as 2:1 metal–ligand complexes have been suggested (Mira et al. 2002; Fernandez et al. 2002; Ryan and Hynes 2008; El-Hajji et al. 2006). On the basis of ESI–MS investigations Fernandez et al. (2002) have reported that quercetin is able to reduce Fe(III) to Fe(II). They observed 1:1 Fe(II) complexes with oxidized quercetin, 2:1 Fe(II) complexes with quercetin and oxidized ligand, and a 2:1 Fe(III) complex with quercetin in methanol–water solution. Fe(III)–quercetin 2:1 complex formed initially, subsequently decomposes through an electron transfer step with the rate constant (Ryan and Hynes 2008) and quercetin binds Fe(II) more tightly than Fe(III) (Guo et al. 2007). The interaction of quercetin with copper ions resulted in the formation of chelates with metal:ligand ratio of 1:1 (Fernandez et al. 2002; El-Hajji et al. 2006; Brown et al. 1998) or 2:1 (Fernandez et al. 2002; Bukhari et al. 2008), although some other stoichiometry such as 1:3 (Mira et al. 2002) or 1:2 (Bukhari et al. 2008) have been also reported. The apparent second-order rate constant of 1:1 quercetin complexation for Cu(I) was higher than for Cu(II) (El-Hajji et al. 2006). In the contrast, Fernandez et al. (2002) reported that quercetin does not appear to form complexes with Cu(I). The higher reducing capacity of quercetin for copper ions than for Fe(III) has been mentioned in a few studies (Mira et al. 2002; Fernandez et al. 2002; El-Hajji et al. 2006). The participation of flavonoids and copper ions in biochemical reactions has become of interest in recent years; enzymes such as Cu-containing quercetin-2,3-dioxygenase catalyse redox processes involving flavonoids (Kaizer et al. 2006).

In this work, we have attempted spectroscopic study for the complexation and oxidation of quercetin in the presence of Cu(II) ions. The study were conducted with various Q:Cu molar ratios and prolonged time.

The scavenging activities of quercetin and the solutions with various quercetin–Cu(II) molar ratios were evaluated by the 2,2-diphenyl-1-picrylhydrazyl radical (DPPH•) scavenging method and from an electrochemical point of view. The interaction of quercetin with metal ions can change the antioxidant properties and some biological effects of the free flavonoid and formed complexes can be used in a range of diseases (Grazul and Budzisz 2009).

Materials and methods

Materials

Quercetin (2-(3,4-dihydroxphenyl)-3,5,7-trihydroxy-4H-1-benzopyran-4-one) by Sigma (Steinheim, Germany) was the highest quality available and as it has very low solubility in water, the solutions of this ligand were prepared in methanol. Copper(II) solutions were prepared in methanol from cupric chloride (POCH, Gliwice, Poland). All solutions were freshly prepared before experiments and used immediately.

Measurements

UV–VIS measurements were carried out at room temperature using Perkin Elmer model Lambda 20 spectrophotometer. Spectra were recorded in the range from 220 to 600 nm with 0.2 nm resolution in 10 mm quartz cells. Data were processed with WinLab software. Quercetin (8×10^{-5} M) and $CuCl_2$ solutions were mixed in three M:L ratios (0.5, 1 and 2). IR spectra were recorded with Nicolet 550 FTIR spectrometer after deposition of complex solution on the surface of KBr pellets in the spectral range of 2,000–400 cm^{-1}.

Mass spectra were acquired using 3200 QTRAP Mass spectrometer equipped with electrospray ionization source. ESI ionization conditions were the following: capillary temperature 450°C, curtain gas pressure at 0.3 MPa, auxiliary gas pressure at 0.3 MPa, discharge current 3 μA, ionization mode source voltage: 4.5 kV. Nitrogen was used as curtain and auxiliary gas. Samples were delivered to mass spectrometer by HPLC equipment (Shimadzu). Sample volume of 20 μl was introduced to water–methanol (70:30, v/v) eluent at a flow rate of 0.5 ml/min. Samples for MS studies were kept at 25°C.

Cyclic voltammetric experiments were performed using one-compartment electrochemical cell equipped with conventional three electrode system: glassy carbon working electrode, platinum wire auxiliary electrode and Ag/AgCl reference electrode. The cyclic voltammograms were recorded in methanol with 0.1 M $LiClO_4$ as supporting electrolyte from −100 to +1,300 mV at a scanning rate of 100 mV/s. The working electrode was carefully polished with 0.05 μm alumina paste and rinsed in deionized water at the end of each cycle.

Evaluation of scavenging activity by the DPPH method

Kinetics of the stable 2,2-diphenyl-1-picrylhydrazyl organic nitrogen radical (DPPH•, from Sigma) for the radical scavenging in the presence of quercetin and variable quantities of copper ions was carried out spectrophotometrically. DPPH• solution (2.9 ml, 2×10^{-5} M) in methanol was mixed with sample solution (0.1 ml). The reduction of DPPH• was followed by monitoring the decrease in absorbance at 539 nm until the reaction reached a plateau. The blank solution of DPPH• was screened to estimate radical decomposition during the time of measurement. The remaining DPPH• concentration in the reaction medium for further calculation of its inhibition was calculated from the calibration curve.

Results

UV–VIS studies

The direct interactions of quercetin with Cu(II) were assessed by UV–VIS spectroscopy. The spectra of free quercetin (Q) and different Cu(II)–quercetin systems (Q:Me = 1:2, 1:1 and 2:1) in methanolic solutions recorded with different times after mixing the components are presented in Fig. 2. Quercetin exhibits two major absorption bands in the UV–VIS region, at 369 nm (band I) representing the B ring absorption (cinnamoyl system) and at 258 nm (band II) due to A ring (benzoyl system). The spectra are related to the $\pi \rightarrow \pi^*$ transitions within the aromatic 3-ring system of the ligand molecule. The addition of Cu(II) to quercetin solution (8×10^{-5} M) produced large hypochromic shifts in band I, particularly significant

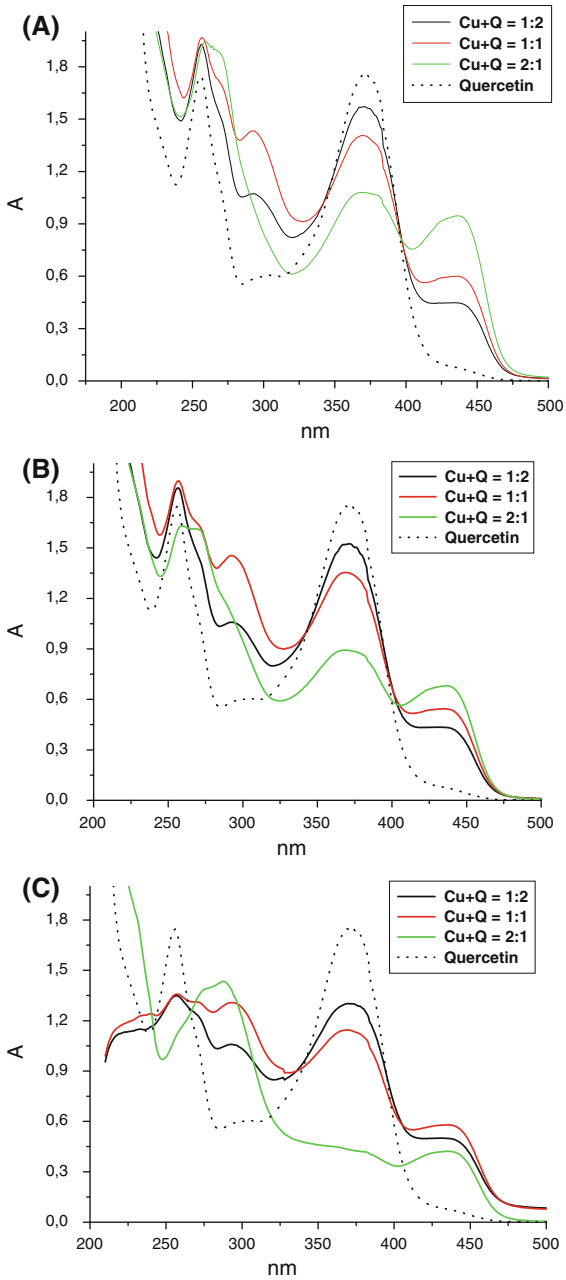

Fig. 2 Absorption spectra of quercetin and Cu–quercetin complexes. **a** Recorded immediately after mixing solutions; **b** after 30 min; **c** after 120 min [Q] = 8×10^{-5} M

when the Cu:Q ratio was 2 (Fig. 2a). Band II of the quercetin spectrum demonstrated hyperchromic shift to 293 nm in the presence of copper ions. The decrease of quercetin absorption band was accompanied by the simultaneous increase of a new characteristic band at

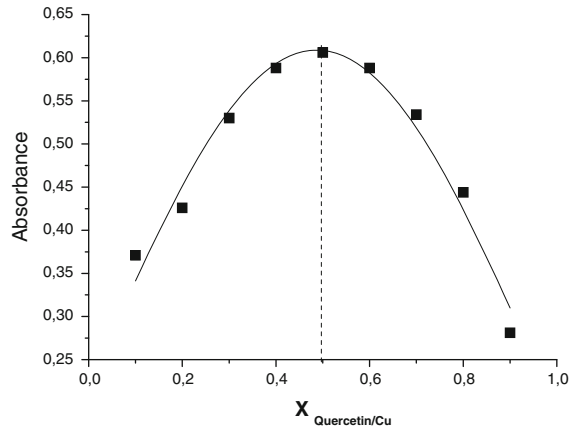

Fig. 3 Jobs curve—the plot of the absorbance at 436 nm versus mole fraction of quercetin

$\lambda = 436$ nm, which is clearly attributable to the complex formation reaction as neither the ligand nor the metal absorb at this wavelength. The absorbance plots at 436 nm against the molar fraction of quercetin have a maximum at $x_{Q/Cu} = 0.5$ (Fig. 3), which suggests that the stoichiometric ratio for the complexation of quercetin and Cu(II) is 1:1. The excess of metal ions promoted the interaction of quercetin with Cu(II) as the absorbance at 436 nm is higher and simultaneously less free ligand is present in a solution in comparison to the other Q:Cu ratios (Fig. 2a).

During prolonged time after mixing copper ions with quercetin solution, the intensity of the observed bands changed, particularly in the presence of the excess of Cu(II) (Fig. 2b, c). The reaction of quercetin with copper ions was accompanied by a decrease in the absorbance at 258 nm with time and this was followed by the appearance of the new peaks at ca. 290–300 nm indicating that the cinnamoyl system has been changed. The peak at 298 nm was also observed for quercetin (8×10^{-5} M) in the presence of $KMnO_4$ solution (4×10^{-5} M) which could be attributed to the oxidation process of quercetin (Fig. 4). It is noteworthy to add that the same spectral data were given for the reaction of quercetin with polyphenolaxidase (Jimenez and Garcia-Carmona 1999), making it likely that the compounds are identical. One of the formed product could be protocatechuic acid.

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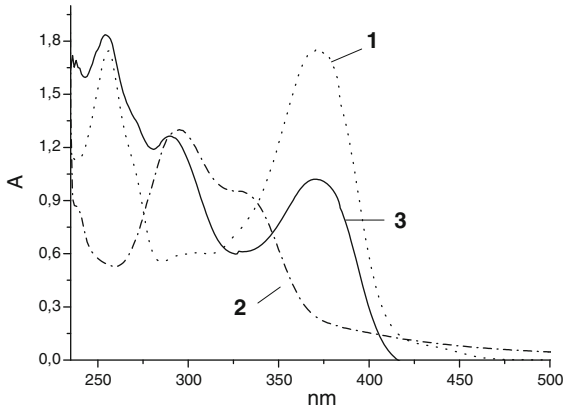

Fig. 4 Absorption spectra of: (*1*) quercetin; (*2*) quercetin oxidized by $KMnO_4$ and (*3*) Cu + Q = 2:1 solution after addition of EDTA [Q] = 8×10^{-5} M

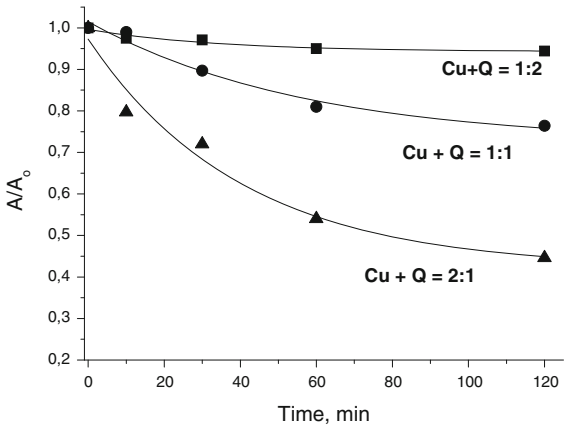

Fig. 5 The effect of time on the relative decrease in absorbance at 436 nm for different Cu(II)–quercetin solutions

the solution in comparison with other Q:Cu ratios (Fig. 2a). This peak gradually has decayed over time and this effect depends on metal:ligand ratio (Fig. 5). The results fitted to a simple kinetic law assuming the first order of complex decomposition gave the rate constants k equals to 2.3×10^{-3} and 5.3×10^{-3} min^{-1} for Cu + Q molar ratio of 1:1 and 2:1, respectively. The decrease of peaks monitored at 436 nm (complex formation) is paralleled by the increase in absorbance at 293 nm typical of the quercetin oxidation products and by the decrease at 369 nm (absorption maximum of quercetin) (Figs. 6, 7).

The UV–VIS spectra of quercetin–copper complex after addition of the excess EDTA are presented in Fig. 4. As could be seen the presence of EDTA

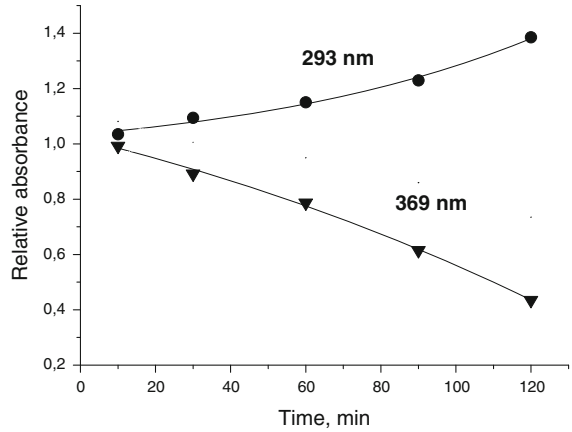

Fig. 6 Absorbance versus time curves for Cu + Q = 2:1 solution detected at 369 nm (quercetin) and 293 nm (oxidation products)

destroyed the complex but did not regenerate the whole original spectrum of free quercetin, which means that it has become oxidized.

IR measurements

As all attempts to obtain the copper–quercetin complexes in the solid state were unsuccessful, even repeated the procedure proposed by Bukhari et al. (2008), the IR spectra were recorded in the 2,000–400 cm^{-1} region after deposition of ligand and complex solution on the surface of KBr pellets.

The ν(C=O) stretching mode from quercetin occurs at 1,605 cm^{-1}, while due to the formation of complex with Cu(II) this band appears at 1,585 cm^{-1}, suggesting that metal coordination occurs through the carbonyl oxygen in C ring and the close hydroxyl group (3-OH or 5-OH). The C–O–H deformation mode observed at 1,264 cm^{-1} in the ligand shifts to 1,275 cm^{-1} in the complex, indicating an increase in bond order normally observed when metal coordination involves the OH group (Bravo and Anacona 2001). Moreover, the presence of the new stretching vibration C_3–O_3 at 1,468 and 1,447 cm^{-1} suggests coordination through 3-OH group, while for free quercetin such bands doesn't exhibit.

ESI–MS studies

Further studies on the complexation of copper by quercetin were carried out in a mass spectrometer

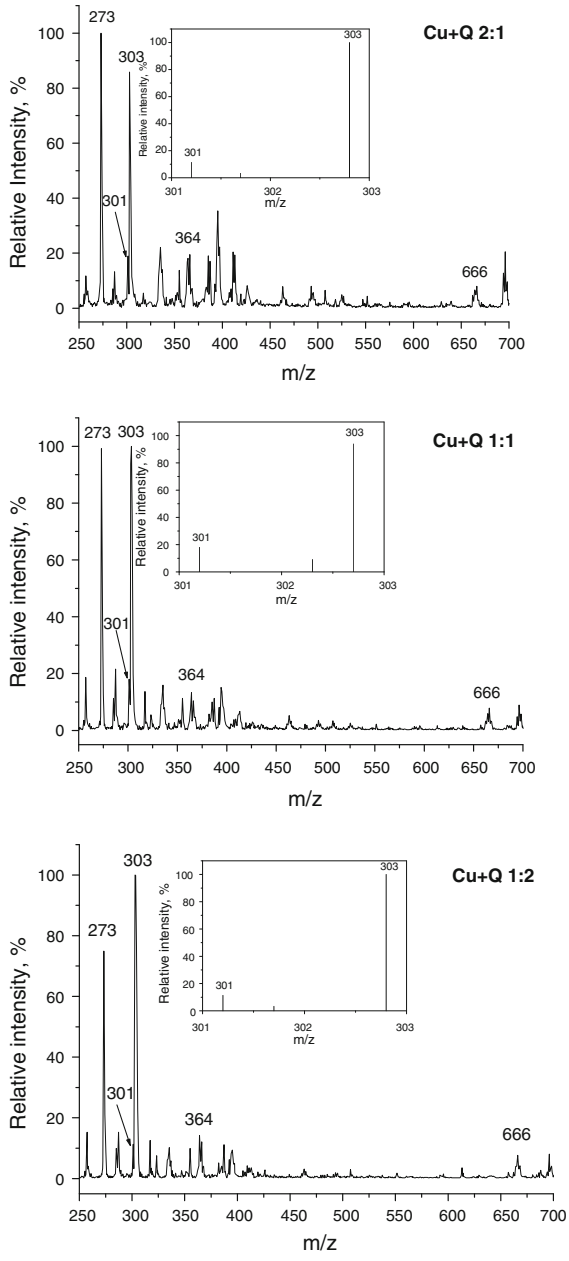

Fig. 7 Electrospray ionization mass spectra in the positive mode of 3×10^{-4} M quercetin solution mixed with different excess of Cu(II)

equipped with electrospray source. Figure 7 shows the mass spectra for mixtures of 3×10^{-4} M quercetin with different excess of Cu(II). The protonated ligand $[Q + H]^+$ was observed at $m/z = 303$ and the species corresponding to the formation of 1:1 complex at $m/z = 364$ and 273. The ion at $m/z = 273$ resulted from the loss of CuCO from the ion $[(Q - H) + Cu(II)]^+$ at $m/z = 364$ is particularly prominent in the presence of the copper excess. The peak at m/z 666 $[(Q - H) + Cu(II) + Q]^+$, which corresponds to the quercetin–copper complex with stoichiometry of 2:1 was also recorded, however, with much less sensitivity (Fig. 8). In all cases, the relative intensities of the isotopic peaks of the copper-containing species agree with the experimental error with the naturally occurring abundances. The base peaks arise from Retro Diels–Alder fragmentation of quercetin (March and Brodbelt 2008; Fabre et al. 2001) were also observed.

The peaks corresponding to the probable formation of Cu(I) complexes with quercetin could not be monitored either in positive or negative ionization mode as they have neutral charge. The reduction of Cu(II) in the presence of quercetin could be follow by recording the spectra of the ligand oxidized products. In the positive ESI–MS these products were detected through the peak at m/z 301 $[(Q - H_2) + H]^+$, which content was gradually increasing during prolonged time (Fig. 8). In addition, the formation of these species could be monitored in the negative mode (data not shown) at m/z 299, m/z 331 and m/z 363, corresponding to the ions $[(Q - H_2) - H]^-$, $[(Q - H_2) + CH_3OH - H]^-$, $[(Q - H_2) + 2CH_3OH - H]^-$, respectively. The main oxidation products of quercetin shown to be the solvent adducts on the *p*-quinomethide

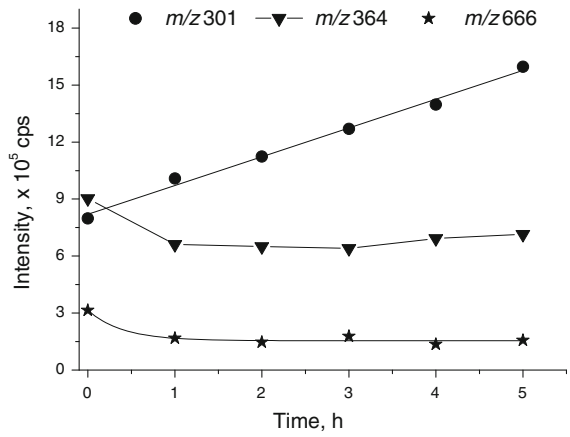

Fig. 8 Changes in the intensity of ESI–MS(+) spectra at m/z 301 $[(Q - H_2) + H]^+$, $m/z = 364$ $[(Q - H) + Cu(II)]^+$ and $m/z = 666$ $[(Q - H) + Cu(II) + Q]^+$ during the time after mixing quercetin solution (8×10^{-4} M) with the excess of Cu(II)

formed upon two-electron oxidation of ligand (El-Hajji et al. 2006). However, the redox reaction is strongly dependent on the conditions in the matrix and those chosen for measurement (Jungbluth et al. 2000).

When Cu(II) is added to the solution of quercetin in the presence of EDTA, no peaks corresponding to the complex formation at $m/z = 364$ and 273 can be detected. Only the protonated ligand $[Q + H]^+$ was observed at $m/z = 303$ as EDTA-bound copper is no available to complex quercetin. Similar results were obtained in UV–VIS study with no absorption band above 400 nm (Fig. 2). The addition of EDTA to the quercetin–Cu(II) complex caused its decomposition but did not regenerate the whole original spectrum of free ligand as its oxidized products were recorded at m/z 301.

Scavenging effects of quercetin–copper complexes

The DPPH test is non-enzymatic method currently used to provide basic information on the reactivity of compounds to scavenge free radicals (Moon and Shibamoto 2009). The reaction of quercetin with DPPH was conducted in methanol, which is more accurate for the prediction of its reactivity in oils, compared to non-alcoholic solvents, such as ethyl acetate (Tsimogiannis and Orepopoulou 2004). The scavenging activities of quercetin and its complex with Cu(II) were evaluated by following the decrease of DPPH• absorbance at 539 nm over time (Fig. 9). A fast decrease in the absorbance of DPPH• was

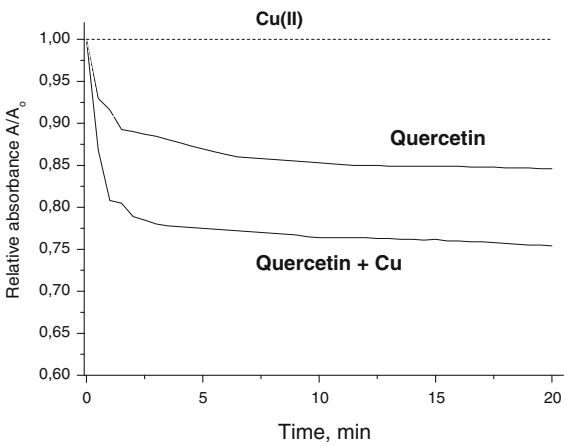

Fig. 9 Kinetics of the change in relative absorbance of DPPH• in the presence of quercetin and quercetin–copper complex

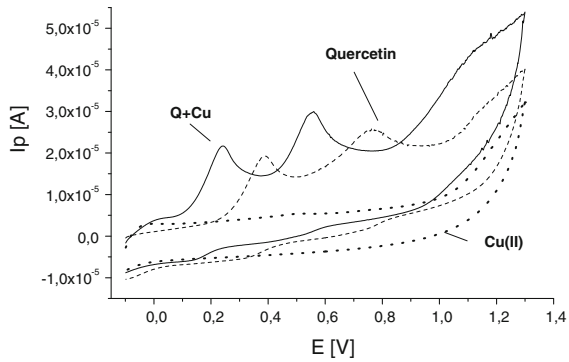

Fig. 10 Cyclic voltammograms of Cu(II), quercetin and quercetin:Cu complex. 0.1 M $LiClO_4$ was used as supporting electrolyte, scan rate 0.1 V/s

observed during the first few minutes of the reaction. This fast step essentially refers to the electron-transfer process from B ring (3′-OH and 4′-OH) to DPPH• and latter slower kinetic reflects the remaining activity in the oxidation–degradation products (Foti et al. 2004). For quercetin in the presence of copper ions DPPH absorbance decays much faster than for free ligand. It suggests that the radical scavenging activity of the Cu–quercetin complex is greater than free quercetin.

The results obtained by the DPPH radical-scavenging method are in good correlation with the oxidation potentials. The cyclic voltammograms of free quercetin and its solutions in the presence of Cu(II) show well-defined peaks and practically no reverse reduction peaks indicating electrochemical process involving two electrons (Fig. 10). The oxidation peak potentials for quercetin–Cu(II) complexes were observed at lower values in relation to those of the free flavonoid. The electrochemical data suggest that the coordination with metal ion makes the oxidation process easier due to the destabilization of the flavonoid structure.

Discussion

Despite a constant increase of knowledge on both positive and negative activities of quercetin, its reactions with copper ions have not been studied extensively. Some papers deal only with metal chelation (Bukhari et al. 2008; Kaizer et al. 2006). No exact experiments were conducted during prolonged time after mixing the components, except the work by

El-Hajji et al. (2006), where interactions of Cu(II) with quercetin concerning complexation and autooxidation in acidic to neutral aqueous solutions have been examined. Our study showed that the reaction of quercetin with Cu(II) resulted in the fast formation of 1:1 metal–ligand complex ($\lambda_{max} = 436$ nm) through the carbonyl oxygen and the close hydroxyl group in A or C ring followed by slow quercetin oxidization to the benzoquinone type products. However, the precise chelating site (3-OH or 5-OH) in quercetin molecule remains still questionable ((Torregiani et al. 2005). Thompson et al. (1976) found that the stability of the complex is higher in the case of chelation via 5-OH; this probably being associated with the stability of the six-membered ring over that of the five-membered ring complexes. From the other hand, under acidic conditions the 3-OH group is thought to be more likely involved in complexation (Tsimogiannis and Orepopoulou 2004). It is quite feasible that Cu(II) ions are six-coordinate with one molecule of ligand as well as chloride and water molecules at the vertices of an octahedron (Bravo and Anacona 2001).

Autoxidation of quercetin in aqueous solutions under physiological pH conditions (pH 7.4 and 37°C) without the presence of metal ions have been reported (El-Hajji et al. 2006). However, in the case of quercetin dissolved in methanol at room temperature, there was no spectral changes recorded over 4 h, which is in agreement with previous reports (Zhou and Sadik 2008; Makris and Rossiter 2000). It suggested that the presence of Cu(II) ions promote quercetin oxidation, probably at the hydroxy groups in B ring. However, the 3-OH moiety, which functions as a chelation site, could also be oxidized. The mechanism of quercetin oxidation is thought to vary with the nature of the oxidizing agent and solvent media, with probable formation of quinone radicals and/or quinone intermediates (El-Hajji et al. 2006; Jimenez and Garcia-Carmona 1999).

The presence of EDTA inhibits complex formation and quercetin oxidation which was confirmed by UV–VIS studies and MS spectra. The complexes of luteolin and rutin with Cu(II) behave different after addition of EDTA as the original spectrum of the ligands were recovered (Brown et al. 1998). These compounds have the differences in the C-ring comparing to quercetin molecule; luteolin lacks the free 3-OH group whereas rutin has 3-glucorhamnoside moiety.

The capacity of flavonoids to act as antioxidant depends on their molecular structure. Among flavones, quercetin, myricetin and luteolin exhibit high DPPH• scavenging activity due to the presence of a catechol group in B-ring (Silva et al. 2002). In addition, the presence of both 2,3-double bond and 3-OH group is of fundamental importance. The interaction of flavonoids with metal ions may change the antioxidant properties and their some biological effects (Mira et al. 2002).

The radical scavenging activity of the Cu–quercetin complex evaluated by DPPH method and cyclic voltammetry was found to be greater than free ligand. There is a relationship between the antioxidant activity and the cyclic voltammetric oxidation peak potential for flavonoids; the lower the potential, the higher the antioxidant activity (Cornard and Merlin 2002).

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