Application of a Potentiometric System with Data-Analysis Computer Programs to the Quantification of Metal-Chelating Activity of Two Natural Antioxidants: Caffeic Acid and Ferulic Acid

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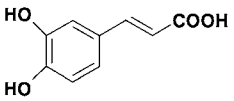
The quantification of metal-chelating activity of caffeic and ferulic acids (**1** and **2**, resp.) was successfully performed by using a potentiometric system with data-analysis computer programs. The method was applied to two phenolic models, which have been systematically reported as antioxidants. Although a chain-breaking mechanism was proposed, several studies pointed out the possibility of complexation of transition metals that can participate in single-electron reactions and mediate the formation of oxygen-derived free radicals. In this work, the complexation properties towards Cu^{II} were investigated by potentiometry with a glass electrode. Acidity constants of the ligands (phenolic acids) and the formation constants of the ligand–metal complexes were evaluated by potentiometry. The modeling of the titration curves and the data treatment were performed with the computer programs Superquad and Best. A detailed quantitative examination of the complexation species formed in the Cu^{II}/caffeic acid (**1**) and Cu^{II}/ferulic acid (**2**) systems is presented together with the formation constants ($\log \beta$). Results have shown that the complexation properties of the two phenolic acids towards the transition metal are quite different: the activity of caffeic acid (**1**) was found higher than that of ferulic acid (**2**). The data are important to get insight into the mechanism of action of antioxidants, and, in this case, could partially explain the efficacy of caffeic acid in the protection of LDL oxidative damage. In addition, the analytical method developed could be applied to quantify the chelating activity of important biological compounds, such as allopurinol, uric acid, cinnamic acids, flavonoids, and anthocianins, and, in that way, could be a valuable tool to understand the mechanisms underlying their protective effects.

Introduction. – Phenolic acids are a well-known family of natural compounds that are present in human diet in representative amounts. This type of compounds has been nowadays an important tool in the research on new effective antioxidants because of their role as inhibitors of deleterious oxidative processes [1][2]. It is currently assumed that the oxidative modification of low-density lipoproteins (LDL) by inducers such as Cu^{II} ions and oxygen radical species could be related to cardiovascular diseases [3][4]. The oxidation of LDL is thought to be an important factor in the pathogenesis of atherosclerosis. There is great interest in the possibility that diets rich in antioxidants may retard the development of atherosclerotic complications such as coronary heart diseases [3][5].

Apart from the purely academic study of their natural occurrence, distribution, biosynthesis, metabolism, and function in plants, phenolic acids are becoming increasingly important in applied science. Recently, much attention has focused on the role and mechanism of several phenolic compounds, such as caffeic and ferulic acids (**1** and **2**, resp.), because of their antioxidant properties. These cinnamic acid

derivatives have been pointed out as preventive chain-breaking antioxidants in the oxidation of LDL, probably through their radical-scavenging activity, which is related to their hydrogen- or electron-donating ability, and to the stability of the resulting phenoxyl radicals [6–9]. They usually react with free radicals formed during initiation or propagation steps of the oxidation process, *e.g.*, alkyl ($R^{\bullet}$), peroxyl ($ROO^{\bullet}$), and alkoxyl ($RO^{\bullet}$) radicals, thus acting as chain-breakers [10–15]. However, other mechanisms of action may be involved, for instance, through chelation of transition metals such as copper or iron, which are well-known catalysts of oxidative stress.



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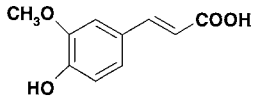

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Although metal–antioxidant interactions might be of therapeutic interest, few studies have been performed to investigate this property even in nonbiological systems. To achieve the objective, the first step is the determination of the acidity constants of the compound, which can be realized by several techniques. Nevertheless, the potentiometric titration with a glass electrode for measuring the H^+-ion concentration and the data treatment by computer programs is one of the most-often-selected methods. This technique can be used as well to evaluate the complexation constants of the ligand with several metals such as Cu^{II}.

The aim of this work was to gain insight into the complexation properties of caffeic and ferulic acids (**1** and **2**, resp.) towards Cu^{II}. The quantification of metal-chelating activity was performed by potentiometry with a glass electrode, and the experimental data were analyzed by using computer programs. A detailed quantitative examination of the complexation species formed in the Cu^{II}/caffeic acid (**1**) and Cu^{II}/ferulic acid (**2**) systems is presented.

Results and Discussion. – Phenolic acids are a well-known family of natural compounds, present in fruit and plant components of our diet. Although the antioxidant *vs.* antiradical activity of caffeic and ferulic acids (**1** and **2**, resp.) has been studied in different model systems, few studies were performed towards gaining insight into their chelating properties towards transition metals. Therefore, knowledge of the stability of the phenolic acid–metal complexes is an important tool in the evaluation of their antioxidant mechanisms.

Acidity Constants. The dissociation constants (pK_a) evaluated for caffeic and ferulic acids (**1** and **2**, resp.) are presented in *Table 1*. Results are means ± standard deviation of at least six independent experiments.

Table 1. *Acidity Constants of Caffeic and Ferulic Acids in Aqueous Solutions*

Phenolic acids	pK_{a1}	pK_{a2}	pK_{a3}
Caffeic acid (**1**)	4.38 ± 0.02	8.58 ± 0.05	11.50 ± 0.10
Ferulic acid (**2**)	4.50 ± 0.03	8.92 ± 0.05	

As expected, the carboxy groups exhibit similar acidic strengths, which are related to the chemical structure of the ligands, *i.e.*, type of the spacer between this function and the aromatic nucleus, and the substituents in the *meta*-position.

It can be observed that different acidity constants were found for the OH group at C(4): $pK_a = 8.92$ for ferulic acid (**2**) and $pK_a = 8.58$ for caffeic acid (**1**). This can be accounted for when we consider that the MeO group at C(3) is a better electron donor than its OH counterpart, lowering, therefore, the reactivity of OH dissociation. The effect of the side chain on the acid–base properties of the catechol group can be noted when comparing the acidity values of caffeic acid (**1**; $pK_{a2} = 8.58$ and $pK_{a3} = 11.50$) with the homologous compound having a fully saturated side chain, dihydrocaffeic acid ($pK_{a2} = 9.41$ and $pK_{a3} = 11.70$) [16]. Comparing the values of pK_{a2}, obtained in the same experimental conditions, it can be suggested that some electron-withdrawing effect of carboxy moiety can act across the C=C bound, as the value of pK_{a2} for caffeic acid (**1**) is lower than for dihydrocaffeic acid. Allowing for the variation in ionic strength and background medium, the differences between the values of acidity constants found for caffeic acid (**1**) and those given in the literature can be considered acceptable [17].

Formation Constants. The formation constants ($\log \beta$) for the binary systems Cu^{II}/caffeic acid (**1**) and Cu^{II}/ferulic acid (**2**) as well as the pH intervals in which the data were collected are presented in *Tables 2* and *3*.

Table 2. *Equilibrium Constants* ($\log \beta$) *Calculated for* Cu^{II}*/Caffeic acid* (**1**) *in Aqueous Solution*[a])

p	*q*	*r*	Species	$\log \beta$	ΔpH
1	1	−1	$[CuLH_2]^{+1}$	1.72+0.1	2.4–6.1
1	1	−2	[CuLH]	6.76 ± 0.1	3.5–7.8
1	1	−3	$[CuL]^{-1}$	12.0 ± 0.1	4.3–8.0
1	2	−3	$[CuL_2H_3]^{-1}$	8.12 ± 0.3	4.0–6.5
1	2	−5	$[CuL_2H]^{-3}$	18.6 ± 0.2	4.4–7.8
1	2	−6	$[CuL_2]^{-4}$	27.5 ± 0.2	6.0–8.0
2	2	−5	$[Cu_2L_2H]^{-1}$	15.1 ± 0.1	4.5–7.7
2	2	−6	$[Cu_2L_2]^{-2}$	20.9 ± 0.5	4.5–8.0

[a]) All constants were calculated with the program Superquad [24] from data obtained potentiometrically at 25° and $I = 0.1$M $NaNO_3$. The symbols *p*, *q*, and *r* are used in the programs to indicate the stoichiometric coefficients associated with the possible equilibria in solution: *p*, coefficient for ligand; *q*, for Cu^{II}; and *r*, for H-atoms.

Table 3. *Equilibrium Constants* ($\log \beta$) *Calculated for* Cu^{II}*/Ferulic acid* (**2**) *in Aqueous Solution*[a])

p	*q*	*r*	Species	$\log \beta$	ΔpH
1	1	−1	$[CuLH]^{+1}$	1.74 ± 0.1	2.4–7.0
1	1	−2	[CuL]	8.30 ± 0.2	5.5–7.8
1	2	−3	$[CuL_2H_2]$	3.03 ± 0.2	3.5–7.0
1	2	−5	$[CuL_2H]^{-1}$	9.46 ± 0.5	5.5–7.8
1	2	−6	$[CuL_2]^{-2}$	16.13 ± 0.2	6.0–8.0

[a]) All constants were calculated with the programs Superquad [24] from data obtained potentiometrically at 25° and $I = 0.1$M $NaNO_3$. The symbols *p*, *q*, and *r* are used in the programs to indicate the stoichiometric coefficients associated with the possible equilibria in solution: *p*, coefficient for ligand; *q*, for Cu^{II}; and *r*, for H-atoms.

Different concentration ratios of metal/ligand were used in the complexation studies of the ligands. From the results obtained, it must be pointed out that, for all ligands, the formation constants determined were independent of the concentration ratio of metal/ligand used.

For the binary system Cu^{II}/caffeic acid (**1**), the model that best fits the data assumes the occurrence of equilibria in solution that correspond to formation of the following species: $[CuLH_2]^{+1}$, $[CuHL]$, $[CuL]^{-1}$, $[CuL_2H_3]^{-1}$, $[CuL_2H]^{-3}$, $[CuL_2]^{-4}$, $[Cu_2L_2H]^{-1}$, $[Cu_2L_2]^{-2}$, and $[Cu_3L_2]$ (L^{3-} represents fully deprotonated caffeic acid). For the system Cu^{II}/ferulic acid (**2**), the model that best fits the data assumes the formation of the following species: $[CuLH]^{+1}$, $[CuL]$, $[CuL_2H_2]$, $[CuL_2H]^{-1}$, and $[CuL_2]^{-2}$ (L^{2-} represents fully deprotonated ferulic acid).

The formation constants obtained were $K^{Cu}_{CuL} = 12.46$ and $K^{Cu}_{CuL_2} = 21.42$ for caffeic acid (**1**), and $K^{Cu}_{CuL} = 5.12$ and $K^{Cu}_{CuL_2} = 10.71$ for ferulic acid (**2**). Formation constants calculated for **1** are in a good agreement with those reported in literature ($K^{Cu}_{CuL} = 12.85$ and $K^{Cu}_{CuL_2} = 22.74$ determined at 30°, $I = 0.1$M $NaClO_4$) [18]. The differences between the values can be related to different concentration ratios metal/ligand and different ionic-strength adjusters used. For the other ligand, no formation constants were found in the available literature.

Comparing the results obtained, it can be stressed that the formation constants for caffeic acid (**1**) are higher than those obtained for ferulic acid (**2**). The difference in the complexation properties can be attributed to the absence of the catechol moiety in **2**, which has a MeO group C(3). Caffeic acid (**1**) acts probably as bidentate ligand through the catechol group, thus forming five-membered chelate rings. In $[CuL_2]$ complexes, both phenolic anions (catechol group) are bound equatorially to the metal ion as bidentate ligands, yielding a square-planar Cu^{II} moiety, to which two H_2O molecules can weakly coordinate in the axial positions. These results allow postulating that a catechol function is an important chemical feature in the complexation properties exhibited by phenolic acids. Comparing the formation constants of caffeic acid (**1**) with some homologous compounds, such as dihydrocaffeic acid and 3,4-dihydroxyphenylacetic acid, it can be concluded that either the presence of the C=C bound in the side chain or the size of the alkyl chain does not affect the complexation properties. The formation constants K^{Cu}_{CuL} and $K^{Cu}_{CuL_2}$ were 12.46 and 21.62 for dihydrocaffeic acid, and 12.25 and 21.20 for 3,4-dihydroxyphenylacetic acid, respectively, values that are very similar with those obtained with caffeic acid (**1**).

The distribution diagrams of the species of Cu^{II}/caffeic acid (**1**) and Cu^{II}/ferulic acid (**2**) systems as a function of pH are presented in *Fig. 1*.

The knowledge of metal-complexation properties of biological compounds could be also an important tool to gain insight into their antioxidant or pro-oxidant mechanisms [18–22]. The complexation of metals may have indirect effects, such as facilitating cell penetration or sparing the compound from inactivating metabolic reactions, and enhancing oxidative pathways. On the other hand, this type of ligand can also change the redox state of the metal, and consequently alter its bioavailability.

The results obtained suggest that caffeic acid (**1**) might inhibit free-radical formation and the propagation of free-radical reactions through the chelation of transition-metal ions. The study also demonstrates that the reactivity of the cinnamic

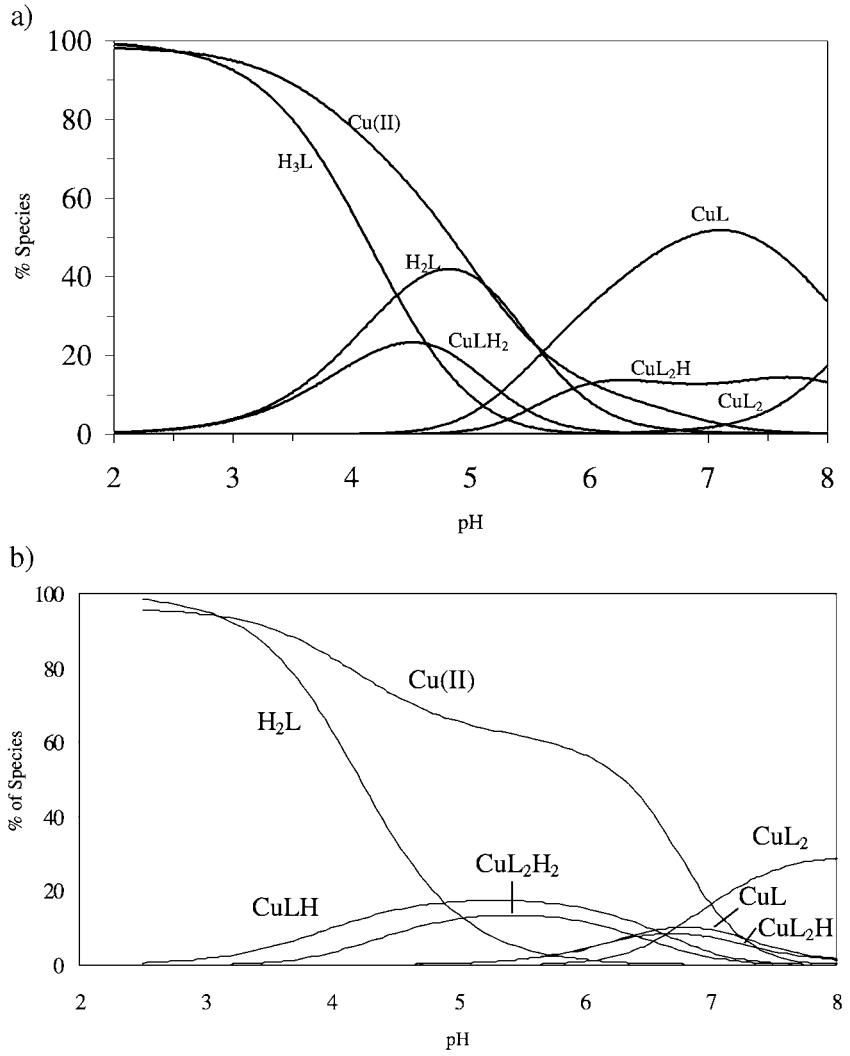

Fig. 1. *Species-distribution diagrams of* a) *Cu^{II}/caffeic acid* (**1**) *and* b) *Cu^{II}/ferulic acid* (**2**) *systems* (1.01 mM caffeic acid and ferulic acid, resp., 1.00 mM Cu^{II}, 1.00 mM HNO_3, and 0.100M KNO_3, at 25°)

acids in protecting LDL against Cu^{2+}-ion-induced oxidation is dependent on their structural properties.

It is, therefore, urgent to develop analytical methods that could lead, in a short time of analysis, to the identification and quantification of the stability of the ligand–metal species.

As the information in this area is rather sparse and not fully understood, the research can also contribute to establish a database suitable for the design of antioxidants with health benefits, *i.e.*, preventive agents in certain disease states, such as cancer or atherosclerosis.

It may be possible that co-administration of two antioxidants acting by different mechanisms may have a synergistic effect, resulting in a higher antioxidant activity, without raising the dose required to obtain the same effect with a single compound. Likewise, an agent that possesses more than one mechanism of action may have a net therapeutic effect greater than the sum of the individual component mechanisms.

Experimental Part

General. All chemicals employed were of anal.-grade purity (purchased from *Aldrich* (copper(II) nitrate, and caffeic and ferulic acids) and *Riedel* (HNO_3, NaOH, and KNO_3)). The H_2O used was double deonized water (conductivity less than 0.1 μS cm^{-1}).

Potentiometric Measurements. Potentiometric measurements were carried out with a *Crison 2002* pH-meter and *2031* burette controlled by a personal computer, which was also used for data manipulation. The electrode assembly was made up of an *Orion 900029/4* AgCl/Ag reference electrode and a *Russell SWL* glass electrode. System calibration was performed according to the method of *Gran* [23] in terms of H^+-ion concentration by strong acid/strong base titrations (HNO_3 (1.00 mM)/NaOH (*ca.* 0.02M)) with solns. having adjusted ionic strengths of 0.1M with KNO_3. Titrations were always carried out under N_2 at 25° in a double-walled glass cell. The potentiometric system is shown in *Fig. 2*.

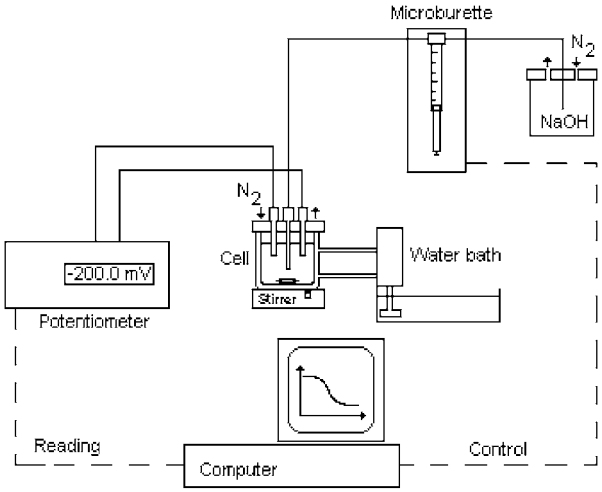

Fig. 2. *Potentiometric system used in the study*

Potentiometric Determination of Acidity and Stability Constants. The acidity constants of the compounds were obtained by titrating 20.00 ml of acidified solns. (1.00 mM HNO_3) of the phenolic acids (0.8 – 1.00 mM) with NaOH (*ca.* 0.02M). Stability constants of Cu^{II} complexes with the ligands were determined by titrating 20.00 ml of aq. solns. of phenolic acids (0.8 – 1.00 mM), HNO_3 (1.00 mM) and copper(II) nitrate (1.00 mM) with NaOH (*ca.* 0.02M). For all solns. the ionic strength was adjusted to 0.1M with KNO_3. System calibration was performed before each determination. The evaluation of acidity and stability constants of the ligands and metal–ligand complexes were performed with data obtained from at least six independent titrations, each with more than 30 points. The experimental titration data were analyzed with the computer programs Superquad [24] and Best [25]. The errors reported were calculated by the method of *Albert* and *Serjeant* [26], as the maximum difference between the logarithm of the average of the antilogarithms of the calculated p*K*a values and their individual values.

Different concentration ratios of metal/ligand were used in the complexation studies (2:1, 1:1, and 1:2).

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