Electrochemical and Theoretical Complexation Studies for Zn and Cu with Individual Polyphenols

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ABSTRACT

Zn and Cu interactions with three selected flavonoids (catechin, quercetin and rutin) have been electrochemically monitored. It has been shown that catechin takes 1 atom of metal per molecule; quercetin takes 2 atoms, and rutin is able to take up to 3 atoms. Not all ligands bind metals equally strong, and weakly bonded metals can be distinguished. Zn shows a sluggish kinetics and, at the same time, the highest conditional formation constants. The method could be applied to a real sample. Theoretical models are proposed for the most favourable compounds.

Keywords: Zn, Cu, polyphenols, flavonoids, complexation, DPASV

INTRODUCTION

Red wines are very much appreciated because of their organoleptic properties such as colour, taste, bouquet … Most of these features depend on many different factors, some of them still unknown, due to the great complexity of wine matrix. It is very well known that the organic fraction of wine is mainly responsible for those organoleptic properties and for the beneficial effect of wine on human health. Within this fraction, polyphenolic compounds play the most important role, since they are involved in the differences found between white and red wines, specially in colour and taste. This group of compounds includes anthocyanins, tannins, flavonoids and phenolic acids as the most representatives.

However, many different authors found interesting the study of the inorganic fraction, less abundant [1] but made up of different elements which play an important role, both on the vine growth and on the nutritional and organoleptic characteristics of wine [2].Total metal content in wine has typically been evaluated through different analytical techniques [2-4] for the role they play, i.e. as micronutrients and flavour enhancers, their toxic relevance, i.e. Pb [2] and others for their cationic contribution [5].

Regarding polyphenol studies, quantification of anthocyanins was done along the first seven days of fermentation of a Tempranillo wine [6]. The results showed the presence of increasing concentration values for that period of time. A more comprehensive study has recently been published [7] in which the fermentation process has been monitored up to a 1-year period of vinification,

and notable metal-polyphenol relationships have been found.   Vivar [8-9] performed a wine sample fractionation and by means of HPLC identified Malvidin-3-glucoside as the most abundant anthocyanine in red wines. Håkansson *et al*. [10] studied the organic reactions involved in the synthesis of those compounds, and Alcalde *et al* [11] isolated new polyphenolic pigments in wine.

Anthocyanins and tannins have shown a marked complexing activity on metals such as Fe, Cu, Al and Mg [12] resulting in small changes in wine colour as proposed by Useglio [13]. Markis [14] reports on the browning rates of quercetin and rutin in the presence of Fe(II) and Cu(II).

Other authors also confirm the relevance of metal-polyphenol complexation processes since they might affect and distort natural existing equilibria in wines, producing a colour modification [4, 15-17]. Dangles *et al*. [16] studied the behaviour of wine at different pHs, showing the existence of a competitive process between the metallic ions and protons to reach the 3'-4'-carbonyl groups on the catechol ring, the proton being displaced by the metallic atoms generating a coloured complex.

Other complexing phenomena involving not only metals and polyphenols, but anthocyanins and polyphenols have been described [12] originating copigmentation processes; this results in final colour changes such as hyperchromic or batochromic effects. Compounds involved in this process could be cynamic acids, flavonols and glycosilated flavones.

In the last few years, a relative large number of publications have appeared in which several complexing mechanisms and spatial structures are proposed for metal-flavonoid compounds. [18-21]. Le Nest *et al.* [20, 21] reported the structure of the possible metallic complexes formed between quercetin and catechin with Zn^{2+}, as well as those of quercetin and rutin with Cu^{2+}, claiming that the catechol moiety is the most probable binding point between this metal and the flavonoid molecule. On the contrary, other reports [22, 23] suggest that the binding site Cu^{2+}-flavonoid corresponds to the 4-oxo-5-OH group of the organic structure. Thermogravimetric and atomic absorption spectrophotometric studies tend to indicate that the 3-OH-4-oxo and 3'-OH-4'-OH moieties are the most likely binding sites of the flavonoids for metals [24]. ESI-MS has also been applied to the elucidation of the chemical structure of these kind of complexes, and the authors also conclude that both hydroxyl moieties are involved even in a cross-linked morphology [25].

Heavy metals have been previously quantified and conditional complex formation constants have also been evaluated in acidified wines by Differential Pulse Anodic Stripping Voltammetry (DPASV) and Anodic Stripping Voltammetry (ASV) [26, 27]. Vasconcelos *et al* have electrochemically monitored the complexation of Pb [28] and Cu [29] in selected red wines at pH 3.5. Cu determination and its speciation in white wines has also been done by electrochemical means [30,31].

Thus, in the last decade or so, attention has been placed on metal complexation by naturally occurring ligands in real samples of wine, but no direct attempt has been done to conclude on the nature of the ligand(s) responsible for the complexation process. Present work is part of a larger project in which an up to 3-year period of vinification of Tempranillo grape variety will be studied and metal and polyphenol contents evaluated. So far, along the first year, a close association of metals as Zn and Cu was found to

occur with cyanydin-3-glucoside when the sample was subjected to an open column fractionation [7]. Catechin resembles very closely the structure of cyanidin-3-glucoside and it is one of the most abundant phenols in wine [32-34]. Accordingly, it was chosen as a model ligand for electrochemical studies directed to find evidence of complexation taking place in wine at the natural pH of 4.0. On the other hand, it was mentioned above that glycosilated flavans are involved in the copigmentation processes taking place in wines. Thus, rutin has been also selected as a model ligand in order to check its behaviour with respect to either Zn or Cu as in the case of catechin. The finding of experimental evidence of a complexing activity of ligands of this type with the assayed metals, would be a factor to take in mind at the hour of changing agricultural practices, so that a possible exogenous contribution of certain selected cations might result in a favourable colour modification. Finally, a third model ligand was considered in which the 3-rutinoside is not present, namely the quercetin, which structure is exactly the same as that of rutin but for the 3-rutinoside. Both electrochemical and theoretical modelling studies will be used in parallel to check and discuss the experimental findings.

It has been shown that all these organic molecules, known for their antioxidant properties and their abundance in red wines, are able to form complexes with different cations in a wide variety of aqueous-organic mixtures. Our aim is to individually study the capacity of complexation of each of the above mentioned ligands for Zn and Cu in simple aqueous solutions (containing 8% ethanol) in which the only fixed parameter is the pH, that is adjusted to 4.0 with an acetate buffer, which does not show any complexation affinity for any of the metals. This is a basic study oriented to a better understanding of the nature of these individual complexes, in the absence of any other competing ligand. Such would be the case of a red wine, in which a myriad of natural occurring ligands would exert a complexing role on these cations. That is the reason why we do not intend to simulate the natural wine matrix, in which case tartaric acid , for example, should be added given its well documented interaction with Cu.

EXPERIMENTAL

Standard Zn and Cu solutions were prepared by adequate dilution in a pH 4.0 acetate aqueous-ethanol (8%) buffer from the Certipure Merck 1,000 ppm stock solutions.

(+)-catechin, 3,3',4',5,7,-pentahydroxiflavane, was purchased from Fluka. Stock concentrated solutions of the compound required sonication for 10 min. Rutin, 3',4',5,7-tetrahydroxiflavone-3-rutinoside, was isolated and purified in the Department of Pharmacy and Pharmaceutical Technology of the University of Navarra. Stock solutions were prepared in methanol and solubility was achieved by means of a large period of 30 min sonication. Quercetin, 3,3',4',5,7-pentahydroxiflavone dihydrate, was purchased from Riedel De Haën, dissolved in ethanol, aided by 30 min sonication.

Two electrochemical equipment have been employed. One consisted of a Metrohm 746 Trace Analyzer coupled with a 747 VA Stand, and was used to monitor Zn. The other one consisted in a Autolab PGSTAT 12 (EcoChemie) in conjunction with a Metrohm 663 VA Stand, that was used for Cu analysis.

Differential Pulse Anodic Stripping Voltammetry was used. Usual procedure involves transfer of ligand to the cell, deaeration for 10 min, and successive spikes of the selected metal. After every spike of metal, a conditioning time (30 min for Zn; 3 min for Cu) is allowed and then the preconcentration (accumulation) step is done. An accumulation time, t_{acc}, of 60 s has been used throughout. Accumulation potentials, E_{acc}, have been -1.200 V for Zn, and either -1.200 V or -0.600 V for Cu. Maximum drop size has been used in both equipment. In the measurement mode, a Pulse Amplitude of 70 mV was superimposed on the staircase for Zn, and a 50 mV pulse amplitude was applied in Cu quantification. A resulting 20 and 25 mV/s scan rate were used for Zn and Cu, respectively.

Theoretical modelling studies were carried out with the aid of an Hyperchem ® software. Two successive optimization steps were employed. The first one is based on molecular mechanics governed mainly by coulomb interactions. The second one is based on quantum mechanics and Zindo/1 semi-empirical methodology.

A 21-day fermented sample of must from Tempranillo (*Vitis vinifera*) variety was chosen as target sample for evaluation of complexing activity in real wine. This very same sample had been previously characterised as published elsewhere [7].

RESULTS AND DISCUSSION

All three assayed ligands, which structures are depicted in Scheme 1, showed a marked affinity for both Zn and Cu, although following different patterns and forming complexes of different abilities as competitors with the mercury electrode. Experiments were carried out in which a known amount of ligand was placed in the electrochemical cell, and then increasing amounts of either Zn or Cu were spiked on the solution. After an adequate equilibration time was elapsed, a 60 s preconcentration step was performed in order to obtain the deposit of the amalgamated metal on the mercury electrode. A subsequent scan on the positive going potentials provokes the oxidative stripping of the metal, that can be evaluated. When ligand is in excess an initial equilibration takes place with the added metal, and the electrochemical accumulation of the metal on the electrode can be written as a competitive process with the complex formation:

$$ML + 2\ e^- \leftrightarrows M(Hg)^0 + L$$
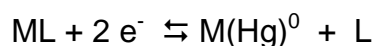
When voltammetric analytical signals are processed according to the algorithms proposed by Langmuir and Scatchard [35], and if linearization of experimental titration data is achieved, it implies the existence of simple 1:1 M:L dissociatable complexes.

Preliminary attempts to record the electrochemical stripping signal of Zn after successive spikes of known amounts of the metal failed to yield good breaks in the titration curve with either of the three ligands, what suggested that Zn-ligand complex formation might require a certain equilibration time to elapse between each fresh addition and the preconcentration step onto the mercury

electrode. Increasing equilibration times were checked and a 30 min period was seen to be necessary in order to electrochemically follow-up the competition of the ligand with the mercury for the Zn. On the other hand, Cu was seen to achieve equilibration easily with the assayed ligands and a short 3 min equilibration period was observed throughout.

<u>Catechin as a ligand</u>

Figure 1 shows the titration curves obtained for catechin with Zn and Cu, in a pH 4.0 acetate buffer. In both instances, two distinct linear portions are observed, corresponding to excess catechin and excess free Zn or Cu, respectively. The break in linearity should mark, approximately, the amount of metal able to fully saturate the available linking sites of the catechin. As it can be seen from Figure 1, the conditional concentration of Zn ([Zn']) that provokes the saturation of the ligand is ca. 1.40 μM that fairly matches the amount of catechin introduced in the cell (1.45 μM). Similarly, for an assayed 0.58 μM concentration of catechin, the titration curve with Cu changes slopes for a labile copper concentration of ca. 0.60 μM. These results point to a 1:1 stoichiometry as most likely. A further check consisted in the linearization of these experimental data according to the Langmuir algorithm [35] that is theoretically deduced and applicable for 1:1 stoichiometries. When the same experimental data were subjected to yet another linearization algorithm, the Scatchard one, that -working as well for 1:1 stoichiometries- is able to detect up to two different populations of ligands, a single straight line was again achieved for both metals, implying that a unique Zn:catechin or Cu:catechin complex was being formed in solution. The data thus obtained are in excellent agreement with those obtained through Langmuir and are listed in Table 1 for both metals.

It is noteworthy that calculations of total available catechin resemble very closely the real known amount of ligand to be present in the cell.

In order to prove that metals deposit freely on the mercury electrode from the acetate buffer solution, titration curves for both Zn and Cu were done in a pH 4.0 acetate buffer. The slopes of the graphs match those of the second linear portions obtained for the studied ligands, indicating that acetate is not affecting the preconcentration of metals on the electrode; that is to say acetate acts not as a complexing agent for either Zn or Cu as previously reported [36].

It was our interest to further check the evidence for a 1:1 stoichiometry by applying a theoretical study with the aid of Hyperchem®. Results suggest that catechin is able to fold over the Zn atom, trapping it in its cavity and stabilising the complex through the π aromatic electronic clouds, as depicted in Figure 2. A similar image is also obtained for the interaction of Cu with catechin. When compared with the values obtained for Zn, conditional stability constants seem to be consistently lower for the case of Cu, which on the other hand presents higher kinetics of complexation. All these findings support the experimental reported evidence [7] that both Zn and Cu associate in a very close fashion with cyanidine-3-glucoside in real must samples.

<u>Quercetin and rutin as ligands</u>

Experiments involving quercetin and rutin followed a similar pathway but, at the same time, showed a marked difference with respect to that of catechin.

Figure 3 collects the titration curves obtained for both ligands with either Zn or Cu. A glance at the breaking points of the linear potions of the graphs indicates that quercetin is able to uptake 2 atoms of either metal per molecule, while rutin is able to allocate up to 3 atoms of either Zn or Cu in its structure. However, algorithms indicate a 1:1 stoichiometry for either metal with rutin but fail to give a simple stoichiometry for any of them with quercetin. Repeated experiments for varying ligand concentrations showed a consistent metal to ligand ratio of 2 and 3 for quercetin and rutin, respectively. As for the theoretical modelling studies, a most favourable complex is obtained for either Zn or Cu with rutin through the 3-rutinoside moiety, whereas in the case of quercetin the picture differs somewhat for Zn and Cu. Although in both instances a 1:1 stoichiometry proves to be energetically the most favourable, the linkage for Cu would be through the 4-oxo-5-hydroxil group, while a dative bond from the aromatic rings would be the most likely situation for the case of Zn. This dative bond would imply *p* orbitals, and for Cu semi-occupied *d* orbitals would be involved. It is a generally accepted fact that Cu forms more stable complexes with natural ligands such as humic acids; nevertheless, in our experiments Zn always shows the larger values for the estimated conditional constants, which could be ascribed to the fact that intervening orbitals are of different type.

There are some reports [21] in which authors support the idea that quercetin and rutin should be in their reduced form in order to better complex metals as those under our consideration. Along our studies, both aged and fresh solutions were used and trouble was not taken to prevent oxidation (if any) of the molecules. Nevertheless, we have proved that rutin is electrochemically reduced on the mercury electrode at ca. -1.170 V. Zn titration experiments were seen to be independent on the preconcentration potential used (either −1.200 V or −1.000 V as to prevent rutin reduction on the surface of the mercury electrode) whereas experiments carried out for Cu showed a marked dependence on the potential used in the preconcentration step. Given the ample margin of working potentials available, two distinct sets of experiments for the complexation study of Cu were done. Thus, preconcentration of Cu in presence of either rutin or quercetin were carried out at both -0.600 V and -1.200 V. In the first situation, we can assume that no ligand is forced to be reduced on the electrode surface, while abundant quercetin and rutin reduced forms could be expected after 60 s preconcentration time under electrolysis at -1.200 V. Figure 4 shows the stripping voltammograms for Cu that had been deposited under those two accumulation potentials.

Two patterns are clearly distinguishable depending on the preconcentration condition used. When no reduction of ligand is forced (preconcentration at -0.600 V), the Cu stripping oxidation peak appears split into two, whereas a single well defined stripping signal is obtained when a very negative potential is used for the preconcentration step (-1.200 V), forcing the reduction not only of the metal but that of the ligand as well. Furthermore, when these experimental peak intensity data are subjected to the linearization algorithms, a good linear correlation is achieved (see Figure 5) in the case of rutin, but not in the case of quercetin.

Obviously, no mathematical treatment of peak intensity data could be done with the split signal. Anyway, transferred charge could be evaluated instead through measurement of the area under the overall curve. Figure 6

shows the Cu stripping transferred charge for both assayed preconcentration potentials with rutin as ligand.

It can be seen that when the reduction of ligand is not favourable (-0.600 V) the charge increases linearly, indicating that not detectable compound is being formed between rutin and Cu; in other words, the spiked Cu on the solution fairly deposits on the mercury without competition from the rutin. A kinetic-dependent response should be discarded since a long period of time elapses from the first to the last addition of standard metal; if any complex had been formed, the charge should reflect this fact. On the contrary, when the preconcentration takes place at -1.200 V, the peak current shows the typical pattern with two different linear portions of increasing slopes. At the same time, these experimental charge data adapt very well to a linear Langmuir plot indicating, once again, the existence of a weakly dissociatable 1:1 Cu:rutin complex in solution. On the other hand, although the voltammograms shape obtained for Cu-quercetin are a mirror image of those just mentioned for Cu-rutin the experimental data show no simple 1:1 Cu:quercetin dissociatable complex, even for a favourable preconcentration potential of -1.200 V. This could indicate that quercetin, when uptaking 2 atoms of Cu, form a 2:1 Cu:quercetin through the catechol and the 4-oxo-5-hydroxil position. As a matter of fact, the theoretical model predicts a more stable form between Cu and quercetin through this moiety.

Recently, there have appeared a good number of papers dealing with the possible structure of these type of complexes [24, 25, 37] . It has been stated that both quercetin and rutin would show a better complexing capacity in their reduced form. This asseveration is in very close agreement with our experimental evidence, in which a reduced ligand is able to compete with the mercury electrode for the Cu, while remains nearly ineffective in its oxidised form. It should be kept in mind that Cu(II) is a powerful oxidising agent for these substances, while Zn(II) is a more redox innocent species. Thus, added Cu may oxidise partially the ligands, and the presence of electrogenerated reduced ligands could probe mandatory in order to get the complex formation. On the contrary, since Zn(II) does not affect the redox state of the ligands, complexing with quercetin and rutin may take place directly with the reduced chemical form.

When compared with the results seen for rutin, we may admit that, effectively, this molecule is able to uptake 3 metal atoms (either of Zn or Cu). One, forming a most stable structure through the 3-rutinoside moiety, as depicted in the theoretical model as well, and another 2 atoms through the same catechol and 4-oxo-5-hydroxyl portions of the molecule, in common with quercetin. However it is noteworthy that while in the case of quercetin no single 1.1 complex is electrochemically detectable for either Cu or Zn, in the case of rutin a 1:1 dissociatable complex is consistently detected for both Zn and Cu. We tend to think that the third atom entering the molecule of rutin is the one linking through the 4-oxo-5-hydroxyl group; this position might be unstabilised for the close vicinity of the 3-rutinoside making it much more weaker than the bond through the catechol group. That would be then the responsible for the Langmuir and Scatchard detectable weak complex.

As a conclusion we may assert that:

- Catechin is able to form a 1:1 complex with either Zn or Cu that is monitored through the linearization algorithms as competitive with the preconcentration of the metals on the mercury electrode.
- Quercetin and rutin are able to uptake 2 and 3-fold of both Zn and Cu, respectively, in their molecules. Quercetin could link 2 metal atoms through the catechol and the 4-oxo-5-hydroxyl moieties, while rutin could take another third metallic atom in the 3-rutinoside.
- When the assayed ligand is rutin, the experimental data are favourably transformed in a linear set of data adjusting very well to a Langmuir or Scatchard picture, proving the existence of an electrochemically detectable 1:1 weak dissociatable complex.
- Quercetin produces not simple 1:1 dissociatable complexes with either Zn or Cu, since no good linearizations are got for the transformed experimental data.

Real wine sample

Once the complexing activity of these ligands with respect to either Zn or Cu has been thoroughly studied in aqueous (8% ethanol) solutions, an attempt has been done to monitor the complexing role of a red wine for these metals. Two 1:10 diluted aliquots corresponding to day 21 of wine fermentation were studied in an acetate pH 4.0 buffer in separate cells. We are aware of the disturbance that dilution might exert on the natural equilibria of the sample, but this is the only way to perform the voltammetric experiments. Similar considerations have been reported by other authors [28]. One of the aliquots was spiked with Zn and the other with Cu. Obtained titration curves can be seen in Figure 7.

Assuming that several natural occurring ligands might be present, a striking feature is that those sets of experimental data adjust extremely well to Langmuir and Scatchard linearization algorithms, in a way that very closely reminds that of catechin, which -at the same time- happens to be most abundant polyphenol in wines. If we look at estimated values of conditional stability constants listed in Table 1, we may suppose that complexing activity of the wine is due mainly to the catechin, although the presence and contribution of other ligands is not precluded. As a matter of fact, that complexing role should be ascribed to all those ligands present that behave as catechin under similar conditions.

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Table 1: Weak dissociatable conditional formation constants (log K$_{ML}$) as evaluated from Langmuir and Scatchard algorithms for Zn and Cu.

	Zn		Cu	
	Langmuir	Scatchard	Langmuir	Scatchard
Catechin	5.52	6.05	4.55	4.65
Rutin	5.67	5.86	5.71	5.81
Wine	5.70	6.03	5.26	5.12

Scheme 1: Molecular structure for: a) Catechin, b) Quercetin and c) Rutin
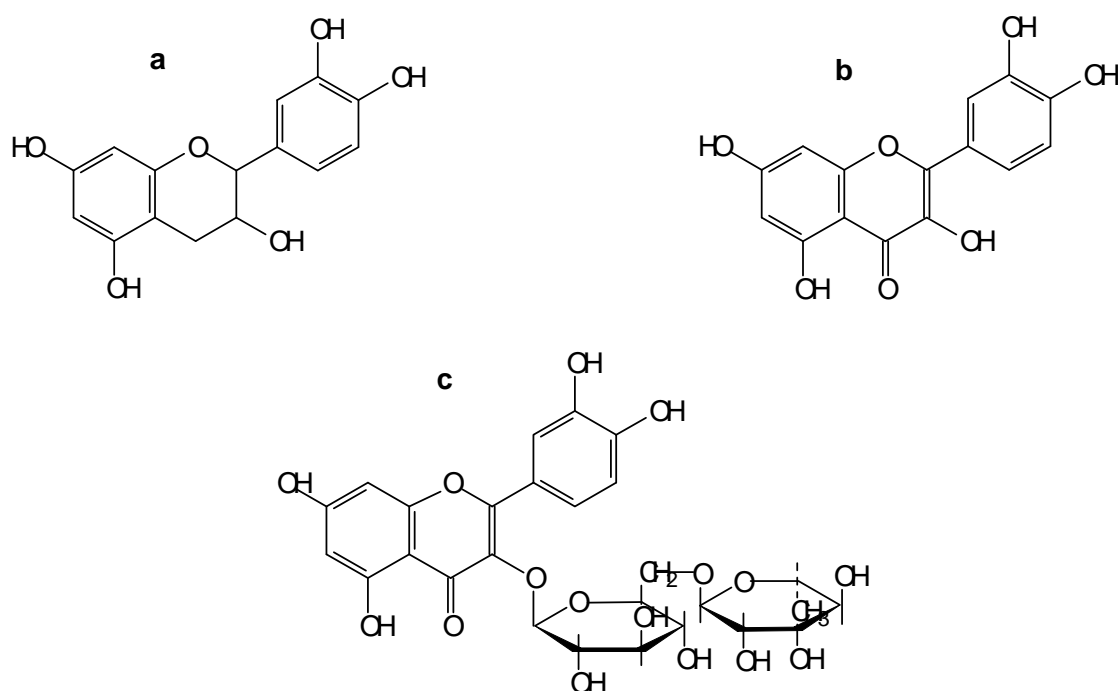
Figure 1: Titration curves for catechin with Zn (a) an Cu (b) at pH 4 acetate buffer. a) assayed catechin : 1.4 μM ; b) assayed catechin : 0.58μM

a

$y = 0.5536x - 0.2219$
$R^2 = 0.9905$

$y = 0.4125x - 0.0069$
$R^2 = 0.9906$

I (μA)

Zn (μM)

b

$y = 0.1473x - 0.036$
$R^2 = 0.9971$

$y = 0.1013x + 4E-05$
$R^2 = 0.9943$

I (μA)

Cu (μM)
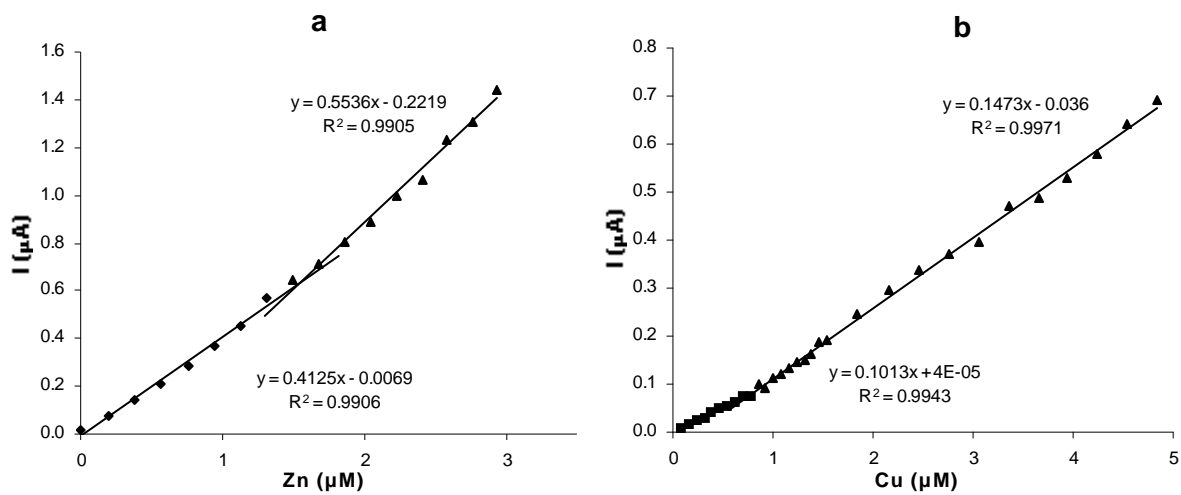
Figure 2: Theoretical Zn-catechin complex structure as depicted by Hiperchem[®]
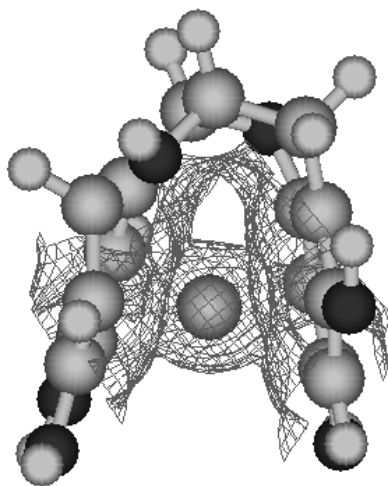
Figure 3: Titration curves obtained in aqueous acetate buffer pH 4 for quercetin with Zn (a) and Cu (b) and for rutin with Zn (c) and Cu (d). Assayed ligand concentration: 1.0 μM
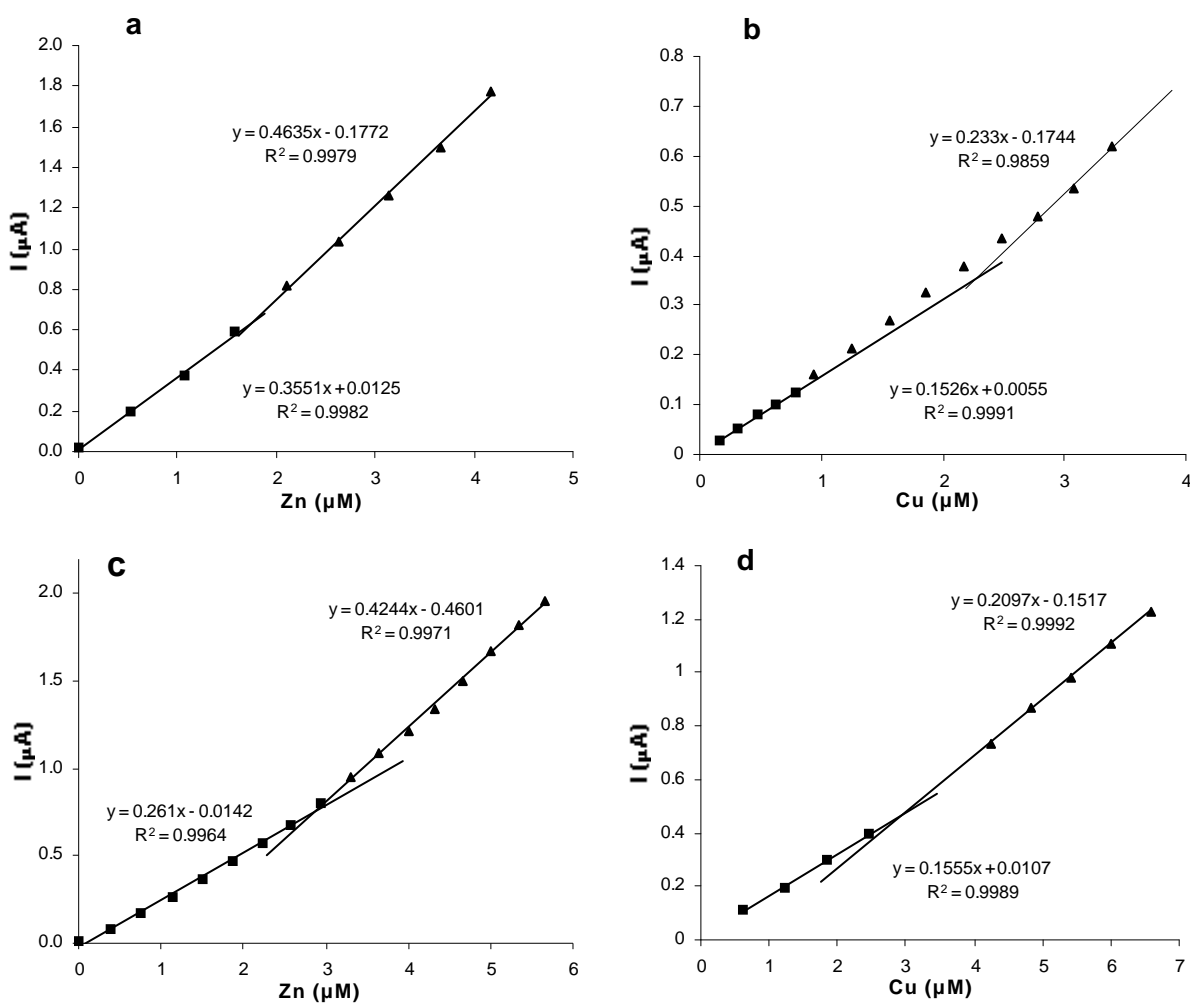

Figure 4: Stripping voltammograms for Cu in 1.0 μM rutin (acetate buffer, pH 4). a) E_{acc} : -0.600 V ; b) E_{acc} : -1.200 V.
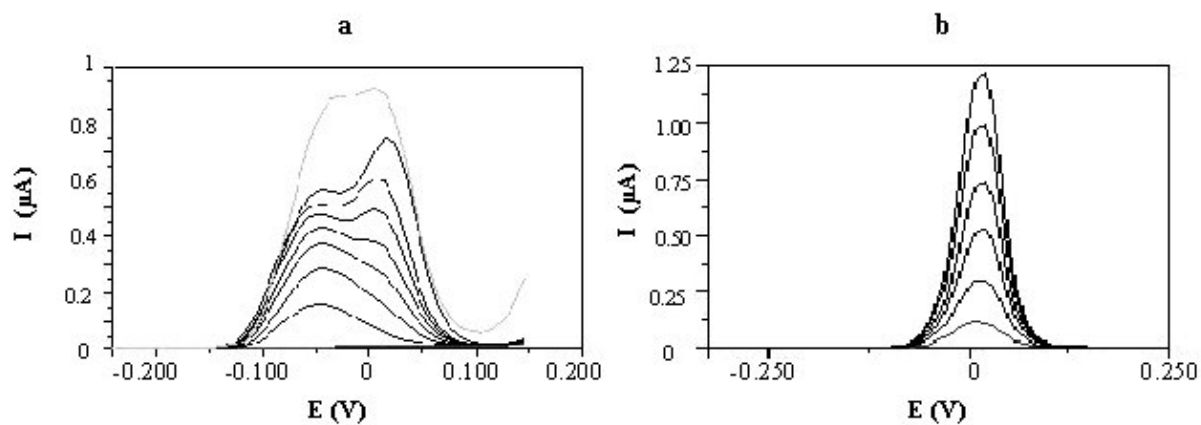
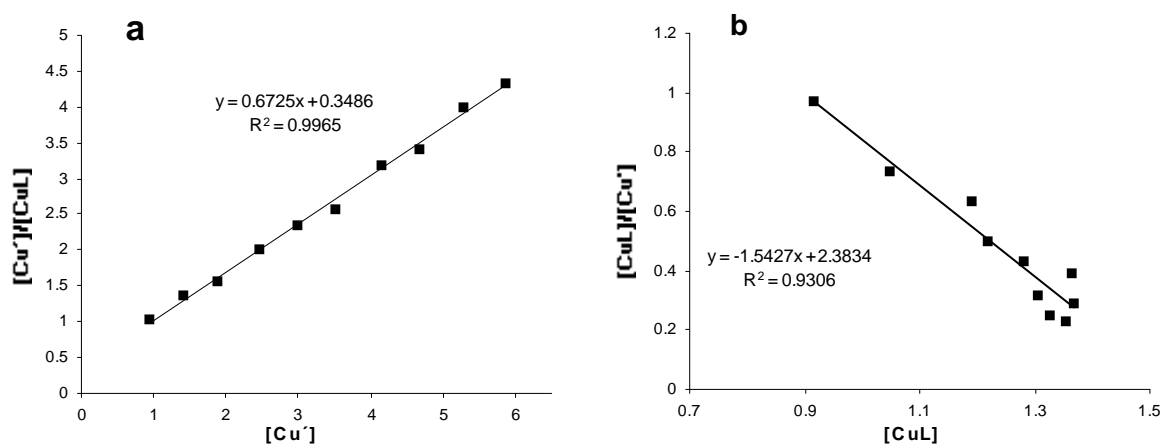**Figure 5:** Langmuir (a) and Scatchard (b) transformed data for Cu-rutin titration curves. E_{acc} : -1.200 V

Figure 6: Transformed Cu stripping charge with E_{acc} : −0.600 V (•) and −1.200 V (▲).
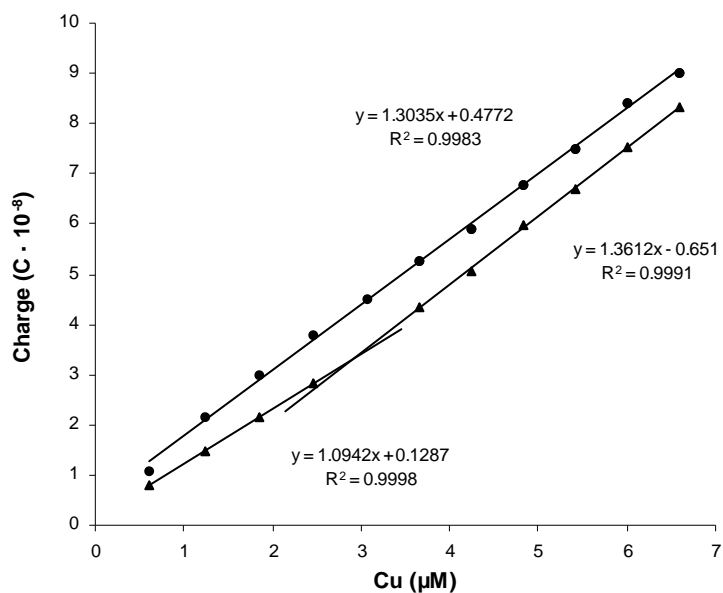
Figure 7: Zn (a) and Cu (b) titration curves on a natural pH 4 wine diluted 1:10 with acetate buffer. Wine samples belong to day 21 of must fermentation.