Role of Polyphenols in Copper Complexation in Red Wines

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Four monovarietal red wines, Tinta Roriz, Tinta Barroca, Touriga Nacional, and Touriga Francesa, were studied for their complexometric properties toward copper. For the purpose, ion selective electrode potentiometric titrations were carried out, and the data were treated by two methods suitable for heterogeneous ligands: the Scatchard plot, which provided the complexation capacity (CC_{total} = maximum number of moles of sites per liter), and the differential equilibrium function (DEF), which provided the conditional stability constants of the sites effectively involved in the metal complexation (K_{DEF}) at a given titration point. The different wines displayed similar properties: CC_{total} ranged from 4.5×10^{-3} (Tinta Barroca) to 4.9×10^{-3} M (Touriga Francesa), and log K_{DEF} values were between 4.2 and 6.5. The range of $\log(|Cu|_{bound}/CC_{total}) = \log \theta$ embraced in the titration was between -0.2 and -1.9. With regard to the natural levels of copper, from 4.4×10^{-7} (Tinta Roriz) to 1.3×10^{-6} M (Touriga Nacional), and ligands (CC_{total}) in the wines, it can be expected that copper will be strongly complexed in the studied wines (log K_{DEF} > 6). Studies carried out, in parallel, for solutions of isolated seed tannins and skin polyphenols (mainly anthocyans) showed that their contribution to the CC_{total} of the wines was <40% for tannins and <2% for skin polyphenols. However, skin polyphenols were shown to be much stronger copper ligands (log K_{DEF} = 6.7, log θ = -1.5) than tannins (log K_{DEF} = 4.5, log θ = -1.5).

Keywords: *Copper; red wines; complexation parameters; polyphenols*

INTRODUCTION

Metal cations existent in wine have different sources: natural, addition, and contamination. Their potential hazardous effects include tartrate precipitation (K and Ca), haze formation (Al, Cu, and Fe), enhancement of oxidation rate (Cu and Fe), and toxic effects (Pb and Hg) (Scollary, 1997). It is recognized that the extent of the effects caused by copper are not determined by its total concentration. In fact, copper hazes have been found in wines in which total copper concentrations were clearly below the recommended level (0.3−0.5 mg/L) (Scollary, 1997). It is expected that advances achieved in the speciation of copper, namely, the identification of the chemical forms under which it is present in the wines, will facilitate the understanding of the haze process and oxidation rate enhancement by copper.

Some electrochemical techniques, such as differential pulse anodic stripping voltammetry (DPASV) (Daniele et al., 1989; Arcos et al., 1993) and potentiometric stripping analysis (PSA) (Jagner and Westerlund, 1980), have been applied to wines with the aim to provide some speciation information in addition to the determination of total metal concentration. Concretely, these techniques have been able to discriminate the fractions of operationally "inert" and "labile" (reactive) copper. Very recent works (Green et al., 1997; McKinnon and Scollary, 1997; Wiese and Schwedt, 1997) gave a step further in this field, as they tried to identify the ligands primarily responsible for copper complexation in table

and fortified wines. It is patent from these works that the most important groups of ligands in wine are large molecules of peptides or proteins and polyphenols, namely, the condensed tannins. These conclusions are compatible with information obtained by us (unpublished results) by computational speciation calculations based on the published (Síllén and Martell, 1974; Martell and Smith, 1974) protonation constants of free amino acids and organic acids found in wines and their Cu(II) complexation equilibrium constants. It was found that the amino acids and organic acids of the wine, at typical enological concentrations and pH, are weak ligands, and therefore they cannot account for the strong Cu(II) complexation verified in wines. In the case of the above-mentioned macromolecules, there is a lack of information about the nature and molar concentration of the wine ligands, as well as about their strength (equilibrium constants) to bind heavy metals, particularly copper ions.

The purpose of the present work was to study the complexometric properties to copper(II) of four different red wines from only one of the following varieties of grapes, cultivated in the Douro's Demarked Region of Portugal: Tinta Barroca, Tinta Roriz, Touriga Nacional, and Touriga Francesa. These wines were produced for research purpose. At the same time, the complexometric properties of the polyphenols isolated from grape seeds (condensed tannins) and skin (mainly anthocyans) from a mixture of the same grapes were also studied, and the results were compared with those of the wines. The complexometric properties were expressed in terms of maximum complexation capacity (CC_{total}), or concentration of the total sites available in the sample, and conditional formation constants of the complexes formed with copper(II). For calculation of the conditional equi-

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Table 1. Polyphenolic Characteristics of the Studied Wines [Mean Results of Two Determinations (RSD < 8%)]

| | wine | | | |
property	Tinta Barroca	Tinta Roriz	Touriga Nacional	Touriga Francesa
total phenolic compounds index (D_{280})[a]	47.0	52.3	66.5	63.2
total tannins[b] (g/L)	2.86	3.63	3.92	3.52
tannin condensation index (dDMACH)[c]	6.06	8.60	9.00	7.40
anthocyans[d] (g/L)	0.44	0.56	0.64	0.40
polymerized anthocyans (%)[e]	89.8	65.6	55.8	51.9

[a] Optical density at 280 nm for 1 cm of optical length (Glories, 1978). [b] Determined according to the method described by Ribéreau-Gayon and Stonestreet (1966). [c] dDMACH = $(d_1 - d_2) \times 10$, where d_1 is the optical density at 640 nm (1 cm of optical length) after reaction with dimethylaminocinnamaldehyde (DMACH) and d_2 is the same as d_1 with DMACH replaced by methanol (Vivas et al., 1994). [d] Determined according to the method described by Glories (1978). [e] Percentage of the optical density at 520 nm that remains after reaction with bisulfite (Glories, 1978).

librium constants, the differential equilibrium function model, which has been considered to be suitable for characterization of complexes involving heterogeneous ligands (Buffle, 1988) was used. These concepts are adequately explained under Data Treatment. As far as we know, this approach of considering the heterogeneity of the ligands of the wine was never applied before to the study of the metal complexation properties of wines.

Due to their aromatic structure, tannins exhibit a fluorescent capability (Guilbault, 1977), which is diminished by binding to Cu(II) (quenching). This property was exploited in this work [fluorometric titration of solutions of tannins with Cu(II) solutions] but with a very reduced success because the analytical window of the technique allowed only results in unrealistic conditions, that is, for ratios of |Cu(II)|/|tannins| too high relative to those present in the wines. Therefore, ion selective electrode potentiometric titration of the samples under study with Cu(II) solutions was the methodology elected for the present study.

MATERIALS AND METHODS

Reagents and Materials. All reagents used were of analytical grade. All solutions were prepared using deionized water with specific conductance of <0.1 μS cm^{-1} and stored in polyethylene containers. All of the material was decontaminated overnight with 20% HNO$_3$ and thoroughly washed with deionized water.

Some relevant polyphenolic characteristics of the four monovarietal red wines studied (V. Freitas, unpublished data) are shown in Table 1. The methods used in these determinations are described in the literature by Glories (1978), Vivas et al. (1994), and Ribéreau-Gayon and Stonestreet (1966). The grapes of Touriga Nacional, Touriga Francesa, Tinta Barroca, and Tinta Roriz varieties were obtained in a large Douro vineyard during the growth season, by random sampling. A mixture with equal numbers of berries from each variety was then used for polyphenolic extraction.

Vinification. The musts used in the vinifications were obtained in the same vineyard. The harvested grapes of each variety (15000 kg) were destemmed, crushed, and then transferred into stainless steel wine vats. The musts, treated with 0.5 mL of a 6% (w/v) solution of SO$_2$ per liter, were fermented during ~7 days with wild yeast to dryness at a temperature between 19 and 24 °C. During fermentation the must was pumped over the cap four times a day. After fermentation, the wine was separated from pomace by filtration and was pumped to another vat, where it underwent malolactic fermentation.

Polyphenol Extraction Procedures. Polyphenolic contents of grape seeds and skins were extracted through a hydroalcoholic extraction, followed by extraction with chloroform to remove lipids and chlorophyll. The experimental details are described elsewhere (Darné and Madero, 1979; Freitas, 1995). The polyphenol residues obtained from grape seeds (condensed tannins) and from skins (mostly anthocyans) were dissolved in an aqueous/ethanolic solution with 0.05 M KNO$_3$, pH 3.5, and 10% ethanol.

Determination of Total Metal Concentration. Total copper concentrations in the wines were determined with a Perkin-Elmer 4100-ZL atomic absorption spectrophotometer with electrothermal atomization provided with a Zeeman background correction system. The wines were diluted 1/4 with 1% HNO$_3$ and determined according to the method of standard additions. Volumes of 15 μL of sample and 5 μL of matrix modifier, 0.05 mg of NH$_4$H$_2$PO$_4$ + 0.003 mg of Mg(NO$_3$)$_2$ (a common procedure to stabilize the metal in the graphite furnace, thus avoiding losses of the metal in the form of volatile compounds, during the pyrolysis step of the atomization program), were used in the analysis. The accuracy of the method was successfully checked by analyzing some BCR reference wines, with results reproducing the referenced values within the associated error.

Spectrofluorometric Titrations. To study the CC$_{total}$ of tannins toward Cu(II), titrations of 50 mL of 20 mg/L tannins in 0.05 M KNO$_3$, pH 3.5, and 10% ethanol were carried out with 10^{-2} and 10^{-1} M Cu(II). To maintain the volume during the titration, the solution of tannins was continuously circulating in the spectrofluorometer (Jasco FP-770 model) cell, through a tube circuit driven by a peristaltic pump (Gilson Minipuls 2). The differential between excitation and emission wavelengths was set to 50 nm. In these optical conditions, tannins displayed a maximum emission at 275 nm. The spectra were recorded with a data interval of 0.5 nm, scan speed of 100 nm/min, slit width of 10 nm, medium PMT voltage, and response time of 2 s. Three replications were carried out.

Potentiometric Titrations. For the study of affinity of Cu(II) ions toward the various wines and isolated polyphenols, potentiometric titrations of wines (or solution of extracts of polyphenols) with a 0.01 M Cu(II) solution were carried out, at 25 °C. The free Cu(II) was measured with the respective ion selective electrode (ISE) from Radiometer, activated with a mixture of Ag$_2$S and CuS prepared as before. As reference electrode, an Orion Ag/AgCl(s) KCl 3M (double junction) was used. A computer-controlled arrangement comprising a decimilivoltimeter (Crison micro pH 2002) coupled to an automatic buret (Crison micro BU 2030) was used. Three independent titrations were carried out for each system under study. The natural levels of Cu in the titrant, determined by AAS, were taken in consideration for the total metal concentration. Calibrations were performed in a 0.05 M KNO$_3$, pH 3.5/10% ethanol (v/v) medium.

DATA TREATMENT

Wine comprises groups of ligands, such as the tannins, the polysaccharides, the peptides, or proteins, and combinations of them, the individual chemical structure of which is unknown or not well-defined. Because of the complexity of the wine matrix and of the lack of information about the molar concentration sites (of both proton and metal ions) of the individual compounds, the well-established computational methods for determination of stoichiometric stability constants cannot be applied. In addition, the wine contains polymeric ligands which may act as heterogeneous ligands, that is, organic substances that display numerous coordination sites which may be different in their nature or not. Owing to electrostatic and steric effects, these substances display a large range of complexation properties. The respective microscopic stability constants decrease with the successive occupation of the sorption sites even when they

are chemically homogeneous. For this reason, metal complexation in wine has to be studied by methods developed specifically for systems that include heterogeneous ligands.

Detailed descriptions of the models used in the present study, Simplex (Nelder and Mead, 1965) for the quenching fluorescence data and Scatchard (Scatchard, 1949) and K_{DEF} (Buffle, 1988; Shuman et al., 1983) for the potentiometric data, can be found in the literature. Only the concepts and equations relevant for the discussion of the data of the present work are summarized here.

Fluorometric Experiments. The addition of a paramagnetic metal ion to a fluorescent ligand, such as tannins, with complex formation, results in reduction of fluorescence (quenching), which is proportional to the decrease of concentration of the free ligand. The fluorescence intensity of the ligand solution (I_F) is given by (Ryan and Weber, 1982a,b)

$$I_F = I_F^L + I_F^{CuL} \quad (1)$$

where I_F^L and I_F^{CuL} are the fluorescence intensities of the free ligand (L) and the complex (CuL), respectively.

If the stoichiometry of the complex formation is assumed as 1:1

$$Cu + L \rightarrow CuL \quad (2)$$
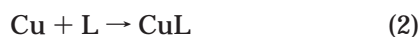
an average conditional stability constant, K_{av}, is defined by

$$K_{av} = |CuL|/(|L| \times |Cu|_{free}) \quad (3)$$

or

$$K_{av} = |Cu|_{bound}/(|L| \times |Cu|_{free}) \quad (4)$$

where $|Cu|_{bound} = |Cu|_{total} - |Cu|_{free} = |CuL|$.

K_{av} is valid only for the experimental conditions used (pH, temperature, ionic strength, and ligand and metal concentrations range), and it is an arithmetic mean of the conditional microscopic equilibrium constants of the different specific sites.

Applying adequate mathematical relations, eq 4 can be converted to

$$I_F = 1 - [1/(2K_{av}CC_{total})][(K_{av}CC_{total} + K_{av}|Cu|_{total} + 1) - [(K_{av}CC_{total} + K_{av}|Cu|_{total} + 1)^2 - 4K_{av}2CC_{total}|Cu|_{total})^{1/2}] \quad (5)$$

where CC_{total} (the total concentration of coordination sites) $= |CuL| + |L|_{free}$.

Having I_F and $|Cu|_{total}$ values available for every titration point, this equation can be solved for K_{av} and CC_{total} by nonlinear regression analysis, using a modified Simplex optimization as described by Nelder and Mead (1965). Calculations were performed using a Qbasic program developed in our laboratories by Luis Ribeiro.

Potentiometric Experiments. The CC_{total} of the samples as well as mean values of conditional stability constants, K_{av}, were determined by Scatchard plot. As before, it is assumed that the complexes formed between the metal and the ligands have a 1:1 stoichiometry (see eqs 2−4).

Applying mass balances, eq 6 is obtained

$$|Cu|_{bound}/|Cu|_{free} = K_{av}CC_{total} - K_{av}|Cu|_{bound} \quad (6)$$

Equation 6 implies that a plot of $|Cu|_{bound}/|Cu|_{free}$ against $|Cu|_{bound}$ displays only one approximately linear zone having a slope of $-K_{av}$, and intercept corresponding to $K_{av}CC_{total} \cdot |Cu|_{free}$ is given experimentally by the ISE measurements. It must be stressed that the K_{av} obtained by the Scatchard method is an average value of the formation constants referring to the different complexation sites occupied in the range of $|Cu|_{bound}/CC_{total} = \theta$ embraced (i.e., to which a linear range was obtained in the Scatchard plot).

In a few cases a curved plot is obtained in which two (or more) distinct linear segments can be found. Such results may be interpreted as indicating the presence of two different types of sites ($i = 1, 2, ...$), which have associated K_{1av} and CC_1 and K_{2av} and CC_2, etc. Obviously, CC_{total} is given by

$$CC_{total} = CC_1 + CC_2 + ... \quad (7)$$

To interpret the potentiometric titration data, the differential equilibrium function (DEF) has also been used. This method provides the values of K_{DEF}, which is a weighted arithmetic mean of individual microscopic formation constants (designated here K_j), but it has a greater and more precise significance than K_{iav} determined by the Scatchard model, because the weighting factor is such that at a given titration point only the sites with very close K_j values influence the K_{DEF} (Altmann and Buffle, 1988). These focused calculations allow one to obtain a K_{DEF} value for each titration point, being though a function of the degree of occupation of the active sites θ. In conditions of constant total ligand concentration (e.g., titration of a ligand with a small volume of a proper metal ion solution), K_{DEF} can be calculated

$$K_{DEF} = -(\alpha^2/|Cu|_{total})[1/(1 + (\alpha - 1)(d \ln(|Cu|_{total}/CC)/d \ln \alpha)] \quad (8)$$

where $\alpha = |Cu|_{total}/|Cu|_{free}$.

K_{DEF} are related to K_{av} and θ through eq 9:

$$K_{DEF} = -d[K_{av}(1 - \theta)]/d\theta \quad (9)$$

In an opposite way, for each selected θ range, a mean K_{DEF}, equivalent to the K_{av}, can be computed by eq 10

$$\overline{K_{DEF}} = \sum_j K_{jDEF}/m \quad (10)$$

where K_{jDEF} is the specific value of K_{DEF} and m is the total number of different K_{jDEF} values considered in the θ range.

The differential calculations of K_{DEF} were performed by the CHEMECT Qbasic program developed in our laboratories by Carlos Gomes.

RESULTS AND DISCUSSION

Fluorometric Experiments. Figure 1 shows that tannins had a fluorometric linear response from ≈0 to 20 mg/L. That was the reason for using 20 mg/L tannins in the titrations, thus allowing full advantage of the linear response range to be taken. A typical set of spectra obtained along the titrations with Cu(II) is presented in Figure 2. The Simplex optimization gave
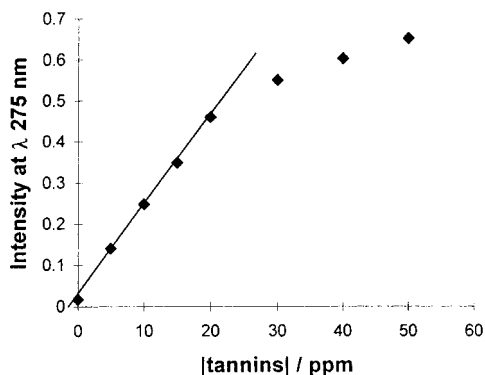
Figure 1. Calibration curve for tannin intensity of emission at 275 nm, with excitation at 225 nm.

Figure 3. Potentiometric calibration (■) and titration curve of Tinta Barroca with Cu(II) (□). In the abscissa axis the |total Cu| refers to the Cu naturally present in wine plus the Cu added.
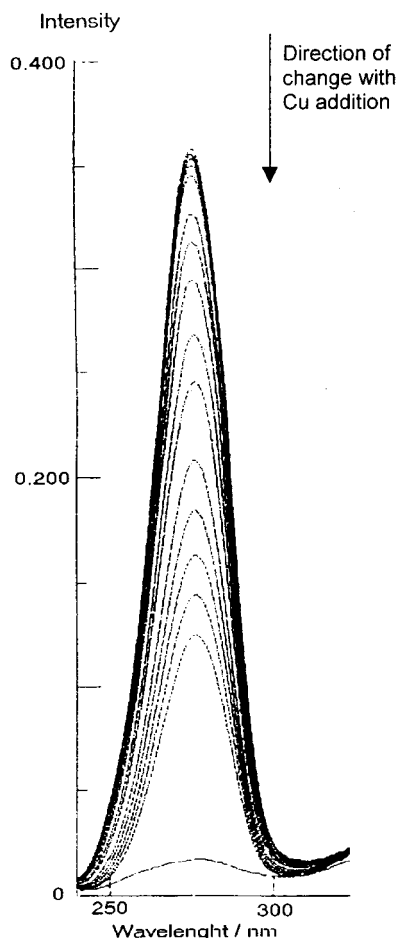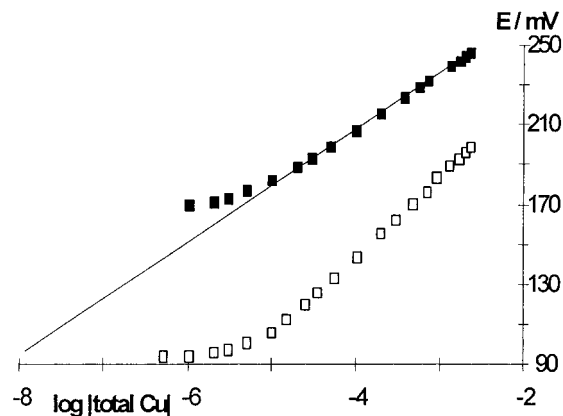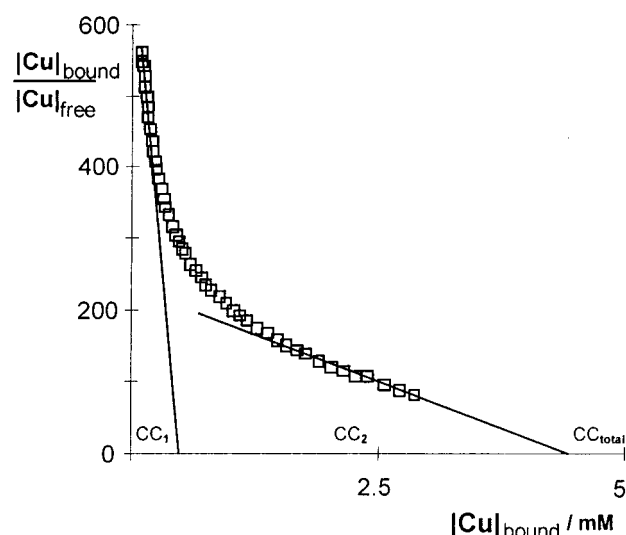

Figure 2. Spectra illustrating the successive quenching obtained along a typical titration of 20 ppm of tannins with Cu(II). Conditions: data interval, 0.5 nm; $\Delta\lambda$, 50 nm; scan speed, 100 nm/min; slit width, 10 nm; medium PMT voltage; response time, 2 s.

Figure 4. Scatchard plot obtained for Tinta Barroca red wine.

a log K_{av} of 2.74 for log θ between 2.11 and 3.19, which means that an excess of metal of 129–7943 times occurred during the titrations. For the CC parameter no convergence was obtained by this iterative method. The log θ values embraced by this technique were much greater than those existent in the studied wines, where a large excess of ligand, that is, log θ between −3.6 and −4.0 (see below how these values where attained), instead of a large excess of metal is found. This fact dictated the inadequacy of the fluorometric technique.

Potentiometric Experiments. Ion selective electrode potentiometry was shown to cover a range of θ values closer to that occurring in wines.

Figure 3 illustrates with a typical example the potentiometric titration curves of wines with Cu(II) obtained in the present work. For comparison purposes, the respective calibration curve was included. It is worth noticing that because the ligands exert a buffer effect toward Cu(II) concentration, in their presence there was an extended range for the practical linear response limit of ∼3 orders of magnitude (from 10^{-5} to 10^{-8} M).

Data of titration curves were first treated through the Scatchard plot. Figure 4 illustrates, for Tinta Roriz (for the remaining wines similar results were obtained), that two approximately linear ranges could be selected. In other words, two different |Cu|$_{bound}$ ranges (corresponding to two θ ranges) compliant with the Scatchard model, where eq 6 could be applied, were observed. This means that, macroscopically, the systems studied could be reduced to two different types of ligand with markedly different coordination strengths, expressed by K_{1av} (the higher value) and K_{2av}. The group of ligands that coordinated the metal ions first (thus corresponding to the lower |Cu|$_{bound}$ values) are those with the highest metal affinity or the stronger sites (K_{1av}). The correlation coefficients of the least-squares linear regression were in most cases ≥0.99 ($n \geq 8$); thus, the linearity of the zones selected on Scatchard plots was considered to be acceptable. The mean values of CC and K_{av} obtained for the different wines and for the grape extracts are

Table 2. Complexation Capacities (CC) of Cu(II) of Four Different Wines and Solutions of Tannin and Skin Polyphenols Isolated from Grapes, Obtained from the Potentiometric Data[a]

	stronger sites			weaker sites			
medium	CC_1 (M)	θ range	log K_{1av}[a]	CC_2 (M)	θ range	log K_{2av}[a]	CC_{total} (M)
Tinta Barroca	4.3×10^{-4}	0.014–0.051	6.0	4.0×10^{-3}	0.38–0.64	4.7	4.5×10^{-3}
Tinta Roriz	3.4×10^{-4}	0.011–0.027	6.1	4.6×10^{-3}	0.24–0.59	4.5	4.9×10^{-3}
Tinta Francesa	3.2×10^{-4}	0.012–0.040	6.4	4.5×10^{-3}	0.38–0.60	4.9	4.8×10^{-3}
Tinta Nacional	3.8×10^{-4}	0.014–0.054	6.2	4.4×10^{-3}	0.33–0.60	4.8	4.8×10^{-3}
tannins, 2 g/L	1.2×10^{-4}	0.009–0.079	5.4	8.6×10^{-4}	0.22–0.78	3.7	9.7×10^{-4}
skin extract, 20 g/L	1.9×10^{-4}	0.017–0.067	6.6	1.3×10^{-3}	0.39–0.67	4.6	1.5×10^{-3}

[a] The table includes the conditional stability constants, K_{av}, obtained by the Scatchard model within the given θ range (see eqs 4, 6, and 7 in the text).

reported in Table 2, which includes the range of θ values embraced in each case.

The four different wines displayed very similar CC_{total} values for Cu(II), between 4.5×10^{-3} and 4.9×10^{-3} M. Table 2 also shows that a solution with 2 g/L tannins extracted from seeds displayed a CC_{total} of 9.7×10^{-4} M, which corresponds to 480 μmol of Cu sites/g of tannins. As the concentration of tannins in the studied wines varied between 2.86 (Tinta Barroca) and 3.92 g/L (Touriga Nacional) (Table 1), the maximum contribution of tannins for the CC_{total} of the wines will be between 1.4×10^{-3} and 1.9×10^{-3} M, respectively. Aggregation of tannin macromolecules, which will increase when their concentration increases, may reduce the sites of tannins available for the metal ion in the wines. Nevertheless, the CC_{total} values of wines were ~3 times higher than the maximum contribution of tannins. Solutions with 20 g/L skin polyphenols (which includes mainly anthocyans) displayed a CC_{total} of 1.5×10^{-3} M, corresponding to 75 μmol/g. Because the concentration of anthocyans in the wines was between 31 (Touriga Nacional) and 50 (Touriga Francesa) times lower than in the studied solution (between 0.40 and 0.64 g/L, see Table 1), the anthocyan contribution for the CC_{total} of the wines is expected to be small, probably <2%. Therefore, abundant Cu(II) complexing agents other than the isolated polyphenols should exist in the wines.

In addition, Table 2 shows that values of K_{1av} between 6.0 and 6.4 were obtained for the stronger sites of the wines that can be measured by potentiometry (the analytical window of observation used). For the weaker sites (K_{2av}) values between 4.5 and 4.9 were found. Tannins exhibited lower K_{av} values (~1 order of magnitude) than the wines, thus showing that stronger ligands have to be present in wines. Skin polyphenols presented a K_{1av} value slightly higher than that of the wines and a K_{2av} similar to that of the wines.

A more detailed discussion of the strength of the ligands is left for the K_{DEF} results of the potentiometric data treatment, which are presented in Figure 5). As in the case of the CC values, the different wines displayed a very similar distribution of the K_{DEF} values. For log $\theta = \log(|Cu_{bound}|/CC_{total}) < -1.5$, which corresponds to $|Cu_{bound}| < 1.5 \times 10^{-4}$ M, a horizontal plateau of log K_{DEF} constants was observed, between 6.0 (for Touriga Nacional) and 6.4 (for Tinta Roriz), which indicates the absence of heterogeneity in this θ range. This may be due to a homogeneous character for the stronger ligands. Electrostatic and steric effects should be reduced because the occupation degree by the metal ion was very low. For log $\theta > -1.5$ the values of K_{DEF} systematically decrease when the degree of occupation of the binding sites increases.

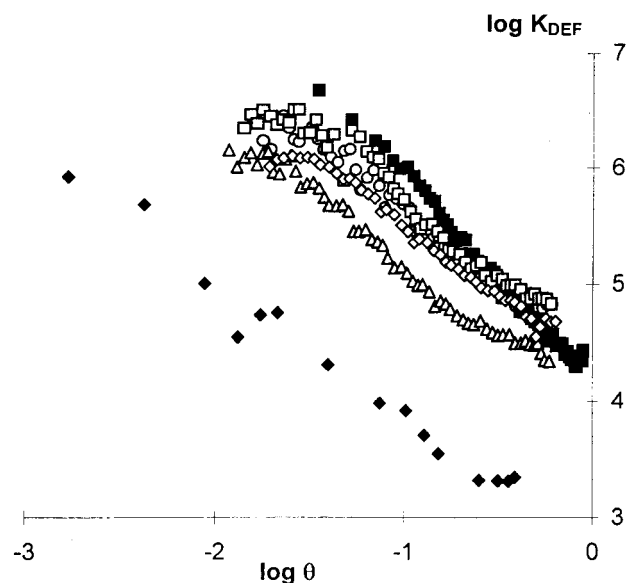Typical values of log K_{DEF} obtained for solutions with 2 g/L tannins and 20 g/L skin polyphenols were included

Figure 5. K_{DEF} obtained from the four wines studied [Tinta Roriz ($\triangle$); Tinta Barroca ($\diamond$); Touriga Nacional ($\bigcirc$); Touriga Francesa ($\square$)] and from tannins (2 g/L) ($\blacklozenge$) and skin polyphenols (20 g/L) ($\blacksquare$), as a function of log θ ($\theta = |Cu|_{bound}/CC_{total}$).

in Figure 5. If identical θ values are considered for the different solutions studied (wines and isolated polyphenols), for instance, log $\theta = -1.5$, and the log K_{DEF} values are compared, it will be observed that K_{DEF} will be markedly lower for Cu(II) tannins than those observed for the wines, whereas for Cu(II) skin polyphenols it will be higher. The results indicate that the ligands isolated from the grape skins, which include mainly anthocyans and in small percentages some tannins, carbohydrate–polyphenol complexes, and other compounds, are much stronger ligands than the tannins isolated from seeds.

The levels of Cu found in the wines ranged from 4.4×10^{-7} to 1.35×10^{-6} M, which (considering $|Cu|_{total} = |Cu|_{bound}$) corresponds to log θ between -3.6 and -4.0. These results clearly indicate that the Cu present in the studied wines will be strongly bound up with organic ligands (log $K_{DEF} > 6$).

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