# Pb and Cu Speciation and Bioavailability in Port Wine

M. A. G. O. Azenha and M. T. S. D. Vasconcelos*

LAQUIPAI, Chemistry Department, Faculdade de Ciências da Universidade do Porto, R. Campo Alegre 687, P-4169-007 Oporto, Portugal

Information about the speciation of Pb and Cu in different types of Port wines (white, single-year and blended aged red, and young red wines) was gathered to estimate their respective bioavailabilities to man. For this purpose, wines were subjected to in vitro simulated gastrointestinal digestion, and the following properties were studied in the wines and its gastric and intestinal digests: (1) average conditional stability constant ($K_{av}$) of the Cu complexes (by potentiometry), of the strongest Pb complexes (those inert to cathodic voltammetry, $K'_{av}$), and of the respective ligand concentrations (CC or $CC_{inert}$); (2) the distribution of the metal among the different groups of compounds of different molecular weights and/or polarities in the different bands separated by reverse phase high-performance liquid chromatography; (3) the total metal concentration present in the wines and the respective fractions present in the soluble and in the dialyzable fractions of the digest (an estimation of the assimilable fraction). The study showed that the complexing affinity for Pb (expressed by either $CC_{inert}$ or $K'_{av}$) of white and very aged red Port wines was lower than for the remainder of the wines. For Cu, the strength of the ligands in the white wines was lower (<log $K_{av}$ values) than in the other wines, but their concentrations (CC) were higher. For Pb, $CC_{inert}$ was much higher after the digestion than for the untreated wines, whereas the log $K'_{av}$ values were ~1 order of magnitude lower. These parameters could not be determined for Cu in the gastrointestinal digests. For all of the studied wines the dialyzable fraction of Pb during the intestinal digestion was low (10−22% of the total Pb) and the dialyzable fractions of Cu were close to 50% of the total Cu.

**Keywords:** *Copper; lead; speciation; Port wine; bioavailability; in vitro digestion*

## INTRODUCTION

The bioavailability of an element present in a food or a drink, such as wine, depends on the capacity of the physical−chemical forms under which it is present at the site of absorption to surpass the intestinal barrier. The intestinal absorption (assimilability) requires that those forms be soluble, although it is not a sufficient condition (Hocquellet, 1997). Only the metal that can surpass the intestinal barrier is potentially bioavailable; only those forms of the element that can efficiently interact with sites of the biological ligands are bioavailable. Therefore, information about the nature and concentration of the wine ligands as well as about their strength (equilibrium constants) to bind heavy metals and, particularly, their fate during gastrointestinal digestion deserves investigation.

There is some evidence that Cu and Pb present in wines are mainly associated with macromolecules (Szpunar et al., 1998; Wiese and Schwedt, 1997; McKinnon and Scollary, 1997), but information about the forms of these metals after the digestion is hard to find in the literature. In a former work (Azenha and Vasconcelos, 2000) we have shown that the Pb present in table wines is susceptible to insolubility and complexation weakening during the intestinal digestion, which was simulated by in vitro methods (faster and less expensive than in vivo methods). In the same work it was demonstrated that Pb and Cu may be much less bioavailable in red wines than in white wines. Nevertheless, the maximum threshold set for Pb by the L'Organisation Internationale de la Vigne et du Vin (OIV) is 200 $\mu$g/L, regardless of the type of wine. This maximum threshold is particularly restraining for the exportation of old Port wines, which have Pb contents that can be significantly >200 $\mu$g/L due to the type of enological procedures (such as lead plumbing) practiced a few decades ago.

Therefore, it is of interest to know the potential bioavailability of Pb and Cu, present in different types of Port wine, which constituted the objective of the present study. For this purpose, the following parameters were determined as before (Azenha and Vasconcelos, 2000): ($A_1$) total Cu and Pb concentrations in the wines (by atomic absorption spectroscopy, AAS); ($A_2$) average conditional stability constants of the Cu complexes (by ion selective electrode, ISE, potentiometry) and of only the stronger Pb complexes (by square wave cathodic voltammetry, SWCV) and the mean concentration of the respective ligands; and ($A_3$) information about the nature of the ligands (by reverse phase high-performance liquid chromatography, RP-HPLC). (B) The wines were in vitro digested under both gastric and intestinal conditions, and studies identical to those mentioned in (A) were carried out in the digests. In this case, the Cu and Pb concentrations were determined only in the soluble fraction. The metal concentrations in the dialyzable fraction of intestinal digests were also determined.

Direct SWCV without a deposition step was the voltammetric procedure selected for the determination of the chemical equilibrium parameters. The measure-

* Corresponding author (telephone +351 22 6082870; fax +351 22 6082959; e-mail mtvascon@fc.up.pt).

10.1021/jf991167i CCC: $19.00 
Published on Web 09/29/2000

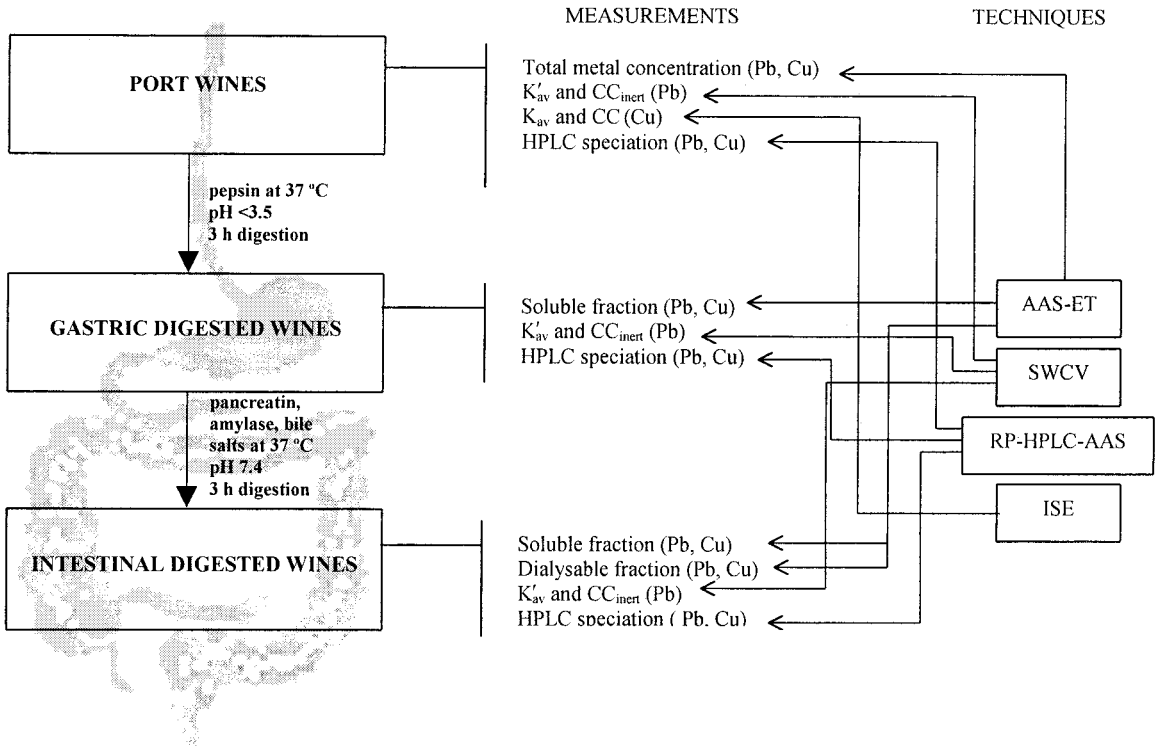


**Figure 1.** Scheme of the experimental methodology used.

ment was carried out just after the electrode renewal (new drop) by applying a rapid scan. This feature makes it less sensitive than anodic stripping voltammetry (the technique most widely used for speciation purposes) to interferences of the wine matrix, namely, in the stripping step, due to $SO_2$, pigments, and surface activity (Scollary, 1997).

## EXPERIMENTAL PROCEDURES

All reagents used were of analytical grade. All of the solutions were prepared in deionized water with conductance <0.1 $\mu$S/cm. The material was previously decontaminated of trace metals, in 20% $HNO_3$ overnight, and thoroughly washed with deionized water before use. All of the procedures for the trace analysis were carried out in a clean laboratory with forced filtered air (HEPA filter), and the manipulations were performed in a laminar flux chamber.

**Wine Samples.** Eleven different Port wines supplied by the Port Wine Institute were used in the present study: 2 white wines (1 sweet and 1 extra-dry) and 9 red wines (4 blended wines with indication of ages of 10, 20, 30, and 40 years, 1 blended Tawny, 1 blended Ruby, 1 dated Port of 1941, and 2 very young red wines). After the bottles were opened, the wines were stored at 18 °C under inert atmosphere.

**Procedures.** The methodology used in the present study is summarized in Figure 1, and the details are given in the following sections.

*Determination of Pb and Cu by AAS-ET.* Total Pb concentrations in the wines were determined by atomic absorption spectroscopy (AAS) using a Perkin-Elmer (Norwalk, CT) 4100-ZL apparatus with electrothermal atomization (ET) and a Zeeman background correction system. The method of calibration curve against aqueous standards (in 1% $HNO_3$) was applied to the wines without any pretreatment, as described elsewhere (Simões et al., 1995). The accuracy of the method was checked by analyzing a BCR reference wine. The same equipment and procedure were used for the determination of Pb in the wine digests and dialysates as well as for the chromatographic fractions.

Total Cu concentrations in wines were determined by AAS with flame atomization on a Phillips Scientific (Cambridge, U.K.) PU 9200X instrument, using the method of calibration curve against aqueous standards (in 0.2% $HNO_3$), after 1:2 wine dilution. The accuracy of the method was checked as for Pb. The concentration of the metals in the wine digests and dialysates and chromatographic fractions was determined by ET-AAS operating with the manufacturer's standard conditions for Cu.

*Cu ISE Titrations of Wine.* For the study of affinity of Cu(II) ions toward the various wines, potentiometric titrations of wines with a 0.01 M Cu(II) standard solution were carried out, at 25 °C. The free Cu(II) concentration was measured with the respective ion selective electrode (ISE) from Radiometer (Lyon, France), activated with a mixture of $Ag_2S$ and CuS prepared as before (Lima and Machado, 1982). As reference electrode, an Orion Ag/AgCl(s) KCl 3M (double junction) was used. A computer-controlled arrangement comprising a decimillivoltimeter (Crison micro pH 2002, Alella, Barcelona, Spain) coupled to an automatic buret (Crison micro BU 2030) was used. The natural levels of Cu in the titrant, determined by AAS, were taken into consideration for the total metal concentration. Calibrations were performed in a 0.05 M $KNO_3$, pH 3.5, 20% ethanol (v/v) medium.

*Pb SWCV Titrations of Wine and Wine Digests.* The cathodic voltammetric measurements consisted of applying a successively more negative potential to the chemical system, the current generated at the surface of the working electrode being recorded. This technique measures only the labile Pb, that is, the fraction of the metal which is free in the solution plus that able to dissociate from a complex within the time scale of the technique.

Measurements were performed with an Autolab PSTAT10 system (Ecochemie, Utrecht, The Netherlands) connected to a 663 VA stand (Metrohm, Herisau, Switzerland). A conventional three-electrode arrangement, consisting of a glassy carbon electrode as a counter electrode, an Ag/AgCl(s), 3 M KCl electrode as reference electrode, and a Metrohm multimode mercury electrode, was used. The instrumental settings were as follows: initial potential, −0.2 V; final potential, −0.9 V; wave frequency, 50 Hz; pulse amplitude, 25 mV; step potential, 2.44 mV.

Wines and gastric digests were properly diluted with 0.05 M $KNO_3$, pH 3.5, 20% ethanol. Intestinal digests (filtered with

0.45 $\mu$m pore size and unfiltered) were properly diluted in water and buffered to pH 7 with 0.3 mL of a solution of 10% $NaHCO_3$ for an aliquot of 10 mL. As titrant, a 0.10 mM $Pb(NO_3)_2$ standard solution with the same background composition as that used to dilute the wines was used, covering a concentration range from 0 to 4 $\mu$M (for wines and gastric digests) or from 0 to 8 $\mu$M (for intestinal digests) of total Pb in the titrated solution. The dilution of wines and digests was necessary to reduce the matrix interference, to avoid the red pigment fouling in the electrochemical cell, and to work at low total Pb concentration levels (otherwise, between samples, deep decontamination measurements would have to be taken). The dilution may alter slightly the aggregation degree of the macromolecular compounds, thus increasing their complexation capacities. Moreover, the values of the average stability constants, which were the purpose of these measurements, also may slightly increase by dilution (Buffle and Altmann, 1987). However, it was the only way that rendered this study possible. The spiking of Pb during wine and gastric digests titrations had a negligible influence on the titrands' pH, it being experimentally verified that a maximum pH decrease of 0.2 unit occurred in the last points of the titrations. Therefore, the titrations were carried out without any addition of a pH buffer.

The sensitivity of the method may be very different in the synthetic medium (with 0.05 M $KNO_3$, 3.5 pH, 10% ethanol, or $NaHCO_3$ buffer) and in the diluted wines and digests, because of the presence of surface active compounds in the wines (polyphenols and other organic ligands) or digests (those existent in wines plus the added enzymes, especially the biliar salts). Therefore, an internal calibration procedure was carried out. The last titration points fit a linear segment, indicating that full saturation of the operationally inert ligands was achieved. The ligand saturation, that is, the absence of operationally inert ligands, implies that any posterior Pb increments in the titration correspond to labile Pb only. Thereby, the slope of that linear relationship provides the internal calibration curve.

*RP-HPLC of the Wines and Wine Digests.* In an attempt to get some information about the nature of the Pb and Cu ligands in Port wine, chromatographic experiments were carried out for three markedly different Port wines (sweet white, young red, and dated 1941, which were also the wines used for the digestion-related studies). The chromatographic conditions used are commonly utilized for separation of large molecules such as peptides, proteins, and tannins. The methodology consisted in detecting the sample components with UV detector, at a wavelength of 215 nm, with collection of chromatographic fractions corresponding to the different components (different retention times) for subsequent AAS-ET analysis. The sample collections were performed with the criteria of minimum sample volume necessary for AAS-ET analysis, collecting as close to the UV peak maximum as possible for the sake of the sensitivity and selectivity of the method. Nevertheless, the method could not provide quantitative information but gave an indication about the distribution of Pb and Cu among the organic components eluted at different retention times.

A Dionex (Sunnyvale, CA) ion chromatography system (model DX 300 IC) including a 50 or 100 $\mu$L loop, a Vydac (Hesperia, CA) $C_{18}$ 218TP54 column (5 $\mu$m, 250 mm $\times$ 4.6 mm) with the corresponding guard column coupled to a UV−visible Konic (Barcelona, Spain) UVIS 204 detector was used. A gradient elution, from 0 to 30% ethanol in 20 mM $KH_2PO_4$, in 90 min, at 0.7 mL/min flux rate, was used. The chromatographic fractions corresponding to peak appearance at 215 nm were collected to appropriate small tubes and analyzed for Pb and Cu within 24 h by ET-AAS.

*Simulation of Gastrointestinal Digestion.* The simulations took place in a Teflon reaction vessel constructed specifically for that purpose, in our laboratories. The reaction vessel was involved by a thin plastic compartment used to circulate water for thermostatization (at 37 °C) of the contents. As an adaptation of the procedure described by Crews et al. (1985), for various solid foods, 50 mL of wine was mixed with 25 mL of gastric juice (10 g/L pepsin, 0.15 M NaCl, 0.02 M HCl) and left with stirring for 3 h. It was necessary to ensure that in the course of reaction the pH of mixture would not rise above 3.5. It was verified by potentiometric monitoring with a glass pH electrode that, in fact, the pH did not achieve that value. After the 3 h reaction time, 12 mL of $NaHCO_3$ at 10% (to buffer the pH at 7.4), 12 mL of bile salts at 1.5 g/L in 0.15 M NaCl, and 12 mL of pancreatic juice, containing 30 g/L pancreatin and 10 g/L amylase, were added to the contents of the reaction vessel and left with stirring for 3 h. All of the enzymes were supplied by Sigma. Enzyme solutions were always freshly prepared. Blank simulations, with 50 mL of deionized water in the place of wine, were regularly carried out for contamination control.

*Determination of the Simulated Solubility of Cu and Pb Present in Digested Wines.* The potentially assimilable fractions of Cu and Pb present in the wine digests were determined as the soluble fractions. Either after the gastric phase or after the intestinal phase of the simulated digestions, two aliquots of 1 mL were collected, and one of the aliquots was filtered with a 0.45 $\mu$m membrane filter. The fraction of the total metals that remained soluble was determined directly by the ratio of electrothermal AAS signals (with blank subtracted) for filtered and unfiltered samples.

*Dialyzability of Pb and Cu in Simulated Intestinal Digests.* In these experiments, a modified gastrointestinal simulation procedure was used: the adjustment of the pH from stomachal to intestinal conditions was achieved gradually (2 mL of $NaHCO_3$ at 10% every 5 min during 30 min), which resembles more accurately the in vivo situation in kinetic terms. During the intestinal phase of the digestions, a 5 mL dialyzer (QuixSep, MFP Inc., San Antonio, TX) filled with a solution of 0.15 M NaCl, equipped with an H1 (heavy metal free) 15000 nominal MWCO membrane (MFP Inc.), was immersed in the vessel to determine the dialyzable fraction of the metals. At the end of the 3 h intestinal phase, the dialyzer was removed and the dialysate was analyzed for Cu and Pb contents by electrothermal AAS. Because the volume outside the dialyzer was much greater ($\sim$20 times) than the volume inside the dialyzer, the decrease of the outside metal concentrations due to dialysis is neglected, and so, the dialyzable fraction was simply determined as the ratio between outer and inner metal AAS signals (with blank subtracted).

**Data treatment of ISE and SWCV Titrations.** Wines and their digests have very complex matrixes, and there is a lack of information about the molar concentration sites (for both proton and metal ions) of various groups of compounds having unknown or poorly defined individual chemical structures (such as tannins, polysaccharides, peptides, or proteins and combinations of them). For this reason, as before (Vasconcelos et al., 1999; Azenha and Vasconcelos, 2000), metal complexation in wine has to be studied by methods developed specifically for systems that include heterogeneous ligands.

*Potentiometric Cu Data.* The total complexation capacity ($CC_{total}$) of the samples as well as mean values of conditional stability constants, $K_{av}$, were determined by Scatchard plot (Scatchard, 1949), assuming a 1:1 stoichiometry of the formed complexes, which is illustrated by the equation

$$[Cu]_{bound}/[Cu]_{free} = K_{av}(CC_{total}) - K_{av}[Cu]_{bound} \qquad (1)$$

When the experimental data fit the Scatchard model, the plot of $[Cu]_{bound}/[Cu]_{free}$ against $[Cu]_{bound}$ is approximately linear, $K_{av}$ being obtained from the slope and $CC_{total}$ from the intercept.

In some 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 (or more) different types of sites ($i = 1, 2, ...$) which have associated $K_{1av}$, $CC_1$, and $K_{2av}$, $CC_2$, etc. Obviously, $CC_{total}$ will be given by

$$CC_{total} = CC_1 + CC_2 + ... \qquad (2)$$

*Voltammetric Pb Data.* From the data of voltammetric titrations the conditional stability constants of the operation-

**Table 1. Total Pb and Cu Concentrations Observed in the Different Wines[a]**

| wine | [Pb] | | [Cu] | |
|---|---|---|---|---|
| | μg/L | μM | mg/L | μM |
| white wines | | | | |
| sweet | 111 (9) | 0.536 (0.043) | 0.33 (0.02) | 5.2 (0.3) |
| extra dry | 65 (6) | 0.31 (0.03) | 0.17 (0.02) | 2.7 (0.3) |
| red wines | | | | |
| young half-dry | 69 (5) | 0.33 (0.02) | 0.14 (0.02) | 2.1 (0.3) |
| young | 108 (7) | 0.521 (0.034) | 0.30 (0.03) | 4.6 (0.5) |
| Tawny | 40 (5) | 0.19 (0.02) | 0.086 (0.05) | 1.4 (0.8) |
| Ruby | 52 (6) | 0.25 (0.03) | 0.14 (0.02) | 2.1 (0.3) |
| 10 years | 107 (7) | 0.516 (0.034) | 0.22 (0.03) | 3.5 (0.5) |
| 20 years | 129 (7) | 0.623 (0.034) | 0.51 (0.04) | 7.9 (0.6)) |
| 30 years | 204 (8) | 0.985 (0.039) | 0.81 (0.05) | 12.8 (0.8) |
| 40 years | 453 (15) | 2.19 (0.07) | 1.58 (0.07) | 24.8 (1.1) |
| dated 1941 | 184 (9) | 0.888 (0.043) | 0.94 (0.05) | 14.7 (0.8) |
| BCR D (liquor) | 124 (9) | | 1.17 (0.05) | |
| certified value | 135 (40) | | 1.150 (0.020) | |

[a] Standard deviations are given in parentheses ($n = 3$).

ally inert complexes, $K'_{av}$, and the respective total concentration of the ligands, $CC_{inert}$, can be estimated by a modified form of eq 1

$$[Pb]_{inert}/[Pb]_{labile} = K'_{av}(CC_{inert}) - K'_{av}[Pb]_{inert} \qquad (3)$$

where $[Pb]_{inert} = [Pb]_{total} - [Pb]_{labile}$, $[Pb]_{labil}$ being the concentration of the metal measured by SWCV (see above) and $[Pb]_{inert}$ the concentration of Pb bound to ligands operationally inert.

$[Pb]_{labile}$ species comprise the concentration of the free metal ion ($[Pb]_{free}$) plus the concentration of all the metal species that are able to dissociate from the respective complexes during the measurement. As $[Pb]_{labile} > [Pb]_{free}$, the $K'_{av}$ is underestimated (the obtained values are lower than the real values). Correction to the real values, that is, conversion of $[Pb]_{labile}$ into $[Pb]_{free}$ is not possible because the concentrations and stability constants of the labile complexes in the wines are unknown. Nevertheless, these underestimated values were useful in the present work as they provided a comparative basis for the complexometric properties of different wines.

## RESULTS AND DISCUSSION

**Pb in Port Wines.** The levels of total Pb obtained for the studied wines are presented in Table 1 and ranged from 40 to 453 μg/L. In general, the oldest wines presented the higher Pb contents, some of them near or above the present OIV maximum limit, 200 μg/L, which was set in 1991 (OIV resolution 7, 1991) and is applicable only from that year on.

Information about the strength of the ligands that form operationally inert complexes with Pb (the strongest ones) existent in Port wines was obtained by SWCV and is shown in Table 2. The values of the concentration of the strongest ligands ($CC_{inert}$) obtained for the wines were very similar, ranging from 5 to 11 μM. However, it was observed that the lowest values, 5 μM, were obtained for the white wine and for the oldest red wine (dated 1941), and the higher value, 11 μM, was obtained for the youngest wine (young red). Red wines are expected to display a higher $CC_{inert}$ owing to their richer matrix in organic ligands (e.g., higher polyphenol and polysaccharide contents), but it is well-known [e.g., Arcos et al. (1993)] that aging causes a decrease of the contents of organic matter in wines either by precipitation or by oxidation processes. This is compatible with the fact that the highest $CC_{inert}$ value (11 μM) has been found in the youngest red Port wine studied and the lowest (5 μM) in the oldest Port wine. Much higher $CC_{inert}$ values for red wines (16 and 28 μM) than for white wines (3−8 μM) have been found for table wines (Azenha and Vasconcelos, 2000). The differences observed among red and white Port wines were not so pronounced as those found before in table wines.

The values of the logarithm of the average conditional stability constants (log $K'_{av}$) of the stronger Pb com-

**Table 2. Average Conditional Stability Constants ($K'_{av}$) of Pb Complexes in Port Wine and Its Digests and Concentrations of the Respective Ligands[a]**

| sample | range [Pb]/μM[b] | $K'_{1av}/M^{-1}$ | log $K'_{1av}$ | $CC_{1inert}$/μM | $CC_{inert}$(total)/μM |
|---|---|---|---|---|---|
| white wines | | | | | |
| sweet | | | | | |
| untreated | 4.5−11 | $1.9\ (0.1) \times 10^8$ | 8.3 | 5 (1) | 5 (1) |
| GD | 5.2−18 | $5.6\ (1) \times 10^7$ | 7.7 | 5 (1) | 5 (1) |
| ID-UF | 1.7−40 | $3.9\ (0.7) \times 10^6$ | 6.6 | 30 (4) | 30 (4) |
| ID-F | 1.2−57 | $3.3\ (0.8) \times 10^6$ | 6.5 | 35 (7) | 35 (7) |
| extra dry[c] | 3.5−10 | $1.8\ (0.6) \times 10^8$ | 8.3 | 4 (2) | 5 (1) |
| red wines | | | | | |
| young half-dry[c] | 7.0−20 | $6.2\ (0.2) \times 10^8$ | 8.8 | 7 (3) | 9 (3) |
| young | | | | | |
| untreated[c] | 8.5−22 | $8.9\ (0.2) \times 10^8$ | 8.9 | 10 (3) | 11 (2) |
| GD | 7.5−18 | $6.4\ (0.2) \times 10^7$ | 7.8 | 8 (2) | 8 (2) |
| ID-UF | 2.5−50 | $4.1\ (1) \times 10^5$ | 6.6 | 45 (11) | 45 (11) |
| ID-F | 2.0−43 | $4.4\ (2) \times 10^5$ | 6.6 | 31 (10) | 31 (10) |
| Tawny | 7.0−22 | $7.5\ (1) \times 10^7$ | 7.9 | 8 (2) | 8 (2) |
| Ruby | 7.0−20 | $7.8\ (0.8) \times 10^7$ | 7.9 | 10 (1) | 10 (1) |
| 10 years[c] | 6.2−25 | $2.9\ (0.3) \times 10^8$ | 8.4 | 8 (3) | 10 (5) |
| 20 years | 6.2−22 | $6.4\ (0.6) \times 10^7$ | 7.8 | 7 (1) | 7 (1) |
| 30 years[c] | 7.0−24 | $1.3\ (0.1) \times 10^8$ | 8.1 | 8 (1) | 10 (2) |
| 40 years[c] | 5.0−20 | $1.5\ (0.2) \times 10^8$ | 8.2 | 6 (1) | 8 (2) |
| dated 1941 | | | | | |
| untreated | 1.0−13 | $3.1\ (0.3) \times 10^7$ | 7.5 | 5 (1) | 5 (1) |
| GD | 1.3−10 | $2.0\ (0.4) \times 10^7$ | 7.3 | 5 (1) | 5 (1) |
| ID-UF | 2.4−50 | $9.4\ (0.3) \times 10^5$ | 6 | 25 (8) | 25 (8) |
| ID-F | 2.4−55 | $3.0\ (0.2) \times 10^6$ | 6.3 | 29 (3) | 29 (3) |

[a] Standard deviations are given in parentheses ($n = 3$). [b] Range of Pb concentration covered by the titration; different dilutions were applied, so the ranges were normalized to correspond to those that would be covered in the respective undiluted samples. [c] Samples where the Scatchard plot gave two linear zones; log $K'_{2av} = 7.6$, extra dry white; 7.8, young half-dry red; 8.2, young red; 7.2, 10 years; 7.2, 30 years; 7.1, 40 years. $CC_{2inert} = CC_{inert}(total) - CC_{1inert}$. GD, gastric digest; ID-UF, intestinal digest unfiltered; ID-F, intestinal digest filtered.

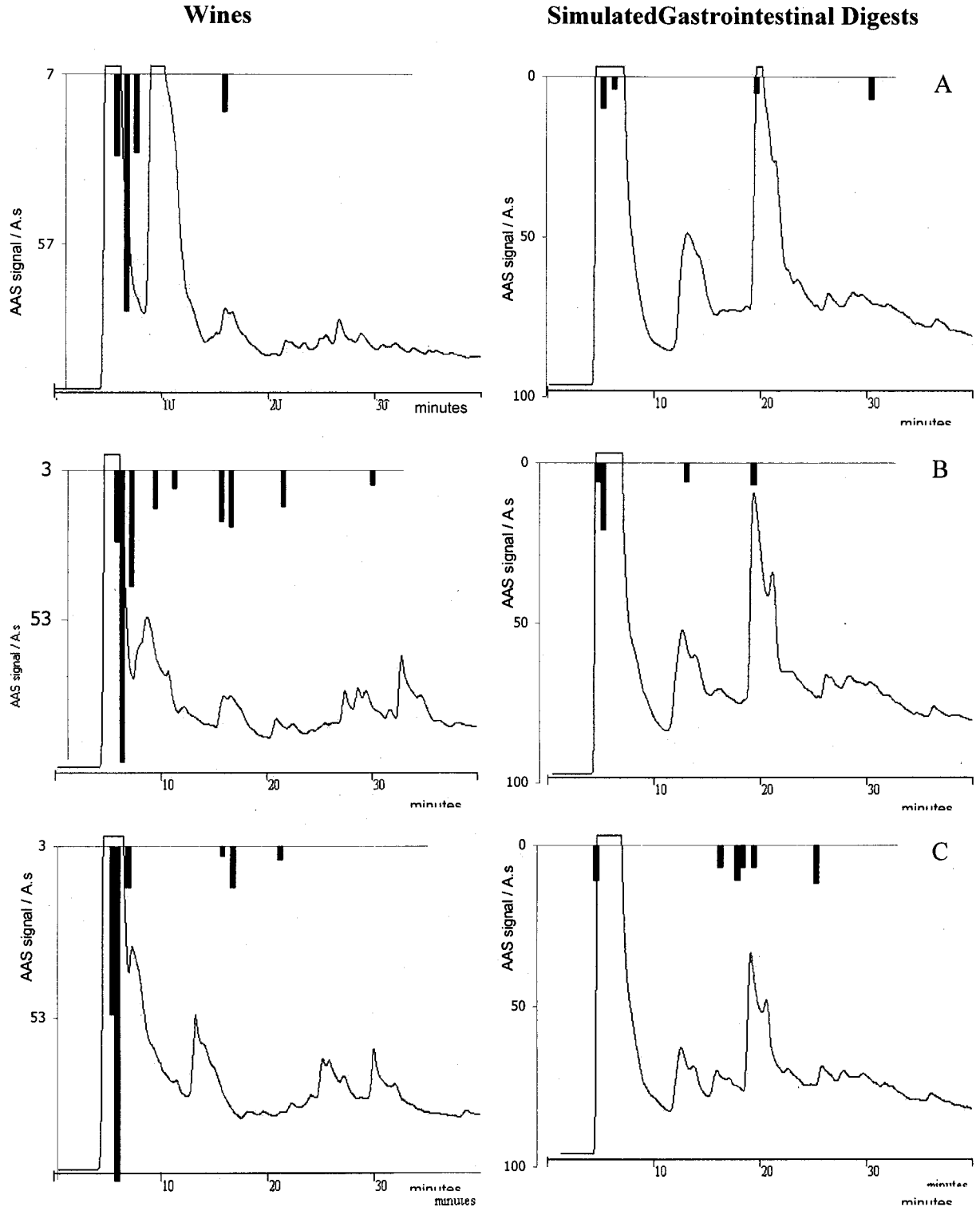


**Figure 2.** RP-HPLC chromatograms with UV and Pb-AAS detection for wines and its in vitro gastrointestinal digests: (A) sweet white Port; (B) young red Port; (C) 1941 Port. The *y* scale applies to the vertical bars, which represent the atomic absorption signal. Chromatographic conditions: Vydac $C_{18}$ 218TP54 column, UV detection (215 nm), 90 min gradient elution (0−30% ethanol in 20 mM $KH_2PO_4$).

plexes ranged between 7.5 and 8.9. The highest values (8.8−8.9) were observed in the young red wines, and the lowest value corresponded to the oldest wine. All of the other wines displayed similar values (between 7.8 and 8.4). Therefore, the process of aging seems to result in a weakening of the Pb ligands present in the wine in a soluble form (that is, not deposited at the bottom of the respective containers). This information is compatible with the higher voltammetric lability, observed for older table wines by Arcos et al. (1993).

On the other hand, the values of $CC_{inert}$ obtained for the wines (5−11 $\mu$M) are greater than the total Pb concentration existent in the wines (0.19−2.19 $\mu$M). Such an excess of ligands, associated with the high stability of the Pb complexes ($K'_{av}$), is compatible with the very low lability of Pb in table wines reported in

the literature (Green et al., 1997; Arcos et al., 1993; Marin and Ostapczuck, 1992).

Typical elution profiles of Pb under RP-HPLC conditions are illustrated in Figure 2 for three very different Port wines selected to undergo these experiments: the sweet white, the young red, and the red dated 1941. Although the procedure did not provide quantitative data, some useful information was obtained, especially for comparison with the experiments with the intestinal digests (see below). It can be observed for all cases that the majority of the Pb was eluted closely after the void volume, which corresponds to 5 min of retention time. Such a low retention indicates that the Pb is mainly associated with compounds that have relatively low molecular weight and/or high ionic character. Other Pb-containing substances, with higher molecular weight and/or apolar character (later eluted), appeared in all cases, although a greater distribution of Pb species along the chromatogram was found for the Port dated 1941, with retention times between 7 and 30 min. Similar results were also obtained before for the table wines (Azenha and Vasconcelos, 2000).

According to Szpunar et al. (1998), the Pb in wines is mainly complexed by the dimer of a pectic polysaccharide, rhamnogalacturonan II (RG-II), which has a high number of negatively charged glycosyl residues. Therefore, these ionized complexes may belong to the group of substances eluted closely to the void volume, despite the relatively high molecular weight of the pectic dimer, ~10 kDa. This information is compatible with the high fraction of Pb that was eluted closely to the void volume in the present work.

On the other hand, studies carried out by McKinnon and Scollary (1997) demonstrated that the majority of Pb is found in complexes with molecular weight >30 kDa, the condensed tannins being pointed out as the most probable ligands for Pb. Coordination of Pb by tannins is compatible with the appearance of the metal with retention times of 10−30 min. However, when identification of the nature of the compounds eluted in those groups was attempted by applying tests of color development for tannin, polysaccharide, or protein fragments, the results were systematically negative, probably because the concentration of the compounds was below the detection limit of the methods, which are not very sensitive.

**Pb in Simulated Gastric Digests.** The fractions of total Pb that remained soluble after in vitro gastric digestion are shown in Figure 3a. This figure shows that for the three wines studied, almost all of the Pb (between 95 and 99%) remained in soluble forms after the gastric digestion and therefore potentially assimilable.

Information about the complexation properties of the gastric digests is presented in Table 2. The values of $CC_{inert}$ were of the same order of magnitude for the three different digested wines (5−8 $\mu$M) and were very close to those observed for the respective wines. The values of $K'_{av}$ were also similar for the different wines (7.3−7.8) and lower than those observed for the untreated wines. The decrease was more marked for the young red wine.

The profiles of Pb chromatographic elution in the gastric digests (data not shown) were similar to those observed for the wines (before digestion).

The different studies carried out in the present work demonstrated that the influence of the gastric phase of

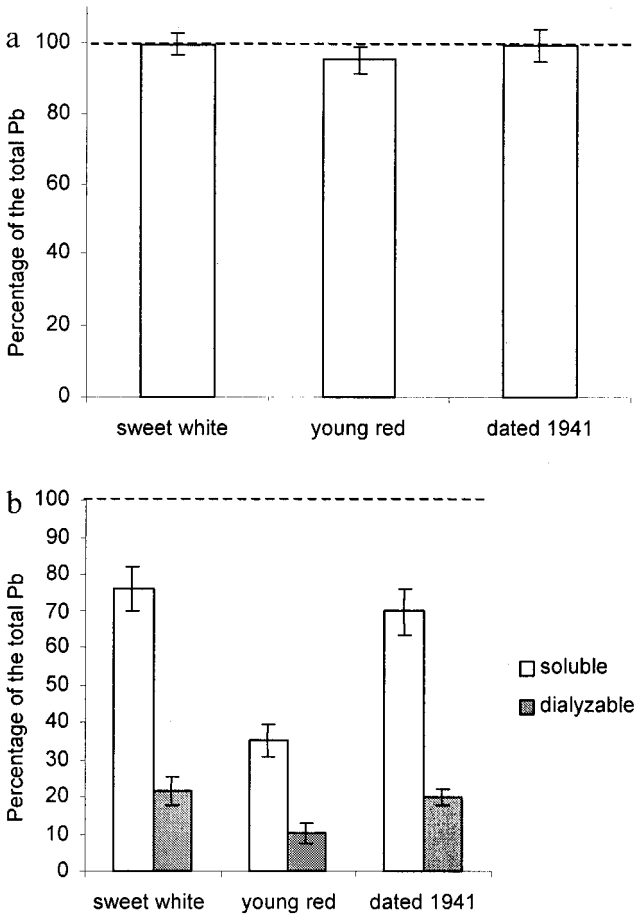


**Figure 3.** Percentage of total Pb that was in soluble forms after (a) gastric and (b) intestinal in vitro digestions of different wines. Dialyzable Pb fractions during intestinal digestion are also shown. Error bars represent the standard deviation of independent replicates ($n = 3$).

digestion on the overall digestion was very limited. Indeed, the percentage of the total metal present in a soluble form after the gastric digestion, the values of both $K'_{av}$ and $CC_{inert}$, and the chromatographic profiles of Pb elution were all similar to those observed for the wines, except for the young red wine. Such results are quite expected, because the pH at the end of the gastric digestion was between 3.0 and 3.3, thus not introducing great changes relative to the pH of the wines (3−4). Moreover, the gastric enzyme, pepsin, hydrolyzes proteins only, which occur in low concentrations in wines. Therefore, it is understandable that wine compounds and Pb speciation remain practically unaltered after gastric digestion.

**Pb in Simulated Intestinal Digests.** The percentages of total Pb in soluble forms after intestinal digestion are shown in Figure 3b. For the Port dated 1941 and the sweet white Ports a high percentage of the Pb (70−76%) remained in soluble forms. For the young red Port, after the intestinal digestion, the soluble Pb dropped to 35% of the total metal. As the Pb in insoluble forms is not bioavailable, these results indicate that the potential assimilability of the Pb present in the young red wine is much lower than the metal assimilability for the other two wines. Therefore, the process of in vitro digestion, which has been always regarded as an element-releasing process for the solid foods [e.g., Crews et al. (1985, 1996) and Walter et al. (1998)], led in the case of wines to a partial insolubilization of Pb, par-

**Table 3. Average Conditional Stability Constants ($K_{av}$) of Cu Complexes in Port Wine and Concentration of the Respective Ligands[a]**

| | stronger sites | | | weaker sites | | | |
|---|---|---|---|---|---|---|---|
| sample | $CC_1$/mM | $\theta$ range | log $K_{1av}$ | $CC_2$/mM | $\theta$ range | log $K_{2av}$ | $CC_{total}$/m$M$ |
| white wines | | | | | | | |
| sweet | 0.3 (<0.1) | 0.011−0.060 | 5.1 | 3.2 (0.6) | 0.429−0.686 | 3.9 | 3.5 (0.6) |
| extra dry | 0.5 (0.1) | 0.020−0.105 | 4.1 | 3.3 (0.5) | 0.410−0.641 | 3.5 | 3.9 (0.6) |
| red wines | | | | | | | |
| half-dry young | 0.2 (<0.1) | 0.0055−0.024 | 5.8 | 3.6 (0.5) | 0.447−0.710 | 4.0 | 3.8 (0.5) |
| young | 0.2 (<0.1) | 0.0068−0.025 | 6.0 | 3.6 (0.5) | 0.500−0.710 | 4.1 | 3.8 (0.5) |
| Tawny | 0.2 (<0.1) | 0.0078−0.026 | 6.1 | 3.5 (0.6) | 0.432−0.729 | 4.2 | 3.7 (0.6) |
| Ruby | 0.2 (<0.1) | 0.0057−0.022 | 5.7 | 3.5 (0.4) | 0.460−0.730 | 3.9 | 3.7 (0.4) |
| 10 years | 0.2 (<0.1) | 0.0091−0.030 | 5.5 | 2.9 (0.4) | 0.437−0.719 | 3.7 | 3.1 (0.4) |
| 20 years | 0.1 (<0.1) | 0.0083−0.023 | 6.9 | 3.4 (0.5) | 0.429−0.743 | 3.9 | 3.5 (0.4) |
| 30 years | 0.2 (<0.1) | 0.010−0.030 | 6.4 | 3.5 (0.7) | 0.460−0.730 | 4.0 | 3.7 (0.7) |
| 40 years | 0.4 (<0.1) | 0.019−0.050 | 5.5 | 3.4 (0.6) | 0.500−0.710 | 3.9 | 3.8 (0.6) |
| dated 1941 | 0.2 (<0.1) | 0.012−0.031 | 6.2 | 3.6 (0.7) | 0.513−0.667 | 3.9 | 3.8 (0.7) |

[a] Standard deviations are given in parentheses ($n = 3$).

ticularly in the case of the young red Port. The concentration of condensed tannins in young red wines is much higher than in white wines. These polyphenolic compounds are considered to be an antinutritional factor (Boisen and Eggum, 1991) because they depress nutrient digestibility. It is also known that the tannins inhibit the bioavailability of Pb from foods in mice (Peaslee and Einhellig, 1977). Therefore, the higher level of tannins found in red wines is compatible with differences of solubility of Pb observed in this work.

Figure 3b also shows that the dialyzed fraction of Pb in the three wines ranges between 10 and 22% of the total Pb, which is much lower than that in the total soluble fractions, especially for the sweet white and 1941 Ports. The results indicates that not all of the Pb in soluble species may be bioavailable.

The values of $K'_{av}$ and $CC_{inert}$ obtained either for the whole unfiltered intestinal digest or for the soluble fraction of the digest are presented in Table 2. For the whole digests log $K'_{av}$ values between 6.0 and 6.6 and $CC_{inert}$ values between 25 and 45 $\mu$M were obtained and were quite similar to those observed for the soluble digest log $K'_{av}$ values between 6.3 and 6.6 and $CC_{inert}$ values between 29 and 35 $\mu$M.

The intestinal conditions caused changes in the $K'_{av}$ values relative to those obtained for wines and gastric digests. Considering the whole digests, a significant decrease (~1 order of magnitude) was observed for all wines. A decrease of $K'_{av}$ values after gastrointestinal digestion, despite the great increase of pH, from 3 to 7.4, indicates that the ligands in the digests are weaker than those in wines. For instance, the carboxylic groups, very common in organic ligands, would be deprotonated at pH 7, which favored the metal complexation. In addition, at pH 7.4, a fraction of Pb may be as soluble or insoluble hydroxo- or tartrate complexes. For the filtered intestinal digests, $K'_{av}$ values similar to those of the unfiltered digests were verified, indicating that the soluble and the insoluble Pb ligands have approximate strengths.

The $CC_{inert}$ values obtained for the intestinal digests were much greater than those obtained for untreated wines and gastric digests, which is related with the deprotonation of organic ligands, eventual hydroxide complex formation, and catabolic activity of the enzymes, which may have fragmentized the macromolecules. For the unfiltered intestinal digest of the young red wine, a much higher $CC_{inert}$ value (45 $\mu$M) was obtained as compared with that obtained for the respective filtered intestinal digest (30 $\mu$M). Although this situation would suggest that a considerable fraction of the Pb ligands is in the insoluble form, we cannot state it because of the high standard deviation values associated with these $CC_{inert}$ values. For the other two cases, similar $CC_{inert}$ values were obtained for the unfiltered and filtered intestinal digests.

Figure 2 illustrates also the typical elution profiles of Pb in the intestinal digests obtained for the three wines. Different from what was observed for the wines and the gastric digests, for the intestinal digests only a small amount of Pb was found in the groups eluted for 5−7 min of retention time. For higher retention times the distribution of the Pb species along the chromatogram also changes very much from the wine to the gastrointestinal digests. Particularly, in the digests it was found that Pb species are associated with compounds with much higher retention times.

The results indicated that the digestion caused a redistribution of the forms of Pb. They also suggest that the ionic and/or low molecular weight forms are those most prone to ligand exchange or insolubilization during digestion.

**Cu in Wines.** The levels of Cu found in the studied wines, presented in Table 1, ranged between 1.4 and 25 $\mu$M, the higher concentrations being observed for the older wines.

Unfortunately, the SWCV method could not be applied for Cu because abnormal peaks were observed. Instead, ISE potentiometry was used, providing the mean values of the conditional stability constants of all the complexes present in the wines ($K_{av}$), not only that of the strongest ($K'_{av}$) as was obtained for Pb. The results (Table 3) indicate that two types of complexation sites, with significantly different strengths ($K_{av1}$ and $K_{av2}$), could be distinguished in all of the studied wines. The concentration of the sites associated with $K_{av1}$, $CC_1$, ranged from 0.1 to 0.5 mM, the higher values being found for the white Ports, 0.3 (sweet white) and 0.5 mM (extra dry white). The remainder displayed lower $CC_1$ values, except the 40 year Port with 0.4 mM.

However, the white Ports displayed the lowest log $K_{1av}$ values (4.1 and 5.1), whereas for the other Ports log $K_{1av}$ ranged from 5.5 to 6.9. No relation between age or wine type and log $K_{1av}$ was found. The values of $CC_2$ (concentration of the sites associated with $K_{2av}$) were similar for all of the wines (2.9−3.6 mM) and much higher than $CC_1$. The values of log $K_{2av}$ were also very similar for all of the wines (3.5−4.2) and 2 orders of magnitude

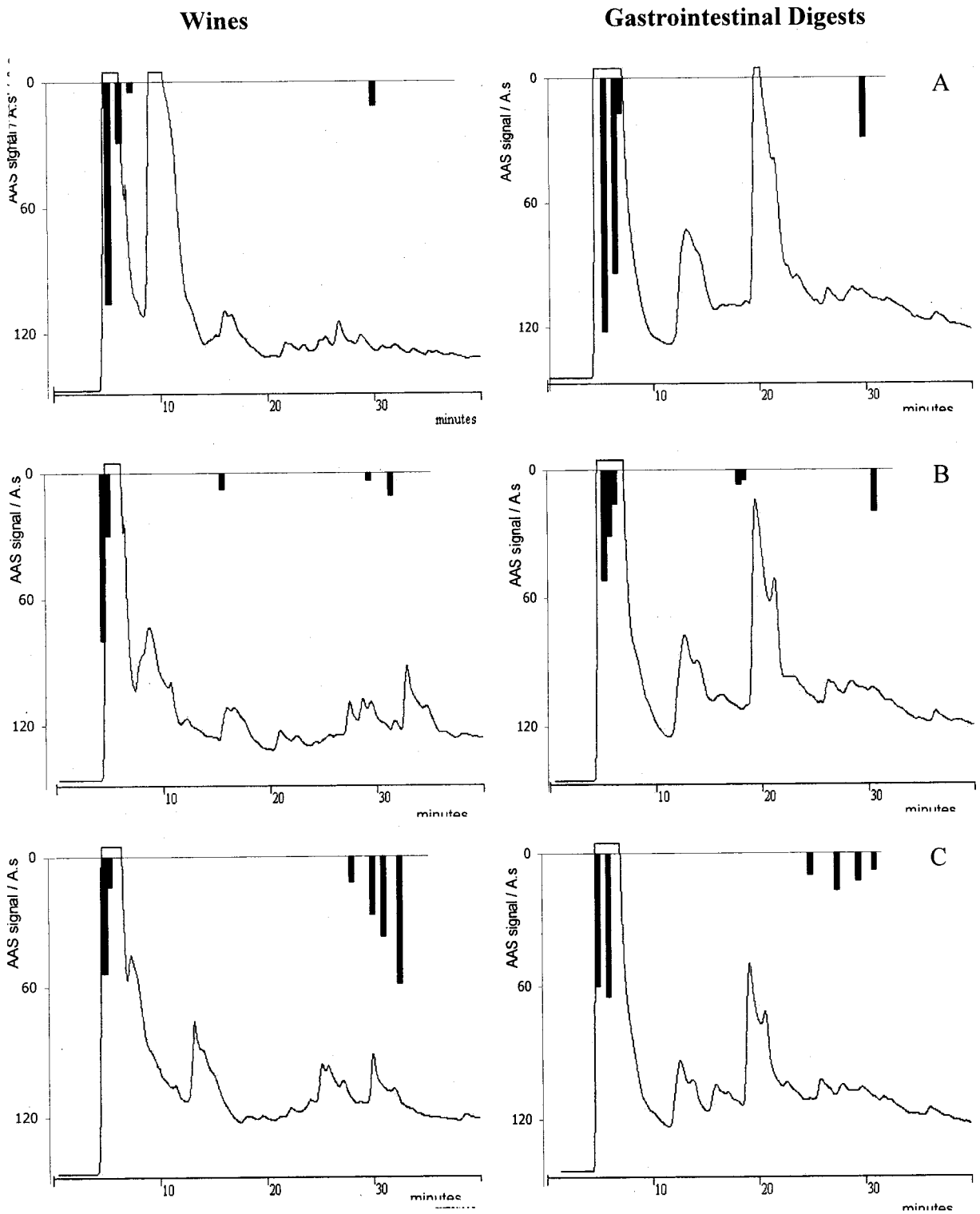


**Figure 4.** RP-HPLC chromatograms with UV and Cu-AAS detection for wines and its in vitro gastrointestinal digests: (A) sweet white Port; (B) young red Port; (C) 1941 Port. The *y* scale applies to the vertical bars, which represent the atomic absorption signal. Chromatographic conditions: Vydac $C_{18}$ 218TP54 column, UV detection (215 nm), 90 min gradient elution (0−30% ethanol in 20 mM $KH_2PO_4$).

lower than log $K_{av1}$. In every case, the values of $CC_1$ were >20 times higher than the total Cu concentration in the respective Port wine, which allows the conclusion that the Cu in wines is practically all complexed by the stronger sites.

The profile of the chromatographic bands (Figure 4) for the young red and sweet white Ports showed that the Cu was mainly associated with compounds eluted closely to the void volume. Minor Cu signals were detected in the fraction with retention times of 26 min (sweet white wine) and 16, 29, and 32 min (young red wine). For the Port dated 1941, similar Cu signals were found for the fractions eluted closely to the void volume and for those highly retained, eluted around 30 min. In different table wines studied before using the same experimental conditions (Azenha and Vasconcelos, 2000), all of the Cu was eluted close to the void volume. Therefore, in the Port wines the Cu seems to be present

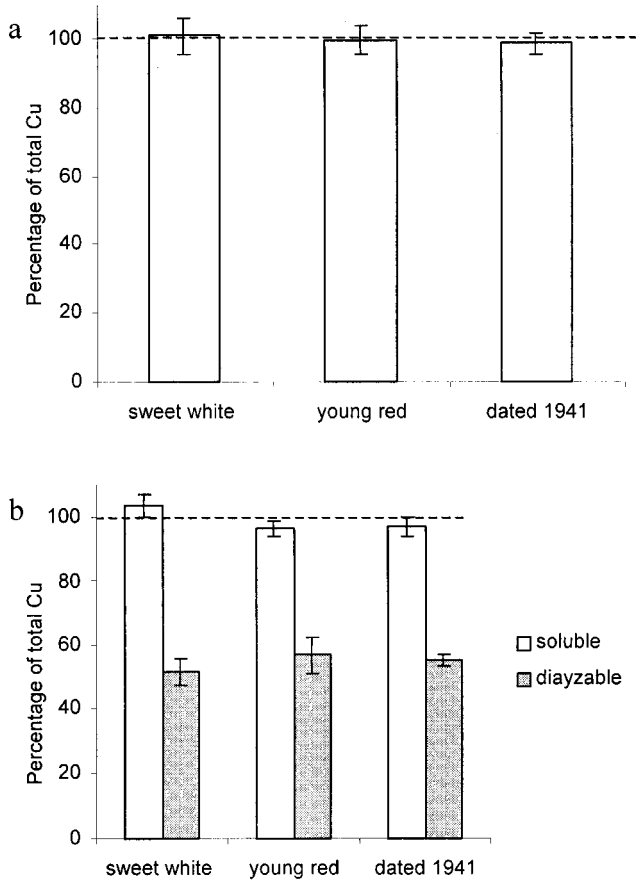


**Figure 5.** Percentage of the total Cu that was in soluble forms after (a) gastric and (b) intestinal in vitro digestions of the different wines. Dialyzable Cu fractions during intestinal digestion are also shown. Error bars represent the standard deviation of independent replicates ($n = 3$).

in forms of higher molecular weight/apolar character than in the table wines.

**Cu in Simulated Gastric Digests.** Figure 5a shows that after the gastric digestion practically all of the Cu remained soluble.

The chromatographic profiles of Cu elution after gastric digestion were similar to those obtained for the wines (data not shown). Therefore, the gastric digestion did not cause any apparent change in these two studied parameters, in the same way as occurred for Pb.

**Cu in Simulated Intestinal Digests.** At the first points of the potentiometric titrations of the intestinal digests with a Cu solution occurred the formation of a precipitate that prevented the determination of both $K_{av}$ and CC values for these digests.

After the intestinal digestion, practically all of the Cu from all of the wines remained in soluble forms (see Figure 5b). However, the Cu in the respective dialyzed fraction was considerably lower in all cases, ~50% of the total metal. This indicates that the kinetics and molecular size of the ligands play an important role in the bioavailability of Cu in wines, as was also observed for Pb. These results were different from those observed before (Azenha and Vasconcelos, 2000) for table wines, in which the dialyzable fraction of Cu was higher for white wines than for a red wine, and similar among the wines of identical color classification.

The chromatographic profiles of Cu elution in the intestinal digests are shown in Figure 4. The elution of Cu occurred at similar retention times as it did in the wines and gastric digests. These results suggest that the type of compounds that bind Cu did not change markedly during the digestion, contrarily to what occurred for Pb in some wines. The Cu fraction eluted close to the void volume was similar to those observed for the wines and its gastrointestinal digests, in opposition to that observed for Pb.

In summary, this work has shown the influence of the speciation at the gastrointestinal level to the final assimilability and bioavailability of an element. Concretely, it was found that a fraction of the Pb was rendered insoluble at simulated intestinal conditions, contrarily to the conventional viewpoint that digestion generally increases mineral availability. Even though the Pb assimilability indices are low, the oldest Port wines have high Pb contents, which may have some bearing on Pb biovailability. The in vitro digestion apparently had slight effects on Cu bioavailability because it remained mostly soluble, and a moderate ( ~50%) assimilability index was found.

## ACKNOWLEDGMENT

We acknowledge the Institute of Port Wine for supplying the Port wines and for a careful reading of this paper by Dr. Paulo Barros; we also acknowledge Dr. Bernard Medina for providing the BCR reference wines and Dr. Victor de Freitas for providing the HPLC column.

## LITERATURE CITED

Arcos, M.; Ancin, M.; Echeverria, J.; Gonzalez, A.; Garrido, J. Study of lability of heavy metals in wines with different degrees of aging through differential pulse anodic stripping voltammetry. *J. Agric. Food Chem.* **1993**, *41*, 2333−2339.

Azenha M.; Vasconcelos, M. Assessement of the in vitro Pb and Cu availability in wines by means of speciation procedures. *Food Chem. Toxicol.* **2000**, in press.

Boisen, S.; Eggum, B. Critical evaluation of in vitro methods for estimating digestibility in simple-stomach animals. *Nutr. Res. Rev.* **1991**, *41*, 141−162.

Buffle, J. Interpretation of complexation equilibria. In *Complexation Reactions in Aquatic Systems: An Analytical Approach*; Ellis Horwood: Chichester, U.K., 1988; pp 195−303.

Buffle, J.; Altmann, S. Interpretation of metal complexation by heterogeneous complexants. In *Aquatic Surface Chemistry*; Stumm, W., Ed.; Wiley: New York, 1987; Chapter 13.

Crews, H.; Burrell, J.; McWeeny, D. Trace element solubility from food following enzymolysis. *Z. Lebensm. Unters. Forsch.* **1985**, *180*, 221−226.

Crews, H.; Clarke, P.; Lewis, J.; Owen, L.; Strutt, P. Investigation of selenium speciation in *in vitro* gastrointestinal extracts of cooked cod by high-performance liquid chromatography-inductively coupled plasma mass spectrometry and electrospray mass spectrometry. *J. Anal. At. Spectrom.* **1996**, *11*, 1177−1182.

Green, A.; Clark, A.; Scollary, G. Determination of free and total copper and lead by stripping voltammetry. *Fresenius' J. Anal. Chem.* **1997**, *358*, 711−717.

Hocquellet, P. Speciation et nutrition, l'approche enzymologique. *Analusis* **1997**, *25*, m25−m27.

Lima, J.; Machado, A. In *Analytical Techniques in Environmental Chemistry*; Albarges, D., Ed.; Pergamon: Oxford, U.K., 1982; Vol. 2, p 419.

Marin, C.; Ostapczuc, P. Lead determination in wine by potentiometric stripping analysis. *Fresenius' J. Anal. Chem.* **1992**, *343*, 881−886.

McKinnon, A.; Scollary, G. Size fractionation of metals in wine using ultrafiltration. *Talanta* **1997**, *44*, 1649−1658.

Peaslee, M.; Einhellig, F. Protective effect of tannic acid in mice receiving dietary lead. *Experientia* **1977**, *33*, 1206.

Scatchard, G. The attractions of proteins for small molecules and ions. *Ann. N. Y. Acad. Sci.* **1949**, *51*, 660−672.

Scollary, G. Metals in wine: contamination, spoilage and toxicity. *Analusis* **1997**, *25* (3), M26−M30.

Simões, M.; Moreira, M.; Granhão, S.; Simões, J. Dosage du plomb dans les vins de liqueur. *Feuillet Vert O.I.V.* **1995**, 1003.

Szpunar, J.; Pellerin, P.; Makarov, A.; Doco, T.; Williams, P.; Medina, B.; Lobinski, R. Speciation analysis for biomolecular complexes of lead in wine by size-exclusion high performance liquid chromatography-inductively coupled plasma mass spectrometry. *J. Anal. At. Spectrom*. **1998**, *13*, 749−754.

Vasconcelos, M.; Azenha, M.; de Freitas, V. Electrochemical studies of complexation of Pb in red wines. *Analyst* **2000**, *125*, 743−748.

Walter, A.; Rimbach, G.; Most, E.; Pallauf, J. Effect of citric acid supplements to a maize-soya diet on the in vitro availability of minerals, trace elements, and heavy metals. *J. Vet. Med. A* **1998**, *45*, 517−524.

Wiese, C.; Schwedt, G. Strategy for copper speciation in white wine by differential pulse anodic stripping voltammetry, potentiometry with an ion-selective electrode and kinetic photometric determination. *Fresenius' J. Anal. Chem*. **1997**, *358*, 718−722.

Received for review October 25, 1999. Revised manuscript received July 13, 2000. Accepted July 19, 2000. We thank JNICT/Programa Ciência (Project 27/M/90) for supporting equipment acquisition. M.A. thanks the Portuguese Foundation for Science and Technology (Lisbon) for a Ph.D. scholarship (BD/5511/95).

JF991167I