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SPECIATION AND BIOAVAILABILITY OF Pb AND Cu IN WINES

INFLUENCE OF Cu ON THE KINETICS OF FERMENTATION BY *SACCHAROMYCES cerevisiae*

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À Migu e ao Dioguinho

À nossa família.

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RESUMO

Estudou-se a influência da concentração de Cu no processo de fermentação alcoólica, nomeadamente no que respeita a fermentação de mostos. A fermentação alcoólica foi estudada num amplo intervalo de concentrações de Cu (desde níveis naturais até 1.0 mM), em dois meios de fermentação diferentes: mostos brancos (que constituem matrizes altamente complexas, contendo diversos ligandos de Cu) e "yeast nitrogen base" (YNB) com glucose. No meio YNB, a produção de etanol e a taxa de fermentação foram altamente influenciadas pela concentração de Cu, sendo que na presença de 0.50 e 1.0 mM Cu a produção de etanol foi dupla da verificada na ausência de Cu adicionado. No entanto, a cinética de fermentação foi mais lenta. Na produção de etanol a partir dos mostos apenas se verificaram efeitos ligeiros com a variação da concentração de Cu, o que significa que o teor de Cu não é responsável por alterações à fermentação, e por isso, encontra-se abaixo de níveis tóxicos relativamente ao desempenho das leveduras. Dum ponto de vista práctico-biotecnológico, os resultados sugerem o interesse em efectuar trabalho futuro sobre a influência do Cu nos meios usados na indústria de produção de etanol, usando a *S. cerevisiae* ou outras células. A explicação bioquímica para o fenómeno observado é um tópico de investigação que fica em aberto, sendo possível que exista uma ligação entre o efeito do Cu e a disponibilidade de Fe, uma vez que o Cu é necessário para o transporte de Fe para o interior das leveduras.

Estudaram-se por potenciometria as propriedades complexométricas para Pb e Cu de quatro vinhos tintos diferentes, produzidos a partir de apenas uma das seguintes castas cultivadas na Região Demarcada do Douro: Tinta Barroca (TB), Tinta Roriz (TR), Touriga Nacional (TN) e Touriga Francesa (TF). Paralelamente estudou-se as propriedades complexométricas dos polifenóis isolados das graínhas (taninos condensados) e das películas (maioritariamente antocianas) duma mistura de uvas das quatro castas, e os resultados foram comparados com os dos vinhos. As propriedades de complexação foram expressas em termos de (a) máxima capacidade de complexação (CC_{total}) dos ligandos dos vinhos, isto é, a concentração total de locais de ligação disponíveis na amostra, e (b) constantes condicionais de estabilidade dos complexos de Pb e Cu (K_{av}). Neste sentido, usou-se o modelo de Scatchard e da Função Diferencial de Equilíbrio, que são considerados adequados à caracterização de complexos envolvendo ligandos heterogéneos. Para Cu este estudo foi alargado a diferentes tipos de vinho do Porto (branco, tawny, ruby, data de colheita, tinto jovem, com indicação de idade).

Para Pb foi possível estudar por voltametria catódica parâmetros adicionais de complexação: a capacidade de formar complexos inertes (CC_{inert}) e uma estimativa das constantes condicionais de estabilidade dos complexos inertes (K'_{av}), os quais foram também determinados para vinhos de mesa (tinto, branco, Verde branco e Verde tinto) e para os vinhos do Porto referidos para o Cu.

O estudo mostrou que os taninos e os extractos da película ligam o Cu. No entanto a existência de outros ligandos abundantes tornou-se evidente por comparação dos parâmetros de complexação determinados para os extractos e para os vinhos. Inferiu-se que o Cu estava fortemente complexado nestes vinhos ($\log K_{av} > 6$). Para os vinhos do Porto tintos também se inferiu um estado fortemente complexado para o Cu ($\log K_{av} > 5.5$). Para o Pb, apesar de algumas limitações envolvidas, a voltametria proporcionou informação mais útil acerca do estado de complexação do Pb nos vinhos (K'_{av} e CC_{inert}) uma vez que esta informação diz respeito aos ligandos mais fortes, que na realidade ligam os metais aos níveis naturais encontrados nos vinhos. Uma vez que os valores de $\log \theta$ (grau de ocupação dos locais de complexação) nos vinhos são menores do que os valores abrangidos nas experiências voltamétricas, o Pb está fortemente complexado nos vinhos ($\log K'_{av} > 7.2$). Estudos paralelos dos taninos isolados e extractos da película mostraram que nos últimos existem ligandos mais fortes que os taninos, provavelmente não antociânicos. Foi mostrado que os polifenóis por si só não justificam a forte complexação do Pb observada nos vinhos. Os valores de K'_{av} obtidos para os diferentes vinhos de mesa foram todos similares variando entre 6.5 e 7.8 e indicaram também uma forte complexação. A CC_{inert} dos vinhos tintos (16-28 μM) foi muito maior que a dos vinhos brancos (3-8 μM). No caso dos vinhos do Porto, os vinhos brancos em geral apresentaram menores valores de CC_{inert} (5 μM) que os tintos (5-11 μM). Nos tintos, os resultados sugerem uma tendência para a diminuição da CC_{inert} com a idade.

Foi também estudado o destino do Pb e Cu presente em alguns dos vinhos de mesa e do Porto quando sujeitos a digestão gastrointestinal *in vitro*. Para tal, recolheu-se informação da solubilidade (condição necessária para a assimilação ocorrer) dos metais no fim das fases gástrica e intestinal, e da dialisabilidade durante a digestão intestinal (tida como um indicador de assimilabilidade). Adicionalmente, efectuaram-se separações cromatográficas de alguns vinhos e respectivos digestos, em condições vulgarmente usadas para macromoléculas, com detecção de Pb e Cu por espectroscopia de absorção atómica, de maneira a obter informação acerca da natureza dos ligandos dos vinhos e eventuais alterações devidas à digestão gastrointestinal. Nos casos em que foi possível, determinaram-se também para os digestos os mesmos parâmetros de complexação que se determinaram para os vinhos.

Os resultados mostraram que no caso de vinhos de mesa tintos e vinhos do Porto tintos jovens o Pb, após digestão gastrointestinal, encontra-se presente maioritariamente em formas insolúveis (não assimiláveis). Diferenças na dialisabilidade das espécies de Pb durante a digestão intestinal observadas para diferentes tipos de vinho, indicam que o Pb pode ser muito menos biodisponível em vinhos de mesa tintos e vinhos do Porto tintos jovens que em vinhos de mesa brancos e vinhos do Porto velhos. As experiências cromatográficas mostraram que a distribuição do Pb entre as diferentes espécies de Pb existente antes e depois da digestão gastrointestinal é substancialmente diferente, evidenciando assim a importância da especiação a nível gastrointestinal para a biodisponibilidade final dos elementos.

A solubilidade do Cu permaneceu alta (quase total) depois da digestão gastrointestinal o que significa que estava praticamente todo numa forma potencialmente assimilável. Os resultados obtidos para a fracção dialisável durante a digestão intestinal mostram uma descida significativa (especialmente para o vinho de mesa tinto) relativamente à fracção solúvel (excepto para o vinho de mesa branco), indicando que nem todo o metal solúvel poderá ser assimilável. O estudo cromatográfico mostrou que a distribuição do Cu entre as várias espécies foi pouco afectada pelo processo digestivo, contrariamente ao que aconteceu com o Pb. Adicionalmente, indicou que nos vinhos do Porto uma fracção do Cu encontra-se numa forma de composto apolares e/ou de grande massa molecular, enquanto que para os vinhos de mesa apenas compostos polares e /ou de baixa massa molecular contendo Cu parecem existir.

SUMMARY

The influence of Cu concentration on the alcoholic fermentation process, namely regarding must fermentation was studied. Alcoholic fermentation was studied in a wide range of Cu concentrations (from natural levels to 1.0 mM) for two different fermentative media, white grape musts (an highly complex matrix which contains several Cu ligands) and yeast nitrogen base (YNB) with glucose. In the YNB medium, the ethanol production and fermentation rate were highly influenced by the Cu concentration, being the yield of ethanol doubled in the presence of 0.50 and 1.0 mM Cu. However, the fermentation rate was slower. As for production of ethanol from musts, only minor effects were observed for the different Cu concentrations, which indicates that Cu levels are not responsible for changes in fermentation, and, therefore, are below any toxic level regarding the yeast performance. From a practical biotechnological point of view, our results envisage the interest to perform future work on the influence of Cu in the media used in the ethanol production industry, when using *S. cerevisiae* or other cells. The biochemical explanation for the phenomena observed is an open research topic, indeed it is possible that there is a link between the effect of Cu and Fe availability, as Cu is required for Fe transport into yeast cells.

The complexometric properties to Pb and Cu of four different red wines produced from only one of the following varieties of grapes, cultivated in the Douro's Demarked Region of Portugal: "Tinta Barroca" (TB), "Tinta Roriz" (TR), "Touriga Nacional" (TN) and "Touriga Francesa" (TF) were studied by potentiometry. Parallelely, 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 complexation properties were expressed in terms of (a) maximum complexation capacity (CC_{total}) of the ligands from the wines, that is, the total concentration of the sites available in the sample, and (b) conditional stability constants of the complexes with Pb or Cu (K_{av}). For this purpose, the Scatchard and the Differential Equilibrium Function models, which have been considered suitable for characterisation of complexes involving heterogeneous ligands were used. For Cu, this study was extended to different types of Port wine (white, tawny, ruby, dated, young red, with age indication). For Pb, additional complexation parameters were possible to study by cathodic voltammetry: the capacity for inert complexation

(CC_{inert}) and an estimation of the conditional stability constants of inert complex formation (K'_{av}) which were also determined for table wines (white, red, white Verde and red Verde) and the Port wines referred for Cu.

The study showed that tannins and skin extracts bind Cu. However the existence of other abundant ligands in wine was made evident by comparison of the complexation parameters determined for polyphenolic extracts and wines. It was inferred that Cu was strongly complexed in these wines ($\log K_{av} > 6$). For red Port wines a strong complexed state for Cu could also be inferred ($\log K_{av} > 5.5$). For Pb, despite some limitations involved, voltammetry could provide more valuable information about the complexation state of Pb in wines (K'_{av} and CC_{inert}) since these information regards the most strong ligands which in fact bound the metals at their natural levels found in wines. Since the values of $\log \theta$ (degree of occupation of the complexation sites) in wines are lower than those embraced in the voltammetric experiments, Pb is strongly complexed in wines ($\log K'_{av} > 7.2$). Parallel studies of isolated tannins and skin extracts showed that the later include much stronger ligands than tannins, probably not anthocyans but other polyphenols or polyssacharides . It was shown that the polyphenols, alone, do not account for the strong Pb complexation observed in wines. The values of K'_{av} obtained for the different table wines were all similar ranging 6.5 to 7.8 and indicated a strong complexation as well. The CC_{inert} of red wines (16-28 μM) was much higher than of the white wines (3-8 μM). In the case of the Port wines, white wines generally displayed lower CC_{inert} values (5 μM) than red wines (5-11 μM). Within the red Port wines the results suggest a trend for a decrease of CC_{inert} with age.

It was also studied the fate of the Pb and Cu present in some of the table and Port wines when they were subjected to *in vitro* gastrointestinal digestion. For that purpose, information about Pb and Cu solubility (necessary condition for the assimilation to occur) at the end of the gastric and intestinal phases and the dialysability during the intestinal digestion (taken as an assimilability indicator) was gathered. Moreover, chromatographic separations in conditions usually set for macromolecules, with Pb and Cu detection by atomic absorption spectrometry, were carried out for some wines and their respective digests, as a way to find information about the nature of the ligands of wine and eventual changes due to the gastrointestinal digestion. In the cases that was possible, the complexometric parameters determined for the wines were also determined for their digests.

The results have shown that in the case of red table wines and young red Port wines a major part of the Pb is present in insoluble forms (not assimilable) at the gastrointestinal digests. Differences in the dialysability of Pb species in intestinal digests observed for different types of wine, indicate that Pb may be much less bioavailable in red table and young red Port wines than in white table and old Port wines. The chromatographic

experiments have shown that the Pb distribution among different Pb-species existent in wines before and after gastrointestinal digestion is significantly different, thus evincing the importance of the speciation at the gastrointestinal level on the final bioavailability of the elements.

The solubility of Cu remained high (almost total) after the gastrointestinal digestion which means it was practically all on a potentially assimilable form. The results obtained for the dialysable fraction during the intestinal digestion shows a significant decrease (especially for the red table wine) relatively to the soluble fraction (except for the white table wine), thus indicating that not all soluble metal may be bioavailable. The chromatographic study showed that the distribution of the Cu among the different species was little affected by the digestive process, contrarily to what was verified for Pb. In addition, it indicated that in Port wines a fraction of Cu is in the form of highly apolar and/or high molecular weight compounds whereas for table wines only polar and/or low molecular weight compounds containing Cu seem to exist.

RÉSUMÉ

On a étudié l'influence de la concentration de Cu dans le processus de fermentation alcoolique, plus particulièrement en ce qui concerne la fermentation des moûts. La fermentation alcoolique a été étudiée pour une gamme ample de concentrations de Cu (depuis les taux naturels jusqu' à 1.0 mM), dans deux milieux de fermentation différents, moûts blancs (qui representent des matrices complexes contenant divers ligands de Cu) et "yeast nitrogen base" (YNB) avec glucose. Dans le milieu YNB, la production d' éthanol et le taux de fermentation ont été largement influencés par la concentration de Cu, la production d' éthanol étant le double en présence de Cu 0.50 et 1.0 mM. Cependant, la cinétique de fermentation a été plus lente. Pour la production d' éthanol à partir des moûts, on a vérifié à peine une influence légère de la variation de la concentration de Cu, ce qui signifie que le taux de Cu n´est pas responsable pour des altérations de la fermentation, et donc se situe au dessous des niveaux toxiques par rapport à l'accomplissement des levures. D'un point de vue pratique-biotechnologique, les résultats suggèrent l'intérêt de poursuivre des études sur l' influence du Cu dans les milieux employés dans l' industrie de production d' éthanol utilisant la *S. cerevisiae* ou autres cellules. L' explication biochimique pour le phénomène observé demeure un sujet de recherche en suspens, étant possible qu' il existe une liaison entre l' effet du Cu et les ressources de Fe, puisque que le Cu est nécéssaire pour le transport de Fe dans l' intérieur des levures.

On a étudié, par potenciométrie, les propriétés complexometriques pour Pb et Cu de quatre vins rouges différents, produits à partir d' un seul des cépages cultivés dans la Région d'Appelation du Douro: Tinta Barroca (TB), Tinta Roriz (TR), Touriga Nacional (TN) et Touriga Francesa (TF). Parallèlement, on a étudié les propriétés complexométriques des polyphénols isolés des pépins (tannins condensés) et des pellicules (en majorité anthocyanes) d'un mélange de grappes des quatre cépages, et les résultats ont été comparés avec ceux des vins. Les propriétés de complexation ont été exprimées en termes de (a) capacité de complexation (CC_{total}) des ligands des vins, soit la concentration totale des lieux de liasons disponibles dans l' échantillon, et (b) constantes condiotionelles de stabilité des complexes de Pb et Cu (K_{av}). Pour ce, on a employé les modèles de Scatchard et de la Fontion Différentielle d' Equilibre, qui sont considérés comme appropriés pour la caractérisation des complexes engageant des ligands hétérogènes. Pour Cu, cette étude a été étendue à différents types de vins de Porto (blanc, "tawny", "ruby", date de cueillette, rouge jeune, avec indication de l' âge). Pour Pb il fût possible, par voltammétrie cathodique, d' étudier des paramètres additionnels de complexation: la capacité de former des complexes inertes (CC_{inert}) et une estimation des constantes

conditionnelles de stabilité des complexes inertes (K'_{av}), lesquels ont également été déterminés pour les vins de table (rouge, blanc, "Verde" blanc et "Verde" rouge) et pour les vins de Porto référés au dessus pour le Cu.

L' étude a montré que les tannins et les extraits de pellicule complexent le Cu. Cependant, l'existence d'autres ligands en abondance s' est montrée evidente lors de la comparaison des paramètres de complexation déterminés pour les extraits et pour les vins. On a conclu que le Cu était fortement complexé dans ces vins (log K_{av} >6). Pour les vins de Porto rouges aussi, on a conclu un état fortement complexé pour le Cu (log K_{av} > 5.5). Pour le Pb, malgré les limitations, la voltamétrie a proporcionné une information plus utile sur l' état de complexation du Pb dans les vins (K'_{av} e CC_{inert}) puisque cette information respecte les ligands plus forts, qui, dans la réalité, lient les métaux aux niveaux naturels rencontrés dans les vins. Etant donné que les valeurs de log θ (degré d' occupation des lieux de complexation) sont inférieures aux valeurs accessibles dans les expériences voltamétriques, le Pb est fortement complexé dans les vins (log K'_{av} >7.2). Des études parallèles des tannins isolés et extraits de pellicule ont montré que dans ces derniers il existe des ligands plus forts que les tannins, probablement non anthocyaniques. Il a été montré que les polyphénols par eux seuls ne justifient pas la forte complexation du Pb observée dans les vins. Les valeurs de K'_{av} obtenues pour les différents vins de table ont été tous semblables variant entre 6.5 et 7.8 et indiquent aussi une forte complexation. La CC_{inert} des vins rouges (16-28 μM) a été beaucoup plus élevée que celle des vins blancs (3-8 μM). Dans le cas des vins de Porto, les vins blancs en général presentent des valeurs inférieures de CC_{inert} (5 μM) par rapport aux vins rouges (5-11 μM). Dans les vins rouges, les résultats suggèrent une tendance pour la diminution de CC_{inert} la avec l' âge.

On a aussi étudié la destination du Pb et du Cu dans certains vins de table et de Porto quand ils sont soumis a une digestion gastrointestinale *in vitro*. Pour cela, on a recueilli l' information sur la solubilité (condition nécéssaire pour que l' assimilation aie lieu) des métaux a la fin des stades gastrique et intestinale, et de la dialysabilité pendant la digestion intestinale (tenue comme un indicateur de l' assimilabilité). Additionnellement, on a éfectué des séparations chromatographiques de certains vins et digestes respectifs, dans des conditions communément employées pour macromolécules, avec detection de Pb et Cu par spectroscopie d'absorption atomique, de façon a obtenir une information sur le caractère des ligands des vins et eventuelles altérations dues à la digestion gastrointestinale. On a aussi determiné pour les digestes, dans les cas où c' était possible, les mêmes paramètres de complexation que pour les vins. .

Les résultats montrent que dans le cas des vins de table rouges et des vins de Porto rouges jeunes, le Pb, après digestion gastrointestinale, se trouve présent sutout sous formes insolubles (non assimilables). Des

différences de la dialysabilité des espèces de Pb pendant la digestion intestinale observées pour différents types de vin, indiquent que le Pb est beaucoup moins biodisponible dans les vins de table rouges et vins de Porto rouges jeunes que dans les vins de table blancs et vins de Porto veillis. Les expériences chromatographiques montrent que la distribution du Pb entre les différentes espèces de Pb avant et après digestion gastrointestinale est substanciellement différente, ce qui met en evidence l' importance de la speciation au niveau gastrointestinale pour la biodisponibilité finale des éléments.

La solubilité du Cu reste elevée (presque totale) après digestion gastrointestinale, ce qui signifie que le Cu se trouvait presque totalement sous une forme potentiellement assimilable. Les résultats obtenus pour la fraction dialysable pendant la digestion intestinale montrent un décroissement significatif (surtout pour le vin de table rouge) par rapport à la fraction soluble (sauf pour le vin de table blanc), ce qui indique que tout le métal soluble n'est pas forcemment assimilable. L' étude chromatographique a montré que la distribution du Cu parmis les différentes espèces a été peu affectée par le processus digestif, contrairement a ce qui advient au Pb. De plus, ceci indique que dans les vins de Porto une portion du Cu se trouve sous la forme de substances apolaires et/ou de masses moléculaires elevées, alors que pour les vins de table, à peine des substances polaires et/ou de masses moléculaires moins elevées contenant du Cu semblent exister.

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LIST OF ABBREVIATIONS AND SYMBOLS

AAS-ET- Atomic absorption spectrometry with electrothermal atomisation

BCR- Bureau Communautaire de Référence

CC_{inert}- Complexation capacity to form inert ligands

CC_{total}- Total complexation capacity

DEF- Differential equilibrium function

DMACH- dimethylaminocinnamaldehyde

DPASV- differential pulse anodic stripping voltammetry

EDTA- Ethylenediamotetracetic acid

HEPA- High efficiency particulate air

HPLC- High performance liquid chromatography

I_F^L- Fluorescence intensity of the free ligand L

I_F^{ML}-Fluorescence intensity of the complex ML

I_F-Total fluorescence intensity of the ligand L

ISE- Ion selective electrode

K'_{av}- Estimation of the conditional stability constant of formation of the inert complexes

K_{av}- Average conditional stability constant of complex formation

K_{DEF}- Conditional stability constants obtained by the differential equilibrium function

MWCO- Molecular weight cut-off

OIV- L'Organisation Internationale de la Vigne et du Vin

PC- Personal computer, IBM compatible

PED- Pulsed electrochemical detector

PMT- Photomultiplier tube

ppm- Parts per million

PSA- Potentiometric stripping voltammetry

RP-HPLC- Reverse phase high performance liquid chromatography

SWCV- Square wave cathodic voltammetry

TB- Tinta Barroca

TF- Touriga Francesa

TN- Touriga Nacional

TR- Tinta Roriz

UV- Ultraviolet

YNB- Yeast nitrogen base

θ- degree of occupation of the complexation sites

CHAPTER 1

GENERAL INTRODUCTION

CHAPTER CONTENTS

1- GENERAL INTRODUCTION

1.1- FRAMING OF THE WORK

In this section some general topics of the presence of metals in the environment, the role of metals in biology, metals in wines, influence of metals on the fermentation process, and others, related with the framing of the present work, will be covered. Special attention will be paid to the particular cases of Cu and Pb which were the metals studied in the present work.

1.1.1- The Metals in the Environment

Metals and metalloid elements are ubiquitous. They occur naturally as ions, compounds and complexes and – to an increasingly relevant degree – in the anthroposphere in a variety of forms.

Their mean concentration in the continental and oceanic earth's crust and in abundant rock species has been estimated (Wedepohl 1991), being Fe, Mg, Ti, Mn, V, Zn, Cr, Cu, Ni and Zr the most concentrated elements. Their concentrations embrace a large range of values, being as high as 70000 ppm of Fe in the oceanic crust, or as low as 0.0025 ppm of Au in the continental crust. The concentration of Cu in these materials range between 4 ppm (in limestones) and 80 ppm (in basaltic and gabbroic rocks), and the concentration of Pb range between 0.89 ppm (in the oceanic crust) and 32 ppm (in granitic rocks).

The most important natural agents of transport of the elements at the earth's surface are water and wind. Beside matter in suspension, rivers and rain carry large amounts of dissolved compounds from continental rock weathering, to the oceans. Rain also moves salt spray in opposite direction from ocean to land. Wind carries much lower concentrations but it compensates with its much greater speed.

The combustion of fossil fuels for the production of energy introduces numerous metals into the atmosphere and subsequently into soils, rivers and oceans. For example the concentration of Cu ranges between 0.7 ppm (in crude oil) and 16 ppm (in hard coal) and the concentration of Pb ranges between 0.3 ppm (in crude oil) and 46 ppm (in hard coal) (Wedepohl 1991).

The metal mineral mining started a little more than 4000 years ago. But the size of the operations was small until fossil energy became available for an exponential growth of mineral mining and processing during the

last century. Three quarters of these metal minerals are processed and mostly consumed in the relatively small highly industrialised countries with a quarter of the world population. The aerial concentration of processing and consumption causes environmental problems. The risk of contamination of soils, rivers and air by toxic trace elements is high in the industrialised countries. Beside the firing of coal and oil, processing of ores and the technical use of several metals are major sources of such contaminations.

1.1.2- The Role of Metals in the Living Systems

Analysis of a great number of plants, animals, as well as organs, tissues and other substances of biological origin have established that the living systems concentrate certain chemical elements and reject others. One could, therefore, speak of a natural selection of the chemical elements by the biological systems, which involves a readjustment of the element distribution on the earth's local scale by utilising energy ultimately provided by the sun (Silva and Williams 1993).

There are eleven elements (H, O, C, N, Na, K, Ca, Mg, P, S, and Cl) that appear to be approximately constant and predominant in all biological systems. In the human body these constitute 99.9 per cent of the total number of atoms present but just four of them (C, O, H, N) correspond to 99 per cent of that total. The very large percentages of H (62.8 %) and O (25.4 %) arise from the high water content of all living systems. The C (9.4 %) and N (1.4 %), next in importance, together with O and H, are the basic elements of the organic structures and metabolites of living systems (Silva and Williams 1993).

However, the organic compounds have limitations at some crucial levels such as their low mechanical resistance and their unsuitability for catalysts of redox reactions. These insufficiencies are overcome by the inclusion of inorganic species, namely metals at trace levels (micronutrients) (Ochiai 1987). Considering the diversity of functions the metals exert, its amount in the living systems is largely disproportionate to its application range (Laurie 1987). Thereby, many metallic ions (such as Mn(II), Fe(II), Fe(III), Cu(II), Zn(II), ...) are essential to normal operation of the living systems.

The essential micronutrients have a relatively narrow range of concentrations where their action to organisms is beneficial. Concentrations below that range cause problems of subnutritional character, and concentrations above exert toxic effects to the organisms. The elements considered non-essential (like Pb and Hg) are generally toxic at lower concentrations than the essential elements (Wood 1984).

1.1.2.1- The Biological Role of Cu

Cu is an element essential to all organisms. For some of them, as the snails, Cu presence in blood is essential, being active in the O_2 transport. It is a component of some enzymes, especially associated with electron transfer systems, such as the ascorbate oxidase, the superoxide dismutase or the cytochrome oxidase, the later being an almost universal enzyme of living organisms. Many Cu oxidases help to produce the extracellular protective matrices of living eukaryotic organisms, such as the chitins, the cuticles of insects, the collagens of many species and resins and lacquers released by plants. In plants, the Cu oxidases (free radical oxidases in this case) are used in a protective manner when the tissues are damaged, producing phenol oxidation (and in man the formation of melanin), "browning" reactions which are the beginnings of protective films of plastic. This "browning" is a protection against further damage, even from light. However, protection by Cu goes further in that both in the cell cytoplasm (superoxide dismutase) and on the outside of cells (ascorbate oxidase) two Cu enzymes scavenge free radicals or perhaps produce weak free radicals to remove strong ones (Silva and Williams 1993). Therefore Cu is mainly associated with protective functions within the eukaryotic cells, as human cells.

1.1.3- The Toxicity of Essential and Non-Essential Metals

All the trace metals, essential or non-essential, exhibit toxicity above the concentration tolerance levels (figure 1.1). The toxic effects of metals include the obstruction of the functional groups of important biomolecules such as enzymes and polynucleotides, removal and/or replacement of essential ions from sites where they exert their biological action, denaturation and inactivation of enzymes and destruction of integrity of the cellular membranes (Gadd 1992).

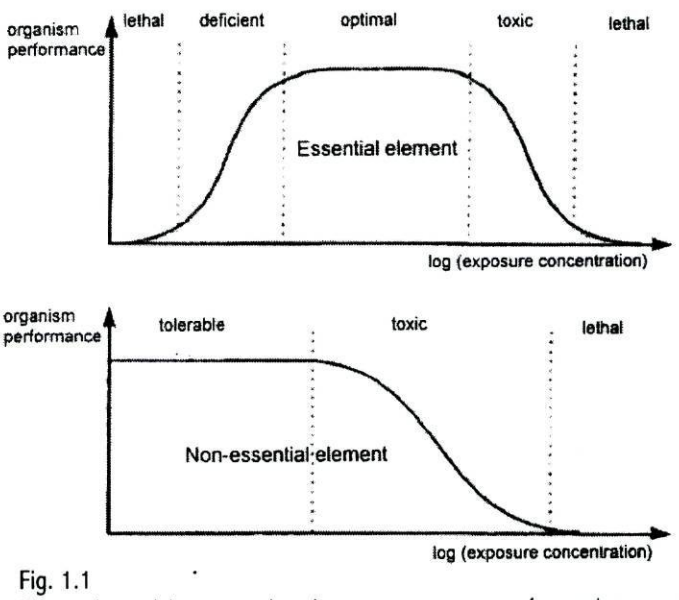

Fig. 1.1
Schematic model representing dose-response curves of organisms exposed to essential and non-essential elements. Adapted from Karman and Jak(1998).

1.1.3.1- The Toxic Effects of Pb on Humans – Routes of Exposure

The clinical diagnosis of lead poisoning is not always easy. Individuals may present a wide variety of symptoms and be in different states of the disease. The wide variety of symptoms is likely due to the fact that several different biological systems may be affected. It is known that Pb affects the peripheral nervous system, the central nervous system, the kidneys, the cardiovascular system, the immunosystem and possibly others (Ewers and Schlipkoter 1991).

In general, typical levels of human Pb exposure may be attributed to four components of the human environment: food, inhaled air, drinking water and dusts of various types.

The respiratory absorption of Pb from air and dusts to enter the bloodstream is a two-part process: (1) deposition of some fraction of inhaled air Pb in the deeper part of the respiratory tract and (2) absorption of the deposited fraction (Ewers and Schlipkoter 1991).

The gastrointestinal absorption of Pb from food and drinking water depends on which physical-chemical forms Pb is present at the absorption site, *i.e.* it depends on Pb speciation. That speciation is governed by the chemical composition of the food or drink that introduced the metal in the gastrointestinal tract and by other food and/or drink ingested at the same meal. For example, citric acid supplements to a maize-soya diet increase the Pb assimilability (Walter *et.al.* 1998).

Food and beverages constitute the major sources of exposure to Pb among adults not employed in Pb-related industries and among children that do not ingest non-food items such as Pb-containing paint chips.

The absorbed Pb enters the bloodstream from where it is distributed to various organs and tissues. Redistribution then occurs in relation to the relative affinity of each tissue for Pb. More than 95 % of the Pb in human blood is bound to erythrocytes under steady state conditions. Most of the erythrocyte Pb is bound within the cell, primarily to haemoglobin. In plasma and extracellular fluids, nearly all of the Pb is bound to proteins, mainly albumin and some high-molecular-weight globulins. It is unknown, which of these binding forms constitutes the active (or bioavailable), diffusible fraction for movement of Pb into tissues and cells (Ewers and Schlipkoter 1991).

Dietary Pb in humans and animals that is not absorbed passes through the gastrointestinal tract and is eliminated with the faeces. Absorbed Pb is mainly excreted in the urine.

1.1.3.2- The Toxic Effects of Cu on Humans – Routes of Exposure

Several diseases, namely the Wilson disease, the liver cirrhosis, tuberculosis, carcinoma, infections, epilepsy, and leukaemia are related with undesirable levels of Cu (either excessive or deficient) in the liver or in blood serum. It is not known exactly if the abnormal levels of Cu are the cause or an effect of the diseases (Berman 1982).

The routes of exposure to Cu include the respiratory absorption in the same way as to Pb and the gastrointestinal absorption. Experimental data support the existence of carrier-mediated transport mechanism for Cu absorption in the intestine. Beside, the chemical composition of the ingested foods and drinks influences the level of Cu absorbed. For example, supplements of metals with chemical properties similar to Cu may reduce Cu absorption (Wapnir 1998).

Subsequently to absorption, the Cu is transported by the bloodstream to the sites where it is required in its biological role. The excretion from the organism of non-absorbed Cu is made essentially via the bile and faeces.

1.1.4- Metals in Wine and Must

As referred in 1.1.1 metals are ubiquitous in the environment. Vineyards, musts and, by consequence, wines are a part of the environment where several metals can be found. The presence of these metals in wine has an oenological importance in the sense that they may influence the quality of the wine, and a nutritive/toxicological importance in the sense that wine is a source of both essential and toxic metals.

1.1.4.1- Metals in the process of alcoholic fermentation

Alcoholic Fermentation of Grape. The process of alcoholic fermentation is generally produced by yeasts of the *Kluyveromyces* and *Saccharomyces* families. Alcohol is obtained at the expense of the hexoses and hexobioses, all of which are transformed into glucose-6-P to enter the cycle of anaerobic glycolysis (figure 1.2). The fermentation is described by the following equation:

$$C_6H_{12}O_6 \rightarrow 2CH_3CH_2OH + 2CO_2 \qquad (1.1)$$

The term "wine" is only applicable to the product of the alcoholic fermentation of the must of the grape. The sugars involved are mainly glucose and fructose (in almost equal quantities in the ripe grape). The yield of ethanol is of 73 –120 g/L or 9 – 15 % (v/v) (Alais and Linden 1991).

White grapes can only yield white wine. Red grapes which have white juice yield a white wine if there is immediate pressing of the grape harvest with separation of the solids before fermentation. Red grapes which have white or red juice yield either rosé or red wine, respectively, if the fermentation is of the whole fruit without pressing of the juice or skins. The skins possess the red pigment or anthocyanins (see 1.1.4.3), which goes into solution when the alcohol develops. For rosé wines, a limited time is allowed for this stage (10-15 hours of maceration) (Alais and Linden 1991).

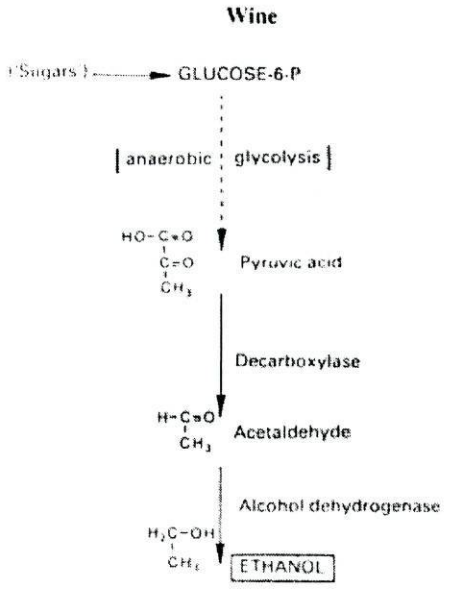

Fig. 1.2
Scheme of wine fermentation. Adapted from Alais and Linden (1991).

Influence of Metals. The metal cations of K, Mg, Ca and Zn are known to directly influence the fermentative metabolism in yeast, and therefore studies for establishing their optimal levels in industrial fermentations were performed (Chandrasena *et.al.* 1997; Walker and Maynard 1997). The influences of other essential metals like Cu in the fermentative process are not known currently.

The presence of trace metals in toxic concentrations can be a significant problem during the fermentation by means of the reduction of the performance of the fermentative microorganism. For example, Ergun *et.al.* (1997) found a fermentative improvement when trace metal strong ligands or sorbers such as EDTA, zeolite X or ferrocyanide were add to sugar beet molasses.

1.1.4.2- Metals in Wine

The products of biological origin always contain diverse minerals beside the organic materials. In grapes, as in most vegetable materials, the predominating minerals are K, Ca, Mg and Na. Other less abundant natural

constituent elements of grape include Cu, Fe, Zn, Mn. Therefore, all these elements appear in wine at considerable levels (table 1.1). At trace levels, a set of other metallic non-essential elements are generally also present in wines: Pb, Al, Rb, Ba, Cd, Cr, Ni, U, Va, Ti, Hg and others.

The metals may have both biological (endogenous metal) and technological (exogenous metal) sources contributing to their composition in a finished wine. While metals such as K and Ca come mainly from endogenous sources, others such as Al, Fe and Cu have significant contribution either from endogenous and exogenous sources. Some other metals such as Pb, Cd and Hg are considered contamination from exogenous sources (Scollary 1997).

Table 1.1

Typical metal composition of wines (in mg/L)*

K	100 – 1700
Mg	50 – 200
Ca	100 – 200
Na	5.0 – 400
Fe	5.0 – 30
Cu	0.2 – 2.0
Mn	0.5 – 15
Zn	0.0 – 0.5
Pb	0.025 – 0.4
Al	0.3 – 1.5
Rb	0.2 – 4.0

*- values presented by Cordonnier (1965)

Sources of Cu in Wine. Beside the Cu naturally present in the grapes, this metal may have several exogenous sources. Cu may be carried over into grape juice from the use of Cu-based vineyard fungicides and pesticides. However, the Cu concentration in freshly fermented wine is generally low owing to the ability of dead yeast cells to take up Cu and to the formation of CuS (favoured by the presence of SO_2) which precipitates (Scollary 1997; Curvelo-Garcia 1988). The common winemaking practice of adding $CuSO_4$ for the removal of H_2S and other sulphidic off-odours frequently results in relatively high Cu concentrations. Contact with Cu, brass and bronze materials used in wineries is another possible exogenous source of Cu in wines (Curvelo-Garcia 1988).

High concentrations of Cu in wines can lead to either haze formation or enhanced oxidation rate. The critical level at which Cu-induced spoilage occurs is still subject of debate. While it is generally recommended to maintain Cu concentrations of less than 0.3-0.5 mg/L, there is evidence that some wines with lower Cu concentrations are subject to Cu-induced spoilage (Scollary 1997).

Sources of Pb in wines. Before fermentation, Pb may come into juice through the atmospheric pollution, by the use of phytosanitary products and by contact with winery material. During fermentation a reduction of Pb occurs via bioremediation through sorption by yeast cells. (Legrand *et.al.* 1991). After the fermentation, additional contamination of Pb may arise from the contact with plastics, paints, capsules and other Pb containing

materials (Legrand *et.al.* 1991; Scollary 1997; Curvelo-Garcia 1988). At the consumer level, there is a potentially high contaminating Pb source, the Pb-containing crystal jars. For example, a Port containing 89 μg/L of Pb was placed in crystal decanters and the Pb content of the wine rose steadily to 3518 μg/L after 4 months (Graziano and Blum 1991). Therefore all the Pb sources are exogenous and a careful choice of vineyard, cellar and consumer domestic practices and materials is necessary for taking wines with Pb levels compliant with the regulated levels.

Concentrations of Pb in wine are regulated by most countries, with maximum concentrations between 50 and 200 μg/L. The L'Organisation Internationale de la Vigne et du Vin (OIV) presently recommends 200 mg/L as the maximum limit for Pb in wines. There is obvious health concerns in the toxicity of Pb absorbed from wine and the regulated levels are intended to reflect this concern. However, this regulation is somewhat arbitrary because is not supported by a scientific toxicological basis. Indeed, Pb became potentially an effective trade barrier, as a country may set a level for imported wine that is considerably lower than the mean of its own domestic wines (Scollary 1997).

1.1.4.3- Metal Speciation and Bioavailability in Wines

Metal speciation designates the determination of the physical-chemical forms in which metals are present in a sample. The term speciation has been used either in a functional sense, referring, for example, to the determination of the species that are bioavailable to a certain organism, or in an operational sense, respecting in this case to the determination of the metal forms which are measurable by a certain physical-chemical method, for example, exchangeable forms, stable or reducible forms of an element in well defined experimental conditions.

As already referred in the present work, some metals have potential harmful effects in wine quality (as haze formation and enhanced oxidation rate) while others are potentially toxic for humans. It is well recognised that these effects are not determined by the total concentration of the metals. Therefore, any advances in metal speciation in wines may facilitate the understanding of spoilage and toxic effects of metals in wine. In the case of the study of potential toxicity of metals ingested within wine, the speciation must be extended also at the gastrointestinal digestion level, especially at the intestinal level where the majority of the assimilation occurs. The forms of metal that can surpass the intestinal barrier may be bioavailable, *i.e.*, able to interact with blood or

other sites where it is taken by the blood stream (this is the toxicological/pharmacological notion of bioavailability; other notions of bioavailability exist; see Dickson *et.al.* 1994).

Metal Complexants Present in Wine

An important feature of metal speciation in wines is to know the complexed forms of the metals, that is, to know which are the metal ligands. A survey of potential metal complexants present in wines is presented next.

In wine, two types of possible complexants can be found: simple low molecular weight compounds whose individual structure and physical-chemical properties are known or may be readily determined (among those properties are the stability constants of complex formation with metals) and macromolecular and/or polymeric compounds which are of difficult characterisation either in structure or physical-chemical properties (also called heterogeneous ligands), including the stability constants of complex formation with metals.

Low Molecular Weight Compounds (Curvelo-Garcia 1988):

Organic Acids

These compounds exist in wines proceeding from grapes, microbial activity, natural chemical processes occurring during must and wine evolution and from technological actions (namely the addition of acid correctives). The principal organic acids are tartaric, malic, citric, succinic, lactic, gluconic, pyruvic and ascorbic, which possess carboxylic functional groups as coordination sites. Wine contains phenolic acids (benzoic acids and cinnamic acids) but these are combined with other compounds. There are also the volatile acids, mainly acetic. The metals face an important competitor for the carboxylic groups present in the organic acids of wine, the proton, because of the acidic character of wines (pH 3-4). On the other hand, some organic acids may be in such high concentrations (for instance 1500-4000, 500-1500, 0-4000 mg/L of tartaric, succinic and malic acids, respectively) that constitute important effective ligands for some metals as Ca and K.

Monomeric and Dimeric Glycosides

The D-glucose and D-fructose (hexoses) are the most important mono-glycosides present in wines. Some pentoses as L-arabinose or D- xilose are also present in wines. Dimeric glycosides as the saccharose, maltose or lactose also appear in wines. All this compounds possess hydroxyl and carbonyl functional groups which may have affinity for hard Lewis acid metals (as K, Na, Ca, Al,...; Nieboer and Richardson 1980).

Polyhydroxyl Compounds

Glycerol (CH_2OH-$CHOH$-CH_2OH) is usually the third most abundant component of wine with levels of 6 to 10 g for 100 g of ethanol. Other less abundant polyhydroxyl compounds are sorbitol, inositol, manitol and others. The functional groups have preference for hard Lewis acid metals as well.

Aminoacids and Small Peptides

Around 30 aminoacids (apolar, polar, acidic and basic) were already identified in wines. This ligands, which possess carboxylic and amine functional groups, have preference for borderline Lewis acid metals such as Fe, Cu, Cd,.... and soft Lewis metals when sulphide functional groups are present (Nieboer and Richardson 1980).

Biogenic Amines

The most noticeable biogenic amine found in wines is histamine, although more than 20 in the total have been already identified. The functional group is the amine, with preference for borderline Lewis acid metals.

Polyphenols (also Robards and Antolovich 1997)

The most important group of polyphenols in wines is the flavonoids. These are aromatic secondary plant metabolites with a C_6-C_3-C_6 flavone skeleton in which the C_3 bridge between the phenyl groups is commonly cyclized with an O atom. Several classes (figure 1.3) are differentiated according to the degree of unsaturation and degree of oxidation of the C_3 segment. Within the various classes, further differentiation is possible based on the number and nature of substituent groups attached to the rings. The range of known flavonoids is therefore

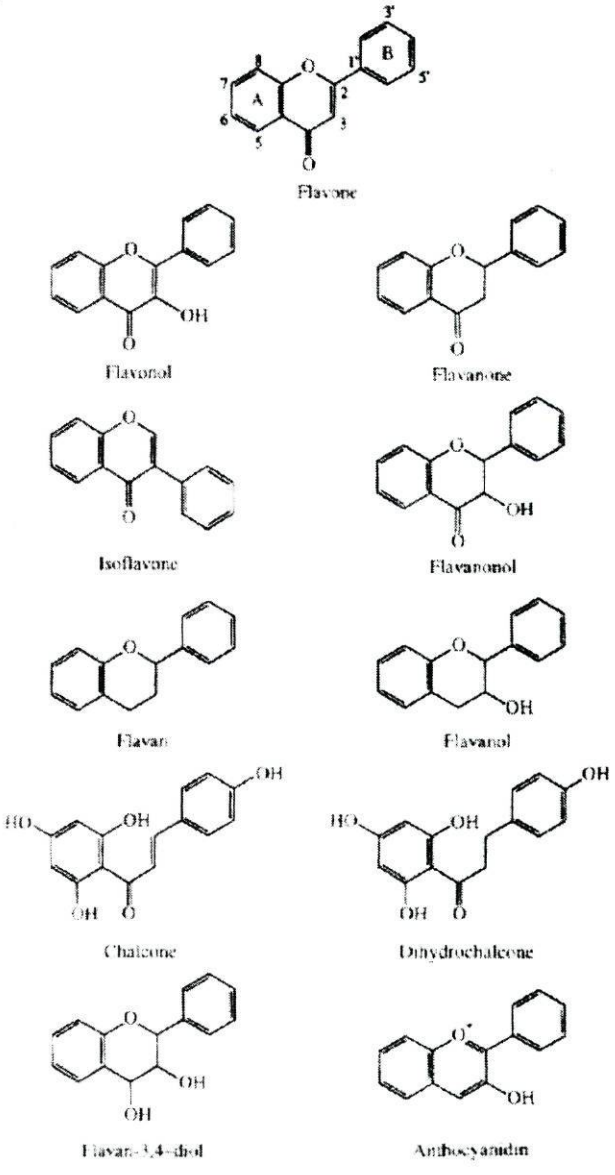

Fig. 1.3
Structures of the various classes of flavonoids. Adapted from Robards and Antolovich (1997)

vast, currently exceeding 5000. Additional structural complexity is introduced by the common occurrence of flavonoids as the O-glycosides in which one or more of the flavonoid hydroxyl groups is bound to a sugar or sugars by an acid labile hemiacetal bond. A common flavonoid such as kaempferol may be found to occur in nature in any one of 214 different glycosidic forms. Besides the flavonoids, wines contain always derivatives of benzoic and hydroxycinnamic acids. The functional groups associated to wine polyphenols are therefore mainly hydroxyl, carbonyl and oxonium, which have preference for hard Lewis acid metals.

High Molecular Weight Compounds

Polysaccharides

These compounds are constituted by a large number of molecules of the same glycoside or of different glycosides. The pectins constitute one of the most important fractions of this group. They are formed essentially by unites of galacturonic acid partially esterified by methanol with unites of glucuronic acid. The pectins originate from the cell walls of yeasts. The gums are as well an important fraction of wine polysaccharides. They are polymers of unites of pentoses (arabinose, rhamnose), hexoses (galactose, mannose) and galacturonic acid. The polysaccharides in wine are a complex mixture of polymers whose molecular weight usually varies from 10 000 to 200 000 (Curvelo-Garcia 1988). Although the functional groups are the same existing in the low molecular weight glycosides, the polysaccharides may assume metal trapping conformations as illustrated in figure 1.4 (Pellerin and O'Neill 1998).

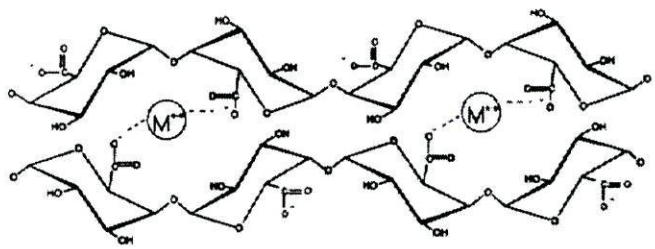

Fig. 1.4
A cross-linkage structure of two non-esterified galacturonan chains, suitable for metal trapping. Adapted from Pellerin and O'Neil (1998)

Peptides and Proteins

The quantity of proteins in wine is very low due to precipitation in various stages of wine production. But the polypeptides, with lower molecular weight, not retained by ultrafiltering membrane represent a high fraction of the nitrogen of wine (Curvelo-Garcia 1988). These compounds seem to originate from yeast proteolysis which continues in wine, thus resulting in continuously smaller peptides (Moreno-Arribas *et.al.* 1996). The peptides with sufficiently long chains may also assume metal trapping conformations.

CONDENSED TANNINS

These compounds, also called proanthocyanidins, are oligomers and polymers of flavan-3-ols. Several classes can be distinguished on the basis of the hydroxylation pattern of the constitutive units. Among them, prodelphinidins, consisting of (epi)gallocatechin units, and procyanidins which are presented in figure 1.5, have been reported in grapes (Cheynier *et.al.* 1997). Flavan-3-ol units may be encountered as 3-O-esters, in particular with gallic acid, or as glycosides. As the other macromolecules referred above the proanthocyanidins may bind metals in trap structures.

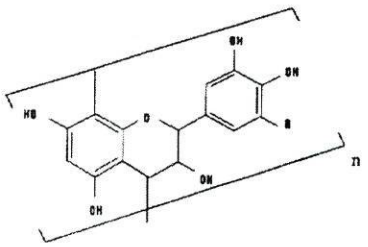

Fig. 1.5
Structure of proanthocyanidins (condensed tannins). Adapted from Cheynier *et.al.* (1997)

PRESENT STATE OF THE KNOWLEDGE

Some work on metal speciation in wines, essentially operational speciation, has already been produced. 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, it has been able to discriminate the fractions of operationally "inert" and "labile" (reactive) Pb and Cu.

Very recent works (Green *et.al.* 1997; McKinnon and Scollary 1997; Wiese and Schwedt 1997; Szpunar *et.al.* 1998) gave a step further in this field, as they tried to identify the ligands primarily responsible for Pb and Cu 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 pectic polysaccharides, peptides or proteins and polyphenols, namely the condensed tannins. However, there is a total lack of information about the quantitative complexometric properties (such as the complexation capacity and the binding strength expressed as stability equilibrium constants) of these groups of ligands or even of the wines as a whole, towards Pb and Cu.

About the bioavailability and speciation of wines at the gastrointestinal level there is no knowledge of published work before the present work.

1.2- AIMS OF THE WORK

The first aim of the present work was the study of the influence of Cu concentration on the alcoholic fermentation process, namely regarding must fermentation. Alcoholic fermentation was studied in a wide range of Cu concentrations (from natural levels to 1.0 mM) for two different fermentative media, white grape musts (an highly complex matrix which contains several Cu ligands) and yeast nitrogen base (YNB) with glucose.

But the central goal of the work was the pursuance to obtain information about the speciation and bioavailability to humans of Pb and Cu in different types of wine.

To accomplish the task of speciating, some complexometric properties (concentration of ligands and conditional stability constants of complex formation) to Pb and Cu of different types of wine were determined by electrochemical methods. Parallelely, the complexometric properties of the polyphenols isolated from grape seeds (condensed tannins) and skin (mainly anthocyans) were also determined. Additional information about metal speciation was seek by performing chromatographic separations of wine, with Pb and Cu detection, in conditions usually set for macromolecules, expecting to obtain some insight into the nature of the Pb and Cu complexes in wines.

Information on the bioavailability to humans of Pb and Cu in wines was gathered by tracking the fate of the Pb and Cu present in wines when they were subjected to *in vitro* gastrointestinal digestion. Concretely, metal solubility and dialysability in the course of the digestion were determined. Moreover, the speciation parameters studied for Pb and Cu in wines were also studied in the course of the digestion, when possible.

1.3- ORGANISATION OF THE DISSERTATION

This dissertation is structured in six chapters.

This current first chapter- General Introduction - is composed of the framing of the work in which some topics related with the work are covered, the presentation of the aims of the work and the strategy for their accomplishment, and the description of the organisation of the dissertation.

The second chapter – Experimental Section - is composed by the description of the aspects regarding the experimental execution, namely, the reagents and solutions used, the assemblies set, the instrumental devices used, the procedures followed and the methods of data handling used.

The third, fourth and fifth chapters contain the results obtained and the respective discussion. The third chapter is dedicated to the study of the influence of Cu concentration in the process of fermentation by *Saccharomyces cerevisiae*. The fourth chapter is dedicated to the speciation and bioavailability of Pb in different types of wine. The fifth chapter is dedicated to the speciation and bioavailability of Cu in different types of wine.

The sixth chapter – Overall Conclusions and Final Remarks– is dedicated to the final conclusions and future research needs.

A list of references covering all six chapters is included next.

The dissertation includes an appendix devoted to the instrumental techniques employed, with references listed therein.

CHAPTER 2

EXPERIMENTAL SECTION

CHAPTER CONTENTS

2- EXPERIMENTAL SECTION

2.1 - GENERAL ISSUES

All reagents used were of analytical grade. All the solutions were prepared in deionised water with conductance < 0.1 μS/cm. The material was previously decontaminated of trace metals, in 20 % HNO_3 overnight and thoroughly washed with deionised water before usage. The trace analysis were carried out in a clean lab with forced filtered air (HEPA filter) and the manipulations were performed in plasticware, inside a laminar flux chamber.

All the experiments were carried out in triplicate. Control blanks were performed for all the procedures involving metal analysis for contamination control (see below).

2.2 - STUDIES RELATED WITH *Saccharomyces cerevisiae*

2.2.1- Media Preparation

Cell Culture

Cells of *S. cerevisiae* wild type DL1 were cultured by inoculation on a medium composed of 0.67 % YNB (yeast nitrogen base), 2 % glucose, 0.004 % uracil, 0.004 % histidine and 0.008 % leucine. Cell growth was carried out at 26 °C with vigorous shaking (120 rpm) for at least 12 hr.

Working Medium

The composition of the working medium used throughout this work differed of that of the culture medium only in the glucose concentration, 100 g/L, and pH, adjusted to 4.0 with concentrated HCl.

Musts and Working Medium Fermentations

All the fermentations occurred in hermetic containers (0.5 L for musts and 1 L for working medium) and were monitored as described below. Fermentations without or with addition of 0.010, 0.10, 0.50 and 1.0 mM of Cu were performed (two replicates for each Cu concentration) either for musts or for the synthetic working medium. In both types of fermentation medium, a suitable volume of cell suspension collected from the culture medium, in the exponential growth phase, was added to obtain a density of 10^6 cell / mL. Musts were produced from berries collected from commercially available white table grapes which were frozen at -20 °C. Before each must fermentation, 900 g of frozen berries were let to defrost for a couple of hours. Then the berries were manually crushed with the help of a pestle, to obtain at least 400 mL of juice, which was the volume introduced in a 0.5 L fermentor. The fermentations in the working medium were carried out in a volume of 1 L.

2.2.2- Determination of the Complexation State of Cu in the Working Medium by DPASV

An Autolab PSTAT10 system (Ecochemie) connected to a 663 VA stand (Metrohm) was used. 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 experiments were performed in plastic reaction vessels (Radiometer, ref. 904-488) to minimise metal adsorption to the surface. The dissolved oxygen was previously removed from the solution by bubbling nitrogen for 5 min. Between experiments, the electrodes were immersed in 20 % nitric acid for 1 min with stirring and then washed with deionised water. The DPASV instrumental settings were as follows: deposition potential, E_d = -0.9 V vs. Ag/AgCl(s), 3 M KCl , 120 s of deposition time, 100 mV pulse height, 60 ms pulse duration, 1 s pulse repetition time, and 5 mV s^{-1} scan rate.

2.2.3- Growth Curves

Aliquots of cultures grown overnight, at the end of the exponential phase, were diluted 1:5 in the working medium with different Cu concentrations: 0.63 μM (natural level), 0.010 mM, 0.10 mM, 0.50 mM and 1.0 mM. The cultures were incubated at 26 °C. The cellular growth was time monitored by the measurement of the absorbance at 600 nm (A_{600}).

2.2.4- Determination of Total Cu Sorbed by Cells and Intracellular Cu

The total Cu (extracellular plus intracellular) associated with the cells was measured before (blank levels) and during the growth experiments described above. For the total Cu determination, 0.5 mL of cell suspension was filtered (0.45 μm pore size) and washed with 5 mL of deionised water. The filters with the cells were then dried at 60 °C for 1-2 hr. The filters were put in 4 mL of 20 % HNO_3 and digested in the microwave oven. For the intracellular Cu determination, the cell suspensions (0.5 mL) were centrifuged, the supernatant was rejected and cells were ressuspended in a solution with 15 % HCl and with 1.2 M sorbitol and left for 10 min. These operations were repeated twice. After the third resuspension, the cells were centrifuged, dried at 60 °C for 1-2 hr and acid digested as for the total Cu determination. The Cu determination in the digested cells was carried out by atomic absorption spectrometry with electrothermal atomization (AAS-ET), in a Perkin Elmer 4100 ZL model with Zeeman background correction. For calibration, aqueous Cu standard solutions with 1 % HNO_3 was used. The accuracy of the determinations was checked by spiking some samples with standard, the percentage of recovery laying in the 93-104 % range.

2.2.5- Determination of Ethanol by Ion Exclusion Chromatography

A Dionex ion chromatography system (model DX 300 IC) including a 50 μL loop, a Bio-Rad Aminex® HPX -87 H column (300 mm x 7.8 mm) with the corresponding guard column coupled to a pulsed electrochemical detector (PED) was used. The PED, comprising a gold working electrode, a Ag/AgCl reference electrode and the stainless-steel body of the detector serving as counter electrode, was operated in the integrated amperometric mode. Table 2.1 shows the phases of the amperometric program used in the PED. The eluant, 2 mM H_2SO_4, was run with 0.7 mL/min flux rate. The chromatographic system was calibrated for ethanol percentage in the range 0 to 0.1 %.

Table 2.1- Phases of the amperometric program used with the pulsed electrochemical detector (PED)

Phase	*E / V*	*Duration / ms*	*Integration interval /ms*
1	+ 0.15	300	100 - 300
2	+ 1.20	200	____
3	- 0.10	200	____

2.2.6- Monitoring the CO_2 Releasing During Fermentation

The experimental assembly used to monitor the mass of CO_2 released during fermentations was set up according to Sablayrolles J. *et al.*, 1987, and consisted, on positioning the fermentation vessel on the measuring plate of a suitable electronic balance (figure 2.1) controlled by a personal computer. Special optimisation effort was necessary as not to exceed the maximum weight measured by the balance (2100 g), what was accomplished by setting a table based support for the stirrer, thus avoiding the stirrer to weigh on the balance. Another support, balanced based, was transversely set to the stirrer support, without physical contact between them, where the fermentor, a small tube serving as security flask against liquid rollback, and a small flask containing concentrated H_2SO_4 (a trap for the water vapour released from the fermentor) were set. The whole system (except the personal computer) was inside a closed camera (to minimise balance disturbance caused by air currents) which was located in an acclimated room with temperature of 18 ºC (musts) or 22 ºC (synthetic working medium).

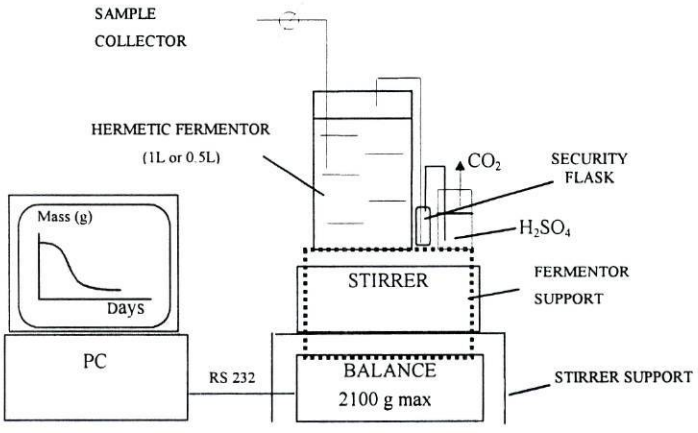

Fig. 2.1
Scheme of the experimental assembly used for monitoring CO_2 releasing during fermentations

2.3 - STUDIES OF SPECIATION AND BIOAVAILABILITY OF Pb AND Cu IN WINES

2.3.1- Wine Samples

The studied wines were divided in three groups, according to the study carried out.

Group 1

Four monovarietal red wines of recommended varieties for the Douro Delimited Region: Touriga Nacional, Touriga Francesa, Tinta Barroca e Tinta Roriz. The grapes were obtained in a large Douro vineyard during the growth season, by random sampling.

VINIFICATION. The harvested grapes of each variety (15 000 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 approximately seven days with wild yeast to dryness at a temperature between 19-24 ºC. 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.

Some relevant polyphenolic characteristics of the four monovarietal red wines studied (Freitas, unpublished data) are shown in table 2.2. The methods used in these determinations are described in the literature by Glories (1978), Vivas N *et al* (1994) and Ribéreau-Gayon and Stonestreet (1966).

Table 2.2. Polyphenolic characteristics of the studied wines (group 1). Mean results of two determinations (RSD < 8 %)

Property	Wine			
	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 (g/L) [b]	2.86	3.63	3.92	3.52
Tannins condensation index (dDMACH) [c]	6.06	8.60	9.00	7.40
Anthocyans (g/L) [d]	0.44	0.56	0.64	0.40
Polimerised 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 by the method described by Ribéreau-Gayon and Stonestreet 1966

c- dDMACH = $(d_1-d_2)x10$, where d_1 is the optical density at 640 nm (1cm 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 by the method described by Glories 1978

e -Percentage of the optical density at 520 nm that remains after reaction with bisulfite (Glories 1978)

Group 2

Seven Portuguese wine samples commercially available were selected for this study: two red wines, one Verde red wine, 2 white wines and two Verde white wines. Verde designates a type of wine exclusively produced in a demarcated region situated in the north-west of Portugal, from unique varieties which origin musts medium rich in sugar but rich in acid, with low pH, resulting in wines medium in alcohol (8.5-11 %).

Group 3

Eleven different Port wines supplied by the Port Wine Institute: 2 white wines (1 sweet and 1 extra-dry) 4 blended wines with indication of age (10, 20, 30 and 40 years), 1 blended Tawny, 1 blended Ruby, 1 dated Port of 1941 and 2 very young red wines.

Regardless of the type of wine, once a bottle was opened it was kept at 18 °C under inert atmosphere.

2.3.2- Polyphenols Extraction Procedures

In this study, a mixture of equal parts of the four varieties of grapes (grapes of the same varieties and collected in the same vineyard as those used to produce the wines of the group 1) was used. Polyphenolic contents of grape seeds and skins were extracted through an 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 polyphenols residues obtained from grape seeds (condensed tannins) and from skins (mostly anthocyans, but also other polyphenols, as tannins, and polysaccharides, in smaller amounts) where dissolved in an aqueous/ethanolic solution with KNO_3 0.05 M, pH 3.5, 10% ethanol.

2.3.3- Determination of Pb and Cu by AAS-ET

Total Pb concentrations in all the wines was determined by atomic absorption spectroscopy (AAS) using a Perkin-Elmer 4100-ZL apparatus with electrothermal atomisation (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 pre-treatment, as described elsewhere (Simões *et.al.* 1995). The accuracy of the method was

checked by analysing some reference wines, BCR C (white wine), BCR D (liquor) and BCR E (red wine) obtained from the EEC's Bureau Communautaire de Référence (BCR). 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 atomisation on a Phillips 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 AAS-ET operating with the manufacturer's standard conditions for Cu.

2.3.4- Determination of Parameters of the Complexation of Cu with Tannin Solution by Fluorimetric Titrations

To study the complexation of tannins, extracted from grapes as described in the above section, towards Cu, titrations of 50 mL of 20 mg/L of tannins in KNO_3 0.05 M, pH 3.5, 10% ethanol were carried out with 10^{-2} M and 10^{-1} M Cu. To maintain the volume during the titration, the solution of tannins was continuously circulating in the spectrofluorimeter (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 this 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, response time of 2 s. Three replications were carried out.

2.3.5- Determination of Parameters of the Complexation of Cu and Pb in Wines and Polyphenolic Extracts by Potentiometric Titrations

This study included Cu titrations (for wines of groups 1 and 3 and to the polyphenolic extracts) and Pb titrations (for the wines of group 1 and polyphenolic extracts). All the experiments were carried out at 25 ºC. As titrant solution it were used standard solutions of Cu (0.01 M) and Pb (0.019 M). The concentrations of free Cu and Pb were measured with the respective ion selective electrodes (ISE) from Radiometer, activated with a mixture of Ag_2S / CuS e Ag_2S / PbS, respectively. As reference electrode a Orion de Ag/AgCl(s) KCl 3M (double junction) was used. A computer controlled arrangement comprising a decimilivoltimeter (Crison micro pH 2002)

coupled to an automatic burette (Crison micro BU 2030) was used. Three independent titrations were carried out for each system under study. The natural levels of Cu and Pb in the titrant, determined by AAS, were taken in consideration for the total metal concentration. Calibrations were performed in a KNO_3 0.05 M, pH 3.5, 10% or 20 % (for the wines of group 3) ethanol (v/v) medium.

2.3.6- Determination of Parameters of the Complexation of Pb in Wine and Wine Digests by Cathodic Voltammetric Titrations

This study was applied to the wines of groups 1, 2 and 3, to some digests of the wines of groups 2 and 3 and to tannin solution. Unfortunately, technical limitation restricted this study to Pb.

The cathodic voltammetric measurements consisted on 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) connected to a 663 VA stand (Metrohm). 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 KNO_3 0.05 M, pH 3.5, 10% (table wines) or 20 % (Port wines) ethanol. Intestinal digests (filtered with 0.45 μm pore size and unfiltered) were properly diluted in water and buffered to pH 7 with 0.3 mL of a solution 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 up to 4 μM (for wines and gastric digests) or from 0 μM up to 8 μM (for intestinal digests) of total Pb in the titrated solution. The dilution factors of the wine used may to some extent alter the aggregation degree of the macromolecular compounds, thus affecting their complexation capacity. However it was the only way that rendered possible to carry out measurements at relatively low Pb levels. Otherwise, that is, with undiluted wine and wine digests, the Pb would be strongly complexed with organic ligands, the labile Pb fraction would be detectable only when the total Pb concentration in wine was very high. Moreover, in undiluted red wine its red pigments tightly adsorb on the electrodes and are difficult to remove. The

spiking of Pb during wine and gastric digests titrations had a negligible influence on the titrands pH, being experimentally verified that a maximum pH decrease of 0.2 units 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, which affect the diffusion current of the metal (polyphenols and other organic ligands) or digests (those existent in wines plus the added enzymes, specially the biliar salts). Therefore, an internal calibration procedure was carried out. The internal calibration was attained with the last titration points, fitting a linear segment, which indicated that the fully saturation of inert ligands was achieved. When inert ligands saturation occurs, all the spiked Pb is in labile forms thus allowing the internal calibration. Thereby, the slope of the linear relationship gave the sensitivity of the measurements, and the current signal was proportional to the excess of Pb in labile form.

2.3.7- Separation of Wine and Wine Digests Fractions by Reverse-Phase High Performance Liquid Chromatography

As an attempt to get some information about the nature of the Pb and Cu ligands in some wines of the groups 2 and 3, and also in their respective digests, chromatographic experiments were carried out. The chromatographic conditions used are commonly utilised for separation of large molecules like peptides, proteins and tannins. The methodology consisted in detecting the sample components with UV detector, at 215 nm wavelength, with collection of chromatographic fractions corresponding to the different components (different retention times), for subsequent AAS-ET analysis. Due to limitations of technical character, the AAS-ET detection was not performed to consecutive fractions of the eluate. Instead, it were only collected all the fractions coincident with the chromatographic peak maxima obtained with the UV detection, regardless of the relative intensity of the peak. Therefore, the method could not provide quantitative information, but gave indication about the distribution of Pb and Cu among the organic components eluted at different retention times.

A Dionex ion chromatography system (model DX 300 IC) including a 50 μL or 100 μL loop, a Vydac C_{18} 218TP54 column (5 μ, 250 mm x 4.6 mm) with the corresponding guard column coupled to a UV-visible detector (Konic UVIS 204) was used. A gradient elution, 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 analysed for Pb and Cu within 24 h, by AAS-ET.

2.3.8- Simulation of the Gastrointestinal Digestion

For the same wines studied by RP-HPLC, an *in vitro* simulation of the gastrointestinal digestion was applied (figure 2.2). 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 termostatisation (at 37 ºC) of the contents. As an adaptation of the procedure described by Crews *et.al.* 1985 for various solid foods, 50 mL of wine were mixed with 25 mL of gastric juice (10 g/L pepsin, NaCl 0.15 M, HCl 0.02 M) and left with stirring for 3 h. It was necessary to ensure that in the course of reaction the pH of the mixture would not rise above 3.5. It was verified by monitoring by potentiometry with a glass pH electrode that, in fact, the pH did not achieve that value in any case. 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 of NaCl and 12 mL of pancreatic juice, containing 30 g/L of pancreatin and 10 g/L of amylase) were added to the contents of the reaction vessel and left with stirring for 3 h. All the enzymes were supplied by Sigma: pancreatin from porcine pancreas grade VI ref. P-1750; bile salts approx. 50% sodium cholate and approx. 50% sodium deoxycholate, ref B-8756; pepsin from porcine stomach mucosa (pepsin A, EC 3.4.23.1) ref. P-7000; α-amylase from porcine pancreas (EC 3.2.1.1) type VI-B. Enzyme solutions were always freshly prepared. Blank simulations, with 50 mL of deionised water in the place of wine were regularly carried out, for contamination control.

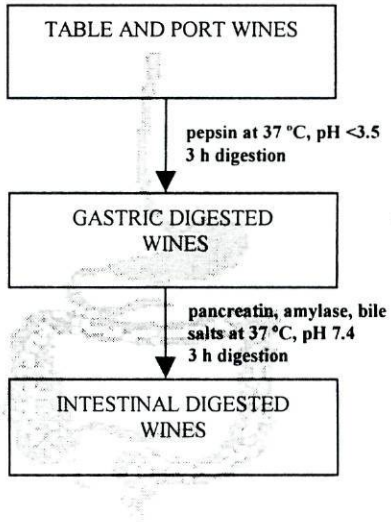

Fig. 2.2
Scheme of the experimental strategy used for simulation *in vitro* digestions of wines

2.3.9- Determination of the 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 the aliquots was filtered with a 0.45 μm membrane filter. The fraction

of the total metals that remained soluble was determined directly by the ratio of AAS-ET signals for filtered and unfiltered samples. Control filters were carried out through the same procedure, using deionised water in the place of wine, and the blank signals when significant, were subtracted to the sample signal.

2.3.10- Dialysability of Pb and Cu During 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), what resembles more accurately the *in vivo* situation in kinetic terms. During the intestinal phase of the digestions, a 5 mL dialyser (QuixSep®, MFP Inc.) filled with a solution of 0.15 M NaCl, equipped with a 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 dialyser was removed and the dialysate was analysed for Cu and Pb contents by AAS-ET. Since the volume outside the dialyser was much greater (approximately 20 times) than the volume inside the dialyser, the decrease of the outside metal concentrations due to dialysis is neglected, and so, the dyalisable fraction was simply determined as the ratio between outer and inner metal AAS signals. Control dialysis were carried out through the same procedure, using deionised water in the place of wine, and the blank signals when significant, were subtracted to the sample signal.

2.4 - DATA TREATMENT

Wines and their digests have very complex matrixes and there is lack of information about the molar concentration sites (for both proton and metal ions) of various groups of compounds whose individual chemical structure is unknown or not well defined (like tannins, polysaccharides, peptides or proteins, and combinations of them). Therefore, the well established computational methods for determination of stoichiometric stability constants of monomeric ligands can not be applied. The polymeric ligands of the wines and their digests may act as heterogeneous ligands. These substances display numerous coordination sites and, owing to differences in the nature of the sites and/or electrostatic and steric effects, their complexation properties change during a titration with a metal ion. Concretely, the microscopic stability constants of the respective complexes 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 description of the models used in the present study can be found in the literature (Scatchard 1949; Buffle 1988; Shuman *et.al.* 1983). Only the concepts and equations relevant for understanding the enological significance of the method are summarised here.

2.4.1- Fluorimetric Data

The addition of a paramagnetic metal ion to a fluorescent ligand, like 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 equation 1 (Ryan and Weber 1982a; Ryan and Weber 1982b):

$$I_F = I_F^{L} + I_F^{ML} \qquad (2.1)$$

where I_F^{L} and I_F^{ML} are the fluorescence intensity of the free ligand (L) (that is, not bound to M) and of the complex (ML), respectively.

The models applied to complex heterogeneous ligands begin by assuming that the stoichiometry of the complex formation is 1:1

$$M + L \rightleftarrows ML \qquad (2.2)$$

an average conditional stability constant, K_{av}, is defined by

$$K_{av} = |ML| / (|L| . |M|_{free}) \qquad (2.3)$$

or

$$K_{av} = |M|_{bound} / (|L| . |M|_{free}) \qquad (2.4)$$

where $|M|_{bound} = |M|_{total} - |M|_{free} = |ML|$

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, equation 2.4 can be converted to

$$I_F = 1-(1/(2.K_{av}.CC_{total}))[(K_{av}.CC_{total}+ K_{av}. |M|_{total}+1)-$$
$$-((K_{av}.CC_{total}+ K_{av}. |M|_{total} +1)^2 -4. K_{av}{}^2.CC_{total}. |M|_{total})^{1/2}] \qquad (2.5)$$

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

Having I_F and $|M|_{total}$ 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 optimisation as described by Nelder and Mead (1965). Calculations were performed using a Qbasic program developed in our laboratories by Luis Ribeiro.

2.4.2- Potentiometric Data

The CC_{total} of the samples as well as mean values of conditional stability constants, K_{av}, were determined by the Scatchard plot. As a starting, unavoidable condition of the method it is assumed that the complexes formed between the metal and the ligands have an 1:1 stoichiometry (see equations 2.2-2.4):

Applying mass balances, equation 2.6 is obtained

$$|M|_{bound}/|M|_{free} = K_{av}.CC_{total} - K_{av}.|M|_{bound} \quad (2.6)$$

Equation 6 implies that a plot of $|M|_{bound}/|M|_{free}$ against $|M|_{bound}$ displays only one approximately linear zone whose slope is $-K_{av}$ and the intercepts correspond to $K_{av}.CC_{total}$. $|M|_{free}$ is given experimentally by the ISE measurements. The slopes and intercepts were computed by linear regression analysis. 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 $|M|_{bound}/CC_{total} = \theta$ embraced (*i.e.* to which a linear range was obtained in the Scatchard plot).

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

$$CC_{total} = CC_1 + CC_2 + ... \quad (2.7)$$

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 by 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). This focused calculations allow 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:

$$K_{DEF} = -(\alpha^2/|M|_{total}) \cdot [1/(1+(\alpha-1).(d\ln(|M|_{total}/CC)/d\ln\alpha)] \quad (2.8)$$

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

K_{DEF} are related with K_{av} and θ through equation 2.9:

$$K_{DEF} = -\frac{d[K_{av}(1-\theta)]}{d\theta} \quad (2.9)$$

In an opposite way, for each selected θ range, a mean K_{DEF}, equivalent to the K_{av} can be computed by equation 2.10:

$$\overline{K_{DEF}} = \frac{\sum_j K_{j\,DEF}}{m} \quad (2.10)$$

where K_{jDEF} are the specific values of K_{DEF} and m is the total number of different K_{jDEF} 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.

2.4.3- Voltammetric Data

The voltammetric method used makes the distinction between two metal fractions, the labile metal, M_{labile}, and the inert metal, M_{inert}. The inert fraction corresponds to the metal strongly complexed which is not reduced

at the electrode surface. The labile fraction corresponds to the free metal plus the complexed metal which dissociates within the time scale of the measurement (labile complexes):

$$|M|_{labile} = |M|_{free} + |ML|_{labile} \quad (2.11)$$

The equilibria involved in the complex formation are:

$$M_{free} + L_i \rightleftarrows |ML_i|_{inert} \quad (2.12)$$

$$M_{free} + L_j \rightleftarrows |ML_j|_{labile} \quad (2.13)$$

The conditional stability constants are given by:

$$K_{inert} = |ML_i|_{inert}/(|M|_{free}.|L_i|) \quad (2.14)$$

$$K_{labile} = |ML_j|_{labile}/(|M|_{free}.|L_j|) \quad (2.15)$$

where $|L_i|$ and $|L_j|$ are the total concentrations of the ligands not bound to the metal.

Combining 2.11 and 2.15 it gives:

$$|M|_{labile} = |M|_{free}.(1 + K_{labile}.|L_j|) \quad (2.16)$$

or

$$|M|_{labile} = \alpha.|M|_{free} \qquad (\alpha = 1 + K_{labile}.|L_j|) \quad (2.17)$$

Combining 2.14 and 2.17 it gives

$$K_{inert} = \alpha . |ML|_{inert} / (|M|_{labile} . |L_i|) \quad (2.18)$$

Applying mass balances the following equation is achieved:

$$|ML_i|_{inert} / |M|_{labile} = - |ML_i|_{inert} . K_{inert} / \alpha + CC_{inert} . K_{inert} / \alpha \quad (2.19)$$

A plot of $|ML_i|_{inert} / |M|_{labile}$ *vs.* $|ML_i|_{inert}$ will then have a slope of $-K_{inert}/\alpha$ and an intercept of $CC_{inert}.K_{inert}/\alpha$, which means that the intercept = $-CC_{inert}$.slope. Thus the CC_{inert} value can be calculated from the intercept and slope values of the Scatchard plot. Sufficient data for computing the value of α is unavailable and, therefore, it remains unknown, but it is certainly higher than 1. Therefore, considering $\alpha = 1$, the slope of the Scatchard plot will provide an underestimation of the real value for K_{inert}, which was designated as K'_{av} throughout the work. The slopes and intercepts were computed by linear regression analysis.

If we consider α approximately constant within a set of wines under study, a comparative basis for the strength of the inert complexes formed in different wines is, based on the respective K'_{av} values can be established.

CHAPTER 3

INFLUENCE OF Cu IN THE PROCESS OF FERMENTATION

CHAPTER CONTENTS

3- Influence of Cu in the Process of Fermentation

3.1- Introduction

Ethanolic fermentation is a complex process involving several metabolic reactions which are described in the literature in some detail (*e.g.* Wills 1990). Nevertheless, the ethanol production is dependent on different factors, such as temperature, pH, or the presence of enzyme modulators or together with specific enzyme inhibitors. Metal ions (K^+, Mg^{2+}, Ca^{2+} and Zn^{2+}) (Walker and Maynard 1997; Chandrasena *et.al.* 1997) can change the rate of glycolysis and, subsequently, the conversion of pyruvate to ethanol. However, the mechanisms underlying the regulation of glycolysis rate and ethanol production by metal ions remains to be elucidated, and it is relevant to know their optimal levels for industrial fermentations. The influence of heavy metals in the fermentative metabolism is even less known but their presence is usually regarded as a problem for the fermentation process, what led to the use of heavy metal sorbents as zeolite X or chelating agents as EDTA in some cases (Ergun *et.al.* 1997). Cu is a heavy metal essential to all organisms, a constituent of some enzymes, such as cytochrome oxydase in the mitochondria (Silva and Williams 1993), superoxide-dismutase in the cytosol and a ferroxidase in the plasma membrane. For Cu and the other micronutrients there is a relatively narrow interval of concentration optimum for the organisms, and at higher concentrations toxic effects occur. Some mechanisms of toxicity of heavy metals towards *S. cerevisiae* are well known (Blackwell *et.al.* 1995), as well as the molecular mechanisms of protection against them (Perego and Howell 1997). In the case of Cu it is known that it is transported inside by Ctr1p (Cu transporter 1 protein) and Ctr3p and appropriately distributed to Cu-requiring proteins, and that cells possess sensors of high Cu concentrations, which activate protecting mechanisms (metallothionein production) and deactivate Cu transport mechanisms (Knight *et. al.* 1996, Santoro and Thiele 1997). The toxicity of copper is dependent on the *S.cerevisiae* strain and growth conditions (Sarais *et al.* 1999, Pearce and Sherman 1999). The observed tolerance has been associated with increased enzyme activities such as Cu,ZnSOD or H(+)ATPase (Fernandes and Sá-Correia 1999).

However, the influence of Cu concentration on the alcoholic fermentation process is not known yet, namely regarding must fermentation, and was the main objective of this work. Alcoholic fermentation was studied

in a wide range of Cu concentrations (from natural levels to 1.0 mM) for two different fermentative media, white grape musts (an highly complex matrix which contains several Cu ligands) and yeast nitrogen base (YNB) with glucose.

3.2- Experimental

This study was conducted both in a laboratory of cellular and molecular biology (Unity of Stress in Microorganisms – Institute of Molecular and Cellular Biology, of Porto University) and in our clean chemistry laboratory devoted to trace and ultratrace metal speciation, where the metal concentration determinations by AAS-ET, the ion chromatography determination of ethanol, and the fermentation monitoring was carried out. The experimental details are described in the previous chapter Experimental Section.

3.3- Estimation of the Bioavailable Cu in the Synthetic Medium

Preliminary work was carried out in order to find a fermentative media with a chemical composition as simple as possible, where the Cu speciation and the evaluation of the bioavailable fraction of the metal would be easier. A yeast nitrogen base (YNB) with uracil, leucine, histidine and glucose was the medium chosen, with minima concentrations to allow a fair growth. The inclusion of sodium tartrate to buffer the pH at 3.5 was considered, but it was rejected after verifying that the tartrate complexed Cu, thus decreasing the bioavailable Cu in the medium. Therefore, instead of pH buffering, the pH of the medium was initially adjusted to 4. It was experimentally verified that during the experiments the pH of the medium varied between 4 and 3, which are typical values of musts and wines.

The total concentration of Cu in the YNB medium is known, 40 μg/L, as indicated in the product's data. DPASV was used to determine the operationally available Cu in the culture medium, as an estimation of its bioavailability. A concentration of 43 μg/L labile Cu was obtained by Cu standard additions, which indicated that all the Cu existent the YNB medium was labile. Moreover, as figure 3.1 shows, the voltammetric peaks of Cu obtained in culture medium without Cu addition (diluted 1:2 with

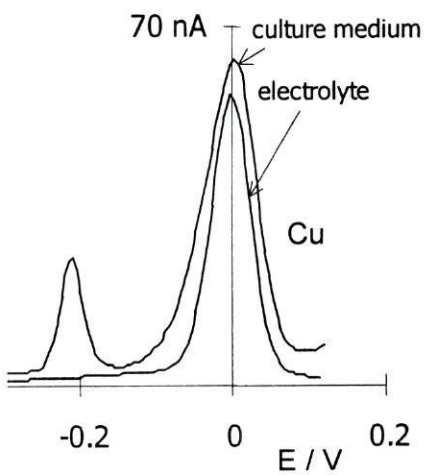

Fig. 3.1
DPASV voltammograms obtained for YNB culture medium diluted 1:2 with electrolyte ($NaClO_4$ 0.5 M at pH 3.5) and for 0.31 μM of Cu in the electrolyte diluted 1:2 with deionised water

a 0.5 M $NaClO_4$ solution with pH 3.5) and those obtained in standard solution (0.25 M $NaClO_4$ solution with pH 3.5 spiked with 0.31 μM of Cu) appeared at identical potentials (around 0 V). A cathodic shift of the peak potential, relative to that of the electrolyte, would occur if complexation of Cu existed (Buffle J. 1988) in the YNB medium, unless surface active substances eventually present in the medium, but not probable in the present case, would mask that shift (Sagberg and Lund 1982). Thereby, all the information available indicates that the Cu existent in the medium (originally present or added to) is not strongly complexed in YNB at pH 3.5 and therefore it is available to yeast cells.

3.4- Growth Curves and Cu Sorption

Figure 3.2 shows the growth curves of cells incubated in the working medium in the presence of different Cu concentrations. Metal levels up to 0.10 mM did not affect growth, whereas for 0.50 mM Cu a marked decrease of the rate of growth occurred. Nevertheless, the final cell density was similar to that reached in the control medium. For 1.0 mM the cells did not grow, indicating marked and rapid toxic effects.

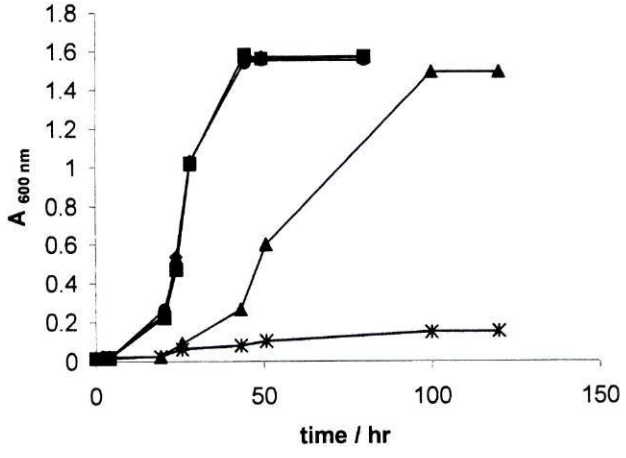

Fig. 3.2
Cell culture growth in synthetic fermentative medium with different Cu concentrations: (-◆-) blank; (-●-) 0.010 mM; (-■-) 0.10 mM; (-▲-) 0.50 mM; (-✱-) 1.0 mM

The total Cu (extracellular + intracellular) sorbed by the cells was determined during the first hours of the cultures growth, for the different initial total dissolved Cu. Figure 3.3 shows the results of a typical experiment. As expected, the higher levels of Cu concentration in the growth medium led to higher metal sorption by the cells. In the media with added Cu 0 (background level 0.63 μM) and 0.10 mM,

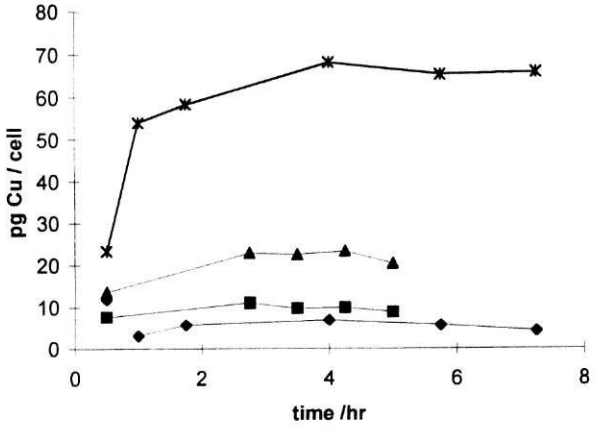

Fig. 3.3
Cu sorption by *S. cerevisiae* cells during culture growth at different Cu concentrations: (-◆-) blank; (-■-) 0.10 mM; (-▲-) 0.50 mM; (-✱-) 1.0 mM

sorption up to 10 pg Cu / cell were observed without toxic effects. In the medium with 0.50 mM Cu, a sorption of about 20 pg Cu / cell was observed in the first hours of growth, and the rate of cell growth was very low. Indeed, the culture density of A_{600} = 1.5 was reached after 4 days, in contrast with control cells that could reach the same density in 2 days. For the medium with 1.0 mM Cu about 65 pg Cu / cell was found, and the cultures did not grow appreciably during the 5 days experiment. In fact, the toxic effects of heavy metals to microorganisms usually correlate well with the amount of accumulated metal by the cells (yeast: Gadd and Mowll 1983; Avery *et.al.* 1996; bacteria: Azenha *et.al.* 1995; microalgae: Leal *et.al.* 1999).

The metal uptake by yeast may be influenced by a number of environmental and experimental factors (Blackwell *et.al.* 1995; Brady and Duncan 1994). Due to that, a meaningful detailed comparison with other results published for yeasts but obtained in conditions different of those used in the present study, was not possible. Nevertheless, it is worth to notice that the levels of Cu sorption obtained in this work are much lower than those reported for *S. cerevisiae* by other authors (e.g. Brady and Duncan 1994), up to 0.7 μmol/mg cell dry mass at 0.50 mM of Cu external concentration (at pH 5.0 - 9.0), where the yeast cells were considered as promising bioaccumulators for removal and/or recovery of heavy metals from waste waters.

The intracellular Cu was determined, after 5 hours of growth, as the Cu not accessible to (and not removed by) an aqueous solution with 15% HCl and 1.2 M sorbitol. For all the dissolved Cu concentrations studied, the quantification of the intracellular Cu turned out to be very inaccurate because the levels found were very low, less than 5 pg Cu / cell, close to the detection limits of the analytical method used (AAS-ET). Cells were able to maintain the normal intracellular concentration (as compared with the control medium) even for extremely high external Cu concentrations, indicating that the major toxic effects of Cu were focused in cell wall. The high capacity of the cell wall of *S. cerevisiae* to bind Cu is well known and occurs mainly at anionic sites of the mannan polymers (Brady *et.al.* 1994). As a result of Cu sorption, membrane damage can occur due to the substitution of mobile cellular solutes, leading to an increased permeability, or by involvement in free radical mechanisms which affects cell integrity by lipid peroxidation of membranes (Blackwell *et.al.* 1995). A damage of the cell wall may contribute for the "normal" intracellular level found, by making some of the intracellular Cu accessible to acid removal.

The results of culture growth and Cu sorption showed that, under the experimental conditions used, *S. cerevisiae* cells were very tolerant to high dissolved available Cu concentrations (up to *ca.* 0.50 mM). Such high levels are unrealistic of the environmental, biological, and food processing points of view.

3.5- Monitoring of the Ethanol Production During the Fermentation Process

A typical ion exclusion HPLC chromatogram obtained for a fermented culture medium is shown in figure 3.4 This includes (upper right corner) the calibration curve of the chromatographic system obtained, signal = f(% ethanol).

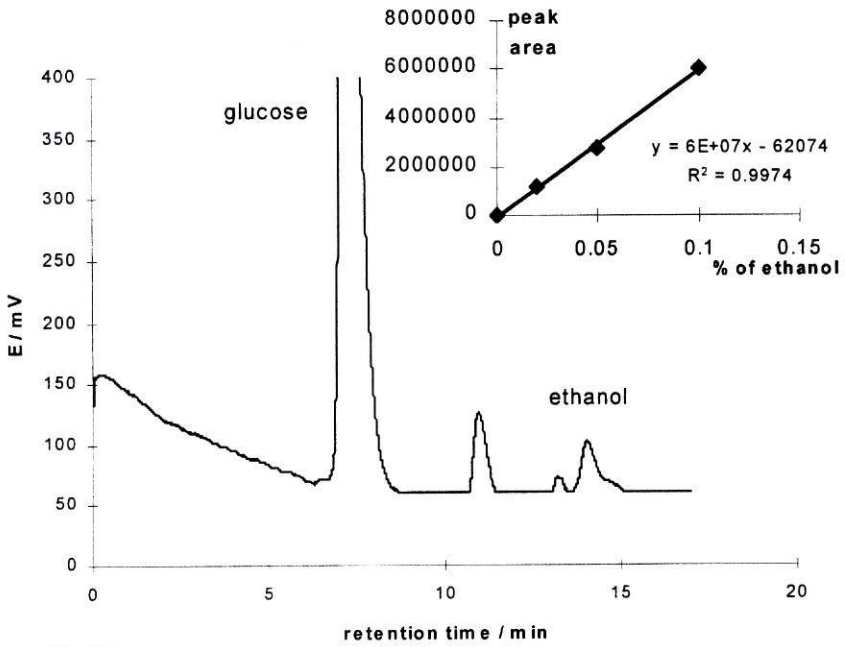

Fig. 3.4
Typical ion exclusion chromatogram obtained from monitoring fermentation of culture medium, using a Bio-Rad-HPX 87H column, 5 mM H_2SO_4 as eluant, at a flow rate of 0.7 mL/min and integrated amperometric detection. On the upper right corner: calibration curve for ethanol quantification

The relationship observed between the percentage of ethanol (v/v) in the fermentation medium and the mass of CO_2 released is presented in figure 3.5 A fairly linear relationship was obtained, in agreement with the results reported in the literature (Sablayrolles *et.al.* 1987; El Haloui *et.al.* 1988; Bely *et.al.* 1990). Therefore, for measuring the release of CO_2, the described system provided an acceptable indirect method of monitoring the ethanol production during the fermentations.

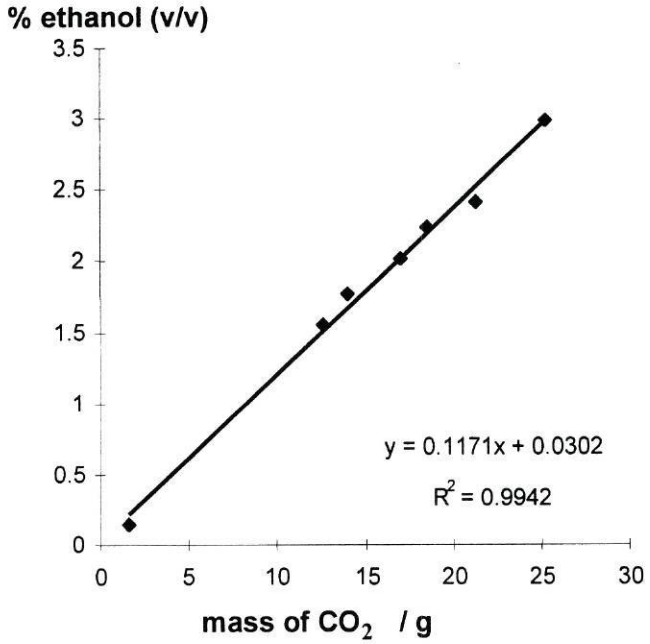

Fig. 3.5
Experimental relationship obtained between the percentage of ethanol (v/v) in the fermentative medium and the mass of CO_2 released. Relation valid for 1L of fermentation medium

3.6- Musts Fermentations

The production of ethanol during fermentation of musts, with different total Cu concentrations, in the range 0.018 - 1.0 mM, is presented in figure 3.6. The value 0.018 mM was the natural total Cu concentration in the musts (determined by acid digestion followed by AAS-ET). It is shown that there were not very marked differences between the percentage of ethanol yielded by the fermentations in the different cases

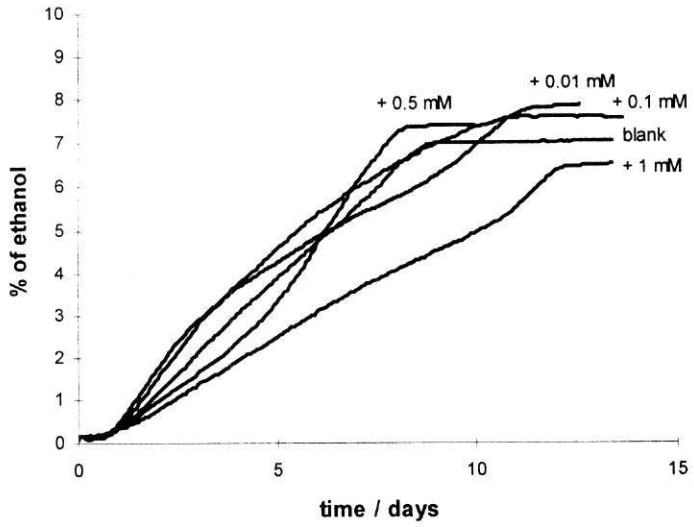

Fig. 3.6
Ethanol production (in v/v percentage) during must fermentations at different Cu concentrations

(6.5 - 7.9 %), even when extremely unrealistic high Cu concentrations were used, especially 0.50 and 1.0 mM. Nevertheless, for 0.028 (0.018 + 0.010 added Cu), 0.12, and 0.52 mM of total Cu, the percentage of yielded ethanol was a little higher than that in the control situation, and for 1.0 mM Cu the kinetics of ethanol production was slower than in all the other situations. Therefore, a slight positive influence in the fermentation process of Cu concentration up to 0.50 mM has occurred, and a slight negative influence was observed for 1.0 mM, likely due to the toxicity of Cu at this level, as occurred in the YNB medium (see below).

The Cu concentration in musts usually lies in the 0.010 - 0.10 mM interval (Curvelo-Garcia 1988) which included the mean value observed in the present study, 0.018 mM. The study carried out for the synthetic medium showed that such Cu levels were not toxic for *S. cerevisiae*. In the musts, similar Cu concentrations were non toxic as well, as expected, because the presence of many organic ligands probably still decreases the bioavailable fraction of Cu by metal complexation. Therefore, Cu concentrations up to at least 0.10 mM, are expected not to affect the normal cell growth in musts.

3.7- Fermentation in the Synthetic Working Medium

Typical results of ethanol production in fermentations of the synthetic medium are presented in figure 3.7. For 0.010 mM Cu concentration, only a slight increase (as compared with the blank fermentation, i.e. that in the medium without Cu addition) on the ethanol production was observed (from 2.0 to 2.2 % ethanol), and no significant differences at the kinetics of the fermentation (6 days to completion). For the Cu concentrations 0.10 mM, 0.50 mM and 1.0 mM, marked effects were observed both in the ethanol production and in the kinetics of the fermentation. Indeed

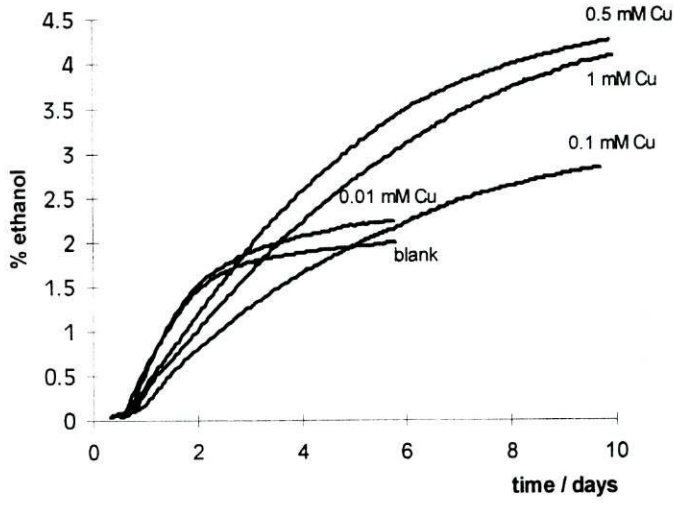

Fig. 3.7
Ethanol production (in v/v percentage) during fermentations in synthetic medium at different Cu concentrations

the ethanol production doubled for the higher metal concentrations, 0.50 mM (4.3 % ethanol achieved) and 1.0 mM (4.1 % ethanol achieved), though the fermentations became clearly slower (more than 10 days to completion).

As referred above, in the synthetic fermentative medium, whose matrix is much simpler than that of the musts, strong Cu complexation does not occur, the Cu being more available to interact with the cells. These interactions, whatever their nature is, notoriously affected the process of fermentation in the synthetic medium, resulting in an increase in the ethanol production and a lower fermentation kinetics.

3.8- Conclusions

In the YNB medium, the ethanol production and fermentation rate were highly influenced by the Cu concentration, being the yield of ethanol double in the presence of 0.50 and 1.0 mM Cu. However, the fermentation rate was slower. As for production of ethanol from musts, only minor effects were observed for the different Cu concentrations, which indicates that Cu levels are not responsible for changes in fermentation, and, therefore, are below any toxic level regarding the yeast performance.

From a practical biotechnological point of view, our results envisage the interest to perform future work on the influence of Cu in the media used in the ethanol production industry, when using *S. cerevisiae* or other cells. The biochemical explanation for the phenomena observed is an open research topic, indeed it is possible that there is a link between the effect of Cu and Fe availability, as Cu is required for Fe transport into yeast cells.

CHAPTER 4

SPECIATION OF Pb IN WINES AND WINE DIGESTS

CHAPTER CONTENTS

4- Speciation of Pb in Wines and Wine Digests

4.1- Introduction

As referred before (section 1.1.4.3) there is still a lot to know about the nature and effective concentration of the wine ligands, as well as about their strength (equilibrium constants) to bind heavy metals, particularly Pb.

The lack of information about the metal speciation in wines at the gastrointestinal level, with direct relation on the bioavailability, is total. The bioavailability of an element depends on the capacity of the physico-chemical forms under which it is present at the site of absorption to surpass the intestinal barrier. The metal that can surpass the intestinal barrier is potentially bioavailable to interact with the biological molecules in blood and other fluids or tissues reached by the metal. The intestinal absorption requires that those forms are soluble, being a necessary condition but not sufficient (Hocquellet 1997). At present, there are well established *in vitro* methods (faster and less expensive than *in vivo* methods) for estimating the element solubility in simple stomach animals (Boisen and Eggum 1991). These methods comprise a two phase simulation of the gastrointestinal physiology, the stomachal and intestinal phases. The stomachal phase is essentially proteolytic by the action of pepsin and HCl at 37 ºC (Boisen and Eggum 1991) and will have a significant importance only if the food being digested is protein rich or medium rich (which is not the case of wine). The intestinal phase, occurring at slightly alkaline pH, and also at 37 ºC, constitutes a more embracing hydrolytic medium, being active the pancreatic juice, biliar salts and amylase enzymes. At this level, proteins and peptides hydrolyse to aminoacids, starch polymers to maltose, maltotriose and α-limit dextrins (which are further hydrolysed to glucose), fats to mixed micelles consisting of monoacylglycerols, long-chain fatty acids, bile salts and phosphoacylglicerols, and carbohydrate residues (like pectins and β-glucans) to monosaccharides (hydrolysis by microflora) (Boisen and Eggum 1991). The soluble fraction of an element at the end of this stage is a useful but insufficient datum for the estimation of assimilable fraction of the element, i.e., that can surpass the intestinal membrane.

It has been demonstrated that the fraction of metal that penetrates dialysis membranes (12-15 KDa molecular weight cut-off) under simulated physiological conditions (in which the pH adjustment from gastric to intestinal levels is achieved gradually by addition of $NaHCO_3$), broadly mimics the events occurring when food leaves the stomach and enters the duodenum (Hazell and Johnson 1987). This method showed to give a good estimate for the *in vivo* assimilable fraction of several mineral and trace elements proceeding from different foodstuffs, *e.g.* Fe in composed meals (Miller *et.al.* 1981a and b), and Zn and Ca from milk- and soy-based formula (Shen *et.al.* 1994). On the other hand, other authors have found that zinc dialysability from a series of foods was not a strong predictor of zinc bioavailability (Hunt *et.al.* 1987). Therefore, valid correlations between *in vivo* and *in vitro* assays are not extrapolable for other nutrients and/or other foods. However, the use of the method of continuous dialysis has been frequently used without *in vivo* validation, *e.g.* for studying the effect of citric acid on the dialysability of heavy metals from a maize-soya diet (Walter *et.al.* 1998), for the dialysability of Fe, Zn and Cu of different types of infant formulas (Garcia *et.al.* 1998) and for the estimation of the bioavailability of minerals and trace elements of breads (Wolters *et.al.* 1993). In such cases the results must be taken as relative indexes for the assimilability, which means that the method provides a good basis for establishing tendencies, comparisons and determination of effects caused by different factors.

Therefore, the methods just described were used in the present work as relative indexes of the solubility and the assimilability of metal intake by simple stomach animals like man, in spite of their limitation: they do not account for the microflora activity and other influencing *in vivo* factors such as the adaptive character of the enzyme secretions face to changes in the substrate intake. As far as we know these methods have never been applied before to the bioavailability of metals in wines.

The first purpose of the present work was to study the complexometric properties to Pb of four different red wines produced from only one of the following varieties of grapes, cultivated in the Douro's Demarked Region of Portugal: "Tinta Barroca" (TB), "Tinta Roriz" (TR), "Touriga Nacional" (TN) and "Touriga Francesa" (TF). Parallelely, 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 complexation properties were expressed in terms of (a) maximum complexation capacity (CC_{total}) of the ligands from the wines, that is, the total concentration of the sites available in the sample, and (b) conditional stability constants of the complexes with Pb. For this purpose, the Scatchard plot and the Differential Equilibrium Function (DEF) models, which have been considered suitable for characterisation of complexes involving heterogeneous ligands (Scatchard 1949; Buffle 1988) were used.

The second purpose of this study was to determine some speciation parameters for different types of table wines (red, white and Verde) and Port wines (white, tawny, ruby, dated, young red, with age indication). In this case the wines were also treated as heterogeneous ligands, and the speciation parameters studied were the estimates of the conditional stability of the Pb inert complexes (K'_{av}) described in the Data Treatment section, the concentration of the ligands that form inert complexes (CC_{inert}) and the chromatographic profiles of Pb elution in conditions usually set for macromolecules, which gave some insight into the nature of the Pb complexes in wines and wine digests.

The third purpose was to study the fate of the Pb present in some of the table and Port wines when they were subjected to *in vitro* gastrointestinal digestion. The same speciation parameters studied for Pb in wines were also studied after gastric and gastrointestinal digestions. Moreover, information about Pb solubility and dialysability after the digestion was gathered.

4.2- Experimental

For the determination of the conditional stability constants of Pb complexes in wines, the potentiometry with Pb ion selective electrode and the square wave cathodic voltammetry (SWCV) were used. The voltammetric technique allowed the under-estimation of the strength and the concentration only of the operationally inert complexes, which are the most stable and so those that really coordinate the metal of the wine, where the metal to ligand concentration ratio is very low. Therefore, SWCV dealt with more realistic metal to ligands concentration ratios, although K'_{av} values lower than the real were systematically obtained, as described in the Data Treatment section.

The Reverse-Phase High Performance Liquid Chromatography (RP-HPLC) experiments were performed in a C_{18} column suitable for peptide, protein and tannin separation, and eventually other macromolecules.

The *in vitro* digestions procedure was optimised from a method applied to solid foods (Crews *et.al.* 1985) and the dialysis experiments were adapted from Hazell and Johnson (1987)

All the experimental details can be found in the chapter Experimental Section.

4.3- Determination of the Complexation Parameters in Wines and Polyphenolic Extracts

4.3.1- Wines of Group 1 and Polyphenolic Extracts

4.3.1.1- Potentiometric Study

The potentiometric titration curves of wines with Pb are illustrated in figure 4.1 (for "TN" wine). For comparison purpose, the respective calibration curve was included in figure 4.1. It is worth to notice that because the ligands exert a buffer effect towards Pb concentration, in the real samples the lower limit of the practical linear response range was extended from $5x10^{-5}$ M (in the calibration) to $5x10^{-7}$ M.

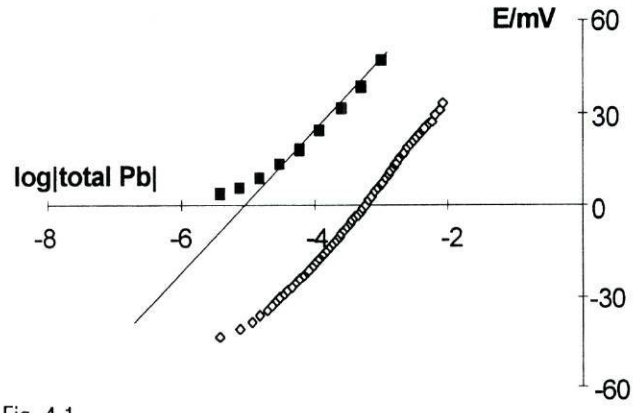

Fig. 4.1
Typical potentiometric calibration (■) and titration (◊) curves obtained for "TN" red wine. In the abscissas axis the |total Pb| refers to the |Pb| naturally present in the wine plus that added.

Data of titration curves were firstly treated through the Scatchard plot, as figure 4.2 illustrates only for "TF" wine, for the sake of simplicity. Two linear zones with markedly different slopes were obtained for "TF", corresponding to groups of ligands of different macroscopic complexation properties. The "TN" wine behaved similarly. This means that, macroscopically, in the

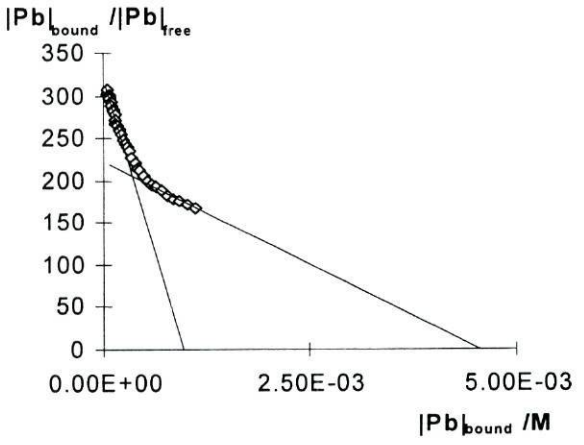

Fig. 4.2
A typical Scatchard plot obtained from potentiometric data for "TF" red wine

experimental conditions studied, the systems could be reduced to two different types of ligand with markedly different coordination strengths, expressed by K_{1av} (the higher value) and K_{2av}. For "TR" and "TB" wines only one linear zone was observed. The group of ligands that coordinated the metal ions first (thus corresponding to the lower $|Pb|_{bound}$ values embraced in the potentiometric experiments), are those with the higher metal affinity or the measured stronger sites (K_{1av}). The mean values of CC and K_{av} obtained for the different wines and for the grape extracts are reported in table 4.1, which includes the range of θ embraced in each case.

One straight observation is that the mean CC_{total} of wines ranged from $4.5x10^{-3}$ M ("TF") to $1x10^{-2}$ M ("TN"). Solutions with 2 g/L of tannins displayed a mean CC_{total} of $2.6x10^{-3}$ M, corresponding to 1.3 mmol of active sites per gram. For solutions of 20 g/L of skins extract, a mean CC_{total} of $5.3x10^{-3}$ M was determined, corresponding to only 0.26 mmol per gram. The relatively low CC_{total} value obtained for the skin extract suggests that the complexation capacity is probably due to the minor non-anthocyanic fraction (containing other polyphenols, as tannins, and also polysaccharides, and possibly combinations of them) which is also obtained by the extraction employed. Moreover, taking into account the concentration of the polyphenols in the wines (table 2.2), it is secure to state that other abundant ligands with Pb complexing ability have to be present in wine, since the Pb CC_{total} found for the wines is much higher. In fact, recent literature refers other types of ligands as main Pb complexants in wines. A work by Szpunar *et.al.* (1998) reported the identification of an important (or even dominant, in some cases) Pb ligand in wines, the dimeric polysaccharide rhamnogalacturonan-II. Another recent work (McKinnon and Scollary 1997) refers proteins and tannins as probable macromolecular Pb ligands in wines.

Table 4.1 also shows that the values of log K_{1av}, which correspond to the stronger sites of the wines that can be measured by potentiometry (the analytical window of observation used), were much higher for "TF" (5.6) and "TN" (5.4) than for "TB" (4.1) and particularly for "TR" (3.0). The values of log K_{2av} (weaker sites) determined for "TF" (4.8) and "TN" (4.9) were still higher than the log K_{1av} obtained for "TB" and "TR". Tannins exhibited log K_{av} values similar to that of the "TR" wine, and lower than those observed for the other wines, indicating that stronger ligands have to be present in wines. Skin polyphenols presented a log K_{1av} value higher than those found in the wines, and a log $K_{2av} = 4$. However the θ ranges in the wines and skins extract titrations were markedly different, which prevent a meaningful comparison of the results.

The values of K_{DEF} obtained from the potentiometric data for the different wines are illustrated in Fig 4.3. As expected, based in the K_{av} values discussed above, "TF" and "TN" displayed higher K_{DEF} values than "TB"

and "TR". The heterogeneity of the systems, given by the respective slope of log K_{DEF} = f(log θ), was small in all cases and practically absent in "TR" and "TB" wines.

Typical values of K_{DEF} obtained for solutions of 2 g/L tannins and of 20 g/L skin extract are also shown in figure 4.3. When an identical occupation degree of the ligands by Pb is considered, for instance log ($|Pb|_{bound}/CC_{total}$) = log θ = -1.0, the log K_{DEF} values for Pb-tannins will be markedly lower than those observed for the wines, whereas for Pb-skin extract they will be higher. These results clearly demonstrate that the skin extract includes much stronger Pb complexing agents than tannins, probably not the anthocyans but polysaccharides and other ligands.

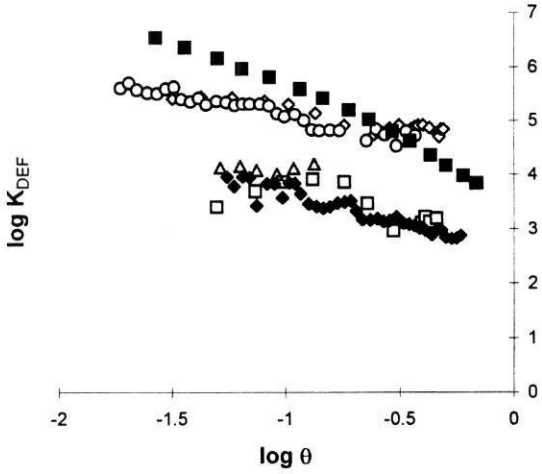

Fig. 4.3
Values of log K_{DEF} obtained from potentiometric data for the four wines studied, "TR" (Δ), "TB" (□), "TN" (o), "TF" (◊); and for tannins (◆) and skin polyphenols (■), as a function of log θ (θ = $|Pb|_{bound}/|L|_{total}$)

The levels of Pb found in the wines ranged from $5.0x10^{-8}$ M ("TF") to $1.3x10^{-7}$ M and ("TB"), which correspond to estimated values of log θ between –4.7 and –5.1 for the different wines, attending to the values of CC_{total} observed. Therefore, from the obtained potentiometric results it can be expected that the Pb present in "TN" and "TF" wines will be strongly complexed (log K_{DEF} > 5.5). The results obtained by potentiometry for "TB" and "TR": log $K_{DEF} \leq 4$ for log $\theta \geq -1.4$ do not allow to predict strong Pb complexation in these wines.

Table 4.1 - Complexation capacities (CC) of Pb displayed by different wines and by solutions of tannins and skins polyphenols isolated from grapes.* Standard deviations of CC are given in brackets.

	POTENTIOMETRY							VOLTAMMETRY					
Titrand [#]	***CC_1/mM***	***log K_{1av}***	***θ range***	***CC_2/mM***	***log K_{2av}***	***θ range***	***CC_{total}/mM***	***CC_{1inert}/mM***	***log K'_{1av}***	***θ range***	***CC_{2inert}/mM***	***log K'_{2av}***	***θ range***
TB	6.3(0.9)	4.1	0.040-0.16	—	—	—	6.3	—	—	—	—	—	—
TR	2.1(0.7)	3.0	0.022-0.12	—	—	—	6.7	$27(10)\times10^{-3}$	7.4	0.0014-0.0075	—	—	—
TF	0.9(<0.1)	5.6	0.015-0.036	3.6(0.6)	4.8	0.12-0.19	4.5	—	—	—	—	—	—
TN	2.1(0.5)	5.4	0.03-0.12	8.3(0.8)	4.9	0.17-0.45	10	$43(11)\times10^{-3}$	7.6	0.0025-0.0062	$25(15)\times10^{-3}$	6.6	0.0062-0.015
Tannins 2g/L	0.5(0.1)	3.4	0.042-0.15	2.1(0.6)	2.9	0.28-0.38	2.6	$1.1(0.3)\times10^{-3}$	7.2	0.00019-0.00038	—	—	—
Skins extract 20 g/L	0.4(0.1)	6.5	0.009-0.051	4.9(0.9)	4.0	0.51-0.74	5.3	—	—	—	—	—	—

* The average conditional stability constant, K_{iav}, obtained by the Scatchard model is also included. $\theta = |Pb|_{bound} / CC_{total}$

[#] TB: Tinta Barroca; TR: Tinta Roriz; TN: Touriga Nacional; TF: Touriga Francesa

4.3.1.2- Voltammetric Study

In order to study the systems in experimental conditions closer to those existing in the wines, where the |metal|/|ligand| ratios are much lower than those allowed in the ISE potentiometric experiments, a more sensitive technique, SWCV, was applied to the samples "TR" and "TN" wines and tannins. The labile Pb was measured (see typical voltammograms in figure 4.4) and the data (figure 4.5) were treated by the Scatchard plots (figure 4.6). This later figure illustrates that two groups of ligands with different macroscopic complexation properties were found for "TN" but not for "TR". Therefore, the voltammetric ligands confirmed a much higher heterogeneity of the ligands in "TN" than in "TR", as observed by potentiometry.

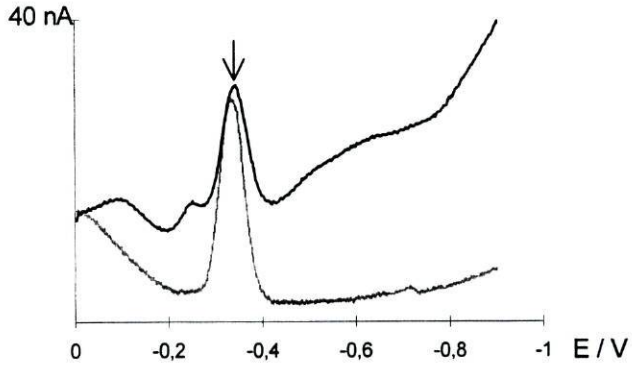

Fig. 4.4
Typical SWCV voltammograms obtained for (—) 1:50 diluted "TR" spiked with 2 μM of Pb and (·····) 2 μM of Pb in KNO3 0.05 M at pH 3.5. The peak referring to the labile Pb is signaled

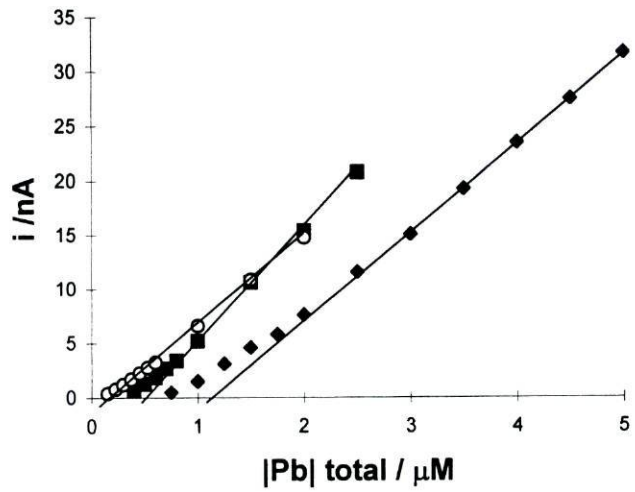

Fig. 4.5
Typical SWCV titration curves obtained for (■) "TR", (◆) "TN" and (o) tannins and (____) the respective calibration curves determined from the last titration points

The mean values of $CC_{i,inert}$ and $K'_{i,av}$ obtained from these plots were included in table 4.1. The occupation degrees presented in table 4.1 for the voltammetric results were estimated as $\theta = |Pb|_{inert} / CC_{total}$, the CC_{total} being that obtained in the potentiometric experiments, which is an estimation of the total concentration of coordination sites present in the samples.

Table 4.1 shows that, relatively to those studied by potentiometry, the θ ranges embraced in the voltammetric experiments were lowered about one order of magnitude for wines and about two orders of

magnitude for tannins. Table 4.1 shows that both wines and tannins displayed some very strong Pb complexants: log $K'_{av} > 7$. As the SWCV gives an under estimation of the K'_{av} values (see data treatment section) the real constants will be even higher. The concentration of ligands associated to these mean macroscopic stability constants (CC_{1inert}= 27.0 μM, "TR", and 43 μM , "TN") were much lower than the total ligand concentration (measured by potentiometry). Even so, they are in large excess relatively to the concentration of Pb present in the wines which was $5.0x10^{-8}$ to $1.3x10^{-7}$ M in the present cases, corresponding to values of θ between 0.00010 and 0.00025. These results clearly indicate that Pb will be tightly bound in all the studied red wines, independently of the variety employed for producing the wine. The "TN" wine displayed higher concentration of ligands and log K'_{av} than the "TR" wine, although in both wines values of log $K'_{av} > 7$ have been observed. An higher value of log K'_{1av} for "TN" is compatible with the higher K_{DEF} values observed for "TN" by potentiometry. K_{DEF} calculations could not be applied to the voltammetric data due to insufficient number of experimental points.

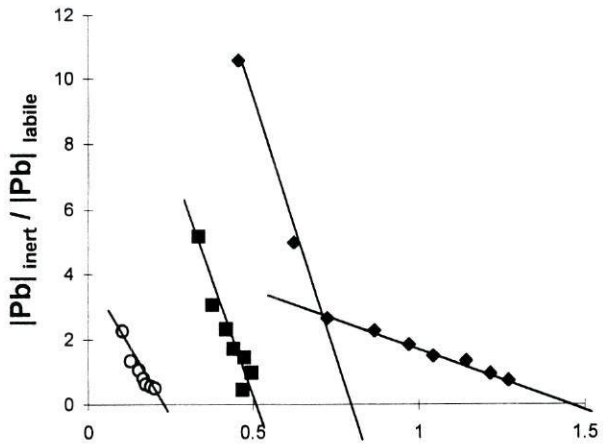

Fig. 4.6
Typical Scatchard plots obtained from voltammetric data for (■) "TR", (◆) "TN" and (o) tannins

4.3.2- Table Wines (Group 2)

The previous study has shown that SWCV information about the strength of the ligands that form operationally inert complexes with Pb (the strongest ones) existent in wines is more useful than the potentiometric information, although some approximations are required. Therefore, only SWCV was applied to the table wines of group 2. The wines were titrated with Pb standard addition to obtain labile Pb current against total Pb concentration. Green *et.al.* (1997) refer the erroneous results which may come from Pb standard addition experiments in wine if spikes are not left to fully equilibrate with the sample. In this work the minimum equilibrating time required to achieve a stable current signal was tested and it was found that 5 minutes were sufficient. Moreover, the results obtained for 5 minutes of stabilisation were compared with batch experiments

equilibrated overnight and the values of K'_{av} and CC_{inert} obtained in both cases were statistically identical. The results are shown in table 4.2. The values of the concentration of the strongest ligands (CC_{inert}) obtained for the white wines ranged from 3 to 8 μM and they were much lower than those obtained for the red wines, between 16 and 28 μM. The values of the logarithm of the average conditional stability constants (log K'_{av}) were similar for red and white wines and ranged between 6.5 and 7.8. They are also similar to the values obtained with the monovarietal wines of group 1.

Table 4.2.

Average conditional stability constants (K'_{av}) of the operationally inert Pb complexes in table wines and concentrations of the respective ligands. Standard deviations are given in brackets (n=3).

Sample	range |Pb|/ μM *	K'_{1av}/ M^{-1}	Log K'_{1av}	CC_{1inert}/μM	CC_{inert}total/μM
Red 1	13 – 155	$2.2\ (0.2)\times10^{7}$	7.3	20 (3)	20 (3)
Red 2	25 – 150	$7.3\ (1)\times10^{6}$	6.9	28 (6)	28 (6)
Red Verde	10 – 55	$2.4\ (0.3)\times10^{7}$	7.4	16 (3)	16 (3)
White 1	3 – 18	3.2 (0.9)	6.5	3 (1)	3 (1)
White 2	6 – 32	$1.9\ (0.5)\times10^{7}$	7.3	8 (2)	8 (2)
White Verde 1	2 – 38	$3.1\ (0.5)\times10^{6}$	6.5	3 (1)	3 (1)
White Verde 2	6 – 14	$5.7\ (1)\times10^{7}$	7.8	8 (2)	8 (2)

* – range of Pb concentration covered by the titration; since different dilutions were applied, the ranges were normalised to correspond to those that would be covered in the respective undiluted samples.

The values of CC_{inert} obtained for the wines are much greater than the total Pb concentration existent in the wines which ranged between 0.085 μM and 0.35 μM for the red wines and between 0.039 μM and 0.148 μM (table 4.3). Such high excess of ligands associated to high values of the conditional stability constants of Pb

Table 4.3

Total Pb concentration observed in the studied table wines. Standard deviations are given in brackets (n=3)

Wine	|Pb| / μg/L	|Pb| / nM	
Red 1	19 (4)	94 (20)	
Red 2	17 (4)	84 (20)	
Red Verde	71 (5)	350 (25)	
White 1	19 (3)	94 (15)	
White 2	7.9 (2.9)	39 (14)	
White Verde 1	30 (5)	148 (25)	Certified
White Verde 2	16 (3)	79 (15)	values / μg/L
BCR C	75 (6)		70 (10)
BCR E	42 (5)		40 (10)

complexes (K'_{av}) are compatible with the very low lability of Pb in wines reported in the literature (Green *et.al.* 1997, Arcos *et.al.* 1993, Marin and Ostapczuck 1992). A relationship between the CC_{inert} and the type of wine was observed: red wines displayed much higher concentration of strong ligands than white wines. These results, which are quite expectable due to the richer matrix existent in the red wines (*e.g.* higher polyphenol and polysaccharide contents), are compatible with other results published by Green *et.al.* (1997), who have also found a much greater complexing capability for red wines, although no quantification was presented.

4.3.3- Port wines (Group 3)

SWCV was also applied to the wines of group 3 (Port wines) and the results are shown in table 4.4 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 value, 5 μM, was obtained for the white and the oldest wine (dated 1941), and the higher value, 11 μM, was obtained for the youngest wine (young red). White wines are expected to display a lower CC_{inert} due to the richer matrix existent in the red wines (*e.g.* higher polyphenol and polysaccharide contents). In the previous study much higher CC_{inert} values for red table wines (16 and 28 μM) than for table white wines (3 to 8 μM) were observed. Therefore, the differences observed for CC_{inert} values in different Port wines were not so pronounced as for the table wines. It is quite known (*e.g.* Arcos *et.al.* 1993) that ageing causes a decrease of the contents of organic matter in wines. This is compatible with the Port wine exhibiting the higher CC_{inert} value (11 μM), being the youngest red wine studied, and the red wine exhibiting the lowest CC_{inert} value (5 μM) being the oldest of the studied wines.

The values of the logarithm of the average conditional stability constants ($\log K'_{av}$) ranged between 7.5 and 8.9. The highest values (8.8 - 8.9) corresponded to the young reds and the lowest value corresponded to the oldest wine. All the other wines displayed similar values (between 7.8 and 8.4). The process of ageing seems, then, to result in a weakening of the Pb ligands, which is compatible with the increased voltammetric lability, observed for increasingly older wine, by Arcos *et.al.* 1993.

The values of CC_{inert} obtained for the wines (5 - 11 μM) are greater than the total Pb concentration existent in the wines which ranged between 0.19 μM and 2.19 μM (table 4.5). Such excess of ligands associated to the high values of the conditional stability constants of Pb complexes (K'_{av}) obtained (which were values higher than those obtained for the table wines), are compatible with the very low lability of Pb in wines

reported in the literature (Green *et.al.* 1997, Arcos *et.al.* 1993, Marin and Ostapczuck 1992), although no Port or other fortified wines were studied.

Table 4.4

Average conditional stability constants (K'_{av}) of the operationally inert Pb complexes in Port wine and Port wine digests and concentrations of the respective ligands. Standard deviations are given in brackets (n=3).

Sample	range |Pb|/ µM *	K'_{1av}/ M^{-1}	Log K'_{1av}	CC_{1inert}/µM	CC_{inert}total/µM
White wines					
Sweet	4.5 – 11	1.9 (0.1)x10^8	8.3	5 (1)	5(1)
*Extra dry***	3.5 – 10	1.8 (0.6)x10^8	8.3	4 (2)	5 (1)
Red wines					
*Young half dry***	7.0 –20	6.2 (0.2)x10^8	8.8	7 (3)	9 (3)
Young **	8.5 – 22	8.9 (0.2)x10^8	8.9	10 (3)	11 (2)
Tawny	7.0 – 22	7.5 (1)x10^7	7.9	8 (2)	8 (2)
Ruby	7.0 – 20	7.8 (0.8)x10^7	7.9	10 (1)	10 (1)
*10 years***	6.2 – 25	2.9 (0.3)x10^8	8.4	8 (3)	10 (5)
20 years	6.2 –22	6.4 (0.6)x10^7	7.8	7 (1)	7 (1)
*30 years***	7.0 – 24	1.3 (0.1)x10^8	8.1	8 (1)	10 (2)
*40 years***	5.0 – 20	1.5 (0.2)x10^8	8.2	6 (1)	8 (2)
Dated 1941	1.0 – 13	3.1 (0.3)x10^7	7.5	5 (1)	5 (1)

* – range of Pb concentration covered by the titration; since different dilutions were applied, the ranges were normalised to correspond to those that would be covered in the respective undiluted samples.

** - 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. $CC_{2inert} = CC_{total\ inert} - CC_{1inert}$.

Table 4.5

Total Pb concentration observed in the studied Port wines. Standard deviations are given in brackets (n=3)

Wine	[Pb] / μg/L	[Pb] / μM	
sweet white	111 (9)	0.536 (0.043)	
extra dry white	65 (6)	0.31 (0.03)	
young half dry red	69 (5)	0.33 (0.02)	
young red	108 (7)	0.521 (0.034)	
Tawny	40 (5)	0.19 (0.02)	
Ruby	52 (6)	0.25 (0.03)	
10 years	107 (7)	0.516 (0.034)	
20 years	129 (7)	0.623 (0.034)	
30 years	204 (8)	0.985 (0.039)	
40 years	453 (15)	2.19 (0.07)	
dated 1941	184 (9)	0.888 (0.043)	certified value / μg/L
BCR D (liquor)	124 (9)		135 (40)

4.4- Information About the Pb Speciation in Wines by RP-HPLC

From the wines of group 2, three (one red, one white and one Verde white) were selected for the HPLC experiments. From the wines of group 3 it were also selected three (the sweet white, young red and dated 1941 Ports).

For the group 2, typical elution profiles of Pb under reverse phase HPLC conditions are illustrated in figure 4.7 for a red and a white wines, and they were similar for the Verde wines. According to the retention times of the fractions that contained Pb, the metal was distributed among three groups of compounds. The first group appeared for retention time between 5-7 minutes (closely after the void volume which corresponds to 5 minutes of retention time). This group corresponds to the compounds with the lowest molecular weight and/or to ionised substances. The second group (appeared for 17-19 minutes of retention time) and the third group (20-25 minutes) correspond to substances with higher molecular weight and/or apolar character.

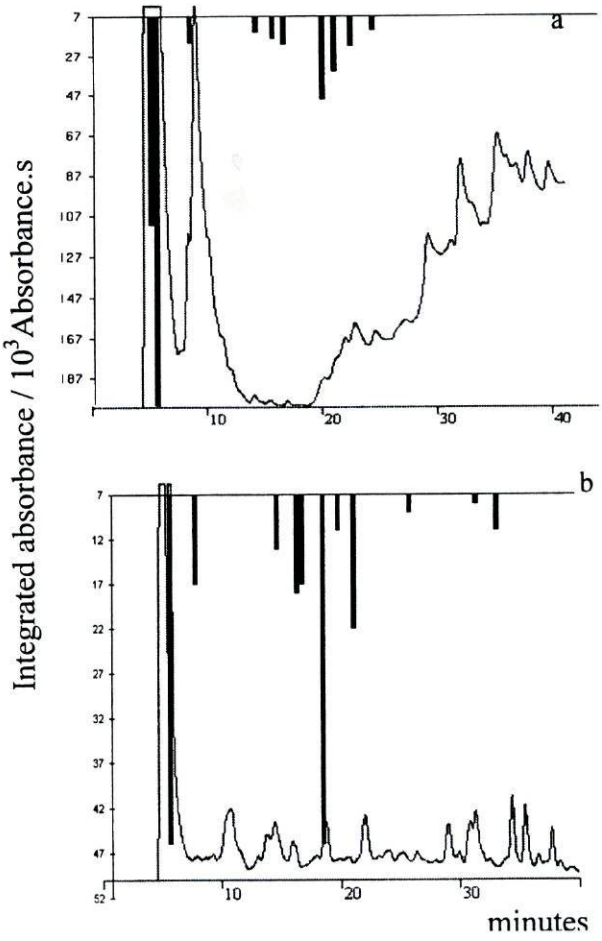

Fig. 4.7
Reverse phase HPLC chromatograms with UV and Pb-AAS detection for (a) a red table wine and (b) a white table wine. The y scale applies to the vertical bars, which represent the atomic absorption signal

For the wines of group 3, typical elution profiles of Pb under reverse phase HPLC conditions are illustrated in figure 4.8 for three very different Port wines selected to undergo these experiments: the sweet white, the young red and the red dated 1941. It can be observed for all the cases that the majority of the Pb was also eluted closely after the void volume, corresponding to the Pb associated with compounds which have relatively low molecular weight and/or high ionic character. Other Pb containing substances, with higher molecular weight and/or apolar character (eluted later), appeared in all cases, although a higher widespread of Pb species along the chromatogram was found for the

dated 1941 Port, with retention times between 7 – 30 minutes. These results are similar to those obtained for the wines of group 2.

According to Szpunar *et.al.* (1998), 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 ionised complexes may belong to the first group of substances (eluted closely to the void volume) despite of the relatively high molecular weight of the pectic dimer, *ca.* 10 KDa, which is compatible with the high fraction of Pb eluted in the first eluted group 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 higher than 30 KDa, the condensed tannins being pointed out as the most probable ligands for Pb. Coordination of Pb by tannins is compatible with the appearing of the metal in the groups eluted later in the separations by chromatography in the present work. However, when it was tried to identify the nature of the compounds eluted in those groups by applying tests of colour development for tannin,

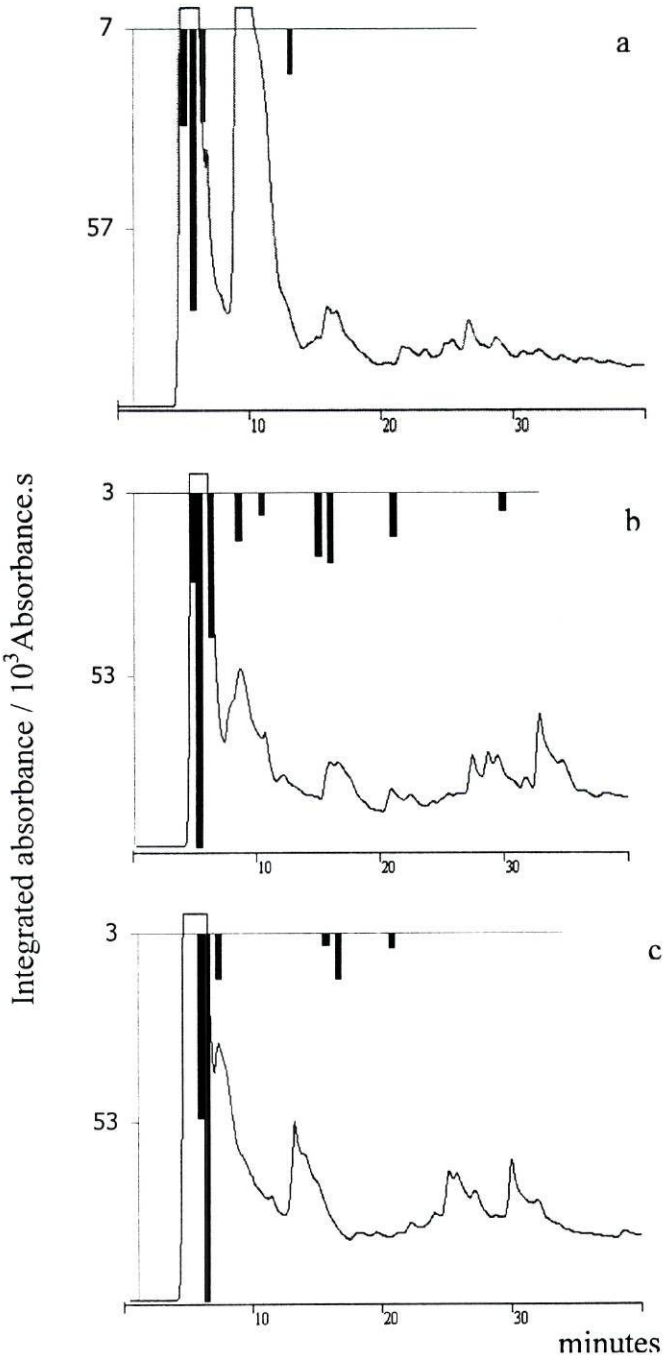

Fig. 4.8
Reverse phase HPLC chromatograms with UV and Pb-AAS detection for (a) a sweet white Port, (b) a young red Port and (c) a dated 1941 Port wines. The y scale applies to the vertical bars, which represent the atomic absorption signal

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. These information has only qualitative character, but it is useful when compared with that obtained after the wine digestion (see below).

4.5- Solubility of Pb After Gastric Digestion

The fractions of total Pb that remained soluble after *in vitro* gastric digestion are shown in figure 4.9a, for the wines of group 2. This figure shows that in all the wines studied, almost all the Pb (between 93 and 103 %) was in soluble forms after the gastric digestion and therefore potentially assimilable.

The fractions of total Pb that remained soluble after *in vitro* gastric digestion are shown in figure 4.9b for the selected Port wines. This figure shows that in the three wines studied, almost all the Pb (between 95 and 99 %) remained in soluble forms after the gastric digestion and therefore potentially assimilable, as in the case of the table wines.

Therefore, no influence of the gastric phase on Pb solubility was found.

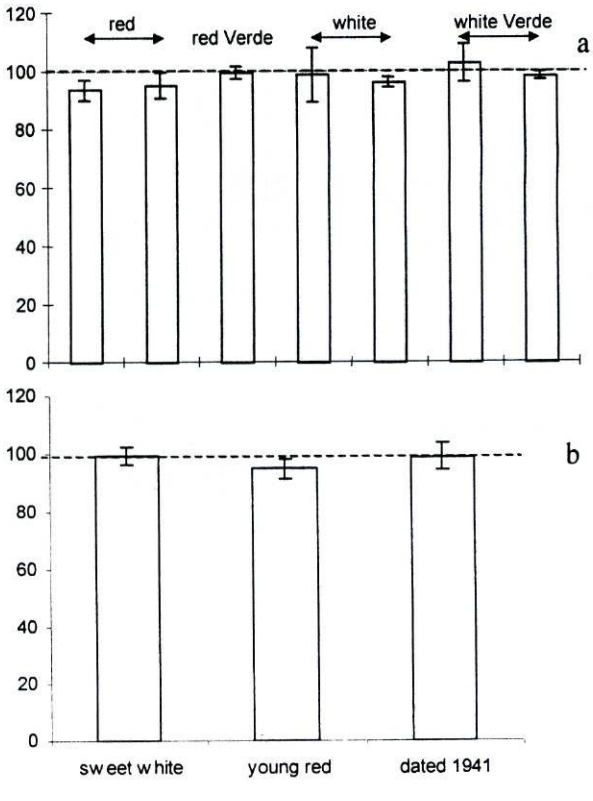

Fig. 4.9
Percentage of the total Pb that was in soluble forms after gastric *in vitro* digestion of different table wines (a) and Port wines (b). The error bars represent the standard deviation of independent replicates (n=3)

4.6- Determination of the Complexation Parameters in the Gastric Digests

Relatively to the complexation properties of the gastric digests of the table wines (group 2), the values of K'_{av} and CC_{inert} obtained for one red, one white and one Verde white wines are presented in table 4.6. Similar

results were obtained for the remaining digested wines. The values of CC_{inert} and log K'_{av} were similar for the different digested wines, and ranged from 9 to 12 μM and from 7.1 to 7.3, respectively.

The results for the Port wines are also included in table 4.6. The values of CC_{inert} were of the same order of magnitude for the three different digested wines, (5 - 8 μM) 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.

Table 4.6

Average conditional stability constants (K'_{av}) of the operationally inert Pb complexes in wine gastric digests and concentrations of the respective ligands. Standard deviations are given in brackets (n=3).

Sample	range |Pb|/ μM *	K'_{1av}/ M^{-1}	Log K'_{1av}	CC_{1inert}/μM	CC_{inert}total/μM
Red Verde	8 – 38	2.1 (0.5)x10^7	7.3	12 (4)	12 (4)
White 2	8 – 32	1.9 (0.3)x10^7	7.3	9 (2)	9 (2)
White Verde 2 **	6 – 16	1.4 (0.1)x10^7	7.1	8 (1)	12 (2)
Sweet white Port	5 – 18	5.6 (1)x10^7	7.7	5 (1)	5 (1)
Young red Port	8 – 22	6.4 (0.2)x10^7	7.8	8 (2)	8 (2)
Dated 1941 Port	1 - 13	2.0 (0.4)x10^7	7.3	5 (1)	5 (1)

* – range of Pb concentration covered by the titration; since different dilutions were applied, the ranges were normalised to correspond to those that would be covered in the respective undiluted samples.

** - samples where the Scatchard plot gave two linear zones; with log K'_{2av}: (white Verde 2: log K'_{2av} = 6.5). $CC_{2inert} = CC_{total\ inert} - CC_{1inert}$.

4.7- Information About the Pb Speciation in Gastric Digests by RP-HPLC

The profiles of Pb elution in the gastric digests (data not shown) were similar to those observed for the wines (before digestion) of both group 2 and 3.

The different studies carried out for the gastric digests demonstrated that the gastric phase of digestion had a very limited influence on the overall digestion. Indeed, the percentage of the total metal present in the soluble fraction of the gastric digests, the values of both K'_{av} and CC_{inert} (except for the young red Port digest) and the chromatographic profiles of Pb elution were all similar to those observed for the wines. Such results probably are a consequence of the gastric enzyme, pepsin, being proteolytic, and of the wines having low protein content. Moreover, the acidic conditions of the gastric fluid do not introduce great changes in wine matrix which is also acidic. Therefore, it is understandable that wine compounds and Pb speciation remain practically unaltered after gastric digestion.

4.8- Solubility of Pb After Gastrointestinal Digestion

The fractions of total Pb that remained soluble after *in vitro* gastrointestinal digestion are shown in figure 4.10a for the table wines (group 2) and in figure 4.10b for the Port wines (group 3).

For the wines of group 2, only for white wines almost all the Pb (93-94 %) remained in soluble forms. For the white Verde wines, after the intestinal digestion, the soluble Pb dropped to values of 74-77%, and for the red wines (including Verde) it was only 36-39 % of the total metal. In the case of Port wines (group 3), for the 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

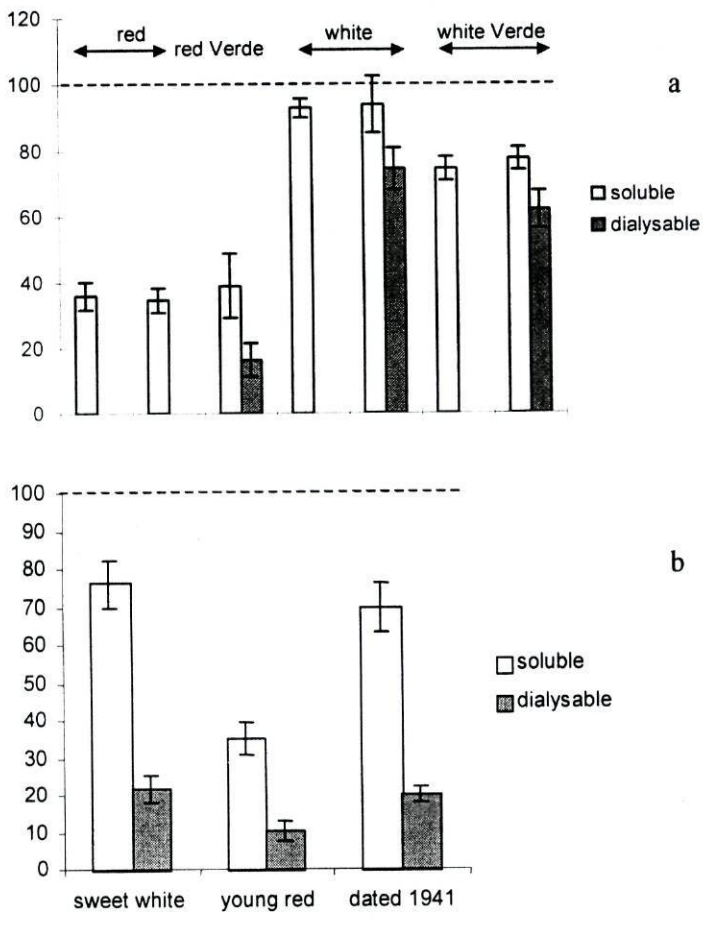

Fig. 4.10
Percentage of the total Pb that was in soluble forms after gastrointestinal *in vitro* digestion of different (a) table wines and (b) Port wines. Dialysable Pb fraction during intestinal digestion is also shown. The error bars represent the standard deviation of independent replicates (n=3)

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 red table wines is much lower than that of the white table wines. It is also shown that the Pb present in Verde white wines is potentially less assimilable than in the other white wines. For the Port wines, the results indicated 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.

One important feature coming from these results is that 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, Crews *et.al.* 1996, Walter *et.al.* 1998), led in the case of wines to a partial insolubilization of Pb. This is particularly manifest for Verde white and red table wines and for the young red Port wine. The concentration of condensed tannins in young red wines is much higher than in white wines. These polyphenolic compounds are considered as an anti-nutritional 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.

4.9- Dialysability of Pb During Intestinal Digestion

Figure 4.10a also shows for a white wine, a white Verde wine and a red wine of the group 2 (table wines), that the dialysed fraction of Pb was lower than the total soluble fraction in the amount of about 10 – 20 % of the total Pb, and figure 4.10b also shows that the dialysed fraction of Pb in three wines of the group 3 (Port wines) ranged between 10 and 22% of the total Pb, which are much lower than that in the total soluble fractions, especially for the sweet white and the dated 1941 Ports. The results indicate that the relative assimilability index for Pb was very reduced, especially in red table wines and in the three types of Port wine.

4.10- Determination of the Complexation Parameters in the Gastrointestinal Digests

In table 4.7 are shown the results of K'_{av} and CC_{inert} obtained for the gastrointestinal digests of table and Port wines. In the case of table wines, the intestinal conditions caused changes in the K'_{av} values relatively to

those obtained for wines and gastric digests. However the comparison has a reduced meaning in this case as the concentration and strength of labile complexes may be very different whether the matrix is wine or gastrointestinal digest (what means that very different α values in equation 2.19 may be expected for wines and their gastrointestinal digests). In fact the great increase of pH, from 3 to 7.4, caused by the intestinal conditions results in the deprotonation of the carboxylic groups and other common functional groups of organic ligands, which favours the metal complexation. Moreover, at pH 7.4, probably a fraction of Pb is in the form of soluble or insoluble hydroxo-complexes. On the other hand, the digestion process may lead to macromolecule fragmentation which may disfavour the metal trapping conformations, what may result in weaker inert complexes. Therefore the results for the intestinal digests can only be comparable among them.

Table 4.7

Average conditional stability constants (K'_{av}) of the operationally inert Pb complexes in wine gastrointestinal digests and concentrations of the respective ligands. Standard deviations are given in brackets (n=3).

Sample	range |Pb|/ μM *	K'_{1av}/ M^{-1}	Log K'_{1av}	CC_{1inert}/μM	CC_{inert}total/μM
Red Verde					
UF**	10 – 110	1.3 (0.3)x10^7	7.1	37 (8)	75 (22)
F	10 – 100	1.1 (0.4)x10^6	6.0	63 (26)	63 (26)
White 2					
UF	4 – 20	2.1 (0.4)x10^6	6.3	26 (5)	26 (5)
F	12 – 30	7.3 (0.7)x10^5	5.9	27 (3)	27 (3)
White Verde 2					
UF	2 – 22	2.9 (0.4)x10^6	6.5	16 (2)	16 (2)
F	2 – 30	1.4 (0.2)x10^6	6.1	19 (3)	19 (3)
Sweet white Port					
UF	1.7 – 40	3.9 (0.7)x10^6	6.6	30 (4)	30 (4)
F	1.2 – 57	3.3 (0.8)x10^6	6.5	35 (7)	35 (7)
Young red Port					
UF	2.5 – 50	4.1 (1)x10^5	6.6	45 (11)	45 (11)
F	2.0 – 43	4.4 (2)x10^5	6.6	31 (10)	31 (10)
Dated 1941 Port					
UF	2.4 – 50	9.4 (0.3)x10^5	6	25 (8)	25 (8)
F	2.4 - 55	3.0 (0.2)x10^6	6.3	29 (3)	29 (3)

* – range of Pb concentration covered by the titration; since different dilutions were applied, the ranges were normalised to correspond to those that would be covered in the respective undiluted samples.

** - samples where the Scatchard plot gave two linear zones; with log K'_{2av}: (white Verde 2 UF: log K'_{2av} = 6.3). $CC_{2inert} = CC_{total\ inert} - CC_{1inert}$.

UF= unfiltered; F= filtered

The values of log K'_{1av} for the filtered intestinal digest were similar for all wines ranging between 5.9 and 6.1. However in the case of the red wine a marked decrease in the K'_{1av} value (about one order of magnitude) was verified from the unfiltered to filtered intestinal digest. These results indicate that the strongest ligands of that red wine are in insoluble forms, in the intestinal digests.

The CC_{inert} values obtained for the intestinal digests were much greater than those obtained for untreated wines and gastric digests, what is related with the deprotonation of organic ligands, eventual hydroxide complex formation and the catabolic activity of the enzymes, which fragmentize the macromolecules, as referred. For the intestinal digest of red wine, much higher CC_{inert} values were obtained as compared with those obtained for the intestinal digests of white wines, either for the whole digest and for the soluble fraction of the digest.

The K'_{av} results are compatible with the solubility of the Pb species observed for intestinal digests. In fact, for the red wines both the strongest ligands and most of the Pb were in insoluble forms.

4.11-Information About the Pb Speciation in Gastrointestinal Digests by RP-HPLC

Figure 4.11 illustrates the typical elution profiles of Pb in the intestinal digests obtained for the (a) red and (b) white table wines. Differently of 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 minutes of retention time. Most of the Pb appeared at 17-25 minutes of retention time.

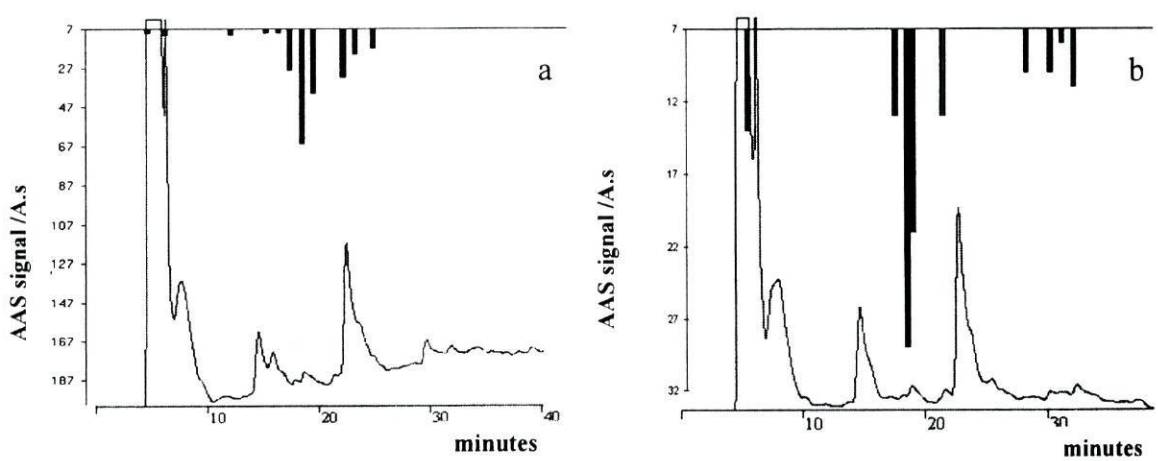

Fig. 4.11
Reverse phase HPLC chromatograms with UV and Pb-AAS detection for the gastrointestinal digests of (a) a white and (b) red wines. The y scale applies to the vertical bars, which represent the atomic absorption signal (integrated absorbance / Absorbance.s)

Figure 4.12 illustrates the typical elution profiles of Pb in the intestinal digests obtained for the three Port wines analysed. As in the case of the table wines only a small amount of Pb was found in the groups eluted for 5-7 minutes of retention time. For higher retention times the distribution of the Pb species along the chromatogram also changed very much from the wine to the gastrointestinal digests. Particularly, in the digests it was found Pb species associated to compounds with much higher retention times.

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

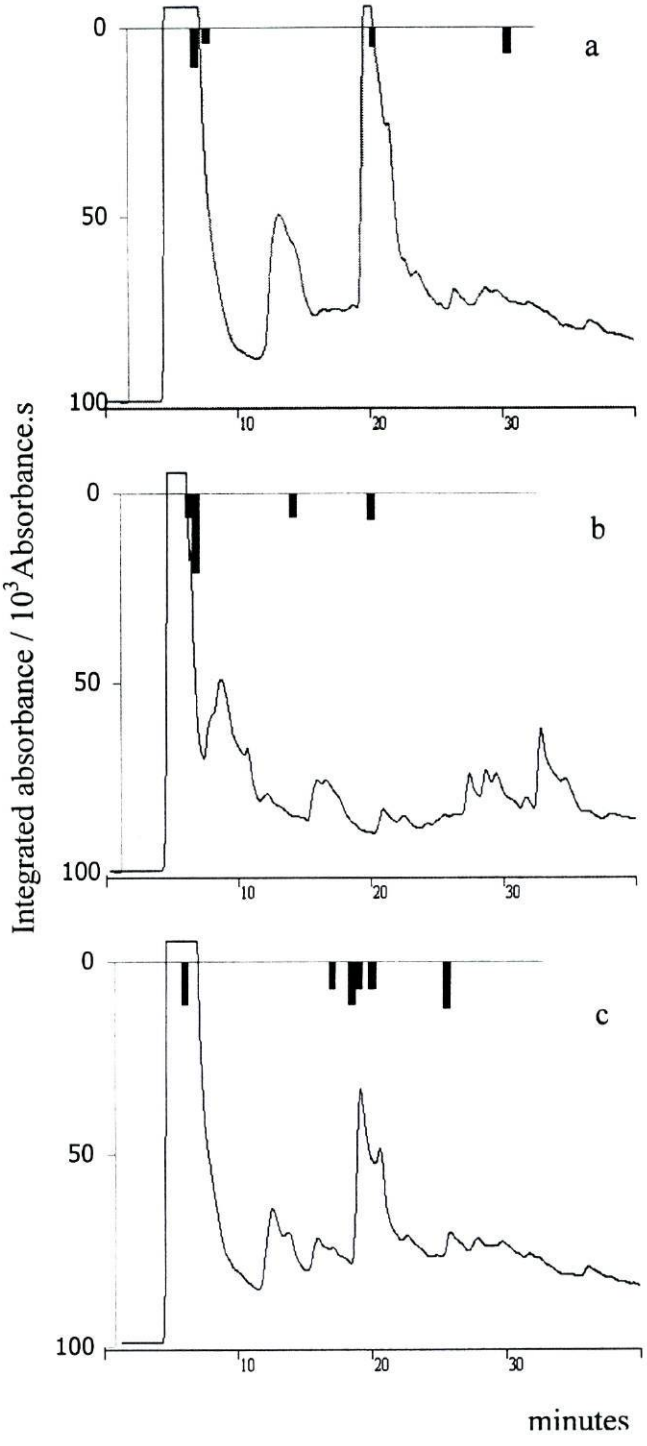

Fig. 4.12
Reverse phase HPLC chromatograms with UV and Pb-AAS detection for the gastrointestinal digests of (a) a sweet white Port, (b) a young red Port and (c) a dated 1941 Port wines. The y scale applies to the vertical bars, which represent the atomic absorption signal

4.12- Conclusions

The study carried out for the Douro's monovarietal red wines (group 1) and polyphenols extracts showed that, despite the limitations involved, voltammetry could provide more valuable information about the complexation state of Pb in wines (K'_{av} and CC_{inert}) than the potentiometry. Since the values of log θ in wines are lower than those embraced in the voltammetric experiments, Pb is strongly complexed in wines (log $K'_{av} > 7.2$). Parallel studies of isolated tannins and skin polyphenols showed that the later are much stronger ligands than tannins. It was shown that the polyphenols, alone, do not account for the strong Pb complexation observed in wines. The values of log K'_{av} obtained for the different table wines (group 2) were all similar ranging 6.5 and 7.8 and indicated a strong complexation as well. The CC_{inert} of red wines (16-28 μM) was much higher than of the white wines (3-8 μM). In the case of the Port wines (group 3), the log K'_{av} ranged from 7.8 and 8.9, being the highest values (8.8 and 8.9) observed for the young red wines. The white wines generally displayed lower CC_{inert} values (5 μM) than red wines (5-11 μM). Within the red Port wines the results suggest a trend for a decrease of log K_{av} and CC_{inert} with age.

The RP-HPLC experiments have shown that the Pb distribution among different Pb-species existent in wines before and after gastrointestinal digestion is significantly different, thus evincing the importance of the speciation at the gastrointestinal level on the final bioavailability of the elements. In the case of red table wines and young red Port wines a major part of the Pb is present in insoluble forms (not assimilable) at the gastrointestinal digests. Differences in the dialysability (taken as an assimilability indicator) of Pb species in intestinal digests observed for different types of wine, indicate that Pb may be much less bioavailable in red table and young red Port wines than in white table and old Port wines.

CHAPTER 5

SPECIATION OF Cu IN WINES AND WINE DIGESTS

CHAPTER CONTENTS

5- Speciation of Cu in Wines and Wine Digests

5.1- Introduction

The most important Cu ligands in wine seem to be macromolecular compounds, as referred in section 1.1.4.3. That fact is compatible with information obtained by us (unpublished results) by computational speciation calculations based on the published (Sillén and Martell 1974; Martell and Smith 1974) protonation constants of free amino acids and organic acids found in wines, and their Cu 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 therefrom they cannot account for the strong Cu complexation verified in wines. In the case of the above referred 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 Cu ions.

Additionally, the human bioavailability of Cu in wines is of interest since Cu is a micronutrient. Therefore a study similar to that carried out for Pb needs to be included, which will give an estimate of the assimilability of this essential metal.

The first purpose of the present work was to study the complexometric properties to Cu of four different red wines produced from only one of the following varieties of grapes, cultivated in the Douro's Demarked Region of Portugal: "Tinta Barroca" (TB), "Tinta Roriz" (TR), "Touriga Nacional" (TN) and "Touriga Francesa" (TF). Parallelely, 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 complexation properties were expressed in terms of (a) maximum complexation capacity (CC_{total}) of the ligands from the wines, that is, the total concentration of the sites available in the sample, and (b) conditional stability constants of the complexes with Cu. For this purpose, the Scatchard plot and the Differential Equilibrium Function (DEF) models, which have been considered suitable for characterisation of complexes involving heterogeneous ligands (Scatchard 1949; Buffle 1988) were used. Due to their aromatic structure, tannins exhibit a fluorescent capability (Guilbault 1977) which is diminished by

binding to Cu (quenching). This property was exploited in this work (fluorimetric titration of solutions of tannins with Cu solutions) but with a very reduced success because the analytical window of the technique allowed only to obtain results in unrealistic conditions, that is, for ratios of |Cu| / |tannins| too high relatively to those present in the wines. Therefore, ion selective electrode potentiometric titrations of the samples under study with Cu solutions was the methodology elected for the present study.

The second purpose of this study was to determine some speciation parameters for different types of table wines (red, white and Verde) and Port wines (white, tawny, ruby, dated, young red, with age indication). In this case the wines were also treated as heterogeneous ligands, and the speciation parameters studied were the conditional stability constants of the Cu complexes (K_{av}) (performed for Port wines only), the concentration of the ligands that form complexes (CC)(performed for Port wines only) and the chromatographic profiles of Cu elution in conditions usually set for macromolecules, which gave some insight into the nature of the Cu complexes in wines and wine digests.

The third purpose was to study the fate of the Cu present in some of the table and Port wines when they were subjected to *in vitro* gastrointestinal digestion. Some speciation parameters (those possible) studied for Cu in wines were also studied after gastric and gastrointestinal digestions. Moreover, information about Cu solubility and dialysability after the digestion was gathered.

Part of the work are already published (Vasconcelos *et.al* 1999).

5.2- Experimental

For the determination of the conditional stability constants of Pb complexes in wines, the potentiometry with Cu(II) ion selective electrode was used, although the fluorimetry was also tested for the tannin solution.

The Reverse-Phase High Performance Liquid Chromatography (RP-HPLC) experiments were performed in a C_{18} column suitable for peptide, protein and tannin separation, and eventually other macromolecules.

The *in vitro* digestions procedure was optimised from a method applied to solid foods (Crews *et.al.* 1985) and the dialysis experiments were adapted from Hazell and Johnson (1987)

All the experimental details can be found in the chapter Experimental Section.

5.3- Determination of the Complexation Parameters in Wines and Polyphenolic Extracts

5.3.1- Monovarietal Table Wines (Group 1) and Polyphenolic Extracts

5.3.1.1- Fluorimetric Study

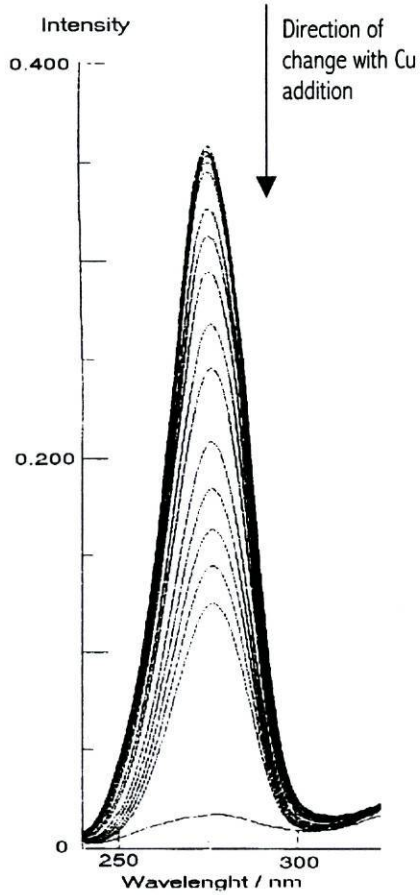

Fig. 5.2
Spectra illustrating the successive quenching obtained along a typical titration of 20 ppm of tannins with Cu.

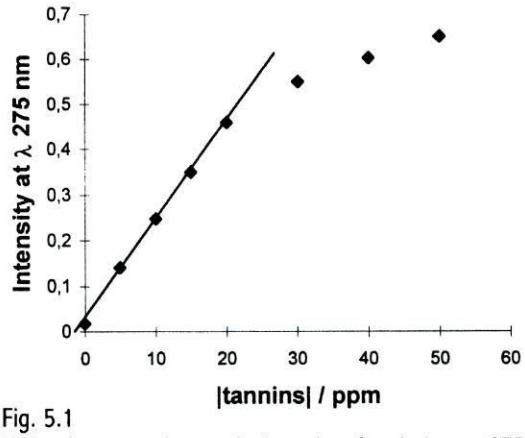

Fig. 5.1
Calibration curve for tannin intensity of emission at 275 nm, with excitation at 225 nm

Figure 5.1 shows that tannins had a fluorimetric linear response from ≈ 0 to 20 mg/L. That was the reason for using 20 mg/L of tannins in the titrations, thus allowing to take full advantage of the linear response range. A typical set of spectra obtained along the titrations with Cu is presented in figure 5.2. The Simplex optimisation gave a mean log K_{av} of 2.74 for log θ between 2.11 and 3.19, what means that an excess of metal of 129 to 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 exists, that is, log θ between -3.6 and -4.0 (see

below how these values where attained), instead of a large excess of metal. This fact dictated the low usefulness of the fluorimetric technique.

5.3.1.2- Potentiometric study

A SWCV method for studying the inert complexes of Cu in wines and digests, similar to that used for Pb, was seek. Several instrumental conditions were tested (frequency of square wave, initial potential, pulse amplitude and step potential), but unfortunately, either the Cu peaks were superimposed by other peaks of the samples, or when a separate peak for the labile Cu was obtained, it was broad and its shape suffered alterations along the titration. 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. Nevertheless, for tannins, the ISE potentiometry allowed to study more realistic θ values than the fluorimetry. Figure 5.3 illustrates with a typical example the potentiometric titration curves of wines with Cu obtained in the present work. For comparison purposes, the respective calibration curve was included. It is worth to notice that because the ligands exert a buffer effect towards free Cu concentration, in their presence there was an extended range for the practical linear response limit of about three orders of magnitude (from 10^{-5} M to 10^{-8} M).

Data of titration curves were firstly treated through the Scatchard plot. Figure 5.4 illustrates, for "Tinta Roriz" (for the remaining wines similar results were obtained), that two approximately linear ranges could be selected. In other words, it were observed two different $|Cu|_{bound}$ ranges (corresponding to two θ ranges) compliant with the Scatchard model, where equation 2.6 could be applied. 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 $|Cu|_{bound}$

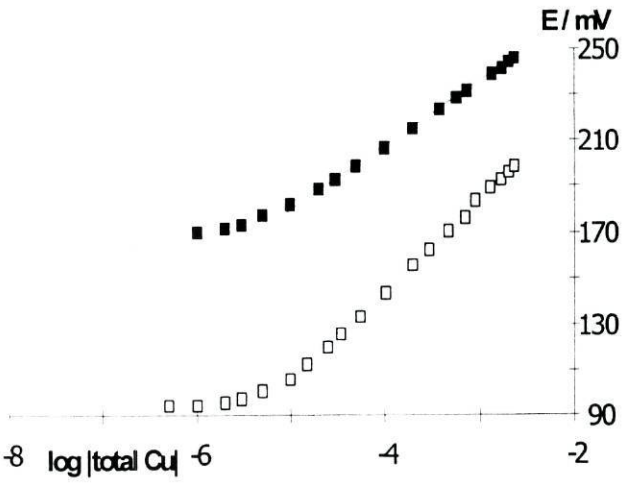

Fig. 5.3
Potentiometric calibration (■) and titration curve of "Tinta Barroca" with Cu (□). In the abscise axis the |total Cu| refers to the |Cu| naturally present in wine plus the |Cu| added.

values), are those with the highest metal affinity or the stronger sites (K_{1av}). The correlation coefficients of the least square linear regression were in most cases ≥ 0.99 ($n \geq 8$), thus, the linearity of the zones selected on Scatchard plots were considered acceptable. The mean values of CC and K_{av} obtained for the different wines and for the grape extracts are reported in table 5.1, which includes the range of θ values embraced in each case.

The four different wines displayed very similar CC_{total} for Cu, between $4.5x10^{-3}$ and $4.9x10^{-3}$ M. Table 5.1 also shows that a solution with 2 g/L of tannins extracted from seeds displayed a CC_{total} of $9.7x10^{-4}$ M, which corresponds to 480 $\mu mol_{Cu\ sites}/\ g_{tannins}$. As the concentration of tannins in the studied wines varied between 2.86 g/L ("Tinta Barroca") and 3.92 g/L ("Touriga Nacional") (table 2.2 in the Experimental Section), the maximum contribution of tannins for the CC_{total} of the wines will be between $1.4\ x10^{-3}$ M and $1.9\ x10^{-3}$ M, respectively. Aggregation of tannins 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} of wines were about three times higher than the maximum contribution of tannins. Solutions with 20 g/L of skins polyphenols (which includes mainly anthocyans) displayed a CC_{total} of $1.5x10^{-3}$ 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 g/L and 0.64 g/L, see table 2.2), the anthocyans contribution for the CC_{total} of the wines is expected to be small, probably lower than 2 %. Therefore, abundant Cu complexing agents other than the isolated polyphenols should exist in the wines.

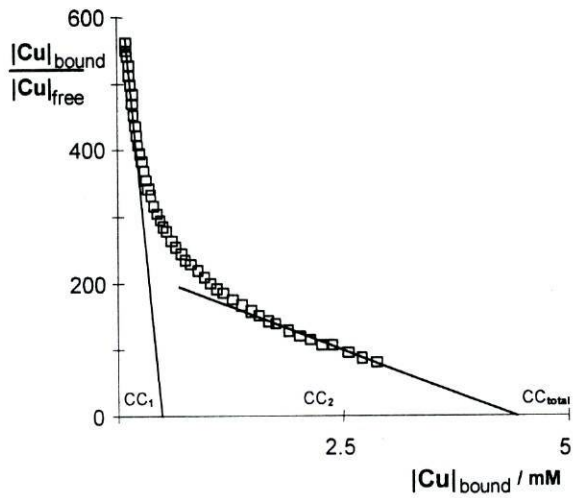

Fig. 5.4
Scatchard plot obtained for "Tinta Barroca" red wine

In addition, table 5.1 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 (about one 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 the wines and a K_{2av} similar to 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 5.5. As in the case of the CC values, the different wines displayed very similar distribution of the K_{DEF} values. For log θ = log ($|Cu_{bound}|$ / CC_{total}) < -1.5, which corresponds to $|Cu_{bound}|$ < $1.5x10^{-4}$ M, a horizontal plateau of log K_{DEF} constants was observed, between 6.0, "Touriga Nacional", and 6.4, "Tinta Roriz", which indicates 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 θ > -1.5 the values of K_{DEF} systematically decreased when the degree of occupation of the binding sites increased.

Typical values of log K_{DEF} obtained for solutions with 2 g/L tannins and of 20 g/L skin polyphenols were included in figure 5.5. If identical θ values are considered for the different solutions studied (wines and isolated polyphenols), for instance log θ = -1.5, and the log K_{DEF} 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 percentage some tannins, carbohydrate-polyphenol complexes, and other compounds, have much stronger ligands than the tannins isolated from seeds.

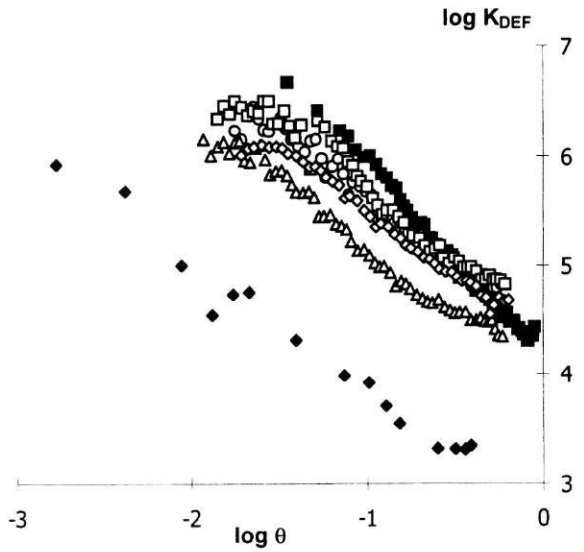

Fig. 5.5
K_{DEF} obtained from the four wines studied, "Tinta Roriz" (Δ), "Tinta Barroca" (◊), "Touriga Nacional" (○), "Touriga Francesa" (□); and from tannins (2 g/L) (◆) and skin polyphenols (20 g/L) (■), as a function of log θ ($\theta = |Cu|_{bound}/CC_{total}$).

The levels of Cu found in the wines ranged from $4.4x10^{-7}$ M to $1.35x10^{-6}$ 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).

Table 5.1

Conditional stability constants (K_{av}) of Cu complex formation and complexation capacities (CC) of four different wines and solutions of tannins and skins polyphenols isolated from grapes, obtained from the potentiometric data. Standard deviations are given in brackets for CC (n=3).

Medium	Stronger sites			Weaker sites			
	CC_1/mM	θ range	$logK_{1av}$	CC_2/mM	θ range	$logK_{2av}$	CC_{total}/mM
T. Barroca	0.4 (0.1)	0.014-0.051	6.0	4.0(0.4)	0.38-0.64	4.7	4.5(0.3)
T. Roriz	0.3 (<0.1)	0.011-0.027	6.1	4.6(0.4)	0.24-0.59	4.5	4.9(0.4)
T. Francesa	0.3 (<0.1)	0.012-0.040	6.4	4.5(0.3)	0.38-0.60	4.9	4.8(0.3)
T. Nacional	0.4(<0.1)	0.014-0.054	6.2	4.4(0.4)	0.33-0.60	4.8	4.8(0.4)
Tannins 2 g/L	0.1(<0.1)	0.009-0.079	5.4	0.9(0.2)	0.22-0.78	3.7	1.0(0.2)
Skins extract 20 g/L	0.2(<0.1)	0.017-0.067	6.6	1.3(0.2)	0.39-0.67	4.6	1.5(0.2)

5.3.2- Port Wines (Group 3)

The levels of Cu found in the studied wines, presented in table 5.2, ranged between 1.4 and 25 μM, being the higher concentrations observed for the older wines.

The results (table 5.3) indicate that two types of complexation sites, with significantly different strength (K_{1av} and K_{2av}) could be distinguished in all the studied wines. The concentration of the sites associated with K_{1av}, CC_1, ranged from 0.1 to 0.5 mM, the higher values being found for the

Table 5.2

Total Cu concentrations observed in different Port wines. Standard deviations are given in brackets (n=3)

Wine	|Cu| / mg/L	|Cu| / μM	
White wines			
Sweet	0.33 (0.02)	5.2 (0.3)	
Extra dry	0.17 (0.02)	2.7 (0.3)	
Red wines			
Young half dry	0.14 (0.02)	2.1 (0.3)	
Young	0.30 (0.03)	4.6 (0.5)	
Tawny	0.086 (0.05)	1.4 (0.8)	
Ruby	0.14 (0.02)	2.1 (0.3)	
10 years	0.22 (0.03)	3.5 (0.5)	
20 years	0.51 (0.04)	7.9 (0.6))	
30 years	0.81 (0.05)	12.8 (0.8)	
40 years	1.58 (0.07)	24.8 (1.1)	
Dated 1941	0.94 (0.05)	14.7 (0.8)	certified value mg/L
BCR D (liquor)	1.17 (0.05)		1.150 (0.020)

white Ports, 0.3 (sweet white) and 0.5 mM (extra dry white). The remainder displayed lower CC_1, except the 40 years Port with 0.4 mM.

However, the white Ports displayed the lowest log K_{1av} values (4.1 and 5.1), while 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 the wines (2.9 to 3.6 mM) and much higher than CC_1. The values of log K_{2av} were also very similar for all the wines (3.5 – 4.2) and two orders of magnitude lower than log K_{av1}. In every case, the values of CC_1 were more than twenty times higher than the total Cu concentration in the respective Port wine, which allows to conclude that the Cu in wines is practically all complexed by the stronger sites.

Table 5.3

Average conditional stability constants (K_{av}) of Cu complexes in Port wine and concentration of the respective ligands. Standard deviations are given in brackets (n=3).

Sample	Stronger sites			Weaker sites			
	CC_1/mM	θ range	$logK_{1av}$	CC_2/mM	θ range	$logK_{2av}$	CC_{total}/mM
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)

5.4- Information About the Cu Speciation in Wines by RP-HPLC

The profile of the chromatographic bands showed that, for all the table wines (group 2), the Cu was mainly associated to groups eluted closely to the void volume. Even for wines enriched with 1 mg/L of Cu no AAS signal was observed for retention times higher than 7 minutes (data not shown).

The profile of the chromatographic bands (figure 5.6) of Port wines (group 3) showed that for the young red and sweet white Ports 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 minutes (sweet white wine) and 16, 29 and 32 minutes (young red wine). For the dated 1941 Port similar Cu signals were found for the fractions eluted closely to the void volume and for those highly retained, eluted around 30 minutes. A comparison with the results for the table wines shows that in the Port wines a fraction of the Cu seems to be present in forms of higher molecular weight/apolar character than in the table wines.

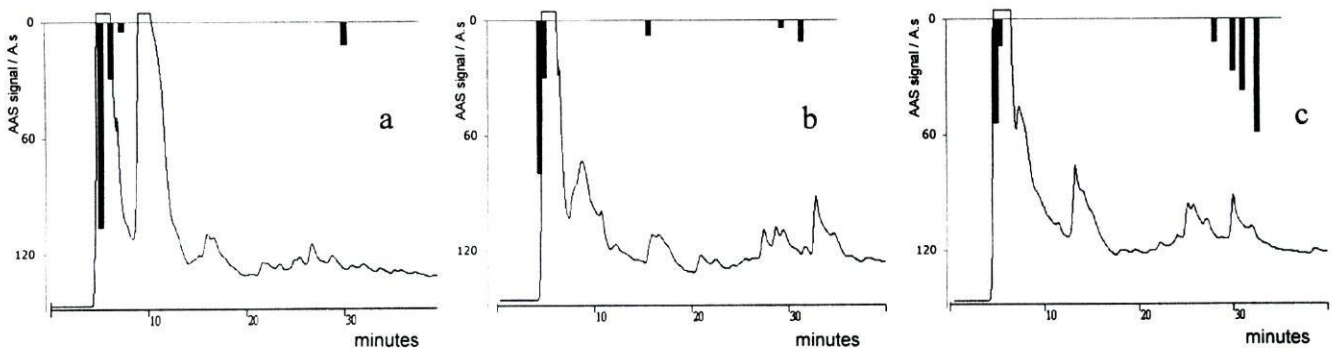

Fig. 5.6
Reverse phase HPLC chromatograms with UV and Cu-AAS detection for Port wines: (a) a sweet white Port, (b) a young red Port and (c) a dated 1941 Port. The y scale applies to the vertical bars, which represent the atomic absorption signal (integrated absorbance / Absorbance.s)

5.5- Solubility of Cu After Gastric Digestion

Figure 5.7a shows that after the gastric digestion of the table wines almost all the Cu remained soluble: approximately 100 % for all the wines, but the red Verde (77 %) and one of the white wines (85 %).

Figure 5.7b shows that after the gastric digestion of the Port wines practically all the Cu remained soluble.

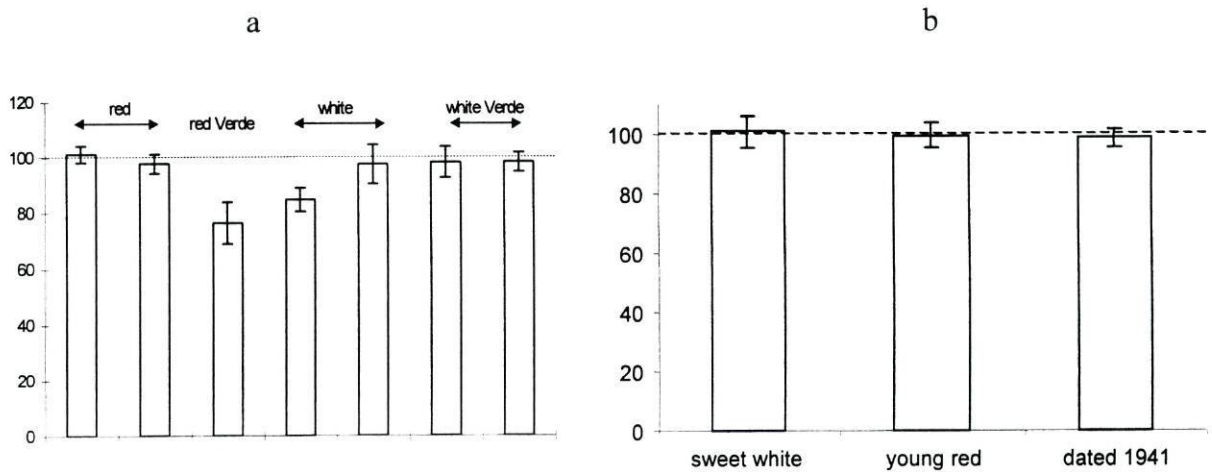

Fig. 5.7
Solubility (%) of Cu after gastric digestion of: (a) table wines and (b) Port wines. The error bars represent the standard deviation of independent replicates (n=3)

5.6- Information About the Cu Speciation in Gastric Digests by RP-HPLC

The chromatographic profiles of Cu elution after gastric digestion of the table and Port wines were similar to those obtained for the wines (data not shown).

Therefore the gastric phase of the digestion was of minor importance, as occurred for Pb.

5.7- Solubility of Cu After Gastrointestinal Digestion

Contrarily to Pb, after the intestinal digestion practically all the Cu from all the table (figure 5.8a) and Port (figure 5.8b) wines remained in soluble forms.

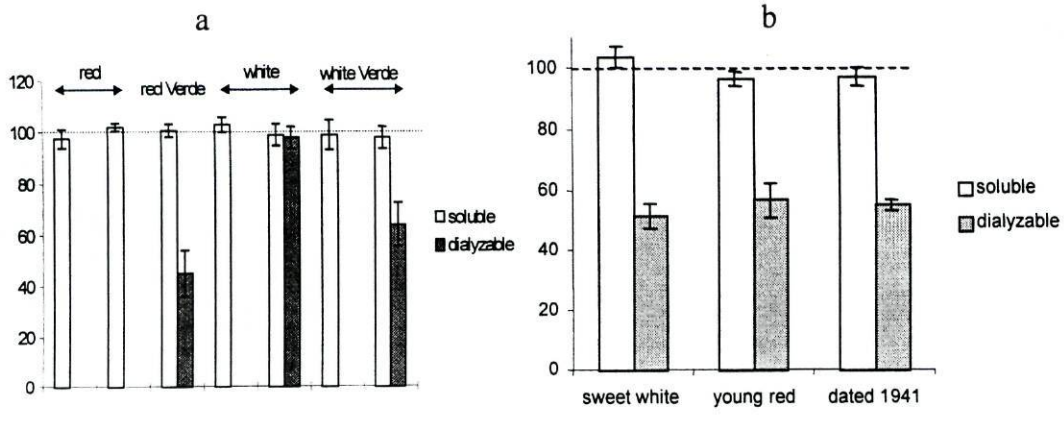

Fig. 5.8
Solubility (%) of Cu after gastrointestinal digestion and dialysability (%) of Cu during intestinal digestion: (a) table wines and (b) Port wines. The error bars represent the standard deviation of independent replicates (n=3)

5.8- Dialysability of Pb During Intestinal Digestion

For the table wines, the Cu in the fraction dialysed during the intestinal digestion was approximately 100 % only for the white wine, being much lower for the other types of wines: 64 % for the white Verde and 45 % for the red wine (figure 5.8a). For all the Port wines the Cu in the dialysed fraction were about 50 % of the total metal (figure 5.8b). It indicates that the kinetics and molecular size of the ligands play an important role on the assimilability of Cu from wines, as was also observed for Pb. While for the table wines the dialysable fraction of Cu was higher for white wines than for a red wine, for the Port wines no distinction whatsoever could be found.

5.9- Determination of the Complexation Parameters in the Gastrointestinal Digests

At the first points of the potentiometric titrations of the intestinal digests with a Cu solution, occurred the formation of a precipitate which prevented the determination of both K_{av} and CC values for these digests.

5.10- Information About the Pb Speciation in Gastrointestinal Digests by RP-HPLC

As before, for the table wines, despite of the high enrichment used for the chromatographic experiments, 1 mg/L, Cu was only detected between 5-7 min of elution, thus not allowing to see any changes in Cu speciation due to the digestive process. However, these results indicate that Cu is present under more ionic and/or lower molecular weight forms than Pb, what is compatible with the higher dialyzable fraction found for Cu in wines.

The chromatographic profiles of the Cu elution in the intestinal digests of Port wines are shown in figure 5.9.

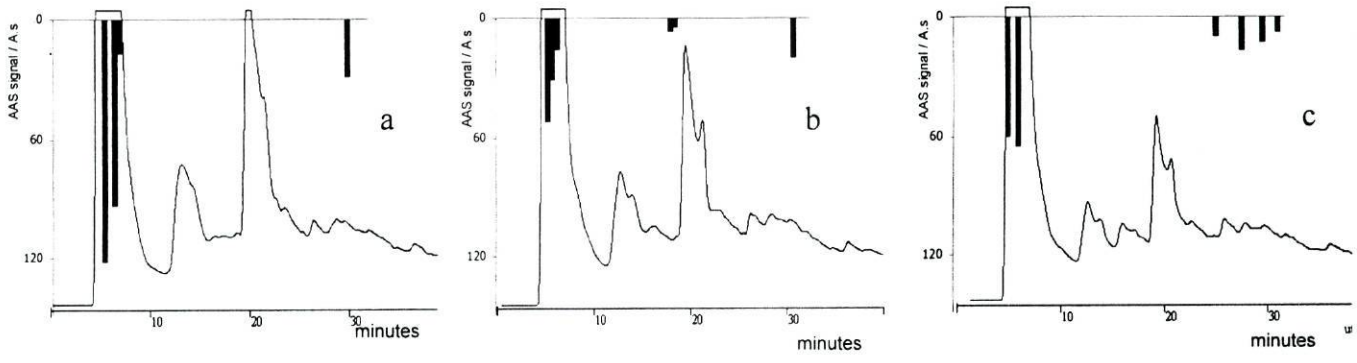

Fig. 5.9
Reverse phase HPLC chromatograms with UV and Cu-AAS detection for the gastrointestinal digests of Port wines: (a) a sweet white Port, (b) a young red Port and (c) a dated 1941 Port. The y scale applies to the vertical bars, which represent the atomic absorption signal (integrated absorbance / Absorbance.s)

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. Once more, in the Port wines gastrointestinal digests a fraction of the Cu seems to be present in forms of higher molecular weight/apolar character than in the table wines gastrointestinal digests.

5.11- Conclusions

The study carried out with the polyphenolic extracts of grapes from Douro and the respective table wines showed that tannins and skin extract bind Cu. However the existence of other abundant ligands in wine was made evident by comparison with the complexation parameters determined for polyphenolic extracts and wines. It was inferred that Cu was strongly complexed in these wines (log $K_{av} > 6$). For red Port wines a strong complexed state for Cu could also be inferred (log $K_{av} > 5.5$).

The RP-HPLC study showed that the distribution of the Cu among the different species was little affected by the digestive process, contrarily to what was verified for Pb. In addition, it indicated that in Port wines a fraction of Cu is in the form of highly apolar and/or high molecular weight compounds whereas for table wines only polar and/or low molecular weight compounds containing Cu seem to exist.

The solubility of Cu remained high (almost total) after the gastrointestinal digestion which means it was practically all on a potentially assimilable form. The results obtained for the dialysable fraction during the intestinal digestion, taken as an estimation of the assimilable fraction, shows a significant decrease (especially for the red table wine) relatively to the soluble fraction (except for the white table wine), thus indicating that not all soluble metal may be bioavailable.

CHAPTER 6

OVERALL CONCLUSIONS AND FINAL REMARKS

CHAPTER CONTENTS

6- Overall Conclusions and Final Remarks

6.1- The Influence of Cu in the Alcoholic Fermentation

High concentrations of Cu in musts were found to have practically no influence on the efficiency of must fermentation by the *Saccharomyces cerevisiae* cells.

An improved ethanol production was verified at Cu high concentrations in YNB medium. In this case, Cu was more bioavailable than in musts due to the absence of strong Cu ligands.

6.2- Speciation and Bioavailability of Pb in Wines

This work demonstrated that it can not be stated that a metal is not bioavailable by judging only by its electrochemical operational inertness. These measurements do not account for the digestive process prior to assimilation. In fact, a redistribution of the Pb among its different species when passing from untreated wines to its gastrointestinal digests was found. Moreover, it was shown that in most wines a significant fraction of the Pb turns insoluble in the intestinal phase of the digestion, therefore being non assimilable.

The estimated assimilable fractions of Pb in wines exhibited very different values for different wine types, thus reinforcing the arbitrary, non scientific based, empirical and sometimes political character of the threshold values set mainly by governmental institutions, which treat all wines as all of a kind in this matter. It could make sense to establish higher threshold limits for Pb in red wines (not very old) than in white wines, as shown by the results of this work. Therefore, this work pointed out an health related advantage of the red table wines, beyond those well recognised presently (*e.g.* Klatsky *et.al.* 1997, Soleas *et.al.* 1997 and Halpern *et.al.* 1998), which is their ability for keeping a low bioavailability of Pb and probably other toxic heavy metals.

6.3- Speciation and Bioavailability of Cu in Wines

Estimations of conditional average stability constants of complex formation in wines, and complexation capacities, were also determined by recurring to heterogeneous ligands concepts. Only a potentiometric procedure was used for the purpose because no suitable cathodic voltammetric procedure was found among the several tested. A cathodic (preferentially adsorptive) voltammetric procedure would be extremely advantageous as it would allow the study of more realistic $|Cu|_{bound}/CC$ ratios.

The Cu species seemed to be more stable to the digestive process than the Pb species. The dialysable fractions obtained indicate that table and Port wines may be a considerable source of dietary Cu for regular consumers.

6.4- Future Research Needs

The present work has opened several lines for future work:

Future research work is necessary as to explain the results of enhancement of ethanol production at high Cu concentrations at a mechanistic level. Possible ways to take advantage of the enhancement of ethanol production can also be attempted.

For the first time, estimations of conditional average stability constants of complex formation in wines, and complexation capacities, were determined, by recurring to heterogeneous ligands concepts. Potentiometric and voltammetric procedures were optimised for the purpose, and its limitations were analysed. A future prolongation of this topic would, preferentially, begin with the seek for a more suitable voltammetric procedure, as the adsorptive cathodic stripping voltammetry with a competition ligand which would allow the calculation of a more accurate vaçue of K'_{av}. The task seems very difficult, as shown by some tries performed, due to the lack of finding an appropriate competition ligand for acidic medium, which should have a known high stability constant of complex formation with Pb and whose voltammetric peak would not overlap with the labile Pb peak or others. Besides that, several other items are prompted for study:

-the speciation and bioavailability of other heavy metals and/or in other wine types or other alcoholic beverages

-the influence of the addition of individual compounds or groups of compounds to wine on the speciation and bioavailability of metals. For example, the addition of tannins to white wine could clarify the hypothesis of the involvement of the tannins in the Pb insolubilization phenomena occurred for the red table wines.

-the bioavailability and speciation of metals in mixed wine-foods digestion.

-the toxicity of the assimilable forms of the toxic metals, by testing some toxicological parameters in a microorganism, such as *Saccharomyces cerevisiae*, that permit some degree of extrapolation to the human organism.

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APPENDIX

PRINCIPLES OF THE INSTRUMENTAL ANALYTICAL TECHNIQUES EMPLOYED

APPENDIX CONTENTS

A1 - ELECTROCHEMICAL TECHNIQUES

If a solution forms part of an electrochemical cell, the potential of the cell, the current flowing through it and its resistance are all determined by the chemical composition of the solution. Quantitative and qualitative information can thus be obtained by measuring one or more of these electrical properties under controlled conditions. The potentiometry with an ion selective electrode and the square wave cathodic voltammetry were the electrochemical techniques used in the present work and will be briefly presented in the next sections.

A1.1- Potentiometry with Ion Selective Electrodes

(Fifield and Kealey 1986)

The electrochemical cell consists of an indicator and a reference electrode, the latter usually being the calomel or silver / silver chloride type. The potential of the indicator electrode is related to the activities of one or more of the components of the solution and it, therefore, determines the overall cell potential. Ideally, its response to changes in the activity should be rapid, reversible and governed by the Nernst equation, which gives the relation between the activities of the species involved in the electrode reactions and the electrode potential. For a general half-cell reaction (oxidation or reduction at an electrode) written as a reduction, *i.e.*,

$$aA + bB + ... + ne^- \rightarrow xX + yY + ... \quad (A1)$$

the equation is of the form

$$E = E^0 - (RT/nF).\ln([X]^x[Y]^y.../[A]^a[B]^b...) \quad (A2)$$

If the activities of all reactants and products are unity, $E = E^0$. Activity and concentration are related by

$$[A] = \gamma_A . |A| \quad (A3)$$

where γ_A is the activity coefficient and $|A|$ is the concentration of the species A. The activity coefficient depends mainly on the ionic strength of the solution. Therefore, if the ionic strength is kept constant over the potentiometric measurements, γ is practically constant, which means that the activity is proportional to the concentration along all the potentiometric measurements. In that case, and at constant temperature, equation A2 becomes:

$$E = K + \ln 10.(RT/nF).\log(|X|^x|Y|^y.../|A|^a|B|^b...) \quad (A4)$$

where concentrations replaced activities and K is a constant which includes the contribution from the coefficients of activity and the potential of the liquid junction (which is formed at the surface of contact between the solution under study and the filling solution at the reference electrolyte).

There are two types of indicator electrode which possess the desired characteristics – metallic and membrane. In this work, a sub-type of membrane (or ion selective) electrodes, the solid state electrodes were used and will be further focused next. These incorporate membranes fabricated from highly insoluble crystalline materials, such as the metal sulphides. These types of membrane show a selective and Nernstian response to solutions containing either the cation or anion of the salts used. Electrodes responding to Pb and Cu (those used in the present work) are made using membranes fabricated from Ag_2S doped with PbS or CuS, respectively. The electrode responds by virtue of the solubility product equilibria involved, the movement of Ag^+ ions within the membrane being the potential-governing factor. In this situation the Nernst equation assumes a simpler form (for divalent cations like Cu^{2+} and Pb^{2+}):

$$E = K' + s.\log|M^{2+}| \quad (A5)$$

where K' is a constant which includes the contribution of the solubility product of the salt MS plus the contributions included for K.

In practice, calibration curves of E *vs.* $\log|M^{2+}|$ performed in conditions of constant temperature and ionic strength are used to obtain K' (as the intercept) and the slope (s) which should be close to the Nernstian theoretical value.

A1.2- Square Wave Cathodic Voltammetry

(Fifield and Kealey 1986; Wang 1985; Bond 1980)

The study of current-potential relations in an electrolysis cell (an electrochemical cell through which current is forced by some external source of energy) where the current is determined solely by the rate of diffusion of an electroactive species is called voltammetry. To obtain diffusion-controlled currents, the solution must be unstirred and the temperature of the cell thermostatically controlled so as to eliminate mechanical and thermal convection. In addition, a high concentration of an electrochemically inert background or supporting electrolyte is usually added to the solution to suppress the migration of electroactive species towards the electrodes by electrostatic attraction.

Typically, the cell comprises a readily polarisable electrode (working electrode), a reference electrode and an auxiliary electrode. By using a small polarisable electrode, conditions can readily be attained wherein the diffusion current is independent of the applied potential and directly proportional to the concentration of electroactive species in the bulk solution. Measurements of such limiting currents form the basis of quantitative analysis. The polarisable microelectrode is usually made the cathod at which the electroactive species are reduced.

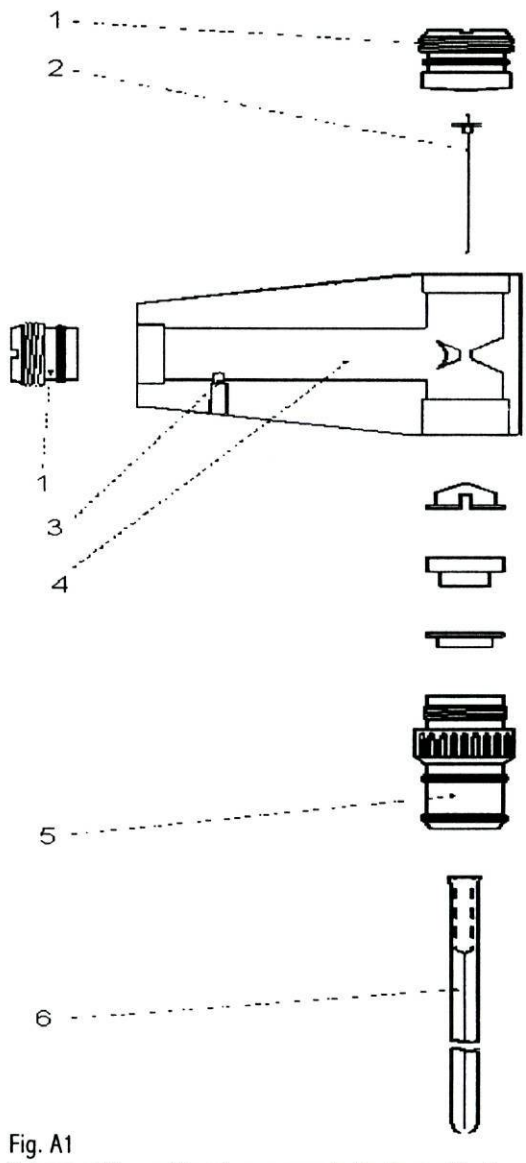

Fig. A1
Scheme of the multimode mercury electrode used in the present work: 1- slotted screws, 2- sealing needle, 3- electrical contact pin for mercury, 4- mercury reservoir, 5- retaining nut, 6- glass capillary

The mercury electrodes are the most commonly found in voltammetric applications, and in this cases the technique is known as polarography. The mercury electrodes used in polarography are characterised by their small surface area, which enhances polarisation and minimise depletion (by electrolysis) of the analyte, thus favouring high sensitivity and reproducibility. They also have wide cathodic potential range. In the present work it was used the hanging mercury drop electrode (HMDE), which is one the different possible mercury electrodes achievable with our voltammetric system (figure A1). The HMDE consists of a small mercury drop hanging at the glass capillary tip, which is renewed after every measurement. Although there are mercury electrodes, like the thin mercury film electrode (TMFE), which offer higher sensitivity than the HMDE, the later is advantageous at several levels: reduced intermetallic interferences, low sensitivity to supporting electrolyte variations or to solution resistance at low concentrated electrolyte, low susceptibility to surfactant activity, and often provides clear resolution of Zn and Cu peaks from the hydrogen evolution and the mercury oxidation background currents, respectively. Therefore, the HMDE is a reliable and versatile type of electrode.

A plot of current flowing in the cell as a function of the applied potential is called a voltammogram (figure A2). At small applied potentials, only a residual current flows in the cell caused by the reduction of trace impurities in the sample solution and by charging of the mercury drop. Above the decomposition potential, at which point the reduction of an electroactive species is initiated, the current increases with applied potential, until it levels off at a limiting value. The difference between the limiting current and the residual current is known as the diffusion current, i_d. If other species in the solution are reduced, additional voltammetric waves will be observed. Finally the current will increase due to reduction of the supporting electrolyte or of the electrode material. In each voltammetric wave, the potential at which the diffusion current reaches half the limiting value is known as the half-wave potential $E_{1/2}$ and is characteristic of the particular electroactive species involved.

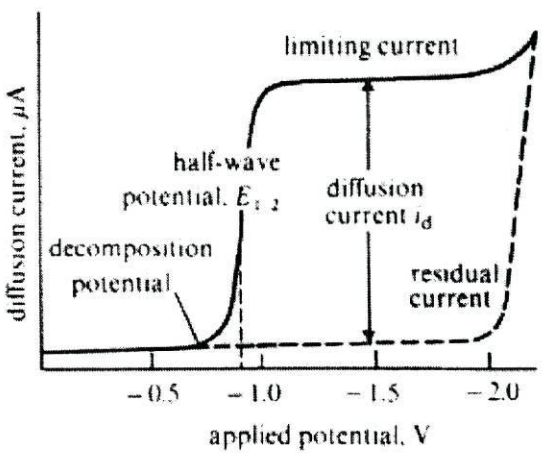

Fig. A2
Voltammogram or voltammetric wave. Adapted from Fifield and Kealey 1986.

When the potential applied to a voltammetric cell exceeds the decomposition potential of an electroactive species, its concentration at the surface of the mercury drop is immediately diminished. A concentration gradient is thereby established and more of that species diffuses from the bulk solution to the electrode surface (Fick's

law of diffusion). The resulting current flow is proportional to the rate of diffusion which in turn is determined by the concentration gradient, *i.e.*

$$i = k.(c-c_0) \qquad (A6)$$

where c and c_0 are the concentrations of the electroactive species in the bulk solution and at the surface of the HMDE respectively, and k is a proportionality constant. By progressively increasing the applied potential, reduction occurs more rapidly, c_0 eventually becomes virtually zero, and the concentration gradient reaches a maximum. At this point, the rate of diffusion and therefore the current flowing in the cell reaches a limiting value, *i.e.*

$$i_d = k.c \qquad (A7)$$

Further increases in the applied potential do not increase the current and the cell is said to be completely polarised. The diffusion current i_d is hence directly proportional to the bulk concentration of the electroactive species.

The earliest types of voltammetry system involved the manual changing of a dc applied potential stepwise. More recently, oscillographic or rapid scan voltammetry, pulse voltammetry, ac voltammetry, square wave voltammetry and other fully automated potential sweeping modes were introduced, primarily to increase sensitivity and to facilitate the resolution of closely-spaced voltammetric waves. The square wave voltammetry (used in the present work) involves the superposition of small square-wave potential amplitude on a staircase ramp (figure A3). By sampling the current just before the square wave changes polarity, the residual or charging current is corrected. This potential-time wave form may be operated at fast scan rates allowing complete scanning in a matter of seconds. The reading of the current signal is carried in a way that the first derivative of the i = f(E) function is obtained. It implies that peaks are obtained instead of voltammetric waves.

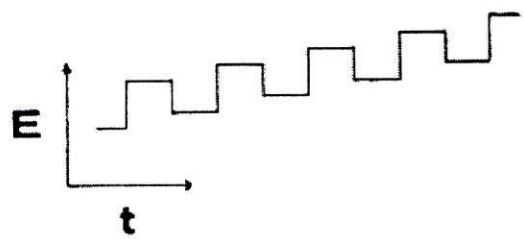

Fig. A3
Potential-time waveform of square wave voltammetry. Adapted from Wang 1985.

A2 - SPECTROMETRIC TECHNIQUES

All spectrometric techniques depend on the emission or absorption of electromagnetic radiation characteristic of certain energy changes within an atomic or molecular system. The energy changes are associated with a complex series of discrete or quantized energy levels in which atoms and molecules are considered to exist (Fifield and Kealey 1986).

Atomic absorption spectroscopy, UV-Vis spectroscopy and fluorescence spectroscopy will be focused in the following sections, as they were techniques employed in the present work.

A2.1- Atomic Absorption Spectrometry (Mermet 1998)

Atomic absorption spectrometry (AAS) is based upon the absorption of radiation by free atoms, usually in the ground state. By selecting a wavelength for a given element that corresponds to an optical transition between atoms in the ground state and atoms in an excited level, the absorption leads to a depopulation of the ground state. The value of the absorption is related to the concentration of the atoms in the ground level, and therefore, to the concentration of the element. By measuring the amount of radiation absorbed, a quantitative determination of the amount of analyte can be made.

The absorption of an incident radiation by an analyte can be calculated by the transmission factor, T, which is the ratio of the transmitted (non absorbed) intensity, I, to the incident intensity, I_0. The absorbance, A, is defined as:

$$A = -\log T = \log (I_0/I) \qquad (A8)$$

A can also be expressed as:

$$A = 0.434.A.(g_m/g_0).(\lambda^4/8\pi c).(1/\Delta\lambda_{eff}).n_0.l \qquad (A9)$$

where A is the transition probability between the ground and excited states, g_m and g_0 are the statistical weights of the excited and ground states, respectively, $\Delta\lambda_{eff}$ is an effective width of the analyte line, *i.e.*, the width corrected by a coefficient, λ is the wavelength associated to the transition, l is the length of the absorption path and c is the concentration of the analyte.

By using the logarithm of A makes it possible to obtain a linear relationship with analyte concentration. This relation is practically linear over a limited range of concentrations because of change in the length of absorption, variation in the line width, atom distribution in the source, etc.

An atomic absorption spectrometer consists of a primary radiation source which produces the radiation to be absorbed, a source of free atoms with an associated sample introduction system, an optical dispersive system, a detector and electronics for data acquisition, processing and editing (figure A4). The presence of free atoms must be obtained in the path between the primary radiation source and the detector.

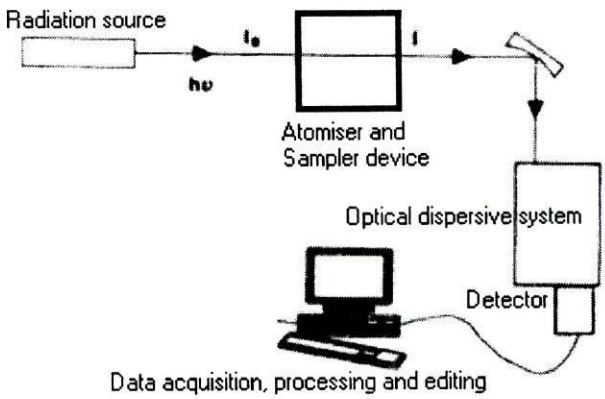

Fig. A4
Principle of atomic absorption spectroscopy. Adapted from Mermet (1998)

The most commonly used radiation sources are the hallow cathode lamp (HCL) and the electrodeless discharge lamp (EDL). The HCL lamp (used in the present work) consists of a hollow cathode made of a highly pure metal whose spectrum is to be produced with an inner diameter in the 2-5 mm range. The cathode and anode are set up in a glass cylinder. A high voltage and a current of up to 30 mA are used to produce a discharge which takes place entirely in the hallow cathode. The glass cylinder is filled by Ar or Ne gas with a pressure in the 1-5 torr region. Narrow, intense lines are emitted following sputtering and excitation processes by the ions of the fill gas. The EDL lamp consists of a sealed silica tube containing the element or salt of interest with Ar as a fill gas. A discharge is sustained by means of a radiofrequency (RF) field through an antenna or a coil. The RF energy served both for the element vaporisation and excitation. The emission arising from the EDL is usually more intense than that emitted from a HCL.

Two types of atomiser are commonly used, the flame and the electrothermal atomiser (furnace) and were both used in the present work. Among the possible flames the air acetylene and the nitrous oxide (N_2O)-

acetylene are the most common. The later is suitable for refractory elements because of the high temperature achieved (> 2600 °C). The sample is nebulised by means of the oxidant flow and a selection of fine droplets is obtained in a spray chamber with paddles or impact bead, where the aerosol is mixed with the fuel gas and finally reaches the burner head (figure A5). A steady absorption process is therefore obtained. However, the residence time of the free atoms in the absorption path is short. This is one of the reasons for the relatively poor sensitivity of flame-AAS along with the low efficiency of the nebuliser-burner system and the dilution of free atoms by the combustion gases.

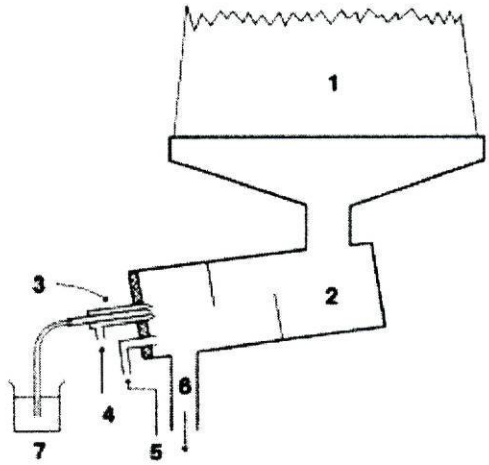

Fig. A5
Principle of flame AAS atomiser. 1: flame, 2: burner, 3: nebuliser, 4: oxidant gas, 5: fuel gas, 6: drain, 7: liquid sample. Adapted from Mermet (1998)

The alternative to the flame is the electrothermal atomiser (ET). This atomiser makes use of an electrically heated refractory material on which the sample is deposited. A transient formation of free atoms is then obtained. One significant advantage of the ETA is the increase in the residence time of the free atoms compared with the flame. Commercially available ETs make use of a cylindrical tube (generally of graphite) also called furnace. At the present it is usual the furnaces to have platforms which consist of a thin graphite plate onto which the sample is deposited. The platform is mainly heated by the radiation from the wall of the tube so that the increase in the temperature of the sample is delayed with that of the wall and the gaseous phase. Therefore, the atomisation of the sample occurs after the wall and the gaseous phase have reached a temperature plateau. An enhancement of sample dissociation is obtained, in particular for volatile elements. Moreover, interference effects are minimised and the volume of sample is quite reduced (10-50 μL). The electrothermal program consists of several progressive heating stages (generally three: drying, ashing and atomisation) (figure A6). The first stage ensures the desolvation of the sample in order to remove the solvent by evaporation. The second stage is the ashing (pyrolysis) of the solid residue remaining after the drying step. The ashing step ensures the removal or simplification of organic or inorganic matrix and consequently represents the most important step of the electrothermal program. The third stage is the atomisation step in which the dissociation of the analyte

molecular species occurs at high temperature (1200 –2700 °C) and a transient formation of free analyte atoms is obtained.

Both flame and furnace AAS can be sensitive to matrix interferences, which correspond to a change in the formation of free atoms. In the case of flame, the limited temperature does not ensure a full dissociation and atomisation of thermally stable compounds in the gaseous phase. The ashing step is a crucial stage in AAS-ET with possibility of loss of volatile species. The main use of chemical modifiers in AAS-ET is to increase the difference in the volatilisation between the analyte and the matrix to obtain for instance a less volatile analyte (analyte modifier) or a more volatile matrix (matrix modifier).

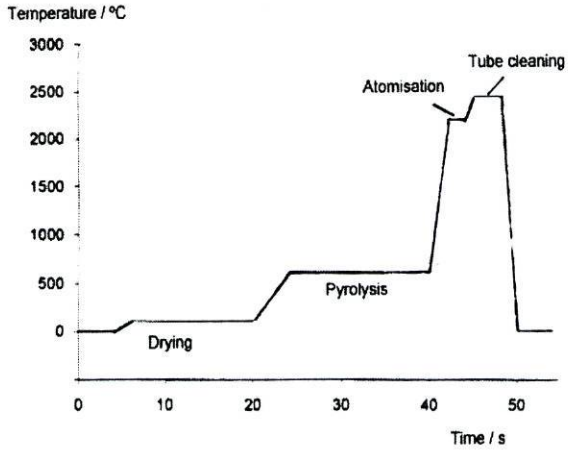

Fig. A6
A typical AAS-ET temperature program.

For example. when using an air-acetylene flame, the absorbance of Ca decreases in the presence of phosphate due to the formation of stable Ca phosphates. It is then necessary to have a buffer such as La which will form a thermally stable compound with phosphate so that Ca will be released to form free atoms. The presence of easily ionised elements such as the alkali elements modifies the equilibrium between ions and neutral atoms. This ionisation interference can be overcome by adding an excess of Cs to produce a large number of electrons so that the presence of an element such as Na with a lower amount than Cs will not significantly change the total number of electrons. Another example, for the AAS-ET, is the use of phosphate (usually $NH_4H_2PO_4$) for turning the analytes less volatile, thus allowing it to be ashed at higher temperatures, implying that more interferences are eliminated prior to measurement of the absorbance (Braun 1987).

In AAS, the possibility of spectral interferences arising from a spectral line of another element within the spectral band pass of the dispersive system is rather small. Moreover, the spectral interferences are well characterised. Far more complex is the presence of non-specific absorptions arising from the components of the matrix. They are either due to light scattering of solid or liquid particles still present in the atomiser or due to molecular bands. This can result in a significant increase in the background and, therefore, an increase in the absorbance of the signal. Diffusion from particles can arise in the flame when high salt concentration solutions are nebulised. It is more often observed in AAS-ET.

Molecular bands are emitted by species, molecules and radicals, either present in the matrix or formed during the atomisation processes. Their structure depends on the spectral bandpass of the dispersive system. Most of the time they look like nonstructured spectra. Because of the excess in absorbance, it is necessary to carry out background subtraction in order to obtain the accurate absorbance of the analyte. Besides the possibility of correcting with a true blank solution, which would be the ideal solution for eliminating the compounds responsible for the excess in absorbance, and for optimising the various heating steps, three methods are currently commercially available for background subtraction: the deuterium lamp method, the Zeeman effect method, and the Smith-Hieftje method. The principal is to measure sequentially the absorbance of the background and that of the analyte and background, the absorbance of the analyte being obtained by subtraction.

A2.2- Ultraviolet – Visible Spectrometry (Kellner 1998)

Most ultraviolet – visible (UV-VIS) spectra are obtained by measuring the intensity of the absorption of monochromatic radiation across a range of wavelengths passing through a solution in a cuvette. The practical wavelength region extends from 190-400 nm (UV range) and from 400-780 nm (VIS range).

In a typical experiment, a light beam of intensity I_0 strikes a sample consisting of a quartz or glass cell containing a solution (figure A7). After passing through the cell, the light beam has a reduced intensity I due to reflection losses at the cell windows, absorption in the sample and, eventually, by scattering at dispersed particles. The run is repeated using an identical cell containing only solvent to compensate for reflection and scattering losses, and the transmittance T is calculated using the following equation:

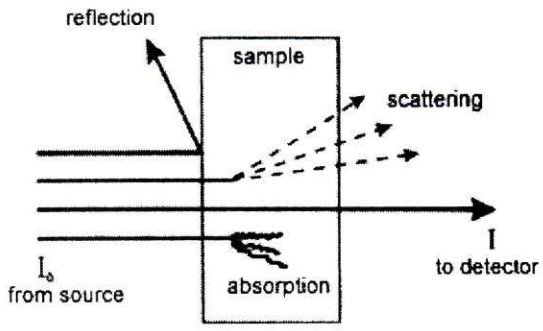

Fig. A7
Intensity loss of a light beam of intensity I_0 by reflection, scattering and absorption. Adapted from Kellner (1998)

$$T = I/I_0 \approx I_{solution}/I_{solvent} \qquad (A10)$$

The intensity of an absorption band, *i.e.*, the absorbance (see equation A9), is proportional to the number of absorbing species in the illuminated part of the cell. The absorbance is proportional to the cell thickness, d [cm], the concentration of the solution, c [mol/L]; and a substance specific proportionality constant ε called the molar absorptivity [$L.mol^{-1}.cm^{-1}$]:

$$A = \varepsilon.c.d \qquad \text{Beer's law} \qquad (A11)$$

For a given system, a linear relationship exists between A and the sample concentration, but usually only for dilute ($c \leq 0.01$ mol/L). At higher concentrations, changes of ε values may occur which lead to deviations from a linear working curve.

The most important use of UV-VIS absorption spectroscopy is in quantitative (trace) analysis of metals, drugs, body fluids, and food due to its sensitivity, reproducibility and ease of operation. Array detector system are furthermore used as flow-through detectors for high performance liquid chromatography (HPLC) due to their high recording speed.

A2.3- Fluorimetric Spectrometry

(Kellner 1998)

The excitation of molecules to higher energy states by the absorption of energy is very rapid (10^{-15} s), and can occur to several vibrational levels of the excited electronic levels. The excited states usually decay rapidly by non-radiational "vibrational relaxation" (10^{-10} s). Fluorescence occurs when a molecule excited to higher vibrational state of an upper electronic level returns via the lowest vibrational level of the excited electronic state to any of the vibrational levels of the ground state (figure A8). In this case, radiation having a longer wavelength than

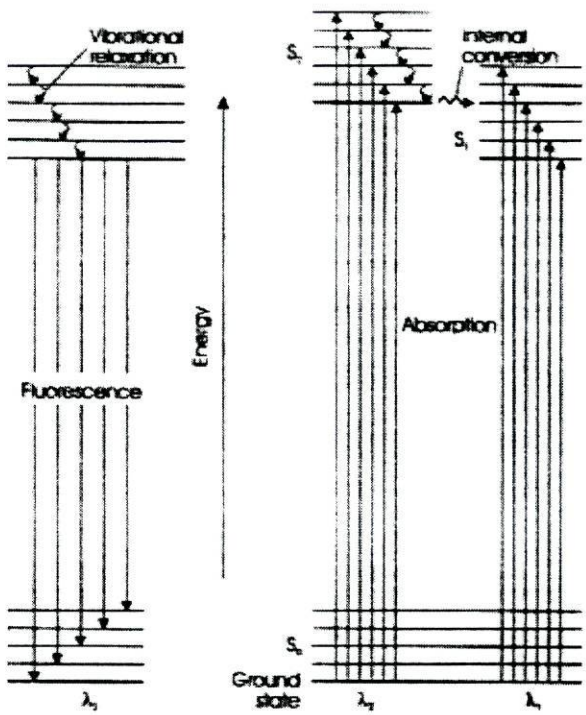

Fig. A8
Energy level diagram to describe absorption, vibrational relaxation and fluorescence. Adapted from Kellner (1998)

wavelength than the exciting radiation is emitted only after 10^{-8}-10^{-6} s. The most intensive fluorescence is found in aromatic compounds with low energy $\pi \rightarrow \pi^*$ transitions (conjugated chromophores).

Instruments for measuring fluorescence are similar to UV-VIS spectrometers, but require a reference beam and detector for background measurements (figure A9). Compared to absorption techniques, fluorescence methods are extremely sensitive (ppb range) and the signal intensities are largely proportional to the concentration of the sample. the number of substances which fluoresce is, however, limited.

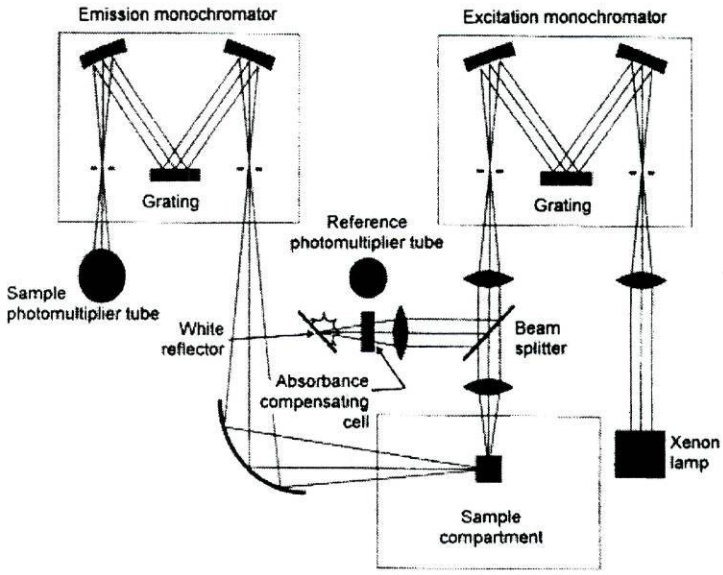

Fig. A9
Schematic of a typical spectrofluorimeter. Adapted from Kellner (1998)

A3 - CHROMATOGRAPHY

Chromatography designates a set of similar separation methods in which the components of a mixture are distributed among two phases: a stationary phase (solid or liquid, adhered to a porous solid medium of support) with high superficial area, and a mobile phase (fluid) that contacts with the stationary phase. The separation results from the differences in the velocity of dragging the components by the mobile phase, given the different interactions with the stationary phase.

A3.1- High Performance Liquid Chromatography (HPLC) (Pombeiro 1991)

This type of chromatography is one the several existent types, and is actually the most used due to its high resolution in a relatively short analysis period. Two sub-types of HPLC were used in this work (see below) and this section will cover the relevant aspects related with the specific techniques used.

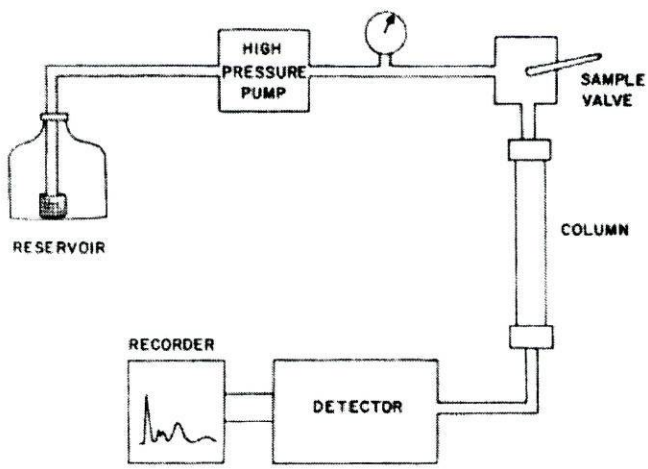

Fig. A10
Schematic HPLC instrumentation. Adapted from McNair H. 1985. in *Inorganic Chromatographic Analysis*, MacDonald J. ed. John Willey & Sons. New York.

The mobile phase is liquid and is driven under high pressure through the stationary phase which consist of columns filled with particles of very reduced size. The sample is introduced near the beginning of the column and its components are dragged by the mobile phase (this process is called *elution*) as shown in the schematic HPLC instrumentation in figure A10. A continuous detection system of the eluate allows obtaining chromatograms as those illustrated in figure A11.

A judicious choice of the composition of the eluant (mobile phase) is crucial for achieving a good resolution. However, in cases were the components of the sample have very different affinity for the eluant, the elution may be very delayed and the resolution for the last components be very decreased, as shown in figure A11a. This problem can be overcome by recurring to several techniques, being gradient elution the most usually utilised. It consists of the continuous and adequate variation of the composition of the eluant (and, therefore, of

its polarity, pH, ionic force), during the chromatographic separation. The gradient must evolve towards a composition with increasing affinity for the most retained components, what, ideally, results in chromatograms as illustrated in figure A11b. In the present work both isocratic and gradient elution were used.

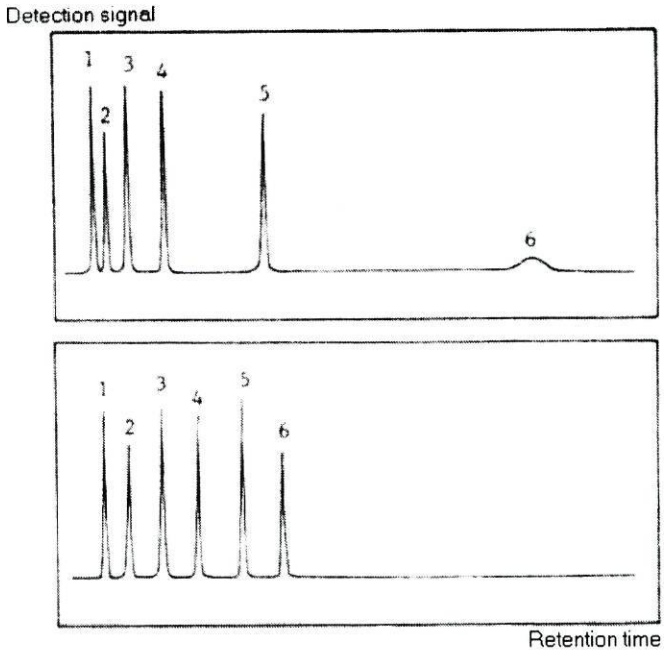

Fig. A11
Separation by isocratic (a) and gradient (b) elution in a mixture with components with a wide range of eluant affinities.

A3.2- Reverse-Phase HPLC

In chromatography, the stationary phase is usually more polar than the mobile phase, the technique being called of normal phase chromatography. However, when the stationary presents a lower polarity than the mobile phase, the technique is called reverse-phase chromatography.

As eluant, it is frequently used water (an highly polar solvent, thus, of reduced strength in the reverse phase technique) or aqueous solutions of a miscible organic solvent, usually methanol, ethanol or acetonitrile. Generally a gradient elution is used, with increasingly organic solvent fraction, thus, evolving towards less polar mobile phase.

The stationary phase most usually used for reverse-phase HPLC is the octadecylsilane (ODS)-C_{18} with a alkyl chain of 18 carbon atoms. The ODS serves as the supporting material.

The reverse-phase HPLC has been increasingly employed due to some advantages over the normal phase HPLC: low cost and ease of attainment of the mobile phase; high stability of the supports for the stationary phase; reliable prediction of elution order by the solubility of the solute in the mobile phase; wide application range.

A3.3- Ion-Exclusion HPLC

The technique of reverse phase can be adapted to the separation of acid or alkaline compounds of high solubility in the aqueous mobile phase. In principle, such analytes would be eluted with excessive quickness originating malformed bands and the impossibility of their separation. However, buffering the solution to a convenient pH allows the control of the ionisation extent, making it possible to perform the separation – technique designated by ion exclusion.

For example, the adjustment of the pH to a sufficiently low value so that the following ionisation equilibrium of the acid HA (analyte to elute) is not excessively displaced to the right side,

$$HA \rightleftarrows H^+ + A^- \qquad (A12)$$

allows the separation of weak or moderate acids.

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