IMPROVING WINE STABILITY WITH PHYTIC ACID

Brent Corey Trela

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มีความเป็นไปได้หลายสาเหตุที่ทำให้ไวน์ไม่มีความอยู่ตัว เป้าหมายของงานวิจัยนี้มุ่งเน้นเฉพาะสาเหตุที่เกิดจากเหล็ก แคลเซียม และเกลือของกรดทาทาริกและโปรตีน (ไบโออัลบูมิน) ซึ่งสิ่งเหล่านี้อาจเป็นสาเหตุก่อให้เกิดการขัดขวางการเปลี่ยนแปลงทางด้านสรีรวิทยาเคมี และออกาโนแลบติก รวมทั้งรสชาติโลหะ การทำให้สีจาง และการเปลี่ยนรสชาติโดยออกซิเจน รวมถึงการเกิดความขุ่นและเกิดตะกอน ซึ่งเป็นการยากที่จะทำการขจัดให้หมดไปในไวน์ งานวิจัยนี้เป็นการแก้ปัญหาโดยวิธีการใหม่ด้วยกรดไฟติก เพื่อป้องกันหรือกระทำให้ไวน์มีความอยู่ตัวมากขึ้น และเป็นที่ยอมรับทางธุรกิจ วิธีดำเนินงานโดยการใส่กรดไฟติกลงในไวน์ เพื่อทำให้เกิดกระบวนการจับกลุ่มประจุบวกในสัดส่วน 1:1 โมลาร์ (เหล็ก:กรดไฟติก) โปรตีนที่ PA:BSA สัดส่วนโมลาร์มากกว่า 6.5 มิลลิโมล และยับยั้งการตกตะกอนของแคลเซียมทราเทรต ตั้งแต่อัตราส่วนโมลาร์ 5:1 (แคลเซียม:กรดไฟติก) จนถึงอัตราต่ำ 10:1 การใส่เกลือแคลเซียม ใส่ในอัตราส่วนของโมลาร์ที่ 5:1 (แคลเซียม:กรดไฟติก) เพื่อตกตะกอนร่วมของกลุ่มเกลือ เหล็กไฟเทต และทำการขจัดตะกอนเหล่านี้โดยวิธีการกรอง วิธีการนี้สามารถแก้ปัญหาต่างๆเหล่านี้ได้อย่างมีประสิทธิภาพในราคาที่ย่อมเยา และสามารถขจัดเหล็ก (> 97 %) และโปรตีน (> 99 %)ได้อย่างปลอดภัย ในขณะที่ทำให้แคลเซียมทราเทตในไวน์ ไวน์ซ่า และเครื่องดื่มอื่นๆ มีความคงทน วิธีการนี้ไม่ทำให้เกิดการเปลี่ยนแปลงในสีและรสชาติของไวน์ ไม่ทำให้เกิดความเป็นพิษในผลิตภัณฑ์ ถึงแม้ว่าจะใส่ลงในปริมาณที่มากเกินก็ตาม วิธีนี้ทำให้เกิดการเปลี่ยนแปลงของกรดและพีเอชเพียงเล็กน้อย และปริมาณแคลเซียมเพิ่มขึ้นเมื่อความเข้มข้นเพิ่มขึ้นหลังจากใส่เกลือแคลเซียมลงไป แต่อย่างไรก็ตาม สิ่งเหล่านี้สามารถนำมากระทำใหม่เพื่อลดผลกระทบดังกล่าว

สาขาวิชาเทคโนโลยีชีวภาพ ลายมือชื่อนักศึกษา

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METAL CHELATION/WINE/PHYTIC ACID/FERRIC CASSE/CALCIUM TARTRATE STABILITY/PROTEIN

There are many potential causes of wine instability. The focus of this work concerns three specific areas: iron, calcium and its tartaric acid salt, and protein (bovine serum albumin). All of these may cause objectionable physiochemical and organoleptic changes including metallic taste, discoloration and oxidative flavor changes, as well as forming hazes and precipitates that can be difficult to treat in wine. This work elucidates the novel use of phytic acid to prevent or treat these problems to make a wine more stable and commercially acceptable. It describes a method in which phytic acid is added to wine to chelate polyvalent iron cations in a 1:1 molar ratio (iron: phytic acid), proteins at phytic acid: BSA molar ratios > 6.5:1 mM, and inhibit calcium tartrate crystallization at molar ratios (Ca:phytic acid) as low as 10:1. The addition of a calcium salt was added at a molar ratio of 5:1 (Ca:phytic acid) to co-precipitate the phytate-iron ion complex and subsequently was removed by filtration. This method overcomes the problems of known methods, for example it effectively, inexpensively and safely removes excessive levels of iron (>97%) and protein (>99%), while stabilizing calcium tartrate in wine, sparkling wine, and other beverages. The method did not change the color or taste, and produces no toxicologically objectionable products even in the case of over clarification. Titratable

acidity and pH were slightly changed after treatments, and calcium concentration increased after calcium salt additions, however, these treatments could be refined to mitigate the described effects.

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Student.s Signature

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Brent Corey Trela

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CHAPTER I

WINE STABILITY AND PHYTIC ACID:

INTRODUCTION AND REVIEW OF LITERATURE

1.1 Stability and fining in wine

1.1.1 Instability common causes in wine

Most wine haze problems are caused by grape particulates and colloids, microbes, **proteins**, **tartrates**, phenolic polymers, polysaccharides and **metals**. Wine clarity problems are not mysterious, and unless a wine has been grossly contaminated by the addition of some foreign material, wine haze is normally the result of one or more of these factors (Table 1.1).

Most white and blush wines will be nearly clear after being protein stabilized with bentonite, but additional clarification steps are often undertaken to produce a bright, clear wine. Additional clarification steps might consist of chill stabilizing, fining with Sparkolloid, or gelatin and Kieselsol fining. After a few months, red wines normally become clear without any fining or filtration treatments because the tannin in red wine acts as a fining agent. Clarity is seldom a problem in red wine. But, long-term bottle stability is always an issue because red wine components polymerize with time and can produce significant bottle deposits unless they are filtered or lightly fined with a proteinaceous material.

Table 1.1 Particulate Causes.

CLEAR WINE	**UNCLEAR WINE**		
	Particles visible using electron microscope	**Particles visible using optical microscope**	**Particles visible to the naked eye**
	Diameter 0.03 µm	**0.3 µm**	**10 µm**
Dissolved particles - Sugars - Organic acids - Salts	Colloidal particles Hydrophobic Colloids: - Nascent tartrate crystals - Metallic hazes Hydrophilic Colloids: - Proteins - carbohydrates, viruses, mucilages, dextrans, etc	Microbial particles - Yeasts (5 – 8 µm) - Bacteria (0.5 – 1 µm)	Deposits - Debris from grapes - Tartrate crystals - Precipitated phenolics - Filtration residues - Sufficiently large colloidal and microbial particles

A wine may be perfectly clear when bottled, however, it may develop a haze unless the wine is completely stable. Sometimes a winemaker bottles a wine without doing stability tests because the wine had been brilliantly clear for several months. But, a few weeks after bottling, the wine develops a haze and precipitates in the bottles.

There are many potential causes of wine instability. The focus of this work concerns three specific areas: the metals iron and copper, calcium ions and their potential to form salts with tartaric acid, and proteins. All of these may cause objectionable organoleptic changes including metallic taste, discoloration and oxidative flavor changes, as well as forming hazes that can be difficult to treat in wine. This work also elucidates the novel use of phytic acid to prevent or fine some of these problems to make a wine more stable and commercially acceptable.

1.1.1.1 Metals

Metals in wine can come from a variety of sources. Soil or dust high in iron can come in directly on harvested fruit (Dupuy et al. 1955). Large gondolas or picking bins made from non-stainless ferrous material, particularly if dejuicing occurs as with mechanically harvested fruit and comes into contact with iron (Hsia et al. 1975). Even damaged stainless steel can rust and contribute iron to wine. Some vineyards employ copper containing vineyard sprays for use as a fungicide that if sprayed too close to harvest may persist on the berries and elevate copper levels in the juice (Hsia et al. 1975). Some wineries may still use bronze winery fittings (e.g. hose connectors) that can be leached by juice or wine acids into the wine. Copper concentration may also be increase from wine treatments such as the treating of sulfides with copper sulfate. Other metals are of concern too; particularly lead that along with copper is also toxicologically objectionable. The low pH and high acidity of juice or wine can dissolve metals that can result in hazes (*casse*), discoloration, catalyze oxidation, as well as leading to objectionable metallic tastes, or outright regulatory bans for excess levels (US TTB; OIV). The prevalent use of stainless steel

and plastic winemaking materials has significantly reduced the iron and copper levels common in earlier eras.

1.1.1.2 Tartrates

Tartrates in wine are commonly potassium or calcium salts of tartaric acid. They can form white crystal deposits that are not a health concern although may detract from their aesthetics. The most common is potassium bitartrate (KHT) (Berg and Keefer 1958), also called cream of tartar, is used in place of lemon juice or vinegar in some recipes, and it is one of the ingredients in baking powder. Calcium tartrate (CaT) is sometimes used in cooking or industrial processes as an anticaking or antifoaming agent among other uses. Tartrate crystals can form in wine that has not been through a stabilization process. Sometimes the crystals are called “wine stones,” but may also be mistaken for broken glass in a bottle of wine, and are objectionable to consumers.

New wines are often supersaturated with potassium bitartrate (Berg and Keefer 1958). The tartrate may precipitate naturally out of the wine during cellaring; however, precipitation may not be complete and may cause long-term potential instability even after bottling. The degree of supersaturation and precipitation is dependent upon concentration of tartrates, temperature, alcohol concentration and pH. Most white and blush wines are stabilized sometime during the course of cellaring. Red wines may also be susceptible and with time may precipitate tartrate-pigment complexes on the sides of bottles in what is often called “lacquering.” There are various methods to test for instability as well as treatments to stabilize wine for potassium bitartrate.

Calcium tartrate supersaturation and spontaneous precipitation, unlike potassium bitartrate, cannot be easily predicted with KHT cold stability tests (McKinnon et al. 1996). Additionally, calcium tartrate precipitation can be slow and may precipitate after bottling (Berg and Keefer 1958; De Soto and Yamada 1963; Postel 1983; Postel et al. 1984; McKinnon et al. 1995). A wine considered stable with respect to potassium bitartrate may, in fact, be very susceptible to calcium tartrate precipitation (Clark et al. 1988).

1.1.1.3 Proteins

Fruit and their resulting wines contain small quantities of proteins, the types and quantities of which are dependent on the cultivar, maturity, and climate (Moretti and Berg 1965; Bayly and Berg 1967). The nature of wine protein instability involves many factors. Under certain conditions protein molecules polymerize or complex with other wine constituents such as polysaccharides and polyphenols into much larger and visible macromolecules that eventually precipitate out of suspension (Vincenzi et al. 2005). Polymerization is slow at normal cellar temperatures, but at elevated temperatures, protein molecules rapidly form large particles (Bayly and Berg 1967). Winemakers often call protein haze "heat instability" because warm storage conditions can trigger the phenomena.

Red wines are seldom unstable with respect to protein. Red wine phenolic compounds react with and bind to proteins during crush and primary fermentation, and the complex precipitates out of solution. White and blush wines usually contain low concentrations of phenolic compounds necessitating the testing and treating of these wines to assure long-term protein stability.

1.1.2 Fining

Fining materials, like filtering, are often used as clarifiers, used to remove haze-causing compounds, but they are also used to modify color, taste and aroma. Conventional filtering, i.e. depth filtration rather than reverse osmosis or nanofiltration, can clarify wine of macromolecules that cause a wine to be cloudy and are likely to eventually precipitate and form deposits. Fining can treat compounds in the wine that do not respond to classical filtering or have not yet reacted or polymerized to form haze-causing macromolecules. Usually, wines undergo filtration and fining, especially with respect to excess protein and tartrates, to provide reasonable assurance of long-term clarity and stability after the wine is bottled.

Bottled commercial wines should be bottled clear and remain clear. The only acceptable deposit in a wine might be colored tannin-anthocyanin matter in a red wine over five years old. A clear wine may be unstable and develop a haze or a deposit upon being subjected to a change in storage conditions such as:

- Aeration (e.g. at bottling)
- Exposure to UV light (e.g. store display)
- Low or high temperatures (e.g. during transport)

It is important to assess a wine's stability or potential instability prior to bottling, and usually easier to prevent spoilage than to cure it.

1.1.3 Health & environment issues

Fining procedures have long relied on the use of natural products that still pose health and safety concerns as well as waste streams that result in loss of product and potential environmental damage. Some traditional filtration and stabilization

materials and techniques may be potentially dangerous to the operator or environment and can be slow, energy intensive and waste producing. Additionally, some fining agents also carry consumer health and safety concerns. These factors are increasingly pressuring wineries to consider new processing options.

Many traditional fining agents are natural products. Consider that despite all the glittering stainless steel, monitoring by sensors, and analysis technology of modern winemaking, ox blood continued to be used, albeit limitedly, as a fining agent in Europe until 1997 when it was banned. New legislation to eliminate its use no doubt resulted from legitimate concerns about bovine spongiform encephalopathy (BSE), commonly called "mad cow disease," which became epidemic among cattle in Britain during the 1990s and which has been associated with the human brain disorder Creutzfeldt-Jakob disease. Consider then that gelatin, derived from hooves and hides of cattle, is still widely used. Animal-derived fining agents include:

- Milk and casein (bovine milk protein)
- Albumin (egg white albumin from chicken, or bovine blood albumin)
- Gelatin (bovine)
- Isinglass (fish air bladders, usually sturgeon)

There is some social and industry movement to reduce or eliminate the use of all animal derived winemaking materials. Apart from the large variation (specificity and efficacy) inherent in natural products, health concerns and consumer trends, particularly the allergen status and consideration to vegetarians and vegans, warrant attention to the choice of winemaking ingredients. Plant proteins might someday provide a substitute for proteinaceous fining agents of animal origin.

The allergen status of food has lead to changes in the regulation and label declaration of some food components for example the listing of sulfites that may in the future extend to other potentially allergenic substances such as egg, fish, and milk products as per international Codex recommendations. There is some debate whether specific fining agents leave residues in the wine that could cause allergic reactions in sensitive individuals. This debate will also likely extend to fining agents derived from plant sources such as gluten. In some countries it is mandatory to label all wines that contain potential food allergens:

- Cereals containing gluten and their products
- Crustacea and their products
- Egg and egg products
- Fish and fish products
- Milk and milk products
- Nuts and sesame seeds and their products
- Peanuts and soybeans, and their products
- Added sulfites in concentrations of 10mg/kg or more
- Apiculture products:
    - Royal jelly
    - Bee pollen
    - Propolis

Other fining and filtration materials are also being scrutinized from health and environmental waste perspectives. Inhalation of diatomaceous earth can lead to silicosis of the lungs (Checkoway et al. 1999). Processes such as protein stabilization through the use of bentonite, produces a portion of wine with high solids that either

goes to waste or requires additional recovery steps such as rotary drum vacuum filtration. Both procedures produce waste streams with cost prohibitive recovery potential, usually just going to disposal, often in landfills.

The development of alternative materials and techniques for wine clarification and stabilization is an ongoing challenge. New approaches may be worth consideration to avoid losses, both in time and materials, as well as the health, safety and environmental concerns associated with conventional techniques. Emerging new techniques may allow the wine industry to produce better wines more safely, cleanly and efficiently, benefiting the producer, the community, the environment and the consumer. Additionally, recycling winery wastes or turning them into valuable products (e.g. cream of tartar) is becoming an essential part of good winemaking practices, cutting costs and further reducing waste disposal effluents.

1.1.4 The principles of fining wine

Wine fining can be defined as the addition of reactive and/or adsorptive substances to remove or reduce the concentration of one or more undesirable constituents (Boutlon, 2005). Often fining is the addition to wine of a colloidal substance that flocculates on contact with wine, and its reactive turbidity causing components, and precipitates to the bottom of the container thus helping either in its clarification, coloration, stabilization, or sensorial properties.

The flocculation of fining agents is due to the action of the pH, tannins, or other conditions in the wine, thus turning the fining agent into a hydrophobic colloid. This reaction requires the presence of minerals, particularly ferric salts, but also calcium, magnesium, sodium and potassium, hence the improvement in flocculation

observed after adding a pinch of salt when adding albumen to red wine. Fining agents generally function by binding with substances through:

- Adsorption: the substance(s) to be removed bind to the surface of the fining agent either by chemical bonds or through ionic interactions:
    - The fining agent and the substance(s) to be removed coalesce forming larger particles that settle in the wine
- Absorption: the substance(s) to be removed are caught within the structure of the fining agent

Wine fining is often a process where a colloidal compound is added to a wine to react with substances that cause turbidity. The two substances (that in the wine and that added) have opposite charges and are attracted to each other. In the process of adsorbing opposing charged molecules, the fining agent flocculates and the resulting coalesced complex is precipitated due to its greater density to form lees. The wine is then usually racked, centrifuged or filtered to separate it from the lees.

In the wine there are positively charged and negatively charged colloids. In order for fining to be effective it is necessary to choose colloids with the opposite charge as the colloid to be removed. Protein fining agents, for example, are electropositive macromolecules that bind with negatively charged colloids (phenolics and flavonoids), which then flocculate and precipitate. Fining agents also function by removing the charge of particles, which then spontaneously flocculate. The flocculation of fining agents is faster at warm temperatures, but sedimentation is better at low temperatures.

Over-fining is for example, when an amount of some proteinaceous fining agent remains unflocculated. This may flocculate later with temperature changes,

time, or with tannins from barrels or corks, and so over-fining should be avoided. Over-fining with respect to residual proteinaceous agents remaining suspended in the wine is unlikely to occur in red wines due to their high tannin content, but can happen in whites with gelatin. Insinglass and casein are less prone to over fining.

Stability tests may be performed before and after fining treatments, on the final blends, and certainly prior to bottling. Blending two stable wines or additions such as sweetener or acid can lead to instability in the final wine.

1.1.4.1 Fining Procedure

Fining can begin at the must stage, though more frequently occurs after fermentation. Fining before fermentation may have the least negative overall organoleptic impact, however usually requires centrifugation or excessive settling times, especially with late harvest or elevated spoilage rate vintages high in glucans and colloids. After fermentation fining often occurs after the wine has lost most of its carbon dioxide. Evolution of carbon dioxide gas tends to maintain particulates in suspension and impede settling.

Fining agents often perform with maximum efficiency at a certain pH and temperature. The age of the wine also affect the fining action of the agent. In all cases rapid introduction and thorough mixing are important to fining efficacy.

1.1.4.4.1 Mixing

It is vital that fining agents are mixed thoroughly and quickly into the wine since, for instance, proteinaceous fining agents coagulate almost instantly on contact with wine. An efficient method of mixing, especially for large volumes where mixing

in tank might be difficult, is to use in-line dosing ("Y" fitting on the pump suction side) during a pumping operation while racking, transferring or mixing (Figure 1.1). The fining agent is slowly introduced to the wine proportionately during the course of pumping, such that by the end of the pumping the fining agent has been completely added. Other methods of mixing include tank paddle mixers, sparging, or in barrels with a barrel mixing dowel or sir rod (Figure 1.2).

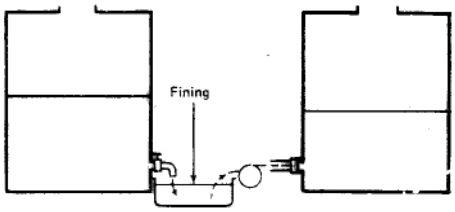


Figure 1.1 "In-line" Dosing.

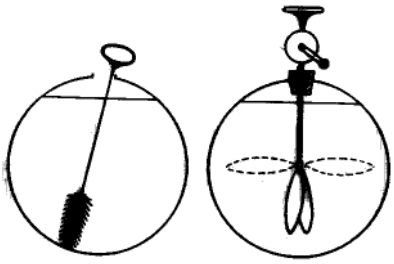


Figure 1.2 Barrel Mixing.

Powdered fining agents should be re-hydrated with water before addition to the wine, re-hydrating in wine often reduces their effectiveness. The contact time between the fining agent and the wine should be no longer than the time it takes for them to react. In practice this may mean as long as it takes for the fining agent to

settle out and form lees in the tank (generally one to two weeks) before being racked. Centrifugation and filtration may be used for more rapid separation.

1.1.5 Fining agents

Current wine fining agents can be classified into one of the following groups:

- Proteins:
  - Gelatin, casein, albumin, isinglass, and gluten
- Earths:
  - Bentonite, Kaolin
- Synthetic polymers:
  - PVPP, nylon
- Polysaccharides:
  - Agar, gum arabic
- Microbial & Enzymatic
  - Yeast
  - Enzymes
- Other agents
  - Carbons
  - Silica gels
  - Chelators
    - Colloidal ferrocyanide & phytic acid
  - Sulfur dioxide
    - Inhibits some enzymes and microorganisms

- Can bind with and render sensorially neutral some spoilage products

The correct choice of fining agent(s) and dose depends on the wine's composition, its colloidal content, and the nature of the particles in suspension. It is best to carry out comparative trials of different agents at different doses, using small quantities to determine the smallest addition required to achieve the desired result. Only fining agents of the highest purity, free from undesirable odors and flavors, should be used. Addition and mixing into the wine should be thorough. The contact time should be limited to only that required to complete the reaction and then be physically removed through settling and racking, centrifugation, or filtration). The selected fining agent should provide the best effect, leave the least volume of lees, and precipitate quickly. The effectiveness of a fining agent depends on the agent, method of preparation and addition, levels of addition, pH, metal content, and temperature. Additionally, a single fining agent can simultaneously affect a number of wine attributes, e.g. gelatin fining to reduce astringency may also reduce color significantly, have a tendency to over-fine, affect relative quality changes among other effects (Table 1.2).

Table 1.2 Comparison of Fining Agents[1] (adapted from Zoecklein 1990).

Color Removal	Tannin Removal	Quantity of Lees	Tendency to Over fine	Clarity and Stability	Quality Impairment
carbon	gelatin	bentonite[2]	gelatin	bentonite	carbon
gelatin	albumen	gelatin	albumen	ferrocyanide	bentonite
casein	isinglass	casein	isinglass	cufex	casein
albumen	casein	albumen	casein	carbon	gelatin
isinglass	bentonite	isinglass	ferrocyanide	isinglass	albumen
bentonite	carbon	ferrocyanide	cufex	casein	isinglass
ferrocyanide	ferrocyanide	cufex		gelatin	ferrocyanide
cufex	cufex	carbon		albumen	cufex

[1]Most to least effect

[2]Usually twice the lees of other agents

1.1.5.1.1 Immobilized Fining Agents

The use of immobilized fining agents is not yet widespread; however there are some advantages to using immobilized agents including:

- Recovery of reactants and products
- More specificity of reactant
- Enhanced activity and stability over soluble form
- Residue minimization
- Lees and wine volume loss reductions.

Almost any wine reactant could be immobilized including:

- Yeast & Bacteria
- Enzymes
- Proteins
- Tannins
- Copper
- Chelators
- Others

For example, metal chelators could be immobilized onto insoluble particulate matrices such as filter sheets for the lowering of metal contents (e.g. Cu and Fe) as an alternative to the use of cyanide containing preparations that may be difficult to remove in the wine. Additionally, copper-containing matrices, including filter sheets, could be used to remove sulfides.

1.1.6 Fining Trials

Fining is often a trade off between achieving a specific goal, such as protein stability, and producing a palatable wine that retains its character. Often there is a range of fining agents and doses that may achieve the same or similar goals, but produce different organoleptic or counter-fining results. Usually different fining agents will act differently on the same wine. Laboratory trials are essential for determining the appropriate agent and dose to use for a given wine.

Laboratory trials usually test a range of fining agent doses to a series of small volume wine samples (see section). After treatment the samples are assessed for results and organoleptic quality. The preferred dose is then scaled to the cellar treatment volume.

For results correlation and agreement between cellar additions and trials, the laboratory trials must be prepared by the same method and under the same conditions as conducted during the cellar application. For consistent results, trial conditions must be equal to those in the cellar including:

- Same reaction temperature
- Same batch of fining agent
- Same method of preparation
- Same method and degree of mixing agent and wine
- Same reaction time

The effectiveness of a fining agent is contingent upon proper preparation. Always prepare the fining agent in exactly the same way for laboratory and winery use. Preparation equipment, temperatures, degree of mixing and timing are critical.

1.2 Iron and copper in wine

Metals, particularly iron and copper transition metals, can accumulate in beverages for a number of reasons. These two elements naturally occur in small amounts in grapes, for example in copper containing enzymes such as oxidases (Hsia et al. 1975). Larger quantities of these elements may accumulate especially from high iron content soils and dust, or copper containing fungicides that remain on the fruit before processing (Dupuy et al. 1955; Rankine 1955; Hsia et al. 1975; Puig-Deu et al. 1994). Metals may also amass from the corrosion of metal processing equipment and storage containers or from any other metal parts that are in contact with the beverage (Hsia et al. 1975). Additionally, the deliberate addition of copper sulfate as a fining

agent to treat sulfides can result in increased copper levels. The average concentration of iron and copper in wine from different regions worldwide has been reported to range from 2.8 to 16 mg/L and 0.11 to 3.6 mg/L respectively (Ough and Amerine, 1988).

These metals can catalyze oxidation reactions as well as complex with wine proteins thereby altering their solubility and stability (Kean and Marsh 1956; Ough et al. 1982). Additionally, the formation of metallo complexes between metal ions and anthocyanins can form colored complexes that lead to darkened discoloration in abraded epidermal cells of fruits such as peaches and nectarines (Cheng and Crisosto 1994; Phillips 1988). Iron levels above 4 mg/L or copper levels above 0.2 mg/L may require treatment to reduce their concentration in order to prevent unwanted cloudiness, oxidation, discoloration, metallic tastes, and metal toxicity (to yeast and humans) (Ough et al. 1982; Cheng and Crisosto 1997). Greenish metallo complexes can form in a 1 : 1 ratio between Fe(III) and cyanidin-3-glucoside, chlorogenic acid, caffeic acid, catechin, or epicatechin (Cheng and Crisosto 1997). Copper ions are reported to increase the production of sulfide ions in synthetic fermentation media (Ashida et al. 1963). Grapes treated with copper-containing fungicides resulted in increased copper content (1-3 mg/L) in grape juices that led to increased H_2S content in wines made from the juices (Eschenbruch and Kleynhans 1974). Several of the issues that can be caused by metals in wines, and a number of methods to reduce metal contamination will be addressed here.

1.2.1 Oxidation catalysts & casse formation

Metal ions such as iron and copper act as catalysts in the oxidation of wine components, although slowly compared to enzymatic oxidation reactions (Cacho et al. 1995). Alcohols are oxidized by hydrogen peroxide in the presence of iron(II) or copper(II) ions and can yield aldehydes and ketones from simple primary and secondary alcohols respectively (Figure 1.3) (Danilewicz, 2003). Metal-catalyzed hydrogen peroxide decomposition occurs through a mechanism known as the Fenton reaction. (Halliwell and Gutteridge1984; Aust et al. 1985; Masarwa *et al.* 1988; Fridovich 1989; Rush et al. 1990; Gunther et al. 1995; Huie and Neta 1999; Danilewicz 2003).

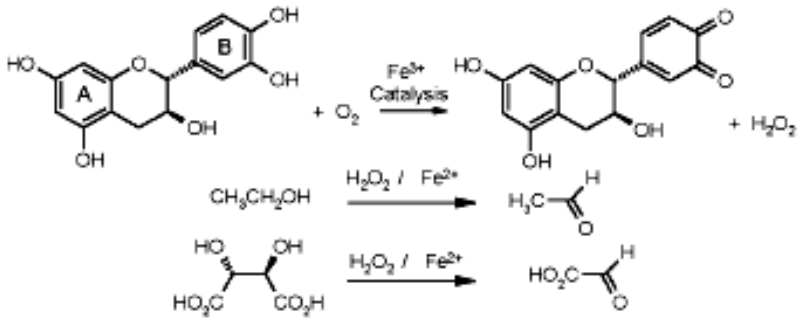


Figure 1.3 Iron catalyzed oxidation of (+)-catechin, ethanol, and L-tartaric acid in wine (Danilewicz 2003).

Iron is present in wine as non-ionic complexed iron (e.g. soluble complexes with organic acids such as citrate, malte, and tartrate), and both Iron(II) and Iron(III) states (Green et al. 1961; Timberlake 1964a,b; Yokoi et al. 1994). The ratio of ionic species depends on the storage conditions, sulfur dioxide content, and the redox-potential of the wine (Costa et al. 2000; Danilewicz 2003). Iron(II) is favored under

low oxygen conditions, whereas aeration favors a larger proportion of the more oxidized Iron(III) form. This is also the form that is more likely to form complexes that result in casse precipitation. Similar to iron, copper is also present in various ratios of the two forms depending on the oxidative condition of the wine, however, in this case it is the more reduced or Copper(I) state that favors casse formation. Reductive conditions such as *sur lies* aging or wine in bottle favors low redox potential and reduction of copper. Table 1.3 shows some common metal states in wine, other oxidation states for these metals are possible, but are less common or rare at wine pH values due to the oxidation-reduction balance (redox state).

Table 1.3 Common oxidation states of iron and copper in wine.

	Oxidation states
Iron: $Fe^{2+} \leftrightarrow Fe^{3+} + e^-$	o In aqueous acid solutions: $Fe[Ar](3d^6 4s^2) \rightarrow [Fe(H_2O)_6]^{2+}$ and $[Fe(H_2O)_6]^{3+}$ (Crichton, 1991) o Iron(II) state, Fe^{2+}, previously *ferrous*, very common o Iron(III) state, Fe^{3+}, previously *ferric*, very common (e.g. rust), most likely to form casse o Iron(IV) state, Fe^{4+}, previously *ferryl*, stabilized in some enzymes (e.g. peroxidases)
Copper: $Cu^{1+} \leftrightarrow Cu^{2+} + e^-$	o Copper(I) state, Cu^{1+}, previously *cuprous*, most likely to form casse o Copper(II) state, Cu^{2+}, previously *cupric*, forms blue or blue-green salts (e.g. copper sulfate) o Copper is the active component of some oxidases (e.g. laccase)

1.2.1.1 Ferric casse

Grapes contain 1 to 5 mg/L of iron; concentrations above this are usually due to soil on the grapes, and from wine processing equipment (Amerine and Ough 1974). During fermentation 45 – 75 percent is absorbed or adsorbed by yeast (Thoukis and Amerine, 1956). In oxidative wine conditions, soluble Fe(II) ions are oxidized into Fe(III) ions that form colloids with phosphate ions (Figure 1.4). The ferric phosphate molecules react with proteins to flocculate and form pale blue deposits in white wines (Chow and Gump 1987; Ribéreau-Gayon et al. 2000). In red wines, Iron(III) reacts directly with phenolics to form blue-black precipitates (Ribéreau-Gayon et al. 2000). Casse formation can occur in white or red wines with iron levels as low as 6 mg/L. High pH and low temperature decreases solubility of colloidal Iron(III) complexes. Solubility was reported as maximum at pH 3.3 (Ribéreau-Gayon et al. 2000).

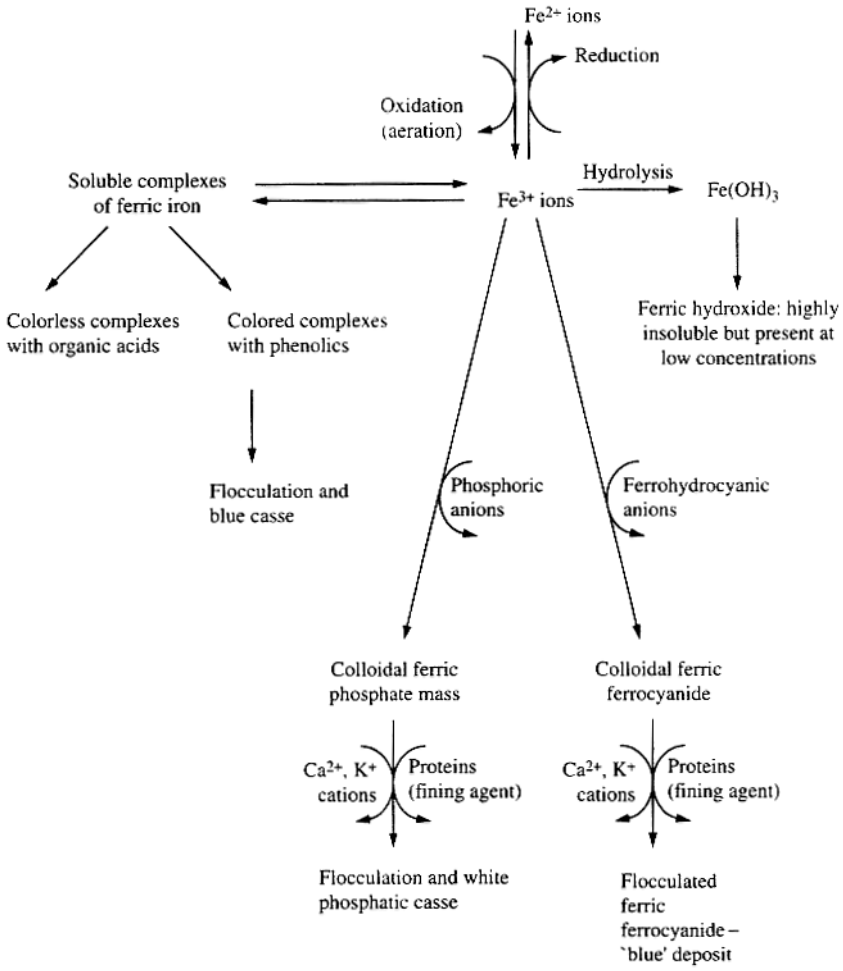


Figure 1.4 Iron reactions in aerated wines (Ribéreau-Gayon et al. 2000).

1.2.1.1.1 Iron sources

- The berry: 1 – 5 mg/L depending upon soil conditions (Amerine and Ough 1974; Dupuy et al. 1955)
- The soil on the berry surface and harvest containers: depends upon soil composition (Dupuy et al. 1955). Much can be settled out.
- Iron containing metals (including damaged stainless steel) in the winery that are in contact with the wine – fittings, etc. (Hsia et al. 1975)

- Typical levels are 2 – 4 mg/L in clear wines and 15 – 25 mg/L in primary lees. Clarify must as soon as possible to reduce levels.

1.2.1.2 Copper casse

The concentration of copper in grape musts averages 5 mg/L (Ribéreau-Gayon et al. 2000), although yeast fix the majority of the copper during fermentation or it is precipitated as copper sulfide. Copper casse can form in wines with copper in excess of 0.2 mg/L, the legal limit for copper ions according to the OIV (2005), in the US the legal limit is 0.5 mg/L (US TTB). The deposit is a reddish-brown hydrosulfide salt of reduced copper [Cu(I)]. Photochemical reaction, proteins (Figure 1.5), amino acids, high temperatures, iron, high pH and high levels of SO_2 can induce copper casse formation. Excess copper can bind to many organic substances and cause a haze, and the concentration of proteins in white wines makes them more prone to this precipitation than red wines. High levels of copper can be sensorially detected at concentrations over 2 mg/L.

Copper casse mechanism (Ribéreau-Gayon et al. 2000):

1. Reduction of copper ions:

   $Cu^{2+} + RH \rightarrow Cu^{+} + H^{+} + R$

2. Reduction of sulfur dioxide:

   $6Cu^{+} + 6H^{+} + SO_2 \rightarrow 6Cu^{2+} + H_2S + H_2O$

3. Formation of copper sulfide, complex with protein causes flocculation:

   $Cu^{2+} + H_2S \rightarrow SCu + 2H^{+}$ (see Figure 1.5)

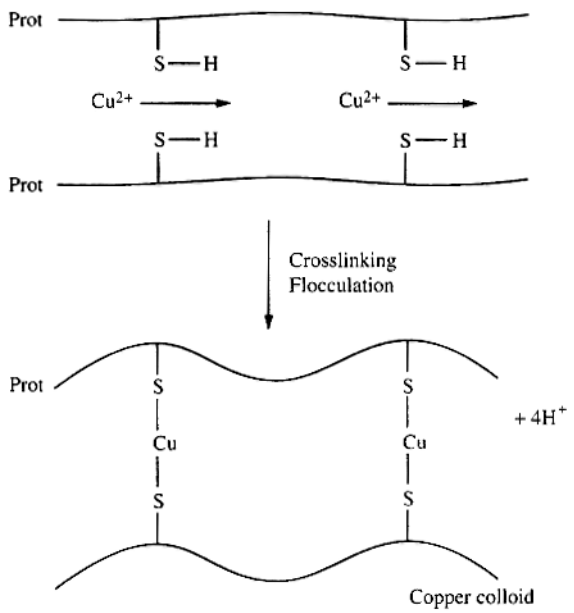


Figure 1.5 Protein cross-linking by copper and copper casse (Ribéreau-Gayon et al. 2000).

1.2.1.2.1 Sources

- Copper containing fungicidal berry spray residues (Hsia et al. 1975)
- Copper containing metals (copper and copper containing alloys) that are in contact with the wine (e.g. valves, fittings, etc., in the winery) (Hsia et al. 1975)
- Copper treatments e.g. fining hydrogen sulfide.

1.2.2 Analysis

Several methods for metals determination exist, but the techniques of atomic absorption and flame emission spectrometry are currently the most sensitive (mg/L to μg/L levels) and rapid. Most metals present in wine can be determined with these

techniques (Caputi and Ueda 1967; Ough and Amerine 1988; Ajlec and Stupar 1989; Lima and Rangel 1990; Aceto et al. 2002).

1.2.2.1 Iron

- Concentration:
  - Colorimetry (various methods)
    - Reaction with potassium thiocyanate (for Fe(III) only)
      - Oxidation with hydrogen peroxide shifts equilibrium to Fe(III)
  - Atomic Absorption Spectroscopy
  - For total iron assay liberate complexed iron with low pH acidification
- Stability:
  - Saturate a half filled bottle of wine with oxygen, or add 5 ml of 10 percent v/v hydrogen peroxide to 1 liter of wine, seal the bottle, and store in refrigerated darkness. Turbidity within 48 hours to one week may indicate the potential for iron casse formation.

1.2.2.2 Copper

- Concentration:
  - Colorimetry using diethyldithiocarbamate or 2,2'-diquinoline (Ough and Amerine 1988)
  - Atomic Absorption Spectroscopy
- Stability:

- Expose a fully filled and sealed bottle of wine to sunlight or ultraviolet radiation for up to seven days. Turbidity may indicate the potential for copper casse formation.

1.2.3 Treatment

Wines may be stabilized with respect to iron and copper by the use of chelating agents, such as commercial resins, ethylenediaminetetraacetic acid (EDTA), pectinic acid, and alginic acid. Removal with ferrocyanide is probably the most efficient method because it precipitates most metal ions, including iron, copper, lead, zinc, and magnesium, although it is ineffective for calcium. Some various metal ion-fining treatments include:

- Yeast and yeast hulls
- Proteins & Colloids
  - Casein
  - Gelatin
  - Gum Arabic
  - Bentonite
- Carbon
- Blending
- Chelators
  - Citric acid
  - Ascorbic acid
  - Calcium phytate
  - Potassium ferrocyanide

- Metal Specific Resins

- Ion exchange

If the metal concentration is below 0.5 – 0.2 mg/L for copper and 7 mg/L of iron, generally no treatment is necessary depending on legal limits for various jurisdictions. If the level of copper is 0.5 – 1 mg/L, a stability test is warranted. If there is no deposit, it may be possible to rely on bentonite and filtering to remove associated complexing colloids (Ribéreau-Gayon et al. 2000).

1.2.3.1 Ferrocyanide

For information on the use of ferrocyanide see section 2.1.1 Ferrocyanide.

1.2.3.2 Hydroxy acids

Iron complexes with hydroxyl acids, such as malic, lactic, tartaric, citric, or tannic acid (Field et al. 1974; Timberlake 1964 a,b; Yokoi et al. 1994), examples of which follow below.

1.2.3.2.1 Citric acid

Citric acid forms a soluble Fe(III) citrate complex (Figure 1.6). Doses of 200 – 300 mg/L can help prevent low levels of iron (15 mg/L) from forming casse (Rankine 1960; Timberlake 1964 a,b; Yokoi et al. 1994; Ribéreau-Gayon et al. 2000), however, it is ineffective with copper. Citric acid has also been shown to prevent and reverse the formation of Fe(III) cyanidin-3-glucoside complexes (Cheng and Crisosto 1997). Colloidal gum arabic (50 – 200 mg/L) may act synergistically with citric acid

to prevent iron casse precipitation (Ribéreau-Gayon et al. 2000). The citric acid content of a bottled wine should not be more than 1 g/L (OIV 2005).

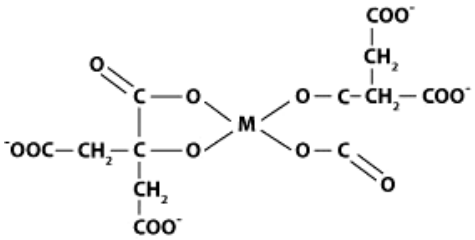


Figure 1.6 Citrate metal chelate.

1.2.3.2.2 Ascorbic acid

Ascorbic acid is a strong reductive agent and antioxidant. It can be used to prevent ferrous iron from becoming oxidized (Bandy and Davison 1987; Hsieh and Hsieh 2000), however due to its reactivity, especially in the ascorbate-driven Fenton reaction, the action is temporary and may favor additional oxidative reactions, and copper casse formation in wines with high levels of copper.

1.2.3.3 Phytate

For information on the use of phytates, see sections 2.2.2 Calcium phytate and 2.2.3 Phytic Acid.

1.2.3.4 Protective colloids

Iron and copper casse may be inhibited by the addition of agents that limit the flocculation of insoluble ferric and copper complexes. Gum arabic acts in this manner. It functions as a protective colloid, restricting haze formation. Because gum

arabic limits the clarification of colloidal material, it can only be safely applied after the wine has undergone all other stabilization procedures. Furthermore, such stabilized iron (and excessive amounts of copper) can still impart an undesirable metallic taste to the wine and also serve as oxidative catalysts that generate off-flavors during storage since not all redox coordination sites are inactivated (Trela and Graf 2005a,b).

The maximum dose of gum arabic should not exceed 0.3 g/L (OIV 2005).

1.2.3.5 Bentonite

Copper casse and iron casse are both precipitated as protein complexes. Reducing the protein concentrations to less than 1 mg/L may prevent their flocculation with proteins.

1.2.3.6 Inducing casse formation

Inducing casse formation could be looked at a form of stabilization since it can bring about the precipitation of casse that can then be removed from the wine before it is bottled. The following examples introduce some methods:

1.2.3.6.1 Oxygenation & tannin addition

Oxygenate the wine until iron turbidity formation. In white wines, 10 – 15 mg/L of oenological tannin is usually added first. The wine is then fined with casein and gelatin, and filtered or clarified by settling. Citric acid is often added to complete the treatment. This method is not commonly used due to the risks of oxidation and loss of aromas.

1.2.3.6.2 Heat treatment

Heat treatment (HTST) in the absence of air followed by rapid cooling and subsequent fining and/or filtering can reduce Cu(II) to Cu(I) that are precipitated along with heat-denatured proteins.

1.2.3.7 Blending

Blend a high metal containing wine with one of negligible content.

1.2.3.8 Lees & yeast

Yeast, yeast hulls, and yeast lees are very effective at absorbing and adsorbing metals (Thoukis and Amerine, 1956), in some cases reducing copper concentration in the final product by up to 90% when metal resistant strains of *Saccharomyces cerevisiae* yeast that accumulate the metal in the cells are used (Brandolini et al. 2002).

1.2.3.9 Sodium sulfide

Sodium sulfide has been proposed as a method of removing copper from wine (Ribéreau-Gayon et al. 2000) through the formation of hydrogen sulfide (H_2S) and insoluble copper sulfide (SCu), however, the generation of H_2S can negatively impact the quality of the wine.

1.2.3.10 Ion exchange

Metal specific ion exchange resins, filter matrixes, and polymers using imidodiacetate functional groups or pyrrolidone and imidazole such as BASF's

Divergan HM (copolymer of 2-imidazolidinone, 1,3-diethenyl-, polymer with 1-ethenyl-1H-imidazole and 1-ethenyl-2-pyrrolidinone) can be used to reduce metal concentration in wine where permitted (Boulton et al. 1996; Palacios et al. 2001). Divergan HM is insoluble in most solvents including wine and is completely removed by filtration. The polymer is intended to be added directly to alcoholic beverages during the maturation process at a recommended use level of 80 grams per 100 liters of beverage (BASF).

1.2.3.11 Supercritical fluids

Supercritical fluids (SCFs) possess properties that are intermediate between liquids and gases. This unique phase is obtained at pressures and temperatures greater than the critical point (Figure 1.7, Table 1.4). Near the critical point of a fluid, minute changes in pressure or temperature significantly alter the physicochemical properties of the SCF (e.g., density, diffusivity, or solubility characteristics) (Yonker et al. 1998; Wai et al. 1998).

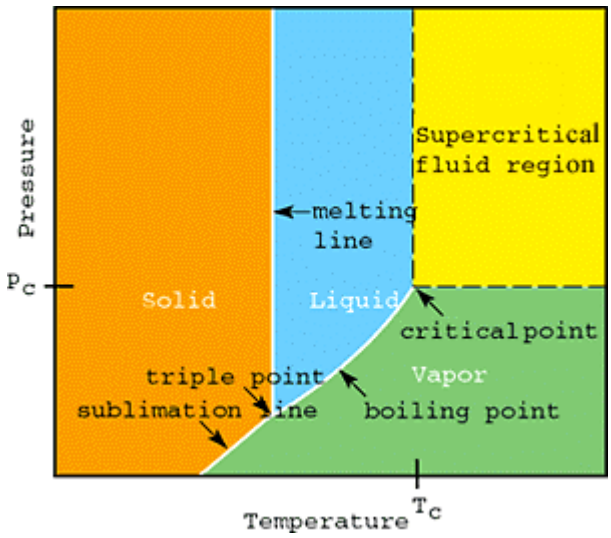


Figure 1.7 Pressure–temperature phase diagram for a pure substance (Fahlman 2002).

Table 1.4 Comparison of the critical constants for commonly used fluids (Fahlman, 2002).

Fluid	Critical Temperature (°C)	Critical Pressure (atm)
Carbon dioxide (CO_2)	31.1	72.8
Methane (CH_4)	–82.1	45.8
Ethane (C_2H_6)	32.3	48.2
Propane (C_3H_8)	96.7	41.9
Argon (Ar)	–122.3	48
Nitrous oxide (N_2O)	36.5	72.5
Water (H_2O)	374.1	218.3

SCFs, especially carbon dioxide (sc-CO_2) and water, are viable replacement solvents for a variety of hazardous organic solvents. By changing the temperature and pressure and, perhaps, adding small amounts of ligands or cosolvents, these two environmentally benign compounds can span a remarkably wide range of solvating power. Supercritical CO_2 is used commercially for the decaffeination of coffee and tea and the extraction of a wide variety of natural oils, spices, flavors and fragrances (Manninen et al. 1997). In the wine industry sc-CO_2 has been used to remove TCA from corks (Anon. 2001). Also of interest are the uses of these two supercritical fluids as solvents for reactions and for some specialty extractions, such as the removal of metals from soils, sludges and aqueous waste streams (Laintz et al. 1992; Saldaña et al. 2005; Brennecke 2005), as well as finding applications in wines.

1.2.4 Other metals

Magnesium is the third most abundant metal in wines after potassium and calcium. Magnesium content in wines ranges between 20 – 240 mg/L (Ough and Amerine, 1988). All magnesium salts are soluble and do not decrease during fermentation and aging (Ribéreau-Gayon et al. 2000).

Other metals including aluminium, boron, nickel, and lead, among others, are found in trace amounts in wines. The ancient Roman aristocracy were often lead poisoned by sapa, a sweet syrup rich in lead acetate (also called sugar of lead, or sugar of Saturn) that was produced by boiling soured wine in lead pots (Nriagu 1985). The Greeks pioneered a related practice earlier during the third century BC, using vinegar to produce pigments from metals; examples of which include lead carbonate (white lead) and mixtures of copper salts to produce green verdigris.

1.3 Tartrates in wine

1.3.1 Introduction

Wines contain 2 – 8 g/L or more tartaric acid, 0.28 – 1.44 g/L potassium, and 0.05 – 0.13 g/L calcium (Ough and Amerine 1988). Tartaric acid is a weak organic acid that in the ripening fruit decreases in concentration as dissociated mono and dibasic (tartrate and bitartrate) salts form. It can precipitate as two main salts: potassium bitartrate (KHT) and sparingly soluble calcium tartrate (CaT) (Figure 1.8).

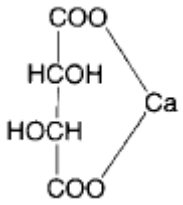


Figure 1.8 Calcium tartrate.

The predominant salt is usually KHT between pH 3.4 and 4, the range in which its solubility is lowest, however with high pH, CaT may be favored. Potassium bitartrate, also called cream of tartar; acts as a mild acid and is often used to remove scale (hard water salts) in coffee pots. It is also used in baking powder (a carbonate plus an acid, usually KHT) or in combination with baking soda (carbonate).

The amounts of potassium, calcium, and tartrate in a berry are both dependent on the cultivar, climate, and degree of maturity (Berg and Keefer 1958; Berg and Keefer 1959). Unlike malic acid, tartaric acid is resistant to metabolic respiration and so its concentration in the berries of a given cultivar is mostly affected by berry swell

during maturation. Tartaric acid, unlike malic and citric acids is also resistant to microbiological metabolism by lactic acid bacteria (LAB) at wine pH values. Potassium concentration, however, is influenced by its uptake from the soil and therefore depends on the growing conditions, particularly rootstock, soil type and moisture, weather conditions, and pH. Potassium and tartrate concentrations in musts and wines vary widely, depending on the growing conditions of the fruit and any given juice or wine may be significantly more or less stable than another (Berg and Keefer 1958; Berg and Keefer 1959). The concentration of calcium ions in a wine is variable, but can be excessively high if there has been contamination from cement containers, filter pads, or through addition e.g. calcium carbonate and calcium bentonite.

All juices and wines differ in their capacity to retain tartate salts in solution as determined by their concentration product (CP), also commonly called solubility product (SP) (De Soto and Yamada 1963). The Solubility Product (K_{sp}) is the thermodynamic constant for the equilibrium that exists between a slightly soluble salt and its ions in a saturated solution. If the wine or juice becomes supersaturated, potassium bitartrate forms crystals and precipitates from solution. Each wine has a unique composition and therefore a unique solubility level. Many juices are saturated with potassium bitartrate; however, the greatest concern is its solubility and precipitation in the wine after bottling. The solubility of potassium bitartrate is dependent primarily upon the alcohol content, pH, the temperature of the wine, and the interactive effects of other wine components. Red wines have up to twice the solubility product of comparable white wines, and much more than model wine solutions of equal ethanol concentration and ionic strength (Abgueguen and Boulton,

1993). This is due to various wine component complexing that increases KHT solubility by the interaction of other wine chemicals. The production of alcohol during fermentation lowers the solubility and often gives rise to a supersaturated solution of KHT that may spontaneously precipitate or have greater potential to precipitate with additional changing conditions such as decreasing temperature, especially after fermentation.

Potassium bitartrate is present in grapes. It is precipitated (along with complexed wine pigments) during fermentation due to its lower solubility in alcohol. In young wines it is usually present at saturated or super-saturated levels. If the wine is cooled, KHT crystallizes and precipitates, until the saturated equilibrium at that temperature is reached.

Supersaturated conditions for CaT are much less common than for potassium bitartrate precipitation. In the wine, excess calcium can form calcium tartrate via the following 1:1 stoichiometric reaction (Clark et al. 1988; Mckinnon et al. 1995):

$$Ca^{2+} + T^{2-} \quad \leftrightarrow \quad CaT(aq)$$

If the concentration of calcium tartrate is sufficiently high, it can precipitate. At a tartaric acid concentration of 6 g/L, the recommended maximum calcium content (based on CP values) in a white wine at pH 4 would be approximately 5 mg/L, and 50 mg/L at pH 3 (Boulton et al. 1996). Many wines naturally contain calcium concentrations of 80 – 100 mg/L, near to instability (Boulton et al. 1996). Calcium tartrate is a concern, however, because conditions are not easily attained for its stabilization, low temperature treatments for potassium bitartrate are ineffective for

calcium tartrate (Postel 1983; Parfent'eva et al. 1984; Postel et al. 1984). Additionally, since calcium is divalent, it is more energetically favored than potassium to participate in colloid flocculation and precipitation, for example, ferric phosphate, and tannin-gelatin complexes (Ribéreau-Gayon et al. 2000).

The occurrence of tartrate crystals (sometimes called “wine stones” or “wine diamonds”) in bottled wines is a natural process that does not affect taste and is not a health concern; however, it can and should be prevented for a number of reasons:

- Aesthetics: crystals are often confused with broken glass, sugar, or chemical residues. Usually unacceptable to consumers.
- Returns: Commercial shipments may be returned, especially in certain export markets.
- Foaming: In sparkling wines, KHT or CAT crystals provide nucleation sites that can cause excess liberation of carbon dioxide resulting in foaming and loss of product when the wine is disgorged or when consumers open bottles.
- pH shift: causes pH changes that may lead to additional instabilities.

1.3.2 Solubility

1.3.2.1 Potassium bitartrate solubility

Potassium bitartrate solubility is affected by:

- Temperature:
    - The cooler the temperature, the less soluble is potassium bitartrate.

- Alcohol content:
    - The higher the alcohol level, the less soluble is potassium bitartrate.
- pH:
    - At a pH of around 3.8 (depending upon alcohol and ion content), the proportion of potassium bitartrate in relation to tartaric acid and tartrate is greatest (~73%).
- Potassium content.
- Wine component complexing factors: (see Chemical Inhibition section 1.3.5.2)
    - Slows down or inhibits crystallization: e.g. phenolics and protecting colloids (polysaccharides, mannoproteins)

1.3.2.2 Calcium tartrate solubility

Calcium tartrate solubility is affected by:

- Temperature
    - Calcium tartrate solubility decreases with decreasing temperature (Table 1.5), but does not enhance crystallization. Calcium tartrate cannot be cold stabilized as per potassium bitartrate (Parfent'eva et al. 1984).

Table 1.5 Solubility of calcium tartrate (g/L) in model solutions (Berg and Keefer, 1959).

Temperature	Ethanol Concentration (%v/v)					
(°C)	10	12	14	16	18	20
-4	0.057	0.048	0.040	0.034	0.028	0.024
0	0.065	0.054	0.046	0.039	0.032	0.027
5	0.076	0.064	0.054	0.045	0.038	0.032
10	0.089	0.075	0.063	0.053	0.045	0.038
15	0.105	0.088	0.075	0.063	0.053	0.045
20	0.124	0.104	0.088	0.074	0.063	0.053

- Alcohol
    - Increasing alcohol content decreases solubility
- pH dependent
    - Higher pH decreases solubility.
    - pH has little, if any, effect on the rate of precipitation, only on the final equilibrium concentration (Clark et al. 1988).

1.3.3 Crystallization rate influences

During fermentation the main factor affecting potassium bitartrate and calcium tartrate solubility is the alcohol content. As it increases, the solubility decreases and potassium bitartrate and calcium tartrate precipitate as saturation threshold values are exceeded. Decreasing temperature also causes potassium bitartrate precipitation, however, cannot be relied upon to precipitate calcium tartrate

at supersaturation levels (Dunsford and Boulton 1981b; Abgueguen and Boulton 1993). Both of these conditions can lead to supersaturation, i.e. a solution that contains more dissolved material (tartrate salts) than could be dissolved by the solvent (wine) under existing conditions. Supersaturated solutions without the activation energy to form crystal nuclei will remain in what is known as a metastable condition. The carbon dioxide in sparkling wines (or beer) is another example of metastable supersaturation. When the seal (cork or cap) is removed exposing the wine to atmospheric pressure conditions, the carbon dioxide fizzes forth. Small particles (nuclei or "seeds") can trigger the separation of the dissolved potassium bitartrate and calcium tartrate from the wine causing precipitation due to lower activation energy required for deposition of the solute onto a solid phase (Pilone and Berg 1965; Grases et al. 1993). In the solid form these seeds can lead to the formation of crystallites or even large single crystals.

Tartrate crystallization occurs in two stages: the induction stage (nucleation) when there is a rapid increase in nuclei, for example, due to chilling, and the crystallization stage when slow crystal growth and development occurs though the transport of solute ions to the growing crystal surface and incorporation into the crystal lattice. Crystallization is limited by:

- The degree of supersaturation and agitation
- Nucleation or availability of focci or nuclei on which the salt may deposit and form crystals
- Diffusion, the rate at which the dissolved potassium bitartrate or calcium tartrate contacts the growing crystal surface
- Crystal surface growth rate

- Complexing factors: polymeric inhibitors, malic acid (Clark et al. 1988)

Nucleation can be slowed or prevented by compounds binding with one or both of the reactants (e.g. Ca, K, and tartrate) that form the salt, or by attaching to the solute salt combination (e.g. CaT or KHT) and blocking nucleus formation (Mckinnon et al. 1994, 1995). Crystal growth can be inhibited by compounds that attach to the crystal face and obstruct further crystal growth. In wines, crystal growth can be slowed by inhibition to such an extent that crystals are prevented from growing to a detectable size during the lifetime of the wine (Mckinnon et al. 1994, 1995)(see 1.3.5.2 Chemical inhibition).

1.3.4 Stability determination

Several of the most commonly used test methods for potassium bitartrate stability in wines include the concentration product test, hold-cold or freeze tests, conductivity measurements, and measurement of the specific cation concentration before and after testing by seeding. Among the cooling or freezing tests, there is a wide range of temperatures and times used. Conductivity measurements require special instrumentation and are generally not a priority for a small start-up winery. Unfortunately with the exception of measuring the concentration of calcium before and after seeding, none of the aforementioned methods are effective to predict calcium tartrate stability. Moreover, there is no industry standard tartrate stability measurement, and indeed, each producer often defines stability differently.

Precipitation of potassium bitartrate or calcium tartrate in the bottle occurs due to incomplete stabilization prior to bottling that might have been prevented by a

more accurate stability evaluation. This section outlines some of the methods used to test for tartrate stability and gives an indication of their limitations.

1.3.4.1 The concentration product test

The CP test was one of the first used tests of potassium bitartrate and calcium tartrate stability (Berg and Keefer, 1958; Berg and Keefer, 1959). It is based on the measurement of potassium, calcium, tartaric acid and ethanol concentrations and pH. The concentration product relationship between tartaric acid and potassium and calcium ions can be expressed quantitatively using the following formulae derived from the solubility product (K_{sp}):

$$\text{Potassium CP} = [K^+]\,[HT^-]$$

$$\text{Calcium CP} = [Ca^{2+}]\,[T^{2-}]$$

The calculation has been simplified and compiled into tables of the ionization of the acid as a function of pH and ethanol content of the wine (De Soto and Yamada 1963; Berg and Akiyoshi 1971). If the calculated CP value obtained exceeds published or privately determined 'safe' values for the particular wine it is considered to be unstable. If the calculated value is less than the 'safe' value, the wine is considered to be stable. (Berg and Keefer, 1958; Berg and Keefer, 1959; De Soto and Yamada 1963; Boulton, 1983)

The test has limitations. The concentration product requires determination of the potassium, calcium, and tartaric acid, and ethanol concentration, as well as pH for each wine (Berg and Keefer, 1958; Boulton, 1983). Additionally CP values determined using model wine solutions do not allow for the variation in the potassium

bitartrate holding or complexing capacity of different wines (particularly red wines) and may therefore overestimate the CP value and a wine's stability at a given temperature.

The concentration products put forth by Berg and Keefer to determine the equilibrium relationship between the activity of potassium bitartrate ions and their solubility at any temperature is now rarely used as a criterion to determine potassium bitartrate stability levels (Müller et al. 1990; Boulton *et al.* 1996).

1.3.4.2 Hold cold & freeze

The hold-cold test relies on the formation of potassium bitartrate crystals as a result of holding the wine sample for a period of time (several days to weeks) at a reduced temperature (often +2°C). The freeze test freezes the wine and after it is allowed to thaw, the presence of crystals after the ice has melted is interpreted as instability in the wine. In each test, if there is no observed precipitation, the wine is generally considered to be stable.

According to Boulton (1983), these tests are essentially crystallization rate tests, based on the formation of crystals that may be dependent on the time allowed for the test. There are many factors that can impact the rate of crystallization if the wine is supersaturated with potassium bitartrate, namely crystal nuclei and growth inhibitors that may significantly slow the rate of crystal formation. Many of these factors are not overcome by the test conditions and can lead to false negatives or positives. Filtration of laboratory samples may remove nuclei and/or inhibitors and affect the rate of crystallization, possibly slowing the formation of crystals in an unstable wine that it is observed as stable under the duration of the test. In the freeze

test, ice formation increases the concentration of potassium bitartrate and alcohol, greatly enhancing nucleation and crystallization making a determination of the long-term bottle stability unclear at best.

1.3.4.3 Conductivity

Many winery laboratories measure the potassium bitartrate stability of a wine by its conductivity using a conductivity meter, however, the differences in conductivity due to calcium tartrate changes are too small to be of use for determining stability with respect to calcium (Boulton et al. 1996). The test sets conditions for rapid crystal growth of potassium bitartrate if the solution is supersaturated at the temperature of interest. This is achieved by cooling the wine sample in a temperature-controlled water bath (easy for 0°C in an ice/water bath, though any temperature could be chosen), seeding with finely powdered KHT, and constant mixing (Boulton, 1983, Boulton et al. 1996). Crystallization of potassium bitartrate and the concomitant removal of free K^+ ions result in decreased conductivity as measured by the conductivity probe and meter. The other conducting species provide a constant background signal.

Changes of less than 5 percent in electrical conductance during the test period may be considered stable (Boulton, 1983, Boulton et al. 1996). Samples passing the test are stable only at (or above) the test temperature, which should be set to the lowest temperature the wine might be anticipated to encounter after bottling.

The advantage of this method is that it provides a final stable conductivity value that is specific for the wine being tested. Complexing factors from other wine

components that may affect potassium bitartrate crystal formation are also overcome by the excess added KHT crystal nuclei that favors complete crystallization.

1.3.4.4 Cation determination

For the determination of calcium and potassium ions (as well as iron, magnesium, and sodium), atomic absorption spectroscopy (AAS) is favored (Amerine and Kishaba 1952; Ough and Amerine 1988). Other methods exist including ion-selective electrode (for K and Ca) (Cardwell et al. 1991), and chemical methods such as complexometric titrations with ethylenediaminetetraacetic acid (EDTA) for calcium (Schreffler and Witzke 1952; Ough and Amerine 1988).

1.3.5 Stabilization and Inhibition

Most wines contain near supersaturated quantities of potassium bitartrate after fermentation, and most white and blush wines require stabilization before bottling. A wine considered stable with respect to potassium bitartrate may be unstable with respect to calcium tartrate. There are two general types of tartrate salt stabilization techniques for wines, physical and chemical:

- Physical: to reduce by refrigeration or by membrane separation a fraction of the salt or ionic species that can crystallize and precipitate.
    - Temperature reduction (not effective for CaT)
        - Batch cooling
        - Scraped surface heat exchange
    - Contact seeding

        - Addition of excess KHT or CaT causing supersaturation and providing nuclei for crystal formation
        - Can be batch process, fluidized bed, or filtration process
    - Ion exchange or electrodialysis
        - Removal of Ca^{+2}, K^{+} and/or T^{2-}
- Chemical: Additives that are inhibitory and slow down the crystallization process. Effectiveness is limited by time.
    - CaT
        - Carboxylic acids: malate, citrate, and phosphate
    - KHT
        - Carboxylic acids: metatartaric acid
    - CaT and KHT
        - Polysaccharides e.g. arabinose, pectins, carboxymethylcellulose
        - Mannoproteins

1.3.5.1 Physical stabilization

Of the physical stabilization methods, cooling, even to the freezing point, is ineffective for calcium tartrate stabilization. Chilling increases the level of potassium bitartrate and calcium tartrate supersaturation in wine, however, not enough to induce formation of calcium tartrate crystals, unlike temperature dependent crystallization of potassium bitartrate (Berg and Keefer 1959; De Soto and Yamada 1963; Pilone and Berg 1965; Parfent'eva et al. 1984).

1.3.5.1.1 Contact/seeding process

Seeding with potassium bitartrate provides sources of nucleation for crystal growth. The technique is more efficient than batch cooling alone; however, potassium bitartrate seed additions can increase material use and cost (Rhein and Neradt 1970). It may be possible to reuse KHT crystals from treated wine, however after repeated use, the crystal size increases, resulting in decreased surface area available for growth and decreased efficiency (Dunsford and Boulton, 1981a). The process of seeding can be combined with other processes for increased efficiency including:

Calcium tartrate stabilization by seeding with CaT (racemic mixture) is slow (about eight hours at 1°C with 4 g/L powdered CaT) (Boulton et al. 1996). It is effective at lowering calcium concentration; however, CaT as a racemic mixture is not approved for use in wine. Calcium tartrate (non racemic) is permitted for use in wine at up to 2 g/L (OIV 2005).

1.3.5.1.2 Ion-exchange

Sometimes ion exchange is used to reduce potassium bitartrate concentration in wine. It uses ion exchange resins that can affect the flavor and ionic balance in the wine. In order to reduce the negative effects, ion exchange is usually performed on a fraction of the total volume that is then recombined with the wine. It is generally not considered a premium wine production practice and may have limitations or prohibitions on its use.

- Wine is passed through a resin bed (of which there are three main types):
    - Replaces potassium with sodium ions with a cation exchange resin

        - Most common resin used. Slight reduction in acidity, increases sodium content
    - Replaces tartrate anion with hydroxyl or other anion with an anion-exchange resin
    - Replaces potassium and tartrate respectively with hydrogen and hydroxyl by cation and anion exchange resin, in effect exchanging potassium bitartrate for water
- Advantages:
    - Extremely efficient
    - Rapid
    - Remove trace metals from wine
    - Reusable
    - Acidity reduction
- Disadvantages:
    - Quality?
    - Sodium concentration increase
    - Acidity reduction

1.3.5.1.3 Electrodialysis

Electrodialysis is a wine stabilization process that was developed by the French National Agronomic Research Institute (INRA). The process is approved for use by the TTB, OIV, and EU. Electrodialysis can be used to remove the potassium and calcium cations and the tartrate anions from wine. It has the benefit of removing

only K^+, Ca^{2+}, and tartrate from wine and does not impact other wine components such as polyphenols, polysaccharides, or volatile flavor compounds.

1.3.5.1.3.1 Process

Electrodialysis uses a unit called an electrodialysis stack composed of two compartments, one for wine and the other for brine. Both compartments are equipped with conductivity probes and meters to control the wine circulation sequences and the brine concentration. Wine conductivity is used as the control parameter. A pump feeds the wine from the cellar tank into the wine compartment from where it circulates through the stack. An electrical potential is applied to electrode leads in the stack that affects the migration of ions in solution: the potassium and calcium cations migrate toward the cathode and the tartrates anions toward the anode. The tartrates can cross the anionic membranes and are removed from the wine because they cannot leave the brine compartments since the next membranes they reach are cationic membranes. Similarly, the cations are removed from the wine through the cationic membranes. With the alternate succession of cationic and anionic membranes in the stacks, the ions are removed from the wine compartments into the brine compartments. When the measured wine conductivity has reached a control point (stable conductivity or percent reduction from original value), that volume of stabilized wine is sent to the reception tank. Another batch of wine is then fed to the wine compartment for processing. This batch process is automated and semi continuous and the small volumes of wine are stabilized in a few minutes. The brine circuit, containing the ions that were extracted, is monitored by measuring the

conductivity and pH and then regulated by dilution to avoid precipitation of potassium bitartrate in the stack. (Eurodia, 2005)

1.3.5.1.3.2 Advantages & properties (Eurodia, 2005)

- Reliability: Properly treated wines are completely stable
- Specific to each wine:
    - The process is adaptable to the characteristics of each wine by removing only the quantity of K^+, Ca^{++}, and tartrate necessary to achieve the tartrate stability level that has been defined in advance
    - The treatment eliminates a fraction of the calcium, making the wine stable relative to calcium tartrate.
    - The treatment eliminates a fraction of the potassium, making the wine stable relative to potassium bitartrate
    - Conductivity drop in wines (wines from various regions and styles)
        - White: ≤ 30 percent
        - Red: 5 – 20 percent
- The process does not alter the wine characteristics (pH, acidity, sugar content, alcohol level, etc.)
    - No significant sensory impact in wines
    - No effect on non-ionic wine components
    - Moderate impact on physico-chemical balance
    - Ethanol decrease ≤ 0.1 percent by volume maximum
    - pH decrease < 0.25
    - Volatile acidity decrease < 0.11 g/L as acetic acid

  - There are no temperature changes
  - There are no additives of any kind
  - No extensive pretreatment is required (only microfiltration).
  - Reduction in consumables required (no filtration additives, no seeding material)
- Low energy cost vs. batch cooling
  - Power consumption is between 0.5 and 1 kWh per 1000 liters of processed wine, including pumping power. This is about 10 times lower than the energy needed for refrigeration, depending on the overall process and the equipment used.
- Low labor costs through automation
- Liquid effluent allows for the recovery of the tartaric values
- Brine volume
  - Waste: ~15 percent of wine volume
- Flow rate (typical)
  - 100 L/h/m^2 of cell, depending on the quantity of ions to be extracted
- Fouling:
  - Red wines foul faster due to higher surface tension
  - Anionic membranes more prone to fouling – flushing can regenerate

1.3.5.2 Chemical inhibition

Chemical components that are naturally present, or are added to a wine can impact nucleation and the rate of tartrate salt crystallization and precipitation. Red wines contain a much higher concentration of polyphenols and pigments than white

wines and therefore contribute a higher holding capacity for tartrate salts than white or model wines. As pigment polymerization occurs through aging, the holding capacity of tartrate declines and may result in delayed CaT or KHT precipitation (Balakian and Berg, 1968). Potassium bitartrate precipitation in red wines often occurs as tartrate-pigment complexes that can form deposits, often called "lacquering," on the sides of bottled wines (Peng et al 1996a). Prevention of lacquering can usually be achieved through KHT stabilization methods (Peng et al 1996b). After KHT stabilization, along with TA/pH effects, some loss in color can also be expected. Other wine components may also act to inhibit potassium bitartrate crystallization including metals, sulfates, proteins, and colloids such as pectins, glucans, among other polysaccharides (Pilone & Berg, 1965). Sweet wines and those infected by *Botrytis cinerea* precipitate more slowly than dry wines. Sulfate is believed to complex free potassium as K_2SO_4 or KSO_4^- (Bertrand *et al.* 1978). Inhibition may come from component complexes with tartrate ions, or with tartrate crystal surface adsorption.

Occasionally, winemakers choose to add chemical inhibitors to prevent potassium bitartrate formation, more often attempts are made to remove these complexing agents (by fining and/or filtration) to facilitate crystallization and subsequent precipitation to achieve stabilization.

Some of the additives used for tartrate salt crystal growth inhibition follow:

1.3.5.2.1 Carboxylic acids

1.3.5.2.1.1 Citrate and malate

Carboxylic acids containing adjacent carboxyl and/or hydroxyl functional groups, such as citric and malic acids, can inhibit the spontaneous crystallization of calcium tartrate (Postel et al. 1984; McKinnon et al. 1996). Citrate is known to chelate iron ions and may function similarly with divalent calcium. Salts of these acids were also shown to increase the solubility of calcium tartrate (Postel 1983; Postel et al. 1984).

1.3.5.2.1.2 Metatartaric acid

Metatartaric acid is a mono or diester of tartaric acid. It is produced by melting tartaric acid at 170°C to form an anhydrous powder whose molecules link to form a polymer like structure. It acts as a potassium bitartrate (not CaT) nucleation inhibitor, however with time it hydrates back to tartaric acid and loses its inhibitory effect. The effectiveness of metatartaric acid by wine storage temperature (Peynaud 1984; Rankine 1989):

- Several years at 0°C
- 18 months at 10°C
- 2 – 3 months at 25°C

The dosage rate is commonly 50 to not more than 100 mg/L (Peynaud, 1984; Rankine, 1989, O.I.V. 2005). Before use, it is dissolved in cold water. The use of hot

water for dissolution, or warm humid storage conditions can hasten its hydrolysis. Due to its temperature dependent nature, its use may be suitable in early-consumed wines. Metatartaric acid is permitted for addition to wine by the O.I.V. and the E.U., although it is not allowed in U.S. wines (ATT).

1.3.5.2.2 Phosphate

Phosphate chelation of calcium is well documented and is known to bind most of the calcium in milk (Molins et al. 1990) as well as tooth enamel (Ferguson 1998). The phosphate content of wine normally varies between 0.05 to 0.9 g PO_4/L, with an average value of 0.29 g/L (Ough and Amerine 1988). Mckinnon et al (1995), showed that phosphate (PO_4) at 0.4 g/L slightly inhibited the induction period and crystallization rate of calcium tartrate, however concluded that at normal wine pH values phosphate (as H_2PO_4) does not bind calcium. They attributed the inhibition of calcium tartrate precipitation by phosphate on changes in ionic strength of the solution. Postel et al (1984), indicated small positive solubility effects on calcium tartrate due to phosphate ions. It is proposed here that phytic acid may produce greater chelation at wine pH values.

1.3.5.2.3 Polysaccharides

Polysaccharides (pectins, arabinose, acaci, gum arabic) are known to inhibit tartrate crystal nucleation (Kohn 1975; McKinnon et al., 1995). Pectins are linear polymers of galacturonic acid covalently bound to some neutral sugars such as arabinose, galactose, mannos and rhamnose by alpha-1,4 glycosidic linkages. Monouronic acids (e.g. galacturonic acid) do not affect calcium tartrate crystallization

(McKinnon et al. 1996). Pectin is known to significantly bind calcium (as well as divalent copper ions) between pH 3.0 – 3.5 (max 4.0) and can cause colloidal gelation of the pectin complex (Torre et al. 1992; McKinnon et al. 1996). Although pectins are a normal constituent in fruits and wine, the prevalent use of pectic enzymes in winemaking significantly reduces their concentration (Robertson et al. 1980).

1.3.5.2.3.1 Carboxymethylcellulose (CMC)

Carboxylmethylcellulose ($C_6H_9OCH_2COONa$ as sodium salt) is a water-soluble polymer colloid similar to pectins that inhibits potassium bitartrate crystal growth similar to metataric acid (McKinnon et al. 1996; Margalit 1997). It is also inhibitory to calcium tartrate crystal growth (Clutton 1974; Stocké and Görtges 1989a,b). It is stable and does not lose efficacy with time or higher temperatures. It is often effectively used at concentrations of 25 – 50 mg/L (Clutton 1974; Stocké and Görtges 1989a,b; Margalit 1997). Carboxylmethylcellulose is not permitted for use in wine, although it has been demonstrated to be a safe additive in foods and is approved for food use by the World Health Organization (Wucherpfennig et al., 1984).

1.4 Proteins in wine

Proteins are high-molecular-weight organic compounds made up of amino acids joined together by peptide bonds. Most wine proteins are either structural (i.e. cellular building blocks) or enzymes. Wine proteins are a mixture of proteins derived from the grape and from microbial populations, particularly yeast, especially after autolysis. Yeast proteins (peptides <10,000 Da) released by yeast excretion and

autolysis have been shown not to be a cause of protein instability in wine (Bayly and Berg 1967), but may be involved in peptide tannin hazes (Boulton et al. 1996). Wine grape derived proteins have isoelectric points between 2.5 and 8.7 (Yokotsuka et al. 1977; Anelli 1977) and molecular weights in the range of 20,000 to 50,000 Daltons (Somers and Ziemelis 1973).

The total concentration of proteins ranges between 20 – 800 mg/L in un-fined juice or wine depending on various conditions including (Bayly and Berg 1967; Tyson et al. 1981; Murphey et al. 1989b; Høj et al. 2001):

- Varietal (Bayly and Berg 1967)
- Soil fertility:
    - Fertile soils increase total nitrogen in the grapes and protein levels
- Climate:
    - Protein content tends to be higher in warmer vintages and regions
- Grape maturity:
    - Increases with ripening (Murphey et al. 1989b)
- Grape infection
    - Grape infection from common grapevine pathogens can increase the concentration of proteins that are highly resistant to low pH and enzymatic or non-enzymatic proteolysis (Waters et al. 2005).
- Processing conditions:
    - The must temperature on settling – warm musts dissolve more protein
    - Higher press pressures may increase protein content

- More skin contact increases proteins; however, increased tannins (including those derived from stems or additions) can reduce quantity. Mechanically harvested fruit may have higher protein concentrations due to maceration between harvest and crush
- The duration of yeast contact, though proteins from yeast do not contribute to instability (*sur lies* aging may actually improve protein stability) (Waters et al. 2005).

Protein solubility is a complex function of the physiochemical nature of the proteins, pH, temperature and the concentration of wine complexing components, including tannin, metal ions (copper in particular) and detergent residues (Kean and Marsh 1957; Moretti and Berg 1965). Any of these factors can cause dissolved wine proteins to precipitate and appear as a fine amorphous colorless haze or deposit. The slow denaturation of wine proteins and subsequent aggregation and flocculation into hazes in white wines is second only to the precipitation of potassium bitartrate as the most common physical instabilities in wine.

The degree of instability varies according to the cultivar, maturity, climate, condition of the fruit and processing, pH, and the interaction with other wine components. Hazes in wine associated with proteins are usually complexes of proteins, polysaccharides and polyphenolics compounds. The total concentration of proteins does not correlate well with heat stability or haze formation, although wines that are stored under warm conditions can cause protein hazes to form (Moretti and Berg 1965). What has been shown is that the pH has a much larger effect on the solubility of various protein fractions and their relative stability in the wine (Bayly and Berg 1967). Heat denaturation, total protein measurement or precipitation tests

are less accurate predictors of potential instability than measuring their solubility at normal wine pH and temperature conditions.

1.4.1 Protein complexes

1.4.1.1 Tannin

Tannins and other polyphenolics bind with and can form hazes and precipitate proteins (Yokotsuka et al. 1983). Red wine polyphenolics can also bind human lipoproteins and protect them from metal ion-dependent and independent oxidation (Ivanov et al. 2001). Proteins in red wines are completely bound by flavonoids and precipitated, however, white wines containing much lower concentrations of phenolics may only bind a minor quantity of proteins (about half of total white wine proteins) and these reacted proteins may be prone to precipitation and haze formation (Somers and Ziemelis 1973a). Some protein-tannin complexes may be soluble, however may also be heat sensitive or slowly haze forming with increasing age and phenolic polymerization.

1.4.1.2 Metals

Copper has long been known to be involved with protein binding (Kean and Marsh 1957) and through the centuries cooks and bakers have used the principle, though perhaps without knowledge of the underlying science. Copper bowls were discovered to produce more stable egg white meringues with creamier foam than those made in ceramic, glass or stainless steel bowls (McGee 1984). Egg whites contain the protein albumin. When whisked in a copper bowl, copper ions migrate

from the bowl and complex with the albumin producing a more stable complex that is less likely to denature (i.e. through over-beating). Denatured proteins coagulate and stiffen. Mechanically whisking denatures egg white proteins; the trick is to produce the right amount that will stabilize the foam and the air trapped within it. Baking solidifies the foam by coagulating the protein. Iron and zinc also form complexes with albumin, however, do not make the foam more stable. Cream of tartar (potassium bitartrate) may be added to egg whites to stabilize the whites also.

1.4.2 Isoelectric point

The isoelectric point (pI) of a protein is the pH at which the protein has an equal number of positive and negative charges, i.e. carries no net electrical charge. Proteins can be in the form of cations at low pH and anions at high pH, but are neutral at the isoelectric point.

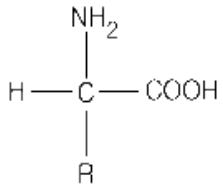


Figure 1.9 Un-ionized Amino Acid.

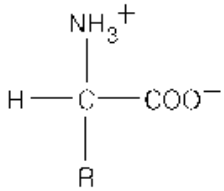


Figure 1.10 Ionized (dipolar) Amino Acid.

For an amino acid with only one amine and one carboxyl group, the pI can be calculated from the molecules pKas:

$$pI = \frac{\sum pK_a}{2}$$

Proteins can be separated according to their isoelectric point in a process known as isoelectric focusing (Hawcroft 1996). Isoelectric focusing is a method of separating proteins based on their relative content of acidic and basic residues. Proteins are introduced into a gel (composed of polyacrylamide, starch, agarose, etc.) that has an established pH gradient or is capable of establishing such a gradient after applying an electrical current. At a pH below the pI, proteins carry a net positive charge. Above the pI they carry a net negative charge. This is similar to electrophoresis, which is the movement of an electrically charged body under the influence of an electric field.

1.4.3 Solubility

The smaller the difference between the juice or wine pH and the isoelectric point of the protein fraction, the lesser the net charge on that protein fraction and the lower the solubility of that fraction. Conversely, the greater the charge on the protein fraction, the greater its solubility and potential affinity to electrostatically bind to fining agents. Therefore, the isoelectric properties of proteins influence not only their tendency to precipitate according to the pH of the solution they are in, but also the efficacy of fining agents on their removal (Dawes et al. 1994).

The importance of the isoelectric point is that it is the pH value at which the solubility of the protein is at a minimum. Protein solubility can vary with salt strength (e.g. potassium bitartrate), sometimes increasing within a range of increasing salt

concentration. Some proteins are up to 10 times more soluble with a shift in pH of only 0.5 units (Boulton et al. 1996). Increasing ethanol decreases protein solubility in all cases. Protein solubility is not determined by the size of the proteins, but by their ionic strength, and the temperature and alcohol content of the wine (Boulton et al. 1996).

1.4.4 Treatment

As previously mentioned, wine protein fractions are most likely to cloud and precipitate when the pH is near their isoelectric point and they have the least charge. This has implications with some wine protein fining agents, particularly bentonite. A stable wine that undergoes any change in pH (e.g. through fining or citric acid addition) can cause instability. Some protein treatments in wine include:

- Fining
    - Bentonite
- Ultrafiltration
- Microbial
    - Enzymatic (proteolytic)
    - Mannoproteins (protective, from *sur lies*) (Waters et al. 2005)
- Heat denaturation

Some protein stabilization treatments can significantly and negatively affect wine sensory attributes. Bentonite in particular has the potential to reduce wine color, aroma and flavor components with large treatment amounts (significant at rates of addition over 0.5 g/L) (Rankine 1989; Main and Main 1994). It is therefore important

to conduct effective stability trials and analysis to determine the least amount of treatment to achieve the desired stability.

1.4.4.1 Bentonite

Bentonite fining remains the most common and effective method for the adsorption and removal of proteins from wine despite the undesired effects of modifying flavor and the removal of some assimilable nitrogen (Ough et al. 1969). The adsorption potential of bentonite is dependent primarily on its cation exchange capacity as determined by it negatively charged plate surfaces (Blade and Boulton 1988). Cationic wine components such as calcium, potassium, magnesium, sodium, hydrogen ions, and the cationic fractions of amino acids and peptides can compete with proteins in wine for ion exchange with bentonite (Blade and Boulton 1988).

Bentonite will selectively remove protein fractions with the largest cationic charge. Protein fractions at or near their neutral isoelectric point are the most unstable and least removed by bentonite (Blade and Boulton 1988). Bentonite efficacy does not appear to be temperature dependent (Berg et al. 1968; Blade and Boulton 1988), however, is affected by the method of addition (Weiss et al. 2001). Unstable proteins near electrostatic neutrality may not be stabilized by bentonite treatment alone or only with excessive amounts that can strip wine character (Bayly and Berg 1967; Hsu and Heatherbell 1987b; Dawes et al. 1994). Additionally, wines fined with bentonite at one pH and that thereafter undergo a pH change (e.g. potassium bitartrate precipitation by chill proofing, MLF, blending, or acidity adjusting) may then become unstable. In most cases, bentonite fining should be performed at the lowest possible pH, so that as much protein as possible is positively charged (Boulton *et al.* 1996).

1.4.4.1.1 Combined Chill Proofing and Bentonite Fining

Most white and blush wines require both potassium bitartrate and protein stabilization treatments. Some winemakers combine both stabilization procedures into a single operation. Often the wine is first fined with bentonite, and then the wine is immediately chilled to about 2°C. The wine is held at the cold temperature for a week or so while the tartrate precipitates. When the excess tartrate is precipitated, the cold wine is racked or filtered off the bentonite and tartrate lees. This combined procedure has some advantages. The tartrate crystals settle on top of the fluffy bentonite lees, forming a crusty layer, and the wine is much easier to rack off the compacted lees. Both procedures are accomplished in a single winemaking operation, so labor and wine manipulations are reduced. However, this method does not anticipate the pH shift likely to occur with chill proofing and may require additional bentonite treatment to fine the potentially newly charged protein fractions.

1.4.4.2 Ultrafiltration

Ultrafiltrations using 10,000 – 20,000 molecular weight cutoff (MWCO) membranes have been used as an alternative to bentonite for the removal of haze causing proteins (Hsu et al. 1987; Peri et al. 1988), however, undesirably removes phenolic components (Peri et al. 1988) and flavor (Flores et al. 1991).

1.4.4.3 Silicon dioxide and tannin

Silicon dioxide (SiO2, Kieselsol, silica gel) is sometimes used in aqueous suspension as an alternative to tannin in the fining of proteins (including counter-fining gelatin) (Hahn and Possmann 1977). Negatively charged silicon dioxide

electrostatically binds with positively charged proteins causing them to flocculate. The extent of binding is determined by the particle size, conformation and charge density (Hahn and Possmann 1977).

Silicon dioxide offers some advantages over bentonite or tannin including reduced risk of overfining, decreased lees volume and faster precipitation including promoting protein-tannin complex precipitation (Hahn and Possmann 1977).

1.4.4.4 Denaturation

Denaturation involves the breaking of many of the weak hydrogen bonds within a protein molecule that are responsible for the highly ordered structure and function of the protein in its natural state (Sikorski 2001). Denatured proteins lose their structure and function. Most become insoluble.

- The function of a protein is determined by its three-dimensional structure
- The structure of a protein is determined by its sequence of amino acids

Denaturation can be caused by heat, extreme pH (acidic or basic), detergents, alcohols, heavy metal salts, reducing agents or certain chemicals such as urea. Some processes of denaturation (Sikorski 2001):

- Alter electrostatic interactions between charged amino acids:
  - pH changes
  - Salt concentration changes
- Alter hydrogen bond strength:
  - Temperature changes
- Break the disulfide (S-S) linkage between cysteines

- Reducing agent presence

As an example of denaturation, consider egg white albumin. When eggs are cooked, the protein-water hydrogen bonds break and albumin unfolds into long chains that, in the heated conditions, begin to protein-protein bond to each other through stronger ionic and covalent bonds (McGee 1984). Eventually the proteins become clustered together densely enough to become opaque. If the albumin in heated further, more water is lost and the protein becomes increasingly rubbery. The same effect is achieved by salting or pickling eggs.

1.4.4.4.1 Thermal Denaturation

Eliminating proteins by thermal denaturation and precipitation with high-temperature short-time treatment also offers the advantage of destroying oxidative enzymes (Boulton et al. 1996):

- Heat wine rapidly to 80 or 90°C for seconds and then rapidly cool and filter.

1.4.4.5 Enzymes

Proteases that hydrolyze proteins into soluble polypeptides to reduce the potential for protein haze formation are being sought. Suitable proteases may also be able to liberate assimilable nitrogen for exploitation by yeast.

1.4.4.6 Other

Haze-protective factors such as yeast mannoproteins (i.e. from *sur lies* aging, or by addition), to wines results in decreased particle size of haze (Waters et al. 2005).

1.4.5 Protein Analysis

The presence of unstable proteins, protein complexes or polysaccharides in a white or blush wine can lead to the development of a haze or deposit that is unacceptable to consumers. If the protein fractions are unstable under conditions the wine might be expected to undergo, they should be fined for those conditions. However, wine should not undergo extremes of temperature and producers should be in a position to assure that the wine never experiences such conditions. Current technology allows the monitoring of excessive temperature (e.g. heat-sensitive bottle label sensors).

Ranges of tests are available for estimating protein or heat stability in wine. These all involve denaturing the protein – by heating, or the addition of acid or alcohol – and are often performed along with bentonite fining trials. Due to the extreme nature of some of these tests, they are not necessarily specific to proteins and may induce other reactions or precipitation including tannin polymerization. As a result some tests can exaggerate the potential for haze formation by causing denaturation and precipitation of all protein fractions. Some common protein assays and haze stability tests include:

- Protein separation and identification
    - Electrophoresis
- Total protein assays:
    - Biuret
    - Coomassie Blue
    - Pierce protein reagent
- Chemical denaturation:

- Trichloroacetic acid
- Phosphomolybdic acid (“Bentotest”)
- Ethanol precipitation
- Tannic acid

- Heat denaturation:
  - Common ranges: 90°C for 1 hour to 50°C for 24 hours

1.4.5.1 Protein Separation & Electrophoresis

Electrophoresis (
Figure **1.**11) is a commonly used technique to separate proteins, lipoproteins, nucleic acid, particles, emulsion grains, or even bacteria on the basis of their net charge in specified buffered media.

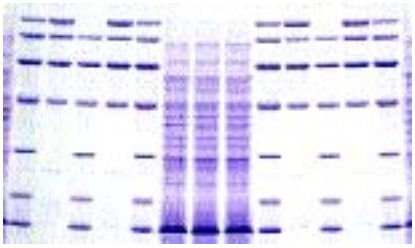


Figure 1.11 Electrophoresis with Coomassie Blue.

Electrophoresis has been important in food science for the study and fractionation of proteins that lead to the high-resolution separation and discovery of many major proteins such as enzymes, hormones, and antibodies. Its cost is relatively low and is commonly used at the preparative level. Furthermore, it furnishes information on the charge, conformation, and the shapes of the analytes. It has been

used to characterize wine proteins (Koch and Sajak 1959; Moretti and Berg 1965; Bayly and Berg 1967; Somers and Ziemelis 1973b; Anelli 1977; Murphey et al. 1989b; Yokotsuka et al. 1977; Tyson et al. 1981; Mesrob et al. 1983; Dubourdieu et al. 1986; Lee 1986; Hsu and Heatherbell 1987a,b; Lamikanra and Inyang 1988; Paetzold et al. 1990; Dawes et al. 1991; Waters et al. 1991; Vincenzi et al. 2005).

Protein Separation Principles:

- Charge on the Proteins
- Shape of the Proteins
- Size of the Proteins
- Electric Field

1.4.5.1.1 Charge on the Proteins

The pI of proteins has implications when running electrophoretic gels. Proteins are sequences of amino acids that can be ionized depending on their acid or base character. The N- and C- terminal and T-groups of the polypeptide can be ionized, contributing to the overall charge (Sikorski 2001). The protein's net electric charge is the sum of the electric charges found on the surface of the molecule as a function of the environment:

- At the pI of a specific protein, the protein molecule carries no net charge and does not migrate in an electric field.
- At pH above the pI, the protein has a net negative charge and migrates towards the anode.

- At pH below the pH, the protein obtains a net positive charge on its surface and migrates towards the cathode.

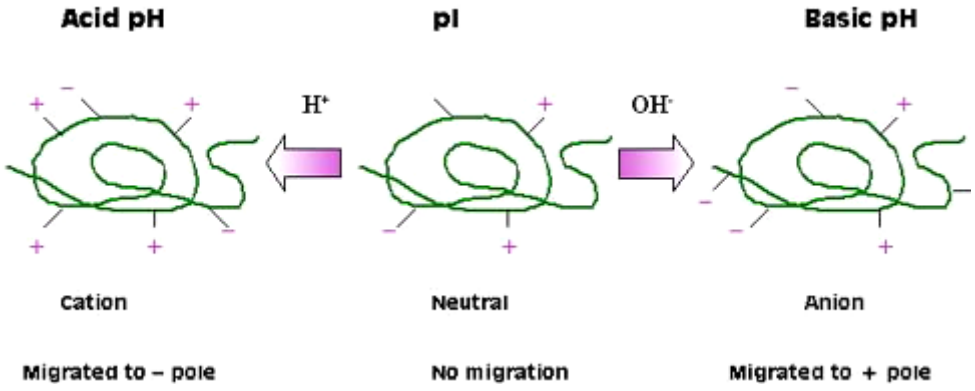


Figure 1.12 Protein Migration by Charge.

The pH of an electrophoretic gel is determined by the buffer used for that gel (Hawcroft 1996). Depending on the pH of the buffer, proteins may carry a charge and can therefore be separated in an electric field (Figure 1.12). When an electric field is applied, proteins will migrate towards their corresponding poles. If the pH of the buffer is above the pI of the protein being run, the protein will migrate to the positive pole (negative charge is attracted to a positive pole). If the pH of the buffer is below the pI of the protein being run, the protein will migrate to the negative pole of the gel. If the protein is run with a buffer pH that is equal to the pI, it will not migrate at all. In this regard, proteins can be separated based on their electric charges.

1.4.5.1.2 Shape of the Proteins

If two proteins carry the same charges they can still be separated by electrophoresis. When starch or polyacrylamide gels are used, proteins can also be

separated based on their difference in shape (Hawcroft 1996). A long, loose protein tends to interact more with the gel network and travels at a slower rate than a globular protein (Hawcroft 1996).

1.4.5.1.3 Size of the Proteins

If two proteins have the same charge but are different in size they can still be separated. The use of SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE) enables the separation of proteins by size. Docium dodecyl sulfate (SDS) is used as a detergent in electrophoresis to dissociate a protein. SDS molecules attach to the protein in a constant ratio giving the proteins an identical charge density. The SDS-polypeptide complex assumes a rod-like shape. The proteins are now ready to be separated based on the difference in shape. The larger the protein, the more it will interact with the gel structure and thus, travel slower in the gel. It is therefore possible to get an estimation of molecular mass by comparing the migration distance to that of a protein with a known mass.

1.4.5.1.4 Electric Field

The rate of separation (v) depends on the electrophoretic mobility (U) and electric field strength (E):

$$v = U * E$$

Increasing U and E can improve the efficiency of electrophoresis, however by increasing the electric field strength (i.e. electric current), increases heat and therefore requires an efficient cooling system.

1.4.5.1.5 Capillary Electrophoresis

One of the major drawbacks of gel electrophoresis is the speed of analysis. Speed can be improved by increasing the electric current of the system, however, a large amount of heat would be generated and an efficient cooling system would be required. The invention of capillary electrophoresis (CE) has solved the heating problem. Silica fused capillaries ranging from 0.150 to 0.375 millimeters in outer diameter efficiently dissipate the heat that is produced. Increasing the electric fields produces very efficient separations and reduces separation times.

In a CE separation, a very small amount of sample (0.1 to 10 nL) is required. The sample solution is injected at one end and an electric field of 100 to 700 volts/centimeter is applied across the capillary. Proteins in the solution migrate through the capillary due to the applied electric field (electrophoresis), differing electrophoretic mobility drive each of the components into discrete bands. Finally, the separated proteins are eluted form on end of the capillary.

Quantitative detectors such as fluorescence and absorbance detectors can be used to identify and quantify the proteins in the solution eliminating the cumbersome staining techniques of gel electrophoresis.

1.4.5.2 Total Protein

The determination of total protein as a measure of the tendency for precipitation (Koch and Sajak 1959; Moretti and Berg 1965; Bayly and Berg 1967; Somers and Ziemelis 1973a; Anelli 1977) is of very limited use since individual protein fractions behave very differently depending on the wine conditions. Additionally, there are wine component interferences (phenol, tannin, and copper)

when measuring total protein assays with Biuret and Coomassie Blue reagents (Godshal 1983; Waters et al. 1991).

1.4.5.3 Coomassie Blue

Dye-binding assays using Coomassie blue are more rapid than some methods (Hsu and Heatherbell 1987a; Murphey et al. 1989a; Murphey et al. 1989b; Dawes et al. 1991), however with the aforementioned limitations.

1.4.5.4 Trichloroacetic Acid & Bentotest Tests

The trichloroacetic acid test (TCA test) (Berg and Akiyoshi, 1961) involves the use of 1 mL of 55 percent TCA added to 10 mL of wine. The solution is then heated in boiling water for two to five minutes, after which it is cooled to room temperature and observed for haze formation after 15 minutes. The "Bentotest" (using phosphomolybdic acid) is similar but performed at room temperature. The difference in clarity before and after the test is determined either visually or with a nephelometer. Both tests chemically denature all wine proteins in an acidic media (pH <1), making no differentiation between stable and potentially unstable proteins. While the test is rapid, its measure of most wine proteins has little bearing on the amount of bentonite that should be used to fine unstable protein fractions.

1.4.5.5 Ethanol Solubility

The ethanol precipitation test causes the precipitation of the least soluble protein fractions and colloids, at wine pH, with ethanol. With constant pH (given wine condition), ethanol content is the agent most responsible for limiting colloidal

solubility. Temperature is a factor, however, it is expected that with modern quality controls in production, transportation, and sales, extreme temperature conditions would not be encountered. Additionally, this test can be effective at showing the influence of pH on solubility simply by varying the pH of the sample in the test.

An example by Boulton et al. (1996) shows a difference between ethanol solubility and thermal denaturation test methods and how each method would affect the amount of bentonite each test indicated would be required to reduce haze formation potential. In one example, the ethanol solubility test indicated more specificity and lower bentonite treatment levels (by about half) than the heat test.

1.4.5.6 Thermal Denaturation

Bentonite addition levels required for wine stabilization are frequently determined by heat denaturation trials (Pocock and Rankine 1973). Heat denaturation tests are a measure of heat labile proteins that may be very soluble under normal wine conditions. The test is not related to protein isoelectric points and may not precipitate those proteins most likely to form a haze and precipitate under normal wine conditions. The prolonged heating can also cause other wine reactions – such as accelerated oxidation and phenolic polymerization – that, even in the absence of air, that can lead to combined protein and polyphenolics complexes that form hazes and precipitates. The test may lead to over-fining compared to other test methods such as protein solubility in ethanol.

Heat treatments for protein stability have often been used to simulate extreme heat conditions to which wine may be exposed. Nowadays, it is easy to expect, and

perhaps even demand, that no wine ever be exposed to these kinds of conditions. That said, heat tests might be appropriate to measure a wine's heat sensitivity.

1.4.5.7 Stability Test Comparisons

Table 1.6 A comparison of alternative stability tests (Boulton et al. 1996)

Wine	pH	Total protein[1] (mg/L)	TCA haze[2] (NTU)[3]	Heat haze (NTU)	Ethanol haze (NTU)
Chardonnay	3.00	8.3	16	3	24
Chardonnay	3.12	12.1	33	9	40
Riesling	3.11	12.5	88	1.1	25
Chardonnay	3.50	12.8	68	28	30
Sauvignon blanc	2.85	14.2	90	16	24
Muscat Canelli	3.44	17.9	100	70	40
Riesling	3.42	18.5	117	38	52
Chardonnay	3.41	19.6	184	52	57
Sauvignon blanc	3.18	24.6	162	25	25
Sauvignon blanc	3.56	29.6	242	120	53

[1]Coomassie Blue assay.

[2]Trichloroaretic acid assay.

[3]NTU = Nephelos turbidity unit.

1.4.5.8 Fining Trials

Determining how much bentonite or any other fining agent to add to achieve haze stability is often estimated by performing fining trials. The goal is to determine the minimum amount to add to achieve stability without affecting wine flavor.

1.5 Phytic acid

1.5.1 Introduction

Phytic acid or myo-inositol hexakis (dihydrogen phosphate) ($C_6H_{18}O_{24}P_6$, CAS 83-86-3) (Figure 1.13) is generally recognized as the primary storage form of phosphorus and insoitol in many plants (Asada 1969), comprising 1-3% of all cereal grains (wheat, corn, etc.), nuts, legumes (beans, lentils, peas), oil seeds (sunflower, canola, sesame) (Camire and Clydesdale 1982a,b; Graf 1983), spores (DeMaggio and Stetler 1985), pollen (Jackson et al. 1982), roots and tubers (Roberts and Loewus 1968; Lásztity and Lásztity 1990) (Table 1.7). Lott et al. (2000) published a comprehensive review of phytic acid and phosphorous in crop seeds and fruits, estimating nearly 35 million metric tones per year of phytic acid production globally from these crops. Phytate metabolism during seed development, dormancy, and germination performs several important physiological functions including storage depot for phosphorous (Asada 1969), cations (Williams 1970), and ion exchange from globoid protein-phytate beads (Ogawa et al. 1979), cell wall precursors (Scott and Loewus 1986), and protection from oxidation (Graf 1986; Graf et al. 1987).

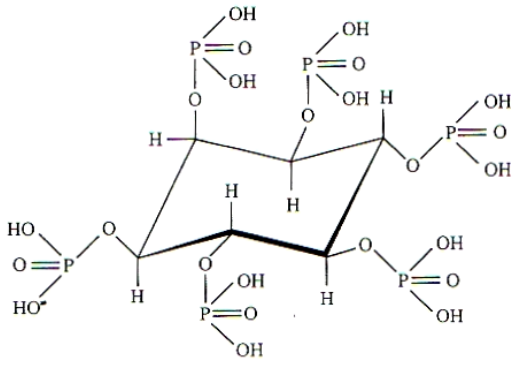


Figure 1.13 Phytic acid.

Table 1.7 Phytic acid concentration in various foodstuffs.

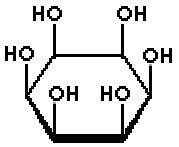
Foodstuff	Phytic Acid g/100g
Hard red wheat bran	6.880 ± 0.185 [a]
Soft white wheat bran	5.027 ± 0.145 [a]
Soy flour (defatted)	2.245 ± 0.115 [a]
Legumes	1.720 max [b]
Split peas	1.679 ± 0.102 [a]
Refined corn bran	1.577 ± 0.251 [a]
Brown rice	1.555 ± 0.192 [a]
Cereals	1.420 [b]
Parsnips	0.818 ± 0.018 [a]
Durum wheat	0.520 [b]
Broccoli	Not detected [a]

[a]Camire and Clydesdale 1982a, originally reported as mg/g.

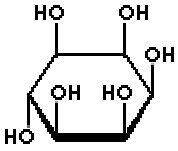
[b]Hídvégi and Lásztity 2002.

Phytic acid contents were found to be higher in the outer coverings of seeds, particularly the globoid granular protein region called the aleurone layer, in the endosperm, than in the whole seeds of monocotyledons (Hídvégi and Lásztity 2002) where it typically accounts for 60-90% of the total seed phosphorus (Lolas et al. 1976). Corn differs from other cereals as more than 80% of phytic acid is concentrated in the germ (Hídvégi and Lásztity 2002). The phytic acid content is influenced by the cultivar and seasonal climatic conditions. Processing and milling of grains into different products modifies the level of phytates depending on the proportion or extraction of the kernel bran where most phytic acid content is located, low extraction white flours contain low phytic acid quantities.

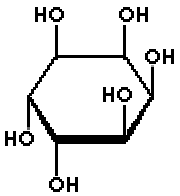
Phytic acid belongs to a group of inositol phosphates found widely in the natural environment. They exist as various inositol isomers (Figure 1.14) in various states of phosphorylation (bound to between one and six phosphate groups), although myo-inositol hexakisphosphate is the most prevalent form in nature (Cosgrove 1962, 1963a,b, 1980) including eukaryotic cells (Sasakawa et al. 1995).



cis-INOSITOL

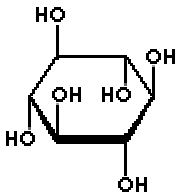


epi-INOSITOL



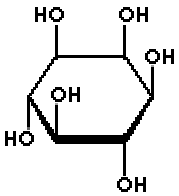
allo-INOSITOL

(CAS RN: 643-10-7)



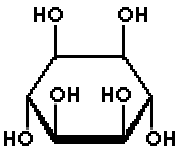
neo-INOSITOL

(CAS RN: 488-54-0)



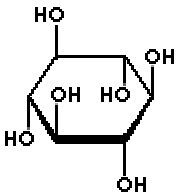
myo-INOSITOL

(CAS RN: 87-89-8)



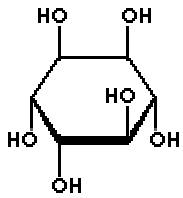
muco-INOSITOL

(CAS RN: 41546-34-2)



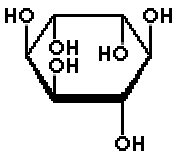
scyllo-INOSITOL

(CAS RN: 488-59-5)



L-(-)-chiro-INOSITOL

(CAS RN: 551-72-4)



D-(+)-chiro-INOSITOL

(CAS RN: 643-12-9)

Figure 1.14 Inositol Isomers.

Inositol phosphates are principally derived from plants and accumulate in soils and aquatic environments (where they may contribute to eutrophication) to become the dominant class of organic phosphorus compounds (Turner et al. 2002a).

Phytic acid has been attributed to high phosphorus excretion by monogastric animals lacking efficient phytases, such as in poultry and swine manure, and the resulting environmental phosphorus pollution of soil and water (Caldwell and Black 1958a,b; Sharpley et al. 1994; Oatway et al. 2001; Sharpley and Moyer 2000). High levels of phosphorus that can accumulate in animal waste-amended soils, or

direct waste runoff can lead to eutrophication of waters. Phosphates also accumulate in municipal sewage where the preferred method of removal is biological through incorporation into microorganisms and where the resulting sludge can then be used for agriculture purposes (Suschka et al. 2001). An alternative or adjunct to using microorganisms to sequester phosphates is the use of alum or Fe(II) or Fe(III) salts (typically chloride or sulphate salts) and the resulting chemical precipitation of phosporus as aluminum or iron phosphates, for example:

$$3FeCl_2 + 2PO_4^{3-} \rightarrow Fe_3(PO_4)_2\ ppt + 6Cl^-$$

$$3FeSO_4 + 2PO_4^{3-} \rightarrow Fe_3(PO_4)_2\ ppt + 3SO_4^{3-}$$

The molar ratio of Fe : P is 3 : 2, and 162.2 g of $FeCl_3$ will react with 95 g $PO_4^{3-,}$ to form 150.8 g $FePO_4$. Precipitation with Fe(III) is most effective in the pH range of 4.5 – 5.0 (Suschka et al. 2001).

The number of substituted phosphate groups on the inositol ring may vary between one and six, which is indicated by the prefixes mono, bis, tris, tetrakis, pentakis and hexakis the preferred nomenclature over di, tri, tetra, penta and hexa usage (IUPAC 1971). The positions of the phosphate groups are denoted by the position number of the carbon in the inositol ring to which they are attached, e.g. *myo*-inositol 1,2,3,4,5,6-hexakis(dihydrogen phosphate) (IUPAC name) (Cosgrove 1966a,b, 1980).

Phytic acid (IP6 or PA) is referred to by a number of different names: Inositol hexakis (dihydrogen phosphate), Alkalovert; D-myo-Inositol-1,2,3,4,5,6-

hexaphosphate; Fytic acid; IP6; InsP_6; Inositol 1,2,3,4,5,6-hexakisphosphate; Inositol hexakis (phosphate); Inositol hexaphosphate; Phytic acid; meso-Inositol hexaphosphate; myo-Inositol hexakis (phosphate); or myo-Inositol hexaphosphate. As a food additive, phytic acid is used as a preservative with E number E391, which falls into the antioxidants and acidity regulators category as determined by the Codex Alimentarius committee following the International Numbering System (INS) for food additives. Posternak (1902) first used the name 'phytic acid' for the phosphorous-containing compounds obtained from various plant seeds that is currently used as an alternative name for the free acid form, while 'phytate' refers to the salt of phytic acid. 'Phytin' is a term occasionally used, especially in older texts, to refer to the Ca–Mg salt of phytic acid (Figure 1.), which forms the dominant inositol phosphate in seeds (Lott and Ockenden 1986) and the aleurone layer of cereal grains such as wheat and rice (Wheeler and Ferrel 1971; Tanaka et al. 1974; Lott and Ockenden 1986).

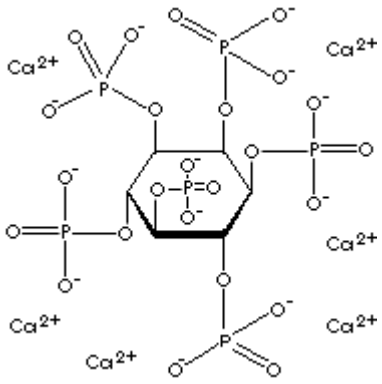


Figure 1.15 Calcium phytate.

Anderson elucidated the structure of phytin in 1914 and Posternak first suggested a gross formula for phytic acid in 1921. Phytate is a white amorphous powder, odorless and tasteless, almost insoluble in water, soluble in dilute mineral acids and in some organic acids. One part phytate dissolves in 10 parts of 1 N hydrochloric acid and forms a clear solution. Upon heating with dilute acids, alkali and water, phytic acid hydrolyzes to give ortho-phosphoric acid and the cyclitol myo-inositol as end products (Cosgrove 1966a,b, 1969). It was further elucidated by potentiometric measurements (Barre 1954), X-ray crystallography (Blank et al. 1971), and ^{1}H-NMR and ^{31}P-NMR spectroscopy (Johnson and Tate 1969, Costello et al. 1976). The chemical properties of inositol phosphates have been comprehensively reviewed by Cosgrove (1980).

Phytic acid possesses an optically inactive axial chair conformation between pH 5 and 12 and an equatorial structure outside this range (Johnson and Tate 1969). Phytic acid has 12 ionizable protons: pK_a values estimated by measuring the chemical shift in ^{31}P nuclear magnetic resonance (^{31}P NMR) spectra with change in pH (Table 1.8, Figure 1.16).

Table 1.8 pKa data for myo-inositol hexakisphosphate (Costello et al. 1976).

pK_1	pK_2	pK_3	pK_4	pK_5	pK_6	pK_7	pK_8	pK_9	pK_{10}	pK_{11}	pK_{12}
1.1	1.5	1.5	1.7	2.1	2.1	5.7	6.9	7.6	10.0	10.0	12.0

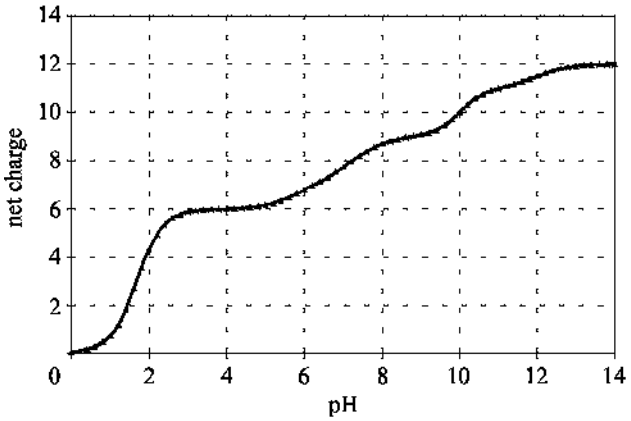


Figure 1.16 Estimated charge on myo-inositol hexakisphosphate over a range of pH values. The calculated charge is based on the pKa data shown in Table 1.8 (Costello et al. 1976).

The general acid dissociation equilibrium of phytic acid is (De Stefano et al. 2002; Seaman et al. 2003):

$$LH_n^{(12-n)-} \leftrightarrow LH_n^{(12-n+1)-} + H^+$$

For a constant ionic strength, this equilibrium can be characterized by the related apparent acidity constant (De Stefano et al. 2002; Seaman et al. 2003):

$$K_n = \frac{\left[LH_{n-1}^{(12-n+1)-}\right]\left[H^+\right]}{\left[LH_n^{(12-n)-}\right]}$$

Phytic acid, containing six orthophosphate moieties with 12 ionizable protons, is a potentially strong metal chelator over a wide range of pH values. There have been several investigations of the interaction between phytic acid and polyvalent cations at

different pH values (Martin and Evans 1986a,b, 1987; Evans and Martin 1991; Luttrell 1992; De Stefano et al. 2002). Phytate-metal chelation affinity varies according to the sequence: Cu(II) ≥ Zn(II) > Cd(II) > Mn(II) > Mg(II) > Ni(II) ≈ Co(II) (Martin and Evans 1987; Bebot-Brigaud et al. 1999). Phytic acid forms complexes with polyvalent metals, the binding constants of which increase with the valency of the cations (Maddaiah 1964; Vohra 1965). The adsorption of phytic acid to iron precipitates in model solutions is greatly influenced by pH; at pH 4.5, the uptake of phytic acid is more than double that at pH 6.5 (Shang et al. 1992). According to Graf and Eaton (1984), many phytate chelates are insoluble with the exception of (metal)1-phytate and (metal)2-phytate. This chelating capacity can remove or inactivate reactive metals, for example with iron(III) and its ability to generate hydroxyl radicals that often adversely affects biological systems and the production or storage of foods and beverages. It also interacts with a variety of other compounds including proteins. Some uses of phytic acid include lipid peroxidation inhibition of cooking oils (Lee and Hendricks 1995); chelating metals in tinned foods, corrosion and rust preventative coatings on metal surfaces (Xiao 1998); as a natural antioxidant in foods (Lee and Hendricks 1997; Lee et al. 1998), and to counter paper corrosion caused by iron gall ink in ancient manuscripts (Botti et al. 2004); among many other food, medical, and industrial applications. The uses of phytic acid are multifarious and are well documented (Johnson and Tate 1969; Blank et al. 1975; Isbrandt and Oertel 1980; Maga 1982; Graf 1983,1986; Martin and Evans 1986a; Sands et al. 1986; Li et al. 1989b; Jensen et al. 1996; Bauman et al. 1999; Bebot-Brigaud et al. 1999; Harland and Narula 1999; Paton et al. 1999; Oatway et al. 2001; Seaman et al. 2003) (see 1.5.2 Pharmacology and 1.5.3 Metal interactions for more information).

1.5.2 Pharmacology

1.5.2.1 Inositol

Inositol (hexahydroxyclohexane; cis-1,2,3,5-trans-4,6-cyclohexanehexol; $C_6H_{12}O_6$) is a cyclic polyalcohol isomer of glucose that is a constituent of many cell phosphoglycerides. Meso- or myoinositol, named for its presence in muscle tissue where it may form complexes with tocopherols for the storage of creatin in muscles (Chatterjea and Shinde 1993), is the major nutritionally active form of inositol. Myo-inositol is a precursor in the phosphatidylinositol cycle, a source of two second messengers in the form of inositol phosphates (diacylglycerol and inositol triphosphate) (Chatterjea and Shinde 1993).

Myo-inositol is an essential growth factor in some yeast (White et al. 1991), fungi, and rodents, but not for most animals, including humans. Although myo-inositol is not regarded as an essential nutrient in humans, it is sometimes classified as a member of the vitamin B-group (thiamine, riboflavin, niacin, pantothenic acid, biotin, pyridoxine, folic acid, inositol, and vitamin B12). Vitamins are organic molecules required in small amounts by mammals, including humans, in their diet, for metabolic purposes (Chatterjea and Shinde 1993). Most of the B-vitamins function as coenzymes or co-factors in many enzymatic reactions. Humans can synthesize myo-inositol endogenously from glucose-6-phosphate, however requirements are primarily supplied through consumption of fruit and cereals, where it occurs in the form of inositol-hexaphosphate (phytic acid) (Holub 1986, 1987). Inositol phosphates can undergo enzymatic hydrolysis. Phytic acid dephosphorylation into absorbable myo-inositol can occur through phytases including 3-phytase (EC 3.1.3.8) present in fungi

and yeast (Stolz et al. 1998), and 6-phytase (EC 3.1.3.26) found in wheat (Turner et al. 2002).

Dietary or supplementary intake of myo-inositol or phytic acid can influence the levels of circulating and bound myo-inositol in the body and may influence certain biological activities (Holub 1986, 1987). Nutritional supplementation of my-inositol may affect behavior and may have antidepressant and antianxiety activities. Clinical studies have reported that inositol is effective in relieving symptoms of depression (Einat et al. 1999), bulimia, panic disorder and bipolar depression (Palatnik et al. 2001). Inositol has been found in double-blind studies to be an effective treatment for obsessive-compulsive disorder (OCD) and is virtually free from side effects (Palatnik et al. 2001). Other studies have reported that high levels of myo-inositol are found in the brains of those afflicted with Down's Syndrome, as well as promoting the formation of amyloid plaques, one of the major components of Alzheimer's Disease (Huang et al. 1999).

1.5.2.2 Phytic acid

Phytic acid is a strong iron and mineral chelator and has often been reported as an antinutritional component in cereals and legumes due to polycationic metal ion (Ca, Co, Cu, Fe, Mg, Zn) intestinal absorption impairment *in vitro* and *in vivo* in animals and humans (Davies and Nightingale 1975a,b; Ellis et al. 1982; Morris and Ellis 1982; Morris 1985; Graf 1986; Graf et al. 1987; Reddy and Pierson 1994; Oatway et al. 2001; Oboh et al. 2003). Iron plays an essential role in many metabolic processes including oxygen transport, oxidative metabolism, and cellular growth (Lynch 1997). However, through mineral chelation, phytic acid has also been

reported to be a beneficial antioxidant in biological systems (Graf et al. 1987) that may someday replace intravenous chelation therapy such as the mineral-chelator EDTA or iron-binding drugs such as desferrioxamine (Desferal) (Lee and Hendricks 1995). Additional reports address the controversy of decreased mineral bioavailability (Graf 1986) and describe health benefits of dietary phytate (Wise 1986; Truelove et al. 1985; Kaufman 1986; Sharma 1986; Thompson 1986). Phytic acid has been reported to be a preventive and therapeutic anticancer agent (Shamsuddin and Ullah 1989; Ullah and Shamsuddin 1990; Shamsuddin 1996; Shamsuddin et al. 1997), including cancer of the colon and rectum (Owen et al. 1996), through its inhibition as an antioxidant to the generation of reactive oxygen species from H_2O_2 by chelating metals (Midorikawa et al. 2001); as a powerful inhibitor of calcium oxalate and calcium phosphate crystallization (March et al. 1999); as a preventive agent against renal calculi formation in the human urinary tract (Sharma 1986); to treat hypercalciuria (Grases et al. 2004) and other pathological calcifications (Midorikawa et al. 2001; Shears 2001); to have involvement with calcium ion influx in cerebellar neurons and on the functional acitivity of the central nervous system (Nicoletti et al. 1989); as an inhibitor of human immune deficiency viruses (HIV), implicated as causative agents of the acquired immune deficiency syndrome (AIDS) (Otake et al. 1989); inhibition of platelet aggregation (Borgo 1983); for protective effect on ischemic heart disease and improved ischemic myocardial region perfusion (Rao and Liu 1991); as well as the control of *E. coli* growth through phytic acid's capacity to bind and remove iron and iron containing nutriles such as hemoglobin (Eaton et al. 1982). In nuclear medicine, technetium-99m (99mTc) labeled-phytic acid is used to perform hepatic scintigraphy (Baker 1986; Gomes et al. 2002). Phytic acid has also

found additional medical and biological applications (Martin and Evans 1986a,b; Luttrell 1992; Martin 1995).

1.5.3 Metal interactions

Phytic acid's physiochemical properties, and biological metal chelation and antioxidant functions have been well documented as described earlier. Phytic acid contains about 28% phosphorus, in the form of 6 phosphoric acid acid groups with 12 dissociable hydrogens (Brown et al. 1961) on the inositol ring complex. These negatively charged phosphate sites bind mainly with alkali metal ions, particularly K(I) and Mg(II) in seeds and fruits (Lott et al. 2000), but may also form salts with other divalent and trivalent cations including Ba(II), Cd(II), Co(III), Cu(III), Fe(II), Hg(II), Mn(II), Ni(II), Pb(II), Sn(II), Sr(II), Al(III), Fe(III), and La(III) (Gersonde and Weiner 1982; Graf and Eaton 1984; Lott 1984; Lott et al. 1995; Rimbach and Pallauf 1998; Grases et al. 2001; Raboy 2001). Phytic acid–base properties and interactions with alkali metal ions, particularly for their use as chelating ligands, have been extensively reported (Barré et al. 1954; Costello et al 1976; Evans et al. 1982; Graf and Eaton 1984; Bieth and Spiess 1986; Bieth et al. 1989; Li and Wahlberg 1989; Li et al. 1989b; Balogi and Làsztity 1991; Siddiqi et al. 1993; Brigando et al. 1995; Bebot-Brigaud et al. 1999; De Stefano et al. 2002), however phytate speciation and determination of its protonation constants in different supporting electrolytes at different ionic strengths, and its complexing capacity with the major cations have been relatively infrequent and inconsistent (Isbrandt and Oertel 1980; Egbewatt and Dill 1987; Làsztity and Làsztity 1988; Li et al. 1989a,b; Li and Wahlberg 1989; Bieth et al. 1989; Evans and Martin 1992; Brigando et al. 1995; Bebot-Brigaud et al. 1999;

Pettit et al. 1999; De Stefano et al. 2002; Vasca et al. 2002). These types of elucidations have been limited by the relative analytical difficulty associated with the extraction, separation and detection of inositol phosphates in samples, using potentiometry and spectrophotometry with NMR and/or calorimetric methods (see 1.5.5 Phytic acid isolation and identification for more information).

Phytic acid-metal chelating affinity increases exponentially with the valency of the cation, for example, phytic acid chelates low levels of iron even in the presence of high calcium concentrations (Trela and Graf 1995a,b). Metal-phytate complexes have variable solubility products (Jackman and Black 1951). All equimolar metal1-phytate chelates are very soluble at any pH (Graf and Eaton 1984), while metal-phytate complexes containing 3 or more cations are extremely insoluble (Graf and Eaton 1984). As the metal to ligand ratio increases, nonstochiometric solid-phase mixtures coprecipite (Wise 1986). A few examples of such insoluble chelates include $(Ca^{2+})_3$-phytate, $(Ca^{2+})_4$-phytate, $(Ca^{2+})_5$-phytate, $(Ca^{2+})_6$-phytate, $(Fe^{2+})_3$-phytate, $(Fe^{2+})_4$-phytate, $(Fe^{2+})_5$-phytate, $(Fe^{2+})_6$-phytate, $(Fe^{3+})_3$-phytate, $(Fe^{3+})_4$-phytate, $(Ca^{2+})_4(Fe^{3+})$-phytate, etc.

Transition metal ions are catalysts for autoxidation (Black 1978). Phytic acid occupies all redox coordination sites on metal ions thereby completely inactivating metal ion catalytic activity, which accounts for its excellent antioxidant potential (Graf and Eaton 1990). Containing highly anionic chelating ligands with antioxidant potential, phytic acid has found many applications, some of which have already been mentioned, but also include as an antioxidant (Lee and Hendricks 1997; Lee et al. 1998) and the elimination of heavy metals such as Pb(II), Hg(II), and radioactive Sr(II) in foods (Binche 1968; Kudo 1969) and blood (Truelove et al. 1985); as a

treatment for acute lead poisoning (Wise 1983); for the industrial extraction of rare earths, or remediation of radioactive (e.g. uranic actinides such as depleted uranium) and toxic metal wastes including environmental contamination to soils, sediments, and colloidal and particulate matter in aquatic systems such as aquifers (Anderson et al. 1974; De Groot and Golterman 1993; Nash et al. 1998b; Ulusoy et al. 2003). The in situ immobilization of contaminated soils using phytic acid has been described (Jensen et al. 1996; Nash et al. 1997, 1998a, b; Seaman et al. 2003). Through a multistep process, phytic acid is surface-applied or injected into the subsurface in a soluble form such as sodium phytate. As phytic acid migrates through the material, it precipitates with the contaminants and native polyvalent cations such as Ca(II) or Mg(II) (Jensen et al. 1996; Nash et al. 1997, 1998a; Seaman et al. 2003). Phytic acid has found use in electrochemical sensors (Vasca et al. 2002), and as an intermediate in the production of fuels (McDermott 1968; Cao 1996). Due to phytic acid's affinity and solubilization of Ca(II) and Fe(III) it has found use as an active ingredient in tooth paste for the inhibition of plaque formation (Iwata and Irano 1998); as a water additive to prevent mineral scale formation in commercial reactors, boilers and cooling towers (Graf 1986); and as a cleaner and rust remover and preventative (Xiao 1998).

1.5.3.1 Phytic acid-Fe(III) ion interactions

Iron is an essential micronutrient for almost all organisms, due in large part to its ability to exist in two redox states Fe(II) and Fe(III) at physiological pH (Graf 1986; Minihane and Rimbach 2002). The ability to exist in two different valency states allows it to function as a catalyst in numerous biochemical reactions, including

similar reactivity in other systems such as food and beverages. Reducing agents such as ascorbic acid, catechols, glutathione, and superoxide anion radical ($O^{\cdot -}$) reduce Fe(III) to Fe(II), which then becomes reoxidized in the presence of dioxygen (O_2) (Graf 1986). Fe(II) is involved in the generation of hydroxyl radicals as part of the Haber-Weiss cycle (Equation 1.1) (Koppenol et al. 1978) and Fenton (Equation 1.2) (Burkitt and Gilbert 1990) reactions that generate reactive oxygen species that have the potential to damage tissue proteins, lipids and DNA, as well as oxidative damage to foods and beverages.

$$HO^{\bullet} + H_2O_2 \rightarrow H_2O + O_2^{\bullet -} + H^+$$

and

$$O_2^{\bullet -} + H^+ + H_2O_2 \rightarrow O_2 + HO^{\bullet} + H_2O$$

Equation 1.1 Haber-Weiss cycle.

It is believed that iron(III) complexes can catalyze the Haber-Weiss reaction: first Fe(III) is reduced by superoxide, followed by oxidation by dihydrogenperoxide.

$$O_2^{\bullet -} + Fe^{3+} \rightarrow Fe^{2+} + O_2$$

$$2O_2^{\bullet -} + 2H^+ \rightarrow H_2O_2 + O_2$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + {}^{\bullet}OH$$

Equation 1.2 Fenton reaction.

The ascorbate-driven Fenton reaction is the iron-salt-dependent decomposition of dihydrogen peroxide, generating the highly reactive hydroxyl radical (Walling 1975). Continuous redox-cycling reactions between iron, oxygen and reductive ascorbate that result in the production of hydroxyl radicals represent an important mechanism of cell injury in biological systems (Koppenol et al. 1978; Burkitt and Gilbert 1990), including induced lipid peroxidation involved in natural aging and in the etiology of several diseases (Halliwell 1981; Halliwell and Gutteridge 1985). Oxygen radicals are produced in ischemic tissues that may result in pathologic processes such as cardiac disease, intestinal hemorrhage and ulceration (Graf 1986). Additional oxygen radical induced pathologies include rheumatoid arthritis (Blake et al. 1981; Blake et al. 1984), cancer (Halliwell and Gutteridge 1985; Shamsuddin et al. 1997), and protein and DNA damage, among others.

Iron induced hydroxy radical formation in foods and beverages can lead to increased deterioration leading to short shelf life. Phytic acid has been shown to prevent hydroxyl radical formation resulting in reduced oxidative tissue damage in biological systems, and the stabilization of lipids (Loury et al. 1968), sorbic acid (Mahoney and Graf 1986), ascorbic acid (Graf 1986), and other food and pharmaceutical system components (Graf 1983). High concentrations of phytic acid inhibit polyphenol oxidase thereby preventing browning and putrefaction of various fruits and vegetables (Graf et al. 1987).

Iron-catalyzed free radical formation has been well documented. Iron chelating agents such as ethylenediaminetetraaceticacid (EDTA), generally in the form of dihydrate disodium salt, nitrilotriaceticacid (NTA), and numerous other chelating agents form complexes with iron(II) ions, however, retain reactive

coordination site and therefore catalyze iron-mediated oxidation via the Haber-Weiss cycle. Phytic acid and desferrioxamine occupy all available iron coordination sites and therefore inactivate iron catalysis (Graf et al. 1984) and hydroxyl radical formation (Hawkins et al. 1993; Phillippy & Graf 1997). In molar ratios of 0.25 phytate-to-iron and above, the generation of hydroxyl radicals via the Fenton reaction was almost completely blocked (Graf et al. 1987) Phytic acid has the ability to remove oxygen via the reduction of Fe(III) by ascorbate, and the oxidation of Fe without the concomitant generation of deleterious hyroxyl radicals (Graf et al. 1984).

Phytic acid forms fairly stable chelates with almost all multivalent cations including iron, which blocks the Fenton reaction and consequent oxidation. Metal corrosion due to iron oxidation can be inhibited by phytic acid (Graf 1983). It is also used in the stabilization of paper processing, and inking formulations (Allison 1983; Morimi 1983). Calcium phytate has been used to stabilize cellulose oxidation of ancient iron-gall ink manuscripts, where the resulting iron(II)-phytate complexes are white and therefore do not affect the color of the ink and paper (Botti et al. 2004).

Phytic acid is examined here as a method to treat excess copper in wines (see chapter II ine stabilization with phytic acid: i. Iron and copper for more information).

1.5.3.2 Phytic acid-Cu(II) ion interactions

Copper is essential in very small amounts in all higher plants and animals (Adult human DRI Tolerable Upper Intake Level of dietary copper from all sources is 10 mg/day), higher amounts are toxic and can inhibit the enzyme dihydrophil hydratase, an enzyme involved in haemopoiesis, or lead to death. It is stored in some

organs such as the liver where it is first transported bound to albumin. Copper is required for the activity of several enzymes including cytochrome c oxidase and superoxide dismutase. It is associated with iron metabolism, elastin and collagen formation, melanin production, integrity of the central nervous system and normal red blood cell formation (Kumar 2002). In hemocyanin, the oxygen-carrying respiratory protein that forms the blood of most mollusks, and some arthropods such as the horseshoe crab, *Limulus polyphemus*, uses copper for oxygen transport instead of iron as in hemoglobin. Oxygenation causes a color change between the colorless Cu(I) deoxygenated form and the blue Cu(II) oxygenated form.

Phytic acid chelates copper with high affinity constants in vitro (Vohra et al. 1965). The chelating ability of phytic acid varies with the metal according to the sequence: Cu(II) > Zn(II) > Cd(II) > Ni(II) ≈ Co(II) (Bebot-Brigaud et al. 1999). Calcium has a potentiating effect on the precipitation of phytate-Cu(II) complexes as a function of PA:Cu(II):Ca(II) molar ratios and pH (Champagne 1987; Lönnerdal 2002). Ca(II) ions compete with Cu(II) ions for binding (Champagne 1987). Aqueous phytate-Cu(II) solutions form soluble complexes up to pH ≈3.3 (Vasca et al. 2002).

As previously mentioned, phytic acid inhibits the generation of hydroxyl radicals through the Fenton reaction with iron. It can also prevent the generation of reactive Cu(II)-hydroperoxide complexes through H_2O_2, by binding tightly to Cu(II) (Midorikawa et al. 2001; Lima-Filho et al. 2004). Low concentrations of copper can inhibit urease, a nickel-dependent metallo-enzyme that catalyzes the hydrolysis of urea to form ammonia (NH_3) and carbon dioxide (Jabri et al. 1995; Lima-Filho et al. 2004). The use of urease in wine can prevent the formation of carcinogenic ethyl carbamate in the alcoholic solution from urea. Phytic acid chelates copper, which

could provide urease protection, however, it also chelates nickel, a property that is inhibitory (Zaborska et al. 2001; Juszkiewicz et al. 2004).

Phytic acid is examined here as a method to treat excess copper in wines (see chapter II ine stabilization with phytic acid: i. iron and copper for more information).

1.5.3.3 Phytic acid-Ca(II) and K(I) ion interactions

In fruit and seeds, phytic acid usually occurs as a K and Mg salts (Lott 1984; Lott et al. 1995; Lott et al. 2000). In biological systems phytate usually forms Ca, Zn and Fe salts. Calcium phytate is often used as a dietary supplement. Martin and Evans (1986a) studied the interaction of phytic acid with Ca(II) by potentiometric titration and by measurement of free Ca(II) concentrations using an ion selective electrode, and concluded that the extent of binding is dependent upon both pH and the calcium to phytic acid ratios. Other metals may be coprecipitated or ex-changed with Ca(II) in $(Ca^{+2})_n$–phytate at concentrations that are insufficient to promote precipitation by themselves (Wise 1986). Additionally, metals such as Pb may be strongly sorbed to precipitated $(Ca^{+2})_n$–phytate without resulting in the stochiometric release of Ca(II) (Wise 1986).

Phytic acid is examined here as a method to treat excess calcium and to stabilize calcium tartrate in wines (see chapter III for more information).

1.5.4 Phytic acid-protein interactions

Phytic acid strongly interacts with proteins in a pH and cation-dependent manner (Cheryan 1980). The interaction of phytate with proteins begins in seeds during ripening, when phytate accumulates in the protein-rich aleurone layer of cereals and protein bodies of legumes (Hídvégi and Lásztity 2002). The formation of protein-phytate globoid crystals and their size is highly dependent on the presence of inorganic cations. Higher amounts of magnesium and calcium favor the formation of large globoid crystals (Lott et al. 1985; Graf 1986), suggesting that higher concentration of divalent cations increases cation–phytic acid interactions instead of protein–phytic acid interactions (Hídvégi and Lásztity 2002). At low pH and low cation concentration, phytate-protein complexes are formed with the basic lysine, arginine, and histidine residues through direct electrostatic interaction (Cheryan 1980). At neutral and basic pH, both phytate and most proteins have a net negative charge, which leads to their dissociation from each other (Cheryan 1980). In the presence of multivalent cations, however, protein-cation-phytate complexes can occur (Cheryan 1980) with various resulting solubilities.

In the preparation of soybean protein isolates, the interaction with phytate has to be considered, especially if preparing pure protein isolates due to the formation of protein-calcium-phytate complexes. Soybean protein isolates are precipitated at a pH near 4.5, the isoelectric region for the major proteins where maximum yields occur (Honig and Walter 1987). When the protein isolate is subsequently neutralized (pH 6-8.5), soluble and insoluble protein-phytate complexes are formed (Honig and Walter 1987) inhibiting the coagulation of soybean protein (Saio et al. 1969). Phytic acid also interacts with and can modify the activity of enzymes. It increases the activity of

alcohol dehydrogenase (Altschuler and Schwartz 1984), however it inhibits trypsin (Singh and Krikorian 1982), alpha amylase (Thompson 1986), and tyrosinase (Graf 1986). Phytic acid extract consumption from sweet potato and commercial phytic acid plus zinc supplement has been shown to lower blood glucose levels with no significant change in the activity of 6-phosphogluconate dehydrogenase, pyruvate kinase, malic enzyme, or ATP-citrate lyase compared to the group fed formulated diets (Dilworth et al. 2005). Lowering of blood glucose levels may be desirable for diabetics (Dilworth et al. 2005).

Phytic acid is examined here as a method to treat and stabilize excess proteins in model wine (see chapter IV. for more information).

1.5.5 Phytic acid isolation and identification

1.5.1.1 Isolation

The most common raw material for phytic acid production is the bran of rice and cereal grains, as well as oil plant cakes obtained as by-products of the food processing and oil-producing industries. As a natural product, it is produced in quantities dependent on the cultivar and growing conditions as well as the methods and conditions of processing, for example, temperature, enzyme, pH, or microorganism influences (Cheryan 1980; Hídvégi and Lásztity 2002). Some raw materials such as legumes contain phytase that can cause partial or complete hydrolysis of phytic acid into intermediary products, such as inositol-mono-, inositol-di-, and inositol-tri-ortho-phosphates. For a review of phytases see Mayini and Markakis (1986). A biosynthetic enzyme for the production of phytic acid has been

patented (Cahoon et al. 2002). Most methods of phytic acid extraction from natural products use acidulated water either with organic acids (formic, trichloroacetic, lactic, oxalic, citric, etc.) (Sarma 1942) or dilute mineral acids (hydrochloric, nitric) (Pavlov et al. 1969; Zakharov 1993). These initial extracts yield phytic acid, proteins, sugars, salts and other extractables, which are then neutralized with mild alkaline base to precipitate amorphous white phytic acid (Pavlov et al. 1969). The precipitated crude product is filtered, washed, and purified by subsequent dissolution and precipitation, boiling with activated charcoal and intermediary treatments to remove specific admixtures (Posternak 1903, Pavlov et al. 1969).

1.5.5.2 Identification

The main limitation of phytic acid and other inositol phosphates analysis has been the availability of suitable analytical techniques. According to Oberleas and Harland (1986) there are no known specific reagents that identify phytate and since inositol phosphates are optically inactive with no characteristic ultra-violet (UV) or visible absorption spectrum, spectrophotometric methods of measure have proven difficult. Under suitable conditions, phytic acid may precipitate all polyvalent cations. This precipitation forms the basis of some classic methods for the determination of phytate deriving from the method of Heubner and Stadler (1914). These methods are based on the extraction, purification, and subsequent precipitation with stoichiometric ratios of ferric ion (e.g. $FeCl_3$) with phytate in dilute acid (e.g. 2% HCl) solution and analysis of phosphorus or iron in the precipitate (Holt 1955; Davies and Reid 1979; Talamond et al. 1998b; Oboh et al. 2003). The interaction of phytic acid with specific cations such as Ca(II) has been studied by potentiometric titration and by

measurement of free Ca(II) concentrations using an ion selective electrode (ISE) (Martin and Evans 1986a). Other methods including qualitative methods: paper chromatography, paper electrophoresis, thin-layer chromatography, ion exchange, and nuclear magnetic resonance (NMR) and X-ray diffraction measurements; and quantitative analytical methods involving titration, precipitation, ion exchange, high performance liquid chromatographic (HPLC), NMR, enzymatic, amperometric and other methods have been reviewed in detail by Oberleas and Harland (1986). Table 1.9 shows the detection limits for inositol phosphates and inositol for a range of analytical techniques (Turner et al. 2002a). This section will outline a few of the most common modern methods of analysis.

Since inositol phosphates lack chromophoric functional groups, spectrophotometric methods of measure often involve digesting or derivatization post-column. Detection of inositol phosphates after chromatographic separation may involve colorimetric determination of molybdate-reactive P (molybdenum blue) following a quantitative hydrolysis step (Cilliers and Van Niekerk 1986; Benson et al. 1996; Kamaya et al. 1998; Turner et al. 2002b) and the determination of inositol (Koning 1994) or phosphate (March et al. 1998). Other HPLC methods involve sample extracts that are first prepurified by passing through an anion-exchange resin to remove inorganic phosphate and concentrate inositol (Cosgrove 1963; Tangendjaja et al. 1980). Some systems incorporate reversed-phase (Camire and Clydesdale 1982a; Graf and Dintzis 1982; Sandberg and Ahderinne 1986; Marquié et al. 1995; Matthaüs et al. 1995), or anion-exchange phase (Rounds and Nielsen 1993; Dorsch et al. 2002) separation, followed by refractive index detection (RFID) (Rimbach et al. 1998; Rimbach and Pallauf 1998), or detection after subsequent derivatization due to

phytate's poor spectrophotometric properties. Derivatization reactions, while sensitive, however, present a potential source of error and may not be 100% efficient and result in partial hydrolysis of phytate (Benson et al. 1996; Talamond et al. 1998a). A study by Oltmans et al. (2005) found good agreement between results obtained through a ferric-precipitation method and those obtained through an HPLC method.

Phytic acid determination using replacement reactions and subsequent fluorimetric measurements was first propsed by Grases et al. (1981). This method was based on phytic acid's action on a metal ion catalysed reaction by the oxidative transformation of 2,2A-dipyridyl ketone hydrazone, catalysed by Cu(II) ion to a fluorescent product. Other fluorimentric detections methods for inositol phosphates have been based on the dissociation of the fluorescent Fe(III)–methylcalcein blue (Irth et al. 1990), or zinc chloranilate (Kamaya et al 1995) complexes. A high performance ion chromatography (HPIC) method developed by Talamond et al. (1998a,b) followed by chemically suppressed conductivity detection (Skoglund et al. 1997) did not require any prepurification and derivatization steps.

The following is an example of phytic acid analysis in soybeans from Israel et al. (2006): Unless otherwise stated, all experiments and procedures can be performed at room temperature. Dried seed samples were ground in a centrifugal grinding mill (speed 15,000 rpm) followed by seiving through a 1.0-mm stainless steel sieve to produce ground samples with a uniform particle size < 0.5 mm. Extraction of the flour was done with 0.5 M HCl in a ratio of 1:20 (w/v) for 1 hour while stirring. A volume of the crude extract from each sample are then centrifuged (e.g. 18,000 x g for 10 min). An aliquot of the supernatant containing pytic acid was then filtered through a

0.45-µm filter. According to the authors, filtered samples can be stored at 4°C for several days before HPLC analysis. HPLC analysis was then performed with a strong anion exchange column equipped with a guard column. Elution of phytic acid was achieved with a 30-min linear gradient of 0.01 M 1-methylpiperazine, pH 4.0 to 0.5 M $NaNO_3$ in 0.01 M 1-methylpiperazine, pH 4.0, at a flow rate of 1 mL min^{-1}, as previously described by Rounds and Nielsen (1993). Wade's color reagent (Wade and Morgan 1955), consisting of 0.015% (w/v) $FeCl_3$ and 0.15% (w/v) 5-sulfosalicylic acid (also at flow rate of 1 mL min^{-1}) and phytic acid eluted from the column, and were mixed in a mixing tee with inline check valves for both eluants installed before the mixing tee to prevent backflow. The postcolumn reaction was allowed to take place in a 0.05- by 210-cm PEEK tubing at the combined flow rate of 2 mL min^{-1}. The absorbance was monitored at 500 nm.

1.5.5.3 Total P, phytate P, inorganic P, and other P

Various analytical methods of measuring phosphate forms also exist and are described below as outlined by Oltmans et al. (2005). If measuring solid samples (e.g. seeds or soils), they are usually dried and then milled to pass through a 20-mm screen. Total phosphorous can then be determined following wet-ashing of a ground sample with a colorimetric assay of digest P (Chen et al. 1956). Inorganic phosphorous can be determined colorimetrically following extraction of a ground sample in 12.5% (w/v) TCA and 92 mM $MgCl_2$. Phytate phosphorous can be extracted in 0.4 M HCl:0.7 M Na_2SO_4 and then determined by ferric-precipitation (Raboy et al. 2000), where the precipitate is wet-ashed, and assayed for P as in the total P analysis. Phytate P can be expressed as its P (atomic weight 31) content to

facilitate comparisons between other P fractions (Dorsch et al. 2002). Other P can then be determined by subtracting phytate P and inorganic P from total P. Other P then represents the sum of nonphytate P and noninorganic P compounds including RNA, DNA, protein, lipids, and starches (Oltmans et al. 2005).

Table 1.9 Detection limits for inositol phosphates and inositol for a range of analytical techniques (Turner et al. 2002a).

Table 3. Detection limits for inositol phosphates and inositol for a range of analytical techniques.
(IMER, immobilized enzyme reactor; HPLC, high performance liquid chromatography; I(2)P, inositol 2-monophosphate; LC–MS, liquid chromatography–mass spectrometry; MRP, molybdate-reactive phosphorus; NMR, nuclear magnetic resonance; P_i, inorganic phosphate as orthophosphate.)

technique	species detected	detection limit	sample type	reference
anion-exchange chromatography/colorimetric	P_i	0.5 μg P g^{-1}	soil	McKercher & Anderson (1968*b*)
gel filtration/colorimetric	MRP	10 μg P l^{-1}	lake sediments	Eisenreich & Armstrong (1977)
^{31}P NMR	IP_6	< 24 mg P g^{-1}	food	O'Neill *et al.* (1980)
HPLC/colorimetric	MRP	30 μg P g^{-1}	plants	Graf & Dintzis (1982)
ion-exchange column/colorimetric	P_i	10 μg P g^{-1}	soil	Appiah & Thomas (1982)
HPLC–IMER/fluorimetric	P_i	15.5 ng l^{-1}	enzymatic products	Meek & Nicoletti (1986)
HPLC/colorimetric	MRP	25 μg P l^{-1}	model compounds	Minear *et al.* (1988)
HPLC/flow injection analysis	MRP	10 μg P l^{-1}	aqueous	Clarkin *et al.* (1992)
HPLC/colorimetric	$FeCl_3$	2.5 μg P g^{-1}	food	Rounds & Nielsen (1993)
flow injection analysis/colorimetric	MRP	27 μg P l^{-1}	aqueous	McKelvie *et al.* (1993)
flow injection analysis/colorimetric	MRP	4 μg P l^{-1}	aqueous	McKelvie *et al.* (1995)
enzymatic/colorimetric	MRP	90 μg P g^{-1}	food and pharmaceutical	March *et al.* (1995)
HPLC/metal–dye detection	inositol phosphates	0.93 μg P l^{-1}	cells and tissues	Guse *et al.* (1995)
HPLC/colorimetric	MRP	0.3 μg P g^{-1}	coastal sediments	Suzumura & Kamatani (1995*b*)
ion-exchange/thermospray LC–MS	inositol	19 mg P l^{-1}	biomedical	Hsu *et al.* (1990)
capillary electrophoresis	I(2)P	200 μg l^{-1}	physiological	Henshall *et al.* (1992)
HPLC-enzymatic/bioluminescence	inositol	31 ng l^{1}	physiological	Prestwich & Bolton (1991)

1.6 Outline of the dissertation

Chapter 2 analyzes the fate of iron and copper in wine and model wine solutions after being treated with phytic acid and calcium carbonate. It investigates the stoichiometry and effect of phytic acid to bind and precipitate different concentrations of iron and copper ions in white, red and model wine solutions.

Chapter 3 analyzes the fate of calcium and potassium in a model wine solution after being treated with phytic acid. It investigates the stoichiometry and effect of phytic acid to bind and precipitate different concentrations of calcium ions in white, red and model wine solutions.

Chapter 4 analyzes the fate of protein in a model wine solution after being treated with phytic acid. It investigates the effect of phytic acid on different concentrations of bovine serum albumin and the effect of phytic acid to bind and precipitate different concentrations of bovine serum albumin in a model wine solution.

The appendix contains additional data regarding each experiment in chapters 1 through 3 that is not intended to be included in the published material.

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CHAPTER II

WINE STABILIZATION WITH PHYTIC ACID: I. IRON AND COPPER

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And associated technology

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2.1 Abstract

Polyvalent metal cations such as iron when present in high concentration in wine and other beverages can adversely affect product quality. They may cause objectionable organoleptic properties including metallic taste, discoloration and oxidative flavor changes, as well as forming hazes and cloudiness. Reducing heavy metal content in beverages, especially wines, has long been desirable. This investigation describes a method in which phytic acid is added to a red, a white and a model wine to chelate polyvalent iron cations in a phytic acid to iron molar ratio of 1:1, which corresponds to 0.018 mM phytic acid or 11.8 mg/L or 0.0012% w/v phytic acid or 0.0024% w/v of 50% w/v aqueous phytic acid for each 1 mg/L of iron present. A calcium salt was added in a sufficient amount to co-precipitate the complex quantitatively, at a calcium to phytic acid molar ratio of 5:1, which corresponds to 9.0 mg/L $CaCO_3$ or 0.0009% $CaCO_3$ for each 1 mg/L of iron that subsequently is removed by filtration. This method effectively, inexpensively and safely removes excessive levels of iron in wine, sparkling wine, and other beverages. This method overcomes the problems of known methods, for example it binds iron to the most complete extent possible, produces no toxicologically objectionable products even in the case of over clarification, and acts selectively on heavy metal ions while being ineffective on copper or potassium ions. Color and phenolic parameters were measured spectrophotometrically and were not greatly affected by phytic acid and calcium carbonate treatments. Acidity measures were slightly changed after treatment however could likely be controlled.

2.2 Introduction

Metals, particularly iron and copper transition metals, can accumulate in beverages for a number of reasons. These two elements naturally occur in small amounts in grapes, for example in copper containing enzymes such as oxidases (Hsia et al. 1975). Larger quantities of these elements may accumulate especially from high iron content soils and dust, or copper containing fungicides that settle and remain on the fruit before processing (Dupuy et al. 1955; Rankine 1955; Hsia et al. 1975; Puig-Deu et al. 1994). Metals may also amass from the corrosion of metal processing equipment and storage containers or from any other metal parts that are in contact with the beverage (Hsia et al. 1975). Additionally, the deliberate addition of copper sulfate as a fining agent to treat sulfides can result in increased copper levels. The average concentration of iron and copper in wine from different regions worldwide has been reported to range from 2.8 to 16 mg/L and 0.11 to 3.6 mg/L respectively (Ough and Amerine 1988).

These metals can catalyze oxidation reactions as well as complex with polyphenols and proteins thereby altering their solubility and stability (Kean and Marsh 1956; Glories 1974; Ough et al. 1982; Gorinstein et al. 1984; Cacho et al. 1995). Additionally, the formation of metallo complexes between metal ions and anthocyanins can form darkened colored complexes (Cheng and Crisosto 1994; Phillips 1988). Iron levels above 4 mg/L or copper levels above 0.2 mg/L in wines (Ough et al. 1982) may require treatment to reduce their concentration in order to prevent unwanted cloudiness, casse formation, metal catalyzed oxidation, discoloration, metallic tastes, premature aging, and metal toxicity (Joslyn and Lukton 1953; Ough et al. 1982; Gorinstein et al. 1984; Cheng and Crisosto 1997).

Wines may be stabilized by removal of iron and copper with chelating agents, such as commercial resins (Palacios et al. 2001; Scholten 2001), ethylenediaminetetraacetic acid (EDTA), pectinic acid, and alginic acid (Joslyn and Lukton 1953; VanBriesen and VanBriesen 2005). Other methods for metal stabilization also exist including adsorption by yeast or yeast hulls (Thoukis and Amerine 1956); complexing with carboxylic acids such as citric acid (Field et al. 1974; Timberlake 1964 a,b; Yokoi et al. 1994); the use of super critical fluids (Fahlman 2002); or blending into a wine with less metal concentration. Removal with ferrocyanide is probably the most efficient method because it precipitates most metal ions, including iron, copper, lead, zinc, and magnesium, however is ineffective for calcium (Joslyn and Lukton 1953), and its use raises toxicological and waste disposal concerns.

2.1.1 Ferrocyanide

Cyanide treatment of wine with potassium ferrocyanide (hexacyanoferrate(II), $Fe(CN)_6K_4$) is known as blue fining and is mainly used to remove iron by precipitation as sparingly soluble Prussian blue. Potassium ferrocyanide reacts with both iron(II) and more slowly with iron(III) to produce white and blue insoluble salts respectively (Castino 1965). Other heavy metal ions (Cu, Zn, Mg, Pb, Mn and Cd) are co-precipitated. Blue fining is currently the most internationally commonly used process for reducing the concentration of heavy metal ions in wine; however, it is prohibited in many countries and is strictly controlled where permitted. Colloidal ferrocyanide preparations (Fessler compound, Cufex, and Metafine) are also used and differ from potassium ferrocyanide in that they are of limited solubility and contain

an excess of iron to minimize residual ferrocyanide in wine (Fessler 1952; Joslyn and Lukton 1953). These colloidal preparations are currently no longer in production.

Ferrocyanide use has some serious disadvantages in terms both of winery technology and, especially, of toxicology. The amount of potassium ferrocyanide required for clarification should be accurately determined by preliminary analytical tests. After blue fining, subsequent testing (Hubach test) for cyanide is highly advisable, and in many countries mandatory, in order to detect residual levels. Residual ferrocyanide may result in the formation of toxic hydrocyanic acid (HCN):

$$Fe(CN)_6^{4-} + H_2O \rightarrow Fe(CN)_5^{3-} + OH^- + HCN$$

Cyanide compounds are highly toxic, causing harm by interfering with the body's use of oxygen (Salkowski and Penney 1994; Hall and Rumack 1986). The lethal dose of cyanide in humans is approximately 50 – 60 mg (Salkowski and Penney 1994), although the consumption of wine is unlikely to cause severe toxicity or death. Furthermore, Prussian blue tends to form a colloidal solution in the wine so that, for example, subsequent fining with silica sol/gelatin is necessary. Colloidal Prussian blue slowly agglomerates and sediments, the consequence being long waiting times with the risk of gradual decomposition of the Prussian blue and the formation of hydrocyanic acid. Equipment that has come into contact with potassium ferrocyanide must be treated very thoroughly with sodium carbonate solution in order to remove excess reagent or adherent Prussian blue (Trela and Graf 2005a,b). In addition, since Prussian blue contains cyanide it is classified as a special waste and its separated solids in the wines must be disposed of appropriately.

Iron and copper casse may be inhibited by the addition of agents that limit the flocculation of insoluble iron and copper complexes. Protective colloids such as gum arabic can restrict haze formation. Because gum arabic limits the clarification of colloidal material, it can only be safely applied after the wine has undergone all other stabilization procedures. Furthermore, such stabilized iron (and excessive amounts of copper) can still impart an undesirable metallic taste to the wine and also serve as oxidative catalysts that generate off-flavors during storage since not all redox coordination sites are inactivated (Trela and Graf 2005a,b).

2.2.2 Calcium phytate

Calcium phytate, $(Ca^{2+})_4$-phytate ($Ca_6C_6H_{12}O_{27}P_6$), has been employed to remove iron(III) from wine (Cordonnier 1952; Negre and Cordonnier 1952; Joslyn and Lukton 1953; Ribereau-Gayon and Peynaud 1953; Deibner and Bouzigues 1954; Nieto 1954; Prillinger 1954; Rotini 1954; Capt 1955; Maveroff and Sanchez 1955; Nieto 1955; Capt 1956a,b,c; Hennig 1956; Leglise 1958). Since calcium phytate preferentially reacts with iron(III), strong aeration of the wine to favor oxidation of Fe(II) to Fe(III) has been recommended (Ribéreau-Gayon et al. 2000). To prevent over addition of calcium that may lead to subsequent calcium tartrate instability, non stoichiometric dosages of calcium phytate, at the rate of 4 – 5 mg/L for every 1 mg/L of iron(III) have been recommended (Cordonnier 1952; Ribereau-Gayon and Peynaud 1953). Iron reductions of between 27 – 90% were observed depending on the extent of aeration of the wine.

This method is very ineffective due to the insolubility of calcium phytate. The oxidation procedure and extensive manipulations may impair wine quality. The

procedure relies on slow adsorption of iron(III) onto colloidal calcium phytate that needs to be kept dispersed by stirring the wine for several days. Additionally, the procedure may increase the calcium content of the wine (20 – 30 mg/L) and lead to instability.

2.2.3 Phytic Acid

Phytic acid (PA) or myo-inositol, hexakis (dihydrogen phosphate) (Figure 2.1) is a strong chelating agent and antioxidant present in all seeds (Graf 1986). Grapes are estimated to contain about 0.18% phytic acid (Lott et al. 2000), although most is likely contained in the seeds. Phytic acid contains six orthophosphate moieties with 12 dissociable protons and therefore it has a high chelation potential for polyvalent cations over a wide range of pH values (De Stefano et al. 2003). The binding affinity increases exponentially with the valency of the cation, which means that phytic acid chelates low levels of iron even in the presence of high calcium concentrations (Maddaiah 1964; Vohra 1965; Wise 1986; Vasca et al. 2002); however, it is ineffective for binding copper or potassium (Trela and Graf 2005a,b). All metal1-phytate complexes are very soluble at any pH, while metal-phytate complexes containing 3 or more cations are extremely insoluble (Graf and Eaton 1984). A few examples of such insoluble chelates include $(Ca^{2+})_3$-phytate, $(Ca^{2+})_4$-phytate, $(Ca^{2+})_5$-phytate, $(Ca^{2+})_6$-phytate, $(Fe^{2+})_3$-phytate, $(Fe^{2+})_4$-phytate, $(Fe^{2+})_5$-phytate, $(Fe^{2+})_6$-phytate, $(Fe^{3+})_3$-phytate, $(Fe^{3+})_4$-phytate, $(Ca^{2+})_4(Fe^{3+})$-phytate, etc. Finally, phytic acid occupies all redox coordination sites on iron and thereby completely inactivates its catalytic activity, which accounts for its excellent antioxidant potential (Graf and Eaton 1990). This chelating capacity can remove or inactivate reactive metals, for

example iron(III) and its ability to generate hydroxyl radicals that often adversely affects biological systems and the production or storage of foods and beverages. It also interacts with a variety of other compounds including proteins. Some uses of phytic acid include lipid peroxidation inhibition of cooking oils (Lee and Hendricks 1995); chelating metals in tinned foods, corrosion and rust preventative coatings on metal surfaces (Xiao 1998); as a natural antioxidant in foods (Lee and Hendricks 1997; Lee et al. 1998), and to counter paper corrosion caused by iron gall ink in ancient manuscripts (Botti et al. 2004); among many other food, medical, and industrial applications. The uses of phytic acid are multifarious and are well documented (Johnson and Tate 1969; Blank et al. 1975; Isbrandt and Oertel 1980; Maga 1982; Graf 1983,1986; Martin and Evans 1986; Sands et al. 1986; Li et al. 1989; Jensen et al. 1996; Bauman et al. 1999; Bebot-Brigaud et al. 1999; Harland and Narula 1999; Paton et al. 1999; Oatway et al. 2001; Seaman et al. 2003), including stabilizing calcium (Trela and Graf 2005a,b; Trela 2006a, submitted for publication) and protein in wine (Trela 2006b, submitted for publication).

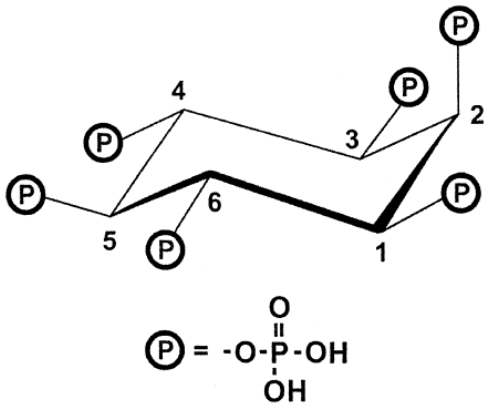

Figure 2.1 Phytic Acid.

The reported use of phytic acid, as opposed to calcium phytate, in wine has been limited and generally was not recommended (Joslyn and Lukton 1953; Capt 1955). Joslyn and Lukton (1953) showed that phytic acid had about the same affect in the removal of iron and copper as calcium phytate. Capt (1955) reported that due to the solubility of Fe(II)-phytate, only 40-70% of the iron could be precipitated resulting in potential casse forming concentrations. In both of these studies Ca was not added to coprecipitate the metal-phytate complex and therefore it is conceivable that soluble metal-phytate complexes remained in the wines.

The present method using phytic acid, coprecipitated after metals reaction with calcium carbonate, provides a novel means for selective removal of heavy metals, especially iron cations, from beverages such as wines and fruit juices. The method overcomes the above-mentioned problems of known methods, for example it binds iron to the most complete extent possible, produces no toxicologically objectionable products even in the case of over clarification, and acts selectively on heavy metal ions.

2.3 Materials and methods

2.3.1 Sample preparation

Samples were prepared using a white, a red, and a model solution (Table 2.1). The white wine was a dry 2005 Torrontes from Davis, California. The red wine was a dry 2004 Merlot from Napa Valley, California. The model wine was made similar in composition to a dry table wine. The model solution, which contained 2.00 g/L KHT

and 12.5% (v/v) ethanol, had a pH of 3.55 and an acidity of 1.00 and 2.00 g/L respectively of titratable and total acidity expressed as tartaric acid to pH 8.2. The model wine solution was then separated into three lots and adjusted for pH with conc. HCl or NaOH. All model wine studies, with the exception of the one described below, used Model 1 wine. Distilled, deionized (18.6 mΩ · cm and 3 ppb total organic carbon [TOC]) ultrapure water (Millipore Synthesis A10 with Quantum EX ultrapure cartridge, MA, U.S.A.) was used to make all solutions.

Table 2.1 Sample wine conditions.

	Varietal	**Location**	**Vintage**	**Alc. % (v/v)**	**TA* (g/L)**	**pH**
Red	Merlot	Napa	2004	14.5	5.33	3.44
White	Torrontes	Davis	2005	13.5	3.51	3.63
Model 1				12.5	1.04	3.55
Model 2				12.5	1.12	3.22
Model 3				12.5	0.78	3.75

* Titratable acidity (TA) expressed as g tartaric acid per liter titrated to pH 8.2

Metal spiked samples (250 mL) were prepared in triplicate from each wine by adding increasing amounts of stock metal solutions: 100 mg/L Cu(II) (copper sulfate, $CuSO_4 \cdot 5H_2O$, J. T. Baker, NJ, U.S.A.), and 1,000 mg/L Fe(III) (ferric chloride hexahydrate, $FeCl_3 \cdot 6H_2O$, Sigma-Aldrich, MO, U.S.A.) solutions. All containerware and reagent bottles were triple rinsed with 0.1 N HCl followed by triple rinsing with water. Samples were stored in 0.5 L bottles at room temperature. The final

concentrations in the samples were: iron at 5, 10 and 20 mg/L; copper at 0.25, 0.50, and 1.00 mg/L; and iron 10 mg/L + copper 0.50 mg/L (Table 2.2).

Table 2.2 Sample metal concentrations added (mg/L).

Metal	Model wine	White wine	Red wine
Fe	0	0	0
	5	5	5
	10	10	10
	20	20	20
Cu	0.25		
	0.50		
	1.00		
Fe + Cu	10 + 0.5		

Phytic acid ($C_6H_{18}O_{24}P_6$, CAS 83-86-3, 50% w/w, Sigma-Aldrich, MO, U.S.A) treatments ranged between metal:PA molar ratios of 1:1 to 6:1 for Fe; 1:1, 1:2, and 1:4 for Cu in the model wines respectively (Table 2.3). The red and white wines were treated with PA on a Fe:PA molar ratio of 1:1. Phytic acid concentrations before and after sample treatments were not measured in this study, although phytic acid analysis is possible using various methods (Talamond et al. 1998; Israel et al. 2006). After addition of phytic acid the wines were mixed and followed 1 day later with addition of calcium (calcium carbonate, $CaCO_3$, Fisher Scientific, NY, U.S.A.) at a Ca:PA molar ratio of 5:1 (Trela and Graf 2005a,b). All samples were centrifuged (15,000 rpm x 3 minutes in 50 mL centrifuge tubes) and then sterile filtered through 4

mm poly(tetrafluoroethylene) (PTFE) 0.45 μm pore size syringe filters before analysis at five days after Ca addition. All samples were stored under nitrogen gas.

Table 2.3 Metal treatment levels with phytic acid and calcium.

	Metal (mg/L)	**Metal (mM)**	**PA (mg/L)**	**PA (mM)**	**Molar ratio Metal:PA (mM)**	**Ca (mg/L)**	**Ca (mM)**	**Molar ratio Ca:PA (mM)**
Fe	5	0.090	59.1	0.090	1:1	17.9	0.448	5:1
	10	0.179	118.2	0.179	1:1	35.9	0.895	5:1
	20	0.358	236.4	0.358	1:1	71.8	1.791	5:1
	20	0.358	118.2	0.179	2:1	35.9	0.895	5:1
	20	0.358	78.8	0.119	3:1	23.9	0.597	5:1
	20	0.358	59.1	0.090	4:1	17.9	0.448	5:1
	20	0.358	47.3	0.072	5:1	14.4	0.358	5:1
	20	0.358	39.4	0.060	6:1	12.0	0.298	5:1
Cu	0.25	0.004	2.6	0.004	1:1	0.8	0.020	5:1
	0.50	0.008	5.2	0.008	1:1	1.6	0.039	5:1
	0.50	0.008	10.4	0.016	1:2	3.2	0.079	5:1
	0.50	0.008	20.8	0.031	1:4	6.3	0.157	5:1
Fe + Cu	10 + 0.5	0.179 + 0.008	123.4	0.187	1:1	37.5	0.934	5:1

2.3.2 Titratable acidity and pH analyses

Wine pH was measured with a pH meter (Accumet 925, Fisher Scientific, PA, U.S.A.; and Orion 81-72 ROSS™ Sure-Flow pH Electrode, Thermo Electron Corporation, MA, U.S.A.). Titratable acidity (TA, tartaric acid in g/L) was measured by placing 10 mL of wine sample into 100 mL deionized water, the solution was degassed under vacuum, and titrated with 0.1 or 0.01 N NaOH to an end point of pH 8.2.

2.3.3 Color analyses

Color was determined by measuring the absorbance at 280, 420 and 520 nm with a Hewlett-Packard (HP) (Palo Alto, CA) 8452A diode array spectrophotometer controlled by Agilent UV-vis Chemstation software (Windows NT) Rev. A.08.08[71] through a 10 mm quartz cuvette (model wine), and a 1 mm flow-through quartz cuvette (red and white wine) attached to a Gilson Minipuls 2 peristaltic pump (Gilson, WI, U.S.A.). Absorbance response of the model wine was increased by measuring the absorbance through a 10 mm path length instead of 1 mm for the red and white wines.

Absorbance measurements were made at 280, 420, and 520 nm for phenolic, browning, and red color measurements respectively. The absorbance measured at 280 nm gave an estimate of the concentration of total phenolics, and in white wine, an indication of the amount of brown pigments. Absorbance at 420 nm estimated the concentration of yellow/brown pigments present in the wine whereas absorbance at 520 nm in the red wine estimated the concentration of red-colored anthocyanins (Iland et al. 2004). Color density ($A_{520} + A_{420}$) describes the intensity of color (red

and yellow/brown pigments). Total phenolics included the concentration of all phenolics present in wine.

2.3.4 Calcium, copper, iron and potassium assay

Quantification of soluble metals was determined by atomic absorption spectrometry (AAS) (Perkin-Elmer Corp., Model 5100 PC, Norwalk, CT) and Inductively Coupled Plasma Atomic Emission Spectrometry (ICP-AES) (Perkin-Elmer Corp., Model ICP/6500, Norwalk, CT) in an air/acetylene oxidizing flame according to the method of Clesceri et al. (1998) for water and waste water with modifications as noted. Cu, Fe, and K were analyzed by AAS and Ca was determined by ICP-AES with vacuum spectrometer against water/alcohol standard solutions. The sample uptake was performed with a 16 x 100 mm autosampler tube. Determination of Ca occurred at a wavelength of 315.8 nm by ICP-AES. Determination of Cu and Fe by AAS occurred at 324.7 nm, slit 0.7L; 248.3 nm, slit 0.2H respectively in absorbance mode (abs), and K at 766.4 nm wavelength in emission mode (EM). The cathode lamp current for all analyses was set to 25 mA. Results were recorded as mg/L of analyte to three significant digits.

Some authors recommend first destroying the organic matter of wine (Ough et al. 1982), including alcohol (OIV), followed by determination against aqueous standards in an air/acetylene oxidizing flame, with deuterium lamp background correction at 213.9 and 248.3 nm, respectively (Costa et al. 2000). In the case of must, dry ashing or wet ashing is recommended, since sugars interfere in the quantification of metals (Puig-Deu et al. 1994). Since these samples were dry, no

ashing was performed. Also, due to linear response among the standards, alcohol was not removed from the wine.

2.3.5 Statistical and data analysis

Samples measurments by atomic absorption, UV spectrophotometer, pH and titratable acidity were prepared and analyzed in triplicate and the results averaged. Data are expressed as means ± SD before analysis of variance (ANOVA) using the Statistical Analysis System PROC GLM procedure (release 8.2; SAS Institute, Cary, NC). Treatment means were separated by the least significant difference test (LSD) at the 5% significance level.

2.4 Results and discussion

2.4.1 Precision

2.4.1.1 Metal and mineral analysis

Metal concentration was determined by the linear relationship plot (curve of best fit) of emission or absorption intensity versus the concentration of the standards. Results are reported to three significant digits on a percentage mg/L basis. Standard curves for metals were obtained with ≥ 0.999 (r^2) linear correlation (Beer's law) of integration response over the concentration range. The detection limit for Ca was 0.5 mg/L, and for Cu, Fe, and K it was 0.05 mg/L respectively. Reproducibility was within 5% for all analytes.

2.4.1.2 UV absorbance

Instrument precision was evaluated by performing 10 replicate absorbance spectra at at each wavelength for each wine type. The coefficients of variation (CV) (Table 2.4), indicate an acceptably high level of analytical reproducibility. The model wine had relatively high CV values due to minimal absorbance at 280 and 420 nm, essentially equivalent to the water blank. Similarly white wine shows very little absorbance at 420 nm accounting for the relatively high CV values at that wavelength. Model wine samples containing PA at concentrations ranging between 5 and 1,000 mg/L had absorbance spectra that were not significantly different than the model wine solution without PA at 280, 420, and 520 nm.

Table 2.4 Instrument precision coefficients of variation (%) in absorbance spectra.

	Model wine			**White wine**			**Red wine**		
nm	**Mean (abs)**	**SD**	**CV (%)**	**Mean (abs)**	**SD**	**CV (%)**	**Mean (abs)**	**SD**	**CV (%)**
280	0.0002	0.0031	1554.84	0.4588	0.0003	0.07	2.6444	0.0408	1.54
420	0.0003	0.0002	58.74	0.0045	0.0003	5.77	0.4102	0.0003	0.06
520							0.6068	0.0002	0.03

2.4.2 Metals

Phytic acid treatment followed by addition of calcium carbonate at a molar ratio of 5:1 (Ca:PA) was effective at lowering the concentration of a model wine containing 20 mg/L Fe at all Fe:PA molar ratios tested. At a molar ratio of 6:1 (Fe:PA), 63% of the Fe was removed, while at 2:1, >95% of Fe was removed and >97% removed at 1:1 (Fe:PA) molar ratio (Table 2.5). At a concentration of 5 mg/L

Fe, a 1:1 (Fe:PA) molar ratio reduced Fe by 91% and was more effective at higher Fe concentrations (Table 2.6). In all cases, including trials in a red and a white wine (Table 2.7), a Fe:PA molar ratio of 1:1, which is equivalent to 11.82 mg PA per 1 mg Fe/L, followed by calcium carbonate treatment, reduced the iron content below 2 mg/L, more than 50% below the 4 mg/L minimum concentration considered safe for the prevention of iron catalyzed wine instabilities (Ough et al. 1982). The pH of the model wine solution had only slight effect on the efficacy of PA at reducing iron content (Table 2.8). The pH tended to increase with increasing $CaCO_3$ treatment doses, while TA tended to decrease. Decreasing the ratio of calcium used to coprecipitate the metal-phytate complex might mitigate the changes in acidity. In all cases the calcium content increased which could be cause for calcium tartrate (CaT) stability issues. The significant rise in calcium concentration indicates excess calcium used in the reaction and similar to moderating the changes in acidity, might be reduced by decreasing the Ca:PA molar ratio used to coprecipitate the metal-phytate complex, which was not investigated in this study. The red wine showed the highest increase in Ca, suggesting that red wine components may compete in precipitating iron-phytate complexes. Phytic acid-calcium treatments did not significantly affect potassium ion concentration (Table 2.5), which is not surprising given its low valency. Similarly, at the low pH and low redox potential of the wine solutions, copper ions (Table 2.9) were not influenced by PA-Ca treatments. Phytic acid might have an effect if Cu(II) is first oxidized to Cu(III), however, the oxidation process may adversely affect the wine.

Table 2.5 Affect of various molar ratios of PA in a model wine containing 20 mg/L Fe.

Molar ratio Fe:PA (mM)	Phytic acid (mg/L)	Phytic acid (mM)	pH	Diff. from Fe:PA 0	TA[a] (g/L)	Diff. from Fe:PA 0	Ca (mg/L)	Diff. from Fe:PA 0	Fe (mg/L)	Diff. from Fe:PA 0	K (mg/L)	Diff. from Fe:PA 0
0	0	0	3.55cd[b]	0.00ab	1.04a	0.00f	<0.5g	<0.5g	20.12a	0.00e	407a	0a
1	236.4	0.358	3.66a	0.11d	0.86e	-0.18b	43.7a	43.7a	0.57e	-19.55a	403a	-4a
2	118.2	0.179	3.60b	0.05c	0.85f	-0.19a	26.9b	26.9b	0.95e	-19.17a	404a	-3a
3	78.8	0.119	3.58bc	0.03bc	0.85ef	-0.19ab	18.5c	18.5c	2.36d	-17.75b	405a	-2a
4	59.1	0.090	3.56c	0.01b	0.96b	-0.08e	13.9d	13.9d	2.94d	-17.17b	402a	-5a
5	47.3	0.072	3.55cd	0.00ab	0.94c	-0.10d	10.6e	10.6e	4.46c	-15.66c	403a	-4a
6	39.4	0.060	3.52d	-0.03a	0.93d	-0.11c	7.1f	7.1f	7.41b	-12.71d	401a	-6a
Average:			3.54	-0.01	0.94	-0.10	10.53	10.53	4.94	-15.18	402	-5
SD:			0.02	0.02	0.01	0.01	3.40	3.40	2.27	2.27	1	1

[a] Titratable acidity (TA) expressed as g tartaric acid per liter titrated to pH 8.2

[b] Means with the same letter within a column are not significantly different ($p \leq 0.05$).

Table 2.6 Affect of PA at 1:1 Fe:PA (mM) in a model wine with various concentrations of Fe.

Fe (mg/L)	pH	Diff. from Fe = 0	TA[a] (g/L)	Diff. from Fe = 0	Ca (mg/L)	Diff. from Fe = 0	Fe 0[b] (mg/L)	Fe 1[c] (mg/L)	Diff.
0	3.55 c[d]	0.00 c	1.04 a	0.00 a	<0.5 d	0.00 d	0.00	0.00	0.00
5	3.59 b	0.04 b	0.84 d	-0.20 b	18.1 c	18.1 c	5.08 c	0.47 a	-4.61 a
10	3.59 b	0.04 b	0.85 c	-0.19 c	25.4 b	25.4 b	10.21 b	0.35 a	-9.86 a
20	3.66 a	0.11 a	0.86 b	-0.18 d	35.4 a	35.4 a	20.12 a	0.57 a	-19.6 b
Average:	3.60	0.05	0.90	-0.14	26.3	19.7	11.8	0.46	-11.3
SD:	0.05	0.05	0.10	0.10	8.7	14.9	7.65	0.11	7.58

[a] Titratable acidity (TA) expressed as g tartaric acid per liter titrated to pH 8.2

[b] Before treatment with PA and Ca.

[c] After treatment with PA and Ca.

[d] Means with the same letter within a column are not significantly different ($p \leq 0.05$).

Table 2.7 Affect of PA at 1:1 Fe:PA (mM) in a red and a white wine with various concentrations of Fe.

Wine	Fe (mg/L)	pH 0[a]	pH 1[b]	Diff.	TA 0 (g/L)	TA 1 (g/L)	Diff.	Ca 0 (mg/L)	Ca 1 (mg/L)	Diff	Fe 0 (mg/L)	Fe 1 (mg/L)	Diff.
W	0	3.63a[d]	3.63a	0.00d	3.51d	3.51d	0.00a	42.1a	42.1d	0.0d	0.17d	0.17d	0.00a
W	5	3.61b	3.50d	-0.11a	3.60c	3.60c	0.00a	42.1a	64.1c	22.0c	4.64c	1.26c	-3.39b
W	10	3.60c	3.59c	-0.01b	3.62b	3.62b	0.00a	42.0a	74.7b	32.6b	8.27b	1.65a	-6.61c
W	20	3.58d	3.62b	0.04c	3.68a	3.66a	-0.02b	42.0a	100.0a	58.1a	15.27a	1.41b	-13.86d
Average:		3.61	3.59	-0.02	3.60	3.60	0.00	42.04	70.22	28.18	7.09	1.12	-5.96
	SD:	0.02	0.06	0.06	0.07	0.06	0.01	0.06	24.07	24.13	6.38	0.66	5.91
R	0	3.44b	3.44d	0.00a	5.33c	5.33a	0.00a	76.9a	76.9d	0.0d	0.98d	0.98a	0.00a
R	5	3.46a	3.45c	-0.01b	5.22d	5.73a	0.52a	76.7a	103.1c	26.4c	6.75c	0.50d	-6.25b
R	10	3.43c	3.47b	0.04c	5.45b	5.35a	-0.10a	76.2a	129.5b	53.3b	20.35a	0.70b	-19.64d
R	20	3.43c	3.49a	0.05d	5.51a	5.79a	0.29a	76.3a	172.6a	96.3a	17.55b	0.57c	-16.98c
Average:		3.44	3.46	0.02	5.38	5.55	0.17	76.5	120.5	44.0	11.41	0.69	-10.72
	SD:	0.01	0.02	0.03	0.13	0.25	0.28	0.3	40.8	41.1	9.09	0.22	9.20

[a] Before treatment with PA and Ca.

[b] After treatment with PA and Ca.

[c] Titratable acidity (TA) expressed as g tartaric acid per liter titrated to pH 8.2

[d] Means with the same letter within a column are not significantly different ($p \leq 0.05$).

Table 2.8 Affect of PA at 5:1 molar ratio (mM) Fe:PA in model wines with differing pH (Fe = 20 mg/L).

pH 0[a]	**pH 1**[b]	**Diff.**	**TA**[c] **0 (g/L)**	**TA 1 (g/L)**	**Diff.**	**Ca 0 (mg/L)**	**Ca 1 (mg/L)**	**Diff.**	**Fe 0 (mg/L)**	**Fe 1 (mg/L)**	**Diff.**
3.75	3.53	-0.22a[d]	0.78	0.87	0.09 a	<0.5	7.6	7.6b	20.00a	4.61a	-15.39 a
3.55	3.55	0.00c	1.04	0.94	-0.10 b	<0.5	10.6	10.6a	20.12a	4.46a	-15.66 a
3.22	3.13	-0.09b	1.12	1.01	-0.11 b	<0.5	10.8	10.8a	20.00a	3.86b	-16.14 b
Average:						<0.5	9.67	9.67	20.04	4.31	-15.73
SD:						0.00	1.78	1.78	0.07	0.40	0.38

[a] Before treatment with PA and Ca.

[b] After treatment with PA and Ca.

[c] Titratable acidity (TA) expressed as g tartaric acid per liter titrated to pH 8.2

[d] Means with the same letter within a column are not significantly different ($p \leq 0.05$).

Table 2.9 Affect of PA in a model wine with differing Cu.

Cu (mg/L)	PA (mM)	Ratio PA:Cu (mM)	pH	Diff. from PA:Cu 0	TA[a]	Diff. from PA:Cu 0	Ca (mg/L)	Diff. from PA:Cu 0	Cu (mg/L)	Diff. from PA:Cu 0
1.00	0	0.00	3.55b[b]	0.00b	1.04b	0.00e	<0.5g	<0.5g	0.82de	0.00b
1.00	0.0157	1.00	3.61a	0.06a	0.90d	-0.14c	1.2e	1.2e	0.83cd	0.01b
0.25	0	0.00	3.55b	0.00b	1.04b	0.00e	<0.5g	<0.5g	0.21bc	0.00b
0.25	0.0039	1.00	3.55b	0.00b	0.85e	-0.19b	1.8d	1.8d	0.21ab	0.00b
0.50	0	0.00	3.55b	0.00b	0.85e	-0.19b	<0.5g	<0.5g	0.41e	0.00b
0.50	0.0079	1.00	3.54b	-0.01b	0.90d	-0.14c	0.5f	0.9f	0.41de	0.01b
0.50	0.0157	2.00	3.61a	0.06a	0.77f	-0.27a	2.7c	2.9c	0.42abc	0.01c
0.50	0.0315	4.00	3.60a	0.05a	0.92c	-0.12d	5.6b	5.9b	0.43a	0.02c
[c]0.50	0	0.00	3.55b	0.00b	1.04b	0.00e	<0.5g	<0.5g	0.41de	0.00b
[c]0.50	0.1869	1.00	3.61a	0.06a	1.16a	0.12f	22.5a	22.4a	0.35f	-0.06a
Average:				0.02		-0.09				0.00
SD:				0.03		0.12				0.02

[a] Titratable acidity (TA) expressed as g tartaric acid per liter titrated to pH 8.2

[b] Means with the same letter within a column are not significantly different ($p \leq 0.05$).

[c] Copper in the presence of 10 mg/L Fe. PA addition for Fe + Cu on 1:1 ratio (mM). After treatment Fe = 0.2 mg/L.

2.4.3 Color

In white wine, iron significantly increased absorbance at 280 nm, and the increasing yellow intensity was perceptible even to the unaided eye (Table 2.10). After PA and Ca treatment the wine showed absorbance that was not significantly different from the control wine without the addition of iron except for iron = 5 mg/L, which was likely an outlier although the trend was consistent. At 420 nm there were

significant increases in absorbance with increasing iron content. After PA treatment, there was no significant difference in absorbace after treatement when compared to the control. These results indicate that PA treatment followed by the additions of Ca can eliminate the color effects at 280 and 420 nm due to iron in white wines.

In red wine, iron did not significantly increase absorbance at 280 nm, however there were some small but significant differences at 420 and 520 nm (Table 2.11) and among color density and hue measurements (Table 2.12). Overall, the differences were small and may be related to the slight changes in pH after treatment.

There were no significant differences among the replicates within a treatment group for both red and white wines.

Table 2.10 White wine absorbance at 280 and 420 nm.

Fe (mg/L)	A_{280} 0[a]	A_{280} 1[b]	Diff. from Fe = 0 (before PA)	A_{420} 0	A_{420} 1	Diff. from Fe = 0 (before PA)
0	0.44972d[c]	0.44759a	0.0021b	0.00455c	0.00594a	-0.0014a
5	0.47233c	0.43836b	0.0114a	0.00703b	0.00413a	0.0004a
10	0.48080b	0.44941a	0.0003b	0.00862a	0.00629a	-0.0017a
20	0.51035a	0.44546a	0.0043b	0.00903a	0.00468a	-0.0001a

[a] Before treatment with PA and Ca.

[b] After treatment with PA and Ca.

[c] Means with the same letter within a column are not significantly different ($p \leq 0.05$).

Table 2.11 Red wine absorbance at 280, 420, and 520 nm.

Fe (mg/L)	A_{280} 0[a]	A_{280} 1[b]	Diff. from Fe = 0 (before PA)	A_{420} 0	A_{420} 1	Diff. from Fe = 0 (before PA)	A_{520} 0	A_{520} 1	Diff. from Fe = 0 (before PA)
0	2.57237a[c]	2.56500b	0.0074a	0.39386b	0.38842a	0.0054b	0.58069bc	0.59149a	-0.0108c
5	2.66740a	2.62349a	-0.0511ab	0.39596b	0.37403b	0.0198a	0.58871b	0.56596c	0.0147a
					a				
10	2.63378a	2.59641b	-0.0240a	0.40487a	0.38232b	0.0115ab	0.60068a	0.57526bc	0.0054ab
20	2.63677a	2.67396a	-0.1016b	0.39183b	0.38962a	0.0042b	0.57867c	0.58384ab	-0.0032bc

[a] Before treatment with PA and Ca.

[b] After treatment with PA and Ca.

[c] Means with the same letter within a column are not significantly different ($p \leq 0.05$).

Table 2.12 Red wine color density and hue in red wine before and after treatment with PA.

Fe (mg/L)	Color density[a] 0[b]	Color density 1[c]	Diff. from Fe = 0 (before PA)	Hue[d] 0	Hue 1	Diff. from Fe = 0 (before PA)
0	0.97455b[e]	0.97991a	-0.0054c	1.47445c	1.52281a	-0.0484c
5	0.98467b	0.93999c	0.0346a	1.48679a	1.51318ab	-0.0387bc
10	1.00555a	0.95757bc	0.0170ab	1.48371ab	1.50470bc	-0.0302ab
20	0.97051b	0.97346ab	0.0011bc	1.47687bc	1.49853c	-0.0241a

[a] Color density = ($A_{520} + A_{420}$).

[b] Before treatment with PA and Ca.

[c] After treatment with PA and Ca.

[d] Color hue = (A_{520}/A_{420}).

[e] Means with the same letter within a column are not significantly different ($p \leq 0.05$).

Contrary to Joslyn and Lukton (1953) and Capt (1955), phytic acid, when coprecipitated with calcium, was highly effective in reducing iron in wine. Phytic acid was added in an amount that forms a soluble chelate with polyvalent cations present in wine, such as iron and calcium. It was added to wine in a molar ratio of 1:1 (PA:Fe), which corresponds to 0.018 mM phytic acid or 11.8 mg/L or 0.0012% w/v phytic acid or 0.0024% w/v of 50% w/v aqueous phytic acid for each 1 mg/L of iron present. Subsequently calcium should be added in a sufficient amount to co-precipitate the iron-phytate complex quantitatively, at a molar ratio of ≤ 5:1 (Ca:PA), which corresponds to 9.0 mg/L $CaCO_3$ or 0.0009% $CaCO_3$ (which provides a calcium concentration of 3.6 mg/L Ca^{2+}) for each 1 mg/L of iron.

The additions of phytic acid and calcium salt was made sequentially to allow for complete chelation of polyvalent cations by phytic acid before co-precipitation by calcium. 118.2 mg phytic acid is able to chelate 10 mg/L iron to form a 1:1 iron-phytate chelate, however, in effect may chelate less iron due to the presence of other polyvalent cations in the wine. Calcium carbonate was used as the preferred calcium salt in order to counteract the acidifying effect of phytic acid, although due to the increase in Ca, slight increase in pH, and decrease in TA, trials with molar ratios < 5:1 (Ca:PA) should be investigated. Any other calcium salt may be employed, such as calcium sulfate. After treatment with phytic acid and calcium carbonate, the wine is then held for some time (from a few hours up to 7 days) to allow for completion of precipitation; it was then filtered and may be suitable for bottling.

Phytic acid and calcium carbonate treatments did not affect copper or potassium concentrations, and only slightly affected color in red wines, while

reducing yellow color absorbance at 280 nm in the white wine. Acidity was decreased slightly, but may be mitigated by slightly reducing the concentration of the $CaCO_3$ treatment after addition of PA.

The advantages of this novel method over other methods include its low cost, the safety of its industrial use, the absence of hazardous wastes during processing, the toxicological safety of the treated wine for human consumption, and the efficacy to remove iron from wine. The treatment could be improved by reducing the amount of calcium added to produce no significant effect on pH, color, and residual calcium content. Simple bench trials using three experienced wine tasters did not notice aroma or flavor impairment after PA-Ca treatment in wines containing no iron. In wines containing 20 mg/L Fe, flavor was improved after PA-Ca, however, the effects on both the sensory perception and the long-term stability of wines treated with PA-Ca need to be determined under actual winemaking conditions and with controlled sensory investigations.

The phytic acid content of grapes, musts and wines has not been well explored and there is a need for a quantitative residual phytic acid analysis after treatment to confirm that phytic acid is completely reacted and precipitated. Additionally, determining the rate of metal-phytate reactions under various wine conditions and the extent of potential metal-phytate-protein interactions would be useful. Phytic acid is not currently approved (OIV) for use in wine and may require petition to regulatory bodies before its use therein.

2.4.4 Conclusions

A method for selective removal of heavy metals from wine and other beverages includes adding phytic acid to the beverage in an amount sufficient to form a chelate with the polyvalent cations present in wine, storing the treated wine for a period of time sufficient to chelate all of the iron and other polyvalent cations, subsequently treating the beverage with a calcium salt in an amount sufficient to co-precipitate the metal-phytate complexes, storing the treated wine for a period of time sufficient to allow for complete metal precipitation, and removing the insoluble metal sludge through microfiltration or by means of any other form of separation.

The method relies on several useful but independent chemical properties of phytic acid and its interactions with metals. Exploitation of these effects in the described method effects almost complete iron removal from wine and additional protection against oxidative catalytic activity of any residual iron. Furthermore, unlike blue fining and other chelation methods described elsewhere, the proposed sequential addition of phytic acid and calcium requires no knowledge of the exact concentration of iron and other metals and of the exact volume of wine inside the tank - the efficacy of the method is premised on the excess of phytic acid relative to iron present in wine, and on the molar ratio of calcium to phytate (5:1). This ratio assures complete precipitation of the mixed metal salt of phytic acid independent of the amount of iron, and it also prevents the accumulation of any added calcium.

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CHAPTER III

WINE STABILIZATION WITH PHYTIC ACID: II. CALCIUM TARTRATE

3.1 Abstract

An alternative method of calcium tartrate (CaT) stabilization for a model wine was investigated at laboratory scale using phytic acid (PA), a form of phosphorus storage in plants. Treatments with increasing doses of PA at concentrations ranging between Ca:PA molar ratios of 1:1 to 10:1, did not result in significant reductions of Ca in a model wine containing 74.7 mg/L (1.86 mM) Ca as determined by atomic emission spectroscopy (ICP-AES). Phytic acid did prevent CaT precipitation in all samples to which it was added and may be effective at concentrations lower than 123 mg/L (0.187 mM), the lowest concentration of PA used in this study. The control model solution without PA showed significant visible precipitation after just two days at room temperature, while none of the samples containing phytic acid showed any precipitation after 1 month, indicating that PA is effective at keeping CaT soluble. However, the effects on both the sensory perception and the long-term stability of wines treated with phytic acid need to be determined under actual winemaking conditions.

3.2 Introduction

Wines contain 2 – 8 g/L or more tartaric acid, 0.3 – 1.4 g/L potassium, and 0.05 – 0.13 g/L calcium (Ough and Amerine 1988). The amounts of potassium, calcium, and tartrate in a berry are both dependent on the cultivar, climate, and degree of maturity (Berg and Keefer 1958; Berg and Keefer 1959). Tartaric acid is a weak organic acid that in the ripening fruit decreases in concentration as dissociated mono and dibasic (tartrate and bitartrate) salts form. It can precipitate as two main salts: potassium bitartrate (KHT) and sparingly soluble calcium tartrate (CaT). Potassium bitartrate precipitation is well documented and the most common in wines, however, many wines naturally contain calcium concentrations near to instability. At a tartaric acid concentration of 6 g/L, the recommended maximum calcium content in a white wine at pH 3.5 would be approximately 0.03 g/L (Berg and Keefer 1959; Boulton et al. 1996), as determined by their concentration products (CP). Additional calcium can come from calcium salts used to treat wines (Clark et al. 1988) or through dissolution from concrete tanks (Boulton et al. 1996). Calcium tartrate solubility decreases with decreasing temperature, and increasing ethanol content (Berg and Keefer, 1959), but does not enhance crystallization. Wine pH has little, if any, effect on the rate of precipitation, only on the final equilibrium concentration (Clark et al. 1988). Calcium tartrate is a concern because conditions are not easily attained for its stabilization and in some instances it may precipitate after bottling (Steele and Kunkee 1979). Low temperature stability tests and treatments for potassium bitartrate are ineffective for calcium tartrate (Parfent'eva et al. 1984; Postel 1983; Postel et al. 1984). A wine considered stable with respect to potassium bitartrate might be unstable with respect to calcium tartrate. Although not a health concern, if CaT precipitates after bottling

the wine is usually recalled and the precipitate removed to prevent cosumer complaint.

There are two general types of tartrate salt stabilization techniques for wines, physical and chemical. Of the physical stabilization methods, cooling, even to the freezing point, is ineffective for calcium tartrate stabilization. Chilling increases the level of potassium bitartrate and calcium tartrate supersaturation in wine, however, not enough to induce formation of calcium tartrate crystals, unlike temperature dependent crystallization of potassium bitartrate (Berg and Keefer 1959; De Soto and Yamada 1963; Pilone and Berg 1965; Parfent'eva et al. 1984). Calcium tartrate precipitation by seeding with non racemic Ca L(+)tartrate is rapid and effective at lowering calcium concentration (Abguéguen and Boulton 1993). Calcium tartrate is permitted for use in wine at up to 2 g/L (OIV 2005). Electrodialysis can be used to remove the potassium and calcium cations and the tartrate anions from wine. It has the benefit of removing only K(I), Ca(II), and tartrate from wine and does not impact other wine components such as polyphenols, polysaccharides, or volatile flavor compounds (Escudier 2002; Eurodia 2005). However, there are several disadvantages with physical methods including high energy costs required for chilling and seed methods, or high initial capital and continued maintenance costs associated with electrodialysis treatment.

Chemical components that are naturally present, or are added to a wine can impact nucleation and the rate of tartrate salt crystallization and precipitation. Red wines contain a much higher concentration of polyphenols and pigments than white wines and therefore contribute a higher holding capacity for tartrate salts than white or model wines. As pigment polymerization occurs through aging, the holding

capacity of tartrate declines and may result in delayed CaT or KHT precipitation (Balakian and Berg, 1968). Other wine components may also act to inhibit tartrate crystallization including metals, sulfates, proteins, and colloids such as pectins, glucans, among other polysaccharides (Pilone & Berg, 1965). Sweet wines and those infected by Botrytis cinerea precipitate more slowly than dry wines. Inhibition may come from component complexes with tartrate ions, or with tartrate crystal surface adsorption. Chemical inhibitors may be an alternative to physical methods to prevent tartrate formation, although more often attempts are made to remove these complexing agents (by fining and/or filtration) to facilitate crystallization and subsequent precipitation to achieve stabilization. Some of the additives that, to varying degrees, inhibit calcium tartrate salt crystal growth include carboxylic acids such as citrate, malate, phosphate, and sulfate (Postel et al. 1984; McKinnon et al., 1995; McKinnon et al. 1996); and polysaccharides such as arabinose, pectins (Kohn 1975; McKinnon et al., 1995; McKinnon et al. 1996), and carboxymethylcellulose (Clutton 1974; Stocké and Görtges 1989a,b). Ferrocyanide preparations, while effective at chelating iron and other metal cations, are ineffective for chelating potassium or calcium ions (Joslyn and Lukton 1953).

Phosphate is known to effectively chelate calcium (Wise 1986). McKinnon et al. (1995) showed that phosphate had a slight inhibitory effect on the induction period and the crystallization rate of CaT in model wines, however concluded this was due to variations in ionic strength since at wine pH, binding between calcium and phosphate did not occur. The phosphate content of wine normally varies between 0.05 to 0.9 g PO_4/L, with an average value of 0.29 g/L (Ough and Amerine 1988). Some of that phosphate content may come from the phytic acid contained in the seed (Lott et

al. 2000). Phytic acid (PA) or myo-inositol, hexakis (dihydrogen phosphate) is a strong chelating agent and antioxidant present in all seeds (Graf 1986). Phytic acid contains six orthophosphate moieties with 12 dissociable protons and therefore it has a high chelation potential for polyvalent cations over a wide range of pH values (De Stefano et al. 2003) making it a suitable candidate for calcium chelation in wines (Trela and Graf 2005a,b). All metal1-phytate complexes are very soluble at any pH, while metal-phytate complexes containing 3 or more cations are extremely insoluble (Graf and Eaton 1984). A few examples of such insoluble chelates include (Ca^{2+})3-phytate, (Ca^{2+})4-phytate, (Ca^{2+})5-phytate, (Ca^{2+})6-phytate, etc. Phytic acid's chelation potential (Maddaiah 1964; Vohra 1965; Graf and Eaton 1984; Graf 1986; Wise 1986; Vasca et al. 2002; Trela and Graf 2005a,b) and application in foods is well documented (Graf and Eaton 1990; Lee and Hendricks 1995; Lee and Hendricks 1997; Lee et al. 1998), including iron chelation (Trela and Graf 2005a,b; Trela 2006a, submitted for publication), and protein stabilization in wine (Trela 2006b, submitted for publication).

The reported use of phytic acid, and calcium phytate, in wine has been limited. Previous studies focused primarily on the metal chelation potential of calcium phytate (Joslyn and Lukton 1953; Capt 1955), and more recently with phytic acid (Trela and Graf 2005a,b; Trela 2006a, submitted for publication), however Joslyn and Lukton (1953) recommended against phytic acid use in wine claiming it was no more effective than calcium phytate. The present method using phytic acid, which is known to precipitate after reaction with calcium ions, was investigated as a stabilizing agent for CaT in a model wine. Phytic acid provides a novel means for selective stabilization of CaT in beverages such as wines and fruit juices. The method

did not significantly bind and precipitate calcium once Ca formed salts with tartrate, however it does appear to inhibit CaT precipitation and produces no toxicologically objectionable products even in the case of over clarification.

3.3 Materials and methods

3.3.1 Sample preparation

Samples were prepared using a model solution made similar in composition to a dry table wine. The model solution, which contained 2.00 g/L KHT and 12.5% (v/v) ethanol, had a pH of 3.55 and an acidity of 1.04 g/L titratable acidity expressed as tartaric acid to pH 8.2. Calcium carbonate (334.7 g of 97% $CaCO_3$) dissolved in a minimum amount of concentrated HCl, was added on a volumetric basis to the model wine solution. The resulting filtered solution contained 72.4 mg/L of calcium had a pH of 3.48 and a titratable acidity of 0.73 g/L after 14 days at room temperature when treatments with PA began. Distilled, deionized (18.6 mΩ · cm and 3 ppb total organic carbon [TOC]) ultrapure water (Millipore Synthesis A10 with Quantum EX ultrapure cartridge, MA, U.S.A.) was used to make all solutions. All containerware and reagent bottles were triple rinsed with 0.1 N HCl followed by triple rinsing with water. Samples were stored in 0.5 L bottles at room temperature.

Treatments were performed with various amounts of phytic acid ($C_6H_{18}O_{24}P_6$, CAS 83-86-3, 50% w/w, Sigma-Aldrich, MO, U.S.A) ranging between Ca:PA molar ratios of 1:1 to 10:1. Phytic acid concentrations before and after sample treatments were not measured in this study, although phytic acid analysis is possible using

various methods (Talamond et al. 1998; Israel et al. 2006). After one week, all samples were sterile filtered through 4 mm poly(tetrafluoroethylene) (PTFE) 0.45 μm pore size syringe filters before analysis. All samples were stored under nitrogen gas.

3.3.2 Titratable acidity and pH analyses

Wine pH was measured with a pH meter (Accumet 925, Fisher Scientific, PA., U.S.A.; and Orion 81-72 ROSS™ Sure-Flow pH Electrode, Thermo Electron Corporation, MA., U.S.A.). Titratable acidity (tartaric acid in g/L) was measured by placing 10 mL of wine sample into 100 mL deionized water, the solution was degassed under vacuum, and titrated with 0.01 N NaOH to an end point of pH 8.2.

3.3.3 Calcium assay

Quantification of soluble calcium was determined by Inductively Coupled Plasma Atomic Emission Spectrometry (ICP-AES) (Perkin-Elmer Corp., Model ICP/6500, Norwalk, CT) with vacuum spectrometer at 315.8 nm wavelength against water/alcohol standard solutions according to the method of Clesceri et al. (1998). The sample uptake was performed with a 16 x 100 mm autosampler tube. The cathode lamp current was set to 25 mA. Results were recorded as mg/L of analyte to three significant digits. Due to linear response among the standards, alcohol was not removed from the wine before analysis.

3.3.4 Statistical and data analysis

Samples measurments by atomic absorption, UV spectrophotometer, pH and titratable acidity were prepared and analyzed in triplicate and the results averaged.

Data are expressed as means ± SD before analysis of variance (ANOVA) using the Statistical Analysis System PROC GLM procedure (release 8.2; SAS Institute, Cary, NC). Treatment means were separated by the least significant difference test (LSD) at the 5% significance level.

3.4 Results and discussion

Metal concentration was determined by the linear relationship plot (curve of best fit) of emission intensity versus the concentration of the standards. Results are reported to three significant digits on a percentage mg/L basis. Standard curves for Ca were obtained with ≥ 0.999 (r^2) linear correlations (Beer's law) of integration response over the concentration range. The detection limit for Ca was 0.5 mg/L. Reproducibility was within 5%.

After the addition of 0.003 M calcium to the model wine solution (0.01 M total tartarate), precipitation consistent with CaT crystalization was observed and explains the 57% difference between the Ca added and the Ca remaining in the model wine after two weeks. The average Ca content among replicates within all PA treated model wines was 72.2 ± 0.7mg/L, which is well within ± 5% of the original Ca concentration of 72.4 mg/L, showing that phytic acid had no significant removal effect on calcium after it formed CaT (Table 3.1). A calcium concentration of 74.7 mg/L at a pH of 3.48 is 40% more than the 30 mg/L recommended by Berg and Keefer (1959), when ignoring the difference in TA, 0.74 g/L in this study versus 6 g/L.

Treatment with PA significantly decreased the pH and increased the TA in all treatments. The significant changes in acidity could be expected to be less pronounced in wines with more buffering capacity, however, interactions with other wine components may also change the stabilization potential of PA on CaT, particularly if the wines contain metals such as iron, or proteins, which are both known to react with PA (Trela 2006a,b, submitted for publication).

Table 3.1 Affect of PA on CaT in a model solution.

Molar Ratio Ca:PA (mM)	**PA (mg/L)**	**PA (mM)**	**pH**	**Diff. from PA = 0 (mg/L)**	**TA[a] (g/L)**	**Diff. from PA = 0 (mg/L)**	**Ca (mg/L)**	**Diff. from PA = 0 (mg/L)**
∞	0.0	0.0	3.48a[b]	0.00i	0.73i	0.00i	72.4b	0.0d
10.0	123.5	0.187	3.36b	-0.12h	0.92h	0.19h	71.5e	-1.0a
8.7	141.2	0.214	3.34c	-0.14g	1.03d	0.30d	72.2cde	-0.2bcd
7.5	164.7	0.250	3.29d	-0.19f	1.00f	0.27f	71.9bcd	-0.6abc
6.2	197.6	0.299	3.27e	-0.21e	0.94g	0.21g	72.3bc	-0.1cd
5.0	247.0	0.374	3.23f	-0.25d	1.02e	0.29e	72.2bc	-0.2cd
3.7	329.4	0.499	3.15g	-0.33c	1.09c	0.36c	71.8de	-0.7ab
2.5	494.1	0.749	3.08h	-0.40b	1.42b	0.69b	73.7a	1.3e
1.2	988.1	1.497	2.75I	-0.73a	1.88a	1.15a	71.6e	-0.8a
Average:			3.22	-0.26	1.11	0.38	72.2	-0.3
SD:			0.21	0.21	0.34	0.34	0.7	0.7

[a] Titratable acidity (TA) expressed as g tartaric acid per liter titrated to pH 8.2

[b] Means with the same letter within a column are not significantly different ($p \leq 0.05$).

While PA did not reduce the concentration of Ca, it did prevent CaT precipitation in all samples to which it was added and may be effective at concentrations lower than used in this study. The control model solution containing no PA showed significant visible precipitation after just two days at room temperature, while none of the samples containing PA showed any precipitation even after 1 month, indicating that PA is effective at preventing CaT crystalization. Phytic acid may be effective at binding and precipitating calcium ions, for example, in wines that were recently deacidified with calcium carbonates.

Determining the effect and rate of Ca-phytate reactions and CaT-phytate interactions, including spontaneous precipitation and calcium binding curves under various wine conditions including ionic strength, alcohol content, and temperature as per McKinnon et al. (1996) would be useful. There is a need to investigate the extent to which PA may be inhibitory to the crystallization kinetics of calcium tartrate and the extent of potential metal-phytate-protein interactions would be useful. Lastly, the effects on both the sensory perception and the long-term stability of wines treated with PA need to be determined under actual winemaking conditions. Phytic acid is not currently approved (OIV) for use in wine and may require petition to regulatory bodies before its use therein.

3.5 Acknowledgments

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CHAPTER IV

WINE STABILIZATION WITH PHYTIC ACID: III. PROTEIN

4.1 Abstract

An alternative method of standard protein (bovine serum albumin) stabilization for a model wine was investigated at laboratory scale using phytic acid (PA), a form of phosphorus storage in plants. Treatments with increasing doses of phytic acid resulted in exponential BSA reductions of >99.9% at PA:BSA molar ratios > 6.5:1 at a BSA concentration of 1,000 mg/L, and linear reduction at a BSA concentration of 200 mg/L. However, the effects on both the sensory perception and the long-term stability of wines treated with phytic acid need to be determined under actual winemaking conditions.

4.2 Introduction

The slow formation of protein hazes is a common instability in white wines and occasionally observed in blush wines. Wine proteins are a mixture of proteins derived from the grape and from microbial populations, particularly yeast, especially after autolysis. Yeast proteins (peptides <10,000 Da) released by yeast excretion and autolysis have been shown not to be a cause of protein instability in wine (Bayly and

Berg 1967), but may be involved in peptide tannin hazes (Boulton et al. 1996). Wine grape derived proteins have isoelectric points between 2.5 and 8.7 (Yokotsuka et al. 1977; Anelli 1977) and molecular weights that range between 20,000 to 50,000 Daltons (Somers and Ziemelis 1973).

The total concentration of proteins ranges between 20 – 800 mg/L in un-fined juice or wine depending on various conditions including varietal, climate, soil conditions, grape maturation, degree of berry infection, and processing conditions (Bayly and Berg 1967; Tyson et al. 1981; Murphey et al. 1989; Høj et al. 2001), including duration of yeast contact (Waters et al. 2005), however, usually range between 20 and 100 mg/L in red and white wines (Fukui and Yokotsuka 2003), with levels in white wines generally below 60 mg/L (Marchal et al. 1997, Ferreira et al. 2000).

The solubility of proteins and hence their stability in wine is a complex function of the physiochemical nature of the proteins, especially their isoelectric point (pI); the pH (Bayly and Berg 1967), temperature, ethanol content (Boulton et al. 1996), and the concentration of wine complexing components, including tannins, polyphenolics, metal ions (copper in particular), and detergent residues (Kean and Marsh 1957; Moretti and Berg 1965; Somers and Ziemelis 1973; Yokotsuka et al. 1983). Any of these factors can cause dissolved wine proteins to precipitate as a fine amorphous colorless haze or deposit.

Hazes in wine associated with proteins are usually complexes of proteins, polysaccharides and polyphenolic compounds. The total concentration of proteins does not correlate well with heat stability or haze formation (Bayly and Berg 1967), although wines that are stored under warm conditions can cause protein hazes to form

(Moretti and Berg 1965). Wine pH has a much larger effect on the solubility of various protein fractions and their relative stability in the wine (Bayly and Berg 1967) as determined by the pI of a protein. The pI of a protein is the pH at which the protein carries no net electrical charge. Proteins can be in the form of cations at low pH and anions at high pH, but are neutral at the isoelectric point. The smaller the difference between the juice or wine pH and the isoelectric point of the protein fraction, the lesser the net charge on that protein fraction and the lower the solubility of that fraction. Therefore, the isoelectric properties of proteins influence not only their tendency to precipitate according to the pH of the solution they are in, but also the efficacy of fining agents on their removal (Dawes et al. 1994). Some treatments include ultrafiltration, heat denaturation, tannin or silicon dioxide (Kieselsol), haze protective mannoproteins (Waters et al. 2005), and enzymes; however, the most common treatment is bentonite fining (Blade and Boulton 1998). Among these treatments some can significantly and negatively affect wine sensory attributes. Ultrafiltration undesirably removes phenolic components (Peri et al. 1988) and flavor (Flores et al. 1991). Bentonite in particular has the potential to reduce wine color, aroma and flavor components with large treatment amounts (Rankine 1989; Dawes et al. 1994; Main and Main 1994).

Phytic acid strongly interacts with proteins in a pH and cation-dependent manner (Cheryan 1980; Okubo et al. 1975). The phosphate content of wine normally varies between 0.05 to 0.9 g PO_4/L, with an average value of 0.29 g/L (Ough and Amerine 1988). Some of that phosphate content may come from the phytic acid contained in the seed (Lott et al. 2000). Phytic acid (PA) or myo-inositol, hexakis (dihydrogen phosphate) is a strong chelating agent and antioxidant present in all seeds

(Graf 1986; Lee and Hendricks 1995). Phytic acid contains six orthophosphate moieties with 12 dissociable protons and therefore it has a high chelation potential for polyvalent cations over a wide range of pH values (De Stefano et al. 2003). Phytic acid's chelation potential (Maddaiah 1964; Vohra 1965; Graf and Eaton 1984; Graf 1986; Wise 1986; Vasca et al. 2002; Trela and Graf 2005a,b) and application in foods is well documented (Graf and Eaton 1990; Lee and Hendricks 1995; Lee and Hendricks 1997; Lee et al. 1998), including iron and calcium stabilization in wine (Trela and Graf 2005a,b; Trela 2006a,b, submitted for publication). The complexation of phytic acid with proteins is well documented within cereals (Cheryan 1980; Reddy et al. 1982; Lott et al. 1985; Hídvégi and Lásztity 2002), and has been known to coagulate soybean proteins (Saio et al. 1969; Honig and Walter 1987), as well as modify the activity of enzymes, including increasing the activity of alcohol dehydrogenase (Altschuler and Schwartz 1984). Phytic acid can be degraded by phytase, which is found in most phytate-containing seeds (Graf 1986).

Phytic acid as a means to treat proteins in wines has not been previously explored. At pH values below the isoelectric point of proteins, the terminal amino, arginyl, histidyl, and lysyl groups can be positively charged and can form electrostatic complexes with negatively charged phytate anion (Cheryan 1980). If the steric conditions are suitable, phytic acid can interact with more than one charged group on a protein or proteins. In one study, the maximum binding of phytic acid to the protein gliadin occured in pH range 2.5–3.5, another protein glycinin precipitated over a pH range of 2.0-5.5 (Hídvégi and Lásztity 2002). At pH values above the isoelectric point of the protein, phytate-protein electrostatic interactions are significantly decreased and binding may not occur (Cheryan 1980; Hídvégi and Lásztity 2002).

Ternary phytate complexes with various solubilities can occur in the presence of proteins and multivalent cations such as metal ions (Tompson 1986) that forms a bridge between a negatively charged group of the protein and the phytate anion, allowing phytate-protein binding at neutral and high pH (Hídvégi and Lásztity 2002).

The reported use of phytic acid, and its calcium salt, in wine has been limited. Previous studies focused primarily on the metal chelation potential of calcium phytate (Joslyn and Lukton 1953; Capt 1955), and more recently with phytic acid (Trela and Graf 2005a,b; Trela 2006a,b, submitted for publication). The present method using phytic acid, precipitates after reaction with BSA and may provide a novel means for the treatment of proteins in beverages such as wines and fruit juices. The method binds and precipitates BSA in a model wine solution, and produces no toxicologically objectionable products even in the case of over clarification.

4.3 Materials and methods

4.3.1 Sample preparation

Samples were prepared using a model solution, which was similar in composition to a dry table wine. The model solution, which contained 2 g/L KHTa and 12% (v/v) ethanol, had a pH of 3.52 and an acidity of 1.0 and 2.0 g/L respectively of total and titratable acidity expressed as tartaric acid to pH 8.2. Distilled, deionized

(18.6 mΩ · cm and 3 ppb total organic carbon [TOC]) ultrapure water (Millipore Synthesis A10 with Quantum EX ultrapure cartridge, MA, U.S.A.) was used to make all solutions. Samples were prepared in ground glass stoppered volumetric flasks the evening before the evaluations were made. All samples were sterile filtered through 4 mm poly(tetrafluoroethylene) (PTFE) 0.45 μm pore size syringe filters before analysis.

4.3.2 Titratable acidity and pH analyses

Wine pH was measured with a pH meter (Accumet 925, Fisher Scientific, PA., U.S.A.; and Orion 81-72 ROSS™ Sure-Flow pH Electrode, Thermo Electron Corporation, MA., U.S.A.). Titratable acidity (tartaric acid in g/L) was measured by placing 10 mL of wine sample into 100 mL deionized water, the solution was degassed under vacuum, and titrated with 0.01 N NaOH to an end point of pH 8.2.

4.3.3 Protein

Bovine serum albumin (BSA), Sigma Chemical Co., was used as the test protein. It has an isoelectric point between 4.3 and 4.6 and a molecular weight of approximately 66,000 daltons. A stock solution of 1,000 mg/L BSA in model wine was made 1 week before dilution with model wine into the test concentrations.

4.3.4 Protein assay

Protein content was assayed by measuring the absorbance at 286 nm with a Hewlett-Packard (HP) (Palo Alto, CA) 8452A diode array spectrophotometer controlled by Agilent UV-vis Chemstation software (Windows NT) Rev. A.08.08[71]

through a 10 mm quartz cuvette. The protein concentration assay method differs from the method of Dawes et al. (1994) and Blade and Boulton (1998), in that the absorbance was measured at 286 instead of 280 due to greater sensitivity and spectrophotometer response. The standard curve was prepared in model wine at concentrations of BSA = 0.0, 250, 500, and 1,000 mg/L.

Phytic acid ($C_6H_{18}O_{24}P_6$, CAS 83-86-3, 50% w/w, Sigma-Aldrich, MO, U.S.A) additions ranged between PA:BSA molar ratios of 2.5:1 to 40:1. Phytic acid concentrations before and after sample treatments were not measured in this study, although phytic acid analysis is possible using various methods (Talamond et al. 1998; Israel et al. 2006). All samples were centrifuged (15,000 rpm x 3 minutes in 50 mL centrifuge tubes) and then sterile filtered through 4 mm poly(tetrafluoroethylene) (PTFE) 0.45 μm pore size syringe filters before analysis. All samples were stored under nitrogen gas.

Samples measurments by UV spectrophotometry and pH were prepared and analyzed in triplicate and the results averaged. Data are expressed as means ± SD before analysis of variance (ANOVA) using the Statistical Analysis System PROC GLM procedure (release 8.2; SAS Institute, Cary, NC). Treatment means were separated by the least significant difference test (LSD) at the 5% significance level.

4.4 Results and discussion

4.4.1 Standard Calibration

Calibration curves for BSA were obtained with ≥ 0.999 (r^2) linear correlation (Beer's law) of integration response with concentration up to 1,000 mg/L, and produced an extinction coefficient of 2.525E3 mg/L • AU.

4.4.2 Detection Limits and Precision

The detection limit was measured as the concentration corresponding to the lowest signal (absorbance) measurable above baseline with a signal-to-noise ratio of 3:1 when done in triplicate with the four BSA standards. The limit of detection for BSA was 8.4 mg/L at 286 nm. Analytical confirmation of the detection limit by measuring standards of concentrations saddling the respective limits was not attempted.

Relevant to all sections of the study, sample variation due to the analytical procedure was determined. Instrument precision was evaluated by performing 10 replicate absorbance spectra at 286 nm with 250 and 500 mg/L BSA standards. The coefficients of variation (CV) were 1.5% and 1.0% at 250 mg/L and 500 mg/L BSA, respectively, indicating an acceptably high level of analytical reproducibility. Samples of model wine containing only phytic acid at concentrations ranging between 5 and 1,000 mg/L were not significantly different than the model wine absorbance spectra at 286 nm.

Table 4.1 Effect of phytic acid in model wine containing BSA (1,000 mg/L).

Ratio PA:BSA	BSA (mg/L)	%
0.0	999.5a[a]	100
1.0	998.2a	99.8
2.0	996.9a	99.7
2.5	637.2b	63.8
5.0	227.5c	22.8
7.5	42.4d	0.04
10	<8.4e[b]	
20	<8.4e[b]	<0.01
40	<8.4e[b]	<0.01

[a] Means with the same letter within a column are not significantly different ($p \leq 0.05$).

[b] Values below the BSA detection limit (8.4 mg/L).

Samples were analyzed one day after phytic acid addition and again after 16 days. The results of various treatments at varying concentrations of PA and BSA (200 – 1000 mg/L) were scaled to 1,000 mg/L of initial BSA. The effect of phytic acid in model wine containing BSA (1,000 mg/L), averages of triplicate values are shown in Table 4.1 and Figure 4.1. Protein content was significantly reduced with phytic acid treatments at PA:BSA (mM) ratios of 2.5 and greater. Treatments produced an exponential reduction in BSA concentration between the range of 2 to 10 mM PA:BSA ($r^2 \geq 0.987$, regression = $2896.5e^{-0.5499x}$). At PA:BSA ratios of 10 mM or

great, BSA was reduced below the limit of detection. There were no significant differences among replicates or between the dates, although some sediment consistent with protein was visible in the treatments at molar ratios of PA:BSA <10 mM at day 16, which may be a cause for concern.

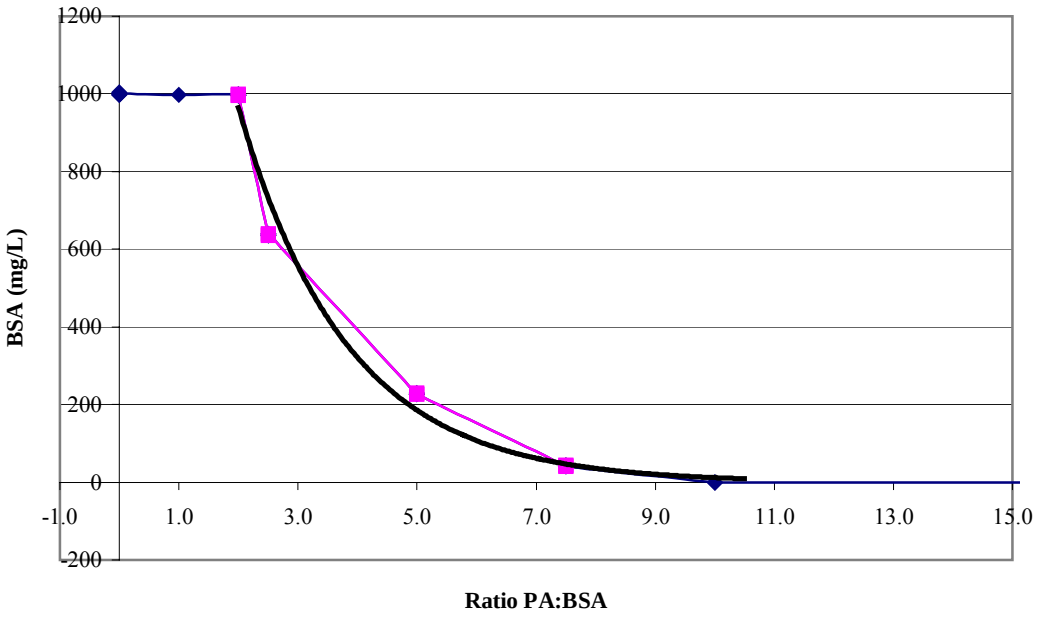

Figure 4.1 Effect of phytic acid in model wine containing BSA (1000 mg/L).

The effect of phytic acid in model wine containing BSA (200 mg/L), averages of triplicate values are shown in Table 4.2 and Figure 4.2. Protein content was significantly reduced with all phytic acid treatments. Treatments produced a linear reduction in BSA concentration between the range of 0 to 5.0 mM PA:BSA ($r^2 \geq 0.999$), which corresponds to BSA concentration of 45 – 200 mg/L. The linearly interpolated x-intercept = 6.5 mM PA:BSA. At PA:BSA ratios of 7.5 and 10 mM,

BSA was reduced below the limit of detection. There were no significant differences among replicates or between the dates, although some sediment consistent with protein was visible in the treatments at molar ratios of PA:BSA <10:1 at day 16. There were no significant differences among pH with increasing PA additions.

Table 4.2 Effect of phytic acid in model wine containing BSA (200 mg/L).

PA added (mg/L)	Ratio PA:BSA (mM)	Ratio BSA:PA (mg/L)	BSA (mg/L)	SD	pH	SD
0	0.0	∞	201.7 a[a]	1.7	3.81 a	0.04
5	2.5	40	127.3 b	0.5	3.79 a	0.02
10	5.0	20	45.3 c	0.3	3.85 a	0.04
15	7.5	13	8.3 d[b]	0.4	3.84 a	0.02
20	10.0	10	8.0 d[b]	0.0	3.85 a	0.03

[a] Means with the same letter within a column are not significantly different ($p \leq 0.05$).

[b] Values below the BSA detection limit (8.4 mg/L).

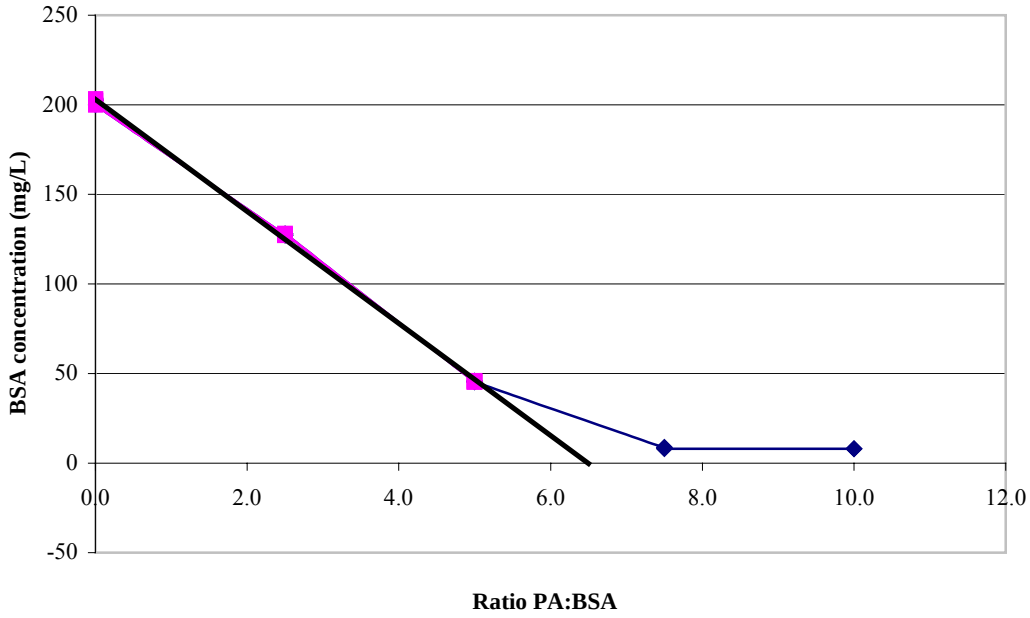

Figure 4.2 Effect of phytic acid in model wine containing BSA (200 mg/L).

At BSA concentrations of 1,000 mg/L there was an exponential decrease in BSA after PA additions. It appears that a minimum of about 2.5 mM PA:BSA is required before significant reduction in BSA is observed. At 200 mg/L BSA, the reduction appears linear up to PA:BSA ratio of 5.0. Comparing both data sets, it appears that the minimum amount of PA require to precipitate all BSA lies somewhere between PA:BSA 6.5 – 10:1 mM and in both cases, at 10 mM PA:BSA, phytic acid is highly effective at precipitating BSA below the limit of detection. At PA:BSA 10 mM, unlike all treatments at lower PA concentrations, there was no visible sediment in samples 16 days after filtration, which is another indication of complete BSA precipitation.

Phytic acid treatments significantly reduced BSA content in a model wine solution. The phytic acid content of grapes, musts and wines has not been well explored and there is a need for a quantitative residual phytic acid analysis after

treatment to confirm that phytic acid is completely reacted and precipitated. Additionally, determining and characterizing the rate of protein-phytate reactions under various wine conditions, especially the influence of pH, temperature, and ethanol content, and the extent of potential protein-phytate-metal interactions would be useful. The formation of ternary phytate complexes among proteins at neutral or negative pI and multivalent cations such as calcium ions is a particularly interesting area of study that may allow the treatment of proteins in wine that are otherwise difficult to treat with bentonite. The adsorption isotherm should be evaluated to determine if it better fits common adsorption models such as the Langmuir adsorption model. The effect of phytic acid on haze forming proteins vs. total proteins in wines should also be investigated. Lastly, the effects on both the sensory perception and the long-term stability of wines treated with PA need to be determined under actual winemaking conditions.

Phytic acid was effective at significantly reducing BSA in model wines at concentrations ranging between PA:BSA molar ratios of 2.5 – 10 mM over BSA concentrations of 1,000 – 200 mg/L. A PA:BSA molar ratio of 10 mM, which is equivalent to 0.1 mg/L PA per 1 mg/L BSA, was effective at reducing >99% of BSA. There were no significant changes in pH with PA additions up to 10 mM in a model wine containing 200 mg/L BSA. Phytic acid is not currently approved (OIV) for use in wine and may require petition to regulatory bodies before its use therein.

4.4 Acknowledgments

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APPENDIX

APPENDIX

1. Preliminary study results in Armenia: Fe and pH

Results obtained from a preliminary study conducted in Armenia, city of Yerevan at Yerevan State University during November 2003:

Samples: Ten wine samples were prepared from one liter of semisweet Armenian red wine from the Areni grape variety (Table 5.1):

•Blank: Iron content of 2.7 mg/L and a pH of 3.3 was stored at room temperature for a total of three days and then filtered through a 0.45 μm Millipore membrane before determining iron content.

•Treatment I: Triplicate samples of the above blank wine were spiked with 35 mg/L Fe(III); the bottles were agitated at room temperature for three days, filtered through 0.45μm membrane before determining iron content.

•Treatment II: Triplicate samples of the above blank wine were treated with 35 mg/L Fe(III) and 330 mg/L of 50% phytic acid; the bottles were stored for 1 day at room temperature and then the wine samples were treated with 125 mg/L $CaCO_3$, agitated for two days at room temperature, filtered through 0.45μm membrane before determining iron content.

•Treatment III: Triplicate samples of the above blank wine were treated with 35 mg/L Fe(III) and 660 mg/L of 50% phytic acid; the bottles were stored for 1 day at room temperature and then the wine samples were treated with 250 mg/L $CaCO_3$,

agitated for two days at room temperature, filtered through 0.45µm membrane before determining iron content.

Table 5.1 Treatment results averages.

	Fe [mg/L]	**pH**
Blank wine (no added iron)	2.7	3.3
Treatment I (iron added)	37.0	3.2
Treatment II (iron plus full-strength phytic acid and calcium added)	2.5	3.3
Treatment III (iron plus half-strength phytic acid and calcium added	0.8	3.3

2. Statement of commercial use or potential for phytic acid in wine

Phytic Acid Use in Wine: A novel and safe method was developed to remove excess iron from wine at a cost of about $0.007 per bottle. Phytic acid is added to wine to chelate iron and other polyvalent cations. A calcium salt is added to co-precipitate the complex that subsequently is removed by filtration. This method effectively, inexpensively and safely removes excessive levels of iron in wine, champagne and other beverages. The advantages of this method over other methods are its low cost, safe to use with no hazardous waste disposal issues. It has no effect on pH, color, and taste with wines containing low iron levels. In wines containing high iron levels taste is improved. There is a protective effect on taste in wines

containing high iron levels; this is especially true if phytic acid is added immediately after fermentation and the calcium carbonate shortly before bottling.

Cyanide treatment of wine is currently the standard and commonly used process in Russia and all former U.S.S.R. Commonwealth of Independent States (CIS countries) for reducing excessive iron levels. However, it is prohibited in many countries and is strictly controlled where permitted. A novel, safe, and inexpensive method was developed to remove excess iron from wine and other beverages. Phytic acid, a natural seed component, is added to wine to chelate iron and subsequently calcium carbonate is added to co-precipitate the complex that can be removed by filtration. The method effects almost complete iron removal from wine. Furthermore, unlike bluefining and other chelation methods, the proposed sequential addition of phytic acid and calcium requires no knowledge of the exact concentration of iron, copper, and other metals and of the exact volume of wine inside the tank - the efficacy of the method depends on the excess of phytic acid relative to iron present in wine, and on the molar ratio of calcium to phytate (5:1). This ratio assures complete precipitation of the mixed metal salt of phytic acid independent of the amount of iron, and it also prevents the accumulation of any added calcium.

The efficacy of this method was tested in the laboratories at Yerevan State University and other laboratories and confirmed by trials with various wineries in Armenia. This very effective method will allow for replacement of the unsafe cyanide treatment currently used in Russia and CIS countries.

Effective treatments should begin at a minimum of 330 mg of phytic acid and 250 mg of $CaCO_3$ per 1 liter of wine should be added to wines with iron concentrations of 25 mg/L or less (330 mg phytic acid is able to chelate 28 mg/L iron

to form a 1:1 iron-phytate chelate, however, in effect may only chelate 25 mg/L of iron due to the presence of other polyvalent cations in the wine and analytical uncertainties). Wines with iron levels between 50 mg/L and 25 mg/L should be treated with 660 mg/L phytic acid and 500 mg/L $CaCO_3$. Wines with very high levels of iron, e.g. above 100 mg/L could be treated with even higher concentration of phytic acid and $CaCO_3$ or they could be treated twice with lower levels, however, the extremely high level of iron is likely to have caused substantial damage and the wine may be better suited for the production of distillation spirits. Wines with iron levels below 7-10 mg/L do not need to be treated as these levels are relatively stable (1). After treatment with phytic acid and calcium carbonate, the wine is then held for some time (from a few hours up to 7 days) to allow for completion of precipitation; it is then filtered and may be bottled.

3. UV spectrophotometer reproducibility

Table 5.2 Reproducibility: Model wine.

Trial	Abs (280nm)	Abs (420nm)
1	-4.12E-03	1.22E-04
2	1.39E-03	2.29E-04
3	7.02E-04	5.04E-04
4	5.10E-03	2.90E-04
5	-2.06E-03	1.22E-04
6	3.71E-03	9.16E-05
7	3.23E-03	2.59E-04
8	-7.02E-04	2.44E-04
9	-3.19E-03	5.95E-04
10	-2.04E-03	3.36E-04
Mean=	0.0002	0.0003
STD =	0.0031	0.0002
CV (%) =	1554.84	58.74

Table 5.3 Reproducibility: Red Wine.

Trial	Abs (280nm)	Abs (420nm)	Abs (520nm)
1	2.6827	0.40984	0.60666
2	2.6365	0.41013	0.60674
3	2.6139	0.41006	0.60667
4	2.6686	0.41025	0.60692
5	2.5944	0.41014	0.60701
6	2.6112	0.41017	0.60655
7	2.6632	0.41020	0.60706
8	2.5922	0.41040	0.60678
9	2.6686	0.41071	0.60666
10	2.7125	0.41058	0.60675
Mean=	2.6444	0.4102	0.6068
STD =	0.0408	0.0003	0.0002
CV (%) =	1.54	0.06	0.03

Table 5.4 Reproducibility: White wine.

Trial	**Abs (280nm)**	**Abs (420nm)**
1	0.45926	4.18E-03
2	0.45857	4.10E-03
3	0.45920	4.29E-03
4	0.45834	4.18E-03
5	0.45906	4.55E-03
6	0.45876	4.76E-03
7	0.45892	4.72E-03
8	0.45905	4.72E-03
9	0.45877	4.70E-03
10	0.45834	4.52E-03
Mean=	0.4588	0.0045
STD =	0.0003	0.0003
CV (%) =	0.07	5.77

Table 5.5 Reproducibility: BSA at 250 and 500 mg/L (AU at 286 nm).

Trial	250 mg/L (AU)	500 mg/L (AU)
1	0.08546	0.19867
2	0.08827	0.19925
3	0.08740	0.19473
4	0.08675	0.19740
5	0.08460	0.19511
6	0.08899	0.19525
7	0.08725	0.19781
8	0.08766	0.19740
9	0.08658	0.19322
10	0.08638	0.19887
Mean =	219.49	496.82
STD =	3.25	5.18
CV (%) =	1.48	1.04

4. BSA limit of detection

Table 5.6 BSA limit of detection.

BSA (mg/L)	**Abs (286 nm)**
0	1.89E-03
0	1.22E-04
0	1.62E-03
Mean =	0.0012
STD =	0.0010
CV (%) =	78.69
250	9.31E-02
250	9.80E-02
250	0.10120
Mean =	0.0974
STD =	0.0041
CV (%) =	4.17
500	0.20056
500	0.20108
500	0.20326
Mean =	0.2016
STD =	0.0014
CV (%) =	0.71

Table 5.6 (continued)

BSA (mg/L)	Abs (286 nm)
1000	0.39246
1000	0.39513
1000	0.39615
Mean =	0.3946
STD =	0.0019
CV (%) =	0.48

Calibration curve results

r^2 =	0.9998
Slope† =	0.00039
y-Intercept =	0.00111

† Slope = calibration equation: Concentration = 2534.7 mg/L * Abs

BSA limit of detection

mg/L*	2.81
3:1	8.4

* Equals the x-intercept (y-intercept/slope)

5. Atomic absorption spectroscopy (AAS) and inductively coupled plasma atomic emission spectrometry (ICP-AES) instrument conditions

Table 5.7 Instrument conditions.

Element	Instrument	Mode	Wavelength (nm)	Slit (mm)	Lamp Current (mA)	Gain
Ca	ICP-AES	Emission	315.8		25	100
Cu	AAS	Absorbance	324.7	0.7	25	100
Fe	AAS	Absorbance	248.3	0.2	25	100
K	AAS	Emission	766.4		25	100

6. Standard metals calibration

AAS and ICP-AES calibration was carried out using a seven-point and four-point calibration respectively with standard solutions of Ca, Cu, Fe, and K. Stock standard calibration solutions were prapared using deionized water (ASTM Type II grade) and 2% (v/v) nitric acid (HNO_3) by volume. Standards were prepared by diluting stock metal solutions (Ca: 2,000 mg/L; Cu and Fe: 1,000 mg/L; and K: 1,000 mg/L) (Appendix) to the following concentrations: 0.0, 20.0, 200.0 and 400.0 mg/L of Ca; 0.0, 0.25, 0.5, 1.0, 2.0, 3.0 and 5.0 mg/L of Cu and Fe; and 0.0, 5.0, 10.0, 20.0, 50.0 80.0, and 100 mg/L of K with 2% (v/v) HNO_3 (Table 5.9).

Table 5.8 Stock and standard solution concentrations as a percent mg/L in 2% (v/v) HNO_3.

	Ca	Cu and Fe	K
Stock Solution	2,000.00	1000.00	1,000.00
Standard 1	0.00	0.00	0.00
Standard 2	20.00	0.25	5.00
Standard 3	200.00	0.50	10.00
Standard 4	400.00	1.00	20.00
Standard 5		2.00	50.00
Standard 6		3.00	80.00
Standard 7		5.00	100.00

7. Stock metal solutions

7.1 Calcium solution, stock, 2,000 mg/L Ca

Suspended 4.9938 g $CaCO_3$ (element Ca fraction = 0.4005), dried at 180°C for 1 hour before weighing, accurately weighed to at least four significant figures, in reagent water and dissolved with a minimum amount of (1:1) HNO_3. Added 10.0 mL concentrated HNO_3 and dilute to volume in a 1 L volumetric flask with reagent water.

7.2 Copper solution, stock, 1,000 mg/L Cu

Dissolved 1.2564 g CuO (element fraction Cu = 0.7989), accurately weighed to at least four significant figures, in a minimum amount of (1:1) HNO_3. Added 10.0

mL concentrated HNO_3 and dilute to volume in a 1 L volumetric flask with reagent water.

7.3 Iron solution, stock, 1,000 mg/L Fe

Dissolved 1.4298 g Fe_2O_3 (element fraction Fe = 0.6994), accurately weighed to at least four significant figures, in a warm mixture of 20 mL HCl (1:1) and 2 mL of concentrated HNO_3. Cool, add an additional 5.0 mL of concentrated HNO_3, and dilute to volume in a 1 L volumetric flask with reagent water.

7.4 Potassium solution, stock, 1,000 mg/L K

Dissolved 1.9069 g KCl (element fraction K = 0.5244) dried at 110°C, accurately weighed to at least four significant figures, in reagent water, and diluted to volume in a 1 L volumetric flask with reagent water.

8. Iron treatment: Phytic acid addition tables

Enter Fe^{+3} concentration here:	20.0 mg/L	Phytic Acid $/kg:	15.00
Enter Volume to treat here:	1000.0 L	$CaCO_3$ $/kg:	0.07

Fe (mg/L)	Fe (molar conc.) in mmoles/ L (mM)	Molar Ratio Fe : Phytic	Molar % Phytic : Fe	Phytic Acid conc. mmoles/ L (mM)	Phytic Acid (mg/L)	Weight Ratio Phytic : Fe	Phytic Acid to treat volume (g)	Phytic Acid Cost $	Molar Ratio Ca : Phytic	Molar % Phytic : Ca	Ca conc. mmoles/ L (mM)	Ca (mg/L)	Weight Ratio Phytic : Ca	$CaCO_3$ (100%) to treat volume (g)	$CaCO_3$ Cost $
20.00	0.358	1:1	1.00	0.3581	236.386	11.82	236.386	4	5:1	0.20	1.791	71.766	3.29	179.226	0.01
20.00	0.358	2:1	0.50	0.1791	118.193	5.91	118.193	2	5:1	0.20	0.895	35.883	3.29	89.613	0.01
20.00	0.358	3:1	0.33	0.1194	78.795	3.94	78.795	1	5:1	0.20	0.597	23.922	3.29	59.742	0.00
20.00	0.358	4:1	0.25	0.0895	59.097	2.95	59.097	1	5:1	0.20	0.448	17.942	3.29	44.806	0.00
20.00	0.358	5:1	0.20	0.0716	47.277	2.36	47.277	1	5:1	0.20	0.358	14.353	3.29	35.845	0.00
20.00	0.358	6:1	0.17	0.0597	39.398	1.97	39.398	1	5:1	0.20	0.298	11.961	3.29	29.871	0.00

Trial/ 1L wine

Fe (mg/L)	Phytic Acid to treat volume (mg/L) Fe:IP6 1:1	$CaCO_3$ to treat volume (mg/L) Ca:Pa 5:1	Phytic Acid to treat volume (mg/L) Fe:IP6 2:1	$CaCO_3$ to treat volume (mg/L) Ca:Pa 5:1	Phytic Acid to treat volume (mg/L) Fe:IP6 3:1	$CaCO_3$ to treat volume (mg/L) Ca:Pa 5:1	Phytic Acid to treat volume (mg/L) Fe:IP6 4:1	$CaCO_3$ to treat volume (mg/L) Ca:Pa 5:1	Phytic Acid to treat volume (mg/L) Fe:IP6 5:1	$CaCO_3$ to treat volume (mg/L) Ca:Pa 5:1
5	59.1	44.8	29.5	22.4	19.7	14.9	14.8	11.2	11.8	9.0
10	118.2	89.6	59.1	44.8	39.4	29.9	29.5	22.4	23.6	17.9
15	177.3	134.4	88.6	67.2	59.1	44.8	44.3	33.6	35.5	26.9
20	236.4	179.2	118.2	89.6	78.8	59.7	59.1	44.8	47.3	35.8
25	295.5	224.0	147.7	112.0	98.5	74.7	73.9	56.0	59.1	44.8
30	354.6	268.8	177.3	134.4	118.2	89.6	88.6	67.2	70.9	53.8
35	413.7	313.6	206.8	156.8	137.9	104.5	103.4	78.4	82.7	62.7
40	472.8	358.5	236.4	179.2	157.6	119.5	118.2	89.6	94.6	71.7
45	531.9	403.3	265.9	201.6	177.3	134.4	133.0	100.8	106.4	80.7
50	591.0	448.1	295.5	224.0	197.0	149.4	147.7	112.0	118.2	89.6

Trial/ 1L wine (Continue)

Fe (mg/L)	Phytic Acid to treat volume (mg/L) Fe:IP6 1:1	$CaCO_3$ to treat volume (mg/L) Ca:Pa 5:1	Phytic Acid to treat volume (mg/L) Fe:IP6 2:1	$CaCO_3$ to treat volume (mg/L) Ca:Pa 5:1	Phytic Acid to treat volume (mg/L) Fe:IP6 3:1	$CaCO_3$ to treat volume (mg/L) Ca:Pa 5:1	Phytic Acid to treat volume (mg/L) Fe:IP6 4:1	$CaCO_3$ to treat volume (mg/L) Ca:Pa 5:1	Phytic Acid to treat volume (mg/L) Fe:IP6 5:1	$CaCO_3$ to treat volume (mg/L) Ca:Pa 5:1
55	650.1	492.9	325.0	246.4	216.7	164.3	162.5	123.2	130.0	98.6
60	709.2	537.7	354.6	268.8	236.4	179.2	177.3	134.4	141.8	107.5
65	768.3	582.5	384.1	291.2	256.1	194.2	192.1	145.6	153.7	116.5
70	827.4	627.3	413.7	313.6	275.8	209.1	206.8	156.8	165.5	125.5
75	886.4	672.1	443.2	336.0	295.5	224.0	221.6	168.0	177.3	134.4
80	945.5	716.9	472.8	358.5	315.2	239.0	236.4	179.2	189.1	143.4
85	1004.6	761.7	502.3	380.9	334.9	253.9	251.2	190.4	200.9	152.3
90	1063.7	806.5	531.9	403.3	354.6	268.8	265.9	201.6	212.7	161.3
95	1122.8	851.3	561.4	425.7	374.3	283.8	280.7	212.8	224.6	170.3
100	1181.9	896.1	591.0	448.1	394.0	298.7	295.5	224.0	236.4	179.2

9. Calcium treatment : Phytic acid addition tables

Enter Ca^{+2} concentration here: 100.0 mg/L Phytic Acid $/kg: 15.00

Enter Volume to treat here: 1000.0 L

Ca (mg/L)	Ca (molar conc.) in mmoles/L (mM)	Molar Ratio Ca : Phytic	Molar % Phytic : Ca	Phytic Acid conc. mmoles/L (mM)	Phytic Acid (mg/L)	Weight Ratio Phytic : Ca	Phytic Acid to treat volume (g)	Phytic Acid Cost $
100.00	2.495	1:1	1.00	2.4951	1646.913	16.47	1646.913	25
100.00	2.495	2:1	0.50	1.2476	823.456	8.23	823.456	12
100.00	2.495	3:1	0.33	0.8317	548.971	5.49	548.971	8
100.00	2.495	4:1	0.25	0.6238	411.728	4.12	411.728	6
100.00	2.495	5:1	0.20	0.4990	329.383	3.29	329.383	5
100.00	2.495	6:1	0.17	0.4159	274.485	2.74	274.485	4
100.00	2.495	7:1	0.14	0.3564	235.273	2.35	235.273	4
100.00	2.495	8:1	0.13	0.3119	205.864	2.06	205.864	3

Trials/ 1L wine

Ca (mg/L)	Phytic Acid to treat volume (mg/L) Ca:IP6 1:1	Phytic Acid to treat volume (mg/L) Ca:IP6 2:1	Phytic Acid to treat volume (mg/L) Ca:IP6 3:1	Phytic Acid to treat volume (mg/L) Ca:IP6 4:1	Phytic Acid to treat volume (mg/L) Ca:IP6 5:1	Phytic Acid to treat volume (mg/L) Ca:IP6 6:1	Phytic Acid to treat volume (mg/L) Ca:IP6 7:1	Phytic Acid to treat volume (mg/L) Ca:IP6 8:1
5	82.3	41.2	27.4	20.6	16.5	13.7	11.8	10.3
10	164.7	82.3	54.9	41.2	32.9	27.4	23.5	20.6
15	247.0	123.5	82.3	61.8	49.4	41.2	35.3	30.9
20	329.4	164.7	109.8	82.3	65.9	54.9	47.1	41.2
25	411.7	205.9	137.2	102.9	82.3	68.6	58.8	51.5
30	494.1	247.0	164.7	123.5	98.8	82.3	70.6	61.8
35	576.4	288.2	192.1	144.1	115.3	96.1	82.3	72.1
40	658.8	329.4	219.6	164.7	131.8	109.8	94.1	82.3
45	741.1	370.6	247.0	185.3	148.2	123.5	105.9	92.6
50	823.5	411.7	274.5	205.9	164.7	137.2	117.6	102.9
55	905.8	452.9	301.9	226.5	181.2	151.0	129.4	113.2
60	988.1	494.1	329.4	247.0	197.6	164.7	141.2	123.5
65	1070.5	535.2	356.8	267.6	214.1	178.4	152.9	133.8
70	1152.8	576.4	384.3	288.2	230.6	192.1	164.7	144.1
75	1235.2	617.6	411.7	308.8	247.0	205.9	176.5	154.4
80	1317.5	658.8	439.2	329.4	263.5	219.6	188.2	164.7
85	1399.9	699.9	466.6	350.0	280.0	233.3	200.0	175.0
90	1482.2	741.1	494.1	370.6	296.4	247.0	211.7	185.3
95	1564.6	782.3	521.5	391.1	312.9	260.8	223.5	195.6
100	1646.9	823.5	549.0	411.7	329.4	274.5	235.3	205.9

10. Data tables

10.1 Protein (BSA) study

Method file: PHYTICRW.M Last update: Date 4/6/06 Time 3:55:23 PM

Information : Default Method

Data File: D:\BRENT\BSASAMP.SD Created: 4/7/06 11:51:50 PM

Overlaid Spectra: {D:\BRENT\Pic_0001.WMF}

Method file: PHYQUANT.M Last update: Date 4/7/06 6:07:29 PM

Information: Default Method

Analyte name: BSA

Calibration equation: Conc. = 2.5248E3 mg/L * Abs

Calibrated at: Date 4/7/06 Time 6:07:29 PM Operator: user

Name	Rep	BSA added (mg/L)	PA added (mg/L)	Abs <280nm>	Abs <285nm>	Abs <286nm>	Abs <420nm>	Dilut. Factor	BSA (mg/L) at A_{286}	pH
P000-10	1	0	10	-3.68E-03	-1.46E-02	-1.48E-02	-9.84E-03	1.0100	-37.783	
P000-10	2	0	10	-8.90E-03	-1.49E-02	-1.76E-02	-1.15E-02	1.0100	-44.825	
P000-10	3	0	10	-2.08E-03	-1.46E-02	-1.48E-02	-1.07E-02	1.0100	-37.666	
P000-100	1	0	100	-4.59E-03	-9.53E-03	-9.87E-03	-8.01E-03	1.0100	-25.175	
P000-100	2	0	100	-6.87E-03	-9.13E-03	-1.03E-02	-7.55E-03	1.0100	-26.304	
P000-100	3	0	100	-2.99E-03	-9.73E-03	-1.14E-02	-1.04E-02	1.0100	-28.989	
P250-00	1	250	0	5.14E-02	8.62E-02	8.55E-02	-1.10E-02	1.0000	215.78	
P250-00	10	250	0	4.90E-02	8.98E-02	8.83E-02	-9.89E-03	1.0000	222.87	
P250-00	2	250	0	4.95E-02	8.71E-02	8.74E-02	-1.08E-02	1.0000	220.68	
P250-00	3	250	0	4.88E-02	8.82E-02	8.67E-02	-1.06E-02	1.0000	219.02	

Name	Rep	BSA added (mg/L)	PA added (mg/L)	Abs <280nm>	Abs <285nm>	Abs <286nm>	Abs <420nm>	Dilut. Factor	BSA (mg/L) at A_{286}	pH
P250-00	4	250	0	5.37E-02	8.36E-02	8.46E-02	-1.18E-02	1.0000	213.59	
P250-00	5	250	0	5.61E-02	8.84E-02	8.90E-02	-9.22E-03	1.0000	224.68	
P250-00	6	250	0	5.00E-02	8.75E-02	8.73E-02	-1.04E-02	1.0000	220.29	
P250-00	7	250	0	5.26E-02	8.87E-02	8.77E-02	-9.80E-03	1.0000	221.33	
P250-00	8	250	0	5.13E-02	8.73E-02	8.66E-02	-1.07E-02	1.0000	218.6	
P250-00	9	250	0	5.74E-02	8.69E-02	8.64E-02	-1.15E-02	1.0000	218.09	
P250-10	1	250	10	4.80E-02	8.06E-02	8.03E-02	-9.14E-03	1.0100	204.71	
P250-10	2	250	10	5.89E-02	8.27E-02	8.39E-02	-8.99E-03	1.0100	213.93	
P250-10	3	250	10	6.05E-02	9.30E-02	9.25E-02	-2.78E-03	1.0100	236	
P250-10	3	250	10	6.11E-02	9.50E-02	9.57E-02	-2.24E-03	1.0100	244.05	
P250-100	1	250	100	1.86E-03	-9.73E-03	-1.11E-02	-1.05E-02	1.0100	-28.21	
P250-100	2	250	100	1.63E-03	-5.49E-03	-5.74E-03	-5.66E-03	1.0100	-14.631	
P250-100	3	250	100	-1.05E-02	-1.41E-02	-1.47E-02	-9.16E-03	1.0100	-37.471	
P250-1000	1	250	1000						-0	
P250-1000	2	250	1000						-0	
P250-1000	3	250	1000						-0	
P500-00	1	500	0	0.11557	0.19756	0.19867	-5.89E-03	1.0000	501.61	
P500-00	10	500	0	0.11496	0.19774	0.19925	-7.95E-03	1.0000	503.07	
P500-00	2	500	0	0.11046	0.19299	0.19473	-8.65E-03	1.0000	491.67	
P500-00	3	500	0	0.11229	0.19752	0.1974	-7.69E-03	1.0000	498.41	
P500-00	4	500	0	0.11168	0.19474	0.19511	-9.26E-03	1.0000	492.63	
P500-00	5	500	0	0.10576	0.19255	0.19525	-9.61E-03	1.0000	492.98	
P500-00	6	500	0	0.11803	0.19667	0.19781	-8.13E-03	1.0000	499.45	
P500-00	7	500	0	0.10783	0.1953	0.1974	-8.50E-03	1.0000	498.41	
P500-00	8	500	0	0.11079	0.19276	0.19322	-8.67E-03	1.0000	487.85	
P500-00	9	500	0	0.1171	0.19726	0.19887	-7.80E-03	1.0000	502.11	

Name	Rep	BSA added (mg/L)	PA added (mg/L)	Abs <280nm>	Abs <285nm>	Abs <286nm>	Abs <420nm>	Dilut. Factor	BSA (mg/L) at A_{286}	pH
P500-10	1	500	10	0.1129	0.19696	0.19933	-4.01E-03	1.0100	508.29	
P500-10	2	500	10	0.10811	0.19643	0.19768	-4.17E-03	1.0100	504.09	
P500-10	3	500	10	0.11588	0.20057	0.20256	-1.51E-03	1.0100	516.54	
P500-10	3	500	10	0.11201	0.19601	0.19617	-6.24E-03	1.0100	500.24	
P500-10	3	500	10	0.1153	0.19587	0.19714	-5.62E-03	1.0100	502.73	
P500-100	1	500	100	-5.26E-03	-1.07E-02	-1.15E-02	-1.00E-02	1.0100	-29.339	
P500-100	2	500	100	-2.55E-03	-7.67E-03	-8.56E-03	-8.19E-03	1.0100	-21.829	
P500-100	3	500	100	-7.78E-03	-1.26E-02	-1.34E-02	-9.61E-03	1.0100	-34.281	
P500-1000	1	500	1000						-0	
P500-1000	2	500	1000						-0	
P500-1000	3	500	1000						-0	
P1000-10	1	1000	10	0.19893	0.37826	0.38919	-5.11E-03	1.0100	992.46	
P1000-10	2	1000	10	0.1899	0.37313	0.38049	-8.36E-03	1.0100	970.28	
P1000-10	2	1000	10	0.19168	0.37518	0.38544	-4.41E-03	1.0100	982.89	
P1000-10	2	1000	10	0.196	0.37421	0.38643	-5.08E-03	1.0100	985.42	
P1000-10	3	1000	10	0.1846	0.37392	0.38361	-7.06E-03	1.0100	978.22	
P1000-100	1	1000	100	2.08E-03	-7.37E-03	-7.16E-03	-1.08E-02	1.0100	-18.249	
P1000-100	2	1000	100	-3.22E-03	-3.58E-03	-5.98E-03	-8.36E-03	1.0100	-15.253	
P1000-100	3	1000	100	-3.91E-03	-1.26E-02	-1.42E-02	-1.01E-02	1.0100	-36.226	
P1000-1000	1	1000	1000						-0	
P1000-1000	2	1000	1000						-0	
P1000-1000	3	1000	1000						-0	
P200-00	1	200	0			8.05E-02		1.0000	203.26	3.77
P200-00	2	200	0			8.01E-02		1.0000	202.34	3.85
P200-00	3	200	0			7.92E-02		1.0000	199.99	3.81
P200-05	1	200	5			5.04E-02		1.0005	127.24	3.77

Name	Rep	BSA added (mg/L)	PA added (mg/L)	Abs <280nm>	Abs <285nm>	Abs <286nm>	Abs <420nm>	Dilut. Factor	BSA (mg/L) at A_{286}	pH
P200-05	2	200	5			5.03E-02		1.0005	127.12	3.81
P200-05	3	200	5			5.07E-02		1.0005	127.97	3.79
P200-10	1	200	10			1.79E-02		1.0010	45.313	3.81
P200-10	2	200	10			1.81E-02		1.0010	45.853	3.88
P200-10	3	200	10			1.79E-02		1.0010	45.313	3.85
P200-15	1	200	15			3.36E-03		1.0015	8.4884	3.85
P200-15	2	200	15			3.52E-03		1.0015	8.9128	3.81
P200-15	3	200	15			3.19E-03		1.0015	8.064	3.85
P200-20	1	200	20			3.19E-03		1.0020	8.068	3.88
P200-20	2	200	20			3.19E-03		1.0020	8.068	3.82
P200-20	3	200	20			3.19E-03		1.0020	8.068	3.85

11. SAS Programing

Statistical Analysis System PROC GLM procedure (MS Windows version 8.2(TS2MO); SAS Institute, Cary, NC, U.S.A.).

11.1 Metals analysis

Data\SAS\Model_Metals_SAS.sas

```
data;
infile 'I:cor Fe3.txt' firstobs=2 delimiter='09'x;
input wine :$12. rep ratio pH_0 pH_2 pH_1 TA_0 TA_2 TA_1
Ca_0 CA_2 Ca_1 Cu_0 Cu_2 Cu_1 Fe_0 Fe_2 Fe_1 K_0 K_2 K_1;
*if wine='5';
diffpH = pH_2 - pH_1;
diffTA = TA_2 - TA_1;
diffCa = Ca_2 - Ca_1;
diffCu = Cu_2 - Cu_1;
diffFe = Fe_2 - Fe_1;
diffK = K_2 - K_1;
proc print;
run;
proc glm;
*wine :$12. rep pH pH_0 pH_1 diffpH TA_0 TA_1 diffTA Ca_0 Ca_1
diffCa Cu_0 Cu_1 diffCu Fe_0 Fe_1 diffFe K_0 K_1 diffK;
class ratio rep;
```

```
model pH_0 pH_1 diffpH TA_0 TA_1 diffTA Ca_0 Ca_1 diffCa Cu_0
Cu_1 diffCu Fe_0 Fe_1 diffFe K_0 K_1 diffK = ratio rep;
Means ratio rep /lsd;
run;
```

11.2 Wine color in response to iron and phytic acid and calcium treatment

Data\SAS\WineColor_SAS.sas

```
data;
infile 'I:WineColor_SAS.txt' firstobs=2 delimiter='09'x;
input wine :$12. rep Iron A280_0 A280_1 A420_0 A420_1 A520_0
A520_1 ColDen_0 ColDen_1
ColHue_0 ColHue_1;
if wine='R';
diff280 = A280_0 - A280_1;
diff420 = A420_0 - A420_1;
diff520 = A520_0 - A520_1;
diffCD = ColDen_0 - ColDen_1;
diffCH = ColHue_0 - ColHue_1;
proc print;
run;
proc glm data;
class iron rep;
```

```
model diff280 diff420 diff520 diffCD diffCH = iron rep;
Means iron rep/lsd;
run;
```

and

```
data;
infile 'I:WineColor_SAS.txt' firstobs=2 delimiter='09'x;
input wine :$12. rep Iron A280_0 A280_1 A420_0 A420_1 A520_0
A520_1 ColDen_0 ColDen_1 ColHue_0 ColHue_1;
if wine='R';
proc print;
run;
proc glm;
*A280_0 A280_1 A420_0 A420_1 A520_0 A520_1 ColDen_0 ColDen_1
ColHue_0 ColHue_1;
class iron rep;
model A280_0 A280_1 A420_0 A420_1 A520_0 A520_1 ColDen_0
ColDen_1 ColHue_0 ColHue_1=iron rep;
Means iron rep/lsd;
run;
```

11.3 BSA treatment

Data\SAS\BSA_SAS.sas

```
data;
infile 'I:BSA_SAS.txt' firstobs=2 delimiter='09'x;
input wine :$12. rep molarBSA PA_ADD BSA;

proc print;
run;
proc glm;
class rep molarBSA;
model BSA=molarBSA rep;
Means molarBSA rep /lsd;
run;
```

11.4 BSA results comparison after two weeks

Data\SAS\BSA_SAS_2wks.sas

```
data;
infile 'I:BSA_2wks.txt' firstobs=2 delimiter='09'x;
input date wine :$12. rep molarBSA PA_ADD BSA;

proc print;
run;
```

```
proc glm;
class date rep molarBSA;
model BSA=molarBSA date rep;
Means molarBSA rep date/lsd;
run;
```

11.5 BSA 200 mg/L to molar ratio of phytic acid

Data\SAS\BSA_SAS_200.sas

```
data;
infile 'I:BSA_200.txt' firstobs=2 delimiter='09'x;
input wine :$12. rep molarBSA PA_ADD BSA pH;

proc print;
run;
proc glm;
class rep molarBSA;
model BSA pH=molarBSA rep;
Means molarBSA rep/lsd;
run;
```

12. SAS Results

Statistical Analysis System PROC GLM procedure (MS Windows version 8.2(TS2MO); SAS Institute, Cary, NC, U.S.A.).

12.1 Metals analysis

12.1.1 Iron

Data\SAS\cor Fe3.rtf

12.1.2 Copper

Data\SAS\cor Cu3.rtf

11.1.3 Calcium

Data\SAS\cor Ca.rtf

The SAS System

The GLM Procedure

Class Level Information

Class	Levels	Values
ratio	9	0 1 2 3 4 5 6 7 8
rep	3	1 2 3

Number of observations 27

Dependent Variables With Equivalent Missing Value Patterns

Pattern	Obs	Dependent Variables
0	0	Cu_0 Cu_1 diffCu Fe_0 Fe_1 diffFe K_0 K_1 diffK
1	27	pH_0 pH_1 diffpH TA_0 TA_1 diffTA Ca_0 Ca_1 diffCa

NOTE: Variables in each group are consistent with respect to the presence or absence of missing values.

The SAS System

The GLM Procedure

Dependent Variable: pH_0

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	10	0	0	.	.
Error	16	0	0		
Corrected Total	26	0			

R-Square	Coeff Var	Root MSE	pH_0 Mean
0.000000	0	0	3.480000

Source	DF	Type I SS	Mean Square	F Value	Pr > F
ratio	8	0	0	.	.
rep	2	0	0	.	.

Source	DF	Type III SS	Mean Square	F Value	Pr > F
ratio	8	0	0	.	.
rep	2	0	0	.	.

The SAS System

The GLM Procedure

Dependent Variable: pH_1

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	10	1.06320000	0.10632000	Infty	<.0001
Error	16	0.00000000	0.00000000		
Corrected Total	26	1.06320000			

R-Square	Coeff Var	Root MSE	pH_1 Mean
1.000000	0	0	3.216667

Source	DF	Type I SS	Mean Square	F Value	Pr > F
ratio	8	1.06320000	0.13290000	Infty	<.0001
rep	2	0.00000000	0.00000000	.	.

Source	DF	Type III SS	Mean Square	F Value	Pr > F
ratio	8	1.06320000	0.13290000	Infty	<.0001
rep	2	0.00000000	0.00000000	.	.

The SAS System

The GLM Procedure

Dependent Variable: diffpH

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	10	1.06320000	0.10632000	Infty	<.0001
Error	16	0.00000000	0.00000000		
Corrected Total	26	1.06320000			

R-Square	Coeff Var	Root MSE	diffpH Mean
1.000000	0	0	0.263333

Source	DF	Type I SS	Mean Square	F Value	Pr > F
ratio	8	1.06320000	0.13290000	Infty	<.0001
rep	2	0.00000000	0.00000000	.	.

Source	DF	Type III SS	Mean Square	F Value	Pr > F
ratio	8	1.06320000	0.13290000	Infty	<.0001
rep	2	0.00000000	0.00000000	.	.

The SAS System

The GLM Procedure

Dependent Variable: TA_0

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	10	0	0	.	.
Error	16	0	0		
Corrected Total	26	0			

R-Square	Coeff Var	Root MSE	TA_0 Mean
0.000000	0	0	0.730000

Source	DF	Type I SS	Mean Square	F Value	Pr > F
ratio	8	0	0	.	.
rep	2	0	0	.	.

Source	DF	Type III SS	Mean Square	F Value	Pr > F
ratio	8	0	0	.	.
rep	2	0	0	.	.

The SAS System

The GLM Procedure

Dependent Variable: TA_1

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	10	2.77566667	0.27756667	Infty	<.0001
Error	16	0.00000000	0.00000000		
Corrected Total	26	2.77566667			

R-Square	Coeff Var	Root MSE	TA_1 Mean
1.000000	0	0	1.114444

Source	DF	Type I SS	Mean Square	F Value	Pr > F
ratio	8	2.77566667	0.34695833	Infty	<.0001
rep	2	0.00000000	0.00000000	.	.

Source	DF	Type III SS	Mean Square	F Value	Pr > F
ratio	8	2.77566667	0.34695833	Infty	<.0001
rep	2	0.00000000	0.00000000	.	.

The SAS System

The GLM Procedure

Dependent Variable: diffTA

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	10	2.77566667	0.27756667	Infty	<.0001
Error	16	0.00000000	0.00000000		
Corrected Total	26	2.77566667			

R-Square	Coeff Var	Root MSE	diffTA Mean
1.000000	0	0	-0.384444

Source	DF	Type I SS	Mean Square	F Value	Pr > F
ratio	8	2.77566667	0.34695833	Infty	<.0001
rep	2	0.00000000	0.00000000	.	.

Source	DF	Type III SS	Mean Square	F Value	Pr > F
ratio	8	2.77566667	0.34695833	Infty	<.0001
rep	2	0.00000000	0.00000000	.	.

The SAS System

The GLM Procedure

ependent Variable: Ca_0

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	10	3.12000000	0.31200000	Infty	<.0001
Error	16	0.00000000	0.00000000		
Corrected Total	26	3.12000000			

R-Square	Coeff Var	Root MSE	Ca_0 Mean
1.000000	0	0	72.43333

Source	DF	Type I SS	Mean Square	F Value	Pr > F
ratio	8	0.00000000	0.00000000	.	.
rep	2	3.12000000	1.56000000	Infty	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
ratio	8	0.00000000	0.00000000	.	.
rep	2	3.12000000	1.56000000	Infty	<.0001

The SAS System

The GLM Procedure

Dependent Variable: Ca_1

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	10	13.38370370	1.33837037	16.89	<.0001
Error	16	1.26814815	0.07925926		
Corrected Total	26	14.65185185			

R-Square	Coeff Var	Root MSE	Ca_1 Mean
0.913448	0.390071	0.281530	72.17407

Source	DF	Type I SS	Mean Square	F Value	Pr > F
ratio	8	11.15851852	1.39481481	17.60	<.0001
rep	2	2.22518519	1.11259259	14.04	0.0003

Source	DF	Type III SS	Mean Square	F Value	Pr > F
ratio	8	11.15851852	1.39481481	17.60	<.0001
rep	2	2.22518519	1.11259259	14.04	0.0003

The SAS System

The GLM Procedure

Dependent Variable: diffCa

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	10	11.83703704	1.18370370	14.93	<.0001
Error	16	1.26814815	0.07925926		
Corrected Total	26	13.10518519			

R-Square	Coeff Var	Root MSE	diffCa Mean
0.903233	108.5902	0.281530	0.259259

Source	DF	Type I SS	Mean Square	F Value	Pr > F
ratio	8	11.15851852	1.39481481	17.60	<.0001
rep	2	0.67851852	0.33925926	4.28	0.0324

Source	DF	Type III SS	Mean Square	F Value	Pr > F
ratio	8	11.15851852	1.39481481	17.60	<.0001
rep	2	0.67851852	0.33925926	4.28	0.0324

The SAS System

The GLM Procedure

t Tests (LSD) for pH_0

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	16
Error Mean Square	0
Critical Value of t	2.11991
Least Significant Difference	0

Means with the same letter are not significantly different.

t Grouping

	Mean	N	ratio
A	3.480	3	0
A			
A	3.480	3	1
A			
A	3.480	3	2
A			
A	3.480	3	3
A			
A	3.480	3	4
A			
A	3.480	3	5
A			
A	3.480	3	6
A			
A	3.480	3	7
A			
A	3.480	3	8

The SAS System

The GLM Procedure

t Tests (LSD) for pH_1

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	16
Error Mean Square	0
Critical Value of t	2.11991
Least Significant Difference	0

Means with the same letter are not significantly different.

t Grouping	Mean	N	ratio
A	3.480	3	0
B	3.360	3	8
C	3.340	3	7
D	3.290	3	6
E	3.270	3	5
F	3.230	3	4
G	3.150	3	3
H	3.080	3	2
I	2.750	3	1

The SAS System

The GLM Procedure

t Tests (LSD) for diffpH

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	16
Error Mean Square	0
Critical Value of t	2.11991
Least Significant Difference	0

Means with the same letter are not significantly different.

t Grouping

	Mean	N	ratio
A	0.7300	3	1
B	0.4000	3	2
C	0.3300	3	3
D	0.2500	3	4
E	0.2100	3	5

F	0.1900	3	6
G	0.1400	3	7
H	0.1200	3	8
I	0.0000	3	0

The SAS System

The GLM Procedure

t Tests (LSD) for TA_0

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	16
Error Mean Square	0
Critical Value of t	2.11991
Least Significant Difference	0

Means with the same letter are not significantly different.

t Grouping

	Mean	N	ratio
A	0.7300	3	0
A			
A	0.7300	3	1
A			
A	0.7300	3	2
A			
A	0.7300	3	3
A			
A	0.7300	3	4
A			
A	0.7300	3	5
A			
A	0.7300	3	6
A			
A	0.7300	3	7
A			
A	0.7300	3	8

The SAS System

The GLM Procedure

t Tests (LSD) for TA_1

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	16
Error Mean Square	0
Critical Value of t	2.11991
Least Significant Difference	0

Means with the same letter are not significantly different.

t Grouping	Mean	N	ratio
A	1.880	3	1
B	1.420	3	2
C	1.090	3	3
D	1.030	3	7
E	1.020	3	4
F	1.000	3	6
G	0.940	3	5
H	0.920	3	8
I	0.730	3	0

The SAS System

The GLM Procedure

t Tests (LSD) for diffTA

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	16
Error Mean Square	0
Critical Value of t	2.11991
Least Significant Difference	0

Means with the same letter are not significantly different.

t Grouping

	Mean	N	ratio
A	0.000	3	0
B	-0.190	3	8
C	-0.210	3	5
D	-0.270	3	6
E	-0.290	3	4

F	-0.300	3	7
G	-0.360	3	3
H	-0.690	3	2
I	-1.150	3	1

The SAS System

The GLM Procedure

t Tests (LSD) for Ca_0

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	16
Error Mean Square	0
Critical Value of t	2.11991
Least Significant Difference	0

Means with the same letter are not significantly different.

t Grouping

	Mean	N	ratio
A	72.43	3	0
A			
A	72.43	3	1
A			
A	72.43	3	2
A			
A	72.43	3	3
A			
A	72.43	3	4
A			
A	72.43	3	5
A			
A	72.43	3	6
A			
A	72.43	3	7
A			
A	72.43	3	8

The SAS System

The GLM Procedure

t Tests (LSD) for Ca_1

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	16
Error Mean Square	0.079259
Critical Value of t	2.11991
Least Significant Difference	0.4873

Means with the same letter are not significantly different.

t Grouping

		Mean	N	ratio
	A	73.7667	3	2
	B	72.4333	3	0
	B			
C	B	72.2667	3	5
C	B			
C	B	72.2333	3	4
C	B			
C	B	D 72.2000	3	7
C		D		

C E D 71.8333 3 6

E D

E D 71.7333 3 3

E

E 71.6333 3 1

E

E 71.4667 3 8

The SAS System

The GLM Procedure

t Tests (LSD) for diffCa

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	16
Error Mean Square	0.079259
Critical Value of t	2.11991
Least Significant Difference	0.4873

Means with the same letter are not significantly different.

t Grouping

	Mean	N	ratio
A	0.9667	3	8
A			
A	0.8000	3	1
A			
B A	0.7000	3	3
B A			
B A C	0.6000	3	6
B C			
B D C	0.2333	3	7
D C			
D C	0.2000	3	4
D C			
D C	0.1667	3	5
D			
D	0.0000	3	0
E	-1.3333	3	2

The SAS System

The GLM Procedure

t Tests (LSD) for pH_0

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	16
Error Mean Square	0
Critical Value of t	2.11991
Least Significant Difference	0

Means with the same letter are not significantly different.

t Grouping	Mean	N	rep
A	3.480	9	1
A			
A	3.480	9	2
A			
A	3.480	9	3

The SAS System

The GLM Procedure

t Tests (LSD) for pH_1

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	16
Error Mean Square	0
Critical Value of t	2.11991
Least Significant Difference	0

Means with the same letter are not significantly different.

t Grouping

	Mean	N	rep
A	3.217	9	1
A			
A	3.217	9	2
A			
A	3.217	9	3

The SAS System

The GLM Procedure

t Tests (LSD) for diffpH

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	16
Error Mean Square	0
Critical Value of t	2.11991
Least Significant Difference	0

Means with the same letter are not significantly different.

t Grouping

	Mean	N	rep
A	0.2633	9	1
A			
A	0.2633	9	2
A			
A	0.2633	9	3

The SAS System

The GLM Procedure

t Tests (LSD) for TA_0

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	16
Error Mean Square	0
Critical Value of t	2.11991
Least Significant Difference	0

Means with the same letter are not significantly different.

t Grouping

	Mean	N	rep
A	0.7300	9	1
A			
A	0.7300	9	2
A			
A	0.7300	9	3

The SAS System

The GLM Procedure

t Tests (LSD) for TA_1

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	16
Error Mean Square	0
Critical Value of t	2.11991
Least Significant Difference	0

Means with the same letter are not significantly different.

t Grouping

	Mean	N	rep
A	1.114	9	1
A			
A	1.114	9	2
A			
A	1.114	9	3

The SAS System

The GLM Procedure

t Tests (LSD) for diffTA

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	16
Error Mean Square	0
Critical Value of t	2.11991
Least Significant Difference	0

Means with the same letter are not significantly different.

t Grouping

	Mean	N	rep
A	-0.3844	9	1
A			
A	-0.3844	9	2
A			
A	-0.3844	9	3

The SAS System

The GLM Procedure

t Tests (LSD) for Ca_0

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	16
Error Mean Square	0
Critical Value of t	2.11991
Least Significant Difference	0

Means with the same letter are not significantly different.

t Grouping

	Mean	N	rep
A	72.90	9	1
B	72.30	9	2
C	72.10	9	3

The SAS System

The GLM Procedure

t Tests (LSD) for Ca_1

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	16
Error Mean Square	0.079259
Critical Value of t	2.11991
Least Significant Difference	0.2813

Means with the same letter are not significantly different.

t Grouping

	Mean	N	rep
A	72.4778	9	1
A			
A	72.2556	9	2
B	71.7889	9	3

The SAS System

The GLM Procedure

t Tests (LSD) for diffCa

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	16
Error Mean Square	0.079259
Critical Value of t	2.11991
Least Significant Difference	0.2813

Means with the same letter are not significantly different.

t Grouping	Mean	N	rep
A	0.4222	9	1
A			
B A	0.3111	9	3
B			
B	0.0444	9	2

12.2 Wine color analysis

Data\SAS\BSA_SAS_Result_ColorRW.rtf

Wine color analysis in response to iron and phytic acid and calcium treatment.

Sample #	Rep. #	Fe (mg/L)	A280 0	A280 1	A420 0	A420 1	A520 0	A520 1	Color Density (A520 + A420) 0	Color Density (A520 + A420) 1	Color Hue (A520 / A420) 0	Color Hue (A520 / A420) 1
W-0	1	0	0.449	0.447	0.004	0.003	0.001	0.000	0.005	0.003	0.208	-0.122
W-0	2	0	0.450	0.452	0.004	0.008	0.001	0.002	0.005	0.010	0.155	0.213
W-0	3	0	0.450	0.444	0.005	0.006	0.001	0.001	0.007	0.008	0.283	0.222
W-5	1	5	0.472	0.439	0.007	0.003	0.003	0.000	0.010	0.002	0.382	-0.147
W-5	2	5	0.472	0.439	0.007	0.005	0.003	0.001	0.010	0.006	0.388	0.210
W-5	3	5	0.473	0.437	0.007	0.005	0.002	0.001	0.010	0.006	0.331	0.242
W-10	1	10	0.484	0.446	0.008	0.004	0.002	0.001	0.010	0.004	0.275	0.173
W-10	2	10	0.479	0.452	0.009	0.008	0.002	0.001	0.011	0.009	0.240	0.174
W-10	3	10	0.479	0.450	0.009	0.007	0.003	0.001	0.012	0.009	0.273	0.178
W-20	1	20	0.508	0.446	0.007	0.004	0.002	0.001	0.009	0.005	0.258	0.204
W-20	2	20	0.513	0.445	0.010	0.004	0.002	0.000	0.012	0.003	0.202	-0.117
W-20	3	20	0.510	0.446	0.010	0.006	0.002	0.001	0.012	0.007	0.250	0.156
R-0	1	0	2.587	2.597	0.392	0.390	0.585	0.594	0.977	0.984	1.491	1.525
R-0	2	0	2.548	2.555	0.400	0.389	0.583	0.593	0.983	0.982	1.459	1.522
R-0	3	0	2.582	2.544	0.390	0.386	0.574	0.587	0.964	0.973	1.473	1.522
R-5	1	5	2.645	2.649	0.401	0.370	0.599	0.563	1.001	0.933	1.493	1.523
R-5	2	5	2.687	2.587	0.398	0.372	0.589	0.561	0.987	0.932	1.482	1.507
R-5	3	5	2.670	2.634	0.389	0.381	0.578	0.574	0.967	0.955	1.485	1.509
R-10	1	10	2.664	2.608	0.402	0.379	0.602	0.573	1.004	0.953	1.498	1.512
R-10	2	10	2.574	2.606	0.407	0.379	0.600	0.570	1.007	0.950	1.474	1.503
R-10	3	10	2.664	2.574	0.406	0.388	0.600	0.582	1.006	0.970	1.479	1.498
R-20	1	20	2.588	2.623	0.393	0.391	0.584	0.584	0.977	0.975	1.487	1.494
R-20	2	20	2.707	2.702	0.397	0.390	0.583	0.583	0.980	0.973	1.468	1.494
R-20	3	20	2.615	2.696	0.386	0.388	0.569	0.585	0.955	0.973	1.476	1.508

12.2.1 White Wine

The SAS System

The GLM Procedure

Class Level Information

Class	Levels	Values
Iron	4	0 5 10 20
rep	3	1 2 3

Number of observations 12

The SAS System

The GLM Procedure

Dependent Variable: A280_0

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	0.00566208	0.00113242	225.23	<.0001
Error	6	0.00003017	0.00000503		
Corrected Total	11	0.00569225			

R-Square	Coeff Var	Root MSE	A280_0 Mean
0.994700	0.468849	0.002242	0.478250

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Iron	3	0.00566158	0.00188719	375.35	<.0001
rep	2	0.00000050	0.00000025	0.05	0.9519

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Iron	3	0.00566158	0.00188719	375.35	<.0001
rep	2	0.00000050	0.00000025	0.05	0.9519

The SAS System

The GLM Procedure

Dependent Variable: A280_1

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	0.00023008	0.00004602	7.63	0.0140
Error	6	0.00003617	0.00000603		
Corrected Total	11	0.00026625			

R-Square	Coeff Var	Root MSE	A280_1 Mean
0.864163	0.551410	0.002455	0.445250

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Iron	3	0.00021158	0.00007053	11.70	0.0064
rep	2	0.00001850	0.00000925	1.53	0.2896

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Iron	3	0.00021158	0.00007053	11.70	0.0064
rep	2	0.00001850	0.00000925	1.53	0.2896

The SAS System

The GLM Procedure

Dependent Variable: A420_0

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	0.00004442	0.00000888	13.90	0.0030
Error	6	0.00000383	0.00000064		
Corrected Total	11	0.00004825			

R-Square	Coeff Var	Root MSE	A420_0 Mean
0.920553	11.02490	0.000799	0.007250

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Iron	3	0.00004092	0.00001364	21.35	0.0013
rep	2	0.00000350	0.00000175	2.74	0.1428

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Iron	3	0.00004092	0.00001364	21.35	0.0013
rep	2	0.00000350	0.00000175	2.74	0.1428

The SAS System

The GLM Procedure

Dependent Variable: A420_1

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	0.00002608	0.00000522	3.83	0.0663
Error	6	0.00000817	0.00000136		
Corrected Total	11	0.00003425			

R-Square	Coeff Var	Root MSE	A420_1 Mean
0.761557	22.22222	0.001167	0.005250

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Iron	3	0.00000758	0.00000253	1.86	0.2376
rep	2	0.00001850	0.00000925	6.80	0.0287

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Iron	3	0.00000758	0.00000253	1.86	0.2376
rep	2	0.00001850	0.00000925	6.80	0.0287

The SAS System

The GLM Procedure

Dependent Variable: A520_0

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	4.6666667E-6	9.3333333E-7	4.20	0.0548
Error	6	1.3333333E-6	2.2222222E-7		
Corrected Total	11	6E-6			

R-Square	Coeff Var	Root MSE	A520_0 Mean
0.777778	23.57023	0.000471	0.002000

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Iron	3	4.6666667E-6	1.5555556E-6	7.00	0.0219
rep	2	0	0	0.00	1.0000

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Iron	3	4.6666667E-6	1.5555556E-6	7.00	0.0219
rep	2	1.128475E-36	5.642373E-37	0.00	1.0000

The SAS System

The GLM Procedure

Dependent Variable: A520_1

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	1E-6	2E-7	0.45	0.8006
Error	6	2.6666667E-6	4.4444444E-7		
Corrected Total	11	3.6666667E-6			

R-Square	Coeff Var	Root MSE	A520_1 Mean
0.272727	80.00000	0.000667	0.000833

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Iron	3	3.3333333E-7	1.1111111E-7	0.25	0.8587
rep	2	6.6666667E-7	3.3333333E-7	0.75	0.5120

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Iron	3	3.3333333E-7	1.1111111E-7	0.25	0.8587
rep	2	6.6666667E-7	3.3333333E-7	0.75	0.5120

The SAS System

The GLM Procedure

Dependent Variable: ColDen_0

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	0.00006442	0.00001288	17.18	0.0017
Error	6	0.00000450	0.00000075		
Corrected Total	11	0.00006892			

R-Square	Coeff Var	Root MSE	ColDen_0 Mean
0.934704	9.196730	0.000866	0.009417

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Iron	3	0.00005825	0.00001942	25.89	0.0008
rep	2	0.00000617	0.00000308	4.11	0.0751

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Iron	3	0.00005825	0.00001942	25.89	0.0008
rep	2	0.00000617	0.00000308	4.11	0.0751

The SAS System

The GLM Procedure

Dependent Variable: ColDen_1

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	0.00005467	0.00001093	2.81	0.1203
Error	6	0.00002333	0.00000389		
Corrected Total	11	0.00007800			

R-Square	Coeff Var	Root MSE	ColDen_1 Mean
0.700855	32.86711	0.001972	0.006000

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Iron	3	0.00001667	0.00000556	1.43	0.3241
rep	2	0.00003800	0.00001900	4.89	0.0551

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Iron	3	0.00001667	0.00000556	1.43	0.3241
rep	2	0.00003800	0.00001900	4.89	0.0551

The SAS System

The GLM Procedure

Dependent Variable: ColHue_0

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	0.04421358	0.00884272	5.70	0.0280
Error	6	0.00931333	0.00155222		
Corrected Total	11	0.05352692			

R-Square	Coeff Var	Root MSE	ColHue_0 Mean
0.826007	14.56946	0.039398	0.270417

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Iron	3	0.04068492	0.01356164	8.74	0.0131
rep	2	0.00352867	0.00176433	1.14	0.3814

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Iron	3	0.04068492	0.01356164	8.74	0.0131
rep	2	0.00352867	0.00176433	1.14	0.3814

The SAS System

The GLM Procedure

Dependent Variable: ColHue_1

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	0.07477367	0.01495473	0.53	0.7510
Error	6	0.17048333	0.02841389		
Corrected Total	11	0.24525700			

R-Square	Coeff Var	Root MSE	ColHue_1 Mean
0.304879	145.9430	0.168564	0.115500

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Iron	3	0.01513967	0.00504656	0.18	0.9078
rep	2	0.05963400	0.02981700	1.05	0.4066

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Iron	3	0.01513967	0.00504656	0.18	0.9078
rep	2	0.05963400	0.02981700	1.05	0.4066

The SAS System

The GLM Procedure

t Tests (LSD) for A280_0

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	6
Error Mean Square	5.028E-6
Critical Value of t	2.44691
Least Significant Difference	0.0045

Means with the same letter are not significantly different.

t Grouping	Mean	N	Iron
A	0.510333	3	20
B	0.480667	3	10
C	0.472333	3	5
D	0.449667	3	0

The SAS System

The GLM Procedure

t Tests (LSD) for A280_1

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	6
Error Mean Square	6.028E-6
Critical Value of t	2.44691
Least Significant Difference	0.0049

Means with the same letter are not significantly different.

t Grouping	Mean	N	Iron
A	0.449333	3	10
A			
A	0.447667	3	0
A			
A	0.445667	3	20

B 0.438333 3 5

The SAS System

The GLM Procedure

t Tests (LSD) for A420_0

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	6
Error Mean Square	6.389E-7
Critical Value of t	2.44691
Least Significant Difference	0.0016

Means with the same letter are not significantly different.

t Grouping

	Mean	N	Iron
A	0.0090000	3	20
A			
A	0.0086667	3	10

B 0.0070000 3 5

C 0.0043333 3 0

The SAS System

The GLM Procedure

t Tests (LSD) for A420_1

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	6
Error Mean Square	1.361E-6
Critical Value of t	2.44691
Least Significant Difference	0.0023

Means with the same letter are not significantly different.

t Grouping

Mean N Iron

A 0.0063333 3 10

A

A 0.0056667 3 0

A

A 0.0046667 3 20

A

A 0.0043333 3 5

The SAS System

The GLM Procedure

t Tests (LSD) for A520_0

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	6
Error Mean Square	2.222E-7
Critical Value of t	2.44691
Least Significant Difference	0.0009

Means with the same letter are not significantly different.

t Grouping

	Mean	N	Iron
A	0.0026667	3	5
A			
A	0.0023333	3	10
A			
A	0.0020000	3	20
B	0.0010000	3	0

The SAS System

The GLM Procedure

t Tests (LSD) for A520_1

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	6
Error Mean Square	4.444E-7
Critical Value of t	2.44691
Least Significant Difference	0.0013

Means with the same letter are not significantly different.

t Grouping

	Mean	N	Iron
A	0.0010000	3	0
A			
A	0.0010000	3	10
A			
A	0.0006667	3	5
A			
A	0.0006667	3	20

The SAS System

The GLM Procedure

t Tests (LSD) for ColDen_0

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	6
Error Mean Square	7.5E-7

Critical Value of t 2.44691

Least Significant Difference 0.0017

Means with the same letter are not significantly different.

t Grouping

	Mean	N	Iron
A	0.0110000	3	10
A			
A	0.0110000	3	20
A			
A	0.0100000	3	5
B	0.0056667	3	0

The SAS System

The GLM Procedure

t Tests (LSD) for ColDen_1

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha 0.05

Error Degrees of Freedom	6
Error Mean Square	3.889E-6
Critical Value of t	2.44691
Least Significant Difference	0.0039

Means with the same letter are not significantly different.

t Grouping

	Mean	N	Iron
A	0.007333	3	10
A			
A	0.007000	3	0
A			
A	0.005000	3	20
A			
A	0.004667	3	5

The SAS System

The GLM Procedure

t Tests (LSD) for ColHue_0

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	6
Error Mean Square	0.001552
Critical Value of t	2.44691
Least Significant Difference	0.0787

Means with the same letter are not significantly different.

t Grouping

	Mean	N	Iron
A	0.36700	3	5
B	0.26267	3	10
B			
B	0.23667	3	20
B			
B	0.21533	3	0

The SAS System

The GLM Procedure

t Tests (LSD) for ColHue_1

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	6
Error Mean Square	0.028414
Critical Value of t	2.44691
Least Significant Difference	0.3368

Means with the same letter are not significantly different.

t Grouping

	Mean	N	Iron
A	0.1750	3	10
A			
A	0.1043	3	0
A			
A	0.1017	3	5
A			
A	0.0810	3	20

The SAS System

The GLM Procedure

t Tests (LSD) for A280_0

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	6
Error Mean Square	5.028E-6
Critical Value of t	2.44691
Least Significant Difference	0.0039

Means with the same letter are not significantly different.

t Grouping

	Mean	N	rep
A	0.478500	4	2
A			
A	0.478250	4	1
A			
A	0.478000	4	3

The SAS System

The GLM Procedure

t Tests (LSD) for A280_1

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	6
Error Mean Square	6.028E-6
Critical Value of t	2.44691
Least Significant Difference	0.0042

Means with the same letter are not significantly different.

t Grouping	Mean	N	rep
A	0.447000	4	2
A			
A	0.444500	4	1
A			
A	0.444250	4	3

The SAS System

The GLM Procedure

t Tests (LSD) for A420_0

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	6
Error Mean Square	6.389E-7
Critical Value of t	2.44691
Least Significant Difference	0.0014

Means with the same letter are not significantly different.

t Grouping	Mean	N	rep
A	0.0077500	4	3
A			
A	0.0075000	4	2
A			
A	0.0065000	4	1

The SAS System

The GLM Procedure

t Tests (LSD) for A420_1

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	6
Error Mean Square	1.361E-6
Critical Value of t	2.44691
Least Significant Difference	0.002

Means with the same letter are not significantly different.

t Grouping	Mean	N	rep
A	0.0062500	4	2
A			
A	0.0060000	4	3
B	0.0035000	4	1

The SAS System

The GLM Procedure

t Tests (LSD) for A520_0

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	6
Error Mean Square	2.222E-7
Critical Value of t	2.44691
Least Significant Difference	0.0008

Means with the same letter are not significantly different.

t Grouping	Mean	N	rep
A	0.0020000	4	1
A			
A	0.0020000	4	2
A			
A	0.0020000	4	3

The SAS System

The GLM Procedure

t Tests (LSD) for A520_1

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	6
Error Mean Square	4.444E-7
Critical Value of t	2.44691
Least Significant Difference	0.0012

Means with the same letter are not significantly different.

t Grouping	Mean	N	rep
A	0.0010000	4	3
A			
A	0.0010000	4	2
A			
A	0.0005000	4	1

The SAS System

The GLM Procedure

t Tests (LSD) for ColDen_0

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	6
Error Mean Square	7.5E-7
Critical Value of t	2.44691
Least Significant Difference	0.0015

Means with the same letter are not significantly different.

t Grouping	Mean	N	rep
A	0.0102500	4	3
A			
B A	0.0095000	4	2
B			
B	0.0085000	4	1

The SAS System

The GLM Procedure

t Tests (LSD) for ColDen_1

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	6
Error Mean Square	3.889E-6
Critical Value of t	2.44691
Least Significant Difference	0.0034

Means with the same letter are not significantly different.

t Grouping

	Mean	N	rep
A	0.007500	4	3
A			
A	0.007000	4	2
B	0.003500	4	1

The SAS System

The GLM Procedure

t Tests (LSD) for ColHue_0

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	6
Error Mean Square	0.001552
Critical Value of t	2.44691
Least Significant Difference	0.0682

Means with the same letter are not significantly different.

t Grouping	Mean	N	rep
A	0.28425	4	3
A			
A	0.28075	4	1
A			
A	0.24625	4	2

The SAS System

The GLM Procedure

t Tests (LSD) for ColHue_1

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	6
Error Mean Square	0.028414
Critical Value of t	2.44691
Least Significant Difference	0.2917

Means with the same letter are not significantly different.

t Grouping

	Mean	N	rep
A	0.1995	4	3
A			
A	0.1200	4	2
A			
A	0.0270	4	1

12.2.2 Red Wine

The SAS System

The GLM Procedure

Class Level Information

Class	Levels	Values
Iron	4	0 5 10 20
rep	3	1 2 3

Number of observations 12

The SAS System

The GLM Procedure

Dependent Variable: A280_0

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	0.01455708	0.00291142	1.19	0.4129
Error	6	0.01468983	0.00244831		

Corrected Total	11	0.02924692

R-Square	Coeff Var	Root MSE	A280_0 Mean
0.497731	1.883113	0.049480	2.627583

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Iron	3	0.01426892	0.00475631	1.94	0.2241
rep	2	0.00028817	0.00014408	0.06	0.9434

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Iron	3	0.01426892	0.00475631	1.94	0.2241
rep	2	0.00028817	0.00014408	0.06	0.9434

The SAS System

The GLM Procedure

Dependent Variable: A280_1

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	0.01914608	0.00382922	2.83	0.1190
Error	6	0.00812283	0.00135381		
Corrected Total	11	0.02726892			

R-Square	Coeff Var	Root MSE	A280_1 Mean
0.702121	1.407264	0.036794	2.614583

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Iron	3	0.01901492	0.00633831	4.68	0.0516
rep	2	0.00013117	0.00006558	0.05	0.9531

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Iron	3	0.01901492	0.00633831	4.68	0.0516
rep	2	0.00013117	0.00006558	0.05	0.9531

The SAS System

The GLM Procedure

Dependent Variable: A420_0

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	0.00041675	0.00008335	5.59	0.0293
Error	6	0.00008950	0.00001492		
Corrected Total	11	0.00050625			

R-Square	Coeff Var	Root MSE	A420_0 Mean
0.823210	0.973462	0.003862	0.396750

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Iron	3	0.00029625	0.00009875	6.62	0.0248
rep	2	0.00012050	0.00006025	4.04	0.0774

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Iron	3	0.00029625	0.00009875	6.62	0.0248
rep	2	0.00012050	0.00006025	4.04	0.0774

The SAS System

The GLM Procedure

Dependent Variable: A420_1

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	0.00047108	0.00009422	5.24	0.0339
Error	6	0.00010783	0.00001797		
Corrected Total	11	0.00057892			

R-Square	Coeff Var	Root MSE	A420_1 Mean
0.813733	1.105201	0.004239	0.383583

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Iron	3	0.00044292	0.00014764	8.21	0.0152
rep	2	0.00002817	0.00001408	0.78	0.4985

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Iron	3	0.00044292	0.00014764	8.21	0.0152
rep	2	0.00002817	0.00001408	0.78	0.4985

The SAS System

The GLM Procedure

Dependent Variable: A520_0

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	0.00121217	0.00024243	12.38	0.0041
Error	6	0.00011750	0.00001958		
Corrected Total	11	0.00132967			

R-Square	Coeff Var	Root MSE	A520_0 Mean
0.911632	0.753671	0.004425	0.587167

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Iron	3	0.00089700	0.00029900	15.27	0.0032
rep	2	0.00031517	0.00015758	8.05	0.0200

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Iron	3	0.00089700	0.00029900	15.27	0.0032
rep	2	0.00031517	0.00015758	8.05	0.0200

The SAS System

The GLM Procedure

Dependent Variable: A520_1

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	0.00114342	0.00022868	9.18	0.0089
Error	6	0.00014950	0.00002492		
Corrected Total	11	0.00129292			

R-Square	Coeff Var	Root MSE	A520_1 Mean
0.884370	0.861993	0.004992	0.579083

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Iron	3	0.00108625	0.00036208	14.53	0.0037
rep	2	0.00005717	0.00002858	1.15	0.3785

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Iron	3	0.00108625	0.00036208	14.53	0.0037
rep	2	0.00005717	0.00002858	1.15	0.3785

The SAS System

The GLM Procedure

Dependent Variable: ColDen_0

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	0.00293250	0.00058650	8.31	0.0114
Error	6	0.00042350	0.00007058		
Corrected Total	11	0.00335600			

R-Square	Coeff Var	Root MSE	ColDen_0 Mean
0.873808	0.853800	0.008401	0.984000

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Iron	3	0.00220600	0.00073533	10.42	0.0086
rep	2	0.00072650	0.00036325	5.15	0.0499

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Iron	3	0.00220600	0.00073533	10.42	0.0086
rep	2	0.00072650	0.00036325	5.15	0.0499

The SAS System

The GLM Procedure

Dependent Variable: ColDen_1

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	0.00300425	0.00060085	7.45	0.0149
Error	6	0.00048400	0.00008067		
Corrected Total	11	0.00348825			

R-Square	Coeff Var	Root MSE	ColDen_1 Mean
0.861248	0.932897	0.008981	0.962750

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Iron	3	0.00284625	0.00094875	11.76	0.0063
rep	2	0.00015800	0.00007900	0.98	0.4285

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Iron	3	0.00284625	0.00094875	11.76	0.0063
rep	2	0.00015800	0.00007900	0.98	0.4285

The SAS System

The GLM Procedure

Dependent Variable: ColHue_0

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	0.00124758	0.00024952	11.58	0.0049
Error	6	0.00012933	0.00002156		
Corrected Total	11	0.00137692			

R-Square	Coeff Var	Root MSE	ColHue_0 Mean
0.906070	0.313614	0.004643	1.480417

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Iron	3	0.00029492	0.00009831	4.56	0.0544
rep	2	0.00095267	0.00047633	22.10	0.0017

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Iron	3	0.00029492	0.00009831	4.56	0.0544
rep	2	0.00095267	0.00047633	22.10	0.0017

The SAS System

The GLM Procedure

Dependent Variable: ColHue_1

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	0.00111442	0.00022288	4.61	0.0449
Error	6	0.00028983	0.00004831		
Corrected Total	11	0.00140425			

R-Square	Coeff Var	Root MSE	ColHue_1 Mean
0.793603	0.460356	0.006950	1.509750

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Iron	3	0.00101492	0.00033831	7.00	0.0219
rep	2	0.00009950	0.00004975	1.03	0.4126

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Iron	3	0.00101492	0.00033831	7.00	0.0219
rep	2	0.00009950	0.00004975	1.03	0.4126

The SAS System

The GLM Procedure

t Tests (LSD) for A280_0

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	6
Error Mean Square	0.002448
Critical Value of t	2.44691
Least Significant Difference	0.0989

Means with the same letter are not significantly different.

t Grouping

	Mean	N	Iron
A	2.66733	3	5
A			
A	2.63667	3	20
A			
A	2.63400	3	10
A			
A	2.57233	3	0

The SAS System

The GLM Procedure

t Tests (LSD) for A280_1

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	6
Error Mean Square	0.001354
Critical Value of t	2.44691
Least Significant Difference	0.0735

Means with the same letter are not significantly different.

t Grouping

	Mean	N	Iron
A	2.67367	3	20
A			
B A	2.62333	3	5
B			
B	2.59600	3	10
B			
B	2.56533	3	0

The SAS System

The GLM Procedure

t Tests (LSD) for A420_0

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	6
Error Mean Square	0.000015

Critical Value of t 2.44691

Least Significant Difference 0.0077

Means with the same letter are not significantly different.

t Grouping

	Mean	N	Iron
A	0.405000	3	10
B	0.396000	3	5
B			
B	0.394000	3	0
B			
B	0.392000	3	20

The SAS System

The GLM Procedure

t Tests (LSD) for A420_1

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha 0.05

Error Degrees of Freedom	6
Error Mean Square	0.000018
Critical Value of t	2.44691
Least Significant Difference	0.0085

Means with the same letter are not significantly different.

t Grouping	Mean	N	Iron
A	0.389667	3	20
A			
A	0.388333	3	0
A			
B A	0.382000	3	10
B			
B	0.374333	3	5

The SAS System

The GLM Procedure

t Tests (LSD) for A520_0

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	6
Error Mean Square	0.00002
Critical Value of t	2.44691
Least Significant Difference	0.0088

Means with the same letter are not significantly different.

t Grouping	Mean	N	Iron
A	0.600667	3	10
B	0.588667	3	5
B			
C B	0.580667	3	0
C			
C	0.578667	3	20

The SAS System

The GLM Procedure

t Tests (LSD) for A520_1

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	6
Error Mean Square	0.000025
Critical Value of t	2.44691
Least Significant Difference	0.01

Means with the same letter are not significantly different.

t Grouping	Mean	N	Iron
A	0.591333	3	0
A			
B A	0.584000	3	20
B			
B C	0.575000	3	10
C			
C	0.566000	3	5

The SAS System

The GLM Procedure

t Tests (LSD) for ColDen_0

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	6
Error Mean Square	0.000071
Critical Value of t	2.44691
Least Significant Difference	0.0168

Means with the same letter are not significantly different.

t Grouping	Mean	N	Iron
A	1.005667	3	10
B	0.985000	3	5
B			
B	0.974667	3	0
B			

B 0.970667 3 20

The SAS System

The GLM Procedure

t Tests (LSD) for ColDen_1

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	6
Error Mean Square	0.000081
Critical Value of t	2.44691
Least Significant Difference	0.0179

Means with the same letter are not significantly different.

t Grouping	Mean	N	Iron
A	0.979667	3	0
A			
B A	0.973667	3	20

B

B C 0.957667 3 10

C

C 0.940000 3 5

The SAS System

The GLM Procedure

t Tests (LSD) for ColHue_0

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	6
Error Mean Square	0.000022
Critical Value of t	2.44691
Least Significant Difference	0.0093

Means with the same letter are not significantly different.

t Grouping

Mean N Iron

A 1.486667 3 5

A

B A 1.483667 3 10

B

B C 1.477000 3 20

C

C 1.474333 3 0

The SAS System

The GLM Procedure

t Tests (LSD) for ColHue_1

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	6
Error Mean Square	0.000048
Critical Value of t	2.44691
Least Significant Difference	0.0139

Means with the same letter are not significantly different.

t Grouping

	Mean	N	Iron
A	1.523000	3	0
A			
B A	1.513000	3	5
B			
B C	1.504333	3	10
C			
C	1.498667	3	20

The SAS System

The GLM Procedure

t Tests (LSD) for A280_0

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	6
Error Mean Square	0.002448
Critical Value of t	2.44691
Least Significant Difference	0.0856

Means with the same letter are not significantly different.

t Grouping	Mean	N	rep
A	2.63275	4	3
A			
A	2.62900	4	2
A			
A	2.62100	4	1

The SAS System

The GLM Procedure

t Tests (LSD) for A280_1

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	6
Error Mean Square	0.001354
Critical Value of t	2.44691
Least Significant Difference	0.0637

Means with the same letter are not significantly different.

t Grouping	Mean	N	rep
A	2.61925	4	1
A			
A	2.61250	4	2
A			
A	2.61200	4	3

The SAS System

The GLM Procedure

t Tests (LSD) for A420_0

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	6
Error Mean Square	0.000015
Critical Value of t	2.44691
Least Significant Difference	0.0067

Means with the same letter are not significantly different.

t Grouping

	Mean	N	rep
A	0.400500	4	2
A			
B A	0.397000	4	1
B			
B	0.392750	4	3

The SAS System

The GLM Procedure

t Tests (LSD) for A420_1

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	6
Error Mean Square	0.000018
Critical Value of t	2.44691
Least Significant Difference	0.0073

Means with the same letter are not significantly different.

t Grouping

	Mean	N	rep
A	0.385750	4	3
A			
A	0.382500	4	2
A			
A	0.382500	4	1

The SAS System

The GLM Procedure

t Tests (LSD) for A520_0

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	6
Error Mean Square	0.00002
Critical Value of t	2.44691
Least Significant Difference	0.0077

Means with the same letter are not significantly different.

t Grouping

	Mean	N	rep
A	0.592500	4	1
A			
A	0.588750	4	2
B	0.580250	4	3

The SAS System

The GLM Procedure

t Tests (LSD) for A520_1

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	6
Error Mean Square	0.000025
Critical Value of t	2.44691
Least Significant Difference	0.0086

Means with the same letter are not significantly different.

t Grouping

	Mean	N	rep
A	0.582000	4	3
A			
A	0.578500	4	1
A			
A	0.576750	4	2

The SAS System

The GLM Procedure

t Tests (LSD) for ColDen_0

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	6
Error Mean Square	0.000071
Critical Value of t	2.44691
Least Significant Difference	0.0145

Means with the same letter are not significantly different.

t Grouping

	Mean	N	rep
A	0.989750	4	1
A			
A	0.989250	4	2
B	0.973000	4	3

The SAS System

The GLM Procedure

t Tests (LSD) for ColDen_1

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	6
Error Mean Square	0.000081
Critical Value of t	2.44691
Least Significant Difference	0.0155

Means with the same letter are not significantly different.

t Grouping

	Mean	N	rep
A	0.967750	4	3
A			
A	0.961250	4	1
A			
A	0.959250	4	2

The SAS System

The GLM Procedure

t Tests (LSD) for ColHue_0

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	6
Error Mean Square	0.000022
Critical Value of t	2.44691
Least Significant Difference	0.008

Means with the same letter are not significantly different.

t Grouping

	Mean	N	rep
A	1.492250	4	1
B	1.478250	4	3
B			
B	1.470750	4	2

The SAS System

The GLM Procedure

t Tests (LSD) for ColHue_1

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	6
Error Mean Square	0.000048
Critical Value of t	2.44691
Least Significant Difference	0.012

Means with the same letter are not significantly different.

t Grouping

	Mean	N	rep
A	1.513500	4	1
A			
A	1.509250	4	3
A			
A	1.506500	4	2

12.3 BSA analysis

12.3.1 BSA treatment

Data\SAS\BSA_SAS_Result_data1.rtf

Name	Replicate	Molar ratio BSA 1: PA (mM)	PA added (mg/L)	BSA (mg/L)
P250	1	0.0	0	863
P250	2	0.0	0	883
P250	3	0.0	0	876
P500	1	0.0	0	990
P500	2	0.0	0	983
P500	3	0.0	0	997

Name	Replicate	Molar ratio BSA 1: PA (mM)	PA added (mg/L)	BSA (mg/L)
P1000	1	0.0	0	982
P1000	2	0.0	0	985
P1000	3	0.0	0	993
P1000	1	1.0	10	992
P1000	2	1.0	10	983
P1000	3	1.0	10	978
P500	1	2.0	20	1017
P500	2	2.0	20	1008
P500	3	2.0	20	1000
P250	1	4.0	40	819
P250	2	4.0	40	856
P250	3	4.0	40	944
P1000	1	10.0	100	0
P1000	2	10.0	100	0
P1000	3	10.0	100	0
P500	1	20.0	200	0
P500	2	20.0	200	0
P500	3	20.0	200	0
P250	1	40.0	400	0
P250	2	40.0	400	0
P250	3	40.0	400	0

Name	Replicate	Molar ratio BSA 1: PA (mM)	PA added (mg/L)	BSA (mg/L)
P1000	1	100.0	1000	0
P1000	2	100.0	1000	0
P1000	3	100.0	1000	0
P500	1	200.0	2000	0
P500	2	200.0	2000	0
P500	3	200.0	2000	0
P250	1	400.0	4000	0
P250	2	400.0	4000	0
P250	3	400.0	4000	0

The SAS System

The GLM Procedure

Class Level Information

Class	Levels	Values
rep	3	1 2 3
molarBSA	12	0 0 0 1 2 4 10 20 40 100 200 400

Number of observations 36

The SAS System

The GLM Procedure

Dependent Variable: BSA

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	13	8227369.361	632874.566	1704.83	<.0001
Error	22	8166.944	371.225		
Corrected Total	35	8235536.306			

R-Square	Coeff Var	Root MSE	BSA Mean
0.999008	4.044661	19.26719	476.3611

Source	DF	Type I SS	Mean Square	F Value	Pr > F
molarBSA	11	8226676.306	747879.664	2014.63	<.0001
rep	2	693.056	346.528	0.93	0.4082

Source	DF	Type III SS	Mean Square	F Value	Pr > F
molarBSA	11	8226676.306	747879.664	2014.63	<.0001
rep	2	693.056	346.528	0.93	0.4082

The SAS System

The GLM Procedure

t Tests (LSD) for BSA

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	22
Error Mean Square	371.2247
Critical Value of t	2.07387
Least Significant Difference	32.625

Means with the same letter are not significantly different.

t Grouping

			molar BSA
	Mean	N	
A	1008.33	3	2
A			

A 990.00 3 0

A

A 986.67 3 0

A

A 984.33 3 1

B 874.00 3 0

B

B 873.00 3 4

C 0.00 3 10

C

C 0.00 3 20

C

C 0.00 3 40

C

C 0.00 3 100

C

C 0.00 3 200

C

C 0.00 3 400

The SAS System

The GLM Procedure

t Tests (LSD) for BSA

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	22
Error Mean Square	371.2247
Critical Value of t	2.07387
Least Significant Difference	16.313

Means with the same letter are not significantly different.

t Grouping

	Mean	N	rep
A	482.333	12	3
A			
A	474.833	12	2
A			
A	471.917	12	1

12.3.2 BSA results comparison after two weeks

Data\SAS\BSA_SAS_Result_2wks.rtf

Date	Name	Replicate	Molar ratio PA:BSA (mM)	PA added (mg/L)	BSA (mg/L)
1	P250-10	1	4	40	819
1	P250-10	2	4	40	856
1	P250-10	3	4	40	944
1	P500-10	1	2	20	1017
1	P500-10	2	2	20	1008
1	P500-10	3	2	20	1000
1	P1000-10	1	1	10	992
1	P1000-10	2	1	10	983
1	P1000-10	3	1	10	978
2	P250-10	1	4	40	830
2	P250-10	2	4	40	829
2	P250-10	3	4	40	820
2	P500-10	1	2	20	999
2	P500-10	2	2	20	995
2	P500-10	3	2	20	996
2	P1000-10	1	1	10	978
2	P1000-10	2	1	10	975
2	P1000-10	3	1	10	975

Date 1: 2006/04/07

Date 2: 2006/04/23

Days: 16

The SAS System

The GLM Procedure

Class Level Information

Class	Levels	Values
date	2	1 2
rep	3	1 2 3
molarBSA	3	1 2 4
Number of observations		18

The SAS System

The GLM Procedure

Dependent Variable: BSA

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	84590.44444	16918.08889	21.77	<.0001
Error	12	9325.33333	777.11111		
Corrected Total	17	93915.77778			

R-Square	Coeff Var	Root MSE	BSA Mean
0.900705	2.952694	27.87671	944.1111

Source	DF	Type I SS	Mean Square	F Value	Pr > F
molarBSA	2	81774.11111	40887.05556	52.61	<.0001
date	1	2222.22222	2222.22222	2.86	0.1166
rep	2	594.11111	297.05556	0.38	0.6903

Source	DF	Type III SS	Mean Square	F Value	Pr > F
molarBSA	2	81774.11111	40887.05556	52.61	<.0001
date	1	2222.22222	2222.22222	2.86	0.1166
rep	2	594.11111	297.05556	0.38	0.6903

The SAS System

The GLM Procedure

t Tests (LSD) for BSA

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	12

Error Mean Square	777.1111
Critical Value of t	2.17881
Least Significant Difference	35.067

Means with the same letter are not significantly different.

t Grouping

molar

	Mean	N	BSA
A	1002.50	6	2
A			
A	980.17	6	1
B	849.67	6	4

The SAS System

The GLM Procedure

t Tests (LSD) for BSA

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	12

Error Mean Square	777.1111
Critical Value of t	2.17881
Least Significant Difference	35.067

Means with the same letter are not significantly different.

t Grouping	Mean	N	rep
A	952.17	6	3
A			
A	941.00	6	2
A			
A	939.17	6	1

The SAS System

The GLM Procedure

t Tests (LSD) for BSA

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05

Error Degrees of Freedom	12
Error Mean Square	777.1111
Critical Value of t	2.17881
Least Significant Difference	28.632

Means with the same letter are not significantly different.

t Grouping	Mean	N	date
A	955.22	9	1
A			
A	933.00	9	2

12.3.3 BSA 200 mg/L to molar ratio of phytic acid

Data\SAS\BSA_SAS_Result_200.rtf

Replicate	Molar ratio PA:BSA (mM)	Weight ratio BSA:PA (mg/L)	BSA added (mg/L)	PA added (mg/L)	BSA (mg/L)	pH
1	0.0	0	200	0	203 a	3.77 a
2	0.0	0	200	0	202 a	3.75 a
3	0.0	0	200	0	200 a	3.81 a
1	2.5	40	200	5	127 b	3.77 a
2	2.5	40	200	5	127 b	3.81 a
3	2.5	40	200	5	128 b	3.79 a
1	5.0	20	200	10	45 c	3.81 a
2	5.0	20	200	10	46 c	3.79 a
3	5.0	20	200	10	45 c	3.81 a
1	7.5	13	200	15	8 d	3.85 a
2	7.5	13	200	15	9 d	3.81 a
3	7.5	13	200	15	8 d	3.85 a
1	10.0	10	200	20	8 d	3.88 a
2	10.0	10	200	20	8 d	3.82 a
3	10.0	10	200	20	8 d	3.85 a

The SAS System

The GLM Procedure

Class Level Information

Class	Levels	Values
rep	3	1 2 3
molarBSA	5	0 2.5 5 7.5 10
Number of observations		15

The SAS System

The GLM Procedure

Dependent Variable: BSA

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	6	85644.00000	14274.00000	19917.2	<.0001
Error	8	5.73333	0.71667		
Corrected Total	14	85649.73333			

R-Square	Coeff Var	Root MSE	BSA Mean
0.999933	1.083483	0.846562	78.13333

Source	DF	Type I SS	Mean Square	F Value	Pr > F
molarBSA	4	85643.06667	21410.76667	29875.5	<.0001

rep	2	0.93333	0.46667	0.65	0.5470

Source	DF	Type III SS	Mean Square	F Value	Pr > F
molarBSA	4	85643.06667	21410.76667	29875.5	<.0001
rep	2	0.93333	0.46667	0.65	0.5470

The SAS System

The GLM Procedure

Dependent Variable: pH

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	6	0.02844000	0.00474000	Infty	<.0001
Error	8	0.00000000	0.00000000		
Corrected Total	14	0.02844000			

R-Square	Coeff Var	Root MSE	pH Mean
1.000000	0	0	3.848000

Source	DF	Type I SS	Mean Square	F Value	Pr > F
molarBSA	4	0.02844000	0.00711000	Infty	<.0001
rep	2	0.00000000	0.00000000	.	.

Source	DF	Type III SS	Mean Square	F Value	Pr > F
molarBSA	4	0.02844000	0.00711000	Infty	<.0001
rep	2	0.00000000	0.00000000	.	.

The SAS System

The GLM Procedure

t Tests (LSD) for BSA

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	8
Error Mean Square	0.716667
Critical Value of t	2.30600
Least Significant Difference	1.5939

Means with the same letter are not significantly different.

t Grouping	Mean	N	molar BSA

A	201.6667	3	0
B	127.3333	3	2.5
C	45.3333	3	5
D	8.3333	3	7.5
D			
D	8.0000	3	10

The SAS System

The GLM Procedure

t Tests (LSD) for pH

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	8
Error Mean Square	0.001055
Critical Value of t	2.30600
Least Significant Difference	0.0612

Means with the same letter are not significantly different.

t Grouping

	Mean	N	molar BSA
A	3.840	3	10
A			
A	3.837	3	7.5
A			
A	3.847	3	5
A			
A	3.790	3	2.5
A			
A	3.810	3	0

BIBLIOGRAPHY

Mr. Brent C. Trela has been a winemaker, educator and designer with progressively responsible management experience in all phases of winery design, production and management, producing consistently high quality award winning wines, and university education since 1994. He specializes in development projects to aid the world's poor and quality of life improvements. His wines have received numerous awards and accolades. His work history has spanned the globe from the U.S.A. where he worked as a winemaker for 10 years in Washington State; started his own international winemaking consultation business called Alert Aesthetics (www.alertaesthetics.com). During the previous 8 years he has been a consulting winemaker and design engineer for new ultra premium wineries in Australia and New Zealand, as well as new vineyard and winery ventures in Thailand, and China. Recently he worked as a senior scientist and advisor to Texas A & M University in a project with the US Embassy and United States Department of Agriculture where he assisted approximately 20 large and small Armenian and Georgian wineries and wine related industries. He initiated and directed new research programs in the areas of indigenous yeast isolation, identification and selection; and of metal chelation in wines resulting in a pending US patent. He has taught chemistry, wine science, wine business and wine appreciation among international organizations and university degree programs. Currently he is a private consultant, and the Program Director of Distance Education in Winemaking for the University of California, Davis Extension.