SOURCES, DISTRIBUTION AND SPECIATION OF ALUMINIUM IN WINE PRODUCTION

Mark Willem Adrianus Hendriks

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School of Molecular Sciences

Faculty of Science, Engineering & Technology

Victoria University of Technology

Melbourne, Victoria, Australia

DECLARATION OF AUTHENTICITY

I hereby declare that the work presented in this thesis is my own and has been carried out in the School of Life Sciences and Technology, Victoria University of Technology (Footscray Park, St. Albans & Werribee Campuses, Melbourne, Australia) and Gribbles Analytical Services (formerly National Analytical Laboratories, Blackburn, Australia) during the period of March 1996 through to June 2001. This thesis contains no material which has been accepted for the award of any other degree or diploma in any university or other institution, and to the best of my knowledge contains no material previously published or written by another person, except where due reference is made.

Mark Hendriks [B.App.Sc.(App.Chem.), BSc.(Hons)]

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In the final years of the last century, I began this great quest. However, as I basked in the enthusiasm and exhilaration of obtaining a scholarship and acceptance to do my PhD, I could not imagine how epic a journey this project and this thesis would be, until now. Much water has passed under the bridge since those early days and I have changed in this great span of time. But like the great voyager, Odysseus, I never lost sight of home, the ultimate goal of one day getting to this very point, writing my last and epochal section of this thesis and closing a door on an incredible yet sometimes frustrating and wearying experience. This is due immeasurably to many people, who, even in the darkest and gravest hours when the task appeared impossible, provided me with the fortitude and resilience to continue.

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"Homo sapiens..... what an indomitable species"
Fourth Doctor, from television series Doctor Who

ABSTRACT

The bioavailability and toxicity of aluminium in humans resulting from dietary intake, including beverages such as wine, has been of political concern over the last 20 years. In order to ascertain its potential bioavailability and toxicity from a dietary source such aa wine a full knowledge of the concentration of aluminium and its speciation must be determined. A complete study of the aluminium profile over the entire wine production process has not been described previously and an investigation of the speciation of aluminium in wine has not been undertaken. This thesis sets out to establish the sources and sinks of aluminium during red and white table wine production and to describe the speciation of aluminium in wine.

Aluminium speciation methods and the workings and merits of the chosen speciation technique, electrospray mass spectrometry (ES-MS), are reviewed in this thesis. The methodology section discusses method development, particularly regarding sample digestion techniques, and the procedures used to combat contamination. Sixteen individual wines from vineyard/wineries near Melbourne for the 1997 and 1998 vintages were sampled and analysed throughout the production process to determine the total aluminium profile. Some of these bottled wines were analysed for aluminium speciation by ES-MS after an extensive method development identifying Al-organic acid species in aqueous and model wine media.

The profile of aluminium during wine production showed that the majority of aluminium in wine is 'naturally' derived from the grapes which showed aluminium concentrations up to 6μg/g. Grape aluminium distribution analysis showed that 57-75% of this grape aluminium is carried over into the must. The most significant change in aluminium concentration occurred during the fermentation process where, on average, 1.1mg/L (70%) is lost from the ferment during this stage. The mechanism remains unclear but the lees appear to be the most likely sink for aluminium. As reported by earlier studies, bentonite fining gave a significant increase in wine aluminium concentration, however the magnitude of the increase was only 20% that of the decrease observed during fermentation. Bentonite fining accounts for the higher mean aluminium concentrations of white table wine as opposed to red table wines, which are 0.90mg/L and 0.43mg/L respectively. The

overall mean concentration for all wines studied was 0.70mg/L. Little difference was observed between the aluminium profiles of red and white wines, and the 1997 versus 1998 vintages.

While aluminium complexes with organic acids in aqueous and model wine solutions could be discerned using ES-MS, these complexes were not evident in diluted and aluminium spiked diluted wines using ES-MS directly in the positive ion mode. However, indirect analyses of organic acid anions in diluted wine showed that the anion intensities decreased with increasing aluminium concentration. This suggested that aluminium is complexed with organic acids of which bitartrate was found to preferentially bind with aluminium in wine. Complexes identified directly in positive ion mode ES-MS in aqueous and model wine solutions suggest the possible species of aluminium with organic acid species in wine at pH 3-3.5 are tetrahedral or octahedral ML_2 or mixed complexes (MLL') with or without water molecules attached. The exception was citrate, for which hydrated tetrahedral or octahedral ML species were observed.

This work allows a better understanding of the aluminium wine production profile that can aid in future reductions in wine aluminium loads and provides a framework for increased knowledge of the bioavailability of aluminium in wine and hence its potential metabolism and toxicity from this beverage to the wine consumer.

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"The only good is knowledge, and the only evil is ignorance"
Herodotus, Greek Historian (484-425BC)

CHAPTER ONE

1. INTRODUCTION

1.1 Relevance and Objectives of This Study

The quality of Australian wines is internationally recognised and in the last 20 years the industry's export sales have grown to make up over half the Australian wine market with an increase from 3% exported in 1980-81 to 51% in 2001-2002 (see Figure 1.1, AWBC 2002). The most rapid growth however has occurred in the last decade, with wine exports increasing rapidly. The export of Australian wines in the 12 months to December 1995 totalled 113.4 million litres netting $A391.5 million (Wiley 1996). In the past 7 years this figure has increased almost 4-fold to 417.3 million litres in the financial year ending June 2002 setting a new export sales record at $A2.000 billion (AWEC & AWBC 2002a). Of the 12 months up until November 2002, the annual export sales figure now tops $A2.191 billion with the industry showing consistent growth of 20-30% over recent years (AWEC & AWBC 2002b). The top ten export destinations for Australian wine show the UK and the US as our major markets as can be seen in Table 1.1. This growth has seen Australia's share of global export volumes triple in the last decade and has been accelerated with the Vision 2025 statement delivered by the Winemakers Federation of Australia in 1996. The industry has set itself the goal 'that by the Year 2025 the Australian wine industry will achieve $4.5 billion in annual sales by being the world's most influential and profitable supplier of branded wines, pioneering wine as a universal first choice lifestyle beverage' (Major 2001). The wine industry in Australia has become a very valuable one, that is basing its growth premise in the quality and marketing of wine as a healthy lifestyle choice in moderation, especially with perceived benefits in reducing cardio-vascular disease (Major 2001). Anything that compromises this perception, or the exploitation of contaminant concentrations as trade barriers by importing countries can potentially threaten a multi-billion dollar industry.

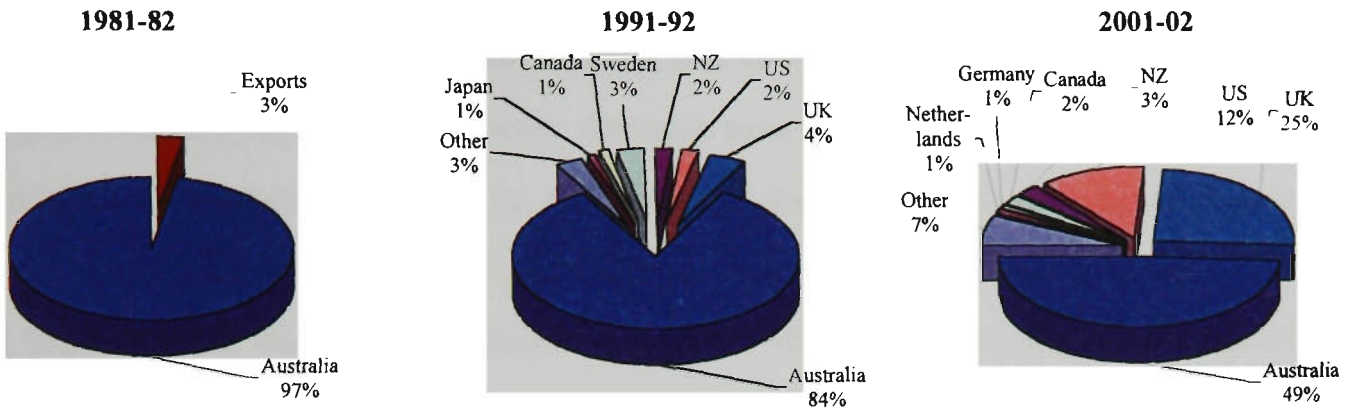


Figure 1.1 Growth of Australian wine export markets over two decades (from AWIIS 2001).

Table 1.1 Summary of export market statistics for Australian wine 2001-02 for 12 months ending November 2002 (from AWEC, AWBC 2002).

Export Destination	Volume (million Litres)	Value ($A million)
United Kingdom	207.3	869.4
United States of America	111.8	691.1
New Zealand	26.4	92.6
Canada	21.6	146.3
Germany	14.8	52.4
Netherlands	9.1	36.3
Denmark	7.8	28.4
Ireland	7.2	39.2
Sweden	7.0	28.7
Switzerland	5.5	31.3
Japan	5.1	30.3
France	4.5	14.1
Belgium-Luxembourg	3.0	13.5
Singapore	2.8	21.7
Norway	2.2	9.4
Other	15.4	86.1
World Total	451.4	2190.8

The aluminium level found in most wines is higher than the WHO drinking water limit of 0.2 mg/L (see Table 1.3 in Section 1.6 for references). Although most current drinking water and wine limits are guidelines for aesthetic purposes only, the amount of aluminium in foods, water and beverages and possible risks it poses to human health has come under scrutiny in the last 20 years (Ganrot 1986; Sherlock 1988; Duffield 1988; Delves et al. 1988; Martin 1988; Pennington & Jones 1988; Orme 1990; UK MAFF 1993; Domingo 1994; Jeffery 1995; Sharpe & Williams 1995; Berthon 1996, 2002, 2002; Scollary 1997; Ysart et al. 1999, 2000; Stauber et al. 1999; Yokel et al. 2001; Soni et al. 2001; Barabasz et al. 2002). There have been several studies on the aluminium content in wine from different countries of which McKinnon (1990) and McKinnon et al. (1992) conducted the most thorough research to date, however only these latter studies included insights into the origins of aluminium from the production process. This was only a minor survey of aluminium concentrations throughout the production process incorporating only five wines looking at 3-4 stages in the winemaking process. Galani-Nikolakaki et al. (2002) on studying Cretan wines, also investigated the aluminium concentrations of grape skins, however suggested that a more thorough research of the aluminium profile over the whole winemaking process was required. Other than these limited studies, no major investigation of the winemaking process has been attempted to comprehensively profile aluminium content from the soil to the bottled wine.

In order to gauge the toxicity of aluminium, its bioavailability is an important factor. This cannot be ascertained in different foodstuffs without having a knowledge of its speciation. It is now well recognised that the speciation has a greater influence on an element's toxicity than its total content (Rubini et al. 2002). 'In terms of acute toxicity, the inorganic forms are believed to be more toxic. However, organically bound species may be capable of crossing biological membranes and contributing to chronic bioaccumulation' (Rubini et al. 2002). Chapter 2 demonstrates that aluminium speciation has been studied extensively in the last decade, the effectiveness and validity of these studies have suffered from the difficulties of identifying individual species due to the limitations of the methods used. Size fractionation studies have intimated possible speciation scenarios in wine, however to this author's knowledge no sophisticated speciation analysis has been attempted to identify aluminium species in wine, let alone the use of Electrospray Mass Spectrometry (ES-MS) to accomplish this. As ES-MS has successfully been used to investigate

aluminium complexes in various media, it is reasonable to assume the technique can be applied to aluminium speciation in wine.

The aims of this study were two-fold. The first was to determine a comprehensive profile of aluminium throughout the winemaking process and to further elucidate and confirm the sources and sinks of aluminium in finished table wine. This aim was to be accomplished by measuring the extractable aluminium concentration in soil and the total aluminium concentration in wine grapes, juice, ferment, unfinished wine over the course of the production process and finished wine, for both white and red cultivars from 5 Central Victorian vineyards/wineries over two vintages (1997-1998). The second aim was to develop an analytical procedure to identify and characterise the speciation of the aluminium in both red and white finished table wines. This would be accomplished by developing aluminium speciation identification with ES-MS on aqueous and model wine solutions. These methods would then be applied to real wine samples produced from the wines analysed for the production profile analysis to determine what aluminium species exist in wine.

The knowledge provided by profiling aluminium in wine production gives the wine industry the ability to take measures in regards to reducing or removing the aluminium content in wines if future legislative, trade or health issues deem that the level of aluminium in wine compromises product quality and safety. The elucidation of the aluminium species in wine will reveal and/or confirm what complexes with aluminium in the wine giving a better understanding of the bioavailability of aluminium to humans. This information can be used to give estimations as to the uptake, metabolism and toxicity of the metal, providing a starting point for any future biological studies on the fate of aluminium in vivo derived from wine consumption.

1.2 Aluminium in the Environment

Aluminium accounts for approximately 8% of the earth's crust, making it the third most abundant element after oxygen and silicon and the most abundant metallic element (see Figure 1.2). With its combination of small ionic radius of 0.52Å, high oxidation potential at +1.66V and high valence charge, Al^{3+} is highly electrophilic and forms very stable compounds (Ganrot 1986; Savory & Wills

1991). Al^{3+} is the hardest 'Lewis' trivalent metal ion and hence hard 'Lewis' bases, in particular, oxygenous ligands, are strongly bound to aluminium by non-covalent bonding that is ionic or electrostatic in nature (Martin 1986,1988; McDonald & Martin 1988; Harris et al. 1996; Yokel 2002). These chemical features underlie the formation and stability of aluminium silicates, hydroxides and oxides in rock, soil and clays (Duffield & Williams 1988; Savory & Wills 1991; Cronan 1994; Jardine & Zelazny 1996).

Because of this stability, the solubilities of these compounds at neutral pH (natural water) are extremely low and renders the majority of aluminium biologically unavailable (Duffield & Williams 1988; Frech & Cedergren 1992; Driscoll & Postek 1996, Barabasz et al. 2002). The low amount of labile aluminium that enters the environment does so by a slow process of dissolution of the aluminium minerals and clays in rocks and soil due to weathering and subsequent water runoff (Ritchie 1995; Lindsay & Walthall 1996) and is the primary source of aluminium to aqueous and biological systems (Driscoll & Schecher 1990). However acidification of soils solubilises Al^{3+} as $[Al(H_2O)_6]^{3+}$ at pH<5.0 and its hydrolysis products $[AlOH]^{2+}$ and $[Al(OH)_2]^{+}$ at pH 3.0-6.0 and can be mobilised into water systems. This can occur in acidic soils with low exchangeable base saturation where neutralisation is incomplete and acidic cations (H^+, Al^{3+}) are transported to surface water by weak and/or strong acid leaching (Driscoll & Schecher 1990; Furrer et al. 1992; Cronan 1994; Driscoll & Postek 1996; Soni et al. 2001). Weak acid leaching occurs naturally in shallow, acidic soils in upland regions under high-flow conditions. Strong acid leaching has come into prominence in the industrial era where burning of fossil fuels containing acidic sulfur and nitrogen oxides has lead to the phenomenon of 'acid rain', particularly in the heavily industrialised areas of North America and Europe (Barabasz et al. 2002), and the nitrification of soils due to modern farming practices. It is strong acid leaching that releases significant amounts of inorganic aluminium into the environment, although soluble organic complexes of aluminium also increase with decreasing pH. Lakes and wetlands act as a sink for this mobilised aluminium (Driscoll & Postek 1996).

Inorganic aluminium can be complexed by organic material in topsoil to form stable compounds that can make up to 50% of monomeric aluminium in the first 10cm of soil and provides another

mechanism for mobilisation of aluminium from soil (Sjoberg et al. 1992; Wolt 1994; Driscoll & Postek 1996). However, aluminium can also combine with solid-phase organic matter and remains virtually immobile under normal conditions (Lindsay & Walthall 1996). It has been shown that prolonged soil acidification results in considerable solubilisation of aluminium from these solid-phase complexes (Berggren et al. 1997). Arnesen (1997) found that fluoride from anthropogenic sources could also induce breakdown of Al-oxides/hydroxides and solubilise aluminium as AlF_x complexes. The solubilisation and transport of aluminium is totally dependent on the speciation of the aluminium which in turn is influenced by pH, ionic strength, availability of soil/sediment aluminium, complexing ligand concentrations and temperature. The concept of speciation is critical to the bioavailability and toxicity of aluminium and is covered in a review of aluminium speciation analysis in Chapter 2. The aluminium cycle in nature is shown schematically in Figure 1.2.

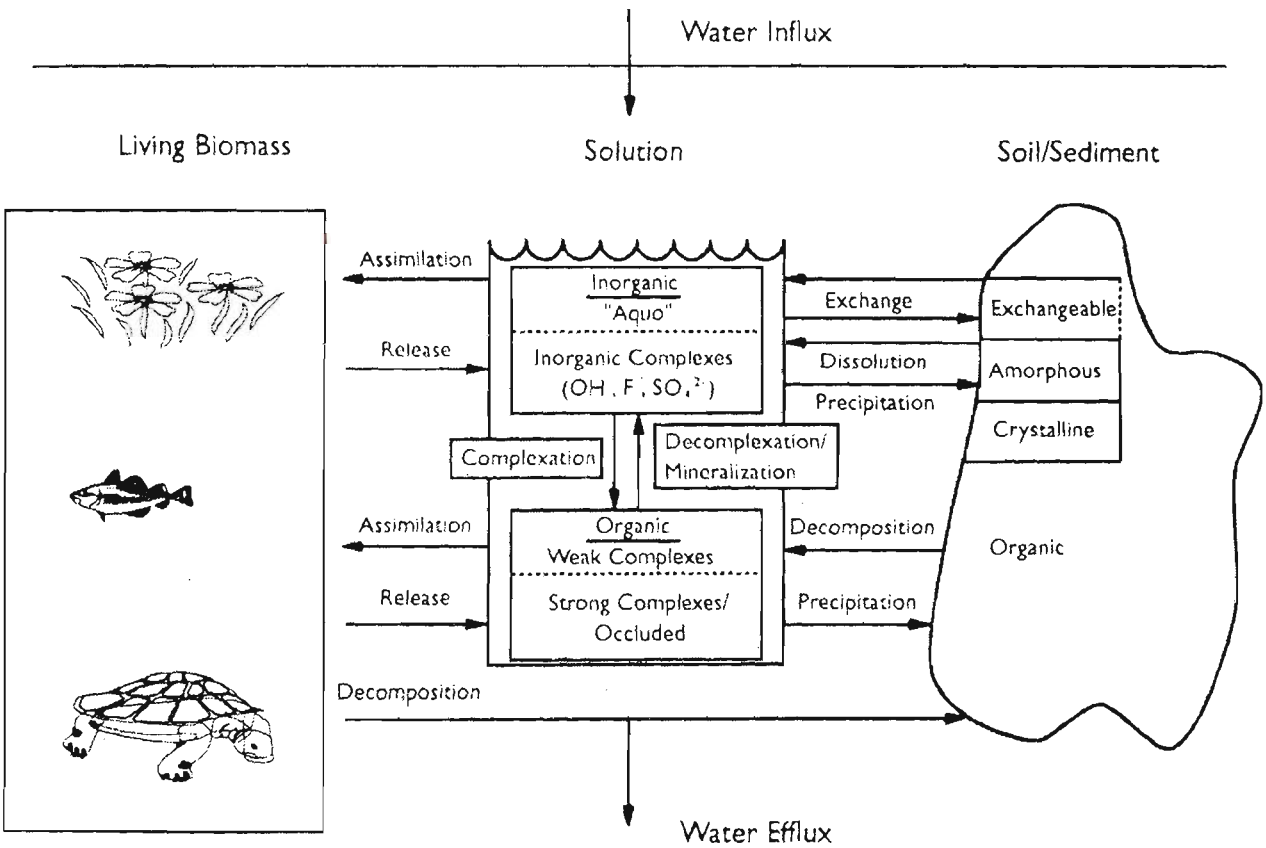


Figure 1.2 A schematic representation of the aluminium cycle (from Driscoll & Postek 1996).

Plants are extremely sensitive to monomeric inorganic species mobilised by lowered soil pH. These species, including Al^{3+}, $[AlOH]^{2+}$ and $[Al(OH)_2]^{+}$, are considered the most toxic aluminium species to biological organisms whereas Al-organic complexes are seen as least toxic (Fishbein 1992; Ross 1994; Kochian 1995; Gensemer & Playle 1999). Higher aluminium concentrations in soils is known

to stunt root growth, branching and cause necrosis of cells in roots and shoots (Blamey & Asher 1993; Wolt 1994; Mohren 1995; Parker 1995). This occurs by interfering with enzyme function and with the uptake of essential nutrients, such as calcium which is required for many cellular processes and relaying of environmental signals, cell division and membrane transport (Cronan 1994; Huang et al. 1995; Kochian 1995; Crawford et al. 1998). The primary site for aluminium-induced toxicity is in the root apex (Kochian 1995). Some plant species have shown a resistance to aluminium. Tea is an example of this, in old tea plants the leaves can accumulate aluminium to a concentration of 2-3% (Flaten 2002). The mechanisms postulated include removal of aluminium from cell systems by complexation with organic acid anions such as citrate and malate, root alterations of the rhizosphere pH to increase pH around the root apices and hence produce insoluble aluminium species, and exclusion of the aluminium from crossing the plasma membrane by binding and immobilisation of aluminium in the root cell walls (Savory & Wills 1991; Kochian 1995; Yokel 2002). Although different plant species vary in their ability to prevent aluminium absorption, and aluminium varies in its mobility from soil to soil, some aluminium uptake past the root plasma membrane and into plant cells will occur. Cottenie et al. (1982) suggested aluminium uptake into aerial parts of plants was a function of the concentration in the soil. However, it is now recognised that total aluminium concentration alone cannot determine absorption and uptake has been shown to be dependent on the speciation of the aluminium (McDonald-Stephens & Taylor 1995). One of the mechanisms postulated for root uptake involves Al^{3+} following the pathways utilised by Fe^{3+} or Mg^{2+} (Kochian 1995) due to the similarity of both ions in terms of charge, size and ligand exchange rates (Jeffery 1995). Once past the root membrane barrier and into the xylem vessels, aluminium could be transported throughout the plant, possibly via iron transport systems, finding its way into the cytoplasm of plant cells. Masunaga et al. (1998) reported that of 77 trees in a rainforest in West Sumatra with elevated aluminium concentrations, the majority had higher aluminium content in the leaves than the bark. It was also found that there was a correlation between leaf aluminium content and leaf phosphorus, sulfur and silicon concentration suggesting that aluminium may have been transported to the leaves as complexes with these elements. Lidon et al. (1998) found that in maize, aluminium can affect the uptake of nitrates by the roots and can reduce the amount of nitrates that are transported to the leaves.

In a review on the bioavailability and toxicity of aluminium to aquatic life, Gensemer & Playle (1999) noted that while aquatic plants and invertebrates generally showed no ill effects due to aluminium, algal growth was reduced under the influence of increased aluminium. Fish were especially sensitive to aluminium where polymerisation at the gills and interference with calcium dependent gill function caused respiratory problems.

1.3 Aluminium in Food & Water

Although most aluminium remains unavailable to biological organisms, its sheer abundance means aluminium is found in soil, water and air and consequently moves up the food chain via plants, animals and liquids into the human diet (Hewitt et al. 1990; Savory & Wills 1991; Frech & Cedergren 1992; Fishbein 1992; UK MAFF 1993; Soni et al. 2001). The sources of aluminium in the human diet are food, water, drugs, cosmetics, aluminium utensils and containers (Rajwanshi et al. 1997; Barabasz et al. 2002), with food acting as the major source of ingested aluminium (Soni et al. 2001). Several studies have been conducted for total aluminium concentrations in foods and foodstuffs. In a comprehensive paper produced for the British Ministry of Agriculture, Fisheries and Food the average daily consumption of aluminium was given as 3.9mg/day or 27mg/week (UK MAFF 1993). This compares well with other estimates of 5-10mg/day (Alfrey 1993), 3.9 and 4.4 mg/day (Delves et al. 1988), 1-20mg/day (Ganrot 1986), 11mg/day (Ysart et al. 1999) and with data in the review by Sherlock (1988) who, finding a striking similarity between the majority of studies conducted in different countries, gave an average of around 5mg Al /day. A more recent British study supports these earlier findings with a reported value of 3.4mg/day (Ysart et al. 2000). A WHO/IPCS (1997) report gave the range of aluminium intake taken from several countries as 0.03-11.5mg/day.

In the 1993 (UK MAFF) paper, it was found that beverages contributed 51% (2.0mg/day) of aluminium to the daily diet and breads and cereals 27% (1.1mg/day). The concentrations ranged from 0.005mg/kg found in ground coffee to 1150mg/kg in sponge flan cases. Delves et al. (1988) on analysing 68 different foods showed concentration ranges of <0.02mg/kg in eggs to 1500mg/kg in some flour mixes. Higher concentrations are found in plant derived food compared with meat

products (Frech & Cedergren 1992) and are probably due to animals virtually excluding aluminium by resistance to uptake in the gastrointestinal tract and poorly accumulating the element. Besides the natural concentrations found in food, other contributors to aluminium in the diet exist. High concentrations of aluminium seen in cereals, bread and pastry have been attributed to aluminium-containing food additives such as antiadherance agents in baked goods (Martin 1988; Delves et al. 1988; Sherlock 1988; Jeffery 1995; Soni et al. 2001). However, additives are not just confined to these foods and are seen to contribute heavily to aluminium in the diet (Ganrot 1986; Sherlock 1988; UK MAFF 1993; Jeffery 1995; Soni et al. 2001). The US Committee on Nutrition (1996) found that infant milk formulas had higher aluminium concentrations than human milk by one to two orders of magnitude, particularly those based on soy protein. This last finding was supported by the work of Ikem et al. (2002) but also found that Ca salts contributed to high aluminium concentrations in infant formulas.

Water is another source of dietary intake, estimated to be 0.2-0.4mg/day (UK MAFF 1993; Soni et al. 2001). The EU and WHO limit for aluminium in water has been set at 0.2mg/L (Uwers 1991). Although these guidelines in some countries are under review (Srinivasan et al. 1999; Stauber et al. 1999), they are primarily set from an aesthetic rather than a toxicological point of view as high water aluminium concentrations produce a 'noticeable opalescence' (Gardner & Gunn 1995). A table of water quality guidelines is given in Table 1.2.

Table 1.2 International water quality standards for aluminium in drinking water (from Srinivasan et al. 1999).

Organisation/Government	Guideline concentrations (mg/l)	Maximum acceptable concentration (mg/l)
World Health Organisation	0.20	----
European Union	0.05	0.20
Belgium	0.05	0.10
Germany	0.05	0.20
Sweden	----	0.10
Switzerland	0.05	0.50
US Environment Protection Agency	0.05	0.20
American Water Works Association Recommended Operating Level	----	0.20
New York State guidelines on Al in filtered water for pilot-plant studies. Minimum percent of recorded values 95% 75% 50%	 < 0.15 < 0.09 < 0.05	 ---- ---- ----
1986 Proposed Illinois Regulation	0.10	----
Finland	----	0.20
Denmark	0.05	0.20
Austria	----	0.20
California Code of Regulations (maximum contaminant level)	----	1.00

Young et al. (1996) tested panels of 6-10 females for taste thresholds of various chemical additives and contaminants including aluminium and found that 4mg/L aluminium sulfate was the lowest concentration at which a taste was registered as musty, mouldy and stale although the mean was 7.4mg/L. However, the presence or absence of taste or odours cannot be correlated with toxicity (Young et al. 1996).

While natural waters generally have low concentrations of aluminium, acid rain can mobilise more aluminium that is soluble. In addition, aluminium in the form of aluminium sulfate, $Al_2(SO_4)_3$, has been used since Roman times as a flocculant to clarify water, removing particulate, colloidal and dissolved substances via coagulation (Ganrot 1986; Martin 1988; UK MAFF 1993; Srinivasan et al. 1999). Gardner & Gunn (1995) found that aluminium in water after aluminium sulfate treatment was in a more labile form as low molecular weight, reactive species compared with that found in

natural waters. Studies of bioavailability of aluminium in water have concluded that the overall uptake of aluminium from drinking water is small (around 1-2% of total daily intake) and that current concentrations of aluminium in water do not pose a significant threat (Srinivasan et al. 1999; Stauber et al. 1999). The latter of these studies suggests that the bioavailable aluminium content of aluminium sulfate treated water is 0.37%, which is similar to uptake from food. A more recent study by Yokel et al. (2001) suggests the bioavailability of aluminium from drinking water is 0.25-0.40% and found that this was not greatly influenced by the presence of food or water hardness. Birchall & Exley (1992) postulated that the amount of bioavailable aluminium from water may be dependent on soluble silicon and proposed an inverse relationship between the two elements. They suggested that waters with higher silicic acid concentrations would minimise the amount of aluminium available for human uptake. This relationship between soluble aluminium and silicon was confirmed in work by Taylor et al. (1995).

Aluminium present in the air results from anthropogenic activities such as exhaust gases of metallurgical activities and naturally sourced aluminium released via the weathering of rocks (Soni et al. 2001; Barabasz et al. 2002). The amount of aluminium thought to be ingested from the air is around 0.2mg/day (Soni et al. 2001).

The use of aluminium cookware, especially in combination with hot acidic foods can also be a factor in dietary aluminium although the aluminium content in food derived from this source appears to be small, however most foods are near neutral pH (Ganrot 1986; Martin 1988; Sherlock 1988; UK MAFF 1993; Mei & Yao 1993). A comprehensive study of aluminium uptake from cookware and packaging materials found that there were cases of high aluminium absorption by some foods from aluminium cookware and storage materials. This is mainly from acidic attack from tomatoes, quince and citrus juices on bare aluminium metal although boiling tap water also leached considerable amounts of aluminium (Muller et al. 1993). It was estimated that over time the aluminium ingested from this source would be negligible when compared with aluminium derived from food. However the use of stainless steel, Teflon coated cookware and lacquered or plastic lined storage containers was found to effectively inhibit aluminium absorption and the prevalence of modern cookware today would preclude most people accumulating aluminium from this source. On

an interesting note it was also found that aluminium concentrations in cola after storage of >400 days in cans ranged from 0.4-0.8 mg/L despite the protective lacquering on the inside of the can. Mei & Yao (1993) reported that complexing reactions of the aluminium ions could play a more important role in increased aluminium food content from cookware than pH or electrochemical corrosion induced by NaCl. This was supported by the work of Bi (1996) who used modelling to show that complexing reactions with various ligands in the food solution, particularly organic acids were central in the leaching of aluminium from cookware and that even though most food was in the pH neutral range, these complexes would still solubilise aluminium (see Section 1.4). This demonstrates the importance of elemental speciation which will be discussed in Chapter 2. However in a review on aluminium derived from cookware, Rajwanshi et al. (1997) reports the wide range of methodologies and results makes it difficult to draw a conclusion on the extent of aluminium leaching from cookware and the amount of bioavailable metal available for human ingestion. Soni et al. (2001) has given an estimated intake of aluminium from cookware as 3.5mg/day.

Ingestion of antacids and buffered analgesics appear to be the greatest oral source of aluminium, with intakes of around 1-5g/day they are 2 to 3 orders of magnitude higher than ordinary dietary intakes (Ganrot 1986; Sherlock 1988; UK MAFF 1993; Jeffery 1995; Soni et al. 2001). It has been estimated medicinal and food sources of aluminium constitute greater than 95% of the total daily aluminium intake by humans (Orme & Ohanian 1990).

1.4 Human Aluminium Uptake/Absorption

Despite its abundance, aluminium has no apparent biological function (Ganrot 1986; Frech & Cedergren 1992; Yokel 2002). Wood (1985) postulated that this could be due to the first organism's biochemistry selecting nutrients that were available in the earth's crust 4 billion years ago. The majority of aluminium was, as it is today, insoluble and therefore unavailable for transport to primitive bacteria. While this is plausible, the most probable explanation lies in the kinetics of ligand exchange rates of Al^{3+}, which are slow compared with other biologically relevant metals (Rubini et al. 2002; Yokel 2002). The sluggish ligand exchange and the stability of aluminium complexes render the ion 'useless as a metal ion at the active sites of enzymes' (Martin 1986;

MacDonald & Martin 1988). It is this lack of biological significance and the opinion that most organisms have had to deal with the presence of aluminium with no apparent adverse effects, that the element was considered biologically inert and non toxic up until the 1970's when toxicity was observed in relation to dialysis patients (Ganrot 1986; Duffield & Williams 1988; Hewitt et al. 1990; Frech & Cedergren 1992; Berthon 1996, 2002). Currently, aluminium is regarded as a biologically active metal and its toxicity to humans, especially its neurotoxicity, is well recognised (Ganrot 1986; Simonsen et al. 1994; Jeffery 1995; Das et al. 1996; Berthon 1996, 2002).

There are three main pathways for aluminium intake: inhalation, parenteral administration via clinical operations and oral ingestion involving the gastrointestinal tract (Klein 1990; Savory & Wills 1991; Alfrey 1993; Rubini et al. 2002). While aluminium-containing dust particles are continuously inhaled, most will be exhaled and some is retained in the pulmonary tissue and does not translocate to other areas of the body (Ganrot 1986; Alfrey 1993). Although increased urinary excretion of aluminium has been observed after prolonged workplace inhalation of aluminium (Gitelman 1995; Fishbein 1992), other than these anthropogenic sources, aluminium intake via inhalation is minimal (Orme & Ohanian 1990; Jeffery 1995). Parenterally administered aluminium has been associated with acute aluminium toxicity seen with Dialysis Encephalopathy, which will be discussed later. However, increased awareness of aluminium toxicity and contamination associated with it has led to dramatic improvement in the use of 'cleaner' equipment and materials. Other medications also transport aluminium into the body, including antacids, buffered aspirins, vaccines and allergen injections (Fishbein 1992). However, for parenterally administered aluminium, "the most common route by far is that of oral-enteral administration. The largest quantities of aluminium are taken in by this route" (Klein 1990). A diagram of a hypothetical model of aluminium ingestion is shown in Figure 1.3. The gastrointestinal tract is generally considered resistive to absorption of aluminium into the blood under normal conditions (Ganrot 1986; Klein 1990; Domingo 1994; Berthon 1996, 2002). Of the average daily amount of aluminium ingested, around 5-10mg, it is estimated that only 8-10μg or 0.1-0.5% is absorbed (Alfrey 1993; Ganrot 1986). The area of aluminium absorption occurs mainly in the duodenum or jejunum (Hewitt et al. 1990; Cunat et al. 2000). The remaining aluminium ingested will be passed through and excreted via the faeces and urinary aluminium is only a fraction of that found in the stools (Gorsky et al.

1979). Most of the absorbed aluminium will be extracted by the kidneys and passed with the urine in normal healthy humans (Hewitt et al. 1990; Berthon 2002).

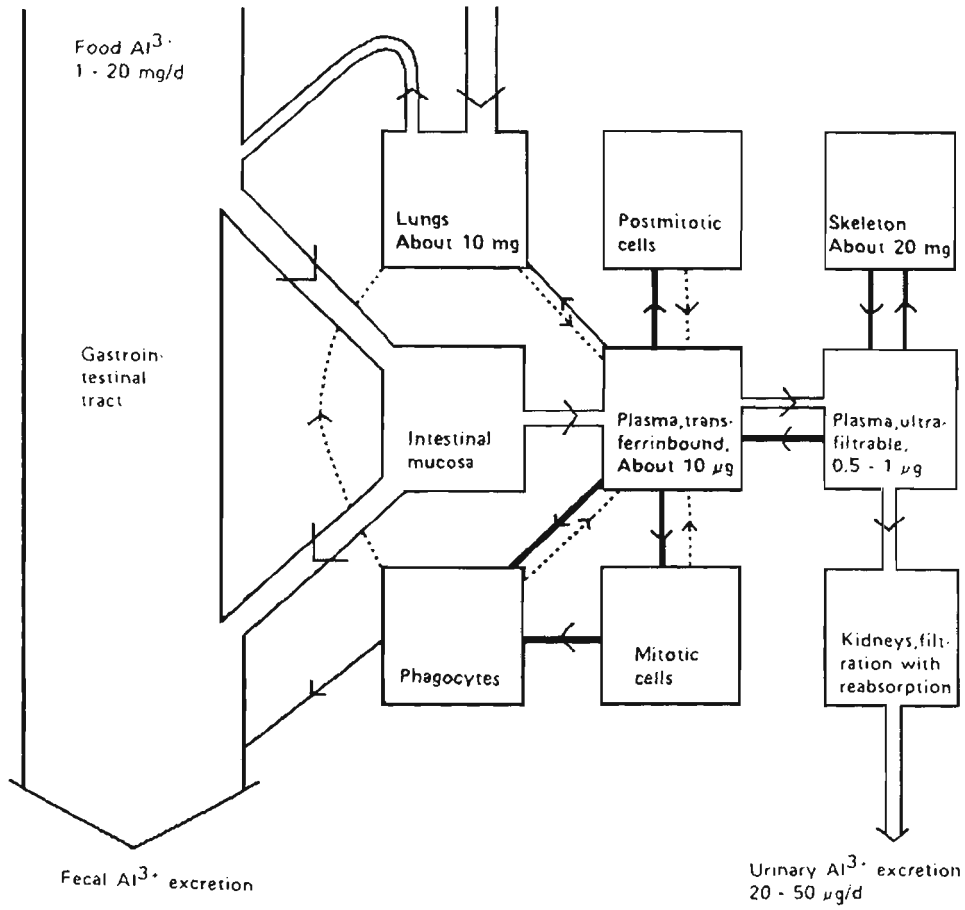


Figure 1.3 A hypothetical model of Al^{3+} metabolism in humans (from Ganrot 1986).

The amount of metal available for absorption depends upon many factors (Klein 1990):

a) the amount ingested in the diet. This could include aluminium that is "naturally" derived from food, from food additives, from the water supply, from cooking in aluminium utensils or from the use of medicinal formulations such as antacids.

b) the chemical form of the aluminium.

c) the pH in different parts of the gastrointestinal tract.

d) the presence and amount of different complexing ligands.

With the administration of large doses of aluminium some absorption will occur (Klein 1990) but this plays only a part in the absorption process. Absorption is thought to take place via passive diffusion when intestinal fluid chemical conditions permit solubilisation and formation of neutral

organic acid complexes, or by a carrier mediated pathway (Alfrey 1993; Jeffery 1995; Berthon 1996, 2002).

In the stomach the pH is around 3, hence solubilised or free Al^{3+} will be the dominant species in solution. Further down the gastrointestinal tract the pH is increased thereby allowing hydroxide ions to precipitate the Al^{3+} mainly as $Al(OH)_3$ (Jeffery 1995; Berthon 1996, 2002). Al^{3+} has been shown to strongly bind to phosphate anions (Janssen et al. 1996). It has been proposed that while Al^{3+} will be in solution in the stomach and proximal duodenum, phosphate will complex with Al^{3+} in the less acidic environment found lower in the gastrointestinal tract to form insoluble $Al(OH)_2H_2PO_4$ thus allowing the majority of aluminium to be passed with the faeces (Ganrot 1986; Martin 1986,1988; Jeffery 1995; Berthon 1996, 2002). Insoluble phosphate complex formation has been suggested as a major factor in preventing gastrointestinal absorption (Martin 1986,1988).

This has been challenged (Powell & Thompson 1993; Berthon 1996, 2002), and even in acidic environments it is believed that the insoluble mucosa absorb and retain Al^{3+}, where precipitation of its salts into the intestinal lumen occurs after cell death (Ganrot 1986). It has been suggested that absorption may include rapid mucosal uptake followed by gradual release into the blood stream (Berthon 1996, 2002). The level of silicon in conjunction with aluminium is also an important factor in accumulation. An inverse relationship between soluble silicon and soluble aluminium has been suggested, with lower labile aluminium concentration in solutions of high silicic acid content due to formation of insoluble aluminosilicates (Taylor et al. 1995). Edwardson et al. (1993) found dissolved silicon in water reduced peak plasma aluminium concentrations by 85%.

Absorption is thought to follow two pathways; a transcellular (through the cells) pathway or a paracellular (between the cells) pathway (Jeffery 1995; Cunat et al. 2000). Transcellular uptake would be a carrier-mediated process which may use the pathways used by calcium, iron or magnesium (Cochran et al. 1990; Savory & Wills 1991; Jeffery 1995; Desroches et al. 2000; Cunat et al. 2000). Paracellular uptake however is now seen as the most likely route of absorption (Alfrey 1993; Berthon 1996, 2002; Desroches et al. 2000; Cunat et al. 2000) utilising the formation of aluminium complexes with dietary organic acids.

Organic acid ligands, especially citrate, are now well regarded as enhancers of aluminium absorption through the gut wall (Van Ginkel et al. 1990; Jeffery 1995; Berthon 1996, 2002; Martin 1986,1988; Alfrey 1993; Domingo 1994). Researchers have found increased urinary amounts and tissue concentrations of aluminium in both rat and human studies after dosing with aluminium and citrate rich foods (Slanina et al. 1985; Taylor et al. 1992). Because citrate has a stronger affinity for Al^{3+} than either phosphate or hydroxide (Martin 1986,1988) it was initially thought that precipitation was precluded by citrate with the formation of a soluble neutral Al-citrate complex at pH 1-4, thereby facilitating absorption through lipid cell walls (Martin 1986,1988; Berthon 1996, 2002). Now it is generally held that citrate delays precipitation until pH>8, allowing aluminium-citrate complexes to be absorbed over most of the gastrointestinal tract (Berthon 1996, 2002; Domingo 1994; Taylor et al. 1992; Jeffery 1995). The mechanism seems to lie in the citrate complex decreasing the tight junction (a barrier between cells formed by closely adjacent cell membranes) resistance between mucosal cells by chelating the calcium necessary for tight junction maintenance (Jeffery 1995; Taylor et al. 1992). Thus citrate facilitates opening the space between cells to allow the aluminium-citrate complex to diffuse along this paracellular pathway (Berthon 1996, 2002). Hence citrate complexation of aluminium is a two edged sword, both mobilising the aluminium throughout the gastrointestinal tract and providing a pathway for absorption (Alfrey 1993), allowing healthy humans to absorb aluminium (Martin 1986, 1988). Other organic ligands such as gluconate, oxalate, succinate, lactate, ascorbate, malate and tartrate have also been shown to enhance gastrointestinal absorption of aluminium (Berthon 1996, 2002; Venturini-Soriano & Berthon 1998; Desroches et al. 2000; Venturini-Soriano & Berthon 2001) thus posing the problem that increased organic acid concentration coupled with increased aluminium intake could overcome the gastrointestinal barrier to absorption into the bloodstream (Slanina et al. 1985). Recently Cunat et al. (2000) studied the deposition of organic and inorganic compounds of aluminium in the gastrointestinal tracts of rats and any subsequent appearance in the blood. They found that ingestion of organic compounds gave a lower amount of aluminium in the gut and increased amount in the blood. Of the ligands studied, Al-citrate gave the highest absorption, followed by Al-gluconate and Al-tartrate. Inorganic forms such as Al-nitrate, Al-chloride and Al-sulfate showed minimal absorption.

A recent paper by Desroches et al. (2000) has suggested that tartaric acid may induce both transcellular and paracellular absorption of aluminium. The mechanisms are thought to act by lowering the pH of the ingesta by releasing two protons and dissolving aluminium salts and as tartrate decrease the concentration of free Al^{3+} shift the precipitation of their insoluble slats to the limit of the pH range of the small intestine. Transcellular diffusion may be also accomplished by complexes formed between aluminium and tartrate. In their study, Desroches et al. (2000) found that the pH of precipitation of aluminium was shifted to 6.66 in the presence of 0.01M tartrate. A concentration of 0.1M tartrate was found to move the pH of precipitation to 7.59. They concluded that tartaric acid could aggravate aluminium bioavailability significantly and although the presence of phosphate reduced the effectiveness of tartrate, it did not remove it entirely and at high aluminium concentrations did not reduce its affect at all. Similar studies on succinic and malic acids showed that these ligands did not increase the bioavailability of aluminium to the same extent as tartaric acid (Venturini-Soriano & Berthon 1998; Venturini-Soriano & Berthon 2001). A schematic of possible pathways of gastrointestinal absorption is shown in Figure 1.4.

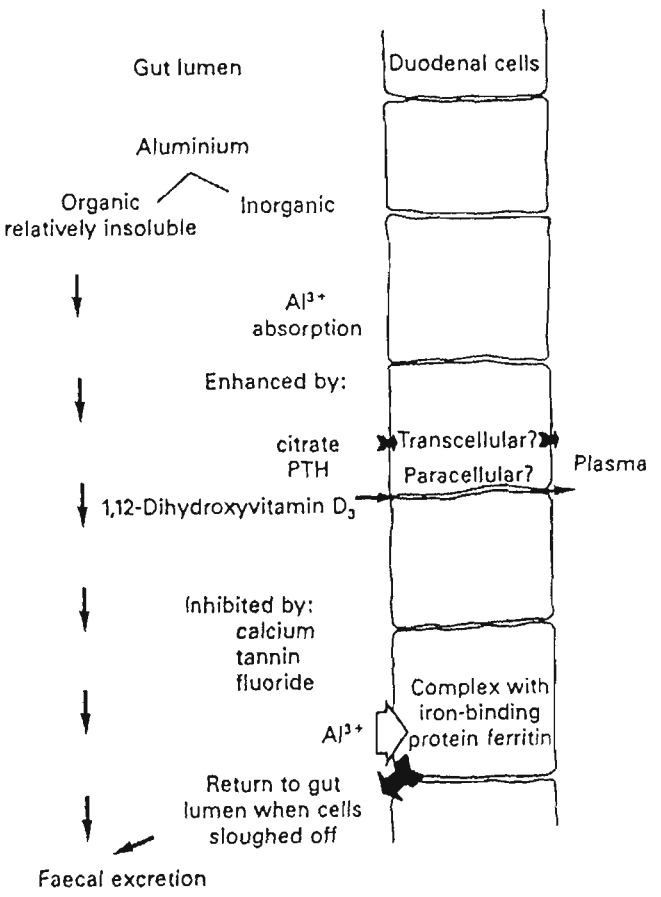


Figure 1.4 Gastrointestinal absorption of aluminium (from Lote & Saunders 1991).

Some researchers have challenged the assertion that aluminium in water and food and in the presence of citrate induces increased blood aluminium concentrations. In evaluating aluminium uptake in a baboon using aluminium dose controlled water and food over a period of 31 days, Turnquest & Hallenbeck (1991) concluded that overall 'there was no association between aluminium intake via food or water and aluminium concentrations in serum and whole blood'. However they admitted that due to behavioural difficulties of the baboon, blood was sampled on an irregular basis, leaving gaps in their data. In a more comprehensive recent analysis of aluminium uptake in 29 human subjects, Stauber et al. (1999) found that added citrate in the controlled diet set to mimic actual concentrations found in food and aluminium sulfate treated water had 'no effect' on absorption of aluminium. The authors attributed the difference from other citrate absorption studies to a use by other researchers of high dosages of aluminium/citrate that may have produced different aluminium species more amenable to gastrointestinal absorption.

1.5 Biological Metabolism

Free Al^{3+} will not exist to any significant degree in the blood plasma, due to the formation of $Al(OH)_3$ at the physiological pH 7.4 (Martin 1986, 1988). Citrate is a major low molecular mass component of the blood plasma and because of its high affinity for aluminium and its solubility at physiological pH it is regarded as the predominant binder of aluminium in the low molecular mass fraction (Martin 1986; Ohman 1988; Van Ginkel et al. 1990; Berthon 1996, 2002). In the bloodstream, citrate acts as a detoxifying agent, as part of the ultrafilterable fraction, allowing excretion via the kidneys which is considered the major pathway of elimination from the blood. Biliary excretion is very small (Klein 1990; Savory & Wills 1991; Alfrey 1993; Berthon 1996, 2002). This has been proposed also for malate and tartrate species (Berthon 1996, 2002), although Corain et al. (1992a, 1994) found in aqueous studies at pH 7.5 that these ligands do not retain aluminium, and a metastable monomeric hydroxy-aquo species is formed. Citrate accounts for only 5-10% of the total plasma aluminium (Ganrot 1986; Harris & Sheldon 1990; Klein 1990) and whether this fraction increases with increased plasma concentration is currently in dispute. The majority of aluminium (80-95%) is thought to reside in the high molecular mass/protein fraction of plasma which is not removed via excretion (Ganrot 1986; Berthon 1996, 2002). As the plasma

aluminium concentration rises, the renal clearance decreases (Berthon 1996, 2002) and consequently the protein concentration increases.

Many authors have discussed protein transport of aluminium in blood plasma, particularly the role of transferrin (Martin 1986, 1988; Van Ginkel et al. 1990; Jeffery 1995). Transferrin is the major iron transport protein. Transferrin has a higher binding affinity for aluminium than does citrate or the other organic acids. Due to their similar charge Al^{3+} and Fe^{3+} behave similarly with transferrin. Citrate can present aluminium to transferrin before excretion. Although the Fe^{3+}-citrate complex will transfer Fe^{3+} to transferrin 100 times more efficiently than Al^{3+}-citrate transfers Al^{3+} to transferrin, only 30% of the binding sites on transferrin will be utilised by Fe^{3+} at any one time. Hence 70% of unbound sites on transferrin are available to Al^{3+} (Martin et al. 1987). The role of transferrin in binding and transporting Al^{3+} has been demonstrated experimentally in the last decade (El-Sebae et al. 1994; Sanz-Medel 1998; Sanz-Medel et al. 2002).

Tartrate and other carboxylates may readily transfer their aluminium to Transferrin because in aqueous solutions at pH 7.5 they have been found to relinquish most or all of their bound aluminium, hence reducing the amount of ultra-filterable (excretable) aluminium when compared with citrate (Corain et al. 1992a; Corain et al. 1994). Because of the above mechanisms, in blood plasma the majority of aluminium will be bound to transferrin, making it the most significant carrier of aluminium in blood (Ganrot 1986; Martin 1986; Van Ginkel et al. 1990; Klein 1990; Harris & Sheldon 1990; Erasmus et al. 1993; Jeffery 1995; Berthon 1996, 2002; Das et al. 1996; Sanz-Medel 1998; Sanz-Medel et al. 2002).

Aluminium not excreted will find its way to organs throughout the body (Berthon 1996, 2002) of which the major ones presently considered are bone and bone marrow, the liver, the kidney, the heart and the brain (Slanina et al. 1985; Ganrot 1986; Hewitt et al. 1990; Klein 1990; Verbeelen 1991; Yokel et al. 1991; Taylor et al. 1992; Winship 1993). The brain has been the organ that has undergone the most scrutiny. The brain has shown slower uptake than the other organs and this may be due to the blood/brain barrier (BBB) (Yokel et al. 1991; Ganrot 1986). However other research has attributed to aluminium an alteration to the BBB by opening the space between the cells,

allowing extracellular uptake (Berthon 1996, 2002; Yokel 1991; Banks & Kastin 1989) and it is in the brain where most organ Al^{3+} is found (Ganrot 1986). Membrane alteration has been attributed to polynuclear aluminium ions (ions containing more than one aluminium) (Flaten & Garruto 1992) but the existence of these ions is still in doubt (Ohman 1988). Uptake into cells occurs mainly by interaction of transferrin bound aluminium and the transferrin receptors on organ cells (Berthon 1996, 2002; Klein 1990). Entry into cells has been proposed by Ganrot (1986) to occur via the Fe^{3+} pathway, albeit slowly. Once bound to cells by the transferrin receptors, Fe mechanisms allow aluminium to be transported into the lysosomes (storage area of unwanted cell substrates and for Fe^{3+}), a possible natural cell defence against aluminium uptake. Al^{3+} could be slowly leaked from the lysosomes into the cytosol (liquid within cells not including the nucleus) and may lead to accumulation in the cell nucleus, where it would be bound strongly.

No evidence of elimination from cells exists, though aluminium may be removed from an organ on cell death (Ganrot 1986; Edwardson et al. 1988). However, long-lived cells such as the neurons of the brain may continue to accumulate aluminium, although slowly, in the nucleus over an entire lifetime (Ganrot 1986; Winship 1993). At least 20-100 times more aluminium is found in neuronal nuclei than for other cells (Ganrot 1986). Accumulation in the brain may take decades to occur and other organs may not accumulate aluminium due to removal in cell death (Ganrot 1986).

1.6 Aluminium Toxicity

Aluminium toxicity has only been considered in the last 20 years, however its neurotoxicity is now well established (Simonsen et al. 1994) even though its pathological role for some diseases is still in dispute. The inorganic forms of aluminium, particularly free Al^{3+} is considered the most toxic forms of the metal (Zatta et al. 2002; Rubini et al. 2002). The toxicity of aluminium is thought to be due to its replacement of Fe^{3+} and Mg^{2+}, inducing disturbances with intercellular communication, secretory functions and cellular growth (Barabasz et al. 2002). Aluminium tends to mimic the physiological behaviour and pathways of Fe^{3+} (Sanz-Medel et al. 2002) and in the case of Mg^{2+}, prevents this element from acting as an anti-oxidant (Zatta et al. 2002). Ganrot (1986) suggested that although normal aluminium content in the organs is low, the difference between this value and its toxic

concentration is considered quite small. In addition, it was postulated that due to the disparity between normal newborn infant brain aluminium concentrations (~0.2mg/kg wet weight) and those in normal elderly humans (~0.6-0.7mg/kg), aluminium will slowly accumulate over a lifetime to eventually reach toxic concentrations within 100-150 years. Ganrot (1986) proposed that this toxic concentration of aluminium could possibly be a natural human age limitation and a part of the aging process.

Aluminium has been shown to alter the permeability of the BBB, possibly allowing xenobiotics or regulated chemicals to enter neuronal cells in greater amounts and potentially cause central nervous system dysfunction (Banks & Kastin 1989; Berthon 1996, 2002). It is thought that aluminium may enhance iron mediated lipid peroxidation in cells in the acidic media of the lysosome and oxidation of cell membranes which contributes to neurodegeneration (Erasmus et al. 1993; Xie & Yokel 1996; Zatta et al. 2002). Other effects of aluminium accumulating in the brain include protein synthesis, axonal transport and neuro-transmitter-related events, enzyme inhibition and astrocyte protection of neuronic cells (Bigay et al. 1987; Sass et al. 1993; Winship 1993). In animals, impaired learning and poor motor coordination was attributed to aluminium loading (Xie & Yokel 1996). In 1999 a court case in the UK ruled in favour of residents who had been poisoned with aluminium by a local water authority when a worker accidentally put too much alum in the drinking water (Poole 1999); effects included 'short term memory loss and loss of concentration'.

Two diseases that are generally accepted as resulting from acute aluminium toxicosis are Dialysis Encephalopathy (D.E.) and Dialysis Osteomalacia which can occur in renal failure patients who are unable to remove aluminium from the blood during dialysis (Ganrot 1986; Savory & Wills 1991). This was first recognised in the 1970's by researchers including Elliot et al. (1978). Aluminium containing medications/antacids administered with citrate or phosphate were at first suspected however it is now recognised that the main source of aluminium into patients is the water contaminated with aluminium when dialysis is conducted (Elliot et al. 1978; Alfrey 1993; Savory & Wills 1991). Patients suffering D.E., resulting from acute aluminium loading in the brain, begin symptoms with speech disturbances, disorientation, hallucinations and increased dementia followed by facial grimacing, convulsions and epileptic seizures culminating in muteness, paralysis, coma

and eventually death in 1-6 months up to 18 months (Ganrot 1986; Klein 1990; Hewitt et al. 1990; Savory & Wills 1991; Verbeelen 1991; Alfrey 1993; Winship 1993). Dialysis Osteomalacia is severe aluminium toxicosis of the bone characterised by retarded mineralisation of newly formed bone tissue and softening of bones resulting in skeletal pain and fractures (Ganrot 1986; Savory & Wills 1991; Alfrey 1993). Both diseases have been combated by use of aluminium free dialysis water and reduction of aluminium/citrate containing foods/medicines (Alfrey 1993). An aluminium chelating agent, desferrioxamine (DFO) has also been used successfully to lower serum bound aluminium (Verbeelen 1991; Berthon 1996, 2002). Another acute toxicosis by aluminium has been reported from alum irrigation used to treat bleeding in the bladder with patients with renal failure. Although still disputed, Perazella & Brown (1993) and Shoskes et al. (1992) in separate cases had renal failure patients after treatment for cancer suffer symptoms similar to D.E. followed by rapid coma and death following alum irrigation. It was found that these patients had very high concentrations of serum aluminium.

The possible role of aluminium in Alzheimer's Disease (AD) has been extremely controversial. Alzheimer's disease is the most common cause of dementia (73%) in Australia and its prevalence increases with age (Jorm & Henderson 1993). The disease is characterised by progressive deterioration of memory and intellect and ultimately death within 2-20 years of the initiation of the disease. AD has been associated with neuronal depletion, the abundance of neurofibrillary tangles (NFT) and senile plaque (SP) formation (Ganrot 1986; Berthon 1996, 2002; Jorm & Henderson 1993; Edwardson et al. 1988) and the depletion of the neurotransmitter making enzyme choline acetyltransferase (Jorm & Henderson 1993) in about 1-10% of neurons in parts of the brain. Aluminium has been targeted since it was found in a high percentage of the central cores of SPs and in NFTs in AD patients (Candy et al. 1986; Savory & Wills 1991; Erasmus et al. 1993; Berthon 1996, 2002; Edwardson et al. 1988). Candy et al. (1986) found that 31 of 40 patients studied with SP had central cores with colocalised aluminium and silicon within the central region. Aluminium and silicon have been identified as aluminosilicates in lipofuscin granules in both SPs and NFTs (Tokutake & Oyanagi 1995). Lipofuscin granules, which normally increase in normal brain aging, appear faster in AD patients (Tokutake & Oyanagi 1995). Tau protein, the major structural protein in SPs and NFTs has shown changes in processing in DE patients that was similar to AD-like

changes after long exposure to aluminium (Harrington et al. 1994a, 1994b). A decrease in choline acetyltransferase in rats after chronic exposure to aluminium has been observed with similar concentrations found in the autopsied brains of AD patients (Erasmus et al. 1993).

Other researchers have countered that aluminium may be just a secondary effect and not play an aetiological role at all (Klein 1990; Hamdy 1990). Major areas of dispute include: NFT's seen in AD differ from those seen in high aluminium toxicity DE patients (Hamdy 1990; Berthon 1996, 2002; Manabe 1994; Yokel et al. 1988), low amounts of transferrin sites (thought to be the point of entry into neurons) have been suggested in sites of NFT's and SP's (Morris et al. 1994), differences in lesions between the diseases and the fact that removal of aluminium from the diet can cure symptoms of DE but not AD (Hamdy 1990). It is now generally considered that the evidence does not support the aluminium inducement of AD (Winship 1993; Soni et al. 2001) and that the initial methodology linking aluminium with senile plaques and neurofibrillary tangles in AD patients is seriously under question (Manabe 1994). Despite the consensus of no causal link between aluminium and AD, research continues. A recent study by Gauthier et al. (2000) found that in a survey of AD sufferers and their exposure to different species of aluminium that there was a significant association between AD and organic monomeric aluminium at the onset of the disease. They suggested that genetic factors might control environmental exposure, particularly to specific forms of aluminium, which have not been studied in association with AD before. In a recent paper Berthon (2002) argues that denying the significance of a correlation between high water aluminium content and Alzheimer's disease overlooks the role of bioavailability and hence the speciation of the aluminium. These latter two papers highlight the need for speciation analysis in the determination of aluminium toxicity. Speciation will be discussed in Chapter 2.

1.7 Aluminium in Wine

As discussed earlier, there has been concern in the last 20 years regarding the toxicity of aluminium and studies have been conducted on its presence in foods, water and beverages and the relationship between their aluminium content and uptake and potential toxicity (Ganrot 1986; Sherlock 1988; McKinnon et al. 1992; Scollary 1997; UK MAFF 1993; Stauber et al. 1999; Ysart et al. 2000). At

present aluminium is perceived as a problem by the wine industry only in terms of aesthetics with respect to the stability (clarity) and taste of the wine. A limit of 3mg/L aluminium has been recommended to avoid these problems (Rankine 1983) although the German limit for aluminium in wine for stability purposes is 8mg/L (Eschnauer & Scollary 1995). The low pH (<4.0) and presence of complexing organic ligands in wine is the perfect environment for the solubilisation of aluminium. The rate of corrosion of metallic aluminium in a white wine of pH 3.07 was measured at 13μmol/year (Kojima et al. 1996). However, this disagrees with the work of McKinnon (1990) and McKinnon et al. (1992) who showed that even at a pH of 3.8 (just below the 'depassivation' pH of 3.9 given by Kojima et al. 1996) a 1cm by 1cm piece of aluminium foil in a white wine dissolved at a rate of 2.06mmol/year. The rate of aluminium dissolution increased with a decrease in the pH. The evidence of this corrosion and uptake by wine can be seen in Figure 1.5. The disparity in results is probably due to the fact that a much slower induction rate was observed over the first 15 days for McKinnon's work, whereas Kojima et al. (1996) observed aluminium corrosion for 1 week only, during which time there is a greater resistance to dissolution by the oxide film.

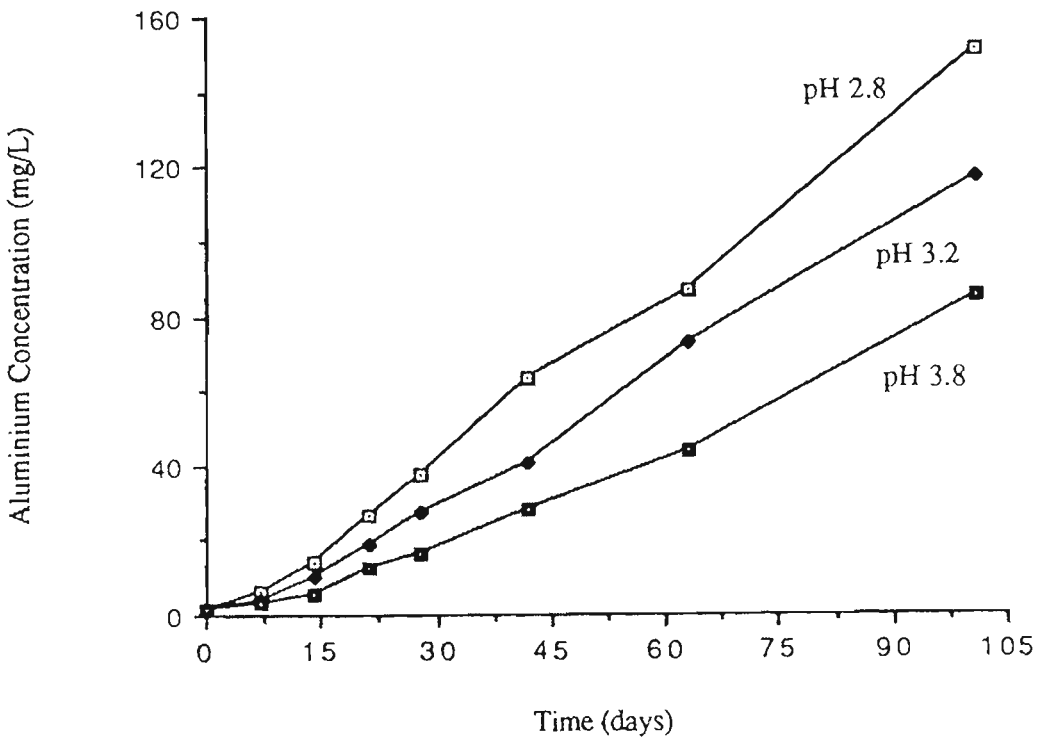


Figure 1.5 Dissolution of aluminium in wine (from McKinnon 1990 and McKinnon et al. 1992).

Despite these concerns, the origins, content and speciation of aluminium have not been thoroughly conducted since 1981. Most published studies have primarily been concerned with the concentration

of aluminium in finished wines. In a study of aluminium quantities in 267 Australian and international wines a range of 0.17-5.55mg/L (mean 0.91mg/L) was found, with higher concentrations found in white and sparkling wines (McKinnon 1990; McKinnon et al. 1992). In 163 German wines a range of 0.063-4.928mg/L (mean 1.12mg/L) of aluminium were observed of which <0.5mg/L was estimated to be of 'natural' origin (Eschnauer & Scollary 1995). Table 1.3 shows the aluminium concentrations found in finished wines in studies conducted in the last 20 years. The data shows that a significant proportion of the wines studied had aluminium concentrations higher than the WHO guideline for drinking water with some results an order of magnitude higher. It should also be noted that in general white wines give a higher aluminium concentration than red wines (McKinnon et al.1992; Seruga et al. 1998; Larcher & Nicolini 2001). The ranges show that some wines can have an elevated aluminium content with the highest given as 8.60 mg/L aluminium. Also interesting to note is the study of 24 Croatian wines by Seruga et al. (1998) that reports mean aluminium concentrations 1.5-3 times higher than concentrations found by any other investigation. In addition, a higher upper range limit at 8.60mg/L was reported and 90% of white and red wines were found to have aluminium concentrations above 1mg/L. These concentrations could reflect the true aluminium concentration in Croatian wines or aluminium contamination encountered in preparation and analysis. The aluminium concentration in traditional Cretan fortified wines known as 'tsikoudia' also displayed a large range with an even higher upper concentration limit of 9.5mg/L (Galani-Nikolakaki et al. 2002) although the production process is different from table wines.

Table 1.3 Reported aluminium concentrations in wine.

Reference	Country	Wine Type	Mean(mg/L)	Range (mg/L)
Hubert (1981)	France	White	1.470	0.49-2.61
		Red	0.944	0.40-1.722
Pennington & Jones (1988)	United States	Red	0.93	----
McKinnon (1990), McKinnon et al. (1992)	Australia *	White	1.16	0.34-5.55
		Red	0.77	0.17-3.81
Larroque et al. (1994)	France	Red	0.763	0.25-2.55
Eschnauer & Scollary (1995)	Germany	Both	1.12	0.063-4.928
Lopez et al. (1998)	Spain	White	0.548	0.189-1.683
		Red	0.651	0.072-1.254
Seruga et al. (1998)	Croatia	White	2.82	0.97-8.60
		Red	2.51	0.62-5.38
Camean et al. (2000)[#]	Spain	Sherry Brandy	0.47	0.02-1.14
Larcher & Nicolini (2001)	Italy	Both	0.63	0.12-1.58
Galani-Nikolakaki et al. (2002)	Crete, Greece	Fortified Wine	-----	0.36-9.5

*** Note: This study included some non-Australian wines.**
[#] Note: Although not a table wine the spirit is derived from wine.

Naturally derived aluminium comes from aluminium taken up by the vine roots from soil and distributed via sap circulation into the berries (Hubert 1981; Enkelmann & Wohlfarth 1994; Scollary 1997; Seruga et al. 1998). Anthropogenic input has been concentrationled at the use of aluminium containers, pesticides, filter aids, glass bottles, additives to wine such as red tannin and fining agents such as bentonite (Hubert 1981; Severus 1988; McKinnon 1990; Enkelmann & Wohlfarth 1994; Eschnauer & Scollary 1995; Scollary 1997; Seruga 1998). Now that aluminium vats, vessels and equipment have been replaced by stainless steel, bentonite is considered a major source of aluminium (McKinnon 1990; McKinnon et al. 1992; Eschnauer & Scollary 1995). Bentonite is a clay used specifically in white wine production to remove proteins and other charged particles which can cause hazing of the wine (Ough 1987; Rankine 1989; Zoecklein et al. 1990). This may explain the higher average aluminium concentrations observed for some white wine (McKinnon 1990; McKinnon et al. 1992).

McKinnon (1990) and McKinnon et al. (1997) found that aluminium was strongly bound to low molecular weight compounds in wine. Tartaric acid is the major low molecular mass organic acid in wine (Rankine 1989; Zoecklein et al. 1990) and a significant proportion will be as the conjugate anion bitartrate at pH 3.0-4.0 (Zoecklein et al. 1990). The ability of the organic acid anions to complex aluminium at the low pH seen in wines (2.5-4.0) suggests that aluminium could be bound to bitartrate (McKinnon 1990, McKinnon et al. 1997; Eschnauer & Scollary 1995). Bitartrate is an organic acid anion that has been shown to enhance gastrointestinal absorption of aluminium (Berthon 1996, 2002; Desroches et al. 2000) and its presence with aluminium in wine has significant implications for the bioavailability of aluminium in wine (Scollary 1997). However, no investigation of aluminium uptake and distribution in the human body from wine has been attempted to this author's knowledge. Complexation, bioavailability, and absorption of aluminium in the gastrointestinal tract relies on the speciation of the aluminium. The concept of speciation and speciation analysis of aluminium will be discussed in Chapter 2.

Seruga et al. (1998) calculated that the average daily intake of aluminium from the wines investigated in that study would contribute 1.345mg assuming that the average wine intake was 0.5L. This daily contribution suggested that wine was a significant source of total dietary aluminium from beverages but was low compared with the WHO tolerable aluminium intake of 7mg per kg of body mass. Despite the fact there have been no toxicological studies of aluminium uptake and metabolism in wine, it has been reported that combined exposure to aluminium and ethanol increases blood and liver concentrations in rats compared with concentrations seen after aluminium dosing alone and the combined exposure altered concentrations of catecholamine in rat brains (Flora et al. 1991). This may point to an increased toxicity risk if aluminium is ingested in the presence of ethanol, the alcohol found in wine (10-14%), posing an additional risk factor to wine drinkers with respect to aluminium. However there has been no supporting evidence that exposure to low amounts of aluminium with alcohol in wine could induce similar affects.

1.8 Total Aluminium Analysis

After appropriate sample collection and preparation, generally by a process of extraction or digestion, there are various instrumental methods employed to determine the total concentration of aluminium in a given sample. The choice of technique is dependent upon the sample matrix and aluminium concentration.

Atomic Emission Spectrometry with the use of Inductively Coupled Plasma as the excitation source has come to the fore in metal analysis in the last decade and is more commonly known by its acronym ICP-AES. The method is also giving way to mass spectrometry, and the power of ICP-MS has been realised in recent years. In ICP-AES the plasma produces a complex emission spectrum of atoms excited at high temperature and its main advantages are the much faster sample throughput due to the inline nature of sample introduction and the ability to perform multi-element analyses, either sequentially or simultaneously (Sturgeon 1992). Initially the considerable start-up and running costs of ICP-AES had restricted its use, however recently the cost of ICP-AES has decreased substantially and has virtually replaced Flame Atomic Absorption Spectrometry (FAAS) in routine metal analysis. Flame based emission techniques are hardly used for aluminium determination in environmental samples as the detection limits are poor (Bloom & Erich 1996). Analysis of aluminium in food has been conducted with ICP-AES (Yang et al. 1994; Sun et al. 1997; Barnes 1997; Dolan & Capar 2002). Sun et al. (1997) determined total aluminium concentrations in seafood and meat following microwave digestion with a mixture of HNO_3-H_2O_2-HF. The method was also utilised by Barnes (1997) to analyse aluminium and 23 other elements in fruit, juice and associated products. In most cases the aluminium concentration was found to be below the limit of quantitation and in some cases below the detection limit. This demonstrates the disadvantage of poorer sensitivity of ICP-AES compared with that of Graphite Furnace Atomic Absorption Spectrometry (GFAAS).

UV-Visible spectrophotometry has been employed to determine aluminium and was the method of choice before FAAS became commonplace (Bloom & Erich 1996). The method relies on the reaction of aluminium with an organic complexing reagent such as Pyrocatechol Violet or 8-hydroxyquinoline to form a complex that can be detected spectrophotometrically. Greater sensitivity

has been gained by preconcentrating the aluminium by extracting the complex into an organic solvent such as MIBK, toluene and butyl acetate. The major disadvantages of this method are the susceptibility to interference by competing organic ligands that can cause an underestimation of the total aluminium and the labour intensive sample preparation. Automation with flow injection methods has reduced the latter problem in recent years. Complexing agents coupled with UV-Vis spectrophotometry has also been used as a detection method for liquid chromatographic systems.

Fluorescence Spectrophotometry has also been utilised for total aluminium analysis working along similar lines to UV-Vis spectrophotometry where a fluorescing reagent is complexed with aluminium and the resulting fluorescence is measured at the appropriate wavelength. Lumogallion has been the most widely used fluorescence reagent for aluminium however 8-hydroxyquinoline-5-sulfonate (HQS) has also been used. Fluoride, iron and phosphate can interfere with the signal and the same problems of organic ligand competition and difficult sample preparation seen in UV-Vis spectrophotometry also apply. However the addition of a surfactant has been found to increase sensitivity (Bloom & Erich 1996). Fluorescence detection has been applied to chromatographic analysis on samples such as tea (Miyahara et al. 1999) after ion exchange separation.

Other less common methods for total aluminium analysis have included gas chromatography (GC) after complexation with volatile chelates, titration with fluoride using a fluoride ion-selective-electrode, liquid chromatography in the forms of ion chromatography, reverse phase HPLC and neutron activation analysis. The last method works by measuring gamma rays emitted from nuclei previously irradiated by neutrons (Bloom & Erich 1996). However, it has major disadvantages in that it requires a nuclear reactor, an experienced analyst and careful handling of material that is highly radioactive for several hours. Wyatt et al. (1993) used this technique to measure aluminium in bone. A HPLC method was developed by Nagaosa & Bond (1992) to determine aluminium in serum via ion exchange and UV-Vis spectrophotometry detection.

Atomic Absorption Spectroscopy (AAS) has been the favoured technique for total aluminium quantitation. In the 1970's and 1980's the main method of metal quantitation was Flame Atomic Absorption Spectroscopy (FAAS). The mechanisms of FAAS is well known, hence it is sufficient in

this section to state that the main premise of metal quantitation using this method relies on the Beer-Lambert law, whereby the concentration of a metal is proportional to its atomic absorbance over a given concentration range.

There are several interferences that can occur in FAAS (Tsalev & Zaprianov 1983; Lajunen 1992; Bloom & Erich 1996) with accompanying methods used to overcome them. Chemical interferences occur when stable compounds which do not decompose in the flame are formed. In the case of aluminium, refractory compounds are formed, hence a hotter flame of a mixture of nitrous oxide as the oxidant and acetylene as the fuel is utilised to decompose these compounds. Interference from ionisation caused by higher flame temperatures can be overcome by addition of an alkali salt. Physical interferences encountered due to analyte matrices altering the nebulisation of the solvent due to viscosity and surface tension are countered by matching the matrix of the standards with the sample. Spectral interferences are encountered when resonance lines of other elements overlap with those of the element under investigation, hence other metals in solution with these overlapping lines can add to the absorbance of the sample. However, overlapping absorption lines are rare. A major interference that occurs with FAAS is known as background absorption, caused by light scattering by molecular species, salt particles or molecular absorption in the flame. This can be accounted for (though not eliminated) with instrumental background. With detection limits in the low ppm range FAAS is not sensitive enough for most trace determinations of aluminium in biological, food, water and beverage analysis (Tsalev 1984; Bloom & Erich 1996). FAAS is generally used where high concentrations prevail such as in soil and sediment samples, however ICP-AES and ICP-MS is now more commonly used for routine metals analysis.

Graphite Furnace or Electrothermal Atomic Absorption Spectroscopy (GFAAS or ETAAS) is 3000 times more sensitive than FAAS for aluminium (Welz 1985) giving detection limits in the low to sub ppb range and requiring significantly less sample (Tsalev & Zaprianov 1983). Because of this GFAAS is considered the best analytical technique for trace aluminium quantitation (Tsalev 1984) and is the most commonly used method for various sample matrices, particularly serum, waters and beverages (Tsalev 1984; Delves et al. 1988, Frech & Cedergren 1992; UK MAFF 1993). GFAAS involves introducing a small sample (~20μL) to a graphite tube that has an inert atmosphere, which

depending on the sample matrix, undergoes a series of temperature ramps over a programmed temporal profile. A drying step removes the solvent (80-400°C for aluminium), an ashing step removes complex matrix interferents via decomposition (1000-1300°C) and a third step involves atomisation where the free atoms are released and absorbance is measured in the same manner as for FAAS (2300-2700°C) (Hubert 1981; Tsalev & Zaprianov 1983; Tsalev 1984;Sullivan et al. 1987; McKinnon 1990; Lajunen 1992; Lu et al. 1994; Vinas et al. 1995). During atomisation the inert gas flow is temporarily ceased in order to allow a longer residence time of the sample in the light path with consequent increase in sensitivity.

Like FAAS, in a particular concentration range absorbance varies linearly with concentration. Peak areas rather than peak heights are generally used because they more correctly relate to the amount of analyte in the sample (Tsalev & Zaprianov 1983; Lu et al. 1994; Ericson 1992). For GFAAS analysis, argon is used as the inert gas because aluminium in the presence of nitrogen forms involatile nitrides which hinder free aluminium formation, depressing aluminium peaks by up to a factor of three (Sturgeon et al. 1976; Tsalev 1984; Tsalev & Zaprianov 1983; Craney et al. 1986; Lu et al. 1994; McKinnon 1990).

Ashing temperatures are critical for interference removal with the maximum possible temperatures required to 'expel as much and as many potential interferents' without losing the analyte (Tsalev & Zaprianov 1983). This does not always succeed and matrix modifiers have been used to remove interferences for elements such as aluminium. A matrix high in chloride is a good example of this interference, it is widely held that chloride forms volatile $AlCl_3$ thereby causing loss of aluminium in the ashing stage and underestimation of the aluminium content. However Tang et al. (1995) proposed that the loss of aluminium more likely occurs at the beginning of the atomisation stage. Modifiers work by converting matrix interferents to a more volatile form, thereby facilitating removal in the ashing stage, or converting the metal under investigation into a more stable form (Tsalev & Zaprianov 1983; Lajunen 1992). The latter modification technique allows a higher ashing temp which can more easily decompose matrix interferents (Tang et al. 1995; Wieteska & Drzewinska 1995). Although choice of modifier should be based on the type of interferent in the matrix, $Mg(NO_3)_2$ has been predominantly used for aluminium determinations (Delves et al. 1988,

Tsalev 1984; Ericson 1992). This has been challenged by Tang et al. (1995), who found virtually no difference between the performance of $Mg(NO_3)_2$, $Ca(NO_3)_2$, $Pd(NO_3)_2$ and $NH_4H_2PO_4$ on the absorbance of aluminium in water, although $Ca(NO_3)_2$ was suggested as a better modifier for samples high in Ca content and for samples in the presence of chloride. It was also found that a small amount of nitric acid in the matrix improved the aluminium signal. The positive effects of $Ca(NO_3)_2$ and HNO_3 have been supported in work by Wieteska & Drzewinska (1995), Pierson & Evenson (1986), Van Landeghem et al. (1994) and Vinas et al. (1995), however Larroque et al. (1994) found that 1% HNO_3 gave no significant difference in calibration curves of aqueous aluminium standards. Other modifiers that have been used for aluminium include phosphoric acid (Craney et al. 1986), ammonium nitrate (Smeyers-Verbeke & Verbeelen 1988), potassium dichromate (Betinelli et al. 1992; Almieda et al. 1997) and H_2O_2 (Vinas et al. 1995). GFAAS is more susceptible to background absorbance interference and background correction is highly recommended (Bloom & Erich 1996).

Because of the sensitivity of GFAAS and the ubiquitous nature of aluminium, serious problems with external contamination arise (Tsalev & Zaprianov 1983; Tsalev 1984; Delves et al. 1988; Ericson 1992). Because of this, careful cleaning and analytical procedures are required with minimal handling steps, ultrapure reagents, precleaned glassware/plasticware and storage away from lab air (Frech & Cedergren 1992; Ericson 1992). Due to the random nature of contamination by aluminium, appropriate sample handling protocols and efficient cleaning procedures need to be diligently adhered to.

GFAAS has been used extensively for serum analysis (Fagioli et al. 1987; Betinelli et al. 1992; D'Haese et al. 1992), where its sensitivity for aluminium in this matrix is unrivalled and remains the method of choice. Other biological analyses of aluminium have included the determination of the concentration of the metal in the dialysate of uraemic patients (Smeyers-Verbecke & Verbeelen 1988; Minoia et al. 1992), bone (Tang et al. 1996) and neuronal tissue (Pierson & Evenson 1986). Craney et al. (1986) applied GFAAS to aluminium concentrations in water. Aluminium content in a wide variety of foods including vegetables and seeds has also been investigated with GFAAS (Sullivan et al. 1987; Vinas et al. 1995; UK MAFF 1993). However, a vigorous sample preparation

is required prior to analysis to break down the solids and avoid any suspensions which can allow carbonaceous residues to build up in the tube and block the light beam. This is usually accomplished by acid digestion although Sullivan et al. (1987) have used a technique of fusion of the sample with a mixture of sodium carbonate and sodium borate followed by dissolution in 10% HNO_3. GFAAS has also been used to quantitate aluminium in beers, where a median concentration of 0.1mg/L with a range of 0.005-6.5mg/L was found from a survey of beers in the United Kingdom (Sharpe & Williams 1995; Sharpe et al. 1995). In the last decade, GFAAS has been adopted as a detection method for 'hybrid' techniques for speciation studies in conjunction with HPLC or IC (Van Landeghem et al. 1994; D'Haese et al. 1995; Yuan & Shuttler 1995; Kozuh et al. 1996). This will be discussed further in Chapter 2.

1.9 Determination of Total Aluminium in Wine

GFAAS has been the analytical method of choice for aluminium determination in wine; the majority of the studies listed in Table 1.3 have used this technique to quantitate wine aluminium. The most comprehensive study to date on aluminium in wine is the work of McKinnon (1990) and McKinnon et al. (1992) who compared different analytical procedures of quantitating total aluminium in wine, namely FAAS, GFAAS, GC after derivatisation, colorimetric and fluorometric analysis. FAAS was found to be too insensitive, requiring pre-concentration and was prone to interferences. It was asserted by McKinnon (1990) and McKinnon et al. (1992) that aluminium determinations in wine using FAAS in the 1970's were incorrect as the concentrations reported were below the limit of quantitation. They also found that derivatisation of aluminium with trifluoroacetylacetonate for GC analysis, pyrocatechol violet (PCV) or eriochrome cyanine R in cetyltrimethylammonium bromide (ECR/CTA) for colorimetric detection, or N-(3-hydroxy-2-pyridyl)salicylaldimine (3-OH-PSA) in fluorimetric analysis all suffered from significant interferences when applied to wine due to the organic acid content successfully competing with these complexing agents. These problems rendered all the above-mentioned methods useless in determining total aluminium in wine. GFAAS on the other hand was 'found to be the only successful analytical technique for the determination of aluminium in wine, in terms of sensitivity, selectivity, precision and minimal sample pre-treatment' (McKinnon 1990; McKinnon et al. 1992). This assessment was supported by Larroque et al. (1994).

Other than the sensitivity of the technique, the major benefit of GFAAS for wine aluminium analysis was that a 20-fold dilution of the wine with water was found to be sufficient to not only bring the concentration of the sample into the linear working range, but also reduced the complex matrix effects to the point that a simple ashing procedure without matrix modifiers could be used. In addition, because of the reduction of the matrix to an effectively aqueous solution, a typical calibration curve without standard additions based on aqueous aluminium standards could be used. Larroque et al. (1994) adopted this approach, using a 50-fold dilution prior to GFAAS analysis. This dilution affords a great advantage by decreasing preparation and analysis times and the risk of contamination compared with methods where acid digestion (Lopez et al. 1998) and matrix modifier such as $Mg(NO_3)$ (Lopez et al. 1998), H_3PO_4 (Hubert 1981) or $K_2Cr_2O_7$ (Almieda et al. (1997) have had to be used. Almieda et al. (1997) also successfully used a ten-fold dilution in the study of aluminium content of sherry brandy using GFAAS, this smaller dilution probably the result of a simpler sample matrix found in distilled products. In all the studies mentioned in Table 1.3 where GFAAS was used, the method was found to be a reliable, reproducible technique for the measurement of total aluminium in wine that 'does not require modern furnace technology' (Larroque et al. 1994). Seruga et al. (1998) used Zeeman background corrected GFAAS to determine the aluminium content of 24 Croatian wines, however other than this the methodology was the same as McKinnon et al.'s (1992).

With the increasing prevalence and obvious benefits of ICP-AES, this technique has been increasingly used for aluminium determination in wine, particularly as part of a multi-element study, an area that is more quickly and easily accomplished with ICP-AES than GFAAS or FAAS. Larcher & Nicolini (2001) used the technique to determine the concentration of 22 mineral elements including aluminium with good accuracy and precision. Aceto et al. (2002) performed a comparison of the atomic spectroscopy techniques for elemental determination in wines. Unlike McKinnon et al. (1992) this study had access to an ICP-AES, and concluded that although both FAAS and GFAAS were suitable for wine analysis, ICP-AES was preferred due to its speed and simplicity of analysis. The study considered GFAAS too slow and prone to interferences, however this is in disagreement with McKinnon et al. (1992) and Larroque et al. (1994). This disparity in opinion about GFAAS

could be due to the lower dilution that was used by Aceto et al. (2002) and that their study was based on an overall element determination basis rather than specifically aluminium.

II

"I will take the ring.....though I do not know the way"
Frodo Baggins, Hobbit from J.R.R. Tolkein's 'The Lord of the Rings'

CHAPTER TWO

2. REVIEW OF ALUMINIUM SPECIATION ANALYSIS

2.1 Introduction

Speciation analysis has presented the science of analytical chemistry with a tremendous challenge. New and improved methods to determine and characterise the speciation of chemical entities at a molecular concentration have dominated the research efforts of analytical chemists worldwide in the last decade. Chemical species, speciation analysis and speciation of an element have been defined by recent IUPAC commissions as follows (Quevauviller 2000; Templeton et al. 2000):

> *Chemical species; species*: <chemical elements>: specific form of a chemical element defined as to molecular, complex, electronic or nuclear structure.
>
> *Speciation analysis*: <elemental analysis>: measurement of the quantities of one or more individual chemical species in a sample.
>
> *Speciation of an element; speciation*: distribution of defined chemical species of an element in a system.

In the early years of metal speciation analysis, 'extractable forms' of metals were included in the term 'speciation', however Quevauviller (1995a, 2000) quite correctly asserts that these forms should not be included as they are 'operationally defined' and differ with varying experimental conditions and instrumental techniques and cannot be easily compared with each other.

Speciation has become important because it is now recognised that bioavailability and toxicity are not wholly based on the total concentration of an element in a given medium and this recognition is demonstrated in regulatory limits now being set for elemental species (Quevauviller 2000). Bioavailability is the ability of a chemical moiety to be absorbed by an organism. In the case of aluminium in humans, it is 'the extent to which aluminium in food and beverages is absorbed across the intestinal wall and into the body's circulatory system' (Sharpe & Williams 1995). The rate and amount of absorption of aluminium in the gastrointestinal tract will vary depending on its chemical forms (speciation) that exists in various dietary sources (Dayde & Berthon 1992; Gardner & Gunn

1995). 'A form of aluminium which is highly bioavailable might thus be expected to exert an effect which is disproportionate to its concentration, relative to less easily absorbed forms' (Gardner & Gunn 1995).

Although a bioavailable entity is not necessarily a toxic one, the toxicity of aluminium is directly influenced by its bioavailability (Driscoll & Schecher 1990; Berthon 1996). The toxicity of an element is 'a function of both the target element and the chemical structure of the compounds considered and it depends on the absorption paths of the element' (Das et al. 1996). As discussed in Chapter 1 the free aluminium aquo-ion $[Al(H_2O)_6]^{3+}$ and its hydrolysed forms, $[Al(OH)(H_2O_5]^{2+}$, $[Al(OH)_2(H_2O)_4]^{+}$ are considered the most toxic forms of aluminium followed by inorganic aluminium complexes, while the complexes it forms with organic acids are considered less toxic (Rubini et al. 2002). As standard practice in the literature for describing the aluminium hydrolysis products the water molecules will be excluded for simplicity throughout the rest of the thesis. However, as discussed in Chapter 1, these Al-organic acid species have been shown to make aluminium more bioavailable by allowing it to be transported through the gut wall into the bloodstream where transferrin can distribute the metal to the organs. Because different chemical species have varying degrees of toxicity and these toxic fractions have varying degrees of bioavailability it is apparent that the determination of total aluminium 'is an inadequate measure for fully understanding the metabolism, elucidating the mechanisms of toxicity, gaining insight into the element's cellular uptake or distribution, or studying the interactions at the concentration of protein binding and tissue deposition' (D'Haese et al. 1995). To have any concept of the bioavailability and toxicity of aluminium in different foodstuffs, beverages and environmental samples, an understanding of the speciation of aluminium in a particular medium is paramount (Rubini et al. 2002).

Speciation analysis is challenging to the analytical chemist because chemical species, especially in aqueous systems, are involved in a system of dynamic equilibrium. To analyse a particular chemical species or group of species the techniques used invariably involve a chemical or physical reaction with these species which in effect alters their equilibrium states and consequently changes their concentrations and/or their chemical form from the original entity that one is trying to analyse.

Essentially, by upsetting the original equilibria of the sample as its method of detection, these techniques make it difficult to identify what is actually measured (MacFall et al. 1995; Berden et al. 1994; Clarke et al. 1992; Bi 1995, Berthon 1996; Pyrzynska et al. 1999). These problems led Clarke et al. (1992) to surmise that 'one might conclude that true speciation is impossible'. In addition many factors influence speciation. These include pH, ionic strength, reaction kinetics, relative concentrations and complexing affinities of cations and anions, temperature and solution contact with particulate phases (Driscoll & Schecher 1990; Vance et al. 1996; Ritchie & Sposito 1995; Martin 1988; Berthon 1996). Any analytical method that affects any of these factors will impact on the solution chemistry and potentially alter the speciation.

The majority of aluminium speciation work has been carried out in aqueous media and the behaviour of aluminium in water is well reported and understood. Due to its small size and high positive charge, Al^{3+} will prefer electrostatic rather than covalent bonding (Martin 1986,1988; McDonald & Martin 1988; Harris et al. 1996; Berthon 1996; Yokel 2002), making it a hard Lewis acid and as a result has a strong affinity for electronegative hard Lewis bases such as O^{2-} and F^-. Because oxygen can be incorporated into multidentate ligands that form metal complexes with high formation and stability constants, complexes with oxygen donor ligands tend to dominate binding with Al^{3+} (Martin 1988; Orvig 1993; Ritchie & Sposito 1995; Berthon 1996).

In aqueous media, pH is a dominant factor in the speciation of a metal. In water at a pH <3 free Al^{3+} is found exclusively as the octahedral hexaaquaaluminium(III) ion, $[Al(H_2O)_6]^{3+}$ in the absence of other ligands. As the pH increases this ion undergoes hydrolysis initially to the mononuclear species $[AlOH]^{2+}$, $[Al(OH)_2]^+$ and $Al(OH)_3$ of which the latter precipitates out of solution. Polynuclear species, such as $[Al_{13}O_4(OH)_{24}(H_2O)_{12}]^{7+}$, can also form although the mechanisms of formation and structure of these species is still not well understood (Bertsch & Parker 1996), however most aluminium speciation work has centred on mononuclear species. In basic solution $Al(OH)_3$ dissolves to form $[Al(OH)_4]^-$, which is the dominant species in alkaline conditions (Orvig 1993). A distribution diagram describing the behaviour of Al^{3+} in an aqueous media with respect to pH is shown in Figure 2.1.

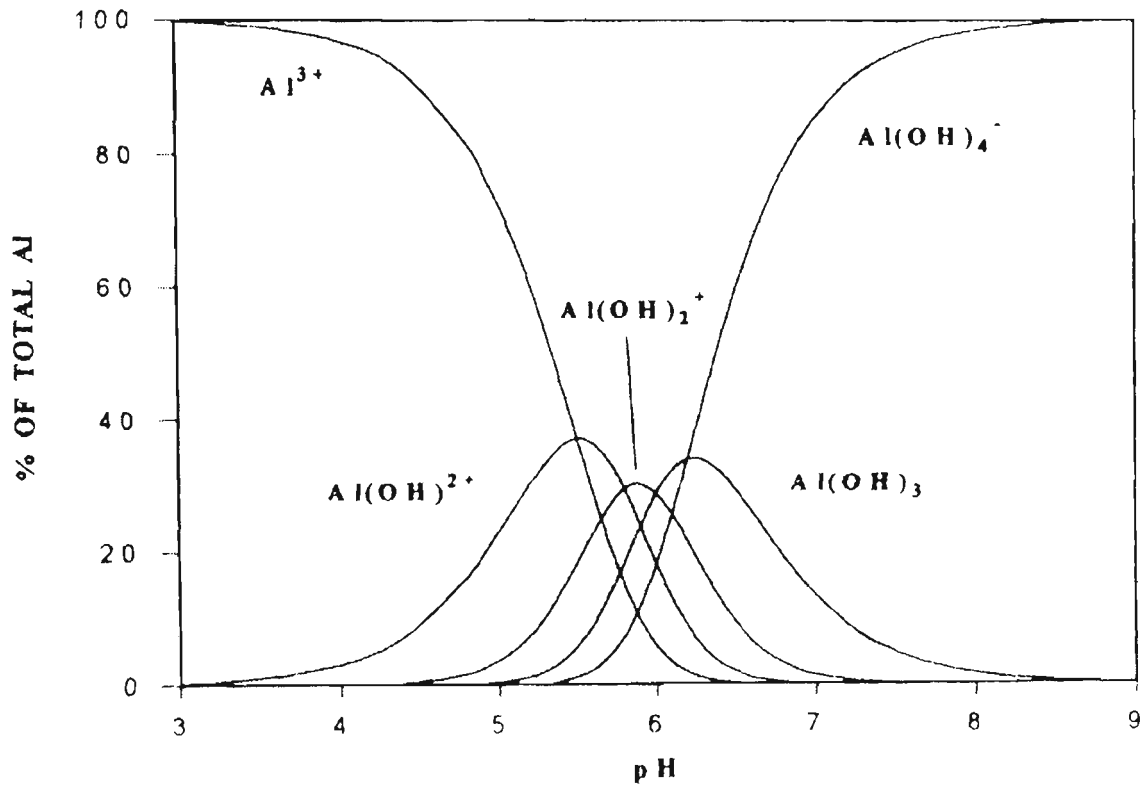


Figure 2.1 Distribution of the Al^{3+} aquo ion and the mononuclear $Al(OH)_n$ species for 1μM total aluminium (from Harris et al. 1996).

Ligands other than OH^- also form complexes with Al^{3+} in water. Due to its strong electronegativity and a similar charge and size compared with OH^-, fluoride can bind very strongly with aluminium, and like hydroxide, form octahedral complexes. Sulfato ligands can form a complex with aluminium but are significantly weaker than those of F^- or OH^- (Nordstrom & May 1996; Martin 1988). Phosphato ligands have been shown in computer modelling to bind strongly to aluminium forming an insoluble neutral complex (Martin 1988) however they have been difficult to characterise experimentally (Nordstrom & May 1996). As described earlier, organic ligands, particularly those with nitrogen or oxygen atoms that can bind as multidentate ligands, form complexes with aluminium in water. These include carboxylic acids, phenols, amines, alkoxides and amino acids although amino acids (Martin 1988) are thought to bind weakly to Al^{3+} (Yokel 2002). Carboxylates are prominent in their preference for Al(III) in biological fluids where aliphatic acids are the strongest chelators and their acids order of binding strength from the strongest are dicarboxylic >>hydroxycarboxylic>carboxylic>>amino acids (Rubini et al. 2002; Salifoglou 2002; Yokel 2002). Nordstrom & May (1996) provide a comprehensive and fully referenced list of organic ligand/aluminium formation constants derived from experimental data which include ionic strength

and temperature of the solutions used (where available). Orvig (1993) provides a less comprehensive list. Anionic ligands will compete with the hydrolysis of aquated Al^{3+}. Harris et al. (1996) state that 'mixed Al(ligand)-(hydroxide) complexes are the rule, rather than the exception, and hydroxo-bridged polynuclear complexes are common'. Thermodynamically, successful complex formation over hydrolysis will rely on the formation constants of the complexes, which in turn are dependent on pH, temperature and ionic strength.

One other consideration that must be taken into account is that while proton transfers in hydrolysis reactions are virtually instantaneous, Al^{3+} is a strong but 'sluggishly labile' cation, hence the reaction kinetics of ligand binding is considered slow compared with that of other cations (Martin 1988; Birchall & Exley 1992; Harris et al. 1996; Nordstrom & May 1996; Berthon 1996; Orvig 1993). The slow kinetics are evident in Martin's (1988) scale of increasing water exchange rates for substitution of inner sphere water from metals at 25°C as follows: $Al^{3+} < Fe^{3+} < Be^{2+}$, $Ga^{3+} << Mg^{2+} << Zn^{2+}$, $Sc^{3+} < Ca^{2+}$, where each inequality sign indicates a 10-fold increase in rate from about 10 sec^{-1} for Al^{3+} and increasing through 7 powers of 10 to about 10^8 sec^{-1} for Ca^{2+}. In terms of the hydrolysis reactions, tetrahedral aluminium is thought to exchange ligands more rapidly than octahedral aluminium and polynuclear species are extremely slow to form, contributing to characterisation difficulties. Chelated ligands exchange even more slowly than monodentate ones (Martin 1988). Complexation kinetics is an important consideration in aluminium speciation analysis. This "sluggishly labile" behaviour of Al^{3+} may work in favour of this project when speciation by a relatively fast technique such ES-MS is attempted (see Section 2.3).

2.2 Summary of Aluminium Speciation Work to Date

The last decade has seen a dramatic increase in developing methods to identify and characterise aluminium species. Aluminium speciation research has generally focused on two main areas: aqueous media, both controlled solutions and environmental samples, and aluminium in the human body, both theoretical considerations and clinical characterisation. The former has become of interest particularly with the mobilisation of Al^{3+} by acid rain and the latter due to toxicological issues outlined in Chapter 1. Natural waters present a broad range of pH's and experimental

conditions, while in the gut pH can also vary considerably depending on the location within the gastrointestinal tract. In the blood and other areas of the body however the temperature and the pH are controlled at 37 C and 7.4 respectively with very little variation tolerated.

Many different analytical techniques have been used in an attempt to identify and characterise aluminium species in environmental, biological and synthetic samples. Initially, instrumental methods used for aluminium could only determine the total aluminium concentration in a sample. These methods include flame, emission and graphite furnace AAS, UV-visible and fluorescence spectrophotometry, neutron activation analysis, liquid chromatography and fluoride-selective electrode (Bloom & Erich 1996). When it was established that the determination of the total concentration of aluminium was an inadequate measure of the element's bioavailability and toxicity, these traditional methods were modified or new techniques were applied.

Due to the problems of direct aluminium species determination two analytical procedures have dominated aluminium speciation research (Pyrzynska et al. 1999, 2000). One is a theoretical approach using computer modelling with thermodynamic data and the other involves experimental techniques to separate fractions of species by parameters of charge, size or affinities for complexing agents. In some cases researchers have used features of both procedures but each is reliant on traditional or modified instrumental methodology developed from those employed for total aluminium determinations. The following review discusses the above-mentioned procedures followed by the instrumental methods employed to provide the data, concluding with the pitfalls of these techniques and the introduction of more direct methods of aluminium speciation analysis.

2.2.1 Theoretical Method of Speciation Determination

This approach has been enhanced by the development of more powerful computers and modelling software. It primarily depends on the use of analytical techniques to ascertain the concentrations of the total aluminium and the major ligands present and using a computer program to make calculations based on this information and available equilibrium formation constants to give a theoretical distribution of particular species. Much of the work using this approach has focused on inorganic monomeric species without taking $Al(OH)_3$ (which precipitates out of solution),

polymeric and organic species into account and has rendered the model distribution incomplete. Furthermore, when factoring other ligands into the model authors have used different ligands, formation constants or ionic strengths of solutions, making it difficult to compare results (Pyrzynska et al. 1999, 2000; Nordstrom & May 1996; Driscoll & Postek 1996). In addition, the calculations are totally dependent on the thermodynamic data, and information derived from this approach is only as good as the quality and completeness of these data (Harris et al. 1996). Theoretical modelling depends on ideal conditions, and natural samples are far from ideal, hence the information derived from this procedure should be considered a guide only, while experiments that factor in more variables and use a more comprehensive database are closer to the real situation. The limitations of computer modelling based on non-standardised techniques and customised programs utilising different formation constant data, coupled with the problems of slow reaction kinetics of polymeric and organic aluminium, has confused the picture of aluminium speciation. These difficulties led Driscoll & Postek (1996) to conclude 'there is considerable uncertainty as to what forms of aluminium are actually represented'.

2.2.2 Experimental Fractionation Method of Speciation Determination

The second procedure grew from the need to determine and characterise aluminium species using actual instrumental data. Because direct speciation analysis of individual species was impossible to perform, an indirect method that characterised particular groupings or fractions of aluminium species was developed by Driscoll (1984). The procedure separates the aluminium in a sample into five fractions; essentially total reactive aluminium (Al_t), total monomeric aluminium (Al_m) and non-labile monomeric aluminium (Al_o) are measured directly and acid soluble aluminium (Al_t - Al_m) and labile monomeric aluminium (Al_m - Al_o) are measured by difference. The fractionation scheme is shown in Table 2.1.

Table 2.1 Basic fractionation scheme for aqueous aluminium.

Sample Treatment	Aluminium Fraction	Fraction Composition
Acid digestion	Total reactive (Al_t)	Acid digestible forms
Without digestion	Total monomeric (Al_m)	Inorganic and organic monomeric complexes
	Acid soluble (Al_t - Al_m)	Colloidal, polymeric and strong organic complexes
Cation-exchange treatment	Non-labile monomeric (Al_o)	Monomeric organic complexes
	Labile monomeric (Al_m - Al_o)	Al^{3+}, hydroxide, sulfate & fluoride complexes

As will be seen further in the review this procedure has been adopted in whole or in part by most researchers since. Many compare their work with Driscoll's, and his methodology has become a *de facto* standard. Again there are limitations to this procedure as the various methods define differing fractions that do not always coincide and are operationally defined, i.e. 'the determined forms depend on the exact procedure used for the determination' (Pyrzynska et al. 2000). This makes direct comparison difficult and the fractions that are separated are not always mutually exclusive of one another (Pyrzynska et al. 2000).

2.2.3 Review of Speciation Analysis Methods

2.2.3.1 Modelling

Computer modelling was initially the first technique to be used for aluminium speciation as direct analysis was unavailable. Software, which was mostly developed by the researchers themselves, was used to calculate the distribution of aluminium species via two methods: one using a database of formation constants of aluminium complexes with major ligands gleaned from several sources in conjunction with simulation software, and the other using a modelling program to interpret information derived from the author's measurements of Al^{3+} and/or the ligands activities.

Martin (1986, 1988) used modelling to comprehensively review the speciation distribution of aluminium in the human gastrointestinal tract and bloodstream. Using data from conditional stability constants, the role of $Al(OH)_3$ and $Al(OH)_2(H_2PO_4)$ in precipitating and removing aluminium from the body was demonstrated. In addition he found that citrate could solubilize aluminium to form a potentially bioavailable neutral complex and that citrate and transferrin were

the major aluminium binders in the bloodstream. Dayde & Berthon (1990) performed a computer simulation of interactions of aluminium with citric, malic, oxalic, succinic and tartaric acids, giving estimations of pH required for dissolution of aluminium salts in the gastrointestinal tract. They found these acids could solubilise $Al(OH)_3$ and $AlPO_4$ over a large pH range especially for lower aluminium concentrations. They expanded this work, focussing on Al-tartrate complexes in the gut, concluding that a dimeric $[Al_2(H_{-1}tart)_2]$ (where H_2tart denotes a tartaric acid molecule with both carboxylic acid protons) species can form over a wide pH range (Dayde & Berthon 1992). $AlPO_4$ and $[Alcit(OH)_2]^{2-}$ were identified as the main bioavailable aluminium species in beer based on modelling data which simulated the speciation of aluminium throughout the gastrointestinal tract (Sharpe & Williams 1995; Sharpe et al. 1995).

Other than biological systems, simulations have also been used to study speciation in natural waters. Bi (1995) conducted an exhaustive study on aluminium aqueous speciation applying a modelling program that included previously ignored data on organic and polymeric aluminium species, long considered too difficult, and investigated the factors influencing various aluminium complexes. He concluded that, below pH 4, polymeric aluminium is a more significant species than previously thought and at a pH of 4-7 the dominant species are Al-F and Al-Org complexes followed by the aluminium hydrolysis products, $[AlOH]^{2+}$ and $[Al(OH)_2]^{+}$, with sulfato and phosphato complexes insignificant. Temperature was found to significantly influence species concentrations and that variations in literature values of the thermodynamic data used for modelling could cause 100% variation in aluminium complex concentration, demonstrating the limitations of the procedure. Tunega et al. (2000) used computer modelling to examine bonding and structures of aluminium acetate complexes. Using this model, they found that monodentate structures are energetically more stable energetically than bidentate carboxylates. This is due to hydrogen bonds in the monodentate structures and steric factors and ring strain in a four membered ring for the bidentate structures.

The earliest method to gather thermodynamic data for speciation modelling utilised potentiometry. This is generally performed using an H^+ Ion Selective Electrode (ISE) measuring pH in titrations of aluminium salts with a base, or monitoring F^- ion activities of aluminium fluoride complexes with a fluoride ISE (Nordstrom & May 1996; Berthon 1996). The former method is more commonly used,

especially for aluminium hydrolysis simulations, as the fluoride method requires aluminium to be the only component present that binds F^- and is prone to interferences that lead to underestimation of aluminium species concentration (Bloom & Erich 1996).

Aluminium(III) in solution as its nitrate salt was titrated via an automated potentiometric technique with potassium bicarbonate by Brown et al. (1985) to investigate aluminium hydrolysis. Using a program to model distributions from the titration data, they concluded that between pH 4-6, $[AlOH]^{2+}$ was the most significant hydroxy ion with $[Al(OH)_2]^+$ becoming important only at higher pH and low aluminium concentration. They characterised a low-molecular mass polymeric species, $[Al_3(OH)_4]^{5+}$ which they suggested only formed at high aluminium and hydroxide concentrations. While their model included a high-molecular mass polymeric species, they could not define its stoichiometry, with too many possibilities fitting the model data. Ohman (1988) used a combination of potentiometry and computer modelling to study the kinetic route for complexation of citric acid (H_3cit) with Al^{3+} in the pH range 3-7. A pre determined amount of OH^- was added to a mixture of aluminium and H_3cit and the pH was observed until stable equilibrium was reached, some solutions taking up to 20 hours to equilibrate. This was repeated many times varying the added OH^-, total Al^{3+} and total H_3cit. $[Al(H_{-1}cit)]^-$ (where H_3cit denotes a citric acid molecule including the three carboxlic acid protons) and $[Al(OH)(H_{-1}cit)]^{2-}$ were considered the main species in freshly mixed solutions which then gradually converted into a polymeric complex, $[Al_3(OH)(H_{-1}cit)_3]^{4-}$; where $H_{-1}cit$ is an anion of citric acid minus four protons.

Other Al-organic acid systems have been more recently investigated. Following on from their earlier modelling work the group of Berthon has extensively investigated the distribution of aluminium complexed with organic acids by potentiometry and then using computer modelling to simulate the speciation of the aluminium throughout the gastrointestinal tract though not without controversial results. In the investigation of simulated gastrointestinal tract aluminium-tartrate speciation they concluded that a $[Al(tart)]^+$ species was dominant at pH 2 and that the neutral species $Al(H_{-1}tart)$ and dimeric $Al_2(H_{-1}tart)_2$ made up 85% of aluminium present at pH 3.66 (Desroches et al. 2000). At higher pH, these species and further polynuclear species continued to predominate up until pH 7. An investigation of malate speciation shows similar trends although at pH 2 the aluminium bimalate

species was dominant and monomeric neutral Al(mal)(Hmal) and Al(H_{-1}mal) made up 39-59% of the available aluminium depending on the malate concentration (Venturini-Soriano & Berthon 2001). Between a pH of 4-7, polymeric aluminium species were also reported. Potentiometric titrations involving Al-succinate complexes in the gastrointestinal tract were also modelled over the pH range 1-8 (Ailley et al. 1996; Venturini-Soriano 1998). They proposed monomeric $[Al(Hsuc)]^{2+}$ and $[Al(suc)]^{+}$ at pH 2-5 and neutral polymeric $Al_4[(H_{-2}suc)_3]$ at pH 5-7 although the existence of the last species is highly unlikely (see next paragraph). As Ohman (1988) found with aluminium-citrate, these studies showed that increased aluminium concentration in the mixture increased the likelihood of polymeric aluminium species. This was particularly the case for succinate which demonstrated polynuclear species with aluminium over the whole pH range with very little monomeric species.

Despite the characterisations, the studies of Berthon's group also show the limitations of the method. It is highly unlikely that doubly and triply deprotonated malate and tartrate would exist in solution at a pH of 2-3.5, even though these ligands can accommodate the removal of 3 and 4 protons respectively from their carboxyl and hydroxyl groups. Furthermore, acid/base solution chemistry would be hard pressed to explain the removal of more than two protons from succinate as postulated in the $Al_4(H_{-2}suc)_3$ species since succinic acid only has two carboxylate protons. The removal of any methylene protons from the carbon chain is extremely dubious, even at a pH of 5-7.

Computer model simulations have also been used as a reference to identify possible species from experimental data derived from other instrumental techniques (Bertsch & Anderson 1989; Gibson & Willett 1991; Danielson & Sparen 1995; Boudot et al. 1994; Mitrovic 1996). Aluminium speciation in serum was investigated by Harris & Sheldon (1990) by titrating solutions of apotransferrin with aluminium nitrate and measuring the UV spectra of resultant complexes to obtain equilibrium constants for use in modelling aluminium complexation behaviour in serum. The study showed 5% of aluminium in serum was bound to citrate, the rest was almost exclusively bound to transferrin.

Wang et al. (2001) described an electrochemical method using derivative adsorption chronopotentiometry with a mercury drop electrode at pH 4.9 and 8.2 to measure Al(III)-PCV

complexes derived from labile and total inorganic monomeric fractions respectively. The same group followed on from this study to develop a novel differential pulse voltammetric (DPV) method using pyrocatechol violet modified electrodes (PCV-CMEs) to determine similar aluminium fractions as Driscoll's method in natural waters (Liu et al. 2001). The electrodes were fabricated using a dip-coating method whereby cleaned and polished graphite electrodes were dipped into a 0.02M PCV solution for a certain time and rinsed with water. Al^{3+} forms a 1:1 or 1:3 electroinactive complex with PCV at pH 4.8 and 8.5 respectively which is absorbed onto the electrode as a monolayer covering the electroactive sites resulting in a decrease in peak current. Quantification is based on a linear relationship between aluminium concentration and the decrease in the oxidation DPV peak current. Three fractions are measured directly by DPV, one after an acid digestion for 24hr then measurement at pH 8.5 for the acid reactive aluminium, one at pH 4.8 which measures labile monomeric aluminium (inorganic forms) and another at pH 8.5 that measures total monomeric aluminium (inorganic and organic forms). Like Driscoll's procedure, acid soluble and non-labile monomeric aluminium can then be measured by difference. Results compared favourably with Driscoll's method.

2.2.3.2 Spectrophotometric reagents

Metallochromic reagents have been used to determine total reactive aluminium by way of binding the aluminium to an organic reagent to form a light absorbing or fluorescent complex that can be detected by UV-Visible or fluorescence spectrophotometry. These methods have been modified to perform speciation analysis with varying degrees of success by reacting a coloured or fluorescing agent in a short period of time with the solution in question and rapidly extracting the Al-organic reagent complex into an organic solvent to avoid interferences from humic substances. Detection and quantitation is then performed by the chosen spectrophotometric method. By varying the contact time of the reagent with the solution, different amounts and fractions of species can be analysed and determined. Iron(III) and other cations that can react with the complexing agents can interfere with the Al-complex and its detection. Hydroxylamine and 1,10-phenanthroline have been used as masking agents to reduce this interference.

Initial methods involved manual batch reactions and extractions after a period of 15-60 seconds (Bloom & Erich 1996; Pyrzynska et al. 1999, 2000). More recently Flow Injection Analysis (FIA) has been used to automate and reduce the reaction and extraction times to 2.5s. The shorter the reaction time the greater the discrimination between the labile monomeric species and organically complexed and polymeric forms of aluminium. Many complexing agents have been used for this purpose. The most commonly used reagent for UV-Vis detection has been 8-hydroxyquinoline (8-HQ) at 390nm, followed by Pyrocatechol Violet (PCV), Tiron and Ferron. Lumogallion and 8-hydroxyquinoline-5-sulfonic acid (8-HQS) have been the most commonly used reagents for fluorescence detection (Bloom & Erich 1996; Nordstrom & May 1996; Pyrzynska et al. 1999, 2000; Yokel 2002). Initial speciation work using 8-HQ required the aluminium complex to be rapidly extracted into chloroform after 15 seconds. Later procedures utilised MIBK to extract the aluminium complex with more rapid extraction times. Since the method has been so widely used, several authors have employed the 8-HQ technique as a validation of their experiments using other speciation techniques, primarily ion exchange procedures.

James et al. (1983) developed the use of 8-HQ with a short sample contact time (15s) that was 'long enough to form the 8-HQ complex with free Al^{3+} and short enough to minimize release of complexed aluminium to 8-HQ'. However, some kinetically labile ligand bound aluminium was also found to be extracted and the fraction was deemed an operationally defined quantity of labile aluminium. With the separate determination of total aluminium, the non-labile fraction could be calculated by difference. This method has been widely adopted and modified since in the same manner as Driscoll's fractionation procedure. Mitrovic et al. (1996), Kozuh et al. (1996, 1997) have used a modified 8-HQ, 15 second extraction methodology of James et al. (1983) to compare their results derived from ion exchange procedures via IC and FPLC. Because the 15 second extraction is prone to extract some of the organically complexed aluminium, more recent studies have used shorter extraction times to capture labile monomeric aluminium while at the same time extracting as little organically complexed aluminium as possible. Boudot et al. (1994) used a 5 second flash extraction of the Al-8-HQ complex with MIBK on spring waters in an attempt to limit extraction of organic aluminium which partially occurs with the widely used 15 second procedure. The complex was then detected using UV-Vis spectrophotometry. For a synthetic Al-fulvate complex, 8% was

extracted by the 8-HQ in the shorter extraction method compared with 20% for the 15 second procedure. Al-fluoride complexes were not included in the extracted fraction.

In the quest for faster reaction and extraction times, recent studies have resorted to Flow Injection Analysis (FIA) to enhance aluminium speciation work using spectrophotometric methods. Clarke et al. (1991,1992) devised an FIA system to automatically react 8-hydroxyquinoline (8-HQ) with aluminium in a sample and terminate the reaction with extraction into $CHCl_3$ in a very short period. With a reaction time of 2.3s they captured an operationally defined 'quick reacting fraction' of aluminium which they equated to Al^{3+}, $[AlOH]^{2+}$, 'probably' $[Al(OH)_2]^{+}$ and sulfato aluminium complexes. Some organic-aluminium complexes were partially extracted but polynuclear aluminium, citrato-Al complexes and fluoro-Al complexes were not extracted. Because the latter complexes were not included in the 'quick reacting' aluminium species, this fraction was not equivalent to Driscoll's labile monomeric aluminium, again highlighting the problems associated with comparing procedures. In addressing this difference Berden et al. (1994) compared the FIA method with Driscoll's manual procedure and found that if the Al-fluoro complexes were excluded from the latter method, the two methods showed good agreement in both synthetic solutions and those of natural water and soil leaching samples. The advantage of the FIA procedure is its quick analysis times, low sample requirements, safety (reducing handling of organic solvents) and a direct measurement of toxic fractions of aluminium compared with measuring by difference of various analytical techniques encountered in Driscoll's procedure.

Clarke & Danielsson (1995) and Danielsson & Sparen (1995) developed the FIA procedure further by investigating simultaneous aluminium and Fe speciation and introducing GFAAS as a quantitative detection method. In the case of the former work, the FIA reacted 8-HQ and 1,10-orthophenanthroline-iodide with aluminium and Fe to speciate 'quick reacting' Al(III) and Fe(III) and Fe(II) using chloroform as the extractant and a diode-array UV-Vis for detector. The latter investigation utilised GFAAS as a detector to effectively lower the detection limit of 'quick reacting' aluminium in near neutral water where sensitivity was poor in the earlier system of Clarke et al. (1991; 1992). Toluene replaced chloroform as the extractant as the latter solvent caused poor reproducibilities in the GFAAS analysis of the extracted solution. The poor reproducibility was

probably due to formation of $AlCl_3$ which is volatile and destabilises the aluminium GFAAS signal. This modified FIA method gave a 50-100 fold lower detection limit, allowing detection of low concentrations of 'quick reacting' aluminium in natural waters.

Fluorescence spectrophotometry has been found to give better sensitivity than UV-Vis however the technique has been applied more as a detection method for other speciation procedures (see 2.2.3.3) rather than as a time dependent reaction study as already discussed in this Section with UV-Vis methodology. FIA has also been combined with fluorescence speciation of aluminium with Sutheimer & Cabaniss (1995) devising an FIA system reacting aluminium in solution with lumogallion. While the method did show greater sensitivity without the need for masking agents as used in UV-VIS spectrophotometry or micelle forming agents required in earlier fluorescence techniques, the fraction of aluminium extracted was relatively non-selective and included not only total labile monomeric aluminium but organically complexed aluminium as well. Only colloidal and polymeric forms of aluminium were excluded from extraction. This makes the procedure difficult to apply as a direct speciation tool.

Both spectrophotometric and fluorimetric procedures have been allied to other speciation methods as detectors and will be discussed in Sub-Sections 2.2.3.3 and 2.2.3.5.

2.2.3.3 Ion exchange

Aluminium speciation by ion exchange can almost be considered as evolving from the spectrophotometric/fluorometric methods described in 2.2.3.2 by adding a separation step prior to detection and using a post column derivatising agent to form a suitable colorimetric/fluorescent complex (Buldini et al. 1997). Driscoll (1984) pioneered the method analysing natural waters using cation exchange to separate the aluminium into fractions or groups of cationic species. Although the method does not completely identify and characterise individual species it provides information on the amount of monomeric labile aluminium which is considered the most toxic fraction to bio-organisms. It relies on monomeric labile aluminium being sorbed onto a cation exchange column, leaving the strongly bound organic and non-labile inorganic aluminium in solution. By analysing the solution before and after sorption the labile monomeric species can be determined by difference.

Initially a batch technique was employed with the chelating resin, later studies performed the separation with a simple column and then ion chromatography and high performance liquid chromatography were utilised. Resins generally used for ion exchange include Amberlite IRA-120, XAD-2 and Chelex 100 (Pyrzynska et al. 1999, 2000). The procedure has been taken further with hybrid systems utilising two or more instrumental techniques at the same time. These will be discussed later in the review. The technique has been modified using anion exchange resins to analyse anionic species at basic pH.

The procedure developed by Driscoll (1984), as outlined in Sub-Section 2.2.2, separated aluminium in natural lake water into five fractions by analysing three separate sub-samples. Total acid-reactive aluminium was determined by filtering the water after contact with acidic solution at a pH of 1.0 for one hour, followed by derivatisation and UV analysis. A second portion that had not been treated with acid was analysed to determine the total monomeric aluminium which included organic and non-labile complexed inorganic forms. The acid reactive fraction was calculated from the difference between the total reactive and total monomeric aluminium concentrations. A third sample of solution was passed through a column packed with cation exchange resin, to sorb labile monomeric aluminium, and the aluminium concentration of the column effluent was analysed. The difference between the organic/non-labile-inorganic monomeric aluminium and total monomeric aluminium gave the amount of labile monomeric aluminium in the water. This last fraction is believed to include Al^{3+} and its hydroxo, sulfato, fluoro and silicato complexes.

This method has been modified and enhanced over the last 20 years. Schintu et al. (2000) used an extended version of Driscoll's method to perform speciation analysis on drinking water by including the determination of particulate and colloidal aluminium fractions. Gan et al. (2001) modified the method by using chloroform as the extractant rather than MIBK and measuring the inorganic monomeric aluminium by fluorescence of the complex with 8-HQ directly rather than as a difference as used in the original method. The aluminium content of the fractions of the modified method was almost identical to that obtained using Driscoll's method. Wu et al. (1996) modified Driscoll's method for the determination of three fractions of aluminium in urine. GFAAS was used to determine total aluminium. Fluorometric analysis using lumogallion as the complexing agent was

used to first measure total labile aluminium (pre column) and the effluent from the urine passed through a column of ion exchange resin (post column). By difference labile aluminium and colloidal, polymeric and protein bound aluminium were determined. A rapid flow rate of 4ml/min was used in the ion exchange column to avoid adsorption of weakly bound Al-Org complexes and overestimation of labile monomeric aluminium. A batch method was used by Pesavento et al. (1998a) to sorb Al(III) from natural waters onto differing amounts of Chelex-100 resin as a titration. After a holding period of 8 hours aluminium was eluted off the resin with HNO_3 and measured with GFAAS. Interaction co-efficients derived from metal ion/resin equilibria gave a measure of metal ion stabilities which can be compared with known ligands. However while it could show the presence of complexes of particular binding strength it could not give information on the nature of the complexes.

The column technique has predominated ion exchange aluminium speciation procedures since the benefits of automated instrumental methods were realised and applied. Ion Chromatography (IC) has long been a useful technique for the analysis of ions in aqueous media including the determination of total aluminium (Jones et al. 1988; Bloom & Erich 1995). These techniques have been modified to investigate aluminium speciation. Tapparo & Bombi (1990) employed IC with an ion exchange column to determine a fraction which amounted to total monomeric and labile polymeric aluminium. Bertsch and Anderson (1989) evaluated IC as an aluminium speciation tool using a separator column with post column derivatisation with Tiron (4,5-dihydroxy-m-benzenedisulfonic acid) as a complexing agent. Subsequent detection was carried out using UV-Vis spectrometry. By comparing their values obtained for fluoro, oxalato, citrato, and uncomplexed aluminium with predicted values from modelling software, they found excellent agreement between the two methods provided sample and eluent pH and ionic strengths were matched. Polynuclear aluminium could not be separated due to a strong interaction with the stationary phase. However it was also found that while uncomplexed and monofluoro aluminium were separated into distinct peaks, difluoro, oxalato and citrato species co-eluted and aluminium complexes with the ligands sulfate, acetate, propionate and benzoate were not stable enough to survive the eluent-column environment and eluted as the hexaaqua species. Busch & Seubert (1999) used a combined size exclusion and cation exchange column in an IC system with detection by reacting the eluent with

Tiron post-column and measuring with UV photometry. With this system they found that weak neutral and negative aluminium species with ligands like fluoride and oxalate partially degraded in the column. However, this effect was found to be sensitive to temperature with minimal degradation observed for these complexes at column temperatures below 10°C. Aluminium-citrate species did not show an appreciable difference with changes in column temperature.

Gibson and Willett (1991) applied IC with a guard column to aluminium speciation in soils. The complexing agent 8-hydroxyquinoline-5-sulfonic acid (8HQS) with cetyltrimethylammonium bromide (CTAB), which enhances fluorescence signal, was introduced to the eluent post column and detected by fluorescence. This method was found to have greater sensitivity than using colorimetric derivatisation and separated the aluminium into three fractions:

1) 'free', hydroxo and sulfato aluminium,
2) doubly charged aluminium complexes eg. $[AlF]^{2+}$ and,
3) singly charged aluminium complexes eg. $[AlF_2]^{+}$, oxalato and citrato aluminium.

However the citrato-Al complex did not fully dissociate in the presence of 8-HQS, demonstrating a constant problem with the use of complexing agents for species detection of aluminium in the presence of strongly binding organic ligands. Using a similar methodology with the same column and post column derivatising agent but different eluent and pH (4.0 instead of 3.0), both fluoro species $[AlF]^{2+}$ and $[AlF_2]^{+}$ were individually separated from labile monomeric and organic complexed aluminium (Jones 1991; Jones & Paull 1992). This method allowed true speciation of Al-fluoro complexes in natural waters and direct determination of Driscoll's labile monomeric aluminium fraction.

Kozuh (1996,1997) used an automated batch column method with Chelex 100 resin as the ion exchange medium to speciate monomeric labile aluminium in the pH range 3.0-8.0 in soil extracts and natural waters. As the sample was passed through a microcolumn containing the ion exchange resin, the aqua Al^{3+}, hydroxo, sulfato and fluoro aluminium complexes were retained on the column. This fraction was subsequently eluted with 1M HCl and the eluate aluminium concentration determined via ICP-AES/GFAAS. Although ICP-AES was initially used to detect aluminium, the GFAAS technique was preferred because it lowered the detection limit by an order of magnitude.

Comparison with the 8-HQ spectrophotometric method and calculated data showed good agreement with the microcolumn technique however the latter method showed greater sensitivity with water and soil extract samples. While this technique was not hampered by surfactants as is the case with the 8-HQ method, high salinity and an excess of alkaline earth metals caused negative interferences. $Al(OH)_3$ sorption on the resin at a pH above 5.0 was alleviated with a pre-washing of the column with dilute HCl prior to the elution step.

Recently Fast Protein Liquid Chromatography (FPLC) has been utilised for aluminium speciation of waters and soil extracts. A strong cation exchange column was employed by Mitrovic et al. (1996) to separate aluminium species with detection after elution by ICP-AES 'off-line'. Linear gradient elution with $NaNO_3$ through the FPLC column gave individual species separation of Al^{3+}, $[Al(OH)_2]^+$ and $[AlOH]^{2+}$ in synthetic aluminium aqueous solutions where charge was deduced from retention times and species from theoretical calculations. This was a significant improvement on other methods which could not discern individual species in the labile monomeric aluminium fraction. When inorganic and organic ligands were present, $[Al(SO_4)]^+$, $[AlF_2]^+$ and negatively charged oxalato and citrato complexes were found to co-elute with $[Al(OH)_2]^+$, while $[AlF]^{2+}$ co-eluted with $[AlOH]^{2+}$. Aluminium-citrato complexes co-eluted with $[AlOH]^{2+}$ and Al^{3+} at lower pH. Like Kozuh et al. (1996,1997), the technique was compared with the 8-HQ method giving good agreement in results of analysis of soil extracts.

While cation exchange methods have been very useful in determining 'toxic' labile monomeric aluminium in waters and soil extracts, they only detect those ions which are positively charged at a pH lower than 7.0. Neutral or negatively charged species are not detectable with cation-exchange (Gibson & Willett 1991; Jones & Paull 1992; Jones 1991) and hence do not give aluminium speciation information in samples where the pH is above 7.0. To do aluminium speciation in alkaline samples researchers turned to anion-exchange procedures. Pesavento et al. (1998b) extended the analysis of aluminium sorption onto cation exchange resin by replacing it with an anion exchange resin. The sorption equilibria of aluminium solutions with this resin were examined by resin titration at pH 7.5 and 6.0. The findings showed that a strong ligand was present in water to bind some of the aluminium but gave no information as to the identity or distribution of any species.

Further work with a combined cation/anionic hybrid method by Pesavento's group is described in Sub-Section 2.2.3.6. Bantan et al. (1998) modified the FPLC technique developed by Mitrovic et al. (1996) to use an anion exchange column to speciate negatively charged aluminium complexes in aqueous solutions of pH 3.0 to 11.0. Coupled with 'off-line' ICP-AES detection negatively charged Al-citrate, Al-EDTA and Al-oxalate were separated from neutral aluminium citrate and Al^{3+} which were adsorbed onto the column. The pH, as expected, was shown to influence the aluminium complex speciation with 100% Al-citrate recovered as a negatively charged complex at neutral pH and $[Al(OH)_4]^-$ could only be separated from Al-citrate at a pH of 11.0. The distribution of the negatively charged complexes closely mirrored the distribution predicted by computer models.

Yamada et al. (2002) developed an FIA method with lumogallion and fluorescence detection that used both acidic and basic ion exchangers to determine not only cationic but also anionic organic acid bound fractions of soil extracts and labile inorganic aluminium. Erdemoglu et al. (2000) also developed a two stage ion-exchange system by placing two different exchange columns in series and measuring part of the eluent by FAAS to measure hydrolysable polyphenolic bound and cationic aluminium species in tea. They reported that around 30% of aluminium was bound to hydrolysable polyphenolics and 10-20% as cationic species.

Few studies have attempted to determine the optimum fractionation method. Wickstrom et al. (2000) compared different combinations of aluminium fractionation techniques. This was accomplished by comparing the performance of different combinations of fractionation analysis (ion exchange, PCV and 8-HQ complex reactions), different reaction times, flow systems (FIA with segmented flow analysis) and determination methods (molecular absorption spectrometry or ICP-AES) against theoretical 'labile aluminium' equilibrium calculations provided by computer modelling. There was a large discrepancy in results using different methods, probably due to the previously mentioned fact that each method fraction is operationally defined. The combination with the best correlation with model determined labile aluminium was found to be the use of an Amberlite column with ICP-AES as the detection method. This study underlines the fact that speciation fractions using different methods are very difficult to compare, however Wickstrom et al. (2000) suggest that this study and others like it could provide correction factors for each method to

overcome this problem. Ginting et al. (2000) also compared the performance in reaction times of the colorimetric reagents PCV and aluminon in the presence of citrate, malate, malonate, oxalate and tartrate. Aluminon gave a better estimate of labile aluminium in the presence of these ligands, particularly citrate and tartrate, in soil and water with the best results obtained with the shortest reaction times.

2.2.3.4 Size exclusion/filtration

The basic tenet of this technique is to separate the aluminium complexes into fractions discriminated by molecular size. In particular it has been a useful technique for clinical aluminium speciation in determining the aluminium complexes in blood and environmental samples where the method can deduce aluminium bound to humic and fulvic acids in soils and colloidal aluminium in solutions. The results obtained using size exclusion are not very specific however as the cut off limits for particular size fractions are operationally defined and do not separate monomeric inorganic aluminium from all forms of organically bound aluminium; in addition the fractions of aluminium filtered can be prone to pH variations, which ultimately changes the speciation and hence the fractions filtered (Bloom & Erich 1996). Like ion exchange and UV-Vis/fluorometric analysis, there remains the question of the filtration upsetting the equilibrium of the solution under investigation causing redistribution of the species (Pyrzynska et al. 1999; Pesavento et al. 1998a; Driscoll & Schecher 1990). There are effectively two aluminium fractions deduced using this technique, high-molecular-mass (hmm) and low molecular mass (lmm) aluminium (Berthon 1986; Sanz-Medel 1998).

Ultrafiltration was used by Yokel et al. (1991) to determine the amount of aluminium bound to transferrin. Aluminium in the form of a buffered lactate complex was introduced to a slight excess of human apotransferrin, pH was adjusted to 7.4 and the solutions were incubated at 37 C over various time intervals. As transferrin is a large protein, at ~77kDa, the aluminium bound to the protein was retained by the filter membranes and the filtrates were analysed for aluminium by GFAAS. The amount of aluminium bound to transferrin was then deduced by the difference between the initial aluminium added and the aluminium in the filtrates. The results showed that virtually all the aluminium presented to transferrin was complexed by the protein. Sanz-Medel

(1998) and co-workers have produced a number of papers outlining their research efforts in the last decade in the area of blood aluminium speciation. Their initial work focused on ultrafiltration and centrifugation (with GFAAS detection) (Sanz-Medel & Fairman 1992), mimicking dialysis to determine what molecular fractions were eliminated during haemodialysis of uremic patients. It was found that only 11 ± 2% of the aluminium was ultrafilterable suggesting around 90% of aluminium is protein bound in serum. However when desferrioxamine (DFO) was introduced to the solution 75% of total serum aluminium was ultrafilterable, confirming other work that suggested that DFO is an effective Al-detoxifying agent by binding aluminium from its high molecular mass protein complexes and allowing the metal to pass through the dialysis membrane.

A size fractionation procedure was conducted by Gardner & Gunn (1995) on drinking water and tea. Four fractions were identified:

1) total (acid-digestible) aluminium including most particulate, colloidal and dissolved species,
2) dissolved and colloidal aluminium passed through a 0.45μm membrane filter,
3) low molecular mass species dialysed through a 1kDa cut off membrane and,
4) low MW (<1000Da) reacted with 8-HQ.

This approach showed that particulate aluminium was removed by normal water treatment procedures but the dissolved aluminium was all low molecular mass species and remained unchanged. In tea, aluminium was found predominantly as high molecular mass species bound to organic ligands. Size fractionation has been combined with chromatographic techniques in an order to attain the benefits of automated analysis in terms of speed, sensitivity and accuracy.

Gel filtration chromatography was utilised by Van Ginkel et al. (1990) to investigate the interaction of aluminium with rat serum constituents. By packing a column with gel to exclude molecular mass fractions, and applying GFAAS and UV-Vis as detectors, it was found that aluminium was associated with a high molecular mass protein bound fraction and a low molecular mass fraction. UV-Vis spectrophotometric and GFAAS analysis of the serum protein and aluminium respectively showed the two coeluted, suggesting aluminium was bound to transferrin in the serum and supporting the work of Yokel et al. (1991). The low molecular mass complex was attributed to citrate.

Size exclusion chromatography with ICP-MS detection was utilised to examine the speciation of aluminium in tea and its simulated gastrointestinal digests (Owen et al. 1992). By passing tea infusions and infusions treated with simulated gastric juice at pH 2.5 and 5.5, the authors found that at the lower pH the majority of the aluminium was low molecular mass species whereas the higher pH saw a significantly higher molecular mass bound aluminium eluted. It was also concluded that of the aluminium in tea infusions, 15% was present as soluble, labile aluminium after treatment with intestinal enzymes. An SEC system was developed by Kerven et al. (1995) to study organic acid complexation of aluminium in soils. A size exclusion resin packed column was used to separate the aluminium size fractions with detection via UV-Vis and ICP-AES. Model aluminium citrate solutions showed two peaks attributable to two unidentified aluminium citrate species. Analysis of soil extracts showed that there was an even distribution of total aluminium in the <5kDa and 5k-10kDa classes with only a small proportion above the 10kDa molecular mass class.

Size fractionation provides some speciation information. Although useful in clinical analysis to ascertain what blood aluminium is protein bound and what proportion of aluminium is bound to lower molecular mass fractions, specificity suffers as the technique cannot characterise individual species. While useful as an initial screening tool, the information provided is far too broad to give comprehensive speciation data. Again, as with the earlier methods discussed, the results are based on operationally defined fractions of a varying array of molecular mass cut-off sizes, making direct comparisons of different studies difficult.

2.2.3.5 Traditional HPLC

HPLC has been applied to aluminium speciation. This method relies on the difference in the polarity of its stationary and mobile phases and the different interactions between these phases of molecules of varying polarity, hence causing component separation. A comprehensive study was conducted by Datta et al. (1990) on the feasibility of HPLC as a means of determining individual Al-citrate complexes. Nine stationary phases and 41 mobile phases were trialled in various combinations to attempt Al-citrate speciation. Despite the exhaustive and rigorous investigation, no one column or mobile phase was able to reproducibly speciate Al-citrate. This was thought to be due to the highly polar and non-covalent complex not being retained on non-polar or slightly polar columns as well as

the total dissociation of the complex on highly polar columns. An Al-citrate species was distinguished using chiral columns, however recovery was poor and worsened with increasing retention times, thought to be due to slow complex elution, making it indistinguishable from the background (Datta et al. 1990). This problem was attributed to aluminium interaction with silicate in the stationary phase supports. It was concluded that for HPLC separation of Al-citrate to be successful a column with a polar hydrophilic stationary phase on a polymer based support was required, however no such commercial column existed at the time (Datta et al. 1990).

2.2.3.6 Hybrid techniques

As can be seen in the preceding Sections, two analytical techniques have been used to speciate aluminium. This has usually involved coupling a separation or reaction step (HPLC, ion-exchange) with detection of aluminium entities usually by an atomic spectrometry method (Sanz-Medel 2002). For the latter method FAAS and GFAAS off-line was used initially, now these techniques have largely been replaced with on-line ICP-AES or ICP-MS (Gonzalez & Sanz-Medel 2000; Ebdon & Fisher 2000; Ackley et al. 2000). As each method has its advantages and disadvantages in characterising particular aluminium species, researchers have realised that a combination of two or more techniques can give better resolution and identification. Most of these techniques have been applied to aluminium speciation in serum at physiological or basic pH. These have been termed 'hyphenated' or 'hybrid' techniques (D'Haese et al. 1995; Das et al. 1996). In essence they involve the use of two instrumental procedures to separate and detect aluminium species.

Sanz-Medel & Fairman (1992) and Sanz-Medel (1998) have described a combined HPLC with off-line GFAAS as part of the latter author's work on aluminium speciation in biological systems. Using a HPLC with an anion exchange column and sodium acetate gradient elution at pH 7.4, protein bound aluminium was separated with the proteins detected by UV-Vis and the aluminium by fraction collection of the column eluant and detection off-line using GFAAS. By superimposing the protein absorbance profile onto the GFAAS fraction profile, the proteins containing aluminium could be determined (see Figure 2.2). This work showed that in the serum, aluminium was virtually exclusively bound to transferrin and confirmed the role of transferrin as the major ligand binder of

aluminium in the blood. Aluminium-citrate was also separated and identified. This technique was modified and improved in subsequent years.

Later work saw HPLC separation replaced with FPLC. FPLC was also used by the group of Milacic (Mitrovic et al. 1996; Kozuh et al. 1996, 1997; Bantan et al. 1998). With the FPLC system, Al-citrate could be separated from Albumin which was not the case with the HPLC system. In addition the technique was made truly hybrid by replacing GFAAS detection with ICP-MS allowing online aluminium detection. This last modification was taken further with the use of double focusing high resolution ICP-MS coupled to the FPLC column to allow lower detection limits. Mitrovic & Milacic (2000) used two coupled systems, one size exclusion separation with UV/ICP-AES detection and the other FPLC with GFAAS detection, to investigate the speciation of aluminium in forest soils. They found this complementary analysis gave more speciation information than one alone. Using this system, they reported that 80-95% of total water soluble aluminium exists as monomeric aluminium species and that 45-55% occurs as low molecular mass complexes such as $[AlF]^{2+}$, Al-citrate and Al-oxalate species.

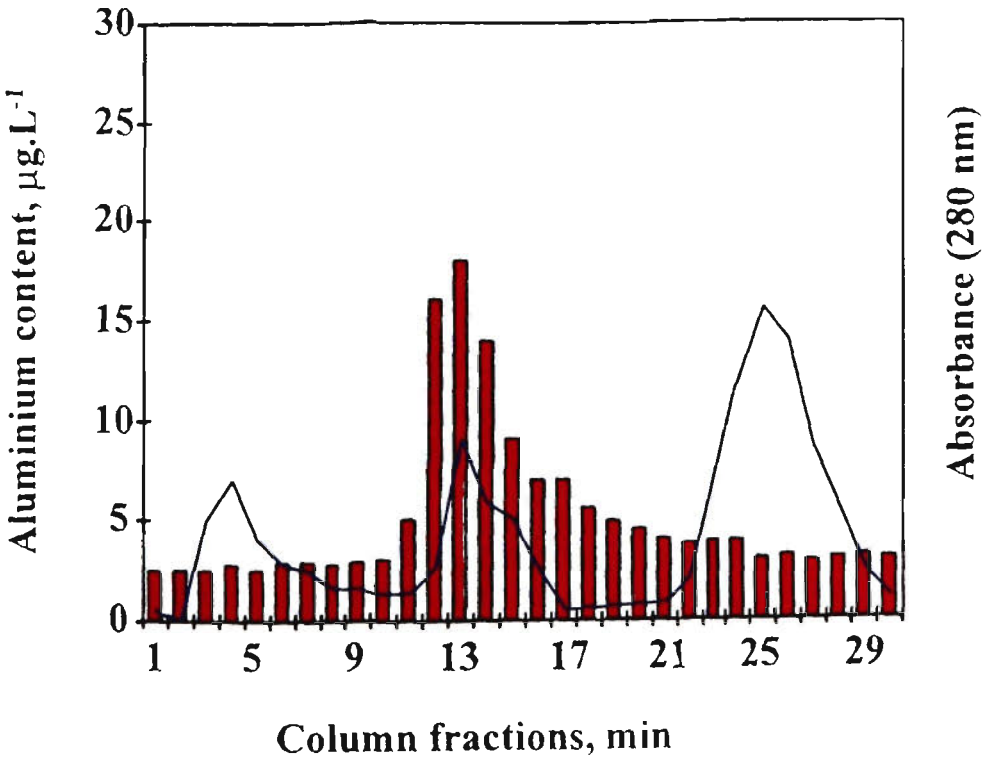


Figure 2.2 Elution profile of proteins (detected by UV at 280nm, blue line) and aluminium (ETAAS, red columns) in diluted serum sample (from Sanz-Medel 1998). Note: the superimposition of the UV-VIS peak and the higher aluminium concentration bar plots signify transferrin bound aluminium.

A similar hybrid approach was taken by Van Landeghem et al. (1994) and D'Haese et al. (1995) by combining HPLC with GFAAS. They used an anion exchange column with gradient elution of 0.05M Tris-HCl to 0.05M Tris-NaCl at a pH of 9.2 to separate proteins which were detected using UV-Vis whilst simultaneously a fraction collector introduced a sample to a GFAAS instrument whereby the aluminium concentration of the fraction was determined. A scavenger column was placed into the eluent stream before the separation column to remove any aluminium contamination from the eluent. Following Datta's (1990) advice a polymer based packing was used to avoid aluminium losses caused by binding to silica. Like the method of Sanz-Medel (1998), the profile of aluminium concentration was superimposed onto the UV-Vis detection profile to yield information about which protein peaks contained aluminium (see Figure 2.3). The work agreed with that of Sanz-Medel (1998) showing aluminium predominantly bound to transferrin in artificial solutions, suggesting 80% of aluminium in serum is bound to transferrin, further confirming what was postulated from previous findings. An aluminium-citrate fraction was also separated.

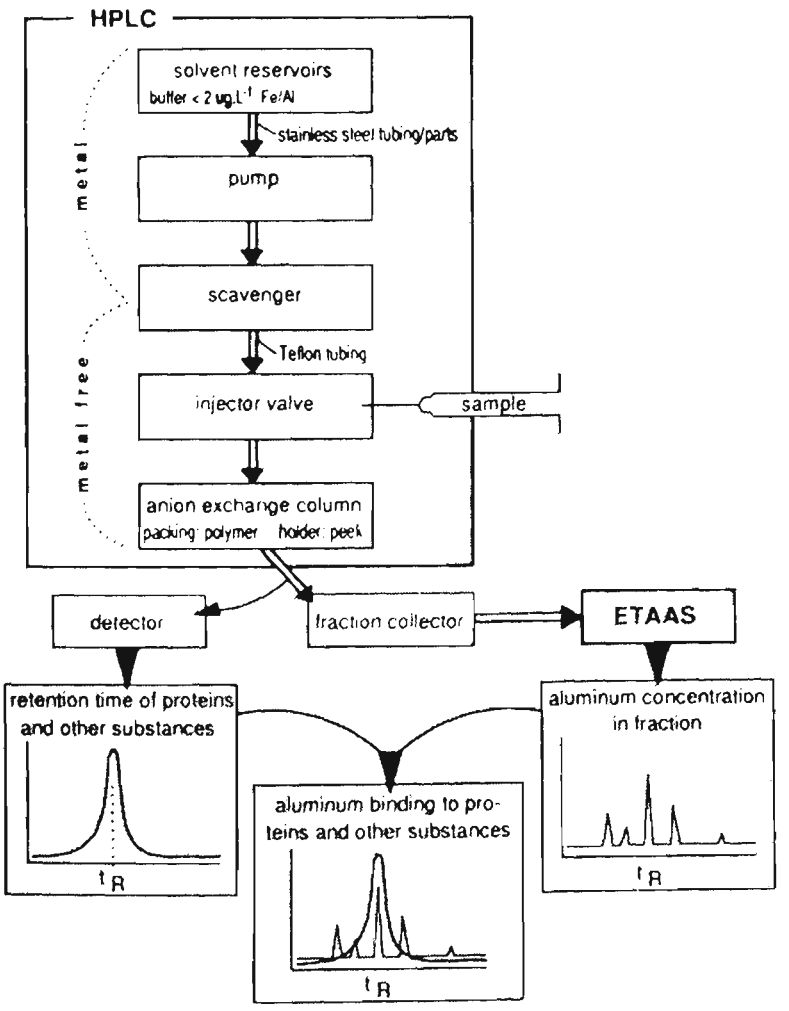


Figure 2.3 Schematic representation of the HPLC/GFAAS hybrid technique (from Van Landeghem et al. 1994).

Yuan and Shuttler (1995) developed a hybrid system combining FIA with GFAAS. The flow injection system was used to pump sample onto a preconcentration column where two packings were studied, 8-quinolinol immobilized on controlled pore glass (8-Q-CPG) and Amberlite XAD-2 resin. For the 8-Q-CPG, aluminium binds to the 8-quinolinol groups and is subsequently eluted with dilute HNO_3 and sampled directly into the graphite furnace for analysis. In the case of the XAD-2, the sample containing the aluminium is premixed and complexed with 8-quinolinol which is retained by the column, subsequently eluted with methanol, and analysed by GFAAS. These methods were applied to water samples. The XAD-2 system gave better preconcentration but could not cope with salt water matrices. The method captures a very broad section of the labile aluminium fraction that, like so many other procedures, depends on the reaction time of aluminium with 8-quinolinol. This makes the species captured operationally defined and difficult to compare with other aluminium fractions analysed using other techniques. In addition the methodology is unable to characterise individual species. By adjusting sample reaction time, as used in other methods involving aluminium reactions with a chelating agent, separating and isolating smaller and more meaningful fractions of labile aluminium may be possible.

Pesavento & Alberti (2000) followed up their earlier work by combining titration with ligands in tap water with the determination of aluminium sorbed on cationic and anionic exchange columns. Due to interferences from other metals, only the presence of very strong ligands bound to aluminium could be detected which was unexpected in normal tap water. However, the method provides limited information as it only detects the presence of binding ligands and cannot determine individual fractions or species.

2.2.3.7 Accelerator mass spectrometry

Day et al. (1994) reviewed the use of accelerator mass spectrometry (AMS) to trace the artificial isotope ^{26}Al in biological systems. In one study, blood from a subject ingesting ^{26}Al-citrate was fractionated down to protein concentration by membrane filtration and chromatography with the ^{26}Al distribution determined by AMS. This work agreed with others that transferrin is the major binder of aluminium in blood plasma. The AMS however is more a mode of detection than a

speciation technique itself and relies on other methods described previously. Another downside is the inaccessibility and rarity of AMS to most researchers and analysts.

2.2.3.8 Nuclear magnetic resonance spectroscopy & infra-red spectroscopy

The major problem associated with the techniques discussed previously in speciating aluminium is the fact that these procedures require a physical or chemical interaction with the entities in solution. These techniques will inevitably change the speciation of the aluminium either by breaking up or derivatising them or by forcing a redistribution of the species to re-establish equilibrium. In doing this, the very entities that one is trying to identify and characterise are changed in the process of investigation. Aluminium species have been characterised by utilising ^{27}Al NMR. Organic-Al complexes have been analysed using ^{1}H and ^{13}C NMR, by determining the structure of the ligands and their bonding to aluminium from the NMR spectra. Infra-red (IR) spectroscopy has also been used in a complementary role with NMR. In a sense these spectroscopic procedures provide a true non-invasive speciation technique as they do not interfere with the species on a molecular concentration and hence do not affect the equilibrium of the system. Both techniques have rarely been used as stand alone tools. In general they have complemented other speciation techniques, particularly potentiometry, providing confirmation of the species present and structural information.

Akitt and Elders(1985) used ^{27}Al NMR to study the hydrolysis of aluminium, in particular the formation of $[AlOH]^{2+}$. This cation forms on dilution of aluminium salt solutions and gives a strong response that broadens the signal from $[Al(H_2O)_6]^{3+}$ due to fast exchange between the two ions. By analysing the spectra of $[AlF]^{2+}$ which gives a broad linewidth similar to $[AlOH]^{2+}$ the authors were able to determine the proportion of the latter cation in the solution and calculate its formation constant. The constant derived was similar to that calculated from potentiometric data. In addition they surmised that $[AlOH]^{2+}$ is the only fast-exchanging hydrolysed species formed.

The investigation of aluminium complexes with organic ligands has dominated NMR aluminium speciation analysis. A polynuclear Al-citrate complex was isolated by Feng et al. (1990) and characterised by ^{27}Al, ^{1}H and ^{13}C NMR and X-ray crystallography. $[Al_3(H\text{-}_1cit)_3(OH)(H_2O)]^{4-}$ was isolated at pH 7.0-9.0 and the structural characterisation allowed further assignment of ^{27}Al NMR

spectra of the Al^{3+}/citric acid system over the pH range 0.3-9.0. It also provided proof of the existence of polynuclear Al-organic complexes. A series of papers by the group of Corain has explored aluminium complexes with organic ligands. Initially investigating Al-lactate complexes using IR, ^{1}H and ^{13}C NMR in acidic and neutral solutions they turned their attention to aluminium complexes containing anions of tartrate, citrate and gluconate using the first two methods (Corain et al. 1992a; Corain et al. 1992b; Sheikh-Osman et al. 1993; Corain et al. 1994). Solutions were prepared to give the complexes in solution and were characterised at pH 2.0-4.0 and 7.5. Lactate showed a rapid exchange between free and metal coordinated states at pH 3.6 and complexes assigned were a mixture of $[Al(lact)_2(H_2O)_2]^+$, $Al(lact)_2(OH)(H_2O)$ and/or $Al(lact)(H_{-1}lact)(H_2O)_2$ with some $Al(lact)_3$. These species agree with potentiometric data at pH 3.6. At pH 7.5 however the spectroscopic evidence showed the majority of lactate not coordinated to aluminium, disagreeing with expected theory of aluminium bound to lactate as $Al(lact)_3$. They postulated this was due to the formation of a metastable complex such as $Al(OH)_3(H_2O)_3$ that slowly precipitates to solid $Al(OH)_3$ over time. Crystals of $Al_2(tart)_3(H_2O)_4$ were synthesised and the spectral data indicated a dicarboxylic tetradentate ligand involved in a dimeric structure attached to two Al(tart)-(H_2O) units. When in solution an $Al_2(H_{-1}tart)_2$ complex was thought to dominate at autogenous pH while at pH 7.5 the aluminium-tartrate complex behaved very similarly to aluminium-lactate in that the majority of tartrate was uncomplexed as predicted by theoretical distribution diagrams. What was not predicted however was the presence of a metastable aluminium complex with water and hydroxy ligands with a fraction of $[Al_2(H_{-2}tart)_2]^{2-}$ instead of precipitation of $Al(OH)_3$. Unlike lactate or tartrate, aluminium-gluconate solutions derived from $Al(gluc)(OH)_2$ were found to form a metastable complex dominated by gluconate again delaying $Al(OH)_3$ precipitation. This also challenged theoretical expectations. A solution derived from $Al_2(cit)_2(H_2O)_6$ showed no free citrate ligand at pH 4.0 or pH 7.5, although at the higher pH the NMR analysis pointed to two structurally different Al-citrate complexes (Sheikh-Osman et al. 1993). Unlike the other ligands observed, the spectrometric evidence agreed with expected behaviour of the Al-citrate system.

Tapparo et al. (1996) extended the work to investigate aluminium speciation with malonic acid. Again utilising ^{27}Al, ^{1}H and ^{13}C NMR, they postulated that at pH 7.5, $[Al(mal)_2(H_2O)_2]^-$ converts to form the predominant complex at that pH, $[Al(mal)_3]^{3-}$. This complex also displayed metastable

characteristics, delaying aluminium hydroxide precipitation. Aluminium complexes with oxalic and citric acids were investigated by Kerven et al. (1995). ^{27}Al NMR spectra of aluminium-oxalate mixtures of ratios 4:1, 1:2, 1:1, and 1:2 at autogenous pH were obtained. The 4:1 solution exhibited the characteristic sharp aluminium hydroxy monomer peak at 0ppm with a broader peak further downfield assigned to an Al-oxalate species. As more oxalate was added the monomer peak disappeared to give three broad but distinct peaks downfield, assigned as 1:1, 1:2 and 1:3 Al-oxalate species. Al-citrate mixtures of similar component ratios as used for oxalate gave broader ^{27}Al NMR peaks, these were assigned to Al(cit) and $[Al(cit)_2]^{-3}$ complexes. The NMR peak integrations disagreed with models using the program GEOCHEM, highlighting possible limitations of modelling software. However this study shows that, although useful in deducing speciation information in terms of aluminium to ligand ratio, ^{27}Al NMR by itself fails to give more detailed charge and structural information.

A study of aqueous Al-citrate speciation was conducted by Lakatos et al. (2001) wherein ^{27}Al, ^{13}C and ^{1}H NMR were used to support time dependent potentiometric titrations under freshly prepared and equilibrium solution conditions. Both equimolar Al:ligand and excess ligand solutions were monitored over the pH range 2.0-8.0. The mononuclear tridentate complexes $[Al(Hcit)]^{+}$, Al(cit), $[Al(H_{-1}cit)]^{-}$ (pH 2-3) and $[Al(H_{-2}cit)]^{2-}$ (pH 7) were characterised in freshly prepared 1:1 Al/ligand solutions along with the bis mononuclear complexes $[Al(cit)_2]^{3-}$ (pH 3-4), $[Al(H_{-1}cit)(cit)]^{4-}$ (pH 5) and $[Al(H_{-1}cit)_2]^{5-}$ (pH 7) in 1:2 solutions. The suggested structure of the mononuclear tridentate complex is shown in Figure 2.4. The 1:1 complexes were found to undergo slow oligomerisation to equilibration, forming a trinuclear species $[Al_3(H_{-1}cit)_3(OH)]^{4-}$ which tended to predominate around pH 4-7. This trinuclear complex formation was shown to decrease with increasing ligand excess due to the bis mononuclear complexes competing with the oligomerisation process.

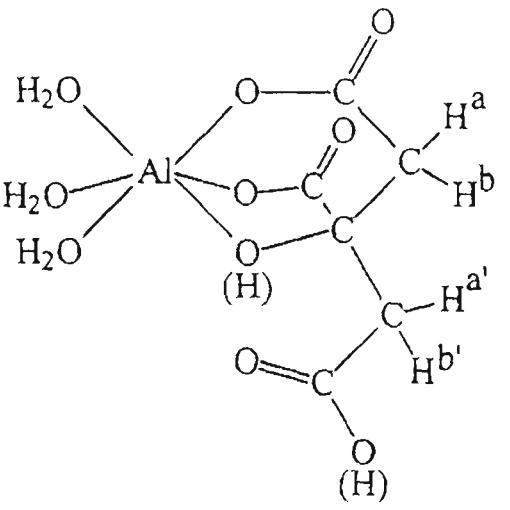


Figure 2.4 Suggested structure of Al-citrate complex system $[AlHL]^{+}$-[AlL]-$[Al\ H_{-1}L]^{-}$ (from Lakatos et al. 2001).

Aluminium-citrate speciation analysis was also attempted by Bodor et al. (2002a) and Bodor et al. (2002b) using ^{1}H and ^{13}C NMR and also characterised the tri-nuclear complex as $[Al_3(H_{-1}cit)_3OH(H_2O)]^{4-}$although unlike Loring et al. (2001) with a water molecule attached. It was reported that the slow ligand exchange of citrate-aluminium species is dependent on the rate of chelate formation.

Aqueous Al-picolinate solutions were characterised by Loring et al. (2000) using Attenuated Total Reflection FTIR and ^{27}Al NMR. The NMR spectra at pH 2.92 gave three peaks, with a sharp peak at 0ppm representing $[Al(H_2O)_6]^{3+}$ and two small very broad peaks assigned to $[Al(Pico)]^{2+}$ and $[Al(Pico)_2]^{+}$ (see Figure 2.5). Spectral subtractions were done for the IR interpretations by using software to remove the individual IR spectra of $[Al(H_2O)_6]^{3+}$ and $H_2(Pico)$ from the IR of the various mixtures of Al^{3+} and picolinate rings and compared with calculated spectra. From this information it was hypothesised that picolinate could complex aluminium via bidentate or bridging coordination through an oxygen of the carboxylate group and the nitrogen of the pyridine ring. Further work was done on aluminium with quinolinate using the same methods and the addition of potentiometry, showing that this ligand preferentially binds aluminium via its ring nitrogen and an oxygen of its ∝-carboxylate (Loring et al. 2001).

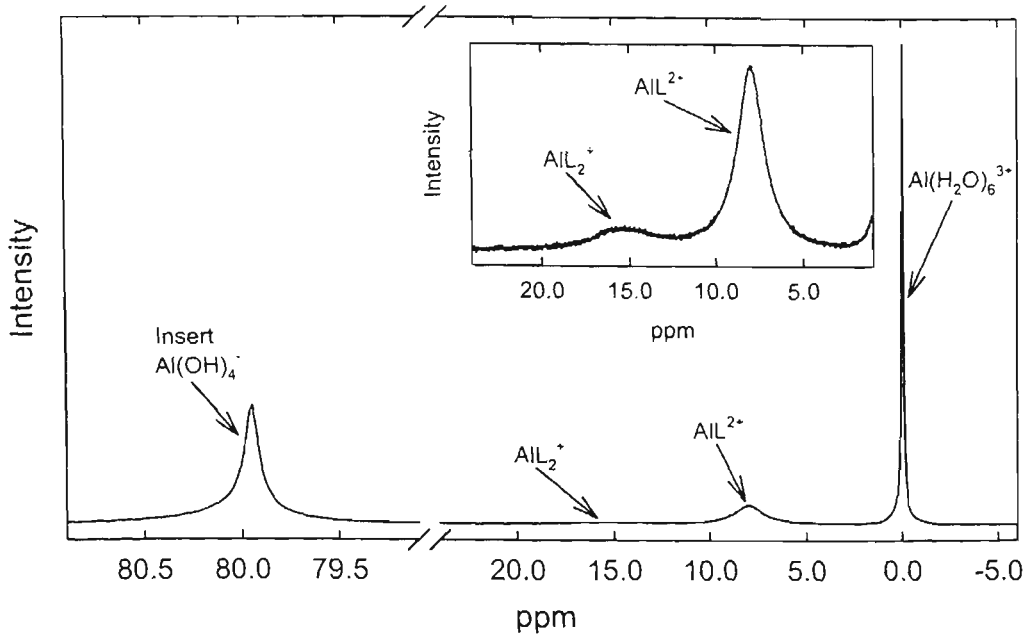


Figure 2.5 Representative ^{27}Al-NMR spectrum of an Al(III)-picolinate solution with a total picolinate concentration of 0.0594M, a total Al(III) concentration of 0.100M, a pH of 2.90, and an I of 1.42M (from Loring et al. 2000).

Not just restricted to controlled solutions, NMR has also been applied to 'real' samples. Bell (1993) utilised proton NMR to investigate human blood plasma and ultrafiltrate. The interpretation of NMR becomes much more complex as the variety of protons and their environments in these samples is substantially increased from simple controlled aqueous solutions. By characterising free citrate peaks, the complexation of aluminium by citrate was indirectly shown with a decrease in the intensity of the citrate peaks when a small spike of aluminium was added to the plasma. The addition of desferioxamine to this solution, a well known binder and detoxification agent for aluminium used medicinally for renal failure patients, restored the intensity of the citrate peaks, indirectly pointing to aluminium being preferentially bound to desferrioxamine and releasing free citrate.

^{27}Al NMR was applied to the characterisation of aluminium complexes with oxalate and fluoride in tea infusions (Horie et al. 1994). By comparing the ^{27}Al spectra of tea infusions with solutions containing known mole ratios of aluminium, oxalic acid and NaF (which gave very similar broad peaks that varied in intensity, broadness and shape depending on component concentrations), they were able to suggest that the speciation of aluminium in six Japanese teas mainly consisted of a complex with stoichiometry Al:oxalate of 1:3 and a further complex containing aluminium, oxalate and fluoride. This analysis again demonstrates the limitations of using ^{27}Al NMR alone, giving

incomplete structural information. This problem was also demonstrated in the work of Masion et al. (2000) using ^{27}Al NMR and X-ray Scattering to analyse flocs formed by coagulation of natural organic matter from lake water with aluminium salts. An attempt was made to equate two peaks with octahedral and tetrahedral aluminium complexes, however it was admitted that the peaks could be due to aluminium monomers and dimers and Al-organic acid oligomers and no actual species could be attributed to a particular peak. The low chemical shifts observed were seen to indicate a low amount of polymeric complex formation. This was thought to be due to the complexation of the aluminium by the organic material.

Recently a multi-NMR study was conducted on aluminium complexes not containing carboxylate ligands. Aluminium-pyrophosphate and Al-fluoride complexes were investigated using ^{27}Al, ^{31}P and ^{19}F NMR in aqueous solution at pH 7.5 (Martinez et al. 1999). From the spectra it was deduced that pyrophosphate binds to an octahedral aluminium by a singly bound oxygen, as a bidentate ligand or two pyrophosphates acting as a double bridge in a dimer. It was also shown that the addition of fluoride to this system produced ternary complexes of similar structure replacing H_2O or OH^- with F^-. A new class of stable complexes was also postulated with a central F bridge in addition to the two pyrophosphate bridges in an aluminium dimer. Champmartin et al. (2001) also used a multinuclear NMR study to discern aluminium complexes with glucose-6-phophate in aqueous solutions. ^{13}C, ^{27}Al and ^{31}P NMR was used in tandem with potentiometry to identify various mononuclear and dimeric species. The use of potentiometry was found to be very complementary to the NMR technique allowing easier speciation and structural identification.

NMR is an extremely powerful analytical tool and its potential for speciation determination is immense as it not only characterises aluminium complexes but also gives information on structure. Another major but most important advantage over other 'speciation' techniques previously described is that NMR does not alter the chemistry and hence the speciation of the sample in question. Although the NMR literature discussed here demonstrates the ability of the technique to characterise aluminium complexes, particularly those with carboxylate ligands, there are some limitations.

^{27}Al NMR has been used extensively in the last decade, however recent work by Kubicki et al. (1999), utilising *ab initio* modelling of Al-carboxylate complex formation and their ^{27}Al NMR shifts, suggests that previously assigned peaks of tridentate and bidentate complexes may actually be monodentate or protonated bidentate complexes. It provides limited structural information and the broad resonance bands make it difficult to resolve and assign particular peaks to given complexes. This problem and its 'dependence on coordination changes inside the metal sphere' led Corain et al. (1992a) to conclude that interpretation of speciation using this technique alone without the support of data derived from other methods should be treated with suspicion. Indeed, as can be seen from the review, those papers that include other forms of NMR such as ^{13}C and ^{1}H give a greater detail of individual species and their structure with greater confidence. However there are limitations to these multi-NMR techniques as well.

Most of the literature describes speciation work done on controlled aqueous solutions generally composed of aluminium and the ligands in question or of previously identified and isolated complexes dissolved in water. 'Real world' samples are not quite as tractable with a matrix that can give very complex spectra as seen in the human blood plasma work of Bell (1993). It is much easier to assign peaks to a ligand or complex structure when one has a good idea of what components and hence possible structures are present. In addition, acquisition and interpretation of spectra require significant experience and expertise. Often the NMR spectra are characterised by broad lines with overlapping complicated spectra or spectra that are 'too simple' (Bodor et al. 2002b). This makes characterising an unknown sample very painstaking and difficult. Another important factor to consider is that most of the speciation work done with NMR is with concentrations of aluminium not seen in nature. Many biological samples contain aluminium at concentrations around 10^{-5}-10^{-6}M which is below the range of most NMR spectrometers. Another problem, particularly for ^{27}Al NMR, is the use of probes containing appreciable amounts of aluminium giving a high background signal that can swamp that of the sample.

MacFall et al. (1995) devised a new NMR probe that significantly reduced the background to resolve peaks at 10^{-6}M (Figure 2.6). This probe was manufactured with three primary components containing as little contaminating aluminium as possible:

1) a probe body replacing the aluminium components of the housing in commercial probes
2) a radio-frequency (rf) coil designed for increased sensitivity to ^{27}Al from the sample, but with reduced background
3) a sample holder specifically machined for the rf coil with separate chambers for the sample and reference, and which is non-reactive with the sample and reference solutions

Innovations like these and the use of more powerful spectrometers (500Mhz and up) will see the detection limits, decrease however speciation work of low concentration aluminium complexes in complex natural matrices continues to be at the edge of NMR detection. Even using the new 'aluminium free' probe, 18-20 hours of NMR signal averaging was required to accomplish detection of 10^{-6}M aluminium complexes.

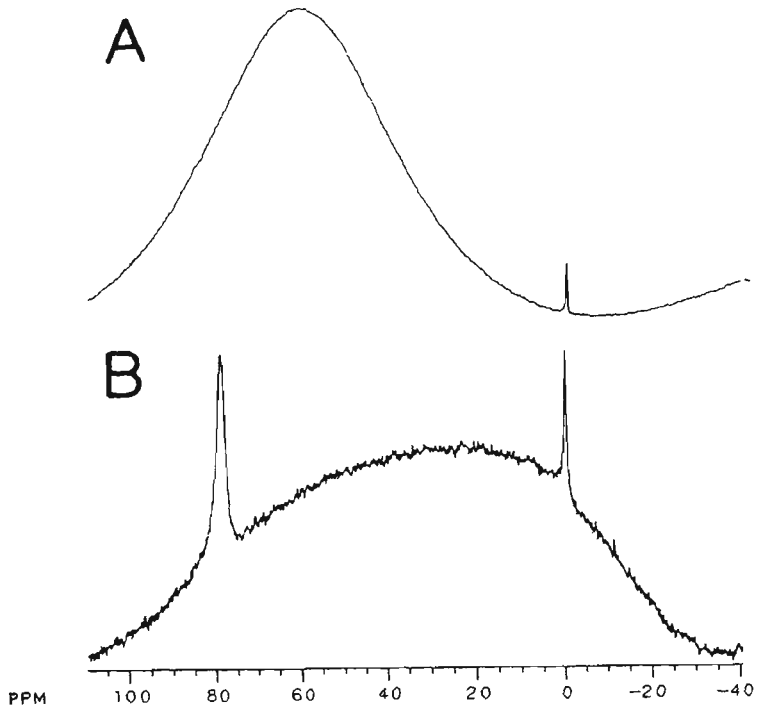


Figure 2.6 ^{27}Al NMR spectrum of; (A) 10^{-4}M pH 3.0 $AlCl_{3(aq)}$ acquired with a commercial 20mm NMR multinuclear probe and 20mm glass NMR tube, and (B) 10^{-5}M pH 3.0 $AlCl_{3(aq)}$ (outer chamber) and 8 x 10^{-4} $Al(OD)_{4\,(aq)}^-$ (inner chamber) acquired with capacitively tuned solenoid probe and dual-chamber sample holder constructed with low aluminium materials. The signal at 0ppm and 79ppm are attributed to $AlCl_3$ and $Al(OD)_4^-$ respectively (from McFall et al. 1995).

2.2.4 Aluminium Speciation Analysis Review Summary

Many instrumental techniques and sampling methods have been utilised to attempt the speciation of aluminium in aqueous systems. These have involved the use of theoretical modelling and physical instrumental analysis, the separation of fractions based on kinetic factors, reactions with complexing

agents and the spectroscopic analysis of atoms. The methods elucidate some species with varying degrees of success however the basic problem of the last two decades, the ability to detect and characterise individual species and their charges at any given pH, temperature or concentration in one analysis remains elusive.

Modelling from potentiometric data can give detailed distribution diagrams but is only as reliable as the formation constants input, and is based on ideal situations, in many cases polymeric and other possible complexes are not included. Reactions with reagents, ion exchange fractionation and separation all involve chemically altering the species and/or the equilibrium (if it has been reached) either removing the very species one is trying to analyse or initiating redistribution of the equilibria or both.

An additional problem is that many of these methods do not identify individual species but groups of species which are operationally defined i.e. largely grouped depending on the analysis and its parameters including time of analysis, reactivity of reagents, experimental conditions such as solution pH, ionic strength, temperature which vary with each technique and operator. The data derived from each investigation rarely has been conducted under a standard set of conditions where a direct comparison can be made. A group of species defined by one technique will contain different sets of individual species from those defined by another investigation using a different technique. Indeed defining species into groups provides only partial speciation information; we may know what classes of species are present but not the identity of the individual species. In addition the potential for overlap, where some species are contained in more than one fraction is very high. Although NMR and IR can provide structural information that does not alter the species or equilibrium it is difficult to interpret with complex systems and struggles to detect aluminium species at biological Al concentrations (Wang et al. 2001; Bodor et al. 2002b). In the case of ^{27}Al NMR the technique requires data from other methods to make reliable interpretations.

The 'holy grail' of aluminium speciation research is to find an ideal technique which can analyse a solution containing aluminium complexes almost instantaneously over a range of physical conditions, without chemical alteration and equilibrium disturbance, providing data that is easy to

interpret and can unambiguously identify complexes and provide information on their charge. Mass spectrometry has been rarely used for aluminium speciation analysis over the last two decades. This technique could satisfy most of the above-mentioned requirements. For speciation Sanz-Medel et al. (1998) suggested that 'on-line, real-time detection' is more desirable and recently utilised various forms of ICP-MS to perform speciation analysis on biological samples. The use of ICP-MS has become more prevalent in recent times and has been the main use of MS technology towards aluminium speciation as a detection mechanism for existing speciation techniques (Ackley et al. 2000). However it provides limited structural and valence information. Section 2.3 will describe a rapidly developing MS technique that can be used as a source of detection for other methods and as a 'stand alone' speciation procedure.

2.3 Electrospray Mass Spectrometry

Although the principles behind Electrospray Mass Spectrometry have been known for almost a century, it was not fully developed into an analytical tool until the mid 1980's. Since that time it has rapidly progressed with many commercial instruments now available and work continues to improve and develop the technique further. The acronym used for the technique varies from author to author with ES, ESI, ESP and ionspray commonly used, the latter describing a modified form of the technique. In this body of work, Electrospray Mass Spectrometry will be abbreviated as ES-MS. This Section will discuss how the method works in its various manifestations, its advantages and disadvantages and how it has been utilised including how it can and has been applied to inorganic and organometallic speciation.

2.3.1 Principles of ES-MS Operation

There are two parts to an ES-MS instrument, the electrospray unit that converts ions and molecules in solution to free gaseous ions and a mass spectrometer that separates the ions based on their mass and charge. The electrospray is the recent addition to MS techniques and will be discussed here in more detail than the mass spectrometer. The description of ES-MS featured in Sub-Sections 4.3.1 to 4.3.4 is derived from a number of sources on the science and engineering of ES-MS (Kebarle & Tang 1993; Stewart & Horlick 1996a; Johnstone & Rose 1996; Niessen 1996; Van Baar 1996; Das

1997; Kebarle & Ho 1997; Van Berkel 1997; McEwen & Larsen 1997; Bruins 1997; Wang & Cole 1997; Stewart 1999; Rubinson & Rubinson 2000; Kebarle 2000; Barnett et al. 2000).

The ES mechanism in its original form relies on the use of an electric field to convert molecules and ions in solution to gas phase ions that can be analysed by a mass spectrometer in a three part process; the production of charged droplets, the shrinkage of these droplets and the emanation of gas phase ions. Solvent evaporation is also involved in the latter two processes.

A peristaltic pump forces analyte in a liquid solvent through a thin capillary until it culminates in a very thin hollow needle that at its tip has a potential difference of around 2-3 kilovolts between it and a counter electrode 1-3cm away in a chamber at atmospheric pressure. The solution at the tip is affected by the electric field whereby positive and negative ions are forced to move under the influence of this field. When the capillary is the positive electrode, positive ions drift towards the meniscus of the solution at the tip and the negative ions move away from the surface. The electrostatic force of the field will cause the ions to drag the solution away from the tip and when the stress of the field balances the surface tension the end of the solution forms into a conical shape known as the 'Taylor cone'. At the apex of the cone a fine jet extends out. Coulombic repulsion between the ions increases as the like ion concentration increases until it reaches what is known as the 'Rayleigh limit' where the force from charge repulsion equals that of the surface tension. When this limit is exceeded droplets break away from the jet and fly through an open volume towards the counter electrode. The process is shown in Figure 2.7.

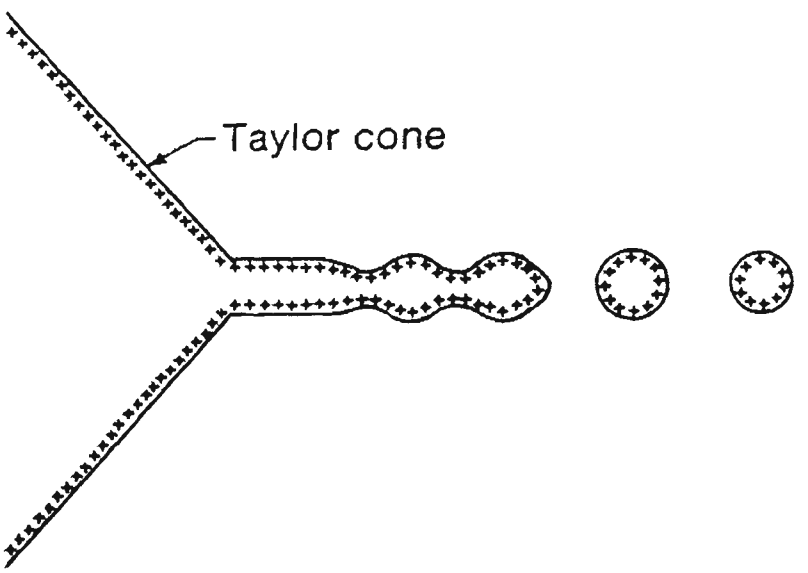


Figure 2.7. Diagram showing the formation of the 'Taylor Cone' and droplet emission (from Kebarle 2000).

The droplets can have 2% of the original mass and 15% of the original charge so the charge is highly concentrated on a smaller surface area where each droplet has the same polarity. As the droplets move towards the counter-electrode the process begins again and smaller droplets are formed, this process of droplet fissioning is repeated forming a 'spray' giving the technique its name. During this flight from the tip to the counter electrode, the neutral and volatile solvent molecules evaporate. Eventually the charge density is sufficiently large to allow quasi-molecular ions to emerge into the gas phase and head towards the counter electrode (see Figure 2.9). The final process of the aerosol giving rise to gas phase ions is a matter of debate and the exact mechanism is still unknown.

The whole process takes place in the timeframe of micro to milliseconds. By changing the polarity of the electric field, the polarity of the ions is changed. Hence the description of the electrospray mechanism would reverse in terms of polarity if the spray tip were the negative electrode. However this mechanism is far from unanimously agreed upon. A competing mechanism suggests that gas phase ions are emitting directly from the 'Taylor Cone' (Kebarle & Tang 1993), while Loscertales & Fernandez de la Mora (1995) suggest the rate of ejection of monovalent dissolved ions is governed solely by solvent evaporation. The exact mechanism of gas phase ion formation is still open to question. The overall electrospray process from capillary to sampling cone is shown in Figure 2.9.

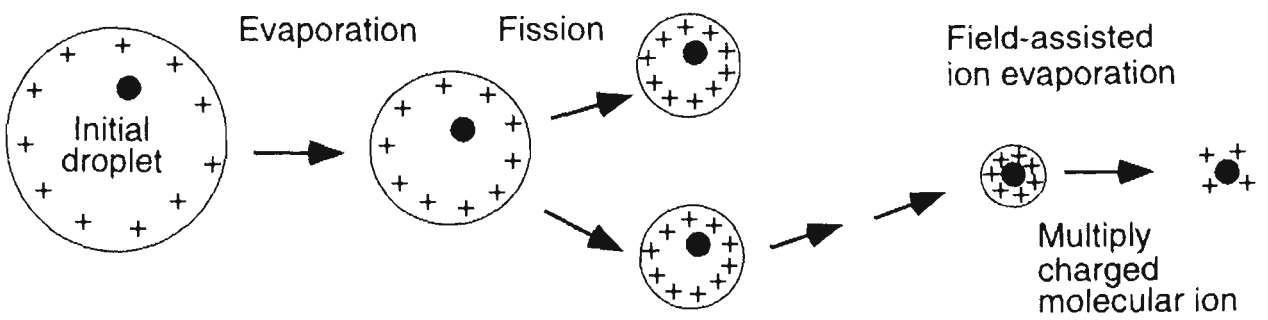


Figure 2.8 Gas phase ion formation due to solvent evaporation and droplet fission processes (from Dass 1997).

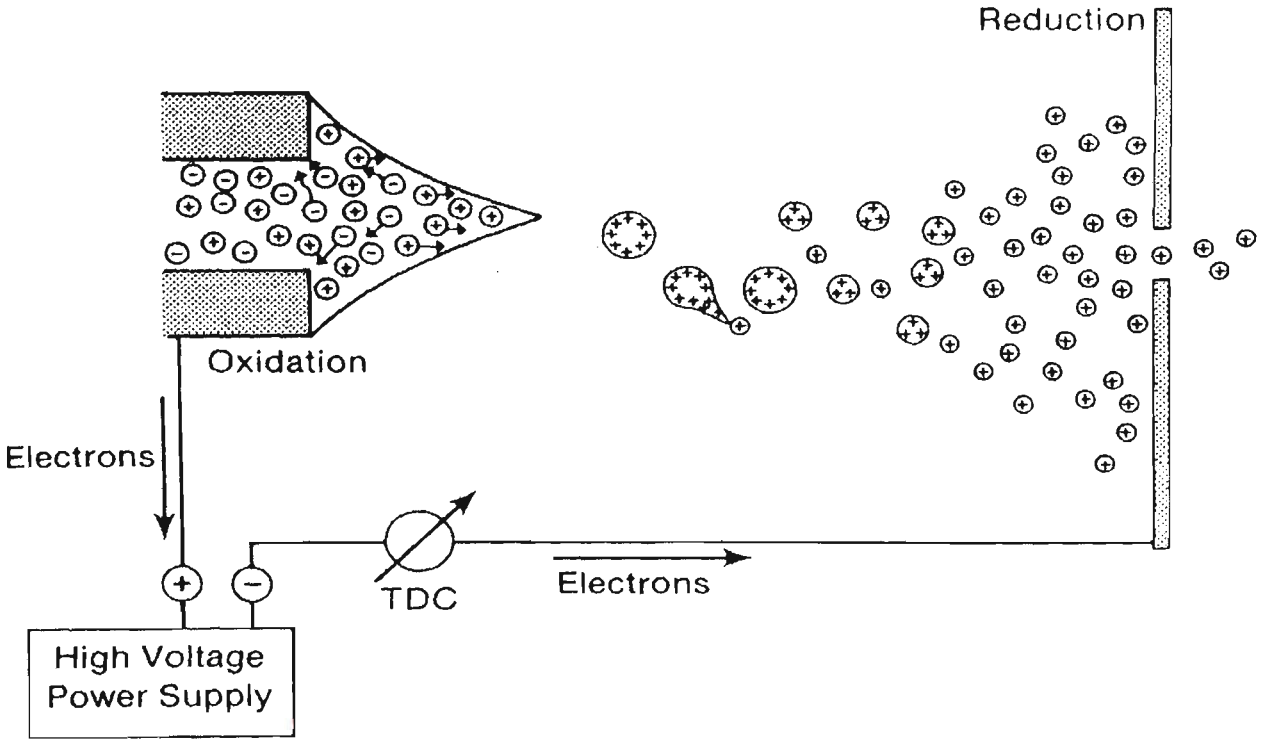


Figure 2.9 A diagram showing an overall view of an unassisted positive ion electrospray process from the capillary tip on the left to the sampling plate on the right. The labelling of redox processes at these points demonstrates the latent electrochemical cell properties of this technique. The Taylor cone shown in Figure 2.8 would emerge from the end of the capillary (from Kebarle 2000).

From this point the most difficult step is the transfer of these gaseous ions from atmospheric pressure into the high vacuum of the mass spectrometer. At the counter electrode a small orifice collects the gaseous ions and three types of sampling devices have been used to transfer the ions into the first vacuum stage. These are a glass capillary with a countercurrent drying gas, a heated transfer capillary and a two capillary system in a zig-zag path to help breakdown ion clusters and a simple sampling cone before the vacuum stage. At this stage these transfer pathways also help evaporate the solvent. Because the ions are transferring from atmospheric pressure to lower pressure, the outlet of the sampling orifice acts as a nozzle from which the gas expands.

The gas then travels to a second orifice known as a skimmer. A mechanical pump creates the vacuum in the region between the transfer capillary and the skimmer. From the skimmer the gas is sampled into a second higher vacuum created by a turbomolecular pump. At this point the remaining solvent vapour and gas is pumped away leaving the ions to go through to the ion collection and focusing lenses of the mass spectrometer before eventually ending up at the mass analyser. Although a two step sampling of ions, lowering of the pressure from the 1 atm electrospray chamber and removal of neutral/volatile analyte and solvent as described above, the

number of incrementally lower pressure chambers, sampling/skimmer cones and focusing lenses vary from instrument to instrument.

Quadrupole mass analysers have been the most commonly used for the electrospray technique. Mass separation is performed by four parallel cylindrical rods where the diagonally opposite rods are electrically connected. Direct current potential and radiofrequency voltage are applied to the rods, this electric field creates a path of stability through which only ions of certain mass to charge ratios (m/z) can pass. Hexapole, Octapole, Ion Trap, Fourier Transform, Time-of-Flight and Magnetic Sector mass detection has also been used for ES-MS.

2.3.2 Electrospray Method Developments

The description in Sub-Section 2.3.1 covers a simple electrospray setup where the desolvation is carried out purely by the electric field. Under these conditions problems which generally lower the ion sensitivity are encountered. The flow rate of sample and solvent has to be very low. This can be a problem when using electrospray as a detector for liquid chromatographic separation. In addition, the surface tension and the amount of electrolyte in the solvent also have to be low. For these reasons water is not recommended as a solvent. Typically, the best solvents are polar organic solvents with a low surface tension and low dielectric constant. Electric discharges from the tip to the counter electrode, especially in the negative ion mode, can also occur due to a high amount of electrolyte. This can be a problem by causing gas phase reactions between ions thereby decreasing sensitivity. Modifications and enhancements have been developed and implemented into more recent electrospray instrumentation to overcome many of the above-mentioned problems.

The most common adaptation to the technique involves the use of a warm nebulising/drying gas, which is generally N_2 (Bacon et al. 1997). The gas is introduced into the solution via a sheath tube around the capillary tip exit. Use of the gas assists spray formation and ion evaporation and can narrow the droplet beam thus increasing the concentration of gaseous ions sampled into the vacuum. This modification has been termed Ionspray (ISP). Because formation of the aerosol and gas phase ions are less reliant on the electric field, higher flow rates, lower electric fields and higher electrolyte concentrations can be tolerated. The last enhancement works by the sheath gas reducing

the concentration of conducting species at the needle tip. Other than pneumatic assistance, mechanical vibration at the capillary tip and the use of a sheath liquid with electron scavengers to reduce electrostatic discharge have also been utilised to improve electrospray efficiency (see Figure 2.10). These enhancements and the utilisation of heated assemblies have allowed the use of water in electrospray solvents. The most common solvents now used are mixtures of 50:50 water/methanol and water/acetonitrile. Importantly, this has also allowed easier interfacing with liquid chromatography.

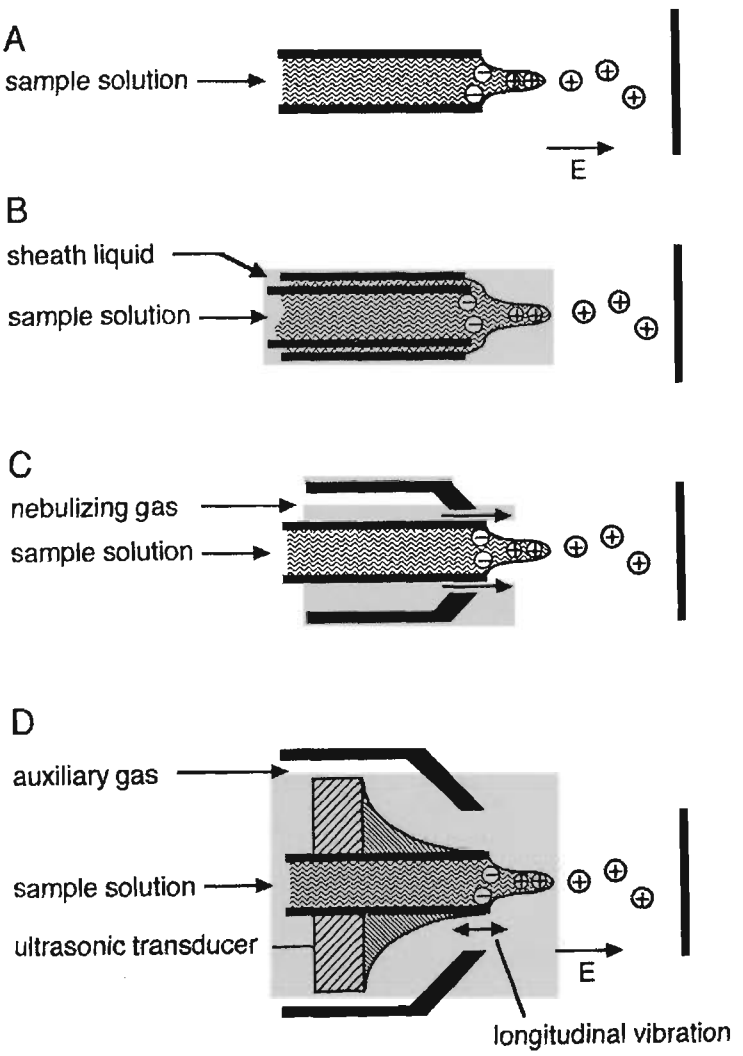


Figure 2.10 Aerosol formation by various methods of electrospray in use today: a) simple ES, b) ES with sheath flow, c) ES with pneumatic assistance and d) ES with ultrasonic assistance (from Bruins 1997).

2.3.3 Information Derived From Electrospray Mass Spectrometry

The two main advantages of electrospray mass spectrometry over other techniques is the 'soft ionisation' of the analyte and multi-charging of large molecules such as proteins. In the case of the former, because the 'free' ions have little excess internal energy left after going through the ion evaporation and gas phase ion production stages, little fragmentation occurs, as is usually the case

with other mass spectrometric processes. Hence, up to a size of 500 daltons, the ions seen in the mass spectrum are singly charged quasi-molecular ions of the analyte and solvent rather than its fragments. Because of this ES-MS is considered the 'softest' ionisation technique available. In the case of the latter, large molecules above 500 daltons form ions of multiple charges. This is a major advantage as multiple charges lower the mass to charge ratio (m/z) allowing molecules that are normally outside the mass range of a quadrupole mass spectrometer to be observed.

The singly charged ions are often observed as adduct ions $[M+H]^+$ and $[M-H]^-$ where M is the molecular mass and H is a proton. Doubly and triply charged ions are rare. Doubly charged ions can sometimes be seen as $[M+2C^+]/2$ and dimers as $[2M+C^+]$, where M is the molecular mass and C is an ionising cation e.g. H^+ and Na^+. These ions can be both inorganic and organic.

Another important advantage of ES-MS is that ion intensity is proportional to the concentration of the analyte in solution. This differs from other ionisation techniques where the ion intensity is proportional to the mass-flow. Because of this, provided that concentration of an analyte is above the minimum, low flow rates can be used. In addition because sensitivity is concentration dependent there is no competition in the ionisation process between differing analytes. However, the relationship of ion intensity to concentration is non-linear and dependent on matrix composition. Linearity can be achieved by the use of a stabiliser and measuring intensity versus concentration ratios with an internal standard.

Mass spectra are normally acquired by averaging a number of scans of the ion cluster as it is detected over time and smoothing the resulting mass peaks observed using computer manipulation.

2.3.4 Influences and Parameters Affecting ES-MS

There are some variables in the technique that influence the intensity of the electrospray and hence ion intensity. The role of the ES delivery design on electrospray efficiency has already been discussed. Also previously discussed, the solvent and its physical characteristics greatly influence the quality of the spray and ion intensity.

Primarily the solvent influenced factors include surface tension, conductivity, viscosity, pH, flow rate and polarity. The surface tension plays a direct role in the 'onset potential' or the potential where the droplet formation occurs. The lower the surface tension, the easier the formation of the 'Taylor cone' and the higher the rate of solvent evaporation from the droplets. The use of surfactants however can swamp analyte signals due to their attraction to the surface of droplets where desolvation takes place, suppressing ion formation. Ionisation is governed by the droplet surface layer (Zhou & Cook 2000). High conductivity suggests a high concentration of electrolytes, which can cause electrostatic discharges as described in 2.3.2 and suppress analyte ions by overwhelming competitive interference with analyte ion formation (Wang & Cole 1994b). However some electrolyte is required or charged droplets will not form (Cole & Harrata 1993; Agnes & Horlick 1994a). Addition of spectator electrolytes, not involved in the association/dissociation of the analyte, was found to have an insignificant effect upon analyte charge distributions in both positive and negative ion ES-MS (Wang & Cole 1994b). Viscosity has a direct influence on droplet size, the smaller the droplets the easier droplet fissioning and gas phase ion formation will be. The pH of the solution can exert an influence on the solution acid-base equilibria and hence the degree of positive and negative charging via protonation/deprotonation of the analyte species. Neutral analytes will more likely spray in protic solvents, whereas ionic compounds can yield better results in aprotic solvents. Although the sensitivity is not proportional to flow rate, higher flow rates can impact upon the electrospray above a certain limit. Too high a flow rate will produce droplets large enough to cause electrical breakdown. Solvent polarity also has an important influence on stable electrospray. A high polarity solvent can increase solvation of electrolytes and hence stabilization of charge separation in solution. This enhances the process of charged droplet formation at the capillary tip. The best polarity is one where the analyte molecules are more able to accommodate the charge. Solvent polarity was identified by Cole & Harrata (1993) as the most important factor governing ion charge states. As discussed previously ion sensitivity is proportional to analyte concentration but only over a small concentration range (Tang & Kebarle 1993; Agnes & Horlick 1994a; Barnett et al. 2000). Above this limit, the sensitivity concentrations off with respect to increased analyte concentration and is thought to be due to an increased competition for charges in droplets leading to a depletion of droplet charge by earlier evaporation of ions of lower charge states. Hence analyte concentration can disturb stable spray formation at higher concentrations. In tandem with the

concentration, solubility also affects ion intensity by influencing the electric field required to remove an ion from the droplet into the gas phase (Cole & Harrata 1993). The most common solvent in recent times has been 1:1 H_2O:acetonitrile due to its high stability for a broad range of analytes and its proton donor/acceptor behaviour (Henderson 1998b). Methanol is also a very popular solvent.

Other than solvent influences, the ion desolvation process can also cause changes to the ions in solution compared with the gas phase ions. The exact identity of preformed ions in solution may not be preserved once converted to the gas phase (Kebarle 2000). The pH can change during the ion evaporation process with a greater concentration of hydrogen ions in smaller drops forcing the acid/base equilibrium of the solution to change. Reactions between ions in the gas phase and at the liquid/gas interface can be caused by the above-mentioned protonation or by complexation to form neutral molecules which cannot be analysed in the mass spectrometer. Gas phase reactions can also produce adduct cluster ions which can contribute to or interfere with ion formation.

There are instrumental parameters that can also influence the mass spectrum observed from electrospray. The most obvious parameter is the polarity of the electric field. This directly influences the charge of the ions sprayed, with the spray tip as the cathode, positive ions are produced and when the tip is the anode, negative ions are formed. The magnitude of the electrospray source potential can also be varied, influencing the quality of the spray. Another variable that can be controlled is the sampling and skimmer nozzle potential or 'cone voltage'. Increased cone voltage can lead to ion fragmentation by focusing the ion beam more tightly and inducing greater collisions between these ions. Fragmentation may be desired if analyte identification through fragment fingerprinting is required.

Prolonged use of the electrospray results in a build-up of contaminants in and around the sampling orifice or tube. A build-up of charge on this contamination can reduce or stop the passage of ions by disturbing the electric field in front of the sampling orifice and prevent ions from being carried by the gas flow into the vacuum. The use of a 'pepperpot' in Micromass instruments reduces contamination by removing a line of sight path between spray source and sampling orifice and

allowing ions and small charged droplets through but retaining big droplets (see Figure 2.11). Even with the use of the 'pepperpot' the build-up of contaminants will still occur and regular cleaning of the sampling cone and chamber is required.

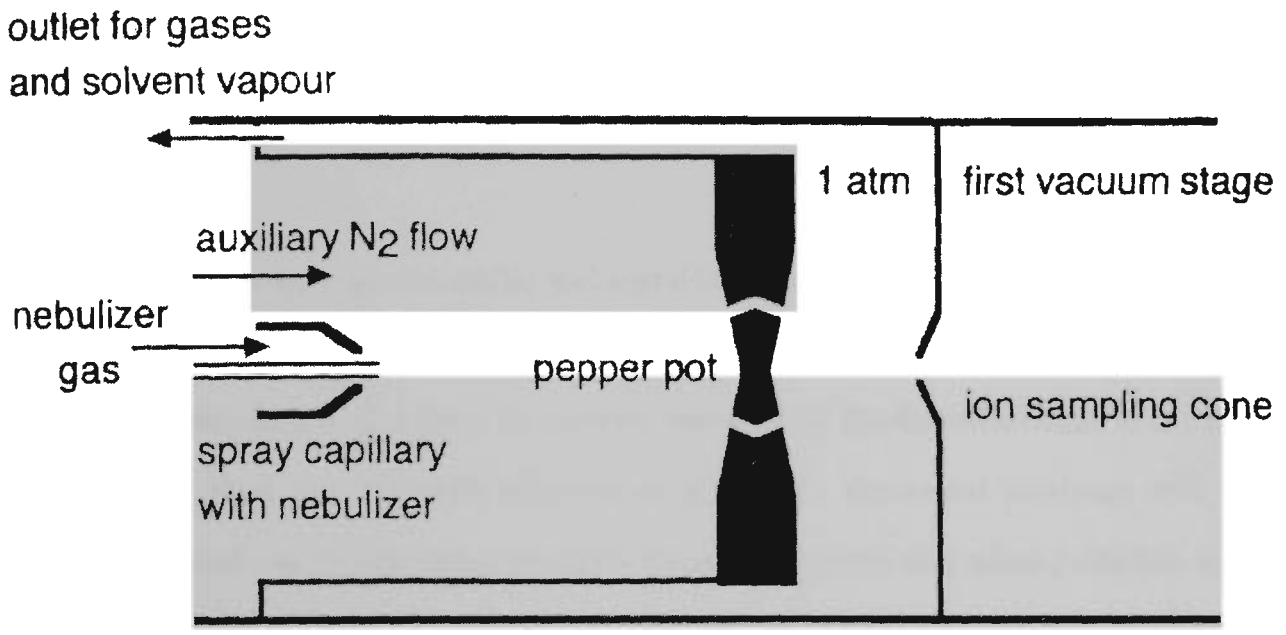


Figure 2.11 Pepper pot used in Micromass mass spectrometers for reduction of contamination of the sampling cone (from Bruins 1997).

2.3.5 Inorganic and Organometallic Speciation Using ES-MS

ES-MS was initially used to determine masses of high molecular mass molecules such as proteins and large polymeric species where multiple charging was a distinct advantage. In the early 1990's researchers began to realise the potential of the technique for elemental and molecular speciation analysis, particularly as previously mentioned enhancements such as N_2 nebulisation improved the scope and sensitivity of the method. The reason why ES-MS has caused so much excitement in the last decade is solely due to the premise that its 'soft' ionisation technique provides quasi-molecular ions that reflect the ions/molecules in the sample solution, providing qualitative and in some cases quantitative speciation of sample analytes (Zoorob et al. 1997; Bacon et al. 1997; Stewart 1999; Barnett et al. 2000). It has been shown that electrospray sources operating in a very soft ionisation mode can preserve low energy associations of weak bonds (Collette et al. 1997). In addition, for some systems, it has been shown that ES-MS can analyse all individual ions/components even undergoing rapid ligand exchange due to ligand exchange ceasing when solution ions enter the gas

phase, effectively providing a true picture of the species identities and proportions in a thermodynamic equilibrium (Colton et al. 1995; Leize et al. 1996; Gatlin & Turecek 1997). This has been a major drawback for speciation methods mentioned previously in this Chapter. One major area that has been developed with regards to ES-MS analysis is the analysis of inorganic and organometallic species where the potential of ES-MS to establish the identity of small metal complexes in biological and environmental media has been increasingly appreciated (Agnes et al. 1994).

A wide range of inorganic, organometallic and metal/organic ligand complexes have been studied using ES-MS in recent years covering almost all elements (Stewart & Horlick 1996a; Stewart 1999). Elemental metal analysis has also been conducted, however as the detection limit is 2-3 orders of magnitude higher than for ICP-MS (Brown et al. 1996) elemental analysis will still be predominantly carried out by the latter method. Complexes involving alkali, alkaline earth and transition metals have been the most commonly analysed with ES-MS (Brown et al. 1996; Zoorob et al. 1997), although the technique has also been applied to the metals of groups 3-6 and some lanthanides and actinides. Of organometallic studies, those involving the first, second and third row transition metals have predominated (Stewart 1999).

There are several methods of analysis of inorganic and organometallic complexes in ES-MS which involve the manipulation of ion formation. Neutral molecules require conversion to ions before being analysed by ES-MS. The following synopsis of these methods is derived from excellent reviews on inorganic and organometallic ES-MS by Colton et al. (1995), Henderson et al. (1998b) and Traeger (2000), the latter two particularly on metal carbonyl speciation. In this Sub-Section M represents the molecular mass of metal or metal complex.

a) Charged species are ideally suited to ES-MS whereby these solution ions are transferred to the gas phase without any further ionisation required. Hence these ions are observed directly.
b) Protonation/deprotonation has been a very common method of ionisation. This occurs with complexes containing O or N atoms providing protonation sites. In the positive ion mode, protonation of basic molecules produce $[M+H]^+$ ions whereas in negative ion mode, acidic species such R-COOH and R-OH can deprotonate to give $[M-H]^-$ ions. Zhou & Cook (2000)

have postulated that the former process occurs due to gas-phase chemical ionisation by precursors such as NH_4^+ in solution or by corona discharge at the sampling orifice, whereas deprotonated ions from acidic solutions in negative ion mode are caused by hydroxide or methoxide in the gas phase derived from negative ion discharges. This ion formation can be particularly helpful for some neutral species that would not otherwise be detected with ES-MS.

c) Metallation can be used where neutral species are not amenable to protonation/deprotonation. Singly charged positive ions such as NH_4^+, Na^+, K^+ and Ag^+ are added to solution to form $[M+Na]^+$ metal adduct ions. Although the alkali metals have been the most commonly used, transition metals have also been employed.

d) Derivatisation of neutral entities by nucleophilic attack has also been used. Alkoxide anions derived from reagents such as NaOMe in alcoholic solvent are most commonly used for this task. The resultant $[M + OMe]^-$ ions are then detectable by ES-MS in the negative ion mode.

e) Quaternization of complexes containing phosphine and arsine can convert the neutral molecule to a cation without affecting the rest of the complex.

f) Conversion of neutral molecules to anions or cations can be achieved by redox reactions via a preceding chemical reaction or by electrochemical processes. The electrochemical nature of ES-MS has been utilised for this purpose. Studies of metal carbonyl complexes have shown the formation of $[M]^+$ ions due to oxidation of the metal centre of neutral species by the electrospray process.

g) Anionic ligands in a neutral complex can be displaced by a neutral ligand to produce a cationic complex.

Although the above methods alter the chemical composition and hence the initial speciation of the analyte, the derivatives are generally close enough to the original to be easily characterised in the resulting mass spectra.

Many studies have been conducted on characterisation of inorganic and organometallic complexes of which metal carbonyl speciation has been very prominent. These studies are listed by reference with the metal species studied and the ES-MS mechanism of gas phase ion production in Table 2.2.

Table 2.2 List of metal and metal complex speciation studies by ES-MS and the mechanisms used.

Reference	Metals/Complexes Analysed	ES-MS Mechanism
Henderson & Nicholson 1995	$Ru_3(CO)_{12}$, $Os_3(CO)_{12}$, $Re_2(CO)_{10}$, $SiFe_4(CO)_{16}$	Add $AgNO_3$: Ag^+ metallation $[M + Ag^+]^+$
Henderson et al. 1996 **Henderson et al. 1998a**	Various neutral mono-, di-, tri-, tetra- and hexa-nuclear metal carbonyls	Add NaOMe in methanol, NaOEt in ethanol, $NaOPr_i$ in propanol: OMe^-, OEt^-, OPr_i^- adduct formation $[M + OMe^-]^-$, $[M + OEt^-]^-$, $[M + OPr_i^-]^-$ Deprotonation: $[M\text{-}H^+]^-$ Add (alkali metal)-OR: alkali metal metallation $[M + (\text{alkali metal})^+]^+$ ES capillary tip oxidation: formation of molecular ion $[M]^{\bullet+}$
Henderson & Evans 1999	Neutral transition metal (M) complexes with halide ligands (X)	Loss of halide ligand: $[M^{n+}X_n - X^-]^+$ Loss of halide + solvent coordination: $[M^{n+}X_n - X^- + \text{solvent}]^+$
Kane-Maguire et al. 1995, 1996	Fe, Cr, Mo, W carbonyl complexes, ferrocene	Low skimmer voltage: molecular ion formation and protonation $[M]^+$ & $[M+H^+]^+$ High skimmer voltage: loss of sequential CO ligands $[M\text{-}(CO)_x]^+$ Oxidation of ferrocene: $[M]^{\bullet+}$
Hopfgartner et al. 1993	Supramolecular complexes of Cu, Co, Eu and Tb with large ligands	Multiply charged cation formation
Lieze et al. 1996	LiCl, NaCl, KCl, CsCl, RbCl	Loss of halide: $[M - Cl^-]^+$
Young et al. 1997	Crown ethers with Li, Na, K	Add alkali metal: alkali metal metallation $[\text{crown ether} + M]^+$
Ralph et al. 1996	Transition metals in bicyclic hexamine 'cage' complex (macropolycyclic ligands: are 3-D complexing agents that encapsulate the metal	Mild ionisation: divalent and deprotonated trivalent ion formation $[M(cage)]^{2+}$, $[M(cage)^{3+} - H^+]^+$
Brown et al. 1996	Ca, Rb, Cs, Ba, V, Cr, Ni, Co, Cu, Zn, U	Bare metal ions: $[M]^+$ Divalent metal charge reduction: $[M^{2+} + e^-]^+$
Zoorob 1997	Cr^{2+}, Cr^{3+}, Co^{2+}	Bare metal ion formation Alkoxide adduct formation
Stewart & Horlick 1996b	Cr^{3+}, Cr^{6+}	Alkoxide adduct formation
Gwizdala III et al. 1997	Cr^{3+}, $Cr_2O_7^{2-}$	Halide adduct formation & mild ionisation process retains oxygen ligands: $[CrO^+ + 2Cl]^-$ Charge reduction & halide adduct formation: $[CrO_3]^-$, $[CrO_3Cl]^-$

Table 2.2 (cont.) List of metal and metal complex speciation studies by ES-MS and the mechanisms used.

Reference	Metals/Complexes Analysed	ES-MS Mechanism
Mollah et al. 2000	Fe^{2+}, Fe^{3+}, Cu^{2+},	Excess HNO_3 or HCl: further halide adduct formation $[M^{n+} + X_{n+1}]^-$ (where X represents Cl^- or NO_3^-) In presence of EDTA: deprotonation of EDTA $[M^{n+} + EDTA^{-(n+1)}]^-$
Baron & Hering 1998	Cu, Pb, Cd, Al, Fe^{3+} with EDTA	Protonation of EDTA: $[M^{n+} + EDTA^{-(n-1)}]^+$
Lover et al. 1997	$Zr(OEt)_4$, $Ti(OEt)_4$, $Al(OEt)_3$, $Si(OEt)_4$	Add NaOEt: OEt^- adduct formation $[M^{n+}(OEt)_n + OEt^-]^-$ For $Si(OEt)_4$: Ligand protonation & removal $[Si(OEt)_3]^+$

Fragmentation has become an important part of ES-MS with the extent of fragmentation controlled by the skimmer 'cone voltage'. The majority of the work described in ES-MS literature has included studies of variations in cone voltage to observe the degree of fragmentation and the distribution of ions produced. It is well known that increasing the voltage on the skimmer cone increases the number of ion/ion and neutral molecule/ion collisions in the gas phase, hence increasing fragmentation (Henderson et al. 1998b). However the number of fragment ions giving structural information is smaller in negative ion mode and differs from those seen in positive ion mode (Straub & Voyksner 1993). In terms of metal complexes the most labile ligand will be removed first followed by less labile ligands as the cone voltage is increased. An example of this is the successive removal of carbonyl ligands from metal carbonyl complexes with increasing voltage (Traeger 2000). Dyson et al. (2000) put forward a fragmentation mapping process by collecting negative mass spectra of methoxide derivatised $Rh_6(CO)_{16}$ at various cone voltages and superimposing the hundreds of mass spectra onto one chart plotting cone voltage against mass-to-charge ratio. In this way a 2-dimensional pattern of CO ligand removal was observed and this technique was postulated as a method of identifying signals of a mixture of compounds. Agnes & Horlick (1995) investigated instrumental operating parameters on metal analyte signals in the mass spectrum and concluded that the curtain gas flow rate and sampling plate voltage between the sampling cone and the capillary tip were the two most important variables influencing metal ions observed. High gas flow rates and high sampling plate voltage lead to the formation of singly charged bare metal ions whereas low

values of both induced the formation of ion-solvent clusters with a m/z reflecting the valence state of cations in solution.

The greatest question that has been asked of ES-MS inorganic and organometallic speciation studies is; "Are the ions observed and their distribution in the mass spectra truly reflective of the molecules/ions in solution?" The majority of authors cited in this Section have claimed this is the case, or close to it, when considering derivatised analogues formed by processes before, during or after ionisation have been easily related to their precursors in solution. Indeed other extensive reviews of inorganic/organometallic speciation using ES-MS have agreed that in general there is an excellent correlation between the ions observed in the gas phase and the nature and distribution of the components in solution and hence the use of ES-MS for analysis of metal complex species is quite feasible (Agnes et al. 1994; Agnes & Horlick 1994b; Colton et al. 1995; Stewart & Horlick 1996a; Gatlin & Turecik 1997; Hieftje 1998; Sanz-Medel et al. 1998; Traeger 2000). However there have been examples where studies of gas phase ion formation with organic analytes showed that in some cases, solution components and equilibrium distribution are not maintained in the electrospray ionisation process (Wang & Cole 1994a; Chillier et al. 1996). The most strenuous challenger of the broad assumption that the ions evolved from electrospray will closely resemble solution components, particularly with respect to inorganic/organometallic complexes, is Van Berkel and associates (Van Berkel et al. 1997; Van Berkel 2000). The primary contention is that most researchers using the technique neglect or are ignorant of the electrochemical nature of the electrospray ionisation process and the influence exerted by the resultant redox reactions. These reactions can affect solution components, and hence ions formed, in two ways:

a) Charge balancing redox reactions in the ES capillary under certain conditions have been shown to alter the initial solution due to oxidation of water in positive ion mode and reduction in negative ion mode. In some cases the pH change has been up to 4 orders of magnitude which would significantly change the equilibrium ion distribution of some acidic and basic analytes. However it was noted that 'electrolytically-induced pH changes will be most prominent in ES-MS scenarios employing low flow rates (i.e. $\leq$ 1.0 $\mu L\ min^{-1}$), non-buffered solutions near neutral pH, and metal spray capillaries or metal contacts to solution comprised of difficult to oxidize material (e.g. platinum or gold)'. The second of these

conditions is in agreement with the observations of Zhou & Cook (2000) who stated that for strongly acidic solutions, pH changes due to the electrospray process would be negligible.

b) Other than pH effects, redox reactions may alter the nature of the metal species in two ways. Firstly, reduction and oxidation in the negative and positive modes respectively can change the valence of the metal, e.g. $Fe^{3+} + e^- \rightarrow Fe^{2+}$, leading to observations of these redox products along with the original ions. Secondly, metal ions can be converted to insoluble product or elemental deposition of the solid onto the emitter can occur, e.g. $Cu^{2+} + 2e^- \rightarrow Cu(s)$, in negative ion mode which, when the ES-MS is switched to positive ion mode, can be liberated into the sprayed droplets. Either reaction can alter the concentration and distribution of the metal species in solution and hence affect the relative ion abundances in the resultant mass spectrum. Investigations by Van Berkel show the concentration of the metal in the solution sprayed may be up to 50% lower than in the initial solution entering the capillary. The susceptibility of metals to undergo redox reactions depends upon the other electrolytes in solution and the potential for reduction/oxidation of the metals; e.g. 'as the potential necessary for reduction of these individual metals becomes more negative, the efficiency of the redox reaction should decrease as other reactions involving the solvent and additives become more energetically favourable' (Van Berkel 2000).

From these observations Van Berkel and colleagues suggest researchers be aware of these processes before assigning identities to ions and expecting them to reflect the solution chemistry. In spite of these reservations, Colton et al. (1995) in their exhaustive review noted that 'in almost every case where the identity of ions in solution has been established by some other technique, such as multinuclear magnetic resonance spectroscopy (NMR) or electrochemistry, the intact ions observed by ES-MS agree with the prior identification'. Wang & Agnes (1999a,b) demonstrated that for metal-EDTA complexes, kinetically labile solution species altered by the electrospray process could be predicted and that only species that react faster than the ~5μs residence time in the evaporating droplet will be significantly affected. They postulated that even if a species reacts fast enough desolvation will only cause a change of concentration of 1 order of magnitude and due to metal complexation being rate limited by water dissociation only the labile alkali metals are expected to give significant changes in ion quantitation from solution to the gas phase.

2.3.6 Aluminium Speciation Using ES-MS

Very few aluminium-specific speciation studies have been conducted using ES-MS. Much of the aluminium work done in ES-MS has been part of a general study, including investigations discussed in Sub-Section 4.3.5. Colton et al. (1995) noted that the ligand $[(C_5H_5)Co(OPO(OEt)_2)_3]^-$ or $[L_{Et}]^-$ formed the strongest complexes with trivalent metal ions including Al^{3+} in aqueous/methanol solvent. The intact ion, $[Al(L_{Et})_2]^+$, was observed in positive ion mode without the need for protonation. In their investigation into metal alkoxides using ES-MS, Lover et al. (1997) characterised $[Al(OEt)_4]^-$ at m/z of 207 in the negative ion mode after derivatisation of $Al(OEt)_3$. Less intense ions assigned to $[Al(OEt)_4]^-$ and sequential adducts of NaOEt and $\{NaAl(OEt)_4\}_n$ were also observed. The primary ion proved to be very robust with 100% relative ion intensity maintained even after the application of high cone voltages up to 90V. It was concluded that Al-alkoxides are primarily trimeric and tetrameric in nature. Baron & Hering (1998) also included aluminium in their study of metal-EDTA complexation with ES-MS (see Figure 2.12). Aluminium-EDTA present as an anion in solution was protonated in the positive ion mode giving the related ion $[Al\text{-}H_2EDTA]^+$ at m/z of 317 with little fragmentation. $[Al(NO_3)_4]^-$ in solutions containing nitric and hydrochloric acids was characterised by Mollah et al. (2000) in the negative ion mode. Charge reduction of the 3+ cation was avoided by stabilising the charge with X^- rather than by delocalisation over neutral solvent molecules.

Deng & Van Berkel (1998) conducted one of the few specific aluminium speciation studies with ES-MS, investigating Al(III)-flavonoid complexes. A 1:2 ion, $[AlL_2]^+$ (L = [flavonoid-H^+]$^-$) predominated in a range of different solvents in ES positive mode except at high Al^{3+} concentration. A 1:1 complex of the form $[AlR(L)]^+$ (where R = H^-, OH^- or CH_3O^-) was also observed in lower abundance. The degree of complex formation was found to be dependent on the Al^{3+}:flavonoid ratio however the best response was observed when methanol was used as the solvent with the dimer signal suppressed for all other solvents used. When aluminium concentration was several times higher than that of the flavonoid the 1:1 complex tended to predominate in the mass spectrum. In another aluminium specific ES-MS study, Gumienna-Kontecka et al. (2000) used ES-MS to complement NMR and potentiometric investigation of 2-(hydroxyimino)propano-hydroxamic acid as a potential aluminium sequestering reagent in biological systems. Although the ES-MS data was

complicated, two peaks corresponding to metallated and protonated adduct ions, $[Al(HL)_3 + K^+]^+$ and $[Al(HL)_3 + H^+]^+$, were assigned in the positive ion mass spectrum at pH 6-8. This confirmed that the complex stoichiometry was 1:3 metal-to-ligand for the predominant complex at high ligand excess preformed in solution.

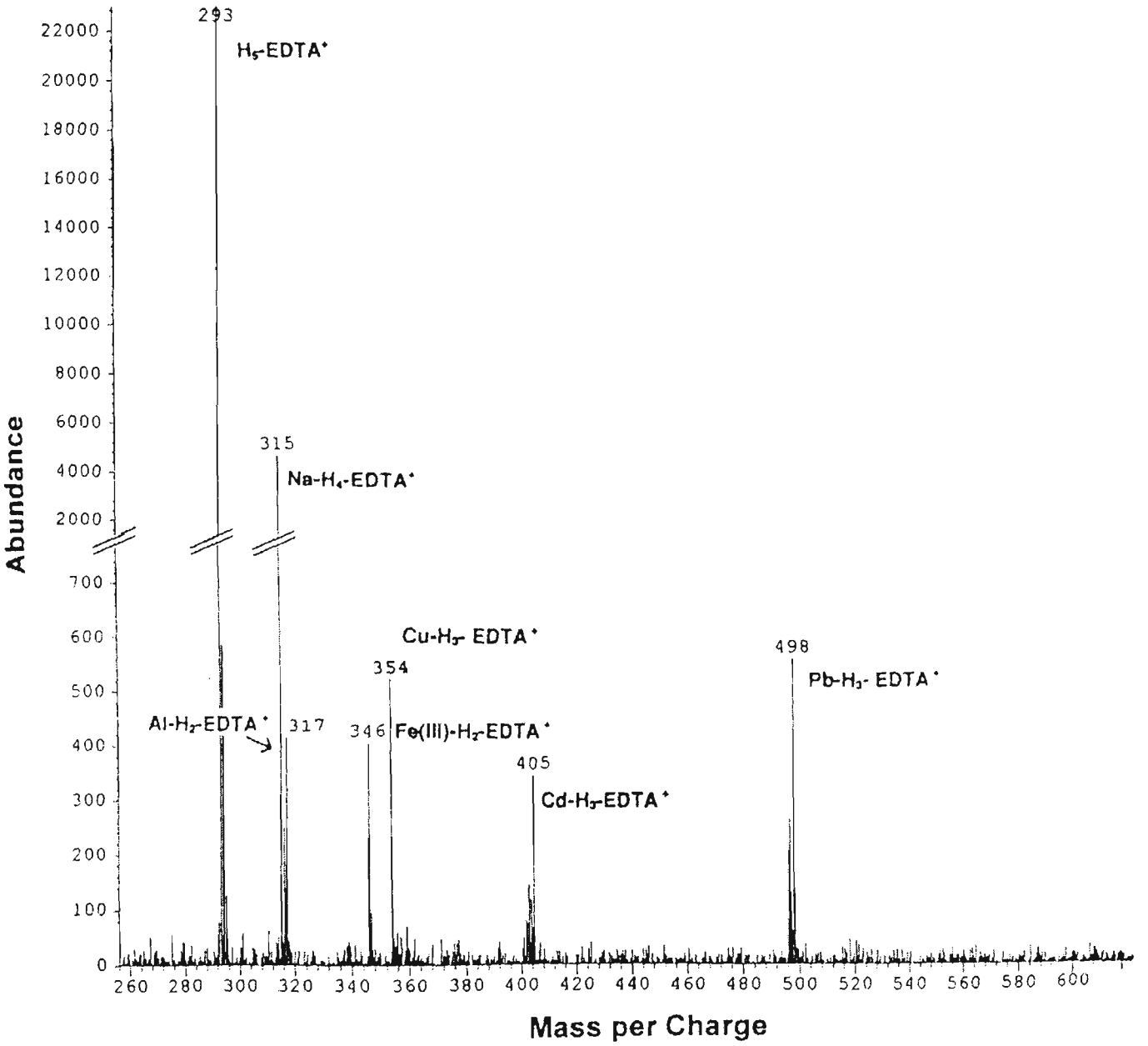


Figure 2.12 Positive ion mode electrospray mass spectrometry (ES-MS) spectrum of a solution of 10^{-4} M Na_2EDTA, and 10^{-5} M each of Al, Fe(III), Cu, Cd and Pb. Uncomplexed EDTA and EDTA-metal complexes are detected as protonated species with a single positive charge. (from Baron & Hering 1998)

2.3.7 ES-MS of Organic Acids

Salifoglou (2002) suggested that new techniques are called for to elucidate aluminium-carboxylate speciation and specifically singled out ES-MS. This has been increasingly used in several studies on organic acids in various media. In general organic acids can be determined as a singly charged negative ion in the negative ion mode without derivatisation. Bartok et al. (1997) developed a

scheme to derivatise organic acids to allow ES-MS speciation analysis in the positive ion mode which was considered more sensitive. Acetic, succinic and benzoic acids were derivatised with N,N'-dicyclohexylcarbodiimide and N,N'-dimethylethylenediamine giving a stable amide derivative with a free tertiary amino group which can be protonated. A singly charged cation was observed in positive ion mode in 1:1 acetonitrile/water. These ions were very stable up to a capillary voltage of 130V. Using this technique these carboxylic acids could be detected at the femtomole concentration. Groundwater organic acids were investigated by McIntyre et al. (1997) using ES-MS in the more traditional negative ion mode. A rather complex 'stegosaurus' pattern was observed in the mass spectrum with a distribution of singly charged ions every second mass-to-charge value from ~200 to 700 Da. The distribution of ions shifted to the lower masses with increased cone voltage. Using this fragmentation data it was concluded that the organic acids in the groundwater consisted mainly of aliphatic polycarboxylic acids. Collette et al. (1997, 2001) studied complexes of polyammonium macrotricyclic ligands with oxalic, malonic, succinic, adipic, maleic and fumaric acids using both negative and positive ion mode ES-MS to give a more comprehensive analysis of all the ions formed. Both singly charged and doubly charged ions were observed in the mass spectrum. Subsequent comparison with theoretical calculations showed good agreement with the results derived from the electrospray method.

ES-MS has also been used as a detector for organic acid speciation. Frauendorf & Herzschuh (1998) used a megaflow ES-MS to detect the effluent from an HPLC separating carboxylic acid standards and those derived from lignite extracts. They successfully characterised 19 organic acid standards as $[M-H]^-$ in negative ion mode after separating the acids in the HPLC column. Gradient elution with 0.01% aqueous formic acid (A) and acetonitrile (B) was used as the mobile phase starting at 100:0 (A:B) with the final concentration at a 50:50 mixture of (A:B) for ionisation in the ES-MS. Cone voltage of –20V caused a small loss of CO_2 from the anions however higher cone voltages created additional losses of CO_2. Loss of H_2O was also observed. Mass spectra of lignite extracts proved more difficult to assign with limited separation by HPLC. Some peaks gave an indication of the presence of hydroxybenzene-carboxylic acids with one to three carboxyl groups. Another example of ES-MS used as a detection method is the work of Johnson et al. (1999). A mixture of four dicarboxylic acids, succinic, maleic, malonic and glutaric were separated using capillary

electrophoresis and the subsequent ions were observed in negative ion mode ES-MS as the negative parent ions $[M-H]^-$ however the detection limits were poor.

2.3.8 Applicability of ES-MS Speciation Analysis

ES-MS has emerged as one of the most exciting new techniques in the last decade, especially for speciation studies. The potential of analysing individual species, their charges and their distributions, even in kinetically labile systems, is immense. This versatility has been extremely difficult to achieve using other speciation techniques. Not only has ES-MS been used for qualitative determinations, it has also been demonstrated that the technique can be used to quantitate species in solution with many investigations demonstrating a linear relationship between ion intensity and analyte concentration over a select concentration range (Tang & Kebarle 1993; Agnes & Horlick 1994a, Stewart 1999). Some limitations have been discussed, however in most cases solution components that have been altered by the process are generally easy to relate to their original precursors in solution. As a stand-alone instrumental technique or as a detector in a hybrid speciation technique, ES-MS is a genuinely useful speciation tool that is complementary and in some cases superior to NMR (Gatlin & Turecek 1997). Much has been made of ES-MS versus ICP-MS and which technique is better suited to speciation analysis. Houk (1998) has suggested that ES-MS and ICPMS are complementary techniques that can be used in tandem, especially for inorganic and organometallic species. ES-MS is still a relatively new technique and while progress to date has been rapid, there is still much to be discovered. The use of the technique in a hybrid system has yet to be fully appreciated and there are numerous chemical systems to be analysed. ES-MS is a significant new weapon in the armoury of analytical chemists in the quest for true speciation and progress in developing this analytical tool will no doubt continue unabated for some time.

2.4 Aluminium/Organic Acid Speciation in Wine

In terms of speciation of aluminium-organic acid complexes in beverages, tea has been the most thoroughly researched, probably because the plant is well-known to accumulate a large amount of aluminium, and the majority of research on tea has centred on high molecular mass polyphenolics (Flaten 2002). Although wine and its chemical components have been well researched there has been little work done in the area of speciation of aluminium in wine. In terms of metal speciation

studies in wine Pb, Cu, Fe, Ca and K composition and species have all been investigated to some degree for either their toxicology or influence over wine stability. Pb has been focused upon in particular as it has been demonstrated that lead derived from atmospheric deposition of organolead from automobiles is easily dissolved and complexed in wine and the adverse health effects of this element are well known (Teissedre et al. 1994a; Lobinski 1994; Lobinski 1995; Scollary 1997). Cu and Fe have been investigated due to their ability to cause wine spoilage by inducing oxidation and haze formation (McKinnon & Scollary 1997; Scollary 1997). Speciation analyses for Pb, Cu and Fe have included ICP-MS, size-exclusion fractionation via filtration and HPLC-ICP-MS, GC/Microwave Induced Plasma-AES, IC-FAAS, stripping potentiometry and gastrointestinal solubility modelling (Barbaste et al. 2001; Muranyi & Papp 1998; McKinnon & Scollary 1997; Szpunar et al. 1998; Lobinski 1993; Ajlec & Stupar 1989; Weise & Schwedt 1997; Green et al. 1997; Azenha & Vasconcelos 2000a; Azenha & Vasconcelos 2000). Studies on K and Ca in wine have focused more on these elements complexation with tartrate and subsequent crystallisation and precipitation during wine production, including flow injection and potentiometric titration speciation analysis (McKinnon et al. 1994; McKinnon et al. 1995; McKinnon et al. 1996; Gerbaud et al. 1996; Minguez & Hernandez 1998; Veruchet et al. 1999a; Veruchet et al. 1999b). Arsenic speciation in wine has also been investigated (Wangkarn & Pergantis 2000; Moreno et al. 2000).

The only significant aluminium speciation work involving wine has been size fractionation using microfiltration. McKinnon (1990) and McKinnon & Scollary (1997) reported filtering several white and red wine samples through filters of 10000, 5000, 1000 and 500Da pore sizes. They found that for all the wine samples studied most of the aluminium passed through the 1000Da filter and more than half passed through the 500Da filter. In the case of the latter finding, it was subsequently discovered that aluminium retention in the 500Da filter was due to membrane affinity for the element rather than size of the aluminium complexes. Muranyi & Papp (1998) performed a similar study for several elements including aluminium. Out of 8 Hungarian wines they studied, 5 had 50-80% pass through a 0.2 micron cellulose nitrate membrane filter and 3 had between 26-40% pass through the same filter. These studies indicate that aluminium is mainly bound to small molecules such as organic acids although the individuality of each wine sample is demonstrated with three wines in the Hungarian study showing aluminium binding to larger molecules. Although the

filtration study provided information on the size of the aluminium complexes and their possible ligands, it could not identify the actual species of aluminium in wine. It was suggested that tartaric acid with its high concentration in wine and potentially high affinity for binding to the Al^{3+} cation could be the ligand bound to aluminium in wine. This theory was supported indirectly with accompanying work using complexing agents for total aluminium concentration determinations in wine by colorimetric and fluorescence analysis (McKinnon 1990). It was found that strong binding ligands (that were postulated to be tartaric acid) interfered with complex formation of aluminium with N-(3-hydroxy-2-pyridyl)salicylaldimine whereby only 1-2% of the total aluminium was detected in wine.

The carboxylic and phenolic acids in wine and grape juice have been more thoroughly investigated due to their importance in contributing to its chemistry, character and flavour of which tartaric, malic and the tannic acids are the most prominent. HPLC and IC have been the most widely used techniques for characterising and quantifying organic acids in grape juice and wine. In particular ion exchange and reverse phase HPLC take advantage of the differing polarities of the acids and their differing Ka values (Almela et al. 1994; Ding et al. 1995; Jun et al. 1996; Mongay et al. 1996; Linget et al. 1998; Escobal et al. 1998; Vonach 1998; Dong 1998; Masson 2000; Castellari et al. 2000; Kordis-Krapez et al. 2001; Buglass & Lee 2001). A typical analysis was carried out by Vas (1997) using an anion exchange column with a 35:65 acetonitrile/50mM KH_2PO_4 mobile phase at pH 5.5 with UV detection. A guard column was required for grape musts and red wines due to their more complex matrices. With this system maleic, succinic, malic, tartaric and fumaric acids were separated. Another technique used to determine organic acids in wine and grape juice that has risen to prominence recently is capillary electrophoresis. Relying on potential difference induced flows through a small capillary, analytes are separated in terms of charge and size with detection typically carried out by UV. Organic acids determined with this technique have included formic, fumaric, succinic, oxalic, malic, tartaric, acetic, lactic and citric acids (Monson et al. 1997; Arellano et al. 1997; Kandl & Kupina 1999; Mallet et al. 1999; Castiniera et al. 2000; Oztekin & Erim 2001; Moreno et al. 2001). Other techniques used in recent years for determination of organic acid species are gas chromatography following derivatisation to esters, infrared and UV spectrometry, FIA-dialysis followed by enzymic derivatisation with detection by UV or fluorescence spectrometry, thin

layer chromatography (TLC) and LC-MS, all with varying degrees of success (Giumanini et al. 2001; Edlemann et al. 2001; Mataix & de Castro 2001; Boido et al. 1999; Cappiello et al. 1999).

Dartiguenave et al. (2000) carried out a study on the buffering capacity of organic acids in wine. The investigation involved titration of mixtures of tartaric, malic, succinic and citric acids at various concentrations with NaOH in aqueous solutions and hydroalcoholic NaOH in model wine solutions (11% v/v ethanol). It was found that alcohol enhanced the buffering effect by a factor of 2.3 for some mixtures and that the difference in buffering capacity of an acid mixture compared with an individual acid solution was indicative of interactions between the organic acids themselves.

Dissolved tannins, a major source of polyphenolic acid in red wine, and their metal complexes have been characterised by ES-MS (Ross et al. 2000). By increasing the ion source temperature from 60 to 120°C and the cone voltage from 30 to 60V multiply-charged ions were reduced to yield mainly singly charged ions. Surprisingly fragmentation was not increased with the higher cone voltage and this is thought to be due to stabilisation by intramolecular hydrogen bonding promoted by greater size and flexibility of the tannic ligands. The tannic acids were observed as two ions confirmed as gallotannins. Alkali, alkaline earth and transition metal complexes with these ligands in 50:50 acetonitrile:water were observed for all the metals studied as 1:1 metal/ligand complexes although the divalent metals also formed a complex containing a nitrato ligand and the alkali metals gave rise to 2:1 metal/ligand complexes. Al^{3+} was found to complex with the tannic acid ligand giving the 1:1 negative ion $[Al\text{-}L\text{-}4H]^-$. The same high cone voltage, elevated temperature ES-MS method was also used by Marmolle et al. (1997) to study polyphenolic speciation with Fe^{3+}. The group of Hayasaka et al. (2001) and Peng et al. (2001) used ES-MS as a detector for LCMS to study grape juice proteins and confirm HPLC work on tannins in grape seeds respectively. The former investigation looked at ways to use LC-ES-MS to determine varietal differentiation in grapes via their protein peaks while the latter used ES-MS to determine the composition of separated peaks of polymeric procyanidins (tannins) eluted from a reverse phase HPLC column.

2.5 Summary of Aluminium Speciation

A survey of the literature has shown that a substantial research effort has gone into speciation analysis in the last two decades. It is now well recognised that total concentration alone cannot indicate element bioavailability and toxicity, so much so that analyses and test limits set by relevant authorities are under greater pressure for review (Srinivasan et al. 1999; Gensemer & Playle 1999). Organizations such as the BCR of the EU have instigated programs to develop standardisation and validation of analyses aimed at elucidating and quantitating particular species of an element and certified reference materials (Sanz-Medel & Fairman 1992; Quevauviller 1995a). Unlike simple elemental analysis, speciation work is inherently more difficult due to the fact that most chemical forms in aqueous solution are involved in a system of dynamic equilibrium where any change to the system in terms of pH, ionic strength, concentration of species, the presence of competing ligands or ions, temperature and a host of other variables will irrevocably change the composition of the solution under study. Aluminium species are no exception.

As has been discussed, a host of analytical instrumental techniques has been applied ranging from computer modelling with formation constants, potentiometric titrations, derivatisation with reagents to form complexes detectable by spectroscopic means (colorimetry, UV-Vis, fluorescence), separation via ion exchange methods, combinations of separation and atomic spectrometry, fractionation by size filtration, IR and NMR. Of these, hybrid techniques coupling two or more instrumental techniques are now considered the best method of speciation elucidation (Sanz-Medel et al. 2002). However, most of these methods can only elucidate a type or fraction of a set of species or identify one or two species. Many of these methods are operationally defined, i.e. the method defines the fraction of species characterised which cannot be compared with data derived from other methods which do not use the same techniques or operational parameters. Most importantly, many of these methods disturb the chemical equilibria between the species or even change the species under investigation, rendering the derived speciation information incomplete or incompatible with the actual situation in a given system. Those techniques used where no interaction with the chemical equilibria are involved such as NMR or IR can be difficult to interpret in a complex matrix, take considerable time to acquire data leading to difficulty in characterising systems that contain fast

exchanging/reacting species or ligands and can lack the required sensitivity to sufficiently resolve individual components of the system. These problems have dogged aluminium speciation research.

A relatively new technique has risen to prominence in inorganic and organometallic speciation in the form of Electrospray Mass Spectrometry. The technique has been shown to characterise individual components in a system, including their valence state and structure, be they elemental or molecular. This is primarily due to the soft ionisation process, leaving molecular ions intact and the very fast solution to gas phase ion transfer, thereby outpacing redistribution in many cases. In addition fast exchange is stopped once these ions enter the gas phase. Although there is still debate over whether the ions in the mass spectrum truly reflect the species in a solution, ES-MS is the closest thing to a universal 'true' speciation analysis and its potential in organometallic and inorganic speciation investigations has been established and continues to be developed. Barnett et al. (2000) suggests the future of elemental speciation analysis lies in two areas, 'hybrid' systems using two or more instrumental techniques, and soft ionisation techniques such as ES-MS. Few aluminium speciation studies have been conducted using this new technique, although what work has been done with aluminium and with other metal species has been encouraging.

III

"Do or do not, there is no try"
Master Yoda, Jedi Master from George Lucas's 'Star Wars' film series

CHAPTER THREE

3. METHODOLOGY

3.1 Introduction

In this Chapter the methodology of the project is presented. Although the work and scope of the project is essentially divided into two parts, one covering the total concentration profile over the production process and the other on the speciation in the bottled wine, the description of the procedures used to derive the experimental data will be presented together. The techniques used for sampling, preparation and analysis of soil, grape, juice, ferment and wine samples are discussed and the methodology for ES-MS speciation of the finished wine will also be covered. In this current study, no distinction has been made between must (solution of grapes crushed but not pressed) and juice (must after pressing), hence both these types of sample are referred to as 'juice'. Method development that made a significant contribution to the method protocols of this study is discussed. However, as most of the speciation work in this study was a method development study in itself, only the general operating and sample pre-treatment procedures are presented in this Chapter, the experiment development will be discussed in Chapter 5.

Aluminium analysis is prone to contamination (Dolan & Capar 2002). Throughout this chapter it will become apparent that utmost consideration was given to the ubiquity of aluminium and the contamination problems associated with this. To reduce contamination effects a 'clean room' specifically designed for low-concentration metals determination is recommended for Al, especially for trace analysis (Wieteska & Drzewinska 1995). Unfortunately this project did not have access to such facilities and hence the methods discussed in this chapter were designed to reduce the sample exposure to extraneous aluminium as much as possible. This was accomplished by use of pure reagents, 'cleaned' labware and minimisation of sample handling to reduce exposure of reagents, labware or sample to air (Tsalev 1984; Frech & Cedergren 1992). Blanks and spiked blanks, subjected to the same conditions and procedures as the samples, were extensively used throughout this study to account for any input of aluminium other than that derived from the sample.

3.2 Reagents

Unless otherwise stated, all reagents used in this study were of Analytical Reagent (AR) purity and all solutions were made up using Millipore Milli-Q® water purified to a resistivity of 18MΩcm. Mineral acids used were derived from concentrated stocks i.e. nitric acid (BDH Analar, 70% v/v), sulfuric acid (BDH Analar, 98% v/v), hydrochloric acid (BDH Analar, 32% v/v) and perchloric acid (BDH Analar, 70% v/v). An exception to AR grade acids was the use of Merck Suprapur™ ultrapure nitric acid (65% v/v) for digestions. This grade of acid has a certified aluminium concentration of no greater than 5µg/L. The concentration of the hydrogen peroxide (Ajax Chemicals) stock used in digestion development was 25% v/v. Aluminium standards were derived from diluting 1000mg/L $Al(NO_3)_3$ BDH Spectrosol™ standard solution (containing 3% nitric acid) or dissolving $AlK(SO_4)_2.12H_2O$ (BDH, Analar 99.5% pure) in water to give 3mg/L in 100ml. Further serial dilutions were made to give required standards in 100ml. The aluminium potassium sulfate stock solution and all standards derived from both stock solutions were prepared fresh on the day of analysis. Daily preparation is required to minimise adsorption of aluminium from dilute non-acidified stock solutions onto the walls of volumetric glassware. This effect can be seen in a chart of storage of aluminium in volumetric glassware over a period of a few days (Figure 3.1) where the aluminium concentration of a stock solution measured with GFAAS (using the method in Sub-Section 3.7.1.3) is shown to decline. Tartaric (L-Tartaric 99.5%, Aldrich, ACS Reagent), malic (DL-Malic 99+%, Aldrich), succinic (99+%, Aldrich ACS Reagent), citric (99.5+%, Aldrich, ACS Reagent) and lactic acid (85+% in water, Aldrich, ACS Reagent) solutions [1000mg/L] were prepared by dissolving the appropriate mass of crystalline AR solid, or in the case of lactic acid an 85% m/v aqueous standard, in water. These organic acid stock solutions and the standards diluted from them in water were prepared fresh on the day of analysis. Argon and nitrogen gases were of high purity grade from BOC gases.

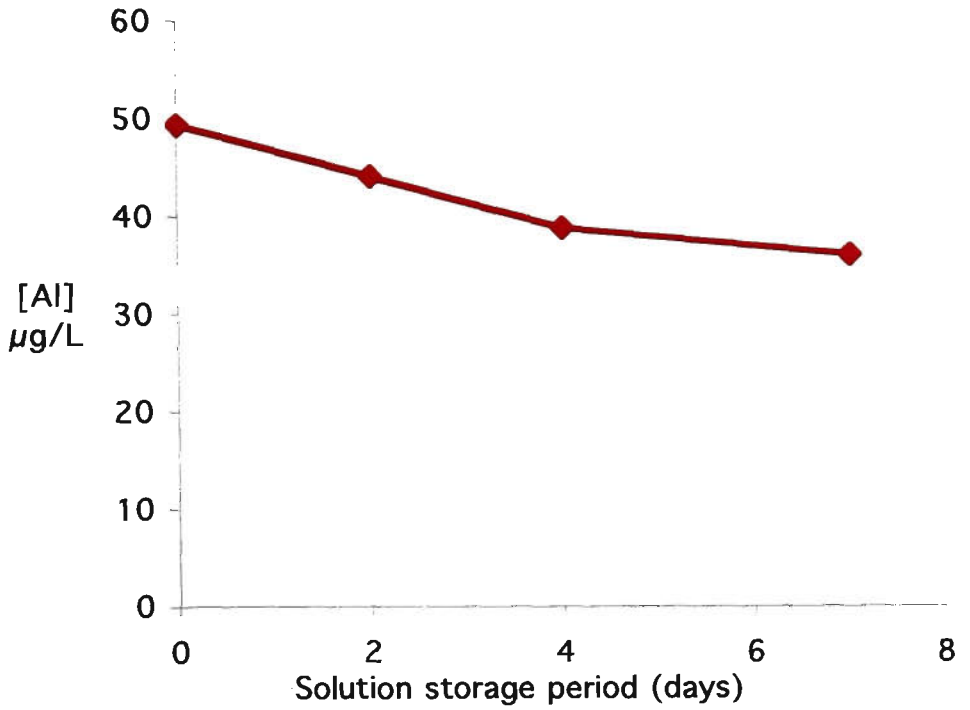


Figure 3.1 Plot of the aluminium concentration of an initially 50 µg/L unacidified aluminium solution stored in volumetric glassware for one week.

3.3 Labware

3.3.1 Labware Cleaning Development

Traditionally in metals analysis, and in the vast majority of papers perused by this author, HNO_3 is the preferred agent used to clean labware, both glass and plastic. Labware used for aluminium analysis is no exception, in fact more so since alumino-silicates can be found in significant quantities in glass and aluminium catalysts are used in the production of polyethylene (Ericson 1992). Concentrations of HNO_3 utilised in 'cleaning' labware have ranged from 10% to concentrated (70%) mostly of AR grade. However, in his work on optimal analytical techniques for aluminium analysis of wines, McKinnon (1990) used EDTA to clean labware because nitric acid was quoted as being unsuitable for removing trace aluminium from plasticware. As this contradicts the majority of studies on aluminium trace analysis it was decided to conduct an analysis of the cleaning solutions and their aluminium leaching potential over time.

Two sets of plastic vials, used for holding GFAAS samples, were prewashed with detergent and were soaked in either a 10% nitric acid bath or a 2% m/v aqueous solution of EDTA similar to that used by McKinnon (1990) for at least 24 hrs. Duplicate analyses of both sets of vials were conducted where vials were removed from each bath, rinsed with Milli-Q® water several times, and

loaded with a blank solution of approximately 2.5% v/v of ultrapure nitric acid made up in Milli-Q® water. This test matrix was used because most of the solutions to be analysed in this study would consist of digests or solutions acidified with ultrapure concentrated nitric acid. The aluminium in these solutions was analysed by GFAAS the results plotted against time as shown in Figure 3.2.

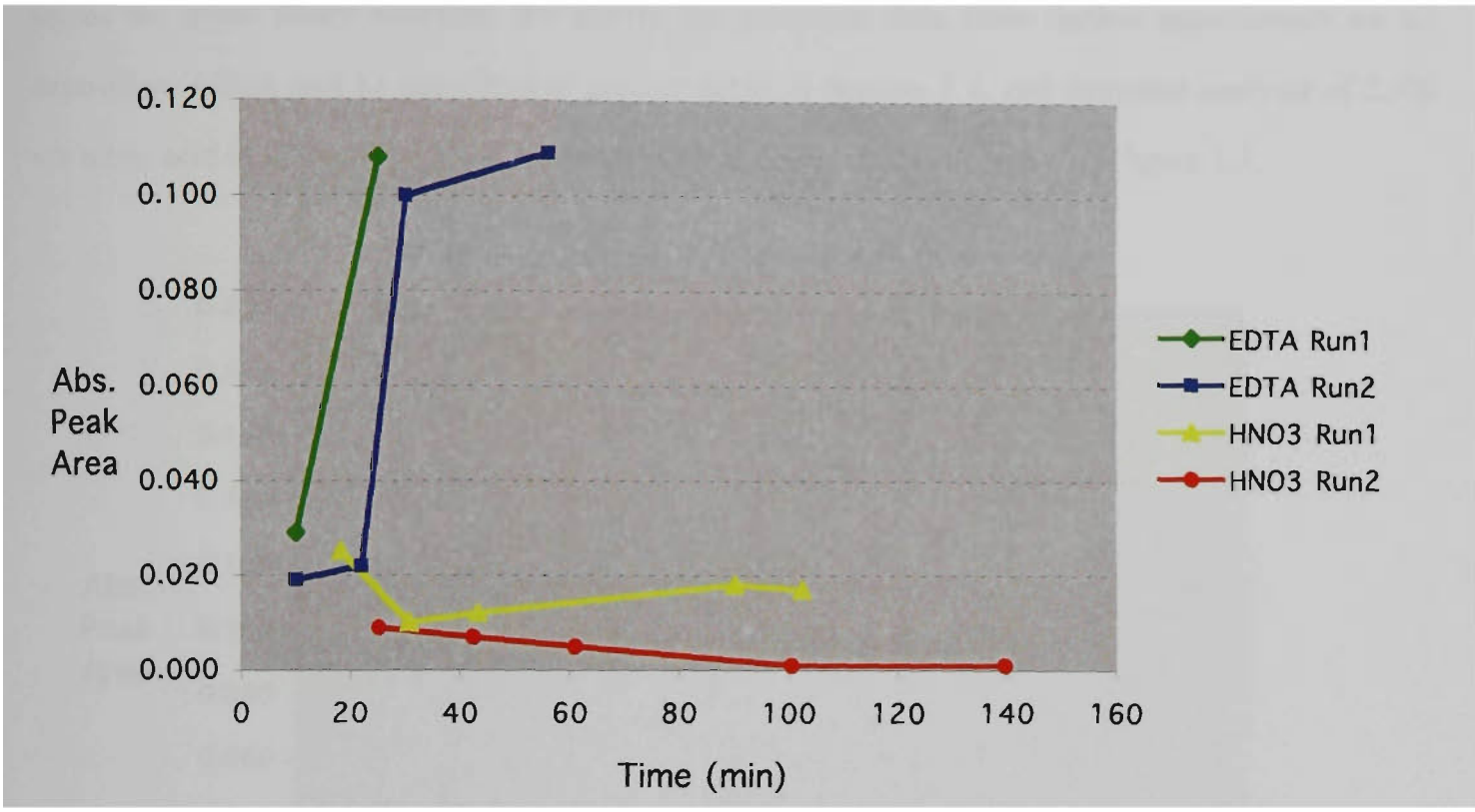


Figure 3.2 Temporal plot of aluminium absorbance (peak area) versus time showing the leaching of aluminium from plastic GFAAS vials 'cleaned' with 2.0% EDTA or 10% nitric acid.

From Figure 3.2, it can be seen that a dramatic increase in aluminium leaching occurs in the first 10-20 minutes for the vials soaked in EDTA. In contrast, the vials soaked in 10% nitric acid showed a static or decreasing aluminium concentration over a greater period of time. These trends were repeated for both sets of vials showing that it is not an instrumental artefact. One or both of the following can explain the sudden rise in aluminium concentration for EDTA soaked vials. One is that extraneous aluminium is depositing from the atmosphere into the vials. It is well known that air contains small aluminium rich particulates that can contaminate samples (Frech & Cedergren 1992; Hamilton 1995). The other is that aluminium is being leached from the vials; this suggests that the EDTA bath has not effectively removed acid exchangeable aluminium from the walls of the plastic vials. Similar initial aluminium concentrations for both sets of vials make solution contamination unlikely.

Contamination from the atmosphere is a random occurrence that is not dependent on the bath used to decontaminate the vials. The repeatability of the trends for both sets of vials suggests that air deposition is not the cause for increasing aluminium content, as these trends are repeatable and not randomly distributed between vials washed by nitric acid or EDTA. Hence, the second possibility seems the more likely scenario. To clarify the situation, data from further experiments on air deposition, which will be described in greater detail in Section 3.4, and temporal analysis of 2.5% v/v nitric acid in untreated vials is superimposed on Figure 3.1 and shown as Figure 3.3.

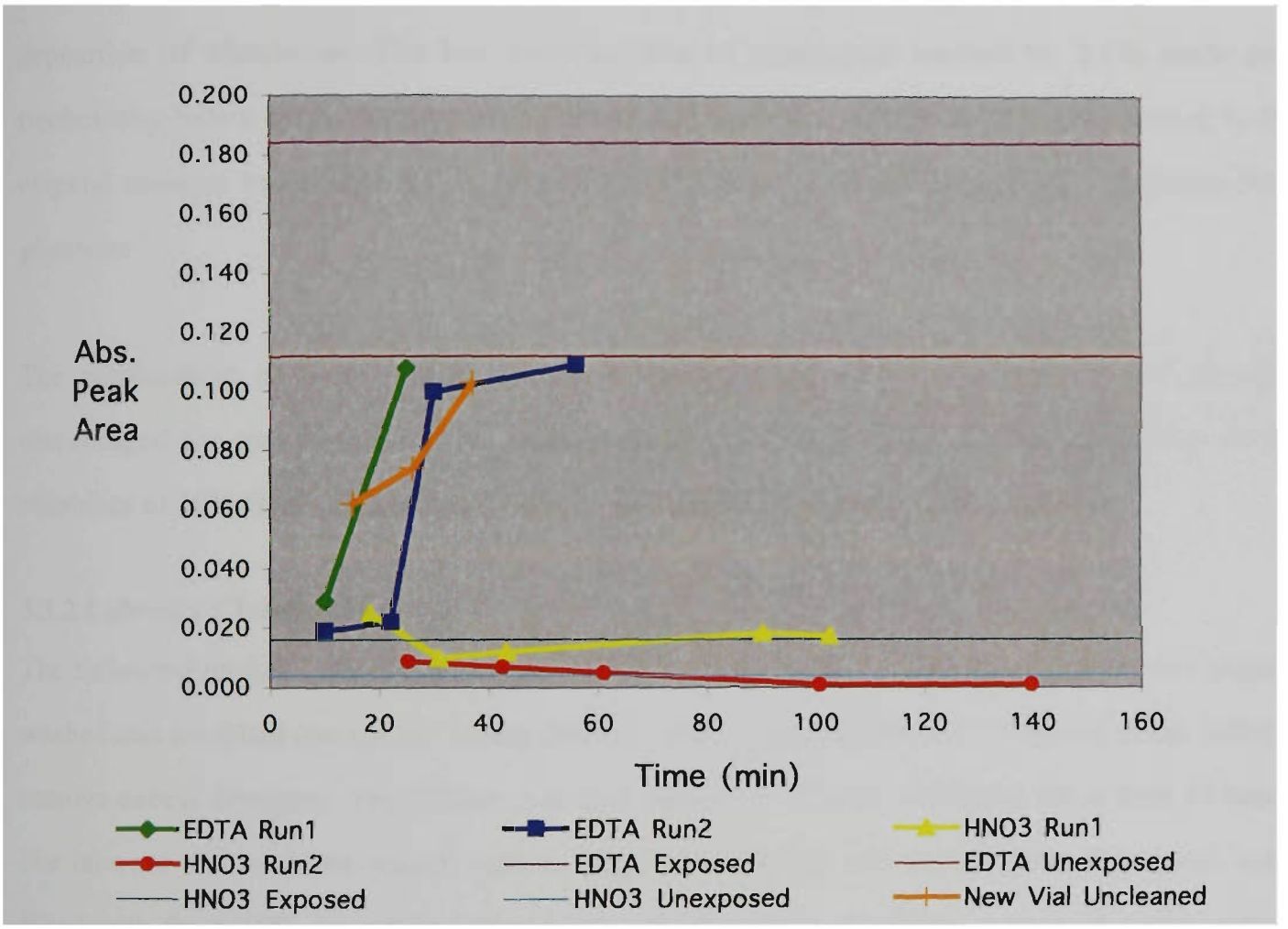


Figure 3.3 As Figure 3.2 but including superimposed temporal data from Section 3.4. This data consists of 2.5% v/v nitric acid solutions in unexposed and 2-day air exposed vials (both EDTA and 10% nitric acid soaked) and a new untreated vial.

The results show that EDTA cleaned vials left to sit for 2 days exposed to the lab atmosphere give a significantly higher aluminium content than unexposed nitric acid cleaned vials. This demonstrates that EDTA has not completely removed the acid exchangeable aluminium. There is also a strong similarity between the aluminium content seen in the final data points of the temporal leaching study and the aluminium content of the acid solution in the vials sitting unexposed to air for 2 days.

The temporal profile of a new untreated vial shows a remarkable similarity to the profile of EDTA cleaned vials.

The correlation of results of three sets of experiments confirms that treating the plastic vials in EDTA does not completely remove acid exchangeable Al. Although the temporal plot of the second set of nitric acid cleaned vials shows higher aluminium leaching, the absorbance peak areas observed are similar to 2 day air exposed vials suggesting that set of vials suffered from air deposition of aluminium. The low concentration of aluminium leached by 2.5% nitric acid (technically below accepted quantitation limits and probably indicative of the aluminium in the original solution itself) shows that 10% HNO_3 will remove acid exchangeable aluminium from glassware.

The combination of these results and the widespread use of HNO_3 in trace metal research, discouraged the use of the EDTA cleaning protocol of McKinnon (1990), and confirmed the reliability of 10% HNO_3 as a pre-treatment for all labware, including plastic and glass.

3.3.2 Labware Cleaning Protocol

The following protocol was adhered to for all labware, both glass and plastic. Labware was initially washed and scrubbed (except for Teflon labware) with detergent followed by rinsing in tap water to remove excess detergent. The labware was then soaked in 10% v/v nitric acid for at least 24 hours. For labware that could be sealed, such as sample bottles and volumetric flasks, the vessels were filled with fresh 10% v/v nitric acid, sealed and allowed to stand for at least 24 hours or until required. After the prescribed period the HNO_3 cleaning solution was drained off and the labware rinsed with Milli-Q® water 3-4 times. Once rinsed, immediately all vessels with lids were sealed and not opened until required. Open containers, e.g. beakers, were placed upside down on a clean bench to minimise the air exposure to the inside of the container.

3.4 Air Contamination

Extraneous deposition from air can cause significant contamination of samples (Tsalev 1984; Frech & Cedergren 1992, Hamilton 1995), particularly for trace aluminium analysis. Typical air fallout

concentrations are given in Table 3.1. As previously described in Sub-Section 3.3.1, a study of possible contamination by aluminium air deposition was conducted in tandem with the evaluation of the cleaning baths.

Table 3.1 Typical air fall-out of aluminium (from Frech & Cedergren 1992).

Location	$\mu g/m^2/day$
Outdoors	179.0
Analytical Lab	8.2
Clean- Air lab	0.9

Two duplicate sets of GFAAS vials were cleaned using the protocol in Sub-Section 3.3.2, half in EDTA and the other half in HNO_3. For both the EDTA and nitric acid cleaned vials, a 2.5% v/v HNO_3 solution was decanted into the vials and half were exposed to the lab atmosphere while the other half were isolated from the lab air. These solutions stood for two days to mimic the usual sample preparation and analysis period. The aluminium concentration of these solutions is given in Figure 3.4.

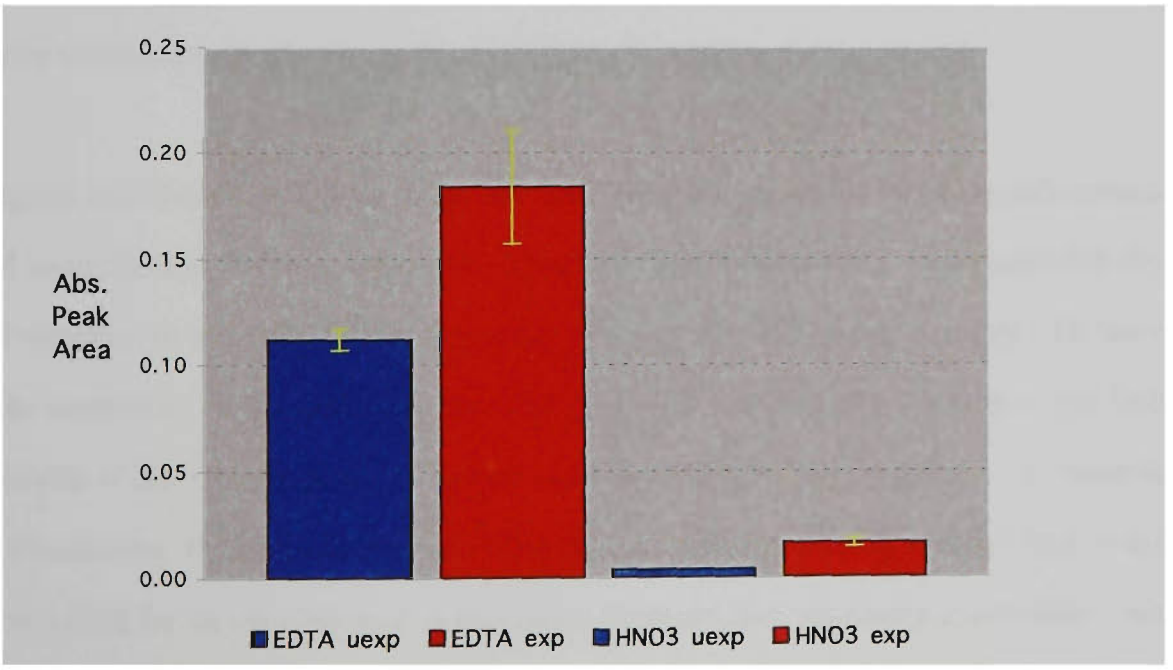


Figure 3.4 Mean absorbance peak areas for air exposed(red) and unexposed(blue) 2.5% v/v HNO_3 solutions in plastic vials.

As can be seen from the plot above, significantly higher aluminium concentrations were observed for solutions exposed to air compared with those shielded from the atmosphere. It is apparent that

solutions in vials using both cleaning methods show the same trend when they are exposed to lab atmosphere demonstrating that there is potential for contamination from lab air where this work will be undertaken.

To combat contamination from laboratory air, precautions were put in place throughout the project. The primary method of reducing air contamination is to reduce the time that samples are exposed to air (Frech & Cedergren 1992; Ericson 1992). This was achieved in the current work by minimising the number and duration of sample handling steps.

3.5 Sampling

3.5.1 Overall Sampling Considerations

The number of samples required for statistical validity and the broad range of sample types required for this project meant a large number of samples had to be collected. Due to the ubiquitous nature of aluminium contamination, all samples had to be collected according to a strict protocol and because there are various types of samples, different protocols were required for different sample types. To ensure adherence to these protocols, the author collected all samples in this project.

A sampling regime and choice of area to undertake field sampling required some considerations. As the author had to undertake frequent trips over a period of several years it was preferable for the vineyards and wineries to be close to the university campus in Melbourne, Victoria. To maintain control over the continuity of the samples from the start of the production process to the finish it was also preferable if the vineyards and wineries were part of the same business, i.e. wineries of modest size processing their own grapes. This last condition would ensure that constant communication could be developed and maintained between the vigneron/winemaker and the author, allowing tighter sampling control and an understanding of individual winemaking production steps crucial to the correct timing of sampling to accurately reflect the processes involved in winemaking.

Consequently a compromise had to be reached between the above-mentioned considerations and satisfactory sample size and coverage. Initially five vineyards/wineries were chosen and agreed to

participate in this study. A confidentiality agreement was entered into between the university and the vineyards/wineries so that their names and locations could not be divulged. The wineries are located to the north and northwest of Melbourne in the Macedon Ranges region which is a cool climate wine region. As the production processes and potential sources of aluminium input differ between red and white wines (McKinnon 1990; McKinnon et al. 1992), it was decided that one white and one red wine variety would be studied from each vineyard/winery. As wine variety depended on the grape cultivars grown at the vineyards, Chardonnay was the predominant white variety examined while Pinot Noir was the main red variety studied. The samples were to be collected for the two vintages of 1997 and 1998. As some varieties, particularly red cultivars take more than two years to go from grape to bottled wine, some final samples from the 1998 vintage were not collected due to time constraints. Samples were collected from the initial five wineries for the 1997 vintage. However for the 1998 vintage this was reduced to three. This was because one winery had blended product with grapes from other unknown sources, rendering the aluminium profile inaccurate; communication difficulties encountered at the other winery compromised sampling schedules, and ultimately the aluminium profile analysis of its wines, beyond redemption.

Due to the confidentiality agreement all samples were coded to indicate location, variety and time of processing/sample collection. Each sample from soil to bottled wine was individually coded upon collection and the amount, nature, type and date of sample were recorded in a logbook per individual wine. The exact relation of the coding to the sample will be explained further in Chapter 4. Sampling protocols were developed, adopted or adapted from established methods which will be referenced where applicable within the methodology described in this section. It must also be mentioned at this point that between trips to different vineyards, all sampling collection utensils and in particular shoes were thoroughly scrubbed and washed with detergent and Milli-Q® water to reduce any potential for spreading *phylloxera* from one vineyard to another (Jackson & Schuster 1997). *Phylloxera* is an aphid-like pest that attacks the roots of non-resistant vine cultivars that can severely damage wine grape vines.

3.5.2 Soil Sampling

Soils were sampled from all five participating vineyards/wineries in 1997. Field sampling of soils was carried out in a systematic way to provide a surface survey of the soil. For each white and red cultivar at each vineyard the area of the vines for each cultivar was mapped out and five sites were chosen based on a zigzag pattern commonly used in soil sampling practices (Rubio & Ure 1993; Ure 1995). Five replicate samples for each vine area were chosen as this is considered the minimum number required for statistical validity (Grimshaw 1989) and further sample collection was limited by logistical concerns. This pattern differed from cultivar to cultivar per vineyard as each group of vines (set up in rows) were distributed differently in a given space. In some instances where the vines were placed in 2 or 3 separate plots, the 5 sampling sites were distributed over these plots where the number of sample sites per plot were proportional to the relative size of the plot. Site selection ensured that no two samples were taken from the same vine row twice or taken on the same line perpendicular to the vine rows. In addition, sites at the ends of rows or adjacent to unhealthy vines were also avoided (Zoecklein et al. 1990). The sampling site maps for each grape/wine set can be seen in Appendix 1. Collection after or during heavy rain was avoided (EPA Victoria 1993) and all the soil samples were collected in the week prior to vintage (grape picking) for each particular grape/wine set during April and May of 1997.

At each sampling site a stainless steel twist auger (Grimshaw 1989; Rubio & Ure 1993; Fiedler et al. 1994; Ure 1995) was used to take a cylindrical sub-sample of soil approximately 4cm diameter by 10-15cm deep (Grimshaw 1989; McGrath 1996) from four evenly spaced areas around the vine row shown in Figures 3.5 and 3.6. This depth profile was chosen because the grapevines derive most of their nutrients and water from the topsoil (Jackson & Schuster 1997). These four samples were taken 30cm from the vine row and 1 metre away from each other with each sampling point the corner of a parallelogram (see Figure 3.6). During sampling, vegetation and other non-soil material remained with the soil to preserve the integrity of the sample. Each sub-sample was approximately 250g and a composite ~1kg sample (EPA Victoria 1993; Quevauviller et al. 1993a; Ure 1995) was created from the four sub-samples and stored in a ziplock polyethylene bag away from the light until pre-treatment and analysis (Grimshaw 1989; EPA Victoria 1993; Quevauviller et al. 1993a; Ure & Rubio 1993; Feidler et al. 1994; Ure 1995). All equipment used throughout the soil sampling

process was thoroughly washed with Milli-Q® water between each grape cultivars sample collection to avoid cross-contamination (EPA Victoria 1993).

Figure 3.5 Photograph of the author showing use of stainless steel auger and sampling position relative to vine rows. Polyethylene sample bags appear at the author's feet in the foreground.

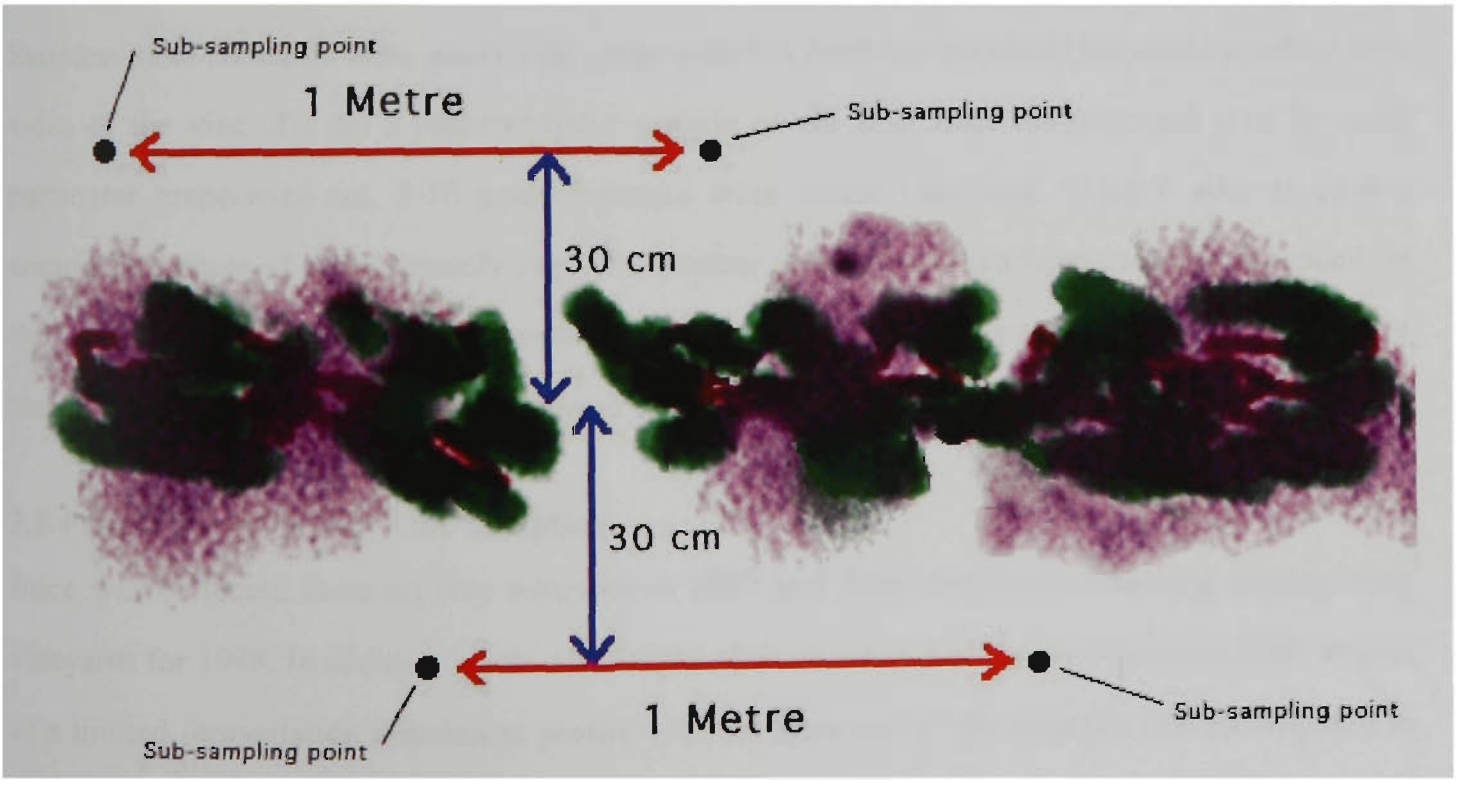


Figure 3.6 Overhead schematic diagram showing the position of sub-sampling soil cores relative to the vine row per sampling site.

3.5.3 Grape Sampling

Sampling of grapes was performed concurrently with the soil sampling in 1997 within a week of vintage. Grapes were collected from vines at exactly the same sites as those used for the soil samples. As soil samples were not collected for the 1998 vintage, the site positions were accurately recorded and a tag was placed on the appropriate vines so that sampling was performed on the same vines as those used in 1997. Grape samples were collected for all five vineyards in 1997 whereas in 1998 grapes were collected from the remaining three vineyards chosen to continue the profiling for the second season. The maps giving sampling site location information for the soil samples shown in the Appendix also represent the sampling site locations where grape samples were taken.

Grape sampling techniques were adapted from sampling suggestions by Zoecklein et al. (1990) who advocate strict berry sampling protocols as grape composition differs with location, within the grape cluster, cluster location on the vine and location of the vine in the vineyard. As mentioned in Sub-Section 3.5.2, sampling sites for soil and hence grapes were chosen where sampling of fruit from the ends of rows and unhealthy vines could be avoided.

Stainless steel secateurs were used to cut grape bunches from the middle of the vine including both sides of the vine. To get a representative sample of the fruit from the vineyard area for each particular grape/wine set, 5-10 grape bunches were taken from each of the 5 sites to give a composite sample of approximately 3kg. The number of bunches taken from each site depended on the bunch and grape size of the particular cultivars sampled. Once cut from the vine, each grape bunch was placed in a plastic bag and stored at ~4 C until homogenisation could be undertaken.

3.5.4 Juice, Ferment and Wine Sampling

Juice was collected from all five wineries in 1997 and from the three remaining participating vineyards for 1998. In addition, a one-off sample of chardonnay juice was collected in 2001 as part of a limited fermentation aluminium profile analysis from one of the wineries that participated in both the 1997 and 1998 sampling regimes. Juice fresh from crushed grapes was collected by one of two methods depending on the vineyard and the equipment available. Where available a Milli-Q® washed stainless steel fife or pail was lowered into either a vat containing the juice or the crusher itself to retrieve a sample and was decanted into a plastic acid washed bottle. Where this equipment

was not available at the winery then a plastic acid washed bottle was immersed into the juice to take a sample.

Ferment and unfinished wine were sampled from four of the five initial wineries in 1997; sampling of the fifth was cancelled due to blending of grape juice from a vineyard outside the control of this study. Ferment and unfinished wine were collected from the three remaining participating wineries in 1998. A one-off sample of Chardonnay ferment was procured in 2001 from one of the wineries participating in both seasons (1997-1998) and was used in an analysis of aluminium sources and sinks during the fermentation process. Ferment and unbottled wine was similarly sampled as per juice, either by appropriately washed stainless steel fife or pail followed by decanting into a polyethylene bottle (see Figure 3.7), or by immersion of the bottle into the liquid to be sampled. In the case of barrel ferment/wine the target sample could only be collected either by washed stainless steel fife or plastic siphon tube followed by decanting into an acid washed polyethylene bottle (see Figure 3.8).

For all samples of juice, ferment and unbottled wine, 500ml to 1 litre of sample was collected into the polyethylene bottles followed by storing the samples in a freezer at or below 0 C until pre-treatment and analysis. Bottled wine was collected by acquiring a bottle of finished wine from the wine maker. All bottled wine samples were collected from four of the five wineries in 1997 the fifth not sampled due to previously mentioned blending problems. As with juice, ferment and unfinished wine, bottled wine samples from the remaining three participating wineries were collected in 1998. However, as the red wines can sit in barrel after final production to age for at least 1 year, the collection of samples of bottled red wines from the 1998 vintage is incomplete. The bottled wine samples were stored at ambient temperature in a cupboard away from the light until pre-treatment and analysis. Ferment and unfinished wine samples were collected for all five wineries in the first season and from three wineries in the second season.

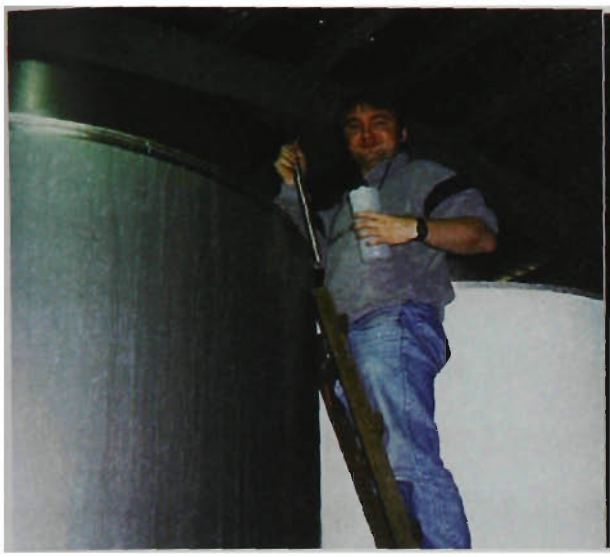


Figure 3.7 Sampling ferment from tank using washed stainless steel fife to decant into clean polyethylene bottle.

Figure 3.8 Sampling unfinished wine from barrel using washed stainless steel fife and collecting into clean polyethylene bottle.

3.5.5 Lees Sampling

The collection of a single sample of lees was conducted at one winery in 2001 by syphoning the lees from the bottom of a barrel after ferment utilising a washed plastic siphon hose. The lees were syphoned into an acid washed polyethylene bottle and stored at or below 0 C in a freezer until pre-treatment and analysis.

3.6 Sample Pre-Treatment for Total Aluminium Analysis

This section covers all the procedures concerning the conversion of the raw sample into a form that is amenable to instrumental analysis. As the sample type varies significantly from soil to fruit to liquid, each sample type will be dealt with separately as in the sampling section. As mentioned earlier, in developing the pre-treatment of samples, it was always kept in mind that the most direct route from sample to analysis was the preferred option. By keeping sample exposure to air and reagents to a minimum and by reducing sample handling steps, the probability of significant sample contamination was minimised as much as possible.

3.6.1 Soil Sample Pre-Treatment

3.6.1.1 Soil pre-treatment considerations

Many methods have been used for extraction of metals from soils and sediments, however it is only in the last decade that general protocols have been standardised (Ure et al. 1993; Quevauviller et al. 1995). Digestions using strong mineral acids have been used extensively (Grimshaw 1989; Ure

1990; EPA Victoria 1993). However, analysis of the resultant solution only provides the total aluminium concentration in the soil rather than that which is available to plants. To determine bioavailable metal in soil, both sequential and single extraction techniques have been developed. Tessier et al. (1979) developed a 5-step sequential extraction technique using progressively more aggressive extractants. Recently this has been modified to 3 steps; 1) dilute acetic acid, 2) hydroxylamine hydrochloride and 3) hydrogen peroxide, however these methods have been mainly applied to sediment analysis (Davidson et al. 1993; Fiedler 1994; Quevauviller et al. 1994). Soil extraction has generally been accomplished with single step extraction procedures of which the most common extractants have been KCl, $CaCl_2$, DPTA, EDTA and acetic acid/ammonium acetate (Lindsay & Norvell 1978; Clayton & Tiller 1979; Ure 1990; EPA 1993; Ure et al. 1993; Quevauviller et al. 1993a, 1995, 1996; Van Raij et al. 1994; Houba et al. 1996; Bosnak & Grosser 1996; McGrath 1996). The latter two methods were adopted for certification of soil material for the EEC (now European Union) Community Bureau of Reference (BCR) due to their good reproducibility (Ure et al. 1993; Quevauviller 1996).

For this current study, extraction of the bioavailable fraction was desired, hence a BCR standard method for bioavailable soil extraction was to be adopted. Preliminary work utilised EDTA due to higher extracted metal concentrations (Ure et al. 1993), however it was subsequently considered that EDTA would be too harsh an extractant, possibly removing aluminium that is more strongly bound in the soil. Hence, it was decided that an adaptation of the milder acetic acid/ammonium acetate extraction proposed by the EEC BCR would be used for this project.

3.6.1.2 Decontamination of 0.43M acetic acid extraction solution

Prior to use in extracting bioavailable aluminium from the soil samples, 50g of Chelex 100 was added to a 1-litre solution of 0.43M acetic acid and stirred for 2 days. A two-fold Milli-Q® water diluted sample of this solution was then analysed using GFAAS with a program described in Sub-Section 3.7.1.3 against a 30µg/L aluminium standard. The results of the solution compared with Milli-Q® water are shown in Table 3.2.

Table 3.2 Aluminium content of Chelex-100 treated 0.43M acetic acid compared with Milli-Q® water.

Sample	Al Abs. Peak Area
Milli-Q® water	0.000*
Chelex 100 treated 0.43M Acetic Acid	0.005

*** This solution is used as the reference blank and hence its absorbance peak area is set to 0.000.**

Table 3.2 shows that there is little difference between the aluminium absorbance peak area of the acetic acid solution and Milli-Q® water, indeed the aluminium content of the acetic acid solution is at the very limit of GFAAS detection. Considering that the concentration of aluminium in the soil extracts were expected to be 1000 times greater than the concentration seen in the blank solutions it was concluded that Chelex-100 successfully removed aluminium from the acetic acid extraction solution to the point where it was insignificant relative to the aluminium content of the soils. This solution was used for aluminium extraction for all soil samples in this study.

3.6.1.3 Pre-treatment for soil pH measurement

Before soil extraction work began, the pH of the soil was measured for later correlation analysis with acetic acid extractable aluminium content. The pH method used was adapted from Australian Standard AS 1289.3.4.1 (Standards Australia 1997). The following procedure describes the method for a site soil sample for one grape/wine set. A sub-sample of soil was produced by shaking the soil in its storage bag and pouring approximately 60g onto a Milli-Q® washed Teflon mat. Stainless steel tweezers were used to remove root and rock matter. The remaining field moist soil was then sieved using a Milli-Q® water washed stainless steel sieve of 2mm mesh size. The sieved material was manually coned and quartered on the Teflon mat with a flat rectangular piece of washed polyethylene of approximate dimensions of 5cm by 10cm. In a 250ml beaker, 75ml of Milli-Q® water was added to a 30g sub-sample of sieved soil, and the resultant suspension was stirred vigorously for five minutes. When stirring was complete, the beaker was covered with a clean watchglass and the solution was left to stand for 1-2 hours. The ensuing pH analysis is described in Sub-Section 3.7.3.1.

3.6.1.4 Bioavailable soil aluminium extraction protocol

Although it is general practice to remove moisture prior to homogenisation by drying in an oven or at 20-40 C on plastic trays in open lab air (Tessier et al. 1979; Davidson et al. 1994; Szpunar et al. 2000), it was decided that the soil sample would not be dried before analysis in order to preserve the soil sample structural and chemical integrity in the soil. However, the field moisture content was measured on a separate sub-sample of soil. This was accomplished by drying the soil in an oven at 110 C to constant mass (Grimshaw 1989).

Soil from the five sites that make up the samples for each grape/wine set were shaken in their respective sample storage bags (EPA Victoria 1993) and approximately 100g of field moist soil was poured onto a washed Teflon mat. Each of the soil samples were cone and quartered (Grimshaw 1989) in the same manner as for the pH study, where a quarter of each of the 5 samples was mixed together and spread out on the mat whereupon vegetation, sod, rocks, roots etc were removed from the soil (EPA 1993) with stainless steel tweezers. The soil was then homogenised by crushing in a mortar and pestle until finely ground and passed through a 2mm pre-washed stainless steel sieve (Grimshaw 1989; Quevauviller et al. 1993a; Rubio & Ure 1993; Ure et al. 1993; EPA Victoria 1993; Fiedler 1994; Davidson et al. 1994; Ure 1995; McGrath 1996; Szpunar et al. 2000).

The homogenised soil was further sub-sampled using the cone and quartering method to give triplicate ~5g composite sub-samples. Each sample was accurately weighed in an acid cleaned polyethylene container and 40.00ml of 0.43M acetic acid (Quevauviller 1996) was added. The samples were agitated using a rotary shaker at 60rpm for 16 hours. After shaking, the suspension was left to settle for several minutes and the extract was decanted and centrifuged at 4250rpm for 10 minutes. To remove small wood particles and other floating debris, the centrifuged extract was filtered through cotton wool and some of this filtrate was decanted into a 25ml volumetric flask until the flask was filled to the mark. The analysis of this solution is discussed in Sub-Section 3.7.2. To account for any contamination from the extraction process including the cotton wool filtering and labware, duplicate reagent/procedural blanks of the 0.43M acetic acid solution underwent the same procedure as the samples.

3.6.2 Grape & Juice Sample Pre-Treatment

Although as samples they are very different, both grape and juice samples were treated in a similar manner. The only difference between the two methods was that the grapes required a more aggressive homogenisation procedure. Homogenisation is required to derive a representative sample of solid plant and food matter (Grimshaw 1989; Markert 1995; Sutton & Heitkemper 2000) and, once this has occurred, the material has to be converted into a form amenable for instrumental analysis. For plant and particularly food material concentrated mineral acid digestion has been the most common method used. As discussed later in Sub-Section 3.7.1.2, the inability of GFAAS to directly determine aluminium in grape juice due to its sugar content meant that the juice, like the grape matter, also required an acid digestion procedure to decompose the organic matter and sugars. The digestion procedure required extensive method development which will be described in this Sub-Section along with the methods employed for individual grape dissection.

3.6.2.1 Acid digestion development

In this Sub-Section it will become apparent that the majority of the work in developing the digestion procedures dealt with the problems of extraneous aluminium contamination and incomplete dissolution. In the case of total metal analysis in plant and food material, these matrices require complete dissolution into a liquid matrix so that the sample can be analysed by most instrumental techniques. This can be accomplished by oxidation of organic matter usually by 'wet digestion techniques' involving concentrated mineral acids. The oxidants and methods used can vary, in many cases a mixture of mineral acids are used with combinations of nitric, perchloric and sulfuric acids and sometimes hydrogen peroxide can also be included (Grimshaw 1989; Novozamsky et al. 1995). Due to risks of explosion, digestions involving perchloric acid and hydrogen peroxide are treated with caution (Grimshaw 1989). Delves et al. (1988) suggested that simple oxidation of foodstuffs with HNO_3 is sufficient to produce clear solutions with little matrix difficulties. Tyler (1994) used HNO_3 digestion for aluminium analysis of a range of plant materials. Novozamksy et al. (1995) states that HNO_3 is the only acid which can be used alone, its advantages are its high purity, solubility of nitrates and oxidising power over a range of temperatures.

For the above reasons it was decided to begin trials of digestions of whole oven-dried grapes and juice using HNO_3 as the only oxidant. Initially AR grade acid was utilised, with 5ml concentrated

HNO_3 added to approximately 0.5g of grape or 2ml of juice in a 50ml conical flask and heated over a period of 1-2 days on a hot plate at a medium heat setting. After 2 days it was observed that the grape did not totally decompose and after repeated experiments with increasing acid content it was realised that a larger amount of acid and a longer time would be required to completely digest a grape. As this was considered too inefficient for a large number of samples, a homogenisation procedure was introduced where maceration of the grapes by blending was conducted followed by rotary shaking before the acid digestion step.

An initial GFAAS examination of digestions of blanks, juices and grapes showed that the absorbance peak area for the reagent/procedural blank was as high as that for the samples, as can be seen in Figures 3.9 and 3.10, suggesting extremely high aluminium contamination. It was thought that as the acid was the major component in the system, other than the sample, it could be the source of this contamination. Hence digestions were repeated using ultrapure grade Merck Suprapur™ nitric acid (S HNO_3) in the same amounts as previously used with AR grade acid. The GFAAS aluminium absorbance peak area signals of the reagent/procedural blank and a homogenised grape sample are shown in Figures 3.11 and 3.12. Comparing Figures 3.11 and 3.12 with 3.9 and 3.10 it can be seen that the digests of blanks and samples from the same source but performed with Suprapur™ nitric acid give a lower aluminium absorbance peak area than those performed with AR nitric acid. In addition the quality of the atomisation with SuprapurWhile this suggested that the higher-grade acid gave less aluminium contamination, it did not account for all the contamination, which was still unacceptably high. However, for all future digestions Suprapur™ grade nitric acid was used.

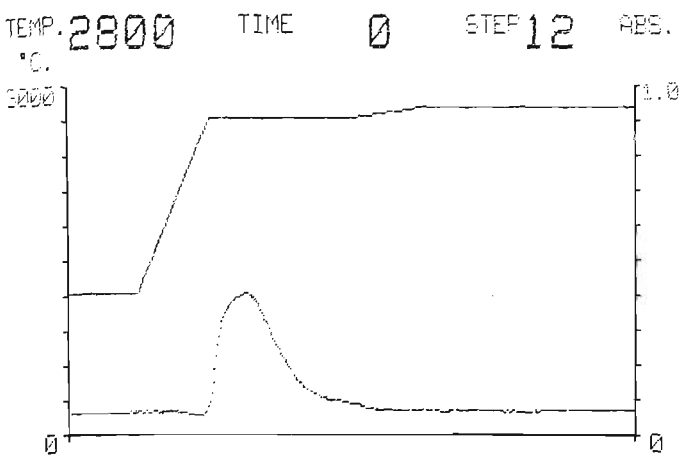


Figure 3.9 GFAAS absorbance signal for a blank digest of 5ml concentrated AR HNO_3 in a glass flask.

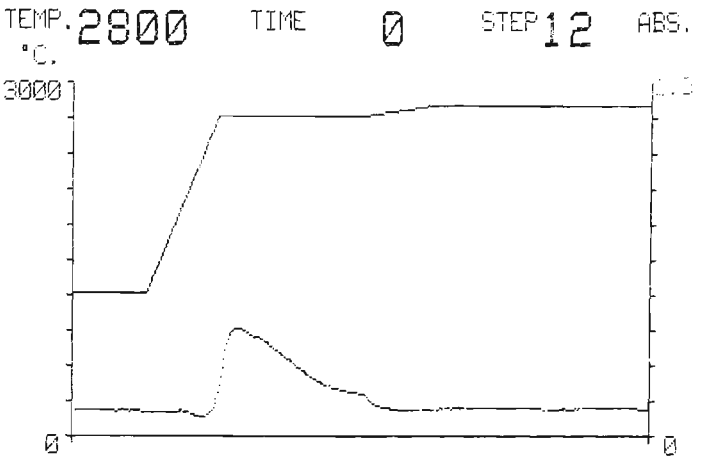


Figure 3.10 GFAAS absorbance signal for a ~0.5g grape digest in 5ml concentrated AR HNO_3 in a glass flask.

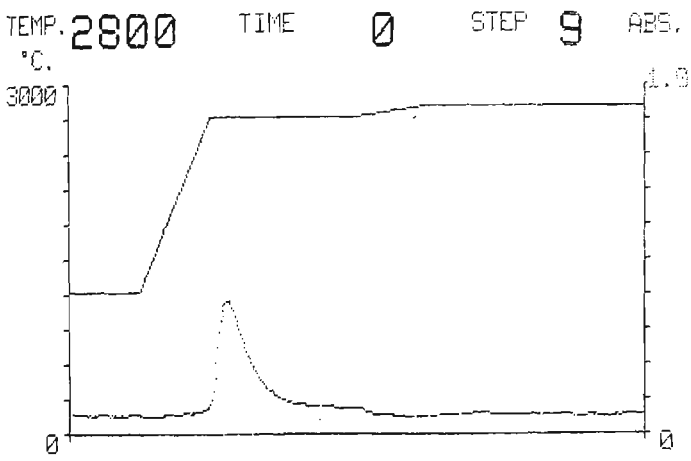


Figure 3.11 GFAAS absorbance signal for a blank digest of 5ml concentrated S HNO_3 in a glass flask.

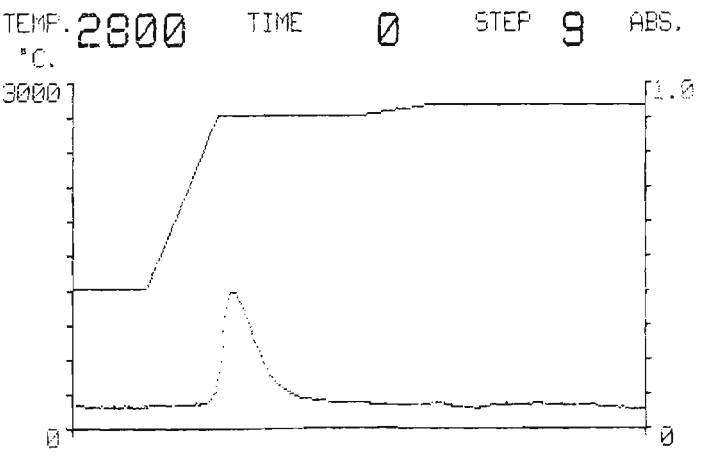


Figure 3.12 GFAAS absorbance signal for a ~0.5g grape digest in 5ml concentrated S HNO_3 in a glass flask.

Other than the acid used for digestion, there are only two ways aluminium contamination could be introduced during the digestion process;

1) contamination from the walls of the flask itself, or
2) air deposition of aluminium particles into the solution via the open neck of the digestion flask.

The latter form of contamination has been shown to occur for dilute HNO_3 solutions left exposed for several days (see Section 3.4) and will be discussed later in this section. The former possibility makes sense when one considers that glass contains alumino-silicates and some glasses, like those used for labware may contain some Al_2O_3 (McTigue et al. 1982). Although, as discussed in Section 3.3, the bathing of labware in 10% HNO_3 was shown to remove the majority of acid exchangeable

aluminium, the solution was dilute and at ambient temperature. However at higher temperatures and concentrations used for digestion, the reactivity and oxidising power is increased (Novozamsky et al. 1995), hence a more vigorous attack on the glassware might occur leaching more aluminium from the glass walls than at ambient temperature with 10% HNO_3. This second scenario appeared to be the more probable cause of the contamination seen in the developmental acid digests.

To combat contamination of this sort, different flasks were used to carry out digestion. Teflon is well known to resist adsorption and does not contain aluminium. Teflon flasks (50ml) were obtained, however because it was recommended by the manufacturer that these flasks should not be heated at temperatures greater than 200 C , they could not be heated directly on a hot plate. To overcome this, the flasks were partially immersed in an oil bath that did not exceed a temperature of 120 C. Due to the lower temperature of this digestion of the sample the amount of concentrated Suprapur™ HNO_3 was increased to 10ml. It was immediately apparent (Figure 3.13) when these digests were analysed by GFAAS that the aluminium concentration in the digestate was significantly lower for the blanks than those observed in Figures 3.9 and 3.11. As can be seen from Figures 3.13 and 3.14 the ratio of sample:blank signal was now high enough to have a measurable signal after accounting for aluminium content from the blank. This experiment confirmed that the glass digestion vessels were contributing aluminium to the final digestate solution.

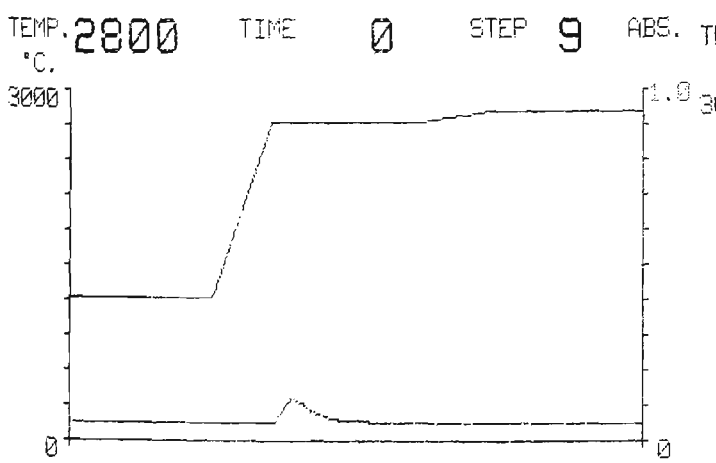


Figure 3.13 GFAAS absorbance signal for a blank digest of 10ml concentrated S HNO_3 in a Teflon flask.

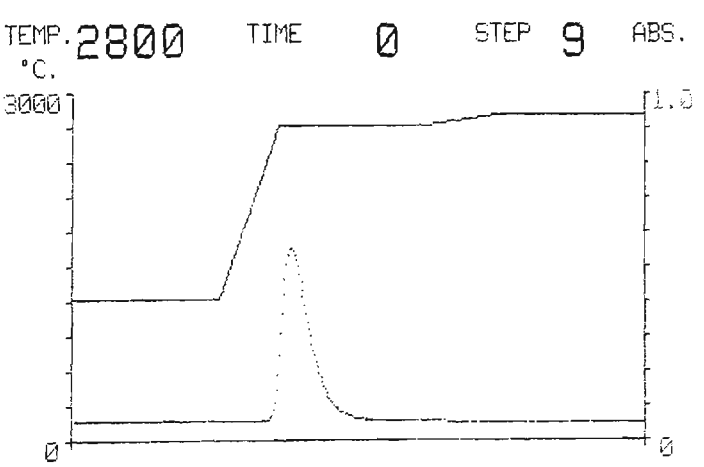


Figure 3.14 GFAAS absorbance signal for a ~0.5g grape digest in 10ml concentrated S HNO_3 in a Teflon flask.

Despite the success in reducing the aluminium contamination, it was observed by close inspection of digests that not all the sample was completely digested, with some transparent particulates

suspended in the digestate when cool. In addition, digestions in the Teflon flasks took much longer to complete, some lasting 2.5 days. To optimise the digestions, the use of different amounts of acid and parallel digests of samples and blanks in Teflon and glass were conducted. During these experiments, it was observed that the glass flasks were giving blank digest aluminium signals more closely resembling those seen in Teflon and contrary to the large signals shown in Figures 3.9 and 3.11. This suggested that as the glass flasks were being used for digestions, progressively less aluminium was being leached from the glassware. To test this hypothesis, a parallel study of five sequential blank digestions of 10ml of Suprapur™ HNO_3 in both glass and Teflon were conducted in duplicate. After each digestion the digestate was tested for aluminium concentration. The results of this experiment are shown in Figure 3.15.

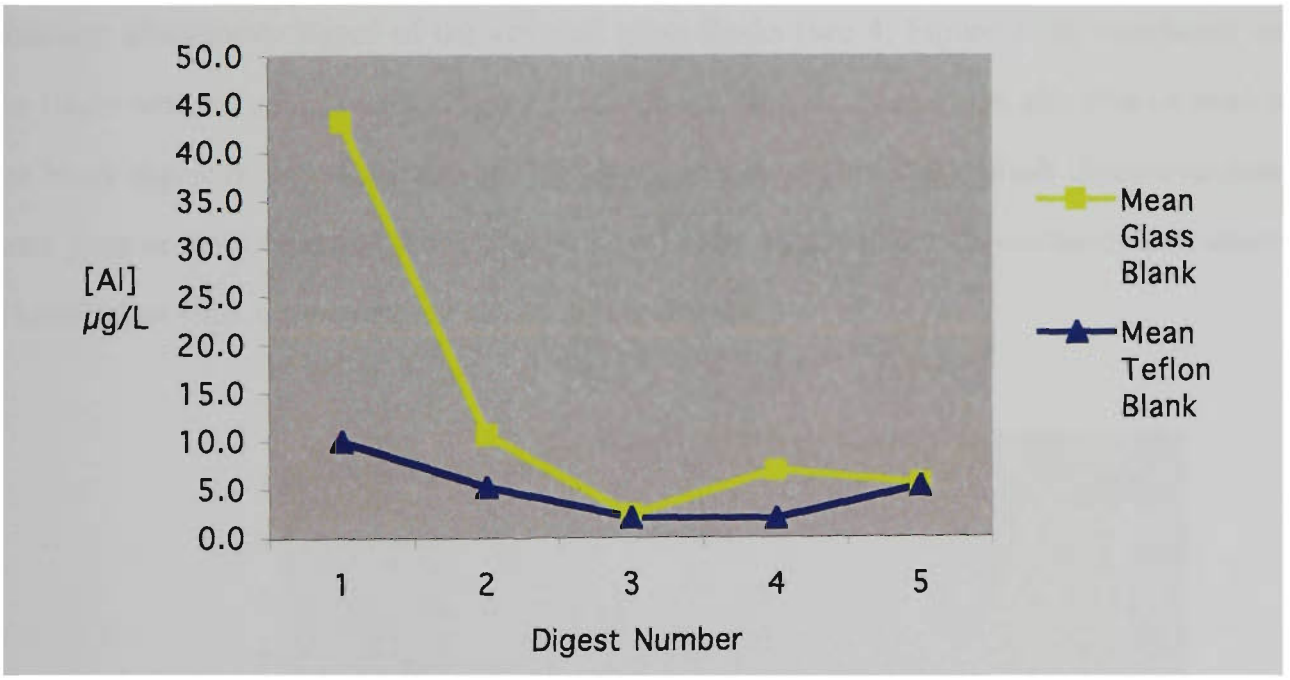


Figure 3.15 Trends in aluminium concentration of digest blanks from parallel studies of sequential digestions for both glass and Teflon flasks.

Figure 3.15 shows that after two digestions glass flasks show a similar aluminium blank concentration compared with that of Teflon flasks and from the third digest on behave similarly to Teflon flasks. This shows that if the glass flasks are pre-treated by undergoing several blank digestions prior to sample digestion the concentration of contamination from the glass should be not significantly greater than that derived from Teflon. From this point, all glass flasks used for digestion were pre-treated by running three blank digestions prior to actual digestions of samples.

The other possibility of contamination mentioned above is air deposition of particles containing aluminium. To examine this contamination another parallel study was conducted where two separate blank digests of 10ml Suprapur™ HNO_3 was carried out in triplicate in pre-treated glass flasks. One set of flasks had no cover over the flask opening and another set had a custom made blank glass 'bauble' covering the top of the opening. These 'baubles' worked by covering the opening and letting the vapour pressure of the digestion solution momentarily lift the bauble slightly to relieve pressure thereby minimising the chance of fallout of aluminium containing particles into the digestion solution. After the digestions the aluminium signal of each digestate solution was determined using GFAAS.

On analysing the digestates it was found that there was no significant difference between the aluminium absorbance signal of the covered glass flasks (see 4, Figure 3.16) compared with the glass flasks with no cover (see 5, Figure 3.16). In addition the aluminium absorbance peak area of these blank digestion solutions was no higher than those observed for blank digestions using pre-treated glass or Teflon flasks (see 3, Figure 3.16). This suggests that the contamination observed in the initial glass flask digests are not due to air deposition.

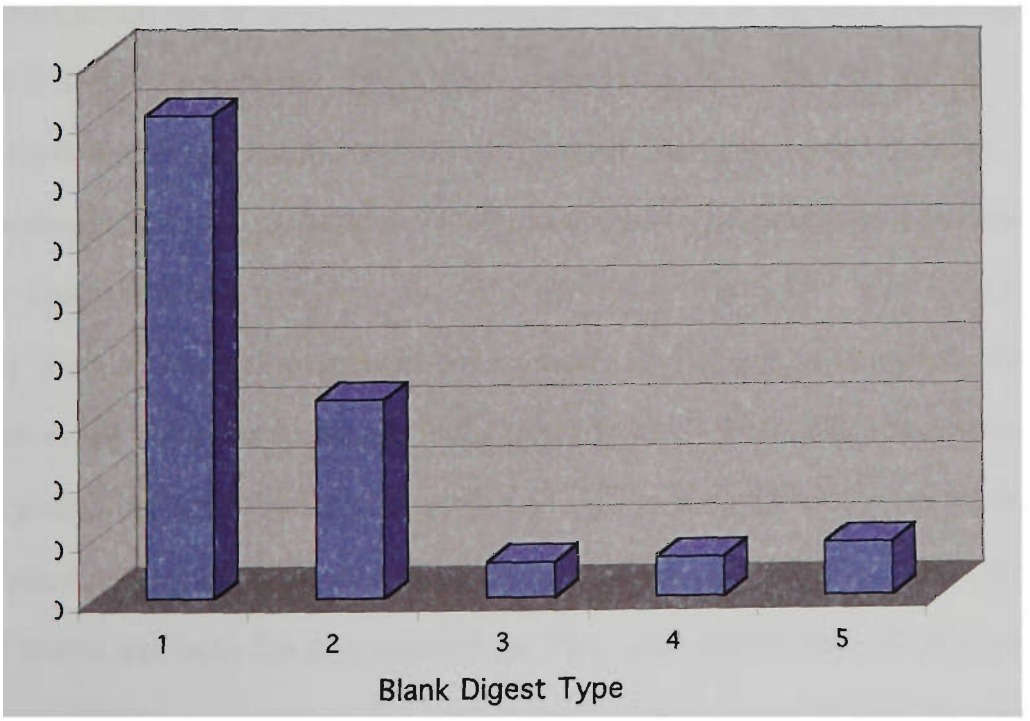


Figure 3.16 Aluminium absorbance peak area signals of various digest blanks: (1) AR HNO_3 in untreated new glass flask, (2) Suprapur™ HNO_3 in an untreated glass flask, (3) Suprapur™ HNO_3 in a Teflon flask, (4) Suprapur™ HNO_3 in a pre-treated covered glass flask, (5) Suprapur™ HNO_3 in a pre-treated uncovered glass flask.

From these contamination studies it was concluded that the aluminium contamination observed for the initial glass flask acid digests was due to the grade of acid and aluminium leaching from the flask walls and not from air deposition. Although the aluminium contamination could now be controlled, the digestion process was still unable to completely dissolve the entire sample within a reasonable time frame. Fine suspended particulates remained in the solution after digestion, particularly when the solution had cooled or was diluted for analysis. Mixtures of 10ml Suprapur™ HNO_3 with 2ml AR $HClO_4$ were trialled for digestion. However, as there was no appreciable improvement in the dissolution capacity and there are explosion risks involved with using perchloric acid (Grimshaw 1989, Novozamsky et al. 1995), further work involving acid mixtures containing perchloric acid were discontinued. Digestions with various mixtures of HNO_3, H_2SO_4 and H_2O_2 have been cited in the literature (Novozamsky et al. 1995, Sun et al. 1997; Barnes 1997) and were also investigated in this study. However, like the mixtures involving perchloric acid, complete dissolution of a grape sample was variable using these oxidizing agents, even though it was found that contamination was not introduced by these agents.

A review of digestion procedures up to this point revealed limitations in the methods. Teflon flasks with Suprapur™ HNO_3 could not be used as direct heating could not be applied, resulting in longer digestions that took 2-3 days to complete. In addition, dissolution of the sample was less complete than for any other method/labware used. Digestion in glass flasks is cheaper and faster, however, contamination was always a pressing concern. In addition, each flask required a painstaking pre-treatment program. This ultimately would increase the amount of Suprapur™ nitric acid used and would substantially increase the pre-treatment time required with only a marginal reduction in contamination which in any case was inconsistent between batches. Dissolution was also incomplete as the open vessels prohibited digestion temperatures higher than the acids boiling point and the use of the 'baubles' to allow a semi-reflux was also hazardous, as some acid condensed on the 'bauble' flowed outside the vessel and onto the hotplate below. This and volatilisation of acid and sample components to the atmosphere can account for sample losses adding an error into the method. The use of other acids complicated the procedure and added longer digestion times for little gain in dissolution efficiency; in addition the use of perchloric acid and hydrogen peroxide unacceptably increased the hazardous nature of the method.

After perusing the literature it was decided to investigate microwave digestion. This method has become popular in the last decade with many commercial units now available. The digestion is carried out at elevated temperature and pressure in Teflon digestion vessels, with the higher temperatures providing increased reactivity and oxidising power (Novozamsky et al. 1995; Sun et al. 1997). This is the main advantage over traditional digestion methods. It means digestions can be completed in a significantly shorter time, usually between 20-40 minutes (Quevauviller et al. 1993b; Yang et al. 1994; Novozamsky et al. 1995). There are several other advantages of microwave digestion. Due to the digestions taking place in closed vessels, environmental contamination is minimized, less acid is required and losses of volatile components is substantially reduced (Quevauviller et al. 1993b; Yang et al. 1994; Sun et al. 1997). Madeddu & Rivoldini (1996) successfully applied the procedure to digest plant material for a wide range of elements. The studies of Yang et al. (1994), Tahan et al. (1995) and Sun et al. (1997) have utilised microwave digestion as sample preparation for the determination of aluminium in bread, seafoods and meat. All these studies reported good accuracy and precision using this preparation technique. Of most relevance to this study, juice samples have been digested using a microwave digestion technique in preparation for analysis with ICP-OES, showing good recoveries and agreement with certified reference materials (Barnes 1997). Dolan & Capar (2002) also used microwave digestion to prepare a variety of food materials for ICP-AES determination of various elements including aluminium. They reported that the method for aluminium was prone to contamination and required extensive assessment of quality control results.

A microwave digestion system was not available at Victoria University and permission was sought and granted to use a system at a commercial laboratory, Gribbles Analytical Laboratories (formerly National Analytical Laboratories). Initially, digestions were performed with a mixture of 10ml Suprapur™ HNO_3 and 2ml AR H_2SO_4 followed by digestion with 3ml H_2O_2 to remove the sugars (Barnes et al. 1997). Two problems were immediately observed when the digestates were analysed for aluminium with GFAAS. It was found that the inclusion of H_2SO_4 broadened the GFAAS aluminium absorbance peak, suggesting different atomisation processes (see Figure 3.17). Hence, further digestion with sulfuric acid was discontinued. The other problem, as suggested by Dolan & Capar (2002),was that despite the use of closed Teflon containers a high blank digestion aluminium

absorbance peak area (0.15-0.20) was observed similar to that seen for new untreated glass flasks (see Figure 3.19).

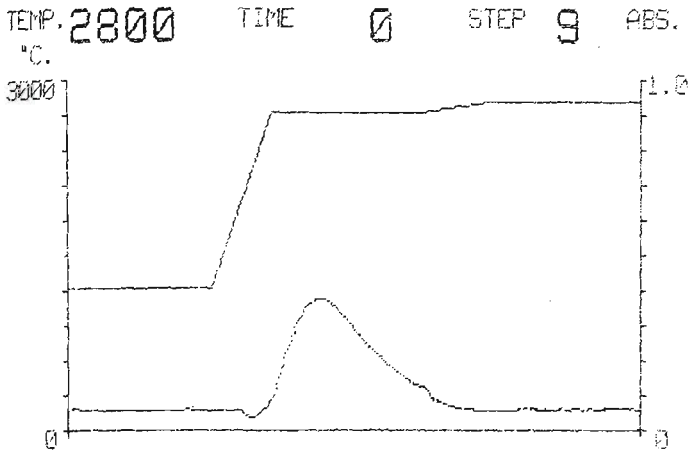


Figure 3.17 GFAAS absorbance signal for a ~0.5g grape microwave digest with 10ml Conc. S HNO_3+ 2ml Conc. AR H_2SO_4 in a sealed Teflon bomb.

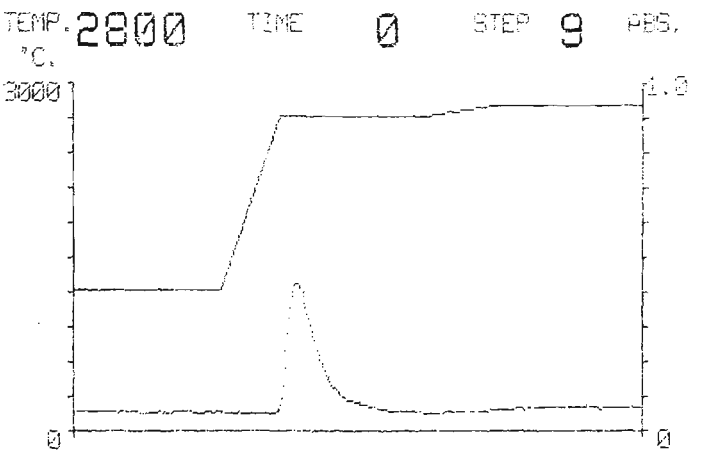


Figure 3.18 GFAAS absorbance signal for a blank digest with 10ml Conc. S HNO_3 in a sealed Teflon bomb (compare with Figure 3.12).

The partially cooled digests were poured into nitric acid treated polyethylene bottles after the microwave digestion. It was considered possible that, like the glass flasks used in traditional digestions of the earlier developmental work, the hot nitric acid was leaching aluminium from the walls of the bottles. This theory was tested by performing a simulated leaching test in the acid soaked polyethylene bottles which consisted of storing 10ml of 1:2 concentrated Suprapur™ HNO_3/Milli-Q® water v/v at ~ 80 C for 30 minutes. These conditions were considered sufficient to simulate the temperature and residual concentration of acid from a microwave digestion. The preparation to analysis time was 1 day, which would be the standard time the digestate would remain in the bottle until analysis for the actual project samples. Two sets of duplicate bottles were subjected to the leaching test; one set were new bottles, the other were pre-cleaned by bathing for two days in the 10% nitric acid bath and rinsed with Milli-Q® water several times. The leaching solutions were decanted from the bottles after approximately one day of storage and diluted to 100ml followed by aluminium analysis with GFAAS. The results of the leaching test are compared with absorbance peak areas of blank digestates of untreated Teflon microwave digestion vessels and pre-treated Teflon flasks from traditional digestion procedures in Figure 3.18.

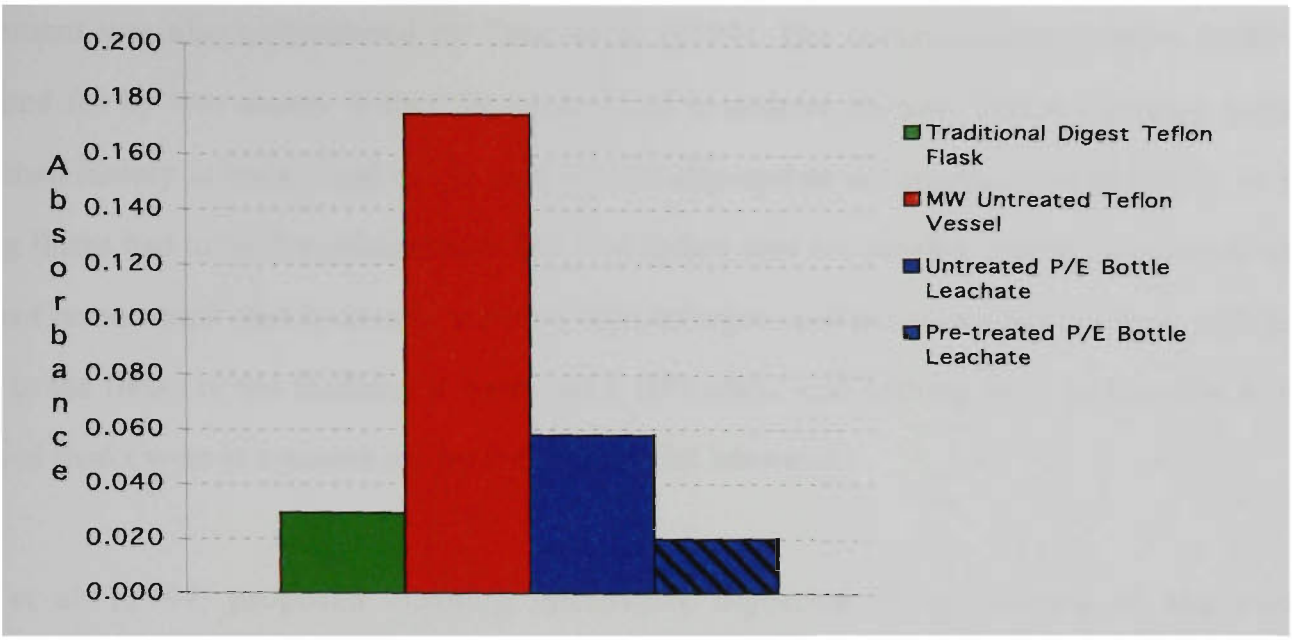


Figure 3.19 Comparison of the aluminium atomic absorbance peak areas of acid digest leachate of new and cleaned polyethylene storage bottles compared with the aluminium atomic absorbance peak areas of untreated Teflon microwave digestion vessels (MW Untreated Teflon vessel) and traditional digest Teflon flasks.

From Figure 3.19 it can be seen that more aluminium is leached from new polyethylene bottles than acid cleaned polyethylene bottles, as would be expected. However, it is also evident that the aluminium concentrations of the leachates are significantly lower than that seen for the untreated Teflon vessel from the microwave digestions. The cleaned bottle leachate had a lower aluminium absorbance peak area than that observed for a digestate solution in a Teflon flask after traditional acid digestion. From these results it can be concluded that while bottle leaching may contribute some aluminium to the digestate, it does not account for the large amount of aluminium contamination observed in the blank digestates of the initial microwave digests.

All the materials used in the digestion process and making up of the GFAAS solutions, including the majority of the vessels in which these solutions come into contact with, have been shown to contribute minimal aluminium contamination when subjected to the appropriate controlling protocols. The author had complete control over every component added to the system. The only entity that was not under the control of the author in the digestion process was the cleaning of the microwave Teflon vessels. It was concluded that like the glass digestion flasks investigated earlier, the Teflon digestion vessels were not cleaned sufficiently to remove all the aluminium from the previous digested sample, hence the remaining aluminium causes the contamination. This

predicament was also encountered by Yang et al. (1994). The contamination problem could be accounted for by two means. Either the project had to acquire its own Teflon digestion vessels where their history is known and can be subjected to appropriate decontamination protocols, or the existing flasks had to be decontaminated. The first option was not possible and the second solution could not be accomplished by simple washing with detergent and the author did not have sufficient access to the flasks or the facilities to carry out a 10% nitric acid bathing prior to digestion as the borrowed flasks were in constant use by the commercial laboratory.

Yang et al. (1994) proposed cleaning microwave digestion Teflon vessels of aluminium contamination by submitting them, containing 8ml of 50% v/v nitric acid, to the same microwave program as for the samples prior to mineralisation of the analyte. Hence this cleaning method was tested for this study by performing a blank digestion pre-treatment of the Teflon digestion vessels prior to sample digestion using the same conditions that samples and blanks would experience. The GFAAS aluminium absorbance peak area of the resultant digest blanks of the digestion vessels after pre-treatment compared with absorbance peak area signals for other digest blanks and samples is shown in Figures 3.20 and 3.21.

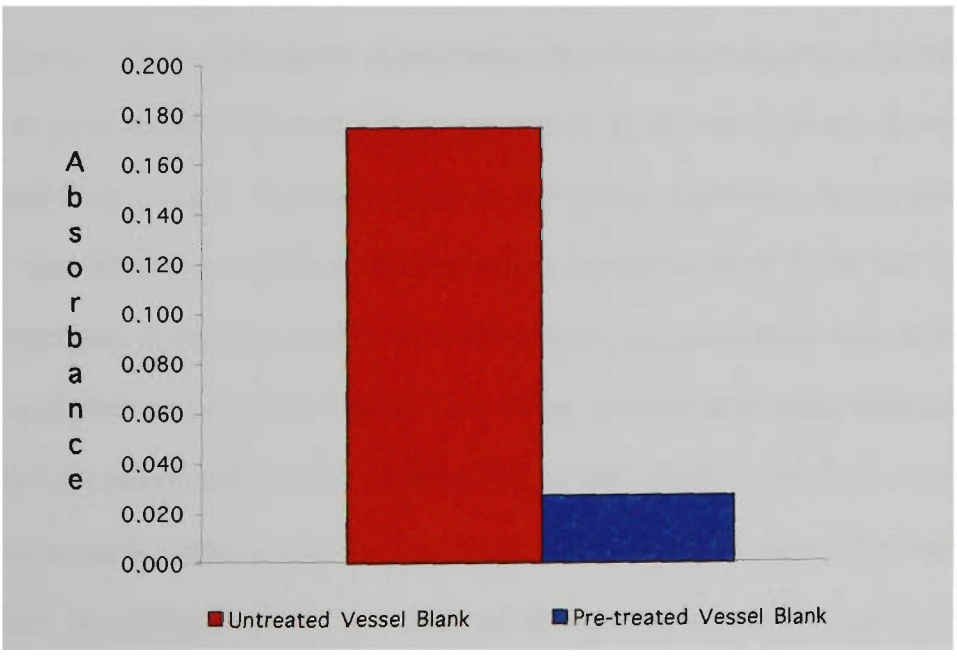


Figure 3.20 Comparison of the aluminium absorbance peak area of blank digestates in an untreated Teflon vessel and a blank digest pre-treated Teflon vessel respectively.

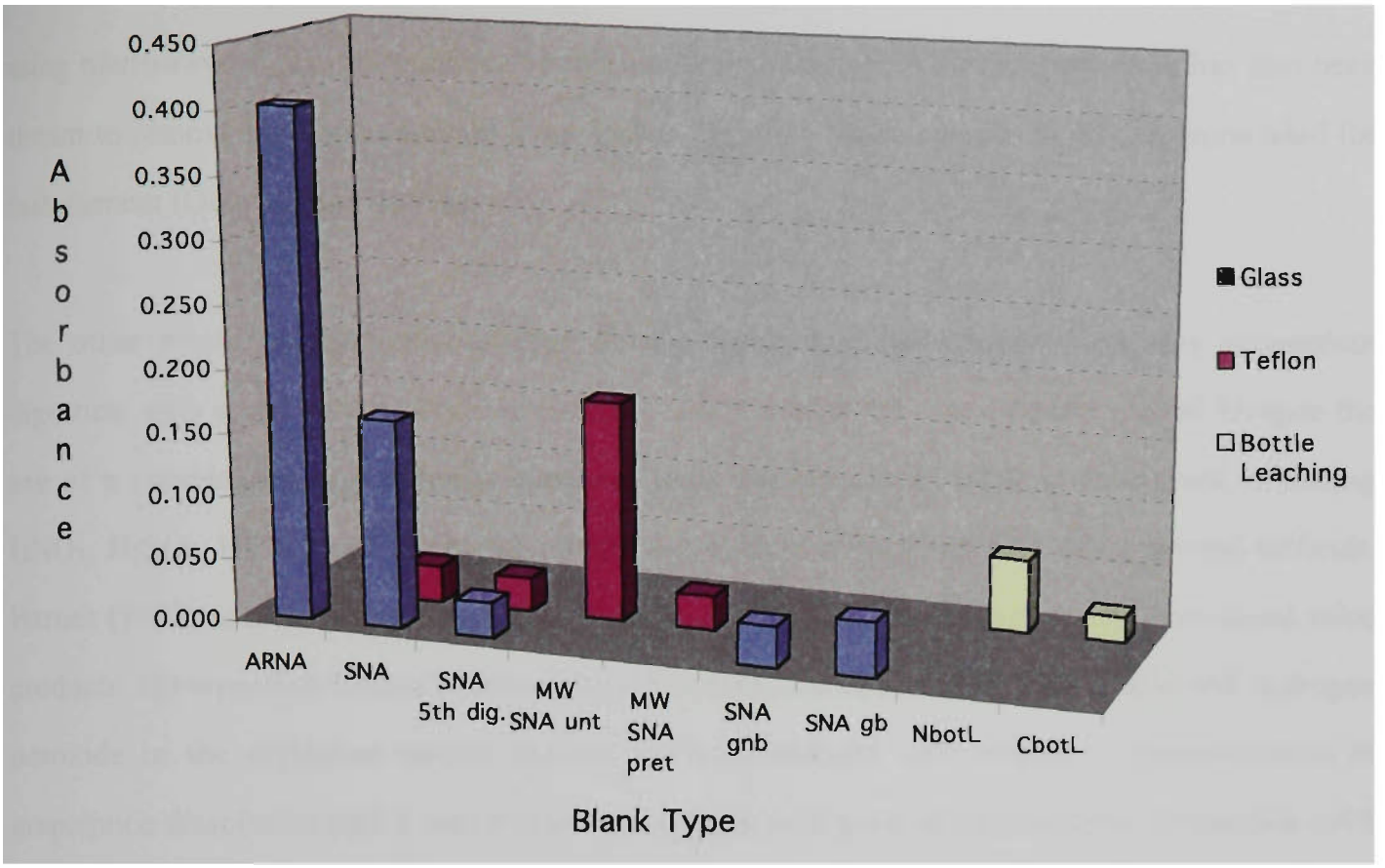


Figure 3.21 Broad comparison of aluminium absorbance peak areas for blank digest solutions showing aluminium contamination over various combinations of digest method categorised by vessel type and including absorbance peak areas from bottle leaching study. Acronyms signify from left to right: ARNA - AR grade nitric acid, SNA – Suprapur™ grade nitric acid, 5th dig – blank digest after 5 blank digestion pre-treatments, MW – microwave digestion, unt – untreated Teflon vessel, pret – pre-treated Teflon vessel, gnb – glass digest with no 'bauble', gb – glass digest with bauble, NbotL – new bottle leachate, CbotL – pre-cleaned bottle leachate.

It is clear from Figure 3.20 that the mean aluminium absorbance peak area of a microwave digest blank carried out in pre-treated Teflon digestion vessels is significantly lower than those that have not been pre-treated with a blank digestion prior to the actual digestion. It can also be concluded from Figure 3.21 that the concentration of aluminium contamination from the Teflon digestion vessels after pre-treatment is commensurate with aluminium contamination seen in traditional blank digests in Teflon and pre-treated glass flasks. This effect is commensurate with the experience of Yang et al. (1994) who advocated careful monitoring of the blank aluminium concentrations over continuing digestions and when a rise in the blank concentration was observed that this pre-treatment procedure be enacted. From the data of the current study it was concluded that the aluminium sample:blank absorbance peak area ratio was sufficiently high enough to suggest that the microwave digestion technique does not significantly introduce aluminium contamination provided the Teflon digestion vessels are appropriately pre-treated before sample digestion. Hence, for this study, a modified method of Yang et al. (1994) was performed prior to each sample digestion when

using microwave digestion to reduce aluminium contamination. A similar procedure has also been shown to remove Hg contamination from Teflon digestion vessels and is highly recommended for that element (Quevauviller 1995b).

The other major problem encountered during digestion development work was incomplete digestion, with small particulates suspended in solution after the digestate had cooled. Despite the use of a combination of oxidising agents in both traditional and microwave digests, including HNO_3, H_2SO_4, $HClO_4$ and H_2O_2, the complete dissolution of grapes and juices proved difficult. Barnes (1997) used a mixture of HNO_3 with H_2SO_4 followed by H_2O_2 to microwave digest juice products. On repeating Barnes' methodology however, the addition of sulfuric acid and hydrogen peroxide to the digestion matrix showed no improvement with respect to completeness of grape/juice dissolution and it was found that sulfuric acid gave an unacceptably broadened AAS signal. The literature (Quevauviller et al. 1993b; Novozamsky et al. 1995; Sun et al. 1997) suggests that these particulates are most likely to be associated with silicates, which do not dissolve in digestions of organic materials using the range of oxidising agents described above. It must also be noted that this problem was not cited by Barnes (1997). Although the amount of particulates remaining in the solutions after microwave digestion was reduced considerably compared with that observed from traditional digestion procedures, there was still some particulates remaining. The concern with silicates is that chemical interference leading to underestimation of aluminium has been demonstrated in aluminium analysis of spruce needles (Quevauviller et al. 1993b).

The use of a mixture of HF with HNO_3 has been recommended and successfully used to completely dissolve organic matrices high in silicate (Quevauviller et al. 1993b; Novozamsky et al. 1995; Madeddu & Rivoldini 1996; Sun et al. 1997). Despite this success, this method of digestion has shortcomings relating to the danger of using HF (Quevauviller 1995b) and is disputed by Yang et al. (1994) who found that HF complicated the GFAAS measurement of aluminium and did not improve the accuracy or precision in sample results. After 10 measurements, HF containing solutions caused a large variation in results and a severe curvature of the calibration line. Yang et al.'s (1994) findings and the following considerations contributed to the decision not to use a HF/HNO_3 mixture to microwave digest the samples:

the high risks of using HF

the hazards of transporting the acid by passenger vehicle to the digestion laboratory,

the cost of a high purity reagent,

the increased safety measures required not only for the author but other members of the commercial laboratory (and possible non cooperation of the commercial laboratory due to the increased risks i.e. workplace safety issues)

possible restrictions on transport, handling and amount of reagent due to HF being a Australian Scheduled Poison

It was found that smaller samples sizes gave lower observable particulate amounts and it was decided that since the contamination risk was reduced considerably, smaller sample sizes would be used to minimise the quantity of particles and that some error in the measurement of aluminium concentration in the samples would be tolerated.

After substantial development it was concluded that microwave digestion using Suprapur™ HNO_3 as the sole oxidising agent in closed pre-treated Teflon vessels would be the method of choice for digesting grape, juice and later yeast and lees samples for this study. This method was found to be optimal in terms of safety, cost, preparation and digestion time, dissolution ability and minimising extraneous aluminium contamination.

3.6.2.2 Grape and juice pre-treatment protocol

The homogenisation procedure for the grape samples differed from juice samples in that they required an extra procedural step before final preparation for digestion. Each approximately 3kg of grape sample from each sample set was manually divided into two with the exception of 2 random bunches which were retained complete on their stalks. One half of the divided pair of sub-samples was thoroughly washed with Milli-Q® water to remove any contamination with soil and dust particles (Grimshaw 1989; Ernst 1995; Sutton & Heitkemper 2000), while the other was left as picked from the vine. These were designated as separate samples thereafter as washed and unwashed grapes. The following methodology applies to both sub-samples. The grapes were stripped by hand from the stalk and homogenised (Grimshaw 1989; Sutton & Heitkemper 2000) by macerating with a Waring™ Commercial stainless steel blender for 2 minutes. The blender was

rinsed with Milli-Q® water before blending the grapes. Between each set of samples and sub-samples the blender was washed with detergent, rinsed several times with tap water followed by several rinses with Milli-Q® water. The blending procedure produced a semi-homogenised pureed composite sample for both washed and unwashed grapes. The puree was then transferred to acid cleaned polyethylene bottles and subsequently stored in a freezer below 0 C until further homogenisation and digestion.

On the day of digestion, the bottled samples of grape and juice were homogenised further by shaking in a rotary shaker for 1 hour after overnight thawing. A triplicate set of sub-samples was then decanted from this homogenised mixture into acid cleaned 125ml polyethylene bottles for transportation to the commercial laboratory for digestion. Also transported to the lab were concentrated Suprapur™ HNO_3 and Milli-Q® water to enable project control of sources of contamination into the digestion system.

Prior to digestion, 24 numbered Teflon flasks were selected for pre-treatment by microwave digestion of 10ml of 70% AR HNO_3 using methods and equipment described in the following paragraph for the samples and the program described in Table 3.3. After cooling, the pre-treatment acid was discarded and the vessels were rinsed several times with Milli-Q® water. These vessels were then used for all subsequent digestions performed on that day.

After the vessels had been pre-treated, approximately 0.5-1.2g of homogenised grape or approximately 2.0-5.0g of juice were sub-sampled by decanting directly from the shaken transport bottle into the Teflon vessel. Each sample was sub-sampled in triplicate into three Teflon vessels and weighed accurately to four decimal places. 10ml of 70% Suprapur™ HNO_3 was then added to each sub-sample and the vessels were assembled and sealed into the rotating carousel. Twelve sub-samples per carousel were then microwave digested with a Milestone MLS-1200 Mega Microwave digestion unit using the heating program shown in Table 3.3.

Table 3.3 Microwave digestion program used for digestions in this study.

Condition	Power (Watts)	Time (mins)
Heating	250	5.0
No Heat	0	1.0
Heating	250	10.0
Heating	450	5.0
Venting	0	5.0

Once the digestion program was complete the carousel was removed from the digestion unit and allowed to cool for 20 minutes. After cooling the vessels were removed from the locking segment and the digestate solutions were quantitatively transferred from the vessels into 125ml polyethylene bottles which were then sealed. Yang et al. (1994) and Dolan & Capar (2002) advocated strenuous quality control assessment for microwave digestions of sample for aluminium determination. Thus for each run of 36 sub-samples, 6 procedural blanks and 6 blanks spiked with freshly prepared aqueous aluminium standard were also digested to determine procedural blanks and recovery studies respectively. Blanks and spikes followed the same protocol as the samples. As discussed in Chapter 4, Sub-Section 4.3.4, the average blank aluminium concentration was 11µg/L or on average about 20% of the sample signal. The partially diluted samples were stored in these bottles for transport back to the laboratory and held overnight for further sample preparation the following day.

The digested sub-samples were quantitatively transferred to 100ml volumetric flasks and diluted to the mark with Milli-Q® water. The diluted samples were immediately transferred back into the same polyethylene bottles used for the original digestates and sealed. The blank and aluminium spike digestates also followed the same dilution and storage protocols. These solutions were then analysed for their total aluminium concentration using instrumental techniques described in Sub-Section 3.7.1.3.

3.6.3 Ferment and Wine Pre-Treatment

3.6.3.1 Pre-treatment considerations for ferment and wine

Wine is a complex matrix of many different compounds. Lopez et al. (1998) used digestion procedures similar to those discussed in the last Sub-Section to break down this matrix into a more

amenable form for instrumental analysis, with both traditional and microwave digestions. However, an obvious advantage of preparing wine samples over those of juice and grape is that all the components of this complex matrix are already homogenised in a close to aqueous solution. Liquids such as milk, fruit juices and beverages such as tea or coffee have been simply diluted and analysed directly for the chosen element with little matrix interferences depending on instrumental techniques used (Delves et al. 1988). This has also been the case for wine. Unlike Lopez et al. (1998), McKinnon (1990) and McKinnon et al. (1992) found that a simple dilution of the wine sample in water was enough to prepare the sample for GFAAS analysis with minimal matrix interferences. Larroque & Cabanis (1994) and Seruga et al. (1998) also used the dilution preparation methodology which was found to be both accurate and precise. Although the pre-treatment protocol of Lopez et al. (1998) is a legitimate procedure, the advantages of the dilution method are obvious; the only component added to the system is ultrapure water presenting minimal interferences, contamination and, most significantly allows minimal sample handling, providing a virtually direct analysis of the sample. However, for this study one deviation from the method of McKinnon (1990) was deemed necessary as the solutions had to be stored for at least an hour while the solutions were transported from the preparation lab to the instrumental lab on another campus. The diluted solutions were acidified with ~0.70% v/v ultrapure nitric acid to prevent adsorption of the aluminium from the wine solution onto the container walls (Szpunar et al. 2000). As 3% v/v HNO_3 is used to stabilise commercial bottled aluminium standards for months, 0.70% v/v was considered adequate for maintaining aluminium stability for several days.

3.6.3.2 Ferment and wine pre-treatment protocol

Wine and ferment samples were thawed overnight prior to the day of analysis. This was followed by rotary shaking the samples in their storage bottles at 60 rpm for at least 1 hour to ensure complete homogenisation. For each sample, triplicate 5.00ml sub-samples were accurately diluted 20 fold to 100ml with Milli-Q® water. Prior to dilution, 1ml of Suprapur™ HNO_3 was added to acidify the sample, giving an acid concentration of 0.70% v/v on dilution to 100ml. After mixing the solution was transferred to 125ml polyethylene bottles for storage until analysis. The analysis of these solutions is covered in Sub-Section 3.7.1.3. A procedural blank in triplicate consisting of acidified Milli-Q® water and a triplicate aluminium spike solution of acidified aluminium standard for

recovery analysis were made up using the same protocol as the samples. These solutions accompanied each discrete batch of samples; as discussed in Chapter 4, Sub-Section 3.4.3 the mean blank aluminium concentration was 4.4µg/L and on average around 15% of the sample aluminium concentration.

3.6.4 Grape Dissection Protocol

To determine the distribution of aluminium in a single grape a dissection procedure was used to isolate distinct parts of a grape for subsequent analysis. It was decided that this experiment would be performed on one white and one red grape set. Both grape sets were subjected to the same procedure.

The few remaining grape bunches retained on the stalk that did not undergo homogenisation (see Sub-Section 3.6.2.2) were removed from the freezer and 10 grapes of various sizes and position on the bunch were removed at random by stainless steel tweezers. The selected grapes were placed in a ziplock polyethylene sample bag and left to thaw overnight. After thawing, the grapes were carefully removed from the bag and placed onto a clean Teflon mat. While holding with a stainless steel tweezers, each individual grape was cut using a clean stainless steel scalpel along the skin from the stalk hole almost to the bottom of the grape as shown in Figure 3.22. The skin was then peeled apart and the flesh, juice and pip were cut away from the skin. The flesh and juice were then gathered together on the mat after the pip was removed. The three components of skin, pip and flesh/juice were then segregated from each other on the mat. This procedure was repeated for each grape and the three separate components were gathered together to form three composite samples of skin, pip and flesh/ juice. Each of these composite samples was then placed in separate ziplock polyethylene bags for transport to the digestion laboratory. Skin samples of 1.0-1.2g, pip samples of 0.5-0.9g and flesh/juice samples of 1.0-1.2g were weighed accurately, digested and prepared for analysis using the same protocol as for grape pulp and juice described in Sub-Section 3.6.2.2. Blanks were also run concurrently with these samples as per Sub-Section 3.6.2.2. The instrumental technique for the analysis of the aluminium concentrations of the dissections is discussed in Sub-Section 3.7.1.3.

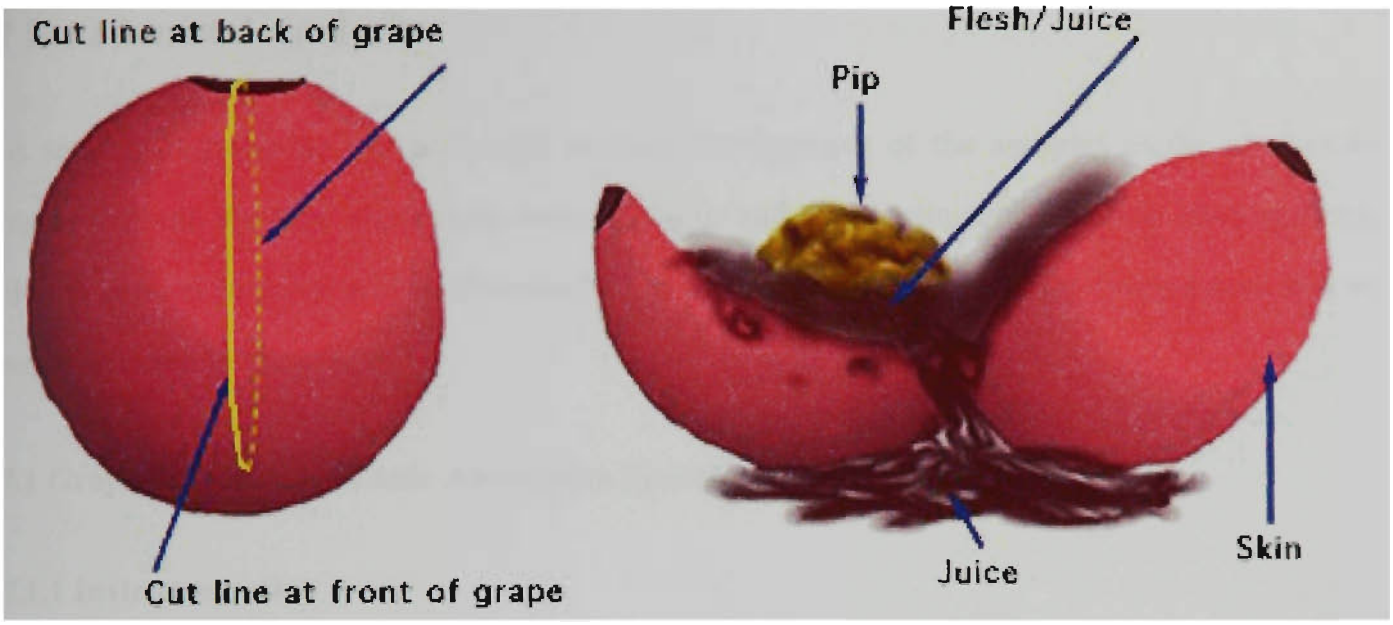


Figure 3.22 Diagram showing initial dissection of the grape and the separate components analysed for aluminium content.

3.6.5 Yeast Lees and Yeast Pre-Treatment

After thawing overnight the lees sample was centrifuged at 4000 rpm for 5 minutes. The liquid was decanted off and the residue was then oven dried at 110 C to remove residual water. The dried residue was then transferred into a ziplock polyethylene sample bag for transport to the digestion laboratory. Triplicate samples of mass 1.0-1.2g were weighed accurately and digested using the protocol described in Sub-Section 3.6.2.2 followed by analysis as discussed in Sub-Section 3.7.1.3. Blanks were also run concurrently with these samples as per the method in Sub-Section 3.6.2.2.

A sample of the initiating yeast from the same batch that was in the lees sample was also collected. The yeast was received in dried small pellet form and ~1.0g samples were accurately weighed, digested and prepared in triplicate concurrently with procedural blanks as per the method described in Sub-Section 3.6.2.2. This was followed by GFAAS analysis using the method outlined in Sub-Section 3.7.1.3.

3.6.6 Wine Pre-Treatment for Speciation Analysis

For speciation analysis of wine using ES-MS, a white wine sample and a red wine sample were 20-fold diluted with 13%v/v ethanol solution before measurement of pH and analysis described in Sub-Sections 3.7.3.2 and 3.7.4 respectively.

3.7 Instrumental Analysis

This section outlines the protocols and method development of the analysis of the pre-treated samples for both the total aluminium determinations and the aluminium speciation investigations. Only the general protocol will be discussed for the latter analyses, as method development will be covered in depth in Chapter 5.

3.7.1 Graphite Furnace Atomic Absorption Spectrometry (GFAAS)

3.7.1.1 Instrumentation

The initial method development work was carried out on a Varian AA1475 atomic absorption spectrometer with a GTA-95 graphite tube atomizer with autosampler. Analyses of all grape, juice, ferment and wine samples collected from the vineyards/wineries in this study were conducted using a Varian SPECTRAA 300 atomic absorption spectrometer with a GTA-96 graphite tube atomizer and autosampler driven by SpectrAA 300/400 Series Worksheet Oriented AA version 01.30.203 software on a PC running Windows 95.

3.7.1.2 Method optimisation

McKinnon (1990) and McKinnon et al. (1992) found that GFAAS was the best analytical method to determine aluminium concentration in wine. A subsequent review of metals determination in wines by Aceto et al. (2002) also suggested GFAAS was a suitable technique for aluminium determination in wines. It was decided to adapt the method of McKinnon (1990) for this study but as the instrument used for this work was different to that used by McKinnon, some method development was required.

McKinnon (1990) and Larroque et al. (1994) both showed that the use of nitrogen gas during drying and ashing gave reduced aluminium absorbance peak areas, with the aluminium absorbance peak area about one third of that observed when argon was used. This effect was linked to involatile nitrides forming in the furnace before atomisation, reducing the quantity of free aluminium atoms during atomisation. An experiment was carried out for this project to confirm this phenomenon where parallel study of an aqueous aluminium standard of 50 µg/L using both nitrogen and argon as

the inert gases was performed. As can be seen in Figures 3.23 and 3.24 there is a large discrepancy between the magnitudes of the aluminium absorbance peaks using the two gases. The aluminium absorbance peak area of the standard using nitrogen as the inert gas was around a fifth that of the aluminium absorbance peak using argon, confirming the observations of McKinnon (1990) and Larroque et al. (1994). Hence all analyses using GFAAS for total aluminium determination was accomplished using argon as the inert gas.

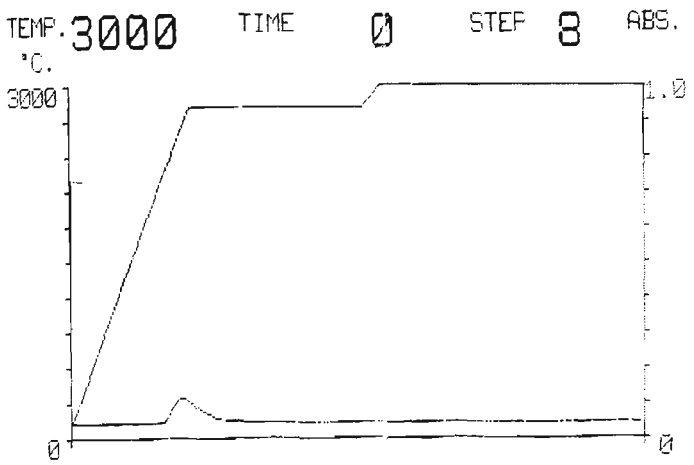


Figure 3.23 GFAAS absorbance signal for a 50µg/L Al standard using nitrogen as the inert gas.

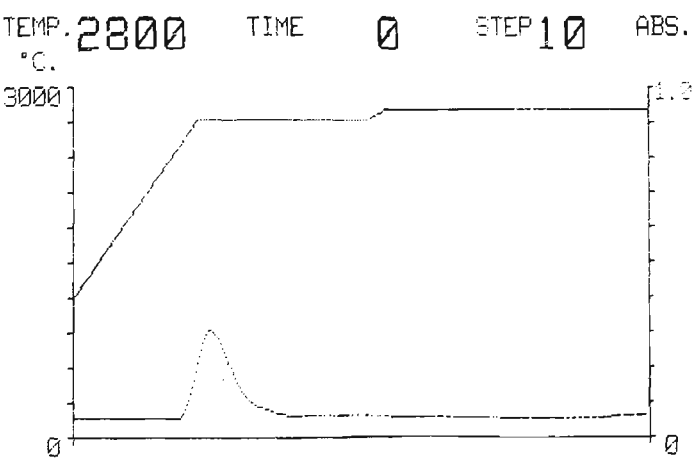


Figure 3.24 GFAAS absorbance signal for a 50µg/L Al standard using argon as the inert gas.

As it had already been demonstrated (McKinnon 1990; Aceto et al. 2002) that the GFAAS method could successfully be applied to wine samples, an investigation was carried out to see if juice samples could also be analysed using this technique. It soon became apparent that during the ashing/atomisation phase a large carbonaceous deposit formed in the furnace, blocking the light path. The evidence of a sweet odour emanating from the graphite furnace during the atomisation phase and reports from the literature suggested that the carbonaceous deposit was due to sugars decomposing in the furnace (Tsalev & Zaprianov 1983; Sullivan et al. 1987; Delves 1987; Vinas et al. 1995). Trials using oxygen to more aggressively break down these sugars during the ashing phase were successful. However, the lifetime of the furnace was dramatically reduced due to the rich oxidative environment rendering the use of oxygen impractical to implement. Hence direct juice analysis with GFAAS was abandoned and the juice samples had to undergo an acid digestion pre-treatment step as discussed in Section 3.6.

Using the dilution method of McKinnon (1990) eliminated the need for a modifier in GFAAS analysis of wine samples, the dilution effectively matching the wine matrix with that of water and minimising interferences. However, with the solutions containing the diluted digestates of grape and juice material there is still a proportion of concentrated acid present in the solution which could influence the aluminium absorbance signal. A simple comparison of peak shapes for both digest solutions and aqueous standards was undertaken. As can be seen in Figure 3.25 there is little difference in the shape of the GFAAS aluminium absorption peaks between an aqueous aluminium standard, a 20 fold diluted wine sample, a 10ml Suprapur™ HNO_3 contaminated digest blank and a solution of a grape digest respectively. This shows there is little matrix interference experienced from the grape digest solution compared with an aqueous standard. This agrees with work by Yang et al. (1994) who also showed that HNO_3 does not change the peak shape and hence affect the GFAAS measurement of aluminium in solutions, even solutions containing up to 20% v/v nitric acid. As the inclusion of modifiers has the potential to introduce further contamination into the system and the omission of the modifier does not appear to significantly alter the atomisation of aluminium from the solutions, no modifier was employed for the analysis of the samples.

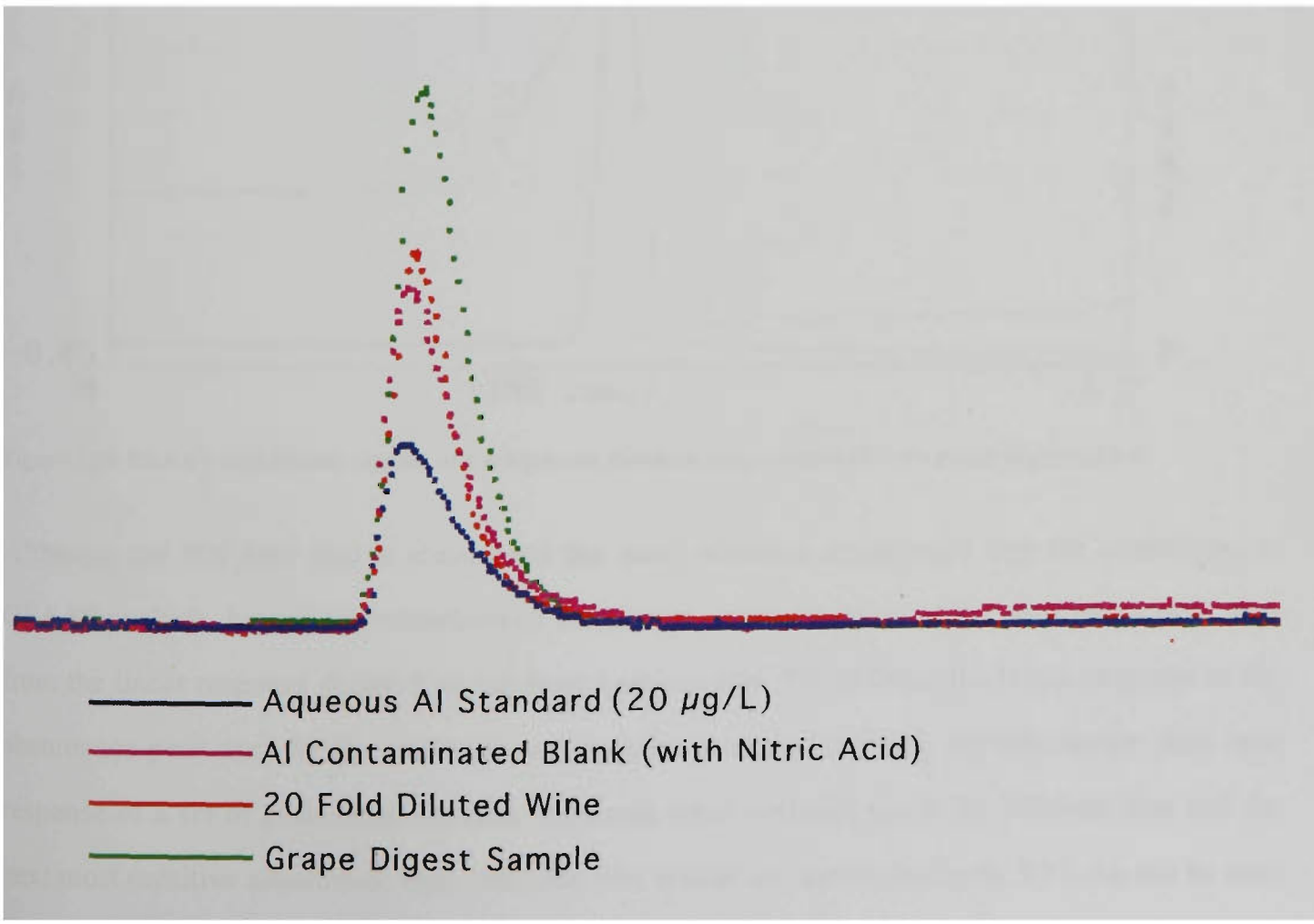


Figure 3.25 Comparison of GFAAS aluminium absorbance peaks for various solution matrices.

As evidenced in Figure 3.25, some tailing is observed with the aluminium absorbance peaks. It was considered that background absorbance could be the source of the tailing and background correction may be required. However, at around 309nm, the deuterium lamp cannot easily match the intensity of the aluminium hollow cathode lamp. With the hollow cathode lamp current reduced from 7mA to 3mA, it was possible to match the two lamp intensities at 309nm. The absorbance signal, both atomic and background, were recorded for a grape digest sample and are shown in Figure 3.26. The results show that a small background signal is observed and is mostly after the aluminium absorbance peak. By controlling the read time in the atomisation stage of the GFAAS program from 2.8s to 1.8s and the use of blank correction to account for background, the GFAAS analysis could be accomplished at the higher lamp current of 7mA without background correction with minimal interference from background absorbance. With these measures in place, subsequent results showed an improved sample signal precision.

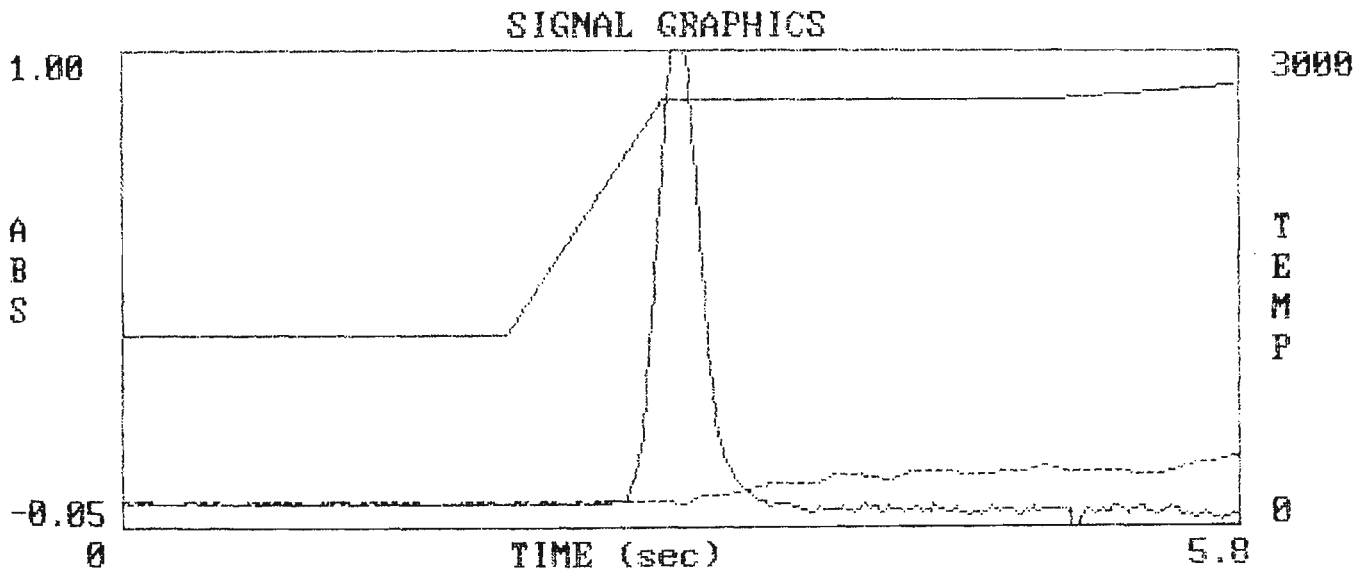


Figure 3.26 GFAAS absorbance signals of background (broken line) compared with grape digest signal.

Although the 309.3nm line is considered the most sensitive absorbance line for aluminium in GFAAS analysis, higher concentrations of aluminium cause deviation of the absorbance peak area from the linear response dictated by the Beer-Lambert law. To optimise the linear response of the absorbance peak area for the standards and samples used in this study, the absorbance peak area response of a set of aluminium aqueous standards were analysed using the 309.3nm line and the next most sensitive absorbance line, 396.2nm. The results are shown in Figure 3.37. As can be seen

from this comparison the 396.2nm gave a better linear response over the aluminium concentration range used in this study, hence the 396.2nm line was used for all GFAAS work in this project.

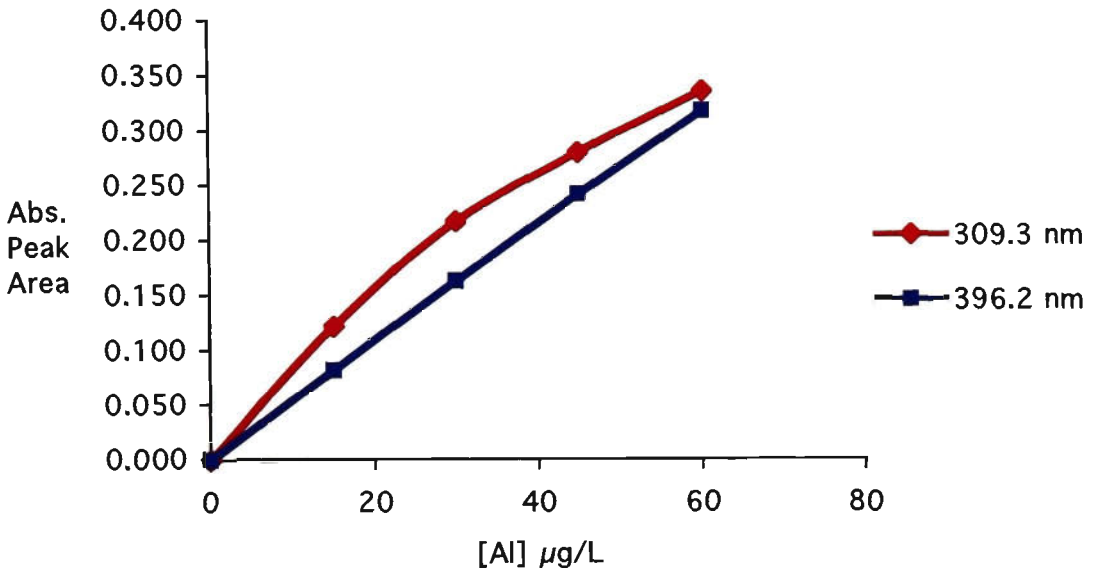


Figure 3.27 Comparison of absorbance peak area responses of an aqueous aluminium standard using the 309.3nm and 396.2nm spectral lines.

Although McKinnon (1990) conducted extensive GFAAS furnace program optimisation, a different instrument meant the three major steps of a graphite furnace program needed to be re-optimised for the current study. However, an examination of the optimum temperatures and times required for the graphite furnace heating program showed little deviation from the methodology of McKinnon (1990) with only minor adjustments to program times required.

The delivery of the sample from the auto-sampler to the furnace must operate repeatably to ensure precision in the analysis. Observation of the delivery of the diluted wine sample revealed that most of the solution was not passing from the capillary into the furnace. On closer inspection, it was noticed that most of the diluted wine samples were depositing around the outside of the capillary above the tip and pouring over the sides of the furnace. This effect is attributed by Aceto et al. (2002) to the presence of ethanol due to changes in viscosity and surface tension, and it was recommended that GFAAS sample should be diluted and a surfactant such as Triton X-10 added. However, to add a surfactant to the diluted wine sample increases the risk of aluminium contamination. To avoid this a novel approach was utilised to increase the hydrophobicity of the auto-sampler capillary by applying a thin film of petroleum jelly to the outer surface of the capillary

tip as shown in Figure 3.28. This was found to be successful in allowing the entire sample to be deposited in the graphite tube. Hence to ensure repeatability of sample delivery, a petroleum jelly film was maintained on the outside of the last ~2cm of the auto-sampler capillary for all GFAAS analyses of the ferment and wine samples in this study.

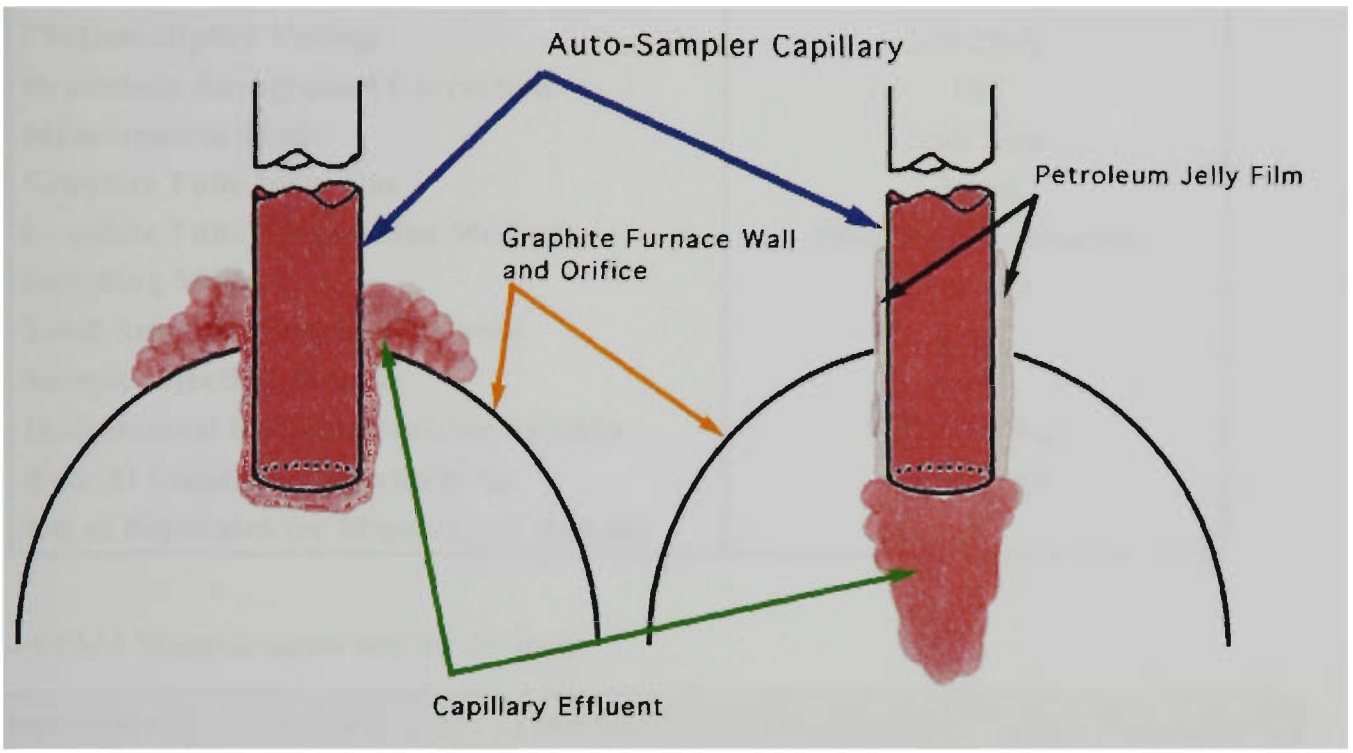


Figure 3.28 Diagram illustrating the flow of a diluted wine sample from the GFAAS auto-sampler capillary with and without a thin film of Petroleum Jelly.

3.7.1.3 GFAAS parameters and program

The parameters and furnace program used for GFAAS analysis in this study are shown in Tables 3.4 and 3.5 respectively. Sample vials were rinsed twice with the sample it was to contain before the solution was decanted into the vial for placement into the autosampler. Each triplicate sample was analysed in triplicate to maximise accuracy and precision. Aqueous aluminium standards were run before each batch of samples analysed that day.

Table 3.4 GFAAS instrumental parameters used for this study.

Instrumental Parameter	Setting
Wavelength	396.2nm
Monochromator Slit Width	0.5nm
Monochromator Slit Height	Normal
Al Hollow Cathode Lamp Current	7mA
Photomultiplier Voltage	240-250V
Deuterium Background Correction	Off
Measurement Mode	Peak Area
Graphite Tube Inert Gas	Argon
Graphite Tube Atomisation Method	Tube Wall Atomisation
Sampling Mode	Automix
Total Automix Injection Volume	20µL
Sample Injection Volume	20µL
Instrumental Blank and mixing solution	Milli-Q® Water
Bulk Al Standard Concentration	60-100µg/L
No. of Replicates for Standards & Sample	3

Table 3.5 GFAAS furnace program used for this study.

Temperature (C)	Program Mode	Time (s)	Argon Gas Flow (L/min)	Absorbance Peak Area Measurement
70	Hold	2	2.0	----
120	Ramp	20	2.0	----
120	Hold	20	2.0	----
1200	Ramp	10	2.0	----
1200	Hold	10	2.0	----
1200	Hold	2	0.0	---
2700	Ramp	0.8	0.0	On
2700	Hold	1	0.0	On
2800	Ramp	0.5	2.0	----
2800	Hold	1	2.0	----

3.7.2 Flame Atomic Absorption Spectrometry (FAAS)

All soil extraction solutions were analysed using a Varian AA1475 atomic absorption spectrometer. The absorbance of all soil extraction solutions and aluminium aqueous standards were determined in triplicate where the reading was noted after a stable signal was acquired. Aluminium aqueous standards were run prior to each batch of samples on that day. In the event of burner clogging, analysis was stopped, the burner cleaned thoroughly and the standards re-measured prior to

resumption of sample analysis. Instrumental parameters are shown in Table 3.6. The blank and standards were made up in 0.43M Acetic Acid to match the matrix of the soil extraction solutions.

Table 3.6 FAAS Instrumental parameters for soil extraction analysis.

Instrumental Parameter	Setting
Wavelength	309.3nm
Monochromator Slit Width	0.5nm
Al Hollow Cathode Lamp Current	7mA
Deuterium Background Correction	Off
Absorbance Measurement Mode	Peak Area
Double Beam	On
Burner	N_2O-Acetylene
Fuel:Oxidant Ratio	2.3:1
Instrumental Blank set at 0.000 Abs.	0.43M Acetic Acid
Al Standard Concentration Range	1-20mg/L
No. of Replicates for Standards & Sample	3

3.7.3 pH Determination

The pH measurement of solutions was accomplished for soil suspensions (Sub-Section 3.6.1.3), for aqueous speciation reference solutions and for wine and diluted wine solutions using a Eutech Cybernetics Activon Cyberscan pH 500 pH meter with a temperature probe and an Orion ROSS® combination pH electrode. Prior to measurement the electrode and meter were calibrated over three pH points using pH 4.00, pH 7.00 and pH 10.00 standard buffer solutions. The meter automatically adjusted pH with respect to the temperature of the solution.

3.7.3.1 Soil pH determination

This methodology was adapted from Australian Standard AS 1289.3.4.1 (Standards Australia 1997). After standing for 1 hour (as mentioned in Sub-Section 3.6.1.3), the solutions were stirred gently for 5 minutes. After stirring, the Milli-Q® water washed calibrated electrode and temperature probe were immersed into the soil suspension and pH recorded after a stable pH reading was reached. This procedure was performed in triplicate for each soil suspension with stirring for a minute between readings.

3.7.3.2 Speciation reference solution, wine and diluted wine pH determination

The washed calibrated electrode and temperature probe were immersed into the gently stirred solution and the pH was recorded after the pH reading had stabilised. For adjusting pH of solutions,

dilute HCl or KOH was added while the solution was under constant stirring until the target pH was reached.

3.7.4 ES-MS Speciation Measurement

The speciation of aqueous reference solutions and diluted wine samples was performed using a Micromass Platform II® electrospray mass spectrometer (ES-MS) with a quadrupole mass selective detector. Solution is delivered to the ES-MS by a JASCO PU-980 peristaltic pump with a 20µL injection loop. The mass spectrometer is driven by Masslynx 2.3 software on a PC running Windows NT 4.0 and is shown in Figure 3.29 with the spray tip. The system uses an ionspray described in Sub-Section 2.3.2 of Chapter 2 with nitrogen as a nebulising/drying gas. The inner section of the electrospray tip and mass spectrometer system used for this study is shown in Figure 3.30. A 50µL syringe was used to inject the solution to be analysed into the injection loop, whereupon the solution plug was introduced into the 50:50 Milli-Q® Water:acetonitrile solvent to be nebulised at the capillary tip in both positive and negative ion modes. Results were obtained and analysed using the Masslynx 2.3 software. As the speciation work was a method development in its own right, a more detailed description of the analysis is discussed in Chapter 5. The general operating parameters of the ES-MS are given in Table 3.7.

Table 3.7 ES-MS general operating parameters for speciation analysis of aqueous solutions and diluted wines.

Parameter	Positive Mode	Negative Mode
Capillary Voltage	+3.5kV	-3.5kV
Counter Electrode Voltage	+0.5kV	-0.5kV
Cone Voltage	+30V	-30V
Skimmer Lens Voltage	+35V	-35V
Ion Energy	1.0V	1.0V
Nebulising Gas	N_2	N_2
Nebulising Gas Flow Rate	20L/hr	20L/hr
Source Temperature	60 C	60 C
Solvent	50:50 CH_3CN/H_2O	50:50 CH_3CN/H_2O
Solvent Flow Rate	0.02ml/min	0.02ml/min
Injection Loop Volume	20µL	20µL

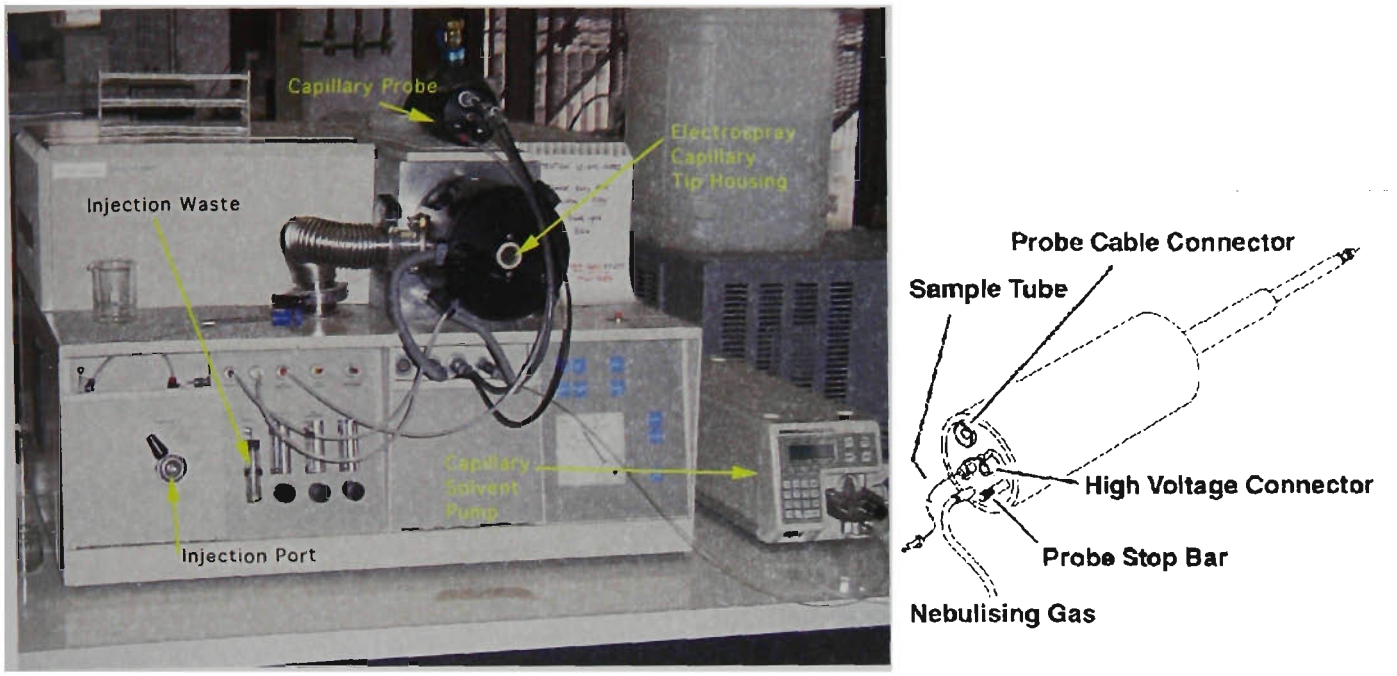


Figure 3.29 The Micromass Platform II electrospray mass spectrometer and diagram of the electrospray probe.

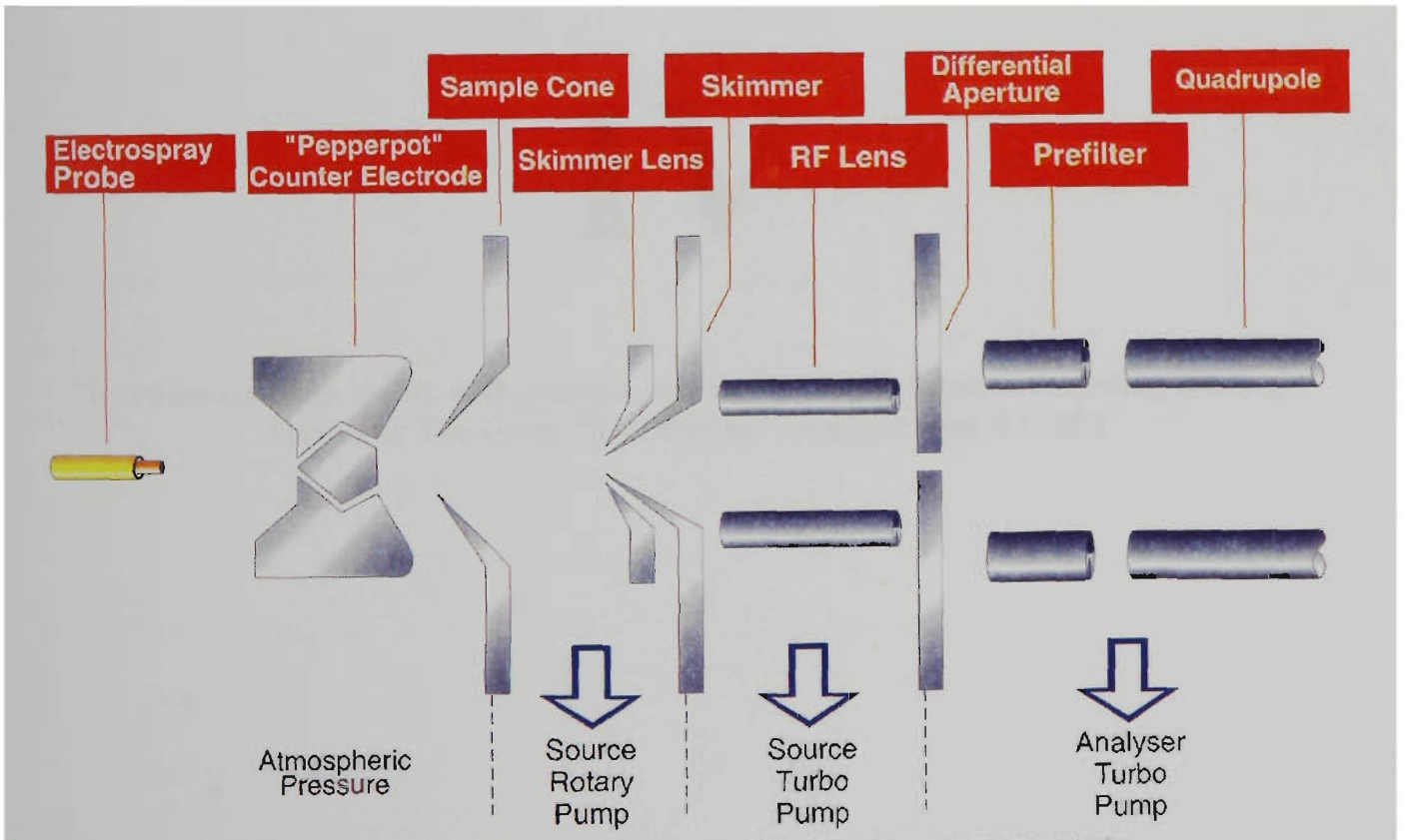


Figure 3.30 Schematic representation of the inner workings of the ES-MS used for this study.

IV

"It is a lovely thing to live with courage, and to die leaving behind everlasting renown"
Alexander The Great, Macedonian Conqueror (356-323BC)

CHAPTER FOUR

4. PROFILE OF TOTAL ALUMINIUM CONCENTRATION OVER THE ENTIRE WINE PRODUCTION PROCESS

4.1 Introduction

As discussed in Chapter 1, the majority of work that has been conducted on aluminium in wine has focused on the total aluminium concentration in the finished table wine using either GFAAS or FAAS. In the last decade McKinnon (1990), McKinnon et al. (1992), Larroque et al. (1994), Eschnauer & Scollary (1995), Lopez et al. (1998), Seruga et al. (1998) and Galani-Nikolakaki et al. (2002) have all published aluminium concentrations in white and red wines from GFAAS analyses, quoting the mean aluminium concentration of wines from 0.548-2.82mg/L. These concentrations are all higher than that recommended by the EU and WHO for drinking water set at 0.2mg/L (Uwers 1991). While useful in determining possible aluminium ingestion upon beverage consumption, the total aluminium concentration does not reveal the sources and sinks of aluminium throughout the wine production process that generated the final concentration. Of the studies mentioned, only McKinnon (1990) and McKinnon et al. (1992) attempted to discern the total aluminium concentration profile throughout the production process. More particularly those studies determined the best technique for determining the concentration of aluminium in a vast array of wine samples; the profile analysis was at best an addendum. Of the five wines that were profiled at the major production stages starting from juice samples, McKinnon and colleagues concluded that bentonite and filters were the major source of aluminium in white wines while red tannin was the main source of aluminium in red wine.

As the aluminium content of food, water and beverages in general has come under increasing scrutiny in the last 20 years (Ganrot 1986; Sherlock 1988; UK MAFF 1993; Muller et al. 1993; Sharpe et al. 1995; Srinivasan et al. 1996; Stauber et al. 1999; Gauthier et al. 2000), a thorough knowledge of the total aluminium profile and the sources and fates of this element in wine will assist any future measures to control its concentration in the finished product. To gain a true profile of the sources and sinks of aluminium throughout the entire production process, one needs to

include the background aluminium derived from the grapes and ultimately the soil to ascertain how much aluminium is derived from 'natural sources'. This cannot be underestimated, as aluminium is a ubiquitous element found extensively in soils and in varying amounts in water and air. The current investigation was intended to expand on the work of McKinnon (1990) and McKinnon et al. (1992) by conducting a more comprehensive analysis of the total aluminium concentration profile throughout the wine production process. The profile would include the extractable aluminium content of the soil, and the total aluminium content of the grapes, juice, ferment, unfinished wine at many of the production steps and ultimately the bottled wine, determined for red and white cultivars at five vineyards/wineries over two vintages.

This chapter presents the results of the total aluminium concentration profile analyses conducted by FAAS and GFAAS as set out in Chapter 3. The results of the individual major production steps will be tabled and discussed, culminating in the overall charting and discussion of the aluminium concentration profile on both an individual wine and overall mean wine basis. In addition, particular case studies of wines with unusual profiles and production steps exhibiting a significant change in aluminium concentration will also be identified and discussed.

4.2 Aluminium Profile Analysis Considerations

Before the results of the aluminium profile analysis can be presented and discussed, a brief overview of the techniques of winemaking need to be addressed to understand why particular samples were taken and analysed. The production techniques differ for red and white wines, however the procedures outlined here are a general overview only. Winemakers use a multitude of different techniques based on individual tastes and requirements and as the origin and vintage conditions of the grapes and the winemaking procedures changes from year to year there is an element of uniqueness for each wine. However, the basic principles of dry table wine making are shown in flowcharts derived from those provided by Rankine (1991) in Figure 4.1 for white wine and Figure 4.2 for red wine. Steps shown in bold are the steps after which samples were generally taken in this investigation.

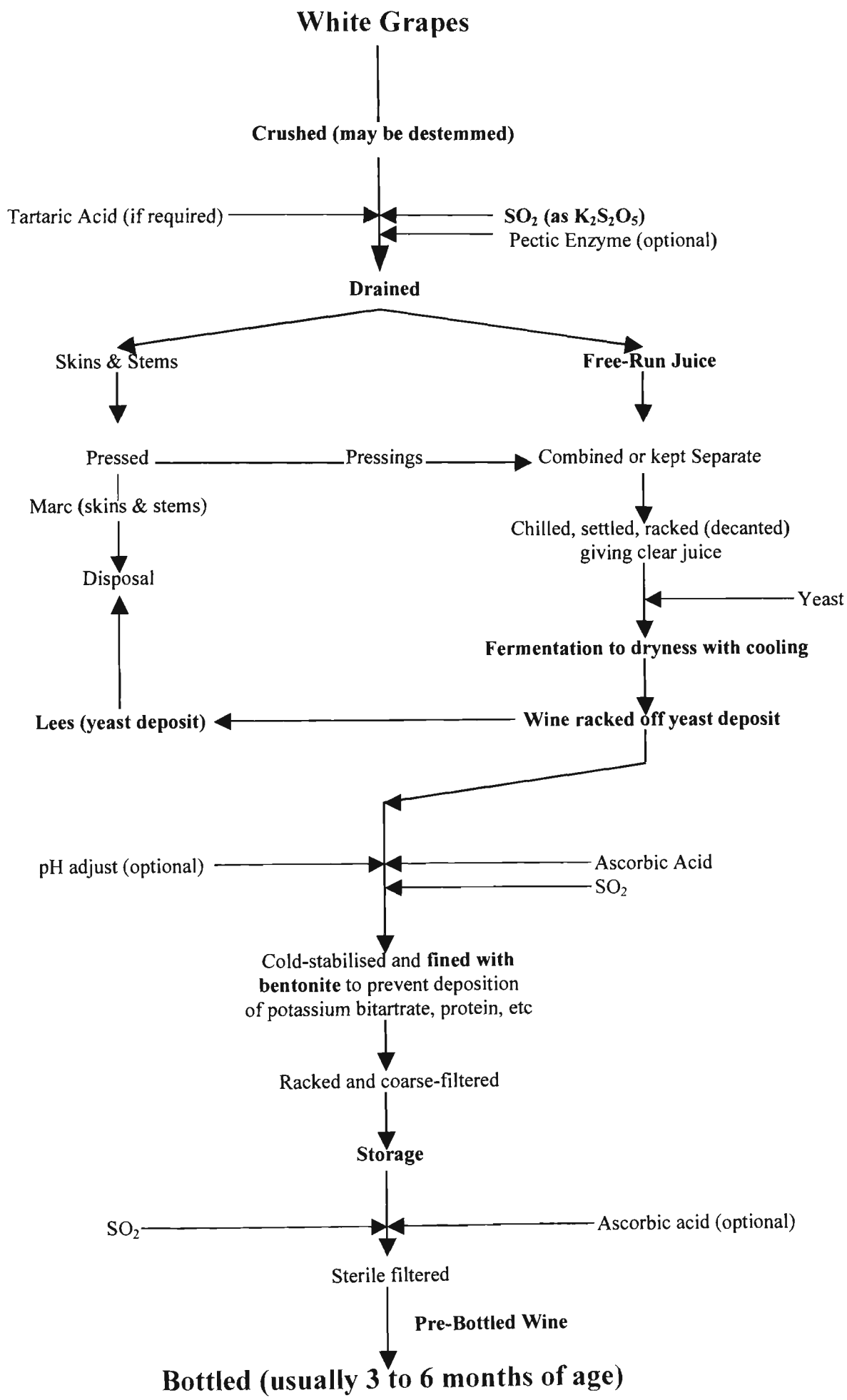


Figure 4.1 Flow chart derived from Rankine (1991) of general procedure of dry white table winemaking. Steps in bold show where samples were taken after the event. Blue text denotes wine additives and treatments. Red text denotes samples taken for single fermentation study in 2001.

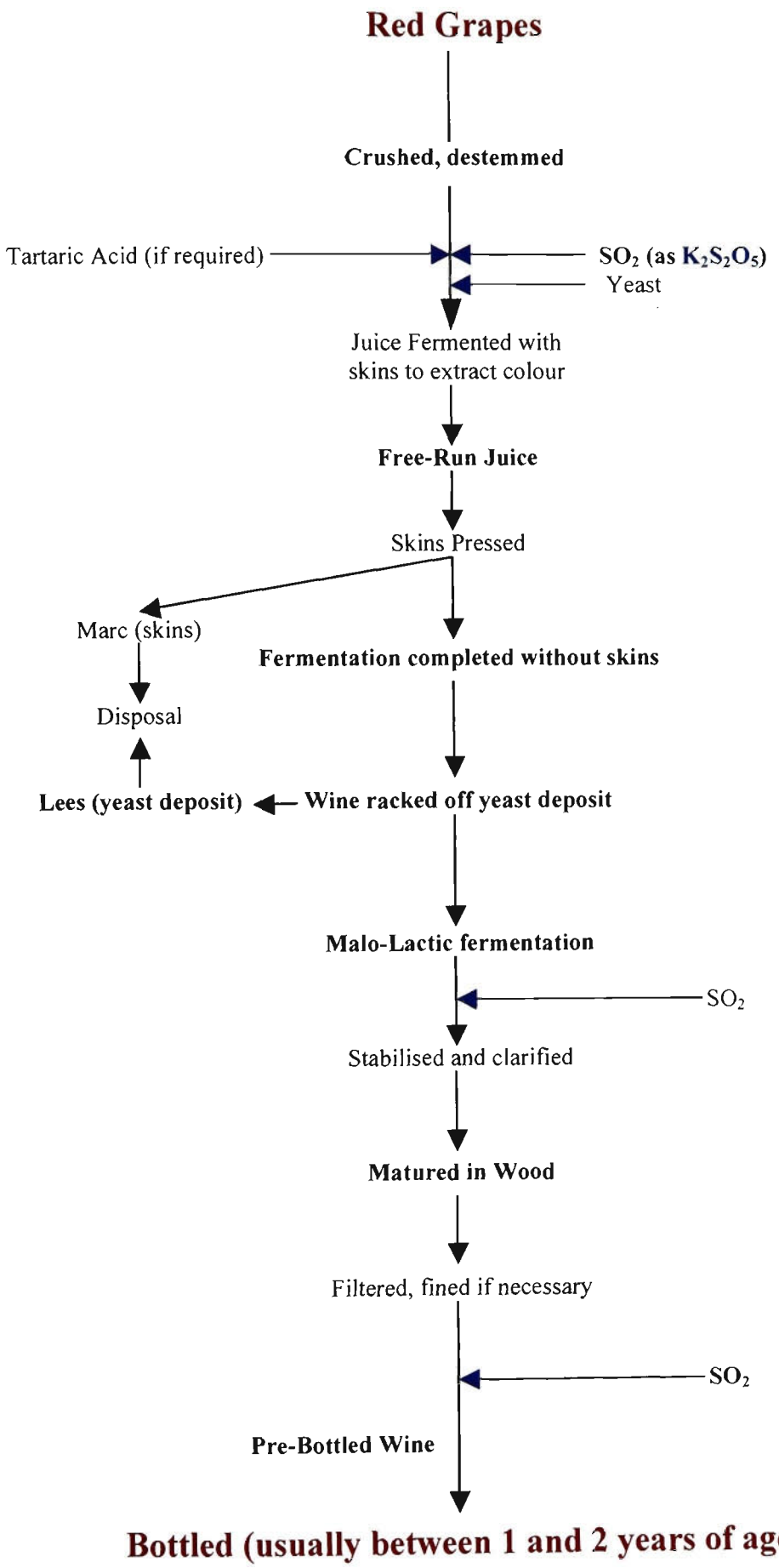


Figure 4.2 Flow chart derived from Rankine (1991) of general procedure of dry red table winemaking. Steps in bold show where samples were taken after the event. Blue text denotes wine additives.

An important note is made here about the term juice used in this Chapter. Must is normally defined as the crushed grapes with the solid material, juice is normally defined as the must with the solids

filtered off. In this study there was no distinction made between must and juice, hence both types of samples are defined in this thesis and this Chapter as 'juice'.

The major difference in making red wines as opposed to white wines is the requirement for the extraction of the colour from the skins in the form of anthocyanidins during the fermentation process (Rankine 1991). Hence, for part or all of the fermentation, the must is still in contact with the skins. Other differences are: a long aging process and malo-lactic fermentation is generally carried out for red wines whereas for white wines aging is not commonly carried out and malo-lactic fermentation is only used with some wine styles. These differences mean that samples did differ in some respects and the processing profile from the ferment concomitantly differs. In addition, bentonite fining is generally restricted to white wines, hence these samples were not taken for the red wines in this study.

Although individual winemakers used techniques or additives that are not mentioned in the above flow diagrams there were common samples taken for all wines. These common samples correspond to the steps that must be undertaken in all winemaking. These were in the majority of cases the grapes, juice, ferment, pre-bottled wine and bottled wine. The flow diagrams allow a greater understanding of how the aluminium concentrations in particular samples relate to the overall winemaking process, and these steps will be referred to later in this Chapter in the discussion of results.

In addition to the understanding of the winemaking process, reference must be made to the sample codes used throughout the study and in the presentation of results that designate sample identity and production step. The first capital letter of the code shown in bold denotes the identity of the wine and the following characters denote the type of sample. Tables 4.1 and 4.2 are presented here to assist in deciphering the sample codes matching the first letter of a code with wine identity and the code characters with the production step respectively. As mentioned previously, there was no distinction made between must and juice in this study hence all samples of these types are defined as juice.

Table 4.1 Matrix of sample identity prefixes with their variety, vineyard/winery and vintage.

Wine Identity Prefix	Vintage	Vineyard-Winery (V-W)	White Variety	Red Variety
A	1997	V-W 1	-----	Pinot Noir
B	1997	V-W 1	Chardonnay	-----
C	1997	V-W 2	Chardonnay	-----
D	1997	V-W 2	-----	Shiraz
E	1997	V-W 3	Chardonnay	-----
F	1997	V-W 3	-----	Pinot Noir
G	1997	V-W 4	Sauvignon Blanc	-----
H	1997	V-W 4	Semillon	-----
I	1997	V-W 5	Chardonnay	-----
J	1997	V-W 5	-----	Pinot Noir
K	1998	V-W 1	-----	Pinot Noir
L	1998	V-W 1	Chardonnay	-----
M	1998	V-W 2	-----	Shiraz
N	1998	V-W 2	Chardonnay	-----
O	1998	V-W 3	-----	Pinot
P	1998	V-W 3	Chardonnay	-----

Table 4.2 Matrix of sample type codes with their corresponding production steps.

Sample Code	Corresponding Production Step
S	Composite **S**oil sample
GUW	**G**rapes **U**nwashed before homogenisation
GW	**G**rapes water **W**ashed before homogenisation
J	Freshly crushed **J**uice with no SO_2
JSO2	Free-run **J**uice treated with **SO_2**
JGe	Free-run **J**uice treated with **Ge**latin
FW	**F**ermented **W**ine
PW	**P**ressed **W**ine
RW	**R**acked **W**ine
maloFW	**malo**-lactic **F**ermented **W**ine
maloFWSO2	**malo**-lactic **F**ermented **W**ine with **SO_2** added
mfW	**m**ilk **f**ined **W**ine
TW	**T**annin added **W**ine
BW	**B**entonite fined **W**ine
PrebotW	**Pre-bot**tled **W**ine
botW	**bot**tled **W**ine
(HY) within code	Sample from **H**igh **Y**ield clone grapes
(LY) within code	Sample from **L**ow **Y**ield clone grapes

The prefix and the code were combined for each sample that was collected. For example, a Pinot Noir washed grape sample that was collected from vineyard/winery 1 for the 1997 vintage is denoted as AGW. A 1998 vintage chardonnay treated with bentonite from vineyard/winery 3 would be denoted PBW. This system was used for each profile sample with two exceptions, those samples used for the grape dissection analysis described in Section 4.6 and the single fermentation study discussed in Section 4.11. These tables can be referred to for all sample identification in tables and charts for the remainder of the Chapter.

4.3 Analytical Quality Control

Quality control tests were performed for the analysis of soil by FAAS, and grape and wine by GFAAS. These will be demonstrated for each sample type by a typical standard calibration plot, repeatability, reproducibility and recovery analysis results. However, the soil results do not include a repeatability or recovery analysis. An analysis of the blanks with respect to the sample concentration will also be included. The methodology of blank, standard and sample analysis was discussed in Chapter 3.

The quality control statistics for repeatability and reproducibility are based on the standard deviation and are expressed as a relative error to the mean in terms of %RSD used for comparison of precision (Miller & Miller 2000; Larcher & Nicolini 2001). As there are numerous individual means, the repeatability and reproducibility for the method is taken as the mean %RSD of all the individual %RSD measurements as used by Almieda et al. (1997) for aluminium determination in port wine by GFAAS.

4.3.1 Soil Quality Control Analysis

A typical FAAS calibration plot for aluminium standards derived from BDH Spectrosol™ 1000mg/L stock solution made up in 0.43M acetic acid over the concentration range 0-20mg/L was found to be linear with an R^2 of 0.999. All samples were within the calibration range.

Table 4.3 shows the repeatability analysis for aluminium acetic acid soil extraction concentrations of all the samples. The repeatability is assessed by the relative error derived from the percentage

relative standard deviation (%RSD) from each sample. An estimate of overall repeatability for the method is shown as a mean relative error derived from the mean of the individual samples %RSD.

Table 4.3 Soil repeatability analysis results.

Soil Composite	%RSD
A	4.1
B	10.5
C	1.0
D	10.1
E	5.7
F	9.1
G	13.4
H	10.8
I	13.8
J	19.0
Mean	9.7

The %RSD of the aluminium extractant concentrations showed a significant amount of variance between sub-samples and accordingly the mean %RSD is almost 10%. However, when it is considered that %RSD is based on a subset of three samples, sensitive to relatively low means and outliers, this result was accepted for this study.

4.3.2 Grape & Juice Quality Control Analysis

Grape and juice samples, blanks and spiked blanks were subjected to the same pre-treatment and analysis. Hence the following analyses are equally valid for juice analyses as for grapes.

4.3.2.1 GFAAS calibration

A typical GFAAS calibration plot for aqueous aluminium standards (containing dilute nitric acid) derived from BDH Spectrosol™ 1000mg/L stock solution over the concentration range 0-60µg/L was found to be linear with an R^2 of 0.9996. All samples were within the calibration range. Similar calibration responses were noted up to 100µg/L. The result is not entirely surprising as the introduction of standard to the GFAAS is done by mechanical means, with a greater reproducibility than manually delivered standards.

4.3.2.2 Instrumental repeatability

A repeatability analysis was conducted by measuring the peak area ten times of two digested grape samples from the method development samples in the GFAAS with the furnace program described in Chapter 3. The repeatability of the instrumental method was found to be excellent with a %RSD of the absorbance peak area of 10 replicates of two samples at 0.62% and 1.73% respectively.

4.3.2.3 Method reproducibility

For each sample of grapes and juices analysed, three sub-samples were separately digested and analysed in triplicate. A %RSD was calculated for each sub-sample based on the mean and standard deviation result for the three analyses. The %RSDs were collated and method reproducibility was established from the mean and median %RSD. The results for grape and juice samples are shown in Table 4.4.

Table 4.4 Grape and Juice reproducibility analysis results.

Sample Type	Grape	Juice
Mean %RSD	14.6	12.6
Median %RSD	10.2	10.3

The mean %RSD for the GFAAS analyses shows the reproducibility of a sample over three discrete analyses is between 12-15%. However, the precision obtained for this study appears to be close to that obtained for other studies using microwave digestion and GFAAS; Yang et al. (1994) considered a %RSD better than 10% as a good result and Dolan & Capar (2002) reported a %RSD of 10% for prune juice. Considering the low concentrations that are being encountered, the absence of a clean room in which to perform sample preparation and analysis, and the requirement to store samples and analyse them over a period of 3-7 days, the precision obtained for this study is acceptable. There is some evidence that the mean %RSDs are skewed by some outliers as the median %RSD is lower at around 10%. The median %RSD shows good agreement between both grape and juice samples.

4.3.2.4 Recovery analysis

The use of standard reference materials was not practicable. To compensate for this a comprehensive aluminium recovery analysis was carried out on blanks spiked with an aluminium

standard that had undergone the same pre-treatment and analysis phases as the samples. This analysis would reflect the accuracy of the methodology in determining the actual aluminium concentration and whether there was any bias.

Grape and juice samples were subjected to microwave digestion prior to subsequent GFAAS analysis. Twelve samples were digested in each batch and of these, three were either blank or spiked blanks. Generally, four batches were digested in one session, so that of 48 analysable solutions, six were blanks and six were spiked blanks. However as the number of spaces in the GFAAS autosampler carousel was limited to 45, only 9 solutions were digested in the fourth batch. Recoveries were conducted for each set of digestions and were calculated by subtracting the mean blank concentrations from the spiked blank concentrations and relating the adjusted concentrations to the actual concentration of the aluminium spike. The mean recovery of the spiked aluminium blanks for both grape and juice methodology was found to be 114% (n=52).

This result implies that there is a bias in the method. The reason for this (as discussed in Section 3.6.2) is that the digestion vessels are contributing some aluminium from previous digestions (despite the blank digest pre-treatment) and the high likelihood of some contamination, despite the precautions and minimal handling steps. The recovery result was not surprising considering the absence of a clean room and that digestions were carried out with concentrated acids in vessels whose prior use was outside the control of this investigation. However, taking these considerations into account and as sample concentrations were well above those of the blanks the recovery result was deemed acceptable.

4.3.3 Wine Quality Control Analysis

The following quality control analysis pertains to all samples that have been defined as wine, whether newly fermented, bottled or a production stage sample somewhere in between. This categorisation is drawn from the fact that all these samples are pre-treated and analysed using the same methodology, whereas the pre-treatment is very different for grapes and juices. For wine samples no digestion method has been employed, however the GFAAS method is virtually unchanged from that employed for grapes and juices.

4.3.3.1 Instrumental repeatability

The repeatability analysis was conducted on two wine samples, I (white) and J (red), that were acidified and 20-fold diluted. Their GFAAS peak area was measured 10 times for each sample. The repeatability of 10 replicates of two diluted wine samples is excellent with %RSD of 2.1% and 2.0% respectively. Both samples show %RSD in good agreement with each other and with those obtained for the grape and juice GFAAS repeatability studies. These results demonstrate that the instrumental methodology for the GFAAS analysis of aluminium concentration in wine is precise within an analysis.

4.3.3.2 Method reproducibility

A test of reproducibility was conducted for 20-diluted wine samples in the same fashion as that for grapes and juice. The %RSD for each individual 20-fold diluted sub-sample was calculated from their mean and standard deviation of three separate GFAAS analyses. These were then collated and the overall mean %RSD determined. The reproducibility result is demonstrated in Table 4.5.

Table 4.5 Wine reproducibility analysis results.

Reproducibility Statistic	Result
Mean %RSD	10.1
Median %RSD	6.6

Again the reproducibility reflects the greater sources of error associated with a greater number of analytical steps as it is not as precise between analyses as the repeatability within an analysis. However, it is better than that for the grapes and juice (see Section 4.3.2.3) due to less likelihood of contamination. Again the lower median %RSD compared with the mean %RSD suggests that the distribution of %RSD data is skewed by some outliers.

4.3.3.3 Recovery analysis

A recovery test was conducted on the same wine samples as used for the repeatability analysis, namely wine I and J. A 30μg/L spike was added to the diluted wine samples and their aluminium concentrations were determined as well as the diluted samples themselves. After subtracting the diluted wine samples aluminium concentration from that of the spiked solutions, the difference was compared with the theoretical solution spike concentration. This analysis is shown in Table 4.6.

Table 4.6 Recovery analysis of diluted wine samples with their corresponding aluminium spiked samples for wines I and J.

Sample	Wine I	Wine J
Wine Mean [Al] (µg/L)	23.8	20.9
Spiked Wine Mean [Al] (µg/L)	54.9	52.8
Mean Experimental Spike [Al] µg/L	31.1	32.0
Actual Spike [Al] (µg/L)	30.0	30.0
Recovery (%)	104	107
Mean Recovery (%)	105	

Table 4.6 shows a good recovery of 105%, demonstrating that the methodology for the pre-treatment and the analysis of wines is more accurate with less bias than that observed for the grapes and juice. This is not surprising given that the matrices of the two sample types differ and the number of sample handling steps is less, with less opportunity for contamination, particularly from digestion vessels as is the case for grapes and juice pre-treatment. There was good agreement between the recoveries of the red and white wines.

4.3.4 Blank Results

Another important QC consideration was the blank response. As is evident in Chapter 3, establishing methods that could demonstrate effective management and minimisation of contamination formed a major part of the method development. Contamination was monitored by accompanying all batches of samples with blanks that were exposed to the same contamination derived from the reagents and encountered throughout the methodology. As the complete elimination of contamination was impossible, it was taken into account by adjusting each sample's aluminium concentration. This was done by subtracting the mean blank aluminium concentration calculated for each sample set from each samples mean i.e. the mean aluminium concentration of 6 blanks in a set of 45 samples is subtracted from the mean aluminium concentration of each sample. This process is repeated for each analysis set of 45 samples. A comparison of the mean blank aluminium concentrations of all sets of samples with the samples average aluminium concentrations is shown in Table 4.7.

Table 4.7 Blank analysis results.

Sample Type	Soil (mg/L)	Grape & Juice (µg/L)	Wine (µg/L)
Mean Blank [Al]	0.58	10.92	4.39
Std Deviation Blank [Al]	0.70	5.09	2.49
Mean Sample [Al]	7.35	51.52	29.71
% Ratio Blank/Sample	7.9%	21.2%	14.8%

The blank analysis results demonstrate that the soil analysis shows the best blank to sample ratio percentage of about 8% while the worst ratio percentage is shown by the grape and juice analysis with just over 21%. The blank to sample ratio of wine is in between, at almost 15%. These differing ratios are easily explained. Wines require less vigorous sample pre-treatment than grapes and juices, and soils require even less pre-treatment again, providing less opportunities for contamination and hence lower blanks. The concentration of the aluminium sample is also a factor as grapes, juices and wines have much lower concentrations than soils therefore the blanks become a more significant component of the analytical signal. While the percentage ratios of blank:sample concentrations are not exceptional, the differences between blank and sample aluminium concentrations are great enough to use blank correction on sample aluminium concentrations and discern the actual concentration with a degree of accuracy and confidence.

4.4 Soil Analysis Results

This section presents the results of the soil sample analysis. As described in Chapter 3, the five soil samples per vineyard were sub-sampled into one composite sample. These composites were subjected to extraction with 0.43M acetic acid and pH measurement to determine the bioavailable (acetic acid extractable) aluminium concentration and any relationship between this concentration and soil pH. The aluminium concentrations were recorded as field moist concentrations. Although dry weight concentrations are more commonly used for official soil analysis (Tessier et al 1979; EPA Victoria 1993; Davidson et al 1994), field moist samples were analysed because the aim was to extract the available aluminium directly and avoid redepositing it onto the dry soil and then re-extracting it. Dry weight aluminium concentrations can be calculated using moisture content values given in Table 4.8. The measurement of soil pH was conducted on all five samples from each

vineyard. From this data a mean soil pH was derived for each vineyard representing the field moist soil pH. The acetic acid extractable soil aluminium concentrations and soil pH measurements are shown in Table 4.8.

Table 4.8 Acetic acid extractable concentrations and pH of vineyard soils.

Vineyard/Winery	Sample	Field Moist Acetic Acid Extractable [Al] (µg/g)	pH	Moisture Content (%)
1	AS	27.1 ±1.1	6.7 ±0.2	3.6
	BS	43.0 ±4.5	5.4 ±0.3	1.9
2	CS	67.1 ±0.7	5.6 ±0.1	2.3
	DS	22.4 ±2.3	5.8 ±0.2	2.1
3	ES	81.5 ±4.6	4.7 ±0.1	10.7
	FS	70.2 ±6.4	4.9 ±0.2	9.6
4	GS	82.5 ±11.1	5.6 ±0.7	14.3
	HS	68.6 ±7.4	5.4 ±0.5	15.5
5	IS	27.9 ±3.9	5.0 ±0.2	16.1
	JS	61.9 ±11.8	5.0 ±0.3	14.4

The acetic acid extractable soil aluminium concentrations range from 27.1µg/g to 82.5µg/g. The average moisture content was 9.0%, however this varied widely from 1.9% to 16.1%. The soil acetic acid extractable aluminium concentrations are compared in Figure 4.3.

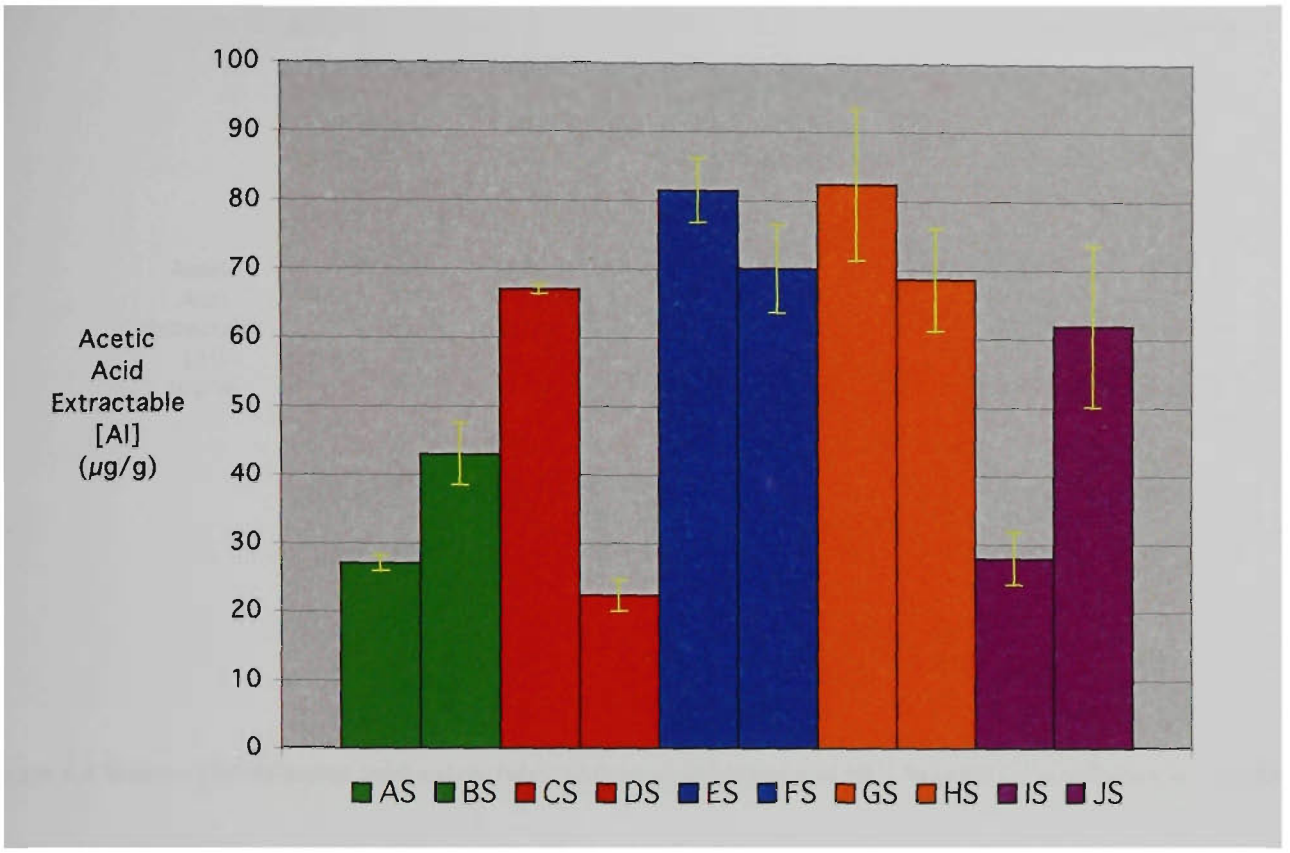


Figure 4.3 Comparison of acetic acid extractable aluminium concentrations (µg/g) of the vineyard soil composite samples (n=5). Colour coding denotes vineyard, hence the bioavailable soil aluminium concentrations for both the white and red wines studied can be compared. The error bars denote the standard deviation of triplicate analyses.

Table 4.9 t-test results of comparison of acetic acid extractable soil aluminium concentrations between soils from cultivars from the same vineyard. Results are presented as significance of difference and confidence concentration.

t-test (comparing soil aluminium concentrations)	t-test Result (two tailed)	Confidence Concentration
Soil [Al] Sample A vs B	Significant difference	97%
Soil [Al] Sample C vs D	Significant difference	99.9%
Soil [Al] Sample E vs F	Significant difference	93%
Soil [Al] Sample G vs H	**Insignificant difference**	**<90%**
Soil [Al] Sample I vs J	Significant difference	96%

As can be seen from the chart (Figure 4.3) and the t-test results (Table 4.9) there is a significant difference between the acetic acid extractable concentrations of soils from the same vineyard with 93% confidence upwards, except for vineyard 4. The pH varied widely as well, with a range of an acidic 4.70 to an almost neutral 6.69. As the solubility of aluminium has been related to soil acidity (Furrer 1992; Wolt 1994; Ritchie 1995; Jardine & Zelazny 1996), a correlation analysis was carried out between the extractable aluminium concentrations and soil H^+ ion concentration. This analysis is shown in a scatter plot in Figure 4.4.

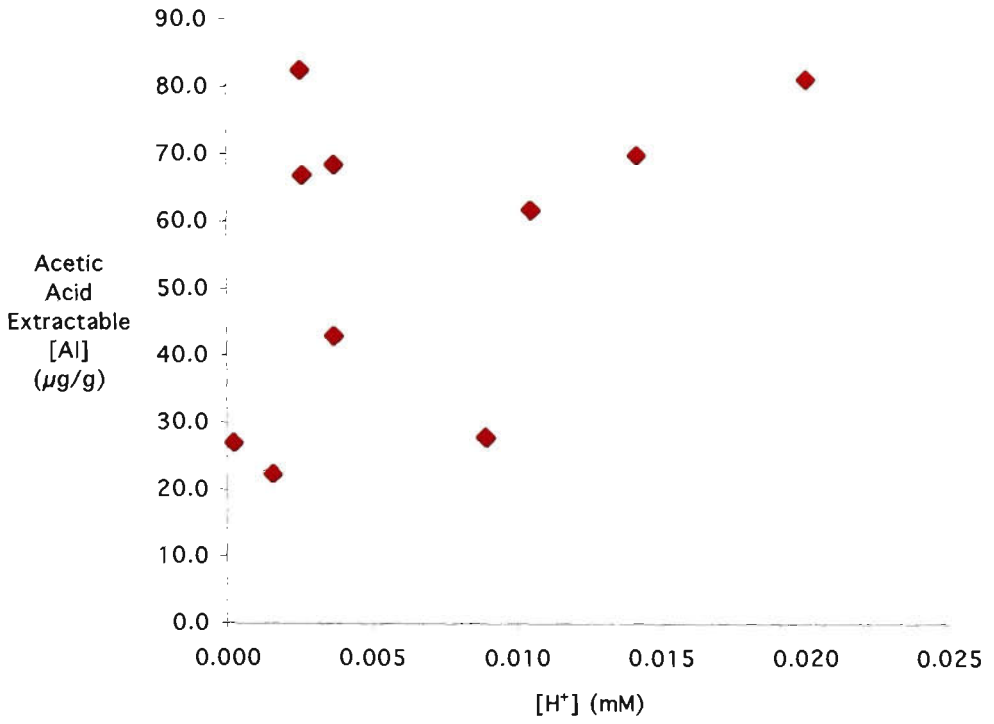


Figure 4.4 Scatter plot of acetic acid extractable [Al] in µg/g versus soil [H^+] in (mM). Correlation R^2 is 0.46.

The scatter plot shows no definitive relationship between the extractable aluminium concentration of the soil and the soil hydrogen ion concentration with a correlation co-efficient of. 0.46, although a trend is perhaps noted as [H^+] increases. This implies that the acidity of the soil is only one of a number of factors including soil type, surface area and extent of irrigation/rainfall that determine bio-available aluminium in the soils studied. This result is not entirely unexpected as the soil pH in all samples measured here is higher than that required to liberate Al^{3+}, and other soil factors such as binding to dissolved organic matter including organic acids would probably have more effect on mobile and bioavailable aluminium soil distribution at the soil pH concentrations encountered in this study (Jansen et al. 2002; Mitrovic & Milacic 2000; Yamada 2002). Xu & Ji (2001) reported the mobilisation of aluminium in soil also relied more on the type of acid and the charge type of the soils rather than the H^+. Further correlation analysis of bioavailable soil aluminium with the concentration encountered in the grapes is discussed in Section 4.5.

4.5 Grape Analysis Results

The results of the analysis of total aluminium concentrations (wet weight) of the homogenised grape samples are presented for both those washed with Milli-Q® water and those remaining in the same state as sampled from the grape vine. This separation of the grapes samples was performed to

determine the amount of aluminium deposited onto the grape from atmospheric and/or anthropogenic sources. The grape samples for each wine studied were homogenised and combined to form a composite sample in the same manner as the soil samples. Thus, these results represent the total aluminium of the whole grape, including skins, pip, pulp, and juice for a given cultivar at one vineyard and are quantified in terms of μg/g. The distribution of the aluminium within a single grape will be discussed in Section 4.6. The results for the total aluminium analysis of all the composite homogenised grape samples from both vintages are presented in Table 4.10.

Table 4.10 Total aluminium concentrations (wet weight) of composite grape samples. White and red wine samples are labelled light green and burgundy respectively.

Unwashed grape sample	[Al] (μg/g)	s.d.	Washed grape sample	[Al] (μg/g)	s.d.
1997 Vintage			**1997 Vintage**		
AGUW	6.1	±1.0	AGW	3.7	±0.9
BGUW	3.2	±0.4	BGW	3.09	±0.04
CGUW	3.1	±0.4	CGW	1.7	±0.6
DGUW	2.6	±0.7	DGW	1.8	±0.8
EGUW	1.8	±0.4	EGW	1.3	±0.2
FGUW	1.9	±0.2	FGW	1.7	±0.5
GLYGUW	2.6	±0.6	--------	-----	------
GHYGUW	3.1	±1.7	GHYGW	2.7	±1.1
HGUW	2.8	±1.4	HGW	2.1	±0.2
IGUW	3.6	±1.2	IGW	2.1	±0.3
JGUW	1.4	±0.6	JGW	1.7	±0.4
1998 Vintage			**1998 Vintage**		
KGUW	6.5	±0.5	KGW	4.3	±0.5
LGUW	4.38	±0.08	LGW	3.4	±0.3
MGUW	2.6	±0.2	MGW	1.1	±0.7
NGUW	1.8	±0.3	NGW	2.1	±0.4
OGUW	4.0	±0.8	OGW	1.3	±0.8
PGUW	2.1	±0.1	PGW	1.4	±0.4

The results show that in most cases the mean aluminium concentrations of the unwashed grapes is higher than the mean aluminium concentrations of the washed grapes. However, the uncertainty of measurement is quite high with samples IGUW, GHYGUW, GLYGUW and GHYGW showing a %RSD greater than the average 21%. The high uncertainty means the overlap of the two sample sets' standard deviation is substantial which could offer an explanation for the two exceptions to the

general trend (**N**GUW→**N**GW; **J**GUW→**J**GW); an alternative possibility is these samples genuinely contained very little deposited aluminium on the skins. The average difference of the overall mean aluminium concentrations for the two sets of samples is 0.94μg/g. A t-test analysis of these two groups of samples shows this difference is significant with 95% confidence. Because of the high uncertainty, a t-test was also carried out on the individual triplicate results as a group. The difference was also significant at greater than 99% confidence (n = 51 for GUW, n = 48 for GW).

The reason for this difference is simple: grapes are exposed to the open atmosphere until crushed and with airborne particles containing aluminium, dust deposits on the skins as the grape matures to vintage and remains on the skins unless washed. This deposition is also random, hence the variation in the difference of the aluminium concentrations. High grape skin deposited aluminium concentrations observed for samples A and K (these are from the same vine stock but different vintages) could be due to the close proximity of a major road to the sampling sites, however this hypothesis is purely speculative. Eschnauer & Scollary (1995) have also suggested that aluminium-containing pesticide sprays could contribute to aluminium concentrations of grapes, however, no analysis of sprays was conducted for this study. A chart showing a comparison of the aluminium concentration of all unwashed grapes with their corresponding washed grapes is shown in Figure 4.5.

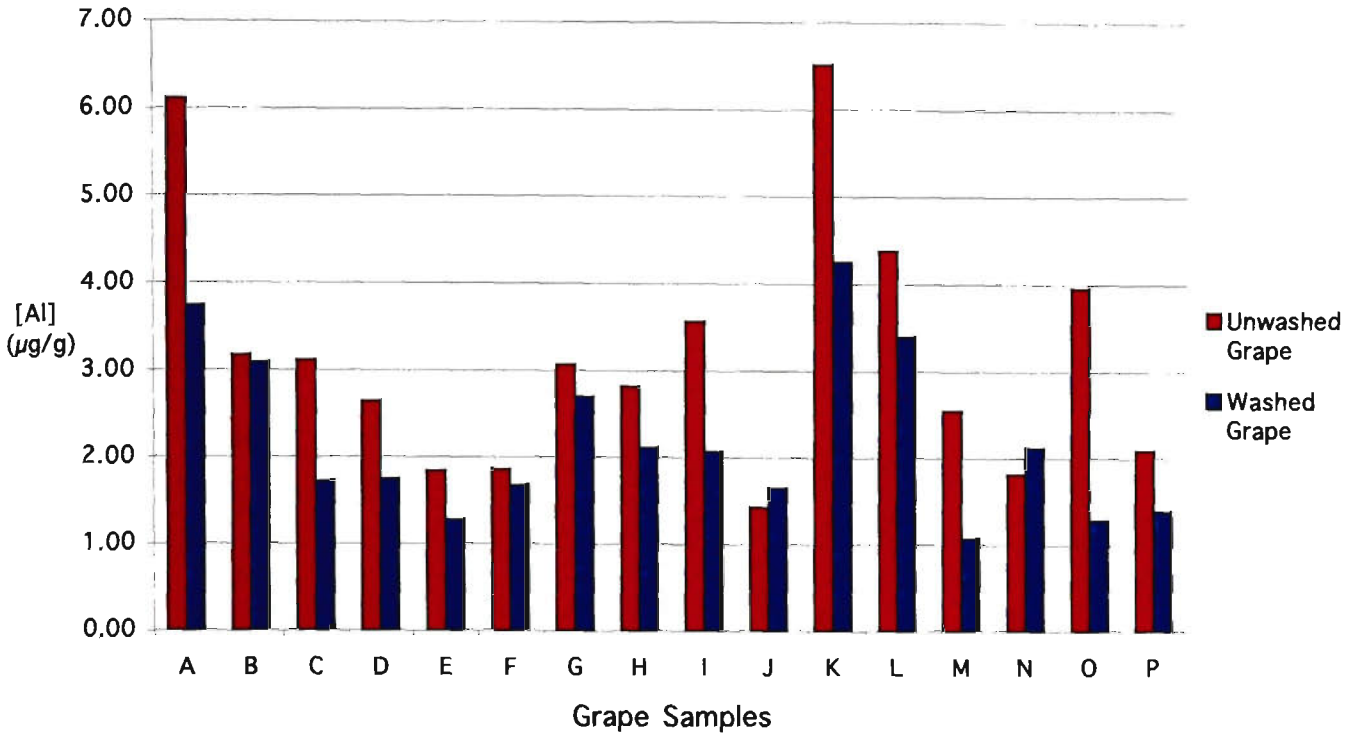


Figure 4.5 Comparison of total aluminium concentrations (wet weight) of unwashed and washed grapes. The comparison for the H sample represents that of the high yield clone grapes. Samples A-J represent the grapes from the 1997 vintage, samples K-P represent those from the 1998 vintage.

With results from both soil and grape samples, a correlation analysis was conducted to see if there was any relationship between the acetic acid extractable soil aluminium concentration and the aluminium concentration of the grape samples. In addition, although there was no discernable relationship between the extractable soil concentration and soil pH a correlation analysis was conducted to determine any relationship of grape aluminium concentration with soil pH. The resultant scatter plots are shown in Figures 4.6 and 4.7.

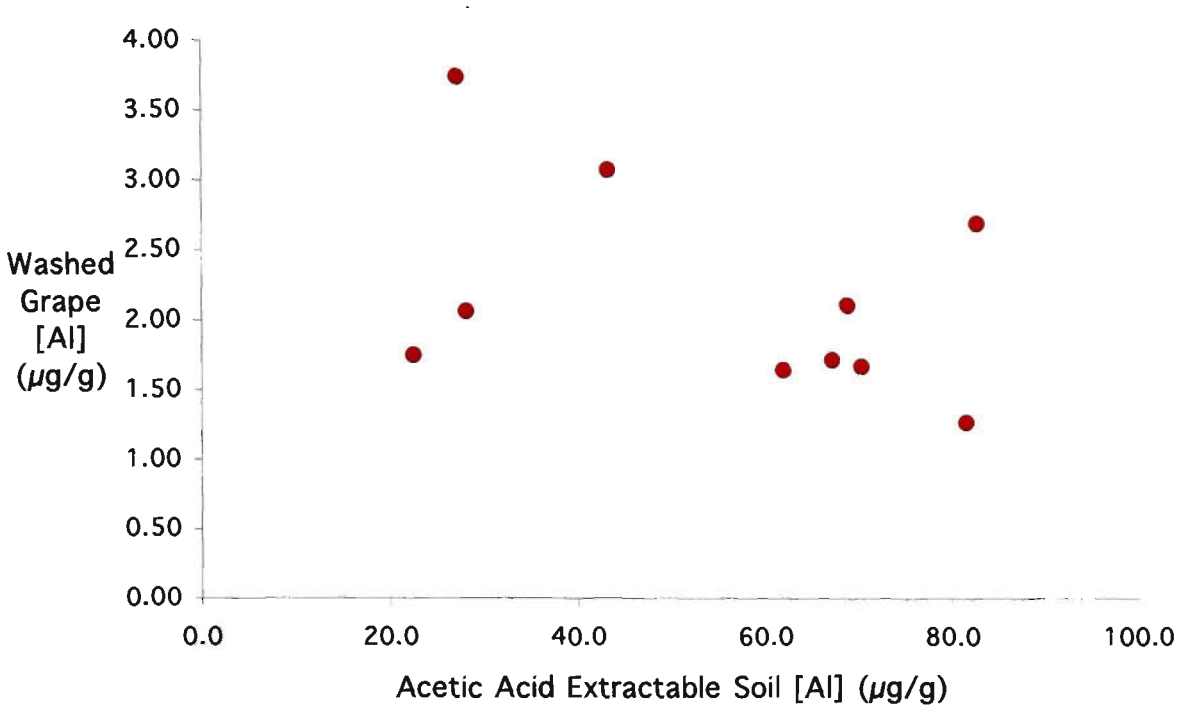


Figure 4.6 Scatter plot of grape [Al] (wet weight) against the corresponding soil acetic acid extractable [Al]. Correlation R^2 is -.40.

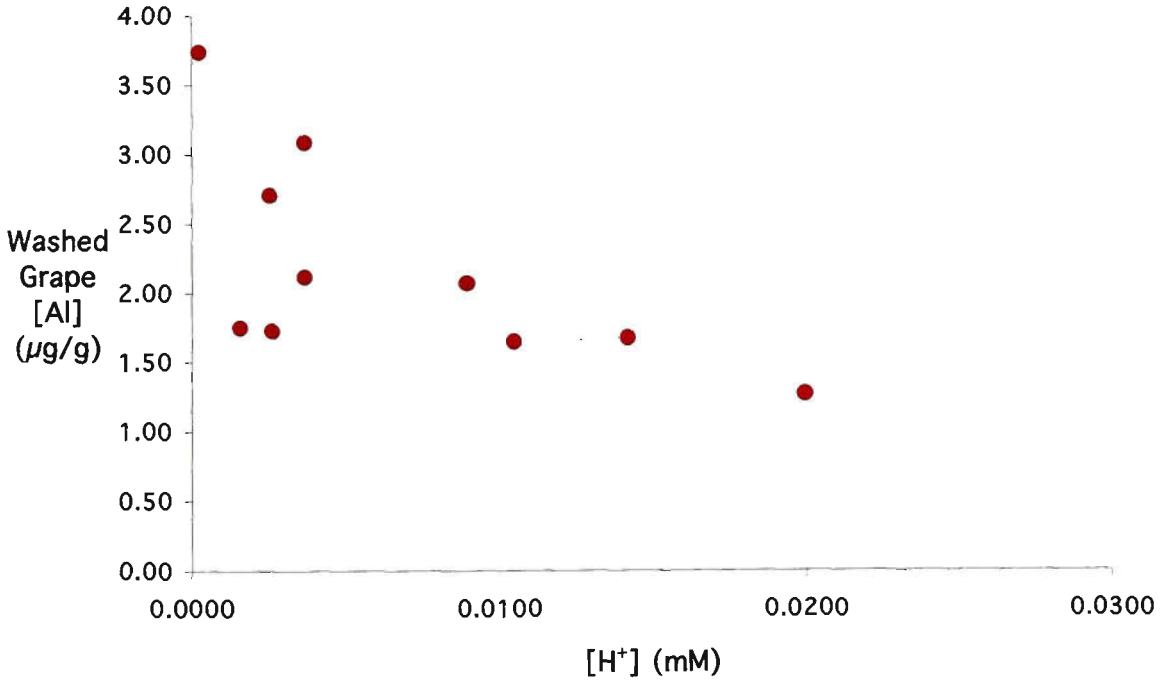


Figure 4.7 Scatter plot of grape [Al] (wet weight) against soil hydrogen ion concentration in mM. Correlation R^2 is –0.64.

The scatter plot of Figure 4.6 shows that there is no significant correlation between the soil acetic acid extractable aluminium concentration and the aluminium concentration of the grapes after washing. However, Figure 4.7 suggests that there is some inverse relationship between soil hydrogen ion concentration and grape aluminium concentration but the correlation was not significant with a correlation co-efficient of –0.64. This inverse nature of the relationship implies that the uptake of aluminium by the vine into the grape is not wholly dependent on free Al^{3+}

concentration, which would be become less available in soils with a higher pH. The results again suggest that uptake of aluminium from the soil to the grape is influenced by more factors than pH driven soluble Al^{3+} concentration and that acetic acid extractable aluminium is a limited estimation of the bioavailable soil aluminium for grape vines and grapes. However it is clear from the aluminium concentrations of the washed grape samples that aluminium is drawn by the grape vine and deposited into the grape, and hence a 'natural' source of aluminium into wine. Its distribution within the grape itself is discussed in the next section.

4.6 Aluminium Distribution in Wine Grapes

The analysis of the washed grapes shows that some of the aluminium in wine originates from natural sources. The majority of the aluminium is contained within the grape itself, transported there and to other organs in the vine from the soil via the roots and sap (Enkelmann & Wohlfarth 1994). However, some is derived from deposition on the skins from natural and anthropogenic sources, thus it is recommended that for fruits the skin be analysed for surface contamination (Enkelmann & Wohlfarth 1994; Sutton & Heitkemper 2000). As will be seen from the overall aluminium profile in Section 4.10, this concentration of aluminium in the whole grapes showed higher concentrations than those observed for the juice and wines which will be discussed in greater detail in later sections. Importantly the concentrations presented in Section 4.5 are for the homogenised whole grape including the skin, pip, pulp/juice similar to that used in a grape lead distribution study by Teissedre et al. (1994b). Referring to the schematic diagrams of wine making shown in Figures 4.1 and 4.2, one can see that the skins and pips are removed from the white juice prior to fermentation, although for red wines the fermentation is carried out on the skins and pips, hence the wine has some contact with these solids. Although lead has shown a propensity for extraction from the solid matter in contact with the juice and ferment (Teissedre et al. 1994b), no similar study has been conducted for aluminium. A study of the extraction of aluminium from the solid material would require close monitoring in a controlled system where all the components masses are known. This type of investigation was not possible for this project; hence, in calculating the amount of aluminium derived from the grape, an assumption was set whereby aluminium bound in solid matter would remain there.

To determine what proportion of aluminium is carried over to the juice, a distribution of the element within the grape was required. A distribution analysis was performed where two sets of triplicate composite samples of grapes randomly drawn from a bunch of red (sample A) and white (sample B) grapes were dissected into three components (namely pulp/juice, pip and skins). These were then digested and analysed for their aluminium concentration (wet weight) as per the method used for the homogenised grape samples. The results of this analysis are presented in Table 4.11.

Table 4.11 Grape dissection [Al] (wet weight) distribution analysis.

Grape Component	A (red) Mean [Al] (µg/g)	B (white) Mean [Al] (µg/g)
Pip	7.5 ±3.5	5.8 ±1.2
Skin	5.6 ±1.4	3.5 ±1.0
Flesh & Juice	6.7 ±2.8	2.6 ±1.4

The contribution, in terms of mass, for the three components was calculated by determining the average mass of each component in a 1g grape from the composite grape component masses, measured as a percentage of the total grape mass. Using the concentrations in Table 4.11 an average mass of aluminium in a theoretical 1g grape was determined from both samples A & B to give an average grape mass independent of cultivar. These results are shown in Table 4.12.

Table 4.12 Mass analysis of aluminium in a theoretical 1g grape derived from the combined mean data of grapes A and B.

Grape Component	Component mass per 1g grape (g)	Mean Component Al mass per 1g grape (µg/g)
Pip	0.10	0.67
Skin	0.33	1.45
Flesh & Juice	0.57	2.70
Total Grape	**1.00**	**4.83**

Table 4.12 shows that the highest mean aluminium concentration per component is observed in the pip samples A and B, however Table 4.12 shows that, as the average pip mass is one tenth of the whole grape, its average actual mass proportion in the grape is the lowest. The average mass of aluminium in flesh and juice was almost double that of the skin average mass. The total concentration of the aluminium calculated in a 1g grape (calculated at 4.83µg/g and derived from

data from grapes A and B) is in agreement with the mean unwashed homogenised grape concentrations of sample A and B which is 4.64µg/g (see Table 4.10).

The distribution of aluminium will be unique for each grape due to different cultivars and varied conditions encountered for that particular vintage giving different yields and grape sizes, which ultimately determine the amount of flesh, juice, pip and skin. An estimate of the aluminium distribution in a grape using the data in Table 4.12 for the three grape compartments studied is presented in Figure 4.8.

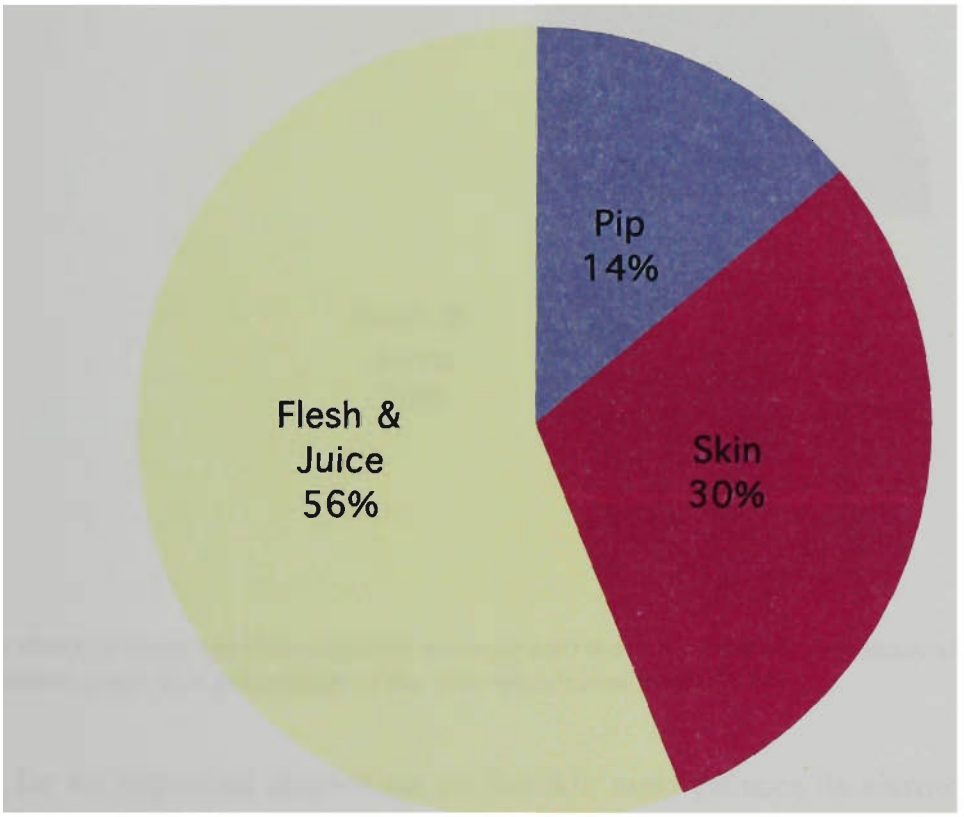


Figure 4.8 Pie chart showing average distribution of the average mass of aluminium in different parts of the unwashed grape as a percentage of the total aluminium load of 4.83µg/g.

The distribution shows that just over half the aluminium content of a grape is in the flesh and juice combined. This differs from the situation of lead, which has been shown to reside mainly in the pips (65%) with only 35% in the pulp (Teissedre et al. 1994b). However, as the dissection analysis was carried out on unwashed grapes to reduce compromises in mass introduced by water, some of the aluminium attributable to the skin would be due to the aluminium derived from air deposition. From the data in Table 4.10, the mean concentration of aluminium from air deposition of sample A and B is 1.23µg/g. Therefore, for a theoretical 1g grape sample, 1.23µg of aluminium attributed to the skin

would not be included in the vine derived aluminium distribution of grapes, leaving 0.22µg/g aluminium in the skins themselves. Hence, the distribution shown in Figure 4.8 is recast in Figure 4.9. The adjusted total concentration of aluminium calculated in a 1g grape is 3.59µg/g which is in close agreement with the average washed grape concentrations of sample A and B which is 3.42µg/g (see Table 4.10).

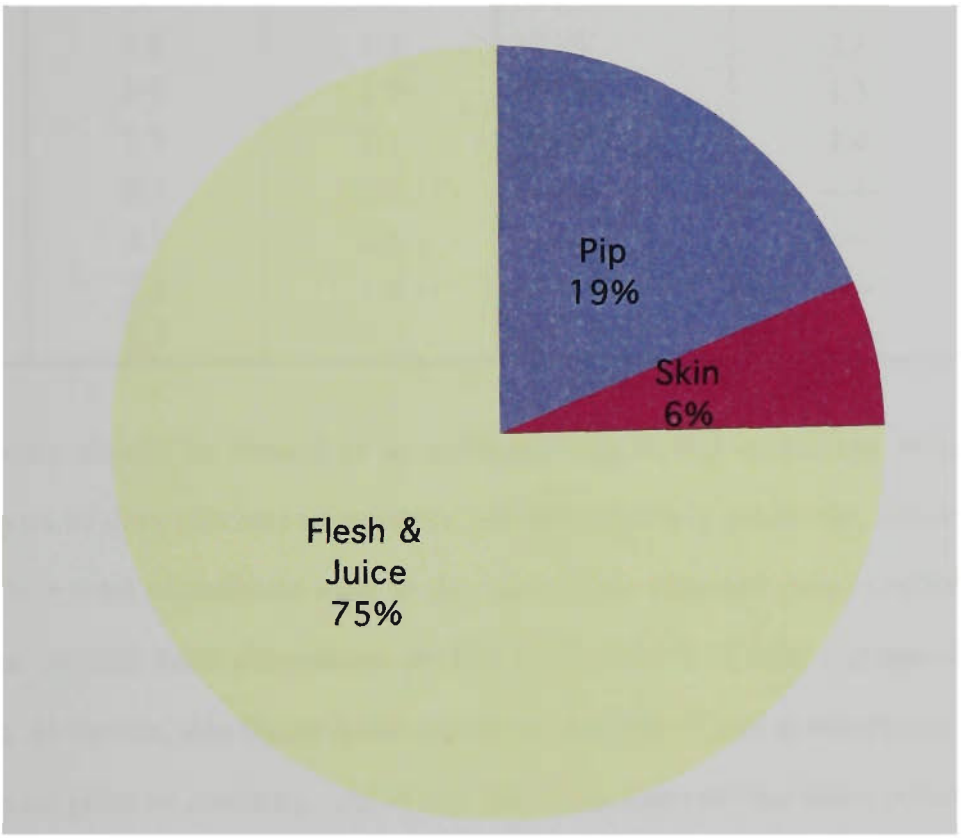


Figure 4.9 Pie chart showing the skin adjusted average distribution of the average mass of aluminium in different parts of the washed grape as a percentage of the total aluminium load of 3.59µg/g.

Accounting for air deposited aluminium on the skin mass reduces its aluminium proportion in a grape and increases that in the other components, most prominently the flesh and juice which now account for three-quarters of the grape aluminium mass. This suggests that of the homogenised grape samples, only 75% of the aluminium in washed grapes can be transferred to the juice. Hence, the washed grape aluminium concentrations need to be adjusted to more closely reflect the aluminium available for transfer to the juice. The mean grape concentration data for the washed grapes presented in Table 4.10 is shown again in Table 4.13 with the data adjusted to account for the available aluminium in the grape samples.

Table 4.13 Adjusted washed grape wet weight aluminium concentrations (assuming 75% of grape aluminium is available to the wine).

Washed grape sample	[Al] (µg/g)	Adjusted [Al] (µg/g)	Washed grape sample	[Al] (µg/g)	Adjusted [Al] (µg/g)
1997 Vintage			1998 Vintage		
AGW	3.7	2.8	KGW	4.3	3.2
BGW	3.09	2.31	LGW	3.40	2.55
CGW	1.7	1.3	MGW	1.1	0.8
DGW	1.8	1.3	NGW	2.1	1.6
EGW	1.3	1.0	OGW	1.3	1.0
FGW	1.7	1.3	PGW	1.4	1.0
GHYGW	2.7	2.0	-----	-----	-----
HGW	2.1	1.6	-----	-----	-----
IGW	2.1	1.6	-----	-----	-----
JGW	1.7	1.2	-----	-----	-----

These adjustments should be treated as an estimate only as the correction is based on the grape dissection analysis of only two sets of samples. An assumption is made that, on average, grapes will yield 75% of their total aluminium load to the juice. This adjusted data is referred to later in the analysis for the overall total aluminium profile in Section 4.10 and compared with that of the unadjusted data. However, this figure holds true provided one of two assumptions are made; that the grapes are washed prior to crushing, and if not, the aluminium on the skins is not released into the juice (for white wines prior to fermentation) or the wine (for red wines during fermentation on the skins). The second assumption is unlikely; as it has been shown that washing with water removes aluminium, then assuming that none of the aluminium would be leached by juice or acidic wine is probably inadequate. If the grapes have not been washed then the percentage of the mass of aluminium derived from the skin lies somewhere between 6-30% and inversely that of the pulp and flesh somewhere between 75-57%. Regardless of the exact figure, the distribution charts demonstrate that the majority of the aluminium in the wine grape is derived from the juice and flesh.

4.7 Juice Analysis Results

The results of the analysis of aluminium concentrations (wet weight) for the juice samples straight from the crusher before sulfur dioxide addition and after sulfur dioxide addition are presented. From this point on there is not a sample for every wine that was made due to different processes or

sampling difficulties. Hence, the juice not treated with metabisulfite was only collected for the 1997 vintage, and of these wines some were not treated with metabisulfite or a sample was unable to be collected. Unique for this study and for the first season, juice C was taken after gelatin addition. For the second season all juice samples collected were those treated with metabisulfite. The concentrations initially reported are in µg/g as the samples were treated as solids. However, with the changing nature of the sample, a density experiment was conducted to convert the concentration units from µg/g to mg/L to more easily compare the juice aluminium concentrations with those in the ferment and the results are presented at the end of this section. The results for the total aluminium analysis of all the juice samples from both vintages are presented in Table 4.14.

Table 4.14 Total aluminium concentrations (wet weight) of juice samples. White and red wine samples are labelled light green and burgundy respectively.

SO_2 free Juice sample	[Al] (µg/g)	s.d.	SO_2 treated Juice sample	[Al] (µg/g)	s.d.
1997 Vintage			**1997 Vintage**		
AJ	2.5	±0.3	$AJSO_2$	3.1	±0.2
BJ	1.6	±0.1	$BJSO_2$	1.1	±0.2
----	-----	-----	$CJSO_2$	1.9	±0.2
----	-----	-----	CJGe*	1.8	±0.3
DJ	2.5	±0.4	----	-----	-----
EJ	1.57	±0.02	$EJSO_2$	0.9	±0.4
FJ	1.8	±0.3	----	-----	-----
----	-----	-----	$GLYJSO_2$	0.7	±0.7
----	-----	-----	$GHYJSO_2$	0.3	±0.2
----	-----	-----	$HJSO_2$	0.6	±0.3
IJ	0.5	±0.1	----	-----	-----
JJ	0.4	±0.1	----	-----	-----
1998 Vintage			**1998 Vintage**		
----	-----	-----	$KJSO_2$	3.1	±0.7
----	-----	-----	$LJSO_2$	3.7	±0.2
----	-----	-----	$MJSO_2$	2.0	±0.1
----	-----	-----	$NJSO_2$	0.23	±0.04
----	-----	-----	$OJSO_2$	2.4	±0.3
----	-----	-----	$PJSO_2$	0.29	±0.02

(*) This sample, although containing SO_2, had gelatin added afterwards. This sample was not included in the means in this table or statistical analyses and should only be compared with the previous sample $CJSO_2$. To reiterate symbol meanings, the first letter denotes the wine cultivar, J denotes juice, SO_2 denotes sulfur dioxide addition, Ge denotes gelatin addition and LY and HY denote low yield clone and high yield clone respectively.

From this set of data there is no conclusive trend although the uncertainty is substantially lower than that observed for grapes. Two t-tests comparing the 1997 data and the overall data bear this observation out, with both tests showing no significant differences between the sample sets at 95% confidence, albeit with such a small data set the value of this analysis is limited. A chart showing the comparison of the total aluminium concentration of all SO_2 free juice with that of the SO_2 containing juice for both vintages is shown in Figure 4.10.

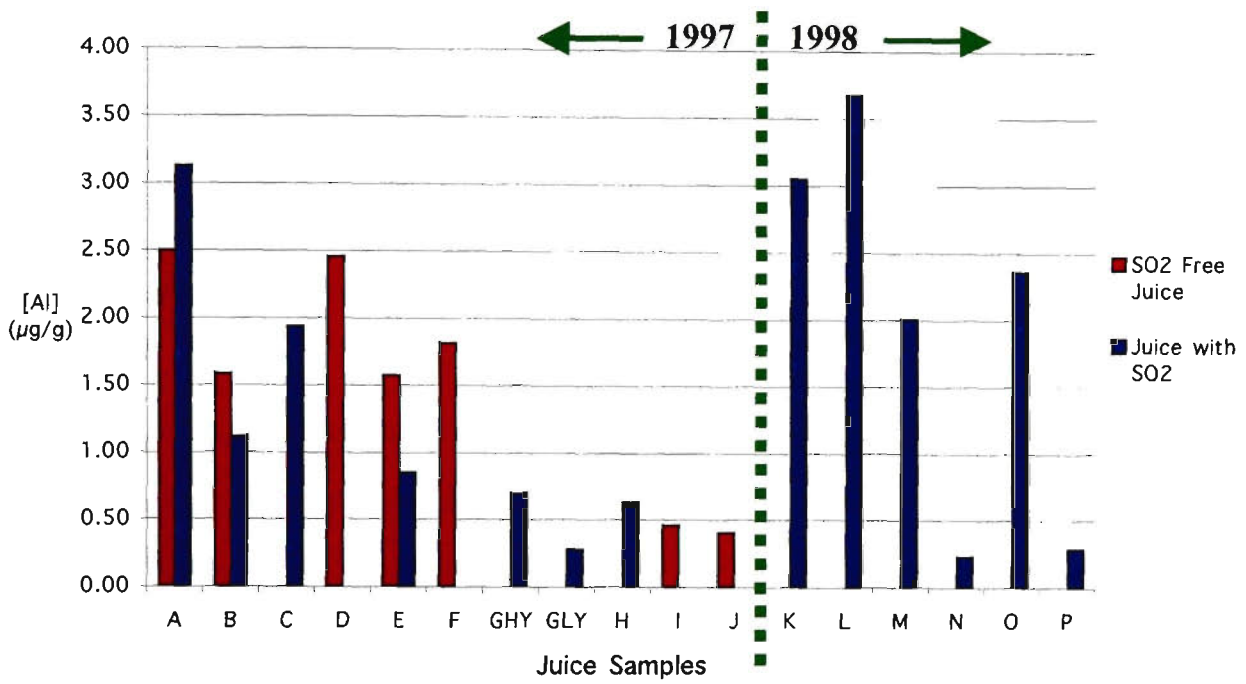


Figure 4.10 Comparison of total aluminium concentrations of SO_2 free and SO_2 containing juices. Samples A-J represent the grapes from the 1997 vintage, samples K-P represent those from the 1998 vintage.

The juice aluminium concentrations, both those of the SO_2 free juice samples and the SO_2 containing samples, were statistically compared with the unadjusted and adjusted aluminium concentrations of the washed grape samples by a t-test. The results matrix for these analyses is shown in Table 4.15.

Table 4.15 t-test results of comparison of grape aluminium concentrations with those of juices. Results are presented as significance of difference and confidence concentration.

t-test (comparing aluminium concentrations)	t-test Result (one tailed)	Confidence Concentration
SO_2 free Juice with washed Grapes	Significant difference	95%
SO_2 free Juice with adjusted washed Grapes	**Insignificant difference**	**95%**
Juice with SO_2 with washed Grapes	Significant difference	93%
Juice with SO_2 with adjusted washed Grapes	**Insignificant difference**	**95%**

The t-test results show that when using the washed grape concentrations that include aluminium from both skin and pip, there is a significant difference between the aluminium concentrations of the grapes and the juice. The mean decrease in aluminium concentration from unadjusted washed grapes to both juice sample sets is 0.65 and 0.68 μg/g. However, when the aluminium concentration of the grape is derived from the adjusted washed grapes the difference between the two sets of samples is not significant. The fact that this trend was observed for both SO_2 free and SO_2 containing juices agrees with the earlier assessment that there is no significant difference between these juice samples. The results also suggest that the aluminium found in the juice is derived from the pulp and juice components of the grape, with any influence from SO_2 insignificant. This is evident in the mean decrease in aluminium concentrations from adjusted washed grape samples to both sets of juice samples of only 0.10-0.13 μg/g.

A density study was also conducted on two juice samples to derive a density of the juice and hence convert the aluminium concentrations found for these samples from a mass based unit to a volume based unit. This was required to compare the juice aluminium concentrations with that of the ferment and wines, which were calculated by volume. The mean density of the juice samples was 1.09 ± 0.02 g/ml. This factor was used to convert the juice samples aluminium concentrations from μg/g to mg/L that are presented in Table 4.16 in the same format as Table 4.14. The converted concentrations are compared with those of the fermented wine samples in Section 4.8.

Table 4.16 Aluminium concentrations of the juice samples (wet weight) presented in Table 4.14 converted from units of µg/g to mg/L.

SO_2 free Juice sample	Converted [Al] (mg/L)	s.d.	SO_2 treated Juice sample	Converted [Al] (mg/L)	s.d.
1997 Vintage			**1997 Vintage**		
AJ	2.7	±0.4	$AJSO_2$	3.4	±0.2
BJ	1.7	±0.1	$BJSO_2$	1.2	±0.2
----	-----	-----	$CJSO_2$	2.1	±0.3
----	-----	-----	CJGe*	2.0	±0.3
DJ	2.7	±0.4	----	-----	-----
EJ	1.7	±0.02	$EJSO_2$	0.9	±0.4
FJ	2.0	±0.3	----	-----	-----
----	-----	-----	$GLYJSO_2$	0.8	±0.8
----	-----	-----	$GHYJSO_2$	0.3	±0.2
----	-----	-----	$HJSO_2$	0.7	±0.3
IJ	0.44	±0.2	----	-----	-----
JJ	0.5	±0.1	----	-----	-----
1998 Vintage			**1998 Vintage**		
----	-----	-----	$KJSO_2$	3.3	±0.8
----	-----	-----	$LJSO_2$	4.0	±0.2
----	-----	-----	$MJSO_2$	2.2	±0.1
----	-----	-----	$NJSO_2$	0.31	±0.05
----	-----	-----	$OJSO_2$	2.6	±0.3
----	-----	-----	$PJSO_2$	0.31	±0.03

*** This sample, although containing SO_2, had gelatin added afterwards.**

4.8 Ferment and Production Wine Analysis Results

4.8.1 Presentation of Results

This section presents a more varied set of samples than the other results sections as the samples address the majority of the wine production stages from primary fermentation until prior to bottling. The types of samples cover a wide range of treatments and additions, including those taken after primary fermentation, pressing of the ferment, malo-lactic fermentation, racking, bentonite fining, milk fining and further metabisulfite addition. As the wine making diversifies extensively during this stage of production, due either to the style of wine (see Figures 4.1 and 4.2) or the individual winemaker's taste, so do the samples collected. Hence, for some production stages the total number of samples may not exceed two or three, making statistical comparison less reliable. In some cases differences between production steps can only be compared on an individual wine basis. Although

the samples are taken when the wine is still a "work in progress", these samples are referred to as wine samples and the concentrations are now presented in terms of mass per unit volume rather than by mass per unit mass as previously used for soils, grapes and juices. As with earlier sample types, comparisons will be made with the samples of the prior production step where possible.

As outlined in the winemaking schematics, Figures 4.1 and 4.2, juice is fermented in two lots, one as free run juice (white wine) and the other with skins or pulp left over from crushing (red wine). The latter ferment is then pressed from the solids. The ferment is denoted as [(sample prefix)FW] while that of the pressed ferment is denoted [(sample prefix)PW]. In some cases a ferment sample was not collected until after pressing. Collection of samples for wines G and H were discontinued after juice collection for reasons discussed in Section 3.5.4. The remote site for Wine I meant, unfortunately, that the ferment sample could not be collected before further processing had occurred. The total aluminium concentrations for the fermented and pressed wines are presented in Table 4.17.

Table 4.17 Total aluminium concentrations of ferment and pressed samples. White and red wine samples are labelled light green and burgundy respectively.

Fermented wine sample	[Al] (mg/L)	s.d.	Pressed wine sample	[Al] (mg/L)	s.d.
1997 Vintage			**1997 Vintage**		
AFW	0.58	±0.05	APW	0.41	±0.01
BFW	0.16	±0.02	----	-----	-----
CFW	0.19	±0.03	----	-----	-----
----	-----	-----	DPW	0.4	±0.2
EFW	4.2	±0.1	----	-----	-----
FFW	0.32	±0.02	FPW	0.26	±0.02
JFW	0.14	±0.02	----	-----	-----
1998 Vintage			**1998 Vintage**		
KFW	0.65	±0.07	----	-----	-----
LFW	0.15	±0.02	----	-----	-----
MFW	0.33	±0.05	MPW	0.27	±0.00
----	-----	-----	OPW	0.32	±0.09
PFW	0.13	±0.01	----	-----	-----

As there are no white pressed ferment samples, no comparison can be made for white wines. Red wines show little difference in their aluminium concentrations from ferment (FW) to pressed wine

(PW). The majority of red wine ferment aluminium concentrations are more than double that of their white counterparts with one exception. The result for wine E shows a very high aluminium concentration that is an order of magnitude higher than any of the other fermented wine sample aluminium concentrations. This suggests a significant aluminium input for this sample which will be discussed in more detail in Section 4.10. The consequence of this high outlier is that the overall and white wine means tabled in Sub-Section 4.10.3 for the free-run ferment become substantially skewed. The data for wine E was removed and the adjusted means are presented in Table 4.27 and plotted in Sub-Section 4.10.3.

There are few data points to make a valid comparison of the aluminium content of the ferment with the pressed wine. However, all three complete sample pairs (samples A, F and M) show a slight decrease in the aluminium concentration after pressing. This may be because solids containing aluminium are removed after pressing. However, a t-test of these three sample pairs showed the difference was not statistically significant at the 95% confidence concentration. T-test analysis was also conducted on the difference in aluminium concentrations of both sets of fermented samples with the aluminium concentration in SO_2 containing and SO_2 free juice samples. In statistical comparisons the data from EFW was omitted, however a closer investigation of this wine will be conducted in Section 4.10. For this test, only sample populations with results for both compared sample sets were tested, hence the population sizes differed for each t-test. The mean and standard deviation data for each sample population compared is shown in Table 4.18. The t-test results matrix of the aluminium concentration differences are shown in Table 4.19.

Table 4.18 Mean and standard deviation data used for the t-tests described in Table 4.19.

Comparison	Juice [Al]	Ferment
Untreated Juice with Ferment (n = 5)	1.63 ± 0.74	0.28 ± 0.18
SO_2 treated Juice with Ferment (n = 7)	2.17 ± 1.20	0.31 ± 0.22
Untreated Juice with Pressed Ferment (n = 3)	2.26 ± 0.38	0.35 ± 0.07
SO_2 treated Juice with Pressed Ferment (n = 3)	2.50 ± 0.57	0.33 ± 0.07

Table 4.19 t-test results of comparison of ferment and pressed wine aluminium concentrations with those of juices. Results are presented as significance of difference and confidence concentration.

t-test (comparing aluminium concentrations)	t-test Result (one-tailed)	Confidence Concentration
Ferment with SO_2 free juice	Significant difference	99%
Ferment with juice with SO_2	Significant difference	99%
Pressed Wine with SO_2 free juice	Significant difference	99%
Pressed Wine with juice with SO_2	Significant difference	98%

The results of the t-test analysis demonstrate that for every comparison permutation of ferment (whether ferment or pressed) with juice (whether SO_2 free or SO_2 containing) the drop in aluminium concentration from juice to ferment sample is statistically significant at the 98-99% confidence concentration. The mean decrease in aluminium concentration from either juice sample type to either ferment sample type was 1.1 mg/L. This suggests that somewhere in the fermentation process, aluminium is being redistributed in the system. This will be discussed in more detail in Sub-Section 4.8.2.

Of the six wines for which McKinnon (1990) conducted a partial aluminium profile analysis from the ferment to the bottled wine, only one had a sample taken after the ferment without the addition of bentonite or other additives. The aluminium content of this sample (ferment of a Cabernet Franc Merlot) was found to be 0.10mg/L. This aluminium concentration is slightly lower than the lowest found in this study, however it is not significantly so and could easily fit within the distribution of results from the current study. The two studies show good agreement as virtually all of the aluminium concentrations in ferment samples in this study are of the same order of magnitude as that for McKinnon's (1990) result.

For some production steps up to the pre-bottled wine only one sample was taken for some of the wines studied. These include a sample taken after SO_2 addition to a pressed wine, one sample each after a red and white tannin addition, two samples taken after malo-lactic fermentation (of which one was followed by an SO_2 added sample), four samples taken after bentonite fining, one after milk fining and one after racking. The aluminium concentrations of these samples are shown in Tables 4.20, 4.21 and 4.22.

Table 4.20 Total aluminium concentrations of malo-lactic fermented samples. White and red wine samples are labelled light green and burgundy respectively.

Fermented wine sample	[Al] (mg/L)	s.d.	SO_2 added malo-lactic fermented wine sample	[Al] (mg/L)	s.d.
1997 Vintage			1997 Vintage		
BmaloFW	0.16	±0.00	BmaloFWSO_2	0.20	±0.01
DmaloFW	0.38	±0.01	----	-----	-----

Table 4.21 Total aluminium concentrations of bentonite fined samples, including the relevant sample taken before this procedure. White and red wine samples are labelled light green and burgundy respectively.

Ferment wine sample	[Al] (mg/L)	s.d.	Bentonite Fined wine sample	[Al] (mg/L)	s.d.
1997 Vintage			1997 Vintage		
BFW	0.16	±0.02	BBW	0.44	±0.01
CFW	0.19	±0.03	CBW	0.56	±0.12
1998 Vintage			1998 Vintage		
LFW	0.15	±0.02	LBW	0.27	±0.02
NJSO_2*	0.21	±0.04	NBW	0.36	±0.04

*** No ferment sample was collected for this wine, hence the last sample result prior to the bentonite sample was used.**

Table 4.22 Total aluminium concentrations of miscellaneous wine samples, including the relevant sample taken before the production step. White and red wine samples are labelled light green and burgundy respectively.

Previous wine sample	[Al] (mg/L)	s.d.	Miscellaneous production wine sample	[Al] (mg/L)	s.d.
1997 Vintage			1997 Vintage		
APW	0.41	±0.01	APWSO2	0.63	±0.02
APWSO2	0.63	±0.02	AredTW	0.51	±0.01
AredTW	0.51	±0.01	AwhiteTW (ApreBotW)	0.66	±0.05
FPW	0.26	±0.02	FRW	0.23	±0.01
1998 Vintage			1998 Vintage		
LFW	0.15	±0.02	Lmf	0.46	±0.03

Comparing the malo-lactic fermented wines (BmaloFW [Al] = 0.16mg/L, DmaloFW [Al] = 0.38mg/L) with their ferment precursors (BFW [Al] = 0.16mg/L, DPW [Al] = 0.4mg/L) shows that there is no change in aluminium concentration during malo-lactic fermentation. As observed for

earlier results concerning SO_2 addition, the addition of metabisulfite to the malo-lactic fermented wine **B**maloFW did not appear to change the aluminium concentration, however with only one sample it is impossible to statistically define this. This general trend for SO_2 addition was not observed for the addition of metabisulfite to pressed wine A. This production step for this sample saw an increase in the aluminium concentration, again with only one sample statistical analysis of aluminium differences was also impossible.

Red wine A, prior to bottling, had first red tannin then white tannin added to it. The addition of red tannin resulted in a slight decrease in aluminium concentration, however with the addition of white tannin there was a slight increase with virtually no difference observed at the end of these procedures. Because these single sample changes cannot be statistically analysed, the results remain inconclusive in determining the influence of tannin on aluminium concentration in this wine; however for this wine this influence is probably minimal. This disagrees somewhat with the findings of McKinnon (1990) and McKinnon et al. (1992), who proposed that tannin was a potential source of aluminium in red wines.

The racked wine for sample F, sampled after the wine was decanted off the lees, predictably showed very little change in aluminium concentration. This result is expected, as any aluminium exchange between solid lees and wine would already be complete and its removal would not alter the concentration in the liquid.

Wine L was unique among the wines studied in that prior to bentonite fining it underwent milk fining. After this process the aluminium concentration increased threefold. As this sample preceded the bentonite fining this result was all the more unusual as the change in aluminium concentration from milk fining to bentonite fining saw a decrease rather than an increase as normally expected. As there was only one sample of its type collected, it is statistically impossible to discern if this increase is the result of contamination, error or aluminium from the milk (samples of the milk used were not able to be obtained). As such the most that can be concluded is that for this sample a net increase was observed in aluminium concentration after milk and bentonite fining was completed.

Because of the commercial nature of the winemaking process and remote sampling sites, there were times when unplanned winemaker intervention meant a sample was not obtained. This occurred with wine N. This was unfortunate as this renders the picture of the change in aluminium concentrations for the four wines where bentonite samples were collected incomplete. However, as there was a drop in aluminium concentration from juice to ferment for virtually every wine studied, the SO_2 added juice aluminium concentration was used as a highest aluminium concentration scenario for a ferment sample. This worse case scenario was used with the assumption that the probability that the ferment aluminium concentration would be less than that of the juice was high, hence a significant increase in aluminium concentration using the juice concentration would probably mean the increase for a ferment sample would also be significant. Table 4.21 demonstrates that every sample that underwent bentonite fining showed an increase in aluminium concentration. The mean change was an increase of aluminium concentration of 0.22mg/L. A t-test analysis of the overall data showed that this difference was statistically significant at a confidence concentration of 98%. This finding confirms the findings of McKinnon (1990) and McKinnon et al. (1992) who also observed an increase in wine aluminium concentration after bentonite fining. The current study was also in agreement with the earlier work in regard to the magnitude of change with both showing aluminium concentration doubling after bentonite fining.

4.8.2 Loss of Aluminium from Wine During Fermentation

As discussed in the last section a significant decrease in aluminium concentration was observed after fermentation of the juice for all the wines investigated in this study with two exceptions. The reason these two wines did not show a decrease in aluminium concentration at this stage is discussed in Sub-Section 4.10.2. The change was the most significant found throughout the wine production process. Although the focus of this investigation was on the aluminium in wine, an examination of the significant loss of aluminium during this process was conducted for completeness. The aluminium trend before and after fermentation did not become apparent until all the samples were analysed for their aluminium concentration, therefore the collection of further samples around this production step was not possible for the 1997 and 1998 vintages. However, a study was conducted on a Chardonnay sample from vineyard/winery 1 for the 2001 vintage focussing on samples taken prior to and after fermentation.

As aluminium due to skins and pips were removed with these components prior to fermentation, there appeared to be no other mechanism for the loss of aluminium other than its removal from the solution into the lees. Therefore, a potassium metabisulfite treated juice sample was taken prior to fermentation and a ferment sample and a sample of the lees on the bottom of the barrel were collected after fermentation (see Sub-Section 3.5.5 for the methodology of the lees sampling). As yeast was the main additive to the juice prior to fermentation, a sample of the dried yeast used in this wine was also collected. This sample would allow the aluminium from this source to be taken into account. An estimate of the yeast treatment rate was also recorded. The aluminium concentrations of the samples are shown in Table 4.23.

Table 4.23 Results of 2001 vintage chardonnay fermentation aluminium analysis.

Sample Type	[Al]	Treatment Rate
Juice	***2.7 ± 1.3 mg/L**	-----
Yeast	12.1 ± 2.0 µg/g dry mass	100mg/L
Lees	39 ± 7 µg/g dry mass	-----
Ferment	**0.09 ± 0.01 mg/L**	-----

***Concentration was converted from µg/g to mg/L using estimated density of 1.09g/ml (see Section 4.7)**

Table 4.23 confirms the decrease in aluminium concentration from juice to fermented wine with an average loss of 2.6 mg/L amounting to about 96% reduction. The concentration of aluminium in the dried lees is an order of magnitude higher than the juice suggesting that aluminium is concentrated in the lees. However, the concentration of aluminium in the yeast means that some of the aluminium in the lees would be attributable to this additive. As the lees can contain any number of substances other than yeast, including particles of grape solids, bacteria, tartrate crystals and metal and protein precipitates, a mass balance would require a substantial investigation of the individual changes in components in several 225L barrels throughout fermentation.

These data can be related to a study by Meierer (1984) who monitored ferments of white juice in the presence of added metal ions, including aluminium. The data for this study (presented in Table 4.24) shows a decrease in aluminium concentration of between 20-30% from the juice to the ferment depending on the amount of aluminium in the juice and implies that the aluminium is distributed between the lees and the wine after fermentation. Meierer (1984) also reported that other

investigations noted decreases even greater than that shown in Table 4.23, up to 70-90%. These findings are in good agreement with the aluminium profile found in this study over the fermentation process (see Section 4.10). Meierer's investigation also noted the difficulties in sampling and assessing yeast lees and attributed the wide variation in the results to these problems which also probably accounts for the absence of a mass balance. As the fermentation process is a dynamic one with interaction within and between liquids and suspended solids, plus the complexity of the components within the system as mentioned in the previous paragraph, the attainment of an accurate mass balance may well be impossible without seriously compromising the system.

Table 4.24 Data of aluminium profile over fermentation from the study of Meierer (1984).

Juice		After Ferment		% Decrease
Total Amount [Al] (mg/L)	Added [Al] (mg/L)	Wine [Al] (mg/L)	Yeast Lees [Al] (mg/L)	Juice to Wine
0.50	---	0.35	0.15	30.0
0.98	0.48	0.75	0.23	23.5
1.94	1.44	1.46	0.48	24.2
5.30	4.80	4.20	1.10	20.8

Thus, although the analysis described in this sub-section was limited in its scope it does show that the ferment lees are a significant sink for aluminium. This agrees with the work of Meierer (1984) and suggests that the lees are a repository for aluminium either through a mechanism of absorption/adsorption or precipitation. It also agrees with the hypothesis of Enkelmann and Wohlfarth (1994), who suggested that aluminium in grapes derived from Ulmasud (a spray of finely ground aluminium based clay used against spores of downy mildew) deposits on vine leaves were preferentially absorbed in must deposit particles. However, Enkelmann & Wohlfarth's (1994) assertion that the fermentation does not influence the aluminium content may be the case for Ulmasud particles but was not found to be the case for the wines examined in this current work.

4.9 Pre-Bottled and Bottled Wine Analysis Results

This section presents the last set of sample results for total aluminium concentrations of the wine production profile. Both wine prior to bottling and the bottled commercial product are presented in the data. In general the pre-bottled wine samples that were collected were not taken after any

particular production step other than aging for a period in stainless steel vats or oak barrels; as shown in Figures 4.1 and 4.2, the red wines sat in storage prior to bottling for at least 6-12 months longer than the white wines. The main reason for taking a sample before bottling was to observe any changes in aluminium concentration during the bottling process and if any leaching of aluminium from the bottle occurred. This latter observation was made by McKinnon (1990), however that result was based on one wine and the result could not be confirmed. The hypothesis has merit as glass contains alumino-silicates (Ericson 1992) and preliminary work from this study has shown that acidic media can extract aluminium from glass (see Chapter 3).

Not all bottled wine samples were collected, in particular the second season red wines are not included because this investigation was completed before bottling of these wines. The aluminium concentrations of the pre-bottled and bottled wines are presented in Table 4.25.

Table 4.25 Total aluminium concentrations of pre-bottled and bottled wine samples. White and red wine samples are labelled light green and burgundy respectively.

Pre-bottled Wine sample	[Al] (mg/L)	s.d.	Bottled Wine sample	[Al] (mg/L)	s.d.
1997 Vintage			**1997 Vintage**		
AprebotW	0.66	±0.05	AbotW	0.50	±0.07
BprebotW	0.40	±0.03	BbotW	0.78	±0.01
----	-----	-----	CbotW	0.49	±0.02
----	-----	-----	DbotW	0.24	±0.01
EprebotW	4.1	±0.1	EbotW(1)	2.5	±0.2
FprebotW	0.40	±0.03	FbotW	0.24	±0.02
----	-----	-----	IbotW	0.53	±0.02
----	-----	-----	JbotW	0.54	±0.02
1998 Vintage			**1998 Vintage**		
----	-----	-----	KbotW	0.66	±0.08
----	-----	-----	LbotW	0.39	±0.04
----	-----	-----	NbotW	0.38	±0.00
OprebotW	0.20	±0.02	----	-----	-----
PprebotW(2)	1.8	±0.1	PbotW(2)	1.22	±0.03

(1) - Bottling for E was done after wine was blended with another product of unknown origin
(2) – Pre-bottled sample was taken after blending wine with another product of unknown origin. Bottled wine E is also a product of this blend.

The results in Table 4.25 show that wine E has maintained its high aluminium concentration since fermentation. The following vintage, wine P, also showed a higher than average aluminium concentration. Like the ferment results, these samples skew the respective mean aluminium concentrations quite heavily as shown in Sub-Section 4.10.3. The result for both wines E and P were not included in statistical considerations when it was discovered that they had been blended with wine from sources outside this study. This was also the case with pre-bottled wine P. As both wines E and P will be discussed in Section 4.10, these samples were collected for completeness of the sample set.

From pre-bottled to bottled wines there is no definitive trend with one sample increasing and the other two decreasing in aluminium concentration. Despite the two sample populations being incomplete, a t-test comparison was conducted on the differences between the group of samples collected both before and after bottling. There was no significant difference found between the aluminium concentrations of the bottled wine compared with the pre-bottled wines. Mean aluminium concentrations of the wines analysed in this study are compared with those found by McKinnon (1990) and McKinnon et al. (1992) in the overall profile analysis in Section 4.10.

4.10 Wine Production Aluminium Profiles

This section summarises the results presented in the last five sections and graphically shows the profile of the total aluminium concentration for all wines. As there was no established link between the acetic acid extractable soil aluminium concentration and the total aluminium concentration in grapes, the soil results have not been included in the profile charts. Profiles of both red and white wines of the same vineyard/winery for both the 1997 and 1998 vintages will be shown on one chart and particular case studies of individual wines showing unusual results will also be discussed. Mean values for discretely independent samples should always be treated with some caution, however they are useful in defining trends that appear to be general to all wines. This in particular will point out the sources and sinks of aluminium in the wine making process. These overall profiles will also be discussed and trends and production steps showing significant change will be discussed and conclusions drawn. Comparisons will also be made with the conclusions of McKinnon (1990) and

McKinnon et al. (1992) and in all profile charts the WHO drinking water limit will also be displayed for comparison purposes.

4.10.1 Total Aluminium Concentration Production Profiles of Individual Wines

The total aluminium concentration profiles of both vintages of each cultivar per vineyard/winery are shown in Figures 4.11-4.18. These profiles also show the adjusted concentrations for washed grapes and juices. For unit comparative purposes, unwashed grape, washed grape and non-modified juice results are given in units of mg/g while that of modified juice and wine results are given in units of mg/L. The aluminium drinking water limit is also given in terms of mg/L. Note the sample codes in the profile charts have been changed for the adjusted samples, with the sample code ending with a lower case (a) denoting a sample where the aluminium concentration has been adjusted as discussed in Sections 4.6 and 4.7.

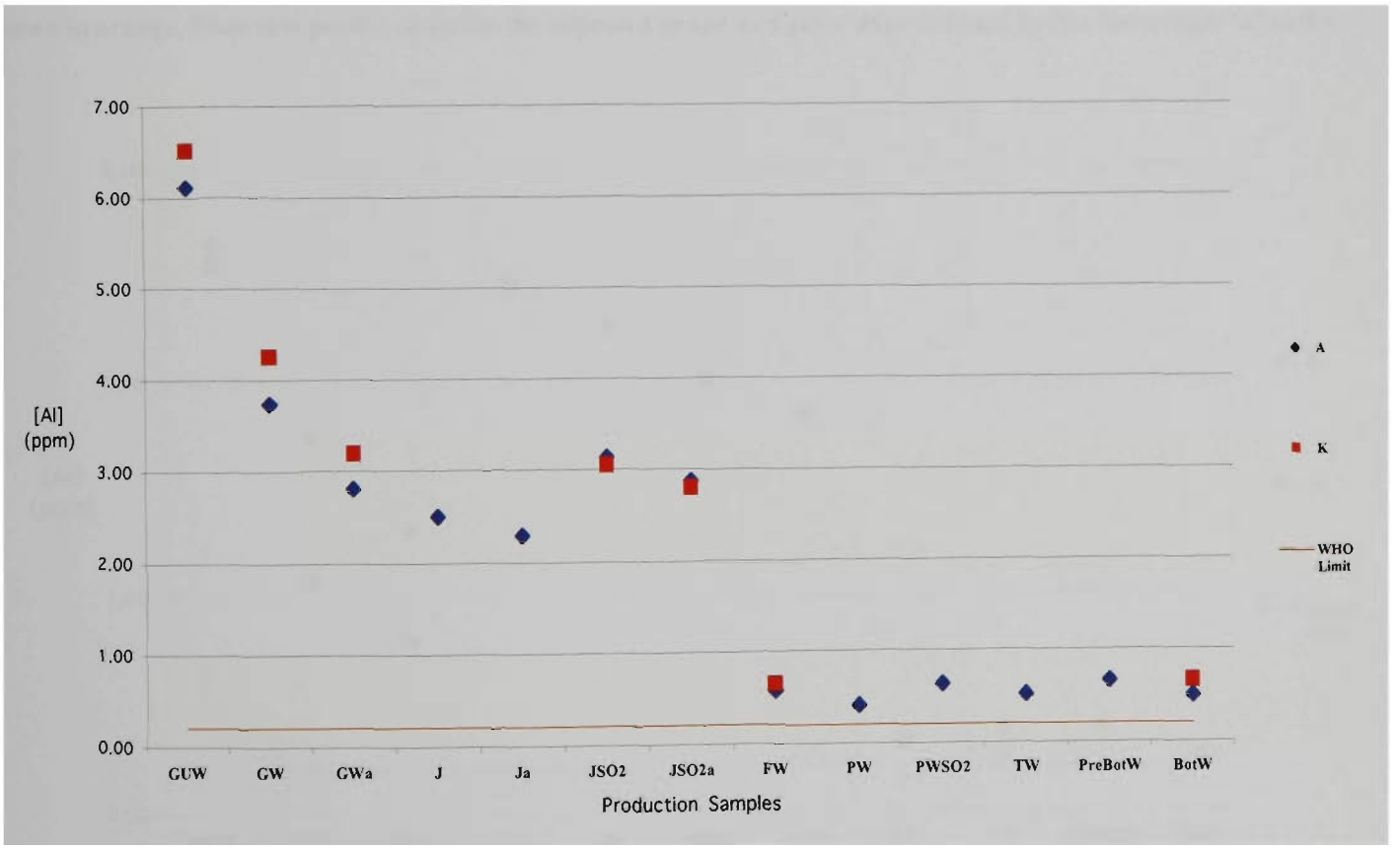


Figure 4.11 Total aluminium concentration profile throughout the wine production process of red wines A (1997) and K (1998) from vineyard/winery 1. The WHO drinking water limit for aluminium concentration is shown in orange. Note this profile contains the adjusted grape and juice data denoted by the lower case 'a' suffix.

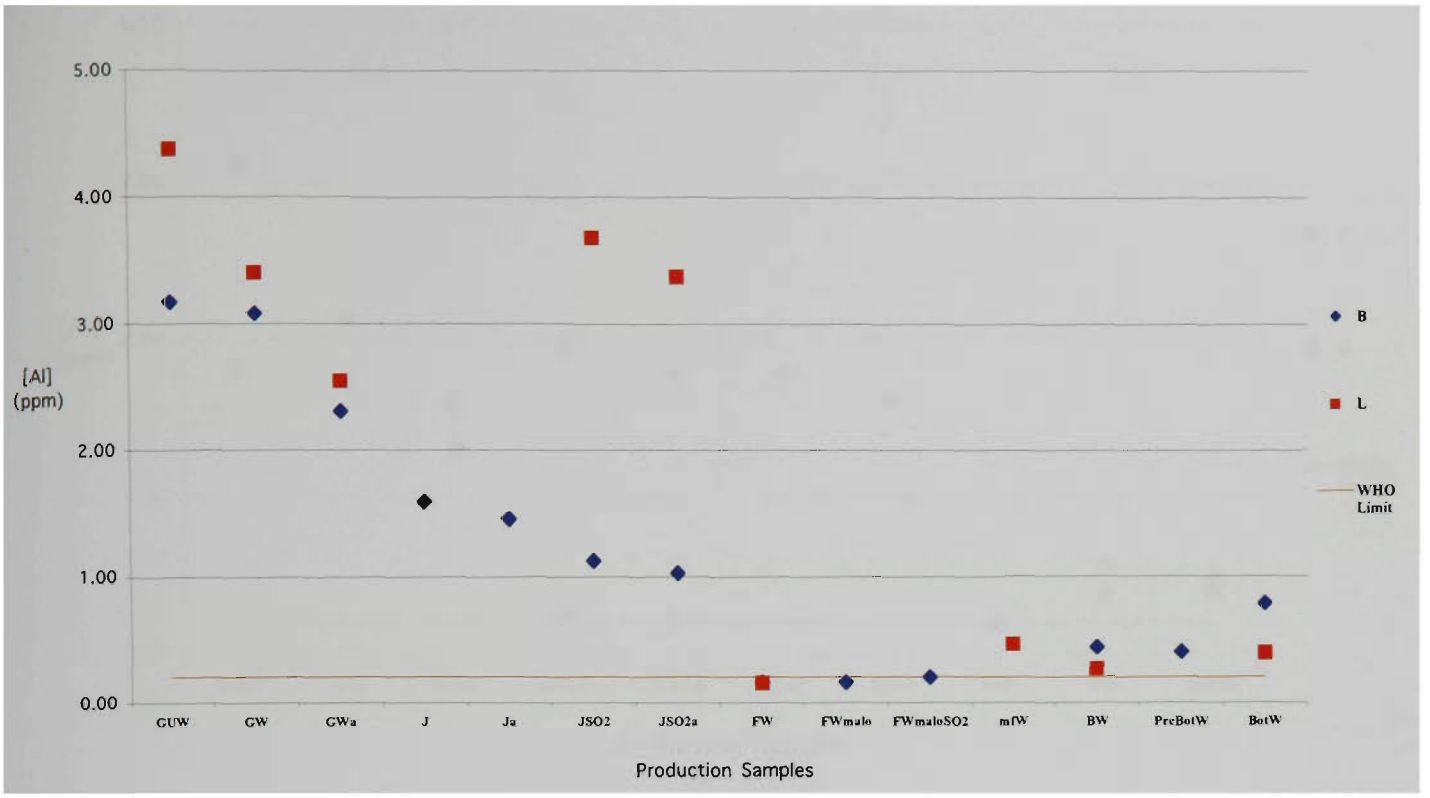


Figure 4.12 Total aluminium concentration profile throughout the wine production process of white wines B (1997) and L (1998) from vineyard/winery 1. The WHO drinking water limit for aluminium concentration is shown in orange. Note this profile contains the adjusted grape and juice data denoted by the lower case 'a' suffix.

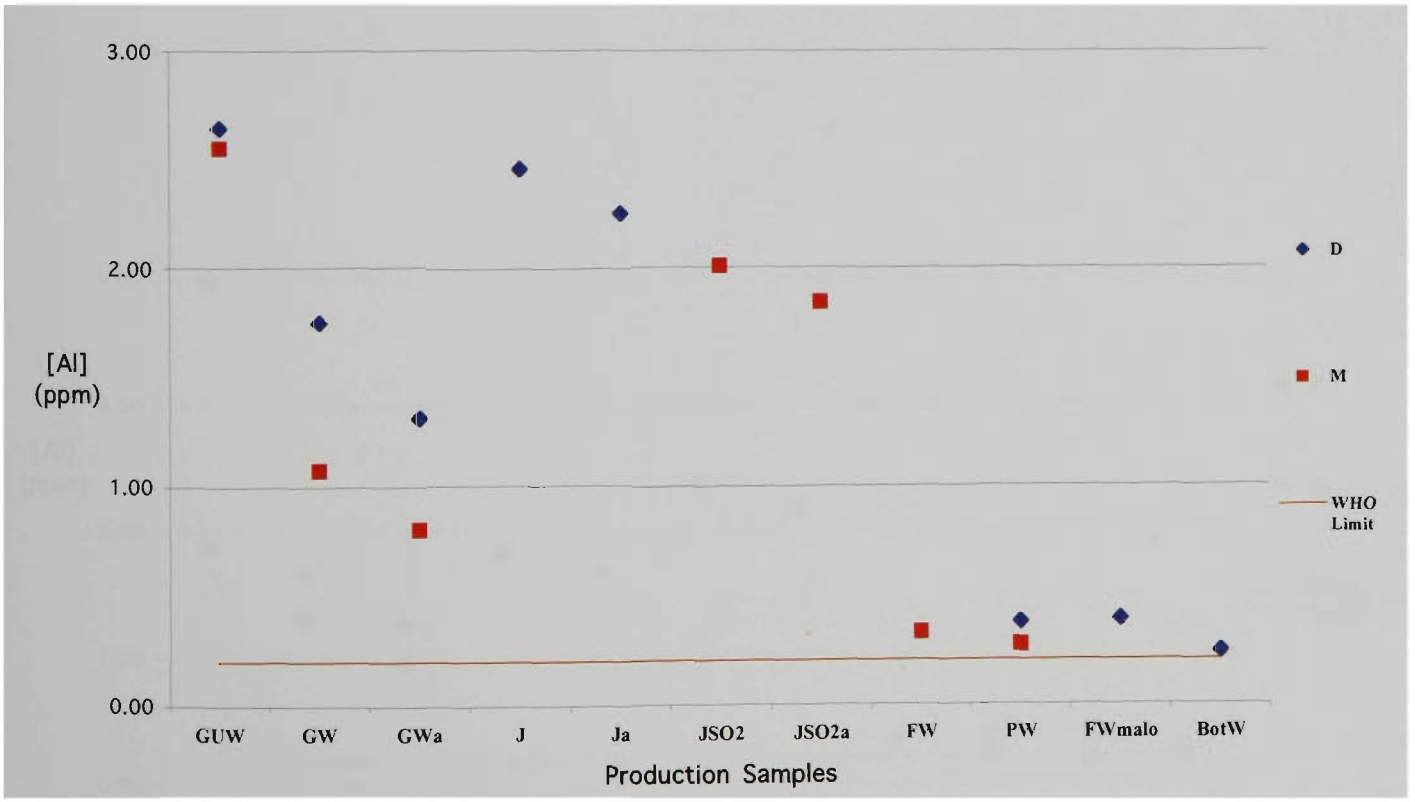


Figure 4.13 Total aluminium concentration profile throughout the wine production process of red wines D (1997) and M (1998) from vineyard/winery 2. The WHO drinking water limit for aluminium concentration is shown in orange. Note this profile contains the adjusted grape and juice data denoted by the lower case 'a' suffix.

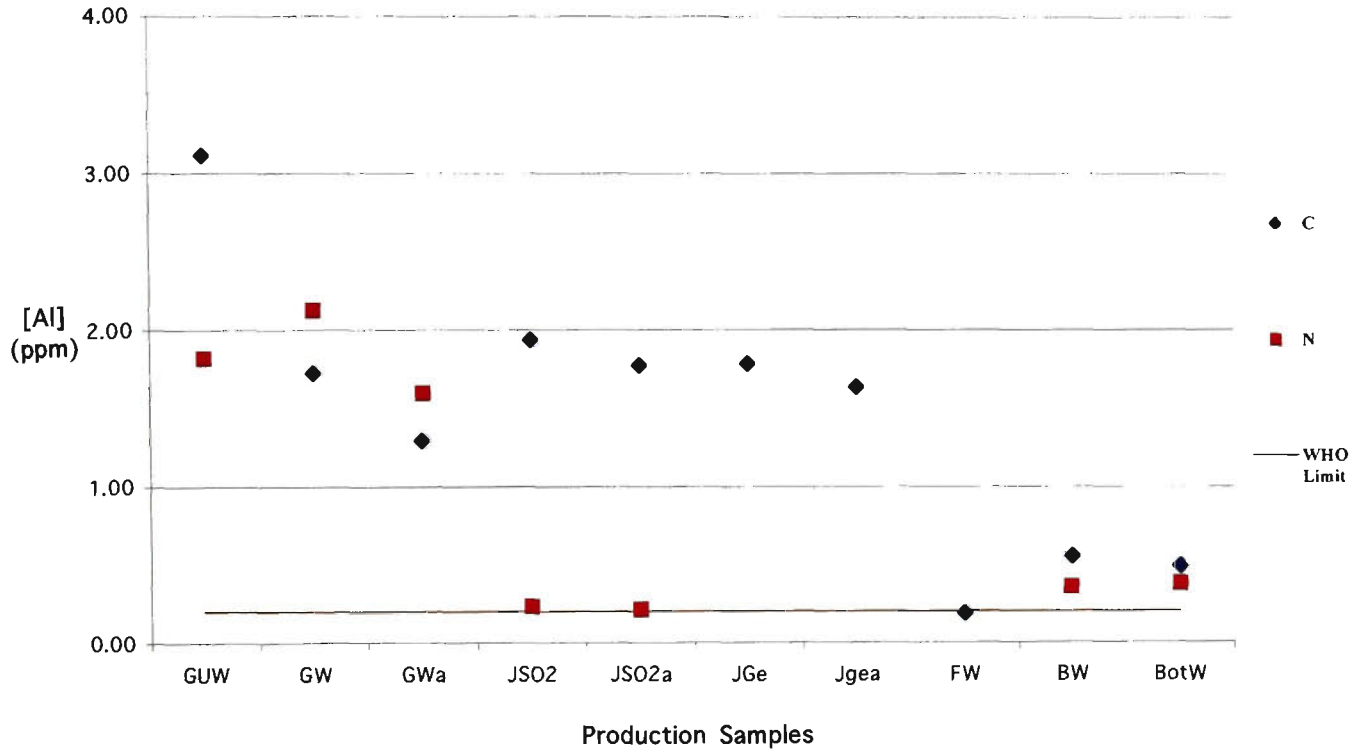


Figure 4.14 Total aluminium concentration profile throughout the wine production process of white wines C (1997) and N (1998) from vineyard/winery 2. The WHO drinking water limit for aluminium concentration is shown in orange. Note this profile contains the adjusted grape and juice data denoted by the lower case 'a' suffix.

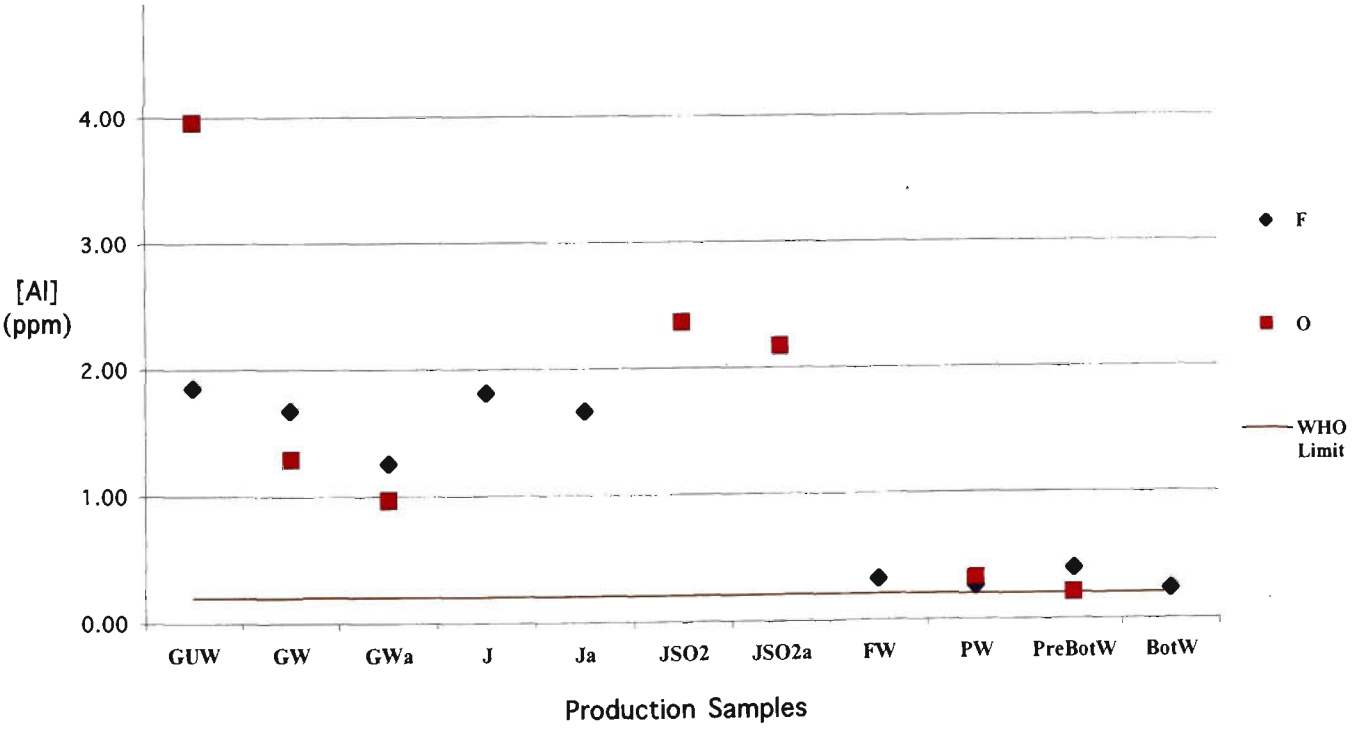


Figure 4.15 Total aluminium concentration profile throughout the wine production process of red wines F (1997) and O (1998) from vineyard/winery 3. The WHO drinking water limit for aluminium concentration is shown in orange. Note this profile contains the adjusted grape and juice data denoted by the lower case 'a' suffix.

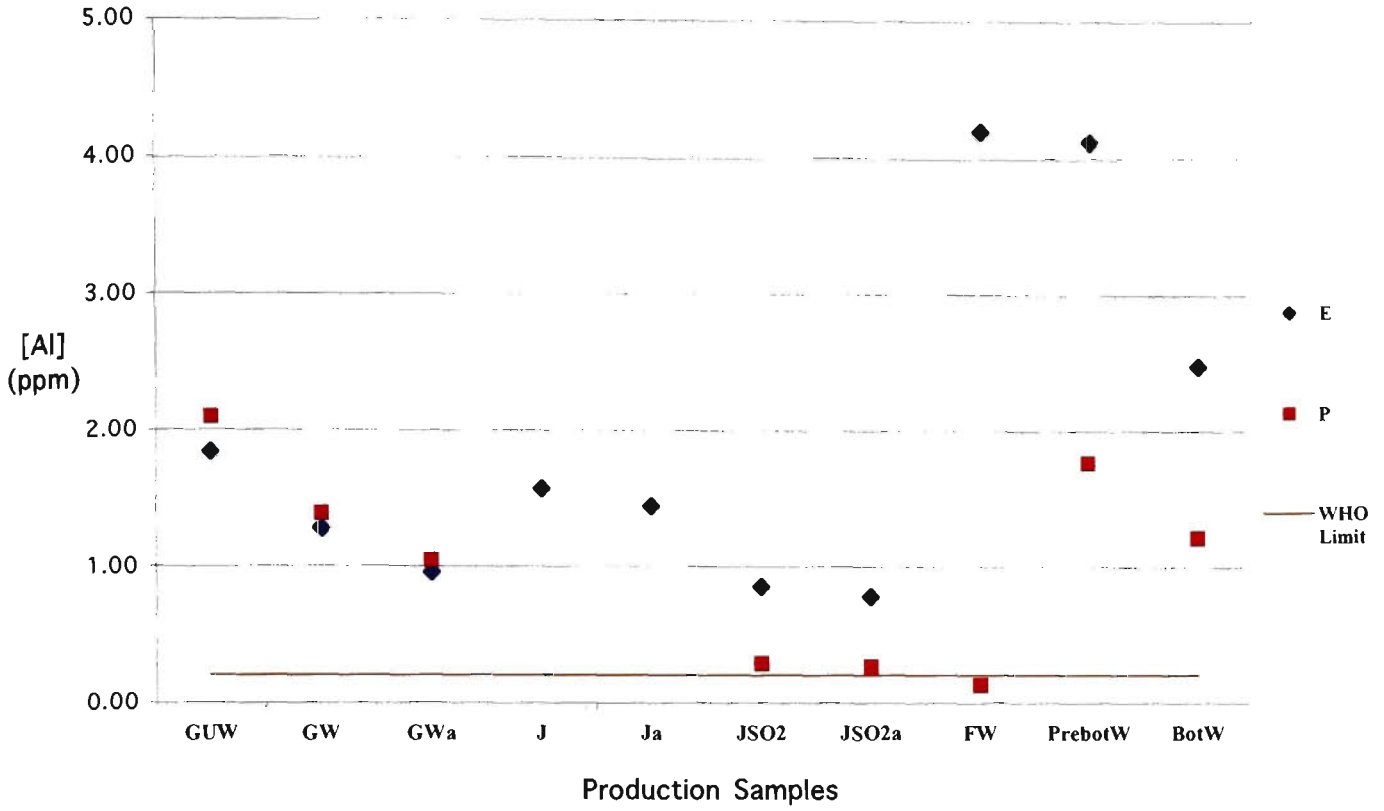


Figure 4.16 Total aluminium concentration profile throughout the wine production process of white wines E (1997) and P (1998) from vineyard/winery 3. The WHO drinking water limit for aluminium concentration is shown in orange. Note this profile contains the adjusted grape and juice data denoted by the lower case 'a' suffix.

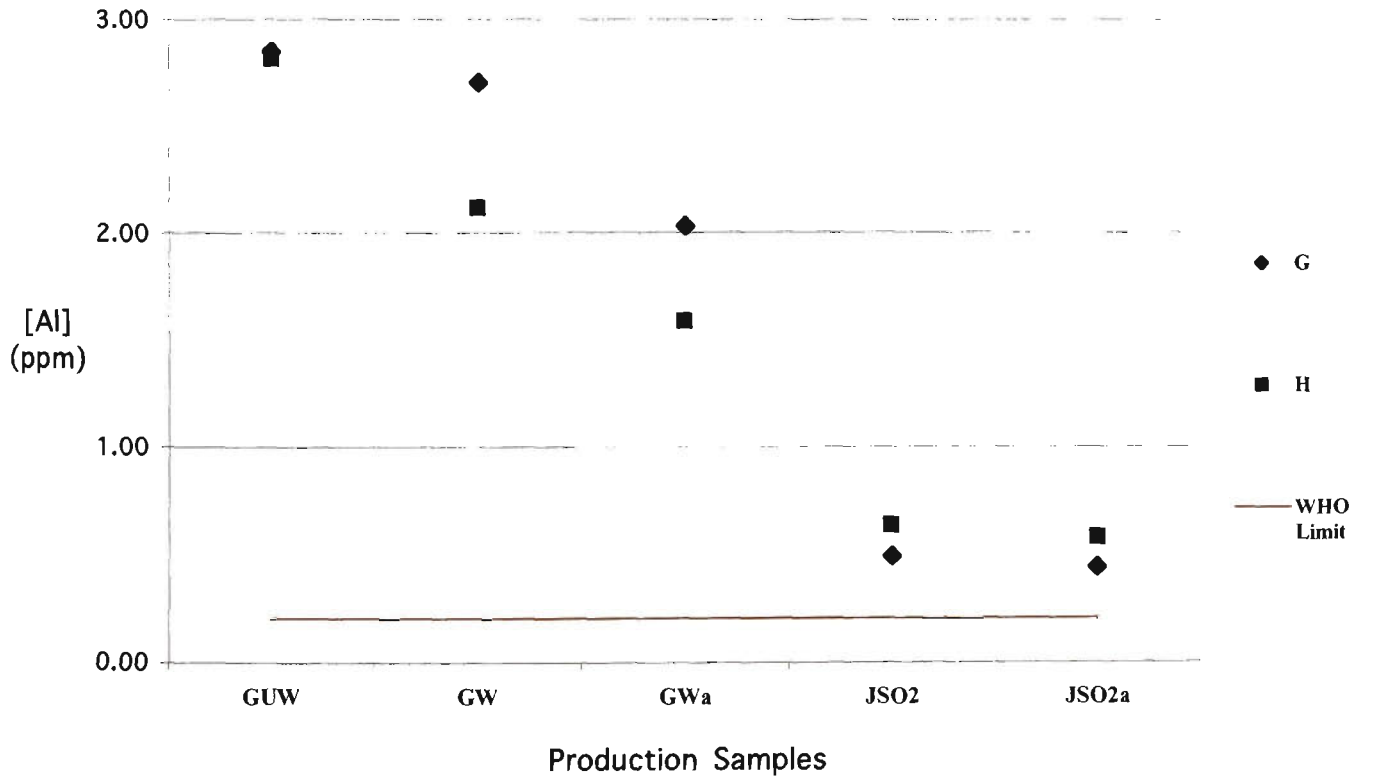


Figure 4.17 Total aluminium concentration profile throughout the wine production process of white wines G and H (1997) from vineyard/winery 4. The WHO drinking water limit for aluminium concentration is shown in orange. Note this profile contains the adjusted grape and juice data denoted by the lower case 'a' suffix. No further sampling was completed after collection of the juice in the first sampling season.

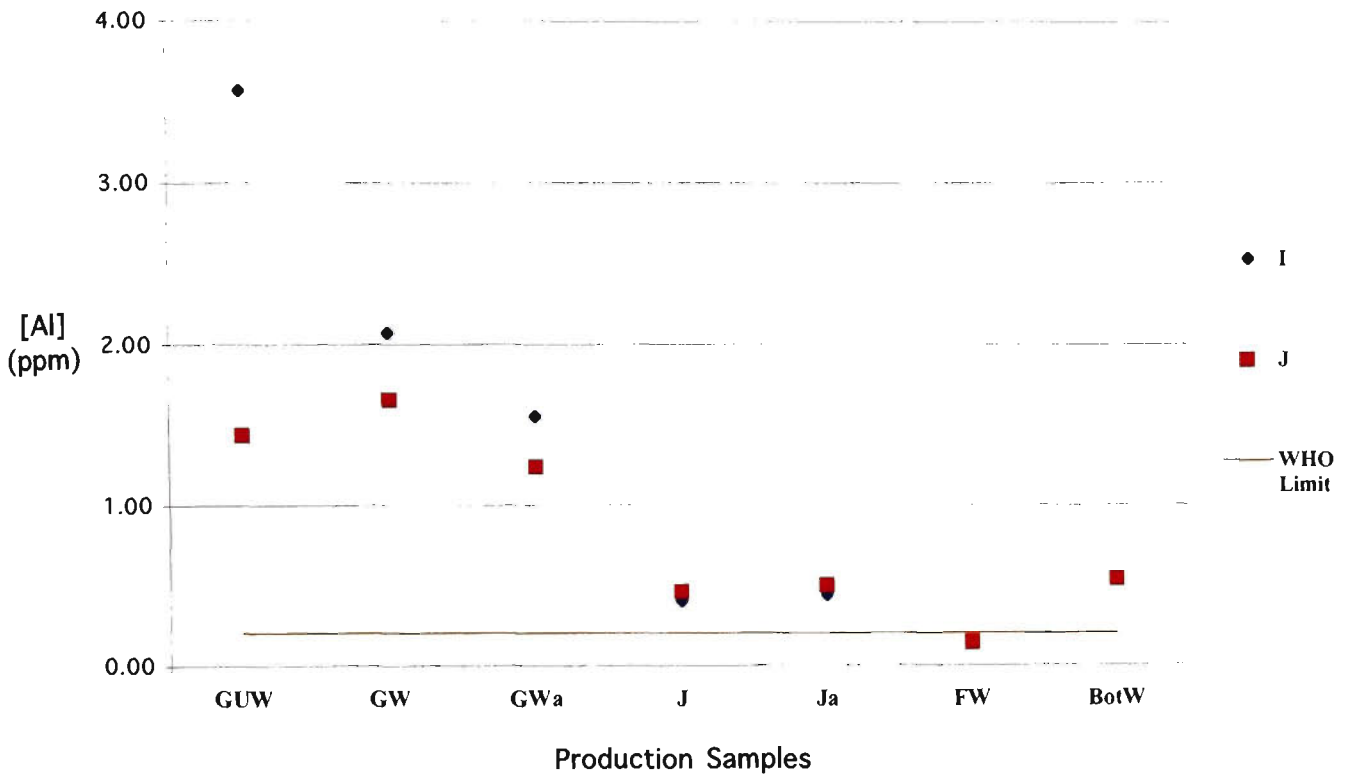


Figure 4.18 Total aluminium concentration profile throughout the wine production process of red wine J and white wine I (1997) from vineyard/winery 5. The WHO drinking water limit for aluminium concentration is shown in orange. Note this profile contains the adjusted grape and juice data denoted by the lower case 'a' suffix. No further sampling was completed after collection of samples in the first sampling season.

The individual aluminium concentration profiles of the wines highlight the discrete nature of each wine, and why interpreting means and statistical results requires some caution. Adding to this individuality is that some of the sample points are absent because of unplanned winemaker intervention. This is unfortunate as it renders some of the profiles incomplete, particularly where ferment samples have been missed as is the case for wines D, I, N, O and P. The profiles allow a longitudinal inspection of the changes in aluminium concentrations for the individual wines. Some particular wine profiles are of interest and will be discussed in more detail in the following sub-section.

4.10.2 Discussion of Individual Wine Aluminium Profiles of Note

Wines A and B demonstrate the inconclusive nature of SO_2 addition with wine A showing an increase in aluminium content while wine B displays a decrease. In wine A the production steps of red tannin and white tannin (the latter sampled as AprebotW) addition showed no significant change in the aluminium content. This is in dispute with the findings of McKinnon (1990) and McKinnon et al. (1992) who concluded that red tannin was a source of aluminium in red wine. In wine B, malo-

lactic fermentation showed no change in the aluminium concentration, however there was a doubling of the aluminium content after bentonite fining. The winemaker's notes showed that the 1998 vintage used a bentonite treatment rate of 0.5g/L, which falls into the average treatment rate of bentonite given by Rankine (1991) of 0.3-0.8g/L. The bentonite result supports McKinnon's (1990) findings in which his limited aluminium production profile work showed a similar increase in aluminium concentrations after similar bentonite treat rates. The winemaker's notes do not provide an explanation for the high aluminium content of SO_2 treated juice in wine L.

The wines of vineyard/winery 2 (Figures 4.13 and 4.14) show a similar inconclusive trend regarding the addition of SO_2. The white wines also showed an increase in aluminium concentration after bentonite fining. The addition of gelatin to the juice of the white Wine C showed no significant change in aluminium content.

The white wines of vineyard/winery 3 (Figures 4.15 and 4.16) provided the most interesting profile of all the wines studied, particularly wine E. The white juice aluminium concentration after SO_2 addition was relatively low compared with similar samples, however rather than the usual decrease in aluminium concentration after fermentation, wine E displayed a massive increase in aluminium content with its ferment having a concentration of over 4mg/L. As discussed in Sections 4.8 and 4.9 this aluminium concentration and its increase was unique amongst all the wine studied.

This trend was not repeated for wine P, but its blended pre-bottled and bottled aluminium concentration were also well above its ferment concentration, second only to wine E. However these samples were taken after blending with a wine from an unknown source. The rise in aluminium concentration is probably the result of blending with a wine with a higher aluminium concentration. Hence, the data for these samples of wine P were not included in the overall profile analyses. The result for wine E was further clouded by the blending of the wine prior to bottling with wine from an unknown source. For completeness this bottled wine was analysed and showed half the aluminium concentration after blending, presumably a product of dilution with a wine of low aluminium concentration. On bottling, the wine was found to have a hazy suspension, and even after filtering still exhibited some haze and was subsequently analysed by The Australian Wine Research

Institute (AWRI 1998). The report concludes that 'some of the results obtained provide an indication the haze was due to a form of iron instability' but it was 'considered unusual that the concentration of iron in the wine was only slightly greater than the normal range observed at the Institute in wines of similar style'. It was 'also considered unusual that the addition of an oxidising agent (hydrogen peroxide) did not lead to a significant increase in turbidity, as is usually the case with wines exhibiting iron instability' (AWRI 1998).

The winemaker's notes were consulted to elucidate the cause of the increase and provide a plausible explanation. According to these and the sampling notes, the winemaker added bentonite to the juice prior to fermentation at a treatment rate of 4.0g/L, this addition at this early stage was unique for this wine compared with all the white wines analysed in this study. Compared with that recommended by Rankine (1991) at 0.3-0.8g/L and the treatment rates recorded by McKinnon (1990) which had a maximum dosage of 1.0g/L (giving an increase in aluminium concentration of 0.6mg/L) for his aluminium profile work, this is a high dose of bentonite in a juice with a recorded pH of 3.28. The bentonite in the juice may not have been significant if exposure to the lees had been relatively short as it generally is for a white wine. However, this wine had a more unusual history than its counterparts. The winemaking notes reveal that this wine stopped fermentation partway through to the puzzlement of the winemaker. It was then restarted and the wine sat in storage on the lees for almost a year before any further work was done on the wine. If one refers to the winemaking scheme of Figure 4.1, the production of white wine from picking to bottling usually takes from 3-6 months and for some of the wines of this study, one year was the maximum. The ferment sample was not collected until after the prolonged process was completed, hence in this time the juice and wine were in contact with this high concentration of bentonite for an extended period. This combination of early production high bentonite dose and prolonged wine contact were the probable cause of the high aluminium content. The AWRI (1998) report indicates that the wine was not tested for aluminium, and though exhibiting some iron instability symptoms had an iron content just above normal wine concentrations. Aluminium is regarded as causing wine haze at higher concentrations, 8mg/L being the limit for wine used in Germany (Eschnauer & Scollary 1995). It could be argued that it was the high aluminium content that contributed to the haze pre and post bottling and the haze was due to aluminium instability rather than iron instability.

Consequently this wine, and possibly the following vintage, are good examples of the influence of bentonite fining on the total aluminium concentration of white table wine. It also suggests that this influence is dependent on the dose of bentonite and the duration of exposure to the ferment/wine. This case supports the overall findings of this study that bentonite can be a major source of aluminium in table wines, particularly white wines, confirming and fully supporting the conclusions of McKinnon (1990) and McKinnon et al. (1992).

Wines from vineyard/winery 5 (Figure 4.18) were also of some interest as these were produced 'naturally' with no SO_2 or other treatment and no bentonite fining. These samples are valuable to compare with wines that had been through various treatments including bentonite. These wines showed the same or higher bottled wine aluminium concentration as treated wines. This result may suggest that the majority of the wines studied display concentrations that are reflective of the aluminum introduced into the wine from natural sources. Sampling could not be continued in the second season to confirm these observations.

4.10.3 Comparison of Bottled Wine Aluminium Statistics

The mean aluminium concentration of the grapes and juices was well above the WHO drinking water limit of 0.2mg/L. However, the wines throughout the production steps showed a much lower aluminium concentration. From the fermentation production step onwards the unfinished wine samples in this study were found to have a mean aluminium concentration that ranged from the drinking water limit to 2.5 times the limit. The bottled mean aluminium concentrations were lower than that noted by McKinnon (1990), however the sample size from this study is over an order of magnitude smaller than that of McKinnon's work. A comparison of basic statistics of bottled wine aluminium concentrations of this study with that of McKinnon's (1990) is presented in Table 4.26 and Figure 4.17. The data from this study include those for wines E and P for completeness.

Table 4.26 Comparison of basic statistics of bottled wine total aluminium concentrations from this study with that of McKinnon (1990).

Variety	Sample Size (This Study)	Sample Size (McKinnon)	Range This Study (mg/L)	Range McKinnon (mg/L)	Mean This Study (mg/L)	Mean McKinnon (mg/L)	Median This Study (mg/L)	Median McKinnon (mg/L)
Red	5	153	0.24-0.66	0.17-3.81	0.43	0.77	0.50	0.67
White	7	67	0.38-2.47	0.34-5.55	0.90	1.16	0.53	0.93
Total	12	220	0.24-2.47	0.17-5.55	0.70	0.91	0.52	0.77

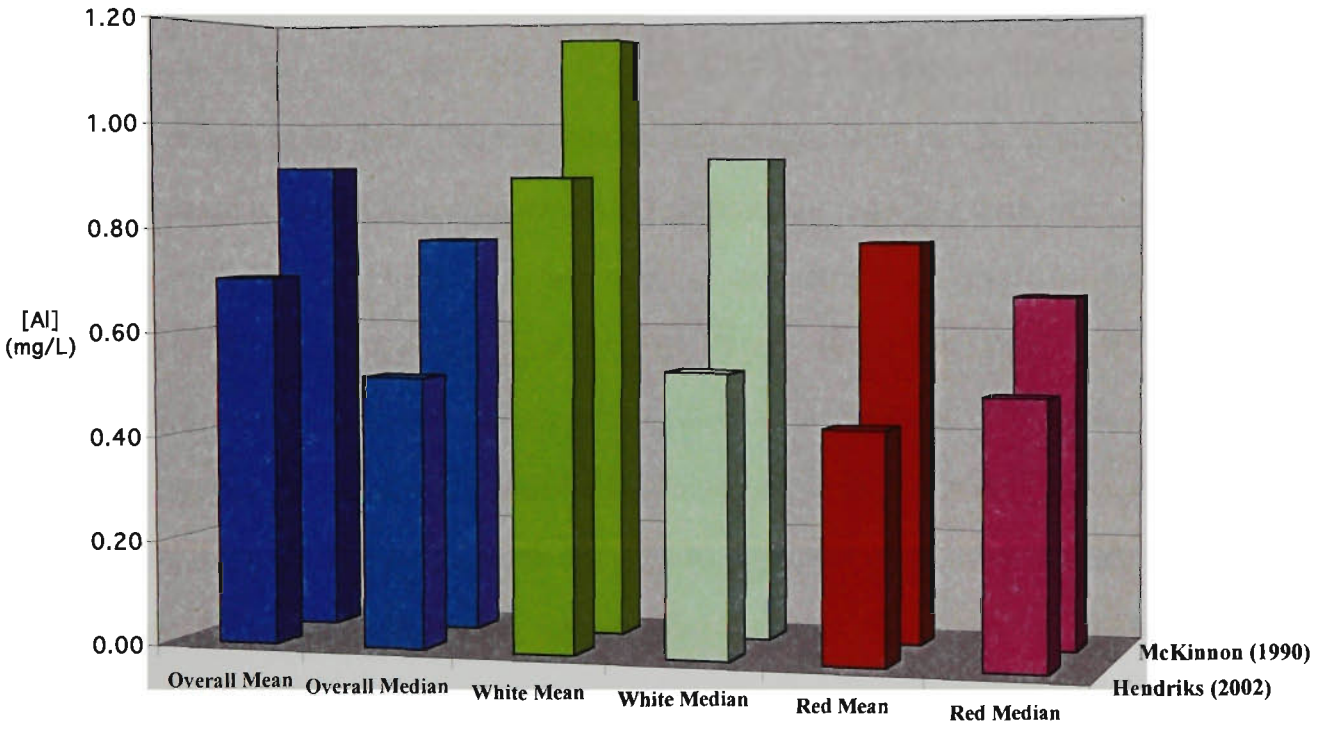


Figure 4.19 Chart comparing the basic aluminium total concentration statistics of bottled wines of the current study with those of McKinnon (1990).

The comparisons demonstrate the lower overall aluminium concentrations derived from this study compared with those of McKinnon (1990). In this study, the median concentrations for the overall, white and red wines give virtually the same result of around 0.50mg/L whereas those of McKinnon show some variation. This difference is most likely due to the small number of samples studied in this work compared with that of McKinnon.

4.10.4 Overall Trends of Wine Production Total Aluminium Profile Analysis

As individual wines are discrete sample sets that are different over vintages, varieties, cultivars and styles, any overall profile analysis using the means of the sample groups are at best speculative with

a good chance of outliers skewing the data. However, as can be seen from the individual wine profiles and the statistical analysis on differences in aluminium concentrations from different sample groups, some general trends can be deduced. The overall wine production total aluminium concentration profile and profiles comparing white with red wines and the two vintages are presented in Table 4.27. Note the overall analysis only includes sample groups with more than one sample. Adjusted data are shown in red.

The plots of the mean aluminium concentration profile for overall data, for vintage comparison and for wine varietal comparison are presented in Figures 4.20-4.23. There are three sets of adjusted mean aluminium concentration data. Those of the washed grapes were recalculated based on grape distribution, while those of the SO_2 free juice and SO_2 containing juice had their concentration units converted from µg/g to mg/L. These first two types of adjusted mean data are included in the following overall profile plots shown in Figures 4.20-4.23 and are distinguished on the x-axis with the sample code followed by a lower case 'a'. The third set of adjusted data were the modified means of ferment, pre-bottled wine and bottled wine recalculated after removal of the outlying data of samples E and P. As these data points were not used in statistical analysis of the data set, only the modified means are shown in the following figures. These sample means are denoted on the x-axis using the conventional sample codes without change. The un-modified mean data of the third adjusted data set are not included in Figures 4.20-4.23. The WHO drinking water limit for aluminium is superimposed on all these figures.

Table 4.27 Overall, vintage and varietal mean aluminium concentrations for sample sets of more than one sample. Note: Grape and juice aluminium concentrations are given in terms of wet weight.

Sample Type	Overall Mean [Al]	1997 Mean [Al]	1998 Mean [Al]	White Mean [Al]	Red Mean [Al]
Unwashed Grape	3.2±0.6µg/g	2.9±0.8µg/g	3.6±0.3µg/g	2.9±0.7µg/g	3.6±0.6µg/g
Washed Grape	2.2±0.5µg/g	2.2±0.5µg/g	2.3±0.5µg/g	2.2±0.4µg/g	2.2±0.7µg/g
Adjusted Washed Grape	1.7±0.4µg/g	1.6±0.4µg/g	1.7±0.4µg/g	1.7±0.6µg/g	1.7±0.9µg/g
SO_2 free Juice	1.6±0.2µg/g	1.6±0.2µg/g	-----	1.20±0.08µg/g	1.8±0.3µg/g
Adjusted SO_2 free Juice	1.7±0.2mg/L	1.7±0.2mg/L	-----	1.4±0.1mg/L	2.0±0.3mg/L
SO_2 Treated Juice	1.6±0.3µg/g	1.2±0.3µg/g	1.9±0.2µg/g	0.9±0.2µg/g	2.6±0.3µg/g
Adjusted SO_2 treated Juice	1.7±0.2mg/L	1.4±0.3mg/L	2.1±0.2mg/L	1.2±0.2mg/L	2.6±0.3mg/L
Fermented Wine	0.68±0.04mg/L	0.93±0.05mg/L	0.32±0.03mg/L	0.96±0.04mg/L	0.40±0.04mg/L
Adjusted Fermented Wine	0.29±0.03mg/L	0.28±0.03mg/L	As Above	0.16±0.02mg/L	As Above
Pressed Wine	0.33±0.06mg/L	0.35±0.06mg/L	0.30±0.05mg/L	-----	0.33±0.06mg/L
Malo-lactic Fermented Wine	0.25±0.01mg/L	0.25±0.01mg/L	-----	-----	-----
Bentonite Fined Wine	0.40±0.05mg/L	0.50±0.06mg/L	0.31±0.03mg/L	0.40±0.05mg/L	-----
Pre-Bottled Wine	1.26±0.06mg/L	1.40±0.05mg/L	0.99±0.08mg/L	2.10±0.09mg/L	0.42±0.03mg/L
Adjusted Pre-Bottled Wine	0.42±0.03mg/L	0.49±0.04mg/L	0.20±0.02mg/L	0.40±0.03mg/L	0.42±0.03mg/L
Bottled Wine	0.70±0.04mg/L	0.72±0.04mg/L	0.66±0.04mg/L	0.89±0.04mg/L	0.43±0.04mg/L
Adjusted Bottled Wine	0.47±0.02mg/L	0.47±0.02mg/L	0.48±0.04mg/L	0.52±0.02mg/L	0.43±0.03mg/L

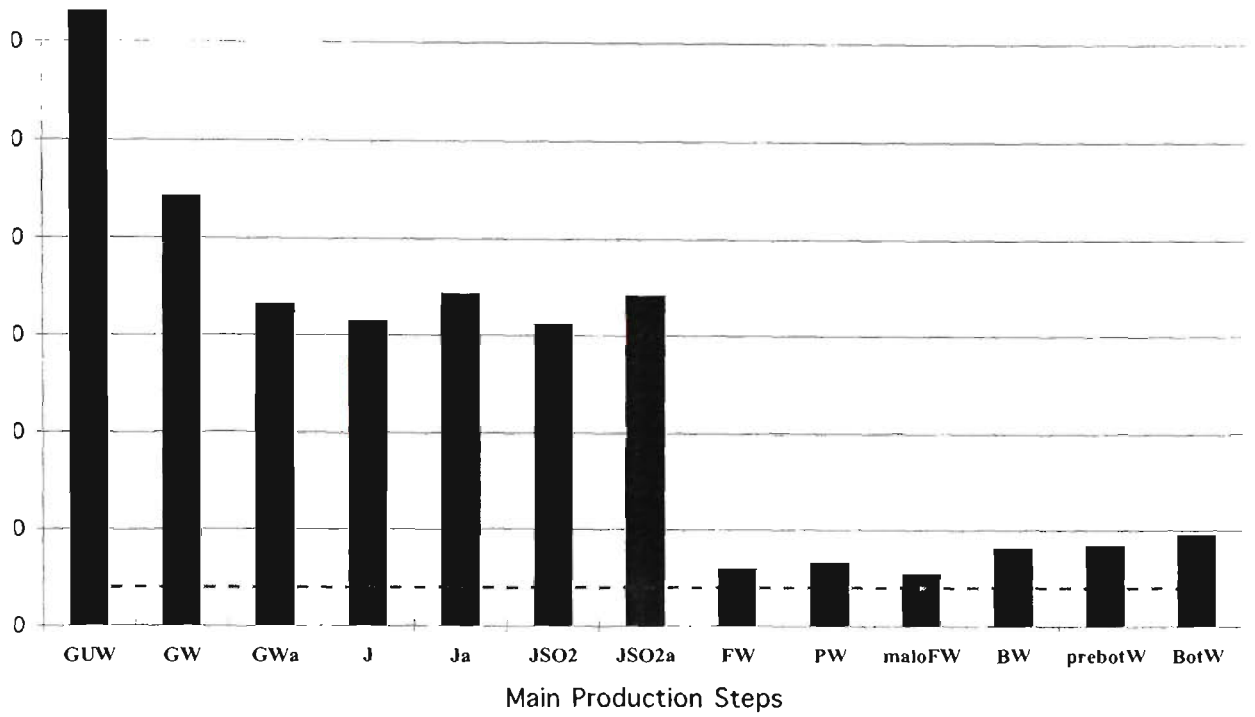


Figure 4.20 Mean total combined vintage (1997 + 1998) aluminium concentration profile over the production process. Adjusted concentrations are shown in green and the WHO drinking water limit is shown as a broken red line. Note the concentration unit ppm covers (μg/g) for grapes and non-adjusted juice with the adjusted juice and remainder in mg/L.

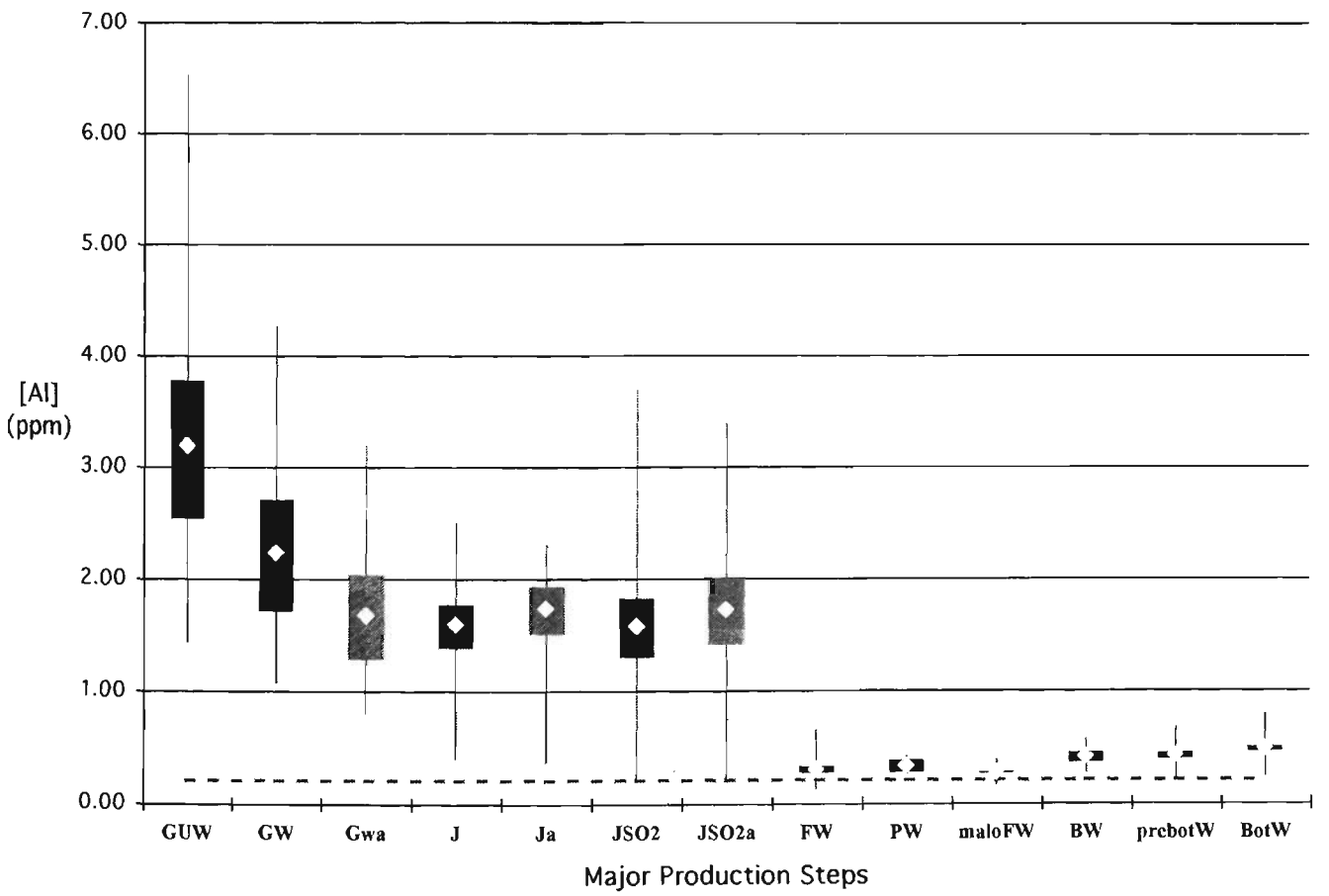


Figure 4.21 Mean total combined vintage (1997 + 1998) aluminium concentration profile over the production process shown as a statistical box plot with the thin line representing the range, the box representing one standard deviation from the mean and the diamond representing the mean. The three green box plots show the adjusted data, the broken red line represents the WHO drinking water limit. Note for grapes and non-adjusted juice the concentration unit ppm refers to μg/g with the adjusted juice and remainder in mg/L.

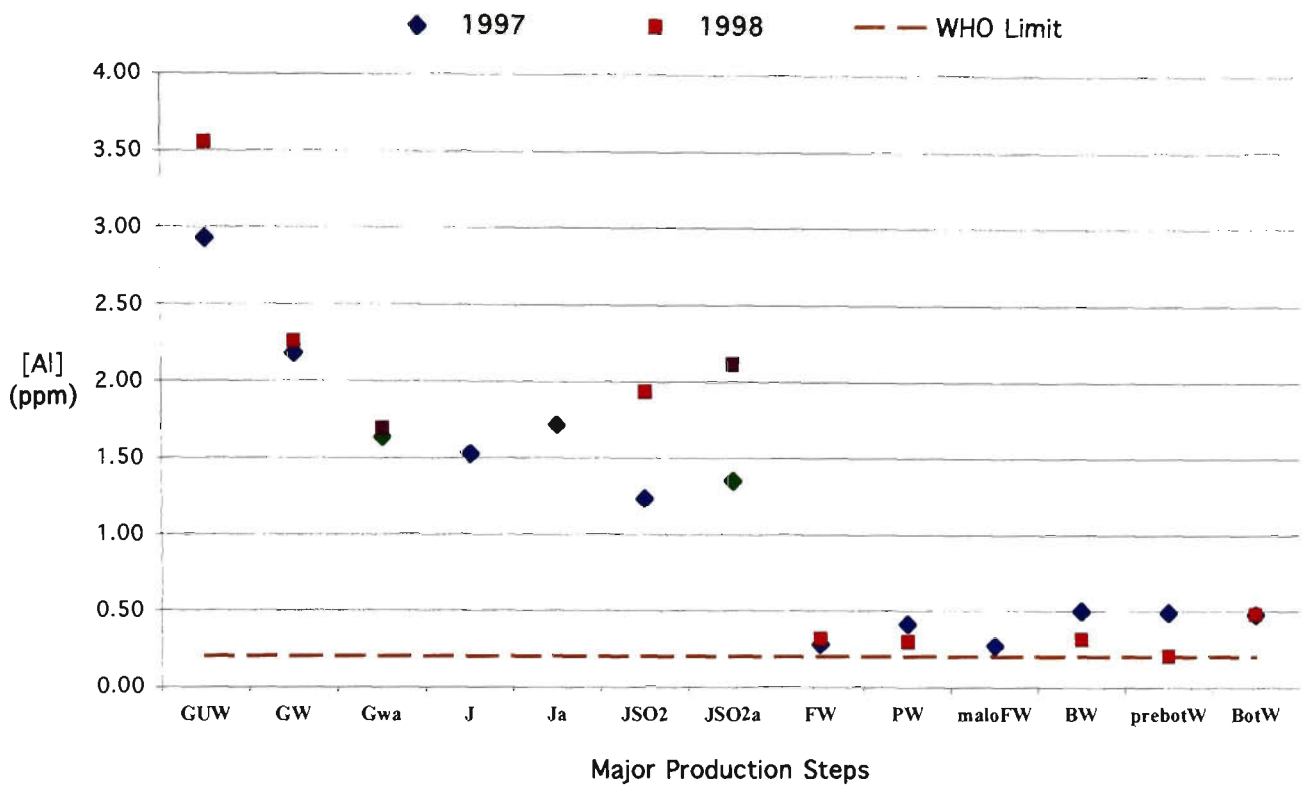


Figure 4.22 Mean total aluminium concentration profiles separating the 1997 and 1998 wines. The data points coloured light green and purple represent the adjusted means, the orange broken line represents the WHO drinking water limit. Note for grapes and non-adjusted juice ppm refers to µg/g with the adjusted juice and remainder in mg/L.

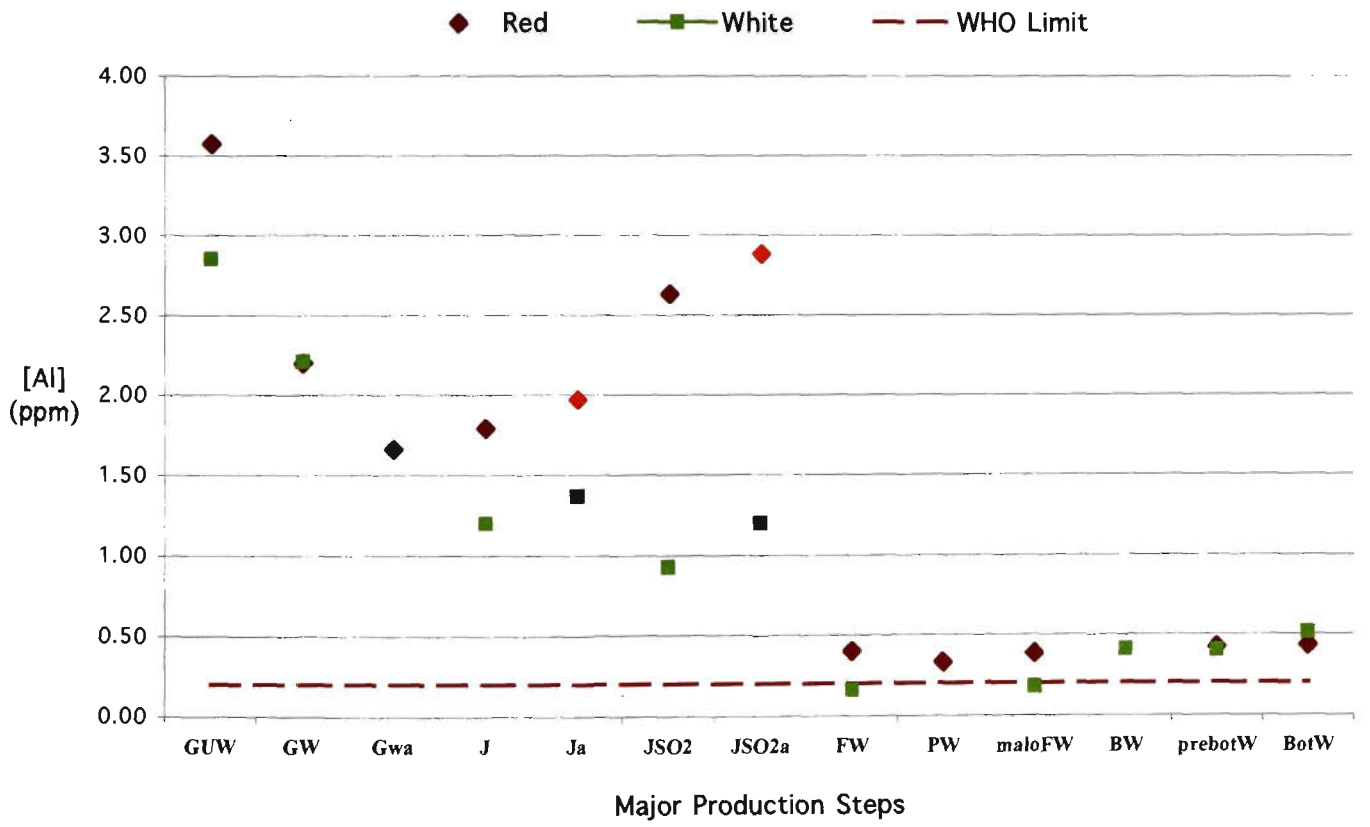


Figure 4.23 Mean total combined vintage (1997 + 1998) aluminium concentration profiles of red and white wines. The data points coloured orange and navy blue represent the adjusted means, the red broken line represents the WHO drinking water limit. Note the concentration unit ppm covers (µg/g) for grapes and non-adjusted juice with the adjusted juice and remainder in mg/L.

The overall total aluminium profile shows three phases of aluminium concentration magnitudes. Figure 4.20 shows a high unwashed grape aluminium concentration compared with washed grape concentrations, which as discussed in Section 4.5 showed a statistically significant decrease when the grapes were washed. Once the washed grape concentration has been adjusted to account for skin and pip aluminium that does not make it into the juice and ultimately the ferment, the mean aluminium concentration stabilises at around 1.5mg/L in juices.

At this point the most significant change that is statistically significant at a 99% confidence concentration in aluminium concentration occurs. A dramatic decrease of approximately 1.1mg/L (or around 70% of naturally sourced) aluminium is observed during the fermentation process, however there is a wide spread in the decrease as the range in the data for the juice aluminium is quite large (see Figure 4.21). This agrees with findings of Meierer (1984), who also noted a wide spread in the data for the decrease in aluminium during the fermentation process and quoted other studies also showing a decrease of aluminium concentration of up to 70-90% from the juice to the wine. From this point in the production process the aluminium concentration remains around 0.4-0.6mg/L until bottling. Pressed ferment and malo-lactic fermentation showed no significant change, however the only other significant change in wine aluminium concentration was observed in white wines after bentonite addition. This statistically significant change (98% confidence concentration) on average doubled the aluminium concentration, fully confirming the conclusions of McKinnon (1990) and McKinnon et al. (1992). As mentioned earlier the case study of wine E, where high exposure to bentonite early in the production process lead to aluminium concentrations around 4mg/L, gives further support to this hypothesis. However, the mean magnitude of change was only a fifth that of the loss of aluminium from the system after fermentation. After this step there is relatively little change in the aluminium concentration, with no evidence to support the hypothesis that significant quantities of aluminium is leached from the glass after the wine is bottled.

The caution in interpreting means is demonstrated in Figure 4.21 with a relatively large range in aluminium concentration shown for all grape and juice samples compared with those of the ferments and wines. However, the aluminium concentrations covered by one standard deviation either side of the mean supports the profile analysis.

The comparison of 1997 with 1998 wines shows a good agreement over two seasons, however the juices with SO_2 addition show a higher aluminium concentration in the second year. The large decrease in aluminium is emphasised again during the fermentation process, with the mean wine aluminium concentrations from ferment to bottling maintaining an aluminium concentration range of 0.20-0.50mg/L. This plot shows the general trends observed for the overall mean aluminium profile is not heavily influenced by one vintage. The red and white wines showed some differences in profile, particularly with the aluminium content of the juices. Red wine is produced with skin included in the early stages and although the difference in the aluminium concentrations for these samples is significant, overall the influence of SO_2 remained inconclusive. These plots also demonstrate the loss of aluminium from the system during fermentation, however after this point the red wines concentration remains unchanged while the white wines show the significant increase in aluminium concentration after bentonite fining.

V

"Because a thing seems difficult for you, do not think it impossible for anyone to accomplish"
Marcus Aurelius, Roman Emperor (121-180 AD)

CHAPTER FIVE

5. THE SPECIATION ANALYSIS OF ALUMINIUM IN WINE USING ES-MS

5.1 Introduction

Knowledge of the bioavailability of the aluminium in wine facilitates an understanding of the metabolism, fate and possible toxicity of the metal after wine consumption. In turn, to understand the bioavailability of aluminium in wine, one must determine its speciation in that matrix. Although aluminium speciation analysis of waters and biological matrices has been well documented (see Chapter 2), there has been little work directed at the determination of the speciation of aluminium in wine. A review of the development of aluminium speciation analysis generally and the use of electrospray mass spectrometry (ES-MS), chosen as the speciation technique for this work, is covered in detail in Chapter 2.

This chapter presents and discusses the results of the analysis of aluminium speciation in wine using electrospray mass spectrometry, including the development of the method from simple aqueous matrices and the theoretical considerations that influenced the direction of the research.

5.2 Speciation Considerations

In order to conduct aluminium speciation analysis in wine one must consider the possibilities for complexation, the availability of labile aluminium and ligands able to bind to it, and their distributions with regard to pH and relative solution concentration. The species and distribution of aluminium found in nature, particularly in aqueous media, have been discussed in detail in Chapters 1 and 2.

The pH of wine varies between 3.0-4.3 (Rankine 1991) and inspection of the distribution chart in Figure 5.1 shows that over this pH range the aluminium in wine would be present as labile Al^{3+}, readily available for complexation with anionic or electron rich ligands.

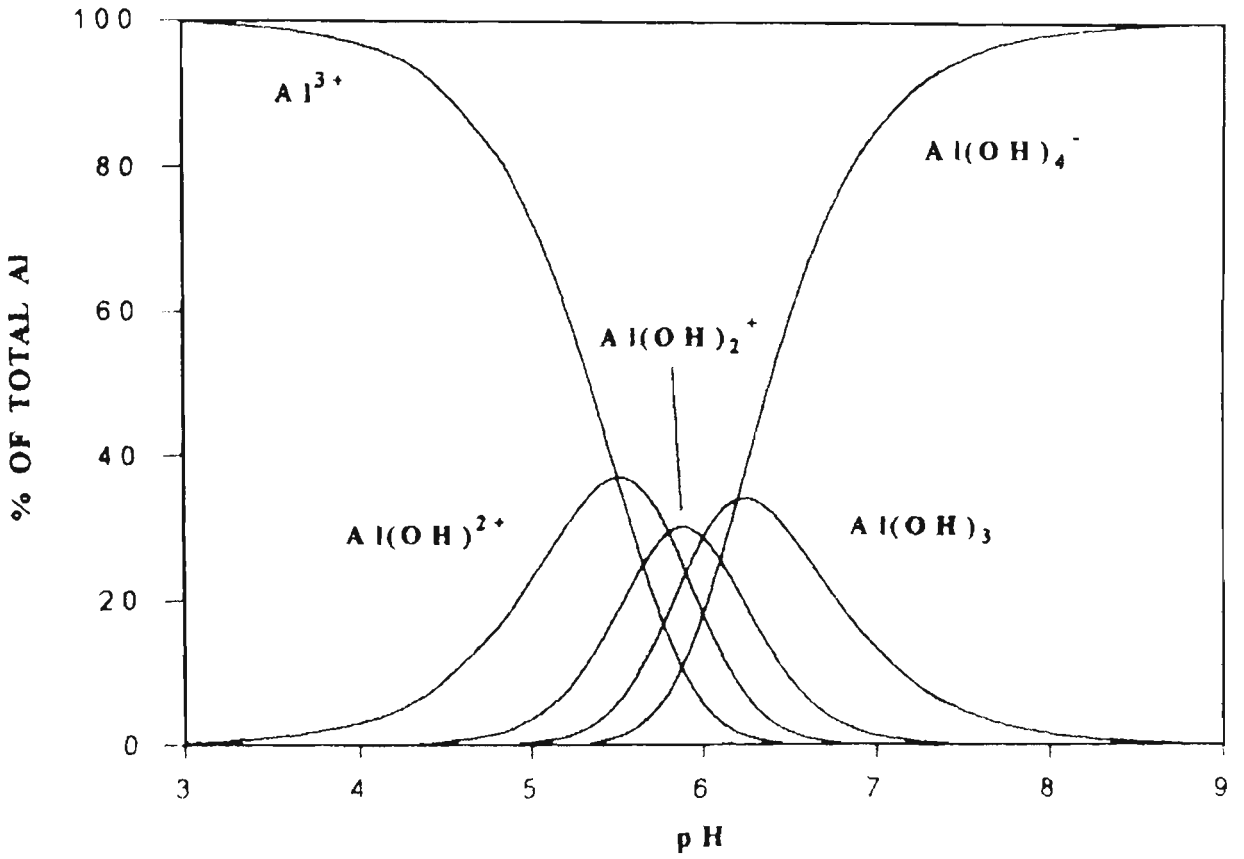


Figure 5.1 Distribution of the Al^{3+} aquo ion and the mononuclear $Al(OH)_n$ species for 1μM total aluminium (from Harris et al. 1996).

Wine is an extremely complex matrix that is aqueous in nature with a significant alcohol content derived from the fermentation of the grape sugars. Two factors contribute to this complexity. Initially the distribution of components reflects that of the grapes which can differ widely in chemical composition 'depending on the variety, the climate and soil in which they are grown, their maturity and soundness' and secondly, due to the fermentation process a large number of volatile and non-volatile compounds are formed that were not present in the grapes (Rankine 1991). Rankine (1991) gives an excellent listing of the possible components encountered in wine grouping them into eight broad classes shown in Table 5.1. From the table it is clearly evident that water and ethanol constitute the majority of a wine, however the number and variety of components that make up the rest is quite large.

Table 5.1 The percentage distribution of broad classes of wine components (from Rankine 1991).

Wine Component Class	Content
Water	70-90 %v/v
Ethanol*	8-14 %v/v
Sugars*	0.01-0.075 %m/v
Acids	0.3-1 %m/v
Bases	0.1-0.3 %m/v
Phenolics, Tannins	0-0.4 %m/v
Volatiles	0-0.2 %m/v
Other non-volatiles	0.5-1 %m/v

***These figures are for dry table wines**

Rankine (1991) also lists the concentrations of major individual wine components. Of the acids, tartaric acid is the most predominant with a concentration between 2-5g/L, and, other than glycerol, is the principal component after water and ethanol. Malic acid can also be found in significant concentration (up to 5g/L) depending on the fermentation process followed by lactic, succinic and citric acids with ascorbic acid, although present in grapes, mostly oxidised by the end of the fermentation process (Rankine 1991). Glucose and fructose are the predominant sugars found in table wine with up to 10% m/v found in sweet varieties, however in dry wines the sugar concentration is significantly lower with less than 0.75g/L total sugar content. Other significant components of wine include glycerol (1-15g/L), the anions sulfate (0.1-3g/L), bisulfite, phosphate and chloride, the cations potassium (0-2.5g/L), sodium (0.02-2.5g/L), calcium and magnesium and the phenolics comprised of anthocyanins and tannins (0.2-4g/L). In lesser amounts are other acids, sugars, alcohols, polyols, anions, cations and phenolics, in addition to esters, carbonyls, aldehydes, nitrogenous compounds such as proteins and amino acids, vitamins and many other compounds in trace amounts. Although simplified, this description of the components of wine demonstrates its chemical complexity and the abundance of a large number of possible ligands for aluminium. Competition for ligand binding with aluminium will depend on the relevant complex formation constants, ligand concentration and wine pH.

When defining target aluminium species to investigate in wine, a number of considerations were taken into account. A major influence in determining target species was the work of McKinnon

(1990). Using size filtration of wine samples followed by GFAAS analysis, it was shown that the majority of aluminium in wine is bound to small molecules, thereby eliminating polyphenolics and proteins as significant aluminium binding ligands in wine. Accompanying work with solvent extraction and colorimetric/fluorimetric analysis showed significant interferences that were attributed to aluminium binding with low molecular mass organic acids (Scollary 1997). This makes sense because, as discussed in Chapter 2, aluminium is a hard Lewis acid that preferentially binds to electron rich Lewis bases, particularly oxygenous multidentate ligands that have high formation and stability constants (Martin 1988; Orvig 1993; Ritchie & Sposito 1995; Berthon 1996; Yokel 2002). Although there are a vast number of potential ligands in wine, binding with organic acids becomes more plausible when one considers the organic acids in wine are aliphatic acids that are the strongest chelators of aluminium (Yokel 2002, Salifoglou 2002), particularly dicarboxylic and hydroxycarboxylic acids (Rubini et al. 2002).

In the case of tartaric acid, an additional consideration is the very high ligand concentration compared with other competing ligands. Tartaric acid at 4g/L has a potential ligand excess of over three orders of magnitude over aluminium at about 0.5 to 4mg/L in wines reported by McKinnon (1990) and this study (see Chapter 4). Potentiometric studies combined with computer modelling simulating gastrointestinal conditions have shown that a ligand excess gives a much greater proportion of ligand bound aluminium compared with free aluminium for malate, succinate and tartrate over the pH range 2-7 (Venturini-Soriano & Berthon 1998, 2001; Desroches et al 2000). In terms of the availability of anionic species of tartaric acid, the distribution chart with respect to pH in Figure 5.2 clearly shows that 45-60% of bitartrate $[Htart]^-$ and 5-45% of tartrate $[tart]^{2-}$ is readily available for complexation over the wine pH range of 3-4. Comparing Figures 5.1 and 5.2 shows that both free Al^{3+} and anionic forms of tartaric acid are available for complexation in wine.

After reviewing all the factors mentioned above, it was considered highly probable that aluminium is bound to anions of tartaric acid in wine, particularly bitartrate. Hence, it was decided to initially target complexes of aluminium and tartaric acid for speciation analysis using ES-MS.

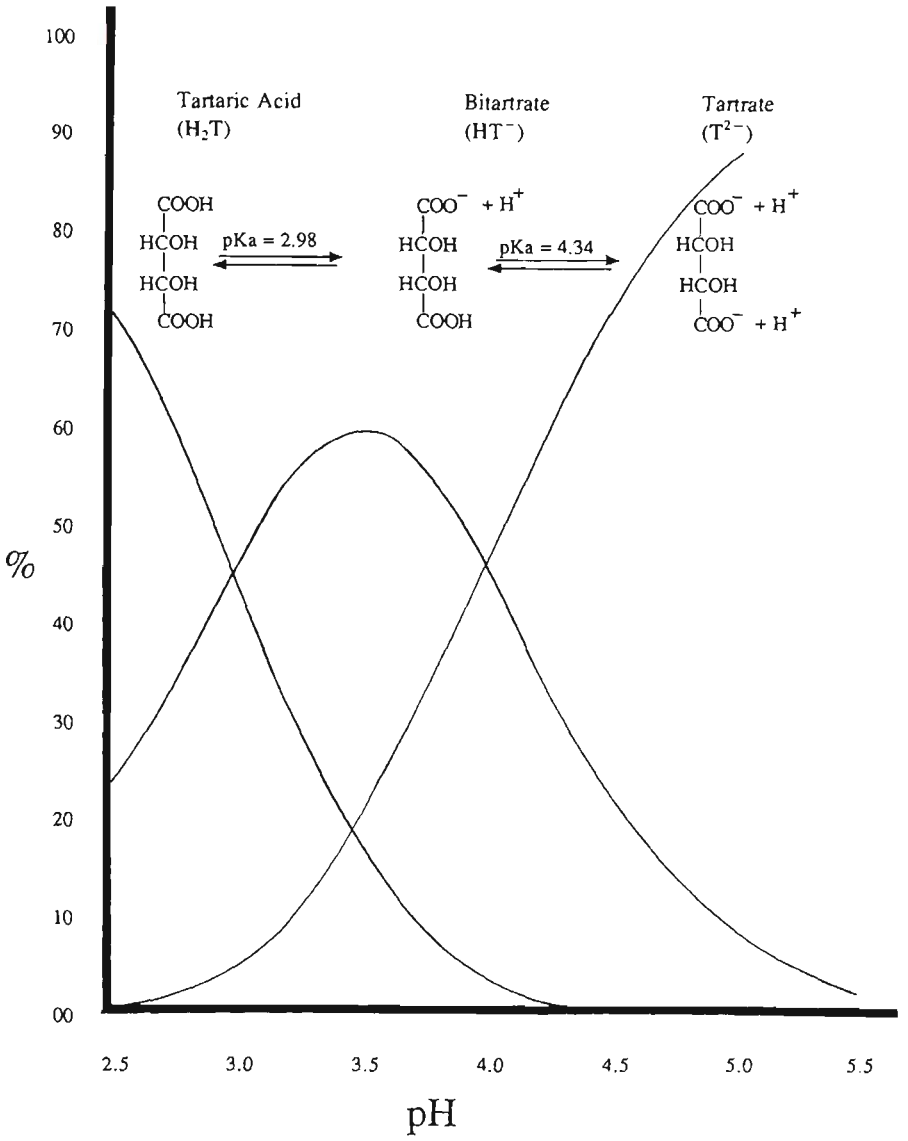


Figure 5.2 Distribution of tartaric acid species in aqueous solution with respect to pH (from Zoecklein et al. 1990).

5.3 Preliminary ES-MS Speciation Investigation of Aqueous Solutions

5.3.1 Method Development Considerations

Due to the complexity of the wine matrix and considering that, to the author's knowledge, no work has been conducted prior to this study regarding aluminium speciation in wine using ES-MS, it was decided to begin the investigation by analysing simple aqueous solutions of the targeted separate components and their simple mixtures. This approach was adopted to discern what possible species could actually be determined with ES-MS in solutions in conditions that could be carefully controlled and served several functions. Firstly, it assisted in optimising the solution and instrumental conditions for the signals attributable to component species and their optimal concentrations for species detection. Secondly, by simplifying the matrix, the mass spectra should be less complex due to the reduced number of contributing ions and hence any true aluminium

tartrate species should be easier to elucidate and confirm. Additionally, the simplified matrix could eliminate interference from other major wine components, such as ethanol, that could disturb complex formation, solution equilibria or instrument performance. Most importantly, this preliminary work was designed to enhance the most likely aluminium/organic acid species and increase confidence in assigning and confirming mass spectral data to aluminium/organic acid species.

Wine is mainly an aqueous solution (refer Table 5.1), hence it was deemed desirous to maintain this solvent matrix as closely as possible throughout the electrospray process to reduce any change in solution equilibria. As water is not normally a solvent easily amenable to simple electrospray (van Baar 1996; Daas 1997), nitrogen gas nebulisation, a heated electrospray capillary and the use of a 50:50 acetonitrile/water solvent is recommended for aqueous analytes (van Baar 1996; Johnstone & Rose 1997). Using these conditions, the operating parameters were adapted from the default settings of the instrument manufacturer and optimised for an acetonitrile/water solvent. As this solvent type was the best compromise in terms of maintaining solution integrity and ES-MS performance, this solvent was used as the carrier solution between injections of the aqueous samples. The operating parameters are given in Chapter 3, Section 7.4.

To aid the reader in interpreting the mass spectral data, all the mass spectra shown in this Chapter display the ion mode in which the ES-MS analysis was carried out and the ion intensity of the largest peak in the top right hand corner. The intensity scale on the y-axis is given as a percentage of the intensity of this largest peak and the mass scale is displayed on the x-axis.

5.3.2 Characterisation of Aluminium Species Sourced from BDH Spectrosol™ Aluminium Nitrate Standard in Aqueous Media

Before investigating mixtures of aluminium and tartrate, the individual components were analysed with ES-MS to aid in assigning ion peaks attributable to these components in a mixture. Aluminium was the first component investigated. 1mg/L-10mg/L ($3.7x10^{-5}$M-$3.7x10^{-4}$M) aluminium solutions at autogenous pH were injected into the ES-MS and the spectra were observed in both positive and negative ion modes. These concentrations were chosen because 1mg/L approximates the average

concentration and 10mg/L is double that of the highest concentration found for aluminium in wine respectively (McKinnon 1990; McKinnon et al. 1992; Chapter 4 of this thesis).

Using the ES-MS positive ion mode, the aluminium solutions were investigated over a mass range of 20-500Da. From these analyses, the positive electrospray mass spectra of the 10mg/L aluminium solution derived from BDH Spectrosol™ standard is shown in Figure 5.3 over a range of 75-400Da.

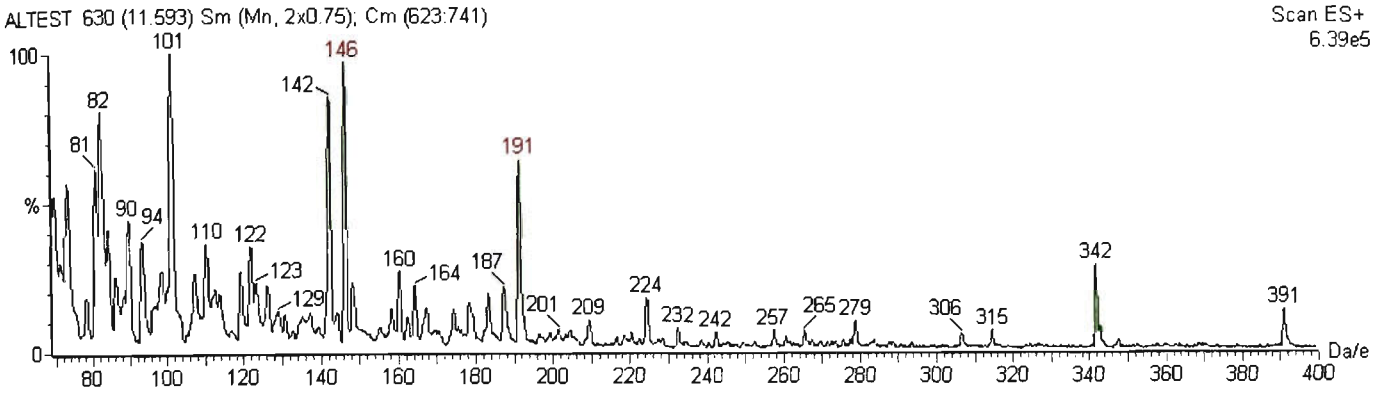


Figure 5.3 Positive mode ES-MS spectrum of 10mg/L (0.37mM) aluminium solution derived from BDH Spectrosol™ aluminium nitrate standard solution. The m/z corresponding to ion peaks attributed to aluminium are shown in red.

From the positive ion mode analyses and as shown in more detail later in this section, only two peaks observed at 146Da and 191Da were distinguished and attributed to an aluminium species in solution using methods developed to attribute and confirm component ion peaks. These methods are discussed in the next section. Despite this analysis, the masses of probable aluminium species could not be matched with these mass numbers. Examination of the same solution in negative ES-MS mode over 100-700Da is demonstrated in Figure 5.4.

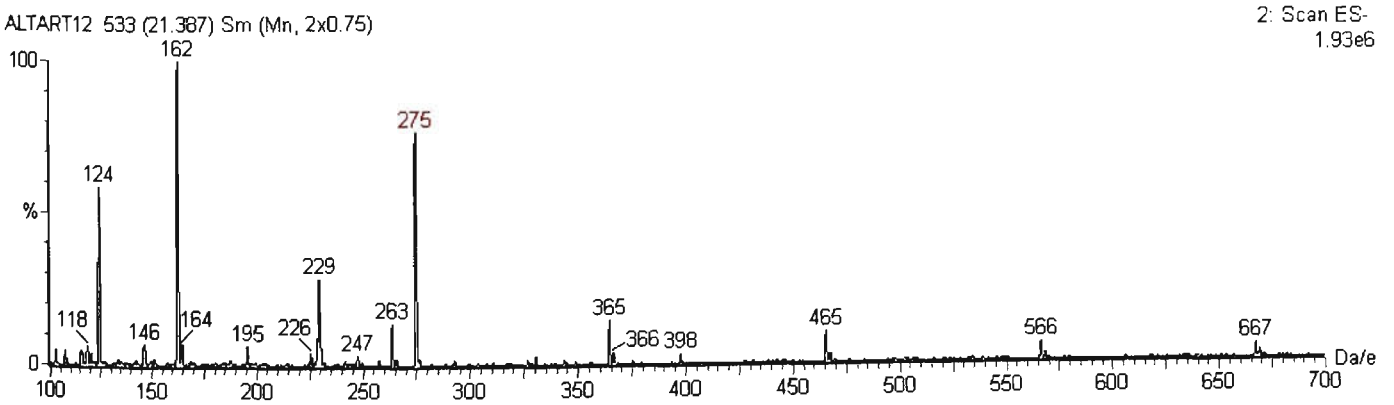


Figure 5.4 Negative mode ES-MS spectrum of 10mg/L (0.37mM) aluminium solution derived from BDH Spectrosol™ aluminium nitrate standard solution. The m/z corresponding to assigned ion peak is shown in red.

Only one peak could be characterised from the negative ion spectrum of dilute aluminium nitrate standard. This peak at 275Da was assigned to the species $[Al(NO_3)_4]^-$. This characterisation was also confirmed using the methods described in the next section.

One would expect that in simple aqueous solutions with the addition of standard compounds under tight controls that aquated aluminium ions known to exist in water would be found. Hence, it was expected that an $[Al(OH)_2]^+$ species at 61Da would predominate in the positive ion spectra and that nitrate would be too weak a ligand to form the tetranitratoaluminate(III) ion at 275Da. The absence of the former and the existence of the latter meant that the results observed for aluminium nitrate solutions did not meet expectations. However, the BDH Spectrosol™ standard contains 0.5% nitric acid for solution stability giving a diluted standard pH of 2. At this pH, aluminium will only exist as $[Al(H_2O)_6]^{3+}$ and OH^- formation would be difficult. The solution pH, combined with the ES positive ion process favouring protonation rather than deprotonation, does minimise the likelihood of a peak due to the hydrolysis product. However, although Al^{3+}, $[Al(H_2O)_6]^{3+}$ or $[Al(H_2O)_4]^{3+}$ could be expected in the positive ion mode at m/z 9, 45 and 33 respectively in an aluminium standard at pH 2, there was no evidence of these peaks in the ES positive ion mode.

The negative ion mode result suggests that the majority of the aluminium is bound to nitrate for solutions derived from this standard. Although nitrate is a weak ligand, the large excess of nitrate from the nitric acid, in concert with the negative ion electrospray process could increase the probability of attachment of nitrate ligands to aluminium. It is probably the former condition that exhibits the most influence on the formation of the tetranitratoaluminate (III) ion as its peak at 275Da only occurs when Al^{3+} and NO_3^- are present with nitrate in excess. In the positive ion mode one could expect the existence of a di-nitrato-aluminium species with the possibility of the addition of associated water molecules numbering from 1 to 4, although as aluminium is known to prefer tetrahedral or octahedral arrangements these would be restricted to 2 or 4 additional molecules. However, no peaks attributable to these species were observed in the positive ion mode.

The unassigned ion peaks at 146Da and 191Da in the positive ion mode mass spectrum of diluted aluminium BDH Spectrosol™ standard could represent the product of polyvalent or ES-MS induced

fragmented polynuclear aluminium species, impurities in the standard or the nitric acid in the standard. However, these scenarios can be effectively discounted as the existence of polynuclear aluminium species at pH around 2-2.5 is questionable (Bertsch & Parker 1996), impurities in the standard are certified to be low, and these peaks are not observed in the positive ion ES mass spectrum of dilute analytical grade nitric acid (see Figure 5.5).

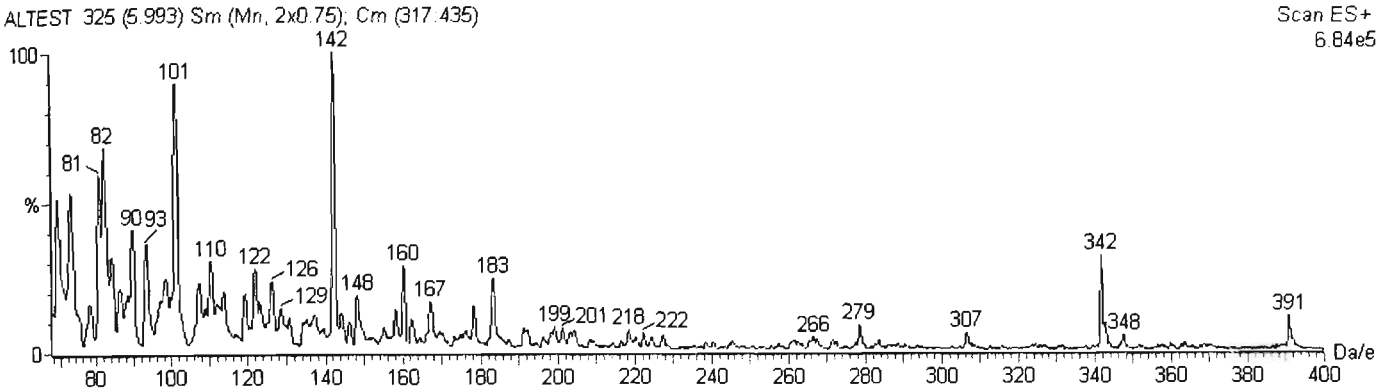


Figure 5.5 Positive ion mode ES-MS spectrum of dilute AR HNO_3.

In summary, the ions attributed and assigned in this section are presented in Table 5.2.

Table 5.2 Summary of attributed and assigned ions presented in Sub-Section 5.3.2.

Peak Ion Mass (Da)	Assigned Species
ES Positive Ion Mode	
146	Unassigned
191	Unassigned
ES Negative Ion Mode	
275	$[Al(NO_3)_4]^-$

5.3.3 Development of Ion Peak Identification and Confirmation Techniques Using ES-MS

In investigating the speciation of dilute BDH Spectrosol™ aluminium nitrate solutions, two methods of ion peak attribution and confirmation were developed. One involves the use of subtraction of the mass spectrum due to the matrix from that of the solution containing the analyte of interest. The other involves the matching of particular ion count peaks of target ion m/z with the injections of particular solutions over time.

5.3.3.1 Mass spectral subtraction

Using the Micromass Masslynx™ software, a mass spectrum of one solution can be subtracted from another. Any ion peaks in the solution of interest that are due to the solution matrix components, such as nitric acid, can be reduced in intensity by the magnitude of the intensity of the same ion peak in the mass spectrum of the matrix solution. This method relies on four assumptions. The first is that the matrix solution is injected in the same analysis under the same conditions as the solution of interest, with enough time between injections to avoid electrospray signal overlap. The second is that the matrix is virtually devoid of the analyte of interest. The third is that the component concentrations in the matrix solution matches or is greater than their concentrations in the solution of interest. The fourth is that the matrices of the solutions are closely matched to avoid differences in the electrospray conditions, and hence ion formation, due to changes in the solution physical properties.

An example of this technique is demonstrated in Figure 5.6. This figure shows the mass spectra of 10mg/L aqueous aluminium solution derived from BDH Spectrosol™ standard and a solution of 0.035% analytical grade nitric acid. The latter was chosen as it is the main matrix component in the aluminium solution and its concentration was derived from calculating the approximate nitric acid content after dilution of the standard. Below these spectra are those of the superimposition of the nitric acid spectrum over that of the analyte solution and the resultant mass spectrum generated from the subtraction of the matrix mass spectrum from the analyte mass spectrum. As can be seen from Figure 5.6, by subtracting the background due to the matrix, the resultant mass spectrum is simplified by removing matrix peaks and emphasising those that are not due to the background but are genuinely introduced by the analyte solution. In the case shown in Figure 5.6, after background subtraction, the unassigned peaks attributed to the dilute aluminium standard at 146Da and 191Da are now the dominant peaks clearly originating from the analyte solution.

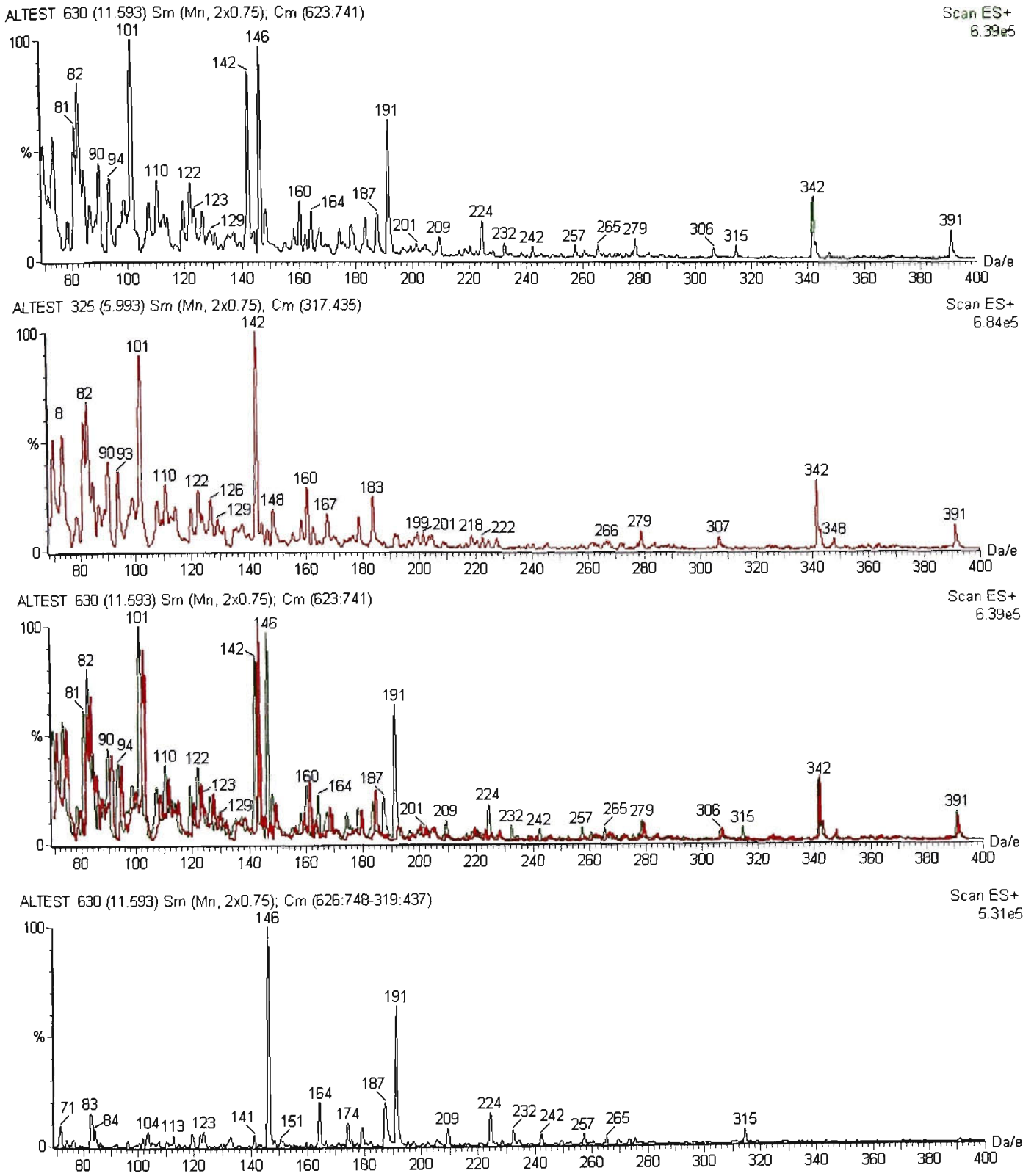


Figure 5.6 A sequential collection of mass spectra in positive ion mode over a range of 100-400Da demonstrating the use of mass spectral subtraction via the Masslynx™ software. The first shows the mass spectrum of a 10mg/L aqueous aluminium solution derived from BDH Spectrosol™ standard. The second shows the spectrum of a 0.035% v/v solution of AR HNO_3, coloured in red. This spectrum is then shown, also in red, superimposed on top and slightly offset to the right of the spectrum for the aluminium standard (shown in green). The final mass spectrum is that derived after subtracting the matrix mass spectrum from the aluminium spectrum. This isolates two major peaks at 146Da and 191Da, and indicates that they are due to the aluminium standard solution and not an artefact of the solution matrix.

Of course, how well the background subtraction works depends on how closely the matrix of the analyte solution can be matched in terms of both solution components and their concentrations. The closer the matrix matching, the better the background peak removal will be.

5.3.3.2 Ion count chromatogram peak matching

For each sample analysed the total ion count or ion intensity over a defined m/z range is monitored by the Masslynx™ software. The Masslynx™ software then plots the intensity with respect to time for the duration of the analysis. The resultant plot is known as a 'chromatogram', however, this term should not be confused with that traditionally used for the technique of chromatography. Although similar in appearance, these chromatograms do not represent the same thing. The ES-MS 'chromatogram' is the representation of ion intensity over time, and the peaks indicate a quantity of ions over a defined mass range detected for different samples by the mass selective detector. Hence from this point on in this chapter, a 'chromatogram' refers to that derived from the application of ES-MS mentioned above, not a separation carried out by chromatographic techniques.

The second technique developed for attributing and confirming mass spectral peaks involves the use of viewing the chromatograms of a single m/z of peaks of interest chosen from the analyte mass spectrum after background subtraction. These peak ion intensities are then matched with the total ion peaks representing the ions of particular analyte solutions. This is also managed by the Masslynx™ software. The software can process the chromatogram of the ion count over the total ES-MS run time for the m/z interval of a single peak with the interval set by the operator. The chromatogram of the single ion count can be viewed on the same page either directly above or below that for the total ion count; indeed several chromatograms of peak m/z intensity can be viewed simultaneously, allowing cross-matching of peaks with analyte injections and hence possible species. An example of this technique is shown in Figure 5.7 presenting the chromatograms of the peaks observed for 10mg/L aluminium derived from BDH Spectrosol™ standard at various pH values compared with the chromatogram of the total ion count in ES positive mode.

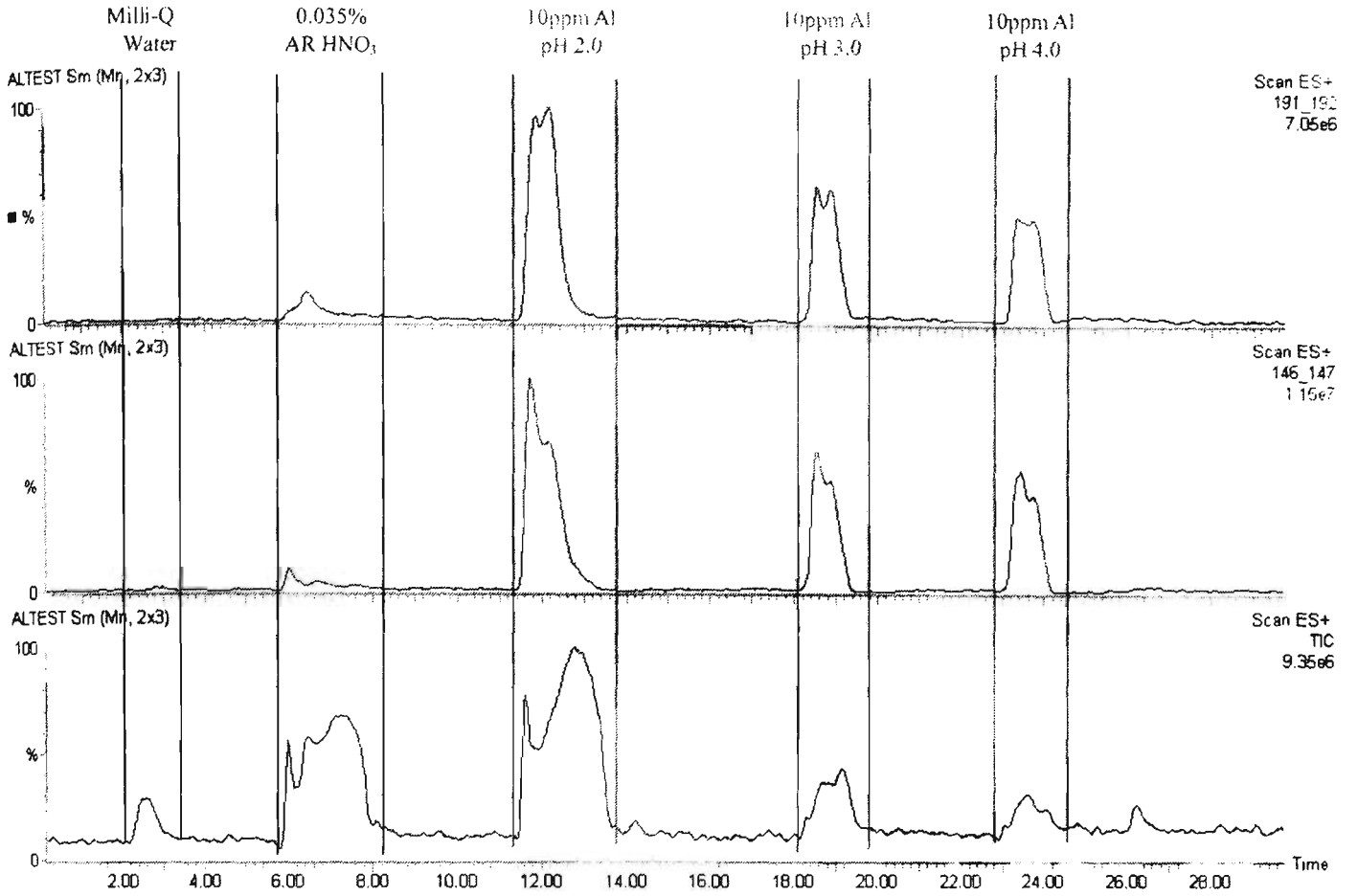


Figure 5.7 Example of ion count chromatogram matching. Forming a 2-dimensional matrix the ion count chromatograms are shown from top to bottom; 191-192Da ion count, 146-147Da ion count, total ion count. These are the intensities for these ion groups over time with the peaks denoting the ion response of an injection plug of a particular solution. These groups are colour coded to match the colour coding of the solution type shown above. Note: the scale of intensity for each ion count chromatogram is not the same.

Figure 5.7 shows that the ions at 146Da and 191Da are absent from Milli-Q™ water, used to prepare all solutions, and in AR HNO_3 exhibits a small response which is insignificant when compared with their response in the aluminium solution at various pH. These observations show that the ions do not result from Milli-Q™ water and insignificantly from AR HNO_3. Both these ions have significant presence in the aluminium standard solution and their absence from the two major matrix components clearly show that these ions are derived from the aluminium standard and not an artefact of the matrix as described in Section 5.3.2.

5.3.3.3 Summary of ES-MS peak isolation and confirmation methods

The two methods developed for peak attribution and confirmation described in sections 5.3.3.1 and 5.3.3.2 demonstrate their ability to enhance the targeting of relevant peaks. Spectral subtraction greatly lowers the number of peaks to be investigated, by reducing the background and enhancing peaks of interest. Chromatogram examination allows the analysis of many ion peaks simultaneously in a single graphic matrix; by a process of cross matching and elimination, ion peaks can be shown

to be unique to a particular solution. Furthermore, mass numbers that correspond to a proposed species gives greater confidence in peak assignment and species characterisation. As these methods were found to be so successful in interpreting mass spectral data, they were adopted for all further speciation work described in this chapter. None-the-less, there are peaks that either could not be assigned or could only be tentatively assigned by these methods and will be noted where appropriate.

5.3.4 Characterisation of Tartaric Acid/bitartrate Species in Aqueous Media

Several solutions of tartaric acid at various concentrations were analysed by ES-MS in the negative ion electrospray mode. Eventually a concentration of 100mg/L was deemed suitable for ES-MS analysis. This was arrived at by considering two factors. First, in wine the concentration of tartaric acid is in the range 2-5g/l (Rankine 1991); however using this concentration in the electrospray would severely overload the instrument, producing massive memory effects. Second, it was also anticipated that real wine samples would need to be diluted around 20-50 fold for the same reason. Assuming these concentrations of tartaric acid in wine hold true, on average, a 35-fold dilution would reduce the concentration of tartaric acid to approximately 100mg/L. Trials demonstrated that this concentration did not overload the system and provided excellent ion intensities using ES-MS. Figure 5.8 shows the mass spectrum between 100-700Da in negative ES mode of a 100mg/L aqueous solution of tartaric acid at autogenous pH.

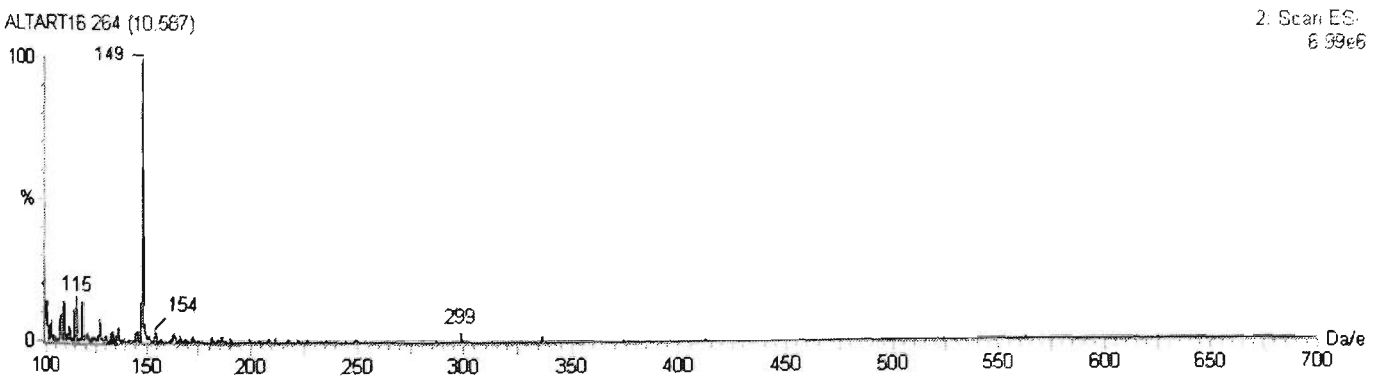


Figure 5.8 Negative mode ES-MS spectrum of 100mg/L (0.67mM) tartaric acid aqueous solution at autogenous pH. The m/z corresponding to assigned ion peak is shown in red.

The spectrum in Figure 5.8 shows that the ES-MS of tartaric acid solution gives a strong peak at 149Da which can be characterised as the bitartrate anion. The existence of this ion was expected. Referring to the distribution diagram in Figure 5.2, at the autogenous pH 3.3 the majority of the

tartaric acid will be present as bitartrate. However, in addition to the bitartrate already in solution, the negative ion mode of ES-MS has probably also induced deprotonation of a carboxylic acid group in tartaric acid. Hence, the 149Da peak most likely represents the sum of bitartrate and tartaric acid. No evidence of a doubly charged tartrate ion at m/z of 74Da was observed. This is a well-recognised example of ES-MS providing molecular ions through maintaining solution ions and deprotonation of acids at low pH (Colton et al. 1995; Henderson et al. 1998b; Traeger 2000).

Throughout this chapter, the following symbolism of organic acid species will be used. In the case of a di-carboxylic acid, the acid is designated as [H_2acid] with the protons denoting those of the carboxyl groups. Anionic species are denoted by showing the removal of protons eg. [Hacid]$^-$ and [acid]$^{2-}$. The removal of a further hydroxyl group proton is designated [H_{-1}acid]$^{3-}$ or the acid minus three protons. This system is used for all organic acids with the number of acid protons shown dependent on the number of carboxyl groups

Table 5.3 Summary of assigned ions presented in Sub-Section 5.3.4.

Peak Ion Mass (Da)	Assigned Species
ES Negative Ion Mode	
149	Htart$^-$

5.3.5 Characterisation of Complexes From Aqueous Mixtures of Aluminium and Tartaric Acid

5.3.5.1 ES-MS of aqueous mixtures of BDH Spectrosol™ aluminium with tartaric acid

With confidence that ions attributable to aluminium nitrate and tartaric acid/bitartrate could be identified in aqueous media at 275Da and 149Da in negative ion ES-MS, carefully controlled aqueous mixtures of aluminium and tartaric acid could now be investigated. To enhance the detection of aluminium/tartaric acid complexes using the methods described in Section 5.3.3, separate solutions of 0.035%v/v nitric acid, 20mg/L aluminium and 100mg/L tartaric acid were analysed (for background subtraction and component peak reference purposes) in conjunction with a set of solutions containing both tartaric acid and aluminium. These latter solutions were used to determine not only potential complexes but to ascertain optimum concentration and metal/ligand proportions with respect to the intensity of ion peaks. The concentration ranges chosen for both

solution components were based on those successfully employed for the previous individual component ES-MS investigations discussed in Sections 5.3.2 and 5.3.4. Hence, the solutions investigated were aqueous mixtures of 10:1 mg/L tartaric acid/aluminium, 20:1 mg/L tartaric acid/aluminium, 50:1 mg/L tartaric acid/aluminium, 100:1 mg/L tartaric acid/aluminium, 100:10 mg/L tartaric acid/aluminium and 100:20 mg/L tartaric acid/aluminium, where the ratios are in mass terms. Initially the pH was not adjusted from autogenous concentrations. The solutions were analysed in both ES negative and positive ion modes in the same run by alternating the polarity of the electrospray each second of scanning. The positive ion ES-MS was found to yield attributable and measurable analyte peaks. The background corrected ES positive mass spectra covering 100-500Da for the tartaric acid/aluminium solutions are shown in order in Figures 5.9 through to 5.14 (note that the ion intensity scales for each mass spectrum are not the same). The mass spectrum of a 20mg/L BDH aluminium standard analysed concurrently with the solutions was used for background removal, as described in Section 5.3.3.2.

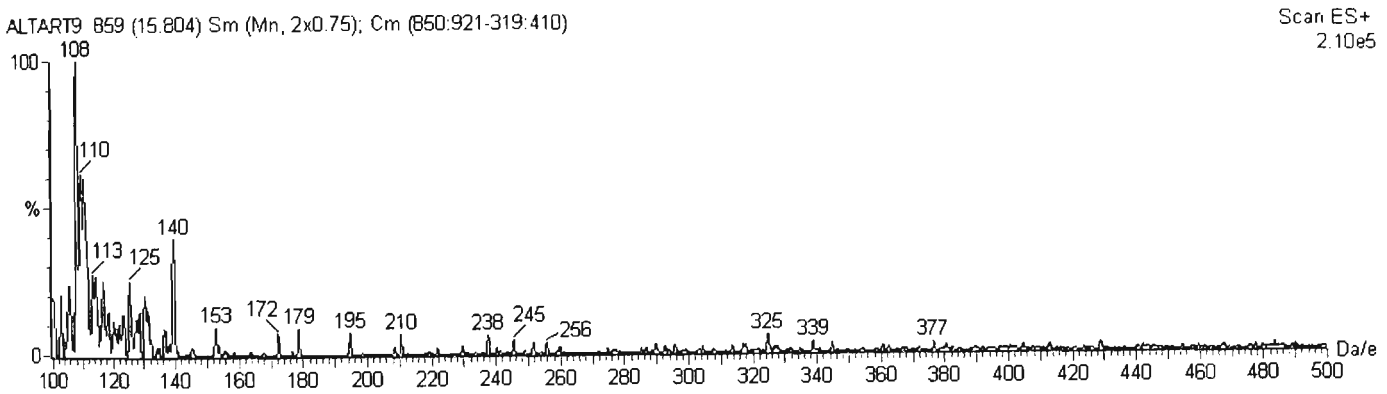


Figure 5.9 Background corrected positive ES-MS spectrum of an aqueous mixture of 10mg/L(0.06mM) tartaric acid and 1mg/L(0.04mM) aluminium(BDH Spectrosol™ aluminium nitrate) over 100-500Da.

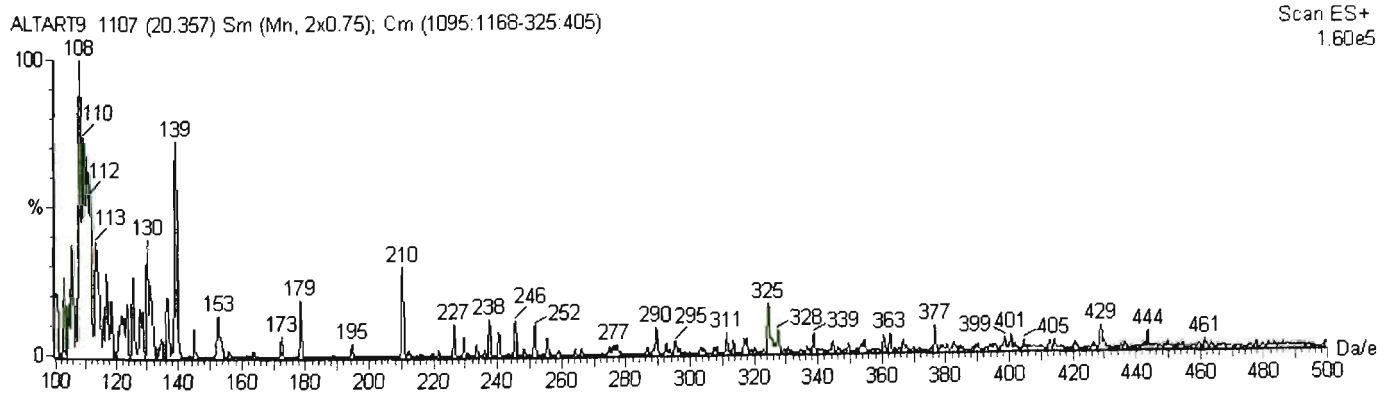


Figure 5.10 Background corrected positive ES-MS spectrum of an aqueous mixture of 20mg/L(0.13mM) tartaric acid and 1mg/L(0.04mM) aluminium(BDH Spectrosol™ aluminium nitrate) over 100-500Da.

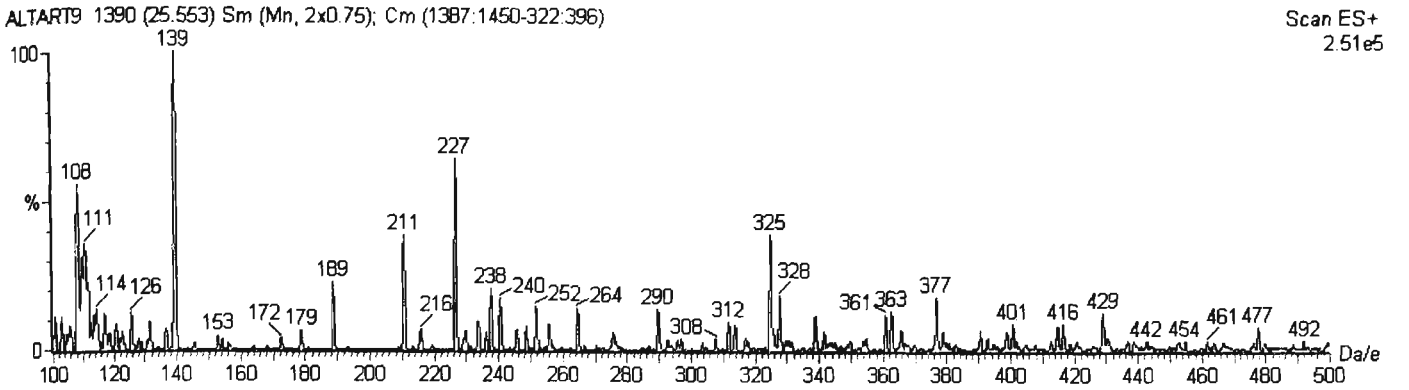


Figure 5.11 Background corrected positive ES-MS spectrum of an aqueous solution of 50mg/L(0.33mM) tartaric acid and 1mg/L(0.04mM) aluminium(BDH Spectrosol™ aluminium nitrate) over 100-500Da.

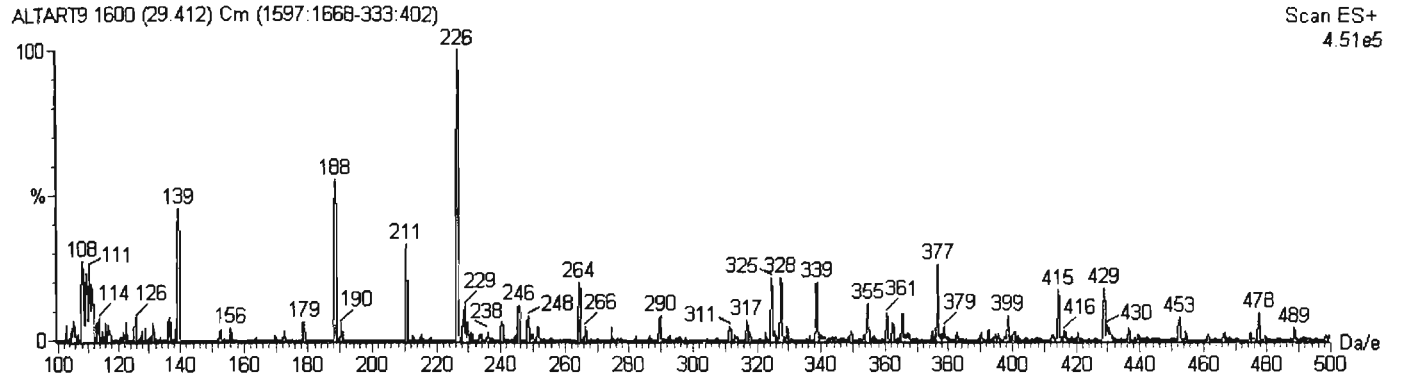


Figure 5.12 Background corrected positive ES-MS spectrum of an aqueous solution of 100mg/L(0.67mM) tartaric acid and 1mg/L(0.04mM) aluminium(BDH Spectrosol™ aluminium nitrate) over 100-500Da.

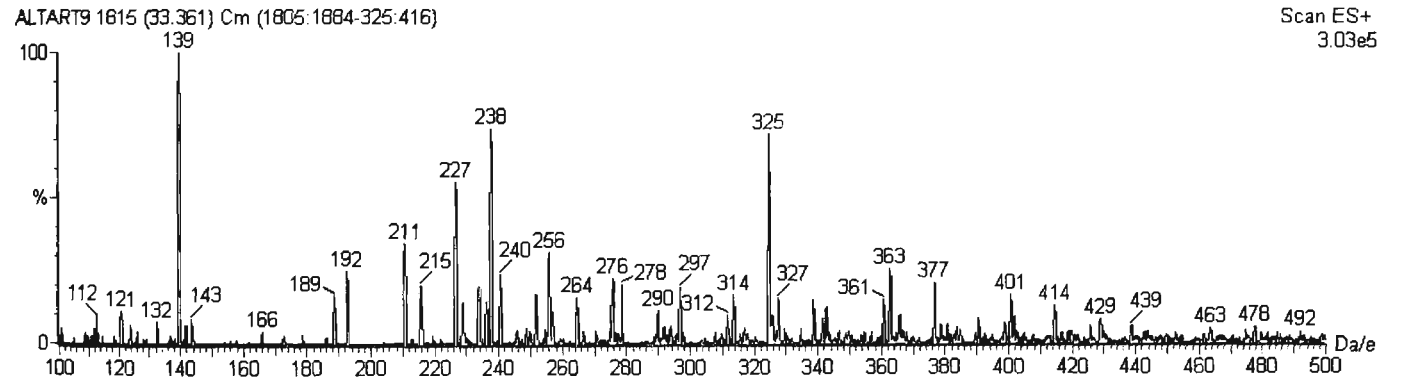


Figure 5.13 Background corrected positive ES-MS spectrum of an aqueous solution of 100mg/L(0.67mM) tartaric acid and 10mg/L(0.37mM) aluminium(BDH Spectrosol™ aluminium nitrate) over 100-500Da.

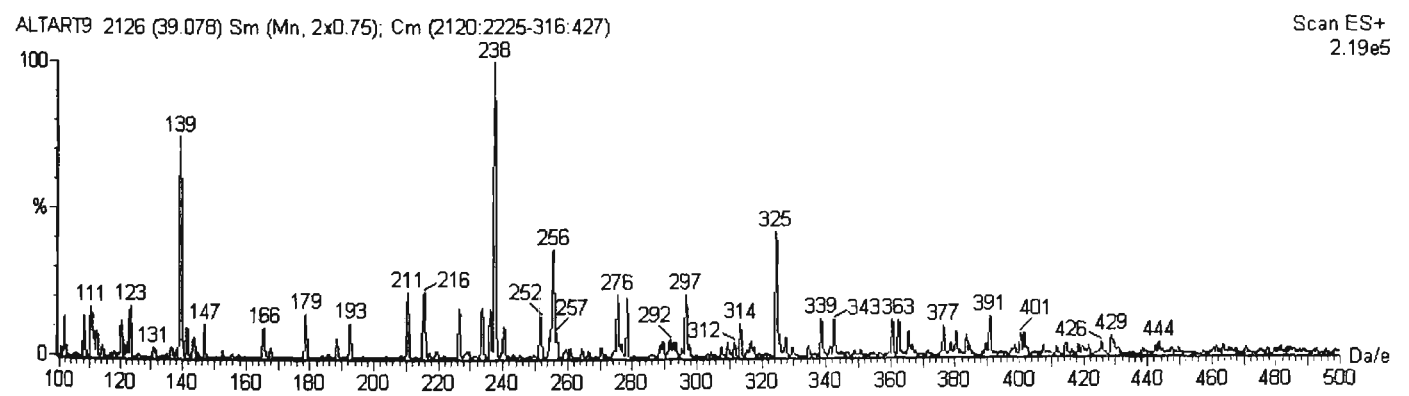


Figure 5.14 Background corrected positive ion ES-MS spectrum of an aqueous solution of 100mg/L(0.67mM) tartaric acid and 20mg/L(0.74mM) aluminium(BDH Spectrosol™ aluminium nitrate) over 100-500Da.

From Figures 5.9-5.14 it can be seen that the mass spectra differ with change in proportion of tartaric acid and aluminium. Of the peaks displayed in these figures two were identified at 238Da and 325Da and assigned as $[Al(Htart)(NO_3)]^+$ and $[Al(Htart)_2]^+$ respectively. Examination of Figures 5.9-5.14 reveals that the peaks at 238Da and 325Da increase as both aluminium and tartaric acid increase. However, the most notable increase in these peaks occurs when the concentration of the aluminium is increased. These trends are shown in the single ion chromatograms of the confirmation analysis of these peaks as shown in Figure 5.15.

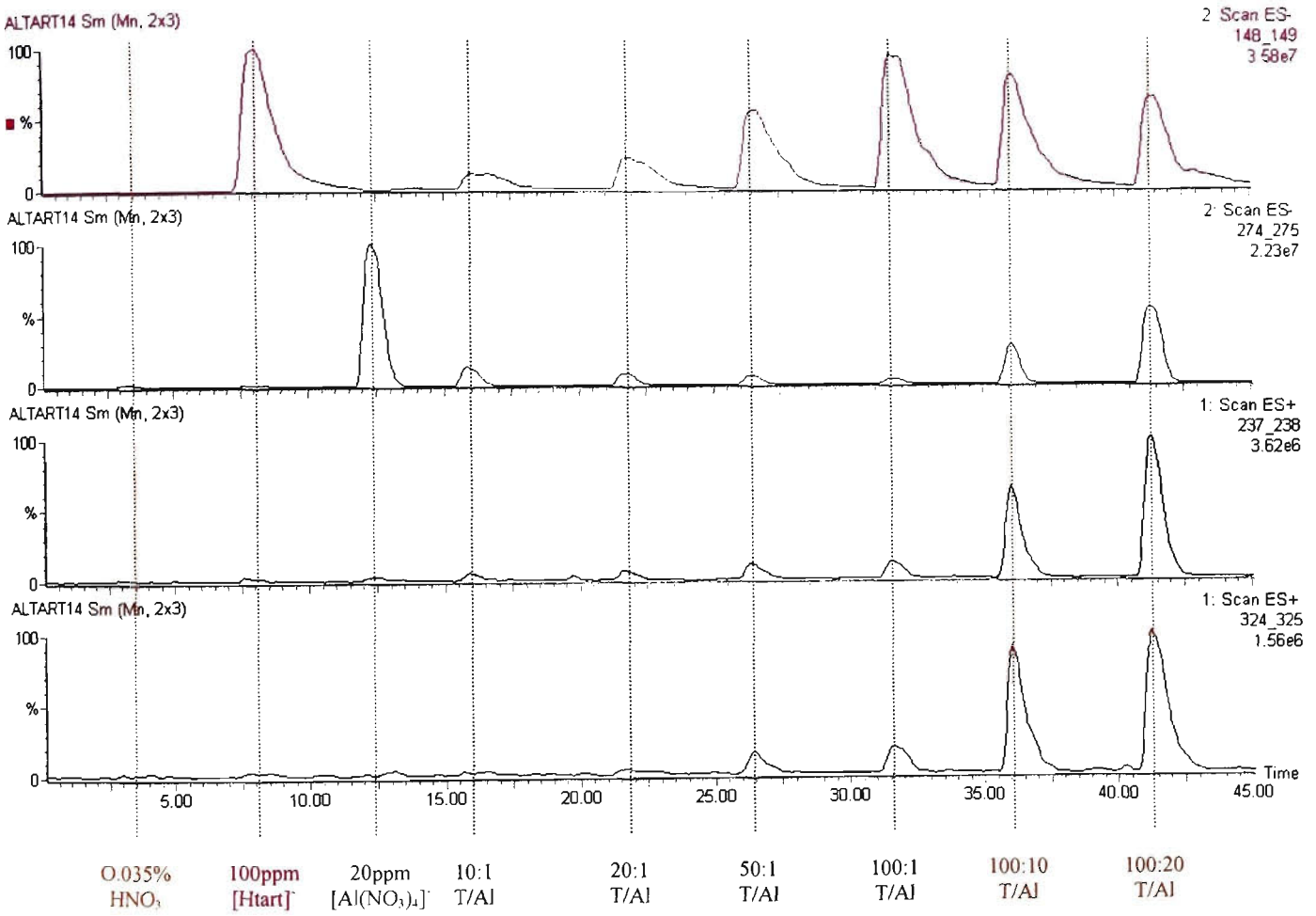


Figure 5.15 Confirmation chromatogram matrix showing ion count profiles of the ions (in order top to bottom) 148Da, 275Da, 238Da and 325Da with respect to ES-MS run time in minutes. Colour coding shows the corresponding electrospray of particular solutions. It must be remembered the top two chromatograms are for electrospray negative ion mode and the bottom two are for electrospray positive ion mode. Note: the scale of intensity for each ion count chromatogram is not the same.

From Figure 5.15 none of the ion counts selected shows a response for dilute AR HNO_3. The $[Htart]^-$ ion 149Da shows the highest response when the 100mg/L tartaric acid solution is injected, however none of the ion counts at 238Da, 275Da and 325Da show a response. This is consistent with 149Da representing $[Htart]^-$. As the concentration of tartaric acid is increased in the solutions with aluminium, the 149Da ion count response increases steadily in proportion to the amount of

tartaric acid added. However, in the last two injections with increasing aluminium concentration the ion count response of the 149Da anion decreases. This effect indicates a reduction in free tartaric acid/bitartrate in the solution, consistent with increased complexation of bitartrate with aluminium.

The ion count response of the 275Da ion representing $[Al(NO_3)_4]^-$ is absent from the 100mg/L tartaric acid solution and is greatest for the 20mg/L $[Al(NO_3)_4]^-$ solution, correlating with the characterisation of that ion as a tetranitratoaluminate(III) anion. The complex ion count responses (238Da, 325Da) are significantly smaller for the aluminium/tartaric acid solutions with an aluminium concentration of 1mg/L. As the tartaric acid concentration is increased, there is a decreased response of the aluminium nitrate ion count (238Da), suggesting the replacement of nitrate by bitartrate. As the aluminium standard concentration is increased, so does the response of the aluminium nitrate ion count. However, the response for this species in the 100:20 mg/L tartaric acid\aluminium solution is half that of the 20mg/L aluminium standard solution on its own, indicating a large proportion of the aluminium is being complexed by bitartrate. The rise and fall of the aluminium nitrate ion count also correlates well with complexation of aluminium with bitartrate.

Both $[Al(Htart)(NO_3)]^+$ (238Da) and $[Al(Htart)_2]^+$ (325Da) follow similar response patterns in Figure 5.15. Their ion counts show no response in the separate solutions of diluted AR HNO_3, tartaric acid and aluminium standard. In the solutions where the components are at their lowest, these species ion count response is negligible. However, with the increase of tartaric acid to 50 and 100 mg/L respectively, low ion count responses become evident. As the tartaric acid concentration is held constant and that of the aluminium is increased, a dramatic increase in the ion count response is observed, with a maximum apparent in the 100:20 mg/L tartaric acid\aluminium solution. The absence of ion count responses in the component solutions, their appearance in the mixture solutions only and their increase in intensity with increasing component concentration, correlated with the peak responses of the component ions, confirm the characterisation of the complex ion peaks.

It was noted that the mixed complex (238Da) containing bitartrate and nitrate ligands had double the ion intensity of the bis-bitartrate complex (325Da). This was surprising as nitrate is considered a much weaker ligand than bitartrate. Hence, solution changes other than component concentrations

were considered. As the mixed and single component solutions were injected at autogenous pH, no control was maintained over this variable. Hence the pH was measured for all the solutions described in this section. The results are tabulated in Table 5.4.

Table 5.4 The solutions analysed in the ES-MS study of aqueous aluminium and tartaric acid speciation and their corresponding autogenous pH. The colour coding shows groups of solutions with similar pH.

Solution	pH
0.035% HNO_3	2.14
100 mg/L Tartaric Acid	3.90
20mg/L Al BDH Standard	2.02
10:1 mg/L Tart\Al mixture	3.29
20:1 mg/L Tart\Al mixture	3.29
50:1 mg/L Tart\Al mixture	3.37
100:1 mg/L Tart\Al mixture	3.35
100:10 mg/L Tart\Al mixture	2.35
100:20 mg/L Tart\Al mixture	2.03

Table 5.4 shows that there is an order of magnitude difference in the hydrogen ion concentrations of the solutions high in aluminium compared with those with a low aluminium concentration. This is because the stock solution contains nitric acid; the higher the concentration of the aluminium from the BDH standard, the more nitric acid is present. Tartaric acid shows a buffering effect for the low aluminium concentration solutions with a pH around 3.3; however with increased nitric acid, this decreases to 2.0-2.3. The pH of the 100:20 mg/L tartaric acid\aluminium solution (pH 2.0) closely matches that of the aluminium standard. This suggests that for this solution of aluminium and tartaric acid, ES-MS background subtraction of 20mg/L aluminium was the most appropriate. The close matching of the pH of the 0.035% nitric acid with that of the aluminium standard shows the estimation of nitric acid required to simulate the effect on pH by the aluminium BDH Spectrosol™ standard is accurate. The table also shows that the solutions with 1mg/L aluminium have a similar pH, demonstrating that comparisons between these solutions are not compromised. The effect of the these pH findings on the trends observed in Figure 5.15 may limit the feasibility of direct comparison of ion intensities of groups of solutions using different aluminium concentrations. Additionally it suggests that the greater intensity of the 238Da peak may be explained by the

increased amount of nitrate introduced by a higher aluminium standard concentration. In a 20mg/L aluminium solution there is approximately 13mM of nitrate, exceeding tartaric acid concentration by almost 20-fold, making nitrate binding to aluminium more competitive.

Table 5.5 Summary of assigned ions presented in Sub-Section 5.3.5.1.

Peak Ion Mass (Da)	Assigned Species
ES Positive Ion Mode	
238	$[Al(Htart)(NO_3)]^+$
325	$[Al(Htart)_2]^+$
ES Negative Ion Mode	
149	$[Htart]^-$
275	$[Al(NO_3)_4]^-$

5.3.5.2 Study of pH and component concentration effects on solution mixture complex ion intensity

Further analysis of these solutions was conducted to ascertain the role of pH on the ion intensities. To do this the same set of solutions described in Sub-Section 5.3.5.1 were all adjusted to the same pH concentrations at pH ~2.0, 2.5 and 3.3 and analysed by positive ion mode ES-MS. Dilute ultrapure nitric acid was used to lower pH and dilute KOH was used to increase pH. The ion intensities of the 238Da and 325Da peaks with respect to tartaric acid/aluminium mixture for different solution pH are shown in Figures 5.16, 5.17, 5.18.

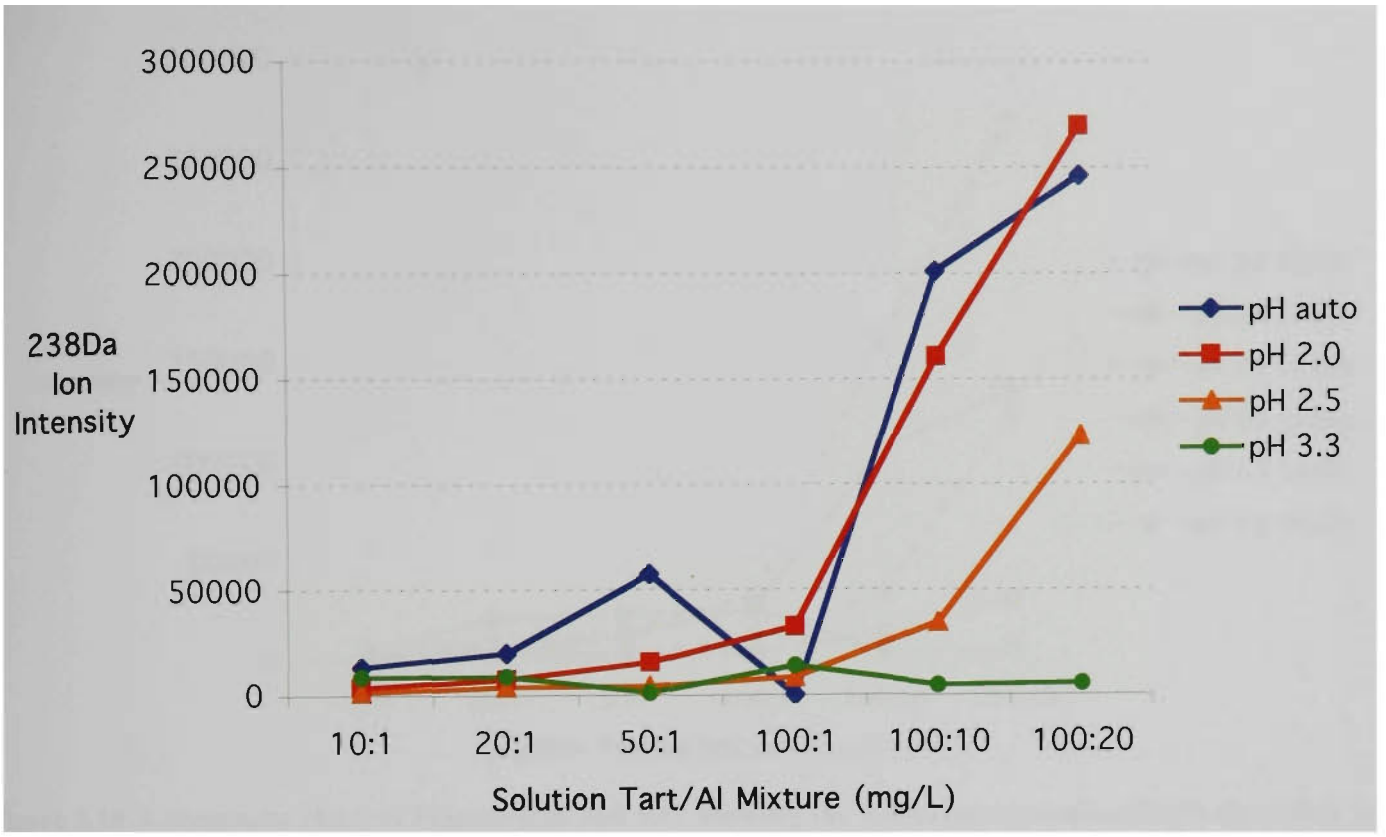


Figure 5.16 Trends of ion intensities of the 238Da peak representing $[Al\text{-}NO_3\text{-}HT]^+$ at varied pH with respect to the tartaric acid\aluminium solution in mg/L. Note: pH auto represents autogenous pH.

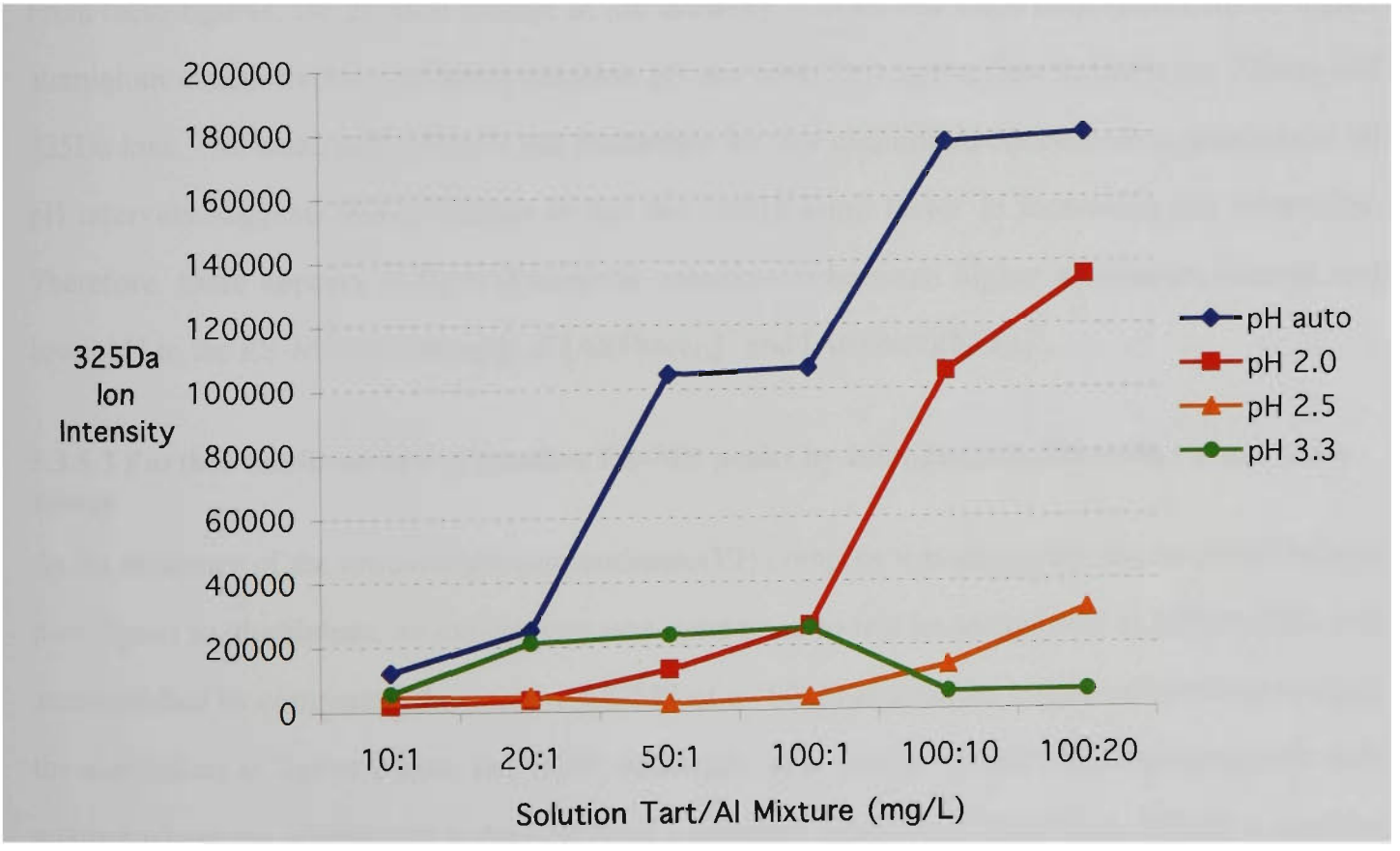


Figure 5.17 Trends of ion intensities of the 325Da peak representing $[Al\text{-}(HT)_2]^+$ at varied pH with respect to the tartaric acid\aluminium solution in mg/L. Note: pH auto represents autogenous pH.

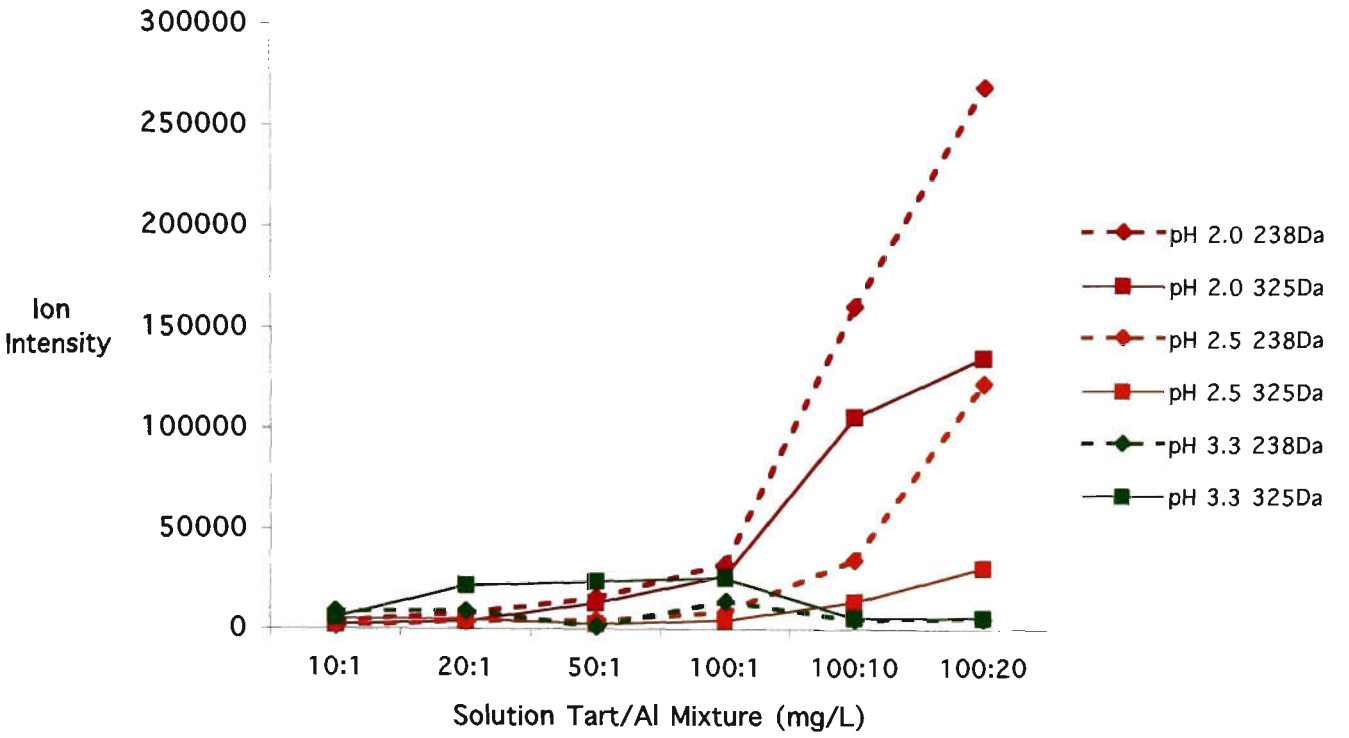


Figure 5.18 A composite chart of Figures 5.16 and 5.17 showing the ion intensity trends of both the 238Da ion (bold dotted lines) and the 325Da ion (thin unbroken lines) at varied pH with respect to the tartaric acid\aluminium solution in mg/L. Note: lines of the same colour represent the trends of both ions at the same pH.

From these figures, the greatest change in ion intensity is observed when both conditions of higher aluminium concentration and lower solution pH are met. This is the case for both the 238Da and 325Da ions. The small difference in ion intensities for low aluminium concentration solutions at all pH intervals suggests that pH alone is not the contributing factor in increasing ion intensities. Therefore, there appears to be a synergistic association between higher aluminium content and lower pH in the ES-MS ion intensity of $[Al(Htart)_2]^+$ and $[Al(Htart)(NO_3)]^+$.

5.3.5.3 Further confirmation of positive ES-MS peaks by introduction of another aluminium source

As the existence of the nitratobitartratoaluminium(III) complex was surprising due to nitrate being a poor ligand to aluminium, an experiment was conducted to test its assignment at 238Da. This was accomplished by comparing the positive ES-MS of a 100:20 aluminium/tartaric acid mixture (where the aluminium is derived from the BDH standard) with another 100:20 aluminium/tartaric acid mixture where the aluminium is derived from a standard solution of $AlK(SO_4)_2.12H_2O$, a standard free of nitrate and nitric acid. The mass spectra of both solutions can be seen in Figure 5.19.

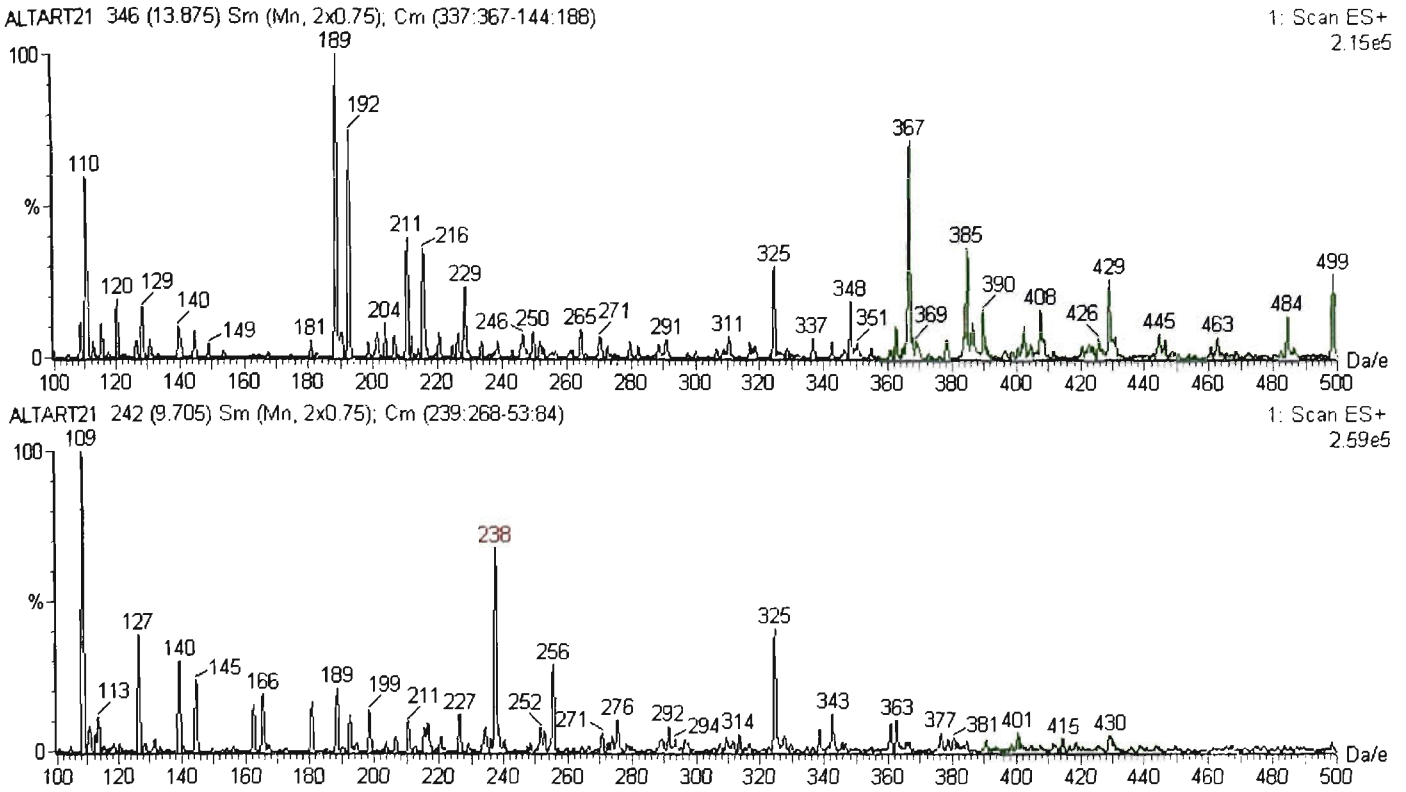


Figure 5.19 Comparison of the mass spectra of 100:20mg/L tartaric acid/Al solutions. The top mass spectrum is from a mixture where the aluminium is derived from $AlK(SO_4)_2.12H_2O$ and the bottom mass spectrum is from a mixture where the aluminium is derived from BDH Spectrosol™ standard. The peaks of the nitratotartratoaluminium(III) and aluminium bis-bitartrate complexes are shown at 238Da and 325Da. Note: the intensity scale is not the same.

Figure 5.19 demonstrates that the solution without nitrate shows no evidence of the nitratobitartratoaluminium(III) complex at 238Da while the aluminium bis-bitartrate complex at 325Da is evident in both solutions. To confirm the role of nitrate the pH of the solution derived from the aluminium potassium sulfate salt was progressively decreased by adding dilute nitric acid until the pH of this solution matched the autogenous pH of the solution mixture where the aluminium was sourced from BDH Spectrosol™ standard. The mass spectra from this experiment are shown in Figure 5.20.

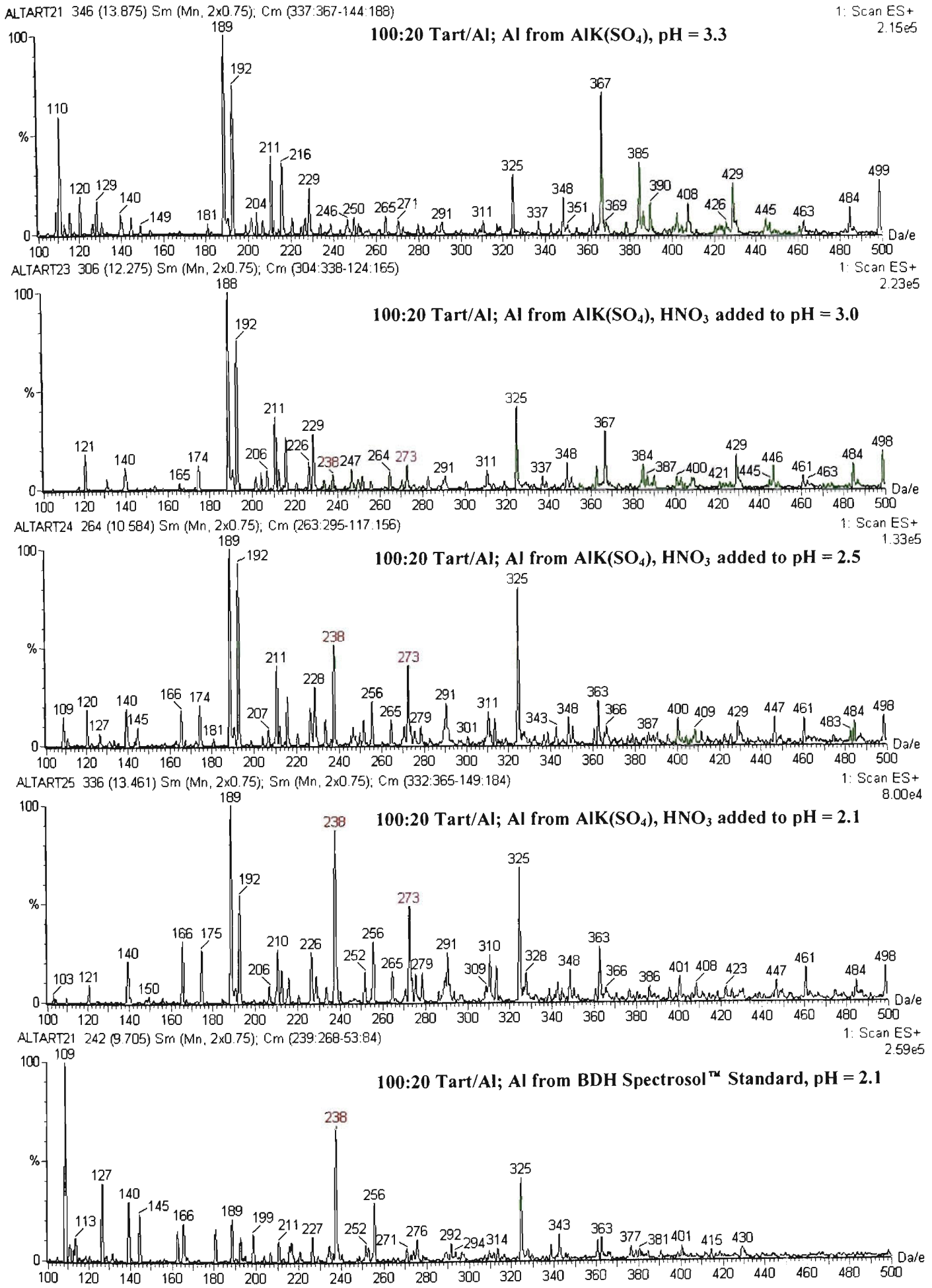


Figure 5.20 Positive ion mass spectra of 100:20 Tartaric acid/aluminium mixtures (with aluminium derived from aluminium potassium sulfate) including those with HNO_3 added progressively to the pH shown. The positive ion mass spectrum of the same mixture but with the aluminium derived from BDH Spectrosol™ standard is shown at the bottom as a comparison. Note: intensity scales are not the same.

Figure 5.20 clearly demonstrates that as nitric acid is added to an aluminium/tartaric acid mixture that does not contain nitrate originally, the 238Da peak appears and becomes more prominent until the ion ratios at pH 2.1 are similar to that observed for the BDH Spectrosol™ containing solution. As the appearance and increase in intensity of the 238Da peak correlates with the addition of nitrate to the aluminium/tartaric acid solution mixture the comparison supports the assignment of the 238Da peak to nitratobitartratoaluminium(III).

In addition, this experiment showed evidence of a mixed complex of aluminium with bitartrate and bisulfate at 273Da assigned to $[Al(Htart)(HSO_4)]^+$. This peak was only observed in sulfate containing solutions at lower pH. Figure 5.20 shows no evidence of this ion in the pH 3.3 solution of 100:20 tartaric acid/(aluminium potassium sulfate) but its peak becomes more prominent in the same solution at lower pH. This bisulfatobitartratoaluminium(III) ion is similar to the nitrato complex observed in BDH Spectrosol™ based solutions.

These experiments also support the existence of the aluminium bis-bitartrate complex. The assigned peak at 325Da was reproducible in separate solutions with aluminium from different sources and hydrogen ion concentrations an order of magnitude apart. This shows that this ion is not just an artefact of mixing tartaric acid with BDH Spectrosol™ aluminium standard and that this peak only occurs when aluminium (from any source) and bitartrate are in the same solution.

It is interesting to note that the ion intensity of the 325Da peak was approximately 65000 for the 100:20 tartaric acid/(aluminium potassium sulfate) solution at an autogenous pH of 3.3. This compares with an ion intensity of 6000 for the same peak for the same mixture and similar pH but with the aluminium sourced from the BDH Spectrosol™ standard (see Figure 5.17). This comparison shows a ten-fold difference in ion intensity at the same pH. This observation supports the hypothesis that pH is not the sole driving factor in ion intensity for this complex in aqueous media. The higher intensity for a tartaric acid/aluminium solution using aluminium potassium sulfate salt at a pH within the range found in wine should favour ES-MS to be used to examine solutions similar to wine as discussed later in Section 5.3.7.

Table 5.6 Summary of assigned ions presented in Sub-Section 5.3.5.3.

Peak Ion Mass (Da)	Assigned Species
ES Positive Ion Mode	
238	$[Al(Htart)(NO_3)]^+$
273	$[Al(Htart)(HSO_4)]^+$
325	$[Al(Htart)_2]^+$

5.3.5.4 Negative ion mode ES-MS of aqueous aluminium/tartaric acid mixtures

The solutions of tartaric acid with aluminium were also analysed in the negative ion mode simultaneously with the positive ion mode ES-MS analysis. The mass spectrum of a 100:20mg/L tartaric acid/Al solution at autogenous pH where the aluminium is derived from BDH Spectrosol™ standard is shown in Figure 5.21.

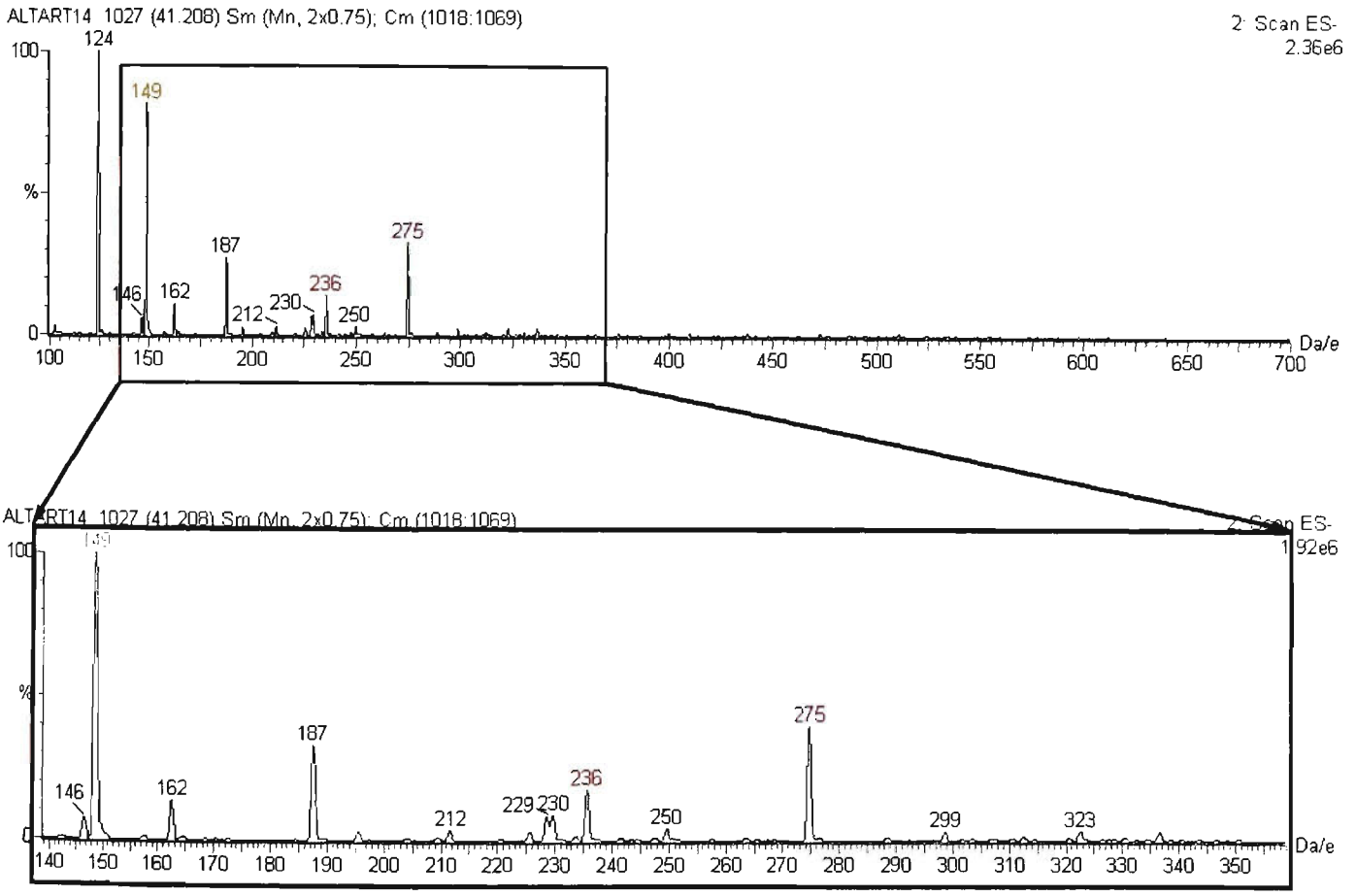


Figure 5.21 Negative ES-MS spectrum of 100:20mg/L tartaric acid/aluminium(BDH Spectrosol™) mixture at autogenous pH of 2.0 with the m/z range 140-360Da expanded in the inset. Assigned peaks are 149Da, 236Da, 275Da and 323Da.

As expected, in negative ion ES-MS mode the anions of the complex precursors, $[Htart]^-$ and $[Al(NO_3)_4]^-$ were observed at 149Da and 275Da respectively. This indicates that even though we see evidence of complexation in the positive ion mode, there is still a significant proportion of the

precursors remaining uncomplexed. Two ion peaks were assigned in the negative ion mass spectrum. One at 236Da was attributed to $[Al(NO_3)(H_1tart)]^-$ where both carboxyl protons and a proton from an alcohol group have been removed from tartaric acid/bitartrate. The other peak at 323Da was assigned to an aluminium bis-tartrate complex, $[Al(tart)_2]^-$, although as can be seen in the confirmation analysis this peak assignment is a tentative characterisation. The attribution analysis for these characterisations is shown in Figure 5.22. This vigorous proton stripping is a function of the electrospray process and does not imply the existence of these anions in the undisturbed solution.

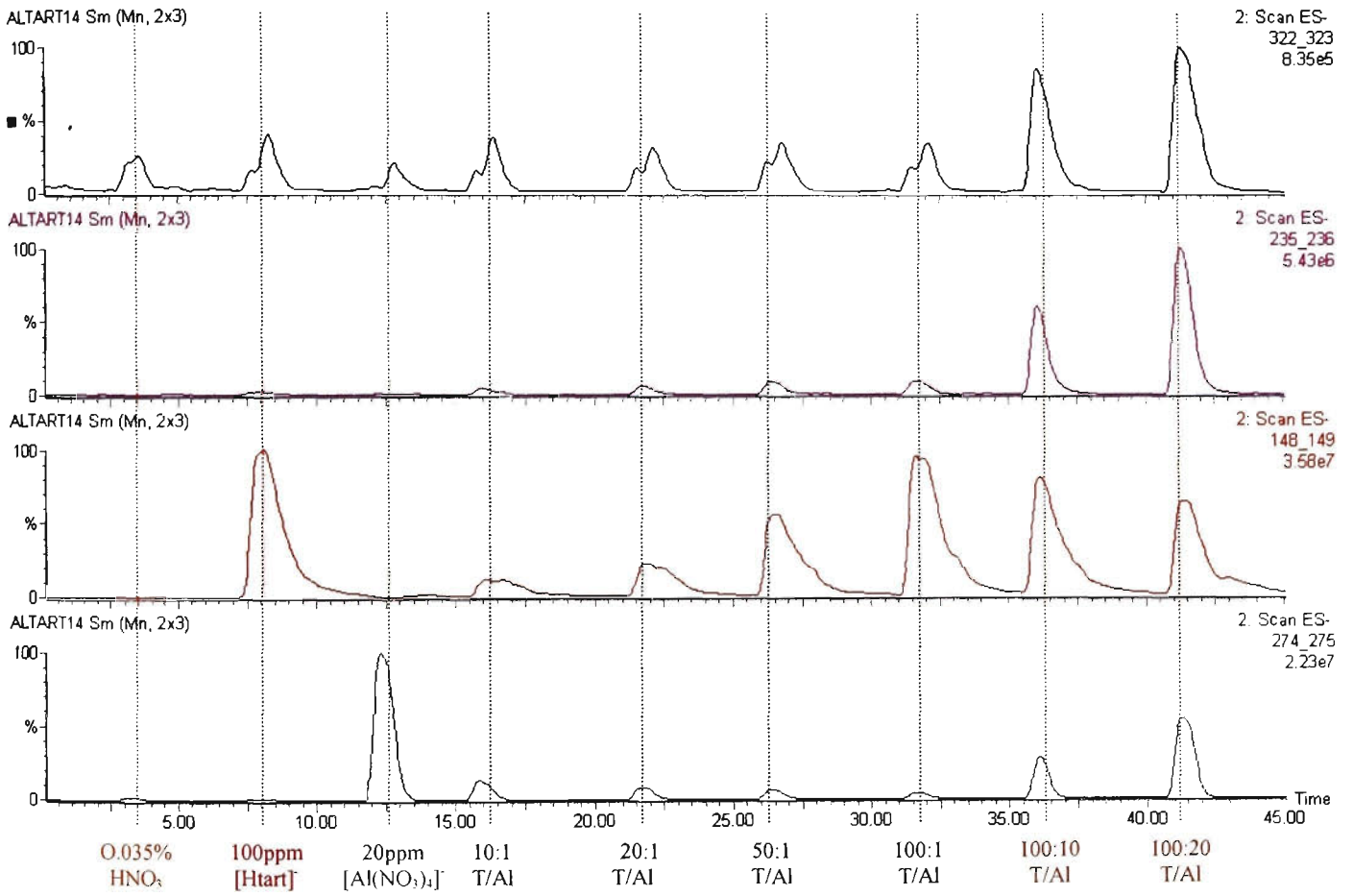


Figure 5.22 Confirmation chromatogram matrix showing ion count response of the ions (in order top to bottom) 323Da, 236Da, 149Da and 275Da with respect to ES-MS run time in minutes. Colour coding shows the corresponding electrospray of particular solutions. All chromatograms are for electrospray negative mode. Note: the scale of intensity for each ion count chromatogram is not the same.

Figure 5.22 demonstrates that the characterisation of the 323Da peak is tentative because the ion count response is measurable in injections 1, 2 and 3 representing the solutions of tartaric acid, 0.035% HNO_3 and the aluminium BDH Spectrosol™ standard respectively, where we would expect little response from an aluminium tartrate complex. However there is a large increase observed in the 323Da ion count response when the aluminium and tartaric acid concentrations are higher. The

characterisation of the 236Da peak is less ambiguous. No ion count response is apparent in the precursor solutions, and a steady increase in response followed by a dramatic rise as firstly tartaric acid and then aluminium is increased, strongly suggests that the 236Da peak is a complex comprising aluminium, nitrate and a deprotonated tartrate; the latter component being a product of the negative ion electrospray process.

As the negative and positive mode mass spectra were acquired at the same time on the same solutions, the role of pH on the intensity of the characterised ions was also investigated in the same manner as for the ions analysed in the ES-MS positive ion mode. A composite chart, similar to that seen in Figure 5.18, presenting the trends of the intensities of both ions over varying pH and mixture composition is shown in Figure 5.23.

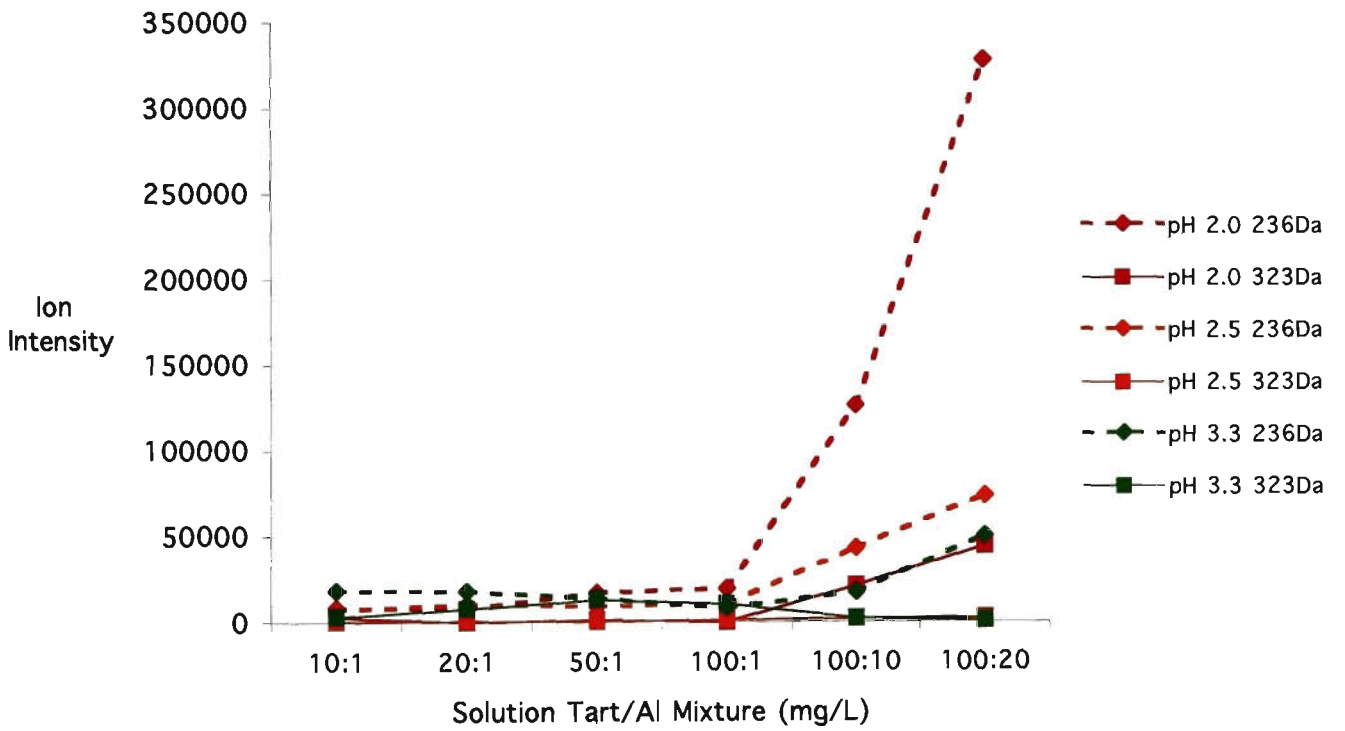


Figure 5.23 A composite chart showing the ion intensity trends of both the 236Da ion (bold dotted lines) and the 323Da ion (thin unbroken lines) at varied pH with respect to the tartaric acid\aluminium(BDH Spectrosol™) solution mixture in mg/L. Note: lines of the same colour represent the trends of both ions at the same pH.

The trends observed for the negative ES-MS ions at various pH closely resemble those of the positive ions. Again, the greater the aluminium concentration and the lower the pH, the greater the intensity of the $[Al(NO)_3(H_{-1}tart)]^-$ ion at 236Da, mirroring the nitrato complex in the positive mode at 238Da with little change at lower aluminium concentration. However, in the negative ion mode

the intensity of the 323Da peak is less responsive at higher aluminium concentrations and pH than the 325Da peak in the positive ion mode (see Figure 5.18). The similarity of the positive and negative ion mode trends makes sense, as the ions are sourced from the same solution ions.

The mechanisms for these complex ion formations in the ES-MS are similar to those seen in the positive mode, except this time being based on deprotonation rather than protonation. In the case of the $[Al(NO_3)(H_{-1}tart)]^-$ ion, the formation of a triply deprotonated tartaric acid would not occur naturally in an aqueous solution at pH 2.0, hence the removal of an hydroxyl proton would be a result of the electrospray process. The deprotonation and solution pH suggests that the original ion in solution was $[Al(Htart)(NO_3)]^+$. The $[Al(tart)_2]^-$ ion is probably the result of the electrospray process deprotonating the bitartrate in $[Al(Htart_2)]^+$. The difficulty of deprotonating the $Htart^-$ anion correlates well with the extremely low intensities of the anions characterised. The slight increase of $[Al(tart)_2]^-$ observed at pH 2.0 would be due to further deprotonation by the negative ion electrospray process (which works best in acidic environments (Johnstone & Rose 1996)), however this effect is minimal compared with that observed for the nitrato complex.

The negative ion ES-MS mass spectrum of a 100:20 mg/L tartaric acid/aluminium solution where the aluminium was sourced from aluminium potassium sulfate was also investigated. This was conducted to see if the same relationships between nitrate concentration, pH, tartaric acid and ultimately different aluminium sources held in the negative ion mode ES-MS as was the case for the positive ion mode as discussed in Section 5.3.5.3. Unlike the aluminium solution sourced from the BDH Spectrosol™ standard, none of the peaks in the negative ion mode mass spectrum of 20mg/L aluminium potassium sulfate could be matched to aluminium species. However, mass spectrum background subtraction of 20mg/L aluminium potassium sulfate was still utilised for all solutions containing this compound. The negative ion ES-MS mass spectrum of this mixture is shown in Figure 5.24.

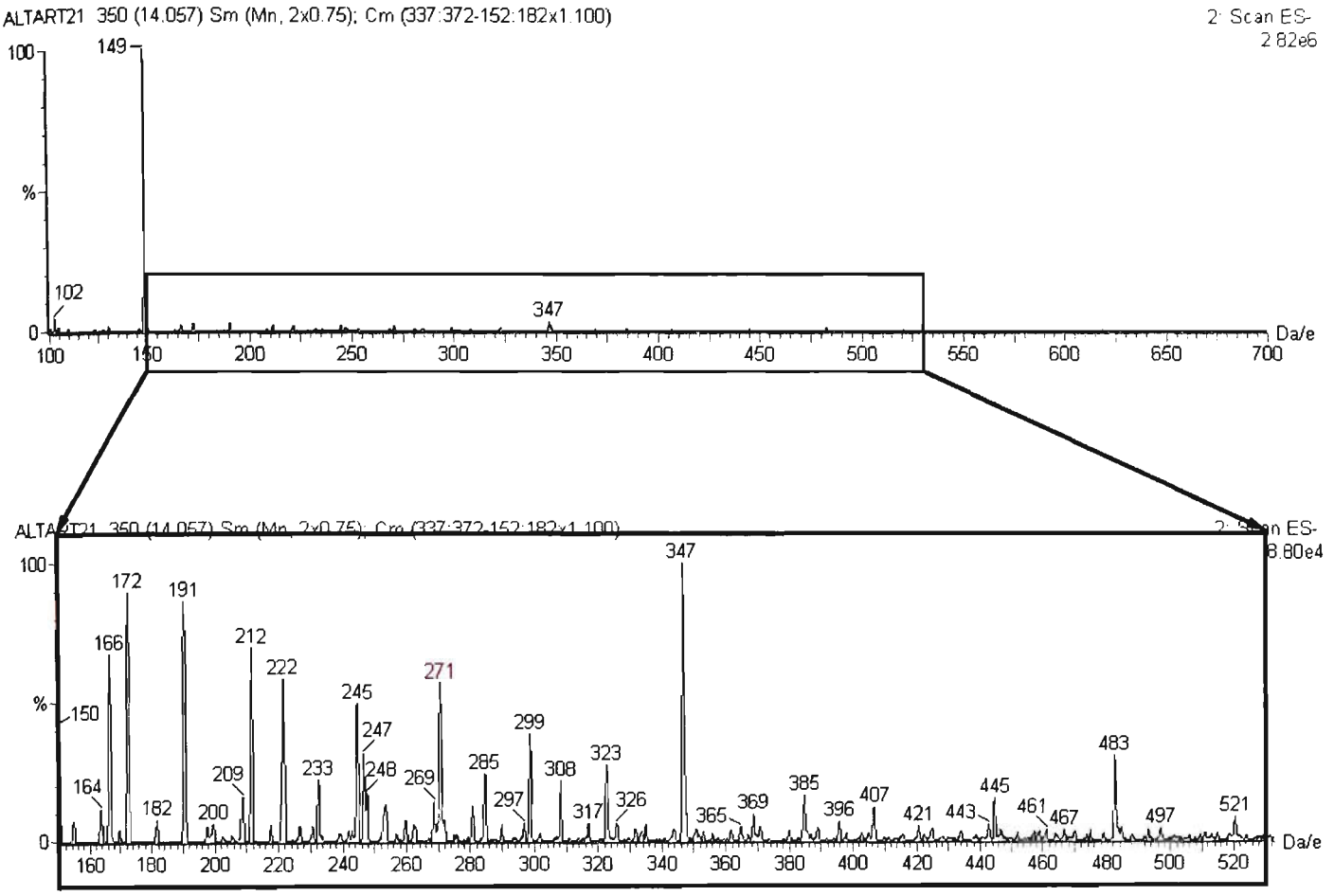


Figure 5.24 Negative ES-MS spectrum of 100:20mg/L tartaric acid/aluminium potassium sulfate mixture at autogenous pH of 3.3. The characterised peak is $[Al(Tart)_2]^-$ (323Da). Note the $[Al(SO)_4(tart)]^-$ ion at 271Da, similar to the bisulfatobitartratoaluminium(III) ion observed in positive ion mode at 273Da (see Figure 5.20). This can be compared with the positive mode mass spectrum in Figure 5.21.

Comparing the negative ion mode mass spectrum in Figure 5.24 with Figure 5.21 demonstrates that using a non-nitrate aluminium stock gives a mass spectrum with no peak at 236Da pertaining to $[Al(NO_3)(H_{-1}tart)]^-$. However, although of lower intensity than most of the other peaks in the mass spectrum, the $[Al(tart)_2]^-$ complex at 323Da is still observed. These observations are identical to those seen in the positive mode analysis of tartaric acid/aluminium solutions with both sources of aluminium and provides further evidence for the identification of the $[Al(Htart)(NO_3)]^+$ complex as its deprotonated species is only observed when aluminium, nitrate and tartaric acid are in the same solution. Similar to the bisulfatobitartratoaluminium(III) complex observed in the positive ion mode (see Figure 5.20) an $[Al(SO_4)(tart)]^-$ complex was observed in the negative ion mode spectrum minus two protons at 271Da. Nitric acid was added in incremental amounts to the solution mixture where aluminium potassium sulfate was used to see if the same trends as seen for the positive ion mode analysis (see Figure 5.20) were observed. The results of this experiment are shown in Figure 5.25.

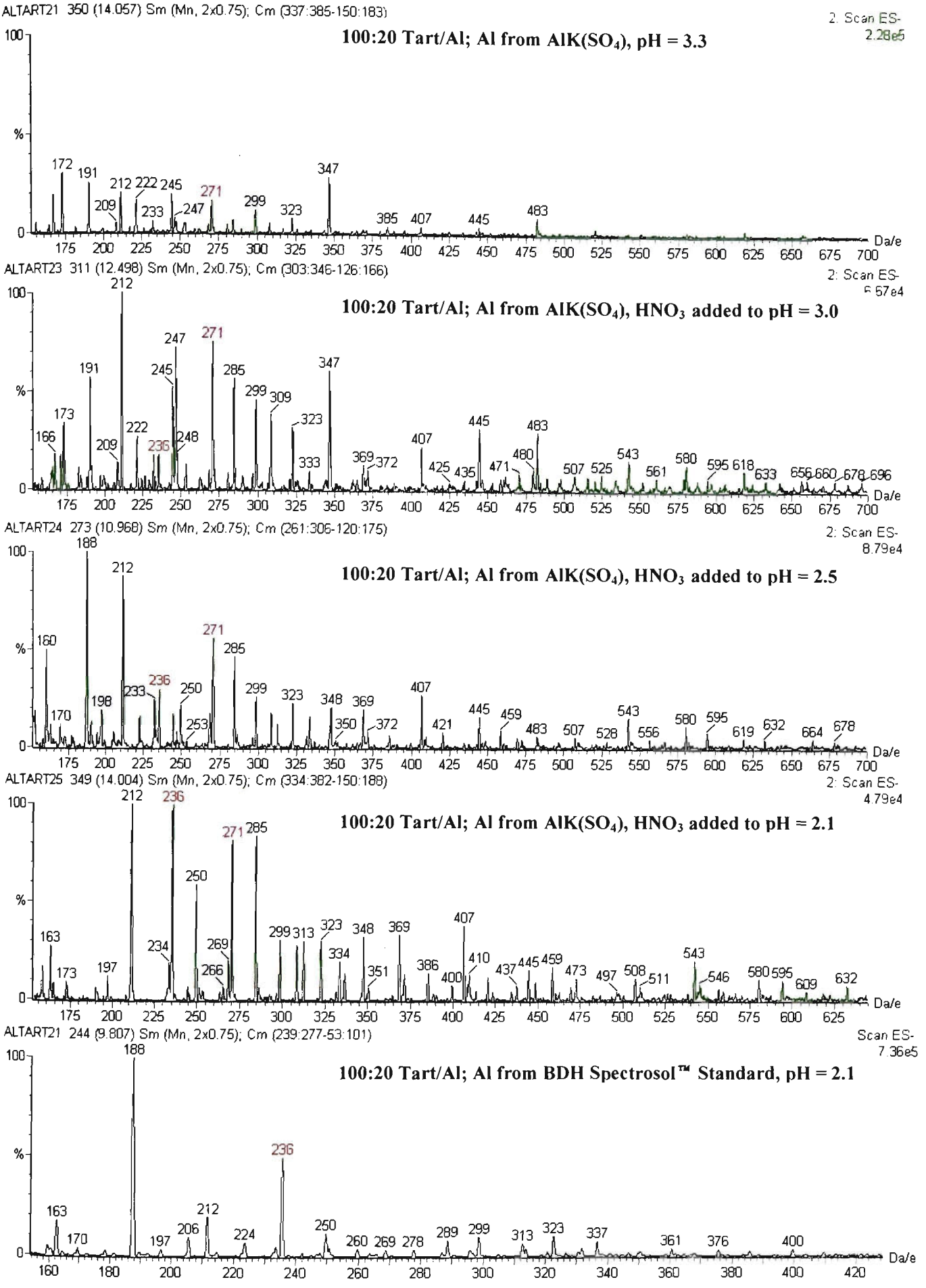


Figure 5.25 Negative mass spectra of 100:20 Al/Tartaric acid mixtures (with Al derived from aluminium potassium sulfate) including those with HNO_3 added progressively to the pH shown. The positive mass spectrum of the same mixture but with the aluminium derived from BDH Spectrosol™ standard is shown at the bottom as a comparison. Note: The vertical and horizontal scales are not identical for all mass spectra, and intensity scales are not the same.

Figure 5.25 displays similar trends observed for the same experiment in the positive ion mode shown in Figure 5.20. As nitric acid is added the 236Da peak increases until, at pH 2.1 the 236Da peak is greater than the 323Da peak. The appearance and increase of the 236Da peak directly correlates with the addition of nitrate into the solution via the introduction of nitric acid, as was the case for the $[Al(Htart)(NO_3)]^+$ complex at 238Da in the positive ion mode (see Figure 5.20). Figure 5.25 also supports the assignment of the $[Al(tart)(SO)_4]^-$ complex, with the corresponding peak at 271Da becoming more prominent with the lowering in solution pH; a trend similar to that observed for the $[Al(Htart)(HSO)_4]^+$ complex in positive ion mode (see Figure 5.20). Unlike its positive ion mode counterpart, the bis-tartratoaluminate(III) anion at 323Da did not show an appreciable difference in ion intensity with change in pH, supporting the hypothesis that this ion is a product of the electrospray process.

It is apparent from the analyses presented that there is a similar pattern observed for the characterised aluminium complexes in both the negative ion and positive ion modes. The types and numbers of ligands remain the same but differ by two protons which are removed from bitartrate, the only source of easily removed protons in the complexes with the exception of $[HSO_4]^-$ ions in bisulfatobitartratoaluminium(III). One could almost refer to these complexes as 'electrospray conjugate pairs', with two protons the difference rather than the one seen in acid/base conjugates. The appearance of these pairs allows more confident identification of particular complexes by providing information on the identity of the original solution ions that, through the electrospray process, become deprotonated and observed in the negative ion mode ES-MS.

Table 5.7 Summary of assigned ions presented in Sub-Section 5.3.5.4.

Peak Ion Mass (Da)	Assigned Species
ES Negative Ion Mode	
149	$[Htart]^-$
275	$[Al(NO_3)_4]^-$
236	$[Al(NO_3)(H_{-1}tart)]^-$
271	$[Al(SO)_4(tart)]^-$
323	$[Al(tart)_2]^-$

5.3.5.5 Optimisation of ES-MS cone voltage

As described in Chapter 2, the manipulation of the cone voltage of the skimmer cone in the interface between the electrospray and the quadrupole mass detector is a major variable used in ES-MS to adjust the concentration of fractionation of gas phase ions (van Baar 1996). Increasing the cone voltage gives a greater amount of fractionation of the original solution ions. To take advantage of one of the main benefits of ES-MS, i.e. soft ionisation providing singly charged molecular ions, the cone voltage must remain relatively low. Hence, it was decided to determine the relationship of an identified ion with the cone voltage and try to optimise the ion intensity. As there was a high degree of confidence in the identification of the $[Al(Htart)_2]^+$ at 325Da and this species was found in solutions containing aluminium from both the BDH Spectrosol™ standard and aluminium potassium sulfate, this ion was chosen to optimise the cone voltage. The default cone voltage which had been used up to this point was set at ±30Volts depending on the polarity of the ES-MS analysis. An experiment was carried out where 100:20mg/L tartaric acid/aluminium mixtures using both sources of aluminium were analysed in positive ion mode ES-MS with the cone voltages varied from 20 to 90 volts. The intensity of the 325Da peak was then measured and plotted against cone voltage. This plot is shown in Figure 5.26.

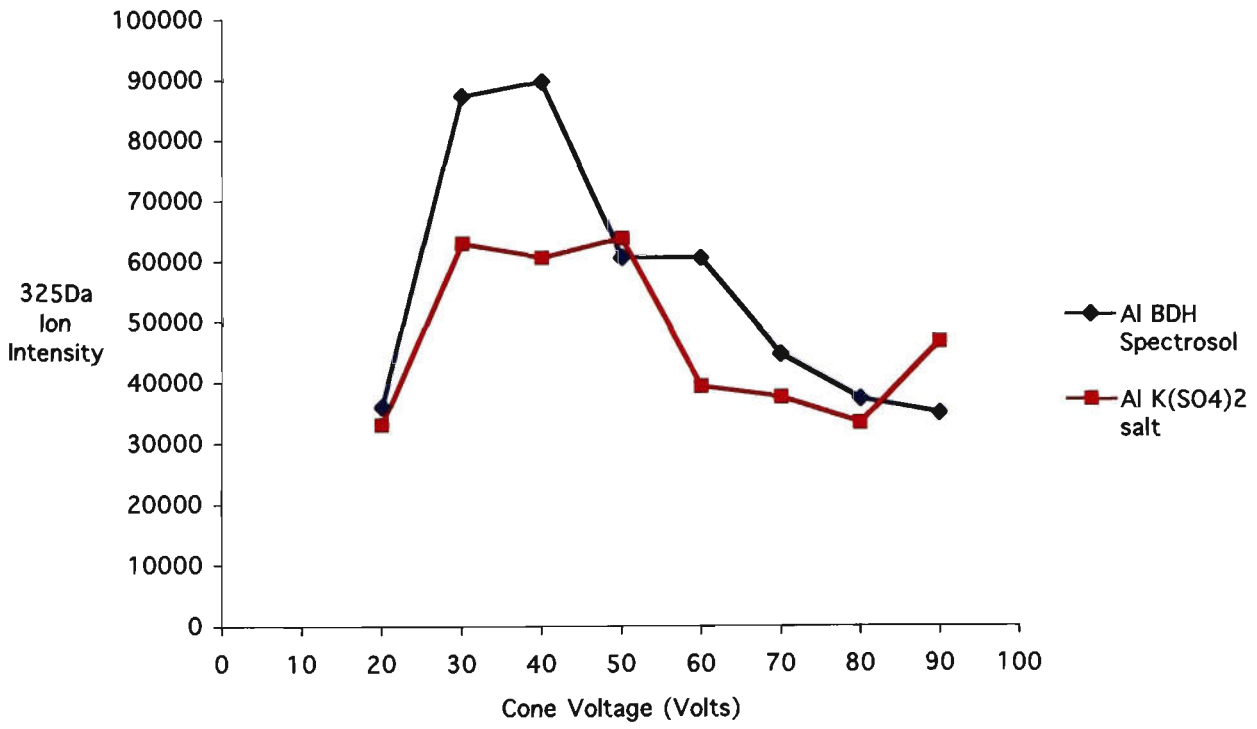


Figure 5.26 Plot of the ion intensity of the peak at m/z 325Da against the cone voltage of the ES-MS skimmer cone for the positive ion mode.

The plot shows that the maximum ion intensity for the 325Da peak is observed when the cone voltage is 30-40V for the solution containing BDH Spectrosol™ aluminium standard and 30-50V for the mixture containing aluminium potassium sulfate. Since the intensities were virtually identical over the 30-40V range the optimum cone voltage was maintained at the default setting of 30V. This cone voltage would also minimise the chance of further fragmentation of molecular ions in solution. All speciation work with ES-MS was carried out using a cone voltage of ±30V.

5.3.6 Characterisation of Complexes From Aqueous Mixtures of Aluminium and Malic Acid

After tartaric acid, malic acid is the next major organic acid found in wine, unless the wine has undergone malo-lactic fermentation. Malic acid has a similar structure to tartaric acid. The difference is an hydroxyl group as shown in Figure 5.27.

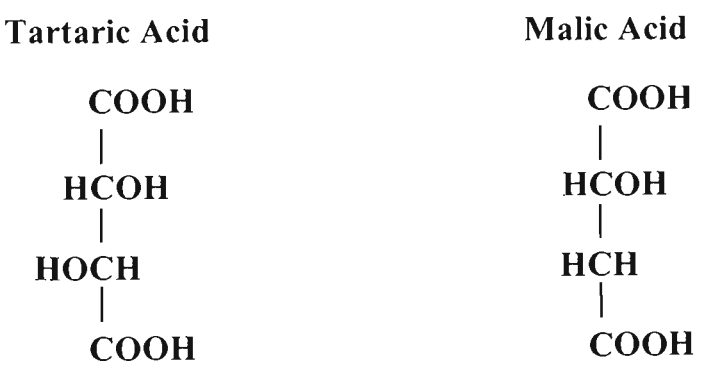


Figure 5.27 Simple molecular structures of tartaric and malic acids.

It was its relative abundance that suggested malic acid was a good candidate as a ligand to bind with aluminium and led to an expansion of the aqueous speciation work to include an investigation of malic acid/aluminium complex speciation by ES-MS. Malic acid proved easy to analyse in aqueous media using ES-MS, with a very strong single peak at 133Da in the negative ion mode representing the bimalate ion. In a similar fashion to that observed for tartaric acid, this species observed in the ES-MS is most likely the sum of residual bimalate in the solution and deprotonated malic acid caused by the electrospray process. The mass spectrum of a 100mg/L (0.75mM) malic acid solution at autogenous pH is shown in Figure 5.28.

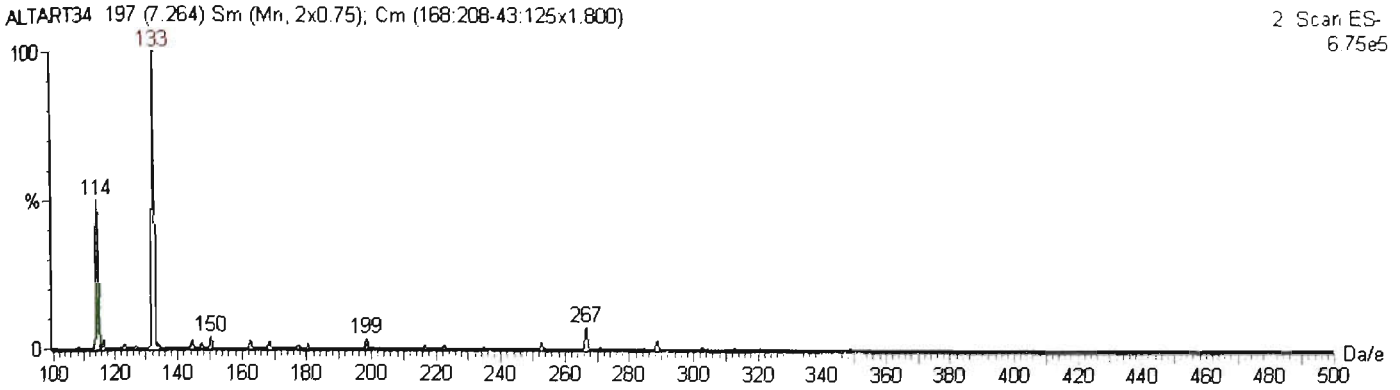


Figure 5.28 Negative ion mode ES-MS spectrum of 100mg/L (0.75mM) malic acid aqueous solution at autogenous pH. The m/z corresponding to assigned ion peak is shown in red.

Mixtures of malic acid and aluminium from both sources were made up at autogenous pH. Hence, two sets of solutions containing 100mg/L (0.75mM) of malic acid and 20mg/L (0.74mM) of aluminium (one set deriving aluminium from the BDH Spectrosol™ standard and the other from aluminium potassium sulfate) were analysed by ES-MS in both positive and negative ion modes. The mass spectra for both solutions in positive and negative ion modes are shown in Figures 5.29 and 5.30 respectively.

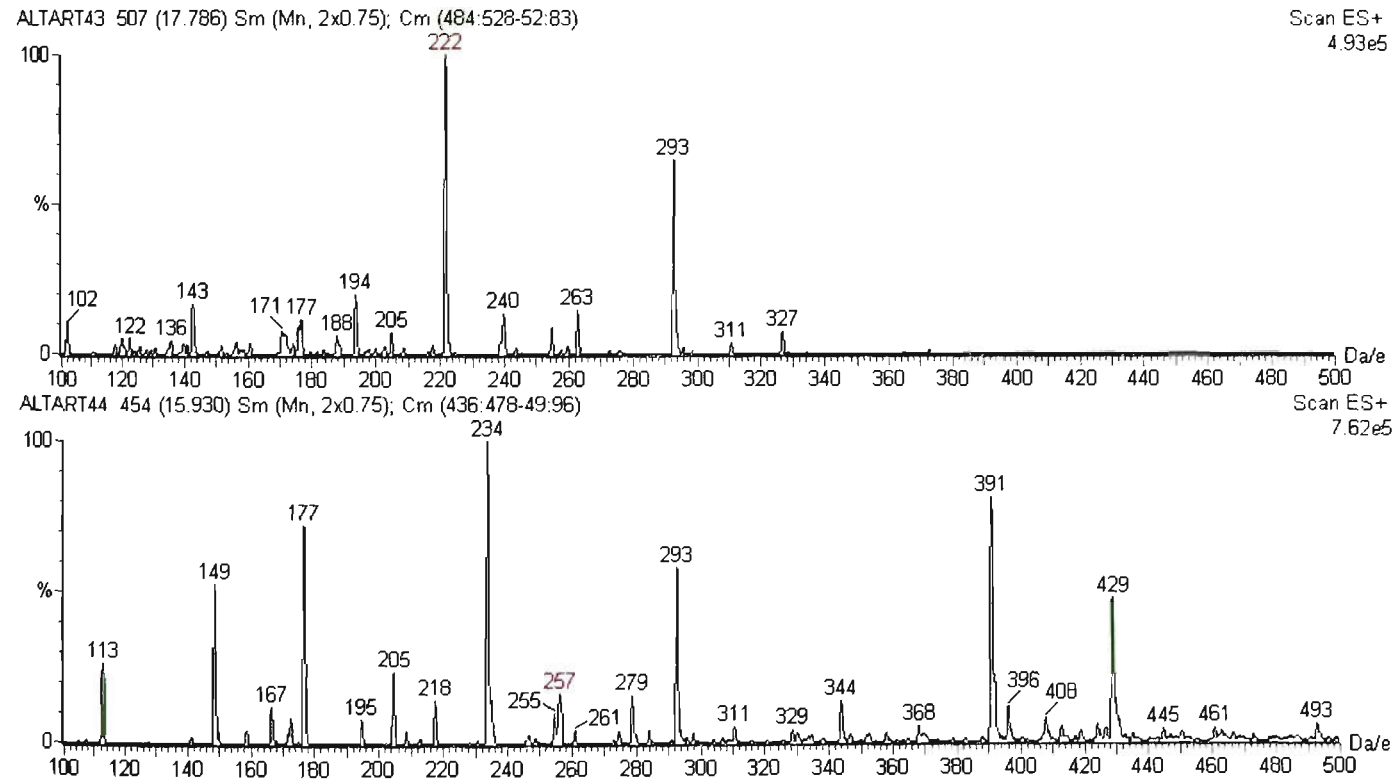


Figure 5.29 Comparison of the mass spectra of 100:20mg/L malic acid/Al mixtures in the positive ion mode. The top mass spectrum is from a solution mixture where the aluminium is derived from a BDH Spectrosol™ standard and the bottom mass spectrum is from a solution mixture where the aluminium is derived from $AlK(SO_4)_2.12H_2O$ standard. Assigned peaks are at 222Da, 257Da and 293Da. Note: the intensity scales are not the same.

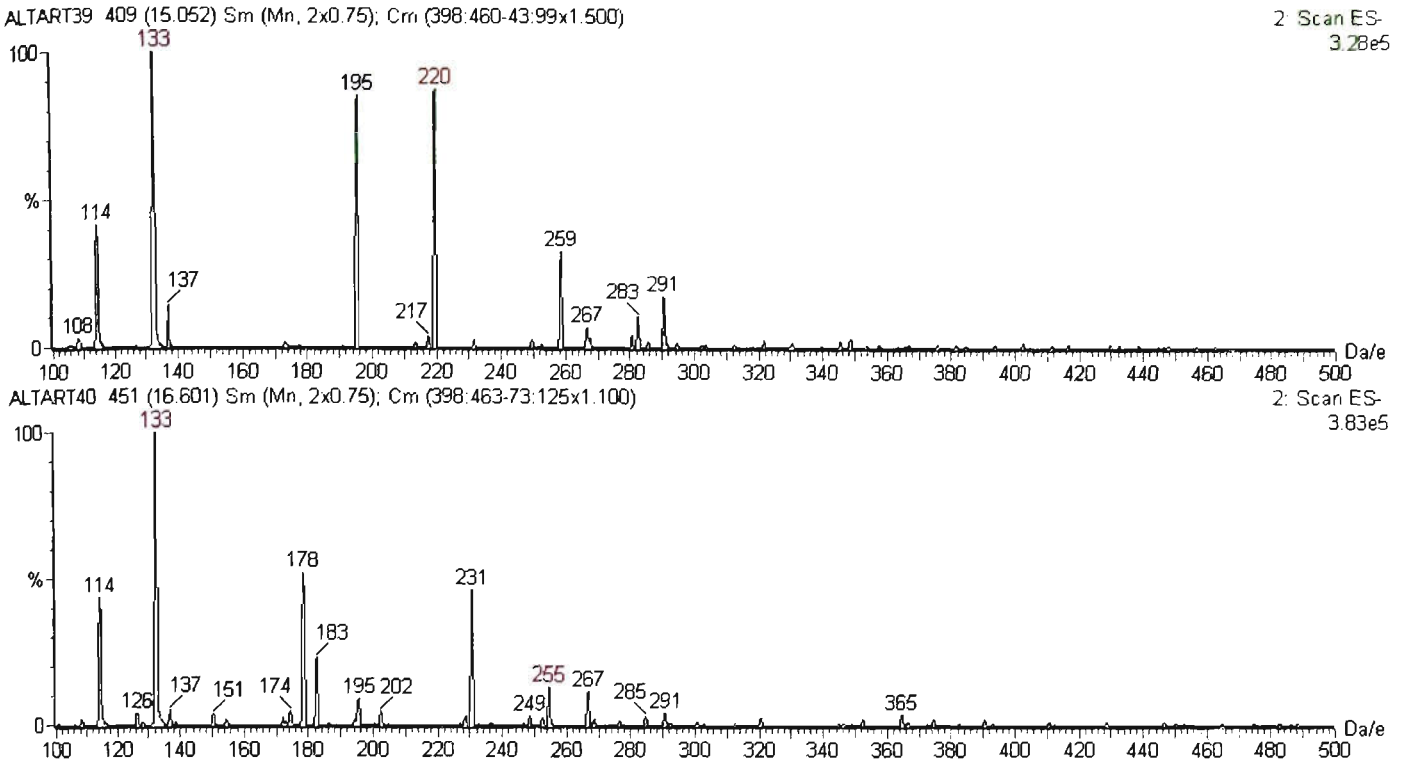


Figure 5.30 Comparison of the background corrected mass spectra of 100:20mg/L malic acid/Al mixtures at autogenous pH in the negative electrospray mode. The top mass spectrum is from a solution mixture where the aluminium is derived from a BDH Spectrosol™ standard and the bottom mass spectrum is from a solution mixture where the aluminium is derived from $AlK(SO_4)_2.12H_2O$ standard. Assigned peaks are at 133Da, 220Da and 291Da. Note: intensity scales are not the same.

The mass spectra for both sets of solutions in negative and positive ion mode show similar peak assignments and patterns as those observed for tartaric acid/aluminium solutions. All the peaks characterised in Figures 5.29 and 5.30 have been confirmed using the same analyses as applied to the tartaric acid/aluminium solutions. In the positive ion mode $[Al(Hmal)_2]^+$ was observed in both solution sets at 293Da, while $[Al(Hmal)(NO_3)]^+$ was observed in the solution containing BDH Spectrosol™ aluminium standard at 222Da and $[Al(Hmal)(HSO_4)]^+$ is evident in the solution containing aluminium potassium sulfate at 257Da. Like the related tartaric acid/aluminium solutions, the species at 222Da and 257Da are due to the nitrate derived from the BDH standard and the sulfate derived from the aluminium potassium sulfate respectively. These respective peaks are not observed in the solutions where nitrate or sulfate is absent, further confirming the binding of these ligands to aluminium as these observations are evident in complexes with both tartaric and malic acids.

The negative ion mode results also mirror the patterns and complexes observed for tartaric acid/aluminium solutions under the same conditions. Along with the uncomplexed bimalate at

133Da, $[Al(mal)_2]^-$ is found in both solution sets at 291Da with $[Al(H_{-1}mal)(NO_3)]^-$ in the solution containing BDH Spectrosol™ standard at 220Da and $[Al(mal)(SO_4)]^-$ in the solution with aluminium potassium sulfate at 255Da. Again, the 'electrospray conjugate pairs' described for the tartaric acid/aluminium are manifest in the ES-MS of the malic acid solutions where these pairs differ by two protons with one complex observed in the negative ion mode and the other in the positive ion mode. The mechanisms of complex formation are probably identical to those discussed for tartaric acid/aluminium mixtures.

Table 5.8 Summary of assigned ions presented in Sub-Section 5.3.6. 'Conjugate pairs' are assigned similar coloured text.

Peak Ion Mass (Da)	Assigned Species
ES Positive Ion Mode	
222	$[Al(Hmal)(NO_3)]^+$
257	$[Al(Hmal)(HSO_4)]^+$
293	$[Al(Hmal)_2]^+$
ES Negative Ion Mode	
133	$[Hmal]^-$
220	$[Al(H_{-1}mal)(NO_3)]^-$
255	$[Al(mal)(SO_4)]^-$
291	$[Al(mal)_2]^-$

5.3.7 Characterisation of Complexes From Aqueous Mixtures of Aluminium, Tartaric and Malic Acid

Comparing the ion intensity data of the complexes between those containing tartaric and malic acids showed that there were differences in complex peak intensity for both positive and negative modes (see Section 5.3.6). Although some of these intensity differences could be attributed to changes in pH, the comparison raised the question of preferential binding of aluminium to one ligand or the other. As electrospray conditions can alter for each separate analysis, indications of preferential binding can more effectively be deduced by analysis of a mixture of all the components in one solution that has the same pH. Hence, an ES-MS analysis of an aqueous solution containing aluminium, tartaric acid and malic acid was conducted to ascertain the complexation distribution pattern in the one solution. Additionally, as both these ligands can be present in wine, this analysis is a logical progression towards investigating the speciation of more complex matrices such as wine.

Aqueous solutions of 100mg/L (0.67mM) tartaric acid, 100mg/L (0.75mM) of malic acid and 20mg/L (0.74mM) of aluminium were analysed in both positive and negative ion modes, with aluminium derived from either Spectrosol™ BDH standard or aluminium potassium sulfate. The results are shown in the mass spectra for positive ion mode in Figure 5.31 and for negative ion mode in Figure 5.32.

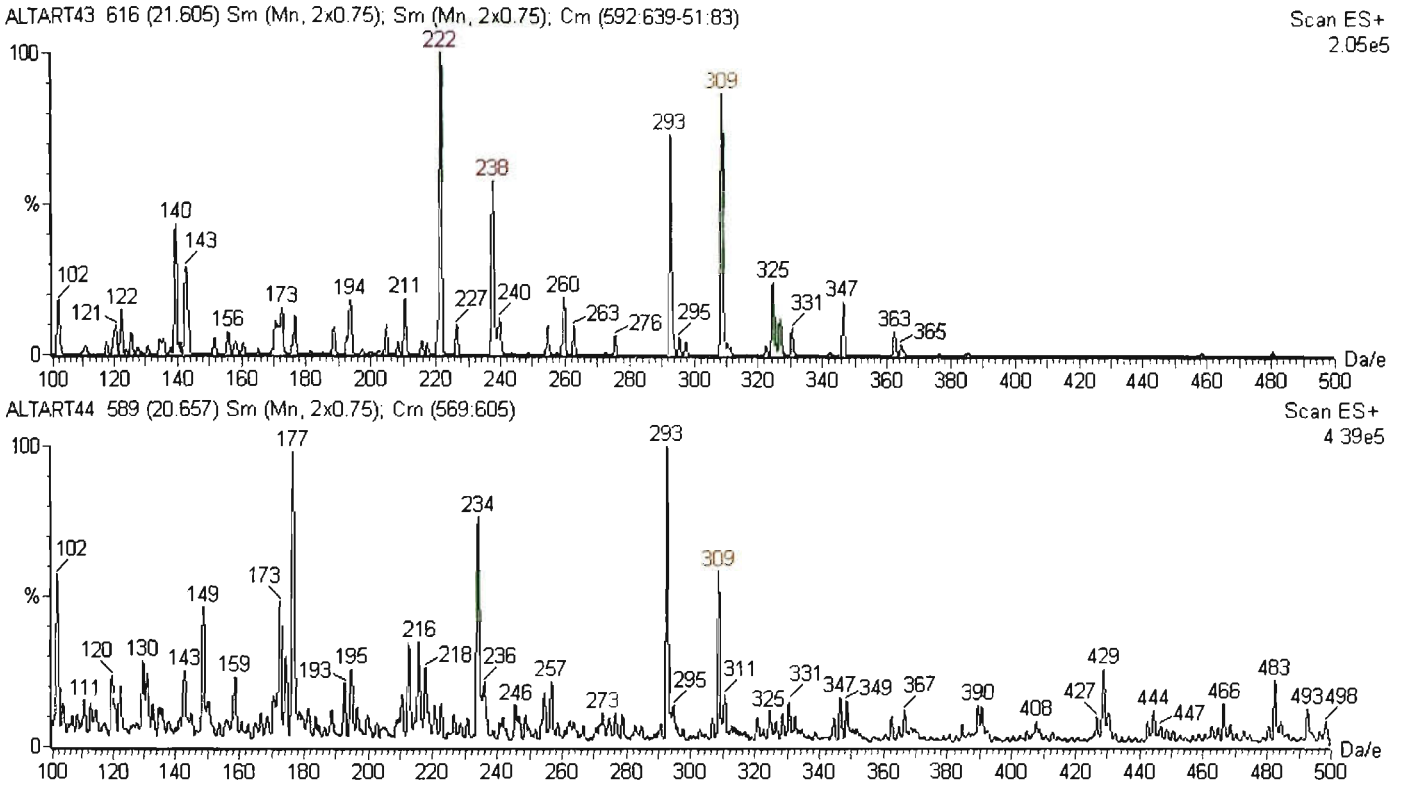


Figure 5.31 ES positive mode mass spectra of aqueous mixtures of 100mg/L (0.67mM) tartaric acid, 100mg/L (0.75mM) malic acid and 20mg/L (0.74mM) aluminium where the top and bottom mass spectra represent mixture solutions containing aluminium derived from BDH Spectrosol™ and potassium sulfate salt standards respectively. Note: intensity scales are not the same.

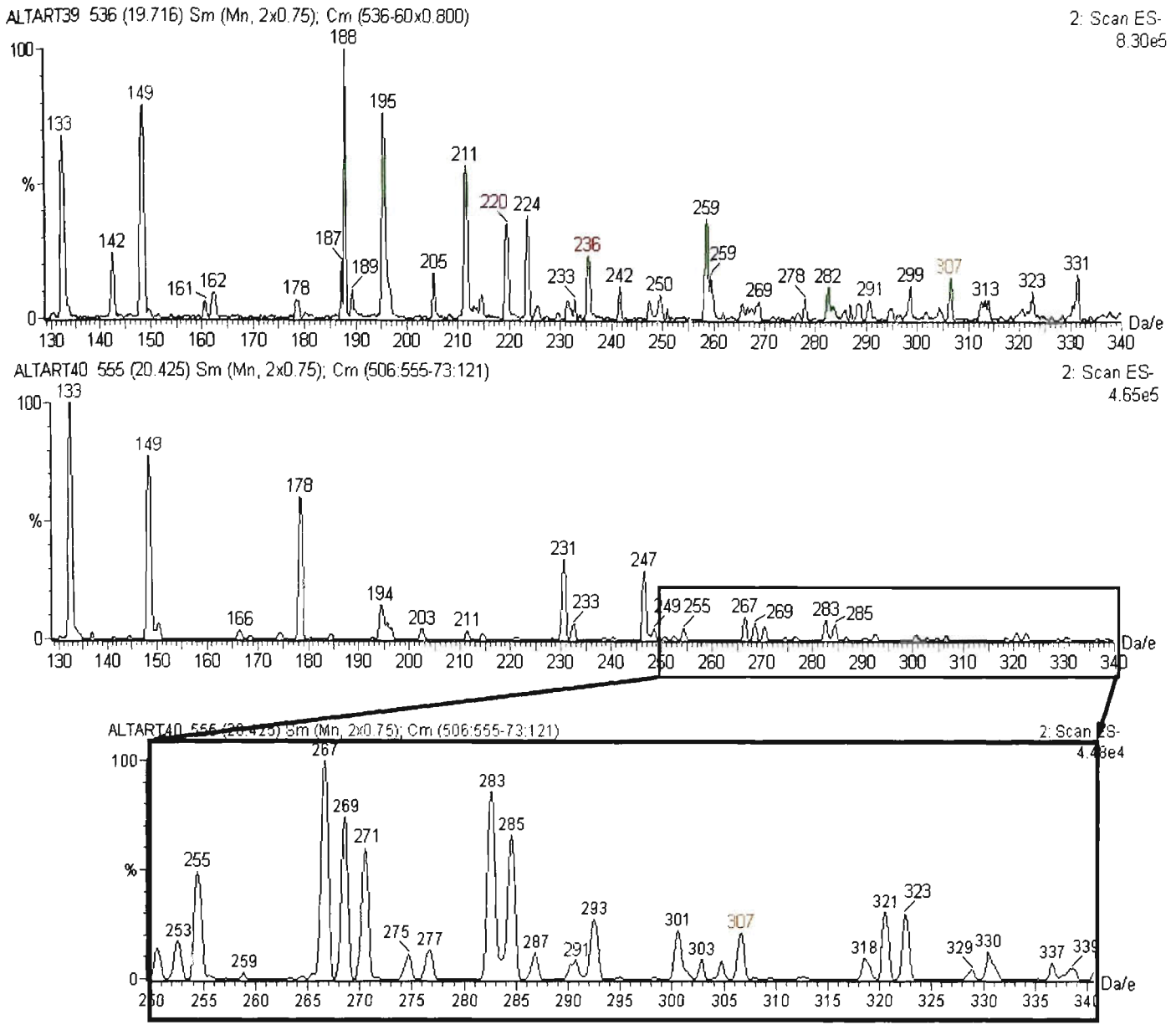


Figure 5.32 ES negative mode mass spectra of aqueous solutions of 100mg/L (0.67mM) tartaric acid, 100mg/L (0.75mM) malic acid and 20mg/L (0.74mM) aluminium where the top and bottom mass spectra represent solutions containing aluminium derived from BDH Spectrosol™ and potassium sulfate salt standards respectively. Note: intensity scales are not the same.

Both figures clearly demonstrate that the aluminium complexes and ES-MS patterns observed for solutions of aluminium with tartaric and malic acid separately are found in the combined solutions in both negative and positive ion mode (see Table 5.9 at end of section for characterisations). In positive ion mode the bis-bitartrate (325Da) and bis-bimalate (293Da) species are observed in both solutions containing aluminium from either source, and the nitrato species (222 & 238Da) are observed in the solution with aluminium derived from the BDH Spectrosol™ standard. Again, a parallel ion pattern is observed in the negative ion mode with the bis-tartrate (323Da) and bis-malate (291Da) species evident in both aluminium sourced solutions and the nitrato species apparent (220Da) in the solution containing BDH Spectrosol™ standard.

Although these ion complex relationships repeat what was evident in the single ligand solutions, after peak attribution analysis it was found that the combined solutions yielded a mixed ligand aluminium complex. This mixed complex was apparent in both positive and negative ion modes with $[Al(Htart)(Hmal)]^+$ observed at 309Da and $[Al(tart)(mal)]^-$ at 307Da respectively. As expected, they were observed in solutions using both aluminium sources, with the solution containing BDH Spectrosol™ standard showing a higher mixed complex ion intensity than for aluminium sourced from aluminium potassium sulfate. Further, the distribution between the bis-bitartrate, bis-bimalate and the mixed ligand complexes also differed significantly between the two different aluminium sourced solutions.

From the positive ion intensities one could conclude that the bis-bimalate complex is more prevalent than the bis-bitartrate complex, suggesting greater competitive aluminium binding by the former. This relationship is reversed in the negative ion mode and may reflect the ease with which protons can be removed from the bis-bitartrate compared with the bis-bimalate complex, as opposed to ion intensity directly related to solution concentration. Overall, the ion intensities suggest that the amount of aluminium bound to bitartrate/tartrate is significantly less than that bound to bimalate/malate, even when the slight initial excess in mole quantities of malic acid is taken into consideration. In the positive ion mode, if bimalate binds more strongly to aluminium than bitartrate, one would expect that aluminium bis-bimalate would show a higher intensity than the mixed complex and the aluminium bis-bitartrate. The distribution of the non-nitrato complexes, purely from visual inspection of the mass spectra, demonstrates that this is the case for the solution containing aluminium derived from the potassium sulfate salt. However, this distribution is not observed in solutions containing aluminium from the BDH Spectrosol™ standard. A possible reason could be the role of nitrate as a competing ligand and strong electrolyte source, and its conjugate, nitric acid, as a key arbiter of solution pH. This difference in ion intensity distribution suggests that the presence of the nitrato complexes complicates the distribution of the aluminium between the two organic acid ligands.

Whilst it was useful and constructive to identify the complexes containing a nitrato ligand, there is little likelihood of such species occurring in wine (Rankine 1991). However, the pH of the solutions

derived from aluminium potassium sulfate and the other ions, K^+ and SO_4^{2-}, are more likely to correspond with wine samples to be used in the latter part of the project. In addition, complexes containing sulfate ligands were significantly less intense in solutions at the pH found in wine and demonstrated less interference with organic acid/Al complex ion intensities. For these reasons, aluminium sourced from BDH Spectrosol™ standard was not used for the remainder of the speciation investigation.

Table 5.9 Summary of assigned ions presented in Sub-Section 5.3.7.

Peak Ion Mass (Da)	Assigned Species
ES Positive Ion Mode	
222	$[Al(Hmal)(NO_3)]^+$
238	$[Al(Htart)(NO_3)]^+$
257	$[Al(Hmal)(HSO_4)]^+$
273	$[Al(Htart)(HSO_4)]^+$
293	$[Al(Hmal)_2]^+$
309	$[Al(Hmal)(Htart)]^+$
325	$[Al(Htart)_2]^+$
ES Negative Ion Mode	
133	$[Hmal]^-$
149	$[Htart]^-$
220	$[Al(H_{-1}mal)(NO_3)]^-$
236	$[Al(H_{-1}tart)(NO_3)]^-$
255	$[Al(mal)(SO_4)]^-$
271	$[Al(tart)(SO)_4]^-$
291	$[Al(mal)_2]^-$
307	$[Al(mal)(tart)]^-$
323	$[Al(tart)_2]^-$

5.4 ES-MS Speciation Investigation of Model Wine Solutions

5.4.1 Model Wine Investigation Considerations

Earlier in this Chapter it was demonstrated that ES-MS could be used to identify aluminium complexes with two major organic acids found in wine for simple aqueous matrices in both positive and negative ion mode. The characterisation of aluminium species with different ligands in the same solution was also encouraging, showing that ES-MS could be used for solutions containing potentially competing aluminium complexing agents. The success of the preliminary work for aqueous solutions meant the techniques performed on aqueous solutions could be applied to a model wine. Studies began on simple solutions to gain confidence in identification of complexes, followed by investigating progressively more complex solutions by adding more of the components found in wine.

The major difference in the model wine solutions was the addition of 12-13% v/v ethanol to the solution matrix. Any influence this has on the ES-MS identification of aluminium speciation had to be investigated before analysis of wine samples could begin. As the model wine investigation progressed, more organic acids were added at concentrations found in wine. To allow for better comparison with the earlier aqueous speciation work the highest organic acid concentration was maintained at 100mg/L and that of the aluminium at 20mg/l.

5.4.2 ES-MS Analysis of Aluminium, Tartaric Acid and Malic Acid Solutions with Ethanol

Solutions of aluminium with malic acid and tartaric acid similar to those described in Section 5.3.7 were made up in a 12% v/v ethanol solution to determine the effects of the alcohol on the electrospray of the aluminium complexes. The 20mg/L aluminium standard used for background subtraction was also made up in 12% v/v ethanol to match the matrices. The positive and negative ion mode mass spectra of an ethanol solution of 100:100:20mg/L tartaric acid/malic acid/aluminium are shown in Figures 5.33 and 5.34.

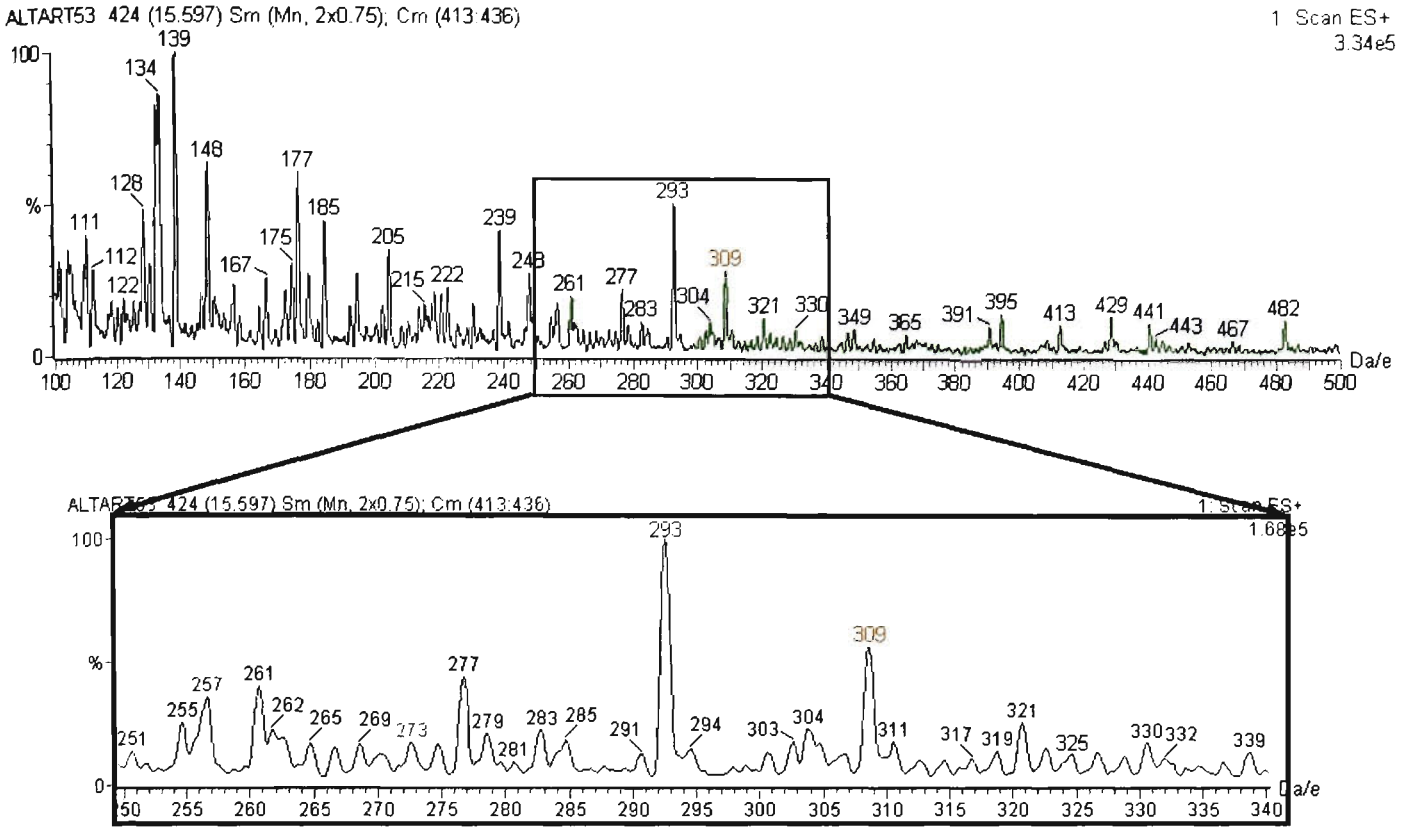


Figure 5.33 Positive ion mode mass spectrum of a 100:100:20mg/L Tart/Mal/Al solution in 12% (v/v) ethanol at autogenous pH.

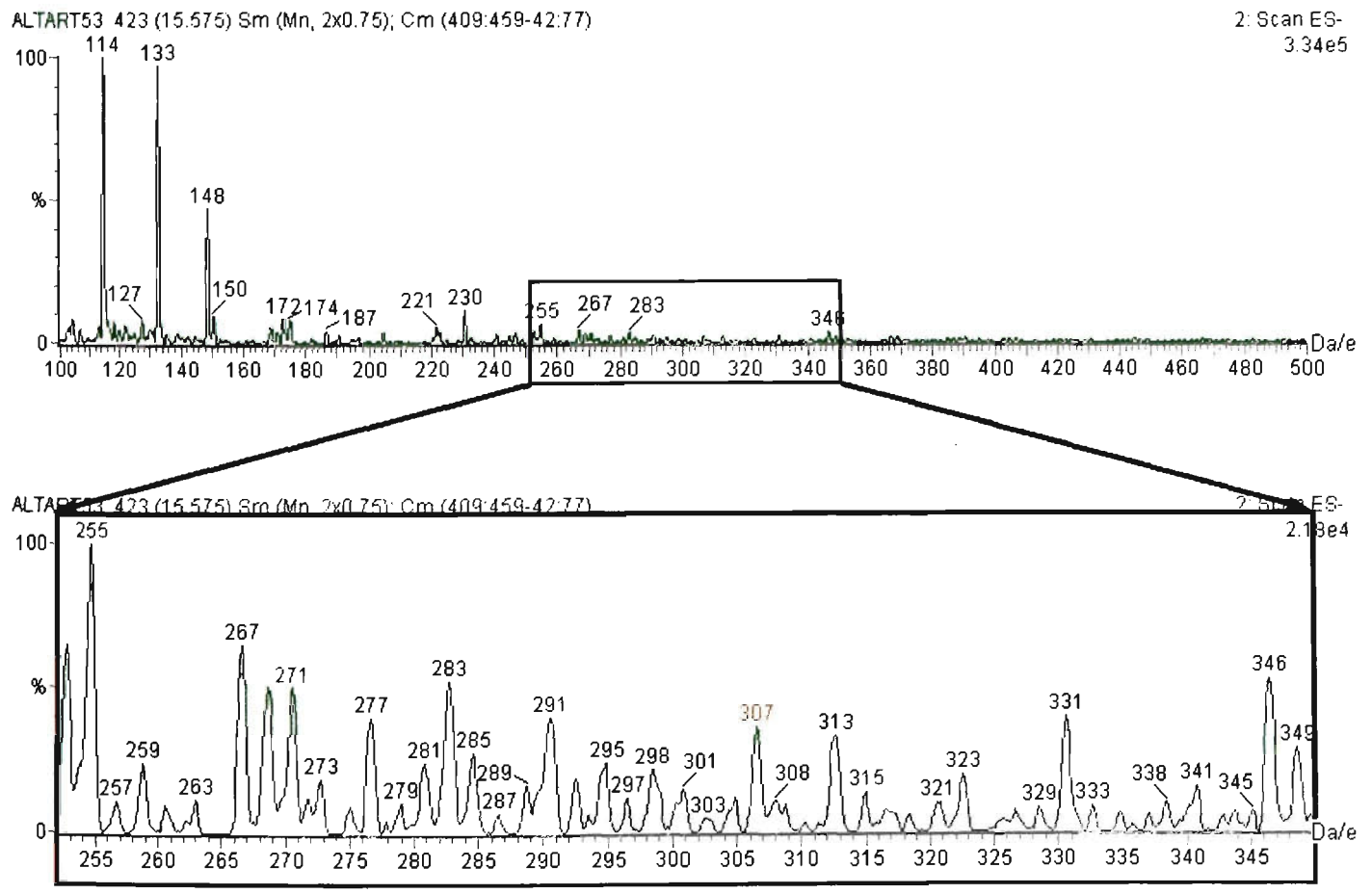


Figure 5.34 Negative ion mode mass spectrum of a 100:100:20mg/L Tart/Mal/Al solution in 12% (v/v) ethanol at autogenous pH.

On initial inspection of the positive ion mode mass spectrum, there appears to be little difference between the 12% v/v ethanol and aqueous solutions. The absolute ion intensities (see Figure 5.35) however, show that ion intensities in 12% v/v ethanol for bis-bimalate and bitartrate/bimalate complexes at 293Da and 309Da are half their intensity in purely aqueous solutions. Curiously, the intensity of the bis-bitartrate complex at 325Da has not changed appreciably. The effects of ethanol on the ions observed in the negative ion mode show some differences in intensity, however the changes are minimal compared with those observed in the positive ion mode.

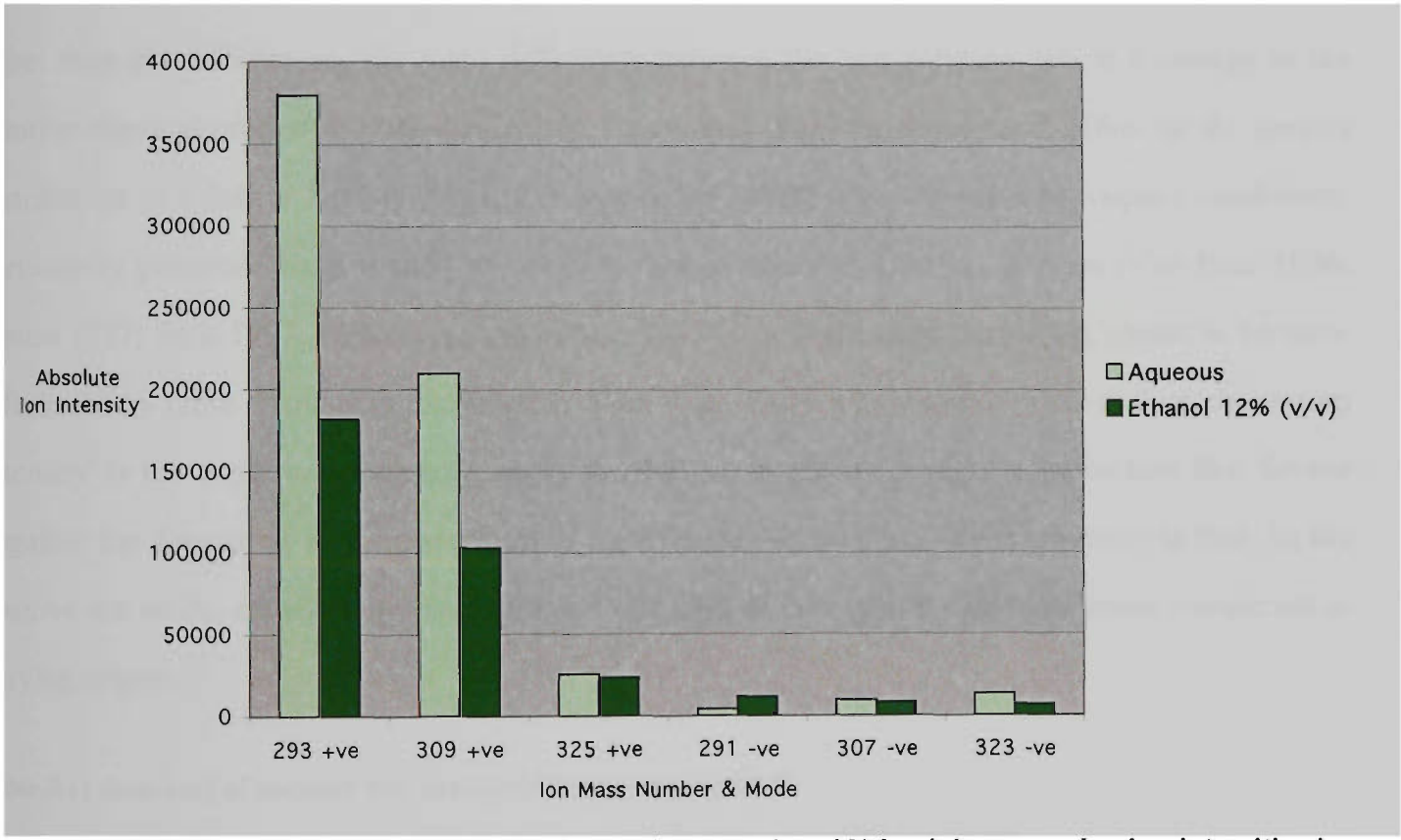


Figure 5.35 Comparison of both positive and negative organic acid/aluminium complex ion intensities in a solution of 100mg/L (0.67mM) tartaric acid, 100mg/L (0.75mM) malic acid and aluminium (0.74mM) for aqueous and 12% (v/v) ethanol matrices.

The effect of ethanol on the intensities of bis-bimalate (293Da) and bitartrate-bimalate (309Da) aluminium complexes could be due to a variety of factors. The pH of the solutions was the first variable to be examined. A comparison of the pH of the ethanolic solutions with the aqueous solutions (Table 5.10) shows that the pH is virtually unchanged by the addition of ethanol.

Table 5.10 Comparison of the pH of aluminium/organic acid solutions in aqueous and 12%(v/v) ethanol matrices.

Solution	pH (Aq. Solutions)	pH (12% Et. Solutions)
20mg/L Al [$K.(SO_4)_2$] salt	4.08	4.07
100mg/L Tartaric Acid	3.89	3.95
100mg/L Malic Acid	3.37	3.42
100:20 mg/L Tartaric Acid/Al	3.19	3.17
100:20 mg/L Malic Acid/Al	3.10	3.16
100:100:20 mg/L Tart/Mal/Al	3.05	3.09

Other than pH differences, the main difference between the two solution sets is a change in the solution physical properties with the addition of ethanol. This may have some effect on the species distribution in solution but the greatest consequence would apply to the electrospray conditions; particularly physical changes such as viscosity, conductivity and surface tension (Van Baar 1996; Bruins 1997; Dass 1997; Rubinson & Rubinson 2000). The malic acid complexes appear to be more influenced by these changes in the solution conditions. The slight increase in bis-malate/aluminium intensity in the negative mode may point to changes in the electrolyte composition that favour negative ion formation and suppression of positive ion formation. The conclusion is that, in the positive ion mode, ethanol suppresses the ion intensity of the organic acid/aluminium complexes to varying degrees.

Table 5.11 Summary of assigned ions presented in Sub-Section 5.4.2.

Peak Ion Mass (Da)	Assigned Species
ES Positive Ion Mode	
293	$[Al(Hmal)_2]^+$
309	$[Al(Hmal)(Htart)]^+$
325	$[Al(Htart)_2]^+$
ES Negative Ion Mode	
291	$[Al(mal)_2]^-$
307	$[Al(mal)(tart)]^-$
323	$[Al(tart)_2]^-$

5.4.3 Considerations for the Development of a Model Wine

The use of 100mg/L concentrations for malic and tartaric acid solutions was arbitrarily set during the preliminary work partially to avoid overloading the electrospray and because this concentration was the estimated tartaric acid concentration after a wine was diluted 35 fold. However, the solutions of mixed ligands had not been made up with the concentration ratios that truly reflected those seen in wine. In addition, as previously discussed in Section 5.2 and presented in Table 5.1, wine is a very complex matrix made up of more than just tartaric and malic acids in water. To further the development of the ES-MS analysis of wine, a model wine more reflective of the major constituents was required. A simple matrix of major constituents and their average concentrations, as described by Rankine (1991) and discussed in Section 5.2 and Table 5.1, was developed and is shown in Table 5.12. From this matrix, a decision was made as to what constituents should be included in the model wine and the concentrations that should be employed. The concentrations of the constituents would be related to the mean of the concentration ranges given by Rankine (1991) and then divided by 35, the dilution factor required to get the tartaric acid concentration in wine down to 100mg/L.

Table 5.12 Matrix of major wine constituents, their mean concentrations in wine and the diluted concentration required in the model wine. Based on data from Rankine (1991).

Component	Mean Wine Concentration (mg/L)	35 Fold Dilution Model Wine(mg/L)	From 20mg/L $AlK(SO_4)_2$ Spike
Tartaric Acid	3500	100.0	---
Malic Acid*	2500	71.4	---
Citric Acid	500	14.3	---
Succinic Acid	1000	28.6	---
Lactic Acid*	1700	48.6	---
Glucose (dry wine)	1000	28.6	---
Fructose (dry wine)	1000	28.6	---
Sulfate	1550	44.3	142.1
Chloride	210	6.0	---
Phosphate	500	14.3	---
Bisulfite	150	4.3	---
Potassium	1250	35.7	30.0
Sodium	1240	35.4	---
Calcium	140	4.0	---
Magnesium	105	3.0	---
Ethanol	12% v/v	12% v/v	---
Aluminium	0.70	---	20.0

*Note: lactic and malic acids would not be used in the same model wine solution. See end of sub-section

The components shown in Table 5.12 labelled in red are the constituents that have been used in solutions previously examined. Fortunately, the use of the aluminium standard derived from the aluminium potassium sulfate stock does not introduce ions or compounds that are foreign to wine. In the case of potassium, the amount of the element introduced by the addition of the aluminium to the model wine is very close to the estimated mean concentration in a 35 fold diluted wine. On the other hand, the sulfate concentration contributed by the aluminium solution is three times the estimated mean concentration in a 35 fold diluted wine. This was not considered a problem, as the intensity of the sulfato/aluminium complexes were generally low compared with the organic acid/aluminium complexes and there had not been evidence of any interference by these sulfato complexes on the complexation of aluminium by organic acids. The malic acid concentration used in previous work was greater compared with its estimated concentration in the diluted wine; hence, the model wine would use 70mg/L malic acid rather than the 100mg/L used previously. To match the original wine matrices as much as possible, the model wine solution was made up in 12% v/v ethanol solution, to minimise the impact on the species distribution. Thus, the original concentration of ethanol was maintained in the 35-fold diluted model wine.

The constituents in Table 5.12 labelled in black were components that were not included in the model wine. These were omitted because they were thought too low in concentration to significantly influence the aluminium speciation in the solution. The latter was the case for sodium and the sugars, fructose and glucose. The sugars were omitted as it was considered they would form deposits and ultimately block the ES-MS system and were unlikely to complex aluminium in preference to the organic acids. Thus, the only new additions to form the model wine are labelled in blue in Table 5.12.

The inclusion of lactic and citric acids and the exclusion of succinic acid in the model wine was based upon both their mean concentration (from Rankine (1991)) and their association constants with aluminium as shown in Table 5.13. These data indicate that citric acid competes very strongly for complexation with aluminium and lactic acid is less competitive, with succinic acid the least competitive. The complexing strength of citric acid is not surprising because of the number, location and variety of functional groups on its five-carbon chain.

Table 5.13 Published aluminium-organic ligand association constants for the major organic acids found in wine (from Vance et al. 1996). Where more than one data set is available, the $\log K_1$ value is a mean of all data.

Organic Acid	Structural Formula	Association Log K_1 (M + L ⇒ ML)
Tartaric Acid	$HO_2CCH(OH)CH(OH)CO_2H$	5.32
Malic Acid	$HO_2CCH_2CH(OH)CO_2H$	5.34
Citric Acid	$HO_2CCH_2CH(OH)(CO_2H)CH_2CO_2H$	8.88
Succinic Acid	$HO_2CCH_2CH_2CO_2H$	3.2
Lactic Acid	$CH_3CH(OH)CO_2H$	4.26

Hence, citric and lactic acids were added to the model wine matrix. The latter because it was still present in significant quantity and could possibly compete for aluminium, the former because, although it showed the lowest estimated mean concentration of all the five acids, its very high association constant meant that it could favourably compete for binding to aluminium even at a low concentration. Succinic acid had the lowest formation constant and a low concentration compared with the other acids rendering it unlikely to successfully compete for binding to aluminium. These factors and the preference for a simple solution system precluded it from being added to the model wine.

The final consideration was the effect of malo-lactic fermentation on the organic acids in wine. This process is carried out on some wines, converting malic acid into lactic acid. Lactic acid is generally found in wine due to this process, so there would not be significant quantities of both acids in the same wine. Thus, it was decided to analyse two discrete model wines, one containing 50mg/L lactic acid and the other 70mg/L malic acid.

5.4.4 Revisiting Analyses of Aluminium Complexes with Tartaric and Malic Acids Using Model Wine Media Ligand Concentrations

With the malic acid concentration in the model wine reduced from 100mg/L (0.74mM) to 70mg/L (0.52mM), a solution of 100:70:20mg/L tartaric acid/malic acid/aluminium in 12% v/v ethanol was analysed by positive and negative ion mode ES-MS. The results were compared with the mass spectra of the solution using 100mg/L malic acid in the same matrix. The positive and negative ion mode mass spectra of this solution are demonstrated in Figure 5.36.

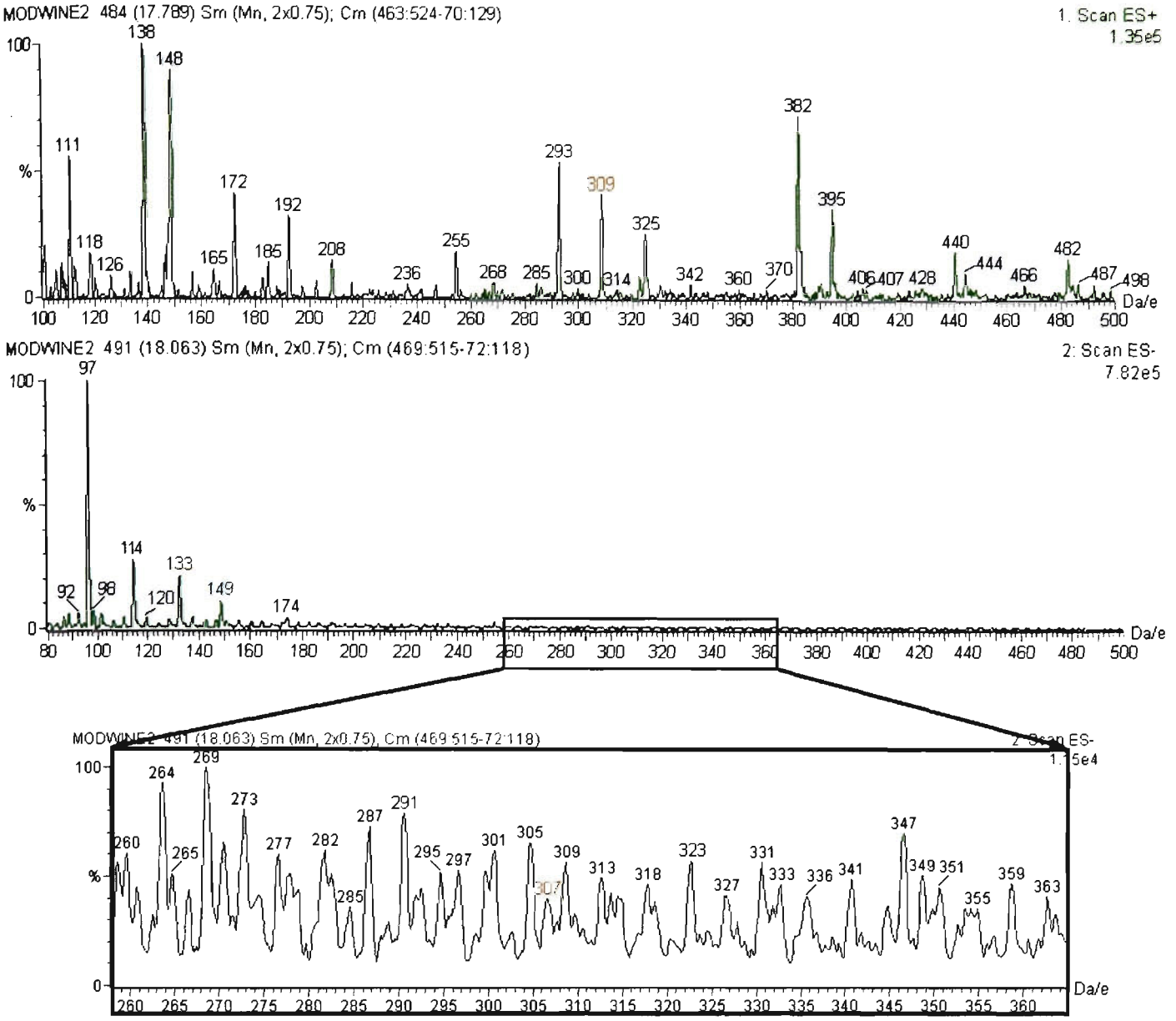


Figure 5.36 Mass spectra of a 100:70:20mg/L tartaric acid/malic acid/aluminium solution in a model wine matrix. Positive and negative ion mode mass spectra are shown top and bottom (with inset) respectively. Note: the m/z and intensity scales are not similar between mass spectra.

In the positive mode, although the distribution remains 293Da>309Da>325Da, the peak at 325Da is significantly more prominent than it was (see Figures 5.33 & 5.34), suggesting that with a molar excess of 0.15mM, more bis-bitartrate is complexing aluminium at the expense of the mixed ligand and bis-bimalate. It was also interesting to note the absence or extremely low intensities of the sulfato complexes with aluminium. This experiment shows that working at concentration ratios more akin to that in wine alters the intensity ratios of peaks, indicative of solution distribution changes compared with mixed solutions with a higher malic acid concentration (see Figures 5.33 & 5.34).

5.4.5 ES-MS of Aluminium Complexes with Lactic and Citric Acids in Model Wine Media

Before carrying out the investigation of a model wine, an ES-MS study was carried out on discrete solutions of aluminium with lactic acid and with citric acid to ascertain any possible complexes that may need to be identified in the model wine. Two solutions, one containing 100mg/L (1.14mM) lactic acid and the other 100mg/L (0.52mM) citric acid, both with 20mg/L aluminium in 12% aqueous ethanol were analysed by positive and negative mode ES-MS. The mass spectra are shown in Figures 5.37 and 5.38.

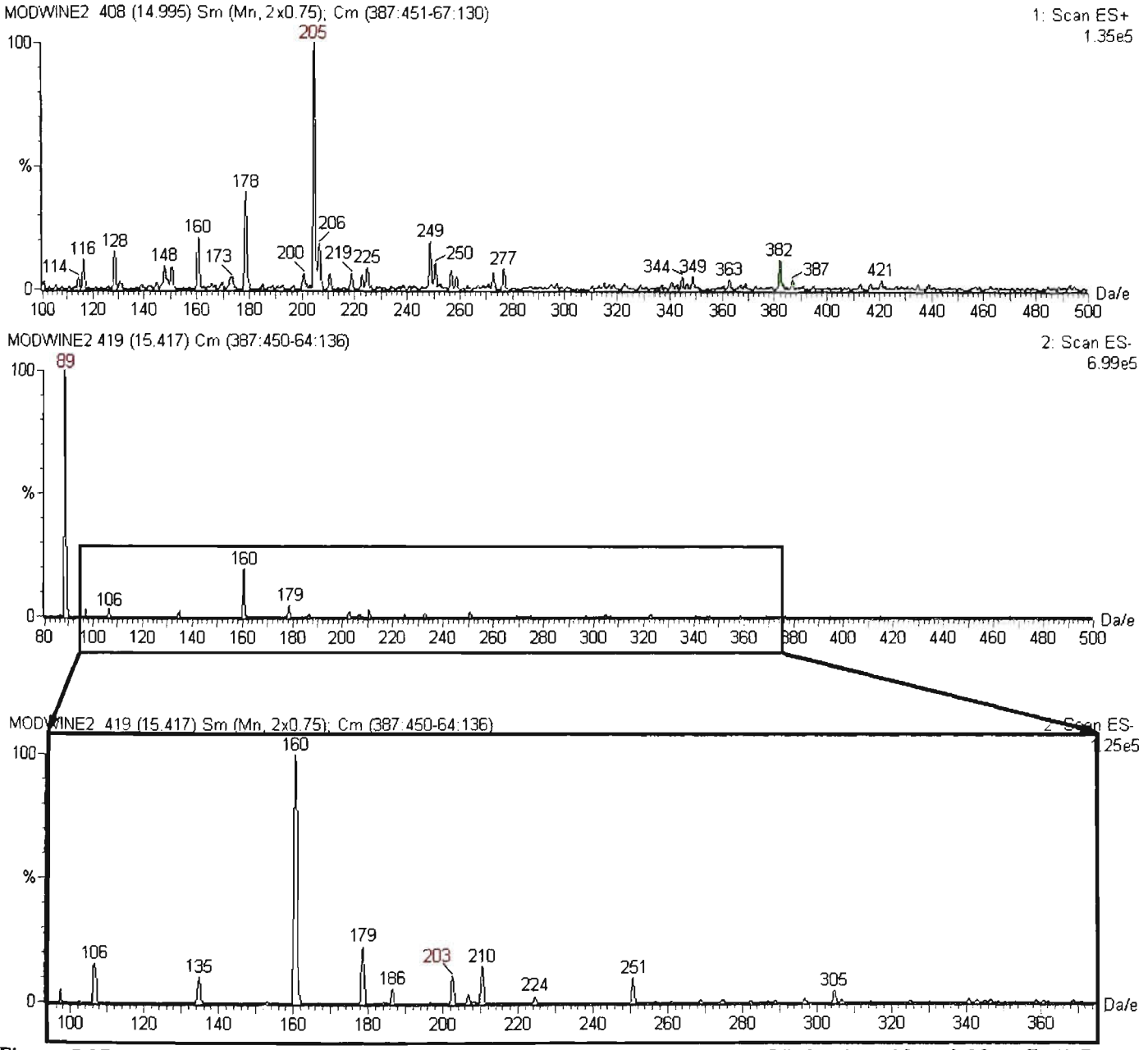


Figure 5.37 Positive and negative mode mass spectra of a 100mg/L (1.14mM) lactic acid and 20mg/L (0.74mM) aluminium solution with the positive mode spectrum at the top. Assigned peaks are at 205Da, 89Da and 203Da. Note: the m/z and intensity scales are different for each mass spectrum.

Lactic acid differs from both tartaric and malic acids by having only one carboxylic acid functional group and is the lowest molecular mass organic acid studied. In the positive ion mode, the intense

peak observed at 205Da was characterised by ion chromatogram peak matching as $[Al(lac)_2]^+$. As demonstrated for the other organic acids studied previously, a 'conjugate' complex was observed in the negative ion mode, though with a much weaker ion intensity, at 203Da. This was characterised as the complex $[Al(H_{-1}lac)_2]^-$. The lactate anion is also observed in the negative mode, with an intense peak at 89Da. This is not surprising as in molar terms, the 100mg/L lactic acid solution is more concentrated than 100mg/L of tartaric or malic acids. There was no conclusive evidence of the existence of sulfate complexes with aluminium and lactate.

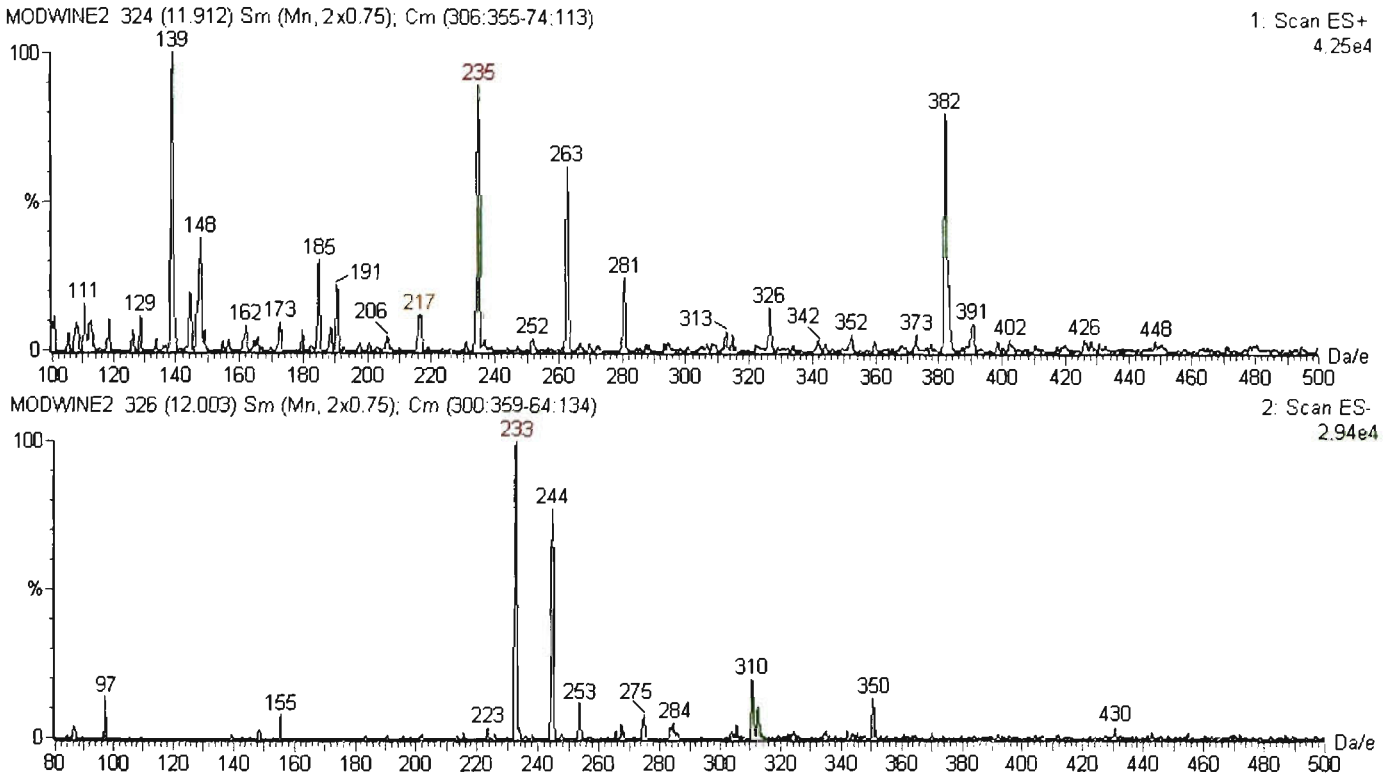


Figure 5.38 Positive and negative mode mass spectra of a 100mg/L (0.52mM) citric acid and 20mg/L (0.74mM) aluminium solution with the positive mode spectrum at the top. Assigned peaks are at 217Da, 233Da and 235Da. Note: the m/z and intensity scales are different for each mass spectrum.

The mass spectrum of the citric acid/aluminium solution was very different from the other organic acids studied in several ways. Two aluminium complexes with citric acid were characterised by ion chromatogram peak matching in the positive mode. The first, an $[Al(Hcit)]^+$ complex at 217Da is similar to the positive ion mode complexes of aluminium with the other organic acids except it has only one ligand. However, the second complex, $[Al(Hcit)(H_2O)]^+$ at 235Da was unusual in this speciation study as no other complex of an organic acid ligand co-complexing aluminium with hydroxide or water ligands could be identified. In the negative ion mode, a 'conjugate' species, probably $[Al(cit)(OH)]^-$, was observed at 233Da. The co-complexing of aluminium with a water/hydroxide ligand by citric acid was unusual in that it was not observed in ES-MS spectra of

mixtures of aluminium with the other organic acids studied. The reason for the attachment of only one citric acid anion to aluminium may lie in the lower concentration of the ligand compared with the other acids, as other studies have indicated that bis-citrate aluminium complexes only significantly occur when the citrate is in excess (Gregor & Powell 1986; Kerven et al. 1995; Rubini et al. 2002). Additionally, citric acid is a larger molecule than the other organic acids studied with three carboxylic acid groups, therefore there is the possibility that steric hindrance may also play a role in excluding mixed organic acid aluminium complexes, whereby only a smaller ligand such as water is allowed to attach to aluminium in conjunction with citrate. There was no conclusive evidence of sulfate complexes with aluminium and citrate.

The negative ion mass spectrum of the citric acid/aluminium solution was also unique amongst the organic acids studied because of a lack of evidence of a peak at 191Da due to $[H_2cit]^-$ from residual solution anions and deprotonated citric acid. This was unusual as all the other acids demonstrated substantial peaks for the uncomplexed acid and anion. The absence of measurable uncomplexed $[H_2cit]^-$ may indicate that all the ligand could be incorporated in a complex with aluminium. Citric acid is known to strongly bind to aluminium (see Table 5.13) and since the aluminium is in molar excess, there is every opportunity for complete binding. The only other less likely explanation is the formation of a stable neutral complex of citric acid with aluminium that is resistant to charge accumulation in the electrospray process. Model wine investigation discussed in Sub-Section 5.4.6 would determine if this observation was reproducible in a solution where the citric acid/aluminium concentration ratio was greatly reduced in a solution with competing ligands close to equimolar concentrations with aluminium.

Table 5.14 Summary of assigned ions presented in Sub-Section 5.4.5.

Peak Ion Mass (Da)	Assigned Species
ES Positive Ion Mode	
205	$[Al(lac)_2]^+$
217	$[Al(Hcit)]^+$
235	$[Al(Hcit)(H_2O)]^+$
ES Negative Ion Mode	
89	$[lac]^-$
203	$[Al(H_{-1}lac)_2]^-$
233	$[Al(cit)(OH)]^-$

5.4.6 ES-MS Analysis of the Model Wine

With an understanding of the types of complexes that could be encountered in this matrix and sufficient confidence in the technique and the methods employed, an analysis of a model wine was conducted. For reasons discussed in Section 5.4.3, two sets of model solution were developed, designated model wine I and model wine II. The composition of these solutions and their constituents concentrations are shown in Table 5.15.

Table 5.15 Components of model wine solutions and their concentrations discussed in Sub-Section 5.4.6.

Model Wine Component	Model Wine I		Model Wine II	
	(mg/L)	(mM)	(mg/L)	(mM)
Tartaric Acid	100	0.67	100	0.67
Malic Acid	70	0.75	---	-----
Lactic Acid	---	-----	50	0.56
Citric Acid	14	0.07	14	0.07
Aluminium ($AlK(SO_4)_2$)	20	0.74	20	0.74
Ethanol	12 (%v/v)	12 (%v/v)	12 (%v/v)	12 (%v/v)

Model wine I contains malic acid but no lactic acid with the reverse the case for model wine II. These model wine designations will be used throughout this sub-section. The positive and negative ion mode mass spectra for both solutions are shown in Figures 5.39 and 5.40 and characterised ions are shown in Table 5.16.

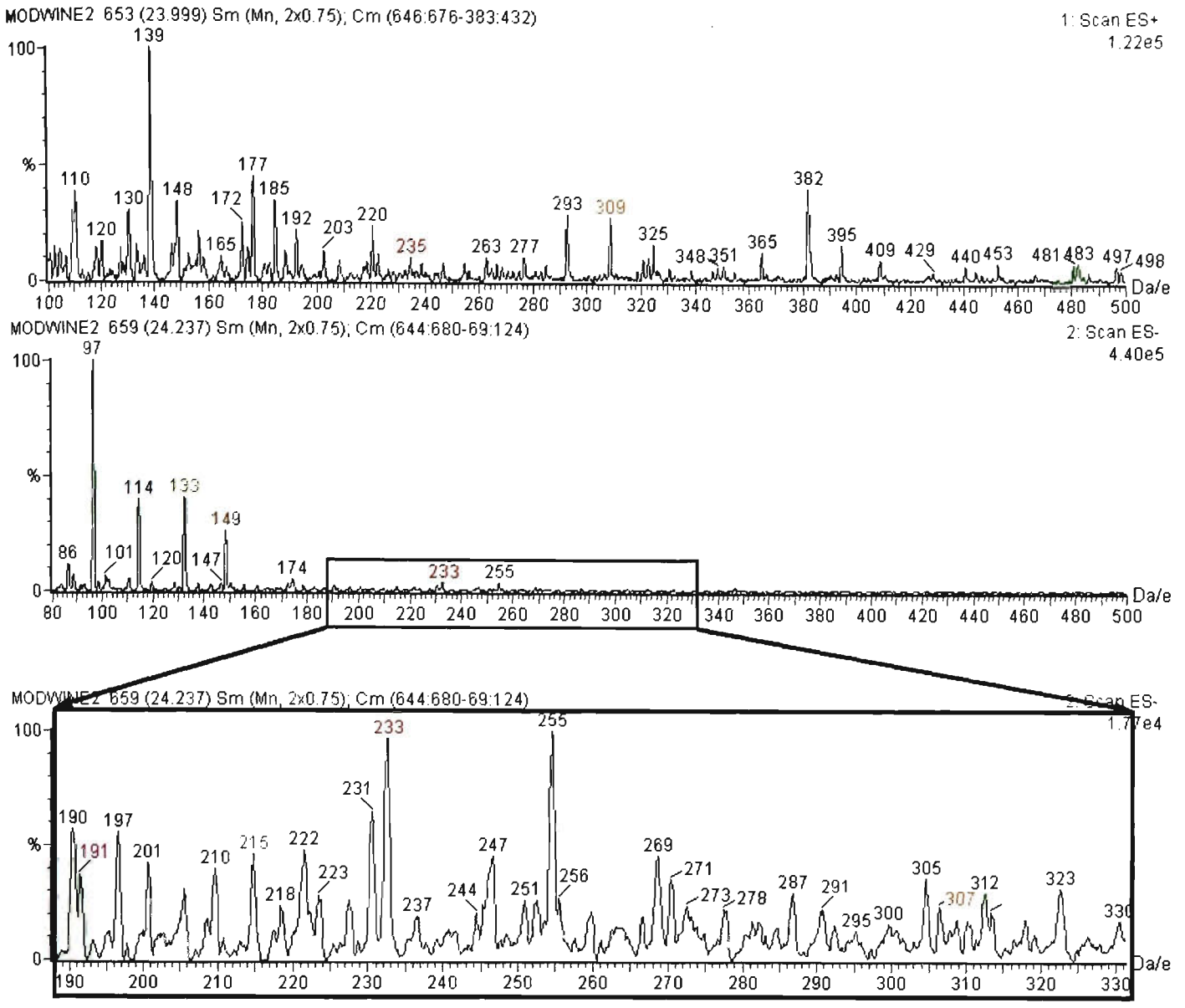


Figure 5.39 Positive and negative mode mass spectra of model wine I. The positive mode spectrum is topmost. Note: intensity scales are not the same.

The peaks characterised in Figure 5.39 represent the complexes that were previously observed in the single ligand solutions. Inspection of the positive mass spectrum shows that even though the concentration of citric acid is one tenth of that of tartaric or malic acid, the 235Da peak is still evident. This appears to be at the expense of the bimalate complexes, which show a relative decrease compared with the bis-bitartrate aluminium complex at 325Da. As discussed previously, this would be due to citric acid strongly complexing with aluminium. Unlike the aluminium citrate solution shown in Figure 5.38, the negative ion mode mass spectrum of model wine I shows some evidence of a $[Al(Hcit)(SO_4)]^-$ complex at 313Da, although its ion intensity is extremely low. The characterised complexes from Figure 5.39 are shown in Tables 5.16 and 5.17.

Table 5.16 Characterised complexes from model wine I featured in Figure 5.39.

Peak Ion Mass (Da)	Assigned Species
ES Positive Ion Mode	
235	$[Al(Hcit)(H_2O)]^+$
293	$[Al(Hmal)_2]^+$
309	$[Al(Hmal)(Htart)]^+$
325	$[Al(Htart)_2]^+$
ES Negative Ion Mode	
97	$[HSO_4]^-$
133	$[Hmal]^-$
149	$[Htart]^-$
191	$[H_2cit]^-$
215	$[Al(H_{-1}cit)]^-$
233	$[Al(H_{-1}cit)(H_2O)]^-$
255	$[Al(mal)(SO_4)]^-$
271	$[Al(tart)(SO_4)]^-$
291	$[Al(mal)_2]^-$
307	$[Al(mal)(tart)]^-$
313	$[Al(Hcit)(SO_4)]^-$
323	$[Al(tart)_2]^-$

The previously assigned ions at 235Da, 293Da, 309Da and 325Da are clearly observed in Figure 5.39, but there was no evidence of $[Al(Hcit)]^+$ at 217Da. The pattern of 'conjugate' complexes similar to those in the positive ion mode less the mass of two protons was again observed in the negative ion mode, although at a much lower ion intensity than their positive ion mode counterparts. Along with $[H_2cit]^-$ at 191Da, a $[Al(H_{-1}cit)]^-$ complex at 215Da was characterised in the negative ion mode but its counterpart was not observed in the positive ion mode. However, these assignments are tentative as their ion intensities are extremely low. In the case of the 191Da peak, its ion intensity is insignificant when compared with the ion intensities of bimalate and bitartrate. This supports the observations described in Sub-Section 5.4.5 for a 100:20 citric acid/aluminium solution which suggested virtually complete complexation of the available citric acid with aluminium. In widening

the m/z range in the negative ion mode down to 80Da, bisulfate from the aluminium standard was also identified at 97Da.

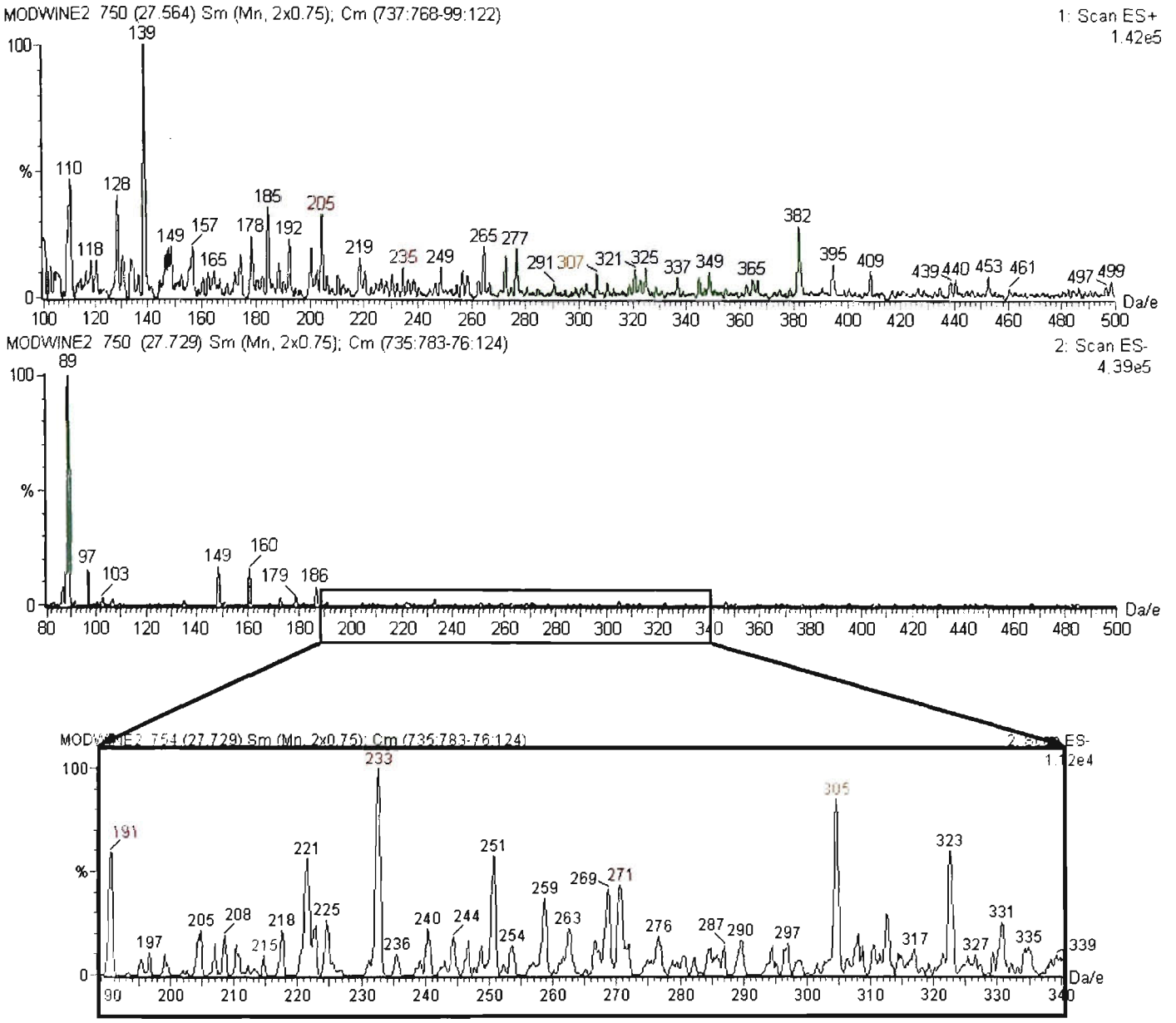


Figure 5.40 Positive and negative mode mass spectra of model wine II. The positive mode spectrum is topmost. Note: intensity scales are not the same.

The mass spectra of the model wine II also demonstrated the same complexes as previously observed in single ligand solutions and additional mixed complexes with lactic acid were also identified. These mixed complexes were manifest in both positive and negative ion mode spectra, with the latter showing 'conjugate' complexes with the same total mass less two protons as previously noted for other aluminium species with organic acids. The characterised complexes from Figure 5.40 are shown in Table 5.17.

Table 5.17 Characterised complexes from model wine II featured in Figure 5.41.

Peak Ion Mass (Da)	Assigned Species
ES Positive Ion Mode	
205	$[Al(lac)_2]^+$
235	$[Al(Hcit)(H_2O)]^+$
265	$[Al(lac)(Htart)]^+$
307	$[Al(lac)(H_2cit)]^+$
325	$[Al(Htart)_2]^+$
ES Negative Ion Mode	
89	$[lac]^-$
97	$[HSO_4]^-$
149	$[Htart]^-$
191	$[H_2cit]^-$
215	$[Al(H_{-1}cit)]^-$
233	$[Al(cit)(OH)]^-$
263	$[Al(lac)(H_{-1}tart)]^-$ or $[Al(H_{-1}lac)(tart)]^-$
271	$[Al(tart)(SO_4)]^-$
305	$[Al(lac)(cit)]^-$ or $[Al(H_{-1}lac)(Hcit)]^-$
313	$[Al(Hcit)(SO_4)]^-$
323	$[Al(tart)_2]^-$

Unlike the solution of lactic acid and aluminium, there was no evidence of the $[Al(H_{-1}lac)_2]^-$ in the negative ion mode mass spectrum of model wine II. However a $[Al(H_{-1}cit)]^-$ complex at 215Da was again observed with no 'conjugate' peak present in the positive ion mode mass spectrum. Once again, the ion intensities of the negative mode peaks at 215Da and 263Da were extremely low making assignments of these peaks tentative.

In the absence of malic acid the relative ion intensities of the other organic acid/aluminium species change in the positive ion mode, with $[Al(lac)_2]^+$ dominating in positive ion mode followed by $[Al(lac)(Htart)]^+$. In the negative ion mode, although the $[Al(cit)(OH)]^-$ complex still dominates, it is closely followed by $[Al(lac)(cit)]^-$ and even the intensity of $[Al(tart)_2]^-$ is significantly improved. The strong intensity of complexes with lactic acid in the positive mode is probably due to the higher

molar concentration compared with tartaric and citric acids. The absence of mixed complexes of citric acid with malic or tartaric acids could suggest the role of ligand size in co-complexing of aluminium by the organic acids. The smaller lactate ligand could allow other larger organic acids to bind to aluminium, while the citrato ligand is sufficiently large and flexible to exclude tartaric and malic acids.

5.5 ES-MS Speciation Investigation of Bottled Table Wine

It has already been discussed throughout this chapter that in analysing wine two modifying factors would be employed to enhance aluminium species detection. One was that due to the large disparity in the concentration of the aluminium and its possible ligands in a wine sample, a spike of aluminium would be required to give measurable peaks for the various complex ions. This would form the base from which an extrapolation could be attempted to discern the species distribution as close as possible to the actual aluminium concentration found in wine. The second was that in order to avoid overloading the ES-MS system, wine would have to be diluted by 20-40 fold. By diluting the wine with a 12% v/v ethanol solution the starting matrix would be matched as closely as possible and the buffering capacity of the organic acids in the wine could maintain the pH and as a consequence the aluminium species distribution as close to that of the original solution. The wines employed for the speciation work were opened just prior to the speciation analysis to avoid any changes in chemistry of the wine due to oxidation and spoilage.

5.5.1 Organic Acids in Wine and the Dilution of Wine

As the requirement for identifiable peaks without overloading the system were the two most critical factors in ES-MS wine speciation investigations, experiments were conducted to determine the most appropriate dilution factor for the wines used in this study. A standard containing 100mg/L each of lactic, succinic, malic, tartaric and citric acids in 12% ethanol was analysed by negative ion mode ES-MS and the mass spectrum compared with that of a 35-fold diluted white wine shown in Figure 5.41.

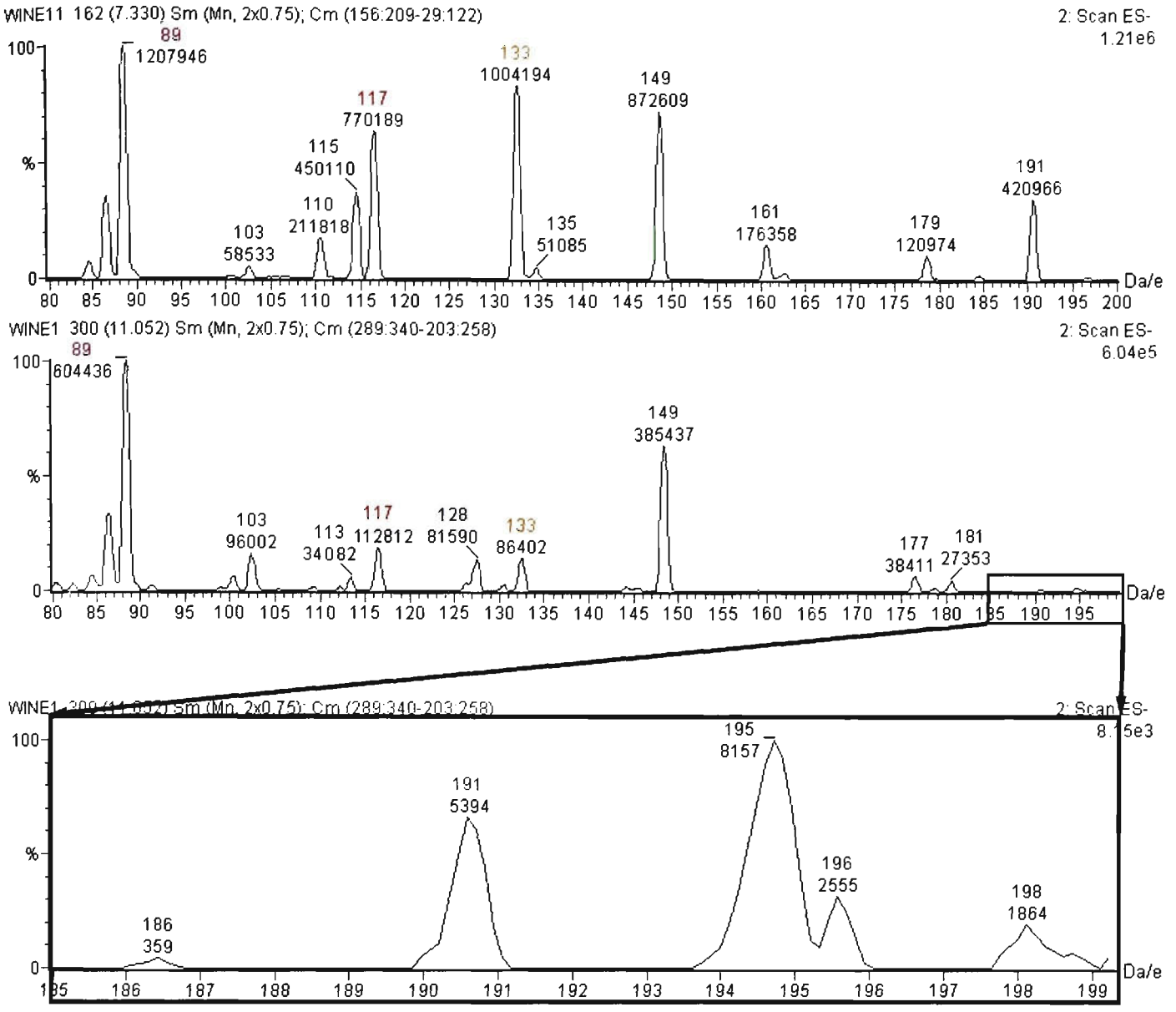


Figure 5.41 Mass spectral comparison of a solution of 100mg/L each of lactic, succinic, malic, tartaric and citric acids in 12% v/v ethanol with a 35-fold diluted white wine in the same matrix. The solution's organic acid anion peaks are at 89, 117, 133, 149 and 191 Daltons respectively. Note: intensity scales are not the same between mass spectra.

Figure 5.41 shows that the intensities of the organic acids in the white wine sample examined after a 35-fold dilution are significantly less than those observed in the standard organic acid solution of 100mg/L in 12% ethanol suggesting that the initial concentrations in the wine were less than that estimated from the data of Rankine (1991). As the preliminary work showed that the instrument could accommodate 100mg/L of any of the organic acids without overloading, a 20-fold diluted wine was analysed in the negative ion mode and the ion intensities compared with the 100mg/L organic acids standard shown in Figure 5.42.

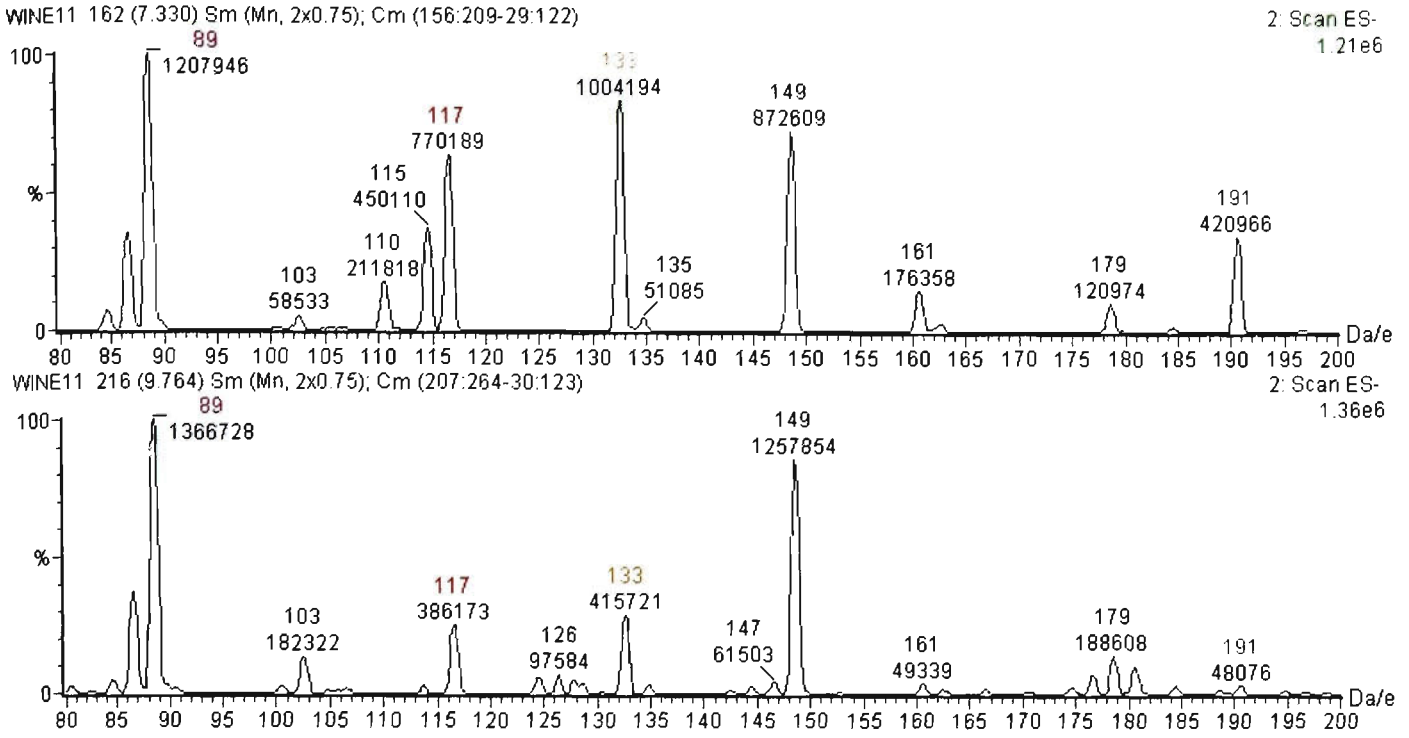


Figure 5.42 Mass spectral comparison of a solution of 100mg/L each of lactic, succinic, malic, tartaric and citric acids in 12% v/v ethanol with a 20 fold diluted white wine in the same matrix. The solution's organic acid anion peaks are at 89, 117, 133, 149 and 191 Daltons respectively. Note: intensity scales are not the same between mass spectra.

Figure 5.42 demonstrates that the 20-fold dilution virtually matches the concentrations of tartaric and lactic acid in both the standard and the wine sample. From this experiment a 20-fold dilution of wine was shown to be a reasonable compromise between delivering a measurable signal without overloading the system with the advantages of reduced dilution further minimising pH changes and possible species redistribution. This dilution was used for the remainder of the study.

Inspection of the mass spectrum of the organic acids standard demonstrates that the various acids do not respond equally in ES-MS. Since MS responds to ion count, the comparisons of intensities have to be based on mole quantities. The absolute ion intensities were converted to relative response for each acid per mM. The highest acid response was set to unity and each relative response was converted into a proportion relative to this response to give an individual anion (mM) response factor. The results are shown in Figure 5.43.

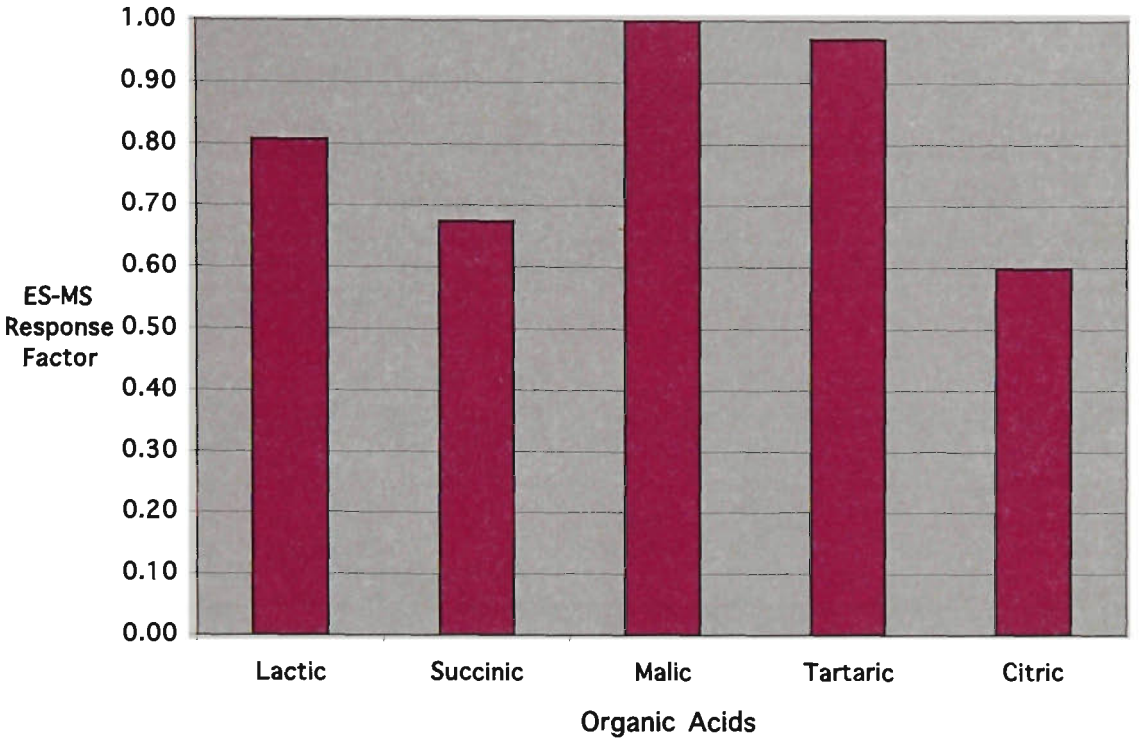


Figure 5.43 ES-MS response factors of the organic acids from an average mixed standard in 12% ethanol for mM solution concentrations. The response factor of each acid is set against that of the organic acid showing the highest response, which is set at unity.

Figure 5.43 shows there are differences in response factors for each organic acid. To determine ion concentrations, each ion's intensity is divided by its molar response factor. Using this method the ion intensities can be directly compared while taking the individual ion's electrospray response into account. Hence, comparisons of ion intensities for the remainder of this chapter are shown after the relative anion response is applied.

Figure 5.44 compares the mass spectrum of the white wine shown in Figure 5.42 with that of the red wine also investigated in this speciation study.

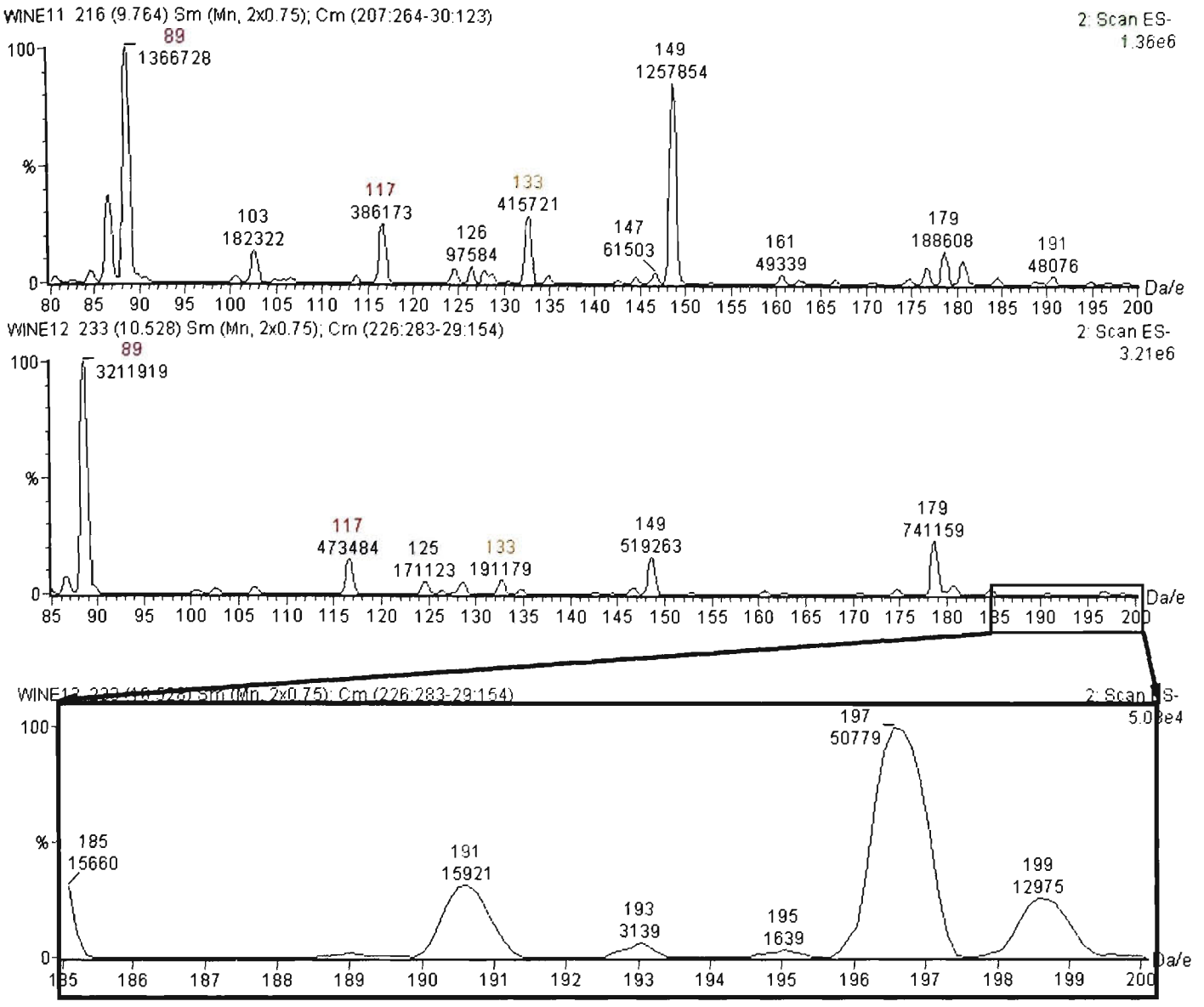


Figure 5.44 Negative ion mode mass spectra of a 20-fold diluted white wine (top) compared with a 20-fold diluted red wine (bottom two) showing the monovalent anion of the organic acids, lactic, succinic, malic, tartaric and citric acids at 89, 117, 133, 149 and 191 Daltons respectively. Note: intensity scales are not the same.

Figure 5.44 demonstrates the significant presence of lactic acid (89Da) in both wines suggesting the wines have undergone malo-lactic fermentation, however not all of the malic acid has been converted. The significant lactic acid content derived from malo-lactic fermentation is expected for the red wine. In fact, about two thirds of red wines produced undergo this process (Rankine 1991). Despite the white wine sampling notes showing no evidence of production driven malo-lactic fermentation the significant presence of lactic acid suggests this wine has undergone a partial malo-lactic fermentation process. In the white wine, one can see that tartaric acid (149Da) and lactic acid (89Da) make up the majority of the organic acid content while malic (133Da) and succinic acids (117Da) are present in moderate amounts with citric acid (191Da) present only at trace concentrations. In the red wine the lactic acid (89Da) makes up the overwhelming proportion of the organic acid content, the intensity of the tartaric, malic and citric acid is half that in the white wine,

while that of succinic acid has doubled to almost equal the intensity of tartaric acid. These observations demonstrate the large variation in the five wine organic acid components, indicating that the concentration estimates of the model wine can be at best estimates only, particularly as the concentration response can be non-linear for ES-MS and extremely dependent on matrix composition (Barnett et al. 2000). Using the organic acid response factor, an estimation of the concentrations of the free organic acids and their monovalent anions can be determined. These concentrations are listed in Table 5.18.

Table 5.18 Approximate concentration of major organic acid/anions in mM for white wine L and red wine F used in the wine speciation study. The concentration ranges suggested by Rankine (1991) are included for comparative purposes.

Organic Acid	White Wine (mM)	Red Wine (mM)	Typical Range (mM)
Lactic	25	59	4.4-33.3
Succinic	8.5	10	4.2-12.7
Malic	6.2	2.8	Trace-37.3
Tartaric	19	7.9	13.3-33.3
Citric	1.2	0.4	Trace-5.2

Table 5.18 demonstrates that the white and red wine organic acid concentrations measured by ES-MS are appropriate when compared with the concentration ranges given by Rankine (1991), except for lactic and tartaric acids in the red wine which were above and below the typical concentration range respectively. These concentrations are approximate and the sensitivity of the instrument can vary between discrete ES-MS sequences. Because of this, concentration estimations carried out in later work were conducted by comparing the ions in a diluted wine with those of a 100mg/L organic acid standard that was made on the same day and analysed in the same sequence as the sample on the ES-MS.

Table 5.19 Characterised complexes presented in Sub-Section 5.5.1.

Peak Ion Mass (Da)	Assigned Species
ES Negative Ion Mode	
89	$[lac]^-$
117	$[Hsuc]^-$
133	$[Hmal]^-$
149	$[Htart]^-$
191	$[H_2cit]^-$

5.5.2 Attempted Aluminium Speciation of Wine Using ES-MS Directly

A 20-fold diluted white wine (sample L) and red wine (sample F) were analysed in negative and positive ion mode ES-MS using identical conditions employed for the model wine solutions. Additional samples spiked with 0.74mM aluminium potassium sulfate were also analysed. The resultant mass spectra covering m/z 200-330/40 are shown in Figures 5.45, 5.46, 5.47 and 5.48.

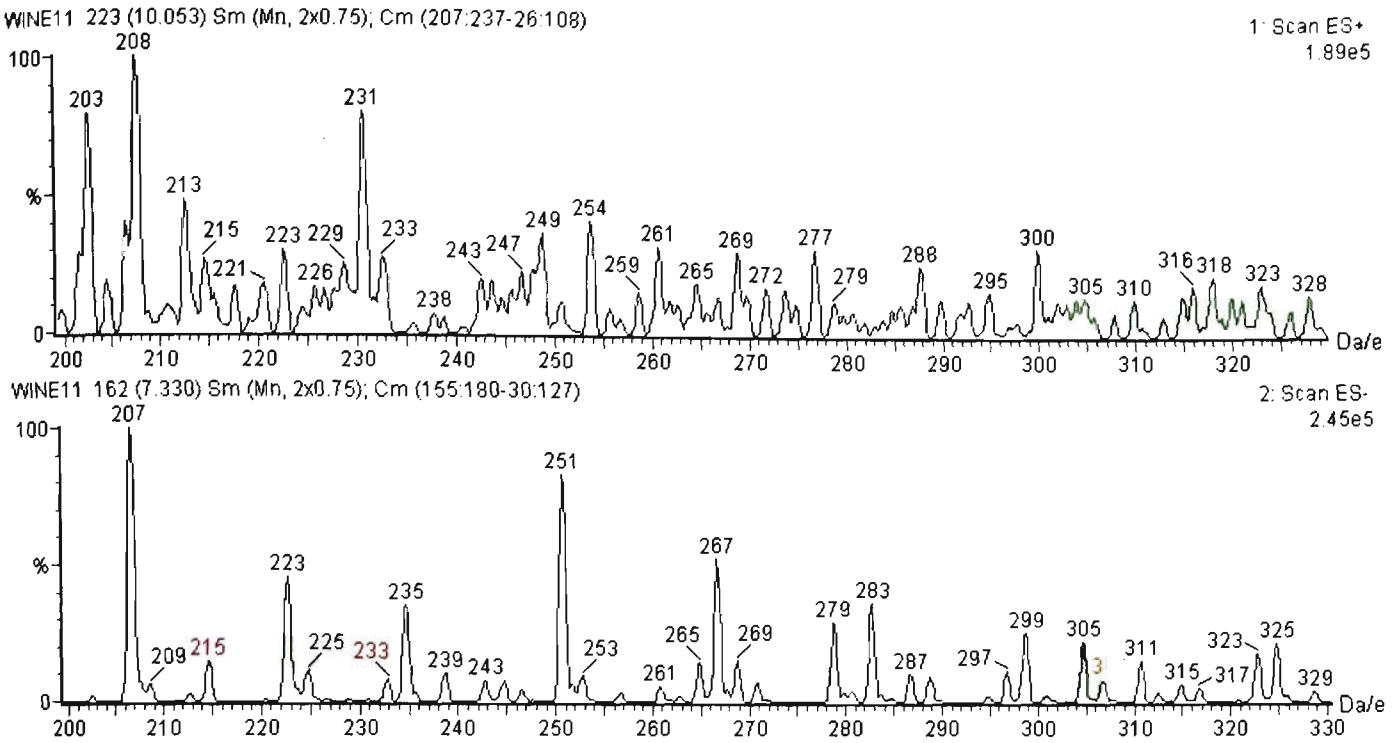


Figure 5.45 Mass spectra of 20-fold diluted white wine L in positive (top) and negative (bottom) electrospray mode. Note: intensity scales are not the same.

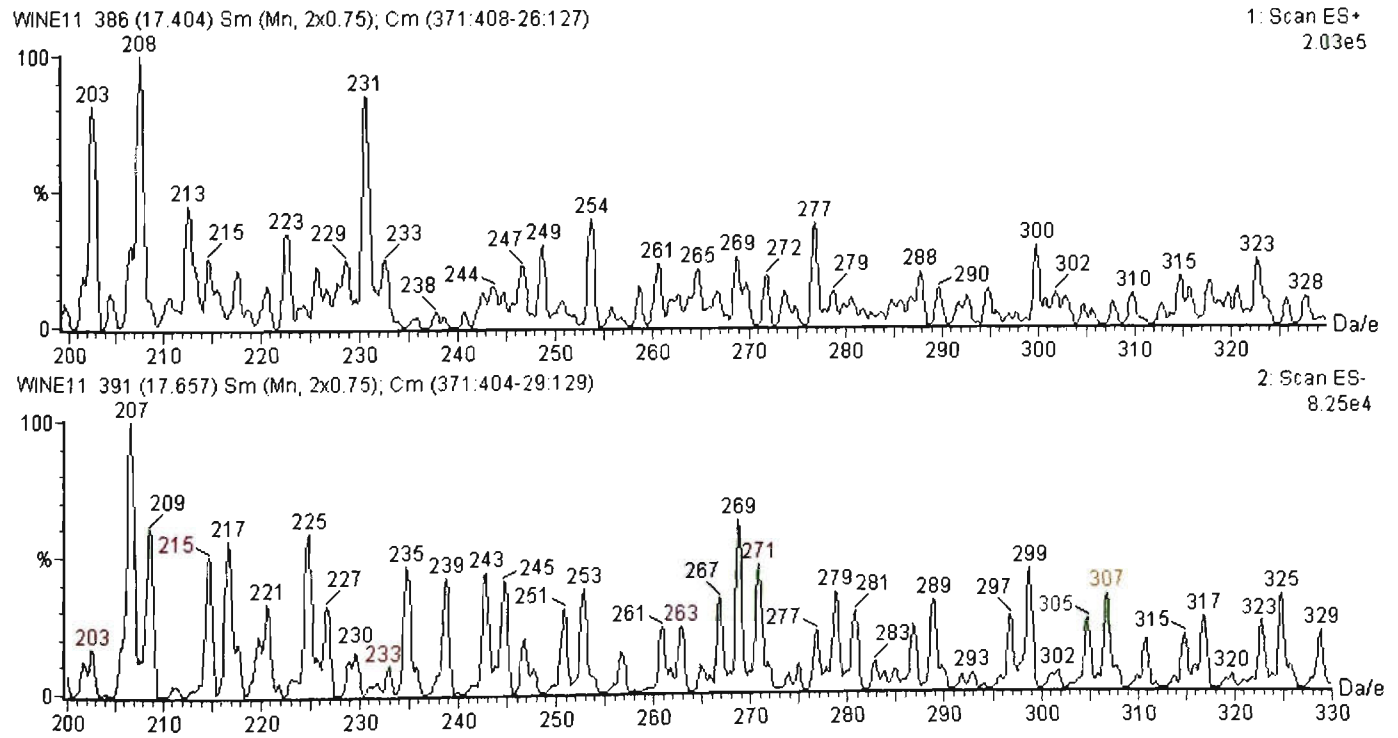


Figure 5.46 Mass spectra of 20-fold diluted white wine L spiked with 20mg/L aluminium in positive (top) and negative (bottom) electrospray mode. Note: intensity scales are not the same.

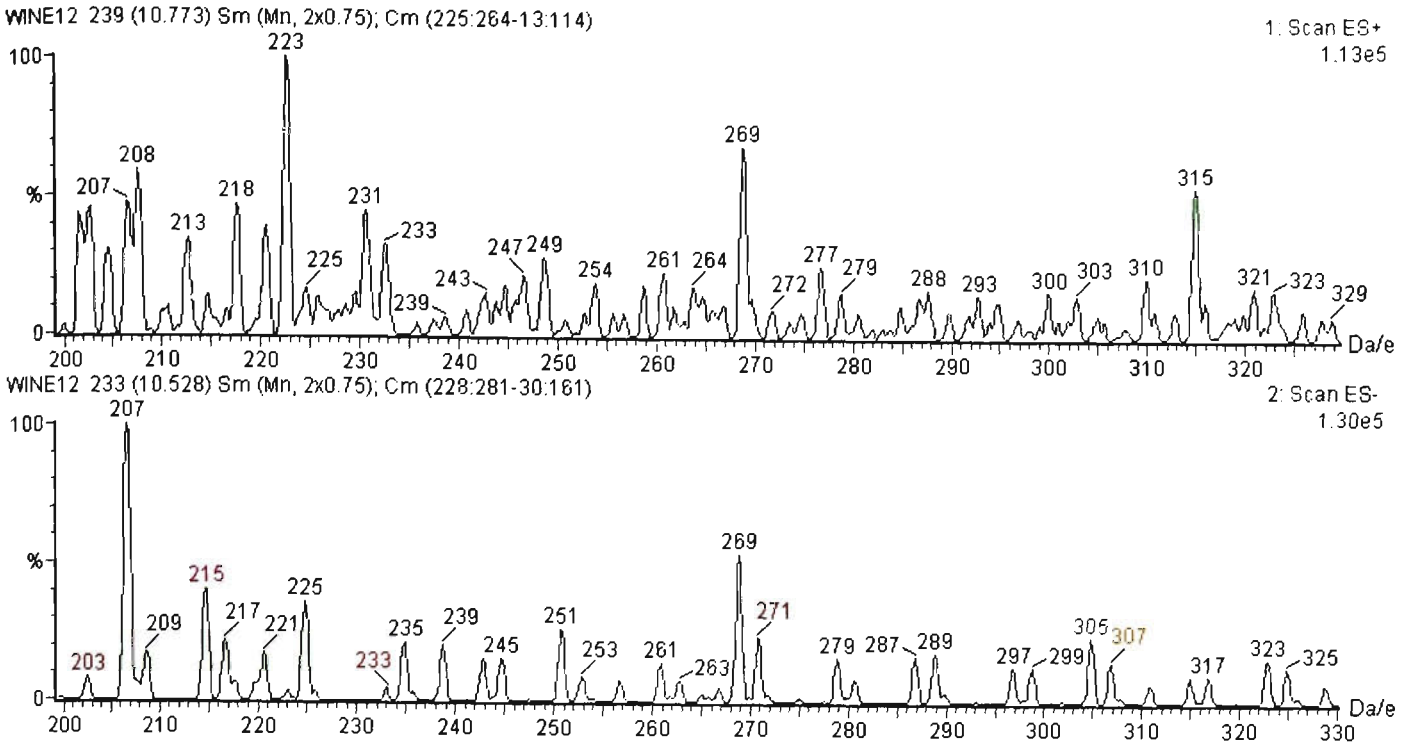


Figure 5.47 Mass spectra of 20-fold diluted red wine F in positive (top) and negative (bottom) electrospray mode. Note: intensity scales are not the same.

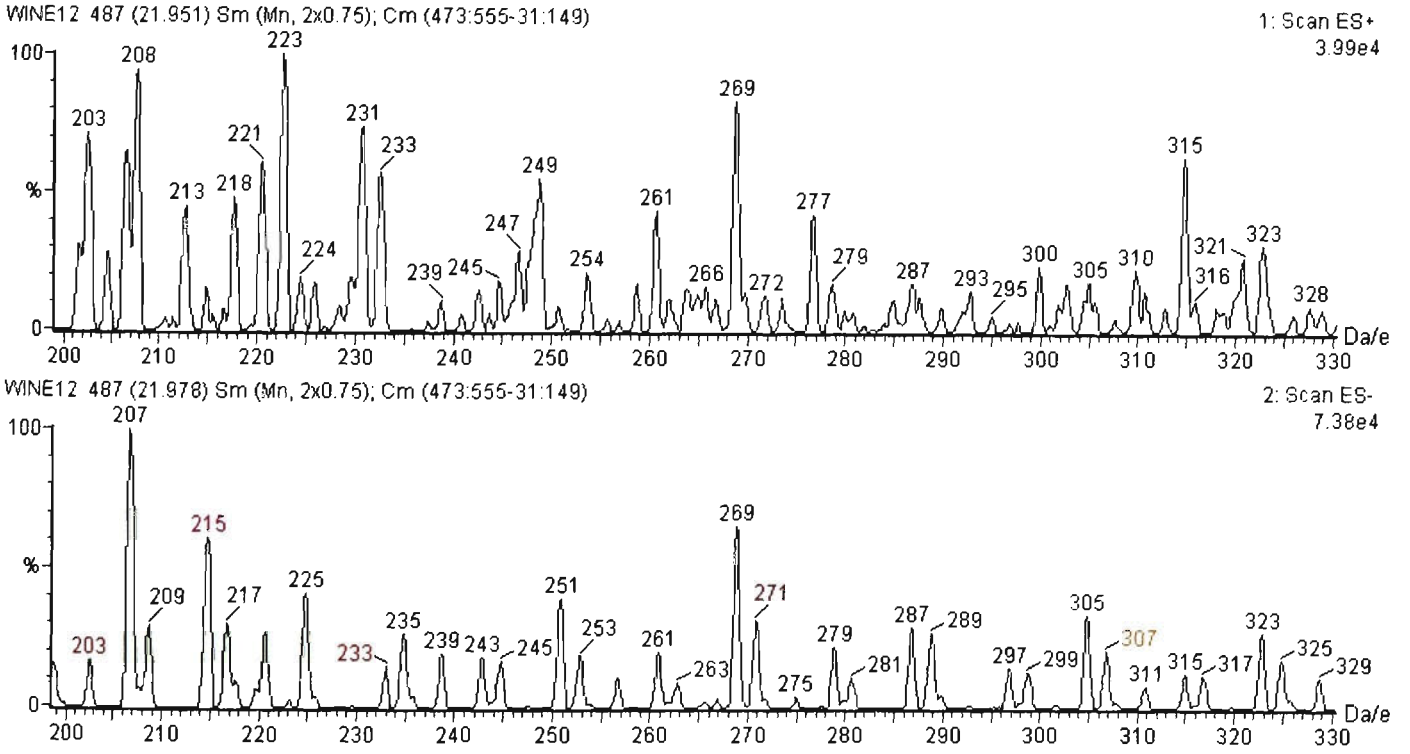


Figure 5.48 Mass spectra of 20-fold diluted red wine F spiked with 20mg/L aluminium in positive (top) and negative (bottom) electrospray mode. Note: intensity scales are not the same.

The most significant result from this experiment was that the positive mass spectra of both the diluted wine sample and the spiked wine sample showed no evidence of the aluminium complex ion peaks that were evident in the model wine and aqueous solutions. A peak at 261Da could possibly be due to an aluminium bis-bisuccinate species, but as this ligand was not included in the earlier

work this cannot be substantiated. Unlike the positive mode spectra, the negative ion mode mass spectra showed some evidence of the aluminium species observed in model wine and aqueous solutions, even though their intensity was low. However, the peaks agree with those observed in the negative ion mode for model wine and aqueous solutions. The low intensities seen in the negative ion mode for all solution matrices has been attributed to the difficulty in forming these ions in the negative ES-MS process. However, the lower negative ion mode peak intensities and the lack of identifiable ions in the positive ion mode for diluted wine matrices suggest that the wine matrix is additionally hindering ES-MS ion formation mechanisms. The peaks with tentative characterisations are presented in Table 5.20.

Table 5.20 Characterised complexes from 20-fold diluted wines and spiked wines featured in Figures 5.46-5.49.

Peak Ion Mass (Da)	Assigned Species
ES Positive Ion Mode	
No ions characterised	
ES Negative Ion Mode	
203	$[Al(H_{-1}lac)]^-$
215	$[Al(H_{-1}cit)]^-$
233	$[Al(cit)(OH)]^-$
263	$[Al(lac)(H_{-1}tart)]^-$ or $[Al(H_{-1}lac)(tart)]^-$
271	$[Al(tart)(SO_4)]^-$
305	$[Al(lac)(cit)]^-$ or $[Al(H_{-1}lac)(Hcit)]^-$
307	$[Al(mal)(tart)]^-$
323	$[Al(tart)_2]^-$

The complexes with peaks at 305Da, 307Da and 323Da were significant in the diluted wine and did not notably change after the addition of aluminium. The peaks at 215Da, 233Da and 263Da, though small, were present in diluted wine and increased after spiking with aluminium. The peak at 203Da was only significant with higher spikes of aluminium above 20mg/L; hence in an unspiked, un-diluted wine this complex would be insignificant. A peak at 271Da corresponding with the species $[Al(tart)(SO_4)]^-$ was observed in diluted wine F and its spiked counterpart. However, this peak was not found in wine L but is clearly evident in the spiked wine L. Due to this disparity in its

appearance in both wines, it is inconclusive as to whether $[Al(tart)(SO_4)]^-$ is found in wine or is a product of the spike with aluminium potassium sulfate.

The absence of identifiable complexes in the positive mode is difficult to explain; especially as these complexes had much higher intensities than their negative ion counterparts in model wine and aqueous media. The absence of these ions in the positive ion mode will be discussed and conclusions drawn in Chapter 6.

5.5.3 Aluminium Ligand Binding Analysis Using an Indirect ES-MS Method

As the direct analysis of aluminium complexes in wine by ES-MS was found to be difficult, another approach was required to determine the dominant aluminium-binding ligand in wine. It has been shown that the organic acids could be determined in the negative ion mode ES-MS and gave extremely high ion intensities in dilute wine solutions (see Figure 5.41). Assuming ion intensity is proportional to the available organic acid anion concentration and that dissociation of organic acid/aluminium species by ES-MS is small, a decrease in the organic acid anion intensity should be observed when aluminium is added to wine if complexation of aluminium by organic acid ligands does occur. Comparisons of the negative ion mode mass spectra of both white and red wine before and after spiking with 0.74mM (20mg/L) aluminium are shown in Figures 5.49 and 5.50.

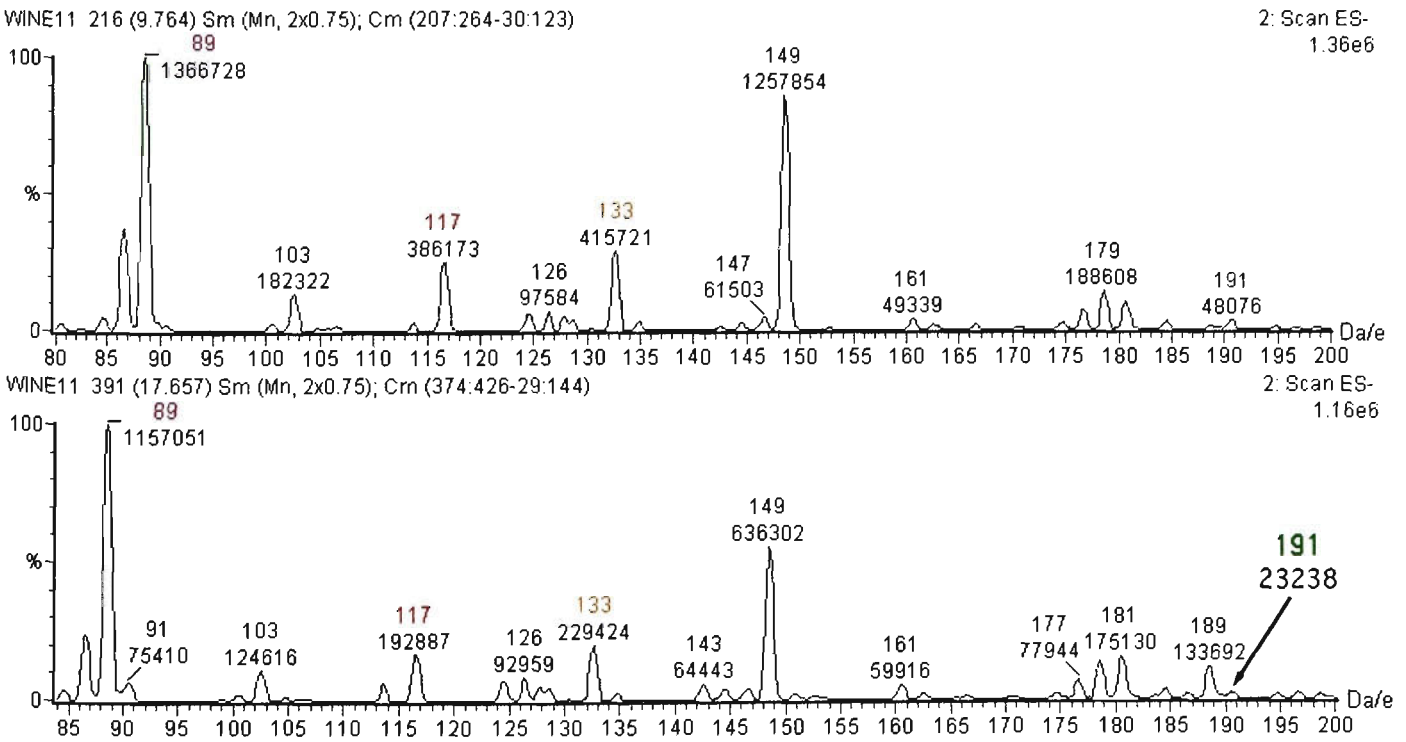


Figure 5.49 Comparison of negative ion mode mass spectra of 20-fold diluted white wine L (top) with the same solution spiked with 20mg/L aluminium (bottom).

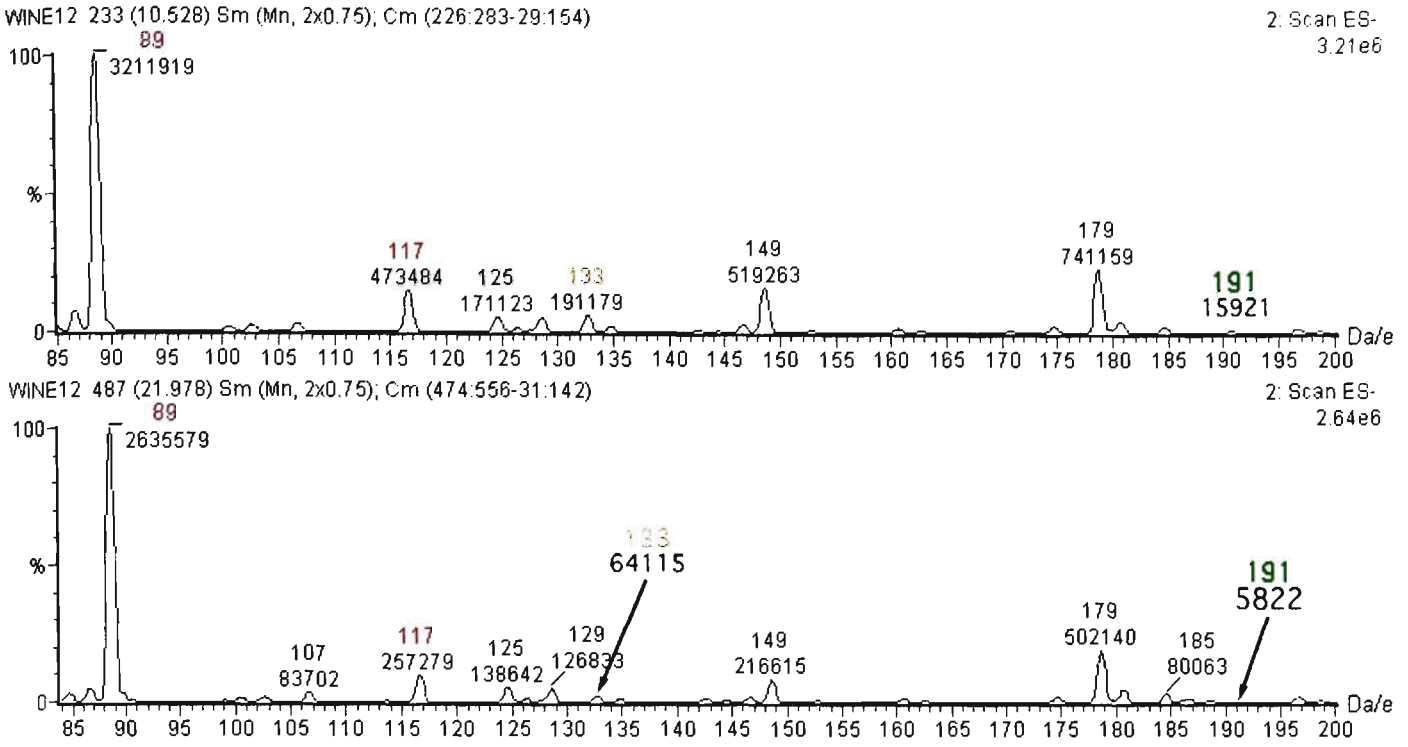


Figure 5.50 Comparison of negative ion mode mass spectra of 20-fold diluted red wine F (top) with the same solution spiked with 20mg/L aluminium (bottom).

For both white and red wine, a decrease in organic acid ion intensity after the addition of aluminium to the solution is evident (see Figures 5.49 & 5.50). In both wines, the greatest decrease is observed for tartaric acid at 149Da, although in the red wine this decrease is not as significant compared with other organic acids as it is in the white wine. These results suggest that free organic acids in the wines are being complexed by the aluminium, although this relationship is difficult to confirm based on only two sets of data. Thus, a more extensive experiment was conducted where diluted wine solutions were spiked with progressively more concentrated aluminium up to 7.4mM for both red and white wines and the organic acid anions intensities were measured using negative ion mode ES-MS. A plot was then produced showing the trend of the organic acid ion intensities versus the aluminium spike concentration in mM; these plots for white and red wine are shown in Figures 5.51 and 5.52.

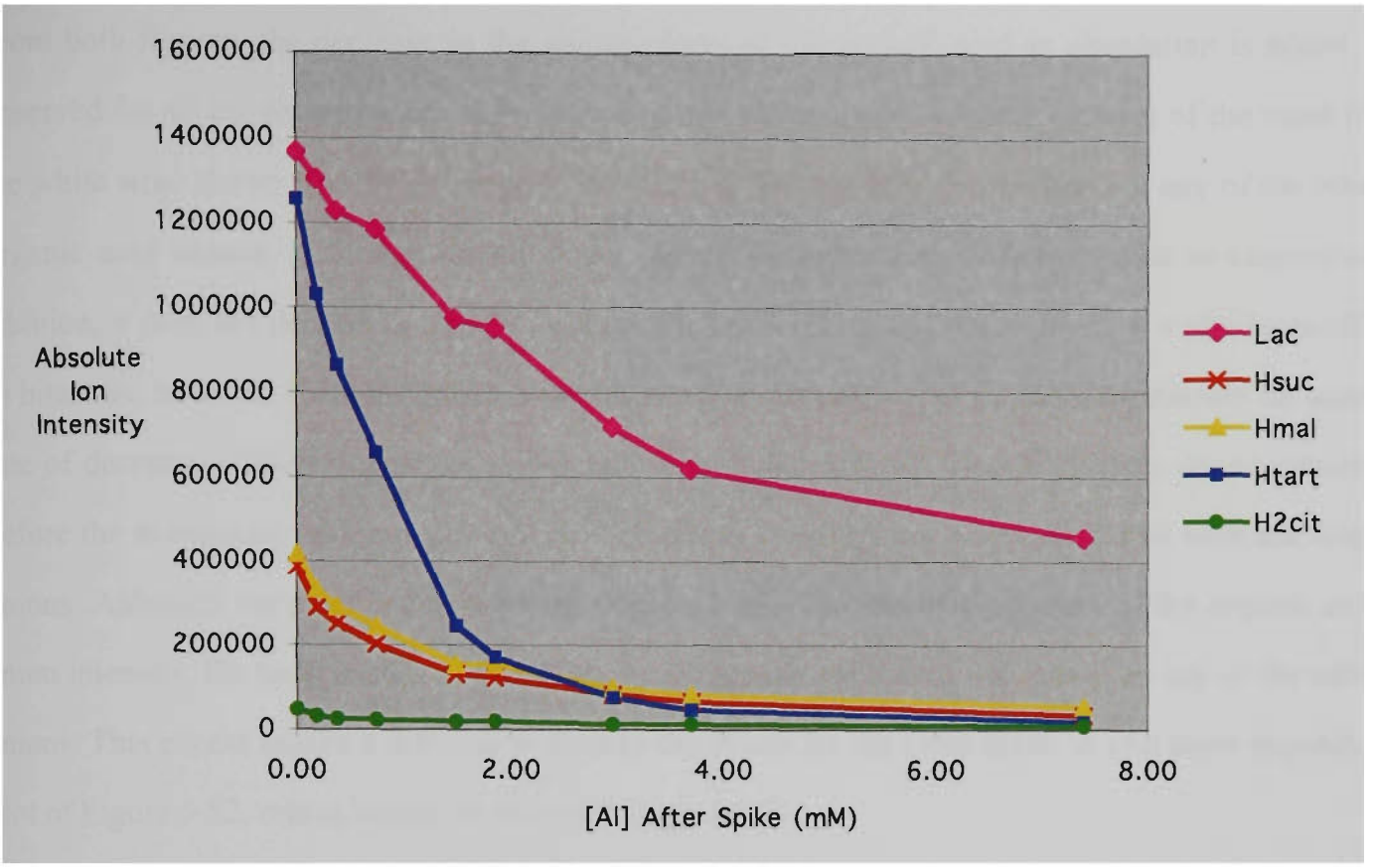


Figure 5.51 Plot of ion intensity of white wine L against concentration of solution aluminium spike in mM.

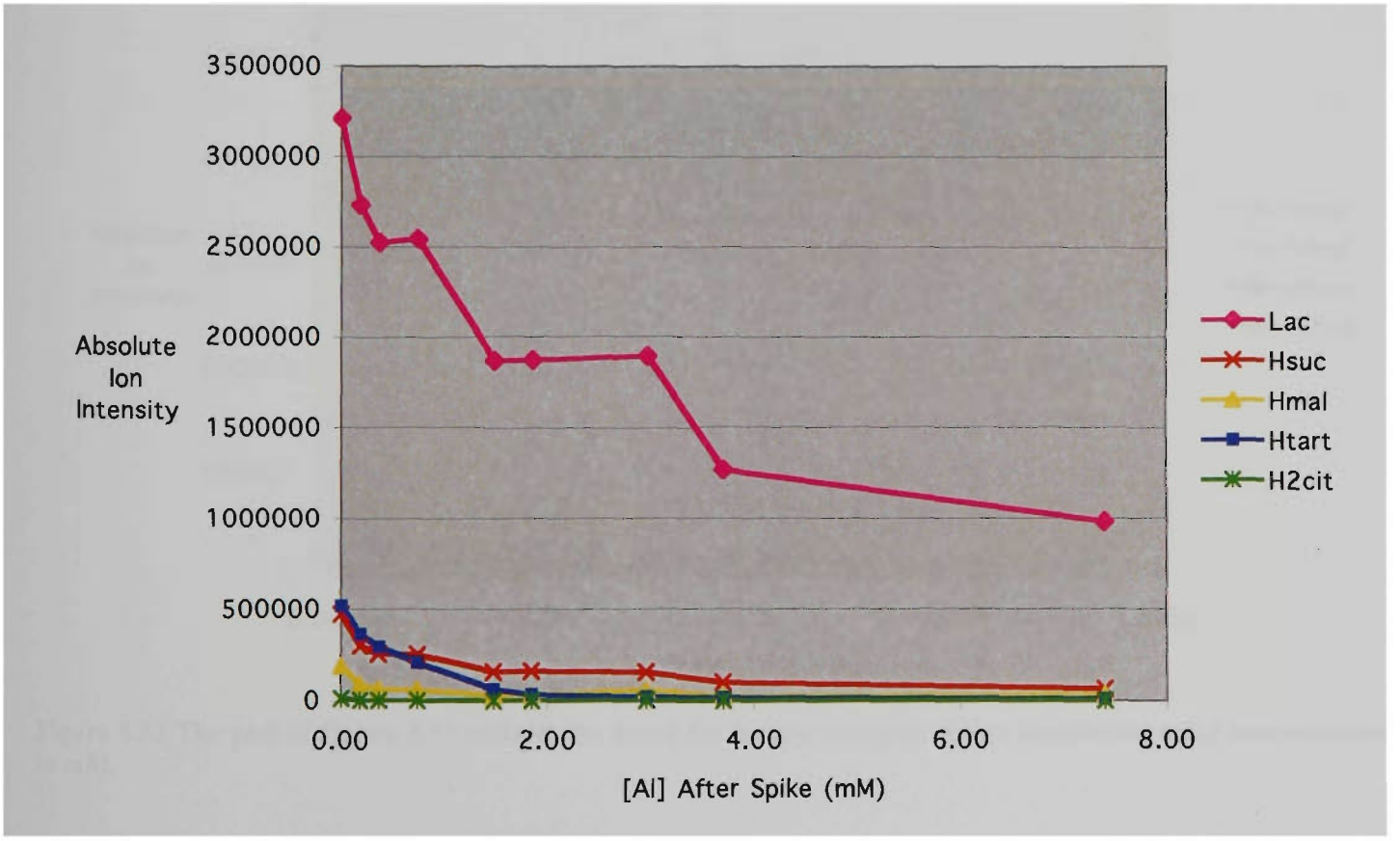


Figure 5.52 Plot of ion intensity of red wine F against concentration of solution aluminium spike in mM.

From both figures, the decrease in the anionic form of the organic acid as aluminium is added is observed for all the organic acids in both the red and white wine. Closer inspection of the trend for the white wine shows that the decrease in bitartrate is significantly greater than for any of the other organic acid anions. Although lactate has a higher intensity than bitartrate prior to aluminium addition, it does not decline as rapidly as bitartrate. Bisuccinate and bimalate show a similar profile to bitartrate however exhibit a much lower starting ion intensity and do not demonstrate the same rate of decrease in their intensities as aluminium is added. $[H_2cit]^-$, already at trace concentrations before the aluminium spiking, shows a decline that is insignificant when compared with the other anions. Although the plot for the red wine (Figure 5.52) also shows a decrease in the organic acid anion intensity, the huge excess of lactate sees a far greater decline in intensity than any of the other anions. This excess makes it difficult to discern the trends for the other acids. A plot scale expanded plot of Figure 5.52, minus lactate, is shown in Figure 5.53.

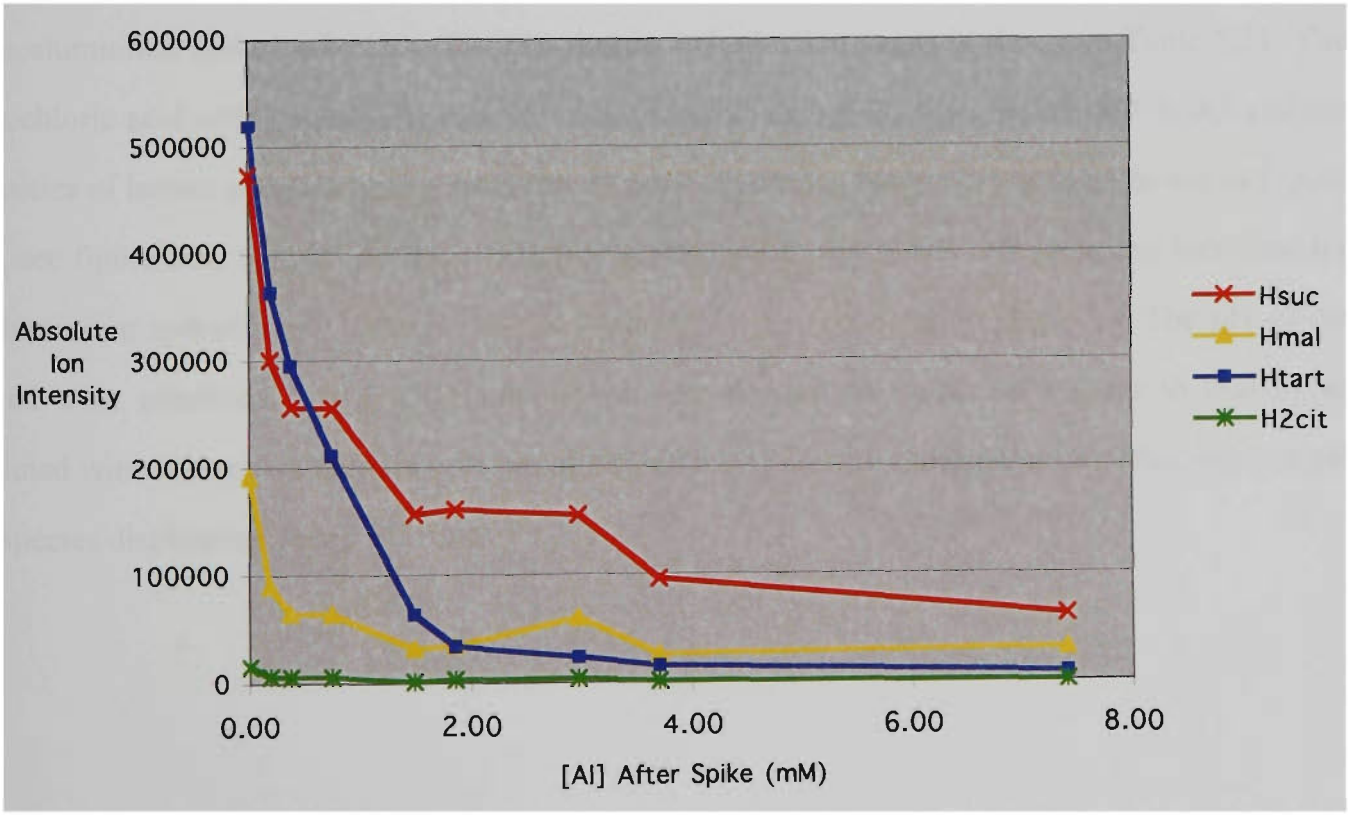


Figure 5.53 The plot of Figure 5.52 without the trend for lactate intensity versus aluminium spike concentration in mM.

Figure 5.53 shows that the ion intensity trend of the bitartrate in red wine is similar to that observed in Figure 5.51 for the white wine. However, the bisuccinate trend is very similar to the bitartrate trend in the red wine, with a similar starting intensity. This anion, along with bitartrate and bimalate,

show a rapid decrease in intensity with the first 10mg/L of aluminium added. $[H_2cit]^-$ again demonstrates insignificant change, primarily due to its very low concentration in the diluted wine.

There are two explanations for the decrease in the organic acid intensities as the aluminium concentration is increased: either these anions are forming complexes with aluminium (something that earlier work suggests is highly probable) or a change in solution chemistry is affecting the electrospray of these anions in the negative ion mode. Other than a change in the electrolyte concentrations, the other major factor known to influence the efficiency of the electrospray is pH. The former has not been shown to affect the organic acid anion intensity in the preliminary work to the same extent as described in Figures 5.51-5.53. However, in the same preliminary study, changes in pH have been demonstrated to affect ion intensity significantly. Hence, an experiment was devised where the pH of freshly prepared aluminium spiked diluted wine solutions was measured and a parallel set of diluted wine samples were treated with dilute HCl until their pH matched those of the aluminium spiked solutions. The aluminium spiked solution pH is shown in Table 5.21. The hydrochloric acid added solutions were subjected to analysis by negative ion mode ES-MS and the intensities of lactate and bitartrate were plotted against hydrogen ion concentration (shown in Figure 5.54, see figure note for plot details). This plot superimposes the trends of lactate and bitartrate for an aluminium spiked white wine shown in Figure 5.51 for comparative purposes. The pH of the diluted wine solutions, particularly after the lower aluminium spikes, is similar to that of an undiluted wine and means that the samples analysed by ES-MS are representative of the solution pH and species distribution found in wine.

Table 5.21 The pH of aluminium spiked diluted wine samples.

Solution Type	Solution pH
Undiluted Wine	3.17
20-Fold Diluted Wine	3.48
20-Fold Diluted Wine with 0.19mM Al	3.30
20-Fold Diluted Wine with 0.37mM Al	3.18
20-Fold Diluted Wine with 0.74mM Al	3.04
20-Fold Diluted Wine with 1.5mM Al	2.92
20-Fold Diluted Wine with 1.9mM Al	2.91
20-Fold Diluted Wine with 3.0mM Al	2.88
20-Fold Diluted Wine with 3.70mM Al	2.87
20-Fold Diluted Wine with 7.40mM Al	2.86

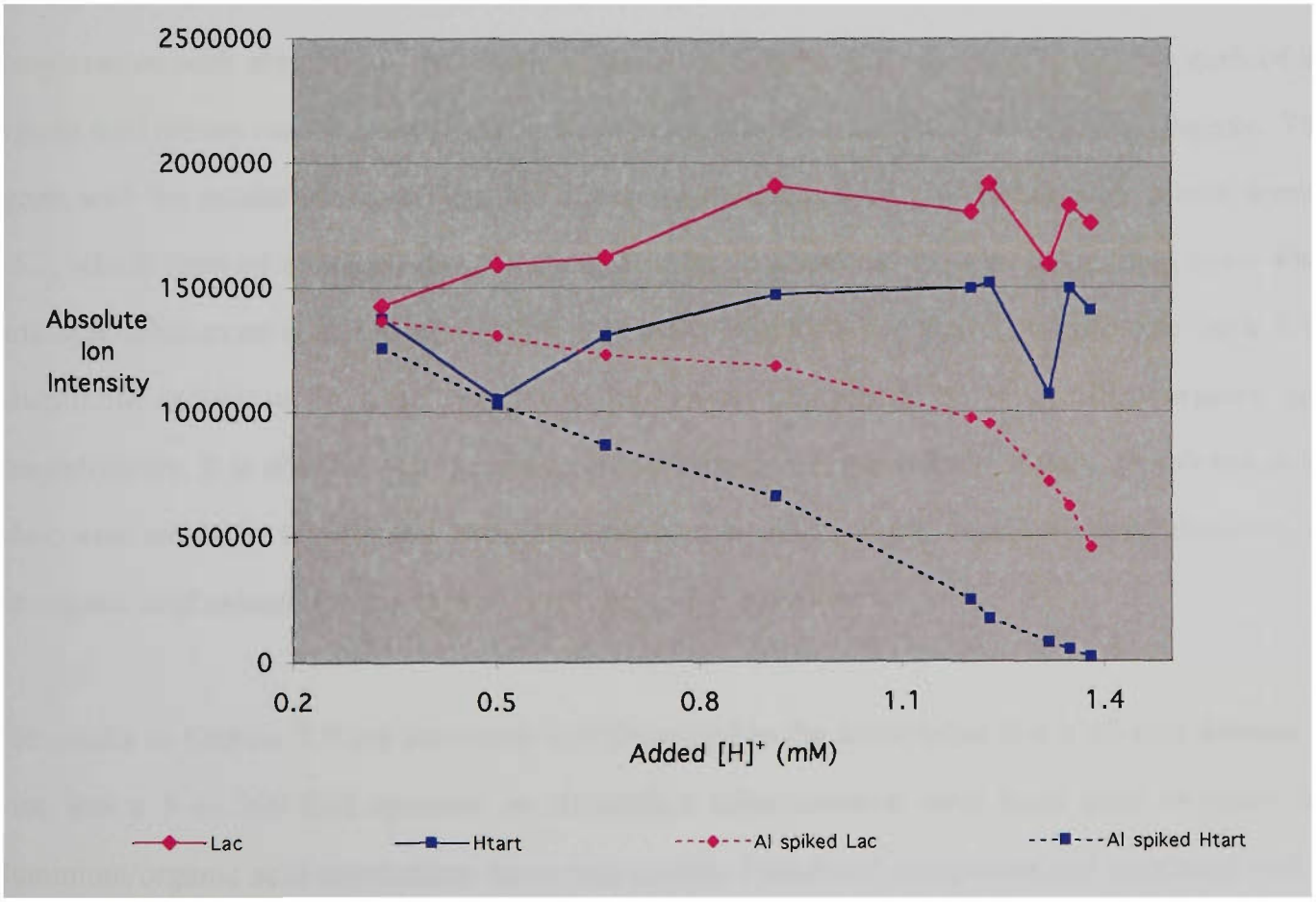


Figure 5.54 Plot is devised as follows: As aluminium potassium sulfate is added to diluted white wine L, the $[H^+]$ (mM) increases. After each aluminium spike the $[H^+]$ is calculated from the solution pH. The diluted white wine is treated with HCl to emulate the pH change caused by addition of aluminium. The negative ion intensity of lactate and bitartrate (full lines) is replotted in terms of added $[H^+]$ from HCl, not increasing aluminium concentration. The negative ion intensity of lactate and bitartrate (broken lines) is superimposed over the first plot against added aluminium from the aluminium potassium sulfate spike.

The plot in Figure 5.54 shows that the change in organic acid anion intensity in solutions with incremental increases in H^+ ions via HCl addition does not match the change in organic acid anion intensity when the H^+ ion concentration is increased by the addition of aluminium. Overall, the ion intensities of lactate and bitartrate in the hydrochloric acid added solutions show no definitive trend, demonstrating little average change in ion intensity over hydrogen ion concentration of 0.2mM to 1.4mM. However, in the aluminium spiked solutions the ion intensities show a continual decrease. Because a decrease in organic anion intensity is only evidenced when aluminium is added to the solution and not when H^+ ions are added, the results show that hydrogen ion concentration is not responsible for the decrease in ion intensity. This supports the proposal that the decrease in ion intensity is due to the complexation of the free ions to form aluminium complexes.

Having established that the decrease in organic acid anion concentration is most probably due to complexation with aluminium, the trends observed in Figures 5.51 and 5.52 show that each of the organic acid anions can be complexed by aluminium in a wine sample, but to varying degrees. This agrees with the results obtained from the direct negative ion mode ES-MS analysis in Sub-Section 5.5.2, which showed evidence that a variety of organic acids can bind to aluminium, even when tartaric acid/bitartrate is dominant in solution as is the case for white wine L as shown in Table 5.18. Aluminium speciation in wine appears to be heavily influenced by the various organic acid concentrations. It is obvious that the predominant ligands with which aluminium complexes in the white wine would be tartaric and lactic acid due to their much higher concentrations. However, all the organic acid anions in wine appear to complex with aluminium.

The results in Section 5.5 are presented and discussed in the knowledge that a 20-fold dilution of wine and a 5 to 200-fold increase in aluminium concentration have been used to make any aluminium/organic acid interactions more measurable. Postulated complexes and structures will be discussed and conclusions drawn in Chapter 6, Sub-Section 6.4.2.

VI

"Brothers……what we do in life, echoes in eternity"
Maximus Decimius Meridius, Roman General from Ridley Scott's film 'The Gladiator'

CHAPTER SIX

6. CONCLUSIONS

6.1 Summary of the Aims and Relevance of this Study

There were two aims of the project: to determine the profile of aluminium concentration throughout the wine production process and to determine the speciation of aluminium in finished wine. The first aim sought to identify the sources and sinks of aluminium in the entire production process and extend the research of McKinnon (1990). The second aim sought to reveal the aluminium containing species in wine.

This chapter summarises the findings of the investigation and draws some conclusions. The discussion and conclusions of the total aluminium concentration production profile analysis and speciation will be dealt with separately, with the former identifying sources and sinks of aluminium throughout wine production and the latter commenting on possible wine aluminium species, their structures and the limitations of the ES-MS technique. An estimate of the average daily intake of aluminium from wines investigated in this study will also be included. Lastly, areas of further research are identified and their relevance briefly discussed.

6.2 Wine Production Aluminium Total Concentration Profile Analysis

The total aluminium concentration profile conducted on wines in this study shows that there is a significant amount of aluminium in the grapes. The grapes contained between 2 to 8 times the concentration of aluminium (in mass terms) found in the wines during any stage of production. This finding contradicts data presented by Eschnauer & Scollary (1995) which suggested that the majority of aluminium in over 50 German wines was introduced by production/anthropogenic activities. The aluminium concentration in grapes can also be divided into natural or anthropogenically sourced. A distribution of the aluminium within a grape showed that between half and two thirds of grape aluminium was contained within the juice and flesh with around 10% in the skin and approximately 15% in the pip. This aluminium would be naturally derived aluminium from

the soil. Although no significant relationship was observed for the acetic acid extractable soil aluminium concentration and soil pH with the grape aluminium concentration, root uptake is the only major route by which significant amounts of aluminium can reach the grapes internally. The lack of a significant correlation probably means that more parameters than pH need to be studied to discern a very complex relationship between soil aluminium chemistry and vine uptake. The remaining aluminium from the grapes is that which is deposited on to the skin. Grape aluminium distribution studies suggest that 25 to 30% of grape aluminium is derived from this source. This was supported by the statistically significant difference found between the aluminium concentration of washed grapes and unwashed grapes and juices. The origin of grape skin adsorbed particles was not the focus of this investigation and thus the source distribution of air deposited aluminium cannot be quantified but is discussed briefly as a point of future research in Section 6.5.

The distribution of the aluminium in the grape provided important information on the aluminium that could be introduced to the juice. If the grapes were washed prior to crushing, the aluminium load was shown to be reduced by almost 25%. This is supported by the change in mean aluminium concentrations in homogenised unwashed and washed grapes from the profile analysis. Therefore, 75% of the remaining grape aluminium in the flesh/juice stays in the juice upon crushing, with the remaining skin and pip aluminium excluded from the juice and removed upon pressing assuming none is extracted into the juice. The calculated aluminium concentration that remains in the juice closely reflects that found for the juice sample in the profile analysis. Hence the grape aluminium load that remains in the juice, as calculated from the grape aluminium distribution, neatly fits the change in concentration for washed grapes to unwashed grapes to juice observed in the profile analysis (Sub-Section 4.10).

However in the majority of cases the grapes are not washed in commercial winemaking. The grape distribution including the skins would then hold and, as the adsorbed skin aluminium can be removed by water washing, it would be likely that juice would also remove the adsorbed aluminium. This scenario means that a greater amount of grape aluminium would be carried forward into the juice which would not match the mean juice content seen in the profile analysis. The lower profile analysis aluminium juice content can probably be reconciled by considering the nature of the

particles on the grape. There is a strong possibility that a good proportion of the dust borne aluminium is found as alumino-silicates which can be resistant to dissolution (Quevauviller et al. 1993b; Novozamsky et al. 1995; Sun et al. 1997). Hence, much of the adsorbed grape aluminium may be removed from the grape and settle in the deposits of the crushed juice and be removed on pressing. This agrees with the findings of Enkelmann and Wohlfarth (1994).

In judging the overall profile of aluminium in grapes and juices it must also be taken into account that there was substantial variability in the mean aluminium concentrations determined by GFAAS with a reproducibility (in %RSD) for these samples of 20%. This high variability does cloud the interpretation of the data to an extent. Despite this variability, whatever the amount of aluminium in juice resulting from grape adsorbed aluminium, the distribution shows that the majority of aluminium in juice originates from the flesh/juice of which most is naturally derived.

The addition of SO_2 to juice did not produce a significant change. From the data of this study the contribution, if any, to aluminium from SO_2 treatment is inconclusive.

The most important finding of the total aluminium profile analysis was the significant decrease in aluminium concentration from the juice (with or without SO_2) to the fermented wine. This decrease was evident for all the wines studied. Juices with an average concentration of around 1.5mg/L were fermented to give wine concentrations between 0.25-0.50mg/L. The average change was 1.1mg/L which, when compared with all other production steps, was a dramatic difference in aluminium content (see Sub-Section 4.10.4). The mechanism of the loss of aluminium from the juice is not altogether clear. A test was carried out to see if it was lost to the lees, the only other significant fraction in fermented wine. However, because the lees samples were taken from a vintage that could not be sampled in very tightly controlled laboratory conditions, quantifying the exact amounts of components in the system proved impossible and meant that a mass balance was unable to be obtained. Aluminium concentrations of the dried lees suggest that aluminium can be deposited in or on the lees, possibly adsorbed/absorbed on the yeast cells. This may account for the loss of aluminium from the ferment and agrees with the work of Meierer (1984) as the most likely sink for aluminium.

Another possible explanation of the sudden decrease in aluminium concentration is the use of cold stabilisation and/or the cold climate around the Macedon Ranges region of Victoria during fermentation. (For these vintages mid-late autumn temperatures are below 10°C during the day). It is well known that wine is usually saturated with potassium bitartrate as well as with calcium tartrate in wines having a high calcium content (Rankine 1991). At cooler temperatures, the solubility limit is exceeded and the metal bitartrate/tartrate precipitate as crystalline deposits. High potassium and calcium content, cooler temperature and increased alcohol content can make the metal bitartrate/tartrate less soluble. This last point is important as a significant amount of impure potassium bitartrate and argols (impure crystals of potassium bitartrate with pigment from the wine) are deposited during fermentation due to the significant increase in alcohol content of the solution (Rankine 1991). Although purely speculative, it may be possible that aluminium co-precipitates under those conditions with potassium bitartrate or calcium tartrate and form part of the lees; some could be bound to the fermentation and/or storage vessel walls. This may be exacerbated by the cooler climate and or cold-stabilisation and account for the loss of solution aluminium during fermentation. Further work in this area will be discussed in Section 6.5.

Whatever the mechanism, this significant change shows that the most fundamental wine production process, fermentation, acts as a principal route for reducing the mainly 'natural' aluminium content of juice to a substantially lower concentration in the wine.

Various production steps are undertaken after the alcoholic fermentation. Samples taken after pressing, further SO_2 addition, malo-lactic fermentation and racking showed no significant change in aluminium concentrations. These results are not unexpected. With pressing and racking, removal of the solids removes aluminium that is already excluded from the wine solution. Malo-lactic fermentation is a natural process not requiring the use of additives and is a conversion of malic acid to lactic acid that does not provide avenues for aluminium loss. Metabisulfite addition to juice or wine showed no significant change in aluminium concentration and probably contributes little if any aluminium. The addition of tannin, either red or white, also showed no appreciable change in the aluminium concentration of wines. This disagrees with the work of McKinnon (1990) who

suggested red tannin was the main source of aluminium to red wine. However, there were insufficient samples for this production step in this study to make a definite conclusion.

Prior to bottling, the only other production step to show a statistically significant change in aluminium concentration was bentonite fining in white wines (Sub-Section 4.10.4). Although with the exception of wine E, the recommended dose of bentonite to wines increased the aluminium concentration by a much smaller magnitude than that observed by McKinnon (1990) and McKinnon et al. (1992). The average change after bentonite fining was around 0.2mg/L or approximately 20% of the change observed for fermentation.

This result combined with the more modest increases in aluminium noted for some other white wines after bentonite fining fully supports the findings of McKinnon (1990), McKinnon et al. (1992), Larroque et al. (1994), Eschnauer & Scollary (1995), Lopez et al. (1998), Seruga et al. (1998) and Larcher & Nicolini (2001). There is little doubt that bentonite fining leads to an increase in the aluminium content of white wines. All the above studies conducted on wines in the last decade have reported that white wines have a higher mean aluminium concentration than red wines that is attributed to bentonite fining. This was also the case for white wines in this study.

There was an expectation that bottling may introduce aluminium into the wine as there are alumino-silicates in and on the surfaces of glass (see Chapter 3) and although these compounds can be resistant to dissolution could still contribute some aluminium into the wine. McKinnon (1990) also suggested that leaching from the bottle surface could contribute aluminium to wine but could not make a conclusion from one data point. The data from this study showed a mixed response, some showing an increase, some showing a decrease, however the change in aluminium concentration was small (generally below 0.1mg/L) with no statistically significant difference noted overall. Despite the greater number of data points obtained for this study, the contribution of aluminium from bottling remains inconclusive.

The production profile analysis for the 1997 and 1998 vintages showed there was good agreement between the two (see Figure 4.22 in Chapter 4). This demonstrates that the changes in aluminium

concentrations are not due to artefacts of the methodology or biased by methods of a particular year. The profile of the white wines and red wines of both years also shows reasonably close agreement except with divergence noted for juice aluminium concentrations (Figure 4.23, Chapter 4), and comparison of the fermented wines shows higher aluminium concentrations for red wines. Although this could suggest leaching from the skins, the difference is probably due to the preceding mean red wine juice aluminium concentration being 1.5 times that of the white wine juice. Even if this difference were due to leaching from the skins, the difference is minimal and demonstrates that although the methods of fermentation differ between white and red wines there appears to be little difference in the transfer of aluminium from grape to juice even though red wines are fermented on the skins. Although this contrasts with McKinnon's (1990) finding that white wine ferments had higher aluminium concentrations, three of the four white wines profiled in that study were bentonite fined prior to the completion of fermentation. Only one of the white wines in this study, wine E, was similarly treated prior to fermentation of the juice (see Sub-Section 4.10.2).

Another interesting finding was the comparison of the bottled wine aluminium concentrations of wines made naturally where no SO_2, bentonite fining or other treatments or additives were used (wine I and J) and the other wines investigated in this study. There was no appreciable difference between the aluminium concentrations of these bottled wines compared with the overall mean. This may suggest that, unless circumstances that significantly increase wine aluminium concentration are encountered (e.g. wine E), the majority of aluminium in wine is derived from natural sources.

In summary, aluminium in wine is primarily derived naturally from the vine which transfers aluminium from the soil to the grape. Combined with more naturally and anthropogenically deposited aluminium on the grape and depending on the lability of the grape skin surface aluminium, approximately 60-75% of the grape aluminium load predominantly from the juice and flesh is released upon crushing to the juice. During fermentation the majority of the aluminium contained in the juice is removed by a process that remains uncertain, but is possibly due to a mechanism of adsorption/absorption into the yeast lees and/or co-precipitation with potassium bitartrate, whereupon the ferment contains on average 70% less aluminium than its precursor juice. This change in aluminium concentration is the most significant in the entire winemaking production

process. The contribution of aluminium, if any, after addition of SO_2 is inconclusive although it is probably small, while the processes of pressing, malo-lactic fermentation and tannin addition appear to have no influence over wine aluminium concentration. Bentonite fining was found to increase aluminium concentrations thereby supporting evidence from earlier studies. However with recommended dosing the increase is not of the same magnitude as the loss observed during fermentation. Finally, although potentially a source of aluminium particularly after long storage, the effect of the bottle on wine aluminium concentration remains inconclusive.

6.3 Assessment of Daily Intake of Aluminium from Wines of this Study

Anderson & Norman (2001) reported that in 1999, 19.6L of wine were consumed per person per year in Australia. Assuming that 30% of the Australian population drinks wine, that equates to 65L consumed by the wine drinker per year. Therefore, the Australian wine consumer drinks 178ml per day. Using the mean aluminium concentrations from Chapter 4, Table 4.27, an estimation of aluminium intake is given in Table 6.1.

Table 6.1 Estimate of aluminium intake from the wines investigated in this study.

	Mean Wine [Al] (mg/L)	Al Intake Assuming 178ml consumed per day (µg/day)	Al Intake Assuming 500ml consumed per day (µg/day)
Overall	0.47	84	240
White Wine	0.52	92	260
Red Wine	0.43	77	220
Wine E	2.5	450	1300

From the second column of Table 6.1, assuming the mean amount of wine consumed per day is 178ml, one can see the average daily intake of aluminium for the Australian wine consumer is less than 0.1mg per day. However, in the case of wines with a higher aluminium concentration such as wine E this amount increases by an order of magnitude. In the case of the blended wine E, the estimated daily intake is close to half a milligram per day. If the original ferment of wine E was used with an aluminium concentration of around 4mg/L the daily intake from this wine would have increased to 0.7 mg/L. Even this "worst case" figure is well below that of Seruga et al. (1998) who

reported an estimated daily intake of aluminium from Croatian wines of 1.345mg per day, based on the consumption of 0.5L of wine per day. From the data of this study, if 0.5L per day is assumed as the volume consumed, then the estimate changes as shown in the third column of Table 6.1.

Using the adjusted consumption volume, wine E now compares with the figure reported by Seruga et al. (1998), however the mean concentration of all wines is still significantly lower than data from Seruga et al. (1998). The daily intake estimate is well below that of the estimated intake from beverages at 2.0mg/day (UK MAFF 1993) and is well below recent estimates of total dietary aluminium intake of 3.4mg/day (Ysart et al. 2000) and 0.03-11.5mg/day (WHO/IPCS 1997). Daily intake of aluminium from the wines of this study will be well within dietary estimates and the recommended WHO daily aluminium intake limit of 1mg/kg body mass/day.

6.4 Aluminium Speciation of Bottled Table Wine by ES-MS

6.4.1 Use of ES-MS for the Speciation Analysis of Aluminium in Wine

ES-MS was used for this project as its soft ionisation allows the determination of molecular ions from species in solution with minimal redistribution of the species directly.

In Chapter 5 of this thesis, it was demonstrated that ES-MS could detect a number of aluminium complexes with organic acids in aqueous and model wine solutions. Complexes were characterised using both positive and negative ion modes. The positive ion mode ions showed a stronger ion intensity and are probably reflective of ions or molecular species in solution. However aluminium/organic acid complexes identified by negative ion mode ES-MS are probably exclusively formed by the ES-MS process itself. The low intensity of the negative ion mode complexes was most likely due to the difficulty in removing protons from the organic acids already attached to aluminium. Despite this, these ions can be related to similarly sourced ions seen in the positive ion mode. Most ions observed in the positive ion mode were also observed in the negative ion mode minus two protons. In Chapter 5 these were termed 'conjugal' species. The observation of these ions in both ion modes supports their characterisation as reflective of actual complexes and not an artefact. Having information from both ion modes gives two points of reference in determining the speciation of these complexes in the original solutions.

Despite the success of the method in aqueous and model wine media, none of the complexes identified in the positive ion mode were observed in solutions of diluted wine which were spiked with aluminium. Some of the ions characterised in the negative ion mode for aqueous and model wine media were observed in diluted wine, however the intensity was extremely low. Considering the successful characterisations of the mass spectral peaks in model wine, the absence of these peaks in diluted wine was disappointing. There could be several reasons for the non-detection of the aluminium-organic acid complex peaks. The most obvious is that aluminium is not bound to any organic acid in wine. Another reason could be the existence of stable neutral species in wine; neutral species are not detected by the MS. Lastly, the complicated matrix of wine may be suppressing or altering the electrospray conditions to a point where these ions cannot be effectively transferred to the gas phase. The mass spectra of diluted wine demonstrated a poor signal to noise ratio, and ethanol was shown to suppress the ES-MS ion intensities even in model wine solutions (see Sub-Section 5.4.2). This poor response and the detection of the ions observed in the negative ion mode suggest that the matrix of the wine is degrading the performance of the ES-MS, particularly in positive ion mode, suppressing the formation and stability of gas phase aluminium/organic acid ions in the electrospray.

Although direct analysis of aluminium speciation in diluted wine proved unsuccessful, the good response of the organic acid anions in the negative ion mode afforded an investigation into the interaction of aluminium with these anions by observing the change in the anions negative ion mode intensity after addition of increasing amounts of aluminium to the diluted wine (see Sub-Section 5.5.3). The peaks due to lactic, succinic, malic, tartaric and citric acids all showed a lowering in ion intensity as the aluminium concentration increased. The reduced intensity is likely caused by the decreased free anion concentration in the solution after complexing with added aluminium. Of these anions, bitartrate appears to be more susceptible to the addition of aluminium thus suggesting that bitartrate may preferentially complex aluminium in the wine solution. This indirect evidence supports the findings of McKinnon (1990) and McKinnon et al. (1992) who also by indirect means showed that a low molecular mass ligand bound aluminium strongly enough to significantly interfere with complexing reagent based colorimetric and fluorometric determination of aluminium in wine. However as the ion intensity response of ES-MS is not necessarily proportional to

concentration and is susceptible to changes in solution conditions other than pH, the indirect evidence is indicative but not conclusive.

In summary, the indirect evidence from the negative ion mode ES-MS suggests that aluminium is bound mainly to anions of low molecular mass aliphatic organic acids in wine, and of these, bitartrate is the predominant complexing ligand.

6.4.2 Discussion of Possible Identities of Aluminium Complexes in Wine

Assuming that aluminium is bound to the organic acids in wine and in the knowledge that ES-MS can provide only limited structural information, the possible formulae of aluminium/organic acid complexes in wine are discussed in the following paragraphs.

Aluminium(III) can form tetrahedral or octahedral complexes, although it prefers a coordination number of six (Rubini et al. 2002; Yokel 2002). Complexes with aluminium by chelating ligands that form six membered rings are considered the most stable (Yokel 2002). The binding strength of the aliphatic carboxylic acids in descending order is di-carboxylic >> hydroxycarboxylic > mono-carboxylic >> amino acids (Rubini et al. 2002).

Lactate was found by ES-MS as a predominantly 1:2 complex or co-complexed with other ligands such as bi-tartrate or bi-malate with aluminium (Chapter 5). As a hydroxycarboxylate, lactate can bind at both the carboxylate and hydroxy groups to form a neutral complex with 3 bi-dentate ligands coordinated around the aluminium with the hydroxy proton still attached (Salifoglou 2002). Further ligand exchange in aqueous solutions can facilitate the formation of $[Al(lac)_2(H_2O)_2]^+$, $Al(lac)_2(OH)(H_2O)$ and $Al(H_{-1}lac)(lac)(H_2O)_2$ at pH 3.5 (Salifoglou 2002). Although the tris-ligand species was not observed in the ES-MS, these above-mentioned ligand exchange products offer a glimpse of the possible solution speciation in aqueous and model wine media with $[Al(lac)_2]^+$ observed in the positive ion mode and its congener species $[Al(H_{-1}lac)_2]^-$ in the negative ion mode (Chapter 5). It would be very difficult to form lactate from lactic acid in the positive ion mode, but lactate can deprotonate in an aluminium complex in the negative ion mode. Because of this the starting complex in the model wine solution is probably either $Al(lac)_3$ and/or its ligand exchanged

counterparts, with the latter species the more likely. In positive ion mode a lactate ligand could be protonated in $Al(lac)_3$ enabling its removal from the complex. Of the positive ligand exchanged species, hydroxy and water ligands are probably removed due to protonation, fragmentation at the sampling or skimmer cone, or by redistribution of charge during the electrospray charged droplet evaporation. Neutral species could undergo protonation of the doubly deprotonated lactate and the removal of the water ligand by the processes mentioned above. In the case of the negative ion mode ES-MS, the lactate ligands in the aluminium complexes are probably deprotonated further and the water/hydroxy ligands removed by the same processes described for the positive ions. However the absence of an $[Al(lac)_2(H_{-1}lac)]^-$ complex and the low intensity of $[Al(H_{-1}lac)_2]^-$ in negative ion ES-MS means that the initial solution species is most likely either tetrahedral $[Al(lac)_2]^+$, octahedral $[Al(lac)_2(H_2O)_2]^+$ and/or $Al(lac)_2(OH)(H_2O)$. Rubini et al. (2002) considered lactate to be a weak ligand in acidic conditions. However aluminium lactate complexes were characterised in model wine solutions in the presence of other organic acids (Chapter 5). Additionally, in the indirect analysis of organic acid anion intensity versus aluminium spike concentration lactate demonstrated an intensity decrease second only to bitartrate (Sub-Section 5.5.3).

Aluminium malate species were characterised in both aqueous and model wine media and observed predominantly as a 1:2 and/or mixed ligand complex with other organic ligands or sulfate and nitrate (Chapter 5). 'Metal ion-promoted deprotonation and coordination of the alcoholic function are more favoured with malic acid' than lactic acid, 'where the presence of another carboxylate group in the β-position allows the tridentate coordination of malate via the formation of a (5+6)-membered joint chelate system' (Rubini et al. 2002). This coordination would fit with the 1:2 and mixed ligand complexes observed in both ion modes of the ES-MS however the low intensity of the $[Al(mal)_2]^-$ complex in the negative ion mode and the strong intensity of the $[Al(Hmal)_2]^+$ in the positive mode suggest that the main ligand bound to aluminium is bimalate in a bidentate fashion. This closely resembles the binding observed for lactate and it is possible that the solution ions and mechanisms are the same and would also explain the case for the mixed ligand complexes. Using the mechanisms explained for lactate, the four possibilities of aluminium malate complexes in solution are: two tridentate bound malates in octahedral coordination $[Al(mal)_2]^-$, two bidentate bound bimalates in tetrahedral coordination $[Al(Hmal)_2]^+$, two bidentate bound bimalates with two

water ligands in an octahedral coordination $[Al(Hmal)_2(H_2O)_2]^+$, or octahedrally coordinated neutral tris-bimalate $Al(Hmal)_3$. Of these, the first and the last are unlikely, due to the low presence of $[Al(mal)_2]^-$ and absence of $[Al(Hmal)_2(mal)]^-$ in the negative ion mode. As aluminium prefers octahedral coordination, and the probability of hydration in aqueous solutions quite high, the second possibility is the most likely. Like the situation for lactate, the water/hydroxy ligands are probably removed via protonation, fragmentation or instabilities caused by charge re-distribution during droplet evaporation in the positive ion mode. A similar mechanism probably occurs in the negative ion mode except that the bimalate ligands are deprotonated. These above-mentioned mechanisms in negative ion and positive ion modes probably also apply for the mixed ligand complexes.

Aluminium complexes with tartaric acid, like those with malic and lactic acids, were predominantly observed in the ES-MS spectra of aqueous and model wine solutions as 1:2 and/or mixed ligand complexes (Chapter 5). The mixed ligand complexes were observed with bimalate/malate, lactate, sulfate and nitrate. Other studies have postulated that aluminium mainly forms mononuclear or binuclear complexes with tartrate as mostly (1:1) or (2:2) stoichiometry with either octahedral or tetrahedral centres (Rubini et al. 2002). Tartaric acid contains two carboxyl and two hydroxy groups of which the latter has been shown to lose its proton for binding to Al^{3+} at a pH even as low as 2 (Rubini et al. 2002). Because of these groups, tartrate has been reported as forming a quadridentate ligand as a bridge in a dinuclear species (Rubini et al. 2002). This diversity of binding groups could allow bitartrate to bind in bidentate fashion at a carboxylate and a hydroxy group. As it is structurally similar to malate, binding as a tridentate species using its other hydroxy or carboxyl group is also a possibility. No evidence of polynuclear aluminium tartrate species was found in the ES-MS mass spectra in either ion mode (Chapter 5). Like bimalate/malate, the absence of a 1:3 ion and the low intensity of the $[Al(tart)_2]^-$ ion in the negative ion mode suggests that complexes of octahedrally coordinated neutral tris-bitartrate $Al(Htart)_3$ and tridentate bound aluminium bis-tartrate are unlikely to exist in solution. Again the ES-MS mechanisms are probably the same as that for lactate, suggesting that tetrahedrally coordinated aluminium bound to bidentate bitartrate $[Al(Htart)_2]^+$ and octahedral aluminium bound to the same ligands but with two added water ligands $[Al(Htart)_2(H_2O)_2]^+$ are the most likely species found in solution. Of these the octahedral species is probably the predominant species in solution, with the ES-MS removing the water molecules in

positive ion mode with the same mechanism in the negative ion mode but with an additional deprotonation of the bitartrate ligands. Using similar mechanisms, mixed ligand complexes with bimalate and lactate would seemingly form in a similar way in ES-MS with octahedral $[Al(Htart)(Hmal)(H_2O)_2]^+$ and $[Al(Htart)(lac)(H_2O)_2]^+$ the precursors in aqueous and model wine solutions.

Citric acid has been the most studied organic acid ligand in complexes with aluminium (Rubini et al. 2002). The mononuclear species $[Al(Hcit)]^+$, $[Al(cit)]$ and $[Al(H_{-1}cit)]^-$ were the earliest species characterised by potentiometric and modelling analyses with a 1:2 complex known to form at high ligand excess (Rubini et al. 2002). This was found to be the case for the ES-MS speciation analysis of model wines, with only mono citrate species observed in either positive or negative ion modes as aluminium was in molar excess in all the solutions studied (Chapter 5). The strong intensity of $[Al(Hcit)(H_2O)]^+$ in the positive ion mode and the presence of $[Al(cit)(OH)]^-$ in negative ion mode suggests the tridentate coordination of citrate. Because of this arrangement and the unfavourable conditions for bis complexation, aluminium citrate species were the only ones to show water ligand binding in the ES-MS (Chapter 5), probably to preserve the tetrahedral or octahedral coordination state that aluminium prefers. The citrate complex in the solution is probably either tetrahedrally coordinated $[Al(Hcit)(H_2O)]^+$ or octahedrally coordinated $[Al(Hcit)(H_2O)_4]^+$, where Hcit is bidentate, with 2-3 water ligands removed by previously discussed mechanisms in positive and negative ion modes. ES-MS studies of model wine solutions showed that only lactate was co-complexed with citrate to aluminium which is most likely formed via the same mechanisms as discussed for other co-complexed organic acid species. The reason for the lack of co-complexing to either bimalate/malate or bitartrate/tartrate is probably due to either electrospray ion instability or steric factors. However, these latter hypotheses are purely speculative.

In summary, the ES-MS speciation analysis of aqueous and model wine solutions suggests that a similar mechanism of gas phase ion formation occurs in the electrospray for all aliphatic organic acid aluminium complexes under the instrumental conditions used in this study. In the positive ion mode it appears to be based on the conversion of hydrated octahedral complexes in solution to tetrahedral coordinated gas phase ions by removal of water molecules. In the negative ion mode, a

similar mechanism occurs with the additional deprotonation of ligands. In general, two organic acid anion ligands are bound in monomeric aluminium complexes, either as 1:2 or mixed ligand complexes with the exception of citrate which does not form 1:2 complexes in the solutions studied. Lactate, bimalate and bitartrate bind to aluminium as bidentate ligands whereas citrate is bound in tridentate fashion. Although the original solution complexes are highly likely to be hydrated and octahedrally coordinated, the possibility of non-hydrated tetrahedrally coordinated aluminium complexes with organic acids cannot be dismissed entirely.

6.5 Future Research

It is inevitable that research of the nature described in this thesis can not only provide answers but also unveil more questions that require further investigation. This final section briefly discusses the areas of further study that arise from the findings of this project. Further work is discussed in two parts, that arising from the total aluminium concentration wine production profile analysis and that from the speciation analysis of aluminium in wine using ES-MS.

6.5.1 Total Aluminium Profile Analysis

The majority of aluminium in wine was found to be derived from the wine grapes of which most originated from the soil and some from air deposition of particulate matter (both natural and anthropogenic). To gain a better understanding of the mechanism of transfer of aluminium from these sources into the grape several areas should be investigated. The optimisation of determining the vine bioavailable aluminium fraction of the soil can be accomplished by investigating different extractants and regimes to find the best correlation between extracted soil aluminium concentrations and the vine and grape. This information can be used to evaluate particular soil types and conditions that affect the uptake of aluminium by the vine. A detailed biochemical investigation of the pathways of aluminium from the root and absorption from air deposition on the vine would also give a better understanding of the mechanism of aluminium transport into the grape. While these would provide a knowledge of the vine-sourced aluminium, an additional study on the sources, distribution and speciation of air deposited aluminium on the grape would give a complete picture of aluminium sourced from the grape.

For the calculation of the distribution of aluminium in wine, an assumption was made that aluminium was not leached from the grape constituents other than the grape juice. This assumption may not be valid, as the leaching capability of wine during bentonite fining has been demonstrated in this thesis (Chapter 5) and in the work of McKinnon (1990) and McKinnon et al. (1992), hence a study investigating possible leaching from crushed grape material and the aluminium contribution from different parts of the grape into the juice should be evaluated to more accurately define the contribution of aluminium from grapes to the juice.

As the decrease in aluminium concentration from juice to ferment was found to be the most significant in the entire wine production process, a comprehensive analysis of the fermentation process and the movement of aluminium in the system is essential for the full understanding of the contribution of naturally sourced aluminium in wine. This should include an investigation of aluminium co-precipitating with potassium bitartrate or calcium tartrate. Crystalline material and lees should be analysed for aluminium content and its speciation determined to see if precipitation in cold climates or during cold stabilisation contributes to the removal of aluminium from the ferment. Speciation analysis of any aluminium found in these crystals or in the lees would prove useful in further understanding the mechanisms of aluminium concentration decrease during fermentation.

As discovered in this study and that of Meierer (1984), these tasks would be painstaking and difficult to carry out. It would require a carefully controlled, closed system fermentation to be carried out with a complete knowledge of the mass and aluminium concentrations of all the constituents prior to fermentation. It would also require the ability to accurately determine the mass of juice, ferment, lees and any deposits on the container walls, as well as their aluminium concentrations. These fermentations would have to be carried out under the complete control of the investigator. This kind of closed system, controlled study would be the logical next step in evaluating future profiles of the total aluminium concentration over the entire wine production process. This type of investigation would avoid the inherent inconsistencies of unplanned winemaker intervention on sample collection and the difficulties in maintaining protocols

throughout the entire process over a lengthy period of time encountered using commercial samples, as was the case with this study.

The profile analysis on commercial wines should be expanded to include wines from different varieties, climates, and geographic regions covering a broad spectrum of commercial operations, from the smallest vineyard/wineries to those of the large winemaking corporations. This would require considerable time and resources, both in manpower and in funding but the greater number of samples would further clarify the overall profile of aluminium concentration by improving statistical precision and elucidate other as yet unknown contributors to wine aluminium.

6.5.2 Speciation of Aluminium in Wine Using ES-MS

It was anticipated that ES-MS would provide an insight into the speciation of aluminium in wine by directly identifying these species without disrupting the equilibrium present in the solution. While this proved possible in simple matrix solutions of water and model wine, direct determination in wines proved elusive. While an optimisation program was developed using these simpler matrices, conditions may not have been optimum for a solution of diluted wine. The success of ES-MS in the simpler matrices provides some optimism of the potential of this technique for speciation in wine. Hence further work should be dedicated to developing new ways of performing ES-MS analysis with a more specific optimisation for aluminium in wine samples.

This could be accomplished in several ways: the use of higher capillary temperatures, different solvents, different potentials not only of the skimmer cone but the sampling cone, addition of various electrolytes at various concentrations, the use of internal standards and a stabiliser for quantitation. In addition, an investigation of the use of fragmentation at higher cone voltages, derivatisation and other ES-MS mechanisms should also be explored. However, technology is ever evolving and new technologies should not be ruled out. Barnett et al. (2000) described a new state-of-the-art electrospray method of ES-FAIMS-MS, where the FAIMS acronym stands for high field asymmetric waveform ion mobility spectrometry. FAIMS acts as an ion filter that can be tuned to transmit selected ions from a complex mixture and when coupled to an ES discriminates 'against background ions', 'simplifying the mass spectra, improving the selectivity of the method, and

lowering the limits of detection' (Barnett et al. 2000). This technology combined with the optimisation of electrospray conditions for wine matrices could allow direct aluminium speciation determination in wines and is worthy of further investigation.

Despite the high detection limits and specialist requirements for interpretation, ^{27}Al NMR along with ^{1}H and ^{13}C used in tandem with ES-MS could still provide useful structural information that can make characterisation of individual species and their coordination easier to determine. NMR is one of few techniques that does not interfere with the solution equilibrium and although high aluminium spikes would be required, the data could be extrapolated back to solutions of lower aluminium content.

Complementary techniques used simultaneously have become increasingly popular in the last ten years (Gonzalez & Sanz-Medel 2000; Ebdon & Fisher 2000; Ackley et al. 2000; Szpunar et al. 2000; Sanz-Medel 2002). The use of separation techniques combined with ICP-MS or other atomic spectrometric determination techniques has proved successful in determining the speciation of aluminium in blood (Sanz-Medel 1998). The complementary use of ES-MS and ICP-MS was advocated by Houk (1998) and other publications have recognised the need for new techniques to elucidate aluminium complexes (Salifoglou 2002; Sanz-Medel 2002). Hence, the use of various combinations of separation or FIA techniques with UV-VIS, ICP-MS or ES-MS detection probably offers the best way forward for future direct aluminium speciation analysis in wine and should be comprehensively investigated and developed to gain an understanding of the bioavailability of wine sourced aluminium.

Finally, as the bioavailability of aluminium and its potential toxicity has been of considerable interest over the last twenty years, biological and biochemical studies would be complemented by information such as that gained from this work. If all of these studies are consolidated, the associated health risk, if any, of the uptake and metabolism of aluminium sourced from the regular consumption of wine would be more fully understood.

6.5.3 Recommendations for Liaison Between University and Industry

It is imperative that the industry contacts that are established are 100% behind the project. It is easier in these days of the internet and mobile phones to keep in touch, however the realities of commercial time lines compared to those of research and education facilities can be a source of friction. The candidate has to maintain a fine balance between successfully completing sampling tasks and avoiding souring of relations with the winemaker. A written letter determining concentration of interest between the university and the winemaker could include the likely demands that cooperating with the university may entail. As the winemaker will bow to commercial/financial necessities, a deal between the university and the winemaker that is more than just purely academically beneficial may provide more incentive to the winemaker to provide more help to the student.

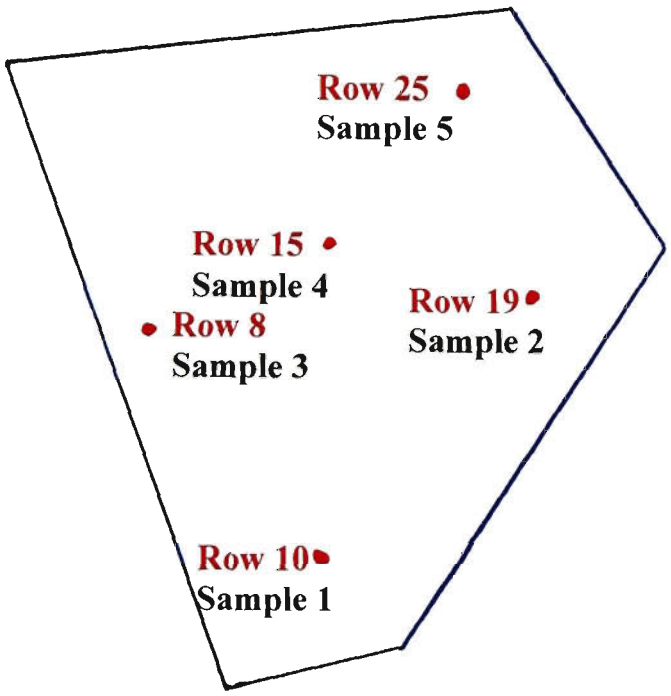
Working as a group with the full support of several students and supervisors on wine projects related to the winemaker may also provide a better and more constant relationship. As a lone student who had to virtually initiate, foster and maintain relationships, including the drawing up of legal documents for confidentiality and do the work that the research requires, this author found that more support from staff, university and a larger group would definitely have improved the cooperation with the vignerons/winemakers involved and provided more manpower for sampling.

It was also the personal experience of this author that those winemakers who had a tertiary educational background were more interested in the outcomes of the research from an academic perspective rather than just a financial one and with this greater interest provided better cooperation, information and reliable sampling in times when the author could not be available at the times sampling was required.

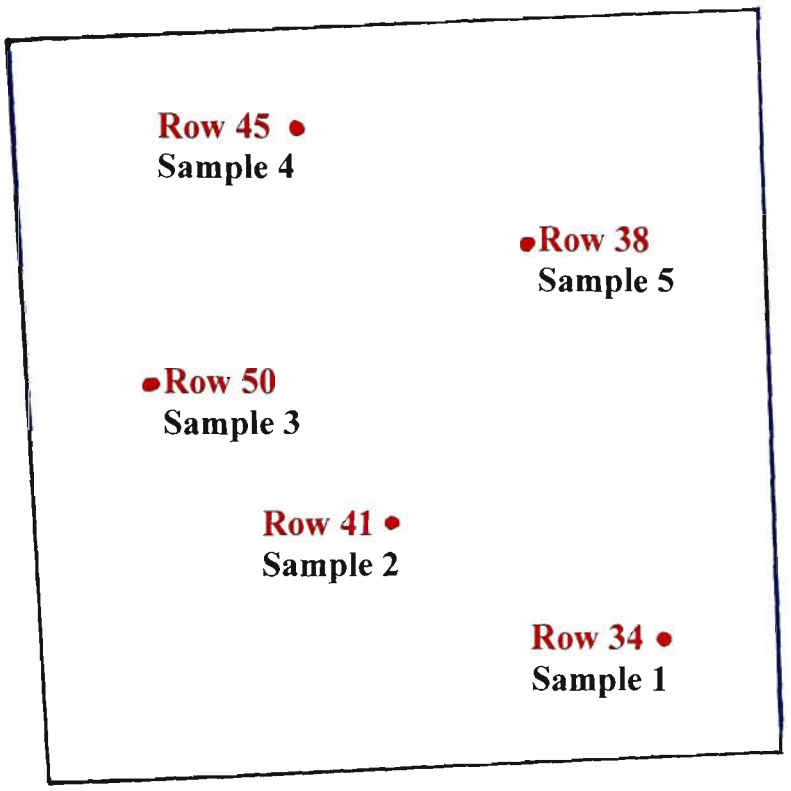
APPENDIX

VINEYARD SAMPLING SITE MAPS FOR 1997 AND 1998 VINTAGES

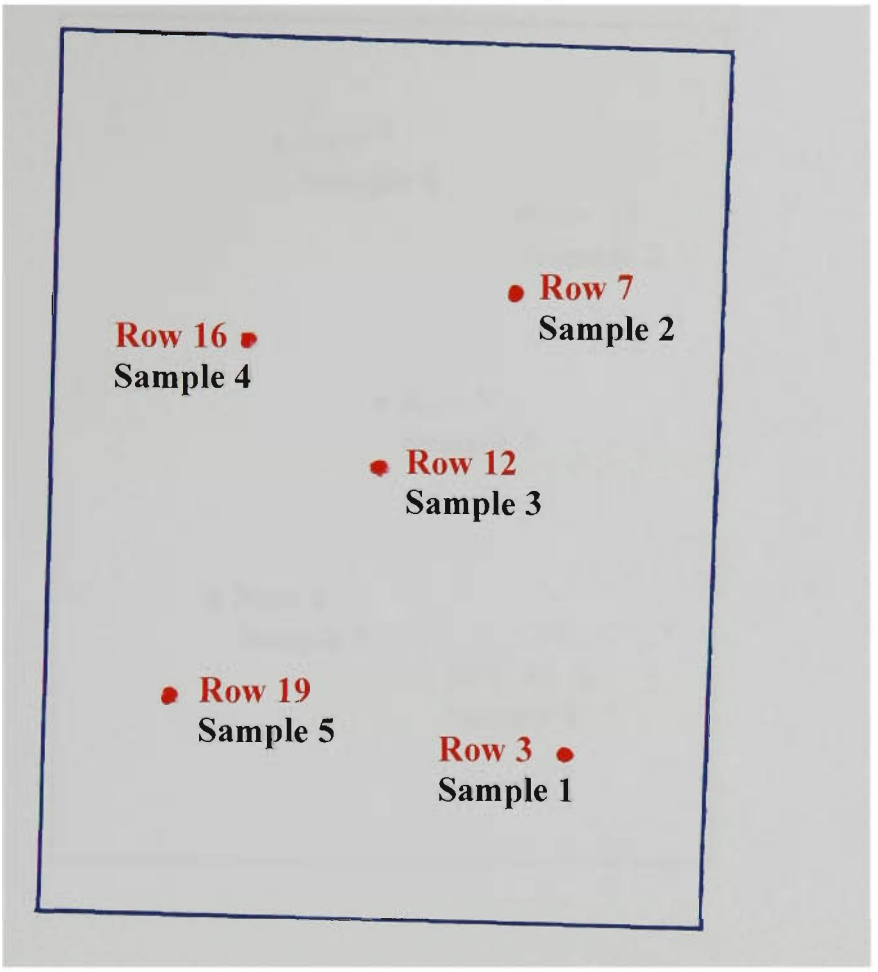
Samples A & K



Samples B & L



Samples C & N



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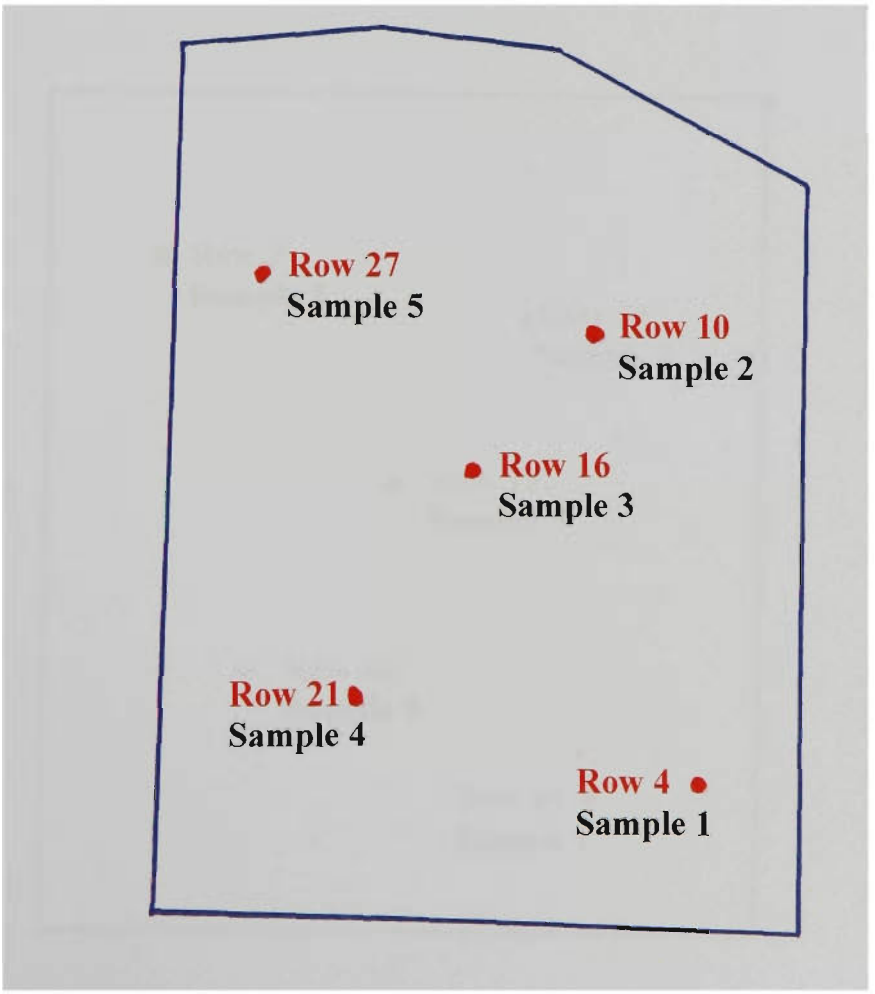
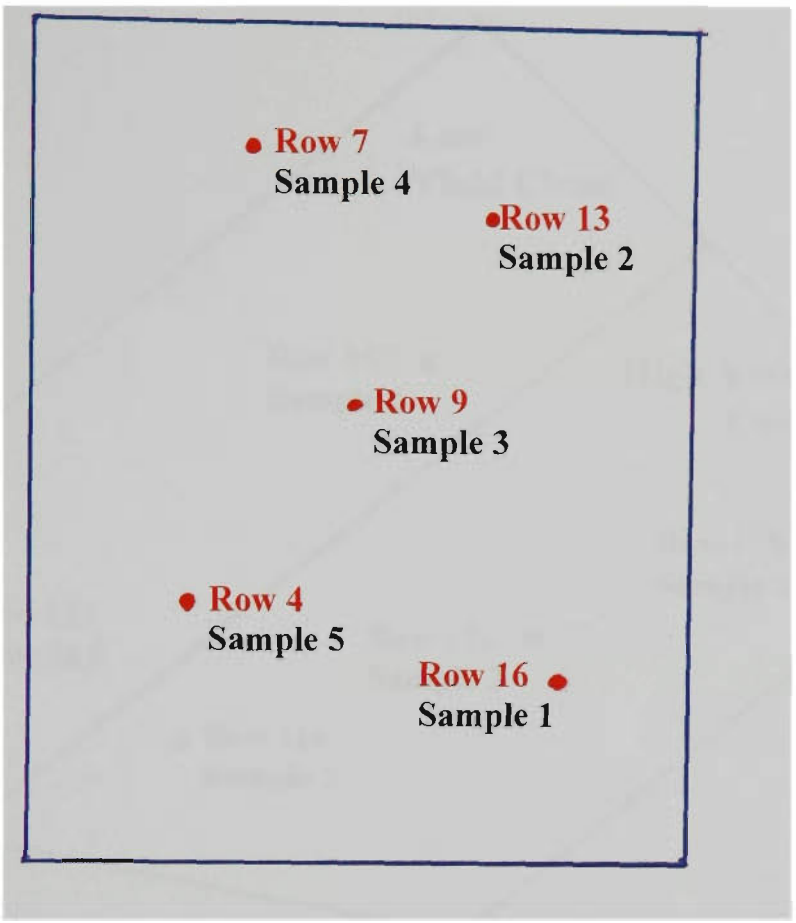


Samples E & P



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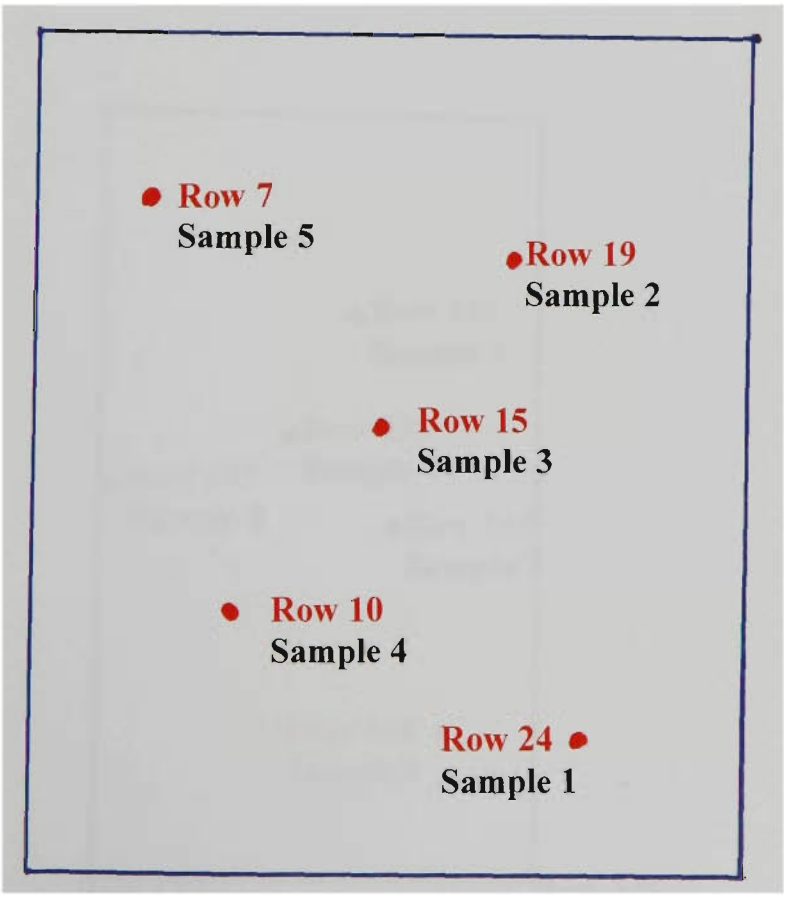

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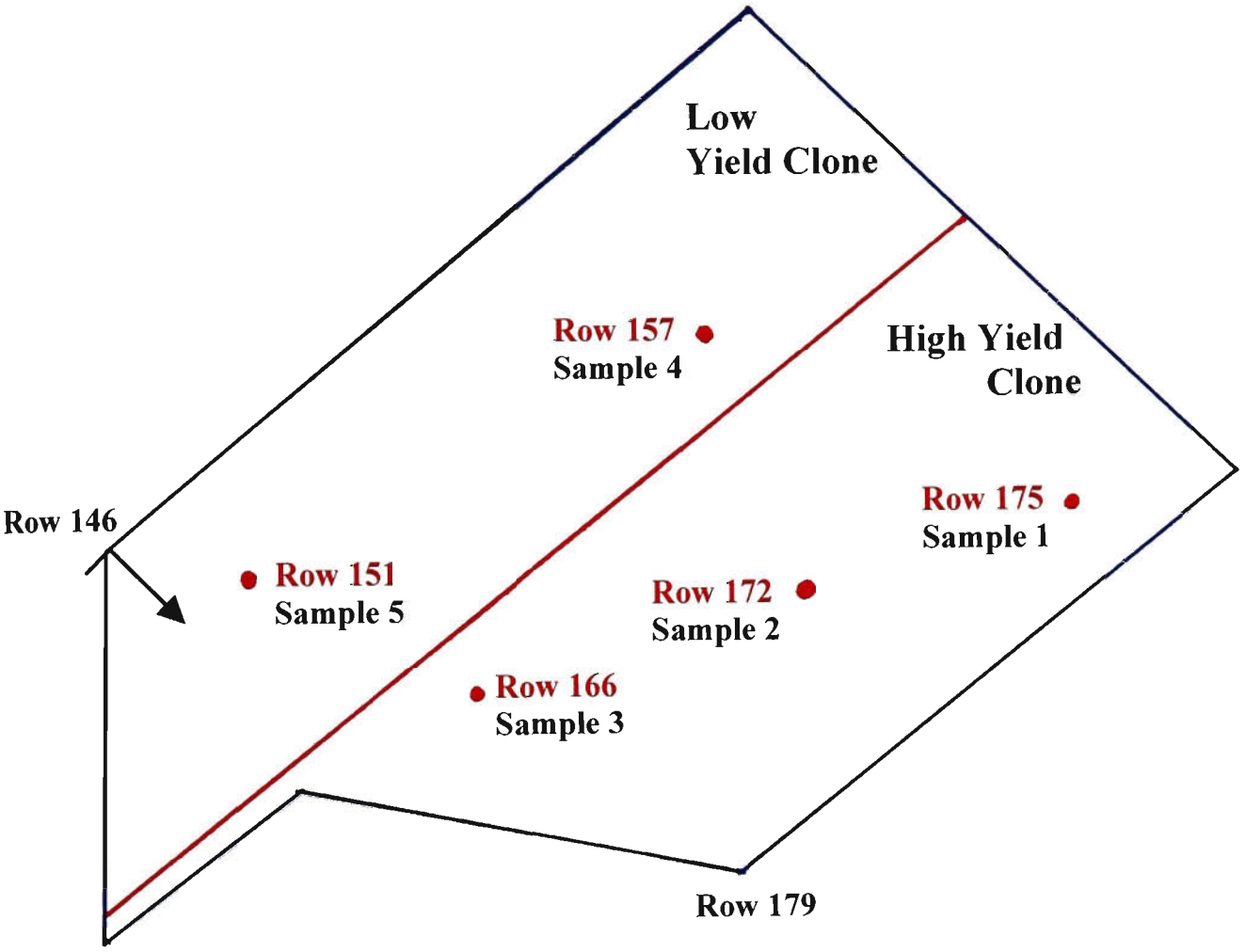

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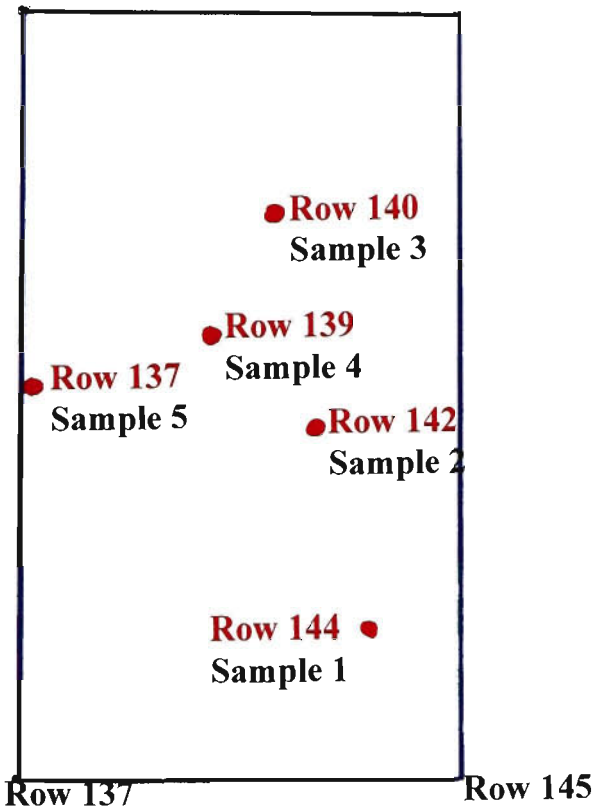
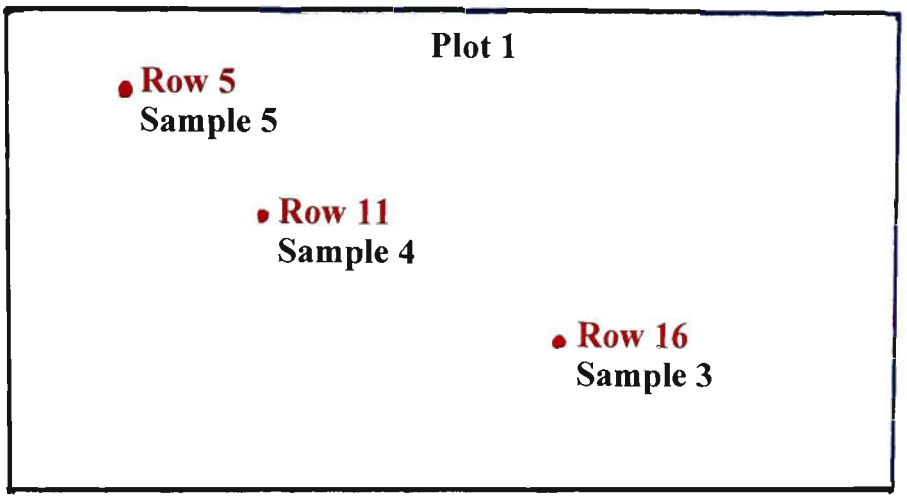
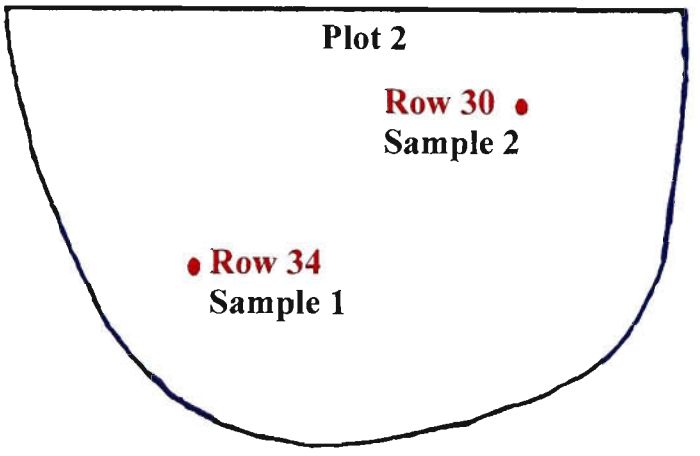


Sample I





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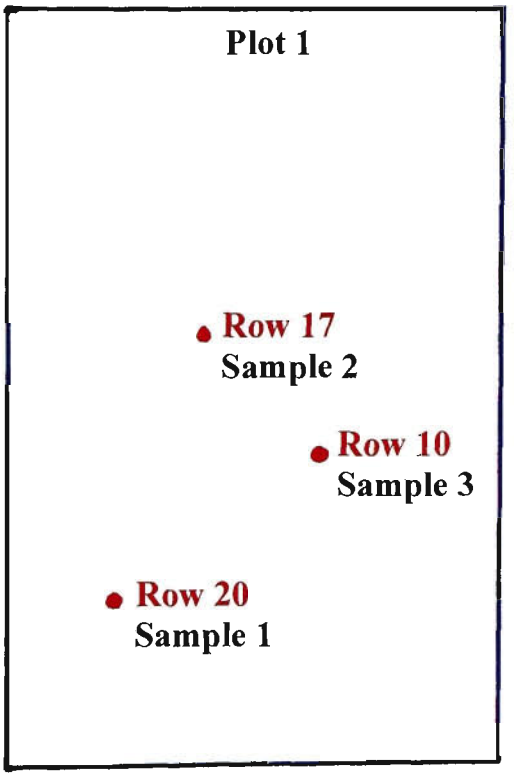

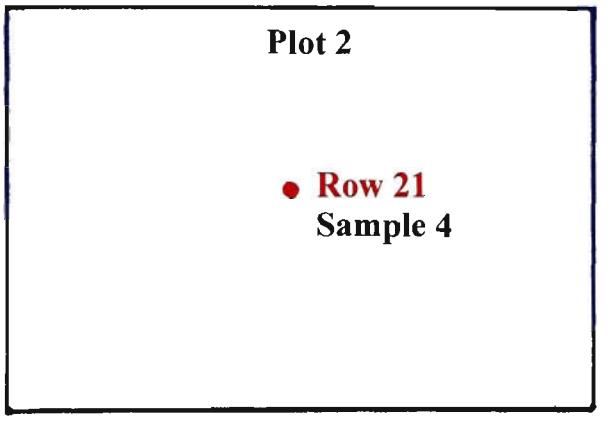

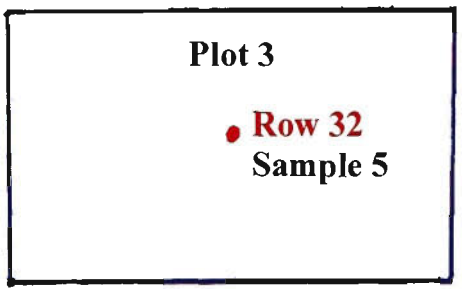

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"The dream has ended: this is the morning"
Aslan, from C.S. Lewis's 'The Last Battle'