Interfacing High Performance Liquid Chromatography with Inductively Coupled Plasma Mass Spectrometry for Speciation Studies

by

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Abstract

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A novel interface for the coupling of high performance liquid chromatography (HPLC) with inductively coupled plasma mass spectrometry (ICP-MS) for the speciation of organometallic compounds has been developed and utilised for a number of diverse applications.

Desolvation of the sample prior to reaching the plasma is shown to facilitate a more versatile coupling of the two instrumental techniques. This has been achieved by first volatilising the aerosol after nebulisation of the sample in a heated cyclone spray chamber. The resulting vapour is removed using a membrane drier and a thermoelectrically cooled condenser. The apparatus developed has been fully optimised to facilitiate the introduction of a range of organic solvents, particularly those used as mobile phases for HPLC separations such as acetonitrile and methanol. This interface also facilitates gradient elutions to be used with ICP-MS for the first time.

The interface developed has enabled the use of HPLC-ICP-MS to speciate the metabolites of a novel platinum based chemotherapy drug, JM-216, in human plasma ultrafiltrate. This approach offers a number of advantages over existing techniques especially in terms of speed of analysis and sensitivity. The platinum containing metabolites derived from this drug have been separated, identified, and compared to those arising from the breakdown of cisplatin and carboplatin in both plasma ultrafiltrate and an aqueous solution. A detection limit of 119 pg of Pt has been achieved for real samples.

The interface has also been used for the speciation of organotin and organolead compounds in environmental samples, and to investigate the speciation of metals in tea liquors. It was found that although the apparatus proved beneficial for both the determination of organotins and the identification of metal containing species in tea, its use adversely affected the analysis of organolead compounds. The results of these latter studies are critically assessed and discussed with respect to HPLC-ICP-MS interface design.

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A Versatile Desolvation System for ICP-MS

Signed.....

Date.....

Chapter 1

1.0 Introduction

Elemental speciation is one of the most important fields in analytical chemistry today. This is because the toxicity, bioavailability, and environmental mobility of an element is strongly dependant on its chemical form (1). The interest is speciation was initiated by a number of poisonings caused by methyl-mercury. This incident in Minamata (Japan), was the result of the ingestion of fish contaminated with methyl-mercury by pregnant mothers, causing severe brain damage to 22 infants born in the area (2). In another case in Iraq, the intake of wheat flour from seed treated with methyl-mercury caused the death of 459 people and the hospitalisation of a further 6530 (3). Since these early cases a number of other elements of interest in environmental and biological studies, including aluminium, antimony, arsenic, chromium, lead, mercury, tin and selenium have been the subject of speciation studies.

In order to undertake speciation studies on environmental samples the separatory powers of chromatography has been increasingly coupled with the sensitivity and selectivity of atomic spectrometric detection. This area has been extensively reviewed, with two major reviews being published in the mid 1980s covered the increasing use of gas chromatography (GC) coupled with atomic spectrometry (4) and liquid chromatography coupled with atomic spectrometry (5). Since then the use of inductively coupled mass spectrometry (ICP-MS) has expanded as a detector for chromatography due to the existence of commercially available instrumentation. The rise of ICP-MS has been charted by two further reviews (6-7). The growing application of supercritical fluid chromatography (SFC) and the commercial availability of a GC microwave

induced plasma atomic emission detector (GC-AED) instrument is recognised by their inclusion in a review by Uden (8).

High performance liquid chromatography (HPLC) is the most widely used chromatographic technique in quantitative chemical analysis (9). It can be used to separate non-volatile, thermally fragile and ionic species as well as solutes of molecular weights of greater than 10,000 Da without the need for alteration of the sample, such as, derivatisation when using gas chromatography. This is a distinct advantage as this may compromise the speciation of the analytes in a given sample. As such, the successful coupling of ICP-MS with the versatility of HPLC gives rise to a powerful tool for speciation studies capable of sensitive element specific detection.

1.1 Considerations for Interfacing HPLC with ICP-MS.

For elemental speciation the Inductively Coupled Plasma Mass Spectrometer is almost an ideal HPLC detection method. The ICP as an ion source is at atmospheric pressure so an elaborate interface, such as, in HPLC-MS is not required to reduce the pressure prior to introducing the sample to the ion source. The use of a mass spectrometer as a detector with such an efficient ion source offers significantly lower limits of detection than optical emission or atomic absorption detectors. However one potential limitation of HPLC-ICP-MS is the low tolerance of the plasma for many commonly used organic solvents in HPLC mobile phases. The behaviour of ICP-MS as a detector for liquid chromatography is therefore dominated by the composition and flow rate of the eluent. This in turn is dependant on the chromatography being used, normal phase HPLC requires the mobile phase to be more polar than the stationary phase so the eluent

is primarily composed of low polarity solvents, such as, hexane. The converse is true for reversed phase chromatography, but to optimise the chromatography, it is often necessary to alter the methods selectivity by adding organic solvents or dissolved salts to the mobile phase. ICPs are intolerant to large quantities of either of these components so mobile phase selection has to be made carefully. The column geometry being used is also an important factor. Varying the column diameter from 8 mm id (wide bore or semi-preparative columns) to 4.6 mm id (standard bore), 3.8 mm id (midbore) and 1-2 mm id (microbore) changes the flow rates possible from 10 ml min^{-1} to 100 µl min^{-1} whilst maintaining the same linear velocity (i.e. the dead time (t_0) of the column divided by the length of the column) of the eluent through the column. Low flow rates have the advantage that less analyte is introduced into the plasma so it is less prone to extinction due to solvent overloading. The disadvantages of this are that high efficiency nebulisers are required to maintain the limits of detection with less sample volume, and specialised microbore equipment is required as the separations are much more sensitive to dead volume effects.

Larger bore columns can be used with conventional HPLC equipment and larger quantities of the analyte can be analysed (2 ml as opposed to up to 500 µl) without overloading the HPLC column and reducing the resolution of the separation (10). However the high flow rate means that the solvent loading of the plasma is increased, causing an additional drop in sensitivity, as well as the possibility that any buffers present in the eluent such as citrate for example, could salt out causing blockages in the interface.

So the most critical part of the ICP-MS for coupling to HPLC is the method of sample transport to the plasma. If too little sample is transported, detection limits are adversely affected, but

efficient transport of sample to the plasma is accompanied by increased solvent transport. So the key is to efficiently transport the sample to the plasma without transporting the solvent.

1.1.2. Problems due to the effect of the eluent composition on the use of ICP-MS as an HPLC detector.

A number of kinds of chromatography require the use of organic solvents, or organic solvent gradients. These include reversed phase chromatography (chapter 3), cation exchange chromatography (chapter 5) and ion pair chromatography (chapters 3 and 4). The problem with the use of organic solvents is that ICPs are intolerant to their use in the eluent. Organic solvents such as acetonitrile, methanol and tetrahydofuran (THF) have a higher vapour pressure than water. This means that when they are nebulised, more solvent than normal is introduced into the plasma. This causes a change in the capacitance of the plasma resulting in a higher than normal reflected power. If the reflected power gets too high the plasma will shut off, tuneable circuits in the torch box can compensate for this, but not entirely.

Further problems occur at the interface between the plasma and the mass spectrometer. When organic solvents are introduced into a plasma, they are atomised but not oxidised. The soot produced builds up on the sampling and skimming cones resulting in a drop in sensitivity as the orifices become blocked. The soot can also build up on the ion lenses causing a further drop in the sensitivity of the instrument. This can only be remedied by cleaning the cones and removing the ion lenses from the vacuum chamber and cleaning them too if a small bleed of oxygen is not added to the plasma.

The use of dissolved salts in HPLC eluents poses a different set of problems. Ion pairing agents and buffers when used in too high concentration can block certain nebulisers, such as, the Meinhard concentric nebuliser. Also the salts can build up at constriction points in the aerosol flow, such as, the injector of the torch or the elbow between the spray chamber and the torch. At these points, solvent vapour can condense around the salt deposits forming large droplets after the spray chamber, if these droplets enter the plasma they can cause a sudden increase in the solvent loading of the plasma resulting in its extinction, so the instrument would have to be reignited and any HPLC run would have to be repeated.

To reduce the negative effects of organic solvents, a number of strategies can be employed all of which are designed to reduce the amount of organic vapour reaching the plasma.

The simplest method is to condense the solvent in the spray chamber by chilling it with a recirculating coolant pumped around a jacket on the spray chamber. This, when used in conjunction with a smaller bore injector in the torch can often be sufficient for most applications. The advantage of this approach is that it requires minimal changes to the normal instrument configuration and is compatible with normal bore LC flow rates of 1.0 to 2.0 ml min^{-1}. A disadvantage is that the smaller bore injector in the torch e.g. is more likely to become blocked if dissolved salts are used in the mobile phase.

An alternative approach is to reduce the amount of solvent entering the plasma by reducing the flow rate used. This can be achieved by interfacing microbore chromatography to a direct injection nebuliser (DIN) (11-13). The DIN is a microconcentric nebuliser that is inserted into the injector of the ICP torch. A fused silica sample transfer line is used to deliver the sample to

the metal nebuliser tip, this assembly is held in a ceramic support tube. The sample is nebulised into the base of the plasma by a concentric gas flow. It is operated at a flow rate of 30 to 100 µl min^{-1} which allows the use of large amounts of organic solvent, such as, 85% methanol in the mobile phase. It is reported that it could be used with gradient chromatography (13). The disadvantages of the DIN are its fragility, and the 30 µm capillary used can become blocked with ion pairing agents. Other disadvantages are that specialised microbore HPLC pumps are required to deliver such low flow rates. In addition, not every packing material or chromatography is designed to work with microbore technology. Usually it's limited to 3 to 5 µm particle sizes, and so strong cation exchange packing materials such as Partisil SCX which is not available in particle sizes less than 10 µm would not be useable.

1.2 The Effect of Solvents on Inductively Coupled Plasmas.

1.2.1 The Effect of Organic Solvents on the physical properties of an ICP.

When compared to an ICP loaded with an aqueous aerosol, the introduction of an organic solvent changes the characteristics of the discharge considerably. Visually, the most apparent feature is the green emission in the aerosol channel resulting from C_2 emission. Accompanying this visual change are changes in the physical properties of the plasma. An ICP loaded with an organic aerosol requires an r.f power increase of 0.5 kW compared to an aqueous ICP (14). The reasons for this are that the organic solvent increases the enthalpy of the carrier gas and species such as C_2, CN, and CO formed by dissociation of the solvent increase the thermal conductivity of the aerosol channel. Blades and Caughlin (15) compared a plasma loaded with water to one loaded with xylene. They found that at a fixed power and height in the plasma the temperature is

lower for an organic aerosol loaded plasma. This correlated with a reduction in the electron number density in the plasma. With an organic aerosol present there is less energy available for ionization of the support gas, again leading to reduced electron number density values.

These two effects can be explained by the different chemical composition of the ICP when loaded with organics compared to an aqueous one. In an water loaded plasma the most abundant molecular species in the central channel is OH. In an ICP loaded with organics it is C_2 (16).

The primary factor that leads to a reduction in temperature and electron number density is the change in thermal conductivity of the aerosol channel due to the transport of energy of reaction through molecular dissociation and ionization.

These species when dissociating absorb energy from the plasma, at lower heights, C_2 molecules at the centre of the aerosol channel diffuse to the hot edge where they are dissociated. This process consumes energy from the surrounding gas, since the dissociation energy of C_2 is 6.5 eV and that of OH is 4.3 eV (15) it is expected that the thermal conductivity of the central channel is higher for an organic ICP. This transfer of energy to the C_2 dissociation equilibrium is why an ICP loaded with an organic aerosol requires about 0.5 kW of additional r.f. power.

In a later paper Weir and Blades (17) investigated the characteristics of an ICP operating with organic aerosols, concentrating on spectral and spatial observations. The response of an ICP to loading with water, methanol, and chloroform was compared. It was noted that the central channel became less diffuse when loaded with water and that the plasma had moved up by 0.5 -

1.5 mm. Similar changes for a plasma loaded with chloroform were observed. By contrast, methanol produced much larger changes. The plasma translated down stream and the central channel dilated indicating that methanol causes a "thermal pinch effect".

The ICP is relatively insensitive to water loading, but methanol loading drastically alters how much energy is available at the central channel. At maximum methanol loading, the discharge has effectively folded in on itself, retracting from the analyte so that the plasma interacts incompletely with the analyte if at all.

1.2.2. Factors Affecting Organic Solvent Load of a Plasma

Since the excitation characteristics strongly depend on the interaction of pyrolysis products produced by the solvent in the plasma, it can be assumed that the solvent load of a plasma is of decisive importance in determining plasma stability.

One of the first studies carried out into the factors affecting the solvent load of a plasma when using organics was carried out by Boorn et al. (18). The extent to which evaporation effects the droplet size of the organic aerosols was demonstrated. Using theoretical evaporation curves for a number of organic solvents, they found that for a 2.5 μm initial diameter droplet with a residence time of 0.7 s in the spray chamber, evaporation must play a considerable part in the improvement of nebulization efficiency when certain organic solvents are used instead of water. This is due to an evaporation induced shift in droplet size distribution to smaller droplets that are less likely to be removed from the spray chamber. With a higher evaporation rate, more of the solvent exists in the vapour phase. Vapours do not pass entirely through the central channel of

the plasma, a certain percentage passes around the base of the plasma. This can influence the energy transfer processes detailed in section 1.2.1. in the energy addition region of the plasma.

In a later paper, Boorn and Browner (19) studied the effects of thirty different solvents on ICPs'. Plasma stability with the solvent was estimated by finding the "limiting aspiration rate" of the solvent, this is defined as the maximum aspiration rate at which a stable plasma operates for 1 hour. These rates correlated with the evaporation factor E for the solvent, showing that evaporation is an important process in determining the tolerance of a plasma to a particular solvent.

E is defined in equations 1.1 and 1.2.

Equation 1.1

$$d = (d_0 - Et)^{\frac{1}{3}}$$

Where d is the droplet diameter (μm) at time t (s) after formation and d_0 is the initial droplet diameter

Equation 1.2

$$E = 48\, D_v \sigma\, P_s\, M^2 (\delta RT)^{-2}$$

Where D_v is the diffusion coefficient for the solvent, M the molecular weight, R the gas constant, T the absolute temperature, P_s the saturated vapour pressure, σ the surface tension and δ the density.

The conclusion was that solvents less volatile than water required no change in operating conditions, where as those of a higher volatility require an increase in r.f. power and a cooled spray chamber to remove solvent vapour so that a stable plasma can be obtained.

Maesson et al. (20) investigated under what conditions stable plasmas could be generated for a variety of organic solvents. Special attention was paid to ignition procedures, aerosol cooling, and the prevention of carbon deposition on the torch. The most difficult solvents were methanol, ethanol, propan-2-ol, toluene, and chloroform. All of these required aerosol cooling to enable the generation of a stable plasma. By using a continuous weighing system the chloroform loading of a plasma measured with aerosol cooling down to -16°C was compared to the water loading without cooling at a liquid flow rate of 0.8 ml min^{-1}. The water loading was found to be 0.02-0.03 g min^{-1}, where as the chloroform loading was 0.2-0.3 g min^{-1}, an increase of an order

of magnitude. This work was continued to study what parameters affect the solvent load of a plasma (21) for chloroform, methanol and water.

By measuring the plasma solvent load at different liquid uptake rates, it was found that the plasma solvent load reaches a plateau at about 1ml min^{-1}. This shows that the liquid uptake rate is too insensitive to be used as a criterium for plasma stability. Maessen *et al.* (20) proposed the use of the "maximum tolerable solvent plasma load" as a more useful criterium. This is defined as a solvent load below which the reflected power does not exceed 75 W and the plasma is stable for 8 hours with minimal operator interference. For chloroform the maximum tolerable solvent plasma load was > 10.7 mg sec^{-1} and for water it was > 0.6 mg s^{-1}. Methanol had a maximum tolerable solvent plasma load of 1.5 mg s^{-1} which meant at a liquid uptake rate of 1 ml min^{-1} the load was too high for the generation of a stable plasma.

The solvent loading as a function of condenser temperature was investigated, at low temperatures (21) (< -20° C) the curves are flat suggesting that reducing the temperature further does not reduce the plasma solvent load. At this temperature all of the solvent is present as an aerosol rather than as a combination of aerosol and vapour. Above -10°C the slope is steep so above this temperature, small temperature increases cause large increases in plasma solvent load. This demonstrates how important it is to keep the spray chamber at a fixed temperature. The conclusion was that solvents could be classified into easy or hard to operate a plasma with, according to their vapour pressure (Table 1.1). Those with a low vapour pressure such as water, xylene, and MIBK are "easy", those with a high vapour pressure such as chloroform, methanol, and ethanol are "hard". The effect of volatility is that through a shift in particle size, the rate of

Table 1.1: The Evaporation factor (E-factor) and saturated vapour pressure for selected solvents (from reference 21)

Solvent	E-factor ($\mu m^3 s^{-1}$)	Vapour Pressure at 20^0C (mm Hg)
Water	13.1	18
Xylene	18.5	4
MIBK	77.3	5
Chloroform	321	105
Methanol	47.2	105
Ethanol	45.6	120

solvent delivery to the plasma is increased and the saturation vapour pressure affects through increased solvent load, the excitation conditions in the plasma.

1.3 Methods of Removing Organic Solvents From the Carrier Stream.

Since the organic solvent load has such a critical effect on the stability of an argon plasma, in order to use high levels of organic solvents in an HPLC eluant it is necessary to reduce the plasma solvent load to tolerable levels.

This has been done in a number of ways. The most common and effective way is to desolvate the aerosol and remove the organic solvent vapour.

1.3.1 Methods of Desolvating an Aerosol

In order to be able to remove the organic solvent from the carrier stream, all of the solvent present must be in the vapour phase so that the remaining analyte particles are dry i.e. desolvated. This can be achieved by volatilising the solvent present in the aerosol phase.

A number of methods have been reported to achieve this, these are:- a heated spray chamber or area after the spray chamber, an ultrasonic nebuliser or a thermospray.

One of the first attempts to use a heated spray chamber to desolvate the aerosol before introduction of the sample to a plasma was made by Veillion and Margoshes (22). A commercial unit designed for use with chemical flames was modified. The spray chamber was heated with heater tape and gave a sample transport efficiency of 35 %. Since then the technology has been modified and is available commercially (23) with a high efficiency nebuliser and gives an analyte transport efficiency of 24 %.

Eastgate *et al.* (24) compared the effects of heating the spray chamber radiatively or conductively whilst designing a commercial system. The result was that conductive heating lead to instability due to irregular evaporation and pooling of the analyte, salt deposits also built up on the inside of the spray chamber where droplets had impacted and then evaporated. Radiative heating was found to be superior, it had a much shorter memory time and the RSDs were improved from an average of 9.3% for conductive heating to 0.83% for radiative heating.

Ultrasonic nebulisers (USNs) have been used since the advent of ICPs (25), this type of nebuliser produces aerosols efficiently that are then easily vapourised in a separate heating chamber (26). USNs are now commercially available from a number of sources. They work by using ultrasound to break the liquid into small droplets. An ultrasonic generator drives a piezo electric crystal at a frequency of 200 kHz to 10 MHz. The longitudinal wave produced propagates towards the liquid air interface where it produces a pressure that breaks the surface into a dense aerosol with a small particle size. This aerosol is then volatilised in a heated tube to produce solvent vapour and desolvated analyte particles (27).

Thermosprays were originally developed as an interface between LC and MS (28). The LC eluent is pumped through a heated capillary where it is vapourised. The temperature of the capillary is precisely controlled to produce a super heated aerosol carried in a supersonic jet of vapour with the analytes retained in the droplets of the aerosol. If the temperature is too high, vapourisation occurs prematurely in the capillary leaving the analyte and any dissolved salts deposited on the inner walls leading to blockage.

The use of a thermospray to interface flow injection analysis (FIA) with ICP-OES was first reported by Koropchak and Winn (29). The authors found that although the jet exiting the thermospray contained aerosol droplets and solvent vapour, free expansion of this led to rapid cooling and condensation causing enlargement of the aerosol droplets in the jet. To prevent this, the jet was allowed to enter a heated expansion chamber to maintain the solvent in the vapour phase. Thermosprays have been found to improve the sensitivity of ICP-OES (30) and once optimised are as stable as pneumatic nebulisers (31).

1.3.2. Methods of Removing the Solvent Vapour.

Before the desolvated analyte can be introduced to the plasma, the solvent vapour has to be removed. This may be achieved by a number of techniques all of which are variations on two main themes, i.e. condensation or removal by a membrane separator.

1.3.2.1 The Use of Condensers to Remove Solvent Vapour.

Veillion and Margoshes (22) used a modified Freidrichs condenser to remove water vapour from an aerosol stream, this was cooled to 10°C and was reported to have removed most of the water vapour present as there was no OH band spectra in the argon plasma discharge. Boumans and DeBoer (26) used a modified reflux condenser to remove water vapour after nebulisation with a USN.

The use of a condenser to remove organic solvent vapour before sample introduction to an ICP was first reported by Maessen and co-workers (20-21), the condenser consisted of a coiled tube immersed in a liquid cooling bath mixture of dry ice and organic solvent to reach temperature down to -20°C. This approach was also used by Wiederin *et al.* (32) and Alves *et al.* (33-34). An alternative approach is to use electronic temperature control using peltier effect coolers. This was first proposed by Weir and Blades (35) and has been adapted for use in commercial instruments (23-24). This approach is more compact and convenient as large coolant baths are not required and temperature control is achieved by altering the power supply to the coolers.

1.3.2.2. The Use of Membrane Driers to Remove Solvent Vapour.

The use of membrane separators to remove solvent vapour was first reported by Gustavsson (36), a silicone membrane was used and the solvent was removed by a vacuum. Organic solvents are soluble in the silicone polymer membrane and permeate through the membrane to the vacuum side. In a later paper it is reported that after optimisation upto 99% of the solvent is

removed (37). Botto and Zhu (38) have reported the use of a microporous PTFE membrane. This is heated to 160°C and the solvent is removed by an argon purge gas flow of 700 ml min^{-1}. This was reported to have removed upto 99.7 % of the solvent.

1.4 Aims of Study

The work described in this thesis is directed towards developing an interface for coupling HPLC to ICP-MS. This is so that acetonitrile and other commonly used organic solvents such as methanol, ethanol and propan-2-ol can be introduced using solvent gradients without the need to continually adjust the instrument to compensate for solvent loading of the plasma. This was achieved by reducing the solvent loading of the plasma to such an extent that it was no longer a major factor in determining the sensitivity of the instrument.

The method used in this study to reduce the solvent loading of the plasma was to desolvate the aerosol and then remove the solvent vapour. In this thesis the use of a heated spray chamber to produce a solvent vapour was investigated. The solvent vapour was successfully removed by using a membrane drier and a condenser in series. Having successfully developed a working desolvation apparatus, its use was applied to a number of chromatographies such as reversed phase, cation exchange and ion pairing chromatography in order to determine its versatility.

Chapter 2. Overview of Instrumentation

2.1 Inductively Coupled Plasma Mass Spectrometry (ICP-MS)

The techniques used in the PlasmaQuad ICP-MS for interfacing an inductively coupled plasma to a mass spectrometer were first developed by Dr Alan Gray in 1979, the VG PQ2 evolved from this pioneering work. The FI Elemental PQ2+ is a continuation of the evolutionary process.

2.1.1 Overview of Principles behind the Inductively Coupled Plasma.

The ICP is generated by coupling energy from a radio frequency generator into a suitable gas via a magnetic field surrounding a water cooled, three turn, copper coil. The gas flows through a quartz torch which consists of three concentric tubes, and is mounted axially in the copper load coil. To start the plasma, the gas streams are seeded with free electrons by a tesla coil. This produces a potential large enough to overcome the dielectric resistance of the gas, the electrons accelerate in the electric and magnetic fields associated with the load coil and ionise the gas in the field. The plasma is sustained by the fluctuating electric and magnetic fields in the load coil which couple energy to the plasma by continuing to accelerate free electrons on the periphery of the plasma into a zone within the load coil. Collisions with other gaseous atoms causes further ionisation so the plasma becomes self sustaining. The ions and electrons flow in the horizontal plane of the coil heating the gas by collisional exchange so a hot "fire ball" is produced. The sample is introduced via an injector gas flow which punches a hole through the plasma producing a torus.

The frequency of the RF generator is crystal controlled at the frequency of 27.12 MHz. The required output level (1350-2000 W) is achieved by a series of transistorised power amplifiers. The RF load coil in the torch box is designed to form the inductance in a tuned circuit and presents a purely resistive 50 ohm load to the generator when the plasma is alight. The inductance of this circuit changes with changing plasma conditions, so a motorised tuning capacitor is used to vary the capacitance and maintain the tuning of the circuit.

Matching by the torch box mechanism ensures that if the current and voltages show phase differences, the reflected power back to the generator is kept to a minimum.

2.1.2 Interfacing an ICP with a Mass Spectrometer

The ICP has long been known to be an efficient ion source, and mass spectrometry is recognised as giving high sensitivity. The problems related to coupling the two stem from the fact that the ICP operates at atmospheric pressure, but a mass spectrometer requires a high vacuum. These problems were overcome by the research groups of Gray and Fassel (39) and Gray and Date (40).

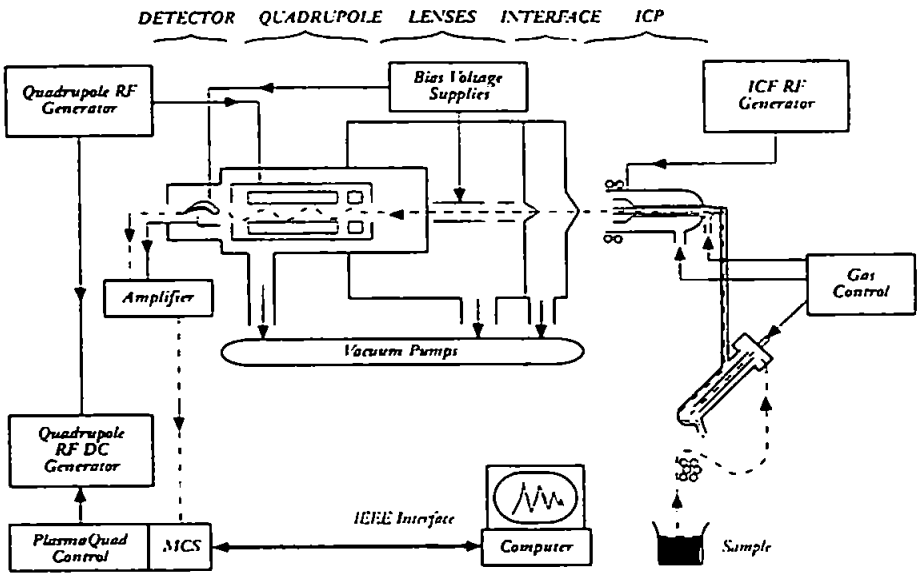
The function of a successful plasma sampling interface is to transfer a representative sample of the plasma to the mass selective detector. The FI Elemental PlasmaQuad has an interface based upon that in reference (40) (Figure 2.1). The plasma is sampled through a water cooled orifice, or sample cone. The size of this orifice is designed to ensure a continuum flow into the vacuum system, this is to prevent the sampling of the high oxide levels found at the boundary layer,

where the hot plasma meets the cooled sample cone. Continuum flow occurs when the ratio of the free mean path of the gas species present to the diameter of the sampling aperture is less than 10^{-2}.

The ions sampled from the central channel of the plasma pass through the aperture into a partially evacuated region called the expansion chamber. Here the sampled gas expands as a supersonic jet enclosed in a shock wave known as the barrel shock. This effectively isolates it from the gas in the expansion chamber. This shock wave has a shock front (the Mach disk) where the expansion of the gas jet is prevented from propagating further by the background gas pressure in the expansion chamber. A second orifice, the skimmer cone protrudes through the mach disk, into the "zone of silence", and skims the ions emerging from the sampler cone, so that a small percentage of the ions pass through this orifice into a second evacuated region, where the ion beam is focused by a series of ion lenses. The focused ion beam passes into a third vacuum stage containing the quadropole where the ions are separated by their mass to charge ratio and are then detected by an electron multiplier.

Further information and background on the principles and development of ICP-MS can be found in references (41-48).

Figure 2.1: A Schematic of the FI Elemental PQ 2+ ICP-MS.



2.2 Data Acquisition

For the analysis of data captured by HPLC-ICP-MS, the software of the data capture device must be capable of displaying the signal from one or more mass channels over a period of time.

Typically, two data capture modes are available with the FI Elemental PlasmaQuad ICP-MS. The first is single ion monitoring, this is when just one mass is monitored over a period of time. In early versions of ICP-MS software, the dwell time, and the number of time channels required were altered until a suitable length of time to acquire the entire chromatogram was attained. In more recent versions of software, the total analysis time is set by the operator, and the computer software automatically sets the dwell time, plus the number of time channels required. The data acquired in this way is then saved as an ASCII text file, it then has to be exported to a third party package for analysis, ie the peaks have to be integrated with a suitable baseline to find the peak areas. Since most business spreadsheets, for example, Excel (Microsoft, Redmond, WA, USA) don't have such a facility, it is necessary to find a scientific spreadsheet with an area under the curve analysis facility. The spreadsheet used for this work was Fig-P (Biosoft, Cambridge, UK).

The second data capture mode is called time resolved analysis (TRA), this is a multimass detection mode, the chromatogram is acquired as discrete time slices during which the quadropole peak jumps between the required masses. Again, the software has evolved over time. In the initial versions, the dwell time, the number of DAC steps, and the number of sweeps per time slice had to be chosen by the operator so that a suitable time per slice could be used (typically 1 s per slice). The acquisition was then aborted and saved when the chromatographic run was complete. In the more modern versions of the software, the time per slice, and the total

analysis time are set by the operator, the other parameters are then set by the computer, or left at default values. Again the problem has been that to analyse the data, it has to be exported to third party packages. In the past, this was complicated by the fact that TRA data files were saved as machine code, so the data for each mass had to be extracted from the file, converted to a single column ASCII text file and then exported. In later versions, the data was saved in the ASCII format, the most modern software is capable of exporting the data in a format such that it can be read by Mass Lynx (FI Organic Mass Spectrometry, Cheshire, UK), an HPLC/GC-MS data analysis package with full chromatographic integration capabilities.

2.3 High Performance Liquid Chromatography (HPLC) Instrumentation

The HPLC pump used is the Star 9010 solvent delivery system (Varian Chromatography Systems, Walnut Creek, California, USA). This is a single piston pump, with pulse damping hardware that can deliver flow in a linear dynamic range 10 μl min^{-1} to 5 ml min^{-1}. The proportioning head can deliver 3 solvents that are then mixed in a separate unit after the pump head. The inert system uses titanium or PEEK (polyetheretherketone) instead of stainless steel components where contact with the eluant occurs. Samples were injected using a chemically inert injection valve (Cheminert Model C 1 valve, Valco Instruments Co. Inc., Houston, Texas) fitted with a 200 μl PEEK injection loop.

2.4 Overview of High Performance Liquid Chromatography

2.4.1 Development and Instrumentation

The general principle of chromatography is that two immiscible phases, one stationary (the stationary phase) and the other mobile (the mobile phase or eluent) (typically a solid and a gas/liquid, respectively) are brought into contact. A sample is introduced into the mobile phase and the solutes in that sample undergo a repeated series of partitions between the mobile and stationary phases. When these phases are properly chosen, the solutes present in the sample separate into distinct bands in the mobile phase and emerge in the order of increasing interaction with the stationary phase (49).

The idea of packing the stationary phase into a column with the mobile phase passing through it (column chromatography) was devised and named by the Russian botanist Mikhail Tswett shortly after the turn of the century (50). He employed this technique to separate various plant pigments such as chlorophylls by passing solutions of these compounds through a glass column packed with finely divided calcium carbonate.

This method for separating mixtures was further improved when Martin and Synge (51) realised that to obtain efficient separations in a short time, very small particles were required with a mobile phase forced through the column at high pressure. However it was not until the 1960s that the technology existed for producing and using packing materials with uniform particle size diameters of 3-10 μm (50). These columns once produced require more sophisticated methods

of mobile phase delivery than the simple gravity fed columns of classic chromatography. A schematic representation of a typical HPLC system can be seen in Figure 2.2.

As can been seen in Figure 2.2, an HPLC system consists of a number of basic components, these are:- i) a mobile phase delivery system ii) a sample introduction system iii) a chromatographic column, and iv) a detector.

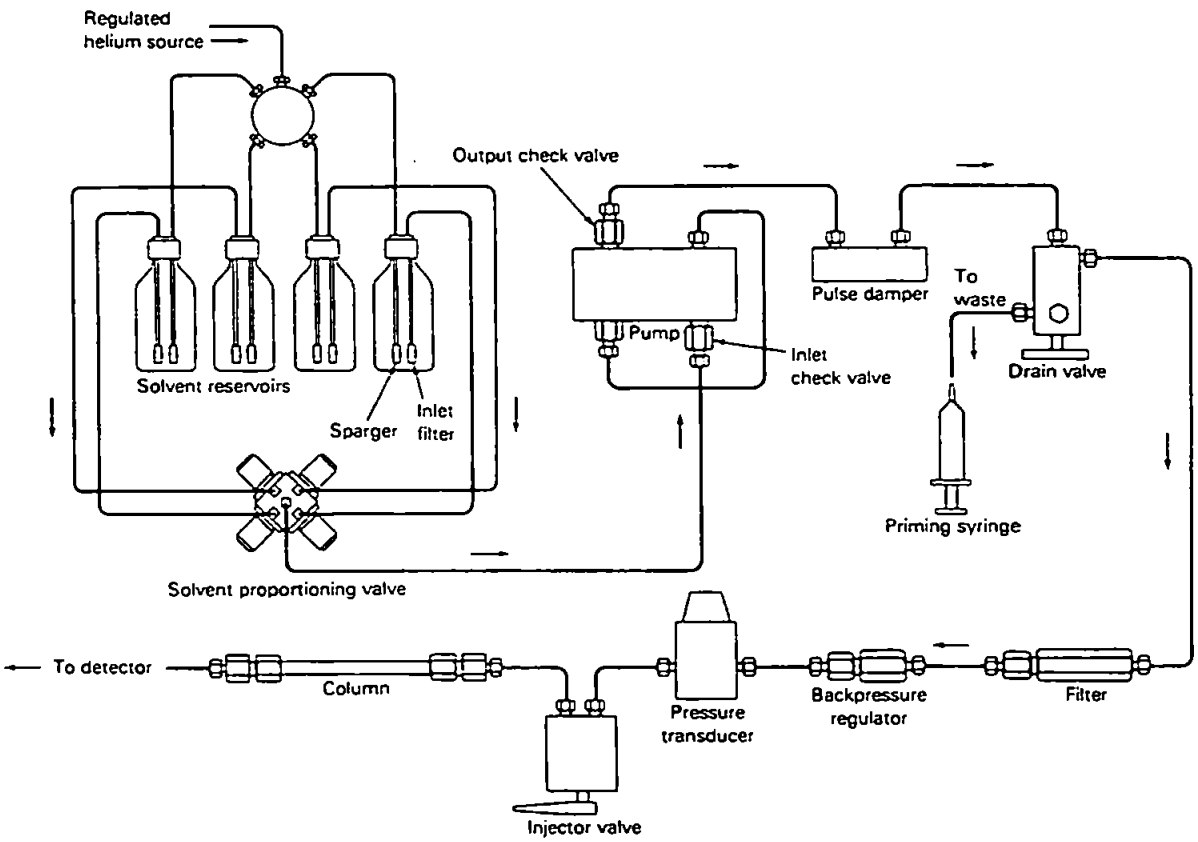
2.4.1.1 The Mobile Phase Delivery System

An HPLC pump should be able to operate at pressures of up to 400 atm (6000 psi), and supply a pulse free output at flow rates ranging from 0.1 to 10 ml min^{-1}. It should also be chemically resistant to the mobile phase.

There are three main ways of achieving this goal. The most common is the use of reciprocating pumps. These produce a pulsed flow which must be damped by one of a variety of methods depending on the manufacturer (50). In essence the solvent is pumped by a motor driven piston and check valves control the flow of solvent in and out of the pump head cylinder. By virtue of their design, this type of pump is readily adaptable to gradient elution and can operate at pressures up to 10,000 psi.

Syringe-type pumps work through a positive solvent displacement by a piston mechanically driven at a constant rate. A disadvantage of this type of pump is that the solvent reservoir has a

Figure 2.2: Schematic Representation of a Typical High Performance Liquid Chromatography System. (From ref 50)



finite capacity (250-500 ml) before it needs to be refilled. However pulse damping is not required with this type of pump (49).

The simplest type of pump is the pneumatic pump, where the mobile phase is held in a collapsible container held in a vessel pressurised by a compressed gas. These pumps are inexpensive and are pulse free, but have a limited capacity and pressure output.

2.4.1.2. Sample Injection System

The most widely used method of sample introduction is based upon the use of a sampling loop configured as in Figure 2.3. To prevent overloading of the column the volumes used must be in the microlitre range up to a maximum of 500 µl. The sample must also be introduced without depressurising the system.

2.4.1.3. Separation Columns

Columns are constructed of stainless steel, PEEK or glass-lined metal tubing. The interior of the column must be smooth with a very uniform bore internal diameter. Column packings consist of particles that are uniformly sized and mechanically stable. Particle diameters lie in the range of 3-10 µm. The particles are retained in the column by frits with smaller pore sizes in each end of the column.

2.4.1.4. Detectors

A wide variety of detectors have been developed for use with HPLC based upon many spectroscopic and physical methods capable of producing a signal proportional to the level of analyte in the chromatographic peak. Ultraviolet-visible spectrophotometers make up 70% of all detection systems in use (49). This type of detector is commercially available in fixed, variable or scanning wavelength detector modes with simultaneous detection of light absorption using a diode array photocell. Other methods include fluorimetric, electrochemical, conductivity and mass spectrometric detection.

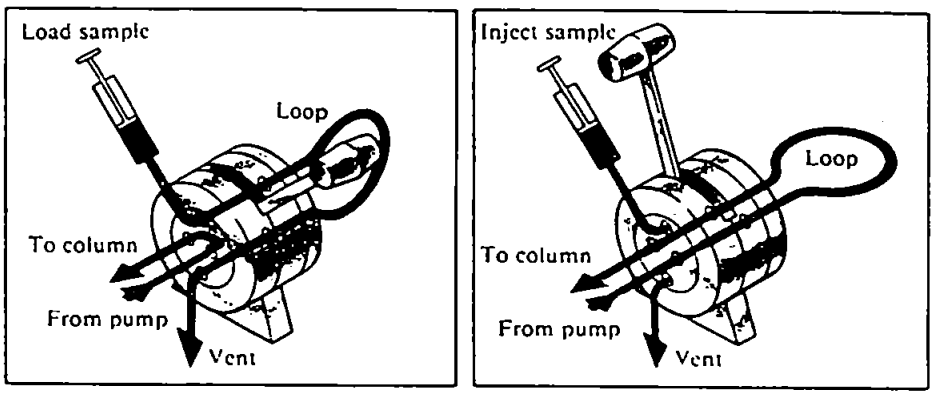
2.4.2. HPLC Separation Mechanisms

Although there are many modes of HPLC separation, only those used in the course of this study will be described in detail. These include reversed phase, ion-pair, and cation exchange chromatography.

2.4.2.1 Reversed Phase Chromatography

Reversed phase chromatography is used to describe the situation when the stationary phase is less polar than the mobile phase. This is opposed to normal phase chromatography where the stationary phase is more polar than the mobile phase.

Figure 2.3: A Typical Sample Introduction Injection Valve for HPLC (From ref 50)



Most reversed stationary phases are n-alkanes or other organic moieties chemically bonded to silica or polymeric supports (52). The alkyl groups come in a variety of chain lengths, usually:- ethyl (C-2), octyl (C-8), or octadecyl (C-18).

In reversed phase chromatography partition occurs between the bonded organic phase and the mobile liquid phase. Water cannot wet the hydrophobic alkyl groups and so does not interact with them in any way. Polar substances prefer the mobile phase and elute first; as the hydrophobic character of the analyte increases, retention increases (52). To reduce retention it is necessary to lower the polarity of the mobile phase.

The accepted mechanism for retention is that only highly polar or ionic solutes can interact with the three dimensional network of intermolecular hydrogen bonds in water. Non-polar molecules are "squeezed out" of the mobile phase and interact with the hydrocarbon moieties of the stationary phase. Therefore in reversed phase chromatography the driving force for retention is the effect of the mobile phase in forcing the solute into the bonded organic layer. In opposition to this effect is the interaction of the solutes polar groups with the mobile phase. As such hydrophobic retention involves the non-polar portions of molecules and can be lessened by adding any organic solvent miscible with water (49). A detailed review of the proposed mechanisms has been given by Dorsey et al. (53).

2.4.2.2. Ion-Pair Chromatography

Ion-pair chromatography can be considered a subset of reversed phase chromatography which can deal with ionised or ionisable solutes on reversed phase columns (54). This method originated in the field of solvent extraction (55). It was found that an ionised compound (A^+_{aq}) that is water soluble can be extracted into an organic solvent by using a suitable counter ion (B^-_{aq}) to form an ion-pair by the equation: $A^+_{(aq)} + B^-_{(aq)} \rightarrow (A^+B^-)_{(org)}$. In ion-pair chromatography, an ion-pairing agent (a large organic counter ion) is added at low concentration (approx 0.005 M) to the mobile phase. The ion-pairing agent is ionised in solution, one ion is retained by the stationary phase providing it with a charge, this charged ion-stationary phase "complex" can then retain and separate organic solute ions of the opposite charge by forming a reversible ion-pair complex. So in effect the ion-pairing agent is loaded onto the neutral stationary phase. The other method for retention is that the analyte ion forms a neutral ion-pair in the mobile phase with the charged organic counter ion and this is retained by the stationary phase. Both mechanisms are thought to play a part resulting in the analyte being retained not only by the interaction of its hydrophobic portion with the stationary phase, but also by its charged portion interacting with the ions loaded onto the stationary phase. This dual mode of retention allows for unique separation not otherwise attainable by either reversed phase or ion exchange chromatography.

Typical ion-pairing agents for cations are anionic surfactants such as alkane sulphonic acids. Anions form ion-pairs with cationic salts such as, tetrabutylammonium salts.

2.4.2.3. Ion Exchange Chromatography

A stationary phase capable of ion exchange has electric charges on its surface (52). Ion exchange chromatography uses the type and degree of ionisation of the column and solutes to achieve a separation. Compounds with charges opposite to that of the column are attracted and retained. Elution is achieved by competitive displacement by an excess of an ion with the same charge as the analyte pushing it off the column (56).

Ion exchange columns are made of two backbone materials, silica or heavily cross linked organic polymers. Bound to this backbone material are charged functional groups which give the columns their separating characters ie. anionic or cationic.

This type of column can be subdivided into two categories, strong or weak ion exchangers. Strong columns possess either permanent charges, or have charges present throughout the full pH range for HPLC operation. Weak columns have functional groups with inducible charges, at one pH they are uncharged, at another pH they are charged. Weak columns have a higher ion exchange capacity than strong ion exchange columns. The exchange capacity of a column can be defined as "the amount of material that can be exchanged by a given amount of stationary phase" and is quoted as a value with the units of microequivalents per gram ($\mu equiv\ g^{-1}$).

2.4.3. Theoretical Principles Behind HPLC

This section deals with some of the basic principles that can be used to describe a chromatographic separation and the factors which affect it.

2.4.3.1. Retention Measurements

Although a peak in a chromatogram can be indentified by its retention time, because this varies with column length and mobile phase flow rate, it is better to locate and identify peaks using the capacity factor (k'), which is given by Equation 2.1.

Equation 2.1 $$k' = \frac{t_R - t_0}{t_0}$$

where t_R= the retention time and t_0= the column dead time.

The capacity factor tells us where the peaks elute relative to an unretained solute. The separation of two peaks relative to each other is described by the selectivity or separation factor (α), which is defined for two peaks as the ratio of the capacity factors. By convention the equation is written such that $\alpha \geq 1$, as in equation 2.2.

Equation 2.2 $$\alpha = \frac{k_2'}{k_1'} = \frac{t_{R2} - t_0}{t_{R1} - t_0}$$

k'_2 =capacity factor of 2nd component, k'_1 =capacity factor of 1st component, t_{R2} = retention of 2nd component, t_{R1} = retention of first component.

The degree of separation of one component from another is given by the resolution (R_s) and is defined by Equation 2.3.

Equation 2.3 $$R_s = \frac{t_{R2} - t_{R1}}{0.5(w_1 + w_2)}$$

w_1 = width of first component, w_2 = width of second component.

When two peaks are just baseline resolved R_s =1.5.

2.4.3.2. Column Performance

One of the problems of chromatography is that a band of solute moving through a column will become dispersed. The longer the solute spends in the column, the more dispersed it becomes. The more efficient the column, the less dispersion will occur i.e. the smaller the value of w_1, w_2, t_{R1}, and t_{R2}. To measure the efficiency of a column quantitative values known as the plate number (N) or plate height (H) are used and are defined by Equations 2.4, 2.5 and 2.6.

Equation 2.4 $$N = 16\left(\frac{t_R}{w}\right)^2$$

Equation 2.5 $$N = 5.54\left(\frac{t_R}{w_{1/2}}\right)^2$$

Where $w_{1/2}$ = peak width at half height

Equation 2.6 $$H = \frac{L}{N}$$

Where L= column length

The plate number is an indication of how well a column is packed, commercial reversed phase columns should have a plate count of about 50,000 plates m^{-1} (55). It is however, primarily a measure of the kinetic contributions to band broadening (dispersion) in the column.

The resolution of two peaks is therefore dependant on the column efficiency (N), the selectivity of the column (α) and the capacity factor of the column for the analytes to be separated, k'_1, and k'_2. From these parameters can be derived the following fundemental resolution equation (Equation 2.7).

Equation 2.7 $$R_s = 0.25\left(\frac{\alpha - 1}{\alpha}\right)\left(\frac{\bar{k}}{1+\bar{k}}\right)N^{1/2}$$

Where $\bar{k}$ = the average capacity factor for the two peaks

A full derivation of this equation has been given by Willard et al. (49).

Equation 2.7 shows that for a desired level of resolution three conditions have to be met:

a) The peaks must be separated from each other ($\alpha > 1$).

b) The peaks must be retained on the column ($\bar{k} > 0$).

c) The column must have a minimum number of plates.

Studying the influence of these three parameters independently can give an idea of what is wrong with a separation if the peaks are not adequately resolved.

As discussed above, a measure of chromatographic selectivity is given by α. At $\alpha = 1$ the peaks overlap, if $\alpha = 2$ then the method is more than adequately selective, so little will be gained by attempting to increase the selectivity further. The value of α is determined by the interaction forces acting on the analytes.

N, the column efficiency increases as a function of better packing, longer column length and optimum flow rate conditions. A column with a high value of N can separate components with similar selectivities.

The capacity factor, k' is dependant only on the strength of the eluent and is effectively a partition ratio between the time spent in the stationary phase relative to time spent is the mobile phase. For adequate resolution the value of k' must be above two, the longer an analyte is interacting with a chromatographic system (the bigger k'), the more separated analytes will become as long as the method is significantly selective ($\alpha > 1$) between the analytes being separated. If a method is not sufficiently selective, then the mobile phase should be changed, or

more drastically, the stationary phase. A more detailed explanation on balancing these three factors can be found in more specialist texts including the work by Lindsay (55).

2.4.3.3. Factors Affecting Dispersion or Band Broadening in a Chromatographic Column.

There are three mechanisms that produce dispersion of a band of solute as it travels through a column, these are:

a) Eddy diffusion and flow dispersion produced by the existence of different flow paths by which a solute can pass through a column. If two solutes had the same velocity, but took different path, one would arrive at a given distance down the column before the other. This effect is reduced by using small particle packing materials.

b) Longitudinal diffusion causes dispersion due to diffusion of the solute in the longitudinal (axial) direction of the column. This effect increases with time in the column, so it is reduced by a rapid flow rate in the mobile phase.

c) Mass transfer effects arise because the rate of the distribution processes (sorption and desorption) of the solute species between the mobile and stationary phases may be slow compared with the solvent velocity.

Ideally, in the time it takes for mass transfer into or out of the stationary phase, a non-interacting solute should have travelled as little as possible, so a low flow rate is required. These

three effects on the number of plates/ or the plate height can be stated as an abbreviated form of the van Deemter equation (Equation 2.8)

Equation 2.8 $$H = A + \frac{B}{u} + Cu$$

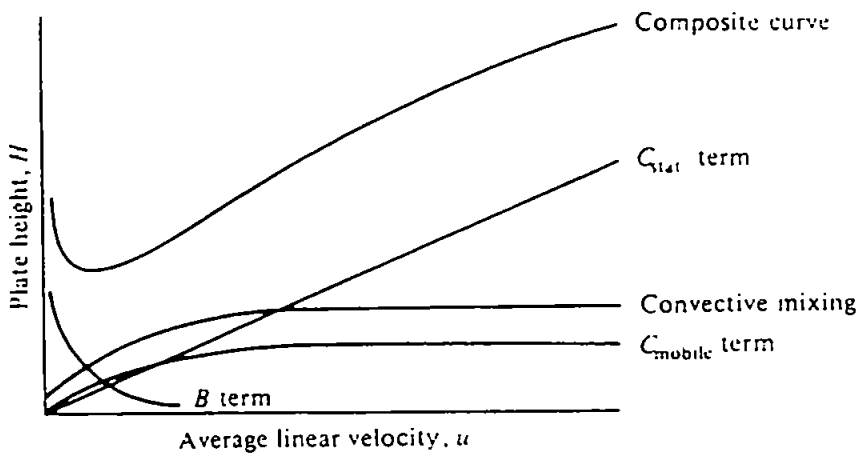
Where u = the average linear velocity, i.e. L/t_m where L is the column length and t_m is the retention time of an unretained species; A= eddy diffusion; B= longitudinal diffusion; and C= mass transfer effects.

When H is plotted against u (Figure 2.4), the minimum in the composite curve gives the optimum flow rate for a given separation.

2.5 Operating Conditions of the HPLC and the ICP-MS

Due to the diverse conditions used for the various studies reported in this thesis for both the ICP-MS and the HPLC, each set of operating conditions is reported in the appropriate chapter together with an explanation for their use. In general however, the aim is to be able to optimise the separation chemistry of the analytes of interest whilst retaining as high a sensitivity as possible when operating the ICP-MS. To this end an interface between the two techniques has been developed and its performance optimised to achieve the above aims.

Figure 2.4: A Van Deemter Plot for a Liquid Chromatographic Column (From ref 49)



Chapter 3. Speciation of Chemotherapy Drugs By HPLC-ICP-MS

3.1. The Discovery of Cisplatin and its Therapeutic Effect.

The discovery that platinum salts can have a biochemical effect was first made by Rosenburg *et al.* (57) whilst investigating the possible effects of an electric field on growth processes in bacteria. He reported that the bacteria *E. Coli* in the presence of certain platinum group metal (PGM) compounds at concentrations of about 1-10 ppm in a cell culture medium caused an inhibition of the cell division process.

The experiments were carried out in a continuous culture apparatus containing a nutrient medium and platinum electrodes. On turning on the current, the turbidity of the chamber began to decrease after an hour. Microscopic examination of the chamber showed that the bacteria had ceased dividing and had begun to elongate.

After eliminating all the traditional reasons for this form of growth, such as the presence of UV light, the authors investigated the possibility that a new chemical species that acted as an oxidising agent was being produced by electrolysis of the culture medium. This possibility was confirmed by a series of tests. Since the culture medium was of a known composition, the various ions present could be tested individually to see which ones reproduced this effect after electrolysis. Positive responses were obtained only for those ions containing chloride. It was known that platinum electrodes can be attacked by an acidified chloride solution during electrolysis to form $[PtCl_6]^{2-}$. It was suspected that a soluble platinum salt was the active agent, and this was later confirmed when the bacterial chamber was inoculated with a 100 ppm solution

of $(NH_4)_2[Pt\ Cl_6]$ and filament growth was observed after 2 hours. Following these experiments, the original medium was tested for Pt (IV) which was found to be present at a concentration of 8 ppm.

In a later paper, Rosenburg *et al.* (58) investigated why platinum had this effect at <10 ppm in the electrolysis medium, but when a platinum solution was added to the medium the effects were only seen at higher concentrations. It was noticed that solutions of $(NH_4)_2[Pt\ Cl_6]$ which had been left standing in the laboratory for a few days were able to cause filament growth at lower concentrations than fresh solutions. However this only occurred to solutions that had been exposed to light and had changed in colour from yellow to colourless.

The explanation put forward for this was that a fresh solution of $(NH_4)_2[Pt\ Cl_6]$ forms $[PtCl_6]^{2-}$ in solution. This ion when present in the nutrient medium and irradiated by light undergoes a ligand exchange reaction where one or more of the chloride ions are replaced by NH_3. The stable neutral species formed $[PtCl_4(NH_3)_2]$ inhibits cell division. This was verified by synthesising the cis and trans form of this complex, however only the cis form was found to be active. The intermediate product in the synthesis, cis Pt (II) Cl_2 $(NH_3)_2$, was also tested and found to cause filamentous growth. Two years later, Rosenburg reported that these compounds inhibited certain cancers in mice (59). The first clinical trials on cisplatin (diamminedichloroplatinum) in 1971 confirmed that it was active against several human tumours and it was approved for use in the UK against testicular and ovarian cancers in 1979 (60).

3.2 The Development of Second and Third Generation Platinum based Chemotherapy Drugs.

It was realised in clinical trials that there was room for improvement in platinum based chemotherapy. Cisplatin as a drug has two main limitations. Firstly it has severe adverse side effects, especially involving the kidneys, gastrointestinal tract and peripheral nerves (61). Secondly, its poor activity (intrinsic resistance) against some common tumour types (e.g. colorectal and non small-cell lung cancers), and its inability to confer lasting remissions in a proportion of patients due to the emergence of (acquired) drug resistance. The drug damages the kidneys by decreasing their filtering capacity, this leads to an elevation of the blood urea nitrogen and a decrease in creatinine clearance. The drug also causes severe vomiting and a loss of appetite that could lead to starvation. An unusual side effect is that it destroys hair cells in the inner ear, leading to a loss of high frequency hearing, and in some cases total deafness (62).

Due to these side effects initial cisplatin analogue development concentrated on the goal of discovering a less toxic but equally effective platinum based drug. During the 1970s a collaborative project between Johnson Matthey and the Institute of Cancer Research studied over 300 complexes and culminated in the discovery of carboplatin [cis-diammine-1,1-cyclobutanedicarboxylatoplatinum (II).] .

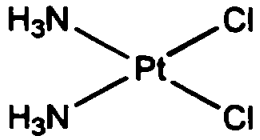
Clinical trials indicated that damage to bone marrow and myelosuppression were dose limiting adverse effects (63). However there was no kidney toxicity, although nausea and vomiting are still associated with this drug but are less severe than for cisplatin. Carboplatin in short was shown to have an equivalent activity to cisplatin with reduced side effects.

Unfortunately carboplatin and cisplatin share a cross resistance, i.e. tumours that have acquired resistance to cisplatin will also be resistant to treatment with carboplatin. To counteract this JM-216 has been developed and is currently undergoing clinical trials. JM-216 is less toxic, is capable of oral administration and has exhibited circumvention of acquired cisplatin resistance.

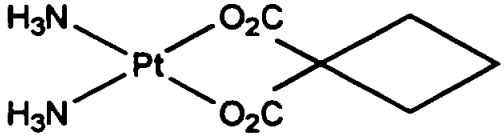
The need for an orally active drug was recognised to reduce the amount of time a patient needed to spend in hospital and improve patient comfort by removing the need for intravenous administration. When carboplatin was given orally, severe gastrointestinal adverse effects and poor absorption was observed.

The problem was to design a drug that was stable when crossing a number of barriers before reaching its target site. A balance between hydrophilic and lipophilic character was required. JM-216 has been found to have these characteristics (64) and entered phase II clinical trials against ovarian and lung carcinomas in 1994. The structures of JM-216 as well as cisplatin and carboplatin are shown in Figure 3.1.

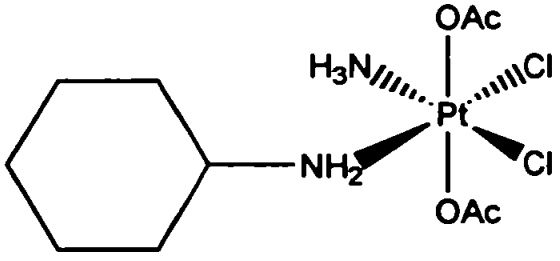
Figure 3.1: Platinum Based Antitumour Agents



Cisplatin



Carboplatin



JM-216
bisacetatoammine dichlorocyclohexylamine platinum (IV)

3.3 The Mechanisms of the Antitumour Capability of Cisplatin and its Analogues.

By the late 1980's a large body of evidence was growing that showed how cisplatin exhibited its biological activity by binding to deoxyribosenucleic acid (DNA) and inhibited replication (65). Both stereoisomers of diamminedichloroplatinum (II) (Cisplatin) have two labile chloride ligands and two ammine ligands that are inert to substitution under biological conditions. It is only the cis isomer that displays anti cancer activity, the trans isomer is toxic and kills cell instead of preventing replication. The implication of this is that the specific reaction or reactions responsible for anti tumour activity require bifunctional attachment to biological molecules.

It was though at the time of cisplatin's discovery that the major target for activity was DNA, early experiments showed that the rate of synthesis of DNA in cells treated with cisplatin was inhibited (66).

Cisplatin and carboplatin form electrophilic reaction intermediates that bind covalently to DNA (67). The spectrum of DNA damage includes bidentate intrastrand crosslinks in which two adjacent adenines and guanines or two adjacent guanines are covalently bound to cisplatin. This accounts for 85% of the damage (68), the rest is in the form of DNA-DNA interstrand crosslinks, and DNA-protein crosslinks. It has been found that the second generation analogues react in a similar manner.

The results of further studies (65) implies that tumour cells are less able to repair DNA damage compared to normal cells, and it is this that accounts for the specific anti tumour activity of cisplatin.

However with the advent of enzyme linked immunosorbent assay (ELISA) a sensitive method exists for the measurement of platinum-DNA adducts in nucleated blood cells and tissues of patients receiving chemotherapy containing platinum drugs. The significance of this is that high adduct levels can be correlated with clinical response (66), i.e. the higher the levels the better the clinical response. High DNA adduct levels are an indication of DNA damage and this further supports the theory that platinum drugs have an anti tumour effect due to the damage they cause to the DNA of tumour cells.

The result of the damage caused by cisplatin to the cell DNA is apparent when the cells attempt to divide. In order to do so effectively, copies of the DNA of the parent cell have to be made so that the daughter cells contain the same genetic information as the parent. If the DNA of the parent is damaged, then it may not be correctly copied resulting in the daughter cells containing DNA with errors that will prevent them from functioning properly and ultimately leading to their "death". Cisplatin also affects other aspects of cellular existence (69), for example, damage to the DNA will also prevent the correct transcription of RNA leading to the incorrect production of proteins. This would prevent enzyme synthesis leading to an effective shutdown of the cell.

3.4 Methods for the Determination of Platinum Based Chemotherapy Drugs and Their Metabolites.

To understand the mode of action of these drugs it became clear that analytical techniques were required that could differentiate between the anti tumour drugs and their transformation products.

This presents quite a challenge, as cisplatin and its analogues are subject to protein binding (70), upto 65% of the administered drug is irreversibly bound to proteins in the body. This inactivates the drug and so only the concentration of the free or ultrafilterable is required. However at typical doses the total level of ultrafilterable platinum falls to 10-50 ng ml^{-1} Pt within 2 hours of administration of the drug. So the lower the limit of detection, the longer the period of time that platinum concentration can be monitored. Thus, to be able to measure the actual species present detection limits lower than this are required.

A number of techniques do exist that are capable of speciating platinum in plasma ultrafiltrate. Most of them use HPLC to separate the biotransformation products from the parent drug followed by detection using atomic spectrometry, such as, electrothermal atomic absorption spectrometry, ICP-AES and ICP-MS. Other techniques include radioactivity detection, UV absorption detection with or without post column derivatisation and electrochemical detection. Recently ELISA has been used to measure the levels of platinum-DNA adducts that give an idea of the chromosome damage caused by these drugs.

3.4.1 Atomic Spectrometry.

The advantage of using atomic spectrometry for detection is that relatively little sample pretreatment is required. Thus urine, plasma and plasma ultrafiltrate samples can be directly injected onto the HPLC column which may then be coupled directly to the chosen detection technique (71).

The most commonly used technique is electrothermal AAS (72-76), which can facilitate limits of detection of 1-10 ng ml^{-1} depending on the matrix. The disadvantage of this approach is that the HPLC eluant usually has to be collected in fractions and analysed off-line.

However, inductively coupled plasmas can be directly coupled on-line with HPLC, and ICP-OES has been used (77, 10) to detect carboplatin and cisplatin in plasma ultrafiltrate. With detection limits of 3.5 ng (35 ng ml^{-1}) and 38 ng of platinum, respectively. However in the work of Alimonti *et al.* (10) to obtain a signal high enough to be able to detect cisplatin and its transformation products, a preparative HPLC column was used with a 2 ml injection loop.

ICP-MS has the advantage of being more sensitive than ICP-OES and will therefore give better detection limits. This technique, has been used to measure total platinum levels (78-80) and transformation products of cisplatin and 3rd generation analogues coupled directly to HPLC (81-82). The detection limits for the determination of total platinum in plasma by ICP-MS are less than 1 ng ml^{-1} (79-80), however for HPLC-ICP-MS a detection limit of 0.1 ng ml^{-1} (1ng ml^{-1}) for cisplatin (81) and 1.32 ng ml^{-1} (26 pg) (82) for platinum have been reported.

Finally Morrison *et al.* (83) have used HPLC with ICP-MS but collected fractions of the eluent and analysed then off-line using an electrothermal vapourisation (ETV) unit to introduce the samples to the plasma. For this method they report a detection limit of 0.1 ng ml^{-1} for a 200 µl injection onto the column.

3.4.2. Spectrophotometric Detection

This is by far the most common detection method for platinum drugs and their metabolites after separation by HPLC.

This mode of detection can be split into two categories, detection of the drug by UV absorption, or the determination of platinum by pre- or post- derivatisation followed by UV absorption detection of the resulting species.

3.4.2.1 Direct Determination by UV Absorption Spectroscopy

Direct UV detection provides detection limits of about 1 $\mu g\ ml^{-1}$ at 280 nm and about 20 $ng\ ml^{-1}$ at 210 nm for cisplatin. The wavelength used is dependant on the chromatographic system used, since there are a number of co-eluting interferences that have to be removed from plasma ultra filtrate if detection below 210 nm is to be used. At wavelengths of 280 nm or higher these interferents do not absorb so they don't present a problem (71).

Carboplatin can be detected at 229 nm with a limit of detection of 0.2 $\mu g\ ml^{-1}$ (84). An advantage of this method is that it can be used to monitor the levels of drugs administered with cisplatin that do not contain platinum such as 5-fluorouacil in the same chromatographic run (85).

The use of diode array detection means that chemical processes involving cisplatin such as binding with amino acids can be monitored. Shearan *et al.* (86) used this method to monitor the interaction of cisplatin with cysteine. The amino acid and cisplatin were monitored at 205 nm, whilst the cysteine-cisplatin complex was monitored at 220 nm simultaneously. The use of diode arrays also allows the simultaneous determination of cisplatin, carboplatin and their aquo complexes (87-88) and their DNA adducts (89).

3.4.2.2. Derivatisation followed by UV Detection

This method requires minimal sample pretreatment of urine or plasma samples since no compounds present in the matrix absorb at the same wavelength as the derivatised platinum species. This technique provides detection limits of better than 25-50 ng ml^{-1}.

This methodology was first proposed by Bannister *et al.* in 1979 (90). The drug cisplatin and its biodegradation products are derivatised directly in the urine by reaction with diethyldithiocarbamate (DDTC). This formed a stable 2:1 DDTC-platinum complex that could be quantitatively extracted from urine with chloroform and detected after HPLC separation at 254 nm with a detection limit of 25 ng ml^{-1}. Unfortunately this method converts all the platinum species present in the urine into the same 2:1 DDTC-platinum complex so only the total platinum species concentration is measured.

To overcome this problem a post column reaction has been employed (91). This approach has maintained the selectivity of HPLC by separating the species present prior to derivatisation. The cisplatin is derivatised with sodium bisulphite after activation with potassium dichromate, and a

detection limit of 40 ng ml^{-1} for cisplatin may be obtained. The method does however have several disadvantages. Three HPLC pumps are required to pump the eluent and the reaction solutions into the reaction coil. In addition, in it original form it was only sensitive to divalent platinum complexes. But this problem has since been overcome so that quadrivalent complexes can also be measured without the dichromate activation step (92-93). This system has two main advantages, only two HPLC pumps are required, and the waste in rendered nonmutagenic after this reaction (94). This methodology can detect cisplatin down to a level of 9 ng with a 2 µl injection loop (93).

3.4.3. Alternative Methods

Electrochemical detection of cisplatin was first described by Bannister et al. (95) who used a hanging drop electrode as an HPLC detector. Parsons et al. (96) used electrochemical detection to measure the rates of hydrolysis of cisplatin and the second generation compounds, iproplatin, carboplatin and tetraplatin after separation of the drugs from their mono and diaquo species. Since these can be detected at a potential of -1.0v versus an Ag/AgCl reference electrode, interferences from blood plasma and urine constituents are minimal as these give a signal at lower electrode potentials. This method gives similar limits of detection to HPLC with post column derivatisation.

Baldew et al. (97) reported the use of a radioactivity detector to determine ^{195m}Pt labelled cisplatin and related complexes by HPLC. They suggest its use as a reference system for other detection systems that are less specific. The detection limit for this technique is 10 ng ml^{-1}.

Cation exchange columns have also been successfully used to separate the drug from its hydrolysis (hydrated) derivatives in urine samples of rats treated with cisplatin (100). Aqueous solutions of cisplatin have also been analysed using an anion and cation exchange column in series (101, 93).

3.4.4.2. Ion Pair Chromatography.

In ion pairing chromatography the analyte is retained as a result of an ion-dipole interaction between the analyte and the surfactant adsorbed onto the stationary phase. Cationic and anionic surfactants can be used to alter the selectivity so that the column has an anion or cation exchange capacity.

Ion pair chromatography was first introduced for the analysis of chemotherapy drugs by Riley *et al.* (74,102) for the determination of intact platinum drugs. A C-18 column has been used with a mobile phase containing hexadecyltrimethylammonium bromide (HTAB), a cationic surfactant. Using this system it was found that between 75-95% of the administered cisplatin was excreted unchanged in urine (74). This chromatographic system has since been used by a number of other workers (Table 3.1).

Although ion pair chromatographies based upon cationic surfactants allow the determination of the levels of intact drug present, they do not allow the quantitation of the potentially important cations in the sample such as the positively charged hydrated species of the drug. For this purpose ion pair systems based upon anionic surfactants are more suitable. These are typically the sodium salts of alkyl sulphates, such as, sodium dodecylsulphate (SDS) and sodium

Table 3.1: Ion Pair Systems Using Cationic Surfactants for the Analysis of Platinum based Chemotherapy Drugs.

Authors	Ref.	Mobile Phase	Compounds
Riley *et al.*	74,102	Citrate Buffer (0.01M; pH 7), 0.1mM HTAB	Cisplatin, carboplatin, iproplatin, DACH-Pt complex
Earhart *et al.*	103	0.005M Tetrabutyl ammonium Chloride in water	Intact vs. non-intact cisplatin
Krull and co-workers.	104,105	Acetate buffer (0.01M; pH 4.6), 0.15mM HTAB, Methanol, NaCl	Cisplatin, carboplatin, iproplatin.
Bannister *et al.*	95	Citrate Buffer (0.005M; pH 6.5), 0.1mM HTAB	Cisplatin, iproplatin
Richmond *et al.*	106	1mM HTACl, made upto 0.1M with KCl	Cisplatin
Marsh *et al.*	91	Citrate Buffer (0.01M; pH 5.25), 0.1mM HTAB	Cisplatin, carboplatin, malonato-Pt, DACH-Pt
Fleming *et al.*	85	2% (v/v) Tetrabutyl ammonium hydroxide in water made to pH 6 with conc. phosphoric acid.	Cisplatin, 5-fluorouracil
Rochard *et al.*	88	5.5×10^{-4}M HTAB (0.01M phosphate buffer pH 7)	Cisplatin, carboplatin

octanesulphonic acid (SOS). Altering the chain length will result in differences in retention time so the actual sulphonic acid used can be optimised.

Ion pair systems based on SDS have been used to quantify the formation of platinum-methionine complexes and the hydrated derivatives of cisplatin (107-108). However the drug and the cationic species of interest had large differences in retention time leading to long acquisition times. Using smaller chain sulphonic acids such as SOS (77) will give sufficient resolution without excessive retention times if a gradient elution is used. Parsons *et al.* (109, 96) has demonstrated the use of heptane sulphonic acid to separate cisplatin from its biotransformation products (109) and cisplatin from its analogues (96). Other ion pair systems used can be seen in Table 3.2.

3.4.4.3. Normal Phase, Reversed Phase and Other Chromatographies.

Chromatographic systems for the determination of platinum drugs have also been based on stationary phases of different polarity and gel permeation packings. Stationary phases ranging from highly polar (silica) to relatively non-polar (octadecyl-bound silica), have been applied to the determination of drugs in their intact form (Table 3.3).

Table 3.2: Ion Pair Systems Using Anionic Surfactants for the Analysis of Platinum based Chemotherapy Drugs.

Authors	ref no.	Mobile phase	Compounds
Daley-Yates *et al.*	107,108	Gradient from 0.005M SDS to acetonitrile:water 9:1	Cisplatin, biotransformation products
Riley *et al.*	110	Phosphate buffer (0.1M; pH2.1) 50mM hexane sulphonic acid, 10-12% acetonitrile	Cisplatin, Pt-methionine complexes
De Waal *et al.*	77	Phosphate buffer (0.01M; pH 2.8), 1mM octane sulphonic acid (a), 5% 2-propanol (b); gradient elution	Cisplatin, biotransformation products
Parsons *et al.*	109	Acetate buffer (0.01M; pH 4.6), 5 or 10mM heptane sulphonic acid	Cisplatin, biotransformation products
Parsons *et al.*	96	Acetate buffer (0.01M; pH 4.6), 5mM heptane sulphonic acid (10 % methanol)	Cisplatin, carboplatin, iproplatin
Kristjansson *et al.*	111	Phosphate buffer (0.04M; pH 2.65), 1.5mM hexane sulphonic acid; phosphate buffer (0.04M; pH 2.65), 6-10mM hexane sulphonic acid, 8% methanol	Cisplatin, hydrolysis products, dimer, trimer
Dedon *et al.*	112	Phosphate buffer (0.05M; pH 2.5), 2.5mM heptane sulphonic acid	Cisplatin and its trans-isomer, carboplatin, iproplatin, Pt complexed with amino acids, thiosulphate, and diethyldithiocarbamate
Baldew *et al*	97	(a) Phosphate buffer (10mM; pH 2.6), 5mM SDS, (b) phosphate buffer (60mM; pH 2.6), 5mM SDS, 25% 2-propanol	Cisplatin, biotransformation products

Table 3.3: Other Chromatographies for Platinum based Chemotherapy Drugs.

Authors	Ref.	Stationary Phase	Mobile Phase	Compounds
Van der Vijgh *et al.*	113	Porasil Silica	Acetonitrile: Water 9:1	Ethylenediammine - Pt (II) malonate
Gaver *et al.*	114	Lichrosorb diol	Phosphoric acid (0.015%): Acetonitrile, 11:89 or 8:92	Carboplatin
Pendyala *et al.*	115	Bondapak phenyl	Methanol:water 1:9 or gradient	Iproplatin, biotransformation products
Cheung *et al.*	116	Lichrosorb amino	Acetonitrile:water 9:1 or 85:15	Cisplatin, iproplatin
Cheung *et al.*	116	Spheri-5 RPM	Water	carboplatin
Elferink *et al.*	117	Sherisorb ODS2	Sodium sulphate solution (0.05M; pH 3 with sulphuric acid), 10-30% methanol	Aqua [1,1-bis (aminomethyl)cyclohexane] sulphato Pt (II) and derived species
Elferink *et al.*	118	Sherisorb ODS2	0.05M sodium perchlorate in water	Carboplatin
Arpalahti *et al.*	119	Technopak C18	Ammonium acetate (0.1M) in water:methanol 95:5	Cisplatin and its trans-isomer after treatment with thiourea

Kizu *et al.*	120	Hitachi gel no 3013-N	Methanol:Water 15:85, 10mM NaCl	Cisplatin
Cheung *et al.*	116	Hamilton PRP-1	Acetonitrile:water 1:9	DACH-Pt complex
Noji *et al.*	121	Toyo Soda G1000PW	Sodium sulphate (0.1M)	Cisplatin and its trans-isomer, DACH-Pt complexes, hydrolysis products
Gullo *et al.*	122	Sephadex G-200	Tris-HCl (0.1M; pH 8), 1M NaCl	Cisplatin, protein bound Pt
Mason *et al.*	123	Sephadex G-15, G-50, DE-52, cellulose, CM sepharose, Sephacryl S-200	Tris-HCl (0.01M; pH 7.4), or Tris-HCl (0.1M; pH 5.6)	Cisplatin, biotransformation products
Repta *et al.*	124	Biogel P2	Water	Cisplatin, biotransformation products
Duncan *et al.*	84	Bondapak NH_2	Acetonitrile:methanol:0.005M sodium perchlorate (pH 2.4)	Carboplatin, JM-10
Shearan *et al.*	86	Alumina	0.05M phosphate buffer, methanol, acetonitrile, tetraethylammonium bromide	Cisplatin, hydrolysis products and interactions with cysteine
Brandšteterová *et al.*	87	Separon C-18	Water or phosphate buffer (pH 7)	Cisplatin, transplatin, carboplatin, oxocarboplatin

Alimonti *et al.*	10	Superose 6 and 12 Preparative grade, HR 16/50	Phosphate buffer (pH 6.8), 0.07M KH_2PO_4, 0.1M NaCl, and $6x10^{-4}$M NaN_3	Cisplatin, carboplatin
Allsopp *et al.*	125	Sherisorb ODS-1 and Apex ODS	Phosphate buffer (0.07M, pH 6.1)	Carboplatin
Cairns *et al.*	82	Hypersil Phenyl	Water:acetonitrile 75:25	JM-216, and impurities in the drug
Hanada *et al.*	126	Hitachi gel no 3013-N	10mM NaCl:acetonitrile 85:15	Cisplatin
Kizu *et al.*	92	MCl gel CDR 10, MCl gel CPK08, inertsil ODS-2	0.1M Na_2SO_4 30%, CH_3CN, 10mM acetate buffer (pH 5.5) or 50mM K_2SO_4 (pH 3.5) or 5% CH_3CN, 10mM acetate buffer (pH 5.5)	Cisplatin, carboplatin, oxaliplatin, oxoplatin, tetraplatin

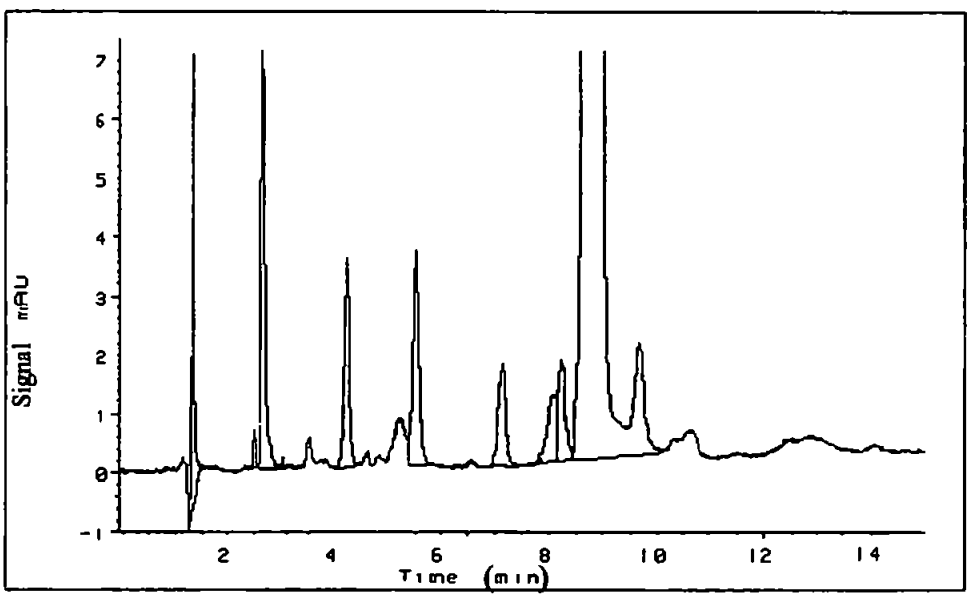
3.5. Development of an HPLC-ICP-MS Method for the Determination of JM-216.

At the commencement of this study, an isocratic (Figure 3.2) and a gradient separation of JM-216 from impurities introduced during the manufacturing process had already been developed by Johnson Matthey with detection based upon UV absorption spectrometry. This was developed as a quality control method for solid samples of the drug. However this method was not sensitive enough for the clinical monitoring of JM-216 in patients treated with this drug.

Therefore it was necessary to develop an element specific detection method for JM-216 for three main reasons. Firstly, a more sensitive method was required to monitor the drug in clinical samples, secondly, there are many compounds in plasma ultrafiltrate that may co-elute with the metabolites of the drug and would interfere with their detection by UV absorption spectrometry. The third reason is that the drug when in the body is completely metabolised, undergoing a number of ligand exchange reactions. This means that some of the metabolites have no chromophores and so are impossible to detect using UV absorption spectrometry.

ICP-MS is a highly selective and sensitive method for the determination of platinum. However due to the problems associated with the introduction of organic solvents into plasmas (as detailed in chapter 1), it was decided that initial studies should use an isocratic separation method for the determination of JM-216 by coupled HPLC-ICP-MS.

Figure 3.2: Isocratic Chromatogram acquired by Johnson Matthey



3.5.1 Experimental

3.5.1.1 ICP-MS Instrumentation

The instrument used was a VG PQ2 (Fisons Instruments, Winsford, Cheshire). The nebuliser used was a "V-groove" type (Ebdon nebuliser, PS Analytical, Orpington, Kent.). The aerosol was sprayed into a standard Scott double-pass spray chamber which was cooled to at least -15°C by pumping propan-2-ol from a refrigerated bath (Techne Refrigerated Bath RB5, Cambridge) into its cooling jacket. The spray chamber cooling was necessary to reduce the reflected power to 25 W so that the plasma could be sustained when the HPLC eluent was introduced. The eluant from the HPLC was introduced to the nebuliser via a 1m length of 0.18mm id PTFE tubing. This was the minimum length possible to prevent possible broadening of the peaks due to dead volume. To prevent the deposition of particulate carbon on the skimmer and sampling cones, oxygen was added to the nebuliser gas flow via a gas blender (Signal Instruments, Surrey) and a second mass flow controller. The operating conditions for the ICP-MS can be seen in Table 3.4.

3.5.1.2 Chromatography

An inert gradient pump was used (Varian Star 9010, Varian Associates, Walnut Creek, California, USA). Samples were injected via an inert injection valve fitted with a 20 µl PEEK sample loop. A phenyl bonded silica analytical column was used, with a mobile phase of 25:75 v/v acetonitrile:water using an isocratic elution program at a flow rate of 1.3 ml min^{-1}. The

Table 3.4: ICP-MS Operating Conditions for the Isocratic Chromatography of JM-216

Forward Power kW	1.5
Argon Flow Rates (l min^{-1})	
Coolant	15
Auxiliary	0.75
Nebuliser	0.75
Oxygen added to nebuliser flow (%)	3
Spray Chamber Temperature (°C)	-15

sample was supplied by Johnson Matthey and consisted of the drug (JM-216) dissolved in the eluent.

3.5.1.3 Data Acquisition

The data was acquired using the single ion monitoring mode of analysis to monitor *m/z* 194. This MSDOS based software on the PQ2 allowed accurate quantitation of the peaks and the possibility to baseline correct peak areas.

3.5.1.4 Results and Discussion

Initial attempts at reproducing the chromatography were unsuccessful, (Figure 3.3). This is because the reproducibility of home packed columns was found to be less than that of commercially available ones. Once an adequately packed column was obtained, good results were achieved. (Figure 3.4). A comparison of the chromatograms obtained by UV and platinum -specific ICP-MS detection show few differences apart from peak intensities (Figures 3.4 and 3.5). The three main impurities are well resolved using both detection techniques, the main peak (JM-216) eluting at 10 mins. Instrumental drift was monitored throughout the experiment over a 3 hour period (Figure 3.6).

3.5.1.5 Quantification of the Method

Since there are no standards available for the impurities found in the drug, and no drug standard of known concentration available at the time, calibration was carried out in the following way.

Before and after each chromatographic run the column was taken off line and flow injection techniques were used to inject standards containing 10, 100 and 1000 ng ml^{-1} of platinum as tetrachloroplatinate. This process was repeated five times and the peak response (area and peak

Figure 3.3: Original Isocratic Elution of JM-216

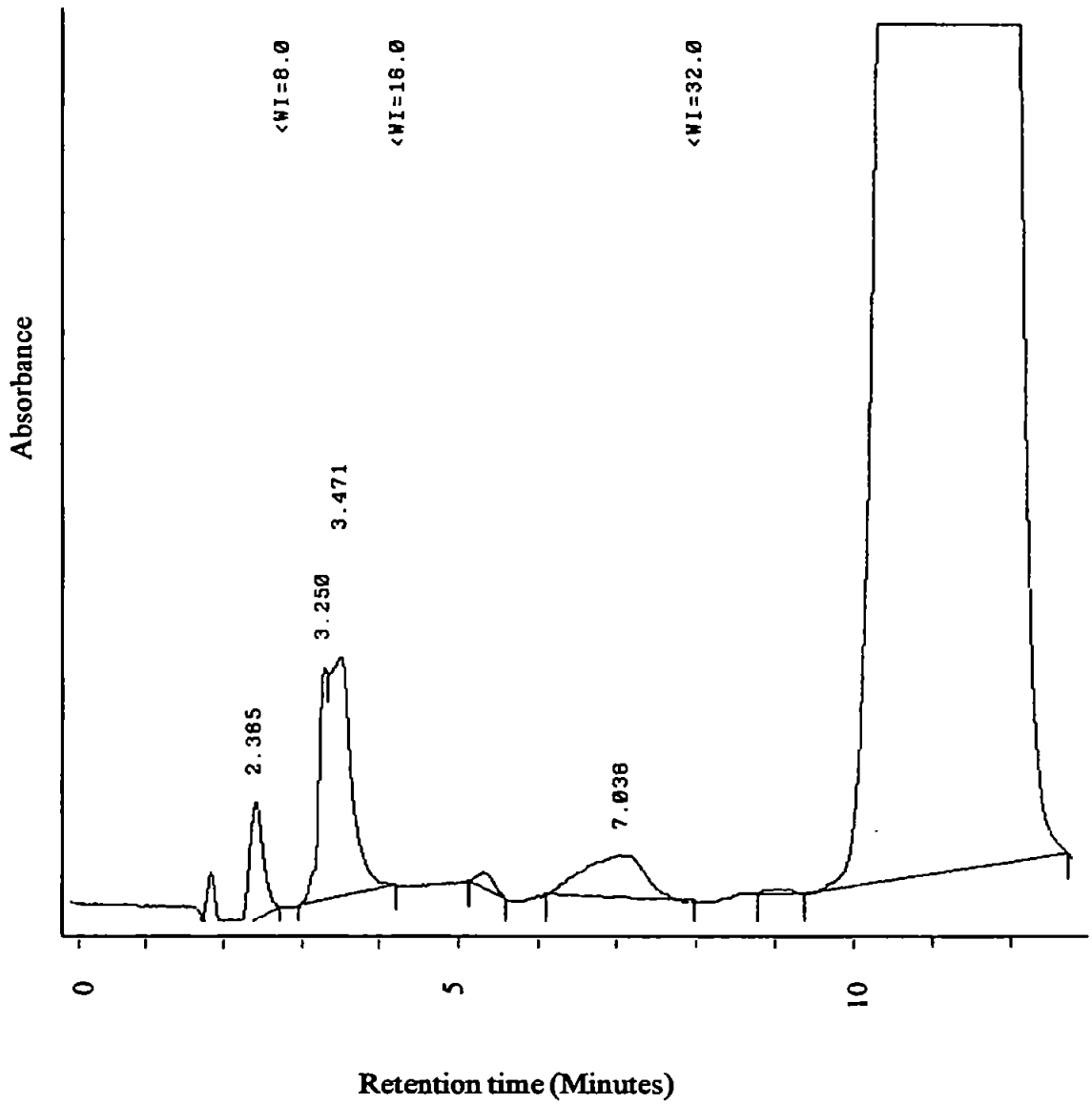


Figure 3.4: An improved isocratic elution of JM-216 with UV detection at 210.4nm

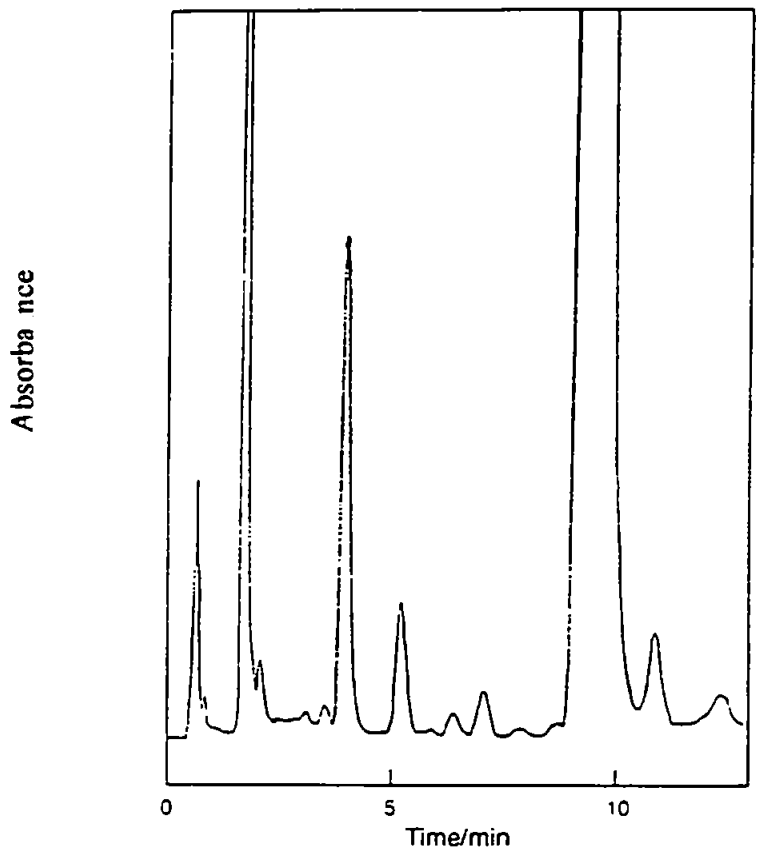


Figure 3.5: An Isocratic Elution of a Solution of JM-216 With ICP-MS Detection at *m/z*194

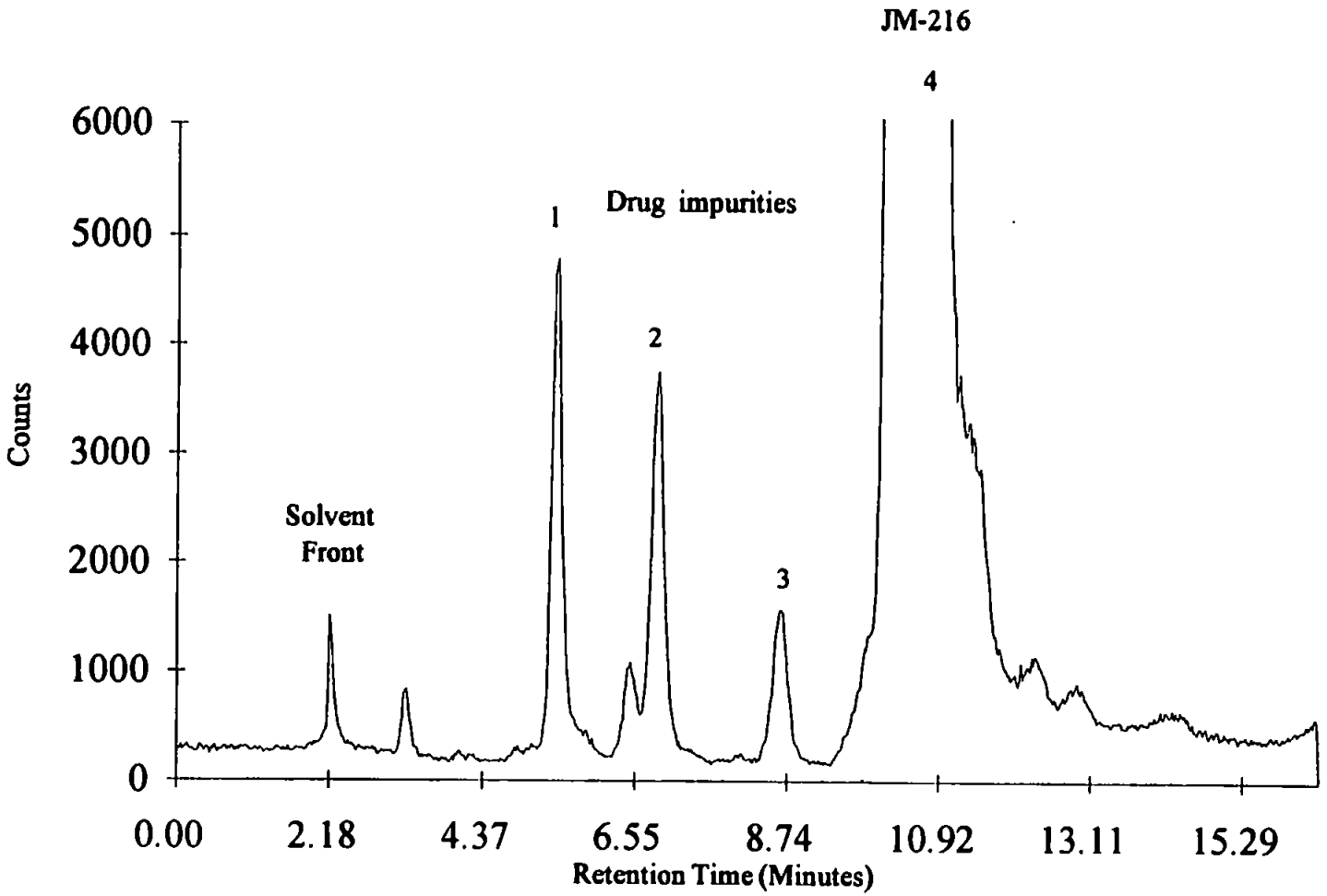
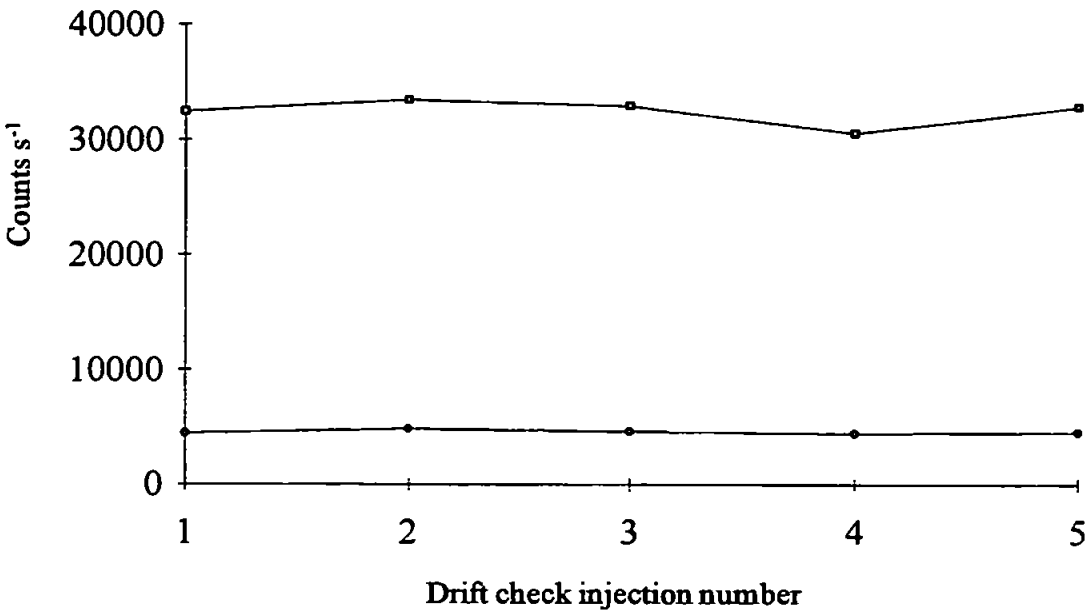


Figure 3.6: Standards containing 10 and 100 ng ml^{-1} were injected after each ICP-MS run in order to monitor instrumental drift (over 5 runs) throughout the experiment



height) recorded in each case. This process was performed for 2 reasons: (i), injections before and after each run will show any drift in the instrumental response between runs; and (ii), the use of three standards covering two orders of magnitude allowed the amounts of each platinum species in the analyte to be determined. Although the measurements of peak height are more precise, the peak areas were used to calibrate the platinum response as the flow injected peaks used for calibration purposes were much narrower, and therefore much higher than the sample peaks as a result of not passing through an analytical column.

The detection limit for platinum was determined as three times the standard deviation of the background noise and was found to be 1.32 ng ml^{-1} representing an actual mass of 26 pg of platinum. The results obtained for the sample are shown in Table 3.5.

Having demonstrated that the use of ICP-MS detection for the isocratic chromatography was possible, it was decided to investigate the tolerance of the ICP-MS to higher levels of organic solvents. The gradient chromatography, as developed by Johnson Matthey (128), requires a gradient of 5:95 to 95:5 water:acetonitrile v/v to be introduced to the plasma at a flow rate of 1.0 ml min^{-1} over 20 minutes.

Solutions of different ratios of acetonitrile:water were made up and introduced to the plasma. The 50:50 solution gave a reflected power of 50 W at a forward power of 1.5 kW, the plasma went out after 5 minutes of operation. In addition further oxygen was required to prevent particulate carbon deposition on the plasma sampling interface. On increasing the power to 1.7 kW the reflected power reduced to 45 W and enabled a longer operation time, but the combination of a higher forward power and increased oxygen caused rapid ablation of the nickel.

Table 3.5: The analytical figures of merit for the platinum species present in the original sample of JM-216 supplied by Johnson Matthey.

Peak Number	Mean Peak Area (Counts s^{-1})	Mean Pt concentration in the sample (ng ml^{-1})	Precision of the peak area data (RSD %) (n=5)	Precision of the Peak height data (RSD %) (n=5)
1	34982	131	35.2	10.2
2	37638	135	30.0	3.2
3	13659	49	24.2	11.4
4	6934990	23900	3.8	3.1

cones. Thus to enable the introduction of acetonitrile levels > 50% v/v required a higher forward power, more oxygen, and in order to prevent cone ablation, platinum tipped cones. However, since the analyte of interest in this study was platinum, the use of platinum tipped cones was clearly undesirable. It was therefore decided to investigate the use of methanol as a mobile phase as this is a solvent much easier to introduce as a gradient into an ICP than acetonitrile. This is because it contains an oxygen atom, so oxygen addition to the plasma to prevent carbon deposition is only necessary above 60% methanol.

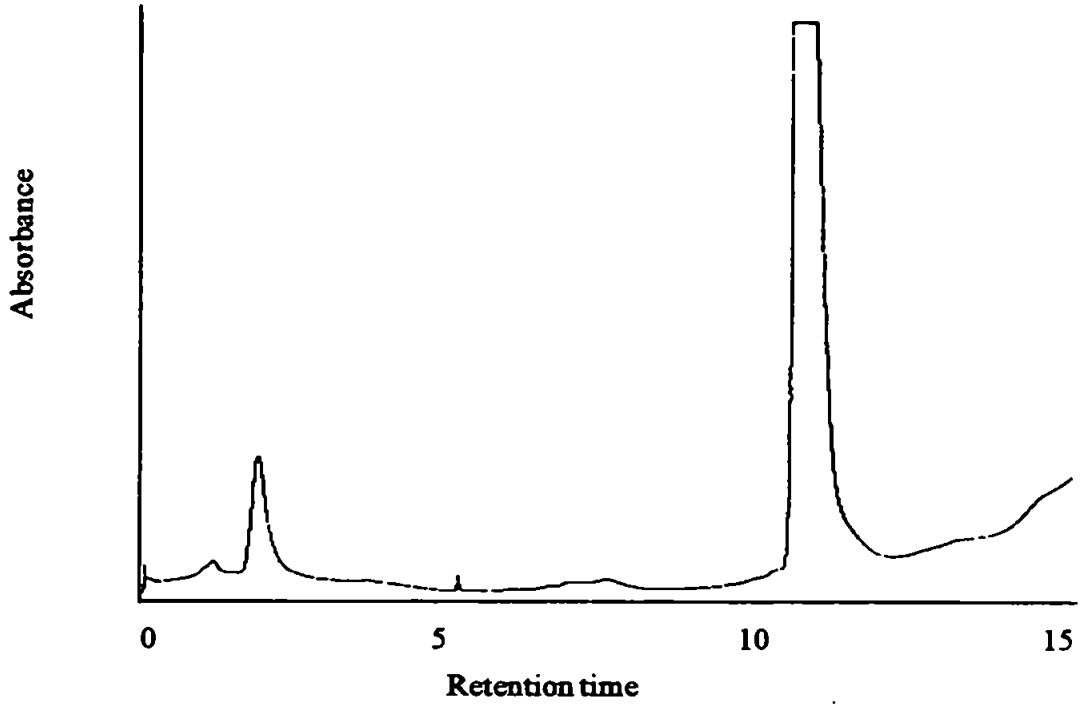
3.5.1.6 Investigation of Methanol as an organic modifier for the chromatography of JM-216

The instrumentation used is as in section 3.5.1.2, again, UV detection was at 210.4 nm. The initial attempt used a gradient of 5% to 95% methanol: water in 20 minutes at a flow rate of 1.0 ml min^{-1}. The result of this can be seen in Figures 3.7 and 3.8.

From these results it can be seen that the chromatography with methanol needs modification to enable the detection and identification of the species present. To improve resolution the gradient was reduced to 5% to 40% methanol: water on 20 minutes. The result of this can be seen in Figure 3.9, although the species present that elute with a retention time less than 5 minutes are resolved, the peak shape is not adequate for ICP-MS detection as this involves a loss of resolution and peak broadening. In an attempt to sharpen the peaks, the gradient was increased to 5% to 70% methanol: water in 20 minutes (Figure 3.10). To maintain the sharpness of the peaks but improve resolution of the species present the gradient program was altered to: 5% to 50% methanol in 5 minutes, then a gradient from 50% to 70% at 5 to 20 minutes (Figure 3.11). From these results it is clear that acetonitrile is the better organic modifier for JM-216 chromatography.

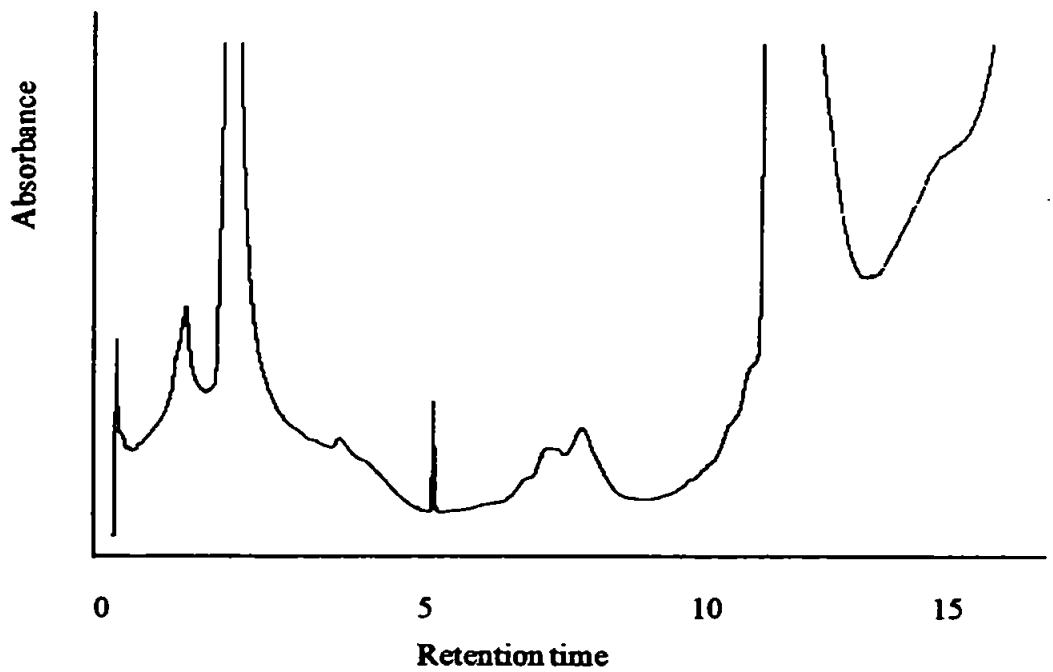
It was therefore decided that the best way forward was to develop an interface that facilitated the use of organic solvents with the HPLC separation achieved using acetonitrile by employing a desolvation step prior to introduction to the ICP-MS.

Figure 3.7: Gradient Elution of JM-216 with methanol as the organic modifier



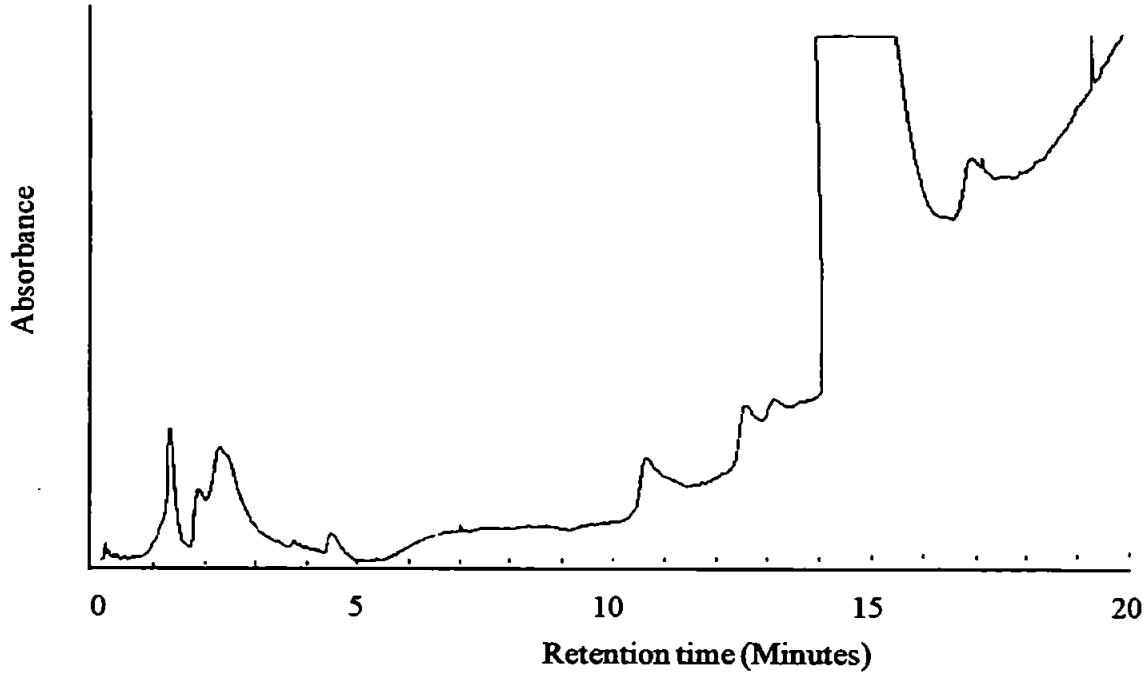
UV detection at 250 nm, absorbance full scale 0.01 AU, attenuation 6

Figure 3.8: Gradient Elution of JM-216 with methanol as the organic modifier (magnification)



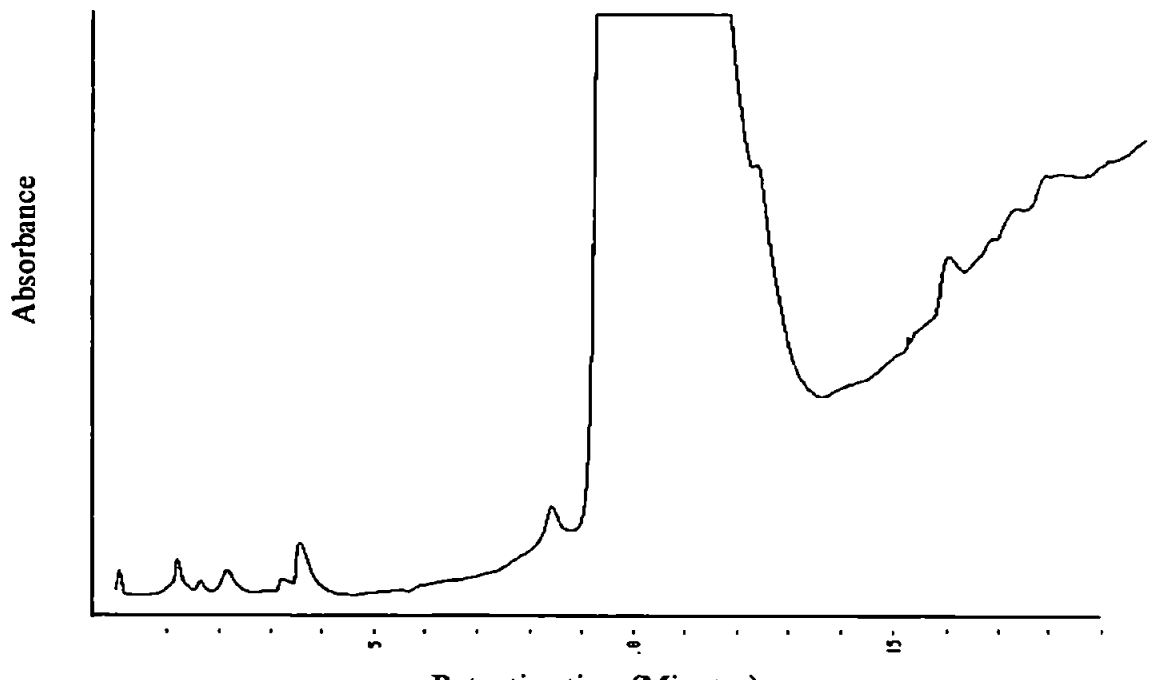
UV detection at 250 nm, absorbance full scale 0.01 AU, attenuation 3

Figure 3.9: Gradient Elution of JM-216 with methanol as the organic modifier (optimisation step 1)



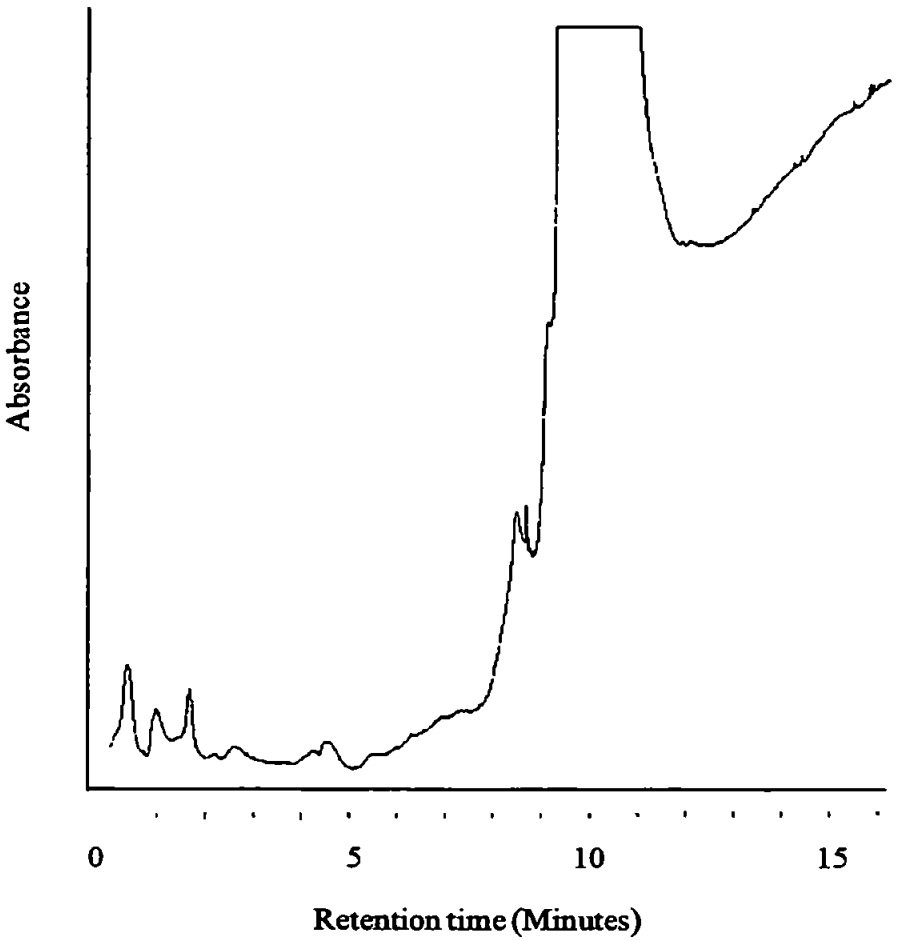
UV detection at 250 nm, absorbance full scale 0.002 AU, attenuation 3

Figure 3.10: Gradient Elution of JM-216 with methanol as the organic modifier (optimisation step 2)



UV detection at 250 nm, absorbance full scale 0.001 AU, attenuation 2

Figure 3.11: Gradient Elution of JM-216 with methanol as the organic modifier (optimisation step 3) with UV detection



Detection at 250 nm, absorbance full scale 0.001 AU, attenuation 5

3.6 Development of the HPLC-ICP-MS Interface.

Previous work already carried out at the University of Plymouth (128-129), suggested that the best approach to desolvating a sample was to use a membrane drier coupled with a cryogenic condenser.

3.6.1. Experimental

3.6.1.1 Instrumentation

The instruments used were a VG Elemental PlasmaQuad 2 and a Fisons Instruments Elemental PlasmaQuad 2+ (Fisons Instruments, Winsford, Cheshire UK). Power for the plasma was supplied from an r.f. generator with a maximum forward power of 2.0 kW at 27 MHz. Nickel sampler and skimmer cones with a 0.7 mm orifice were used. The instrument operating conditions are shown in Table 3.6. An ICP-AES instrument was also used for some of the early development work (Varian Liberty 200, Varian UK Warrington). Power to the plasma was supplied by an r.f. generator operating at 40.68 MHz.

Table 3.6: ICP-MS Operating Conditions with Desolvation

Forward Power kW	1.5
Argon Flow Rates (l min^{-1})	
Coolant	15
Auxiliary	0.75
Nebuliser	0.6

3.6.1.2 Reagents and samples

The acetonitrile was HPLC grade (Rathburn Chemicals Ltd, Walkerburn, Scotland). The water used was Milli-Q 18 MΩ (Millipore, Molsheim, France). The samples and plasma ultrafiltrate were supplied by The Institute of Cancer Research (Sutton, Surrey, UK), and solid samples of the drugs for use as standards were supplied by the Johnson Matthey Technology Centre (Sonning Common, Berkshire, UK).

3.6.1.3. Chromatography

An inert gradient pump was employed (Varian Star 9010, Varian Associates, Walnut Creek, California, USA). Samples were injected using a chemically inert injection valve (Cheminert Model C1 valve, Valco Instruments Co. Inc., Houston, Texas) fitted with a 200 µl PEEK sample loop. The analytical columns employed were a 250mm x 4.6mm i.d.

PEEK column (Alltech UK, Carnforth, Lancashire) packed in house with Hypersil Phenyl 5µm bonded silica (Shandon Scientific Ltd, Runcorn, Cheshire), a 250 x 4.6 mm id column packed with PLRP-S (Polymer Laboratories Ltd, Shropshire, UK.) and a 250 x 4.6 mm id column packed with Hypersil ODS 5 µm bonded silica (Jones Chromatography). A 1 cm guard column was fitted to each packed with the same stationary phase as the analytical column.

3.6.1.4 Data Acquisition

Initially data was acquired by single ion monitoring of *m/z* 195. The data was then exported into Microsoft Excel version 4.0 (Microsoft, Redmond, WA, USA) where the individual data points were converted to a single column text file. From here the data was either charted or imported into Biosoft Fig-P (Biosoft, Cambridge, UK), charted and quantified. For the later work the data was acquired using the TRA mode of analysis in the instrumental software monitoring *m/z* 194 mix chilled to -10^{0}C and pumped via a recirculating refrigerated bath (Neslab endocal RTE-100, Neslab Instruments, Inc., Basingstoke, Hampshire, UK).

3.6.2. Design and Optimisation of the Desolvation Apparatus

The original design of cryogenic condenser used by Hartley *et al.* (128-129) had a number of design flaws that needed to be remedied. The first problem, was that the solder junctions on the peltier coolers kept on melting. To solve this, 2 water cooled aluminium cooling blocks were used to cool the hot faces of the peltier coolers. These blocks had a much higher thermal inertia than the individual copper cooling plates used previously, so any flow variations in the coolant did not have any major effect. The aluminium blocks also proved more effective at removing heat. Another reason why the peltier coolers electrical connections kept on melting, was that an a.c. not a d.c. power supply was used. For this work, a d.c. power supply was built specifically for the peltier coolers as they are described as "electrically fragile".

Another part of the original design that was changed was the aluminium block the glass u-tube was held in. It's thickness was reduced so that less power was needed to reduce the temperature of the gas inside the glass u-tube. The result of this redesign was that at a power supply of 16 v a static air temperature of -36°C could be achieved inside the glass u-tube with a maximum solder junction temperature of 9°C with water cooling of the aluminium blocks.

3.6.2.1. Determination of optimal desolvation configuration for the apparatus.

The first desolvation step would have to be tolerant of high solvent vapour loads varying from predominantly water to mostly acetonitrile. To determine which desolvation method was most tolerant, a 25:75 % v/v acetonitrile: water mix was nebulised into the membrane and the condenser separately. To generate a solvent vapour, in order to desolvate the initial aerosol, the solvent mix was nebulised with a Meinhard nebuliser into a cyclone spray chamber heated with an infrared heat lamp. The water and acetonitrile were mixed and pumped at a flow rate of 0.5 ml min^{-1} using the gradient HPLC pump. The nebuliser gas flow rate used was 0.65 l min^{-1}. The membrane purge gas flow used was 2.0 l min^{-1}.

3.6.2.1.1 Results

When the initial solvent mix was nebulised into the membrane drier, the membrane irreversibly collapsed after 5 minutes of operation with a considerable level of condensation on its' inner and outer walls. At this point it was assumed that some desolvation was necessary to reduce the solvent vapour concentration prior to membrane desolvation.

However, when the initial solvent mix was nebulised into the condenser, it became blocked with ice in under 2 minutes. Since the freezing point of acetonitrile is lower than that of water, it was decided to find out at what water percentage in the solvent mix freezing occured at. To ascertain this, 100%, 95%, 80% and 70% acetonitrile/water solutions were nebulised. With 100% and 95% acetonitrile, no ice was seen to form, with 80% acetonitrile, an approximately 1 mm layer of ice had formed after 5 minutes. The 70% mix resulted in total blockage within 4 minutes. Since it was known that for gradient elution of the drugs, an initial solvent mix of 5% acetonitrile was required, a way of reducing the water content needed to be found before the condensation phase could be used.

On consultation with PermaPure Inc. the manufacurers of the membrane driers, it was discovered that the driers should be able to cope with the solvent loads coming out of the spray chamber. This was the case as long as the solvent remained in the vapour phase and condensation on the membrane was prevented. Since it was solvent condensing on the membrane walls that caused it to collapse initially.

To overcome the condensation problem, an extra I.R. lamp was employed to heat the gas input line for the membrane drier in conjunction with aluminium foil reflectors to direct the infrared radiation back onto the rest of the membrane. With this arrangement, after careful positioning of the lamps, no condensation was seen on the membrane with nebulisation of a 25:75 acetonitrile:water mix.

The condenser was then connected after the membrane drier and mixtures of water and acetonitrile varying from 100% acetonitrile to 5% acetonitrile were nebulised to see if

blocking of the condenser with ice now occured with membrane desolvation prior to sample introduction to the condenser. The result of this was that blocking up with ice only occured after 10 minutes at a mix composition of 5% acetonitrile. It was therefore decided to use this configuration in future experiments.

3.6.2.1.2 Experimental

A known mass (200 g) of 25:75 acetonitrile:water was nebulised using a Meinhard nebuliser with an argon flow of 0.65 l min^{-1} into a cyclone spray chamber radiatively heated with two, 250 W infra-red lamps. The resulting vapour was passed through a membrane drier attached to the spray chamber and purged with a counter flow of argon at a flow rate of 2 l min^{-1}. The membrane drier is heated to 75^0C to prevent condensation on the membrane that can cause it to collapse. The membrane drier removes most of the water, so that ice formation does not occur when the aerosol stream passes into the condenser that operates at -20^0C where the vapour is condensed.

Two membrane driers were used in this one 25 cm long, the other 50 cm long, both consisting of a Nafion® tubular membrane held in a fluoro carbon polymer outer casing supplied by Perma Pure (Toms River, New Jersey, USA) (Figure 3.12).

Two condenser designs were used in this study, both consisted of 6 peltier heat pumps powered by a DC supply (Figures 3.13 and 3.14). The hot faces of these are cooled by 2 liquid cooled aluminium blocks.

The liquid from the spray chamber drain and peltier condenser drain was collected in pre-weighed beakers. From the material collected at the spray chamber drain, the mass of vapour entering the system was calculated. The mass collected at the Peltier condenser drain indicated how much solvent vapour had been removed, and the mass change in the silica traps indicated how much vapour was left in the carrier stream. Thus, knowing the mass of liquid entering the system, and the mass of liquid exiting the system, the mass removed by the membrane could be calculated by difference.

Figure 3.12: A Schematic of the Perma Pure Membrane Drier

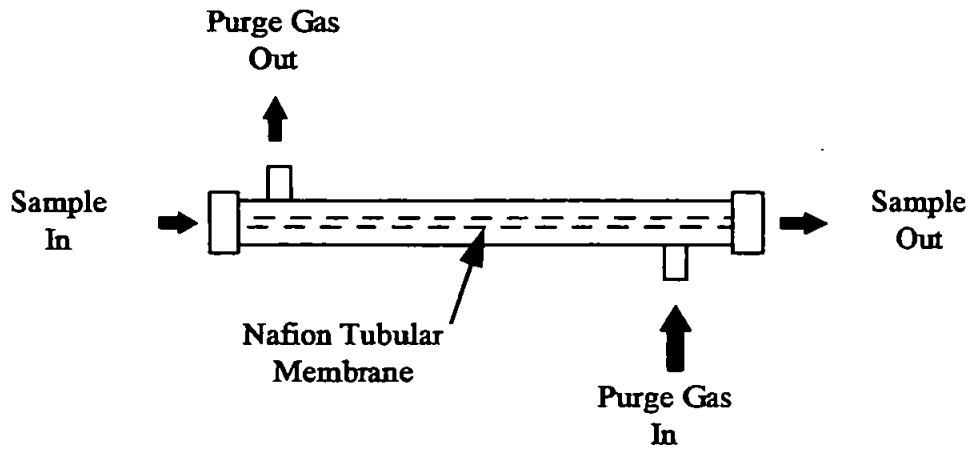


Figure 3.13: First peltier condenser design

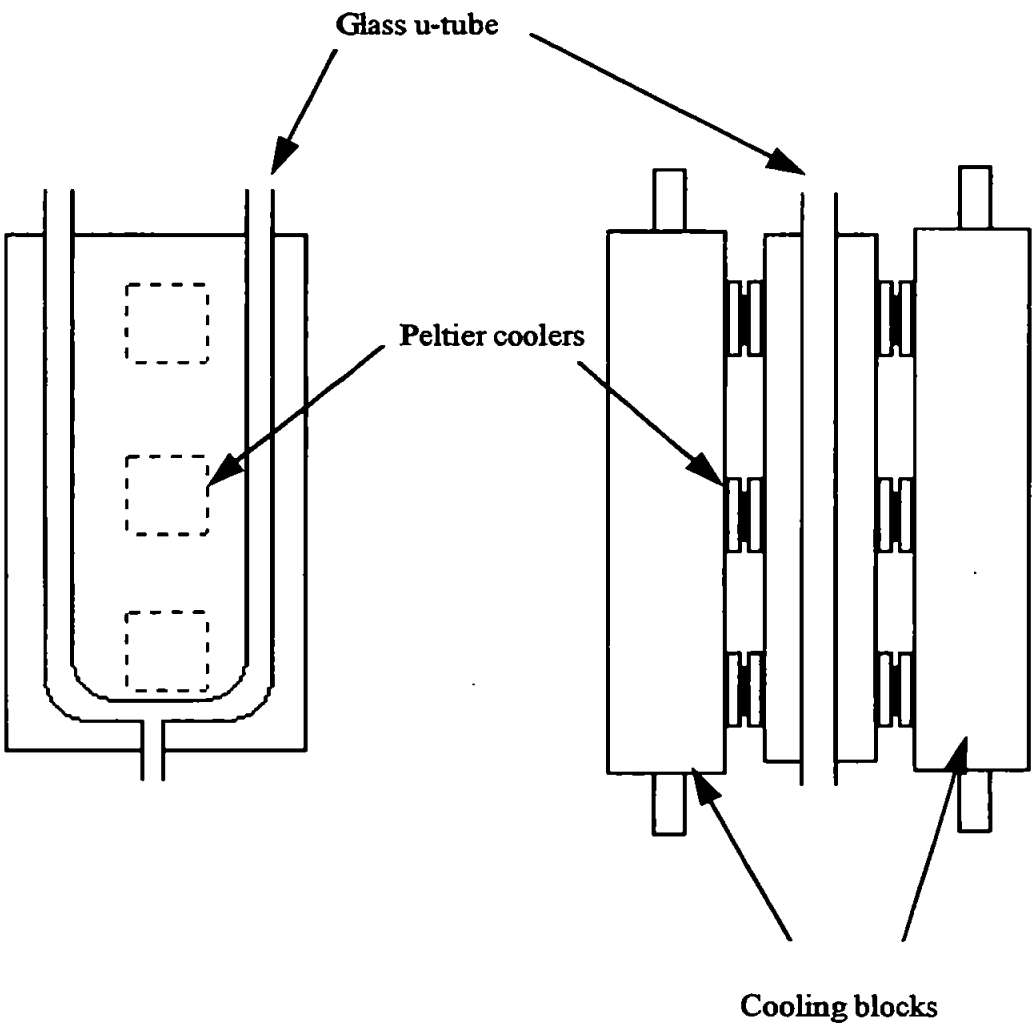
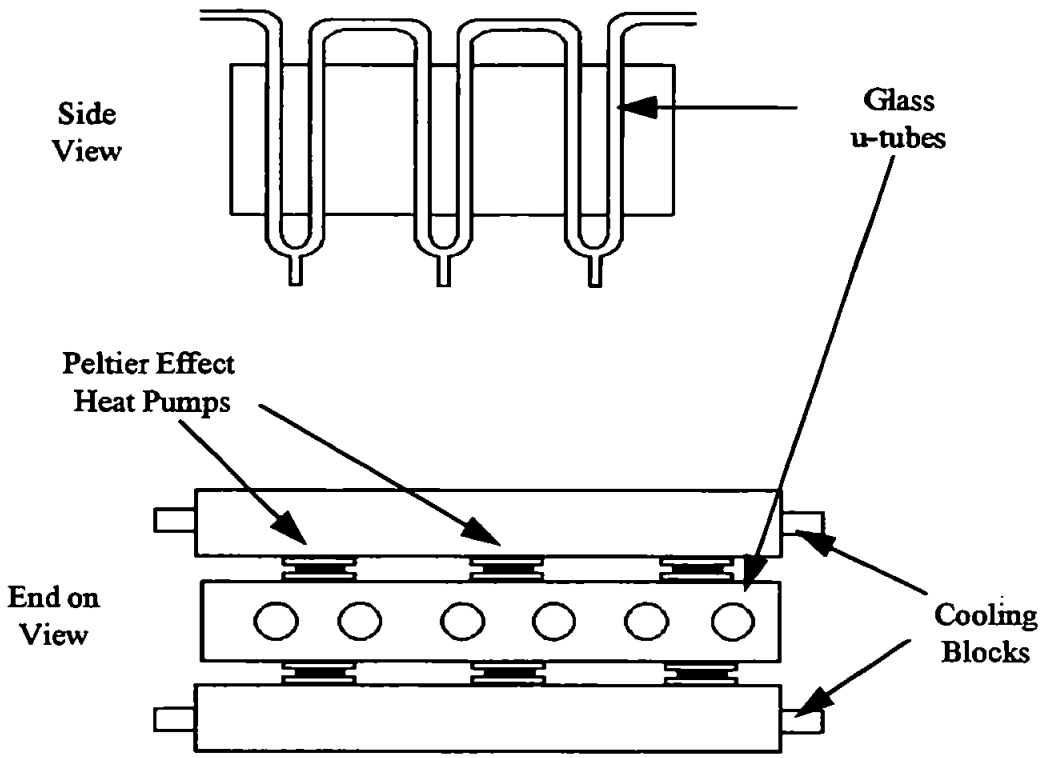


Figure 3.14: A Schematic of
The Improved Peltier Driven Condenser



3.6.2.2 Results

3.6.2.2.1 Evaluation of the Initial Conditions: The initial conditions used were a nebuliser gas flow of 0.7 l min^{-1}, with a membrane purge gas flow rate of 2 l min^{-1}. The result of using these conditions can be seen in Table 3.7. From these results it can be seen that 10.3286 g of solvent exited the spray chamber (146.8069 - 136.4738) resulting in a transport efficiency of 7.0%. Of these 10.0000 g, 2.9932 g were collected by the peltier condenser and 2.6057 g were collected by the silica gel traps resulting in a total of 5.5989 g collected, therefore 4.7342 g must have been removed by the membrane drier. This shows that the membrane removed 45.8% of the solvent, and the peltier condenser removed a further 28.9% with an efficiency of 53.4% (ie 2.9932/5.5989 x 100) resulting in a total desolvation efficiency of 74.7%.

3.6.2.2.2 Optimisation of the nebuliser gas flow rate: Using the same experimental apparatus as in section 3.4.1.1. A known mass of solvent was nebulised, the liquid collected at the drain was weighed and from this the transport efficiency of the spray chamber and nebuliser can be determined. The results of this can be seen in Table 3.8. The optimum nebuliser flow, with respect to transport efficiency, was found to be 0.6 l min^{-1} (Figure 3.15).

3.6.2.2.3 Evaluation of a longer membrane: To further improve the potential desolvation efficiency of the apparatus, a 50 cm long membrane drier was obtained. The results of it's use can be seen in Table 3.9.

Table 3.7. Masses collected to evaluate the initial operational conditions of the desolvation apparatus.

Mass nebulised (g)	Mass collected at spray chamber drain (g)	Mass collected at the peltier condenser drain (g)	Mass change in u-tube moisture traps (total g)
146.8069	136.4738	2.9932	2.6057

Table 3.8: Optimisation of the nebuliser gas flow

Nebuliser gas flow rate (l min^{-1})	Mass nebulised (g)	Mass collected (g)	Tranport efficiency (%)
0.6	55.09	44.81	18
0.65	72.32	61.40	15
0.7	93.09	79.79	14
0.75	108.41	93.89	13

Figure 3.15: Optimisation of the Nebuliser Gas Flow With Respect to Transport Efficiency

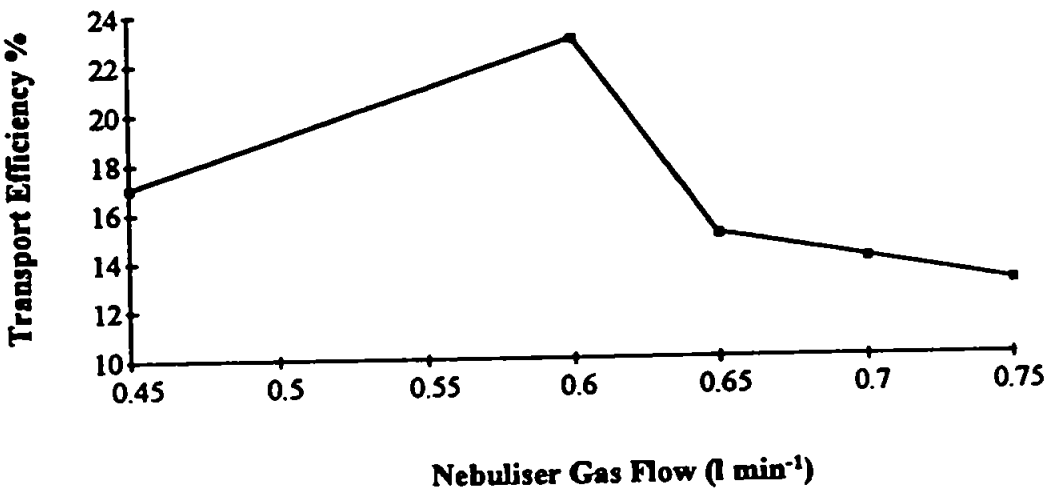


Table 3.9: The effect of using a longer membrane drier.

Mass nebulised (g)	Mass collected at the spray chamber drain (g)	Mass collected at first moisture trap (g)	Mass collected at second moisture trap (g)
78.55	65.48	4.04	0.08

From these results it can be seen that a transport efficiency of 16.6% was achieved along with a desolvation efficiency of 68.5%.

To ensure heating of the entire length of the membrane, further reflectors had been made and an additional 2 IR lamps employed. There was concern however that the silica gel moisture traps were not trapping all the moisture leaving the system, so an additional 2 u- tubes were obtained along with new silica gel to ensure that the desolvation efficiency was not being over-estimated. It was also possible that silica gel did not absorb acetonitrile vapour. To test this, a known mass of acetonitrile (61.3 g) was placed in a dessicator with a known mass of silica gel (55.4 g). This was left for 12 hrs at room temperature, after this time period, the mass of acetonitrile left had reduced by 8.2 g and the mass of the silica gel had increased by 4.1 g. A colour change from royal blue to dark blue was also observed that proved reversible when the silica was dried in an oven. This showed that silica gel is capable of absorbing acetonitrile reversibly.

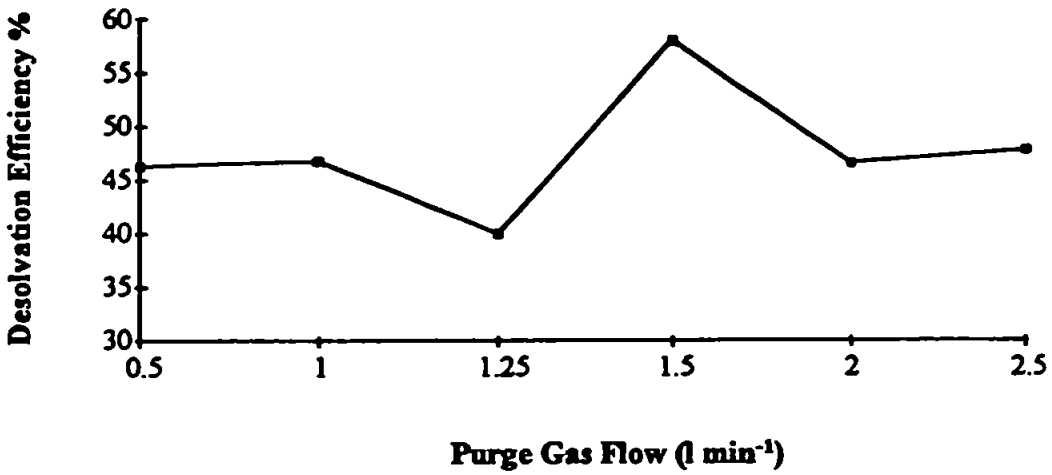
3.6.2.2.4 Optimisation of the membrane drier: The new silica gel, and 4 u-tube configuration were used in conjunction with the 50 cm long membrane drier in this study. The nebuliser gas flow was kept constant at 0.6 l min^{-1}, and the purge gas flow was varied between 0.5 and 2.5 l min^{-1}. The results of this can be seen in Table 3.10. and Figure 3.16.

From these results it can be seen that the optimum purge gas flow rate is 1.5 l min^{-1}. The fact that in all these experiments, the final u-tube moisture trap didn't increase in mass shows that all the vapour exiting the membrane drier was successfully trapped.

Table 3.10: The effect of altering the purge gas flow rate on the desolvation efficiency of the membrane drier.

	Purge gas flow rate (l min)				
	0.5	1.0	1.5	2.0	2.5
Mass nebulised (g)	100.64	102.13	79.62	65.29	45.24
Mass collected at spray chamber drain (g)	75.74	76.89	57.36	50.16	34.90
Mass collected in u-tube 1 (g)	7.78	8.12	6.76	6.43	5.29
u-tube 2 (g)	4.92	4.89	2.53	1.64	0.11
u-tube 3 (g)	0.65	0.40	0.02	0.01	0.01
u-tube 4 (g)	0.00	0.00	0.00	0.00	0.00
Transport efficiency %	24.7	24.7	27.9	23.2	23.0
Desolvation efficiency %	46.3	46.8	58.0	46.6	47.8

Figure 3.16: The Effect Of Purge Gas Flow Rate on the Desolvation Efficiency of the Membrane Drier.



3.6.2.2.5. Evaluation of the new peltier condenser: During the course of the above experiments, the peltier condenser had been redesigned (Figure 3.14). The new design solved a number of problems with the original design. Icing up commenced primarily at the bottom of the u-tube where the drain was placed due to the increased residence time of liquid at this point. Leaving the bottom of the U-tube and drain at room temperature by placing them outside the aluminium blocks completely prevented ice blockages occuring at acetonitrile percentages above 2% in the solvent mix. The tops of the glass U-tubes were also placed outside the aluminium block so that heating of the solvent aerosol between condensation steps could take place. The main aim of this design though, was to increase the length of tubing being cooled so that more solvent could be condensed out prior to sample introduction to a plasma. The results of these modifications can be seen in Table 3.11.

From these results it can be seen that the new design is a significant improvement on the previous design increasing the desolvation efficiency of the condenser from 54% to 74%.

3.6.2.2.6. Determination of the fate of the solvent mix components: Having determined that the desolvation apparatus was removing significant quantites of solvent. It was decided to investigate the fate of the solvent at various points in the desolvation apparatus. The aim of this was to see if desolvation reduced or increased the percentage of acetonitrile in the final effluent, i.e. the vapour stream that finally entered the plasma.

Table 3.11: Evaluation of the new peltier condenser

Mass nebulised (g)	162.33
Mass collected at spray chamber drain (g)	122.37
Mass condensed by peltier condenser (g)	12.47
Mass collected by silica traps (g)	4.27
Transport efficiency (%)	24.6
Membrane desolvation efficiency (%)	58.1
Peltier condenser desolvation efficiency (%)	74.5
Total desolvation efficiency (%)	89.3

3.2.2.6.1. Experimental

A 30% acetonitrile: water solution was continuously nebulised. The spray chamber drain and the membrane effluent were collected in the first experiment. In the second experiment, the spray chamber drain, peltier drain and final effuent were collected. The membrane and final effluent were trapped by placing the 4 u-tubes in a refrigerated bath containing a propanol water mix at a temperature of -20°C. The condensate collected in each was combined and mixed. Measurement of the acetonitrile content in each was taken using Fourier Transform Infra-red (FT-IR) absorption at 2265 cm^{-1}, using a trough Attenuated Total Reflectance (ATR) liquid sample cell. The instrument was calibrated against standards of 5%, 30% and 70% acetonitrile:water mixtures. A water sample was taken as the blank, and this was subtracted from the spectra of the samples and standards.

The results of the two experiments can be seen in Tables 3.12 and 3.13.

From these results it can be seen that the desolvation apparatus not only efficiently removes solvent vapour from the aerosol stream, but it also reduces the organic solvent loading by preferentially removing the organic solvent.

Table 3.12: The effect of the membrane drier on the acetonitrile content of the eluent.

5% Standard (AU)	30% Standard (AU)	70% Standard (AU)	Spray chamber drain (AU)	Membrane drier effluent (AU)
-0.00342	-0.1777	-0.4178	-0.0780	-0.1608
Acetonitrile content (%)			15.2	28.9

Table 3.13: The effect of desolvation on the final acetonitrile content of the eluent.

30% standard (AU)	70% standard (AU)	Spray chamber drain (AU)	Peltier condenser drain (AU)	Final Effluent (AU)
-0.1735	-0.4624	-0.0598	-0.1710	-0.0914
Acetonitrile content (%)		14.3	29.7	18.6

3.6.3. Evaluation of the Desolvation Apparatus as an Interface for HPLC-ICP-MS

Having optimised the desolvation apparatus to remove as much solvent as possible with the available apparatus. It was decided to evaluate whether it was effective enough to be a viable interface for coupled HPLC-ICP-MS.

3.6.3.1 Experimental

The first step was to measure the effect of desolvation on the organic solvent loading of the plasma. A series of acetonitrile:water mixes were nebulised into an ICP-AES and the carbon 247.857 nm emission line monitored to measure the carbon loading of the plasma. The results obtained as shown in Figures 3.17-3.19 show that desolvation has dramatically reduced the carbon loading of the plasma.

The apparatus was then attached to an ICP-MS and a series of 100 ppb platinum standards in different eluent compositions were prepared. These were nebulised with the desolvation apparatus in place and oxygen was added to the nebuliser gas via a gas blender (Signal Instruments, Surrey), whilst the reflected power, and the counts were monitored. The lens stack was retuned for each standard. This was necessary to take into account the varying sample matrix and composition of the plasma since varying levels of oxygen were required to prevent carbon deposition on the sampler and skimmer cones.

3.6.3.2 Results

From the data in Table 3.14 it is possible to see that gradient elution should be possible. The reflected power was found to be stable, and the amount of oxygen and extraction lens settings constant at an eluent composition above 50% acetonitrile. This meant little or no adjustment was necessary during the introduction of a solvent gradient.

3.6.4. The Effect of Introducing a Solvent Gradient into the ICP-MS with Desolvation of the Aerosol

3.6.4.1 Experimental

An inert gradient HPLC pump was employed to supply a solvent gradient of 95:5 to 30:70 % v/v H_2O:acetonitrile in 25 minutes at a flow rate of 1 ml min^{-1}. A 100 ppb aqueous solution of platinum was flow injected via an injection valve fitted with a 200 µl injection loop at various stages in the gradient. The extraction lens setting was maintained at 1.4, and 4% oxygen was added was to the nebuliser gas flow. This was sufficient to prevent carbon deposition at 30:70 % v/v H_2O:acetonitrile.

3.6.4.2 Results

From Figure 3.20 it is possible to see that although the signal does vary throughout the experiment, the change is not dramatic. The injection of standards at various eluent compositions during calibration, makes correction possible. There was no carbon deposited on

Table 3.14: The Effect of Increasing the Acetonitrile Content of the Eluent on the ICP-MS after Desolvation.

% Acetonitrile	Reflected Power	Extraction Lens Setting	Percent oxygen added	Counts for 100 ppb Pt
0	0	2.9	0	50000
25	5	1.6	4	40000
50	10	1.4	5	30000
70	10	1.4	5	30000

Figure 3.17: The Effect of the use of a Peltier Driven Condenser on Plasma Loading

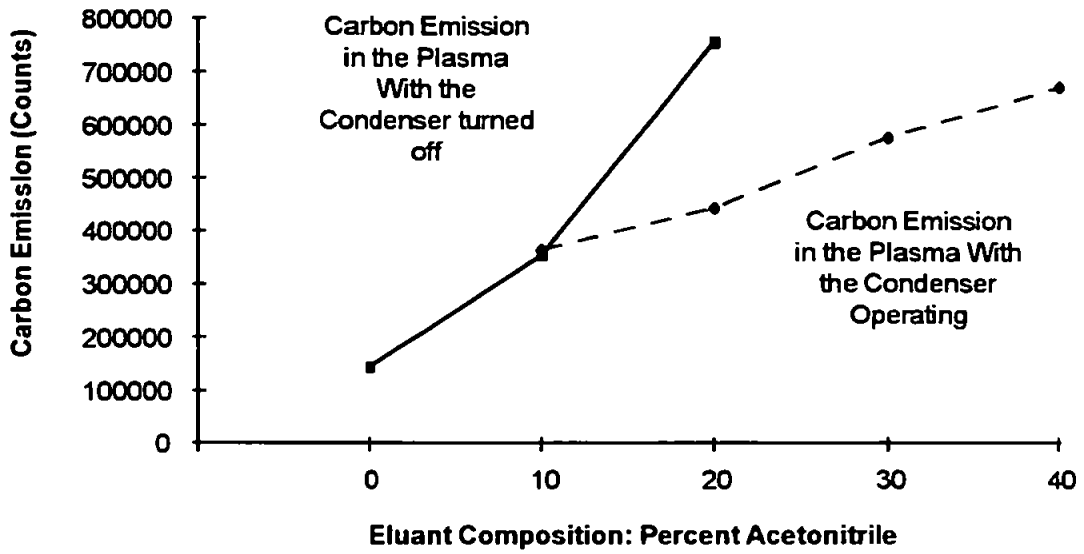


Figure 3.18: The Effect of a Membrane drier on Carbon loading

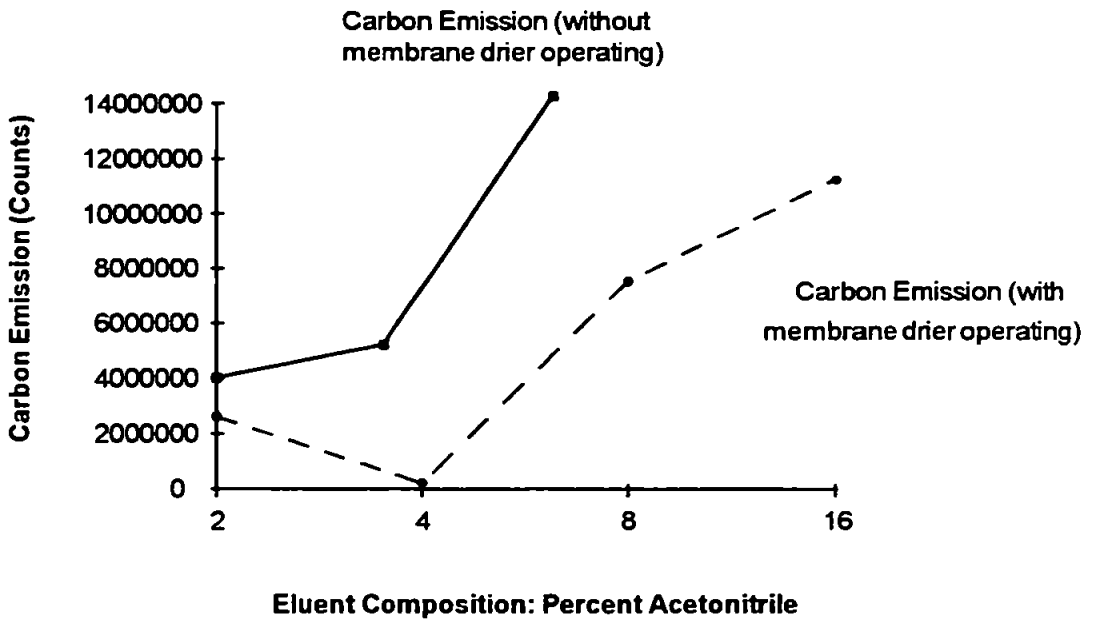


Figure 3.19:The Effect of the Membrane and the Condenser working in Series on the Carbon Loading of a Plasma

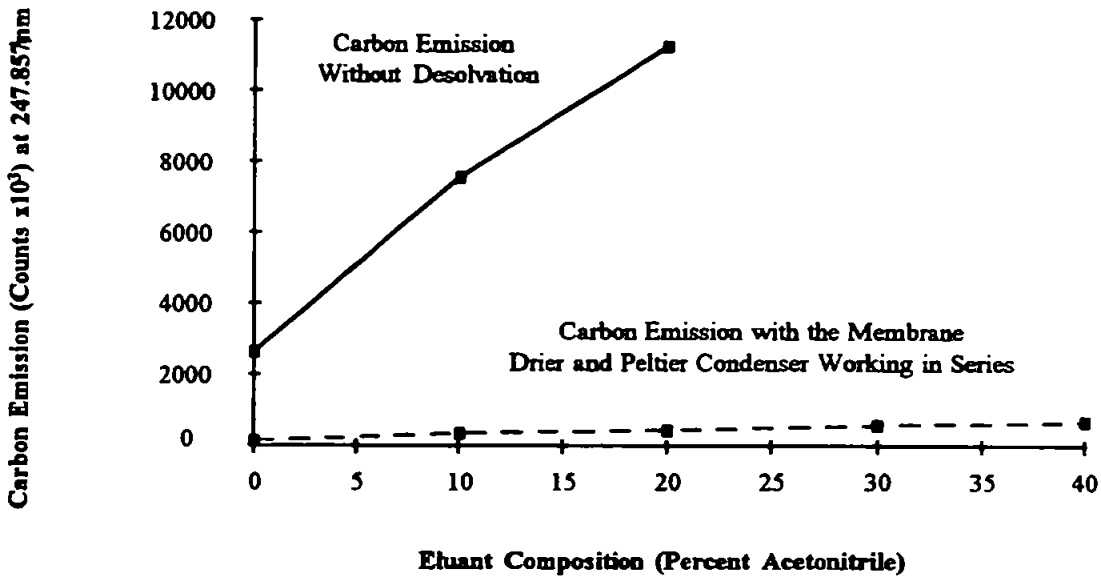
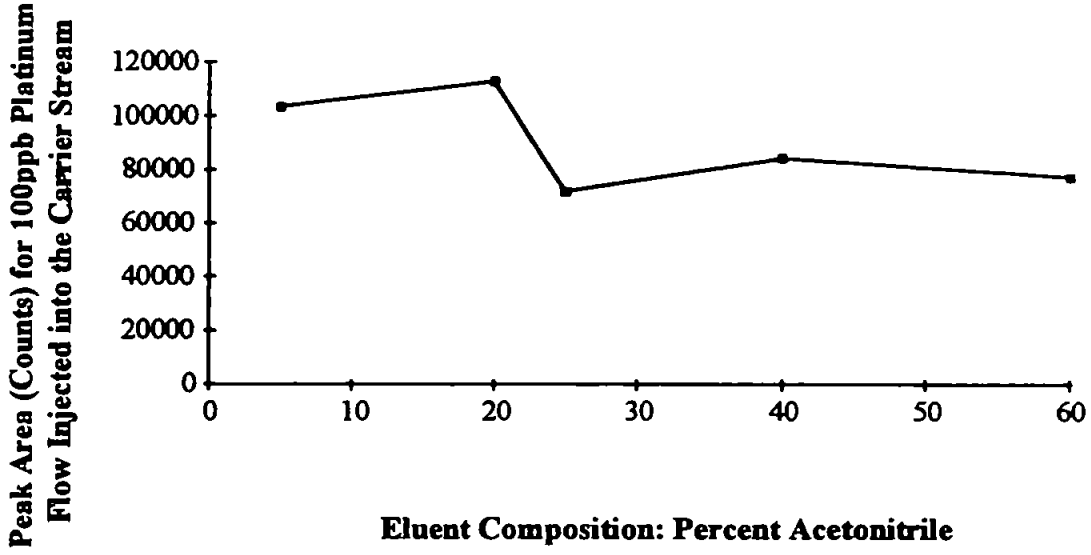


Figure 3.20: Platinum Signal Variation With Increasing Acetonitrile Content of the Mobile Phase With Desolvation and ICP-MS Detection



the sampler cone, and the expansion pressure was constant at 2.2 mbar. This suggests fixing the oxygen percentage in the nebuliser gas at a constant value was not detrimental to the instrument.

3.6.5. Gradient Elution HPLC-ICP-MS

A 0.027 mg ml^{-1} solution of JM-216 was made up in the eluent and injected onto the phenyl column. The sample was eluted with a solvent gradient of 5:95 to 70:30 acetonitrile:water in 25 mins at a flow rate 1.0 ml min^{-1}.

3.6.5.1 Quantification

Calibration was carried out by flow injecting 10, 25, 50, and 100 ng ml^{-1} standards into the eluent stream post-column. This was done after each chromatographic run as the column equilibrated.

3.6.5.2 Results and Discussion

Comparison of the chromatograms obtained by UV and platinum specific ICP-MS detection (Figures 3.21 and 3.22) show that peak broadening does occur. This is probably caused by the large dead volume of the desolvation system, although the gradient chromatography enables the peaks to remain well resolved. The results obtained for the sample are shown in Table 3.15. The detection limit, based on 3 times the standard deviation of the background noise, was found to be 0.6 ng ml^{-1}, representing an actual mass of 120 pg of Pt. The peaks seen before the main drug peak (Figure 3.21) are impurities in the drug, i.e isomers of JM-216, and are not removed by the purification process.

Figure 3.21: Gradient Elution of a sample of JM-216 with UV detection at 210.4 nm

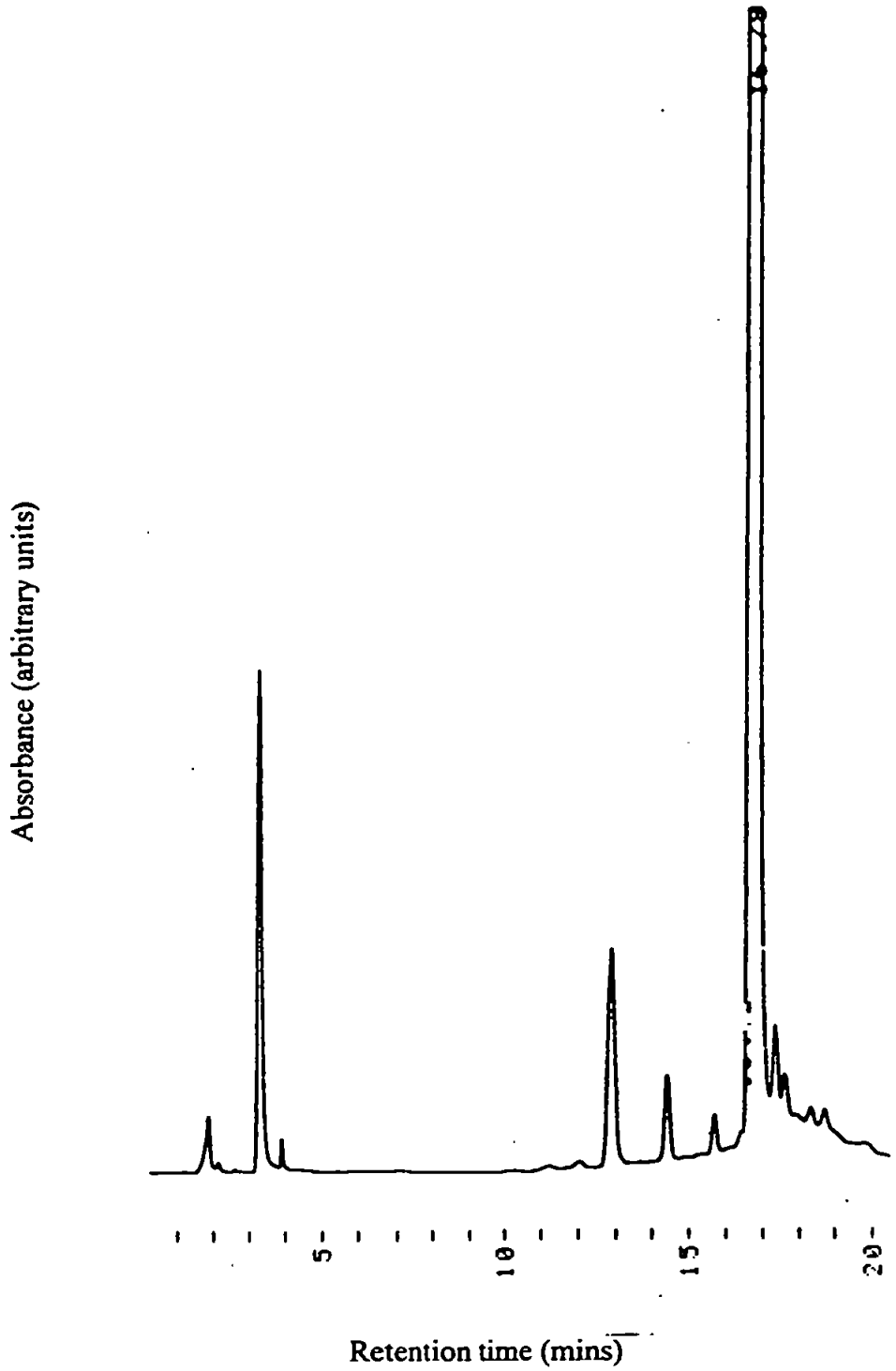
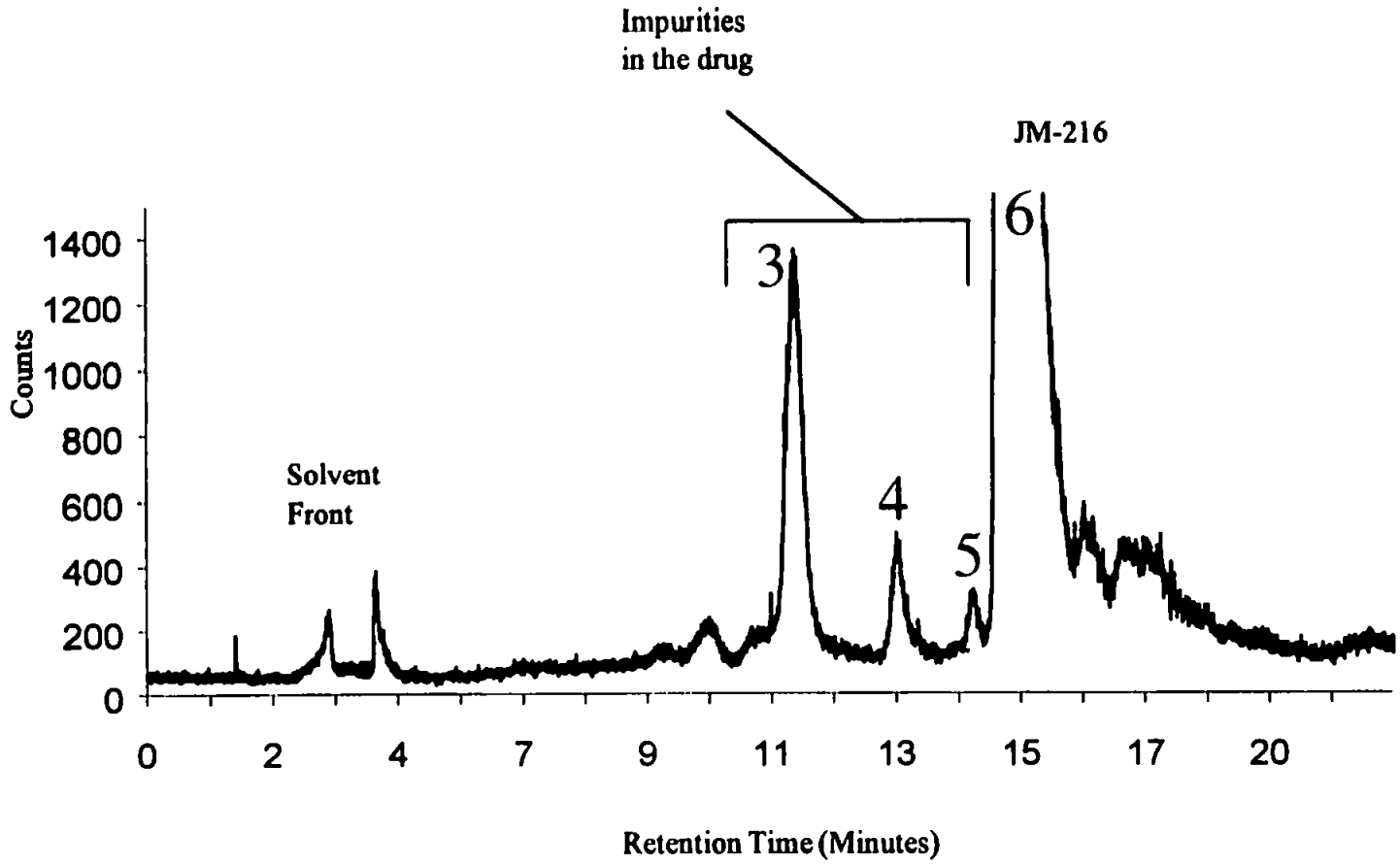


Figure 3.22: Gradient Elution of a JM-216 Solution with ICP-MS Detection at *m/z* 195



Chromatographic Conditions:
The analytes were retained on a 250 x 4.6 mm id column packed with Hypersil Phenyl.
A two eluent system was employed, eluent 1 was water, eluent 2 was acetonitrile.
A linear gradient of 95:5 to 30:70 eluent 1: eluent 2 in 25 minutes was employed at a flow rate of 1.0 ml min^{-1}.

Table 3.15: The Analytical Figures of Merit for the Platinum Species Eluted by the Gradient Chromatography.

Peak Number	Mean Peak Area (Counts s^{-1})	Average Concentration (ng Pt ml^{-1})	Precision of Peak Area Data/RSD (%) (n=5)
3	172720	41.9	10
4	44135	9.1	14
5	11926	2.42	9
6	15825067	3790	5

3.6.6 The Effect of Adding an Additional Argon Flow After Desolvation.

Since the optimum nebuliser flow with respect to transport and desolvation efficiency is 0.6 l min^{-1}, it was suspected that sensitivity was being lost due to inadequate punching of the plasma. To counter this, the effect of adding an additional argon flow after the desolvation apparatus was investigated.

3.6.6.1. Experimental

Three 100 ppb standard solutions were made up, these were aqueous indium and platinum standards plus a platinum standard in a 30% acetonitrile matrix. As these were nebulised, additional argon gas was then added using the glassware in Figure 3.23 after the desolvation apparatus in 0.1 l min^{-1} increments. The gas flow was then adjusted to find the optimum.

3.6.6.2 Results

Figure 3.24 shows that the addition of a make up gas to the original nebuliser gas flow enhances sensitivity by almost an order of magnitude. Figure 3.25 shows the peaks from a 100 ppb aqueous platinum standard flow injected into the plasma with and without an additional gas flow. This illustrates the gain in sensitivity possible as well as showing that adding the gas after the desolvation device does not cause any significant peak broadening.

Figure 3.23: Glassware designed in house to add a sheath Gas Flow After Desolvation to Allow better Punching of the Plasma

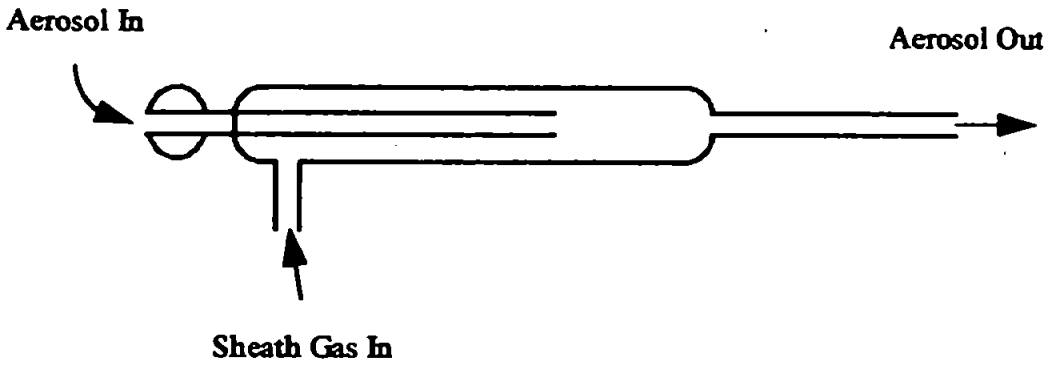


Figure 3.24: Effect of a Sheath Gas on the Platinum Signal For a 10 ng ml^{-1} Standard in Aqueous and Organic Solvents.

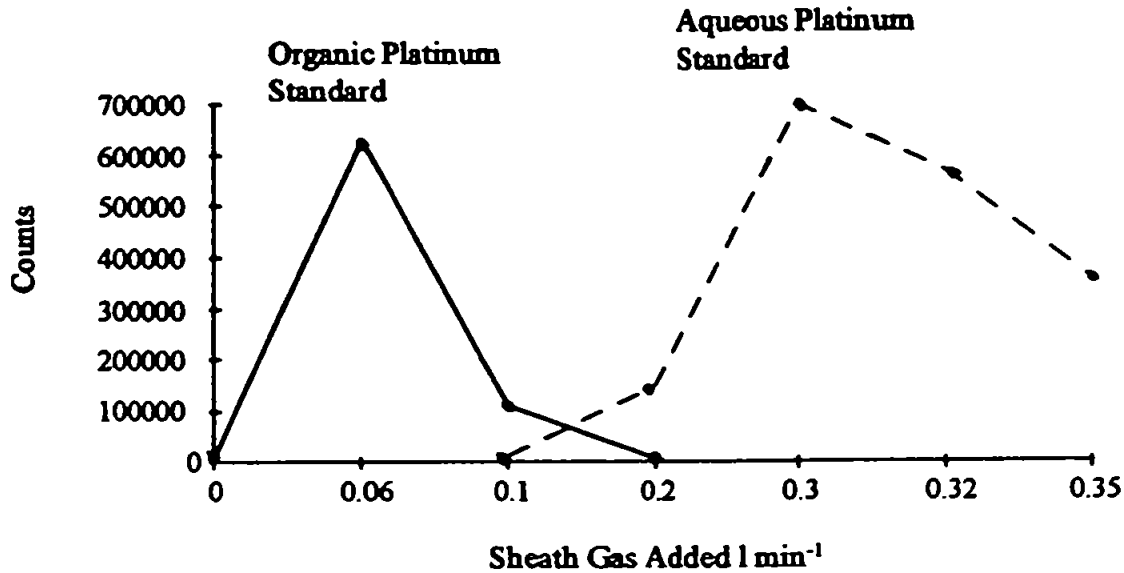
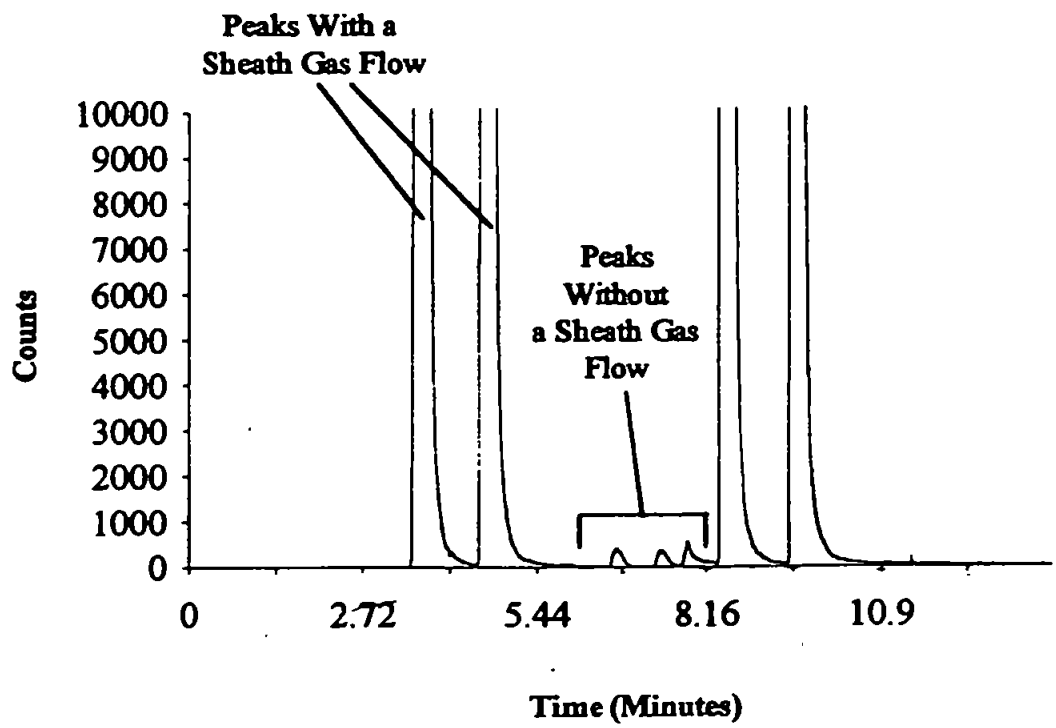


Figure 3.25: Flow Injected Peaks of a 100 ng ml^{-1} Platinum Standard With and Without a Sheath Gas Flow



3.7. Analysis of Real Samples of JM-216 in Human Plasma Ultrafiltrate.

Having proven the desolvation apparatus as an HPLC-ICP-MS interface, it was decided to analyse samples of human plasma ultrafiltrate taken from patients treated with JM-216. The HPLC method used had to be altered as the metabolites of the drug are ionic in nature and would therefore interact with the free silanol groups present in a silica based column resulting in band broadening and excessive retention times. A polymer based column was used so that the only retention mechanism would be hydrophobic interaction of the organic groups present in the metabolites with the stationary phase.

3.7.1 Experimental

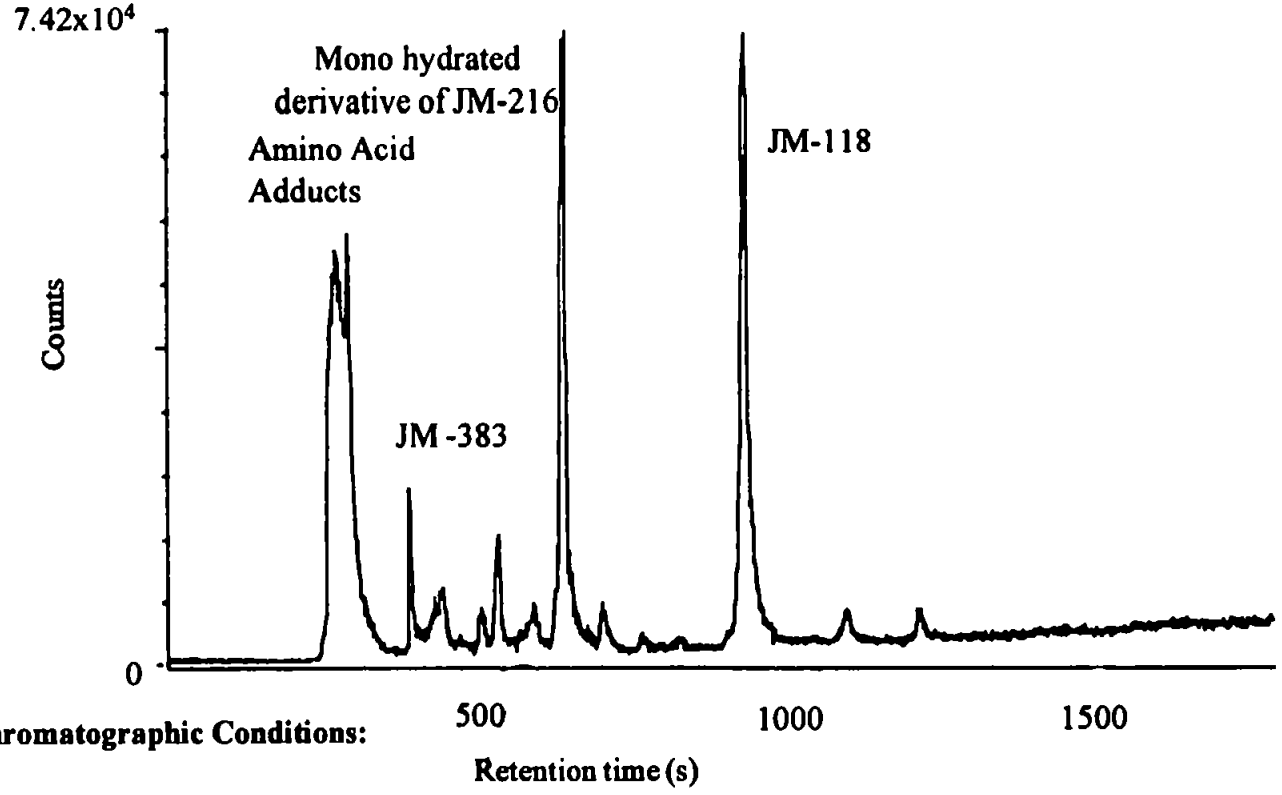
The samples were supplied and prepared by the Institute of Cancer Research and consisted of plasma ultrafiltrate taken from a patient treated with JM-216 during the clinical trials. The samples were taken three hours after administration of the drug and were stored frozen until required.

The samples were diluted 1:50 in the eluent and injected onto the PLRP-S column. The elution consisted of a solvent gradient of 15:85 to 90:10 acetonitrile:water in 30 minutes at a flow rate of 0.6 ml min^{-1}. The samples were quantified by making up standards of the drug in the concentrations of 270, 135, and 27 ng ml^{-1} as the drug, and injecting them onto the column after each chromatographic run.

3.7.2 Results

Figure 3.26 shows that the drug is completely metabolised in the body and converts into a number of compounds. Figure 3.27 shows that none of the platinum containing metabolites have a chromophore making detection of them by UV absorption spectroscopy impossible. The results obtained for the sample used in this study are shown in Table 3.16. The detection limit based on three times the standard deviation of the background noise was found to be 0.60 ng ml^{-1} representing an actual mass of 119 pg of Pt. This represents an improvement in the LOD from 1.3 ng ml^{-1} to 0.6 ng ml^{-1} compared to previous studies (83).

Figure 3.26: A Plasma Ultrafiltrate Sample of a Patient Treated with JM-216 With ICP-MS Detection at *m/z* 195



Chromatographic Conditions:

A two eluent system was employed, eluent 1 was water, eluent 2 was acetonitrile.A solvent gradient of 85:15 to 10:90 eluent 1: eluent 2 over 30 minutes was employed to separate the analytes at a flow rate of 0.6 ml min^{-1}. The analytical column was a 250 x 4.6mm id column packed with PLRP-S.

Figure 3.27: Gradient Elution of a plasma ultrafiltrate sample of a patient treated with JM-216 with UV detection at 210.4 nm

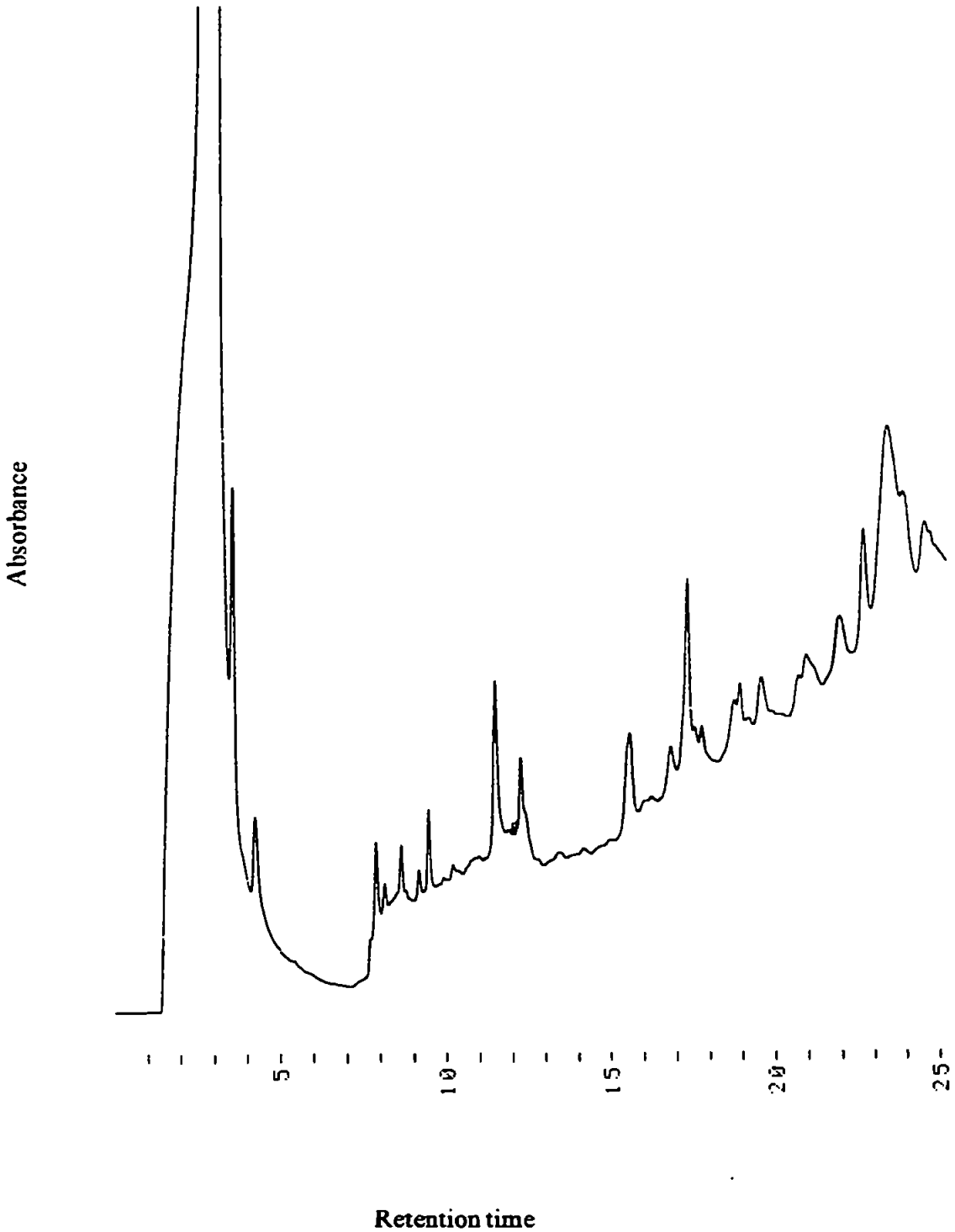


Table 3.16: Analytical Figures of Merit for the Platinum Species Present in the Plasma Ultrafiltrate Sample

Peak Retention Time (s)	Mean Peak Area (Counts)	Average Concentration of Platinum in Metabolite Peak (diluted sample) (ng ml^{-1})	Average Concentration of Platinum in Metabolite Peak (Actual sample) ng ml^{-1})	Precision of Peak Area data (RSD) (%) (n=3)
396	108366	10	500	9
447	103083	9	450	36
647	654744	75	3750	16
712	55422	5	250	8
942	1049186	92	4600	8

3.8. Separation of Cisplatin and Carboplatin in Water and Spiked Samples of Human Plasma Ultrafiltrate.

Having produced a successful chromatographic separation for JM-216, a novel third generation drug from its metabolites in plasma ultrafiltrate, it was decided to compare the results obtained with the separation of cisplatin and carboplatin in water and spiked plasma ultrafiltrate samples.

The chromatographic conditions used were based on those used by De Waal et al. (77) to analyse rat plasma for cisplatin by HPLC-ICP-AES.

3.8.1 Experimental

3.8.1.1 Instrumentation

The instrument used was a FI Elemental PQ2+ turbo, fitted with a standard Scott type spray chamber cooled to -5°C by pumping a water diethylene glycol mix into its cooling jacket. The nebuliser was a concentric nebuliser, operated at a gas flow rate of 0.82 l min^{-1} (Glass Expansion Pty, Australia).

3.8.1.2 Standard and Sample Preparation

Each drug was dissolved in 100 ml of water to make up a stock solution, (28 μg ml^{-1} for cisplatin and 48 μg ml^{-1} for carboplatin). The single and mixed standards were made up by

diluting the stock solutions 100 fold. Artificial samples were then prepared by spiking 0.5 ml of plasma ultrafiltrate with 200 µl of the stock solution. These samples were then incubated in a water bath at 30°C for approximately 1 hour to simulate chemical conditions in the body. 0.1 ml aliquots of the samples were then diluted 100 fold in the eluent and injected onto the column.

3.8.1.3 Chromatography

An inert gradient pump was used and the analytes were retained on a Hypersil ODS analytical column (Jones Chromatography, Mid Glamorgan, UK).

A three eluent elution system was used. Eluent 1 was a 1 mM Sodium octane sulphonic acid (SOS) solution in 10 mM phosphate buffer at pH 3.0. Eluent 2 was a 1 mM SOS solution in 60 mM phosphate buffer at pH 3.0, and eluent 3 was propan-2-ol.

The elution conditions employed were:

100% eluent 1 0 to 5 minutes, a linear gradient to 95:5 (eluent 2: eluent 3) from 5 to 10 minutes, and an isocratic elution of 95:5 eluent 2: eluent 3 from 10 to 26 minutes at a flow rate of 1 ml min^{-1}. The column was then re-equilibrated for 20 minutes at the start conditions.

3.8.2 Results and Discussion

Figures 3.28 to 3.30 show that cisplatin and carboplatin are well resolved using this chromatographic system. The extra peak on the cisplatin standard chromatogram is probably an

hydrolysis product, a similar but smaller peak can be seen on the carboplatin standard chromatogram indicating that it is more stable in solution that cisplatin. Figure 3.31 shows a chromatogram of human plasma ultrafiltrate spiked with cisplatin, apart from the drug peak, three other peaks can be seen. Two of these are present in the standard chromatogram but the first peak is only seen in the ultrafiltrate sample and is probably a cisplatin-amino acid complex. The plasma ultrafiltrate sample spiked with carboplatin (Figure 3.32) has a number of peaks apart from the main drug peak. The first has the same retention time as cisplatin, suggesting that carboplatin breaks down to cisplatin in plasma. The other peaks of interest have the same retention times as the hydrolysis products of cisplatin. The sample of plasma ultrafiltrate spiked with cisplatin and carboplatin (Figure 3.33) has the same peaks present. To verify the existence of hydrolysis products of the drugs, the standards were kept in a refrigerator in the dark overnight and injected onto the column the next day. The results can be seen in Figures 3.34-3.36, The cisplatin drug peak has substantially been reduced, and two large peaks with the same retention times as the suspected hydrolysis products can be seen. The carboplatin standard shows little change and carboplatin can therefore be assumed to be much more stable in solution than cisplatin. The mixed standard result shows both drug peaks as well as the two peaks associated with the hydrolysis of cisplatin. There is however an unknown peak as well.

3.8.3 Conclusions.

The HPLC-ICP-MS interface has proved itself to be capable of handling large amounts of organic solvents in the mobile phase, so that when the eluent is introduced into the plasma, no detrimental affects are observed. It is now possible to optimise the chromatography of organo-

Figure 3.28: A Standard solution of cisplatin

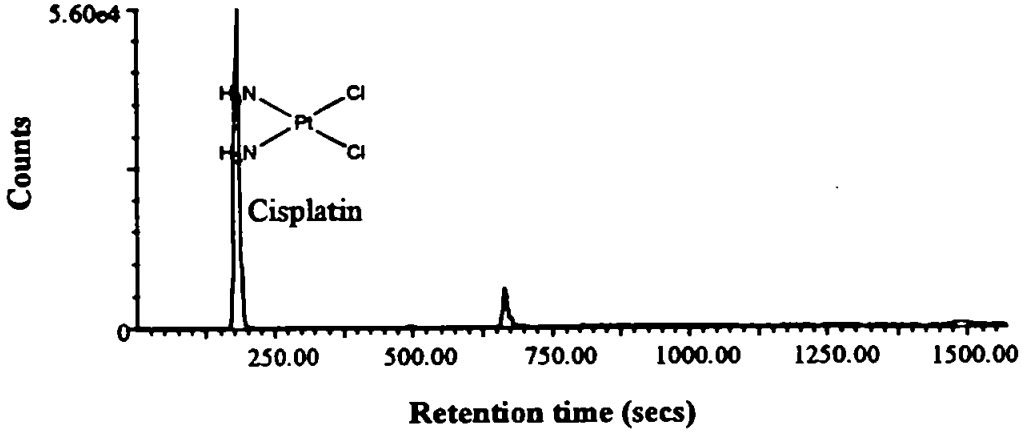


Figure 3.29: A Standard solution of carboplatin

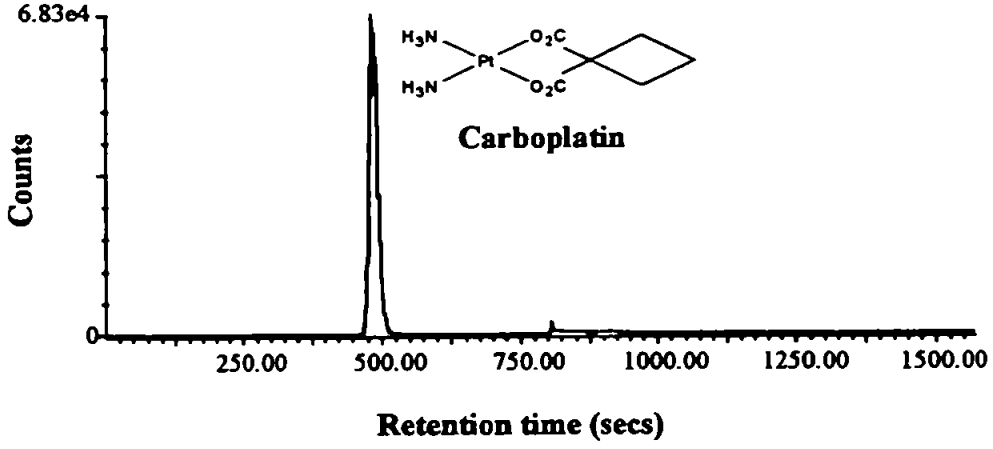


Figure 3.30: A Mixed Standard solution of cisplatin and carboplatin

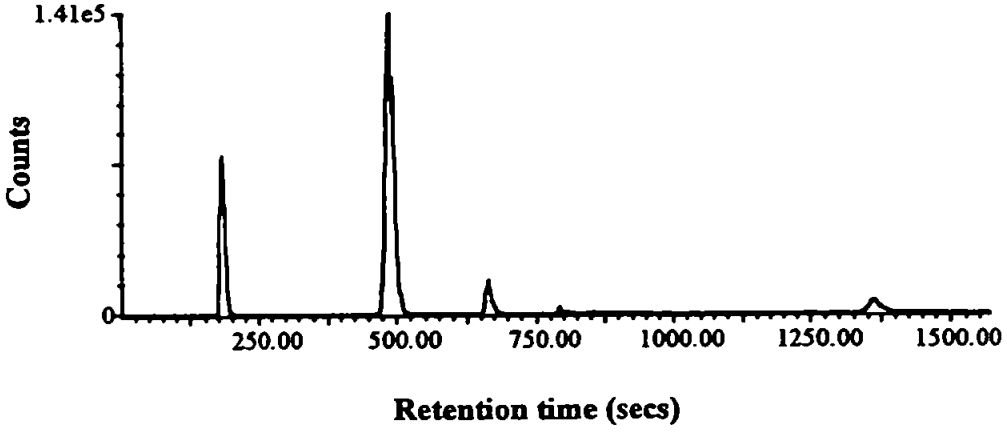


Figure 3.31: Human plasma ultrafiltrate spiked with cisplatin

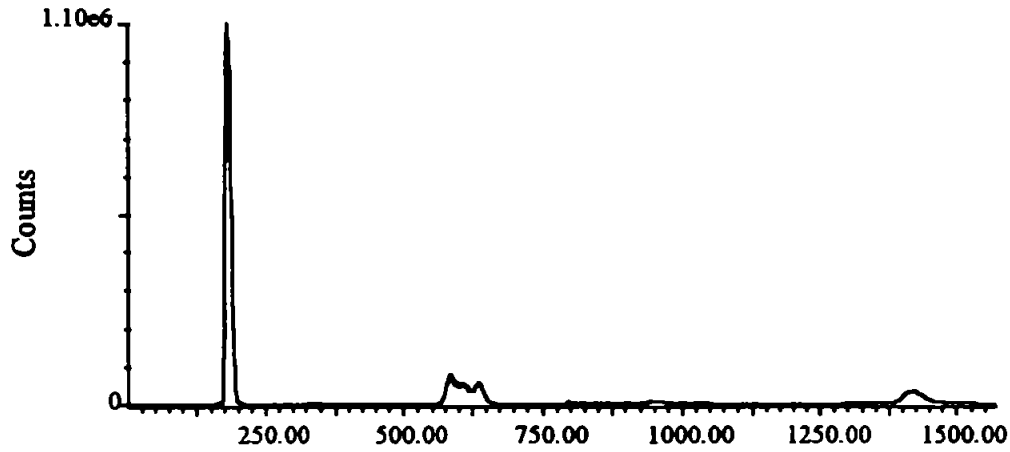


Figure 3.32: Human plasma ultrfiltrate spiked with carboplatin

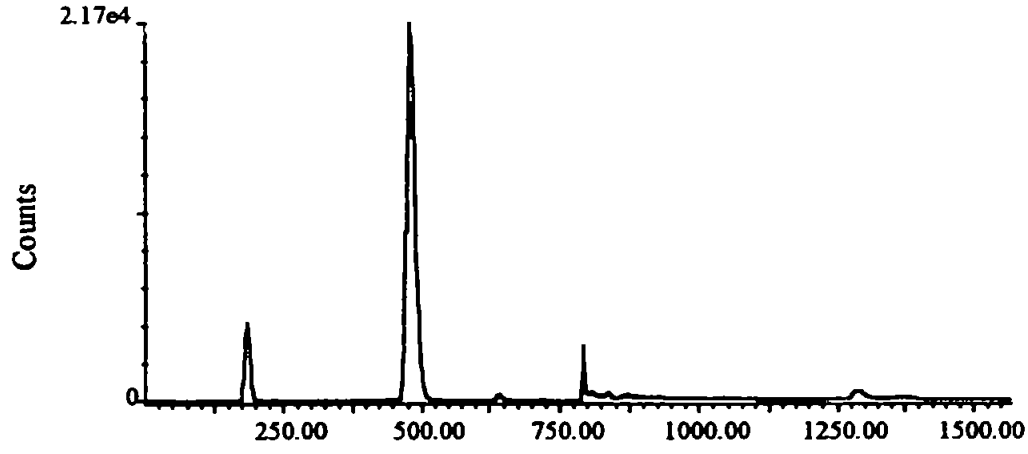


Figure 3.33: Human plasma ultrafiltrate spiked with cisplatin and carboplatin

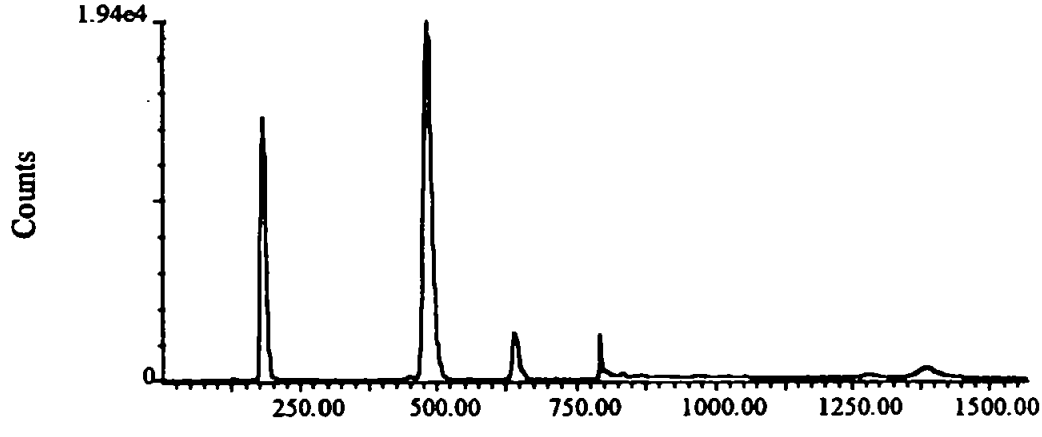


Figure 3.34: An aged cisplatin standard

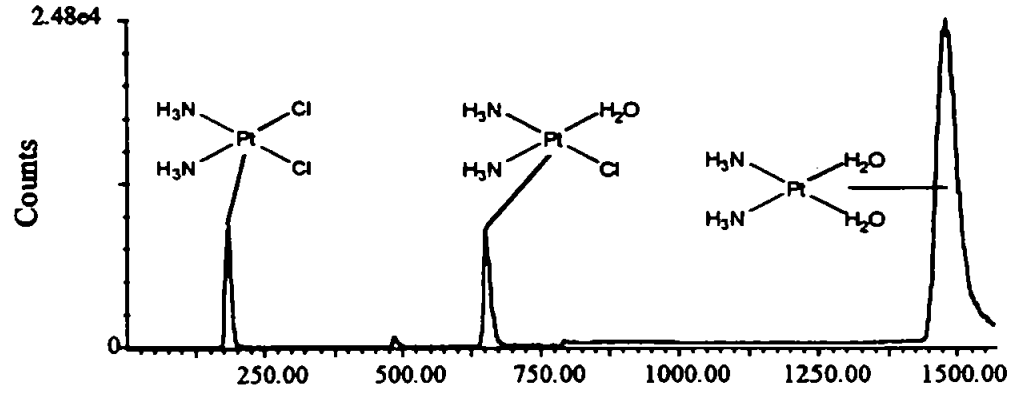


Figure 3.35: An aged standard of carboplatin

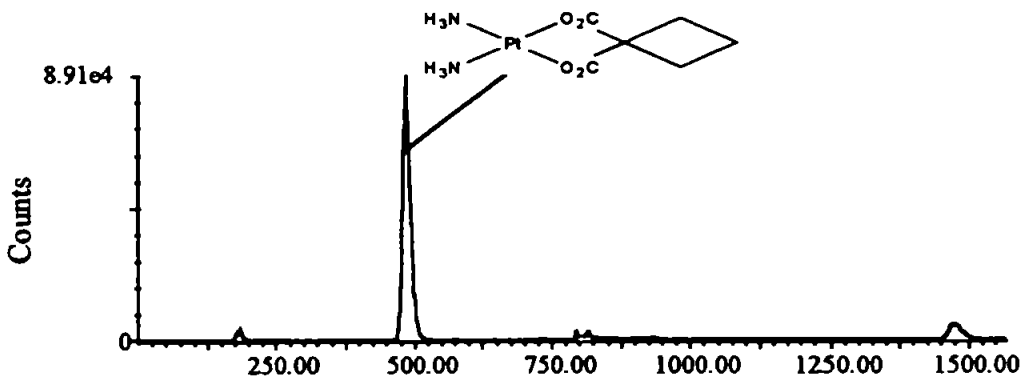
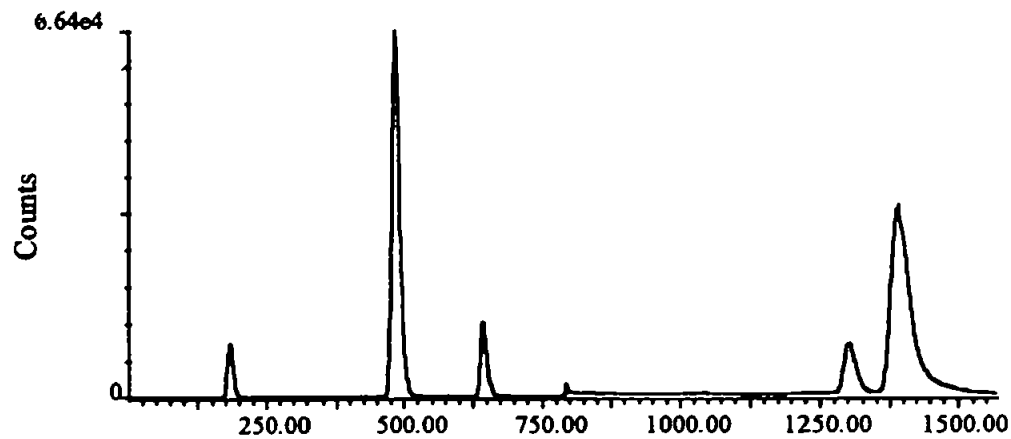


Figure 3.36: An aged mixed standard of cisplatin and carboplatin



metallics without having to take into account the limitations of the ICP-MS as a detector. Thus this interface offers the potential to enhance the flexibility of ICP-MS as an HPLC detector.

Without the ability to use a solvent gradient it would not have been possible to separate the metabolites of JM-216. Figure 3.19 shows the importance of using an element specific detector, none of the metabolites of JM-216 have UV chromophores, so detection would be impossible without a platinum detector. The drug converts to a number of active compounds in the body (Figure 3.18), the advantage of JM-216 is that cisplatin is not one of its biodegradation products, as it is for carboplatin (Figure 3.24), cisplatin has a number of toxic side effects so its formation is undesirable.

The use of ICP-MS as a detector means that chromatograms can be acquired on-line, reducing the acquisition times considerably from those using off-line detection such as HPLC-ETAAS. ICP-MS therefore offers extremely sensitive, specific and rapid detection for the main platinum based chemotherapy drugs and their metabolites, as well as for the new third generation compounds currently undergoing clinical trials.

4.0 Organolead Speciation

4.1 Introduction

Lead is a global pollutant due to the extensive use of tetraethyl and tetramethyllead as an antiknock agent in petrol (2). This is because tetra-alkyllead compounds are easily oxidised to lead oxide, the antiknock agents work by advancing as a fine mist in front of the flame front in the combustion cylinder scavenging the peroxy radicals responsible for premature combustion of the fuel (3). This use remains the largest application of organolead compounds and represents 5-7% of the global lead consumption, estimated to be 3-3.5 Mtonnes in 1982 (2).

The toxicity of organolead is much higher than that of inorganic lead and diminishes in the sequence $R_4Pb > R_3Pb^+ > R_2Pb^{2+} > Pb^{2+}$. Although it is known that high level accidental exposure to organoleads can be fatal the effects of long term exposure at lower levels is not as certain but is blamed for a number of metabolic and neurophysical disorders in children (2).

It is this concern that has prompted the development of analytical methods to speciate lead in environmental samples. The most commonly used separation technique for organolead compounds is gas chromatography (GC) (2). The species are then detected by a number of techniques, that include atomic absorption spectroscopy (AAS), quartz furnace AAS (QF-AAS), microwave induced plasma atomic emission spectroscopy (MIP-AES) and mass spectrometry (MS) (2).

An HPLC-ICP-MS method for the speciation of lead has been developed at the University of Plymouth (130), so it was decided to evaluate the applicability of the HPLC-ICP-MS interface to this method and compare its use to the conditions used without desolvation. The advantage with an HPLC method is that the analytes do not require derivatisation. This is a very time consuming sample preparation step that is prone to interferences. The use of ICP-MS allows the application of on-line isotope dilution analysis, this methodology should allow more accurate and reproducible quantitation of organolead species in the environment.

4.2 Experimental

4.2.1. Instrumentation

The ICP-MS used for this study was a FI Elemental PQ2+. Data was acquired by single ion monitoring of *m/z* 208. The Varian 9010 inert HPLC pump (Varian Associates, California, USA) was used as the solvent delivery system to the HPLC column (details in Section 4.2.3).

4.2.2. Reagents and Standards

The ion pairing agent used in this work was sodium pentanesulphonic acid (Eastman Kodak, UK, HPLC grade). The buffer was made up of sodium acetate and acetic acid (AnalaR and aristar grade respectively, BDH/Merck, Poole, UK.). Methanol was used as the organic modifier (HPLC grade, Rathburn, UK.).

Standards of trimethyllead and triethyllead were prepared from the solid (Alfa products, Johnson Matthey, Royston) and dissolved in a 60:40 methanol: water mix.

4.2.3. Chromatography

The analytes (trimethyl and triethyllead) were separated on a 250 x 4.6 mm id stainless steel column packed in house with 5 μm Hypersil ODS (Jones Chromatography, Mid Glamorgan). A two eluent system was employed:-

Eluent 1: 4mM sodium pentanesulphonic acid/ 0.1M acetate buffer (pH 4.6).

Eluent 2: Methanol

A flow rate of 1.0 ml min^{-1} was employed with an isocratic elution of 40:60 eluent 1:eluent 2.

4.3. Results and Discussion

4.3.1. Chromatography Without Desolvation

In order to accurately quantify any advantage in using the desolvation apparatus as well as check the robustness of the current method, it was decided to repeat the work carried out in these laboratories by Brown *et al.* (130). This involved simply interfacing the HPLC to the ICP-MS using a length of PTFE tubing connected to the nebuliser. The operating conditions used for the ICP-MS are presented in Table 4.1

A Scott type double pass spray chamber chilled to 5 °C with a thermostated water/ethylene glycol mix pumped through a coolant jacket was used to reduce the solvent loading of the plasma. The eluant from the HPLC column was nebulised using a glass concentric nebuliser

Table 4.1: ICP-MS operating conditions for lead chromatography.

Forward Power (W)	1800
Reflected Power (W)	25
Gas Flows (l min^{-1})	
Nebuliser	0.8
Auxiliary	0.75
Coolant	15.0

(Glass Expansion, Australia). Oxygen was added to the nebuliser gas flow as required (2-3%) to prevent carbon deposition on the sampler and skimmer cones.

It should be noted that the forward power is much higher than that normally used for organic solvents (1500W). The forward power was increased to 1800W so that the reflected power was below 30W. It was necessary to keep the reflected power below this value, as the ICP-MS used has a safety cutoff point that prevents operation of the rf generator when the reflected power is above this value. These conditions are not normally required, but at the time of the experiment there was a problem with the torch box rf tuning circuit as well as a problem with the rf generator itself.

A platinum tipped sampler cone was used as at forward powers above 1500W argon/oxygen plasmas ablate the sampler cone at a much higher rate than normal.

The results in Figures 4.1-4.4 show that trimethyllead and triethyllead are well resolved and that inorganic lead gives only a poor response at the 100 ng g^{-1} level. The chromatographic blank, an injection of the mobile phase (Figure 4.2) shows a small inorganic lead peak only.

Since most of the lead in the environment is as inorganic lead (2-3) the effect of a large inorganic lead concentration on the chromatography was investigated. Figure 4.5 shows the effect of spiking the standard mix with 1 μg g^{-1} of inorganic lead. The peaks are poorly resolved and are distorted. This is probably due to the inorganic lead taking up many of the ion exchange sites created by the ion pairing agent in the mobile phase.

Figure 4.1: A 100 ng g^{-1} Test Mix of Inorganic, Triethyl and Trimethyllead

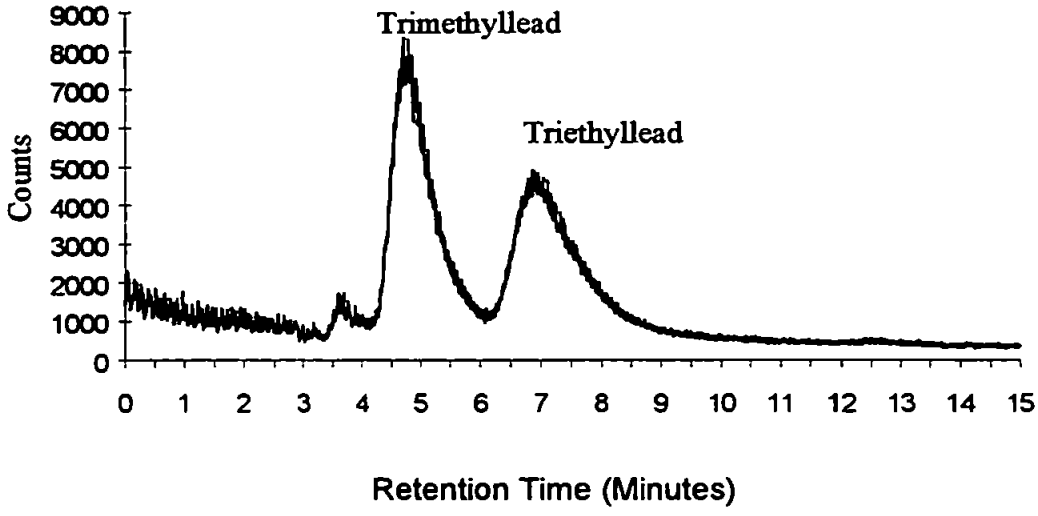
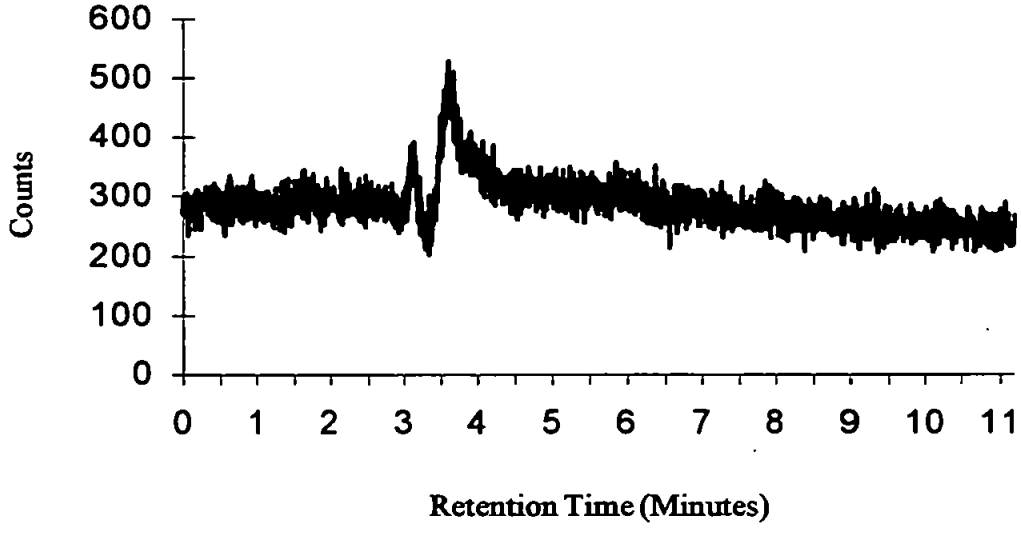


Figure 4.2: Blank Injection



Chromatographic Conditions:

The analytes were separated on a 250 x 4.6mm id column packed with Hypersil ODS. A two eluent system was employed:- Eluent 1: 4mM sodium pentane sulphonic acid 0.1M acetate buffer (pH 4.6) Eluent 2: methanol. A flow rate of 1.0ml min^{-1} was employed with an isocratic elution of 40:60 eluent 1: eluent 2

Figure 4.3: Trimethyllead 100 ng g^{-1} Standard

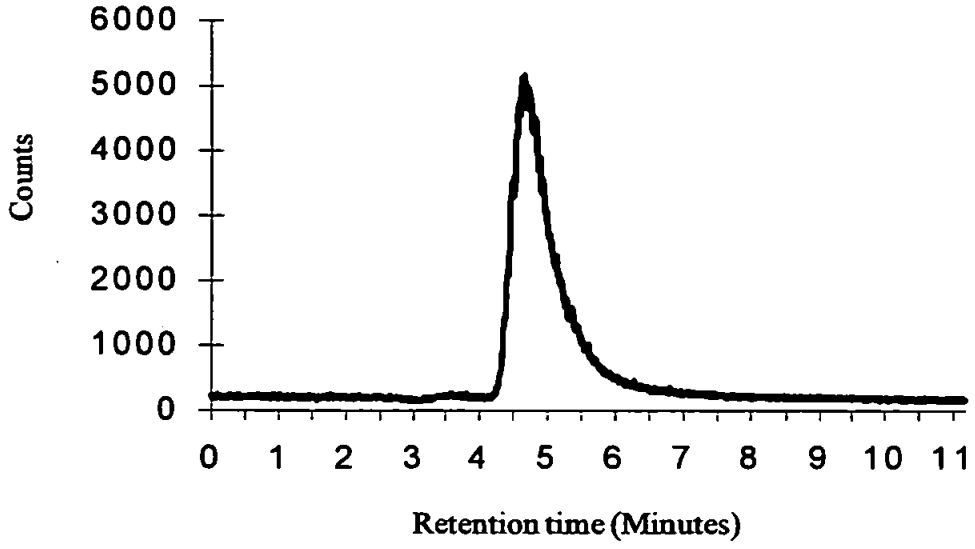
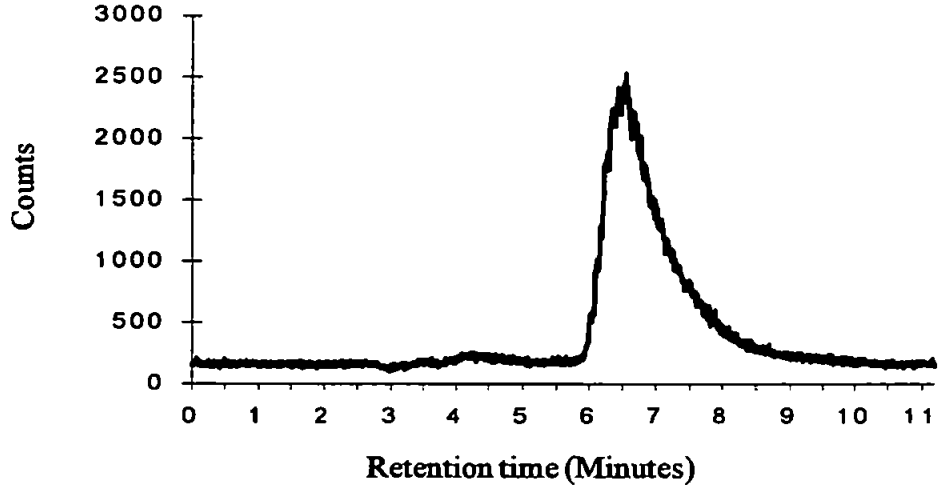


Figure 4.4: Triethyllead 100 ng g^{-1} Standard



Chromatographic Conditions:

The analytes were separated on a 250 x 4.6mm id column packed with Hypersil ODS. A two eluent system was employed:- Eluent 1: 4mM sodium pentane sulphonic acid 0.1M acetate buffer (pH 4.6) Eluent 2: methanol. A flow rate of 1.0ml min^{-1} was employed with an isocratic elution of 40:60 eluent 1: eluent 2

The effect of altering the elution conditions was studied (Figures 4.6 and 4.7). The best peaks were obtained with a step gradient. The elution conditions used are an isocratic elution of a 70:30 mix of eluent 1: eluent 2 for 11.5 minutes, with a step to 40:60 eluent 1: eluent 2, followed by an isocratic elution from 11.51 to 30 minutes. Although the resolution is improved, the analytes are not baseline resolved. So it was decided to use the original conditions to keep the analysis time per sample at 15 minutes.

4.3.2. Chromatography With Desolvation

With the HPLC-ICP-MS desolvation interface in place, the plasma could be operated at 1500W with a reflected power of 7W. The chromatographic conditions used are as shown in Section 4.2.3.

Figure 4.8 shows the injection of the test mix of 100 ng g^{-1}, inorganic, trimethyl, and triethyllead made up in a 60:40 methanol: water mix. This chromatogram cannot really be assigned as the "peak" is of too low intensity and is too broad to be of any use. A fresh test mix of the same analytes at the same concentration was made up in the mobile phase (40: 60 eluent 1: eluent 2) and injected (Figure 4.9). The chromatogram obtained showed poor sensitivity but the peaks were adequately resolved showing that the pH of the sample is important. The instrument ion lenses were retuned and the gas flows carefully adjusted to give a signal maxima with a 100 ng g^{-1} inorganic lead tune solution in 60:40 methanol : water. The test mix was reinjected (Figure 4.10). The peak intensities were still much lower than those previously attained.

Figure 4.5: The Effect of a 10 ppm Inorganic lead spike on the Separation of Trimethyl and Triethyllead

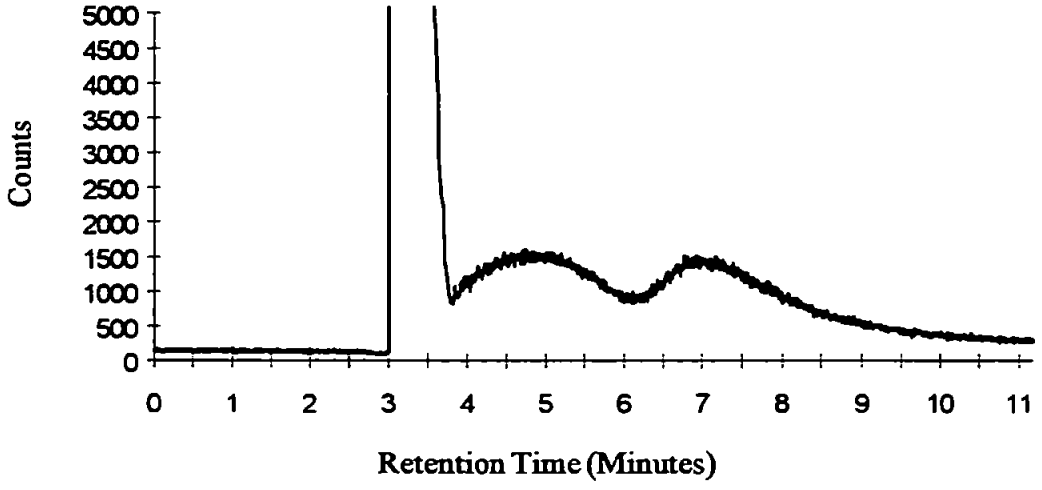
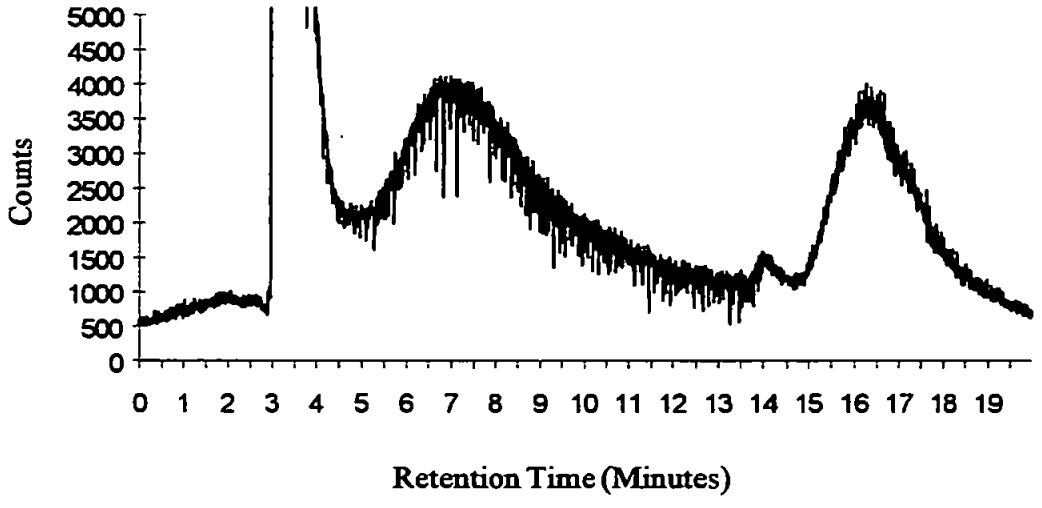


Figure 4.6: A Gradient Separation of Trimethyl and Triethyllead After Being Spiked with 10 ppm Inrganic Lead



Chromatographic Conditions:

The analytes were separated on a 250 x 4.6mm id column packed with Hypersil ODS.

A two eluent system was employed:-

Figure 4.5: Eluent 1: 4mM sodium pentane sulphonic acid 0.1M acetate buffer (pH 4.6)

Eluent 2: methanol, with an isocratic elution of 40:60 eluent 1: eluent 2

Figure 4.6: A step gradient of 40:60 eluent 1:eluent 2 from 0 to 9.5 mins with a step to 60:40 eluent 1:eluent2 from 9.5 to 20 mins.

A flow rate of 1.0ml min^{-1} was employed with both separations.

Figure 4.7: A separation of Trimethyl lead from a 10 ppm Inorganic lead spike.

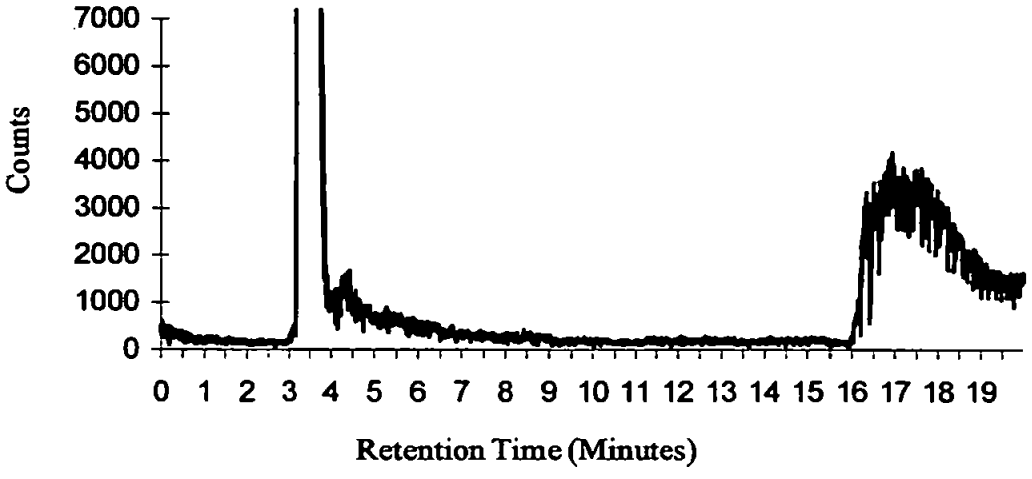
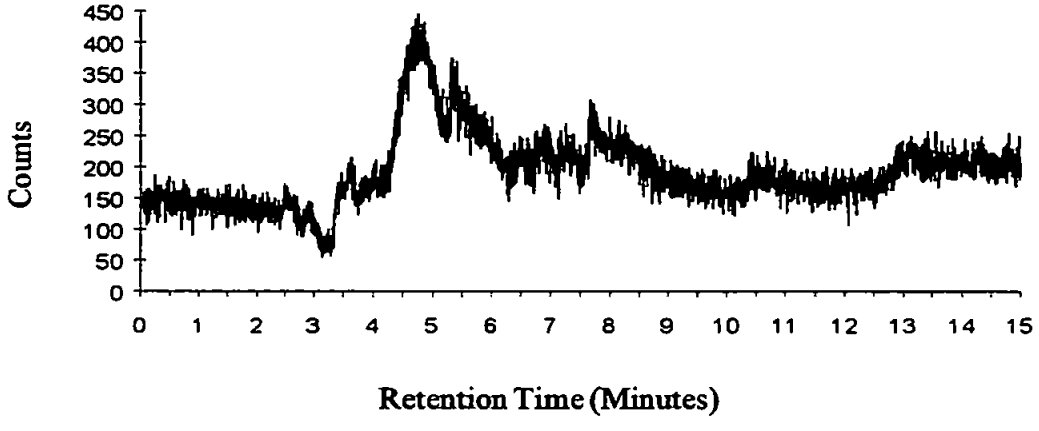


Figure 4.8: A 100 ng g^{-1} Test mix with ICP-MS detection after Desolvation



Chromatographic Conditions:

The analytes were separated on a 250 x 4.6mm id column packed with Hypersil ODS. A two eluent system was employed:- Eluent 1: 4mM sodium pentane sulphonic acid 0.1M acetate buffer (pH 4.6). Eluent 2: methanol

Figure 4.7: A step gradient of 20:80 eluent 1:eluent 2 from 0 to 11.5 mins with a step to 60:40 eluent 1:eluent2 from 11.5 to 30 mins.

Figure 4.8: An isocratic elution of 40:60 eluent 1: eluent 2

A flow rate of 1.0ml min^{-1} was employed with both separations.

Figure 4.9: The 100 ng g^{-1} Organolead Test Mix made up in the mobile phase

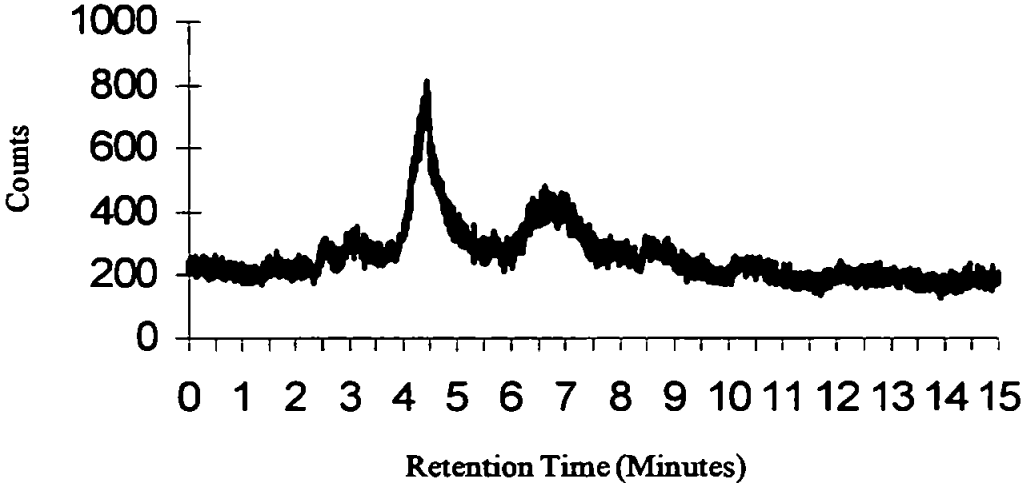
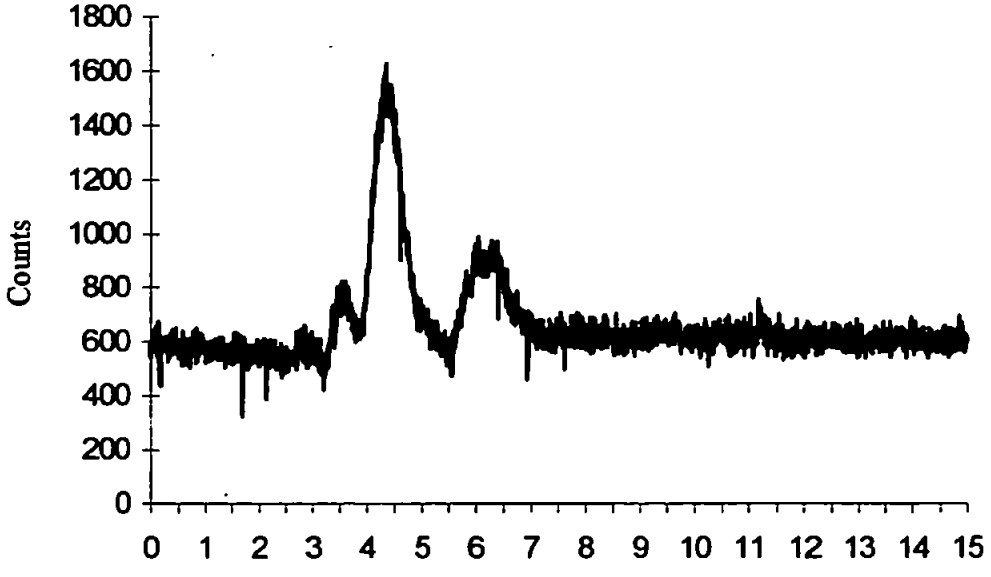


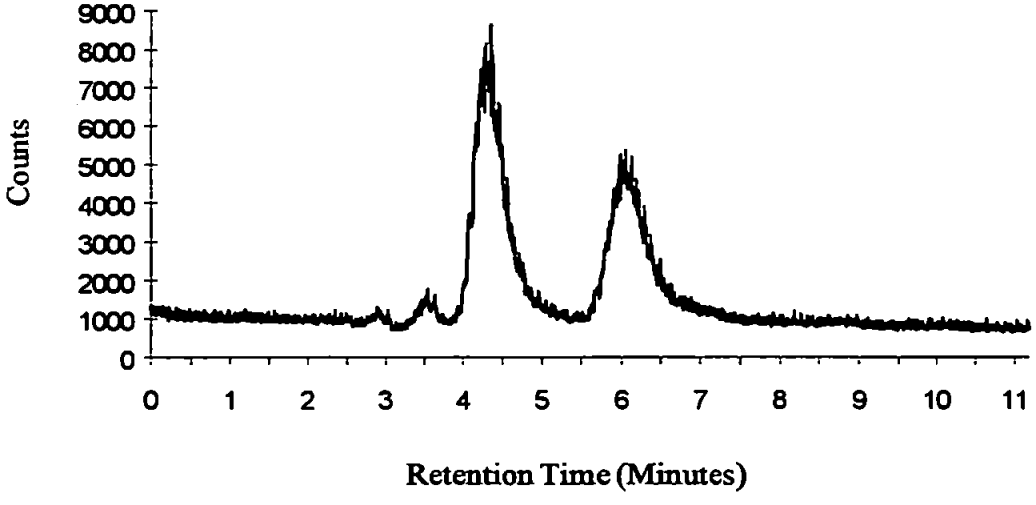
Figure 4.10: The 100 ng g^{-1} test mix with desolvation after careful optimisation of the instrument



Chromatographic Conditions:

The analytes were separated on a 250 x 4.6mm id column packed with Hypersil ODS. A two eluent system was employed:- Eluent 1: 4mM sodium pentane sulphonic acid 0.1M acetate buffer (pH 4.6) Eluent 2: methanol. A flow rate of 1.0ml min^{-1} was employed with an isocratic elution of 40:60 eluent 1: eluent 2

Figure 4.11: Separation of the organolead species after the removal of the desolvation apparatus



Chromatographic Conditions:

The analytes were separated on a 250 x 4.6mm id column packed with Hypersil ODS. A two eluent system was employed:- Eluent 1: 4mM sodium pentane sulphonic acid 0.1M acetate buffer (pH 4.6) Eluent 2: methanol. A flow rate of 1.0ml min^{-1} was employed with an isocratic elution of 40:60 eluent 1: eluent 2

To ensure this was not an instrument artefact of the ICP-MS, the HPLC-ICP-MS interface was removed and the mix reinjected using the conditions described in Section 4.2.2.

Figure 4.11 shows conclusively that the reduction was due to the desolvation apparatus. This is possibly due to the formation of the acetate salts of the analytes in the aerosol that would adhere to the large surface area of the desolvation apparatus.

To test this, two 100 ng g^{-1} inorganic lead standards were measured, one made up in a 60:40 methanol:water mix, the other in the mobile phase. The non-buffered standard gave a signal of 3.5×10^5 counts, whereas the buffered standard gave a signal of 1.8×10^5 counts, a 50% drop in signal. This showed that the presence of acetate buffer was causing a drop in signal. However, since little difference was seen when the conditions in Section 4.2.2. were used, it was decided to try and determine the fate of lead in the desolvation apparatus.

4.3.3. Mass Balance Experiment to Determine the Fate of Lead in the Desolvation Apparatus

In order to evaluate the fate of the lead whilst using the desolvation apparatus, 100 ml of a 10 μg g^{-1} inorganic lead standard was made up in the mobile phase and nebulised. The individual components and all tubing was then washed in 2% nitric acid. The washings were made upto 100 ml and analysed by ICP-MS in peak jumping mode.

4.3.3.1. Results

The results in Table 4.2 show that 12% of the lead nebulised and transported (ie not taken into the drain) had adhered to the walls of the desolvation apparatus. The greatest component was found to be adhered to the walls of the cyclone spray chamber. From the mass of liquid collected at the spray chamber drain, the transport efficiency was calculated as 15%.

4.4. Conclusions

To explain a 50% drop in signal, when only 12% of the analyte is lost due to impact and adhesion to the walls of the apparatus an assumption must be made. It must be assumed that analyte is lost in a similar fashion when a methanol water mix solution is nebulised, but that for that solution, the losses are only half of the losses that occur when an acetate buffered solution is nebulised. This possible explanation is supported by the high background of 1000 counts s^{-1} and the long wash out time after tuning the instrument before a return to this background level (~ 3 minutes) with a water/methanol mix standard. However the drop in signal due to the use of the desolvation apparatus means its use would be detrimental and would seriously effect the sensitivity of the present analytical method for trimethyl and triethyllead .

The results show that with a chilled double pass spray chamber, the solvent loading of the plasma is reduced sufficiently for stable plasma operation, and so further desolvation is not required. It was reported in previous work (130) that the use of a double pass spray chamber causes excessive band broadening of the chromatographic peaks resulting in the need for a gradient elution program.

Table 4.2: The Fate of Lead in the Desolvation Apparatus.

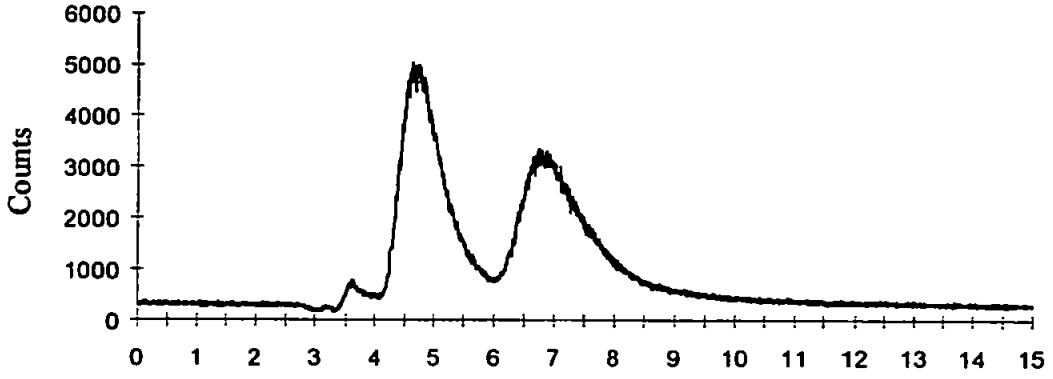
Section of Desolvation Apparatus	Lead Conc^{n} in wash solution (ng g^{-1})	Total in 100 ml (μg)	As a percentage of the total mass nebulised
Spray chamber	134	13.4	8.9
Membrane Drier	28	2.8	1.9
Peltier condenser	21	2.1	1.4

The results obtained in this study do not support this observation, and it is possible that in the earlier work, the column was not allowed to equilibrate for a long enough period of time before injection of the analytes. This view is supported by the difference in resolution between the two chromatograms in Figure 4.12. In Figure 4.12a the column was allowed to equilibrate for three hours, in Figure 4.12b the column was equilibrated for six hours.

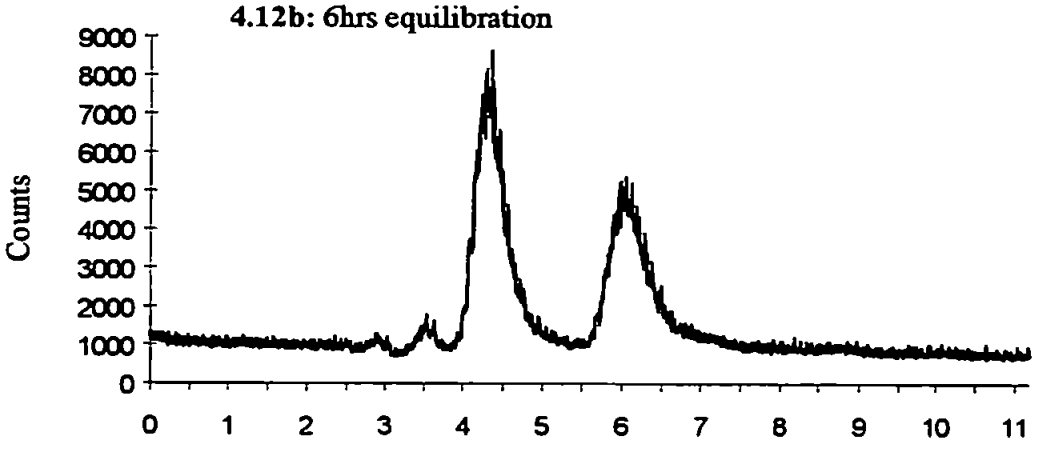
The results in Section 4.3.1 show that problems may be encountered in the future when analysing real samples with an ionic matrix or high inorganic lead content. Due to the large surface area of the desolvation system, any analyte that has an affinity for glass or the nafion membrane will be removed to such an extent that the sensitivity of the instrument will be adversely affected. It is important that when developing a new method, that the instruments sensitivity for the analyte in its matrix is checked with and without desolvation. Further work is required to discover whether any other HPLC eluent systems have this affect. If so this would be a major limitation to the use of desolvation in the future.

Figure 4.12: The effect of column equilibration time on the separation of trimethyl and triethyllead by HPLC

4.12a: 3 hrs equilibration



4.12b: 6hrs equilibration



Chromatographic Conditions:

The analytes were separated on a 250 x 4.6mm id column packed with Hypersil ODS. A two eluent system was employed:- Eluent 1: 4mM sodium pentane sulphonic acid 0.1M acetate buffer (pH 4.6) Eluent 2: methanol. A flow rate of 1.0ml min^{-1} was employed with an isocratic elution of 40:60 eluent 1: eluent 2

5.0 Organotin Speciation

5.1 Introduction

Having successfully demonstrated the applicability of desolvation to the speciation of platinum, it was decided to apply the technique to other HPLC separations being developed at Plymouth for environmental samples. The use of desolvation had not proved successful for the speciation of organolead compounds, so it was decided to apply desolvation to the speciation of organotin compounds in the environment. It was hoped that the problems seen in Chapter 4 were unique to lead analysis, and that the use of a buffered mobile phase would not affect the chromatography of organotins when using desolvation.

The first organotin compound was prepared in 1852 by Lowig (131), but the commercial significance of these compounds was not recognised until the 1940s in the USA and the 1950s in Europe. The use of di-organotin compounds as PVC stabilisers was the first commercial application of this class of compounds. Since then organotin compounds have found a large number of applications. (Table 5.1)

As a result of their extensive use, it was estimated that by 1976, 4300 tonnes of organotin compounds had escaped into the environment (132). A large fraction of this was as biocides and algicides in antifouling paints.

The toxicity of organotins is a function of the number of alkyl groups attached to the central tin atom, with maximum biological activity being attained at n=3. Using the formula R_nSnX_{4-n}

Table 5.1: Commercial Applications of Organotin Compounds (Adapted from references 131 and 3)

Application	Function	Principle Compounds Used
PVC Stabilisation	Heat and Light Stabilisers for Rigid PVC	di-alkyltin di-isooctylthioglycolate (alkyl = methyl, butyl, octyl, 2-butoxy-carbonylethyl) dialkyltin maleate (alkyl = methyl, butyl, octyl) mono-alkyltin tri-isooctylthioglycolate (alkyl = methyl, butyl octyl, 2-butoxy-carbonylethyl)
Polyurethane foams and RTV silicones	Homogenous catalysts	dibutyltin diacetate dibutyl tin di-octoate dibutyltin dilaurate
Transesterification reactions	Homogeneous catalysis	butanestannoic acid dibutyltin diacetate dibutyltin oxide
Glass treatment (scratch resistance)	Precursor for forming tin (IV) oxide films on glass	dimethyltin dichloride butyltin trichloride

		methyltin trichloride
Poultry management	Anthelminthic	dibutyltin dilaurate
Wood preservative	Fungicide	bis(tributyltin) oxide tributyltin naphthenate tributyltin phosphate
Agricultural chemicals	Fungicide Insecticide Miticide Antifeedant	triphenyltin acetate triphenyltin hydroxide tricyclohexyltin hydroxide fenbutatin oxide 1-tricyclohexylstannyl-1,2,4,-triazole
Antifouling paints	biocide	triphenyltin chloride triphenyltin fluoride bis(tributyltin) oxide tributyltin chloride tributyltin fluoride tributyltin acrylate polymers
Materials protection (stone, leather and paper)	Fungicide Algicide Bactericide	bis(tributyltin) oxide tributyltin benzoate
Disinfectant	Bacteriostat	tributyltin benzoate
Moth proofing of textiles	Insecticide	triphenyltin chloride

leading to R_3SnX (3). The alkyl group is of less importance but nevertheless has an affect with toxicity following the pattern:

ethyl > methyl > propyl = butyl > phenyl ≥ octyl (132).

Due to their high toxicity, they are now on the EEC blacklist of dangerous substances after a conference on the subject in 1980. This in turn has lead to a great interest in their levels and speciation in the environment.

The techniques developed so far to speciate organotins in the environment combine a separation stage such as HPLC or GC followed by a specific or selective detection system, for example ICP-MS, MIP-AES, or mass spectrometry (2, 132, 133). For this study the use of HPLC followed by atomic spectrometric detection was investigated as this methodology has been studied extensively at Plymouth (134-138). Recently the focus of attention has been on the use of ICP-MS detection.

With respect to organotin speciation HPLC has two main advantages:

i) a derivatisation step is not necessary so their is no chance that the speciation is altered in anyway.

ii) the stationary and mobile phases can be optimised to obtain the best separation.

As detailed in Chapter 1, interfacing HPLC to ICP-MS can be problematic, the mobile phase used for the organotin chromatography contains a high organic solvent content as well as a high

dissolved salt content. Both of these components can extinguish the plasma, and so the successful application of desolvation to this analysis would hopefully offer an advantage.

5.2 Experimental

5.2.1. Standard and Sample Preparation

Standards (100 and 500ng g^{-1}) were prepared from the pure compounds (Aldrich Chemical Company, Gillingham, England) and dissolved in the mobile phase by weight as displacement pipettes are not calibrated for use with organic solvents. The sample analysed was a certified reference material (CRM) PACS-1 (Bureau of Analysed Samples Ltd, Middlesbrough, England). This is a harbour marine sediment certified for tributyltin (TBT) (1.27 ± 0.22 mg kg^{-1} as tin), dibutyltin (DBT) (1.16 ± 0.18 mg kg^{-1} as tin) and monobutyltin (MBT) 0.28 ± 0.17 mg kg^{-1} as tin).

5.2.2. Extraction Procedure

The organotin compounds were extracted from the sediment using the following procedure.

25 ml of Aristar grade glacial acetic acid was added to 0.5 g of sediment. This was shaken for four hours and then centrifuged, the supernatant liquid decanted off, and the procedure repeated. The sediment was washed with a further 5 ml of acid, which was later combined with the two extracts. To this was added 20 mls of 18 MΩ deionised water. This aqueous phase was then extracted three times with 10 ml of toluene, the organic phase being collected in a round bottom

flask and rotary evaporated to dryness. The analytes were redissolved in 2 ml of the mobile phase and injected onto the column.

5.2.3. Chromatography

An inert gradient pump was employed, and the samples and standards injected via an inert injection valve fitted with a 200 µl loop. The analytical column used was a 25cm x 4.6 mm id stainless steel column packed in house with 10 µm Partisil SCX strong cation exchange bonded silica stationary phase (Whatman, Maidstone, England). The column was fitted with a 3 cm guard column packed with the same material. The mobile phase was a three eluant system and consisted of-

Eluent 1:- Methanol.

Eluent 2:- Citrate buffer (pH 3.4).

Eluent 3:- Citrate buffer (pH 5.8).

The elution Conditions were as follows. 70:30 eluent 1: eluent 2 (v/v), Isocratic elution 0 to 2.5 mins, step to 85:15 eluent 1: eluent 2, isocratic 2.5 to 5 mins, step to 85:15 eluent 1: eluent 3, isocratic 5 to 15 minutes. A flow rate 1.0 ml min^{-1} was used.

5.2.4. Instrumentation

A FI Elemental PQ2+ ICP-MS was used to acquire the data by single ion monitoring of *m/z* 120. The desolvation apparatus was operated under optimum conditions as specified in Chapter 3.

5.3. Results and Discussion

The use of desolvation gave a number of advantages to the analysis of organotins by HPLC-ICP-MS. For an eluant methanol percentage of 70%, the instruments reflected power was reduced from 25-30 W to 11 W which could be reduced to 5 W with manual retuning of the torch box r.f. circuit.

Hardly any C_2 emission could be seen in the plasma. Carbon only started to build up on the cones after 5 minutes of operation (as opposed to immediately) and this could be prevented entirely by adding 3% oxygen to the nebuliser gas flow.

In addition, less time was required between injections as the reflected power never got too high (i.e. above 30 W). Normally it was necessary to wait until it had reduced after the last elution step, as this had the highest methanol content. This saved about five minutes per analysis.

Another advantage was that the sheath gas flow completely prevented the build up of salts in the injector of the torch. The build up of salts normally causes the plasma to extinguish after about 3 hours of operation. Prevention of such a build up meant that the instrument did not need to be shut down whilst the torch injector was cleaned.

5.3.1. Quantification of the Method

Figure 5.1 shows the separation of a mixture of TBT, triphenyltin (TriPhT), DBT and MBT. It can be seen that TBT and TriPhT were not fully separated and so single standards of these two

compounds were also injected (see Figures 5.2 and 5.3) to elucidate their elution order. The extract of the sample PACS-1 was then injected (Figure 5.4) and quantified, the results of which can be seen in Table 5.2.

The high value for MBT can be explained by the peak in the blank (Figure 5.5) eluting at the same time as MBT, this peak can also be seen in Figures 5.1 and 5.2 and could possibly be MBT from the break down of the standards. The results for DBT and TBT can be explained by incomplete extraction from the sample. This could be corrected for by spiking the CRM with standards of the compounds and finding the extraction efficiency. Further work is required to improve the sample preparation, especially the extraction from sediment of these analytes. There are a number of steps used where losses could occur.

The acetic acid extraction does not seem to be very effective for all the analytes. The back extraction to toluene is suspect as the method requires the extraction of ionic organometallic compounds from a polar matrix into a much less polar solvent. It is necessary to elucidate at which steps losses are occurring so appropriate steps can be taken. If losses are occuring primarily at the back extraction stage, it will be necessary to improve the efficiency of this step, this could be done by using ion-pairing agents. These would form a non polar ion-pair with the analyte that would have a greater affinity for the toluene phase than the analyte alone. Whether this method would also extract more interferences would have to be carefully investigated.

Table 5.2: Analytical figures of merit for the HPLC-ICP-MS determination of tin species in PACS-1.

Compound	Concentration in Extract (ng g^{-1} as Tin)	Resulting Amount in Sediment Sample (ng as Tin)	Certified Value in 0.5g of the Sediment (ng as Tin)
MBT	910	1820	146
DBT	223	446	607
TBT	327	654	663

Figure 5.1:Standard Mix of TBT, DBT, MBT, TriPhT and Sn

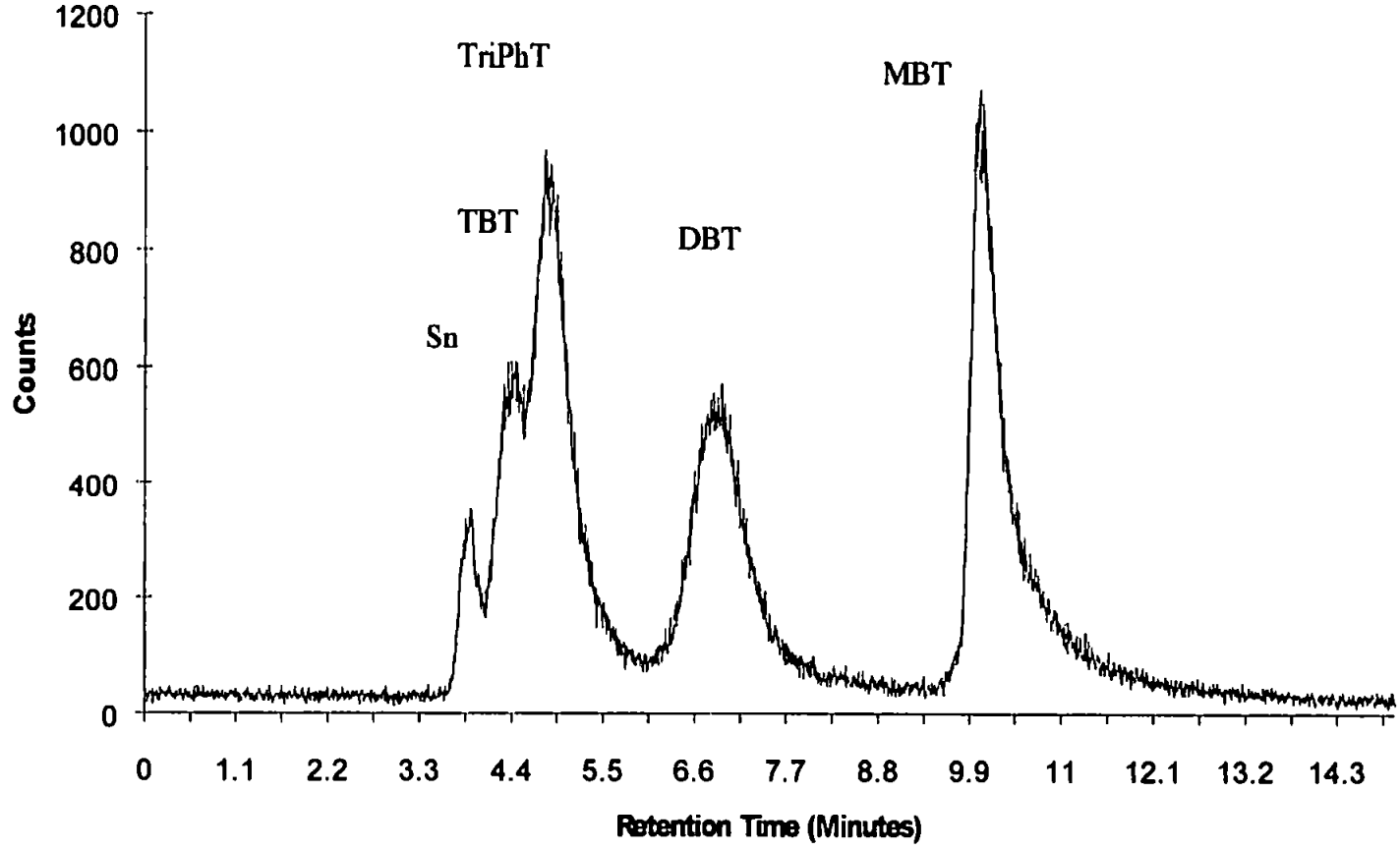


Figure 5.2: A 111 ng g^{-1} TBT Standard

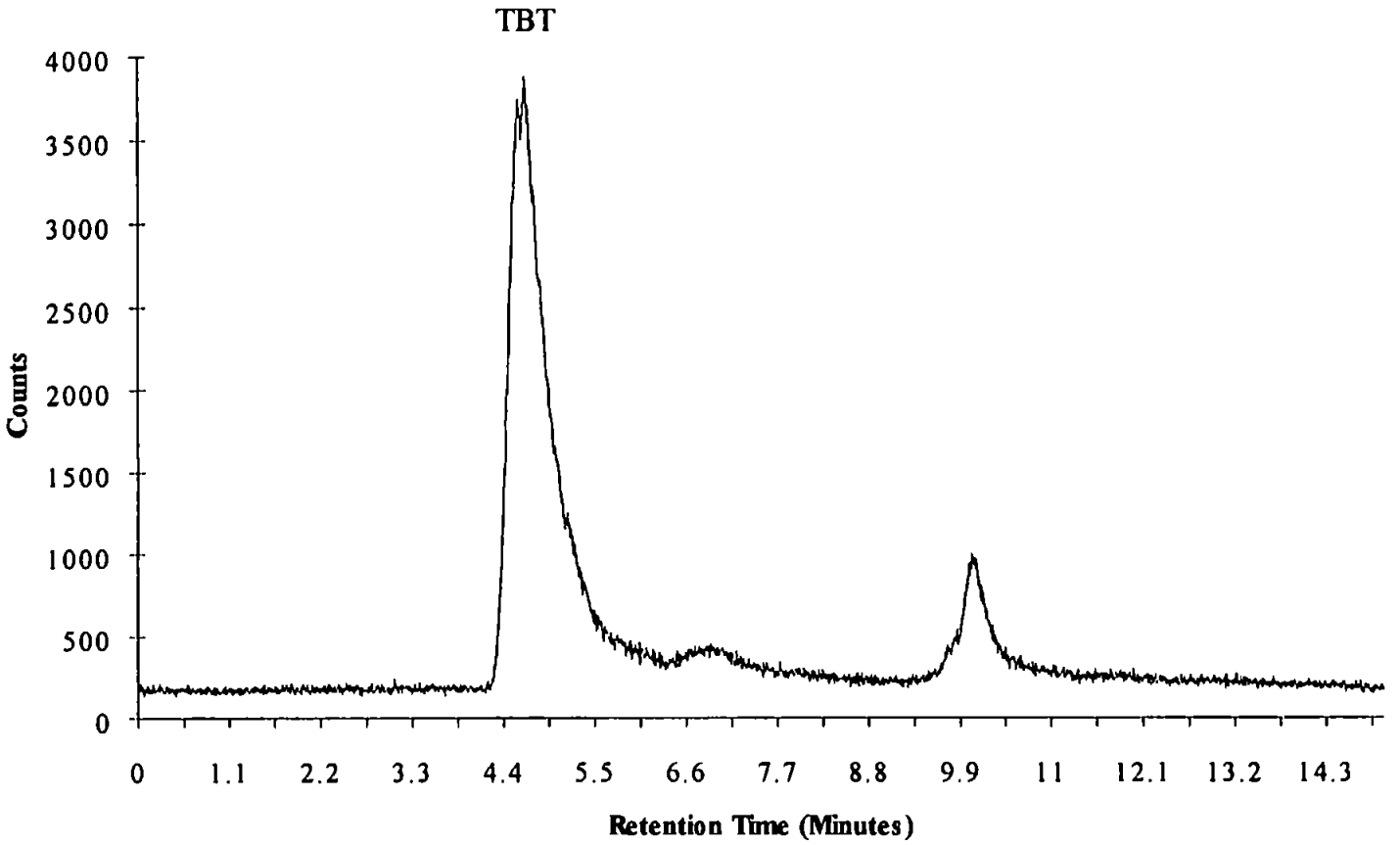


Figure 5.3: A 94 ng g^{-1}TriPhT Standard

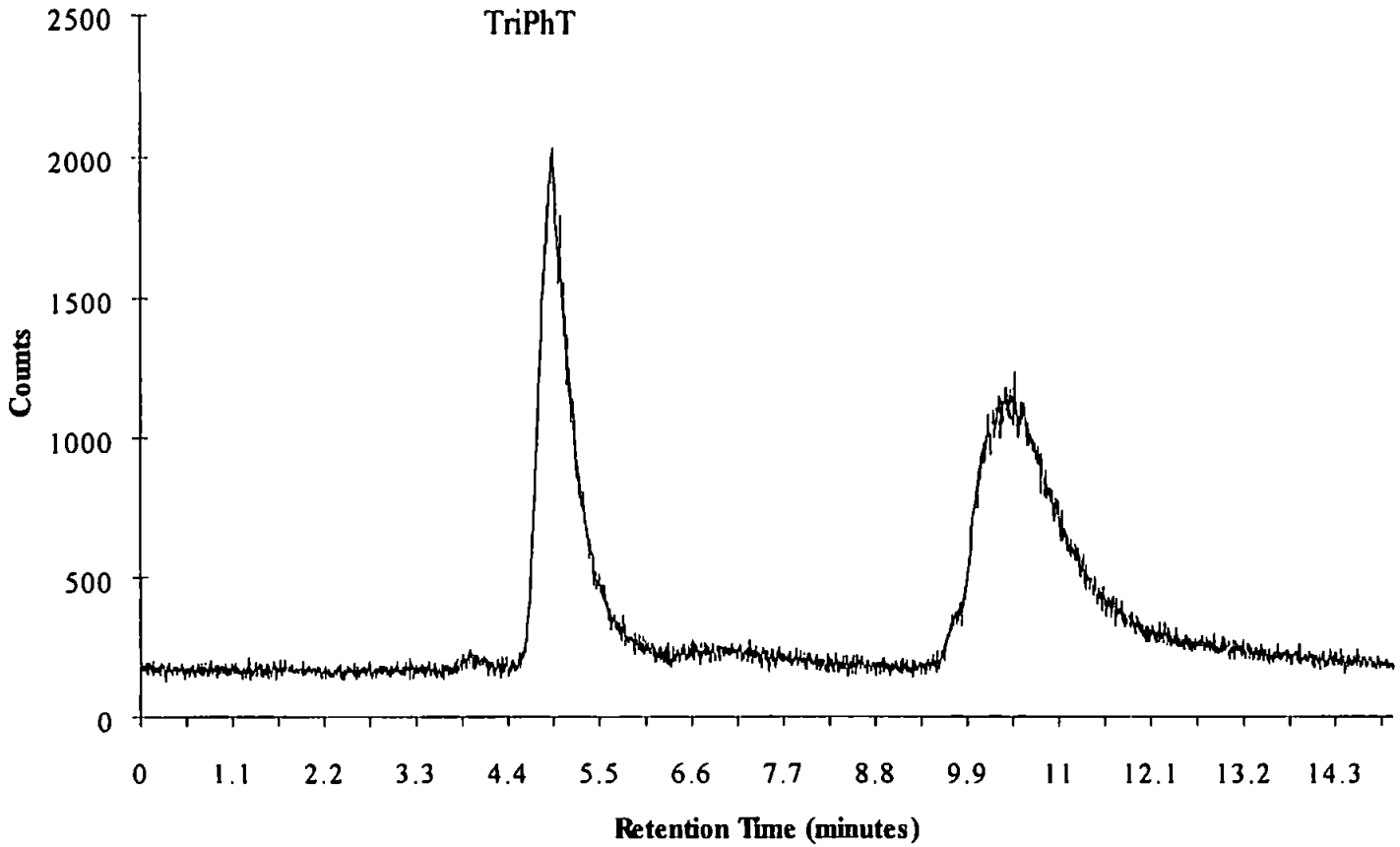


Figure 5.4: Speciation of Tin in PACS-1 CRM

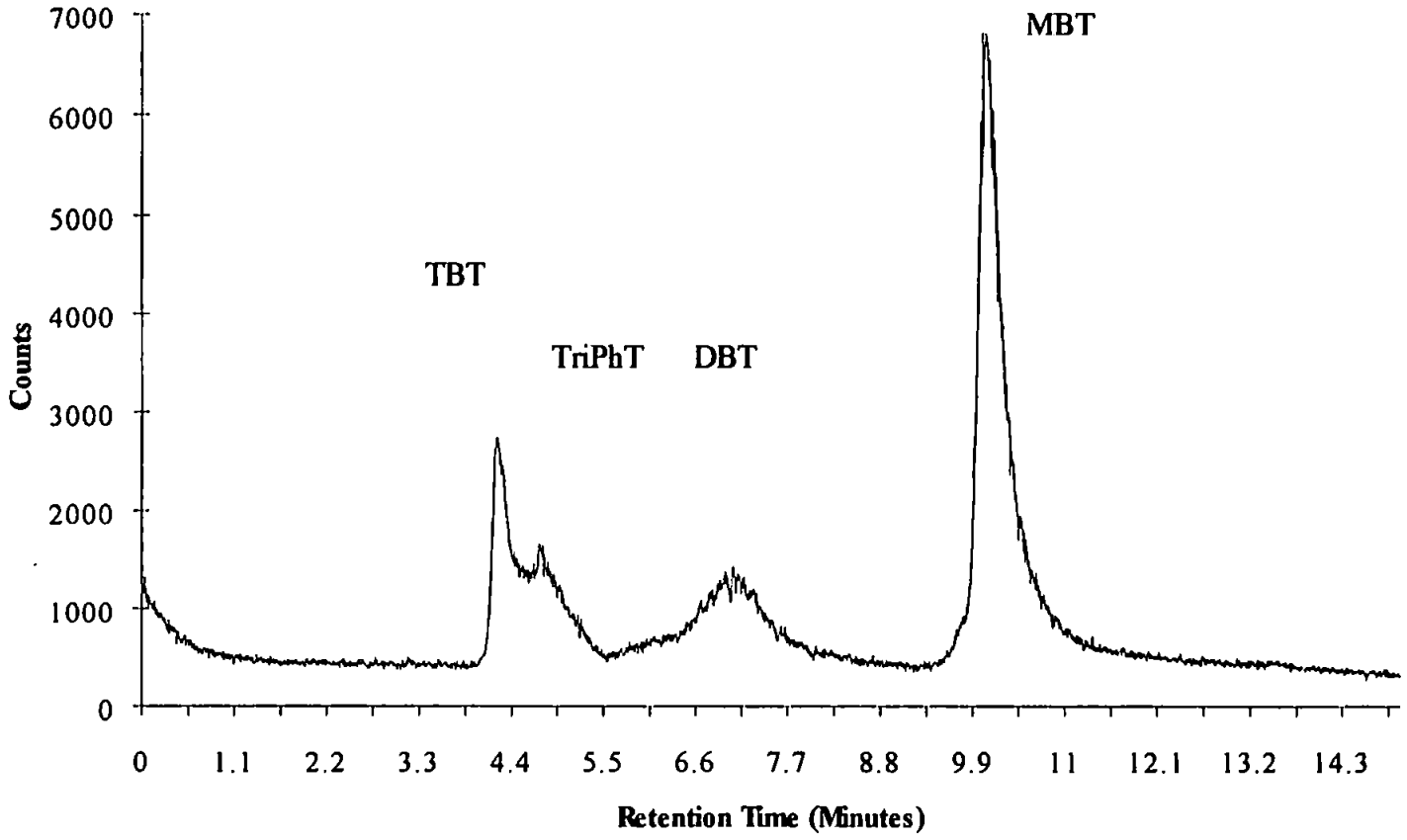
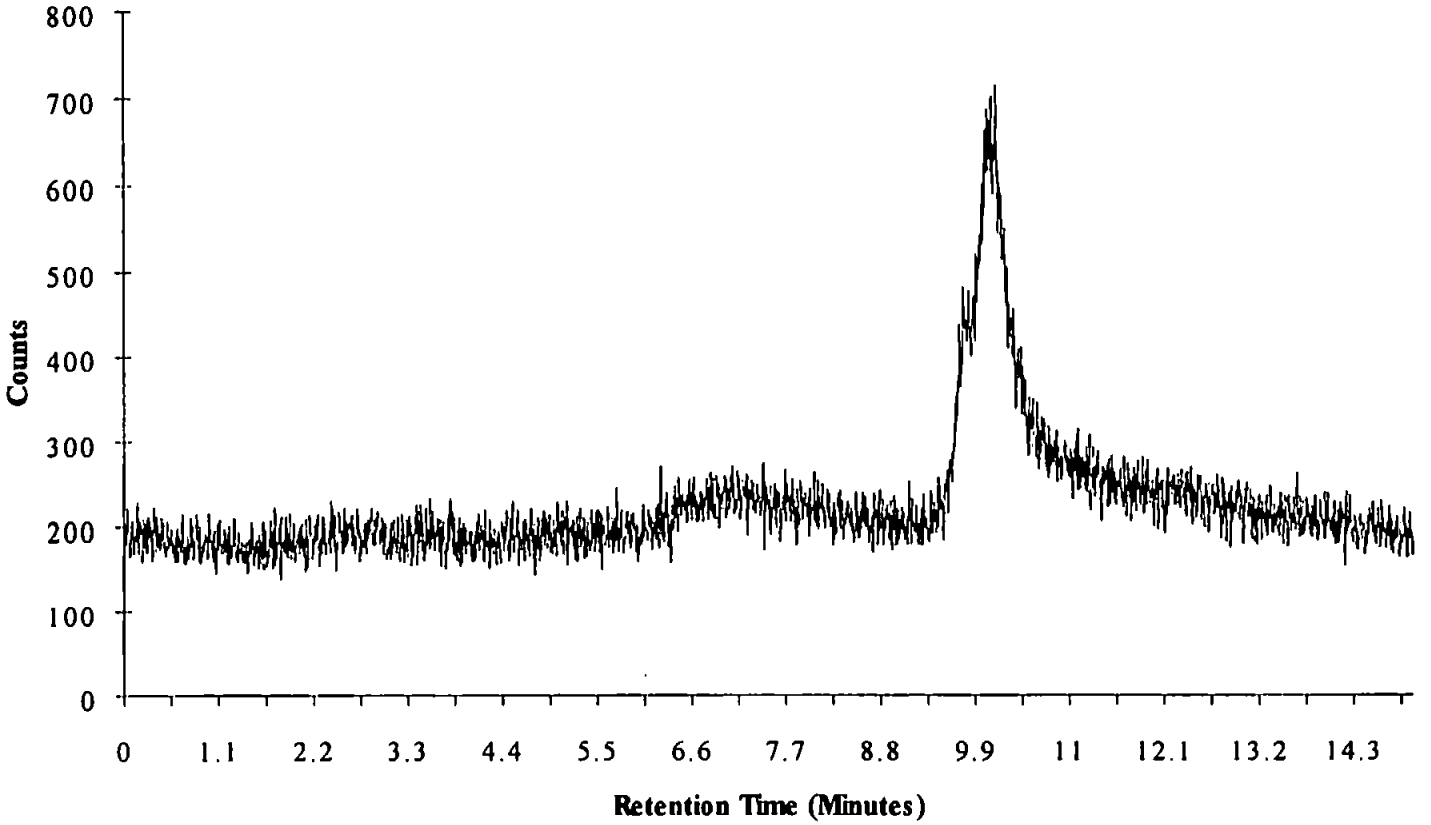


Figure 5.5: Blank Sample Injection



5.4. Micellar Chromatography.

Since tributyl and triphenyl tin were not well resolved using cation exchange chromatography, it was decided to try micellar chromatography to achieve this following the work of Inoue *et al.* (139).

The use of micellar liquid chromatography (MLC) is developing as a useful tool to determine a variety of compounds. Most of the separations involve the use of an organic modifier together with a surfactant (140). In MLC the retention of solutes usually decreases with increasing micelle concentration and the amount of organic modifier in the mobile phase. Optimisation may therefore be achieved by variation of these two parameters.

MLC-ICP-MS was first used to separate and detect organotins by Suyani *et al.* (141). The authors reported that for ICP-MS detection the sodium dodecyl sulphate (SDS) concentration should be kept below 0.1 M which corresponds to 3% total dissolved solid. At this level they found that salt deposition occurred at the constricted area of the torch injector. This problem was solved by using a torch with a demountable injector that had a 3 mm id tapering to 2 mm at the tip. However, the separation of butyltin compounds was not reported since with C-18 columns the capacity factors were found to be too high leading to excessive retention times and resulting in very broad peaks.

Inoue *et al.* (139) also reported problems with torch clogging, but found it did not occur to the same extent if the Tris [Tris (hydroxymethyl)aminomethane] salt of dodecyl sulphate was used in conjunction with a tapered torch with a 1.2 mm bore injector tip. It was decided to use the

sodium salt in this study, and to evaluate the use of the HPLC-ICP-MS interface to prevent problems due to the mobile phase.

5.5 Experimental

5.5.1. Reagents and Standards

The standards were prepared from the pure compounds in methanol by weight. The reagents used were: sodium dodecyl sulphate (Aldrich), ammonium nitrate AnalaR grade (BDH/Merck), acetic acid, aristar grade (BDH/Merck) and ethanol (Rathburns).

5.5.2. Chromatography

An inert gradient pump (Varian 9010) was employed fitted with a 250 x 4.6 mm id PEEK column packed with 5 μm hypersil phenyl bonded silica. This column was fitted with a 1 cm guard column packed with the same stationary phase.

A two eluent system was employed in an attempt to resolve the analytes. The initial chromatographic conditions were:-

Eluent 1: 0.05 M SDS / 0.05 M NH_3NO_3 / 3% acetic acid.

Eluent 2: Ethanol.

Isocratic elutions were employed with an initial composition of 85% eluent 1: 15% eluent 2 at a flow rate of 1.0 ml min^{-1}.

5.5.3. HPLC-ICP-MS Interface.

This was operated close to the optimum conditions reported in Chapter 3. However in this work the sheath gas was added at 0.2 l min^{-1} and the nebuliser gas flow was adjusted to obtain a signal maxima (0.4 - 0.5 l min^{-1}). The sheath gas was maintained at this value to prevent salt deposition in the injector of the torch.

5.6. Results and Discussion

Figure 5.6 shows the results obtained with the conditions in Section 5.5.2. After 30 minutes the only compound to have eluted was MBT at a retention time of 1.8 minutes. This supports the finding of Suyani *et al.* (141) who reported that butyl tins have a too high capacity on reversed phase columns. The phenyl column is less hydrophobic than a C-18 column, but the π-π interaction with the analytes still give it a high capacity factor.

Inoue *et al.* (139) investigated four stationary phases. These were: trimethylsilane bonded silica, butyl group bonded silica, phenyl bonded silica and cyanopropyl group bonded silica. These workers found that although the phenyl column was not the most hydrophobic column of those used, it gave the highest capacity factors, and could separate the organotin species. The authors found that the butyl column gave the best separation. However, since a butyl column was not available for this study, work continued with the phenyl column.

To reduce the retention times it was decided to employ a very short column, i.e. the guard column and increase the ethanol percentage (eluent 2) to 25%. The result of this can be seen in Figure 5.7. To elucidate the peak elution order, single standards were injected at this same set of conditions (Figure 5.8).

Under these conditions, all the compounds were eluted within 350 seconds. To resolve the compounds, it is necessary to sharpen the peaks and to retain them longer on the column by increasing the selectivity and improving the efficiency of the current method. Following this study the primary concern was that the phenyltin compounds (mono- and di- substituted) were showing excessive tailing. This was attributed to interaction with the free silica groups available on the stationary phase.

The effect the mobile phase components have on the separation was investigated by increasing the concentration of each sequentially. Three sets of eluent 1 were prepared, each containing an increased concentration of one of the components. From the results in Figures 5.9-5.14 it can be seen that the best peak shapes are given using the original eluent composition (eluent 1).

As increasing the concentrations yielded no useful effect, the effect of reducing the acetic acid concentration was evaluated. This resulted in excessive peak tailing for all but the tri- substituted tin compounds and MBT which eluted as a double peak on the solvent front (Figure 5.15). It is thought that the alkyl tins may be converted into their acetate salts in the presence of acetic acid. Thus the interaction with the silica column would be depressed. However, elevated acetic acid levels may depress micelle formation resulting in the effects seen in Figures 5.9B-5.14B. As such the acetic acid concentration and consequently pH are critical factors that need to be optimised further.

Figure 5.6: A Micellular Liquid Chromatogram of an Organotin Compound Mix.

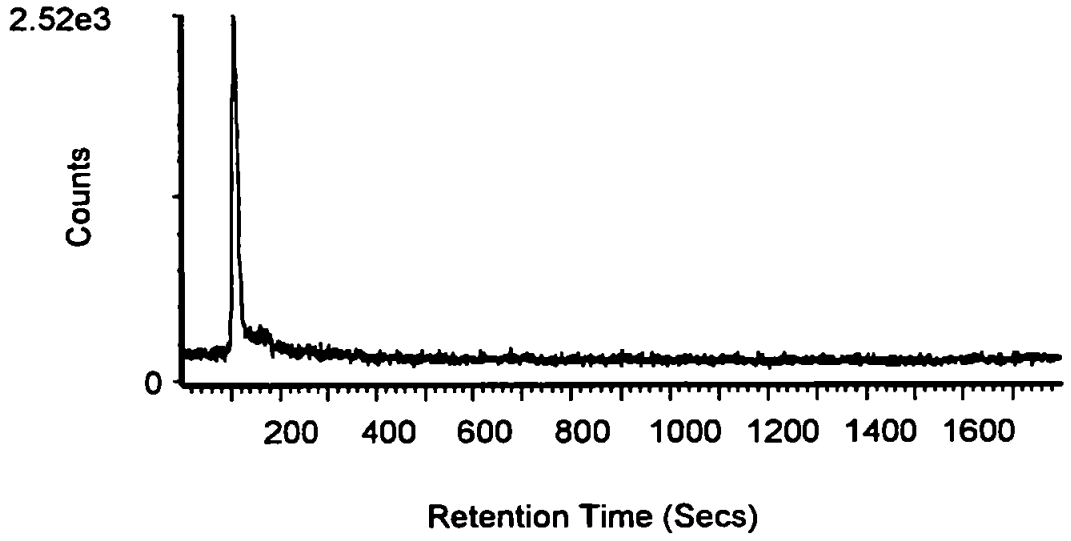
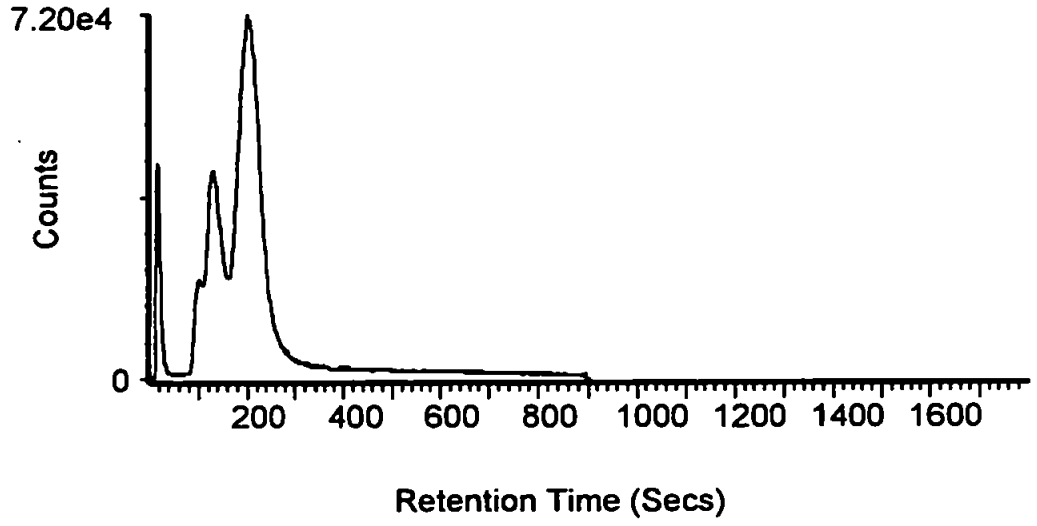
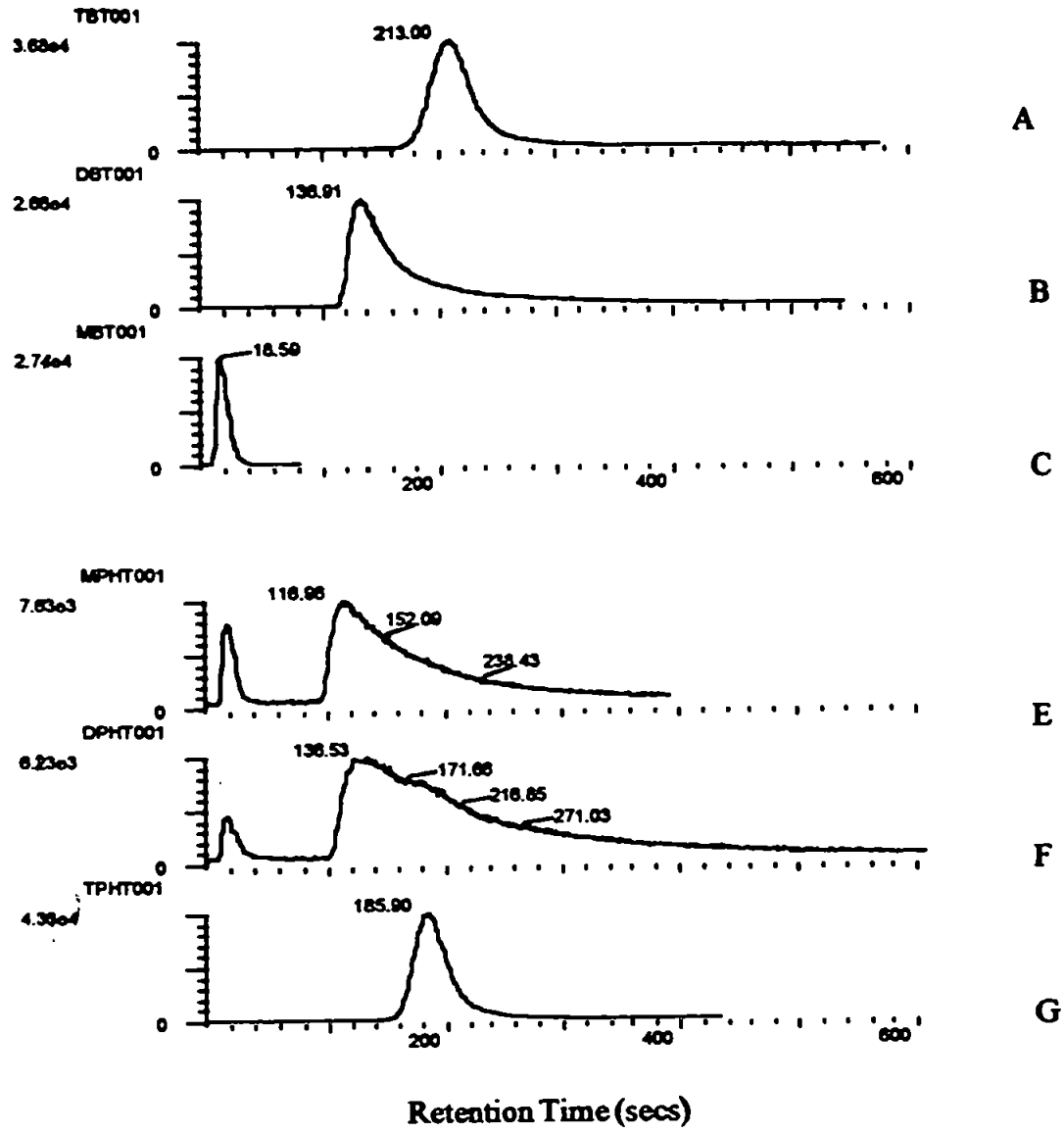


Figure 5.7: A Micellular Liquid Chromatogram of an Organotin Compound Mix with altered conditions.



Chromatographic Conditions: Fig 5.6. A 250 x 4.6 mm id PEEK column packed with Hypersil phenyl bonded silica fitted with a 1cm guard column was used to separate the analytes. **Fig 5.7.** Only the guard column was used.
A two eluent system was employed :- Eluent 1: 0.05M SDS / 0.05M NH3NO3 / 3% acetic acid. Eluent 2: Ethanol. Isocratic elutions were employed with an initial composition of 85% eluent 1: 15% eluent 2 at a flow rate of 1.0 ml min-1(Fig 5.6) and 75% 1: 25 % 2 (Fig 5.7).

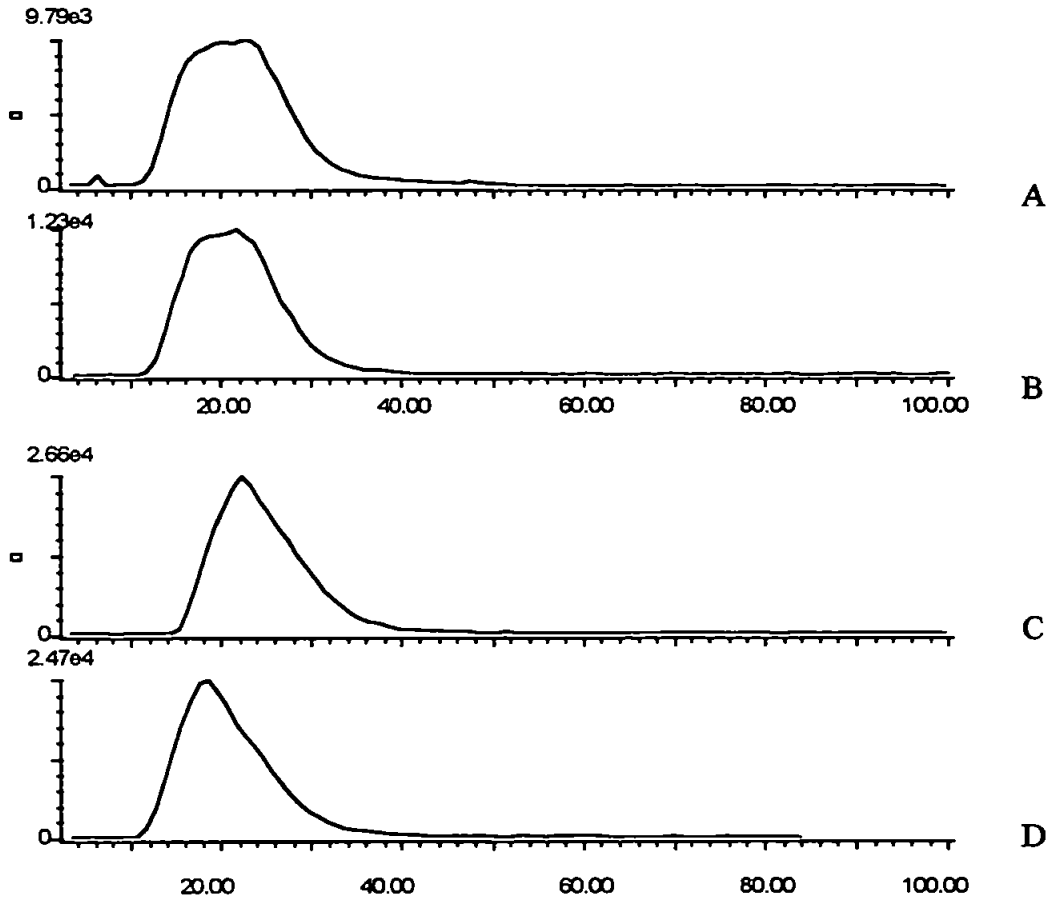
Figure 5.8: Single Standard Injections to Elucidate Elution Order in Figure 5.7



A= Tributyltin B=Dibutyltin C=Monobutyltin E= Monophenyltin F= Diphenyltin G= Triphenyltin. The first peaks in E and F are inorganic tin impurities from break down on column of the organotin species.

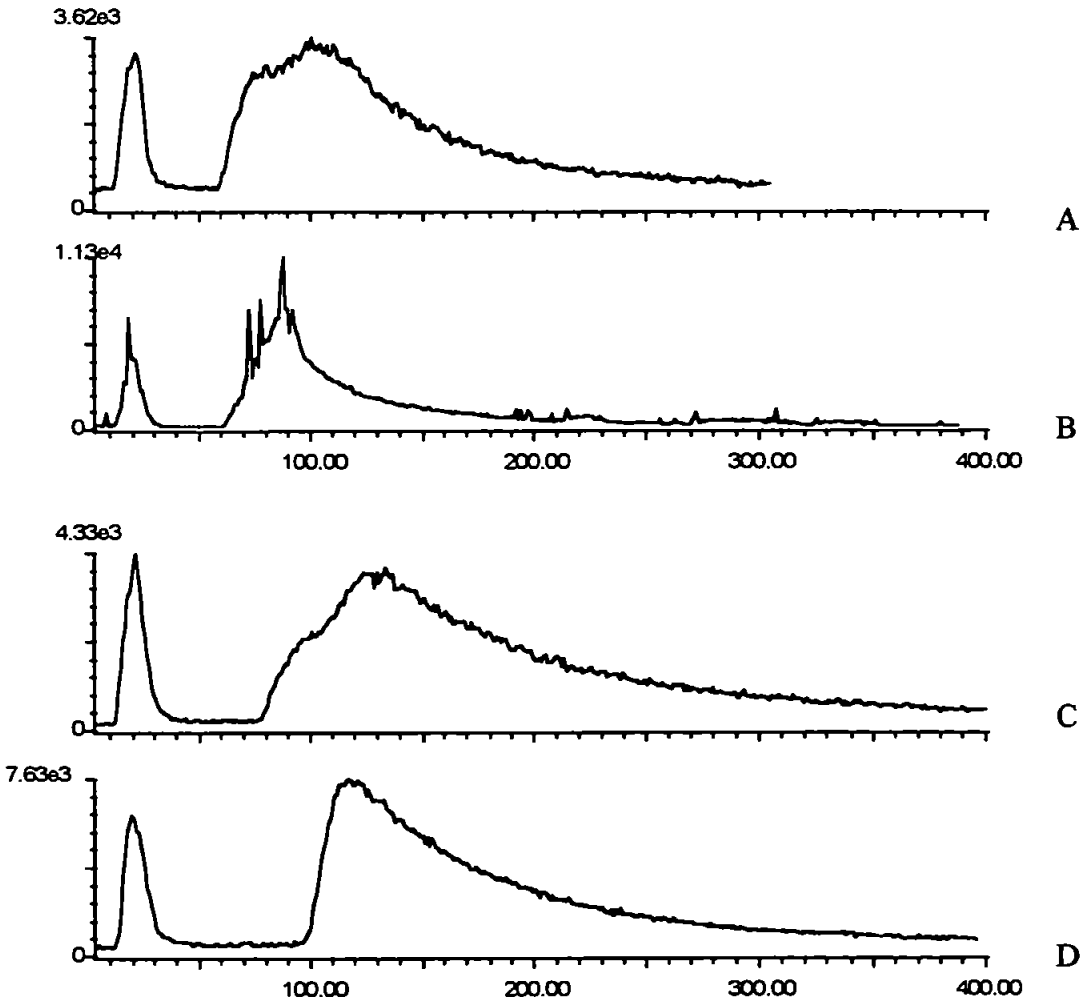
Chromatographic Conditions: A 1 cm guard column packed with Hypersil phenyl was used to separate the analytes.
A two eluent system was employed:- Eluent 1: 0.05M SDS/ 0.05M HN3NO3/ 3% acetic acid. Eluent 2: Ethanol. An isocratic elution was employed with a composition of 75 % Eluent 1: 25% Eluent 2 at a flow rate of 1.0 ml/min.

Figure 5.9: The Effect of Changing the Mobile Phase Composition on the Peak Shape of Monobutyltin



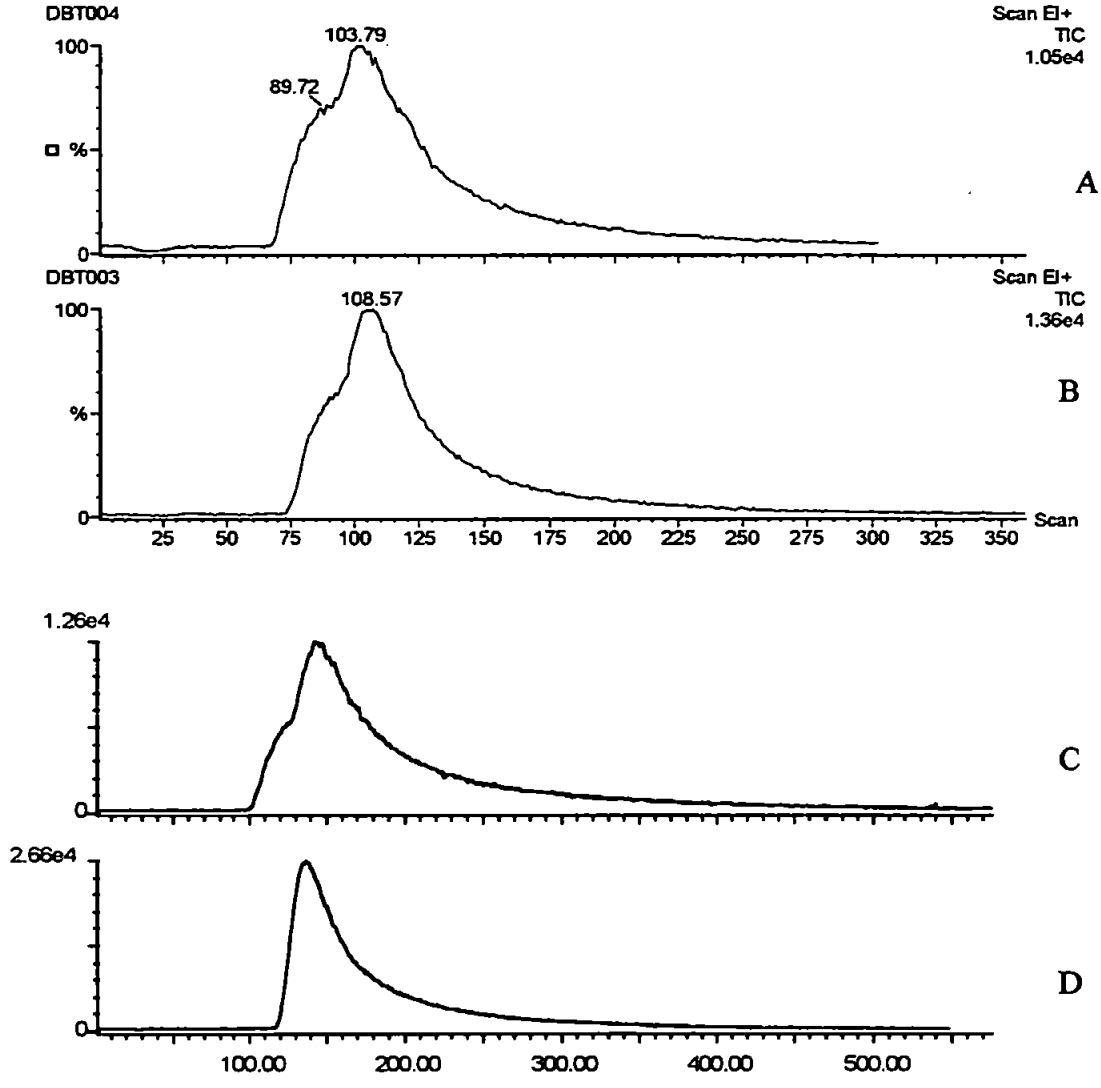
Chromatographic Conditions: A 1cm guard column packed with Hypersil phenyl was used to separate the analytes.
A two eluent system was employed :-
(A)= Eluent 1: 0.1M SDS / 0.05M NH_3NO_3 / 3% acetic acid. Eluent 2: Ethanol.
(B)= Eluent 1: 0.05M SDS / 0.05M NH_3NO_3 / 6% acetic acid. Eluent 2: Ethanol.
(C)= Eluent 1: 0.05M SDS / 0.1M NH_3NO_3 / 3% acetic acid. Eluent 2: Ethanol
(D)= Eluent 1: 0.05M SDS / 0.05M NH_3NO_3 / 3% acetic acid. Eluent 2: Ethanol
All at an eluent composition of 75% eluent 1: 25% eluent 2 and a flow rate of 1.0ml min^{-1}.

Figure 5.10: The Effect of Changing the Mobile Phase Composition on the Peak Shape of Monophenyltin



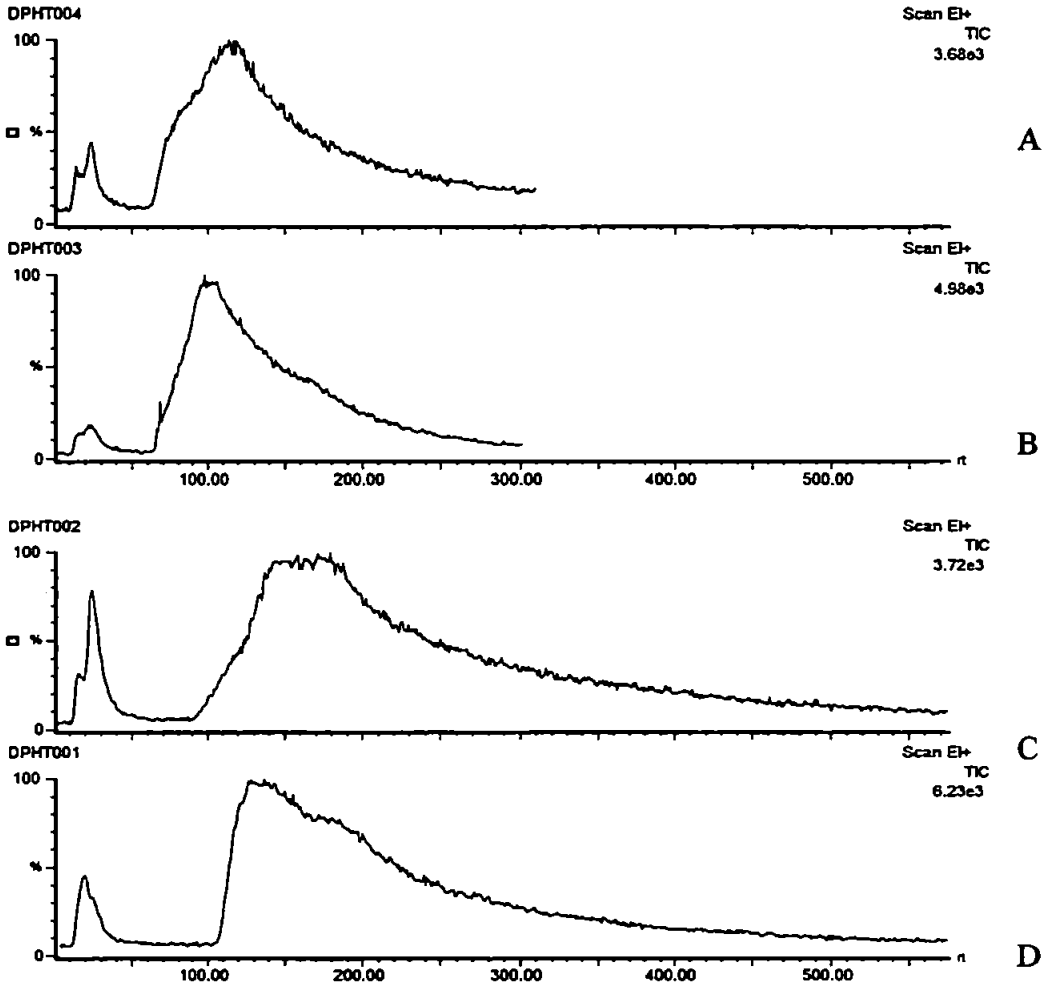
Chromatographic Conditions: A 1cm guard column packed with Hypersil phenyl was used to separate the analytes.
A two eluent system was employed :-
(A)= Eluent 1: 0.1M SDS / 0.05M NH_3NO_3 / 3% acetic acid. Eluent 2: Ethanol.
(B)= Eluent 1: 0.05M SDS / 0.05M NH_3NO_3 / 6% acetic acid. Eluent 2: Ethanol.
(C)= Eluent 1: 0.05M SDS / 0.1M NH_3NO_3 / 3% acetic acid. Eluent 2: Ethanol
(D)= Eluent 1: 0.05M SDS / 0.05M NH_3NO_3 / 3% acetic acid. Eluent 2: Ethanol
All at an eluent composition of 75% eluent 1: 25% eluent 2 and a flow rate of 1.0ml min^{-1}.

Figure 5.11: The Effect of Changing the Mobile Phase Composition on the Peak Shape of dibutyltin



Chromatographic Conditions: A 1cm guard column packed with Hypersil phenyl was used to separate the analytes.
A two eluent system was employed :-
(A)= Eluent 1: 0.1M SDS / 0.05M NH_3NO_3 / 3% acetic acid. Eluent 2: Ethanol.
(B)= Eluent 1: 0.05M SDS / 0.05M NH_3NO_3 / 6% acetic acid. Eluent 2: Ethanol.
(C)= Eluent 1: 0.05M SDS / 0.1M NH_3NO_3 / 3% acetic acid. Eluent 2: Ethanol
(D)= Eluent 1: 0.05M SDS / 0.05M NH_3NO_3 / 3% acetic acid. Eluent 2: Ethanol
All at an eluent composition of 75% eluent 1: 25% eluent 2 and a flow rate of 1.0ml min^{-1}.

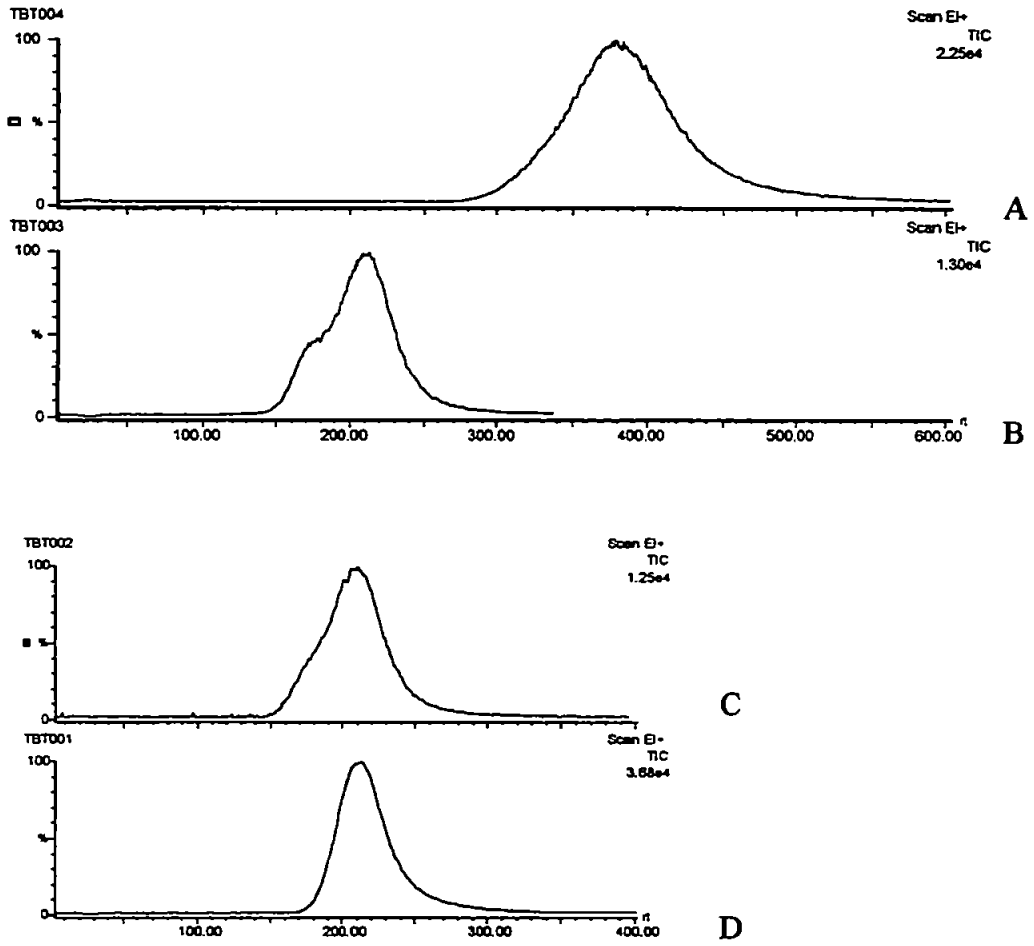
Figure 5.12: The Effect of Changing the Mobile Phase Composition on the Peak Shape of Diphenyltin



Retention Time (secs)

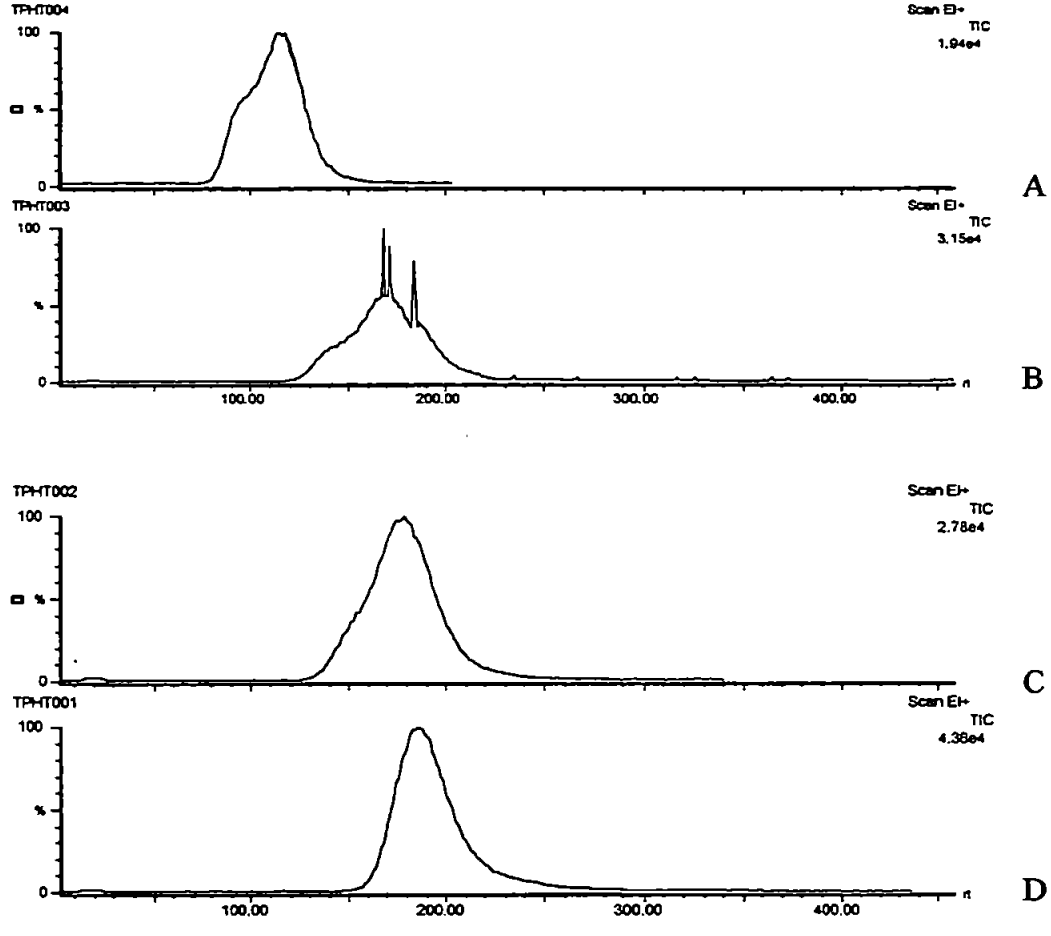
Chromatographic Conditions: A 1cm guard column packed with Hypersil phenyl was used to separate the analytes.
A two eluent system was employed :-
(A)= Eluent 1: 0.1M SDS / 0.05M NH_3NO_3 / 3% acetic acid. Eluent 2: Ethanol.
(B)= Eluent 1: 0.05M SDS / 0.05M NH_3NO_3 / 6% acetic acid. Eluent 2: Ethanol.
(C)= Eluent 1: 0.05M SDS / 0.1M NH_3NO_3 / 3% acetic acid. Eluent 2: Ethanol
(D)= Eluent 1: 0.05M SDS / 0.05M NH_3NO_3 / 3% acetic acid. Eluent 2: Ethanol
All at an eluent composition of 75% eluent 1: 25% eluent 2 and a flow rate of 1.0ml min^{-1}.

Figure 5.13: The Effect of Changing the Mobile Phase Composition on the Peak Shape of tributyltin



Chromatographic Conditions: A 1cm guard column packed with Hypersil phenyl was used to separate the analytes.
A two eluent system was employed :-
(A)= Eluent 1: 0.1M SDS / 0.05M NH_3NO_3 / 3% acetic acid. Eluent 2: Ethanol.
(B)= Eluent 1: 0.05M SDS / 0.05M NH_3NO_3 / 6% acetic acid. Eluent 2: Ethanol.
(C)= Eluent 1: 0.05M SDS / 0.1M NH_3NO_3 / 3% acetic acid. Eluent 2: Ethanol
(D)= Eluent 1: 0.05M SDS / 0.05M NH_3NO_3 / 3% acetic acid. Eluent 2: Ethanol
All at an eluent composition of 75% eluent 1: 25% eluent 2 and a flow rate of 1.0ml min^{-1}.

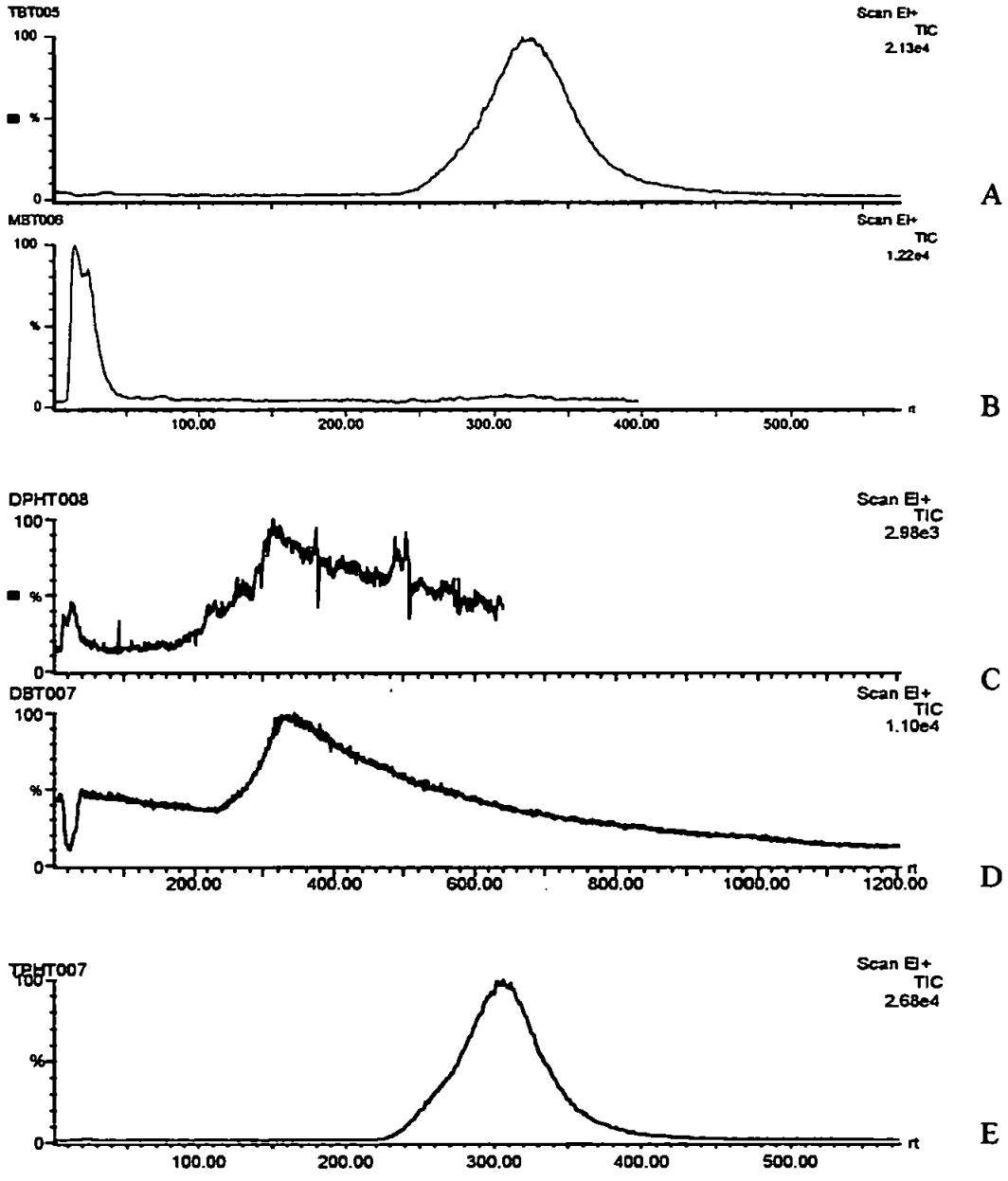
Figure 5.14: The Effect of Changing the Mobile Phase Composition on the Peak Shape of triphenyltin



Retention Time (secs)

Chromatographic Conditions: A 1cm guard column packed with Hypersil phenyl was used to separate the analytes.
A two eluent system was employed :-
(A)= Eluent 1: 0.1M SDS / 0.05M NH_3NO_3 / 3% acetic acid. Eluent 2: Ethanol.
(B)= Eluent 1: 0.05M SDS / 0.05M NH_3NO_3 / 6% acetic acid. Eluent 2: Ethanol.
(C)= Eluent 1: 0.05M SDS / 0.1M NH_3NO_3 / 3% acetic acid. Eluent 2: Ethanol
(D)= Eluent 1: 0.05M SDS / 0.05M NH_3NO_3 / 3% acetic acid. Eluent 2: Ethanol
All at an eluent composition of 75% eluent 1: 25% eluent 2 and a flow rate of 1.0ml min^{-1}.

Figure 5.15: The Effect of Reducing the Concentration of Acetic Acid in the Mobile Phase



A= Tributyltin, B=monobutyltin, C=diphenyltin, D=dibutyltin,E=triphenyltin

Chromatographic Conditions: A 1cm guard column packed with Hypersil phenyl was used to separate the analytes.
A two eluent system was employed :-
Eluent 1: 0.05M SDS / 0.05M NH_3NO_3 / 1% acetic acid. Eluent 2: Ethanol.
At an eluent composition of 75% eluent 1: 25% eluent 2 and a flow rate of 1.0ml min^{-1}.

However since the main purpose of this study was to investigate the usefulness of the HPLC-ICP-MS interface when applied to this application, and not refine the chromatography to fully optimise this separation, the study was stopped at this stage.

5.7. Conclusions

Following the use of two separation techniques for organotin compounds in sediments a number of conclusions can be drawn.

For the micellular liquid chromatography (MLC), it was found that the eluent composition that gave the best peak shape for the individually injected standards, TBT and TriPhT were not separated on the phenyl column that was available. In addition to this, diphenyltin (DPhT) and monophenyltin (MPhT) tailed excessively, due to interactions with free silanol groups on the stationary phase. This seriously affected the efficiency of the method being used. This would be remedied by more closely controlling the pH to alter the ionisation state of the silica stationary phase support. The acetic concentration in the eluent affects the degree of micelle formation and so is an important parameter to optimise. Alternatively, the use of polymer based or more endcapped silica based columns would eliminate or reduce the interaction of the analyte with the silanol groups preventing the excessive tailing of the di- and monophenyl tin compounds.

To separate the organotin compounds using MLC, the selectivity of the method needs to be altered. A longer less hydrophobic column may be necessary, such as a C-4 butyl bonded silica column (139), however one was not available for this study.

At the moment the use of strong cation exchange columns offers the best resolution of the analytes and the most robust separation. How robust the MLC method would be when looking at real samples has to be investigated. Would the matrix from a sediment extract effect micelle formation to such an extent that the separation would not occur? This is quite possible, and as such it is suggested that the use of ion exchange or mixed mode columns with ion exchange and reversed phase capacities are the best options for the speciation of organotins in real samples. This is view is supported by the results obtained in Chapter 4, where the use of ion-pairing agents was shown to be susceptible to matrix effects.

The HPLC-ICP-MS interface has demonstrated its utility by showing that it can successfully reduce the solvent loading of the plasma. Thus the use of high percentages of alcohols in the mobile phase is possible using ICP-MS detection. In addition the sheath gas can significantly reduce or totally eliminate the build up of salts in the torch injector even at levels of 0.1M dissolved solid concentration in the mobile phase. The use of a sheath gas may be used to aid on going studies on the speciation of organotin compounds by HPLC-ICP-MS, as currently a larger bore injector for the ICP torch is required to prevent clogging due to the high levels of dissolved salts in the mobile phase. With the use of a sheath gas a smaller bore injector may be used to reduce the solvent loading of the plasma resulting in a greater tolerance of the plasma for the mobile phase.

6.0 Organometallic Species in Tea

6.1 Introduction

Tea is a commodity valued by assessing flavour and appearance. Together with coffee, wine and certain spirits, tea belongs to a group of plant products of considerable economic importance where visual qualities are an integral part of assessment. Tea beverages are prepared from the leaves of the tea bush *Camellia sinensis*, second only to water, tea is the most widely consumed drink in the world today. Chinese mythology records that in 2737 BC, the Emperor Shen Nung first discovered the merits of tea drinking. The first true accounts of the use and horticulture of tea in China dates back to the writings of Kou P'o, in 350 AD. Tea was introduced in Japan in 600 AD, in Europe in 1610, and in the American colonies in 1650.

Early attempts to determine tea quality by chemical analysis, undertaken in conjunction with chemical investigations of the nature of the important flavour constituents, resulted in a scheme which determined the important flavour components colourimetrically. This demonstrated that the appearance of a liquor is strongly correlated with the flavour, suggesting that many of the flavour components are also responsible for the visual properties.

There are three main types of tea which are determined by the manufacturing process used in their production, green (unfermented), oolong (partially fermented), and black (fully fermented) (142). The manufacturing process is designed to either prevent or allow tea polyphenols to be oxidised by catalytic enzymes. The manufacture of green tea involves the rapid steaming or pan firing of freshly harvested leaves to inactivate the enzymes, thus preventing fermentation, the leaves are then rolled and dried. In the production of oolong and black teas, the leaves are first

allowed to wither after harvest until the moisture content is reduced by 55-60%. This results in a concentration of the polyphenols in the leaves and a deterioration in the leaf structure. Oolong tea is prepared by rolling the tea leaves and then firing them shortly afterwards to prevent further oxidation and fermentation as well as to dry them. Black tea is prepared by crushing the withered tea leaves, these are then rolled, after which warm air is circulated to enhance the fermentation processes. The fermented tea leaves are then fired to inactivate the enzymes and dry the leaves.

Black tea pigments are formed in this fermentation stage of black tea manufacture by the oxidation of simple polyphenols to more complex, condensed polyphenols. There are thought to be two main classes of polyphenols: the theaflavins and the thearubigins.

Thearubigins is a name assigned originally to a heterogeneous group of orange-brown weakly acid pigments formed by oxidative transformation of flavanols, and to a certain extent other polyphenols during the manufacture of black tea. They are an inhomogeneous mixture of pigments with molecular masses in the range 700-400000 Daltons. Thearubigins have been claimed to be the most abundant polyphenolic fraction of black tea. They contribute to taste, depth of colour, and body of a tea brew, and thus influence the quality as evaluated by tea tasters.

Theaflavins are benzotropolone compounds of known structure, they have been synthesised by chemical oxidation, but a detailed examination of the oxidation products of green tea polyphenolics has not been carried out as yet.

Tea is heavily consumed in the UK, so the determination of minor and trace elements in drinking tea and tea leaves is therefore important for estimating the daily intake of these elements in the UK diet (143). As only the free metal ions are bioavailable for the human body (144), the speciation of metals in tea would enable us to determine what percentage of the total metal content was bioavailable.

6.2 Initial Method Development

The method used was developed by Bailey *et al.* (145) for the analysis of the non-volatile, water soluble constituents of black tea. Target elements for speciation studies present in the teas were identified by carrying out a semiquantitative analysis of each tea using the ICP-MS. The teas were then analysed by HPLC-ICP-MS with data acquisition in the TRA analysis mode. A UV detector was placed in-line between the analytical column and the nebuliser, monitoring absorption at 280 nm to identify the organic constituents present in the tea.

6.2.1. Sample preparation

Boiling ultrapure water (25 ml) was added to 2 g of the tea, this mixture was then infused with swirling for 3 min. The hot solution was filtered to remove the tea leaves and allowed to cool. Once cool, the solution was turbid, so it was filtered through a 5 μm syringe filter prior to injection.

6.2.2 Chromatography

The liquor was analysed by HPLC using a 250 x 4.6 mm id analytical column packed with Hypersil ODS 5 μm bonded silica (Jones Chromatography). A 1 cm guard column was fitted packed with the same stationary phase. A binary gradient elution system was employed, eluent 1 was 2% aqueous acetic acid, and eluent 2 was acetonitrile. A linear gradient from 8 to 31% eluent 2 over 50 minutes, with a flow rate of 1.0ml min^{-1} was employed. Samples were injected using a chemically inert injection valve (Cheminert Model C 1 valve, Valco Instruments Co. Inc., Houston, Texas) fitted with a 200 μl PEEK sample loop.

6.2.3. Results and Discussion.

Black and green teas were analysed to see what variations if any arose in the metal speciation due to the fermentation processes required to produce black teas. It was decided to concentrate on the characterisation of three teas. These were; Sainsburys Kenyan tea, Twinings English Breakfast, and Yunnan Pu-Erh tea. The first two are black teas from Africa, and a blend from India respectively, the latter is a Chinese green tea.

6.2.4. HPLC With UV detection analysis of teas.

Comparison of the first seven minutes of the UV chromatograms of the three teas shows one significant difference. The black teas (Kenya, and English Breakfast) (Figures 6.1 and 6.2 respectively) have a large peak eluting at 4.8 minutes.

Figure 6.1: A chromatogram of Kenyan tea with UV detection at 280 nm

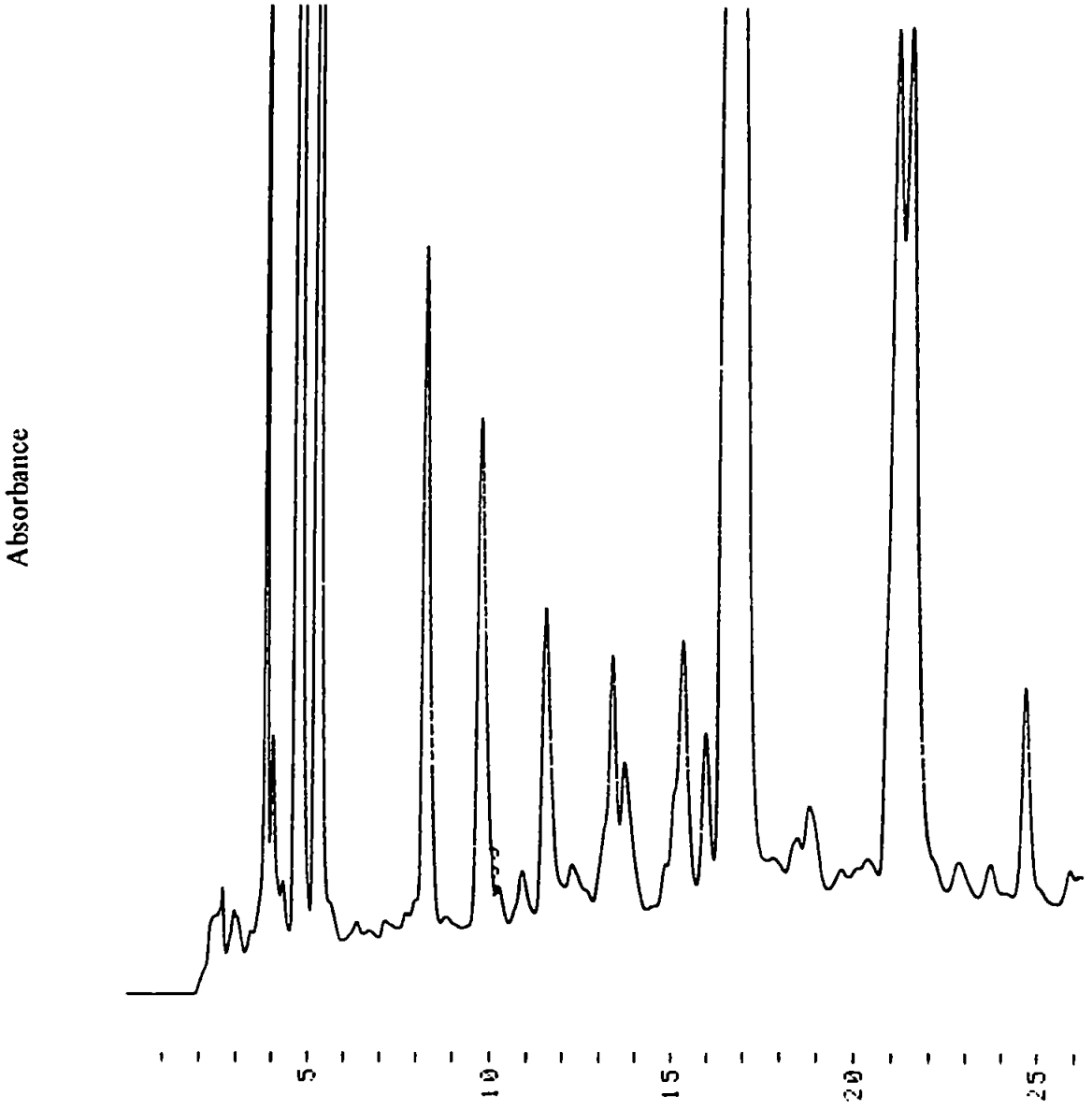


Figure 6.2: A chromatogram of English Breakfast tea with UV detection at 280 nm

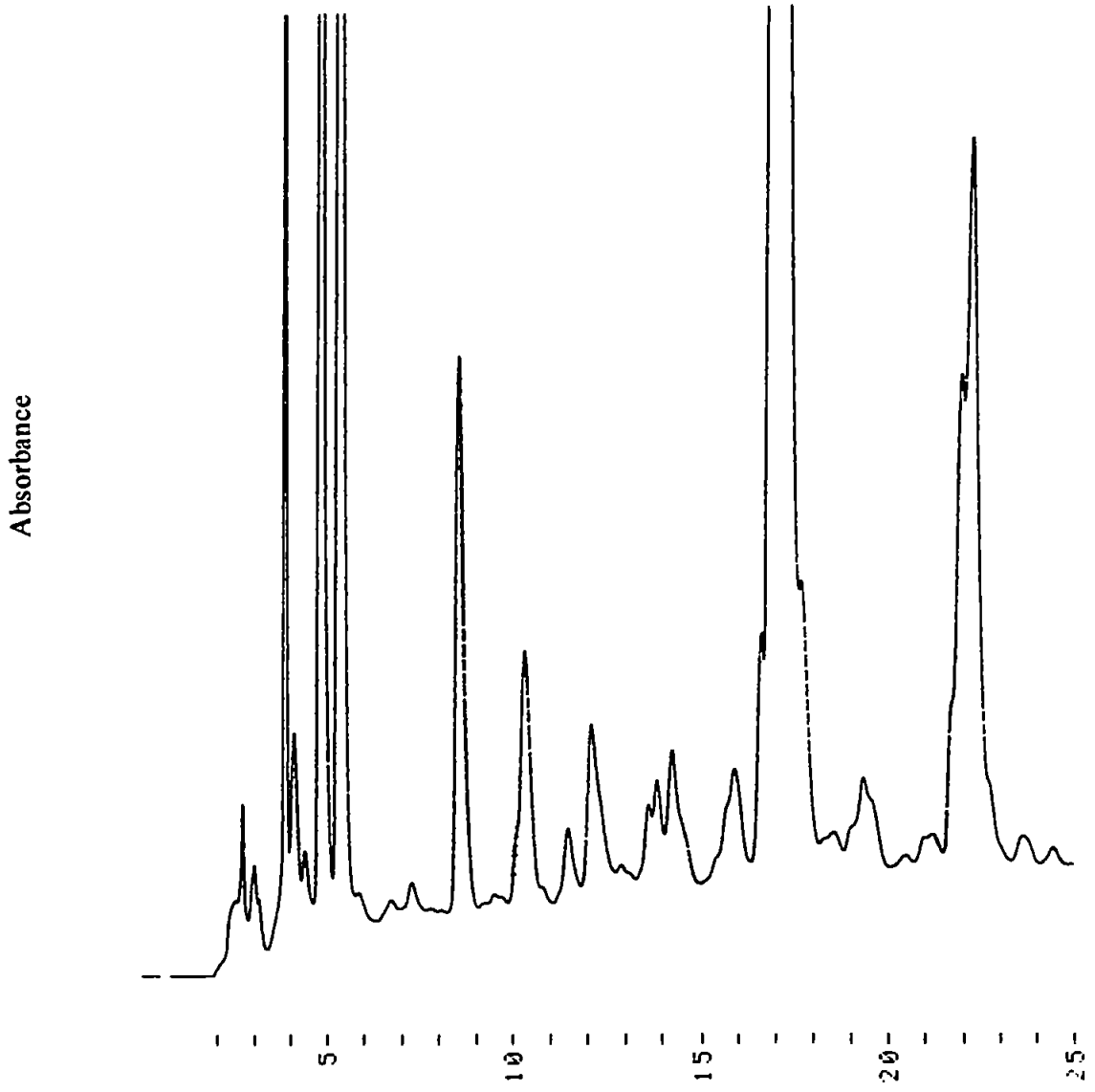
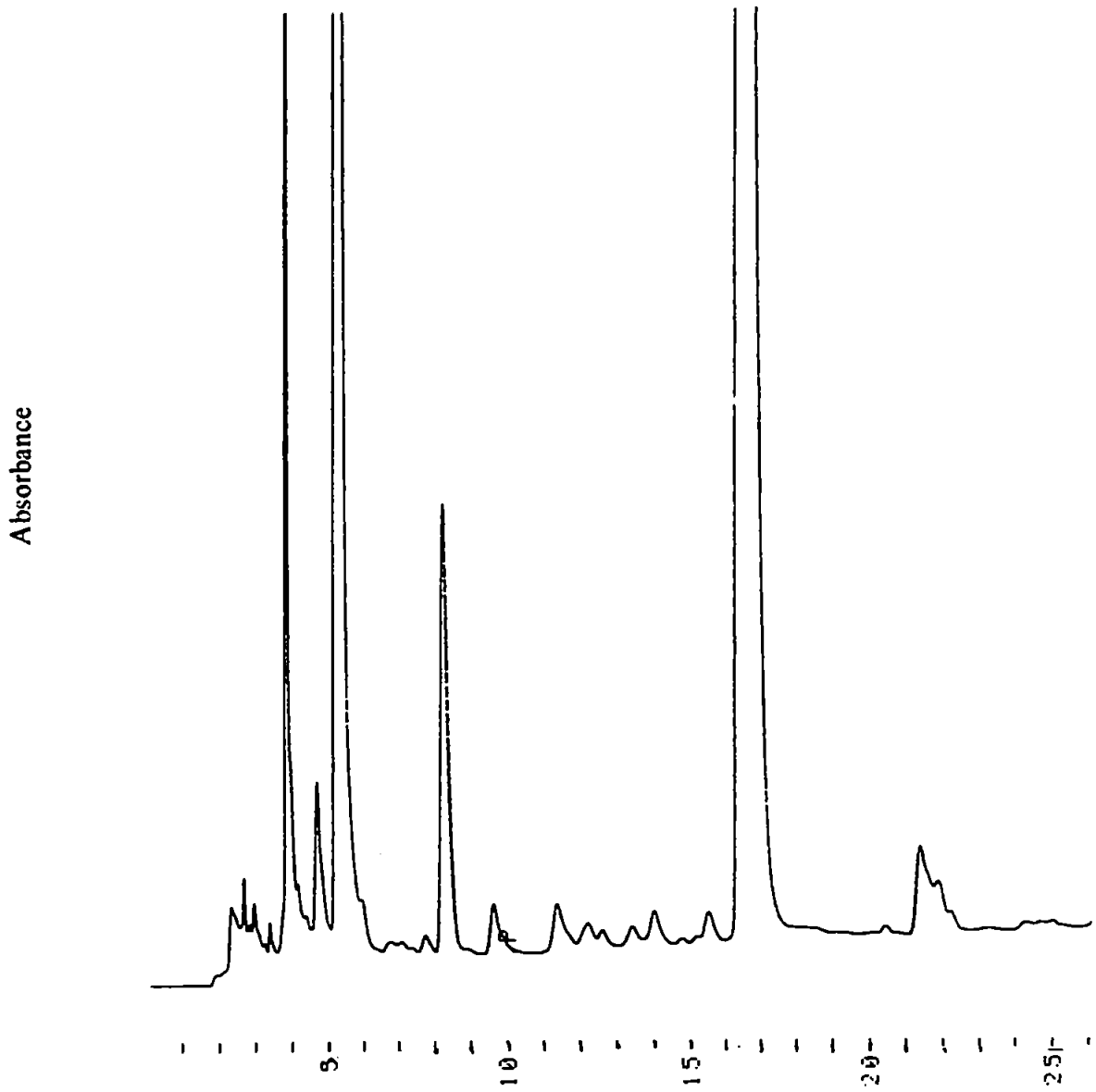


Figure 6.3: A chromatogram of Yunnan Pu-Erh tea with UV detection at 280 nm



where as the green tea (Pu-Erh) (Figure 6.3) does not. This compound has been identified as gallic acid, the 2 major peaks eluting either side of this compound are common to all three teas. The first with a retention time of 4 minutes is theogallin, the second of these eluting at 5.5 minutes is theobromine (145). Comparison up to ten minutes, shows that all three have a common peak eluting at 8.5 minutes, this is 5-O-caffeoylquinic acid, the peak at 10 minutes is caffeine, which is present in all three as well but is lowest in Pu-Erh, and most concentrated in the Kenya tea.

6.2.5. HPLC-ICP-MS Analysis of Tea

The target metals chosen after semiquantitative analysis of the three teas of interest were As, Bi, Cd, Cu, Hg, Mn, Mo, Ni, Pb, Sn, Sr, Te, V and Zn. The semiquantitative results of these analyses can be seen in Table 6.1. These metals were analysed using 3 different element menus depending on the dilution required to stop the detector going overrange causing a protection trip to come into operation. Looking at the mass chromatograms of Pu-Erh and English breakfast, it is possible to see that most of the elements elute with the solvent front at about 2.5-3 minutes (Figures 6.4 -6.7), the only exceptions being Pb, Sn in Yunnan Pu-Erh and Hg in English Breakfast (Figures 6.8-6.10).

In Pu-Erh, Pb displays some speciation, with a major peak at 2.8 minutes, 4 minor peaks accompany this peak, 2 eluting before it, and 2 after it. These have retention times of 1.8, 2.6, 3.7, and 4.0 minutes, respectively. Tin has a major peak at 3.0 minutes and a minor peak at 3.8 minutes.

Table 6.1. Results of Semiquantitative Analysis of tea liquor in mg l^{-1}.

Element	Kenyan	Pu-Erh	English Breakfast
As	0	0	236
Bi	1	0	174
Cd	0	0	1690
Cu	998	238	354
Hg	0	557	4350
Mn	120000	8440	76.9
Mo	3	0	615
Ni	917	171	333
Pb	361	39	267
Sn	0	0	424
Sr	332	77	103
Te	0	0	6610
V	137	3	0
Zn	6080	374	727

Figure 6.4: Speciaton of metals in Yunnan Pu-Erh tea, total ion current mass chromatogram

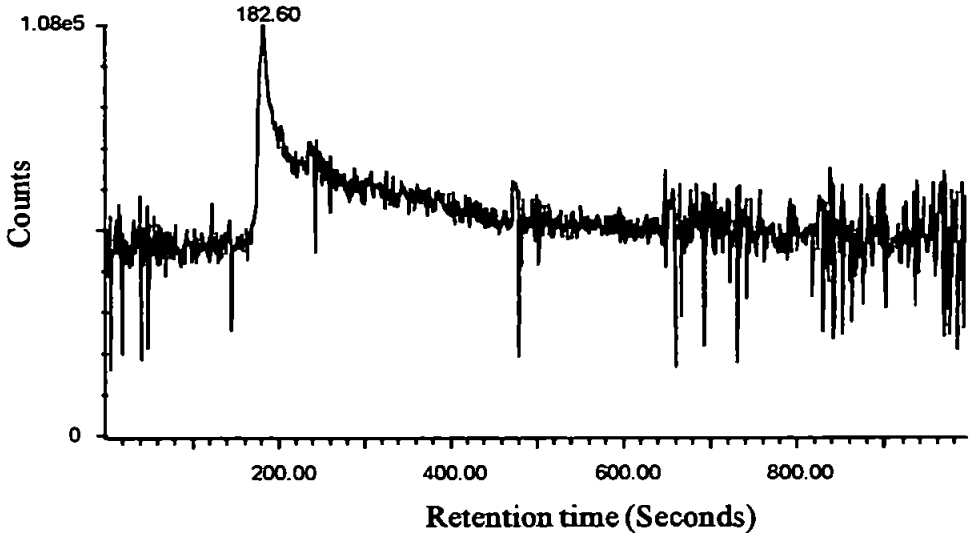
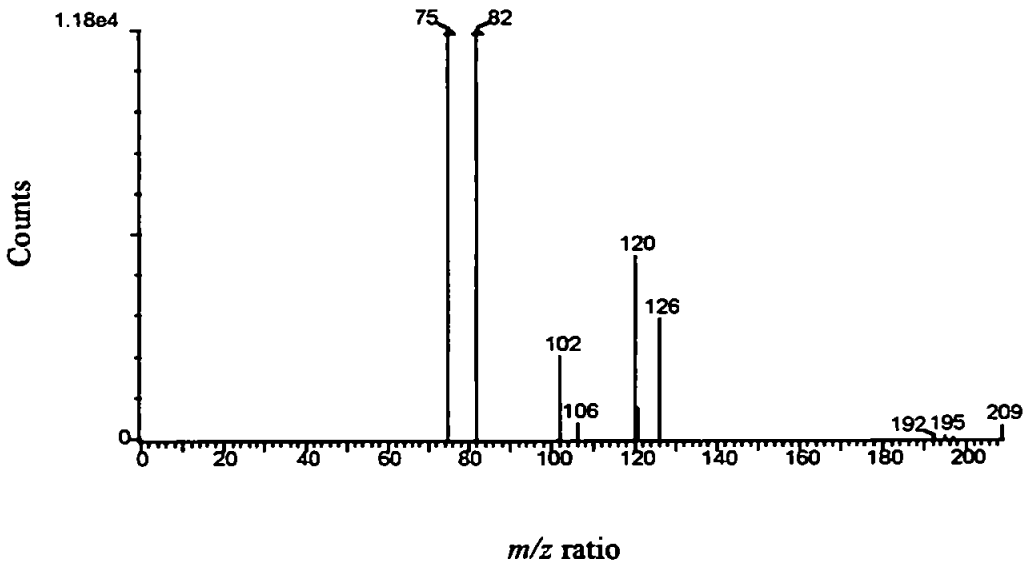


Figure 6.5: Mass spectrum of TIC peak in chromatogram of Yunnan Pu-Erh tea



Chromatographic Conditions: A 250 x 4.6 mm id analytical column packed with Hypersil ODS was used. A 1cm guard column was fitted packed with the same stationary phase. A binary gradient elution system was employed, eluent 1 was 2% aqueous acetic acid, and eluent 2 was acetonitrile. A linear gradient from 8 to 31% eluent 2 over 50 minutes, with a flow rate of 1.0ml min-1 was employed to separate the analytes

Figure 6.6: Speciaton of metals in English Breakfast tea, total ion current mass chromatogram

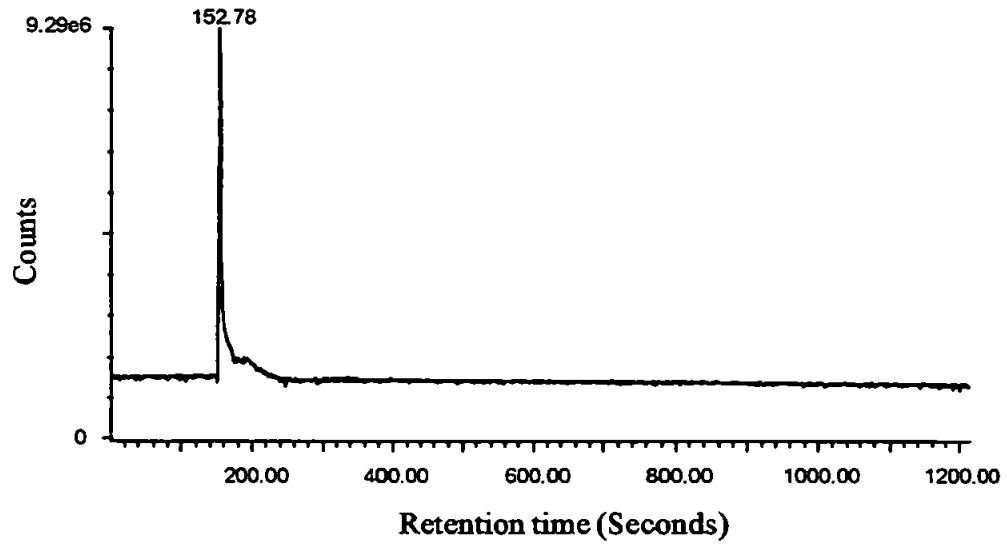
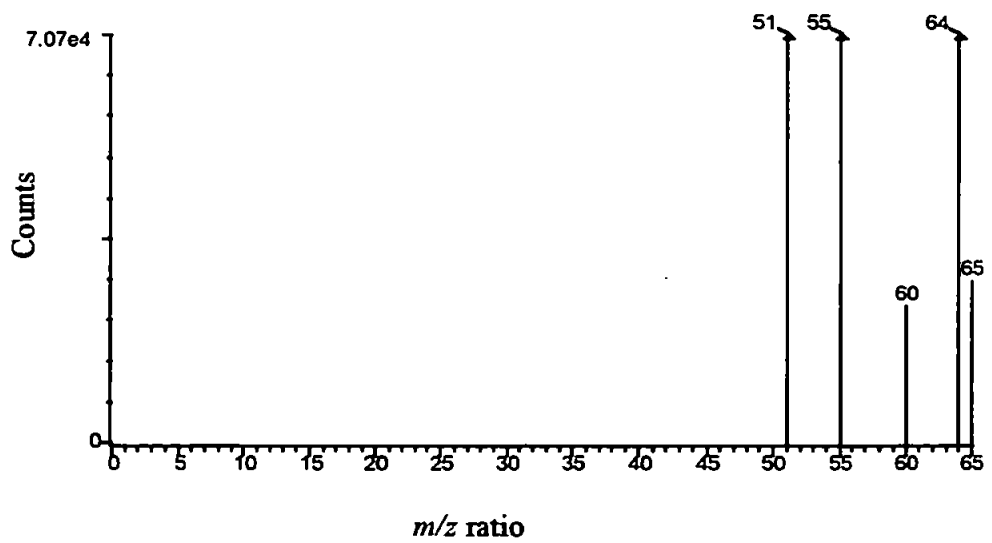


Figure 6.7: Mass spectrum of TIC peak in chromatogram of English Breakfast tea



Chromatographic Conditions: A 250 x 4.6 mm id analytical column packed with Hypersil ODS was used. A 1cm guard column was fitted packed with the same stationary phase. A binary gradient elution system was employed, eluent 1 was 2% aqueous acetic acid, and eluent 2 was acetonitrile. A linear gradient from 8 to 31% eluent 2 over 50 minutes, with a flow rate of 1.0ml min-1 was employed to separate the analytes

Figure 6.8: Speciation of Lead in Pu-Erh Green China Tea.

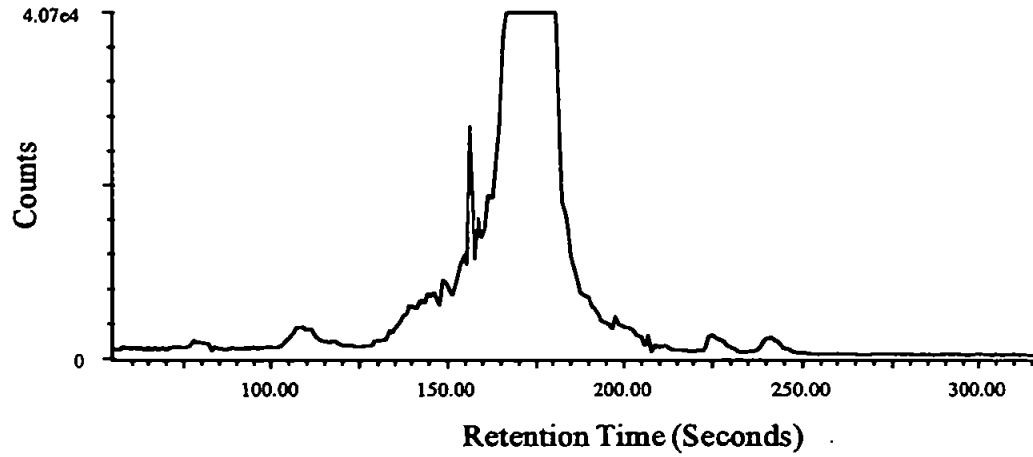
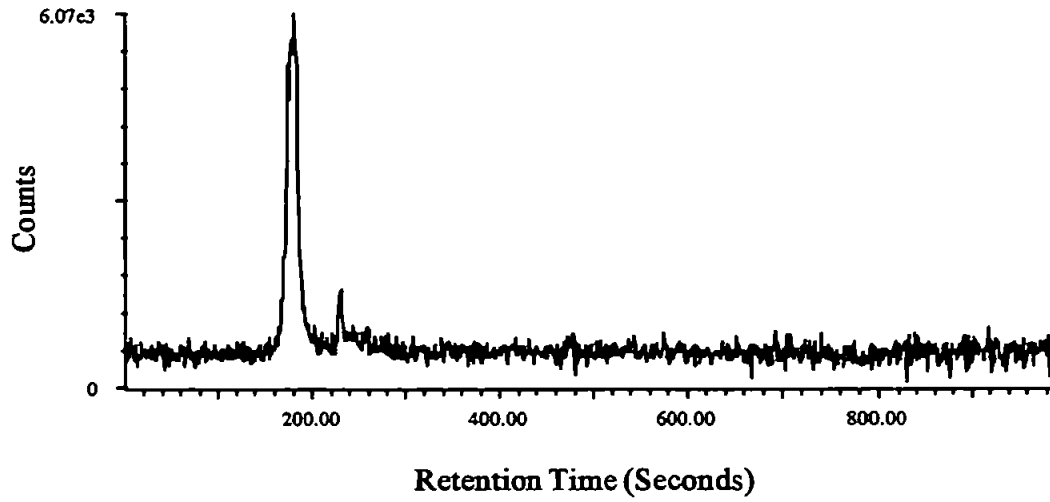
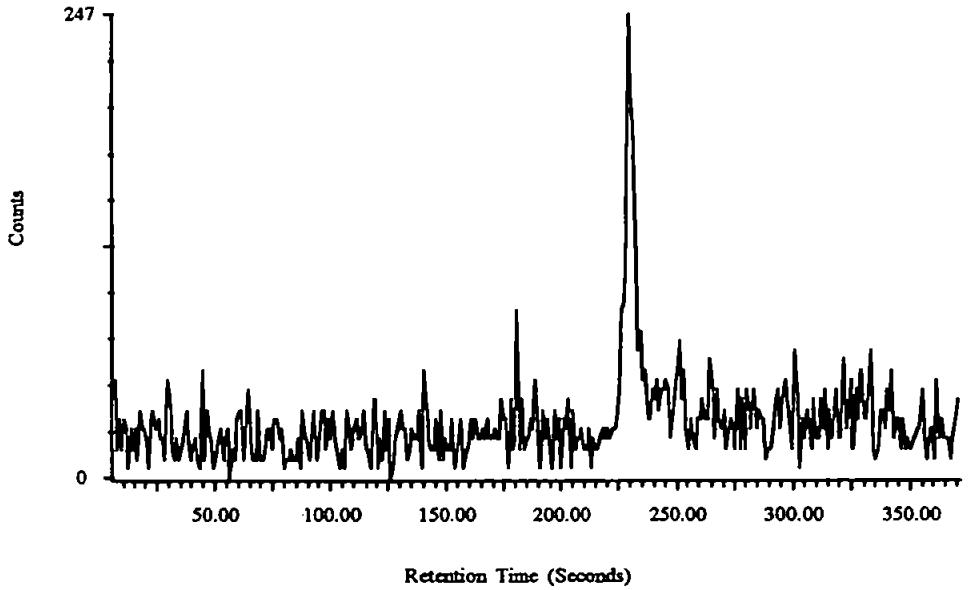


Figure 6.9: Speciation of Tin in Pu-Erh Tea.



Chromatographic Conditions: A 250 x 4.6 mm id analytical column packed with Hypersil ODS was used. A 1cm guard column was fitted packed with the same stationary phase. A binary gradient elution system was employed, eluent 1 was 2% aqueous acetic acid, and eluent 2 was acetonitrile. A linear gradient from 8 to 31% eluent 2 over 50 minutes, with a flow rate of 1.0ml min-1 was employed to separate the analytes

Figure 6.10: Speciation of mercury in English Breakfast tea.



Chromatographic Conditions: A 250 x 4.6 mm id analytical column packed with Hypersil ODS was used. A 1cm guard column was fitted packed with the same stationary phase. A binary gradient elution system was employed, eluent 1 was 2% aqueous acetic acid, and eluent 2 was acetonitrile. A linear gradient from 8 to 31% eluent 2 over 50 minutes, with a flow rate of 1.0ml min-1 was employed to separate the analytes

In English Breakfast tea, the only element eluting after the solvent front was mercury, with a retention time of 3.8 minutes. From the initial results it is possible to see that there is little correlation between the metals present in tea, and the flavanoids present, using the current chromatographic conditions. Since the metals are probably only loosely bound to the flavanoids. It is possible that interaction of the metals with the free silanol groups on the ODS stationary phase, reduces the correlation by separating the metals from the polyphenols they may have originally been associated with.

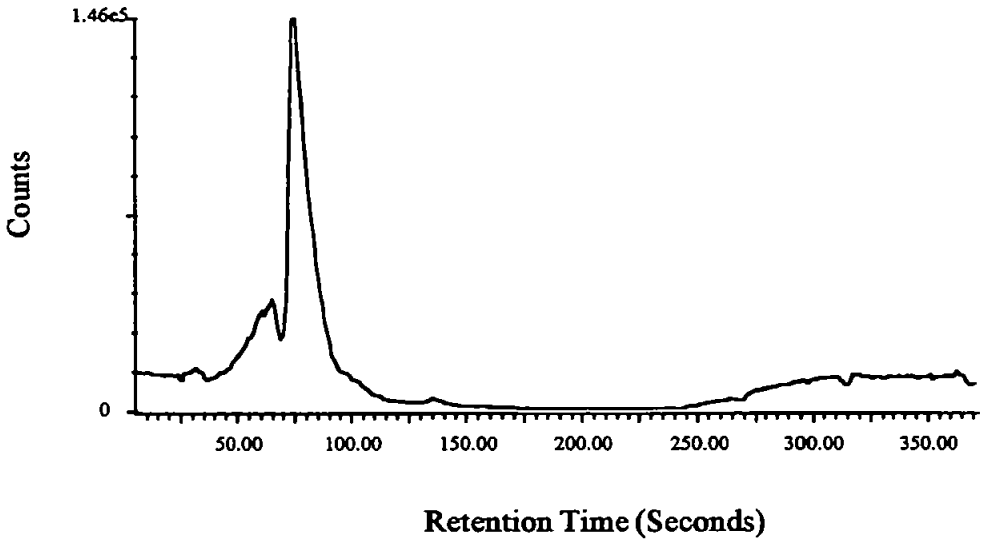
6.3 Optimisation of the Speciation of Metals in Tea by HPLC-ICP-MS

Having had little success with the initial conditions that were used to separate the flavanoids present in tea. It was decided to optimise the separation with respect to metal speciation in tea.

As it was suspected that part of the problem was metal interaction with the free silanol groups present in the ODS column. It was decided to try the elution conditions as in Section 6.1.2 using a 150x4.6 mm id column packed with Hamilton 5 μm PRP-1.

With these conditions, Kenyan tea showed no change, with all the elements eluting with the solvent front. English Breakfast had very little change except for slight evidence of speciation for lead (Figure 6.11). However, the green Pu-Erh tea showed speciation for a number of elements, these were, Cd, Pb and Sr (Figures 6.12 - 6.14). Encouraged by these results it was decided to attempt to optimise the separations attained for these elements in the green tea. The optimum conditions thus found would then be used to analyse all the teas for the target elements.

Figure 6.11: The Speciation of lead in English Breakfast tea separated on a PRP-1 column with a solvent gradient elution



Chromatographic Conditions: A 150 x 4.6 mm id analytical column packed with Hamilton PRP-1 was used. A 1cm guard column was fitted packed with the same stationary phase. A binary gradient elution system was employed, eluent 1 was 2% aqueous acetic acid, and eluent 2 was acetonitrile. A linear gradient from 8 to 31% eluent 2 over 50 minutes, with a flow rate of 1.0ml min-1 was employed to separate the analytes

Figure 6.12: Speciation of cadmium in Pu-Erh Tea.

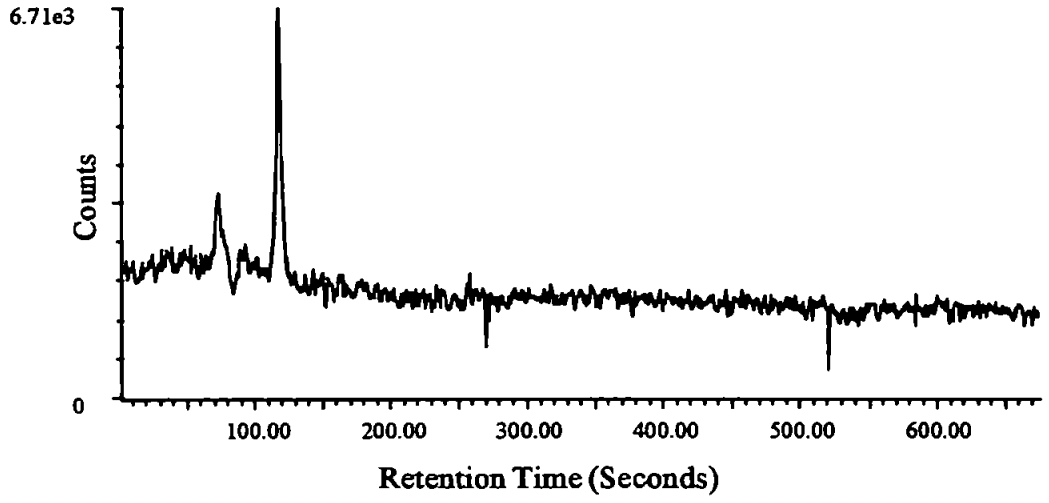
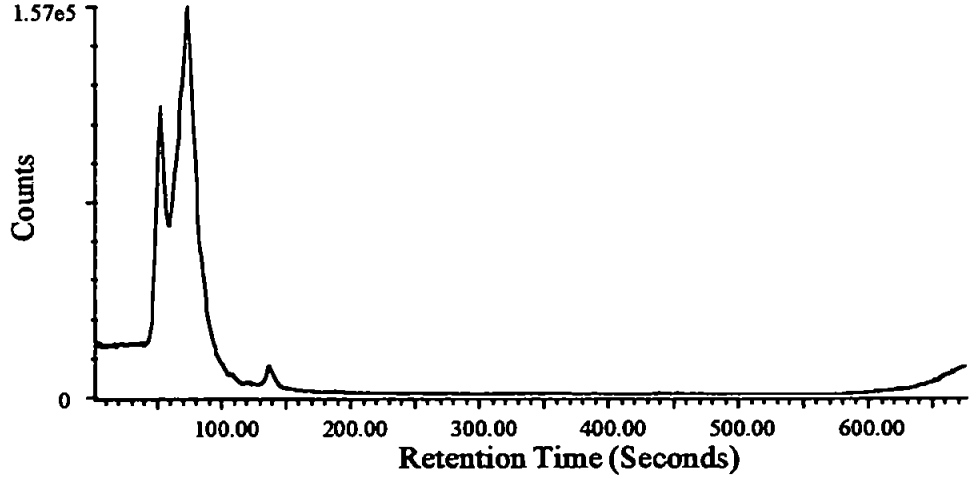
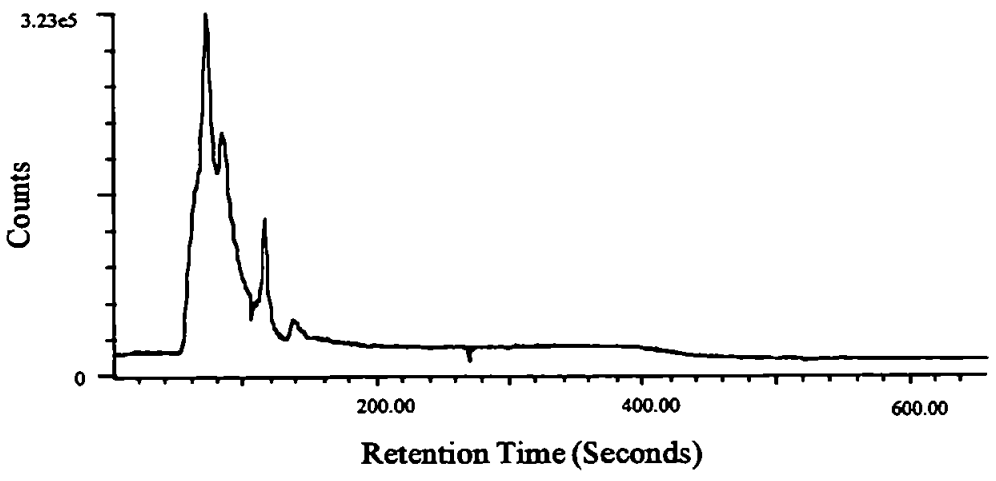


Figure 6.13: The Speciation of Lead in Pu-Erh Tea.



Chromatographic Conditions: A 150 x 4.6 mm id analytical column packed with Hamilton PRP-1 was used. A 1cm guard column was fitted packed with the same stationary phase. A binary gradient elution system was employed, eluent 1 was 2% aqueous acetic acid, and eluent 2 was acetonitrile. A linear gradient from 8 to 31% eluent 2 over 50 minutes, with a flow rate of 1.0ml min-1 was employed to separate the analytes

Figure 6.14: The Speciation of Strontium in Tea on a PRP-1 Column With a solvent gradient



Chromatographic Conditions: A 150 x 4.6 mm id analytical column packed with Hamilton PRP-1 was used. A 1cm guard column was fitted packed with the same stationary phase. A binary gradient elution system was employed, eluent 1 was 2% aqueous acetic acid, and eluent 2 was acetonitrile. A linear gradient from 8 to 31% eluent 2 over 50 minutes, with a flow rate of 1.0ml min-1 was employed to separate the analytes

6.3.1 Experimental

The HPLC column used was the Hamilton PRP-1 column, a series of isocratic elutions were carried out at the following eluent compositions:-

Eluent 1:	Eluent 2
90	10
70	30
50	50
30	70

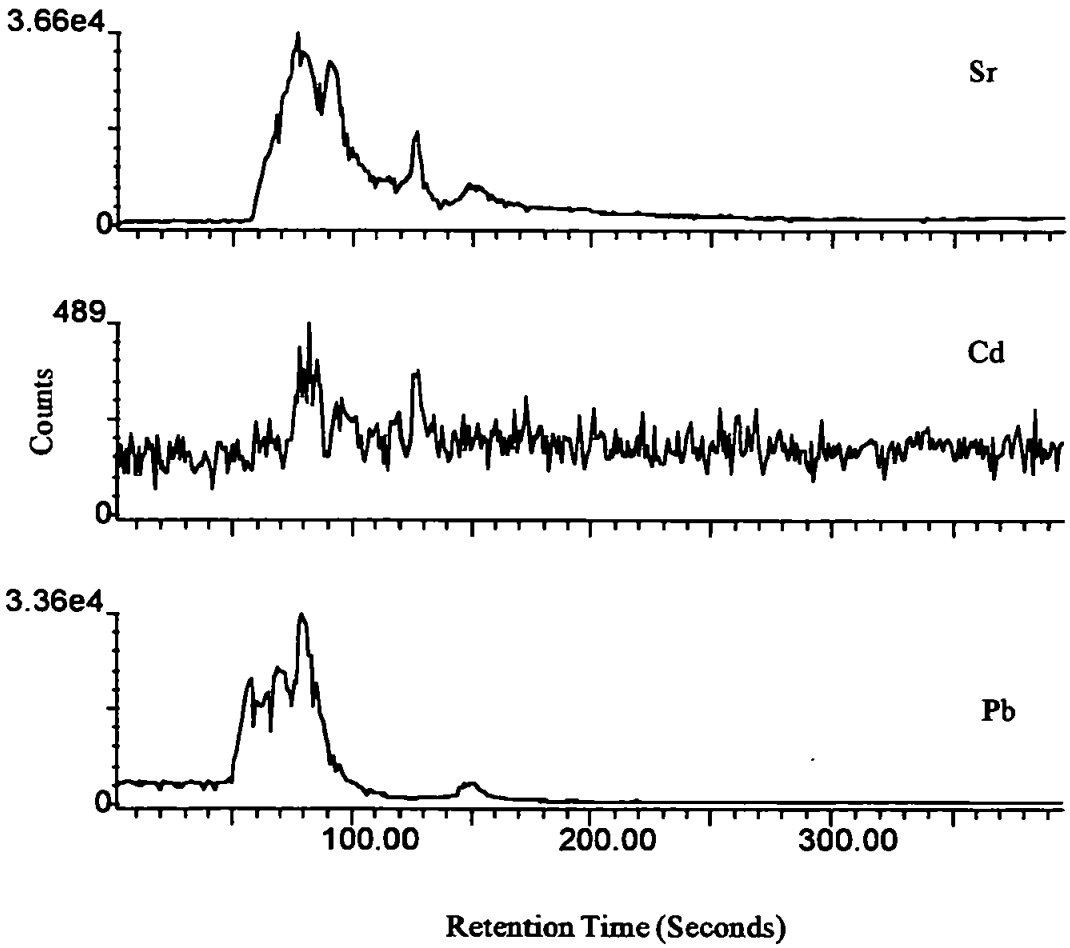
6.3.2 Results and Discussion

It can be seen from Figures 6.15 - 6.22, that the best elution conditions were achieved with the eluent compositions of 90:10 and 90:30 eluent 1: eluent 2 for the elements present in the sample, i.e. Cd, Cu, Mn, Ni, Pb, Sr and Zn. It was decided to analyse the samples of English Breakfast and Kenyan tea with the eluent composition of 90:10 eluent 1: eluent 2. The results can be seen in Figures 6.23 to 6.26, comparison of the black teas with the green tea shows a number of differences.

For the two black teas, strontium at *m/z* 88 has three peaks in the mass chromatogram (Figures 6.23 and 6.25). The green china tea has a less clearly defined speciation (Figure 6.15), however the results show that increasing the acetonitrile content of the mobile phase to 30% results in a more clearly defined chromatogram showing two distinct peaks (Figure 6.16). A similar effect

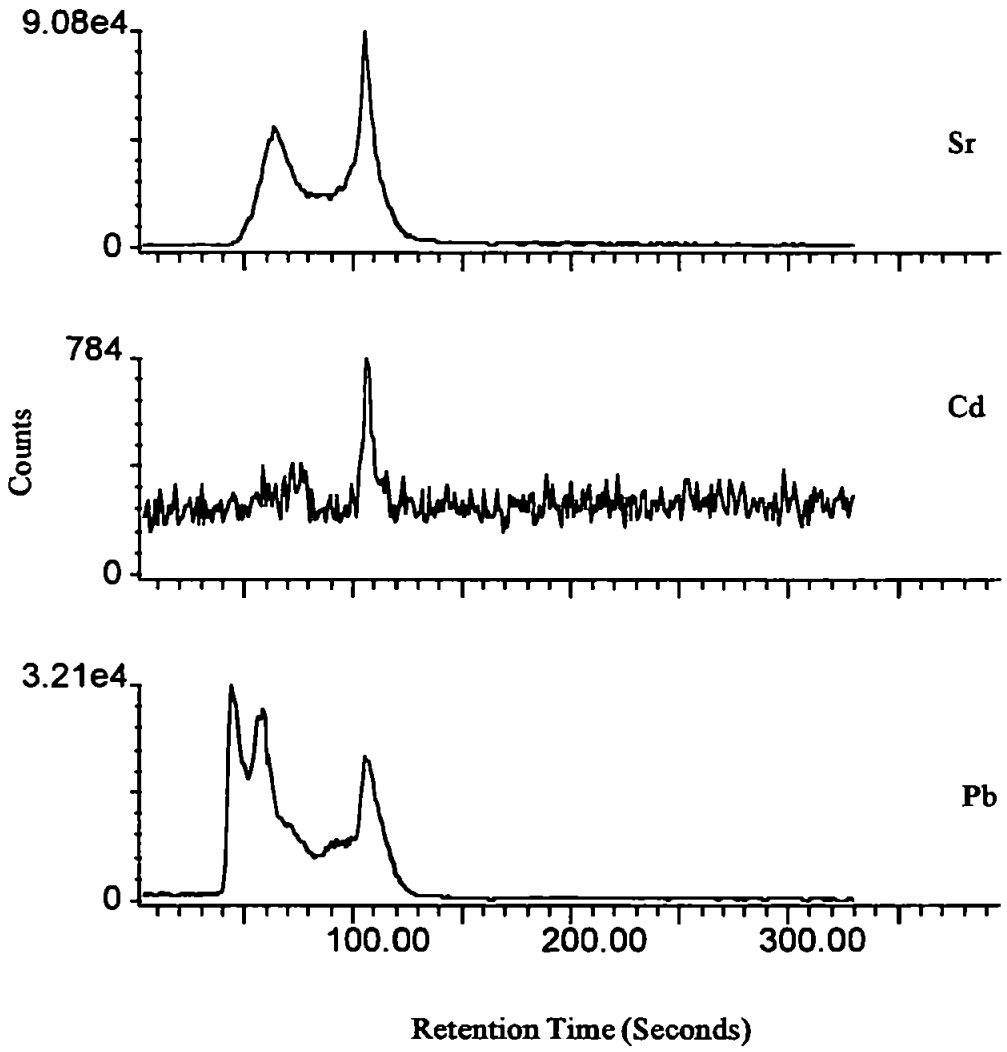
Figures 6.15- 6.18: The effect of eluent composition on the speciation of Sr, Cd and Pb in Yunnan Pu-Erh green China tea

Figure 6.15: Optimisation of Chromatography to Speciate Sr, Cd and Pb in Yunnan Pu-Erh green China tea



Chromatographic Conditions: A 150 x 4.6 mm id analytical column packed with Hamilton PRP-1 was used. A 1cm guard column was fitted packed with the same stationary phase. An isocratic elution system was employed, eluent 1 was 2% aqueous acetic acid, and eluent 2 was acetonitrile. The eluent composition employed was 90:10 eluent 1: eluent 2 , with a flow rate of 1.0ml min-1.

Figure 6.16: Optimisation of Chromatography to Speciate Sr, Cd and Pb in Yunnan Pu-Erh green China tea



Chromatographic Conditions: A 150 x 4.6 mm id analytical column packed with Hamilton PRP-1 was used. A 1cm guard column was fitted packed with the same stationary phase. An isocratic elution system was employed, eluent 1 was 2% aqueous acetic acid, and eluent 2 was acetonitrile. The eluent composition employed was 70:30 eluent 1: eluent 2 , with a flow rate of 1.0ml min-1.

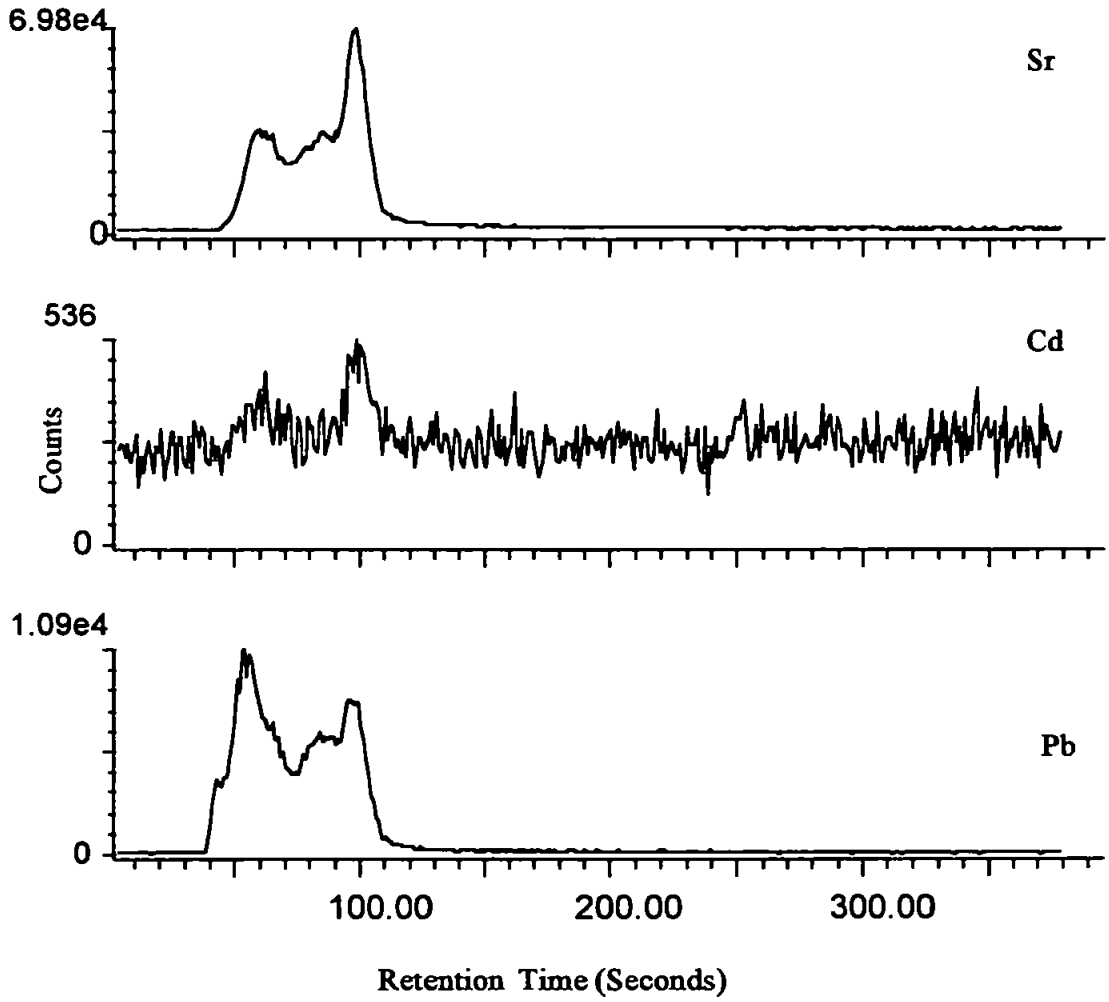
can be observed for the other elements that were monitored. For the black teas, speciation occurs at an acetonitrile content of 10%, but for the green tea, at that eluent composition, only single peaks (*m/z* 55, 60, 64 and 65) or large multicomponent peaks are seen (*m/z* 88 and 208). Increasing the eluent compositon to 30% acetonitrile produces chromatograms closer in nature to those obtained for the black teas (see Figures 6.20, 6.24 and 6.26).

Examination of the chromatograms with UV absorption detection does not immediately help explain this. At the mobile phase composition of 90:10 eluent 1: eluent 2. The chromatograms of English Breakfast (Figure 6.27) and Yunnan Pu-Erh (Figure 6.28) appear to be similar, whilst the chromatogram for the Kenyan tea (Figure 6.29) is different.

Closer examination shows that the peaks in the green tea that correspond to those in the black tea have a shorter retention time. For example, the major peaks in Yunnan Pu-Erh elute at 1.30, 1.68, 2.12 and 6.50 minutes. Those in English Breakfast elute at 1.30 (solvent front), 1.72, 2.23 and 6.62 minutes. The Kenyan tea chromatogram has some features that correspond to those peaks at 1.33, 1.82, 2.30 and 6.65 minutes. The components in the black teas clearly have more affinity for the column suggesting that the components are larger or more organic in nature than those in green tea.

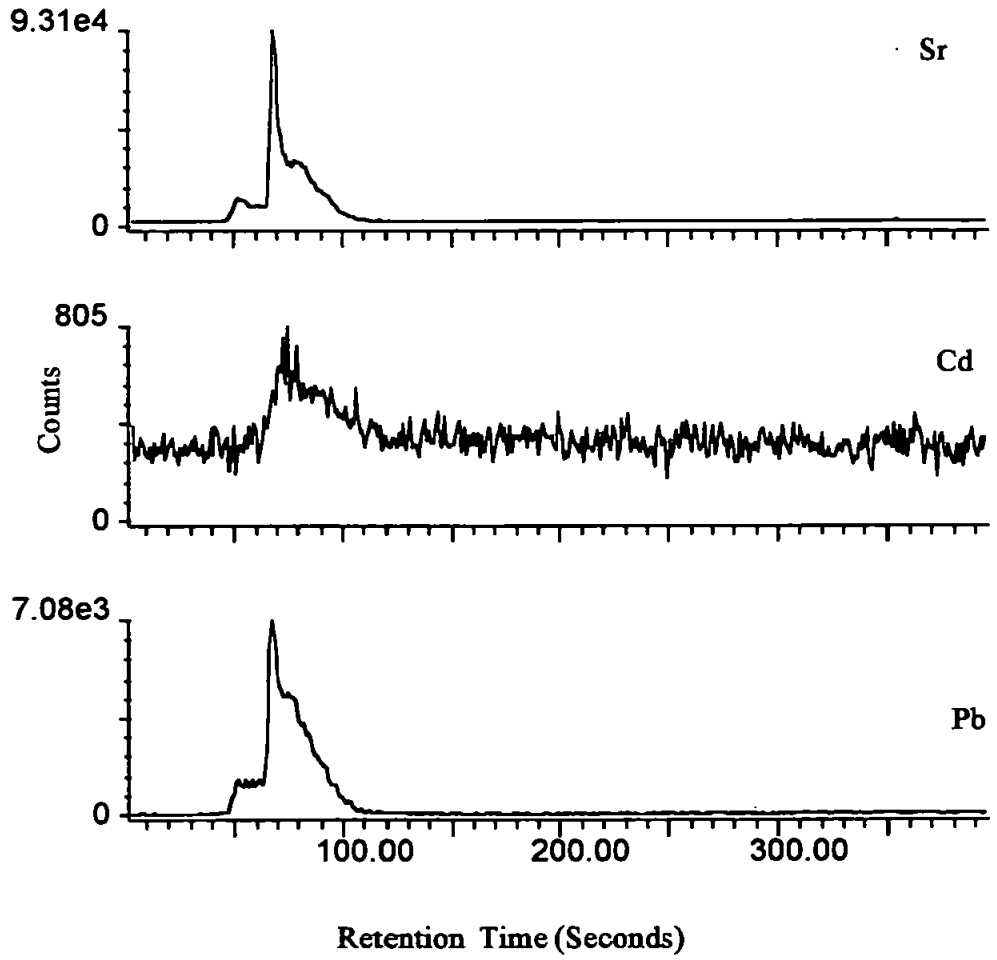
This is due to the different production processes. In the green tea, the flavanoids are simple polyphenols. But the fermentation process used to make black tea causes the flavanoids to polymerise into more complex, condensed polyphenols with a much higher molecular weight. So the black tea flavanoids have a higher affinity for a reversed phase than do those in green tea. So to increase the affinity the green tea flavanoids have for the column, it is necessary to increase the organic modifier content of the mobile phase.

Figure 6.17: Optimisation of Chromatography to Speciate Sr, Cd and Pb in Yunnan Pu-Erh green China tea



Chromatographic Conditions: A 150 x 4.6 mm id analytical column packed with Hamilton PRP-1 was used. A 1cm guard column was fitted packed with the same stationary phase. An isocratic elution system was employed, eluent 1 was 2% aqueous acetic acid, and eluent 2 was acetonitrile. The eluent composition employed was 50:50 eluent 1: eluent 2 , with a flow rate of 1.0ml min-1.

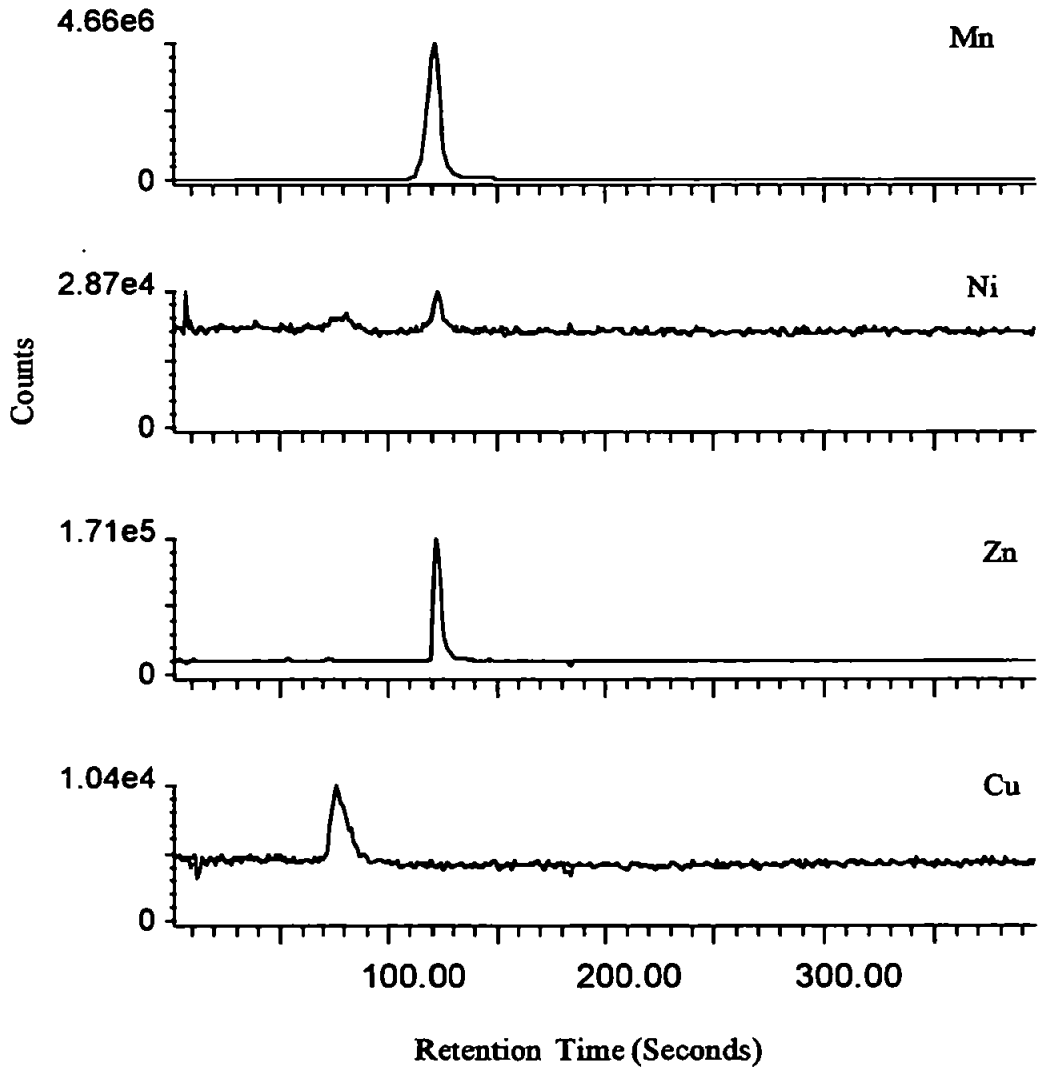
Figure 6.18: Optimisation of Chromatography to Speciate Sr, Cd and Pb in Yunnan Pu-Erh green China tea



Chromatographic Conditions: A 150 x 4.6 mm id analytical column packed with Hamilton PRP-1 was used. A 1cm guard column was fitted packed with the same stationary phase. An isocratic elution system was employed, eluent 1 was 2% aqueous acetic acid, and eluent 2 was acetonitrile. The eluent composition employed was 30:70 eluent 1: eluent 2 , with a flow rate of 1.0ml min-1.

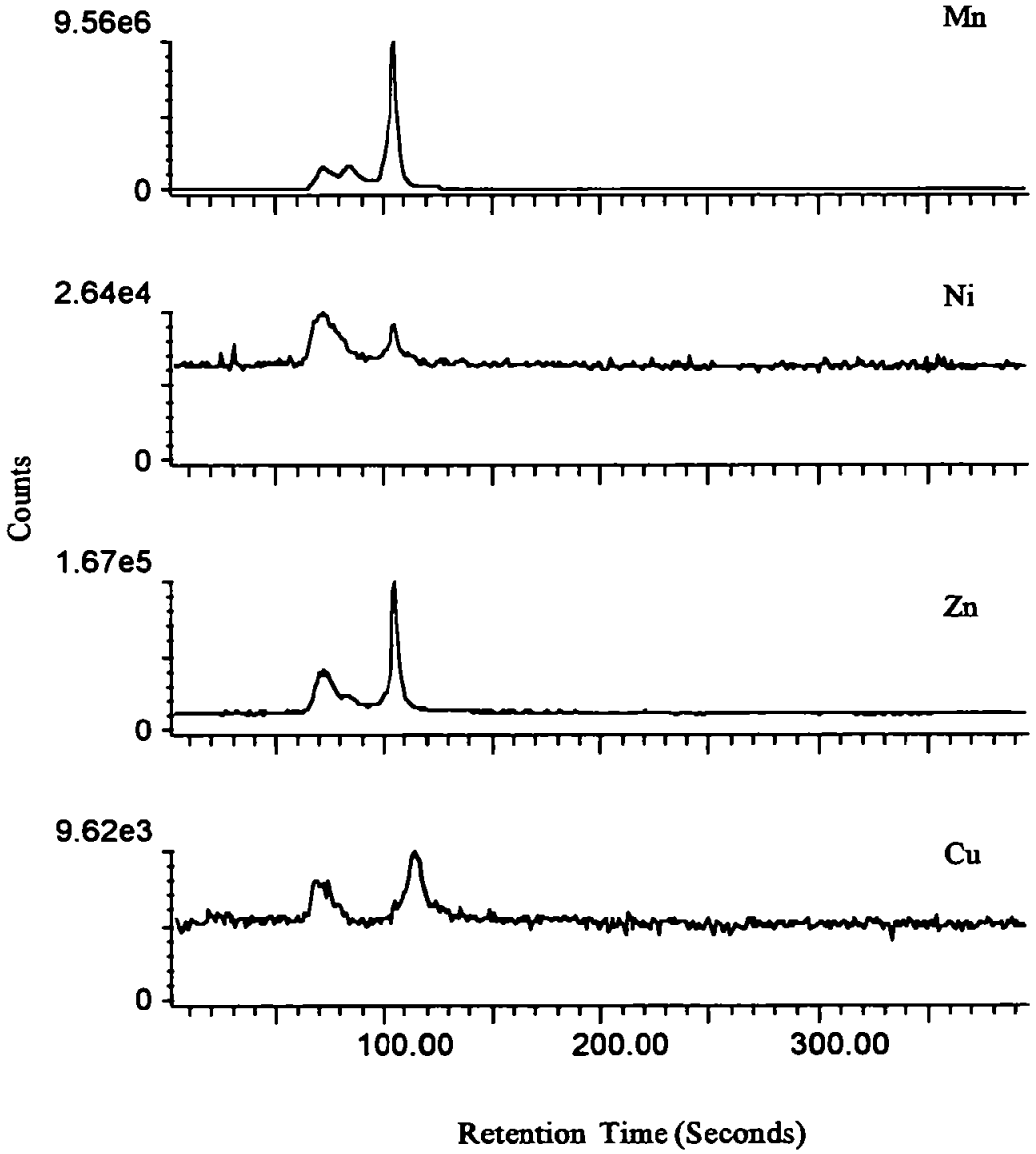
Figures 6.19- 6.22: The effect of eluent composition on the speciation of Mn, Ni, Zn and Cu in Yunnan Pu-Erh green China tea

Figure 6.19: Optimisation of Chromatography to Speciate Mn, Ni and Zn and Cu in Yunnan Pu-Erh green China tea



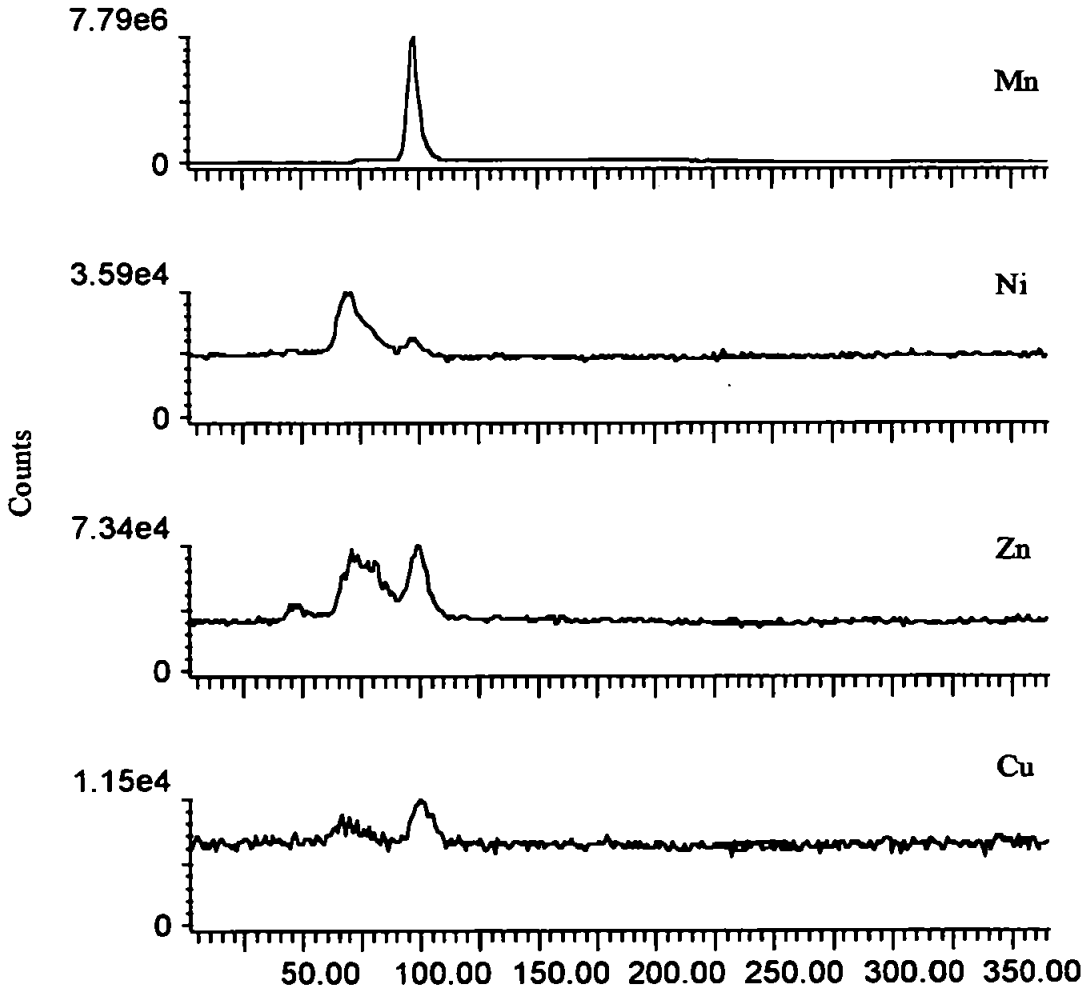
Chromatographic Conditions: A 150 x 4.6 mm id analytical column packed with Hamilton PRP-1 was used. A 1cm guard column was fitted packed with the same stationary phase. An isocratic elution system was employed, eluent 1 was 2% aqueous acetic acid, and eluent 2 was acetonitrile. The eluent composition employed was 90:10 eluent 1: eluent 2 , with a flow rate of 1.0ml min-1.

Figure 6.20: Optimisation of Chromatography to Speciate Mn, Ni and Zn and Cu in Yunnan Pu-Erh green China tea



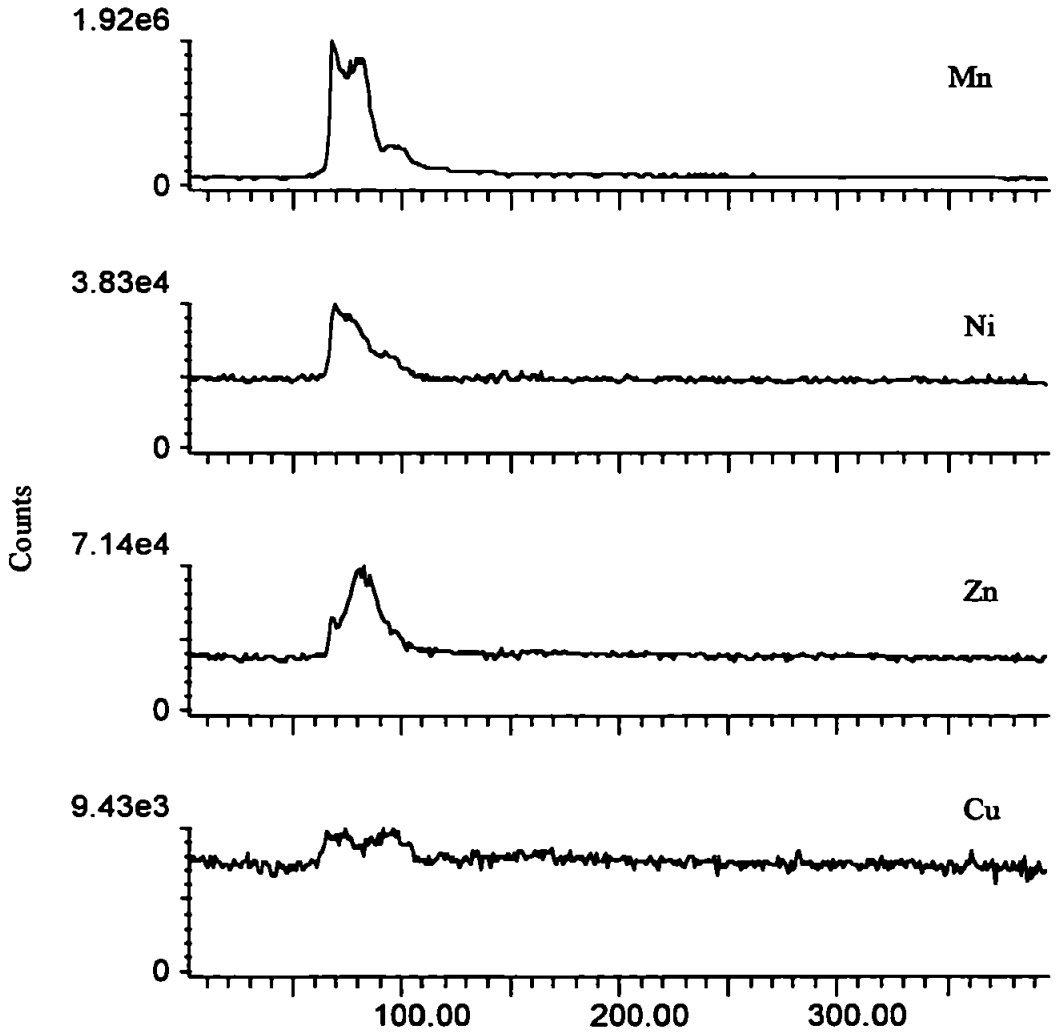
Chromatographic Conditions: A 150 x 4.6 mm id analytical column packed with Hamilton PRP-1 was used. A 1cm guard column was fitted packed with the same stationary phase. An isocratic elution system was employed, eluent 1 was 2% aqueous acetic acid, and eluent 2 was acetonitrile. The eluent composition employed was 70:30 eluent 1: eluent 2 , with a flow rate of 1.0ml min-1.

Figure 6.21: Optimisation of Chromatography to Speciate Mn, Ni and Zn and Cu in Yunnan Pu-Erh green China tea



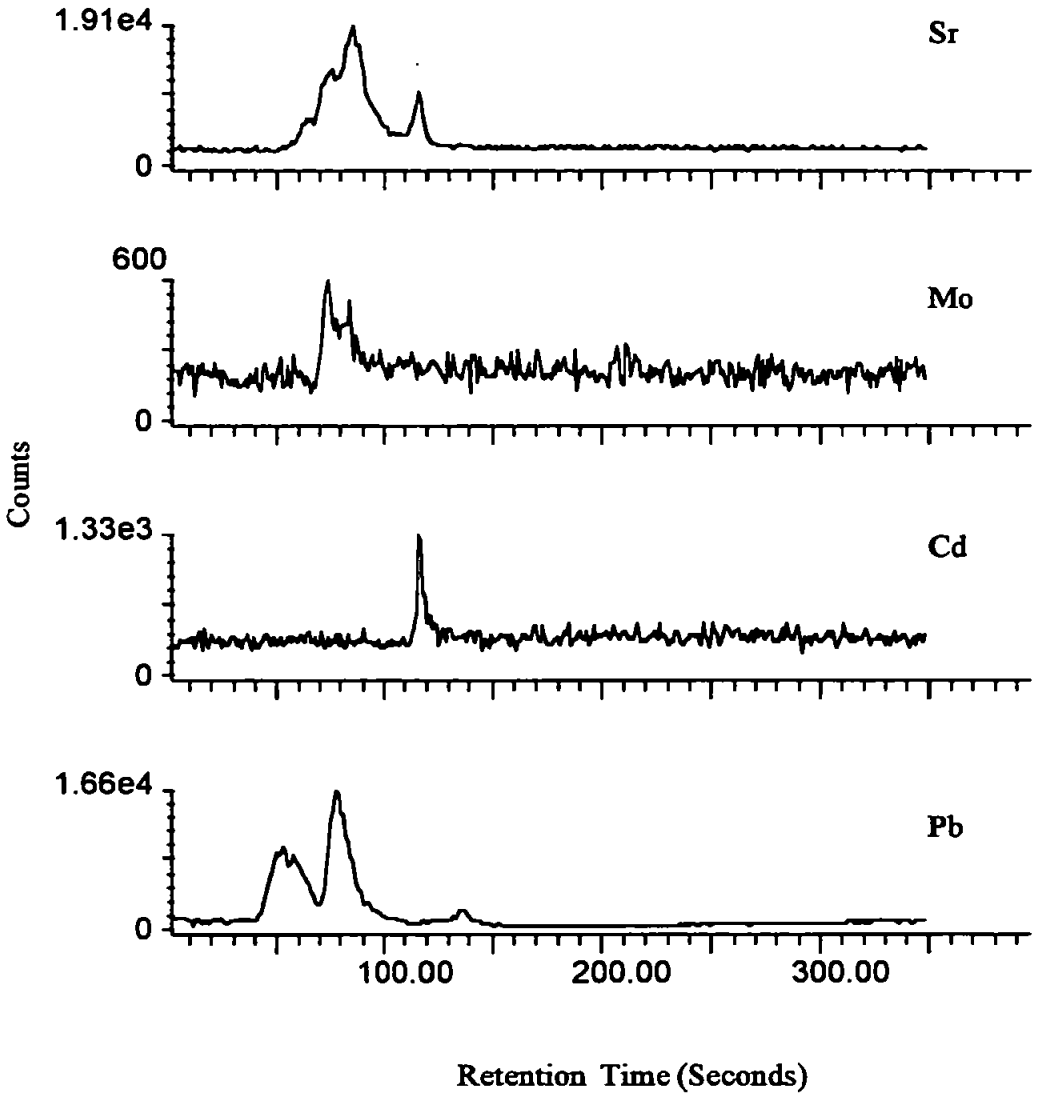
Chromatographic Conditions: A 150 x 4.6 mm id analytical column packed with Hamilton PRP-1 was used. A 1cm guard column was fitted packed with the same stationary phase. An isocratic elution system was employed, eluent 1 was 2% aqueous acetic acid, and eluent 2 was acetonitrile. The eluent composition employed was 50:50 eluent 1: eluent 2 , with a flow rate of 1.0ml min-1.

Figure 6.22: Optimisation of Chromatography to Speciate Mn, Ni and Zn and Cu in Yunnan Pu-Erh green China tea



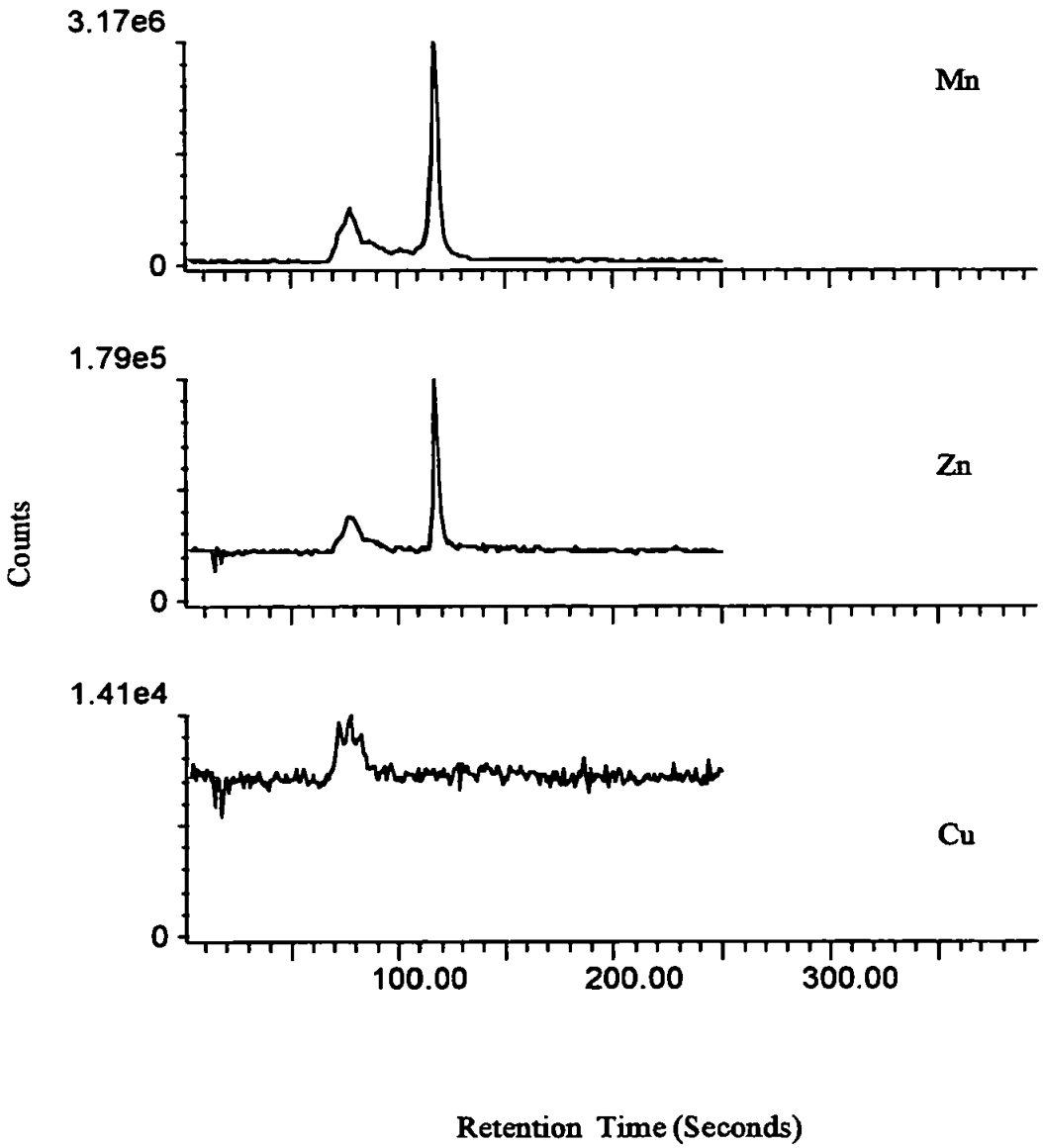
Chromatographic Conditions: A 150 x 4.6 mm id analytical column packed with Hamilton PRP-1 was used. A 1cm guard column was fitted packed with the same stationary phase. An isocratic elution system was employed, eluent 1 was 2% aqueous acetic acid, and eluent 2 was acetonitrile. The eluent composition employed was 30:70 eluent 1: eluent 2 , with a flow rate of 1.0ml min-1.

Figure 6.23: Speciation of Sr, Mo, Cd and Pb in English Breakfast Tea with the optimised HPLC conditions



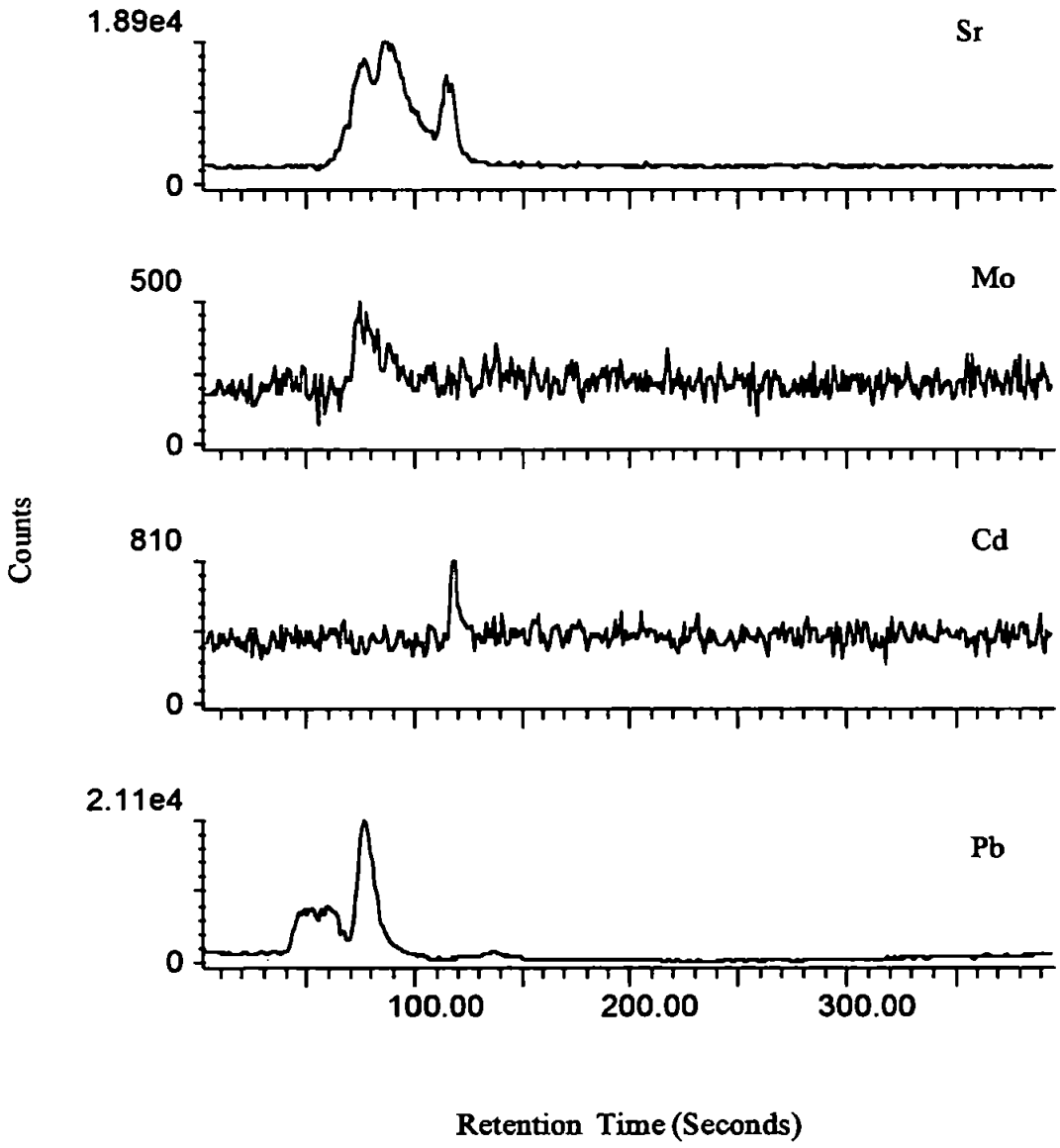
Chromatographic Conditions: A 150 x 4.6 mm id analytical column packed with Hamilton PRP-1 was used. A 1cm guard column was fitted packed with the same stationary phase. An isocratic elution system was employed, eluent 1 was 2% aqueous acetic acid, and eluent 2 was acetonitrile. The eluent composition employed was 90:10 eluent 1: eluent 2 , with a flow rate of 1.0ml min-1.

Figure 6.24: Speciation of Mn, Zn and Cu inEnglish Breakfast Tea with the optimised HPLC conditions



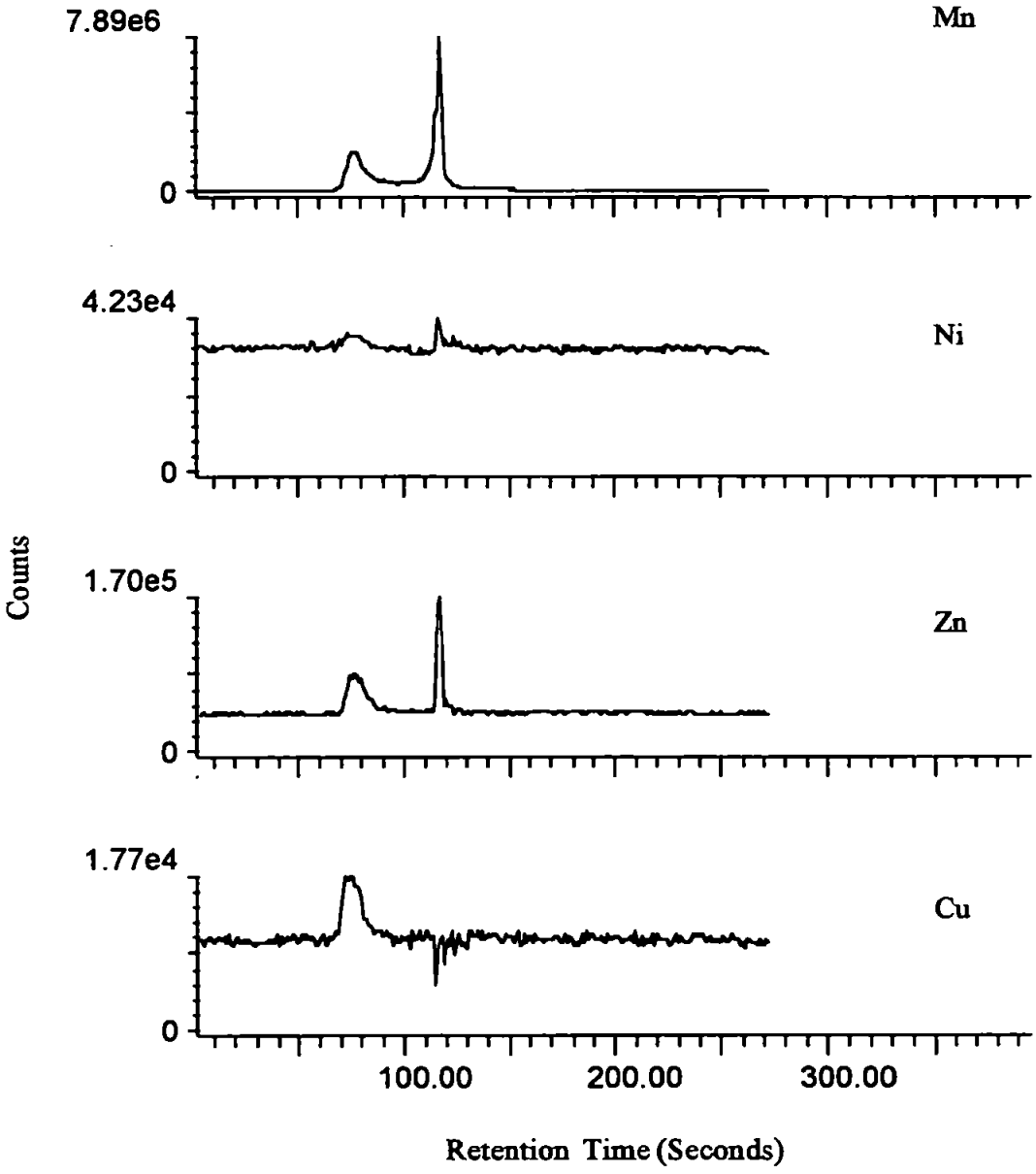
Chromatographic Conditions: A 150 x 4.6 mm id analytical column packed with Hamilton PRP-1 was used. A 1cm guard column was fitted packed with the same stationary phase. An isocratic elution system was employed, eluent 1 was 2% aqueous acetic acid, and eluent 2 was acetonitrile. The eluent composition employed was 90:10 eluent 1: eluent 2 , with a flow rate of 1.0ml min-1.

Figure 6.25: Speciation of Sr, Mo, Cd and Pb in Kenyan Tea with the optimised HPLC conditions



Chromatographic Conditions: A 150 x 4.6 mm id analytical column packed with Hamilton PRP-1 was used. A 1cm guard column was fitted packed with the same stationary phase. An isocratic elution system was employed, eluent 1 was 2% aqueous acetic acid, and eluent 2 was acetonitrile. The eluent composition employed was 90:10 eluent 1: eluent 2 , with a flow rate of 1.0ml min-1.

Figure 6.26: Speciation of Mn, Ni, Zn and Cu inEnglish Breakfast Tea with the optimised HPLC conditions



Chromatographic Conditions: A 150 x 4.6 mm id analytical column packed with Hamilton PRP-1 was used. A 1cm guard column was fitted packed with the same stationary phase. An isocratic elution system was employed, eluent 1 was 2% aqueous acetic acid, and eluent 2 was acetonitrile. The eluent composition employed was 90:10 eluent 1: eluent 2 , with a flow rate of 1.0ml min-1.

Figure 6.27: A chromatogram of English Breakfast tea with UV detection at 280 nm

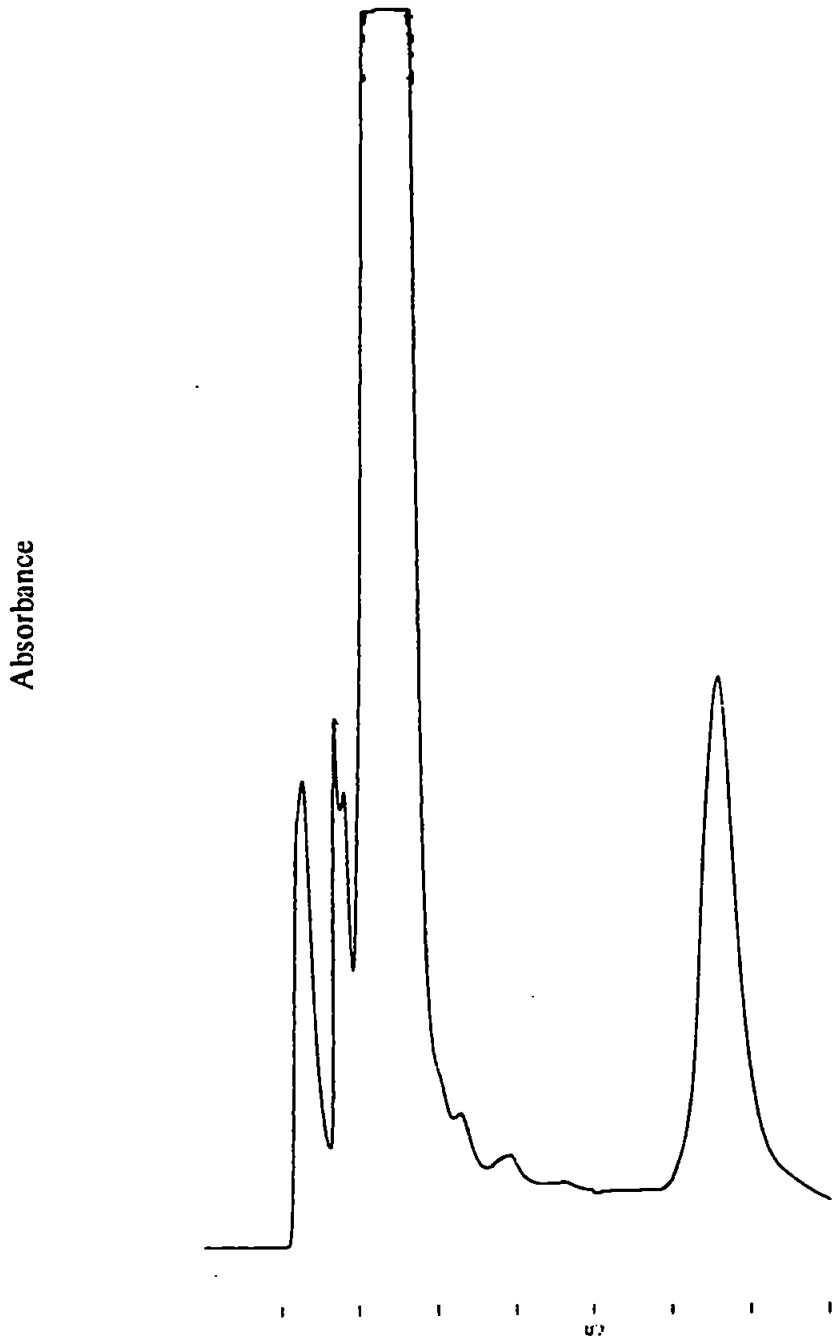


Figure 6.28: A chromatogram of Yunnan Pu-Erh tea with UV detection at 280 nm

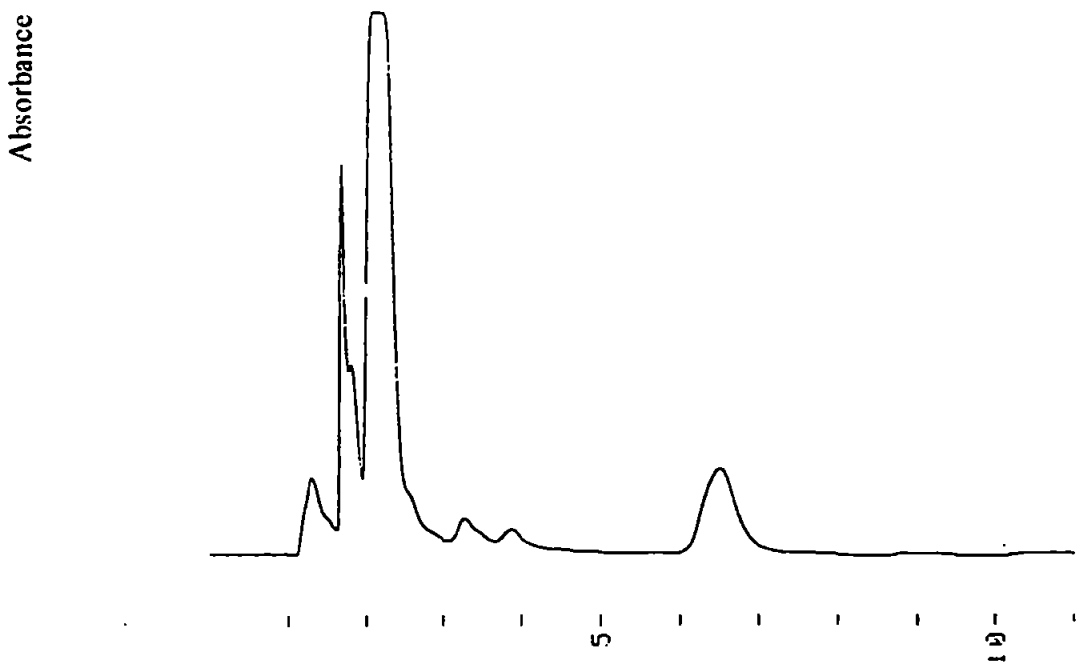
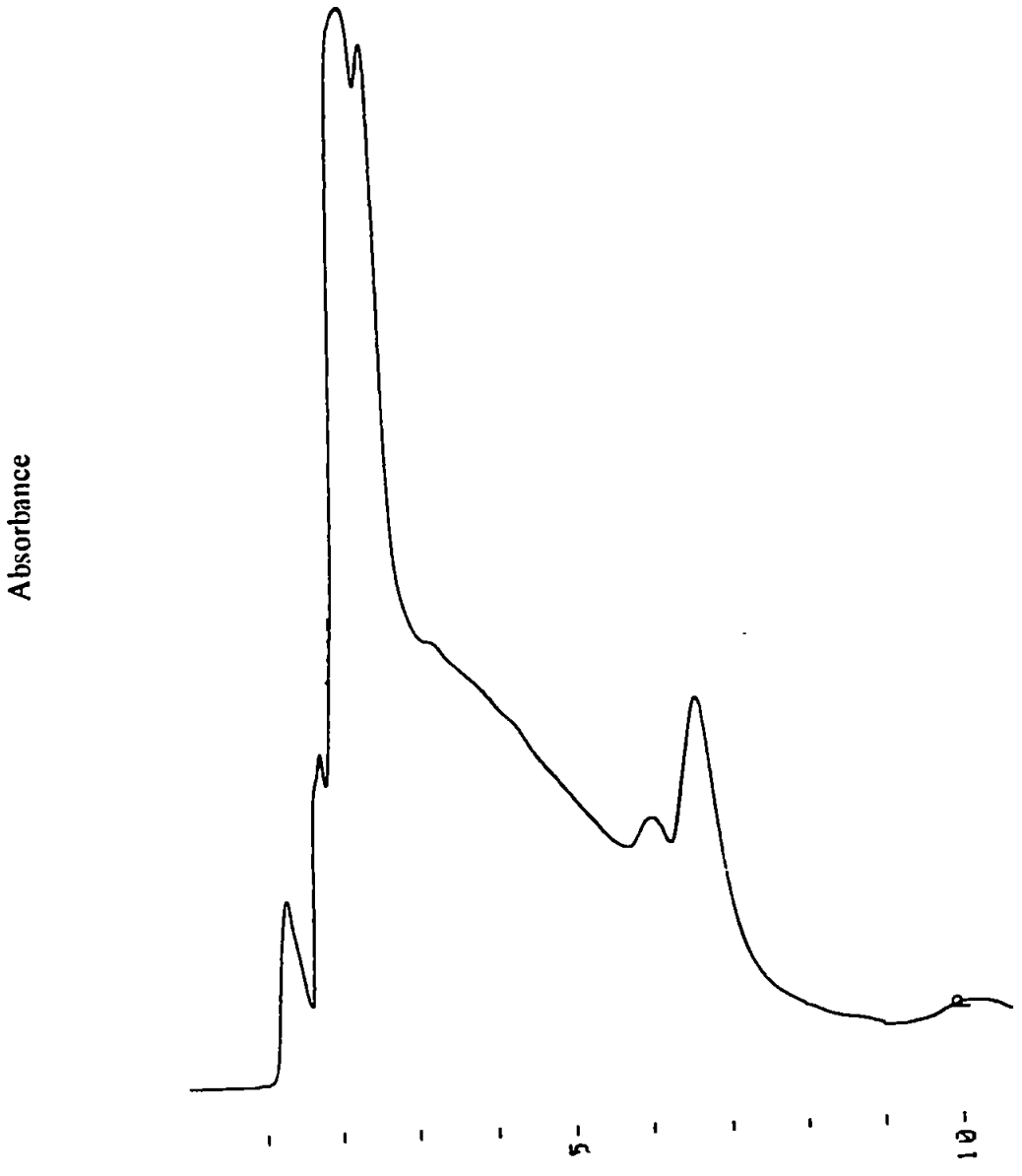


Figure 6.29: A chromatogram of Kenyan tea with UV detection at 280 nm



6.4 Conclusions

To successfully speciate metals in tea, it is necessary to eliminate any ionic interactions the metals may have with the stationary phase thus destroying any association a metal may have had with a particular flavour component in tea. This can be achieved by using a polymer based instead of silica based stationary phase. This prevents the metals associating with the free silanol groups.

The optimal chromatographic conditions for separating the organometallic are not those optimal for the separation of the organic components. This may make the positive identification of the organic components with which the trace metals are associated problematic. This study has, however. shown that coupled HPLC-ICP-MS is a powerful technique for demonstrating the presence of organometallic complexes amongst the flavour components of tea. Further work may elucidate the significance of these complexes for tea chemistry.

Chapter 7

7.0 Conclusions

The priniciple aim of this study, to produce and fully characterise a versatile interface to enable the use organic solvents with coupled HPLC-ICP-MS has been achieved. The interface developed has enabled the introduction of a range of organic solvents including acetonitrile into an ICP. In addition it facilitates the use of solvent gradients without the need to compensate for solvent loading of the plasma, and thus removes past restrictions on the chromatography employed in metal speciation studies.

The use of a heated cyclone spray chamber to produce a vapour improved the transport efficiency of pneumatic nebulisation from about 2-3% to up to 20%. The use of a hydrophilic membrane drier removed a significant proportion of the water vapour present and 58% of the total solvent vapour generated in the spray chamber. The removal of the water vapour facilitated the use of a low temperature condensation stage by preventing the build up of ice which blocks the aerosol path to the plasma. This condensation staged removed a further 74% of the remaining solvent to give a total desolvation efficiency of 89%. It was this high desolvation efficiency that consequently allowed the use of acetonitrile solvent gradients with ICP-MS detection in the analysis of a novel chemotherapy drug JM-216.

The use of a platinum specific detector to determine the metabolites of platinum based chemotherapy drugs is a useful tool since the metabolites no longer possess the chromophore that allowed the spectrophotometric detection of the drug and its impurities. Prior to the

development of the interface described in this work, the analysis was carried out by coupled HPLC-ETV-AAS. This approach was time consuming since fractions of the eluant had to be collected and then analysed individually off-line. The methodology reported in this thesis uses ICP-MS detection on-line and results in a considerable time saving as well as improving the available detection limits from 5ng ml^{-1} to 0.6ng ml^{-1} as platinum in the metabolites.

The applicabilty of the interface to tackle other analytical problems was also evaluated by employing it in the speciation of organolead and organotin compounds, as well as in a study to determine the possible speciation of metals in tea.

The use of desolvation in the speciation of organolead compounds was unsuccessful in that, it is believed that a salt was formed between the organolead compound and the eluent. Once formed this new species has a greater affinity for the walls of the desolvation device than the organolead compounds alone. As a result a 50% drop in signal was observed which seriously compromised the limit of detection. For the speciation of organolead compounds, which do not require a solvent gradient, it was found that it was best to use a cooled double pass spray chamber.

The use of desolvation in the speciation of organotin compounds however offered two practical advantages. Firstly, the reduced solvent loading of the plasma resulted in the easier coupling of HPLC with the plasma. The reflected power was reduced from almost 30 watts to 5W, and thus alleviated the problem of activating safety trips (> 30W) and shutting down the instrument. The second advantage was that coupled to the use of a sheath gas, build up of salts

in the injector of the plasma torch could be prevented, and thus negated the need to regularly clean the torch, which again necessitates shutting down the instrument.

Finally, the ability of the interface to facilitate the use of organic solvent gradients, with ICP-MS detection, enabled a viability study to investigate the speciation of metals in tea. The flavanoids in several tea samples were separated by HPLC and then coupled to ICP-MS detection to indentify any correlation between the metals and the flavanoids present. Although only a preliminary study the use of a polymer based column and optimisation of the chromatography with respect to metal speciation showed that metals were indeed associated with the flavanoids present in tea liquour.

In conclusion the use of desolvation has been shown to enhance the flexibility of ICP-MS as a detection method for HPLC. With desolvation, it is possible to optimise the chromatographic separation without regard for the effect of the chosen eluent on the analytical performance of the detection system. This is a great advantage to the chromatographer, and should improve the applicability of ICP-MS to speciation problems in the future.

7.1 Suggestions for Future Work

During the course of this study a number of areas have been indentified for further study. Firstly the performance of the interface could potentially be improved by a number of methods. The nebulisation efficiency could be improved by using either a thermospray or ultrasonic nebuliser. The use of these devices in conjuction with a heated spray chamber would however result in a larger quantity of solvent vapour. This could be countered by improving the

efficiency of the membrane drier. One possiblity could be to obtain a membrane drier encased in a steel holder. This could be conductively heated along its entire length to a temperature closer to that of the maximum operating temperature of the membrane. At the present time, the membrane temperature is limited to about 70°C by the outer casing which starts to melt above this temperature. The membrane however can be operated at temperatures up to 100°C without any adverse effect. This would improve the desolvation efficiency considerably, since it would prevent condensation occuring in the membrane preventing the passage of vapour through the membrane. A further weakness of the present design is that not all the membrane is heated to the maximum temperature due to the directional nature of the infra-red lamps. This results in both hot and cold spots where the desolvation efficiency is high and low respectively. Complete heating of the membrane would result in its entire length working at maximum efficiency.

Insulation of the condenser stage of the interface would facilitate its operation at lower temperatures. Reduced condenser temperatures result in a higher condensation rate and thus lead to a greater desolvation efficiency. Another advantage of this modification would be a reduction in the amount of solvent vapour as opposed to aerosols reaching the plasma.

A number of areas of improvement can also be indentified to further optimise the performance of the interface for the applications described. For example, further work is necessary to indentify the exact cause of the loss of signal during the analysis of organolead compounds. The chemical nature of the salt formed needs to be identified since this would dictate the affinity for glass and plastic. It may be possible to reduce this effect by using a plastic spray chamber and a larger bore membrane to reduce collisions of the analyte with the walls of the membrane.

The speciation of organotin compounds by HPLC is an analytical problem that has yet to be adequately solved. The use of mixed mode columns with a different capacity and selectivity to the cation exchange columns being used may provide a possible solution. Alternatively the use of micellular chromatography with less hydrophobic columns may provide a better separation than that achieved on the phenyl column used in this work and would benefit from further investigation.

Having achieved a separation of the metal containing components in tea by HPLC-ICP-MS, the flavanoids that are associated with the metals need to be indentified. It may be possible to do this using a different detection method such as a diode array UV absorption detection, or by coupling HPLC with mass spectrometry.

Possible future applications of the interface could include the detection of the metabolites of other metal containing drugs such as gold in arthritus drugs.

Environmental problems that are only now starting to be investigated, such as the presence of platinum in the environment due to the use of catalytic converters could benefit from the use of this interface. At the moment the chemical form of platinum from this source is not known, if its speciation in the environment could be elucidated, the scale of the problem could be ascertained. If it remains in the inorganic state where it is not bioavailable, then the problem is not serious. However if it complexes with the oils and other organic materials associated with the combustion engine, forming organoplatinum species, the problem could be very great as organoplatinum compounds are known to have a biological effect.

Chapter 8

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