Analysis of Aqueous Matrices Using Supercritical Fluid Extraction in Conjunction with Chromatographic, Spectroscopic and Mass Spectrometric Techniques.

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A thesis submitted in partial fulfilment of the requirements
for the degree of Doctor of Philosophy in the
University of Glamorgan

2004

CERTIFICATE

I hereby certify that the work described in this thesis is the result of my own investigations except where otherwise indicated.

Signed.....Candidate

Signed .....Director of Studies

DECLARATION

I hereby declare that this work has not already been accepted for any degree and is not concurrently submitted in candidature for any degree.

Signed.....Candidate

ACKNOWLEDGEMENTS

Firstly I would like to take this opportunity to thank Dr E.D. Ramsey for his support, guidance and friendship, and for giving me access to his wide-ranging knowledge and experience, and also his time which was freely given and greatly appreciated.

I would also like to record my thanks to Brian Evans and Ieuan Davies for their expert technical support throughout this work.

Only when the work was near completion and the enormity of the task become apparent did I realise the demands that had been made on my family. Without their continual support, patience and encouragement the completion of this work would have proved impossible.

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ABSTRACT

The use and supply of various organic solvents for analytical chemistry is coming under scrutiny due to their environmental impact. International legislation now prevents the supply of "ozone depleting" solvents for laboratory purposes. Consequently alternative analytical methods to those which had previously relied upon the use of a range of organic solvents need to be developed. Supercritical fluids exhibit some properties associated with gases and liquids, and in particular their solvating characteristics are equivalent to a wide range of conventional organic solvents. Environmentally benign carbon dioxide in its supercritical fluid state can be utilised to imitate the solvating power of a range of organic solvents from non polar pentane through to more polar pyridine.

There are many reports detailing the use of supercritical fluid extraction for the isolation of target compounds from a very wide range of solid matrices. The objective of these studies was to develop direct liquid supercritical fluid extraction procedures using carbon dioxide to isolate, concentrate and quantify target analytes from aqueous media. These investigations involved using off-line and on-line supercritical fluid extraction procedures with final analytical detection and quantification being accomplished using a range of chromatographic, spectroscopic and mass spectrometric methods.

Within chapters 2 and 3, an alternative infrared method for determining the quantity of oil in process and discharge waters is described. The results of quantification studies involving various oils and hydrocarbons indicate that the custom built supercritical fluid extraction system developed for these investigations directly coupled with an infrared spectrometer provide an alternative method to traditional liquid-liquid extraction procedures that involve the use of ozone depleting and/or toxic organic solvents.

Chapter 4 describes how direct aqueous supercritical fluid extraction was used to continuously isolate free testosterone as it was liberated during the enzymic digest of a testosterone-β-D-glucuronide solution incubated with *Helix pomatia* glucuronidase. Other studies described within this chapter that also involved the use of direct aqueous supercritical fluid extraction with off-line gas chromatography-mass spectrometry describe procedures for determining trace levels of organophosphate pesticides and polyaromatic hydrocarbons in aqueous samples.

The development and use of on-line direct aqueous supercritical fluid extraction coupled with supercritical fluid chromatography-mass spectrometry using atmospheric pressure chemical ionisation for the analysis of phenols at the ppb level is described in Chapter 5.

Chapter 6 describes the use of direct aqueous supercritical fluid extraction coupled on-line with liquid chromatography-mass spectrometry using ammonia chemical ionisation for the analysis of three veterinary drugs at the ppb level. Results of off-line and on-line studies involving electrospray tandem mass spectrometry with high pressure liquid chromatography for the analysis of a range of ionophores at the low ppb level following their isolation using direct aqueous supercritical fluid extraction are also presented.

Table of Contents

Chapter 3

Hydrocarbons Quantification

Abbreviations

APCI	Atmospheric pressure chemical ionisation
ASME	American society of mechanical engineers
CFC	Chlorofluorocarbon
CI	Chemical ionisation
DAD	Diode array detector
DCP	Dichlorophenol
EI	Electron impact
EPA	Environmental protection agency
FAO	Food and agriculture organisation
Freon 113	1,1,2-trichloro-1,2,2-trifluoroethane (TCTFE)
FTIR	Fourier transform infra-red
GC	Gas chromatography
GC-MS	Gas chromatography Mass spectrometry
HMSO	Her Majesties Stationary Office
HPLC	High performance (pressure) liquid chromatography
IMCO	Intergovernmental maritime consultative organisation
IOC	International Olympic committee
i.d.	Inner diameter
IR	Infra red
K	Kelvin
LC	Liquid chromatography
LC50	Median lethal concentration
LLE	Liquid liquid extraction
LT_{50} / LT_m	Median lethal time
MPa	Mega pascal (10^6 pascals)
OCP	Organochlorine pesticide
o.d.	outer diameter
ODS	Octadecyl silane
OPP	Organophosphorus pesticide
Pa	Pascal (N m^{-2} or kg m^{-1} s^{-2})

PAH	Polycyclic aromatic hydrocarbon
Pc	Critical pressure
PCB	Polychlorinated biphenol
PCP	Pentachlorophenol
PEEK	Poly(ether-ether-ketone)
psi	pounds per square inch
PTFE	Polytetrafluoroethene
S/N	Signal to noise
SF	Supercritical fluid
SFC	Supercritical fluid chromatography
SFE	Supercritical fluid extraction
SIM	Selective ion monitoring
SOP	Standard operating procedure
SPE	Solid phase extraction
Tc	Critical temperature
TPHs	Total petroleum hydrocarbons
TSP	Thermospray
UNESCO	United nations educational, scientific and cultural organisation
UV	Ultra violet
UV/Vis	Ultra violet / visible
VOC	Volatile organic compound
WHO	World health organisation

Introduction to Supercritical Fluid Extraction

1.1 Supercritical Fluids

1.1.1 Historical developments of supercritical fluids.

Supercritical fluids were first "discovered" by Cagnaird de la Tour[1] in 1822. The solvating properties of supercritical fluids were first documented by Hannay and Hogarth[2-4] in 1879 and 1880, and in 1954, Francis[5] published a paper on the extraction potential of supercritical carbon dioxide for a variety of organic and inorganic compounds.

In 1961 Klesper[6] was the first to demonstrate supercritical fluid chromatography, and built a system which separated porphyrins using chlorofluoroalkanes as the mobile phase. The technique was referred to as "high pressure chromatography" and it was not until Sie and Rijners[7] in 1967 that this technique was referred to as supercritical fluid chromatography (SFC).

In 1964 Zosel[8] applied for a patent for the use of supercritical fluids in process engineering, and later constructed a plant to decaffeinate coffee by the technique.
Analytical supercritical fluid extraction (SFE) did not occur until the mid 1980's, and before 1986 only two papers on SFE had been recorded in Chemical Abstracts.

1.1.2 Properties of Supercritical Fluids

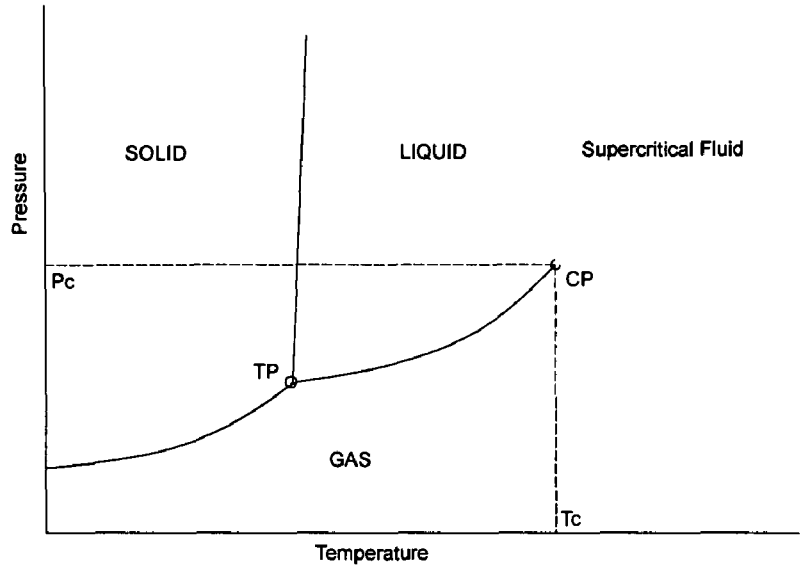

Fig 1.1 Phase diagram of a single substance

Any pure substance, which is above its critical temperature (Tc) and critical pressure (Pc), is considered to be in a supercritical state.

Fig 1.1 represents a generalised phase diagram for a single substance / component, if we move along the liquid, gas equilibrium boundary curve from TP (the triple point) to CP (the critical point), increasing both temperature and pressure, the liquid becomes less dense due to thermal expansion, and the gas becomes more dense due to the increasing pressure. At the critical point the densities of the two phases become identical and the distinction between gas and liquid disappear and the substance becomes a supercritical fluid.

The most frequently used compound, for supercritical fluid applications, is carbon dioxide, which has a critical temperature (Tc) = 304.1 K (31 °C) and a critical pressure (Pc) = 1078 psi (7.38 MPa). Fig 1.2 shows the variation of the density of carbon dioxide with pressure and temperature. Appendix A shows the critical parameters for other fliuids.

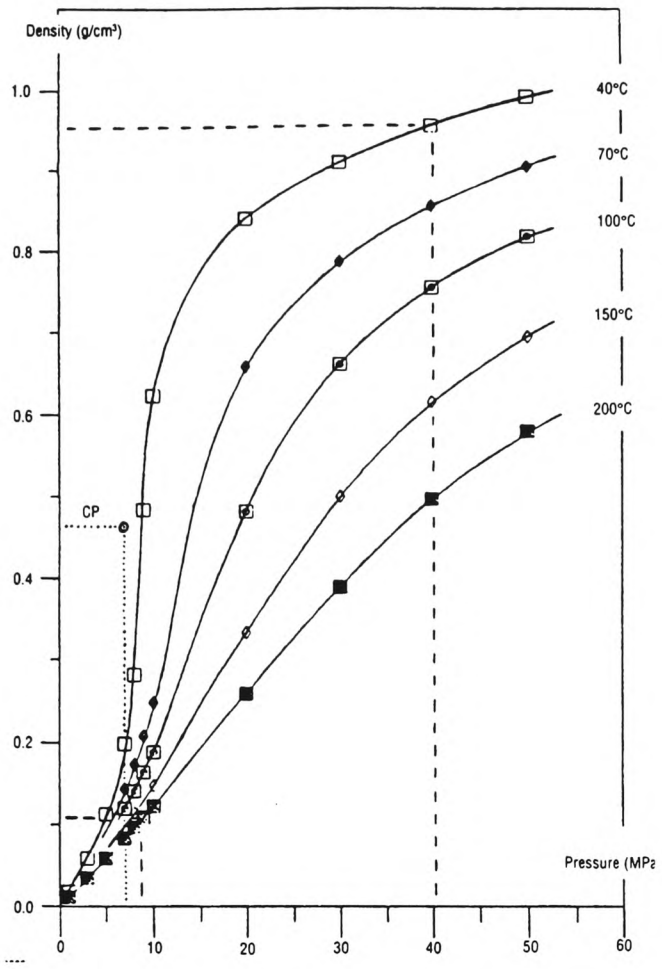

Fig 1.2 Density variation of carbon dioxide versus temperatures and pressure [9-11]

Carbon dioxide has several favourable attributes which make it popular for supercritical fluid extraction

a. relatively low critical temperature (304.1 K)

b. relatively low critical pressure (1078 psi)

c. unreactive and prevents oxidation during use

d. low toxicity

e high purity at low cost

Supercritical fluids have physical attributes between those of typical liquids and gases

a. the viscosity – more gas like

b. the diffusivity – more gas like

c. the density is more characteristic of a liquid

and so contribute to greater mobility and mass transfer during extraction, increasing extraction rates.

	Density (10^3 kg m^{-3})	Viscosity (mPa s)	Self-diffusion coefficient (10^4 m^2 s^{-1})
Gas 303 K, 1 atm	(0.6-2) x 10^3	(1-3) x 10^2	0.1-0.4
Supercritical fluid Near T_c P_c Near T_c $4P_c$	 0.2-0.5 0.4-0.9	 (1-3) x 10^{-2} (3-9) x 10^{-2}	 0.7 x 10^{-3} 0.2 x 10^{-3}
Liquid 303 K, 1 atm	0.6-1.6	0.2-3	(0.2-2) x 10^{-5}

Table 1.1 Typical physical property values for gases, liquids and supercritical fluids [12]

For liquid solvents, their solvent strengths change very little with changing extraction conditions, this is not the case for a supercritical fluid, where its density and solvating power can be changed by altering the temperature and pressure in the extraction zone. Although increasing the pressure increases the density of the supercritical fluid, increasing the temperature has the converse effect.

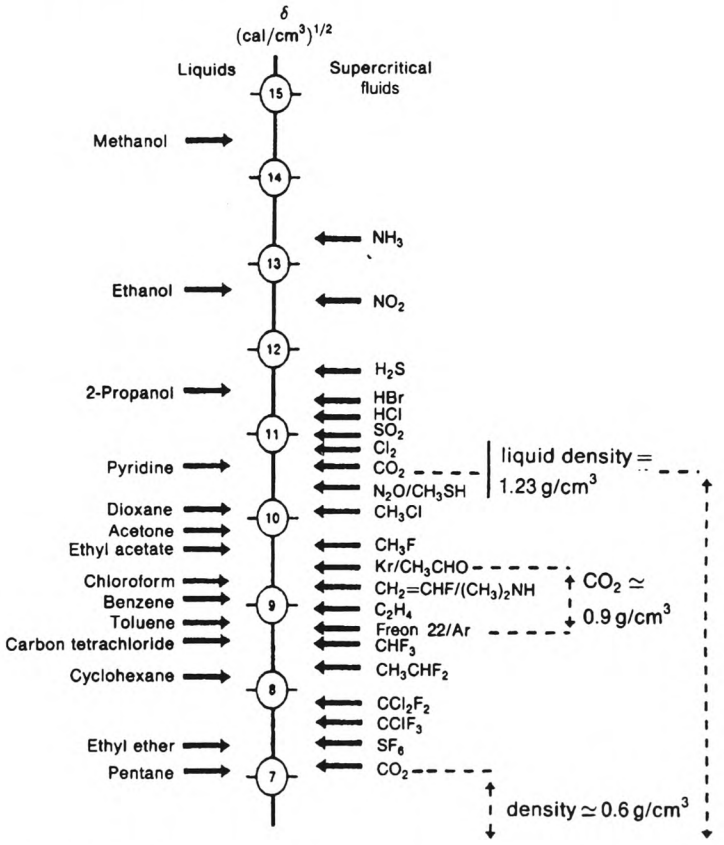

Fig 1.3 Solvating potential of different supercritical fluids [13]

Fig 1.3 illustrates that the solvating characteristics of supercritical carbon dioxide can be controlled such that it can be used to imitate a wide range of organic solvents from pentane through to pyridine.

This ability to alter the density, and so solvating power, of the supercritical fluids can be used beneficially to preferentially isolate, in theory, an analyte from a mixture, although in practice this can be very difficult to achieve without the addition of a chromatographic separation stage.

Carbon dioxide is the most widely used supercritical fluid for the reasons outlined earlier, as the molecule is non-polar it is classified as a non-polar solvent, although it has a limited ability to solvate polar solutes, and can be used as a solvent for most organic molecules.

If extraction of more polar solutes is required it is common practice to add a small quantity of a modifier component to the carbon dioxide. An example of an organic modifier for this purpose would be a lower alcohol, but it is important to ensure that the modified supercritical fluid remains as a single phase. Phase diagrams of many binary mixtures have been determined as a three dimensional plot of pressure, temperature and mole fraction. [14]

1.2 SFE Instrumentation

All preparative SFE systems generally have the same basic components,

a. Source of solvent
b. Pump delivery system
c. Heated extraction vessel
d. Restrictor module (although not for SFE infrared analysis for oil in water)
e. Analyte collection system.

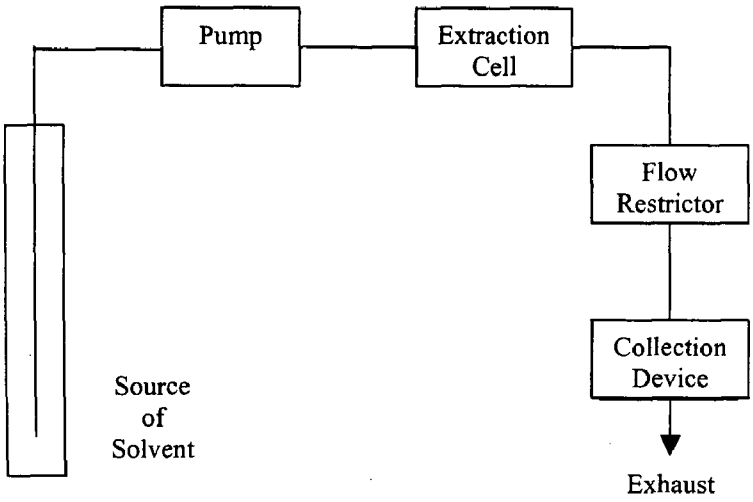

Fig 1.4. Configuration of off-line SFE system

1.2.1 Source of solvent

As already stated, the most frequently used solvent for supercritical extraction is carbon dioxide, this can be supplied to the pump system from a standard gas cylinder with a liquid take off head.

Usage of other solvents such as nitrous oxide (N_2O), which is a more polar solvent, have been reported. A few examples of analytical SFE include:
Polyaromatic hydrocarbons (PAHs) from sediments,[15] pharmaceutical products from animal feed,[16] phenolic nicotine and other related compounds from cigarette ash,[17] aliphatic and aromatic amines. [18-19]

One reported problem with nitrous oxide as a solvent, is its ability to act as an oxidant, which can explosively oxidise organic material.[20] Its use should be avoided, and a warning concerning its use has been reported.[21]

Other reported solvents include chlorodifluoromethane ($CHClF_2$), Camel *et al,* [22] Hawthorn *et al,* [23] and trifluoromethane (CHF_3).[24-26] The use of chlordifluoromethane has now been stopped in line with the phase out of chlorofluorocarbons (CFCs). Trifluoromethane as a solvent was noted to be less efficient for extraction than chlorodifluoromethane or methanol modified carbon dioxide.

The use of sub-critical water has been reported by Yang *et a l*[27] for the extraction of polychloronated biphenyls (PCBs) from environmental solids, the polarity of sub-critical water is strongly influenced by temperature.
The main problem of SFE using SF-H_2O is the range of analytes that can be extracted. They should not be thermally labile at the temperatures concerned (> 523 K) and there is difficulty in handling the extracts, due to dilution by water.
Supercritical water is corrosive, and the range of analytes which are extractable is limited, this makes it of little practical use as a solvent.

1.2.2 Pump delivery system

The pump delivery system must be capable of providing a liquid to be converted into a supercritical fluid at constant pressure in the range of 1450 to 11600 psi, and at sufficient total volumes (up to 500 cm^3) and flow rates(up to 25 cm^3 min^{-1}).
Unlike HPLC pump systems, for SFE there must be a capability of delivering compressible liquids under differing pressures and flow rates reproducibly.

Along with the primary pump, a secondary pump can also be included into the system to deliver the modifier solvent, if and when required.
The pump should ideally have a feed back system connected to the restrictor module to allow control of the input pressure and flow.

1.2.3 Syringe pumps

Early work in the field of supercritical fluids relied upon the use of modified syringe pumps. These modifications allowed greater accuracy and precision, with enhanced mechanical performance. The materials used for the piston seals also had to be modified to resist ingression of the supercritical fluids into the seal, and subsequent distortion resulting in failure of the delivery system.

A further problem was the leaching of the seal material into the supercritical fluid, with the resultant contamination of the collected analyte. The material used in most applications is now graphite filled Teflon®.

The advantage of the syringe pump is the stability through a wide range of flow rates, from $\mu l\ min^{-1}$ to 90 $cm^3\ min^{-1}$, while the major limitation was originally the volume of SF which could be provided. This problem was subsequently resolved by the addition of a second syringe pump, which could be filling while the first was delivering, and connected by a continuous flow connection, to ensure uninterrupted flow.

1.2.4 Reciprocating piston pumps

As with syringe pumps, HPLC reciprocating pumps can be adapted for use in supercritical fluid systems, from their original application of HPLC. These pumps were originally designed for room temperature delivery, and the pump head needed adaptation to allow liquid gases to be pumped. This required the heads to be chilled to approximately 273 K to enhance the pumping capability and also to stop cavitation and vapour formation in the pump head.

A further problem encountered with piston pumps are the pressure surges caused when the pump delivers the liquefied gases, this can be removed with the addition of a pulse dampener.

One advantage of the reciprocating piston pump over the syringe pump is the continuous delivery of supercritical fluid without the addition of a second pump, although the addition of a modifing solvent clearly requires a secondary pump.

1.2.5 Heated extraction vessel

The design of the extraction vessel will, to a great extent, depend upon the matrix being studied. The majority of reported SFE applications have, so far, been directed towards solid matrices, which is surprising considering a large proportion of environmental and biological samples are aqueous based. A further review of aqueous extraction techniques will be given later.

The initial extraction vessels used for solid phase SFE, were based on stainless steel HPLC column bodies which were able to withstand the pressures required for supercritical fluid extraction, and could be easily placed in an oven to enable SFE operation.

A stainless steel frit is retained in each end of the column, with typical pore sizes of 2–5 μm, to retain the solid sample. Typical analytical SFE procedures required internal vessel volumes to be of the order of 0.5 to 50 cm^3, with the recommendation being that the vessel volume exceeds the sample volume by a minimum of 10 %.[28]

1.2.6 Restrictor Modules

Restrictor modules or flow control devices are incorporated into the extraction system to maintain the back pressure required for supercritical fluid extraction to be achieved. Their construction must be rugged, able to operate in the temperature range required, provide a constant even flow for a wide range of modified and unmodified fluids and allow easy connection to the analyte trap.

1.2.7 Fixed flow restrictors

The original basic restrictors were constructed from crimped stainless steel HPLC tubing constructed around a heated block, to stop freezing, and plugging, occurring due to the Joule –Thompson cooling effect on expansion of the SF solvent, and of decompression along the length of the restriction.

This type of restrictor is known as a "fixed flow" restrictor, and was superseded by the use of fused silica "linear flow" capillaries.

These restrictors are fabricated from appropriate lengths of small internal diameter capillary tubing, with the restriction provided being dependent on the length and internal diameter of the capillary.

Fused silica restrictors are particularly difficult to heat consistently throughout their length, and are always prone to plugging.

Tapered restrictors Fig. 1.5, have also been prepared,[29] and although they reduce the length for decompression to occur, they require a high degree of skill to prepare, and reproducibility is a great problem.[30]

Fig. 1.5 Tapered restrictor

Frit restrictors, Fig. 1.6, where a porous frit was used to produce the required back pressure, have been developed. [31-33] Because of the reduced length where decompression takes, they are less prone to plugging compared to fused silica, but are relatively more expensive.

Fig 1.6 Frit restrictor

The development of coaxial heated stainless steel restrictors[34] Fig. 1.7, has extended the range of applications that can now use this type of restriction.

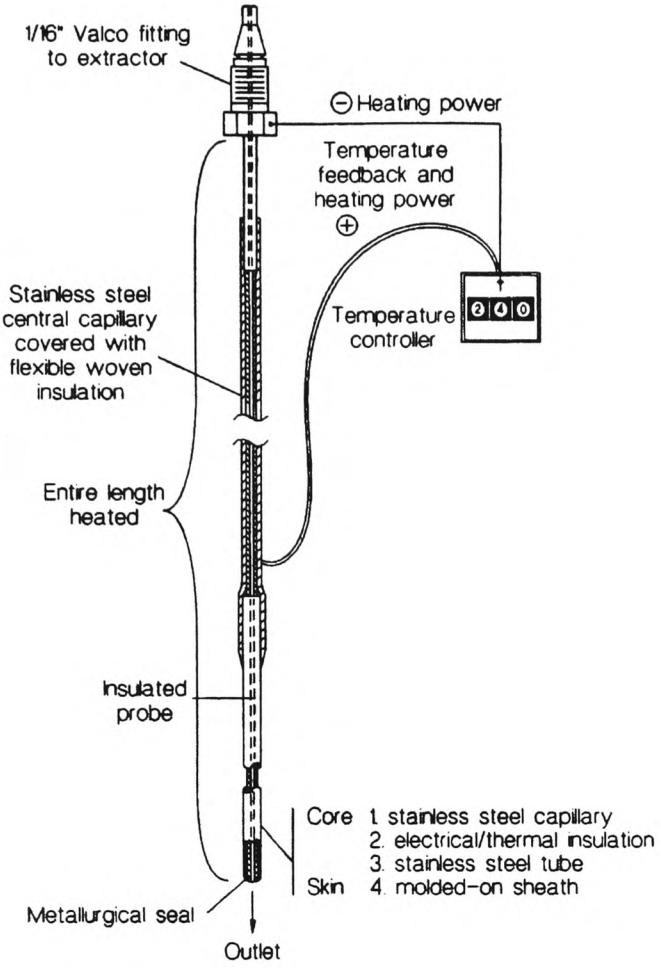

Fig. 1.7 Coaxial heated stainless-steel restrictor

1.2.8 Variable flow restrictors

Early designs for this type of restrictor was based on PEEK tubing being manually constricted by means of a clamping device Fig. 1.8, and showed an improvement over the earlier fixed restrictors. As the decompression region was short, plugging was not as problematic, as any plug could be easily removed by opening the restriction. This proved to be a cheap and easily replaced option.

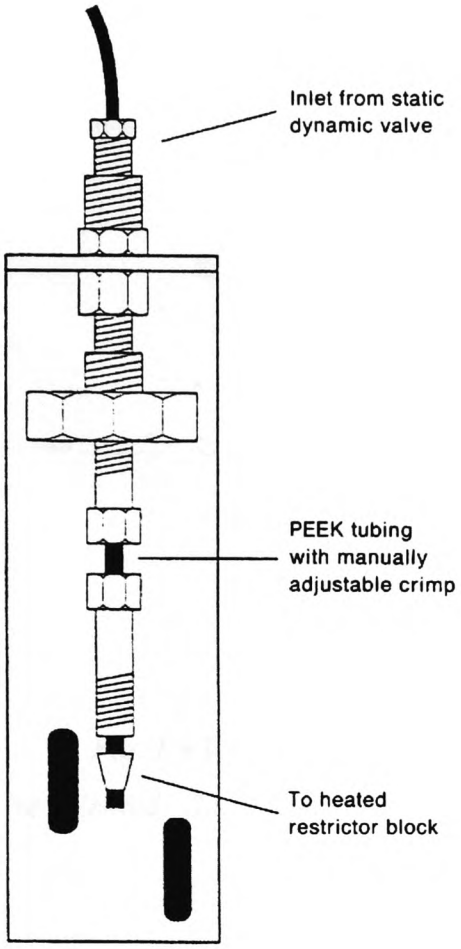

Fig. 1.8 Manual restrictor (PEEK crimped)

1.2.9 Hewlett-Packard restrictor

Hewlett-Packard[35] introduced a variable restrictor, with integrated solid phase trap. The system was based on a low dead volume solenoid valve, where the valve was computer controlled and relied on controlling the flow through an orifice and diaphragm. The restrictor was based around a variable orifice, where the diameter of the orifice can be varied by changing the position of a valve needle within a conic seating Fig. 1.9.

A pressure transducer regulates the position of a solenoid driven needle assembly. On the formation of a blockage , the orifice widens and the blockage material is blown free. As the pressure then drops, the orifice then closes to maintain the SFE target pressure.

The Hewlet Packard 7680A SFE system incorporates a variable orifice restrictor and temperature controlled sorbent trap for reduced temperature analyte trapping, which can be automatically rinsed and the fractions collected in vials.

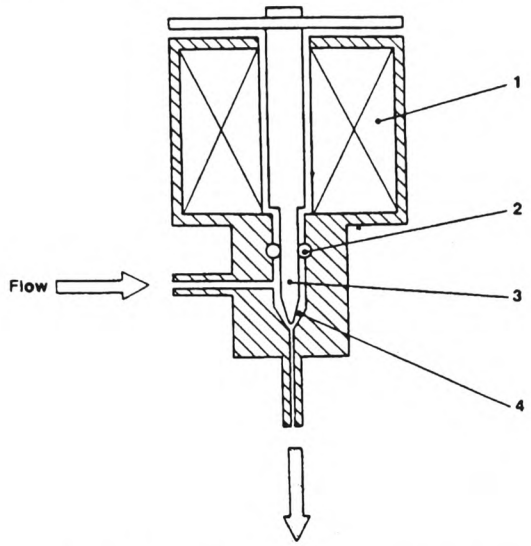

Fig. 1.9 Variable orifice restrictor

1-needle drive solenoid; 2-needle seal; 3- valve needle; 4- valve seal

1.2.10 Isco restrictor

Isco[36] developed a manual restrictor Fig. 1.10, based on a long thin needle within a heated stainless steel barrel, covered in an inert high temperature polymer. Because the whole barrel is heated, the fluid remains supercritical to the very tip where decompression occurs.

They later incorporated this manual design restrictor into a fully automated supercritical fluid extraction system, Fig 1.11.

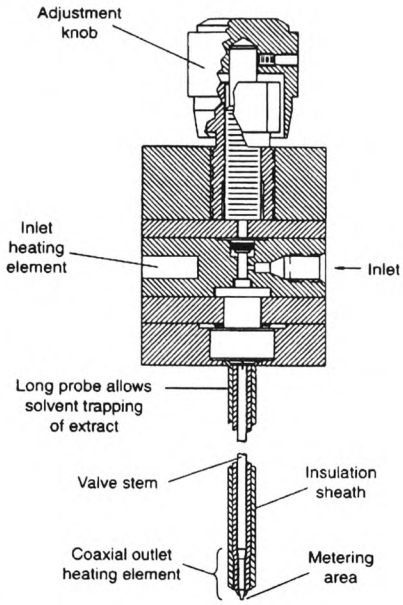

Fig. 1.10 Isco manual restrictor

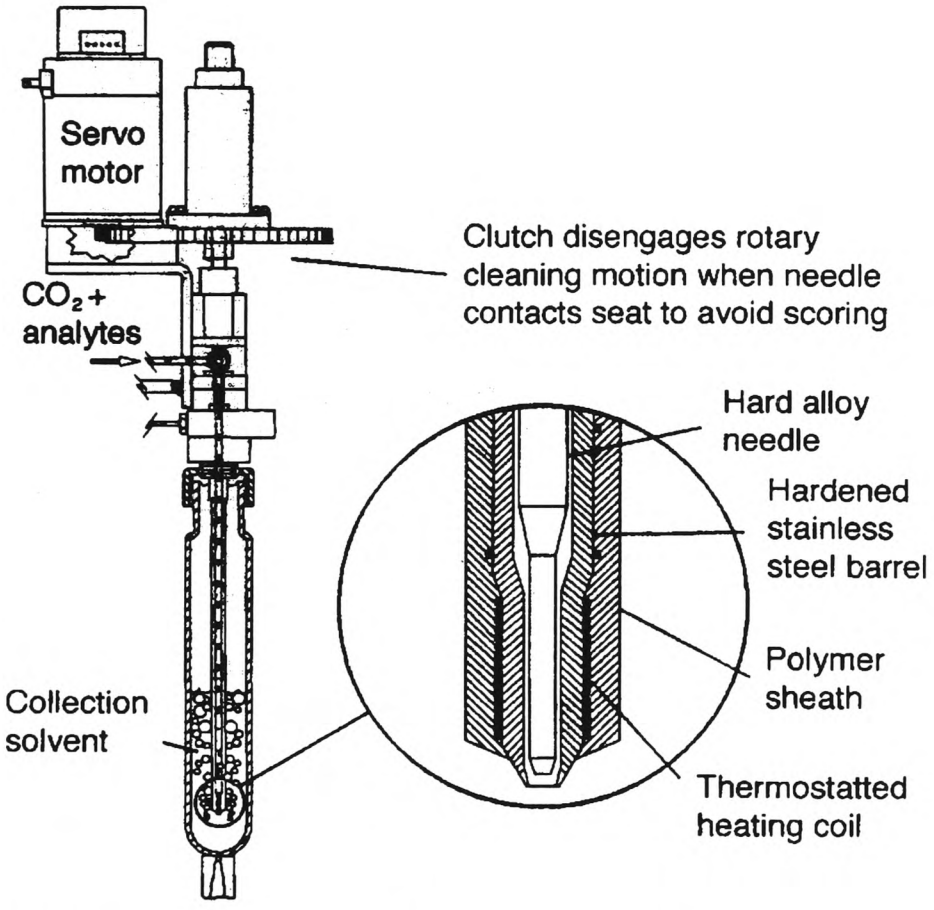

Fig. 1.11 Isco automated restrictor for direct liquid analyte trapping

1.2.11 Suprex Automated restrictor

This automated restrictor was developed by Suprex[37] and is based on a computer controlled blunt needle device, with a heated body, and can be easily interfaced to a variety of solid and liquid collection traps.

1.2.12 Analyte trapping systems

Once extraction has be achieved, quantitative reproducible analyte trapping has to be achieved. Trapping can be sub-divided into two categories,

Off-line trapping

When the whole of the analyte is trapped and physically transported to the analytical instrumentation.

On-line trapping

When the SFE system is directly interfaced to the analytical instrumentation, with the analyte being delivered to the input of the analysing device.

On-line trapping and subsequent analyte delivery is further developed under the heading of hyphenated techniques later.

1.2.13 Off-line trapping

Off-line collection broadly splits into two categories,

1.2.14 Liquid trapping

This is achieved by allowing the supercritical fluid and analyte, to decompress into a small volume of solvent, generally organic. The major problem with liquid trapping is solvent volatility, and several workers have examined the relationship between

collection solvent, [38-40] solvent depth and temperature,[41] restrictor depth,[42] and trapping efficiency.

Considerable work has been applied to the method of solvent containment, whether open or closed flask containers[43] should be used, and the use of liquid trapping for analytes, other than non-volatile or semi-volatile, was developed by Jamerson[44] where he pressurised the collection flask to 30 psi to control the bubble size and improve trapping efficiencies.

Another method of improving the trapping of analytes is described by Wenclaviak *et al*[45] on five groups of analytes, PAHs, alkanes, nitro-PAHs, fatty acid esters and chlorinated hydrocarbons. Here Fig 1.12 a Dewar condenser, held at –25 °C, connected to the top of the collection vial, containing ethyl acetate as the collection solvent. Losses were reduced from 15% to 2% m/m for this range of analytes.

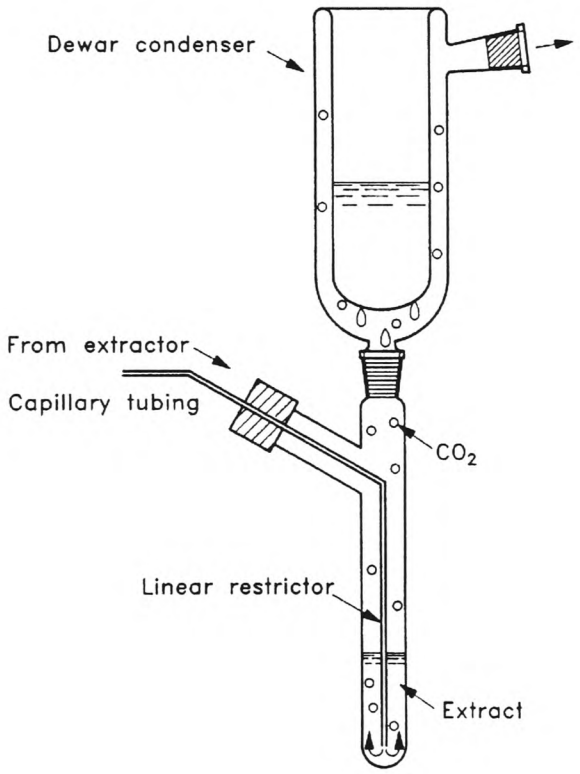

Fig. 1.12 Cryotrap

1.2.15 Solid phase trapping,

In solid phase trapping the supercritical fluid and analyte are passed through a bed of small diameter collection beads (40 – 100 μm diameter), with the beads being cooled for optimum trapping efficiencies. (A variety of different stationary phase HPLC sorbents ranging from, e.g. C_8, C_{18}, amino, to stainless steel beads have been used).

Once trapping is complete the sorbent beads are rinsed with an organic solvent, and the analyte eluted quantitatively into a collection vial.
Comparisons on the efficiencies of different sorbent traps have been made,[46-49] and for different analytes.[50-51]

1.2.16 Solventless trapping

It is sometimes possible to collect the analyte quantitatively without the use of a solvent or solid phase trap. The supercritical fluid and analyte are simply decompressed into a vial containing only glass wool. This technique has been used for fat extraction,[52] PCBs, PAHs, gasoline and diesel fuel by Miller *et al* .[53]

1.2.17 On-line trapping

On-line trapping and associated analysis by a range of analytical techniques will be reviewed later in hyphenated analytical techniques.

1.3 Solid Matrices

The versatility of the supercritical fluid extraction procedure has led to a wide number of applications being reported. [54-60]

According to Bayona,[61] to attain satisfactory and reproducible extraction profiles, sample preparation is extremely important. The sample preparation can be sub-divided into:

a. Grinding

To produce a regular particle size matrix for extraction, it was noted that if volatile analytes were present in the matrix, that the grinding should carried out at low temperatures to avoid losses.

b. Sieving

Since the extraction profile often has a kinetic contribution linked to particle size, the necessity to control this parameter by limiting the range of particle sizes in the matrix is essential.

c. Homogenising

Homogenisation, along with the previous stage of sieving, is important in obtaining reproducible results.

d. Drying

The water content of a solid sample covers a large range from trace level, to major component. At low levels of approximately 1–2%, water promoted extraction, for some matrix /analytes, but at higher levels the effects are unpredictable. To improve extraction efficiency and reproducibility, and avoid problems such as restrictor blockage due to ice formation, the samples need to be dried before extraction.

This can be achieved in several ways depending on the water content of the sample. It is extremely important to reduce the loss of volatile

components during drying, and so freezing drying has been used to reduce this problem. Freeze drying itself has inherent problems, and so the use of drying agents has been investigated, with Burford *et al*[62] publishing details on the 21 different drying agents.

Others who have reported in this field are Lee *et al,*[63] Murugaverl *et al,*[64] and Souk and King,[65] all reporting on tissue samples. While Valverde-Garcia and Fern◈ndez-Alba,[66] and Lehotay *et al*[67] reporting on drying fruit and vegetable samples.

Analytes	Matrix	Drying agent	Reference
Polychlorinated biphenyls	Fish tissue	Basic Al_2O_3	Lee *et al* (1995)[68]
Carbamates	Tissue	Hydromatrix	Murugaverl *et al* (1993)[69]
Alachlor, carbofuran, atrazine Benomyl, 2,4-dichlorophenoxyacetic acid	Meat	Hydromatrix	Souk and King (1994)[70]
Methamidophos	Vegetables	Magnesium sulphate	Valverde-Garcia and Fernandez-Alba (1995)[71]
Pesticides (40)	Fruit and Vegetables	Hydromatrix-Dry ice	Lehotay *et al* (1995)[72]

Table 1.2 Selected drying procedures used for solid sample preparation

Extraction of the analyte from the matrix, and delivery to the point of collection, or analysis, is dependent of three parameters.

a. Solute solubility

The solute must be soluble in the supercritical solvent, if low solubilities are experienced the addition of a modifier solvent may be considered.

b. Matrix

The solute, however soluble in the solvent, must have the ability to be extractable from the matrix. This may involve the interaction with, and desorption through, the matrix material, and can be the limiting factor in the extraction procedure, i.e could be strong relative to analyte/matrix interactions. It has been seen,[73-74] that although SFE is capable of extracting the target analyte from the sample, in many cases the analyte is not completely extracted. This result is observed when SFE is compared against liquid/liquid extractions, and the reason for the low extractability is given as strong analyte/matrix interactions. The analyte is therefore strongly held by the matrix, and effectively "locked" into the structure.

c. Diffusion rates

The diffusivity of the solvent into the matrix, and the solution out of the matrix, is generally not the rate limiting step. The rate of transportation will be dependent on several factors, including:

i. Matrix material – permeability, analyte/matrix interactions.
ii. Particle, or pore, size.
iii. Water content

The effects of each of the above parameters, solute solubility, matrix effects and diffusion rates, can be summarised in the following graph,

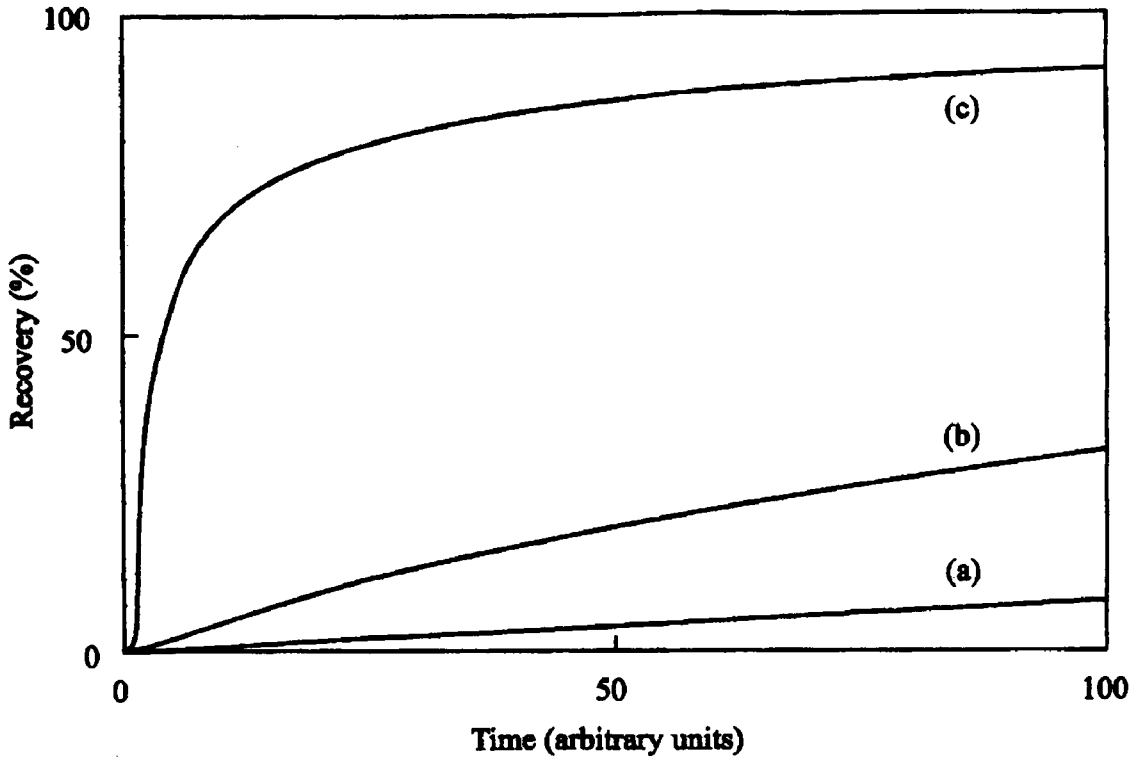

Fig 1.13 (a) diffusion controlled ; (b) significant matrix effects; (c) poor solubility of analyte[75]

1.4. Liquid Matrices

1.4.1 Introduction

Liquid matrices can be sub-divided into two broad general classifications;

i. **Aquatic samples**

These samples are typically analysed for organic pollutants .

ii. **Biological samples**

These samples are typically analysed for drugs and their metabolites.

One of the earliest reported applications for the SF extraction of organic compounds, at the parts per billion level, from an aqueous matrix, was by Ehnthold[76] in 1983.

The apparent low complexity of an aqueous matrix, compared with solid matrices, leads to a puzzling position where the majority of reported applications have involved extractions of solid matrices. This situation has arisen because of several factors:

a. Most commercial instrumentation has been developed for extraction of solid matrices.

b. The solubility of many organic compounds are large enough in aqueous solution, to prohibit their efficient extraction by SFE.

c. Restrictor icing, this proves to be a particular problem with aqueous extractions because of the co-extracted and transported water freezing in the restrictor at the point of decompression due to the Joules-Thompson cooling effect.

These restrictions, in general, have contributed to the difficulty in implementing a successful strategy for aqueous supercritical fluid extraction.

1.4.2. Practical considerations

The successful implementation of aqueous supercritical fluid extraction, will be determined by four considerations;

i. The solubility of the analyte in water and supercritical carbon dioxide (as already noted, carbon dioxide is used in the majority of applications).

ii. Whether the extraction is a direct or indirect extraction process.
Whether the analyte is to be extracted directly from the aqueous matrix, or first loaded onto a support material for extraction.

iii. The volume of aqueous medium to be extracted.
This will be directly dependent upon the concentration levels of the analyte present, and/or vessel volume limitations.

iv. Whether the extraction is to be conducted under dynamic, constant flow of fluid, or static, a fixed volume of fluid, is to be attempted.

1.4.3 Solubility of analyte.

The first criteria is the determination of the solubility of the analyte to be extracted in the supercritical fluid of choice.
Several workers have reported in this field, including Bartle *et al*[77] published detailed tables listing the solubilities of compounds in SF-CO_2, Francis[78] reported solubilities for different compounds based on their functional groups in near critical CO_2 (900 psi , 298 K), and Taylor[79] for naturally occurring compounds.

The work of Luque de Castro[13] (Fig 1.3) gives a guide to the relationship between the solvating powers of supercritical fluids and conventional solvents. Although this gives a good guide to solubility in supercritical fluids, information about the distribution coefficient of the analyte in SF-CO_2 would also be needed to design a robust SFE method.

The distribution coefficients for complex mixtures of phenols has been reported by Roop and Akgerman,[80] for chlorinated hydrocarbons by Sengupta *et al.*[81] Experience at the University of Glamorgan[82] has been that analytes which are sparingly soluble in water , and are compatible with GC can be successfully extracted from water.

1.4.4 Direct or indirect extraction

There are two modes of extraction;

a. <u>Direct extraction</u>

Where the analyte is extracted directly from the aqueous medium. This has the bonus of simplicity in not requiring the need of prior loading onto support material, but suffers from the need of a fixed volume custom built extraction vessel, and design safety considerations for the vessel.

b. Indirect extraction

Where the aqueous sample is first loaded onto a support material such as an solid phase extraction (SPE) cartridge or disk.

Koester and Clement[83] reported that, with no particulates present, extraction times for a 1 g cartridge are of the order of 1 to 2 hours per litre of solution.

These times can be reduced by the use of SPE disks, where the typical flow rates are of the order of 20 to 50 cm^3/min, because of the increased cross-sectional area of the sorbent material. There are reported comparative extraction efficiencies studies of SPE cartridges and disks. Ezzel and Ritcher[84] studied SFE of five organochlorine pesticides (OCPs) and phthalate esters from water.

Methods of support other than SPE cartridges or disks have be reported. Pawliszyn and Alexandrou[85] used HPLC guard columns for the extraction of polychlorinated benzenes in water. Zegers *et al* [86] extracted three OPPs from water using a C_{18} pre column. Bengtsson *et al* [87] extracted pesticides from river water using loose C_{18} sorbent material held on a glass-fibre disk.

Edder *et al*[88] packed a vessel with slurried silica based HPLC stationary phase for the extraction of morphinic alkaloids from urine.

Murugaverl and Voorhees [89] pre-mixed soybean oils with dry C_{18} sorbent material, and this was then loaded on top of a sorbent packed extraction column, the ability of the sorbent to selectively retain lipids was then examined.

Although introducing a further level of manipulation, the use of SPE and other support media allow the loaded material to be analysed by commercial SFE systems which have been developed for solid matrices.

1.4.5. Volume of liquid sample

The extractable liquid volume becomes a further choice where direct and indirect extraction options exist. The choice of aqueous volume will be determined by the level of dissolved analyte in the sample, and the required detection limits of the

subsequent analytical technique, often resulting in volumes between 100 and 1000 cm^3 needing to be extracted.

1.4.6 Dynamic and /or static extraction

Static extraction occurs when a fixed volume of supercritical fluid is introduced into the sample and allowed to equilibrate.

Dynamic extraction allows the continuous flow of fresh supercritical fluid through the sample, and allows even analytes with poor SF–matrix distribution coefficients to potentially be extracted quantitatively, given sufficient time and volume of supercritical fluid.

Some of the theory behind the extraction processes has already been discussed, and includes;

a. the analyte solubility in the supercritical fluid.

b. the distribution coefficients between the supercritical fluid and matrix.

c. the analyte diffusivity of the SF-analyte solution.

Most methods include a mixture of static and dynamic extraction, where the initial static stage is used to promote SF solubility by establishing analyte-modifier interactions. A static SFE stage is also frequently used in procedures involving *in situ* derivatisation of the analytes prior to extraction.

1.4.7 Vessels for indirect liquid supercritical extraction

Indirect supercritical extraction is, as stated previously, more time consuming, because of the additional extraction loadings onto a support material. Once extraction/loading has been completed, supercritical extraction is then relatively

straight forward, and can be accomplished using extraction systems designed for solid sample extraction.

A further advantage of indirect extraction is the variable volume of aqueous solution which can be analysed, so analyte enrichment can be achieved to match the requirements of the analytical technique, and the detection level required for the analyte.

Modifications have been made to commercial extraction vessels to allow the SPE cartridge or disks to be accommodated. Tang *et al*[90] reported a method for the indirect analysis of polyaromatic hydrocarbons (PAHs) from water. An SPE method requiring the incorporation of an aluminium derivatisation reactor within a SFE vessel for the extraction of organotin compounds from aqueous samples has been reported by Alzaga and Bayona.[91]

HPLC guard columns, equipped with 2 μm stainless steel frits, have been used as extraction vessels by Furton and Rein[92] to study the effect of vessel geometry on the quantitative SFE studies of PAHs loaded onto octadecylsilyl-bonded silica (C_{18}) sorbent material. This study established that extraction efficiencies, for the largest PAHs, could be doubled by decreasing the diameter to length ratio of the extraction vessel from 1:20 to 1:1.

Later studies by Furton and Lin[93] reported the relationship of extraction efficiencies and vessel dimensions, for polychlorinated biphenyls (PCBs) laoded onto C_{18} and phenyl sorbents. Furton *et al* [94] reported the relationship between extraction efficiencies, vessel orientation and void volume.

Indirect extractions can also be achieved using small volume direct extraction vessels, the analysis of PAHs in water have been reported by Ong et al[95] by placing a C_{18} loaded sorbent in a vessel designed for direct liquid SFE.[96-97]

1.4.8 Vessels for direct liquid supercritical extraction

Some of the earliest direct liquid supercritical fluid extractions were reported by Hendrick and Taylor using an 8 cm^3 volume extraction vessel, (Keystone Scientific, Bellefonte, PA). The same group have also reported the extraction and analysis of diisopropyl methylphosphonate and triproidine,[98] phosphonates, drugs and phenols[99] from aqueous samples.

This vessel suffered from the problem of resealing, and was replaced by a modified 6.94 cm^3 vessel, which incorporated the use of finger tight fittings and Kel-F ferrules, for the extraction of phenols[100] and nitrogenous bases[101] from aqueous solutions. This modification was reported to improve the resealing of the vessel, and improve re-usage, than the original stainless steel fittings.
This type of vessel was also used by Mulcahey and Taylor[102] for the extraction of sulphamethoxazole and trimethoprim from liquid drug formulations.
Use of small volume extraction vessels have also been reported by Ong *et al*[103] for the extraction of cholesterol from horse blood serum, by Liu and Wehmeyer[104] for the extraction of flavone and ketorolac from dog plasma.

Larger direct aqueous extraction vessel have been constructed. Laintz *et al*[105] (sample volume 14.9 cm^3) for the extraction of Cu (II) ions from solution. By Brewer and Sengupta *et al*[106] for the study of distribution coefficients of chlorinated hydrocarbons between water and SF-CO_2 , using a 30 cm^3 extraction vessel.

Brewer and Kruus[107] have constructed SFE vessels for the analysed of 25–75 cm^3 aqueous samples of pentachlorophenols.
Barnabas[108] used a SFE extraction vessel for the extraction of 45 cm^3 of aqueous organochlorine pesticides (OCPs) solution, this vessel was later utilised by Kane *et al*[109] for the extraction of surfactants from water.
Shimoda *et al*[110] using a 120 cm^3 volume SFE vessel, reported the extraction of volatile organic compounds (VOCs) from water
Roop and Akgerman[111] used a 300 cm^3 SFE vessel for a report on the distribution coefficients of phenols between SF-CO_2 and water.

At the University of Glamorgan, direct aqueous SFE vessels of volumes 150 cm^3, 300 cm^3 and 1000 cm^3 capacity have been used.

1.5 Analysis of Supercritical Fluid Extracts

1.5.1 Introduction

Although the density, and therefore the solvating power, of a supercritical fluid can be altered by adjustment of the pressure and temperature, and can therefore, in theory, selectively extract a range of analytes from a matrix. In practice after extraction, final analysis generally includes a chromatographic technique..

Most separation techniques have been used with SFE, these include;

i. Supercritical Fluid Chromatography (SFC).
ii. High Performance Liquid Chromatography (HPLC).
iii. Gas chromatography (GC).

A detailed description of the coupling of supercritical fluid extraction apparatus with these separation techniques will be addressed in subsequent chapters dedicated to such studies.

Generally if the analytes are volatile and thermally stable , the best separation technique is gas chromatography.
If the analytes are thermally labile the best separation techniques are supercritical fluid chromatography or high performance liquid chromatography.

These techniques can be associated with the SF extract either directly (on-line) or indirectly (off-line).

In the off-line process, the SF extract is collected separately, and then physically introduced into the chromatograph. The extraction and separation stages are totally independent of each other.

The on-line process takes the output from the SFE stage and directs it into the input of the chromatograph, via a trap or sample loop system. Here the extraction and separation stages are coupled, to form an integrated process.

The advantage of directly coupling the output from the extraction stage with the input of the separation/analysis stages are ;

a. Minimisation of sample handling.
b. Elimination of analyte losses during the transfer stage.
c. Reduction of analyte degradation
d. Reduction of analyte contamination
e. Time profile per analysis

1.5.2 Mass Spectrometric Analysis

As mass spectrometry contributes the majority of the detection methods applicable to the research areas described within this thesis, a brief introduction to the relevant mass spectrometry ionisation techniques used will be included at this point.

There are several ionisation techniques available to the analytical chemist, and the selection of an applicable method will depend upon the overall analytical profile.
The ionisation techniques used in this reported work are:

- Electron Impact ionisation (EI)
- Chemical ionisation (CI)
- Atmospheric Pressure Chemical Ionisation (APCI)
- Electrospray

1.5.2.1 Electron Impact Ionisation

Electron impact ionisation is still the most widely used ionisation technique, the system produces a stable ion beam impinging on vaporised samples, whose spectra provide reproducible fragmentation patterns, which are applicable to library search procedures.

An applied voltage, between the filament and source block assembly, accelerates electrons emitted by a heated filament located in a vacuum across the ionisation chamber.

The voltage is continuously variable, generally between 5 to 100 eV, with the normal applied voltage being 70 eV, as the maximum ion yield is obtained around this voltage.

Mass spectra can be obtained at voltages down to the ionisation energy of the compound under investigation, and lower voltages are frequently used to limit the amount of compound fragmentation.

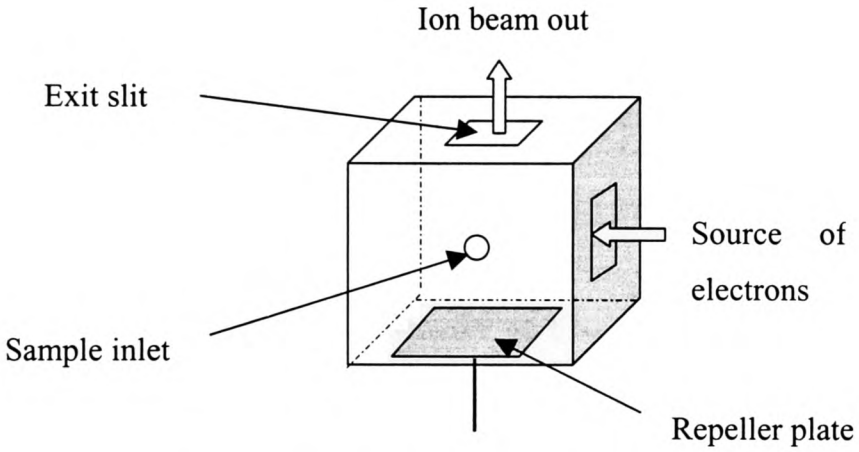

Fig 1.14 Electron Impact Ionisation Source

A volatilised compound is introduced into the ionisation region, which is evacuated to a pressure of approximately 10^{-6} torr (1.33×10^{-4} Pa) via the sample inlet, and when it interacts with an electron, which has energy greater than its ionisation energy, ionisation occurs. Sometimes converting the neutral species into a positively charged ion, (it is estimated that only 1 in 10^3 to 10^4 sample molecules are ionised in this manner).

$$M + e^- \longrightarrow M^{+\bullet}_{(g)} + 2e^-$$

Resulting in the production of the molecular ion (the ion indicative of the molecular mass of the ionised sample).

Electron attachment is also possible resulting in the formation of negatively charged ions.

$$M + e^- \longrightarrow M^-$$

The probability of electron capture resulting in the formation of a negative ion is approximately 10^2 times less than for electron removal and positive ion formation.

The molecular ion $M^{+\bullet}_{(g)}$ may fragment along several pathways;

$$M^{+\bullet}_{(g)} \longrightarrow D_1^{+\bullet}{}_{(g)} + N_{1(g)}$$

(Odd Electron Ion ⟶ Odd Electron Ion + Neutral)

Or

$$M^{+\bullet}_{(g)} \longrightarrow D_2^{+}{}_{(g)} + R_1^{\bullet}{}_{(g)}$$

(Odd Electron Ion ⟶ Even Electron Ion + Radical)

Both $D_1^{+\bullet}{}_{(g)}$ and $D_2^{+}{}_{(g)}$ are known as "daughter ions", and may still posses sufficient internal energy to undergo further fragmentation and produce second generation "grand-daughter" ions, etc.

For Odd Electron Daughter Ion

$$D_1^{+\bullet}{}_{(g)} \longrightarrow GD_1^{+\bullet}{}_{(g)} + N_{2(g)}$$

(Odd Electron Daughter Ion ⟶ Odd Electron Grand-Daughter Ion + Neutral)

Or

$$D_1^{+\bullet}{}_{(g)} \longrightarrow GD_2^{+}{}_{(g)} + R_2^{\bullet}{}_{(g)}$$

(Odd Electron Daughter Ion ⟶ Even Electron Grand-Daughter Ion + Radical)

For Even Electron Daughter Ions

$$D_2^{+}{}_{(g)} \longrightarrow GD_3^{+}{}_{(g)} + N_{3(g)}$$

(Even Electron Daughter Ion ⟶ Even Electron Grand-Daughter Ion + Neutral)

Electron impact ionisation is regarded as a "hard" ionisation technique, and sometimes result in no molecular ions remaining intact.

After ion formation has occurred they are repelled from the ion chamber by the applied voltage on the repeller plate, and into the mass analyser

When operated in positive ion mode, the repeller plate is given a positive charge therefore only repelling the positively charged ions. Any negatively charged ions are attracted towards the plate and discharged, and along with the neutral species pumped out of the chamber. The positively charged ions are produced, and then expelled from, the ion source in approximately 10^{-6} sec.

This ionisation technique is regarded as being relatively inefficient as it results in few positive ions being produced, further ion/molecule interactions take place, producing a variety of ions.

1.5.2.2 Chemical Ionisation

Ionisation can also be induced into a neutral molecule by collision with a charged species.

For effective ionisation to occur, the chemical ionisation gas pressure in the ionisation chamber must be in the region of 0.1 to 1 torr (2×10^{-3} to 2×10^{-2} psi). These

relatively high pressures impose a difficulty in the ionisation chamber with the high applied potentials, causing arcing.

While these relatively high pressures have to be maintained within the ion source to allow sufficient collisions and ion/molecule interactions to take place, a much lower pressure of 10^{-5} to 10^{-6} torr (1.8×10^{-7} to 1.8×10^{-8} psi) must be maintained outside the ion source region, in the mass analysis region. The separation of these two different pressure regions posses additional problems.

The most common reagent gases for chemical ionisation are methane, 2-methylpropane (isobutane) and ammonia.
If methane is used as the reactant gas the following steps occur;

Methane is ionised by electron impact at a pressure of approximately 0.1 to 1 torr (2×10^{-3} to 2×10^{-2} psi) .

$$CH_{4(g)} + e^- \longrightarrow CH_4^{+\bullet}{}_{(g)} + 2e^-$$

Because of the relatively high pressures within the ionisation chamber, there is a high probability of further collisions with other neutral methane molecules resulting in additional ionised products;

$$CH_4^{+\bullet}{}_{(g)} + CH_{4(g)} \longrightarrow CH_3^{\bullet}{}_{(g)} + CH_5^{+}{}_{(g)}$$

These reactant gas ions can further react with neutral molecules (M) causing ionisation to occur.

$$CH_5^{+}{}_{(g)} + M \longrightarrow CH_{4(g)} + (MH)^{+}{}_{(g)}$$

The protonated ion $(MH)^+$ will have an m/z ratio one amu greater than the molecular ion, and is known as the quasi-molecular ion. N.B. also $[M+C_2H_5]^+$ adduct species possible

With ammonia CI, $NH_4^+{}_{(g)}$ is formed as the reactive ion, and as well as the protonated sample molecule being produced, if the source pressure is high, sample adduct ions are formed ($M+NH_4^+$) and are observed at 18 amu above the molecular ion.

Carefully selection of the CI reagent gas can allow some control of the sample fragmentation, with ammonia offering a "softer" ionisation procedure to methane due to its higher proton affinity value.

At the relatively high pressures employed with the chemical ionisation technique, electron capture also becomes more efficient, and so the production of negative ions increases, and is equally as likely as positive ion production.
This has resulted in negative ion chemical ionisation becoming a popular technique.
The sensitivity of positive ion CI is comparable with positive ion EI, while negative ion CI has the suggested sensitivity several times greater than either of them.
Unlike EI, CI is a much more difficult technique to control, i.e. source pressure and temperature, this has an affect on the fragmentation of the sample molecule, and therefore its reproducibility, and hence no commercial CI libraries are available.

1.5.2.3 Atmospheric Pressure Chemical Ionisation (APCI)

(also known as a Heated Nebulizer Interface)

We have discussed, with CI, that the efficiency of ion formation is related to the collision rate of the reagents. As the ionisation efficiency increases with source pressure, it would seem a logical progression to increase the source pressure even higher and so create an even more efficient ionising process.
This has been achieved with APCI, where the ionisation process is carried out at atmospheric pressure.

Ionisation at atmospheric pressure, although efficient, introduces the additional problem, as mentioned for CI, of isolating the high pressure ionisation region, from the low pressure mass analysis region, but still allowing the transmission of the ions.

The design of an interface between these two regions allowed the development of a system where not only ions created at atmospheric pressure can be analysed, but also polar analytes available in solution. Hence the creation of a direct coupling between HPLC and mass spectrometric analysis.

Early methods of ion production used ^{63}Ni (beta emitter) as the ionisation source. More commonly a high voltage corona discharge needle is used, where the incoming solvent molecules are ionised, e.g. H_3O^+ .

These ions undergo ion/molecule interaction producing quasi-molecular ions.

$$H_3O^+ + M \longrightarrow (MH)^+$$

This ionisation process takes place within the region between the discharge needle and the nitrogen gas curtain, and the ions, of the appropriate charge, are attracted towards the orifice by an applied potential on the interface plate.

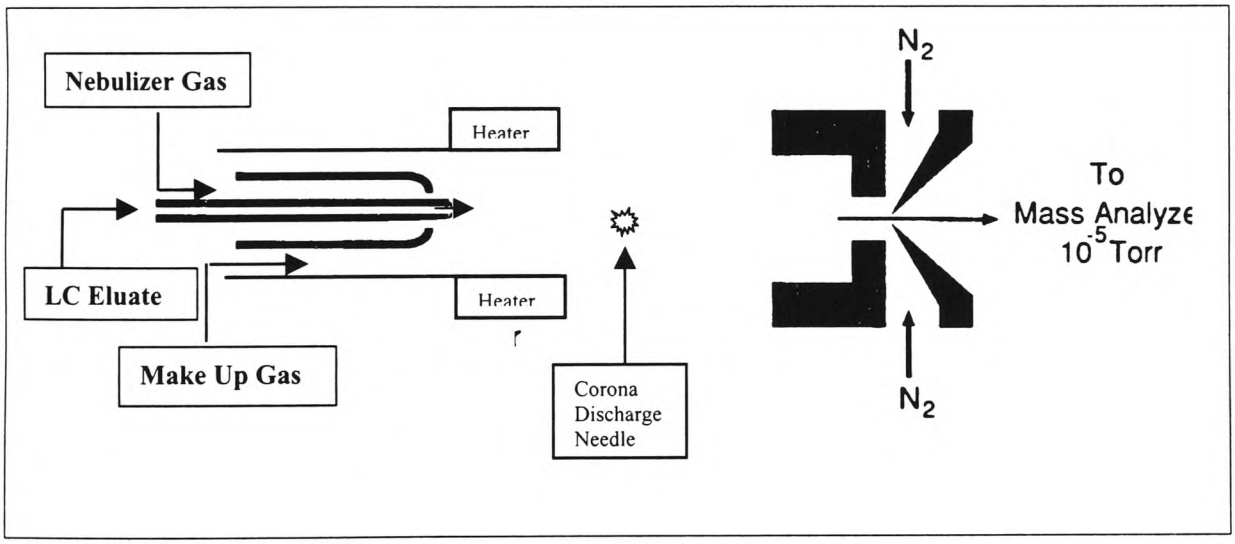

Fig 1.15 Atmospheric Pressure Chemical Ionisation Source

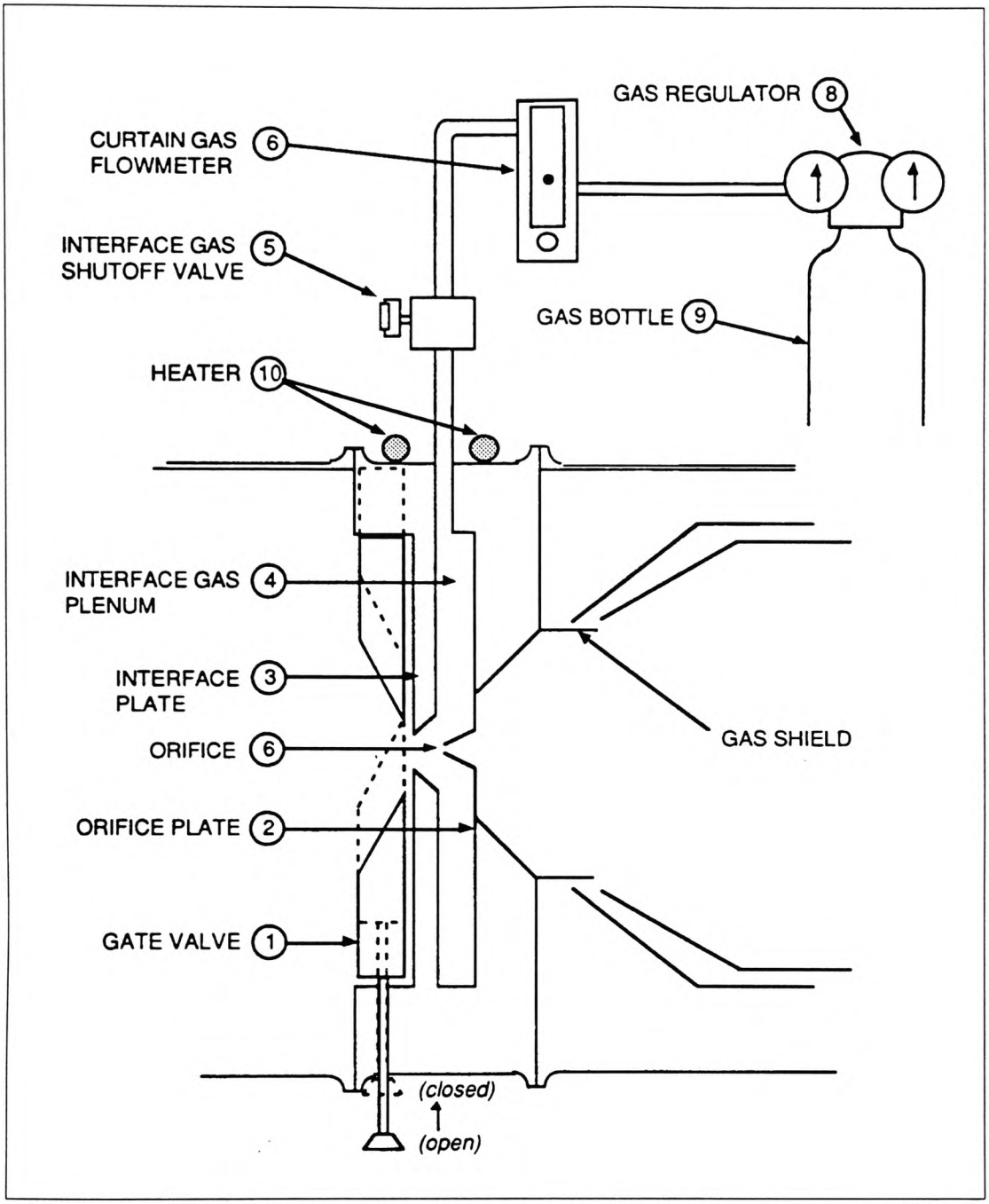

Fig 1.16 Perkin Elmer SCIEX API III APCI to Vacuum Interface

In the APCI interface the LC eluent is fed from the column to the ionising region through a micro bore tube, and nebulization is achieved with the aid of a coaxial nebulizing gas and heating. This produces a vapour of solvent and analyte molecules within the ion source.

A chemical ionisation plasma of solvent derived ions is produced by a discharge electrode. These clusters react with a basic site in the analyte molecule producing $(MH + nH_2O)^+$ solvent clusters.

Water molecules can cluster around the ions reducing the sensitivity of the technique and need to be reduced. This can be achieved in several ways;

a. By the application of accelerating voltages at the discharge needle within the APCI source, the clusters undergo collision induced dissociation which strips the water molecules from the ionised sample.

b. The use of curtain gas flow across the analyser side of the inlet orifice, this has the effect of "de-clustering" water molecules from the ionised sample. It also helps to maintain the stability of the ion/molecule products as they are transferred from the ionising region into the high vacuum mass analysis region. A high purity inert gas such as nitrogen is generally employed for this task.

$$(MH + nH_2O)^+ \xrightarrow{N_2} (MH)^+ + nH_2O$$

During their flight ion-solvent clusters fall apart due to collision with $N_{2(g)}$ molecules, if the interface plate voltage is set high, then fragmentation of the molecular ion can occur by collision induced dissociation (CID).

Advantages of APCI

Allows the coupling with the un-split output from a 4.6 mm i.d. HPLC column, with mobile phase flow rates and composition of up to 1.5 cm^3 min^{-1}, and 100% water, as well as the use of volatile and involatile buffer, up to a concentration of 0.1 M.

1.5.2.4 Electrospray (ES)

Ion evaporation is the mechanism of ion production from a liquid and into the gas phase, and the process was proposed by Iribarne and Thomson [112] in 1976.

When a charged droplets evaporates in air, a critical point is reached when it becomes kinetically and thermodynamically favourable for ions at its surface to evaporate and disperse into the air, and is independent of how the droplets were originally formed. The rate of ion emission is dependent upon the solvation energy of the individual ions, so that one type of ion may preferentially evaporate if it has a low solvation energy.

As a result of this ion evaporation a dry particle of residual involatile components derived from the solution remains. If this stage is reached before the critical ion evaporation limit, then the net charge remains on the particle, and no gas phase ions will be produced.

This can happen for several reasons,

a. the initial droplet had too low a charge
b. if there was to much involatiles in the solution

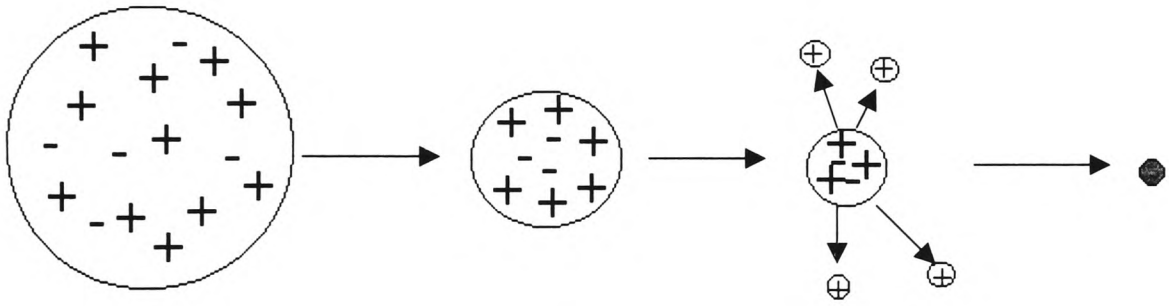

Fig 1.17 Ion Evaporation

A:- The original droplet containing ions of both polarities, one predominating

B:- As the solvent evaporates the electric field increases and the ions move to the surface.

C.:- At a critical field value, the ions are emitted from the drop.

D:- Involatile residue remains as a dry particle.

The ES interface differs from the APCI interface in the way in which the ions are produced. In ES the mobile phase passes through a silica capillary which isolates the ionisation process from the liquid chromatography pump.

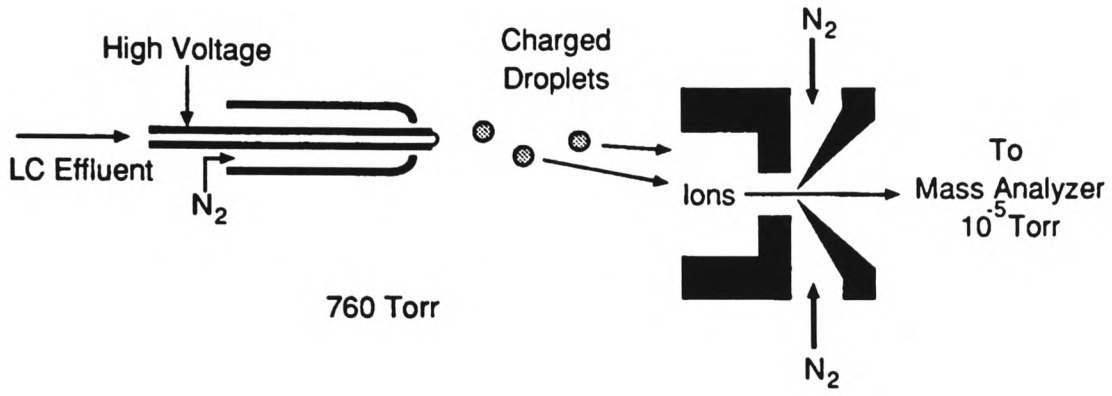

Fig 1.18 Electrospay interface for P.E. SCIEX API III LC/MS/MS System

The spray interface and the ion source region are held at a potential difference of several kV. As a result of this high voltage a charged aerosol is produced, these droplets then evaporate during flight, producing ions, as described above.
As in APCI ion/solvent clusters are produced which can be disintegrated by the curtain gas.

Advantages of Electospray
Good instrument stability
Possible analysis of both high and low molecular weight compounds
Detection of both positive and negative ions.
Gradient elution with HPLC
Use of buffered mobile phases.

All four of these ionisation techniques have been used with this reported work, and specific analytical parameters will be included within the relevant chapters.

In addition to these ionisation methods, tandem mass spectrometry was also used and a brief introduction of this is also given

1.5.2.5 Tandem Mass spectrometry (MS-MS)

Tandem mass spectrometry has several advantageous features. These include the elimination of sample clean-up and preparative procedures;

Therefore reducing analysis time, and also allows compounds not normally amenable to mass spectrometric analysis to be analysed.

A mass spectrometer having a triple quadrupole geometry basically consists of a quadrupole mass filter (Q1) followed by a quadrupole collision cell (Q2) which is usually an enclosed rf-only cell which allows total ion transmission. This is followed by a second quadrupole mass filter (Q3). Between the three quadrupoles are focussing, and pre-filter assembles, which include short rf-only quadrupoles, or electrostatic lenses. The two independent mass analysing quadruploews (Q1 and Q3) can be used to select parent and daughter ions for reactions occurring in the reaction cell (Q2).

Triple quadrupole mass spectrometers can be operated in six basic modes,

- **Daughter products of a specific parent ion.**
  Q1 is set to transmit only a selected parent ion, while Q3 is scanned to yield spectra of all daughter ions produced in the collision cell Q2.

- **Parent ions of a specific daughter ion.**
  In this arrangement the first quadruploe Q1 is scanned, and Q3 is set to only allow specific daughter to be transmitted.. This means that spectra of all parent ions produced in Q2 are recorded.

- **Constant Neutral Loss**
  Specific neutral fragments produced in the collision cell, Q2, are recorded by scanning both Q1 and Q3 with a fixed difference (offset) in mass which is equal to the mass of the neutral fragment produced. With the selected Q3 mass less than the selected Q1 selected mass
- **Constant Neutral Gain.**
  Similar in setup to constant neutral loss. Both Q1 and Q3 are scanned by a fixed difference (offset), for the neutral gained by the parent in the reaction cell Q2. Here the selected Q3 mass is greater than the selected mass in Q1.

- **Multiple reaction monitoring (MRM)**

Selected transmissions of ions are monitored by setting both Q1 and Q3 to the appropriate mass values. A sequence of ion transitions can be monitored by rapid cycling through a programmed set of mass values. MRM is equivalent two a double stage single ion monitoring (SIM) setup.

- **Conventional Mass Spectra**

  This can be achieved in one of two ways. Either operating Q1 and Q2 in rf-only mode allowing the transmission of all ions into Q3 which is scanned. Or operating Q2 and Q3 in rf-only mode, but scanning Q1.

Appendix A

Critical data for pure components

	T_c (K)	P_c (MPa)		T_c (K)	P_c (MPa)
Helium	5.19	0.23	Hydrogen chloride	324.7	8.31
Hydrogen	33.0	1.29	1,1,1 Trifluoroethane	346.25	3.76
Neon	44.4	2.76	Hydrogen bromide	363.15	8.55
Nitrogen	126.2	3.39	Chlorodifluoromethane	369.3	4.97
Carbon monoxide	132.9	3.50	Propane	369.8	4.25
Argon	150.75	4.87	Dichlorodifluoromethane	385.0	4.14
Oxygen	154.6	5.04	Dimethyl ether	400.0	5.24
Methane	190.4	4.60	Ammonia	405.55	11.35
Krypton	209.45	5.50	Chlorine	416.9	7.98
Hexafluoroethane	293.0	3.06	Acetone	508.1	4.70
Trifluoromethane	299.3	4.86	Methanol	512.6	8.09
Chlorotrifluoromethane	301.95	3.87	Ethanol	513.9	6.14
Carbon dioxide	304.15	7.38	n-Heptane	540.3	2.74
Ethane	305.4	4.88	Benzene	562.2	4.89
Nitrous oxide	309.65	7.24	Toluene	591.8	4.10
Monofluoromethane	315.0	5.60	Water	647.3	22.12

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Chapter 2

Hydrocarbon Extraction

2.1 Introduction

The analysis of oil and grease in discharge and process water has traditionally been carried out by liquid/ liquid extraction using carbon tetrachloride or freon as the solvent, followed by quantification using infrared spectroscopy or gravimetric analysis.

With international concern over stratospheric ozone depletion, the Convention for the Protection of the Ozone layer was initiated in Vienna in March 1985. This led to the introduction of principles for protection of the ozone layer, without laying down the much needed detailed phase out of ozone depleting solvents. This base was expanded in September 1987 when 27 countries became signatories to the "Montreal Protocol on Substances that Deplete the Ozone Layer",[1] which required the reduction of certain chlorofluorocarbons (CFCs) to 50% of their 1986 level.[2,3] The number of signatories has now increased to 173, with amendments to the protocol being added in London in 1990, Copenhagen in 1992, Montreal in 1997, and Beijing in 1999.[4,5] Other substances have been added to the original protocol list, these include carbon tetrachloride, 1,1,1-trichloroethane, hydrofluorocarbons, halons and methyl bromide.
It was also recognised that for certain uses, alternative solvent systems were not yet available and so a list of exemptions was agreed in the 6th meeting of the signatories in Nairobi in 1994.[6] Included in this exemption list were laboratory and analytical uses for extraction and calibration purposes, in particular the use of restricted solvents for the extraction and analysis of oil, grease and total petroleum hydrocarbons (TPHs) in water.

In July 1998 the Open-Ended Working Group of the Parties to the Montreal Protocol [7] assembled a proposal for the 10th meeting of the parties which include that Freon 113 and carbon tetrachloride, for the determination of oil, grease and TPH in water, should be removed from the global exemption list for laboratory and analytical uses from 2001. Over concern for a replacement solvent/process the 11th meeting of the parties

in 1999,[8] decided that the removal of these solvents would take place as of 2002. This meant that methods of analysis of oil, grease and TPHs, which required the use of these restricted solvents, would now be obsolete, and therefore alternative analytical procedures would have to be developed.

The basis of this section of reported work, is the development and implementation of an alternative system, and methodologies for the extraction of oil and grease and their subsequent quantification. This work reports on the phased development of a suitable aqueous extraction system, based on the use of supercritical fluid carbon dioxide, and the analysis of the extracted hydrocarbons using infrared spectroscopy for detection and quantification.

It is well documented that supercritical fluid carbon dioxide can imitate solvents such as hexane, carbon tetrachloride and Freons, depending upon the density used for the extraction. These solvents are those used, and previously used, for the extraction of oil and grease from water. Like carbon tetrachloride and Freon-113 (1,1,2-trichloro-1,2,2-trifluoroethane), supercritical fluid carbon dioxide is infrared transparent in the region used for the detection and quantification of extracted hydrocarbons.

Organic analytes give infrared spectra which are characteristic of the fundamental groupings of which they are composed. In the case of hydrocarbons, those compounds consisting of only carbon and hydrogen, the characteristic appearance of the spectra can be assigned to their C-H stretching and bending modes.
The absorbance peaks used in this work appear between 3150 and 2800 cm^{-1}, and can be assigned as symmetric and antisymmetric stretching modes. A further feature of these spectra being the linear relationship between the intensities of these bands and the quantities of material present.

This chapter tracks the progress from the simplest extraction system, based upon a simple "T" design extraction vessel, allowing the dynamic flow extraction of hydrocarbons and detection by off-line infrared spectroscopy to be investigated. This gave an indication of the feasibility of the system to extract hydrocarbons from an aqueous matrix, and guided the design for a second extraction system.

Progress is reported for the second generation extraction system, the welded cell, where the complexity, and flexibility, of the cell allowed other important extraction parameters to be examined. These results were used as the basis for the design of the final extraction system design.

2.2 The Environmental Impact of Hydrocarbons

There have been many international conventions and directives proposed for the protection of the environment from hazardous substances, these include watercourses both fresh and marine.

The EEC drew up a list, in May 1976, of the most hazardous substances under the EEC Council Directive 76/464, [9] which aimed at controlling the discharge of these listed chemicals into the aquatic environment. Contained within List 1 of this directive alongside Organohalides, Organometallics and other metallic compounds are " Persistent mineral oils and hydrocarbons of petroleum origin".

The inclusion of hydrocarbons, indicates the extent of the environmental impact this class of compounds can have, and Article One of this directive states that these restrictions shall apply to water which includes;

- Inland surface water
- Territorial waters
- Internal Coastal waters and
- Ground water

The extent of a pollutants' environmental impact is generally divided into three classifications,

- Toxicity
- Persistence
- Bioaccumulation.

therefore the total impact of a hydrocarbon mixture is extremely difficult to quantify, as each component of the mixture has a differing measure on each classification .

2.2.1 Toxicity

Toxicity relates to how poisonous a substance is, or how large a dose is required to kill an organism, the more toxic the substance, the smaller the dose required. If organisms are exposed to a toxin, not all die at the same time, and to quantify the toxicity of the substance a value of Median Lethal Time is determined. This is the time for 50% of the species under investigation to die, and is written as LT_{50} or LT_m . The lethal time depends upon the concentration of the toxic, the higher the concentration the shorter the mortality period, and there may be a concentration below which the substance is not toxic. Therefore LT_{50} values are not very useful statistically, and it is more usual to determine the concentration of the toxin at which 50% of the organisms are killed within a specified time. This time is generally 48 or 96 hours, and the toxicity is then recorded as the Median Lethal Concentration and is written as 48 h LC_{50} .

Using these criteria the effects can be categorised as follows, (because the definitions are very vague, substantial overlap occurs).

Acute:- Relatively large or severe effect caused by a short term exposure to a toxic environmental agent.

Chronic:- A poisonous effect produced by a long period of exposure to a moderate level of a toxic agent, and causing measurable biochemical or anatomical damage, but not death.

Long term:- A period of time that is longer than several life spans of an organism, or of the dominant organism in an ecosystem.

Sub-Lethal:- An exposure which does not bring about death of the organism.

Lethal:- The exposure that results in the death of the organism under study, in the concentrations delivered in the observation time frame.

The toxicological effects of hydrocarbons in water are very dependent upon its physical state, and if it has the form of a fine distribution, emulsion, or solution the toxic behaviour of such materials can increase considerably.

2.2.2 Toxicity of specific hydrocarbons

It is difficult to determine with consistency the toxic effects of a hydrocarbon mixture simply from the total quantity of hydrocarbon present, as each hydrocarbon will contribute differently to the overall toxicity profile.
The major difficulties in assessing the environmental impact of hydrocarbon discharges, is the determination of the hydrocarbons present in the mixture, and their quantities or concentrations.

Generally it is agreed that aromatics of low boiling point and relatively high solubilities, with LC_{50} values (24-96 hours) of the order 15 mg/l, are the most toxic. High volatility n- and iso-alkanes can also be regarded as having the same order of toxicity.
According to the classification proposed by *IMCO/FAO/UNESCO/WHO, medium to long chain aliphatic hydrocarbons (C_{15}-C_{30}) can be regarded as being practically non-toxic with their problematic environmental effects coming from their physical properties.

*(IMCO - *Intergovernmental Maritime Consultative Organisation*)
(FAO - *Food and Agriculture Organisation of the United Nations*)
(UNESCO - *United Nations Educational, Scientific and Cultural Organisation*)
(WHO - *World Health Organisation*)

Toxicity of hydrocarbons has been reported to increase along the sequence, [10]

Alkanes < Alkenes < Aromatics < Alkyl substituted Aromatics

with work carried out on cycloalkanes and cycloalkenes[11] seeming to suggest that they are more toxic than their straight chain equivalents, and in some cases more toxic than aromatics.

Lethal effects upon some marine organisms tend to suggest that the more toxic hydrocarbons have effect within the 0.3 – 100 ppm range, but there are reports of these effects being observed in juveniles of the species, as low as 0.1 ppm.[12-22]

2.2.3 Persistence (Biological degradability)

Persistence is a measure of the resistance to breakdown of a substance, and indicates the time that it remains in the environment in its original state.

It is well documented that there are several physical changes which occur quickly and effect hydrocarbons when they enter a body of water. These include:

- Absorption
- Spreading
- Evaporation
- Solvation
- Emulsification
- Dispersion
- Sinking

These effects are followed by weathering and ageing processes which begin to take place.

As with toxicity, persistence testing has very little standardisation of analytical methodology, and so the measure of persistence is generally difficult to ascertain. It is generally accepted that virtually all organic material which is derived from bio-

synthesis (this includes hydrocarbons) can be degraded, and that several different mechanisms are involved in this breakdown process. These include:

- Degradation by micro-organisms
- Evaporation
- Adsorption onto solid surfaces
- Photochemical reactions

The rate of degradation of the organic compounds depends not only on its chemical structure and composition but also on:

- Physical conditions in the degradation zone
- pH
- Temperature
- Oxygen availability
- Salinity (if marine environment)
- Available energy sources
- Nutrients present
- Presence of suitable micro-organisms
- Solubility of the pollutant.

The rate of degradation of differing hydrocarbons has been examined,[23] and in general aliphatic compounds are degraded most readily, and within this classification medium to long chain and saturated hydrocarbons are degraded at a higher rate than the lower molecular weight ones, especially those which are unsaturated.

Branched hydrocarbons also differ from straight chained with degradation often only proceeding up to the branching point.

Aromatic compounds seem to have greater resistance to breakdown, and polycyclic aromatics, especially if they contain over three rings, can persist for relatively long periods without degradation, and this trend may be reflected in their subsequent solubilities.

Some of the breakdown products caused by degradation can be as toxic as the original pollutant, particularly those products formed by chemical oxidation under the influence of ultraviolet radiation. [24]

There are naturally occurring organic substances which are relatively persistent in the environment, but produce little or no toxic damage[25](Appendix B), and so persistence on its own is not the only parameter to be considered when trying to estimate environmental impact.

2.2.4 Bioaccumulation

Bioaccumulation is the inability of organisms to breakdown and excrete toxic materials resulting in a build up in concentrations of these toxins. In the case of hydrocarbons, which have a low solubility in aqueous fluids, this accumulation tends to occur in the fats and lipids of the body, and also in target organs such as the liver. Work on the solubility of different classes of hydrocarbons has been reported [26-27] (Appendix C).

2.3 Overview of experimental work

This work was initiated to :-

1. Examine the established method of extracting hydrocarbons in water. This extraction method has historically relied on liquid - liquid extraction with an infrared transparent solvent for hydrocarbon quantification.

2. Investigate the quantitative techniques used in analysis; including infrared methods of analysis.

3. To determine the parameters applicable to these methods,
    a. Precision
    b. Accuracy
    c. Bias
    d. Sensitivity
    e. Selectivity

4. To develop a method of extraction and analysis based on supercritical fluid carbon dioxide as an alternative solvent.

Several problems needed to be overcome before extraction and quantification of organic analytes from an aqueous matrix with supercritical fluids could be achieved;

1. Control and containment of the high pressures associated with supercritical fluids.

2. Problems associated with interference of infrared analysis by water, both by water which is transported in the supercritical stream, and also by water which has been solublised within it.

And for the quantification of hydrocarbons,

3. Choice of a suitable hydrocarbon, or hydrocarbon mixture, for use as a quantification standard.

4. Whether extraction, and hence monitoring and quantification, should be completed under static or dynamic conditions.

2.3.1 Initial work

Hendrick and Taylor [28-29] reported some of the earliest work on supercritical fluid extraction of aqueous samples, using a cylindrical extraction system of length 10 cm,

i.d. of 1 cm, and an internal volume of 8 cm^3, for the analysis of diisopropyl methylphosphonate, triprolidine, phosphonates, drugs and phenols.

To determine the feasibility of the hydrocarbon extraction process using supercritical carbon dioxide, a feasibility study extraction system was constructed. This consisted of an empty HPLC column with $1/16^{th}$ o.d. inch stainless steel HPLC tubing passing through the end fittings and extending to the opposite end of the tube, (Fig 2.1), and located in a temperature controlled oven at 50 °C.

The carbon dioxide was delivered to the extraction column from a Gilson HPLC system, the pump head fitted with a cooling jacket, and the flow parameters computer controlled, to give a carbon dioxide flow rate of approximately 3 ml min^{-1}. The restrictor was a length of $1/16^{th}$ inch stainless steel tubing which had been "crimped" to allow a back pressure of approximately 3.1 Kpsi, and wrapped around a heating cartridge to stop freezing as the carbon dioxide expanded exited the tubing.

The end of this tubing was immersed in 1,1,2-trichloro-1,2,2-trifluoroethane (FREON-113) contained within a round bottom flask, and acted as the collection solvent allowing solvation of the dynamic flow extracted decane.

This arrangement allowed the column to be filled with a known volume of hydrocarbon spiked water, and when clamped in a vertical position, supercritical carbon dioxide to be passed through it. The exit tube was above the level of the water and hence stopped its egress with the SF-CO_2.

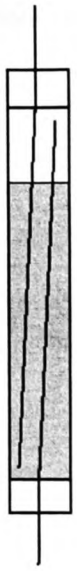


fig. 2.1 Hydrocarbon extraction system

The hydrocarbon chosen for extraction was decane, as this was relatively involatile, and could therefore be trapped easily during the feasibility study.

To allow quantification, a series of standard decane solutions were prepared in FREON-113, in the range 17 to 136 ppm. These standard solutions were then scanned in a Perkin Elmer 1700 Fourier Transform Infra Red (FTIR) spectrometer using a 20 mm pathlength infrared quartz cell, between 3200 and 2500 cm^{-1} at a resolution of 4 cm^{-1} and 20 scans per sample.

The absorbance of the peak located at approximately 2930 cm^{-1} was then measured, using a baseline constructed between 3100 and 2800 cm^{-1}. This data was then utilised too construct a Lambert-Beer calibration graph where the absorbance is linearly related to the concentration of the analyte within the concentration levels chosen.

Five extracts were collected sequentially in 10 minute periods, resulting in a total extraction time of 50 minutes.

The problem of solvent loss during the extraction phase was initially overcome by continually adding solvent to the flask to compensate. These solutions were then

quantitatively prepared in a volumetric flask, before being scanned by FTIR, and their absorbance at 2930 cm^{-1} determined.

Later extraction solvent losses were reduced by the addition of a condenser attached to the flask and filled with liquid nitrogen (Fig 2.2.).

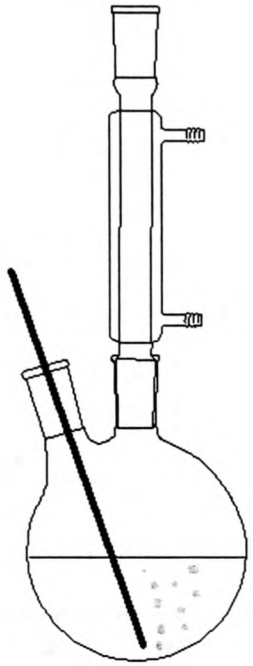


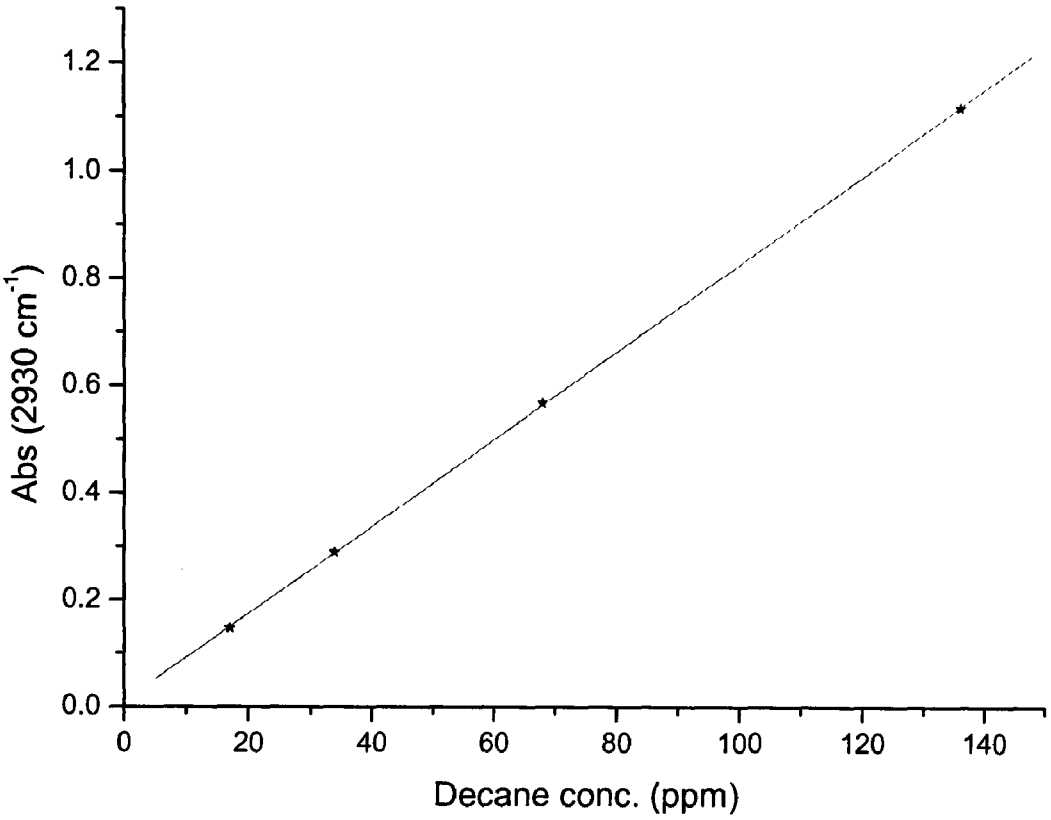
Fig. 2.2 Supercritical fluid solvated hydrocarbon collection system

2.3.2. Initial results

a. Calibration results for 4 decane spiked FREON-113 solutions

Decane concentration (ppm)	Absorbance @ 2930 cm^{-1}
17	0.147
34	0.29
68	0.57
136	1.12

b. Calibration graph for 4 decane spiked FREON-113 solutions

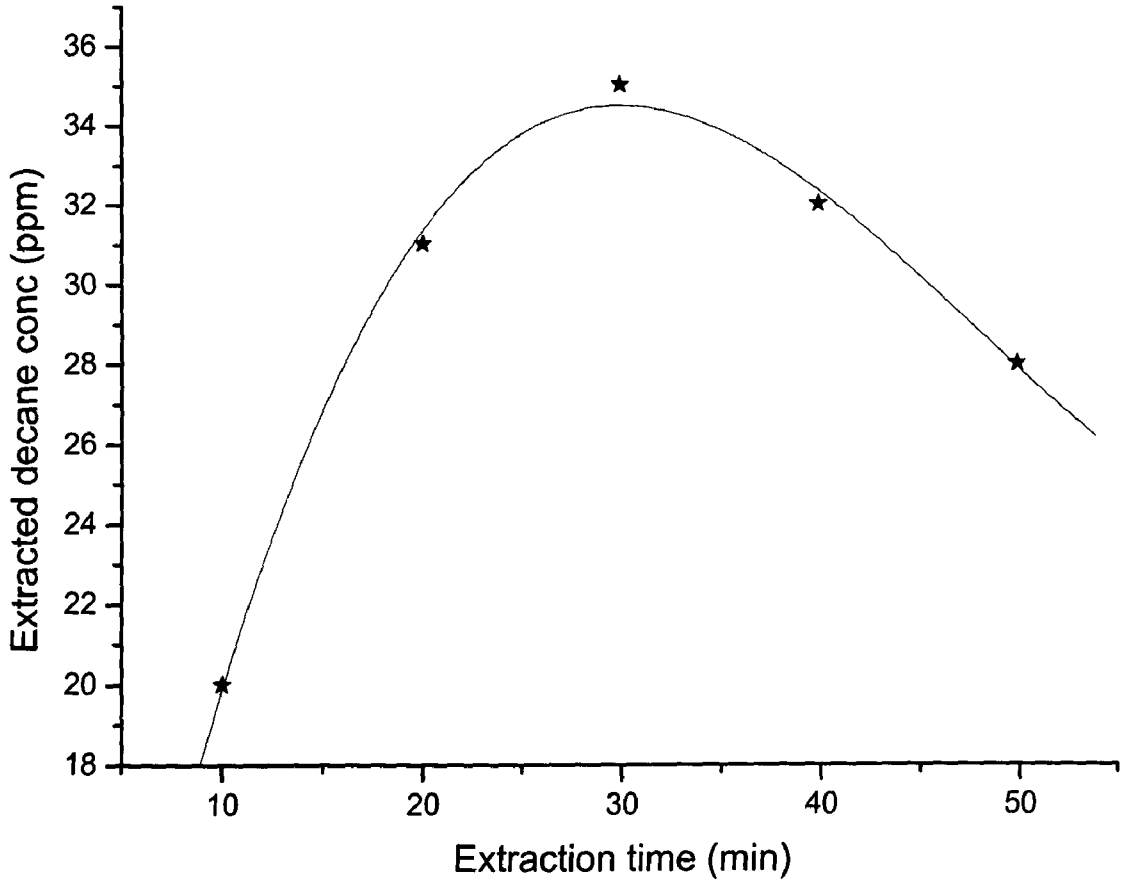


Correlation coefficient = 0.99998

c. Extraction results for the 5 consecutive spiked water supercritical fluid extractions

Extraction period (min) (10 min)	Absorbance @ 2930 cm^{-1}	Concentration of extracted decane (ppm)
Start (0) - 10	0.176	20
10 - 20	0.262	31
20 – 30	0.299	35
30 – 40	0.272	32
40 - 50	0.241	28

d. Extraction profile for the 5 consecutive spiked water supercritical fluid extractions



This graph suggested that the maximum extraction efficiency occurs approximately in the 20 to 40 minute period.

These results indicated the feasibility of aqueous extraction by supercritical carbon dioxide, but the lack of system design flexibility would not allow a detailed examination of the extraction parameters.

2.3.3 Development of an aqueous extraction system, the "T" cell

For this section of the study, an aqueous extraction system was constructed from commercially available high pressure components,, for the extraction, and analysis of hydrocarbons (Fig.2.3).

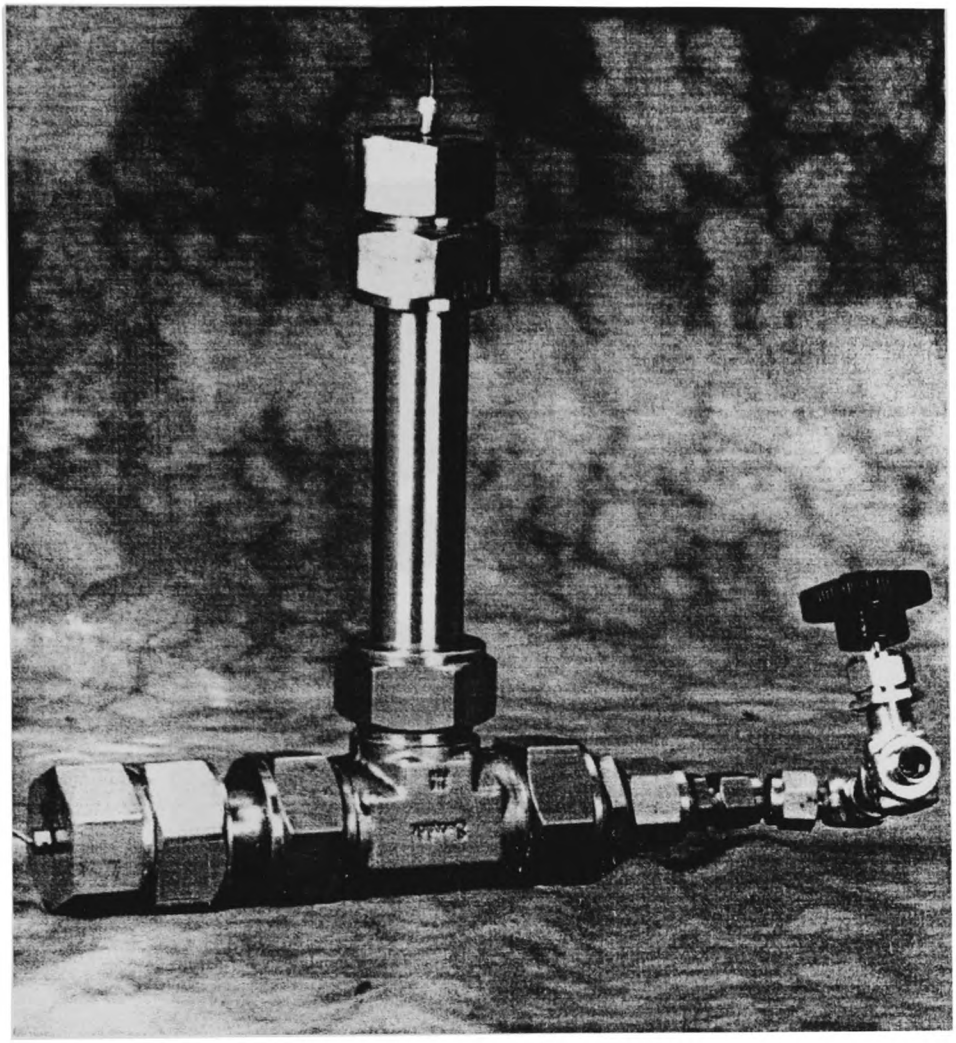


Fig. 2.3 Second generation supercritical fluid extraction system

The system was constructed from 2.54 cm i.d. stainless steel tubing, had an internal volume of 150 cm^3, pressure tested to 41.4MPa, was housed in a Pye 104 gas chromatography oven, to maintain constant thermal conditions.

The inlet and outlet tubes were $1/16^{th}$ o.d. inch stainless steel tubing fitted with stainless steel diffusers, these helped create a diffused stream of SF-CO_2 on the inlet side, and help prevent the egress of water with the analyte solubilised SF-CO_2 on the outlet.

A Gilson 308 pump (Anachem, Luton, Bedfordshire, UK) fitted with a chilling head connected to a Neslab RTE 110 recirculator, operated at -10°C, was used to supply liquid carbon dioxide (Messer UK, Reigate, Surrey, UK) at the required pressure to the extraction system. The outlet of the extraction vessel was connected to a high

pressure FTIR cell fitted with quartz windows with an aperture of diameter 2mm, and a pathlength of 8mm, Fig.2.4.

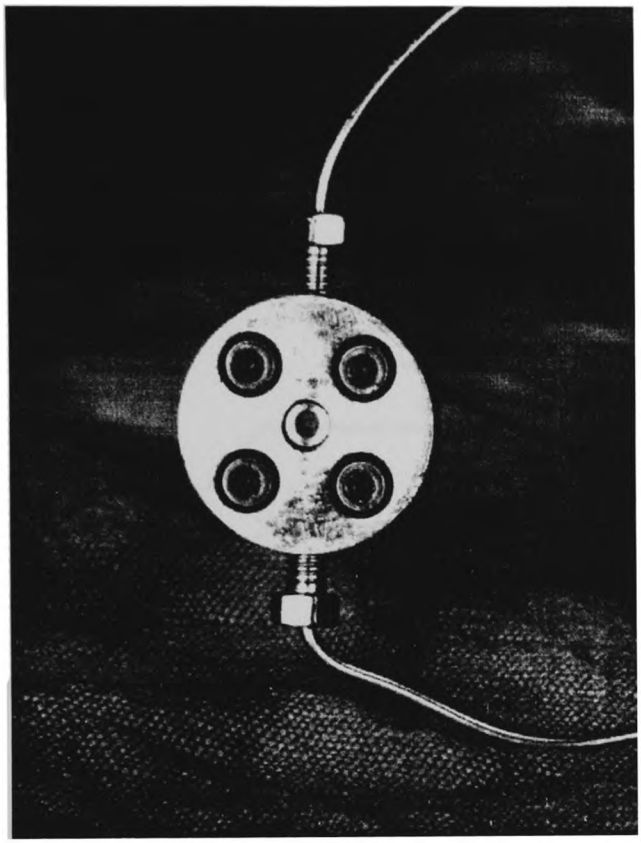


Fig 2..4 IR observation cell

This cell was housed in a Nicolet Magna-IR 550 FTIR spectrometer (Nicolet Instruments, Warwick, UK), which was used for data collection and analysis, using Series TGA/IR software.

The output from this cell was connected to a Gilson 821 pressure regulator, and then to a C-18 HPLC column to trap the extracted hydrocarbons.

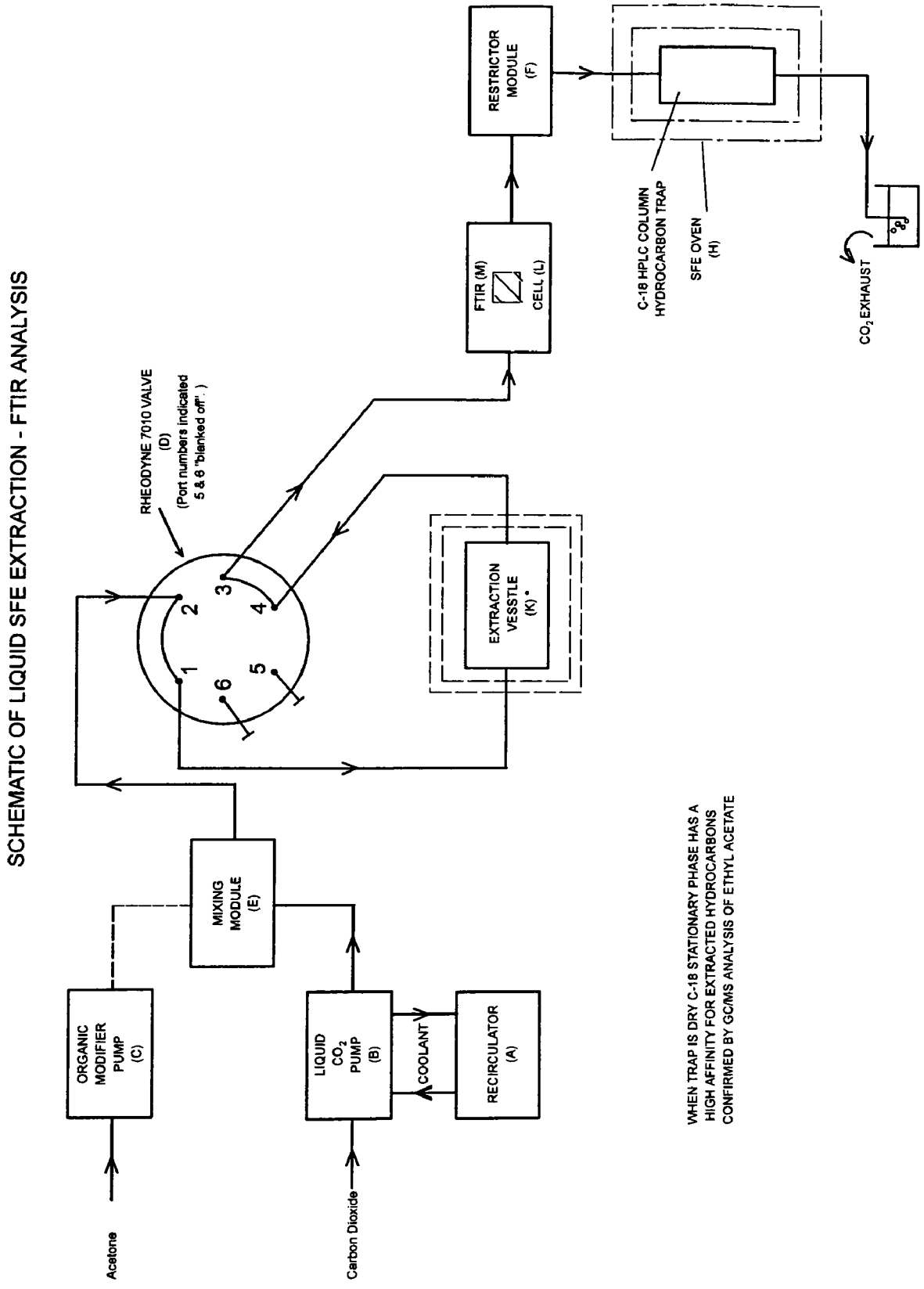


Fig.2.5 SFE extraction phase

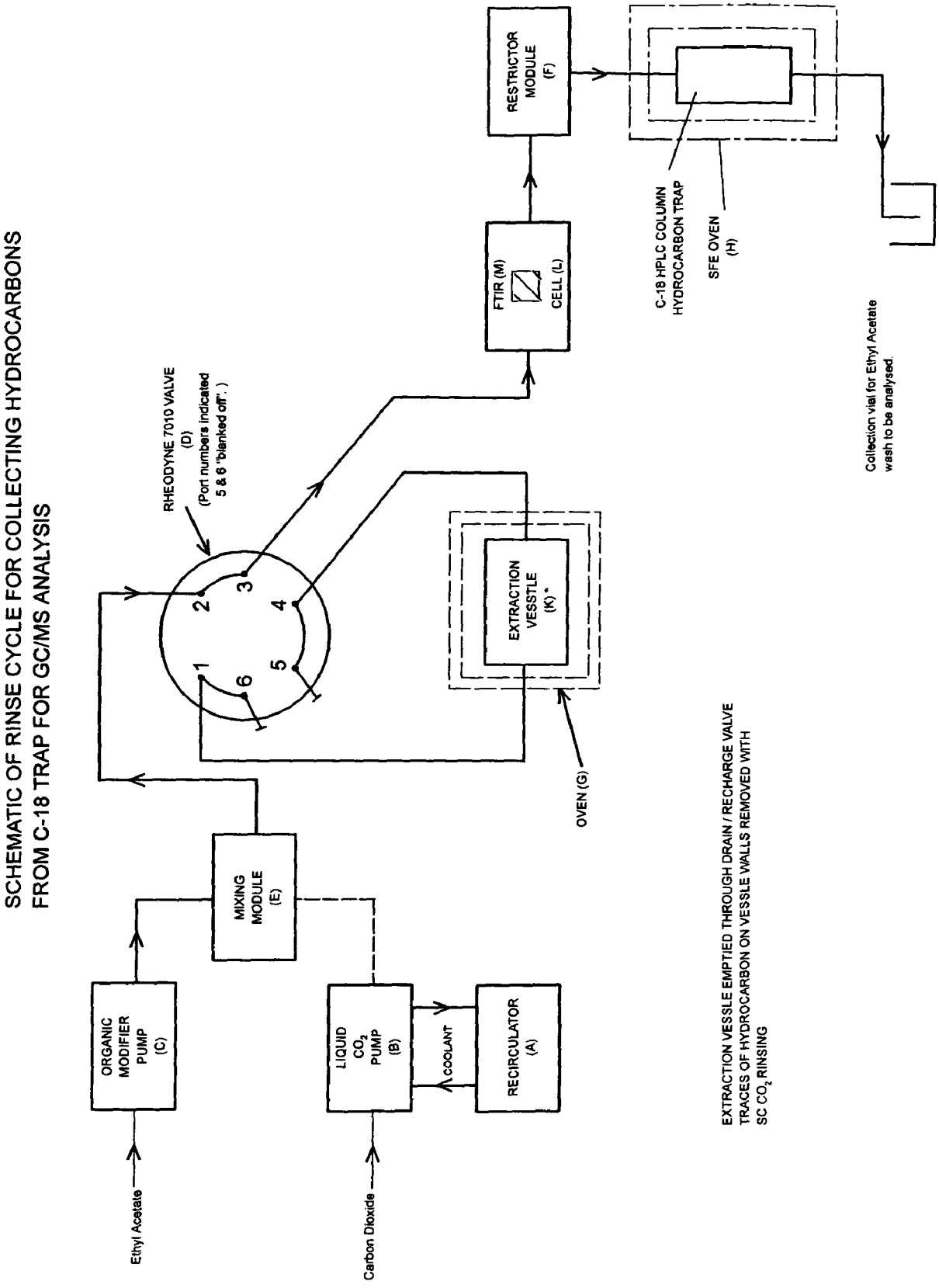


Fig. 2.6 SFE collection phase

Analytical grade dodecane (Sigma, Poole, Dorset,UK) was selected as the hydrocarbon for the study, on the basis of work previously carried out on % recoveries and volatilities of a range of different chain length hydrocarbons Fig.2.7.

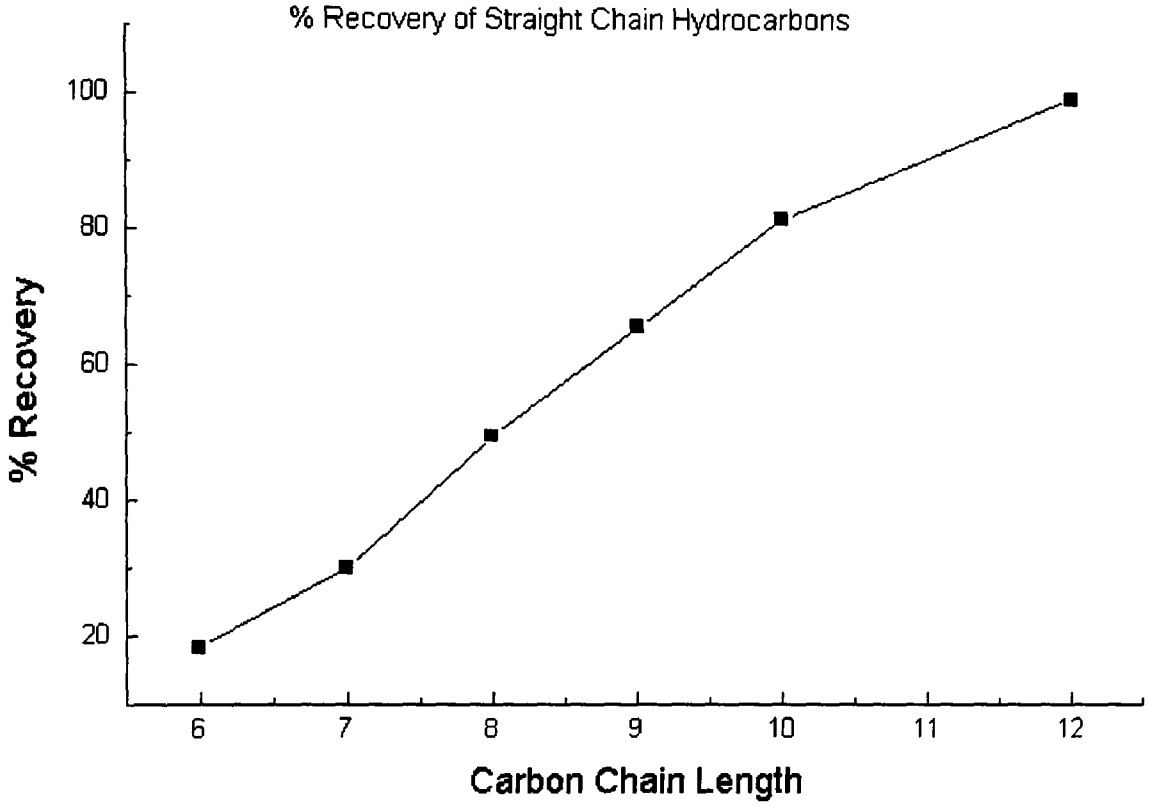


Fig. 2.7 Hydrocarbon recoveries

A set of standard solutions of known concentrations were prepared using analytical grade Freon113 (Sigma, Poole, Dorset,UK) as the solvent. These solutions had a concentration range of 6-200 ppm when 20 µl were injected into 50 cm^3 of water.
For each analysis the extraction cell was loaded with 50 cm^3 of deionised water and 20 µl of the standard dodecane solution. The vessel was then vigorously shaken for 5 min and allowed to stand for 30 min under ambient temperature and pressure conditions.

The extraction procedure was then initiated, with the extraction parameters set as

- extraction pressure = 13Mpa,
- temperature 50 °C, and
- CO_2 flow rate = 2.5 cm^3 min^{-1}

The spectral data were acquired, under dynamic extraction conditions, when the pressure regulator opened allowing a constant backpressure of 13 MPa, and collected in the range 4000 – 400 cm^{-1}, at a resolution of 4cm^{-1}. Each spectra consisted of 5 scans, at a sampling interval of 3.37 s. A total collection time of 20 min was adopted resulting in a total of 356 spectra for the complete data set.

A reference spectrum was acquired prior to the extraction, using 20 μl of pure Freon-113 and 50 cm^3 of deionised water.
Between analytical runs the extraction cell was emptied and cleaned in situ with supercritical carbon dioxide under the condition specified above

2.3.4 Infrared analysis

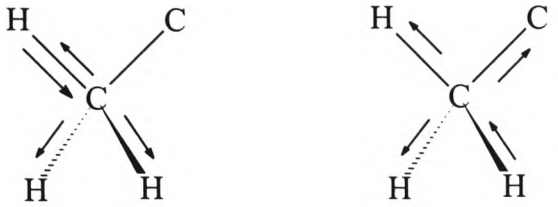
Infrared spectroscopy is based on the ability of a molecule to display a dipole change when irradiated with radiation of the correct energy to induce this change. The wavelength of radiation which corresponds to this energy range can be calculated from the Bohr equation

$$Energy = h\nu$$

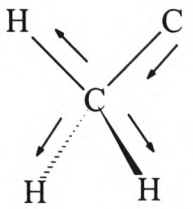
where h is Planck's constant, and ν is the frequency of the associated radiation.
The analysis of the extracted hydrocarbons was confined to the monitoring of the infrared absorption bands in the CH stretching region 3200 - 2500cm^{-1} (3 to 4 μm). These absorptions can be attributed to the symmetric and asymmetric stretch frequencies of the CH_3, CH_2, and CH groups as shown in fig. 8.

For hydrocarbons these modes occur at approximately;

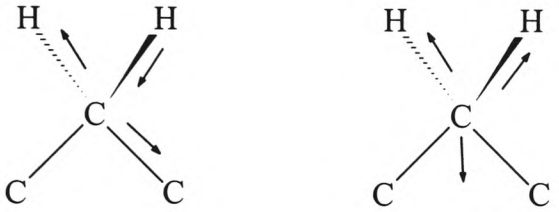
CH_3 (Asymmetric stretch) ≈ 2960 cm^{-1}
CH_2 (Asymmetric stretch) ≈ 2930 cm^{-1}
CH_3 (Symmetric stretch) ≈ 2870 cm^{-1}
CH_2 (Symmetric stretch) ≈ 2860 cm^{-1}



CH_3 Asymmetric Stretch



CH_3 Symmetric Stretch



CH_2 Asymmetric Stretch CH_2 Symmetric Stretc

fig.2. 8 Hydrocarbon stretching modes

The basis for the quantification of hydrocarbons using spectroscopic methods, as with I.R. spectroscopy, is the Beer Lambert Law, where:

$$A = \varepsilon c l$$

and

A = Absorbance = $\log_{10}$ (Io/I)

Io = radiation entering the sample

I = radiation transmitted by the sample

ε = molar absorbtivity

c = concentration

l = sample path length through which the radiation travels.

For a particular absorption band in the infrared spectrum, ε remains a constant, as does the pathlength when the same sample cell is used for each sample. This reduces the Lambert Beer Law to

$$A \sim c$$

and so a calibration graph of concentration against absorbance gives a linear response.

2.3.5 Infrared spectrum of supercritical carbon dioxide

The infrared spectrum of supercritical carbon dioxide is shown, Fig. 2.9 , this shows absorption peaks in the regions 3820-3500cm^{-1}, 2530-2150cm^{-1}, and 1450-400cm^{-1}.

This results in a region between 3500-2530cm^{-1} free of absorption peaks, and allows an unhindered view of the CH stretching region, Fig 2.10.

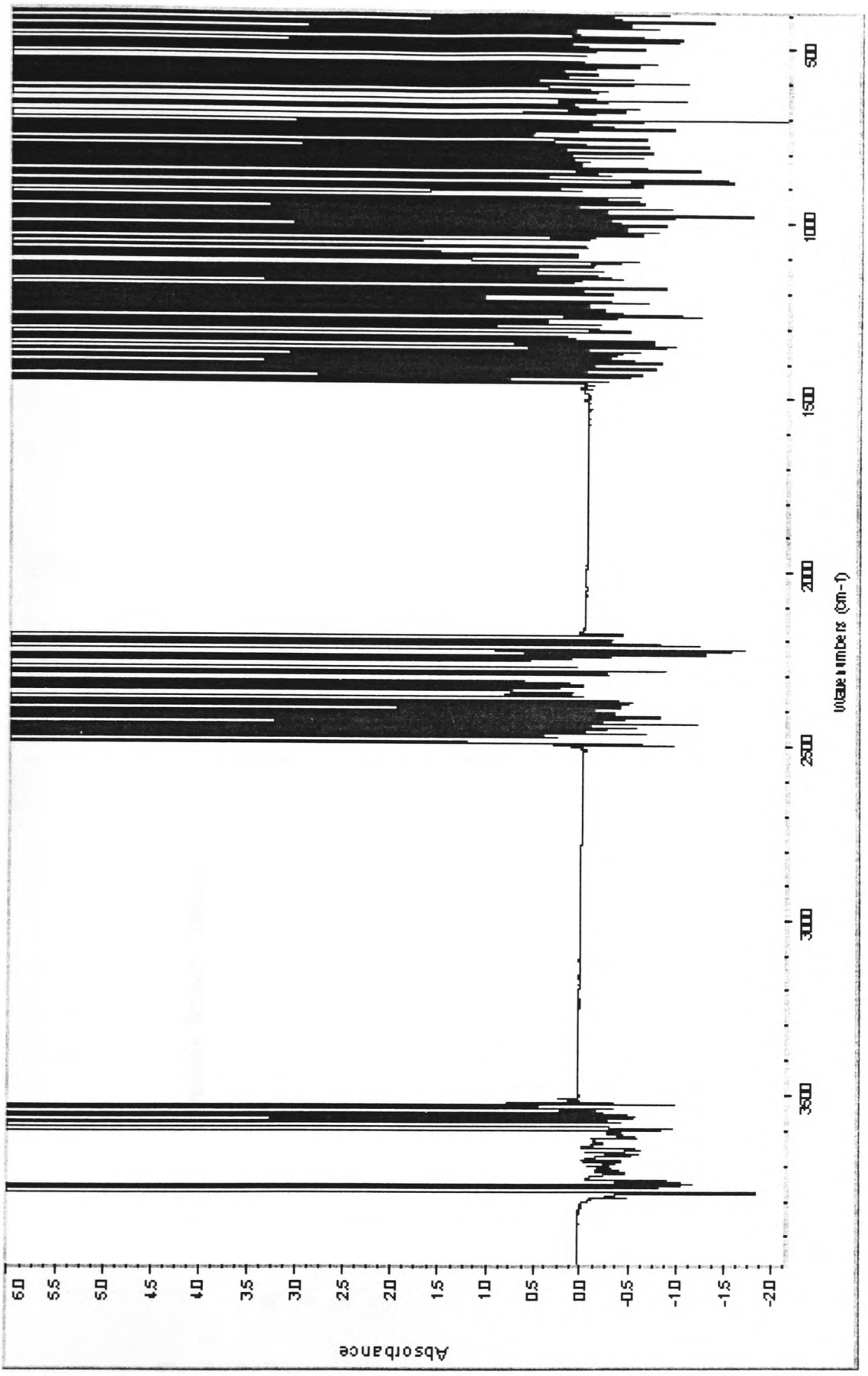


Fig.2. 9 Characteristic supercritical carbon dioxide IR spectrum

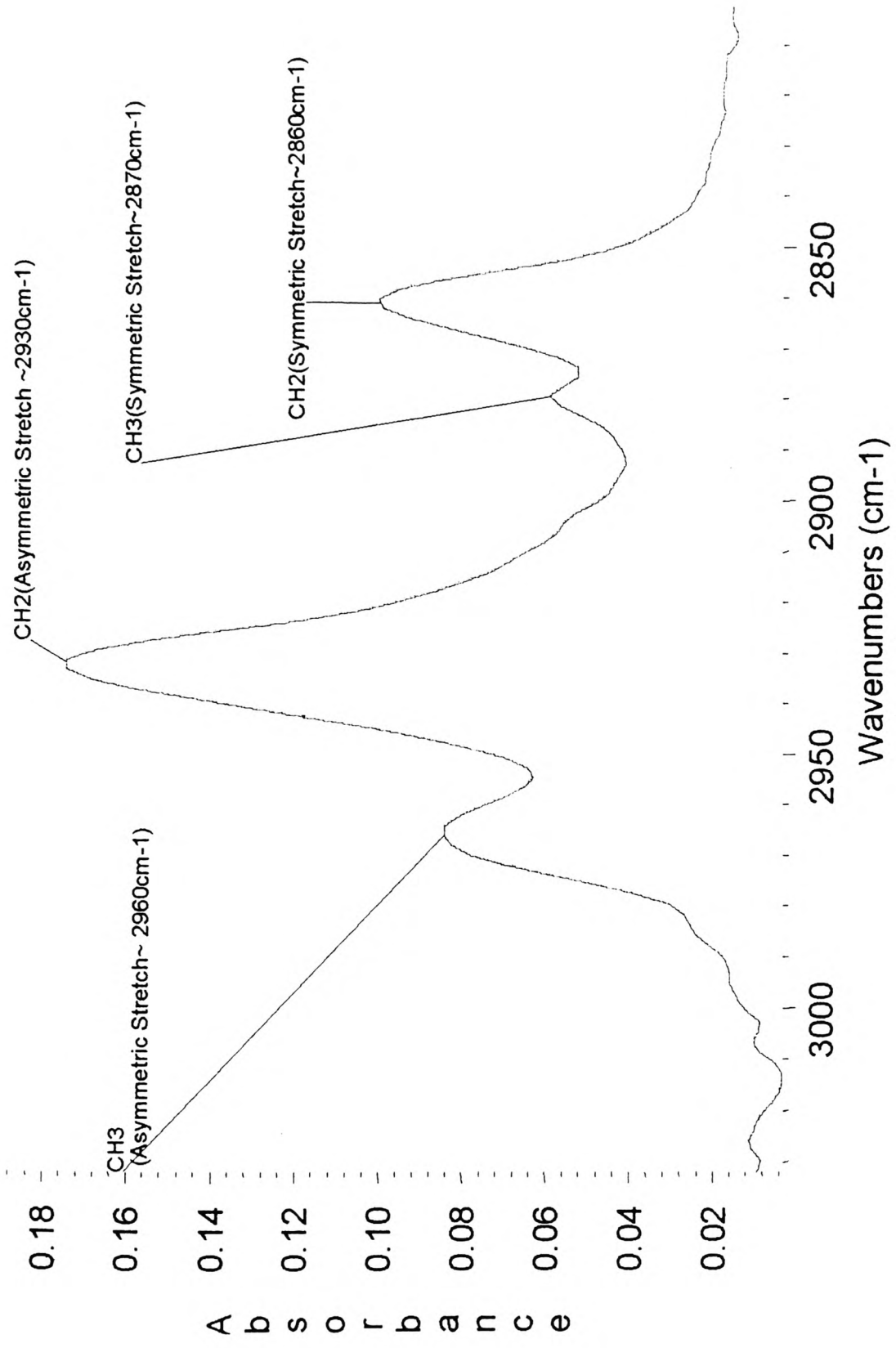


Fig. 2.10 Characteristic hydrocarbon infrared spectrum

2.3.6 Results obtained with the "T" cell

During supercritical fluid extraction of the dodecane from the aqueous solution, it was possible to continuously monitor the CH stretching region, this was achieved using the Series TGA/IR software previously mentioned.
This proved invaluable as initially individual spectra had to be collected over a period of time, and a graph of absorbance / time constructed to monitor the system extraction efficiency.

The data shown in Fig 2.11 & 2.12, are the results of the SFE-FTIR analysis of a 100 ppm dodecane spiked water sample.

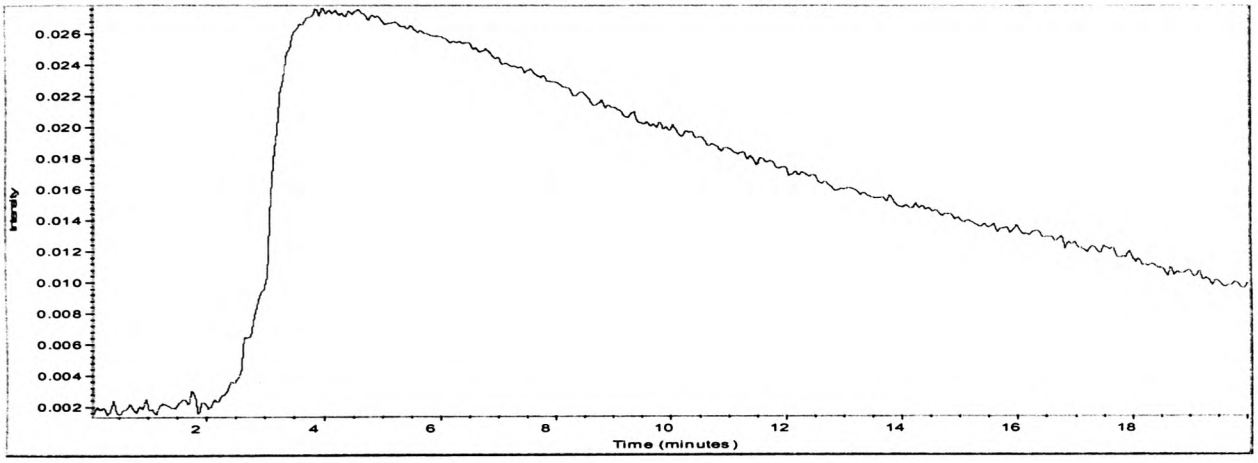


Fig 2. 11 Gram-Schmidt reconstruction of the data set showing the total absorbance in the range 4000 – 400cm^{-1}

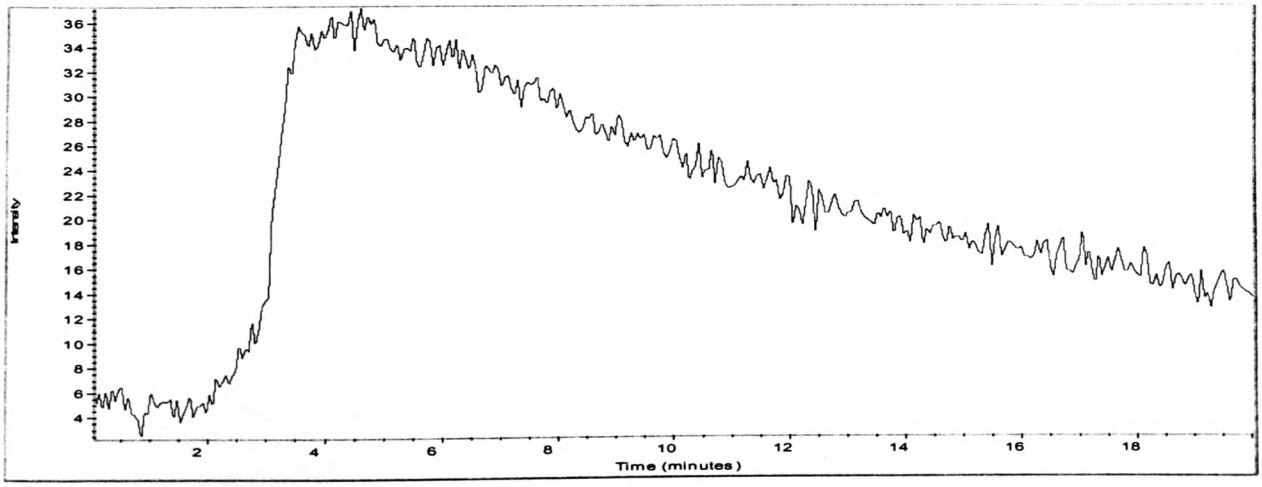


Fig 2. 12 Constructed chemigram for the absorbance in the CH stretching region 3000 –2800cm^{-1}

This data shows that the maximum hydrocarbon absorbance, and hence concentration, occurred after approx. 3.9 min. The initial delay in the response was due to transportation of the hydrocarbon SF-extract to the IR cell, and was shown to be in direct correlation to the flowrate of supercritical carbon dioxide used in the analytical run, at a pre-determined extraction pressure.

As the extraction proceeded, fresh supercritical CO_2 passed through the extraction cell, and the associated absorbance value decreased, as is clearly shown on the chemigram above.

Using the extraction profile obtained for the 100 ppm dodecane sample, a series of water samples spiked with dodecane in the concentration range 12.5 to 200 ppm were extracted, to determine the linearity of response.
The samples were extracted under identical conditions to the 100 ppm dodecane sample, and the absorbances for the asymmetric CH_2 stretching frequency at 2930cm^{-1} for the extraction period between 3.8 and 4.0 min. co-added.

2.3.7. Analysis of Results

The S/N (signal to noise) ratio at a concentration of 12.5 ppm, Fig 2.13, was measured as 3:1, and this was taken to be the lowest concentration dodecane sample that could produce a reliably measurable absorbance peak.

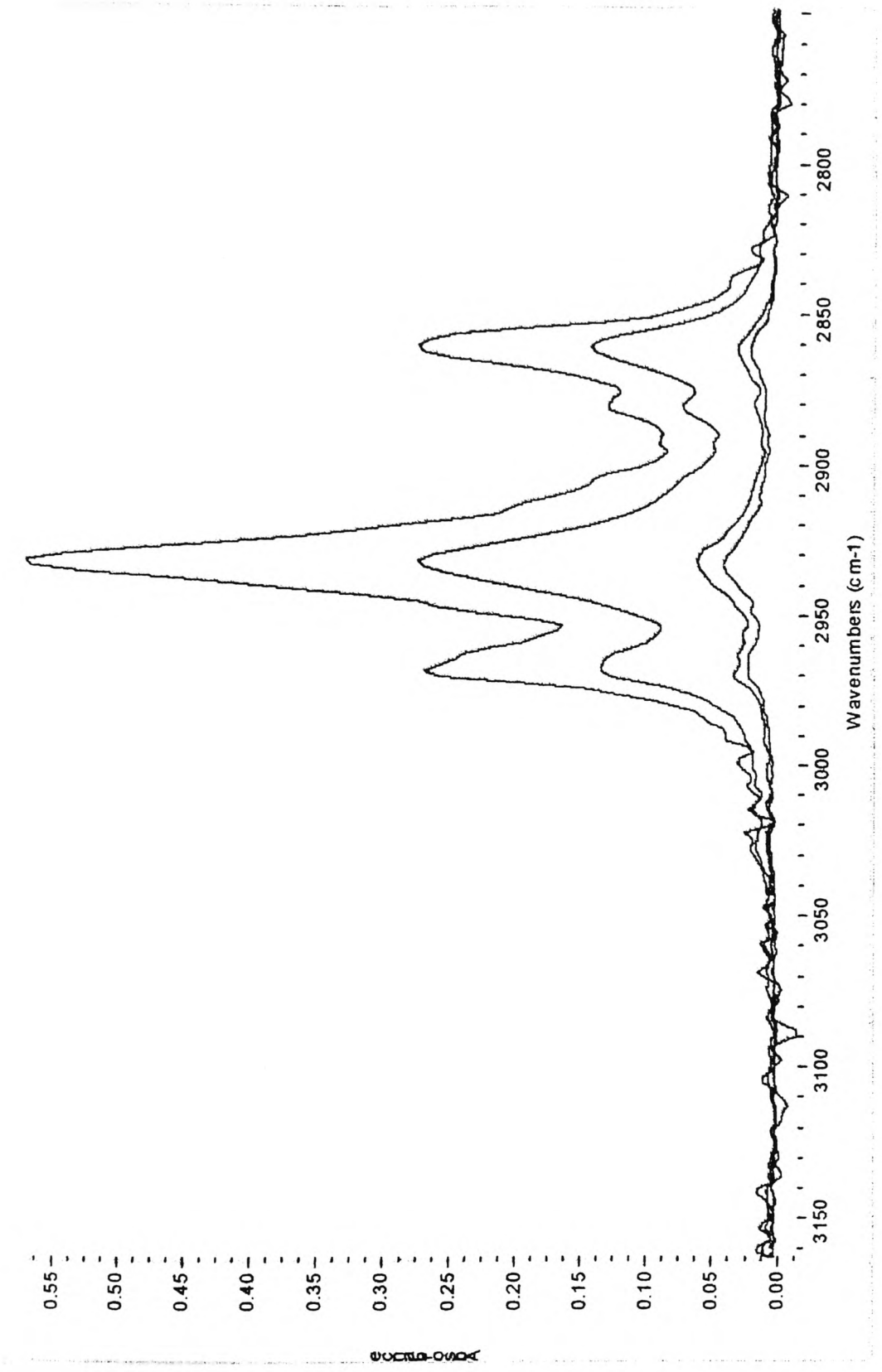


Fig. 2.13 SFE/FTIR spectra of 200 – 12.5ppm dodecane summed over a period of 3.8 – 4.0 min

Fig 2.14 below shows the calibration data for the dodecane samples in the concentration range 200 – 12.5 ppm. This initial data indicated a linear response between the concentration and absorbance of the CH_2 asymmetric stretching frequency (≈2930 cm^{-1}), as predicted from the Lambert- Beer law.

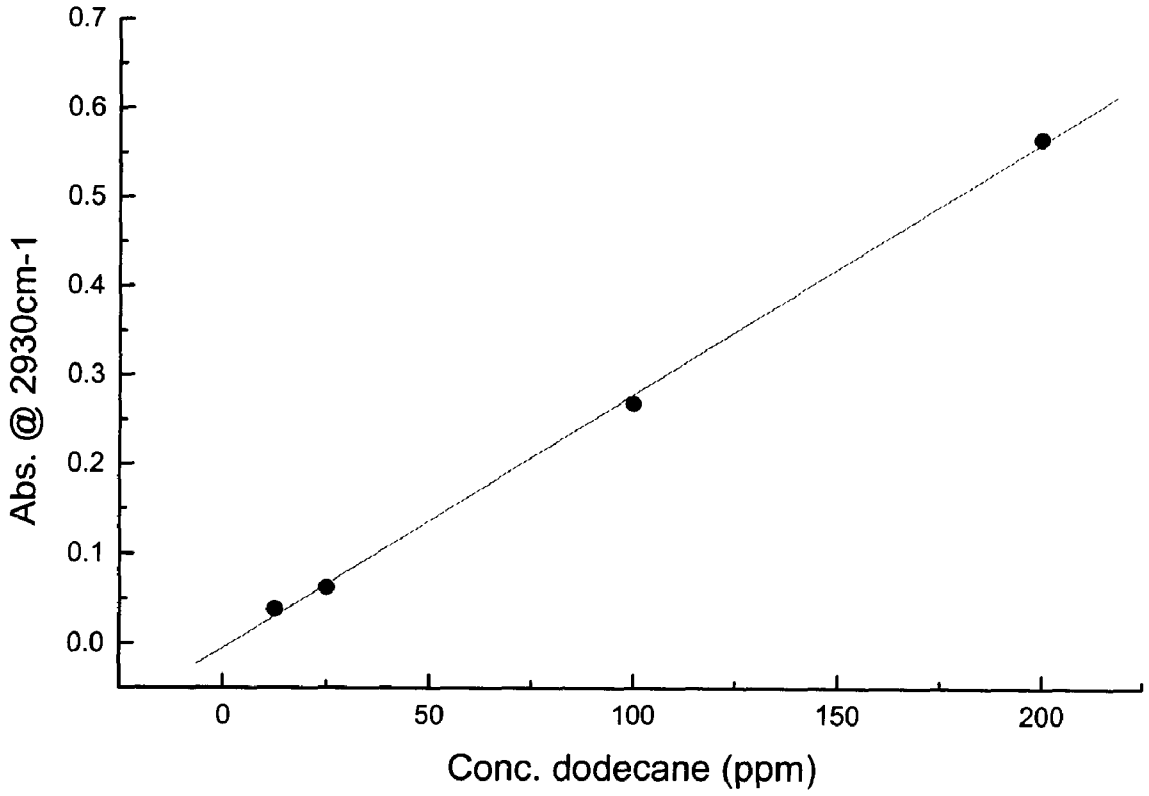


Fig. 2.14 Dodecane calibration graph

Correlation coefficient = 0.9995

2.3.8 Conclusions from experimental results

Even with the lack of an optimised SFE vessel design, the results were sufficiently encouraging to deem this phase a success. The following points had been answered;

i. Hydrocarbons were sufficiently solubilised in SF-CO_2 to allow their extraction from an aqueous matrix.

ii. Extraction could be achieved without the carryover of prohibitive quantities of water which would have made the system non-viable.

iii. Even using a dynamic flow of SF-CO_2 to extract the hydrocarbon, sufficient reproducibility was attained to allow a linear response between the hydrocarbon absorbance value and concentration, reliable quantification has been shown to be possible.

2.3.9 Further development of extraction system

The following two points were seen as shortcomings of the "T" SFE vessel system, and would have to be improved in future developments.

1. Water carryover

One of the major problems encountered with this extraction system was water carryover, even the inclusion of a 20 micron stainless steel filter on the top draw-off line failed to completely prevented the problem. This drawback was attributed to two separate problems with aqueous extractions using supercritical fluids.

a) water is solubilised in SF carbon dioxide in the range 0.3 to 3%, depending on its density [36].

b) simple transportation of the water with the SF-CO_2 flow.

This resulted in the following problems:

i. a periodical build up of water droplets on the quartz infrared cell window which caused instability in the infrared response.

ii. blockage in the restrictor module as the water froze due to the Joule Thompson cooling effect, causing inconsistent and interrupted flow.

To alleviate problem (i) without having to disrupt the compression fittings of the IR cell, an extraction cell by-pass valve was fitted which enabled acetone to be pumped through the IR cell from the second "modifier" pump. The acetone was then flushed from the IR cell by SF-CO_2 until baseline stability was regained. This arrangement allowed minimal system disruption.

To alleviate problem (ii) the exhaust line from the restrictor was heated using a thermostatically controlled cartridge heater, of 100 watts power rating.

2. Filling and cleaning the extraction cell.

Because of the problem of air displacement from the vessel, filling, and hence cleaning, became a slow process. As the system could be pressurised, emptying was not problematic.

These two problems would have to be considered in the preparation of the next generation of extraction system, if continuing progress towards a system suitable for routine use was to be achieved.

2.4 Development of the second extraction system, welded 300 cm^3 volume vessel.

2.4.1 Construction and design considerations.

Using the experience gained from hydrocarbon extraction with the "T" SFE vessel, a second cell was designed with the shortcomings of the last considered.

A system was custom built, of welded stainless steel construction and had an internal volume of 300 cm^3

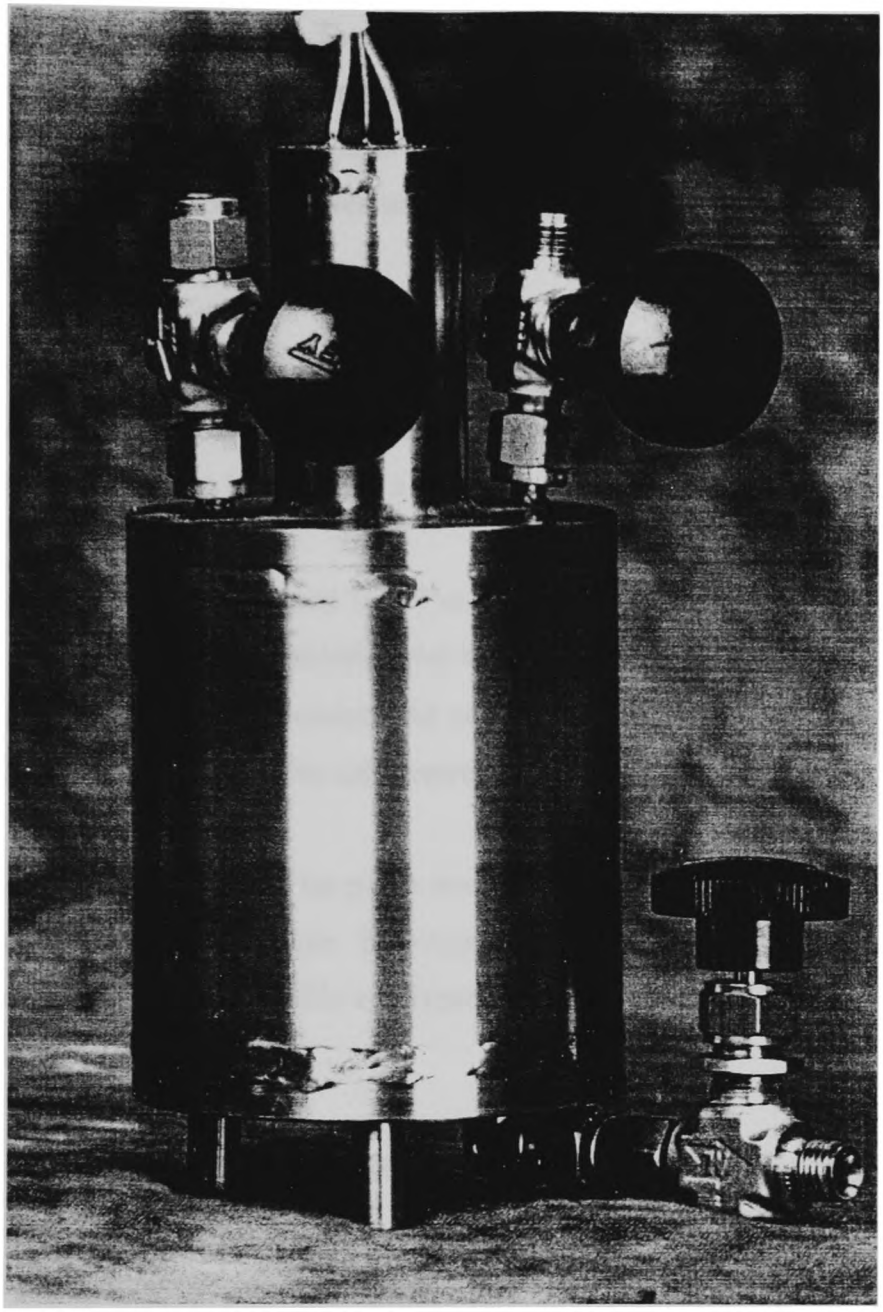


Fig. 2.15 300 cm^3 extraction vessel

The design, Fig 2.15, included a large diameter body with a vortex mixer installed at the bottom to allow a more controllable introduction of the SF-CO_2 to be achieved. A smaller diameter upper section was welded to the main body, where the 1/8 in.o.d. draw off tubes were positioned. These draw off tubes were protected by a series of baffles arranged within the neck of this tube to prevent any egress of water.

The filling and cleaning inefficiencies experienced with the "T" SFE vessel were negated in the new system by the inclusion of three Whitey high pressure valves, two

at the top, and one at the bottom. These allowed quick and easy filling and cleaning to be achieved. During the filling cycle, an off-axis funnel was attached to one of the top Whitey valves, while the other acted as a vent allowing expelation of the air. During the drain cycle a drain tube was fitted to the bottom valve allowing the contents to be easily, and controllably, expelled.

To clean the extraction system, the vessel was charged with water, pressurised, and then drained, this was repeated several times, to ensure complete removal of analyte, and checked between runs by scanning a blank water sample.

The extraction system was housed in a Pye 104 gas chromatography oven, the SF-CO_2 inlet line (1/16 inch o.d. stainless steel tubing) connected to a Gilson 303 HPLC pump, whose pump head was maintained at –5 °C by a Neslab recirculator chiller system, enabling liquid CO_2 to be delivered to the pump head.

Situated between the vessel and the pump was a Gilson 802C pressure regulator, used to set the target extraction pressure. The output from this regulator was fed to a chart recorder where the pressure profile and reproducibility of the extraction process was monitored.

The addition of a Rheodyne 7125 HPLC valve, fitted with a 20 µl injection loop, between the pressure regulator and the extraction vessel allowed the accurate addition of known concentrations of hydrocarbon solutions for detailed calibration and quantification studies.

On the basis of simplicity and reproducibility, it was decided that the second extraction system would be operated in a "stopped flow" method of analysis. This meant that the flow of analtye solubilised in CO_2 would be halted during the analysis period. This had several benefits;

a. the removal of the restrictor, which had caused problems in the "T" SFE vessel by icing up due to the water carry over, and the cooling effect caused by the Joule Thompson effect.

b. The removal of inaccuracies due to inconsistent flow.

c. The errors associated with time dependant extraction of the hydrocarbons.(see chemigram in section 2.3.6), Fig.2.11 & 2.12.

The selection of a "stopped flow" method of analysis while removing certain problems introduced an additional one, inconsistency in the diffusive flow from the extraction cell into the IR cell. As there was no longer any SF-CO_2 and solubilised hydrocarbon flowing through the IR cell during analysis, it meant that diffusion alone could not ensure a representative aliquot of the final SF-CO_2 solution was being analysed. The effect of this is shown in Fig 2.16 where it is evident that under static flow conditions, slow diffusion characteristics become important in terms of spectral stability.

This problem was overcome by the addition of an expansion column after the IR cell, and isolated from the system until the target pressure was attained. At this pressure the isolation valve was opened and sufficient SF-CO_2 and solvated analyte, allowed to expand into this pre evacuated column to ensure consistency of concentration to allow reproducible results to be obtained. This incorporation and operation of the modified SFE system, including the expansion cell, are summarised in Figs 2.17 and 2.18.

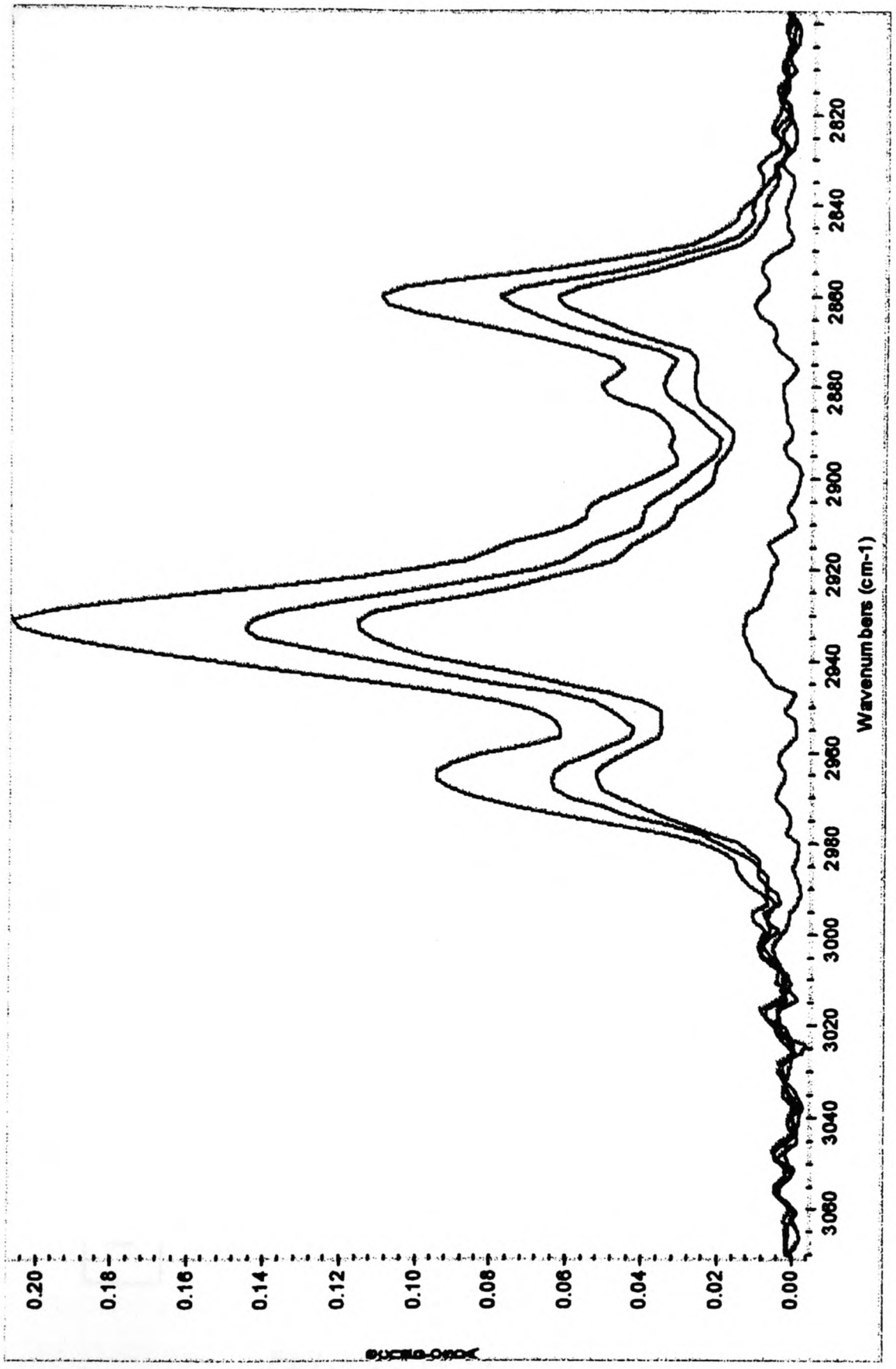


Fig.2.16 Spectra showing the effect of diffused transportation of SF-CO_2 solubilised dodecane (t=0.5, 1.0, 2.0, 4.0 min) (bottom to top)

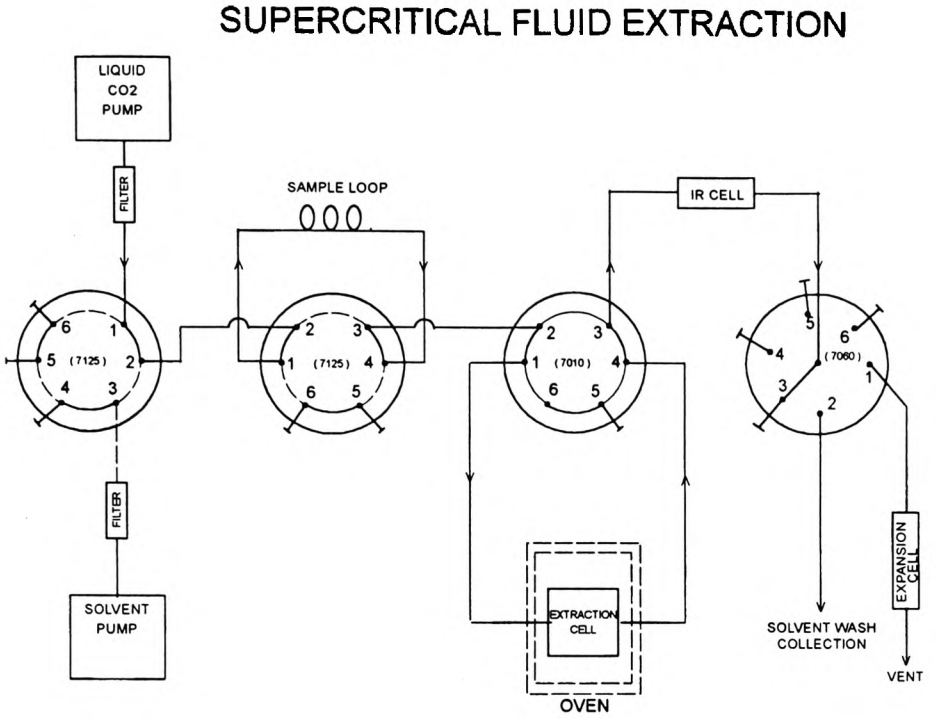


Fig. 2.17 SFE extraction cycle

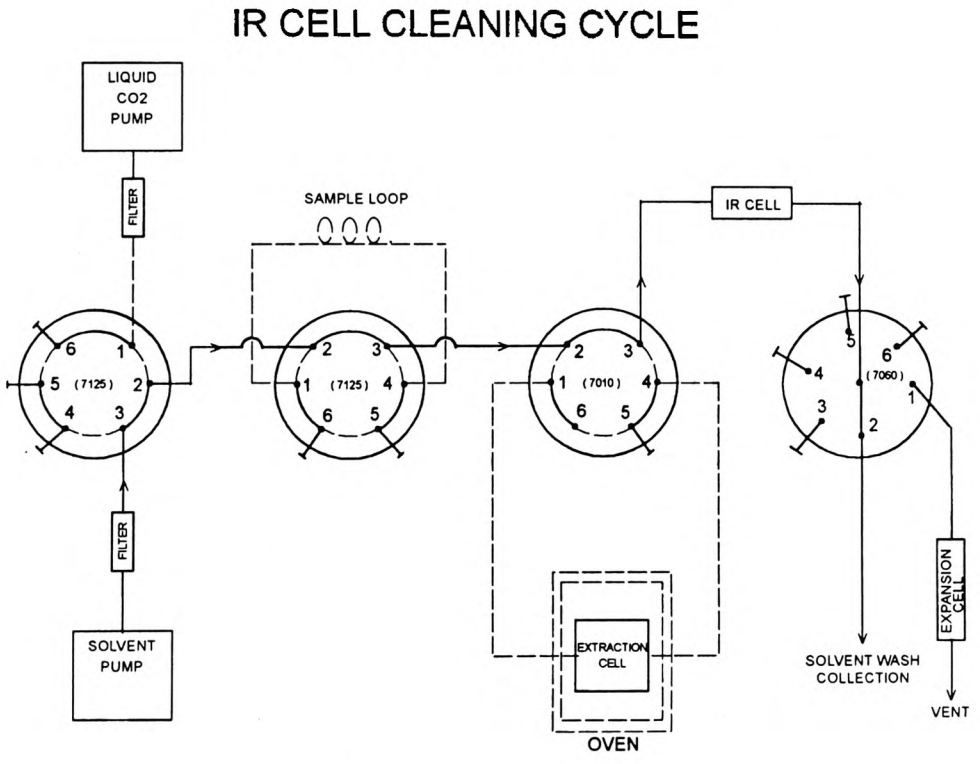


Fig.2.18 SFE cleaning cycle

2.4.2 Experimental data obtained using second generation extraction system.

The extraction system was assembled as shown, fig 16 and 17, and the Standard Operating Procedure (SOP) developed. This SOP formed the basis for all hydrocarbon extractions completed with this system.

This phase of extraction development included analysis of the following,

a. Linearity of IR absorbance with hydrocarbon concentration.
b. Reproducibility of results
c. Detection limits
d. Pressure effects, including extraction efficiency at varying SF pressure.

2.4.3 Procedure for SFE-FTIR hydrocarbon analysis

The following experimental parameters were used:-

- Oven temp = *(55)* ℃
- Recirculator temp = *(-5)* ℃
- Flow rate *8* cm^3 min^{-1}
- Target pressure *2500* psi

The pump head take approximately 1 hour to cool to -5 ℃ -

Ensure background spectra are collected prior to running hydrocarbons, and extraction cell has been cleaned prior to use.

1. Open liquid CO_2 cylinder
2. Set valve 7125 [A] to CO_2 (turn clockwise).
3. Load extraction cell with 250 cm^3 of deionised water
4. Allow 15 min. to equilibrate.
5. Load required hydrocarbon sample into 20 µl loop.
6. Start pressurising the vessel (turn valve 7010 [C] clockwise)

7. Immediately inject required hydrocarbon sample into extraction cell (turn valve 7125 [B] clockwise).
8. Allow vessel to reach required extraction pressure.
9. Allow 5 min to equilibrate
10. Alter value 7060 [D] to open expansion volume (turn one place clockwise).
11. Allow the pressure to recover.
12. Collect spectra.
13. Turn valve 7010 [D] back to original position (one place anti-clockwise)
14. Turn valve 7010 [C] anti-clockwise to isolate extraction cell.
15. De-pressurise extraction cell by slowly opening one of the top valves on the cell.
16. Turn valve 7010 [D] clockwise two places to flush IR cell with fresh CO_2 and open bottom valve on expansion column to depressurise. Empty water from extraction cell.
17. Fill extraction cell with 250 cm^3 of deionised water, and allow to stand for 5 min.
18. Empty water from extraction cell. And repeat from point 2 for a new sample.

One of the major drawbacks with the "T" SFE vessel, was the lack of accurate information about the vessel extraction pressure, and reproducibility of pressurisation. Both of these problems could be attributed to the inconsistency of performance of the restrictor system, i.e. icing up causing the flow to be further restricted or stopped, resulting in a large oscillation of pressure around the pre-set extraction pressure.

With the addition of a pressure transducer to the new system, the pressurisation profile and reproducibility of attainment of the target pressure were monitored.

It is seen from the experimental pressure profile, Fig 2.19, that, as expected, the volume of water introduced into the extraction system has a direct effect on the pressurisation time, an additional 50 cm^3, reducing the pressurisation time by approximately 6 min. It can also be seen that the profile is not linear, and so doubling the extraction pressure does not mean simply doubling the pressurisation period.

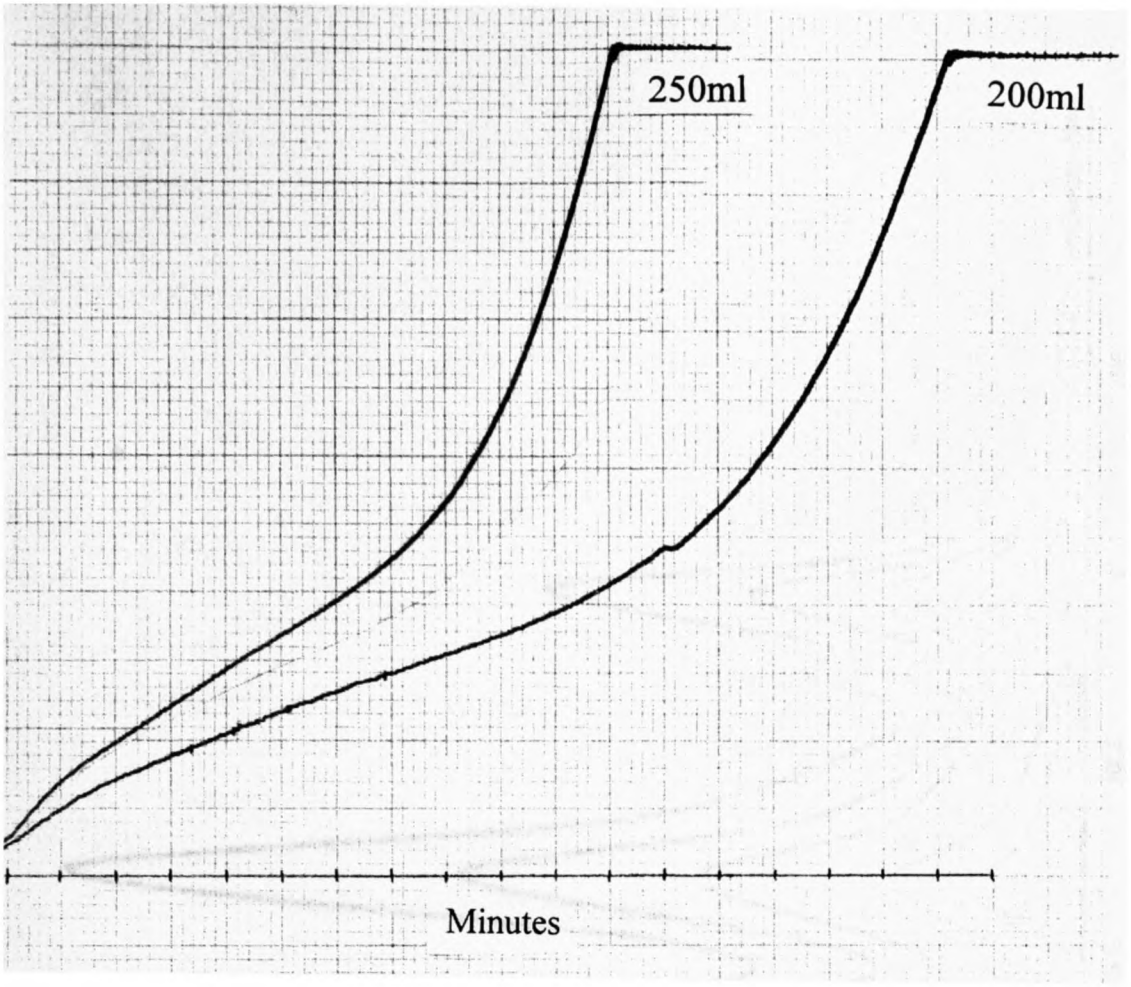


Fig. 2.19 CO_2 pressurisation profile with an extraction volume of 200 and 250 cm^3

a. Linearity of absorbance with hydrocarbon concentration

The linearity of absorbance as a response to hydrocarbon concentration was investigated using a series of dodecane standard solutions in the concentration range of 0.94 to 15 ppm, all analysed using the SOP described earlier.

The spectra, Fig 2.20 and 2.21, show the absorbance of dodecane in the region 3100 to 2750 cm^{-1}.

The absorbance values associated with the concentration range of the dodecane samples were then measured, and a graph of concentration / absorbance constructed. This graph, Fig 2.21, indicated that the range of concentrations used obeyed the Lambert- Beer law, and gave a linear response.

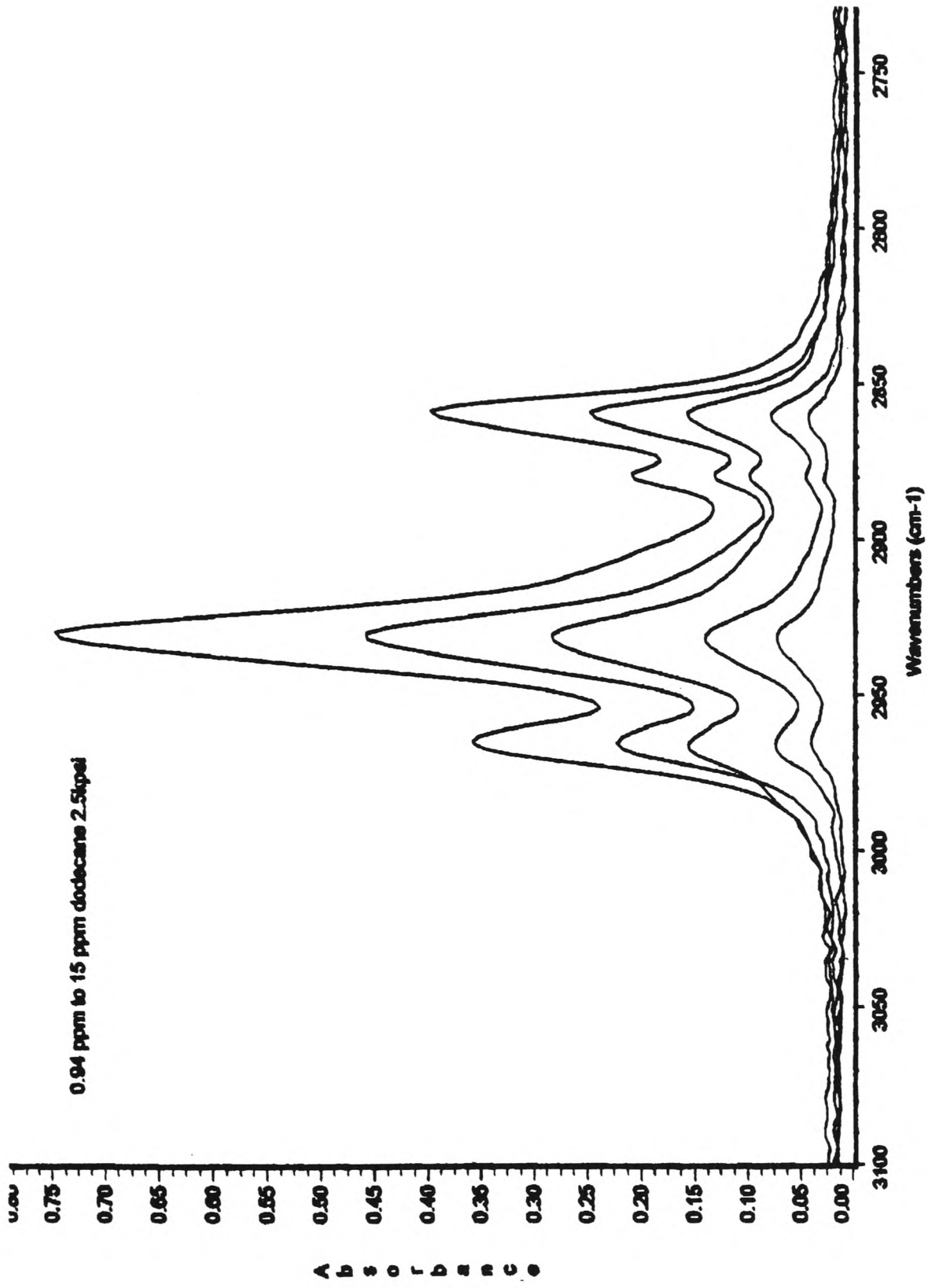


Fig. 2.20 Linearity of absorbance with dodecane concentration in the range 0.94 to 15 ppm (bottom to top)

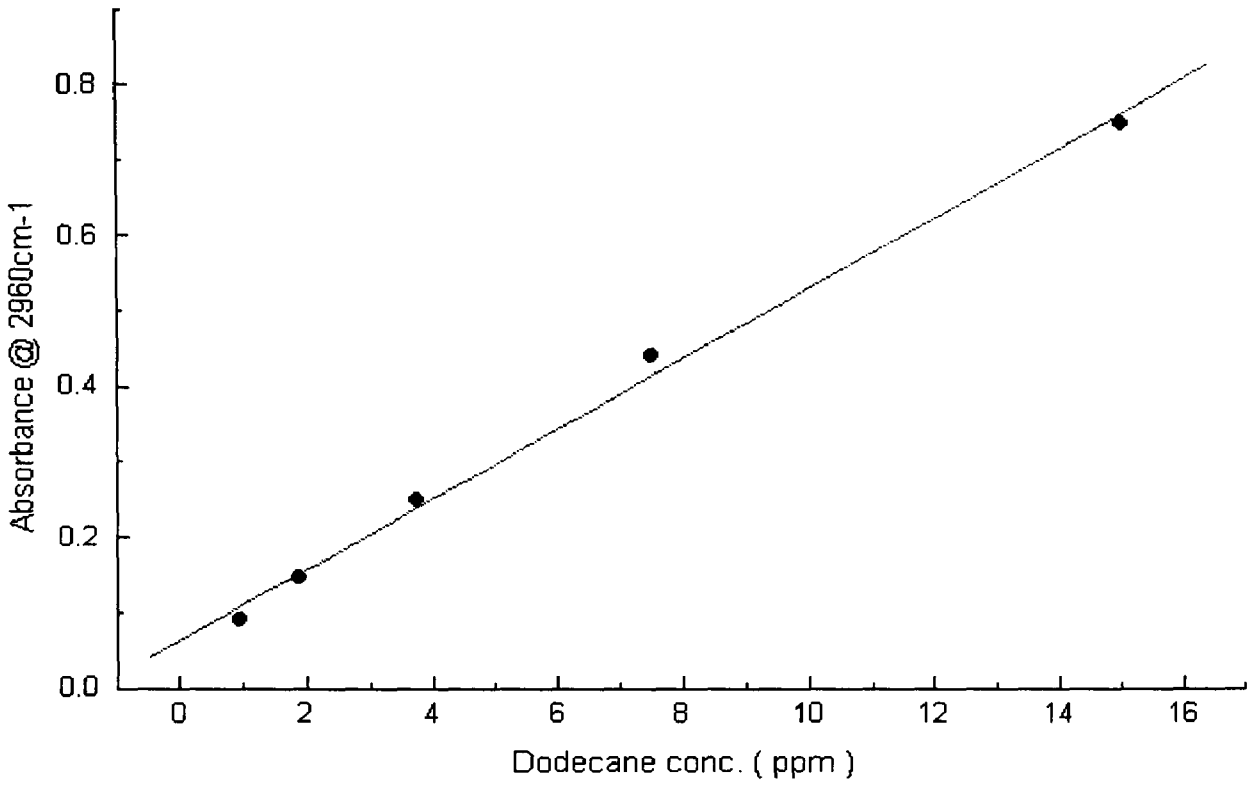


Fig 2. 21 Dodecane calibration graph

correlation coefficient = 0.9976

b. **Reproducibility of the system**

Fig 2.22, shows the reproducibility of multiple extractions (N=5) of 15 ppm dodecane solutions under identical conditions, Standard deviation = 0.05417.

c. Detection limits

While it was important to observe the response of the extraction system with respect to linearity and reproducibility, it was equally important to estimate the lower levels of detection.

It was shown that a linear response was achieved to a concentration level of below 1 ppm Fig 2.23. The signal to noise ratio of this lowest concentration extraction was measured at 23:1 and on this response it would have be possible to obtain a resolvable spectra at approximately 0.1 – 0.2 ppm.

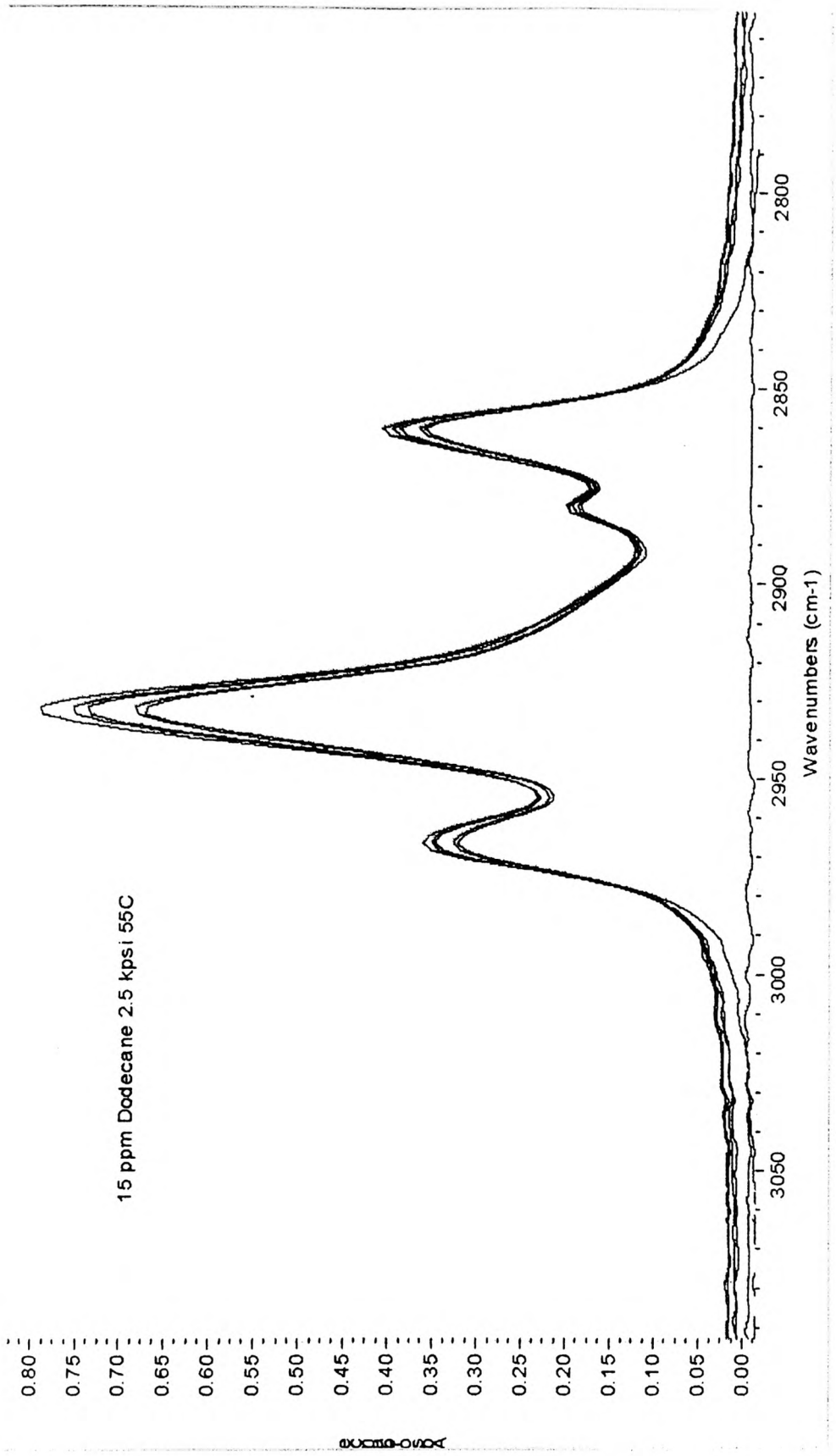


Fig. 2.22 Dodecane reproducibility

Repeat extractions of 15 ppm dodecane solutions at 2.5 kpsi and 55 ℃ (N=5)

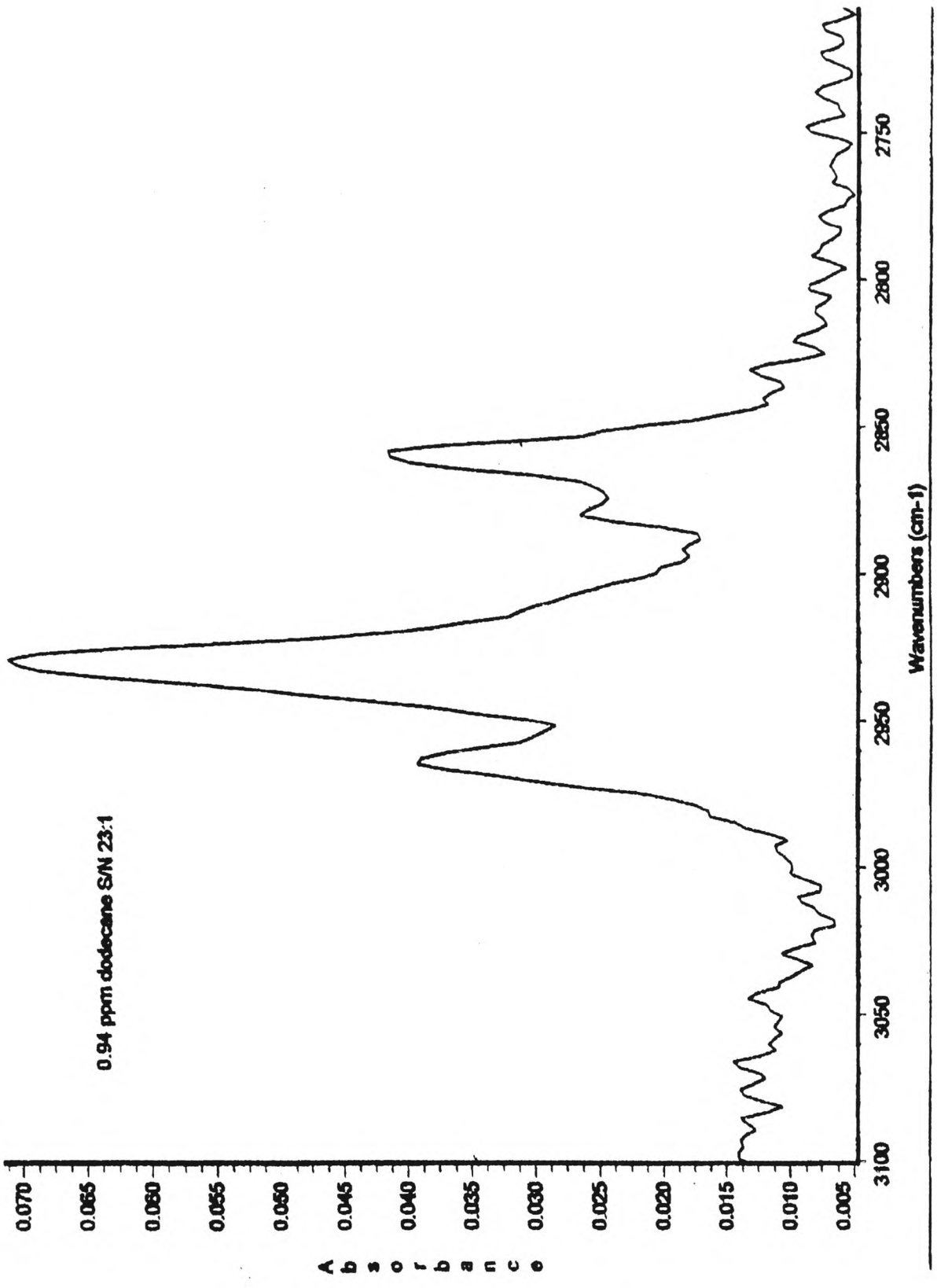


Fig. 2.23 Absorbance of 0.94 ppm dodecane solution extracted at 2.5 kpsi and 55 °C

d. Pressure effects and the extraction efficiency with respect to varying pressure

Multiple extractions were performed on a 15 ppm dodecane solution at varying pressure, to determine any discriminatory effects. Fig 2.24, shows that there was some evidence of extraction efficiency variation over the range 1.5 to 4.0 kpsi.
A compromise situation was adopted where extractions were carried out at 2.5 kpsi, this resulted in a reasonable pressurisation period, and a mean absorbance value of only 9% lower than for 4.0 kpsi.

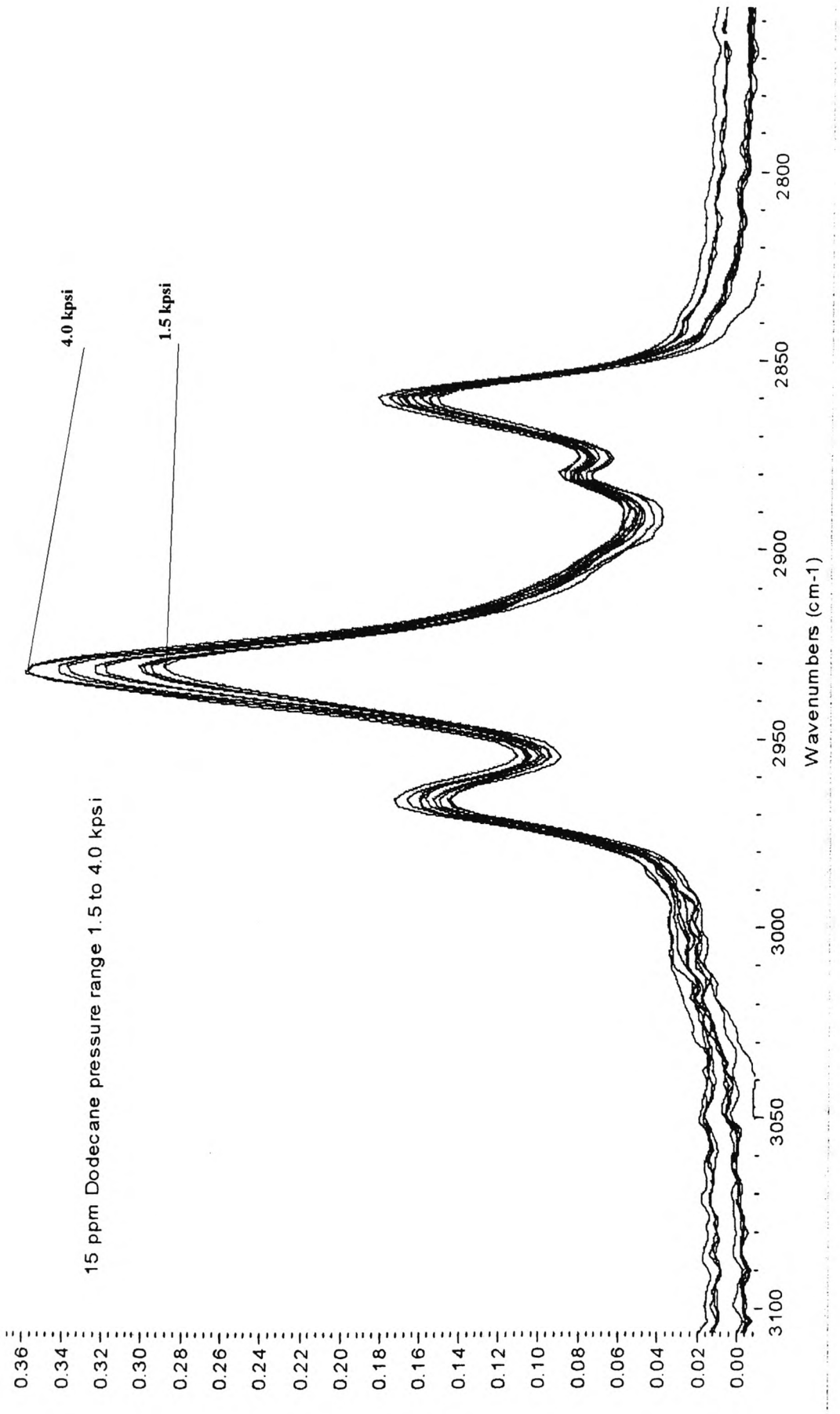


Fig. 2.24 15 ppm dodecane extractions in the pressure range 1.5 – 4.0 kpsi

2.4.4 Overview of the performance of the system, and design implications for the next vessel

During the process of hydrocarbon analysis using this system, three potential problems were identified which needed to be addressed in the next system.

1. Hydrocarbon carryover from high concentration samples.
2. The inability to introduce the whole water sampling bottle into the system.
3. The vessel heating arrangement

1. Hydrocarbon carryover

When samples of 30 ppm dodecane were analysed, it was observed that the following blank water sample indicated trace level cross-contamination arising from the previous sample Fig 2.25. This was envisaged as a problem of re-condensation of the solubilised hydrocarbon on to the vessel walls during the depressurisation stage of the SF-CO_2. The next vessel should therefore have an associated removable top, to allow cleaning with a volatile solvent, i.e. propanone, if necessary.

2. Incomplete sample transfer

One major drawback with this system has been the inability to accept the bottled sample into the vessel for extraction, thus resulting in residual hydrocarbon remaining on the walls of the bottle after the water sample was poured into the vessel. Again the addition of a removable top to the next vessel meant that this problem could be overcome.

3. Vessel heating arrangement

The present system was housed in a Pye 104 G.C. oven, this was clearly not suitable and so an alternative method of heating for the next extraction vessel was required. An obvious alternative was a band heater with integral thermocouple and temperature controller.

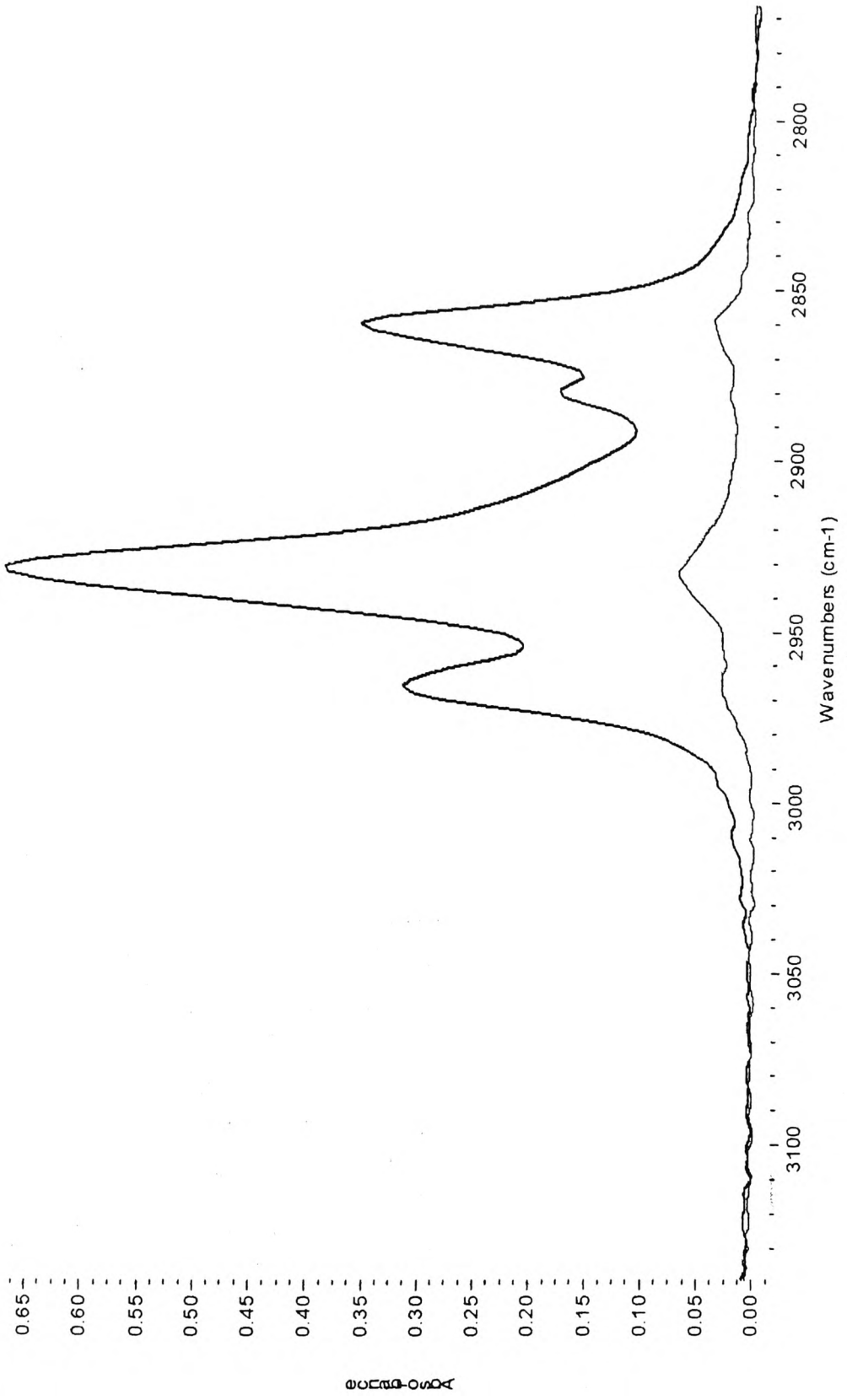


Fig.2. 25 Carryover spectra of 30 ppm dodecane extraction and blank showing hydrocarbon

2.5 Development of a third extraction vessel, the removable top vessel.

2.5.1 Construction and design considerations

Utilising the points raised for consideration from the last vessel, this extraction system was designed to overcome the inadequacies of the welded vessel, namely

i. the ability to introduce, and therefore extract, the complete bottled sample.

ii. the ability to clean the inside of the vessel with a volatile solvent, if necessary, thus reducing hydrocarbon carry over.

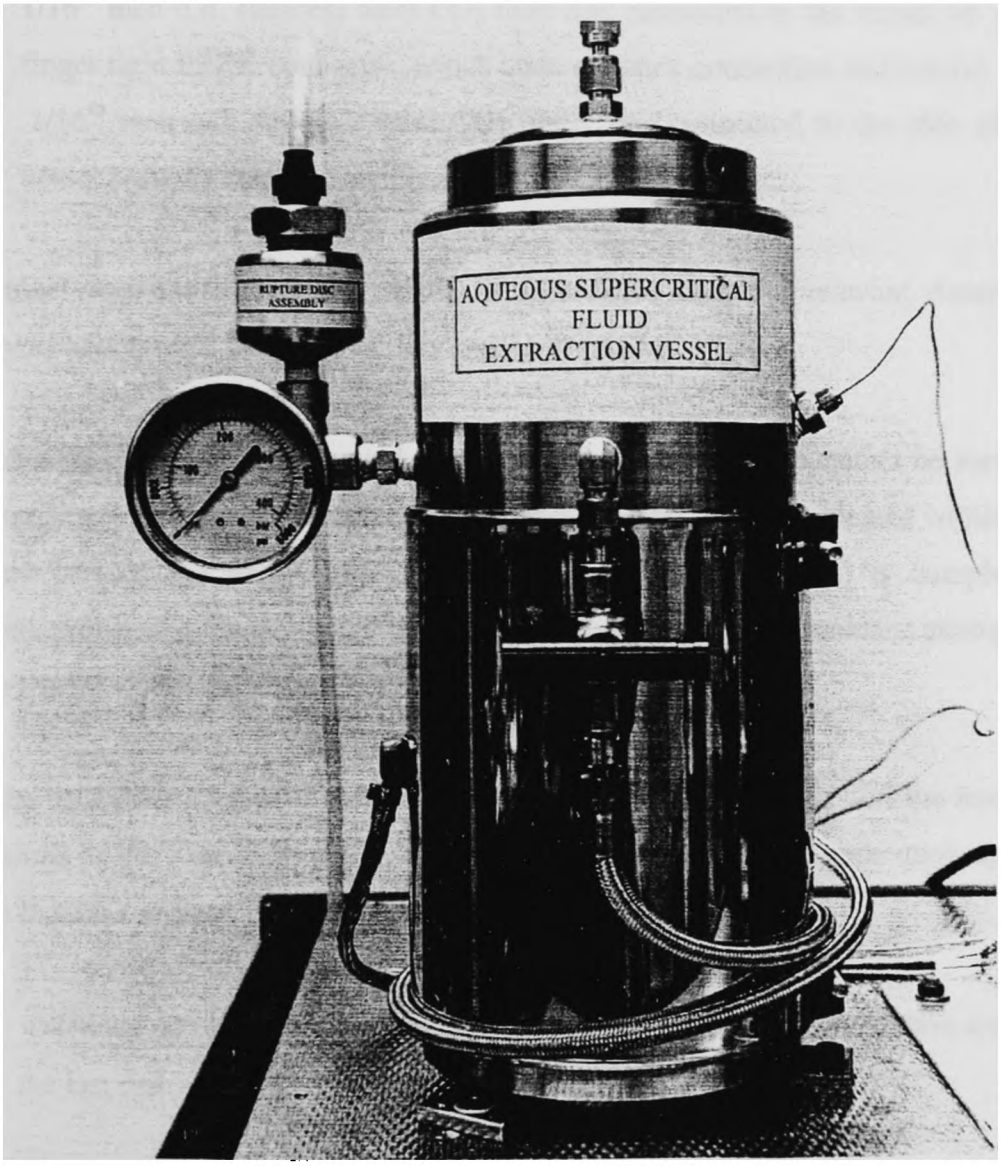


Fig. 2.26 Fourth generation SFE vessel

From Fig 2.26, it can be seen that the new extraction system consisted of the following parts,

i. a pressure vessel body, constructed from a single piece of stainless steel, and pressure tested to 6000 psi
ii. a removable lid, which allows the easy insertion of a 500 cm^3 sample bottle.
iii. de-pressurisation valve, connected to a stainless steel braided outlet tube
iv. integral analogue pressure gauge
v. rupture disk assembly, rated at 4000 psi
vi. integral heating jacket, controlled by a temperature control unit
vii. $1/16^{th}$ inch o.d. stainless steel CO_2 inlet line connected to the vessel lid via a finger tight PEEK connector, which allowed quick connection and release
viii. $1/16^{th}$ inch o.d. stainless steel CO_2 outlet line connected to the side of the vessel body by standard stainless steel HPLC fittings.

The vessel was constructed and tested in accordance with the relevant American Society of Mechanical Engineers safety standards (ASME VIII)

Another safety feature, incorporated into the extraction cell, which cannot be seen on the photograph, is the inclusion of a de-pressurisation port, which is located within the threaded part of the vessel body, and is activated before the lid is completely removed, preventing the explosive release of the vessel top should someone attempt to release the lid whilst the vessel was still pressurised.

With the difference in profile between the neck of the sample bottle and the internal dimensions of the extraction cell, a plug of supercritical carbon dioxide took up the void in the neck region. This led to two problems,

i. extended pump up time, equivalent to the difference in sample volume seen in the last cell.

ii. the dilution factor introduced by this additional carbon dioxide.

To counteract these problems, a pair of aluminium collars were produced Figs 2.28 and 2.29, which could be

a. placed over the neck of the bottle, and act as a filler

b. screw onto the neck of the bottle to act as

i. a filler

ii. a funnel to contain any excess aqueous sample that may be displaced upwards out of the bottle during extraction.

To further reduce the internal void volume, a PTFE inner lining sleeve was fabricated. This allowed a tight fit between the bottle and the vessel walls.

An additional design feature incorporated into this extraction cell was the inclusion of a vortex mixer at the bottom of the delivery tube. This formed the incoming carbon dioxide into a cone and helped keep the aqueous sample within the sample bottle during the pressurisation period. It also allowed better mixing, and hence, a longer contact period between the carbon dioxide and the aqueous sample, thereby improving the efficiency of extraction

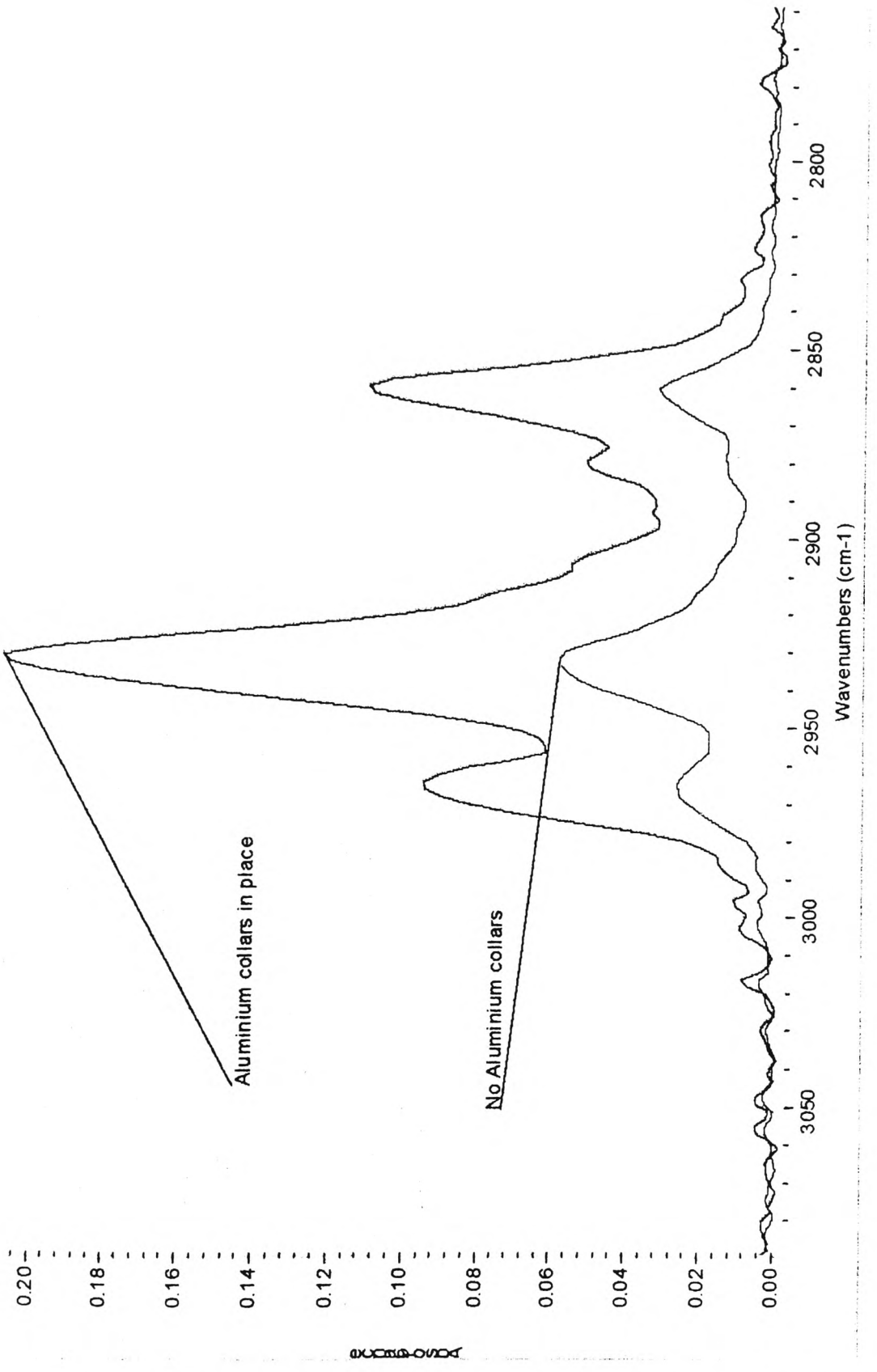


Fig. 2.27 Effect of the aluminium collar / fillers on intensity of Absorbance of identical dodecane standards

Fig.2. 28 500 cm^3 sample bottle and aluminium collars

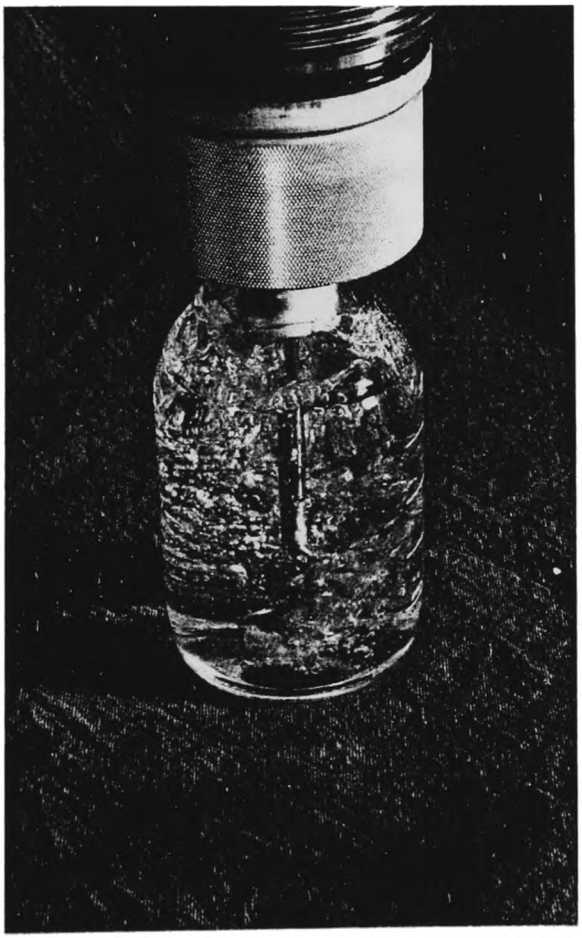


Fig.2. 29 Sample bottle, with aqueous sample, showing the vortex action of the incoming CO_2

The initial configuration of the new system was very similar to the welded extractor, where the ability to clean the IR cell with propanone between extractions in the event of water carryover was retained.

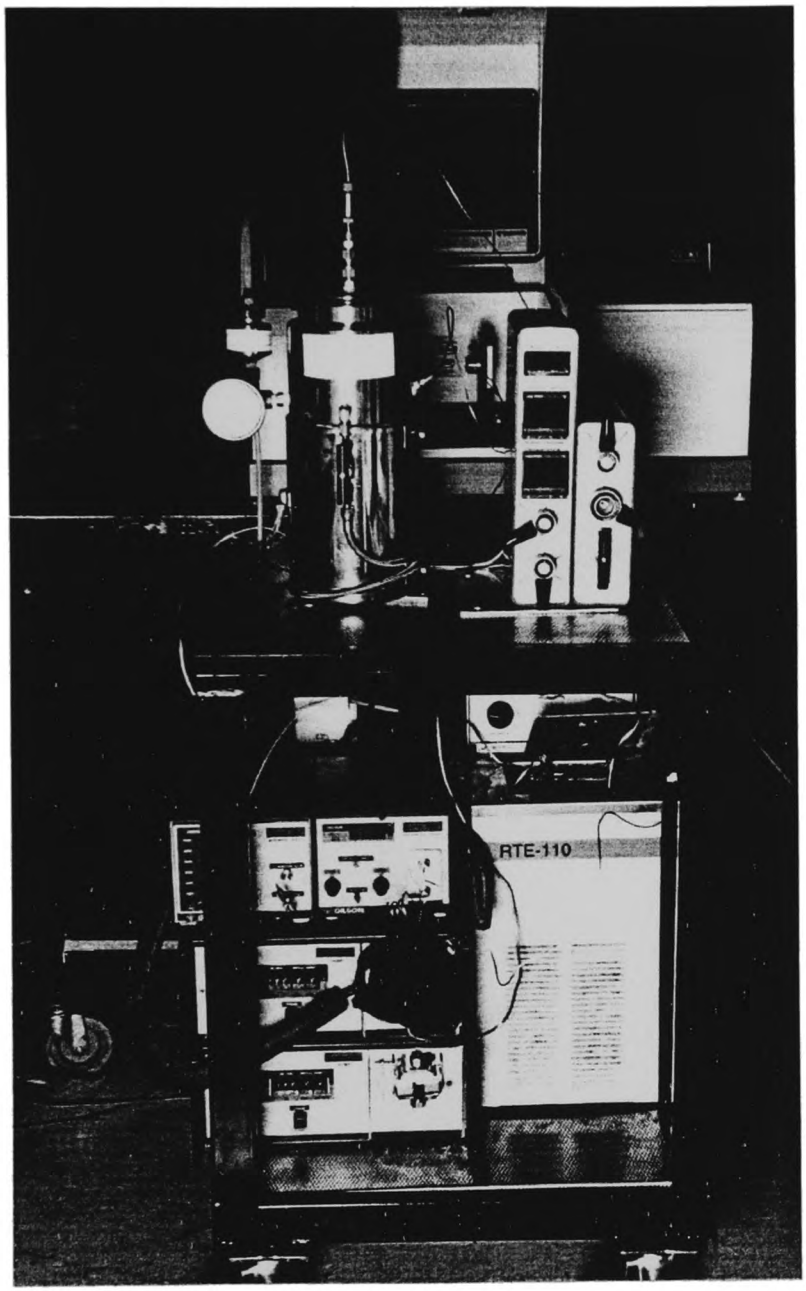


fig.2. 30 SFE system showing all constituent parts

As the lid of the vessel was threaded a suitable, hydrocarbon-free, lubricant had to be found. This initially proved difficult as even silicon-based lubricants, fig 31, contained some hydrocarbons. Numerous lubricants were examined, and eventually a perfluorinated polyether lubricant FOMBLIN RT15 (Rocol Ltd, Swillington, Leeds, UK) was found to be hydrocarbon free, fig 32,, and so allowed an absorption free zone in the region of interest.

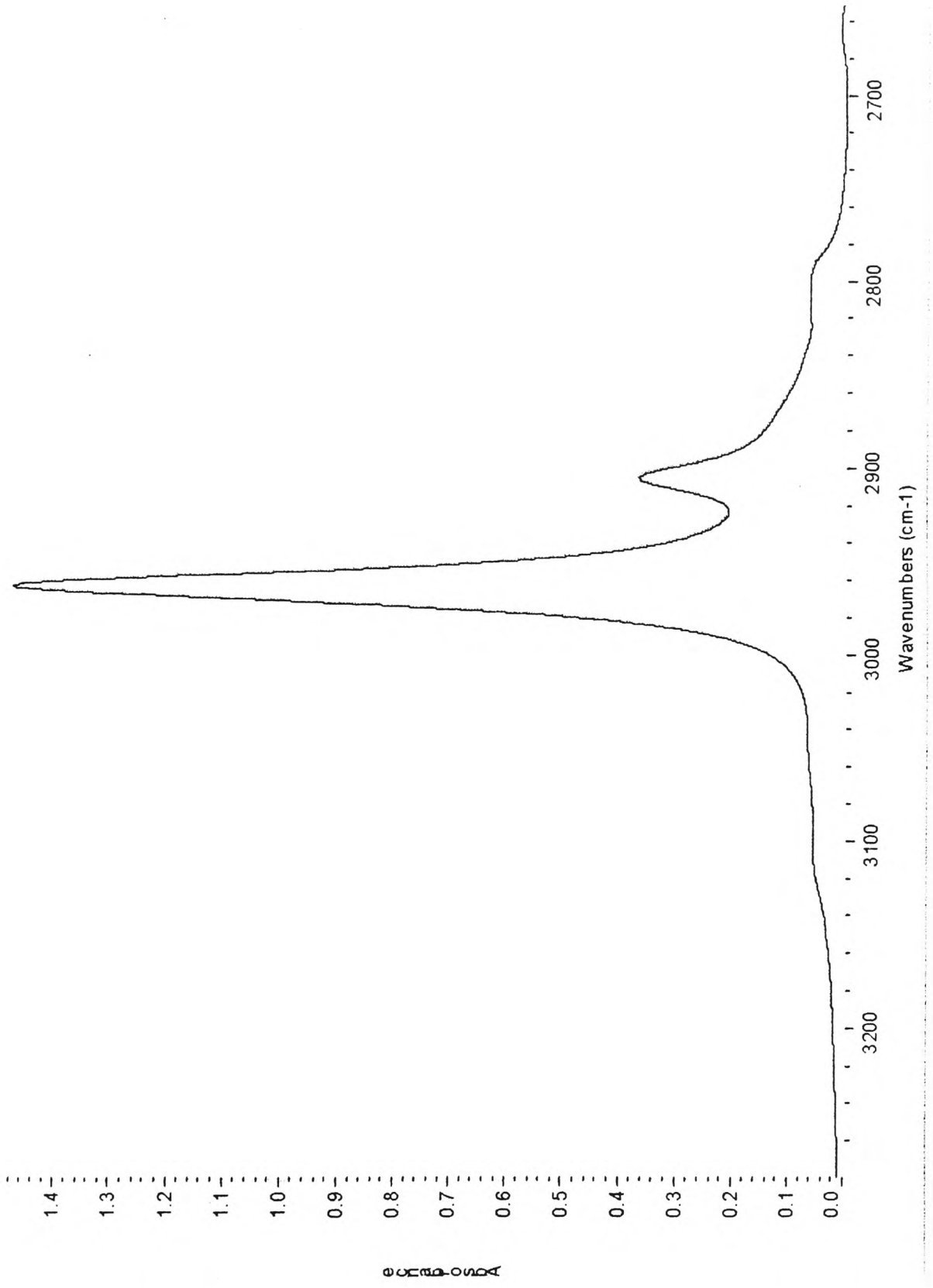


Fig.2. 31 Silicon grease showing CH stretching absorbances

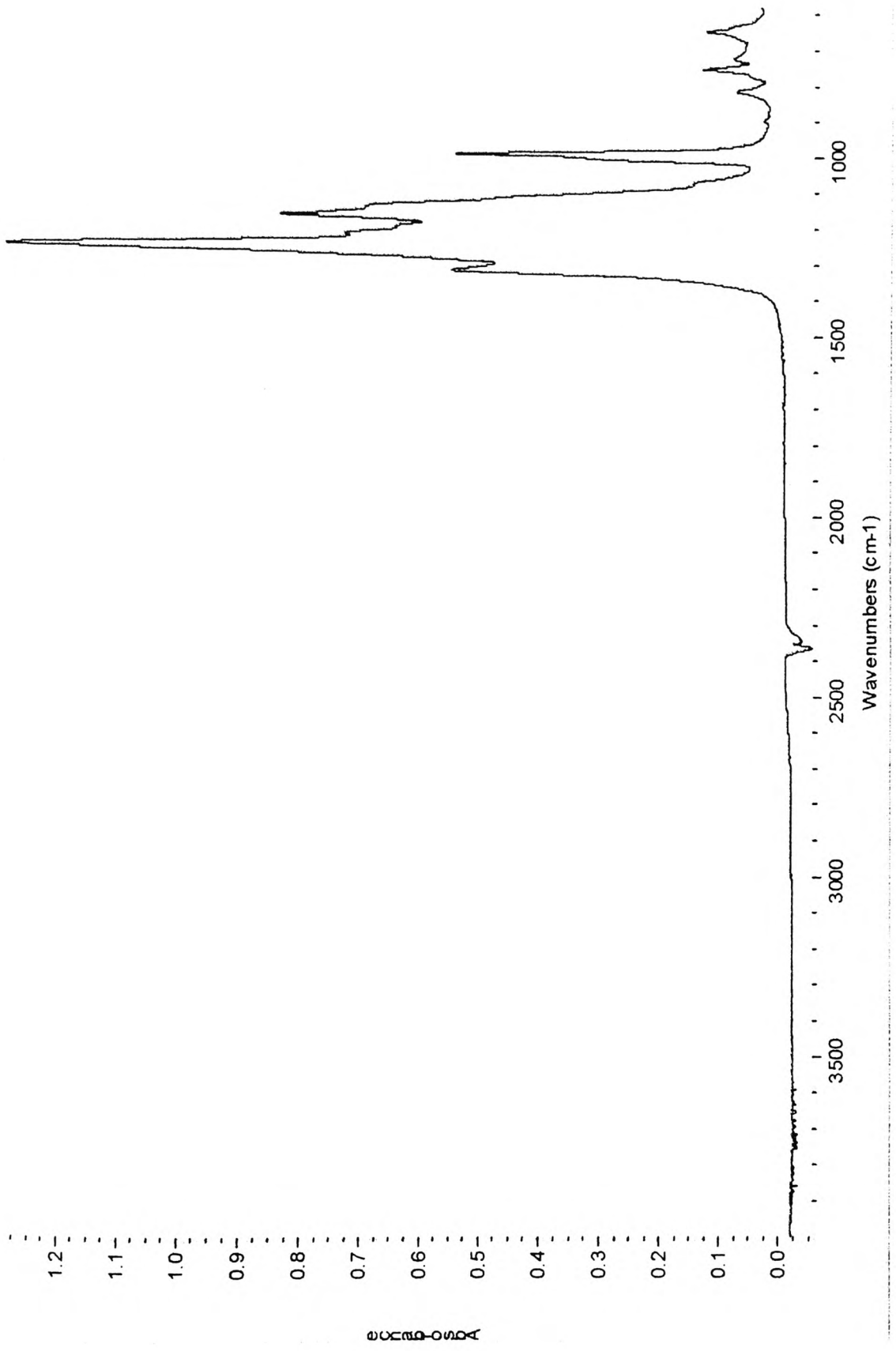


Fig.2. 32 Fomblin RT15 (showing no interference from CH stretching frequencies)

2.5.2 Development of an extraction protocol

Before starting extractions a suitable background was required. There were two options for collection,

a. by-passing the extraction cell, only pressurising the IR cell

b. pressurising the extraction cell, and then allowing the carbon dioxide to enter the IR cell

Both methods had advantages and disadvantages

a. by only pressurising the IR cell, a background spectrum could be obtained in seconds, the problem with this method was the integrity of the spectra.

b. by pressurising the SFE vessel, we were sure that the spectrum obtained would provide a true representation of the cell background, even though this method would take a lot longer.

The former method was tried, the resultant background spectra showed traces of hydrocarbon Fig 2.33.

The spectrum of the sealing O-ring showed a similar IR profile to the hydrocarbon present in the background Fig 2.34, and there was also some evidence that hydrocarbon contamination was arising from the PTFE liner.

Although these hydrocarbon signals were extremely small, with a maximum absorbance in the region of 0.005 Absorbance units, and because maximum sensitivity was required, it was decided that all background spectra would be obtained using the complete extraction system.

To reduce the hydrocarbon "outgassing" several alternative O-rings were evaluated, but did not show any improvement in performance, and because of the effect of the decompression stage, some of them ruptured, causing mechanical integrity failure.
A standard procedure for the analysis of solutions using this new extraction cell was developed, and is shown in Fig 2.35.

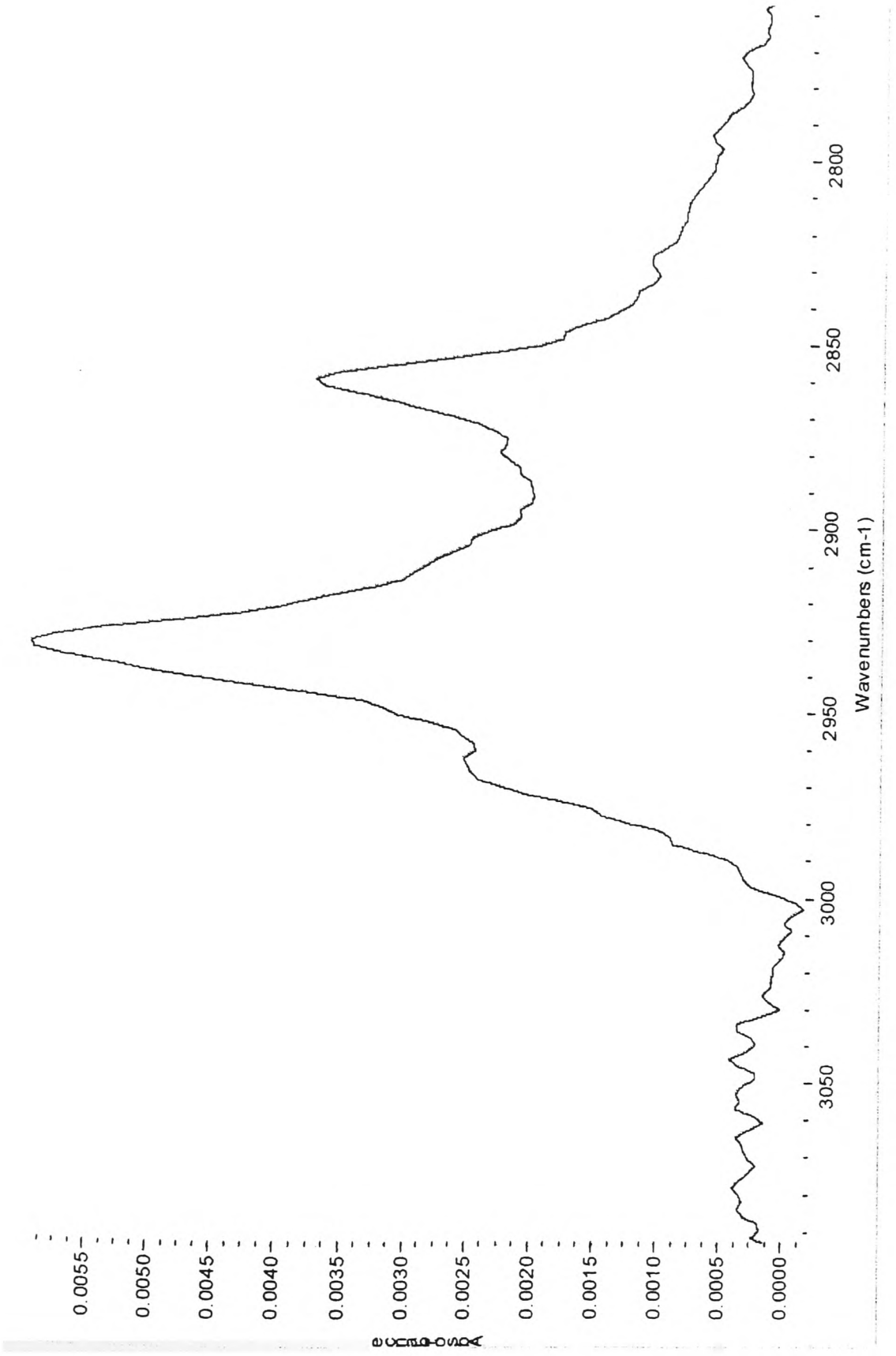


Fig.2. 33 Background spectrum ratioed against IR cell only

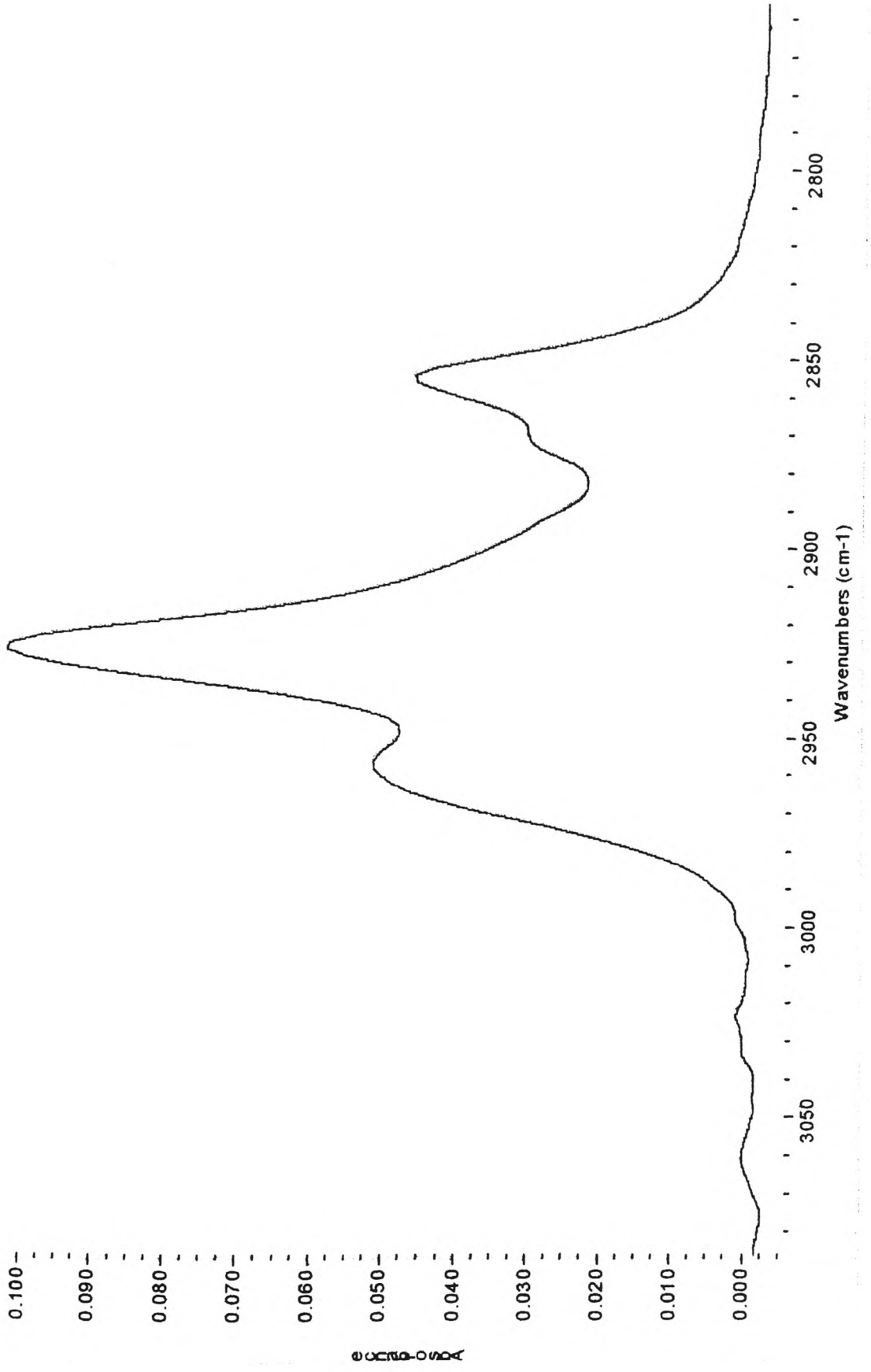


Fig.2. 34 Spectrum of O-ring (extracted with CCl_4)

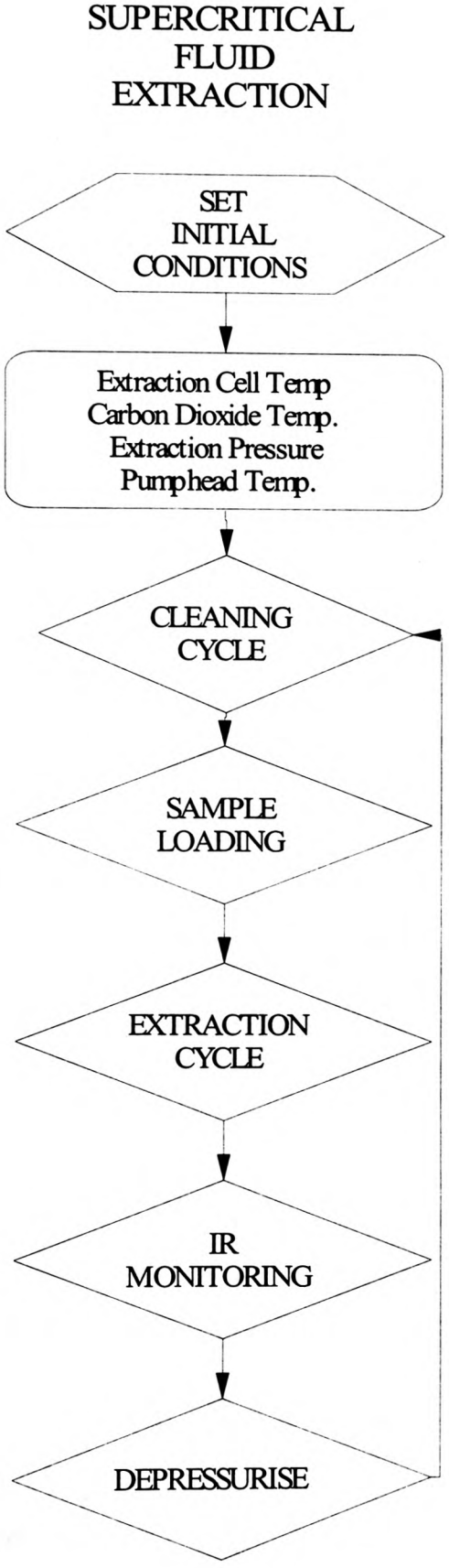


Fig. 2.35 Standard Operating Procedure

The pressurisation profile was developed from the previous extraction system, but it became apparent that the mixing and SF-CO_2 characteristics were quite different.

Consideration of the overall profile of the extraction period was made by including,

a. incubation period (external)
b. incubation period (internal)
c. extraction temperature
d. mixing characteristics - interfacial extraction
e. transportation to IR cell

a. Incubation period (external)

As the integrity of the whole sample could now be maintained, by the inclusion of the bottled sample into the extraction system, this parameter took on extra importance.

We observed the effect on the sample of,

i. incubation of the sample in a water bath for fixed periods.
ii. pre-sonication of the samples prior to extraction, to improve efficiency.

These points had been identified as possible aids to extraction.

The early results we obtained indicated that because of the warming effect of the pre-heated SF-CO_2 that there was no perceivable difference in incubating the samples externally prior to extraction

b. Incubation period (internal)

We considered the need for a period of incubation when the sample bottle was introduced into the extraction system. This would allow the sample to attain the extraction temperature before extraction proceeded. Again it was observed that an internal incubation period prior to extraction did not increase the extraction efficiency.

c. Extraction temperature

With the introduction of a thermocouple port in the top of the SFE vessel, it was now possible to monitor the internal temperature of the sample and SF-CO_2. The time

taken for the sample to attain this target temperature was monitored, with and without the flow of SF-CO_2 . Th results are summarised in Fig 2.36.

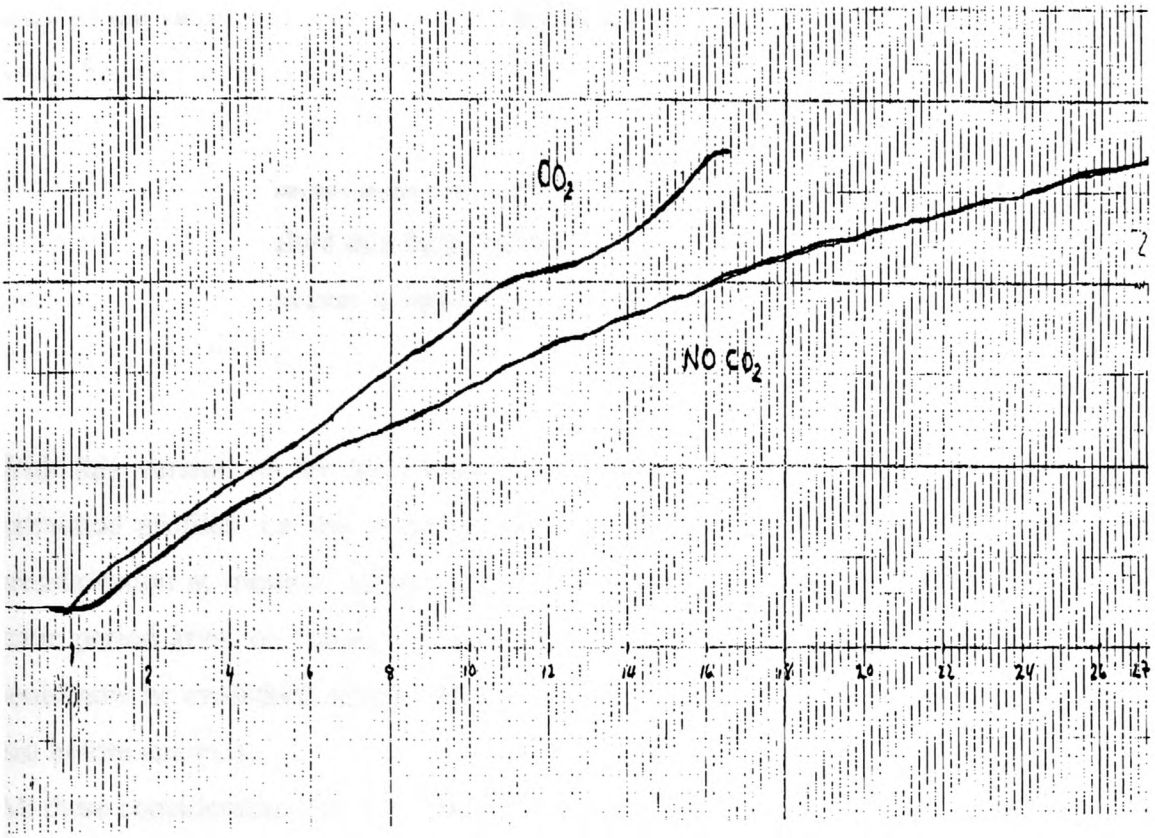


Fig 2.36 Temperature profile of aqueous sample

From the above graph it is seen that the sample attained the target temperature of 45 °C approximately 10 min quicker when the SF-CO_2 was flowing (liquid CO_2 temp 55 °C flow rate 6 cm^3 min^{-1}). This was expected even though the incoming CO_2 could have had a cooling effect, when it initially decompresses into the SFE vessel chamber. Using several extraction temperatures between 308 K and 328 K as the observed range, it did not appear to have any significant effect on the extraction efficiency, and so 313 K was chosen, which was above the Tc value for carbon dioxide.

d. Mixing characteristics – interfacial extraction

As the SF-CO_2 had to be in contact with the hydrocarbon for extraction, the longer the contact time the greater the extraction. In the previous system the pressurisation period involved a constant flow at maximum flow rate, this meant that the "T" SFE

vessel sytem had relied on the SF-CO_2 coming into contact with the hydrocarbon only during the pumping period. This was where the maximum disruption to the aqueous hydrocarbon sample occurred, therefore the SF-CO_2 was incorporated into the main body of the sample instead of remaining on the surface where interfacial extraction would be at a maximum.

Flow rate also had a direct effect on bubble size, matrix disruption, and contact time. A large flow rate resulted in a large bubble size, minimising the contact surface area, and contact time. However a large flow rate had the advantage of providing better mixing characteristics.

With this version of the SFE system, the option of allowing various mixing and extraction profiles, i.e. the introduction of a gradual increase in flow rate, to allow pressurisation at minimal surface disruption could be studied. The introduction of a static period after, or during, pressurisation to allow additional interfacial extraction could now be evaluated, along with the option of a gentle swirl to maximise mixing just before analysis.
All these considerations were evaluated as the pressurisation profile was developed.

e. Transportation to IR cell

From the experiments performed with the "T" SFE vessel, it was found that it was disadvantageous to allow the SF-CO_2 plus solubilised hydrocarbon to simply diffuse into the IR cell, as this led to inconsistencies in spectral responce, and a time delay. Again an expansion cell post IR cell was introduced to draw the SF-CO_2 / hydrocarbon from the SFE vessel cell through the IR cell. Expansion cell dimensions became an important consideration

i. too small a volume and the effect would be incomplete,
ii. too large and you introduce a dilution factor

The effect of having no mixing, and no expansion cell, was demonstrated when the inlet to the extraction system was altered to introduce the SF-CO_2 onto the top of the aqueous hydrocarbon sample instead of passing through it.

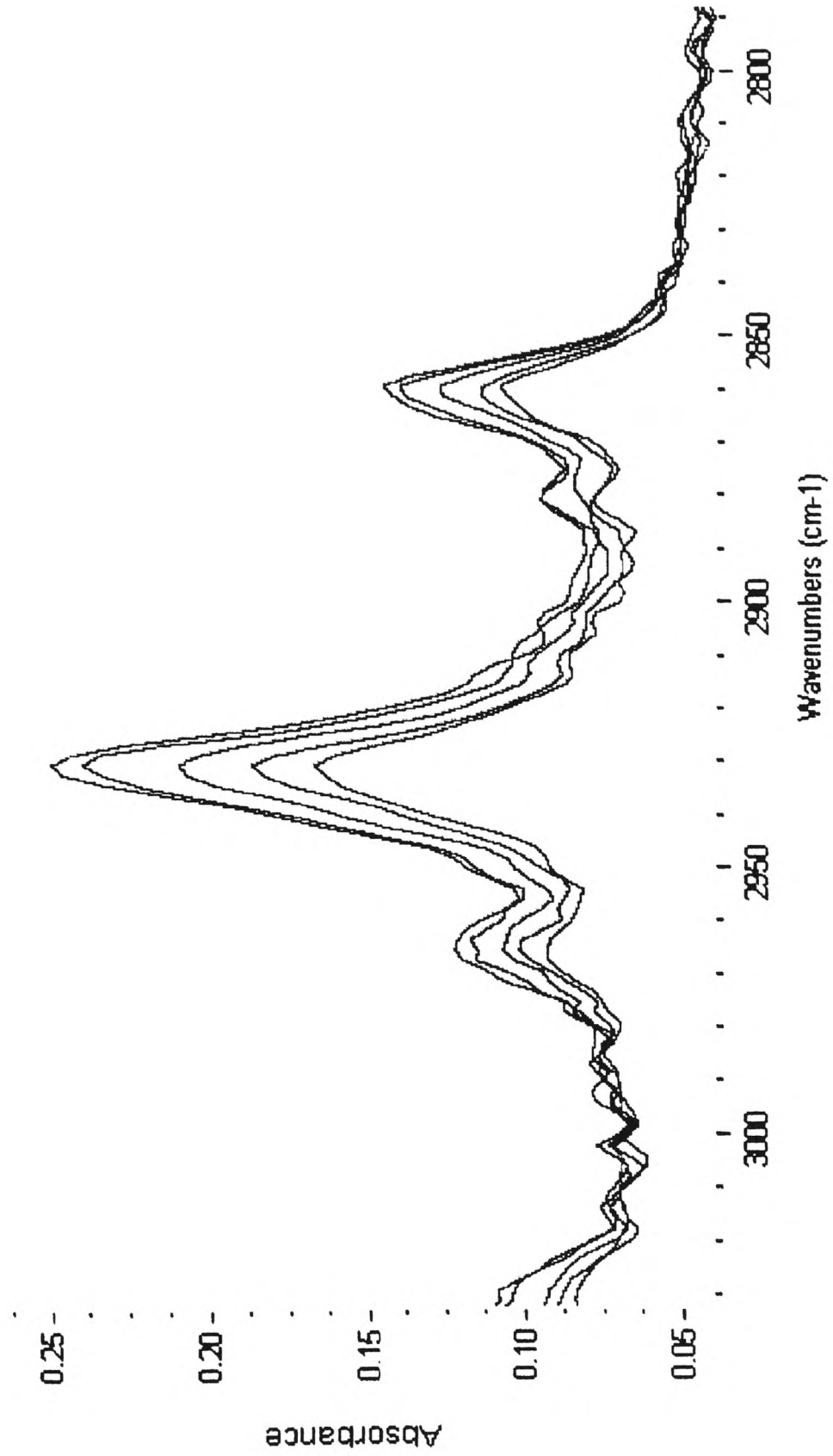


Fig. 2.37 25 ppm Hexadecane sample (no mixing- no expansion cell)after a delay period of 1, 5, 15, 30, 40 min (bottom to top)

It was clearly seen that simply relying on matrix / solvent interactions, and then diffusion for transportation, introduced a long time delay before maximum absorbance (solubility) occurred.

2.5.3 Development of a Standard Operating Procedure (SOP)

There were several categories which needed to be considered during the initial experimental stage,

a. Sensitivity
b. Linearity
c. Reproducibility
d. Selectivity
e. Volatility
f. Contamination – carry over – upper quantifiable limit.

All of these points were considered, and used to optimise the performance of the extraction system. Other parameters included in this optimisation process were temperature, and pressure.

The first objective was the preparation of a standard operating procedure (SOP) which would be used to examine all samples.

The pressurisation profile within the SOP was,

Time (min)	Flow rate (cm^3 min^{-1})
0.00	20
8.50	20
8.60	0.0
13.10	0.0
13.20	1.0
13.30	20.0
20.00	20.0

- The liquid CO_2 was pumped at maximum rate for the first 8.50 min, between 8.60 and 13.10 min the sample was allowed to equilibrate.
- At 13.20 min the liquid CO_2 was slowly introduced at a low flow rate to "stir" the aqueous sample and the SF-CO_2 / solubilised hydrocarbon.
- At 13.30 min the system was then pressurised, to its pre-set extraction pressure, at maximum flow rate.
- On reaching its extraction pressure the pump stopped, and the sample allowed to equilibrate again for 1 min.
- After this period the valve isolating the extraction cell from the IR cell was opened allowing the solubilised hydrocarbon to flow into the IR cell.
- The expansion cell was then opened, and the pump restarted to allow the extraction pressure to be attained.
- The IR spectrum was then collected.

2.5.4 Experimental data obtained using the removable top cell

a. **Sensitivity**

The spectra in Fig 2.38 shows the infrared response for dodecane samples in the concentration range of 1 to 40 ppm for 500 cm^3 spiked water samples. For the 1 ppm sample a signal to noise ratio of 26 :1 was calculated, and an associated absorbance value of 0.044 was obtained.

b. Linearity

The same set of data was used to examine the linearity of response, against concentration. The data was plotted and had a correlation coefficient of 0.9908, Fig 2.39.

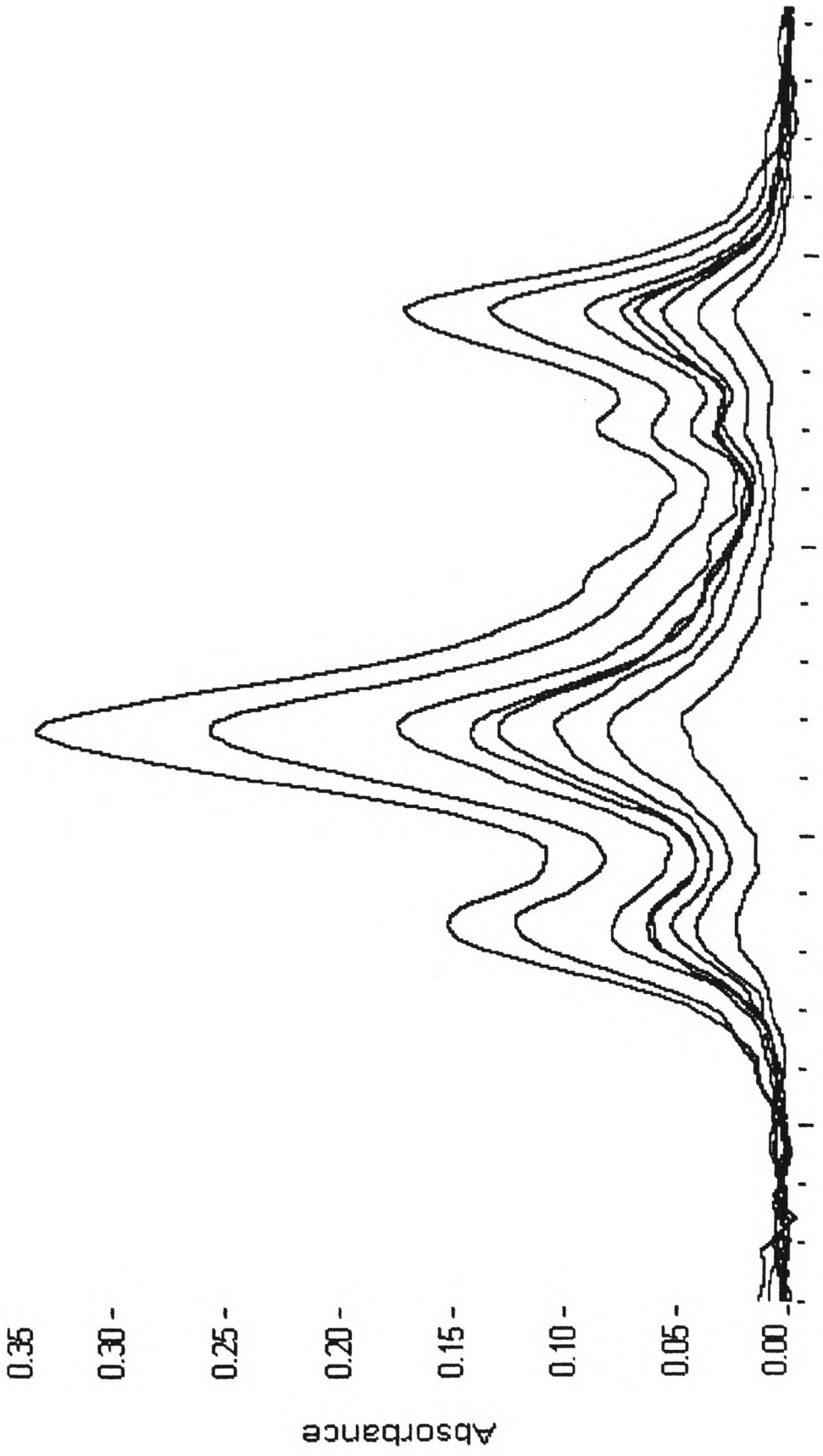


Fig 2. 38 Dodecane extractions (1- 40 ppm) (bottom to top)

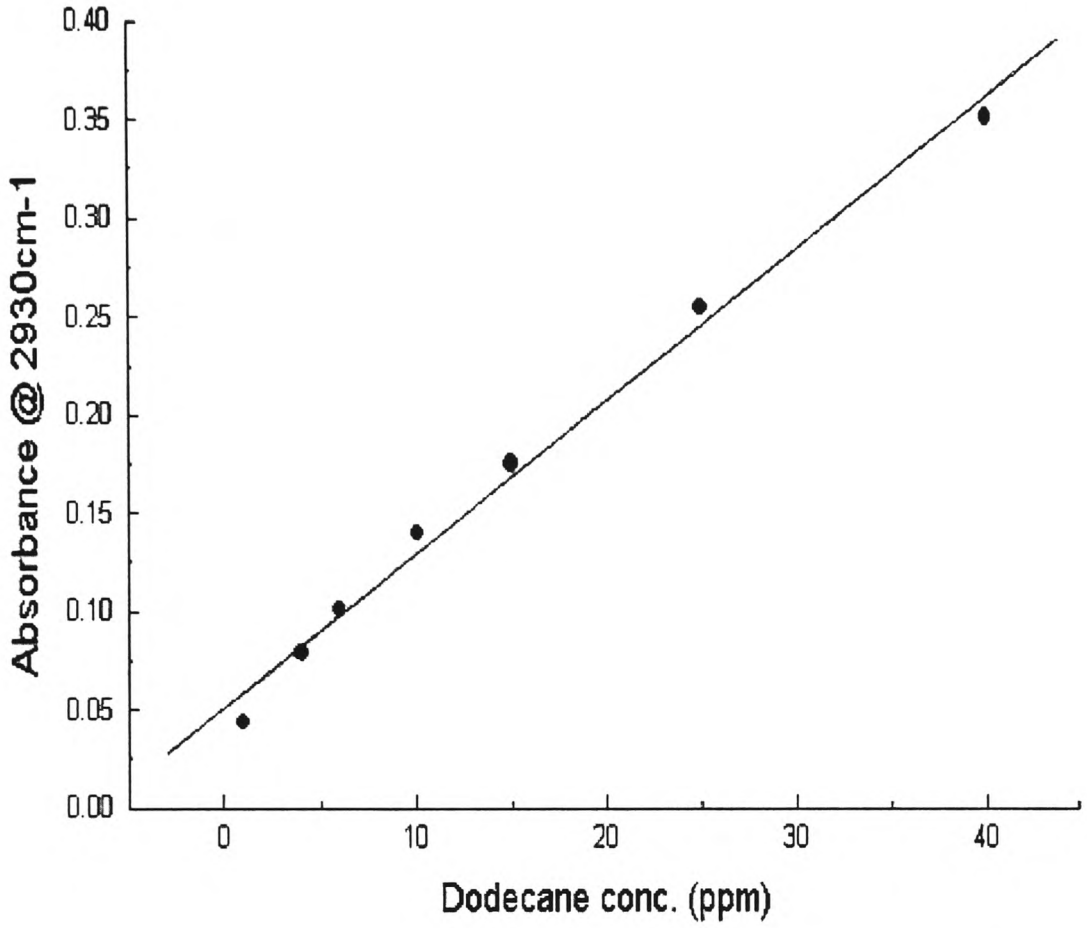


Fig. 2.39 Linearity of Absorbance with concentration for dodecane samples in the concentration range 1 – 40 ppm in 500 cm^3 spiked water sample

correlation coefficient of 0.9908

c. Reproducibility

Sets of 500 cm^3 water spiked samples with hexadecane were prepared and extracted to examine the reproducibility of the extraction process.

i. 10 ppm hexadecane samples, Fig 2.40

ii. 60 ppm hexadecane samples, Fig 2.41

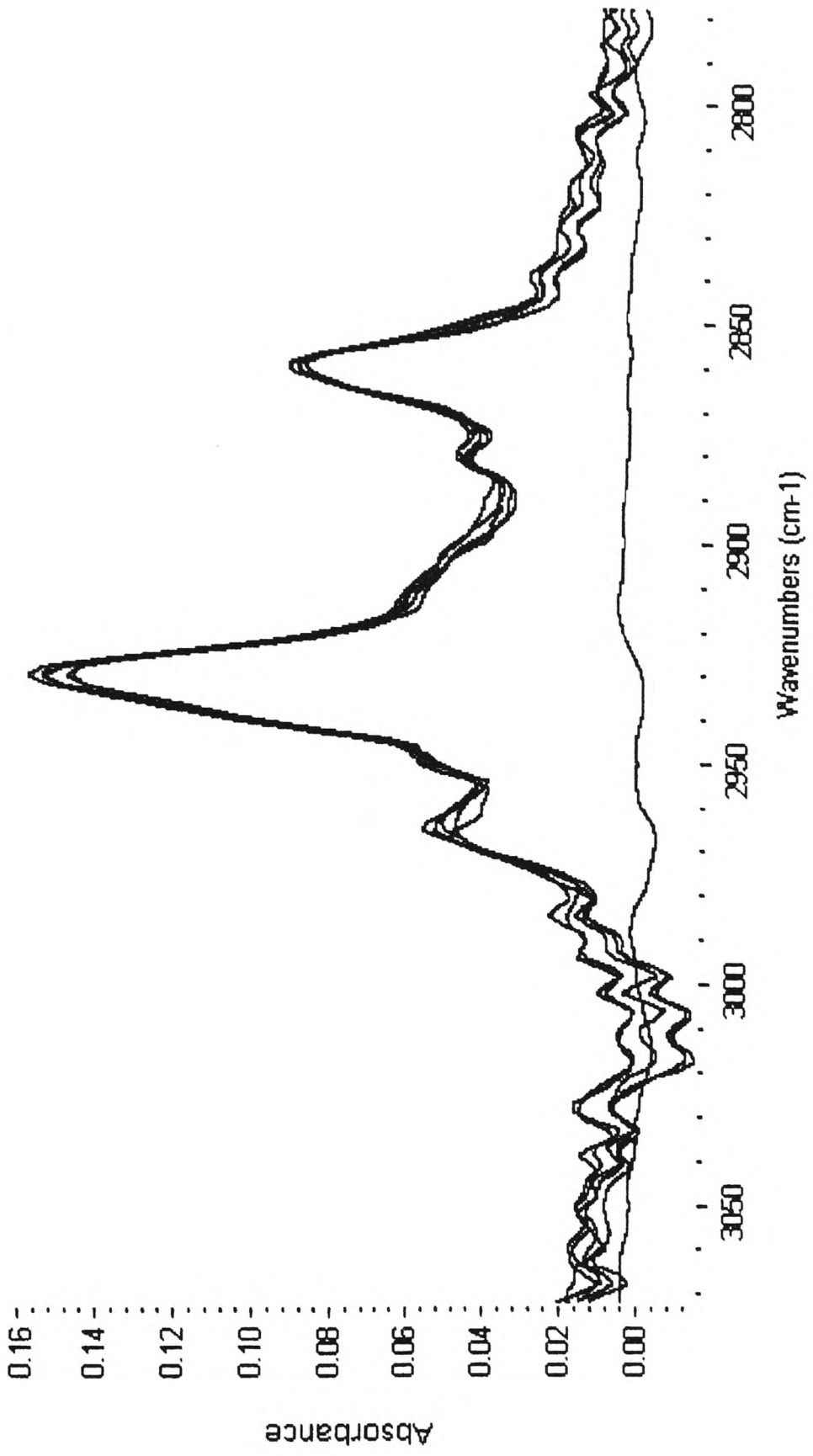


Fig. 2.40 10 ppm dodecane extracted standards

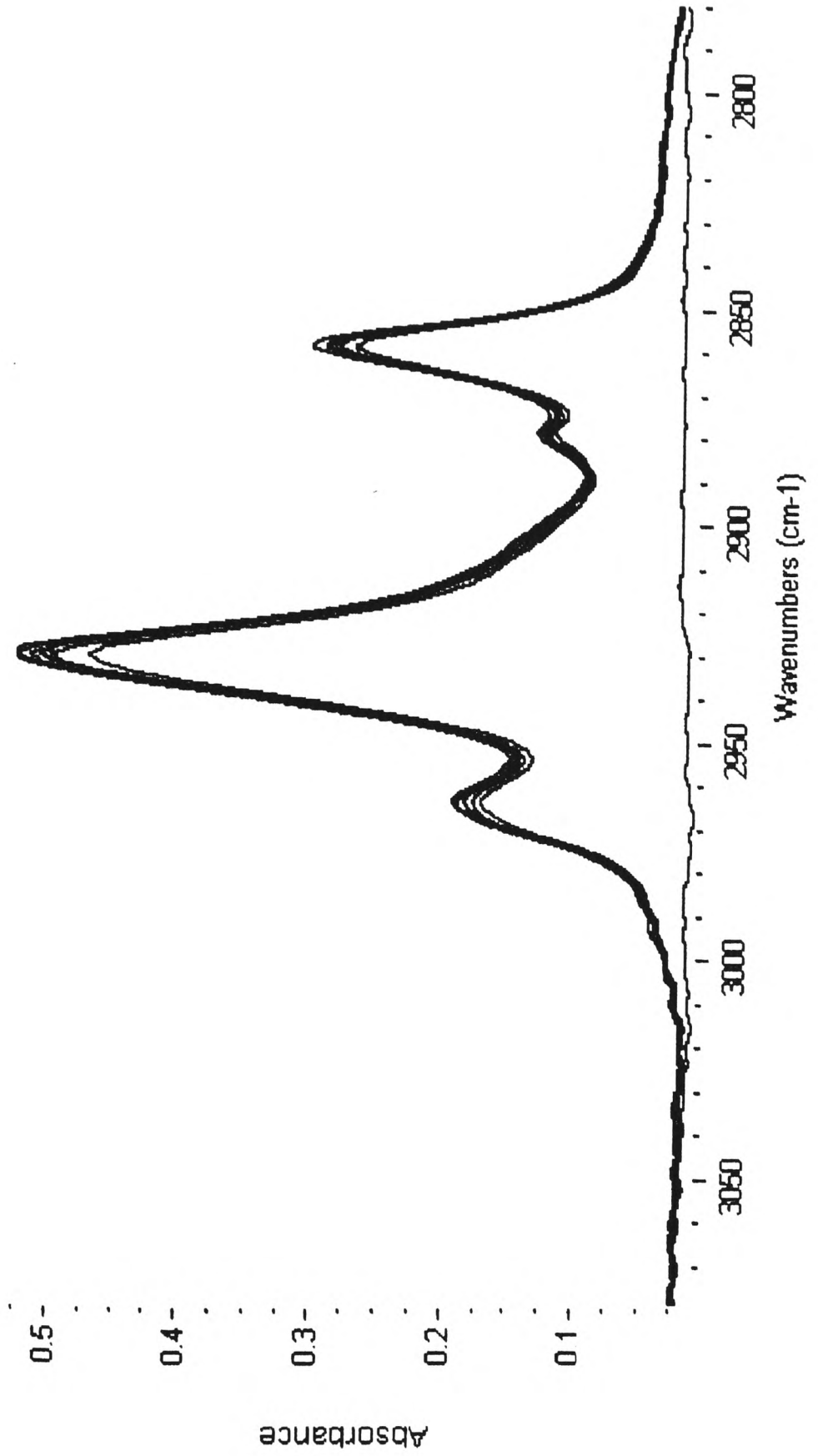


Fig. 2.41 60 ppm dodecane extracted standards

Two different concentration standards were selected to ensure the extraction process was reproducible across a concentration range.

The standard deviations for these extractions were,

10 ppm =0.0037

60 ppm = 0.0193

d. Selectivity (polar and non-polar hydrocarbons)

It became apparent during the course of this investigation, there was a need to demonstrate selectivity within the extraction process. The main analytical point being the ability to individually quantify polar, and non-polar hydrocarbons, as these are regularly analysed separately.

The investigation was conducted on a mixture of vegetable oil and benzene. The selectivity was achieved by the introduction of a 10 micron silica particle packed guard column, positioned between the SFE vessel outlet and the IR cell, and selectable via a valve. The column demonstrated the ability to remove polar hydrocarbons, such as vegetable oil, while allowing the SF-CO_2 solubilised benzene to pass through, Fig 2.42. By careful manipulation of the sample the total hydrocarbon content, and the polar hydrocarbon content, could be determined from the analysis of the same sample.

A:- 15µl Vegetable oil, 30µl Benzene, –Pre cleanup i.e. no silica gel treatment

B:- 15µl Vegetable oil, 30µl Benzene, –Post cleanup i.e. with silica gel treatment

The spectra clearly show the ability of the new extraction system to remove polar hydrocarbons, by the use of a silica column, while allowing the non-polar hydrocarbon content of a water sample to be analysed. Analysis of total oil content meant the the polar hydrocarbons could be calculated by difference.

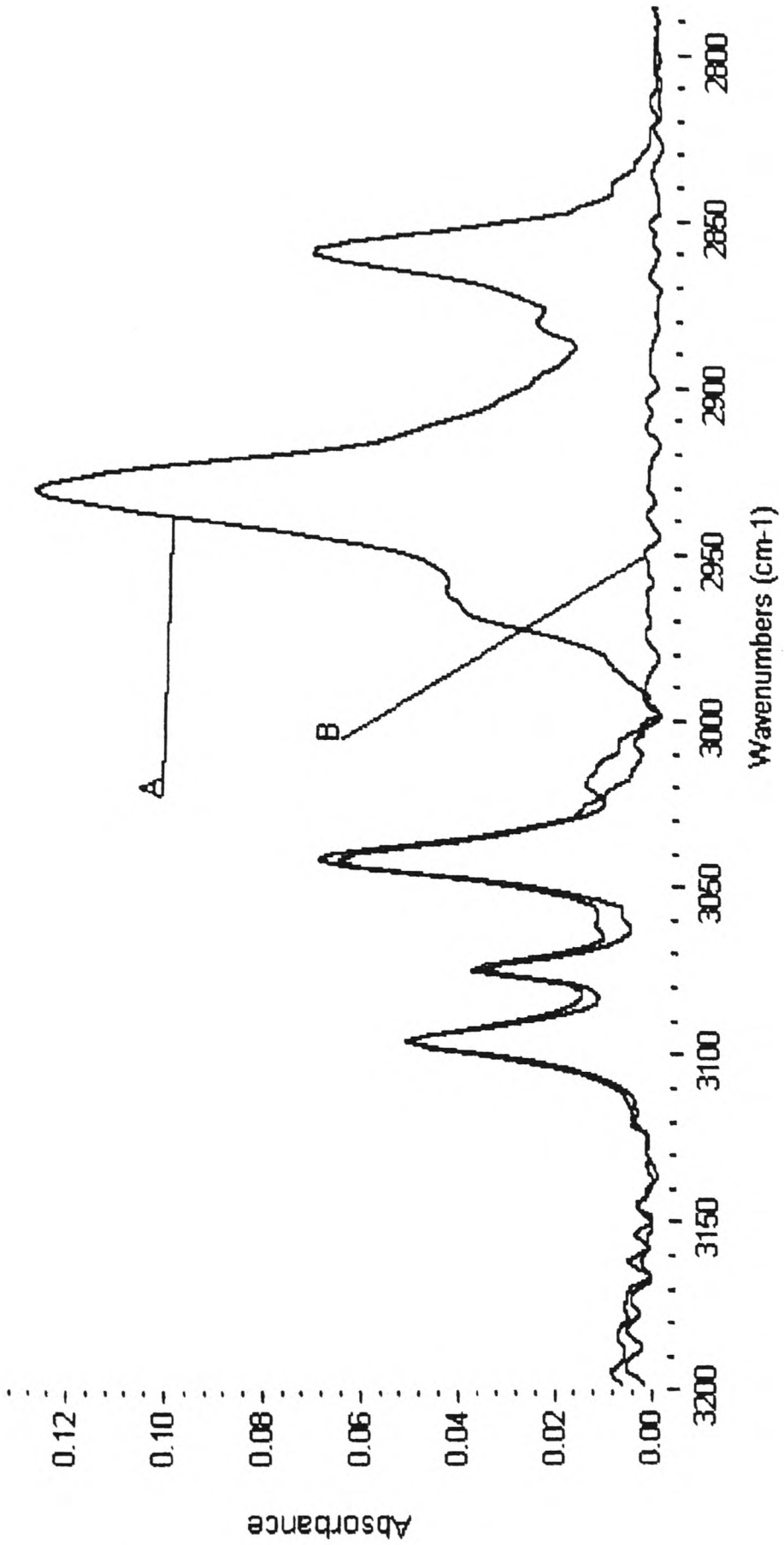


Fig 2 42. Vegetable oil and Benzene, pre (A) and post (B), silica cleanup

e. Volatility

The Fig 2.43 and 2.44 show the linear response, and correlation coefficient of a range of benzene samples, showing the extraction systems ability to handle volatile hydrocarbons.

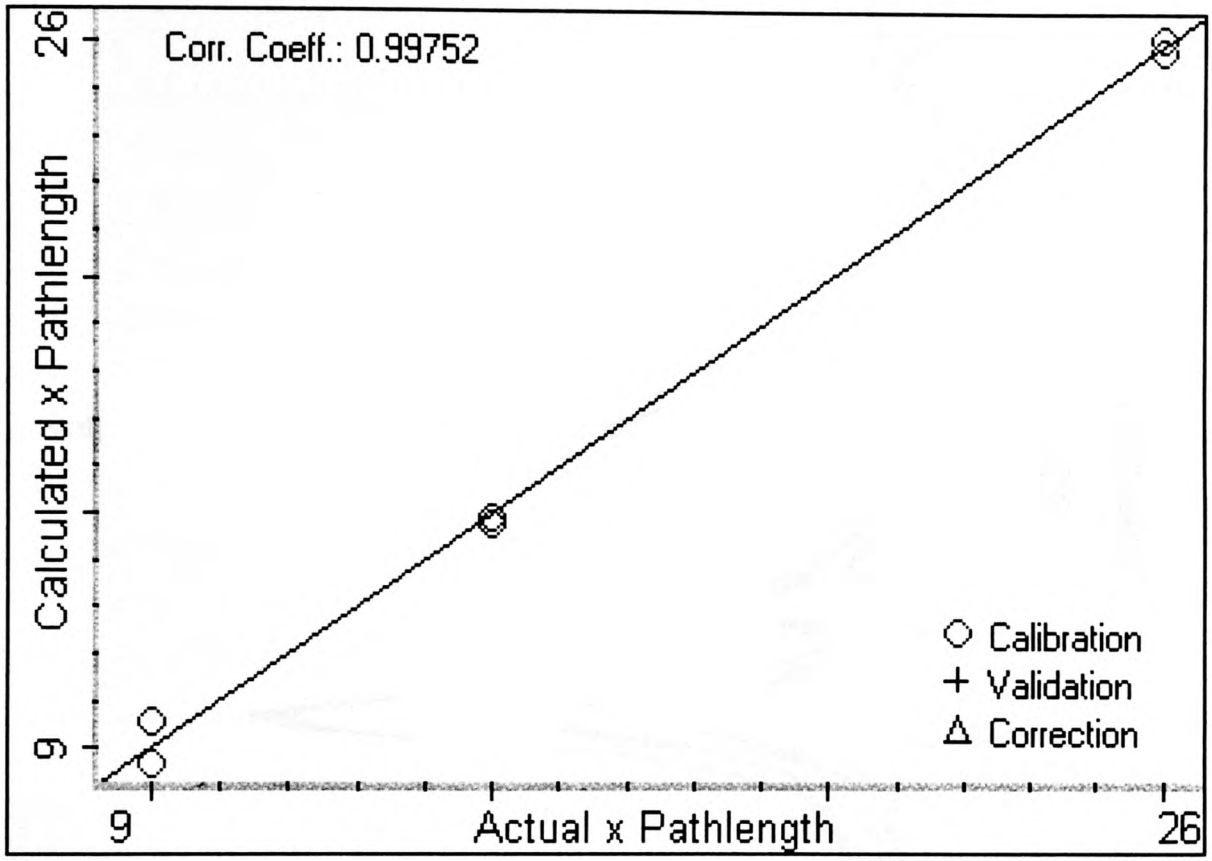


fig.2. 43 Calibration graph of 17.6, 26.4 and 44 ppm Benzene

f. Contamination

Contamination falls into two categories,

i. contamination due to carry over from high concentration samples previously extracted.

ii. external contamination.

The former had a direct bearing on the performance of the extraction process. It places a limit on the upper concentration limit, it also has an effect on the lowest detectable limit.

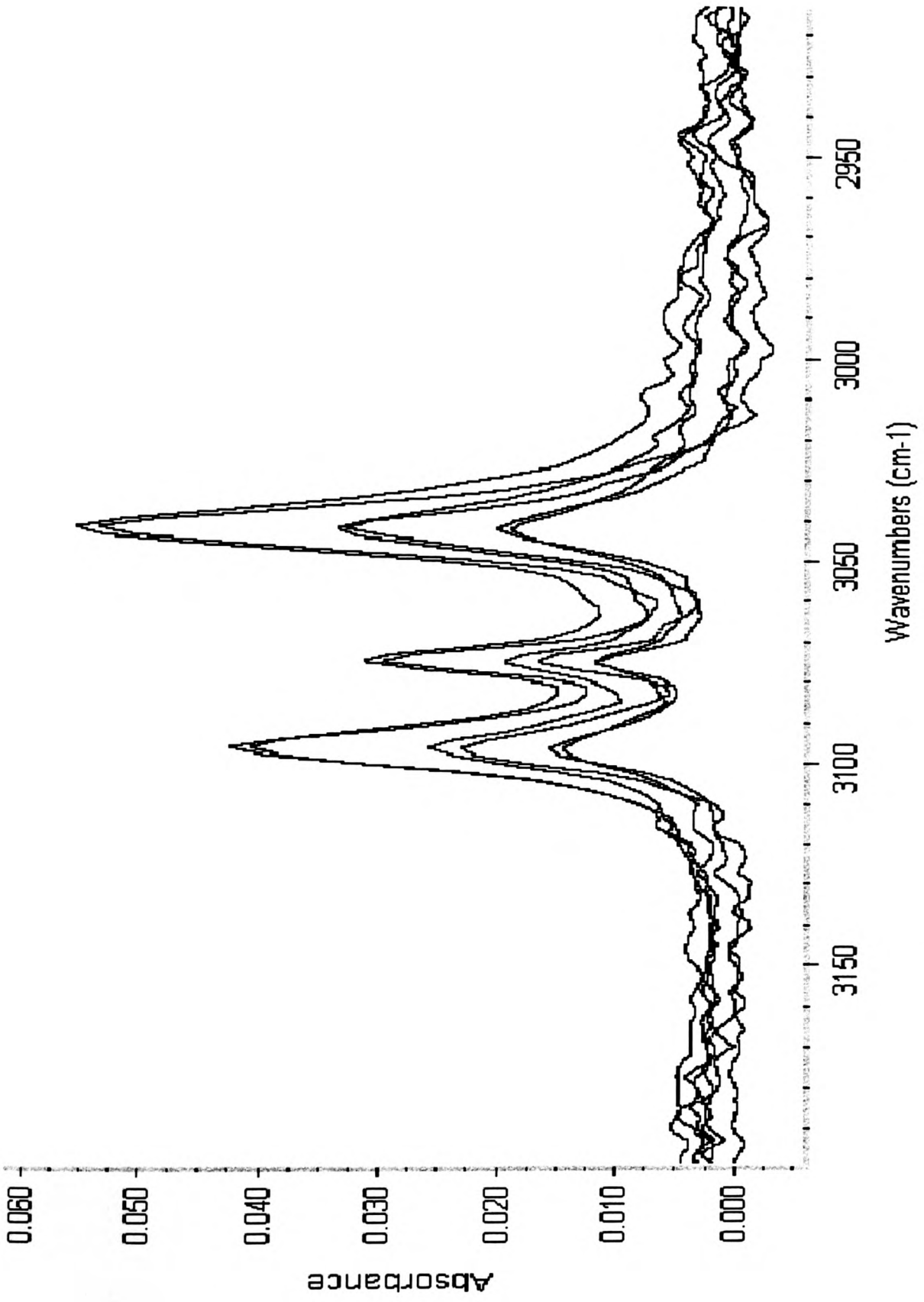


Fig. 2.44 17.6, 26.4 and 44 ppm Benzene

The later category limits the minimum quality threshold allowable, i.e. particulate content.

i. Contamination due to hydrocarbon carry over

Results obtained from the welded extraction system (section 2.4.4) , indicate that carry over of hydrocarbon could be problematic. Having already completed extractions of 60 ppm dodecane samples, there appeared to be no detectable carry over in this extraction system. Although, at this point, carryover did not seem to be problematic, future work with less volatile hydrocarbons would require vigilance.

ii. External contamination

Consideration was given to particulate contamination of "real" hydrocarbon contaminated aqueous samples. Samples for extraction and analysis that were obtained from industrial sources were frequently contaminated with particulate matter. We investigated the effect of particulate contamination on the efficiency of the extraction process by adding soil to a hydrocarbon contaminated aqueous sample. The results are summarised in Fig 2.45, where;

It was seen that the particulate matter had a negligible effect on the quality of the spectra by comparing A and B. In spectra C some evidence of extraction of organic material from the soil can be seen.

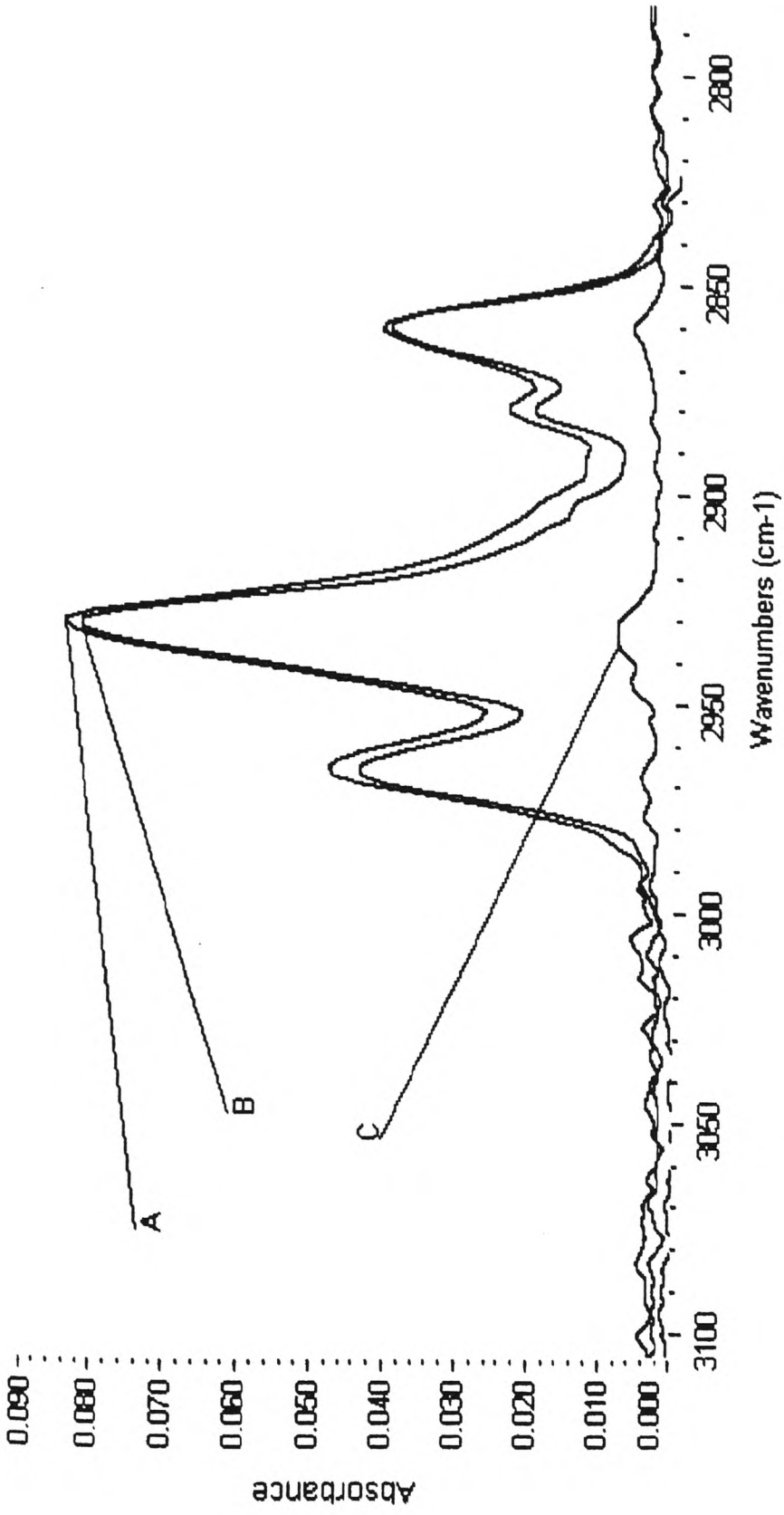


Fig. 2.45 Spectra showing the effect of particulate matter on extraction A:- 14.6 ppm decane + 10000 ppm particulates (soil) B:- 14.6 ppm decane C:- 10000 ppm particulates (soil)

2.6 Conclusion

It can be seen from the results presented in this study, that the SFE-IR system has the potential to offer a replacement procedure to the present liquid / liquid extraction (LLE) methods. Aligned with this, is the opportunity to negate the reliance on ozone depleting, and often toxic, solvents such as Freon-113.

The results have shown that it is possible to reproducibly extract hydrocarbons from an aqueous sample and using IR spectroscopy quantify them. It has also been demonstrated that the application is robust to suspended particulate matter, and has the potential to determine polar and non-polar hydrocarbons in the same sample.

In the next chapter a study is made of the current LLE analysis methods, for oil and grease in water, related to accuracy, repeatability and performance level required for the SFE-IR system to replace these existing methods.

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Appendix B

Persistence of Paleobiochemicals and Other Long Lived Natural Materials

(Alexander 1973)

Material	Source	Age of Material (years)
Tanned Leather	Clay soil	1.9×10^3
Wood	Peat deposits	$> 1.9 \times 10^4$
Mud	Lake bottom	2.4×10^4
Acacia Wood	River bed	3.0×10^4
Fungus Spores	Lake sediment	3.0×10^4
Bound Amino Acids	Marine sediment	3.0×10^7
Succinic acid, terpenoid resin	Amber	4.0×10^7
Bound Amino Acids	Anthracite coal	2.5×10^8
Fatty Acids, Alkanes	Shale	3.5×10^8
Chitin	*Hyolithellus* fossil	5.5×10^8
Porphyrins	Sedimentary rocks	3×10^9

Appendix C

Table 1

Solubilities in water of Alkanes, and Branched - Chain Alkanes at 25° C ±1.5

Hydrocarbon	Solubility (g of Hydrocarbon / 10^6g Water)
Methane	24.4 ± 1.0
Ethane	60.4 ± 1.3
Propane	62.4 ± 2.1
n- Butane	61.4 ± 2.6
Isobutane	48.9 ± 2.1
n- Pentane	38.5 ± 2.0
Isopentane	47.8 ± 1.6
2,2-Dimethylpropane	33.2 ± 1.0
n- Hexane	9.5 ± 1.3
2-Methylpentane	13.8 ± 0.9
3-Methylpentane	12.8 ± 0.6
2,2-Dimethylbutane	18.4 ± 1.3
n-Heptane	2.93 ± 0.02
2,4-Dimethylpentane	4.06 ± 0.29
n-Octane	0.66 ± 0.06
2,2,4-Trimethylpentane	2.44 ± 0.12
2,2,5-Trimethylhexane	1.15 ± 0.08

Table 2

Solubilities in Water of Alkenes, Dienes, and Branched-Chain Alkenes at 25°C ± 1.5

Hydrocarbon	Solubility (g of Hydrocarbon / 10^6g Water)
Ethene	131 ± 10
Propene	200 ± 27
1-Butene	222 ± 10
2-Methylpropene	263 ± 23
1-Pentene	148 ± 7
2-Pentene	203 ± 8
3-Methyl-1-butene	130 ± 14
1-Hexene	50 ± 1.2
2-Methyl-1-pentene	78 ± 3.2
4-Methyl-1-pentene	48 ± 2.6
2-Heptene	15 ± 1.4
1-Octene	2.7 ± 0.2
1,3-Butadiene	735 ± 20
2-Methyl-1,3-butadiene	642 ± 10
1,4-Pentadiene	558 ± 27
1,5-Hexadiene	169 ± 6
1,6-Heptadiene	44 ± 3

Table 3

Solubilities in Water of Alkynes and Diynes, at 25°C ±1.5

Hydrocarbon	Solubility (g of Hydrocarbon / 10^6g Water)
Propyne	3640 ± 125
1- Butyne	2870 ± 101
1-Pentyne	1570 ± 33
1-Hexyne	360 ± 17
1-Heptyne	94 ± 3
1-Octyne	24 ± 0.8
1-Nonyne	7.2 ± 0.5
1,6-Heptadiyne	1650 ± 25
1,8-Nonadiyne	125 ± 3

Table 4

Solubilities in Water of Cyclic and Aromatic Hydrocarbons, at 25°C ±1.5

Hydrocarbon	Solubility (g of Hydrocarbon / 10^6g Water)
Cyclopentane	156 ±9
Cyclohexane	55 ± 2.3
Cycloheptane	30 ± 1.0
Cyclooctane	7.9 ± 1.8
Methylcyclopentane	42 ± 1.6
Methylcyclohexane	14 ± 1.2
1-cis-2-Dimethylcyclohexane	6 ± 0.8
Cyclopentene	535 ± 20
Cyclohexene	213 ± 10
Cycloheptene	66 ± 4
1-Methylcyclohexene	52 ± 2
1,4-Cyclohexadiene	700 ± 16
4-Vinylcyclohexene	50 ± 5
Cycloheptatriene	620 ± 20
Benzene	1780 ± 45
Methylbenzene	515 ± 17
1,2-Dimethylbenzene	175 ± 8
Ethylbenzene	152 ± 8
1,2,4-Trimethylbenzene	57 ± 4
Isopropylbezene	50 ± 5

Chapter 3

Hydrocarbons Quantification

3.1 Introduction

Oil, grease and total petroleum hydrocarbons (TPHs) measurements are required for the safe and efficient discharge of industrial and waste water into the environment. Although providing a gross measurement of these substances, they are essential if these processes are to be managed successfully. A wide variety of analytical procedures have been developed for this purpose,[1] and they can be broadly sub-divided into two categories;-[2]

i. integral procedures which involve gravimetric, infrared (IR), ultraviolet (UV) and fluorescence spectroscopic techniques.

ii. differential methods which involve gas chromatography (GC), or gas chromatography combined with mass spectroscopy (GC-MS), and high performance liquid chromatography (HPLC)

Infrared and gravimetric procedures, for the analysis of oil in water, have become firmly established industrially,[3,4] although not as accurate as GC and GC-MS methods of analysis, they are far less time consuming or analytically challenging.

Gravimetric methods of analysis such as the US Environment Protection Agency (EPA) Method 413.1,[5] and Standard Method 5520B,[6] rely of liquid-liquid extractions (LLE) using 1,1,2-trichloro-1,2,2-trifluoroethane (Freon 113), drying with magnesium sulphate, and evaporation of the solvent, before the residual oil is weighed. This results in the loss of the more volatile components.

An alternative gravimetric method EPA 1664,[7] has been developed where the extraction solvent used is hexane. This method also allows for the use of solid phase extraction (SPE) to be implemented as a replacement for LLE, and the results of a comparative study using SPE cartridges for the analysis of oils, grease and TPHs has been published.[8]

Methods based on UV and fluorescence spectroscopic procedures for the determination of oil, grease and TPHs in water rely on the determination of the aromatic content of the oils, with the total hydrocarbon content being estimated from this value on a proportional basis.[9]

In principle any solvent which replaces LLE solvents currently in use would have to have the following characteristics;

i. be non-absorbing in the C-H stretching region of 3030-2930 cm^{-1}
ii. capable of efficient extraction of the hydrocarbon from water
iii. environmentally friendly
iv. non-toxic
v. non-flammable
vi. have low cost at high purity
vii. have no disposal costs

This section of work reports on these parameters for supercritical fluid carbon dioxide, along with its quantitation capabilities.

There are numerous reported methods for the analysis of aqueous hydrocarbon samples, by infrared spectroscopy, (Appendix D).
The basis for quantification of hydrocarbons, or other analytes which give rise to absorption spectra, is the Lambert Beer law, already discussed in section 2.3.4. This gives a linear relationship between the quantity of absorbing species and the intensity of absorbance for the corresponding radiation.

As the extinction coefficient, also refered to as molar absorbtivity, remains constant for a specific absorption, by varying the concentration of the analyte, and monitoring the absorbance response using the same pathlength cell, a linear graph can be constructed which allows the concentration of an unknown sample to be determined by interpolation.

For hydrocarbons the absobance peaks used in the quantification are generally;

CH_3 – Assymetric stretch ~ 2960 cm^{-1}
CH_2 –Assymetric stretch ~ 2930 cm^{-1}
CH Aromatic ~ 3030cm^{-1}

Many workers have reported on this methodology. [10-14]
Infrared quantification ideally relies on a set of standard solutions, of the analyte under analysis, being prepared and analysed, under identical conditions, and a calibration graph of their associated absorbance and concentration being constructed. Applying the unknown analyte absorbance to this calibration graph allows its concentration to be determined by interpolation.

There are several problems associated with this quantification technique;

a) The availability of the specific hydrocarbon for calibration graph construction.
b) The analyte being a multi component mixture.

This reported work analyses the consequences of these problems, and additionally looks at the application to on-line and off-line analysis.

3.2 Off-line Quantification (no extraction phase, known single component hydrocarbon)

The relative intensities of the infrared absobances are related to the carbon chain length and extent of branching of the hydrocarbon being studied.

The next four figures, Figs 3.1 to 3.4, show the effect of carbon chain length, and branching, upon the ratios of the intensities of the absorbance peaks of hydrocarbons. The spectra are of different hydrocarbons prepared as solutions in carbon tetrachloride, with a concentration of approximately 5 mg in 100 cm^3 of solvent, and analysed in a 2 cm pathlength IR quartz cell.

It can be observed from the spectra that the ratio of the peaks at 2960 cm^{-1} (CH_3) and 2930 cm^{-1} (CH_2) decreases as the chain length increase, and increases with the increase in branching.

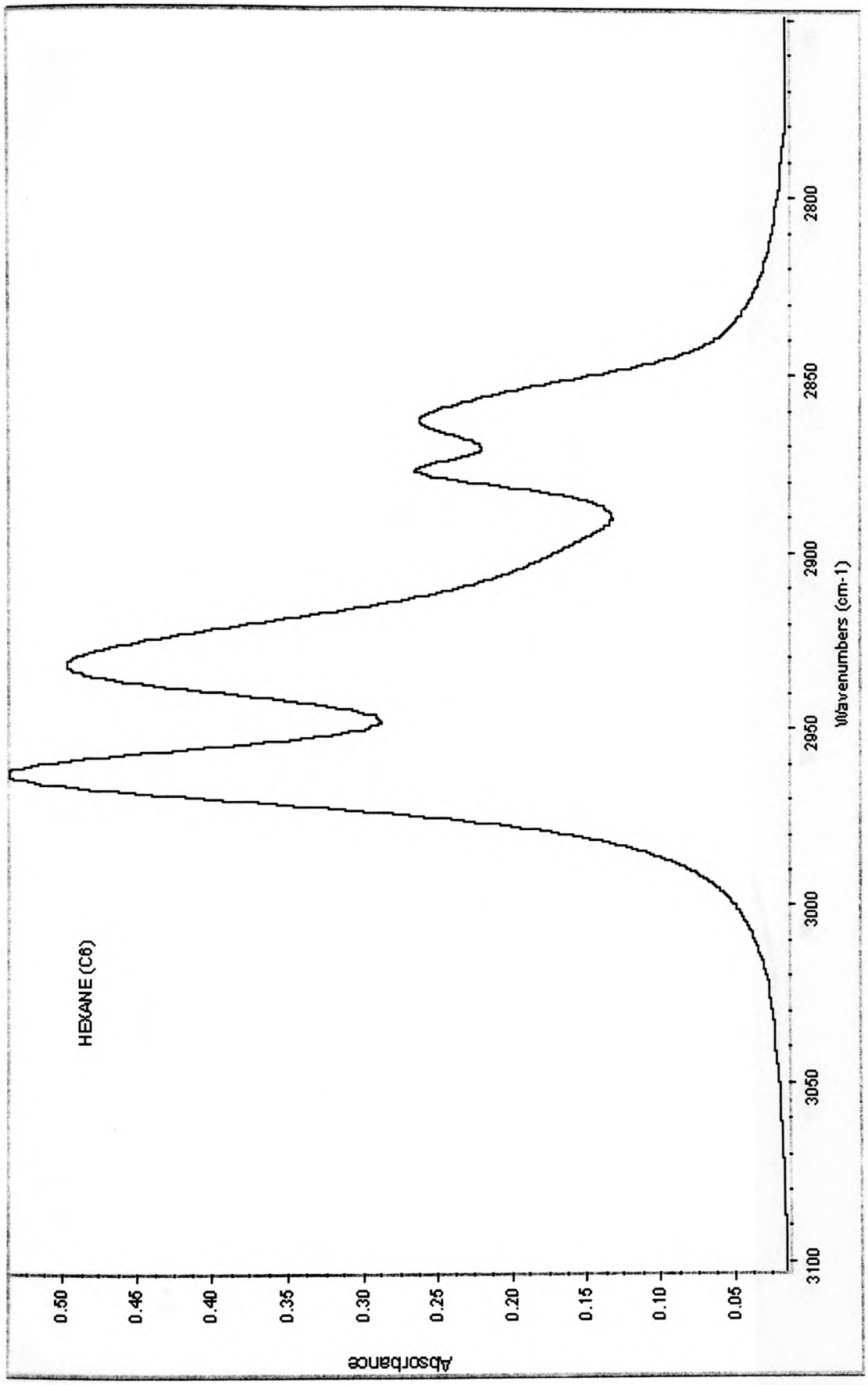

Fig 3.1 Hexane – Carbon chain = 6

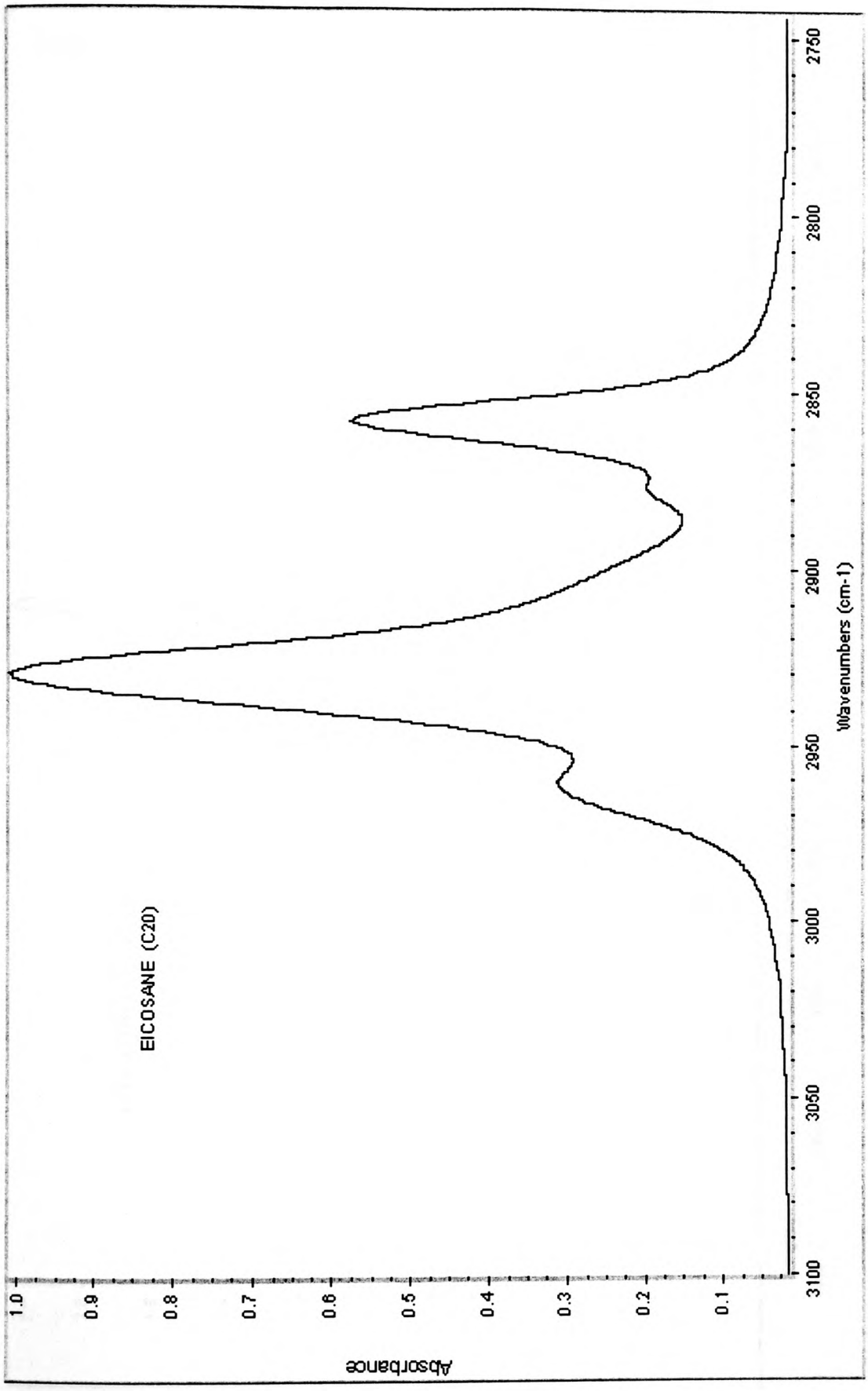

Fig.3.2 Eicosane- carbon chain = 20

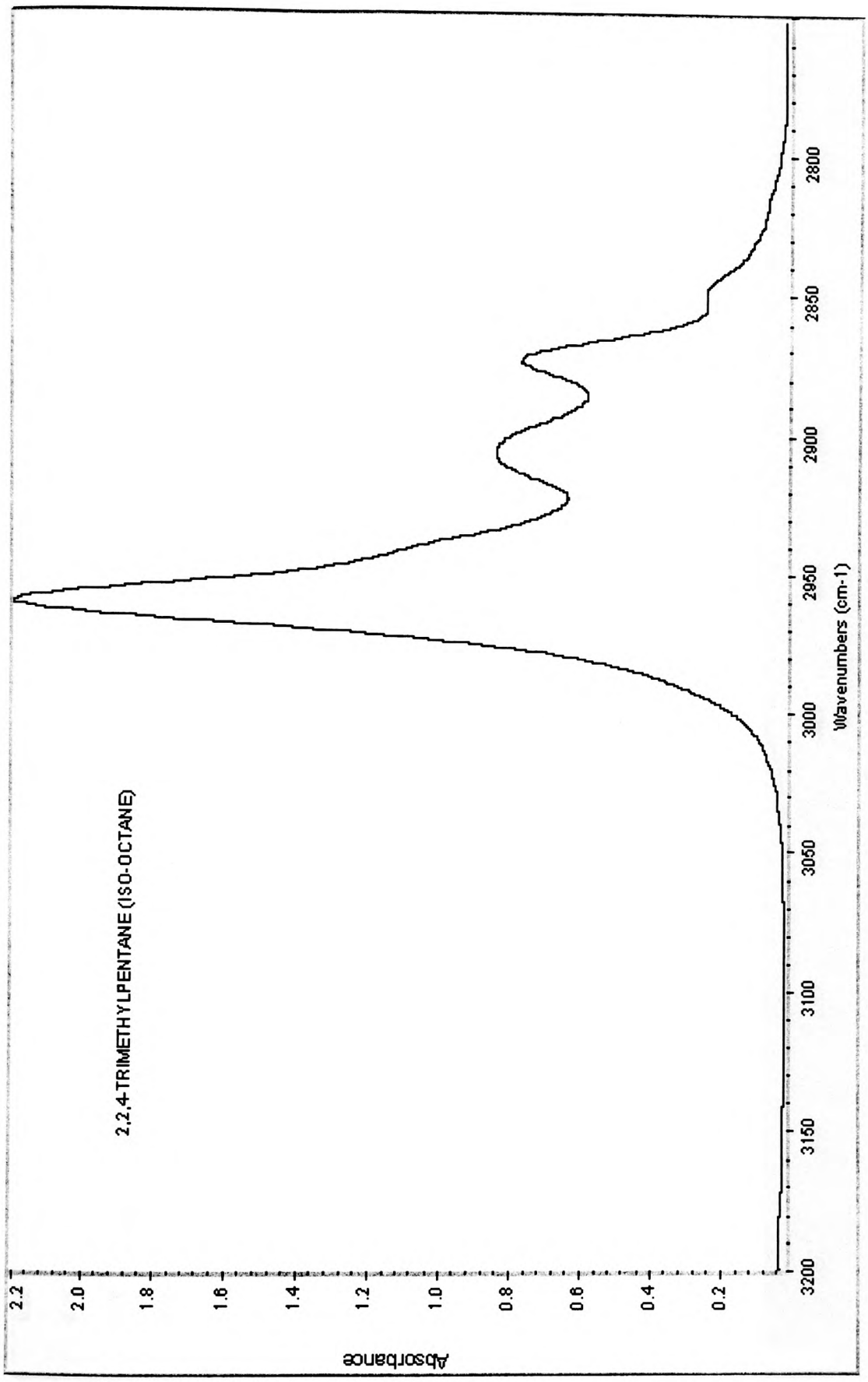

Fig 3.3. 2,2,4-trimethylpentane (iso-octane)

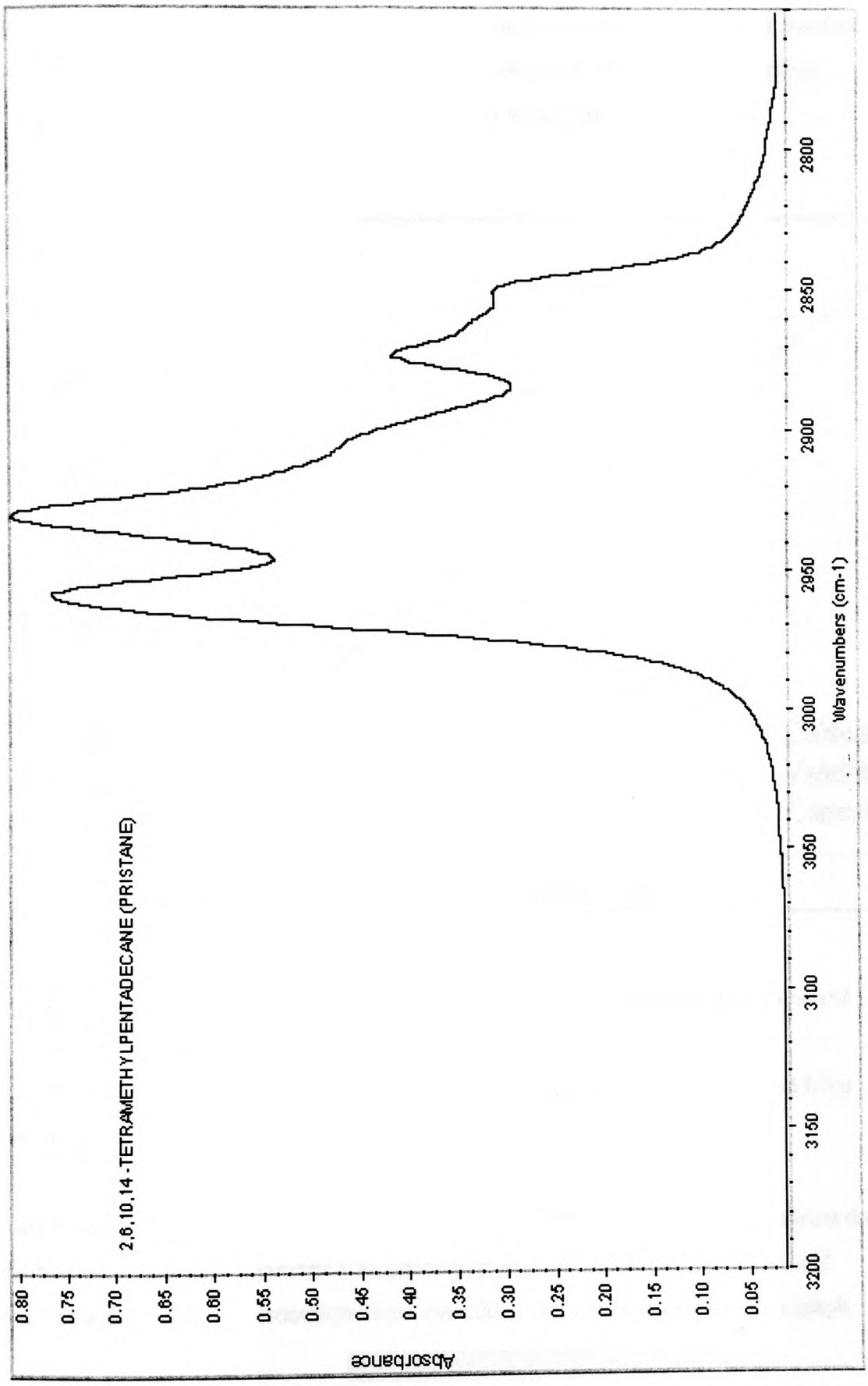

Fig 3.4. 2,6,10,14-tetramethylpentadecane (pristine)

As stated, when quantifying, the first consideration is the choice of standard material used to produce the Beer Lambert plot. In ideal conditions the standard solutions should always be prepared from the analyte under examination.

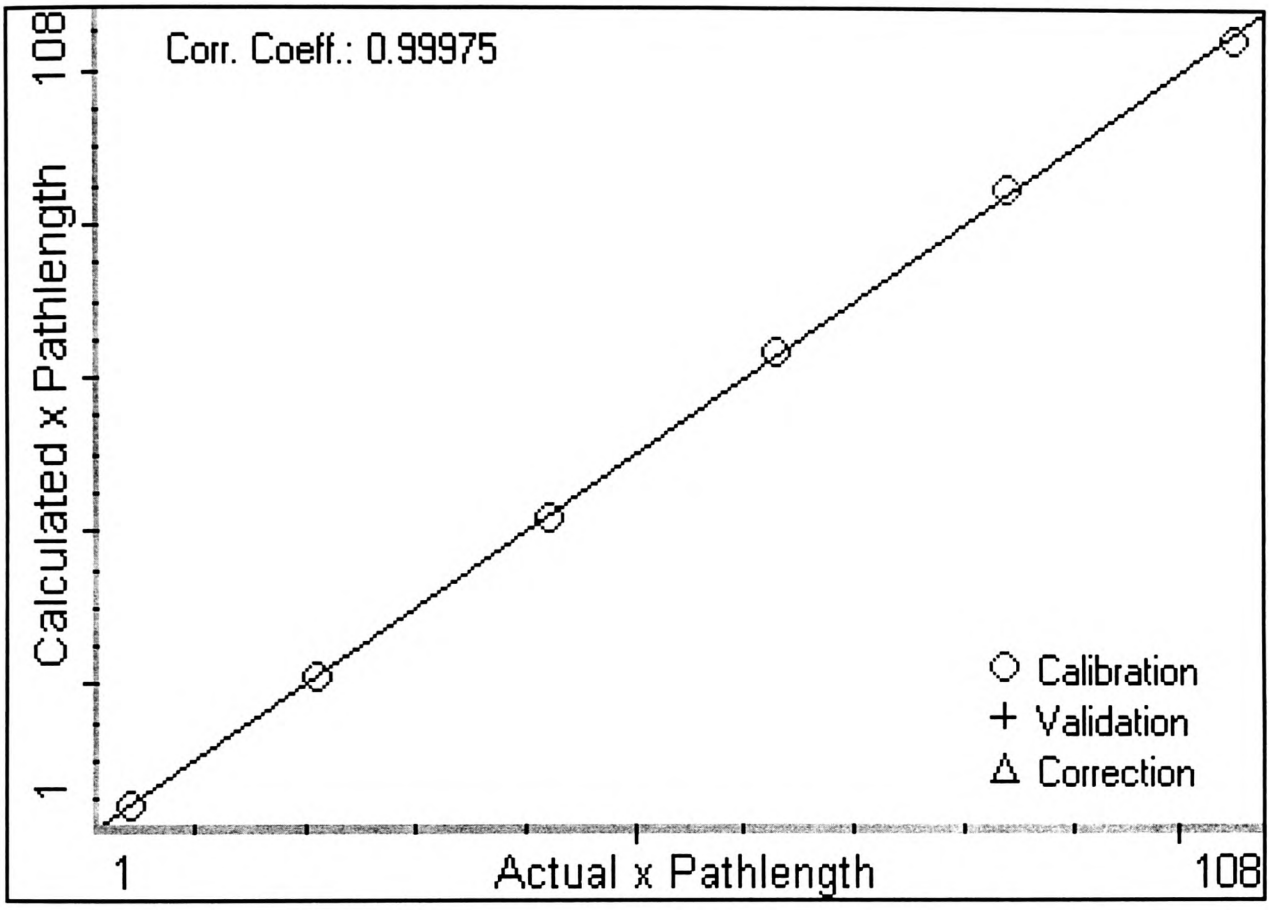

Fig 3.5. Calibration graph – offline analysis using known hydrocarbon standard

The method becomes problematic, when the calibration plot is not constructed from the hydrocarbons being analysed.

This is seen when solutions of hexane in carbon tetrachloride are used to construct the calibration graph, the assymetric CH_2 stretching peak at ~2930 cm^{-1} are used, and other straight chained hydrocarbons concentrations are interpolated from this graph.

Hydrocarbon	% accuracy
Hexane	99.94
Heptane	124.07
Octane	153.44
Nonane	154.81
Decane	165.18
Dodecane	183.94

Where N = 25 for each hydrocarbon

This situation arises because as the chain length of the hydrocarbon increase the CH_2 intensity increases relative to the intensity of the CH_3 absorbance peak of hexane. This results in hydrocarbons with longer chain lengths than hexane, having a positive enhancement to their theoretical concentration value. The opposite effect would be seen if the hexane CH_3 assymetric stretching peak ~2960 cm^{-1} had been chosen.

A more comprehensive list of the adverse effect caused by the use of non-representative hydrocarbons can be seen in Appendix E.

The results in Appendix E, clearly shows that unless the appropriate hydrocarbon is used in the calibration procedure errors, both positive and negative, will occur in the analyte quantification. These results were obtained for a single hydrocarbon, the problem is compounded if a hydrocarbon mixture is analysed.

In an attempt to reduce this effect multiple wavelengths can be used instead of a single wavelength. This was firstly suggested by Simad,[15] and later refined by Whittle.[16,17]

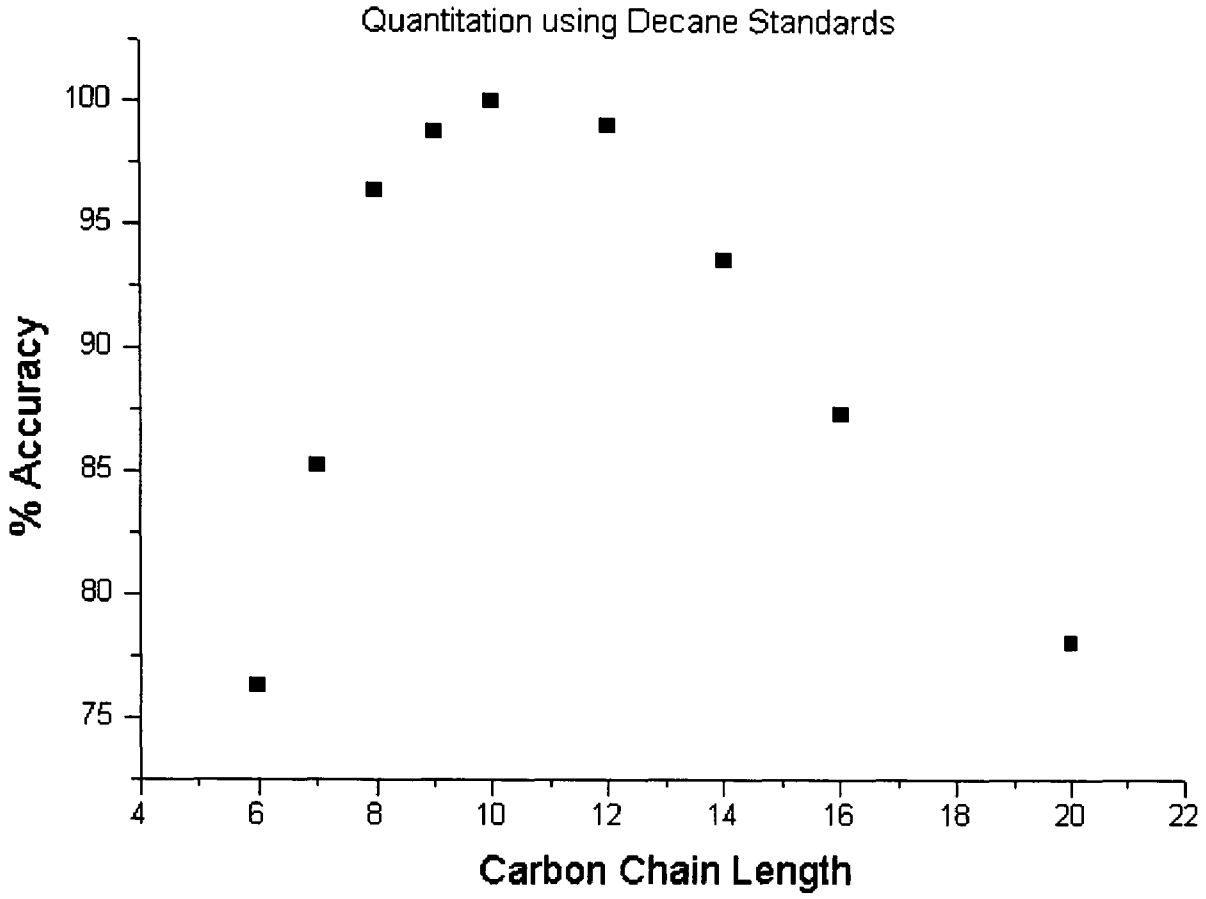

Fig 3.6. The effect of quantification of a range of hydrocarbons using decane as standards, and quantified using multiple wavelengths

3.3 Quantification of a multiple component aliphatic hydrocarbon mixture using a single hydrocarbon calibration procedure.

The quantitative problem of hydrocarbon mixtures can be demonstrated if a range of hydrocarbon solutions are prepared from differing proportions of hexane (carbon chain = 6) and eicosane (carbon chain = 20).,

Figs 3.7 and 3.8 show the single hydrocarbons infrared spectra, concentration 50 and 75 ppm of hydrocarbon in carbon tetrachloride, while Fig 3.9 shows the effect of the binary mixtures on their infrared spectra. All spectra obtained from 2 cm pathlength IR quartz cell.

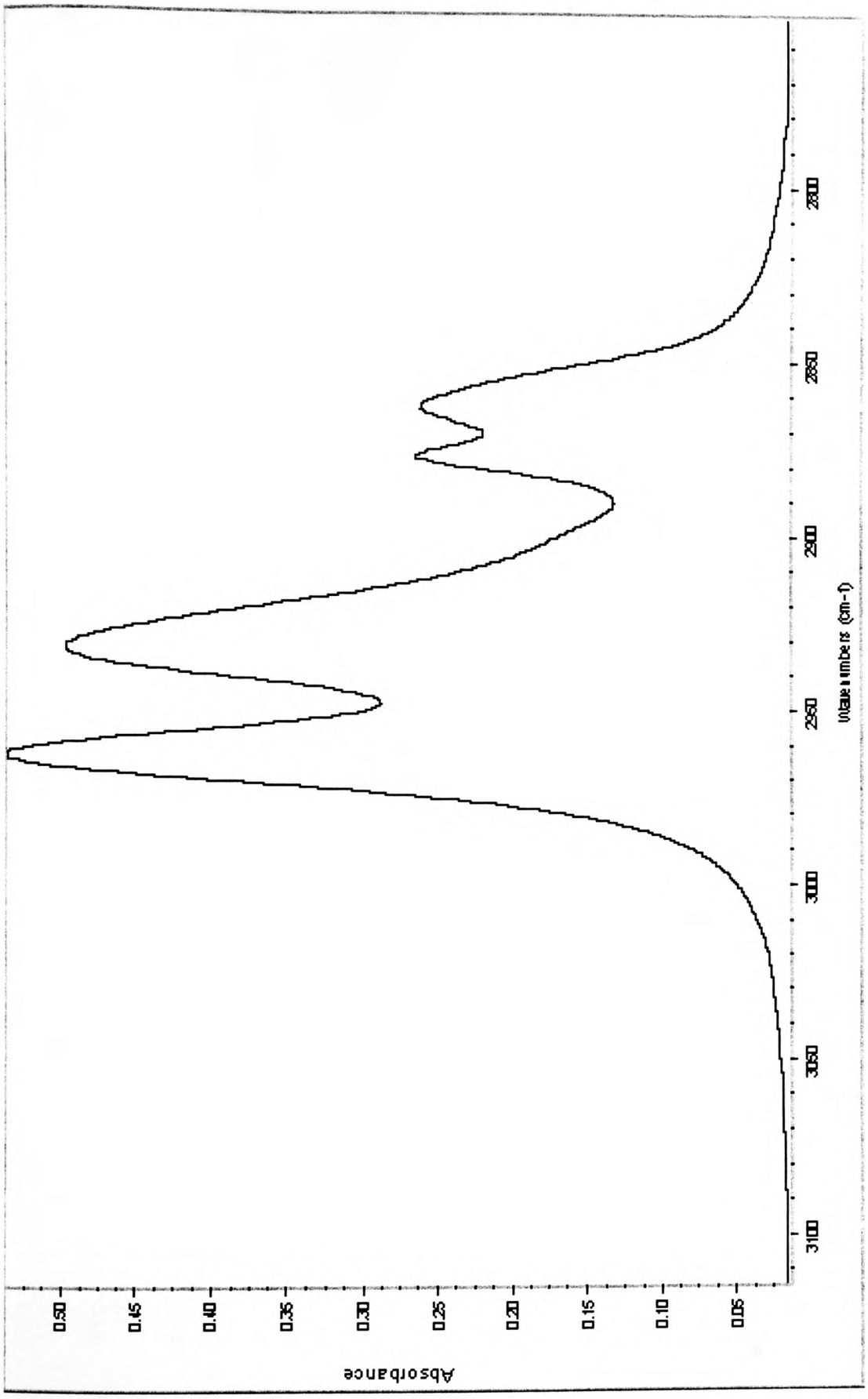

Fig. 3.7 50 ppm Hexane solution in carbon tetrachloride

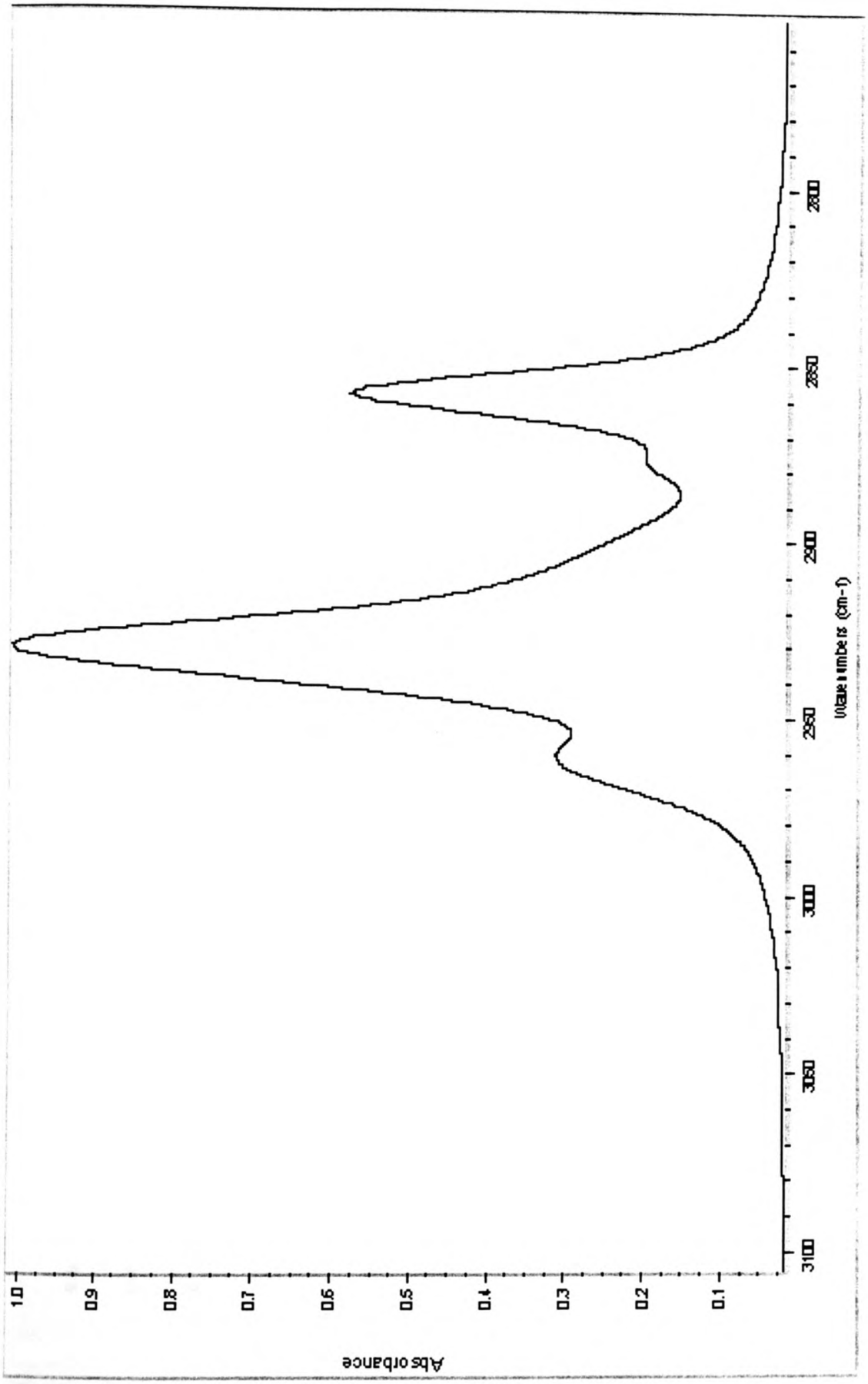

Fig. 3.8 75 ppm Eicosane (carbon chainlength=20) solution in carbon tetrachloride

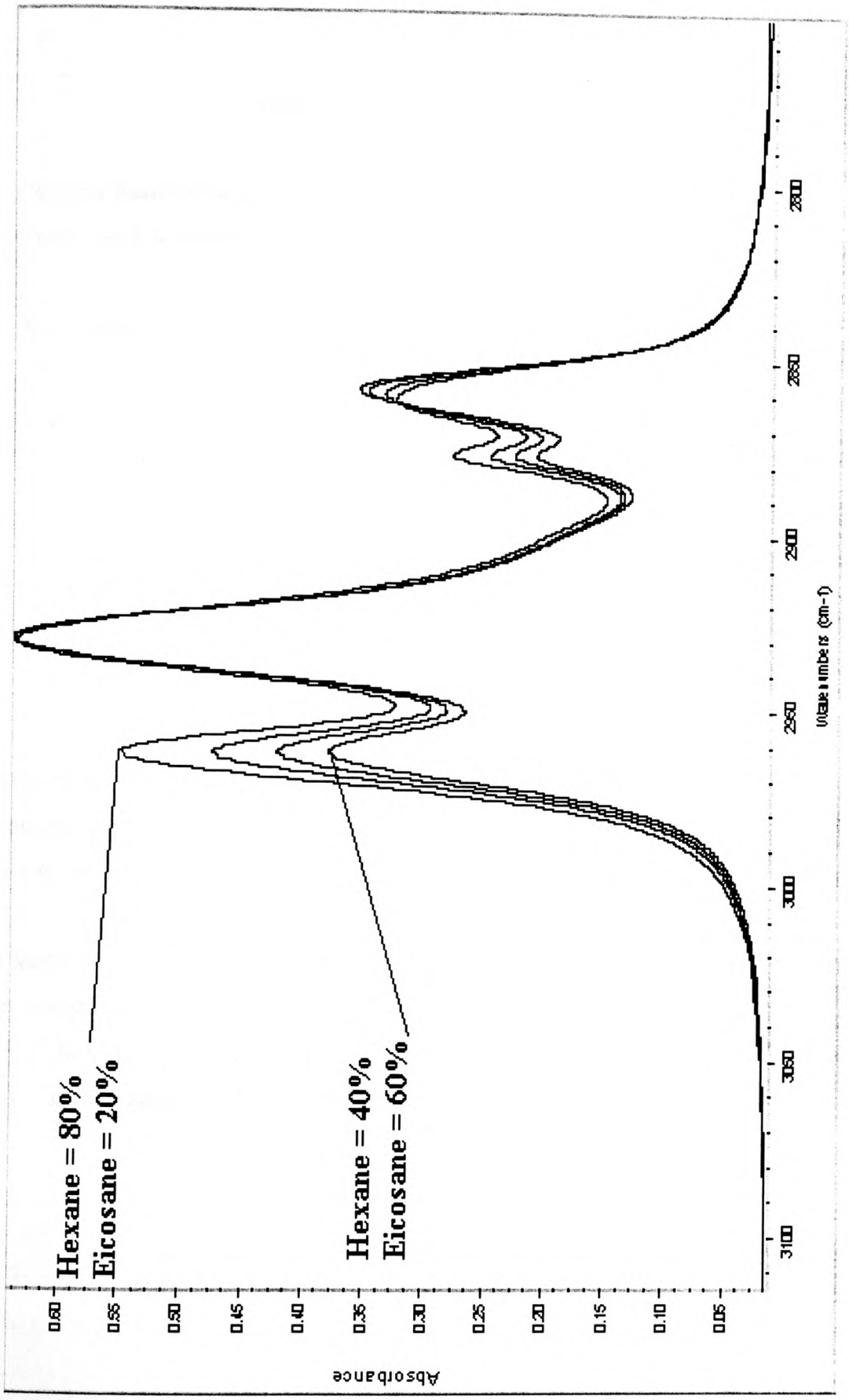

Fig. 3.9 Composite hydrocarbon solution mixtures, prepared from hexane and eicosane (Concentrations in Table 3.1)

In Fig 3.9 the asymmetric stretching peak at 2930 cm^{-1} has been normalized to show the relative effect on the ratio of the CH_3 and CH_2 peaks. Also the total hydrocarbon concentration for these mixtures remained constant, at 60 ppm.

If we now quantify these hydrocarbon mixtures against a single hydrocarbon standard (hexane) the following results are obtained, using 2930 cm^{-1} peak for quantification.

Binary Hydrocarbon mixture Hexane : Eicosane (%)	Quantified against Standard Hexane standards	Quantified against Standard Eicosane standards
80 : 20	67	41
60 : 40	73	45
50 : 50	81	50
40 : 60	87	54

Table 3.1. Quantification of a hydrocarbon mixture

This table shows the errors introduced by varying the composition of the constant concentration hydrocarbon mixture, with, as expected the quantification against hexane being over reported, and against eicosane being under reported.

Clearly the inability to quantify the unknown composition solutions against either of its constituent hydrocarbons has serious consequences on the accuracy of the quantification. Unfortunately this is the case in the vast majority of IR based hydrocarbon quantification procedures.

3.4 Quantification of unknown hydrocarbons (where a general hydrocarbon mixture is used)

There are numerous standard infrared methods (Appendix D), and all follow similar procedures for the analysis of hydrocarbons in water.

There are associated errors with any analytical technique, and as the number of steps within a procedure increase so does the inherent errors. In the analysis of aqueous hydrocarbon effluent the procedure can be split into several distinct steps, each with their own associated errors;

a. Sampling
b. Extraction of the hydrocarbon
c. Selection of reference material
d. Quantification

a. Sampling :-

The sample taken must accurately represent the bulk of the sample being examined.

b. **Extraction :-**

The extraction process must be complete, with no analyte remaining in the aqueous phase. The majority of extraction methods use carbon tetrachloride or Freon 113 (1,1,2-trichloro1,2,2-trifluoroethane). As of 1st January 2002 these solvents can no longer be supplied for the determination of hydrocarbons in water. It is however permitted to run down existing stockpiles and / or revert to an alternative extraction solvent such as trichloroethene.

c. Reference Material

Selection of the reference material can have a significant influence on the accuracy of the quantification, as already seen.

d. Quantification

The overall accuracy of the results obtained by the quantification will depend on all of the above factors. Any new analytical technique must have an overall error no greater than those already encountered with the present methods.

All the infrared methods of analysis are associated with the use of Chloroflurocarbons (CFCs) as solvents, these have three clear problems.

a. the environmental damage caused by these solvents .
b. the spiraling costs associated with their purchase , and disposal
c. lack of availability.

These conditions must be taken into account if any new analytical methods is to be successfully introduced.

The work reported in this chapter has concentrated on three standard, conventional solvent based, methods namely:

a. ASTM Std. D3921 1985
b. DIN H38 409 H18 1981
c. HMSO Std. DoE 1983

Quantification using these three standard methods is now reported.

3.4.1 ASTM Method (D 3921 – 80)

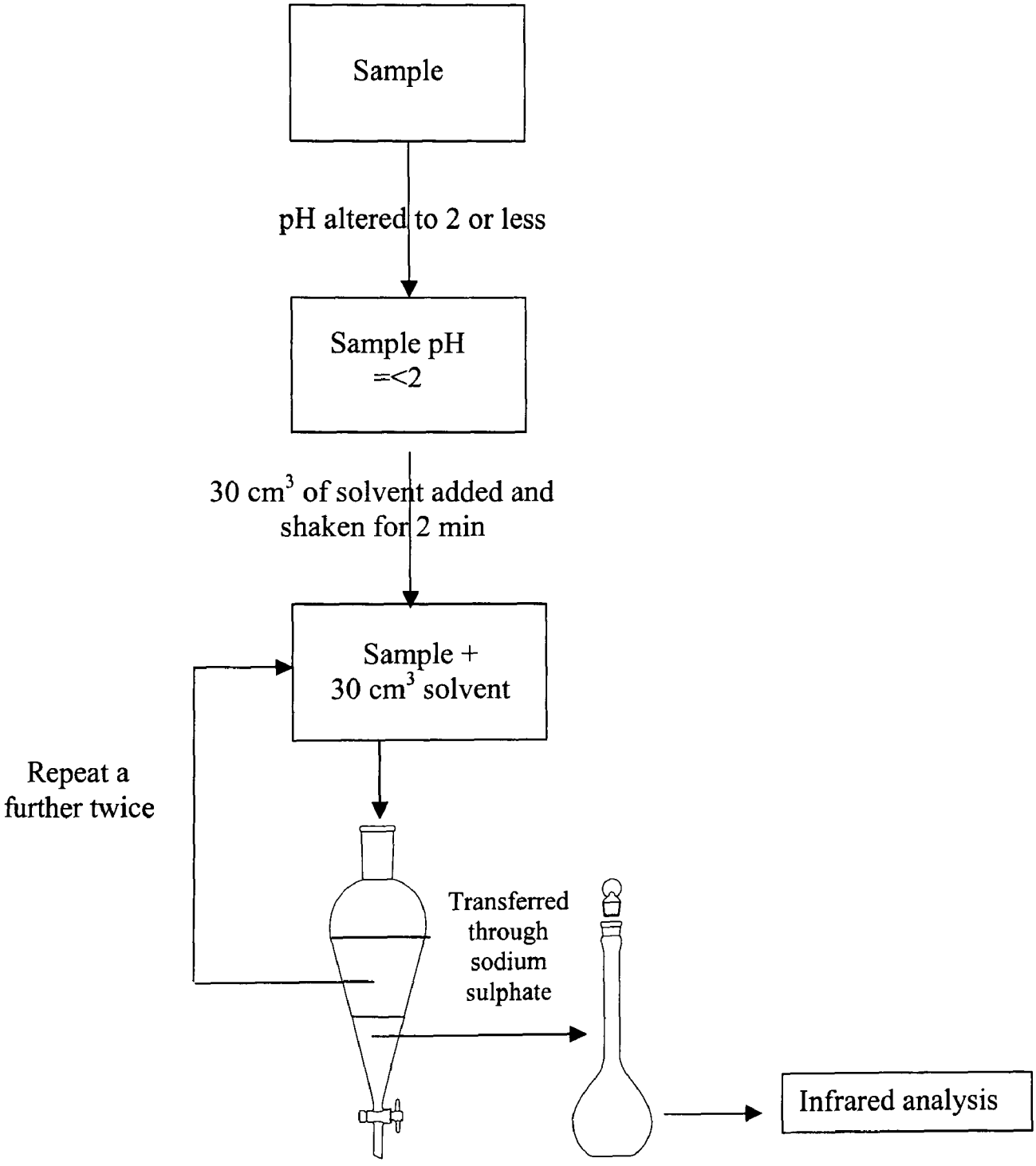

Fig. 3.10 ASTM procedure for extractable hydrocarbon analysis

This method[18] covers the concentration range of 0.5 to 100 mg dm^{-3}, and classifies oils and greases as those components which are extractable by this method, and solvent, and can be measured by infrared spectroscopy. The procedure for extraction and analysis is shown diagrammatically in Fig. 3.10.

3.4.1.1 Extraction Procedure

A 750 cm^3 representative aqueous sample is collected and acidified to pH 2 or below using sulphuric acid or sodium hydrogen sulphate. A 30 cm^3 aliquot of 1,1,2-trichloro-1,2,2-trifluoroethane (Freon-113) is then added, the sample bottle cap quickly replaced, and shaken vigorously for 2 min. The aqueous sample and solvent are then transferred to a separating funnel and allowed to settle.

The bottom organic layer is then transferred to a 100 cm^3 volumetric flask, passing through sodium sulphate, to remove any moisture, which has previously been solvent washed to remove impurities.

The aqueous layer is then returned to the original sample bottle, and the extraction process repeated a further twice. The three solvent extracts are then made up to 100 cm^3 volumetrically, and passed through silica gel before being analysed by infrared spectroscopy. The silica gel is used to remove all polar non hydrocarbon extracted materials.

3.4.1.2. Calibration

The method recommends the use of the hydrocarbon under analysis for the calibration procedure, failing this a standard calibration mixture of *iso*-octane (2,2,4-trimethylpentane) and cetane (hexadecane) should be used.

The original calibration mixture consisted of *iso*-octane, hexadecane and benzene, because of the health hazards associated with benzene this has now been eliminated and to allow a relationship between the old and new methods to be established a scale up factor of 1.4 is now introduced.

The calibration mixture is prepared by accurately mixing 15 cm^3 of each hydrocarbon (*iso*-octane and hexadecane) in a glass stoppered bottle, and mixed well. The calibration standards are then prepared by dilution of this mixture.

Calibration standard A :- 1 cm^3 of the original standard mixture is volumetrically prepared in 100 cm^3 of solvent. The calculated concentration of this solution is then multiplied by the benzene compensation factor of 1.4.

Further calibration standards are then volumetrically prepared from this solution to give a range of approximately 1 to 100 mg / 100 cm^3 of solvent.

These volumetric solutions are then analysed by infrared spectroscopy in the range 3200 to 2700 cm^{-1} and the maximum absorbance value at approximately 2930 cm^{-1} is noted.

A calibration graph of absorbance value against concentration is then plotted.

The unknown concentration hydrocarbon is then analysed using the same procedure as the calibration standards, and the concentration interpolated from the calibration graph.

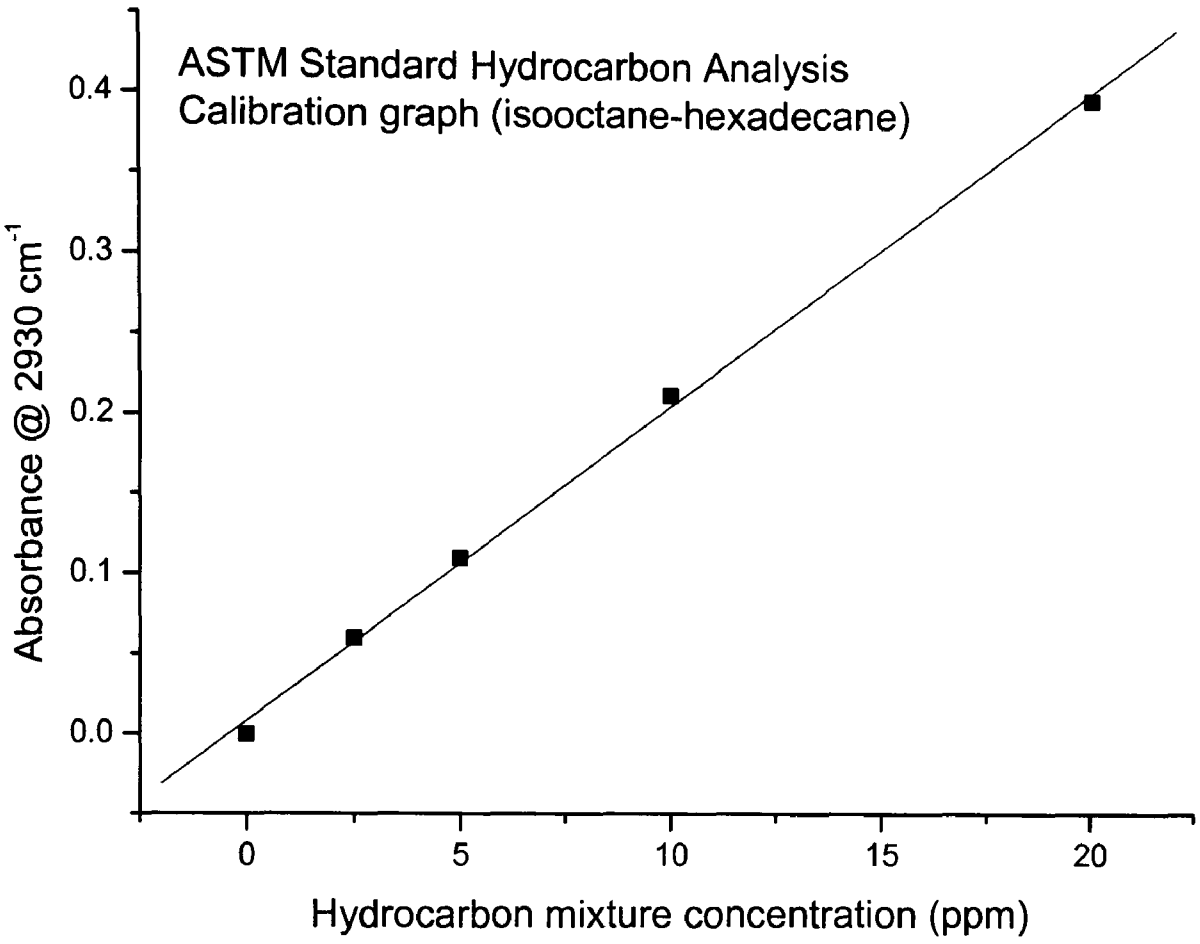

Fig.3.11 ASTM calibration graph

Hydrocarbon	Concentration (ppm)	Calculated Concentration (ppm)	% Error
Hexane	21.0	6.23	-70.3
Heptane	21.6	8.26	-61.7
Octane	13.0	6.7	-48.5
Nonane	13.5	7.7	-42.9
Decane	13.5	8.3	-38.5
Dodecane	12.9	7.7	-40.3
Tetradecane	13.5	6.9	-48.9
Eicosane	10.0	13.4	+34.0

Table 3.2. Different hydrocarbon quantified against the ASTM method

The values in Table 3.2, indicate the problems associated with an unrepresentative reference standard.

3.4.2 DIN H38 409 H18 1981

This is a standard method[19] of hydrocarbon analysis by infrared spectroscopy, formulated by the Technical Water Group and the Association of German Chemists, it is the easiest of the standard methods to implement, as it requires no initial calibration stage, but relies on the use of pre-determined coefficients.

The method covers a wide contamination range, quantitatively down to 0.1 mg/l, and semi-quantitatively between 0.1 and 0.01 mg/l.

Fig. 3.12 shows diagrammatically the implementation of the method, it is important to realise that if the polar contaminants cannot be removed in the close cycle penultimate stage, then this method cannot be used. (In the diagram note that TCTFE is an alternative representation for Freon-113)

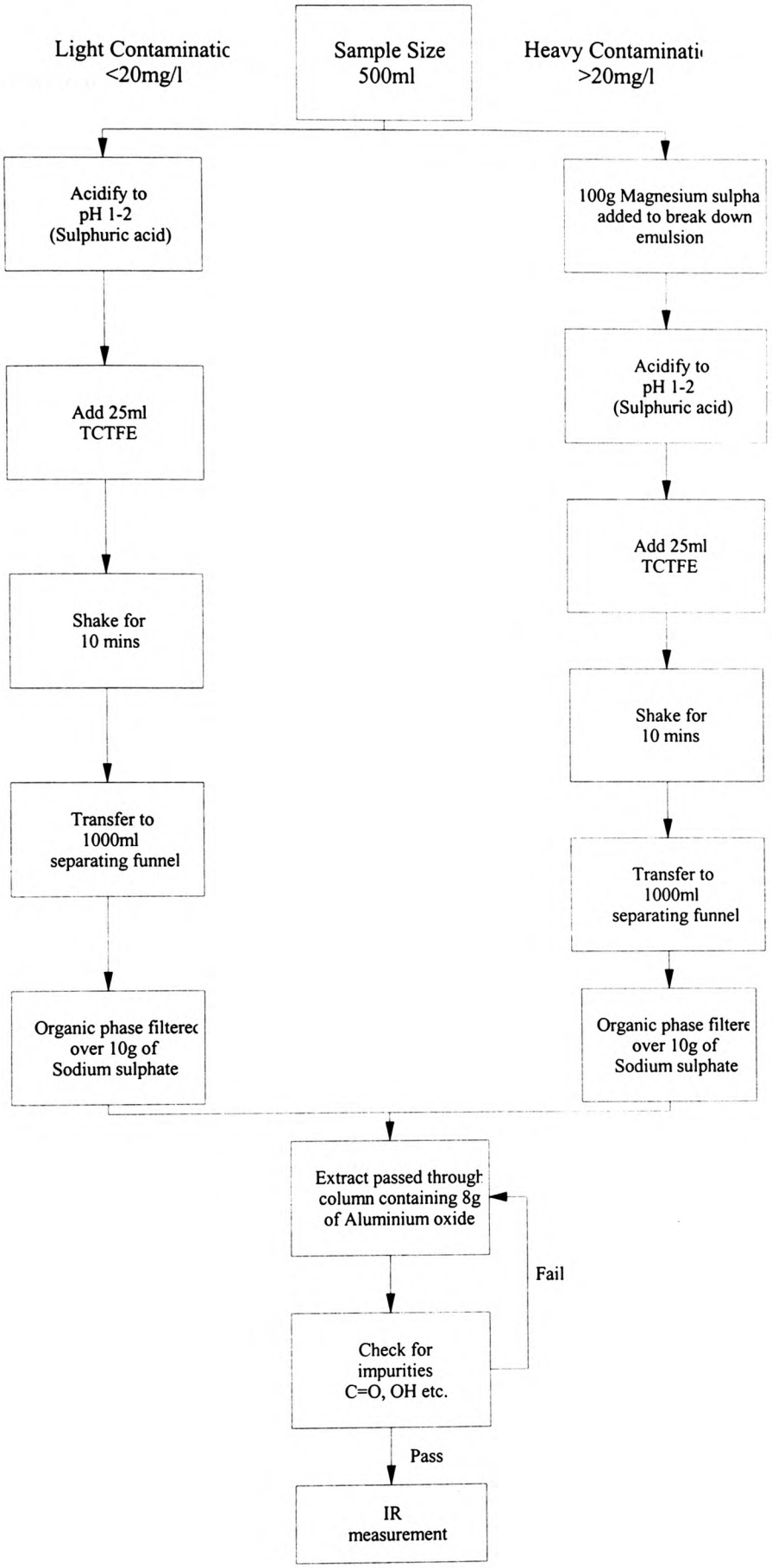

Fig. 3.12 Quantitative steps for the DIN standard analytical method

3.4.2.1 Calculation of hydrocarbon concentration using DIN method

This method subdivides hydrocarbons into three classes;

a) Petrol engine fuels:- containing a high proportion of CH_3 groups.

b) High aromatic fuels:- where the ratio of the aromatic CH groups and the CH_2 group > 0.23.

c) Middle distillate fuels:- contains predominantly CH_2 groups.

Because of the relatively low absorbtivities of the aromatic CH stretching frequencies, (3030 cm^{-1}) this method is not entirely ideal for hydrocarbons in b), and other analytical techniques should be used.

Depending upon the profile of the hydrocarbon under investigation, hydrocarbon concentrations can be calculated using the following formulae.

Petrol engine fuels

$$Conc = \frac{1.3\,Ve\,(\frac{E_1}{C_1} + \frac{E_2}{C_2} + \frac{E_3}{C_3})}{Vp\;\;d}$$

Mineral fuels

$$Conc = \frac{1.4Ve(\frac{E_1}{C_1} + \frac{E_2}{C_2})}{Vp\;\;d}$$

where

Ve = volume of solvent used for extraction (cm^3)

Vp = volume of water extracted (dm^3)

E_1 = CH_3 absorption @ $\approx$ 2960 cm^{-1}

C_1 = CH_3 group extinction coefficient (8.3 $\pm$ 0.3)

E_2 = CH_2 absorption @ $\approx$ 2930 cm^{-1}

C_2 = CH_2 group extinction coefficient (5.4 $\pm$ 0.2)

E_3 = CH absorption @ $\approx$ 3030 cm^{-1}

C_3 = CH group extinction coefficient (0.9 $\pm$ 0.1)

d = solution pathlength

Using the mineral oil equation, no aromatic contribution, the data for a series of aliphatic straight chained and branched hydrocarbons was used to calculate their theoretical concentration.

The standard equation for Petrol engine fuels was then applied to the absorbance data for several aromatic hydrocarbons allowing calculation of their concentrations.

Table 3.3 shows results, each standard was replicated, (N=4), and the mean values used in the calculations.

Hydrocarbon	Actual Conc (ppm)	A2960 cm^{-1}	A2930 cm^{-1}	Calculated Conc DIN (ppm)	% Error	Mean. Error %
Hexane	105	0.527	0.485	108.10	2.95	
	84	0.424	0.393	87.80	4.52	
	63	0.317	0.294	65.70	4.29	6.40
	42	0.213	0.202	44.60	6.19	
	21	0.106	0.097	21.80	3.81	
	4.2	0.023	0.023	4.90	16.67	
Heptane	108	0.542	0.631	129.40	19.81	
	86.4	0.440	0.518	105.80	22.45	
	64.8	0.332	0.388	79.50	22.69	17.65
	43.2	0.218	0.254	52.10	20.60	
	21.6	0.106	0.126	25.60	18.52	
	4.32	0.018	0.022	4.40	1.85	
Octane	131	0.685	0.932	181.50	38.55	
	98	0.494	0.678	131.70	34.39	
	65.5	0.361	0.498	96.50	47.33	38.98
	33	0.174	0.238	46.25	40.15	
	13	0.062	0.093	17.48	34.46	
Nonane	135	0.635	0.976	183.17	35.68	
	101	0.463	0.726	135.47	34.13	
	67.5	0.331	0.519	96.75	43.33	39.69
	34	0.162	0.254	47.42	39.47	
	13.5	0.065	0.107	19.69	45.85	
Decane	135	0.607	1.039	189.12	40.09	
	101	0.456	0.788	143.12	41.70	
	67.5	0.312	0.545	98.65	46.15	46.28
	34	0.161	0.283	51.19	50.56	
	13.5	0.061	0.117	20.64	52.89	
Dodecane	129	0.523	1.102	190.47	47.65	
	96.75	0.407	0.841	145.99	50.89	
	64.5	0.269	0.574	98.97	53.44	51.25
	32.25	0.133	0.288	49.58	53.74	
	12.9	0.052	0.113	19.42	50.54	
n-tetradecane	135	0.426	1.029	172.67	27.90	
	81	0.268	0.675	112.25	38.58	
	54	0.176	0.444	73.87	36.80	
	40.5	0.134	0.332	55.37	36.72	36.58
	27	0.094	0.231	38.59	42.93	
	13.5	0.044	0.111	18.39	36.22	
	5.4	0.022	0.043	7.51	39.07	
n-hexadecane	167	0.054	1.257	212.66	27.34	
	125.25	0.396	1.107	180.50	44.11	
	83.5	0.273	0.751	122.80	47.07	40.56
	41.75	0.129	0.364	59.16	41.70	
	16.7	0.053	0.146	23.81	42.57	

Eicosane	129	0.295	1.001	157.81	22.33	
	103	0.238	0.797	126.07	22.40	
	77.5	0.179	0.595	94.20	21.55	22.01
	51.6	0.118	0.396	62.65	21.41	
	25.8	0.060	0.200	31.57	22.36	
Iso-octane	274.5	1.442	0.561	195.74	-28.69	
	183	1.006	0.377	120.50	-34.15	
	91.5	0.546	0.188	70.85	-22.57	-23.53
	36.6	0.233	0.081	30.29	-17.24	
	9.15	0.059	0.021	7.78	-14.97	
Pristane	127	0.752	0.797	169.17	33.20	
	95	0.586	0.622	132.02	38.97	
	63.5	0.387	0.411	87.09	37.15	43.29
	31.75	0.214	0.228	48.27	52.03	
	12.7	0.089	0.092	19.70	55.12	
Reference Oil	64	0.193	0.337	60.99	-4.70	
	51.2	0.158	0.275	49.85	-2.64	
	38.4	0.120	0.207	37.56	-2.19	0.64
	25.6	0.082	0.143	25.88	1.09	
	12.8	0.045	0.075	13.75	7.42	
	6.4	0.020	0.038	6.71	4.84	

Hydrocarbon	Actual Conc (ppm)	A3030 cm^{-1}	A2960 cm^{-1}	A2930 cm^{-1}	Calculated Conc DIN	% Error	Mean Error %
Benzene	166.0	0.339	0.000	0.000	242.3	46.0	
	124.5	0.241	0.000	0.000	172.3	38.4	
	83.0	0.161	0.000	0.000	115.0	38.6	37.2
	41.5	0.076	0.000	0.000	54.6	31.5	
	16.6	0.031	0.000	0.000	21.9	31.8	
Toluene	174.0	0.174	0.057	0.091	140.2	-19.5	
	130.5	0.131	0.047	0.074	106.7	-18.3	
	87.0	0.088	0.031	0.049	71.4	-18.0	-21.4
	43.5	0.041	0.017	0.026	33.7	-22.5	
	17.4	0.015	0.006	0.011	12.4	-29.0	
Ethyl benzene	149.0	0.180	0.311	0.175	174.9	17.4	
	112.0	0.136	0.232	0.136	131.7	17.6	
	75.0	0.094	0.164	0.093	91.2	21.6	26.2
	37.0	0.048	0.084	0.052	47.2	27.6	
	15.0	0.021	0.041	0.034	22.0	46.6	

Table 3.3 Results of quantification for DIN method

The errors associated with this method, compared with the ASTM method, are better for the shorter chainlengths, +6.4% compared to –70%. From octane to eicosane the results are of the same order of magnitude, the difference being the ASTM method understate the value, while the DIN method overstates the concentration.

3.4.3 HMSO method of quantifying hydrocarbons in water

As stated, where the quantification of an aqueous hydrocarbon sample is required, the procedure should, where possible, use the same hydrocarbon as a reference. This is frequently not possible, and in these cases a "representative" hydrocarbon has to be used as a substitute.

The HMSO method[20] uses three separate hydrocarbons to represent an unknown, one to represent those compounds rich in CH_3 groups, where the compound has considerable branching.
One representing compounds rich in CH_2 groups, straight chained compounds, and a third to represent any hydrocarbon which has an aromatic component.

These hydrocarbons are analysed individually, and attributed coefficients which are representative of the intensity of their absorbance at these specified characteristic wavelengths, for a known concentration.

3.4.3.1 Determination of offline HMSO coefficients

To calculate the concentration of an unknown hydrocarbon, "representative" hydrocarbons have to be used to calculate coefficients which can be applied to the following equation;

$$c = xA_{2930} + yA_{2960} + z\left(A_{3030} - \frac{A_{2930}}{F} \right)$$

where;

c = unknown concentration

A_{3030} = Abs. @ 3030 cm^{-1}

A_{2960} = Abs. @ 2960 cm^{-1}

A_{2930} = Abs. @2930 cm^{-1}

x, y, and z are the representative coefficients

and $F = \frac{A_{2930}}{A_{3030}}$ for n-hexadecane.

Representative hydrocarbons used are:

n-Hexadecane for CH_2 rich compounds

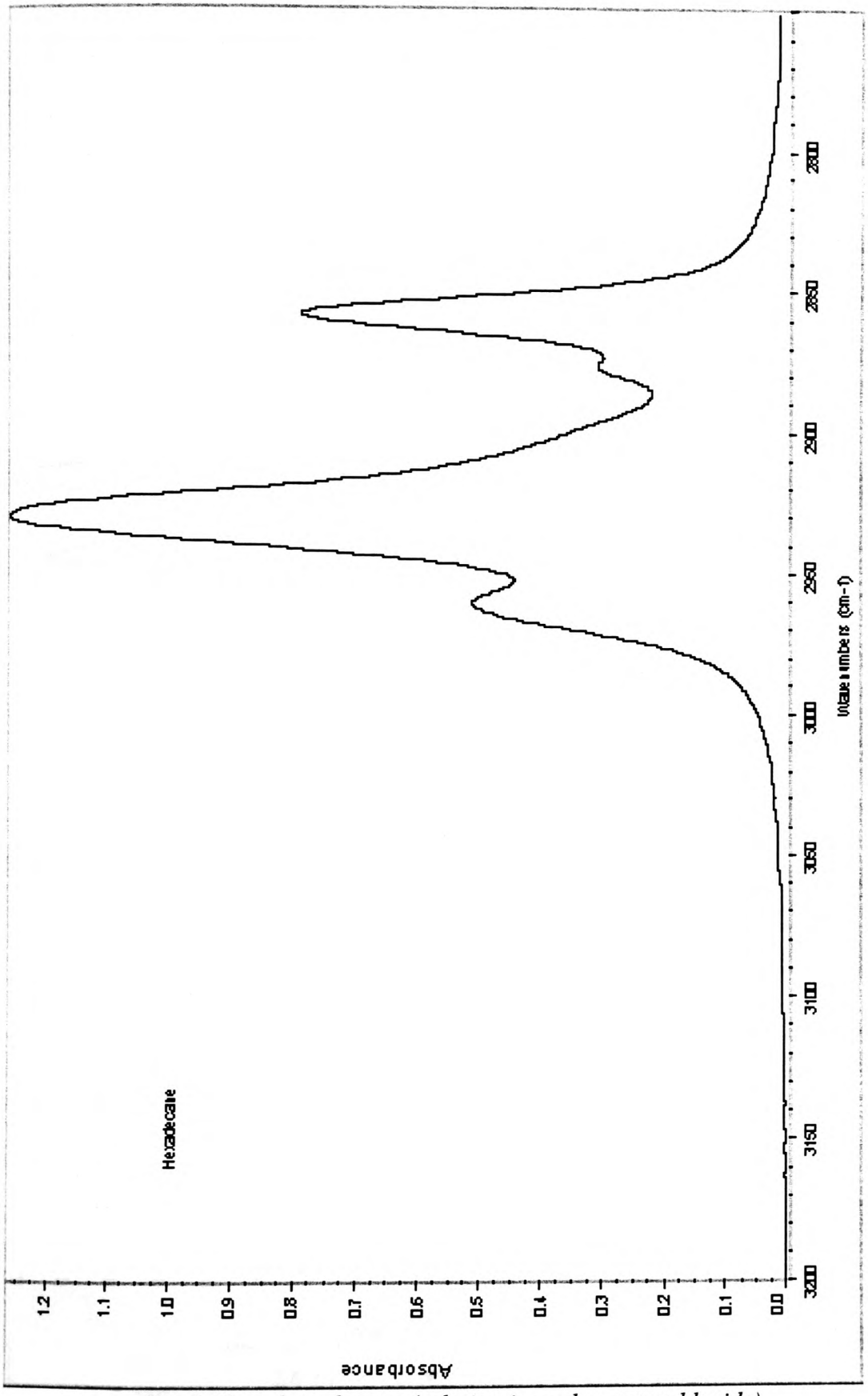

Fig.3.13 n- hexadecane (solution in carbon tetrachloride)

Pristane (2,6,10,14-tetramethylpentadecane) for CH_3

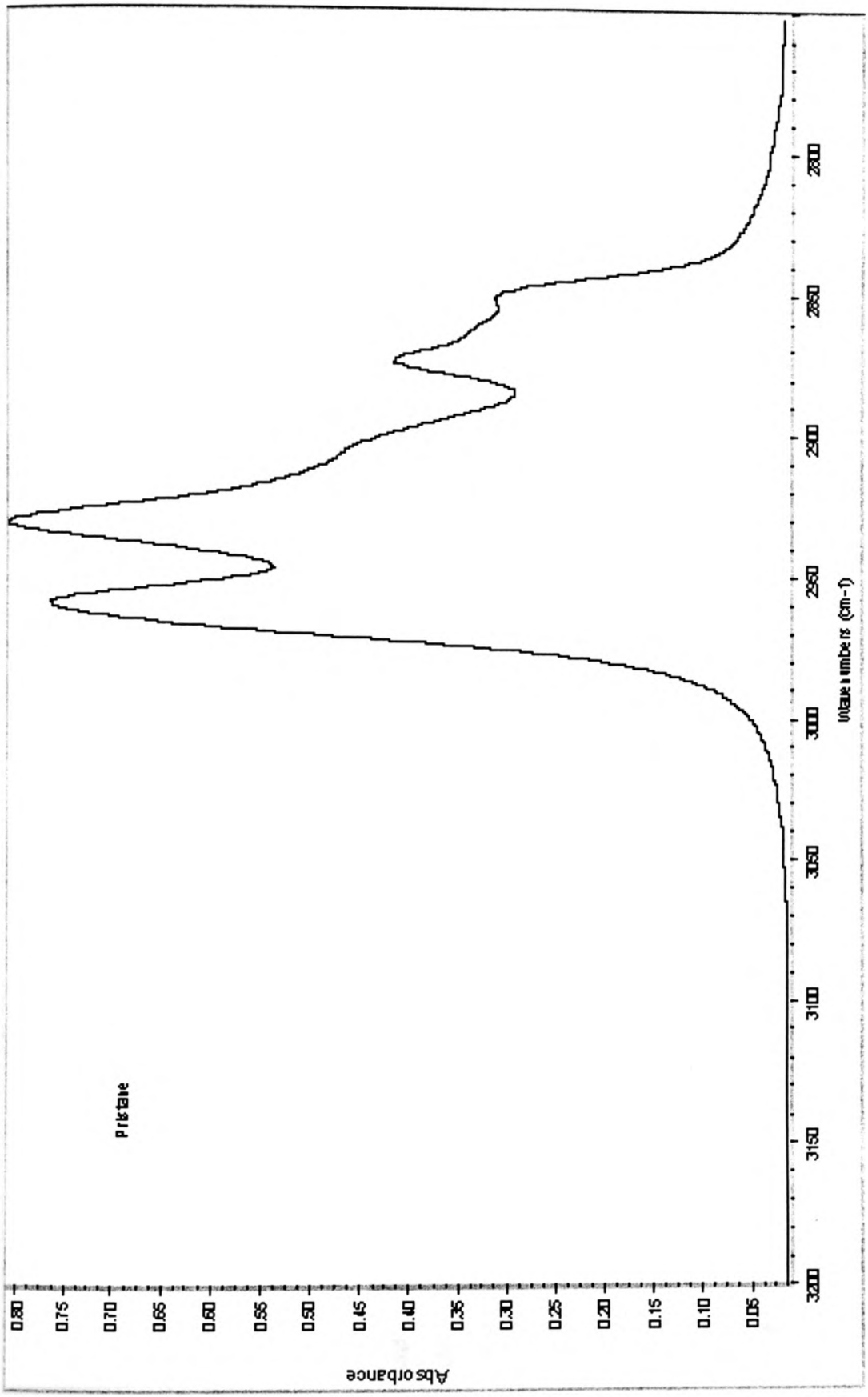

Fig 3.14 pristane (2,6,10,14- tetramethylpentadecane)(solution in carbon tetrachloride)

Ethyl benzene for aromatic CH

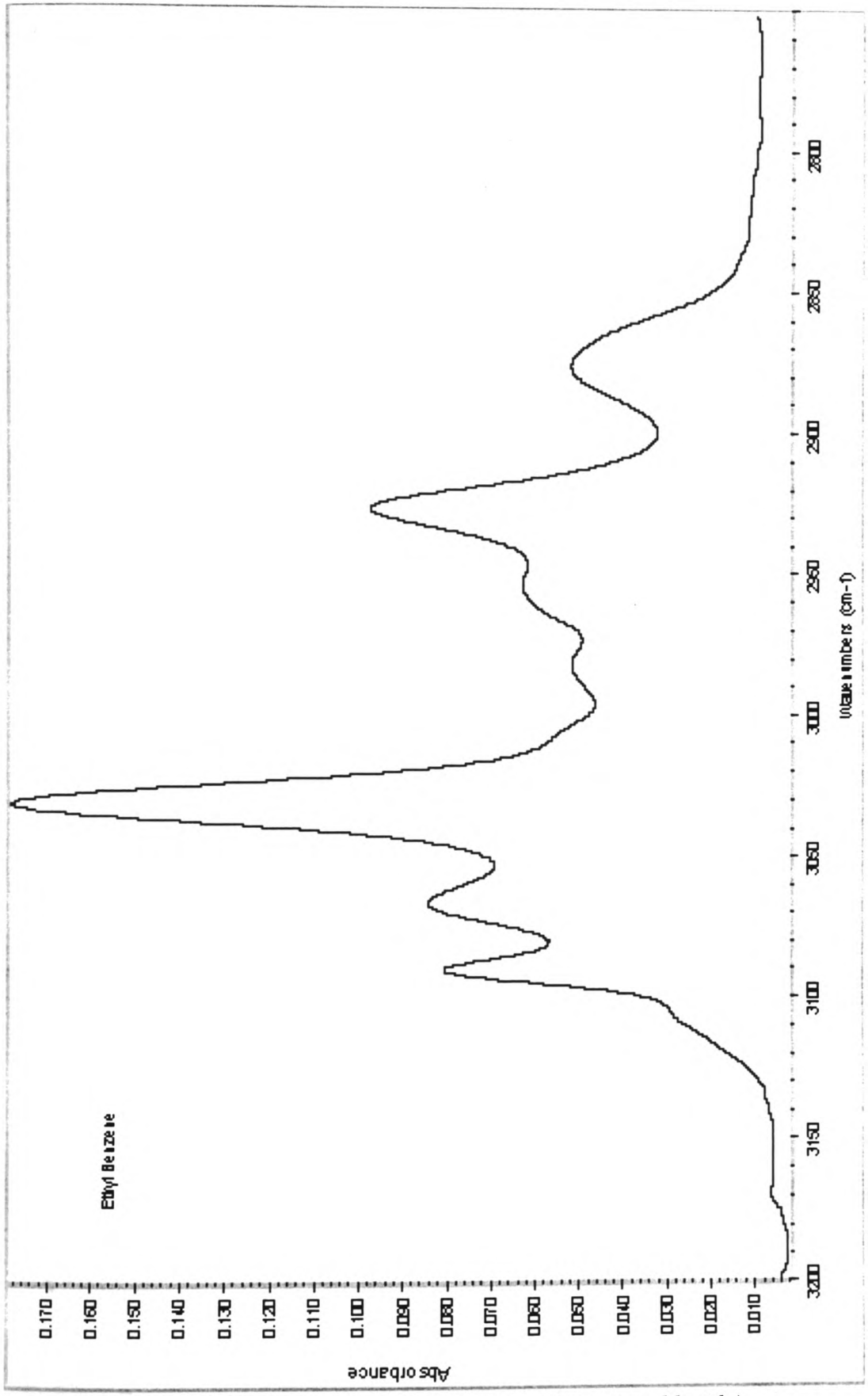

Fig 3.15 ethyl benzene(solution in carbon tetrachloride)

3.4.3.2 Calculation of coefficients

The following parameters were used in the calculations;

Hydrocarbon	Concentration (ppm)	Absorbance @ 2930 cm-1	Absorbance @ 2960 cm-1	Absorbance @ 3030 cm-1
Hexadecane	167	1.254	0.499	0.009
Pristane	127	0.793	0.748	-
Ethyl benzene	171	0.092	0.055	0.169

To calculate x and y, simultaneous equations are formed for hexadecane and pristine, as neither have an aromatic contribution.

$$\textbf{Conc.} = \mathbf{x(A_{2930}) + y(A_{2960})}$$

$$\mathbf{167 = x(1.254) + y(0.499)}$$
$$\mathbf{127 = x(0.793) + y(0.748)}$$

$$\mathbf{x = 113.49}$$

$$\mathbf{y = 49.47}$$

Calculated value for F (hexadecane)

$$\mathbf{F = A_{2930} / A_{3030}}$$

$$\mathbf{F = 1.254 / 0.009}$$

$$\mathbf{F = 139.33}$$

Now using ethyl benzene to calculate z

$$c = xA_{2930} + yA_{2960} + z\left(A_{3030} - \frac{A_{2930}}{F} \right)$$

$$171 = 113.49(0.092) + 49.47(0.055) + z\left(0.169 - \frac{0.092}{139.33} \right)$$

$$\mathbf{z = 937.85}$$

x = 113.49 **y = 49.47** **z = 937.85** **F = 139.33**

These values were then used in the equation for the following hydrocarbon solutions.

Hydrocarbon	Actual Conc (ppm)	A3030 cm-1	A2960 cm-1	A2930 cm-1	Calculated HMSO Conc	% Error	Mean error
Hexane	105		0.527	0.485	81.10	-22.76	-20.11
	84		0.424	0.393	65.52	-22.00	
	63		0.317	0.294	49.00	-22.22	
	42		0.213	0.202	33.41	-20.45	
	21		0.106	0.097	16.28	-22.46	
	4.2		0.023	0.023	3.75	-10.79	
Heptane	108		0.542	0.631	98.39	-8.89	-10.35
	86.4		0.440	0.518	80.54	-6.78	
	64.8		0.332	0.388	60.47	-6.68	
	43.2		0.218	0.254	39.59	-8.36	
	21.6		0.106	0.126	19.53	-9.60	
	4.32		0.018	0.022	3.38	-21.79	
Octane	131		0.685	0.932	139.69	6.64	7.14
	98		0.494	0.678	101.41	3.48	
	65.5		0.361	0.498	74.34	13.50	
	33		0.174	0.238	35.60	7.88	
	13		0.062	0.093	13.55	4.19	
Nonane	135		0.635	0.976	142.21	5.34	8.64
	101		0.463	0.726	105.32	4.28	
	67.5		0.331	0.519	75.21	11.42	
	34		0.162	0.254	36.87	8.44	
	13.5		0.065	0.107	15.35	13.71	
Decane	135		0.607	1.039	147.90	9.56	14.67
	101		0.456	0.788	111.99	10.88	
	67.5		0.312	0.545	77.26	14.46	
	34		0.161	0.283	40.11	17.96	
	13.5		0.061	0.117	16.26	20.47	
Dodecane	129		0.523	1.102	150.90	16.98	19.82
	96.75		0.407	0.841	115.54	19.42	
	64.5		0.269	0.574	78.48	21.68	
	32.25		0.133	0.288	39.18	21.50	
	12.9		0.052	0.113	15.42	19.54	

n-tetradecane	135		0.426	1.029	137.88	2.14	9.25
	81		0.268	0.675	89.84	10.91	
	54		0.176	0.444	59.12	9.47	
	40.5		0.134	0.332	44.27	9.32	
	27		0.094	0.231	30.84	14.21	
	13.5		0.044	0.111	14.72	9.02	
	5.4		0.022	0.043	5.92	9.71	
n-hexadecane	167	0.016	0.541	1.257	169.45	1.47	12.88
	125.25	0.009	0.396	1.107	145.22	15.95	
	83.5	0.006	0.273	0.751	98.75	18.26	
	41.75	0.000	0.129	0.364	47.62	14.06	
	16.7	0.000	0.053	0.146	19.15	14.66	
Eicosane	129		0.295	1.001	128.20	-0.62	-0.99
	103		0.238	0.797	102.29	-0.69	
	77.5		0.179	0.595	76.41	-1.40	
	51.6		0.118	0.396	50.83	-1.49	
	25.8		0.060	0.200	25.61	-0.74	
Iso-octane	274.5		1.442	0.561	135.00	-50.82	-46.56
	183		1.006	0.377	92.51	-49.45	
	91.5		0.546	0.188	48.32	-47.19	
	36.6		0.233	0.081	20.67	-43.53	
	9.15		0.059	0.021	5.32	-41.82	
Pristane	127		0.752	0.797	127.66	0.52	8.09
	95		0.586	0.622	99.63	4.88	
	63.5		0.387	0.411	65.72	3.50	
	31.75		0.214	0.228	36.44	14.77	
	12.7		0.089	0.092	14.83	16.78	
Reference Oil	64		0.193	0.337	47.75	-25.39	-19.12
	51.2		0.158	0.275	39.02	-23.78	
	38.4		0.120	0.207	29.39	-23.46	
	25.6		0.082	0.143	20.26	-20.84	
	12.8		0.045	0.089	12.33	-3.71	
	6.4		0.020	0.038	5.28	-17.56	

Hydrocarbon	Actual Conc (ppm)	A3030 cm-1	A 2960 cm-1	A2930 cm-1	Calculated HMSO Conc	% Error	Mean error
Benzene	166	0.339			317.84	91.47	80.03
	124.5	0.241			226.02	81.54	
	83	0.161			150.90	81.81	
	41.5	0.076			71.56	72.43	
	16.6	0.031			28.70	72.88	
Ethyl benzene	174	0.174	0.057	0.091	175.81	1.04	-1.98
	130.5	0.131	0.047	0.074	133.35	2.19	
	87	0.088	0.031	0.049	89.27	2.61	
	43.5	0.041	0.017	0.026	41.88	-3.73	
	17.4	0.015	0.006	0.011	15.31	-12.02	
Toluene	149	0.180	0.311	0.175	203.31	36.45	45.45
	112	0.136	0.232	0.136	153.01	36.62	
	75	0.094	0.164	0.093	105.84	41.12	
	37	0.048	0.084	0.052	54.57	47.49	
	15	0.021	0.041	0.034	24.84	65.58	

Table3.5 Quantitative results for aromatic and non-aromatic hydrocarbons

From these results in can be seen there is a general trend.

There is a reasonable correlation for the straight chained hydrocarbon which gets progressively worse for the branched and aromatic hydrocarbons, except for those used in the calculation of the coefficients.

Hydrocarbon	ASTM Method Mean % Error	DIN Method Mean % Error	HMSO Method Mean % Error
Hexane	-70.3	6.40	-20.11
Heptane	-61.7	17.65	-10.35
Octane	-48.5	38.98	7.14
Nonane	-42.9	39.69	8.64
Decane	-38.5	46.28	14.67
Dodecane	-40.3	51.25	19.82
n-tetradecane	-48.9	36.58	9.25
n-hexadecane	-	40.56	12.88
Eicosane	+34.0	22.01	-0.99
Iso-octane	-	-23.53	-46.56
Pristane	-	43.29	8.09
Reference oil	-	0.64	-19.12
Benzene	-	37.2	80.03
Toluene	-	-21.4	45.45
Ethyl benzene	-	26.2	-1.98

Table 3.6 Summary of hydrocarbon quantification results for the three standard methods, using conventional solvents

3.4.3.3 Determination of on-line HMSO coefficients using Supercritical Fluid Extraction (SFE)

The basis of this reported work is for the extraction and analysis of hydrocarbons from aqueous media using supercritical carbon dioxide. After analysis of the results obtained by the three standard methods using conventional solvents, Table 3.6, it was noted that the best results were obtained using the HMSO method. This method was now used to analyse supercritical fluid extracted hydrocarbons by first calculating the coefficients of SF-CO_2 extracted standard hydrocarbons, hexadecane, pristine and ethyl benzene, extracted from 500 cm^3 water samples at 2500 psi.

Reference hydrocarbon absorbances

n-Hexadecane (50µl in 500 cm^3 water)

File name	Abs. @ 3030 cm^{-1}	Abs. @ 2960 cm^{-1}	Abs. @2930 cm^{-1}
EDR452	0.012	0.230	0.661
EDR454	0.009	0.232	0.671
EDR456	0.009	0.255	0.734
EDR459	0.002	0.266	0.655
EDR461	0.010	0.265	0.775
Mean	0.008	0.245	0.699
Standard deviation	0.004	0.018	0.053

Pristane (50µl in 500 cm^3 water)

File name	Abs. @ 3030 cm^{-1}	Abs. @ 2960 cm^{-1}	Abs. @ 2930 cm^{-1}
EDR463	0.004	0.385	0.370
EDR480	0.001	0.387	0.356
EDR483	0.004	0.384	0.342
EDR485	0.003	0.411	0.368
EDR486	0.002	0.416	0.370
Mean	0.001	0.397	0.361
Standard deviation	0.003	0.016	0.012

Ethyl benzene (50µl in 500 cm^3 water)

File name	Abs. @ 3030 cm^{-1}	Abs. @ 2960 cm^{-1}	Abs. @ 2930 cm^{-1}
EDR492	0.058	0.107	0.034
EDR493	0.062	0.116	0.039
EDR494	0.057	0.111	0.037
EDR495	0.063	0.120	0.039
Mean	0.060	0.114	0.037
Standard deviation	0.003	0.006	0.002

3.4.3.4 Calculation of On-line SFE coefficients

a. Concentrations in ppm

Hydrocarbon	Density (g/cm^3)
n-hexadecane	0.773
Pristane	0.785
Ethyl benzene	0.876
Diesel	0.851
Gasoline	0.668
Kerosene	0.784
White spirit	0.782
Engine Oil	0.88 (See below)

Engine oil

Because of its viscosity, and in order to use a syringe to spike the water samples, engine oil was prepared as a solution in benzene.
(5g of engine oil dissolved in 10 cm^3 of benzene)

As n-hexadecane and pristane have no aromatic contribution, they again can be used to solve the x and y coefficients from the simultaneous equations below.

n-Hexadecane

Calculation of concentration in ppm from µl

50 µl in 500 cm^3 water = 100 µl in 1000 cm^3 water = 0.1 cm^3/l = 0.1 * 0.773 = 0.0773

g/l =77.3 mg/l

=77.3 ppm

$$77.3 = x(0.6992) + y(0.2496)$$

Pristane

Calculation of concentration in ppm from µl

50 µl in 500 cm^3 water = 100 µl in 1000 cm^3 water = 0.1 cm^3/l = 0.1 * 0.785 = 0.0785

g/l = 78.5 mg/l

= 78.5 ppm

$$78.5 = x(0.3612) + y(0.3966)$$

x = 59.117

y = 144.092

Substituting these valves into the equation for ethyl benzene gives the coefficient z.

Ethyl benzene

Calculation of concentration in ppm from µl

50 µl in 500 cm^3 water = 100 µl in 1000 cm^3 water = 0.1 cm^3/l = 0.1 * 0.876 = 0.0876

g/l = 87.6 mg/l

= 87.6 ppm

$$87.6 = x(0.03725) + y(0.1135) + z\left(0.060 - \frac{0.0373}{83.238}\right)$$

z = 1159.384

$$F = \frac{0.6992}{0.0084} = \mathbf{83.286}$$

Online HMSO coefficients for concentrations in ppm	
x	59.117
y	144.092
z	1159.384
F	83.286

3.4.3.5 Application of on-line HMSO coefficients to calculate the concentration of "standard" hydrocarbons

Five different commercially available hydrocarbons were now used as "standards" to assess the applicability of the coefficients to "real" , multi-component, samples. Hydrocarbons used were:-

a. **White spirit**
b. **Kerosene**
c. **Gasoline**
d. **Diesel**
e. **Engine oil**

Measurement of absorption peaks of "standard" oils.

(All statistics based on n =5, except diesel where n=4)

A. **White spirit**

Density = 0.782g/cm^3

10 µl = 0.01*0.782 g/500 cm^3 = 0.00782 g/500 cm^3 = 0.0156 g/100 cm^3

=15.6mg/1000 cm^3

= 15.6ppm

Conc. µl	Conc. ppm	Abs. @ 3030cm^{-1}	Abs. @ 2960 cm^{-1}	Abs. @ 2930 cm^{-1}
10 mean	15.6	0.0014	0.05252	0.0785
S.D.		0.0009	0.0055	0.0099
25 mean	39.0	0.0056	0.1524	0.1980
S.D.		0.0026	0.0073	0.0093
50 mean	78.0	0.0076	0.2890	0.3602
S.D.		0.0070	0.0194	0.0223
100 mean	156.0	0.0138	0.5798	0.6870
S.D.		0.0075	0.0220	0.0314

B. **Kerosene**

Density = 0.784 g/cm^3

10 µl = 0.01*0.784 g/500 cm^3 = 0.00784 g/500 cm^3 = 0.0158 g/100 cm^3 = 15.8 mg/1000 cm^3

= 15.8 ppm

Conc. µl	Conc. ppm	Abs. @ 3030 cm^{-1}	Abs. @ 2960 cm^{-1}	Abs. @ 2930 cm^{-1}
10 mean	15.8	0.0026	0.0688	0.1096
S.D.		0.0013	0.0056	0.0089
25 mean	39.5	0.0040	0.1702	0.2612
S.D.		0.0028	0.0192	0.0272
50 mean	79.0	0.0070	0.3490	0.5240
S.D.		0.0055	0.0279	0.0400
100 mean	158.0	0.0118	0.6950	1.0452
S.D.		0.0086	0.0260	0.0579

C. **Gasoline**

Density = 0.668g/cm^3

10 µl = 0.01*0.668 g/500 cm3 = 0.00668 g/500 cm^3 = 0.0134 g/100 cm^3 = 13.4 mg/1000 cm^3 = 13.4 ppm

Conc. µl	Conc. ppm	Abs. @ 3030 cm^{-1}	Abs. @ 2960 cm^{-1}	Abs. @ 2930 cm^{-1}
10 mean	13.4	0.0022	0.0520	0.0384
S.D.		0.0018	0.0093	0.0080
25 mean	33.5	0.0084	0.1504	0.0974
S.D.		0.0038	0.0137	0.0067
50 mean	67.0	0.0056	0.2588	0.1440
S.D.		0.0026	0.0116	0.0144
100 mean	134.0	0.0182	0.5950	0.3440
S.D.		0.0102	0.0357	0.0239

D. **Diesel**

Density = 0.851 g/cm^3

10 µl = 0.01*0.851 g/500 cm^3 = 0.00851 g/500 cm^3 = 0.0170 g/100 cm^3 = 1.0 mg/1000 cm^3 = 17.0 ppm

Conc. µl	Conc. ppm	Abs. @ 3030 cm^{-1}	Abs. @ 2960 cm^{-1}	Abs. @ 2930 cm^{-1}
10 mean	17.0		0.059	0.0939
S.D.			0.0035	0.0057
25 mean	42.5		0.1486	0.2350
S.D.			0.0072	0.0102
50 mean	85.0		0.2970	0.4694
S.D.			0.0240	0.0343
80 mean	136.0		0.475	0.7511
S.D.			0.0122	0.0174

E. Engine oil (as the engine oil was used as a solution in benzene, 3030cm^{-1} contribution unknown)

Density = 0.8711 g/ cm^3 (*Note* used as 5 g/10 cm^3 benzene solution)

5 g/10 cm^3 = 0.5 g/ cm^3 = 0.05 g/100 µl = 0.005 g/10 µl =5 mg/10 µl

10 µl = 0.005 g/500 cm^3 = 0.010 g/1000 cm^3 = 10.0 mg/1000 cm^3 = 10.0 ppm

Conc. µl	Conc. ppm	Abs. @ 3030 cm^{-1}	Abs. @ 2960 cm^{-1}	Abs. @ 2930 cm^{-1}
10 mean	10.0		0.0316	0.0621
S.D.			0.0042	0.0072
20 mean	20.0		0.0604	0.1148
S.D.			0.0035	0.0056
45 mean	45.0		0.1016	0.1934
S.D.			0.0064	0.0132
70 mean	70.0		0.1818	0.3458
S.D.			0.0177	0.0315

Calculation of hydrocarbon concentrations

A. White spirit (Using x = 59.117 y = 144.092 z = 1159.384)

Actual Conc. (ppm)	Calculated	% error	Mean Error
15.6	12.21	-21.74	-18.79
39.0	33.66	-13.68	
78.0	62.94	-19.31	
156.0	124.16	-20.41	

B. Kerosene (Using x = 59.117 y = 144.092 z = 1159.384)

Actual Conc. (ppm)	Calculated	% error	Mean Error
15.8	16.39	3.75	2.57
39.5	39.97	1.18	
79.0	81.27	2.87	
158.0	161.93	2.49	

C. Gasoline (Using x = 59.117 y = 144.092 z = 1159.384)

Actual Conc. (ppm)	Calculated	% error	Mean Error
13.4	9.76	-27.14	-24.44
33.5	27.43	-18.12	
67.0	45.80	-31.64	
134.0	106.07	-20.84	

D. Diesel (Using x = 59.117 y = 144.092 z = 1159.384)

Actual Conc. (ppm)	Calculated	% error	Mean Error
17.0	11.79	-30.62	-34.83
42.5	26.47	-37.72	
85.0	52.53	-38.20	
136.0	91.41	-32.79	

E. Engine oil (Using x = 59.117 y = 144.092 z = 1159.384)

Actual Conc. (ppm engine oil)	Calculated	% error	Mean Error
10	4.81	-51.88	-58.38
20	9.08	-54.62	
45	15.28	-66.05	
70	27.33	-60.96	

Summary of % Error

Hydrocarbon	**% Error**
White Spirit	-18.78
Kerosene	2.57
Gasoline	-22.44
Diesel	-34.83
Engine Oil	-58.38

3.4.3.6 Using graphical methods to optimise HMSO coefficients

In an attempt to improve the errors associated with the HMSO method, a data matrix was assembled where the total error for all 5 hydrocarbons was determined for a range of x and y values using the data analysis software package Origin (Microcal Software, Inc. Northampton, MA, USA).

Analysis resulted in a new set of total hydrocarbon coefficients .

(see appendix F for detailed calculations.)

these coefficients were

x = 68 y =179

(As there was no discernible aromatic contribution for the hydrocarbons examined, the z coefficient was omitted)

These calculated values when represented graphically, gave a feel for the overall trend of the errors, against the x and y values, fig 3.16.

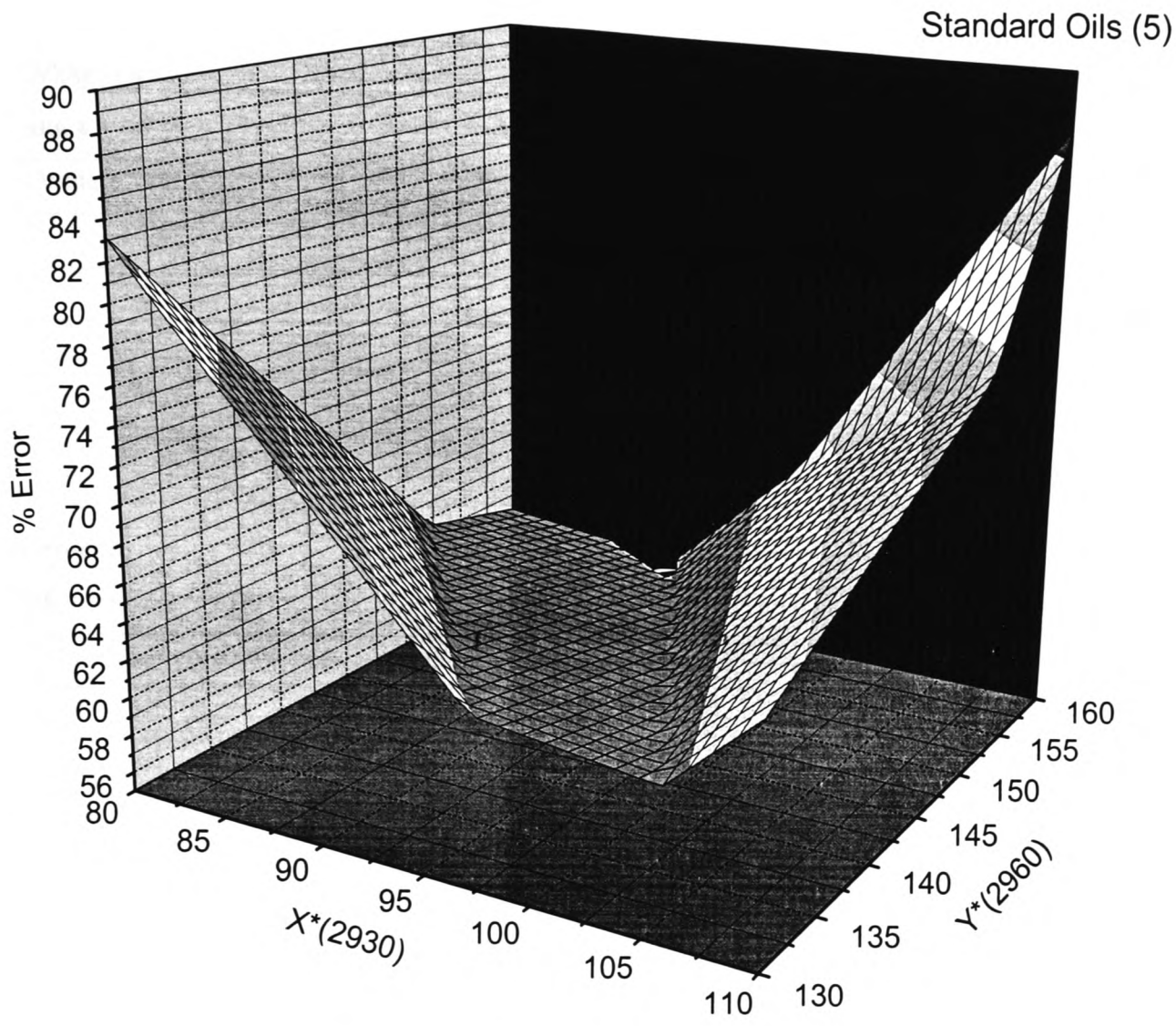

Fig 3.16 Graphical representations of errors associated with calculated coefficients

Applying these coefficients, to each of the 5 hydrocarbons gave the minimum total modulus error for all 5 hydrocarbons, calculated to be 47.66%.

A. White spirit

(Using x = 68 y = 179)

Actual Conc. (ppm)	Calculated	% error	Mean Error
15.6	14.74	-5.52	-1.71
39.0	40.74	4.47	
78.0	76.22	-2.28	
156.0	150.50	-3.53	

B. Kerosene

(Using x = 68 y = 179)

Actual Conc. (ppm)	Calculated	% error	Mean Error
15.8	19.77	25.11	23.78
39.5	48.23	22.09	
79.0	98.10	24.18	
158.0	195.48	23.72	

C. Gasoline

(Using x = 68 y = 179)

Actual Conc. (ppm)	Calculated	% error	Mean Error
13.4	11.92	-11.05	-7.56
33.5	33.54	0.13	
67.0	56.12	-16.24	
134.0	129.90	-3.06	

D. Diesel

(Using x = 68 y = 179)

Actual Conc. (ppm)	Calculated	% error	Mean Error
17.0	16.95	-0.32	0.01
42.5	42.58	0.19	
85.0	85.08	0.10	
136.0	136.10	0.07	

E. Engine oil

(Using x = 68 y = 179)

Actual Conc. (ppm engine oil)	Calculated	% error	Mean Error
10	9.88	-1.21	-14.60
20	18.62	-6.91	
45	31.34	-30.36	
70	56.06	-19.92	

Because of the opposing quantification effects of kerosene and diesel it was decided to exclude one of these to improve the overall accuracy of the method.

a. Using 4 hydrocarbons excluding KEROSENE
b. Using 4 hydrocarbons excluding DIESEL

A. Excluding KEROSENE

The HMSO coefficients were recalculated for only 4 of the hydrocarbons, this led to

x = 91 y = 160

This gave a total (for the sum of all 4) error of 35.57%, (from the matrix) and individual errors of :

A. White spirit

(Using x = 91 y = 160)

Actual Conc. (ppm)	Calculated	% error	Mean Error
15.6	15.55	-0.34	2.31
39.0	42.40	8.72	
78.0	79.02	1.31	
156.0	155.29	-0.46	

B. Diesel

(Using x = 91 y = 153)

Actual Conc. (ppm)	Calculated	% error	Mean Error
17.0	15.2	-10.58	-16.16
42.5	40.0	-20.03	
85.0	67.6	-20.43	
136	117.5	-13.60	

C. Gasoline

(Using x = 91 y = 160)

Actual Conc. (ppm)	Calculated	% error	Mean Error
13.4	11.81	-11.83	-9.44
33.5	32.93	-1.71	
67.0	54.51	-18.64	
134.0	126.50	-5.59	

D. Engine oil

(Using x = 91 y = 160)

Actual Conc. (ppm engine oil)	Calculated	% error	Mean Error
10	10.71	7.07	-7.66
20	20.11	0.55	
45	33.86	-24.77	
70	60.56	-13.49	

B. Exclusion of DIESEL

The HMSO coefficients were recalculated for 4 of the hydrocarbons, excluding diesel, this led to

x = 80 y = 153

This gave a total (for the sum of all 4) error of 56.65%, (from the matrix) and individual errors (from the spreadsheet) of

A. White spirit

(Using x = 80 y = 153)

Actual Conc. (ppm)	Calculated	% error	Mean Error
15.6	14.32	-8.23	-5.53
39.0	39.16	0.40	
78.0	73.03	-6.37	
156.0	143.67	-7.90	

B. Kerosene

(Using x = 80 y = 153)

Actual Conc. (ppm)	Calculated	% error	Mean Error
15.8	19.29	22.12	20.45
39.5	46.94	18.83	
79.0	95.32	20.65	
158.0	189.95	20.22	

C. Gasoline

(Using x = 80 y = 153)

Actual Conc. (ppm)	Calculated	% error	Mean Error
13.4	11.03	-17.70	-15.25
33.5	30.80	-8.05	
67.0	51.12	-23.71	
134.0	118.56	-11.53	

D. Engine oil

(Using x = 80 y = 153)

Actual Conc. (ppm engine oil)	Calculated	% error	Mean Error
10	9.80	-1.97	-15.42
20	18.43	-7.87	
45	31.02	-31.07	
70	55.48	-20.74	

(See Appendix G for detailed calculations)

	Maximum Total Modulus % Error	x =	y =
All 5 hydrocarbons	47.66	68	179
Excluded Hydrocarbon			
Kerosene	35.57	91	160
Diesel	56.65	80	153
White spirit	50.95	91	160
Engine Oil	53.50	80	153
Gasoline	50.30	98	130

Table3.6 Total modulus errors for excluded hydrocarbons

It can be seen from the results in table 3.6 that the least error was attributed to the calculations which excluded the kerosene component.

3.4.4 Quantification of On-line samples by Off-line calibration standards

It would be more efficient to be able to create off-line calibration standards, and hence coefficients, and use these to quantify on-line extracted hydrocarbon samples. To enable this procedure, standard hydrocarbon standards of n-hexadecane, pristane and ethyl benzene were prepared in carbon tetrachloride, and their absorbances measured in a 2 cm quartz infrared cell.

n-Hexadecane =0.0021 g in 25 cm^3 CCl_4 =0.084 g in 1000 cm^3 CCl_4 = 84 ppm
Pristane =0.0020 g in 25 cm^3 CCl_4 =0.080 g in 1000 cm^3 CCl_4 = 80 ppm
Ethyl benzene =0.0022 g in 25 cm^3 CCl_4 =0.088 g in 1000 cm^3 CCl_4 = 88 ppm

File name	Concentration	A_{3030}	A_{2960}	A_{2930}
n-hexadecane EDR550	0.0021 g in 25 cm^3 CCl_4 (84 ppm)	0.006	0.245	0.721
Pristane EDR555	0.0020 g in 25 cm^3 CCl_4 (80 ppm)	0.003	0.391	0.412
Ethyl benzene EDR558	0.0022 g in 25 cm^3 CCl_4 (88 ppm)	0.102	0.190	0.111

The coefficients for these off-line calibration standards were then determined,

3.4.4.1 Calculation of coefficients

n-Hexadecane

$$84 = x(0.721) + y(0.245)$$

Pristane

$$80 = x(0.412) + y(0.391)$$

x = 73.183

y = 127.49

F = 120.17

Ethyl benzene

$$88 = x(0.111) + y(0.190) + z(0.102 - 0.111/120.17)$$

z = 550.481

3.4.4.2 Standard hydrocarbons extracted via HMSO (Blue Book) method

To determine the errors associated with carbon tetrachloride extractions, aqueous solutions of the standard hydrocarbons were prepared, and extracted using aliquots of carbon tetrachloride, then spectroscopically examined under the same conditions as the standards.

Application of the pre-determined off-line coefficients would then allow quantification, and a determination of the associated solvent extraction errors.

This would then give an idea of the efficiency of carbon tetrachloride solvent extractions compared with carbon dioxide in its supercritical phase.

3.4.4.3 Calculation of actual concentration of hydrocarbon.

100 ul volume contains the following mass of hydrocarbon

Gasoline = 0.0668 g

Diesel = 0.08505 g

Kerosene = 0.07840 g

Each sample contains 5 ul of hydrocarbon in 500 cm^3 water. Assuming 100% extraction efficiency, this mass is extracted into the 5 cm^3 CCl_4 solvent extract.

Mass in 5 ul sample

Gasoline = 0.0668/20 = 0.00334 g in 25 cm^3 CCl_4 = 0.1336 g in 1000 cm^3 CCl_4 = 133.6 ppm

Diesel = 0.08505/20 = 0.00425 g in 25 cm^3 CCl_4 = 0.1701 g in 1000 cm^3 CCl_4 = 170.1 ppm

Kerosene = 0.07840/20 = 0.00392 g in 25 cm^3 CCl_4 = 0.1568 g in 1000 cm^3 CCl_4 = 156.8 ppm

Standard	File Name	A_{2930}	A_{2960}	A_{3030}	HMSO Conc.	Actual Conc.	%Error	Mean Error
Gasoline	edr572	0.402	0.478	0.042	111.64	133.6	-16.44	-9.59
Gasoline	edr573	0.457	0.566	0.048	129.94	133.6	-2.74	
Diesel	edr574	1.045	0.530	0.026	153.57	170.1	-10.05	-9.66
Diesel	edr575	1.049	0.538	0.025	154.32	170.1	-9.28	
Kerosene	edr576	1.243	0.746	0.014	188.09	156.8	19.95	21.60
Kerosene	edr577	1.269	0.764	0.016	193.26	156.8	23.25	

Table 3.7 5 μl hydrocarbon in 500 cm^3 water, then extracted with 5 cm^3 CCl_4

These errors are a consequence of only the solvent extraction stage. To demonstrate the additional problems associated with the application of the predetermined coefficients, the same procedure was repeated using the standard as the reference material

.

3.4.4.4. Off-line calibration of on-line hydrocarbon solutions

A set of calibration standards were prepared to allow the calibration of an on-line sample (SFE) by a set of off-line (CCl_4) calibration standards.

Solutions of the reference hydrocarbons were prepared as CCl_4 solutions and used to quantify SFE samples, all extraction carried out at 2500 psi. Quantification of the SF-extracts were

then carried out at both the absorbances, 2930 and 2960 cm^{-1} to determine if there were any differences between the spectrum in carbon tetrachloride solution and SF-CO_2.

A. Gasoline

i. 50 µl in 500 cm^3 water

50 µl of gasoline in 500cm^3 water = 0.1 cm^3 in 1000 cm^3

Density = 0.668 g/cm^3

Mass of gasoline present = 0.0668 g/ 1000 cm^3 = 66.8 ppm

SF-CO_2 hydrocarbon extract filename	Abs. @ 2966cm^{-1}	Abs. @ 2937cm^{-1}
EDR210	0.251	0.144
EDR211	0.276	0.160
EDR212	0.249	0.146
EDR219	0.286	0.166
EDR229	0.262	0.149
	Mean Abs. = 0.265	Mean Abs. = 0.153

ii. CCl_4 solution

EDR562 contains 0.0032 g in 25 cm^3 CCl_4

= 0.128 g / 1000 cm^3 = 128 mg / 1000 cm^3 = 128 ppm

Abs. For 128ppm @ 2960 cm^{-1} = 0.507

Therefore theoretical conc. @ 2960 cm^{-1} for 0.265 = $\frac{0.265}{0.507} * 128\ ppm = 66.90\ ppm$

% error = +0.15 %

Abs. For 128 ppm @ 2929 cm^{-1} = 0.307

Therefore theoretical conc. @ 2960cm^{-1} for 0.153 = $\frac{0.153}{0.307} * 128\ ppm = 63.80\ ppm$

% error = -4.5 %

B. Kerosene

i. 50 µl in 500 cm^3 water

50 µl of kerosene in 500 cm^3 water = 0.1 cm^3 in 1000 cm^3

Density = 0.874 g/cm^3

Mass of kerosene present = 0.0874 g/ 1000 cm^3 = 87.4 ppm

SF-CO_2 hydrocarbon extract filename	Abs. @ 2964 cm^{-1}	Abs. @ 2933 cm^{-1}
EDR72	0.311	0.477
EDR74	0.360	0.540
EDR76	0.390	0.584
EDR80	0.333	0.498
EDR82	0.341	0.508
	Mean Abs. = 0.347	Mean Abs. = 0.521

ii. CCl_4 solution

EDR551 contains 0.0023 g in 25 cm^3 CCl_4

= 0.092 g / 1000 cm^3 = 92 mg / 1000 cm^3 = 92 ppm

Abs. For 92 ppm @ 2957 cm^{-1} = 0.357

Therefore theoretical conc. for 0.347 = $\frac{0.347}{0.357} * 92\ ppm = 89.42\ ppm$

% error = +2.31 %

Abs. For 92 ppm @ 2927 cm^{-1} = 0.559

Therefore theoretical conc. for 0.521 = $\frac{0.521}{0.559} * 92\ ppm = 85.75\ ppm$

% error = -1.89 %

C. White spirit

i. 50 µl in 500 cm^3 water

50 µl of white spirit in 500 cm^3 water = 0.1 cm^3 in 1000 cm^3

Density = 0.782 g/cm^3

Mass of white spirit present = 0.0782 g/ 1000 cm^3 = 78.2 ppm

SF-CO_2 hydrocarbon extract filename	Abs. @ 2964cm^{-1}	Abs. @ 2933cm^{-1}
WS50X1	0.303	0.380
WS50X4	0.297	0.365
WS50X6	0.259	0.336
WS50X7	0.289	0.347
WS50X8	0.288	0.361
	Mean Abs. = 0.287	Mean Abs. = 0.358

ii. CCl_4 solution

EDR553 contains 0.0021g in 25 cm^3 CCl_4

= 0.084 g / 1000 cm^3 = 84 mg / 1000 cm^3 = 84 ppm

Abs. For 128 ppm @ 2960 cm^{-1} = 0.321

Therefore theoretical conc. for 0.287 = $\frac{0.287}{0.321} * 84\ ppm = 75.10\ ppm$

% error = -3.96 %

Abs. For 128 ppm @ 2927 cm^{-1} = 0.401

Therefore theoretical conc. for 0.358 = $\frac{0.358}{0.401} * 84\ ppm = 75.00\ ppm$

% error = -4.1 %

D. Diesel

i. 20μl in 500cm^3 water

SF-CO_2 hydrocarbon extract filename	Abs. @ 2962 cm^{-1}	Abs. @ 2933 cm^{-1}
EDR367	0.124	0.184
EDR369	0.118	0.189
EDR371	0.116	0.193
EDR373	0.120	0.192
EDR376	0.122	0.187
	Mean Abs. = 0.120	Mean Abs. = 0.189

20 μl of diesel in 500 cm^3 water = 0.04 cm^3 in 1000 cm^3

Density = 0.851 g/cm^3

Mass of diesel present = 0.0340 g/ 1000 cm^3 = 34.0 ppm

ii. CCl_4 solution

EDR552 contains 0.0023 g in 25 cm^3 CCl_4

= 0.092 g / 1000 cm^3 = 92 mg / 1000 cm^3 = 92 ppm

Abs. For 92 ppm @ 2956 cm^{-1} = 0.302

Therefore theoretical conc. for 0.120 = $\frac{0.120}{0.302} * 92\ ppm = 36.6\ ppm$

% error = +7.53 %

Abs. For 92 ppm @ 2927 cm^{-1} = 0.502

Therefore theoretical conc. for 0.189 = $\frac{0.189}{0.502} * 92\ ppm = 34.6\ ppm$

% error = +1.76 %

E. Engine oil

i. 70 µl of Engine oil solution in 500 cm^3 water

Engine oil solution contained 5 g of engine oil in 10 cm^3 benzene

Density of engine oil =0.8711 g/cm^3

Therefore 5 g of engine oil has a volume of $\frac{5.0}{0.8711} = 5.74\ cm^3$ or 57.4% v/v

SFE solutions contain 70 µl of engine oil solution, and therefore contain 40.18 µl of engine oil in 500 cm^3 of water, or 80.35 µl in 1000 cm^3 of water.

Therefore there is $\frac{80.36}{1000} * 0.8711 = 0.0700\ g$ **= 70 ppm**

SF-CO_2 hydrocarbon extract filename	Abs. @ 2962 cm^{-1}	Abs. @ 2933 cm^{-1}
EDR150	0.182	0.331
EDR156	0.184	0.350
EDR158	0.209	0.390
EDR162	0.206	0.373
EDR165	0.174	0.323
	Mean Abs. = 0.191	Mean Abs. = 0.353

ii. CCl_4 solution

EDR554 contains 0.0021 g in 25 cm^3 CCl_4

= 0.084 g / 1000 cm^3 = 84 mg / 1000 cm^3 = 84 ppm

Abs. For 128 ppm @ 2956 cm^{-1} = 0.235

Therefore theoretical conc. for 0.235 = $\frac{0.191}{0.235} * 84 ppm = 68.3\ ppm$

% error = -2.4 %

Abs. For 128 ppm @ 2930 cm^{-1} = 0.441

Therefore theoretical conc. for 0.353 = $\frac{0.353}{0.441} * 84 ppm = 67.2\ ppm$

% error = -4.0 %

Summary of off-line calibration of on-line hydrocarbon samples

Hydrocarbon	% Error using Abs. @ 2960 cm^{-1}	% Error using Abs. @ 2930 cm^{-1}
Gasoline	+0.15	-4.5
Kerosene	+2.31	-1.89
White spirit	-3.96	-4.1
Diesel	+7.53	+1.76
Engine oil	-2.4	-4.0

Table 3.8 Errors associated with on-line aqueous SF-CO_2 extracted hydrocarbons calibrated against off-line CCl_4 hydrocarbon standards

The data in Table 3.8 shows the errors associated with the off-line calibration of on-line SF-CO_2 extracted samples. This data indicates the ability to quantify the SF-CO_2 extracts, without the need for on-line extraction of the standards. It also shows, when compared to Table 3.7, the detrimental effect of the HMSO coefficients against the use of the analyte as the standard, even with the additional SF-CO_2 extraction phase, Table 3.9. Unfortunately, as already stated in section 3.1 it is generally very difficult to obtain the extractable hydrocarbon(s) to use as standards, and so the use of the coefficients is a useful compromise.

Hydrocarbon	% Error using CCl_4 extraction and HMSO coefficients (Table 3.7)	% Error using SF-CO_2 extraction and CCl_4 hydrocarbon solutions as standards (Table 3.8)
Gasoline	-9.59	-2.18
Kerosene	+21.60	+0.21
Diesel	-9.66	+4.65

Table 3.9 Comparison of quantitation results for two methods of hydrocarbon extraction and quantitation .

3.4.5. The use of SF-CO_2 extracted standard hydrocarbons as calibrants against SF-CO_2 extracted hydrocarbons.

As we have already shown in the section 2.4.4, the use of coefficients is a necessary compromise when the extractable hydrocarbons are not available as standards, it is also necessary when there are multiple extractable hydrocarbons, section 3.3.

If the extractable hydrocarbon is available for use as a calibrant it can be used to quantify the extracted hydrocarbon, and this application, and results are reported in this section.
In order to establish linearity of response, and potential for performance of the SF-CO_2 extraction system, the hydrocarbons used in section 2.4.4 were again used.

These were:
Gasoline
White spirit
Kerosene
Diesel and
20W/50 Engine oil

Repeat 500 cm^3 water samples (N=5), were spiked with an appropriate quantity of hydrocarbon, and the sample extracted with SF-CO_2 at a pressure of 2500 psi. The quantitation for white spirit, kerosene, diesel and engine oil was achieved using the CH_2 asymmetric stretching frequency at 2930 cm^{-1}, while the CH_3 asymmetric stretching frequency at 2960 cm^{-1} was used for gasoline as its major constituents are branched alkanes. The spectra of each series of hydrocarbon, is shown in Figs 3.17 to 3.21.

Calibration graphs were now constructed using standard quantitative FTIR software, by plotting actual against calculated levels of oil, using partial least squares linear fit, the graph for white spirit is shown in Fig 3.22.

The results for these SF-CO_2 extracted hydrocarbons are shown in Table 3.10. With the exception of engine oil, all the hydrocarbons showed a linear dynamic range above 130 ppm, with engine oil showing an upper linear dynamic range in the region of 70 – 100 ppm.

The reduction in the upper range limit for engine oil can be attributed to the specific properties associated with the formulation of this oil, as it is required to adhere strongly to metal surfaces to give the required protection.

One of the problems occasionally noticed with the highest level concentration samples, was hydrocarbon carry over. This was attributed to the deposition of the solubilised hydrocarbon on the extraction vessel walls with decompression of the SF-CO_2 during the venting cycle. This problem was eliminated by the introduction of a large volume aluminium plug. This plug was inserted into the vessel, in place of the sample bottle, and allowed a rapid cleaning cycle to be run (approximately 3 min), with any hydrocarbon carry over being removed before the next extraction.

The valving configuration also allowed the pressurisation of the IR cell independently, allowing conformation of cleanliness before continuing with another extraction.

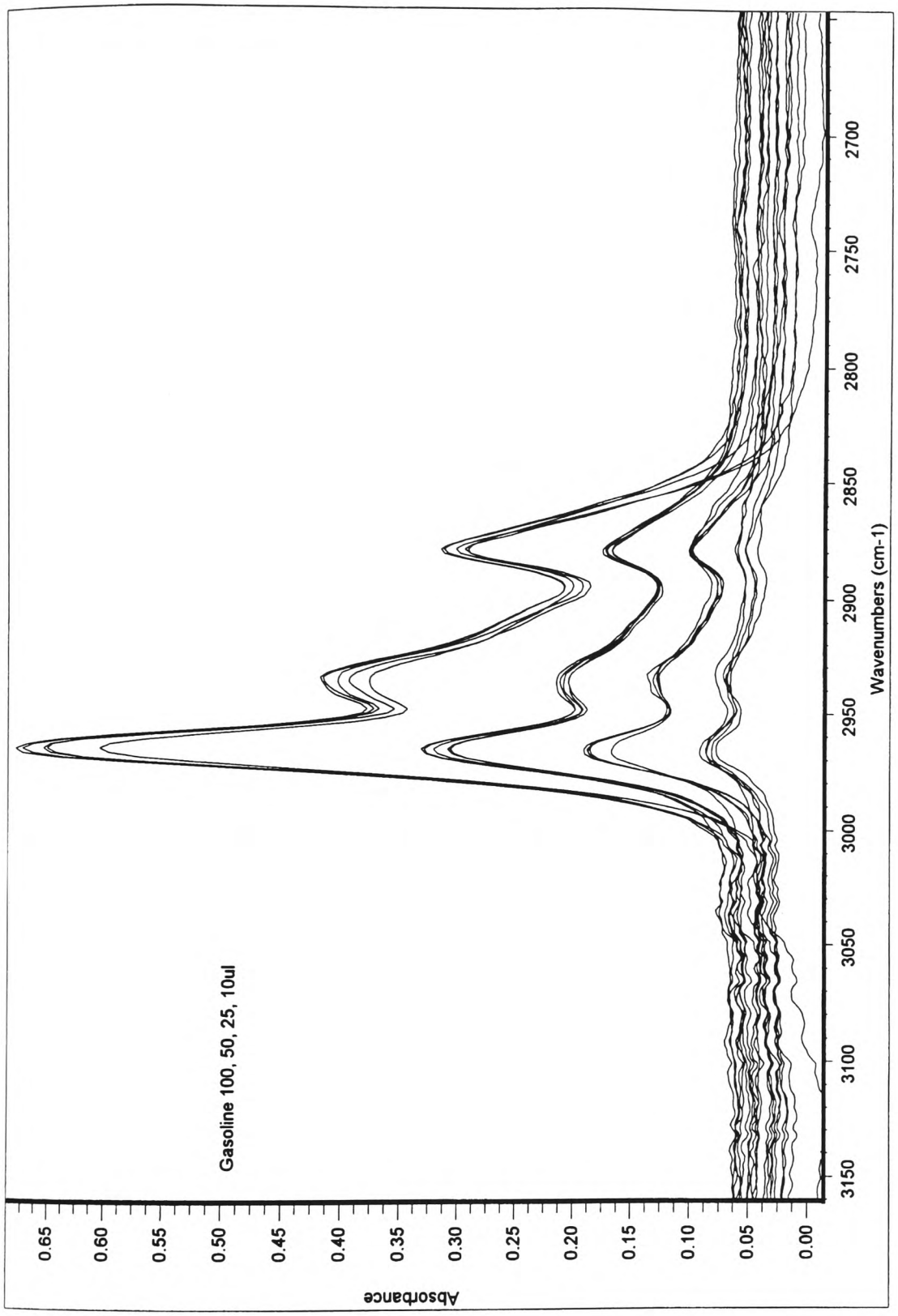

Fig. 3.17 On-line SFE-FTIR spectra obtained for 4 sets (N=5 per set) of 500 cm^3 water samples spiked with different levels of Gasoline

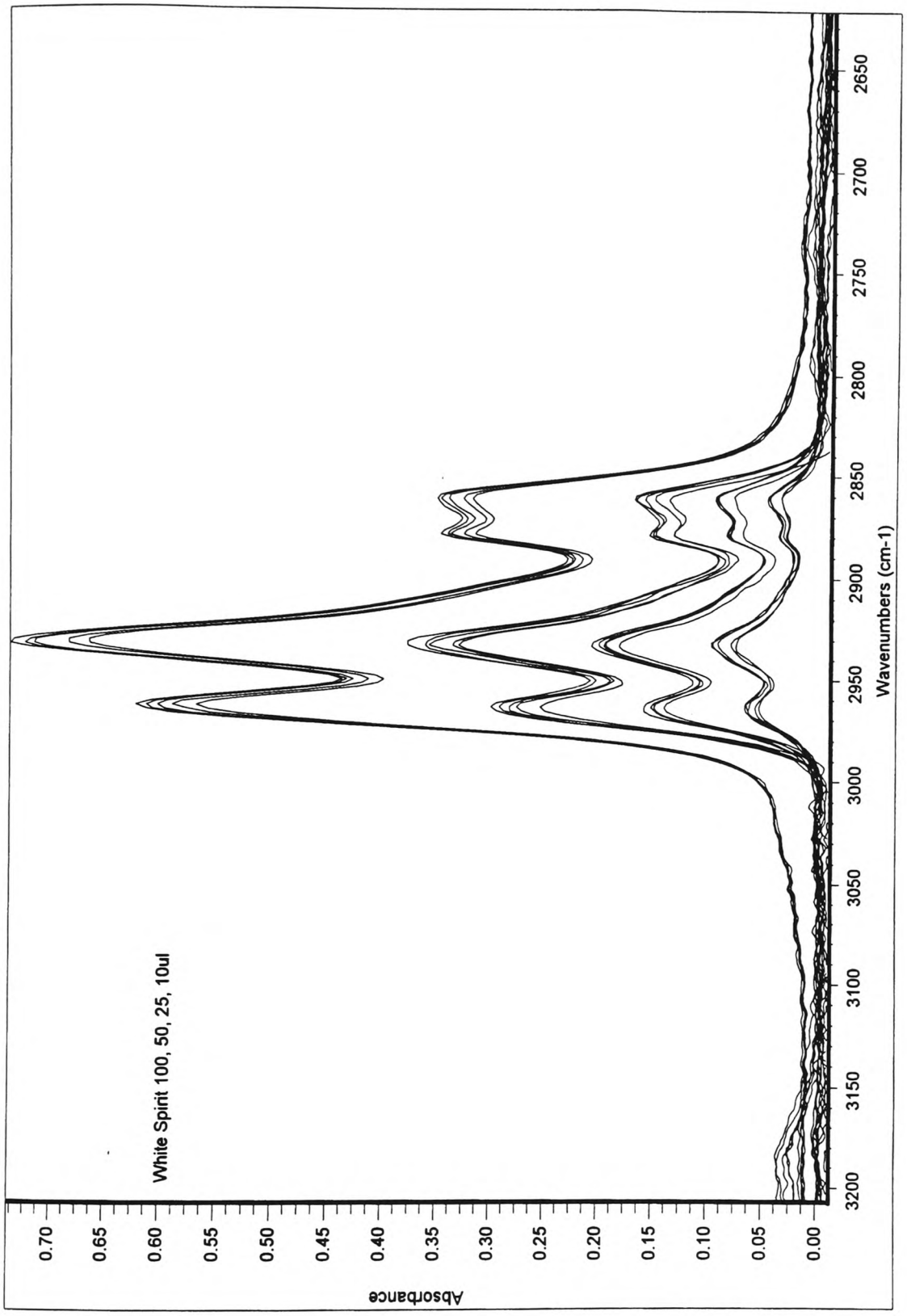

Fig. 3.18 On-line SFE-FTIR spectra obtained for 4 sets (N=5 per set) of 500 cm^3 water samples spiked with different levels of White spirit

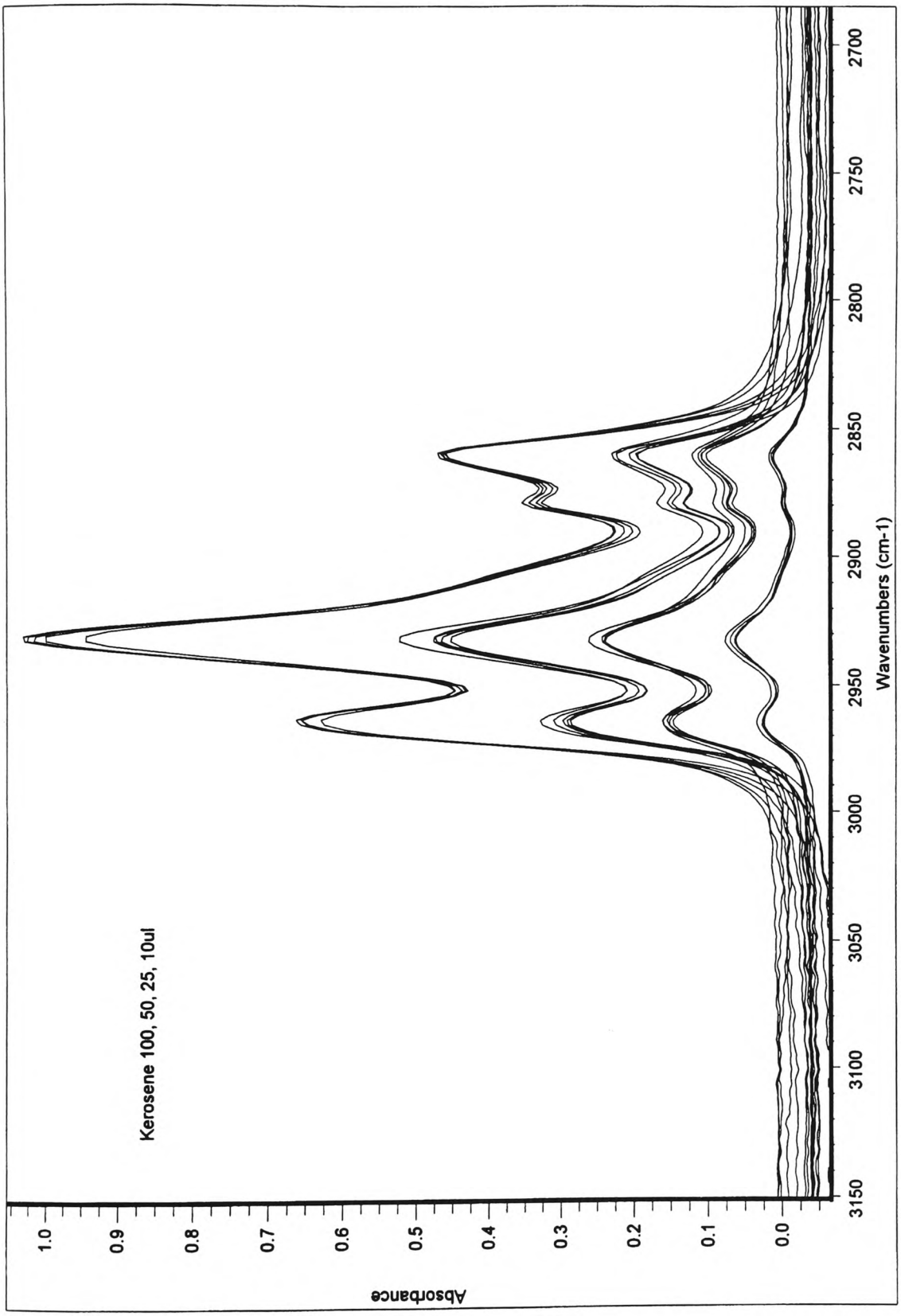

Fig. 3.19 On-line SFE-FTIR spectra obtained for 4 sets (N=5 per set) of 500 cm^3 water samples spiked with different levels of Kerosene

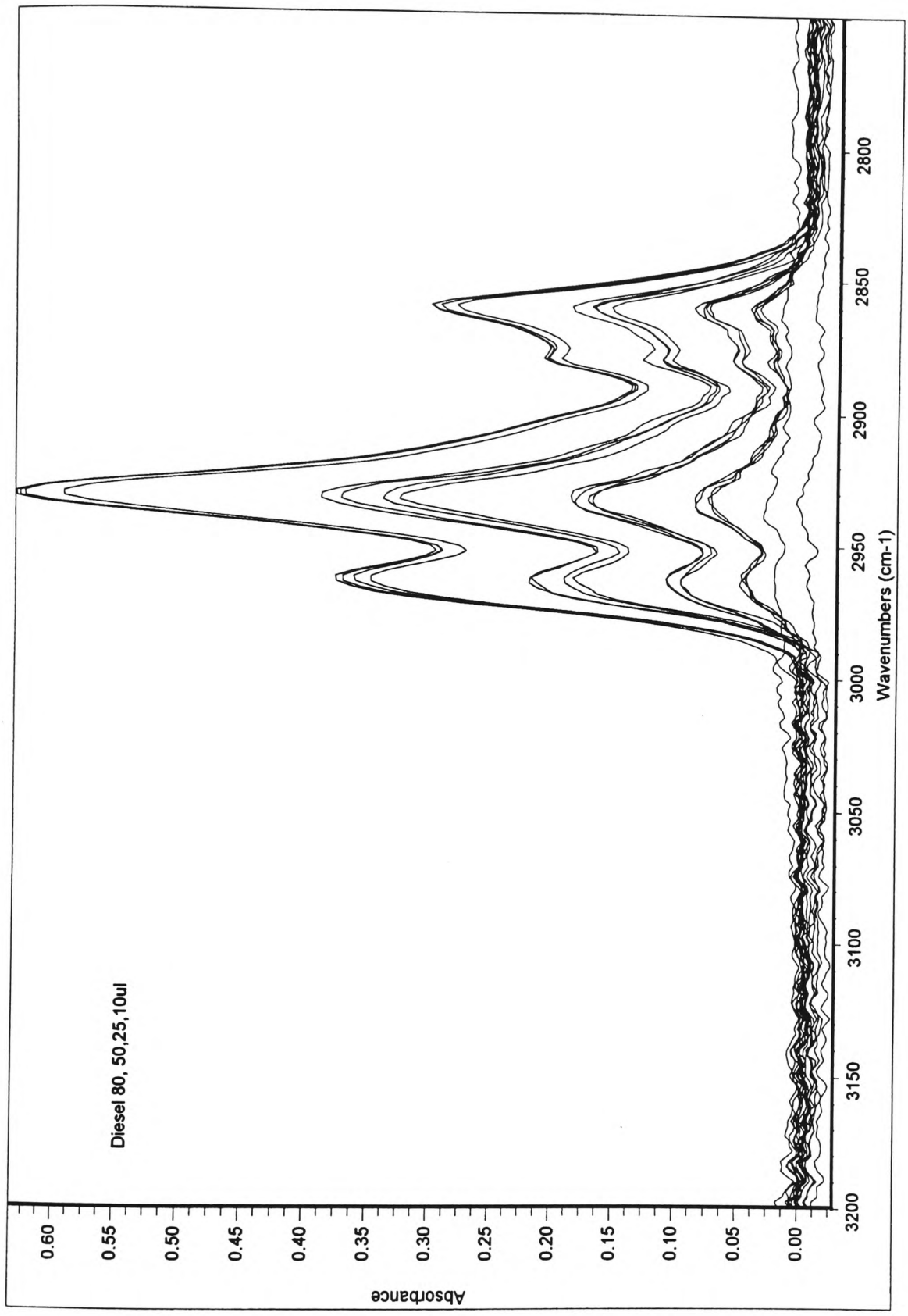

Fig. 3.20 On-line SFE-FTIR spectra obtained for 4 sets (N=5 per set) of 500 cm^3 water samples spiked with different levels of Diesel

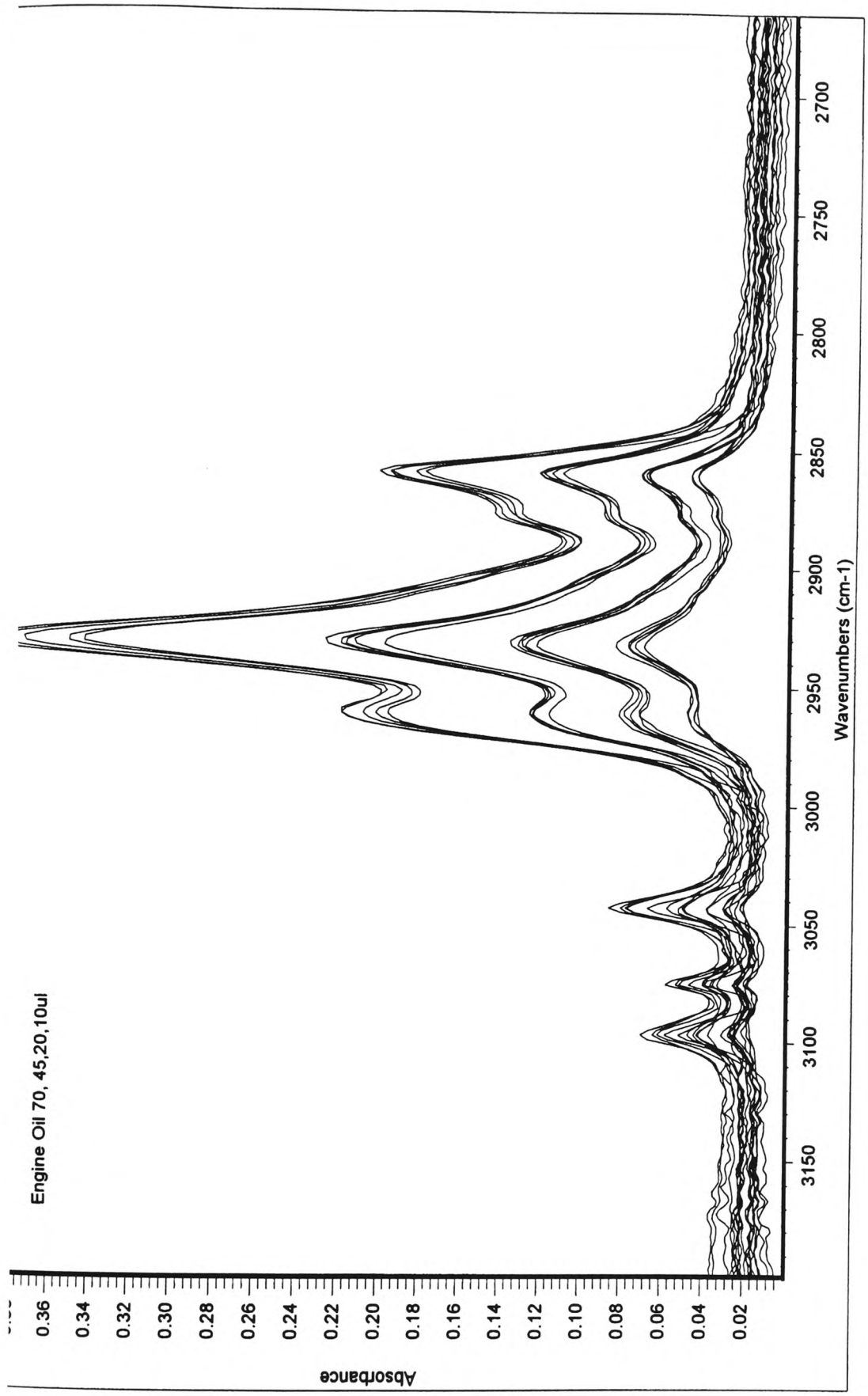

Fig. 3.21 On-line SFE-FTIR spectra obtained for 4 sets (N=5 per set) of 500 cm^3 water samples spiked with different levels of Engine Oil

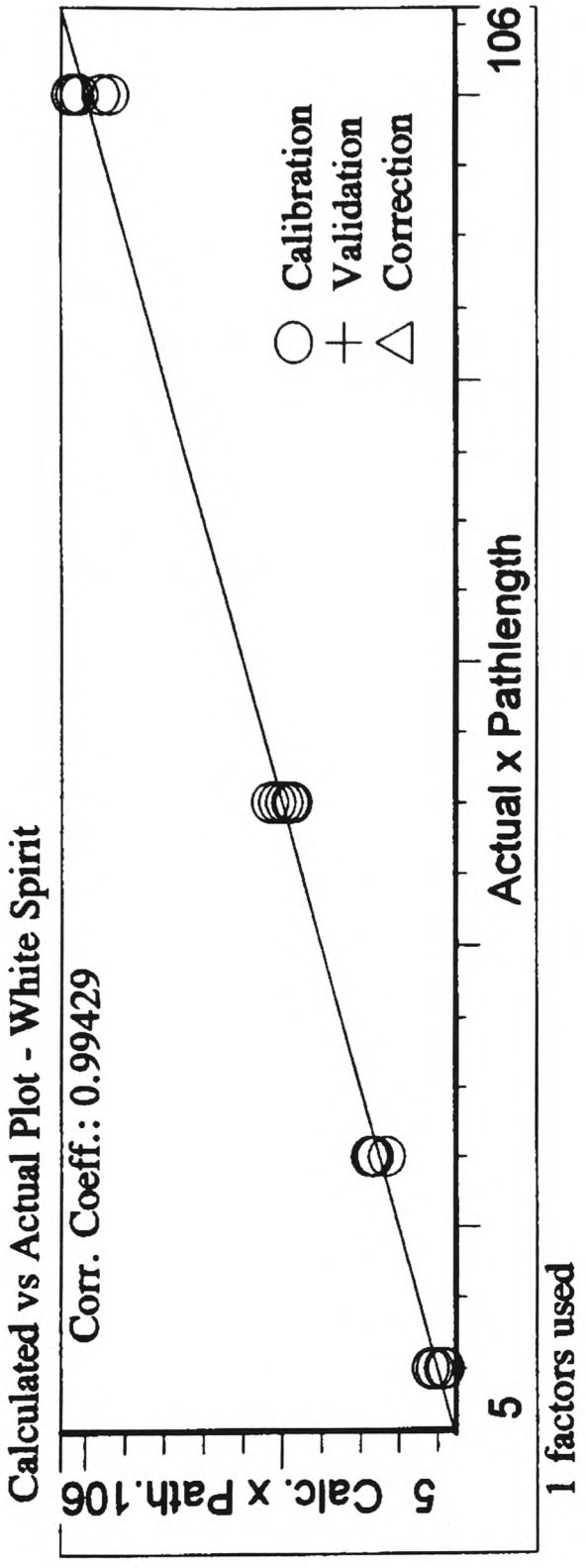

Fig 3.22 Graph obtained for the calculated against actual number of microlitres of white spirit spiked into 500 cm^3 of water

Oil	Actual amount of oil in 500 cm^3 sample (ppm)	Calculated amount of oil in 500 cm^3 sample (ppm)	Correlation coefficient	Accuracy	Mean accuracy
Gasoline	134	135.2	0.9829	99.1	90.8
	67	60.7		90.6	
	33.5	36.1		92.4	
	13.4	15.9		81.0	
White spirit	156	155.5	0.9943	99.7	97.5
	78	77.4		99.2	
	39	40.8		95.2	
	15.6	14.9		96.0	
Kerosene	162	161.4	0.9912	99.6	98.3
	81	81		100.0	
	40.5	40.3		99.6	
	16.2	17.2		94.0	
Diesel	137.6	139.2	0.9909	98.9	94.3
	86	82.4		95.8	
	43	41.1		95.6	
	17.2	19.4		87.0	
Engine oil 20W/50	70	71.6	0.9546	97.7	87.8
	45	38.6		85.8	
	20	23.1		84.5	
	10	11.7		83.0	

Table 3.10 SF-CO_2 extracted oils quantified against the same oil

3.4.6. Quantification of On-line samples by a rapid Off-line calibration procedure using Brent Delta crude oil

Brent Delta crude oil was used for this part of the work, as it is the off-shore oil industry where a large proportion of this work is applicable. Before starting the quantification it was necessary to ensure that SF-CO_2, under the conditions employed, could efficiently solubilise and extract the oil.

The first objective was to prove that it was possible to extract Brent crude oil. This was achieved by "doping" filter paper with some of the oil, placing it in the extraction vessel and extracting it. The extraction was carried out at 2500 psi, and at its conclusion, the "doped" filter paper was removed and the residue further extracted with hexane. This residue sample along with the sample of Brent Delta crude oil were then analysed using a Hewlett-Packard 5890 gas chromatograph equipped with an HP-5MS 30 m x 0.3 mm internal diameter column of 0.25 μm film thickness. Interfaced to the GC system was a Hewlet-Packard 5971A MSD (Mass Spectrometer).

The GC-MS chromatographic profile of Brent crude oil is shown in Fig 3.23 below.
The retention times for C10,16 and 20 were inferred from a standard hydrocarbon mixture, Fig 3.24, and also indicated on the chromatograph is the theoretical retention time for C30, based upon homologue counting.
From this chromatograph it can be deduced that Brent Delta crude oil has carbon compounds that slightly exceed 30 carbons in chain length.

The hexane extracted Brent Delta crude oil residue following SFE is shown in Fig 3.25.

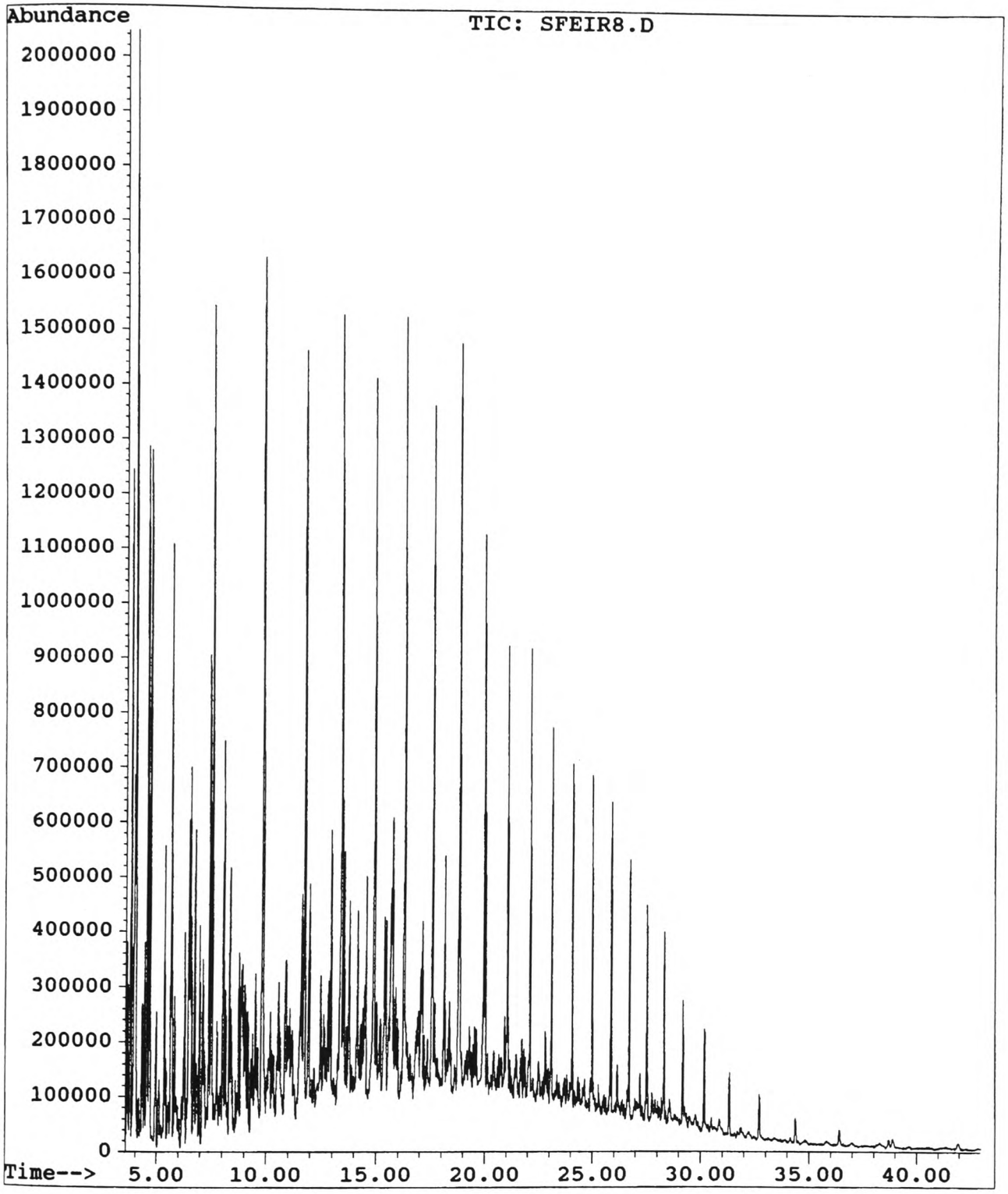

Fig 3.23 GC-MS analysis of Brent Delta crude oil

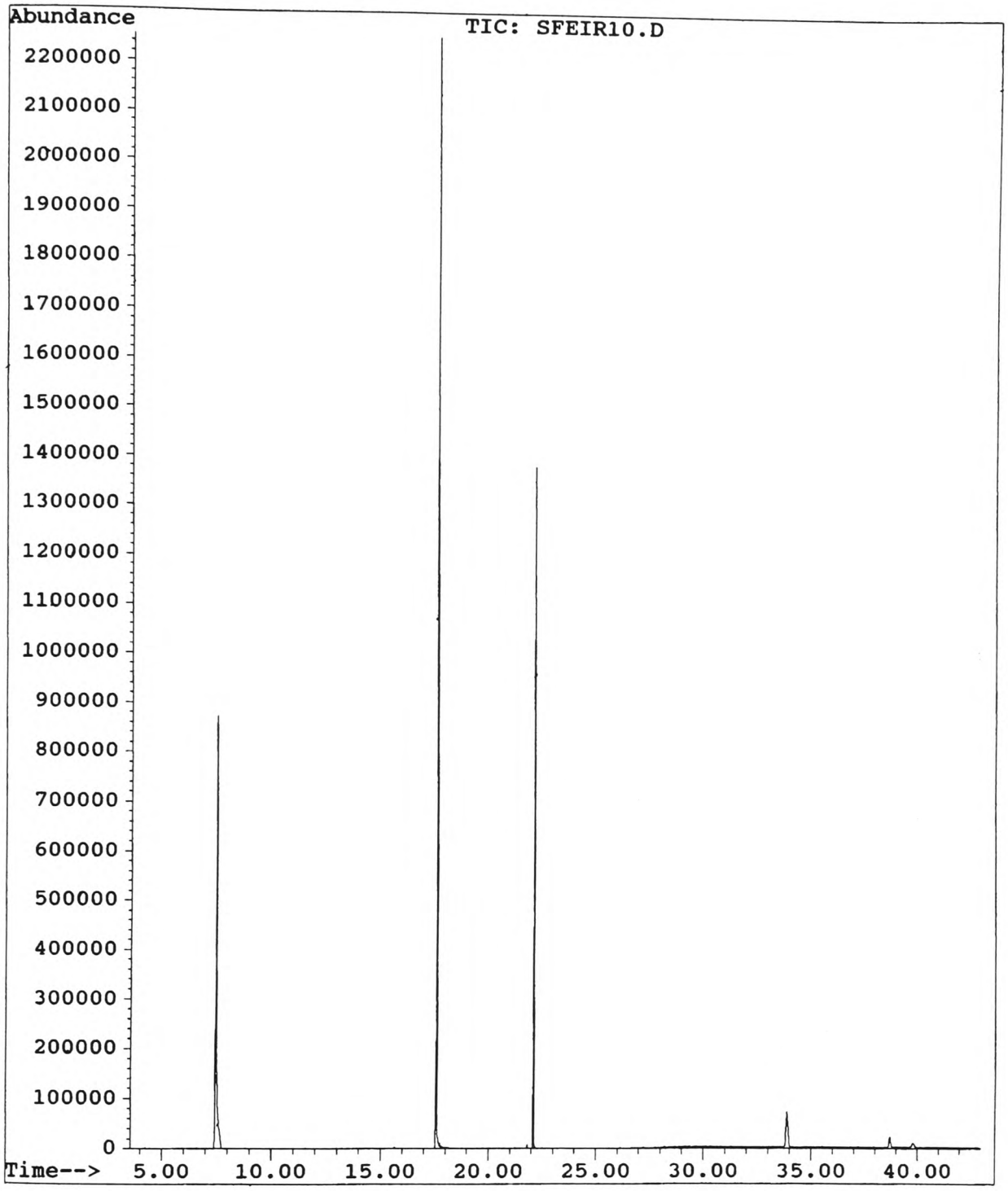

Fig 3.24 Chromatogram of C10, C16 and C20 hydrocarbons

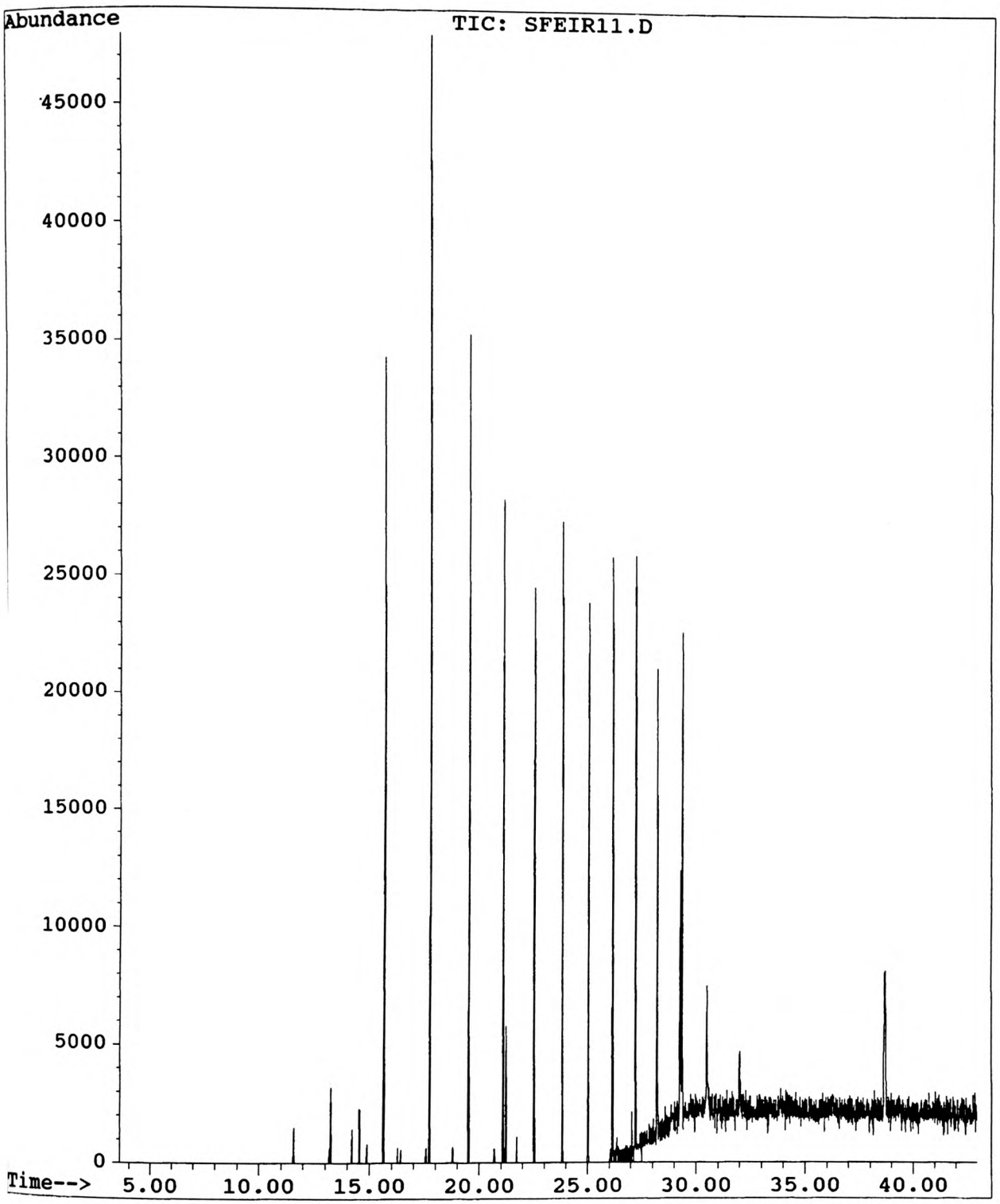

Fig 3.25 Post SF extraction residual sample of Brent Delta crude oil

By comparing both the chromatograms it can be seen that the extraction efficiency of SF-CO_2 is very good, the abundance in Fig 3.23 has a range up to 2 x 10^6, while the residue spectra, Fig 3.25, has an abundance up to 4.5 x 10^4.

To alleviate the need to produce any calibration solutions, as in 3.4.4.4, a method was developed where small volumes of hydrocarbons were injected, via the injection loop on the valve, directly into the SF CO_2 stream, en-route to the IR cell by-passing the SFE vessel.

A 10 µl glass syringe was used for the development of the off-line quantitation process, and multiple injections from 0.5 to 3.5 µl in 0.5 µl increments were analysed. The resultant IR spectra are shown in Fig 3.26.

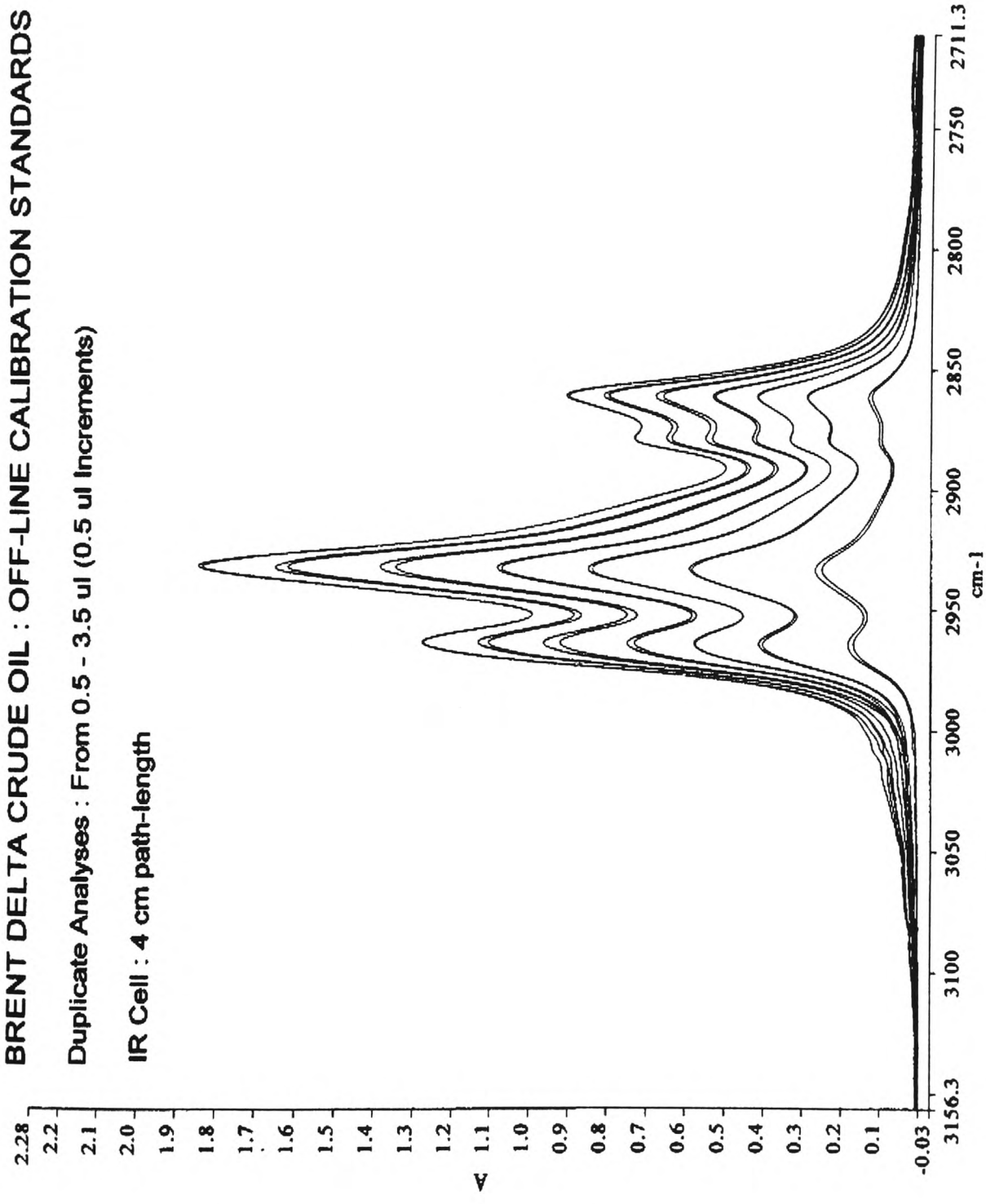

Fig 3.26 Off-line Brent Delta crude oil calibration standards

The absorbances for these samples were measured at 2933 cm^{-1} and a calibration graph drawn of volume of Brent Delta crude oil analysed, against mean absorbance at 2933 cm^{-1}, Fig 3.27.

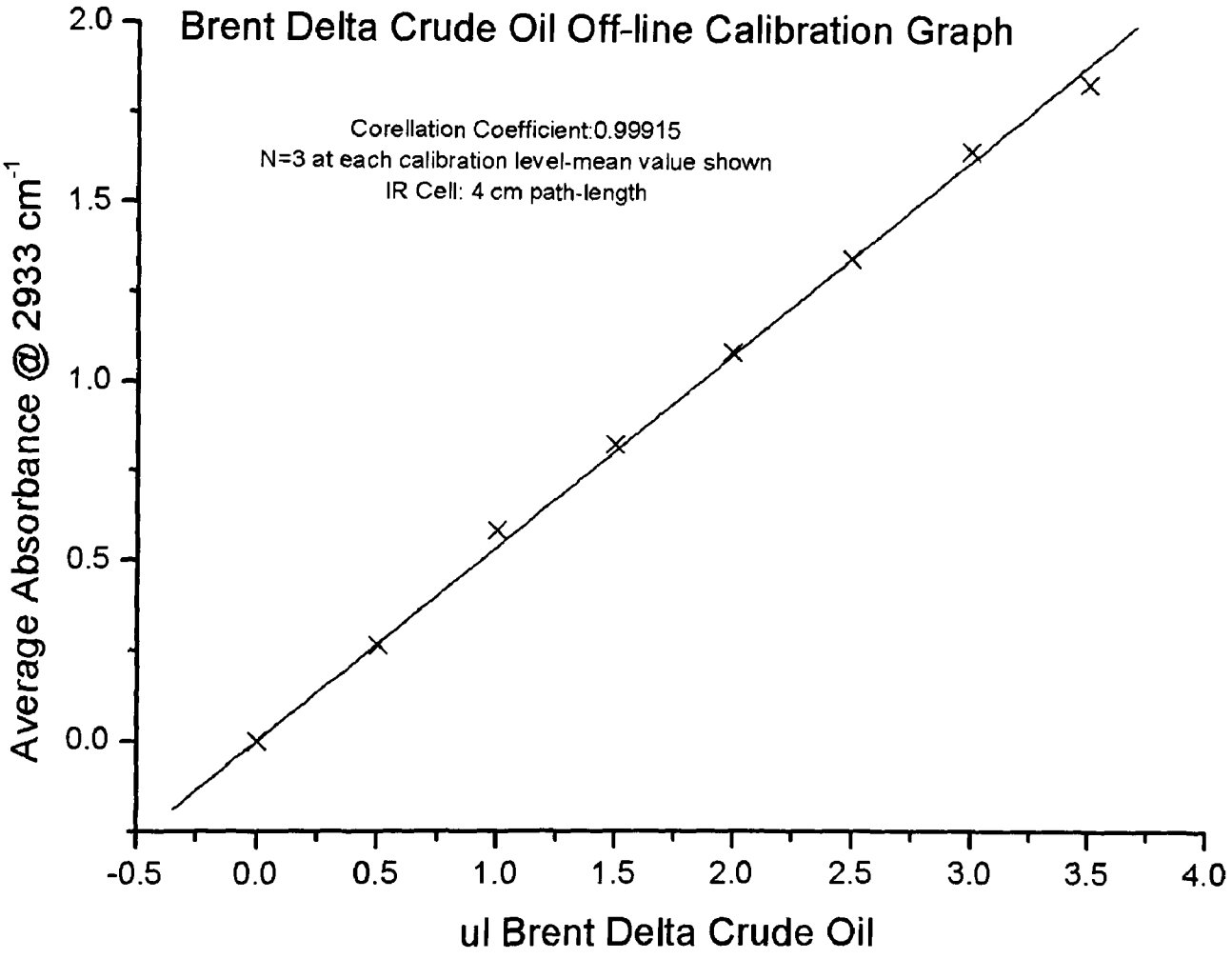

Fig 3.27 Off-line calibration graph of Brent Delta crude oil.

A conversion factor could now be applied to these values, to convert them from µl direct injection, to equivalent µl in 500 cm^3 of water, Fig 3.28. This was determined by using the above calibration graph to quantify known amounts of Brent Delta crude oil in 500 cm^3 spiked water samples.

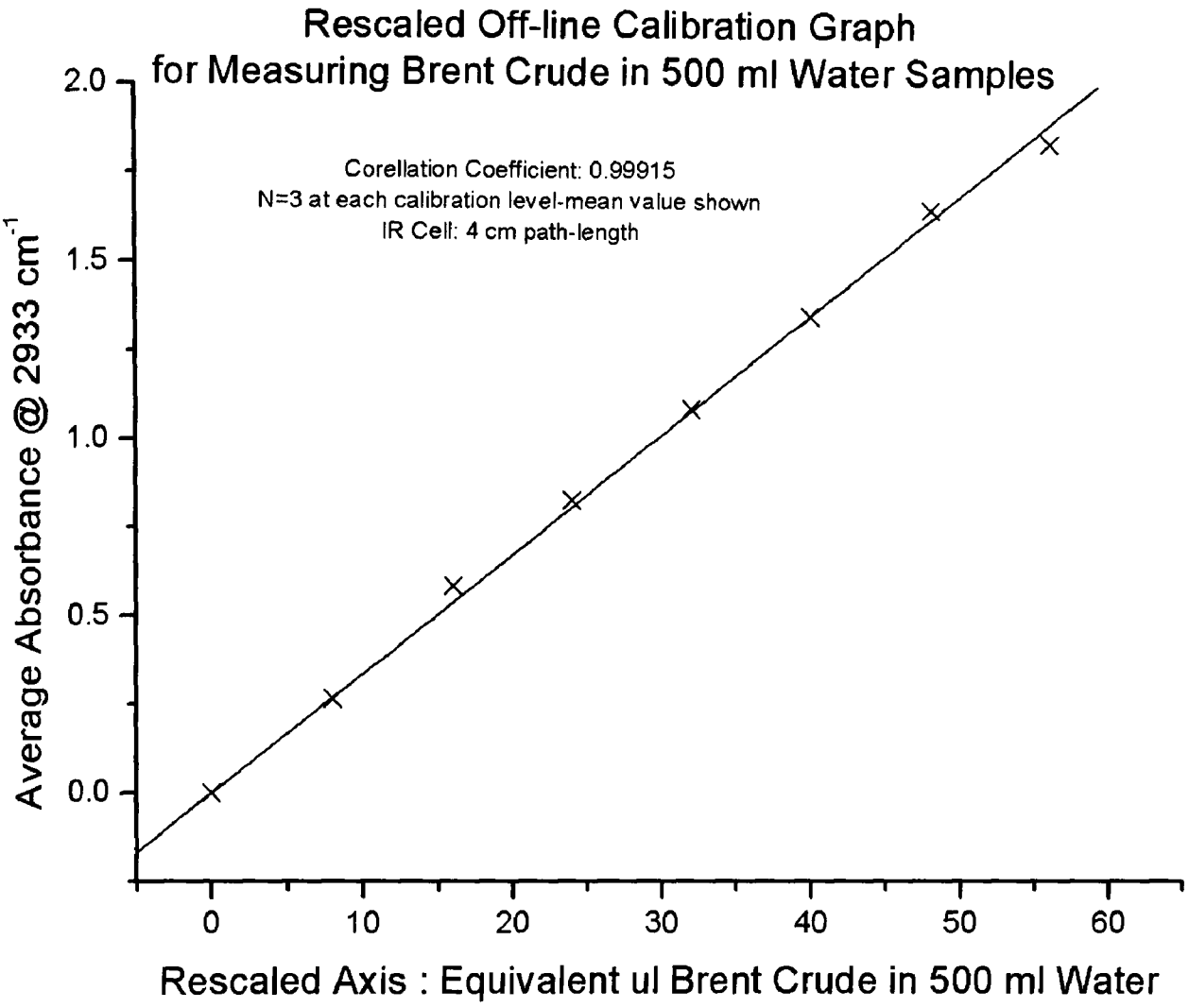

Fig 3.28 Re-scaled calibration graph of Brent Delta crude oil in 500 cm^3 water sample

Multiple samples (N=6) of Brent Delta crude oil, 10, 20 and 40 µl, were then spiked into 500 cm^3 of water, and extracted by SF-CO_2, Fig 3.29. These extracted samples were then quantified against the off-line calibration graph.

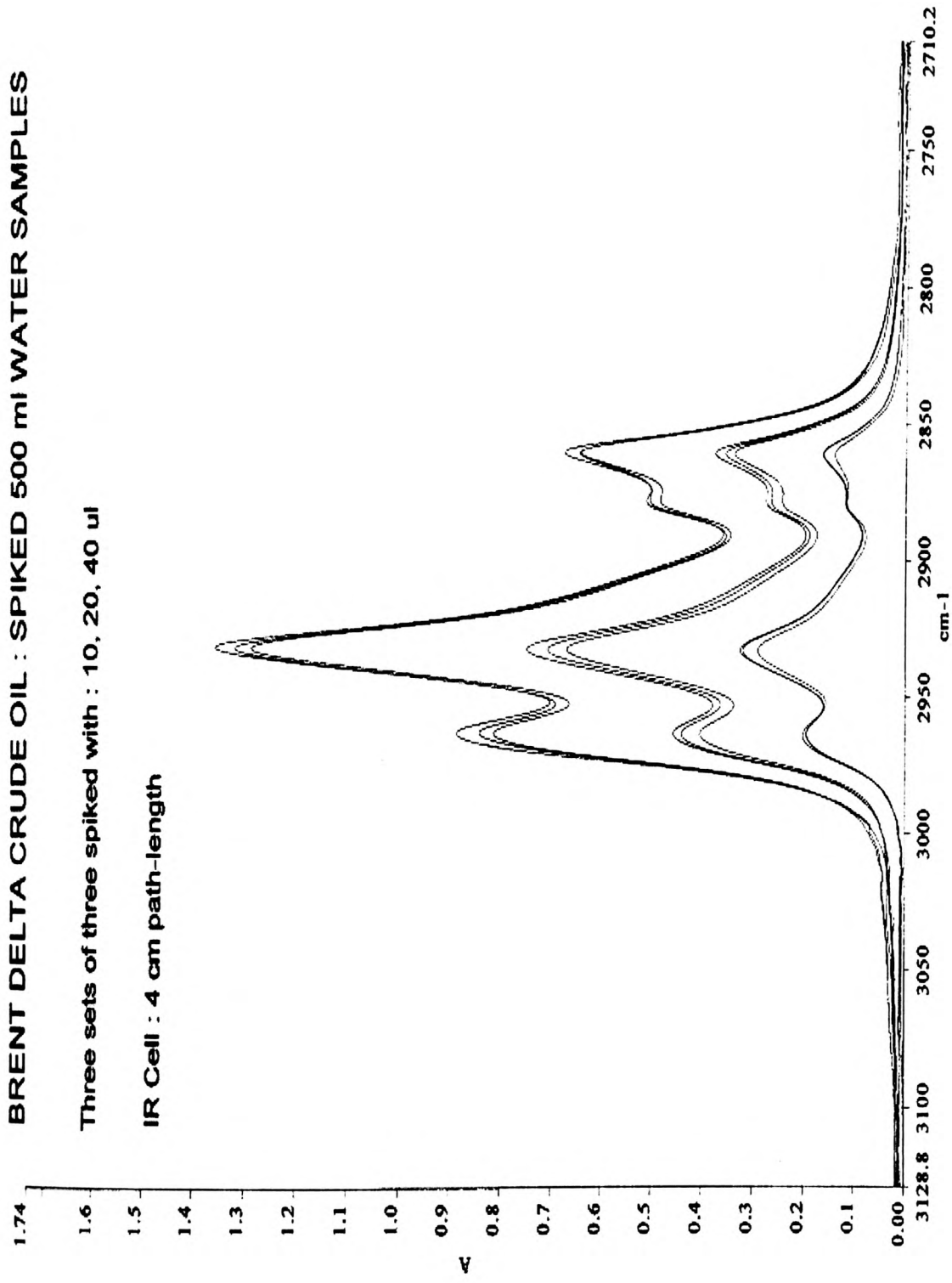

Fig 3.29 Brent Delta Crude oil spiked 500 cm^3 water samples

When quantified against the calibration standards the following values were obtained.

Actual concentration (µl)	Mean calculated concentration (µl)	% Error
10	9.4	-6
20	20.5	+2.5
40	40.2	+0.5

Table 3.11 quantitation of Brent Delta crude oil by on-line rapid calibration procedure

3.4.7 Conclusions

It has been shown in this section of work that the SFE system has the ability to extract and quantify a wide range of hydrocarbons using SF-CO_2 under the conditions employed. A comparison of the three standard methods for hydrocarbon quantification has been made, and that , in the majority of cases, the HMSO method, proved the more accurate, Table 3.6.

The HMSO method of quantification has been shown to be applicable to the SFE system, and has been applied to a range of SF-CO_2 extracted hydrocarbons. Further an alternative method of determining the HMSO coefficients has been successfully implemented, section 3.4.3.6.

As discussed in the introduction the three standard methods evaluated are applicable where the standard reference hydrocarbons are not available for quantification. The benefit to the quantitation where these standards are available can clearly be seen in Table 3.10.

An off-line calibration method was introduced, section 3.4.4.4, using standards prepared in carbon tetrachloride. This proved relatively easy to implement, and the results obtained were good when compared to carbon tetrachloride extractions and quantification using HMSO coefficients Table 3.9.

Even though the off-line calibration proved successful it had two disadvantages,

i. there was still the requirement for volumetric preparation of the standards solutions,

ii. the use of ozone depleting solvents which are in the process of, or have already been, being phased out.

To overcome these problems, a rapid calibration procedure was developed, section 3.4.6. This allowed direct injection of the standards into the IR cell *via* the SF-CO_2 stream, and missed the extraction vessel. This procedure gave results comparable with off-line calibration, Table 3.11.

Overall the SFE system has proved to be reliable, gave comparable, or better, results than the quantification procedures currently being used, and allows rapid calibration and quantification. Each individual calibration sample could be completed in less than three minutes, without the need of volumetric preparation, and hydrocarbon extraction by SF-CO_2 achieved in approximately 15 minutes. The clean cycle, to ensure no hydrocarbon carryover at high concentration levels, can be completed in approximately 5 minutes, and overall the system is more time efficient than the traditional methods. In addition it also has a high level of automation, and allows any of the processes to be initiated with the push of a button.

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Appendix D (Methods of Hydrocarbon Analysis)

Method	Country	Detection level (ppm)	Sample volume (l)	pH	Solvent	Volume	Agitation	Absorbent	Wavenumber (cm^{-1})	Calibration	Accuracy	Precision
API 733-58	U.S.A.	1	3	5	CCl_4	100 +100	Shaking machine	-	2930,2860	A	±10% known oil ± 20% with A	± 10%
DEV H 17/18 (1971)	GERMANY	0.1	1	-	CCl_4	25	Stirrer	Florisil	3030,2960, 2930	Absortivities	Not suitable for aromatics	-
CONCAWE	NETHERLANDS	0.1	3	5	CCl_4	50	Shaking machine	Florisil	2960, 2930	Absorbtivities	Less accurate for gasolines	Better than 10%
UNICHIM (1975)	ITALY	0.5	Variable	5	CCl_4	50	Shaking machine	Florisil	2960, 2930	Absorbtivities	-	at 2ppm level st = 0.24 so = 0.06
NBN (1977) T91-502	BELGIUM	0.2	1	5	CCl_4	50	Magnetic stirrer	Florisil	3030, 2960, 2930	Absorbtivities	Less suitable for aromatics	-
EPA 1978 418.1	U.S.A.	≤ 1	1	< 2	Freon 113	30+30+30	Hand	Silica gel	2930	B	50% for gasoline	-
IMCO (1978)	U.K.	0.1	1	≤ 2	CCl_4	50 + 50	Hand	-	2930	Known oil	Loss of volatiles	-
AFNOR T90-203/79	FRANCE	-	0.9	5	CCl_4	50	Magnetic stirrer	Florisil	2930	Known oil	-	-
PARIS COMM. (1979)	FRANCE	0.1	1	< 2	CCl_4 / Freon 113	50 + 50	Hand	-	2930	Known oli	Loss of volatiles	-
SS028145 (1979)	SWEDEN	0.1	1 - 2	< 2	CCl_4	50	Shaking machine	Al_2O_3	2960, 2930	Known oil or A	± 5 - 10% for A Extreme cases ± 30 - 40%	Often better than ± 2.5%
RIZA	NETHERLANDS	0.5	0.8	2	CCl_4	15 + 15	Shaking machine	Florisil	2930	A	Partial for aromatics	-
ASTM D 3921-80	U.S.A.	0.5	0.75	≤ 2	Freon 113	30+30+30	Hand	Silica gel	2930	Known oil or C	Volatiles lost	At x ppm st = 0.160x +0.329 so= 0.141x +0.048
ALPHA(1980) Parts 502B,502E	U.S.A.	0.2	1	< 2	Freon 113	30+30+30	Hand/shaking machine	Silica gel	2930	Known oil or A	Gasoline not measured	-
NEN 6673 (1981)	NETHERLANDS	0.05	0.8	2	CCl_4	15+15	Shaking machine	Florisil	2930	A	Less suitable for aromatics	-
DIN 38409 H18 (1981)	GERMANY	0.1	0.5	1 - 2	Freon 113	25	Shaking machine	Al_2O_3	3030, 2960, 2930	Absorbtivities or A	Less suitable for aromatics	-

Appendix E

Hydrocarbon Standard Solution (Actual conc ppm)	Hydrocarbons used as standard solutions Calculated concentration when quantified against these standards (ppm) using the CH_2 stretching peak (2930 cm^{-1})											
↓	Hexane	Heptane	Octane	Nonane	Decane	n-Dodecane	Tetradecane	Pristane	iso-Octane	Benzene	Toluene	Ethyl benzene
Hexane (63.0)	62.96	58.85	58.44	57.80	55.83	58.43	64.69	85.12	33.28	4.84	3.81	16.29
Heptane (64.8)	80.35	65.36	58.88	58.93	59.71	60.79	59.76	74.95	28.12	3.44	4.12	13.93
Octane (65.5)	100.21	75.73	65.10	62.48	57.56	60.90	52.15	63.90	21.76	5.04	1.64	9.74
Nonane (67.5)	103.95	75.91	68.80	67.46	62.31	62.52	53.37	64.11	20.68	5.75	1.01	8.79
Decane (54.4)	89.76	67.97	71.42	72.34	54.80	53.95	56.31	66.14	21.30	4.95	1.97	9.53.
n-Dodecane (64.5)	118.04	79.35	71.10	72.19	67.33	64.90	49.16	56.11	17.49	3.88	1.97	7.87
Tetradecane (54.0)	102.6	79.76	72.08	70.74	62.20	64.69	53.80	73.79	25.22	6.42	0.65	10.19
Pristane (63.5)	42.68	51.04	62.30	62.14	45.84	63.42	45.50	62.36	21.07	8.18	1.58	6.59
iso-Octane (36.6)	73.70	88.01	106.6	106.40	81.98	110.7	81.19	108.00	37.90	8.85	3.37	21.64
Benzene (83.0)	3.00	4.87	3.55	6.56	1.09	2.68	1.55	8.72	8.81	83.43	30.73	18.24
Toluene (87.0)	130.00	151.20	183.10	183.90	132.00	184.90	140.60	160.10	65.24	82.09	90.46	105.70
Ethyl benzene (75.0)	138.00	150.5	173.80	165.7	117.7	152.70	105.60	165.00	77.40	5.94	23.72	75.70

Appendix F

Variables used for graphical optimisation of the 5 oils

White spirit (39ppm)	(abs(((((0.198*x)+(0.152*y))-39)/39)*100))
Kerosene (39.5ppm)	(abs(((((0.261*x)+(0.170*y))-39.5)/39.5)*100))
Gasoline (33.5)	(abs(((((0.0974*x)+(0.1504*y))-33.5)/33.5)*100))
Diesel (42.5ppm)	(abs(((((0.1813*x)+(0.1093*y))-42.5)/42.5)*100))
Engine oil (20ppm)	(abs(((((0.1148*x)+(0.0604*y))-20)/20)*100))

Appendix G

Detailed calculations of 4 hydrocarbon coefficient determination

Diesel excluded

X=80 Y=153

Hydrocarbon	Conc. (ppm)	Abs(2930)	Abs(2960)	Abs(3030)	2960*X	2960*Y	Cal. Conc (ppm)	%Error	Mean Error
White spirit	15.6	0.0785	0.05252	0.0014	6.28	8.03556	14.32	-8.23	-5.53
	39	0.198	0.1524	0.0056	15.84	23.3172	39.16	0.40	
	78	0.3602	0.289	0.0076	28.816	44.217	73.03	-6.37	
	156	0.687	0.5798	0.0138	54.96	88.7094	143.67	-7.90	
Kerosene	15.8	0.1096	0.0688	0.0026	8.768	10.5264	19.29	22.12	20.45
	39.5	0.2612	0.1702	0.004	20.896	26.0406	46.94	18.83	
	79	0.524	0.349	0.007	41.92	53.397	95.32	20.65	
	158	1.0452	0.695	0.0118	83.616	106.335	189.95	20.22	
Gasoline	13.4	0.0384	0.052	0.0022	3.072	7.956	11.03	-17.70	-15.25
	33.5	0.0974	0.1504	0.0084	7.792	23.0112	30.80	-8.05	
	67	0.144	0.2588	0.0056	11.52	39.5964	51.12	-23.71	
	134	0.344	0.595	0.0182	27.52	91.035	118.56	-11.53	
Engine oil	10	0.0621	0.0316		4.968	4.8348	9.80	-1.97	-15.42
	20	0.1148	0.0604		9.184	9.2412	18.43	-7.87	
	45	0.1934	0.1016		15.472	15.5448	31.02	-31.07	
	70	0.3458	0.1818		27.664	27.8154	55.48	-20.74	

X=91 y=160

Hydrocarbon	Conc.(ppm)	Abs(2930)	Abs(2960)	Abs(3030)	2960*X	2960*Y	Cal. Conc (ppm)	%Error	Mean Error
White spirit	15.6	0.0785	0.05252	0.0014	7.1435	8.4032	15.55	-0.34	2.31
	39	0.198	0.1524	0.0056	18.018	24.384	42.40	8.72	
	78	0.3602	0.289	0.0076	32.7782	46.24	79.02	1.31	
	156	0.687	0.5798	0.0138	62.517	92.768	155.29	-0.46	
Gasoline	13.4	0.0384	0.052	0.0022	3.4944	8.32	11.81	-11.83	-9.44
	33.5	0.0974	0.1504	0.0084	8.8634	24.064	32.93	-1.71	
	67	0.144	0.2588	0.0056	13.104	41.408	54.51	-18.64	
	134	0.344	0.595	0.0182	31.304	95.2	126.50	-5.59	
Diesel	17	0.083	0.0478		7.553	7.648	15.20	-10.58	-16.16
	42.5	0.1813	0.1093		16.4983	17.488	33.99	-20.03	
	85	0.367	0.214		33.397	34.24	67.64	-20.43	
	136	0.631	0.3755		57.421	60.08	117.50	-13.60	
Engine oil	10	0.0621	0.0316		5.6511	5.056	10.71	7.07	-7.66
	20	0.1148	0.0604		10.4468	9.664	20.11	0.55	
	45	0.1934	0.1016		17.5994	16.256	33.86	-24.77	
	70	0.3458	0.1818		31.4678	29.088	60.56	-13.49	

Chapter 4

Application of Direct Aqueous Supercritical Fluid Extraction Procedures As Off-line Sample Preparation Technique for Gas Chromatography-Mass Spectroscopy Studies

4.1 The Supercritical Fluid Extraction and Quantification of Testosterone by off-line Gas Chromatography- Mass Spectrometry

4.1.1 Introduction

The vast majority of reported work for the extraction of analytes by supercritical fluids has been from solid matrices. This is surprising as it would appear that extraction from aqueous samples, both environmental and biological, should be straightforward. The potential for extraction of organic analytes at the parts-per-billion level from water using SF-CO_2 was first reported by Ehntholt et al [1] as early as 1983. The reasons for the slow development of SFE of aqueous samples could be given as:

i. commercially available equipment was developed for solid matrices
ii. the development of a reliable restrictor module that would resist blockage by ice formation resulting from extracted water being frozen by Joule-Thomson cooling
iii. the solubilities of many organic compounds are sufficiently high in water to prohibit their useful recoveries by SF-CO_2.

To develop a SFE system for analytes in aqueous media, several points need to be considered:

i. the solubility of the analyte to be extracted in water

Several workers have published details in this area, [2-5] further details of analyte SF distribution coefficients have been reported by Roop and Akgerman,[6] and Sengupta et al. [7] As a general indication,[8] any analyte which is sparingly soluble in

water, and which are directly amenable to Gas Chromatography can be successfully extracted from water.

ii. direct or indirect SF extraction.

Direct extraction involves the extraction being conducted directly from the aqueous media. Indirect extraction requires the analyte to be loaded onto a support medium, such as a solid phase extraction (SPE) cartridge or disk.

Direct has the advantage of less stages, and simplicity, but requires the construction of an extraction vessel to contain the aqueous sample during the extraction period.

Indirect extraction has the disadvantage of additional extraction stages, loading of the analyte onto the support material, but has the advantages of support material selection. Careful selection can increase the analyte extraction efficiency, and also the support material can be directly loaded into a commercially available solid phase extraction vessel.

iii. the volume of liquid to be extracted

Often water samples need to be large in volume , typically 100 cm^3 to 1000 cm^3, to ensure sufficient quantity of analyte is extracted for detection, Koester and Clement [9] have published a review of the analysis of organic pollutants in drinking water.

iv. whether dynamic, static, or a combination of both techniques are to be employed.

A continuous stream of supercritical fluid is employed in dynamic extraction, whereas a fixed volume of supercritical fluid is allowed to equilibrate with the analyte solution in static extraction.

Several workers have reported on the extraction of analytes from aqueous samples including Hendrick and Taylor [10,11] for diisopropyl methylphosphonate, triprolidine, phosphonates, drugs and phenols, Combs *et al*[12] also for the extraction of phenols, and Mulcahey and Taylor[13] extracted sulphamethoxazole and trimethoprim from liquid drug formations. Lin *et al*[14,15] extracted Th and UO_2^{2+} ions and Wang *et al*[16,17] extracted Zn(II), Cd(II), Pb(II) and Cu(II). Ong *et*

al[18,19] extracted cholesterol from horse blood serum, while Liu and Wehmeyer[20] extracted the drugs flavone and ketorolac from dog plasma.

This section will report on the direct extraction of testosterone section 4.1, organophosphorus pesticides section 4.2, and polyaromatic hydrocarbons, section 4.3, from water, with subsequent analysis and quantification of the analytes off-line by Gas Chromatograph- Mass Spectrometry.

Testosterone (17β-hydroxy-4-androsten-3-one) (Fig 4.1.1) is a naturally occurring male hormone which has been misused in sport.

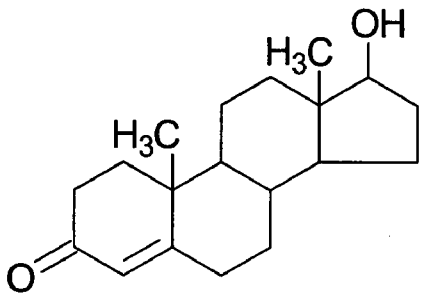


Fig 4 1.1 Testosterone

This compound can increase lean muscle mass which results in artificial enhancement of strength and stamina, and there were allegations that it was being used in the sporting field as early as the 1950s.

When testosterone is administered orally it is absorbed in the gut and deactivated by metabolism in the liver, resulting in oxidation of the 17β-hydroxyl group to produce androstene-3,17-dione.

In 1967 the International Olympic Committee (IOC) established a Medical Commission to deal with the widespread misuse of drugs in top level sport. The following year the commission banned the practice of doping, and following this IOC accredited laboratories were established and dedicated to the testing for banned

substances or their metabolites in athletes urine, as blood sampling was considered too invasive. Testosterone was added to this list of banned substances in 1983.

Consequently this has led to the development of surveillance procedures based upon GC-MS [21,22] and radioimmunoassay[23] for the determination of testosterone levels in urine samples as a means of dope testing.

This approach was adopted by the IOC in 1983, where GC-MS is used to determine the testosterone (T) to epitestosterone (the inactive 17α epimer of testosterone) (E) urinary ratio, with the Atlanta Olympic games in 1996 marking the first occasion where every specimen was screen by gas chromatography.

Healthy males, produce 30 times more testosterone endogenously than epitestosterone, but excrete only approximately 1% of testosterone unchanged as compared with 30% for epiterosterone. Consequently this leads to a urine ratio of approximately 1:1 for T/E ratio. Therefore athletes who have not received administered testosterone, generally have a T/E ratio of approximately 1, although studies have shown that a small number of males can have naturally elevated levels. [24]

The IOC considers a T/E ratio of greater than six suspect, and work is continuing to develop a method that will allow a more sensitive method of testosterone determination, and one avenue of research is based on the carbon isotopic ratio. The method[25,26,] is based on the observation that the carbon isotope ratios of urinary testosterone is lowered, relative to epitestosterone, for administered testosterone when compared with naturally metabolised testosterone.

Since the vast majority of testosterone is excreted in the form of glucuronic acid Fig 4.1.2) and sulphate conjugates, which are too polar to be analysed by GC-MS, methods for analysis include the use of glucuronidase for the enzymatic liberation of testosterone prior to analysis.

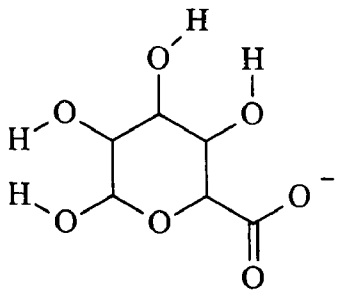


Fig 4.1.2 Glucuronic acid anion

The outline procedure for the current methods [8,9,10] include the following steps,

i. enzymatic hydrolysis of the conjugates

ii. extraction of the free steroids

iii. derivatisation

iv. analysis by GC-MS

These procedures also require the use of solid phase extraction (SPE) and subsequent liquid – liquid extraction preparative stages.

This work reports the direct enzymatic liberation of testosterone from the hydrolysed mixture of testosterone-β-D-glucuronide, and subsequent dynamic extraction of the liberated testosterone using direct supercritical fluid extraction.

The liberated testosterone was then trapped by decompression of the supercritical fluid extract onto a octadecyl silane (ODS) HPLC column, and the testosterone then recovered from the ODS column by solvent rinsing, and the recovered testosterone quantitatively determined using GC-MS, using isotopically labelled testosterone as an internal standard.

4.1.2 Supercritical Fluid Extraction

A patented stainless-steel vessel of internal volume of 300 cm^3 and pressure tested to 41.3 MPa, was used for the supercritical extractions.

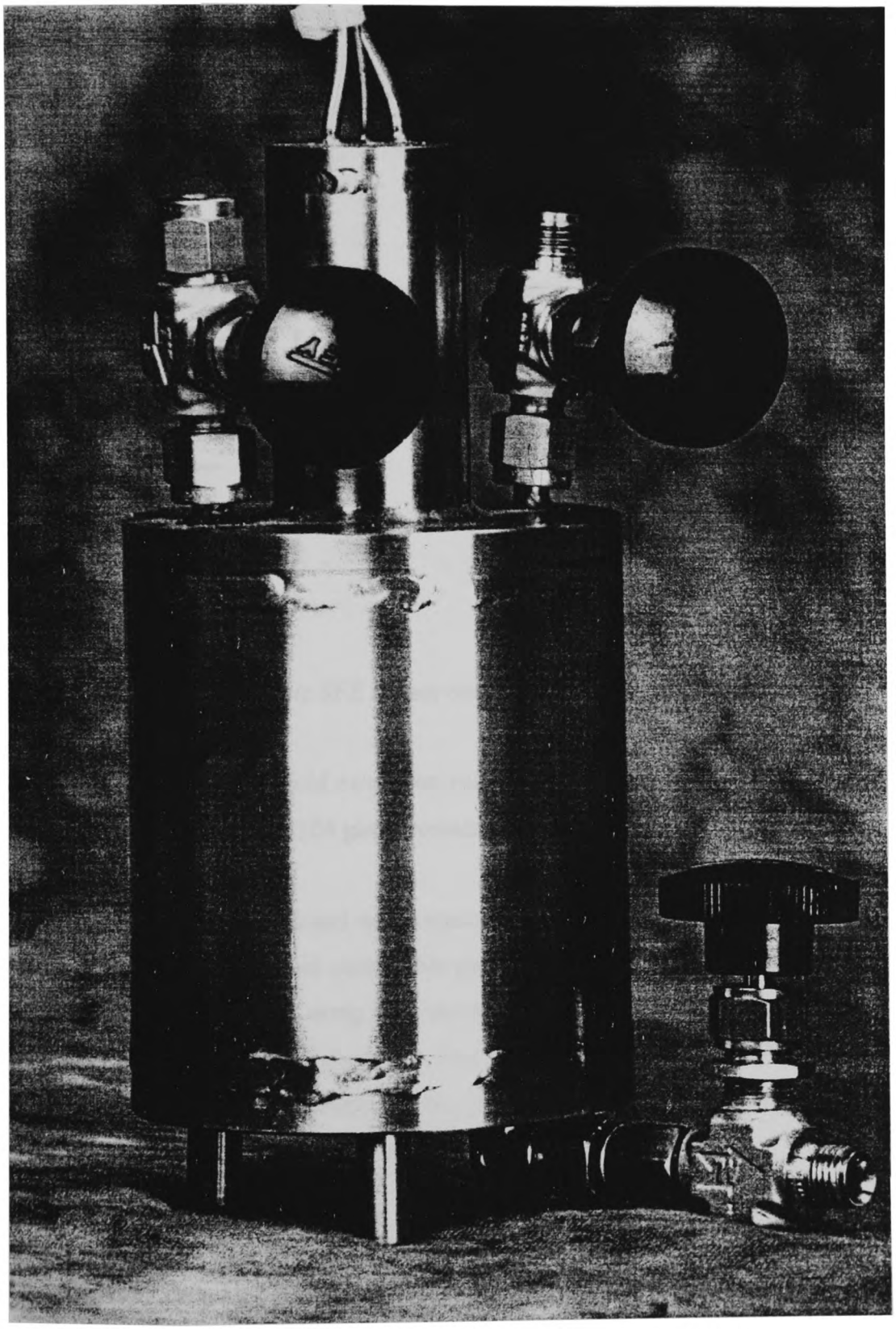


Fig 4.1.3 300 ml Supercritical Fluid Extraction Vessel

The extraction vessel shown in Fig 4.1.3, was configured as illustrated in Fig 4.1.4

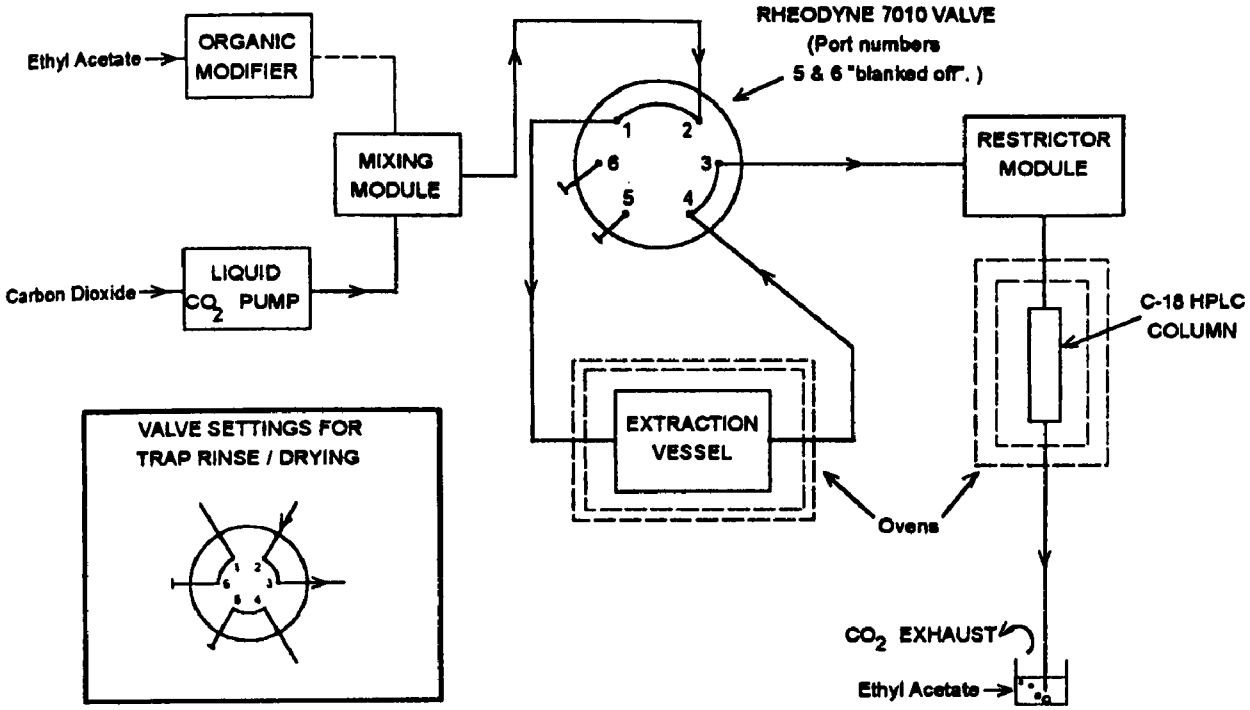


Fig 4.1.4 Dynamic SFE system configuration (Extraction mode)

To allow the supercritical fluid extraction vessel to be thermostatically controlled at 55 °C, it was hosed in a Pye 104 gas chromatography oven

The inlet to the extraction vessel was connected to a Gilson 308 reciprocating pump fitted with a supercritical fluid compatible piston seal, and pump head cooling jacket *via* a Rheodyne 7010 valve, using high pressure 1/16 inch stainless steel tubing and fittings. The pump head cooling jacket was connected to a Neslab RTE 110 liquid coolant recirculator which maintained the head at –10 °C, and ensured the liquid carbon dioxide remains liquefied allowing efficient delivery.

The output from the vessel was connected, via the Rheodyne 7010 valve, to a Gilson 821 pressure regulator module which allowed predetermined programmable backpressures to be selected. The output from the restrictor module was then

connected to a Spherisorb 250 x 4.6 mm diameter, 5 μm S5ODS HPLC column, which was used as the analyte trap, contained within a Gilson 831 oven and temperature regulated at 60 °C. The exhaust from this column was then passed through a beaker containing ethyl acetate, this solvent was checked after each subsequent extraction to ensure all extracted testosterone adhered to the column.

Before commencing the extraction, the ODS column was thoroughly washed with ethyl acetate at 1 ml min^{-1} for 10 min, and then dried *in situ* using carbon dioxide at a flow rate of 3 ml min^{-1} for 10 min, by selecting the required rinse configuration on the Rheodyne valve, and by-passing the extraction vessel.

Stock solutions of :

a. testosterone-β-D-glucuronide sodium salt (3 mg accurately dissolved in 500 cm^3 deionised water)
b. testosterone (6 mg accurately dissolved in 500 cm^3 Analar ethyl acetate)
c. 16,16,17-$^2H_3$-testosterone - internal standard - (4 mg accurately dissolved in 500 ml Analar ethyl acetate)

were then prepared.

A 10 ml aliquot of the standard solution of testosterone-β-D-glucuronide sodium salt was pipetted accurately into the supercritical extraction vessel, along with 220 ml of dilute hydrochloric acid to adjust the sample pH to 5.2), and 1.5 ml of the enzyme HP-2-β-gluuronidase, (type H-2; crude solution from Helix Pomatia with a β-gluuronidase activity approximately 100,000 units cm^{-3} at pH 5.0, 30 min assay).

The extraction cell was regulated at 55 °C for the duration of the extraction, with digestion and extraction being initiated by ensuring the back pressure regulator was set at 24.1 MPa, and the pump was programmed to deliver liquid carbon dioxide at 4 cm^3 min^{-1} .

The first 15 min duration of the extraction was required for the vessel to attain its target pressure, at this point the restrictor opened allowing the carbon dioxide to flow through the vessel. The extraction procedure was continued for a further 120 min

dynamic SFE, after which time the vessel was isolated and the temperature raised to 100 °C for 15 min to denature the enzyme.

After the digestion/extraction phase was complete, the Rheodyne valve was set to rinse/drying, and the carbon dioxide flow rate set at 0 cm^3 min^{-1}. The back pressure regulator was set at 0 MPa pressure, this allowed the vessel to depressurise onto the ODS column before rinsing with solvent.

Once depressurised, the ODS column was rinsed with 7 cm^3 of Analar ethyl acetate, delivered from a HPLC pump, at a flow rate of 1 cm^3 min^{-1}. The solvent, and extracted testosterone, were collected into a 10 cm^3 volumetric flask and allowed to equilibrate at room temperature.

2.5 cm^3 of the isotopically labelled 16,16,17-$^2H_3$-testosterone, of concentration 8 ng μl^{-1}, internal standard was then added to the volumetric flask, and quantitatively prepared to 10 cm^3 with Analar ethyl acetate.

The extraction vessel was then carefully emptied, and cleaned by subsequent extractions of deionised water.

Quantification of the extracted testosterone was then completed using off-line GC-MS.

4.1.3 Quantification of Extracted Testosterone

Analysis was performed using a Hewlett-Packard 5890 gas chromatograph equipped with an HP-5MS 30 m x 0.3 mm internal diameter column of 0.25 μm film thickness. Interfaced to the GC system was a Hewlet-Packard 5971A MSD (Mass Spectrometer), this analytical arrangement was used for all the analyses, and all were completed using high purity helium as the carrier gas.

To enable GC-MS studies to be performed on total extracted testosterone, and to determine extraction profiles, standard solutions of testosterone were prepared in the concentration range 0.75 – 12 ng μl^{-1}, from the original stock solution, with each containing 2 ng μl^{-1} 16,16,17-$^{2}H_3$-testosterone internal standard. Using splitless injection, 3 μl of these samples was injected onto the column.

The gas chromatograph temperature profile used for all analyses was;
2 min at 100 °C, then to 290 °C at 20 °C min^{-1}, with the final temperature held for 10 min.

To improve the sensitivity of the technique the quantification was carried out in selected ion monitoring mode (SIM), this allowed the detection of specified ions, with an increase on the gain of the detector, being unaffected by other ions present.

```
File        : C:\CHEMPC\PROJECTS\TEST1.D
Operator    :
Acquired    : 20 Mar 98    2:49 pm using AcqMethod EDR2
Instrument :    5971 - In
Sample Name:
Misc Info   : testosterone
Vial Number: 1
```

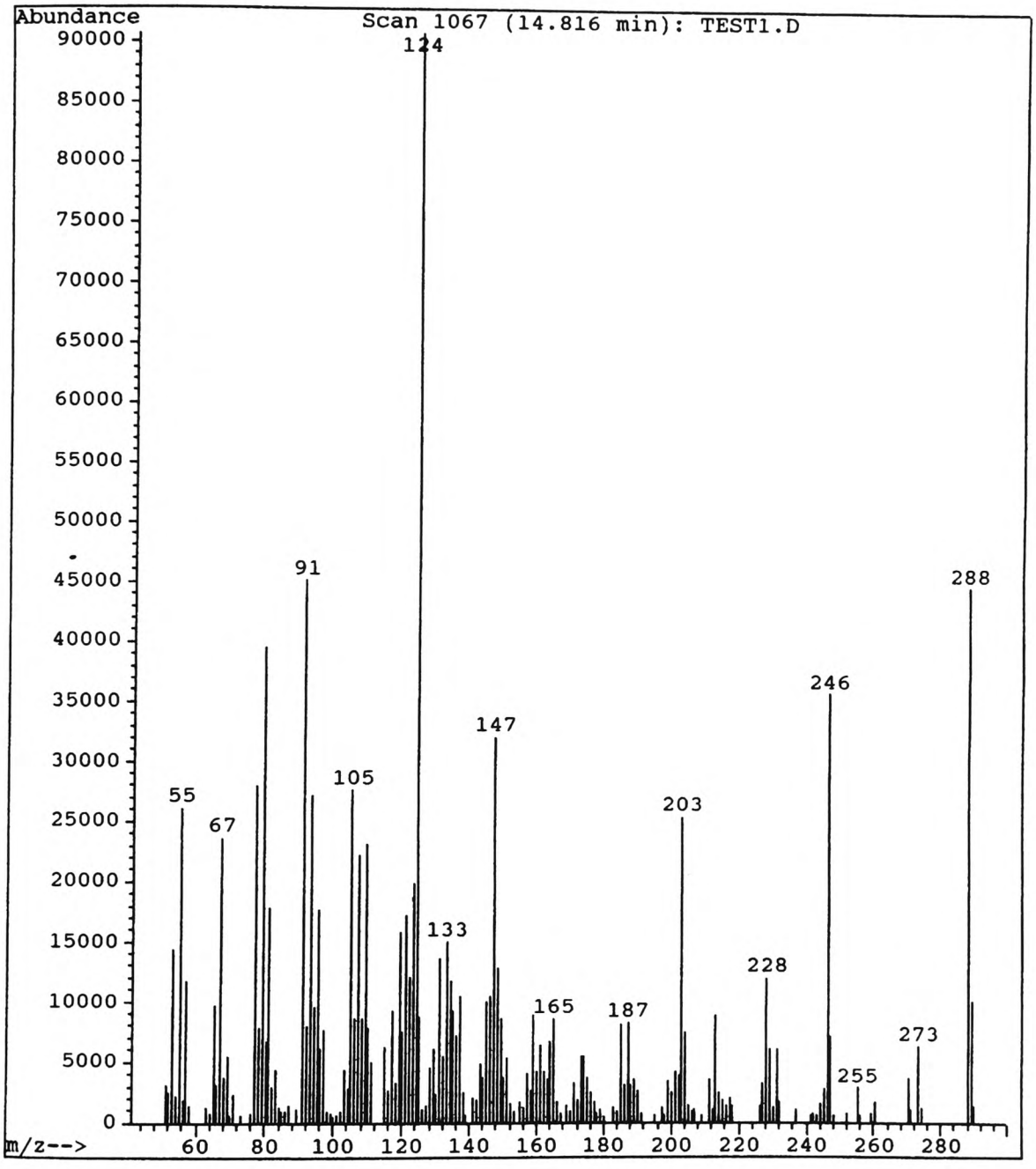


Fig 4.1.5 Mass Spectrum of Testosterone (Full Scan)

```
File       : C:\HPCHEM\1\DATA\BM2.D
Operator   :
Acquired   : 18 Feb 102    1:56 pm using AcqMethod EDR2
Instrument :    5971 - In
Sample Name:
Misc Info  : Testosterone Std - D3
Vial Number: 1
```

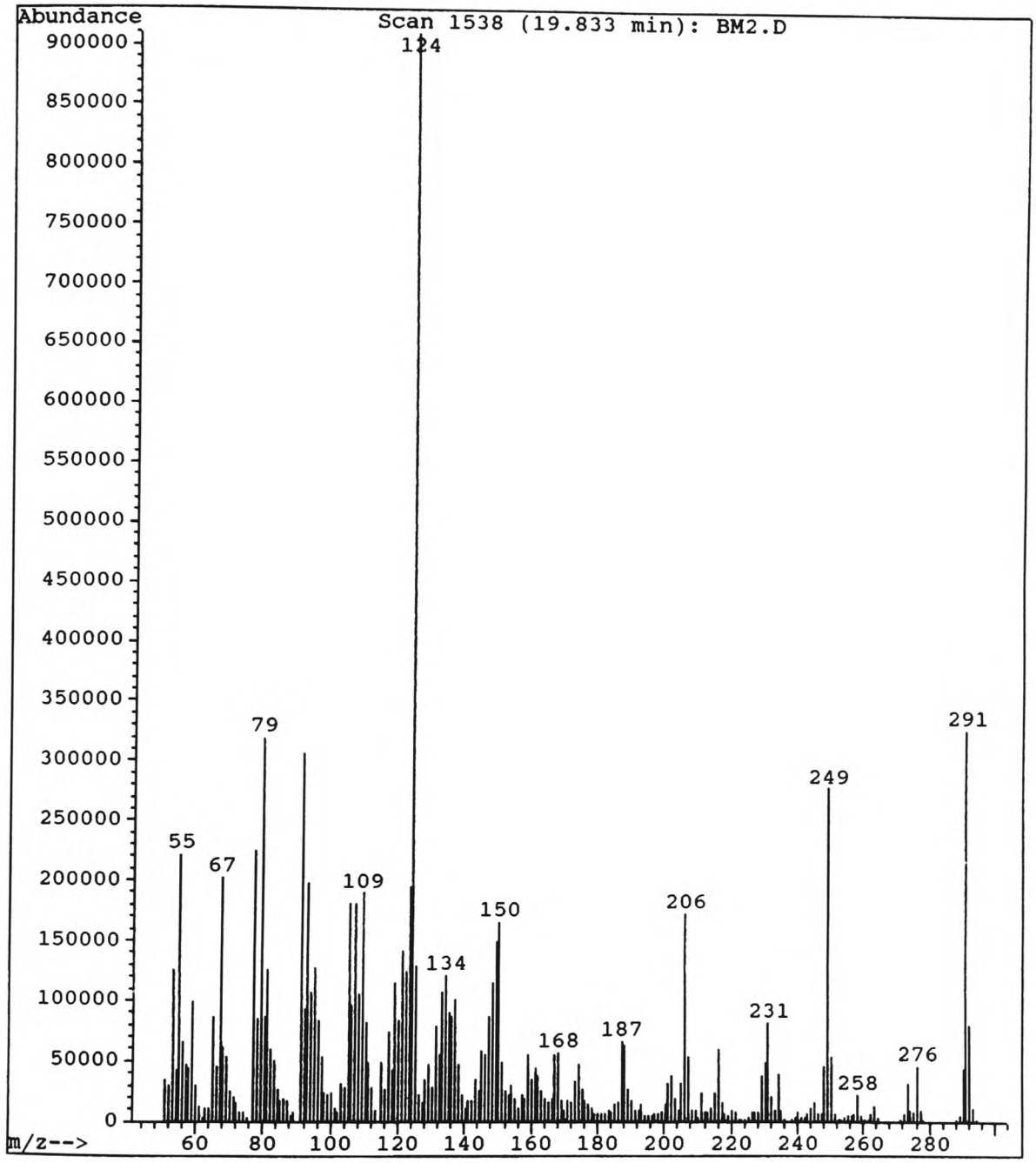


Fig 4.1.6 Mass Spectrum of 16,16,17-$^2H_3$-testosterone (Full Scan)

The calibration graph was constructed using the following molecular and qualifier ions for the analyte and internal standard;

	Molecular Ions (m/z)	Qualifier Ions (m/z)
Testosterone	288	246
16,16,17-$^{2}H_3$-testosterone	291	249

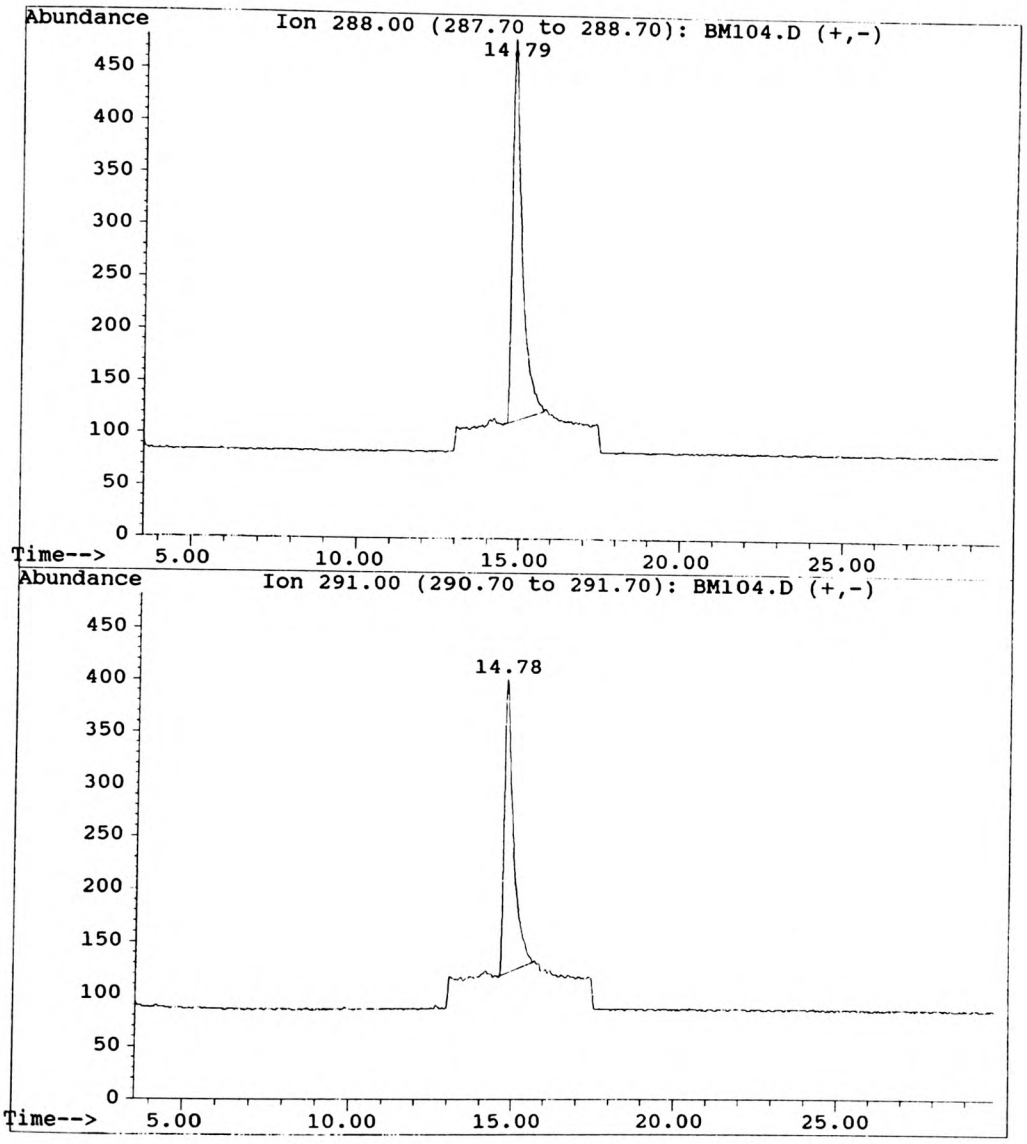


Fig 4.1.7 SIM chromatogram of testosterone (top) and $^{2}H_3$-testosterone internal standard (bottom)

```
File        : D:\CHEMPC\DATA\EDR103.D
Operator    :
Acquired    : 21 Jun 96  10:20 am using AcqMethod EDR3
Instrument :   5971 - In
Sample Name: TESTOSTERONE DYNAMIC EXTRACT
Misc Info   :
Vial Number: 4
```

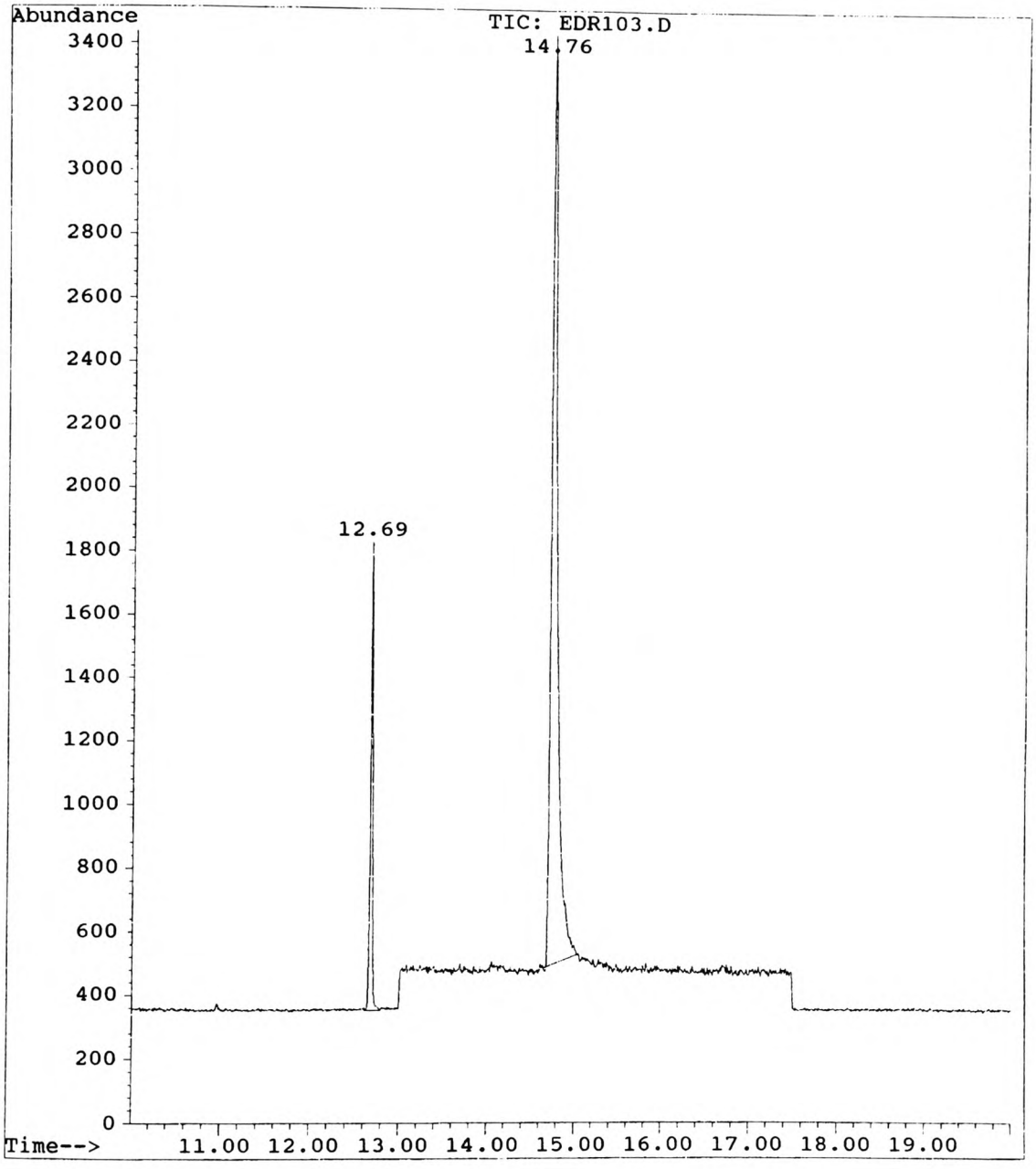


Fig 4.1.8 Summed ion chromatogram obtained for the SIM analysis of liberated testosterone

The profile in Fig 4.1.8 shows a co-extracted species with a retention time of 12.69 min, which was also found to produce ions with m/z ratios of 246 and 249. To

overcome this problem, and to enhance the ratio of the 14.76 and 12.69 min peaks, the electron multiplier value was increased by 500 V in the time frame 13 – 17 min.

The five standard testosterone solutions containing between 0.75 – 12 ng μl^{-1} and 2 ng μl^{-1} $^2H_3$-testosterone internal standard, were analysed under the same parameters as the extracted samples, and a calibration graph constructed, Fig 4.1.9.

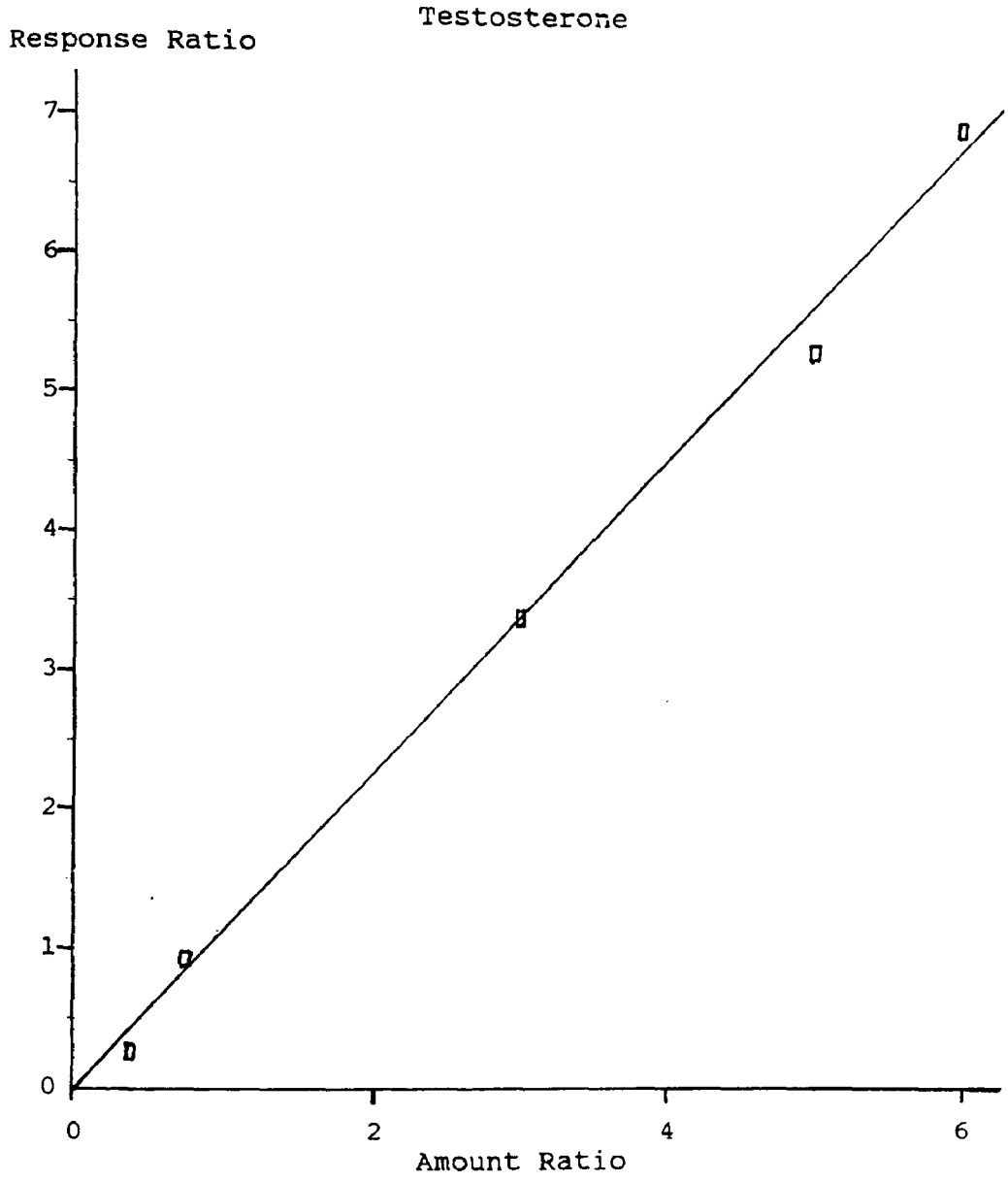


Fig 4.1.9 Calibration graph of standard solutions of testosterone used to establish recoveries by dynamic aqueous SFE derived from the quotient of response

$$\frac{[\,testosterone\,]}{[\,d^3 - testosterone\,]}$$

4.1.4 Quantification Results

To establish an extraction profile for testosterone, a standard solution was dynamically extracted, trapped and quantified at 20 min intervals. It was determined from this work that 96% of the testosterone was extracted over a 120 min extraction period.

Using the calibration data, and assuming 100% enzymatic hydrolysis of the testosterone-β-D-glucuronide, it was established that 61.7% of the theoretically available testosterone had been extracted after 120 min of dynamic SFE.

In order to establish the efficiency of the dynamic extraction process for the extraction of the liberated testosterone, it was necessary to quantify any remaining testosterone in the enzymatic digest.

The cooled contents of the extraction process were diluted with 100 cm^3 of de-ionised water. Two 200 cm^3 aliquots of ethyl acetate solvent were then added, originally 50 cm^3 had been used but found to be unsuccessful. The large volumes of organic and aqueous solvents were found to result in emulsion formation, which required the addition of sodium chloride to assist separation of the two phases.

The ethyl acetate solvent, after separation, was blown down to dryness under a stream of dry nitrogen gas, and the residue re-dissolved in 2.5 cm^3 of the $^2H_3$-testosterone internal standard. Quantification of this solution indicated that a further 8.4% of the liberated testosterone remain un-extracted in the enzymatic digest when extraction was terminated.

Taking into account the liberated testosterone which remained after the SFE was concluded, 8.4%, it was calculated that 70.1% testosterone-β-D-glucuronide had been enzymatically hydrolysed after 135 min, and that the dynamic SFE had been shown to be 88% efficient in isolating the liberated testosterone.

Further Studies and Conclusions

Although the study had provided satisfactory results a number of issues which may have affected testosterone recovery were considered;

a. Trapping efficiency of the testosterone on the ODS column?
b. Could testosterone-β-D-glucuronide be hydrolysed by dilute hydrochloric acid?
c. Could testosterone-β-D-glucuronide be extracted by dynamic supercritical fluid extraction without hydrolysis?

To investigate a. The ethyl acetate solvent, through which the carbon dioxide was exhausted, was quantitatively prepared and analysed, and showed no quantifiable levels of testosterone present after elution.

To investigate points b and c. A standard solution of testosterone-β-D-glucuronide was subjected to dynamic SFE for 120 min in the absence of β-glucuronidase, with an ODS HPLC column serving as the analyte trap.Analysis of the ODS trapping column ethyl acetate rinse solution, showed no quantifiable levels of liberated testosterone could be detected. This indicated that testosterone-β-D-glucuronide was stable to hydrolysis by dilute mineral acids.

The ethyl acetate solution was then "blown down" to dryness under a dry nitrogen gas stream, and re-dissolved in 10 cm^3 hydrochloric acid, pH 5.2. Then 50 μl of β-glucuronidase was then added and the solution incubated for 24 hours at 37 °C. The enzymatic digest was then extracted with two 20 cm^3 aliquots of ethyl acetate which were combined and " blown down " to dryness again under a dry nitrogen gas stream. The residue was then re-dissolved in 1 cm^3 of ethyl acetate and analysed as previous samples.

Since GC-MS SIM analysis failed to detect any testosterone, it was concluded that no testosterone-β-D-glucuronide had been extracted, i.e. the metabalite is too polar, making it unsuitable for isolation from aqueous matrices using SF-CO_2.

4.2 Direct Aqueous Supercritical Fluid Extraction of Organophosphorus Pesticides and Quantification by off-line Gas Chromatography- Mass Spectrometry

4.2.1 Introduction

Organophosphates were first discovered over 150 years ago, however their widespread use and synthesis did not start until the 1920s in Germany where they were used as insecticides, and chemical warfare agents. The use of organophosphate increased in the late 1950s as the use of organochlorine pesticides declined with concerns over their environmental effects.[27-31]

Organophosphates have also been, and continue to be, used therapeutically. They were used as early as 1870 to treat glaucoma, and in the 1930 for muscle disorders. In 1986 testing began on Alzheimer suffers, and organophosphates were released for clinical use in 1993, work continues on their use for general dementia disorders.[32]

Organophosphate insecticides share common structures and common mechanisms of toxicity, and some chemical properties (Fig 4.2.1).

Organophosphates contain a central phosphorus atom which is double bonded to either a sulphur or oxygen atom, attached alkyl groups (R1, R2) which are usually methyl or ethyl groups, they also have a leaving group which is specific to the individual organophosphate.

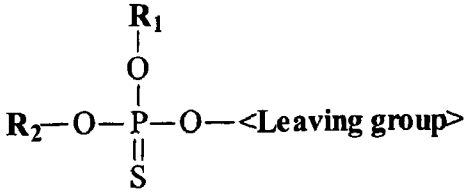


Fig 4.2.1 General structure of organophosphates

Organophosphates are generally highly lipid soluble and are easily absorbed through the skin, mucous membranes, and respiratory system.

Their toxicity[33-36] is related to their ability to inhibit the action of an enzyme in the nervous system. This enzyme, acetylcholinesterase, in turn deactivates the neurotransmitter acetylcholine. This neurotransmitter is present, and necessary, in different parts of the nervous system to enable transmission of stimuli between nerves, or between nerves and organs.

It is normally released in short sharp bursts which provide the required level of stimulation, with the effect of organophosphates inhibiting the acetylcholinesterase, the levels of acetylcholine increases, and therefore the level of stimulation increases, with increased nerve or organ response.

The various sites where acetylcholine is present as a neurotransmitter, and where abnormally high levels can disturb their normal function, can explain the various acute symptoms associated with OPPs. This includes the autonomic nervous which regulates the continuous and "automatic" functions of many organs and glands.

Acetylcholine is also present in the junction between nerves and muscles and in some synapses (nerve to nerve connections) in the brain. Increased levels in these areas cause increased response and leads to the resulting symptoms. These symptoms can include in mild exposure, headaches, weakness and dizziness, moderate exposure include vomiting and muscle tremors, and for severe exposure, convulsions and cardiac failure.

Organophosphates undergo chemical changes in the body and in the environment.
One change, which takes place in the liver, is the substitution on the double bond of the phosphorus from sulphur to oxygen. Example shown thionazin (Fig 4.2.2).

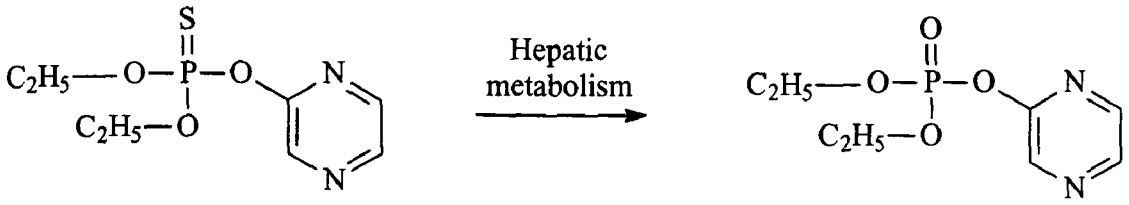


Fig 4.2.2 Hepatic metabolism of organophosphorus compounds (example thionazin)

This resultant activated metabolite is even more potent at inhibiting the cholinesterase enzyme than the parent organophosphate.

Another important metabolic route for the organophosphates in the body, and in the environment, is the hydrolysis and production of a dialkylphosphate and leaving group, thionazin again used as an example (Fig 4.2.3)

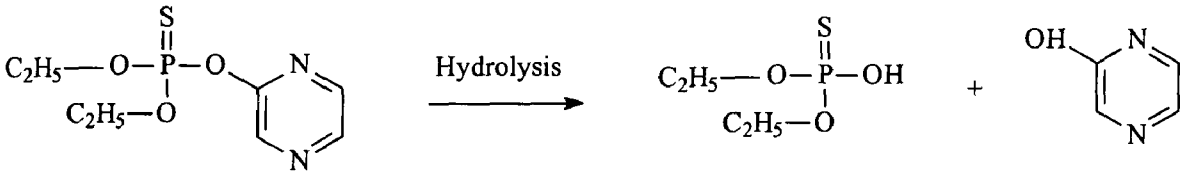
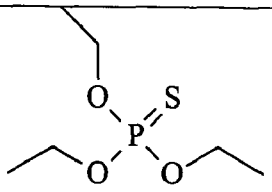
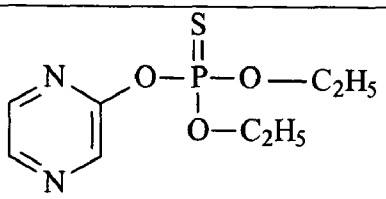
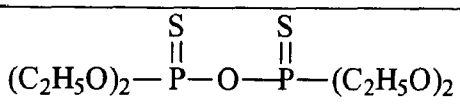
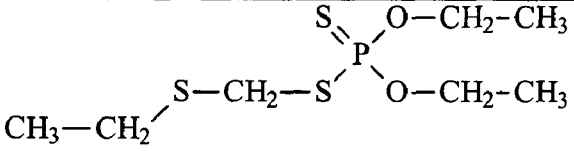
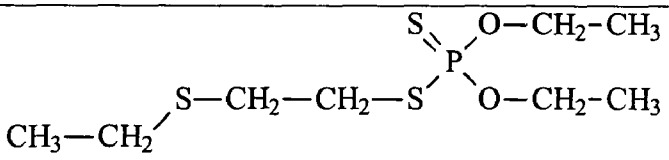
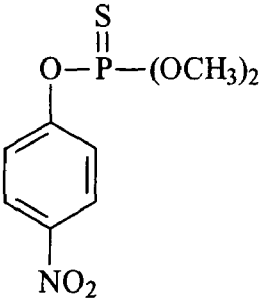
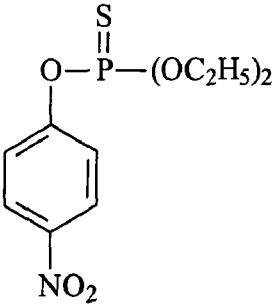


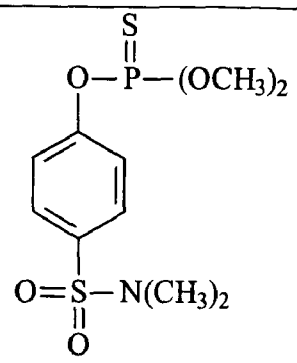
Fig 4.2.3 Hydrolysis of organophosphates (example thionazin)

This reaction results in a decrease in toxicity as the metabolite does not inhibit cholinesterase enzymes. These compound are eliminated from the body in the urine.

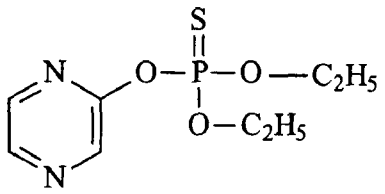
These compounds are used as biomarkers, detection and quantification can indicate the specific organophosphate exposure and exposure level.

Organophosphorus pesticides used in this work were;

OPP	Structure	Molecular Mass (M_r) g mol^{-1}
O,O,O-triethyl phosphorothioate		198.22
Thionazin		220.11
Sulfotepp		322.14
Phorate		260.22
Disulfoton		274.23
Methyl parathion		263.11
Ethyl parathion		291.13

Famphur		325.19

i. Thionazin (O,O-diethyl-O-(2-pyrazinyl)phosphorothioate



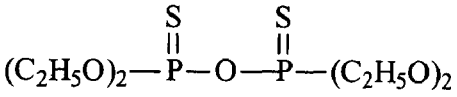
Thionazin is an insecticide which is effective against a wide range of plant parasites, including those that attack bulbs, buds, leaves and roots, as well as soil dwelling root maggots and aphids.

When released it is generally found atmospherically in the vapour phase, but is rapidly destroyed by reaction with photochemically produced hydroxyl radicals, and has a predicted half life of approximately 4 hours.

If released into the soil it is highly mobile, and the half-life of a 10 – 100 ppm concentration is of the order of 2-6 weeks, and the removal process includes degradation, hydrolysis, and leaching, but appears to be removed more rapidly from alkaline than acidic soils.

If released into water the degradation half-life has been experimentally determined at 30 days at pH 7 and 25 °C.

ii. Sulfotep (O,O,O,O-tetraethyl dithiopyrophosphate)



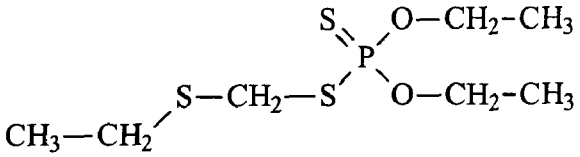
Sulfotep is an insecticide / miticide which has restricted use, and is not registered for any food or feed crops. It is registered for the control of ornamental plant pests, and is only registered for commercial greenhouse use, being formulated into an impregnated smoke fumigant.

Toxicological data shows sulfotep is a neurotoxin, and to be toxic to wildlife, fish and aquatic invertebrates, as well as birds, but as it is only registered for indoor use ecological exposure is expected to be minimal.

Because of the adverse toxic effects of sulfotep its manufacture was stopped in September 2002, and its use from September 2004. Laboratory test have shown sulfotep to have low mobility rates in soils, and because of its low volatility and stability is expected to have a long half-life, being fairly resistant to hydrolysis.

If released into water it has a model volatilization half life of 23 days, and hydrolysis is not expected to be a significant process. With atmospheric release sulfotep predominates in the vapour phase, and its half life to hydrolysis is calculated as 2.2 hours

iii. Phorate (O,O-diethyl S-ethylthiomethyl phosphorodithioate)



Phorate is an insecticide and acaricide, and is used to control sucking and chewing insects, and is used on root and field crops and also in pine forests.

Toxicological data shows that phorate is highly toxic via the oral, inhalation and dermal routes, being readily absorbed through the skin and gastrointestinal tract. The breakdown products of phorate in mammals are more toxic than the pesticide itself, and have greater anticholinesterase activity.

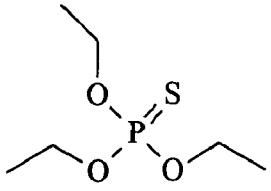
Phorate has long residence times in mammals, with its main target organ being the nervous system, liver, kidney, lung, brain and glandular tissue and has been shown to retain greater than 60 % of an administered dose, 6 days after administration.

Phorate is moderately persistent in soil with a reported representative half-life of 60 days, but differing soil conditions, ie. organic matter, pH, moisture content, can cause this value to vary between 2 and 173 days. The longer persistence in soil, and its moderate soubility in water, can lead to greater plant uptake, but no reported plant persistence has been demonstrated in field trials.

The half life of phorate in acidic waters, depending upon its temperature, can be between several days and several weeks, with the half life in basic waters being less.

Phorate can be biodegraded by microorganisms or by hydrolysis, producing non-toxic water soluble degradation products.

iv. O,O,O-triethyl phosphorothioate



O,O,O-triethyl phosphorothioate is an insecticide used to kill a wide range of mites and insects on contact, and has been used on a wide range of vegetation including fruit and

flowers. It has also been used as a residual spray for farm buildings against house flies, and administered directly onto cattle to control botflies.

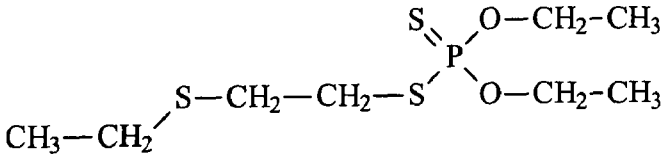
It is moderately toxic by ingestion, inhalation and dermal absorbtion, and as is common with all organophosphates readily absorbed through the skin.

O,O,O-triethyl phosphorothioate is biodegradable , and undergoes rapid degradation in the environment. It is highly soluble in water and therefore may be subject to considerable leaching. It is degraded by hydrolysis, especially in alkaline soils, reported 77% degradation in 2 weeks, and will evaporate from dry soils surfaces, up to 40% in 2 weeks.

It has low persistence in soil and representative half-lives are reported as between 2.5 to 16 days, depending on conditions.

In water it is subject to significant hydrolysis, especially in alklaline conditions, with half-life values of 3.7 days at pH 9, and 118 days at pH 7.

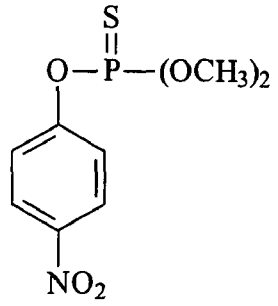
v. Disulfoton (O,O-diethyl S-2-ethylthioethyl phosphorodithioate)

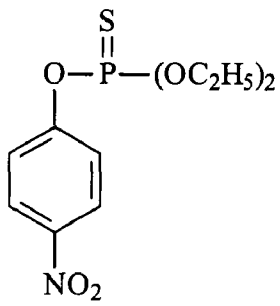


Disulfoton is a selective organophosphate pesticide particularly targeting sucking insects. It is highly toxic to all mammals by all routes of exposure, and is rapidly absorbed through the skin. Like all organophosphates it inhibits cholinesterase, and particularly affects the respiratory and central nervous system, with complete recovery from acute poisoning taking a minimum of 7 days.

When applied to the soil disulfoton is strongly bound, and is actively taken up by the plant roots and distributed throughout the plant. Mobility decreases as organic content increases, and metabolites can persist longer than disulfoton, and is rapidly hydrolysed in alkaline water.

vi. Methyl Parathion (O,O-dimethyl O-4-nitrophenyl phosphorothioate)
vii. Ethyl Parathion (O,O-diethyl O-4-nitrophenyl phosphorothioate)





Methyl and ethyl parathion are both insecticides used at controlling both sucking and biting pests on agricultural crops. They are highly toxic via the oral and dermal routes. The effects have been studied in human volunteers, with mild cholinesterase inhibition being seen in dosage levels above 24 mg day^{-1} over a four week period.

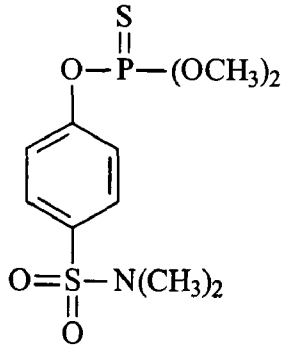
The parathions are rapidly absorbed into the blood stream, with maximum blood concentration appearing between 1 – 2 hours after administration. They do not accumulate in the body but are excreted within 24 hours as the phenolic metabolites.

Parathiones have low persistence in soil with reported half-lives of 1 – 30 days, with a typical value being 5 days, with the rate of degradation being increased by increased temperature and sunlight.

They degrade rapidly in water with total degradation occurring within 2 to 4 weeks, where photolysis is the main breakdown route.
Uptake in plants is rapid, with total metabolism being achieved in about 4 days.

Due to increasing concerns about health issues associated with parathions, their use was banned from May 2001.

viii. Famphur
(4-dimethoxyphosphinothioyloxy-*N*,*N*-dimethylbenzenesulfonamide)



Famphur is used to control grubs, lice and flies, and is directly applied as a solution onto cattle.

In soil famphur has moderate mobility, and may be absorbed into the soil, and will undergo slow hydrolysis in moist conditions.

In water famphur hydrolyses with an experimental half-life of 115 days under neutral conditions, while only 60 days under basic conditions.

4.2.2 Direct Aqueous Supercritical Fluid Extraction Studies of Organophosphorus Pesticides

For extraction of the OPP mixture a micro extraction system, with an internal volume of 25 cm^3, was assembled as shown in Fig 4.2.4.

The inlet to the micro extraction vessel was connected to a Gilson 307 reciprocating pump fitted with a supercritical fluid compatible piston seal, via a Rheodyne 7010 valve, using high pressure 1/16 inch o.d. stainless steel tubing and fittings. The pump head cooling jacket maintained the head at −10 °C, and ensured the liquid carbon dioxide remains liquefied allowing efficient delivery. The 1/16 inch o.d. stainless steel tubing carrying the carbon dioxide to the pump from the cylinder was coiled in a Gilson HPLC column oven maintained at 100 °C.

The configuration of the Rheodyne valve allowed the carbon dioxide to be delivered either to the bottom of the vessel, or via the top and through the 1/16 inch o.d. delivery tube and into the sample.

The output from the extractor was connected to a water trap, which isolated any water aerosol that may be carried from the sample in the carbon dioxide stream. A Druck pressure transducer was connected to the output of the trap, and connected to a Tescom® pressure display unit, and allowed the extraction pressure within the extraction vessel to be constantly monitored.

The output from the pressure transducer was connected to a second Rheodyne 7010 valve, which allowed the carbon dioxide to be exhausted through a linear silica tube back pressure restrictor, and into a flask containing ethyl ethanoate which was used for the analyte solvent trap.

The OPP standard solution was purchased from Supelco, and consisted of each of the eight OPPs at a concentration of 2000 μg cm^{-3} in an 80:20 hexane : propanone solvent mixture.

The commercial 1 cm^3 standard vial of OPP solution was further diluted by dissolution of the contents quantitatively in 50 cm^3 of ethanol (standard solution). This standard solution was further quantitatively diluted 1:100 in ethanol for use in selective ion monitoring (SIM) .

The extraction vessel was thoroughly cleaned and a 5 cm^3 glass vial loaded with the extraction solution. The extraction solution consisted of 3.5 cm^3 de-ionised water and 100 μl of the OPP standard solution (1:50 dilution of commercial standard). The vial was placed inside the extraction vessel, loading from the bottom, and allowed the stainless steel delivery tube to protrude into the solution. The vessel was then sealed.

Valve D was configured to allow the carbon dioxide to enter via the bottom of the extraction vessel which, on reaching the target extraction pressure, was switched to direct the carbon dioxide through the extraction solution. This was developed to help contain the extraction solution within the glass vial during the pressurisation phase of the extraction. Initial extraction procedures introduced the carbon dioxide from the top through the solution, and this occasionally resulted in the initial surge of the gas forcing the solution from the vial.

The back pressure, and hence the supercritical carbon dioxide density, was regulated by the use of a length of silica tubing, which was carefully manufactured to give a back pressure of 3100 psi, which was monitored by the pressure transducer at the output of the vessel. The output end of this restrictor was immersed in a 10 cm^3 volumetric flask containing ethyl ethanoate.

The carbon dioxide delivery pump flow rate was set at 3 cm^3 min^{-1} with the initial extractions carried out over a 30 min period after the extraction pressure limit was reached.

4.2.3 Experimental Results

Analysis and quantitation of the standard and extracted organophosphates was completed using a Hewlett-Packard 5890 gas chromatograph equipped with an HP-5MS 30 m x 0.3 mm internal diameter column of 0.25 μm film thickness. Interfaced to the GC system was a Hewlett-Packard 5971A MSD (Mass Spectrometric Detector), this analytical arrangement was used for all the analyses, and all were completed using high purity helium as the carrier gas.

Before the analytical phase of the extraction was attempted, the standard solution containing the eight organophosphates was chromatographically, and mass spectrometrically analysed, and the individual mass fragmentation patterns of the individual analytes determined.
This was completed to allow identifiable mass peaks to be resolved for selective ion monitoring, which would allow increased sensitivity when analyzing individual anlayte components.

The following sequence of chromatograms and mass spectra were completed for the individual organophosphates.

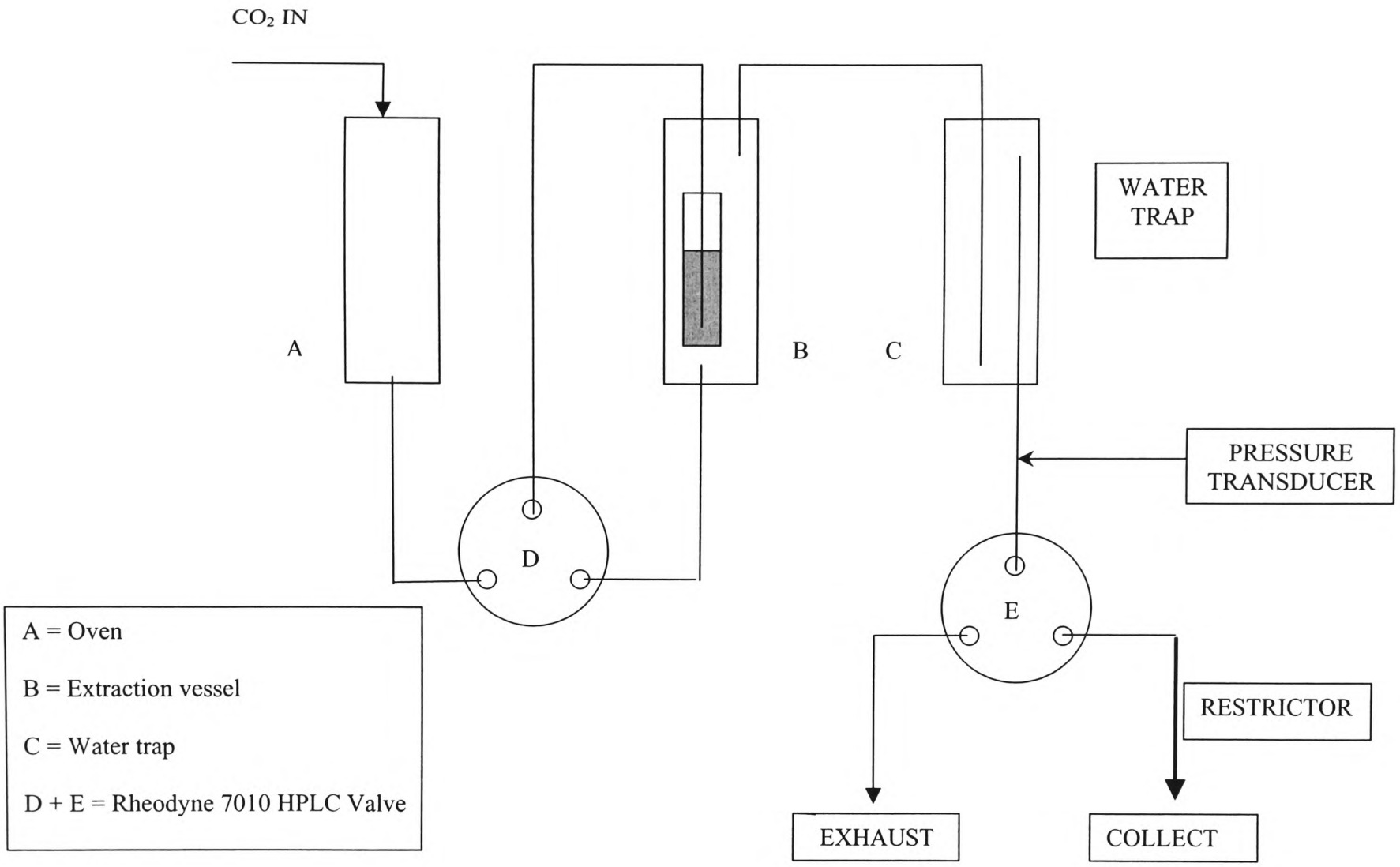


Fig 4.2.4 schematic of Supercritical Fluid Extraction (SFE) system

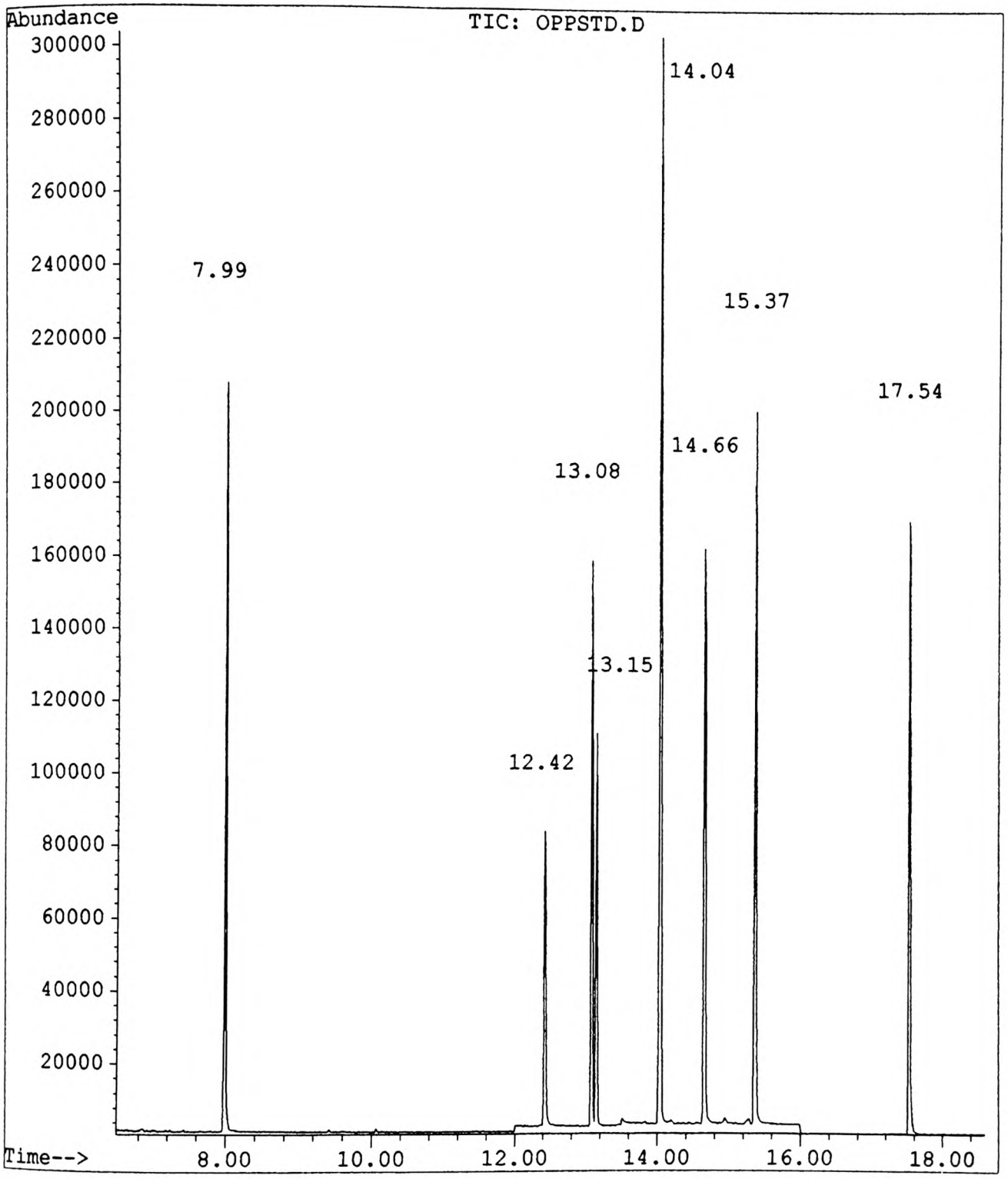


Fig 4.2.5 GC-MS profile of the OPP standard mixture

Organophosphate	Retention time (min)
O,O,O-triethyl phosphorothioate	7.99
Thionazin	12.42
Sulfotepp	13.08
Phorate	13.15
Disulfoton	14.04
Methyl parathion	14.66
Ethyl parathion	15.37
Famphur	17.54

Full Scan EI Mass Spectra of Organophosphates

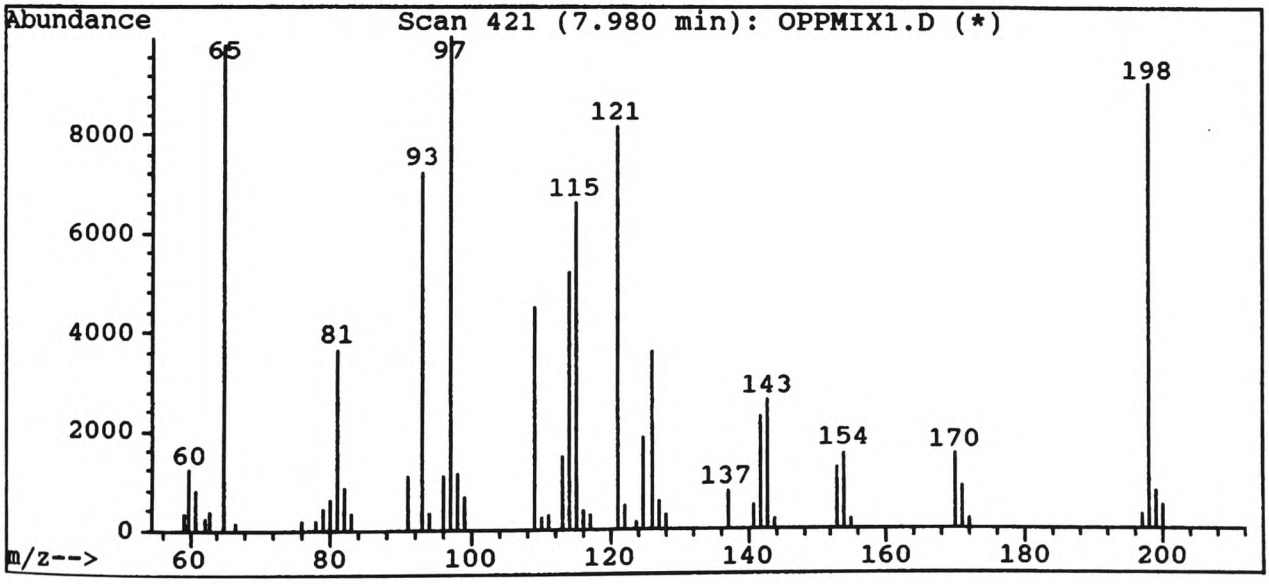


Fig 4.2.6 O,O,O-triethyl phosphorothioate

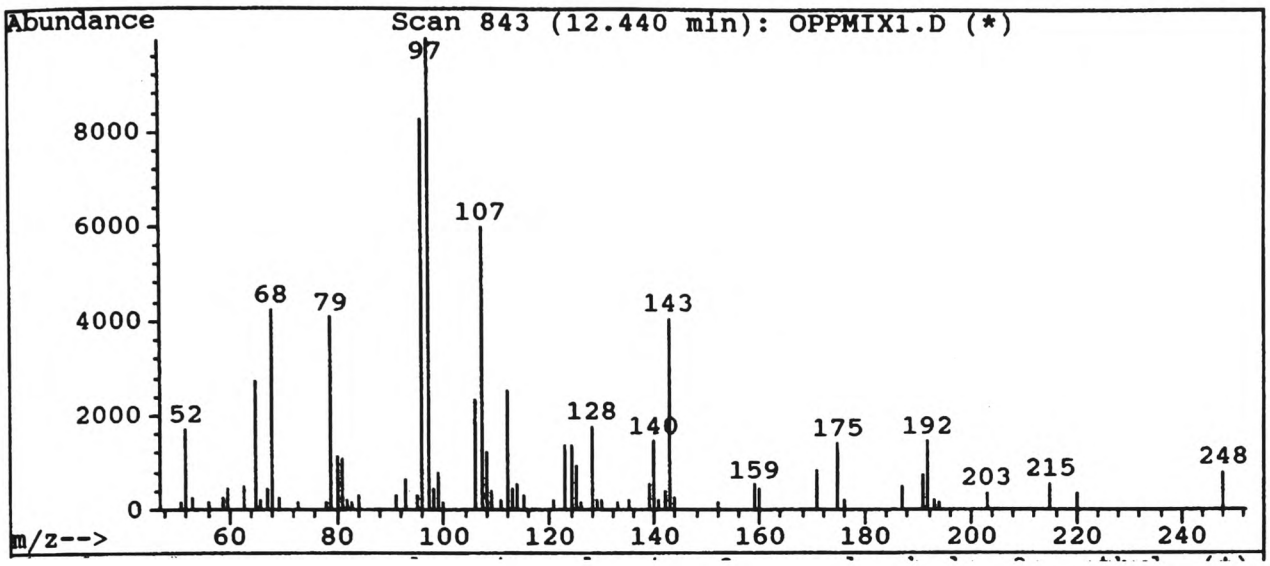


Fig 4.2.7 Thionazin

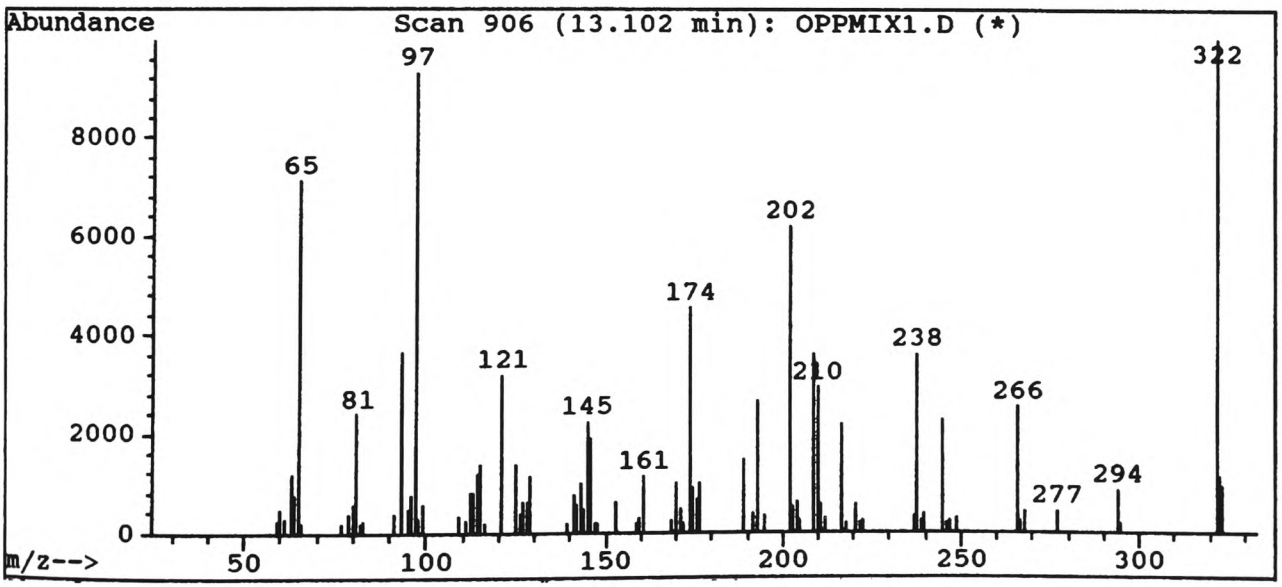


Fig 4.2.8 Sulfotepp

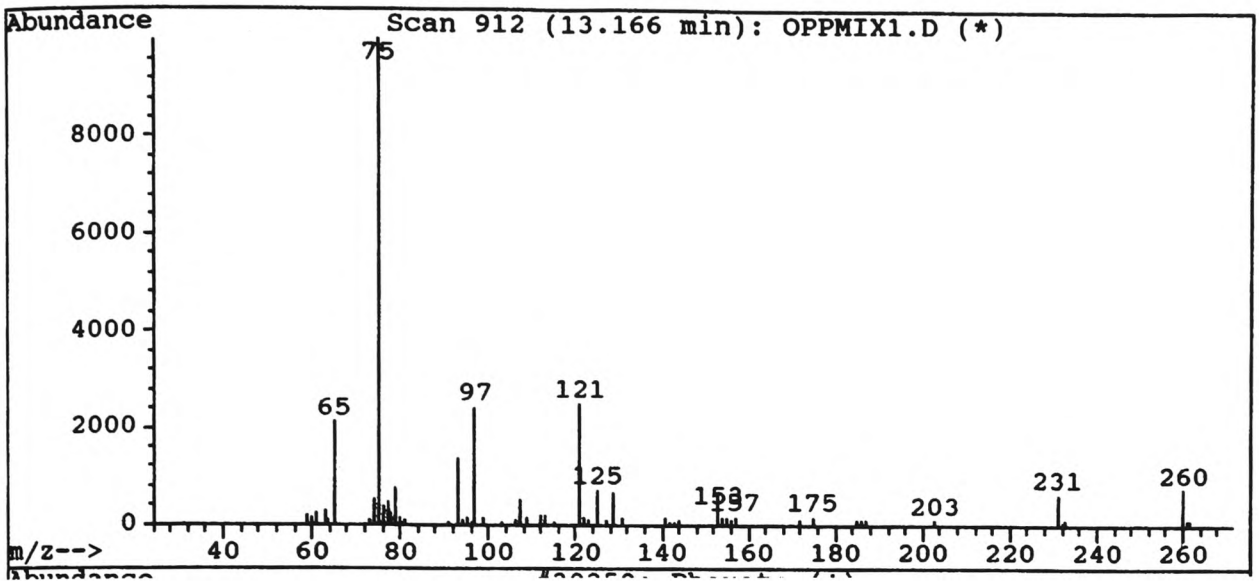


Fig 4.2.9 Phorate

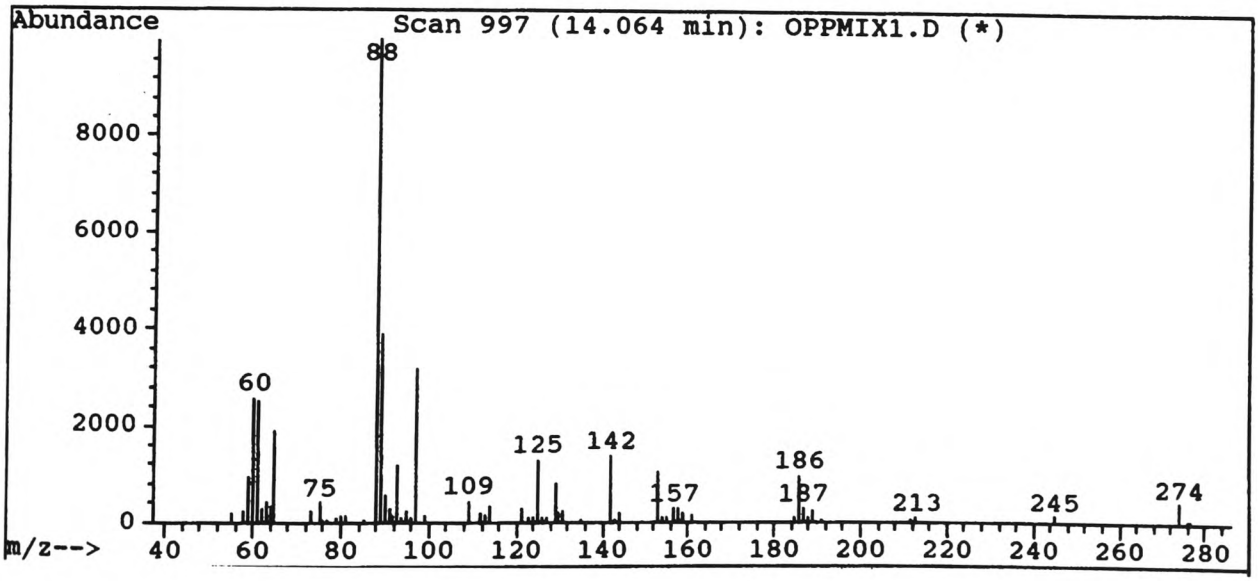


Fig 4.2.10 Disulfoton

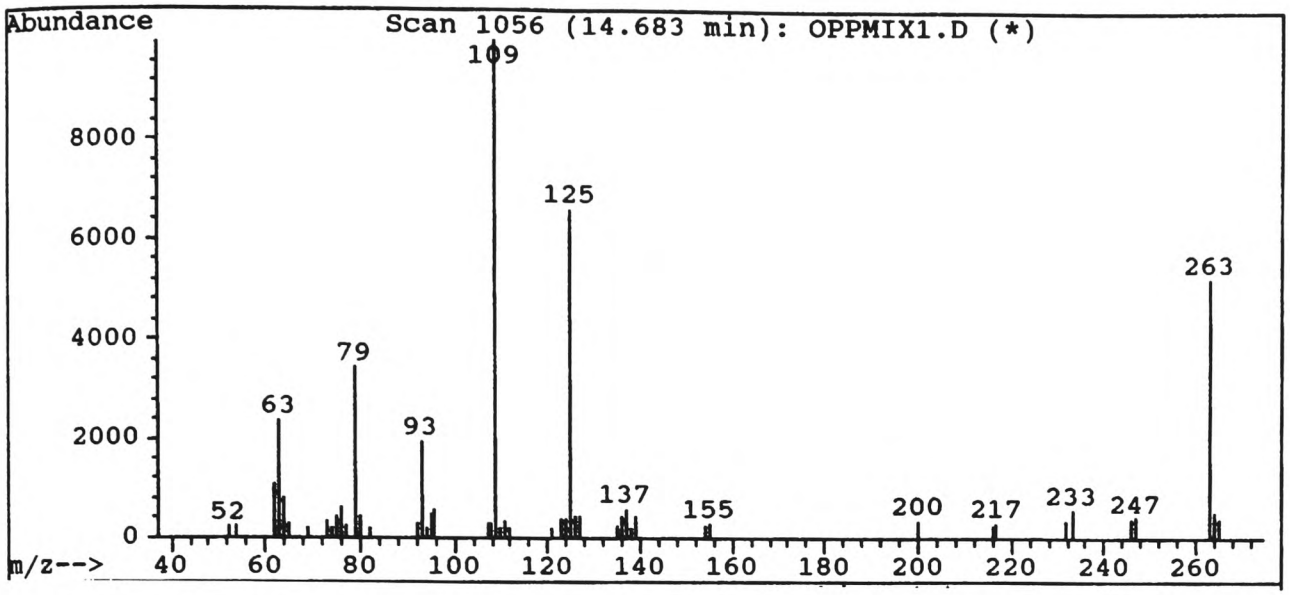


Fig 4.2.11 Methyl Parathion

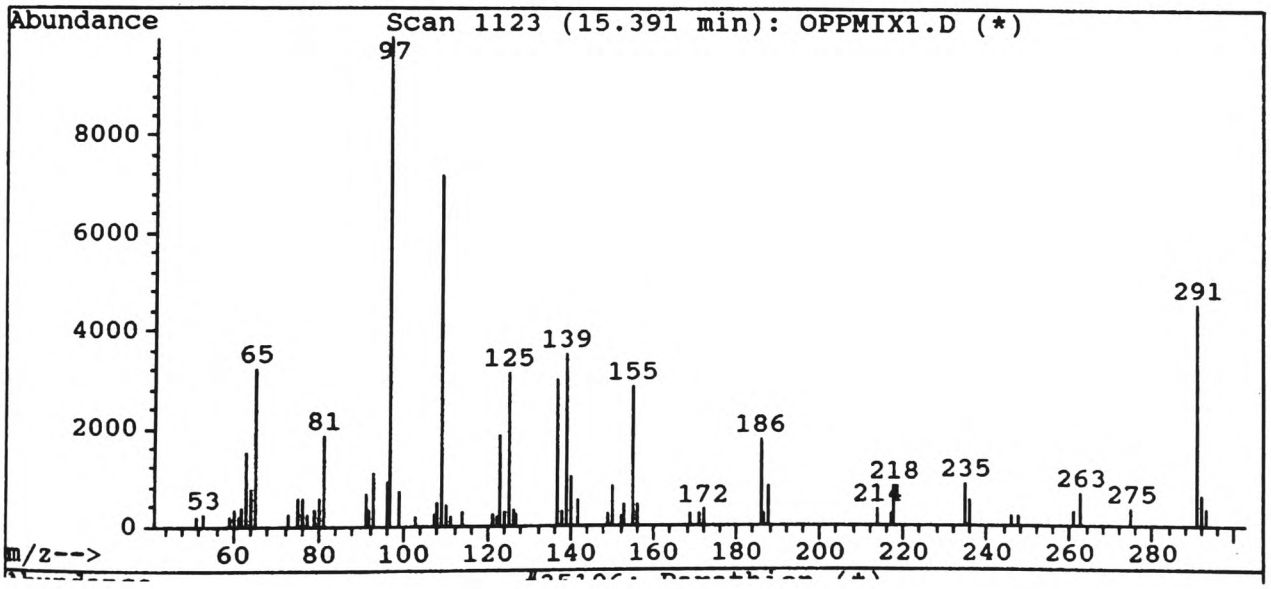


Fig 4.2.12 Ethyl Parathion

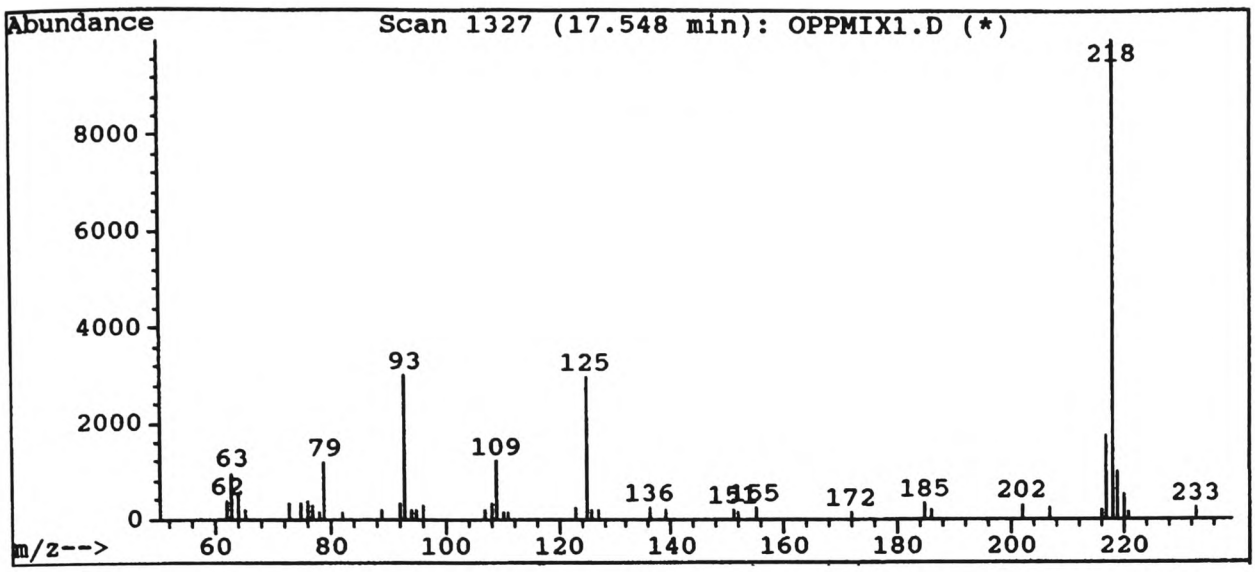


Fig 4.2.13 Famphur

Analysis of these spectra resulted in the following ions and qualifier ions being selected for the identification of the analytes.

Organophosphate	Selected ions	Qualifier ions
O,O,O-triethyl phosphorothioate	198	97 , 121,
Thionazin	192	107, 143,
Sulfotepp	322	202,
Phorate	260	97, 121,
Disulfoton	186	88, 142,
Methyl parathion	263	109, 125,
Ethyl parathion	291	139, 186,
Famphur	218	125,

To increase the sensitivity further for the middle six eluted analytes, thionazin to ethyl parathion, which gave the lowest responses, the gain on the GC-MS electron multiplier was increased

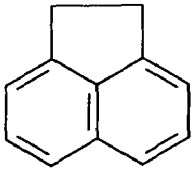
within the region 12 –13.5 min, then further increased between 13.51 - 16 min, and then reset to the initial value after this point. This can clearly be seen on the chromatograms (e.g. Fig 4.2.14) as a series of steps on the baseline.

Group Number	Time Period (min)	Ions in group
1	3.5 - 12	198, 121, 97
2	12 – 13.5	192, 143, 107, 322, 202, 260, 97, 121
3	13.51 - 16	186, 142, 88, 263, 125, 109, 291, 186, 139
4	16 – to end	218, 125

The initial extraction was completed over a 30 min period, after the pre-selected extraction pressure was reached. The ethyl ethanoate analyte solution, was then blown down to dryness under a dry nitrogen stream, and re-dissolved in 500 μl of ethanol, before GC-MS analyses were undertaken. This trial was run to ensure that all the organophosphates were extractable under the selected conditions (Fig 4.2.14).

This extraction confirmed that all eight organophosphates were extractable under the selected conditions, but in order to quantify the extraction efficiency an internal standard was required. This would be added to the extracted analytes as they were being volumetrically prepared, and give a reference against which all the individual components could be quantified.

Acenaphthene was chosen as the internal standard for GC-MS quantification, as this had a retention time within the range of the organophosphates, was thermally labile, available in high purity, and readily soluble in the selected solvent. The I.S. solution contained 3.40 mg in 50 cm^3 ethanol. A GC-MS chromatogram showing the addition of acenaphthene to the standard OPPs standard mixture is shown in Fig 4.2.15.



Acenaphthene $M_r = 154.21\ g\ mol^{-1}$

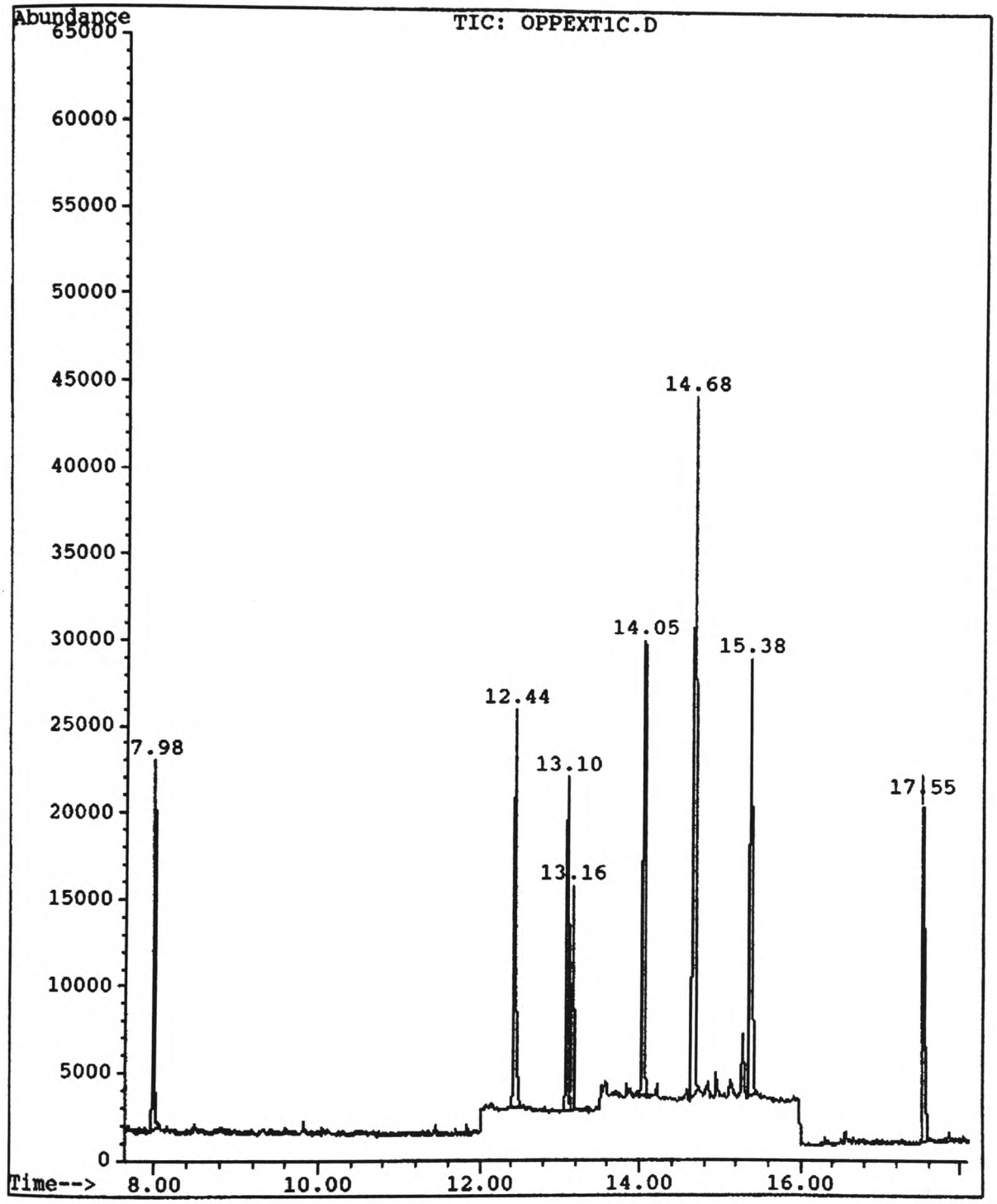


Fig 4.2.14 Trapped analytes after 30 minutes of dynamic SF-extraction of a 3.5 cm^3 aqueous solution of the OPPs mixture

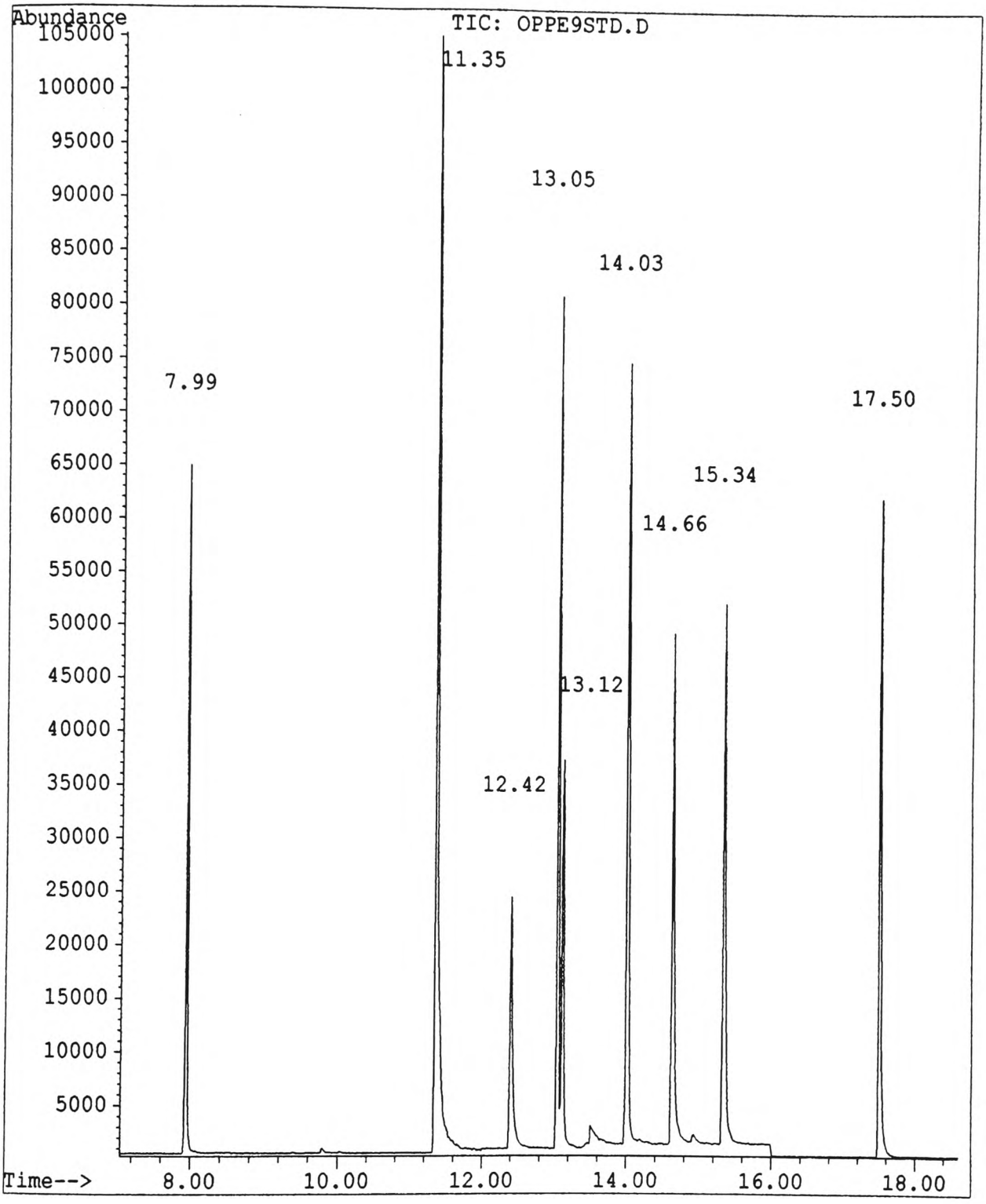
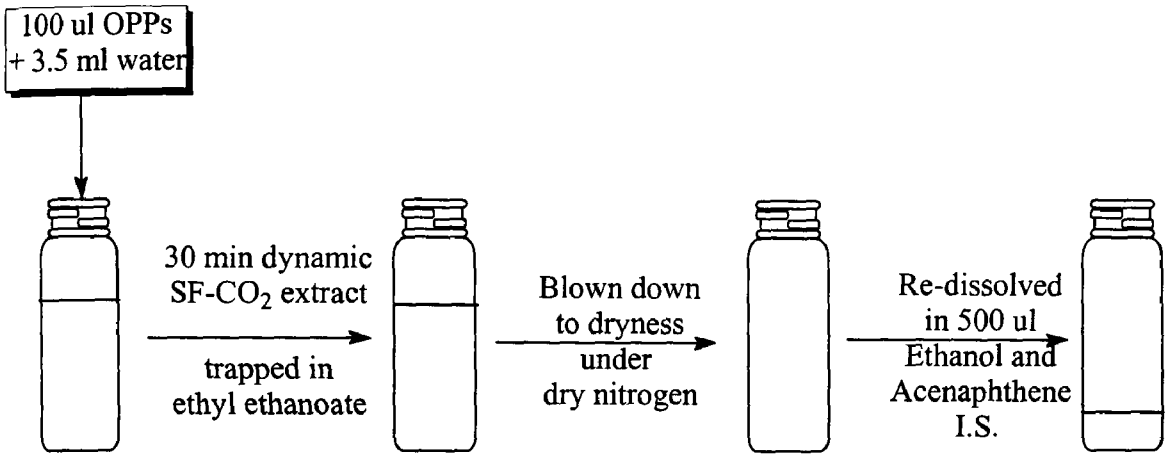


Fig 4.2.15 Organophosphates and Acenaphthene Internal Standard (R.T. = 11.35 min)

Further extractions were carried out with 60 minute extraction periods, with no observed increase in extraction efficiency. It was therefore concluded that the extraction was complete in the initial 30 minute period.



When the extracted samples were quantified against the non-extracted standard it was observed that the percentage of organophosphates being detected were variable. The table of results below show the mean of 3 extractions.

Organophosphate	Retention Time (min)	% Detected by GC/MS
O,O,O-triethyl phosphorothioate	7.99	48.2
Thionazin	12.42	46.3
Sulfotepp	13.08	44.6
Phorate	13.15	49.4
Disulfoton	14.04	56.8
Methyl parathion	14.66	44.8
Ethyl parathion	15.37	31.2
Famphur	17.54	22.4

The extraction and trapping processes now needed to be examined to establish where the losses were occurring.

Parameters considered whch could be giving rise to low OPPs recoveries were:

a. Not extracting efficiently
b. Losing OPPs during the blowing down phase
c. The OPPs were not re-dissolving during the addition of the ethanol
d. Losing OPPs during the trapping phase,

For **a.** the SF-CO_2 extracted OPP solution was back extracted with ethyl ethanoate, blown down and re-dissolved in a small volume of ethyl ethanoate before being analysed (Fig 4.2.16). Essentially this result indicated that no OPPs had been left in the aqueous solution.

This would indicate the OPPs had been extracted by the SF-CO_2 under these conditions, with the only OPP just being detected in the chromatogram being ethyl parathion (R.T. = 15.3 min).

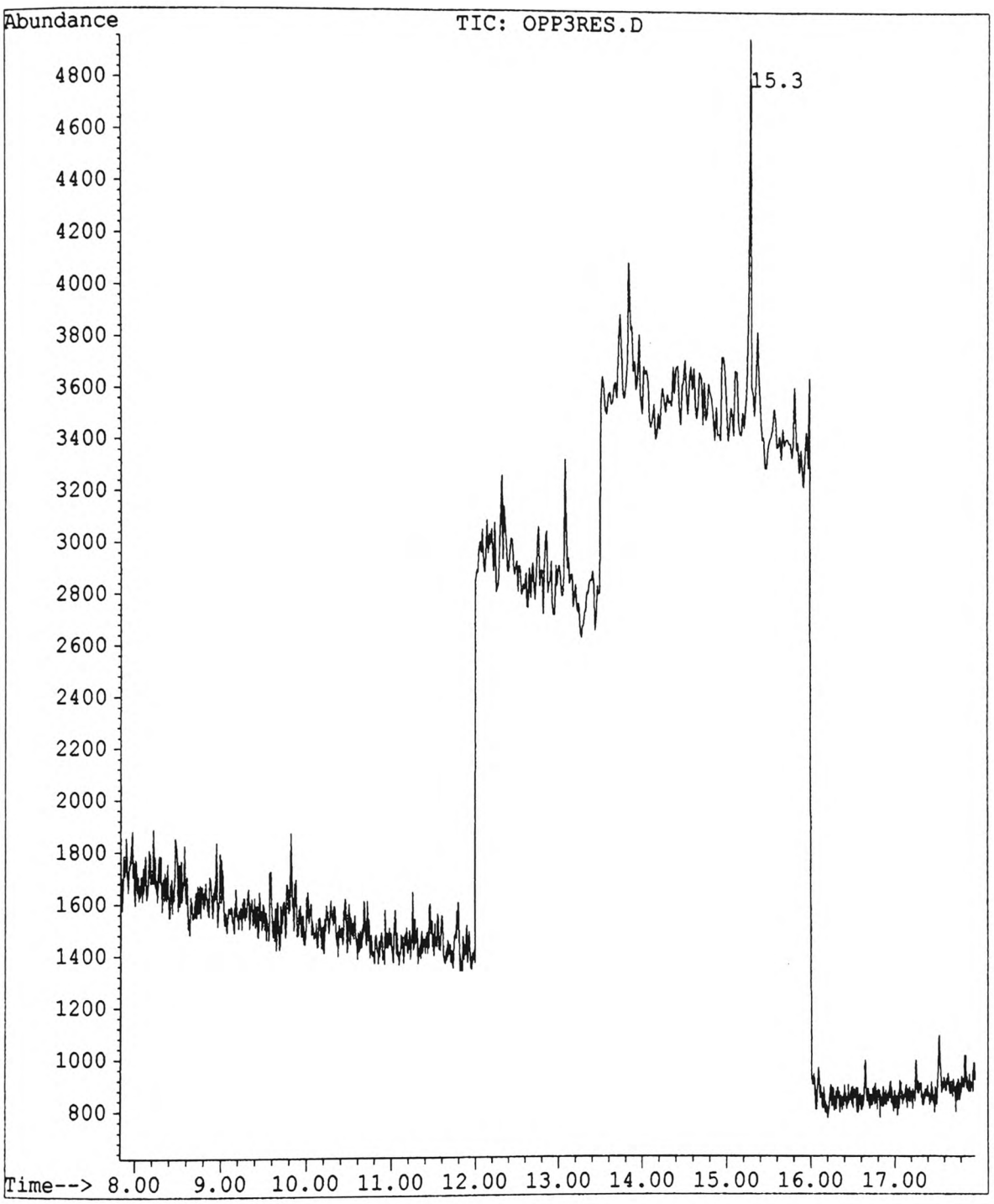


Fig 4.2.16 Solvent back extraction of SF CO_2 extracted sample

Concerning point **b.** A sample of the extracted and trapped OPP solution which had been blown down to dryness and then re-dissolved in ethanol (Fig 4.2.17), was compared to the identical aliquot of the solution which was not blown down. The quantities of OPPs in each solution were then quantified.

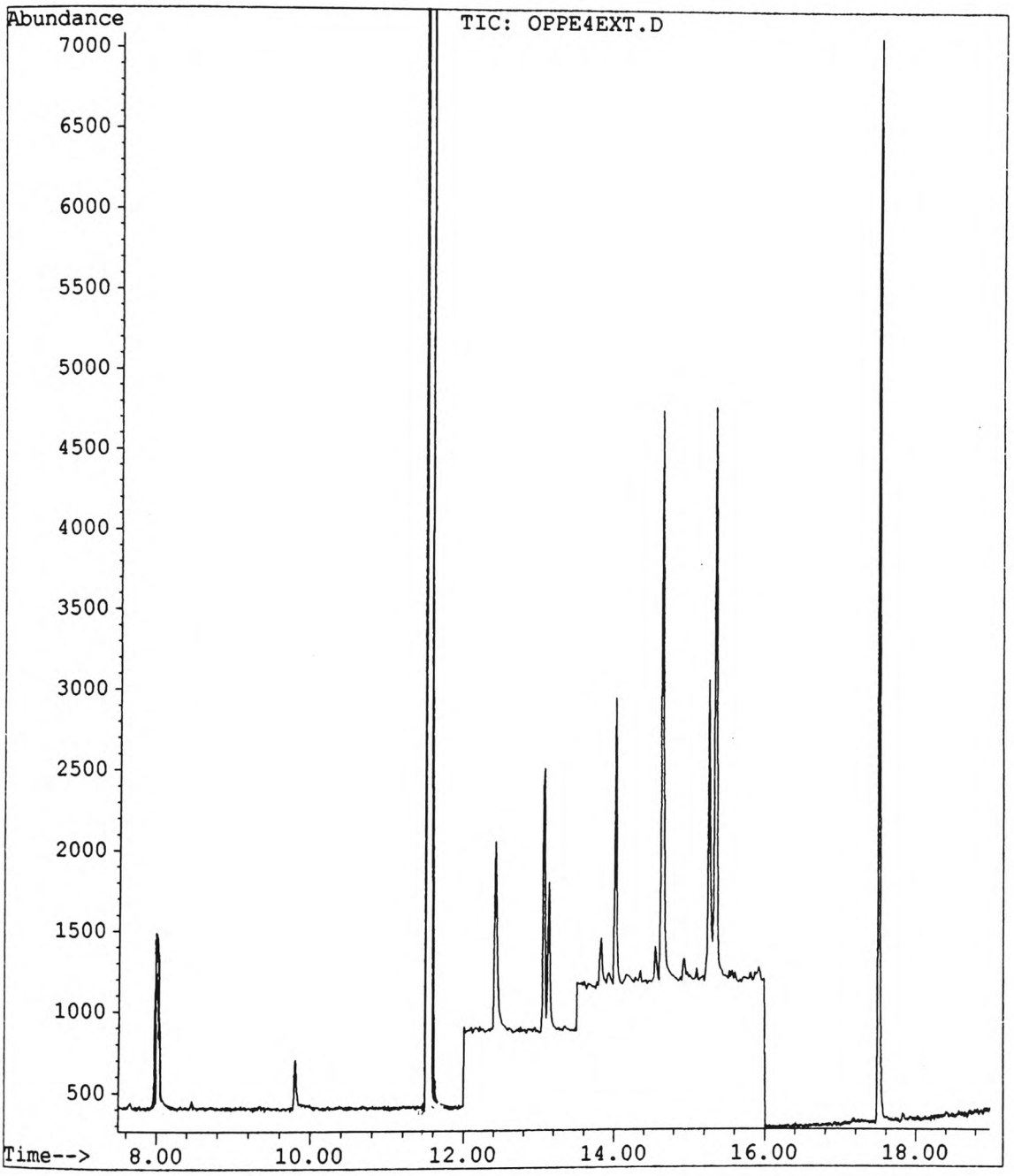


Fig 4.2.17 Re-dissolved "blown down" OPP standard solution

From the quantification it could be seen that the losses of the OPPs became less as their retention time increased. The table shows the mean results of 3 samples

Organophosphate	Retention Time (min)	% Loss due to blowing down to dryness
O,O,O-triethyl phosphorothioate	7.99	36.20
Thionazin	12.42	25.81
Sulfotepp	13.08	24.42
Phorate	13.15	24.14
Disulfoton	14.04	16.55
Methyl parathion	14.66	10.67
Ethyl parathion	15.37	10.15
Famphur	17.54	6.71

These results would seem to be reasonable as they follow the respective volatilities of the OPPs, with the lowest retention time indicative of the highest volatility and therefore the greatest loss due to blowing down.

Concerning point c. The collection vial after the analytes were re-dissolved in ethanol, was then re-extracted with ethyl ethanoate, this would indicate whether any of the analytes were left undisolved by the ethanol (Fig 4.2.18).
This chromatograph shows that Famphur (R.T. = 17.53) was the least soluble of all the OPPs and that the majority are efficiently solubilised by ethanol.

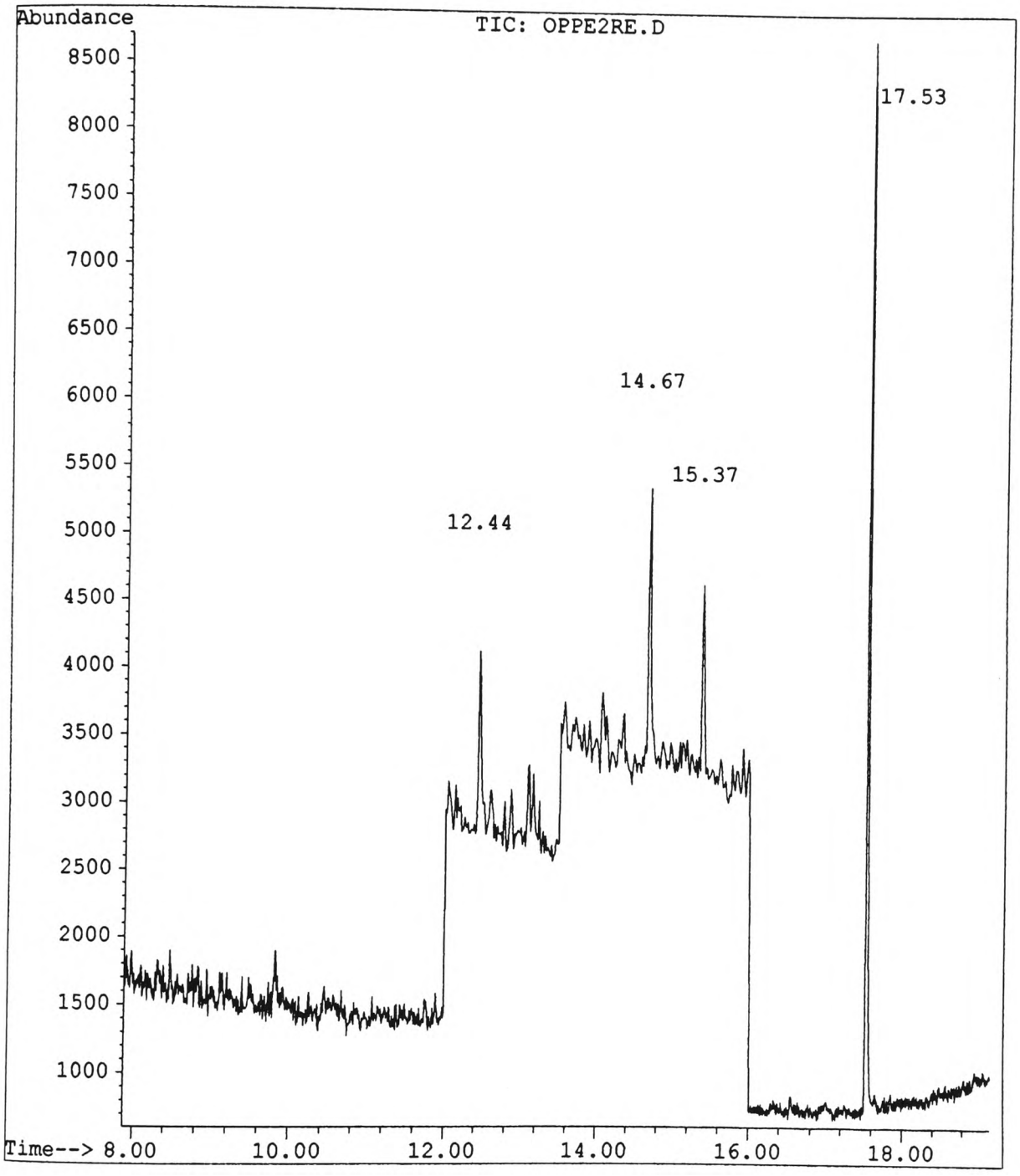


Fig 4.2.18 OPP extracted collection vial re-extracted with ethyl ethanoate

The table below shows the mean results of 3 samples

Organophosphate	Retention Time (min)	% Loss due to re-dissolving in Ethanol
O,O,O-triethyl phosphorothioate	7.99	1.5
Thionazin	12.42	21.6
Sulfotepp	13.08	10.8
Phorate	13.15	9.2
Disulfoton	14.04	8.6
Methyl parathion	14.66	36.7
Ethyl parathion	15.37	38.0
Famphur	17.54	67.2

On quantitation is was calculated that 67.2% of the Famphur remained undisolved in the collection vial.

Concerning point **d.** The solvent trap was replaced by a Phenomenex Lun r 5u 150 x 4.60 mm 5 micron C18 HPLC column. This was designed to investigate whether the OPPs were retained in the solvent trap. This was achieved by connecting the outlet of the silica restrictor to the inlet of the column. The SF carbon dioxide and OPP analytes were decompressed onto the column and trapped.

On completion of the extraction period, the system was allowed to de-pressurise before the column was removed and washed with ethyl ethanoate to elute the analytes, and the internal standard quantitatively added, prior to the combined solutions being volumetrically made up.

Because it was uncertain what volume of solvent was needed to completely elute the analytes from the column, sequential 1 cm^3 volumes were used and collected for analysis. (Figs 4.2.19 & 4.2.20)

The chromatograms clearly show that the OPPs are effectively eluted with 1 cm^3 of ethyl ethanoate.

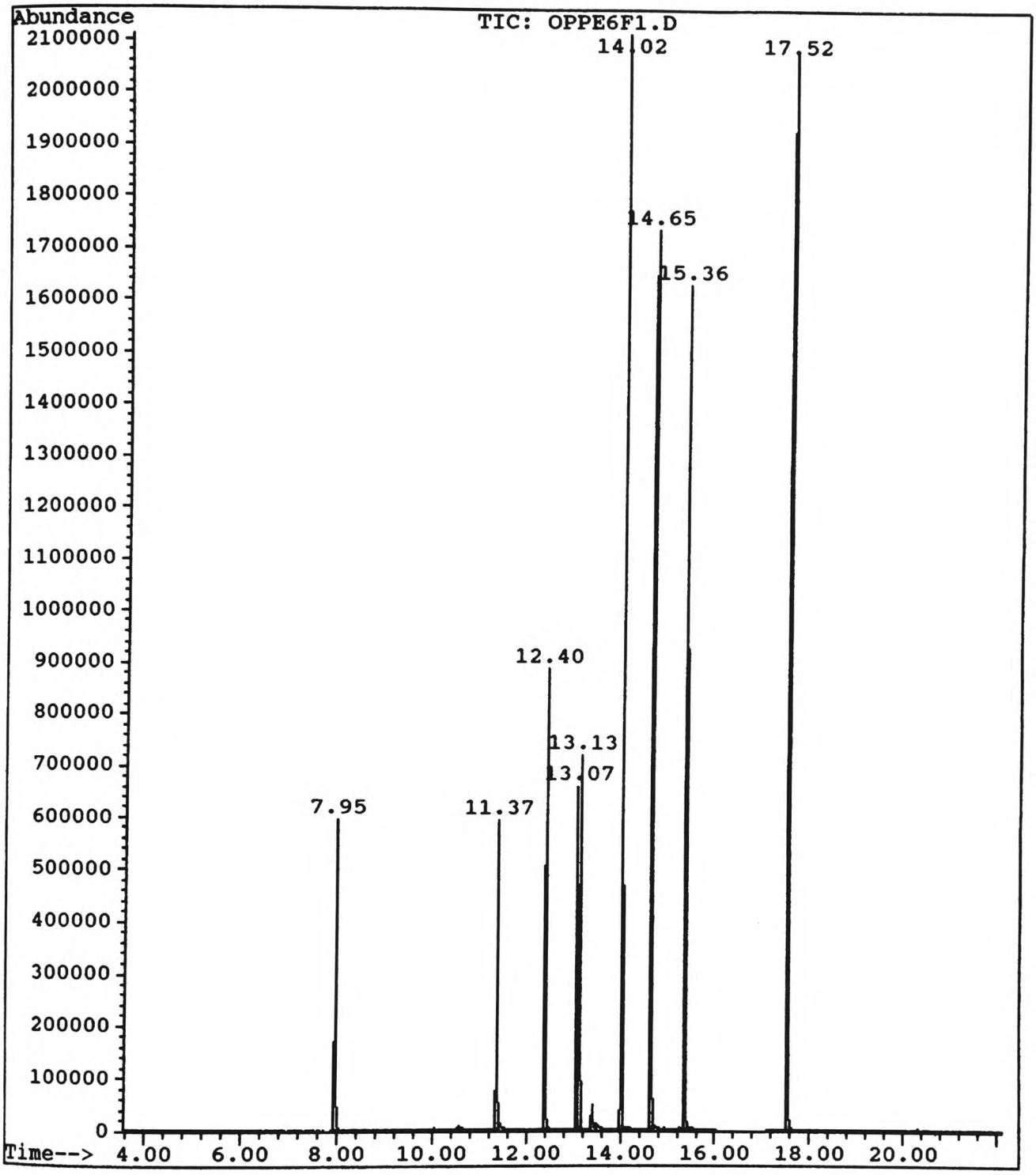


Fig 4.2.19 GC-MS SIM analysis for the first fraction (1 cm^3) C18 trapping column wash

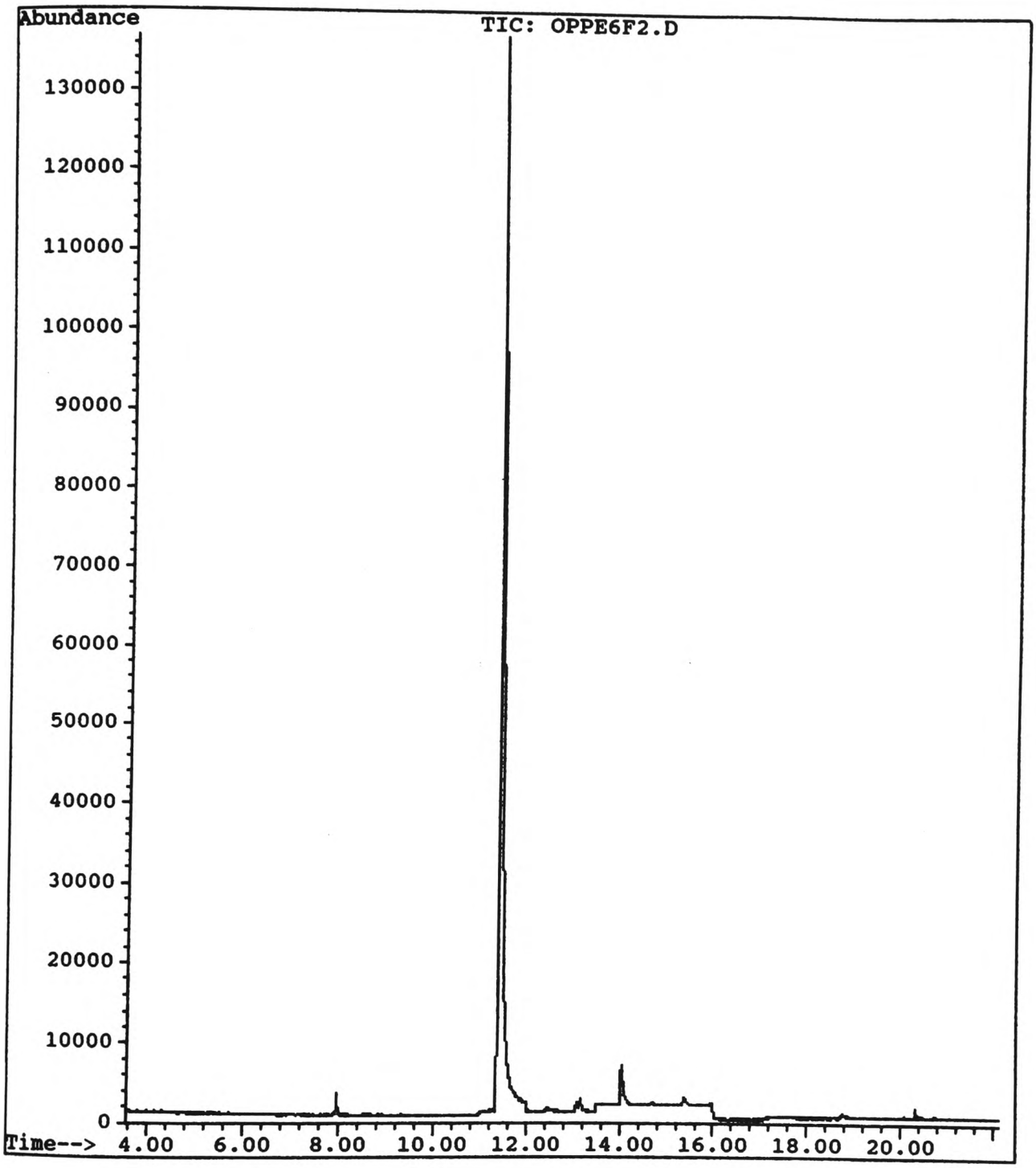


Fig 4.2. 20 GC-MS SIM analysis for the second fraction (1 cm^3) C18 trapping column wash. Only acenaphthene I.S. seen

When quantified and the mean calculated for 3 extractions, the following values were obtained

Organophosphate	Retention Time (min)	% Detected using C18 column as trap
O,O,O-triethyl phosphorothioate	7.99	72.89
Thionazin	12.42	74.86
Sulfotepp	13.08	75.56
Phorate	13.15	70.24
Disulfoton	14.04	83.38
Methyl parathion	14.66	73.15
Ethyl parathion	15.37	79.30
Famphur	17.54	88.72

Summary of Results

A summary of the results obtained for the detected quantities and the losses is shown below. Also included are the quantitative results for trapping using a C18 column.

It can be seen that the lowest recovery was for ethyl parathion, which showed the largest peak in the residual chromatogram (Fig 4.2.16).

The highest level of recovery was for famphur, which showed the highest loses on re-disolving in ethanol. Clearly it can now be see that ethanol was not a good choice of solvent for this compound.

When the initial detection levels, using the solvent trap, are compared with the C18 column, the discrepancy between them appears great, but once the identified losses are included, the values are not that different. It would seem reasonable to summise that if further work was to be attempted a C18 column trap should give enhanced results.

Organophosphate	Retention Time (min)	% Detected by GC-MS	% Loss due to blowing down to dryness	% Loss due to re-dissolving in Ethanol	Total quantities of OPPs extracted	% Detected using C18 column as trap
O,O,O-triethyl phosphorothioate	7.99	48.2	36.20	1.5	85.9	72.89
Thionazin	12.42	46.3	25.81	21.6	93.7	74.86
Sulfotepp	13.08	44.6	24.42	10.8	79.8	75.56
Phorate	13.15	49.4	24.14	9.2	82.7	70.24
Disulfoton	14.04	56.8	16.55	8.6	82.0	83.38
Methyl parathion	14.66	44.8	10.67	36.7	92.2	73.15
Ethyl parathion	15.37	31.2	10.15	38.0	79.4	79.30
Famphur	17.54	22.4	6.71	67.2	96.3	88.72

It can also be observed from the results, that none of the OPPs were quantified to give 100% recoveries. This possibly arises from the fact that mean values were used in the calculations, and some of the losses were not quantified, i.e. the residual losses.

Also the silica restrictor was difficult to control, particularly with reference to the end immersed in the solvent, which periodically blocked due to icing caused by the cooling effect of the depressurising carbon dioxide. This made extraction difficult to replicate.

4.3 Direct Aqueous Supercritical Fluid Extraction of Polyaromatic Hydrocarbons and Quantitation by off-line Gas Chromatography-Mass Spectrometry

4.3.1 Introduction

Polyaromatic hydrocarbons (PAHs), are normally described as three or more benzene ring systems bonded together. The number of individual PAHs is considerable as there are many permutations for the modes of ring connections, and also for the addition of side chains.

PAHs are not generally synthesized, but are by-products of industrial processes. Some of the first figures for their worldwide production were produced in 1976 [37] by Sues and are given in *Table 4.3.1.*

Source	Quantity (x 10^3 tonnes/year)	% of Total
Heating / Power production	260	51
Industrial producers	105	20
Incineration and burning	135	28
Transport	4.5	0.9

Table 4.3.1 Production of PAHs (Sues 1976)

In the early 1980s in the UK, PAH emissions were of the order of 1000 tonnes per year, and it was estimated that individuals were likely to inhale quantities in the order of 1.5 μg / day, when the recommended acceptable limit was 0.2 μg/m^3.

PAHs are also found in low levels in a variety of sources, including mineral oils at a mean level of ng/g of oil. They are formed when motor fuel is combusted in an engine, at a level of approximately 50 μg/km traveled, but this level drops to 0.05 – 0.3 μg/km

when catalytic converters are fitted. It has also been estimated that air travel produces approximately 10 μg/min traveled.

PAHs are found in most watercourses as the fallout of the above processes, and also as runoff from bitumen treated roads, with the estimated use of these products at approximately 6 x 10^7 tonnes / year. Some levels within watercourses have been estimated for the 1980s by the International Research on Cancer[38] (IARC) and are shown below, in Table 4.3.2.

Watercourse	**Thames**	**Factory effluent**	**Industrial sewage**	**Domestic sewage**
Concentration of PAHs (ng/l)	130 - 140	100 - 2200	100 - 3400	1 -250

Table 4.3.2 PAH concentration in watercourses (IARC)

PAHs are also found in food products, and Table 4.3.3 again shows figures produced by IARC[39]

Source	**Concentration (μg/kg)**	**Source**	**Concentration (μg/kg)**
Charcoal cooked meat	8	Flour	4
Margarine	1 – 36	Toasted bread	0.5
Sausages	4 – 50	Lettuce	3 - 12
Roasted coffee	1 - 13		

Table 4.3.3 PAH concentration in foodstuffs (IARC)

Mean exposure limits within a house has been measured at 0.1 –0.6 ng/m^3, which rises to 0.4 – 1.8 ng/m^3 if the occupants are smokers.

The corresponding health effects related to exposure to PAHs have been known for a significant time, with the first related link being made by Percival Pott[40] in 1775 with the exposure of chimney sweeps to domestic soot. He noted the prevalence of skin cancers to this trade and it has now been quantified that the amount of PAHs in soot is approximately 0.2%.

In 1875 von Volkman demonstrated the link between skin cancer and workers in the German coal tar industry. The link between this industry and lung cancer was again made in 1947 by the Kennaways, and this work was extended by Sir Richard Doll *et al*[41,42] 1965 and onwards.

Lloyd[43] identified a lung cancer excess amongst coke oven workers exposed to PAHs in the steel industry. Gibbs and Horowitz[44] in 1979 reported an increased risk for lung cancer in Soderberg aluminium smelter workers, and Gibbs[45] demonstrated in 1985 a tar-dose related risk for this cancer in the same cohort. Bladder cancer was added to the list of PAH-related diseases in aluminium smelters with the studies of Thériault et al.[46]

A review of the cancinogenic nature of PAHs was made by Sir Ernest Kennaway [47] in 1995.

A summary of this study are shown in *Table 4.4.4.*

Cancer type	**Increase in deaths / 100,000**	
	Gas workers	National rates
Lung	3.82	2.13
Bladder	0.40	0.17
Skin and scrotum	0.12	0.02

Table 4.4.4 PAH carcinogens (Kennaway 1995)

The toxicity of the PAHs varies widely, and Benzo-[a]-pyrene (Fig 4.3.1) is regarded as the most dangerous, because it is extensively distributed and also extremely carcinogenic.

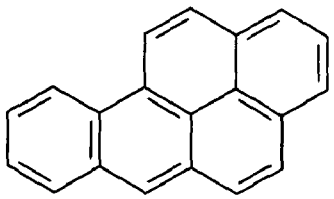


Fig 4.3.1 Benzo-[a]-pyrene

PAHs are extremely stable, sparingly soluble in water and have relatively high boiling points, they are therefore persistent in all spheres of the environment, and this results in a high risk of bioaccumulation.

Breakdown rates for this family of compounds are extremely low, having half-lives in water sediments of 5-10 years, and for soils 2-700 days, but this figure is for metabolism by micro-organism and not degradation.
Because of their carcinogenic nature the EU has set an allowable limit in drinking water of 0.2 μg/l, and the World Health Organisation (WHO) recommended level is 5% of this value at 0.01 μg/l.

When PAHs enter the body they undergo an enzymatic change to produce a reactive epoxide (Fig 4.3.2) which can further react with guanine of DNA and so inhibit synthesis and introduces defects and mutagenic effects, which are related to its carcinogenic properties.

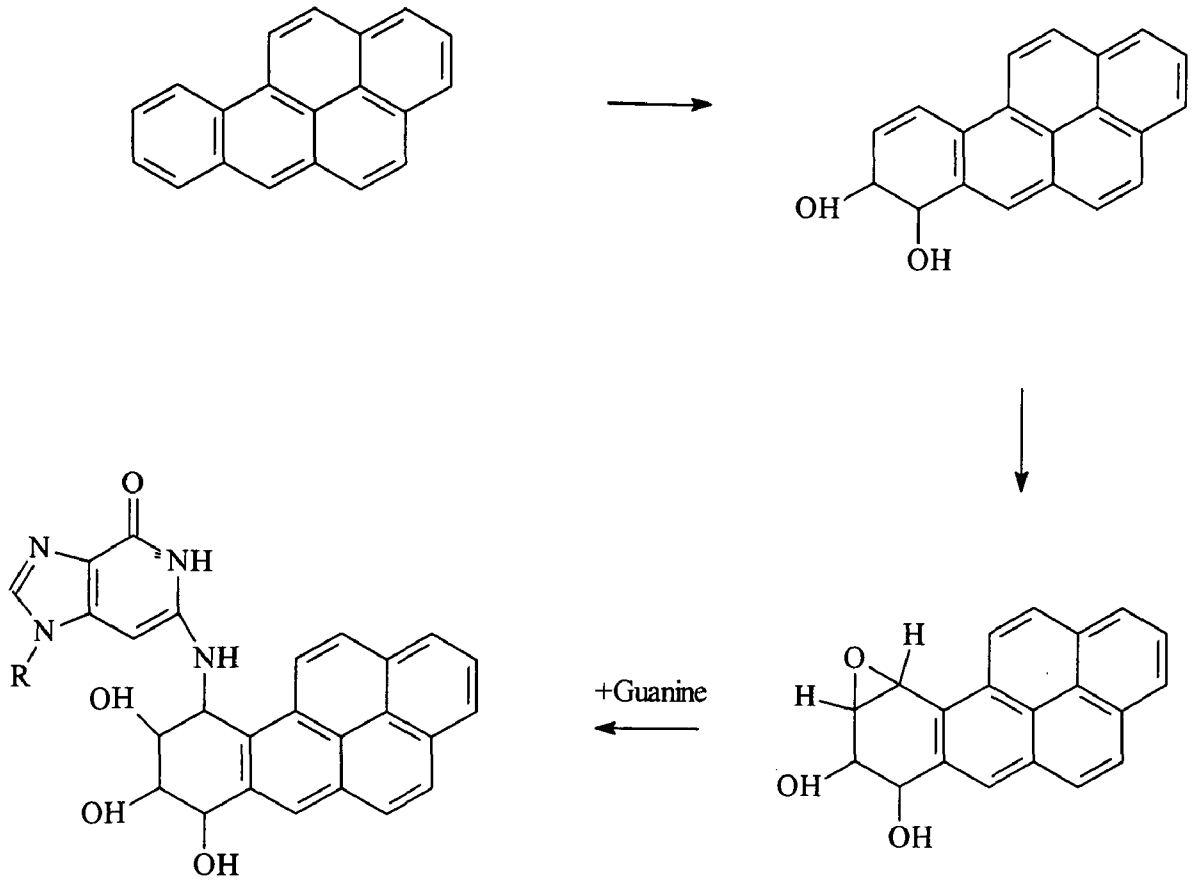


Fig 4.3.2 PAH reaction with DNA Guanine

Another route for metabolism within the body is for the phenols formed in the mechanism in Fig 4.3.2, to become conjugated with water soluble metabolites and so be excreted from the body. One such conjugate is the tripeptide glutathione-glutamyl cysteinyl glycine GSH, (figs 4.3.3 + 4.3.4).

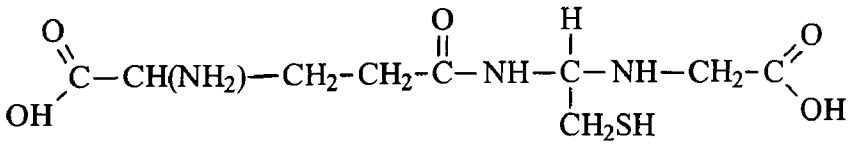


Fig 4.3.3 Glutathione-glutamyl cysteinyl glycine(GSH)

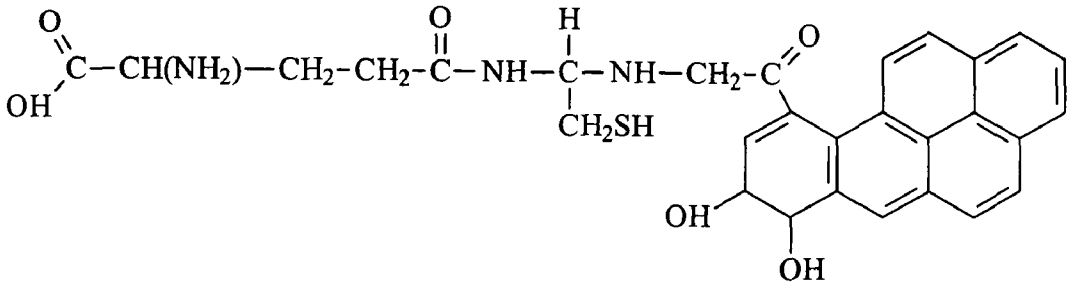
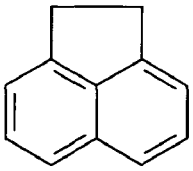


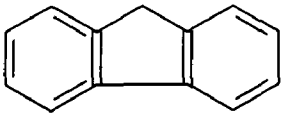
Fig 4.3.4 Conjugation between GSH and PAH

The PAHs used in this study were as follows;

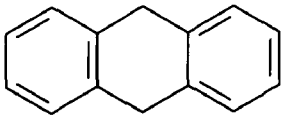
Acenaphthene (154.21 g mol^{-1})



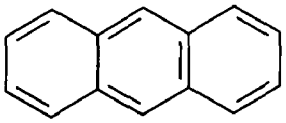
Fluorene (166.22 g mol^{-1})



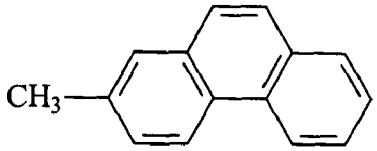
9,10-dihydroanthracene (180.25 g mol^{-1})



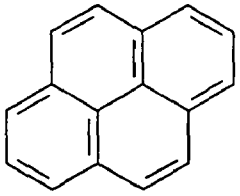
Anthracene (178.23 g mol^{-1})



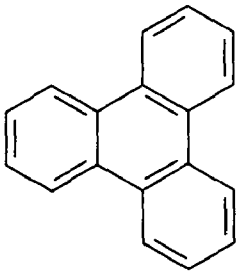
2-methylphenanthrene (192.26 g mol^{-1})



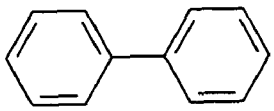
Pyrene (202.25 g mol^{-1})



Triphenylene (228.29 g mol^{-1})



Biphenyl (Internl standard) (154.21 g mol^{-1})



4.3.2 Supercritical Fluid Extraction

For extraction of the PAH mixture, a similar micro extraction system set-up was used as for the OPPs extraction in chapter 4.2, but the configuration was modified to incorporate a C18 trapping column, based upon previous findings for the pesticides.

The inlet to the micro extraction vessel was connected to a Gilson 307 reciprocating pump fitted with a supercritical fluid compatible piston seal, *via* a Rheodyne 7010 valve, using high pressure 1/16 inch stainless steel tubing and fittings. The pump head cooling jacket maintained the head at –10 °C, and ensured the liquid carbon dioxide remains liquefied allowing efficient delivery. The 1/16 inch o.d. stainless steel tubing carrying the carbon dioxide to the pump from the cylinder was coiled in a Gilson HPLC column oven maintained at 100 °C.

As before the configuration of the Rheodyne valve allowed the carbon dioxide to be delivered either to the bottom of the vessel, or via the top and through the 1/16 inch o.d. delivery tube and into the sample.

The output from the extractor was connected to a water trap, which isolated any water that may be carried from the sample in the carbon dioxide stream. A Druck pressure transducer was connected to the output of the trap, and connected to a Tescom pressure display unit, and allowed the extraction pressure within the extraction vessel to be constantly monitored.

The output from the pressure transducer was connected to a second Rheodyne 7010 valve, which allowed the carbon dioxide to be exhausted through a fused silica tube back pressure restrictor, the output from the restrictor was connected to a Phenomenex bondclone 10 C18 300 x 3.9 mm trapping column, which allowed efficient trapping of the analytes. The exhaust from this column was passed through ethyl ethanoate.

A standard solution of all seven PAHs was quantitatively prepared by dissolving known masses of each in a single 25 cm^3 aliquot of a 4:1 ethanol: propanone solution.

Polyaromatic hydrocarbon	Mass dissolved in 25 ml Ethanol / propanone solvent
Triphenylene	1.4 mg
Anthracene	1.5 mg
Pyrene	2.1 mg
9,10-dihydroxyanthracene	1.5 mg
Acenaphthene	1.5 mg
Fluorene	1.9 mg
2-methylphenanthrene	1.5 mg

The extraction vessel was thoroughly cleaned and a 5 ml glass vial loaded with the extraction solution, which consisted of 3.5 ml de-ionised water and 100 µl of the PAH standard solution.

The vial was placed inside the extraction vessel, loading from the bottom, this allowed the stainless steel delivery tube to protrude into the solution. The vessel was then sealed.

Valve D (see Fig 4.2.4) was configured to allow the carbon dioxide to enter via the bottom of the extraction vessel which, on reaching the target extraction pressure, was switched to direct the carbon dioxide through the extraction solution. This helped contain the extraction solution within the glass vial during the pressurisation phase of the extraction

The back pressure, and hence the supercritical carbon dioxide density, was regulated by the use of a length of silica tubing, which was manufactured to give a back pressure of 3400 psi, and monitored by the pressure transducer at the output of the vessel. The output end of this restrictor was connected to a C18 trapping column, and the exhaust from the column led into a 10 cm^3 volumetric flask containing ethyl ethanoate.

The carbon dioxide delivery pump flow rate was set at 3 cm^3 min^{-1} with the initial extractions carried out over a 30 min period after the extraction pressure was reached.

4.3.3 Experimental Results

Analysis and quantitation of the extracted PAHs was completed using a Hewlett-Packard 5890 gas chromatograph equipped with a HP-5MS 30 m x 0.3 mm internal diameter column of 0.25 μm film thickness. Interfaced to the GC system was a Hewlett-Packard 5971A MSD (Mass Spectrometric Detector), this analytical arrangement was used for all the analyses, and all were completed using high purity helium as the carrier gas.

Before the analytical phase of the extraction was attempted, the standard solution containing the PAHs was chromatographically, and mass spectrometrically analysed, and the individual mass fragmentation patterns of the analytes determined.

This was completed to allow identifiable mass peaks to be resolved for selective ion monitoring, which would allow increased sensitivity when analyzing individual anlayte components.
The following sequence of chromatograms and associated mass spectra were completed for the individual polyaromatic hydrocarbons

```
File        : G:\CH222\PAHSTD.D
Operator    :
Acquired    : 27 Jul 100    5:24 pm using AcqMethod BM3
Instrument :    5971 - In
Sample Name: Std PAH Mix
Misc Info   :
Vial Number: 9
```

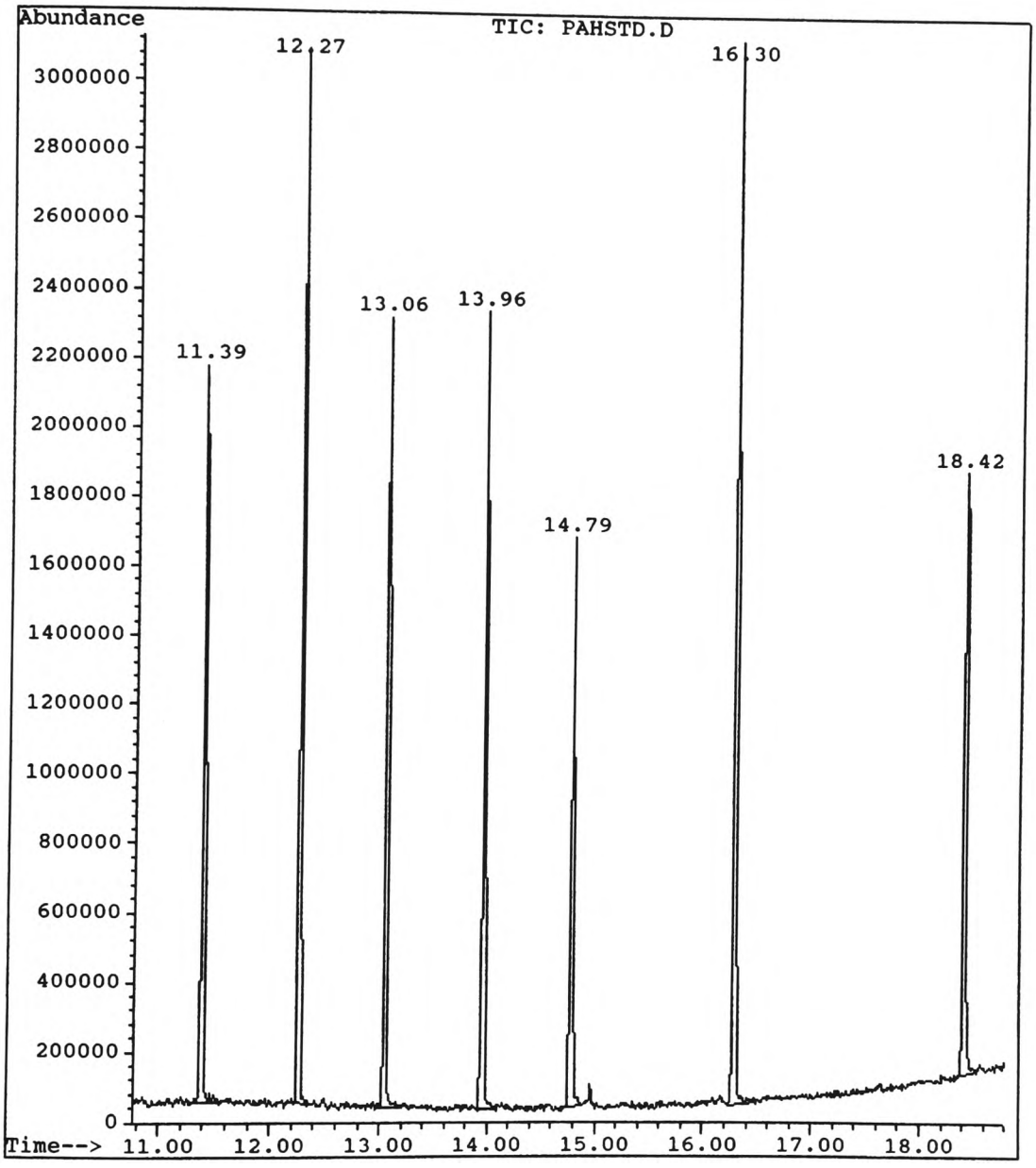
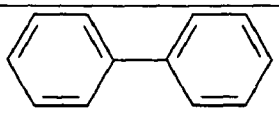
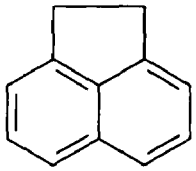
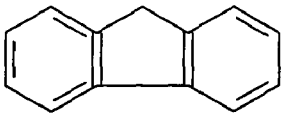
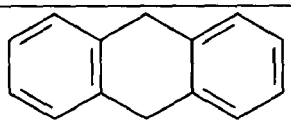
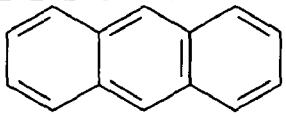
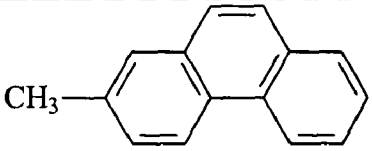
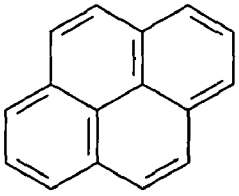
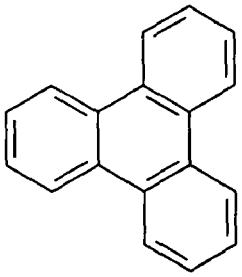


Fig 4.3.5 Gas chromatogram of standard PAH mixture Internal Standard not included.

N.B. Biphenyl acting as an internal standard, had a retention time of 10.33 min.

Retention time (min)	Polyaromatic hydrocarbon	Structure
10.33	Biphenyl (I.S.)	
11.39	Acenaphthene	
12.27	Fluorene	
13.06	9,10-dihydroanthrecene	
13.96	Anthracene	
14.79	2-methylphenanthrene	
16.30	Pyrene	
18.42	Triphenylene	

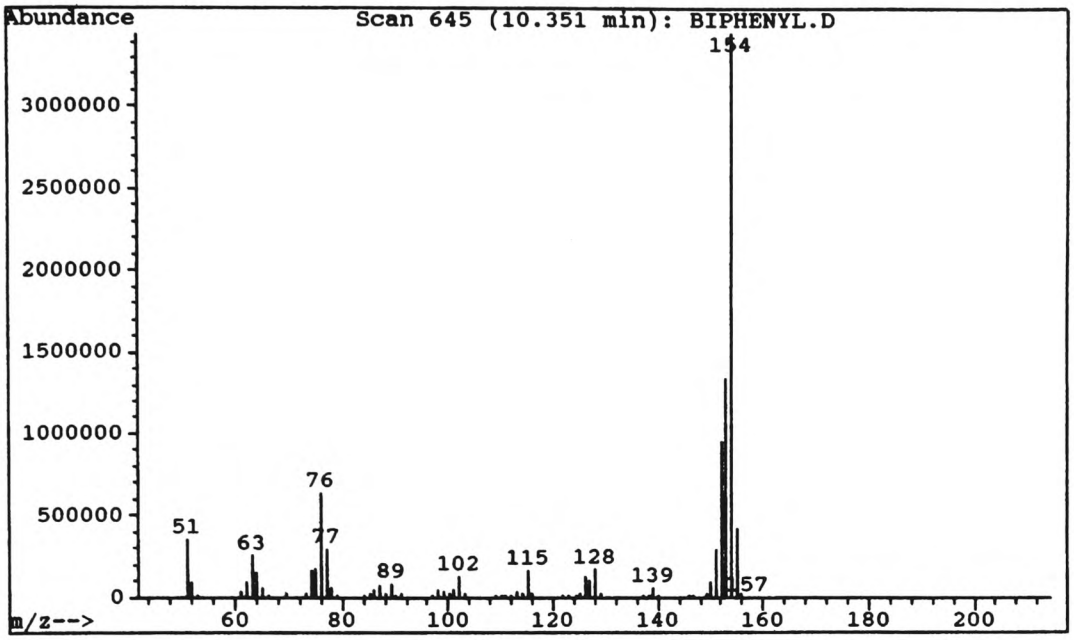


Fig 4.3.6 Full scan mass spectrum of Biphenyl (I.S)

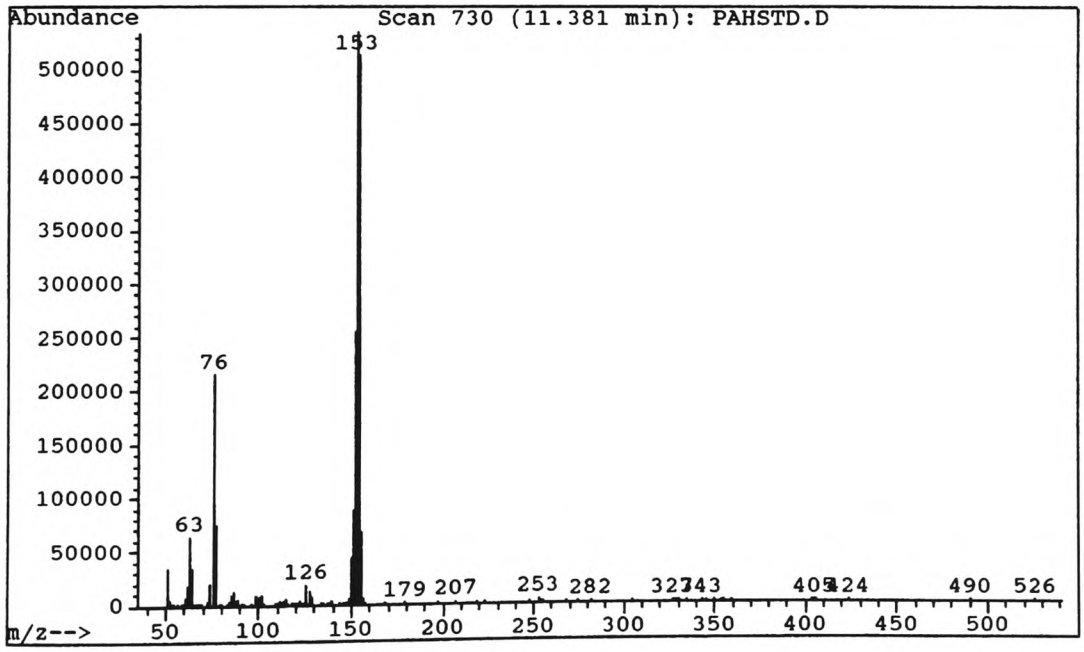


Fig 4.3.7 Full scan mass spectrum of Acenaphthene

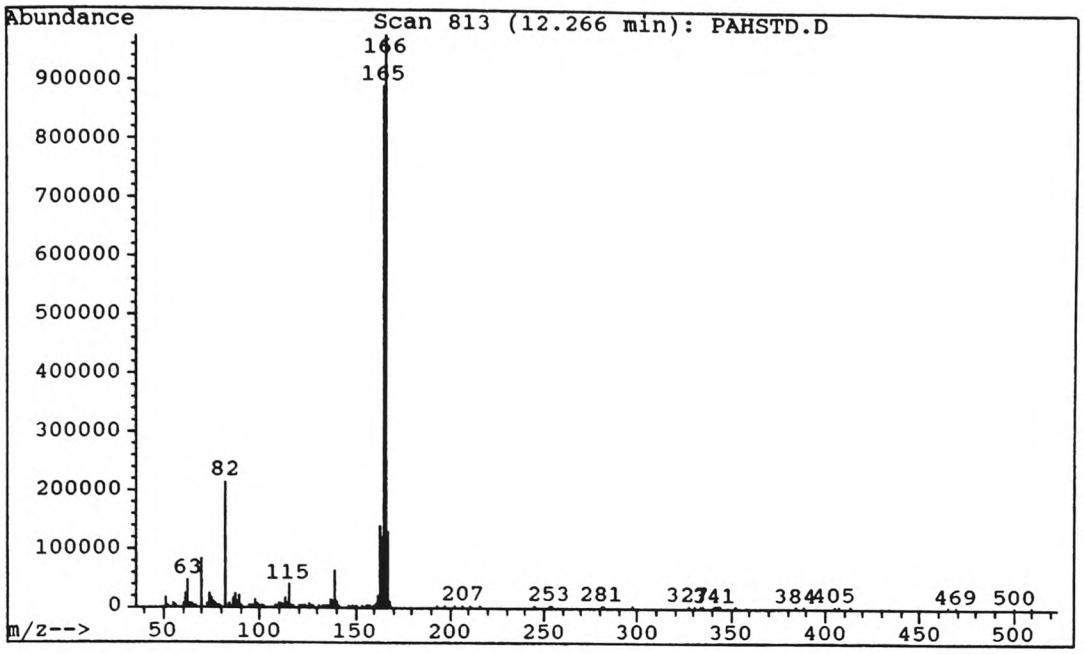


Fig 4.3.8 Full scan mass spectrum of Fluorene

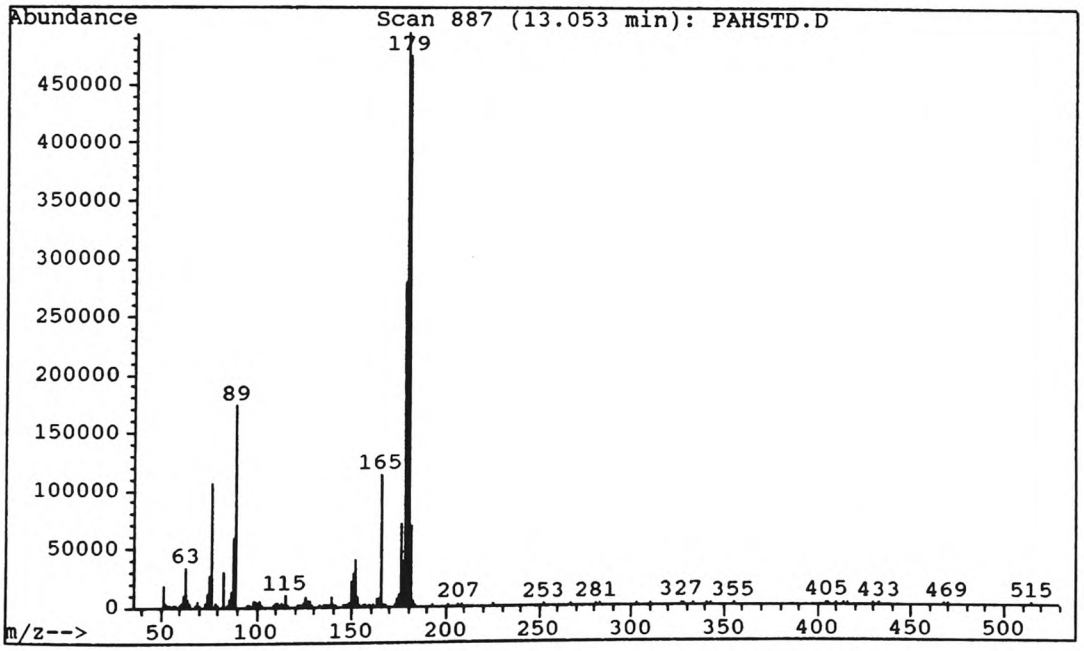


Fig 4.3.9 Full scan mass spectrum of 9,10-dihydroxyanthracene

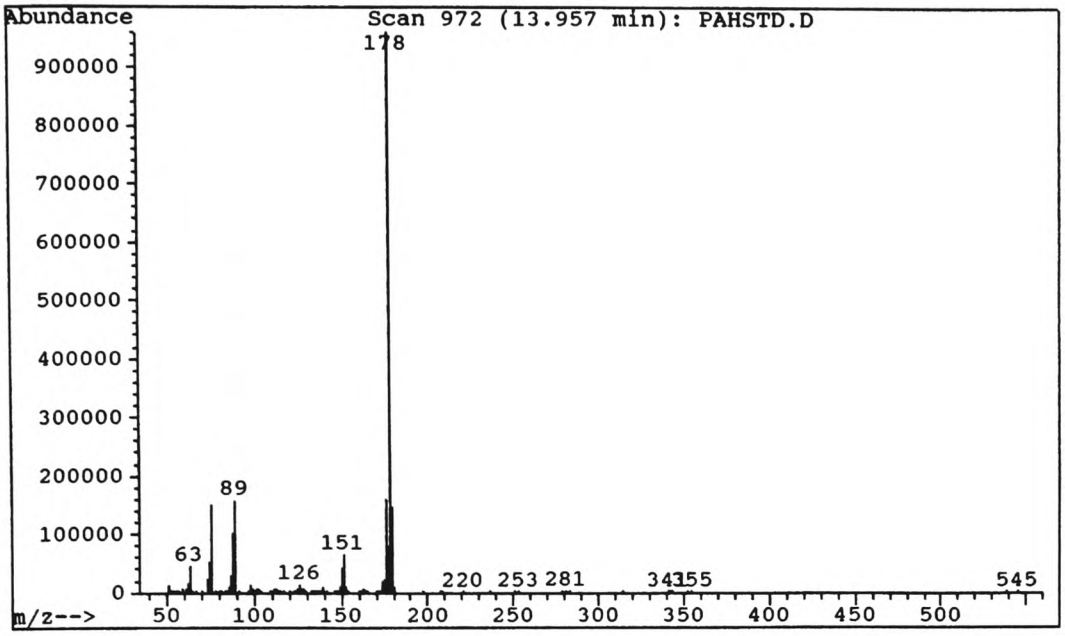


Fig 4.3.10 Full scan mass spectrum of Anthracene

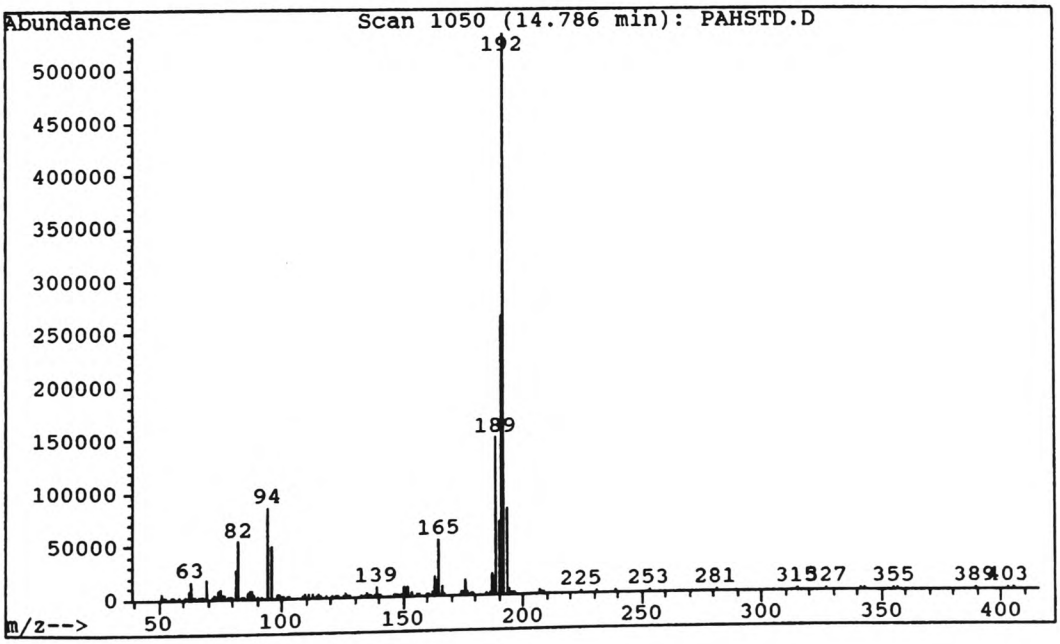


Fig 4.3.11 Full scan mass spectrum of 2-methylphenanthrene

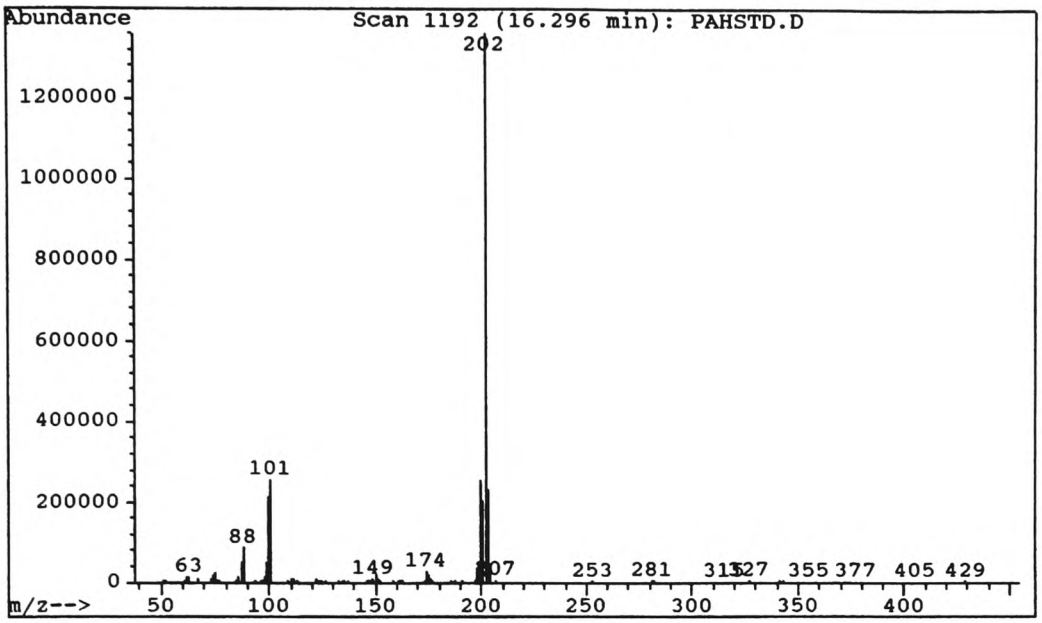


Fig 4.3.12 Full scan mass spectrum of Pyrene

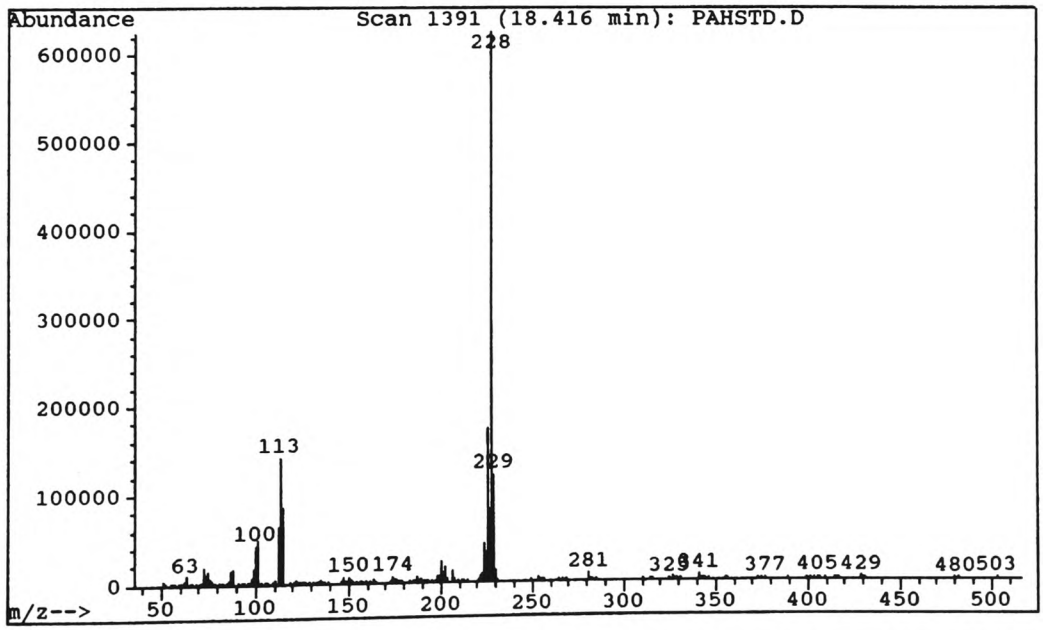


Fig 4.3.13 Full scan mass spectrum of Triphenylene

Analysis of these spectra, resulted in the selection of representative ions to be used in single ion monitoring, which allowed increased sensitivity during the detection phase.

Retention Time (min)	PAH	Selected Ion
10.33	Biphenyl (I.S.)	154
11.39	Acenaphthene	153
12.27	Fluorene	166
13.06	9,10-dihydroanthrecene	179
13.96	Anthracene	178
14.79	2-methylphenanthrene	192
16.30	Pyrene	202
18.42	Triphenylene	228

A difference between the detection phases for the PAHs, and the OPPs as described in the last section, is no requirement for an increase in the detector sensitivity during the run, as the response of all the analytes was adequate.

The extractions were carried out over a 30 min period after the required extraction pressure was attained. At the end of this period the pump was stopped and the supercritical carbon dioxide allowed to de-pressurise through the C18 column for a further 15 minutes.

After this period the C18 column was quantitatively washed with two 1ml aliquots of ethyl ethanoate. A 100μl aliquot of the internal standard, biphenyl, was also volumetrically added, and the individual PAHs quantified.

File : G:\CH222\PAHE2EXT.D
Operator :
Acquired : 8 Aug 100 9:45 am using AcqMethod BM6
Instrument : 5971 - In
Sample Name: PAH ext2 + 100ul Biphenyl IS
Misc Info : 3.5ml water + 100ul PAH mix + 100ul IS
Vial Number: 11

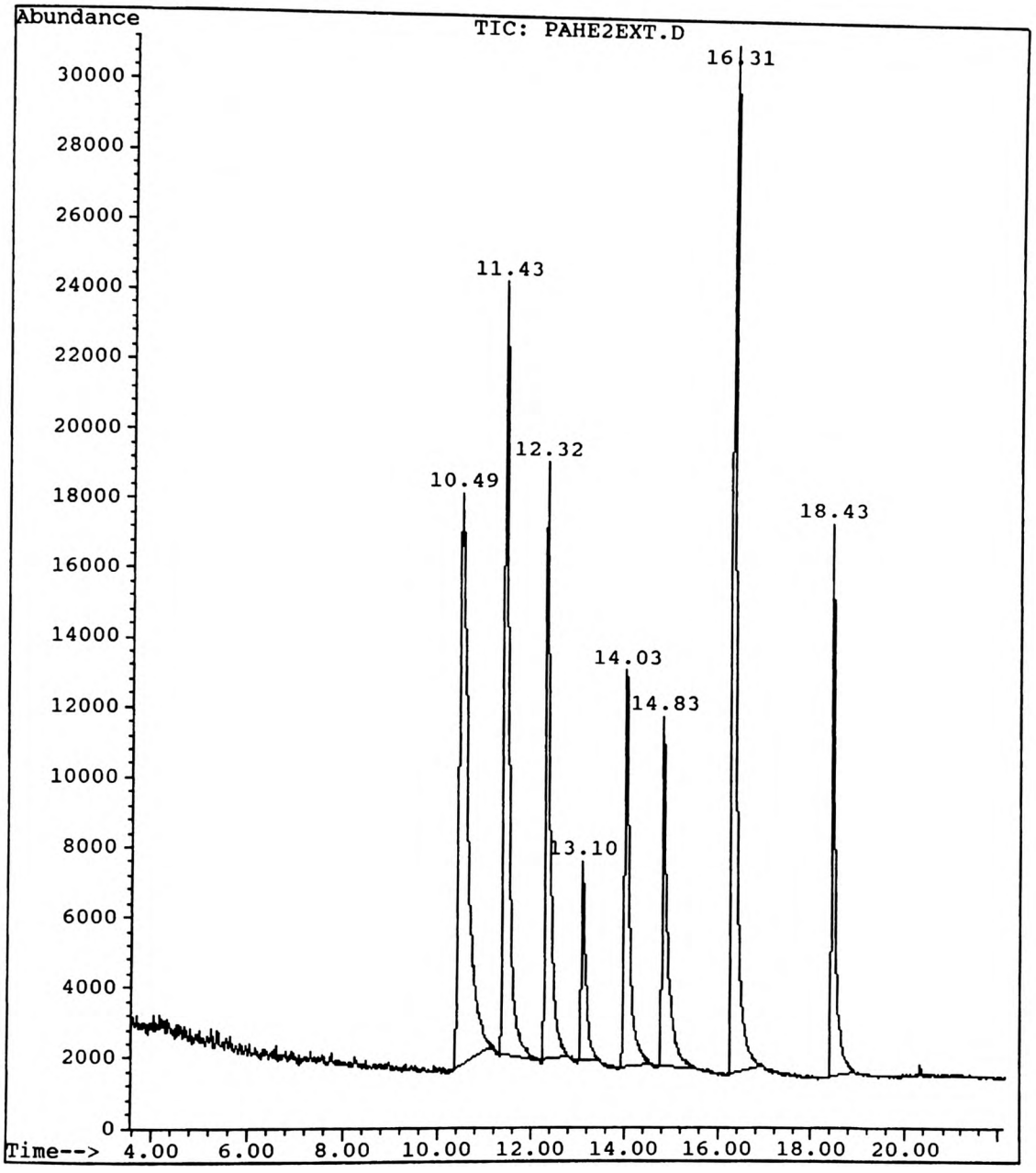


Fig 4.3.14 Chromatogram of PAH/ ethyl ethanoate solution obtained following 30 min dynamic extraction

File : D:\PAHE2RES.D
Operator :
Acquired : 8 Aug 100 10:15 am using AcqMethod BM6
Instrument : 5971 - In
Sample Name: PAH Ext2 Residual water
Misc Info : Check extraction eff.
Vial Number: 12

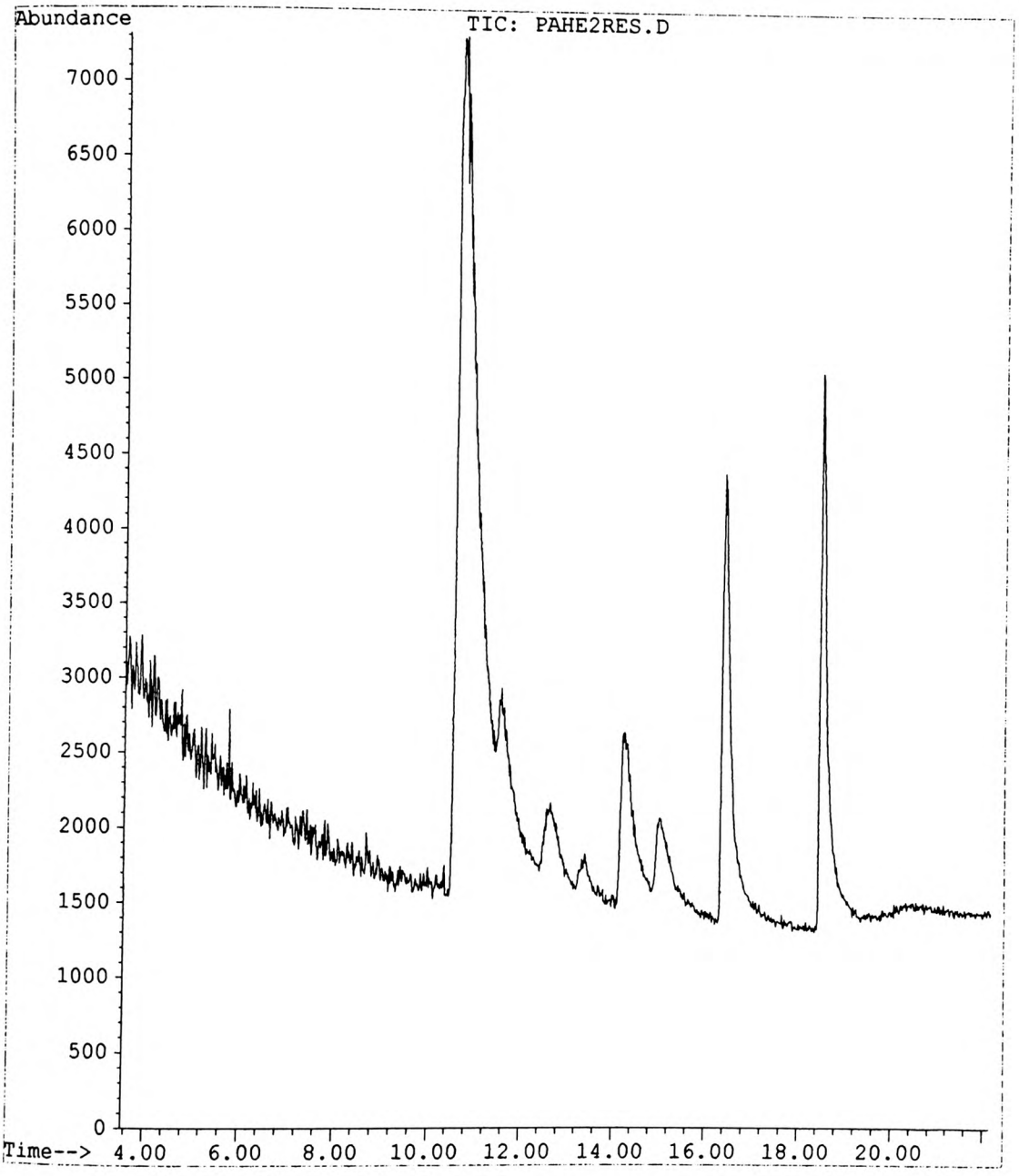


Fig 4.3.15 Chromatogram of Residual water

File : D:\PAHE2W2.D
Operator :
Acquired : 8 Aug 100 10:45 am using AcqMethod BM6
Instrument : 5971 - In
Sample Name: PAH Ext2 second col. wash
Misc Info : check full recovery from column
Vial Number: 13

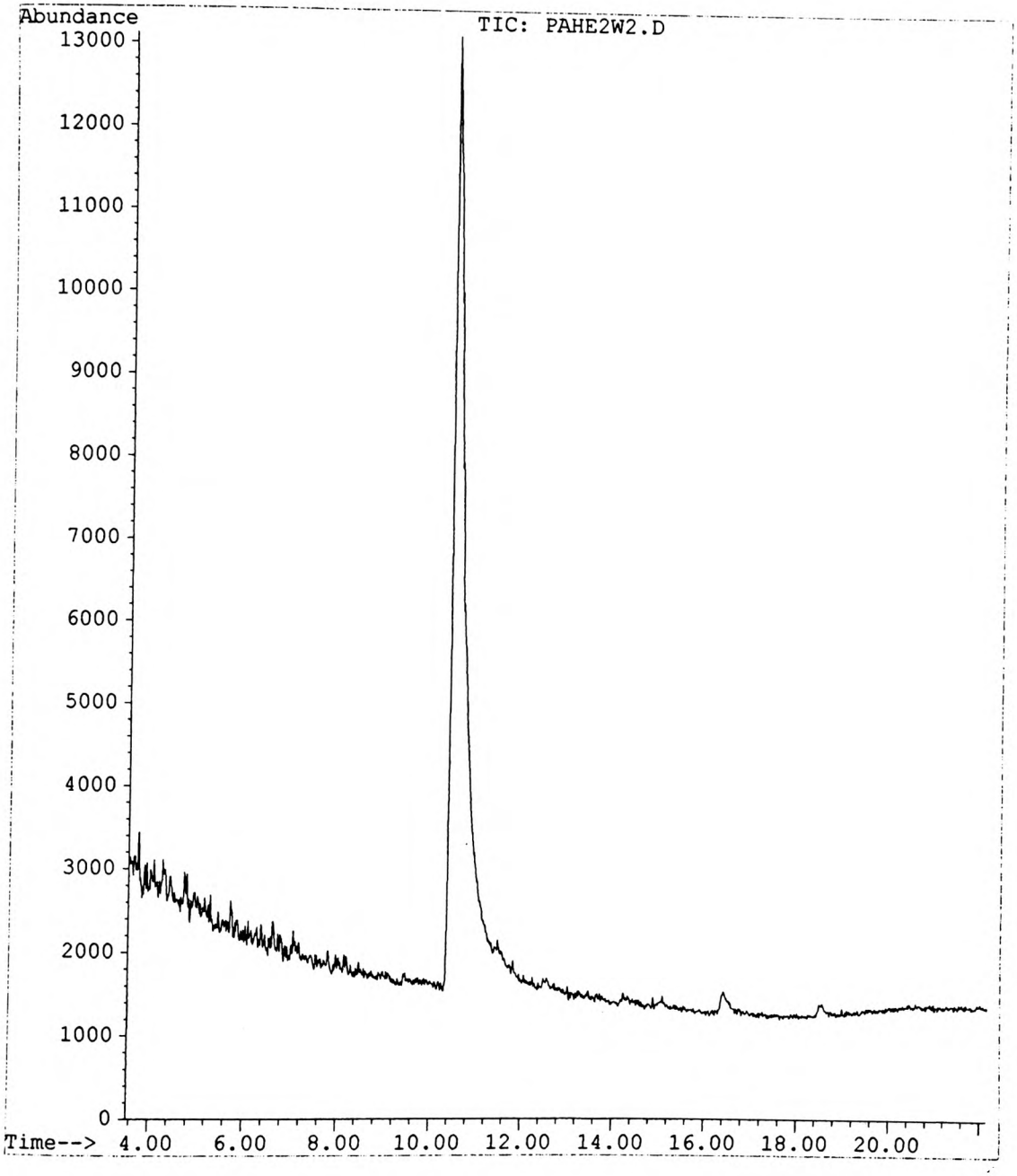


Fig 4.3.16 Chromatogram of Second 1 ml Aliquot of Ethyl Ethanoate

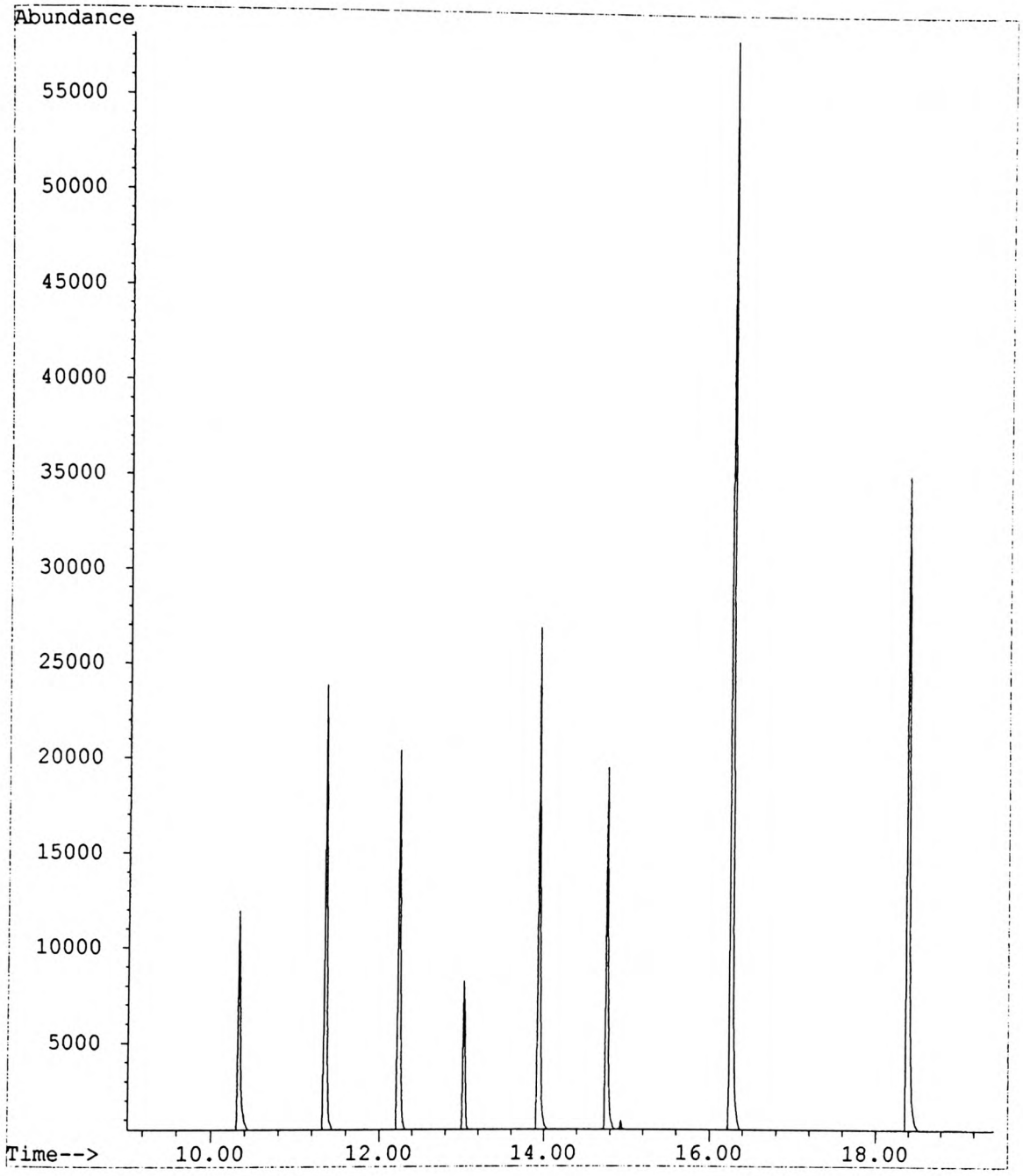


Fig 4.3.17 SIM Spectra of Ethyl Ethanoate solvent trap (post column)

4.3.4 Experimental Conclusions

It can be seen from Table 4.3.5 that, as with the OPP extractions, none of the PAHs are quantified to 100%.

The trapped quantities are comparable between the PAHs and the OPPs when both used a C18 column to trap the components.

The major difference between the two sets of analytes was the residue left in the water. For the OPPs there was little evidence of residual sample, but for the PAHs significant quantifiable levels acenaphthene (5.1%), pyrene (6.3%) and triphenylene (6.6%) remained.

This would suggest that these components were the most water soluble, the most polar of the PAHs, and therefore most difficult to extract with SF-CO_2 which is a essentially a non-polar solvent. This is difficult to envisage as all the PAHs are non-polar and hence should not differ greatly in their solubilities. Data of solubilities for the PAHs is difficult to obtain, and so this theory is difficult to prove, however the solubilities in water should not be that different to be significant.

The use of mean values for three separate extractions is again going to play a part in the overall inaccuracies, and the fact that repeatability of the restriction is difficult, must make a contribution.

Retention time (min)	PAH	Extracted %	Residual Water %	Trapped in solvent %	Total Quantities of PAHs extracted %
11.39	Acenaphthene	85.3	5.1	1.1	91.5
12.27	Fluorene	88.1	3.9	0.8	92.7
13.06	9,10-dihydroanthracene	67.1	3.3	0.4	70.8
13.96	Anthracene	72.2	7.5	2.1	81.8
14.79	2-methylphenanthrene	70.7	5.0	1.7	77.4
16.30	Pyrene	79.4	6.3	3.1	88.8
18.42	Triphenylene	73.7	6.6	1.3	81.6

Table 4.3.5 Quantitative results for the mean of three PAH standard solution extractions

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Chapter 5

Application of Supercritical Fluid Extraction coupled on-line with Supercritical Fluid Chromatography-Mass Spectrometry

The Supercritical Fluid Extraction and Analysis of 2,4-dibromophenol, 4-nitrophenol and 2,4,5-trinitrophenol by on-line Supercritical Fluid Chromatography- Atmospheric Pressure Chemical Ionisation Mass Spectrometry

5.1 Introduction

Supercritical fluid extraction is not generally selective enough to specifically isolate an individual analyte from a matrix without a further cleanup stage prior to qualitative or quantitative analysis. Therefore SFE is generally allied with an associated chromatographic technique. The combination can by either off-line, where the supercritical fluid extraction is a separate process to the chromatography, and where the extracted species are trapped into a suitable solvent, or solid phase sorbent material, before being injected into the chromatographic instrumentation, see chapter 4. SFE can also be directly coupled to the chromatographic instrumentation, generally via a switching valve, to form an integral extraction and analysis process.

SFE was first combined with thin layer chromatography by Stahl and Schiltz [1] in 1976, gas chromatography by Smith and Hawthorn, [2,3] and HPLC by Unger, [4] coupling to Supercritical Fluid Chromatography followed later in the mid 1980's separately by Sugiyama *et al*, [5] and Skelton *et al.* [6]

SFE is generally coupled directly to SFC via a switching valve, and can be coupled to either a chromatographic system based on a packed, packed capillary or capillary chromatography column, the main difference between these classifications is the length and diameter, [7] as shown it table 5.1

	Capillary	Packed capillary	Packed
Internal diameter	25 – 100 μm	0.25 – 1 mm	2 – 4.6 mm
Length	3 – 20 mm	10 –100 cm	10 – 25 cm

Table 5.1

The early preference was for the coupling of capillary columns over packed column, this was due to primarily two reasons,

a. the pressure drop over the column length,
b. the column surface coating which tends to be more inert when compared to the silica based coatings associated with packed columns.

Several workers have reported on the comparison of capillary, packed and micropacked columns coupled to SFE. [8-12] One of the main advantages of coupling packed column SFC to the SFE system, is the load capacity of the column. Packed columns have the ability to be loaded with considerably more material than a capillary column, and therefore there is no need for extract sample splitting between the SFE and SFC stages. This is particularly important when large initial volumes of sample are required which allows higher analyte sensitivity. The alternative view is that with the low flows associated with capillary columns makes them more amenable to direct coupling to GC, because of their compatibility with the associated GC detectors.

5.1.1 Sources

Chlorophenols are a group of compounds that are used in a number of industries and products, including the pesticide industry.
Nitrophenol are intermediates in the synthesis of azo dyes and a number of pesticides, mainly insecticides and herbicides.

The main source of emissions are air emissions by volatilization during the manufacture of products. In 1991, approximately 6.5 tonnes of chlorinated phenols and 0.5 tonnes of nitrophenols were released by this mechanism TRI .[13]

Air emissions are also found as flue gas condensates, emissions from waste burners, and incinerator ash, and has been detected in the combustion of hazardous waste, coal, wood, and solid waste. Nitrophenols have been detected in the exhaust gases of light-duty gasoline and diesel vehicles, where applicable catalytic convertion reduced the nitrophenol emissions to about 8% of this value. They are also generated in the atmosphere during the photochemical degradation of aromatic compounds such as benzene and toluene in the presence of nitric oxide or hydroxyl radicals and nitrous dioxide. Chlorinated phenolic compounds and may be found in both industrial and domestic wastewaters

Nitrophenols emitted into the troposphere will stay predominantly in the gaseous phase and are rapidly removed by nitration. The major portion of airborne nitrophenols are particle bound, and can be washed out to surface waters and soil by wet and dry deposition. Because of their removal from air and their insignificant volatility, nitrophenols are not considered to contribute directly to the depletion of the stratospheric ozone layer or to global warming.

5.1.2 Medical Effects

Exposure to high levels of substituted phenols can cause damage to the liver and immune system. These compounds are thought to be highly toxic and have been shown to be carcinogenic.

In a study comparing 50% toxicity levels of chlorophenols,[14] pentachlorophenol (PCP) was found to be the most toxic with the toxicity decreasing as the number of chlorine substituents decreased.

Workers exposed to pesticides that contain chlorophenols have developed acne and mild injury to their livers, and there is evidence to suggest that people with long term exposure to chlorophenols may have slightly higher incidences of cancer.

Recommended drinking water levels are less than 0.04 milligrams per liter (0.04 mg/l) for 2-chlorophenol, 0.03 mg/l for 2,4-dichlorophenol, and pentachlorophenol has been given a guideline for drinking water by the World Health Organization of 10 mg/l .[15]

5.1.3 Environmental Effects

Chlorophenols can enter the environment when they are being made or used as pesticides. Most chlorophenols released to the environment go into water, with small amounts entering the air, they also stick to soil and sediments at the bottom of lakes, streams, and rivers. Low levels of chlorophenols in water, soil, or sediment are broken down and removed from the environment in a few days to weeks by microorganisms. In the air, sunlight helps destroy these compounds and rain washes them out . Half-lives in the air range from 21.2 to 212 hours for 2,4-DCP depending on the rate of reaction with hydroxyl radicals, and 170 hours for 2,4,6-TCP.[16]

Due to their inherent toxicity and relative persistence, the pesticide industry substituted the use of chlorine with phosphorous, in many instances. This raw material substitution has produced a significant decrease in persistence, and a large drop in the environmental release of chlorinated phenols.

Environmental releases of nitrophenols are mostly to ambient air, surface waters, and to a smaller extent soil. Half-lives of nitrophenols in water ranged from 14.5 to 27.3 days, indicating a slow rate of volatilization.[17-20] Measured half-lives for the photochemical decomposition of nitrophenols in water exposed to sunlight ranged from 2.8 to 13.7 days,[21-22] and longer with increasing pH.. Anaerobic degradation, even of high initial nitrophenol concentrations, was observed to be >90% removal of 2- and 4-nitrophenol (350-650 mg/litre) over a period of 10 days. [23]

The fate and transport of these compounds is largely dependent on whether they are in a neutral or ionic form. This will in part depend on the pH of the aqueous phase which it enters. With a pKa near ambient pH values, it is very likely that the chlorophenols will exist as an ionic species, this increases water solubility and mobility in the aqueous phase, a lower degree of sorption to solid phases, and a decrease in their ability to be vopourised. In their neutral form, chlorinated phenols exhibit relatively low water solubility (although the phenol group does tend to form hydrogen bonding), moderate to high sorption, and in most cases, high volatility.

As the number of chlorine atoms substituted onto the ring increases, the pKa of the chlorophenol decreases. Since the pH of natural waters can easily vary from 6.0 to 8.0 and the pKa's of chlorophenols range from 4.7 to 7.68, they are likely to be found in their ionic form in the environment. For food chain uptake, 2,4-dichlorophenol has a bioaccumulation factor (BCF) between 1.0 and 2.41 depending on the species. However, PCP has a higher tendency to bioaccumulate, with BCF's up to 10000 for some fish species,[24](ATSDR 1994).

The ground water half-life also may vary from 133 to 1032 days, depending on the subsurface conditions. Pentachlorophenol has a half-life in water ranging from 20 to 200 days. The half-life for 2,4-DCP in sediment is estimated to be between 47 and 116 days, and the half-life for 2,4,6-TCP in soil is estimated at 1700 hours, with the dominant processes for degradation being photolysis and biodegradation.

The solubility of 2-chlorophenol in water is approximately 20,000 ppm (mg/l), dichlorophenol is approximately 4,500 ppm, trichlorophenol 434 ppm, and pentachlorophenol 14 ppm, this represents the trend in this chemical family as the number of chlorine substitutions increase.[25]

Data indicates that the time needed for natural processes to remove 95% of PCP, 2,4,6-TCP and 2,4-DC are 27 years, 30 months, and 15 months, respectively.[26]

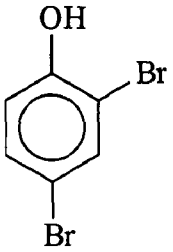
5.2 Experimental

The theory behind the principles of Atmospheric Pressure Chemical Ionisation (APCI) are outlined in section 1.5.2.3, and the experimental set-up for the interface is illustrated in Figs. 1.15 and 1.16.

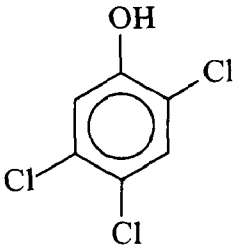
The phenols used in this work were

a. 4-nitrophenol (139.11 g mol^{-1})

b. 2-4,dibromophenol (251.92 g mol^{-1})



c. 2,4,5-trichlorophenol (197.45 g mol^{-1})



An aqueous solution of the three phenols, 2,4-dibromophenol, 4-nitrophenol and 2,4,5-trichlorophenol, was prepared, and its pH adjusted to 4.2 with hydrochloric acid. The resulting solution had a final concentration of 40 ppb for each component. Under acidic conditions the extent of dissociation of the phenols in the solution is suppressed, this theoretically, should increase recovery.

Before SF extraction of the phenols commenced, GC-MS analysis of the individual phenol components dissolved in ethyl ethanoate was carried out. This was achieved using 5 µl splitless injections, and quantification of these standard phenol solutions in the range of 0.2 to 1 ng μl^{-1} was completed, using anthracene at a level of 0.4 ng μl^{-1} as an internal standard. This allowed the construction of calibration graphs of the quotient of response of the phenols against that for anthracene, and would allow quantiification of the SFE recoveries of each phenol to be quantified.

All the GC-MS analyses were carried out using a Hewlett-Packard 5890 gas chromatograph, fitted with a HP-5MS 30 m x 0.2 mm column of 0.25 μm film, and using helium as the carrier gas. Interfaced to the GC system was a HP 5971A mass selective detector operating in single ion monitoring mode (SIM), using electron ionisation.The temperature profile for the chromatographic analyses was, 2 min at 60 °C, and then to 290 °C at a rate of 15 °C min^{-1} with the final temperature held for 15 min.

Also before SFE-SFC-MS studies were commenced, the individual phenols, at an equivalent level of 500 ng, were subjected to study by direct valve loop injection, without the chromatographic separation stage at a pressure of 30.89Mpa using high purity grade Methanol as an organic modifier, details of the flow rate and modifier concentration are given in Fig 5.6. Chromatography was then followed by APCI analysis in both positive and negative ion mode. All three phenols provided excellent response in negative ion APCI mode, with each spectrum dominated by the $[M - H]^-$ ion, (Figs 5.1 to 5.3) The positive ion response for all three phenols was disappointing, and so all further SFE-SFC-MS studies were conducted in negative ion mode.

A stainless steel extraction vessel of 300 cm^3 internal volume, as described in section 2.4.1. and shown in Fig 2.15, housed in a Pye 104 gas chromatograph, was use for all the supercritical fluid extractions. A Hewlett-Packard G 1205A supercritical fluid chromatograph, was used to supply liquid carbon dioxide for the extractions, and SFC-MS studies.

A Rheodyne 7010 valve was used to connect or isolate the vessel from the multi-valve switching system.During dynamic aqueous SFE (Fig 5.4) the restrictor module was situated pre column, using the SFE multi-valve configuration, this allowed the extracted phenols to be trapped on the ODS column after depressurisation of the supercritical fluid solvated phenolic extract.

At the end of the 45 min extraction period the SFC-MS multi-valve configuration (Fig 5.5) was selected, allowing the restrictor module to be situated post-column to enable SFC-MS studies of the trapped phenols to be completed.

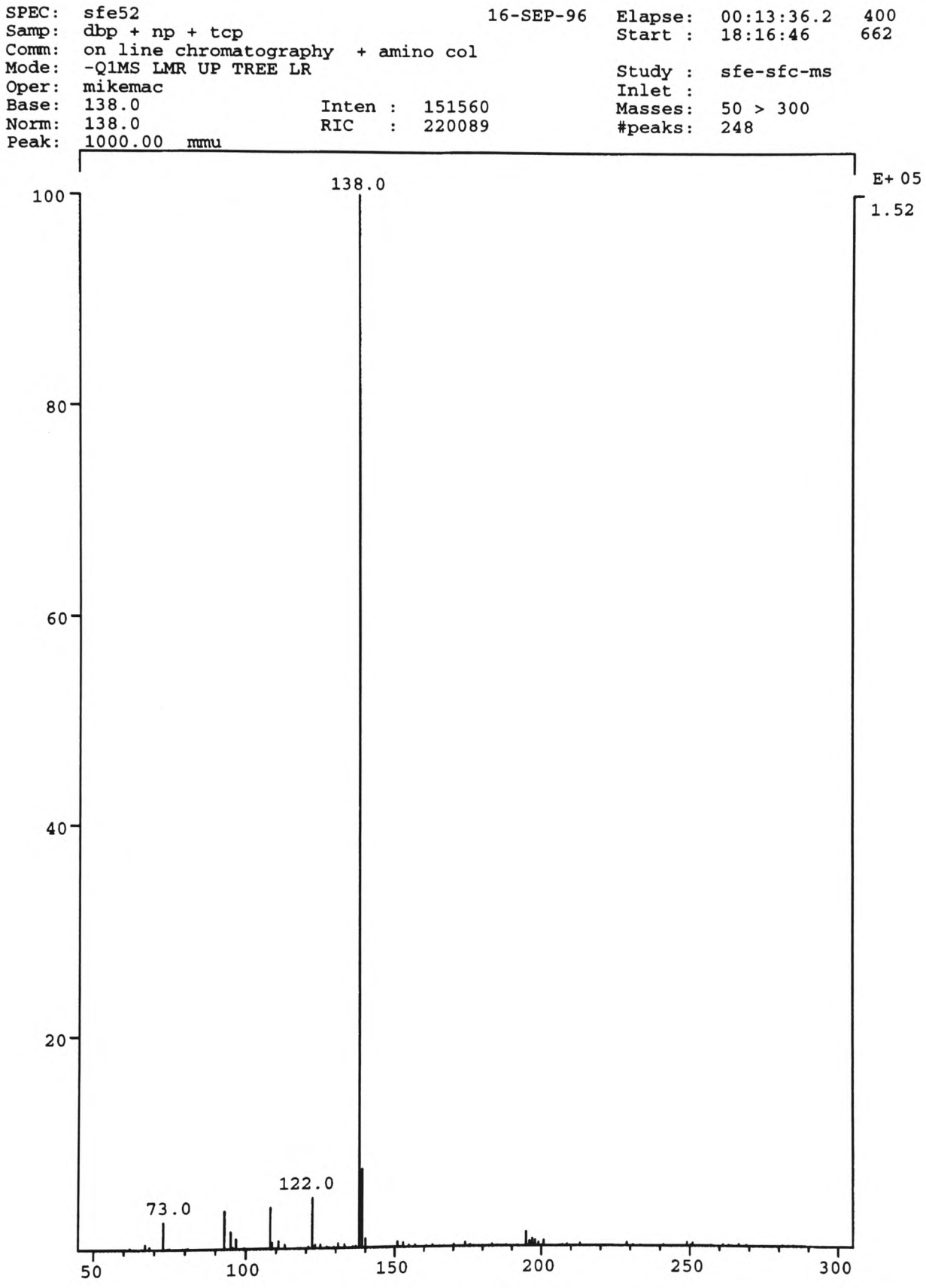


Fig 5.1 4-nitrophenol negative ion APCI mass spectrum

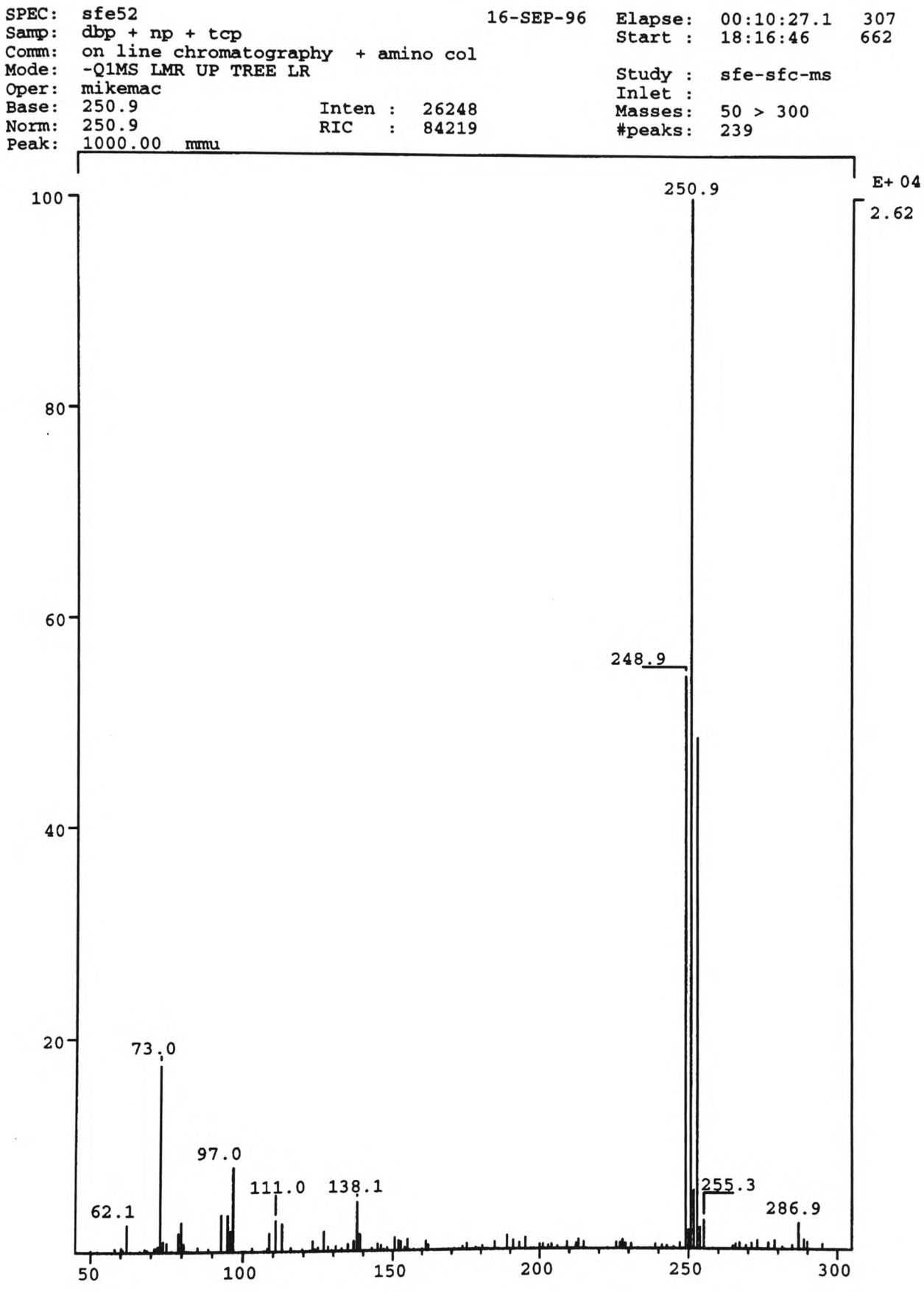


Fig5.2. 2,4-dibromophemnol negative ion APCI

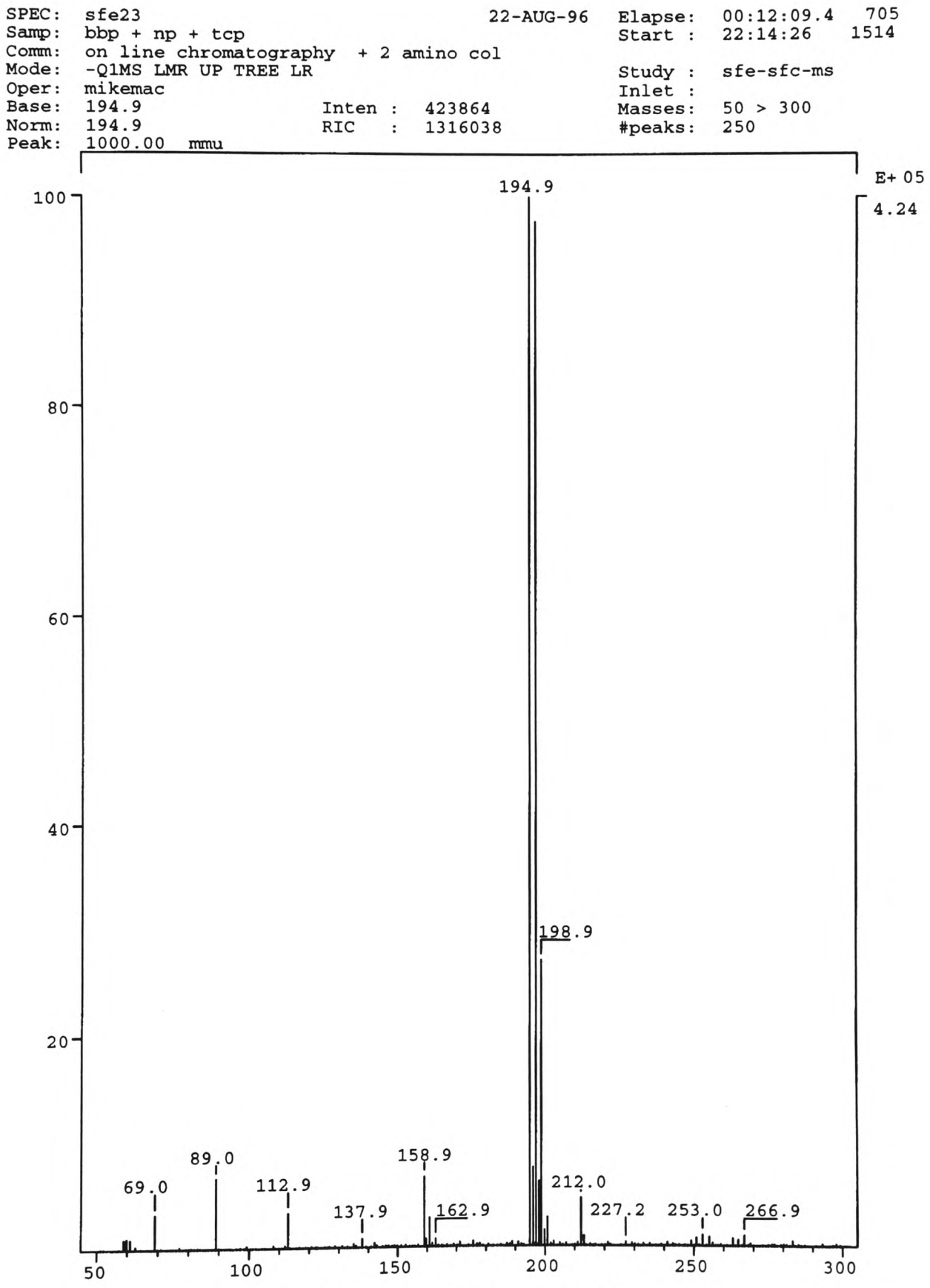


Fig 5.3. 2,4,5-trichlorophenol negative ion APCI

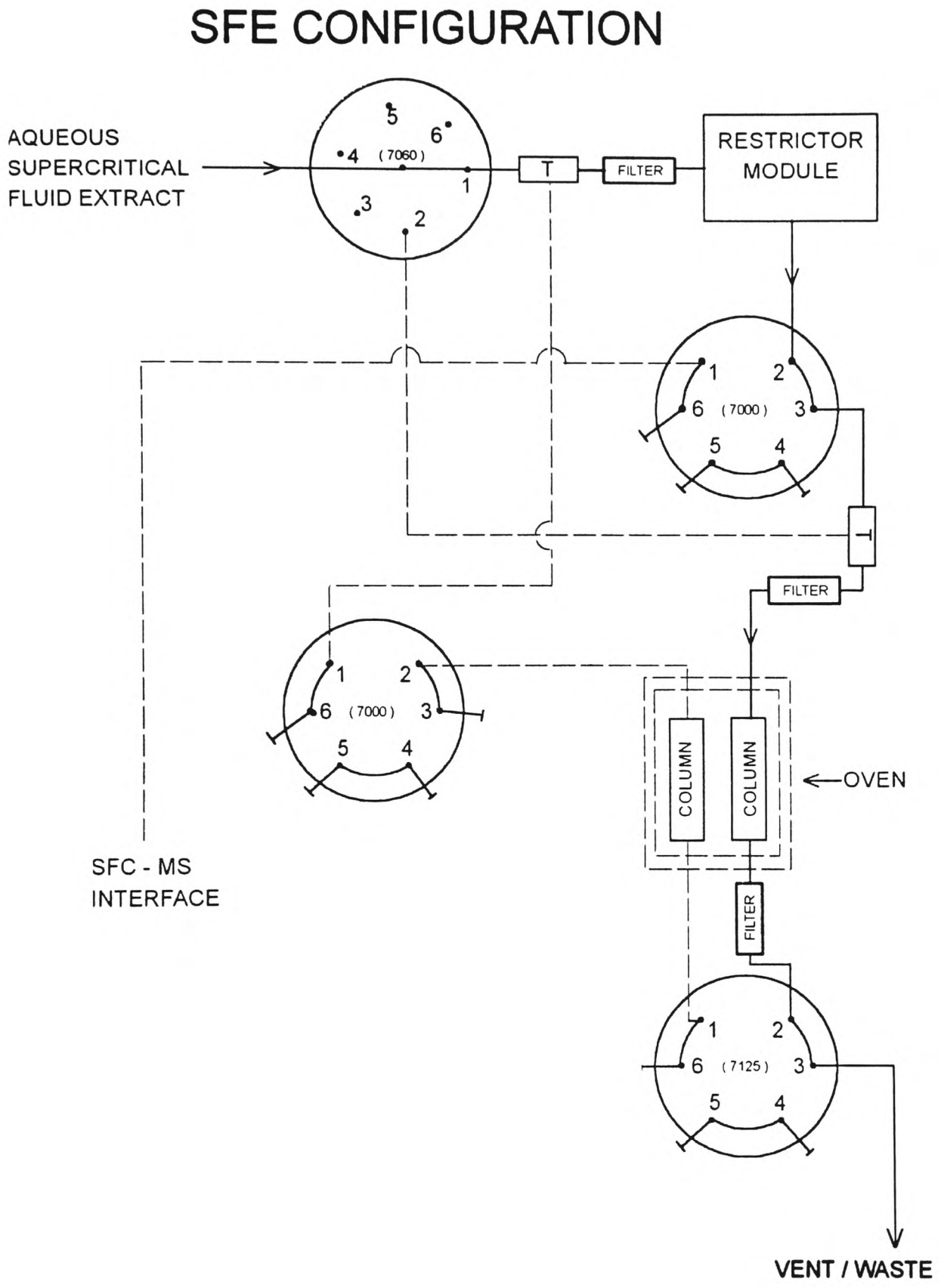


Fig 5.4 Supercritical Fluid Extraction configuration

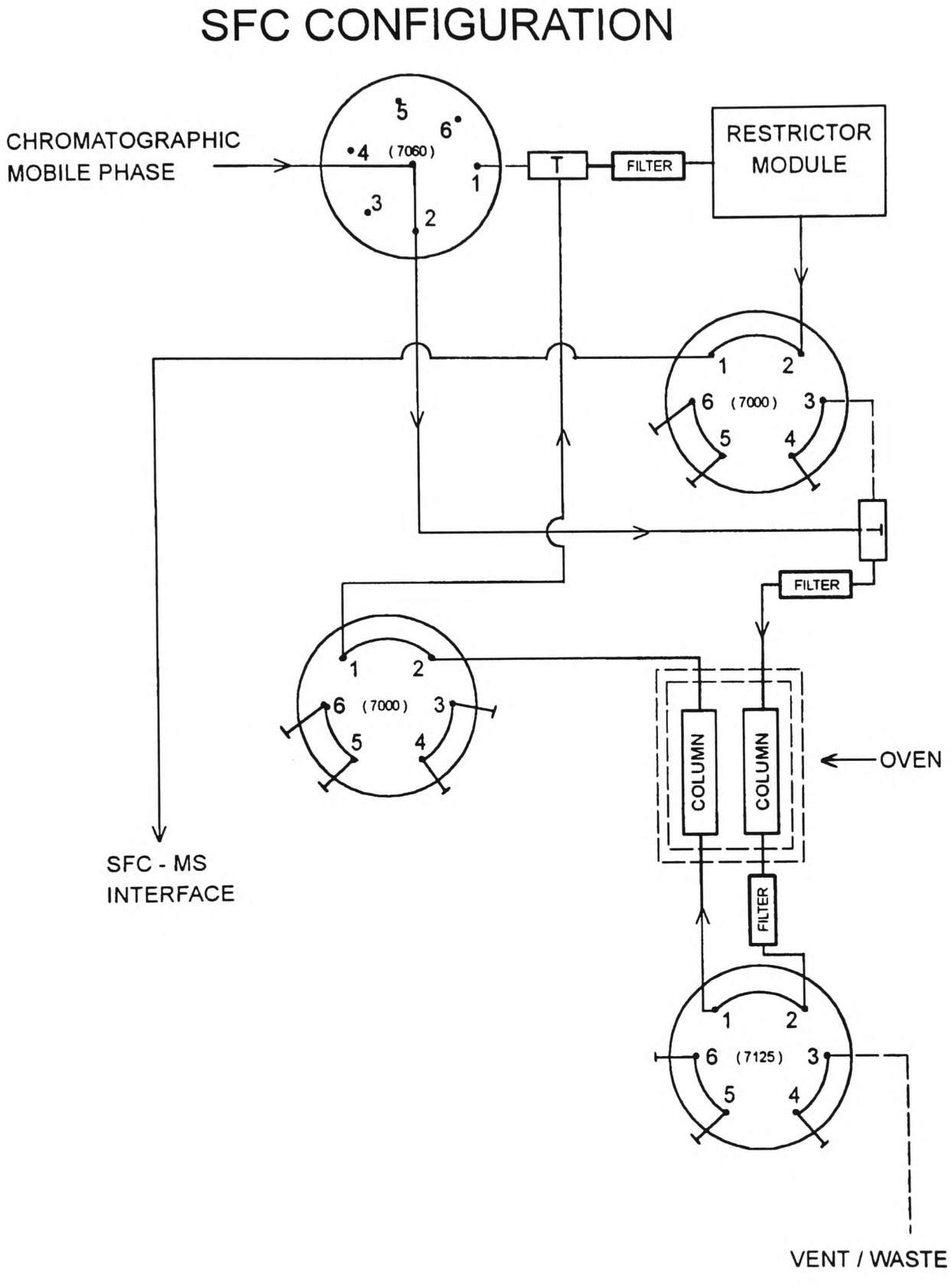


Fig 5.5 Supercritical Fluid Chromatography configuration

Initially a single amino column was used, with both trapping and separating procedures being carried out on this column. When these results were compared to the direct SFC procedure the results were seen to be poor and clearly degradation of chromatographic integrity had occurred. This degradation was attributed to imprecise trapping on the column, resulting in a reduced column length for chromatographic separation.

To overcome this problem a second dedicated chromatographic amino column was incorporated into the multi-valve configuration, allowing good separation of the phenol analytes to be achieved. For the SFE-SFC-MS extraction and analysis, a 250 cm^3 aliquot of the phenol solution was volumetrically transferred to the extraction system, resulting in 10 μg of each phenol being present, this was extracted for 45 min by dynamic aqueous SFE at a pressure of 32.33 MPa and carbon dioxide flow rate of 4 cm^3 min^{-1}, before the vessel was isolated and the multi-valve configuration switched to SFC-MS.

All SFC and SFC-MS studies were conducted at 30.89 MPa, with high purity methanol as an organic modifier for supercritical carbon dioxide. The mobile phase rate was set at 1.5 cm^3 min^{-1}, and the organic modifier programme set as Fig 5.6. Also the associated UV/Visible detector was set at 220 nm for all analyses.

Level	Ramp rate (% methanol)	% Methanol	Hold time (min)
0		0	2
1	1.0	1	1
2	2.0	10	2
3	10.0	30	40

Fig 5.6 Organic modifier programme

The SFE-SFC-MS results for all the phenols at the 40 ppb level are shown Figs 5.7 and 5.8, and despite the poor S/N response in full scan mode, each could readily be detected by monitoring their appropriate $[M - H]^-$ ion.

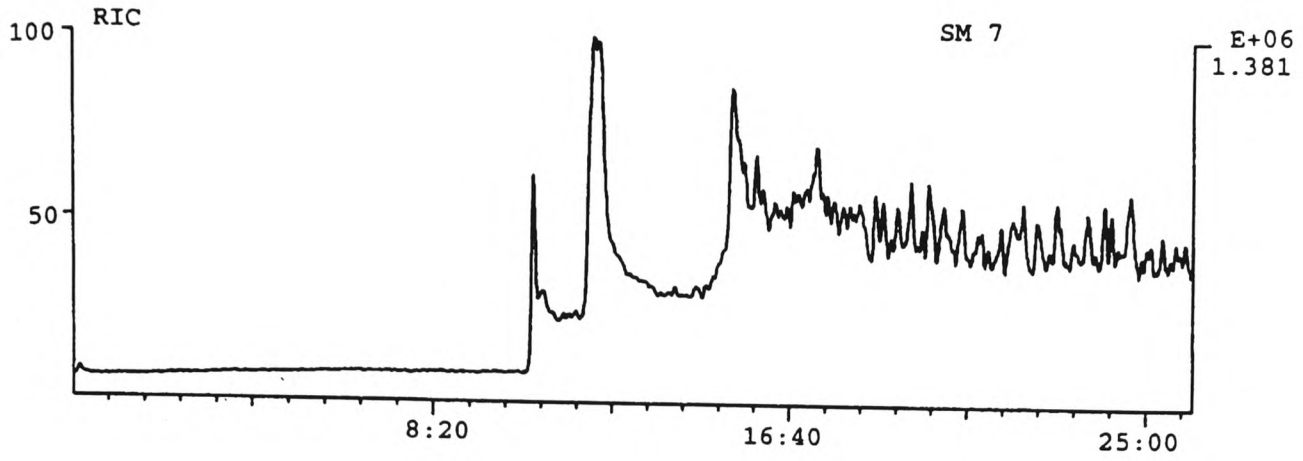


Fig 5.7 SFE-SFC-MS Full Scan Negative Ion Atmospheric Pressure Chemical Ionisation Spectrum of Phenol Mixture

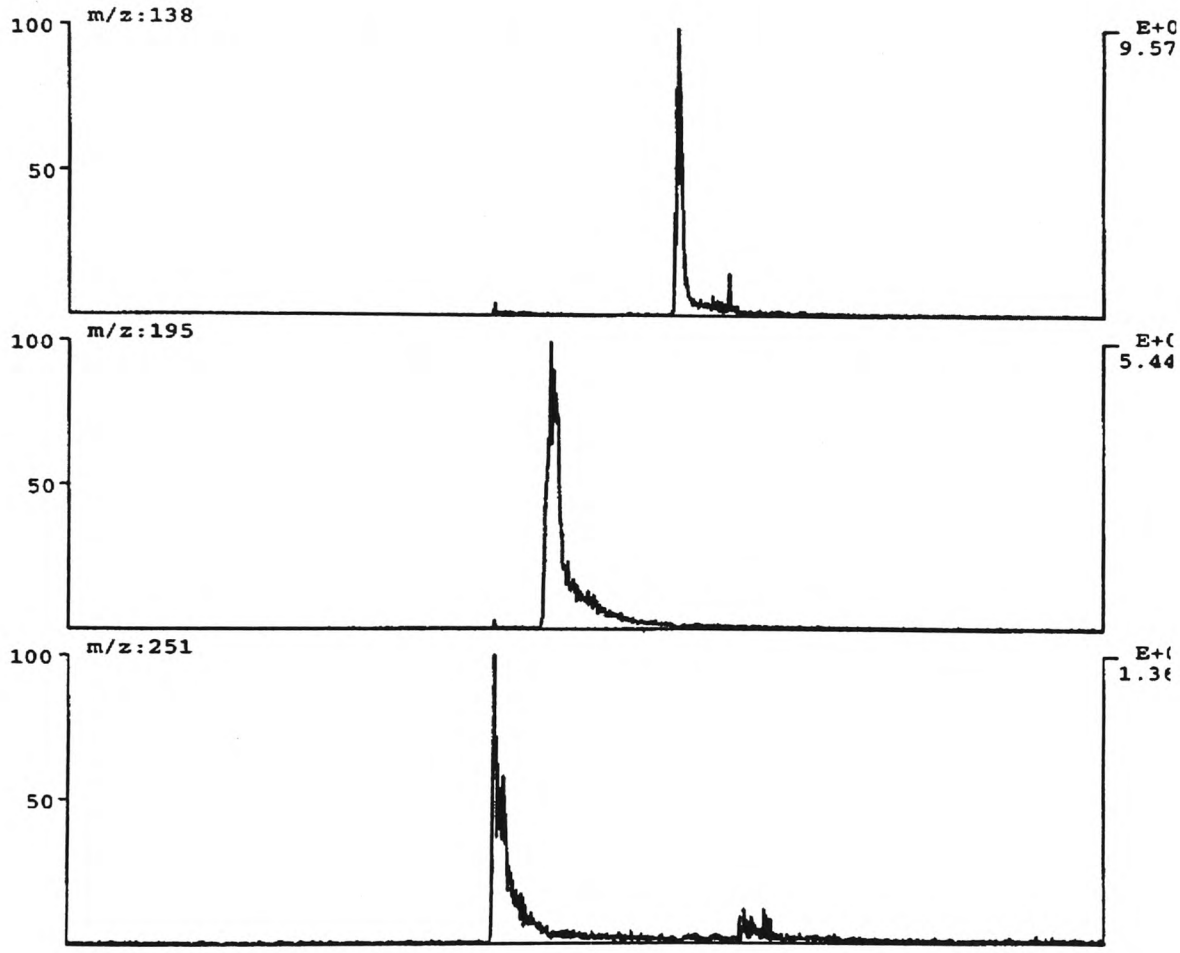


Fig 5.8 Individual Ion Profile Responses for $[M-H]^-$ Species for 4-nitrophenol (m/z 138), 2,4,5-trichlorophenol (m/z 195), 2,4-dibromophenol (m/z 251), (top to bottom)

In order to establish individual extraction efficiencies, the vessel was emptied and flushed with 100 cm^3 of deionised water. The combined washings were acidified to pH 2 using hydrochloric acid, and then extracted with 2, 100 cm^3 aliquots of ethyl acetate.

The combined ethyl acetate fractions were then combined and blown down to almost complete dryness with a stream of nitrogen, before being transferred to a 5 cm^3 volumetric flask. The contents were then volumetrically prepared to 5 cm^3 using an ethyl acetate solution containing an internal standard of anthracene, equivalent to 0.4 ng μl^{-1}.

Quantitative GC-MS studies were then carried out as before, and the extraction efficiencies of each analyte calculated against the calibration graphs produced before the extractions.

5.3 Results and Conclusions

Calculations based on these results showed the following extraction efficiencies.

2,4-dibromophenol	88%
2,4,5-trichlorophenol	81%
4-nitrophenol	67%

These results correlate to the retention times for these phenols, as both are related to the polarity of the phenols. This work has demonstrated the ability of SFE-SFC-MS to quantitatively extract, separate and quantify phenols at a level of 40 ppb. Quantification was achieved by production of the $[M-H]^-$ ion under the conditions employed for APCI. It is anticipated that further refinement of the technique should allow detection levels at the ppt level.

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Chapter 6

Application of Direct Aqueous Supercritical Fluid Extraction Procedures As On-line Sample Preparation Techniques for High Pressure Liquid Chromatography-Mass Spectrometry Studies

6.1 The Supercritical Fluid Extraction and Quantification of Estrone, Hexestrol and Zeranol by on-line High Pressure Liquid Chromatography –UV/Visible Diode Array Detection- Mass Spectrometry

6.1.1 Introduction

The separation of aqueous soluble analytes has frequently been carried out by chromatography after their extraction from the aqueous medium by liquid / liquid extraction (LLE). The requirement for the extraction and analysis of drugs and their metabolites, now has a very important role in the pharmaceutical and toxicological fields.

As many water soluble compounds tend to be either too polar, involatile or thermally labile for their analysis by gas chromatography, high pressure liquid chromatography (HPLC) tends to be the analytical tool of choice.

When the extraction phase is carried out off-line by LLE, there is little problem in completing the separation phase, the extracted, and concentrated, solution is simply introduced onto the HPLC column and separation initiated.
If extraction and analysis are to be carried out on-line, there needs to be coupling between these stages. This may sound straight forward, but there is a major complication, and that is the respective phases of these systems. Howard and Taylor[1] have published a review concerning on-line and off-line applications of SFE up to 1993, Rees, [2] has also reported on these applications, and has shown that SFE-HPLC has the potential to further augment the technique for samples which are,

- involatile and or thermally labile
- light or air sensitive
- present in ultratrace levels
- to be analysed using combined techniques

Further work with on-line coupling of SFE with chromatographic techniques have been reported by Vannoort *et al*, [3] Jinno and Saito,[4] Chester et al,[5] and Stuart et al, [6] and since the early work of Unger and Roumeliotis [7] little has changed in the basic configuration.

HPLC requires gas free solvents to carry out the separation as the presence of gas bubbles in the solvent will disrupt the continuity of separation, resulting in band broadening. To this end HPLC solvents are regularly de-gassed before use. Additionally the presence of gas bubbles provides erratic detectoir response generally resulting in a highly unstable baseline.

Supercritical fluid extraction relies in the solubilisation of the analytes in the supercritical fluid, which may also contain a modifier. To recover the analytes the supercritical fluid is decompressed when it returns to its gaseous state. For the reasons cited above, the presence of this gas will clearly have the potential to impinge on the efficiency of the chromatography stage.

To overcome this problem the decompressed gas must be flushed from the column before the chromatographic stage, leaving the analytes on the column for later separation, and analysis.

The second problematic area that had to be overcome, was the interfacing of the chromatographic stage to the spectrometric stage. As already stated, HPLC trends to be used for analytes which are too polar, involatile or thermally labile for GC, unfortunately one of the prerequisites for electron ionisation and chemical ionisation mass spectrometry is the ability to produce volatilised sample.
The problem with the interfacing is the elimination of the mobile phase from the HPLC system. A flow rate of 1 ml min^{-1} water results in 1244 ml min^{-1} vapour flow at

atmospheric pressure, and this is extremely difficult for a standard MS vacuum system to handle.

A further problem is that buffers and salts which are often added to the mobile phase, are also involatile and prove a further difficulty.

Several different LC-MS coupling methods have been developed, these include,

a. Removal of the mobile phase prior to spectrometric analysis, e.g. moving belt.

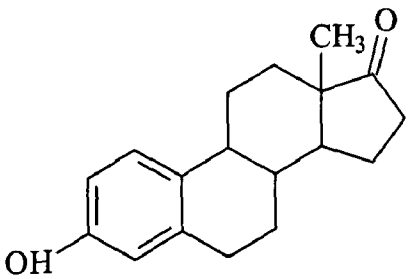
b. Reducing the flow by the introduction of a flow splitting device, this has the disadvantage of reducing the sensitivity e.g. continuous flow Fast Atom Bombardment (FAB).

c. Allowing the entire mobile phase or fraction of flow to enter the spectrometer, e.g. Electrospray (ES). This has a working flow range of approximately 0.2 ml min^{-1} which can be extended using turbospray, while Thermospray (TSP) and Atmospheric Pressure Chemical Ionisation (APCI) have a working flow range of about 1 ml min^{-1}.

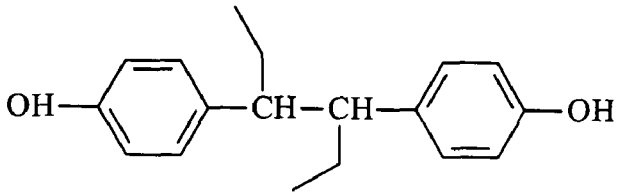
For this reported work a Finnigan MAT 4000 series quadrupole mass spectrometer equipped with a Finnigan MAT moving belt interfaced was coupled to a Shimadzu HPLC-DAD system was used

The analytes used in this study were

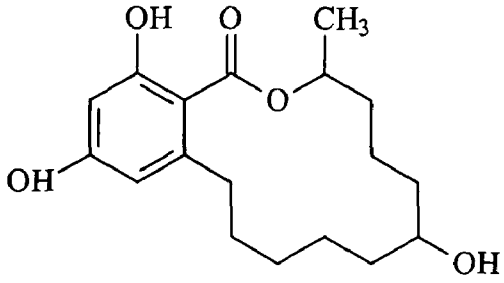
a. ESTRONE



b. HEXESTROL



c. ZERANOL



6.1.2 Experimental

The initial work, before supercritical fluid extraction, consisted of an off-line HPLC study of the three drugs each dissolved in methanol, prepared by the addition of 10 mg of each drug volumetrically in 5 cm^3 of solvent. The valve setting positioned for SFE allowed the analysis to be conducted using only an octadecylsilyl-modified silica (ODS) column.

Once extraction was completed, the ODS column could be linked to an aminopropyl (amino) column using the valve configurations shown in Fig 6.17 and 6.18.

The set-up was then changed to include both columns in series, thus allowing the determination of whether both, ODS and amino columns could be used for this analysis.

A gradient programme was developed to ensure rapid elution of the drugs off the non-polar ODS column with band focusing onto the polar amino column, during the period when the mobile phase was 100% heptane.

The principle reason for developing the coupled HPLC column system was that the trapping column alone proved ineffective when chromatography was attempted. This was due to the analytes being unfocused when trapped on the column, which could lead to band broadening, and poor chromatographic integrity.

Fig 6.1.1 shows two 10 μl injections using the coupled ODS-aminopropyl column system, with a total sample quantity of 40 μg each, with a 5 min interval after the initiation of the gradient programme. The fact that the chromatogram consists of single peaks demonstrates the efficiency of band focusing by the amino column, prior to the onset of the gradient programme as described in the previous paragraph. This is a major advantage given the imprecise zone trapping on the ODS column.
The results of these two chromatographic set-ups were then compared, amino column by itself and also the coupled amino-ODS column system, and showed that no chromatographic integrity was lost by the addition of the ODS column.

Figures 6.1.2 to 6.1.4 show the individual UV/Visible spectra of the individual compounds.

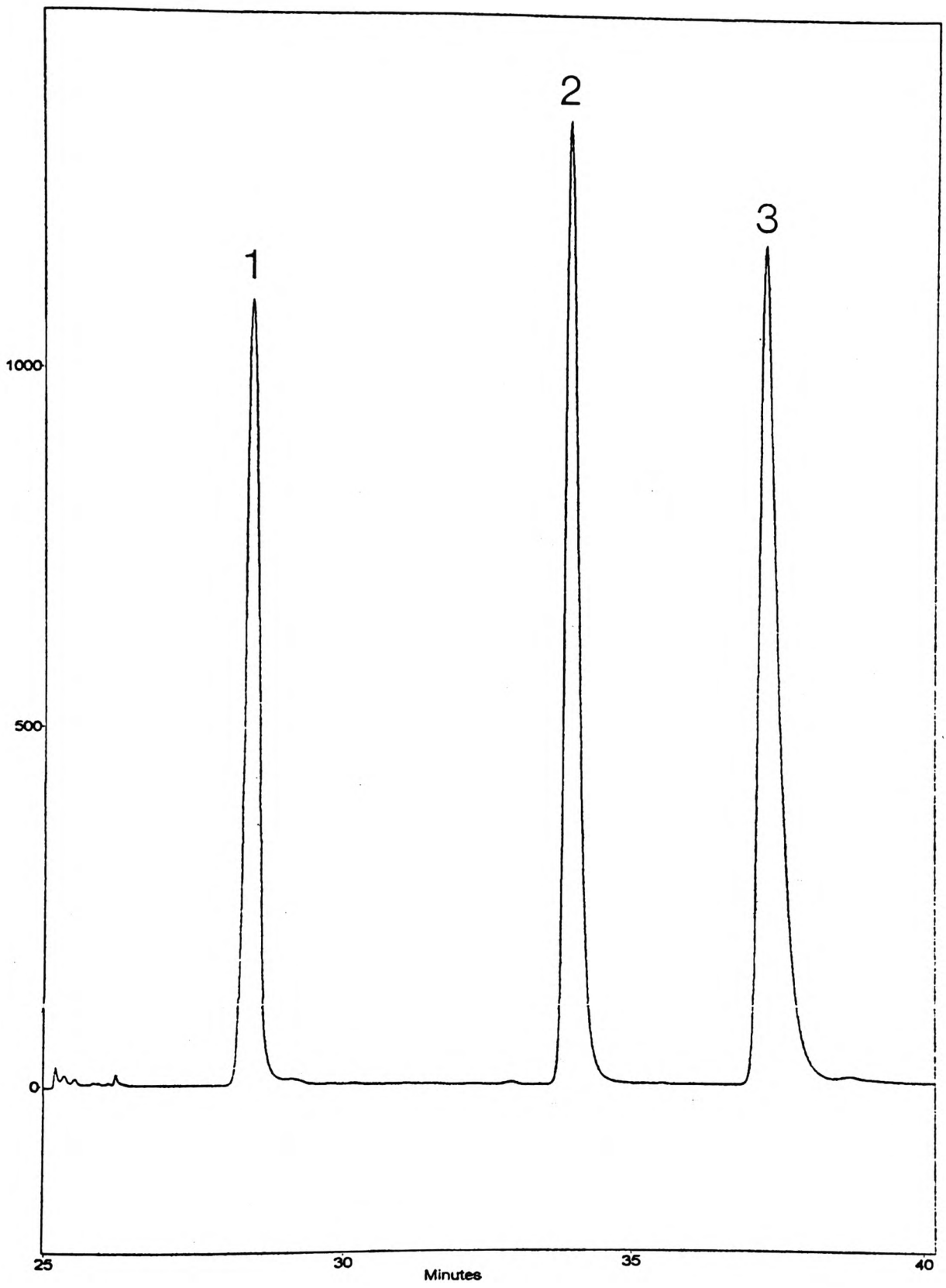


Fig 6.1.1 Off-line LC analysis of drug mixture using coupled ODS-amino columns.
Detection UV/VIS DAD chromatogram at 281 nm.
Peaks 1, estrone; 2, hexestrol; 3, zeranol

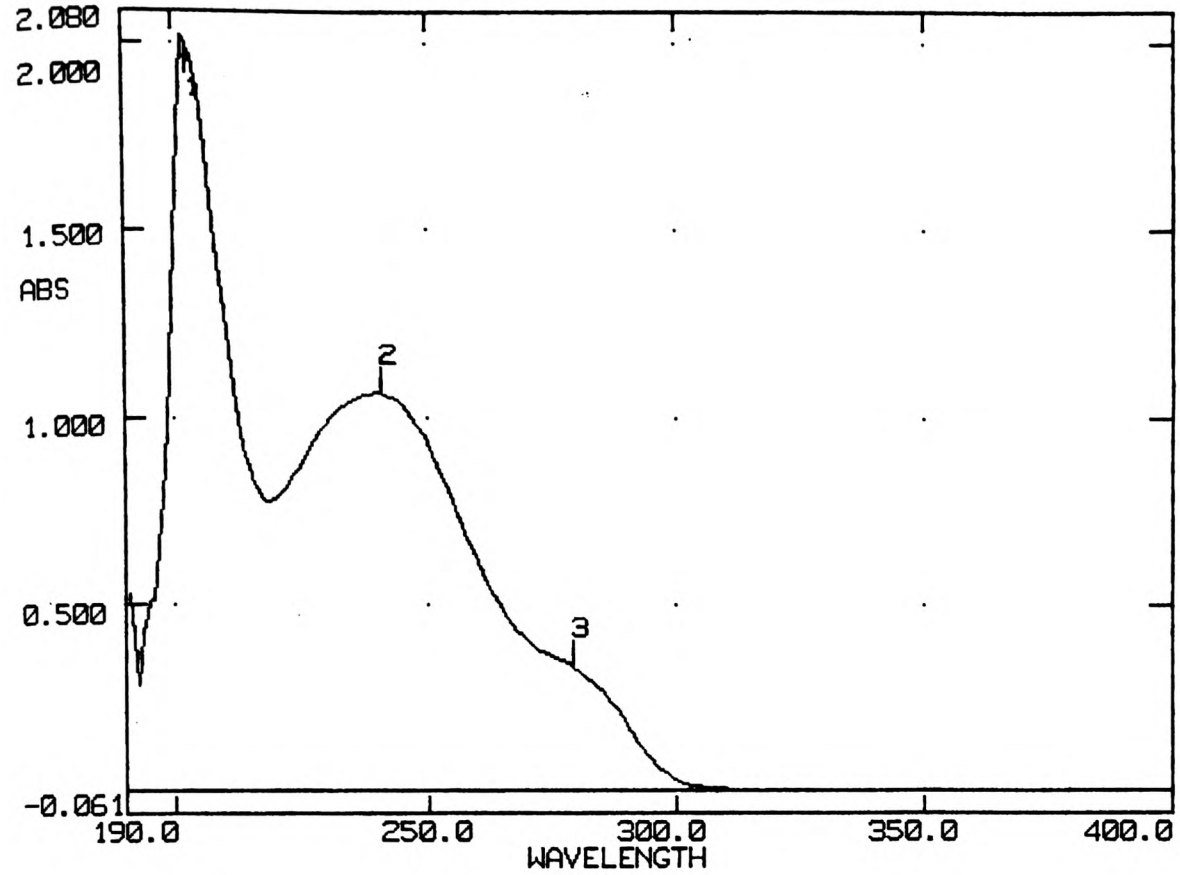


Fig 6.1.2 HPLC UV/Visible spectrum of Estrone

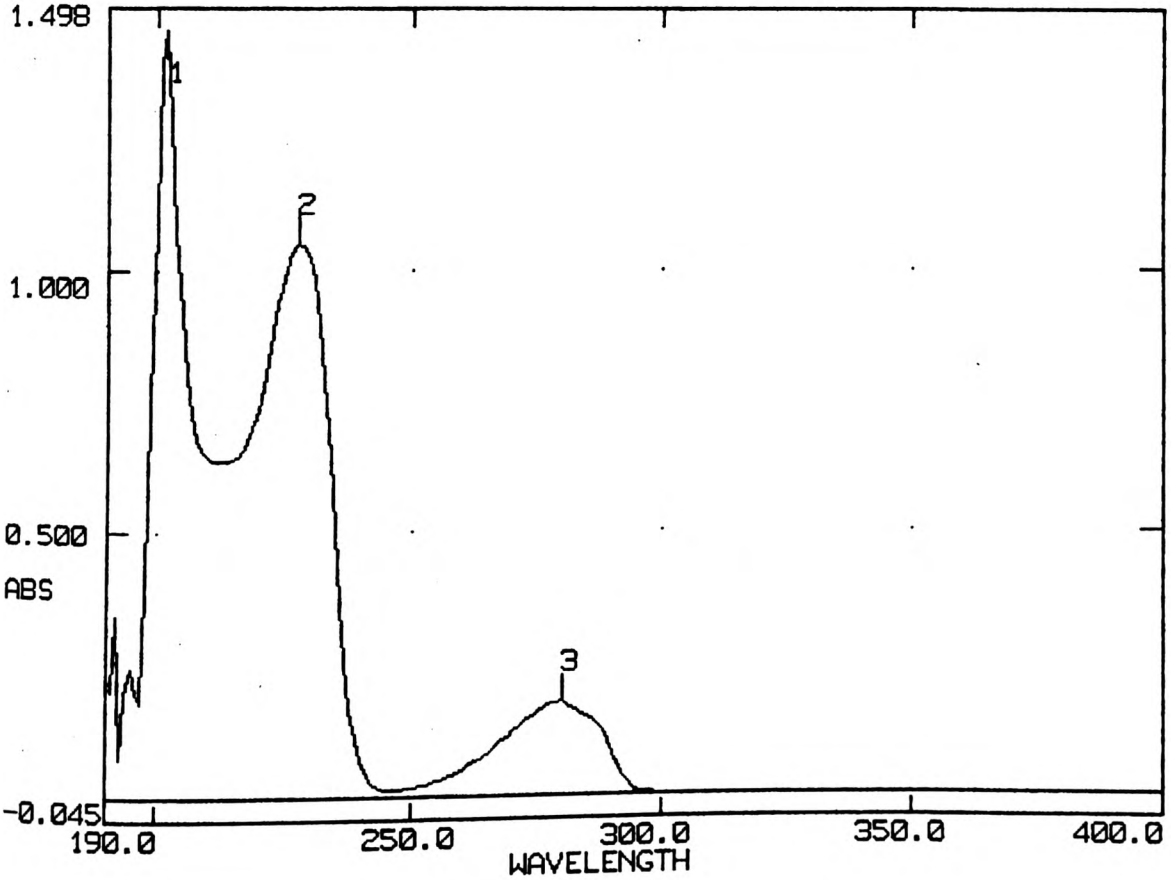


Fig 6.1.3. HPLC UV/Visible spectrum of Hexestrol

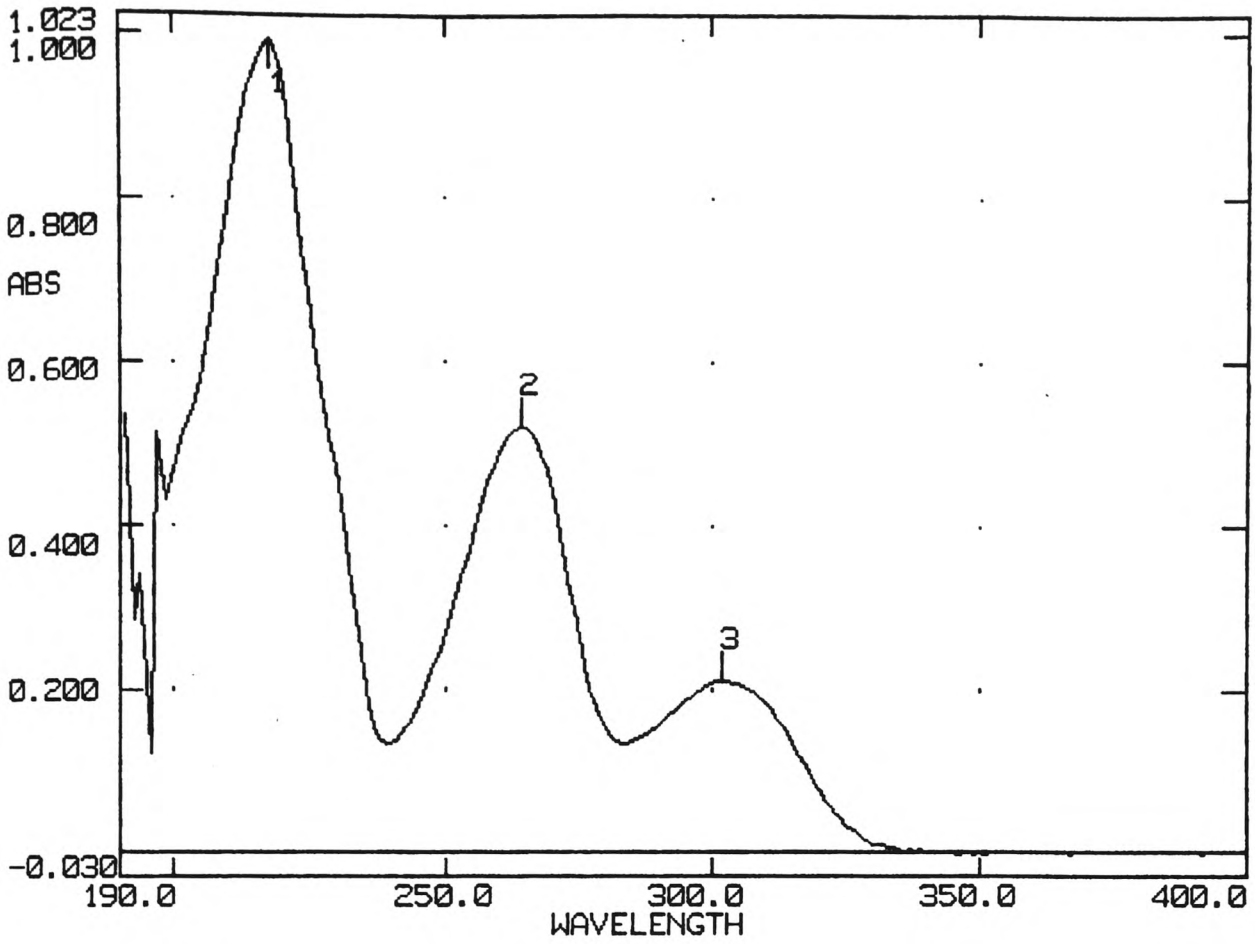


Fig 6.1.4. HPLC UV/Visible spectrum of Zeranol

Mass spectra of each of the compounds were also obtained by the direct application of the analyte solutions onto the moving belt. This work was carried out under ammonia CI ionisation, Figs 6.1.5 to 6.1.7

Details of chemical ionisation techniques have been introduced in section 1.5.2.2.

Chemical ionisation using ammonia can be useful in limiting levels of fragmentation, and can result in the formation of a sample adduct ion formation $(M+NH_4)^+$ ion, which is observed at 18 amu above the molecular ion. The spectra also show the protonated molecular ions for the drugs for estrone $(M+H)^+$ m/z = 271, zeranol $(M+H)^+$ m/z = 323, and the most abundant ion for hexestrol was m/z = 135. This ion was not the protonated molecular ion, but was a result of symmetrical splitting of the molecule. Single ion monitoring, studies involved the detection of the ions at m/z 271 for estrone, m/z 323 for zeranol and m/z 135 for hexestrol.

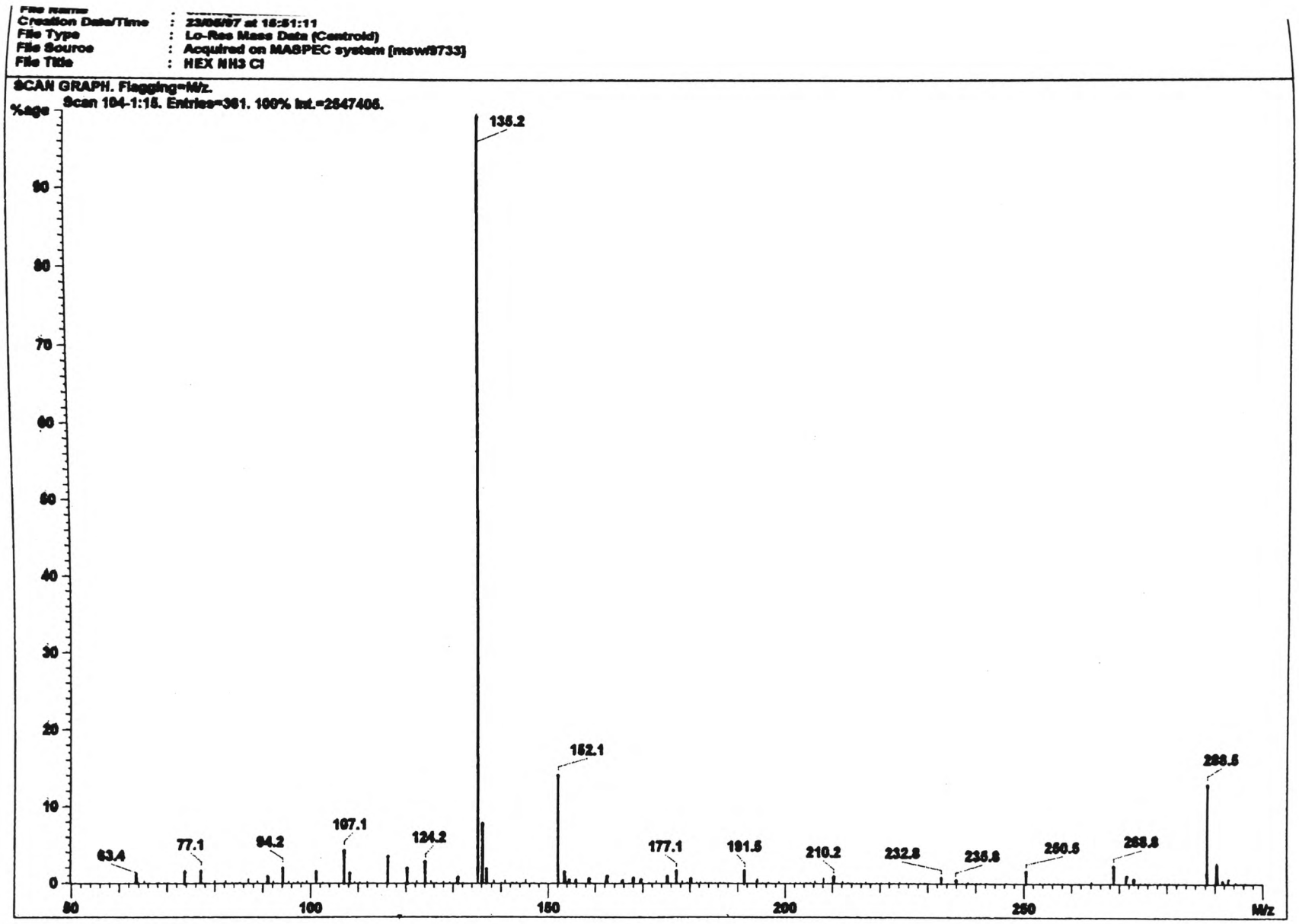


Fig. 6.1.5 Ammonia CI mass spectrum of Hexestrol

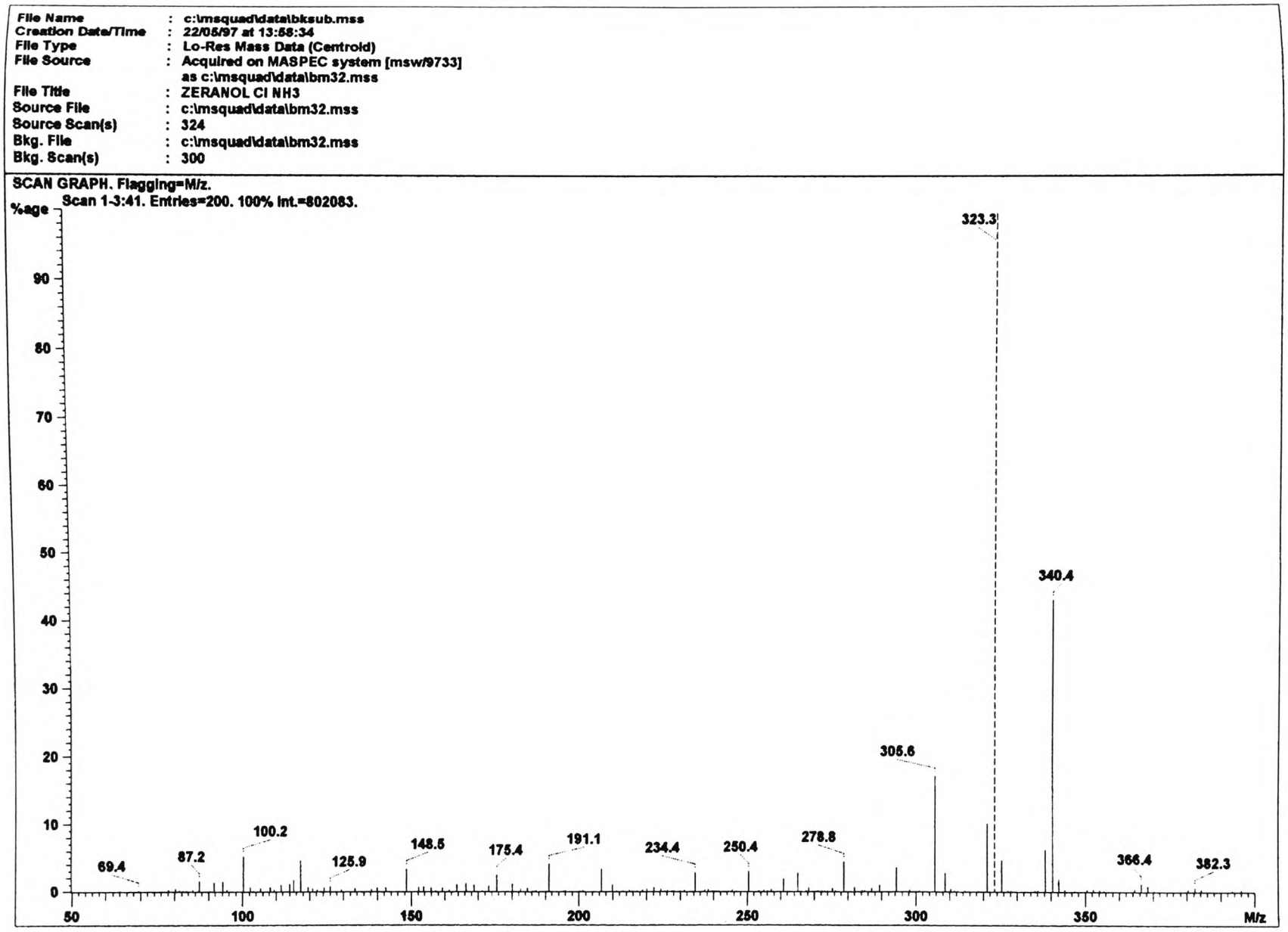


Fig 6.1.6 Ammonia CI mass spectrum of Zeranol

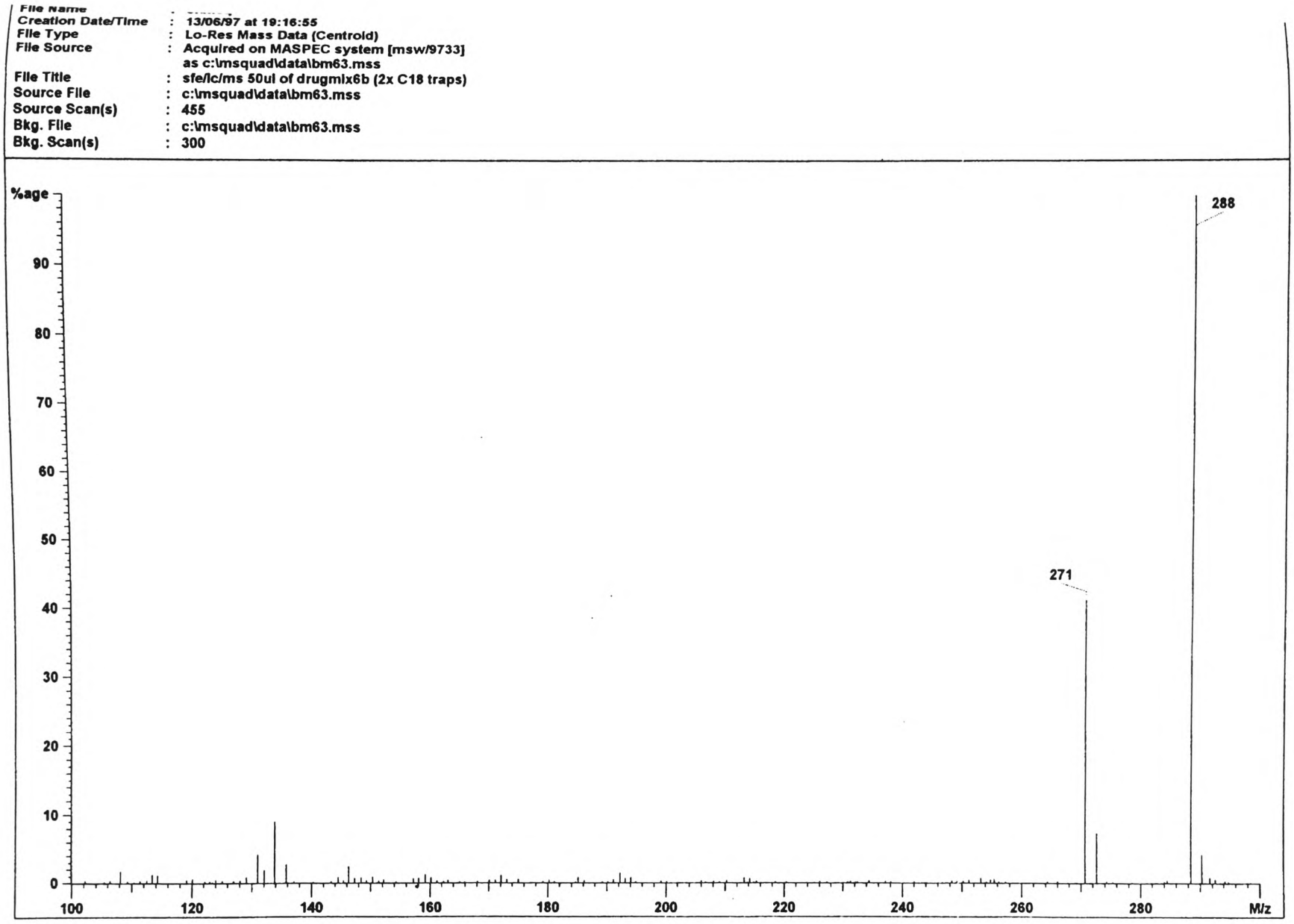


Fig. 6.1.7. Ammonia CI mass spectrum of Esterone

system described in section 2.4.1. and shown in Fig 2.15. It had an internal volume of 300 cm^3 and was housed in a Pye 104 gas chromatography oven.

This SFE vessel was coupled into the LC-MS system by the development of a novel Rheodyne ten port switching configuration shown in Figs 6.1. and 6.1.9.

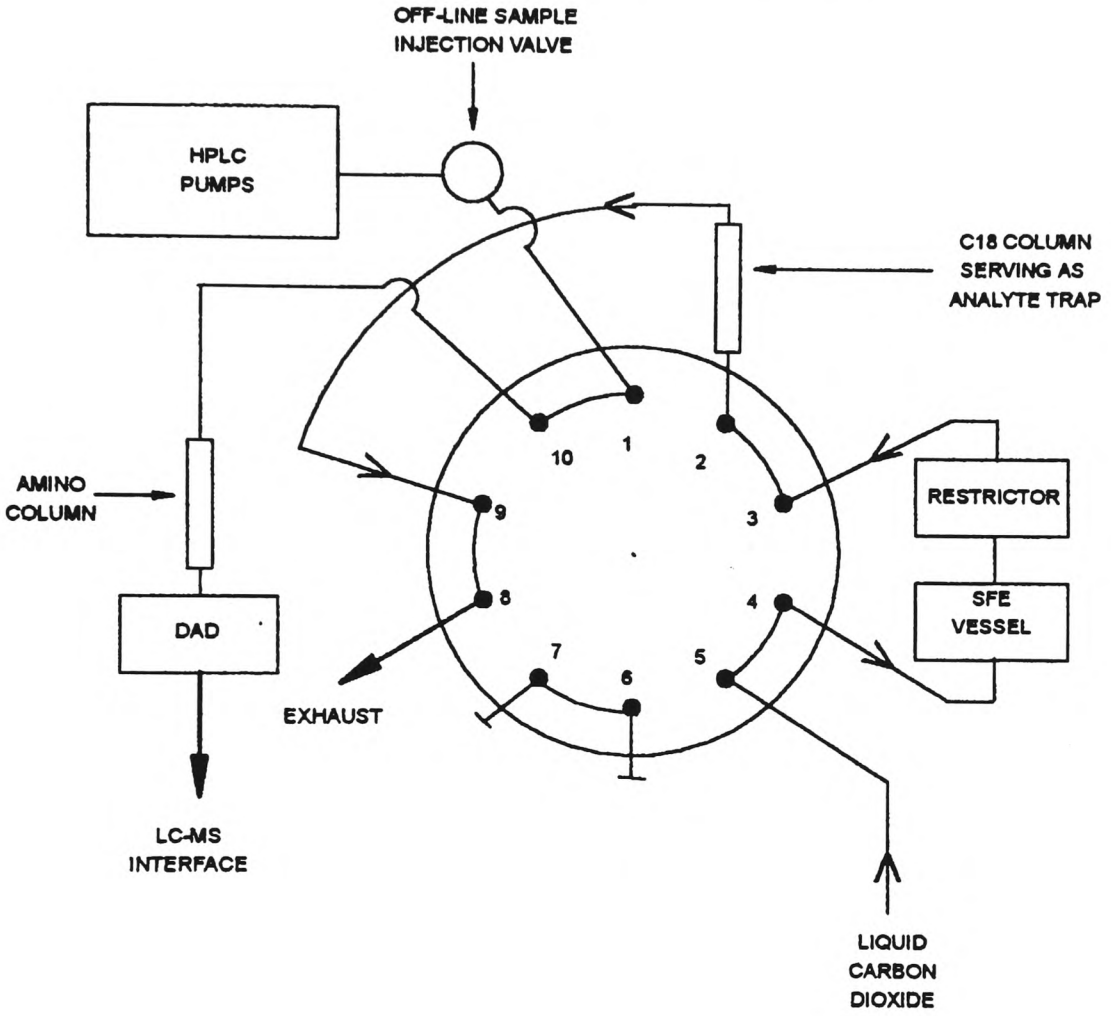


Fig. 6.1.8 Ten port valve direct aqueous SFE configuration, used for trapping extracted analytes.

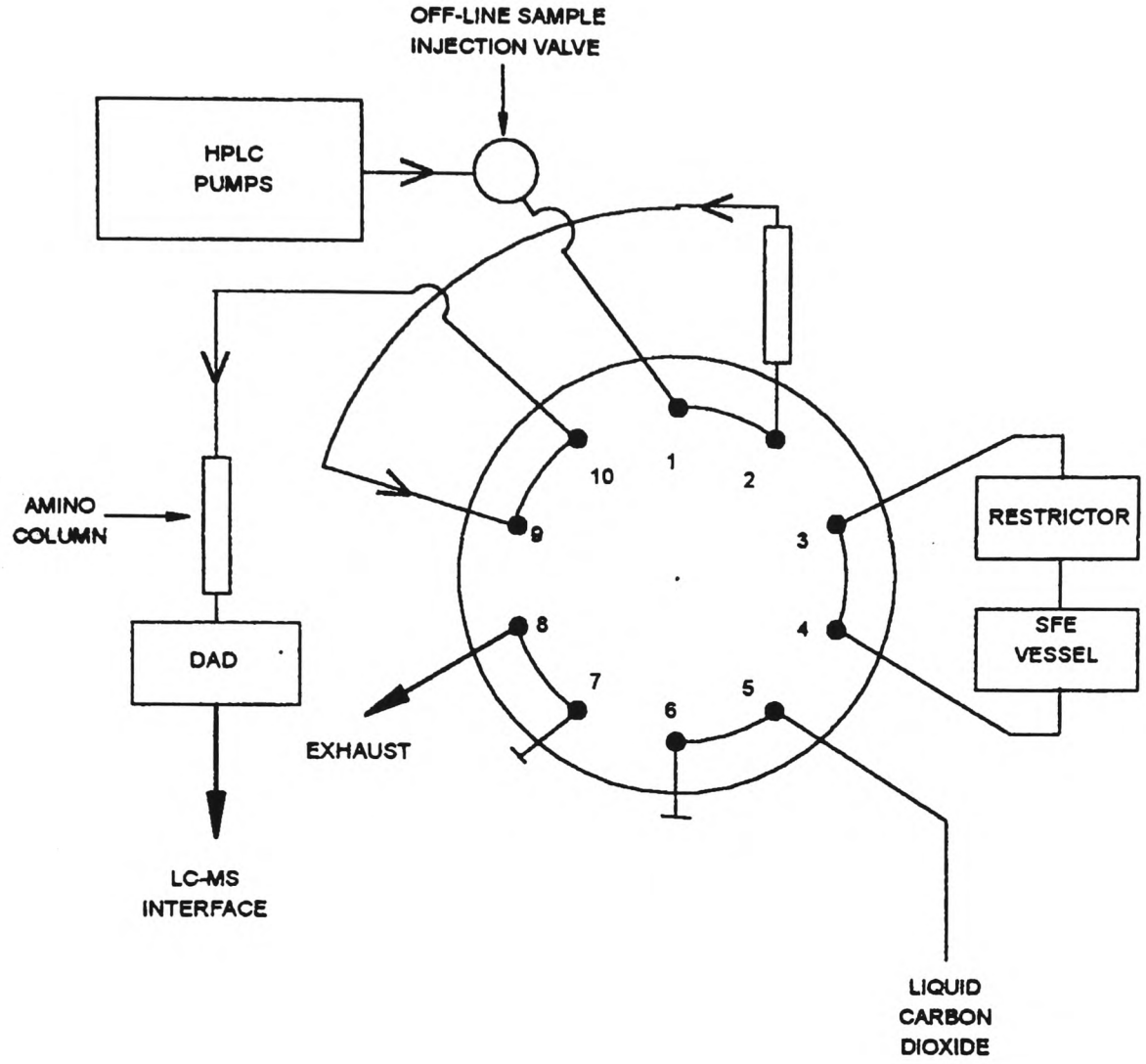


Fig 6.1.9 Ten port valve LC-MS configuration, used for analysing the extracted analytes.

Liquid carbon dioxide was supplied to the extraction vessel by a Gilson 303 pump equipped with a cooling head attached to a Neslab RTE 110 recirculator and operated at –10 °C. The output from the vessel was connected to a Tescom Model 26-1722-24-084A restrictor.

The valve configuration allowed the ODS column to be connected to an amino column which was interfaced to a Finnigan MAT 4000 series quadrupole mass

spectrometer equipped with a Finnigan MAT moving belt LC-MS interface , of the type shown below in Fig.6.1.10, for the LC-MS analysis.

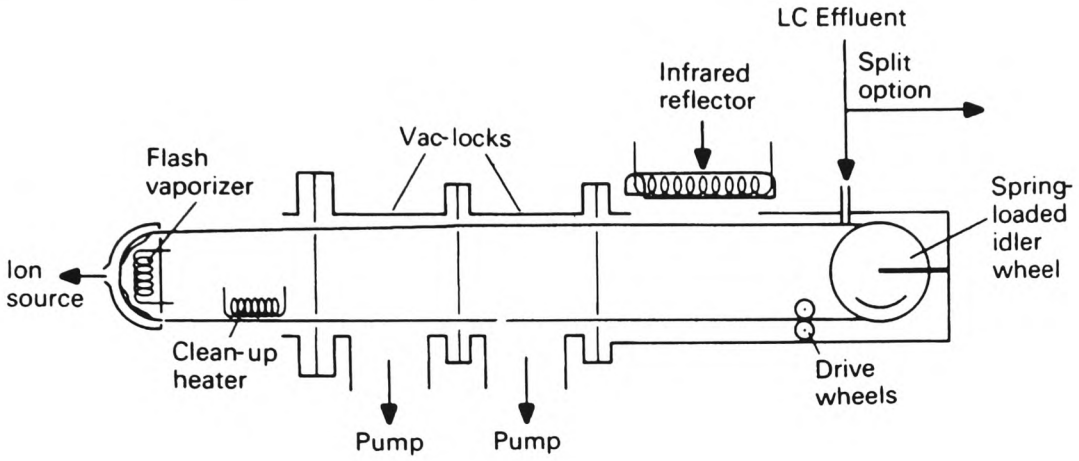


Fig. 6.1.10 Finnigan MAT moving belt LC-MS interface

The LC eluent was sprayed onto a polyamide belt via a nebulizer, this results in an even aerosol spray coating, and assists the removal of the mobile phase as it passes under the infrared heater. The belt then moves through two sets of vacuum locks, and into the high vacuum side of the interface. At this point the analyte is subjected to flash volatilisation by means of a tip heater, and the volatilised analyte enters the mass spectrometer ion source and is subsequently analysed.
The belt then passes a clean-up heater and wiper, before having more solution deposited on it.

The EI studies were conducted at 70 eV with an ion source temperature of 200 °C. The CI SFE-LC-MS studies were conducted using high purity ammonia as the reagent gas at a source housing pressure of 2.5 MPa using an ion source temperature of 150 °C.

Liquid chromatography was performed using a Shimadzu HPLC system with high purity heptane and ethanol as the mobile phase.

Before SFE, the ODS column, which had previously been flushed with heptane, was dried with carbon dioxide, and the amino column conditioned with pure heptane, to remove any traces of carbon dioxide gas.

An aqueous solution of the three drugs estrone, hexestrol and zeranol was prepared so that each component was present at a level of 200 ppb. A 250 cm^3 aliquot of the solution, equivalent to 50 μg of each drug, was transferred into the extraction vessel. The valve configuration was switched to SFE mode, and the extraction performed at 50 °C, with a carbon dioxide extraction pressure of 24.1 MPa and flow rate of 8 cm^3 min^{-1} for 30 min.

At the end of the extraction the carbon dioxide flow rate was set to zero, and the column allowed to depressurise for a further 5 min before the valve was switched to the LC-MS configuration, Fig. 6.1.9.

The LC-MS analysis was conducted with a gradient programme of pure heptane for 12 min, which effectively band focused the drugs onto the amino column, then to heptane : ethanol (65 : 35) at 42 min, followed by a linear profile at a flow rate of 1 cm^3 min^{-1}, with the UV/Vis detector set at 281 nm.

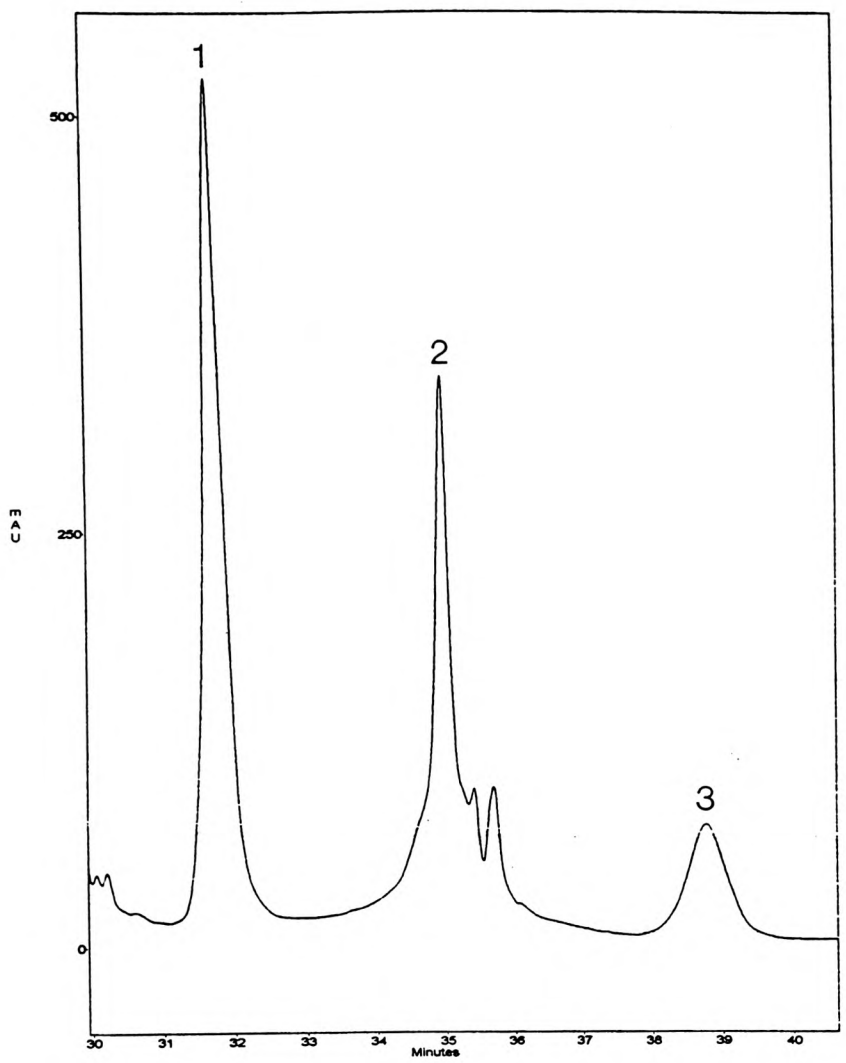


Fig 6.1.11 SFE-LC analysis of 250 cm^3 water sample spiked with 200 ppb of each drug. DetectionUV/VIS DAD chromatogram at 281 nm.
Peaks: 1, estrone; 2, hexestrol; 3, zeranol

The increase in the retention time between the off-line and on-line analysis is attributed to the time required to purge the gaseous carbon dioxide through the coupled columns, and the peak splitting associated with the hexestrol peak may be due to traces of co-extracted water.

The SFE-LC-MS spectra, both full scan and ion profiles of the $[M + H]^+$ for estrone and zeranol, and for the m/z 135 hexestrol for the drug mixture are shown in Fig 6.1.12.

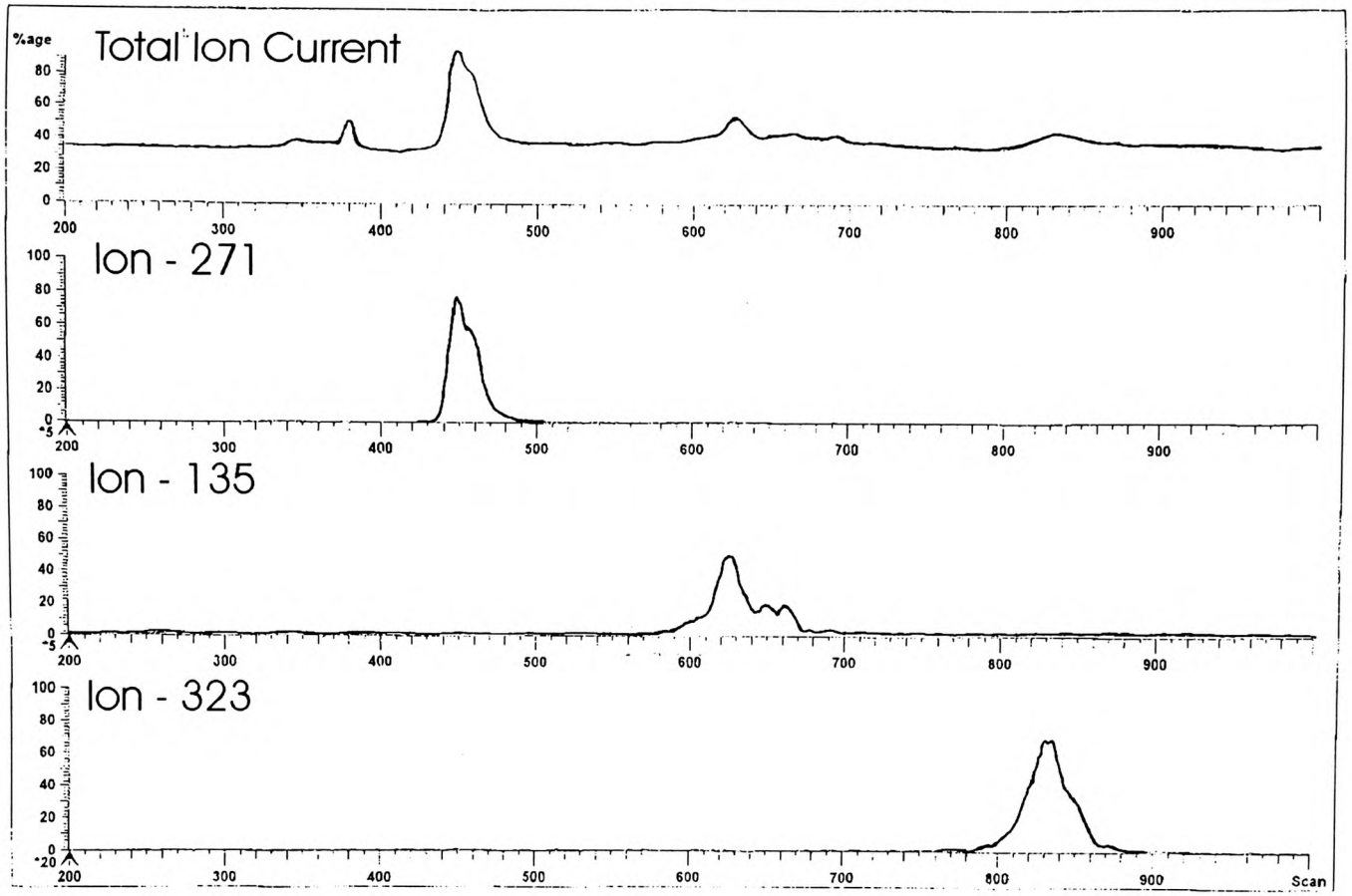


Fig. 6.1.12 Full scan ammonia CI total ion chromatogram for 250 cm^3 aqueous sample containing 200 ppb of each drug. Single ion profiles of $(M+H)^+$ for estrone(m/z 271), hexestrol (m/z 135) and zeranol (m/z 323)

6.1.3 Conclusion

Direct coupling of SFE with HPLC is demonstratively more difficult than coupling to GC or SFC (Supercritical fluid chromatography), but illustrates the ability to selectively extract analytes in preference to interfering co-species. The potential for a rapid extraction, clean up procedure with minimal sample handling has been demonstrated using this technique

This study has further demonstrated the feasibility of direct aqueous SFE-LC-MS. The extraction of 250 cm^3 of an aqueous solution, in conjunction with on-line UV/VIS diode array and selected ion monitoring mass spectrometry analysis.

The use of a coupled octadecylsilane (ODS) amino column, combined with full scan ammonia chemical ionization , allowed both trapping and analysis, of an aqueous solution of estrone, hexestrol and zeranol at the 200 ppb level.

6.2 The Supercritical Fluid Extraction and Quantification of the Ionophores Monensin, Lasalocid, Salinomycin and Narasin by on-line High Pressure Liquid Chromatography–UV/Visible Diode Array Detection- Tandem Mass Spectrometry

6.2.1 Introduction

Ionophores are carboxylic polyether antibiotics, and were originally utilised to treat coccidiosis,[8] an intestinal disease suffered by poultry and cattle, which is caused by a protazoa, and as growth promoters.[9] The major damage to livestock arises from the rapid multiplication of the parasite in the intestinal wall, and results in the subsequent rupture of the cells in the wall lining. One of the side effects of infestation is reduced food consumption, and in serious cases can lead to death. Spread of the infection is rapid especially in cattle between 1 to 12 months of age.

Ionophores are extensively used within poultry and beef farming industries, and are administered orally, monensin being the first ionophore to gain approval , in 1971, for use with poultry. [10]

In 1975 it gained approval from the United States Food and Drug Administration (FDA) for use in confined cattle to improve feeding efficiency, and by the end of 1999, seven ionophores were approved for use with livestock in the USA. These were Laidlomycin, Lasalocid, Maduramicin, Monensin, Narasin, Salinomycin, and Semduramicin. Their usage is now worldwide, and at present there are no limits on polyether residues in food.

Ionophores are a structurally diverse family of compounds. They have oxygen atoms spread throughout their structures which can create "cavities" to trap cations. Ionophores also have polar and non-polar regions which can enhance this trapping ability, and this allows them to interact with cell membranes. It is this ability that allows the compounds

to interact with bacteria, protozoa and fungi in the gut, and so alter the pattern of digestion.

With the use of ionophores, cattle can obtain 20% more metabolisable energy, and a mean increase on weight production of 12%, along with an increase in milk production and fertility.

The benefits of administering ionophores has three main effects[11]

1. Increased efficiency of energy metabolism.
2. Improved nitrogen metabolism
3. Decreased digestive disorders.

The benefits to the producer is more efficient weight gain through improved nutritional intake, and a lower mortality rate due to a reduction in digestive disorders.

Ionophores reduce certain bacteria within the digestive tract, thereby boosting the productions of others which help increase the efficiency of dry feed, and help improve the nitrogen uptake from such feed. [12-15]

The ionophores used in this study were, Salinomycin (Fig 6.2.1), Monensin (Fig 6.2.2), Lasalocid (Fig 6.2.3), and Narasin (Fig 6.4).

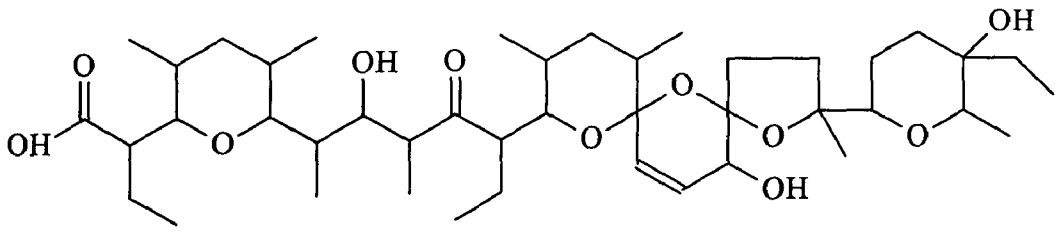


Fig 6.2.1 SALINOMYCIN

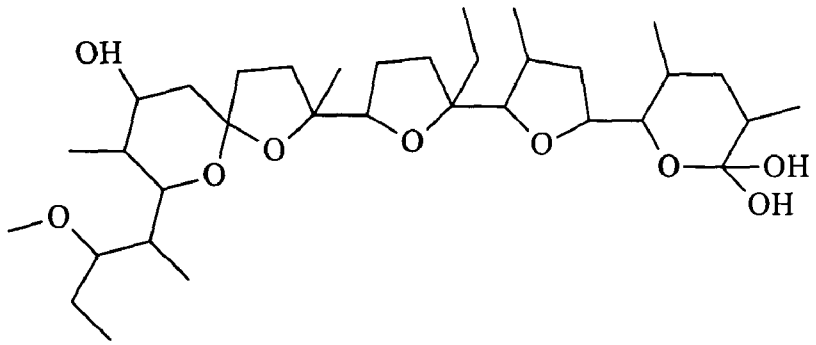


Fig 6.2.2 MONENSIN

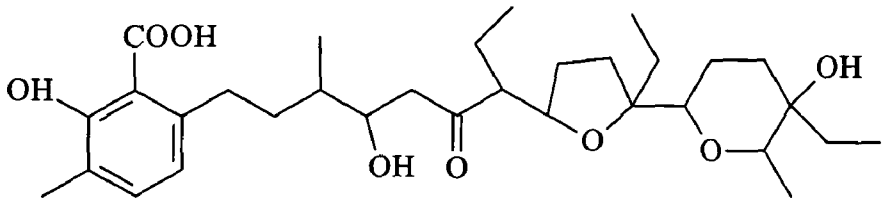


Fig 6.2.3 LASALOCID

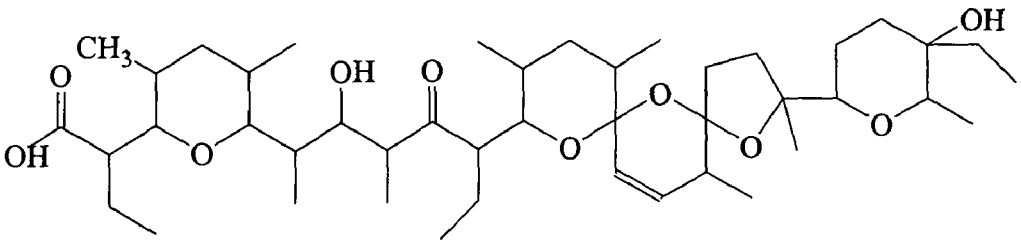


Fig 6.2.4 NARASIN

6.2.2 Development of an off-line SFE procedure for analyzing ionophores extracted from aqueous solutions using LC-MS-MS for detection

The initial phase of this reported work required the mass spectrometric analysis and determination of the HPLC separation parameters for all four ionophores. All spectrometric analysis was carried out on a Perkin Elmer SCIEX API III LC-MS-MS system, fitted with an Apple Macintosh data system.

The sample, which was essentially a liquid stream of polar compounds, entered the system through an articulated ion spray inlet, and was introduced either, in the initial stage by being infused using a syringe pump, or after the separation and analyse parameters had been determined, from the outlet of the high performance liquid chromatograph (HPLC) system. Depending on the input flow rate, the input stream may need reducing. This was achieved by splitting the stream and allowing only a limited portion (10 $\mu l\ min^{-1}$) to enter the source region and be ionized, with the remainder of the flow was exhausted.

The API III system, Fig 6.2.6, consists of two scanning quadrupoles (Q1 and Q3) separated by an open structure quadrupole collision cell (Q2). The liquid sample was introduced into the ion source, where "soft" ionization, electrospray generates ions (generally including high relative abundance protonated molecular ions). The first mass analyser (Q1), separates these electrospray formed ions according to their mass to charge ratio (m/z), and allows them to pass to the collision cell (Q2). Here the selected precursor ion are fragmented by collision with Argon gas, and the fragmented ions produced (product ions) which passed into Q3, where they are scanned to produce a spectrum. Either all ions produced in Q1 can be transmitted, or specifying a given RF/DC voltage(s) only a single ion(s) can be transmitted.

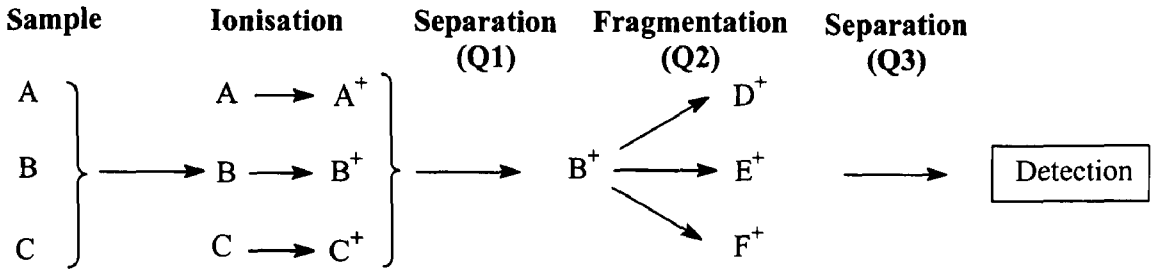


Fig 6.2.5 Sample Fragmentation and Isolation of Product Ions

The technique of MS-MS is well suited to mixture analysis as long as the parent ions (or target ions) have different m/z values. This can enable the product ion spectrum to be produced with little or no pre-analysis separation being required.

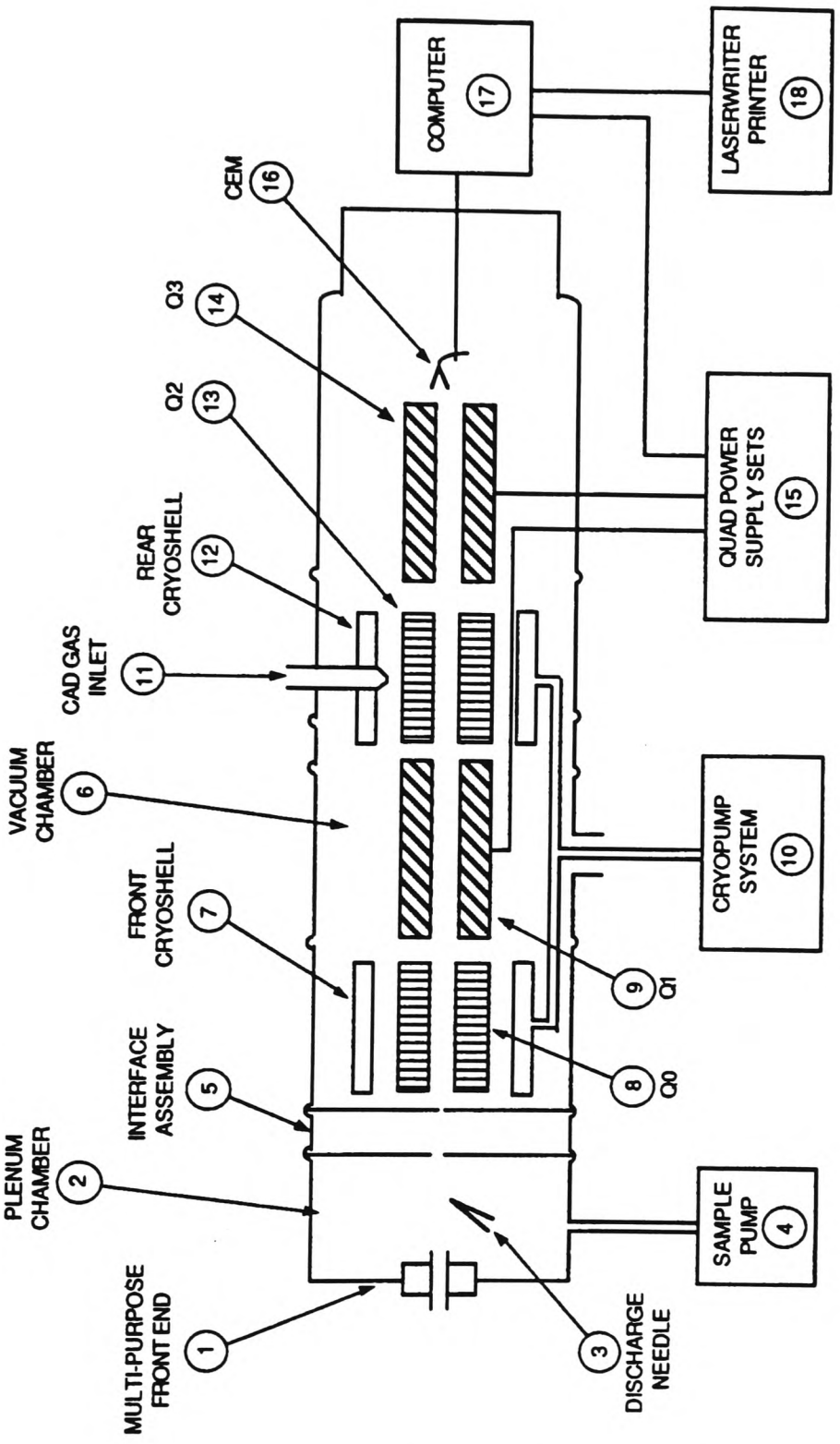


Fig 6.2.6 Schematic Diagram of Triple Quadrupole Mass Spectrometer

Stock solutions of each ionophore was prepared by dissolving a known quanitity of each in 10 cm^3 of methanol . These stock solution were further diluted to give working solutions. These stock solutions were introduced at a rate of 10 $\mu l\ min^{-1}$ into the articulated ion spray interface using a syringe pump.

Ionophore	Stock solution concentration	Working solution concentration
Monensin	0.7 mg in 10 cm^3	100 μl of stock solution in 2 cm^3
Lasalocid	0.9 mg in 10 cm^3	100 μl of stock solution in 2 cm^3
Salinomycin	1.1 mg in 10 cm^3	100 μl of stock solution in 2 cm^3
Naracin	0.9 mg in 10 cm^3	100 μl of stock solution in 2 cm^3

The working solution of each ionophore was directly introduced into the LC-MS-MS system using a syringe pump with a flow rate of 10 $\mu l\ min^{-1}$, and the resulting parent and daughter ion spectra of each ionophore are shown in Fig 6.2.7 to 6.2.14.

The analysis of the data obtained for this initial off-line screening, resulted in the selection of the following parent and daughter ions to be used when analysis of the ionophores was performed using multiple reaction monitoring (MRM). Collision energies of Q2 were investigated as part of this process of ion pair selection.

Ionophore	Parent Ion (m/z) (Q1)	Daughter Ion (m/z) (Q3)
Monensin	693	461
Lasalocid	613	377
Salinomycin	773	431
Naracin	787	431

The next phase of this reported work was the investigation of :-

a. the trapping efficiency of the HPLC columns for the four ionophores,
b. the chromatographic parameters and separation profile for the ionophores.

Monensin infused using standard MS conditions

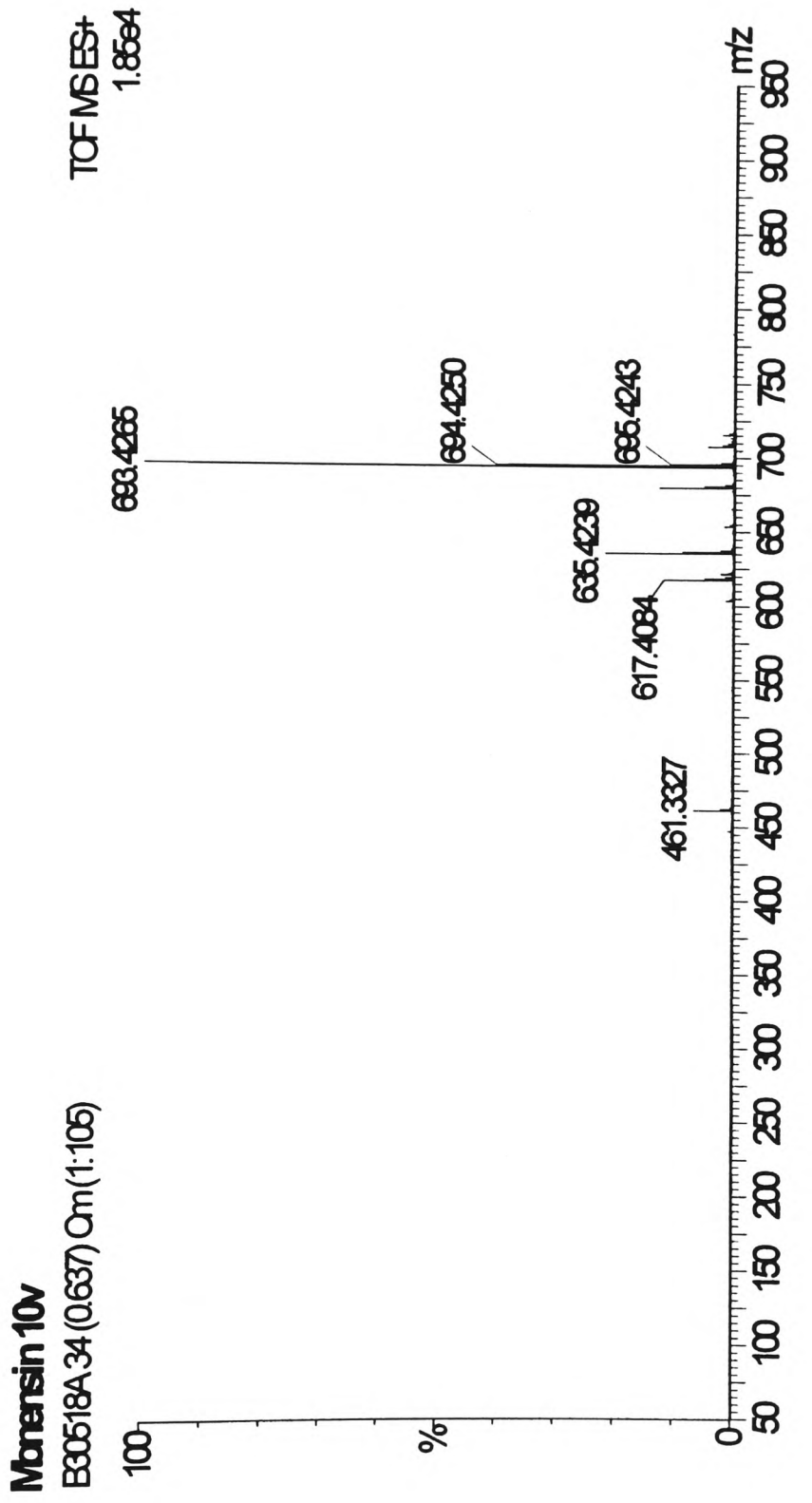


Fig 6.2.7

Lasalocid infused using standard MS conditions

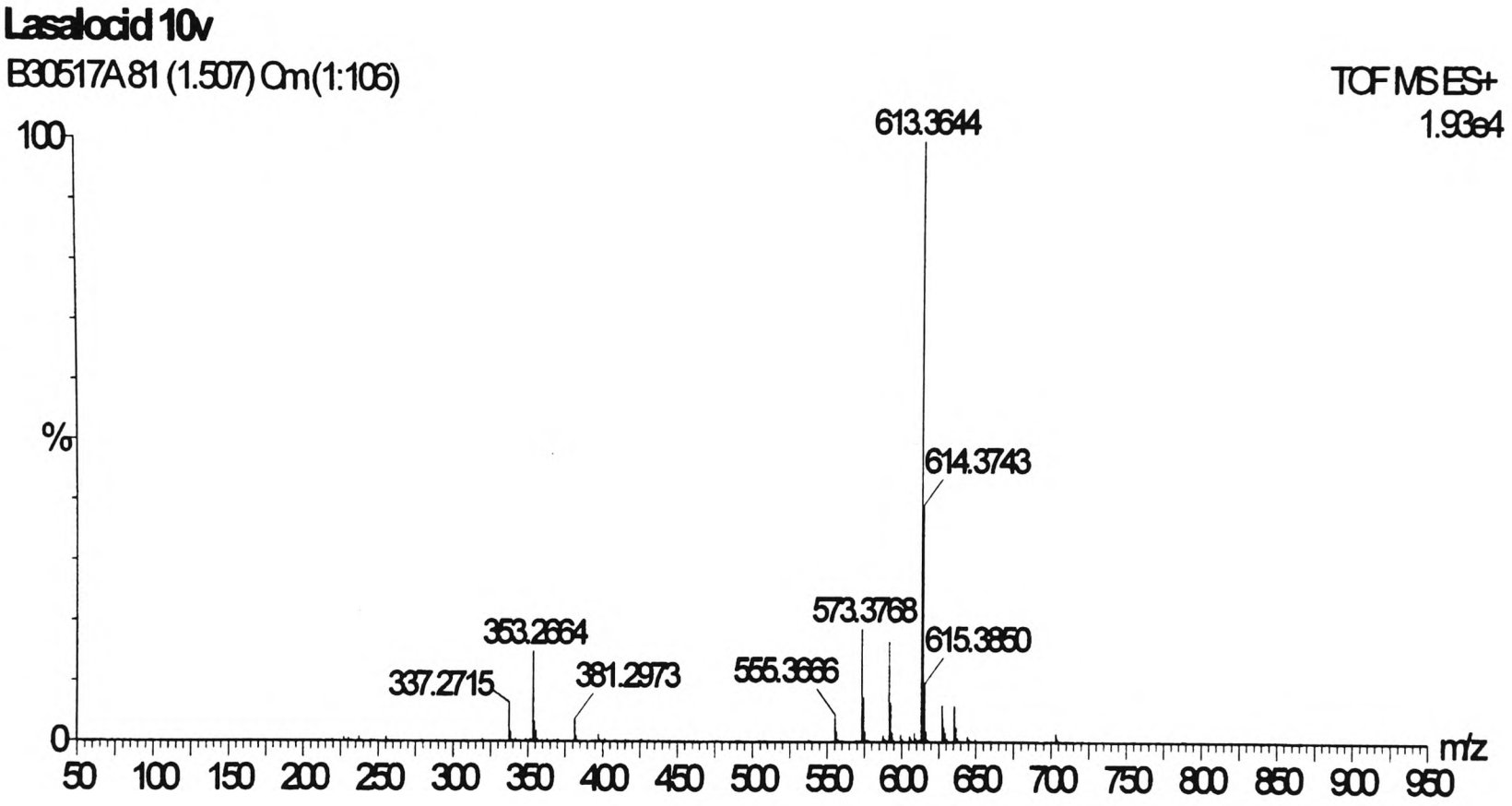


Fig 6.2.8

Salinomycin infused using standard MS conditions

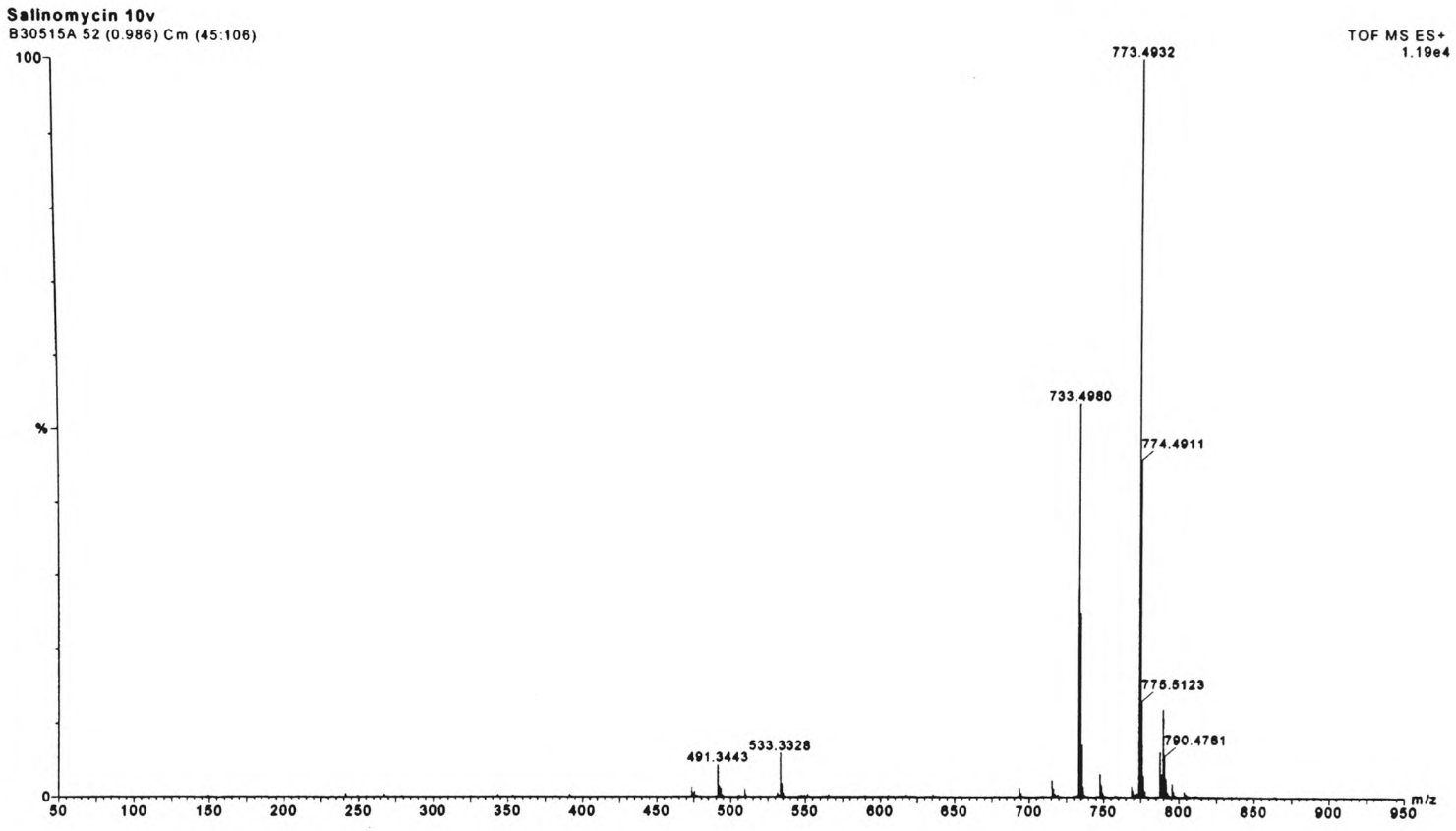


Fig 6.2.9

Narasin infused using standard MS conditions

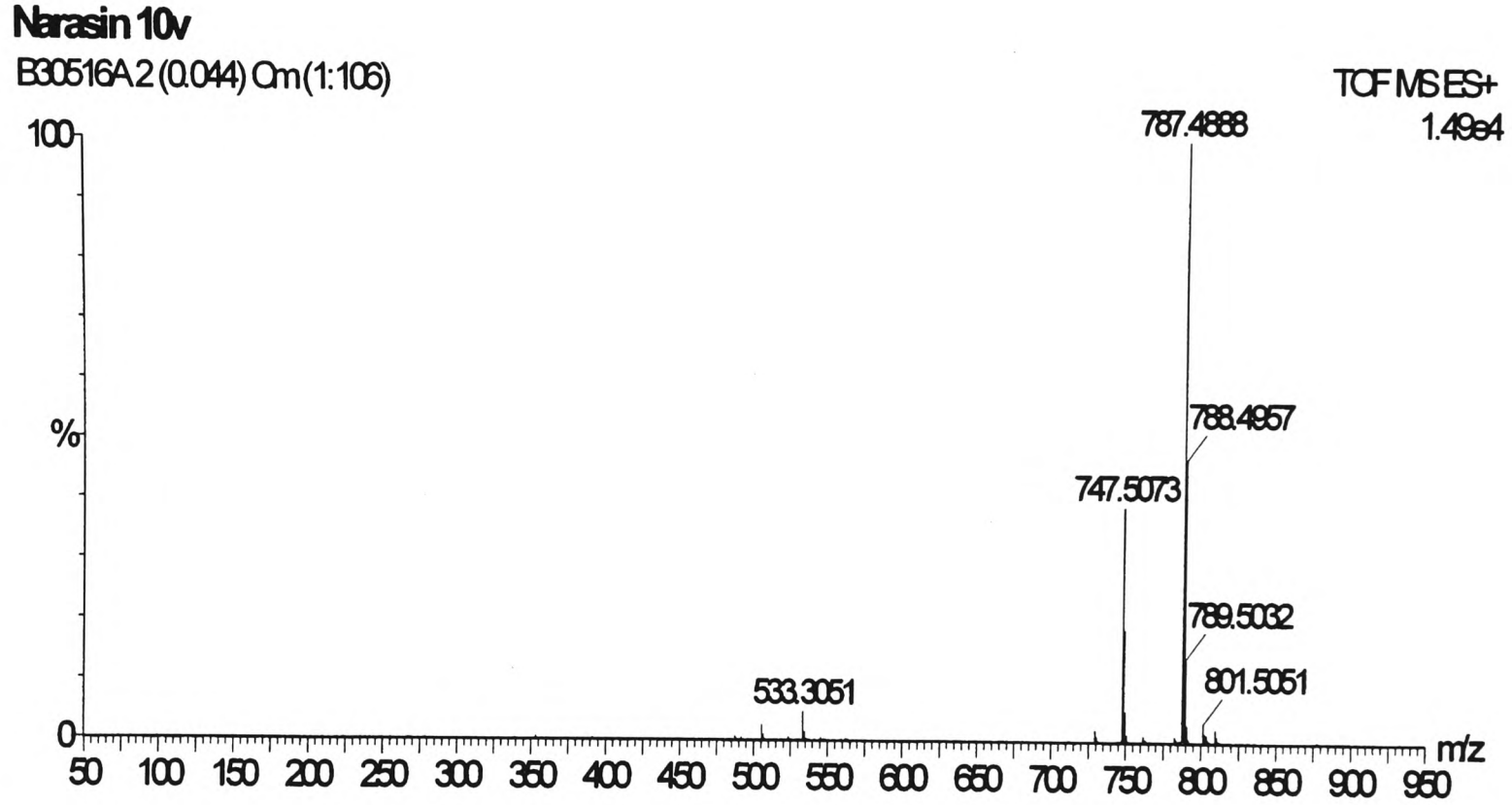


Fig 6.2.10

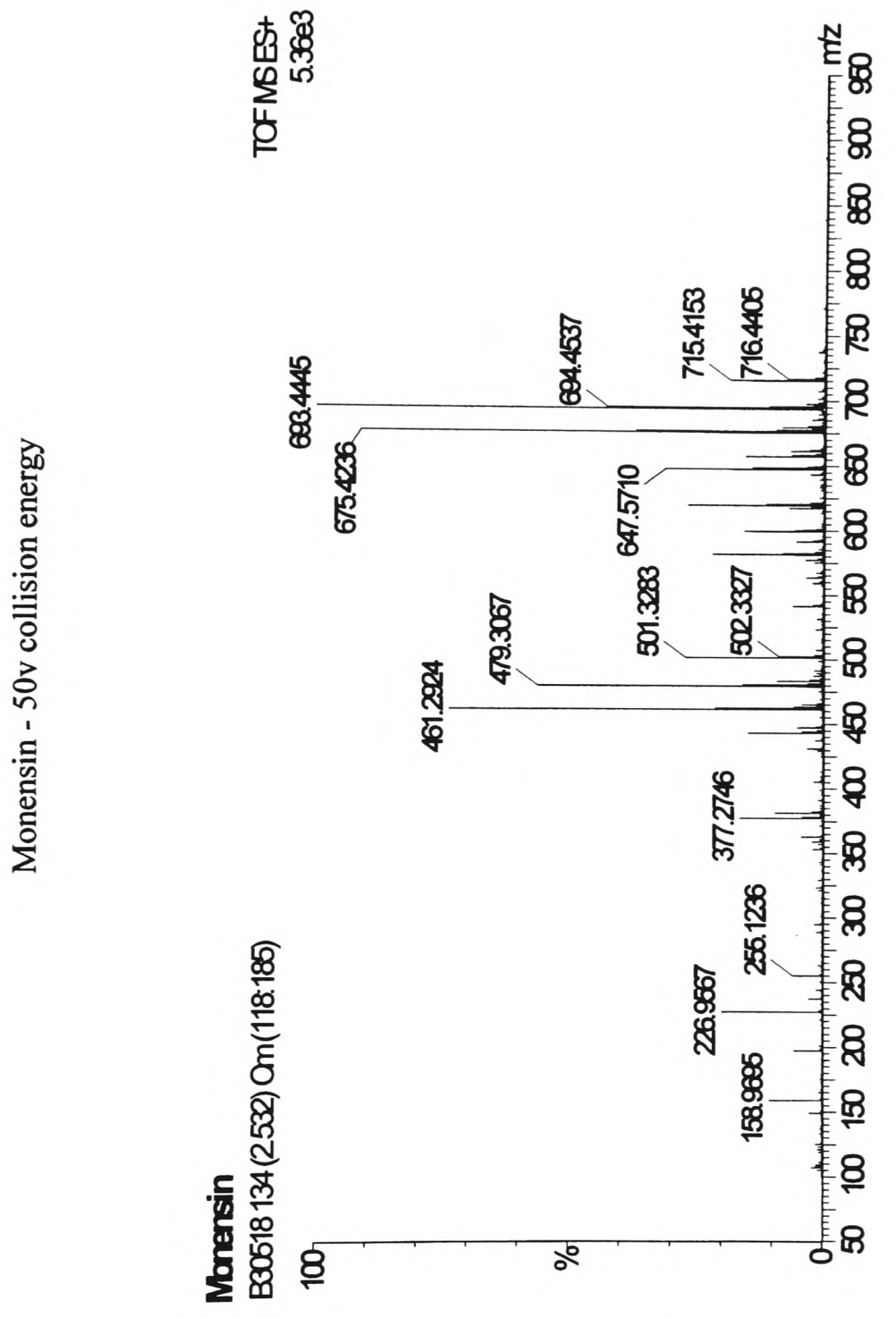


Fig 6.2.11 Monensin daughter ion spectrum

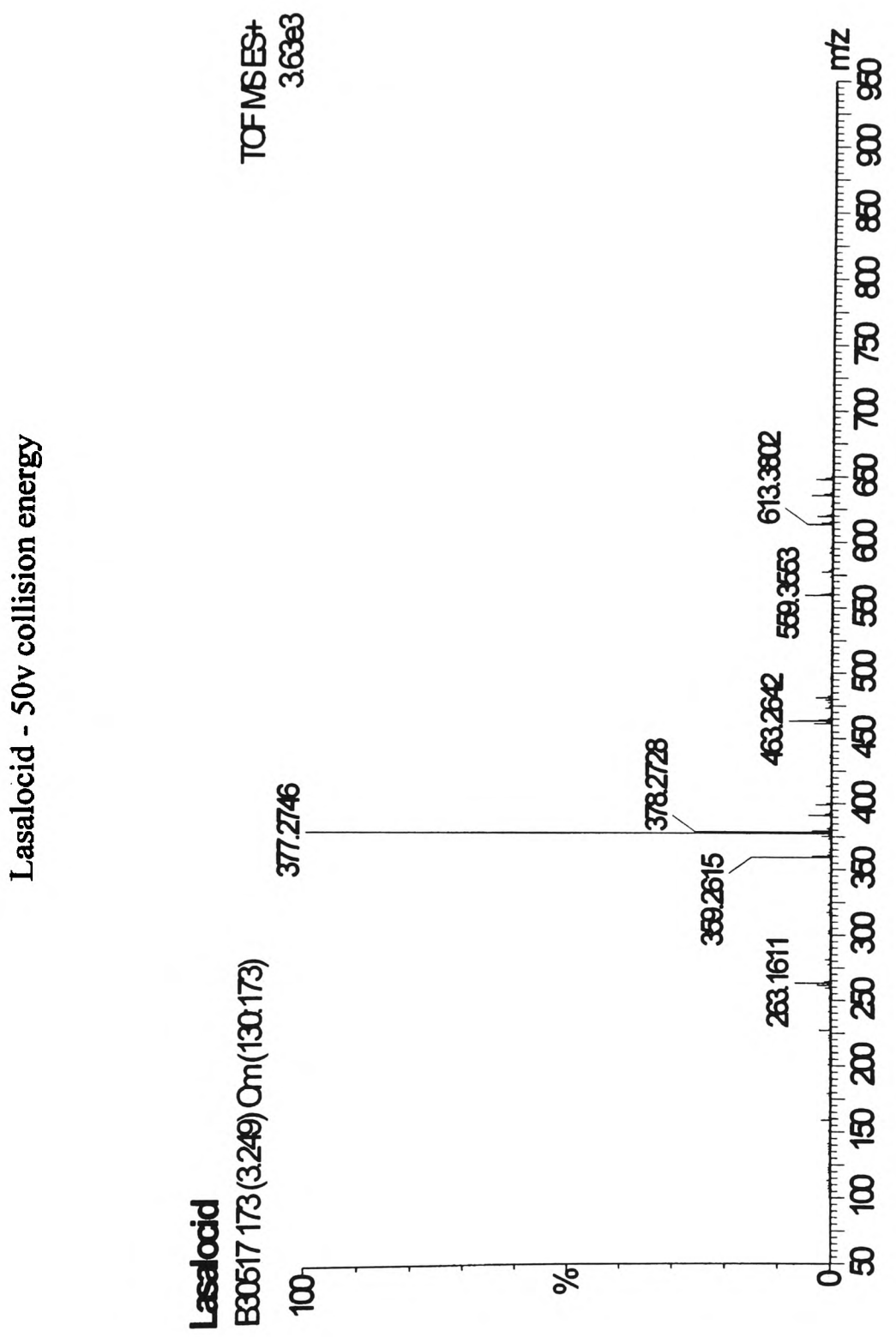


Fig 6.2.12 Lasalocid daughter ion spectrum

Salinomycin - 50v collision energy

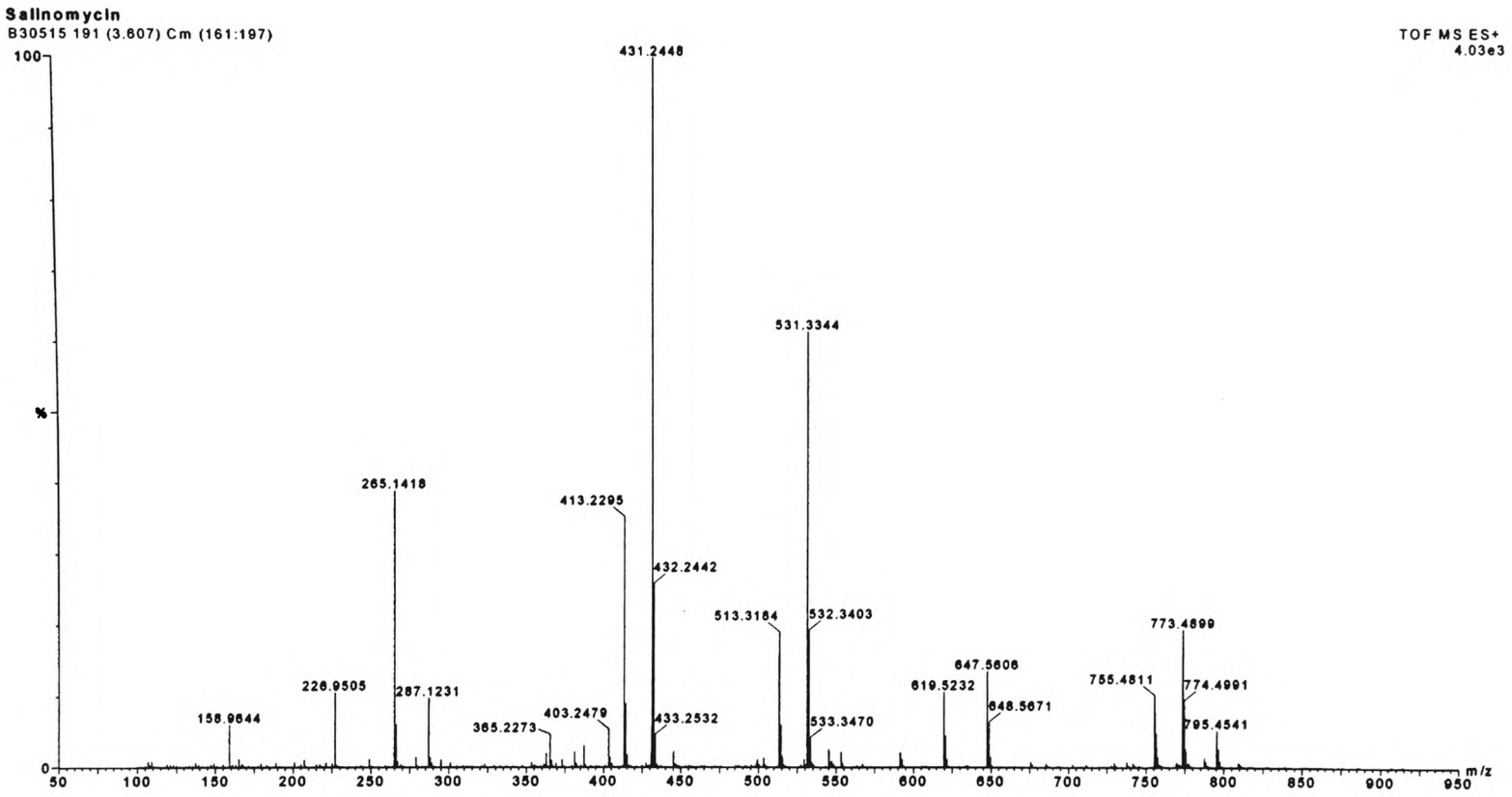


Fig 6.2.13 Salinomycin daughter ion spectrum

Narasin - 50v collision energy

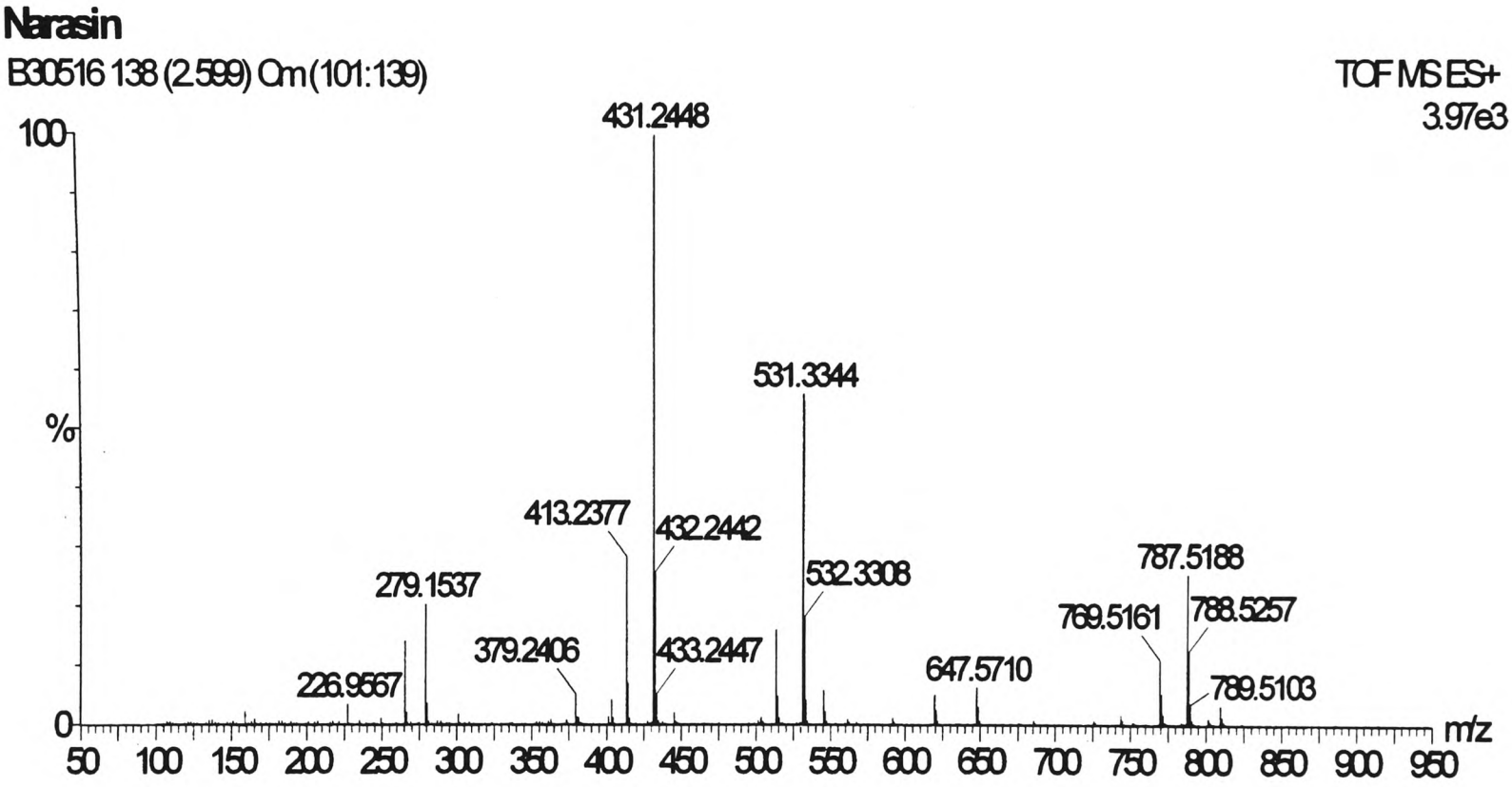


Fig 6.2.14 Narasin daughter ion spectrum

For the separation and analysis of the samples a Shimadzu HPLC system was used which consisted of a SCL-10A system controller, two LC-10AS HPLC pumps and a SPD-M10AVP diode array detector.

The mobile phase phase consisted of the following solvents :-

Solvent A = 90% : 10% water : methanol with 0.1% methanoic acid,
Solvent B = 100% methanol + 0.1% methanoic acid.

The pre-extraction phase of this work involved the development of an efficient trapping system, and initially a single Phenomenex Luna® C1, 4 mm diameter x 3.0 mm guard column was used. To test the efficiency of this column to trap the ionophores, a single 20 µl injection of the 40 ppb ionophore solution was injected onto the column via a Rheodyne 7125 valve, Fig 6.2.15.

In this initial run the mobile profile was 0% B for approximately 38 min (2500 scans), and then to 92% B, with a flow rate of 1 cm^3 min^{-1}. This demonstrated the efficiency of trapping of the ionophores while the mobile phase profile was held at 0% B, and then showed the effective elution of the sample after solvent B was increased to 92%.

Although it was demonstrated that the C1 column was efficient for trapping the ionophores, it had to be seen if refocusing of the ionophores could be achieved, as refocusing of the extracted ionophores would be required to maintain the integrity of the chromatography, if they were not trapped in a discrete zone. To investigate refocusing on a single C1 column, two 20 µl aliquotes of the 40 ppb ionophores solution was injected onto the column, with an interval between injections of 2 minutes, Fig 6.2.16. The mobile phase profile was also altered, and the composition of the mobile phase increased from its initial 0% B to 60% B.

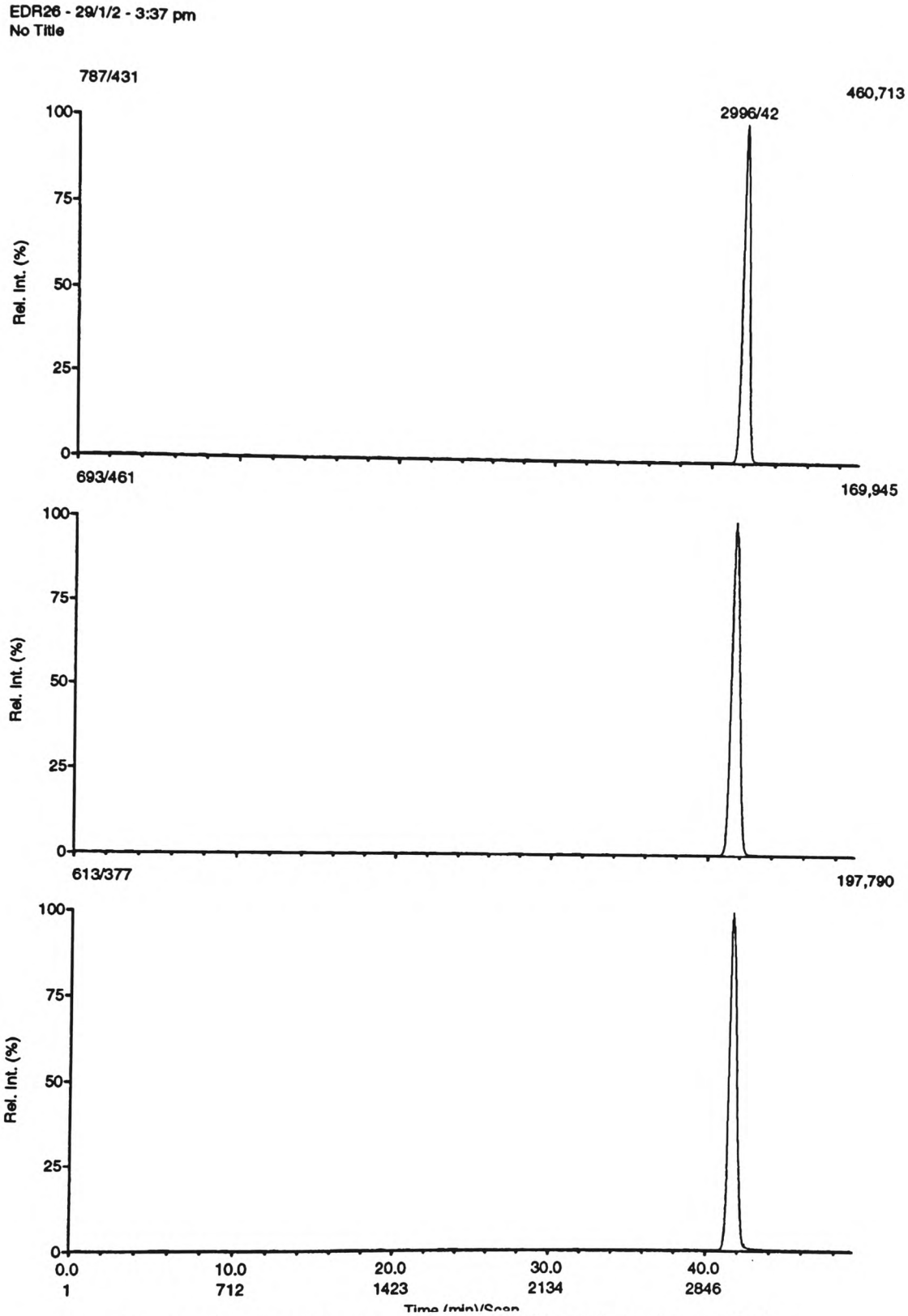


Fig 6.2.15 20 μl single injection of a 40 ppb ionophore solution

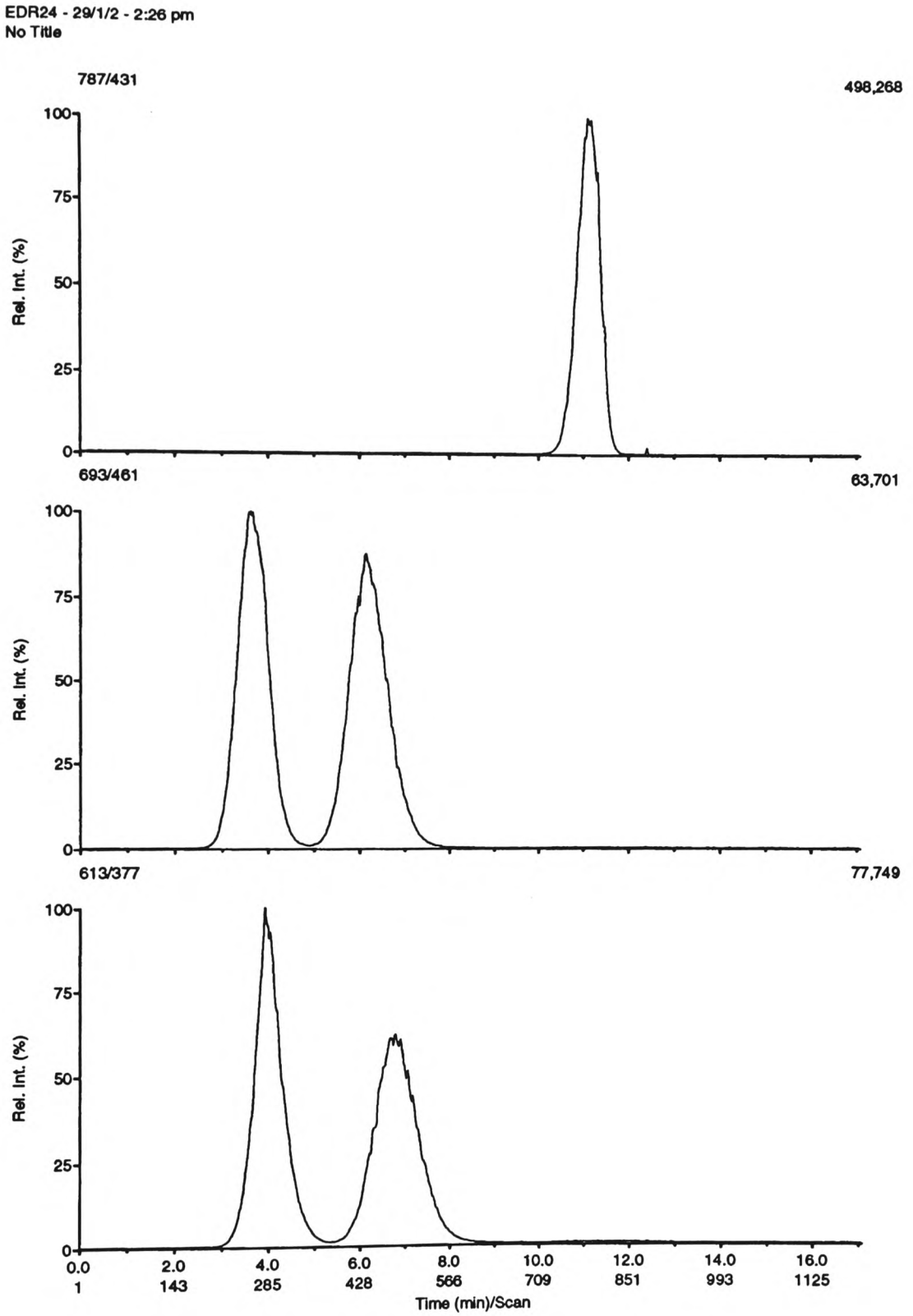


Fig 6.2.16 Double injection of 40 ppb ionophore solution on single C1 column (2 min apart)

Fig 6.2.16 shows that while Narasin was refocused the other ionophores remained unfocussed, and so a single C1 column while an efficient trap, failed to effectively refocus all the ionophores.

To investigate what improvement could be made to the refocusing, a second C1 column was directly coupled to the first, and again a double injection separated by 2 min made, Fig 6.2.17.

Fig 6.2.17 shows that there was no improvement in refocusing with the addition of a second C1 column. Therefore it was necessary to use an alternative column to improve the refocusing of the ionophores. For this purpose a Hypersil C18 50 mm x 4.6 mm diameter column was used.

A double injection of the 40 ppb ionophore solution was injected onto this column, using the same mobile phase profile, 60% B for 6 min then to 92% B, and the same injection delay of 2 minutes, Fig 6.2.18.

Fig 6.2.18 shows that the single C18 column is capable of refocusing the ionophore solution. These experiments demonstrate that C1 pre-column(s) could be effectively used for trapping supercritical fluid extracted ionophores from aqueous solutions. However the C1 column alone could not band focus the extracted ionophores. However it was demonstrated that a C18 column had the capability to refocus the ionophores since injections made onto the column two minutes apart, resulted in ion chromatograms showing only single peak profiles using a step gradient.

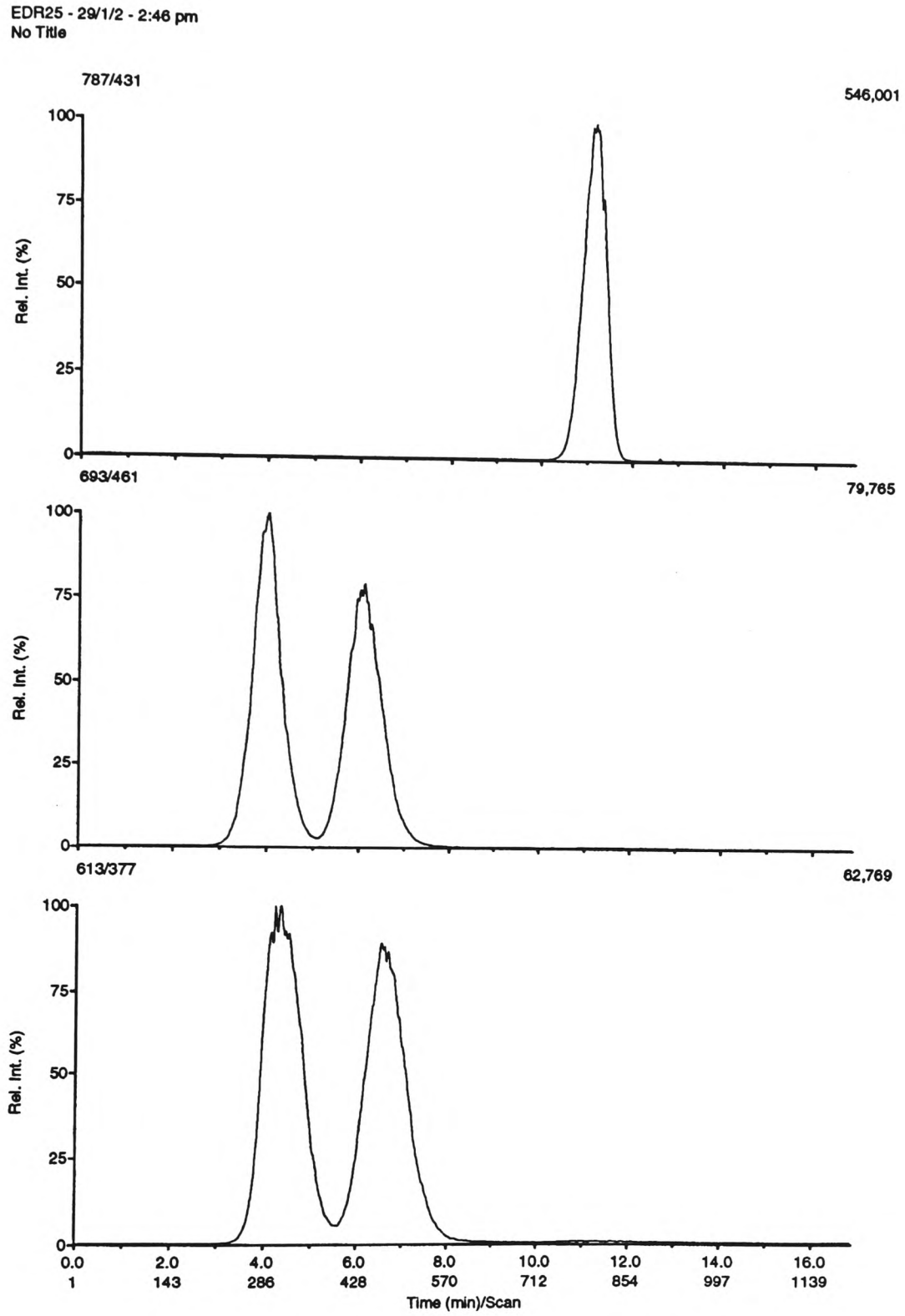


Fig 6.2.17 Double 20 µl injection of 40 ppb ionophore solution onto linked C1-C1 columns. (Top to bottom- Naracin, Monensin, Lasalocid)

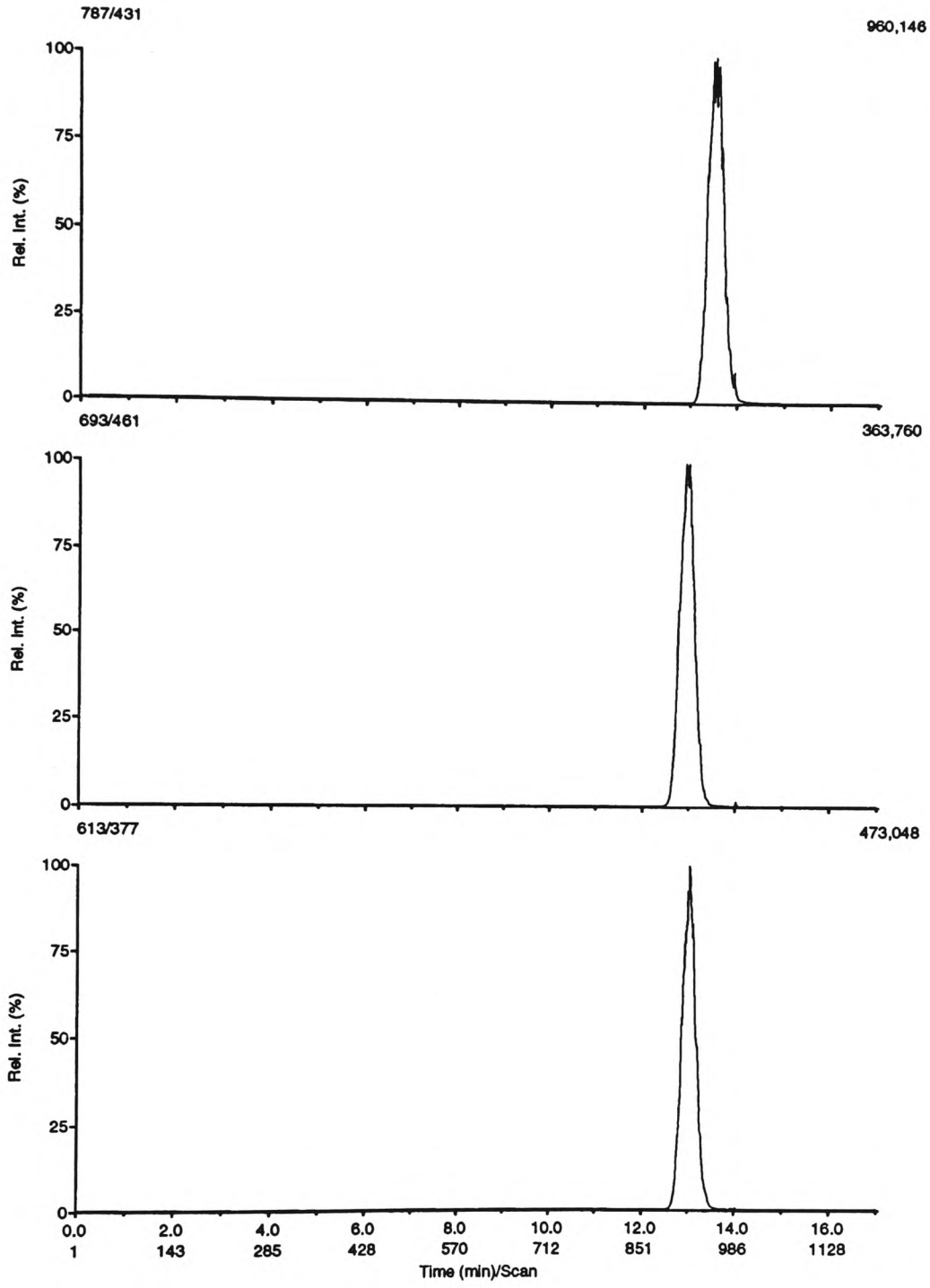


Fig 6.2.18 Single C18 column double injection of 40 ppb ionophore solution. MRM analyses, ion pear for each ionophore channel shown.

(Top to bottom- Naracin, Monensin, Lasalocid)

Therefore the trapping and chromatography was achieved using two Phenomenex Luna® C1 4 x 3.0 mm guard column directly coupled to a Hypersil C18 50 mm x 4.6 mm diameter column.

After the extraction had finished, analysis was completed on

a. the ionophores trapped on the columns,
b. the methanol, for any ionophores not trapped on the columns
c. the residual extraction solution

These off-line SFE recovery results are tabulated below.

Extraction Run No	Narasin % Recovery	Salinomycin % Recovery	Monensin % Recovery	Lasalocid % Recovery
1	92.3	89.2	74.6	86.7
2	87.6	88.1	78.1	82.2
3	90.3	83.1	76.4	83.8
4	88.6	84.2	74.5	85.6
5	89.3	83.8	69.6	76.0
Mean Extraction value	89.6	85.7	74.6	82.9

Ionophore	Extracted %	Column Trap %	Methanol Trap %
Narasin	89.6	69.2	2.5
Monensin	74.6	55.8	11.6
Salinomycin	85.7	62.6	3.6
Lasalocid	82.9	73.9	7.5

The principle of trapping and refocusing had been established and the second stage of the experimental work was commenced, namely the direct coupling of the extraction system to the Sciex API III MS-MS system.

This work was essentially subdivided into three sections,

- extraction and trapping onto the two C1 columns, Fig 6.2.19
- de-gassing of the C1 columns by flushing the columns with 100% water, and conditioning of the C18 column using a mobile phase of 60% methanol, 40% water and 0.1% methanoic acid, Fig 6.2.20
- refocusing of the analytes onto the C18 column and subsequent elution and analysis by MS-MS. Fig 6.2.21.

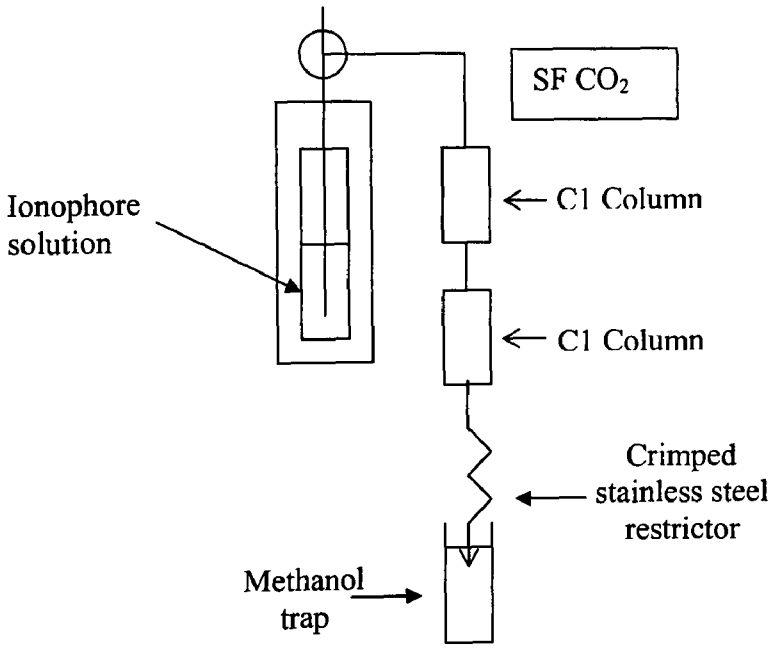


Fig 6.2.19 SFE and trapping onto x2 C1 columns

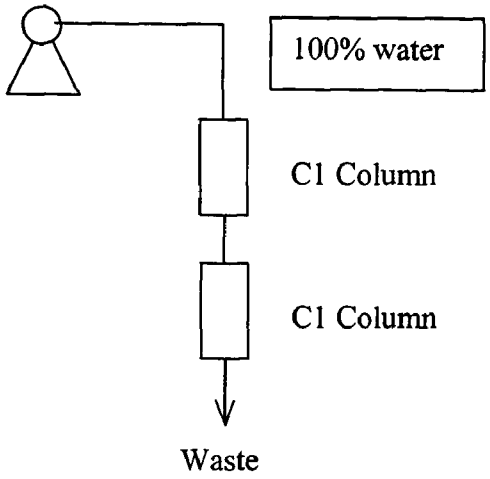


Fig 6.2.20 De-gassing of the 2x C1 columns

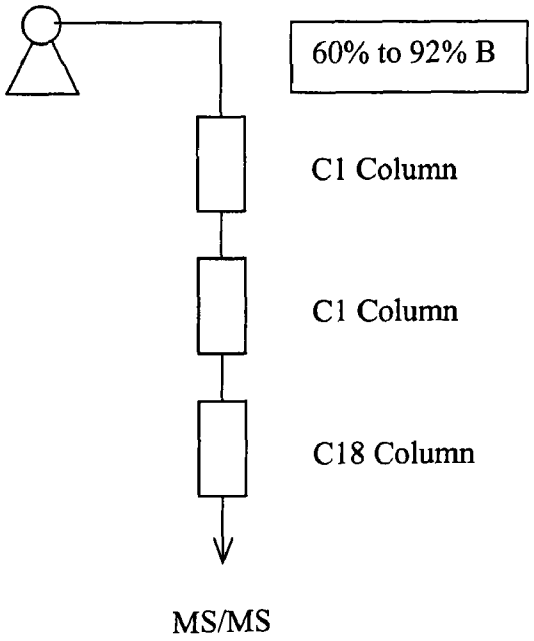


Fig 6.2.21 Elution of the analytes from the C1 columns and subsequent refocusing and MS-MS analysis

6.3 The development of an on-line coupled SFE-LC-MS-MS method for analysing ionoiphores extracted from aqueous solutions

Following the successful application of off-line SFE-LC-MS-MS method it was decided to develop an on-line method. Extractions of the ionophore mixture solution were carried out using the extraction setup shown in Figs 6.3.1 and 6.3.2. The system was based around a 10 port HPLC valve configuration, and allowed the chromatography to be developed independently while the ionophores were being extracted. To ensure the C1 columns were effectively trapping the ionophores the exhaust from the C1 columns was directed through methanol which was analysed at the end of the extraction period for the presence of any ionophores. For HPLC using the on-line system described below:

Solvent A : 100% Water : 0.1 % Methanoic acid.

Solvent B : 100 % Methanol : 0.1 % Methanoic acid

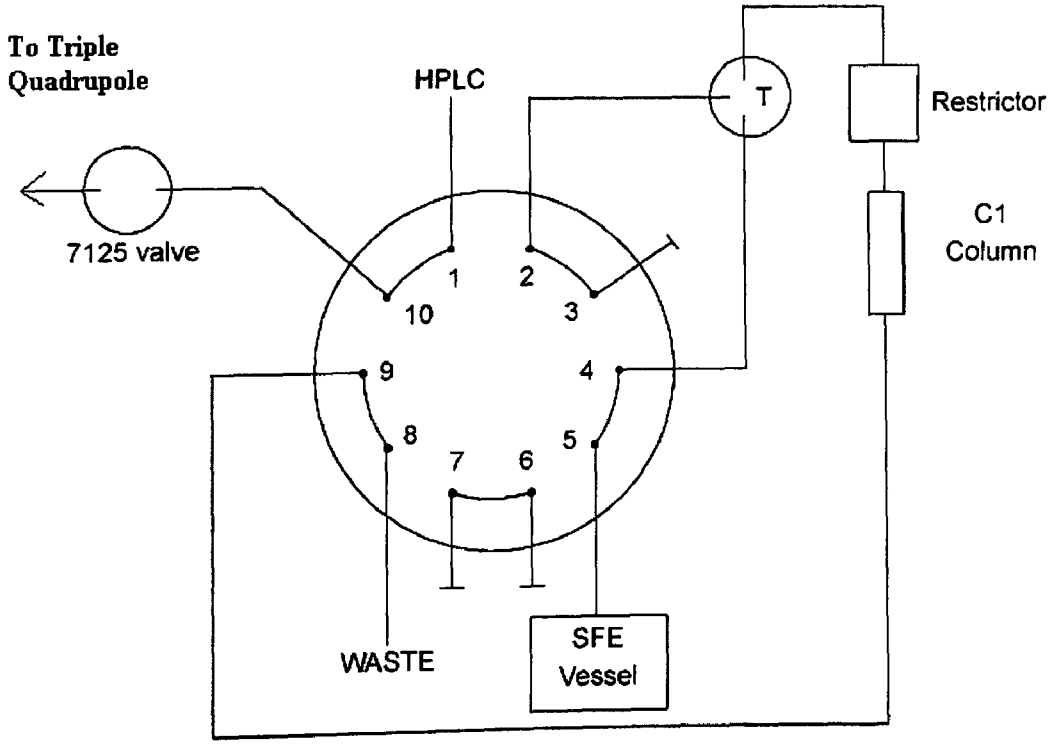


Fig 6.3.1 Valve configuration for SFE

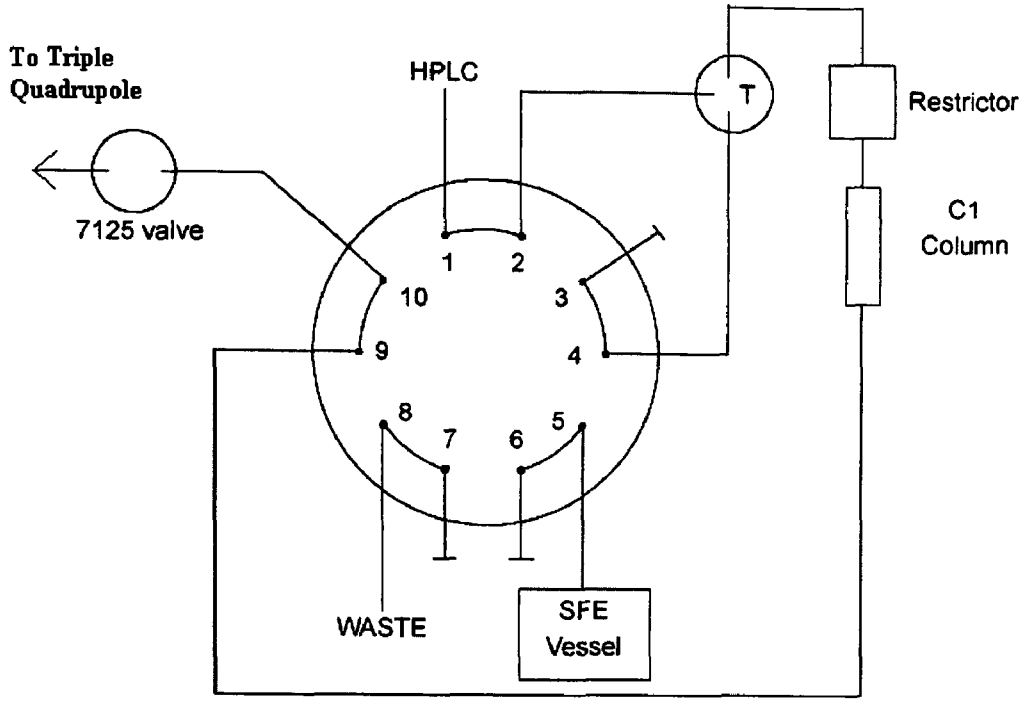


Fig 6.3.2 System setup for LC or LC-MS-MS

During the SFE stage, the valve configuration shown enables the simultaneous LC-MS or LC-MS-MS analysis of mixtures. Prior to using the valve configuration shown in Fig. 6.3.2 the C18 column was conditioned using the initial mobile phase composition used for the step gradient procedure described in section 6.2. Also the C1 column was purged with 100 % solvent A to remove traces of carbon dioxide gas prior to coupling to the C18 column.

In the configuration shown in Fig 6.3.2 the trapped ionophores were then eluted from the C1 trapping column and directed into the LC-MS-MS system via the band focussing C18 chromatography column situated between the output of the Rheodyne 7125 valve and the inlet of the triple quadrupole mass spectrometer.

One problem encountered during the extraction phase, was icing of the crimped stainless steel restrictor tubing before it entered the C1 trapping column. To alleviate this problem

the restrictor tubing was coaxially placed inside another piece of high pressure stainless steel tubing which was attached to a "T" piece, Fig 6.3.3. This allowed warm air to be circulated around the outside of the restrictor tubing which stopped the freezing.

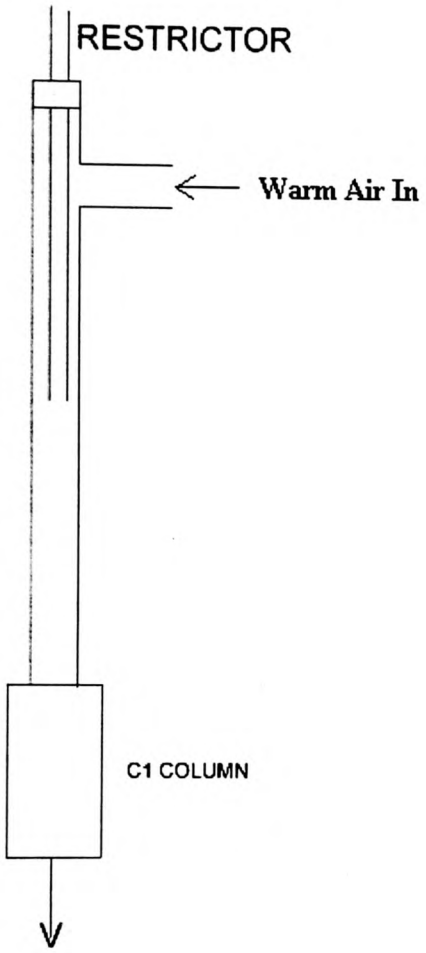


Fig 6.3.3 Coaxial restrictor tube configuration

Before condensing with the on-line SFE, a standard solution of all four ionophores was prepared at a concentration level of 40 ppb (20 µl of the working solutions in 4 ml of water) whose compositions are those specified in section 6.2.2. were analysed by LC-MS-MS with the results shown in Fig 6.3.4

The MRM ion chromatogram profiles indicated that the salinomycin and narasin had started to degrade, as the salinomycin secondary peak interfered with the resolution of the primary peak it was decided that salinomycin would be omitted from the SFE-LC-MS-MS studies of the work. The additional peak due to degradation of the narasin did not interfere with the primary peak and so the primary peak of narasin was used as an internal standard for the quantification of monensin and lasalocid.
The additional peak associated with monensin was the result of an isomer, and did not interfere with the analysis of the sample.

The SFE conditions used in the on-line SFE-LC-MS-MS studies of the 40 ppb solution of monensin and lasalocid were:-

Extraction pressure 2500 psi and flow rate of liquid carbon dioxide 2 cm^3 min^{-1}.

Initially the CO_2 was introduced via the bottom port of the vessel, and at 2000 psi the input was changed as to enter the top and directly into the solution. Fifteen minutes after the target pressure was reached the extraction was stopped, the vessel allowed to de-pressurise through the C1 column, the C1 column was then purged with solvent A to remove gaseous carbon dioxide. To allow quantification to be achieved samples of the ionophore solution were injected via the Rheodyne 7125 valve at know concentrations of 15, 65 and 105 % based on 100 % recoveries of each ionophores from aqueous solution, and contained narasin as an internal standard.

The ionophore solution was then re-extracted for a further 2 x 15 minutes periods allowing an extraction profile to be constructed.

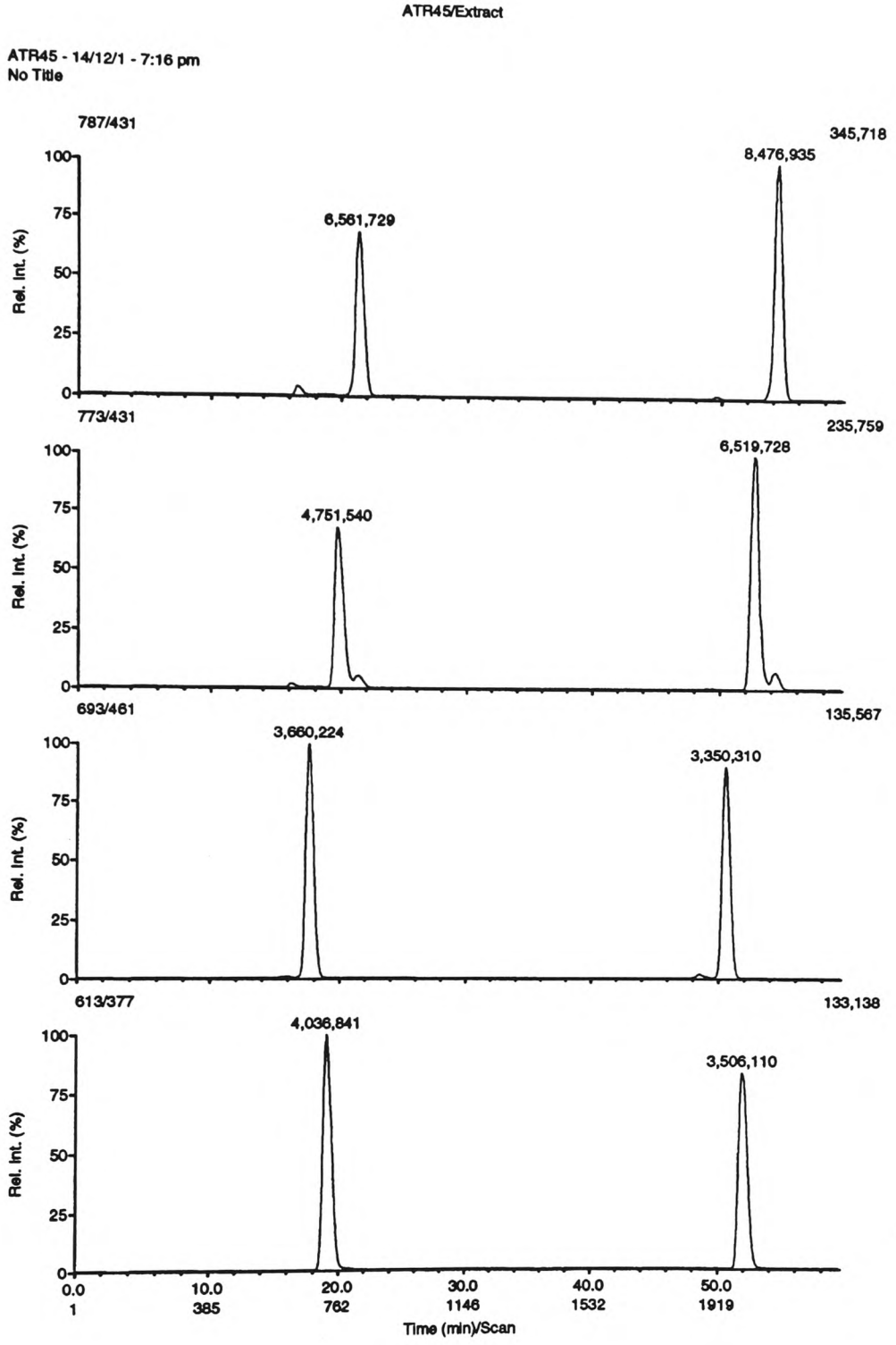


Fig 6.3.4 The MRM ion profiles obtained for a 40 ppb solution of each of the following ionophores.

Top to bottom, Narasin, Salinomycin, Monensin and Lasalocid.

6.3.1 Results

The table below includes the mean data for two identical extraction

	Ratio of Ionophore peak intensity to Narasin Internal Standard								
Extraction Period	15 min			30 min			45 min		
	15%	65%	105%	15%	65%	105%	15%	65%	105%
Monensin	0.0531	0.2445	0.3904	0.0541	0.2422	0.4052	0.0505	0.2335	0.3584
Lasalocid	0.0603	0.3211	0.5308	0.0595	0.3182	0.5375	0.0615	0.3024	0.4995
% Extraction									
Monensin	52.64			16.22			6.89		
Lasalocid	38.66			18.89			8.66		
Total Extraction %									
Monensin	75.75 %								
Lasalocid	66.21 %								

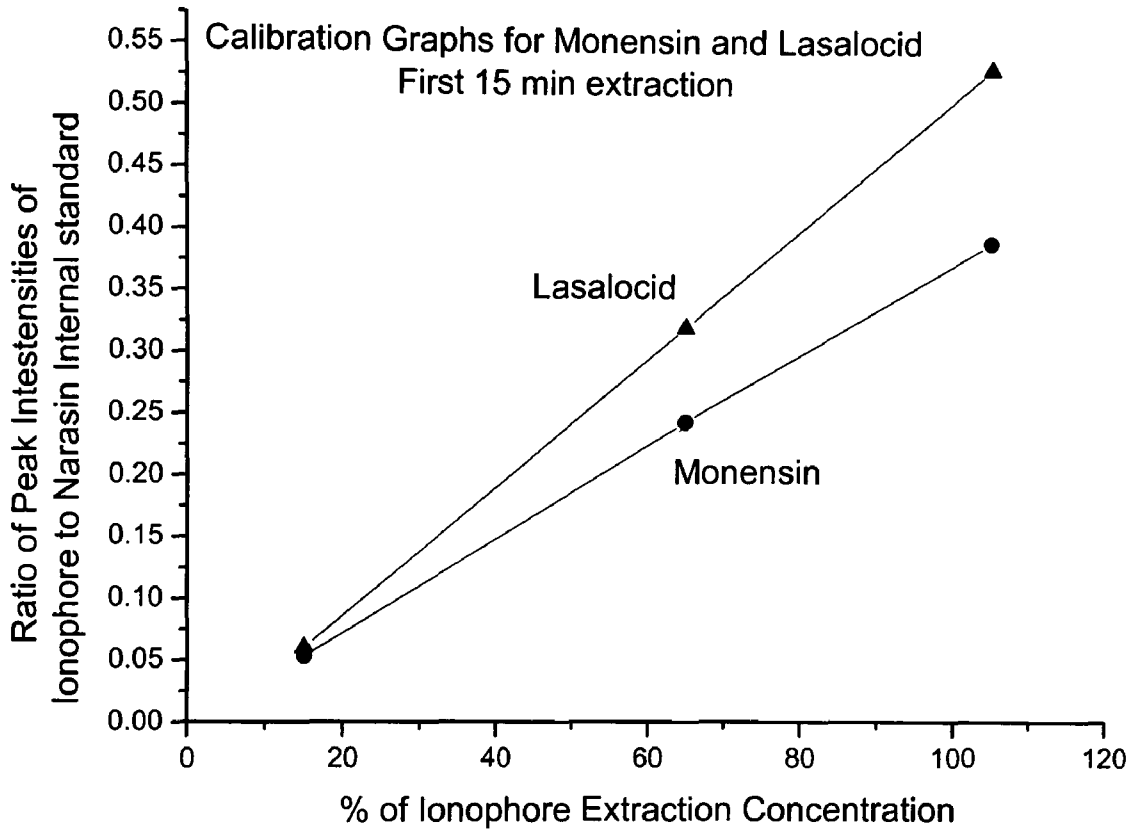


Fig 6.3.5 Lasalocid and Monensin calibration graphs for the first 15 min extraction

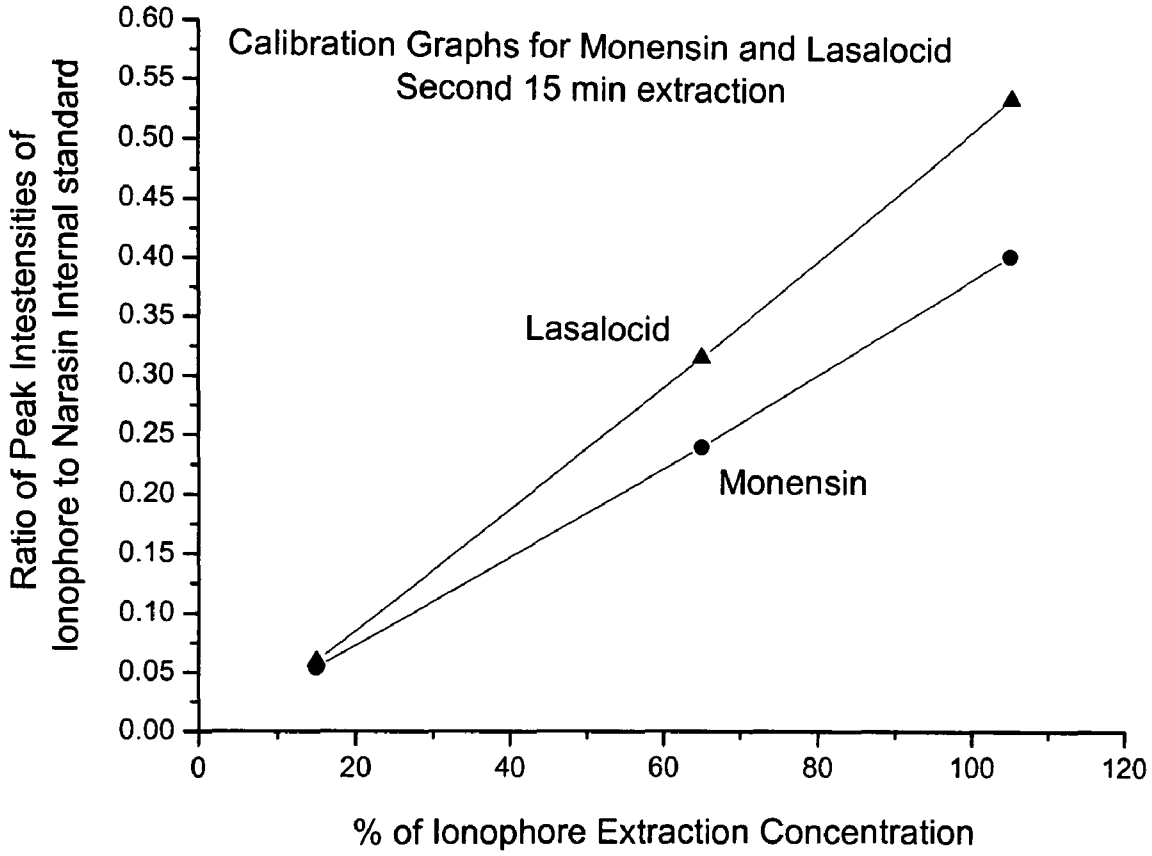


Fig 6.3.6 Lasalocid and Monensin calibration graphs for the second 15 min extraction

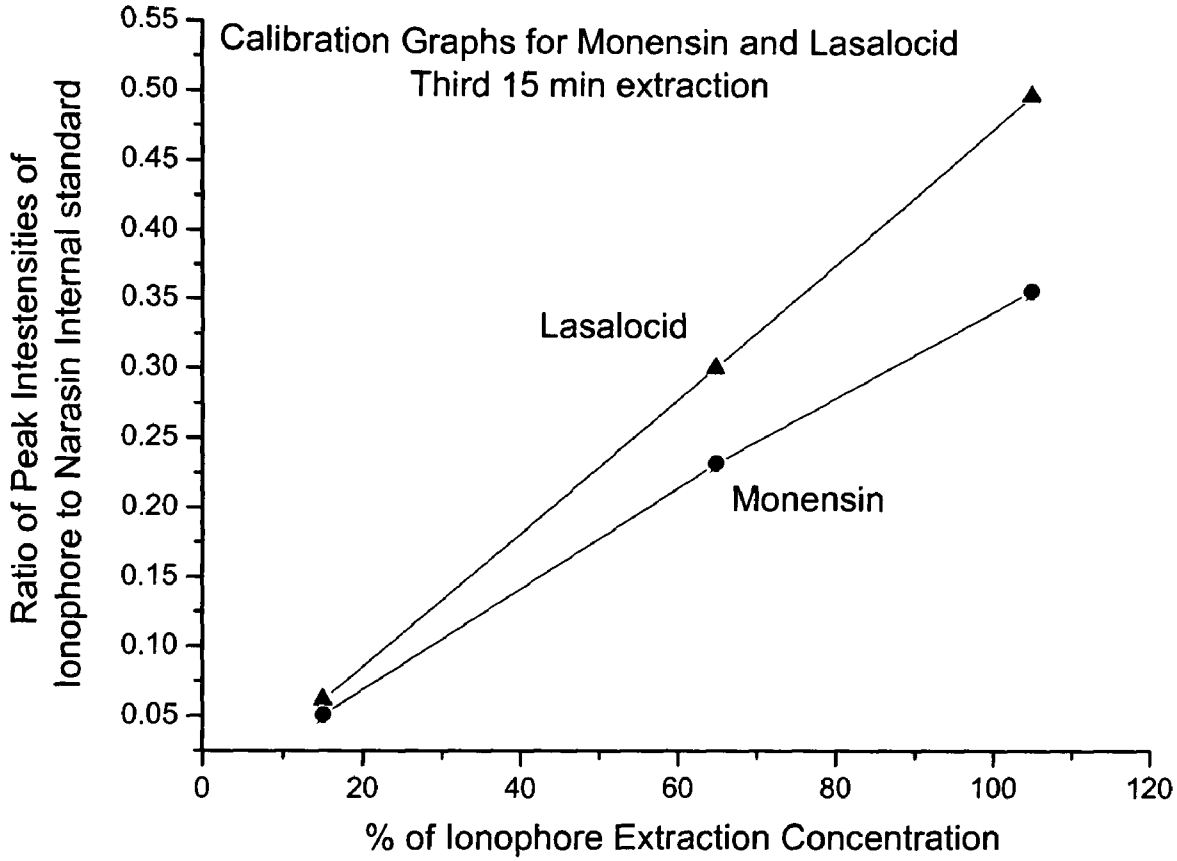


Fig 6.3.7 Lasalocid and Monensin calibration graphs for the third 15 min extraction

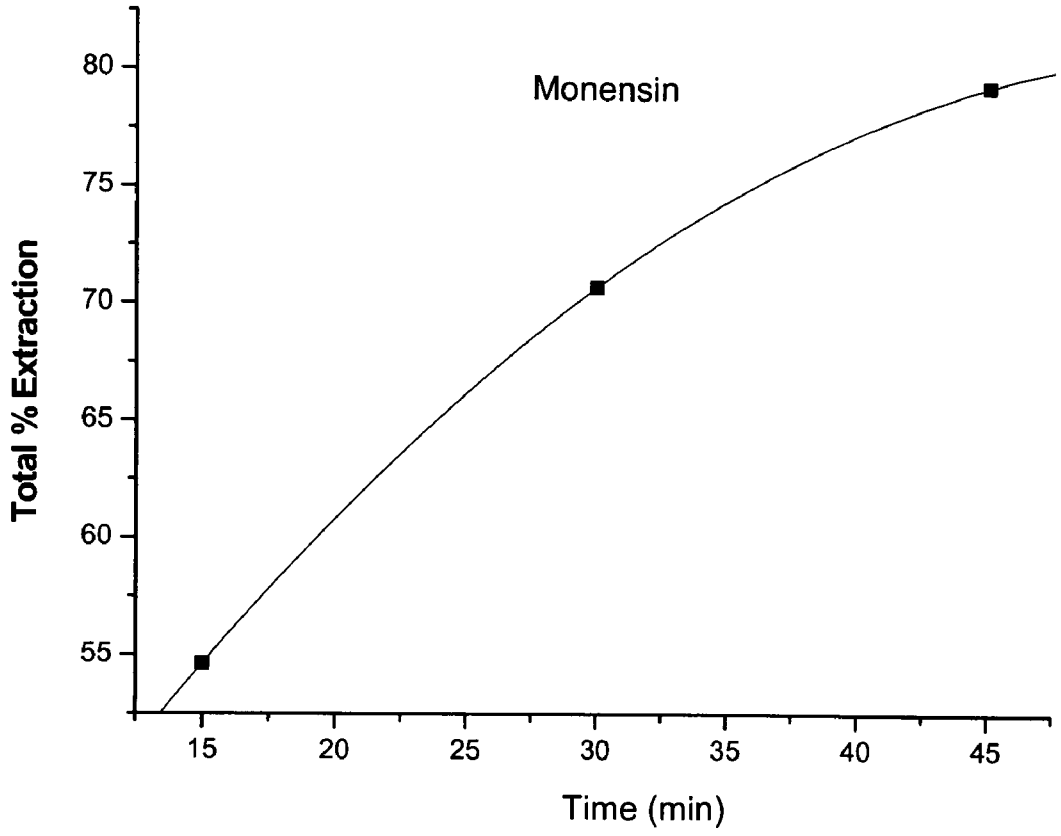


Fig 6.3.8 Extraction profile for Monensin for 3 x 15 minute extractions using the on-line SFE-LC-MS-MS method

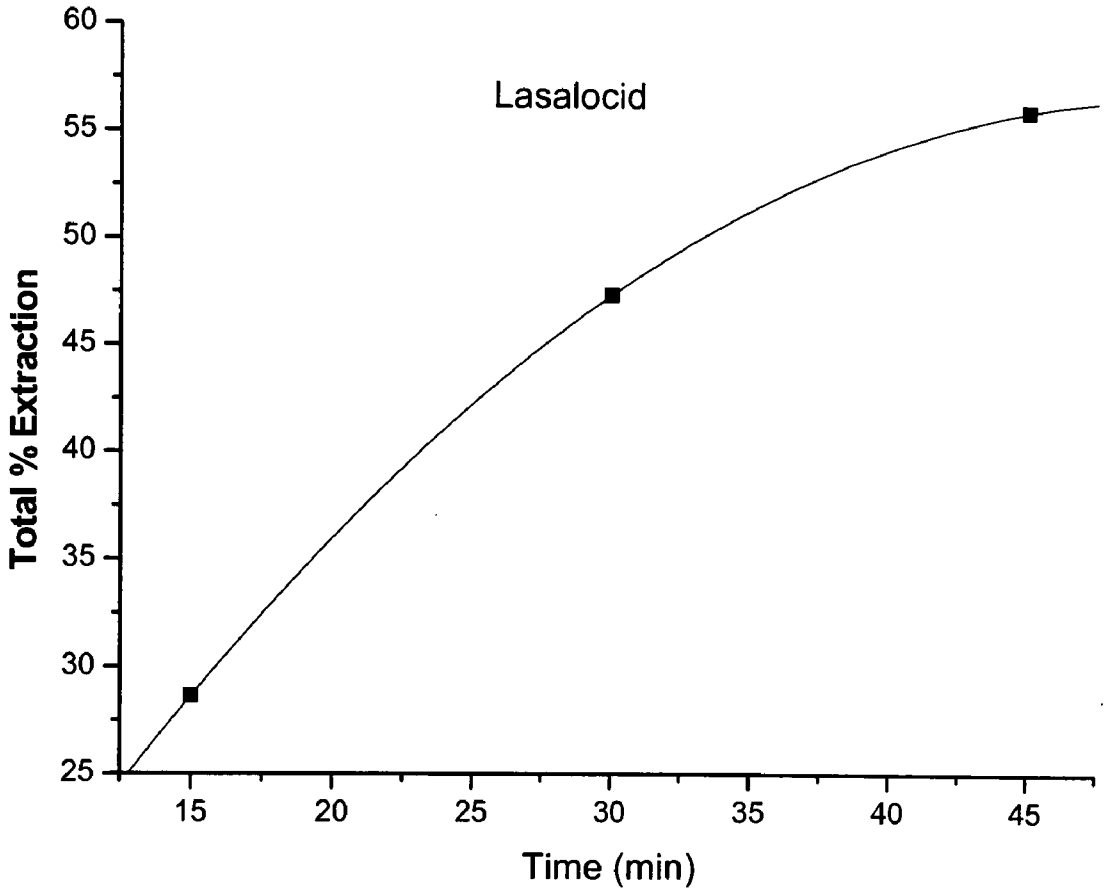


Fig 6.3.9 Extraction profile for Lasalocid for 3 x 15 minute extractions using the on-line SFE-LC-MS-MS method

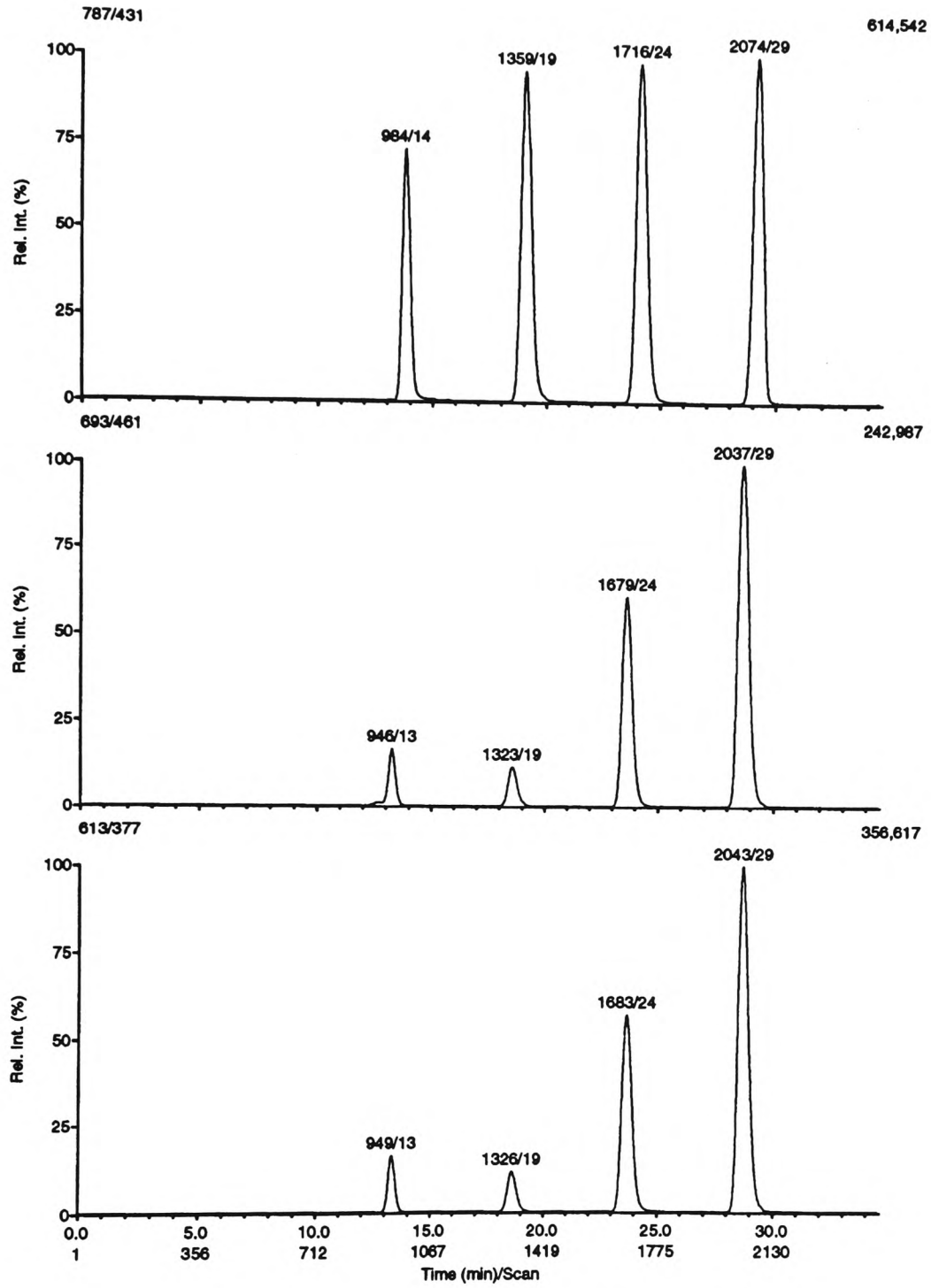


Fig 6.3.10 MRM analyses showing the SFE-LC-MS-MS ion chromatogram profiles for Monensin and Lasalocid during the first 15 min SFE period. Also the 3 subsequent MRM ion chromatograms for individual injections equivalent to 15, 65 and 105 % of total extractable ionophore.

Top to bottom Narasin (internal standard), Monensin and Lasalocid.

LC-MS-MS analyses of the residual water samples were then performed. This showed that the mean of the two extractions resulted in 87.48 and 86.25 % of monensin and lasalocid respectively had been extracted from the original 40 ppb aqueous samples.

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Chapter 7

Conclusions relating to the results of all the studies described within this work

Supercritical fluid extraction has been shown to be well suited for the extraction and isolation of a variety of organic species from aqueous samples. The results of these studies have also shown that SFE is flexible with respect to the range of analytical techniques and equipment to which it can be directly coupled.

As solvent legislation continues to become more restrictive, it may well be that SFE will start to become more generally accepted in analytic laboratories.

PUBLICATIONS

Communications

Hydrocarbons in Water: Analysis Using On-line Aqueous Supercritical Fluid Extraction–Fourier Transform Infrared Spectroscopy

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An aqueous supercritical fluid extraction vessel has been directly coupled to an FTIR spectrometer. This combination has enabled the analysis of dodecane in water ranging from 200 to 12.5 ppm.

The Montreal Protocol treaty[1] set out a series of deadlines for the phasing out of production of ozone depleting solvents in Europe and North America. Amongst these solvents are those which have been widely used in IR spectroscopy, *e.g.*, chlorofluorocarbons and carbon tetrachloride. Within the European Economic Community it is still possible for laboratories to purchase these solvents if their proposed use falls within the 'essential' category and the supplier has obtained a licence from the European Commission.[2]

An important environmental application involving the use of IR spectroscopy is the determination of the total hydrocarbon content in plant and process water prior to discharge. These procedures[3] require the use of 1,1,2-trichloro-1,2,2-trifluoroethane (Freon 113) or carbon tetrachloride for the extraction of the hydrocarbons. The total hydrocarbon content in the original water sample, which may range from 100 to 1 ppm, is then calculated[3] using the absorbance values obtained for the CH_3, CH_2 and CH vibrations. FTIR spectrometers, using quartz cells of typical pathlength 20 mm, are now widely used for this measurement.

Supercritical fluid extraction (SFE), which generally uses carbon dioxide as extraction fluid, has become an established sample preparation procedure.[4] At present most SFE methods provide an alternative to traditional Soxhlet and sonication procedures used for the extraction of solid matrices. Relatively few reports[5,6] have described the use of direct aqueous SFE since current commercial SFE systems do not provide this capability. The use of both solid phase extraction cartridges and solid phase extraction membranes has afforded an indirect means for the SFE of a range of target analytes from water which include explosives,[7] pesticides[8] and sulfonyl urea herbicides.[9]

This paper presents details of the direct coupling of an aqueous SFE system to an FTIR spectrometer to evaluate the potential of on-line SFE–FTIR spectroscopy for the rapid determination of hydrocarbons in water without the use of any ozone depleting extraction solvents.

Experimental

A stainless-steel SFE vessel (patented) with an internal volume of 150 ml, fabricated in our laboratory using 2.54 cm id stainless-steel tubing and pressure tested to 41.4 MPa, housed within a Pye 104 gas chromatograph (Unicam, Cambridge, UK) was used for all aqueous extractions. A Gilson 308 pump (Anachem, Luton, Bedfordshire, UK) equipped with a cooling head connected to a Neslab RTE 110 recirculator (Jencons Scientific, Leighton Buzzard, Bedfordshire, UK), operated at −10 °C, was used to supply liquid carbon dioxide (MG Gas Products, Reigate, Surrey, UK) to the extraction vessel. The outlet of the vessel was connected to a high pressure FTIR cell of 10 mm pathlength, constructed with quartz windows. The FTIR cell outlet was connected to a Gilson 821 pressure regulator. Aqueous extractions were performed at 50 °C at a pressure of 13.8 MPa with a flow rate of liquid carbon dioxide of 2.5 ml min^{-1}. A Nicolet Magna-IR 550 spectrometer (Nicolet Instruments, Warwick, UK) equipped with Series TGA/IR software was used for acquisition and processing of SFE–FTIR data. Spectra were acquired over the range of 4000–400 cm^{-1}, using a resolution of 4 cm^{-1}, at the rate of 5 scans per spectrum with a sample interval of 3.37 s. A collection time of 20 min was used, resulting in 356 spectra being acquired in a data series.

Studies were conducted using serial dilutions of a stock solution of dodecane with Freon 113 (Fisons, Loughborough, Leicestershire, UK) as solvent. The concentrations of these solutions equated to the introduction of 200–6 ppm dodecane when 20 μl were injected into 50 ml of water. For each SFE–FTIR study, the extraction vessel was loaded with 50 ml of de-ionized water and 20 μl of standard dodecane solution. The vessel was vigorously shaken for 5 min and then allowed to stand for 30 min under conditions of ambient pressure and temperature. The vessel was equilibrated at 50 °C for 15 min prior to extraction. Acquisition of SFE–FTIR data was started at the point when the restrictor valve was opened to provide a stable back-pressure of 13.8 MPa. Baseline SFE–FTIR data was acquired using 20 μl of pure Freon 113 and 50 ml of de-ionized water. Between aqueous extractions the empty vessel was cleaned *in situ* with supercritical fluid carbon dioxide using the previously specified conditions.

Results and Discussion

The Gram–Schmidt reconstruction and associated chemigram for the 3000–2800 cm^{-1} range, obtained for the SFE–FTIR analysis of a 100 ppm dodecane spiked water sample are shown in Figs. 1(*a*) and (*b*), respectively. These results indicate that the extraction of dodecane proceeds very rapidly with the maximum SFE–FTIR response obtained for dodecane being achieved after approximately 3.9 min. The initial 2 min delay in detecting the presence of extracted dodecane is attributed to the time required to flush the delivery line connecting the SFE extraction vessel to the high pressure FTIR cell. This view is substantiated by the fact that the time required for the onset of detection of dodecane was proportional to carbon dioxide flow

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rate at a given extraction pressure. After 3.9 min the quantity of extracted dodecane passing through the FTIR cell declined as the 100 ml void volume of the extraction vessel became purged with freshly introduced supercritical fluid carbon dioxide.

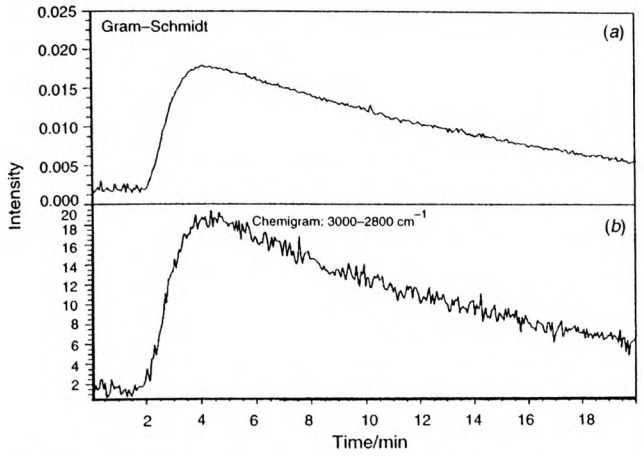

Fig. 1 (*a*) The Gram–Schmidt reconstruction obtained for the on-line SFE–FTIR analysis of a 50 ml water sample spiked with dodecane at the 100 ppm level, and (*b*) the associated chemigram obtained for 3000–2800 cm^{-1}.

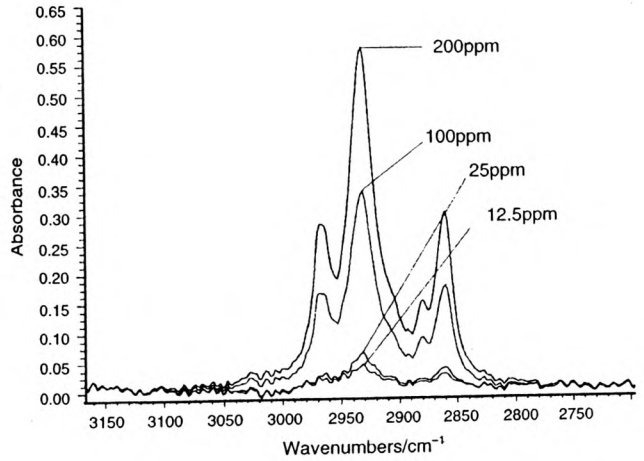

Fig. 2 The SFE–FTIR spectra obtained for dodecane spiked water samples at the 200–12.5 ppm level. Dodecane extraction 13 MPa, 50 °C, flow 2.5 ml min^{-1}.

The SFE–FTIR spectra, summed over the period 3.8–4.0 min, obtained for water samples spiked with 200–12.5 ppm dodecane are shown in Fig. 2. The S/N decreased when greater integration times were used since the concentration of dodecane declined. Preliminary results suggest that a linear relationship exists between the quantity of dodecane used to spike the water and the SFE–FTIR absorbance value recorded for the CH_2 asymmetric stretching frequency at 2930 cm^{-1}. The detection limit (S/N = 3 for the absorbance recorded at 2930 cm^{-1}) for dodecane was 12.5 ppm using the current SFE–FTIR system. Similar results have been obtained for spiked water samples using octane and tetradecane as test compounds at the 200–12.5 ppm level.

At present we are conducting a further series of investigations, using redesigned SFE equipment, aimed at establishing optimum temperature and pressure conditions for the SFE–FTIR analysis of hydrocarbons in water. We aim to provide a detailed account of these studies in due course.

Conclusion

Potential exists for the rapid on-line analysis of hydrocarbons in water using SFE–FTIR spectroscopy with carbon dioxide as extraction fluid. In order to make this technique competitive with established IR procedures for monitoring hydrocarbons in effluents prior to discharge, it will be necessary to determine accurately the total hydrocarbon content in water down to 0.5 ppm.

We acknowledge the technical support provided by M. I. Littlewood, Dr. D. James and D. Roberts (Nicolet Instruments, Warwick, UK).

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Paper 6/02127A
Received March 26, 1996
Accepted April 24, 1996

Dynamic Aqueous Supercritical Fluid Extraction of the Enzymic Hydrolysis of Testosterone-β-D-glucuronide. Analysis of Liberated Testosterone by Gas Chromatography–Mass Spectrometry

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The enzymic hydrolysis of testosterone-β-D-glucuronide has been performed under aqueous SFE conditions. Liberated testosterone was continuously extracted and trapped onto an ODS HPLC column. Results of quantitative GC–MS indicate that 70.1% of testosterone-β-D-glucuronide was hydrolysed after 135 min of enzymic digestion resulting in 88% of the free testosterone being trapped after 120 min of dynamic aqueous SFE.

Testosterone is a naturally occurring male hormone which has been misused in sport. Administration of this compound can increase lean muscle mass which results in artificial enhancement of strength and stamina. Consequently this has led to the development of procedures based upon GC–MS[1,2] and radioimmunoassay[3] for the determination of testosterone levels in urine samples as a means of dope testing. The approach adopted by the International Olympic Committee (IOC) in 1983, involves the use of GC–MS to determine the testosterone (T) to epitestosterone (E) urinary ratio. The IOC considers a T/E ratio of greater than six to be suspect. Since the vast majority of testosterone is excreted in the form of glucuronic acid and sulfate conjugates which are too polar to be analysed by GC–MS, methods[1–3] include the use of glucuronidase for the liberation of testosterone prior to analysis. These procedures also require the use of solid phase extraction and this aspect of steroid sample preparation has been previously reviewed.[4]

Relatively few publications have described direct aqueous SFE on the analytical scale since current commercial SFE instruments do not provide this capability. Applications of direct SFE of water-based matrices have been reviewed.[5] More recently we have reported[6] the direct coupling of an aqueous SFE system to an IR spectrometer for the analysis of hydrocarbons in water.

This paper provides details of a novel application of dynamic aqueous SFE which involves the isolation of testosterone from the enzymic hydrolysis of testosterone-β-D-glucuronide reaction mixture.

Experimental

A schematic of the aqueous SFE system is shown in Fig. 1. A stainless-steel SFE vessel (patented) with an internal volume of 300 ml, pressure tested to 41.3 MPa, housed within a Pye 104 gas chromatograph (Unicam, Cambridge, UK) was used for all aqueous supercritical extractions. A Gilson 308 pump (Anachem, Luton, Bedfordshire, UK) equipped with a cooling head connected to a Neslab RTE recirculator (Jencons Scientific, Leighton Buzzard, Bedfordshire, UK), operated at −10 °C, was used to supply liquid carbon dioxide (MG Gas Products, Reigate, Surrey, UK) to the extraction vessel. The outlet of the extraction vessel was connected to a Gilson 821 pressure regulator which in turn was connected to a Spherisorb 250 × 4.6 mm S5ODS HPLC column (Fisons Scientific Equipment, Loughborough, Leicestershire, UK). The column was housed within a Gilson 831 oven maintained at 60 °C. During SFE the carbon dioxide from the ODS column outlet was exhausted through a beaker containing ethyl acetate. A Rheodyne 7010 valve (Supelco, Poole, Dorset, UK) was used to isolate the extraction vessel during the ODS column rinse and drying cycles. Prior to any dynamic SFE experiments the extraction vessel was isolated and the ODS column was dried *in situ* using carbon dioxide at a rate of 3 ml min^{-1} with the restrictor module set at 0 MPa.

An aqueous solution of testosterone-β-D-glucuronide sodium salt (Sigma, Poole, Dorset, UK) equivalent to 6.02 ng μl^{-1} was prepared. A 10 ml aliquot of this stock solution was transferred into the extraction vessel followed by 220 ml of pH 5.2 aqueous hydrochloric acid solution and 1.5 ml of HP-2 β-glucuronidase from *Helix pomatia* (Sigma). Using the configuration shown in Fig. 1 continuous SFE of the enzymic digest was performed at 55 °C at a pressure of 24.1 MPa with a flow rate of liquid carbon dioxide of 4 ml min^{-1}. In order for the vessel to become pressurized a period of approximately 15 min was required during which time no dynamic extraction occurred since the restrictor module remains completely sealed until the target pressure is attained. After 135 min, resulting in dynamic SFE for 120 min, the vessel was isolated and heated at 100 °C for 15 min to denature the enzyme. A Gilson 306 pump was used to deliver HiPerSolv ethyl acetate (Merck, Poole, Dorset, UK) at a flow rate of 2 ml min^{-1} to rinse trapped material from the ODS column with 7 ml of ethyl acetate into a 10 ml calibrated flask. The calibrated flask had been previously loaded with 2.5 ml of isotopically labelled 16,16,17-$^2H_3$-testosterone (Sigma) ethyl acetate solution, concentration 8 ng μl^{-1}, acting as internal standard. Once cooled the contents of the calibrated flask were made up to 10 ml with ethyl acetate for GC–MS analysis.

The above procedure was also performed, in the absence of the enzyme, using (i) 10 ml of the stock aqueous testosterone-β-D-glucuronide solution and (ii) 10 ml of standard aqueous testosterone solution, concentration 10 ng μl^{-1}.

All GC–MS studies were performed in selected ion monitoring (SIM) mode using a Hewlett-Packard 5971A mass-selective detector, operated using electron ionization, interfaced to a HP 5890 gas chromatograph equipped with a HP 6890

series autosampler. Five calibration standards ranging from 0.75–12 ng μl^{-1} testosterone were prepared such that each solution also contained $^2H_3$-testosterone at the 2 ng μl^{-1} level. All GC–MS analyses were performed using a HP-5MS 30 m × 0.2 mm column of 0.25 μm film thickness with helium (MG Gas Products) serving as carrier gas. The gas chromatograph temperature programme was 2 min at 100 °C then to 290 °C at 20 °C min^{-1}, with the final temperature held for 10 min. Splitless 3 μl injections were made at 250 °C. The quotient of the responses obtained for the molecular ions of testosterone and $^2H_3$-testosterone, *m/z* 288 and 291 respectively, were used for calibration and quantitation with ions at *m/z* 246 and 249 serving as qualifiers.

Results and Discussion

In order to determine the time required to obtain efficient recovery of testosterone by dynamic aqueous SFE, a standard solution of testosterone was first extracted. The quantity of material isolated on the ODS trap was analysed at 20 min intervals following the onset of dynamic SFE. Using the previously specified conditions it was determined that approximately 96% of the testosterone could be recovered after 120 min of dynamic SFE. A five-point GC–MS calibration graph is shown in Fig. 2. Analysis of the concentrated ethyl acetate through which exhaust carbon dioxide had been vented indicated no carry over of testosterone.

A standard solution of testosterone-β-D-glucuronide was subjected to dynamic SFE for 120 min in the absence of β-glucuronidase in order to establish whether (i) testosterone-β-D-glucuronide could hydrolysed by dilute mineral acid and (ii) whether testosterone-β-D-glucuronide could itself be extracted by dynamic aqueous SFE. Analysis of the ethyl acetate ODS trap extract by GC–MS indicated that testosterone-β-D-glucuronide was stable to dilute acid hydrolysis since no free testosterone could be detected. This having been established, the ethyl acetate was then blown to dryness, the residue was redissolved in 10 ml of pH 5.2 dilute hydrochloric acid and incubated with 50 μl β-glucuronidase at 37 °C for 24 h. The enzymic digest was extracted with two 20 ml aliquots of ethyl acetate which were then combined and blown to dryness under a stream of nitrogen. The residue was then dissolved in 1 ml of ethyl acetate. Since GC–MS analysis failed to detect any testosterone it was concluded that no testosterone-β-D-glucuronide had been extracted.

Following the above experiments a solution of testosterone-β-D-glucuronide was incubated with glucuronidase for 135 min with the reaction mixture being simultaneously subjected to SFE for a period of 120 min. After this time the vessel was isolated by means of the appropriate Rheodyne valve setting and the enzyme was denatured by heating the vessel at 100 °C for 15 min. The results of the SIM GC–MS analysis obtained for the dynamic SFE enzyme hydrolysis are shown in Fig. 3. A large peak was also obtained in the total ion chromatogram, Fig. 3(*a*), for a co-extracted compound at retention time 12.69 min, since it provided ions at *m/z* 249 and 246. In order to prevent the signal for this compound being dominant, the electron multiplier default value was increased by 500 V through the time interval 13–17.5 min to promote the testosterone response. No interferences were detected in the *m/z* 288 and *m/z* 291 SIM channels, Fig. 3(*b*) and (*c*) respectively, used for the determination of testosterone recovery. Results of subsequent quantitative GC–MS indicated that approximately

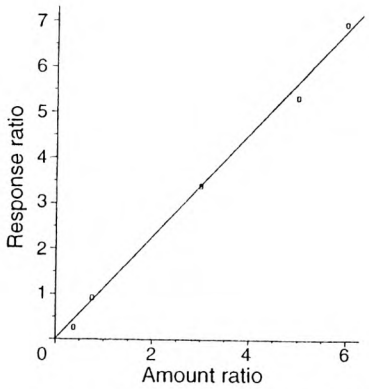

Fig. 2 Five point calibration graph used for quantification of dynamic aqueous SFE testosterone recoveries. Curve fit: linear/origin with r = 0.996. Internal standard : $^2H_3$-testosterone.

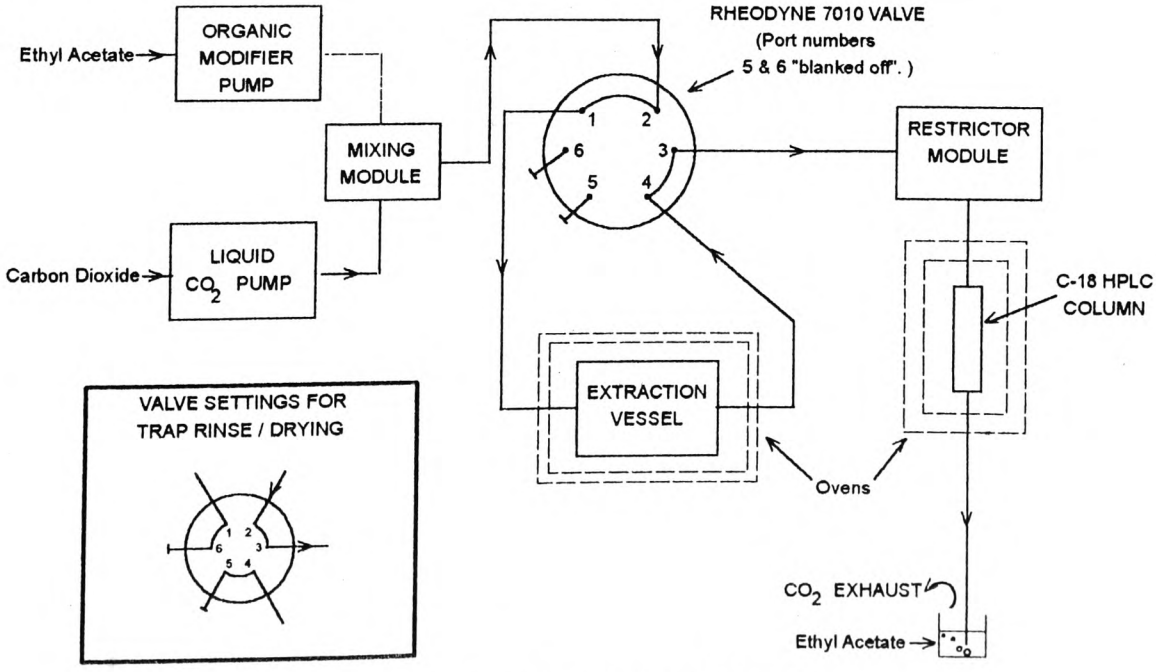

Fig. 1 Schematic of dynamic aqueous SFE system.

61.7% of the theoretically available testosterone, assuming 100% enzymic hydrolysis efficiency, had been extracted after 120 min dynamic SFE.

In order to establish the efficiency of dynamic SFE for the isolation of liberated testosterone it was necessary to determine the quantity of any remaining free testosterone in the enzymic digest. The cooled contents of the extraction vessel were diluted with 100 ml de-ionized water and then extracted with two 200 ml aliquots of ethyl acetate. This procedure involving relatively large volumes of aqueous and organic phase became necessary since initial attempts to extract testosterone from the undiluted enzyme digest with 50 ml of ethyl acetate were unsuccessful. An emulsion was formed in the organic phase which could not be separated *via* the addition of sodium chloride. The 200 ml ethyl acetate fractions were then combined and blown to dryness under a stream of nitrogen. The residue was redissolved in 2.5 ml of $^2H_3$-testosterone internal standard solution and 7.5 ml of ethyl acetate. GC–MS analysis indicated that a further 8.4% of liberated testosterone was still present at the time at which dynamic aqueous SFE had been stopped. Hence it was determined that 70.1% testosterone-β-D-glucuronide had been enzymically hydrolysed after 135 min incubation and that dynamic SFE had been 88% efficient in isolating the liberated testosterone. A longer enzymic incubation period under conditions of dynamic SFE may provide a means of improving the final total recovery of testosterone.

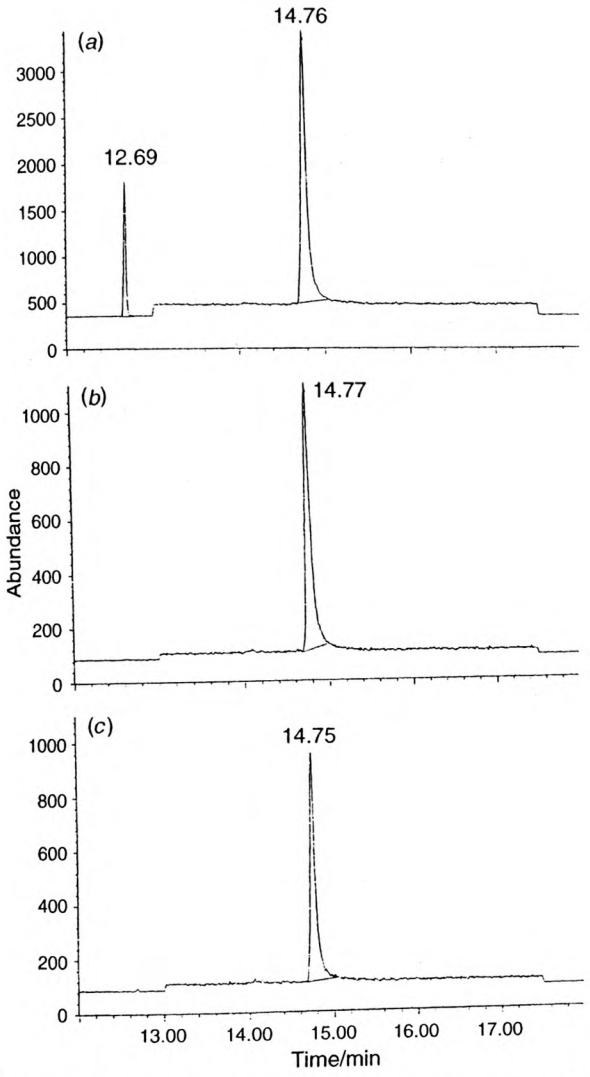

Fig. 3 (*a*) GC–MS total ion chromatogram obtained for the SIM analysis of liberated testosterone isolated after 120 min dynamic aqueous SFE. Electron multiplier gain increased by 500 V during the period 13–17.5 min. (*b*) Integrated molecular ion response obtained for testosterone (M_r = 288). (*c*) Integrated molecular ion response obtained for $^2H_3$-testosterone (M_r = 291).

Conclusion

The feasibility of dynamic aqueous SFE for the isolation of liberated testosterone from the enzymic digestion of testosterone-β-D-glucuronide has been established. The SFE vessel used in these experiments had been designed for another application involving the analysis of hydrocarbons in water.[6] Despite the large internal volume of the vessel, the kinetics of the enzymic hydrolysis under the SFE conditions employed meant that free testosterone was efficiently extracted with respect to time. Depending upon the nature of specific aqueous SFE applications, we are currently considering fabricating several vessels of much smaller volume.

We wish to thank Dr. E. Houghton and Dr. S. A. Westwood (Horseracing Forensic Laboratory Ltd., Newmarket, UK) for their technical advice. We also acknowledge the support provided by Dr. D. James, D. Roberts and M. I. Littlewood (Nicolet Instruments, Warwick, UK).

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Paper 6/04823D
Received July 9, 1996
Accepted July 30, 1996

Analysis of Phenols in Water at the ppb Level Using Direct Supercritical Fluid Extraction of Aqueous Samples Combined On-line With Supercritical Fluid Chromatography–Mass Spectrometry

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A high pressure multivalve switching system has provided a means of interfacing a direct aqueous SFE vessel to a packed column SFC-MS system. Using full scan negative ion atmospheric pressure chemical ionization, the combined SFE–SFC–MS instrumentation enabled the analysis of phenols in water at the 40 ppb level.

A recent review article[1] indicates, from the number of applications cited, that SFE continues to represent a growth area in analytical chemistry. Amongst the potential benefits associated with SFE is the reduction in the number of sample handling stages and the possibility of its direct coupling to a wide range of separation techniques,[2,3] of which SFC was first described.[4] Both of these features serve to promote high sample throughput.

At present most of the published reports dealing with SFE involve the study of solid matrices. Strategies for the SFE analysis of liquid based matrices can be divided into two categories:[5] (*i*) indirect procedures which require the loading of the liquid sample onto support media such as solid phase sorbents or solid phase membrane extraction discs prior to SFE, or (*ii*) direct SFE whereby the liquid matrix is directly extracted with a supercritical fluid in the absence of a support medium. Few studies involving the use of liquid SFE–SFC have been reported. Amongst these, indirect aqueous SFE–SFC has enabled the analysis of prostaglandins[6] and mitomycin C[7] whereas direct aqueous SFE–SFC procedures have been described for the analysis of phenols,[8] diisopropyl methylphosphonate[9] and two organic bases.[9]

This report represents the first account of the analysis of organic contaminants in water using a direct aqueous SFE–SFC–MS system. A novel high pressure multivalve arrangement was designed to enable these studies. The SFE vessel used in these studies has a relatively large internal volume. This feature addresses a previously identified[10] hindrance to the development of direct aqueous SFE sample preparation procedures involving trace analysis of organics in water. Large volume samples, typically 100 ml to 1 l need to be extracted to recover sufficient amounts of compounds to enable their detection by established analytical procedures.[10] The direct aqueous SFE–SFC–MS system described within this paper readily facilitated the detection of three phenols each at the low ppb level using full scan negative ion atmospheric pressure chemical ionization (APCI).

Experimental

A stainless-steel vessel (patented) with an internal volume of 300 ml, pressure tested to 43.1 MPa, housed within a Pye 104 gas chromatograph (Unicam, Cambridge, UK) was used for all aqueous supercritical fluid extractions. A Hewlett-Packard G 1205A supercritical fluid chromatograph was used to supply liquid carbon dioxide (BOC, Manchester, UK) to the extraction vessel and perform all SFC–MS studies. A Rheodyne 7010 valve (Supelco, Poole, Dorset, UK) was used to connect or isolate the vessel from a multivalve switching system in a manner previously described.[11] Schematics of the multivalve system configurations (Rheodyne valve model numbers included) used for all SFE-SFC-MS studies are shown in Figs. 1(*a*) and (*b*). The tee pieces were Valco ZTIC (Phase Separations, Deeside, Clwyd, UK), all filter assemblies were Valco ZUFR1 with Valco TSS110 stainless-steel tubing providing all connections. The multivalve system incorporated two Apex amino RP 5 μ 150 × 4.6 mm columns (Jones Chromatography, Hengoed, Mid Glamorgan, UK). The primary function of the first column was to serve as an analyte trap. Both columns were housed within the supercritical fluid chromatograph oven. Prior to SFE, both columns connected in series *via* the SFC–MS valve settings, Fig. 1(*b*), were dried *in situ* at 60 °C using carbon dioxide at a rate of 1.5 ml min^{-1} at a pressure of 30.89 MPa. At the end of the drying cycle the valves were switched to the SFE configuration, Fig. 1(*a*), such that the second amino column, now primed with carbon dioxide, became isolated.

The supercritical fluid chromatograph was interfaced to a Finnigan MAT (San Jose, CA, USA) TSQ 700 series triple quadrupole mass spectrometer. The APCI interface vaporiser heater was 500 °C with the heated capillary at 250 °C using a corona current of 5 μA. The APCI interface was modified[12] to facilitate SFC–MS using a 1/16" od stainless-steel tube of 0.005" id acting as a final restriction stage. The column eluant was split such that 25% was supplied to the APCI interface.

An aqueous solution of 2,4-dibromophenol, 2,4,5-trichlorophenol and 4-nitrophenol was prepared, pH adjusted to 4.2 *via* the addition of hydrochloric acid, such that each phenol was present at the 40 ppb level. A 250 ml aliquot of this solution, equivalent to 10 μg of each phenol, was transferred into the SFE vessel. Using the multivalve configuration shown in Fig. 1(*a*) direct aqueous SFE was performed at 65 °C at a pressure of 32.33 MPa with a flow rate of liquid carbon dioxide at 4 ml min^{-1} for a period of 45 min. During SFE the temperature of the oven housing the amino columns was maintained at 30 °C. At the end of the extraction period the mutivalve system was switched to enable SFC–MS operation.

All SFC and SFC-MS studies were conducted at 30.89 MPa with high purity grade methanol (Fisons, Loughborough, Leicestershire, UK) serving as organic modifier. The mobile phase flow rate was 1.5 ml min^{-1} and the steps of the organic modifier programme are given in Table 1.

The temperature programme used for SFC and SFC–MS studies was 1 min at 60 °C then to 70 °C at 10 °C min min^{-1} held for 1 min, then to 90 °C at a rate of 20 °C min^{-1} with this final temperature held until analyses were completed. The supercritical fluid chromatograph UV detector was set at 220 nm for off-line SFC and SFE–SFC studies.

Off-line direct valve loop APCI analyses, interface parameters as previously described, of each phenol were conducted at 10.89 MPa using carbon dioxide with 25% v/v methanol at 1.5 ml min^{-1}.

All GC–MS studies were performed in selected ion monitoring mode using a Hewlett-Packard 5971A mass-selective detector, operated using electron ionization, interfaced to a HP 5890 gas chromatograph equipped with a HP 6890 series autosampler. Three calibration standards containing each phenol, whose respective concentrations ranged from 0.2 to 1 ng μl^{-1}, were prepared with ethyl acetate as solvent. Each solution also contained anthracene, acting as internal standard, at the 0.4 ng μl^{-1} level. All GC–MS analyses were performed using a HP-5MS 30 m × 0.2 mm column of 0.25 μm film thickness with helium (MG Gas Products, Reigate, Surrey, UK) serving as carrier gas. The gas chromatograph temperature programme was 2 min at 60 °C then to 290 °C at 15 °C min^{-1}, with the final temperature held for 15 min. Splitless 5 μl injections were made at 250 °C. The quotient of responses obtained for the molecular ions of: 2,4-dibromophenol (M_r = 252), 2,4,5-trichlorophenol (M_r = 196) and 4-nitrophenol (M_r = 139) with that for anthracene (M_r = 178) were used for calibration and determination of individual phenol SFE recoveries.

Table 1 Organic modifier programme

Level	Ramp rate (% methanol)	% Methanol	Hold time/ min
0		0	2
1	1.0	1.0	1.0
2	2.0	10	2.0
3	10.0	30	40.0

Results and Discussion

In order to develop a combined dynamic aqueous SFE–SFC–MS system a high pressure multivalve switching arrangement incorporating an amino column was designed. During dynamic aqueous SFE the restrictor module was situated pre-column by selection of the appropriate valve settings as shown in Fig. 1(*a*). This arrangement resulted in the trapping of extracted phenols onto the column following decompression of supercritical fluid carbon dioxide. At the end of the extraction period, with the aqueous SFE vessel isolated,[11] the multivalve configuration was switched, Fig. 1(*b*), so that the restrictor module became situated post-column to enable SFC–MS analysis of trapped phenols.

Initial studies were conducted to investigate whether a single amino column, acting as an analyte trap, could also provide an efficient means of separating the phenols in the following SFC stage. These investigations indicated significant degradation of chromatographic performance when the results of direct SFC were compared to those obtained for off-line SFE–SFC. This is attributed to the creation of an imprecise trapping zone at some point along the column whose own back pressure serves to provide an *in situ* restriction. Due to this factor the effective column length available for SFC following the trapping stage of SFE was reduced, adversely affecting column separation efficiency. In order to circumvent this problem a second

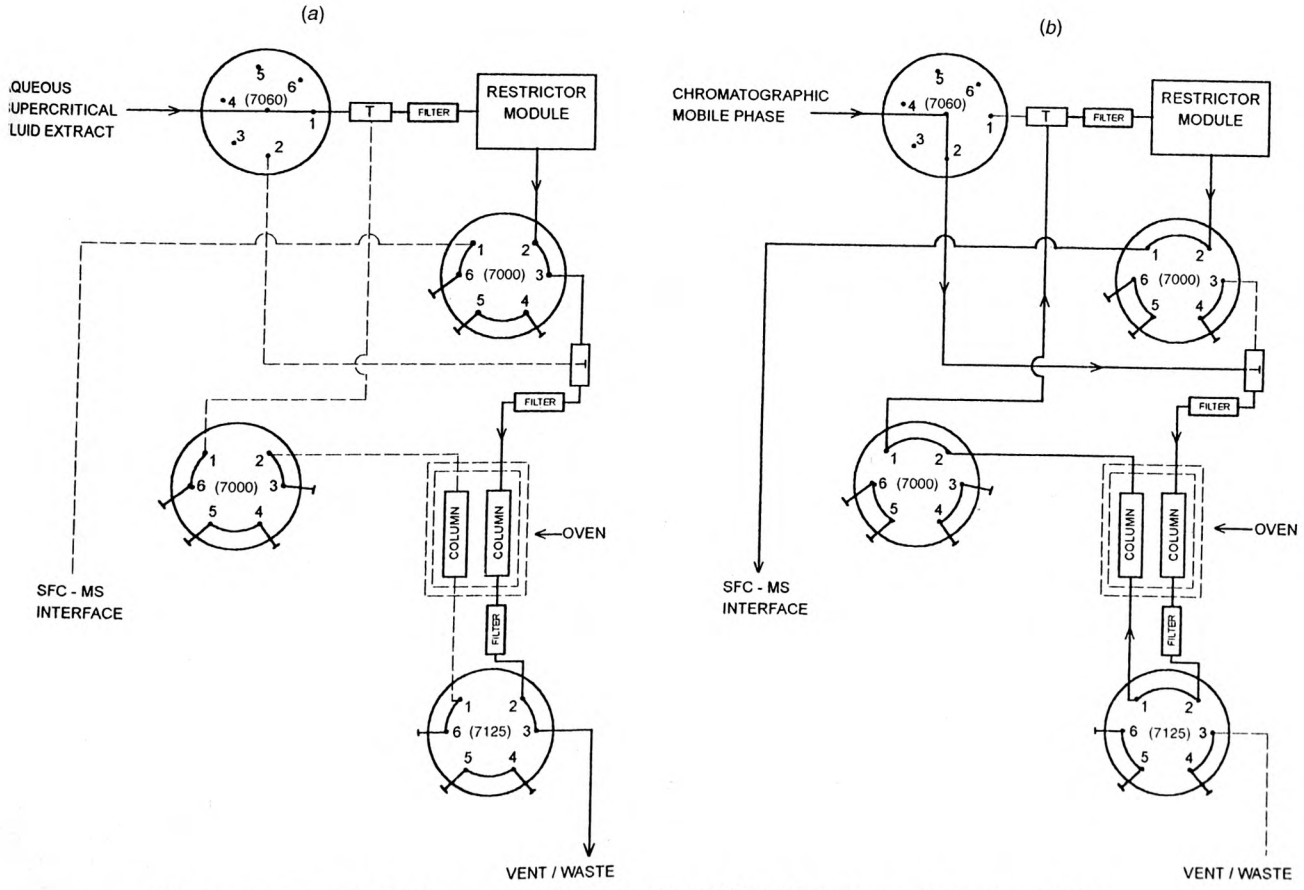

Fig. 1 Configurations of high pressure multivalve system used for (*a*) direct aqueous SFE and (*b*) SFC–MS.

dedicated chromatographic amino column was incorporated into the multivalve system as shown in Figs. 1(*a*) and 1(*b*). During SFE this column, which had been previously conditioned, was isolated by the appropriate valve settings from the trapping column. This arrangement using a second amino column resulted in retention of chromatographic integrity.

Prior to any SFE–SFC–MS studies, each of the three individual phenols were subjected to direct APCI analysis in positive and negative ion modes *via* direct valve loop injections, equivalent to 500 ng of each phenol, without any intervening chromatography. All three phenols provided an intense response in negative ion APCI mode with each mass spectrum being dominated by the presence of an $[M - H]^-$ ion. Since none of the three phenols provided a good response using positive ion APCI, with the previously specified conditions, all further SFE–SFC–MS studies were conducted in negative ion mode.

In order to establish the feasibility of dynamic aqueous SFE–SFC– MS for the analysis of phenols in water, the vessel was loaded with 250 ml of water spiked with each phenol at the 40 ppb level. The water had been previously acidified, pH adjusted to 4.2 with hydrochloric acid. The results of a previous direct aqueous SFE study[12] involving a range of phenols (including three chlorinated species) indicated that lowering of pH suppressed phenol dissociation which served to promote SFE recovery. After 45 min direct aqueous SFE the vessel was isolated and the valves were switched to allow SFC–MS analysis.

The SFE–SFC–MS negative ion APCI total ion chromatogram obtained for the analysis of the three phenols at the 40 ppb level is shown in Fig. 2(*a*). Despite all the phenols' full scan response providing poor S/N, each could be readily detected by inspection of the respective $[M - H]^-$ ion profiles, Figs 2(*b*)–(*d*). Acidification of the mobile phase may help reduce peak tailing which was also observed in off-line SFC studies of the three phenols. The full scan SFE–SFC–MS negative ion APCI mass spectrum obtained for 2,4,5-trichlorophenol is shown in Fig. 3. No attempt was made to determine the full scan negative ion APCI detection limit of each phenol in these preliminary SFE–SFC–MS studies. The intensity of the mass spectrum obtained for each phenol suggests that determining the presence of these compounds at levels lower than 40 ppb should be readily achievable in full scan mode.

In order to determine individual phenol extraction efficiencies, the vessel was emptied and flushed with 100 ml of deionised water. The pH of the combined aqueous fractions was adjusted to 2 *via* the addition of concentrated hydrochloric acid and then extracted with two 100 ml aliquots of ethyl acetate. The combined ethyl acetate fractions were then blown almost completely to dryness under a stream of nitrogen before being transferred to a 5 ml calibrated flask. The contents of the flask were then made up to 5 ml using ethyl acetate and an ethyl acetate solution of anthracene. The final concentration of anthracene acting as internal standard was equivalent to 0.4 ng μl^{-1}. Results of quantitative GC–MS indicated the following recoveries: 2,4-dibromophenol 88%, 2,4,5-trichlorophenol 81% and 4-nitrophenol 67%. These extraction efficiencies correlate with the phenol SFC retention times, both parameters relating to polarity. It should be possible to improve the SFE recoveries for each phenol by using a longer extraction period or by using a pump capable of delivering a flow of liquid carbon dioxide greater than 4 ml min^{-1} to the vessel.

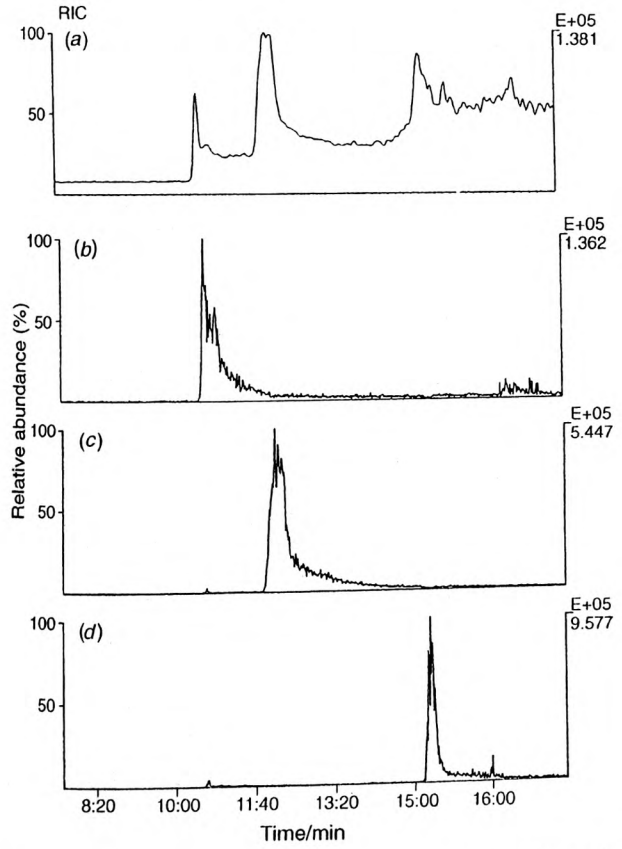

Fig. 2 (*a*) Total ion SFE–SFC–MS chromatogram obtained for a 250 ml water sample, spiked with three phenols each at the 40 ppb level. Ion profile responses obtained for the $[M - H]^-$ species of individual phenols; (*b*) *m/z* 251 for 2,4-dibromophenol (*c*) *m/z* 195 for 2,4,5-trichlorophenol; and (*d*) *m/z* 138 for 4-nitrophenol.

Conclusion

This study has established the feasibility of direct aqueous SFE–SFC–MS. The relatively large sample capacity of the vessel helped facilitate the detection of three phenols each at the 40 ppb level. The SFE–SFC–MS full scan negative ion APCI mass spectrum obtained for each phenol was characterized by the

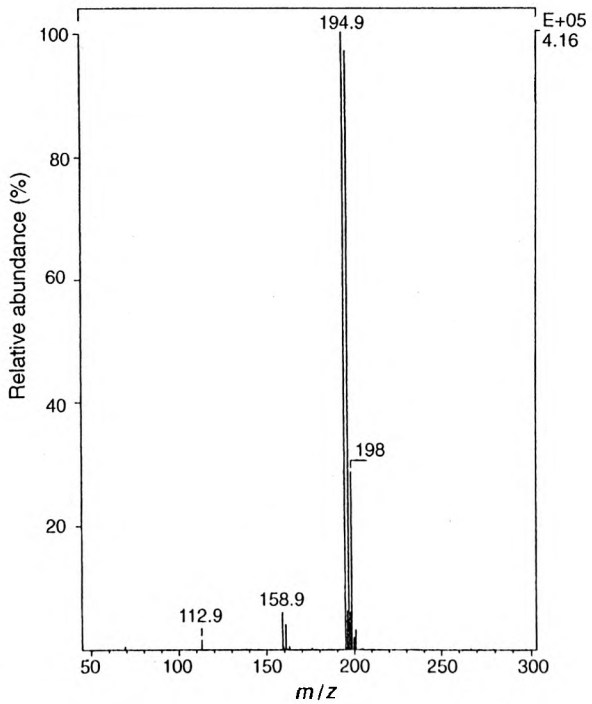

Fig. 3 SFE–SFC–MS negative ion APCI mass spectrum obtained for 2,4,5-trichlorophenol (M_r = 196).

production of an intense $[M - H]^-$ species. By resorting to selected ion monitoring negative ion APCI, it is anticipated that ppt or lower detection limits should be possible for each phenol using the current SFE–SFC–MS system.

We wish to acknowledge the continued technical support provided by Dr. D. James, D. Roberts and M. I. Littlewood (Nicolet Instruments, Warwick, UK).

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Paper 6/07237B
Received October 23, 1996
Accepted November 19, 1996

Drugs in Water: Analysis at the Part-per-billion Level Using Direct Supercritical Fluid Extraction of Aqueous Samples Coupled On-iine With Ultraviolet–Visible Diode-Array Liquid Chromatography–Mass Spectrometry

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A high pressure ten port switching valve has provided a means of interfacing a direct aqueous SFE vessel to an LC–MS instrument equipped with a UV/VIS diode-array detector. Analyte trapping and analysis were performed using a coupled octadecylsilane (ODS)–amino column system. Using full scan ammonia chemical ionization, the combined SFE–LC–MS instrumentation enabled the analysis of estrone, hexestrol and zeranol in water each at the 200 ppb level.

The analysis of drugs and their metabolites in biological fluids represents an essential role in pharmaceutical and toxicology studies. Often many drugs are only present at ultratrace levels within such complex liquid matrices. Accordingly sample preparation procedures have been developed for the recovery of sufficient quantities of drugs to enable their detection using established analytical techniques. Liquid–liquid extraction (LLE) and solid phase extraction (SPE) are now widely used sample preparation procedures for drug analysis in liquids. SPE offers several advantages over LLE which include: ease of automation, high degree of analyte selectivity and on-line coupling with chromatography. Fully automated on-line SPE procedures for the analysis of drugs in plasma involving confirmation by LC–MS[1] and GC–MS[2] have also been developed.

Supercritical fluid extraction is attracting considerable attention as an alternative sample preparation procedure[3] which can also be directly coupled to a range of chromatographic techniques.[4,5] At present the majority of published SFE applications involve the analysis of solid matrices. Amongst these, SFE has been used for the analysis of a range of drug residues in animal tissues which include: anabolic steroids,[6,7] nitrobenzamide antimicrobial agents[8] and sulfa drugs.[9]

Analysis of liquid matrices by SFE can be divided into two categories:[10] (*i*) indirect procedures which involve the loading of the liquid onto support media such as SPE cartridges or SPME discs or (*ii*) direct SFE whereby the liquid is directly extracted. Indirect aqueous SFE has been used for the analysis of a range of drugs including: mitomycin C,[11] prostaglandins,[12] mebeverine alcohol,[13] opiates[14] and β-blockers.[15] Direct aqueous SFE has been used for the analysis of sulfamethazine[16] and the continuous extraction of testosterone liberated from the enzymic hydrolysis of testosterone-β-D-glucuronide.[17]

Although SFE can be coupled to a wide range of chromatographic techniques very few reports involving on-line confirmation of the detection of drug residues in biological samples using mass spectrometry have been published. Examples of such studies include the SFE–SFC–MS–MS determination of anabolic steroids in porcine tissue[6] and SFE–SFC–SFC–MS analysis of 'the digitalis-like factor' in peritoneal dialysate.[18]

Recently we reported the combination of direct aqueous SFE–SFC–MS using APCI for the analysis of phenols in water at the 40 ppb level.[19] The development of such on-line sample preparation procedures provide considerable potential for the reduction of the number of sampling handling stages, with associated errors, prior to final analysis. As far as we are aware the results described within this paper represent the first account of the coupling of a direct aqueous SFE vessel to an LC–MS system. In order to facilitate these studies a novel high pressure ten port switching valve configuration has been designed. The direct aqueous SFE–LC–MS system has enabled the analysis of three drugs in water each at the 200 ppb level.

Experimental

A stainless-steel vessel (patented) with an internal volume of 300 ml, pressure tested to 41.3 MPa, housed within a Pye 104 gas chromatograph (Unicam, Cambridge, UK) was used for all aqueous supercritical fluid extractions. A Gilson 303 pump (Anachem, Luton, Bedfordshire, UK) equipped with a cooling head connected to a Neslab RTE 110 recirculator (Jencons Scientific, Leighton Buzzard, Bedfordshire, UK) operated at −10 °C, was used to supply liquid carbon dioxide (MG Gas Products, Reigate, Surrey, UK) to the extraction vessel. The outlet of the extraction vessel was connected to a Tescom Model 26-1722-24-084A restrictor (Tescom Corporation, Elk River, Minnesota, USA). A Rheodyne 7610-400 ten port switching valve (Anachem) was used to connect the restrictor outlet to a Techsphere 5ODS 250 × 4.6 mm column (HPLC Technology, Macclesfield, Cheshire, UK) during SFE as shown in Fig. 1(*a*). At the end of the aqueous SFE period the valve was switched to the position shown in Fig. 1(*b*) resulting in the ODS column becoming coupled in series to a Nucleosil 5 μm amino 150 × 4.6 mm column (Sigma-Aldrich, Poole, Dorset, UK). A Finnigan MAT (San Jose, CA, USA) 4000 series quadrupole mass spectrometer equipped with Finnigan MAT moving-belt and Mass Spectrometry Services (Manchester, UK) data system was used for SFE-LC-MS studies. The moving belt vaporizer was set at 250 °C with liquid chromatographic eluant being applied to the belt using a Finnigan MAT spray depositor. The EI studies were performed at 70 eV with an ion source temperature of 200 °C. The CI SFE–LC–MS studies were conducted using high purity grade ammonia (BOC, Manchester, UK) as reagent gas, admitted to give a source housing pressure of 2.5 mPa using an ion source temperature of 150 °C.

Liquid chromatography was performed using Shimadzu LC-10AS pumps (Shimadzu, Milton Keynes, UK) interfaced to a Shimadzu SCL-10A system controller. A Shimadzu SPD-M10AVP diode-array detector, operated over the scan range 200–500 nm, was used for all studies. All LC data acquisition and system control was performed using Shimadzu Class VP

software. HPLC grade heptane and ethanol were supplied from Fisons (Loughborough, Leicestershire, UK). The gradient programme used for all studies was pure heptane for 12 min then to heptane–ethanol (65 + 35) at time 42 min following a linear profile at flow rate 1 ml min^{-1}.

The incorporation of a Rheodyne 7125 injection valve (Anachem) into the system, as shown in Fig. 1, facilitated off-line LC studies. A standard mixture of estrone, hexestrol and zeranol was prepared by dissolving 10 mg of each compound in 5 ml of methanol. Prior to the onset of aqueous SFE the ODS column was thoroughly dried by purging with carbon dioxide and the amino column was conditioned with pure heptane.

An aqueous solution of the three drugs was prepared such that each was present at the 200 ppb level. A 250 ml aliquot of this solution, equivalent to 50 μg of each drug, was transferred into the SFE vessel. Using the valve configuration shown in Fig. 1(*a*) direct aqueous SFE was performed at 55 °C at a pressure of 24.1 MPa with liquid carbon dioxide flow rate of 8 ml min^{-1} for a period of 30 min. At the end of the extraction period the flow rate of liquid carbon dioxide was set to zero, which resulted in closure of the restrictor as a drop in SFE vessel pressure below the target of 24.1 MPa was sensed. The ODS column was allowed to depressurize for approximately 5 min before the valve was switched to the position shown in Fig. 1(*b*) and LC–MS analysis was commenced using the gradient programme.

Results and Discussion

In order to develop a combined direct aqueous SFE–LC–MS system a novel high pressure ten port valve configuration was designed as shown in Fig. 1. During direct aqueous SFE the valve was set to the position shown in Fig. 1(*a*). With this arrangement extracted drugs were trapped onto an ODS column following decompression of supercritical fluid carbon dioxide.

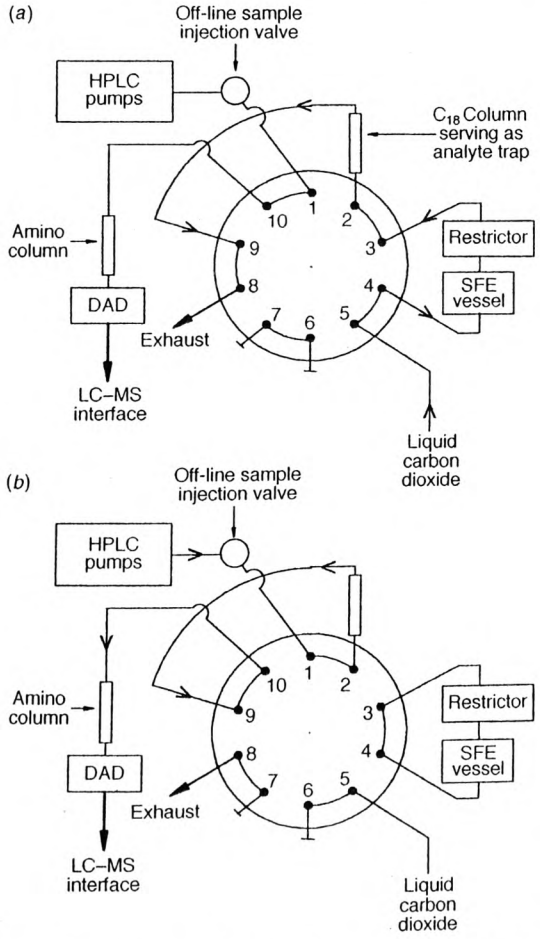

Fig. 1 Configurations of ten port high pressure valve used for (*a*) direct aqueous SFE and (*b*) LC–MS.

Initial studies involving off-line LC analyses of a mixture of the three drugs were conducted. The valve setting shown in Fig. 1(*a*) allowed analysis of the drugs using only the amino column. The drugs were also analysed using coupled ODS-amino columns with the valve position shown in Fig. 1(*b*). Off-line sample introduction was made *via* a standard six port injection valve. The purpose of these studies was to establish whether the coupled ODS-amino column system could be successfully used for the analysis of the drugs. A gradient programme was designed to rapidly elute trapped drugs from the non-polar ODS column with band focusing, onto the relatively polar amino column during the period when pure heptane acted as mobile phase. Comparison of the chromatograms obtained for the analyses of the drugs using: (*i*) only the amino column and (*ii*) the coupled ODS-amino columns, indicated no significant difference in chromatographic performance between the two systems. The chromatogram shown in Fig. 2 was obtained from the injection of two 10 μl samples of standard drug mixture, equivalent to a total of 40 μg of each drug, onto the coupled ODS-amino columns. The injections were made 5 min apart following commencement of the gradient elution programme. The fact that each component gave rise to a single peak serves to demonstrate the band focusing effect of the amino column within the system. This feature is advantageous given the adverse effect imprecise SFE analyte trapping can exert upon on-line chromatographic performance.[19]

The moving belt was the first commercially available LC–MS interface to provide true library searchable EI mass spectra. It has now been almost entirely replaced by particle beam LC–MS for the provision of such information. One commonly cited[20] reason for the decline of the moving belt interface is its mechanical complexity. In our experience this has not presented a problem. In order to assess the most appropriate ionization technique for the analysis of the three drugs, off-line studies were undertaken. A 1 μl methanolic solution of each drug (equivalent to 1 μg of each compound) was directly introduced onto the moving belt interface for EI and ammonia CI analyses. The EI mass spectra obtained for estrone (M_r = 270) and zeranol (M_r = 322) both provided a molecular ion and structurally significant fragment ions. The EI mass spectrum obtained for hexestrol (M_r = 270) did not provide a molecular ion and was dominated by the presence of a fragment ion at *m/z*

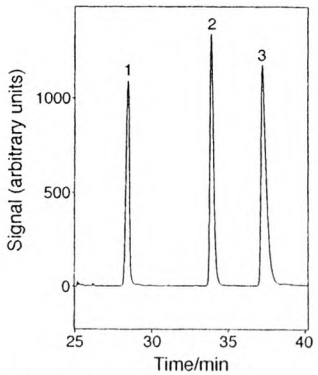

Fig. 2 Off-line LC analysis of drug mixture using coupled ODS-amino columns. Detection: UV/VIS DAD chromatogram selected at 281 nm. Peaks: 1, estrone; 2, hexestrol; 3, zeranol. Total sample size: 40 μg each drug introduced *via* two 10 μl injections of standard drug mixture. The time delay between injections was 5 min after commencement of gradient programme.

135. Ammonia CI was selected for subsequent SFE–LC–MS analyses on the basis that estrone and zeranol both provided classical CI mass spectra which were dominated by the presence of protonated and ammonium adduct molecular ions. Such spectra are ideal for the analysis of known target compounds. The ammonia CI mass spectrum obtained for hexestrol although providing a weak ammonium adduct molecular ion was again dominated by the presence of a fragment ion at *m/z* 135.

In order to establish the feasibility of direct aqueous SFE–LC–MS, the vessel was loaded with 250 ml of water spiked with each drug at the 200 ppb level. With the valve configuration shown in Fig. 1(*a*) the aqueous sample was subjected to SFE for 30 min. It was noted that the external surface of the final 5 cm length of the outlet end of the ODS column rapidly formed a layer of ice during this period. Once the SFE vessel had been isolated the ODS column was allowed to depressurize for a period of 5 min. The ten port valve was then switched to allow LC–MS analysis of the extracted drugs. The gradient programme used had a 12 min period during which pure heptane was delivered to the coupled ODS-amino column system. Apart from band focusing, the initial isocratic step of the programme was also designed to purge residual carbon dioxide gas from the coupled column system. At the end of the isocratic period, the elutropic strength of the mobile phase was increased *via* the addition of ethanol, in order to chromatograph the drugs.

The chromatogram obtained at 281 nm for the SFE–LC–MS analysis of the drugs at the 200 ppb level is shown in Fig. 3 with the corresponding UV/VIS spectrum obtained for zeranol shown in Fig. 4. The increase in retention time observed for each drug, relative to the off-line analysis shown in Fig. 2, is attributed to the time required to purge gaseous carbon dioxide through the coupled columns. With the chromatographic system used, hexestrol appears to have been only partially resolved from several co-extracted compounds. The direct aqueous SFE–LC–MS ammonia CI total ion chromatogram obtained for the drugs at the 200 ppb level is shown in Fig. 5(*a*). The $[M + H]^+$ ion profiles obtained for estrone and zeranol are shown in Figs. 5(*b*) and (*d*), respectively along with the *m/z* 135 ion profile characteristic of hexestrol in Fig. 5(*c*). The SFE–LC–MS ammonia CI mass spectrum obtained for each drug was of comparable quality to that previously described following the off-line studies. Fig. 6 shows the full scan ammonia CI SFE–LC–MS mass spectrum obtained for estrone. The hexestrol *m/z* 135 ion profile shows high fidelity with the corresponding section of the chromatogram, shown in Fig. 3, obtained for this compound. At present we are unable to suggest the identity of any co-chromatographing impurity, following examination of individual ammonia CI and UV/VIS spectra recorded in the region of the hexestrol peak. We have therefore concluded that the on-line chromatographic peak obtained for hexestrol peak is probably split possibly due to selective poor focusing and/or the influence of co-extracted water. Neither the estrone or zeranol peaks whose respective retention times bracket that of hexestrol showed any indications of splitting.

Although no attempt was made to estimate individual drug recoveries during these studies, it was noted that extraction efficiencies for the three drugs correlate with chromatographic retention times as previously reported.[19] Given the band focusing effect of the coupled column system, potential exists for the introduction of an internal standard post-SFE *via* the off-line sample injection valve. The development of such a

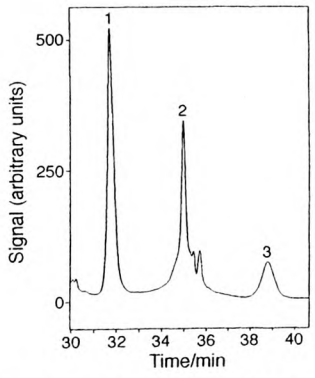

Fig. 3 The SFE–LC analysis obtained for a 250 ml water sample spiked with each drug at the 200 ppb level. Detection: UV/VIS DAD chromatogram selected at 281 nm. Peaks: 1, estrone; 2, hexestrol; 3, zeranol.

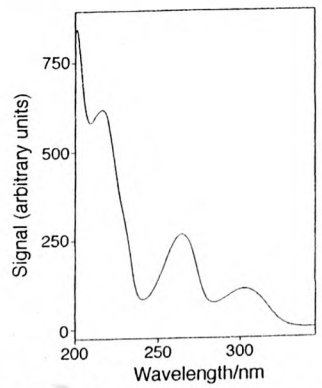

Fig. 4 SFE–LC UV/VIS DAD spectrum obtained for zeranol. Sample: 250 ml water spiked at the 200 ppb level.

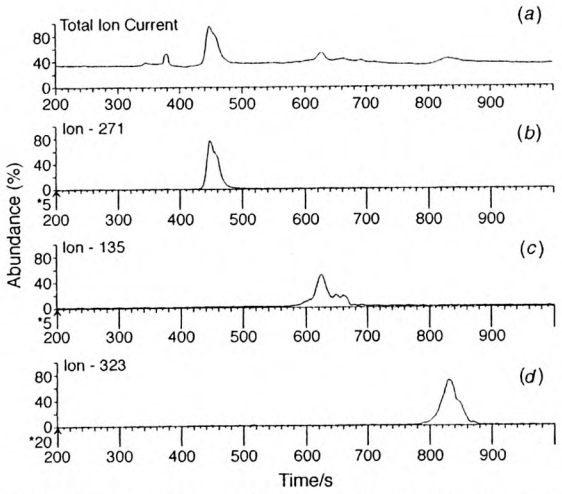

Fig. 5 (*a*) Full scan ammonia CI total ion SFE–LC–MS chromatogram obtained for a 250 ml water sample, spiked with three drugs each at the 200 ppb level. Ion profiles obtained for: (*b*) estrone $[M + H]^+$ species at *m/z* 271; (*c*) hexestrol fragment ion at *m/z* 135; and (*d*) zeranol $[M + H]^+$ species at *m/z* 323.

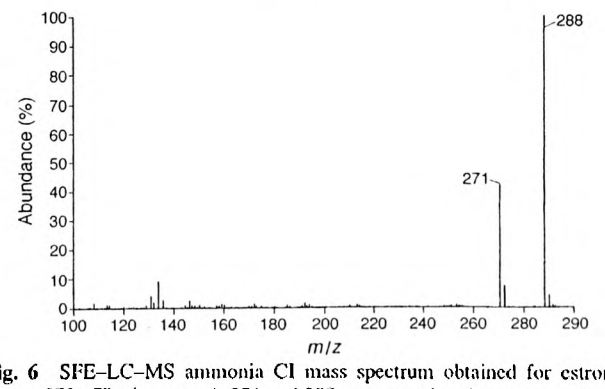

Fig. 6 SFE–LC–MS ammonia CI mass spectrum obtained for estrone (M_r = 270). The ions at *m/z* 271 and 288 correspond to the protonated and ammonium ion adduct molecular species respectively.

procedure should facilitate determination of target compound extraction efficiencies. We hope to report the results of such studies in due course.

Conclusions

This study has demonstrated the feasibility of direct aqueous SFE–LC–MS. The combination of on-line UV/VIS diode-array and mass spectrometric detection provide a high degree of specificity for analysis of target compounds. The relatively large sample capacity of the SFE vessel readily facilitated the detection of each of the drugs at the 200 ppb level. With this aqueous SFE–LC–MS system it should be possible to detect each of the drugs at the ppt level by the use of selected ion monitoring procedures. Further direct aqueous SFE studies, involving a range of LC–MS techniques including particle beam, APCI, ESI and dynamic FAB, should be possible using appropriate columns with the valve system that has been developed.

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Paper 7/04901C
Received July 9, 1997
Accepted August 14, 1997

Analysis of oil in water at the low ppm level using direct supercritical fluid extraction coupled on-line with infrared spectroscopy

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Received 4th June 1998, Accepted 15th July 1998

The continued development of direct aqueous SFE instrumentation has enabled the on-line SFE-FTIR analysis of automobile diesel in water at the low ppm level. The integrated system requires minimum sample handling stages with analysis of a 500 ml water sample being completed within 15 min. Diesel-spiked water samples provide a linear response for absorbance values measured at 2932 cm^{-1} ($\nu_{asymmetric}$ CH_3), through the range 17.2–137.6 ppm. Method accuracy for determining spiked diesel concentrations within this range was greater than 85% with RSD 3–8.6% (n = 4 for each set of spiked calibration standards). The technique allows analyses to be performed with samples containing high levels of particulates and is also suitable for the determination of volatile aromatics and hydrocarbons. The detection limits for benzene and n-decane are 17.6 and 4 ppm, respectively.

Introduction

Parties to the Montreal Protocol Treaty are committed to the control and eventual phase out of the use of substances that deplete the ozone layer. In support of these efforts, the United States Environmental Protection Agency (EPA) has approved a supercritical fluid extraction (SFE) method for the analysis of total petroleum hydrocarbons (TPH) in soil. The SFE procedure (EPA Method 3560) was developed[1,2] to provide an alternative to Soxhlet methods (*e.g.*, EPA Method 3450) which involve the extraction of TPH from soil using 1,1,2-trichloro-1,2,2-trifluoroethane (Freon 113) prior to analysis. Method 3560 involves extracting a soil sample with supercritical fluid carbon dioxide at 340 atm and 80 °C for 30 min dynamic SFE, with tetrafluoroethene being used for analyte collection. An off-line silica gel cleanup stage is then used to remove polar organics and vegetable oils from the extract prior to TPH determination. The use of tetrafluoroethene, which is transparent in the infrared region of interest, enables the use of Fourier transform infrared (FTIR) spectroscopy (*e.g.*, EPA Method 8440) for final TPH quantification. The field portability of Method 3560 has been established[3] in surveys involving contaminated land and remediation programmes.

Sample preparation procedures for IR methods[4,5] for the analysis of oil in water typically involve the extraction of 500–1000 ml acidified and salted water samples with 25–50 ml of Freon 113 or carbon tetrachloride. At present, both of these restricted organic solvents continue to be available to laboratories performing oil in water analyses since this application falls within the essential use category.[6] As with Method 3560 a silica gel cleanup stage can be used to remove the extracted polar organic species prior to the IR determination of water oil content. An alternative technique to IR for the nonspecific determination of oil in water involves the use of gravimetry. In accordance with the phase out of ozone depleting solvents, the EPA has developed Method 1664. This method uses n-hexane to replace Freon 113 as extraction solvent, with an optional silica gel cleanup stage, for the gravimetric determination of oil and grease and petroleum hydrocarbons in industrial effluents and discharge water.

Supercritical fluid carbon dioxide efficiently absorbs IR radiation in the regions 3800–3500 cm^{-1}, 2500–2150 cm^{-1} and below 900 cm^{-1}. Since few organic compounds absorb in these regions, windows of opportunity exist for the development of on-line SFE-FTIR analytical procedures. The weak Fermi resonance bands observed for supercritical fluid carbon dioxide at approximately 2070, 1944 cm^{-1} and 1400-1200 cm^{-1} do not provide a source of interference for the IR determination of hydrocarbons. The principles and applications of combined on-line SFE-FTIR have been reviewed[7,8] and one such study[9] has described the analysis of TPH in soil with this technique.

Recently we described the potential of direct aqueous on-line SFE-FTIR for the analysis of oil in water using supercritical fluid carbon dioxide.[10] The continued development of direct aqueous SFE instrumentation has resulted in the fabrication of an on-line SFE-FTIR system specifically designed for the rapid screening of oil in water at the low ppm level in 500 ml water samples. The system does not require the use of any organic solvents. Minimal sample handling stages serve to ensure sample integrity whilst reducing exposure of the analyst to any toxic hydrocarbons present within the samples. Furthermore, since the whole sample bottle is placed within the chamber of the SFE vessel, all supercritcal fluid extractable hydrocarbons are accounted for in the analysis. Additionally, the system does not require the use of a SFE flow restrictor. This eliminates blockage problems which can be caused by extracted water freezing and/or analyte deposition at the point of supercritical fluid decompression during dynamic SFE. The use of direct aqueous SFE is particularly suitable for samples containing high levels of particulates. The direct aqueous SFE-FTIR system has also been developed to enable the rapid analysis of volatile hydrocarbons. This report illustrates these points by describing the on-line SFE-FTIR analysis of automobile diesel and volatile hydrocarbons in water and soil contaminated water.

Experimental

A custom-built stainless-steel vessel (patented) with an internal volume of 1 l, equipped with integral heaters and thermocouple assembly was used for all SFE-FTIR studies. The SFE vessel was constructed in accordance with the American Society of Mechanical Engineers ASME VIII (NCS) standards. Once fabricated, the SFE vessel was pressure tested to 42 MPa before being fitted with a 28 MPa rupture disc assembly prior to use. A Gilson 307 pump (Anachem, Luton, Bedfordshire, UK)

equipped with a Gilson supercritical fluid chromatography refrigeration unit, was used to supply liquid carbon dioxide (Messer UK, Reigate, Surrey, UK) to the extraction vessel. The SFE vessel was interfaced to the FTIR spectrometer using a high pressure FTIR cell of 8 mm pathlength, constructed with quartz windows. The SFE system control module houses all valves, pressure transducers and heater controls necessary for operation. The exhaust from the SFE vessel and FTIR cell were vented into a fume cupboard *via* an appropriate length of rubber hose. Aqueous extractions were performed using a vessel temperature of 40 °C with a flow rate of liquid carbon dioxide of 25 ml min^{-1} until a SFE pressure of 17.22 MPa was attained. At the end of the extraction period, FTIR data is automatically acquired. A Nicolet Magna-IR 550 spectrometer (Nicolet Instruments, Warwick, UK) equipped with OMNIC software was used for acquisition and processing of SFE-FTIR data. Spectra were obtained over the range of 4000–400 cm^{-1}, using a resolution of 4 cm^{-1}, with 200 scans summed per analysis.

Samples of automobile diesel and garden compost were obtained from a local garage and garden centre. The compost was dried to constant weight by heating at 120 °C. Analytical grade *n*-decane, *n*-heptane, *n*-hexane and benzene standards were obtained from Sigma (Poole, Dorset, UK). Analyses were performed using 500 ml water samples contained within 500 ml graduated Schott bottles (Merck, Lutterworth, Leicestershire, UK). The water samples were incubated at 40 °C using a Grant SE20D water bath (Grant Instruments, Cambridge, UK) for at least 15 min prior to use. Spiked samples were prepared by injecting diesel or hydrocarbon standards into the bulk of the warm water samples. The samples were analysed by placing the open sample bottle into the SFE vessel which was then sealed. Baseline SFE-FTIR data was acquired using a 500 ml deionized water sample. Blank 500 ml deionized water samples were analysed using the SFE-FTIR system after the analysis of each spiked water sample.

Results and discussion

In order to investigate the linearity of response for the direct aqueous SFE-FTIR system, diesel-spiked water samples were analysed. Fig. 1 shows the on-line SFE-FTIR spectra obtained for 500 ml water samples spiked with 10–80 µl (17.2–137.6 ppm) diesel fuel. Analyses were performed in quadruplicate using sets of freshly prepared spiked water samples. It was assumed that the spiked water samples were thoroughly agitated *in situ*, since approximately 2 min were required to reach supercritical fluid pressure (7.37 MPa) following the start of liquid carbon dioxide delivery to the loaded SFE vessel. Data acquisition was automatically initiated once the target pressure of 17.22 MPa had been reached, resulting in analyses being completed within 15 min. The quality of the on-line SFE-FTIR data was evaluated using the four absorbance values obtained at 2932 cm^{-1} ($\nu_{asymmetric}$ CH_3) for each set of diesel-spiked water samples. Using this data with standard FTIR quantitative software, Fig. 2 shows the graph of the calculated number of microlitres of diesel against the actual number of microlitres of diesel in the spiked 500 ml water samples. A correlation coefficient of 0.9909 was obtained using a partial least squares curve fit with the origin considered as a data point. The results of the quantitative data analyses are summarised in Table 1. From these results it can be established that method accuracy was greater than 85% for determining the average level of diesel associated with each set of spiked samples. Method precision was 3–8.6% RSD throughout the range studied. It was noted that the pH of extracted water samples was approximately 3.6, due to *in situ* acidification arising from the production of carbonic acid. This is potentially useful since current IR methods for oil in water determination require an acidification stage.[4,5]

In order to determine whether cross contamination occurred, blank water samples were analysed using the SFE-FTIR system after each spiked water sample. Carryover could be detected following the analyses of 50 µl (86 ppm) and 80 µl (137.6 ppm) diesel-spiked water samples. Wiping the surfaces of the SFE vessel chamber with clean tissue paper following the removal of sample bottles after extraction failed to eliminate the carryover. The SFE-FTIR results obtained for second blank water samples, which were analysed in series, indicated that all diesel residues were removed from the system after the first blank water extractions. Visual inspection of extracted water samples which had been spiked with 80 µl (137.6 ppm) diesel, indicated the presence of a thin film of diesel on the water surface. This is attributed to the deposition of diesel from supercritical fluid solution during the SFE vessel vent cycle rather than incomplete extraction. This view is substantiated by the spectra shown in Fig. 1 whose absorbance values obtained at 2932 cm^{-1}, confirm that the extraction of diesel is not solubility limited through the range studied.

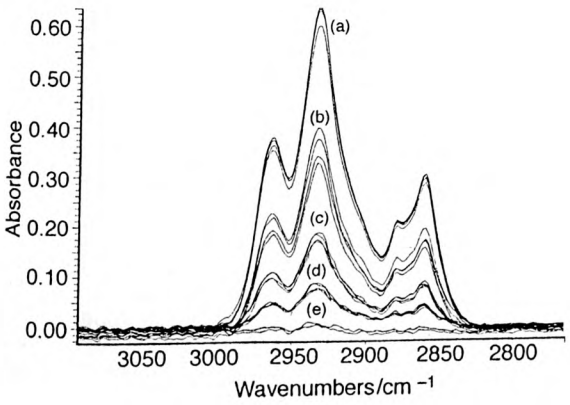

Fig. 1 On-line SFE-FTIR spectra obtained for four sets (n = 4 per set) of 500 ml water samples spiked with the following levels of diesel: (a) 137.6 ppm; (b) 86 ppm; (c) 43 ppm; and (d) 17.2 ppm. The results obtained for two blank 500 ml water samples are shown in (e).

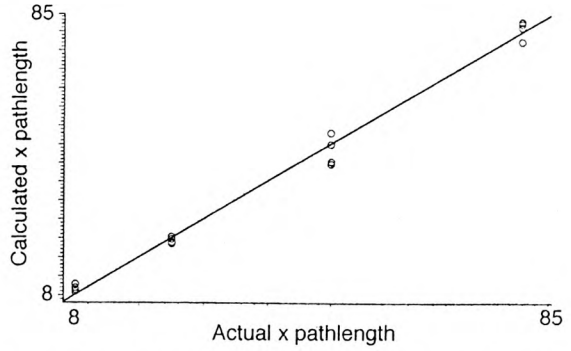

Fig. 2 Graph obtained for the calculated number of microlitres of diesel against actual number of microlitres of diesel in the 500 ml spiked water samples. The FTIR high pressure cell pathlength was 8 mm. Calculated *vs.* actual plot - diesel; r: 0.99089.

Table 1 Summary of quantitative results obtained for the on-line SFE-FTIR analyses of diesel-spiked water samples. Quadruplicate analyses were performed for each level of spiking[a]

Actual number of microlitres of diesel in 500 ml spiked water samples	Average calculated number of microlitres of diesel in 500 ml spiked water samples	Standard deviation expressed in microlitres of diesel per 500 ml spiked water samples
80 (137.6)	80.9 (139.15)	2.4 (4.1)
50 (86)	47.9 (82.4)	4.1 (7.1)
25 (43)	23.9 (41.1)	1.0 (1.7)
10 (17.2)	11.3 (19.4)	0.9 (1.55)

[a] Concentration values expressed in ppm are shown in parentheses.

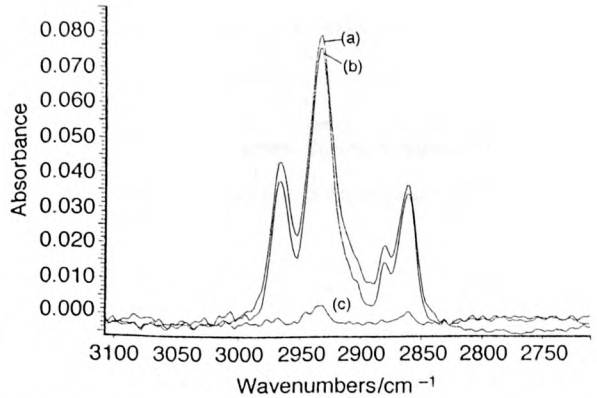

Fig. 3 On-line SFE-FTIR spectra obtained for 500 ml water samples containing: (a) 14.6 ppm *n*-decane and 5 g compost; (b) 14.6 ppm *n*-decane and (c) 5 g compost.

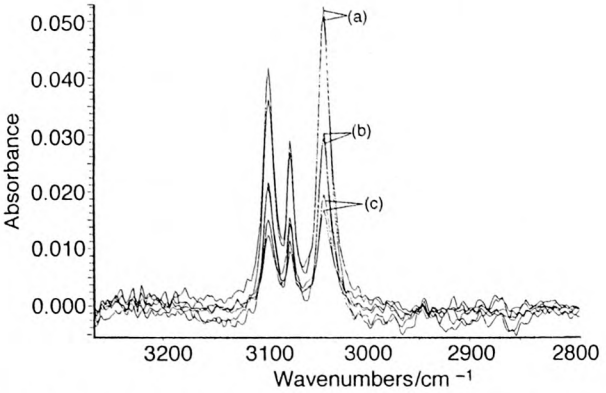

Fig. 4 On-line SFE-FTIR spectra obtained for three sets (n = 2 per set) of 500 ml water samples spiked with the following levels of benzene: (a) 44 ppm; (b) 26.4 ppm; and (c) 17.6 ppm.

Unlike indirect liquid SFE procedures, which typically involve loading the sample onto solid phase extraction cartridges or discs,[11,12] direct liquid SFE procedures are more convenient for samples containing high levels of particulates since no filtration step is necessary. This is an important consideration given that industrial effluents can contain varying levels of suspended particles. In order to demonstrate the ability of the direct aqueous SFE-FTIR system to analyse samples containing high levels of particulates a series of experiments were performed. Fig. 3 shows the on-line SFE-FTIR spectra obtained for *n*-decane from two 500 ml water samples containing: (i) 10 µl (14.6 ppm) *n*-decane and (ii) 14.6 ppm *n*-decane and 5 g dried compost. The spectra obtained show high fidelity. Similar results have been obtained for hydrocarbon spiked water samples containing 10000 ppm clay.

The loss of volatile hydrocarbons has been reported with Method 3560 using tetrafluoroethene as collection solvent during dynamic SFE.[1,3] In order to recover the volatile petroleum hydrocarbons it is necessary to modify the extraction conditions[1] (reducing extraction: pressure, temperature and time) and use a cooled octadecyl-bonded silica sorbent trap onto which the supercritical extract is decompressed. The trap can then be rinsed with tetrafluoroethene to provide an extract suitable for IR analysis. With Method 1664, the loss of volatiles during removal of the extraction solvent *n*-hexane, serves to limit the scope of this gravimetric procedure. Consequently Method 1664 is not applicable for the determination of materials which volatilize below 85 °C. Also partial losses of petroleum fuels from gasoline through to light fuel oil may occur during the removal of *n*-hexane. Since the direct aqueous SFE-FTIR system used in the current studies requires only approximately 30 s to place and completely seal the sample bottle within the extraction vessel, the technique is well suited for the analysis of volatile hydrocarbons. Fig. 4 shows the on-line SFE-FTIR results obtained for sets of analyses of 500 ml water samples spiked with 10–25 µl (17.6–44 ppm) benzene. The quality of the data was evaluated using the two absorbance values obtained at 3043 cm^{-1} for each of the three sets of benzene-spiked water samples. A graph of calculated benzene concentration against actual concentration provided a correlation coefficient of 0.9944, using a partial least squares curve fit with the origin considered as a data point. A further example involving the analysis of volatile hydrocarbons is shown in Fig. 5 which shows the SFE-FTIR analyses of two 500 ml water samples spiked with 20 µl (35.2 ppm) benzene and 10 µl (13.7 ppm) *n*-heptane. Although *n*-hexane (13.7 ppm in 500 ml water) could be readily detected using the SFE-FTIR system, its high volatility meant that non-reproducible results were obtained. It seems likely that incubating the water samples at temperatures lower than 40 °C should afford a means of quantification for *n*-hexane spiked water samples.

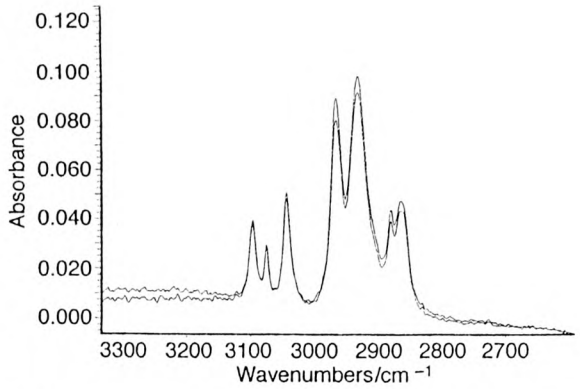

Fig. 5 On-line SFE-FTIR spectra obtained from duplicate analyses of 500 ml water samples spiked with 35.2 ppm benzene and 13.7 ppm *n*-heptane.

At present the current detection limits for benzene and decane are 17.6 ppm and 4 ppm, respectively. Further development work is in progress to improve sensitivity. During the course of one year operating the SFE-FTIR system, it has been established that a cylinder containing 34 kg liquid carbon dioxide enables the SFE-FTIR analyses of more than one hundred and fifty 500 ml water samples. The supercritical fluid chromatography refrigeration unit, used to cool the pump for providing liquid carbon dioxide, has a specification which enables the remote location of liquid carbon dioxide supply cylinders outside the laboratory. At present the analysis of a 500 ml water sample requires approximately 15 min. It is hoped that future analyses will be performed in less than 10 min, by providing higher flow rates of liquid carbon dioxide to the SFE vessel.

Conclusions

The SFE-FTIR system that has been developed offers considerable potential for the determination of oil levels in industrial effluents destined for discharge. The technique is environmentally clean, rapid and allows for a high level of automation.

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Paper 8/04219E

The Analyst
FULL PAPER

Development of an automated method for determining oil in water by direct aqueous supercritical fluid extraction coupled on-line with infrared spectroscopy

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Received 2nd August 2000, Accepted 13th October 2000
First published as an Advance Article on the web 21st November 2000

A direct aqueous supercritical fluid extraction (SFE) system was developed which can be directly interfaced to an infrared spectrometer for the determination of oil in water. The technique is designed to provide an environmentally clean, automated alternative to established IR methods for oil in water analysis which require the use of restricted organic solvents. The SFE-FTIR method involves minimum sample handling stages, with on-line analysis of a 500 ml water sample being complete within 15 min. Method accuracy for determining water samples spiked with gasoline, white spirit, kerosene, diesel or engine oil was 81–100% with precision (RSD) ranging from 3 to 17%. An independent evaluation determined a 2 ppm limit of quantification for diesel in industrial effluents. The results of a comparative study involving an established IR method and the SFE-FTIR method indicate that oil levels calculated using an accepted equation which includes coefficients derived from reference hydrocarbon standards may result in significant errors. A new approach permitted the derivation of quantification coefficients for the SFE-FTIR analyses which provided improved results. In situations where the identity of the oil to be analysed is known, a rapid off-line SFE-FTIR system calibration procedure was developed and successfully applied to various oils. An optional in-line silica gel clean-up procedure incorporated within the SFE-FTIR system enables the same water sample to be analysed for total oil content including vegetable oils and selectively for petroleum oil content within a total of 20 min. At the end of an analysis the SFE system is cleaned using an *in situ* 3 min clean cycle.

Introduction

In response to growing international concern over the depletion of stratospheric ozone, the Vienna Convention for the Protection of the Ozone Layer led to an agreement opened for signature in March 1985. Although the potential severity of the problem was recognised, this document set out broad principles for ozone layer protection rather than imposing obligations upon nations to control and reduce the manufacture of ozone depleting substances. Building on this achievement, in September 1987, representatives from 27 countries signed the 'Montreal Protocol on Substances that Deplete the Ozone Layer'. This protocol committed every signatory nation, by 1999, to reduce its manufacture of certain chlorofluorocarbons (CFCs) by 50% of their level in use in 1986.[1,2] Years of negotiation fostered by the United Nations Environment Programme (UNEP) has now resulted in 173 parties to the Montreal Protocol with its London (1990), Copenhagen (1992), Montreal (1997) and Beijing (1999) amendments.[3,4]

Since its implementation, substances other than CFCs have also become subject to control measures under the Montreal Protocol, *e.g.*, carbon tetrachloride, 1,1,1-trichloroethane, hydrofluorocarbons (HFCs), halons and methyl bromide.[4] However, it was also recognised that certain essential uses for restricted solvents existed for which practical substitutes/technologies were not immediately available. As a consequence, in 1994, a list of global essential use exemptions was agreed at the 6th Meeting of the Parties to the Montreal Protocol.[5] Amongst these exemptions were those identified for laboratory and analytical uses. These included the use of restricted solvents for equipment calibration, use as extraction solvents, diluents or carrier solvents for chemical analysis and other critical analytical and laboratory purposes. In particular, the availability of restricted solvents for the determination of oil, grease and total petroleum hydrocarbons (TPHs) in surface and sea-waters and industrial and domestic aqueous waste fell within the essential use exemption category.

Oil, grease and TPH analyses provide gross measurements of water contamination and are vital for the successful management of industrial discharges and waste-water treatment. A variety of analytical procedures have been developed for the determination of oil in water.[6] This situation can be attributed to the wide range of physical and chemical properties of the constituent components within crude oils and associated products. The analytical procedures can be broadly divided into two classes,[7] those based upon (i) integral procedures which involve gravimetry and infrared (IR), ultraviolet (UV) and fluorescence spectroscopic techniques and (ii) differential procedures which involve gas chromatography, gas chromatography combined with mass spectrometry and high performance liquid chromatography. From an industrial perspective, integral methods based upon IR and gravimetric procedures have become very firmly established as the general means by which oil in water determinations are performed.[8,9] Differential methods, although more accurate, are relatively time consuming, expensive and require a higher level of technical skill.[6]

Gravimetric methods such as US Environmental Protection Agency (EPA) Method 413.1[10] and Standard Method 5520B[11] involve liquid–liquid extraction (LLE) of water samples using 1,1,2-trichloro-1,2,2-trifluoroethane (Freon 113). The extract is then dried using magnesium sulfate prior to the Freon 113 being evaporated, leaving the residual oil to be weighed. In general,

 DOI: 10.1039/b006286n

gravimetric procedures do not provide a means of characterising the composition of extracted oil and also fail to account for volatile hydrocarbon species which are lost during the evaporation of the extraction solvent. Methods involving IR confirmation represent the most widely accepted procedures for the determination of oil, grease and TPH in water.[8,9] Although IR procedures such as Her Majesty's Stationery Office (HMSO)[12] and American Society for Testing and Materials (ASTM) Method 3921[13] provide relatively low sensitivity for aromatic hydrocarbons compared with UV and fluorescence methods, IR spectroscopy is able to cover the whole range of hydrocarbons using relatively simple, inexpensive instrumentation suitable for routine use in industrial laboratories. As with gravimetric procedures, IR methods involve LLE and extract drying stages. The choice of extraction solvent is, however, extremely limited since IR methods involve measuring absorbance values of specific C–H vibrations of extracted hydrocarbon species through the 3030–2930 cm^{-1} region. Hence IR methods have been developed using either Freon 113 or carbon tetrachloride as interference-free extraction solvents. A major drawback of traditional gravimetric and IR methods is that they are labour intensive, involving a number of 'wet chemistry' stages. This situation can be further compounded since these methods generally include an option whereby co-extracted polar species, such as vegetable oils, can be selectively removed from mineral oils in the LLE extract *via* an appropriate sorbent treatment procedure. The treated extract can then be assayed for petroleum hydrocarbon content.

In July 1998, the 17th Meeting of the Open-Ended Working Group of the Parties to the Montreal Protocol[14] forwarded a series of draft proposals for consideration at the 10th Meeting of the Parties to the Montreal Protocol to be held later that year. Amongst these was the proposal that Freon 113 and carbon tetrachloride should be eliminated from the global exemption for laboratory and analytical essential uses in 2001 and subsequent years for determining oil, grease and TPH in water, *i.e.*, both of these substances should enter the Negative List for this application. As a result of extended negotiations reflecting concern over the availability of alternative methods, a decision reached at the 11th Meeting of the Parties to the Montreal Protocol in 1999 eliminates the use of ozone depleting solvents for determining oil, grease and TPH in water from 2002.[15] Hence IR and gravimetric methods for determining oil, grease and TPH in water using ozone depleting solvents are obsolescent. Consequently, many existing methods will need to be revised, re-evaluated or alternatives found.

A gravimetric procedure, EPA Method 1664, which uses hexane as an alternative extraction solvent, has been specifically developed for the determination of oil, grease and TPH in aqueous samples.[16] Method 1664 is performance-based, thereby permitting alternative extraction and concentration techniques provided all performance specifications and quality control stages incorporated within the procedure are met. Hence Method 1664 permits the use of solid phase extraction (SPE) as an alternative to LLE. The results of a comparative gravimetric study involving SPE cartridges specifically developed for determining oil in water and LLE procedures have been published.[17] The SPE procedure allowed more rapid analyses despite additional steps being necessary to avoid SPE cartridge blockage when aqueous samples contained high levels of particulates. In part, Method 1664 was developed to provide a low cost alternative to instrument based techniques for determining oil, grease and TPH in water.[18] However, Method 1664 is not just a simple replacement gravimetric method for those based on Freon 113 LLE; its successful implementation depends upon satisfying extensive quality control parameters which can be difficult to achieve consistently owing to a range of problems.[19]

Methods based on UV and fluorescence spectroscopy for determining oil, grease and TPH in water involve determining the amount of aromatic hydrocarbons present in the sample, with the total petroleum hydrocarbon content being calculated on a proportional basis.[7,20] Hence, successful quantitative studies require prior knowledge of the levels and compositions of constituent hydrocarbons in the oils and greases being analysed. Additional problems may arise owing to the presence of interfering species, *e.g.*, UV methods can be affected by nitrates[20] and plant pigments can interfere with fluorescence methods.[7] Although potentially very sensitive in appropriate situations, these techniques are best suited for effluents containing an aromatic content and cannot be used for alkane-based oils and greases or vegetable oils.

Tetrachloroethene has been suggested as a substitute LLE solvent for established oil in water IR methods.[18] However the toxicity and ozone depleting properties of tetrachloroethene represent significant problems which serve to limit its long term suitability as an alternative solvent for IR spectroscopy. A field portable IR analyser has been developed whose operational wavelength can be factory-adjusted for specific applications.[21,22] The system can be supplied to permit the determination of oil in water using a hexane LLE procedure. After LLE, an aliquot of the hexane extract can be placed in a sample well or applied to a disposable IR card. Once the hexane has evaporated, the infrared filtometer provides a means of determining the amount of oil extracted by measuring the absorbance at 2930 cm^{-1}. Although being more specific than gravimetric procedures, this non-scanning IR technique does not allow the characterisation of extracted oils.

In principle, an ideal LLE solvent for measuring oil, grease and TPH in water *via* IR spectroscopy should be non-absorbing throughout the 3030–2930 cm^{-1} region and be capable of efficiently extracting hydrocarbons from aqueous samples whilst being environmentally friendly. Additionally, the solvent should be non-toxic, non-flammable and freely and cheaply available at high purity with no disposal costs. Supercritical fluid carbon dioxide matches all of these criteria. A method based on supercritical fluid extraction (SFE) using carbon dioxide has been developed (EPA Method 3560) for the determination of TPH in soil.[23] The technique involves off-line IR analysis (EPA Method 8840) of extracted hydrocarbons, after collection of the SFE restrictor output in a small volume of tetrachloroethene. The underlying theory and principles of SFE coupled on-line with IR spectroscopy (SFE-IR) have been reviewed,[24,25] with several reports describing this technique being applied for TPH determination in soil.[26,27] Recently we reported the development of direct liquid SFE-FTIR instrumentation, specifically developed for the determination of oil in industrial effluents and discharge waters.[28] The system is based on a stop-flow IR measurement of the SFE extract. This eliminates the use of a restrictor device and associated blockage problems which may arise due to extracted water freezing and/or analyte deposition at the point of decompression during dynamic SFE of aqueous matrices.[29] The automated system has been developed to allow rapid unattended analyses of 500 ml water samples with minimum sample handling. Since the whole sample bottle is placed within the SFE vessel, losses of hydrocarbons which can result from glass adherence during sample transfer stages are eliminated. The method is also suitable for the determination of volatile hydrocarbons and is not affected by the presence of very high levels of particulates in water samples.[28]

The objective of this study was to evaluate further the applicability of the direct aqueous SFE-FTIR method for determining a range of oils and comparing its level of performance against that achievable using an established LLE IR method. The development of a rapid system calibration procedure and an optional in-line sorbent clean-up stage for the specific determination of petroleum hydrocarbons in the presence of co-extracted vegetable oils serves to improve sample throughput further.

Experimental

A custom-built stainless-steel vessel with an internal volume of 1 l, equipped with integral heaters and thermocouple assembly, was used for all SFE-FTIR studies. The SFE vessel was constructed in accordance with the American Society of Mechanical Engineers ASME VIII (NCS) standards. Once fabricated, the SFE vessel was pressure tested to 42 MPa before being fitted with a 28 MPa rupture disc assembly prior to use. A Gilson Model 307 pump (Anachem, Luton, Bedfordshire, UK) equipped with a Gilson supercritical fluid chromatography refrigeration unit was used to supply liquid carbon dioxide (Messer UK, Reigate, Surrey, UK) to the extraction vessel. The SFE vessel was interfaced to the FTIR spectrometer *via* a high pressure FTIR cell of 8 mm pathlength, constructed with 2 mm diameter quartz windows. In practice, provided that a suitable sample mount is available, the direct aqueous SFE system can be linked to most manufacturers' IR spectrometers in under 2 min. The SFE system control modules house all valves, pressure transducers and heater controls necessary for operation. An *in situ* clean-up column was incorporated into the SFE system between the SFE vessel and the high pressure FTIR cell. The clean-up column consisted of a finger-tight 3 ml capacity high pressure SFE vessel (Thar Designs, Pittsburgh, PA, USA) loosely packed with 100–80 mesh silica gel (Jones Chromatography, Hengoed, Glamorgan, UK) and was plumbed into the system using a high pressure selection/isolation valve.

Fig. 1 shows the direct aqueous SFE system (patents pending). The exhaust from the SFE vessel and FTIR cell were vented into a fume cupboard *via* an appropriate length of rubber hose. Aqueous extractions were performed using a vessel temperature of 40 °C with a flow rate of liquid carbon dioxide of 25 ml min^{-1} until an SFE pressure of 17.22 MPa was attained. In the event that a liquid carbon dioxide cylinder becomes empty during an analysis, a time-out alarm on the SFE system is activated and the cylinder can be changed without affecting the results. A Nicolet Magna-IR 550 spectrometer (Nicolet Instruments, Warwick, UK) equipped with OMNIC software was used for acquisition and processing of SFE-FTIR data. Spectra were obtained over the range 4000–400 cm^{-1}, using a resolution of 4 cm^{-1}, with 200 scans summed per analysis.

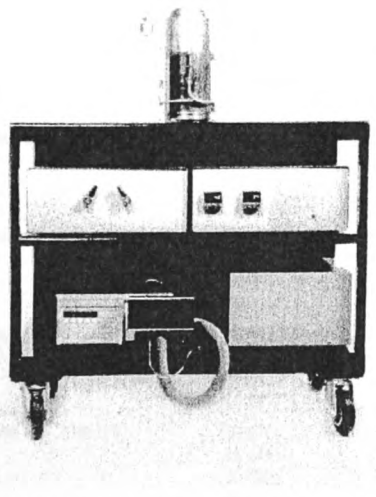

Fig. 1 The direct aqueous SFE system designed for oil in water determination using on-line IR spectroscopy. Base tier: liquid carbon dioxide pump (left), pump-head refrigeration unit (right). Middle tier: valve control module (left), electronic control module (right). Top tier: 1 l capacity SFE vessel.

Automobile gasoline, diesel and 20W/50 engine oil were obtained from a local garage. Samples of kerosene and white spirit were supplied by Hyder (Runcorn, Cheshire, UK). IR spectroscopic grade carbon tetrachloride and tetrachloroethene were obtained from Sigma-Aldrich (Poole, Dorset, UK). Analytical-reagent grade benzene, hexadecane, pristane and ethylbenzene standards were obtained from Sigma-Aldrich. Analyses were performed using 500 ml water samples contained within 500 ml graduated Schott bottles (Merck, Lutterworth, Leicestershire, UK). Spiked samples were prepared by injecting oil samples into the bulk of the water sample. Owing to its viscosity, engine oil was diluted with benzene (5 g in 10 ml benzene solution) to facilitate the use of a syringe in preparing spiked water samples. All samples were prepared and stored at room temperature prior to analysis. The samples were analysed by placing the open sample bottle in the SFE vessel, which was then sealed. Unattended analysis is initiated *via* a single start switch on the SFE electronic control module. As previously described, spiked water samples are thoroughly agitated and acidified *in situ* during the initial stages of SFE.[28] Baseline SFE-FTIR data were acquired using a 500 ml deionized water sample. At the end of each analysis the sample bottle was replaced with an aluminium plug which almost completely filled the sealed SFE vessel void volume. With this arrangement it is possible to clean the SFE vessel, high pressure IR analysis cell and all ancillary plumbing *in situ* using supercritical fluid carbon dioxide within 3 min. Alternatively, the SFE system valve settings can isolate the SFE vessel such that only the high pressure IR cell and associated transfer lines can be cleaned *in situ*. Off-line analyses of carbon tetrachloride or tetrachloroethene solutions of hexadecane, pristane, ethylbenzene and oil standards were performed using a quartz cuvette of 1 cm pathlength with the same acquisition parameters as used for SFE-FTIR analyses.

Results and discussion

Evaluation of the applicability of the direct aqueous SFE-FTIR procedure for determining a range of different oils

Recently, we described the potential of direct aqueous SFE-FTIR for the determination of automobile diesel in 500 ml water samples.[28] In order to evaluate further the performance of the SFE-FTIR procedure as a general means for determining oils in discharge effluents, a range of water samples spiked with various species of oil were analysed. The oils selected were gasoline, white spirit, kerosene and engine oil. These oils were amongst those originally used to develop[30,31] the HMSO method.[12] Each oil was analysed at four different spike levels (n = 5 for each level). Fig. 2(a) shows the results obtained for white spirit. The quality of the on-line SFE-FTIR data obtained for white spirit, kerosene and engine oil was evaluated using the five absorbance values obtained at 2932 cm^{-1} ($\nu_{asymmetric}$ CH_2) for each set of spiked water samples. For gasoline, whose major constituent components are branched alkanes, the five most intense absorbance values obtained at 2960 cm^{-1} ($\nu_{asymmetric}$ CH_3) were used for each set of spiked water samples. Using these sets of data with standard OMNIC FTIR quantitative software, graphs were constructed such that calculated levels of oil present were plotted against actual levels present in the spiked 500 ml water samples. All these graphs were constructed using a partial least squares curve fit with the origin considered as a data point. Fig. 2(b) shows the graph obtained for white spirit.

The results for the oils are summarised in Table 1. With the exception of engine oil, all other oils studied exhibited linear dynamic ranges in excess of 130 ppm. The upper linear dynamic

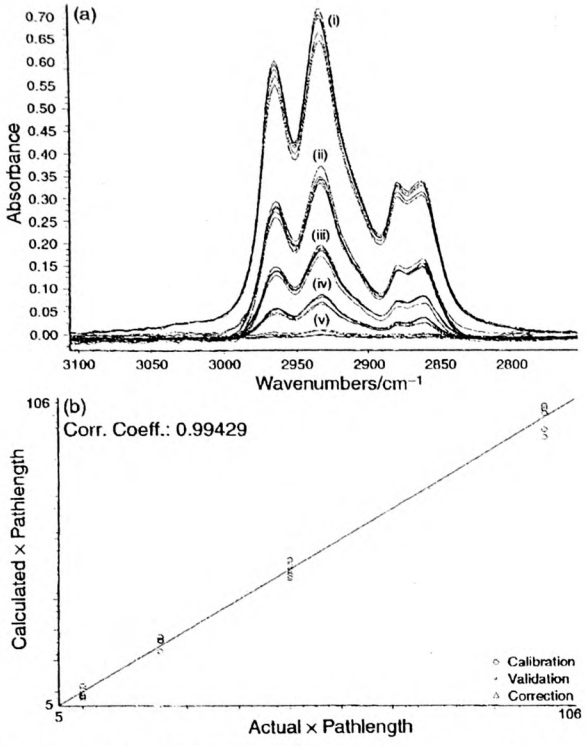

Fig. 2 (a) On-line SFE-FTIR spectra obtained for four sets (n = 5 per set) of 500 ml water samples spiked with the following levels of white spirit: (i) 156; (ii) 78; (iii) 39 ppm; and (iv) 15.6 ppm. The results for four blank water samples are shown in (v). (b) Graph obtained for the calculated number of microlitres of white spirit against actual number of microlitres in the 500 ml spiked water samples. The FTIR high pressure cell pathlength was 8 mm. Calculated *vs* actual plot, white spirit; r = 0.9943.

range for engine oil using the SFE-FTIR system lies in the region of 70–100 ppm. The lower linear dynamic range associated with engine oil is attributed to its complex formulation which contains components specifically designed to adhere strongly to metallic surfaces. In practice, we believe the SFE efficiency to be almost complete within the linear dynamic range for each of the oils. This view is supported by the fact that repeated SFE-FTIR analyses of 500 ml water samples initially spiked with 25 μl of oil failed to provide IR spectra for the oils.[28] Additionally, we found that no increase in spectral intensity for any of the five oils could be gained by increasing the extraction pressure from 17.22 to 25.83 MPa at 40 °C. Consequently, these experiments serve to confirm that the oils are almost completely extracted at 17.22 MPa since more dense, higher solvating strength supercritical fluid carbon dioxide[32] did not improve extraction efficiencies.

As reported previously,[28] carryover can sometimes occur owing to oil deposition within the SFE vessel when the supercritical extract is decompressed during the vent cycle. This problem tends to arise when oil levels in aqueous samples exceed 40 ppm and/or the oil has a high affinity for metal surface adherence. In practice, cross-contamination can be rapidly eliminated by placing an aluminium plug in the SFE vessel following each analysis. The aluminium plug almost completely fills the chamber within the sealed vessel, enabling an SFE clean cycle to be completed within 3 min. *In situ* IR monitoring can be used to confirm the efficiency of the clean cycle. In the case of gasoline and white spirit, no carryover problems occurred after analysing water samples spiked with 134 and 156 ppm, respectively.

In order to evaluate the performance of the SFE-FTIR oil in water analyser for real samples, two diesel contaminated discharge water samples were supplied and independently analysed using the system by an industrial company (Hyder). The results for the two samples are shown in Fig. 3 along with the analysis obtained for a freshly prepared diesel spiked water sample. These results serve to confirm the capability of the SFE-FTIR procedure for quantifying oil levels in industrial effluents.

Table 1 Summary of quantitative results obtained for the on-line SFE-FTIR analyses of oil spiked water samples.[a] The results for diesel have been published previously[28]

Oil	Actual amount of oil in 500 ml spiked water samples/μl	Mean calculated amount of oil in 500 ml spiked water samples/μl	Correlation coefficient[b]	Standard deviation,[c] expressed as amount of oil in 500 ml water samples/μl	Accuracy (%)[d]
Gasoline	100 (134)	100.9 (135.2)	0.9829	6.7 (8.9)	99.1
	50 (67)	45.3 (60.7)		2.5 (3.4)	90.6
	25 (33.5)	26.9 (36.1)		3.0 (4.1)	92.4
	10 (13.4)	11.9 (15.9)		1.3 (1.8)	81
White spirit	100 (156)	99.7 (155.5)	0.9943	4.4 (6.9)	99.7
	50 (78)	49.6 (77.4)		2.5 (3.8)	99.2
	25 (39)	26.2 (40.8)		1.7 (2.7)	95.2
	10 (15.6)	9.6 (14.9)		1.6 (2.6)	96
Kerosene	100 (162)	99.6 (161.4)	0.9912	3.5 (5.6)	99.6
	50 (81)	50 (81)		5.1 (8.0)	100
	25 (40.5)	24.9 (40.3)		3.3 (5.3)	99.6
	10 (16.2)	10.6 (17.2)		1.1 (1.8)	94
Diesel	80 (137.6)	80.9 (139.2)	0.9909	2.4 (4.1)	98.9
	50 (86)	47.9 (82.4)		4.1 (7.1)	95.8
	25 (43)	23.9 (41.1)		1.0 (1.7)	95.6
	10 (17.2)	11.3 (19.4)		0.9 (1.6)	87
Engine oil, 20W/50	70 (70)	71.6 (71.6)	0.9546	6.3 (6.3)	97.7
	45 (45)	38.6 (38.6)		3.0 (3.0)	85.8
	20 (20)	23.1 (23.1)		1.7 (1.7)	84.5
	10 (10)	11.7 (11.7)		1.6 (1.6)	83

[a] Concentration values expressed in ppm are shown in parentheses. [b] The correlation coefficients are derived from four point calibration graphs, where for each oil the calculated number of microlitres are plotted against actual number of microlitres in spiked 500 ml water samples. For oils other than diesel, n = 5 for each individual spike level. For diesel, n = 4 for each individual spike level. [c] Calculated using $n - 1$ degrees of freedom, here and thereafter. [d] Accuracy (%) = 100 − | 100 − [(mean calculated value/actual value) × 100] |, here and thereafter.

The high pressure IR analysis cell used throughout these studies was a modified preparative supercritical fluid chromatography UV cell which provided 2 mm diameter quartz windows and 8 mm pathlength. It is expected that improved detection limits should be achievable using an IR analysis cell of greater dimensions. An increase in IR cell window diameter should provide an improved signal-to-noise ratio whereas a longer pathlength would increase the sensitivity. Such high pressure IR cells suitable for the analyses of supercritical fluids have been developed.[33]

Comparison of the infrared HMSO method with the SFE-FTIR procedure for determining oil in water

In 1951, Simard *et al.*[34] first described an IR method in which a three component synthetic oil mixture of isooctane, hexadecane and benzene (37.5 + 37.5 + 25 by volume) could be used for the determination of oils in waste water. The procedure involved summing the absorbances which were attributed to the vibrations of CH_2, CH_3 and aromatic CH groups, respectively, and relating these values to those obtained for oil which had been extracted from water using carbon tetrachloride. It became apparent that the accuracy of this IR method could be greatly improved by using calibration standards prepared from a sample of oil identical with that which was to be measured.[35] Often, however, this is not possible, *e.g.*, the sample may have been obtained from a site in which numerous oil species have become mixed following discharge from several sources. Under these circumstances, a three component synthetic oil is still widely used as the calibration standard for the IR procedure despite associated errors of ±20% being quoted.[30] A recent report[36] indicated that errors substantially larger than this figure can occur using summed IR absorbance values. Whittle *et al.*[30,31] developed an IR method in which the molar absorptivities of the CH_3 (2960 cm^{-1}), CH_2 (2930 cm^{-1}) and the aromatic CH (3030 cm^{-1}) groups are taken into account to correct for the errors associated with simple addition of unbiased absorbance values. The procedure, which formed the basis for the HMSO IR method,[12] involves constructing three equations for three reference compounds, each rich in either CH_3, CH_2 or CH aromatic protons. A typical set of reference compounds include hexadecane, pristane and ethylbenzene. The equation is as follows and is applied to each set of IR absorbance values for standard solutions of the reference compounds:

$$c = xA_{2930} + yA_{2960} + z(A_{3030} - A_{2930}/F)$$

where: c = concentration in mg l^{-1}, constant for each reference compound, x, y and z = coefficients related to the molar absorptivities of the CH_2, CH_3 and aromatic CH groups, respectively, A = absorbance value at the specified wavelength and F = a correction factor and can be calculated[30,31] from hexadecane absorbances by basing the correction on the absorbance at 2930 cm^{-1} and letting $F = A_{2930}/A_{3030}$.

Once the values of the x, y and z coefficients have been determined, the HMSO equation is applied to the absorbance values of the oil to be measured and its concentration is calculated. In order to evaluate the accuracy of a similar approach for the SFE-FTIR procedure, a comparative study was undertaken. An initial series of analyses were performed to solve the values of x, y and z using standard carbon tetrachloride solutions of hexadecane, pristane and ethylbenzene according to the HMSO method. These values are subsequently referred to as the HMSO coefficients. Following these experiments, the values of SFE-FTIR x, y and z coefficients were determined using mean SFE-FTIR absorbance values for 500 ml water samples spiked with 80 ppm of each of the three reference compounds (n = 5, for each compound). The two sets of coefficients were then appropriately used to calculate the levels of oil in carbon tetrachloride standard solutions and the 500 ml spiked water samples (compositions are given in Table 1) used in the previous section. The quantitative results are summarised in Table 2.

It is considered that the results obtained for each oil's carbon tetrachloride standard solution, calculated using the HMSO coefficients, represent those obtainable in an ideal situation, *i.e.*, these results cannot be adversely affected by any manipulation errors and/or incomplete carbon tetrachloride extraction efficiencies, had the HMSO method been applied to spiked water samples. A recent report[36] described surprisingly low extraction efficiencies for various oils including the components of a synthetic oil mixture using a carbon tetrachloride LLE procedure. The results in the second column of Table 2 are subsequently referred to as minimum HMSO errors hereafter.

As shown in Table 2, application of the HMSO coefficients to the SFE-FTIR analyses obtained for kerosene, diesel and engine oil provide quantification results whose errors closely match the corresponding minimum HMSO errors for these oils. However, the errors associated with the SFE-FTIR quantification results obtained for gasoline and white spirit derived using the HMSO coefficients do not correlate with the minimum HMSO errors. When the SFE-FTIR derived coefficients were applied to the SFE-FTIR analyses for the oils, column four in Table 2, the results for kerosene and engine oil provided a good match with the minimum HMSO errors. With the exception of diesel, the results obtained for the SFE-FTIR analyses of the other oils are approximately equivalent using either the HMSO coefficients or the SFE-FTIR coefficients.

The results in Table 2 indicate that for all oils, a greater error occurs when the SFE-FTIR derived coefficients are applied to the results of SFE-FTIR analyses of oils in water relative to the corresponding minimum HMSO errors. These three sets of results serve to demonstrate that application of coefficients derived from reference compounds can introduce significant errors for various species of oils and their mixtures. According to one report,[37] a reference oil recommended in EPA Method 8440 for samples of unknown petroleum hydrocarbons resulted in analyses having significantly larger errors than any recorded

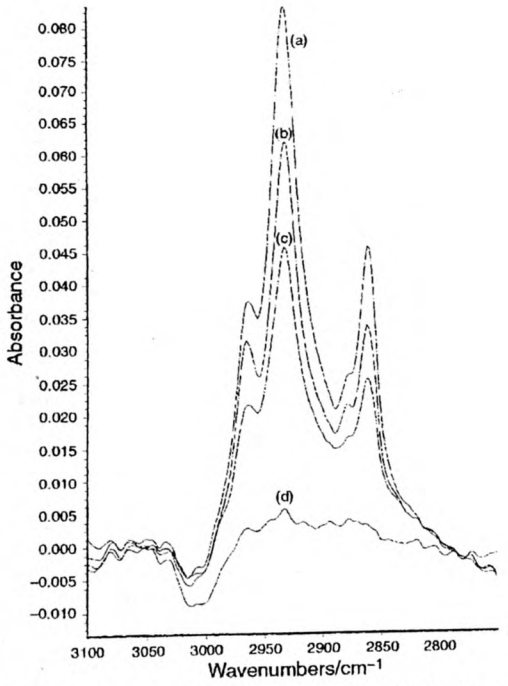

Fig. 3 SFE-FTIR analyses obtained for: (a) 500 ml of industrial effluent containing 4 ppm diesel, (b) 500 ml of water spiked with 3 ppm diesel, (c) 500 ml of industrial effluent containing 2 ppm diesel and (d) 500 ml of blank water.

in Table 2 using either the HMSO coefficients or the SFE-FTIR coefficients.

In order to determine optimum x and y coefficients to apply to the on-line SFE-FTIR data, the SFE-FTIR absorbance values of the oils themselves were used in conjunction with Microcal Origin™ software. The z coefficient was not evaluated for the five oils since their IR spectra indicated a low aromatic content. With this approach, a response surface was constructed in which the summed error associated with the SFE-FTIR results of oil in water analyses for each of the five oils was expressed as a function of varying x and y values. The summed error was based on modulus values for each oil in order to prevent errors of different sign being self-cancelling. From the response surface shown in Fig. 4, the values obtained for x and y which resulted in the minimum summed error for the five oils were reapplied to the results obtained for the SFE-FTIR analyses. As shown in column five of Table 2, with the exception of kerosene this approach provided lower mean errors than the minimum HMSO errors.

Development of a rapid off-line calibration procedure for the SFE-FTIR system

Despite the widespread use of three component synthetic oils to derive coefficients for calculating the levels of oil in aqueous samples, it is well known that more accurate IR analyses can be performed using calibration standards prepared from the actual oil which is to be measured.[12,13,30,35,36] Although preparing calibration graphs using SFE-FTIR results obtained from water samples spiked with the oil to be quantified poses no significant problems, the fact that a single analysis takes approximately 15 min means that time could be saved by developing a rapid off-line calibration procedure. Accordingly, a series of experiments were performed using 500 ml water samples spiked with white spirit and standard tetrachloroethene solutions of white spirit. The SFE-FTIR analyses of 500 ml water samples spiked with 50 μl white spirit provided IR spectra whose CH_2 ($\nu_{asymmetric}$) absorbance values approximate to that obtained for a standard solution of 5 μl white spirit in 100 ml of tetrachloroethene. The results of these analyses allowed the derivation of a dilution factor. Following this experiment, an off-line, five point calibration graph was prepared (r = 0.9995) using standard tetrachloroethene solutions of white spirit. The off-line calibration graph was constructed such that it would permit the SFE-FTIR determination of white spirit in 500 ml water samples through the range 12.5–156 ppm range *via* interpolation of CH_2 ($\nu_{asymmetric}$) absorbance values. It was noted that the maximum values obtained for C–H absorbances in the SFE-FTIR spectra of the oils showed a small shift to higher wavenumbers relative to the corresponding wavenumbers for their tetrachloroethene standard solutions. In practice, this did not pose a problem since the OMNIC™ quantitative software facilitates specifying a maximum absorbance value within a range of wavenumbers. Table 3 summarises the results for the determination of white spirit in water obtained using the off-line calibration graph. In order to test the general applicability of this approach, sets of 500 ml water samples each spiked with one of the five oils were analysed *via* SFE-FTIR and quantification was performed using the off-line system calibration procedure. The quantitative results are summarised in Table 4. High levels of accuracy were obtained for quantifying the other four oils using off-line tetrachloroethene calibration standards whose concentrations were based on the dilution factor previously determined for white spirit. These results further confirm almost identical SFE efficiencies for all of the five oils studied.

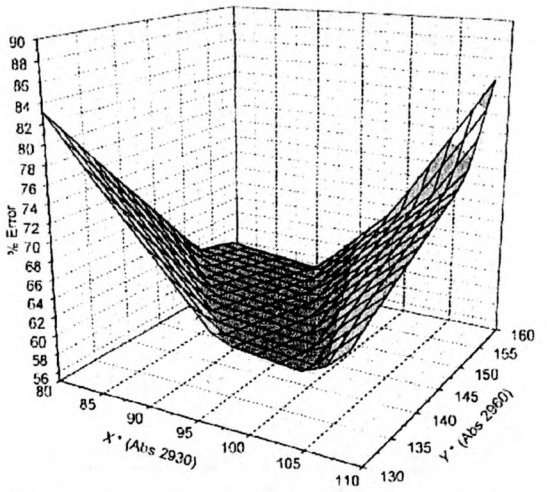

Fig. 4 Response surface obtained using Mirocal Origin software, in which sets of summed modulus errors associated with five oils are expressed as a function of x and y coefficient values applied to their CH_2 ($\nu_{asymmetric}$) and CH_3 ($\nu_{asymmetric}$) IR absorbance values, respectively, using the HMSO calculation.[30]

Table 3 Determination of white spirit spiked into 500 ml water samples using the off-line calibration procedure. Results derived using absorbance values at 2932 cm^{-1}. For each individual spike level, n = 3[a]

Actual amount of white spirit in 500 ml water sample/μl	Mean calculated amount of white spirit in 500 ml water sample/μl	Standard deviation, expressed as amount of white spirit in 500 ml water sample/μl	Accuracy (%)
100 (156)	100 (156)	2.3 (3.6)	100
50 (78)	51.5 (80.3)	0.7 (1.1)	97.1
20 (31.2)	18.8 (29.3)	2.8 (4.3)	94.2

[a] Concentration values expressed in ppm are shown in parentheses.

Table 2 Comparison of the application of x, y and z coefficients for determining oil levels in standard solutions of carbon tetrachloride and on-line SFE-FTIR analyses

Oil	HMSO coefficients applied to carbon tetrachloride standards[a] (% mean error)	HMSO coefficients applied to on-line SFE-FTIR analyses[b] (% mean error)	SFE-FTIR coefficients applied to on-line SFE-FTIR analyses[b] (% mean error)	Graphically optimised coefficients applied to on-line SFE-FTIR analyses[b] (% mean error)
Gasoline	−9.5	−27.8	−24.4	−7.6
White Spirit	−6.5	−18.2	−18.8	−1.7
Kerosene	−0.9	4.8	2.8	21.1
Diesel	−18.4	−19	−34.8	0
Engine oil, 20W/50	−26.1	−26.1	−28.9	−14.6

[a] Errors derived from duplicate analyses of carbon tetrachloride oil standards. Oil concentrations: 130–160 ppm. [b] Errors derived from 20 spiked 500 ml water samples for each oil, except for diesel where the error associated with 16 samples is quoted. The compositions of the spiked water samples are given in Table 1.

The dilution factor was based on determining the concentrations of white spirit in tetrachloroethene solution whose CH_2 ($\nu_{asymmetric}$) absorbance values matched those obtained for SFE-FTIR analyses of white spirit spiked water samples. Under these circumstances, a lower quality match was obtained for the white spirit CH_3 ($\nu_{asymmetric}$) absorbance values. The CH_3 ($\nu_{asymmetric}$) absorbance values obtained for the tetrachloroethene white spirit solutions were of lower intensity than those obtained for the on-line SFE-FTIR analyses when the CH_2 ($\nu_{asymmetric}$) absorbances were matched. However, as shown in Table 4, a good correlation exists between the quantitative results obtained for each oil using absorbance values obtained at 2960 and 2932 cm^{-1}, applying the same dilution factor initially derived from matching white spirit CH_2 ($\nu_{asymmetric}$) absorbances. These results indicate that the CH_3 ($\nu_{asymmetric}$) to CH_2 ($\nu_{asymmetric}$) absorbance ratios for each of the five oils obtained at 17.22 MPa are approximately equivalent to the corresponding ratios obtained for their tetrachloroethene standard solutions. Hence the oils' high pressure IR spectra are not significantly influenced by pressure to the extent that separate CH_2 ($\nu_{asymmetric}$) and CH_3 ($\nu_{asymmetric}$) dilution factors must be determined for the off-line calibration method.

Analysis of mineral oils in the presence of vegetable oils

Gravimetric and IR methods for the determination of oil, grease and TPH in water generally include an optional sorbent clean-up procedure to remove selectively co-extracted polar species, such as vegetable oils, from the LLE extract prior to final analyses.[8–13,16,31] In order to provide similar capability, an optional high pressure silica gel clean-up stage was incorporated into the SFE-FTIR system. In practice, the SFE-FTIR clean-up column can be selected by a single valve switch. Once this option is selected, the supercritical fluid extract flows through the silica gel column en route to the high pressure IR analysis cell. In order to evaluate the effectiveness of the in-line sorbent clean-up procedure, a series of SFE-FTIR analyses of water samples spiked with diesel, benzene and sunflower oil were performed. As shown in Fig. 5, the high pressure silica gel clean-up procedure is highly effective in selectively removing supercritical fluid carbon dioxide dissolved vegetable oil. The aromatic C–H absorbances for benzene are observed at 3150–3000 cm^{-1}. The results obtained for benzene serve to confirm previous findings[28] that the SFE-FTIR method is suitable for the determination of volatile hydrocarbons, which represents a limitation of gravimetric procedures. Following these initial experiments, 500 ml water samples spiked with different levels of diesel and sunflower oil were analysed using the SFE-FTIR in-line clean-up procedure. The outcome of these analyses is summarised in Table 5, in which the off-line calibration procedure was applied to the results obtained for diesel. Inspection of Table 5 clearly demonstrates that diesel can be accurately quantified using the in-line sorbent clean-up procedure when water samples are also contaminated with vegetable oil.

Since the void volume of the clean-up vessel and ancillary plumbing is low, it is possible to perform two analyses for a single water sample very rapidly, *i.e.*, total oil content and petroleum oil content. With this procedure, the small volume of SFE extract first analysed is vented from the system and the IR analysis cell and associated plumbing are then cleaned using the appropriate stages of the SFE-FTIR system clean cycle. A second analysis of the sample's SFE extract can then be performed with or without the silica gel clean-up being selected. The second analysis is very rapid since the SFE vessel remains pressurised during the partial clean cycle. As a result, the total time required to perform both assays for a single sample is less than 20 min. Once the analysis is complete, the high pressure clean-up column can be rapidly removed, cleaned and reloaded with fresh silica. In practice, this can be performed within the 3 min required to complete the full SFE-FTIR system's clean cycle.

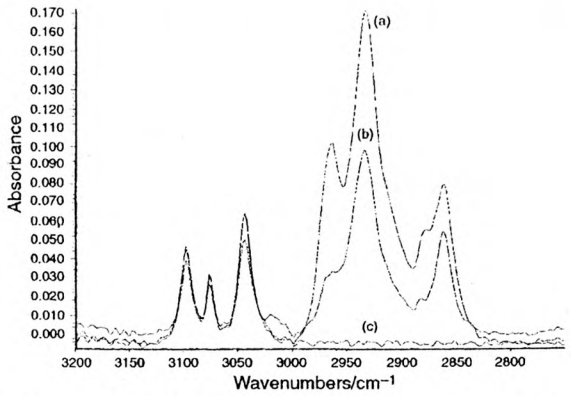

Fig. 5 SFE-FTIR spectra obtained for 500 ml water samples spiked with (a) diesel + benzene + sunflower oil (20 + 30 + 15 µl) with in-line silica gel clean-up, (b) benzene + sunflower oil (30 + 15 µl) without in-line silica gel clean-up and (c) benzene + sunflower oil (30 + 15 µl) with in-line silica gel clean-up.

Table 5 Summary of quantitative SFE-FTIR analyses for diesel, obtained for 500 ml water samples spiked with diesel, sunflower oil and benzene using the on-line silica clean-up column. Results derived using off-line calibration procedure applied to the absorbance values obtained at 2932 cm^{-1}

Amount of diesel + sunflower oil + benzene used to spike 500 ml water samples/µl	Actual level of diesel in spike (ppm)	Calculated level of diesel in 500 ml water sample[a] (ppm)	Accuracy (%)[a]
5 + 15 + 30	8.6	(i) 7.5; (ii) 7.9	(i) 87.2; (ii) 91.9
10 + 15 + 30	17.2	(i) 16.5; (ii) 16.3	(i) 95.9; (ii) 94.8
20 + 15 + 30	34.4	(i) 34.6; (ii) 35.2	(i) 99.4; (ii) 97.7

[a] Results for duplicate analyses shown.

Table 4 Quantitative determination of various oils using the off-line calibration procedure. Dilution factor: as determined for white spirit. Calculations performed using SFE-FTIR absorbance values obtained at (i) 2960 and (ii) 2932 cm^{-1}. For each individual spike level, n = 5, except for diesel, where n = 4

Oil	Concentration of oil in 500 ml spiked water samples (ppm)	Mean calculated concentration of oil in 500 ml spiked watersample (ppm)	Standard deviation (ppm)	Accuracy (%)
Gasoline	67	(i) 66.9; (ii) 63.8	(i) 4; (ii) 4	(i) 99.9; (ii) 95.2
White spirit	78	(i) 75.1; (ii) 75	(i) 4.4; (ii) 3.5	(i) 96.3; (ii) 96.2
Kerosene	81	(i) 87.3; (ii) 83.3	(i) 7.7; (ii) 6.6	(i) 92.2; (ii) 97.2
Diesel	86	(i) 90.5; (ii) 86	(I) 7.4; (ii) 7.7	(i) 94.8; (ii) 100
Engine oil, 20W/50	70	(i) 68.3; (ii) 67.2	(i) 5.6; (ii) 5.4	(i) 97.6; (ii) 96

Conclusions

The SFE-FTIR procedure affords a very rapid, simple and environmentally clean method for determining oil in discharge waters and effluents. Unlike established gravimetric and IR methods which rely upon LLE, the SFE-FTIR method involves an absolute minimum of sample handling stages. Once the sample bottle is sealed within the chamber of the SFE vessel, analysis is performed by depressing a single button on the instrument control panel. At the end of an analysis, the SFE-FTIR system can be cleaned within 3 min, during which *in situ* IR monitoring provides a means to ensure that no cross-contamination between water samples can occur.

As with other IR methods, more accurate analyses can be performed using calibration standards prepared from the actual oil to be analysed. When this is not possible, coefficients may be derived from reference compounds following the HMSO method. These coefficients can then be applied to the SFE-FTIR results. In practice, more accurate SFE-FTIR analyses can be obtained using coefficients calculated from the SFE-FTIR analyses of the various oil types likely to be encountered. With the exception of this improvement, the data generated using the SFE-FTIR system can be interpreted using established/preferred procedures. When the identity of the oil to be analysed is known, a rapid off-line system calibration procedure can be used to improve sample throughput further. With this exception, the SFE-FTIR procedure does not expose the analyst to any toxic and/or flammable organic solvents. It is envisaged that the use of permanently sealed cuvettes containing off-line calibration solutions would effectively minimise the normal hazards associated with using organic solvents.

The incorporation of an optional in-line silica gel clean-up stage permits the specific determination of petroleum hydrocarbons when aqueous samples also contain vegetable oils. Using the silica gel option it is possible to perform two assays for a single 500 ml water sample within 20 min for (i) total mineral and vegetable oils and (ii) mineral oil content.

An inexpensive cylinder containing 34 kg of liquid carbon dioxide allows the analyses of more than 150 500 ml water samples using the SFE-FTIR procedure. In practice, the liquid carbon dioxide cylinder can be remotely located outside the laboratory. The SFE-FTIR system operation costs are highly competitive compared with established IR and gravimetric methods, which require the use of relatively expensive organic solvents for LLE with associated disposal costs.

In summary, the development of a direct aqueous SFE system which can be directly interfaced to an IR spectrometer provides an alternative, environmentally clean, cost-effective method for determining oil in water. The instrumentation is easy to use, rugged and has been developed in close collaboration with several industrial laboratories which routinely perform oil in water analyses.

Acknowledgements

We thank Nicolet Instruments for the extended loan of a Magna-IR 550 spectrometer and financial support. We also thank Dr P. J. Whittle (Hyder) and Dr N. Winstone-Cooper (British Steel, Port Talbot, UK) for their expert advice.

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