# SUPERCRITICAL FLUID EXTRACTION AND ANALYSIS OF EXTRACTS FROM SELECTED MELIACEAE SPECIES

BY

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## Abstract

The thesis outlines an investigative study of the supercritical fluid extraction (SFE) of organic natural products from two Meliaceae species. Extractions from ground wood, bark and seeds of the selected species were studied. Qualitative and quantitative studies were performed using a number of techniques. In the qualitative study, both off-line and on-line combination of micro-SFE with capillary gas chromatography (SFE-GC) were performed on the wood and bark of *Cedrela toona* to show the applicability of the technique in the analysis of semi-volatile components from plant matrices. Results for the on-line and off-line SFE-GC were comparable and indicated that the on-line studies could be used to predict results obtainable from large scale SFE. Dynamic SFE of the limonoid (cedrelone) from the wood of *Cedrela toona* using pure and modified carbon dioxide was studied on the basis of a theoretically-derived dynamic extraction model. Extracts were collected for different lengths of time in order to investigate the extraction kinetics of this compound. The theoretically-derived model which involved extrapolating data obtained from shorter extraction times was used to estimate the amount of the compound present in the wood. The estimated quantitative results were found to be comparable to exhaustive extractions using liquid hexane as well as methanol-modified carbon dioxide. Further, data from the SFE extractions were fitted into the model to produce a characteristic kinetic curve from which factors controlling the extraction of the specific compound could be predicted. Following the dynamic extraction study, a statistical optimisation strategy was used to propose a model equation for the extraction of cedrelone from the wood of *Cedrela toona*. As the preliminary step, a screening design utilising a two-level, three-factor full factorial analysis was used to study the effects of the variables pressure, temperature and the length of extraction time on the yields of cedrelone from the wood of *Cedrela toona*. By a statistical analysis of variance (ANOVA) at the 95% confidence level on the preliminary data collected, pressure and temperature were found to be the significant variables influencing the yields of the compound. Finally response surface methodology using the central composite design was used in an attempt to determine the maximum response space for the pressure and temperature effects by plotting a 3-dimensional response surface graph. An ANOVA study at the 99% confidence level was then employed to establish the true model equation for the extraction.

## Preface

This thesis presents work carried out in the division of Applied Chemistry of the Department of Chemistry and Applied Chemistry, University of Natal, Durban , from September 1993 to December 1995 under the supervision of Professor Mark W. Raynor and Doctor Dulcie A. Mulholland. Parts of chapter 1 and the whole of chapter 2 of this thesis have been published in *Phytochemical Analysis* (1996) as a comprehensive review entitled " Analytical supercritical fluid extraction of natural products ". The work outlined in chapter 4 and part of chapter 6 was presented at the 17th International Symposium on Capillary Chromatography and Electrophoresis in Wintergreen, Virginia, USA, in May 1995 and has also been accepted for publication (1995) in the *Journal of Micocolumn Separations*. Finally the work in chapter 5 has also been submitted to the *Journal of Chromatographic Science* to be considered for publication.

William K. Modey

We certify that the above statements are correct.

Professor Mark W. Raynor

Dr Dulcie A. Mulholland

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| | |
|---|---|
| critical temperature | $T_c$ |
| critical pressure | $P_c$ |
| atmosphere | atm |
| supercritical fluid extraction | SFE |
| supercritical fluid chromatography | SFC |
| newtons second per square metre | $Ns/m^2$ |
| centimetre squared per second | $cm^2/sec$ |
| namely | viz. |
| polycyclic aromatic hydrocarbon | PAH |
| tetrachloro-dibenzo-dioxin | TCDD |
| degree celsius | °C |
| kilopascal | kPa |
| megapascal | MPa |
| pound per square inches | psi |
| kilogram per cubic metre | $kg.m^{-3}$ |
| high performance liquid chromatography | HPLC |
| ultraviolet | UV |
| Fourier transform infrared | FTIR |
| mass spectrometry | MS |
| tandem mass spectrometry | MS-MS |
| thin layer chromatography | TLC |
| gas chromatography | GC |
| internal diameter | i.d. |
| outer diameter | o.d. |
| micrometre | $\mu$m |
| flame ionisation detector | FID |
| microlitre | $\mu$l |
| direct current | dc |
| radiofrequency | rf |

| | |
|---|---|
| Octadecylsilyl | ODS |
| microgram per gram | $\mu$g/g |
| milligram per gram | mg/g |
| leaf protein concentrate | LPC |
| alpha | $\alpha$ |
| beta | ß |
| miceller electrokinetic capillary chromatography | MECC |
| minute/minutes | min/mins |
| millilitre per minute | mL/min |
| nuclear magnetic resonance | NMR |
| mass to mass ratio | m/m |
| gallium | Ga |
| gadolinium | Gd |
| gram per millilitre | g/mL |
| polytetrafluoroethylene | PTFE |
| polyetheretherketone | PEEK |
| hour(s) | h |
| film thickness | $d_f$ |
| perfluorotributylamine | PFTBA |
| chemical shift | $\delta$ |
| dichloromethane | DCM |
| mass to charge ratio | m/z |
| wavenumber | $cm^{-1}$ |
| nanogram | ng |
| relative standard deviation | RSD |
| concentration | conc. |
| internal standard | IS |
| response factor | RS |
| analysis of variance | ANOVA |
| degree of freedom | d.f |

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# CHAPTER ONE

# INTRODUCTION

## 1.1 Introduction and aims

In the classical methods of sample extraction, large volumes of organic solvents are refluxed over the sample-containing matrix for long periods of time in an extraction device giving rise to both disposal and health problems to the laboratory analyst, and this process is time consuming. Therefore the need for an alternative sample preparation technique is important considering the environmental effects and sample preparation time associated with the classical methods. Supercritical fluid extraction (SFE) offers an alternative approach to the extraction of compounds including natural products form matrices. The high mass transfer rate of supercritical fluids, the ability to change the solvating power through density changes, and the non-toxic nature of most substances commonly employed as supercritical fluids in extraction, makes the technique an attractive alternative to the classical methods of sample preparation. Further, since extractions can be performed at low temperatures, it reduces the chances of sample degradation. This chapter is intended to introduce the basic principles and techniques of supercritical fluid extraction as an alternative route to sample preparation in contemporary analytical chemistry as well as the fundamental principles of gas chromatography and supercritical fluid chromatography employed in this study. The chapter is directed towards providing an insight into the objectives of this project.

The aim of the project was to investigate the possibility of using SFE in sample preparation (extraction) from plants of the Meliaceae family, and in particular the extraction of triterpenoid compounds known to be present in this family of plants.

SFE is distinct from the traditional methods in that the enhanced solvating power of substances above their critical point is employed. In the applications to be discussed, both SFE and classical techniques are compared from a qualitative and quantitative point of view. Different matrices have been tested including ground wood, bark and seeds from different plant species belonging to the Meliaceae family. A dynamic extraction model is employed in a study of the SFE of a specific compound from a ground wood sample. Also a comprehensive review outlining areas where supercritical fluids have been employed in the extraction of natural products is discussed.

## 1.2 Definition of a supercritical fluid

A supercritical fluid is a substance existing at temperatures and pressures above its critical temperature and pressure. Above its critical temperature, the substance does not condense or evaporate to form a liquid or a gas. A supercritical fluid is a fluid whose properties change from gas-like to liquid-like as the pressure increases. **Figure 1.1** (page 3) shows the phase diagram of a single substance. If a liquid and a gas are in equilibrium and one moves along the gas-liquid coexistence curve toward the critical point C, by increasing both the temperature and pressure, the liquid becomes less dense because of thermal expansion and the gas becomes more dense as the pressure rises. At the critical point, C, the densities of the two phases become identical and the distinction between the gas and the liquid disappears. The substance becomes a supercritical fluid. The region of interest for supercritical fluid extraction is the area above the critical point where densities, solubilities, viscosities and diffusivities are intermediate between those of typical gases and liquids. This means that with the correct choice of temperature and pressure above or around the critical point, supercritical fluids can be used as solvents for extraction.

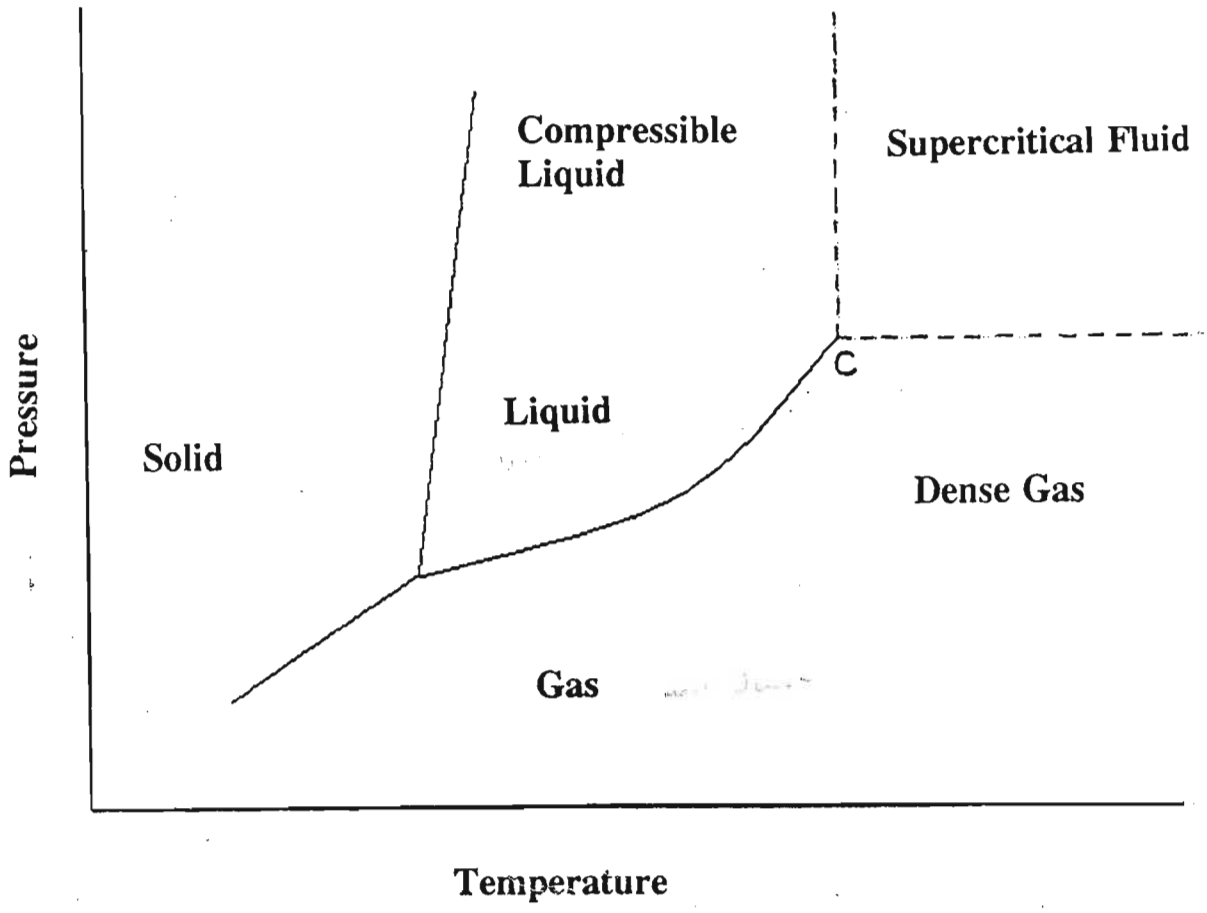



**Figure 1.1** Pressure-temperature phase diagram of $CO_2$ showing the critical point (C), above which supercritical conditions exists.

### 1.3 Selecting a supercritical fluid

Several substances have been used as supercritical fluids both in extractions and as a chromatographic mobile phase. **Table 1.1** below shows typical examples of substances and their critical parameters. From **Table 1.1**, it may be seen that a variety of supercritical fluids are available, but, supercritical carbon dioxide has been the choice for

most SFE studies, primarily because of its attractive practical characteristics: relatively low critical temperature and pressure, non-toxic, inert and high purity at low cost. In general, carbon dioxide is an excellent extraction medium for non-polar to moderately polar species including polycyclic aromatic hydrocarbons (PAHs) [1], plant flavours [2], triterpenoids [3] etc.

**Table 1.1** Commonly-used substances for supercritical fluid extraction and their critical parameters.

| Substance | $T_c$/°C | $P_c$/atm. | Dipole moment(Debye) |
|---|---|---|---|
| $CO_2$ | 31.3 | 72.9 | 0.0 |
| $N_2O$ | 36.5 | 72.5 | 0.2 |
| $NH_3$ | 132.5 | 112.5 | 1.5 |
| $H_2O$ | 374.0 | 227.0 | 1.9 |
| Xe | 16.6 | 58.4 | 0.0 |
| $CH_3OH$ | 240.0 | 78.0 | 1.7 |
| $CHF_3$ | 25.9 | 46.9 | 1.7 |
| n-$C_4H_{10}$ | 152.0 | 37.5 | 0.0 |
| $CHClF_2$ | 96.0 | 49.1 | 1.4 |

Difficulties are experienced when fairly polar analytes need to be quantitatively extracted. The solvent of choice would be a fluid with higher solvent strength, but the use of more polar fluids is severely limited by practical considerations. Supercritical ammonia would be very attractive from a solvent strength point of view, but it is difficult to pump (it tends to dissolve pump seals), it is chemically reactive, and it is likely to be too dangerous for routine use. Supercritical methanol is also an excellent solvent but is less attractive because of its high critical temperature (**Table 1.1**) and because it is a liquid at ambient conditions, which complicates sample concentration after extraction. Despite its excellent characteristics as an SFE fluid, the routine use of $CHClF_2$ is also not likely because of the negative environmental effects. However, for some analyte/matrix combinations, extraction efficiencies can be increased using fluids that would not have been expected to

yield good efficiencies based on their solubility parameters. For example, $N_2O$ (dipole moment, 0.2) gives more rapid extractions of some samples than can be achieved with $CO_2$ (dipole moment, 0.0) [4,5] as demonstrated for the extractions of tetrachlorodibenzo-p-dioxins (TCDDs) and PAHs. These results are surprising given the similarity of $N_2O$ and $CO_2$ in physical properties and solubility parameters ($N_2O$ does have a small dipole moment, $CO_2$ does not). The results from [4] for the SFE of TCDDs from incinerator fly ash are particularly interesting. $N_2O$ yielded good recoveries from untreated fly ash, whereas $CO_2$ yielded good recoveries only after the ash surface was modified by acid treatment, demonstrating that displacement of the analytes from the sorptive sites on the fly ash and not differences in their solubility was responsible for the increased efficiencies demonstrated by $N_2O$. Criteria for selecting a supercritical fluid have recently been discussed [6]. Because of the practical difficulties in using polar fluids such as ammonia and methanol for SFE, extractions of highly polar analytes have most often been done using carbon dioxide containing a few percent of an added modifier. This will be described in section 1.5.

## 1.4 Useful properties of supercritical fluids as extraction solvents

The combined gas-like and liquid-like solvating characteristics have led to considerable interest in their use as mobile phases in supercritical fluid chromatography (SFC) [7], but their use for analytical scale extraction has only recently received attention. The solvent strengths of supercritical fluids approach those of liquid solvents as their density is increased [8]. Although supercritical fluids do not have any advantage over liquid solvents in terms of solvating power, comparison of several other characteristics with liquid solvents demonstrates the potential for SFE to approach the idealized goals for analytical extraction. A good solvent for extraction should have the following characteristics:

(i) Since the object is to separate an analyte from a matrix, the solvent should be able to dissolve the desired material better than it dissolves other constituents.

(ii) A suitable solvent should also have a high capacity for the analyte of interest.

(iii) An ideal solvent should also be stable and unreactive to the proposed system (ie. inert to both solute and matrix and stable under the conditions of the process).

(iv) From an economic perspective, a desired solvent should not be corrosive to the equipment and should be relatively inexpensive.

(v) The solvent should be non-toxic.

The factors given above are some of the criteria behind the investigations of supercritical fluids as extraction solvents. However, other properties from an extraction point of view include the diffusion coefficient, the density and viscosity of the supercritical fluid. Mass transfer limitations ultimately determine the rate at which an extraction can be performed. Supercritical fluids have solute diffusivities an order of magnitude higher ($10^{-4}$ vs $10^{-5}$ $cm^2/s$) and viscosities an order of magnitude lower ($10^{-4}$ vs $10^{-3}$ $Ns/m^2$) than liquid solvents, therefore they have much better mass transfer characteristics, and hence faster extraction. The precise control of the density in SFE is a simple concept. Solvent strength is a function of the density of the supercritical fluid. There is no separation system with so much flexibility of solvent strength available by simple physical manipulations other than SFE. At constant temperature, the density and hence the solvent strength increases as the pressure increases. **Figure 1.2** on page 7 is a pressure-density isotherm for supercitical $CO_2$. It is clear from this figure that at a constant pressure, an increase in the fluid temperature results in a decrease in density and hence in solvating power. However, this phenomenon does not continue indefinitely. Eggers et al. [9] reported that the solubility of soyabean oil finds a maximum between 500 and 1000 atmospheres of pressure. This maximum tends to shift up as the temperature increases. Another aspect of the pressure-solubility relationship bearing mention is the observation that when solubility is plotted against pressure, the isotherms intersect at between 300 to 400 atmospheres [10]. At pressures below this inversion point, solubility decreases with increasing temperature, while solubility increases with increasing temperature at pressures above this inversion point.

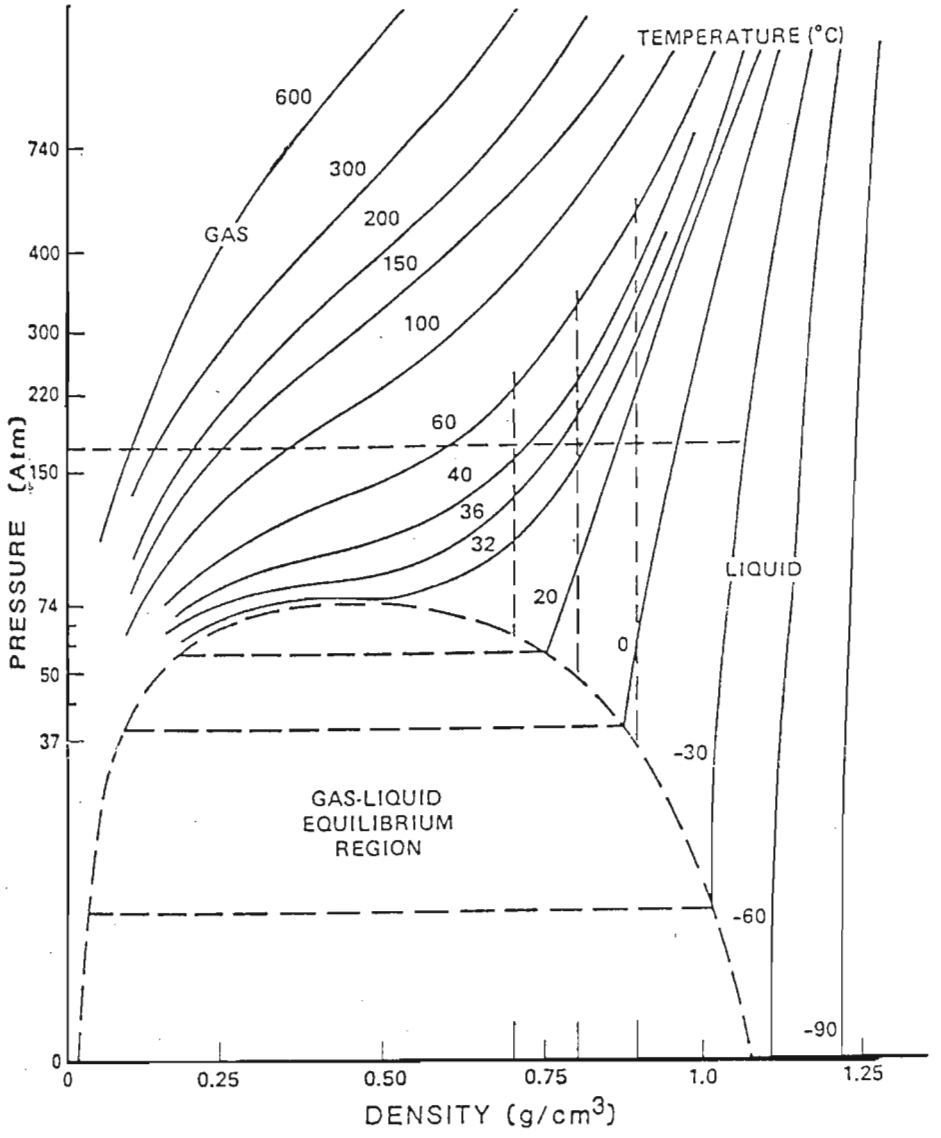



**Figure 1.2** Effect of pressure changes on the density of supercritical $CO_2$ at different temperatures.

This apparent conflict is understood when we remember that the solubility is also a function of the volatility of the solute. Solute solubility in the fluid alone does not guarantee a successful extraction as the rate of extraction also depends on the rate of

diffusion out of the matrix. Even though the increase in temperature lowers the density of the fluid, it exponentially raises the vapour pressure of the solute and hence the solute diffusion coefficient. This can make the optimisation of the pressure-temperature parameters complex, but, if one works from density figures instead of pressure, the problem is averted because at constant density, an increase in temperature increases solubility in every case [11].

## 1.5 Use of Modifiers

Most analytical SFE techniques are accomplished by supercritical $CO_2$ due to its practical advantages viz. low critical parameters, non-toxic, inert and high purity at low cost. However, due to its practical limitation in the extraction of polar analytes, the most practical approach to increasing its solvent strength is by the addition of secondary solvents or modifiers, generally in the range of 1-10 % by volume. Usually, these are classical solvents such as those listed in **Table 1.2** or a material that displays some special interaction with the analyte or with the matrix or both.

Zhuze was the first to describe the effects of modifiers in SFE [12], but the extraction of caffeine from coffee remains the historical model for the use of modifiers in SFE. Several research groups have demonstrated the effectiveness of organic modifiers in SFE. Supercritical $CO_2$ modified with methanol has been shown to give better yields than pure $CO_2$ in several papers [5, 13, 14]. However, methanol-modified $CO_2$ does not always increase extraction efficiency. Extraction selectivity is lowered as well in some applications [15], and hence different modifiers may often be required.

**Table 1.2** Examples of commonly-used modifiers in SFE applications.

| Modifier | $T_c$ (°C) | $P_c$ (atm) | Molecular mass | Dielectric constant at 20°C |
|---|---|---|---|---|
| Methanol | 239.4 | 79.9 | 32.04 | 32.70 |
| Ethanol | 243.0 | 63.0 | 46.07 | 24.30 |
| Propan-1-ol | 263.5 | 51.0 | 60.10 | 20.33 |
| Propan-2-ol | 235.1 | 47.0 | 60.10 | 19.30 |
| Hexan-1-ol | 336.8 | 40.0 | 102.18 | 13.30 |
| 2-Methoxyethanol | 302.0 | 52.2 | 76.10 | 16.93 |
| Tetrahydrofuran | 267.0 | 51.2 | 72.11 | 7.58 |
| 1,4-Dioxane | 314.0 | 51.4 | 88.11 | 2.25 |
| Acetonitrile | 275.0 | 47.7 | 41.05 | 37.50 |
| Dichloromethane | 237.0 | 60.0 | 84.93 | 8.93 |
| Chloroform | 263.2 | 54.2 | 119.38 | 4.81 |
| Water | 374.1 | 217.6 | 18.01 | 80.1 |
| Carbon disulphide | 279.0 | 78.0 | 76.13 | 2.64 |

Hawthorne et al. reported that there was no increase in extraction efficiency of nitro-PAHs from diesel exhaust particulate matter when methanol-modified $CO_2$ was used compared to pure $CO_2$. However, toluene-modified $CO_2$ yielded much higher recoveries (> 100%) compared to the Soxhlet extractions [16], a clear indication that different modifiers suit different extraction processes. Although there is little in the literature to aid in selecting a modifier, a logical first choice for a modifier would be a substance that is itself a good solvent for the target analyte. Choosing a modifier may rely on trial and error, thus making rapid methods for surveying the effectiveness of several modifiers very difficult.

There are several methods for adding modifiers to a primary supercritical fluid, varying considerably in cost, convenience and reliability. The simplest method is to pipette a small volume of the modifier into the extraction cell before the extraction process begins. This method of introducing modifiers has its own disadvantages since it is not suitable for dynamic SFE processes because the modifier is swept away from the cell during that mode of extraction. This method requires that a period of static extraction is performed prior to the dynamic stage. However, this method greatly simplifies the testing of many different modifiers in a short time. A second method of introducing modifiers is to purchase premixed cylinders of modifier and primary fluid. This approach is particularly useful if the optimal composition of the modified fluid is known and the analyst wishes to extract many samples under the same conditions. On the other hand, this approach is clumsy and expensive for selecting modifiers since a new cylinder must be purchased for each different modifier. Claims of consistent delivery concentrations for these products in analytical applications have been at variance with the experience of a number of workers.

The work of Schweighardt and Mathias [17] confirmed the findings of inconsistent performance of these premixed products and traces its origin to a shifting vapour-liquid equilibrium which changes the concentration of the modifier in the liquid phase as the cylinder contents are drawn. As the liquid phase is drawn, $CO_2$ (the more volatile component) vaporizes disproportionately occupying the liberated volume. As a result, the

concentration of the modifier in the liquid phase increases. Such changes not only affect solubility properties of the fluid, but instrument performance as well. In SFE they may result in the extraction of undesirable sample components or reduced extraction efficiency for the analytes of interest or both. In certain cases, the change in modifier concentration may degrade performance to such an extent that the method becomes unusable [18]. Also, as well as increasing modifier concentration as cylinder contents are withdrawn, this change causes unexpected and significant drops in cylinder delivery pressure [17]. As a result, instruments that depend on a specific cylinder pressure to fill their reservoirs will require increasingly longer fill cycles which in turn reduces throughput.

Probably the best, and most expensive method that is presently available for generating a modified supercritical fluid is the use of a dual pumping system. One pump handles only pure $CO_2$ while the second pump provides a constant volume ratio of the modifier. The modifier concentration can be changed immediately. Unlike the second method of introducing modifiers, since the modifier never comes into contact with the primary fluid pump, the possibility of contaminating the primary fluid pump is absent. With any method of adding modifiers to primary fluids, it must be remembered that the critical temperature of the mixed solution is higher than that of the pure fluid, and therefore the temperature of the extraction cell should be raised to ensure that a single phase supercritical fluid is present during the SFE to avoid probable solute partitioning between the two phases that might coexist. Although appropriate phase diagram data are available for $CO_2$/methanol mixtures [19] as in **Table 1.3** (page 14) and **Figure 1.3** (page 13), data on critical temperatures for most $CO_2$/modifier combinations are generally not available except a few compiled by Page et al. [20]. For example, **Figure 1.3** (page 13) shows the relationship between the critical temperature, pressure and mole fraction of $CO_2$-methanol mixtures. If a methanol mole fraction of 0.2 in $CO_2$ was required for extraction, it would be necessary to operate above 150 bar and 80°C in order to maintain a homogeneous supercritical fluid [21]. The way modifiers operate in increasing extraction efficiencies is not well known, but whatever the mechanism may be, it has something to do with the analyte-matrix complex by promoting rapid desorption of the solute into the fluid or

preventing readsorption of the solute onto the matrix, and may also enhance the solubilising properties of the fluid. It is possible that the modifier swells the matrix and facilitates diffusion of the analyte from the matrix, or that the modifier competes with the analyte for matrix sites so that the more effective the modifier is in displacing the analyte from the matrix site, the more efficient the extraction process becomes. Also, the choice of modifier is highly dependent on the type of sample matrix and analyte to be extracted.

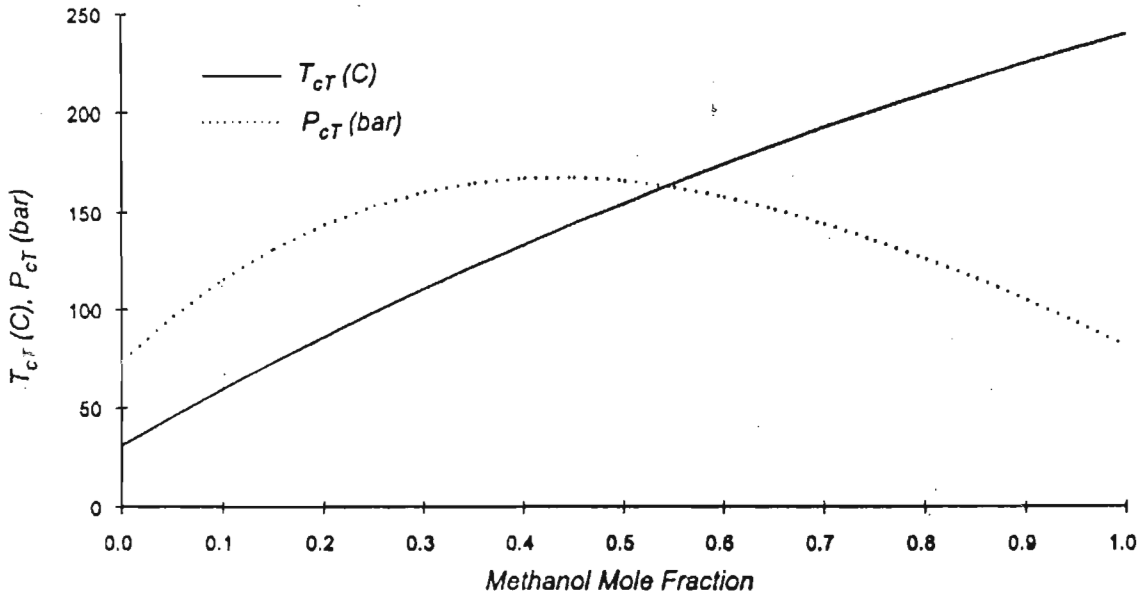



**Figure 1.3** Relationship between the critical temperature, pressure and mole fraction of $CO_2$-methanol mixtures according to Saito and Nitta. [21].

**Table 1.3** Isothermal phase equilibria and densities for $CO_2$ + methanol mixtures according to Brunner et al. $\rho$ is density, and x and y are mole fractions [19].

| $\frac{p}{MPa}$ | $x$ or $y$ | $\frac{\rho}{kg \cdot m^{-3}}$ | $\frac{p}{MPa}$ | $x$ or $y$ | $\frac{\rho}{kg \cdot m^{-3}}$ | $\frac{p}{MPa}$ | $x$ or $y$ | $\frac{\rho}{kg \cdot m^{-3}}$ |
|---|---|---|---|---|---|---|---|---|
| | | | $\{xCO_2+(1-x)CH_3OH\}(l) = \{yCO_2+(1-y)CH_3OH\}(g)$ | | | | | |
| | | | | $T = 298.15$ K | | | | |
| 1.73 | $x$ 0.1214 | 816 | 5.14 | 0.4729 | 866 | 6.23 | 0.9693 | 768 |
| 1.75 | 0.1258 | 815 | 5.29 | 0.4957 | 867 | 5.88 | $y$ 0.99279 | — |
| 2.38 | 0.1723 | 830 | 5.53 | 0.5554 | 872 | 5.08 | 0.99289 | — |
| 3.02 | 0.2289 | 841 | 5.72 | 0.6288 | 865 | 3.96 | 0.99297 | — |
| 3.61 | 0.2792 | 850 | 5.75 | 0.6538 | 861 | 2.91 | 0.99185 | — |
| 4.11 | 0.3323 | 857 | 5.92 | 0.8097 | 849 | 1.97 | 0.9894 | — |
| 4.43 | 0.3642 | 859 | 5.94 | 0.8109 | 848 | 0.97 | 0.9812 | — |
| 4.85 | 0.4200 | 866 | 6.04 | 0.9299 | 797 | 0.41 | 0.9588 | — |
| | | | | $T = 323.15$ K | | | | |
| 0.99 | $x$ 0.0413 | 774 | 9.28 | 0.6581 | 795 | 7.99 | 0.9758 | — |
| 1.96 | 0.0946 | 784 | 9.32 | 0.6950 | 757 | 7.50 | 0.9765 | 200 |
| 2.52 | 0.1197 | 788 | 9.50 | 0.7293 | 754 | 5.96 | 0.9809 | — |
| 3.42 | 0.1632 | 794 | 9.53 | 0.8172 | 679 | 5.17 | 0.9796 | 111 |
| 4.75 | 0.2368 | 813 | 9.55[a] | $x=y$ 0.8404[c] | 657[c] | 3.99 | 0.9792 | — |
| 5.98 | 0.3045 | 817 | 9.53 | $y$ 0.8606 | 630 | 2.00 | 0.9676 | — |
| 7.70 | 0.4344 | 821 | 9.47 | 0.9460 | — | 1.00 | 0.9412 | — |
| 8.81 | 0.5529 | 803 | 9.10 | 0.9628 | — | | | |
| 9.19 | 0.6378 | 809 | 8.77 | 0.9689 | 298 | | | |
| | | | | $T = 373.15$ K | | | | |
| 2.01 | $x$ 0.0392 | 711 | 15.41 | 0.6197 | 632 | 12.95 | 0.8870 | — |
| 3.81 | 0.0880 | 719 | 15.42 | 0.6664 | 588 | 11.05 | 0.9048 | — |
| 4.84 | 0.1185 | 723 | 15.42[a] | $x=y$ 0.6735[c] | 585[c] | 10.31 | 0.9076 | 214 |
| 5.84 | 0.1445 | 721 | 15.42 | $y$ 0.6805 | 574 | 8.06 | 0.9109 | — |
| 8.37 | 0.2262 | 723 | 15.42 | 0.6959 | 570 | 6.62 | 0.9111 | 218 |
| 9.07 | 0.2456 | 718 | 15.32 | 0.7577 | 515 | 3.97 | 0.8820 | — |
| 11.74 | 0.3429 | 719 | 15.31 | 0.7695 | 492 | 2.83 | 0.8523 | 43.5 |
| 12.71 | 0.3968 | 708 | 14.06 | 0.8500 | — | | | |
| 14.75 | 0.5185 | 682 | 13.44 | 0.8727 | 338 | | | |
| | | | | $T = 423.15$ K | | | | |
| 3.67 | $x$ 0.0460 | 645 | 14.80 | 0.3529 | 593 | 15.60 | 0.6370 | 362 |
| 5.89 | 0.0942 | 640 | 15.13 | 0.3753 | 593 | 13.93 | 0.6974 | 274 |
| 6.95 | 0.1192 | 640 | 15.97 | 0.4353 | 540 | 10.79 | 0.7409 | 184 |
| 10.45 | 0.2033 | 629 | 16.08 | 0.4750 | 505 | 8.64 | 0.7340 | 135 |
| 11.62 | 0.2386 | 625 | 16.13[a] | $x=y$ 0.5178[c] | 466[c] | 6.33 | 0.6894 | 92 |
| 12.80 | 0.2748 | 614 | 16.06 | $y$ 0.5684 | 426 | 3.81 | 0.5747 | 51 |
| | | | | $T = 473.15$ K | | | | |
| 7.52 | $x$ 0.0682 | 523 | 12.93[a] | $x=y$ 0.270[b] | 360[b] | 7.94 | 0.3186 | 122 |
| 10.40 | 0.1383 | 497 | 12.92 | $y$ 0.2880 | 343 | | | |
| 12.80 | 0.2396 | 406 | 10.98 | 0.3779 | 197 | | | |

[a] Critical pressure.
[b] Critical values, obtained by graphical interpolation.
[c] Critical values, determined experimentally.

## 1.6 Methodology in SFE

Analytical-scale SFE can be broadly divided into two general approaches, off-line and on-line techniques. Off-line refers to any method where the analytes are extracted using SFE and collected in a device independent of the chromatograph or other measuring instrument [22], while the on-line techniques utilise direct transfer of the extracted analytes to an analytical instrument such as a chromatograph with an appropriate detector [23] for the analysis of the extract. To perform off-line SFE, only the SFE step must be successful, and the extract can be analysed by a variety of techniques. For an SFE to be successful, the target analytes must be controlled through three essential steps. First the analyte must be partitioned from the sample matrix into the bulk supercritical fluid, which is controlled by the physicochemical interactions of the analytes,matrix and the fluid. These interactive forces are analyte diffusion in the matrix, analyte solubility and analyte-matrix interactions. Secondly, the solute must be transported sufficiently by diffusion from the interior of the matrix in which it is contained. Thirdly, the analyte must be sufficiently collected from the supercritical fluid in a form that is compatible with the subsequent analytical method.

These steps are interdependent to some degree. Partitioning of the analyte from the matrix to the bulk fluid is essentially a physicochemical problem, eg. finding the SFE conditions that can best move target analytes from the matrix active sites, including pressure and temperature ranges, and maybe the need for a modifier. Steps two and three are essentially plumbing parameters. These may involve the fluid flow rates required to sweep the analytes from the extraction cell, the maximum size of extraction cell that is useful and the type of device used to control the fluid flow rate. The step that is more difficlt experimentally (collecting the analytes after SFE) is nearly always performed by depressurising the supercritical fluid through a flow restrictor into a collection device.

## 1.7 Static and dynamic modes of extraction

There are two principal ways of performing SFE. The sample can be extracted statically by holding the cell under pressure for a certain period of time before the extract is swept into a suitable collection device, or dynamically by continually exposing the analytes to

a fresh stream of supercritical fluid and accumulating the extract in a suitable collection device. Static extraction proves convenient in a situation where modifiers are introduced in the extraction process to enable a single phase fluid to be formed. The choice for either a static or dynamic mode of extraction is controversial. Anderson and co-workers [10] argue in favour of dynamic extraction methods for several reasons. Firstly, the potential for saturation of a component with limited solubility in a static solvent pool may hinder complete recovery of the analyte. In a dynamic system, the analyte is continually exposed to a fresh stream of solvent. Secondly, in a static situation, extraction times may be longer because the movement of components must rely only on diffusion, as opposed to the mass action of the carrier in dynamic systems. Finally, this same dependence on diffusion may lead to an incomplete recovery of compounds with low diffusion coefficients. While there have been reports of static systems yielding better recoveries than dynamic systems [24], the researchers believe that if the flow dynamics are optimised for the cell dimensions and sample quantities, to prevent chanelling or compaction of the sample, dynamic systems will prove superior in most cases. Since there is no strong evidence in support of either method, it is a matter of personal preference.

## 1.8 Instrumentation for Off-line SFE

Off-line SFE is conceptually a simple experiment to perform, and requires only relatively basic instrumentation. **Figure 1.4** on page 17 is a schematic diagram of the main components of an off-line SFE system. **A**, is a source of fluid; **B**, is a pump to pressurise the fluid in the liquid state; **C**, is a cooling unit to cool the pump head to temperatures of between 8 - 15°C to liquify the fluid; **D**, is a personal computer to control the pump pressure; **E**, is an inlet valve; **F**, is the extraction cell housed in either a GC oven or an external heating system; **G**, is an outlet valve that could be used to control static extractions; **H**, is a linear restrictor to control the pressure in the cell and also the flow rate; **I**, is a collection device; **J**, is either a 1/8 inch or a 1/16 inch stainless steel tube and **K** is a 1/16 inch stainless steel tube.

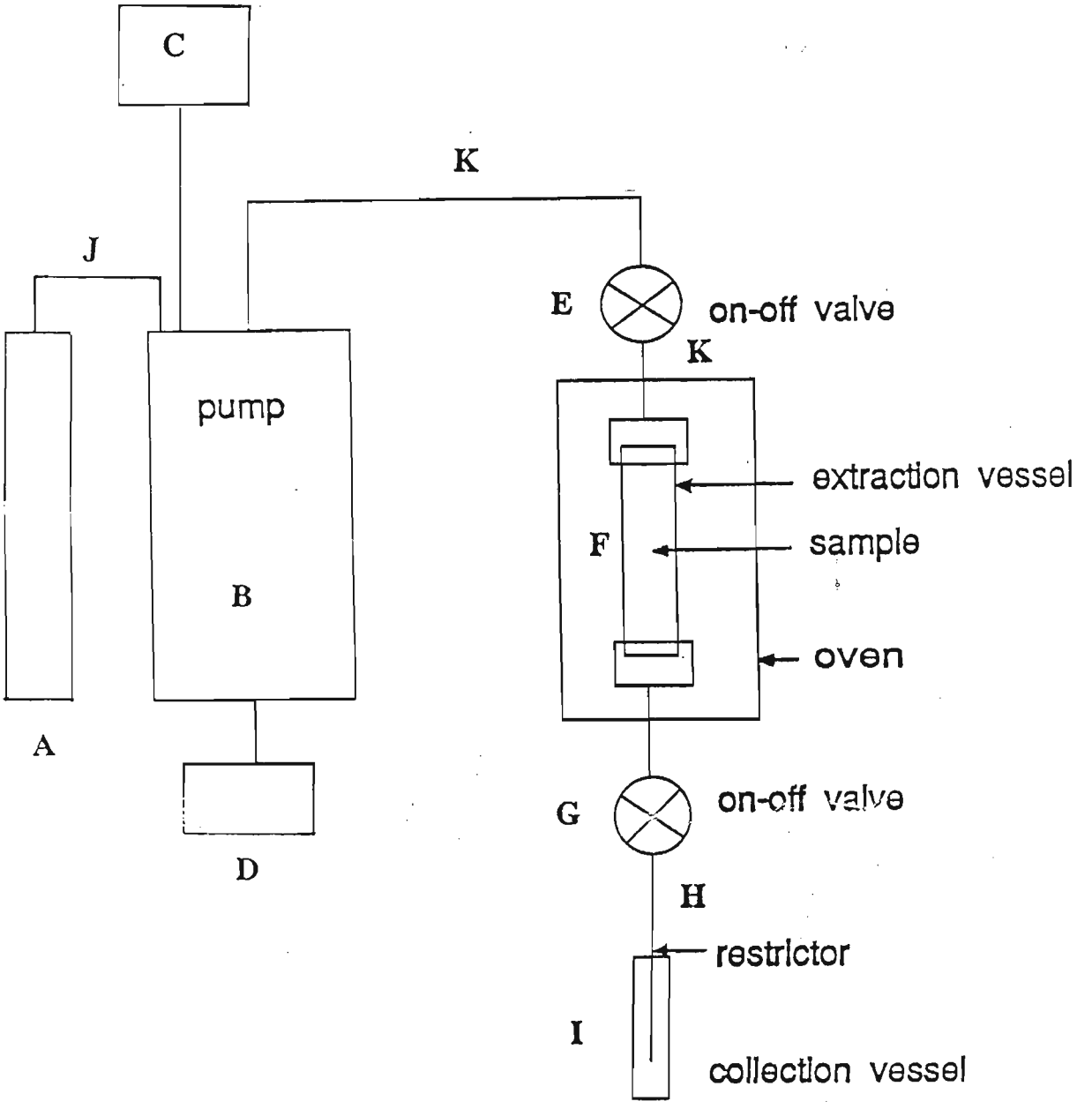



Figure 1.4 A typical instrumental set-up for off-line supercritical fluid extraction.

There may be modifications to this simple design depending on plumbing requirements, including a second pump supplying modifier and additional switching valves.

In the dynamic mode, the sample is continually supplied with fresh supercritical fluid and

the extracted analytes are constantly swept into the collection device. In this mode, both the inlet and outlet valves are open. For static SFE, the outlet valve is closed and the cell is pressurised. Following an appropriate extraction time, the analytes are recovered from the static extraction by opening the valve at the outlet of the cell and performing a short dynamic extraction.

### 1.9 SFE Pumps

The majority of pumping systems used for SFE are similar to HPLC pumps and are based on either syringe or reciprocating piston design, although alternative approaches such as a thermal pumping system have been reported [25]. The upper pressure rating of the pump should be at least 400 atm (ca. 5800 psi) and it should be capable of providing at least 2 ml/min of pressurised fluid at the upper pressure limit. The pump should recover to full pressure, after presurising the extraction cell, in a very short time. Since syringe style pumps require refilling by the operator, such pumps should be capable of being filled and returned to the operating pressure in a few minutes.

### 1.10 Extraction Cells

In the past, many investigators have utilised tube fittings or HPLC columns as extraction cells [12], but recently, commercially available extraction cells with finger-tight fittings have become available. There are several sizes ranging from 0.5 ml to about 1 litre capacity,(JASCO, Hachioji, Japan; and Keystone Scientific, Bellefonte, PA, USA). Therefore, off-line SFE can now be applied to large sample sizes as compared to the past years where extractions were limited to small-sized samples. **Figure 1.5** on page 19 shows typical vessels employed in SFE.

### 1.11 Restriction Devices

As discussed above, the SFE pump is responsible for providing a constant pressure of the extraction fluid. The flow rate of the supercritical fluid is controlled by the restrictor placed at the outlet of the extraction cell. Two general types of restrictors have been used for the majority of off-line SFE studies. The first type, linear restrictors, are generally

made by simply attaching a short piece of small inner diameter fused silica capillary tubing to the cell outlet. Changing the inner diameter and length, changes the flow rate. The larger the internal diameter, the higher the flow rate and vice versa. The disadvantages are that linear restrictors are subject to plugging when samples are wet or contain very high concentration of the extractable analytes. However, they are inexpensive

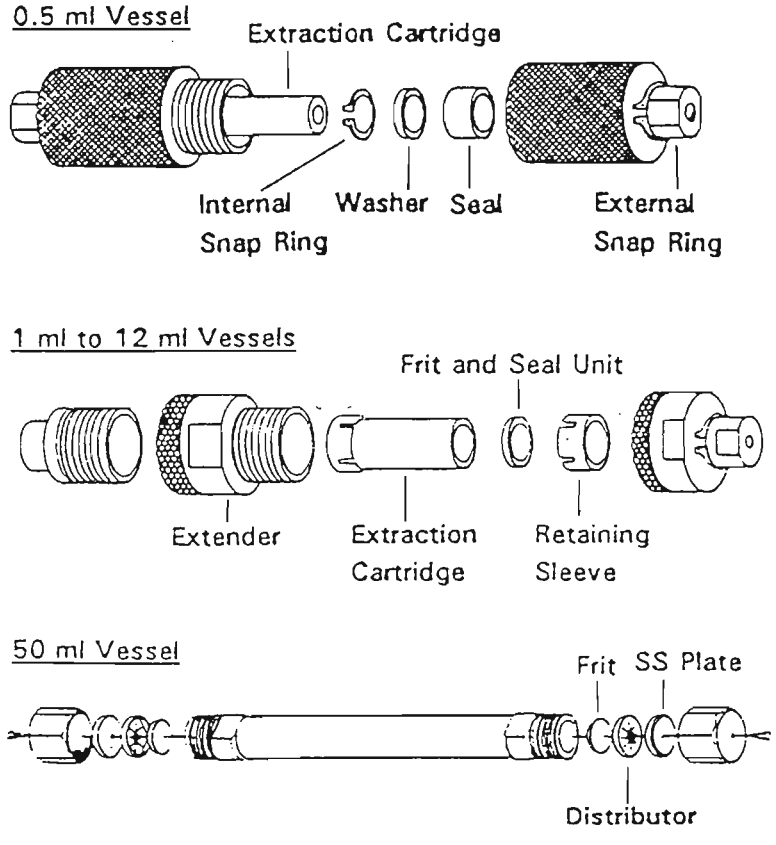



**Figure 1.5** Typical stainless steel vessels employed in SFE

and can be routinely replaced at little cost. Secondly, variable restrictors and back pressure regulators can also be employed to control the pressure of the fluid during the extraction. They are rather more expensive than linear restrictors.

## 1.12 Analyte Collection and Recovery

Methods for recovering the analytes from the supercritical fluid have depended on depressurising the fluid through the restrictor followed by collecting the analytes from the gaseous fluid stream. The three general approaches for collecting analytes off-line after depressurisation are thermal trapping, sorbent trapping and trapping in a liquid solvent. Linear restrictors have been used for all three methods but, commercially-available variable restrictors have proved compatible with thermal and sorbent trapping.

By thermal trapping, sometimes called cryogenic trapping, the SFE effluent is simply depressurised into a cooled vessel. For quantitative purposes, it is suitable for non-volatile organics only since the high gas flow rate causes losses of even moderately volatile analytes. Non-volatile analytes may also be lost through aerosol formation [25]. The remaining two methods have much better potential for quantitative recovery of highly and moderately volatile analytes. Trapping on a sorbent is achieved by depressurising the supercritical fluid onto the sorbent trap. Once the SFE is complete, the trapped analytes are recovered from the trap by eluting them with a small volume of a liquid solvent. Examples of common substances used as trap materials are, cyanopropyl silica, Tenax and diol-bonded silica. This procedure has the advantage of allowing the analyst to choose the sorbent packing that is best for the target analytes as well as the possibility of selective collection on the trap. A typical example of selective trapping of a specific component was demonstrated by Schaeffer et al. [26] when they used a cation-exchange resin to selectively trap the alkaloid, monocrotaline from *Crotalaria spectabilis*. Even though the cation-exchange resin has the possibility of selectively trapping analytes of interest. it should be recognised that the sorbent trapping and the subsequent elution introduce additional steps to the SFE experiment which may or may not affect extraction yields. Solid phase sorbents have been used as efficient traps for off-line SFE [25,27].

Lastly, analytes may be collected by depressurising the SFE effluent directly into a small vial containing a few millilitres of liquid solvent. Though it does not provide the selectivity that sorbent trapping does, solvent trapping avoids the additional steps required by sorbent trapping. Direct solvent collection is very common with many researchers [28]. Howard and co-workers [25] reported a tandem collection system using sorbent and solvent traps in the extraction of thiocarbamate pesticides in apples. Hirata and Okamoto [29] also noted that, in order to trap polymer additives after decompression of an extract-laden supercritical fluid, the restrictor had to be connected to two trapping tubes in series. The first trap was empty and the second packed with silica. Most of the analytes were trapped efficiently in the first trap, but about one-third of the analytes were trapped on the silica. McNally and Wheeler [30] initially tried to adsorb sulphonylureas on two guard columns packed with C-18 and silica. They observed that the major fraction of the analytes was deposited in the back-up collection flask inserted after the guard columns rather than in the columns themselves. Therefore, tandem trapping systems may be necessary with some applications.

## 1.13 Instrumentation for on-line SFE

### 1.13.1 SFE coupled with spectroscopic and spectrometric detectors

SFE can be coupled with a variety of detection and separation techniques. One of the oldest and by modern standards, crudest techniques for determining the extraction yield is a gravimetric analysis, in which the mass of the extract and the mass of the sample are compared with each other [31]. The problem in using gravimetric analysis is that, intrinsically it is an off-line process, which means extra sample manipulations and thus an increased analysis time and higher cost. Monitoring by UV detection requires the presence of a chromophore in the extracted analytes. This illustrates one of the advantages of using $CO_2$ as the supercritical fluid as it is transparent down to about 190 nm. Wright et al. [32] used a closed-loop system with recirculation and on-line UV detection to monitor the effects of ultrasound during the extraction of chrysene from adsorbents or of caffeine from roasted coffee beans. By using absorbance detection spectra, functional

group information can be obtained [33]. For compounds exhibiting unique regions of absorbance in the infrared region, directly coupling the SFE effluent to an FTIR can be of great importance. The recovery and detection of n-tetracosane from a solid matrix by directly coupled SFE-FTIR was carried out by Kirschner and Taylor [34].

In addition to UV and FTIR detection, mass spectrometric detection is a valuable alternative for obtaining structural information. Kalinoski et al. [35] used on-line SFE with chemical ionisation MS detection and collision-induced dissociation tandem MS (MS-MS) for the rapid identification of ppm levels of several trichothecene mycotoxins with minimum sample handling. The limitation of SFE-MS is the possibility of overloading the mass spectrometer with coextracted compounds when complex samples are analysed [35]. The result is that often sophisticated techniques such as tandem MS may be necessary to obtain the necessary selectivity and sensitivity. A cheaper and more attractive alternative is to perform some form of chromatography between extraction and detection, and for the purpose of this discussion, on-line SFE- chromatographic techniques will be discussed.

### 1.13.2 SFE-TLC

On-line SFE-thin layer chromatography (SFE-TLC) provides a rapid and simple insight into the extraction performance. The strength of this technique is that the extract is deposited directly on a thin layer chromatographic plate. Both one- and two-dimensional chromatography can be performed, ie., SFE can be combined with the development of the TLC plate in either one or two directions, after which the components of interest can be detected on or isolated from the support material for further study. Stahl et al. [36,37,38] developed a mini-extraction apparatus for the desorption of a supercritical fluid extract on a moving thin-layer chromatographic plate. Both $CO_2$ and $N_2O$ were used as supercritical fluids and a wide range of naturally-occurring materials were studied. Limitations of SFE-TLC are that quantification is difficult and that the stability of components on the support material or in the presence of oxygen may be a problem.

Further, the resolution of TLC is low compared with that of HPLC, GC and SFC, and at high pressures above 300 atm problems are encountered such as stripping of the support material by the supercritical fluid caused by the increased velocity of the expanding fluid [37].

### 1.13.3 SFE-GC

Hawthorne and Miller [39] were the first to couple SFE directly with on-line GC when they successfully performed a qualitative analysis of automobile-exhaust organics collected on Tenax. Since then, the number of applications and publications involving on-line SFE-GC has continued to increase [40,41]. On-line SFE-GC techniques based on the collection of the extracted analytes in the stationary phase of the capillary column have been the most successful [5,42]. The variations in this approach include depressurising the SFE effluent directly inside the GC column through a conventional on-column injection port [42], or depressurising the effluent inside a split/splitless injection port [5] as shown in **Figure 1.6** on page 24. The problem with the SFE-GC via an on-column injector is that involatile components in the extract remain at the head of the column and eventually degrades its chromatographic performance. Use of a split/splitless injector in the split mode for SFE-GC eliminates this problem as only the volatile analytes enter the column. Involatile material collects in the flash vaporiser which is easily cleaned [42]. Apart from collecting the extracted analytes directly onto the stationary phase of the capillary column, the analytes can be trapped in a precolumn unit before the column [10]. On-line SFE-GC with a split system has been investigated in this work for the analysis of the wood and bark of *Cedrela toona* (Meliaceae). The work is described in chapter three and the results discussed in chapter four.

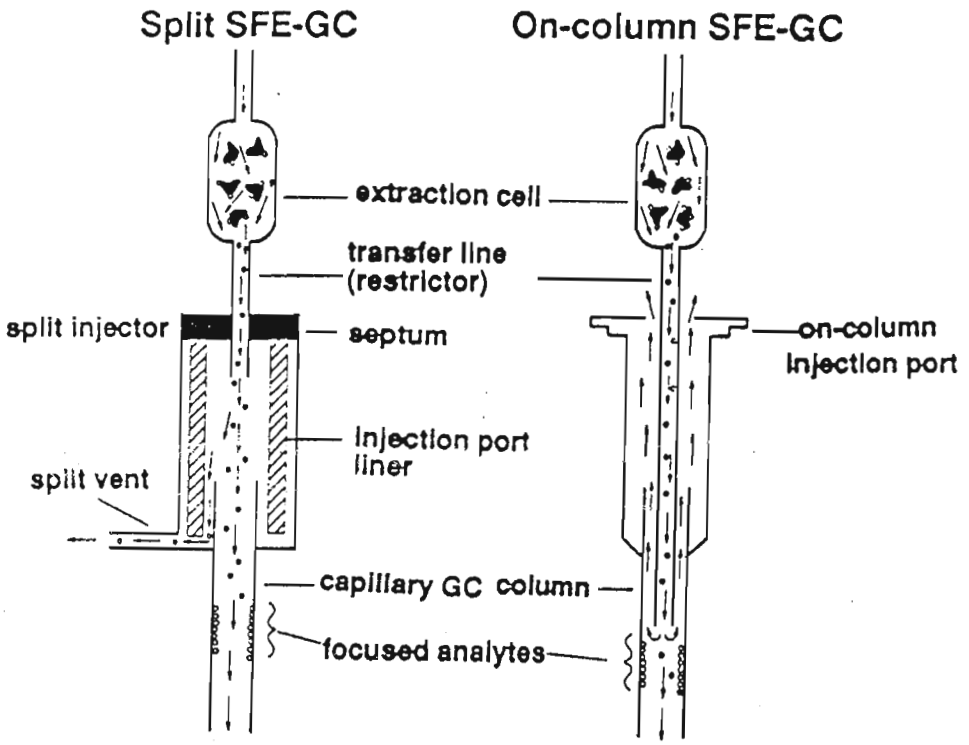



**Figure 1.6** Commonly-used methods for on-line coupling of SFE to GC.[16].

### 1.13.4 SFE-SFC

When the sample contains thermally unstable, reactive or involatile compounds, SFE-SFC is recommended. SFE can be combined with several forms of SFC, ie., with conventional packed columns (1 - 4.6 mm i.d) [22], with capillary columns (10 - 250 $\mu$m i.d) [43] and has been done more recently with packed capillary columns (200 - 530 $\mu$m i.d, 3 - 10 $\mu$m particles) [44]. On-line SFE-packed capillary SFC is an interesting development in comparison with SFE-capillary SFC, because of a higher loadability and shorter analysis time. In comparison with SFE-packed column SFC, the advantages are a lower pressure drop, higher efficiency (theoretical number of plates) and lower flow rates, resulting in an easier interfacing with FID or MS instruments. The instrumentation for SFE-SFC ranges from relatively simple systems to more complex arrangements involving switching valves and multiple pumps [45]. Most systems comprise a thermostated extraction cell, a switching valve, a cryogenically-cooled trap and a chromatographic oven housing the column, and a detector. A typical system is shown in **Figure 1.7** on page 26. The outlet of the cell is connected to a flow restrictor which is, in turn, connected to an accumulating trapping system. The trap normally comprises an uncoated deactivated fused silica retention gap housed in a cryogenically-cooled tee. However, the extract may also be trapped on a sorbent [46]. During extraction, the tee is vented to atmosphere and the extract is concentrated within the transfer line or trap. After extraction is complete, the valve is switched and supercritical fluid is introduced into the side arm of the tee to transfer the extract on to the column for separation.

An obvious advantage of SFE is that it is an ideal way to introduce a sample into an SFC system. In most cases the solvent for extraction is the same as the mobile phase for the separation, thus the criteria for a successful coupling of different techniques is fulfilled, that is the output characteristics from the first instrument and the input characteristics of the second are compatible.

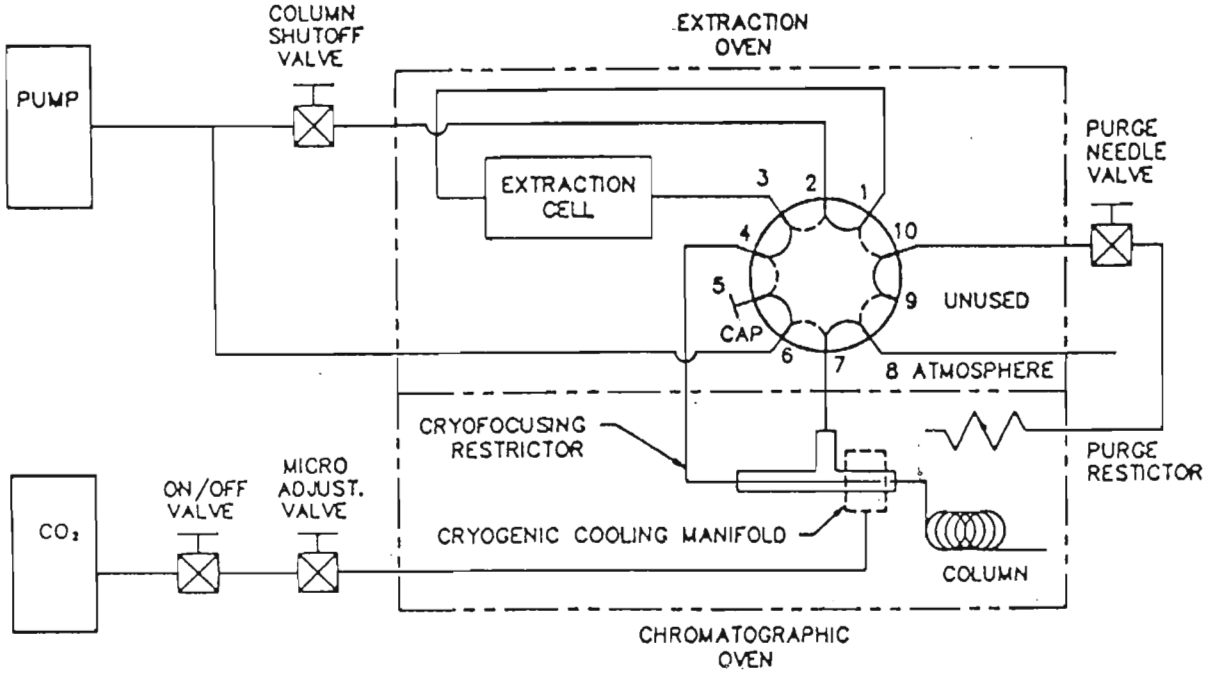



**Figure 1.7** On-line coupling of SFE to SFC using a cryogenically-cooled tee.

### 1.13.5 SFE-HPLC

To date, not much work has been reported on the on-line coupling of SFE to an HPLC system. Unger and Roumeliotis [47] described the first coupling device allowing on-line HPLC of supercritical fluid extracts. This system was used to monitor the kinetics of the extraction of valtrate from *Radix valerianae*. Nair and Huber [48] also described the on-line SFE-HPLC analysis of ground tablets for ibuprofen. The SFE unit consisted of a constant-pressure pump to transfer the $CO_2$ to a preheater, a heated vial containing the sample, a fixed volume injection valve and finally an analytical column. This system of coupling SFE directly to an HPLC system promises to be interesting particularly in the

analysis of naturally-occurring compounds from plant matrices. Unfortunately, a SFE-HPLC interface is not as straight forward as it is in the case of either GC or SFC. The main problem with this interface lies in coupling a sample preparation technique where gas is produced in the interface to a chromatographic technique with a liquid mobile phase. According to Dolan and Snyder [49], pumping of the mobile phase becomes difficult and erratic when gas is present in the plumbing of the HPLC system.

## 1.14 Sample injection techniques in gas chromatography

On page 23 a brief mention was made of the two commonly used injection techniques (split and on-column injections) employed in gas chromatography. However, in this section, both techniques will be discussed in more detail than described earlier. Sample injection in GC analysis is highly critical since poor injection techniques can reduce column resolution and the quality of quantitative data. The sample must be injected as a narrow band onto the head of the column and contain a composition truely representative of the original mixture. Also the amount of sample injected must match the capacity of the column to prevent overloading the stationary phase hence affecting the performance of the column.

### 1.14.1 Split injection

This is a method whereby the injected sample can contain components at concentrations similar to those normally used in packed column work. To prevent overloading of the column, the sample after undergoing flash vaporisation in the heated injection region, is split into two unequal portions. A larger portion is vented from the system while a smaller portion is carried onto the column. The size of the split may be 10:1 - 50:1 for large-bore open tubular columns or 50:1 - 500:1 for narrow-bore columns, and is controlled by a variable valve attached to a vent line leading away from the injection region. **Figure 1.8** on page 28 shows a design for an injector that is operated in the split mode. Although the split method of injection does prevent column overloading, the fraction which reaches the column may not be representative of the original sample. Since it is a flash vaporisation

technique, higher molecular weight components of the sample in contact with the metal surface of the syringe plunger are not expelled from the syringe with the same efficiency as compounds whose boiling points are at or below the injection temperature. Since low injection volumes are typically used for capillary columns, split descrimination may result. To prevent this, inlet liners are employed. They are designed to provide efficient heat transfer and thorough mixing to the sample to minimise discrimination.

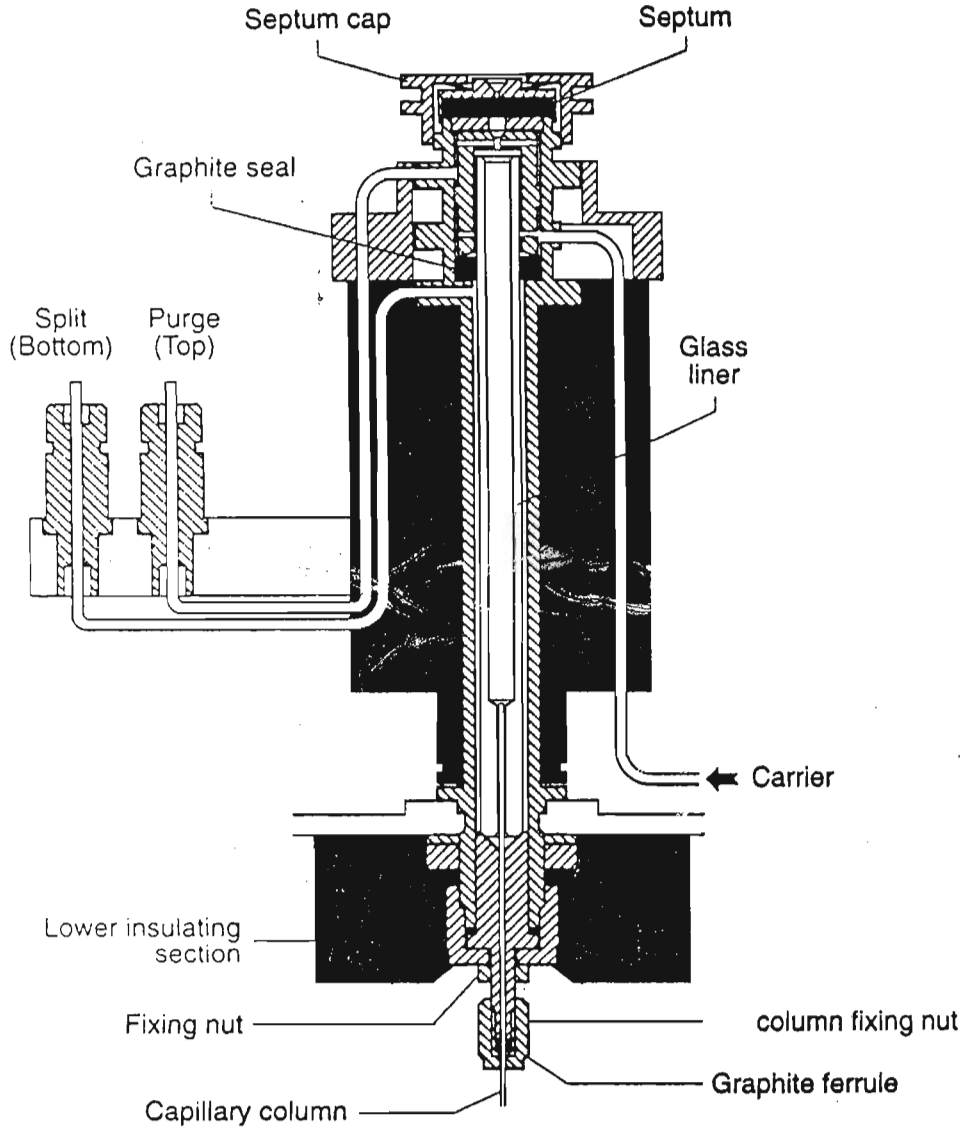



**Figure 1.8** A typical design of a split injection system: [109].

### 1.14.2 On-column injection

As described in section 1.14.1 above sample discrimination may occur as flash vaporisation is required for split injection. Also, at the injection temperatures usually employed, it is possible to observe decomposition of thermally-labile components. These problems can be overcome by injecting the sample directly into the capillary column through a cooled injection port. Small volumes of solvent can be reproducibly injected since all of the sample reaches the column. As no sample splitting is performed, quantitative recovery of high-boiling samples is possible. This method of injection for capillary columns was not popular among early injector designs due to the mechanical difficulties of aligning the syinge needle with the column. Since typical inner diameters of capillary columns are 0.25 to 0.35 mm, almost perfect needle alignment is required. This is made possible by carefully engineered adaptor kits that guide the needle into the capillary column. Injector designs which are suitable for on-column work have only recently become commercially available. A unique secondary cooling facility ensures complete sample transfer from the syringe to the capillary column and is also effective in reducing peak distortion. The comparative descrimination of the two injection methods is shown in **Table 1.4** on page 30. It is clear that each technique has its own merits and demerits. However, most of the extracts to be dicussed in later chapters were analysed by on-column manual injection using a Hamilton 10 $\mu$L syringe.

## 1.16 The mass spectrometer as an invaluable GC detector

Like the flame ionisation detector (FID), detection by the mass spectrometer involves the ionisation of molecules. However, mass spectrometry (MS) is quite different both in its operation and the mode of ionisation. If the components separated by chromatography or obtained in a pure form by any other method are injected into a high vacuum where the molecules can freely move in the evacuated space, they can be broken into their constituent fragments as ions by a stream of fast moving electrons. If the ions are separated according to their masses, then a definite pattern of the abundance of the ions at each mass will be found. This pattern known as the mass spectrum, is unique to a compound and can be used to identify the compound.

**Table 1.4.** A comparison between split and on-column injections in GC.

| | Split injection | On-column injection |
|---|---|---|
| Application | Principal components only | Trace and principal components |
| Maximum conc-centration | Depends on split ratio | 100 ng/component |
| Precision | Poor | Excellent |
| Injection temp. | 250-320°C | Injector not heated |
| Initial column temperature | Variable | Near solvent boiling point |
| Advantages | Control of split ratio prevent column overloading | "Cool injection reduces sample discrimination. Thermally unstable compounds can be analysed. Excellent precision and accuracy. Direct quantification possible. |
| Disadvantages | Possible sample discrimi-nation during split. Flash vaporisation. Indirect quantification. Poor for trace analysis. | Non-volatile components will accumulate at the head of column. Some solvents could damage certain columns. |

Hence, given the separating power of chromatography and the identifying power of mass spectrometry, a direct combination of these two techniques gives an incredible ability to identify the components of a mixture. The mixtures can come from biological tissues such as the wood and bark of plants. The wealth of information pouring out of the GC-MS

combination demands the use of a computer dedicated to specific tasks such as extraction, storing and analysis of the essential data collected. The achievements of GC-MS computer instrumentation are quite impressive, and advantage was taken of this unique ability in the analyses and identification of compounds in extracted mixtures from *Cedrela toona* (Meliaceae).

Instrumentation in GC-MS

Mass spectrometry can be divided into two separate processes, ionisation and mass separation and recording of the ions formed. A typical GC-MS instrumental design is shown in **Figure 1.9**. For the purpose of this chapter, attention will only be focussed on the mode of ionisation and the technique of mass separation. Different methods of ionisation can be combined with the different techniques of mass separation, but emphasis will be centred around electron ionisation and quadrupole mass separation.

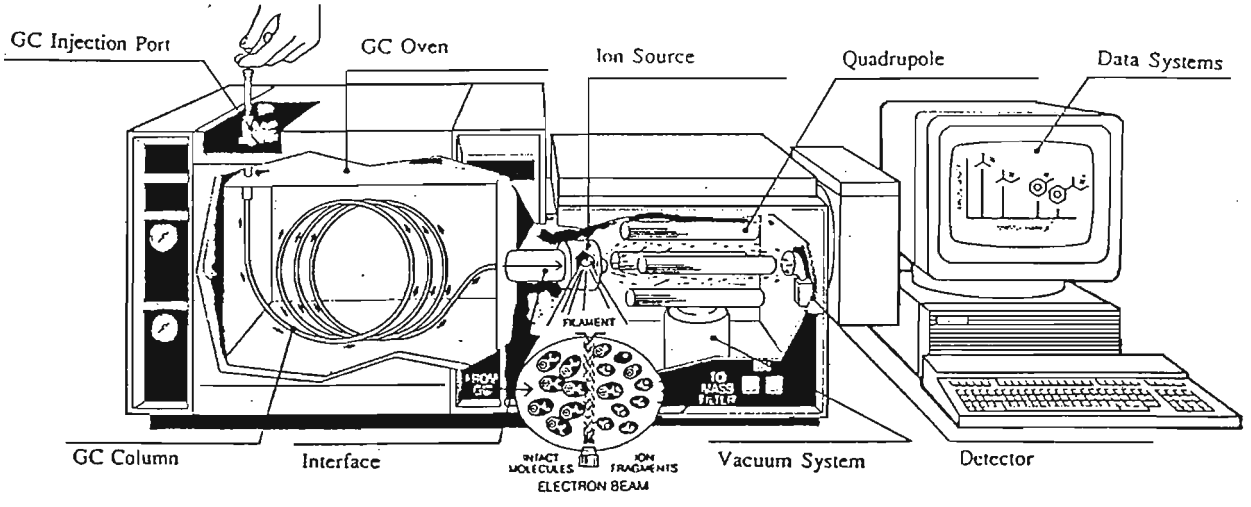



**Figure 1.9** A diagram showing a typical GC-MS instrumentation.

Ionisation techniques

Two forms of ionisation techniques are commonly employed in mass spectrometry: electron impact ionisation and chemical ionisation. The ionisation of molecules takes place in a low pressure chamber at about $10^{-5}$ to about $10^{-8}$ Torr. In electron impact ionisation, electrons from a hot wire filament will be focussed across the chamber and attracted to an electrode at a high positive voltage of about 70 Volts. This gives each electron an energy of about 70 electron Volts. When sample molecules are introduced to the ion source they are bombarded by electrons which collide with and expell an electron from the outer shell of the molecule in a very fast process. The resulting molecular ion is left with excess internal energy and starts to fragment according to different pathways, each with a fixed probability of occurence. This results in the production of all the positive ions conceivable from breaking bonds in the molecular ion. The low sample pressure in the chamber has an important consequence in producing representative and reproducible mass spectra. Because of the higher mean free path of an ion or molecule, the molecules and ions formed behave independently and do not collide with each other. This is the basis of the withdrawal and separation of ions of differnt masses without the disturbing effects of collision and reaction. However, the relative abundances of the ions formed by electron bombardment depend on the energy of the ionising electrons and the temperature at which ionisation occurs. Ion formation can be viewed as a reaction between an energetic electron and a molecule in which the product molecular ion is left with excess internal energy. The molecular ion therefore decomposes through a series of sequential reactions producing various ions and neutral fragments. The extent of these reactions depends on the temperature at which they occur and therefore the relative abundances of ions so produced in the mass spectrum is temperature dependent. Therefore comparison of unknown mass spectra with published spectra must take into account the temperature at which each was produced. The disadvantage of using electron impact ionisation is that many molecules do not have stable molecular ions and as a result the relative abundance of the molecular ion may be so small that positive identification cannot be made. To complement the structural information obtained from electron impact mass spectrometry, a soft ionisation technique can be employed to gain molecular weight information.

Quadrupole mass analysers

The quadrupole mass analyser is known for its high sensitivity and the ability to scan rapidly at millisecond intervals at variable resolution. These qualities are well suited for coupling with a gas chromatograph particularly when open tubular capillary columns with their narrow peaks are used. In the quadrupole mass spectrometer (example of which is shown in **Figure 1.9** on page 31) ion separation is accomplished by passing the ion beam through the centre of four rods to which voltage is applied. The theory of mass separation in quadrupole mass analysers is based on hyperbolic electric fields, ideally formed by conducting surfaces of the same geometry. However, it is difficult to manufacture rods with hyperbolic surfaces, but some quadrupole mass spectrometers use round rods to simulate a hyperbolic electrical field. The separation of the masses is accomplished by simultaneously applying both a direct current (dc) and a radiofrequency (RF) alternating current voltage to the rods. Mass resolution is determined by the ratio of the dc to RF voltage. Resolution is normally adjusted so that only an ion with a particular nominal mass to charge ratio will pass selectively through the rods to the detector. To obtain a mass scan, the dc and RF voltages are varied while maintaining a constant dc/RF ratio.

# CHAPTER TWO
# APPLICATION OF SFE TO NATURAL PRODUCTS
# LITERATURE REVIEW

## 2.1 Introduction

In view of increasing environmental concerns about the use of liquid solvents in the extraction of natural products, there has been growing interest in alternative and reliable sample extraction techniques using supercritical fluids. Supercritical fluids have been used for many years for the selective extraction of selected compounds from bulk samples. The extraction of caffeine from coffee and bitter acids from hops are well known processes performed on an industrial scale. In the former case, the aim is to remove a specific component (ie. caffeine) from large quantities of the bulk matrix to increase its commercial value. In the latter case, the reverse is true and SFE is used to extract specific compounds of high commercial value (bitter acids) from the bulk hops matrix. Analytical-scale supercritical fluid extraction is concerned more with the extraction of analytes of interest from a bulk matrix as a sample preparation step prior to their characterisation by other analytical methods such as chromatographic, spectrometric, spectroscopic and gravimetric techniques. It is therefore potentially very useful for the extraction of natural products prior to structural characterisation.

SFE is gaining acceptance as an alternative to Soxhlet extraction. Much of the current interest in using analytical-scale SFE stems from the need to replace conventional liquid extraction methods with sample preparation methods that are more efficient, easier to automate, faster and safer to use. Many of the properties of supercritical fluids such as carbon dioxide have facilitated advances in these areas [50,51]. Thermally-labile compounds can be extracted at low temperatures. In general, the transport properties of supercritical fluids (high diffusivity and low viscosity) favour high mass transfer rates resulting in improved extraction efficiency and reduced extraction times. By selecting the fluid polarity and/or density, the solvating power of the fluid can be adjusted enabling the

possibility of class-selective extraction. Carbon dioxide is a non-toxic and relatively cheap extraction fluid which can be easily removed from the extraction components by decompression into a suitable collection device. Extracts can also be analysed on-line by coupling the SFE directly with another analytical instrument such as a gas chromatograph [39], supercritical fluid chromatograph [45], Fourier transform infrared spectrometer [34] etc.

Up to now, much of the published work on analytical SFE has been primarily concerned with environmental applications such as total petroleum hydrocarbons [52], pesticide residues [24], polycyclic aromatic hydrocarbons [1], polychlorinated biphenyls [41] and chlorinated dioxins [4] in soils and sediments. This chapter reviews the successes and failures of supercritical fluids in the extraction of natural products, and has been divided into a number of natural product categories. Reference is made to both off-line and on-line analytical SFE methodologies.

## 2.2 Triterpenoids

Triterpenoids and sterols are an important group of compounds which have been investigated to a limited degree using analytical SFE. Most of the work has concentrated on moderately-polar steroids such as cholesterol, stigmasterol, testosterone and cortisone. Wong and Johnstone [53] obtained solubility and vapour pressure data for the biomolecules cholesterol, stigmasterol and ergosterol in supercritical carbon dioxide with and without a modifier. Although these sterols have similar structures, the solubility of cholesterol in carbon dioxide at 35°C and 257 atm was approximately three times greater than that of stigmasterol and 50 times that of ergosterol. When supercritical carbon dioxide was modified with several mole percent of methanol or ethanol the solubility of the sterols increased by up to two orders of magnitude. These studies show that from a qualitative point of view, it is possible to isolate these sterols from their natural sources using SFE. In studies using ascending pressure profile extraction, Bradley showed that milk fat contained in the extraction vessel was stripped of 90% of its cholesterol using

supercritical carbon dioxide [54]. Ong et al.[55,56], also extracted and analysed cholesterol from egg yolk and blood serum using supercritical $CO_2$ at 45°C and 177 atm for about 60 minutes. The results were comparable to classical methods for cholesterol analysis.

Young and Games [46], used supercritical fluid extraction and chromatography with carbon dioxide to isolate the fungal metabolite ergosterol from flour, mouldy bread and mushroom caps. SFE was performed at 40°C at a density of 0.90g/ml with sorbent trapping using an octadecylsilyl (ODS) material. The ergosterol was washed off the sorbent with methanol and chromatographed by packed column SFC on a Spherisorb amino 3 $\mu$m column using $CO_2$ with 10% methanol and a UV detector at 282 nm. Observed levels of ergosterol ranged from 0.08 $\mu$g/g in cake flour to 14.3 mg/g in freeze-dried mushroom caps. The authors suggested the possibility of this technique being a fast laboratory method to determine levels of fungal infection in foodstuffs.

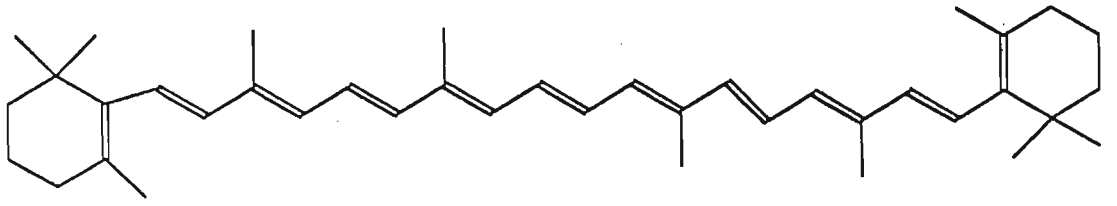
Li et al. [57], utilised supercritical Freon-22 for the extraction and chromatography of the steroids estrone, testosterone, estriol, cortisone, methyl-testosterone and hydro-cortisone from spiked glass wool and compared these results with those obtained using carbon dioxide as a fluid. Extractions were performed with $CO_2$ at 50°C for 30 minutes and with Freon-22 at 100°C for 15 minutes using pressures up to 180 atm. The extraction efficiency with Freon-22 was significantly better than that with $CO_2$, however, Freons are not recommended as extraction fluids due to their adverse environmental effects.

Many triterpenoids are also present in the wood and bark of certain plants. Joo and co-workers [3] have reported the extraction of various phytochemicals from plant materials in Korea using $CO_2$ at 40°C. They found a significant amount of the triterpene $\alpha$-amyrin acetate in the bark of the mulberry tree *Morus alba* (Moraceae). As will be seen in chapter three, three phytosterols were extracted and identified from the bark of *Cedrela toona* (Meliaceae) by GC-MS.

## 2.3 Carotenoids

Carotenoid compounds have become a focus of many studies in SFE firstly because of their possible role in reducing the incidence of certain cancers in humans, secondly because of the provitamin A activity of ß-carotene and thirdly because of the anti-oxidant activity of ß-carotene [60]. Favati et al.[58] utilised supercritical carbon dioxide to extract carotene and lutein from alfalfa leaf protein concentrate (LPC) using pressures of 100-700 atm at 40°C and gaseous flow rates of 5-6 litres/min. Over 90% of the carotene contained in LPC was removed at extraction pressures in excess of 300 atm. However, the removal of lutein from the LPC required higher extraction pressures (700 atm) to attain 70% recovery. Extractions were run on 45-50 g samples of LPC and chromatographic analysis was performed using HPLC. SFE provided the possibility of obtaining a selective extraction of natural colourants free of solvent residuals for food dyes. Tonucci and Beecher [59] also used supercritical carbon dioxide with various modifiers for the analytical extraction of carotenoids from foodstuffs, specifically, tomato paste, canned pumpkin, spinach, red palm oil, butter and cheese. Static and dynamic extractions were investigated using carbon dioxide modified with 1% methanol, ethanol or isopropanol at pressures of 136-680 atm and temperatures of 40, 55 and 70°C. HPLC was used to confirm the presence of specific carotenoids including lycopene. Extraction yields were reported to be poorest for tomato paste (24%), although a 78% yield could be obtained by using modified carbon dioxide. The carotenoids were almost completely extracted from high fat foods (96% yield), however some of the fat was also co-extracted.

Spanos et al. [60] reported the extraction of ß-carotene from sweet potatoes using carbon dioxide at 41°C and 414 atm. They found that the extract obtained contained about 94% ß-carotene but that the yield varied depending on the moisture content, the method of sample dehydration and the particle size. Extraction from ground potato gave around 44 $\mu$g of ß-carotene per gram of fresh sample. Oven drying of the sample prior to extraction resulted in an increase in carotenoid extracted (77 $\mu$g/g dried sample). The highest yield obtained was 228 $\mu$g/g for the freeze-dried sample of the ground potato. The structure of ß-carotene is shown on the next page.



**ß-carotene**

Marsili and Callahan [61] compared SFE using carbon dioxide with classical solvent extraction using ethanol/pentane for the extraction of carotenes from a number of vegetable samples. The SFE method proved favourable in terms of both extraction yield and speed. Lorenzo et al. [62] and Subra and Boissinot [63] investigated the SFE of carotenoids from algae. Subra and Boissinot used a controlled step-wise pressure gradient with supercritical carbon dioxide on the Miditerranean brown algae *Dilophus ligulatus*. This algae exhibits a wide range of anti-fungal activities. The pressure was stepped from

80 to 100, 150, 200 and 250 atm at temperatures of 35, 45 and 55°C. Different fractions in terms of colour and composition were obtained depending on the density of the fluid. Analysis by high performance thin layer chromatography and HPLC showed the presence of ß-carotene and many unidentified components.

## 2.4 Flavour, fragrance and other terpenoid compounds

SFE has been most successful for the isolation of plant volatiles and other terpenoids as indicated by the number of publications in this field. In 1983, Stahl and Keller [64] utilised supercritical carbon dioxide to extract the sesquiterpenoid bitter principles, calamus, acorone and isoacorone from *Acorus calamus*. These compounds are thermally

unstable and partially decomposed using steam distillation. High pressure extraction with carbon dioxide at 90 atm and 40°C eliminated decomposition problems and improved the yield of the bitter compounds. Pellerin [65] compared the extraction of lavandin flowers by supercritical $CO_2$, steam distillation and Soxhlet extraction using hexane and ethanol. SFE gave a similar distribution of components in the extract to those obtained by liquid extraction, but at higher yields. The SFE conditions were not reported. Steam distillation resulted in compounds such as myrcene and *trans*-ocimen indicating that hydrolysis of some of the extract components had occurred in this process. A high level of linalool due to the hydrolysis of linanyl acetate was also identified in the distilled essential oil. Work reported by Barton et al. [66] on the extraction of volatile compounds from *Mentha piperita* and a closely related species designated "spearmint" resulted in similar conclusions to those of Pellerin. The flavour and fragrance of the $CO_2$ mint extracts (60-180 atm, 24-43°C, 4-9 hours) were closer in quality to actual mint leaves compared with mint oils extracted by conventional steam distillation. Simandi et al. [67] also reported SFE of the leaves of *Mentha piperita* and obtained similar results to Barton et al. [66]. The SFE extract was found to contain more of the fragrance characteristic compound menthofuran than the distilled oil. In addition, they extracted the flowers of *Lavandula intermedia* using a step-wise pressure programme. Extraction yields from *Lavandula* by SFE were 84-128% of those obtained by steam distillation depending on extraction pressure. The high levels of linalool in the distilled oil resulting from the decomposition of natural constituents was in agreement with the results of Pellerin [65]. These studies appear to be a major breakthrough in the flavour and fragrance industry since peppermint and spearmint are popular flavours used in a variety of sugar confectioneries, chewing gums, toothpaste, pharmaceuticals and other applications. It is clear that the most natural and true-tasting extracts are obtained by techniques which utilise low temperature and avoid degradative heat processes and reactive solvents.

Moyler [68] has reviewed the published literature of $CO_2$ extraction yields, major components, odour profile, minimum perceptible threshold, major flavour and fragrance applications of 42 natural botanical extracts. 228 references cover the essential oils from

ambrette seed, angelica seed, aniseed, anise star, basil, capsicum, caraway, cardamom, carob, carrot root and seed, cassia bark, cinnamon, pepper, vanilla and many others. SFE has been reported to be very useful for the selective extraction of a number of essential components from hops. Verschuere et al.[69] utilised SFE with carbon dioxide for the selective extraction of the essential oils myrcene, caryophyllene, humulene and the $\alpha$ and ß bitter acids of hops, *Humulus lupulus*. At a density of 0.2 g/ml and a temperature of 50°C, 98% of myrcene, 91% of humulene and 95% of ß-caryophyllene were consequently extracted selectively in the first 15 minutes with no bitter acid components. The bitter acids became soluble in the $CO_2$ at 0.25 g/ml and were completely extracted at about 0.9 g/ml. The essential oils were analysed by capillary GC and the bitter acid fractions by micro-liquid chromatography and miceller electrokinetic capillary chromatography (MECC). Langezaal et al.[70] also used supercritical $CO_2$ in the extraction of hop components and their results were in complete agreement with those of Verschuere et al. [69]. Among the combination of parameters tested, that of 40°C and 200 atm was found to be optimum for the extraction of both the bitter components and the volatiles [70].

SFE has been applied to the extraction of a number of herbs, many of which have medicinal properties. Ma et al. [71] used analytical SFE with $CO_2$ at 200 atm and 50°C followed by GC-MS to separate and determine the volatile components in various herbs used traditionally in Chinese medicine. Three kinds of herbs, frankincense, myrrh and *Evodia rutaecarpa* were extracted and analysed. The GC-MS profile in **Figure 2.1** on page 41 showed that the myrrh extract contained large amounts of sesquiterpenes.

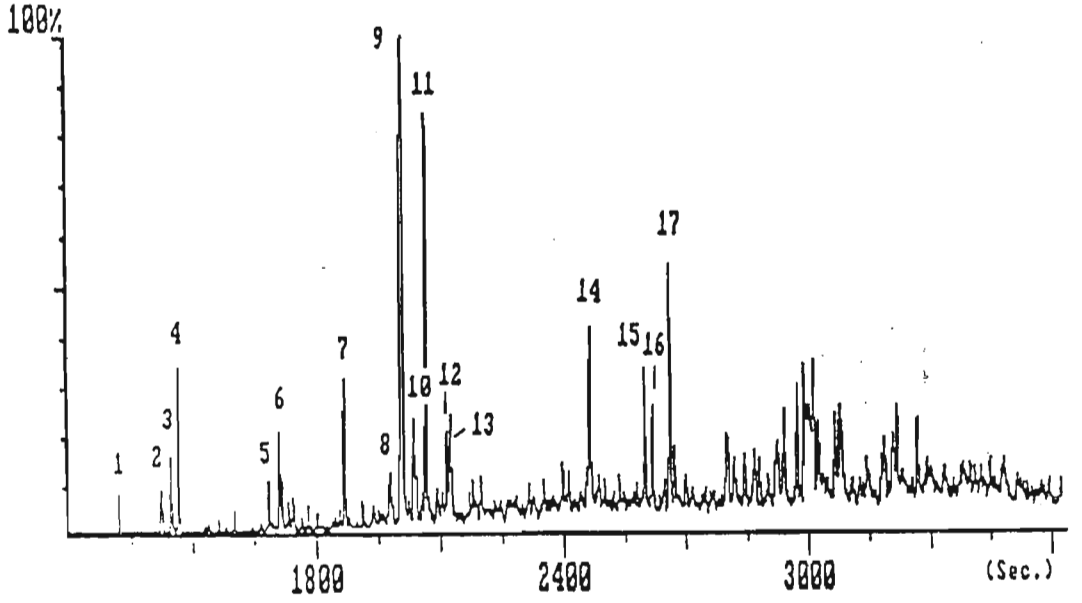



**Figure 2.1** GC/MS total ion chromatogram of a $CO_2$ extract (200 atm, 50°C) from myrrh according to Ma et al. Some of the compounds numbered are: 1 = δ-elemene; 4 = ß-elemene; 7 = elemol; 9 = curzerenone; 12 = ß-eudesmol; 13 = α-eudesmol. [71].

Among the approximately 150 components separated from the *Evodia* extract, there were four main groups of components: monoterpenes; sesquiterpenes; diterpenes and aliphatic hydrocarbons. The results revealed the potential of SFE as an analytical tool for the study of medicinal plants. Smith and Burford [72] utilised SFE and gas chromatographic techniques for the determination of the sesquiterpene lactone, parthenolide, from the medicinal herb feverfew, *Tanacetum parthenium*. SFE conditions were 250 atm, 45°C at 0.85 ml/min fluid flow rate. Extracts were collected in a cryogenically-cooled flask at -17°C using liquid nitrogen. The addition of methanol or acetonitrile as a modifier to the

carbon dioxide gave increased yields of parthenolide, but less selectivity. The extraction conditions and sample collection techniques were based on the optimum conditions obtained from an initial study of the extraction of a range of terpenoids including the sesquiterpene lactone, santonin, from a model plant matrix [73]. Manninen et al.[74] used SFE and SFC with $CO_2$ for the extraction and analysis of some of the commonly used herbal drugs in Finland, *Matricaria recutila*, *Thymus vulgaris* (thyme), *Ocimum basilicum* (basil), *Mentha piperita* (peppermint), and *Dracocephalum moldavica* (dragonhead).

Hawthorne et al. [2] developed a method for the on-line extraction and analysis of flavour and fragrance compounds by SFE coupled to a gas chromatograph. Extracted components were transferred directly from the SFE cell into the gas chromatographic column via an on-column injector. This method was demonstrated on a variety of samples including herbs, spices, orange peel and spruce needles. The on-line SFE-GC results were comparable to the off-line analysis of a variety of SFE extracts which were injected manually into the GC as shown in **Figure 2.2** on page 43.

In the SFE-GC analysis of thyme, the herb was extracted with $CO_2$ at 80 atm, 45°C and the extract was transferred into the GC column where it was cryogenically trapped at -30°C. Flame ionisation and mass spectrometric detection were used to identify compounds such as borneol, thymol and carvacrol. This method has a number of advantages; rapid extraction with high sensitivity and elimination of sample handling with associated possible contamination.

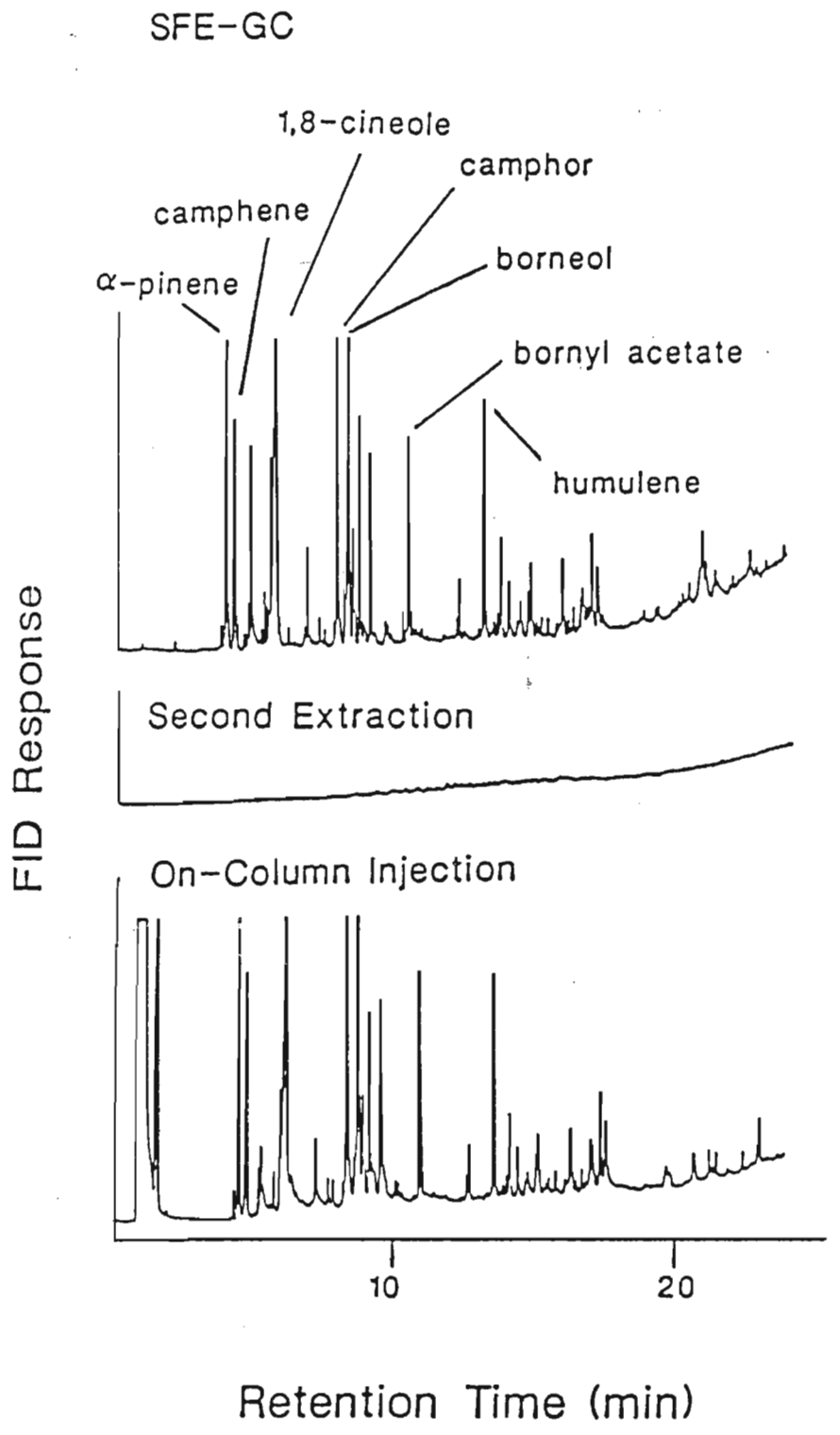
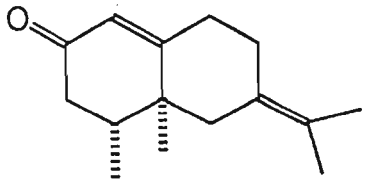
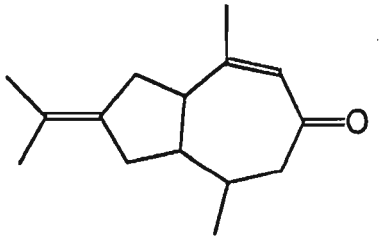



**Figure 2.2** Comparison of chromatograms generated by on-line SFE-GC-FID analysis of rosemary and standard on-column injection of a methylene chloride extract according to Hawthorne et al. The middle chromatogram shows the result of a second SFE-GC-FID analysis of the same sample. [2].

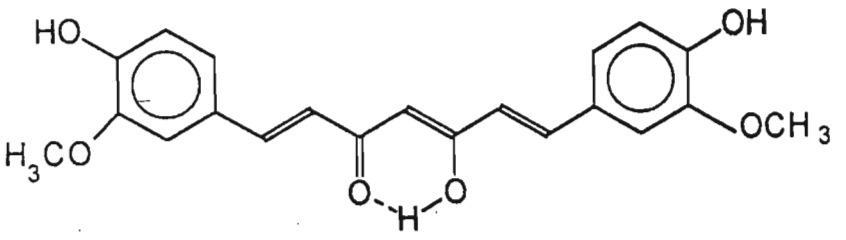
Sanagi et al. [75] used SFE and SFC in the analysis of tumeric, the ground rhizome of the plant *Curcuma longa*, a species of the Zingiberaceae family, which is native to southern Asia. It is valued for its yellow-colouring components, its use as a spice as well as being an ingredient in traditional medications and cosmetics. The main coloring materials in tumeric and its oleoresin are curcumin and two related minor curcuminoids, demethoxycurcumin and bisdemethoxycurcumin. Tumeric also yields about 5% of an orange-yellow, volatile oil composed mainly of the sesquiterpenes turmerone and artumerone. In the SFE of turmeric, pure $CO_2$ at 60°C and 250 atm removed the low polarity components. However, $CO_2$ modified with 20% methanol was required to extract the more polar curcuminoids and under optimised conditions, gave more than 90% recovery of curcumin, the major component. Structures of the main compounds extracted from tumeric are shown on the next page. Blatt and Ciola [76] used SFE and GC in the analysis of essential oils from the roots of the vetiver plant *Vetivera zizaniodes*. GC-MS was used in the identification of compounds including $\alpha$ and ß-vetivones whose structures are shown below.



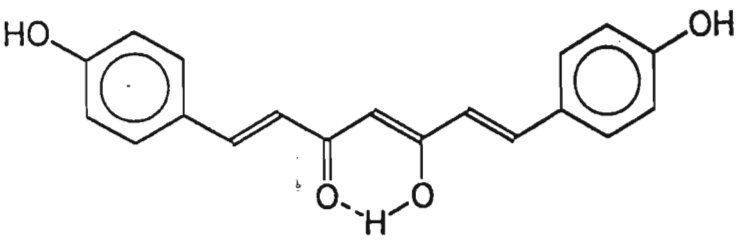

alpha-vetivone



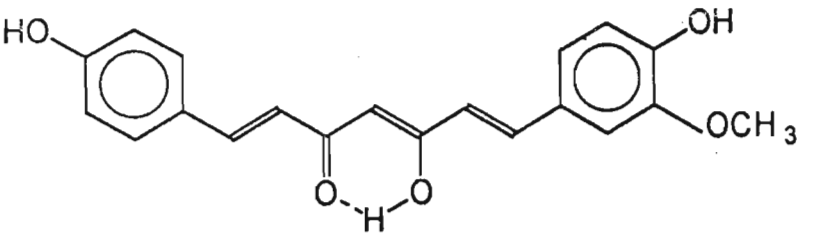

beta-vetivone




curcumin




bisdemethoxycurcumin



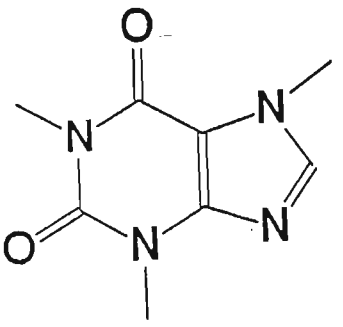

demethoxycurcumin

Several researchers have investigated SFE for analysis of aroma compounds in fruits. Kerrola et al. [77] used on-line SFE-GC in the analysis of carvone and limonene in caraway fruits of various origins. Extractions were performed at 97 atm and 50°C.

Polesello et al. [78] also investigated the possibility of SFE as a preparative tool for strawberry aroma analysis. From their findings, it was possible to recover the majority of the aroma compounds which were also obtained by the classical extraction method. Fractionation of lemon peel oil by SFE-preparative SFC has also been investigated by Yamauchi et al. [79] and Sugiyama et al. [80]. The SFE results indicated that $CO_2$ extracts were similar in composition to cold-pressed oil, but contained less limonene, 10 times the concentration of alcohols and 3-5 times the concentration of linanyl acetate. Four separate fractions were collected after injection of the extract onto the preparative SFC. The fractions were analysed by GC-MS. Fraction 1 contained mainly limonene, ß-pinene and gamma-terpinene. Fraction 2 contained mainly neryl acetate and geranyl acetate. Fraction 3 contained geranial, neral and terpineol as well as small amounts of other aldehydes and alcohols. Fraction 4 comprised of higher molecular weight aromatic compounds.

## 2.5 Alkaloids

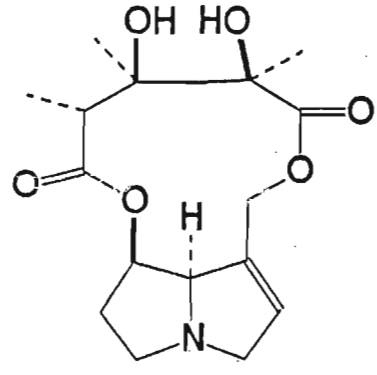
SFE has been used quite extensively for the extraction of alkaloids on both analytical and industrial scales. Those studies focusing on analytical SFE will be dealt with in this section. Certain classes of alkaloids have been extensively investigated using SFE, many others are yet to be investigated. The most common alkaloid which has been extracted is caffeine whose structure is shown on the next page.




caffeine

Elisabeth et al. [81] isolated caffeine from coffee powder using SFE and preparative supercritical fluid chromatography. 2.0 g of roasted coffee powder gave 0.49 g of extract using $CO_2$ at 40°C and 196 atm as the extraction fluid. Structural confirmation was by FTIR and FT-NMR. Sugiyama et al. [22] demonstrated directly coupled SFE-SFC for the extraction and analysis of green coffee beans and showed that various compounds could be extracted including caffeine. They also investigated extraction conditions such as pressure, temperature, water content and extraction time for caffeine extraction by off-line SFE-HPLC. After extraction of the powdered beans (with 20% added water) at 200 atm and 40°C for 15 minutes, extracts were chromatographed on a C-18 HPLC column. Caffeine was detected with a multi-wavelength UV detector. Ndiomu and Simpson [82] compared SFE with liquid extraction for the isolation of caffeine from kolanuts. With $CO_2$ at 60°C and 1050 psi (ca 70 atm) (0.160 g/ml) an extraction yield of 53.7% caffeine was reported, whereas with tetrahydrofuran and methanol, yields of 99.0% and 99.4%

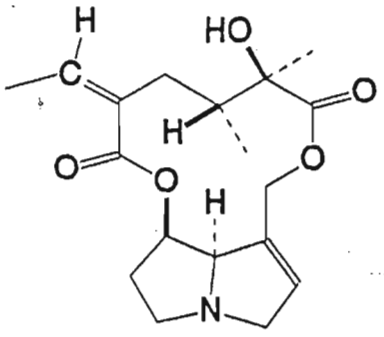
respectively were reported. Extract analysis was by reverse phase HPLC with UV detection. The low SFE recoveries were attributed to two factors. Firstly, the extraction was carried out at a comparatively low density although the system was operated at its maximum pressure and secondly, the kolanuts were dried before extraction. The presence of water in the sample matrix has been reported to improve the extraction yield of alkaloids [83]. Schaeffer et al. [26,84] successfully extracted monocrotaline, a hepatoxic pyrrolizidine alkaloid from the seeds of *Crotalaria spectabilis* using supercritical carbon dioxide with 5-10 mol % ethanol at 103.4 atm and 221.5 atm and 35-55°C. The structure of monocrotaline is shown below.



monocrotaline

The presence of highly soluble lipid material in the seeds resulted in a low monocrotaline percentage in the extracts (approximately 24% m/m). To improve the yield, they incorporated a cation-exchange resin trap after the SFE to selectively trap the alkaloid in preference to the co-extracted lipid material. The reported yield of monocrotaline using this technique increased to 95% m/m, and this indicates that the method could be attractive for the extraction of other alkaloids, drugs and other biological compounds.

Bicchi et al. [85] employed off-line SFE-capillary GC analysis for the isolation of pyrrolizidine alkaloids including senecionine and seneciphylline from two *Senecio* species, *Senecio cordatus* and *Senecio inaequidens*. SFE was performed on a home-made apparatus using methanol-carbon dioxide as the extraction fluid at 55°C and 150 atm. The structure of senecionine is shown below.




senecionine

Comparing the SFE extraction to the classical methanol extraction, SFE required a smaller amount of sample and gave a quicker extraction, a simplified fraction clean-up and a higher recovery. A capillary GC/FID separation of an SFE extract of *Senecio cordatus* is shown in **Figure 2.3** on the next page.

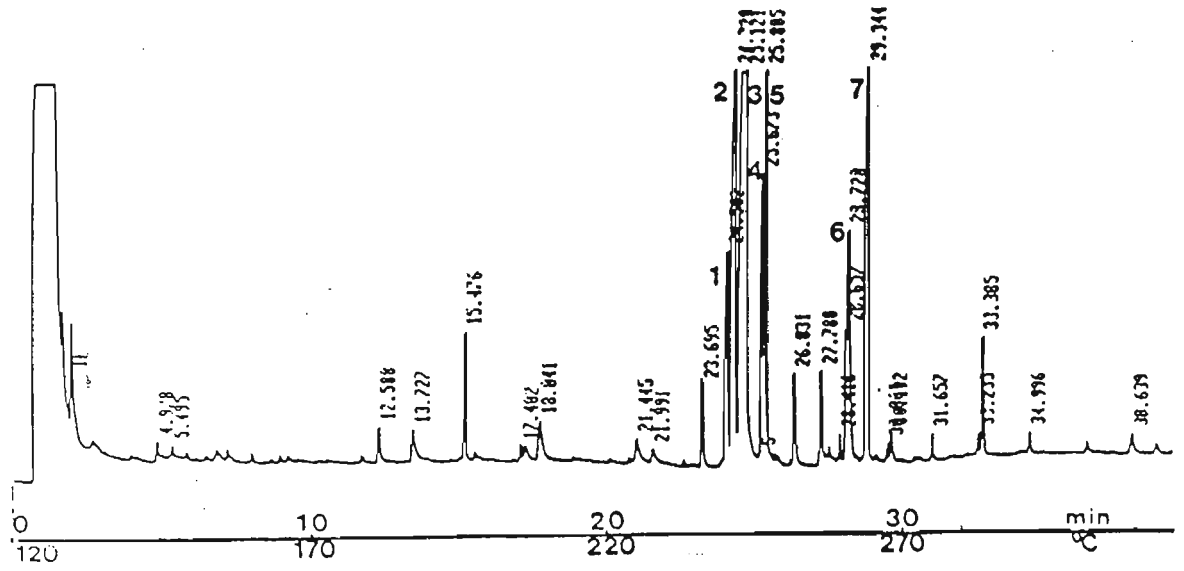



**Figure 2.3** Capillary GC/FID pattern of Senecio cordatus pyrrolizidine alkaloid fractions extracted by off-line supercritical fluid extraction according to Bicchi et al. 1 = senecivernine; 2 = senecionine; 7 = jacozine; 3 = seneciphylline. [85].

Janicot et al. [83] attempted to extract the alkaloids, thebaine, codeine and morphine from poppy straw using $CO_2$ at 200 atm and 40.5°C but were unsuccessful. Therefore the influence of various polar modifiers were considered. Kinetic extraction curves for morphine showed that 50% methanol in $CO_2$ was necessary to achieve quantitative yields in less than 20 minutes. A mixture of 25% methanol, 0.22% methylamine and 0.34% water had the same effect as 50% methanol in the carbon dioxide. However, the authors reported that in spite of its strong extraction power, the methylamine-water mixture had a major drawback. They found that morphine in the presence of the amine degraded in

the presence of light. Hence, $CO_2$-methanol-water mixtures were investigated. Increasing the water content in the extraction fluid dramatically enhanced the extraction rate for thebaine as shown below in **Figure 2.4.**

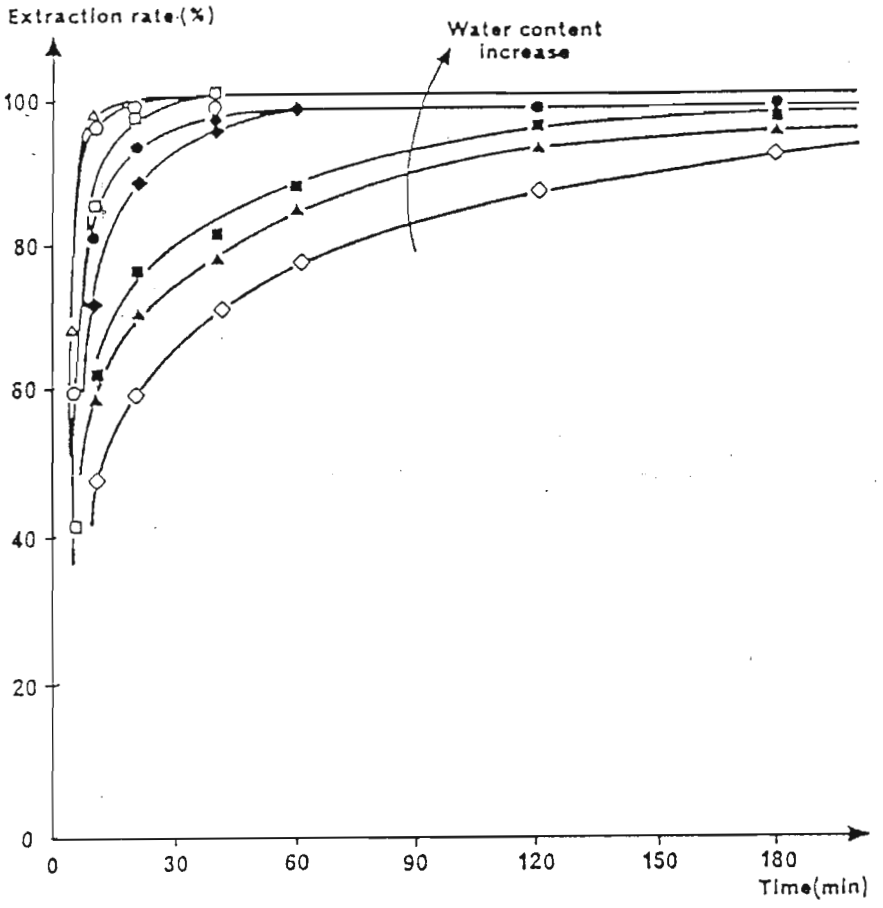
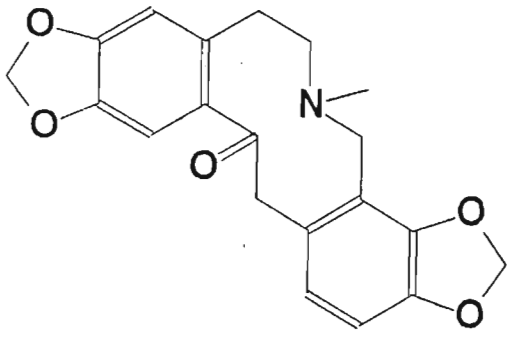



**Figure 2.4** Influence of water on the extraction curves of thebaine at constant mass flow-rate according to Janicot et al.. SFE was performed at 200 atm and 40.5°C using $CO_2$-methanol-water mixtures. Maximum water content was 18 % w/w. The ratios of $CO_2$ - methanol-water content in the direction of the arrow are: 50:50:0; 50:49.5:0.5; 50:49:1; 50:46:4; 50:44:6; 50:40:10; 50:36:14; 50:32:18 w/w/w. [83].

Sharma et al. [86] used supercritical $CO_2$ at 60°C and 544 atm for the extraction of moist snuff over a 20 minute period. A variety of compounds including nicotine were identified in the extracts by GC/FID and GC/MS. When 1.0 ml of methanol was directly added to the snuff before commencement of the SFE, compounds such as benzyl alcohol, benzothiazole and cotinine were identified. These compounds were not present in extracts obtained using pure $CO_2$. Bugatti et al. [87] used supercritical $CO_2$ in the extraction of the isoquinoline alkaloids, O-methylcariachine, protopine, α-allocryptopine, escolzine, californidine, sanguinorine and chelerythine from the aerial parts of *Eschscholtzia californica* (papaveraceae). Extractions were performed at 40°C using pressures from 80 to 300 atm. A reverse phase HPLC with UV photodiode detector was used for the identification of components in the SFE fractions. The structure of protopine is shown below.




**protopine**

Queckenberg and Frahm [88] reported the extraction of a range of alkaloids from Amaryllidaceae plants using modified $N_2O$ fluids. SFE enabled a fast, simple and selective isolation of the alkaloid fractions including the phenanthridone class and was found to be superior to the classical liquid extraction. Although there appears to be a growing interest in the use of SFE for the isolation of alkaloids, more research in its application to other classes of alkaloids is still required before it becomes universally accepted as an alternative sample preparation method. Morrison and MacCrehan [89] recently demonstrated the possibility of using SFE as a screening tool in forensic investigations by extracting cocaine from human hair. While urine analysis can detect only relatively recent drug exposure, hair analysis has the potential for providing long-term information about drug usage. A variety of $CO_2$-modifier mixtures were investigated as fluids. The best recoveries were obtained by spiking the extraction vessel with 100 $\mu$L of a 85:15 v/v mixture of water/triethylamine or 100$\mu$L of a 76:10:14 v/v mixture of methanol/water/triethylamine and extracting the hair sample with $CO_2$ at 400 atm and 110°C for 10 minutes statically followed by a 15 minute dynamic extraction period. The extracts were analysed by capillary GC with a nitrogen phosphorus detector using n-propylbenzoylecgonine as internal standard.

## 2.6 Lipid materials

Pure and modified supercritical carbon dioxide are also suitable extraction media for many lipid materials such as vegetable oils, fatty acids, fatty acid esters, phospholipids and tocopherols. In early reports of vegetable oil extraction by SFE the method showed promise for industrial scale preparations. Friedrich et al.[90] extracted oil from 1 kg samples of soyabeans using supercritical $CO_2$ at 50°C and pressures of 200-680 atm. The extracted oil was lighter in colour and was also found to contain less iron and about one-tenth the phosphorus content obtained by hexane extraction on the same sample. In terms of yield, SFE gave 18.3% and hexane gave 19.0% oil. Other quality indicators such as peroxide value and unsaponifiables were similar. Christianson et al.[91] also used supercritical carbon dioxide on dry-milled corn germ at 340-540 atm and 50°C for the extraction of oil. The oil was found to be lower in free fatty acids and refining loss and

was lighter in colour compared with commercial expeller-milled oil. Total unsaponifiable and tocopherol contents were similar in both oil types. In both of these studies, the extraction efficiency was dependent on the contact time of $CO_2$ with the sample matrix, size of the ground seeds and also the pressure and temperature of extraction.
Supercritical carbon dioxide has shown advantages for the extraction of oils from grape seeds. A 3 hour extraction with $CO_2$ at 350 atm and 40°C gave similar yields to a 20 hour Soxhlet extraction with hexane, according to Gomez et al. [92]. The SFE extract had a lower percentage of free fatty acids and unsaponifiables than the solvent extract and did not require degumming or alkali refining.

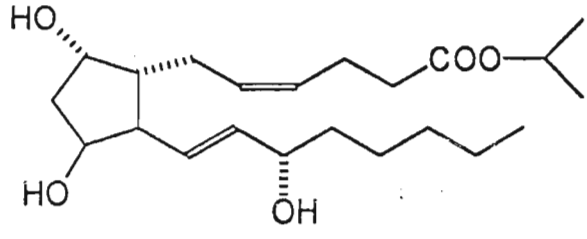
Off-line SFE with GC analysis of extracts has been used to study the extraction of triglycerides from tea seeds by Swift et al. [93]. Pharmaceutically, tea oil is used in Asian countries as a vehicle for long-acting injections and is of current interest for the development of an antimalarial injection containing tea seed oil and artimisinine. Lower molecular weight components were extracted at 60°C and 93 atm whereas larger amounts of triglycerides were extracted at 350 atm using $CO_2$.

The use of SFE in tandem with SFC and other instrumental techniques has been reported more recently for the qualitative and quantitative analysis of lipid materials.
Tocopherols in wheat germ have been extensively studied by Saito et al. [94,95]. Using an analytical SFE-SFC system, 3 g of wheat germ containing approximately 0.03% tocopherols and 10% oil by weight was extracted at 250 atm and 40°C. On-line separation of tocopherols was achieved on a 20 cm x 20 mm i.d. silica packed column using supercritical carbon dioxide with ethanol modifier as mobile phase. The eluent was monitored with a multi-wavelength UV detector and the tocopherol content in each fraction collected was determined.

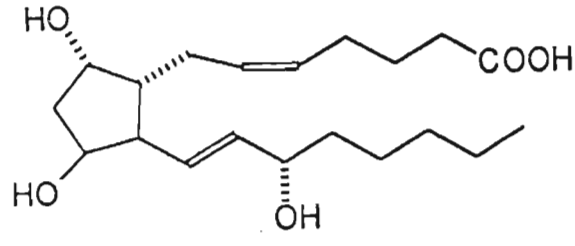
α-Tocopherol was extracted and analysed by Ikushima et al. [96] using on-line SFE-FTIR. Extractions were performed with $CO_2$ at 186, 117 and 83 atm at 50°C. A heated transfer line was used to transfer the extracts to a specially designed high pressure flow cell with

a Ga-Gd-garnet windows housed in the FTIR spectrometer in order to obtain infrared spectral information and quantification of α-tocopherol using absorbance for the characteristic band at 1053 $cm^{-1}$.

SFE-packed column SFC has been investigated by Sugiyama et al.[97] for the determination of lipid peroxide levels in foods and fats. They found good agreement between the SFE-SFC results and those previously obtained by potentiometry. SFE-SFC provided an oxygen-free environment for the analysis of these sensitive and thermally labile compounds. Prostaglandins have also been extracted and analysed using supercritical fluids. They are biologically active metabolites derived from C-20 polyunsaturated fatty acids containing a substituted cyclopentane ring. They possess a strongly diversified physiological activity by modulating the action of hormones rather than acting as hormones. Typical prostaglandin structures are shown below.




**Prostaglandin $F_{2\alpha}$ isopropyl ester**

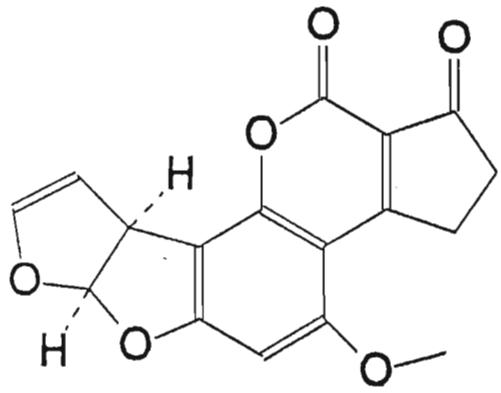



**Prostaglandin $F_{2\alpha}$**

The need for a sensitive and highly selective analytical method that is mild for these labile compounds has made prostaglandins one of the most challenging classes of biological compounds to analyse. Koski et al.[98] used SFE with $CO_2$ and open-tubular column SFC for the trace analysis of prostaglandins in aqueous solutions without derivatisation. Aqueous prostaglandin samples were extracted from adsorbents onto which the samples had been loaded using $CO_2$ at 0.800 g/ml and 35-50°C. The extract was then trapped in a solute trap cooled with liquid $CO_2$ prior to SFC analysis. Eleven prostaglandin standards were separated in 35 minutes.

## 2.7 Mycotoxins

One of the major interests in natural product analysis is the characterisation and determination of mycotoxins, and SFE has proved to be useful in this regard. Most of the work reported has focused on the extraction of aflatoxin B1 from naturally-contaminated samples. Aflatoxin B1 whose structure is shown below is a secondary metabolite from the fungus *Aspergillus flavus* and *Aspergillus parasiticus*.




Aflatoxin B1

Aflatoxin B1 is of particular interest due to its carcinogenicity and its presence in most agricultural commodities such as corn and peanuts. Engelhardt and Hass [15], initially investigated the use of carbon dioxide for the extraction of aflatoxin B1 from animal feeds. They showed that lipid constituents could be removed first from the peanut meal in 30 minutes using carbon dioxide at 200 atm and 40°C and that 31.2% of the aflatoxin B1 could be extracted in 1 hour by increasing the pressure to 650 atm. They predicted that at densities above 1.072 g/ml (ie. pressures greater than 820 atm at 40°C) approximately 50% yield of aflatoxin B1 could be obtained. The addition of methanol as a modifier enabled extraction at lower pressures, with recoveries from spiked peanut meal samples approaching 100%. However, a decline in selectivity was also observed as shown in **Figure 2.5** on the next page.

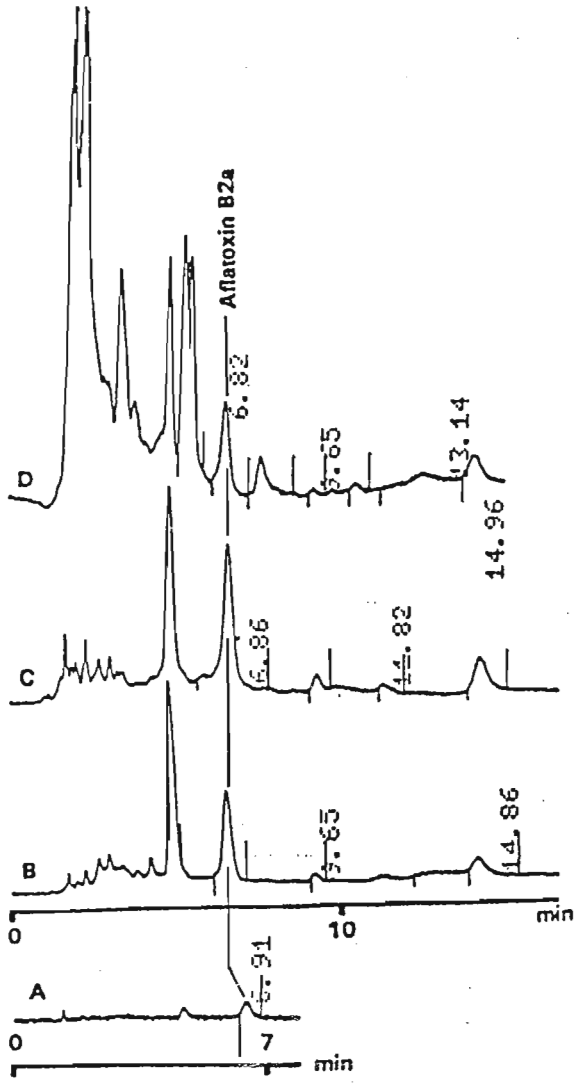
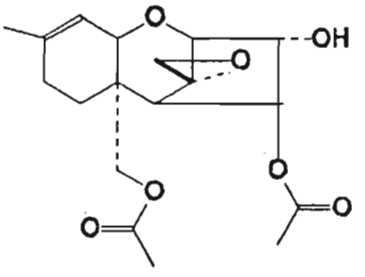



**Figure 2.5** Effect of increasing methanol concentration on the extraction selectivity from a peanut meal sample according to Engelhardt and Hass: (A) $CO_2$; (B) $CO_2$ + 2.9 vol. % methanol; (C) $CO_2$ + 5.6 vol. % methanol; (D) $CO_2$ + ~ 30 vol. % methanol. [15].

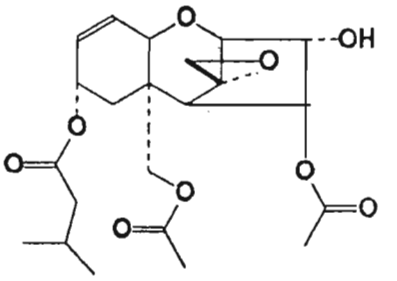
Selim [99], also reported that addition of 3% methanol to the carbon dioxide increased extraction efficiency, but when added at levels greater than 3%, other interfering components were extracted making quantitative analysis difficult. A mixed modifier of acetonitrile and methanol (2:1) was reported to give better recoveries with fewer interfering components, although the recovery obtained was related to the sample mesh size, the smaller the matrix particle the higher the recovery. Selim also reported the

extraction of fumonisin B1, which is structurally related to aflatoxin B1 using acetic acid as the modifier. Pressures from 2000 to 15000 psi (~ 133 atm to 1000 atm) and modifier additions up to 20% v/v were investigated.

Kalinoski et al.[35] used on-line supercritical fluid extraction-chemical ionisation mass spectrometry and collision-induced dissociation tandem mass spectrometry for the rapid identification of several trichothecene mycotoxins in wheat samples at ppm levels. Deoxynivalenol, diacetoxyscirpenol and T-2 toxin were selectively extracted with $CO_2$ at 61 °C by ramping the pressure from 100 atm to 300 atm at 1-5 atm/min. Structures for the mycotoxins diacetoxscirpenol and T-2 toxin are shown below. The restrictor effluent was aspirated directly into the chemical ionisation source and positive ion mass spectra using ammonia as the reagent gas were obtained. Although the reported work on SFE of mycotoxins in the literature is limited, the studies described above indicate that SFE may be useful for selectively extracting other microbial secondary metabolites in complex matrices.




diacetoxyscirpenol




T-2 toxin

## 2.8 Miscellaneous applications

SFE and combined SFE methods have been applied to "micro" samples and may be important in the future. King demonstrated the possibility of isolating and identifying triglycerides and fatty acid composition of single seeds [100]. He also suggested that insect pheromones could be analysed using low temperature SFE-SFC. He demonstrated the feasibility of this approach by extracting and analysing an intact dried fruit beetle, *Carcophilus hemipterus*, a pest that attacks most agricultural commodities. The beetle was extracted with $CO_2$ at 200 atm and 45°C for 1 minute and the extract analysed on-line by capillary SFC. The extract was thought to comprise mainly a high molecular weight hydrocarbon wax.

SFE has also been useful for the extraction of taxanes. Ezzell and Park [101] used SFE with $CO_2$ modified with various molar percentages of methanol at 600 atm to extract taxol from the bark of the pacific Yew tree *Taxus brevifolia*. Taxol is now being used for the treatment of ovarian, breast, lung and skin cancers and is now being used commercially. Unfortunately the yield obtained is known to be approximately 1 g/10 kg of bark which has motivated the production of taxol synthetically. Heaton et al. [102] applied SFE-SFC to the analysis of taxicin from *Taxus baccata*. SFE gave comparable yields to those obtained with liquid solvents such as methanol or carbon tetrachloride.

Smith et al. [103] investigated the SFE of eugenol from pimento berries. Freshly ground pimento berries were extracted with $CO_2$ at 200-300 atm and 40-80°C. At 200 atm and 40°C extracts gave a clean HPLC chromatogram which contained only eugenol (66% recovery). On increasing the temperature to 80°C at 200 atm, a recovery of 75% eugenol was obtained. Addition of 10% methanol to the $CO_2$ only resulted in a 5% increase in recovery, but the inclusion of 1% trifluoroacetic acid in the extraction fluid increased the extraction recovery to 90%. Extraction yields could not be increased any further. Heikes [104] used SFE with GC-MS for the formulation of a rapid, accurate and specific method for the determination of safrole and other related allylbenzenes in unbrewed sassafras root bark (*Sassaras albidum*). Samples were extracted in a static-dynamic mode with $CO_2$ at 690 atm and 80°C with methanol as modifier.

# CHAPTER THREE

# EXPERIMENTAL TECHNIQUES

## 3.1 Introduction

In chapters one and two, the basic principles of SFE and a literature review of the extraction and characterisation of natural products by SFE were presented. From the documented work it is clear that the technique is successful as an alternative to the classical extraction methods in most of the application areas reviewed in chapter two. The objective of this chapter is to describe the various extraction systems used in the study as well as the experimental details of the analytical techniques employed for the analysis of the various plant extracts.

## 3.2 Extraction Systems

### 3.2.1 Construction of the home-made SFE unit

In order to extract various natural products, an SFE system was constructed using various commercially available components. **Figure 3.1** shows a photograph and a schematic diagram of the SFE unit which was employed for off-line studies. The following sections describe the components of the system and how the unit was constructed in detail.

Pump

A Lee Scientific series 501 supercritical fluid chromatography syringe pump as shown in **Figure 3.2** (Dionex Corp., Sunnyvale, CA, USA) was used for fluid delivery. The syringe pump had a cylinder capacity of 150 mL and could deliver a fluid up to 400 atm in pressure. An electronically-actuated valve within the pump was switched so as to connect the pump cylinder with either the gas cylinder or the extraction vessel. The contents of the pump could also be vented to the atmosphere if required. The pump pressure was controlled by a personal computer using Lee scientific software (version 1.7). Direct introduction of a liquid from a high pressure tank into a cooled pump cylinder

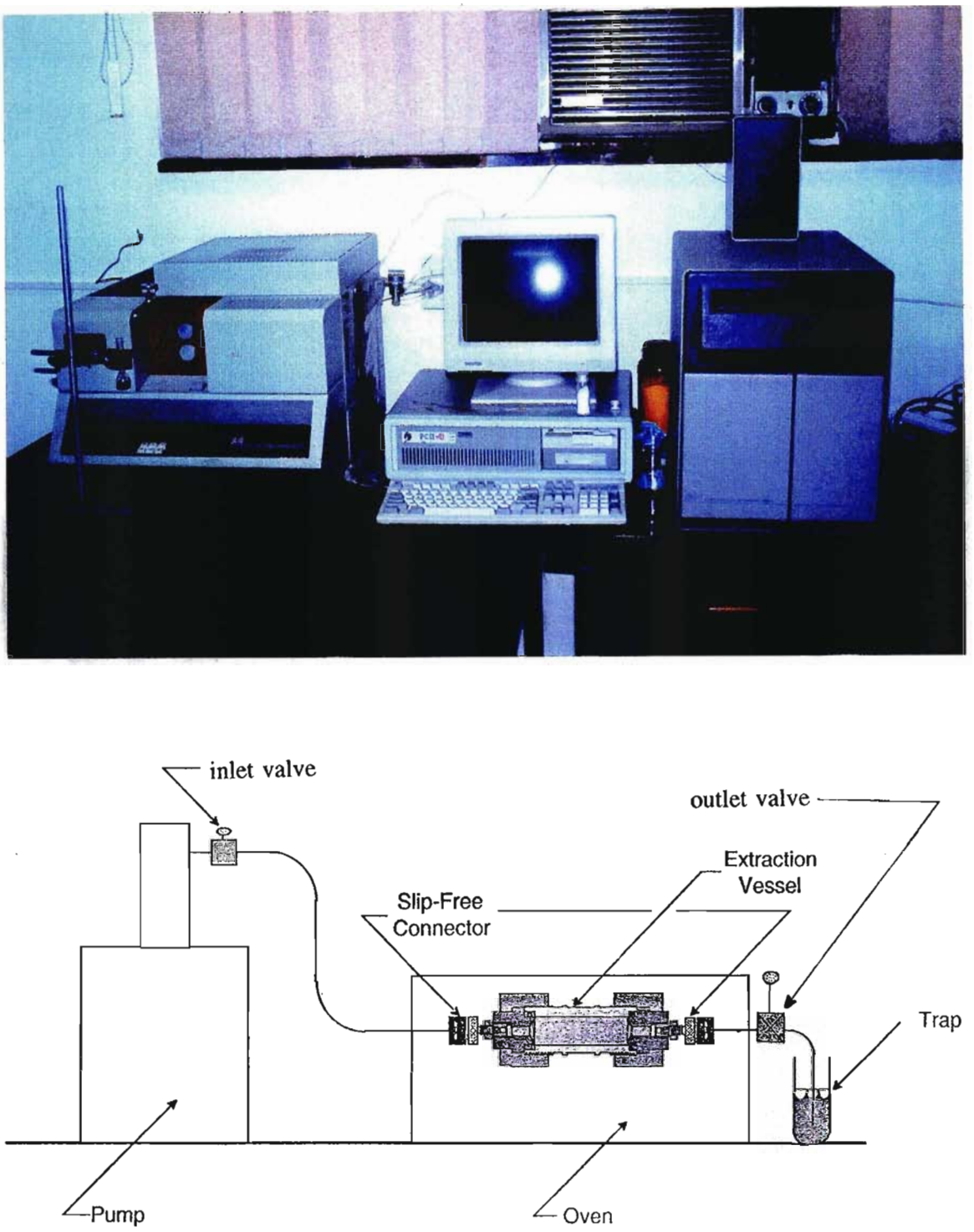



**Figure 3.1** A photograph (top) and a schematic diagram (bottom) of the home-made SFE system.

head has been the recommended method for filling most SFC pumps with $CO_2$. It is usually necessary to cool the pump head when working with eluents that are gaseous at ambient temperatures. Cooling reduces the tendency of the liquid eluent to undergo gasification in the pump head and also enhances the pumping efficiency. As an added advantage, cooling results in a maximum fill of the pump cylinder.

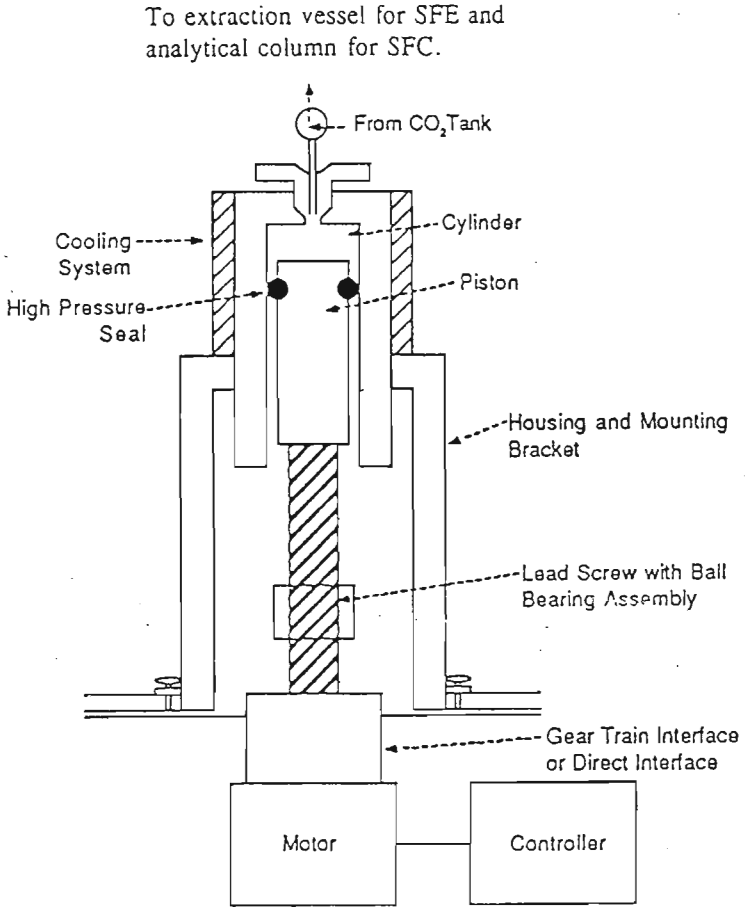



**Figure 3.2** Schematic diagram of a syringe pump. Modified from reference 105.

The pump cylinder jacket was therefore cooled by circulating cold water at between 8-10°C around it. This cold water was generated by a cooling coil (Grant Inst., Cambridge, UK) placed inside a circulatory water bath filled with water. A piece of rubber tubing was connected from the outlet of the water bath to the 1/4" brass bulkhead fitting on the pump labelled coolant inlet. Another piece of rubber tubing was connected

from the coolant outlet bulkhead fitting on the pump back to the inlet of the water bath to ensure the constant flow of the cooling solvent around the pump cylinder jacket. Both the pump cylinder jacket and the coolant transfer lines were insulated using a cut-to-fit polymer foam material. This step was essential to prevent excessive air moisture condensation and loss of cooling efficiency.

Extraction vessels

Two extraction vessels were used for the off-line SFE work. The first vessel investigated was an empty HPLC column which comprised a 300 x 6.5 mm i.d. stainless steel tubing with high end caps. Both end caps housed 3 $\mu$m frits to ensure that the solid matrix was retained within the vessel and only components that were soluble in $CO_2$ could pass out of the vessel. A diagram showing the end fitting of this vessel is shown in **Figure 3.3**.

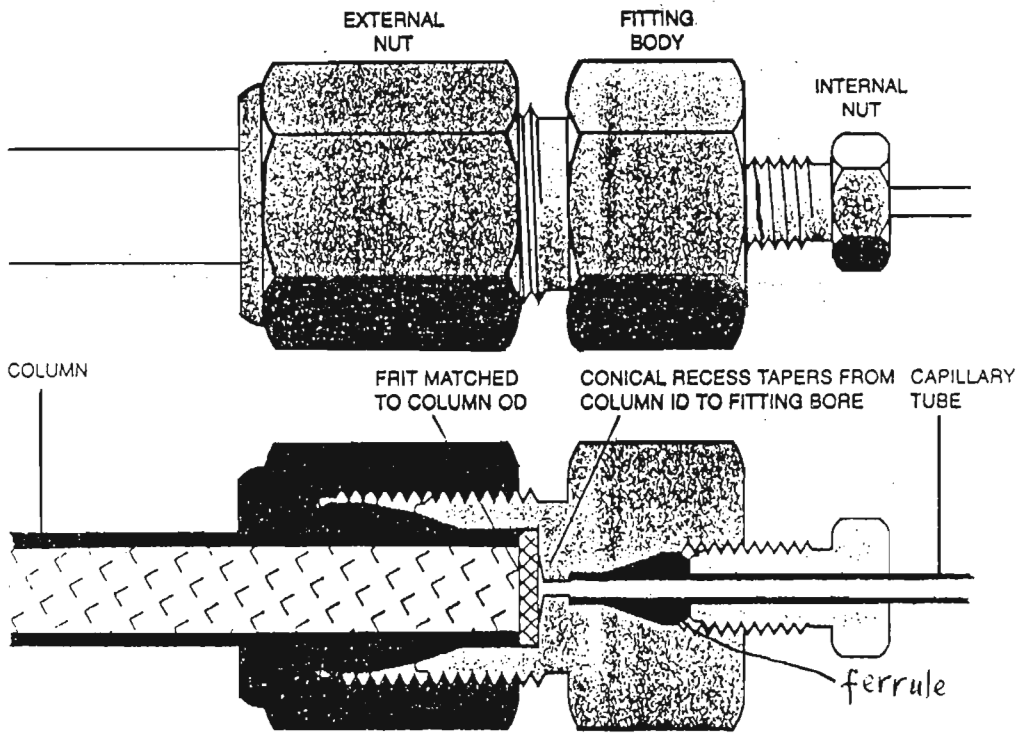



**Figure 3.3** External column end fitting of the empty HPLC column with removable frit.

Unfortunately, with constant opening and closing of the vessel, the ferrule slowly deformed and this resulted in leaks. Therefore a commercially available Keystone stainless steel SFE vessel of dimension 150 x 14 mm i.d. (24 mL) was purchased (Keystone

Scientific, Bellefonte, PA, USA). **Figure 3.4** below shows a diagram of the vessel with high pressure end caps. Both end caps housed a 5 $\mu$m bed support and a spring-loaded polytetrafluoroethylene (PTFE) cap seals. The vessel had a maximum pressure rating of 666 atm (10 000 psi). The extraction vessel was housed in a Perkin-Elmer sigma 3B gas chromatographic oven for temperature control.

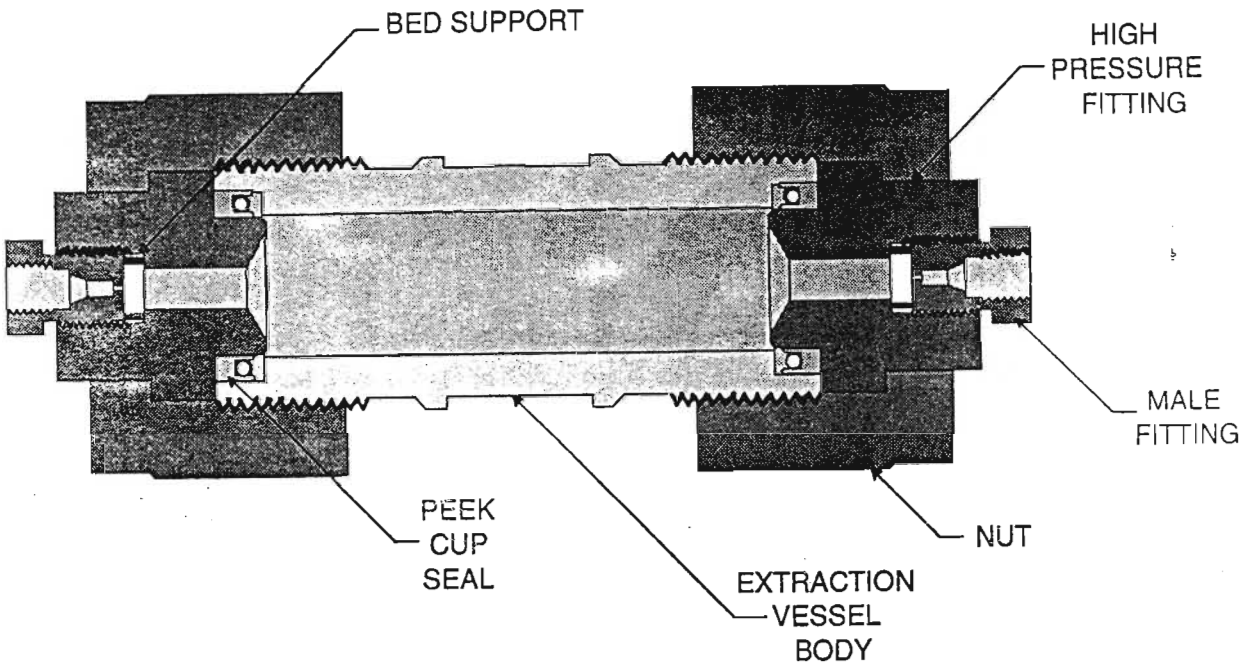



**Figure 3.4** A diagram of a commercially available stainless steel extraction vessel with fingertight end caps. [106].

Plumbing

The plumbing was accomplished as follows: A 1/16" o.d. stainless steel tubing soldered onto a short 1/8" o.d. tubing was used to connect the high pressure tank containing SFE/SFC grade liquid $CO_2$ (Air Products and Chemicals Inc., Allentown, PA, USA) to the 1/16" bulkhead fitting labelled gas source at the back of the series 501 pump. The ends of the stainless steel tubing were pushed through (a) a 1/16" nut and a 1/16" stainless steel Swagelok ferrule (Swagelok Co., Solon, OH, USA) at the end for connection to the pump, and (b) a 1/8" nut and a 1/8" brass ferrule at the end to the high pressure $CO_2$ tank.

Both ends were then connected to the pump and tank respectively. A piece of 1/16" o.d stainless steel tubing with a 1/16" nuts and ferrules fitted at both ends was connected from the 1/16" bulkhead on the pump labelled carrier fluid to a 4-port valve (Valco Inst., TX, USA) fixed outside the GC oven. This served as an on/off valve to control the entry of the fluid into the extraction vessel. A short piece of 1/16" o.d. stainless steel tubing was again connected from the 4-port valve to the extraction vessel housed in the GC oven. Finally, the outlet of the extraction vessel was connected to a high pressure 2-way valve (Supelco, Bellefonte, PA, USA) placed outside the oven. This served to control extractions either in the static or dynamic mode. A 30 cm x 50 $\mu$m i.d. deactivated fused silica capillary (SGE, Australia) for fluid decompression and also as a back pressure regulator was placed on the outlet of the 2-way high pressure valve. One end of the capillary was threaded through a short 1/16" o.d. polyetheretherketone (PEEK) sleeve which was then pushed through a 1/16" nut and stainless steel ferrule in that order before connecting to the 2-way pressure valve. The end of the capillary restrictor was immersed in a volumetric flask containing liquid solvent for solute trapping.

### 3.2.2 The ISCO SFE system

An ISCO SFE system (Model SFX 220, Isco Inc, Lincoln, Nebraska, USA) was evaluated during the course of this study to investigate the effect of adding methanol to the extraction vessel on the extraction yield of a compound from *Cedrela toona*. A photograph of the system is shown in **Figure 3.5**. It consisted of an ISCO 260D syringe pump with a capacity of 260 mL and a maximum pressure of 500 atm, a SFX 220 controller and a SFX 220 extractor which had an in-built temperature controller and was capable of housing two 10 mL extraction cartridges. For extraction, the stainless steel sample cartridge (**Figure 3.6a**) was loaded with sample, clipped into the knurled chamber cap (**Figure 3.6b**) and hand-tightened into the temperature controlled chamber (**Figure 3.6b**). The design of the housing provided a tight seal and prevented leakage of the $CO_2$ fluid. The extractor also housed the electronically-controlled inlet and outlet valves to control the flow of the $CO_2$ fluid as required for any particular experiment. Thus both static and dynamic extractions could be performed in parallel or sequentially using both extraction

chambers. The pump pressure and valve switching were controlled by the SFX 220 controller which allowed either manual or programmed valve switching. Metal and fused silica linear restrictors with an internal diameter of 50 $\mu$m were connected to the outlet valve and inserted in the collection vials held conveniently on a rack attached onto the side of the extractor as shown in **Figure 3.6c**. The extraction chambers could be easily vented by switching the outlet valve to a purge line. Once depressurised, the cartridge was loosened by hand, cleaned and reloaded for the next extraction.

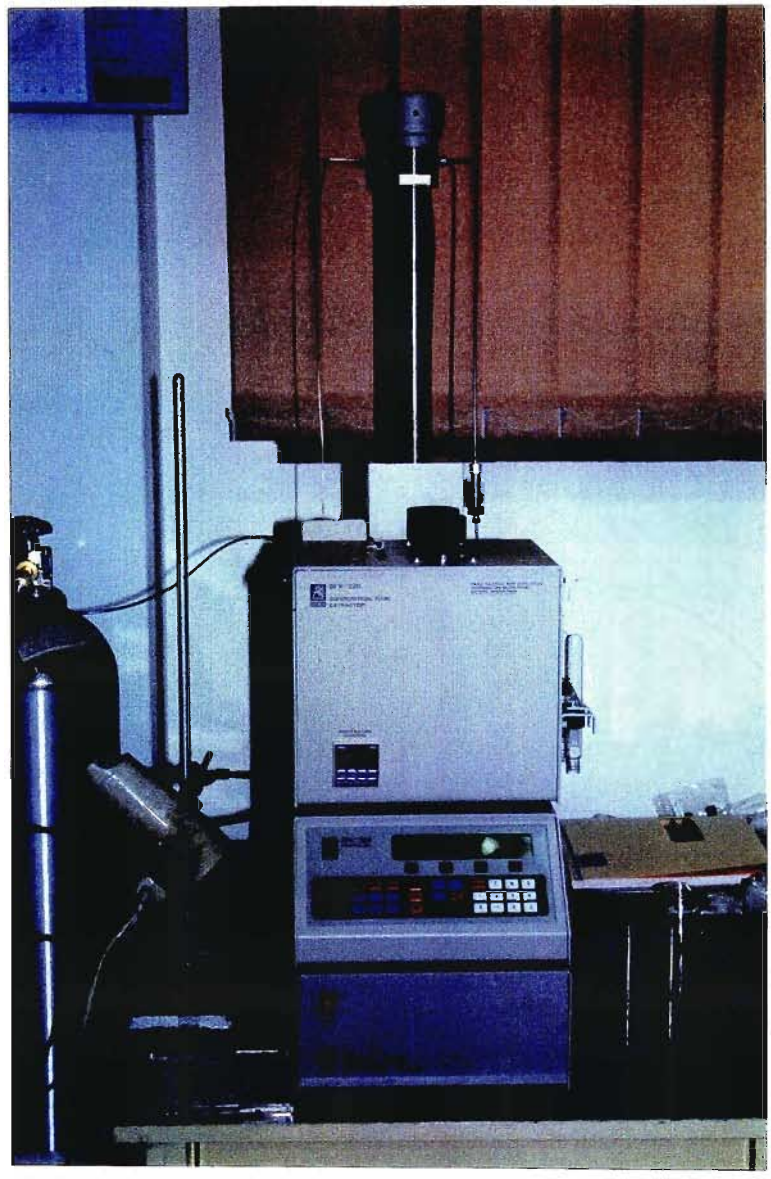
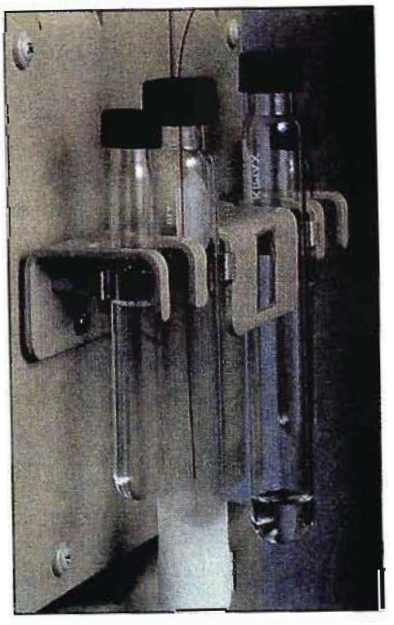


**Figure 3.5** A photograph of the ISCO model SFX 220 SFE system .

A


B



C

**Figure 3.6** A photograph showing (a) typical 10 mL extraction vessels, (b) clipping and tightening the loaded extraction vessels into the temperature controlled chamber, and (c) collection vials for analyte trapping.

In the case of SFE of *Cedrela toona*, the cartridge was loaded with a 0.6 g sample of the ground wood and 40 $\mu$L of methanol was spiked into the sample. The cartridge was sealed and the valves were actuated manually to perform a static extraction for a set period of time at 400 atm and 40°C. After the static extraction period, extractions were then performed dynamically and extracts were collected for analysis. This will be discussed later in chapter five.

### 3.2.3 Soxhlet Extraction

In order to compare SFE with classical solvent extraction, a Soxhlet apparatus shown in **Figure 3.7** was used for multiple simple solvent extraction of the plant material. The apparatus consisted of a 250 mL round bottomed flask, an extraction head and a reflux condenser. Plant material to be extracted was placed in a 100 mm x 30 mm i.d. cellulose extraction thimble (Whatman Ltd). The flask containing 150 mL of extraction solvent was heated with a heating mantle to evaporate the solvent which condensed in the reflux condenser and dripped into the thimble. When the solvent level rose to the top of the siphon, the extracts in solution were transferred to the flask and this procedure was repeated for 4 to 5½ h for exhaustive extraction.

The Soxhlet extraction technique was employed in all the classical extractions performed in this study. Extractions were performed on 3.0 g samples of pulverised wood and seeds of the Meliaceae species *Cedrela toona* and *Melia azedarach* respectively using UV spectroscopic grade hexane (Baxter Healthcare Corp., USA) and analytical grade dichloromethane (BDH Chemicals, UK) as extraction solvents for 4 to 5½ h periods depending on whether extracts were required for qualitative or quantitative comparison.

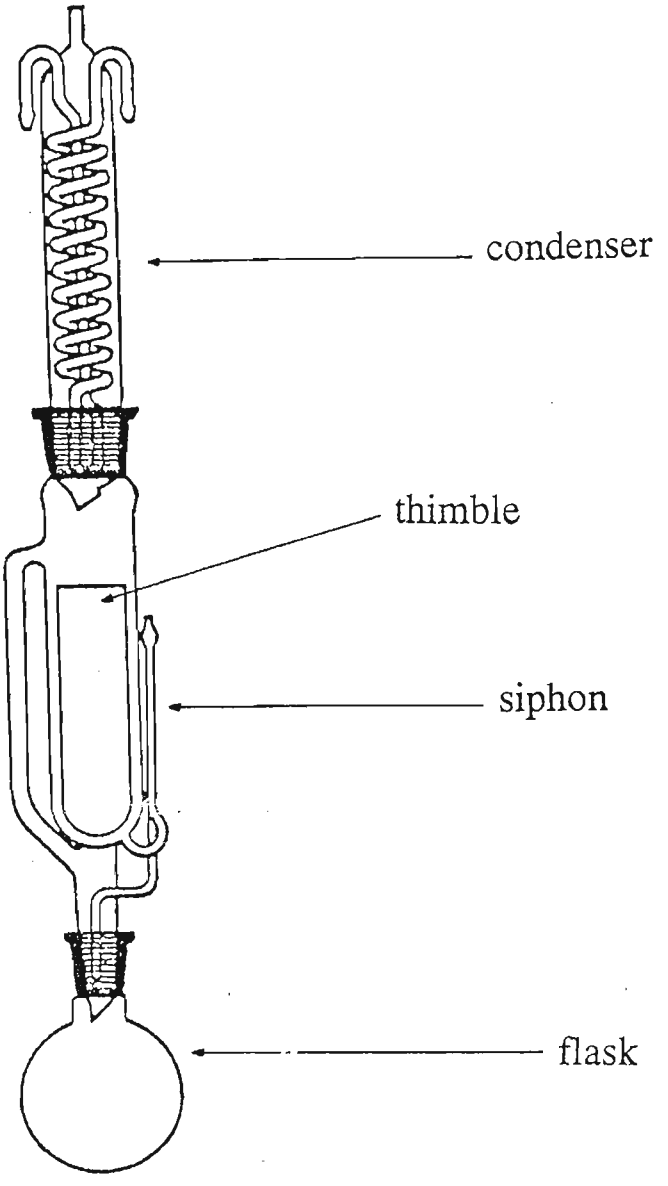



**Figure 3.7** A Soxhlet apparatus for classical solvent extraction. Diagram taken from reference 107.

Using the rotary evaporator with a water bath temperature of 70°C, the solvent was evaporated from the extracts. The gummy residues were made up in dichloromethane for subsequent GC and SFC analysis.

## 3.3 Gas Chromatography of Extracts

Two categories of compounds were encountered in this study: those semi-volatile components from the wood and bark of *Cedrela toona* that were amenable to GC analysis and the involatile components from the seeds of *Melia azedarach* for which GC analysis was impossible. GC analyses were performed on the former using either a FID or an MS detector. Extracts were obtained either by liquid solvent extractions or a combination of off-line and on-line supercritical $CO_2$ extraction techniques.

### 3.3.1 Off-line GC methodology

GC-FID analyses of extracts were performed on a model 8160 gas chromatograph (Fisons Instrument, Italy) equipped with a manually-controlled OC-V71 on-column injector, a temperature programmable oven, a capillary column and a flame ionisation detector (FID) as shown in **Figure 3.8.**

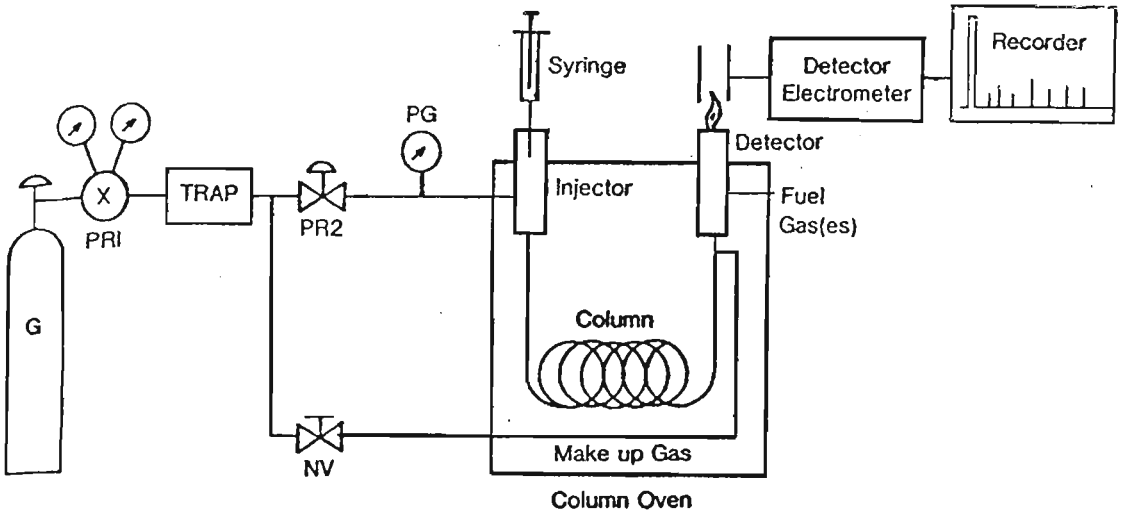



**Figure 3.8** A schematic diagram of a temperature programmable gas chromatograph. Key: G, high-pressure gas supply cylinder; PR1, two-stage pressure regulator; PR2, pressure regulator or flow controller; PG, pressure gauge for column inlet pressure; NV, needle valve for adjusting the make-up gas flow rate. [108].

On-column GC analysis and Installation of the capillary column

The on-column injector was used to introduce sample aliquots directly into the capillary column as shown in **Figure 3.9.**

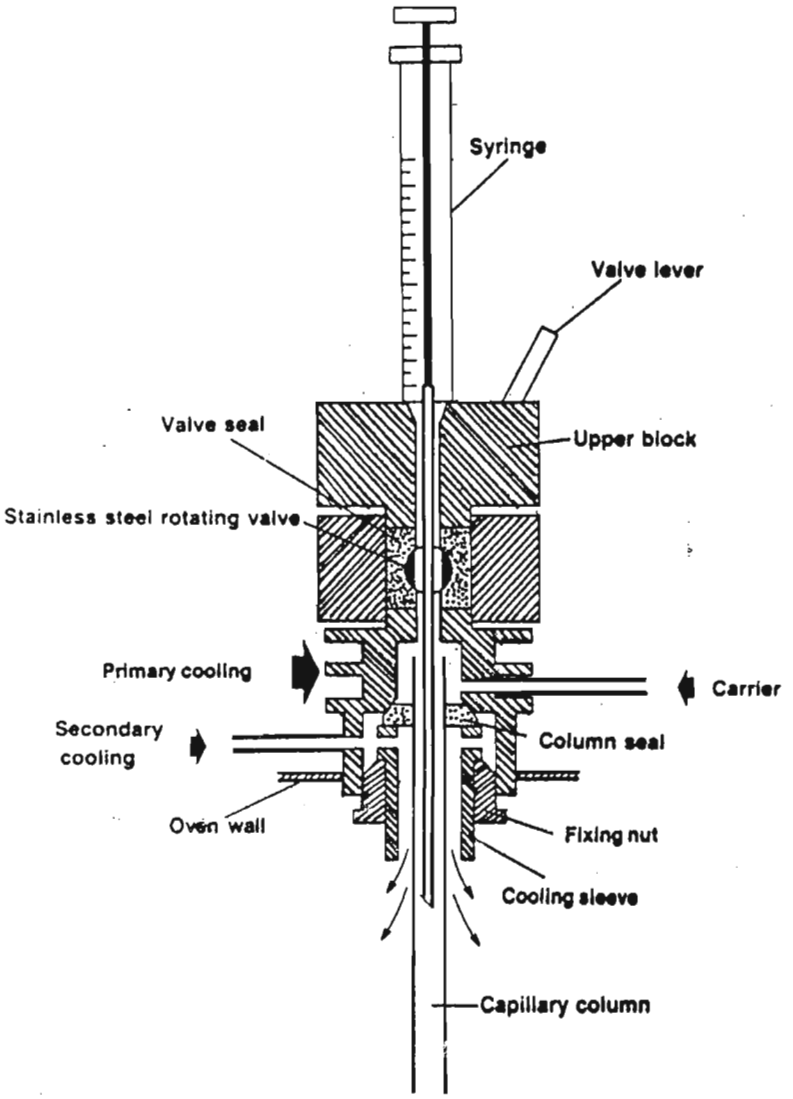



**Figure 3.9** A diagram showing an on-column manual injection onto a capillary column. [109].

This was achieved by inserting the syringe needle into the upper portion of the injector to form a seal and then shifting the valve lever operating the internal stainless steel rotating valve so that the needle could be inserted into the capillary column. Once the injection had been made, the procedure was reversed. During this period of injection, the secondary cooling gas (compressed air) was forced through the base of the injector to maintain the temperature of the injector base and capillary column below the boiling point

of the solvent. This was considered important to prevent sample discrimination and thermal breakdown of labile analytes. 0.5 $\mu$L injections were made manually into a 25 m x 0.25 mm i.d. fused silica capillary coated with poly (5% diphenyl/95% dimethylsiloxane) (commercially named PTE-5) ( $d_f$ = 0.25 $\mu$m, Supelco, Bellefonte, PA, USA). The capillary column was fitted in the oven on a column support and installed using specially designed graphitised vespel ferrule, a backwasher sleeve for secondary cooling and a column retaining nut as shown in **Figure 3.10.**

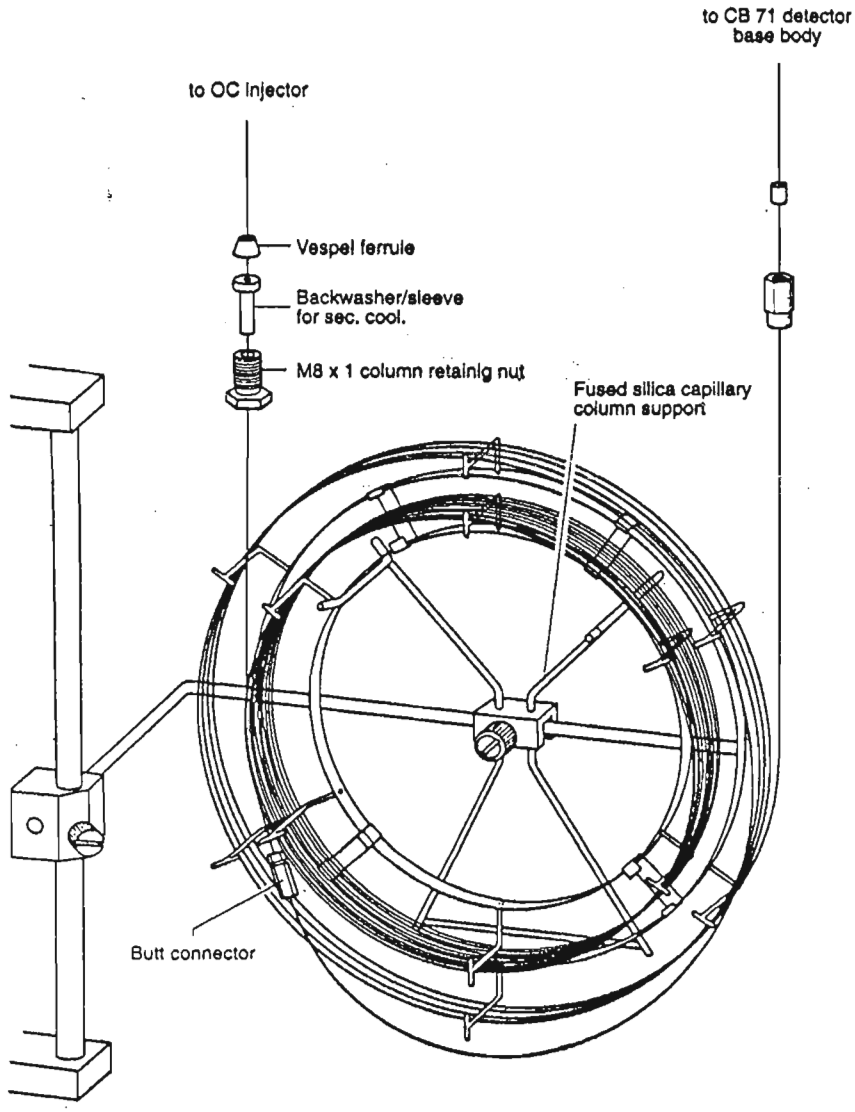



**Figure 3.10** Installation of a fused silica capillary column for on-column injection. [109].

The most difficult aspect of the installation was attainment of the right alignment between the needle and the column. The easiest way found was to loosen the retaining nut slightly and insert the needle into the capillary column. While the needle remained in the capillary column, the retaining nut was then tightened to ensure that correct alignment was attained. Helium was used as the carrier gas at a column head pressure of 100kPa. Flow rates from 0.5 mL/min to 1.5 mL/min were investigated.

Oven temperature program

Injections were performed at an oven temperature of 60°C and held for one minute. A multi-ramp temperature program which is typically useful for on-column injection was employed. The temperature was quickly ramped to 150°C at 18°C/min and held for another minute. It was finally ramped to 320°C at 7°C/min and held for 20 to 25 minutes.

Flame ionisation detection and data collection

The flame ionisation detector was heated to 320°C and operated using hydrogen (30 mL/min), air (300 mL/min) and nitrogen make-up gas (25 mL/min). For quantitative work using this detector, the flame was lit and allowed to stabilise overnight for a 8-10 h period before injections were made. The voltage output from the detector was amplified by an electrometer connected to either a Varian 4270 or a Vista 401 integrator (Varian Inst. Group, CA, USA) on which chromatograms were recorded.

Capillary GC-MS analyses

The off-line supercritical $CO_2$ extracts from the wood and bark of *Cedrela toona* were also analysed by capillary GC-MS. 2.5 g each of the wood and bark were extracted at 400 atm and 40°C for 50 minutes. Extracts were trapped in dichloromethane and subsequently analysed on a HP 5890 (II) gas chromatograph and a HP 5971 mass selective detector (Hewlett Packard, Avondale, PA, USA). One end of the PTE-5 column was inserted into the split-splitless injection port and sealed with a Vespel ferrule and fixing nut while the

other end was pushed into the interface and also sealed with a Vespel ferrule and fixing nut. The pressure inside the ion source and analyser were then reduced to $10^{-4}$ torr using a high vacuum pump accessory. The mass spectrometer was then tuned with perfluorotributylamine (PFTBA) using the software algorithm called autotune for maximum sensitivity consistent with good peak shapes and required mass calibration.

1.0 $\mu$L split injections were then performed at an injector temperature of 250°C and at a split ratio of 100:1. The oven temperature program was as described in section 3.3.1. A solvent delay time of 5 minutes was set to avoid the occurrence of abnormal pressures in the ion source causing erratic filament operation. The ion source was operated in the electron impact mode with the temperature maintained at 250°C. The mass analyser was the quadrupole type which operated in the scanning mode with a mass scan range from 50 to 550 amu. Data capture and analyses were performed using the HPChemStation software that included a Wiley database of authentic mass spectra.

### 3.3.2 On-line SFE-GC

On-line SFE-GC analyses of the wood and bark from *Cedrela toona* were carried out on a Lee Scientific series 600 SFC/GC instrument fitted with a micro-extraction unit. As shown in **Figure 3.11a**, the instrumental components were well-positioned for a direct on-line combination of micro-SFE with capillary GC.

An 80 mg sample of either the pulverised wood or bark was placed in the micro-extraction vessel of dimension 2.5 cm x 4.0 mm i.d. (Valco Instruments, Houston, USA) which was housed in the external heating block. The inlet of the extraction vessel was connected to the syringe pump via an on/off valve, and the outlet was connected using a 1/16" o.d.stainless steel tubing to an 8-port switching valve as shown schematically in **Figure 3.11b** on page 76.

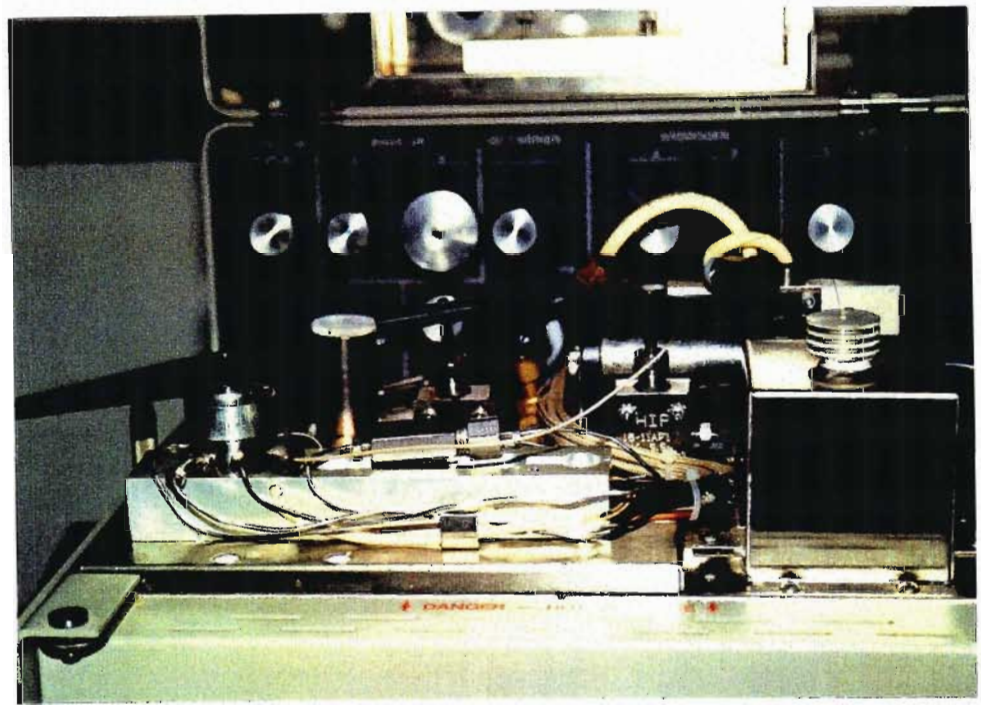


**Figure 3.11a** A photograph showing the positioning of the switching valve (left), the external heating block (centre) housing the micro-extraction vessel, and the direct insertion of the capillary restrictor into the split injection port (right) on the series 600 SFC/GC system.

The valve could be switched so that there was no flow through the extraction vessel (static extraction) or so that the extract was directed via a 30 cm x 10 $\mu$m i.d. fused silica capillary restrictor (SGE, Australia) as in **Figure 3.11b** (dynamic extraction). The restrictor was inserted through the silicone septum with the help of a hypodermic needle into the glass liner of the heated split-splitless injection port (**Figure 3.11b**).

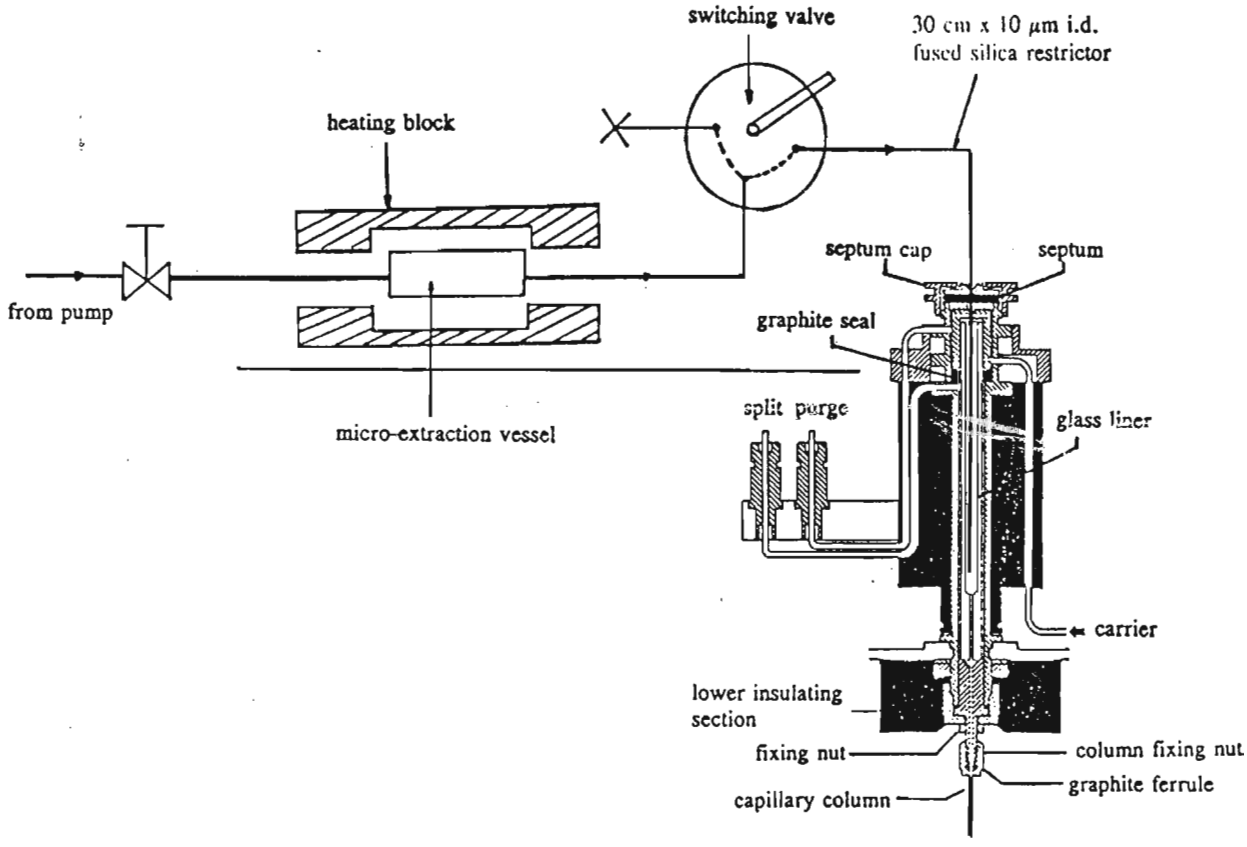



**Figure 3.11b** A schematic diagram showing the on-line coupling of SFE to capillary GC via a split-splitless injection port.

The capillary restrictor was positioned at approximately 1.5-2.0 cm above the PTE-5 coated capillary GC column. The split ratio of the injector was measured using a bubble flow meter and set at 200:1 using helium as the carrier gas. The flow rate through the column was 0.5 mL/min and the vent flow was 100 mL/min. The septum purge flow rate was set at 0.5 mL/min.

SFC/SFE grade carbon dioxide (Air Products, PA, USA) was delivered from the series 600 syringe pump at 300 atm to the extraction vessel maintained in the heating block at 50°C. Using the switching valve, $CO_2$ extracts were transferred dynamically for a one minute period into the heated split-splitless injector where on decompression at the restrictor outlet, the compounds were volatilised in the glass liner. The sample was split according to the split ratio, and the aliquot entering the capillary column was condensed as a narrow band at the head of the column. Depending on the split ratio, the amount of extract entering the analytical column could be effectively controlled to avoid overloading and peak broadening.

Injection port temperatures of 200°C, 250°C and 300°C were used to investigate the transfer efficiency, and oven temperatures of 40°C, 50°C and 60°C were employed to investigate the trapping efficiency of the analytes. The operating conditions of the oven and FID were similar to those described in section 3.3.1.

## 3.4 Capillary supercritical fluid chromatography of extracts

Open-tubular supercritical fluid chromatography with FID, UV and Fourier transform infrared microspectrometry detectors were used in the analyses of the extracts from the wood and seeds of *Cedrela toona* and *Melia azedarach* respectively. A Lee scientific series 600 SFC/GC system comprising a high pressure syringe pump, a timed-split injection valve, oven, FID and controller was used for this work. The system is shown in **Figure 3.12**. The pump is similar to the model 501 described in section 3.2. The pump cylinder jacket and injection valve were cooled by circulating a continuous stream of cold water at about 8°C around the jacket to ensure efficient pumping of the mobile phase. The

oven was equipped with a Valco AC14W timed-split injector with a 200 nL internal volume rotor. The valve loop was filled with sample using a 50 $\mu$L blunt-tipped syringe. The valve was pneumatically actuated using a high pressure flow of helium from an external cylinder. 100 ms timed-split injections were performed to transfer the analytes onto the analytical column. The analytical column was a 10 m x 50 $\mu$m i.d. fused silica capillary coated with a 0.25 $\mu$m film of SB-Biphenyl-30 (Dionex). One end of the analytical column was butt-connected to a tapered restrictor (which was made in-house) and the other end to the AC14W injector.

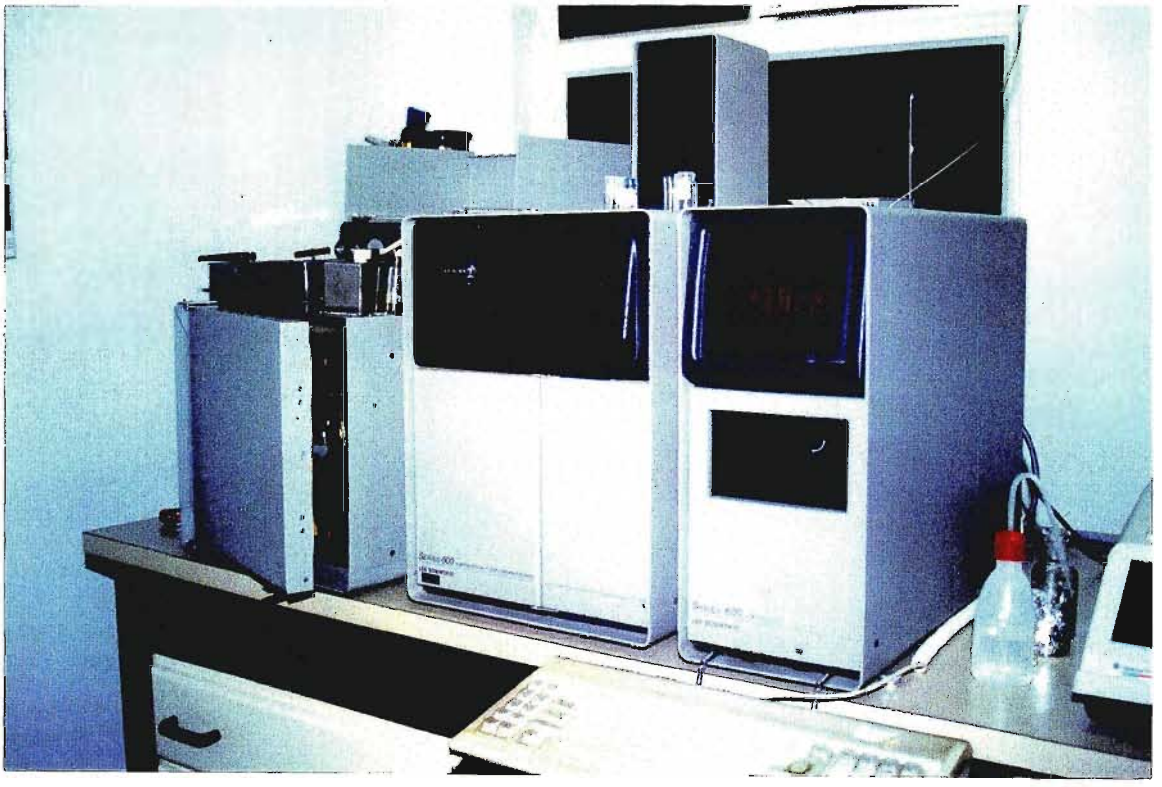


**Figure 3.12** A photograph showing the Lee Scientific series 600 SFC/GC system.

Pressure program

A pressure programme at 100°C was used for the chromatographic separation. Injections were performed at 120 atm and held for 5 minutes followed by a 10 atm/min ramp to 400 atm and held for 10 minutes.

### 3.4.1 Construction of the tapered restrictors

Tapered restrictors are commonly made from short lengths of a 50 $\mu$m i.d. deactivated fused silica capillary tubing. The end of a length of a 50 $\mu$m i.d. capillary tubing was held in the tip of a micro-Bunsen burner flame. A small tension was then applied by hand on both sides of the heated zone. As the tubing softened, it was pulled to a hair-like taper and removed from the flame. The bare hair-like taper was then carefully coated with a thin film of polyimide resin. A short 320 $\mu$m i.d. fused silica capillary sleeve was threaded around the capillary restrictor from its other end and eventually positioned around the taper to protect it. A small amount of the resin was then applied to the base of the sleeve to give the restrictor added strength. **Figure 3.13** on the next page shows the schematic of the steps involved in the fabrication of a tapered restrictor. The restrictor was then placed in an oven at about 100°C to cross-link the polyimide resin. On curing, the restrictor taper glued to part of the inner wall of the sleeve.

### 3.4.2 Connecting the tapered restrictor to the analytical column and FID

The technique for connecting the restrictor to the analytical column involved butting the restrictor to the column using a zero dead volume stainless steel butt connector. The restrictor orifice was then readily adjusted by cautiously cutting back the taper with a pair of scissors to obtain a gaseous flow rate (at room temperature and a column pressure of 150 atm) of 1.5 ml/min. When used with the FID, the restrictor was inserted into the basebody of the FID and sealed with a graphite ferrule and fixing nut. The FID was then heated to 320°C for analysis. The FID operated in the same way as already described in section 3.3.1.

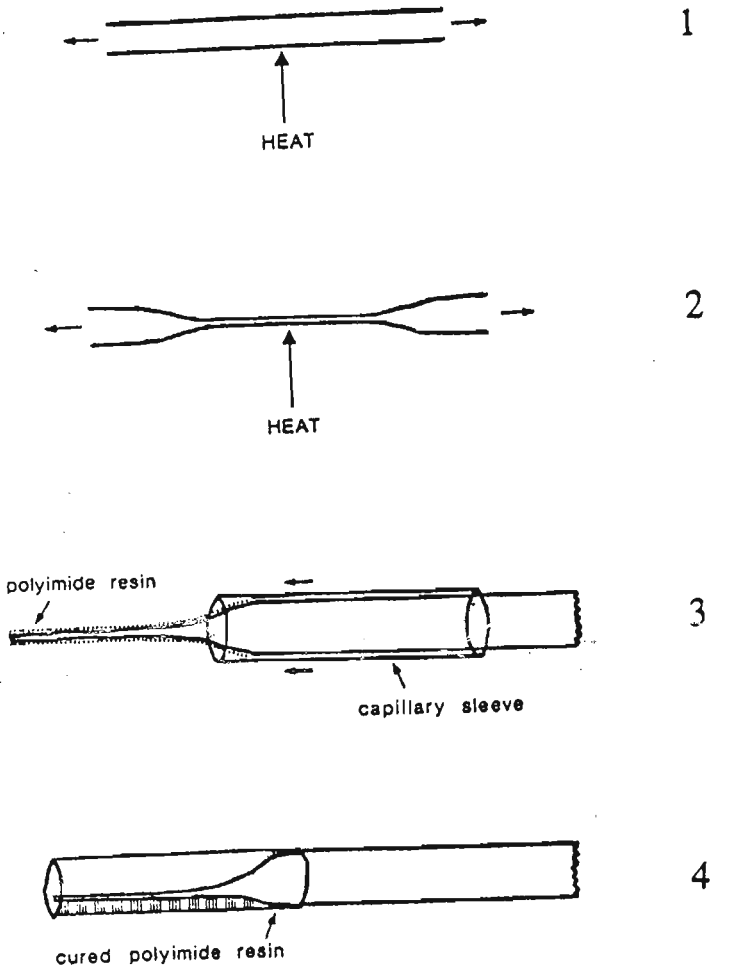



**Figure 3.13** Schematic steps showing the preparation of a tapered capillary restrictor. (1) a micro-Bunsen burner is used for localized heating of the capillary, (2) tension is applied and the capillary is pulled into a hair-like taper, (3) polyimide resin is applied to the taper which is subsequently positioned within a capillary sleeve, (4) the resin cures and glues the taper to part of the inner wall of the sleeve. [110].

### 3.4.3 UV detection

The on-line ultraviolet (UV) detection was accomplished as follows : About 2.0 cm length section of the polymer coating of a 250 $\mu$m i.d. open tubular fused silica capillary was removed to serve as the detector cuvette. Two butt connectors were then used to connect the detector capillary cuvette directly to the SFC column and the tapered restrictor as shown in **Figure 3.14** below. The pump pressure was then set to 400 atm at which time the detector and the Varian 4270 integrator linked to monitor its output were zeroed. The pump pressure was then brought to 120 atm before analysis commenced. In this way fluctuations in the detectors response due to changes in the refractive index of the mobile phase on pressure-programming were eliminated.

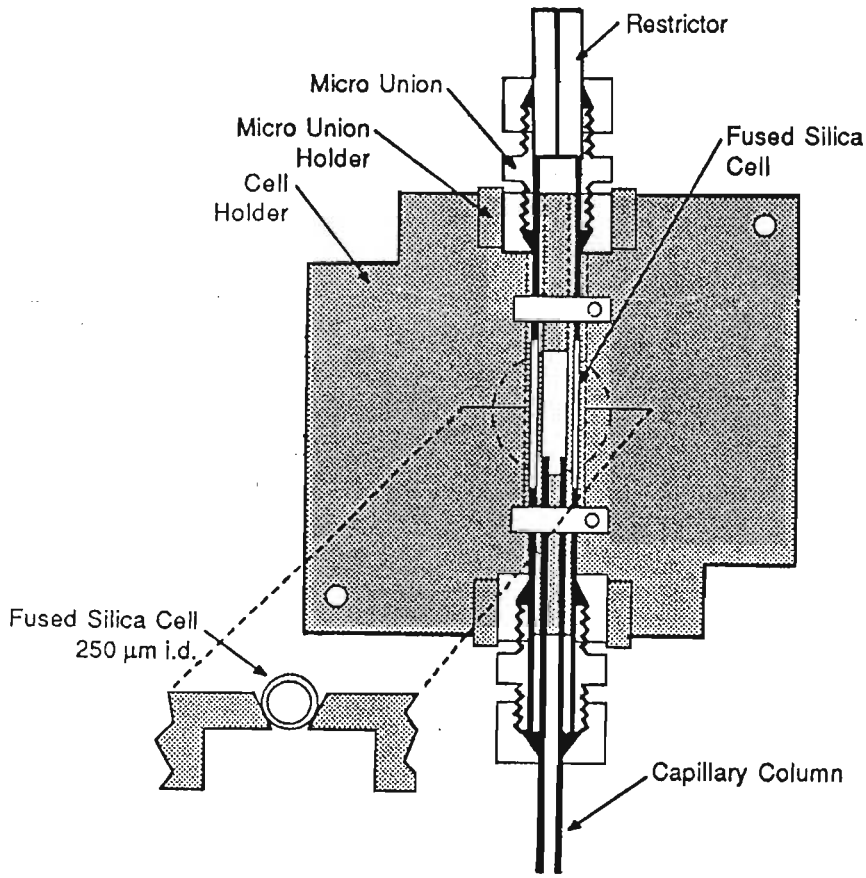



**Figure 3.14** Schematic diagram of a "pseudo" on-column UV flow cell for open tubular column SFC. Modified from reference [105 ].

### 3.4.4 SFC-FTIR Microspectrometry

A mobile phase elimination interface was used to collect nanogram levels of involatile analytes on a KBr disc. Separated components from the analytical column were deposited onto the KBr disc as the $CO_2$ depressurised from the restrictor outlet and evaporated. Once collected, the analytes were positioned in the infrared beam of an FTIR microscope accessory as shown in **Figure 3.15** and infrared spectra were then recorded. The instrumental components and the conditions required for this work are described in detail below.

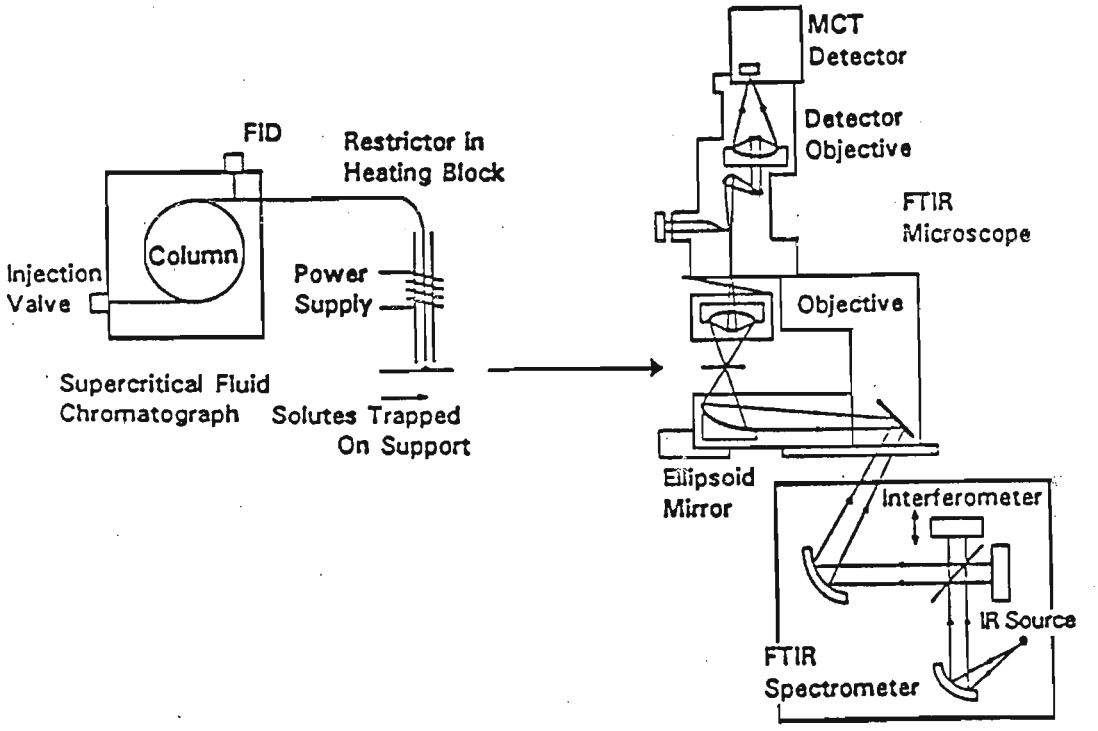



**Figure 3.15** Schematic view of the SFC mobile phase elimination interface and the off-line FTIR microspectrometry system. [105 ].

The heated transfer line

The column effluent was split between a tapered restrictor installed in the FID and the heated transfer line using a butt connector and a graphite ferrule as shown in **Figure 3.16**. The transfer line was a 50 $\mu$m i.d. deactivated fused silica capillary with a tapered restrictor. An FID:transfer line split ratio of 1:1 was set by cutting back the restrictors to produce gaseous flow rates of 1.5 ml/min each at a column pressure of 150 atm and room temperature as described in section 3.4.2.

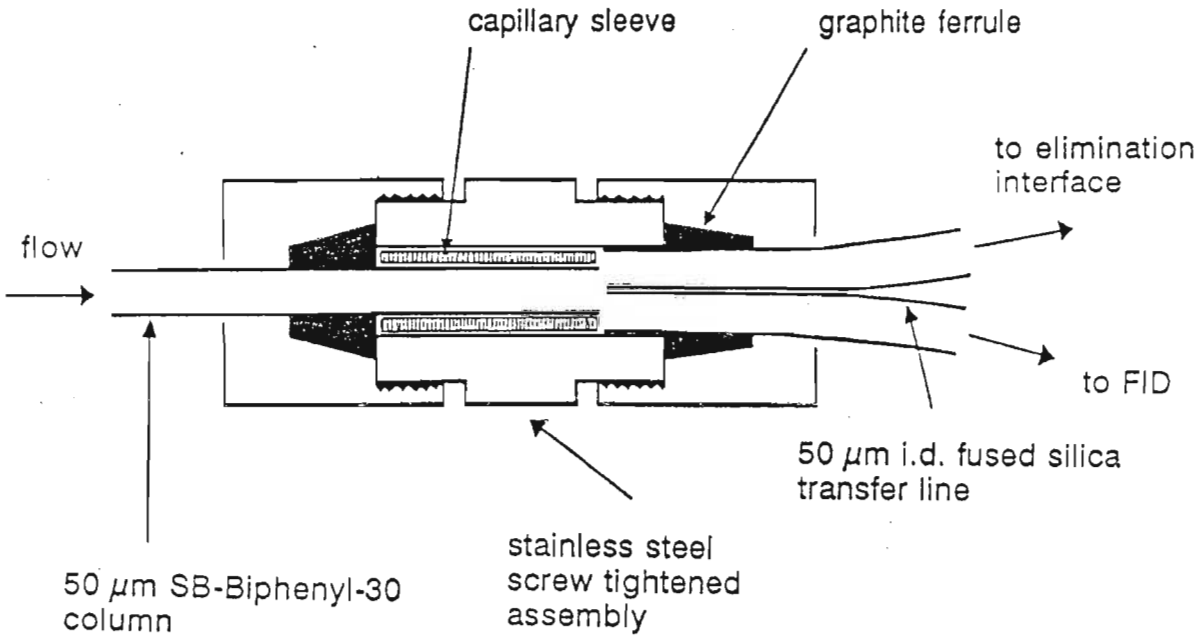



**Figure 3.16** Cross sectional diagram showing the butt connection of the column outlet to two 50 $\mu$m i.d. transfer lines. [111].

The transfer line was pushed through a piece of 1/16" o.d. stainless steel tubing maintained at 150°C using the heating assembly shown in **Figure 3.17**. The end of the transfer line was positioned approximately 1-3 mm above the surface of the KBr disc.

Since the length of the transfer line was about twice that of the FID restrictor, analytes detected by the FID arrived about 25-30 seconds later through the heated transfer line restrictor.

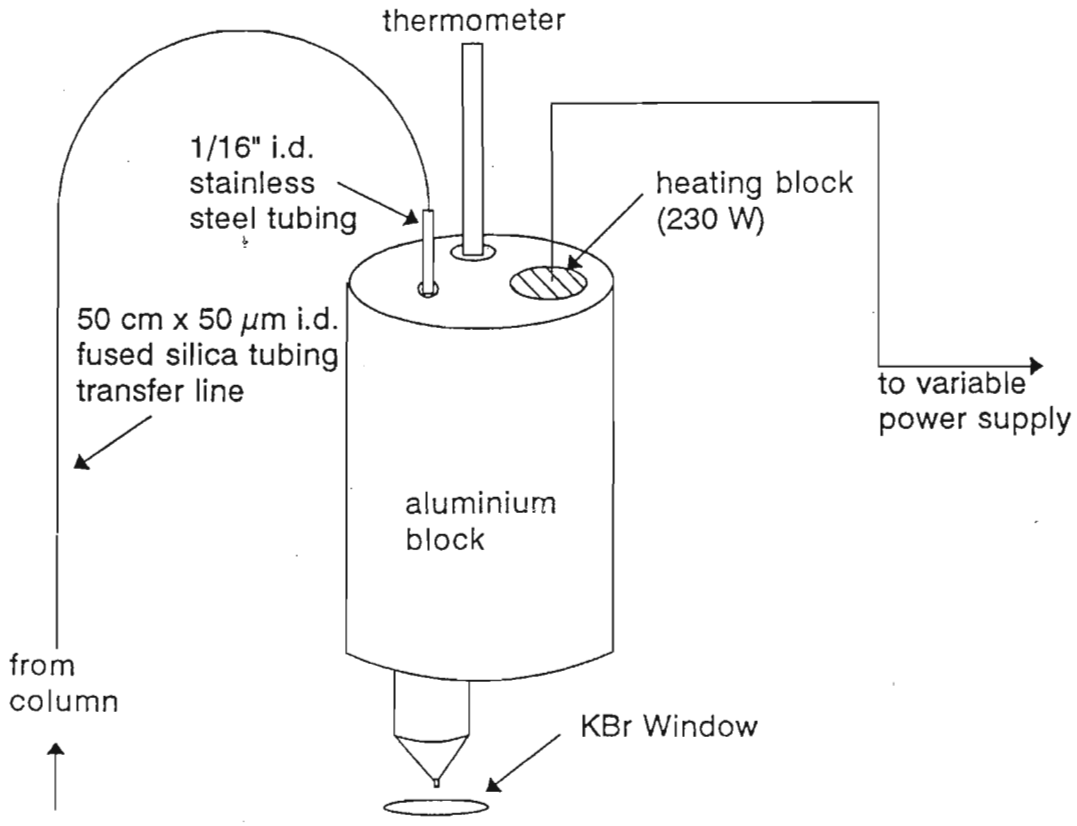



**Figure 3.17** Schematic diagram of the restrictor heating assembly used for the heated transfer line interface. [112].

The KBr disc was therefore positioned underneath the restrictor outlet 20-25 seconds later. **Figure 3.18** shows a typically-spotted KBr disc ready to be placed in the infrared microscope accessory.

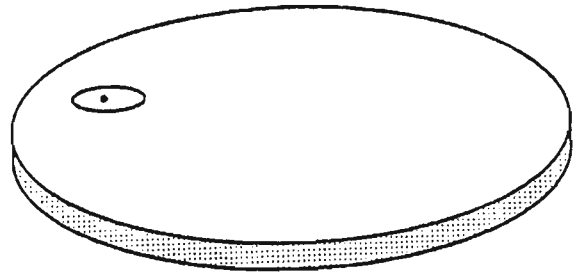


**Figure 3.18** A typically-spotted KBr disc to be focussed in the infrared beam.

FTIR Microspectrometry

The fraction that was deposited on the KBr disc was analysed by off-line FTIR using the microscope accessory. The microscope was an advanced analytical microscope (Spectra-Tech, Stanford, MA, USA) attached to a model 2020 Galaxy FTIR spectrometer (Mattson Inst., USA). The microscope is equipped with a mercury cadmium telluride (MCT) photoconductive detector which required cooling with liquid nitrogen. The background interferogram was recorded using a clean area of the window. Spots were subsequently positioned in the beam focus of the microscope by using the transmission viewing mode while the sample spectrum was collected with the microscope in the transmission sampling mode by taking 400 scans at 4 $cm^{-1}$ resolution.

## 3.5 Nuclear magnetic resonance analysis

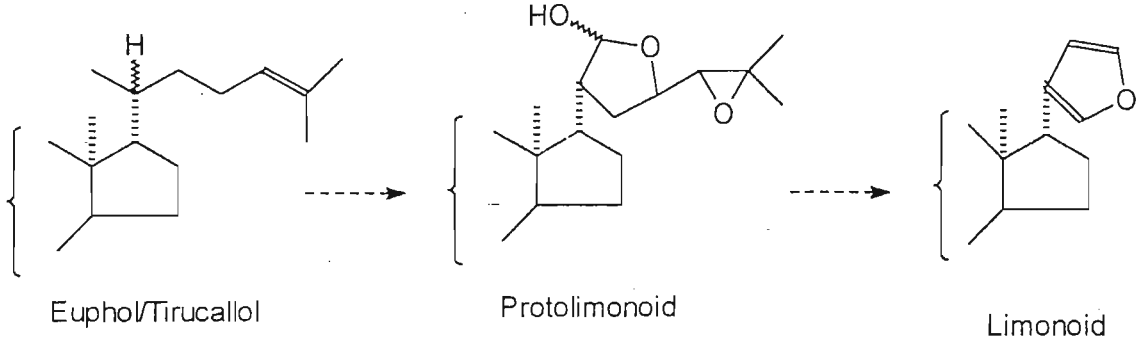
Proton and carbon-13 NMR spectra of compounds were obtained using $CDCl_3$ as the solvent at 300 MHz on a Gemini 300 NMR spectrometer (Varian Inst., Palo Alto, CA, USA).

# CHAPTER FOUR

# ANALYSES OF EXTRACTS FROM CEDRELA TOONA AND MELIA AZEDARACH

## 4.1 Introduction

*Cedrela toona* and *Melia azedarach* are plants belonging to the family Meliaceae. The Meliaceae family is known to contain a group of compounds called limonoids. Structurally, the limonoids are derived from tetracyclic triterpenoids similar to euphol or tirucallol by a series of oxidative changes interspersed with molecular rearrangements. In this process, the eight carbon side chain is converted to a C-17$\alpha$ substituted furan ring and four carbon atoms are lost. Many limonoids are available in relatively large quantities, eg. the timber of some species may yield 1 % of an isolated crystalline limonoid while a single tree of *Entandrophragma angolense* may contain more than 100 kg of the limonoid gedunin, much of it easily recoverable from the timber mill offcuts. The biological advantage for the plants producing such large amounts of material is unclear. However, it has been shown that many limonoids are active as insect antifeedants [114], although most are not directly insecticidal. The effect is so powerful that insects will starve rather than eat leaves treated with certain limonoids, and it is possible that this is the biological advantage of the limonoids to the Meliaceae. There are also a number of oxidised intact triterpenoids known, which by their biological occurrence and oxidation pattern appear to be biochemical precursors of the limonoids, and are therefore known as protolimonoids. A hypothetical view of the conversion of a triterpenoid to a limonoid via the intermediate protolimonoid stage is shown schematically below.



Protolimonoids, limonoids and other tetracyclic triterpenoids may be isolated from the wood, bark, leaves or seeds of Meliaceae species, but their precise distribution varies from plant to plant. Thus, it is possible that a protolimonoid will be present in the seed of the plant but not in the wood or vice versa. In this chapter, both classical and supercritical $CO_2$ extracts obtained from the wood, bark and seeds of the selected Meliaceae species are qualitatively analysed employing techniques outlined in chapter three. In particular, extracts were obtained from the wood and bark of *Cedrela toona* and the oil-rich seeds of *Melia azedarach*. Where possible, attention was focussed on whether comparable extracts could be obtained using supercritical fluid extraction (using pure carbon dioxide) and classical Soxhlet extraction with hexane.

## 4.2 Experimental

### 4.2.1 Sample Preparation

For the purpose of this work, a branch from the tree of *Cedrela toona*, which was identified by the Natal herbarium, was obtained locally and the bark was separated from the wood. Both parts were subjected to solar drying for six weeks after which the parts were ground into fine particle sizes for easy loading into the extraction vessel for SFE, and also into a thimble for the classical Soxhlet extraction. Seeds of *Melia azedarach* were also obtained locally, but a different drying procedure was used. The seeds were dried in an oven set at 45°C for two weeks. After this period the seeds were then finely ground for a similar reason as above.

### 4.2.2 Off-line SFE extractions from *Cedrela toona*

Total extractions of the wood

The home-made SFE system described in section 3.2.1 was used for all the off-line extractions. 3.0 g of the ground wood was placed in the empty HPLC extraction vessel and extracted at a pressure of 350 atm and a temperature of 40°C for 50 minutes in a dynamic mode. Extractions were performed using pure SFC/SFE grade carbon dioxide (Air Products and Chemicals, Allentown, PA, USA). The supercritical fluid was decompressed into about 3.0 mL of dichloromethane (BDH Chemicals Ltd., UK) in a 10

mL volumetric flask using a 30 cm length of a 50 $\mu$m i.d. deactivated fused silica capillary (SGE, Australia). The restrictor tubing was heated to avoid plugging using hot air delivered from a hand-held drier. This extract was analysed by on-column capillary GC-FID, capillary GC-MS, capillary SFC-UV and capillary SFC-off-line FTIR microspectrometry described in Chapter 3.

Selective extractions of the wood

An extraction was performed on a 3.0 g sample of the wood at 40°C and different pressures of $CO_2$ in order to selectively extract specific compounds from the wood. The extracts were trapped in dichloromethane and monitored by capillary gas chromatography as described in section 3.3.1. The selectively-extracted fractions that were ultimately obtained were analysed by proton nuclear magnetic resonance spectroscopy described in section 3.5.

Off-line extractions of the bark

3.0 g of the pulverised bark of the plant was also dynamically extracted at 390 atm and 40°C for 50 minutes and the extracts from the linear restrictor was trapped as described for the wood extract. The extract was analysed by capillary GC-MS as described in section 3.3.1.

### 4.2.3 Off-line SFE from the seeds of *Melia azedarach*

The seeds of *Melia azedarach* contained a high percentage of oil so modifications to the extraction techniques in section 4.2.2 were made. A sorbent with a high oil affinity was introduced into the system to increase the adsorption of the oil and thereby improving the extraction selectivity. 2.5 g of silica gel 9385 (Merck, Germany) was placed in the fingertight stainless steel extraction vessel (Keystone Scientific) and a stream of supercritical $CO_2$ was passed through this sample at 390 atm and 50°C for 30 minutes. This procedure was to ensure that the silica gel was free from any contaminants prior to extraction of the seed. After this initial step, 3.0 g of the pulverised seed was placed on top of the silica bed in the extraction vessel and extraction was performed at 390 atm and

50°C for 60 minutes. The extracts were depressurised through a crimped 1/16" o.d. stainless steel tubing. Hexane, a good solvent for oils, was chosen as a trapping solvent for the extracts decompressed through the stainless steel tubing. A white solid appeared in the course of the extraction indicating the presence of an extract insoluble in hexane. The hexane was decanted after the extraction, and the white solid was dissolved in dichloromethane and subsequently analysed by capillary supercritical fluid chromatography using FID as described in section 3.4.

### 4.2.4 Soxhlet extractions

Extraction of *Cedrela toona*

Extractions were performed on 3.0 g samples of the pulverised wood using the Soxhlet apparatus shown in **Figure 3.3**. The sample was placed in the cellulose thimble and extracted with 150 mL hexane for 4 hours as described in section 3.2.3. After rotory evaporation of the hexane, the residual sample was dissolved in dichloromethane and analysed by on-column capillary GC-FID (section 3.3.1) and capillary SFC-UV (section 3.4 and 3.4.3).

Extraction of *Melia azedarach*

Extraction of the seeds of *Melia azedarach* was initially performed with 150 mL of hexane for 4½ hours to preferentially remove the oil from the seeds. This was followed by an extraction with 150 mL of dichloromethane for 5½ hours. The dichloromethane extract was concentrated to about 10 mL and subsequently fractionated using classical column chromatography. However, no attempt was made at fractionating the hexane extract due to the high oil content.

### 4.2.5 Fractionation of the dichloromethane extract

Preparing the column

The column used was a 38 x 2.5 cm i.d. glass tube fitted with a tap and a head pressure accessory. A small tuft of cotton-wool was placed at the bottom of the column followed by a 1.0 cm high bed of acid washed sand. A quantity of silica gel 9385 support material (Merck, Germany) was poured into the column to a height of approximately 15 cm. The

silica bed was then covered with a 1.0 cm top layer of the acid washed sand. At this stage the column was then washed with a 60:30:10 % V/V/V solvent mixture of dichloromethane:ethyl acetate:methanol respectively.

Elution

The concentrated dichloromethane extract was poured onto the top layer of the column and allowed to pass slowly through the sand bed into the silica-packed material. A solvent mixture of 60:35:10 V/V/V dichloromethane:ethyl acetate:methanol was then poured onto the column and a nitrogen head pressure was applied from a high pressure cylinder to enhance the elution rate. Fractions were collected and aliquots from the collected fractions were spotted on TLC plates and subsequently separated in a thin layer chromatographic tank containing a 60:40 V/V dichloromethane:methanol mixture as the mobile phase. The TLC plates were developed in a tank saturated with iodine vapour and the fractions that produced identical patterns on the developed TLC plates were combined.

Analysis of the combined fractions

The solvent was evaporated off on a water bath and the residue obtained was analysed by proton- and carbon-13 nuclear magnetic resonance spectroscopy as described in section 3.5.

### 4.2.6 On-line SFE-GC analyses of *Cedrela toona*

80 mg samples of the wood and bark of *Cedrela toona* were each analysed by on-line SFE-GC using a flame ionisation detector as described in section 3.3.2.

## 4.3 Results and Discussions

### 4.3.1 Preliminary GC analysis

In the early stages of this study, it was necessary to investigate capillary gas chromatography as a separation method for the qualitative and quantitative analysis of extracts from *Cedrela toona*. As a rule of thumb, Hawthorne [51] indicated that most compounds extracted with carbon dioxide are volatile enough to be analysed by gas

chromatography. However, it was necessary to confirm that this was true. Preliminary GC analysis of an SFE extract using helium as the mobile phase at a flow rate of 0.5 mL/min and an injection aliquot of 1.0 $\mu$L resulted in peak broadening and poor resolution of the later eluting components, as shown in **Figure 4.1a** on page 93. Because of the poor resolution for the later eluting peaks, there was room for further improvement. An attempt was made to improve the chromatographic efficiency by optimising the carrier gas flow rate and also the sample aliquot injected into the column.

Initially, the carrier gas flow rate was maintained at 0.5 mL/min and the sample aliquot was decreased from 1.0 $\mu$L to 0.4 $\mu$L. There was no significant improvement in the separation of the later eluting components. Therefore the carrier gas flow rate was increased from 0.5 mL/min to 1.5 mL/min, and the sample aliquot was then decreased from 1.0 $\mu$L to 0.4 $\mu$L. A 0.4 $\mu$L to 0.5 $\mu$L sample aliquot injection and flow rates of 1.2 mL/min to 1.5 mL/min showed significant improvement in the separation efficiency. A typical chromatogram obtained using a carrier gas flow rate of 1.2 mL/min and a 0.5 $\mu$L aliquot injection is shown in **Figure 4.1b** on page 93 for comparison. This indicated that as the temperature was programmed from 60°C to 320°C the viscosity of the carrier increased and lowered the linear velocity into an inefficient zone of the Van Deemter curve. Using the high column head pressure maintained the column efficiency for the later eluting components. This application highlights the importance of maintaining a constant flow rate or linear velocity over the course of the temperature programme. Once efficient chromatography was obtained, the GC methodology was used to investigate whether comparable extracts could be obtained using both SFE and classical hexane extraction. **Figure 4.2** on page 94 shows the GC chromatograms of the two extracts which from a qualitative basis were identical. Quantitative comparison of these two extracts will be discussed in Chapter 5.

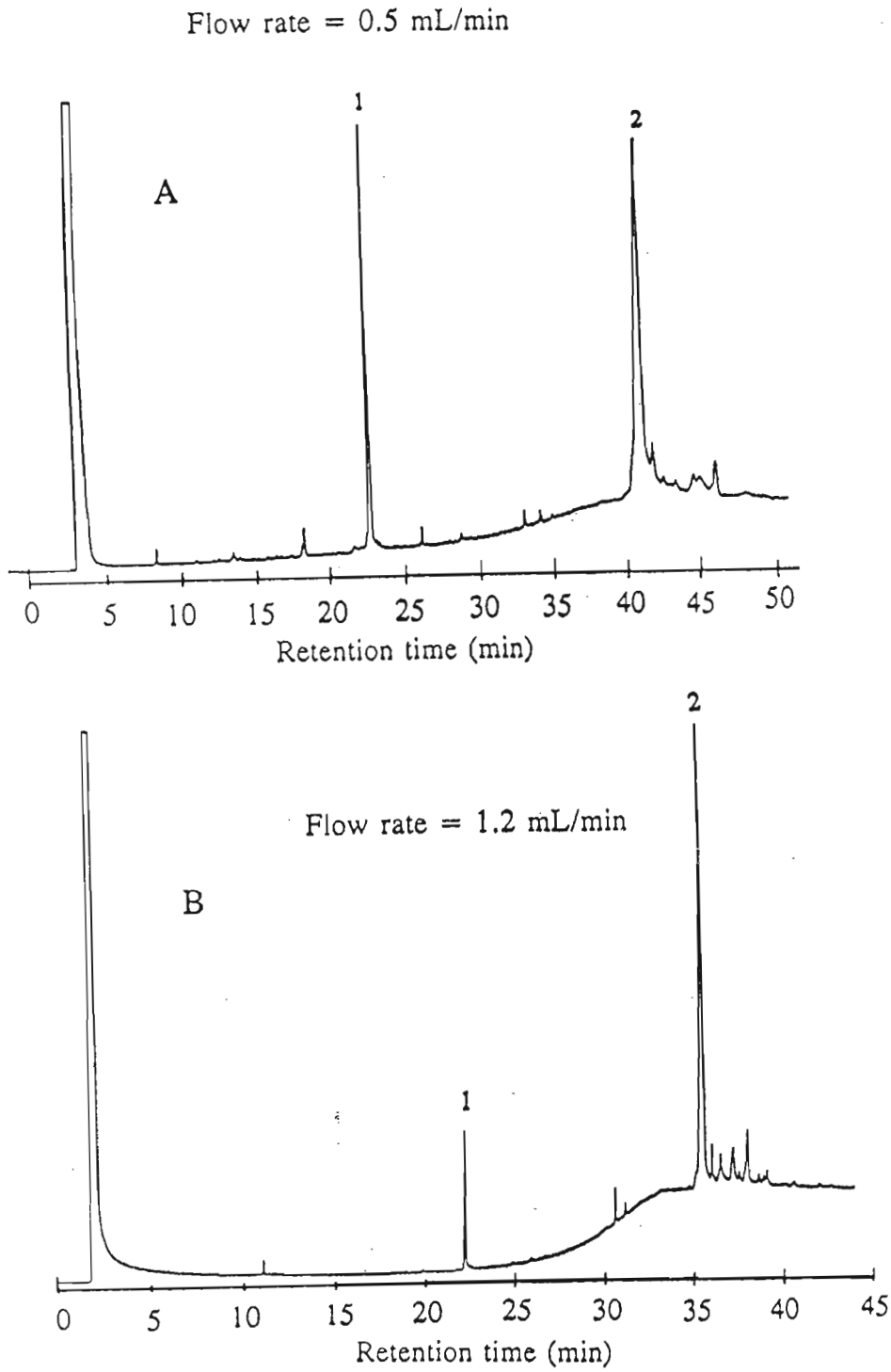



**Figure 4.1** Typical gas chromatograms of extracts obtained by SFE at 350 atm and 40°C. Conditions: Column, 25 m x 250 $\mu$m i.d. fused silica capillary coated with PTE-5 ($d_f$ = 0.25 $\mu$m); Temperature program, 60°C (1 min) then to 150°C at 18°C/min and held for 1 min. 150°C to 320°C at 7°C/min and held for 17-25 min. Carrier gas was helium and FID was at 320°C. (A) 1.0 $\mu$L aliquot injection, (B) 0.5 $\mu$L aliquot injection.

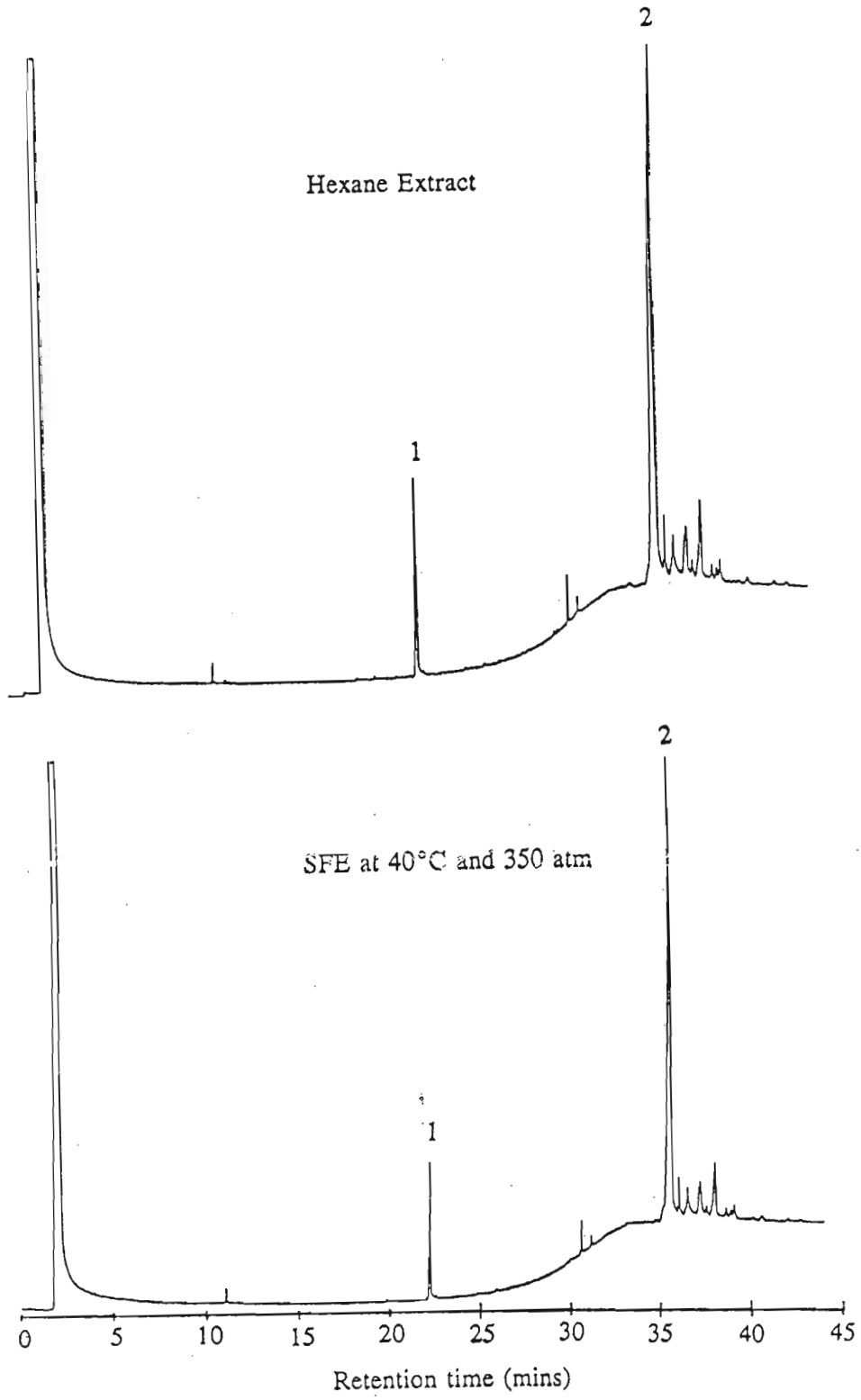



**Figure 4.2** Capillary gas chromatograms of 0.5 $\mu$L injections of extracts from the wood of *Cedrela toona* by SFE and classical Soxhlet extraction using hexane. Carrier gas was helium at a flow rate of 1.2 mL/min. FID was at 320°C. Column and temperature program are as in Figure 4.1. Compound 1 is geranyl geraniol, and 2 is cedrelone.

### 4.3.2 Capillary SFC and proton-NMR analyses

Capillary supercritical fluid chromatography of the wood extracts using UV detection at 207 nm was also performed to confirm that there were no additional involatile components in the extracts. Since pressure programming of the $CO_2$ influences the refractive index of the supercritical fluid mobile phase and hence affects the performance of the detector, it was necessary to zero both the detector and the integrator at the maximum analysis pressure (400 atm). Once this was done, the pump pressure was then brought back to the initial pressure setting (120 atm) before initiating the chromatographic analysis. Typical chromatograms that were obtained for both the SFE (A) and hexane (B) extracts are shown in **Figure 4.3** on page 96 with a descending baseline. Both chromatograms were identical in terms of peak distribution and retention time, and it was again clear that two major components were present in the extracts. Detection at 207 nm, which is the maximum absorption wavelength for the furan ring, was used to identify the presence of limonoids in these extracts. Thus far, the SFC chromatograms showed two major compounds absorbing at 207 nm and hence likely to be limonoids. Therefore, it was necessary to establish whether or not these compounds were limonoids. This was carried out by selectively extracting each of the compounds for further analysis by proton nuclear magnetic resonance (NMR) spectroscopy.

From the proton-NMR spectrum, specific signals can be used to confirm whether the compounds are limonoids or not. Selective extractions were performed on the wood and the extracts obtained were monitored by capillary gas chromatography, and at 80 atm and 40°C (density = 0.300 g/mL), it was possible to selectively extract the compound labelled 1 (in **Figure 4.1**) from that labelled 2. After exhaustive extraction of component 1, the pressure was raised to 110 atm at 40°C (density = 0.696 g/mL) on the same sample and compound 2 was obtained without any trace of compound 1. The variable solvating power of the supercritical fluid resulting from changes in fluid density provides the mechanism for the selective extraction of components. **Figure 4.4** on page 98 shows the GC chromatograms for each of these extracts under identical GC conditions. The trapping solvent was evaporated, and without any further purification step, the proton NMR

spectra were recorded for these fractions using $CDCl_3$ as a solvent. **Figure 4.5** on page 99 shows the proton-NMR spectrum for the extract obtained at 110 atm against a standard spectrum of cedrelone, a limonoid isolated from the wood of *Cedrela toona* [115]. The structure of cedrelone is shown on page 97.

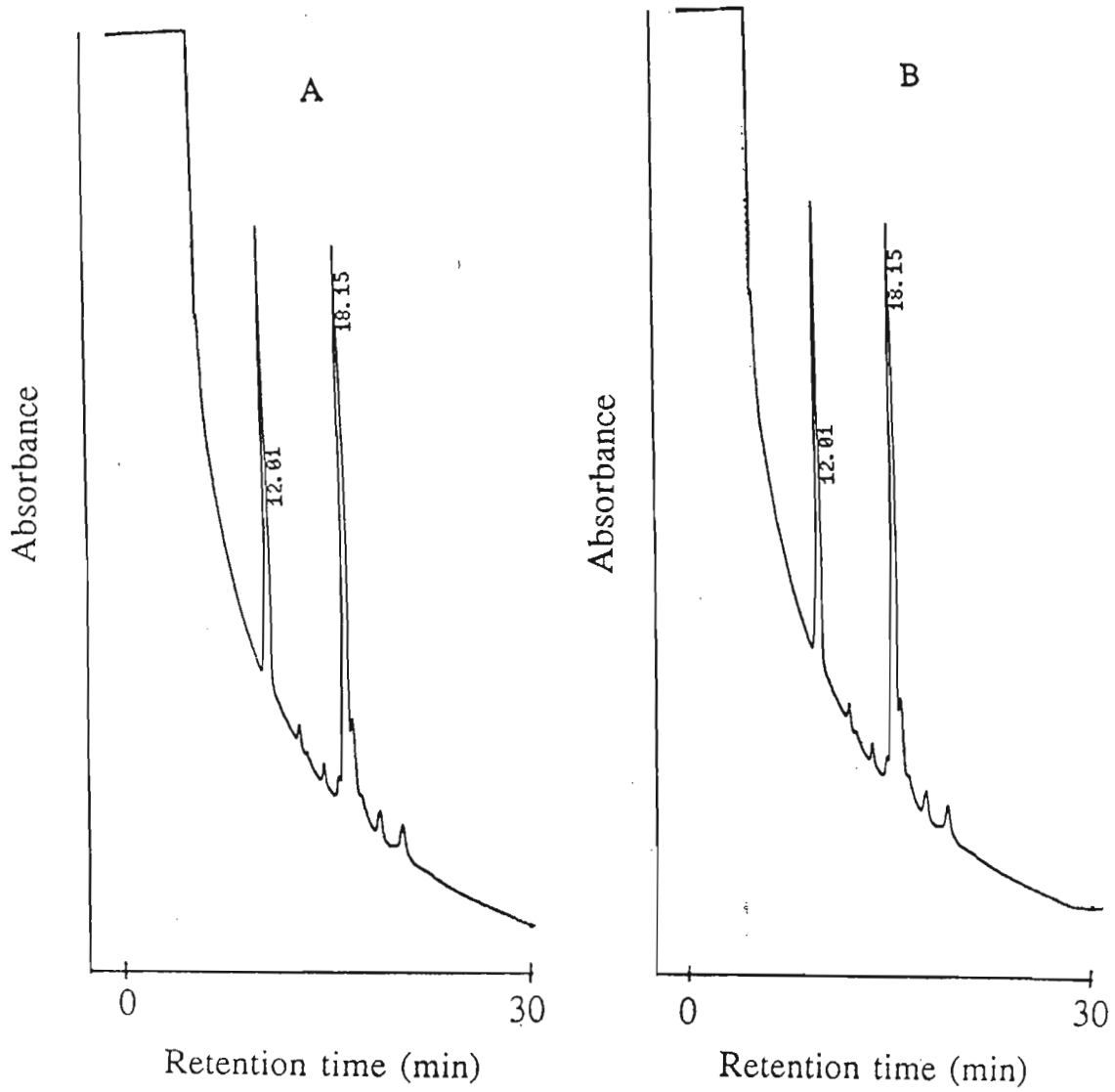
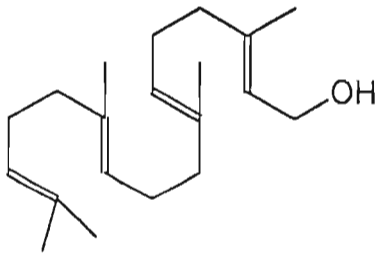
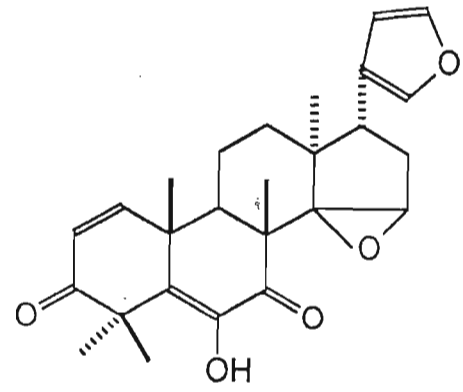



**Figure 4.3** Typical SFC chromatograms for, (A) the SFE extract at 350 atm and 40°C, and (B) a Soxhlet extract. Conditions: Pressure program, 120 atm (5 min) then to 400 atm at 10 atm/min; Oven temperature, 100°C; UV detection at 207 nm; Column, 10 m x 50 $\mu$m i.d. fused silica capillary ($d_f$ = 0.25 $\mu$m) coated with SB-Biphenyl-30.

The signals at $\delta$ 7.4, 7.3 and 6.4 ppm are the characteristic signals for the furan protons in limonoids. These signals were used to confirm that compound 2 was a limonoid. Also, by comparing the sample spectrum with that of the standard, it was evident that the extracted compound at 110 atm showed all the cedrelone resonances and also the presence of impurity signals between $\delta$ 4.0 and $\delta$ 6.0. **Figure 4.6** on page 100 shows the proton spectrum of the compound extracted at 80 atm together with the spectrum of a compound from the same wood identified as geranyl geraniol [115], a diterpenoid whose structure is shown below. Both spectra lacked the typical furan protons characteristic of limonoids. There were also impurity signals at $\delta$ 0.85, 1.25, 7.5 and 7.7 in the sample spectrum that were absent in the reference spectrum. This was not of primary importance since the main aim was to confirm whether the compound was a limonoid. Quite apart from the proton-NMR spectrum, the low elution temperature (240°C-250°C) during the GC analysis was also indicative that the compound was not likely to be a triterpenoid. However, despite the presence of impurity signals, the sample spectrum when superimposed on the standard spectrum showed all resonances related to geranyl geraniol. Interestingly, geranyl geraniol has reportedly been isolated by classical techniques from *Cedrela toona* by Nagasampagi et al. [116].




geranyl geraniol




cedrelone

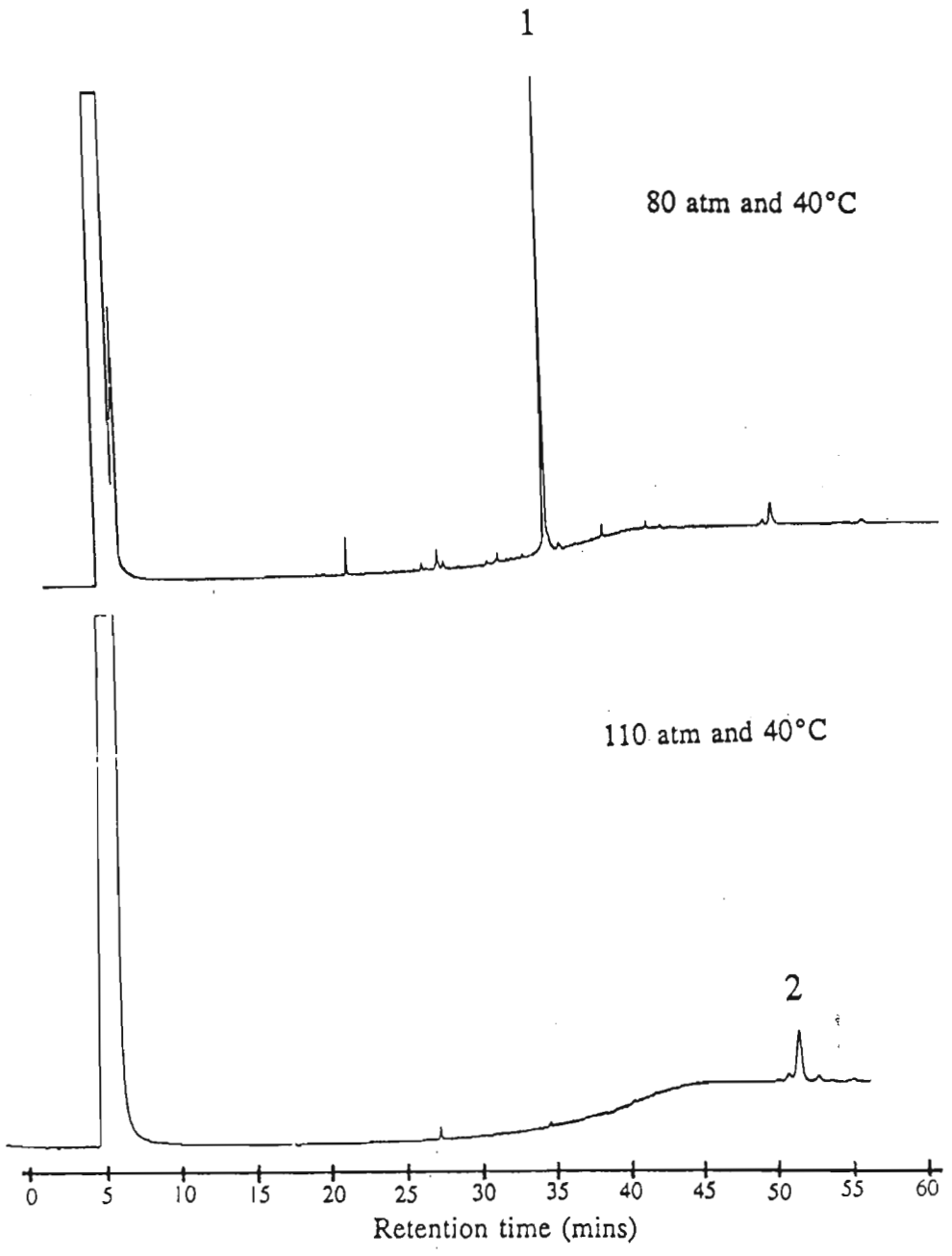



**Figure 4.4** Capillary gas chromatograms of 1.0 $\mu$L aliquot injections of the selectively extracted fractions from the wood of *Cedrela toona*. GC conditions were as in Figure 4.2 except that the carrier gas flow rate was 0.5 mL/min. Key: 1 is geranyl geraniol, and 2 is cedrelone.

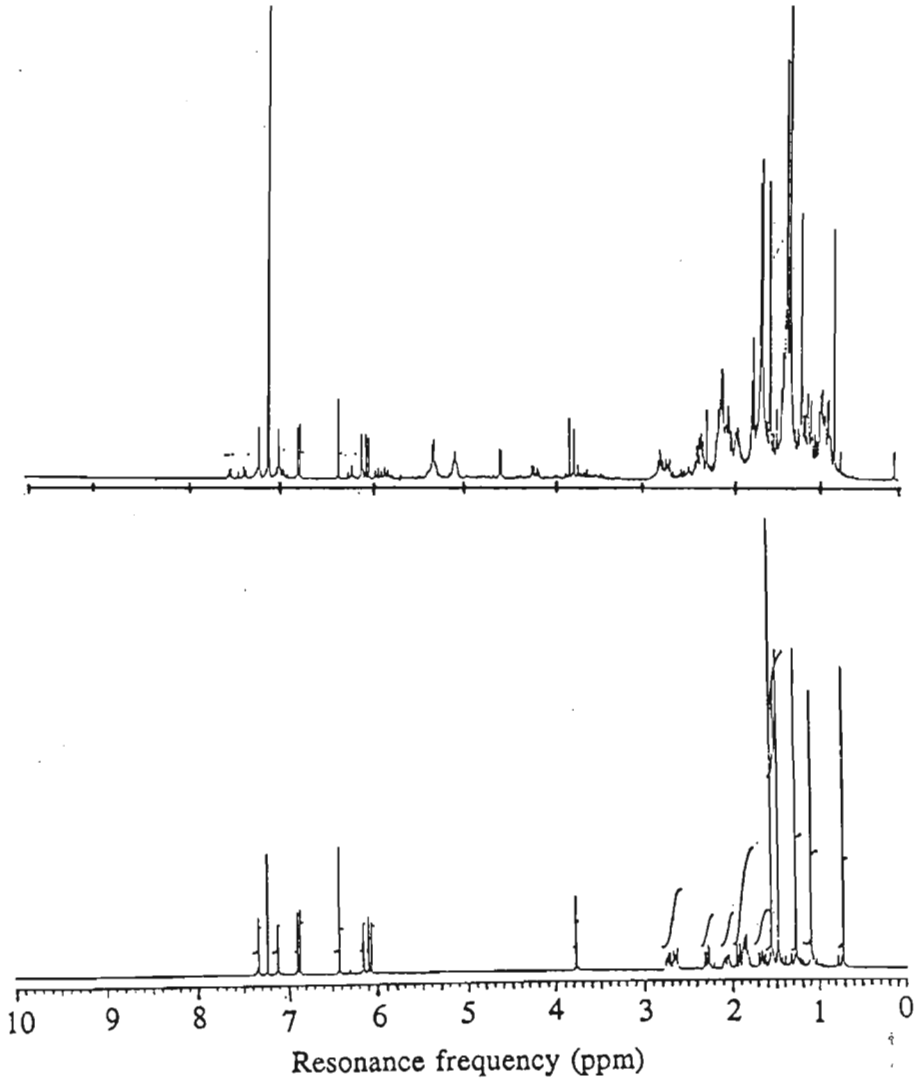



**Figure 4.5** Proton-NMR spectrum for the selectively extracted compound labelled 2 (top), and a standard spectrum of cedrelone (bottom) according to reference 115. $CDCl_3$ was used as solvent.

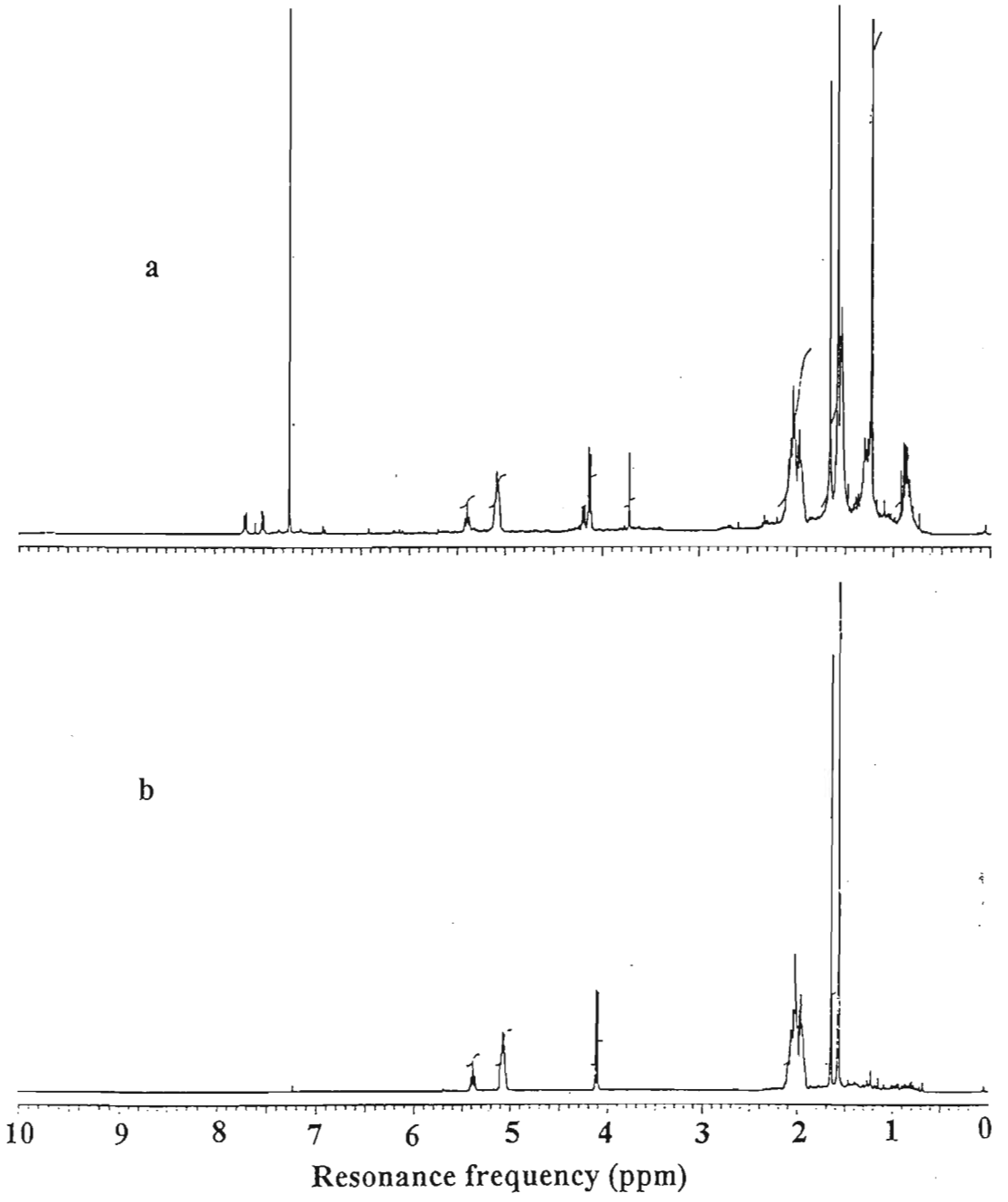



**Figure 4.6** (a) Proton-NMR spectrum for the selectively extracted compound labelled 1, and (b) Proton-NMR spectrum for geranyl geraniol according to reference 115.

### 4.3.3 Capillary GC-MS analyses

As the major focus of this work was on the isolation and characterisation of triterpenoid compounds, further work concentrated on the limonoid. Capillary GC-MS was performed on the wood extract to confirm the structure of the limonoid by means of molecular mass and its fragmentation pattern. The total ion chromatogram of the off-line SFE extract from the wood together with the mass spectrum of the identified limonoid is shown in **Figure 4.7** on page 102. The mass spectrum is consistent with the structure particularly the molecular ion at m/z 422. It was also consistent with the standard mass spectrum of cedrelone on page 103 (shown in **Figure 4.8**) obtained by a direct insertion probe [115].

Capillary GC-MS analyses of the supercritical $CO_2$ extracts from the bark of *Cedrela toona* gave completely different results from those obtained from the wood. Four major compounds were identified, three of which were phytosterols and the fourth was identified as cedrelone. **Figure 4.9** on page 104 shows the total ion chromatogram of the SFE extract from the bark, together with that of the sample and an authentic spectrum of one of the compounds identified using the Wiley library as stigmast-4-ene-3-one. They both show the molecular ion at m/z 412 as well as the base peak at m/z 124. The Wiley library identified three phytosterols as: stigmasterol, dihydrostigmasterol (ß-sitosterol) and stigmast-4-ene-3-one. A fourth compound was identified as cedrelone by a comparison of its mass spectrum with that of cedrelone obtained from the wood. In all the spectra obtained, for example that in **Figure 4.10** on page 105 showing the mass spectrum of the sample peak labelled 2 (top) in **Figure 4.9** and an authentic spectrum of stigmasterol from the Wiley library, sample spectra were marred by background ions particularly the ions of m/z 191, 207, 281 and 429 from the stationary phase. These ions are those resulting from " bleeding" of the stationary phase of the capillary column at the high elution temperatures required for the compounds, and complicate mass spectra. Stationary phase bleeding can be problematic as silicone oligomers from the liquid stationary phase tend to coelute with the sample components during high temperature gas chromatography.

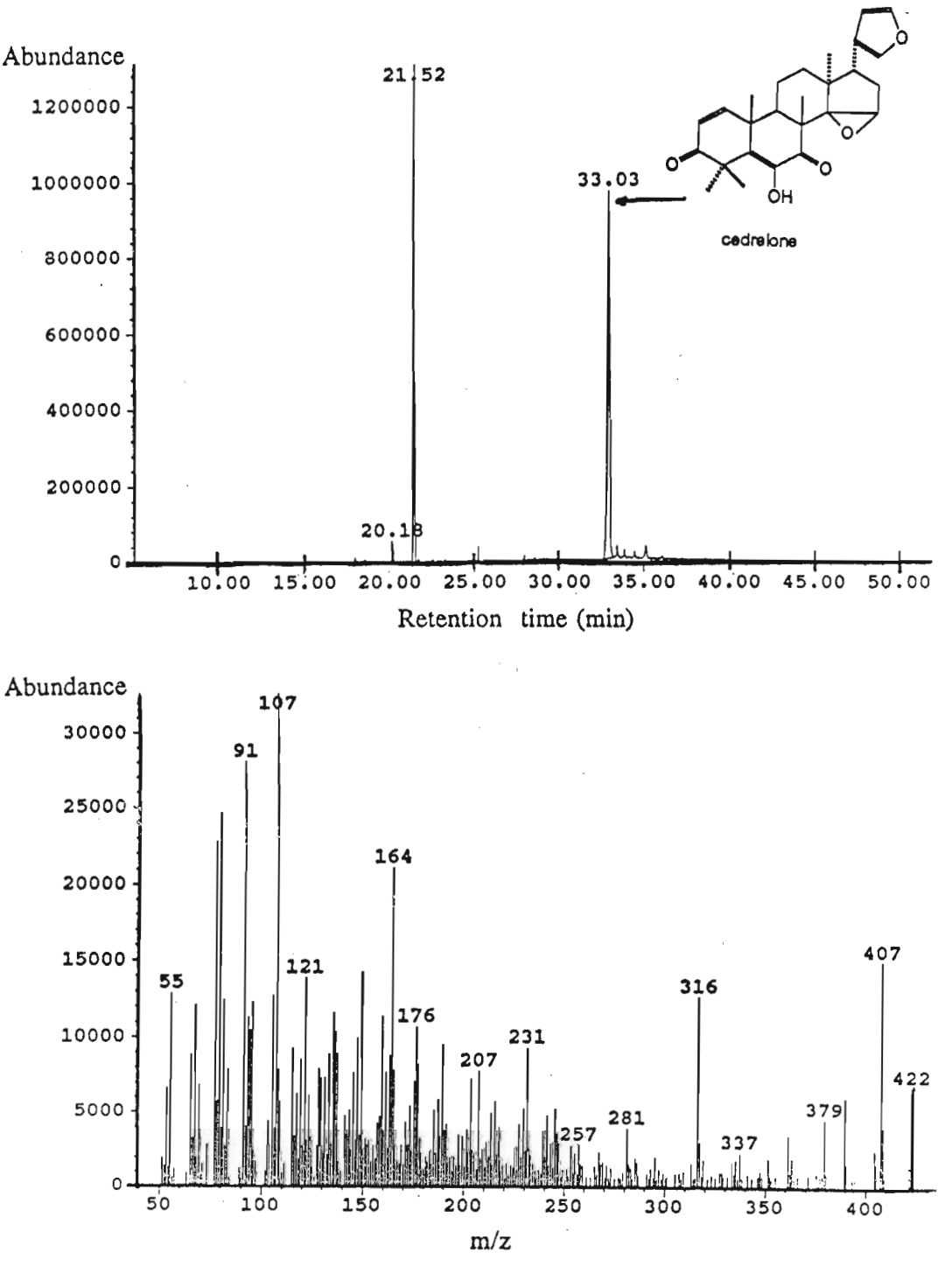



**Figure 4.7** Total ion chromatogram of the $CO_2$ extract at 350 atm and 40°C from the wood of *Cedrela toona* (top), and the mass spectrum for cedrelone (bottom).

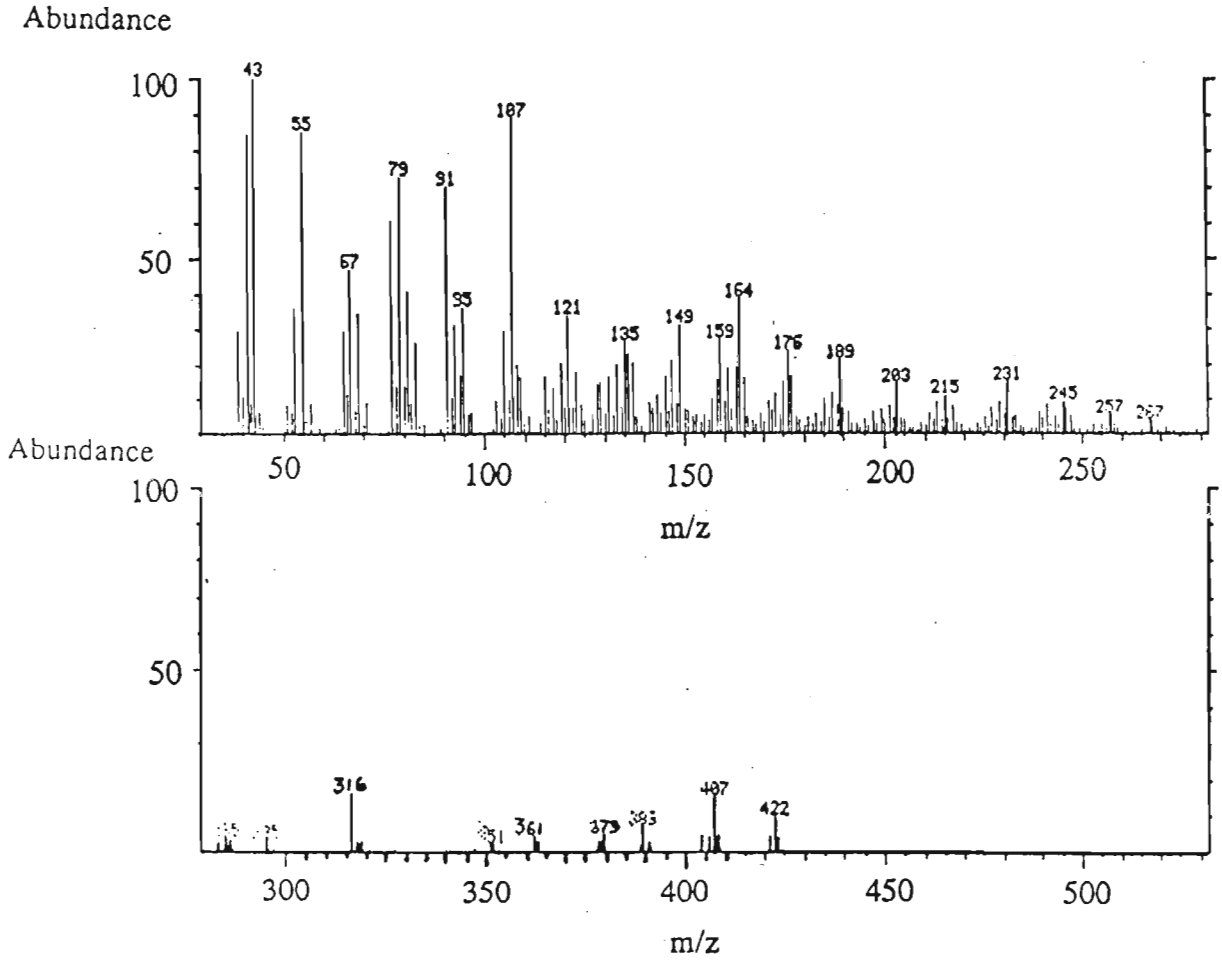



**Figure 4.8** A standard mass spectrum of cedrelone obtained by a direct probe insertion according to reference 115.

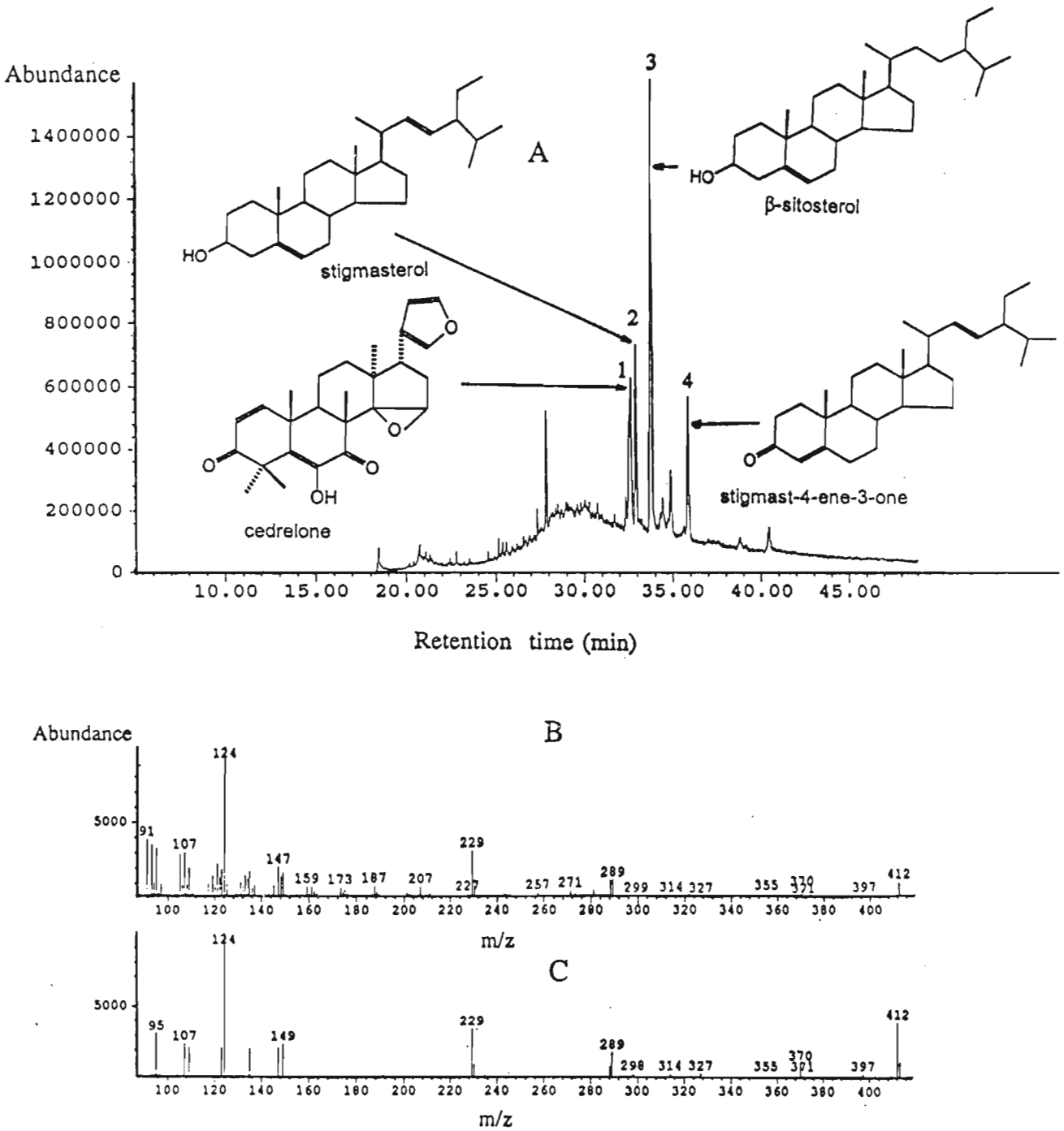



**Figure 4.9** (A) Total ion chromatogram of a $CO_2$ extract at 390 atm and 40°C from the bark of *Cedrela toona*, (B) mass spectrum of the compound labelled 4, and (C) a library spectrum of stigmast-4-ene-3-one.

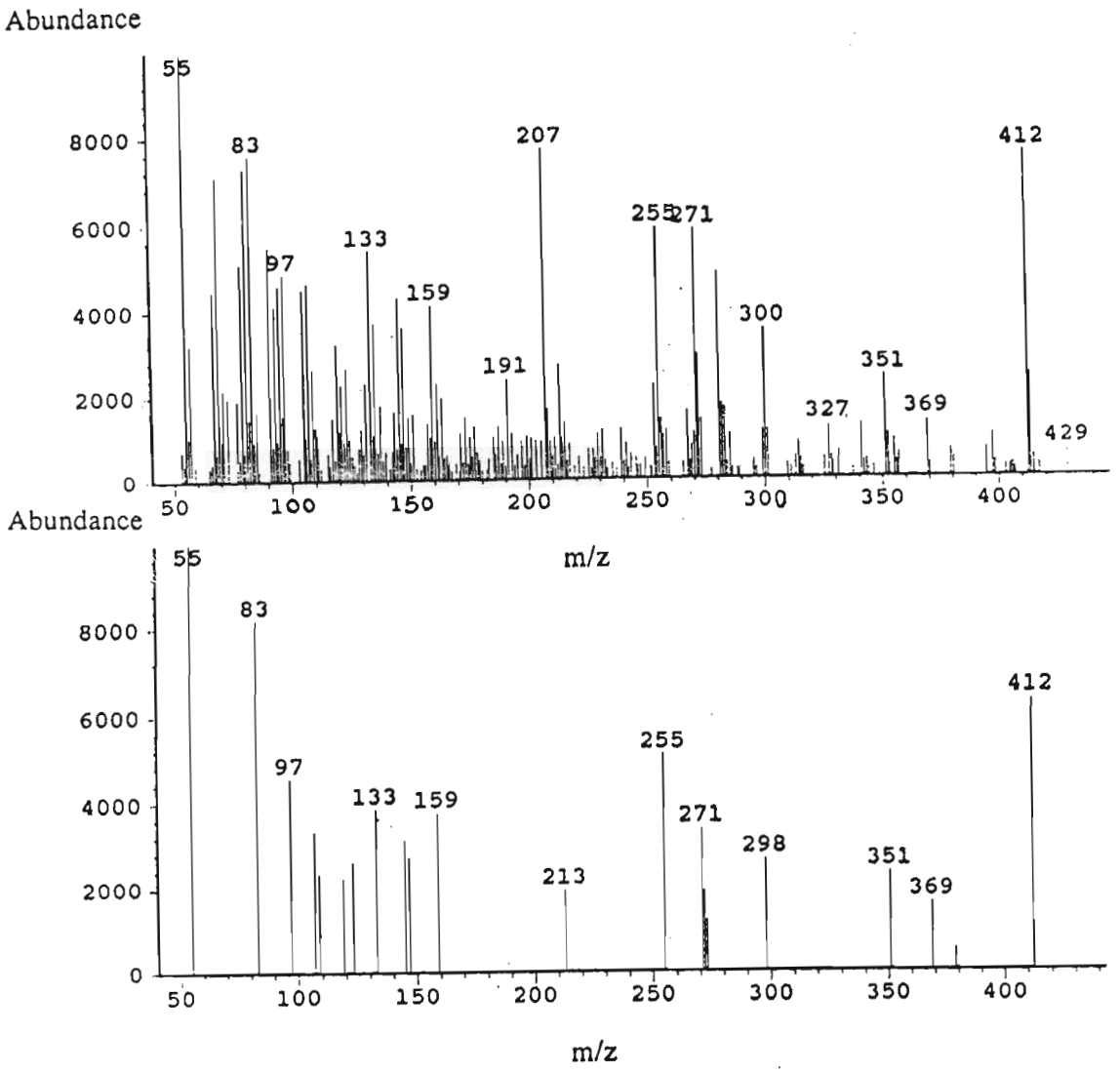



**Figure 4.10** Mass spectrum of the compound labelled 2 in Figure 4.9 (top) and a library spectrum of stigmasterol (bottom).

### 4.3.4 Infrared analysis

Since Fourier transform infrared spectrometry also provides structural information, FTIR microspectrometry was coupled to SFC for further identification of the various functional groups present in the compound. For this technique to be successful, a more concentrated wood extract was injected onto the SFC using flame ionisation detection. The chromatogram obtained is shown in **Figure 4.11** on page 107. On elution, 50-100 ng of the component labelled 2 (supposed to be cedrelone) was deposited on the KBr disc in order to obtain a reasonable infrared spectrum. **Figure 4.12** on page 108 shows the infrared spectrum obtained for cedrelone. The bands at 1165 and 875 $cm^{-1}$ are attributable to the C-H stretching and out-of-plane C-H deformation frequencies respectively for the furan ring. Bands at 3050 and 1541 $cm^{-1}$ are also indicative of the C-H stretching frequency of alkenes, and the alkene double bond stretching frequency respectively. Bands at 1246 and 941 are also characteristic of cyclic epoxides. The broad hydrogen-bonded O-H stretching frequency at 3360 $cm^{-1}$ and the rather low carbonyl stretching frequency at 1622 $cm^{-1}$ are characteristic of an enolic system. Also, the strong band at 1682 $cm^{-1}$ may be attributable to an $\alpha$,ß-unsaturated carbonyl stretching frequency. However, the intense nature of the 1682 $cm^{-1}$ band could be indicative of a possible overlap between the two carbonyl stretching frequencies at positions 3 and 7. It is clear that the infrared spectrum reflects the functional groups present in the molecule. The infrared spectrum of a standard sample of cedrelone (prepared as a thin film on KBr) was recorded on a Nicolet Impact 410 FTIR spectrometer (Nicolet, Madison, USA) and shown in **Figure 4.13** on page 109. Comparing this with the sample spectrum shown in **Figure 4.12**, both spectra are identical in pattern except for slight frequency shifts that exist between them. By the solvent elimination technique, interference due to the mobile phase was eliminated (except the "hunch" on the baseline at around 2350-2400 $cm^{-1}$ which are $CO_2$ absorptions resulting from incomplete background subtraction of $CO_2$ present in the atmosphere).

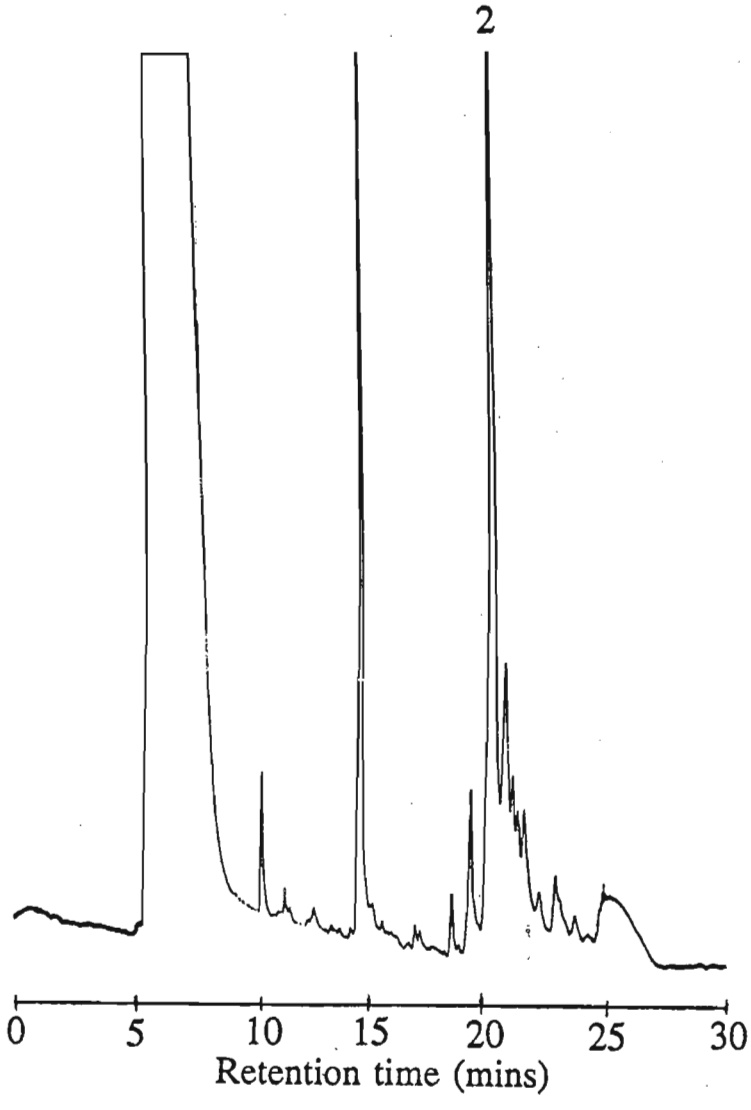



**Figure 4.11** Capillary SFC chromatogram of a concentrated $CO_2$ extract at 350 atm and 40°C from the wood of *Cedrela toona*. Compound labelled 2 was deposited on a KBr disc after eluting through the heated transfer line. SFC conditions were as in Figure 4.3. FID was at 320°C.

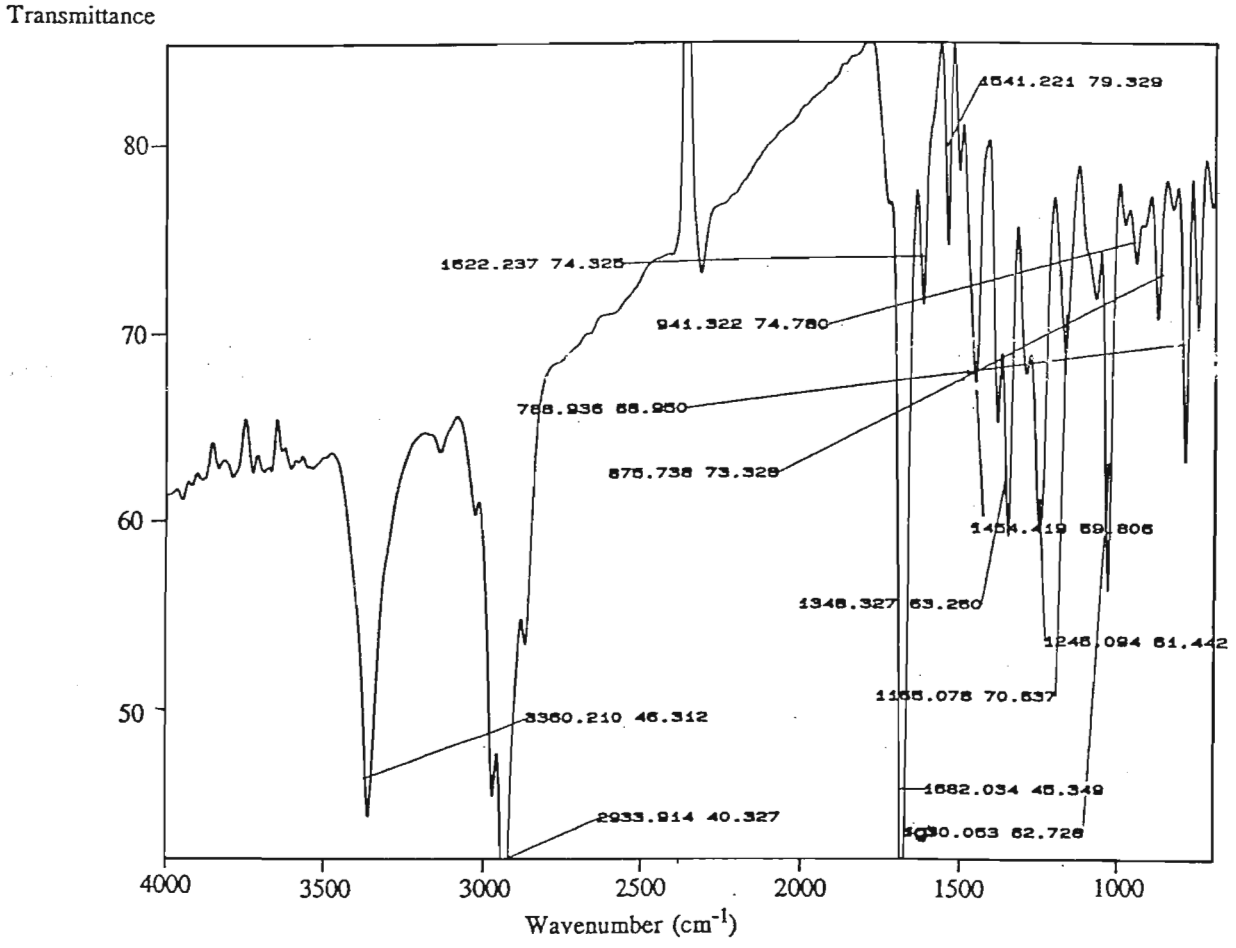



**Figure 4.12** Infrared spectrum for compound labelled 2 in Figure 4.11.

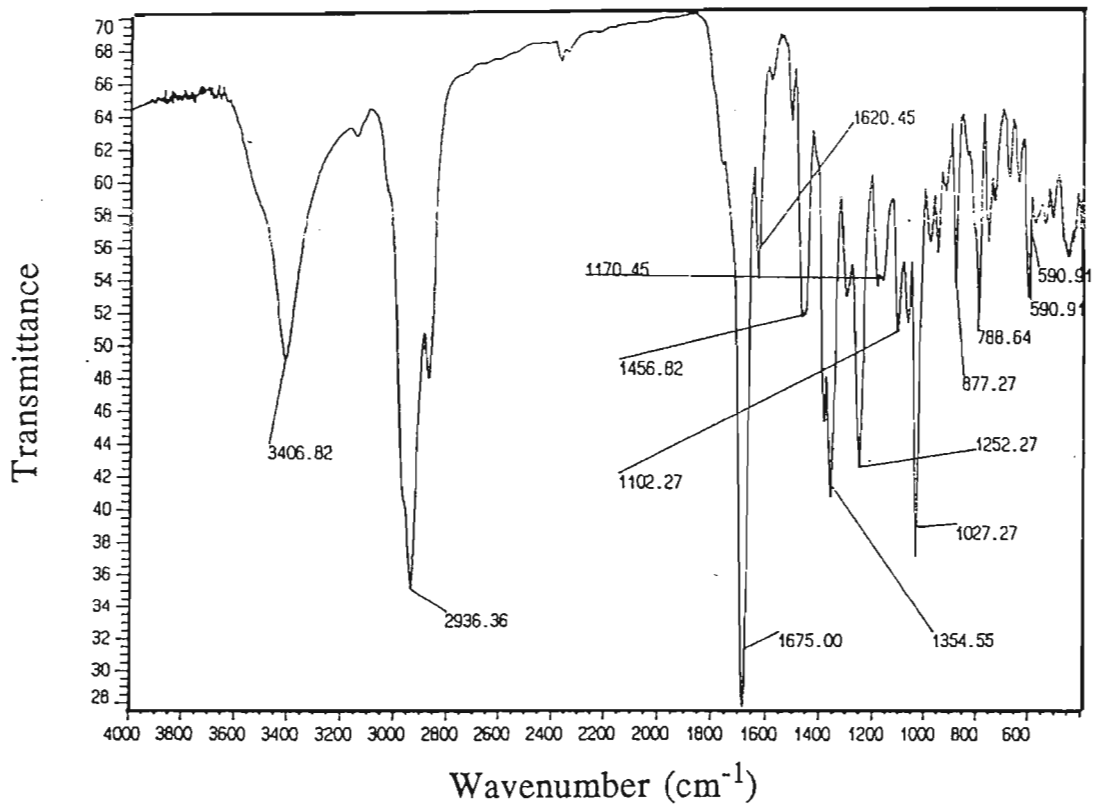



**Figure 4.13** Standard infrared spectrum of cedrelone recorded from a thin film on KBr.

### 4.3.5 On-line SFE-GC analyses of the wood of *Cedrela toona*

Effect of Injection port temperature

Initially, the effect of the injection port temperature (from 200°C-300°C) on the on-line analysis of the wood of *Cedrela toona* was investigated using an oven temperature of 50°C. Preliminary indications were that at an injection port temperature of 200°C, cedrelone which was the target compound under study was not sufficiently volatilised to be focussed into the GC column. **Figure 4.14** on page 111 shows typical chromatograms (from one minute dynamic extractions) obtained during the preliminary study. Injection port temperatures of 200, 250 and 300°C were investigated. Contrary to the chromatogram obtained at a port temperature of 200°C, port temperatures of 250 and 300°C were high enough to volatilise the compound. However, there were variations in the retention time reproducibility of cedrelone at an injector temperature of 200°C on the one hand, and at 250 and 300°C on the other. Since GC operating conditions were identical except for the difference in the port temperature, it is possible that the incomplete volatilisation of this compound may have caused the slight increase in retention (at 200°C than at 250 and 300°C) and hence the observed discrepancy.

Effect of oven trapping temperature

The next stage of the study was to investigate the effect of oven trapping temperature on the chromatographic efficiency. An injection port temperature of 250°C was used for the this study as excessive port temperatures needed to be avoided to prevent thermal degradation of compounds. Using a fixed port temperature of 250°C, the effect of oven temperatures of 40°C, 50°C and 60°C on the chromatographic efficiency from the one minute dynamic extracts were then studied. It was clear that none of the temperatures gave better chromatographic efficiency than the others. Cryogenic trapping of the analytes onto the stationary phase of the capillary column was not necessary as the analytes of interest were only semi-volatile.

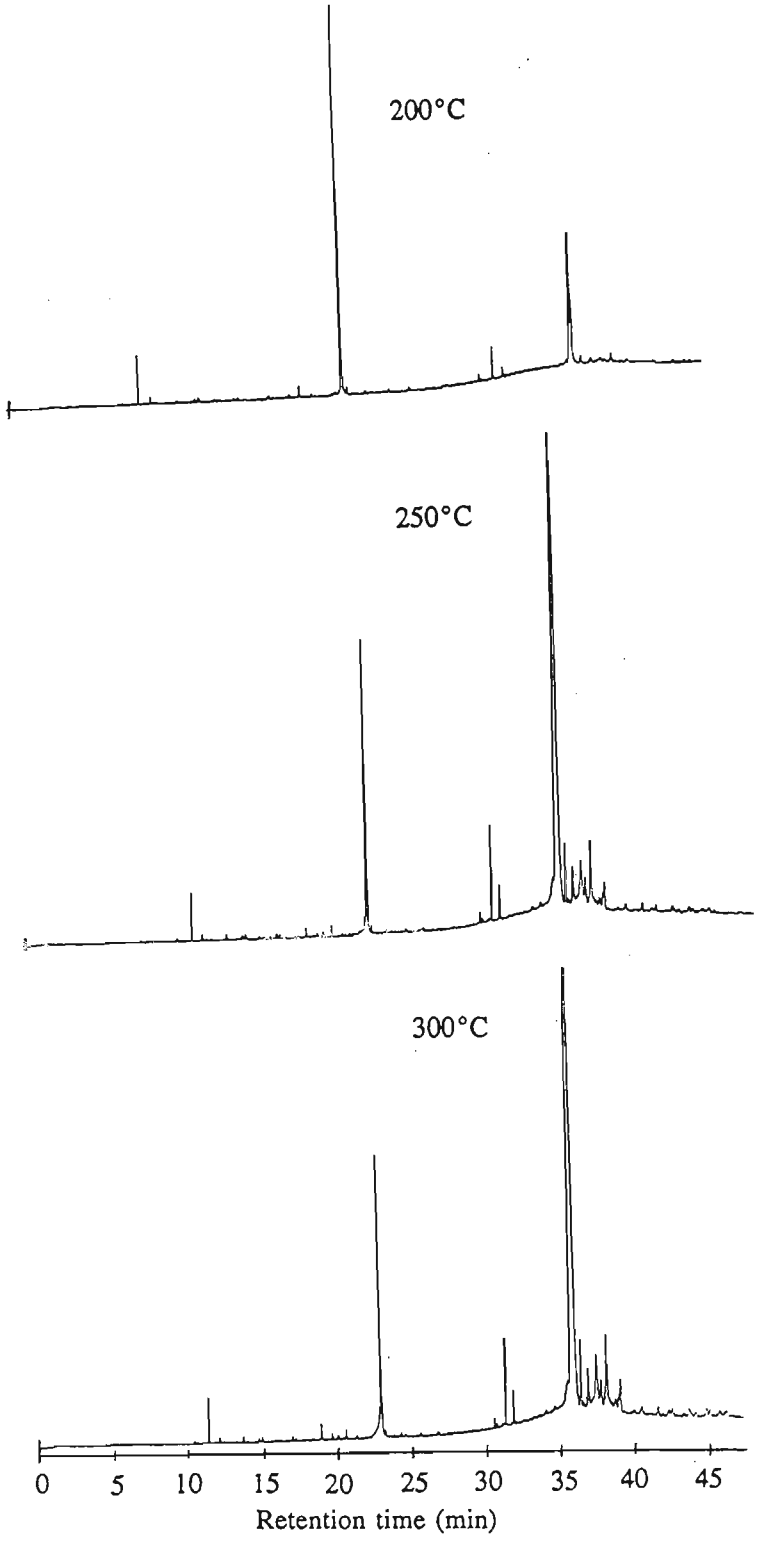



**Figure 4.14** Capillary gas chromatograms showing the effects of injection port temperatures on the on-line chromatographic efficiency of extracts from the wood of *Cedrela toona*. Trapping temperature was at 50°C and FID at 320°C.

Exhaustive extraction and peak area reproducibility

Second and third one minute extractions were also carried out to ascertain whether the initial one-minute extraction period was enough to achieve exhaustive extraction of the 80 mg sample of the ground wood. **Figure 4.15** on page 113 shows the chromatographic profiles with effluent trapping at 60°C. There was no reason why trapping could not have been performed at either 40°C or 50°C since the preliminary study had shown that the chromatographic performances at the three temperatures were identical. It was also necessary to investigate the peak area reproducibility for cedrelone using the on-line analysis technique. The average raw peak areas for cedrelone (three injections) in the first and second one minute extractions were 2089304 and 287184 respectively. About 88% cedrelone could be extracted in the first one minute since no extracted compounds were found in the third minute as seen from **Figure 4.15**. There were no extractable components in the matrix after the second one minute extraction. This was confirmed by the fact that when the extraction pressure was stepped up to 350 atm and 400 atm, no detectable analytes were observed from the chromatograms obtained and therefore it was reasonably clear that exhaustive extractions were achieved in the first two extractions at 300 atm. Peak area reproducibilities for cedrelone by the on-line SFE-GC technique were comparable with a typical off-line on-column manual injection as shown in **Table 4.1**.

**Table 4.1** Comparison of peak area reproducibilities of an on-line SFE-GC and an on-column manual injection for cedrelone.

| Extract | On-line SFE-GC (split injection) | Off-line SFE/GC (on-column injection) |
|---|---|---|
| 1 | 1997720 | 147037952 |
| 2 | 2105830 | 141931328 |
| 3 | 2089304 | 150913792 |
| Mean | 2064284 | 146627691 |
| RSD % | 3.30 | 2.51 |

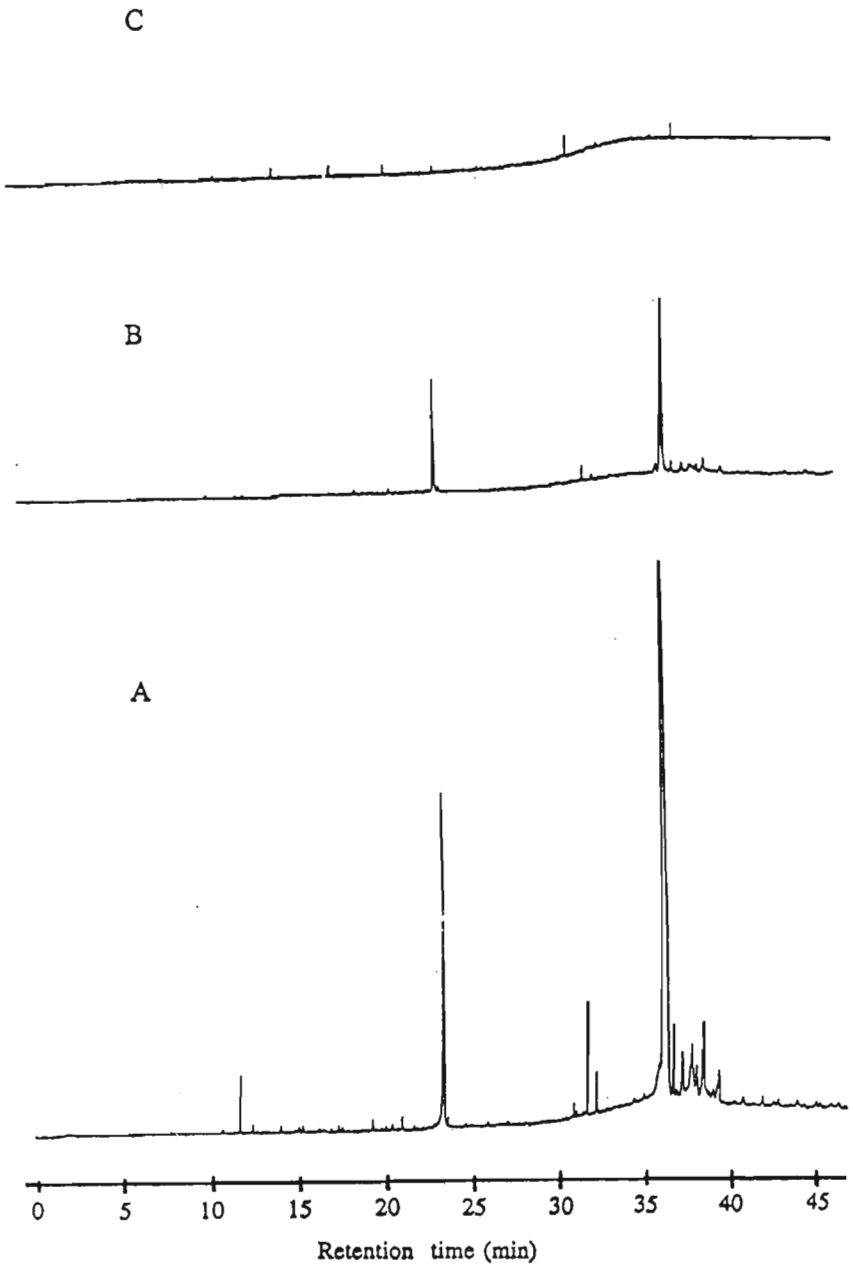



**Figure 4.15** Capillary gas chromatograms showing the exhaustive extraction of analytes from the wood of *Cedrela toona*. Key: (A) first 1 minute dynamic extract, (B) second 1 minute dynamic extract, and (C) third 1 minute dynamic extract, all on the same sample. Trapping temperature was at 60°C.

According to the relative standard deviation (RSD) figures in **Table 4.1**, it is possible to quantify the analyte using the on-line methodology. By choosing appropriate conditions for an on-line study, exhaustive extractions could be obtained in a relatively shorter time. With the combination of a small sample size of 80 mg, a high gaseous flow rate of 150 ml/min at a pressure of 150 atm, and a high split ratio of 1:200, exhaustive extractions are achieved in the shortest possible time. This may be in direct contrast to typical off-line extractions where either as a result of the large sample sizes employed, or factors which are not very clear due to the complexity of the true nature of the mechanism of SFE, higher fluid densities and a combination of other factors may be required to achieve exhaustive extractions.

### 4.3.6 On-line SFE-GC analysis of the bark of *Cedrela toona*.

The bark was also analysed on-line using an 80 mg sample size. Trapping temperatures of 40°C, 50°C and 60°C were investigated at a fixed injection port temperature of 250°C. **Figure 4.16** on page 115 shows the capillary GC chromatograms obtained at various oven temperatures. From the chromatograms, it is clear that a trapping temperature of 60°C was not good enough as seen from the low peak heights. It is not clear whether this was due to sample losses during the trapping process. Despite this minor problem, these on-line studies, have shown that the 80 mg samples used in the studies were a true representation of the matrix composition. This may not always be the same for other sample matrices and it should therefore be mandatory to perform an off-line investigation alongside the on-line study.

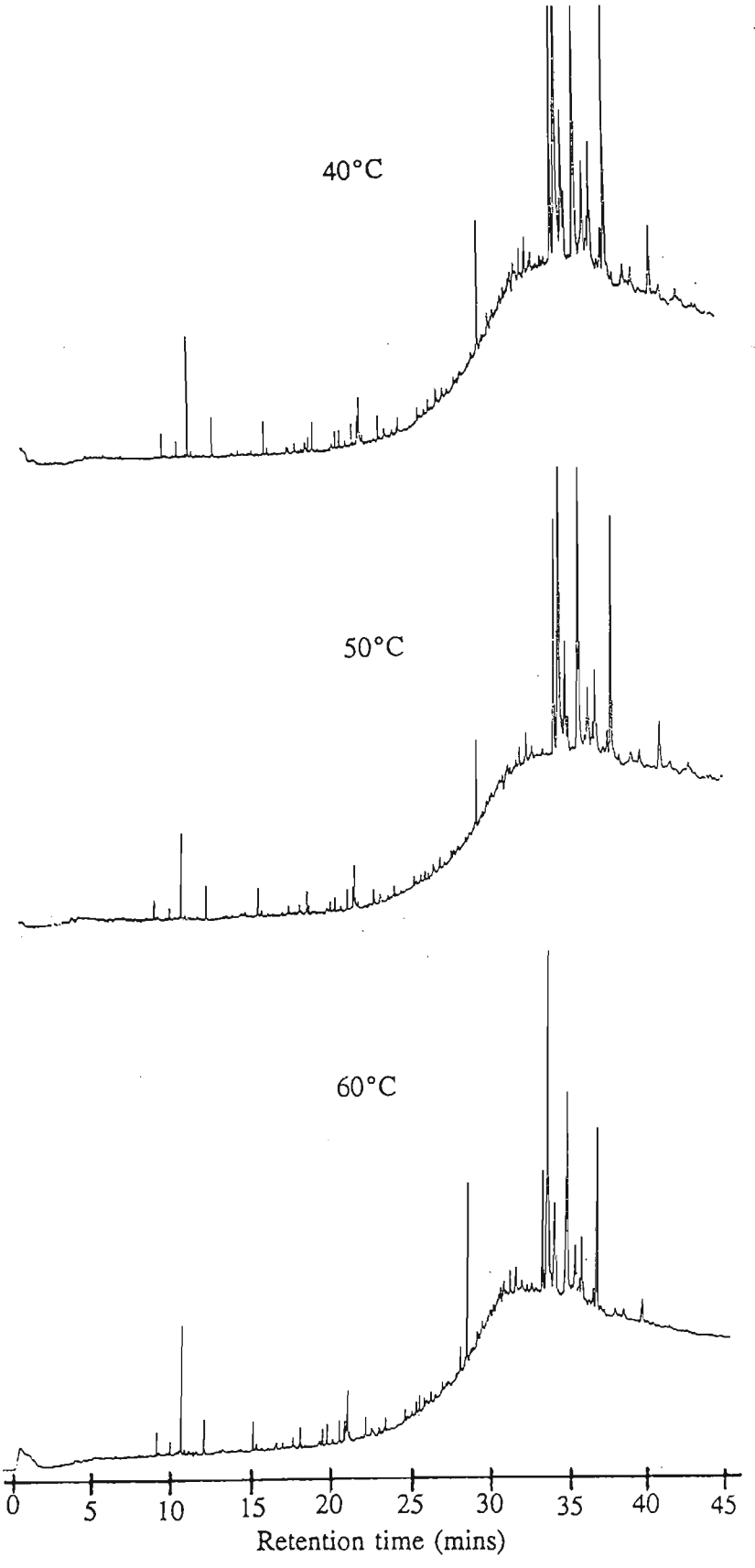



**Figure 4.16** Capillary gas chromatograms showing the effects of oven trapping temperatures on the on-line chromatographic efficiency of extracts from the bark of *Cedrela toona*. Injection port temperature was maintained at 250°C, and the FID was at 320°C.

### 4.3.6 Extraction and analysis of *Melia azedarach*

Extraction

The extraction of the seeds of *Melia azedarach* was the most difficult of all the extractions performed in this study in view of their high oil content. In the supercritical fluid extraction, fine particles of silica gel 9385 were used as a bed for the ground seed sample. The reason for this was to trap selectively the oil that might be coextracted with the analyte of interest. The selective extraction of the analytes without the oil by adjusting the $CO_2$ density alone is a very tricky procedure which may or not be possible depending on the relative solubilities of the two. Since a portion of the oil would be coextracted with the target analytes, a far more practical approach was to add a sorbent with a high fat affinity into the extraction vessel with the hope of trapping the coextracted oil. Despite this approach, the use of a linear deactivated fused silica capillary restrictor placed at the extraction cell outlet was not successful because it got blocked quickly. Thus part of the coextracted oil traversed the silica trap into the capillary restrictor. Therefore a crimped 1/16" o.d. stainless steel tubing was used as the flow restrictor at the outlet of the extraction vessel. Dynamic extractions were then successful, and extracts were trapped in hexane. In the course of the extraction, the initially clear trapping solvent became turbid, and at the end of the extraction period a white crystalline solid was formed in the hexane. On settling, the hexane was decanted and the solid residue left at the bottom of the container was dissolved in dichloromethane and analysed by capillary supercritical fluid chromatography on an SB-Biphenyl-30 column.

NMR and SFC analysis

The above observations showed that it was possible to initially extract the seeds with hexane to remove the oil without having to extract the components of interest. Following the hexane extract, dichloromethane extraction could then be performed to isolate the analytes of interest. This proposed two-stage liquid solvent extraction, firstly with hexane followed by dichloromethane on a 3.0 g sample of the ground seed was performed. The dichloromethane extract was concentrated and fractionated on a silica gel 9385 column with an initial solvent mixture of 60:35:5 v/v/v dichloromethane:ethyl acetate:methanol

respectively. A combined fraction was obtained using this elution mixture, and was analysed by proton and carbon-13 NMR spectroscopy as well as capillary SFC. The proton NMR spectrum of this combined fraction gave no indication of a limonoid, however, the carbon-13 NMR spectrum shown in **Figure 4.17** on page 118 compared well with an authentic spectrum of melianone, a protolimonoid isolated from *Melia azedarach* [117,118,119]. The carbon-13 NMR spectrum shows evidence of an epimeric mixture as there are two doublets at $\delta$ 102 and $\delta$ 98 which are both assigned to C-21. The hemiacetal ring opens and closes in solution, resulting in two possible isomers. The pair of doublets and singlets at approximately $\delta$ 66 and $\delta$ 58 represent C-24 and C-25 respectively and confirm the presence of the epoxide ring. The fact that resonances occurred in pairs indicated that both isomers were present. Capillary supercritical fluid chromatography on an SB-Biphenyl-30 column was performed at this stage to confirm whether or not both isomers were present in solution. Interestingly, a pair of well-resolved compounds was obtained as shown in **Figure 4.18** on page 119 for both the supercritical $CO_2$ extract and the combined fraction obtained from the classical dichloromethane extract. It is possible that these are the pair of diastereomers giving rise to the pair of doublets in the carbon-13 spectra in **Figure 4.17**. The spectrum also exhibits a singlet at $\delta$ 217 which is the characteristic region for keto groups assigned to C-3. The singlet and doublet at $\delta$ 146 and $\delta$ 118 respectively represent C-8 and C-7 of the alkene double bond.

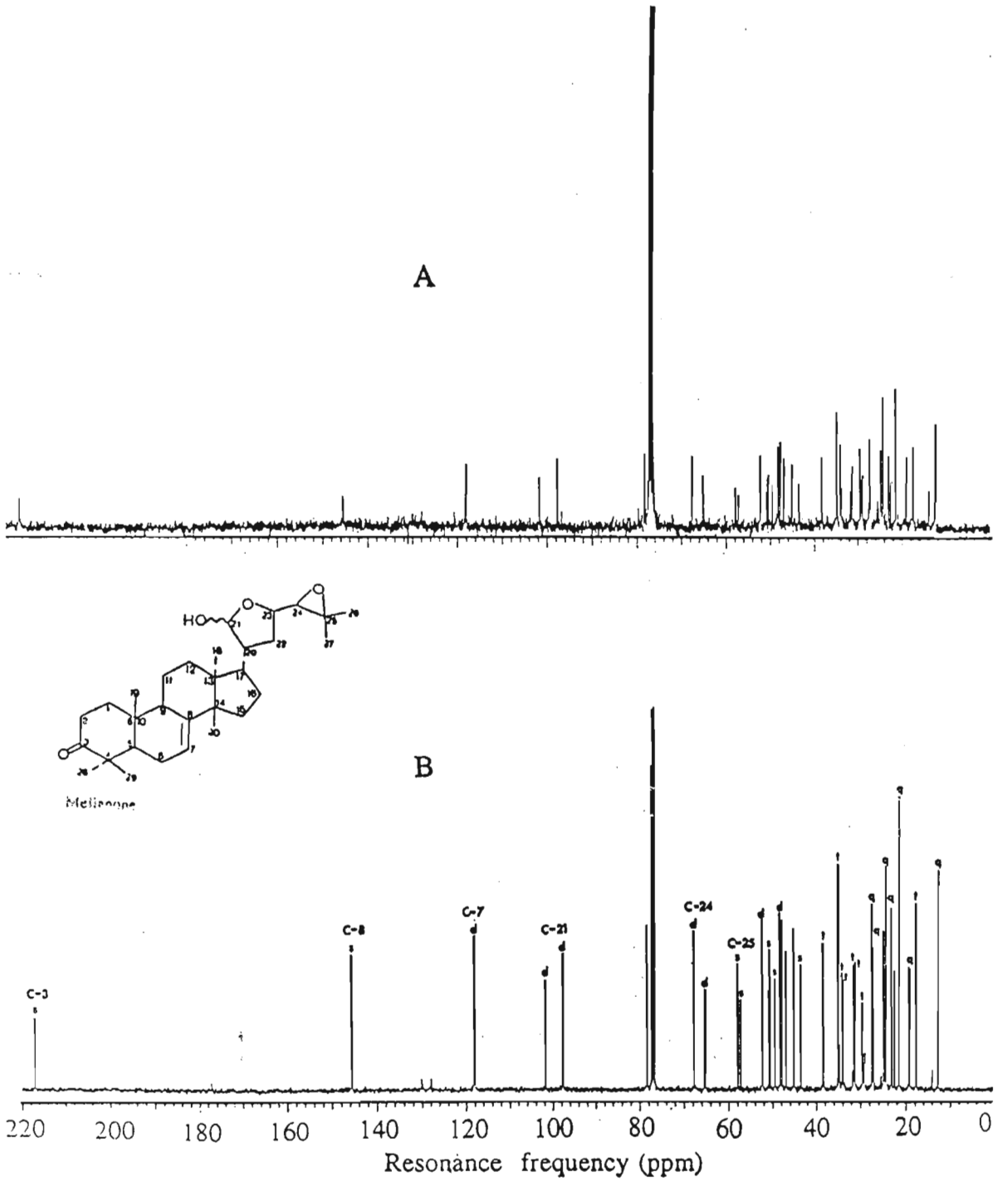



**Figure 4.17** Carbon-13 NMR spectra for melianone. Key: (A) sample spectrum, (B) authentic spectrum according to references117-119.

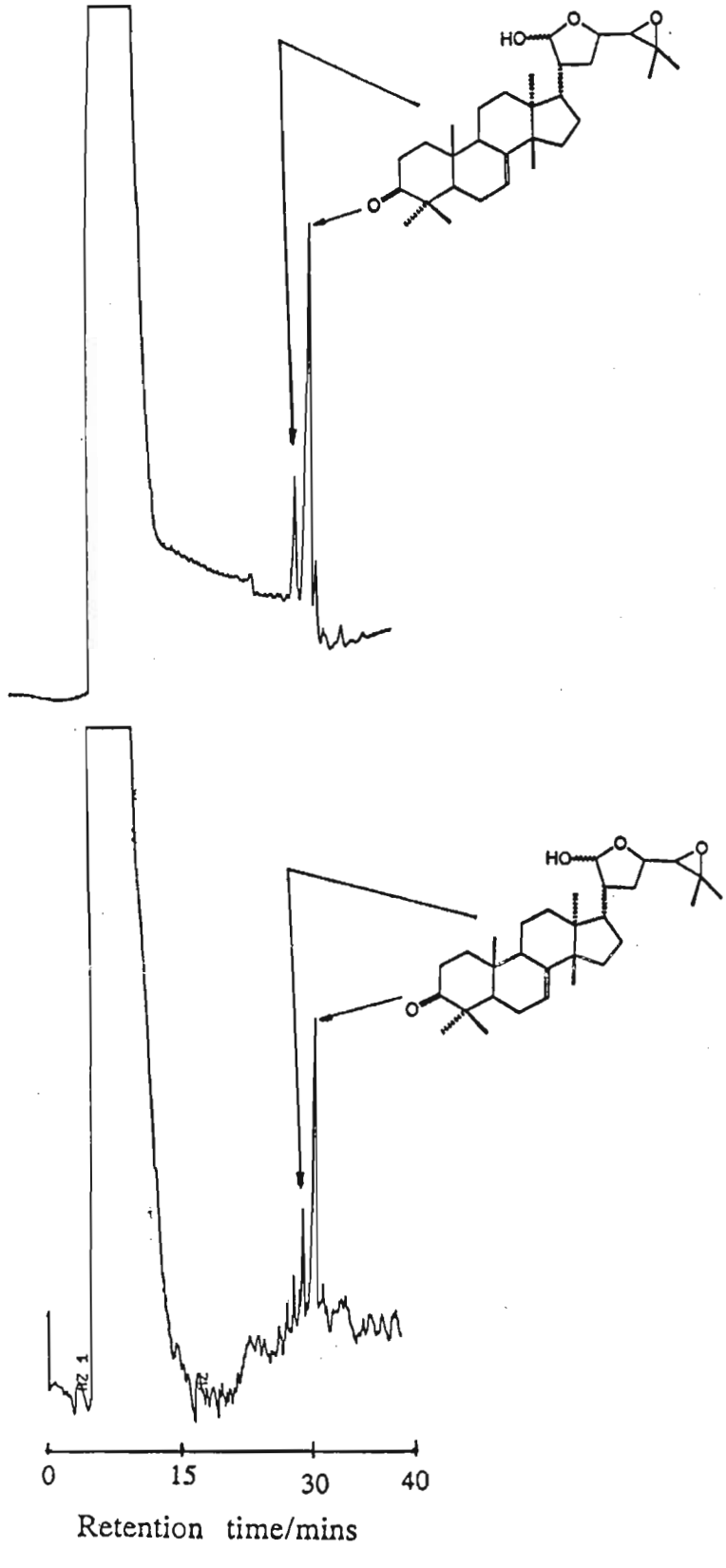



**Figure 4.18** Capillary SFC chromatograms of extracts from the seeds of *Melia azedarach*. Key: (top),SFE extract at 390 atm and 50°C; (bottom),an eluted fraction obtained from a liquid solvent extraction in a Soxhlet apparatus.

## 4.4 Conclusions

In this chapter attempts at utilising both off-line and on-line supercritical fluid extraction -capillary gas chromatography were investigated, as well as sample identification using both proton and carbon-13 NMR spectra. The benefits of directly coupling SFE to GC were that no sample handling was required between the extraction and chromatography step; a shorter analysis time was achieved; the possibility of sample contamination was avoided and the extraction effluents were reproducibly transferred onto the capillary column for direct analysis. Also, when using flame ionisation detection, no detector responses (solvent peaks) appeared for SFE grade $CO_2$, thus making it possible for the determination of volatile components that are often masked by liquid solvents. However, the limitations of coupling SFE to GC are defined by the volatility constraints of high molecular weight solutes in complex matrices.This study has also shown that compound identification using carbon-13 NMR spectra are easier to use than the proton-NMR spectra as traces of impurity compounds such as organic solvents that are not completely evaporated from the sample give rise to background signals making spectra interpretation often difficult if not impossible. Also, the elimination of the use of large volumes of liquid solvents in all the preparation steps leading ultimately to the structural elucidation of the compound is not completely impossible. Pure samples for spectroscopic analyses could be obtained from the crude extract without the use of classical column chromatography. To obtain the infrared spectrum for a pure compound at the nanogram level, the SFC-off-line FTIR microspectrometric technique described in section 3.4.4 was possible. However, since larger sample sizes are required for NMR spectroscopic studies, a rather different chromatographic approach was required. Preparative or semi-preparative supercritical fluid chromatography of the SFE extract using on-line UV detection could have been performed so that eluting components from the end of a capillary restrictor (attached to the outlet of the flow cell) could be collected after passing through the UV flow cell, for subsequent spectroscopic analyses.

# CHAPTER FIVE

# APPLICATION OF A DYNAMIC EXTRACTION MODEL IN THE STUDY OF THE SFE OF CEDRELONE FROM CEDRELA TOONA.

## 5.1 Introduction

From preliminary studies discussed in chapter four, it is clear that SFE with $CO_2$ gives comparable extracts to classical solvent extraction with hexane, but in a fraction of the time. This may be attributed to a combination of gas-like mass transfer properties and liquid-like solvating characteristics of the supercritical fluid. However, as the sample matrix and particle size may also affect the extractions, it is clear that SFE of any particular solute from a sample matrix is a complex process involving many interacting factors.

One of the characteristics of SFE is that most of the compound of interest is removed from in and around the surface of the matrix during a short period as extraction begins, but subsequently, the extraction rate tails off dramatically. This is thought to be due to solute adsorption on the matrix surface and because some of the solute remains held in the structure of the matrix and is only extracted slowly with time. Thus complete extraction is rarely accomplished. This is independent of the manner in which the solutes are distributed and results in quantification problems in analytical extractions. However, if a suitable extraction model could reliably predict the amount of solute present without exhaustively extracting the compound, the quantification problems could be resolved. Further, such a model should be able to predict the factors controlling the extraction of a particular solute, ie. whether the extraction is controlled by diffusion processes or by solubility limitation.

Various models for SFE, mostly based on mass balance have been proposed [120-123]. However, as most models require a knowledge of the matrix characteristics, which in the case of natural products is difficult to understand (because samples are never spherical and vary in particle size), it is difficult to model an extraction. In many cases the simple first

order extraction rate law or " hot ball " model applies [120]. The model describes a solid sphere of radius $r$ with a uniform initial concentration of a material that is immersed into a fluid in which the concentration of extracted material is zero. The model also assumes that the rate of flow of the fluid is so high that the fluid remains infinitely dilute, and also the solute is uniformly distributed throughout the matrix. It is then necessary to solve the diffusion equation for the system with appropriate boundary conditions. The problem is mathematically similar to that of the immersion of a hot sphere into a cold fluid (hence the name "hot ball model") for which the solutions are given by Carlslaw and Jaeger [124] which in a later publication, Crank [125] translated the equations into diffusion terms. Adaption of the published solutions [126, 127] leads to the following equation for the ratio of the mass, $m$, of extractable material that remains in the matrix sphere after extraction for time, $t$, to that of the initial mass of extractable material, $m_o$,

$$m/m_o = (6/\pi^2) \sum_{n=1}^{\infty} (1/n^2) \exp\left(\frac{-n^2\pi^2 Dt}{r^2}\right) \quad \text{. . . . . 5.1}$$

where **n** is an integer and **D** the diffusion coefficient of the material in the sphere. Equation 5.1 may be simplified by defining a quantity $\mathbf{t_r}$, which is proportional to time for any given system (and is therefore a reduced or scaled time) by the following equation:

$$t_r = \pi^2 Dt/r^2 \quad \text{. . . . . 5.2}$$

In terms of the scaled time, equation 5.1 becomes

$$m/m_o = 6/\pi^2 \left[\exp(-t_r) + 1/4 \exp(-4t_r) + 1/9 \exp(-9t_r) + \ldots\ldots\right] \quad \text{. . 5.3}$$

The solution is therefore a sum of exponential decays, and at long times the later (more rapidly decaying) terms will decrease in importance and the first exponential term in the square brackets will become dominant. A plot of ln ($m/m_o$) against the length of extraction time or a quantity proportional to time becomes linear at long times as shown in **Figure 5.1**. The initial steep fall represents the period where the majority of the analyte is extracted.

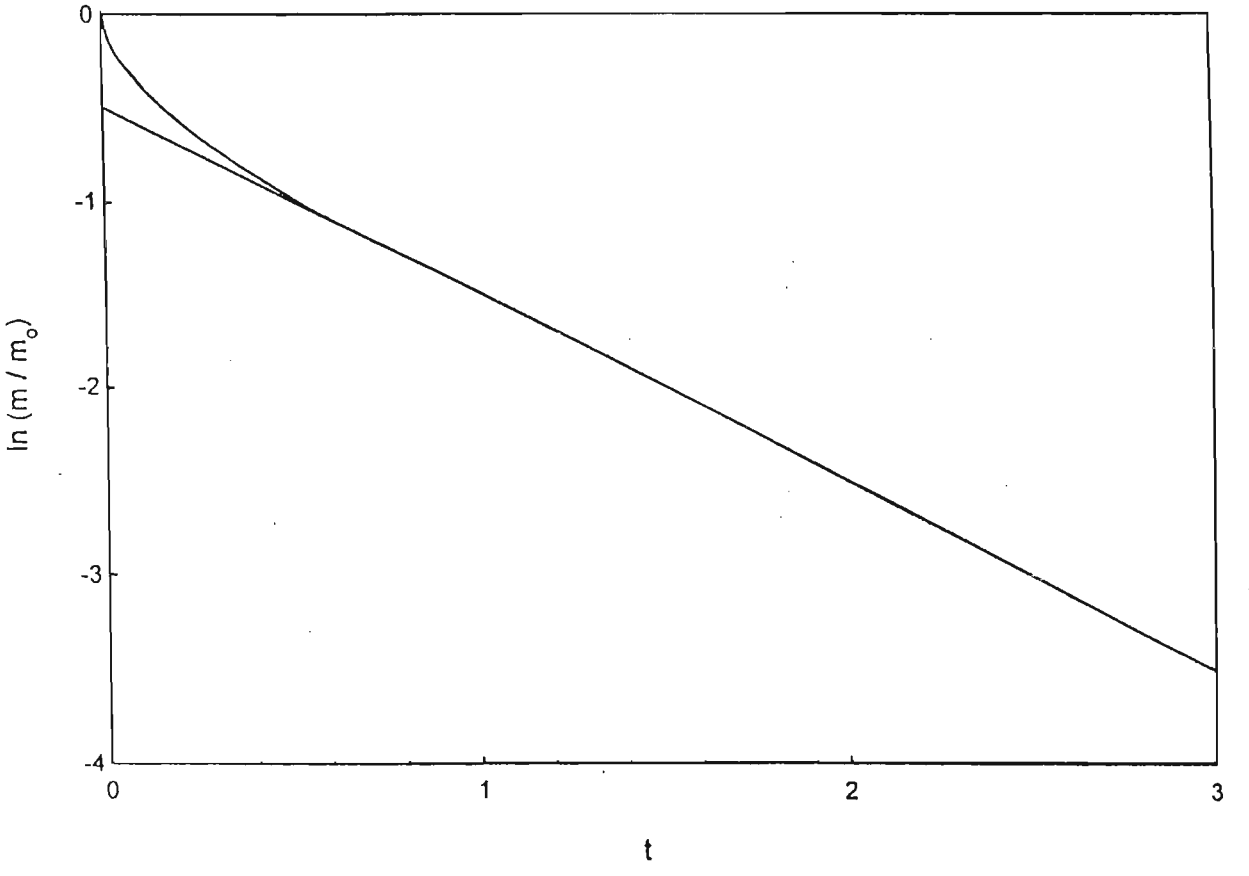



Figure 5.1 Theoretical extraction curve for a perfect sphere.

The exponential behaviour of the extraction after this initial period means that extrapolation may be used to obtain the actual mass of analyte present without exhaustively extracting the sample. The interception of the linear portion of the kinetic plot on the ln ($m/m_o$) axis carries information related to particle size and distribution, as well as the nature of the extraction-controlled processes, either by solubility limitation or diffusion controlled processes. The linear portion of the curve is indicative of extraction from deeper within the matrix and is the most time-consuming part of the extraction.

If the extraction is carried out to obtain an extracted mass $\mathbf{m_1}$, followed by two successive extractions to obtain extracted masses $\mathbf{m_2}$ and $\mathbf{m_3}$, then from equation 5.3, the model predicts that the mass of solute $\mathbf{m_0}$ in the sample is given according to equation 5.4:

$$m_o = m_1 + \frac{m_2^2}{m_2 - m_3} \quad \text{5.4}$$

A number of studies have been undertaken to evaluate this model. Walker et al. investigated the model for the SFE of pinene, camphene, camphor and borneol from rosemary [128], and found that the results compared well with the mean of three exhaustive extractions to give $\mathbf{m_0}$. Liu et al. [129] also used the model to determine the amounts of polycyclic aromatic hydrocarbons from a spiked soil sample, and Subra et al. [130] tested whether this method could be used to model the supercritical fluid extraction of two major carotenes present in carrots.

In this chapter the extraction and subsequent quantification by off-line GC of the limonoid cedrelone from the wood of *Cedrela toona* (Meliaceae) using pure supercritical $CO_2$, methanol-modified $CO_2$ and liquid hexane will be discussed. The "hot ball" model is used to estimate the value of $\mathbf{m_0}$ (the amount of cedrelone present in the wood) by extrapolating data obtained from shorter extraction times using pure $CO_2$ from equation 5.4. The calculated value of $\mathbf{m_0}$ is then compared to those from exhaustive extractions using

methanol-modified $CO_2$ and liquid hexane to investigate the possibility of using this model in quantitative predictions. Further, the nature of the kinetic plot of ln $(m/m_0)$ against time is made use of in an effort to understand the factors controlling the SFE process.

## 5.2 Experimental

### 5.2.1 Sample preparation

The test sample used was the ground wood of *Cedrela toona* (obtained locally), from a branch which had been dried under solar conditions for six weeks. The wood was separated from the bark, ground into a powder (the particle size of which was not characterised) and extracted using various fluids as described below.

### 5.2.2 Extractions

Pure $CO_2$ extractions

Extractions for the kinetic studies based on 3.0 g of the ground wood, were performed dynamically on the home-assembled SFE unit described in section 3.2.1 using the 24 ml Keystone fingertight stainless steel vessel. Supercritical fluid extractions using SFC/SFE grade $CO_2$ (Air Products and Chemicals, Allentown, PA, USA) were conducted at pressures of 300 and 350 atm at 40°C. 30 cm lengths of a 50 and 75 $\mu$m i.d. deactivated fused silica capillary (SGE, Australia) were used as the extraction cell outlet restrictors. The restrictors were kept from plugging by heating with hot air from a hand-held drier. The compounds extracted were collected for several time intervals by purging the extraction cell effluent into about 5.0 ml of analytical grade dichloromethane for subsequent analysis by GC.

Methanol-modified $CO_2$ extractions

Methanol-modified $CO_2$ extractions were performed using the ISCO SFE system described in section 3.2.2. 40 $\mu$L of methanol was spiked into a 0.6 g sample in the 10 mL extraction vessel prior to extraction. A 30 cm length of a 50 $\mu$m i.d. deactivated fused silica capillary (SGE, Australia) was used as the extraction cell outlet restrictor. A 30 minute static extraction at 400 atm and 40°C (during which time the valves were manually

actuated) followed by a 40-minute dynamic extraction under the same conditions was performed. Extracts were collected in the suitably-placed vials shown in **Figure 3.6c** containing 5.0 mL of dichloromethane for subsequent analysis by GC.

Soxhlet extraction

3.0 g of the ground wood sample was placed in a 100 mm x 30 mm i.d. cellulose thimble and extracted exhaustively with 150 ml of hexane in a Soxhlet apparatus for 5½ hours in a manner described in section 3.2.3. After the 5½ hour period, the hexane was evaporated and the contents in the flask were dissolved in 5.0 ml analytical reagent dichloromethane (BDH Chem. Ltd., UK) for subsequent analysis by capillary GC.

### 5.2.3 Quantification of extracted analytes by gas chromatography

An internal standard method was used for quantifying the amounts of cedrelone in the extracts. Initially, standard solutions containing known concentrations of a standard sample of cedrelone and a fixed concentration of the internal standard (methyl palmitate) were prepared in dichloromethane to generate a five point calibration curve using capillary gas chromatography. The calibration curve was used to determine the response factor for cedrelone relative to the internal standard.

Procedure

Different volumes of a stock solution of standard cedrelone (obtained from Professor D.A.H. Taylor) prepared in dichloromethane (2.1 mg/mL) and a fixed volume from a stock solution of methyl palmitate (Sigma Chem.) (2.0 mg/mL) in dichloromethane were prepared to a total volume of 4.0 ml as shown below in **Table 5.1** on the next page.

**Table 5.1.** Mixtures of cedrelone and methyl palmitate standards used for the calibration curve.

| | volume of cedrelone/mL | Volume of internal standard/mL | volume of DCM*/ml |
|---|---|---|---|
| (1) | 0.25 | 1.00 | 2.75 |
| (2) | 0.50 | 1.00 | 2.50 |
| (3) | 1.00 | 1.00 | 2.00 |
| (4) | 1.50 | 1.00 | 1.50 |
| (5) | 2.00 | 1.00 | 1.00 |

*DCM = dichloromethane

For each sample prepared according to **Table 5.1**, the ratio of peak area was determined and plotted against the ratio of concentration using statgraphics software to determine the response factor for cedrelone (being the slope of the linear curve obtained) relative to the internal standard using equation 5.5 below.

$$\frac{analyte\ peak\ area}{IS\ peak\ area} \quad R = \frac{analyte\ conc.}{IS\ conc.} \qquad \text{5.5}$$

where **IS** is the internal standard. The set of samples in **Table 5.1** were analysed within one working day. Once the response factor was determined, methyl palmitate was then added to the extracts obtained from section 5.2.2 prior to their analysis by capillary gas chromatography to determine the amount of cedrelone in the extracts.

Capillary GC analyses

Capillary gas chromatography of the extracts was performed on the model 8160 gas chromatograph (Fisons Instruments, Milan, Italy) as described in section 3.3.1. After extract dilution and addition of the methyl palmitate internal standard, 0.5 $\mu$L aliquots were injected manually via the on-column injector and analysed.

## 5.3 Results and discussions

Initially, it was necessary to verify whether or not the internal standard used in this work coeluted with any of the extracted compounds from the wood. **Figure 5.2** on the next page shows a typical GC chromatogram of the wood extract obtained at 350 atm and 40°C in which the internal standard was introduced. From this chromatogram, it was confirmed that none of the extracted compounds coeluted with the internal standard (labelled A) and enabled quantification studies to proceed. Following this preliminary study, the standard solutions prepared as shown in **Table 5.1** were then analysed to obtain a linear calibration curve. The representative chromatograms obtained for solutions 2-5 are shown in **Figure 5.3.** on page 130. From these chromatograms, peak area ratios of cedrelone and the internal standard were calculated based on the integrated peak areas for both compounds. **Table 5.2** below shows the concentration and peak area ratios obtained from the standard solutions prepared from **Table 5.1** above.

**Table 5.2** Data for the concentration and peak area ratios for the calibration curve.

| Solution | Concentration ratio | Peak area ratio |
|---|---|---|
| (1) | 0.53 | 0.47 |
| (2) | 1.05 | 1.00 |
| (3) | 2.10 | 1.70 |
| (4) | 3.15 | 2.56 |
| (5) | 4.20 | 3.64 |

A linear regression was performed using the Statgraphics software programe. The slope of the curve was 0.84, and this corresponded to the response factor for cedrelone relative to the internal standard methyl palmitate in accordance with equation 5.2. The correlation coefficient obtained from this data was 0.997 with an intercept of 0.028. Thus the equation for the curve was of the form $y = 0.84x + 0.028$. There was therefore a significant correlation between the data obtained and the corresponding linear curve obtained as shown in **Figure 5.4** .

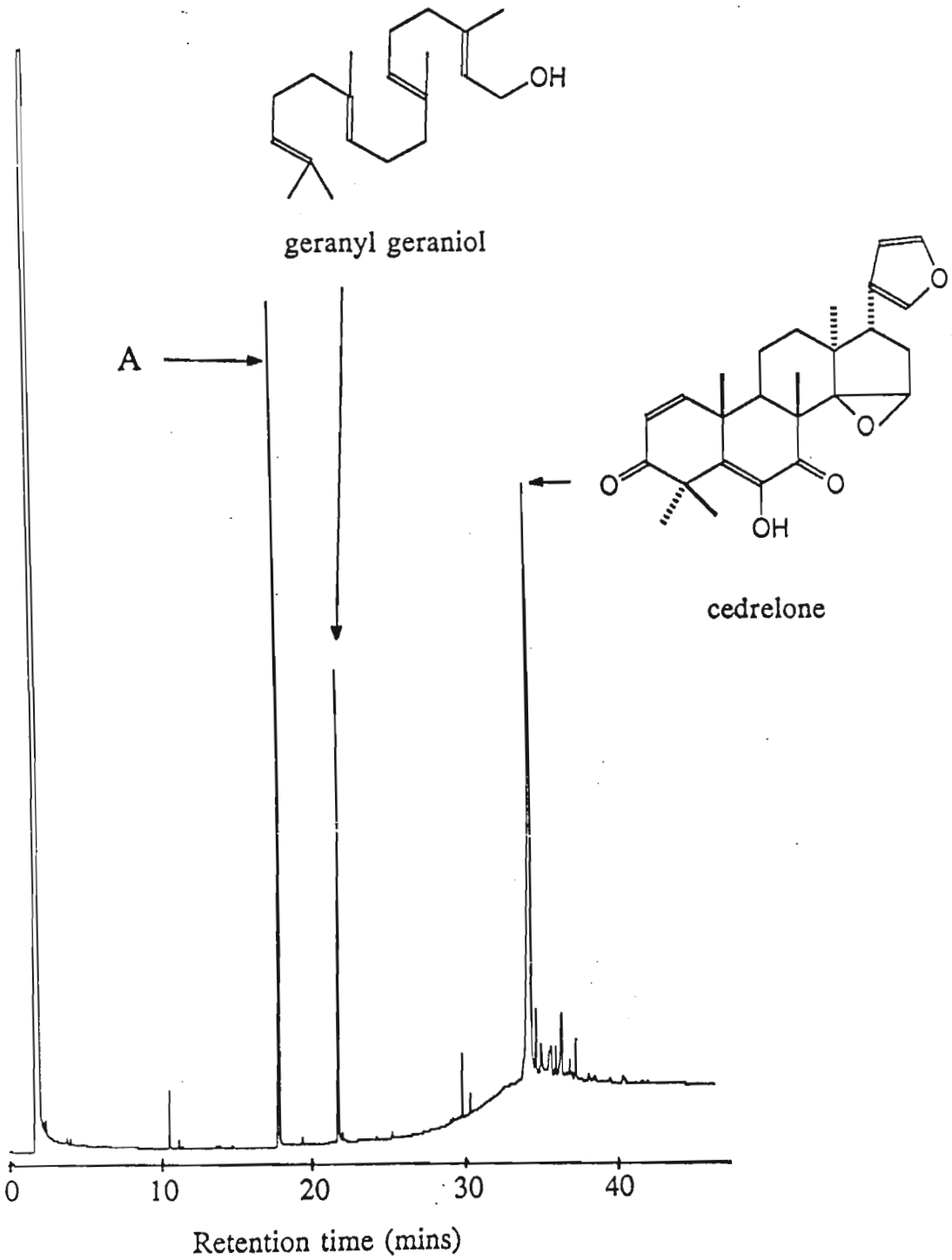



**Figure 5.2** A typical GC chromatogram of a $CO_2$ extract of the wood of *Cedrela toona* at 350 atm and 40°C. Conditions : column; 25 m x 250 $\mu$m i.d. fused silica capillary column coated with PTE-5 ($d_f$ = 0.25 $\mu$m). Temperature program; 60°C (1 min) to 150°C at 7°C/min, then to 320°C at 7°C/min and held for 17 min. FID at 320°C. Peak A is methyl palmitate (internal standard).

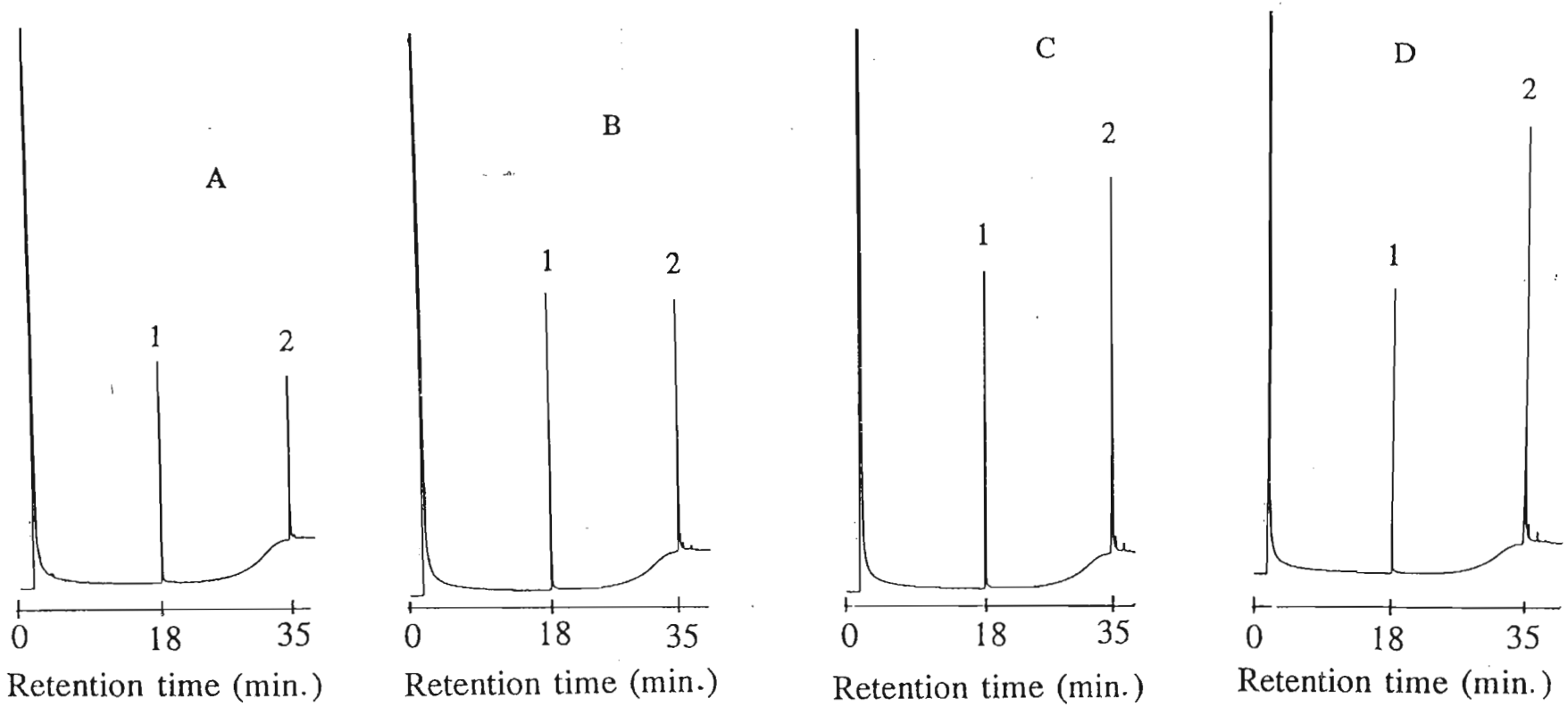



**Figure 5.3** Representative chromatograms for the standard solutions used in the calibration procedure. Key: A, B, C and D are chromatograms for solutions 2, 3, 4 and 5 respectively in Table 5.1.

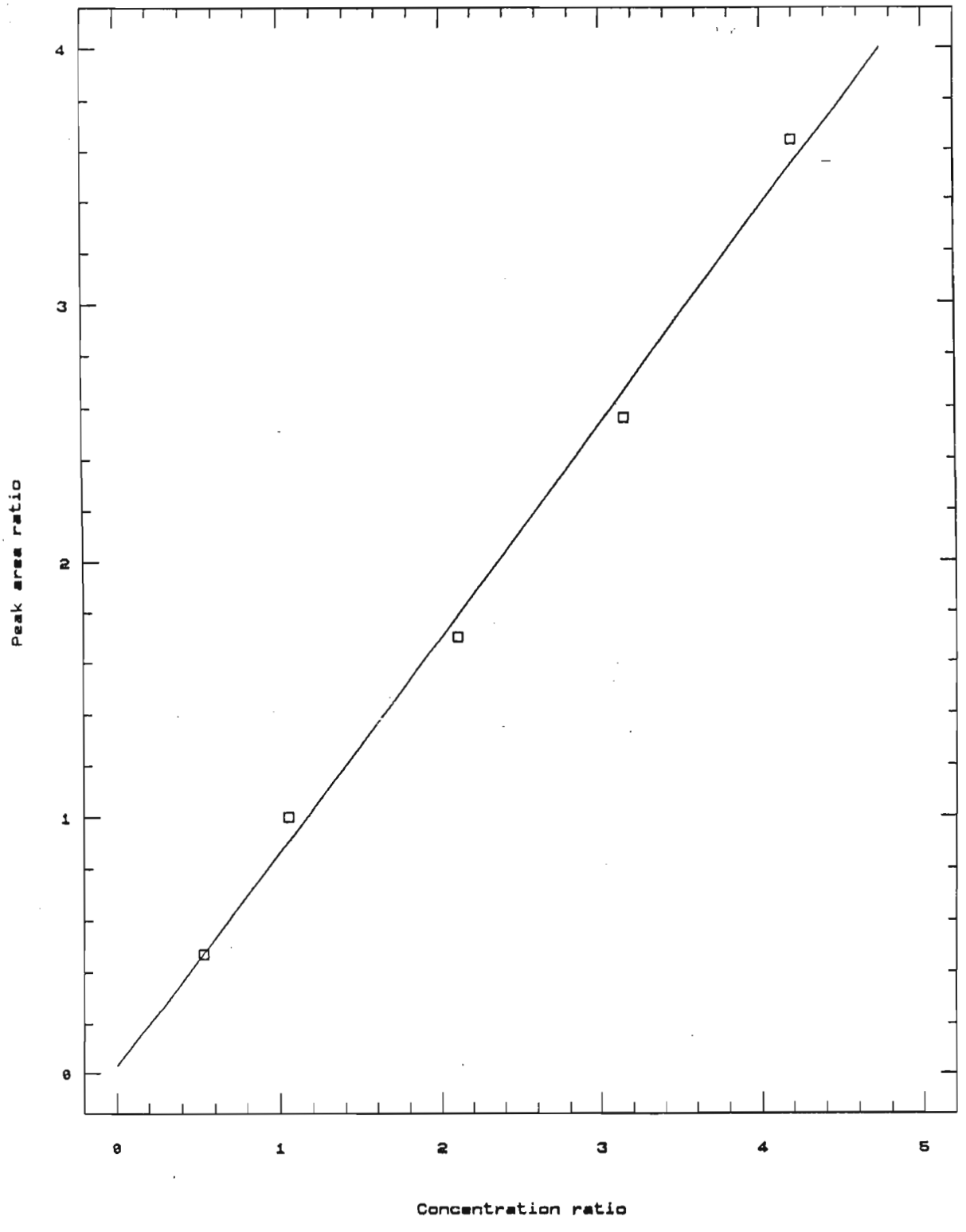



Figure 5.4 A five-point internal standard linear calibration curve used to determine the response factor for cedrelone.

Once the response factor for cedrelone was obtained, kinetic and quantitative studies based on the "hot ball" model were investigated for the extraction of cedrelone from the ground wood. According to the model, the exponential behaviour of the extraction after the initial steep period, opens up the possibility of using extrapolation to obtain quantitative analytical information in a shorter time than would be required for exhaustive extraction. Three successive extractions were performed on three samples using $CO_2$ at 350 atm and 40°C (with a 30 cm x 75 $\mu$m i.d. fused silica capillary restrictor) firstly for 10 minutes to obtain ($\mathbf{m_1}$) followed by two successive 5 minutes dynamic extractions to obtain $\mathbf{m_2}$ and $\mathbf{m_3}$ respectively. Equation 5.1 was then used to determine $m_o$ for cedrelone in the wood. The results are shown in **Table 5.3**.

**Table 5.3**. Data for the three successive extractions in the determination of $m_o$

| Sample | $m_1$ (mg/g) | $m_2$ (mg/g) | $m_3$ (mg/g) | $m_o$ (mg/g) |
|---|---|---|---|---|
| 1 | 2.49 | 0.15 | 0.017 | 2.67 |
| 2 | 3.37 | 0.054 | 0.040 | 3.59 |
| 3 | 3.21 | 0.035 | 0.013 | 3.27 |
| mean | 3.02± 0.38 | 0.080± 0.050 | 0.023± 0.012 | 3.18±0.38 |
| RSD % | 12.6 | 63.7 | 51.7 | 11.9 |

The 11.9% RSD for the measurement of $\mathbf{m_0}$ represents the total reproducibility of the extraction process. Variations in measuring $\mathbf{m_1}$, $\mathbf{m_2}$ and $\mathbf{m_3}$ are higher than in $\mathbf{m_0}$, and this is caused by variations in the extraction profile from run to run. Also, since the major portion of the sample is extracted during the $\mathbf{m_1}$ period, the variation in estimating $m_1$ becomes smaller than in the tailing sections of the extraction ($\mathbf{m_2}$ and $\mathbf{m_3}$). Unfortunately, Walker et al. [128] who used this model in a quantitative study of the supercritical fluid extraction of flavour and fragrance compounds from lavender and rosemary did not show the variations in the measurement of the $\mathbf{m_1}$, $\mathbf{m_2}$, $\mathbf{m_3}$ and $\mathbf{m_0}$ values for comparison. However, results by Liu et al. [129] showed a trend similar to that reported in **Table 5.3**, where variations in measuring $\mathbf{m_1}$, $\mathbf{m_2}$ and $\mathbf{m_3}$ were higher than in measuring $\mathbf{m_0}$.

Similarly, variations in $\mathbf{m}_1$ were lower than in $\mathbf{m}_2$ and $\mathbf{m}_3$. After the $\mathbf{m}_o$ value was determined, extractions were then performed at different lengths of time with $CO_2$ at 300 atm and 40°C using a fresh 3.0 g wood sample each time. A 30 cm x 50 $\mu$m i.d. fused silica capillary was used as the restrictor at the extraction cell outlet. Amounts of cedrelone extracted ($m_e$) were calculated and used to determine the m values from the relation $m = m_o - m_e$ . **Table 5.4** shows the $m/m_o$ values obtained which were then fitted into the kinetic model. Each timed-extract was performed twice and both extracts were analysed in a day. **Figure 5.5** shows the kinetic plot of ln ($m/m_o$) against the length of extraction time. Characteristically, the curve falls steeply initially with the rate of fall finally assuming linearity. Although the initial steep fall appears as a relatively small feature in terms of length of extraction time, in reality, it represents the period where majority of the material from the matrix is extracted. On calculating the percentage cedrelone extracted from the point where linearity is attained (at the eighth minute of extraction) one finds that about 66 % of the analyte is extracted within the initial portion of the curve.

**Table 5.4** Data used to obtain the kinetic plot

| Time (mins) | $m_e$ (mg/g) | RSD % | m(mg/g)* | $m/m_o$ | $\ln(m/m_o)$ |
|---|---|---|---|---|---|
| 2 | 1.05 | 1.73 | 2.13 | 0.67 | -0.40 |
| 4 | 1.55 | 5.05 | 1.63 | 0.51 | -0.68 |
| 6 | 1.91 | 2.53 | 1.27 | 0.40 | -0.82 |
| 8 | 2.09 | 2.59 | 1.09 | 0.34 | -1.08 |
| 10 | 2.23 | 1.65 | 0.95 | 0.30 | -1.22 |
| 12 | 2.35 | 4.67 | 0.83 | 0.26 | -1.36 |
| 14 | 2.48 | 1.72 | 0.70 | 0.22 | -1.50 |
| 16 | 2.57 | 2.29 | 0.61 | 0.19 | -1.64 |

* $m = m_o - m_e$

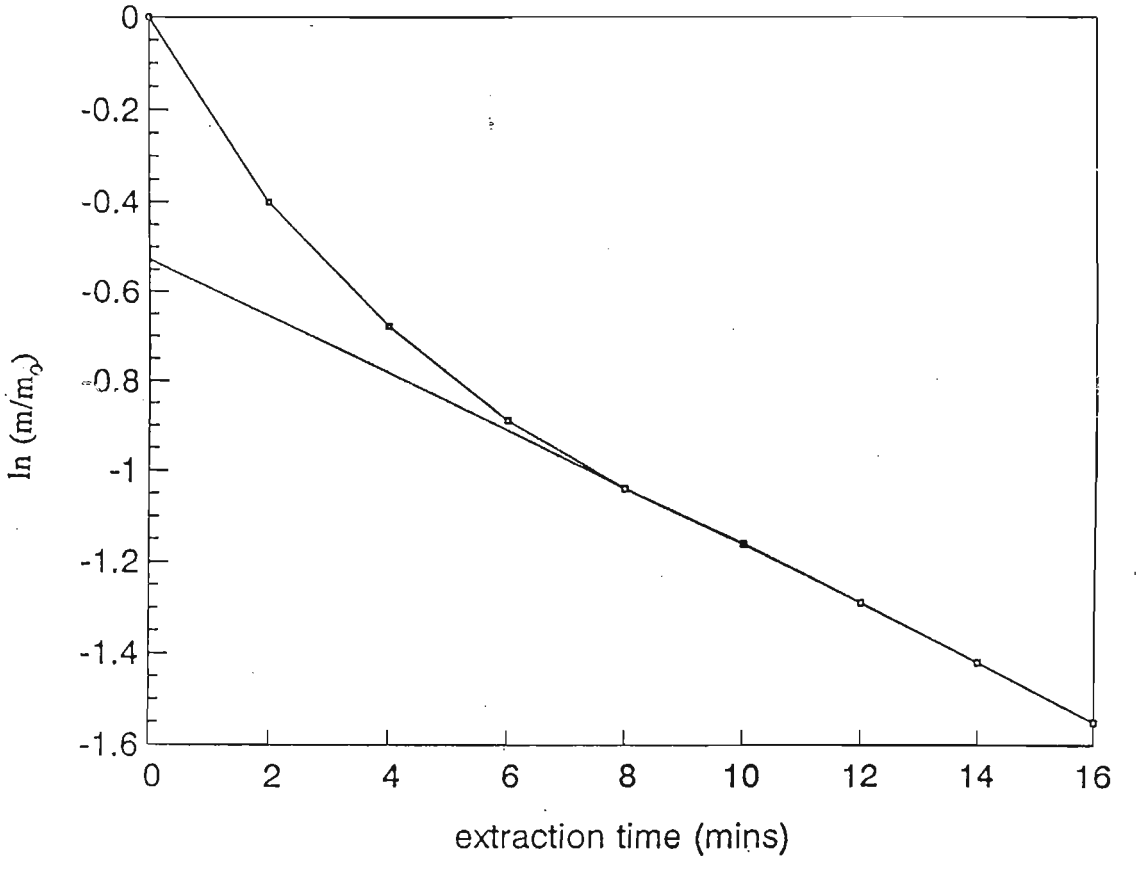



**Figure 5.5** Graph of ln ($m/m_o$) against extraction time for cedrelone from *Cedrela toona*.

The most interesting feature of this plot is the intercept of the linear portion of the kinetic curve on the ln ($m/m_o$) axis. The theoretical model predicts a value of -0.5 assuming that the particles are spherical and have a uniform size, and that the solutes are uniformly distributed. It also predicts that the extraction is controlled by diffusion. The extrapolation of the linear portion of the kinetic plot in **Figure 5.5** intercepts the ln ($m/m_o$) axis at just below the theoretical value at approximately -0.53. Within limits of experimental error, it could be predicted that the rate of extraction was not limited by solubility but rather controlled by diffusion. Thus any factor that increases the diffusion of the solute out of the matrix such as temperature will improve the extraction yield of cedrelone from the wood. In chapter six, factorial analysis, a statistical optimisation technique will be used to confirm the statistical significance (at the 5 % probability level) of the effect of temperature on the extraction yield of cedrelone from the wood of *Cedrela toona*. From **Figure 5.5**, there is also the possibility of a uniform distribution of the solute in accordance with the model, but in reality this is not likely to be the case due to the large variations in the $\mathbf{m_I}$ values in the data shown in **Table 5.3**. In a kinetic study elsewhere, Walker et al. [10] showed similar results from the SFE of camphene from lavender where the intercept of the linear portion of the curve was at -0.48. The intercepts of the linear portions of the curves for other real systems, however may be different from the theoretical value. For example in the SFE of camphor from rosemary by Walker et al. [128], the intercept was -2.36 as shown in **Figure 5.6**. These deviations are explained in terms of (a) the irregular shapes of the matrix particles which lowers the intercept, (b) the non-uniform extractable solute distribution which may either lower or raise the intercept and (c) solubility limitation which raises the intercept. Although the actual particle sizes were unknown in the extraction of cedrelone from *Cedrela toona*, it is possible that the model could be used to study the effect of particle size on the extraction kinetics of cedrelone. Subra et al. [130] who extracted carotenes from carrots found that a non-uniform particle size led to various extracted amounts as well as different kinetic profiles depending on the particle size. They concluded that different mass transfer mechanisms were involved depending on particle size. In the case of the powdered matrix,

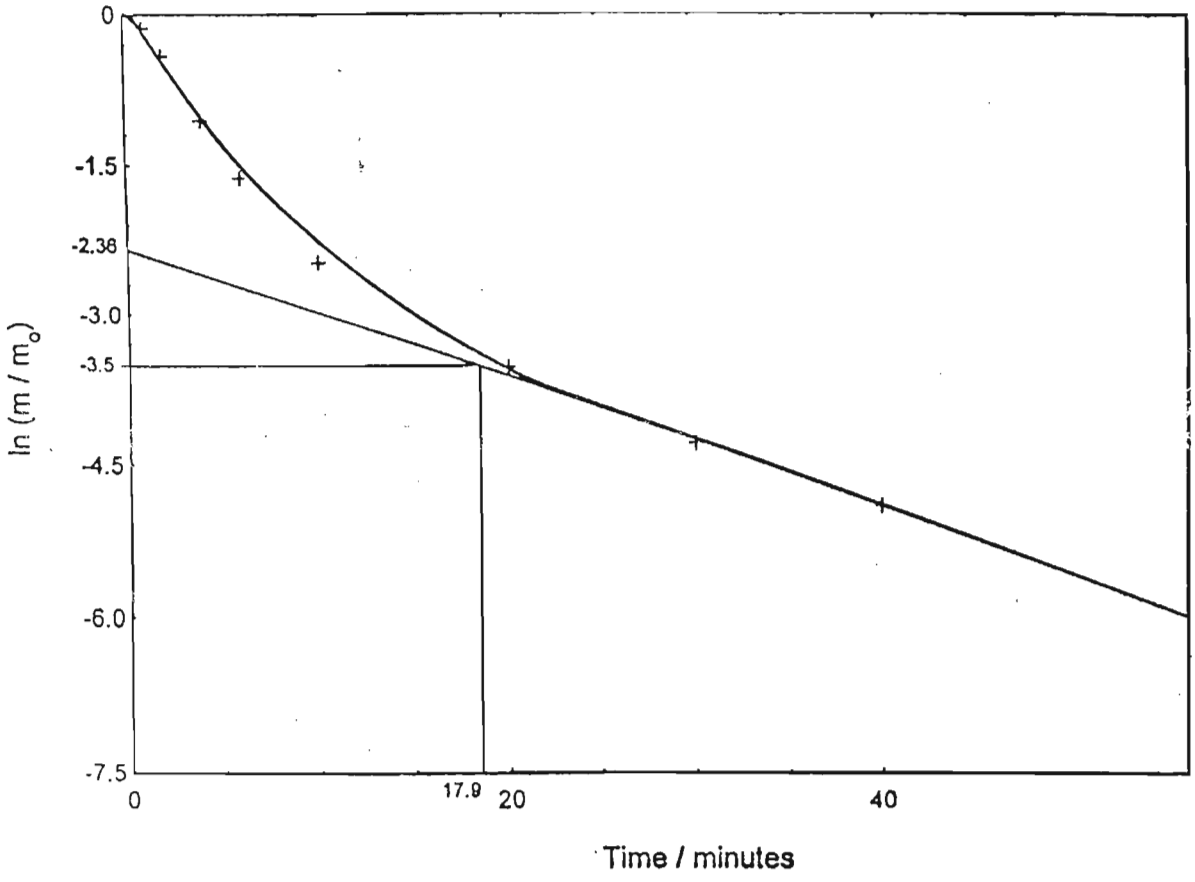



**Figure 5.6** Graph of ln ($m/m_o$) against extraction time for camphor from rosemary according to Walker et al.[128].

most of the carotenes were removed at the beginning of the extraction by a simple washing process and less were extracted by diffusion processes, whereas for the larger particles, diffusion processes played a significant role and became the limiting step.

The amounts of cedrelone obtained by the various exhaustive extractions were also compared with that obtained using equation 5.1 to ascertain the usefulness of this model in the quantification of natural products from plant matrices. **Table 5.5** shows the data for the amounts of cedrelone extracted by the different methods, and shows a significant correlation between the various methods. Each of the three extractions per technique were performed and analysed in a day. The amount of cedrelone in the 5½ h exhaustive extraction using hexane in a Soxhlet apparatus compared favourably with the results obtained using the kinetic model. However, the 4-hour hexane extract resulted in a lower yield for the target analyte. Also, since a preliminary study had shown that small concentrations of methanol improved the extraction yield of the target analyte, an exhaustive extraction with a 0.4 mol % methanol-modified supercritical $CO_2$ was performed and results also compared with that from the kinetic model.

**Table 5.5.** Comparative data for the amounts of cedrelone in mg/g by various extraction techniques.

| Soxhlet extraction (hexane, 4 hours) | Soxhlet extraction (hexane, 5½ hours) | Model | $CO_2$ with methanol |
|---|---|---|---|
| 2.65 | 2.88 | 2.67 | 3.41 |
| 3.06 | 3.18 | 3.59 | 3.18 |
| 2.61 | 3.63 | 3.27 | 3.14 |
| Mean 2.77± 0.20 | 3.23± 0.31 | 3.18± 0.38 | 3.24± 0.19 |
| RSD 7.34% | 9.54% | 11.9% | 3.64% |

## 5.4 Conclusion

From these studies, it has been possible to understand to a certain extent, the supercritical fluid extraction of a target compound from a plant matrix using the dynamic extraction model. By the exponential nature of this model, an extrapolated equation could then be used in a quantitative study to predict the amount of a target compound present in a particular matrix. Results from this work and that from a published literature indicate that the predicted amounts using this model are not different from results obtained by exhaustive extractions.

# CHAPTER SIX

# SYSTEMATIC MULTIVARIATE OPTIMISATION OF SFE OF CEDRELONE FROM *CEDRELA TOONA*

## 6.1 Introduction

It is clear from the literature review in chapter 2 that there has been active research in supercritical fluid extraction of natural product compounds. The diffusivities of supercritical fluids are much greater than those of liquids, but their viscosities are similar to those of gases. These properties frequently result in higher extraction efficiencies in SFE than may be obtained typically from liquid-liquid extraction. Further, in SFE, the extraction efficiency can be controlled by the temperature, pressure, length of extraction time, composition of the extraction solvent, as well as the gradients of these parameters. Consequently, selecting the optimum conditions for SFE is extremely complex. The most common approaches for the optimisation of SFE conditions are usually based on simple univariate sequential optimisation procedures.

The use of one parameter optimisation schemes would not be effective in locating the true optimum parameter zone in some cases and as a result, a simultaneous multivariate optimisation approach (factorial analysis) is preferable. Factorial analysis is becoming a way of simultaneously optimising several controlling factors in analytical chemistry. In the early stages of experimentation, screening design is used to narrow the number of potential variables from a relatively larger number to a relatively small number. Response surface methodology is then used to describe and locate the region of maxima for the interacting factors. Bicking [131] used a two-level (each factor at two levels usually known as "low" and "high"), two-factor ($2^2$) factorial design approach to optimise the temperature and pressure for the SFE of an amine hydrochloride. Ho and Tang [132] reported using a two-level, three-factor factorial design involving pressure, temperature and the length of extraction time, for the optimisation of the SFE conditions of polycyclic aromatic hydrocarbons and organochlorine pesticides from environmental matrices. Li et al. [133] also utilised factorial analysis to optimise the factors, temperature, initial

pressure and pressure gradient for the extraction of Vitamin E in corn oil. Systematic optimisation schemes for the SFE of natural product compounds from plant tissues have rarely been investigated and thus a systematic approach was used in an attempt to optimise the factors pressure, temperature and length of extraction time, for the SFE yields of cedrelone from *Cedrela toona*. In the early stages of the experimentation, screening design was used to test the statistical significance of these factors on the extraction yields of cedrelone at the 95% confidence level (5% probability level). Following the preliminary study, response surface methodology was used in an attempt to determine the location of the maximum response. An analysis of variance (ANOVA) data obtained from the response surface methodology was then used to generate a second order statistical model that best fitted and described the extraction of cedrelone from *Cedrela toona*.

## 6.2 The theory of optimisation (response surface methodology)

One fairly general definition of a response surface is a graph of a response as a function of one or more factors. Whatever this response may be, most response methods work best when the response and the factors vary in a continous manner, that is, they may take any value within a specific range. Response surface methods can be used to answer questions such as:

(a) How is a particular response affected by a set of variables over a specified region?

(b) Which settings of the variables yield a maximum or minimum response ?

The system offers predefined designs for two to eight factors in nine to eighty-seven runs. The development of a response surface is based on the application of a matrix solution to be described below in sections 6.2.1 and 6.2.2. These sections are not intended as a comprehensive insight into matrix algebra, but should enable one to understand the mathematical basis of optimisation strategies.

### 6.2.1 One Factor Study

In the case where only one factor is changed at different levels one could consider an example where the effect of reactant concentration on the reaction yield is investigated

using reactant concentrations of 1%, 2%, 3% and 4%. If the product yield was determined as a percentage of the theoretical maximum, then the experimental result could be shown in **Table 6.1** below:

**Table 6.1** Experimental results for a hypothetical one-factor study at four levels.

| Yield (%) | Reactant Conc. (%) |
|---|---|
| 10 | 1 |
| 15 | 2 |
| 20 | 3 |
| 23 | 4 |

It is then possible to define a design matrix E for this experiment which contains the levels specified in each of the runs, so that E is denoted by the equation:

$$E = \begin{vmatrix} 1 \\ 2 \\ 3 \\ 4 \end{vmatrix} \quad \ldots\ldots\ldots\ldots\ldots\ldots \quad \textbf{6.1}$$

A first order statistical model of the type $y_i = ß_o + ß_1 x_{1i} + r_i$ (where $y_i$ is the response for the ith run, $ß_o$ is the y intercept at factor level, $x_1 = 0$, $ß_1$ is the straight line slope for factor $x_1$, $x_{1i}$ is the level taken by factor $x_1$ in the ith run, $r_i$ is the residual error for the ith experimental run) can be applied so that each response ($y_i$) can be represented as a linear sum of the effects due to an intercept $ß_o$, a slope $ß_1$, and a residual error $r_i$. The observed response is considered to be a combination of a response due to the intercept $ß_o$, a response due to the run being carried out at a particular level of factor $x_1$, plus a residual error. Thus,

$$10 = 1 \times ß_o + 1 \times ß_1 + r_1$$

$$15 = 1 \times ß_o + 2 \times ß_1 + r_2$$

$$20 = 1 \times ß_o + 3 \times ß_1 + r_3$$

$$23 = 1 \times ß_o + 4 \times ß_1 + r_4$$

These equations are viewed as four simultaneous equations with two unknown parameters ($ß_0$ and $ß_1$) and four unknown residuals ($r_1$, $r_2$, $r_3$ and $r_4$). To solve the above equations, it is possible to express the values in matrices such that,

$$\begin{vmatrix} 10 \\ 15 \\ 20 \\ 23 \end{vmatrix} = \begin{vmatrix} 1 & 1 \\ 1 & 2 \\ 1 & 3 \\ 1 & 4 \end{vmatrix} \begin{vmatrix} \beta_o \\ \beta_1 \end{vmatrix} + \begin{vmatrix} r_1 \\ r_2 \\ r_3 \\ r_4 \end{vmatrix} \quad \ldots\ldots\ldots\ldots \quad \mathbf{6.2}$$

thus in the form $Y = Xß + R$ , where Y is the vector of responses, R is the vector of residuals, and X is the matrix of parameter coefficients. Clearly, it is impossssible to determine the six variables $ß_0$, $ß_1$, $r_1$, $r_2$, $r_3$ and $r_4$ in four simultaneous equations. However, the technique of "least squares" which minimises the sum of squares of the residuals and estimates the parameters $ß_0$ and $ß_1$ can be used. The generalised matrix solution for a set of b parameters that estimate the ß parameters while at the same time minimising the sum of squares of the residuals is given by $B = (X^tX)^{-1} X^tY$, where B is a column vector that estimates $ß_0$ and $ß_1$, hence called the least squares estimate. $X^t$ is a transpose matrix of X which premultiplies X to give $X^tX$.

The transpose of X, ie. $X^t$ is given below as,

$$X^t = \begin{vmatrix} 1 & 1 & 1 & 1 \\ 1 & 2 & 3 & 4 \end{vmatrix}$$

which premultiplies X to form the matrix $X^tX$, and Y to form $X^tY$, ie.,

$$X^t X = \begin{vmatrix} 1 & 1 & 1 & 1 \\ 1 & 2 & 3 & 4 \end{vmatrix} \begin{vmatrix} 1 & 1 \\ 1 & 2 \\ 1 & 3 \\ 1 & 4 \end{vmatrix} = \begin{vmatrix} 4 & 10 \\ 10 & 30 \end{vmatrix} \quad \ldots\ldots\ldots \quad \mathbf{6.3}$$

$$X^t Y = \begin{vmatrix} 1 & 1 & 1 & 1 \\ 1 & 2 & 3 & 4 \end{vmatrix} \begin{vmatrix} 10 \\ 15 \\ 20 \\ 23 \end{vmatrix} = \begin{vmatrix} 68 \\ 192 \end{vmatrix} \quad \ldots\ldots\ldots\ldots \quad \mathbf{6.4}$$

The determinant of $X^tX$ is calculated as (4 x 30 - 10 x 10) = 20, hence the inverse of the $X^tX$ matrix, $(X^tX)^{-1}$ is given as,

$$(X^t X)^{-1} = \begin{vmatrix} 30/20 & -10/20 \\ -10/20 & 4/20 \end{vmatrix} = \begin{vmatrix} 1.5 & -0.5 \\ -0.5 & 0.2 \end{vmatrix} \quad \ldots\ldots\ldots \quad \mathbf{6.5}$$

The least squares equation can now be used to determine the parameters $ß_o$ and $ß_1$, thus

$$B = \begin{vmatrix} 1.5 & -0.5 \\ -0.5 & 0.2 \end{vmatrix} \begin{vmatrix} 68 \\ 192 \end{vmatrix} = \begin{vmatrix} 6.0 \\ 4.4 \end{vmatrix} = \begin{vmatrix} \beta_o \\ \beta_1 \end{vmatrix} \quad \ldots\ldots\ldots \quad \mathbf{6.6}$$

The maximum response is 6.0, and the slope on the $x_1$ axis is 4.4.

### 6.2.2 Two-factor Study

The matrix algebra can be extended to a two-factor response surface design where a model can be designed with or without interaction between the parameters. For instance, an experiment could be performed to study the effect of pressure and temperature on the amount of solute extracted in mg/g using a $2^2$ response surface design with three centrepoints, with pressure ranging from 200 atm to 300 atm, and temperature ranging from 40°C to 60°C. The "high" and "low" values for the parameters in the coded form are shown in the **Table 6.2** on page 144.

**Table 6.2** "High", "centre" and "low" values for a hypothetical 2-factor study.

| | -1 | 0 | +1 |
|---|---|---|---|
| Pressure/atm ($x_1$) | 200 | 250 | 300 |
| Temp/°C ($x_2$) | 40 | 50 | 60 |

The codes -1, 0 and +1 denote the variables at their "low", "centre" and "high" levels respectively. A two-level, two-factor factorial analysis with three centrepoints will produce seven runs in total. A design matrix with the observed yields could be as shown in **Table 6.3** below.

**Table 6.3** A design matrix for a 2-factor study.

| Pressure | Temperature | Yield |
|---|---|---|
| -1 | -1 | 2.0 |
| 1 | -1 | 2.6 |
| -1 | 1 | 3.2 |
| 1 | 1 | 3.4 |
| 0 | 0 | 2.7 |
| 0 | 0 | 2.8 |
| 0 | 0 | 2.9 |

Assuming that the experiment satisfies a model equation of the form shown below, $y_i = ß_o + ß_1x_{1i} + ß_2x_{2i} + ß_{12}x_{1i}x_{2i} + r_i$, with the only interaction in the model being that of $x_1x_2$, the matrix of parameter coefficients for this model in the coded form ($X_*$), and the vector of responses obtained (Y), are then as follows:

$$X_* = \begin{vmatrix} x_o & x_1 & x_2 & x_1x_2 \\ 1 & -1 & -1 & 1 \\ 1 & 1 & -1 & -1 \\ 1 & -1 & 1 & -1 \\ 1 & 1 & 1 & 1 \\ 1 & 0 & 0 & 0 \\ 1 & 0 & 0 & 0 \\ 1 & 0 & 0 & 0 \end{vmatrix} \qquad Y = \begin{vmatrix} 2.0 \\ 2.6 \\ 3.2 \\ 3.4 \\ 2.7 \\ 2.8 \\ 2.9 \end{vmatrix}$$

An equation of the form Y = Xß + R can be generated as in equation 6.7,

$$\begin{vmatrix} 2.0 \\ 2.6 \\ 3.2 \\ 3.4 \\ 2.7 \\ 2.8 \\ 2.9 \end{vmatrix} = \begin{vmatrix} 1 & -1 & -1 & 1 \\ 1 & 1 & -1 & -1 \\ 1 & -1 & 1 & -1 \\ 1 & 1 & 1 & 1 \\ 1 & 0 & 0 & 0 \\ 1 & 0 & 0 & 0 \\ 1 & 0 & 0 & 0 \end{vmatrix} \begin{vmatrix} \beta_o \\ \beta_1 \\ \beta_2 \\ \beta_{12} \end{vmatrix} + \begin{vmatrix} r_1 \\ r_2 \\ r_3 \\ r_4 \\ r_5 \\ r_6 \\ r_7 \end{vmatrix} \quad \ldots\ldots\ldots\ldots \mathbf{6.7}$$

Thus, there are seven simultaneous equations with four unknown variables ($ß_o$, $ß_1$, $ß_2$ and $ß_{12}$) and seven unknown residuals ($r_1$ - $r_7$). The least squares technique could then be used to determine the ß and r values as in equation 6.8.

$$(X_*{}^t\, X_*)^{-1} \quad (X_*{}^t\, Y) = B = \begin{vmatrix} \beta_o \\ \beta_1 \\ \beta_2 \\ \beta_{12} \end{vmatrix} \quad \ldots\ldots\ldots\ldots \mathbf{6.8}$$

In real situations, the uncoded matrices are rather used with the parameters at their various levels. Undoubtedly, with the increasing number of interactions involving the

experimental parameters, solutions to such matrices are very difficult, and are best performed using computer programmes. A suitable 3-dimensional response surface plot could then be produced. Depending on the nature of the computer programme, canonical analysis could be performed to determine the stationary points for the 3-dimensional plot. In the subsequent sections, a Statgraphics computer programme [134] (Version 5.1) is used to systematically locate the region of maximum in a study of the effect of extraction pressure and temperature on the yields of cedrelone from *Cedrela toona*.

## 6.3 Experimental

Sample

The dried and ground wood of *Cedrela toona* described in chapters 4 and 5 was used for this study.

SFE and analyte quantification

Dynamic supercritical fluid extractions of 3.0 g samples using pure $CO_2$ were performed according to **Table 6.4** on page 147 and **Table 6.5** on page 149 using the home-made SFE system described in section 3.2.1. Extracts were trapped in dichloromethane and a known amount of an internal standard (methyl palmitate) was added to the extract, and the solution made up to a total volume of 5.0 mL in dichloromethane. The amount of cedrelone in each extract was analysed by on-column capillary gas chromatography. Each experimental run was performed and analysed in one day.

### 6.3.1 The screening design

Formulation of the Design Matrix

It was necessary to initially specify that one response variable (yield) and three factors (pressure, temperature and time) at their "high" and "low" levels were required for the experiment. In order to proceed, the "experimental design" submenu was selected. From this submenu, the "screening design" option was selected and the system displayed the "design list" screen. On the "design list" screen, the "new file" line was selected, and an appropriate file number (104) was typed and entered as the number assigned to the experiment. On entering this file number, the "design specification data entry" screen was

displayed. It was specified that one response variable and three factors were required for the experiment, and the F6 (Go) key was pressed. The "variable definition data entry" screen was displayed and names were assigned to the factors (ie. temperature, pressure and time) as well as well as the "low" and "high" values for each factor (ie. 110-250 for pressure, 40-50 for temperature, and 30-60 for time) with appropriate units assigned (celsius, atm and minutes for temperature, pressure and time respectively). A name (Yield) and an appropriate unit (mg/g) was also assigned to the response variable. Each factor was also specified as "continuous (that is they may take any value within the specified range). On completing this screen, the F6 (Go) key was pressed and the system displayed the "design selection" screen. The 8-run design was selected by highlighting and entering the factorial 2^3 design option. The system displayed the "design options data entry" screen on which it was specified that two centrepoints were required at the end of the block. The randomize field was toggled to **NO** to have the system store the runs in a standard order in the experiment file. The F6 (Go) key was pressed, the system updated the screen and redisplayed a pop-up menu. To view the design, the "display design" option was highlighted and entered. **Table 6.4** below shows the design matrix obtained from the factor levels specified.

**Table 6.4** The design matrix for the 3-factor study with two centrepoints at the end of the block.

3-factor study
Response: Yield

| Run | Pressure/atm | Temperature/celsius | Time/minutes |
|---|---|---|---|
| 1 | 110 | 40 | 30 |
| 2 | 250 | 40 | 30 |
| 2 | 110 | 40 | 60 |
| 4 | 250 | 40 | 60 |
| 5 | 110 | 50 | 30 |
| 6 | 250 | 50 | 30 |
| 7 | 110 | 50 | 60 |
| 8 | 250 | 50 | 60 |
| 9 | 180 | 45 | 45 |
| 10 | 180 | 45 | 45 |

The Esc key was pressed to return to the "design option" screen and the pop-up menu. To save the experiment, the "save experiment" option was selected, the experiment was saved as STATC.104, and the system redisplayed the "screening designs list" screen with the pop-up menu. Having created the design matrix in Table 6.4, it was necessary to perform each of the SFE experiments in the design and obtain the yield (response) in each case. The concentration of cedrelone in the extracts was quantified by GC as described in section 5.2.3.

Data Analysis

After the response data were obtained, the experimental file was retrieved from the "design list". To enter the various responses, the "enter data" option from the pop-up menu on the "screening designs list" screen was selected and the system displayed the variables in the "data editor". The Tab key was pressed to move the cursor to the yield variable which was empty, and the yields obtained from the experimental runs were entered. The F6 (Go) key was then pressed and the system redisplayed a pop-up menu from which the "save and exit" option was selected. The "screening designs list" screen was then redisplayed. The "analyse data" option was selected from this screen, and the system then displayed the "analyse screening design data" entry screen. The F6 (Go) key was pressed to accept the default values which included ignoring interactions higher than the order two. From the pop-up menu redisplayed, the "curvature check" option was selected and the system displayed an analysis of variance (ANOVA) table shown in **Table 6.7** on page 152.

### 6.3.2 Response surface design

Formulation of the Design matrix

Having established the significant variables (ie. pressure and temperature) influencing the extraction of cedrelone, the response surface methodology was employed to systematically optimise or locate the region of maximum yield. In order to proceed, the "response surface designs" was selected from the "experimental designs submenu", and the system displayed the "response surface designs list" screen. The "new file" line was selected (highlighted and entered), a number 204 was assigned to the new experiment and the enter

key was pressed. The system displayed the "design specification data entry" screen, and it was specified on this screen that one response variable and two factors were required in the design. The F6 (Go) key was pressed and the system displayed the "variable definition" screen. The screen was customized by assigning names (pressure and temperature) to the factors, and the "low" and "high" values for each factor (pressure, 200-390; temperature, 40-75) as well as their units (atm and celsius respectively). The screen was completed by assigning a name (Yield) and a unit (mg/g) to the response variable. The F6 (Go) key was then pressed and the system displayed the "design selection list" screen. The "central composite face centred" option was selected and the system displayed the "design options data entry" screen. The randomize field was toggled to **NO** to have the system store the runs in a standard order in the experiment file. It was also specified on this screen that two centrepoints were required in a random placement. The F6 (Go) key was pressed and the system updated the screen and redisplayed a pop-up menu with "display" and "save" options. The "display" option was selected and the system displayed the design matrix shown in **Table 6.5** below.

**Table 6.5** Design matrix for a 2-factor central composite design with randomly-placed centrepoints.

| Run | Temperature/celsius | Pressure/atm |
|---|---|---|
| 1 | 57.5 | 295 |
| 2 | 57.5 | 390 |
| 3 | 75.0 | 295 |
| 4 | 57.5 | 200 |
| 5 | 40.0 | 390 |
| 6 | 75.0 | 200 |
| 7 | 75.0 | 390 |
| 8 | 40.0 | 200 |
| 9 | 40.0 | 295 |
| 10 | 57.5 | 295 |

The Esc key was pressed and the system redisplayed a pop-up menu. The "save and exit" option was selected in order to perform the SFE experiments at the various parameter levels.

Data Entry and Analysis

After obtaining data for the various responses, the STATC.204 file was retrieved for data entry. On the "response surface designs list" screen with the pop-up menu, the "enter data" option was selected and the "data editor" screen appeared. The Tab key was pressed to move the cursor to the yield variable which was empty. In this variable, the yields obtained from the experimental runs were entered. The F6 (Go) key was pressed and a pop-up menu was redisplayed. The "save and exit" option was selected and the system updated the STATC.204 file and redisplaying the "response surface designs list" screen with the pop-up menu. The "analyze data" option was selected with the system displaying the "analyze response surface data entry" screen. The F6 (Go) key was pressed to accept the default settings which included ignoring interactions higher than the order two. From the pop-up menu redisplayed, the "display anova" option was selected with the system performing and displaying an analysis of variance table (**Table 6.9**) (page 156).

The Esc key was pressed to return to the "analyze response surface" screen and the pop-up menu. The "plot response" option was selected with the system redisplaying another pop-up menu of plot options. The "response surface plot" option was selected with the system displaying the "response surface plot data entry" screen. On this screen both temperature and pressure were selected by toggling the field to the left of each factor to display an asterisk. The "hold at" field was irrelevant because both factors were being plotted. The F6 (Go) key was then pressed with the system displaying the "surface plotting options" screen. The F6 (Go) key was pressed again to accept the default values and the system displayed the 3-dimensional response surface plot (**Figure 6.1**) (page 154).

## 6.4 Results and discussions

### 6.4.1 The screening design

In the early stages of the optimisation of the conditions for SFE, screening design was used to narrow the number of potential variables affecting the extraction of a target analyte from the matrix. The statistical significance of the factors (extraction pressure, temperature and length of extraction time) that are potentially capable of influencing the

supercritical fluid extraction efficiency were simultaneously investigated based on a $2^3$ factorial analysis at the 5% probability level. This method required fewer runs and could detect and estimate any interaction which the classical univariate optimisation was incapable of doing. Two centrepoints were included in the experimental runs giving a total of ten runs in the design matrix as shown in **Table 6.4**. **Table 6.6** shows a summary of the design specifications and the experimental conditions which produced the yields for cedrelone from the ground wood.

**Table 6.6** Design matrix and observed responses of cedrelone

(a) Two-level factorials

| Variables | | Low | High |
|---|---|---|---|
| Pressure/atm | (P) | 110 | 250 |
| Temp/°C | (T) | 40 | 50 |
| Time/mins | (t) | 30 | 60 |

(b) Two-level factorials design and their responses (yield)

| Expt # | P | T | t | Yield (mg/g) |
|---|---|---|---|---|
| 1 | 110 | 40 | 30 | 0.0500 |
| 2 | 250 | 40 | 30 | 1.98 |
| 3 | 110 | 40 | 60 | 0.0520 |
| 4 | 250 | 40 | 60 | 2.07 |
| 5 | 110 | 50 | 30 | ND* |
| 6 | 250 | 50 | 30 | 1.79 |
| 7 | 110 | 50 | 60 | 0.00460 |
| 8 | 250 | 50 | 60 | 1.81 |
| 9 | 180 | 45 | 45 | 0.840 |
| 10 | 180 | 45 | 45 | 0.870 |

* None detected

To obtain significant information from this data, the data needed to be transformed into rather more meaningful analytical data. Therefore, computer-aided analysis of variance (ANOVA) was performed on this data to separate the total variations in the set of observations into components that could be associated with the factors whose real effects

were unknown. **Table 6.7** shows the results of the ANOVA on the preliminary data collected in **Table 6.6**.

**Table 6.7** Analysis of variance on the preliminary data collected from **Table 6.3**.

| Effect | SS* | DF[a] | MS[b] | F-ratio | P-value[c] |
|---|---|---|---|---|---|
| A(pressure) | 7.1129 | 1 | 7.1129 | 12829 | 0.0001 |
| B(Temp) | 0.0375 | 1 | 0.0375 | 67.56 | 0.0145 |
| C(Time) | 0.00169 | 1 | 0.00169 | 3.07 | 0.2220 |
| AB | 0.01554 | 1 | 0.01554 | 28.03 | 0.0339 |
| AC | 0.001336 | 1 | 0.001336 | 2.41 | 0.2607 |
| BC | 0.000568 | 1 | 0.000568 | 1.02 | 0.4180 |
| Total error | 0.0011088 | 2 | 0.0011088 | | |

* = sum of squares b = mean squares
a = degrees of freedom c = probability level

From **Table 6.7**, it can be seen that the probability levels for pressure, temperature and pressure-temperature interacting effect were 0.01%, 1.45% and 3.39% respectively. As these levels were below the 5% significance level, they were considered the significant variables influencing the extraction of cedrelone. Extraction time and its interacting effect with pressure and temperature were 22.2%, 26.07% and 41.8% respectively. As these probability levels were above the 5% significance level they were considered insignificant variables influencing the extraction of cedrelone. The data could also be analysed from the point of view of the variance ratios (F-ratios). The critical F-ratio for 2 and 1 degrees of freedom (d.f) at the 5% probability level was 18.5 (Appendix A). From Table 6.7, the F-ratios for pressure, temperature and pressure-temperature effect are 12829, 67.56 and 28.03 respectively. Therefore, since the F-ratios for these variables exceeded the critical value of 18.5, they were considered significant in contrast to the F-ratios for time and its interacting effects which were less than the critical value of 18.5 at the 5% probability level. The significance of pressure indicates that solubility of the solute out of the matrix

is very crucial in influencing extraction yield. However, the significance of temperature indicates that diffusion out of the matrix is important. This result confirmed the conclusions drawn from the kinetic plot in chapter 5 (**Figure 5.5**, page 134) that diffusion processes controlled the extraction of cedrelone from *Cedrela toona*. Therefore, it is possible that these two factors compete for overall control on the extraction and that the possibility of a threshold exists between the two parameters.

### 6.4.2 Response surface methodology

To model an extraction, it is necessary to have a knowledge about the matrix characteristics. The results of chapter 5 describing the application of the "hot-ball model", as well as that from the screening design, have provided a basis for a modeling strategy from a statistical perspective. To achieve this objective, a two-factor central composite design involving pressure and temperature was performed as described in section 6.3.2. A wide range of values were chosen for both pressure and temperature. Interactions higher than the order two were ignored in the study. If $x_1$ and $x_2$ represent temperature and pressure respectively, the matrix of parameter coefficients for this study will involve ten rows (for ten experimental runs), and not less than six columns (for six or more parameters that include $x_o$, $x_1$, $x_2$, $x_1x_1$, $x_2x_2$, $x_1x_2$, etc.). Clearly, solutions involving such complex matrices can best be performed using computer programmes.

Supercritical $CO_2$ extractions based on the $2^2$ central composite design matrix in **Table 6.5** (page 149) was performed. **Table 6.8** on page 154 shows a summary of the factor levels and observed responses (yields) that were obtained by capillary gas chromatography of the extracts. Extractions were performed dynamically for 45 minutes, midpoint between the range chosen for the preliminary screening design. The data in **Table 6.8** on page 154 was analysed using the Statgraphics computer package to generate a 3-dimensional response surface shown in **Figure 6.1** on page 154.

Table 6.8 Factor levels and observed responses for the two-factor central composite design.

| Temp./celsius | Presssure/atm | Response/mg $g^{-1}$ | RSD/% |
|---|---|---|---|
| 57.5 | 295 | 2.59 ± 0.090 | 2.95 |
| 57.5 | 390 | 3.29 ± 0.005 | 0.13 |
| 75.0 | 295 | 2.17 ± 0.079 | 3.07 |
| 57.5 | 200 | 2.30 ± 0.020 | 0.73 |
| 40.0 | 390 | 3.31 ± 0.095 | 2.18 |
| 75.0 | 200 | 1.76 ± 0.094 | 4.48 |
| 75.0 | 390 | 2.43 ± 0.030 | 0.86 |
| 40.0 | 200 | 2.36 ± 0.230 | 5.10 |
| 40.0 | 295 | 2.69 ± 0.160 | 4.93 |
| 57.5 | 295 | 2.59 ± 0.090 | 2.95 |

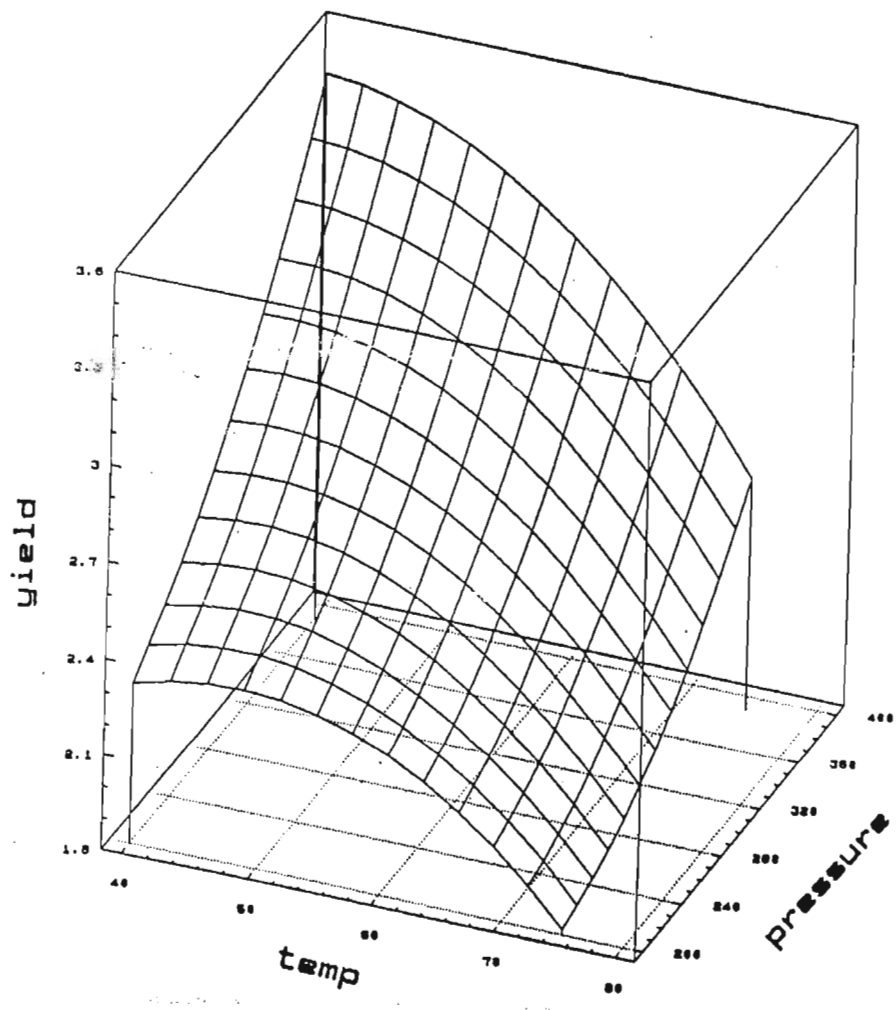



Figure 6.1 Response surface plot for temperature and pressure effect on the extraction of cedrelone from *Cedrela toona*.

Unfortunately, the package did not have the capability to perform canonical analysis in order to determine coordinates for the stationary point. However, the response surface did show that maximum extractions were obtainable at an extraction temperature of approximately 46°C, and a pressure above 400 atm. From the response surface, it is also noticeable that the extraction yield increases initially up to 46°C and begins to decrease beyond the threshold temperature. It should be remembered that the extraction rate of a solute in a supercritical fluid is also a function of the volatility of the solute. Solute solubility in the fluid alone does not guarantee a successful extraction as the rate of extraction also depends on the rate of diffusion out of the matrix. Even though increasing the extraction temperature effectively reduces the solvating power (density) of the fluid as seen in **Figure 1.2** on page 7, its effect on increasing the vapour pressure of the solute and hence the solute diffusion coefficient, subsequently results in an increase in the extraction rate as can be seen in **Figure 6.1**, but beyond a threshold point the decreased solvating strength of the fluid controls the overall extraction rate.

It is therefore necessary that the pressure and temperature for the extraction of a solute be optimised simultaneously in order to establish a true optimum parameter zone particularly for natural products where knowledge concerning analyte-matrix characteristics are unknown. That is not to say that pressure and temperature are the only factors worth mentioning. Since analyte-matrix interactions vary from solute to solute, so would the significant factors influencing the yield or recovery of a particular solute from a matrix. For instance, in the extraction of organochlorine pesticides and polycyclic aromatic hydrocarbons from environmental matrices by Ho and Tang [132], in which pressure, temperature and length of extraction time were investigated, the preliminary study indicated that pressure and length of extraction time (and not pressure and temperature) were the statistically significant factors influencing the recoveries of the compounds. It is possible that solubility and matrix effects unlike solubility and diffusion out of the matrix controlled the the yields of these compounds.

### 6.4.3 Formulation of a Predictive Model using ANOVA

Analysis of variance (ANOVA) was performed on the data in **Table 6.8** to estimate the effects of the variables as well as the interaction effects. **Table 6.9** shows results for the analysis of variance performed.

**Table 6.9** ANOVA for yield - 2 factor study

| Effect | sum of squares | DF | Mean square | F-Ratio | P-value* |
|---|---|---|---|---|---|
| A:temp | 0.80666667 | 1 | 0.8066667 | 72.52 | 0.00 |
| B:pressure | 1.31601667 | 1 | 1.3160167 | 118.31 | 0.00 |
| AB | 0.05760000 | 1 | 0.0576000 | 5.18 | 0.08 |
| AA | 0.12190476 | 1 | 0.1219048 | 10.96 | 0.02 |
| BB | 0.04342976 | 1 | 0.0434298 | 3.90 | 0.11 |
| Total error | 0.04449524 | 4 | 0.0111238 | | |

* probability value

The analysis of variance data can be used to decide whether a model equation should be accepted as a reasonable fit for the data at a particular probability level. At P = 0.05, the critical variance ratio for 4 and 1 degrees of freedom from Appendix A is given as 7.71. Therefore, since the F-ratios for temperature (72.5), pressure (118.3) and the temperature -temperature interacting effect (10.96) exceed the critical value, they provide with 95% confidence a model that fits the data. If A is denoted by $x_1$, B by $x_2$ and the residual errors by r, then at the P = 0.05 probability level, a model that fits the extraction is given as:

$$y_i = \alpha_0 + \alpha_1 x_{1i} + \alpha_2 x_{2i} + \alpha_3 x^2_{1i} + r_i \quad \ldots\ldots\ldots \quad 6.9$$

where $\alpha_0$ is the intercept, $\alpha_1$ is the slope on the temperature axis and $\alpha_2$ is the slope on the pressure axis. However, at the P = 0.01 probability level, the critical F-ratio for 4 and 1 degrees of freedom is 21.2 (Appendix B). Therefore only A (F-ratio = 72.5) and B (F-ratio = 118.3) are significant variables that fit the data and there is a lack of fit for the $\alpha_3$ terms at the 1% probability level. Therefore the best statistical model that fits the

extraction of cedrelone from the wood of *Cedrela toona* is given as:

$$y_i = \alpha_0 + \alpha_1 x_{1i} + \alpha_2 x_{2i} + r_i \quad \ldots\ldots\ldots\ldots \quad \mathbf{6.10}$$

where $x_{1i}$ and $x_{2i}$ describes the diffusional and solubility behaviour respectively of the extraction. A regression equation for this model can be generated which could be used to predict the amount of solute extracted at a particular level of temperature and pressure. The regression coefficients for the extraction data were computed and are shown in **Table 6.10** below.

**Table 6.10** Regression coefficients for yield - 2 factor study.

| | |
|---|---|
| Constant | = -1.716 |
| A: Temp. | = 0.086 |
| B: Pressure | = 1.613 |
| AB: | = -7.218 |
| AA: | = -7.460 |
| BB: | = 1.510 |

Since the ANOVA (in **Table 6.9**) at the 99% confidence level indicated that A, B, and AA were the only significant variables in this model, it therefore follows that the data satisfies a regression equation of the form $y = -1.716 + 0.086x_1 + 1.613x_2 - 7.460x_1x_1$, where y is the yield, $x_1$ is the temperature, and $x_2$ the pressure.

This idea has not been tested yet, but in the future, the validity of this modeling technique will be tested against observed data. Since the regression equation satisfies this model, observed and predicted responses using the regression equation must not be significantly different.

## 6.5 Conclusion

Even though modeling the supercritical fluid extraction of natural products from plant matrices appears to be very difficult because analyte-matrix interactions are difficult to understand, the use of a systematic multivariate optimisation approach makes it possible to derive statistical models (based on least squares estimates and analysis of variance) that fit and describe the dynamics of a natural product extraction. The technique may go a long way as an important contribution towards the use of statistical optimisation schemes for the SFE of natural products. Factorial analysis has only been famous for its use in locating true optimum parameter zones, but its use (in this study) in modeling the supercritical fluid extraction of natural products is undoubtedly an innovative study.

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# APPENDIX A according to reference 135.

$$F(F) = \int_0^F \frac{\Gamma\left(\frac{m+n}{2}\right)}{\Gamma\left(\frac{m}{2}\right)\Gamma\left(\frac{n}{2}\right)} m^{\frac{m}{2}} n^{\frac{n}{2}} x^{\frac{m}{2}-1} (n+mx)^{-\frac{m+n}{2}} dx = .95$$

| n \ m | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 12 | 15 | 20 | 24 | 30 | 40 | 60 | 120 | ∞ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 161 4 | 199 5 | 215 7 | 224 6 | 230 2 | 234 0 | 236 8 | 238 9 | 240 5 | 241 9 | 243 9 | 245 9 | 248 0 | 249 1 | 250 1 | 251 1 | 252 2 | 253 3 | 254 3 |
| 2 | 18 51 | 19 00 | 19 16 | 19 25 | 19 30 | 19 33 | 19 35 | 19 37 | 19 38 | 19.40 | 19.41 | 19 43 | 19 45 | 19 45 | 19.46 | 19 47 | 19 48 | 19 49 | 19 50 |
| 3 | 10 13 | 9 55 | 9 28 | 9.12 | 9 01 | 8 94 | 8 89 | 8 85 | 8 81 | 8.79 | 8.74 | 8.70 | 8 66 | 8 64 | 8 62 | 8 59 | 8 57 | 8 55 | 8 53 |
| 4 | 7 71 | 6 94 | 6.59 | 6.39 | 6 26 | 6 16 | 6.09 | 6 04 | 6 00 | 5.96 | 5.91 | 5 86 | 5 80 | 5 77 | 5 75 | 5 72 | 5 69 | 5 66 | 5 63 |
| 5 | 6 61 | 5 79 | 5.41 | 5 19 | 5.05 | 4 95 | 4.88 | 4 82 | 4.77 | 4.74 | 4 68 | 4.62 | 4 56 | 4 53 | 4 50 | 4 46 | 4 43 | 4 40 | 4 36 |
| 6 | 5 99 | 5 14 | 4 76 | 4 53 | 4.39 | 4.28 | 4.21 | 4 15 | 4 10 | 4 06 | 4 00 | 3.94 | 3 87 | 3 84 | 3 81 | 3 77 | 3 74 | 3 70 | 3 67 |
| 7 | 5 59 | 4 74 | 4 35 | 4 12 | 3 97 | 3.87 | 3 79 | 3 73 | 3 68 | 3 64 | 3 57 | 3 51 | 3 44 | 3 41 | 3 38 | 3 34 | 3 30 | 3 27 | 3 23 |
| 8 | 5 32 | 4 46 | 4 07 | 3 84 | 3 69 | 3 58 | 3.50 | 3 44 | 3 39 | 3 35 | 3 28 | 3 22 | 3 15 | 3.12 | 3 08 | 3 04 | 3 01 | 2 97 | 2 93 |
| 9 | 5 12 | 4 26 | 3 86 | 3 63 | 3.48 | 3 37 | 3 29 | 3 23 | 3.18 | 3.14 | 3.07 | 3 01 | 2 94 | 2 90 | 2 86 | 2 83 | 2 79 | 2 75 | 2 71 |
| 10 | 4 96 | 4 10 | 3 71 | 3 48 | 3.33 | 3.22 | 3 14 | 3.07 | 3.02 | 2.98 | 2.91 | 2.85 | 2.77 | 2.74 | 2.70 | 2 66 | 2 62 | 2 58 | 2 54 |
| 11 | 4 84 | 3 98 | 3 59 | 3 36 | 3 20 | 3 09 | 3 01 | 2 95 | 2 90 | 2.85 | 2 79 | 2.72 | 2 65 | 2 61 | 2 57 | 2 53 | 2 49 | 2 45 | 2 40 |
| 12 | 4 75 | 3 89 | 3 49 | 3 26 | 3.11 | 3 00 | 2 91 | 2 85 | 2.80 | 2.75 | 2 69 | 2 62 | 2 54 | 2 51 | 2 47 | 2 43 | 2 38 | 2 34 | 2 30 |
| 13 | 4 67 | 3 81 | 3.41 | 3 18 | 3.03 | 2 92 | 2.83 | 2 77 | 2.71 | 2 67 | 2 60 | 2 53 | 2 46 | 2.42 | 2 38 | 2 34 | 2 30 | 2 25 | 2 21 |
| 14 | 4 60 | 3 74 | 3 34 | 3.11 | 2 96 | 2.85 | 2 76 | 2 70 | 2 65 | 2 60 | 2 53 | 2 46 | 2 39 | 2 35 | 2 31 | 2 27 | 2 22 | 2 18 | 2 13 |
| 15 | 4 54 | 3 68 | 3 29 | 3 06 | 2.90 | 2.79 | 2 71 | 2 64 | 2.59 | 2 54 | 2.48 | 2 40 | 2 33 | 2.29 | 2.25 | 2 20 | 2 16 | 2 11 | 2 07 |
| 16 | 4 49 | 3 63 | 3 24 | 3 01 | 2.85 | 2.74 | 2 66 | 2 59 | 2 54 | 2 49 | 2.42 | 2.35 | 2.28 | 2 24 | 2 19 | 2 15 | 2 11 | 2 06 | 2 01 |
| 17 | 4 45 | 3 59 | 3 20 | 2 96 | 2 81 | 2 70 | 2 61 | 2.55 | 2 49 | 2.45 | 2 38 | 2 31 | 2 23 | 2 19 | 2.15 | 2 10 | 2 06 | 2 01 | 1 96 |
| 18 | 4 41 | 3 55 | 3 16 | 2 93 | 2 77 | 2 66 | 2 58 | 2 51 | 2 46 | 2 41 | 2.34 | 2 27 | 2 19 | 2 15 | 2 11 | 2 06 | 2 02 | 1 97 | 1 92 |
| 19 | 4 38 | 3 52 | 3 13 | 2 90 | 2 74 | 2 63 | 2 54 | 2 48 | 2 42 | 2 38 | 2 31 | 2 23 | 2 16 | 2.11 | 2 07 | 2 03 | 1 98 | 1 93 | 1 88 |
| 20 | 4 35 | 3 49 | 3 10 | 2 87 | 2 71 | 2 60 | 2 51 | 2 45 | 2 39 | 2 35 | 2 28 | 2 20 | 2 12 | 2 08 | 2 04 | 1 99 | 1 95 | 1 90 | 1 84 |
| 21 | 4 32 | 3 47 | 3 07 | 2 84 | 2 68 | 2 57 | 2 49 | 2 42 | 2 37 | 2 32 | 2 25 | 2 18 | 2 10 | 2 05 | 2 01 | 1 96 | 1 92 | 1 87 | 1 81 |
| 22 | 4 30 | 3 44 | 3 05 | 2 82 | 2 66 | 2 55 | 2 46 | 2 40 | 2 34 | 2 30 | 2 23 | 2 15 | 2 07 | 2 03 | 1 98 | 1 94 | 1 89 | 1 84 | 1 78 |
| 23 | 4 28 | 3 42 | 3 03 | 2 80 | 2 64 | 2 53 | 2 44 | 2 37 | 2 32 | 2 27 | 2 20 | 2 13 | 2 05 | 2 01 | 1 96 | 1 91 | 1 86 | 1 81 | 1 76 |
| 24 | 4 26 | 3 40 | 3 01 | 2 78 | 2 62 | 2 51 | 2 42 | 2 36 | 2 30 | 2 25 | 2 18 | 2 11 | 2 03 | 1 98 | 1 94 | 1 89 | 1 84 | 1 79 | 1 73 |
| 25 | 4 24 | 3 39 | 2 99 | 2 76 | 2 60 | 2 49 | 2 40 | 2 34 | 2 28 | 2 24 | 2 16 | 2 09 | 2 01 | 1 96 | 1 92 | 1 87 | 1 82 | 1 77 | 1 71 |
| 26 | 4 23 | 3 37 | 2 98 | 2 74 | 2 59 | 2 47 | 2 39 | 2 32 | 2 27 | 2 22 | 2 15 | 2 07 | 1 99 | 1 95 | 1 90 | 1 85 | 1 80 | 1 75 | 1 69 |
| 27 | 4 21 | 3 35 | 2 96 | 2 73 | 2 57 | 2 46 | 2 37 | 2 31 | 2 25 | 2 20 | 2 13 | 2 06 | 1 97 | 1 93 | 1 88 | 1 84 | 1 79 | 1 73 | 1 67 |
| 28 | 4 20 | 3 34 | 2 95 | 2 71 | 2 56 | 2 45 | 2 36 | 2 29 | 2 24 | 2 19 | 2 12 | 2 04 | 1 96 | 1 91 | 1 87 | 1 82 | 1 77 | 1 71 | 1 65 |
| 29 | 4 18 | 3 33 | 2 93 | 2 70 | 2 55 | 2 43 | 2 35 | 2 28 | 2 22 | 2 18 | 2 10 | 2 01 | 1 94 | 1 90 | 1 85 | 1 81 | 1 75 | 1 70 | 1 64 |
| 30 | 4 17 | 3 32 | 2 92 | 2 69 | 2 53 | 2 42 | 2 33 | 2 27 | 2 21 | 2 16 | 2 09 | 2 01 | 1 93 | 1 89 | 1 84 | 1 79 | 1 74 | 1 68 | 1 62 |
| 40 | 4 08 | 3 23 | 2 84 | 2 61 | 2 45 | 2 34 | 2 25 | 2 18 | 2 12 | 2 08 | 2 00 | 1 92 | 1 84 | 1 79 | 1 74 | 1 69 | 1 64 | 1 58 | 1 51 |
| 60 | 4 00 | 3 15 | 2 76 | 2 53 | 2 37 | 2 25 | 2 17 | 2 10 | 2 04 | 1 99 | 1 92 | 1 84 | 1 75 | 1 70 | 1 65 | 1 59 | 1 53 | 1 47 | 1 39 |
| 120 | 3 92 | 3 07 | 2 68 | 2 45 | 2 29 | 2 17 | 2 09 | 2 02 | 1 96 | 1 91 | 1 83 | 1 75 | 1 66 | 1 61 | 1 55 | 1 50 | 1 43 | 1 35 | 1 25 |
| ∞ | 3 84 | 3 00 | 2 60 | 2 37 | 2 21 | 2 10 | 2 01 | 1 94 | 1 88 | 1 83 | 1 75 | 1 67 | 1 57 | 1 52 | 1 46 | 1 39 | 1 32 | 1 22 | 1 00 |

$F = \frac{s_1^2}{s_2^2} = \frac{S_1}{m} \Big/ \frac{S_2}{n}$, where $s_1^2 = S_1/m$ and $s_2^2 = S_2/n$ are independent mean squares estimating a common variance $\sigma^2$ and based on $m$ and $n$ degrees of freedom, respectively.

# APPENDIX B according to reference 135.

$$F(F) = \int_0^F \frac{\Gamma\left(\frac{m+n}{2}\right)}{\Gamma\left(\frac{m}{2}\right)\Gamma\left(\frac{n}{2}\right)} m^{\frac{m}{2}} n^{\frac{n}{2}} x^{\frac{m}{2}-1} (n+mx)^{-\frac{m+n}{2}} dx = .99$$

| $n$ \ $m$ | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 12 | 15 | 20 | 24 | 30 | 40 | 60 | 120 | ∞ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 4052 | 4999.5 | 5403 | 5625 | 5764 | 5859 | 5928 | 5982 | 6022 | 6056 | 6106 | 6157 | 6209 | 6235 | 6261 | 6287 | 6313 | 6339 | 6366 |
| 2 | 98.50 | 99.00 | 99.17 | 99.25 | 99 30 | 99.33 | 99.36 | 99.37 | 99.39 | 99 40 | 99.42 | 99 43 | 99 45 | 99 46 | 99 47 | 99 47 | 99 48 | 99 49 | 99 50 |
| 3 | 34.12 | 30.82 | 29.46 | 28.71 | 28 24 | 27.91 | 27.67 | 27.49 | 27.35 | 27 23 | 27 05 | 26 87 | 26.69 | 26 60 | 26 50 | 26 41 | 26 32 | 26 22 | 26 13 |
| 4 | 21.20 | 18.00 | 16.69 | 15.98 | 15 52 | 15.21 | 14.98 | 14.80 | 14.66 | 14 55 | 14.37 | 14 20 | 14 02 | 13 93 | 13 84 | 13 75 | 13 65 | 13 56 | 13.46 |
| 5 | 16.26 | 13.27 | 12.06 | 11.39 | 10 97 | 10.67 | 10.46 | 10.29 | 10.16 | 10 05 | 9.89 | 9.72 | 9.55 | 9 47 | 9 38 | 9 29 | 9 20 | 9 11 | 9 02 |
| 6 | 13.75 | 10.92 | 9.78 | 9.15 | 8 75 | 8.47 | 8.26 | 8.10 | 7.98 | 7 87 | 7.72 | 7 56 | 7.40 | 7 31 | 7.23 | 7.14 | 7 06 | 6 97 | 6 88 |
| 7 | 12.25 | 9.55 | 8.45 | 7.85 | 7.46 | 7.19 | 6.99 | 6.84 | 6.72 | 6 62 | 6.47 | 6 31 | 6.16 | 6.07 | 5.99 | 5.91 | 5 82 | 5.74 | 5 65 |
| 8 | 11.26 | 8.65 | 7.59 | 7.01 | 6 63 | 6.37 | 6.18 | 6.03 | 5.91 | 5 81 | 5.67 | 5 52 | 5.36 | 5.28 | 5.20 | 5.12 | 5 03 | 4 95 | 4 86 |
| 9 | 10.56 | 8.02 | 6.99 | 6.42 | 6 06 | 5.80 | 5.61 | 5.47 | 5.35 | 5 26 | 5.11 | 4.96 | 4.81 | 4.73 | 4 65 | 4.57 | 4.48 | 4.40 | 4.31 |
| 10 | 10.04 | 7.56 | 6.55 | 5.99 | 5 64 | 5.39 | 5.20 | 5.06 | 4.94 | 4 85 | 4.71 | 4 56 | 4.41 | 4.33 | 4 25 | 4.17 | 4 08 | 4 00 | 3 91 |
| 11 | 9.65 | 7.21 | 6.22 | 5.67 | 5 32 | 5.07 | 4.89 | 4.74 | 4.63 | 4 54 | 4 40 | 4.25 | 4 10 | 4 02 | 3.94 | 3.86 | 3.78 | 3 69 | 3 60 |
| 12 | 9.33 | 6.93 | 5.95 | 5.41 | 5 06 | 4.82 | 4.64 | 4.50 | 4.39 | 4.30 | 4 16 | 4 01 | 3 86 | 3.78 | 3 70 | 3 62 | 3 54 | 3.45 | 3.36 |
| 13 | 9.07 | 6.70 | 5.74 | 5.21 | 4 86 | 4.62 | 4.44 | 4.30 | 4.19 | 4 10 | 3.96 | 3 82 | 3 66 | 3.59 | 3 51 | 3.43 | 3 34 | 3 25 | 3.17 |
| 14 | 8.86 | 6.51 | 5.56 | 5.04 | 4 69 | 4.46 | 4.28 | 4.14 | 4.03 | 3 94 | 3.80 | 3.66 | 3 51 | 3 43 | 3.35 | 3.27 | 3.18 | 3 09 | 3.00 |
| 15 | 8.68 | 6.36 | 5.42 | 4.89 | 4 56 | 4.32 | 4.14 | 4.00 | 3.89 | 3 80 | 3.67 | 3.52 | 3 37 | 3.29 | 3.21 | 3 13 | 3 05 | 2 96 | 2.87 |
| 16 | 8.53 | 6.23 | 5.29 | 4.77 | 4 44 | 4.20 | 4.03 | 3.89 | 3.78 | 3 69 | 3.55 | 3 41 | 3 26 | 3 18 | 3.10 | 3 02 | 2 93 | 2 84 | 2.75 |
| 17 | 8.40 | 6.11 | 5.18 | 4.67 | 4 34 | 4.10 | 3.93 | 3.79 | 3.68 | 3.59 | 3.46 | 3 31 | 3 16 | 3 08 | 3 00 | 2.92 | 2 83 | 2 75 | 2.65 |
| 18 | 8.29 | 6.01 | 5.09 | 4.58 | 4 25 | 4.01 | 3.84 | 3.71 | 3.60 | 3 51 | 3.37 | 3 23 | 3.08 | 3 00 | 2.92 | 2.84 | 2.75 | 2 66 | 2.57 |
| 19 | 8.18 | 5.93 | 5.01 | 4.50 | 4 17 | 3.94 | 3.77 | 3.63 | 3.52 | 3 43 | 3.30 | 3.15 | 3.00 | 2.92 | 2 84 | 2.76 | 2 67 | 2 58 | 2.49 |
| 20 | 8.10 | 5.85 | 4.94 | 4.43 | 4 10 | 3.87 | 3.70 | 3.56 | 3.46 | 3 37 | 3.23 | 3 09 | 2 94 | 2 86 | 2.78 | 2 69 | 2 61 | 2 52 | 2 42 |
| 21 | 8.02 | 5.78 | 4.87 | 4.37 | 4.04 | 3.81 | 3.64 | 3.51 | 3.40 | 3 31 | 3.17 | 3 03 | 2 88 | 2.80 | 2.72 | 2 64 | 2 55 | 2.46 | 2 36 |
| 22 | 7.95 | 5.72 | 4.82 | 4.31 | 3 99 | 3.76 | 3.59 | 3.45 | 3.35 | 3.26 | 3.12 | 2 98 | 2 83 | 2.75 | 2 67 | 2 58 | 2 50 | 2.40 | 2.31 |
| 23 | 7.88 | 5.66 | 4.76 | 4.26 | 3.94 | 3.71 | 3.54 | 3.41 | 3.30 | 3.21 | 3 07 | 2 93 | 2.78 | 2.70 | 2 62 | 2 54 | 2.45 | 2 35 | 2 26 |
| 24 | 7.82 | 5.61 | 4.72 | 4.22 | 3 90 | 3.67 | 3.50 | 3.36 | 3.26 | 3.17 | 3.03 | 2.89 | 2.74 | 2.66 | 2.58 | 2 49 | 2.40 | 2 31 | 2.21 |
| 25 | 7.77 | 5.57 | 4.68 | 4.18 | 3.85 | 3.63 | 3.46 | 3.32 | 3.22 | 3 13 | 2.99 | 2 85 | 2.70 | 2 62 | 2.54 | 2.45 | 2 36 | 2 27 | 2.17 |
| 26 | 7.72 | 5.53 | 4.64 | 4.14 | 3 82 | 3.59 | 3.42 | 3.29 | 3.18 | 3.09 | 2.96 | 2 81 | 2 66 | 2 58 | 2.50 | 2.42 | 2 33 | 2 23 | 2.13 |
| 27 | 7.68 | 5.49 | 4.60 | 4.11 | 3 78 | 3.56 | 3.39 | 3.26 | 3 15 | 3.06 | 2 93 | 2 78 | 2 63 | 2 55 | 2 47 | 2 38 | 2 29 | 2 20 | 2 10 |
| 28 | 7.64 | 5.45 | 4.57 | 4.07 | 3.75 | 3.53 | 3.36 | 3.23 | 3 12 | 3.03 | 2.90 | 2.75 | 2.60 | 2.52 | 2.44 | 2.35 | 2.26 | 2.17 | 2 06 |
| 29 | 7.60 | 5.42 | 4.54 | 4.04 | 3.73 | 3.50 | 3.33 | 3.20 | 3 09 | 3.00 | 2 87 | 2.73 | 2 57 | 2.49 | 2 41 | 2.33 | 2 23 | 2 14 | 2 03 |
| 30 | 7.56 | 5.39 | 4.51 | 4.02 | 3 70 | 3.47 | 3.30 | 3.17 | 3 07 | 2 98 | 2 84 | 2 70 | 2 55 | 2 47 | 2 39 | 2 30 | 2 21 | 2 11 | 2 01 |
| 40 | 7.31 | 5.18 | 4.31 | 3.83 | 3 51 | 3.29 | 3.12 | 2.99 | 2 89 | 2 80 | 2.66 | 2 52 | 2 37 | 2 29 | 2.20 | 2 11 | 2 02 | 1 92 | 1 80 |
| 60 | 7.08 | 4.98 | 4.13 | 3.65 | 3 34 | 3.12 | 2.95 | 2.82 | 2 72 | 2 63 | 2 50 | 2 35 | 2 20 | 2 12 | 2.03 | 1.94 | 1 84 | 1 73 | 1 60 |
| 120 | 6.85 | 4.79 | 3.95 | 3.48 | 3 17 | 2.96 | 2.79 | 2 66 | 2 56 | 2 47 | 2 34 | 2 19 | 2 03 | 1.95 | 1.86 | 1.76 | 1 66 | 1 53 | 1 38 |
| ∞ | 6.63 | 4.61 | 3.78 | 3.32 | 3 02 | 2.80 | 2.64 | 2.51 | 2 41 | 2 32 | 2 18 | 2 04 | 1 88 | 1.79 | 1.70 | 1 59 | 1 47 | 1.32 | 1.00 |

$F = \frac{s_1^2}{s_2^2} = \frac{S_1}{m} \Big/ \frac{S_2}{n}$, where $s_1^2 = S_1/m$ and $s_2^2 = S_2/n$ are independent mean squares estimating a common variance $\sigma^2$ and based on $m$ and $n$ degrees of freedom, respectively.



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