BLDSC no :- DX88172

LOUGHBOROUGH
UNIVERSITY OF TECHNOLOGY
LIBRARY

AUTHOR/FILING TITLE

SANAGI, M M

ACCESSION/COPY NO.

040013068

VOL. NO. CLASS MARK

LOAN COPY

13 JUN 1990
5 JUL 1991
1992
3 JUL 1992
- 2 JUL 1993
- 2 JUL 1993
- 1 JUL 1994
28 JUN 1996
27 JUN 1997
20 MAR 1998
26 JUN 1998
25.6.99

040013068 8

THIS BOOK WAS BOUND BY
BADMINTON PRESS
18 THE HALFCROFT
SYSTON
LEICESTER LE7 8LD
0533 602918

SELECTIVITY AND APPLICATIONS OF SUPERCRITICAL FLUID CHROMATOGRAPHY

by

M. MARSIN SANAGI

A Doctoral Thesis
submitted in partial fulfilment of the
requirements for the award of the Doctor of Philosophy
of the Loughborough University of Technology

November, 1988

Supervisor: Dr. R.M. Smith
Department of Chemistry

© by M.M. SANAGI, 1988

Loughborough University
of Technology Library
Date Oct. 89
Class
Acc. No. 040013068

Dedicated to

my wife, Noriah,

my children, Faridah, Hanizah and Jamilia,

and my parents.

ACKNOWLEDGEMENTS

First and foremost I wish to give all the Praise to Almighty God, for with His mercy has given me the strength and time to complete this work.

I would like to thank my supervisor, Dr. Roger M. Smith, for his help and guidance throughout this study. He has taught me his professionalism and the profound art of research, some of which is reflected in this work. For our many lengthy and enjoyable discussions, I am very grateful.

I wish to thank the Department of Chemistry technical staff, especially to J.C. Kershaw, J. Swithenbank, A.G. Stevens, A.F. Bower, and A.J. Greenfield for their invaluable assistance and technical skills. I would also like to thank my friends for useful discussions and for fostering the spirit of cooperation.

I would like to thank Professor L. Oliveros-Belardo for the essential oil sample, Polymer Laboratories, Beckman Laboratories, and Phase Separations for generous gifts of packed columns, and V.G. Evans for furnishing results of his SFC work on cyano-silica columns. I would also like to thank Universiti Teknologi Malaysia and the Public Services Department, Malaysia, for a study leave and financial supports.

Finally, I wish to extend my thanks, for more than words can express, to my wife, Noriah, and my family for their encouragement, and patience throughout the present study.

ABSTRACT

Supercritical fluid chromatography (SFC) has been undergoing tremendous developments in recent years as an attractive technique that is complementary to both gas and liquid chromatography. The main aim of this study has been to examine selectivity in SFC by using retention indices as the means of recording retention and to explore the potential applications of this novel technique.

A simple supercritical fluid chromatograph was constructed based on a Pye-Unicam gas chromatograph and a HPLC pump. SFC separations were carried out using HPLC packed columns with pure or modified carbon dioxide as the mobile phase and using ultraviolet and flame ionization detection. The effects of different parameters on the retention of compounds of different functional groups have been studied.

At a given temperature, the capacity factors of the compounds generally decreased with increases in pressure (density). Little change in selectivity was observed between compounds in a homologous series but selectivity differences were observed between compounds of different functionalities and anomalous behaviour was seen for acetophenone on a cyano-silica column. Temperature can also be used to selectively modify the retention of the analytes. The use of methanol or acetonitrile as the organic modifier generally improved the peak shapes and reduced the retentions, and this was particularly marked for polar compounds. Similar trends in retentions were observed for PS-DVB, ODS-, and cyano-silica columns although the compounds were generally more retained on the PS-DVB column, suggesting that the selectivity was controlled mainly by the properties and compositions of the mobile phase.

Using different sets of homologues, alkylarylketones, n-alkanes, and alkylbenzenes, the possibility of the application of retention indices in SFC was investigated. The use of retention indices have been shown to have advantages over capacity factors, but unlike HPLC, retention indices in SFC are much more susceptible to selectivity changes caused by variations in the operating parameters.

Studies were carried out to demonstrate the viability of SFC for the separation of several drug groups, barbiturates and benzodiazepines. With no modifier in the mobile phase, these compounds were highly retained on the columns, particularly on ODS- and cyano-silica. Successful separations of the drug compounds were achieved with methanol or acetonitrile as the modifier in the mobile phase. The application of the SFC system was extended to the separations of several terpenes and the leaf essential oil of *Cymbopogon martinii* (Roxb.) Wats on a PS-DVB column. The results were compared with those obtained using GC and GC-MS methods and it was evident that the GC methods gave better resolutions and sensitivity and would still be the method of choice.

CONTENTS

Page

ACKNOWLEDGEMENTS iv
ABSTRACT v

CHAPTER 1 INTRODUCTION 1

1.1 Supercritical fluids and SFC 4
1.1.1 Definition 4
1.1.2 Properties of supercritical fluids 5
1.2 Retention and selectivity dependency in SFC 7
1.2.1 Density 7
1.2.2 Pressure 8
1.2.3 Temperature 9
1.2.4 Stationary phases 10
1.2.5 Mobile phases 14
1.2.6 Column pressure drop 17
1.2.7 Mechanisms of solute retention 17
1.3 SFC instrumentation 21
1.3.1 Commercial vs. home-made instruments 21
1.3.2 Detectors 22
1.3.3 Delivery systems 23
1.3.4 Pump head cooling 24
1.3.5 Introduction of modifier 27
1.3.6 Reproducibility 28
1.3.7 Programmed elution in SFC 29
1.4 The present project 31

CHAPTER 2 EXPERIMENTAL 33

2.1 Reagents 33
2.1.1 Solvents 33
2.1.2 Standard compounds 33
2.1.3 Test compounds 34
2.2 Apparatus 34
2.2.1 High performance liquid chromatography 34
2.2.2 Chromatographic columns 35

2.2.3 Column packing apparatus 35
2.2.4 Spectroscopy 36
2.3 Procedure 36
2.3.1 Sample preparation 36
2.3.2 Calculations 36
2.3.3 Calculation of supercritical fluid carbon dioxide density 37

CHAPTER 3 INSTRUMENTATION 39

3.1 Introduction 39
3.2 General description 39
3.3 Mobile phase delivery 42
3.3.1 Mobile phase 42
3.3.2 High pressure pump 45
3.3.3 Connections and tubing in the delivery system 48
3.4 Chromatographic oven 49
3.5 Sample introduction 49
3.6 Analytical column 51
3.7 Pressure and eluent flow controller 51
3.8 Detectors 54
3.8.1 UV detector 54
3.8.2 Flame ionization detector 55

CHAPTER 4 PRELIMINARY APPLICATION STUDIES 58

4.1 Introduction 58
4.2 Packing of columns 58
4.2.1 Practical considerations 58
4.2.2 Packing procedure 60
4.3 Operation and evaluation of the instrument 61
4.3.1 Start-up procedure 61
4.3.2 Pump performance 62
4.3.3 Operating temperature and pressure 63
4.3.4 Effect of pressure-drop 63
4.3.5 Restrictors and eluent flow rates 65
4.3.6 Flow rate determinations 67
4.3.7 Baseline stability 69
4.3.8 Determination of void volume 71

4.4 Reproducibility of injection and detection 72
4.4.1 Effect of sample loading 72
4.4.2 Quantitative analyses 74
4.4.3 Reproducibility of retention time and peak area 74
4.5 Effect of flow rate on retention and efficiency 79
4.6 Preliminary SFC separations on ODS-silica column 82
4.6.1 n-alkanes 83
4.6.2 Aromatic compounds 85
4.7 Preliminary SFC separations on PS-DVB column 87
4.7.1 Homologous compounds 87
4.7.2 Alcohols 89
4.7.3 Amines 81
4.7.4 Fatty acids and the esters 91
4.8 Effect of modifier in SFC with flame ionization detection 94

CHAPTER 5 SELECTIVITY IN SFC 95

5.1 Introduction 95
5.2 Retention indices 96
5.2.1 Establishment of a retention index scale 98
5.3 Selectivity and retention on PLRP-S column 115
5.3.1 Model compounds 115
5.3.2 Effect of pressure 116
5.3.3 Effect of density 122
5.3.4 Effect of temperature 124
5.4 Comparison of selectivity on different stationary phases in SFC 130
5.5 Effect of mobile phase composition on selectivity in SFC 139
5.5.1 Inroduction 139
5.5.2 Methanol as modifier on PLRP-S column 139
5.5.3 Acetonitrile as modifier on PLRP-S column 146
5.5.4 Changes in conditions 150
5.6 Anomalous retention behaviour of acetophenone on cyano-silica 159
5.7 Conclusions 164

CHAPTER 6 APPLICATION OF SFC TO THE ANALYSIS OF BARBITURATES 166

6.1 Introduction 166
6.1.1 Barbiturates 166
6.1.2 Analysis of barbiturates 168
6.1.3 Retention indices in drug analysis 170
6.1.4 Aim of the study 171
6.2 SFC separations of barbiturates on PS-DVB column with carbon dioxide as the mobile phasse 172
6.3 SFC separations of barbiturates on PS-DVB column with modified carbon dioxide as the mobile phase 176
6.3.1 Effect of diferent modifiers 176
6.3.2 Chromatographic behaviour of barbiturates on PLRP-S column using methanol as the modifier 177
6.4 Comparison of SFC separations of barbiturates on PS-DVB and ODS-silica stationary phases with methanol as the modifier 185
6.4.1 Retention and selectivity 185
6.5 Conclusions 189

CHAPTER 7 APPLICATION OF SFC TO THE ANALYSIS OF BENZODIAZEPINES 191

7.1 Introduction 191
7.1.1 Benzodiazepines 191
7.1.2 Analysis of benzodiazepines 191
7.1.3 Retention indices in benzodiazepine analysis 194
7.1.4 Aim of the study 195
7.2 SFC separations of benzodiazepines with carbon dioxide as the mobile phase 195
7.3 SFC separations of benzodiazepines on PS-DVB column with modified carbon dioxide as the mobile phase 198
7.3.1 Effect of different modifiers 198
7.3.2 Chromatographic behaviour of benzodiazepines on PS-DVB column using methanol as the modifier 200
7.4 Comparison of SFC separations of benzodiazepines on different stationary phases with methanol as the modifier 203

7.4.1 SFC separations of benzodiazepines on ODS-silica column 203
7.4.2 SFC separations of benzodiazepines on cyano-silica column 207
7.5 Conclusions 210

CHAPTER 8 APPLICATION OF SFC, GC, AND GC-MS TO THE ANALYSIS OF TERPENES: THE ANALYSIS OF *CYMBOPOGON MARTINII* (ROXB.) WATS 212

8.1 Introduction 212
8.1.1 Terpenes and essential oils 212
8.1.2 Essential oil preparation methods 213
8.1.3 Analysis of terpenes and essential oils 214
8.1.4 Retention indices in essential oil analysis 217
8.1.5 Aim of the study 218
8.2 *Cymbopogon martinii* (Roxb.) Wats 218
8.3 Experimental 219
8.3.1 Materials 219
8.3.2 Sample preparation 220
8.3.3 GC and GC-MS instrumentation 220
8.3.4 Methods 221
8.4 GC and GC-MS analysis 222
8.4.1 Gas chromatography of solvents
8.4.2 GC separations of the essential oil of *Cymbopogon martinii* (Roxb.) Wats and terpene standards 223
8.4.3 GC-MS separations of the essential oil of *Cymbopogon martinii*
8.5 SFC separations of terpenes and essential oil 235
8.6 Comparison of SFC, GC, and GC-MS methods in the analysis of essential oils and terpenes 239
8.7 Conclusions 240

CHAPTER 9 CONCLUSIONS AND SUGGESTIONS FOR FURTHER WORK 241

REFERENCES 248
APPENDIX 263

CHAPTER 1

INTRODUCTION

Although supercritical fluid chromatography (SFC) was first demonstrated as early as 1962 [1], it was only recently that the advantages have been fully realized and there has been a widespread interest in the technique [2-6]. This resurgence of interest is indicated in the sudden and exponential increase in papers since the early 1980s (Figure 1.1) [7]. The principles, instrumentation, applications and the present state of SFC have been extensively reviewed in a number of articles [8-17]. The recent publications in SFC, include books devoted on SFC and related subjects [2-4], a book compilation of more than 300 SFC chromatograms with the accompanying important operating parameters [5], and a comprehensive bibliography of SFC and related articles [6].

SFC is showing considerable potential as an alternative instrumental analytical technique complementing high performance liquid chromatography (HPLC) and gas chromatography (GC). It has been gaining acceptance in a wide range of areas in analytical as well as industrial chemistry, particularly for petrochemicals and pharmaceuticals [2]. Nevertheless, there is a potentially vast area for new applications with improvements in this technique to enable separations of more complex mixtures or matrices.

The common goal of all analytical chromatographic methods is to be able to resolve and determine individual components found in mixtures. One of the ways to achieve resolution in chromatographic techniques is by changing the selectivity. Unlike HPLC or GC, the density of the mobile phase in SFC (determined by the combination of pressure and

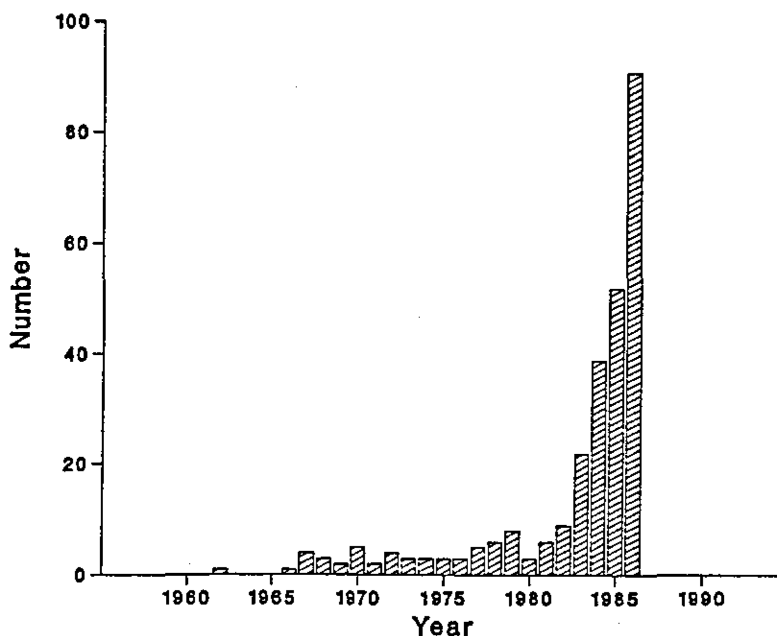


FIGURE 1.1. Distribution of published articles on SFC (by year) from 1962 to 1986 based on a survey, July, 1987.

temperature) plays an important role in dictating the retention of individual compounds [2]. This gives an additional flexibility in optimizing separations in SFC and enables it to offer an alternative selectivity to the other techniques. However, a recent literature survey reveals that, as yet, there is only a limited amount of information on the chromatographic retention behaviour of solutes in SFC [7]. Therefore, some knowledge in this area would serve as a valuable guide to achieve sound supercritical fluid chromatographic separations.

As with HPLC and GC the retention and selectivity in SFC are dependent on the conditions of the separation, including pressure, column temperature, stationary phase, and proportion of modifiers as well as the flow rate of the mobile phase [18]. Inevitably, as with HPLC and GC, some of the experimental conditions are going to be difficult to transfer exactly between different laboratories, or even between

instruments in the same laboratory. Differences in the stationary phases, even if nominally equivalent, are expected to cause corresponding problems in reproducibility, particularly for packed columns, to those already well known in HPLC [19]. Although many samples in SFC have been pure compounds, as the method develops it will be more used for qualitative analyses, possibly combined with other instrumental techniques such as mass spectrometry (SFC-MS) [20-22], and infrared spectroscopy (SFC-FTIR) [23-25]. As a consequence it will become necessary to compare results between standards or between laboratories and retention will need to be recorded by a robust and independent method, independent of the operating conditions. Because they are interpolated relative measurements, retention indices are considerably more robust to changes in conditions of separations and have found wide applications for identification and comparison studies in both GC [26,27] and HPLC [19,28]. These concepts are potentially also applicable to SFC.

The present study set out to develop a packed column supercritical fluid chromatograph with flame ionization (FID) and ultraviolet (UV) detectors in conjunction with carbon dioxide as the primary mobile phase. The constructed system has been used in solute retention and selectivity studies and the importance of a number of parameters including pressure, temperature, stationary phase, and mobile phase composition has been investigated. This work has led to a detailed investigation of the application of retention indices in SFC and a comparison of different reference homologous series. The application of the SFC system to the analyses of several groups of compounds, including barbiturates, benzodiazepines, and terpenes have also been studied. In this introduction the principles of supercritical fluids and SFC are

described briefly and previously reported methods to develop home-made packed column SFC systems are reviewed. The parameters which have been reported to govern solute retention and selectivity in SFC are then discussed.

1.1 SUPERCRITICAL FLUIDS AND SFC

1.1.1 Definition

By definition, SFC is a chromatographic technique which uses a supercritical fluid as the mobile phase. In turn, a supercritical fluid is a substance which has been raised above its critical temperature and critical pressure. The critical temperature, T_c, of a gas is the temperature above which it cannot be liquefied no matter high the pressure and the critical pressure, P_c, is the lowest pressure which will liquefy the gas at its critical temperature [29].

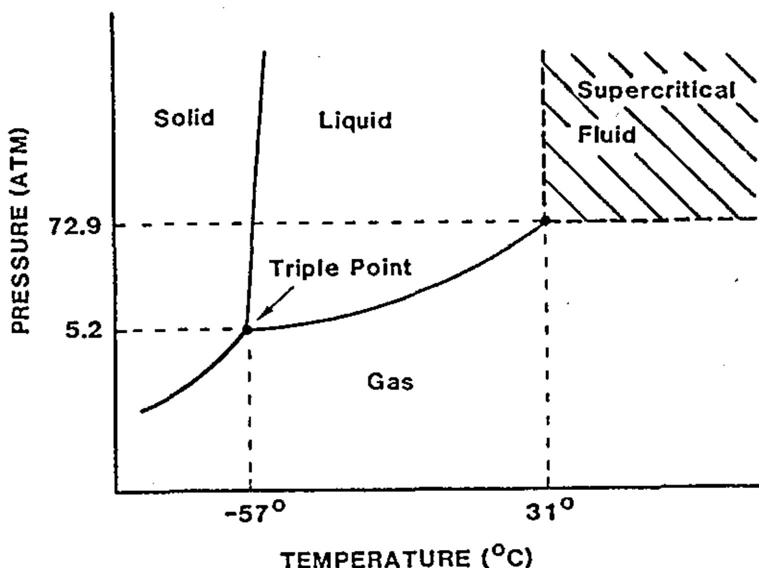


FIGURE 1.2. Phase diagram of carbon dioxide.

Figure 1.2 illustrates the phase diagram of carbon dioxide. The supercritical fluid area is bordered by dashed lines, indicating a smooth transition of properties and that no phase changes occur on crossing these lines.

1.1.2 Properties of supercritical fluids

There are many compounds which can be used as the mobile phase in SFC. A few of these are listed in Table 1.1 together with their physical properties, critical temperature, pressure, and density [15].

The physical properties of a supercritical fluid are generally intermediate between those of a gas and a liquid (Table 1.2) [30]. Compared to liquids, supercritical fluids offer higher analyte diffusivities. Under normal operating conditions, this higher solute diffusion in the mobile phase increases equilibrium speed. Hence higher efficiencies per unit time and consequently faster analyses can be obtained. However, supercritical fluids have lower diffusivities compared to gases, and therefore, smaller inside diameters are required in capillary SFC than those commonly used in capillary GC. The viscosities of supercritical fluids in typical SFC separations are much lower than those of liquids, and can approach those of a gas. Thus, a longer column can be used to achieve higher efficiencies, or alternatively, very fast analyses may be possible with SFC using micro-particulate packed columns and pressure drops similar to those observed in HPLC [31].The solvent strength of supercritical fluids allows the analysis of nonvolatile, high molecular weight compounds, under moderate conditions with an advantage over GC for the separation of thermally unstable substances [32].

TABLE 1.1. Critical properties of potential mobile phases for SFC.*

Substance	T_c (°C)	P_c (atm)	ρ_c (g/cm³)
Helium	-268	2.24	0.070
Xenon	16.6	57.6	1.113
Carbon dioxide	31.1	72.9	0.466
Nitrous oxide	36.4	71.5	0.452
Sulphur hexafluoride	45.5	37.1	0.738
Ammonia	132.4	111.3	0.235
Diethyl ether	193.5	35.9	0.265
n-Pentane	196.5	33.3	0.237
Methanol	239.4	79.9	0.272
Tetrahydrofuran	267.0	51.2	0.322
Acetonitrile	274.8	47.7	0.253
Water	374.1	217.6	0.322

* Data taken from Ref. [15].

TABLE 1.2. Comparison of physical properties of liquids, gases and supercritical fluids by order of magnitude.*

Phase	Density (g/cm³)	Diffusivity (cm²/s)	Viscosity (g/cm sec)
Gas @ 1 atm, 21 °C	10^{-3}	10^{-1}	10^{-4}
Supercritical fluid	0.3 - 0.8	10^{-3} - 10^{-4}	10^{-4} - 10^{-3}
Liquid	1	$<10^{-5}$	10^{-2}

* Data taken from Ref. [30].

1.2 RETENTION AND SELECTIVITY DEPENDENCY IN SFC

In theory, two properties are responsible for determining the retention of a solute in SFC. These are the volatility of the solute and its solubility in the mobile phase [8].

Several parameters can be manipulated in order to affect the retention and selectivity in SFC [18]. Among the most important are temperature, pressure, density, stationary phase, and the nature and composition of the mobile phase. In addition, the eluent flow rate can be varied to obtain optimum separation. The effect of these parameters have been extensively studied in a number of laboratories [30,33-35] but the amount of information available on the subject is still considerably less than that for HPLC or GC.

1.2.1 Density

The density and pressure of a given mobile phase are related to temperature as a parameter in a non-linear fashion by an equation of state. Typical relationships between density and pressure at four different temperatures are shown in Figure 1.3 for carbon dioxide as the mobile phase. Near the critical point, the slope of the isotherm is very low, which means that the density is very sensitive to changes in pressure. At very high temperatures or very high presssures, the curves become more linear.

Since the solvating power of a supercritical fluid is more or less directly proportional to its density [18], the retention of the solute can therefore be controlled as a function of pressure and temperature. Increasing density generally decreases the capacity factor (k'), but

the slope of the decrease is not the same for different classes of compounds [36]. Thus, changes in density can cause selectivity changes in SFC separations.

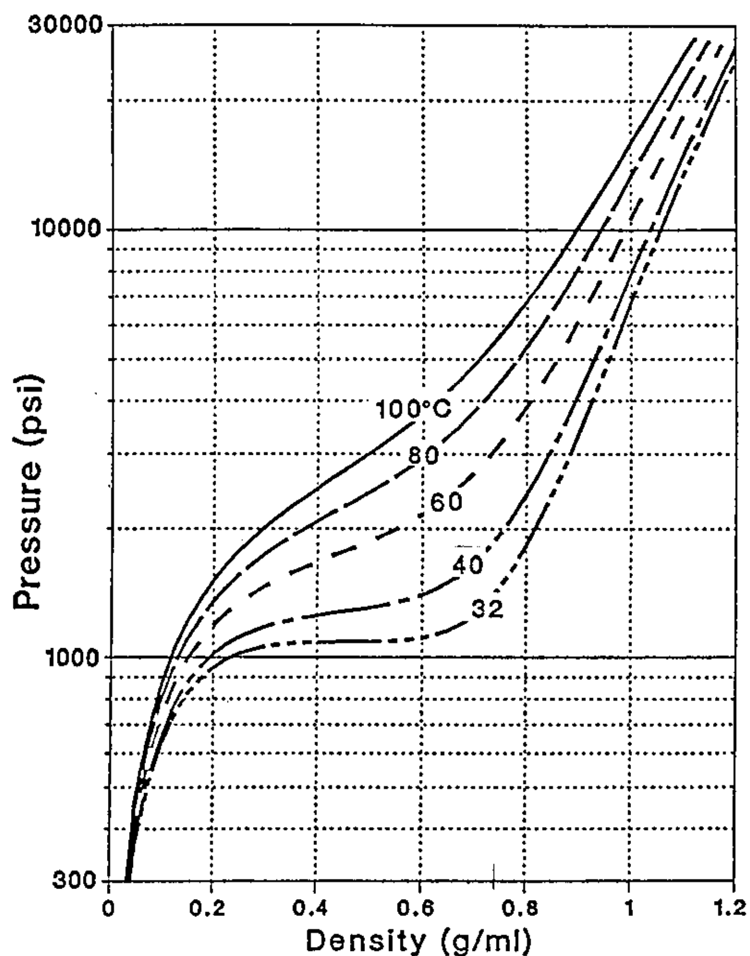


FIGURE 1.3. Pressure-density isotherms for carbon dioxide.

1.2.2 Pressure

The influence of column pressure on separations in SFC is somewhat similar to that of density. Increasing pressure may show a similar effect on capacity factors of different analytes as does increasing

density [36-40]. At a constant temperature, increasing pressure decreases the capacity factors of the solutes and at the same time, the differences in capacity factors usually become smaller and hence a decrease in resolution can be observed [36].

1.2.3 Temperature

The effects of temperature on efficiency, selectivity and resolution have been investigated in a number of studies [34,40-42] and have been well discussed in the literature [2].

Because of the close connection of temperature with pressure and density, the temperature dependences can be considered under either constant density or constant pressure conditions. At constant density, the dependence of retention with temperature can be calculated by the Van't Hoff thermodynamic equation [43]. A plot of log capacity factors against reciprocal absolute temperature showed a straight line relationship [44].

Changing the temperature at constant pressure causes variations in the eluent density. Plots of log capacity factors against temperature at constant pressure have characteristic rather complex shapes with pronounced maximum above the critical temperature [36]. This behaviour is explained by two opposing effects [36]: firstly, by a decrease in the eluent density and hence a decrease in the substrate solubility on increasing the temperature at constant pressure, and secondly, by the opposing effect of an increase in vapour pressure of the substrate and an increase in solubility. The second effect overcompensates for the first above a certain temperature. This behaviour has been observed when analyzing triglycerides [42] and metal acetylacetonates [45] with carbon

dioxide as the mobile phase and in the analysis of dialkyl phthalates [46] with n-pentane as the eluent.

The effect of temperature on retention can, however, differ depending on the nature of the substrate [36] and thus selectivity may also be altered by changing the temperature.

1.2.4 Stationary phases

For solutes to be separated, they must have a reasonable affinity for the stationary phase and individual solutes must have different capacity factors or else they elute simultaneously. Also, the stationary phase must be both chemically and physically stable under the conditions of separation. Immobilized stationary phases, preferably by chemical bonding, are needed for use in SFC because the solvating power of supercritical fluids is often sufficient to cause migration of conventional polymeric stationary phases [47].

Both packed columns and open-tubular columns have been used in SFC in almost equal number [7]. The comparison of characteristics and advantages, and the choice between these types of column for use in SFC have been comprehensively discussed in the literature [12,48-52].

Packed columns have several advantages over open-tubular columns. These include greater sample capacity and may, under suitable operating conditions, develop more theoretical plates per unit time [50]. Alteration of selectivity using the wide range of stationary phases and preparative scale SFC is possible.

The use of capillary columns in SFC imposes more stringent instrumental requirements compared to packed column SFC, particularly on the mobile phase pumping system. Usually, flow rates in the order of

μl/min are required and pulseless operation is more critical. The sample introduction system, used in conjunction with capillary columns, must be capable of injecting small amounts of sample onto the column, which is usually achieved by employing a sample splitter to give effective injection volume of less than about 100 nl. Proper connections and tubings have to be used to minimize extra-column band broadening in the chromatographic system. Nevertheless, for the separation of complex mixtures, capillary SFC is probably the method of choice since it offers much higher efficiencies.

(a) Open-tubular columns

Most "bonded" stationary phases employed in conventional GC open-tubular capillary columns such as methylphenylpolysiloxane, are not truly chemically bonded but rather are cross-linked. Peroxides and azo-compounds, such as azo-t-butane, are used as free-radical initiators to cross-link the stationary phases inside the capillary columns, which gives these stationary phases considerable resistance to column bleeding at high temperatures [53].

The recent tremendous developments in capillary SFC have owed much to the advances in open-tubular column technology in GC. Many capillary GC columns have been used in SFC, and among these, polysiloxane-based stationary phases usually bonded to the wall, or cross-linked, have been predominant and SE-54 has been the most popular material [7]. However, the long-term stability of these stationary phases when used under SFC conditions is still not fully known. Nonetheless, because of the lower solute diffusion coefficients in SFC compared to GC, columns with internal diameters smaller than GC columns are required in capillary SFC to obtain the minimum HETP at comparable speed of analysis [36].

Consequently, many commercial SFC columns are made with internal diameters of less than 100 μm (typically 100 or 50 μm) [54].

Investigators are still continuing the search for better stationary phases for capillary SFC [55] and it has been suggested that ideally, the polymeric phases should be covalently bonded to the column wall of the capillary column [18]. Such investigations has resulted in recent years in the introduction of new stationary phases including n-octylmethylpolysiloxane [56], and siloxanes containing more than one of the side groups, methyl, vinyl, phenyl, and cyanoethyl [57], and monomeric and polymeric liquid crystal stationary phases [58]

(b) Coated-silica columns

A variety of HPLC packing materials with chemically bonded stationary phases are commercially available. These materials are suitable for use with supercritical fluids and offer different selectivities to achieve the required separation.

The most widely used stationary phases in packed column SFC have been pure silica and octadecyl-bonded silica [7]. Other types of stationary phases used include amino-, cyano-, and octyl-bonded silicas [7].

Despite the number of published papers on SFC a current literature search reveals that there have been only limited studies of selectivity in SFC, involving different stationary phases. In a recent paper [35] Mourier et al. studied retention and selectivity on three stationary phases, bare silica, octadecyl-bonded silica, and Pirkle's phase for enantiomeric separations with modified and unmodified carbon dioxide as the mobile phase. In another paper Levy and Ritchey [59] when working with supercritical carbon dioxide as the mobile phase reported that a silica stationary phase provided the poorest separation and peak shapes

and the longest retention for PAHs compared to cyano- and diol-bonded columns. These workers observed that the peak shapes for PAHs with supercritical carbon dioxide mobile phase were broad and assymmetrical but on the addition of small amounts (2% w/w) of dimethylsulphoxide (DMSO) reduced retention and improved separation for all three columns were obtained, particularly for the silica column.

(c) Polymer columns

One of the solutions to the problems associated with specific interactions in silica-based packing materials (due to unreacted silanol groups) is to perform the chromatographic separation using polymer-based stationary phases. Porous polymer packings for chromatography can be classified into microporous gels (xerogels) and macroporous gels (aerogels) [60]. The aerogels cover a wide range of pore sizes and have good mechanical stability. The rigidity of the macroporous gels makes packing easier and they undergo limited or no swelling with solvents and thus they are suitable for use in reversed-phase HPLC. Two commercially available macroporous poly(styrene-divinylbenzene) (PS-DVB) copolymer columns are the Hamilton PRP-1 [61] and Polymer Laboratories PLRP-S [62] materials.

Because of the absence of polar-polar interactions, and the excellent characteristics with respect to surface homogeneity compared to bonded silica stationary phases [18], we can expect PS-DVB columns to be suitable for the elution of polar solutes. The combination of these characteristics with good mechanical stability makes polymer column attractive for use in SFC. However, so far, only a few reports have described the use of PS-DVB copolymer stationary phases in SFC [63-65].

1.2.5 Mobile phases

(a) Choice of mobile phases

A wide range of compounds can be converted to supercritical fluids and the potential of many have been examined as main eluents and modifiers in SFC (see Table 1.1). Carbon dioxide has been the single most widely used mobile phase, n-pentane comes next, followed by hexane [7]. Other compounds that have been used as the mobile phase in SFC include nitrous oxide, ammonia, and the hydrocarbons, butane, propane, ethane, and methane.

There has been considerable interest in the use of modifiers in SFC. Polar organic modifiers are mainly used to elute polar molecules, or substrates of high molecular weight for faster analysis [36], and to improve peak shapes for better sensitivity [50]. In practice, there are many possible organic modifiers, some of which are listed in Table 1.1. Alcohols have been the most widely used mobile phase organic modifier used in SFC, and among them, methanol has been predominant [7]. Other modifiers include dioxane, acetonitrile, tetrahydrofuran, di-isopropyl ether, diethyl ether, water, and formic acid.

The selection criteria for modifier in SFC have often been based on the polarity and interaction classification scheme proposed by Snyder [66] for HPLC. The interaction classification takes into consideration the selectivity or relative ability of the solvent to engage in hydrogen bonding or dipole interactions. Using this scheme, Randall [67] outlined the criteria for selecting a modifier for SFC with carbon dioxide eluents. Using the same principles, Fields et al. [68] selected three solvents (isopropanol, acetonitrile, and dichloromethane) which represent a wide range of interactions and polarity.

(b) Effect of modifier

In addition to the various parameters discussed previously, a modifier can be used to alter the physical properties and hence the solvating power and selectivity of supercritical fluids. This can be accomplished by using a chemically different fluid system and/or changing the concentration of the modifier [69]. A number of reports have described the effect of organic modifiers on solute retention and selectivity [34,63,68-74]. Packed columns have been used in many of these studies [63,70-73], with carbon dioxide as the primary mobile phase. By selecting the modifier and varying the quantity, it is possible to tailor the properties of the mobile phase to enhance the separation.

Numerous studies [35,70,71,75] have shown that the addition of small amounts of organic modifiers (less than 1%) to the primary supercritical mobile phase drastically reduced solute retention in both open-tubular and packed columns. In addition, it appears that the effect of the addition of modifiers, such as methanol and acetonitrile, added to carbon dioxide is much greater for packed columns than for capillary columns [20,74,76]. Generally these effects have been attributed to increased solubility of solutes in the mobile phase and/or modification of the stationary phase . Nonetheless, there has been some evidence which suggests that the drastic changes in retention observed for packed columns are primarily due to surface and stationary phase modification rathar than to changes in fluid solubilities [69,70]

Blilie and Greibrokk [70] demonstrated that retention of the polystyrene and polynuclear aromatic hydrocarbons (PAHs) on a silica column decreased with the use of straight chain alcohols modifiers with

increasing alkyl chain length up to 1-hexanol. In another study, Levy and Ritchey [71] obtained similar effects in the separation of a mixture of aromatic compounds on a cyano-substituted silica column. These workers suggested that the long lipophilic alkyl chain of 1-hexanol can either access the active sites (free silanols) and the bonded cyano groups better than a short chain alcohol (methanol), or there was an increased solubility of the solutes in the longer chain alcohols.

In a series of studies using open-tubular columns, Yonkers et al. [34,44,74] demonstrated that the addition of polar modifiers (methanol, acetonitrile, or 2-propanol) to supercritical carbon dioxide drastically altered the selectivity of the column for analytes with polar substituents, while selectivity remained constant for non-polar compounds. Thus they concluded that selective interactions had been occurring between the polar substituents and a polar organic modifier and that the modifier was changing the solvent power of the mobile phase with increased selectivity for the more polar compounds. These workers also suggested that the drastic changes in retention observed for packed columns are as a result of surface and stationary phase modifications. In a study using a capillary column and three different modifiers, 1-propanol, acetonitrile, or dichloromethane with a carbon dioxide mobile phase, Fields et al. [68] suggested that selective interactions between the solute and the mobile phase contributed to the retention mechanism. These investigators have also noted that for all solutes (PAHs), the magnitude of decrease in capacity factors (increase in solvating power) with modifier was in the order 2-propanol > acetonitrile > dichloromethane.

1.2.6 Column pressure drop

One of the factors which affects retention in SFC is the pressure drop across the column. In SFC, the pressure drop is generally small for an open-tubular column but much higher for packed column. The effect of the column presure drop on the capacity factors and efficiency in packed column SFC has been studied and discussed in detail by Schoenmakers [50].

The pressure drop across the column, ΔP, increases with increasing flow rate (linear velocity, u). Schoenmakers [50] has demonstrated that the plot of the observed capacity factors against pressure drops across the column give a maxima, ΔP(opt), which is the optimum value at which the highest possible number of theoretical plates can be obtained. Working below the optimum pressure drop will result in reduced efficiencies at lower flow rates. At very high pressure drops, the density at the outlet may start to decrease and the solubility of the solute may become the limiting factor. This maximum permissible pressure drop over the column at a given temperature and a given inlet pressure is denoted as ΔP(max). For maximum efficiencies, packed columns in SFC should be operated between ΔP(opt) and ΔP(max) [50].

1.2.7 Mechanisms of solute retention

Retention in SFC depends upon both solute solubility in the fluid and solute interaction with the stationary phase. The solute retention mechanisms in SFC are rather complex and not fully understood. Several workers have developed thermodynamic models for solute retention as a function of temperature and pressure [30,37,44,76-79]. The thermodynamic

models assume that the effect of pressure upon the interaction of the solute with the stationary phase is negligible.

(a) Mechanisms with unmodified carbon dioxide

Until now, the mechanism of separation using supercritical carbon dioxide is still not clear. Carbon dioxide is a relatively non-polar eluent. Using the classification scheme of eluents by Snyder [66], carbon dioxide shows a polarity similar to that of hexane. However, in practice, carbon dioxide sometimes behaves like a polar eluent, sometimes as a non-polar eluent [36] and as a consequent can exhibit normal phase or reversed phase characteristics depending on the column used.

(b) Mechanisms of modifier effect

Several workers have studied solute retention and the mechanism of the effect of modifier in SFC. Wheeler and McNally [80] observed that packed and capillary SFC yield similar retention characteristics to those found in normal phase liquid chromatography, i.e., non-polar molecules elute first, more polar molecules more highly retained. These workers used SB-Cyanopropyl-25 and SE-33 capillary columns with carbon dioxide as the mobile phase for capillary SFC and Vydac silica and Partisil 10 ODS-2 standard HPLC packed columns with methanol-modified (2% and 5%, respectively) carbon dioxide as the mobile phase for packed column SFC.

Using carbon dioxide as the primary mobile phase and an ODS-silica column, Board et al. [63] suggested three possible mechanisms, namely: (a) the modifier competes with the solute for active sites (free silanols) on the stationary phase; (b) the modifier interacts with the

solute in the mobile phase, forming stable species which are more soluble in the mobile phase; and (c) the modifier interacts with the carbon dioxide, forming clusters of short range order in the mobile phase which have higher solubilizing power for the solute. These workers have suggested that any of the above mechanisms may be significant, but that mechanism (a), the interaction of modifier and stationary phase predominates at modifier concentrations up to 1%. They have also shown that the modifiers are able to significantly affect the retention time even on stationary phases without free silanol groups such as polystyrene-divinylbenzene (PS-DVB), and have further suggested that different mechanisms may operate with each combination of solute and stationary phase. These investigators chromatographed dimethyl phthalate on a PRP-1 column at various methanol concentrations up to 1% and observed no changes in retention time [63]. This result suggested that the mechanism involving the modification of the stationary phase predominates on reversed phase bonded silica columns.

In comparing PRP-1 and ODS packings, Morin et al. [64] reported that the retention of n-alkanes on ODS can be explained by an apolar-apolar partition mechanism. However, on a polymer stationary phase, retention increases for solutes with π electron-acceptor groups, and that these compounds are retained by π-π interactions similar to an apolar-apolar partition mechanism.

Recently, Lochmuller and Mink [81] studied the adsorption isotherms of modifier (ethyl acetate) on silica in supercritical carbon dioxide. They have found that the maximum stationary phase coverage by the modifier occurred at approximately 1% (g/ml) of the modifier in the mobile phase, with a maximum stationary phase coverage of approximately 0.031 (g/g). The former value corresponds to the concentration below

which, modification of the stationary phase by the modifier has been found to be primarily responsible for the observed changes in retention [69]. They have also noted that the changes in modifier concentration in the stationary phase as a function of pressure are small in comparison to those obtained as a result of changes in modifier concentration in the mobile phase.

Levy and Ritchey [59] suggested the other possible competing mechanisms for modifier effect are hydrogen-bonding, occurring at low modifier concentrations, and overall polarity effects at higher levels, which are due to a lack of sites for hydrogen bonding. In addition, they suggested that the saturation of overall surface coverage of the modifiers on stationary phases occurs at a moderately high level, depending on the modifier (about 6% w/w).

Whilst the actual mechanisms are still unclear, there is no question about the importance of modifiers in SFC, particularly for separation of the more polar compounds. This has been clearly demonstrated in numerous cases. Blilie and Greibrokk [70] have shown that although arachidonic acid, was completely adsorbed on silica column with 5% acetonitrile in carbon dioxide it could be eluted with 0.1% formic acid plus 5% acetonitrile. With this modifier, UV detection of the compound at 190 nm may be possible [70]. They further noted that carboxylic acids and benzoic acid, were strongly retained and showed severe tailing on silica column, with pure carbon dioxide as the mobile phase. Esters and methyl stearate also resulted in considerable tailing. These effects disappeared with low concentrations (1%) of methanol. In another study, Crowther and Henion [72] used methanol-modified carbon dioxide as the mobile phase in the separation of the polar drugs, cocaine, caffeine, and methocarbamol, with UV and mass spectrometric

(MS) detection. Berry et al. [82] used a packed column SFC-MS in the analysis of carbamate and organophosphorus pesticides, steroids, and ergot alkaloids. Kalinoski et al. [20] performed capillary SFC-MS using propanol-modified carbon dioxide as the eluent for the separations of several classes of compounds including herbicides, carbamate pesticides, and polymeric polyethylene glycol.

1.3 SFC INSTRUMENTATION

1.3.1 Commercial vs. home-made instruments

Although commercial dedicated SFC instruments have been available from a number of manufacturers since 1982 [83] it appears that reported work on SFC using commercial SFC instruments has been rather limited. This can probably be explained by a number of factors [7]; (a) primarily, these commercial SFC instruments have only recently been widely offered as established equipment, attractive to the pharmaceutical and petroleum industries; (b) users have the alternative of constructing their own SFC systems by making often non-permanent modifications to conventional GC or HPLC instruments; (c) SFC has not yet been widely accepted as a routine analytical technique in laboratories, hence maintaining the low demand for commercial SFC systems; (d) commercial SFC systems including the associated computers are relatively expensive; and finally, (e) many users in new technology areas are reluctant to release commercial details of their applications (particularly on commercial instruments). Many workers have therefore turned to home-assembled SFC instruments to carry out their SFC studies.

1.3.2 Detectors

One of the main reasons for the growing acceptance of SFC as a chromatographic technique is its compatibility with a wide range of detection systems [84,85]. Most GC and HPLC detectors have been successfully adapted for use in SFC with little or no modification. Common spectroscopy detectors such as ultraviolet and infrared detectors, normally only require flowcells, which can withstand high pressures. These are commercially available, although some workers have also designed their own [16].

Conventional FID detectors can be used with minimal alterations which are needed to overcome problems relating to restrictor blockage and spiking caused by effluent condensation [86,87]. The modifications include the installment of an extra heater at the base of the detector [86,88] and the adjustment of the flame tip and the gas flows [89,90]. Chester [91] has demonstrated that in capillary SFC, the combination of high temperatures at the detector block (400 °C) and the use of a restrictor with tapering outlet can accomplish a smooth transition of the solute molecules into the flame which enables the extension of the detectable molecular weight range.

There has been considerable interest in the use of SFC with mass spectrometric or Fourier transform mass spectrometric detectors (FTMS) [14,20-22,82,93], and infrared or Fourier transform infrared detectors (FTIR) [23-25,64,94,95]. These detectors have excellent capabilities for on-line detection and identification of compounds.

Other detectors used in SFC include the fluorescence spectrophotometric detector [96,97] flame photometric detector (FPD) [98], thermionic detector (TID) [99], and photoionization detector (PID)

[100]. Despite having the potential of being highly sensitive, other established selective chromatographic mass detectors such as electron capture detector and electrochemical detectors have not been widely used in SFC.

1.3.3 Delivery systems

Mobile phase delivery and control is a major function in SFC. In many cases the pumping systems have been assembled by modification of commercial HPLC components.

The majority of work on SFC has used carbon dioxide as the primary eluent. Commercial liquefied gases such as carbon dioxide are normally available from the cylinder. The gas in the liquid state can be drawn out of the cylinder either by using an aductor (dip) tube, or by turning the cylinder upside down [101]. A fine filter (less than 5 μm) placed in line before the pump will free the liquid from any coarse particles from the cylinder, otherwise these may contaminate the pump and the SFC system. In certain cases when carbon dioxide is used as mobile phase, it may be necessary to place, prior to the injector, an additional purifier such as an adsorbent trap, (usually made of activated carbon or alumina) to improve the carbon dioxide quality. This extra column can also act as an efficient pulse damper [101].

Pressures greater than that supplied by the gas cylinder can be achieved by adding an extra head pressure such as from pressurized helium (typical pressures, 1500 psi) [102]. This method has mainly been used to enhance the filling of syringe pumps with liquid carbon dioxide [102]. Another method to achieve high pressures is by pressurizing the cylinder by careful heating. Although there are obviously limitations to

this technique, pressures of up to 150 bar can be conveniently obtained by heating a carbon dioxide cylinder to about 55-60 °C with the necessary pressure control obtained by means of a pressure valve [103,104]. However, the vast majority of work in SFC, has used high pressure pumps to achieve higher pressures. For packed column SFC, reciprocating pumps have usually been used. These pumps besides having the advantage of being able to deliver unlimited volumes with a continuous flow of mobile phase, may also incorporate eluent gradient capability [105]. Problems with pulsating flow associated with these pumps can be reduced to some extent by the use of pulse dampers. Because the pump works against a restrictor in order to maintain the desired pressure in the column, pressure control is generally more important than flow control. As a consequence, many conventional HPLC pumps have to be modified for pressure control before use [101,106].

Because of the much lower flow rate requirements, in most open-tubular, packed capillary, and packed microbore column SFC, syringe pumps have been used. These pumps have the principle advantage of delivering a pulseless flow, thus offering better baseline stability and higher sensitivity of detection than reciprocating pumps. Furthermore, they are generally reliable and easy to operate [105], but it is more difficult to use modifiers unless two pumps are used.

1.3.4 Pump head cooling

It has usually been necessary to cool the pump head of reciprocating pumps when working with eluents that are gaseous at ambient temperatures and pressures. Cooling generally reduces the tendency of the liquid eluent to undergo gasification or cavitation in

the pump head. Gere et al. [106] noted that cooling the pump heads to zero °C or below greatly enhanced the pumping efficiency.

Several techniques for cooling the pump heads have been described or suggested in the literature. An interesting approach suggested by Bruno is to cool the pump components by using a simple vortex refrigeration method [107]. He used a vortex tube which can be operated by a source of compressed air and temperatures as low as -15 °C are easily reached. This technique, however, has not gained acceptance in SFC, probably because of its limited cooling applicability to small-scale components and its rather noisy operation.

Greibrokk et al. [101] described the modification of a Waters Associates 6000A pump. Cooling channels were drilled in the solid outer parts of both pump heads and two additional check valves (inlet and outlet) were placed in a home-made block of stainless steel also containing cooling channels. Cooling of the pump heads and the check valves was accomplished by running cold methanol (-8 °C) through the cooling channels.

In their SFC investigations, Gere, Board, and McManigill [106] cooled the two liquid pumping heads of a HP 1084B liquid chromatograph by placing them in close thermal contact with clamp-on heat exchangers, through which a water-methanol mixture from an external chiller was circulated. Simpson, Gant, and Brown [108] accomplished cooling of the pump heads of Perkin-Elmer Series 10 pumps by circulating ice water through a heat exchanger plate in thermal contact with the pump heads. The heat exchanger they used was a hollow copper plate with coolant inlet and outlet ports and it was secured to the pump head by the head retaining screws which were passed through appropriately placed holes in solid areas of the exchanger. Greibrokk et al. [109] described in detail

the construction of a clamp-on cooling unit for a Waters 590 pump. The cooling unit was made from aluminium and consisted of three pieces with the centre piece precisely cut to fit accurately between the two pump heads. Cooling channels were drilled through each separate part and cooling was effected by circulating cold methanol. Similar techniques were also reported by several workers. Rawdon [89] used a "cooling cap" through which cold methanol was circulated. Mourier, Eliot, Caude, Rosset, and Tambute [110] used a "clamp-on" heat exchanger through which cold ethylene glycol solution was circulated.

Several papers have indicated that in the case of syringe pumps, cooling is not always essential. A number of workers [86,92,111] reported the use of Varian 8500 high pressure syringe pumps without any cooling. However, cooling can be employed to enhance the filling of the pump during a refill cycle. Doehl et al. [112] in their SFC studies used an ISCO µLC-500 syringe pump which was equipped with a cooling coil through which was circulated methanol at -15 °C during the pump refill procedure. This syringe pump has become the basis of the delivery systems in many of the present commercial SFC instruments and is suitable for microbore and capillary packed columns which require a low mobile phase throughput.

In the process of cooling the pump heads, it is important to provide good insulation to the entire cooling system to prevent excessive air moisture condensation and loss of cooling efficiency. Cooling units can be insulated using cut-to-fit polystyrene foam, and expanded polystyrene hoses can provide insulation to the tubing of the cooling system [7].

Cooling the pump head and components may create adverse effects on the pump performance. Some makes of pump seals, when operated under cold

conditions tend to develop mobile phase leaks and fail to perform satisfactorily. Greibrokk et al. [109,112] faced these problems with a Milton Roy Metric metering pump and an ISCO μLC-500 syringe pump. As a result, for the latter pump, cooling was only turned-on during the refilling procedure. Similar problems have been encountered in the present study with a Kontron 410 LC pump.

1.3.5 Introduction of modifier

Organic modifiers can be introduced into the eluent system in a number of ways. Several workers [59,71,72,74] used commercially available gas cylinders with carbon dioxide doped with methanol, 2-propanol or other modifiers (e.g. 10% w/w). These cylinders can be used as received. However, to permit more accurate addition of small amounts of modifier to a carbon dioxide mobile phase, further dilution is possible by plumbing the doped carbon dioxide into one pump of a dual headed pump and unmodified carbon dioxide into the other [72]. Alternatively, mixtures of modifiers in carbon dioxide can be made by combining them in a lecture bottle or a syringe pump [34,74].

The main method reported in the literature for adding modifier to carbon dioxide is by direct on-line mixing by the use of another pump. This can be accomplished with the use of a microflow pump and a T-piece of tubings [70], or a home-made high pressure low-volume mixer [101]. A T-piece mixing system may be adequate for most analytical purposes as suggested by the low retention time variations of less than 2% RSD [70]. Alternatively, mixing can be effected using a commercial mixing cartridge (e.g. Brownlee Mixer-52), inserted in-line prior to injection valve [113].

1.3.6 Reproducibility

Reproducibility is one of the principal variables in a chromatogram. It is largely a combination of good design of the hardware and manipulative skill. The former factor includes injector design and pump precision.

Good pump performance is very important to obtain high reproducibility in SFC. Some concern has been expressed that the pump refilling method can significantly affect reproducibility. Porter et al. [10:] reported that low precision and adverse chromatographic performance, including peak splitting and high retention of larger molecular weight compounds were obtained when a carbon dioxide cylinder with helium head pressure was used. Using this pump refilling technique and pressure gradient with a timed-split injection method onto a capillary column, these workers obtained peak area and retention time precisions of 21.5% and 3.77% RSD, respectively for n-docosane. They suggested that these relatively poor precisions developed as helium became entrained in the carbon dioxide. On the other hand the use of a cylinder with straight carbon dioxide and pump head cooling jacket have been demonstrated to provide a means of obtaining both complete fill of the pump and excellent precision. With this technique they obtained peak area and retention time precision of 1.76% RSD and 0.10% RSD respectively for same test compound [102].

Subsequently, Schwartz et al., using a helium pump-filling unit, have presented data with excellent precisions in peak area and retention time, both for capillary and packed columns [114] which somewhat contradict the work of Porter et al. Using a similar system with an Applied Biosystems pump, they obtained retention time

precision of 0.097% RSD for trilaurin using an open-tubular column, and peak area reproducibility of 1.9% RSD for n-hexatriacontane using a microbore packed column [114]

In comparing packed column and capillary column separations of herbicides and pesticides as analytes, Wheeler and McNally [52] reported peak area precisions of between 0.8 and 4.3% RSD (5 runs) for 4.6 mm i.d. packed column while for capillary column it was slightly worse, between 2.8 and 5.4% RSD. In another study, White et al. obtained retention time and peak area precisions of 3.10% and 1.75% RSD respectively, using open-tubular column and provitamin D as the test compound [115].

1.3.7 Programmed elution in SFC

As in HPLC and GC, programming techniques can be used in SFC for the elution of samples that contain a wide range of different solutes. Three main types of gradients can be used in SFC, temperature, pressure (density), and eluent composition [36,116,117]. Another gradient method, which is often neglected, is flow rates which can be used effectively to change the analysis time [116]. It is possible to combine any of these gradient methods and thus there are manifold choices of gradient programming for improving separations. Gradient elution has been a subject of a recent review by Schmitz and Klesper [118].

Pressure gradients in SFC can be accomplished in different ways, depending on the method of supercritical fluid delivery. For an SFC system that uses a reciprocating pump, a pressure programmer can be employed. The pressure programmer described by Jentoft and Gouw [119] consisted of a cylinder of high pressure nitrogen, variable motors, and

pressure regulators which essentially controlled the inlet pressure to the pump. Van Lanten and Rothman [120] described a pump pressure control design which involved electronics controlled by a microcomputer, applicable to almost any commercial HPLC pump.

Density programming in SFC is comparable to temperature programming in GC and gradient elution in HPLC [12]. Increasing the pressure or decreasing the temperature of the supercritical fluid causes the density to increase, and thus its solvating power. Considerable amounts of work has been carried out on density-programmed SFC by means of pressure-programming [121-124] and negative or inverse temperature programming [125-127].

One of the interesting facets of density-programming in SFC is that the linear-velocity changes can have significant effects on the chromatographic efficiency. Fields and Lee [41] obtained increased efficiency using low densities and high temperatures at constant linear velocities on capillary column SFC. With n-alkanes and polynuclear aromatic hydrocarbons as model compounds using simultaneous pressure and increasing temperature gradient, their early results showed improved separations compared to those obtained under separate pressure-programmed or temperature-programmed SFC conditions, However, as noted by Schoenmakers [50], the resolution in a programmed elution can be no better than in an elution with constant conditions and optimum capacity factors.

The solvating power of the mobile phase may be enhanced by adding a modifier to the primary mobile phase and this can be accomplished either by mixing the eluents before starting the chromatographic run or by adding the modifier on-line, usually by means of a second pump (see earlier sections). The latter technique is more widely used and it also

allows the composition of the mobile phase to be changed during a run. However, problems arise with the use of modifier in the carbon dioxide mobile phase [36]. Firstly, the use of most organic modifier prohibits the use of FID because of strong resulting background noise. Secondly, there is some uncertainty about the actual composition and the physical state of the mobile phase, i.e. whether it remains in supercritical fluid conditions. This is partly because there are rather complex, non-linear relationships between the critical properties and the eluent composition. In addition, as yet, there are very little few data about the interdependence of temperature, pressure, and density of mixed eluents which makes it more difficult to perform density programming with mixed eluents.

1.4 THE PRESENT PROJECT

As a novel separation technique, SFC has attracted considerable interest among chromatographers. A combination of possible high efficiency, short analysis times, and a wide range of mobile phase and stationary phases makes it a favourable alternative instrumental technique to GC and HPLC. The literature reviewed suggests the feasibility to construct a home-made SFC system.

In the work presented here, a simple, low-cost SFC instrument has been designed and constructed based on preliminary practical as well as theoretical considerations. Suitable operating conditions have been established for the separation of a number of analytes.

The retention of different sets of homologous series of compounds (n-alkanes, alkylarylketones, and alkylbenzenes) have been compared as the basis of potential retention index scales in SFC. In order to gain

better understanding of selectivity and solute retention behaviours in SFC, separations were carried out using selected model compounds which are characteristic of different interactions. Comparison of the chromatographic retentions of these compounds were on ODS-silica, cyano-silica, and PS-DVB polymer packing materials . The separations were examined at different temperatures, pressures, and modifier concentrations. The effect on the retention of the analytes and the selectivity changes have been examined to determined the range over which the retention indices would be most robust. The SFC system has also been applied to the analysis of selected analytes of interest including barbiturates, benzodiazepines, and terpenes.

This thesis is divided into nine chapters. The experimental design and construction of the supercritical fluid chromatograph is described in Chapter 2 and the practical evaluation and assessment of the assembled SFC instrument are described in Chapter 3. Chapter 4 deals with preliminary applications which include the establishment of the SFC operating conditions and the separation of a number of selected analytes. This is followed by the results and discussion of retention and selectivity in Chapter 5, which also examines the application of retention indices in SFC. Chapters 6, 7, and 8 deal with the application of the technique to the analysis of barbiturates, benzodiazepines, and terpenes, respectively, and the last also includes GC and GC-MS analyses of essential oils. The final chapter concludes the experimental results and suggests possible future work.

CHAPTER 2

GENERAL EXPERIMENTAL

In this Chapter, general experimental details and chemicals are described. The components and operation of the supercritical fluid chromatograph and the instrumentation used for the GC and GC-MS analyses will be described in detail in Chapter 3 and Chapter 8, respectively.

2.1 REAGENTS

2.1.1 Solvents

The carbon dioxide used was industry grade (99.98%) supplied by BOC Ltd., Brentford, Middlesex, U.K. Acetonitrile was far UV grade supplied by ROMIL Ltd., Shepshed, Leics, U.K. Carbon tetrachloride was UV/IR grade from Blackford Wells Ltd., Coalville, U.K. Chloroform (pesticide grade), and water, methanol, isopropanol, dichloromethane, and pentane (HPLC grade) were supplied by FSA Laboratory Supplies, Loughborough, U.K. Acetone was LR grade distilled in the laboratory.

2.1.2 Standard compounds

Alkylarylketones: acetophenone and propiophenone were from BDH, Poole, U.K.; butyrophenone and valerophenone were from Koch-Light Laboratories, Colnbrook, Bucks, U.K.; hexanophenone, heptanophenone, octanophenone, decanophenone, dodecanophenone, tetradecanophenone, hexadecanophenone and octadecanophenone were from Aldrich Chemical, Gillingham, Dorset, U.K. n-Alkanes: dodecane, tetradecane hexadecane,

octadecane, eicosane, docosane, tetracosane, (each 99%) were from Sigma Chemical, Poole, U.K.; tridecane (99%) and pentadecane (98%) were supplied by Aldrich Chemical. Others were laboratory grade obtained from a range of suppliers and were of different purity ranging typically from 97-99%.

2.1.3 Test compounds

Alkylbenzenes; toluene, ethylbenzene, propylbenzene, and butylbenzene were reagent grade obtained from Koch-Light and Aldrich. Benzaldehyde, benzamide, benzoic acid, benzylamine, benzyl alcohol, p-cresol, methyl benzoate, nitrobenzene, N-propylbenzene were laboratory grade obtained from different suppliers.

Samples of drug compounds barbiturates and benzodiazepines were from the reference collection at the Central Research Establishment, Home Office Forensic Science Service, U.K.

2.2 APPARATUS

2.2.1 High performance liquid chromatography

The liquid chromatograph used in column testing consisted of an Applied Chromatography Systems (ACS Laboratories, U.K.) with a Rheodyne 7010 injection valve fitted with a 5 µl sample loop. The liquid chromatographic separations of the test compounds were carried out at ambient temperature. The chromatograms were recorded on an Omniscribe chart recorder and a Hewlett-Packard 3390A integrator.

2.2.2 Chromatographic columns

In the preliminary work on SFC, columns of different lengths and internal diameters were slurry packed in the laboratory with Spherisorb ODS-2 5μm (see Section 4.2). These include straight or coiled columns (2.1 mm i.d.) of 30 to 60 cm in length and a straight column (200 x 3 mm i.d.). The latter was found to give better efficiency and was subsequently used in the initial stages of the work involving ODS stationary phases. Other columns were also used in the study and the descriptions of the columns are given below (Table 2.1).

TABLE 2.1. Columns used in the present study.

Dimension	Packing material	Manufacturer	Batch no.
200 x 3 mm i.d.	Spherisorb ODS-2, 5 μm	Phase Separations	820019
250 x 4.6 mm i.d.	Ultrasphere ODS, 5 μm	Beckman-Altex	T608035
150 x 4.6 mm i.d.	PLRP-S, 5 μm	Polymer Labs.	5mRPS1067
250 x 4.6 mm i.d.	Ultrasphere CN, 5 μm	Beckman-Altex	2UEC201N
250 x 4.6 mm i.d.	Ultrasphere CN, 5 μm	Beckman-Altex	3UEC122N

2.2.3 Column packing apparatus

A Haskel air driven fluid pump (Haskel Engineering, Burbank, California, U.S.A.) with compression ratio of 122:1 was used. Either a 30 cm x 8 mm i.d. or a 60 cm x 8 mm i.d. stainless steel packing vessel (reservoir) was used.

2.2.4 Spectroscopy

Ultraviolet spectra were measured and recorded using a Uvikon 810 spectrophotometer (Kontron). Infrared spectra were were obtained using a Pye-Unicam SP3 spectrophotometer (Philips Scientific).

2.3 PROCEDURE

2.3.1 Sample preparation

Most of the compounds were studied as solutions in dichloromethane. Tetrachloromethane and methanol have also been used as alternative solvents. Typical concentrations were about 2.5 mg/ml for the n-alkane, alkylbenzene, and alkylarylketone mixtures, and about 5-12 mg/ml for the test compounds and the drug compounds. There was no pretreatment of the prepared samples before injections.

2.3.2 Calculations

Capacity factors (k') were calculated from the equation

$$k' = (t_r - t_o) / t_o \quad (2.1)$$

where t_r is the retention time of the solute and t_o is the retention time of the unretained peak. Retention indices based on either the n-alkanes or the alkylarylketones were calculated by fitting the log capacity factors against carbon number x 100 for the standard compounds to a linear correlation using a least squares routine and then interpolating the log capacity factor values for the test compounds. The linear least squares routine for the retention index calculations were performed on either an Apple II or an Amstrad

6128 microcomputer using a simple program written in BASIC.

2.3.3 Calculation of supercritical fluid carbon dioxide density

Numerous equations of state have been proposed to describe pressure-volume-temperature (PVT) data for carbon dioxide in the literature [79,128-130]. For the calculations in the present study, the analytical IUPAC equation of state has been exclusively used (Equation 2.2) [129,130]. This equation closely describes PVT data for carbon dioxide apart from the region very close to the critical point [130].

$$Z_m = \frac{PV_m}{RT} = \frac{PM}{\rho_m RT} = 1 + \rho_r \sum_{i=0}^{9} \sum_{j=0}^{6} b_{ij}\left(\frac{1}{T_r} - 1\right)^j (\rho_r - 1)^i \qquad (2.2)$$

where Z_m is the compressibility coefficient of the mobile phase, P the pressure, T the temperature, V_m the molar volume of the mobile phase, R the gas constant, M the molar weight of carbon dioxide, ρ_r the reduced density (ρ/ρ_c), T_r the reduced temperature (T/T_c) and b_{ij} are constants.

The critical properties for carbon dioxide used in the calculations were: T_c = 31.05, P_c = 72.865, ρ_c = 0.466 g/ml. The molecular weight of carbon dioxide, M = 44.009 g/mole and the gas constant, R = 0.0821 l atm mol^{-1} K^{-1}. The values of the coefficients b_{ij} used in the calculations are shown in Table 2.2.

Calculations were performed using a program written in BASIC on an Apple II microcomputer. The density value corresponding to specific pressure and temperature values were determined by using this program.

The pressure-density-temperature curves plotted from data obtained from the calculations are illustrated in Figure 1.4 (Chapter 1).

TABLE 2.2. Coefficients (b_{ij}) for equation of state describing the physical properties of carbon dioxide (Equation 2.2) [130].

i	j = 0	j = 1	j = 2	j = 3
0	-0.725854437	-1.68332974	0.259587221	0.376945574
1	0.447869183	1.26050691	5.96957049	15.4645885
2	-0.172011999	-1.83458178	-4.61487677	-3.82121926
3	0.00446304911	-1.76300541	-11.1436705	-27.8215446
4	0.255491571	2.37414246	7.50925141	6.61133318
5	0.0594667298	1.16974683	7.43706410	15.0646731
6	-0.147960010	-1.69233071	-4.68219937	-3.13517448
7	0.0136710441	-0.100492330	-1.63653806	-1.87082988
8	0.0392284575	0.441503812	0.886741970	0
9	-0.0119872097	-0.0846051949	0.0464564370	0

i	j = 4	j = 5	j = 6
0	-0.670755370	-0.871456126	-0.149156928
1	19.4449475	8.64880497	0
2	3.60171349	4.92265552	0
3	-27.1685720	-6.42177872	0
4	-2.42663210	-2.57944032	0
5	9.57496845	0	0
6	0	0	0
7	0	0	0
8	0	0	0
9	0	0	0

CHAPTER 3

INSTRUMENTATION FOR SFC

3.1 INTRODUCTION

The components fundamental to every SFC instrument are the mobile phase delivery system, sample introduction device, column temperature regulator (usually a gas chromatography oven), analytical column, mobile phase pressure flow controller, and solute detection device. Most of the components can be taken from GC or HPLC, often with little or no modification required.

In the present work, considerable time was spent on constructing and evaluating a packed column supercritical fluid chromatographic system based on a Pye 104 gas chromatograph and a conventional HPLC reciprocating pump. This chapter describes the overall SFC system and discusses the practical considerations of selecting the suitable materials and components for the SFC system.

3.2 GENERAL DESCRIPTION

A schematic diagram of the final SFC system is shown in Figure 3.1. Liquid carbon dioxide was obtained from a standard cylinder with a dip tube. It was then passed through a valve, a frit filter, and another valve before it was passed through a 7 μm in-line filter (Nupro). The filters helped purify the carbon dioxide mobile phase from any solid materials before it entered the pump, thus minimizing the accumulation of impurities on the pump check valves. Pressures higher than the

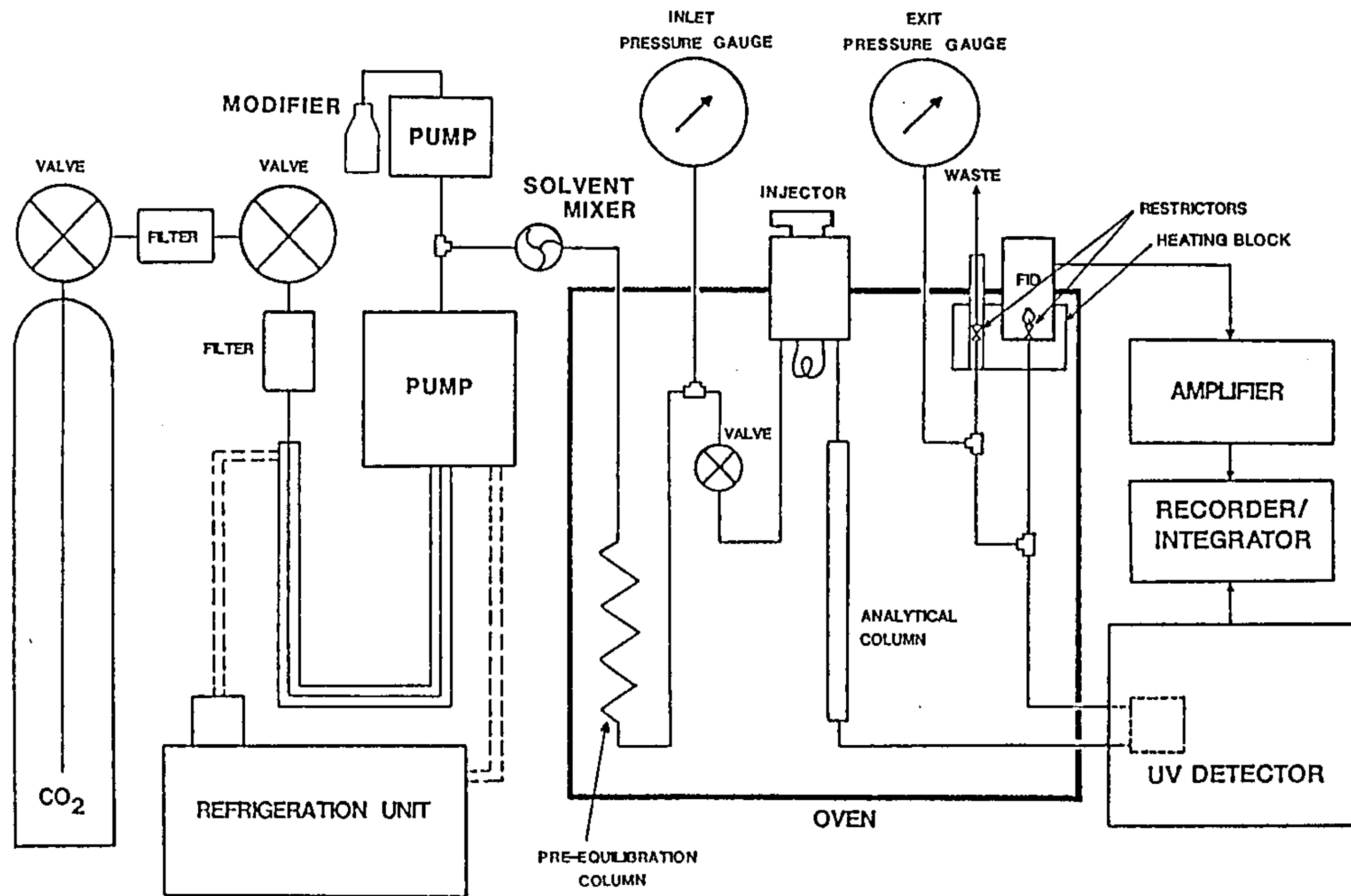


FIGURE 3.1. Schematic diagram of the SFC system.

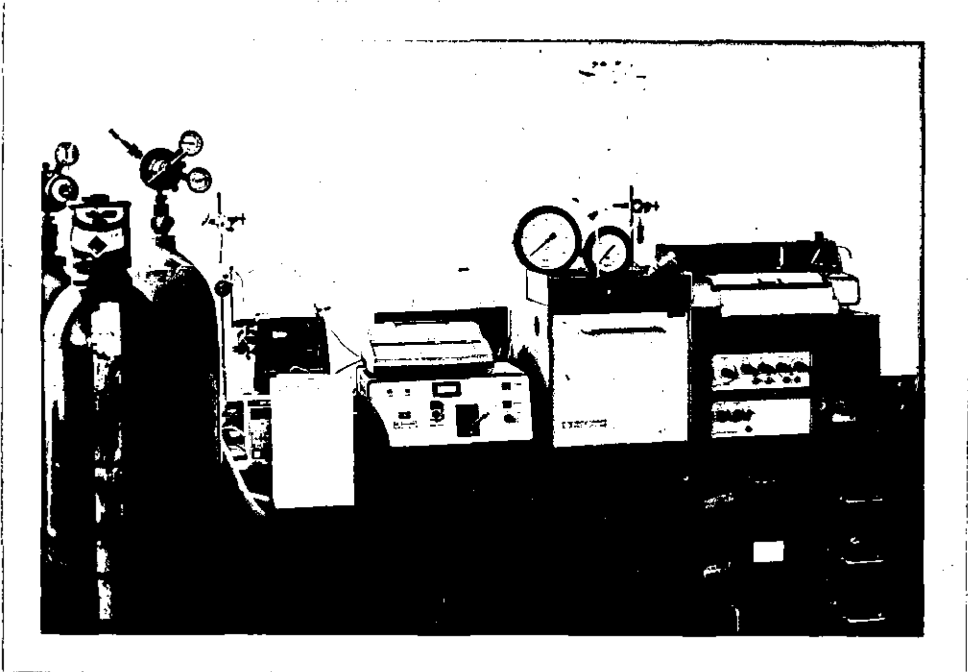


FIGURE 3.2. Photograph of the overall SFC system.

critical pressure were achieved by using a modified Jasco BIP-1 HPLC pump (see below). The organic modifier (if used) was added to the pressurized carbon dioxide using a Pye-Unicam XPS LC pump through a T-piece. The fluid was then passed through a solvent mixer (ACS Ltd.) into a length of 1/16 inch tubing located in the oven unit of a Pye 104 gas chromatograph which acted as a heat exchanger. A shut-off valve was placed prior to the injector. Samples were injected into the preheated carbon dioxide mobile phase using a Rheodyne valve mounted on top of the oven at ambient temperatures and then the flow was passed to a packed analytical column in the oven. The eluent pressures prior to and after the column were monitored using pressure gauges (SSD and Budenberg). The column effluent was passed through a UV detector and a T-piece effluent splitter in the oven was used to allow a small fraction of the effluent (about 60-100 ml/min of gas) to flow to the FID. The main effluent stream was passed to waste through a short length of stainless steel tubing as a back pressure restrictor and into a heated expansion chamber. The expansion chamber was made from a copper tube (1/8 inch o.d.) which was placed in close contact with a heating block at the base of the FID. This simple device eliminated problems with carbon dioxide condensation in the waste restrictor. A photograph of the overall SFC system is shown in Figure 3.2.

3.3 MOBILE PHASE DELIVERY SYSTEM

3.3.1 Mobile phase

There are a few aspects to consider in selecting a suitable SFC mobile phase. A main requisite for the use of any substance as a mobile phase in SFC is that it be chemically stable under the conditions to

which it will be subjected. Other aspects include detector compatibility, critical constants, polarity, and safety [101]. The fluid for mobile phase in SFC must be of acceptable high purity. Organic impurities found in the mobile phase can result in high or noisy background signals which make the chromatography meaningless. The choice of mobile phase also depends on the type of compounds to be analyzed. Although many compounds can potentially be used as supercritical fluids, (see Chapter 1, Table 1.1), some are better for certain types of compounds; for example, ammonia is a better solvent for more polar compounds [8], while hexane is a better mobile phase than carbon dioxide for the separation of (non-polar) polystyrene [72].

Carbon dioxide offers many practical advantages for use in SFC. Its near-ambient critical temperature (31 °C) makes it favourable for use with thermally unstable compounds. It is non-toxic, non-polluting, non-flammable, relatively inexpensive, and readily available in high purity [9]. Furthermore, carbon dioxide is virtually transparent in the UV [9] and has windows in the IR spectral regions [64] which allows the use of the corresponding spectroscopic detection methods.

However, there is a drawback in using carbon dioxide as an SFC mobile phase; it has low polarity which means that it has a somewhat limited solvent strength. While it is favourable for the separation of non-polar compounds such as paraffins, polynuclear aromatic hydrocarbons (PAH), and oligomers, in the case of the separation of very polar compounds the addition of polar modifiers to the mobile phase is necessary [131].

Chemical stability of the solvents is also important if they are used as modifier in SFC. Asche [132] reported that methanol, 1-propanol, cyclohexane, di-i-propylether, benzene, tetrahydrofuran, and ethyl

acetate possess adequate stability when exposed to chromatographic temperatures as high as 300 °C. However, he noticed that chlorinated solvents (carbon tetrachloride, dichloromethane, dichloroethane) can undergo decomposition from reaction of residual water and air at high temperatures to give phosgene and hydrochloric acid. Another solvent of interest, acetonitrile, has been found to decompose forming ammonia only when using alumina as stationary phase [132]. Nevertheless, at mild temperatures typically used in SFC with carbon dioxide as the mobile phase, it is expected that these adverse effects will be negligible.

In the present SFC work, carbon dioxide has been exclusively used as the primary mobile phase. Industry (technical) grade carbon dioxide (purity >99.98%) has been used without prior purification, other than the fine filtering and no significant difficulties with solute detection were encountered.

Two common solvents widely used in HPLC, methanol and acetonitrile have been used as modifiers in the present work. The UV cut-off absorbances of these modifiers are at about 210 and 200 nm respectively which allow the use of UV detection. Methanol and acetonitrile solvents come from two different selectivity groups described by Snyder [66] with different interactions and polarity (Table 3.1).

TABLE 3.1. Classification of solvents according to the scheme proposed by Snyder [66].

	Methanol	Acetonitrile
Group	II	VIb
X_e (proton acceptor)	0.48	0.31
X_d (proton donor)	0.22	0.27
X_n (dipole interaction)	0.31	0.42

3.3.2 High presure pump

A modified Jasco BIP-1 HPLC pump (Jasco Ltd., Tokyo, Japan) was used in the later and major part of this work. The two cylindrical pump heads, the check valves, and the solvent mixers were cooled to prevent gasification of the carbon dioxide mobile phase. This was accomplished by enclosing the components in a box through which cold (about -20 to -5 °C) ethylene glycol-water mixture was run (Figure 3.3). The cooling box was made from a diecast waterproof electrical box, which was large enough to accomodate the pump heads, check valves, and solvent mixers. A metal box was preferred to a plastic one because of its strength. The back part of the box was precision-cut to fit the pump heads. Water-tight seals between the pump heads and the box were provided by

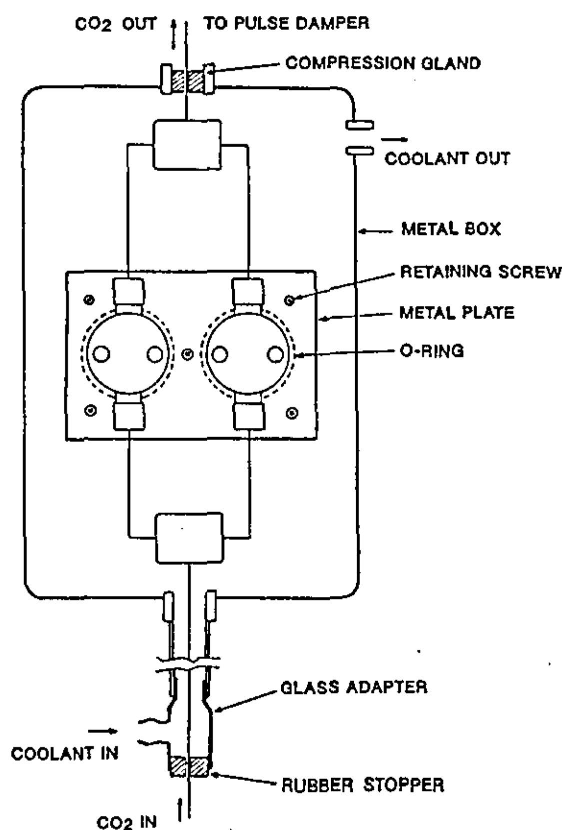


FIGURE 3.3. Schematic diagram of the pump heads, check valves, and solvent mixers cooling unit for the Jasco BIP-1 HPLC pump for the pumping of liquid carbon dioxide.

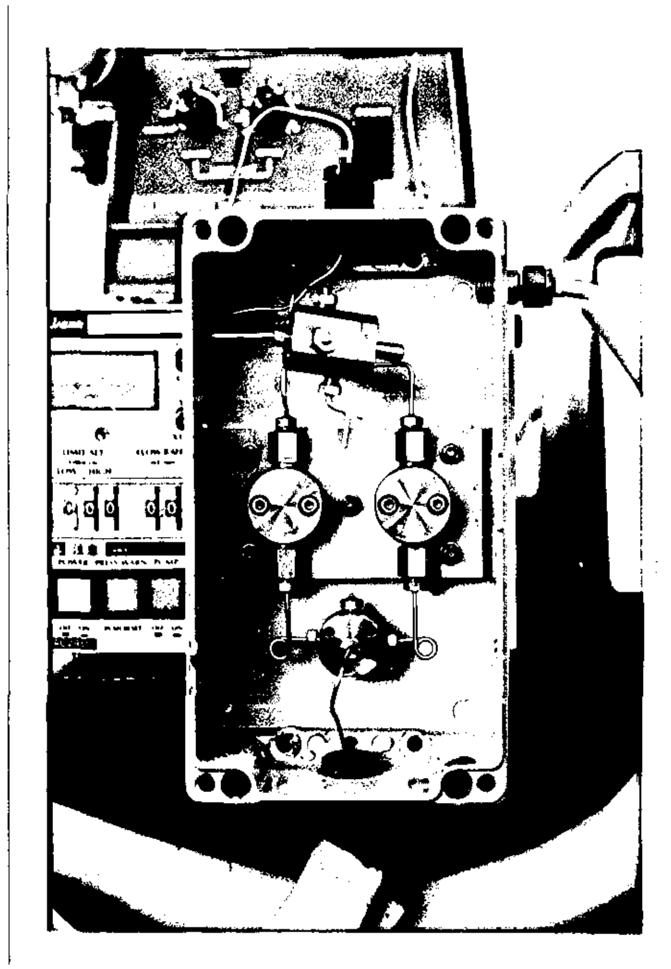


FIGURE 3.4. Photograph if the pump heads, check valves, and solvent mixers cooling unit for the Jasco BIP-1 HPLC pump.

O-rings. Inlet and outlet ports on the box allowed the circulation of the coolant. There was a direct contact between the coolant and the pump heads (Figure 3.4) and therefore, cooling of the latter was very effective. Because the cooling box has a water-tight front lid which can easily be opened, servicing of the inlet and outlet valves and the mixers was possible without dismantling the entire cooling assembly. The cooling efficiency was further improved by cooling a major portion of the carbon dioxide inlet tubing prior to the pump. This was made possible by enclosing the length of tubing in a rubber tube (3/8 inch i.d.), one end of which led to the coolant pump, and the other attached directly to the inlet port of the cooling box. The cooling solution in the reservoir was cooled by a refrigeration unit (L&C Refrigeration, Bognor, U.K.). In order to prevent excessive air moisture condensation and loss of cooling efficiency, the cooling unit was insulated using cut-to-fit polystyrene. Expanded polystyrene hoses provided insulation to the cooling system tubing.

Similar modifications were also carried out in an earlier part of this work on a Jasco Familic 300S HPLC pump (Jasco Ltd., Tokyo, Japan) which has three pump heads [7,65], and a single-headed Pye-Unicam XPS HPLC pump (Pye-Unicam, Cambridge, U.K.) [88].

Another method was used in earlier attempts to cool the single pump head of a Pye-Unicam XPS pump and the single pump head of a Kontron 410 HPLC pump. This cooling unit consisted of a length of copper tubing (1/4 inch i.d.) coiled around the pump head, through which cold ethylene glycol-water solution was circulated. Although this method was relatively simple, it has proved to give inefficient cooling to the pump head and was not successful. This was probably because the cooling coil did not provide as good contact with the pump head for efficient heat

transfer compared to some of clamped-on cooling units reported in the literature [108-110]. Consequently this cooling method was abandoned in favour of the cooling box.

3.3.3 Connections and tubing in the delivery system

Stainless steel HPLC tubing (usually 0.15 mm i.d., 1/16 inch o.d.) was used throughout the SFC high pressure system. Prior to the injector, a shut-off valve (Whitey) was connected to the system. The shut-off valve allowed the maintaining of high pressures downstream while changing columns or restrictors. The outlet pressure gauge was connected via a 1/16 inch T-connector (Swagelok) to the waste effluent flow path after the splitter (see Figure 3.1), and therefore, it did not become a source of band broadening.

The post column effluent splitter consisted of a modified 1/16 inch T-piece. The hole of the T-piece were drilled to a suitable size which is just enough to allow the insertion of 1/16 inch stainless steel tubing. This splitter showed lower extra-column dead volume effects compared to that previously experienced with a 1/8 inch T-piece splitter, as indicated by improved peak shapes and resolution. In the early part of the preliminary SFC work, PTFE ferrules were used in the various joints. However, apparently, these ferrules could not withstand prolonged use under normal SFC conditions (e.g. oven temperature of 60 °C and column pressures of 2000 psi) and they tended to burst. Subsequently, the PTFE ferrules were replaced with ones made of stainless steel.

In order to enhance the mixing of the modifier with the carbon dioxide mobile phase before entering the column, a mechanical solvent

mixer with about 4 ml internal volume (ACS Ltd.) was used in most of the separations with modified carbon dioxide mobile phases.

3.4 CHROMATOGRAPHIC OVEN

The chromatographic oven establishes the operating temperature of the supercritical fluid in the analytical column. The chromatographic oven should show good temperature stability which is important in maintaining controlled supercritical conditions. The oven used in this work was salvaged from an abandoned Pye-Unicam Series 104 gas chromatograph (Pye-Unicam, Cambridge, U.K.). The oven compartment had enough room to accommodate the tubing and connections, and a straight packed column up to about 30 cm long (Figure 3.5). The existing ports on the top and the side of the oven provided convenient inlet and outlet passages for the tubing. The oven temperature controller, the circulating fans, and the detector and signal amplifier were still in good working conditions and needed no modification. The oven temperature can be read directly using a thermometer. The oven can be operated under normal isothermal SFC conditions with a temperature stability of ± 0.5 °C.

3.5 SAMPLE INTRODUCTION

Packed column SFC does not place the same stringent requirements on the sample introduction system as experienced in capillary SFC, which must be capable of delivering very small amounts of sample (<10 nl) [50,111]. Conventional HPLC injection devices have been commonly used in packed column SFC [32].

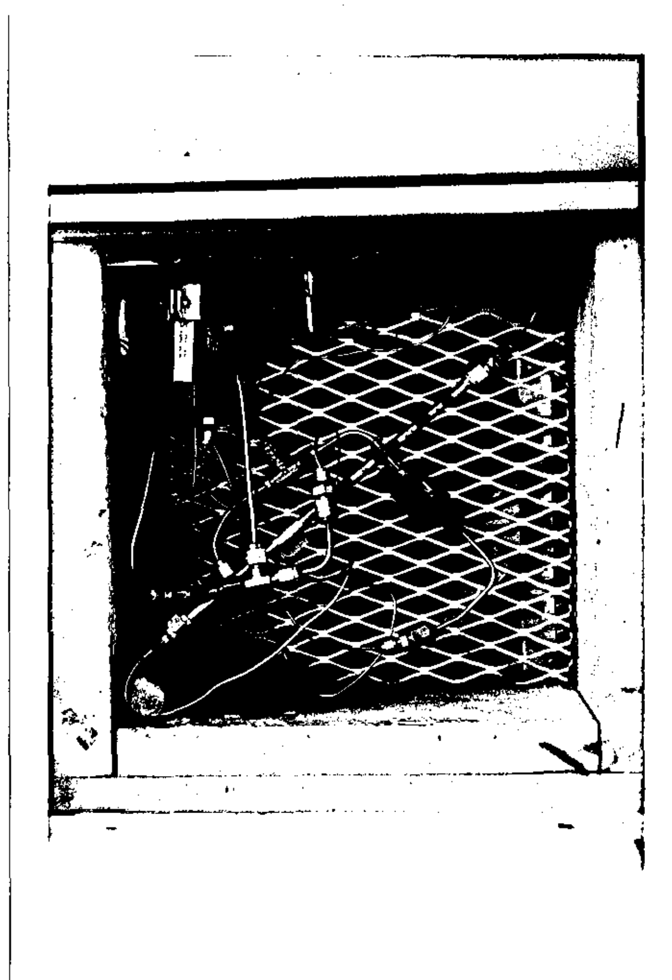


FIGURE 3.5. Photograph of the oven compartment of the SFC system.

Three sample injection valves have been used in this study. These were a Rheodyne 7010 with a fixed volume 5 µl loop, a Rheodyne 7125 with a 20 µl loop, and a Rheodyne 7410 with a fixed volume internal loop (1 µl) in combination with model 7012 loop filler port (all were from Rheodyne, Berkeley, U.S.A.). The injection valves were mounted on a panel on top of the oven and were operated at ambient temperature. Injections up to 8 µl were performed using the Rheodyne 7125 injection valve with a syringe (Scientific Glass Engineering, Milton Keynes, U.K.) and this injector was found to give good reproducibility (see later Chapter) and was therefore used extensively in the present SFC work.

3.6 ANALYTICAL COLUMN

Packed columns have been used throughout the present SFC study primarily because they are readily available with a wide range of stationary phases and particle sizes. Furthermore, they are also compatible with UV and FID detectors which were used in this work, although an efluent splitter was required for the latter. Three types of stationary phases have been used, namely, ODS-silica, cyano-silica, and PS-DVB materials.

3.7 PRESSURE AND ELUENT FLOW CONTROLLER

Two pressure gauges, namely SSD and Budenberg (Manchester, U.K.) were salvaged from an old syringe pump (ACS Ltd., U.K.).

Because the density of the mobile phase is strongly dependent on pressure, it is essential to get accurate pressure readings. The two pressure gauges were calibrated using a "Dead Weight Tester" (Budenberg)

in the Mechanical Engineering Department Laboratory, Loughborough University of Technology. It was found that both pressure gauges were still in good working conditions (linear pressure readings) although their original scale readings had considerable deviations from the true values. Because of this, new scales which correspond to the true values were affixed. The SSD pressure gauge (maximum pressure limit of about 5000 psi) was installed prior to the column while the Budenberg pressure gauge (maximum pressure limit of about 3400 psi) was installed after the column. The accuracy of the pressure gauges was estimated to be in the order of ± 20 psi.

The column pressure and mobile phase flow rate can be controlled using a pressure regulator or restrictors. Two restrictors have been used in the SFC system; one leads to the FID, and the other leads to waste. The restrictors were made simply by crimping and/or filing the tip of a short length of ordinary stainless-steel tubing (0.15 mm i.d.). In normal SFC operations, the waste restrictor was primarily used to adjust the eluent flow rate, column pressure, and pressure drop across the column.

In the early stage of the SFC system development, in order to prevent condensation of carbon dioxide mobile phase, the waste-flow restrictor tip was laid against the cartridge heaters at the base of the FID. This orientation, however, did not allow direct and continuous monitoring of the eluent flow through the restrictor. In such conditions, flow rate measurements required the restrictor tip to be moved away from the heated zone to permit the insertion of the tube leading to the bubble flow-meter. In those instances, the restrictor temperature decreased rapidly which resulted in eluent condensation or partial restrictor blockage. In our experience, the waste-flow

measurements always caused inevitable interruptions in the SFC separations.

Subsequently, a small, heated expansion chamber was designed and installed to the effluent waste restrictor (Figure 3.6). It was constructed using a modified (drilled through) 1/8 - 1/16 inch stainless steel reducing union (Swagelok) and a copper tube (1/8 inch o.d.). A length of the restrictor is inserted into a length of 1/8 inch copper tubing, which was placed in a close contact with a heating block at the base of the FID. This simple device eliminated problems with carbon dioxide condensation in the waste restrictor, and allowed direct flow rate measurements throughout the SFC operation. Since the connection of the restrictor to the expansion chamber was non-permanent (with the use of PTFE ferrule), different restrictors with different flow rate characteristics can be used interchangeably.

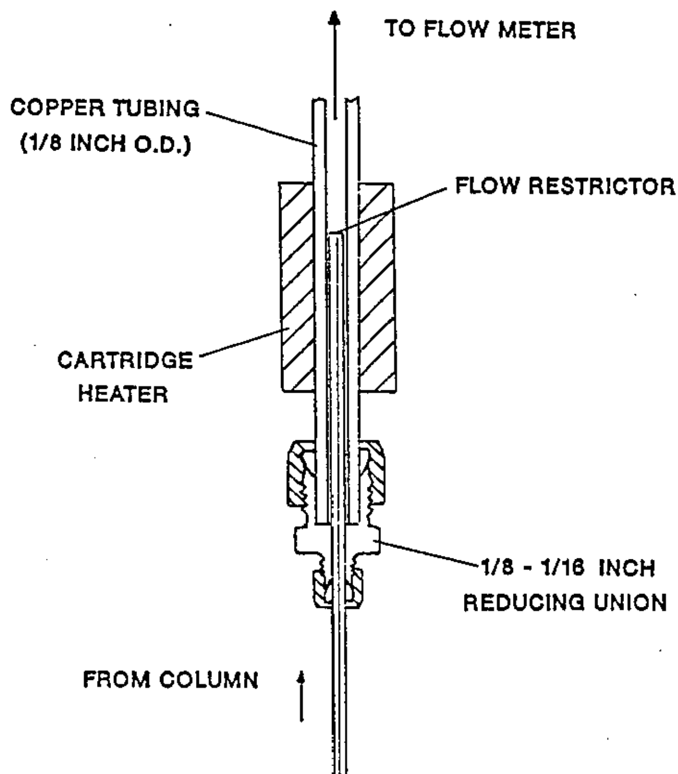


FIGURE 3.6. Schematic diagram of the heated expansion chamber for the restrictor.

3.8 DETECTORS

Among several choices to be made when fabricating a supercritical fluid chromatograph, that of detector may be considered first, since most other decisions depend on the detector chosen. To select a suitable detector for SFC, a number of factors have to be considered including the nature of the analytes, mobile phase availability, operating conditions, as well as cost.

Most conventional GC and HPLC detectors can be readily interfaced to SFC with little or no modification and this has become one of the advantages of SFC. FID and UV detectors have been widely used in SFC, but obviously there are also needs for structural identification techniques in SFC and there has been considerable interest in the use of SFC/MS [14,20-22,82,84,93] and SFC/FTIR [23-25,64,84,94,95] with both capillary and packed columns.

3.8.1 UV detector

Unlike the FID, the UV detector responds only to compounds that have a UV-absorbing chromophore. The UV detector can provide compound-specific information when tuned to an absorption wavelength that is characteristic for the analyte of interest [84]. In this respect, a variable wavelength UV detector or a photodiode-array detector can be used to obtain spectra to assist in compound identification.

Interfacing the UV detector with an SFC instrument that uses standard HPLC columns is simple and straightforward. Because of the large flow rate associated with these columns, there is little concern

about the dead volume. In contrast, capillary columns can impose stringent detector dead volume limitations [50,84].

ACS UV detector model 750/12 with variable wavelength selector or model 750/11AZ with fixed-wavelength filter wheel (ACS Ltd., Macclesfield, U.K.) were used in this study. Each detector was equipped with a high pressure flow cell (8 µl) which can withstand pressures up to 6000 psi. The connection from the analytical column to the UV detector was made using a Shandon zero dead volume union. No difficulties in solute detection or excessive background signals have been encountered while using these detectors.

3.8.2 Flame ionization detector

FID has been extensively employed in many types of supercritical fluid chromatographic separations, with carbon dioxide as the most widely used mobile phase. In the late 60s and 70s, SFC with packed columns and FID had been widely used [133-135], and with the numerous advances in column technologies in late 70s and 80s, reports of capillary column SFC with flame ionization detection have been increasing in number [7].

Combining SFC with FID offers several advantages. First, solutes entering the flame are more easily desolvated and decompose into various detectable species. Secondly, FID gives little response to commonly used fluids like carbon dioxide or nitrous oxide. Thirdly, FID gives similar response to almost all organic compounds including those which do not contain chromophores or fluorophores, and consequently, FID acts as a universal detector for organic solutes. However, FID is not directly compatible with most organic mobile phases and modifiers because they

give high background signals. Other favourable properties of FID include a wide linear range (10^7), high sensitivity (pg range), and it is very stable and reliable [136]. Considering the distinct advantages offered, and its ready availability, FID has been chosen as one of the detection methods used in the present SFC study.

The flame ionization detector used was from an old Pye-Unicam Series 104 gas chromatograph. This FID was found to be able to operate with considerably high carbon dioxide mobile phase flow rates. The FID flow rates under typical SFC conditions were usually in the range of 60 to 100 ml/min (gas). Higher flow rates (ca. >200 ml/min) tend to cool the FID rapidly and extinguish the flame of the FID. This is unlike the case of capillary SFC where make-up gas may have to be used to improve the transfer of sample to the flame [137].

Under supercritical fluid chromatographic conditions, the transition from the chromatographic column to atmospheric pressure should occur along the shortest possible path. In a prolonged exit section, heavy or polar substrates may be deposited due to condensation [138]. In the SFC system used in the present work, the FID restrictor was made by crimping the very tip of a length of stainless steel tubing (0.15 mm i.d.) with a pair of pliers and smoothing out the tip with a file or a grinding stone. The tip of the restrictor was placed as far inside the FID as possible. However, it could not be pushed completely into the FID flame because the FID nozzle inlet size is smaller than the outside diameter of the restrictor.

In was observed early in the present SFC instrumental development that the expansion of the carbon dioxide mobile phase through the restrictor in the FID could lower the temperature of the restrictor as well as the FID. This consequently created the partial restrictor

blockages and detection problems discussed previously (see Section 1.3.2). In order to minimize the problems, a heating block consisting of two heating cartridges was installed at the base of the detector around the restrictor which were used to elevate the temperature of the the FID and the restrictors.

CHAPTER 4

SYSTEM EVALUATION AND PRELIMINARY APPLICATION STUDIES

4.1 INTRODUCTION

Many supercritical fluid chromatographic systems are home-made of individual designs and often the operating procedure of one instrument is quite different from the others. The aims of the early experiments in the present study were to establish suitable operating conditions for separations and to acquire familiarity and skills in handling the instrument and gain some in-depth knowledge of the different factors involved. This chapter describes the general operation of the SFC system and discusses the results of the preliminary application studies. The preparation and evaluation of columns of different sizes packed with Spherisorb ODS-2 are also described in attempts to achieve high column efficiencies.

4.2 PACKING OF COLUMNS

4.2.1 Practical considerations

The separating ability (efficiency) of the analytical column plays an important role in most chromatographic analyses. In order to separate complex mixtures such as some biological and environmental materials, very large plate counts as found in capillary GC (e.g. 100,000-200,000) are often required. However, for packed column, it is difficult to increase the overall efficiency merely by connecting a number of shorter conventional column or using a extra-long column, because of the

tendency for the column to develop a very high pressure drop. This in turn create problems with excessive detection times and detection of dilute peaks [139].

Conventional HPLC packed columns have been used in this study for a number of reasons. Firstly, the use of packed columns in SFC imposes less stringent instrumental requirements compared to capillary SFC such as on the mobile phase pumping system (see Section 1.2.4). Secondly, packed columns can be easily interfaced with both FID and UV detectors although effluent splitting may be required for the former. Thirdly, these columns are readily commercially available in different sizes and with a wide selection of stationary phases, and alternatively, these columns can be prepared in the laboratory.

In the attempts to obtain the maximum possible number of plates, small-diameter columns (2.1 mm i.d.) of different lengths up to 60 cm were tested in the initial part of this study. Several factors were considered when choosing the analytical packed column, which included: (a) thermal equilibrium can be achieved more rapidly using small diameter columns; (b) small-diameter columns are more readily adaptable for use with FID when operated under unsplit effluent conditions; (c) small-diameter columns are advantageous for analyses which have small absolute sample size constraints; and (d) provided that the packing efficiency is not considerably reduced, a longer column is more advantageous and gives higher total column efficiency for better separations. However, there are also some disadvantages associated with long, small-diameter columns including: (a) the longer or the smaller the column the more difficult it becomes to pack efficiently and uniformly; and (b) the longer the column, the greater the pressure drop or pressure gradient across the column.

A considerable number of packing techniques have been suggested in the literature, but the results of a given packing technique remains virtually unpredictable. Because there are many factors involved in packing a column that can affect the efficiency of the packed column [140], it has been generally accepted that optimum results can be obtained only by trial and error with a variety of packing methods and practice. Dense packing is normally considered to give stability and uniform packing and thus better efficiency and this can best be achieved by an "upwards" low-viscosity-solvent packing procedure [140].

4.2.2 Packing procedure

The columns were packed by a slurry method following the general procedures recommended by a HPLC column manufacturer (Shandon Southern). The packing material was suspended in either isopropanol or methanol and dispersed by manual shaking or ultrasonic vibration for about five minutes. It was found that methanol as the initial packing solvent tended to give fast packing times and satisfactory results, which supports the suggestions made by Verzele et al. [140].

TABLE 4.1. Effect of column dimension and shape on efficiency for columns prepared with identical packing procedures and tested on HPLC system.

Eluent: methanol-water 70:30; solute: biphenyl; UV detection, 254 nm.

Dimension (mm)	Column shape	Efficiency (plates)
500 x 2.1	coiled	2300
250 x 2.1	straight	6100
200 x 3.0	straight	9000

Coiled or bent columns (500 x 2.1 mm i.d.) were found to be more difficult to pack efficiently than straight columns (250 x 2.1 mm i.d.) when tested in an HPLC system (Table 4.1). However, it was realized that besides the inherent difficulty in packing because of the narrow bore, the 1/8 inch o.d. 2.1 mm i.d. columns have an additional disadvantage as they were easily bent because of their thin walls. An accidental drop onto the floor for instance, may be likely reduce the homogeneity and efficiency. Narrow-bore columns with rigid thick-walled columns, as used in standard HPLC seemed attractive alternatives. Consequently, a straight column (200 x 3 mm i.d) was packed and it showed reasonable total efficiency of about 9000 theoretical plates (for biphenyl) when tested using the HPLC system. This column was therefore used in many of the subsequent SFC separations on ODS-silica.

4.3 OPERATION AND EVALUATION OF THE INSTRUMENT

4.3.1 Start-up procedure

Chilling of the coolant in the coolant reservoir was normally commenced several hours (> 4) before beginning SFC operations. To save time during the day, this was sometimes done by leaving the refrigeration unit on overnight. The temperature of the coolant used for cooling the pump heads in the SFC operation was usually in the range of -20 to -5 °C. Adequate cooling of the pump heads could normally be achieved in about 10 to 15 minutes after coolant circulation was started. At that point, slight moisture condensation might be observed around the exposed cooling tubing and as the cooling proceeded this could turn into a layer of ice.

After initiating the cooling of the modified pump heads, the rest of the equipment i.e. the oven, recorder/integrator, detectors, and FID catridge heaters was then switched on and set to the desired conditions. Upon opening the valves on the carbon dioxide cylinder, an increase of pressures was immediately observed from the pressure gauges. The modified pump was set to the constant-pressure mode of operation and the pressure control was set to zero pressure or a pressure lower than the pressure of the carbon dioxide cylinder (<5 kg/cm^2). This was to avoid excessive initial pumping action, hence unnecessary strain on the pump. After switching on the pump, the pressure was increased stepwise using the pressure controller switch. The increase of pressure in the chromatographic system became more rapid after the fluid passed the critical pressure.

4.3.2 Pump performance

In most parts of this work, the modified Jasco BIP-1 HPLC pump has been used. In the constant-pressure mode of operation the relationships between the set pump-pressure and the inlet and the outlet pressures of the column were linear (Figure 4.1). This meant that in the constant-pressure mode conditions, the pump was capable of delivering liquid carbon dioxide at the set pressure with good retention time reproducibility. On the other hand, under constant-flow mode of operation, the pump could not maintain a reasonably constant pressure over the length of time for an analysis (say 10 minutes). The pressure drifts observed were probably due to inefficient pumping, which could be as a result of trapped gases in the pump heads. A similar poor performance was also experienced earlier when using either a modified

Pye-Unicam XPS or a Kontron 410 HPLC pump, both of which can be operated only in a constant-flow mode.

4.3.3 Operating temperature and pressure

Except in a few instances, the SFC operations were performed under isoconfertic (constant density) conditions, which effectively meant that the separations were carried out under constant pressure and temperature conditions. In order to take advantage of mild separation capability in SFC offered when using carbon dioxide as the mobile phase, relatively low operating temperatures in the range of 40 to 100 °C were used in this work.

At a constant temperature, the density of supercritical carbon dioxide increases nonlinearly with pressure (see Section 1.2). As the temperature and/or pressure increase, pressure-density isotherms exhibit smaller change in density with the change in pressure. Solute retention in SFC is largely dependent on the density of the mobile phase. Therefore, it would be advantageous to work in the region of the supercritical fluid where the change in density with either temperature or pressure is minimal to achieve better retention time reproducibility as any minor changes would have only a minor effect on the separation.

4.3.4 Effect of presure-drop

The pressure-drop across a packed column in SFC has been known to have a significant effect on the separation. Schoenmakers et al. [38,50] have clearly demonstrated that the greatest variations in retention with pressure-drop occur under conditions where the variation of retention

with pressure is greatest. Thus, to minimize these effects, the SFC separation should be performed in the region of least density-change per pressure-change. The maximum operating pressure would nevertheless be judged by the instrumental limitations.

Investigations were carried out to determine the relationships between the column inlet and outlet pressures, set pump pressure, and pressure drop across the column. The experiments were performed under constant temperature conditions, using a PS-DVB column (PLRP-S, 150 x 4.6 mm i.d.) and a fixed restrictor with carbon dioxide as the mobile phase. It was observed that both the inlet and outlet pressures increased almost linearly with increasing pump pressure and their differences (pressure drop across the column) also increased; from about 40 psi at a set pressure of 1800 psi to about 150 psi at a set pressure of 3500 psi (Figure 4.1). These results showed that the pressure drop

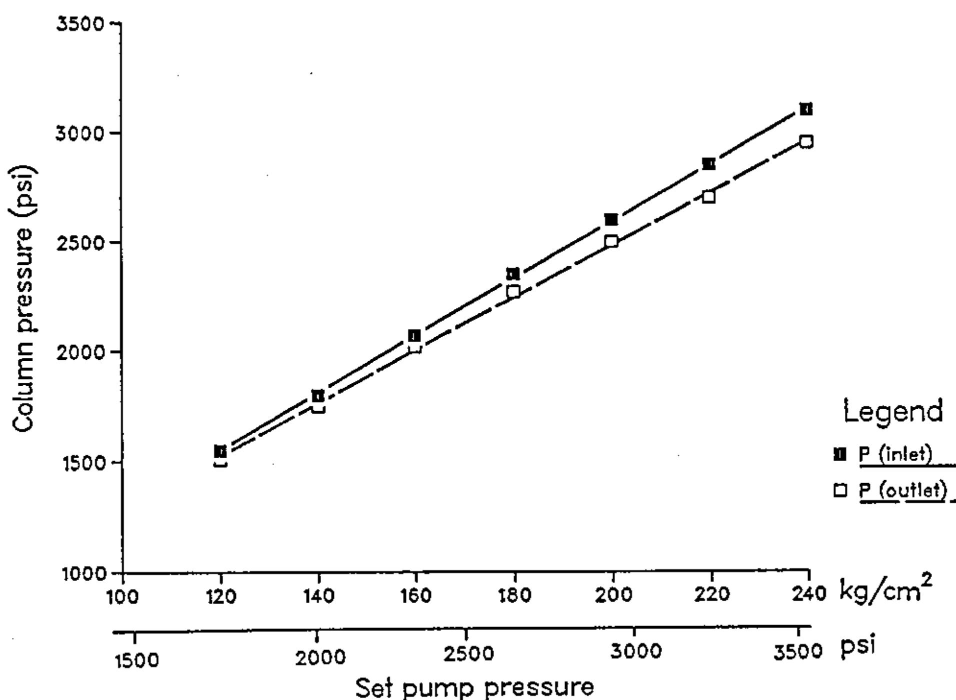


FIGURE 4.1. Variation of inlet and outlet column pressures with set pump pressure using a fixed restrictor. Column (150 x 4.6 mm i.d.), PLRP-S 5 µm; 60 °C.

across the column was quite low and thus it could be increased further in order to optimize the efficiency and shorten the analysis times. This could be effected by increasing the flow rate through the restrictor or increasing the pressure.

4.3.5 Restrictors and eluent flow rates

The SFC instrument design allowed us to maintain stable mobile phase flow rates under isobaric conditions. The flow rate and pressure in the system was therefore governed primarily by the restrictors. It was of interest to investigate the effect of the restrictors on the flow rates and pressure drop across the column.

A series of fixed restrictors of different orifice sizes with different flow rate characteristics have been prepared (see Section 3.5). The waste restrictors were interchangeable and could be used to effect different flow rates. Total plugging of the restrictors during separation experiments has not happened during the course of this investigation but sudden changes in permeability due to partial plugging occurred occasionally, especially when separating higher molecular weight and nonvolatile samples. Similar effects have also been observed by other workers [141].

The relationships between the typical column outlet pressure and effluent flow rate through two restrictors, which have different orifice sizes, restrictor 1 and restrictor 2, are shown in Figure 4.2. The plots show almost linear relationships. The deviations from linearity can probably be explained by the non-linear dependency of the density of the supercritical fluid with pressure. These characteristics have been reported by Grob [103] using Pt/Ir restrictors and also by Smith et al.

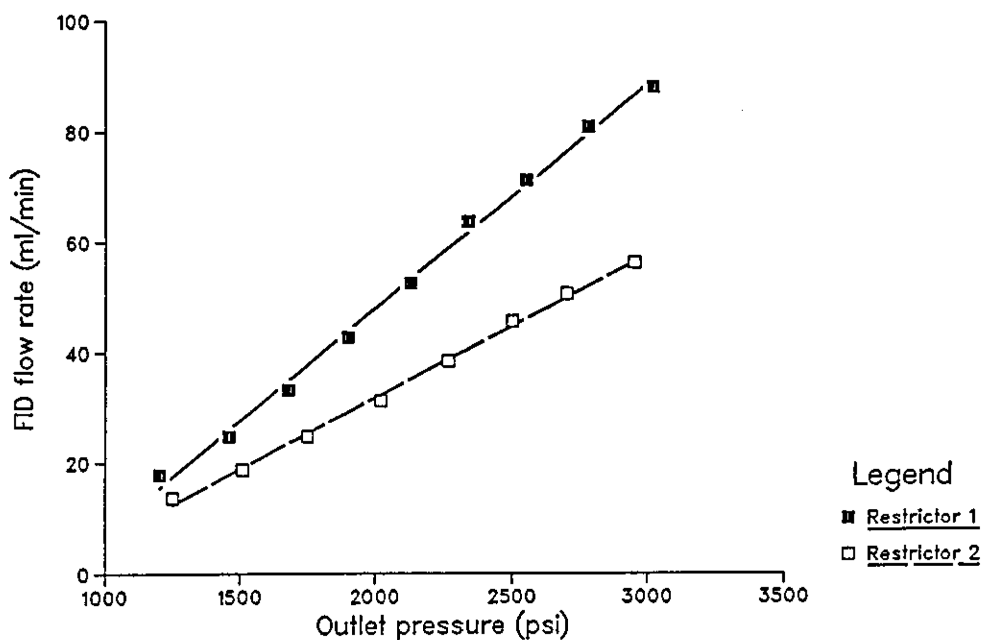


FIGURE 4.2. Variation of FID flow rate (ml/min, gas) with column outlet pressure using two different restrictors. Conditions: as in Figure 4.1.

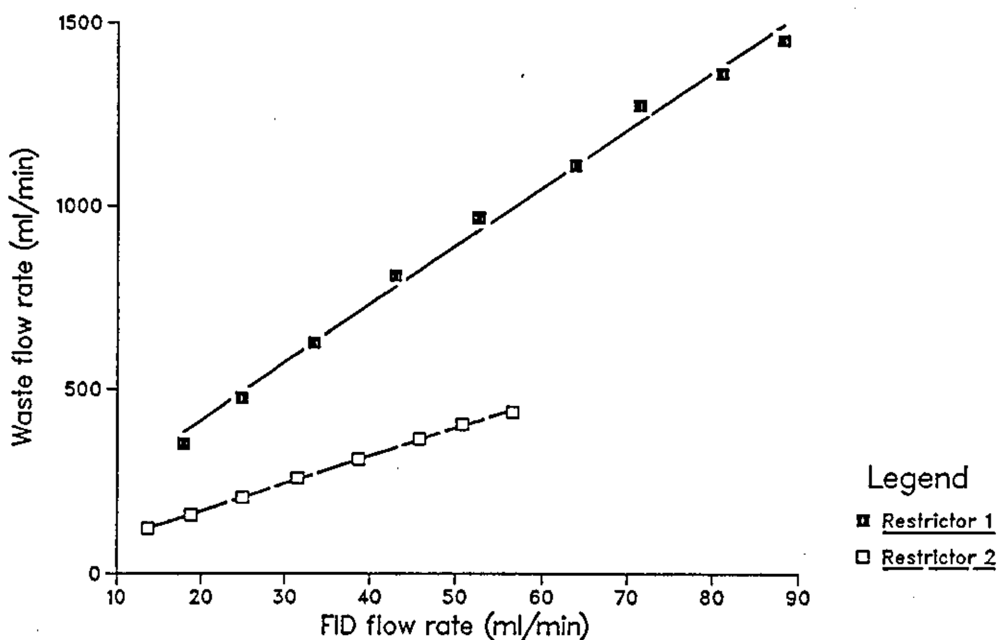


FIGURE 4.3. Relationship between FID flow rate and waste flow rate for two different FID restrictors. Conditions: as in Figure 4.1.

[122] who examined the pressure-linear flow rate characteristics of individual restrictors made from capillary columns.

In the course of the present work, detector flow rates in the range of about 50 to 100 ml/min (gas, NTP) have been found to be most favourable for the FID. The effluent split ratios between the FID and waste for a particular set of restrictors, were found to be largely independent of pressure and the total effluent flow rates. This is clearly demonstrated by the linear plots of FID restrictor flow rates against waste restrictor flow rates for two different FID restrictors (Figure 4.3). Therefore, after having established the split ratio, it was possible to calculate the FID restrictor flow rate at any time during the SFC operation (under split-effluent conditions) by measuring the waste restrictor flow rate.

4.3.6 Flow rate determination

In HPLC, the mobile phase density is practically constant irrespective of the pressure and temperature. In SFC, however, the mobile phase is compressible and the density shows a strong dependency on pressure, temperature and composition. As the flow rate in SFC is dependent on these parameters, it is difficult to determine accurately the mobile phase flow rate in terms of its volume. It is perhaps because of these reasons that most reports, in describing SFC separations, have used either the linear velocity [142], the flow setting and average pressure (hence density) [59,71], or simply by specifying the operating pressure and temperature [70], without actually giving the eluent flow rate in the column.

In the present work, the volume flow rates (gas) of the eluent

measured at room temperature were determined. The volume flow rates were readily converted to mass flow rates by using the ideal gas equation:

$$PV = nRT \tag{4.1}$$

where the P is the pressure (atm), V the gas volume (litre), n the number of moles of gas, R the ideal gas constant (1 atm mol^{-1} K^{-1}), and T the temperature (K). The variables used were T = 298 K (25 °C), P = 1 atm, and the molecular weight of carbon dioxide 44 g/mol.

In the major part of the present work pure carbon dioxide was pumped by using a modified Jasco BIP-1 HPLC pump and the modifier was pumped by using a Pye-Unicam XPS HPLC pump. The Pye-Unicam XPS pump has a minimum liquid delivery rate of 0.1 ml/min and was found able to deliver the organic modifier at typical SFC operating pressures with good accuracy (RSD of 5%). On passing the modifier-containing effluent through the expansion chamber tube and the length of the bubble flow meter tube which was maintained at ambient temperatures, the modifier condensed before or on reaching the flow meter because of its higher boiling point, thus leaving the carbon dioxide gas. From the volume of gas measured, the mass of carbon dioxide was then calculated. The approximate values of the weight percentage of the modifier in the mobile phase was determined from the the equation:

$$[m1 / (m1 + m2)] \times 100 \tag{4.2}$$

where m1 is the mass of the modifier, and m2 is the mass of carbon dioxide.

On changing the modifier concentration, the system was allowed to equilibrate, indicated by a constant detector signal. Equilibrium was usually achieved after about 10 minutes. Randall [67] reported an equilibrium time on silica and ODS-silica columns of only about 2-5 minutes judging from the retention time stability.

4.3.7 Baseline stability

With carbon dioxide as the mobile phase, very stable baselines were obtained using both the UV and flame ionization detectors. For the FID, this suggests that the cartridge heaters installed at the base of the detector was able to maintain adequate high temperature to the restrictor in the FID and eliminated the noisy baseline associated with eluent condensation. The background noise of the UV detector however, increased slightly at lower wavelengths but remained very low levels (Figure 4.4). The baseline tracings of the UV detector at various AUFS settings are shown in Figure 4.5.

In order to improve the elution of polar compounds organic modifiers have been used. Unfortunately, the use of these modifiers with the FID, caused problems of strong background signals and unstable baselines. These problems have led to a reduced sensitivity of the detection. For instance, noisy FID baseline signals were observed when methanol (4%, w/w) was added to the mobile phase (Figure 4.6). Unstable baselines for the UV detector were also observed which corresponds to the modifier pump stroke cycles (Figure 4.7a). In addition to the major fluctuations, the baselines also show very weak but more frequent fluctuations which correspond to the carbon dioxide pump stroke cycles which were clearly visible when operating at sensitivities higher than 0.2 AUFS UV-detector setting. The above effects were largely reduced by using a mechanical solvent stirrer with internal volume of about 4 ml (Figure 4.7b). This has led to the conclusion that the major cause of the baseline fluctuations was fluctuations in the modifier concentration.

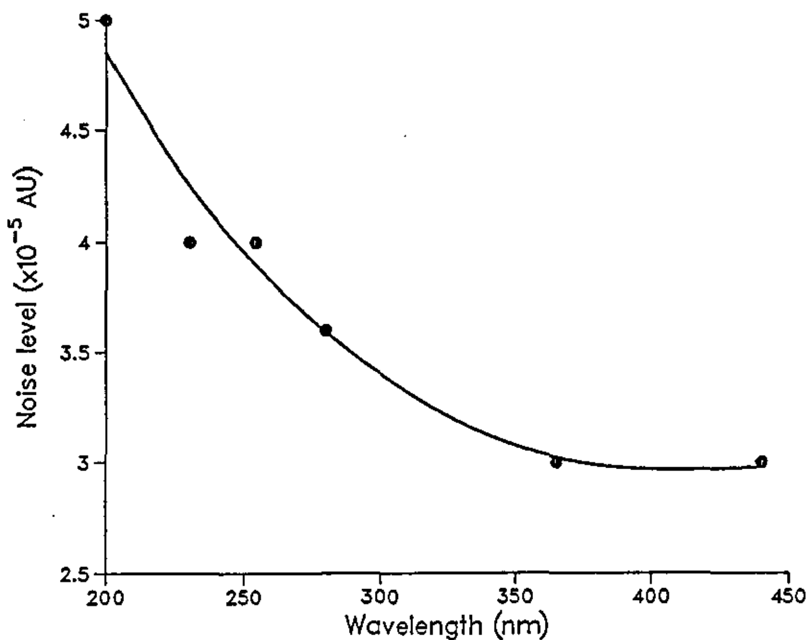


FIGURE 4.4. Background noise of the UV detector at different wavelengths. Conditions: carbon dioxide mobile phase, mean pressure 2285 psi.

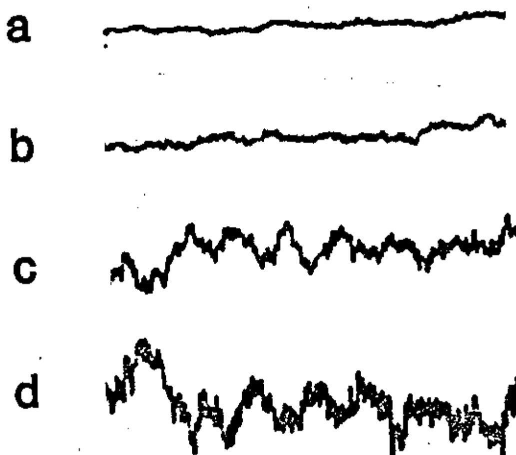


FIGURE 4.5. Baseline tracings of the UV detector at different AUFS settings, a = 0.1, b = 0.05, c = 0.02, and d = 0.01. Conditions: UV detector, 200 nm; column PLRP-S 5 μm; carbon dioxide eluent; 60 °C; chart speed 1 cm/min.

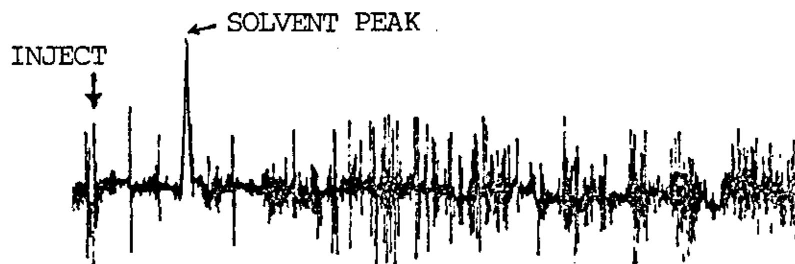


FIGURE 4.6. Tracings of the FID signals with 4% methanol in the mobile phase. Injected sample 1 µl solution of amylobarbitone in methanol. Conditions: column PLRP-S 5 µm; mean pressure 2260 psi; 60 °C; FID, attenuation 50 x 10^2, chart speed 1 cm/min.

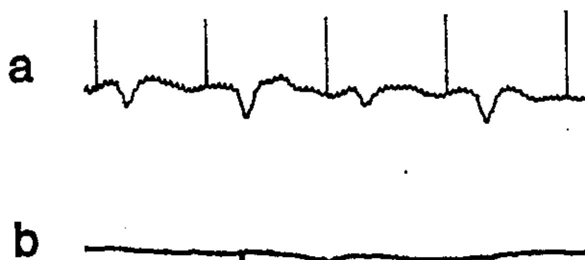


FIGURE 4.7. Baseline tracings of the UV detector with 4% methanol in the mobile phase: (a) without a solvent mixer, and (b) with a solvent mixer. Other conditions: column PLRP-S 5 µm; mean pressure 2260; UV detection, 254 nm, 0.2 AUFS; chart speed 1 cm/min.

4.3.8 Determination of void volume

In order to calculate the capacity factors of the analytes, it was necessary to determine the void volume (t_o). It was observed early in the present work that the retentions of some common solvents were different on different columns, particularly when operating at low pressure conditions. On an ODS-silica column at a pressure of less than about 1600 psi, it was found that the order of elution was pentane, hexane < acetone, dichloromethane, chloroform < methanol < carbon tetrachloride. For separation on an ODS-silica column, hexane was used as the void volume marker. On the PS-DVB column, acetone was found to be least retained among the common solvents. At higher pressures greater

than of about 2000 psi, however, all these solvents co-eluted. In those cases, the void volume was obtained using acetone or the sample solvent (dichloromethane) peak. However, this was not the case on a cyano-bonded silica column when hexane was found to be significantly less retained than polar solvents, even at high pressures. Therefore, hexane was used as the void volume marker and with UV detection the void volume was obtained using the negative peak which corresponded to the FID signal for hexane in the absence of modifier.

4.4 REPRODUCIBILITY OF INJECTION AND DETECTION

4.4.1 Effect of sample loading

One of the drawbacks of carbon dioxide as a mobile phase is that because it has low polarity, it is difficult to elute polar compounds. The separation of polar compounds were typically characterized by broad and tailing peaks. These effects are illustrated in Figure 4.8a for the separation of citronellol on ODS-silica column. The retention peak maximum decreased with increase in injection amount. This led to poor retention time reproducibility and sensitivity, which is known in GC and HPLC [143]. It was however found that if the injection amount was kept constant, good retention time reproducibility was obtained. It was therefore felt that if a constant sample size was used, retention studies on the polar compounds can be carried out without over complicating the results.

The separation of a non-polar compounds such as the n-alkanes on the ODS-silica column gave symmetrical peak shapes and virtually constant retention times with varying injection amounts (Figure 4.8b).

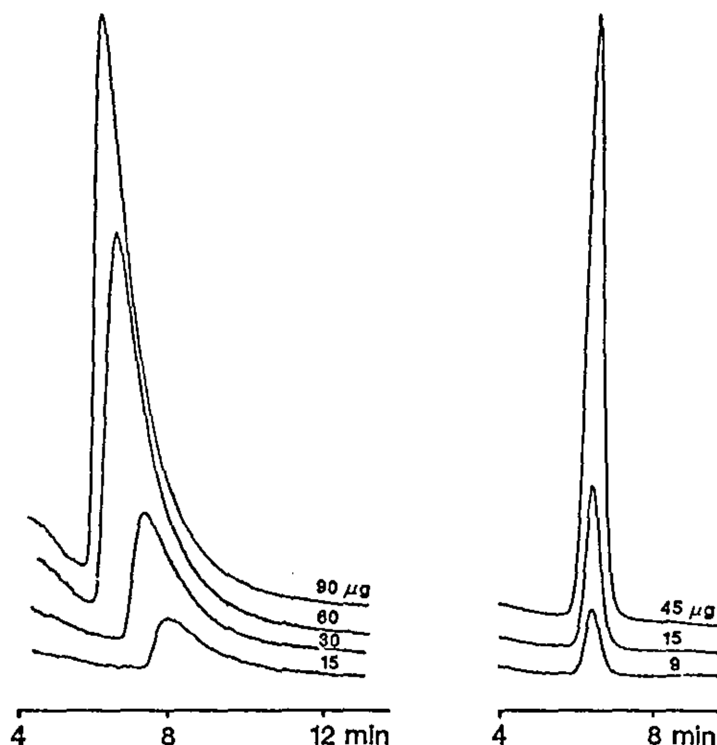


FIGURE 4.8. Variation of peak shape with injected sample amount for (a) citronellol and (b) tetradecane on ODS-silica with carbon dioxide as the mobile phase. Conditions: column (200 x 3 mm i.d.), Spherisorb ODS-2 5 μm; flame ionization detection.

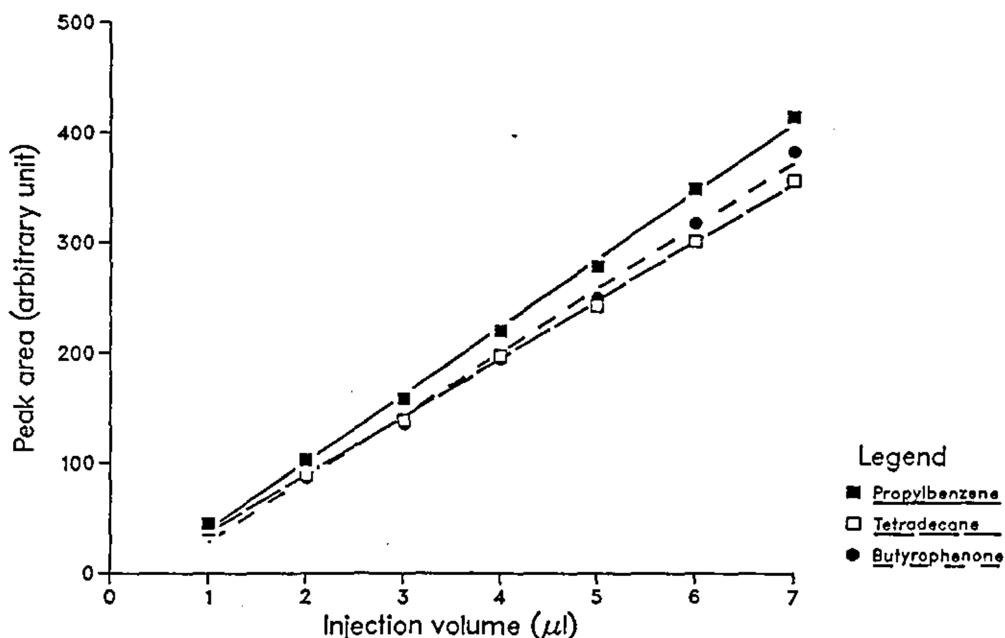


FIGURE 4.9. Relationship between peak area and injection volume. Conditions: column PLRP-S 5 μm; carbon dioxide mobile phase; mean pressure 2245 psi; 60 °C; flame ionization detection; injection valve Rheodyne 7125. Sample: propylbenzene, tetradecane, and butylbenzene, 4.88, 5.33, and 5.81 mg/ml, respectively, in dichloromethane.

4.4.2 Quantitative analyses

Sample injection techniques have been found to play an important role in determining the reproducibility of the peak area. Rapid injection techniques appeared to give maximum peak areas and better reproducibility. This has been seen to be more critical when using fixed volume loops (1 and 5 µl) injection valves (Rheodyne models 7010 and 7410). This was probably because of incomplete filling of the loop with the sample or because a portion of the sample in the loop was expelled to waste during valve switching. Injections of up to 7 µl samples using a "solvent-flush" injection technique wherein all the sample goes into the loop was found to give satisfactory reproducibility using a Rheodyne model 7125 injection valve with a 20 µl sample loop.

4.4.3 Reproducibility of retention time and peak area

Experiments were carried out to determine the reproducibility of retention time and peak area of the SFC system. A 50 ml stock mixture was prepared containing approximately 8 mg/ml each of propylbenzene, tetradecane, and butyrophenone in dichloromethane. Solutions having 80, 60, 40, and 20 percent concentrations of that of the stock solution were prepared by dilution of the appropriate amount of the stock solution with dichloromethane. A 2% concentration of solution was prepared by dilution of a known volume of the 20% concentration solution with the solvent. Triplicate injections were carried out for each injection volume of sample assay using a Rheodyne 7125 injection valve. This valve was placed outside the chromatographic oven and at ambient temperature. Sample separations were carried out on a PLRP-S column using carbon

dioxide as the mobile phase.

The retention time reproducibility of this SFC system is shown in Table 4.2. The data were obtained from 20 consecutive injections and they showed good reproducibility with the RSD values which were in the range of 0.76 to 1.56%. However these results were somewhat inferior compared to those obtainable using commercial capillary SFC reported in the literature [102]. A capillary SFC with syringe pump and "time-split" injection methods gave excellent reproducibility with typical RSD values of 0.996% and 0.121% for docosane and triacontane respectively [102].

In order to examine the effect of injection volume to peak area, a set of experiments were performed by injecting different amounts of the sample mixture (60% concentration) and the measured peak areas were plotted against injection volume (Figure 4.9). It was observed that under the operating conditions the peak area increases linearly over injection volume range of 1 to 7 µl. The least squares analyses of the plots (Table 4.3) show good correlation coefficient: 0.9993 for propylbenzene, 0.9995 for tetradecane, and 0.9994 for butyrophenone but none of the lines passes through the origin suggesting a constant systematic sample loss or measurement error.

TABLE 4.2. Retention time reproducibility of the SFC system.

Conditions: column (150 x 4.6 mm i.d.), PLRP-S 5 µm; carbon dioxide mobile phase; 60 °C; mean pressure 2245 psi; flow rate 1150 ml/min (gas); effluent split ratio 1:18, flame ionization detection.

Compound	Retention time (t_r), n = 20		
	Mean (min)	SD($\times 10^{-2}$)	% RSD
Propylbenzene	1.88	1.43	0.76
Tetradecane	2.45	3.82	1.56
Butyrophenone	3.25	3.17	0.98

TABLE 4.3. Least squares analysis of the correlations between peak area and injection volume and sample amount.

Conditions: as in Table 4.2.

	Propylbenzene	Tetradecane	Butyrophenone
Peak area vs. injection volume*			
Correlation coefficient	0.9993	0.9996	0.9994
Slope	61.53	52.90	57.46
Intercept	-21.50	-15.77	-28.25
Peak area vs. sample amount**			
Correlation coefficient	0.9998	0.9995	0.9994
Slope (x 10^{-3})	10.92	8.88	8.01
Intercept	-13.41	-14.12	-12.9

* n = 7, range 1-7 µl (concentrations: ca. 4.8 mg/ml each).
** n = 5, injection volume 3 µl.

TABLE 4.4. Retention time reproducibility of the SFC system.

Conditions: column PLRP-S 5 µm; mean pressure 2175 psi; 60 °C; flow rate 1050 ml/min (gas) split ratio 1:18; flame ionization detection.

Compound	Day 1		Day 2		Day 3		Overall	
	Mean t_r' (min)	RSD (%)	Mean t_r' (min)	RSD (%)	Mean t_r' (min)	RSD (%)	Mean t_r' (min)	RSD (%)
Decane	0.700	2.47	0.650	3.32	0.645	2.00	0.662	4.42
Tridecane	1.660	1.20	1.545	2.21	1.528	2.85	1.570	4.22
Hexadecane	3.457	1.59	3.230	2.25	3.218	2.69	3.287	3.88

In another set of experiments, sample mixtures of different concentrations were used at a fixed injection volume of 3 μl. The peak area values were plotted against the injected sample amount (Figure 4.10). Again, within the experimental limits the peak area increased linearly with sample amount and correlation coefficients of greater than 0.9994 were obtained (Table 4.4). These results confirm the linear response in the detector and the absence of discrimination in the detector and effluent splitter. The results also suggest that the injection valve, a Rheodyne 7125 with a 20 μl sample loop used in conjunction with a variable-volume syringe could be used in the SFC system to give reasonably good reproducibility. None of the regression lines passes through the origin; they intercept the x axis at about 1.5 μg. The possible reasons could be a constant systematic error in injection or measurement procedures or a constant systematic sample loss in the separation which in turn could result from slight irreversible adsorption of solutes on the stationary phase

In general the peak area reproducibility results shown above were better than if the sample loop and part of the injection valve were placed in the heated part of the chromatographic oven. This was believed to be partly because the heating of the sample loop could cause rapid volatilization of the sample matrix, causing expulsion of portions of the sample from the sample loop prior to injection. Thus in these cases, poor peak area reproducibility can be expected. This observation was however empirical, as a detailed evaluation of the different positioning and performance of injection valve used was not carried out.

A Rheodyne 7410 injection valve with internal fixed sample loop has also been tried out but it did not appear to give significantly better reproducibility than the Rheodyne 7125 valve.

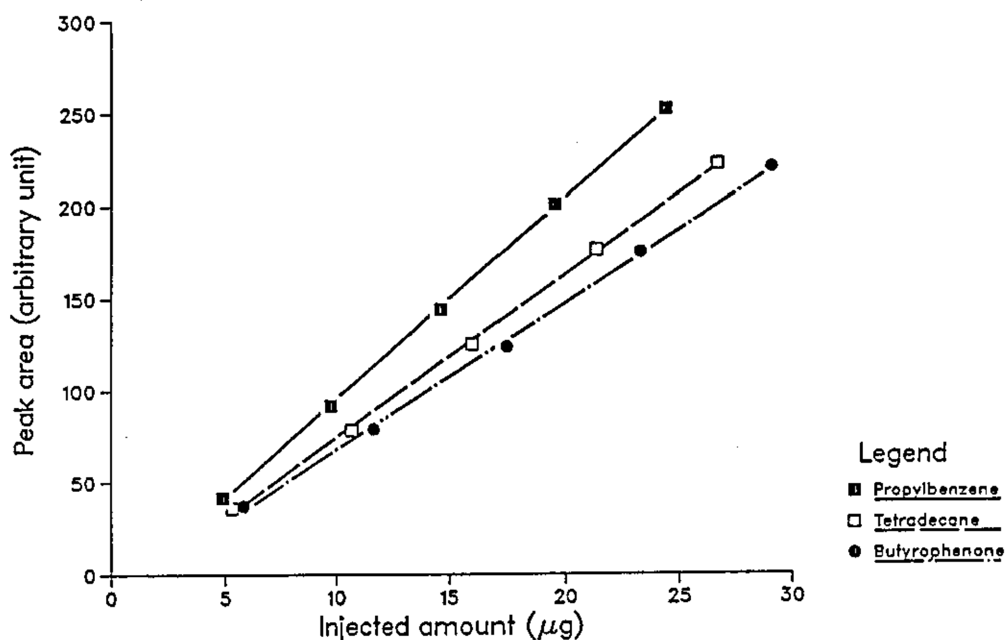


FIGURE 4.10. Relationship between peak area and injected sample amount. Injection volume 3 μl, using samples of different concentrations. Other conditions as in Figure 4.9.

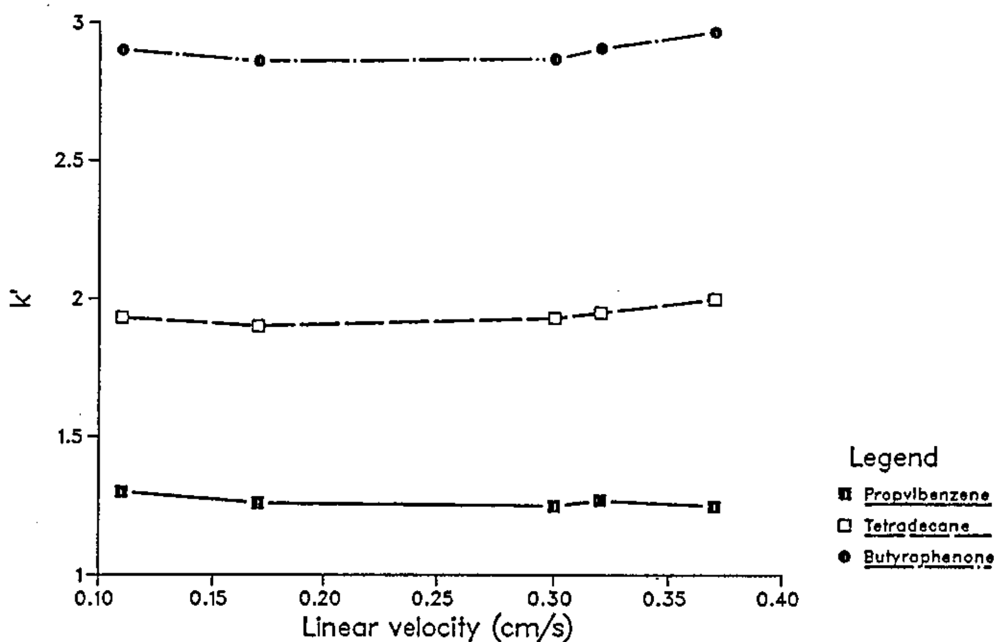


FIGURE 4.11. Variation of capacity factor with linear velocity of the eluent. Column PLRP-S 5 μm; carbon dioxide mobile phase; 60 °C.

Under the operating conditions indicated in Table 4.2, the detection sensitivity of propylbenzene with FID and carbon dioxide as the mobile phase was determined to be 162 ng with a signal-to-noise ratio of 3:1. The attenuation of the FID electrometer was 20 and the effluent split ratio was 1:18. The detection limit was considerably higher, compared to typical detection limit values obtained in GC (<1 pg) [144].

In another set of experiments, the retention times of six n-alkanes, decane to hexadecane were determined in quadruplicate. The experiment was repeated on three different days with the same sample mixture and the same operating conditions. The mean adjusted retention times ($t_r' = t_r - t_o$) and the RSDs were calculated for each day and the overall mean t_r' and RSD for the three-day analyses were determined (Table 4.4).

The results show that the repeatability was excellent, with RSD ranging from 1.20% to 3.33%. Similarly, the reproducibility on a day to day basis was good, with RSDs in the range of 3.88% to 4.42%. although they are high compared to those typically obtainable from conventional HPLC systems (e.g., five determinations carried out on different days on an ODS-silica column with methanol-aqueous buffer 40:60 eluent, RSDs: butyrophenone = 2.63%, nitrobenzene = 2.82%, barbitone = 4.05%) [145].

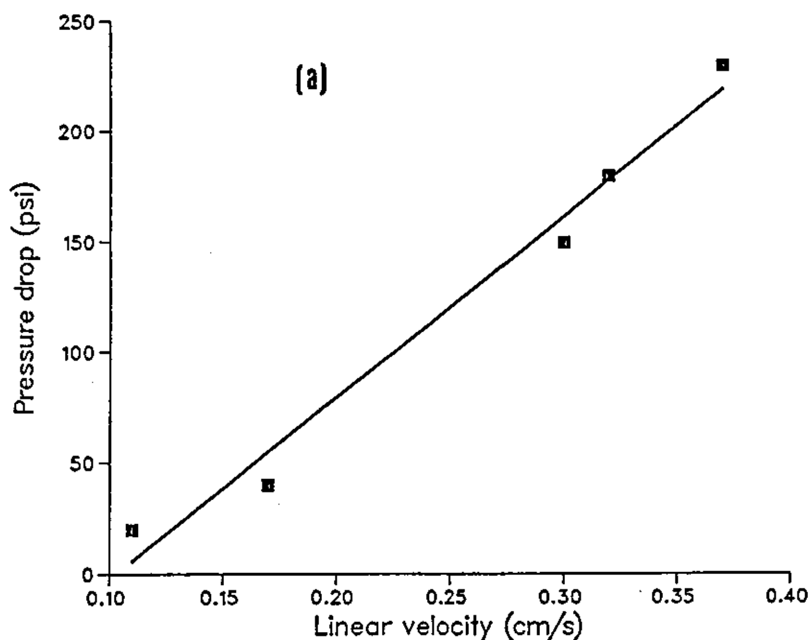
4.5 EFFECT OF FLOW RATE ON RETENTION AND EFFICIENCY

In order to determine the effect of mobile phase flow rate on retention, a mixture of propylbenzene, tetradecane, and butyrophenone was separated on a PLRP-S column using carbon dioxide as the mobile phase. The pumping pressure (thus inlet pressure) was kept constant at

180 kg/cm² while the flow rate through the column was varied by changing the restrictor opening. The linear velocity, u, values were calculated from equation: $u = L/t_o$. The capacity factors were determined and were plotted against linear velocity (Figure 4.11). The capacity factors of each of the solutes show litle variation with changes in linear velocity of the eluent ranging from about 0.11 to 0.37 cm/s. Under the SFC conditions, these were roughly equivalent to 400 to 1400 ml/min (gas) or 0.8 to 2.8 ml/min of liquid carbon dioxide. The plots indicate that the capacity factors remained virtually constant with variation in the flow rate.

Small changes in pressure-drop across the column were observed on increasing the flow rate, with typical changes from 40 psi to 230 psi at flow rates of about 600 ml/min to 1400 ml/min (gas) respectively (Figure 4.12a). However, the pressure drop as percent of pressure increased (from 1 to 10%) with increase in eluent flow rate (Figure 4.12b). The increase in pressure-drop did not appear to significantly change the retentions or relative retentions of the solutes.

However, the pressure drop may contribute significantly to changes in the column efficiency. It was therefore of interest to determine the effect of flow rate on column efficiency. Figure 4.13 shows van Deemter plots (HETP vs. u) for two test compounds tetradecane and propylbenzene. HETPs were determined from $H = L/N$, where $N = 5.54 \times (t_r/w_{1/2})^2$ and the peak width at half height values, $w_{1/2}$, were determined manually from the chromatograms. The plots in general show minimum HETP at linear velocities ca. 0.3 to 0.35 cm/s. These values compare very closely with those of Gere et al. [106] who obtained linear velocities of about 0.3 to 0.5 cm/s for a column packed with 5 μm particles, and those of Mourier et al. [146] who reported an optimum linear velocity for packed



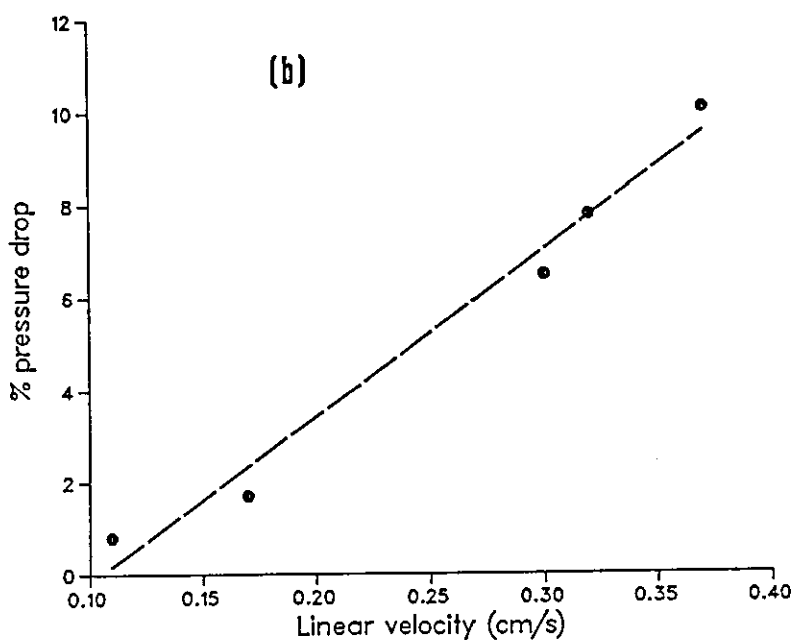


FIGURE 4.12. Variation of (a) pressure drop and (b) percent pressure drop across the column (based on mean pressure) with linear velocity of the eluent. Conditions: as in Figure 4.11.

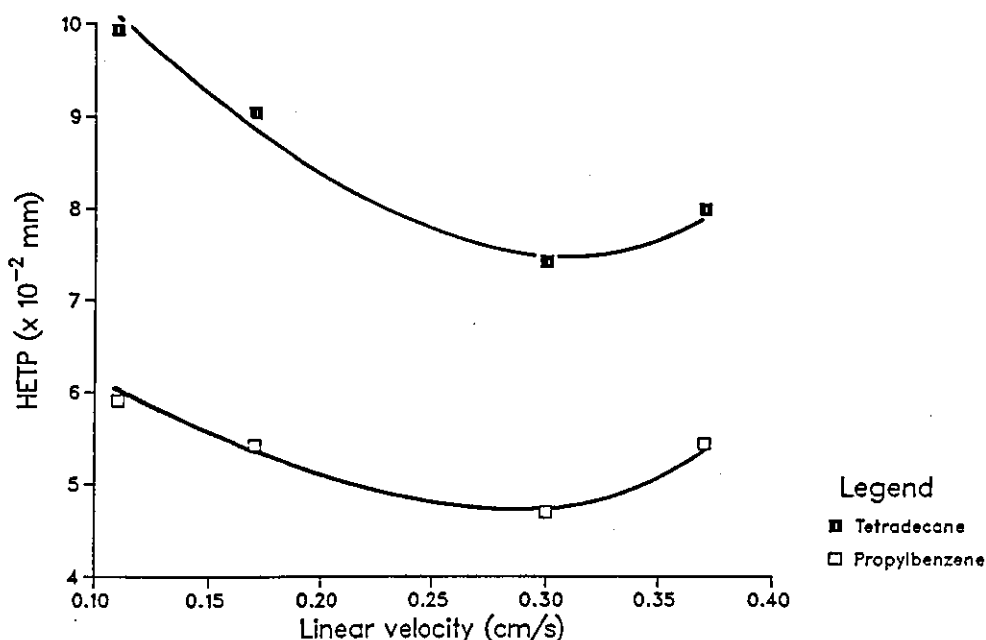


FIGURE 4.13. Variation of HETP of the column with eluent linear velocity. Conditions: as in Figure 4.11.

column of about 0.5 to 0.7 cm/s with carbon dioxide as the mobile phase. The results suggest that higher velocities and hence shorter run times (i.e. higher flow rate by less restriction) can be employed before experiencing a significant drop in the column efficiency.

4.6 PRELIMINARY SFC SEPARATIONS ON ODS-SILICA COLUMN

The majority of the supercritical fluid chromatographic separations were performed under constant pressure and temperature conditions. Careful efforts have been taken to ensure the chromatographic separations were done under controlled and identical conditions both for the standards and the test compounds. In general, the column temperature was quite easily controlled. Although the column inlet pressures stayed virtually constant during the time of analysis (under constant-pressure mode of operation), the outlet pressure and the pressure drop across the

column were more difficult to control with great precision on a day-to-day basis. This was probably because of changes in the size of the orifice of the restrictor.

This section describes the preliminary SFC separations performed on an ODS-silica column. The test compounds includes hydrocarbons, polynuclear aromatic compounds, alcohols, amines, fatty acids and esters.

4.6.1 n-Alkanes

The feasibility of SFC separation using a packed column and flame ionization detection was demonstrated by one of the chromatograms obtained in the initial experiments (Figure 4.14) [88]. The separation was carried out on a Spherisorb ODS-2 column using a modified Pye-Unicam XPS single-head HPLC pump to deliver the carbon dioxide mobile phase. For a homologous series of alkanes, the compounds were eluted in the order of increasing molecular weight, and thus the separation follows a reversed-phase type of mechanism. The peak widths and the distance between the peaks increase with time but apparently, good peak shapes were maintained. It is interesting to note that rapid baseline fluctuations were observed when using the single-head pump, even with the associated pulse damper connected. Such fluctuations are clearly visible in the chromatogram and they were most probably caused by pressure fluctuations in the mobile phase due to the pump strokes.

An experiment was carried out to determine the feasibility of performing pressure gradient using this SFC system. Using the Pye-Unicam XPS HPLC pump, the pressure of the carbon dioxide mobile phase was increased by manually increasing the set flow rates at fixed time

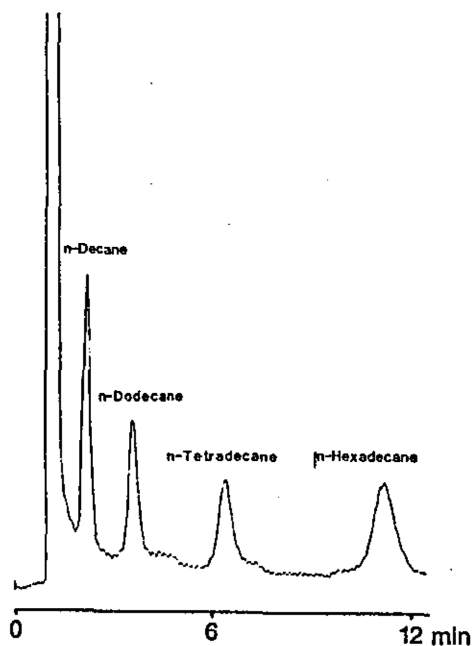


FIGURE 4.14. Isoconfertic SFC separation of n-alkanes on ODS-silica column with carbon dioxide mobile phase using the modified Pye-Unicam XPS single-head HPLC pump. Column Spherisorb ODS-2 5 μm; mean pressure 1805 psi; 60 °C; flow rate 472 ml/min (gas); flame ionization detection.

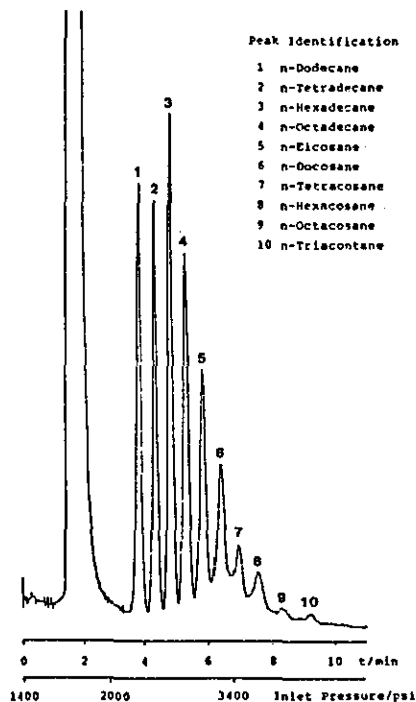


FIGURE 4.15. Pressure-gradient SFC separation of n-alkanes on ODS-silica column with carbon dioxide mobile phase. The mean column pressure and the eluent flow rate varies; other conditions as in Figure 4.14.

intervals. Separation of straight-chain hydrocarbon homologues ranging from dodecane to triacontane was successfully achieved at 60 °C in less than 10 minutes, with improved peak shapes and resolution, by increasing the column pressure gradually from 1400 to 3400 psi (Figure 4.15). This result demonstrates, qualitatively, the advantages of pressure (density) gradient in SFC and confirms the results obtained by previous investigators [121-127]. However, it was difficult to effect the pressure gradients with good precision by simple manual control and such separations are bound to produce poor reproducibility.

4.6.2 Aromatic compounds

Similar satisfactory results were obtained under isoconfertic conditions for polynuclear aromatic hydrocarbons: biphenyl, naphthalene, phenanthrene, and fluoranthene (Table 4.5), and the homologous series of alkylarylketones: acetophenone to heptanophenone (Table 4.6). Generally, these compounds showed good peak shapes. Each set of compounds were eluted in the order of increasing molecular weight and the capacity factors of each of the solutes decreased with increased pressure.

At constant temperature and pressure, homologous series of alkanes and alkylarylketones showed predictable changes in solute retention. Graphs of log capacity factor against carbon-number for these homologous series gave almost linear relationships (see Chapter 5). This behaviour has also been previously demonstrated in SFC by a number of workers for homologous series of alkyl phthalates [147], alkanes, and polynuclear aromatic hydrocarbons [30] and is a well known in HPLC and GC [19].

TABLE 4.5. Capacity factors of aromatic compounds.

Conditions: column (200 x 4.6 mm i.d.), Spherisorb ODS-2, 5 μm; 60 °C; carbon dioxide mobile phase, flame ionization detection.

Compound	Formula	Capacity factor	
		Mean column pressure (psi)	
		1960	2130
Naphthalene	$C_{10}H_8$	1.06	0.88
Biphenyl	$C_{12}H_{10}$	1.33	1.00
Phenanthrene	$C_{14}H_{10}$	4.44	3.38
Fluoranthene	$C_{16}H_{10}$	8.62	6.00

TABLE 4.6 Capacity factors of alkylarylketones.

Conditions: as in Table 4.5.

Compound	Capacity factor	
	Mean pressure (psi)	
	1584	1780
Acetophenone	1.50	0.81
Propiophenone	2.00	1.11
Butyrophenone	2.16	1.29
Valerophenone	2.76	1.57
Hexanophenone	3.41	1.79
Heptanophenone	4.29	2.14

4.7 PRELIMINARY SFC SEPARATIONS ON PS-DVB COLUMN

In an attempt to improve peak shapes and avoid the shifts in retention time for some compounds with sample size (Section 4.4), a study was carried out using a PS-DVB (PLRP-S) copolymer column with carbon dioxide as the mobile phase. Unlike silica-based packing materials, PS-DVB has no bonded alkyl groups or residual silanol sites and we do not expect it to give specific interactions with polar compounds.

4.7.1 Homologous compounds

(a) n-Alkanes

The PS-DVB column was found to be more retentive than the ODS-silica columns and as a result, the operating pressure had to be increased to give reasonable capacity factors and analysis times. The more retentive nature of the polymer column towards the hydrocarbons can be explained mainly because the polymeric packing is more apolar than the ODS-silica packing materials [64]. The separation of n-alkanes, nonane to hexadecane on the PS-DVB polymer column gave good peak shapes (Figure 4.16). The efficiency of the column under the operating conditions was good (e.g. tridecane, N = 5500). The elution order of the n-alkane homologous series was according to their increasing molecular weight. This suggests that the separation on the polymer column also follows a reversed phase type of retention mechanism.

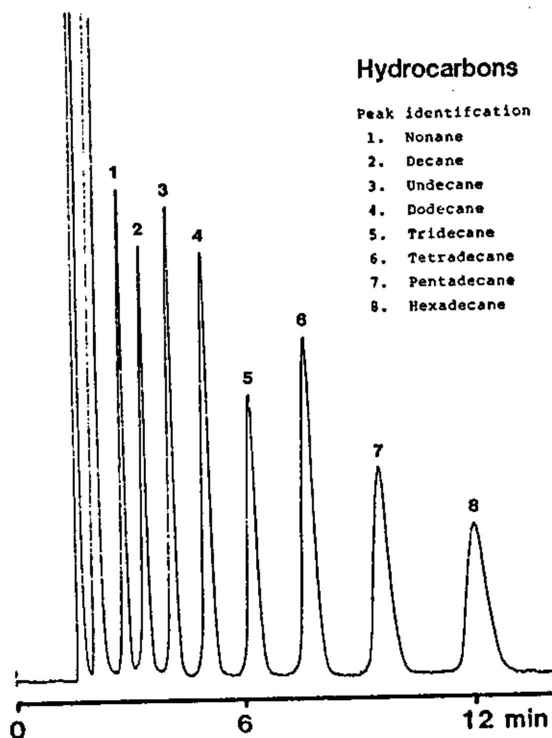


FIGURE 4.16. SFC separation of n-alkanes on PS-DVB column with carbon dioxide mobile phase and flame ionization detection. Column PLRP-S 5 μm; mean column pressure 1920 psi; 60 °C; flow rate 750 ml/min (gas).

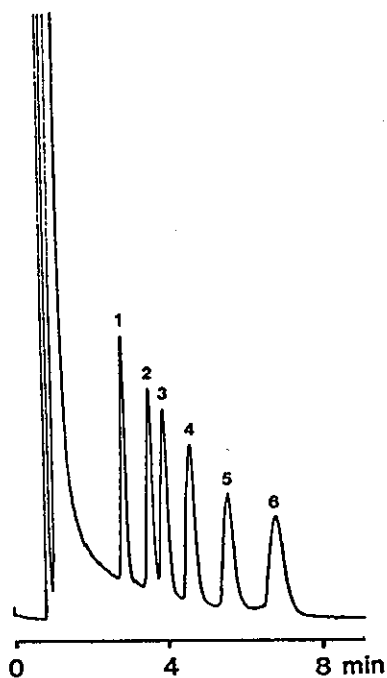


FIGURE 4.17. Separation by SFC of a mixture of alkylarylketones on PS-DVB column with carbon dioxide mobile phase and flame ionization detection. Mean column pressure 2270 psi; other conditions as in Figure 4.16. Peak identification: 1. acetophenone, 2. propiophenone, 3. butyrophenone, 4. valerophenone, 5. hexanophenone, 6. heptanophenone.

(b) Alkylarylketones

The separation of alkylarylketone homologues, acetophenone to heptanophenone on the PS-DVB column with carbon dioxide as the mobile phase gave good peak shapes (Figure 4.17) and the compounds were eluted according to increasing molecular weight. However, propiophenone was seen to be anomalous and was more retained on the column than expected. This behaviour was consistently observed in later investigations (Chapter 5).

(c) Alkylbenzenes

Similar separations of alkylbenzene homologues, toluene to butylbenzene were also carried out on the PS-DVB column (Figure 4.18). and these compounds gave good peak shapes.

4.7.2 Alcohols

Alcohols have been one of the most difficult classes of compound to analyze by GC [144]. This difficulty has also been. observed in packed column SFC with carbon dioxide as the mobile phase On an ODS-silica column the alcohols were highly retained which resulted in extremely broad peaks. This can be explained since the alcohols have relatively high polarity and an ability to interact with surface active sites on silica by hydrogen bonding. However, this effect was not completely eliminated using a PS-DVB polymer column, which does not contain silanol groups. On the PS-DVB column w-arylalkanols, from benzyl alcohol to 5-phenyl-1-pentanol again exhibited tailing peaks (Figure 4.19).

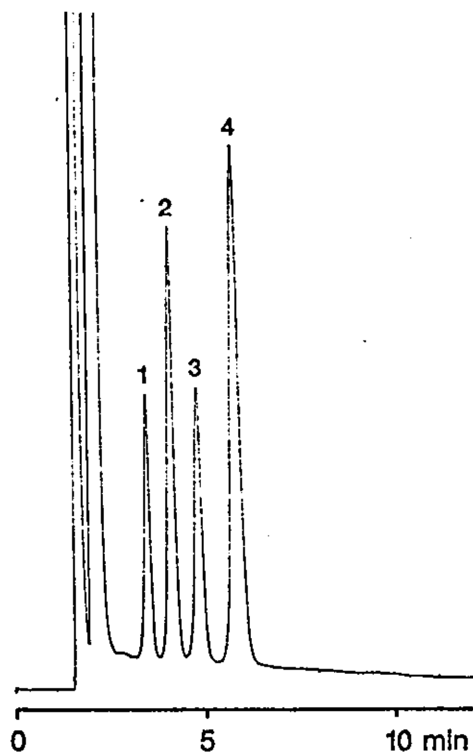


FIGURE 4.18. Separation by SFC of a mixture of alkylbenzenes on PS-DVB column with carbon dioxide mobile phase and flame ionization detection. Conditions: as in Figure 4.16. Peak identification: 1. toluene, 2. ethylbenzene, 3. propylbenzene, 4. butylbenzene.

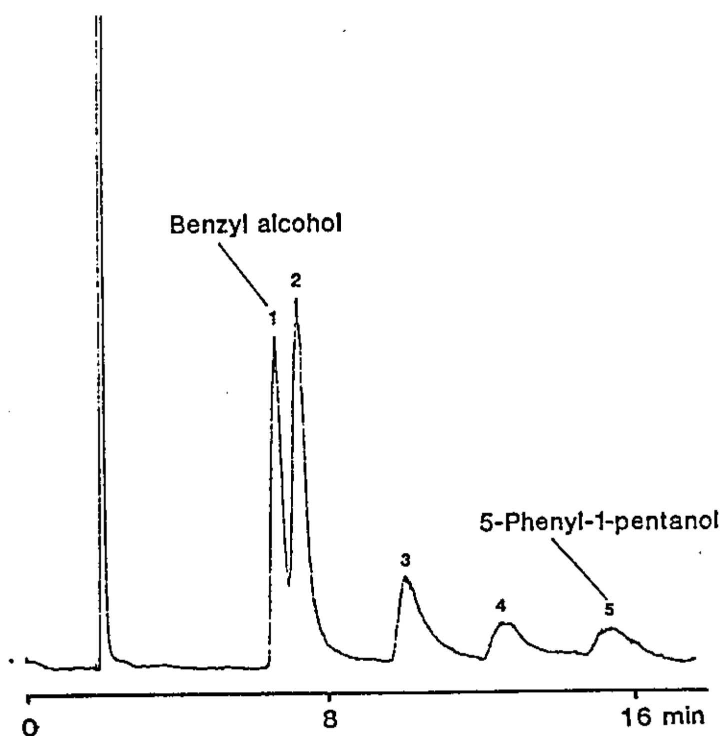


FIGURE 4.19. SFC separation of w-arylalkanols on PS-DVB column with carbon dioxide mobile phase and flame ionization detection. Column PLRP-S 5 μm; mean column pressure 1985 psi; 60 °C.

4.7.3 Amines

The amines are strongly basic compounds. In GC and HPLC these compounds often give problems with irreversible adsorption on the silica surface or peaks exhibiting extreme tailing [144,148]. Derivatization of the amino groups for example, as acyl derivatives have been recommended in GC [149].

There have been suggestions that the analysis of primary or secondary amines can create problems with carbon dioxide as the mobile phase because supercritical carbon dioxide can react with the amino group to form carbamates which are not soluble in supercritical carbon dioxide [149]. In this study, two aromatic amines were successfully separated on the polymer columns (Figure 4.20). and they gave good peak shapes. The reason was probably that the aromatic amines are weak bases and reactions of these compounds with carbon dioxide has not occurred to a considerable extent or that the reaction simply has not taken place in the packed column SFC. This is in accordance with a recent report which pointed out that carbon dioxide has poor affinity for moderately basic aromatic amines (e.g. aniline, $K_b = 4.2 \times 10^{-10}$) to form salts as compared to stronger bases such as ammonia ($K_b = 10^{-5}$) and aliphatic amines ($K_b = 10^{-4}$) which do form salts with carbon dioxide [150]. Subsequently, the more polar benzylamine was also separated (Chapter 5).

4.7.4 Fatty acid and the esters

Glycerides, lipids, and fatty acids are important substances in natural products, food products, bacterial identification, etc. [149]. These group of compounds are generally difficult to detect using

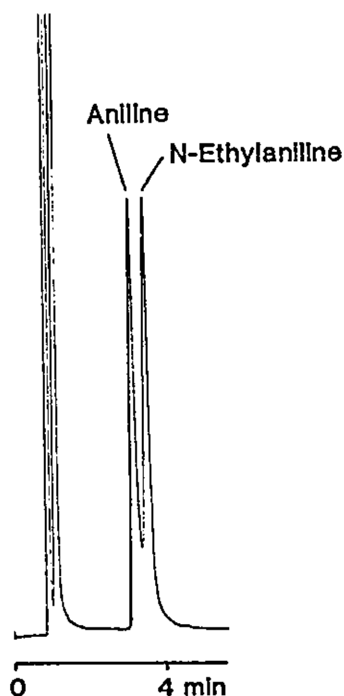


FIGURE 4.20. Separation of amines on PS-DVB column with carbon dioxide mobile phase and flame ionization detection. Column PLRP-S 5 µm; mean column pressure 2725 psi; flow rate 1160 ml/min (gas); 60 °C.

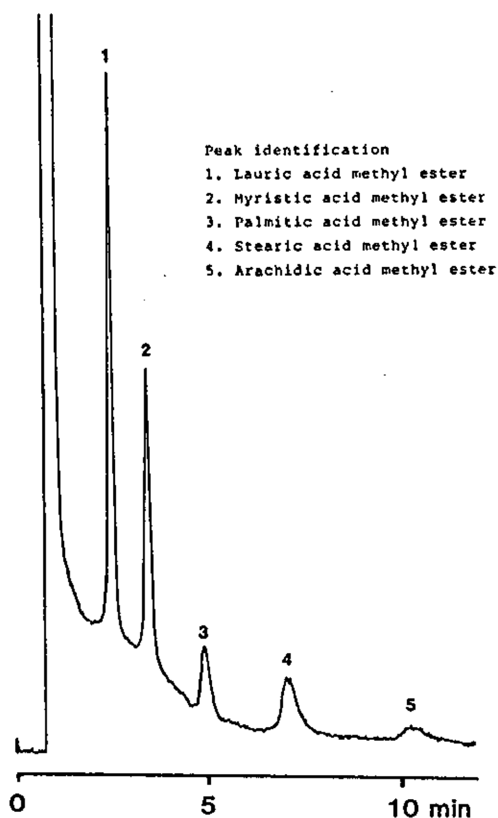


FIGURE 4.21. SFC separations of fatty acid methyl esters on PS-DVB column with carbon dioxide mobile phase and flame ionization detection. Column PLRP-S 5 µm; mean column pressure 2490 psi, 60 °C.

spectroscopic detection methods because of their poor chromophores. In HPLC, lipids can be characterized after hydrolysis and esterification to prepare UV absorbing esters of their fatty acids [151,152]. Because of their high molecular-weight and their polar nature, fatty acids and lipids are not very suitable for analysis by GC, except after prior derivatization to esters.

In order to examine the retention behaviour of this group of compounds, separations were carried out on the PLRP-S column with carbon dioxide as the mobile phase. Several fatty acids methyl esters were chromatographed with supercritical carbon dioxide as the mobile phase (Figure 4.21). These compounds generally gave good peak shapes. The separation of a oleic acid methyl ester and palmitic acid is shown in Figure 4.22. The ester showed good peak shape but the fatty acid showed poor peak shape and was much more retained which indicates the selectivity of the column towards polar compounds. The fatty acid also tended to give problems with flame ionization detection which were characterized by spiky signals.

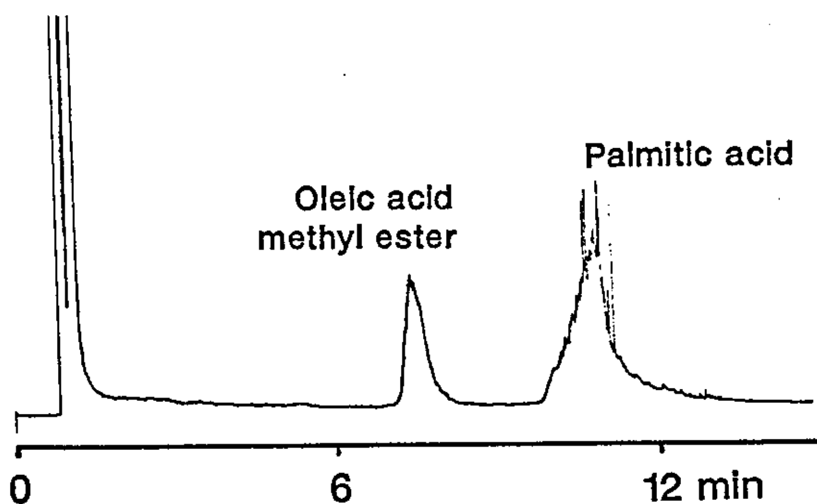


FIGURE 4.22. SFC separation of oleic acid methyl ester and palmitic acid on PS-DVB column and flame ionization detection. Column PLRP-S 5 µm; mean column pressure 2510 psi; 60 °C.

4.8 EFFECT OF MODIFIER IN SFC WITH FLAME IONIZATION DETECTION

Previous reports [70,72] have suggested the potential addition of small amounts of organic modifier (e.g., methanol), as modifier to the carbon dioxide mobile phase can give better separation of polar compounds. However, on trial runs, this caused problems with the background signal from the flame ionization detection (see Section 4.3.7). Further detailed studies on the effect of modifiers were subsequently carried out when a UV detector was available and are described in the next chapter.

CHAPTER 5

SELECTIVITY IN SUPERCRITICAL FLUID CHROMATOGRAPHY

5.1 INTRODUCTION

Good instrumentation is a prerequisite for performing sound chromatographic separations. However, good instrumentation alone will not provide the required separation without a sensible choice of mobile and stationary phases and operating conditions. In SFC, the closely related variables of density, temperature, and pressure, as well as the mobile phase and stationary phase can all affect the retention of the solute. It is therefore necessary to understand the retention behaviour of the solutes in order to optimize the supercritical fluid chromatographic separation in a similar manner to GC and HPLC [18,153]. At present, however, very limited information on solute retention behaviour in SFC is available.

In GC the separation selectivity is primarily a function of the column stationary phase and the volatility of the solute. In HPLC the selectivity and resolution of the separation depends primarily on the specific composition of the mobile phase, whereas the retention times depend heavily on the overall polarity of the mobile phase. As has been mentioned previously (Section 1.1), SFC is expected to show characteristics of both GC and HPLC. The main aim of the present work was to investigate how solute retention and selectivity behave in SFC compared to the other techniques.

The establishment of retention indices in SFC would be valuable to act as a comparison scale for relative retention properties and could be

useful for characterizing different stationary phases. If retention indices are largely independent of small variations in operating conditions, results of analyses measured in one laboratory could be more readily directly compared with those from another laboratory. Moreover, retention indices can be used as a qualitative identification method, complimentary with structural identification detection methods such as SFC-MS and SFC-FTIR.

5.2 RETENTION INDICES

In GC and HPLC the use of relative measurements is well established as a method to reduce the effects of small changes in the operating conditions. These measurements can either be relative to a single standard or to a retention index scale of a series of homologous standard compounds.

In GC an excellent retention index system has been proposed by Kovats, [154] which is theoretically based on the linear relationship between the logarithm of the retention volume and the number of carbon atoms in a homologous series of n-alkanes under isothermal operating conditions ($\log k' = A \times Cn + B$). The retention index value being defined as the carbon number of the n-alkane x 100 and the values for test compounds being calculated by interpolation between standards. A similar concept has also been used in reversed phase HPLC but usually alkylarylketones [155], 2-alkanones [156], or 1-nitroalkanes [157] have been used as standard compounds as the alkanes are highly retained compared to most analytes and the n-alkanes cannot be detected by commonly used spectroscopic detectors.

In SFC, as in HPLC, capacity factors have been primarily used as

the method for recording retentions but these values are prone to problems in the determination of the column void volume [19]. This is partly because of the measured volume often includes any extra column dead volume of the instrument such as the connections, which will differ between experimental systems. Although in theory, capacity factors should be independent of flow rates and column dimensions, in practice they vary significantly between laboratories even using the same column materials and this can lead to poor interlaboratory reproducibility [19]. Another problem in HPLC is the lack of a standard method for measurement of the void volume [158]. In SFC the measurement of the column void volume will present similar problems. A number of nominally unretained compounds have been used as dead volume markers including dichloromethane [71,74], benzene [34,159], pentane [64], and acetone [65,88] but as in HPLC, no standard method has been adopted. In addition some workers have based their measurements on the peak maximum and some on the leading edge of the peak [69].

The present study investigates the application of retention indices either based on alkylarylketones or n-alkanes for use in SFC to monitor separations using porous PS-DVB copolymer and ODS- and cyano-bonded silica columns. So far little use has been made of retention indices in SFC except for a study by Wright [76] who interpolated between n-decane and n-pentadecane to calculate retention indices of acetophenone, N-ethylaniline, naphthalene, 1-decanol, and p-chlorophenol test compounds.The study examined the retention and selectivity effects of various mobile phases (carbon dioxide, nitrous oxide, ethane, and methanol-modified carbon dioxide) using capillary columns. Preliminary notes from this laboratory have described the application of retention indices based on alkylarylketones and n-alkanes to the comparison of the

separation of barbiturates on columns packed with ODS-Spherisorb and PLRP-S stationary phases [65], and a detailed investigation of retention indices in SFC [160,161] and their application to the monitoring of selectivity under various operating conditions [161] have been reported.

5.2.1 Establishment of a retention index scale

The selection of a retention index scale for SFC separations will depend on similar criteria to those in HPLC [155] of which the most important are their similarity to the range of analyte retentions and the compatibility with the detector. In contrast to reversed phase HPLC separations on the same columns, the retentions of hydrocarbons in SFC with supercritical carbon dioxide as the eluent are much shorter compared to the retentions of more polar analytes (see later Sections). This marked difference in selectivity is presumably because the carbon dioxide mobile phase is a much stronger solvent compared to the stationary phase and retention is on the basis of solubility in the mobile phase rather than hydrophobicity. However, the mechanism of retention is not fully understood at present.

For work with a flame ionization detector the obvious choice of standards is the n-alkanes because this gives the possibility of direct comparison with GC retention. As in GC there is also the advantage that a large number of n-alkane homologues are readily available and they are chemically inert and stable on storage. However, it has been found that for any polar analytes, carbon dioxide is unsuitable as a mobile phase in SFC unless an organic modifier such as methanol or acetonitrile is added (Section 5.5). In this case the background signal of a flame ionization detector can become very noisy and the sensitivity will be

considerably reduced. For most samples it will therefore be necessary to use spectroscopic detection with a high-pressure flow cell and the alkanes will not be detectable. An alternative series of unsaturated standards such as the alkylarylketones, which have been used in HPLC, or the alkylbenzenes will thus be needed [19].

In both cases, it is first necessary to confirm that under isobaric conditions there is a linear relationship between log of capacity factor values for the standards and their carbon numbers. The capacity factors of a series of alkylarylketones from acetophenone to octanophenone, hydrocarbons from dodecane to tetracosane, and alkylbenzenes from toluene to butylbenzene were therefore examined on a PLRP-S poly(styrene-divinylbenzene) column and the relationship between the log capacity factors and the retention indices (carbon number x 100) were determined (Table 5.1). Representative plots of log capacity factors vs. carbon number x 100 for the three homologous series are illustrated in Figure 5.1. There was a close correlation for the n-alkanes (coefficients > 0.9992) indicating their suitability as a retention index scale. Less linear relationships were obtained for the alkylarylketones and alkylbenzenes (coefficients > 0.9959). These results were comparable to those reported by Morin et al. [64] who obtained a correlation coefficient greater than 0.995 for the SFC separations of homologous 2-alkanones (2-pentanone, 2-hexanone, 2-heptanone, 2-nonanone, 2-decanone, and 2-undecanone) on PS-DVB (PRP-1) polymer column with carbon dioxide as the mobile phase.

The plots of log capacity factors vs. carbon number x 100 of the alkylarylketones (and n-alkanes, for comparison) suggested that much of the deviation was due to acetophenone and propiophenone and this deviation appeared to occur consistently over a range of operating

TABLE 5.1. Relationship between log capacity factors and retention indices (carbon number x 100) for alkylarylketones, n-alkanes, and alkylbenzenes on PS-DVB column at different pressures.

Conditions: column (150 x 4.6 mm i.d.), PLRP-S 5 μm; carbon dioxide mobile phase; flame ionization detection; temperature 60 °C.

Mean column pressure (psi)	Density (g/ml)	Correlation coefficient	Slope x 10^{-4}	Intercept
Alkylarylketones (acetophenone - octanophenone) Number of points: 7				
2010	0.557	0.9974	9.2410	-0.3036
2260	0.625	0.9969	8.4229	-0.3348
2515	0.673	0.9965	7.8787	-0.3664
2765	0.707	0.9959	7.4810	-0.3924
Alkylarylketones (butyrophenone - octanophenone) Number of points: 5				
2010	0.557	0.9995	9.8752	-0.3842
2260	0.625	0.9995	8.9879	-0.4068
2515	0.673	0.9994	8.3801	-0.4304
2765	0.707	0.9994	7.9628	-0.4540
n-Alkanes (dodecane - tetracosane) Number of points: 7				
2010	0.557	0.9997	10.4613	-1.0165
2260	0.625	0.9995	9.6038	-1.0229
2515	0.673	0.9993	9.0186	-1.0292
2765	0.707	0.9992	8.5737	-1.0305
Alkylbenzenes (toluene - butylbenzene) Number of points: 4				
2010	0.557	0.9989	10.0524	-0.7095
2260	0.625	0.9986	9.6537	-0.7121
2515	0.673	0.9986	9.0162	-0.7125
2765	0.707	0.9988	8.4661	-0.7068

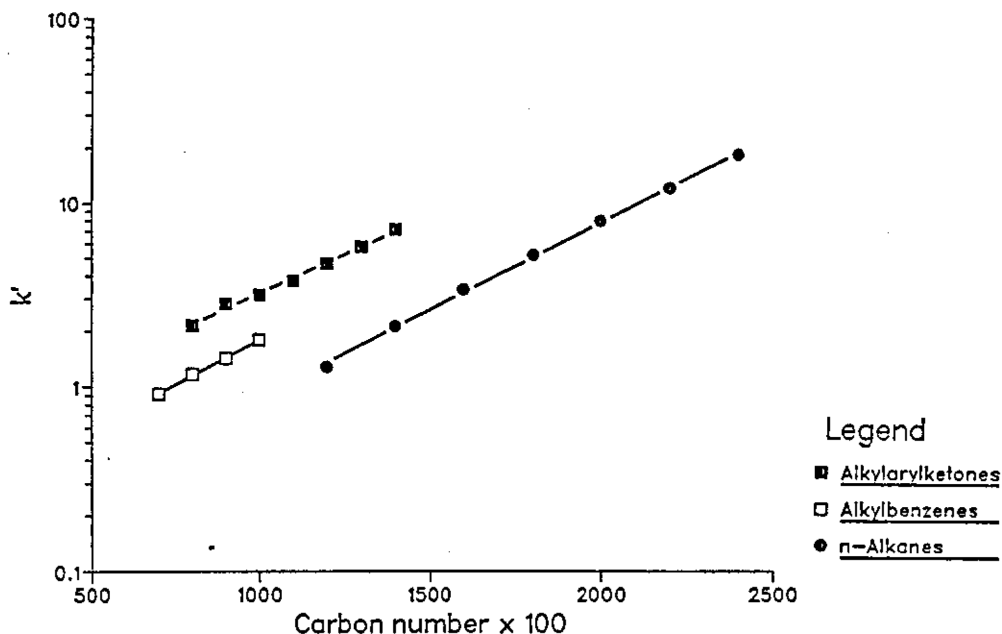


FIGURE 5.1. Relationship between log capacity factors and retention indices (carbon number x 100) for alkylarylketones, n-alkanes, and alkylbenzenes on PS-DVB column with carbon dioxide mobile phase. Column PLRP-S 5 μm; mean column pressure 2260 psi; 60 °C.

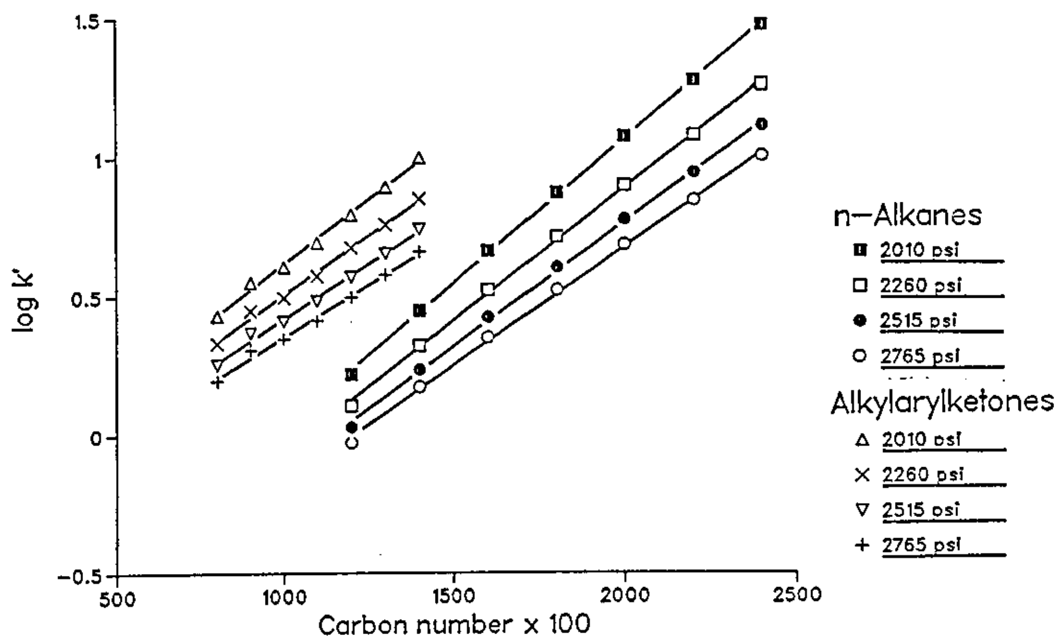


FIGURE 5.2. Variation of log capacity factors with carbon number x 100 for alkylarylketones and n-alkanes on PS-DVB column at different pressures with carbon dioxide mobile phase and 60 °C.

pressures (Figure 5.2). If these two homologues were omitted from the least squares calculations, the correlation was significantly better (> 0.9994) and comparable to the n-alkanes (Table 5.1). This anomalous behaviour of the alkylarylketones is clearly demonstrated further by the retention index (RI) values (see Section 5.3.2, Table 5.10) in which the larger ketones all gave retention indices close to the nominal values (carbon number x 100) but the two ketones, acetophenone (RI = 817 to 827) and propiophenone (RI = 949 to 959) showed marked deviations and thus should probably not included in the set of retention index compounds. These ketones also showed deviations when their separations were examined in reversed phase HPLC on this PS-DVB materials [158] although the effects were larger in that case for acetophenone.

For different homologous series, if the addition concepts of chromatographic retention is correct, the methylene increment (increase in log k' with the addition of a $-CH_2-$ group) should be constant and thus the slope of the correlations should be the same. However, it is of interest to point out that small differences were found between the different series (Table 5.1). The slopes of the correlations for the alkylarylketones, n-alkanes, and alkylbenzenes were similar but not identical in each case suggesting that the addition of a methylene group was not having an equal effect on the retention of the three homologous series. Small differences in slope have also been observed in separations under different operating conditions (see below) and similar small differences have also been found in HPLC comparisons [155,158].

The least squares analyses of the plots of log capacity factors vs. retention indices (carbon number x 100) of the alkylarylketones, n-alkanes, and alkylbenzene at different operating temperatures ranging from 40 to 100 °C gave linear relationship (Table 5.2). In each case,

TABLE 5.2. Relationship between log capacity factors and retention indices (carbon number x 100) for alkylarylketones, n-alkanes, alkylbenzenes at different temperatures.

Conditions: column PLRP-S 5 µm; carbon dioxide mobile phase; mean column pressure 2515 psi; flame ionization detection.

Temperature (°C)	Density (g/ml)	Correlation coefficient	Slope x 10^{-4}	Intercept
Alkylarylketones (acetophenone - octanophenone) Number of points: 7				
40	0.811	0.9951	7.5711	-0.4138
60	0.673	0.9965	7.8787	-0.3664
80	0.519	0.9976	8.9058	-0.3256
100	0.406	0.9985	10.4178	-0.3043
Alkylarylketones (butyrophenone - octanophenone) Number of points: 5				
40	0.811	0.9992	7.9618	-0.4643
60	0.673	0.9994	8.3801	-0.4304
80	0.519	0.9996	9.5468	-0.4077
100	0.406	0.9998	11.1047	-0.3911
n-Alkanes (dodecane - tetracosane) Number of points: 7				
40	0.811	0.9994	8.5093	-0.9979
60	0.673	0.9993	9.0186	-1.0292
80	0.519	0.9997	10.1674	-1.0440
100	0.406	0.9998	11.6618	-1.0271
Alkylbenzenes (toluene - butylbenzene) Number of points: 4				
40	0.811	0.9984	8.2835	-0.6668
60	0.673	0.9986	9.0162	-0.7125
80	0.519	0.9993	10.1443	-0.7277
100	0.406	0.9994	12.1377	-0.7978

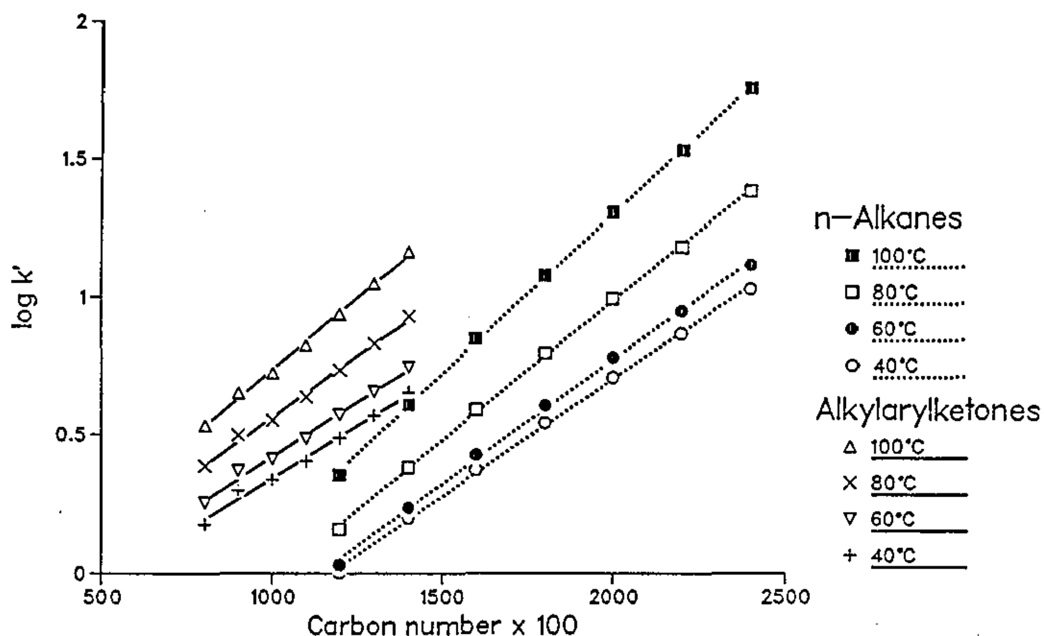


FIGURE 5.3. Variation of log capacity factors with carbon number x 100 for alkylarylketones and n-alkanes on PS-DVB column at different temperatures. Carbon dioxide mobile phase. Column PLRP-S 5 µm; mean column pressure 2515 psi; 60 °C.

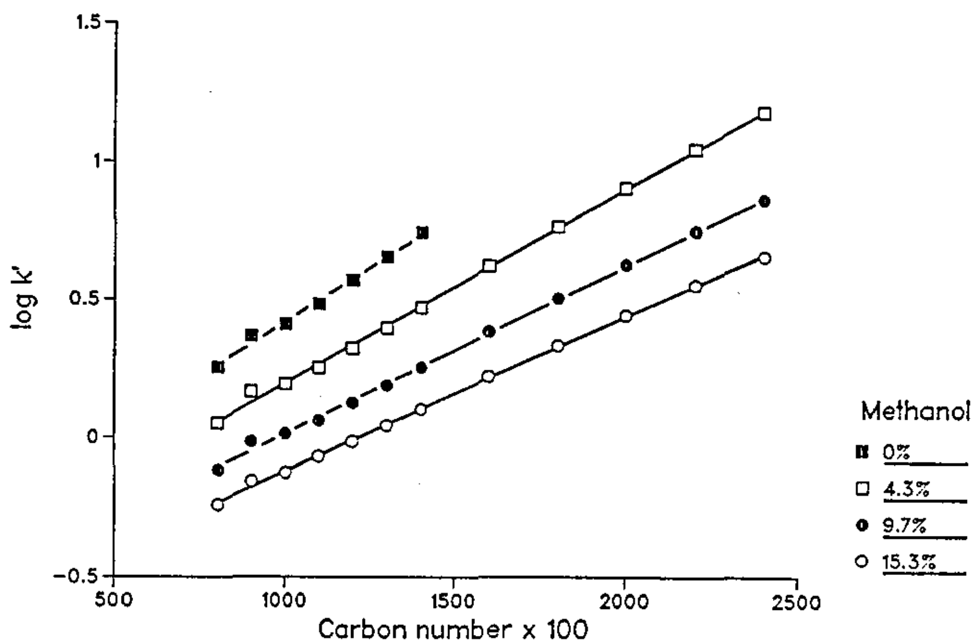


FIGURE 5.4. Variation of log capacity factors with carbon number x 100 for alkylarylketones on PS-DVB column with different methanol concentrations. Column PLRP-S 5 µm; primary eluent carbon dioxide; mean column pressure 2515 psi; 60 °C.

similar correlation was observed to those obtained at different pressures (see above). It is interesting to note that for each series, the correlation became greater as the temperature was increased. Marked deviation was always observed for acetophenone and propiophenone as demonstrated by the plots of log capacity factors vs. carbon number x 100 for this series, whereas the n-alkanes showed better linearity (Figure 5.3). However, correlations comparable to the n-alkanes were obtained if these two compounds were omitted from the calculations (Table 5.2).

SFC separations have also been carried out on the PS-DVB column using a modifier in the mobile phase. Because UV detector had to be used, the n-alkanes were not examined and because the retention were greatly reduced with the use of modifier, a number of higher homologues (decanophenone - octadecanophenone) were also studied. Close correlations of log capacity factors and the carbon number for the alkylarylketones and alkylbenzenes were observed with methanol (Table 5.3) or acetonitrile (Table 5.4) as the modifier. Deviation was observed consistently for acetophenone and particularly more markedly for propiophenone, at different modifier concentrations. This was clearly demonstrated by the separations using methanol as the modifier over the concentration range of 0-15.3% (Figure 5.4). With these two compounds omitted from the least squares calculations, correlations greater than 0.9998 and 0.9995 were obtained for methanol and acetonitrile as the modifier, respectively.

In order to further investigate the linearity of the relationship between log capacity factors and carbon number for the homologous series, the corresponding set of experiments were carried out using two ODS-silica columns. Over a pressure range of 1665 to 2470 psi with

TABLE 5.3. Relationship between log capacity factors and retention indices (carbon number x 100) for alkylarylketones and alkylbenzenes on PS-DVB column at different methanol concentrations.

Conditions: column, PLRP-S 5 μm; primary mobile phase, carbon dioxide; mean column pressure 2515 psi; 60 °C; UV detection, 254 nm.

Methanol (% w/w)	Correlation Coefficient	Slope $\times 10^{-4}$	Intercept
Alkylarylketones (acetophenone - octadecanophenone) Number of points: 12			
0*	0.9965	7.8787	-0.3664
4.3	0.9993	7.0580	-0.5072
9.7	0.9993	6.1074	-0.5955
15.3	0.9997	5.6176	-0.6807
Alkylarylketones (butyrophenone - octadecanophenone) Number of points: 10			
0**	0.9994	8.3801	-0.4304
4.3	0.9999	7.1713	-0.5288
9.7	0.9998	6.1787	-0.6092
15.3	0.9999	5.6465	-0.6863
Alkylbenzenes (toluene - butylbenzene) Number of points: 4			
0	0.9986	9.0162	-0.7125
4.3	0.9983	7.7454	-0.7598
9.7	0.9971	6.4645	-0.7534
15.3	0.9970	5.6055	-0.7133

* Based on acetophenone - octanophenone, n = 7.
** Based on butyrophenone - octanophenone, n = 5.

TABLE 5.4. Relationship between log capacity factors and retention indices (carbon number x 100) for alkylarylketones and alkylbenzenes on PS-DVB column at different acetonitrile concentrations.

Conditions: column, PLRP-S 5 μm; primary mobile phase, carbon dioxide; 60 °C; mean column pressure 2515 psi; UV detection, 254 nm.

Acetonitrile (% w/w)	Correlation coefficient	Slope $\times 10^{-4}$	Intercept
Alkylarylketones (acetophenone - octadecanophenone) Number of points: 12			
0*	0.9965	7.8787	-0.3664
4.3	0.9991	6.5948	-0.5370
9.0	0.9994	5.5668	-0.6517
14.0	0.9992	4.9042	-0.8086
Alkylarylketones (butyrophenone - octadecanophenone) Number of points: 10			
0**	0.9994	8.3801	-0.4304
4.3	0.9995	6.6656	-0.5506
9.0	0.9997	5.5951	-0.6573
14.0	0.9995	4.8738	-0.8031
Alkylbenzenes (toluene - butylbenzene) Number of points: 4			
0	0.9986	9.0162	-0.7125
4.3	0.9986	7.2093	-0.7768
9.0	0.9963	5.7176	-0.7609
14.0	0.9923	4.8725	-0.7947

* Based on acetophenone - octanophenone; n = 7.

** Based on butyrophenone - octanophenone; n = 5.

carbon dioxide as the mobile phase, the compounds were found to be generally less retained on the ODS-silica than on a PS-DVB column and in some cases, the first few smaller members of the alkylarylketone series (acetophenone, propiophenone, and butyrophenone) were not fully resolved and thus they were not included in the calculations (Table 5.5). Close correlations were observed for the alkylarylketones, n-alkanes and alkylbenzenes over the pressure range (Table 5.5), again indicating their potential use as retention index standards. The log capacity factors for the alkylarylketones, valerophenone to octadecanophenone were plotted against the carbon number x 100 and they showed good linearity (Figure 5.5). Similar plots of data obtained using a Spherisorb ODS-2 column under different pressure conditions for acetophenone - heptanophenone (Figure 5.5) showed marked deviations from linearity for acetophenone and propiophenone. However, unlike on the PS-DVB column, acetophenone showed more marked reduced retention than predicted from the least squares fit, whereas propiophenone appeared to show significantly less deviation.

The alkylarylketones showed a close correlation between log capacity factor and the carbon number on elution with methanol (Table 5.6) or acetonitrile (Table 5.7) as the modifier and coefficients greater than 0.9972 were achieved in each case. The correlations for both series decreased with increasing percentage modifier in the mobile phase, (e.g., alkylbenzenes with acetonitrile as the modifier, correlation coefficients = 0.9992 to 0.9665). This is probably mainly because of the resulting decrease in retention and resolution of the homologues as the concentration of the modifier in the eluent was increased and consequent difficulty in accurately measuring the retention time. The log capacity factor values were plotted against the

TABLE 5.5. Relationship between log capacity factors and retention indices (carbon number x 100) for alkylarylketones, n-alkanes, and alkylbenzenes on ODS-silica columns at different pressures.

Conditions: carbon dioxide mobile phase; 60 °C; UV (254 nm) and flame ionization detection.

Mean column pressure (psi)	Density (g/ml)	Correlation Coefficient	Slope x 10^{-4}	Intercept
Alkylarylketones (valerophenone - octadecanophenone) Number of points: 9				
1665	0.395	0.9999	9.5078	-0.6760
1950	0.535	0.9999	8.1040	-0.7420
2160	0.601	0.9998	7.2828	-0.8077
2470	0.666	0.9998	6.9435	-0.8545
Alkylarylketones (acetophenone - heptanophenone)* Number of points: 6				
1580	0.352	0.9939	8.8099	-0.5221
1780	0.458	0.9907	8.0501	-0.7047
Alkylarylketones (valerophenone - heptanophenone)* Number of points: 4				
1580	0.352	0.9996	9.8586	-0.6486
1780	0.458	0.9974	7.1643	-0.6015
n-Alkanes (decane - tetracosane) Number of points: 8				
1665	0.395	0.9986	10.0083	-0.9801
1950	0.535	0.9984	8.6055	-1.0302
2160	0.601	0.9978	7.7774	-1.0520
2470	0.666	0.9978	7.3154	-1.0593
Alkylbenzenes (toluene - butylbenzene) Number of points: 4				
1665	0.395	0.9992	12.8822	-1.2337
1950	0.535	0.9992	11.4664	-1.2655
2160	0.601	0.9998	10.5683	-1.2915
2470	0.666	0.9992	11.3673	-1.4743

Column: Ultrasphere ODS 5 µm, (250 x 4.6 mm i.d.), except those marked with asterisks (*) which denote Spherisorb ODS-2, 5 µm (200 x 3 mm i.d.).

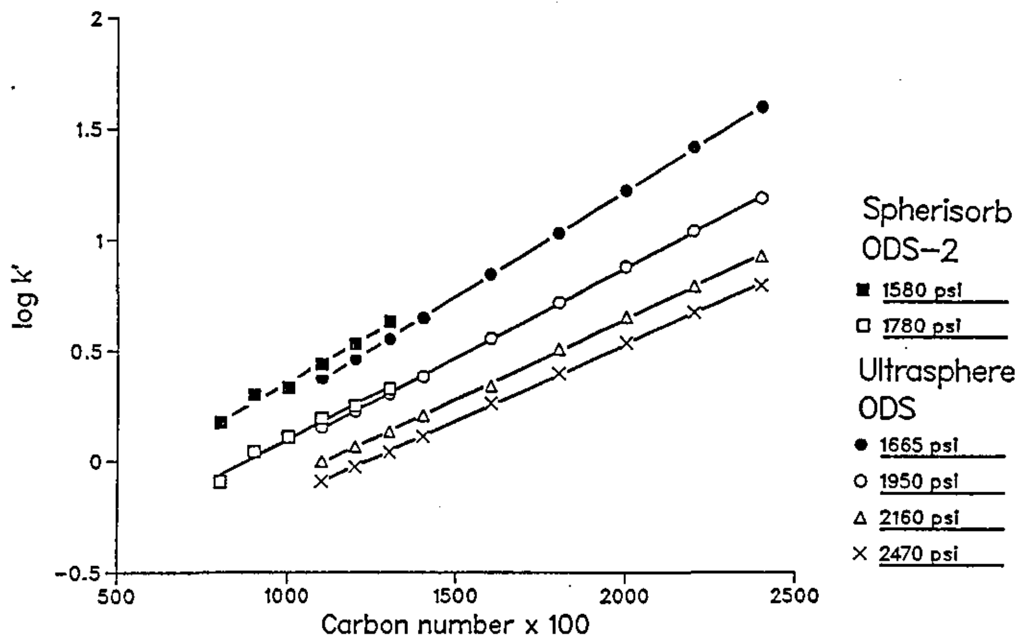


FIGURE 5.5. Variation of log capacity factors with carbon number x 100 on ODS-silica columns for alkylarylketones at different pressures. Columns: Spherisorb ODS-2 5 μm and Ultrasphere ODS 5 μm; carbon dioxide mobile phase; 60 °C.

TABLE 5.6. Relationship between log capacity factors and retention indices (carbon number x 100) for alkylarylketones and alkylbenzenes on ODS-silica column at different methanol concentrations.

Conditions: column, Ultrasphere ODS, 5 μm; primary eluent: carbon dioxide; mean pressure: 2470 psi; 60 °C; UV detection, 254 nm.

Methanol (% w/w)	Correlation coefficient	Slope $\times 10^{-4}$	Intercept
Alkylarylketones (valerophenone - octadecanophenone)			
Number of points: 9			
0	0.9998	6.9435	-0.8545
4.0	0.9991	6.2858	-1.0269
8.3	0.9987	5.8521	-1.1257
12.7	0.9987	5.6183	-1.1688
16.4	0.9972	5.6838	-1.2757
Alkylarylketones (acetophenone - octadecanophenone)*			
Number of points: 12			
4.2	0.9991	7.0389	-0.9746
8.4	0.9989	6.1740	-1.0614
Alkylarylketones (butyrophenone - octadecanophenone)*			
Number of points: 10			
4.2	0.9996	6.9295	-0.9544
8.4	0.9993	6.0561	-1.0395
Alkylbenzenes (toluene - butylbenzene)			
Number of points: 4			
0	0.9992	11.3673	-1.4743
4.0	0.9998	8.3026	-1.2087
8.3	0.9896	5.9658	-1.0524
12.7	0.9878	4.7607	-0.9390

* Mean column pressure, 1950 psi, other conditions the same.

TABLE 5.7. Relationship between log capacity factors and retention indices (carbon number x 100) for alkylarylketones and alkylbenzenes on ODS-silica column at different acetonitrile concentrations.

Conditions: column, Ultrasphere ODS 5 μm; primary eluent, carbon dioxide; 60 °C; mean pressure 2470 psi; UV detection, 254 nm.

Acetonitrile (% w/w)	No. of points	Correlation coefficient	Slope x 10^{-4}	Intercept
Alkylarylketones (valerophenone - octadecanophenone)				
0	9	0.9998	6.9435	-0.8545
3.7	9	0.9993	6.2556	-1.0589
7.1	9	0.9983	5.8852	-1.2031
13.4	9	0.9972	5.9374	-1.3401
Alkylbenzenes (toluene - butylbenzene)				
0	4	0.9992	11.3673	-1.4743
3.7	4	0.9997	8.8025	-1.3044
7.1	3	0.9835	4.8623	-0.9996
13.4	3	0.9665	5.4598	-1.0983

TABLE 5.8. Relationship between log capacity factors and retention indices (carbon number x 100) for alkylarylketones on cyano-silica column at different pressures.

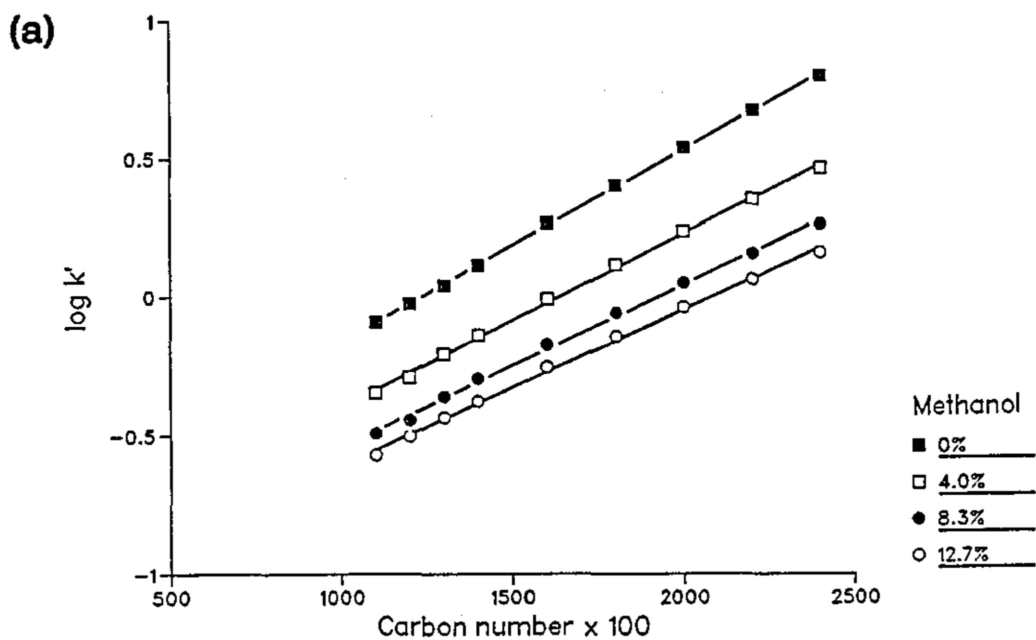
Conditions: column, Ultrasphere CN, 5 μm (250 x 4.6 mm i.d.); carbon dioxide mobile phase; 60 °C; UV and flame ionization detection.

Mean column pressure (psi)	Density (g/ml)	Correlation coefficient	Slope x 10^{-4}	Intercept
Alkylarylketones (heptanophenone - octadecanophenone) Number of points: 7				
1945	0.533	0.9986	3.5348	0.3482
2200	0.611	0.9987	2.7896	0.3102
2460	0.664	0.9983	2.3068	0.2565
2735	0.704	0.9972	1.9303	0.2504

carbon number x 100 for valerophenone - octadecanophenone (Figure 5.6a) and acetophenone - octadecanophenone (Figure 5.6b) at various methanol concentrations. Marked deviation was observed for acetophenone (Figure 5.6b) but significantly less deviation was seen for propiophenone. This is reflected by the retention index values for these compounds: acetophenone, RI = 726 and 722 (nominally 800); propiophenone, RI = 902 and 888 (nominally 900))(see Chapter 6, Table 6.8)

The separations of the homologous series have also been carried out on cyano-silica packing materials. On the cyano-silica column, with supercritical carbon dioxide as the eluent, the n-alkanes, the alkylbenzenes and the smaller alkylarylketones were very poorly retained and unresolved. Particularly anomalous retention behaviour was observed for acetophenone (see Section 5.6). In these cases, retention index calculations were therefore based on the larger alkylarylketones, heptanophenone - octadecanophenone and in each case a close correlation (> 0.9972) was obtained (Table 5.8).

In conclusion, it has been shown that the alkylarylketone, n-alkane, and alkylbenzene homologous series generally give good linear relationships between the log capacity factors and the carbon number under various conditions of separation, thus confirming their predictable retention behaviour. Marked deviation was observed for acetophenone and propiophenone, and if these compounds were omitted from the least squares calculations, better correlations (comparable to the n-alkanes) were obtained. Using the results it would be possible to calculate the retention indices based either on alkylarylketones, n-alkanes or alkylbenzenes. Although the correlation of n-alkanes is more linear, it is more limited in its application particularly if modifiers are present in the eluent. Furthermore, on a polar column,



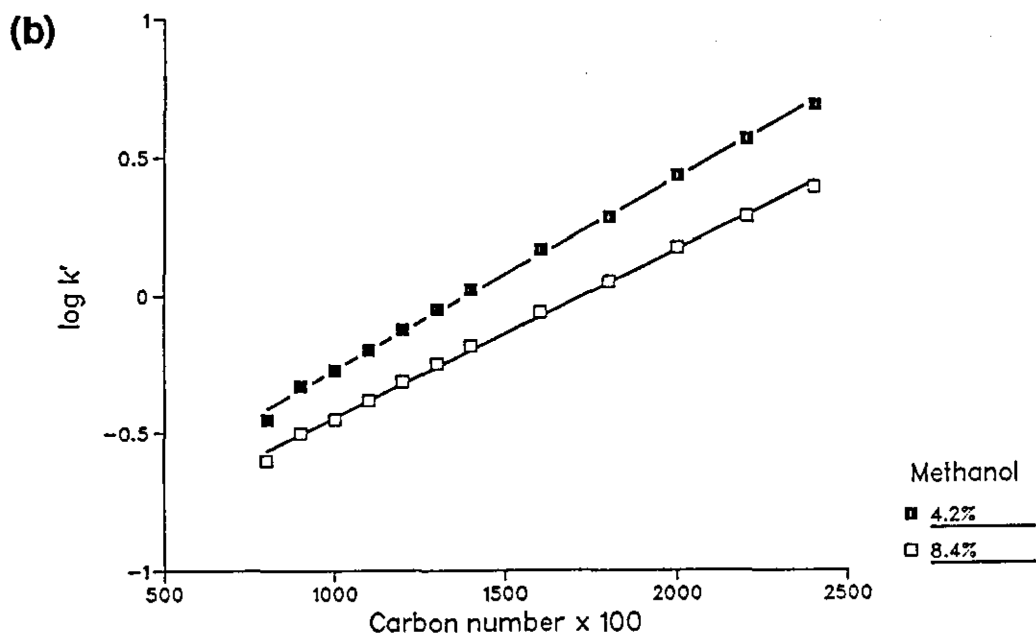


FIGURE 5.6. Variation of log capacity factors with carbon number x 100 for alkylarylketones on ODS-silica column at different methanol concentrations. Conditions: column Ultrasphere ODS 5 μm; 60 °C; mean column pressure: (a) 2470 psi (b) 1950 psi.

such as cyano-silica with supercritical carbon dioxide as the main eluent, the alkanes and the alkylbenzenes show very little retention and often coeluted with the solvent peak (see later Sections) and thus, in such cases they are unsuitable as retention index scales. Therefore, in the majority of this study, retention indices have been based on alkylarylketones with the omission of acetophenone and propiophenone (if present) from the calculation and in each case for the compounds used there was a close relationship.

5.3 SELECTIVITY AND RETENTION ON PLRP-S COLUMN

In HPLC, PS-DVB and ODS-silica column materials showed considerable selectivity differences. The former showed a relatively much lower retention for analytes which contained hydroxy or phenolic groups compared to less polar compounds [162]. Therefore, it was of interest to examine the retention characteristics of these columns under SFC conditions.

5.3.1 Model compounds

A set of model compounds were selected from compounds with different functional groups, which have been found to be characteristic of different interactions on HPLC [28,145,155]. Besides the alkylarylketones, n-alkanes, and alkybenzenes, six other compounds, benzaldehyde, methyl benzoate, benzyl alcohol, nitrobenzene, p-cresol, and N-propylaniline have been used. Three other compounds, benzoic acid, benzamide, and benzylamine were selected although these have not been commonly used as column test compounds in reversed phase HPLC mainly

because of their sensitivity to pH changes [28]. All the models are aromatic compounds which can be easily detected with a spectroscopic detector.

5.3.2 Effect of pressure

In general, the retention of a compound in SFC decreases with increases in pressure (Table 5.9). Typical plots of capacity factors vs. pressure for the SFC separation of alkylarylketones and alkylbenzenes are illustrated in Figure 5.7. The relationship between log capacity factor and pressure is nearly linear (Figure 8.8). This is because the solvating power, which is roughly proportional to density, increases non-linearly with increases in pressure (see Figure 1.3). The retention of the compounds in a homologous series did not change relative to each other with changes in pressure as illustrated by the well spaced (and almost linear) plots of log capacity factors vs. pressure for alkylarylketones and alkylbenzenes. The retention behaviour of alkylbenzenes was very similar to that of alkylarylketones suggesting that similar factors affected the alkylbenzene and alkylarylketone groups. However, other groups of compounds may exhibit different retention behaviours and selectivities.

In order to determine the selectivity changes on the PLRP-S column with pressure, the retention indices of the alkanes, alkylarylketones, alkylbenzenes, and the model compounds based on alkylarylketones were determined (Table 5.10). The retention indices of alkylbenzenes were virtually constant with increases in pressure (Figure 5.9). However, the n-alkanes showed a decrease in retention indices as the pressure increased. This indicates that the n-alkanes were selectively less

TABLE 5.9. Capacity factors of alkylarylketones, n-alkanes, alkylbenzenes, and model compounds on PS-DVB column at different column pressures.

Conditions: column, PLRP-S 5 μm; carbon dioxide mobile phase; 60 °C; flame ionization detection.

Compound	Capacity factor			
	Inlet pressure (psi)			
	2050	2320	2580	2850
	Outlet pressure (psi)			
	1970	2200	2450	2680
	Mean pressure (psi)			
	2010	2260	2515	2765
	Calculated density (g/ml)			
	0.557	0.625	0.673	0.707
Acetophenone	2.71	2.15	1.80	1.57
Propiophenone	3.58	2.82	2.34	2.04
Butyrophenone	4.07	3.15	2.59	2.23
Valerophenone	4.98	3.77	3.06	2.61
Hexanophenone	6.27	4.67	3.73	3.16
Heptanophenone	7.90	5.76	4.56	3.81
Octanophenone	10.07	7.16	5.57	4.61
Dodecane	1.67	1.28	1.07	0.94
Tetradecane	2.83	2.12	1.73	1.50
Hexadecane	4.65	3.37	2.68	2.27
Octadecane	7.55	5.23	4.05	3.37
Eicosane	12.07	8.04	6.05	4.91
Docosane	19.14	12.19	8.95	7.11
Tetracosane	30.42	18.57	13.20	10.29
Toluene	1.05	0.91	0.82	0.76
Ethylbenzene	1.39	1.17	1.04	0.95
Propylbenzene	1.71	1.42	1.24	1.12
Butylbenzene	2.20	1.79	1.55	1.38
Benzaldehyde	2.07	1.71	1.51	1.37
Methyl benzoate	2.42	1.96	1.70	1.54
Benzyl alcohol	2.97	2.40	2.13	1.89
Nitrobenzene	3.29	2.65	2.31	2.05
p-Cresol	4.49	3.63	3.25	2.92
N-Propylaniline	4.72	3.81	3.31	2.95
Benzoic acid	8.41	6.75	5.61	4.88
Benzamide	13.52	10.38	8.70	7.57
Benzylamine	24.62	17.83	14.26	12.30

TABLE 5.10. Retention indices of alkylarylketones, n-alkanes, alkylbenzenes, and model compounds based on alkylarylketone standards on PS-DVB column at different pressures.

Conditions: as in Table 5.9.

Compound	Retention index*			
	Inlet pressure (psi)			
	2050	2320	2580	2850
	Outlet pressure (psi)			
	1970	2200	2450	2680
	Mean column pressure (psi)			
	2010	2260	2515	2765
	Calculated density (g/ml)			
	0.557	0.625	0.673	0.707
Acetophenone	827	823	819	817
Propiophenone	949	953	955	959
Butyrophenone	1006	1006	1007	1007
Valerophenone	1095	1094	1093	1093
Hexanophenone	1196	1197	1196	1197
Heptanophenone	1298	1299	1299	1299
Octanophenone	1405	1404	1404	1404
Dodecane	614	573	550	538
Tetradecane	847	815	798	790
Hexadecane	1065	1039	1025	1017
Octadecane	1278	1252	1239	1232
Eicosane	1484	1460	1447	1438
Docasane	1687	1661	1649	1640
Tetracosane	1891	1864	1851	1842
Toluene	412	406	411	423
Ethylbenzene	534	530	534	542
Propylbenzene	624	623	626	633
Butylbenzene	737	734	739	747
Benzaldehyde	709	710	725	740
Methyl benzoate	777	777	788	804
Benzyl alcohol	867	876	904	918
Nitrobenzene	913	924	946	962
p-Cresol	1050	1075	1123	1155
N-Propylaniline	1072	1099	1133	1161
Benzoic acid	1325	1375	1407	1435
Benzamide	1534	1583	1634	1674
Benzylamine	1798	1844	1891	1939

* Based on alkylarylketones, butyrophenone - octanophenone.

TABLE 5.11. Retention indices of n-alkanes, alkylarylketones, alkylbenzenes, and model compounds based on n-alkanes standards on PS-DVB column at different pressures.

Conditions: as in Table 5.9.

Compound	Retention index*			
	Inlet pressure (psi)			
	2050	2320	2580	2850
	Outlet pressure (psi)			
	1970	2200	2450	2680
	Mean pressure (psi)			
	2010	2260	2515	2765
	Calculated density (g/ml)			
	0.557	0.625	0.673	0.707
Acetophenone	1385	1412	1425	1431
Propiophenone	1501	1533	1551	1563
Butyrophenone	1554	1583	1600	1608
Valerophenone	1638	1665	1680	1687
Hexanophenone	1734	1762	1776	1784
Heptanophenone	1830	1857	1871	1879
Octanophenone	1930	1956	1968	1976
Dodecane	1184	1178	1175	1172
Tetradecane	1404	1405	1405	1408
Hexadecane	1609	1614	1616	1617
Octadecane	1811	1813	1815	1817
Eicosane	2005	2007	2008	2008
Docosane	2197	2196	2196	2196
Tetracosane	2389	2386	2384	2383
Toluene	994	1022	1046	1065
Ethylbenzene	1107	1138	1161	1176
Propylbenzene	1194	1224	1246	1261
Butylbenzene	1300	1328	1351	1366
Benzaldehyde	1274	1324	1338	1360
Methyl benzoate	1338	1369	1396	1419
Benzyl alcohol	1423	1461	1504	1525
Nitrobenzene	1466	1506	1543	1566
p-Cresol	1595	1648	1708	1745
N-Propylaniline	1616	1671	1717	1750
Benzoic acid	1856	1929	1972	2005
Benzamide	2053	2123	2183	2227
Benzylamine	2302	2368	2421	2473

* Based on n-alkanes, dodecane - tetracosane.

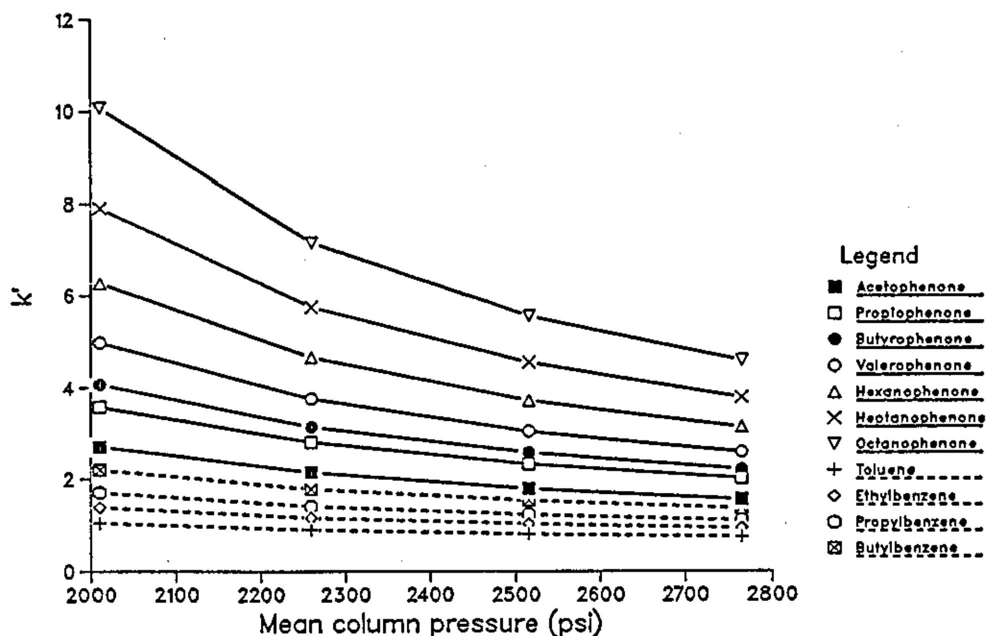


FIGURE 5.7. Variation of capacity factors as a function of pressure for alkylarylketones and alkylbenzenes on PS-DVB column. Conditions: as in Table 5.9.

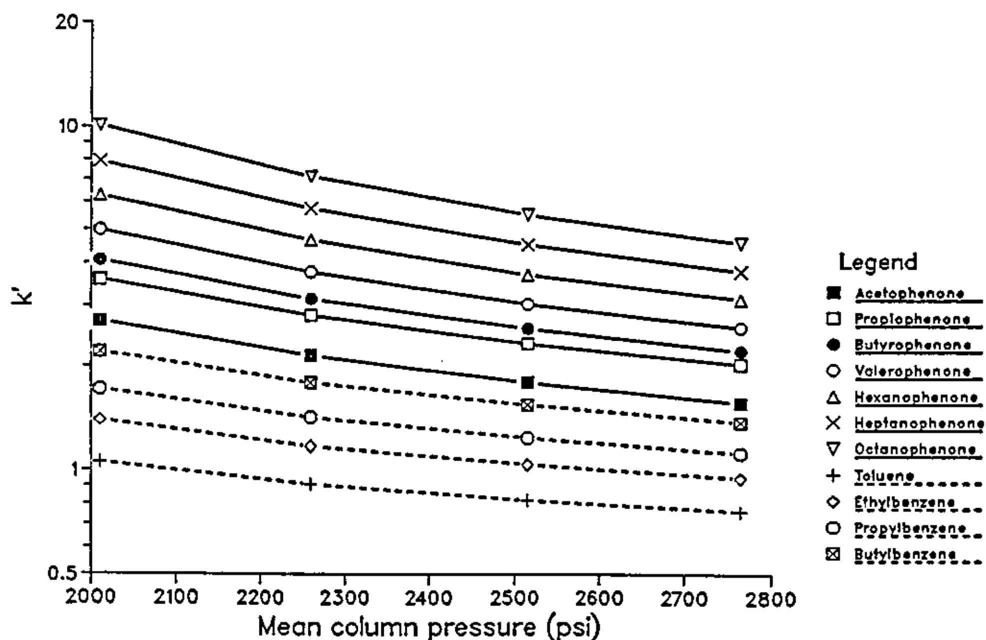


FIGURE 5.8. Variation of log capacity factors as a function of pressure for alkylarylketones and alkylbenzenes on PS-DVB column. Conditions: as in Table 5.9.

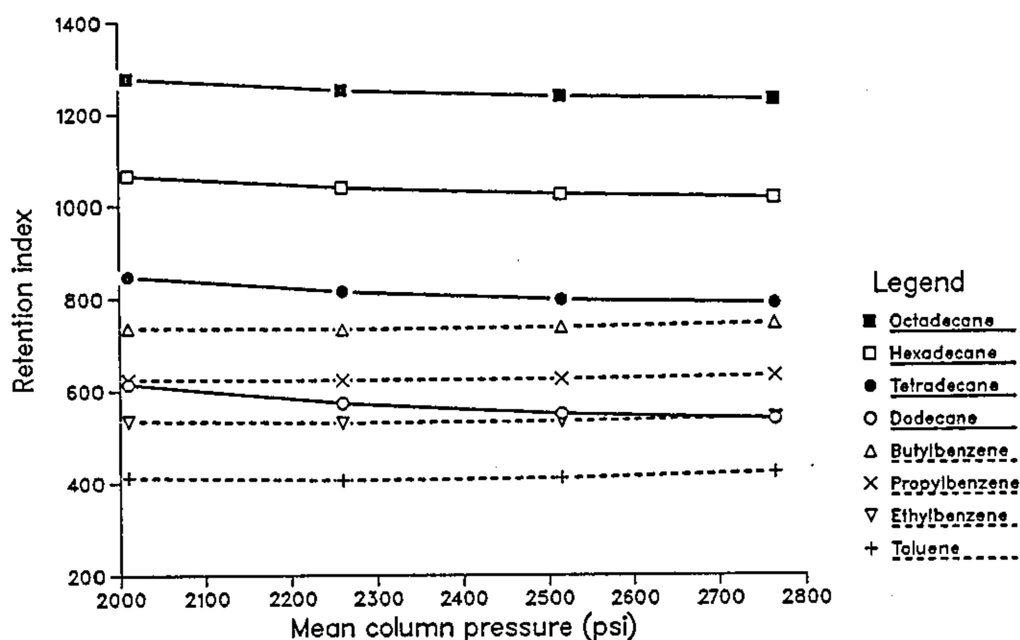


FIGURE 5.9. Retention index values based on alkylarylketones, butyrophenone - octanophenone for n-alkanes and alkylbenzenes separated on PS-DVB column at different pressures. Conditions: as in Table 5.9.

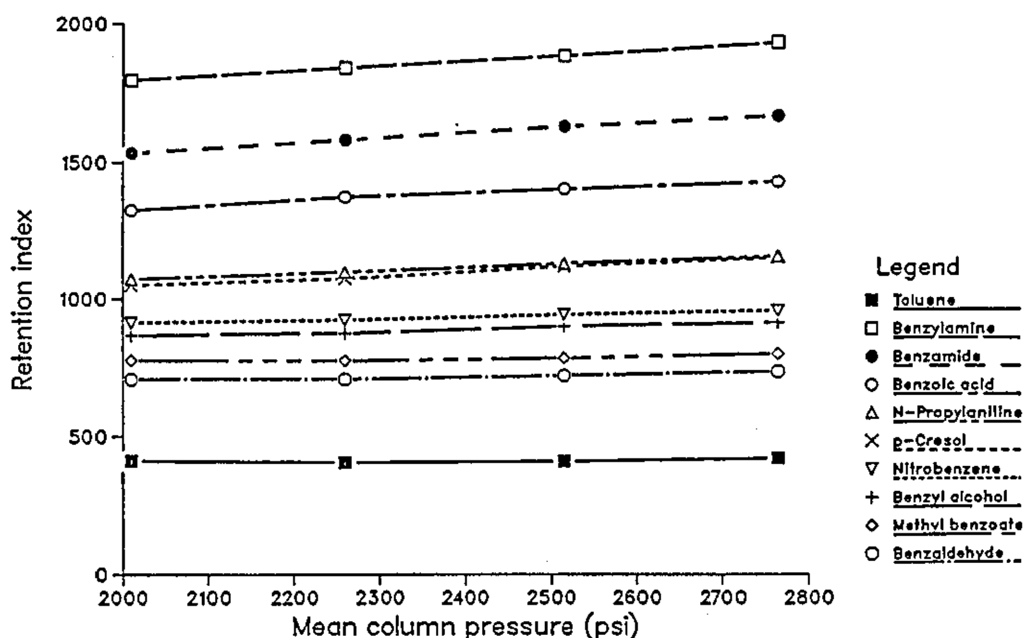


FIGURE 5.10. Retention index values based on alkylarylketones, butyrophenone - octanophenone for model compounds separated on PS-DVB column at different pressures. Condition: as in Table 5.9.

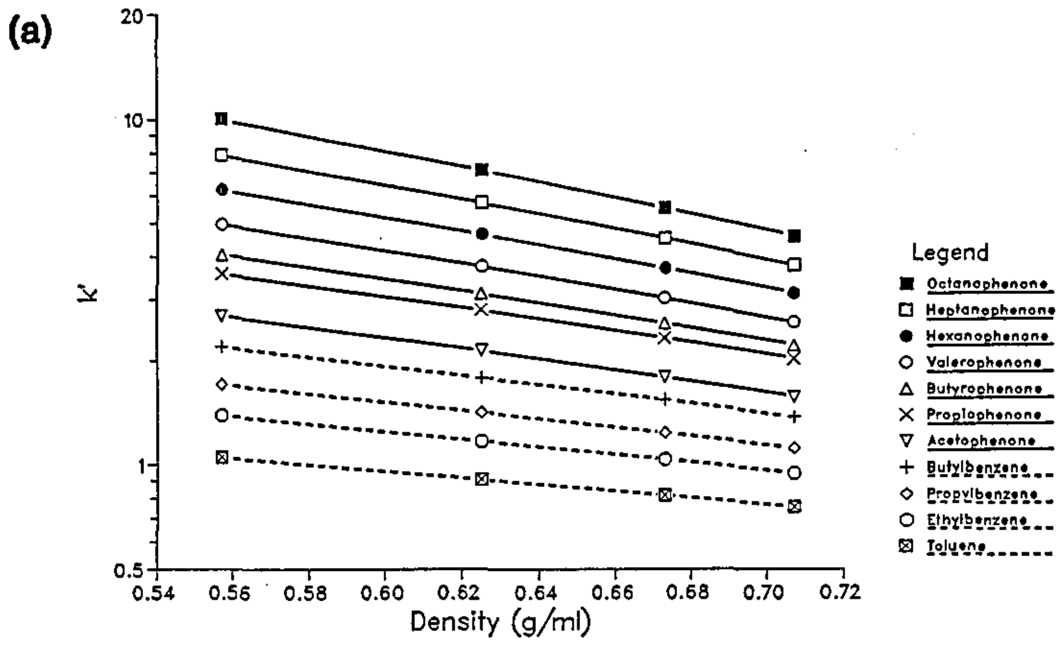
retained on the column relative to the retention index standards as the pressure increased. The results also suggest that increased pressure (hence density), increased the selective solvation power of the carbon dioxide mobile phase for the aliphatic hydrocarbons.

The retention indices based on alkylarylketones of the model compounds were plotted as a function of pressure (Figure 5.10). The compounds showed different retention patterns. The retention indices for benzaldehyde and methyl benzoate were almost constant with increase pressure, which suggests that they have similar retention behaviour to alkylarylketones. On the other hand, the more polar compounds such as benzylamine and benzoic acid were more retained relative to alkylarylketones with increasing pressure. This can be attributed to the decreasing relative solubility of the polar compounds in the mobile phase resulting from increased pressure.

From this set of results, the retention index values based on n-alkanes of the test compounds were also determined (Table 5.11). The retention indices of the alkylarylketones, alkylbenzenes, and the model compounds generally increased with increasing pressure indicating the differences in the retention behaviour of these compounds compared to the n-alkanes.

5.3.3 Effect of density

The density of the carbon dioxide mobile phase corresponding to each set of operating temperature and pressure conditions was calculated using Equation 2.1. The plots of log capacity factors against density (Figure 5.11) showed better linearity compared to the plots of log capacity factor against pressure (Figure 5.8). The linear relationship



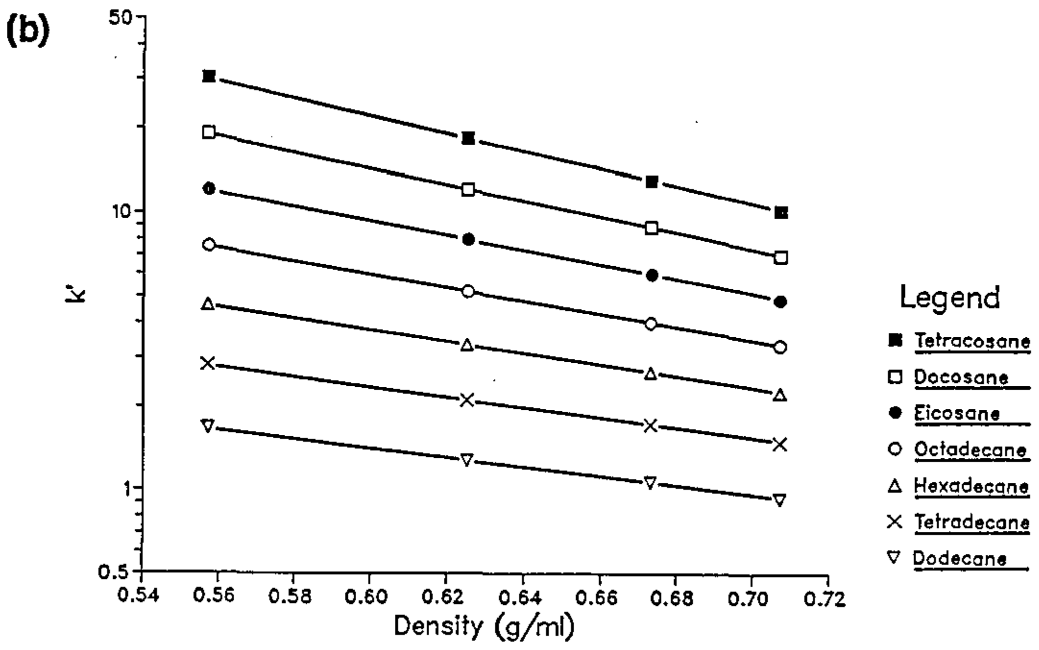


FIGURE 5.11. Variation of log capacity factors with density (due to pressure) on PS-DVB column with carbon dioxide mobile phase for (a) alkylarylketones and alkylbenzenes and (b) n-alkanes. Conditions: as in Table 5.9.

of such plots has also been reported previously for 2-alkanones [64], fatty acid phenyl esters, and fatty alcohol phenyl esters [163].

5.3.4 Effect of temperature

Experiments were carried out to determine the effect of temperature on the retention and selectivity of model compounds on the PLRP-S column. Keeping the pressure constant, the temperature was altered over the temperature range of 40 to 100 °C, and the capacity factors of the test compounds were determined (Table 5.12). All the compounds showed a marked increase in capacity factors with increasing temperature. This can be explained as under constant pressure conditions above the critical point, increasing temperature effectively decreases the density of the mobile phase.

Over the temperature range, there was clearly a nonlinear correlation between the logarithm of the capacity factors and the reciprocal of the absolute temperature (Van't Hoff-type plots) for the selected compounds (Figure 5.12). A complex temperature-dependent retention behaviour with a maxima has been observed by several workers [45,46,127,164] and this has been explained by Leyendecker [36] as the results of two opposing effects, namely, by a decrease in the density (hence solvation power) of the eluent with increase in temperature and the opposing effect of an increase in the vapour pressure (hence solubility) of the substrate (see Section 1.2.3). However, no maxima was observed from the plots (Figure 5.12) probably because the temperature range was too limited.

In order to determine the selectivity changes with temperature, the retention indices of the test compounds were calculated based on

TABLE 5.12. Capacity factors of alkylarylketones, n-alkanes, alkylbenzenes and test compounds on PS-DVB column at different temperatures.

Conditions: column, PLRP-S 5 μm; carbon dioxide mobile phase; mean column pressure 2515 psi; flame ionization detector.

Compound	Capacity factor			
	Temperature (°C)			
	40	60	80	100
	Reciprocal temperature (10^3/K)			
	3.19	3.00	2.83	2.68
	Calculated density (g/ml)			
	0.811	0.673	0.519	0.406
Acetophenone	1.50	1.80	2.43	3.41
Propiophenone	2.00	2.34	3.14	4.47
Butyrophenone	2.18	2.59	3.57	5.30
Valerophenone	2.55	3.06	4.34	6.70
Hexanophenone	3.08	3.73	5.43	8.69
Heptanophenone	3.71	4.56	6.80	11.26
Octanophenone	4.51	5.57	8.56	14.68
Dodecane	1.01	1.07	1.44	2.27
Tetradecane	1.58	1.73	2.42	4.07
Hexadecane	2.37	2.68	3.92	7.08
Octadecane	3.51	4.05	6.28	12.08
Eicosane	5.13	6.05	9.93	20.43
Docosane	7.42	8.95	15.30	34.32
Tetracosane	10.78	13.20	24.43	57.74
Toluene	0.81	0.82	0.95	1.12
Ethylbenzene	1.01	1.04	1.23	1.52
Propylbenzene	1.19	1.24	1.52	1.95
Butylbenzene	1.45	1.55	1.94	2.61
Benzaldehyde	1.27	1.51	2.04	2.72
Methyl benzoate	1.45	1.70	2.34	3.32
Benzyl alcohol	1.87	2.13	2.77	3.65
Nitrobenzene	1.95	2.31	3.19	4.42
p-Cresol	3.19	3.25	3.96	4.86
N-Propylaniline	3.02	3.31	4.26	5.81
Benzoic acid	5.54	5.61	6.96	7.99
Benzamide	7.78	8.70	11.14	14.12
Benzylamine	12.50	14.26	20.70	32.23

TABLE 5.13. Retention index values for alkylarylketones, n-alkanes, alkylbenzenes and test compounds based on alkylarylketone standards on PS-DVB column at different temperatures.

Conditions: as in Table 5.12.

Compound	Retention index*			
	Temperature (°C)			
	40	60	80	100
	Reciprocal temperature (10^3/K)			
	3.19	3.00	2.83	2.68
	Calculated density (g/ml)			
	0.811	0.673	0.519	0.406
Acetophenone	806	819	831	832
Propiophenone	960	955	948	938
Butyrophenone	1008	1007	1005	1004
Valerophenone	1093	1093	1095	1096
Hexanophenone	1196	1196	1197	1198
Heptanophenone	1299	1299	1299	1299
Octanophenone	1405	1404	1404	1403
Dodecane	587	551	593	672
Tetradecane	834	798	829	901
Hexadecane	1055	1025	1048	1117
Octadecane	1268	1239	1263	1327
Eicosane	1475	1447	1472	1532
Docosane	1677	1649	1668	1735
Tetracosane	1880	1851	1881	1938
Toluene	486	411	406	395
Ethylbenzene	589	534	522	515
Propylbenzene	677	626	617	614
Butylbenzene	786	739	728	727
Benzaldehyde	712	725	751	743
Methyl benzoate	787	788	815	822
Benzyl alcohol	924	904	890	859
Nitrobenzene	949	946	955	933
p-Cresol	1215	1123	1053	971
N-Propylanilne	1186	1133	1086	1041
Benzoic acid	1516	1407	1310	1165
Benzamide	1702	1634	1524	1388
Benzylamine	1961	1891	1805	1710

* Based on alkylarylketones, butyrophenone - octanophenone.

TABLE 5.14. Retention index values for alkylarylketones, n-alkanes, alkylbenzenes, and model compounds based on n-alkane standards on PS-DVB column at different temperatures.

Conditions: as in Table 5.12.

Compound	Retention index*			
	Temperature (°C)			
	40	60	80	100
	Reciprocal temperature (10^3/K)			
	3.19	3.00	2.83	2.68
	Calculated density (g/ml)			
	0.811	0.673	0.519	0.406
Acetophenone	1381	1425	1406	1338
Propiophenone	1525	1551	1516	1439
Butyrophenone	1570	1600	1570	1502
Valerophenone	1650	1680	1654	1589
Hexanophenone	1746	1776	1750	1686
Heptanophenone	1842	1871	1845	1782
Octanophenone	1942	1968	1944	1881
Dodecane	1176	1175	1182	1185
Tetradecane	1407	1405	1404	1403
Hexadecane	1614	1616	1610	1609
Octadecane	1813	1815	1811	1809
Eicosane	2007	2008	2007	2004
Docosane	2195	2196	2192	2198
Tetracosane	2386	2384	2392	2391
Toluene	1070	1046	1007	9222
Ethylbenzene	1178	1161	1116	1036
Propylbenzene	1260	1246	1205	1130
Butylbenzene	1362	1351	1309	1237
Benzaldehyde	1293	1338	1330	1253
Methyl benzoate	1363	1396	1391	1328
Benzyl alcohol	1491	1504	1462	1363
Nitrobenzene	1515	1543	1522	1433
p-Cresol	1764	1708	1615	1470
N-Propylaniline	1737	1717	1645	1536
Benzoic acid	2046	1972	1856	1654
Benzamide	2220	2183	2057	1867
Benzylamine	2462	2421	2321	2174

* Based on n-alkanes decane - tetracosane.

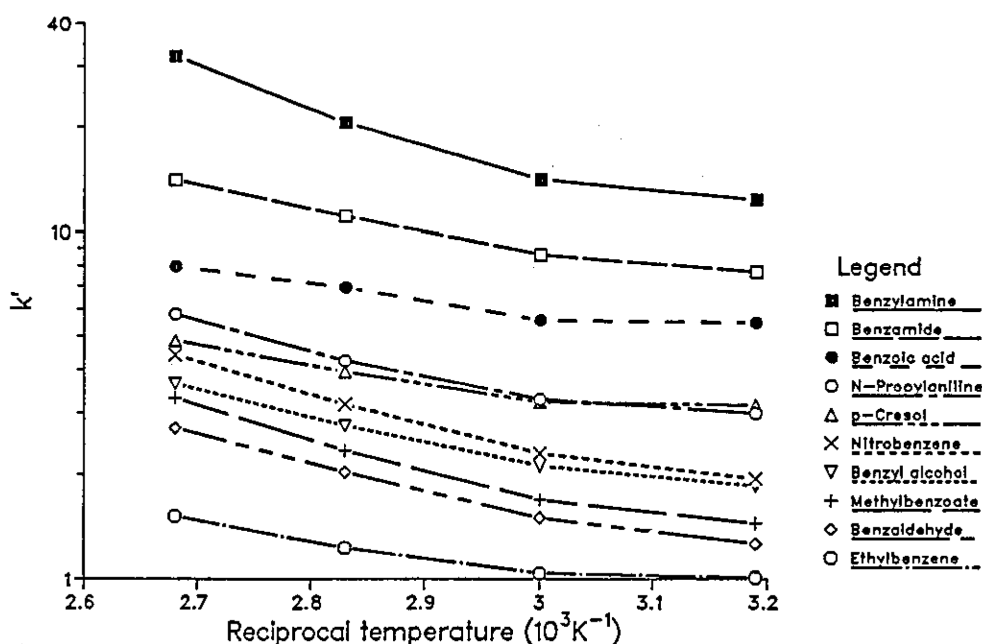


FIGURE 5.12. Variation of log capacity factors with temperature for model compounds on PS-DVB column with carbon dioxide mobile phase. Conditions: as in Table 5.12.

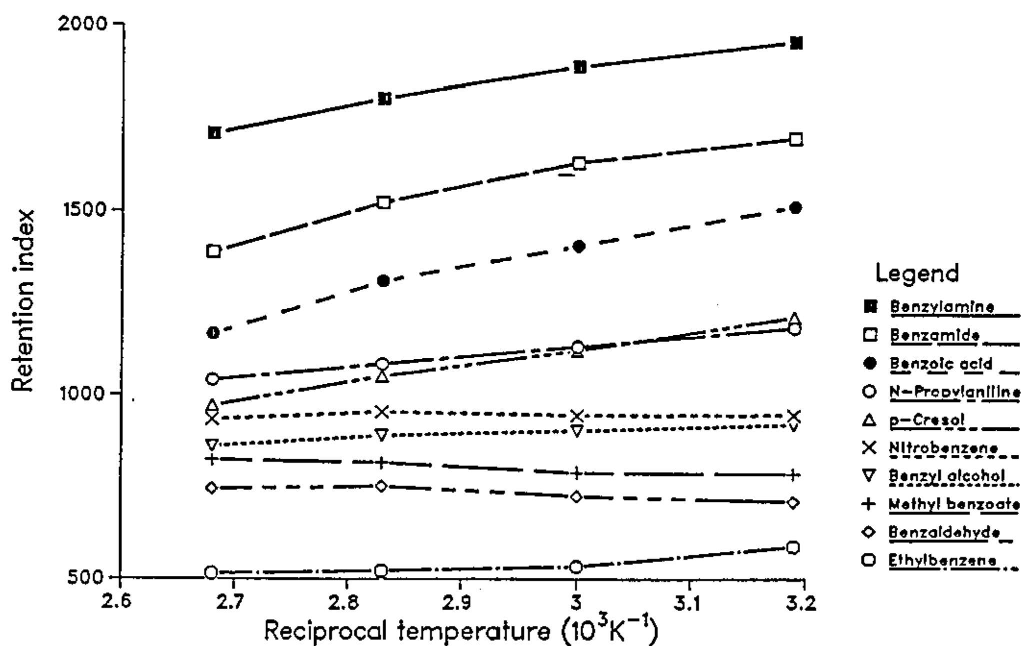


FIGURE 5.13. Retention index values based on alkylarylketones, butyrophenone - octanophenone, for model compounds separated on PS-DVB column at different temperatures. Conditions: as in Table 5.12.

alkylarylketones (Table 5.13) and n-alkanes (Table 5.14). The retention index values for several test compounds were plotted against reciprocal of absolute temperature (Figure 5.13). The results showed that changes in temperature can bring about different selectivities between compounds of different functionalities, and at some points, the elution order of compounds can be reversed. The retention indices of the polar compounds (benzylamine, benzamide, benzoic acid, and p-cresol) decreased more markedly with increasing temperature than the non-polar compounds suggesting that the retention indices of the polar compounds are more susceptible to variations in the temperature. For the n-alkane retention index scale, the effect was even larger (Table 5.14).

The changes in selectivity on the PLRP-S column with carbon dioxide as the mobile phase was in general less pronounced at high temperatures (low 1/T values) (Figure 5.13) suggesting that the property of the fluid was becoming less important in affecting the retention behaviour and that volatility (increased migration rate on the stationary phase) and GC type of mechanism(s) were becoming increasingly involved. This also corresponds to the flatter region (see Figure 1.3) where relatively smaller changes in density are observed with small changes in temperature. In HPLC, the retention indices of the test compounds on same column (PLRP-S) were largely independent of the temperature and were therefore much more robust than capacity factors as a method of recording retentions [158].

In order to examine the retention behaviour with respect to density caused by the variation of temperature, the densities for the eluent at different temperature conditions (40-100 °C) were calculated (see Table 5.12). The densities were plotted against the log capacity factors of the model compounds and poor linear correlations were obtained

(Figure 5.14). Almost linear correlations have been reported by Gere et al. [165] who plotted log capacity factors against density at different temperatures (35, 55, and 100 °C) for homologous series of polynuclear aromatic compounds (PAH), naphthalene, anthracene, pyrene, and coronene.

5.4 COMPARISON OF SELECTIVITY ON DIFFERENT STATIONARY PHASES IN SFC

One role of retention index systems is their application to the comparison of the selectivity of different column materials. This has been widely used in the Rohrschneider [166,167] and McReynolds [168] constants in GC. A related approach has been examined in HPLC [28,169] although the results are more sensitive to the eluent composition than the stationary phase. In this study, the retention index scales based on the alkylarylketone standards were used to compare the selectivities of different column materials (PLRP-S, Ultrasphere ODS, and Ultrasphere CN) with changes in the operating parameters.

First, the capacity factors for the test compounds on the ODS-silica column were measured at different pressures (Table 5.15) and compared with those obtained on the PLRP-S column (see Table 5.9). It was noted that under very similar pressure conditions (e.g., 2470 psi on ODS-silica and 2515 psi on PLRP-S), all the compounds showed greater retention on the PLRP-S column (e.g., k' on PLRP-S: valerophenone = 3.06, p-cresol = 3.25, and the corresponding k' on Ultrasphere-ODS: 0.81 and 1.08, respectively). This was probably due to the greater available adsorptive surface area on the polymer packing material and is also seen in HPLC [158].

From the same set of results, the retention indices were determined (Table 5.16) and compared with those obtained on PLRP-S column (see

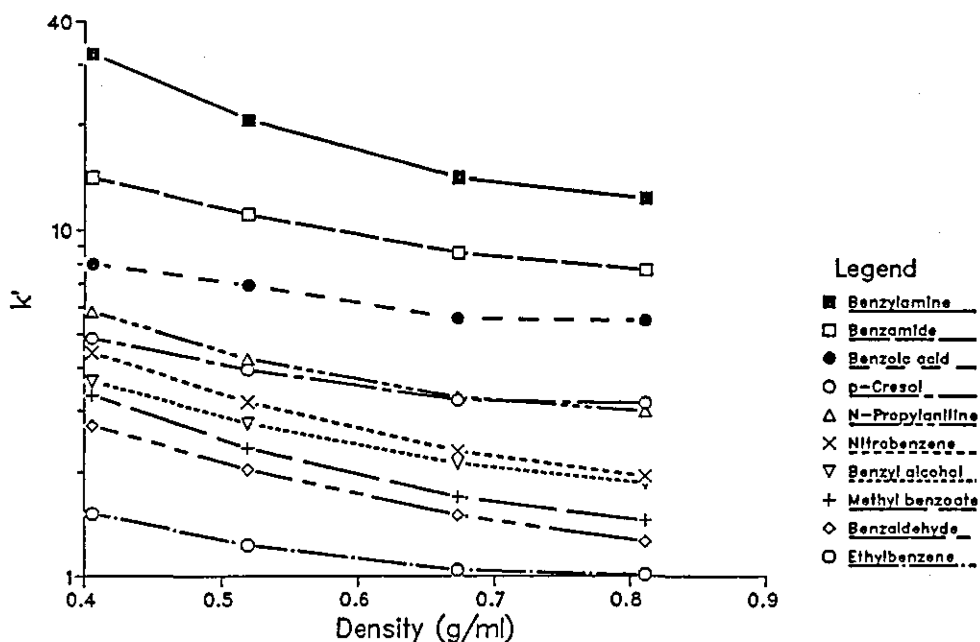


FIGURE 5.14. Variation of log capacity factors with density (due to temperature) for model compounds on PS-DVB column. Conditions: as in Table 5.12.

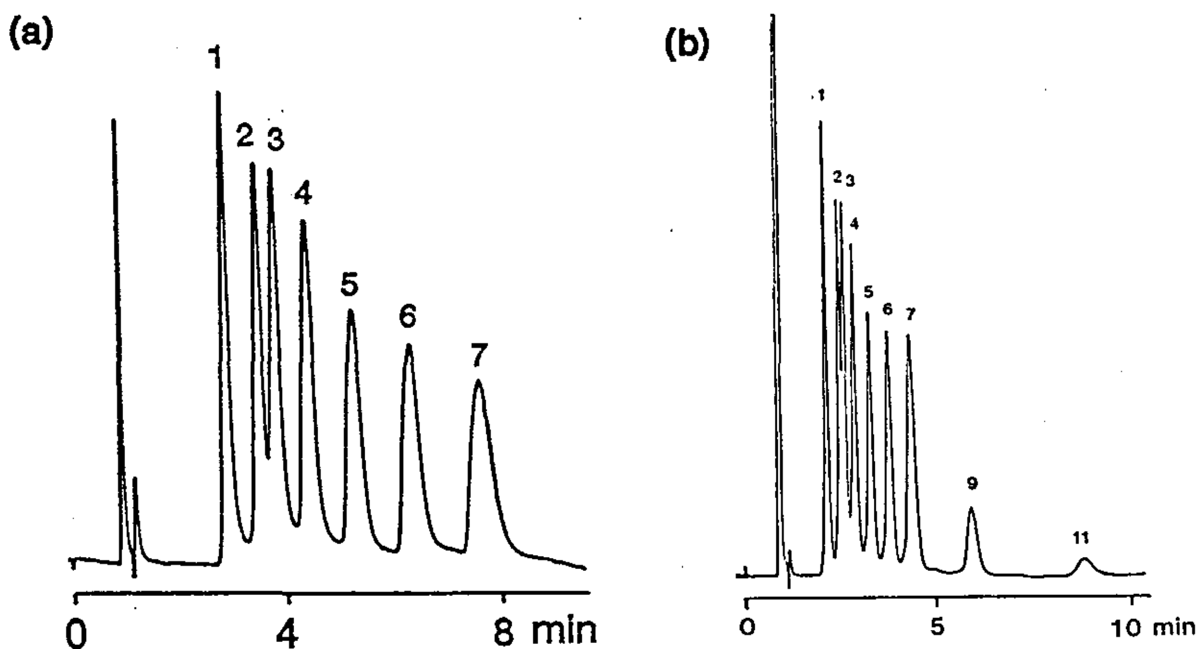


FIGURE 5.16. Comparison of the separation of alkylarylketones on PS-DVB column (a) with pure carbon dioxide and (b) with methanol (4% w/w) as the modifier in the mobile phase. Temperature 60 °C; mean pressure 2200 psi. Numbers denote the alkyl chain carbon number.

TABLE 5.15. Capacity factors of alkylarylketones, n-alkanes, alkylbenzenes, and model compounds on ODS-silica column at different pressures.

Conditions: column, Ultrasphere ODS 5 μm; carbon dioxide mobile phase; 60 °C; UV and flame ionization detections.

Compound	Capacity factor			
	Inlet pressure (psi)			
	1750	2050	2300	2620
	Outlet pressure (psi)			
	1580	1850	2020	2320
	Mean pressure (psi)			
	1665	1950	2160	2470
	Calculated density (g/ml)			
	0.397	0.535	0.601	0.666
Valerophenone	2.39	1.43	1.00	0.81
Hexanophenone	2.91	1.69	1.16	0.95
Heptanophenone	3.58	2.02	1.37	1.10
Octanophenone	4.45	2.43	1.62	1.30
Decanophenone	7.04	3.62	2.21	1.84
Dodecanophenone	10.81	5.26	3.25	2.52
Tetradecanophenone	16.95	7.65	4.53	3.47
Hexadecanophenone	26.28	11.10	6.30	4.74
Octadecanophenone	40.05	15.66	8.57	6.32
Decane	0.97	0.62	0.48	0.43
Dodecane	1.70	1.01	0.77	0.66
Tetradecane	2.83	1.56	1.15	0.97
Hexadecane	4.56	2.37	1.66	1.37
Octadecane	7.20	3.46	2.34	1.90
Eicosane	11.25	4.99	3.25	2.57
Docosane	16.92	7.15	4.43	3.48
Tetracosane	25.56	10.19	6.15	4.68
Toluene	0.46	0.34	0.28	0.21
Ethylbenzene	0.64	0.46	0.36	0.28
Propylbenzene	0.85	0.59	0.46	0.36
Butylbenzene	1.12	0.76	0.58	0.46
Benzaldehyde	1.14	0.80	0.56	0.47
Methyl benzoate	1.24	0.82	0.62	0.51
Benzyl alcohol	4.62	2.66	1.84	1.47
Nitrobenzene	1.02	0.66	0.48	0.36
p-Cresol	2.63	1.71	1.35	1.08
N-Propylaniline	2.43	2.12	1.16	1.00
Benzoic acid	-	-	-	-
Benzamide	-	-	-	-
Benzylamine	-	7.01	4.83	3.95

TABLE 5.16. Retention index values of alkylarylketones, n-alkanes, alkylbenzenes, and model compounds based on alkylarylketone standards on ODS-silica column at different pressures.

Conditions: as in Table 5.15.

Compound	Retention index*			
	Inlet pressure (psi)			
	1750	2050	2300	2620
	Outlet pressure (psi)			
	1580	1850	2020	2320
	Mean pressure (psi)			
	1665	1950	2160	2470
	Calculated density (g/ml)			
	0.397	0.535	0.601	0.666
Valerophenone	1109	1107	1109	1100
Hexanophenone	1199	1197	1199	1195
Heptanophenone	1294	1293	1295	1291
Octanophenone	1393	1391	1397	1395
Decanophenone	1602	1615	1582	1613
Dodecanophenone	1798	1806	1812	1809
Tetradecanophenone	2004	2006	2009	2008
Hexadecanophenone	2204	2205	2206	2204
Octadecanophenone	2397	2390	2390	2383
Decane	695	657	673	699
Dodecane	954	921	949	972
Tetradecane	1186	1154	1191	1210
Hexadecane	1405	1377	1410	1427
Octadecane	1613	1580	1617	1630
Eicosane	1816	1777	1812	1821
Docosane	2003	1970	1996	2011
Tetracosane	2191	2160	2191	2195
Toluene	355	337	348	246
Ethylbenzene	506	496	501	428
Propylbenzene	634	628	643	583
Butylbenzene	764	765	784	740
Nitrobenzene	721	690	666	583
Benzaldehyde	772	795	763	753
Methyl benzoate	809	809	826	803
N-Propylaniline	1116	1317	1196	1230
p-Cresol	1163	1203	1288	1281
Benzyl alcohol	1410	1440	1473	1473
Benzylamine	-	1959	2048	2089
Benzoic acid	-	-	-	-
Benzamide	-	-	-	-

* Based on alkylarylketones, valerophenone - octadecanophenone.

Table 5.10). In order to calculate the retention indices of some of the test compounds such as benzaldehyde, methyl benzoate, nitrobenzene, and the alkylbenzenes, considerable extrapolation of the calibration curve was frequently required. The corresponding values are therefore probably subject to large experimental errors and thus small differences in the variables (e.g. temperature or pressure) may not be significant. Clearly, there are major changes between the selectivities of the columns. The retention of polar (compared to the non-polar) compounds are greater on the ODS-silica column than on the PS-DVB column and this is in the opposite direction to those observed in reversed phase HPLC (Table 5.17).

In SFC, with carbon dioxide as the mobile phase, the non-polar compounds were eluted very rapidly and the polar analytes were markedly retained suggesting that an effectively normal phase or adsorption type interactions might have contributed significantly to the SFC separation. A more complex interaction was clearly taking place on the ODS-silica column as the peak shapes of the benzyl alcohol and phenols were severely distorted and they showed considerable tailing (see Figure 4.19) and the retention also varied with sample loading as described previously (Section 4.4). In subsequent studies, these effects were removed by the use of modifiers but then, the retentions were markedly different (see following Sections).

Experiments have also been carried out using a cyano-Ultrasphere column using carbon dioxide as the mobile phase (Tables 5.18). As has been mentioned earlier this column showed relatively weak retention of the non-polar compounds compared to the corresponding separations on the ODS-silica or polymer columns. Under the experimental conditions the n-alkanes were virtually unretained and coeluted with the the void

TABLE 5.17. Comparison of retention indices of selected model compounds on PS-DVB and ODS-silica columns in reversed phase HPLC [158] and SFC.

Conditions	Column	Retention index		
		Toluene	Nitrobenzene	p-Cresol
Reversed-phase HPLC*				
Methanol-water (90:10)	PLRP-S	933	873	428
Methanol-buffer (90:10)	PLRP-S	937	881	494
Methanol-buffer (90:10)	ODS-2	1128	853	682
Acetonitrile-water (70:30)	PLRP-S	1061	875	568
Acetonitrile-buffer (70:30)	PLRP-S	1034	875	596
Acetonitrile-buffer (70:30)	ODS-2	1050	853	716
Packed-column SFC**				
CO2, P(inlet) 2050 psi	PLRP-S	412	913	1050
CO2, P(inlet) 2050 psi	ODS	337	690	1203

* Ref. [158], buffer, 0.02 M phosphate, pH 7.

** This work; 60 °C, columns: PLRP-S and Ultrasphere ODS.

TABLE 5.18. Capacity factors of alkylarylketones and model compounds on cyano-silica column at different pressures.

Conditions: column, Ultrasphere Cyano 5 μm; carbon dioxide mobile phase; 60 °C; UV and flame ionization detections.

Compound	Capacity factor			
	Inlet pressure (psi)			
	2050	2320	2580	2870
	Outlet pressure (psi)			
	1840	2080	2340	2600
	Mean pressure (psi)			
	1945	2200	2460	2735
	Calculated density (g/ml)			
	0.533	0.611	0.664	0.704
Acetophenone	7.74	6.19	5.03	4.70
Propiophenone	5.30	4.10	3.28	2.98
Heptanophenone	6.40	4.66	3.55	3.12
Octanophenone	6.98	4.97	3.77	3.30
Decanophenone	8.00	5.80	4.27	3.67
Dodecanophenone	9.86	6.52	4.78	4.02
Tetradecanophenone	11.57	7.49	5.25	4.39
Hexadecanophenone	13.43	8.45	5.83	4.70
Octadecanophenone	15.42	9.36	6.35	5.10
Nitrobenzene	2.04	1.34	1.10	0.99
Benzaldehyde	4.02	3.25	2.65	2.46
Methyl benzoate	4.27	2.99	2.42	2.23
Benzyl alcohol	9.47	6.62	5.00	4.20
p-Cresol	16.21	10.87	9.17	8.11

TABLE 5.19. Retention index values for alkylarylketones and model compounds based on alkylarylketone standards on cyano-silica column at different pressures.

Conditions: as in Table 5.18.

Compound	Retention index*			
	Inlet pressure (psi)			
	2050	2320	2580	2870
	Outlet pressure (psi)			
	1840	2080	2340	2600
	Mean pressure (psi)			
	1945	2200	2460	2735
	Calculated density (g/ml)			
	0.533	0.611	0.664	0.704
Acetophenone	1529	1725	1929	2185
Propiophenone	1064	1084	1124	1159
Heptanophenone	1296	1283	1274	1263
Octanophenone	1403	1385	1388	1388
Decanophenone	1570	1623	1620	1627
Dodecanophenone	1826	1806	1831	1835
Tetradecanophenone	2023	2023	2011	2032
Hexadecanophenone	2207	2210	2207	2184
Octadecanophenone	2376	2369	2369	2370
Nitrobenzene	-110	-658	-931	-1315
Benzaldehyde	725	720	729	728
Methyl benzoate	798	594	553	505
Benzyl alcohol	1777	1831	1919	1932
p-Cresol	2437	2602	3060	3411

* Based on alkylarylketones, heptanophenone - octadecanophenone.

volume marker (hexane) while on the other hand, polar compounds benzoic acid, N-propylaniline, benzylamine, and benzamide were very retained and were not eluted in a reasonable time.

The examination of the alkylarylketones showed that under similar pressure conditions, on the cyano-silica column, these compounds were generally more retained than on the ODS-silica column. However, it was also observed that the lower members of the series were largely unresolved over the range of experimental conditions and were thus not included in the retention index calculations (Table 5.19). One striking feature observed on the cyano-silica column was the markedly anomalous retention of acetophenone (RI = 1529 to 2185). It was eluted between octanophenone and decanophenone at mean column pressure of 1945 psi (Figure 5.15). The retention of this compound increased relative to the other homologues with increasing column pressure and subsequently, this anomalous retention behaviour has been investigated in a greater detail (Section 5.6).

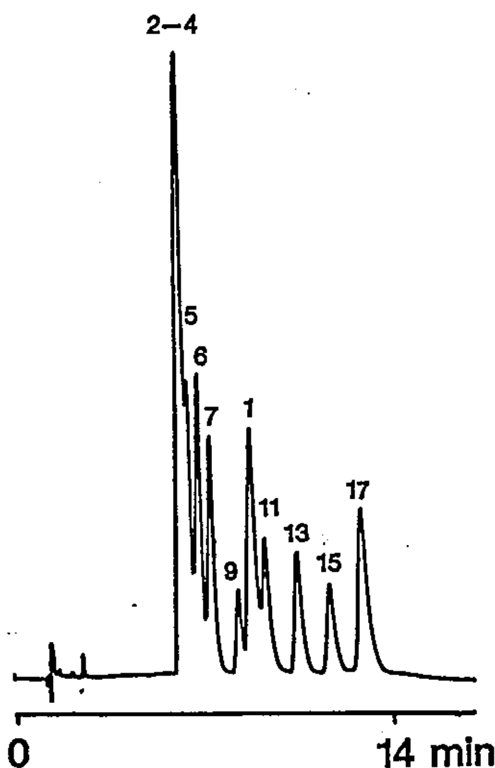


FIGURE 5.15. SFC separation of alkylarylketones on cyano-silica column with carbon dioxide mobile phase. Column Ultrasphere CN 5 µm; mean pressure 2200 psi; 60 °C; flow rate 920 ml/min (gas); UV detection, 254 nm. Numbers denote the alkyl chain carbon number (e.g., 1. acetophenone, 17. octadecanophenone).

5.5 EFFECT OF MOBILE PHASE COMPOSITION ON SELECTIVITY IN SFC

5.5.1 Introduction

One of the drawbacks of carbon dioxide is that because of its low polarity, it is difficult to elute polar compounds such as benzyl alcohol and benzamide from many columns. The separations of these compounds using pure carbon dioxide as the mobile phase are typically characterized by very broad and tailing peaks which lead to poor sensitivity and reproducibility of retention time and peak area (Section 4.4). The use of organic modifiers in the carbon dioxide mobile phase has therefore been investigated.

5.5.2 Methanol as modifier on PS-DVB column

In HPLC, the solute retention is normally very sensitive to the composition of the eluent and even minor differences can cause large changes. In order to examine the effect of modifier in SFC, a series of separations was carried out on a PS-DVB column with different concentrations of methanol over the range 0-15.3% and the capacity factors were measured (Table 5.20). The addition of methanol as a modifier was found to drastically reduced the retention, particularly of the more polar compounds, and it generally gave better separation and peak shapes of polar compounds. These effect are illustrated by the SFC separations of several test compounds, alkylarylketones (Figure 5.16), benzoic acid, nitrobenzene, and benzaldehyde (Figure 5.17), benzamide, N-propylaniline, and methyl benzoate (Figure 5.18) and benzyl alcohol and p-cresol, benzylamine, benzamide, and methyl benzoate (Figure 5.19) using unmodified or methanol-modified carbon dioxide as the mobile phase

TABLE 5.20. Capacity factors of alkylarylketones, alkylbenzenes and model compounds on PS-DVB column at different methanol concentrations.

Conditions: column, PLRP-S 5 μm; primary mobile phase: carbon dioxide; mean column pressure 2515 psi; 60 °C; UV detection, 254 nm.

Compound	Capacity factor			
	Methanol (% w/w)			
	0	4.3	9.7	15.3
Acetophenone	1.80	1.13	0.77	0.58
Propiophenone	2.34	1.47	0.97	0.70
Butyrophenone	2.59	1.57	1.04	0.75
Valerophenone	3.06	1.79	1.56	0.86
Hexanophenone	3.73	2.12	1.34	0.97
Heptanophenone	4.56	2.51	1.55	1.11
Octanophenone	5.57	2.98	1.80	1.27
Decanophenone	-	4.22	2.43	1.67
Dodecanophenone	-	5.86	3.22	2.17
Tetradecanophenone	-	8.12	4.28	2.80
Hexadecanophenone	-	11.25	5.65	3.60
Octadecanophenone	-	15.28	7.33	4.58
Toluene	0.82	0.60	0.49	0.47
Ethylbenzene	1.04	0.74	0.59	0.55
Propylbenzene	1.24	0.86	0.67	0.61
Butylbenzene	1.55	1.03	0.78	0.71
Benzaldehyde	1.51	1.03	0.68	0.55
Methyl benzoate	1.70	1.07	0.75	0.59
Benzyl alcohol	2.13	1.23	0.67	0.36
Nitrobenzene	2.31	1.51	1.05	0.78
p-Cresol	3.25	1.60	0.75	0.39
N-Propylaniline	3.31	2.06	1.38	1.03
Benzoic acid	5.61	1.35	0.66	0.44
Benzamide	8.70	2.45	0.95	0.59
Benzylamine	14.26	7.06	4.04	2.63

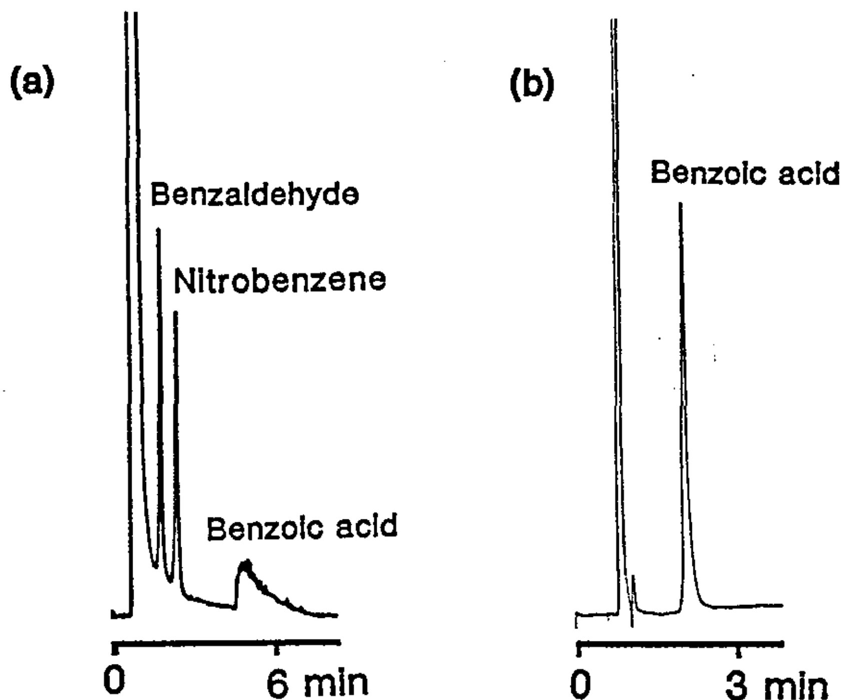


FIGURE 5.17. Effect of modifier on the separation of model compounds on PS-DVB column. Conditions: column PLRP-S 5 μm; mean column pressure 2515 psi; 60 °C; (a) carbon dioxide mobile phase; flame ionization detection; (b) 4.3% methanol in carbon dioxide mobile phase; UV detection, 254 nm.

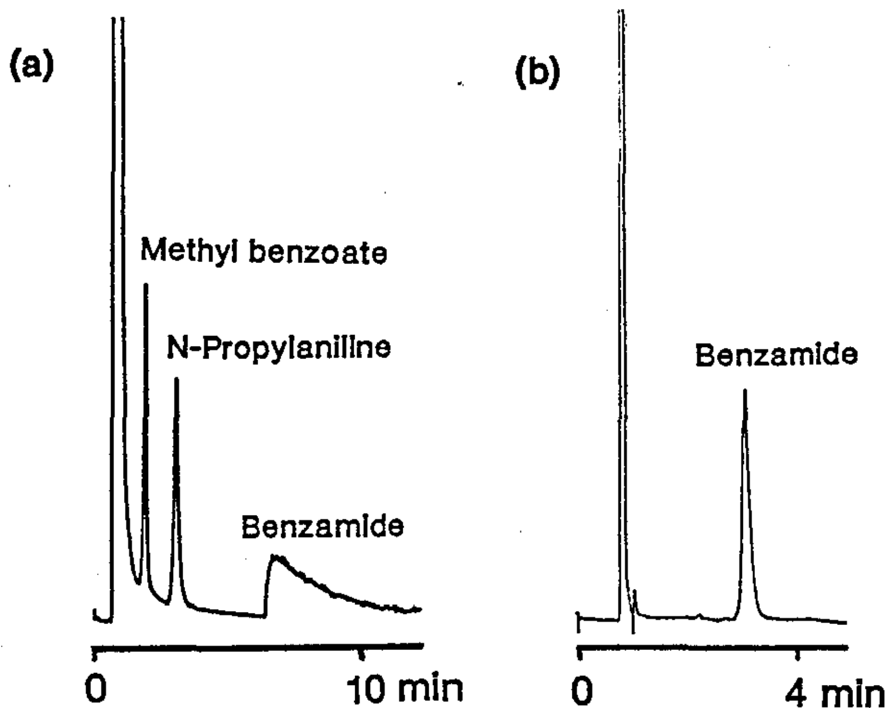


FIGURE 5.18. Effect of modifier on the separation of several model compounds on PS-DVB column. Conditions: as in Figure 4.17.

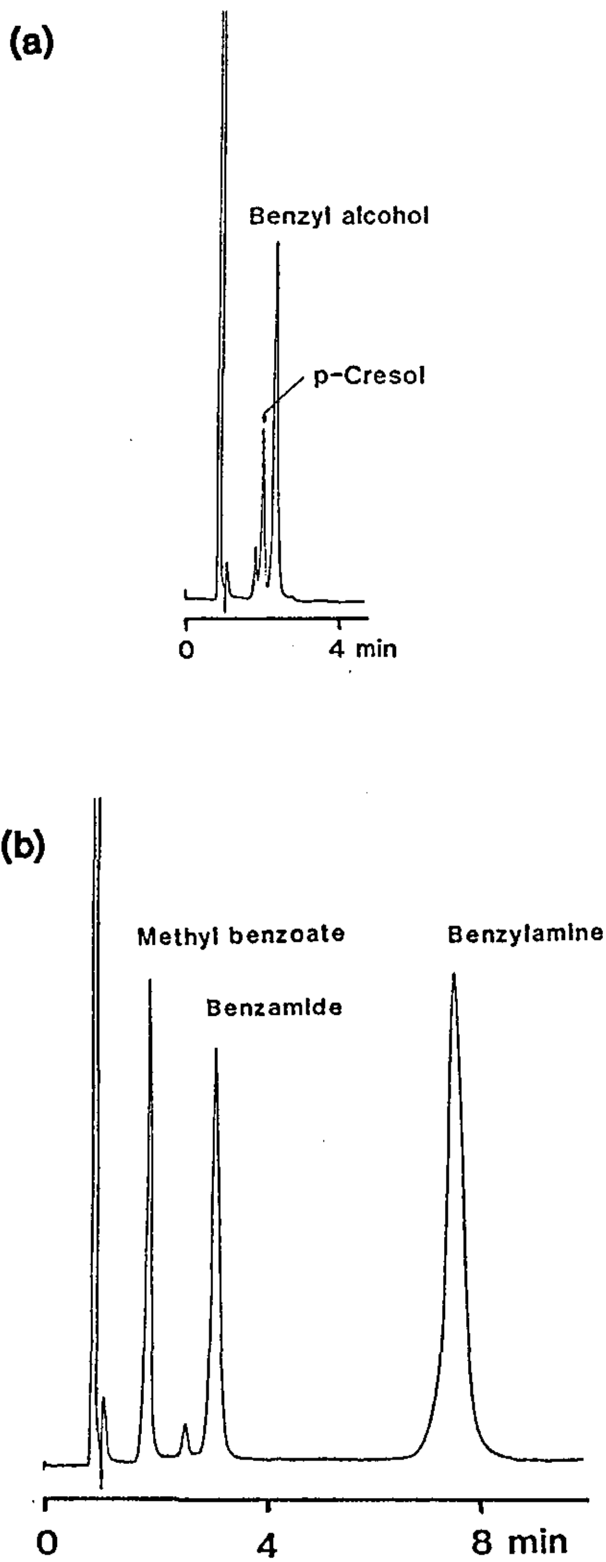


FIGURE 5.19. SFC separation of several model compounds on PS-DVB column with 4.3% methanol in the mobile phase. Conditions: column PLRP-S 5 μm; mean column pressure 2515 psi; UV detection, 254 nm.

on a PS-DVB column. As expected, the capacity factors showed marked reductions on addition of methanol (from 0 to to 4.3%), particularly for the more polar compounds, benzoic acid, benzamide, benzylamine, and the alcohols. On further increasing the methanol concentration in the mobile phase, the capacity factors continued to decrease. These results confirm the previous reports on the drastic effects of the addition of small amounts of modifier to carbon dioxide mobile phase on packed column SFC [70,71]. These suggested that the modifier altered the stationary phase. However, several workers [74,76] have found that in capillary SFC, the modifier does not change the retention as markedly as in packed column SFC.

The retention indices of model compounds were calculated based on alkylarylketone standards over the methanol concentrations 0-15.3% (Table 5.21) and the results are plotted (Figure 5.20). Selectivity changes were observed when changing the amount of modifier in the mobile phase. Generally, the retention indices of the more polar compounds decreased with increasing modifier concentration, whereas those of the nonpolar compounds were virtually unchanged or increased slightly. It was interesting to note that the amines, N-propylaniline, and even benzylamine which has appreciable basicity showed little changes in retention relative to alkylarylketones. In contrast, benzoic acid, benzamide, p-cresol, and benzyl alcohol, all of which are oxygen-containing compounds and capable of hydrogen-bonding or are acidic in nature showed marked decreases in retentions relative to the alkylarylketones as the methanol concentration in the mobile phase was increased.

TABLE 5.21. Retention index values of alkylarylketones, alkylbenzenes and model compounds based on alkylarylketone standards on PS-DVB column at different methanol concentrations.

Conditions: as in Table 5.20.

Compound	Retention index*			
	Methanol (% w/w)			
	0	4.3	9.7	15.3
Acetophenone	819	811	797	790
Propiophenone	955	970	964	939
Butyrophenone	1007	1017	1010	994
Valerophenone	1093	1091	1088	1100
Hexanophenone	1196	1192	1192	1194
Heptanophenone	1299	1295	1295	1297
Octanophenone	1404	1398	1401	1401
Decanophenone	-	1609	1610	1610
Dodecanophenone	-	1808	1809	1810
Tetradecanophenone	-	2005	2007	2007
Hexadecanophenone	-	2203	2203	2201
Octadecanophenone	-	2388	2396	2385
Toluene	411	428	490	641
Ethylbenzene	534	553	619	760
Propylbenzene	626	645	704	837
Butylbenzene	739	758	810	947
Benzaldehyde	725	737	714	753
Methyl benzoate	788	779	785	810
Benzyl alcohol	904	860	700	436
Nitrobenzene	946	989	1017	1026
p-Cresol	1123	1022	784	499
N-Propylaniline	1133	1176	1212	1241
Benzoic acid	1407	917	694	584
Benzamide	1634	1281	949	814
Benzylamine	1891	1921	1968	1958

* Based on butyrophenone - octadecanophenone, except for 0% methanol concentration, which was based on butyrophenone - octanophenone.

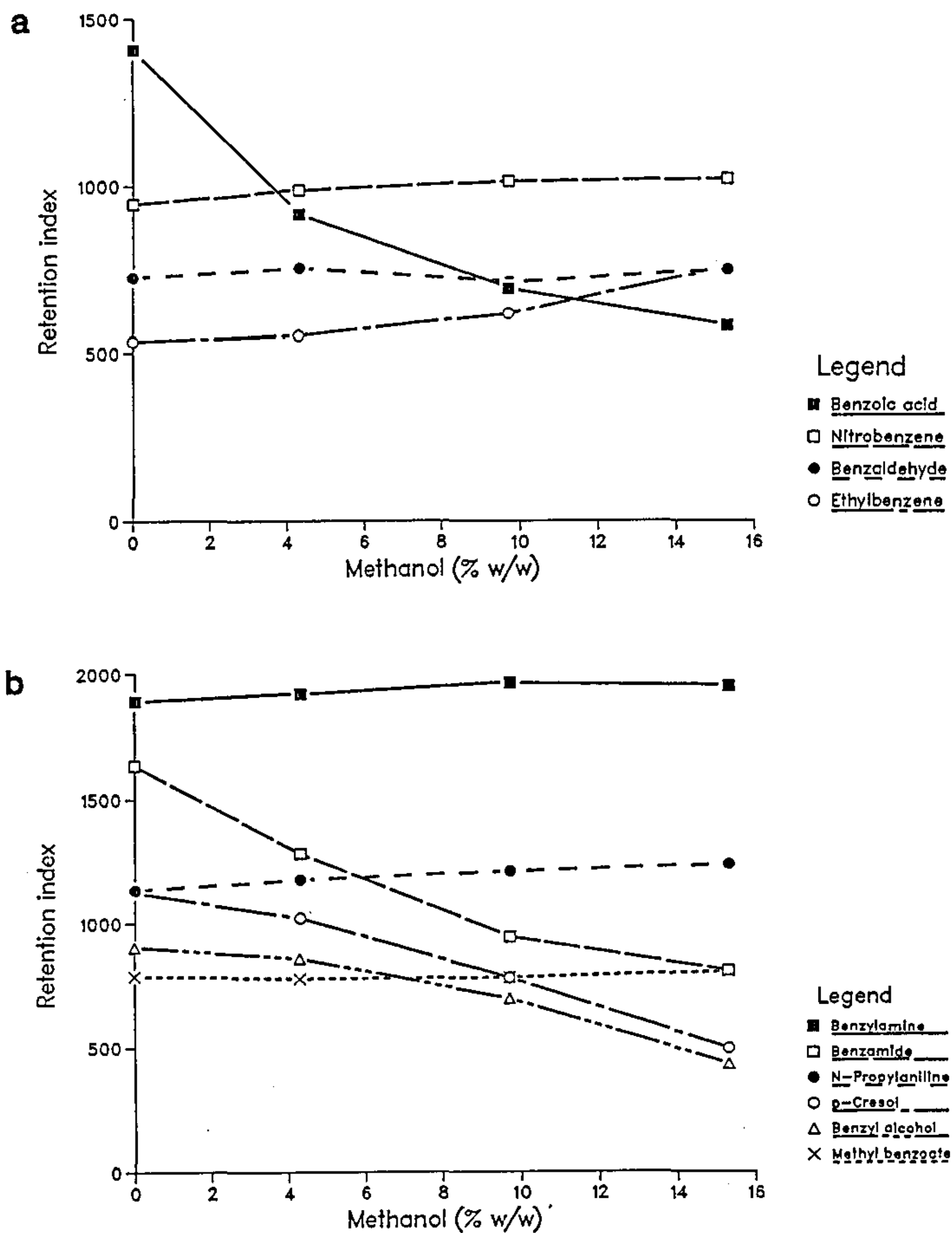


FIGURE 5.20. Variation of retention indices (based on alkylarylketones, butyrophenone - octadecanophenone) with methanol concentration for model compounds separated on PS-DVB column. Conditions: as in Table 5.20.

5.5.3 Acetonitrile as modifier on PLRP-S column

A corresponding series of experiments has been carried out under equivalent operating conditions on the PS-DVB column using acetonitrile as the modifier over the concentration range of 0-14% (w/w). The decrease in the capacity factors of the alkylarylketones and alkylbenzenes with increasing acetonitrile concentration in the mobile phase (Table 5.22) was larger than the effect of methanol and the retention times were shorter. Similar trends were also observed for the model compounds, benzaldehyde, methyl benzoate, nitrobenzene, N-propylaniline, and benzylamine. However, marked differences from the methanol behaviour were clearly observed with the remaining model compounds, benzyl alcohol, p-cresol, benzoic acid, and benzamide. The capacity factors of these compounds in each eluent composition were greater than with the corresponding nominal values with methanol, suggesting that these polar compounds are selectively more retained on the column compared to the less polar compounds and that acetonitrile is a less capable modifier compared to methanol for eluting these compounds. The elution of the polar compounds (e.g. benzoic acid and benzylamine, Figure 5.21) with 4.3% acetonitrile in the mobile phase

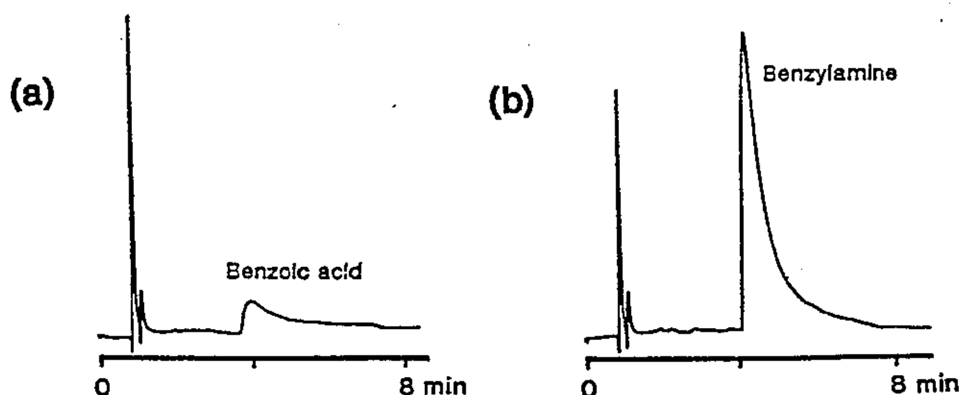


FIGURE 5.21. SFC separations of model compounds, (a) benzoic acid and (b) benzylamine on PS-DVB column with 4.3% acetonitrile in the mobile phase. Mean column pressure 2515 psi; 60 °C.

TABLE 5.22. Capacity factors of alkylarylketones, alkylbenzenes and model compounds on PS-DVB column at different acetonitrile concentrations.

Conditions: column, PLRP-S 5 µm; primary eluent: carbon dioxide; mean column pressure 2515 psi; 60 °C; UV detection, 254 nm.

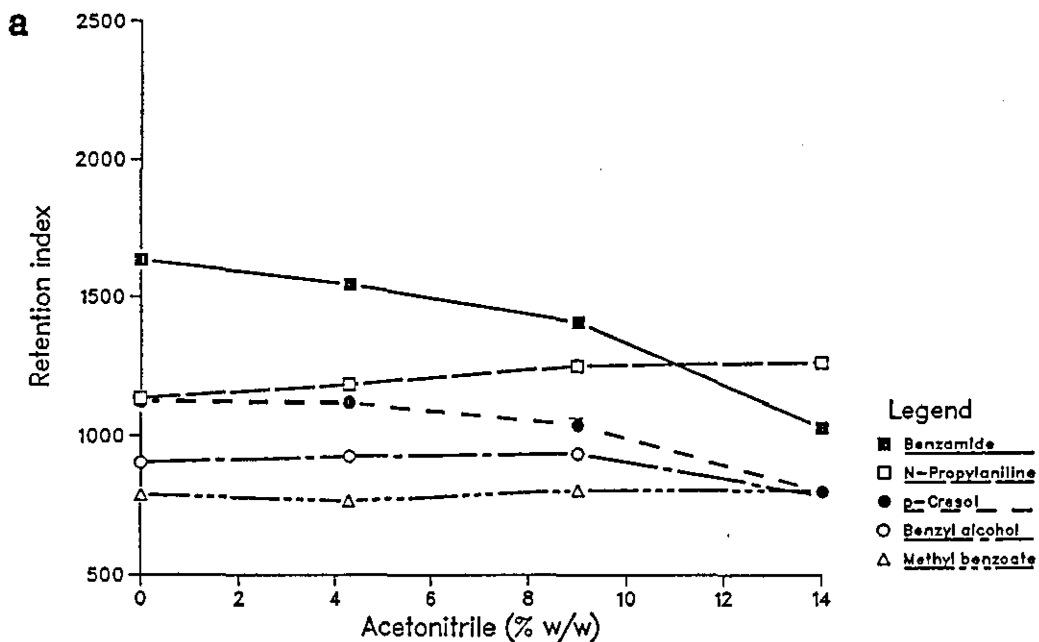
Compound	Capacity factor			
	Acetonitrile (% w/w)			
	0	4.3	9.0	14.0
Acetophenone	1.80	0.96	0.60	0.37
Propiophenone	2.34	1.23	0.75	0.44
Butyrophenone	2.59	1.32	0.80	0.48
Valerophenone	3.06	1.51	0.89	0.54
Hexanophenone	3.73	1.76	1.03	0.59
Heptanophenone	4.56	2.07	1.18	0.68
Octanophenone	5.57	2.43	1.34	0.77
Decanophenone	-	3.33	1.76	0.97
Dodecanophenone	-	4.51	2.27	1.21
Tetradecanophenone	-	5.76	2.92	1.50
Hexadecanophenone	-	8.55	3.75	1.85
Octadecanophenone	-	11.10	4.74	2.27
Toluene	0.82	0.53	0.43	0.35
Ethylbenzene	1.04	0.64	0.51	0.40
Propylbenzene	1.24	0.74	0.56	0.43
Butylbenzene	1.55	0.88	0.65	0.50
Benzaldehyde	1.51	0.83	0.56	0.31
Methyl benzoate	1.70	0.91	0.62	0.39
Benzyl alcohol	2.13	1.17	0.74	0.38
Nitrobenzene	2.31	1.16	0.71	0.42
p-Cresol	3.25	1.57	0.84	0.39
N-Propylaniline	3.31	1.73	1.11	0.65
Benzoic acid	5.61	2.74	1.45	0.56
Benzamide	8.70	3.03	1.36	0.52
Benzylamine	14.26	6.20	3.30	1.59

TABLE 5.23. Retention index values for alkylarylketones, alkylbenzenes, and test compounds based on alkylarylketone standards on PS-DVB column at different acetonitrile concentrations.

Conditions: as in Table 5.22.

	Retention index*			
	Acetonitrile (% w/w)			
	0	4.3	9.0	14.0
Acetophenone	819	796	781	755
Propiophenone	955	961	949	920
Butyrophenone	1007	1004	998	994
Valerophenone	1093	1094	1087	1092
Hexanophenone	1196	1195	1197	1176
Heptanophenone	1299	1299	1300	1307
Octanophenone	1404	1404	1404	1410
Decanophenone	-	1610	1614	1621
Dodecanophenone	-	1808	1812	1818
Tetradecanophenone	-	1967	2007	2008
Hexadecanophenone	-	2224	2200	2197
Octadecanophenone	-	2394	2383	2377
Toluene	411	412	525	720
Ethylbenzene	534	536	646	825
Propylbenzene	626	629	722	894
Butylbenzene	739	742	840	1030
Benzaldehyde	725	707	719	607
Methyl benzoate	788	767	807	806
Benzyl alcohol	904	927	938	786
Nitrobenzene	946	923	912	883
p-Cresol	1123	1120	1041	802
N-Propylaniline	1133	1183	1253	1267
Benzoic acid	1407	1483	1465	1125
Benzamide	1634	1547	1411	1034
Benzylamine	1891	2015	2101	2059

* Based on butyrophenone - octadecanophenone, except for 0% acetonitrile concentration, which was based on butyrophenone - octanophenone.



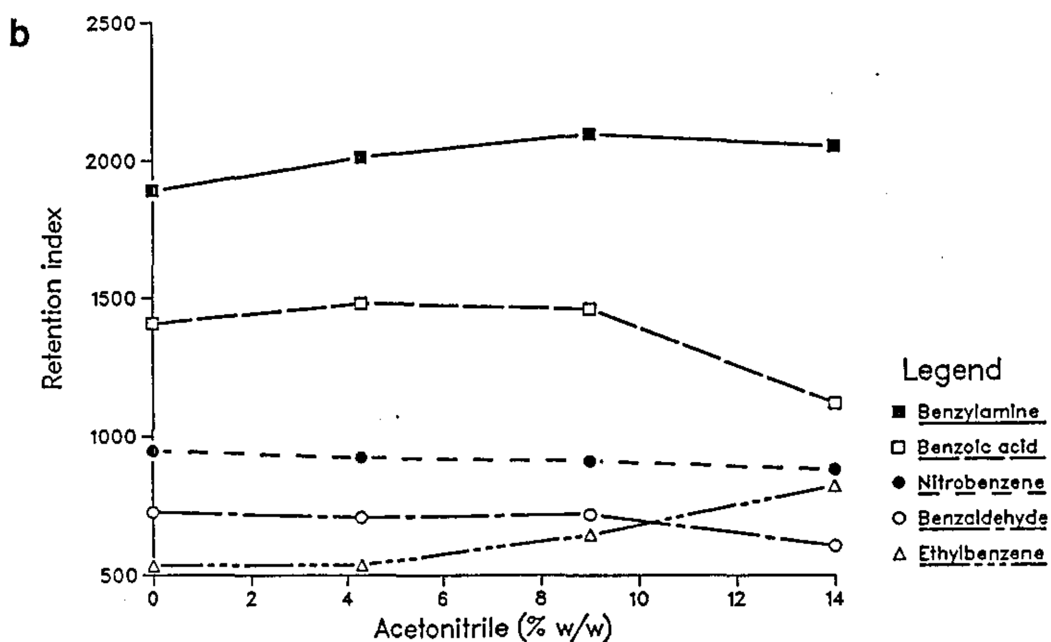


FIGURE 5.22. Variation of retention indices (based on alkylarylketone, butyrophenone - octanophenone) with acetonitrile concentration for model compounds separated on PS-DVB column. Conditions: as in Table 5.22.

gave tailing peaks which were worse than those with methanol as modifier (see Figures 5.17 and 5.18).

These effects were reflected in the relatively higher retention indices of the polar compounds with acetonitrile as the modifier (Table 5.23). Different selectivities were observed, particularly for the oxygen-containing compounds. On changing from methanol (see Figure 5.20) to acetonitrile (Figure 5.22), the decrease in the retention indices of these compounds with modifier concentration became smaller. Thus, modifiers can be used to influence selectivity in SFC.

5.5.4 Changes in conditions

Section 5.4 described the comparison of the selectivity between different stationary phases when pure carbon dioxide was used as the mobile phase. In order to further investigate the selectivity behaviour in SFC, these experiments were extended by carrying out the separations of the test compounds on ODS-silica column using methanol or acetonitrile as the modifier.

The capacity factors of the alkylarylketones, alkylbenzenes, and the model compounds on an Ultrasphere ODS column using methanol as the modifier were measured (Table 5.24) and compared with those obtained on the PS-DVB column with nominally the same eluents (Table 5.20). Again, the n-alkanes were not examined with modified carbon dioxide eluents as they are not detectable with a UV detector. On changing from zero to four percent methanol in the modifier, the capacity factors of the alkylarylketones on the ODS column decreased markedly, and they continued to decrease as the percentage of methanol was increased further. On the same column over the range of methanol concentration

TABLE 5.24. Capacity factors of alkylarylketones, alkylbenzenes, and model compounds on ODS-silica column at different methanol concentrations.

Conditions: Ultrasphere ODS 5 μm; primary eluent, carbon dioxide; mean pressure 2470 psi; 60 °C; UV detection, 254 nm.

Compound	Capacity factor				
	Methanol (% w/w)				
	0	4.0	8.3	12.7	16.4
Valerophenone	0.81	0.45	0.32	0.27	0.21
Hexanophenone	0.95	0.51	0.36	0.31	0.25
Heptanophenone	1.10	0.62	0.44	0.37	0.30
Octanophenone	1.30	0.73	0.51	0.42	0.34
Decanophenone	1.84	0.99	0.67	0.56	0.45
Dodecanophenone	2.52	1.31	0.88	0.72	0.58
Tetradecanophenone	3.47	1.73	1.23	0.92	0.74
Hexadecanophenone	4.74	2.26	1.445	1.16	0.93
Octadecanophenone	6.32	2.92	1.838	1.45	1.16
Toluene	0.21	0.24	0.23	0.24	
Ethylbenzene	0.28	0.29	0.27	0.29	
Propylbenzene	0.36	0.34	0.31	-	
Butylbenzene	0.46	0.42	0.34	0.34	
Benzaldehyde	0.47	0.23	0.18	0.16	
Methyl benzoate	0.51	0.29	0.22	0.20	
Benzyl alcohol	1.47	0.24	0.15	0.14	
Nitrobenzene	0.34	0.30	0.23	0.19	
p-Cresol	1.08	0.31	0.04	0.09	
N-Propylaniline	1.00	0.48	0.36	0.30	
Benzoic acid	--	0.55	0.38	0.25	
Benzamide	--	0.75	0.24	0.13	
Benzylamine	3.95	1.07	0.69	0.54	

0-12.7% (w/w) there was virtually no change in the retention of the alkylbenzenes. This is the expected behaviour for nonpolar compounds on a silica-based column since the competition for active sites is not important and at low modifier concentration it would not appreciably change the density of mobile phase. Small changes in capacity factors on the ODS column were also observed for nitrobenzene, benzaldehyde, and methyl benzoate on the addition of methanol to the mobile phase. Marked changes were however observed for the alcohol and phenol, benzoic acid, benzamide, and the amines. Benzoic acid and benzamide which were not eluted with carbon dioxide as the mobile phase were eluted with 4.3% methanol in the eluent in reasonably short times although they still showed slight tailing peaks (Figure 5.23). With methanol as the modifier, the other test compounds exhibited good peak shapes.

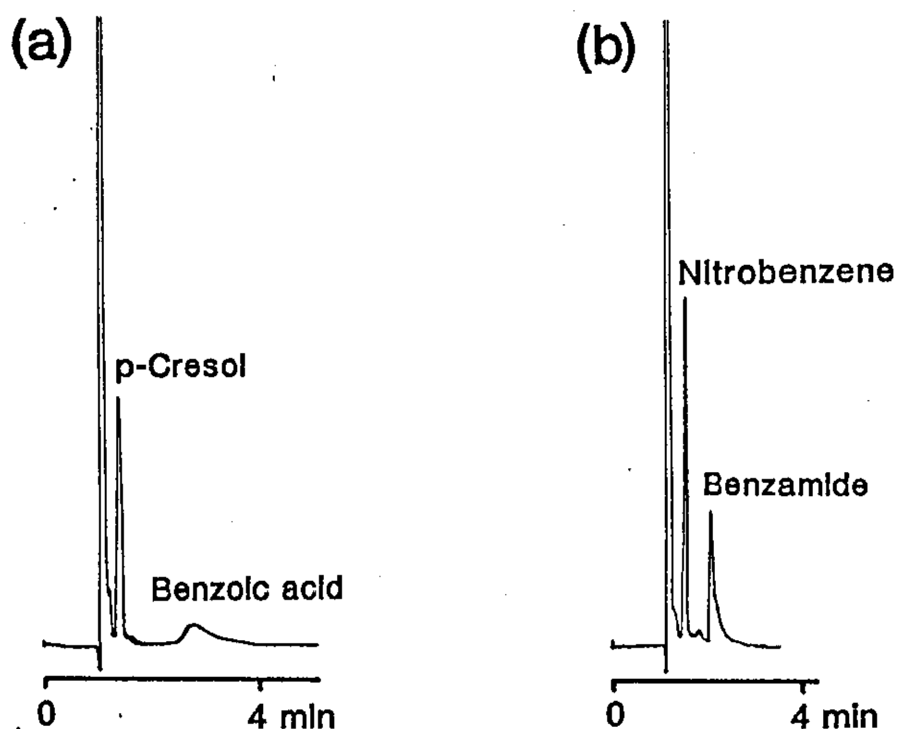


FIGURE 5.23. SFC separations of model compounds, (a) p-cresol and benzoic acid and (b) nitrobenzene and benzamide on ODS-silica column with 4% methanol in the mobile phase. Conditions: as in Table 5.24.

The retention indices on the ODS column with methanol as the modifier were determined (Table 5.25 and Figure 5.24) and compared to those obtained on the PLRP-S column (see Table 5.21 and Figure 5.20). In general, similar selectivities were observed in both cases suggesting that the selectivity was controlled mainly by the nature of the mobile phase. The retention indices of the alkylbenzenes increased steadily with increasing methanol concentration both on the Ultrasphere ODS and PLRP-S columns but to a lesser extent on the latter column. A marked difference was observed for benzylamine which showed sharply reduced indices on the ODS-silica column on the addition of methanol probably because of the interaction of the compound with free silanol groups in the packing material with carbon dioxide as eluent.

Another set of experiments were performed on the Ultrasphere ODS column with different concentrations of acetonitrile in the mobile phase. The capacity factors of the homologous series and the test compounds were determined (Table 5.26) and compared to those obtained on the PLRP-S column under same pressure conditions (see Table 5.22). Similar trends in retentions were observed but generally the test compounds were more retained on the PLRP-S column.

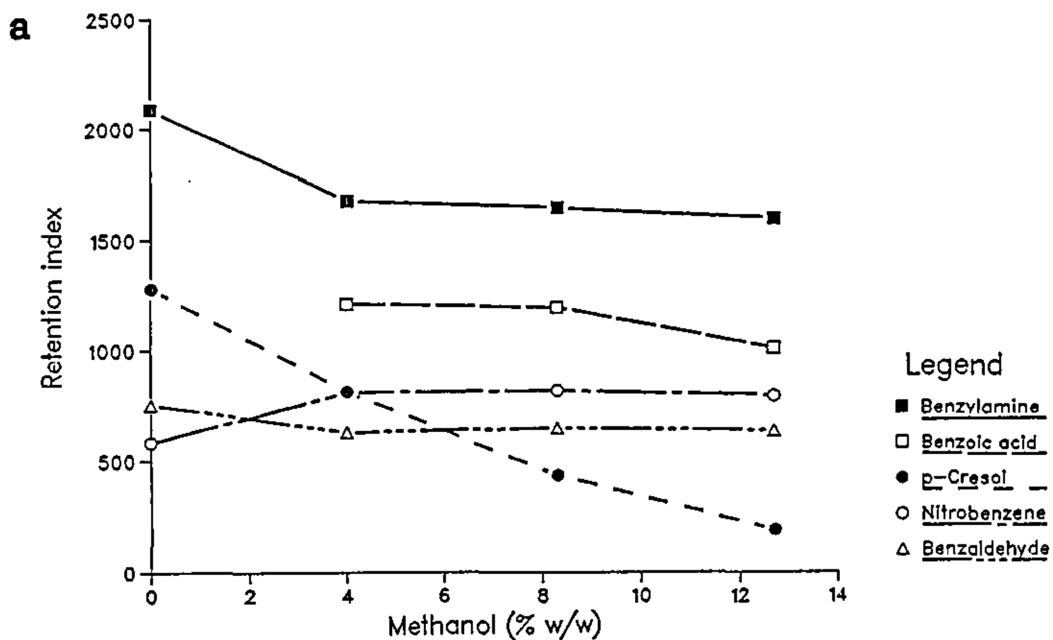
Marked differences were observed with the polar compounds. Within the experimental limitations, benzoic acid and benzamide were eluted with extremely broad peaks and thus retention time determinations of these compounds were not possible. One marked difference is in the retention of benzylamine. The retention indices of this compound on the ODS column (Table 5.27 and Figure 5.25) decreased drastically on changing from 0 to 3.7% acetonitrile whereas on the PLRP-S column the index values changed only slightly. It is possible that the marked reduction in the retention of benzylamine occurs at much lower

TABLE 5.25. Retention index values for alkylarylketones, alkylbenzenes, and model compounds on ODS-silica column at different methanol concentrations.

Conditions: as in Table 5.24.

Compound	Retention index*			
	Methanol (% w/w)			
	0	4.0	8.3	12.7
Valerophenone	1100	1083	1080	1065
Hexanophenone	1195	1171	1163	1185
Heptanophenone	1291	1307	1309	1306
Octanophenone	1395	1414	1420	1412
Decanophenone	1613	1623	1627	1631
Dodecanophenone	1809	1821	1824	1825
Tetradecanophenone	2008	2010	2011	2015
Hexadecanophenone	2204	2196	2194	2194
Octadecanophenone	2383	2374	2370	2367
Toluene	246	633	823	958
Ethylbenzene	428	771	955	1110
Propylbenzene	583	898	1062	-
Butylbenzene	740	1031	1127	1249
Benzaldehyde	753	630	651	639
Methyl benzoate	803	771	793	821
Benzyl alcohol	1473	656	535	503
Nitrobenzene	583	809	820	797
p-Cresol	1281	813	483	193
N-Propylaniline	1230	1124	1170	1154
Benzoic acid	-	1214	1200	1015
Benzamide	-	1430	849	473
Benzylamine	2089	1679	1650	1604

* Based on alkylarylketones, valerophenone - octadecanophenone.



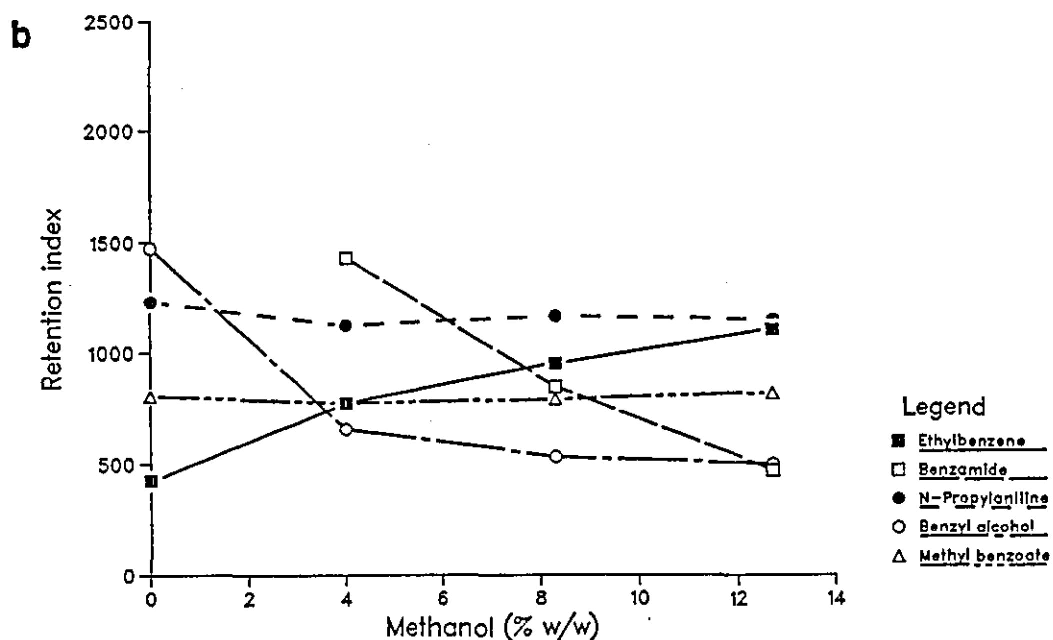


FIGURE 5.24. Variation of retention indices (based on alkylarylketones, valerophenone - octadecanophenone) with methanol concentration for model compounds separated on ODS-silica column. Conditions: as in Table 5.24.

TABLE 5.26. Capacity factors of alkylarylketones, alkylbenzenes, and model compounds on ODS-silica column at different acetonitrile concentrations.

Conditions: column, Ultrasphere ODS 5 µm; primary eluent, carbon dioxide; 60 °C; mean pressure 2470 psi; UV detection, 254 nm.

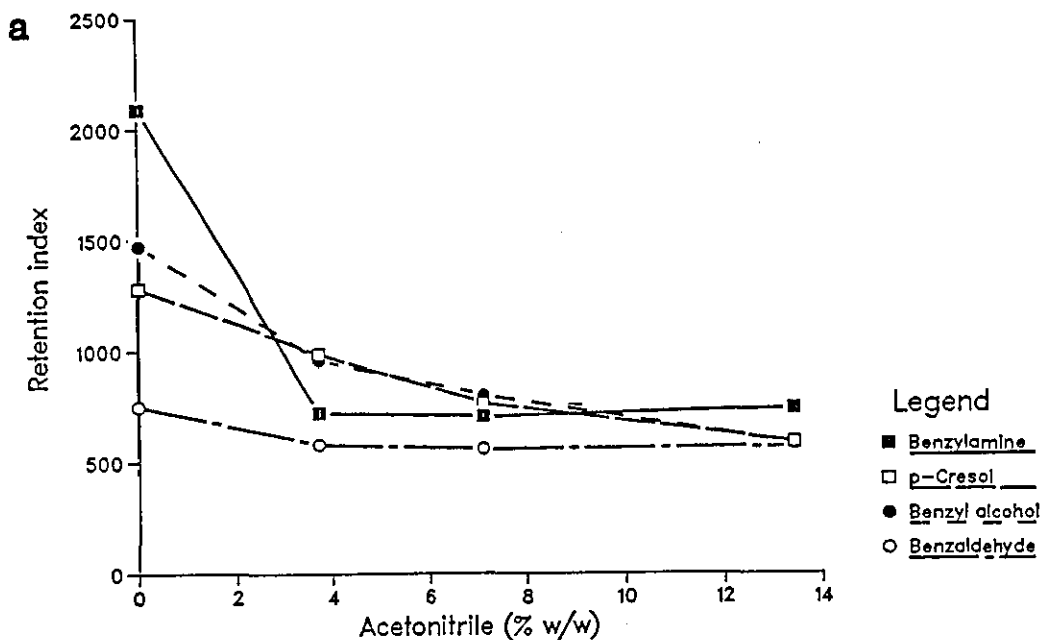
Compound	Capacity factor			
	Acetonitrile (% w/w)			
	0	3.7	7.1	13.4
Valerophenone	0.81	0.41	0.26	0.19
Hexanophenone	0.95	0.49	0.31	0.23
Heptanophenone	1.10	0.57	0.37	0.27
Octanophenone	1.30	0.66	0.43	0.32
Decanophenone	1.84	0.90	0.57	0.43
Dodecanophenone	2.52	1.20	0.75	0.56
Tetradecanophenone	3.47	1.59	0.94	0.72
Hexadecanophenone	4.74	2.07	1.23	0.91
Octadecanophenone	6.32	2.67	1.55	1.14
Toluene	0.21	0.21	0.21	0.19
Ethylbenzene	0.28	0.25	0.25	0.23
Propylbenzene	0.36	0.31	-	-
Butylbenzene	0.46	0.38	0.30	0.28
Benzaldehyde	0.47	0.20	0.13	0.10
Methyl benzoate	0.51	0.26	0.18	0.15
Benzyl alcohol	1.47	0.35	0.19	0.10
Nitrobenzene	0.36	0.24	0.15	0.10
p-Cresol	1.08	0.36	0.18	0.10
N-Propylaniline	1.00	0.50	0.34	0.28
Benzoic acid	-	-	-	-
Benzamide	-	-	-	-
Benzylamine	3.95	0.25	0.16	0.13

TABLE 5.27. Retention index values for alkylarylketones, alkylbenzenes and model compounds on ODS-silica column at different acetonitrile concentrations.

Conditions: as in Table 5.26.

Compound	Retention index*			
	Acetonitrile (% w/w)			
	0	3.7	7.1	13.4
Valerophenone	1100	1075	1056	1046
Hexanophenone	1195	1192	1187	1179
Heptanophenone	1291	1304	1307	1310
Octanophenone	1395	1405	1421	1424
Decanophenone	1613	1620	1633	1641
Dodecanophenone	1809	1920	1931	1938
Tetradecanophenone	2008	2013	2000	2018
Hexadecanophenone	2204	2197	2196	2190
Octadecanophenone	2383	2374	2369	2355
Toluene	246	592	906	1023
Ethylbenzene	428	730	1033	1185
Propylbenzene	583	880	-	-
Butylbenzene	740	1012	1163	1313
Benzaldehyde	753	582	561	573
Methyl benzoate	803	765	587	845
Benzyl alcohol	1473	962	807	587
Nitrobenzene	583	693	634	601
p-Cresol	1281	989	771	594
N-Propylaniline	1230	1209	1248	1326
Benzoic acid	-	-	-	-
Benzamide	-	-	-	-
Benzylamine	2089	726	710	748

* Based on alkylarylketones, valerophenone - octadecanophenone.



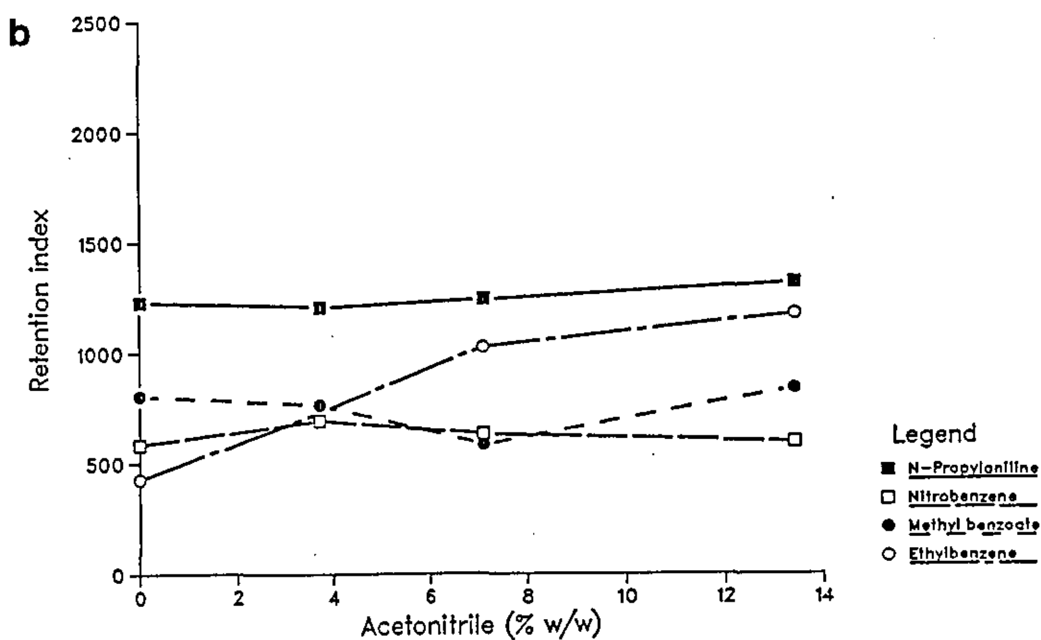


FIGURE 5.25. Variation of retention indices (based on alkylarylketones, valerophenone - octadecanophenone) with acetonitrile concentration for model compounds separated on ODS-silica column. Conditions: as in Table 5.26.

concentrations of acetonitrile (well below 4%) as suggested by the flat curve above this point.

Because of time constraints, a limited amount of work has been carried out on a cyano-silica column with a modifier in the mobile phase. Separations of several model compounds were carried out under the same pressure conditions using 8.0% and 12.8% methanol in the mobile phase and the capacity factors were determined (Table 5.28). Under the conditions of nominally identical methanol concentration in the mobile phase, the retention times and the capacity factors of the compounds on this column were longer and larger than those on an ODS-silica column (see Table 5.26). With methanol as the modifier, unlike on an ODS-silica column, the separations of polar compounds such as p-cresol, benzoic acid, and benzamide as well as the less polar compounds, on the cyano-silica column gave sharp, symmetrical peaks (Figure 5.26). With pure carbon dioxide as the mobile phase, separations of the polar compounds on the cyano-silica column showed tailing peaks (Figure 5.27). This suggested that interactions of some of the polar compounds with unreacted silanol groups in the column were largely eliminated by the addition of small amounts of organic modifier such as methanol. Some further work on SFC using cyano-silica packing materials and a modifier has subsequently been carried out in this laboratory by V.G. Evans [170].

5.6 ANOMALOUS RETENTION BEHAVIOUR OF ACETOPHENONE ON CYANO-SILICA

In the course of this study, anomalous retention behaviour of acetophenone has been observed on a cyano-silica column (Section 5.4). It was initially thought that this was due specifically to problems with deterioration of the sample as benzaldehyde (RI = 720 to 729) and

TABLE 5.28. Capacity factors of model compounds on cyano-silica stationary phase at two different methanol concentrations.

Conditions: column, Ultrasphere-CN, 5 μm; mean pressure: 2475 psi; 60 °C.

Compound	Capacity factor*	
	Methanol (% w/w)	
	8.0	12.8
Benzaldehyde	0.26	0.24
Methyl benzoate	0.22	0.17
Benzyl alcohol	0.79	0.56
Nitrobenzene	0.29	0.24
p-Cresol	0.72	0.51
N-Propylaniline	0.27	0.22
Benzoic acid	0.69	0.49
Benzamide	2.59	0.15
Benzylamine	0.54	0.45

* mean value of two determinations.

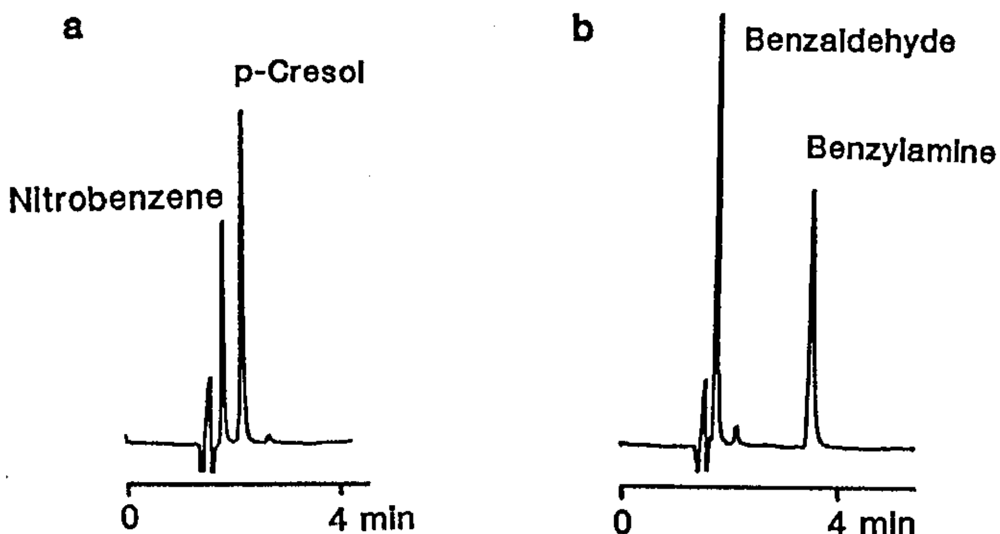


FIGURE 5.26. Separations by SFC of model compounds, (a) nitrobenzene and p-cresol and (b) benzaldehyde and benzylamine on cyano-silica column with 12.8% (w/w) methanol in the mobile phase. Conditions: as in Table 5.28.

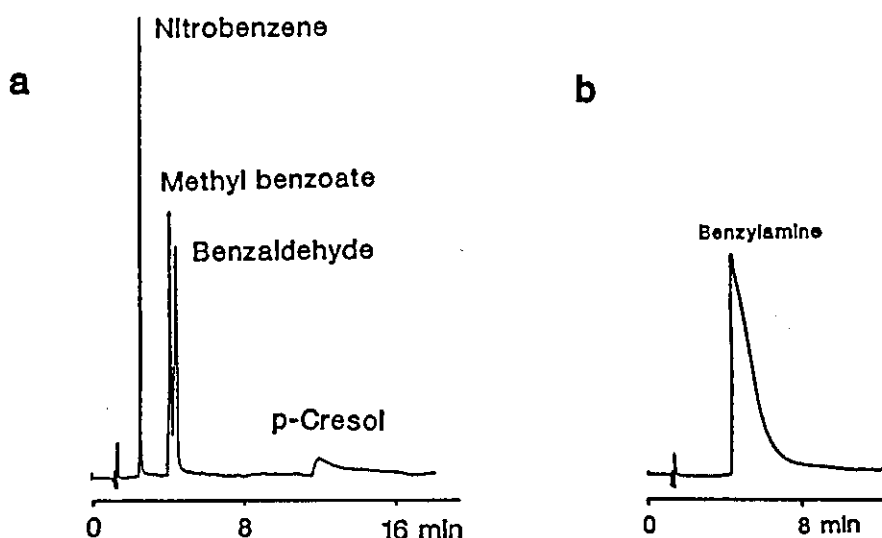


FIGURE 5.27. SFC separation of model compounds (a) nitrobenzene, methyl benzoate, benzaldehyde, and p-cresol and (b) benzylamine on cyano-silica column with carbon dioxide as the mobile phase. Conditions: column Ultrasphere CN 5 μm; mean column pressure 2440 psi; 60 °C.

propiophenone (RI = 1064 to 1159) which are expected to be eluted before and after acetophenone (RI = 1529 to 2185) did not show particularly abnormal retention under the same conditions (see Table 5.19). However, on using another acetophenone sample from another source, this anomaly persisted, and subsequent UV and IR analyses on the original acetophenone confirmed its identity, suggesting that this effect was not due to the sample.

In order to investigate this problem further, separations were carried out on another column (250 x 4.6 mm i.d.) packed with the same make of stationary phase (Ultrasphere CN 5 μm particles) under identical conditions with carbon dioxide as the mobile phase. However, similar anomalous retention of the compound was also observed (Table 5.29).

The results has led to the postulation that a specific reaction could be taking place between acetophenone and the particular cyano-silica column used. As the usage history of the column was not fully known, the next approach was to use a different brand, of preferably unused cyano-silica column. However, because of time limitations, this, and further investigations on this phenomenon have not been carried out in the present work.

The phenomenon was examined further in a subsequent study undertaken by V.G. Evans [170]. In his work, the alkylarylketone homologues were separated under identical SFC conditions but using two newly-packed columns (250 x 4.6 mm i.d.) packed with cyano-Spherisorb 7 μm particles and another packed with Shandon CPS-Hypersil 5 μm particles. His results have shown that the retention of acetophenone on the cyano-Spherisorb column behaved anomalously in a very similar manner to that previously seen on the cyano-Ultrasphere column. Acetophenone was eluted between hexanophenone and octanophenone, and its retention on

TABLE 5.29. Capacity factors of benzaldehyde and alkylarylketones on cyano-bonded silica columns obtained in this study and from Ref. [170] Conditions: mobile phase, carbon dioxide; 60 °C.

Column	Density (g/ml)	Capacity factor						
		Compounds*						
		0	1	2	3	4	5	6
Data from this work								
Cyano-Ultrasphere (2 UEC 201N)	0.533	4.06	7.90	5.37	5.64	-	7.30	8.49
Cyano-Ultrasphere (3 UEC 122N)	0.533	7.15	15.23	-	10.96	-	13.89	16.47
Data from ref. [170]								
Cyano-Spherisorb (19/277)	0.440	5.40	10.78	8.49	8.64	10.78	13.43	-
Cyano-CPS-Hypersil (12/1423)	0.429	1.04	1.38	-	1.45	-	-	-

* Compounds: 0. benzaldehyde, 1. acetophenone, 2. propiophenone 3. butyrophenone, 4. hexanophenone, 5. octanophenone, 6. decanophenone

the column increased relative to the other homologues with increasing pressure (density) (Table 5.29). However, no anomalous retention behaviour of acetophenone was observed on the Shandon CPS-Hypersil; it was eluted between benzaldehyde and butyrophenone and its retention did not vary significantly with changes in eluent density (Table 5.29).

Although further investigations may still be required, these early results suggest the presence of highly steric or probably specific interaction(s) between acetophenone and particular cyano packing materials which are not common to the other members of the homologous series.

5.7 CONCLUSIONS

The effect of pressure, temperature, density, composition of the mobile phase, and stationary phase on the retention of the retention index standards and the model compounds have been studied. Any one or a combination of these factors can be used to vary the selectivity of the system so as to optimize the separation.

The use of modifiers in SFC has been shown to have tremendous potential for generating highly selective and specific mobile phase. From this study, certain trends of solute behaviour have been revealed which will allow better predictions of retention characteristics for various analytes with diverse interactions.

Retention indices have been used as a convenient means to record the selectivity changes with changes in the operating parameters. As a method of recording retention, n-alkane and alkylarylketone homologous series have been shown to be less sensitive to changes in the variables compared to capacity factors and they have good potential for use in

SFC. Unlike the alkanes, the alkylarylketones are detectable by UV detection and can be employed as the retention index standards in separations in which organic modifier is used and flame ionization detection is not possible. However, the retention indices in SFC are still more susceptible to changes in selectivity than in HPLC.

CHAPTER 6

APPLICATION OF SFC TO THE ANALYSIS OF BARBITURATES

6.1 INTRODUCTION

6.1.1 Barbiturates

The barbiturates are a group of drug compounds which are derivatives of barbituric acid, formed by replacement of both hydrogen atoms at position 5 by alkyl, aryl, or alicyclic groups (Table 6.1). Many of the barbiturates are also known by names which end with the suffix "al", which has been used to denote hypnotics [171]. The barbiturates are the most widely used of the sedative hypnotics and anticonvulsants which makes them useful in the asymptomatic treatment of epilepsy, and they can also be employed intravenously to effect surgical anaesthesia [171].

Although variations in the pharmacological properties of the various barbiturates depend on the nature of the 5-substituted entities [171], the chemical group of major importance is the imido hydrogens. It has also been long recognized that in chromatographic analyses, adsorption could result from hydrogen bonding of the imido-protons of the barbiturates with Si-OH and Si-O-Si groups in the stationary phase [172].

All the barbiturates are colourless crystalline compounds with melting points between 96 and 205 °C, and they are not very soluble in water. Barbiturates are generally prescribed as the sodium salts which are water-soluble. Acidification of solutions of the alkaline sodium salts solutions forms the free acids which can be extracted with organic

TABLE 6.1. Structures and molecular weights of Barbiturates

General structure

(General structure: pyrimidine-2,4,6-trione ring with substituents R_5 and R'_5 at position 5, R_1 on N-1, and H on N-3; ring positions numbered 1–6.)

Generic name	Substituents			Mol. Wt.
	R_5	R'_5	R_1	
Barbitone	CH_3CH_2-	CH_3CH_2-	H	184.20
Butobarbitone	CH_3CH_2-	$CH_3CH_2CH(CH_3)-$	H	212.25
Talbutal	$CH_2=CHCH_2-$	$CH_3CH_2CH(CH_3)-$	H	224.26
Amylobarbitone	CH_3CH_2-	$CH_3CH(CH_3)CH_2CH_2-$	H	226.28
Pentobarbitone	CH_3CH_2-	$CH_3CH_2CH_2CH(CH_3)-$	H	226.28
Phenobarbitone	CH_3CH_2-	phenyl (C_6H_5-)	H	232.24
Quinalbarbitone	$CH_2=CHCH_2-$	$CH_3CH_2CH_2CH(CH_3)-$	H	238.29
Heptabarbitone	CH_3CH_2-	cyclohepten-1-yl	H	250.30
Methohexitone	$CH_2=CHCH_2-$	$CH_3CH_2C{\equiv}CCH(CH_3)-$	CH_3-	262.31

solvents for further analyses [171].

The barbiturates in the present investigation have a broad range of UV maximum absorbances which is also dependent on the type of solution (e.g. in alkaline solution barbitone λ max 244 nm and phenobarbitone λ max 253 nm [148]). Nevertheless all the compounds are easily detectable by a UV detector at 254 nm and this method has been used in previous work on HPLC [145,173].

6.1.2 Analysis of barbiturates

Sensitive and reliable methods for the determination of barbiturates are of great interest in the therapy of epilepsy and in barbiturate toxicology. In such cases instrumental methods are particularly important but classical procedures are often used in conjunction [148,174]. Besides chromatographic techniques, numerous methods have been used for the determination of barbiturates, including ultraviolet and infrared spectrophotometry, fluorometry, NMR spectroscopy, mass spectrometry, as well as enzymic and radioimmunoassays [148,175]. The literature contains several methods for the separation of barbiturates based on GC [176] and HPLC [145,173,174]. However, apart from the preliminary note from this laboratory [65], no previous studies have reported the separation of barbiturates using SFC.

(a) Gas chromatography

Gas chromatography offers high-efficiency fast separations combined with selective and sensitive detectors, such as the nitrogen-phosphorus detector (NPD) suitable for the analysis of drug compounds. However, the analysis of barbiturates by GC is difficult because of adsorption on the

column. During the GC separation of these compounds, adsorption of sub-microgram quantities on the chromatographic column is considered common [176]. The consequences of adsorption are the loss of material, column contamination, unsatisfactory peak profiles (tailing), and retention time variations with sample loading [176].

In the early work on barbiturate analysis using GC, Cieplinski [177] incorporated high molecular-weight inorganic acids into the stationary phase to neutralize active sites in the column and reduce peak tailing. Another way to combat these problems is by using a well deactivated column for the GC separation. This was demonstrated by Sandra et al. [178] who successfully chromatographed free barbiturates by GC on a HCl-leached glass capillary column, silylated at 400 °C and coated with OV-1. However, most GC analysis of barbiturates extracts have been carried out by derivatization (usually to give N,N-dialkyl derivatives) prior to analyses. The primary objective in the derivatization has been the reduction of polarity of the free acid due to the conversion from secondary to tertiary imides.

(b) High performance liquid chromatography

HPLC has been widely used in the analysis of barbiturates and other drug compounds [175,179]. Since barbiturates are weak acids, early liquid chromatographic separations were obtained on ion exchange resins. Anders and Latorre in 1970 separated five barbiturates using a gradient eluent with an anion exchange column [180].

Bowman et al. [181] analysed phenobarbitone by HPLC on Waters Bondapak ODS column using methanol-water (40:60) as eluent. A suitable method for the determination of trace amounts of barbiturates in blood and saliva has been developed by Tjaden et al. [182]. Extracts were

analyzed on a 100 x 2.8 mm i.d. column of methylsilyl silica using methanol-water (50:50) as eluent and UV detection at 220 nm. Detection limits of around 3 ng were obtained

In 1981, Gill et al. developed and described a HPLC assay for identification and quantification of barbiturates in blood samples at therapeutic levels and applied it to forensic casework [183]. Since then, numerous reports have decribed the analysis of barbiturates by HPLC, including that of Haginaka et al. [184] who used a hollow-fibre membrane for post-column pH modification.

6.1.3. Retention indices in drug analysis

Accurate determination and identification of drug samples is paramount in forensic and diagnostic drug analyses. Confidence can be improved by using a combination of different analytical techniques. Common in all chromatographic techniques, the identity of the unknown sample can be deduced by direct comparison with an authentic standard sample or sometimes with multiple standards. However, in most HPLC applications, this method has often proved to be time-consuming [185]. Capacity factors (k') have been widely used to record retention in HPLC but this method has been found to be difficult to reproduce between different laboratories or in different systems in the same laboratory. This has prompted a series of investigations on the use of retention indices and other methods as alternatives to capacity factors for recording the results of drug separations by HPLC [186-191]. In a recent article on the application on of retention indices to drug identification, Smith et al. [185] concluded that reproducibility results can be improved by the use of relative method of recording

retentions. They further remarked that retention indices relative to a homologous series such as the alkylarylketones offer a more versatile reference scale that is almost totally independent of small changes in eluent composition and separation conditions [185]. However, in a study on barbiturates, retention indices based on a drug internal standard has been found to be best because it can compensate for differences in retention caused by ionization [145].

6.1.4 Aim of the study

In toxicological drug analysis, accurate, sensitive, and high-speed analytical methods are most desirable. The capability of high-speed analysis and the compatibility with many detectors offered by SFC, makes it attractive as an alternative chromatographic technique in drug analysis. The present study investigates the application of packed column SFC to the separation of barbiturates. Nine barbiturates with different structures and pharmacological properties have been used in the study (Table 6.1). The influence of variations caused by the column packing material and the mobile phase to the selectivity of the separation have been examined. The study also set out to compare whether alternative methods to capacity factors for recording retention values, such as the retention indices, might be more robust and less susceptible to the variations in conditions. The applicability of retention indices in SFC to the analysis of barbiturates was therefore examined. Supercritical fluid chromatographic retention and selectivity of the barbiturates were compared with results reported in the literature for HPLC methods.

6.2 SFC SEPARATIONS OF BARBITURATES ON PS-DVB COLUMN WITH CARBON DIOXIDE AS THE MOBILE PHASE

The barbiturates contain secondary imido groups which contribute to their high polarity. It was therefore of interest to determine whether the high polarity of a weakly acidic analyte would permit separations to be carried out on either a non-polar poly(styrene-divinylbenzene) (PS-DVB) or ODS-silica, with its residual silanols, using non-polar supercritical carbon dioxide as the mobile phase. However, when samples were injected onto the ODS-bonded silica column (200 x 3 mm i.d., packed with Spherisorb ODS-2 5 μm particles), none of the barbiturates under investigation were eluted with carbon dioxide as the mobile phase over a range of operating pressures [65]. This can probably be explained by strong specific interactions between the polar functional groups and residual free silanol groups on the stationary phase. This assumption is consistent with previous suggestions [70,112] in SFC that imido and other polar substituents, carboxy and amino groups, contributed strongly to retention on silica adsorption systems.

The separation was therefore studied using a PS-DVB polymer column which should not show polar-polar interactions. On this column the barbiturates were eluted at a moderate pressure with reasonable retention times. The capacity factors of the barbiturates generally decreased with pressure (Table 6.2). Unlike reversed phase HPLC, when elution is in the order of increasing molecular size [145], the capacity factors of the compounds did not always correspond with the molecular weight order. For instance, talbutal (MW = 224.26) was eluted after pentobarbitone and amylobarbitone (both MW = 226.28). Phenobarbitone and heptabarbitone appeared to be particularly strongly retained while the capacity factors

of the other barbiturates were very close to each other.

The retention indices based on n-alkanes and alkylarylketones were determined (Table 6.2). Although in the SFC separation the retention indices of the barbiturates on both scales decreased with increased pressure, the changes in retention indices based on alkylarylketones appeared to be greater than the corresponding values based on n-alkanes, suggesting selectivity differences of the groups with pressure and solvating power of the fluid. It was also seen that the retention index values based on alkylarylketones were generally greater than the corresponding values reported in the literature for HPLC on ODS-silica (e.g. methanol-aqueous buffer (40:60), barbitone RI = 579.0, quinalbarbitone RI = 930.4) [145].

Unfortunately, even when using a polymer column, peak tailing and adsorption remained a significant problem with all the barbiturates. This can be demonstrated by the separation of barbitone and quinalbarbitone (Figure 6.1). In contrast, peak tailing was not observed for the n-alkane or the alkylarylketone retention index standards (Chapters 4 and 5). Since silanol groups are absent from the polymer column, this means that the tailing effects observed for the barbiturates cannot be solely because of solute-silanol interactions, and that a more complex mechanism may be involved in the retention of these polar compounds.

Previous reports [70,112] and results obtained in the present study (Chapter 5) have suggested that the addition of organic modifier, such as methanol or acetonitrile, as modifier to the carbon dioxide mobile phase can give an improved separation of polar compounds. The separations of the barbiturates were then studied using modified carbon dioxide mobile phase.

TABLE 6.2. Capacity factors and retention indices based on n-alkanes and alkylarylketones for barbiturates at two different column pressures.

Conditions: column (150 x 4.6 mm i.d.), PLRP-S 5 µm; carbon dioxide mobile phase; 60 °C; flame ionization detection.

Compound	Pressure: 2200 psi			Pressure: 2660 psi		
	k'	RI-HC	RI-AAK	k'	RI-HC	RI-AAK
Barbitone	5.32	1771	1207	3.17	1746	1154
Metohexitone	6.16	1835	1274	3.43	1786	1195
Amylobarbitone	6.55	1862	1301	3.57	1805	1216
Butobarbitone	6.51	1860	1299	3.70	1822	1234
Pentobarbitone	6.56	1863	1303	3.78	1833	1245
Talbutal	7.50	1922	1364	4.30	1896	1312
Quinalbarbitone	8.34	1968	1412	4.54	1923	1340
Phenobarbitone	-	-	-	13.28	2448	1894
Heptabarbitone	-	-	-	13.69	2463	1909

Note: retention indices,
RI-HC = based on n-alkanes, dodecane - octacosane.
RI-AAK = based on alkylarylketones, butyrophenone - heptanophenone.

TABLE 6.3. Comparison of capacity factors of selected barbiturates with methanol and acetonitrile as the modifier on PS-DVB column.

Conditions: Column PLRP-S 5 µm; primary eluent carbon dioxide; 60 °C; mean column pressure 2220 psi; UV detection (254 nm).

Compound	Capacity factor			
	Methanol (% w/w)		Acetonitrile (% w/w)	
	4	9	4	9
Barbitone	1.34	0.67	2.11	0.87
Amylobarbitone	1.74	0.78	2.72	-
Phenobarbitone	4.87	1.91	7.99	2.74

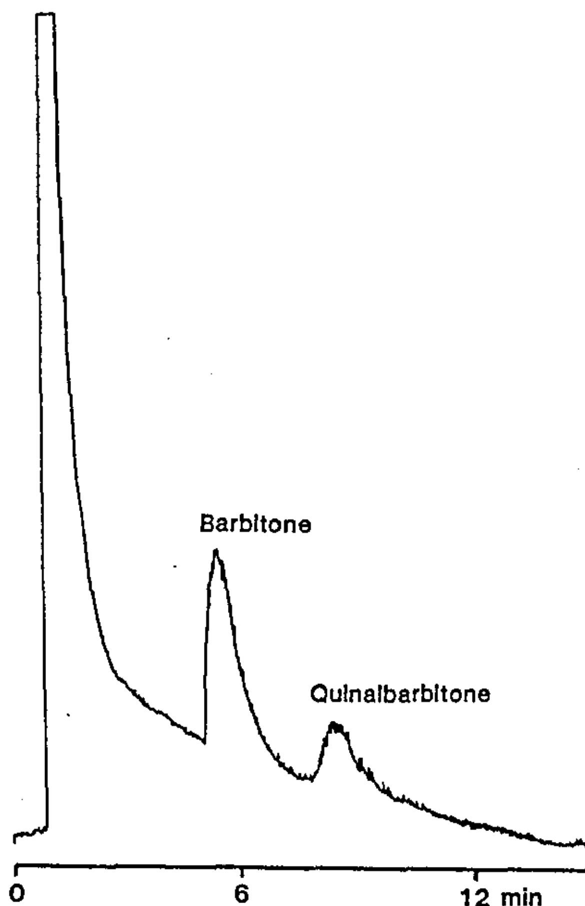


FIGURE 6.1. SFC separations of barbiturates, barbitone and quinalbarbitone on PS-DVB column with carbon dioxide as the mobile phase. Column PLRP-S 5 μm; mean column pressure 2200 psi; 60 °C; flame ionization detection.

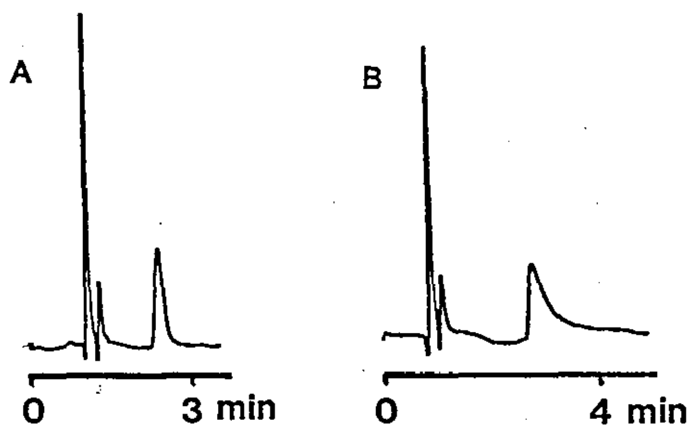


FIGURE 6.2. SFC separations of barbitone on PS-DVB column with different modifiers in the mobile phase, (a) methanol (4% w/w) and (b) acetonitrile (4% w/w). Conditions: as in Table 6.3.

6.3 SFC SEPARATIONS OF BARBITURATES ON PS-DVB COLUMN WITH MODIFIED CARBON DIOXIDE AS THE MOBILE PHASE

On initial trial runs it was clear that the addition of small amounts (4%) of modifier, such as methanol caused problems with the background signal from the flame ionization detector (see Section 5.6). Consequently, a UV absorption detector with a high-presssure flow cell was employed in this work and the samples were detected at 254 nm. Other wavelengths can also be used to optimize the detection of specific barbiturates, e.g. Gill et al. [183] detected 5 barbiturates using UV detection at 240 nm.

6.3.1 Effects of different modifiers

It was of interest initially to find out whether similar effects would be observed using chemically different modifiers. Three barbiturates, barbitone, amylobarbitone, and phenobarbitone, were first chromatographed using carbon dioxide modified with 4% methanol and under nominally identical conditions, another set of separations were performed using acetonitrile as the modifier. The capacity factors of the barbiturates were calculated and compared (Table 6.3). It was found that the capacity factors of the barbiturates obtained using methanol as the modifier appeared to be smaller than the corresponding values obtained using acetonitrile as the modifier.

In addition, with nominally the same modifier concentration, in all cases, significantly better peak shapes were observed with methanol as the modifier as compared to acetonitrile. This is illustrated in Figure 6.2 for the separation of barbitone. These results suggest that

interactions can occur between the analytes and the mobile phase and probably because being more polar, methanol had better capability to increase the solvating power of the eluent for the polar analytes compared to acetonitrile. These also suggest that the separation of barbiturates can be improved by optimizing the quantity as well as the nature of the modifier in the mobile phase. Further analyses of the barbiturates were then carried out using methanol as the modifier.

6.3.2 Chromatographic behaviour of barbiturates on PLRP-S column using methanol as the modifier

In order to study the chromatographic behaviour, the separations of the barbiturates and the alkylarylketone standards were carried out on the PLRP-S column with different methanol concentrations over the range of 4.0-14.6% (w/w).

All the barbiturates were eluted within a reasonably short time with good peak shapes using 4% methanol in the mobile phase as illustrated in Figure 6.3a for the separation of barbitone and phenobarbitone. Decreased retention time and sharper peak shapes were observed for the separation of barbiturates using higher concentrations of methanol in the mobile phase. The separation of quinalbarbitone and heptabarbitone on the PLRP-S column using 9% methanol in the mobile phase was achieved in less than four minutes with good peak shapes (Figure 6.3b). Faster analysis times were obtained using higher percentages of methanol in the mobile phase. Figure 6.3c illustrates the separation of barbitone, methohexitone, and phenobarbitone on the same column with 14.6% methanol in the eluent, which shows good peak shapes.

The capacity factors of the barbiturates and alkylarylketone

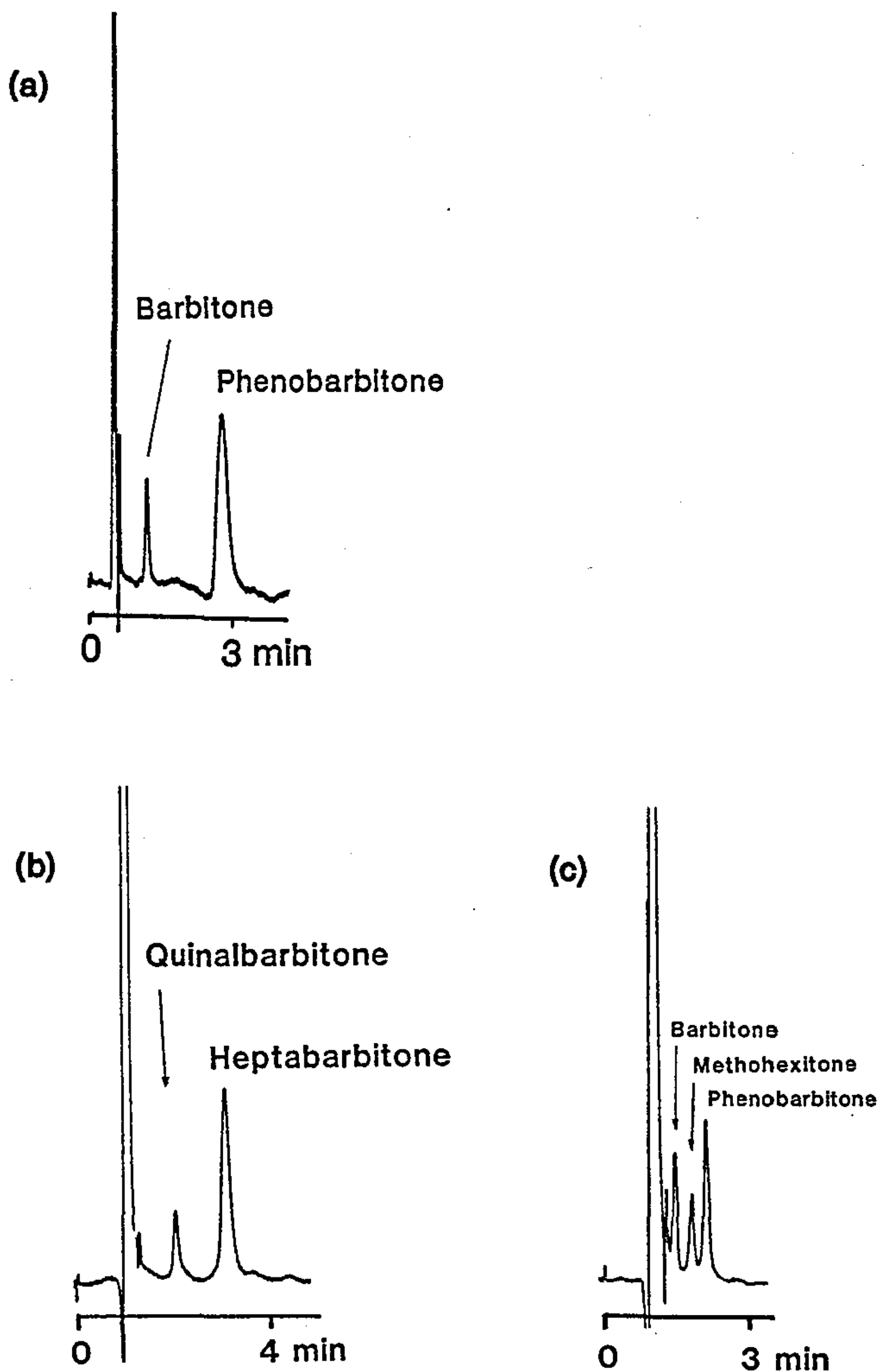


FIGURE 6.3. Effect of methanol concentration on the separation of barbiturates on PS-DVB column. Conditions: column PLRP-S 5 µm; mean column pressure 2200 psi; 60 °C; UV detection (254 nm). Methanol concentration: (a) 4%, (b) 9%, and (c) 15%.

standards were calculated (Table 6.4). The retentions of the barbiturates on the PLRP-S column were reduced drastically with the addition of modifier in the mobile phase (from 0 to 4%) (Figure 6.4). This can probably be attributed mainly to the modification of the stationary phase. Further increasing the methanol concentration in the mobile phase resulted in a continuous but gradual decrease in the capacity factors suggesting increasing solubility of the solutes in the mobile phase. Similar effects have been observed previously (Chapter 5) for other polar compounds.

Phenobarbitone and heptabarbitone which contain a phenyl and unsaturated alicyclic substituents respectively, were found to be more retained than barbiturates with straight-chain hydrocarbon substituents of comparable or higher molecular weights (e.g. quinalbarbitone or methohexitone) (Table 6.4). This effect suggests greater interactions can occur between the stationary phase and the barbiturates with electron-rich unsaturated alicyclic or aromatic substituents.

It was also observed that talbutal which has an allyl substituent was considerably more retained than other barbiturates with similar molecular weights (pentobarbitone and amylobarbitone), again suggesting that unsaturated hydrocarbon substituents tend to increase the retention on the polymer column.

Pentobarbitone was eluted in about the same time as amylobarbitone. Because these compounds have the same molecular formulae, the small differences in retention for these compounds were probably due mainly to differences in steric hindrance to adsorption which is recognized as an important and frequently encountered phenomenon in SFC [64]. One of the substituents (R_5', Table 6.1), in pentobarbitone is a sec-pentyl group whereas for amylobarbitone contains an iso-pentyl group.

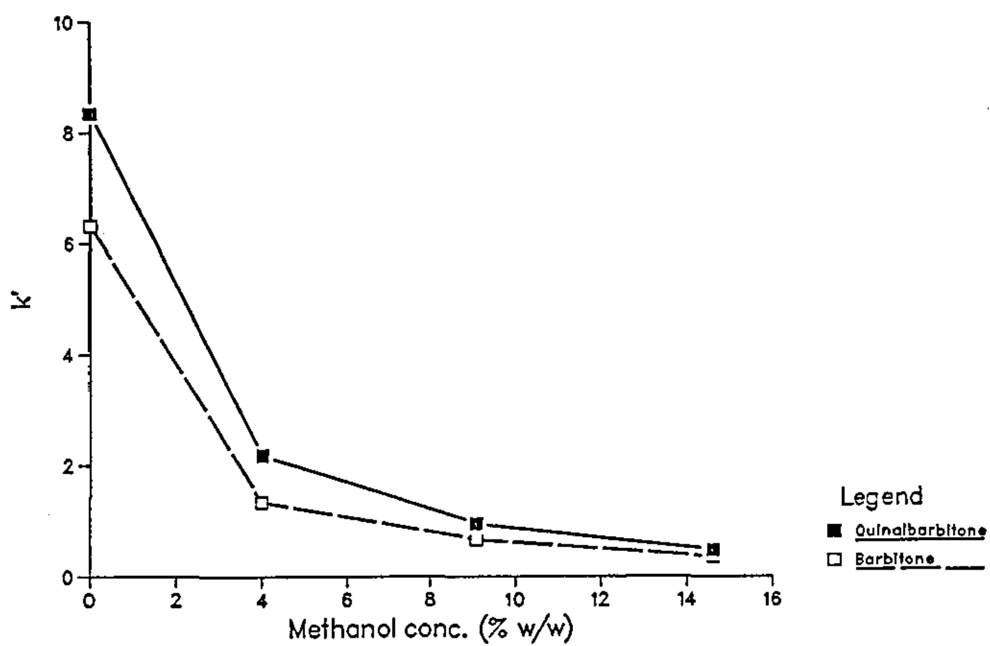


FIGURE 6.4. Variation of capacity factors with methanol concentration for two barbiturates, quinalbarbitone and barbitone on PS-DVB column. Conditions: as in 6.4.

TABLE 6.4. Capacity factors of barbiturates and alkylarylketones at different methanol concentrations on PS-DVB column.

Conditions: column (150 x 4.6 mm i.d.), PLRP-S 5 µm; primary eluent carbon dioxide; mean column presure 2200 psi; 60 °C; UV detection (254 nm).

Compound	Capacity factor			
	Methanol (% w/w)			
	0	4.0	9.1	14.6
Barbiturates				
Barbitone	5.32	1.34	0.67	0.37
Butobarbitone	6.51	1.73	0.78	0.39
Amylobarbitone	6.55	1.74	0.78	0.40
Pentobarbitone	6.56	1.75	0.79	0.39
Talbutal	7.50	2.06	0.92	0.47
Quinalbarbitone	8.34	2.19	0.95	0.48
Methohexitone	6.16	2.27	1.11	0.69
Phenobarbitone	>24	4.87	1.91	0.91
Heptabarbitone	>24	5.07	1.98	0.98
Alkylarylketones				
Acetophenone	2.27	1.33	0.89	0.67
Propiophenone	2.99	1.70	1.07	0.78
Butyrophenone	3.41	1.85	1.17	0.87
Valerophenone	4.15	2.11	1.36	0.99
Hexanophenone	5.21	2.56	1.59	1.14
Heptanophenone	6.57	3.06	1.85	1.31

Although generally the retention of the barbiturates decreased with the addition of methanol to the mobile phase, a marked difference in the retention behaviour was observed for methohexitone. With pure carbon dioxide mobile phase, methohexitone was eluted relatively quickly (k' = 6.1), second only to barbitone (Table 6.4). However, with the addition of methanol in the mobile phase, only a minor decrease in the retention was observed for this compound, compared to the other barbiturates and under these conditions it was eluted after quinalbarbitone. This marked behaviour can probably be explained if its molecular structure is considered. Methohexitone, unlike the other barbiturates studied, has a methyl group substituted on imide nitrogen at the 1-position (Table 6.1) which effectively reduced the polarity and the acidity of the compound. In reversed phase HPLC, increasing the number of methyl groups has the effect of increasing the retention which explains the relatively high capacity factor values of methohexitone compared to the other barbiturates (e.g. methanol-buffer (40:60) eluent, methohexitone k' = 25.68, heptabarbitone k' = 6.14, barbitone k' = 0.74) [145].

Previous workers [34,74] have suggested that selective interactions can occur in SFC between the polar substituents of the analytes with a polar organic modifier and that the modifier changes the solvent power of the mobile phase with increased selectivity for the more polar compounds. Therefore, because of the lower polarity of methohexitone, it was selectively more retained on the column compared to the other barbiturates as reflected in its smaller decrease in capacity factors with increasing percent methanol (Table 6.4). This trend suggests that packed column SFC with carbon dioxide mobile phase yields complex retention characteristics which in addition to the reversed phase type

separation, normal phase type of separation can also be observed in which non-polar analytes elute first and compounds with more polar groups are more highly retained.

In order to determine the selectivity behaviour of the separation, the retention indices based on alkylarylketone standards were calculated (Table 6.5). The retention indices of the barbiturates generally decreased significantly over the methanol concentration 0-14.6% suggesting differences in selectivity between the barbiturates and the alkylarylketone standards. Except for methohexitone, the observed changes in the retention indices (ΔRI) of the barbiturates were very large (greater than 795 units). Great decreases in retention indices have also been observed earlier for polar model compounds, benzoic acid benzamide, and for alcohols (Chapter 5). Methohexitone, being less polar than the other test barbiturates was less affected by variations in the modifier concentration which was reflected in the significantly smaller variations in the retention index values (ΔRI = 432).

In the study of the barbiturate analysis using HPLC, relative capacity factors compared to a standard barbiturate (quinalbarbitone) have been found to be even more reproducible than retention indices [185]. Therefore, similar analysis was performed on the SFC results and the relative capacity factors of the barbiturates were calculated (compared to quinalbarbitone, relative capacity factor = 100) (Table 6.6). It was seen that in general better reproducibility was obtained using relative capacity factor compared to a barbiturate standard although the changes were still significantly greater than in HPLC. This is what one would expect since the the use of a barbiturate standard would also compensate for differences in the degree of specific and steric interactions of the analytes with the stationary phase. However,

TABLE 6.5. Retention indices of barbiturates based on alkylarylketone standards at different methanol concentrations on PS-DVB column.

Conditions: as in Table 6.4.

Compound	Retention index*			
	Methanol (% w/w)			
	0	4.0	9.1	14.6
Barbiturates				
Barbitone	1207	829	641	378
Butobarbitone	1299	974	740	424
Amylobarbitone	1302	976	734	442
Pentobarbitone	1303	978	742	433
Talbutal	1364	1072	848	567
Quinalbarbitone	1412	1106	867	570
Methohexitone	1274	1126	966	842
Phenobarbitone	>1908	1558	1321	1040
Heptabarbitone	>1922	1582	1342	1089
Alkylarylketones				
Acetophenone	820	825	820	811
Propiophenone	945	964	942	924
Butyrophenone	1005	1010	1002	1006
Valerophenone	1094	1094	1099	1096
Hexanophenone	1198	1196	1200	1196
Heptanophenone	1303	1296	1299	1299
Octanophenone	-	1397	1400	1399
Decanophenone	-	1606	1601	1603

* Based on alkylarylketones, butyrophenone - decanophenone, except for 0% methanol, which was based on butyrophenone - octanophenone.

TABLE 6.6. Effect of methanol concentration on the relative capacity factors of barbiturates on PS-DVB column.

Conditions: as in Table 6.4.

Compound	Relative capacity factor*			
	Methanol (% w/w)			
	0	4.0	9.1	14.6
Barbitone	63.8	61.2	70.5	77.1
Butobarbitone	78.1	79.0	82.1	81.3
Amylobarbitone	78.5	79.5	82.1	83.3
Pentobarbitone	78.7	79.9	83.2	81.3
Talbutal	89.9	94.1	96.8	97.9
Quinalbarbitone	100	100	100	100
Methohexitone	73.9	104	117	144
Phenobarbitone	>288	222	201	190
Heptabarbitone	>288	232	208	204

* Compared to quinalbarbitone (relative capacity factor = 100).

marked variations were observed for methohexitone, phenobarbitone, and heptabarbitone which reflects differences in the selectivities of these compounds relative to quinalbarbitone.

6.4 COMPARISON OF SFC SEPARATIONS OF BARBITURATES ON ODS-SILICA AND PS-DVB STATIONARY PHASES WITH METHANOL AS THE MODIFIER

As has been described earlier, with pure carbon dioxide as the mobile phase, the barbiturates were very strongly retained on an ODS-silica column. Investigations was therefore carried out to examine the use of methanol-modified carbon dioxide as the mobile phase.

A similar series of separations to the study on PS-DVB, was performed on an Ultrasphere ODS column with two different methanol concentrations in the mobile phase. Sharp peaks were observed for the barbiturates as illustrated by the separations of barbitone and heptabarbitone (Figure 6.5a) and of amylobarbitone and phenobarbitone (Figure 6.5b). However, slight tailing was observed for some peaks, particularly for the more retained compounds such as phenobarbitone and heptabarbitone suggesting that the specific interactions between the polar analytes and the stationary phase have not been totally eliminated.

6.4.1 Retention and selectivity

The capacity factors were measured with two different eluent compositions (4.2% and 8.4% methanol) (Table 6.7). Identical mobile phase compositions and very similar operating conditions to the experiments with PLRP-S, were used (except that the mean column pressure

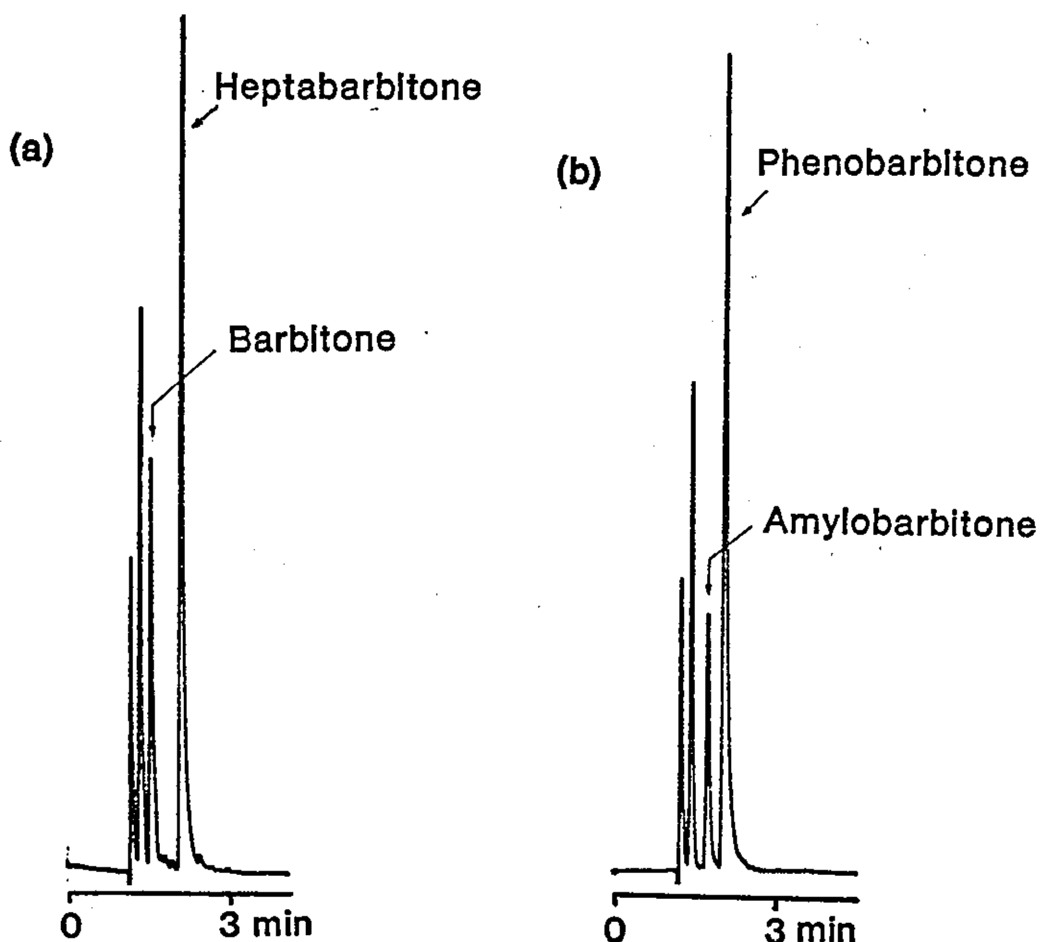


FIGURE 6.5. SFC separations of barbiturates, (a) barbitone and heptabarbitone and (b) amylobarbitone and phenobarbitone on ODS-silica column with 4.2% methanol in the mobile phase. Conditions: column Ultrasphere ODS 5 µm; mean column pressure 1950; 60 °C; UV detection (254 nm).

TABLE 6.7. Capacity factors of barbiturates and alkylarylketones on ODS-silica column at different methanol concentrations.

Conditions: column (250 x 4.6 mm i.d.) Ultrasphere ODS 5 μm; primary eluent carbon dioxide; mean presure 1950 psi; 60 °C; UV detection, 254 nm.

Compound	Capacity factor	
	Methanol (% w/w)*	
	4.2	8.4
Barbiturates		
Barbitone	0.30	0.16
Butobarbitone	0.37	0.17
Amylobarbitone	0.42	0.17
Pentobarbitone	0.37	0.17
Talbutal	0.41	0.22
Quinalbarbitone	0.46	0.24
Methohexitone	0.51	0.28
Phenobarbitone	0.65	0.28
Heptabarbitone	0.78	0.35
Alkylarylketones		
Acetophenone	0.35	0.25
Propiophenone	0.47	0.32
Butyrophenone	0.53	0.35
Valerophenone	0.63	0.42
Hexanophenone	0.75	0.49
Heptanophenone	0.89	0.57
Octanophenone	1.06	0.66
Decanophtnone	1.48	0.88
Dodecanophenone	1.93	1.13
Tetradecanophenone	2.76	1.51
Hexadecanophenone	3.73	1.95
Octadecanophenone	4.97	2.50

* With 0% methanol, all barbiturates were fully retained .

TABLE 6.8. Retention index values for barbiturates and alkylarylketones based on alkylarylketone standards on ODS-silica column at different methanol concentrations.

Conditions: as in Table 6.7.

Compound	Retention index*	
	Methanol (% w/w)**	
	4.2	8.4
Barbiturates		
Barbitone	619	398
Butobarbitone	751	437
Amylobarbitone	831	467
Pentobarbitone	747	437
Talbutal	825	631
Quinalbarbitone	893	681
Methohexitone	953	793
Phenobarbitone	1111	793
Heptabarbitone	1223	964
Alkylarylketones		
Acetophenone	726	722
Propiophenone	902	888
Butyrophenone	984	970
Valerophenone	1090	1088
Hexanophenone	1199	1202
Heptanophenone	1307	1311
Octanophenone	1413	1417
Decanophenone	1622	1623
Dodecanophenone	1790	1087
Tetradecanophenone	2013	2012
Hexadecanophenone	2202	2196
Octadecanophenone	2382	2374

* Based on alkylarylketones, butyrophenone - octadecanophenone.

** With 0% methanol, all barbiturates were fully retained .

was slightly lower than for the PLRP-S column). Under these conditions, the barbiturates as well as the alkylarylketones were generally less retained on ODS-silica than PLRP-S columns.

In order to compare the selectivity behaviour of separation on the two different stationary phases, the retention indices of the barbiturates on Ultrasphere ODS column based on alkylarylketone standards were calculated(Table 6.8). The retention indices of the barbiturates were smaller on the ODS-silica column and decreased with increasing methanol concentration in the mobile phase, but the values were smaller than those obtained on a PS-DVB column (Table 6.5) using similar mobile phase compositions, suggesting a difference in selectivity between the two stationary phases.

The elution order of the barbiturates was the same except that on the Ultrasphere ODS column, the retention index values of amylobarbitone were consistently greater than those of pentobarbitone, whereas the reversed order was observed on the PLPR-S column. This indicates the presence of selectivity differences between the two compounds on the different stationary phases, which was probably due mainly to steric hindrance to adsorption differences caused by the difference in their structural formulae (Table 6.1).

6.5 CONCLUSIONS

The SFC separations of barbiturates on a PS-DVB copolymer and ODS-silica stationary phases have been examined. With no modifier in the carbon dioxide mobile phase, none of the barbiturates under investigations were eluted from an ODS-silica column over a range of pressures but elution were possible using a PLRP-S column with

reasonable analysis times.

The separation of the barbiturates on both stationary phases were possible when polar modifiers were used. A drastic decrease in retention was observed for all analytes on the addition of a small proportion (4%) of modifier to the mobile phase and a further decrease in the retention was observed with further increases in modifier concentration. Within the limits of the experimental conditions, better peak shapes and larger decreases in retention of the barbiturates were observed for methanol as the modifier compared to acetonitrile.

The selectivity of the barbiturates on PLRP-S and ODS-silica stationary phases has been compared and small but significant variations were observed. The selectivity among the barbiturates did not change much with variations in modifier concentration.

It is evident that for the analysis of barbiturates, the large changes in retention indices with methanol as the modifier in the mobile phase (Tables 6.5 and 6.8) means that unlike HPLC, retention indices based on the alkylarylketones could not effectively compensate for small differences in the elution power of the mobile phase. Relative capacity factors based on a barbiturate internal standard have been found to be more reproducible but still showed some changes.

CHAPTER 7

APPLICATION OF SFC TO THE ANALYSIS OF BENZODIAZEPINES

7.1 INTRODUCTION

7.1.1 Benzodiazepines

Benzodiazepines are an important class of pharmacologically active compounds and they are widely used in clinical practice as hypnotics, muscle relaxants, and anticonvulsant agents [171,192-194], much like the barbiturates (Chapter 6). Some of these drugs (e.g., diazepam) are also used in anaesthesia when it is desired to minimize cardiovascular effects [193].

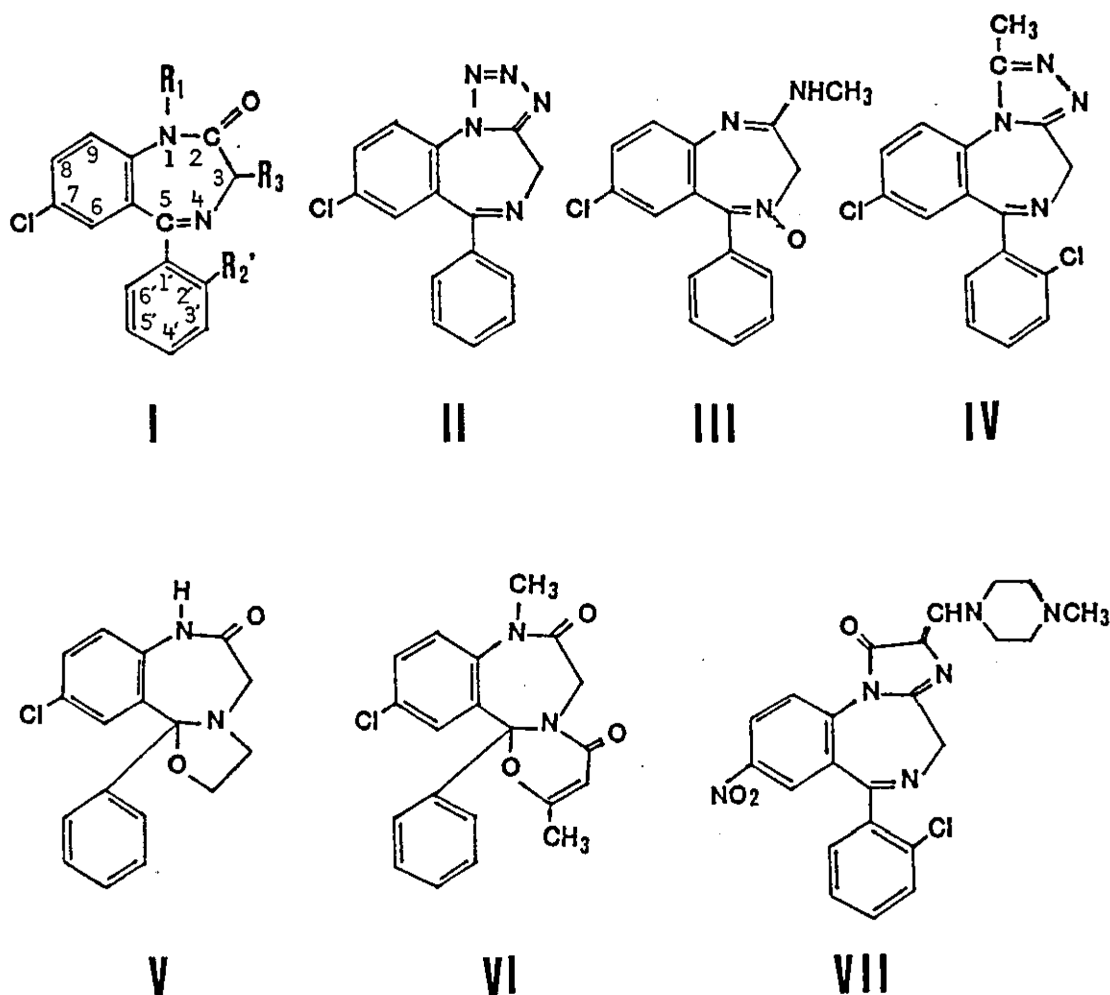
Benzodiazepines have structures (see Table 7.1) which usually have an electron-withdrawing group (typically halogen or nitro) at position 7 and many also have another electron-withdrawing group (usually Cl or F) at 2'-position. The chemical and pharmacological properties of benzodiazepines have long been known [192]. The benzodiazepines are generally insoluble in water and are supplied for injection dissolved in a mixture of organic solvents [194]. Long-term treatment with larger than normal doses of the benzodiazepines may lead to psychic and physical dependence [171].

7.1.2 Analysis of benzodiazepines

Rapid and reliable methods of the identification of drug compounds such as the benzodiazepines are very important in drug toxicology, preventive medicine, and forensic science. Techniques for the

TABLE 7.1. Structures and molecular weights of Benzodiazepines.

Structures:



Compound	Structure	Substituents			Mol. wt.
		R_1	R_3	R_2'	
Desmethyldiazepam	I	H	H	H	270.72
Diazepam	I	CH_3	H	H	284.76
Estazolam	II	-	-	-	294.75
Chlordiazepoxide	III	-	-	-	299.75
Temazepam	I	CH_3	OH	H	300.76
Lorazepam	I	H	OH	Cl	321.16
Lormetazepam	I	CH_3	OH	Cl	335.19
Triazolam	IV	-	-	-	343.22
Cloxazolam	V	-	-	-	349.21
Ketazolam	VI	-	-	-	368.82
Loprazolam	VII	-	-	-	464.91

determination of benzodiazepines have been extensively reviewed [193,195,196]. Recently, several new assays suitable for the determination of diazepam and another benzodiazepine (medazepam) have also been been reviewed [194]. Several chromatographic procedures for the separation of benzodiazepines have been described [179].

GC has found wide applications in the analysis of drugs and their metabolites and often offer better sensitivity and specificity than other non-chromatographic techniques such as colorimetric, spectrometric, and spectrometric assays [148]. GC techniques, combined with highly selective detection methods such as ECD, NPD, or CI-MS have been extensively used in the determination of benzodiazepines [193]. The presence of an electronegative group in the 7-position of the molecule and for some compounds, a halogen in the 2' position of the 5-phenyl ring contribute greatly to ECD response. However, GC methods usually require extensive sample preparation procedures including derivatization (silylation, esterification, or alkylation) and extraction which can both be laborious and time consuming [193]. The high temperatures required for GC are also unsuitable for some heat-labile benzodiazepines such as temazepam [197].

HPLC techniques have been widely used for quantitative analysis of drugs in preparation of pharmaceuticals, therapeutic drug monitoring, and toxicology [196] and this method is suitable for thermally unstable substances such as the benzodiazepine, chlordiazepoxide and its metabolites [193]. The analyses of the benzodiazepines by HPLC commonly employs UV, fluorescence, or electrochemical detection methods [196]. A HPLC method for the determination of lorazepam in human blood plasma has been developed by Gunawan et al. [198]. The method used a RP-ODS Microsorb column with methanol-water (70:30) eluent and UV detection

at 230 nm. However, poor peak shapes (tailing) have often been reported for basic drugs by HPLC on ODS-silica columns, which is most probably due to the presence of residual silanol groups on the surface of the column materials [199]. One way to improve the peak shapes has been to add amine modifiers to the eluent [200]. Recently, Pietrogrande et al. [201] reported a study of the retention behaviour of 16 benzodiazepines in normal phase HPLC on silica, cyano, and amino phases. These workers suggested that benzodiazepine molecule acts as a proton donor towards the adsorbent sites of the phases [201].

The recent technological developments in both packed and open tubular column SFC have shown SFC as a potentially powerful chromatographic technique applicable to a wide range of compounds and problems encountered in the pharmaceutical industry [2]. However, a literature search reveals that no previous work on the analysis of benzodiazepines by SFC has been reported.

7.1.3 Retention indices in benzodiazepine analysis

In GC, Kovat's retention indices based on n-alkanes have been employed in the qualitative analysis of benzodiazepines but the retention indices of the benzodiazepines have been found to be dependent on column temperature [202]. Similar concepts have also been applied in HPLC analysis of several drug compounds but by using alkylarylketones [155] or alkan-2-ones [58] as the retention index standards. A discussion of the use of retention indices in drug analysis has been presented earlier (Chapter 6).

7.1.4 Aim of the study

The purpose of the present study was to examine the feasibility of analyzing benzodiazepines by SFC. The chromatographic behaviour of eleven benzodiazepines (Table 7.1) with different structures, molecular weights, and pharmacological properties have been studied on different stationary phases and with different mobile phase compositions. The application of retention indices in SFC to the analysis of benzodiazepines was also examined.

7.2 SFC SEPARATIONS OF BENZODIAZEPINES WITH CARBON DIOXIDE AS THE MOBILE PHASE

Benzodiazepines have basic properties because of the presence of amino or imino moieties in the molecule. It was of interest therefore to determine the feasibility in SFC for the separation of benzodiazepines on an ODS-silica stationary phase with its residual silanol groups using carbon dioxide as the mobile phase. However, on injection of the samples onto the column (200 x 3 mm i.d., Spherisorb ODS-2 5 μm), none of the benzodiazepines were eluted within reasonable pressure and time limits. On the other hand, hydrocarbons, alkylarylketones as well as several other aromatic test compounds were readily eluted (see Chapter 5). Irreversible adsorption has also been observed previously on ODS-silica in the SFC separation of weakly acidic barbiturates (Chapter 6). Doehl et al. [112] have reported similar strong retention or total adsorption on ODS-silica with supercritical carbon dioxide mobile phase for polar compounds such as the polymer additives Armostat 400 (mol. wt. 228 and 334) which have two amino and hydroxyl groups. In HPLC, the separation

of benzodiazepines on ODS-silica column has been reported to give broad and tailing peaks [176]. These results are consistent with the assumption that the observed retention is mainly caused by strong specific interactions between the solutes and the unreacted silanol groups on the bonded stationary phase.

Since silanol groups are absent on a polymer stationary phase, it was then of interest to examine the separation of the benzodiazepines on a polymer column, which should be free of any specific polar-polar interactions with the stationary phase. It appeared that the benzodiazepines were still strongly retained ($k' > 30$) on the polymer column. In preliminary work, a few of the benzodiazepines, diazepam (Figure 7.1), lorazepam, and temazepam were eluted from a PLRP-S column with carbon dioxide mobile phase at a mean column pressure of 2675 psi, but with long analysis times (more than 20 minutes) and with very poor (tailing) peak shapes. Moreover, the retention time and peak shapes were dependent on the sample amount which results in poor reproducibility of retention time and peak area.

There are two possible ways to improve the analysis times and peak shapes: firstly, by using higher pressures for the separation (limited by the chromatographic instrument) and secondly, by adding a modifier such as methanol to the mobile phase. Previous results of the separation of drug compounds (barbiturates, Chapter 6), have shown that the latter method gives much greater improvement of retention and peak shapes. Similar trends can also be expected for the benzodiazepines and therefore, subsequently, separations of the benzodiazepines were carried out using methanol-modified carbon dioxide as the mobile phase.

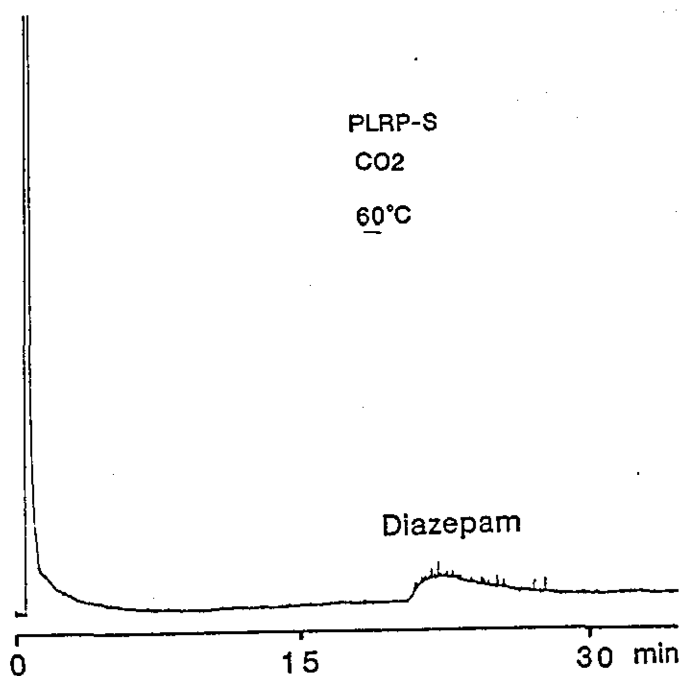


FIGURE 7.1. SFC separation of diazepam on PS-DVB column with carbon dioxide mobile phase. Column (150 x 4.6 mm i.d.) PLRP-S 5 µm; mean column pressure 2675 psi; flow rate 1400 ml/min (gas); 60 °C; flame ionization detection.

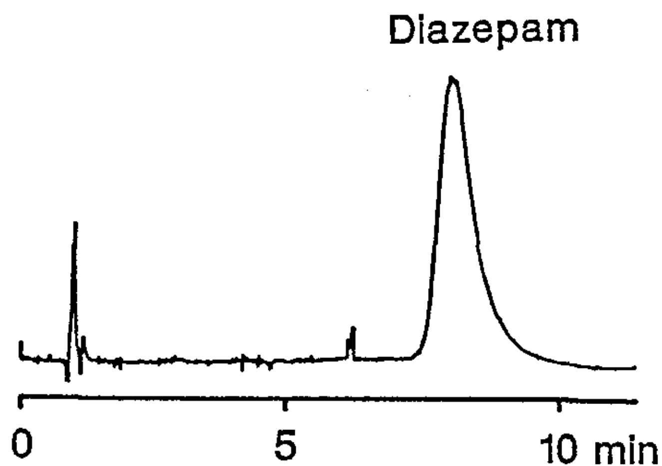


FIGURE 7.2. SFC Separation of diazepam on PS-DVB column with 9% acetonitrile in the mobile phase. Column PLRP-S 5 µm; mean column pressure 2200 psi; UV detection, 254 nm.

7.3 SFC SEPARATIONS OF BENZODIAZEPINES ON PS-DVB COLUMN WITH MODIFIED CARBON DIOXIDE AS THE MOBILE PHASE

7.3.1 Effect of different modifiers

It has previously been observed that different modifiers can show different effects on retention characteristics and peak shapes [70,71] (also see Chapters 5 and 6) particularly for packed columns. In the present work, a brief examination was carried out to determine the effect of the addition of methanol or acetonitrile modifier in carbon dioxide mobile phase on the chromatographic behaviour of a selected benzodiazepine (diazepam).

The capacity factors of diazepam at 9% and 15% concentrations of methanol and acetonitrile were determined and compared (Table 7.2). The trend for each of the modifiers was for decreasing capacity factor with increasing modifier concentration. It was also observed that for this particular system with diazepam as the solute, there was no marked difference in the retention when comparing the modifiers at identical concentrations. Methanol was then used as the modifier in the subsequent separations of the benzodiazepines.

TABLE 7.2. Comparison of capacity factors of diazepam with different modifiers and modifier concentrations on a PS-DVB column.

Conditions: column (150 x 4.6 mm i.d.), PLRP-S 5 μm; primary eluent carbon dioxide; pressure 2220 psi; 60 °C; UV detection, 254 nm.

Modifier	Capacity factor	
	Modifier concentration (% w/w)	
	9	15
Methanol	7.99	4.90
Acetonitrile	7.94	4.71

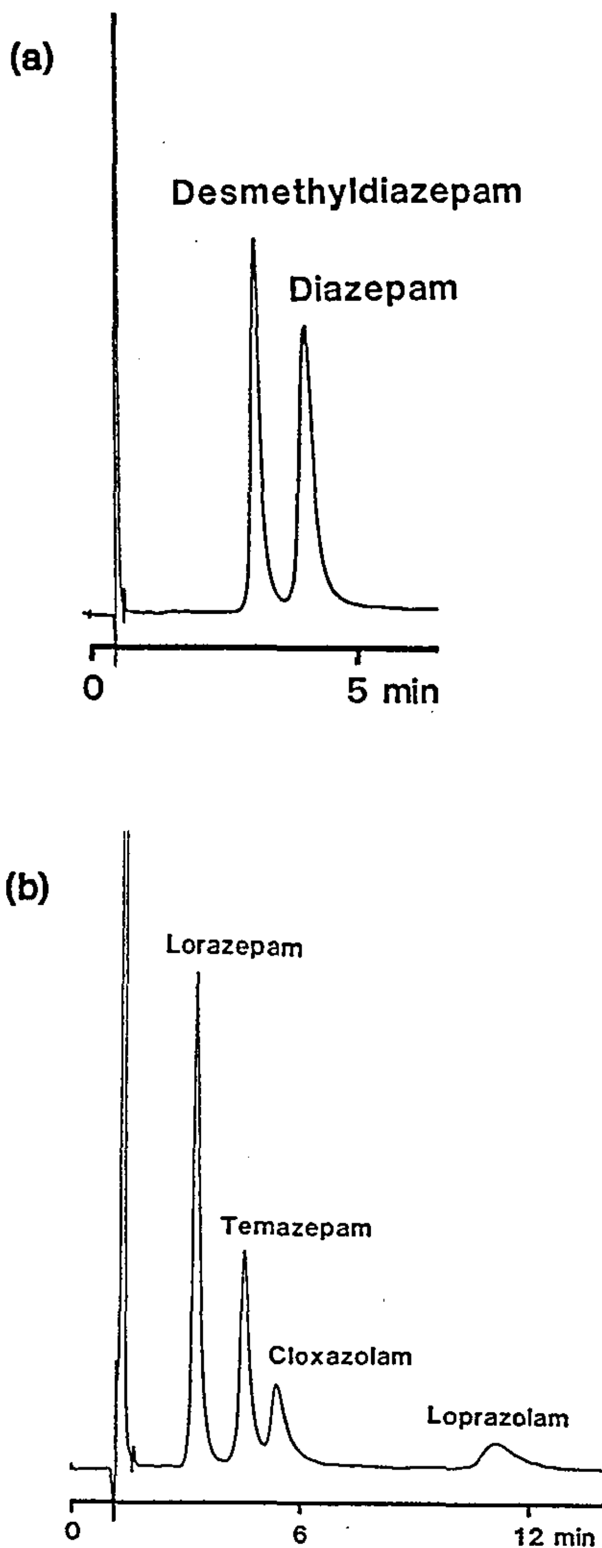


FIGURE 7.3. SFC separations of benzodiazepines, (a) desmethyldiazepam and diazepam and (b) lorazepam, temazepam, cloxazolam, and loprazolam on PS-DVB column with 15% methanol in the mobile phase. Column PLRP-S 5 μm; mean column pressure 2515 psi; UV detection, 254 nm.

Good peak shapes were observed for the SFC separation of diazepam on the PLRP-S column with both methanol and acetonitrile as the modifiers. In both cases, symmetrical peak shapes were generally maintained although some of the peaks were rather broad. The dramatic improvement in peak shapes with the use of modifier in the mobile phase is demonstrated by the separation of diazepam with acetonitrile as the modifier (Figure 7.2) and the separation of diazepam and desmethyldiazepam (Figure 7.3a) and lorazepam, temazepam, cloxazolam, and loprazolam (Figure 7.3b) with methanol as the modifier (as compared to Figure 7.1).

7.3.2 Chromatographic behaviour of benzodiazepines on PS-DVB column using methanol as the modifier

The retention behaviour of the benzodiazepines on PS-DVB stationary phase was examined over a methanol concentration range of 4.3-15.3% (w/w). Except loprazolam, all the benzodiazepines were eluted within a reasonable time with symmetrical peak shapes using 4.3% methanol in the mobile phase. Further decreases in retention were observed for the benzodiazepines using higher methanol concentrations in the mobile phase. With 15.3% methanol concentration, loprazolam was eluted from the column in less than 12 minutes with reasonable peak shape (Figure 7.3b).

The capacity factors of the benzodiazepines at different methanol concentrations were determined (Table 7.3). In general, the capacity factors of the compounds decreased with increasing methanol concentration in the mobile phase. With same percentages of methanol the capacity factors were spread over a range of values (e.g. for 15% concentration, they range from 1.40 for lorazepam to 7.27 for loprazolam) suggesting that many of the benzodiazepines may be able to

TABLE 7.3. Capacity factors of benzodiazepines on PS-DVB column with different methanol concentrations.

Conditions: column (150 x 4.6 mm i.d.) PLRP-S 5 μm; primary eluent carbon dioxide; mean pressure 2515 psi; temperature 60 °C; UV detection, 254 nm.

Compound	Capacity factor		
	Methanol (% w/w)*		
	4.3	9.7	15.3
Desmethyldiazepam	15.61	4.46	1.82
Lormetazepam	16.24	5.57	2.20
Lorazepam	16.48	4.10	1.40
Temazepam	16.84	5.62	2.28
Chlordiazepoxide	18.45	4.81	2.39
Diazepam	18.68	6.11	2.80
Ketazolam	20.44	6.22	2.25
Cloxazolam	24.70	6.70	3.03
Triazolam	28.57	5.78	1.94
Estazolam	32.82	6.83	2.23
Loprazolam	-	26.24	7.27

* With 0% methanol, all compounds had capacity factors > 30.

TABLE 7.4. Retention indices of benzodiazepines at different methanol concentrations on PS-DVB column.

Conditions: as in Table 7.3.

Compound	Retention index*		
	Methanol (% w/w)		
	4.3	9.7	15.3
Desmethyldiazepam	2401	2037	1677
Lormetazepam	2425	2193	1822
Lorazepam	2434	1978	1472
Temazepam	2448	2200	1850
Chlordiazepoxide	2503	2090	1887
Diazepam	2510	2258	2007
Ketazolam	2565	2270	1840
Cloxazolam	2679	2323	2068
Triazolam	2768	2219	1727
Estazolam	2852	2334	1834
Loprazolam	-	3283	2741

* Based on alkylarylketones, butyrophenone - octadecanophenone (see Chapter 5, Table 5.20)

be separated from each other in a single analysis. This was demonstrated by the separation of four benzodiazepines, lorazepam, temazepam, cloxazolam, and loprazolam (Figure 7.3b).

Under the experimental conditions indicated in Table 7.3, the elution order on the polymer column of the benzodiazepines which possess similar structures were in the order of increasing molecular weight, e.g., desmethyldiazepam was eluted before diazepam. However, this elution order may not necessarily be followed for some of the benzodiazepines with different functionalities. This was observed particularly for the hydroxyl-containing compounds (lormetazepam, temazepam, and lorazepam) which were eluted before diazepam or chlordiazepoxide which have lower molecular weights. This retention behaviour suggests that with methanol modified carbon dioxide as the eluent, the less polar compounds were selectively more retained on the column.

It was also observed that with low methanol concentrations (4.3%), estazolam and triazolam which contain imino and amino ring attached to positions 1 and 2 (see Table 7.1) were more retained than cloxazolam and ketazolam with comparable molecular weights. However, apparently, this retention trend was reversed at higher methanol concentrations (15.3%) where estazolam and triazolam were eluted earlier than cloxazolam and ketazolam.

Slightly reduced retentions were observed for lormetazepam with a chloro group substituted at the 2'-position compared to those of the base molecule, temazepam (e.g. with 4.3% methanol, lormetazepam, k' = 16.24; temazepam, k' = 16.84). This suggests that the presence of an electron withdrawing group on the molecule reduces the retention of the compounds on the column.

The retention indices of the benzodiazepines were calculated based on the alkylarylketone standards in order to examine the selectivity behaviour of the separation on the polymer column (Table 7.4). In general, the retention indices of the benzodiazepines decreased with increasing methanol concentration, indicating they were selectively less retained on the column relative to the alkylarylketones. This was probably because of the increasing solubility of the benzodiazepines in the mobile phase with increasing methanol concentration. The changes in the retention indices of the benzodiazepines over the methanol range of 4.3-15.3% were very large (>500 units). Similar trends have also been observed for the separation of barbiturates (Chapter 6) and some polar model compounds benzoic acid, benzamide, and alcohols (Chapter 5). It is evident that in SFC analysis of benzodiazepines on a PS-DVB column, the retention indices based on the alkylarylketone standards are highly sensitive to selectivity caused by changes in eluent compositions and therefore, may not be particularly suitable as a means of recording retentions of the drug compounds.

7.4 COMPARISON OF SFC SEPARATIONS OF BENZODIAZEPINES ON DIFFERENT STATIONARY PHASES WITH METHANOL AS THE MODIFIER

7.4.1 SFC separations of benzodiazepines on ODS-silica column

As mentioned earlier, with pure carbon dioxide as the mobile phase, the benzodiazepines were fully retained on an ODS-silica column. Investigations were therefore carried out to examine the SFC separations of benzodiazepines on an ODS-silica (Ultrasphere ODS) stationary phase with the use of methanol as the modifier in the carbon dioxide mobile

phase. Different methanol concentrations in the mobile phase ranging from 4-16.4% were used. With a high percentage of methanol concentration in the mobile phase, good peak shapes were observed for the separation of the benzodiazepines (except lormetazepam, lorazepam, and temazepam). This is illustrated by the separations of diazepam, ketazolam and desmethyldiazepam with 12.7% methanol (Figure 7.4a-c) which demonstrate reasonably good peak shapes. In contrast, under identical conditions, lormetazepam, lorazepam, and temazepam showed very poor (tailing) peak shapes (Figure 7.4d-f) which had not been observed previously on PS-DVB column. This chromatographic behaviour can probably be explained by the strong polar-polar interactions of the hydroxyl groups of these compounds with free residual groups on the ODS-silica stationary phase.

In order to study the retention behaviour of the benzodiazepines on the ODS-silica, the capacity factors were calculated (Table 7.5). It can be seen that for each methanol concentration, the elution order of the benzodiazepines was not always according to increasing molecular weight. In general, with nominally identical methanol concentrations and operating conditions, the benzodiazepines were less retained on the ODS-silica column than they were on PS-DVB stationary phase (Table 7.3). The overall retention order was also different from that which had been observed for PS-DVB column. On the ODS-silica, greater retentions were observed at lower methanol concentrations (4.0%) for the hydroxyl-containing benzodiazepines (lorazepam, temazepam, and lormetazepam) as well as for chlordiazepoxide, estazolam, triazolam and loprazolam. However, marked decreases in retention were observed for these compounds on increasing the methanol concentrations up to 16.4% which again suggests the increasing solubility of these relatively more polar compounds in the mobile phase with increasing percentage of methanol.

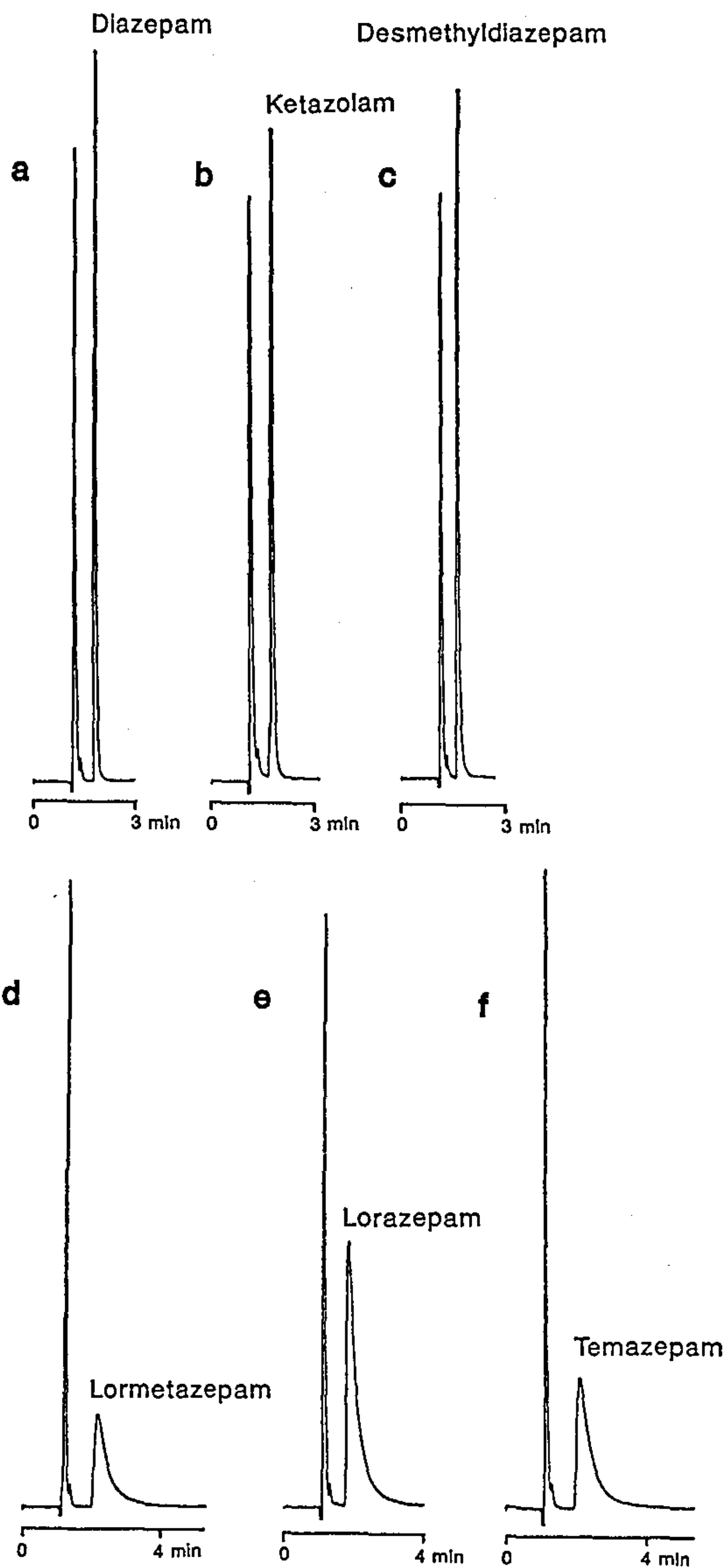


FIGURE 7.4. SFC of benzodiazepines on ODS-silica column with 12.7% methanol in the mobile phase. Column Ultrasphere ODS 5 μm; mean column pressure 2470 psi; UV detection, 254 nm.

TABLE 7.5. Capacity factors of benzodiazepines on ODS-silica column at different methanol concentrations.

Conditions: Column (250 x 4.6 mm i.d.), Ultrasphere ODS 5 μm; primary eluent carbon dioxide; mean column pressure 2470 psi; 60 °C; UV detection, 254 nm.

Compound	Capacity factor			
	Methanol (% w/w)			
	4.0	8.3	12.7	16.4
Ketazolam	1.54	0.76	0.51	0.31
Diazepam	1.56	0.75	0.50	0.31
Desmethyldiazepam	1.64	0.65	0.40	0.23
Cloxazolam	2.88	1.15	0.73	0.58
Chlodiazepoxide	5.12	1.19	0.66	0.46
Lormetazepam	5.15	1.39	0.79	0.44
Estazolam	5.63	1.05	0.52	0.28
Temazepam	5.86	1.54	0.83	0.48
Triazolam	6.19	1.08	0.53	0.26
Lorazepam	7.91	1.44	0.61	0.35
Loprazolam	-	-	-	3.17

* With 0% methanol, all compounds were fully retained.

TABLE 7.6. Retention index values of benzodiazepines on ODS-silica column with different methanol concentrations.

Conditions: as in Table 7.5.

Compound	Retention index*			
	Methanol (% w/w)**			
	4.0	8.3	12.7	16.4
Ketazolam	1934	1765	1554	1350
Diazepam	1939	1710	1545	1342
Desmethyldiazepam	1976	1599	1378	1115
Cloxazolam	2365	2025	1834	1831
Chlodiazepoxide	2761	2050	1760	1656
Lormetazepam	2766	2165	1897	1612
Estazolam	2828	1957	1569	1258
Temazepam	2856	2245	1938	1690
Triazolam	2893	1979	1582	1203
Lorazepam	3063	2195	1700	1447
Loprazolam	-	-	-	3127

* Based on alkylarylketones, valerophenone - octadecanophenone, (see Chapter 5, Table 5.24).

** With 0% methanol, all compounds were fully retained.

The retention indices of benzodiazepines on ODS-silica column were determined in order to compare the selectivity of the separation (Table 7.6). In general, the retention indices of the benzodiazepines on the ODS-silica column decreased with increasing methanol concentration but the retention index values were smaller than those obtained on a PS-DVB column (Table 7.4) suggesting a difference in selectivity between the stationary phases. Marked decreases in retention indices were observed particularly for triazolam, estazolam, chlordiazepoxide, and the hydroxyl-containing benzodiazepines.

7.4.2 SFC separations of benzodiazepines on cyano-silica column

In the earlier part of this work (Chapter 5), it has been observed that some of the model compounds such as benzoic acid, benzamide, and benzylamine were fully retained on a cyano-silica column with carbon dioxide as the mobile phase, but they were readily eluted from the column with the addition of methanol to the mobile phase. It was thus of interest to determine the possibility of separating the basic drug benzodiazepines on the cyano-silica column. The separations of the benzodiazepines on a cyano-silica column (Ultrasphere CN) were examined only briefly because of the time limitation.

With no modifier in the mobile phase, none of the benzodiazepines were eluted over a range of pressures and time limits. Separations were then carried out on a Ultrasphere-cyano column with 12.8% methanol in the carbon dioxide mobile phase. All the benzodiazepines (except loprazolam) were then eluted readily from the cyano-silica column within reasonable time limits (less than 12 minutes). The capacity factors of the benzodiazepines were determined (Table 7.7). With nominally

TABLE 7.7. Capacity factors of benzodiazepines on cyano-silica stationary phase with methanol (12.8% w/w) in the mobile phase.

Conditions: column (250 x 4.6 mm i.d.), Ultrasphere CN, 5 μm; primary eluent carbon dioxide; mean pressure 2470 psi; temperature 60 °C; UV detection, 254 nm.

Compound	Capacity factor*
Diazepam	1.25
Ketazolam	1.30
Temazepam	1.58
Desmethyldiazepam	1.63
Lormetazepam	1.70
Lorazepam	2.39
Chlodiazepoxide	2.83
Cloxazolam	4.76
Estazolam	5.19
Triazolam	6.42
Loprazolam	>19

* With 0% methanol, all compounds were fully retained.

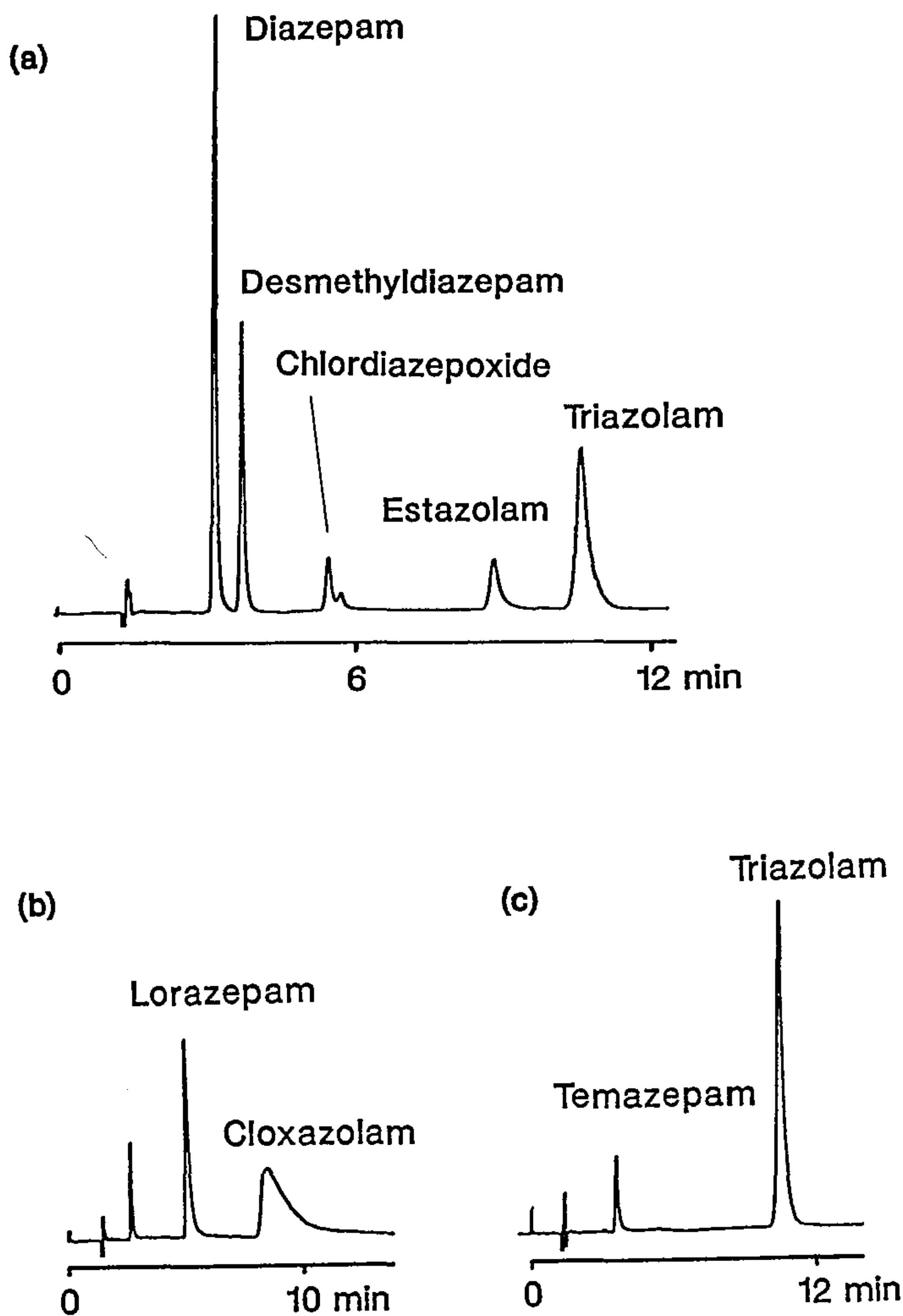


FIGURE 7.5. SFC Separation of several benzodiazepines on cyano-silica column with 12.8% methanol in the mobile phase. Column Ultrasphere CN 5 μm; mean column pressure 2470 psi; UV detection, 254 nm.

identical methanol concentrations, the benzodiazepines were more retained on the cyano-silica column compared to the ODS-silica column (Table 7.5) and the order of elution was not retained. On the cyano-silica column with under 12.8% methanol in the mobile phase, strong retentions were observed for loprazolam, triazolam, cloxazolam, and estazolam whereas diazepam was the least retained.

The separations on the cyano-silica column with 12.8% methanol generally gave good peak shapes for most of the benzodiazepines. This is demonstrated by the separations of diazepam, desmethyldiazepam, chlodiazepoxide, estazolam, and triazolam (Figure 7.5a) and temazepam and triazolam (Figure 7.5c). However, poor peak shapes were observed for cloxazolam (Figure 7.5b). Unlike the separation on ODS-silica column, on a cyano-silica column the separation of benzodiazepines containing hydroxyl groups such as temazepam (Figure 7.5c) and lorazepam (Figure 7.5b) gave reasonably good peak shapes.

Although further work may be required, the present limited investigations has demonstrated the feasibility of the separation of benzodiazepines on a cyano-silica column using an organic modifier such as methanol in the mobile phase.

7.5 CONCLUSIONS

The chromatographic behaviours of eleven benzodiazepines have been examined on PS-DVB, ODS- and cyano-bonded silica stationary phases using different modifier and modifier concentrations. With no modifier none of the benzodiazepine under investigation were eluted from ODS-silica or cyano-silica columns over a range of pressures, and on a PS-DVB column, these compounds were very retained and exhibited poor peak shapes.

With the use of a modifier (methanol) in the carbon dioxide mobile phase, all the benzodiazepines (except loprazolam) were eluted readily from the three columns with considerably shorter analysis times and improvements in peak shapes.

Some changes in selectivity among the benzodiazepines were observed with increasing methanol concentrations on PS-DVB or ODS-silica columns depending on the functionalities of the compounds. On the ODS-silica column, the benzodiazepines containing hydroxyl groups appeared to be selectively more retained on the column at low methanol concentrations and their retention decreased relatively more with increasing methanol concentrations.

Selectivity differences between PS-DVB, ODS-silica, and cyano-silica columns were observed for the separation of the benzodiazepines using methanol modified carbon dioxide as the mobile phase. These selectivity differences could be used as a future guide to optimize the separations of the drug compounds.

Unlike HPLC, retention indices based on alkylarylketone standards for the analysis of benzodiazepines in SFC were very susceptible to variations of selectivity caused by changes in the elution power of the mobile phase. This was particularly evident with the large changes in retention indices with methanol concentration (Tables 7.4 and 7.6).

CHAPTER 8

APPLICATION OF SFC, GC, AND GC-MS TO THE ANALYSIS OF TERPENES: ANALYSIS OF *CYMBOPOGON MARTINII* (ROXB.) WATS ESSENTIAL OIL

8.1 INTRODUCTION

The present study was set out to investigate the feasibility of the application of packed column SFC to the analysis of terpenes and essential oils. This section introduces some of the properties of terpenes and the essential oils. Then, techniques that have been used for the analysis of these groups of compounds are reviewed.

8.1.1 Terpenes and essential oils

Essential oils are substantially volatile, water-immiscible portions of the secondary metabolites of plant products [203]. They are normally prepared by distillation processes from specified parts of plants of defined origin and species, or from the pericarp of citrus fruits, when the essential oil will contain a proportion of non-volatile products [203].

Essential oils are normally complex mixtures containing tens and sometimes hundreds of components [204,205]. Although some constituents of essential oils may contain nitrogen or sulphur, most of them belong to the "terpenes" and "sesquiterpenes" groups and their oxygenated compounds [204]. The terpenes, also called "monoterpenes" have 10 carbons atoms, while sesquiterpenes have 15 carbon atoms. The term "terpenes" is also used in a broader sense to refer to all compounds

which have a distinct architectural and chemical relation to the simple isoprenoid molecule [204]. Other essential oil constituents include straight-chain compounds and benzene derivatives. Compounds having more distant relationships with the terpenes but still having features which link them with the terpene structures are sometimes called "terpenoids" [204]. Essential oil constituents can further be classified according to their functional groups, including hydrocarbons, alcohols, aldehydes, esters, ketones, phenols, and ethers [206].

Many essential oils and terpenes are known to be chemically unstable and can undergo changes such as intermolecular rearrangement, polymerisation, oxidation, hydrolysis, and thermal decomposition which can become a problem in their analysis. These changes are most rapid with very small samples, even when they are kept in dark and low temperatures. For instance, a gradual decomposition of zingibrene to ar-curcumene [207] on storage at 7 °C, and polymerisation of myrcene [208] have been reported.

8.1.2 Essential oil preparation methods

Numerous methods have been used for the isolation of essential oils from the plant materials including steam or water distillation, solvent extraction, extraction with cold fat (enfleurage), extraction with hot fat (maceration), and cold expression [204,205]. In addition, supercritical fluids (usually of carbon dioxide), can potentially be applied in the extraction process to yield volatile oils that are substantially free from the non-volatile components normally associated with extraction by conventional solvents [87]. This method of extraction using supercritical fluids, known as SFE, has been widely used in many

food industries and it has been successfully combined on-line with analytical chromatographic techniques such as SFC [2].

8.1.3 Analysis of terpenes and essential oils

There has been tremendous progress in the techniques used in the analysis of essential oils in the past decade [205]. The quality and the characteristics of an essential oil is usually established using various analytical techniques ranging from physicochemical and non-specific chemical methods, to chromatographic methods. Among the chromatographic techniques, GC has by far been the most widely used technique in the analysis of these compounds [205]. However, no reports have described the use of SFC in the analysis of essential oils or terpenes apart from the preliminary report from this laboratory [88]. Some of the chromatographic techniques are discussed below.

(a) High performance liquid chromatography

The application of HPLC to essential oil analysis has been restricted by its limited resolution compared with capillary GC and also, to a lesser extent, by the lack of a sensitive universal detector [209]. Nevertheless, HPLC has been used with some success in semipreparative separation of terpenoids [210] as well as in selective determination certain components of essential oils such as eugenol [211] using ultraviolet and electrochemical detections. Recently, Cartoni and Coccioli [212] reported the analyses of essential oils from lemon, bergamot, and orange using HPLC with microbore columns (1 mm i.d., 5 µm particles) and UV detection at different wavelengths. These

investigators tentatively identified the non-volatile components of the essential oils by using HPLC-MS [212].

(b) Gas-liquid chromatography

The terpenes have boiling points ranging from 150 °C to over 350 °C and all but a few, such as the triterpenes have ideal vapour pressures suitable for GC [204]. A very detailed study of the GC of naturally occurring hydrocarbons by Klouwen and ter Heide in 1962 [213] has provided the basis for much subsequent analyses. The early results obtained in GC analysis of terpenes have been thoroughly discussed and reviewed in the literature [214-216]. This subject has recently been up-dated [205] showing the general refinement and diversification of the GC technique.

With the advent of capillary or open tubular columns in the past several years, there has been a remarkable improvement in the capabilities of GC both in respect to resolving power and speed of elution [217]. Using capillary columns in conjunction with temperature-programmed gas chromatography (TPGC), a very high number of components in a complex mixture such as the essential oil can be resolved [218].

However, the relatively high temperatures used in GC may cause problems for the analysis of some of the thermally unstable essential oil constituents. High injection temperatures usually employed in GC (above 120-150 °C), may dehydrate compounds like linalool [219], camphene hydrate [220], or α-terpineol [221].

(c) Gas chromatography - mass spectrometry

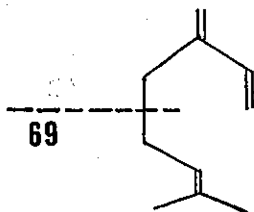
A mass spectrometer bombards a substance under investigation with

an electron beam inside an ionizing region of low pressure at about 10^{-6} mm Hg, and quantitatively records the results as a mass spectrum of positive ion fragments [222]. Separation of the positive ion fragments is on the basis of mass (strictly, mass/charge, but most of the ions are singly charged). Mass spectrometry has become one of the most powerful analytical tools and considerable progress has been made in the interfacing of mass spectrometers with chromatographic systems, particularly with open tubular capillary column gas chromatography. The instrumentation, operation, applications, and fundamentals of MS and GC-MS have well been documented in the literature [222-226].

Compound identification in mass spectrometry is either by interpretation from the theory of mass spectra and the rules for the fragmentation of organic ions in the gas phase, or by empirical spectrum matching. The former technique is known to be slow and tedious and generally requires considerable experience. On the other hand, the latter approach involves searching a collection of mass spectra to find a similar or exact match with an experimental mass spectrum. Although this technique has been greatly facilitated by the use of large and mini-computers, it still suffers from some major problems including the limited availability of pure standards [226]. Some compounds such as the terpenes are not available from any supplier, probably because they have only been isolated in a particular laboratory in very small quantities.

Because the detailed processes that occur in the fragmentations of organic compounds are not fully known, it is difficult to generalize about fragmentation mechanisms among the terpenes. Nonetheless, several general rules for predicting prominent peaks in a spectrum have been put forward by Silverstein, Bassler and Morrill [222]. Most mass spectra of the terpenes show prominent peaks, which often form the base peak at m/z

41, 42, and 43 which are most likely to be fragment ions C_3H_5, C_3H_6, and C_3H_7, respectively. Some of the compounds can undergo simple fragmentation, as examplified by myrcene:



This gives a prominent peak at m/z 69, which is commonly found in the mass spectra of terpenes such as geraniol, nerol, farnesol and citronellal. Most of the sesquiterpene and a few monoterpene hydrocarbons are expected to give fragments corresponding to $M-43^+$ and in many cases they form the base peak in the spectra, such as those of α-terpinene, α-copaene, α-cubebene and guaiene.

8.1.4 Retention indices in essential oil analysis

In GC, the use of retention indices is a well established approach to the qualitative identification of the components of complex mixtures such as essential oils and has been comprehensively reviewed [27,144,205]. To get a more reliable identification, the retention indices of a compound can be determined on more than one stationary phases of different polarities [227].

The Kovats' retention index scale has been widely used in the analysis of essential oils [144]. This index system is based on the constant increment of the logarithms of the retention times of the n-alkane homologues under isothermal conditions. A modified form of the Kovats' retention index is usually used in TPGC by assuming a linear relationship between the elution temperature and the n-alkane carbon

number [144]. However, recently, there have been reports of investigations of the use of isothermal retention indices for the identification of compounds separated by linear temperature-programmed gas chromatography [228] and a proposal for a method of calculating temperature-programmed retention indices based on an extended Kovats' definition [229].

8.1.5 Aim of the study

The purpose of the present work has been to investigate the viability of the application of packed column SFC to the analysis of terpenes and essential oils as compared to the more established techniques, GC and GC-MS. Separation of the components of the leaf essential oil of *Cymbopogon martinii* (Roxb.) Wats and a number of terpene standard compounds were first carried out using GC and GC-MS techniques on both non-polar and polar stationary phases. The samples were then examined by SFC on a PS-DVB column with carbon dioxide as the mobile phase and flame ionization detection. Retention index methods based on the Kovats retention indices based on n-alkanes were used for qualitative identification of the components. Based on the mass spectral and retention index comparisons, several major components present in the oil were identified. The results of these investigations are described.

8.2 *CYMBOPOGON MARTINII* (ROXB.) WATS

Cymbopogon is a plant genus belonging to the Graminale or grass family, the largest family in the plant order Graminales. Some species of this genus give (by steam distillation) essential oils of commercial

value [230]. One of the species, *Cymbopogon martinii* (Roxb.) Wats, grows wild in many regions of India and several varieties are cultivated commercially as a source of palmarosa or ginger-grass oils which are used in soap and perfumery [230-233].

A number of reports have described the analysis of the essential oils of *Cymbopogon martinii* [231-234]. Randriamiharisoa and Gaydou [231] identified 69 components including nine newly identified components in palmarosa (*Cymbopogon martinii*) essential oil from Madagascar using capillary GC by means of Kovats' retention indices and GC-MS identification techniques. They found that the major components of the oil were geraniol, linalool, and geranyl acetate. In subsequent work [232] these workers studied the composition of the hydrocarbon fraction of the essential oil and identified 11 monoterpenes, 28 sesquiterpenes, and 16 n-alkanes. In another study, Bottini et al. [233] isolated a novel constituent (dihemiacetal bis-monoterpenoid) from *Cymbopogon martinii* and identified the compound by means of x-ray diffraction crystallography.

8.3 EXPERIMENTAL

This Section describes the general experimental details and the instrumentation for the GC and GC-MS analyses. The SFC instrumentation has been described in Chapter 3.

8.3.1 Materials

Leaf essential oil of *Cymbopogon martinii* (Roxb.) Wats (2 ml, prepared by steam distillation in February, 1986) was provided by the

Natural Science Research Laboratory, Philippine Woman's University, Manilla, Philippines. The n-alkane standards and the terpene reference compounds were laboratory grade, obtained from a range of commercial suppliers. Methanol and hexane were HPLC grade and dichloromethane and chloroform, were laboratory reagents obtained from FSA Laboratory Supplies, Loughborough, U.K.

8.3.2 Sample preparation

The terpenes and essential oil samples were stored under refrigeration to minimize sample deterioration. A fraction of the yellowish, clear essential oil of *Cymbopogon martinii* sample was diluted with dichloromethane to make solutions of concentration of about 20 mg/ml for use in the chromatographic analyses. Similarly, solutions of the reference terpenes were prepared by dissolving the appropriate amount of the samples in dichloromethane to give concentrations typically of about 10 to 20 mg/ml. There was no sample pretreatment prior to the chromatographic analyses.

8.3.3 GC and GC-MS instrumentation

The GC analyses were performed using a Carlo Erba Fractovap series 2150 gas chromatograph (Carlo Erba Instrumentation, Italy), in a split injection mode (split ratio 1:9). Samples (0.1 to 0.5 µl) were injected using 5 µl SGE syringe (SGE, Australia) using a solvent flush method in which virtually all the sample was injected into the injection port. The syringe was scrupulously washed with acetone by filling and emptying 10-20 times between injections of different samples. Two capillary fused

silica columns were used (25 m x 0.33 mm i.d.), coated with 0.5 μm film of stationary phases: dimethyl polysiloxane (BP-1) and polyethylene glycol (BP-20) (SGE, Australia). Flame ionization detection was used with hydrogen and oxygen flow rates of about 30 and 300 ml/min respectively. The carrier (helium) flow rate kept constant at 2.1 ml/min. The chromatograms were recorded on a Servoscribe chart recorder and a Hewlett-Packard 3390 integrator or a Spectra Physics 4270 integrator.

A Kratos MS 80/DS-55C mass spectrometer which is directly coupled to a Carlo Erba 4200 capillary gas chromatograph was used in the GC-MS analyses. The chromatographic separations in the GC-MS analyses employed different, but matching columns (BP-1 and BP-20) and were performed under identical operating conditions to those used in direct GC analyses. The mass spectrometer was operated with electron impact ionization at an ionization potential of 70 eV, under scanning mode to give low resolution mass spectral information useful for analyte identification.

8.3.4 Methods

In the isothermal GC analyses, dichloromethane peaks were taken as the dead volume indicators used in the adjusted retention time calculations. The Kovats' retention indices of the standard compounds and the analytes were determined from the least squares fit of straight line curves of the plot of log adjusted retention times, against retention indices (carbon number x 100) for the n-alkane standards by using a computer program written in the laboratory. Retention indices in the linear temperature-programmed GC were determined using the equation

$$I = 100 \frac{t_{r(U)} - t_{r(X)}}{t_{r(X+1)} - t_{r(X)}} + 100X \qquad (8.1)$$

where $t_{r(U)}$ is the retention time of the unknown compound, and X and (X+1) are the carbon number of the consecutive alkanes eluted before and after the unknown. Except when noted otherwise, the hydrocarbon standards mixtures were coinjected with the test samples and thus, retention indices of the compounds were obtained from an internal standardization method.

For peak identification, the retention indices of the unknown peaks were compared with the retention indices of the standard compounds as well as with those obtained in the literature [235-238]. Verification of the identities of the peaks were accomplished by "fingerprinting matching" or comparison of the mass spectra of the peaks with available published reference mass spectra [238-244]. In the present work, no attempts were made to obtain the mass spectra of the standard reference compounds.

8.4 GC AND GC-MS ANALYSIS

8.4.1 Gas chromatography of solvents

Experiments were performed to determine the purity of the solvents used in the analysis. The solvents, methanol, dichloromethane, and hexane were examined by GC on the BP-1 column at 150 °C (isothermal) Under the above-stated operating conditions they were found to be acceptably pure with no significant interfering peaks.

8.4.2 GC separations of the essential oil of *C. martinii* and and terpene standards

In order to establish the chromatographic behaviour of the terpenes, separations of the available terpene reference compounds were carried out using capillary GC under isothermal conditions. The retention indices of the terpenes were determined using both polar (BP-20) and non-polar (BP-1) columns (Table 8.1) and compared to the literature values [235-238]. The retention index values found in the literature for a few other selected compounds which may be of interest in the study are also listed in the table. The Kovats' retention indices of the standard reference compounds on both BP-1 and BP-20 columns were generally in good agreement with the corresponding literature values.

The sample of the leaf essential oil of *Cymbopogon martinii* (Roxb.) Wats was subjected to rigorous GC investigation. Better separations of the essential oil components were observed using the BP-1 (non-polar) column compared to those obtained using the more polar column (BP-20) as illustrated by the representative chromatograms (Figures 8.1 and 8.2, respectively). This was possibly due to the BP-20 column which might have deteriorated.

Good separations, particularly for the early eluting, more volatile components were achieved using the non-polar column by temperature programming with a low initial temperature (Figure 8.3). The BP-20 capillary column, however, was again found to ~~be~~ give tailing peaks and poorer resolution.

The retention indices of the major components of the essential oil sample separated by isothermal GC on the BP-1 column

TABLE 8.1. Kovats' retention index (RI) values determined in this study for selected monoterpenes and sesquiterpenes on non-polar and polar stationary phases compared to published literature values.

Temperatures (°C) are in brackets.

Compound	RI on non-polar columns		RI on polar columns	
	BP-1 (160 °C)	Literature values	BP-20	Literature values
pinene	941	928-931a;932(100)b	1027(100)	1023-1035g
methylheptenone	964(100)	-	1360(120)	-
p-cymene	-	1014-1017a;1015(90)b	-	1268-1282g
limonene	1044	1026a;1028c;1022(85)b	1213(100)	1204-1213g;1204h
cineole	1051	1000(100)b	1222(100)	-
γ-terpinene	-	1052a;1053(100)b;1056c	-	1244-1256g;1247h
linalool	1094	1089(90)b;1097c	1548(100)	1540-1547g;1533h
citronellal	1141	1132a;1136(115)b;1143c	1517(100)	1481-1485g;1491h
isopulegol	1160	1133(110)b	1578(100)	1564g
4-allylanisole	1190	-	1797(180)	-
citronellol	1216	1210a;1216(100)b;1192c	1803(140)	1764g;1754h
nerol	1220	1218(75)b;1217a	1823(165)	1788-1800g
carvone	1226	1223(125)b	1815(140)	-
neral	1237	1217a;1220(120)b	1750(140)	1678g
geraniol	1241	1237-1245a;1237(120)b	1888(140)	1836-1845g
geranial	1261	1245a;1260(120)b	1797(140)	1730g
trans-anethole	1283	-	1957(180)	-
safrole	1285	1269	2011(180)	1883g
piperonyl aldehyde	1321	-	2370(180)	-
citronellyl acetate	-	1336a;1335(140)b;1335c; 1335(140)b	-	1671g;1662h
eugenol	1340	1335-1341a	2185(140)	2161g
geranyl acetate	1359	1359a;1361(135)b;1353c	1795(165)	1753-1764g;1754h
vanillin	1371	-	2673(180)	-
eugenol methyl ether	1375	-	2048(140)	-
α-copaene	-	1382-1388a;1401d	-	1503-1510g;1522i
β-elemene	-	1391-1394a;1410e	-	1540a
β-farnesene	-	1429e	-	1668f
isoeugenol	1432	1423a	2402(165)	-
β-caryophyllene	1436	1427-1431a;1455c;1445d	1619(165)	1597-1615f; 1632h;1618i
longifolene	-	1440d	-	1600i
α-cedrene		1445d	-	1598i
β-cedrene	-	1455d	-	1625i
α-humulene	-	1461-1466a;1477d	-	1681i
ε-muurolene	-	1474d	-	1676i
β-selinene	-	1506d	-	1767e
γ-muurolene	-	1506e	-	1725i
α-muurolene	-	1508d	-	1726i
δ-cadinene	-	1521-1525a;1526d	-	1770-1775g;1784f
γ-cadinene	-	1524d	-	1762i
elemol	-	1540a	-	2078g
nerolidol	1551	-	2090(180)	-
calarene	-	1560d	-	1618i

a Methyl polysiloxane, TPGC [235]
b Non-polar stationary phases [238]
c SF96, 100 °C [236]
d SF96, 170 °C [237]
e Apiezon L, 155 °C [237]
f Polyethylene glycol 20M, 156 °C [237]
g Polyethylene glycol 20M, TPGC [235]
h Polyethylene glycol 20M, 75 °C [236]
i Polyethylene glycol 20M, 132 °C [237]

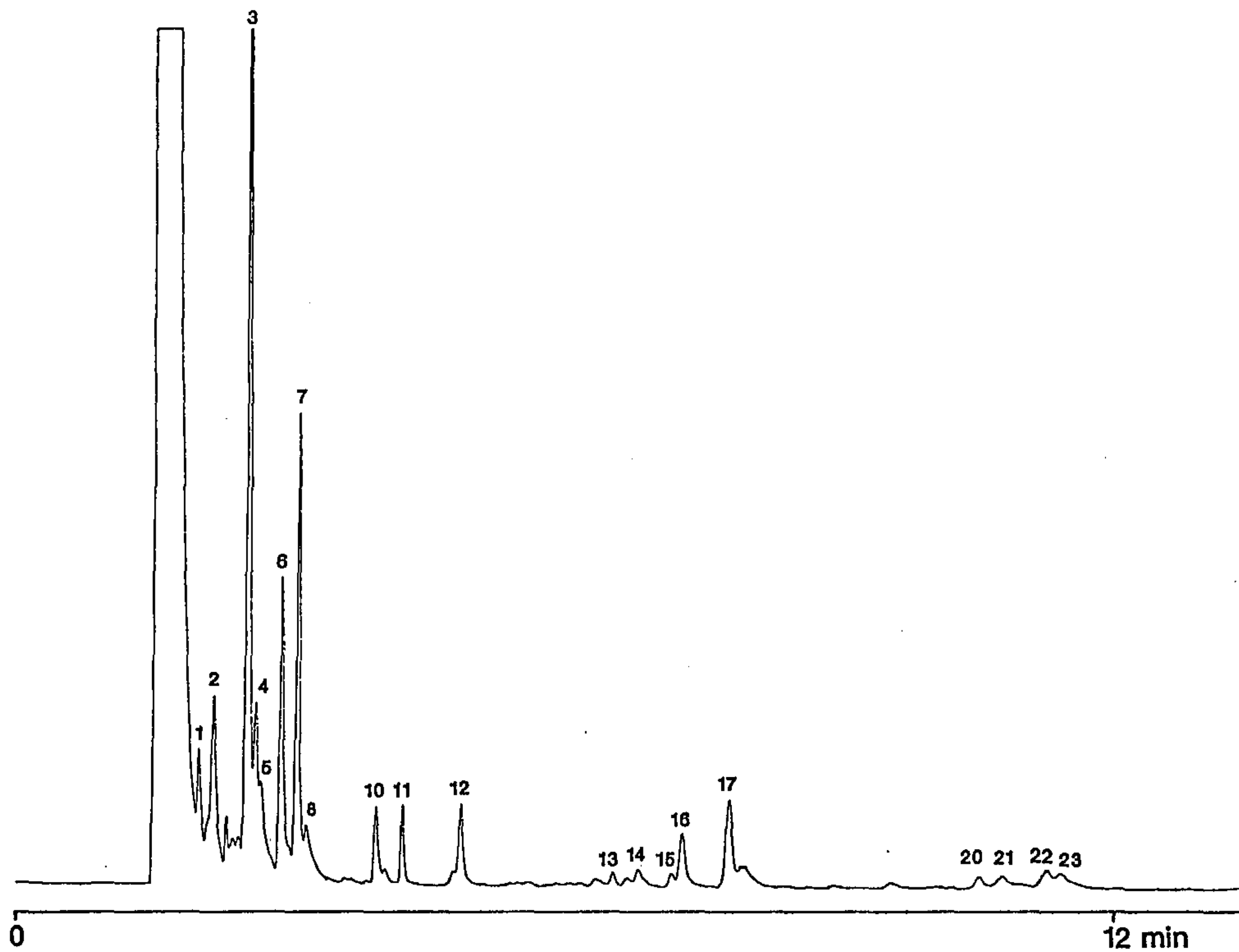


FIGURE 8.1. Isothermal GC separation of *Cymbopogon martinii* (Roxb.) Wats essential oil on non-polar column (BP-1). Column (25 m x 0.33 mm i.d) dimethyl polysiloxane 0.5 μm; temperature 160 °C; carrier gas (helium) flow rate, 2.1 ml/min. See Table 8.2 for peak identification.

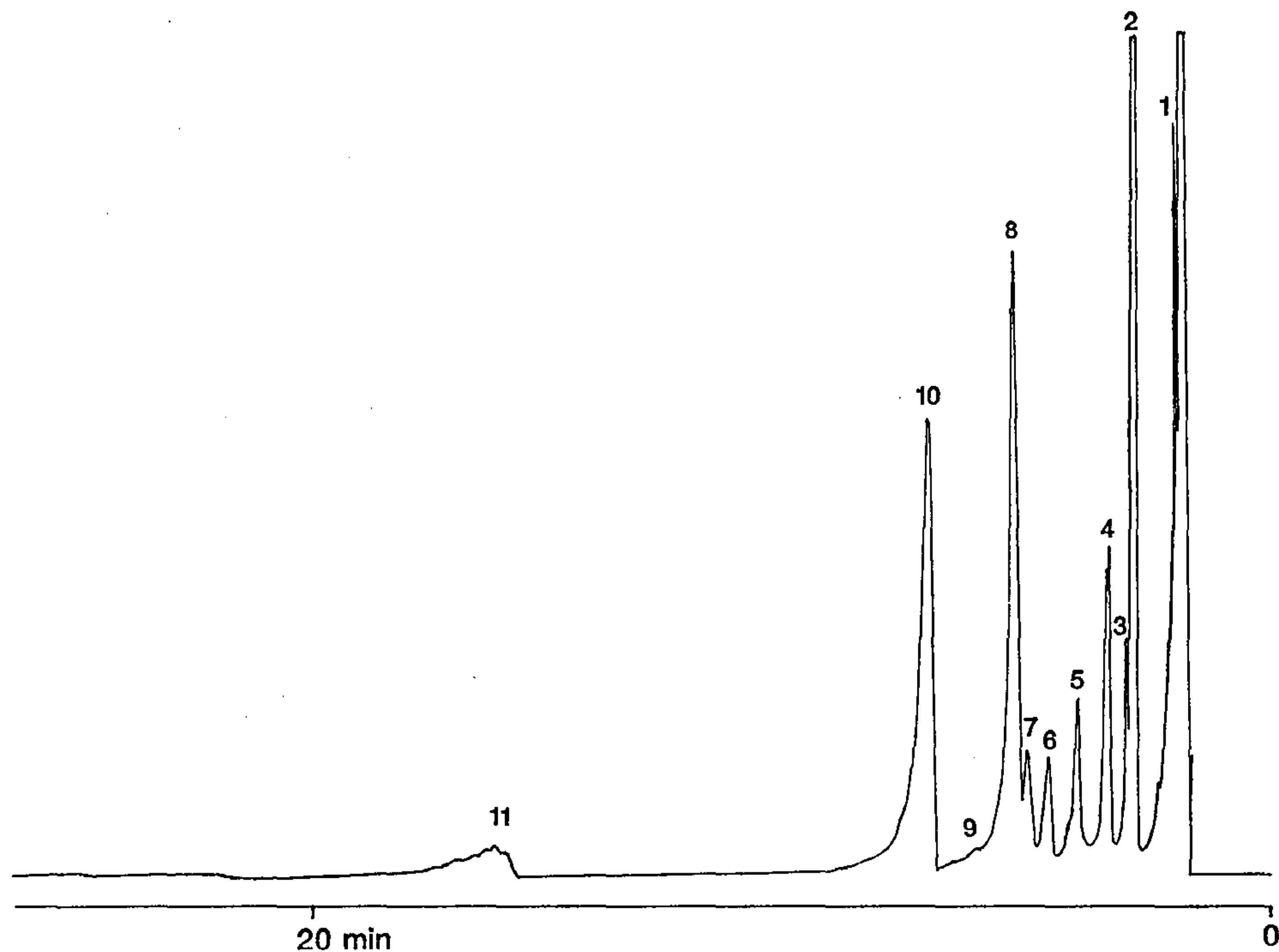


FIGURE 8.2. Isothermal GC separation of *Cymbopogon martinii* (Roxb.) Wats essential oil on polar column (BP-20). Column (25 m x 0.33 mm i.d.) polyethylene glycol 0.5 μm; temperature 140 °C; carrier gas (helium) flow rate, 2.1 ml/min. See Table 8.3 for peak identification.

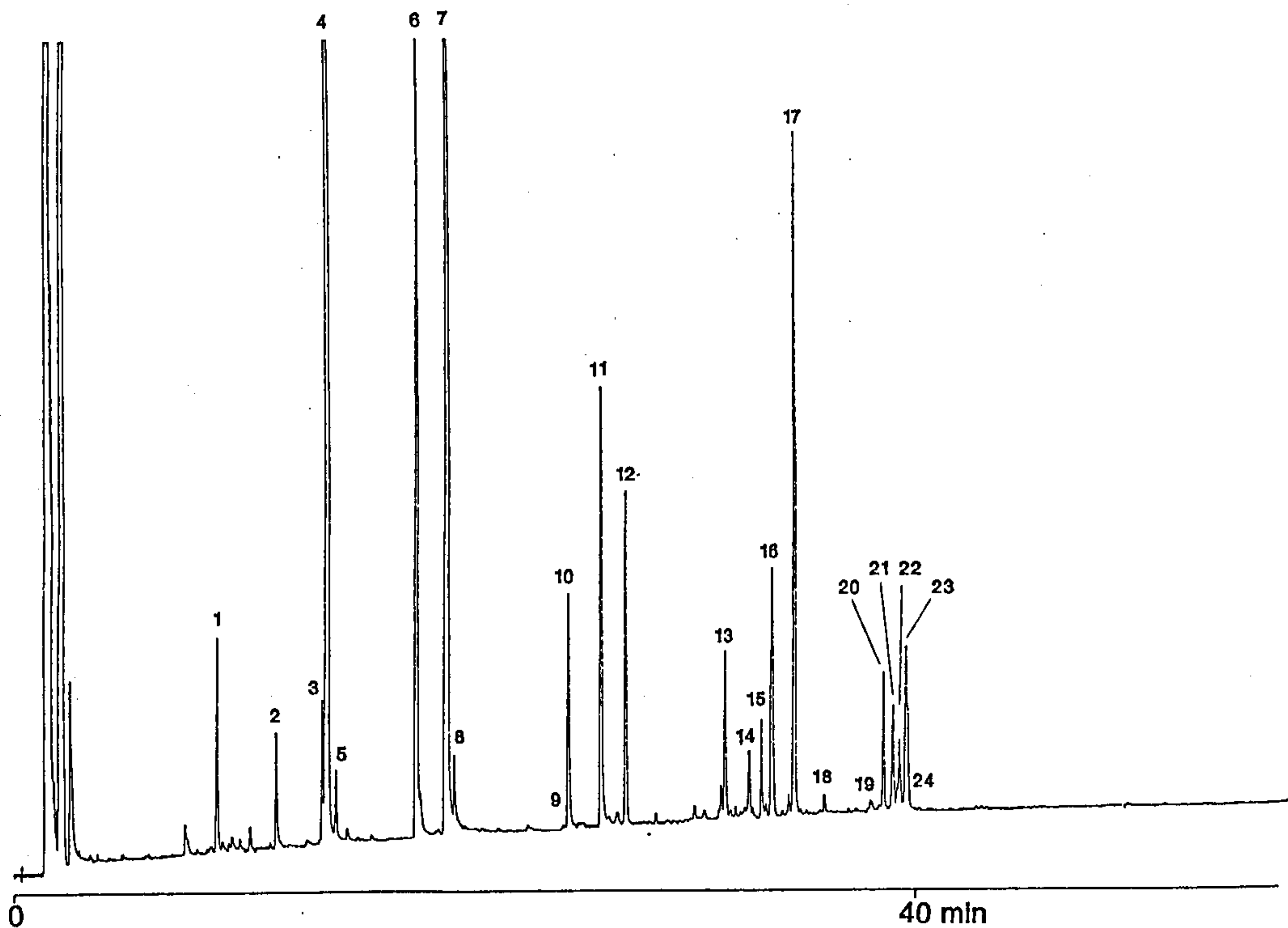


FIGURE 8.3. Temperature-programmed GC separation of *Cymbopogon martinii* (Roxb.) Wats essential oil on non-polar (BP-1) column. Temperature: 70 to 200 °C at 2 °C/min. Carrier gas (helium) flow rate, 2.1 ml/min. See Table 8.4 for peak identification.

(160 °C) and the BP-20 column (140 °C) were determined (Tables 8.2 and 8.3, respectively). The identities of some of the peaks in the chromatograms are given based mainly on retention index comparisons with the retention indices of the reference compounds obtained from isothermal GC (Table 8.1). However, only a few of the peaks could be identified.

Separation of the essential oil components by TPGC on the BP-1 column was carried out and then the results were analyzed in order to identify the peaks. The percent peak areas obtained from the chromatograms gave a good approximation of the relative abundance of the constituents of the essential oil. It was possible to identify several compounds present in the volatile oil on the basis of their retention indices and to a small extent, the percent relative area comparisons in the polar and non-polar columns (Table 8.4). The largest constituent of the essential oil was citronellal (30.0%), followed by geraniol (21.0%) and citronellol (10.0%).

These results match poorly with values reported in the literature for the essential oil of *Cymbopogon martinii* (Roxb.) Wats (palmarosa oil) from Madagascar [231]. Using the combination of Kovats' indices and GC-MS techniques of identification, Randriamiharisoa and Gaydou [231] have successfuly identified 69 components in the palmarosa oil of which the most abundant components are geraniol (76.3-82.8%), geranyl acetate (5.09-11.80%) and linalool (2.26-3.91%), ß-caryophyllene (1.00-1.76%), and limonene (0.15-2.16%) [231]. These marked differences in the relative abundances suggest that the essential oils in these two cases were possibly from different varieties of *C. martinii*.

TABLE 8.2. Kovats' retention indices and identities of major peaks of *Cymbopogon martinii* (Roxb.) Wats and standard compounds separated on BP-1 column at 160 °C.

Conditions: as in Figure 8.1.

Peak No.	RI of *C. martinii*	RI of std. cpd.	Compound
1	982		
2	1039	1044	limonene
3	1142	1141	citronellal
4	1160	1160	isopulegol
5	1174		
6	1214	1216	citronellol
7	1240	1241	geraniol
8	1255		
9	1336	1340	eugenol
10	1344		
11	1361		
12	1406		
13	1484		
14	1493		
15	1519		
16	1523		
17	1542		
20	1620		
21	1626		
22	1637		
23	1640		

TABLE 8.3. Kovats' retention indices and identities of major peaks of *Cymbopogon martinii* (Roxb.) Wats and standard compounds separated using isothermal GC on BP-20 column.

Temperatures are in brackets. Other conditions: as in Figure 8.2.

Peak No.	RI *C. martinii*	RI of std. cpd.	Compound
1	1376		
2	1539	1517(100)	citronellal
3	1577	1548(100)	linalool
4	1631		
5	1696		
6	1750		
7	1783		
8	1797	1803(140)	citronellol
9	1840	1795(165)	geranyl acetate
10	1886	1888(140)	geraniol
11	2104		

TABLE 8.4. The identities of major peaks in the temperature-programmed gas chromatographic separation of *Cymbopogon martinii* (Roxb.) Wats, separated on non-polar (BP-1) stationary phase.

Conditions: as in Figure 8.3.

Peak no.	% Relative abundance*	RI of *C. martinii*	Ref. RI	Compound
1	2.07	1018	1026a	limonene
2	1.03	1081	1089(90)b	linalool
3	1.28	1124	1133(110)b	isopulegol
4	29.98	1129	1132a	citronellal
5	0.64	1136	-	-
6	10.00	1208	1210a	citronellol
7	21.02	1234	1237a	geraniol
8	0.45	1239	-	-
9	0.14	1330	1335a	eugenol
10	2.51	1333	1336a	citronelyl acetate
11	4.84	1360	1359a	geranyl acetate
12	3.16	1380	1391a	β-elemene
13	1.66	1464	-	-
14	0.76	1485	1506e	γ-muurolene
15	0.70	1500	-	-
16	3.37	1508	1521a	δ-cadinene
17	7.43	1526	1524d	-
18	0.17	1553	-	-
19	0.06	1594	-	-
20	1.24	1609	-	-
21	1.11	1618	-	-
22	0.93	1625	-	-
23	2.52	1631	-	-

* Percentages were calculated from peak areas.

a Methyl polysiloxane, temperature-programmed GC [235]
b Non-polar stationary phases; temperatures are in brackets [238]
d SF96, 170 °C [237]
e Apiezon L, 155 °C [237]

8.4.3 GC-MS separations of the essential oil of *C. martinii*

Attempts were made to identify as many constituents of the essential oil as possible by mass spectral analysis. The separation of the essential oil sample was carried out under identical conditions to the GC study. The total ion scan of a GC-MS separation of the essential oil (Figure 8.4) showed good resolution and matched very closely with the chromatogram obtained from a TPGC analysis using FID (Figure 8.3).

The parent ions in the GC-MS spectra, which give the molecular weights of the individual compounds were readily determined. This was made simple primarily because the terpenes can be conveniently grouped according to their respective molecular weight. Thus, for an observed parent ion of m/z 136, we can be reasonably certain that the compound has a molecular formula $C_{10}H_{16}$ and then the mass spectra of the unknown peak could be compared with the mass spectra of the group of compounds, particularly the monoterpenes, which have same molecular formula. Similarly, m/z of 134, 150, 152, 154, 156, 196, 198, 204, and 222 are the parent peaks for terpenes with molecular formulae $C_{10}H_{14}$, $C_{10}H_{14}O$, $C_{10}H_{16}O$, $C_{10}H_{18}O$, $C_{10}H_{20}O$, $C_{12}H_{20}O_2$, $C_{12}H_{22}O_2$, $C_{15}H_{24}$, and $C_{15}H_{26}O$, respectively.

In some cases when the parent peaks were very small, and an extra information was required to lead to identification. The information sought could come from the other mass spectra, from the fragmentation pattern, and the retention data of the GC-MS peak.

Several compounds have been successfully identified by comparing the GC-MS spectra with available reference mass spectra [238-243] as well as by retention index comparisons. By combining the two methods of identification the assignment of many of the peaks in the TPGC

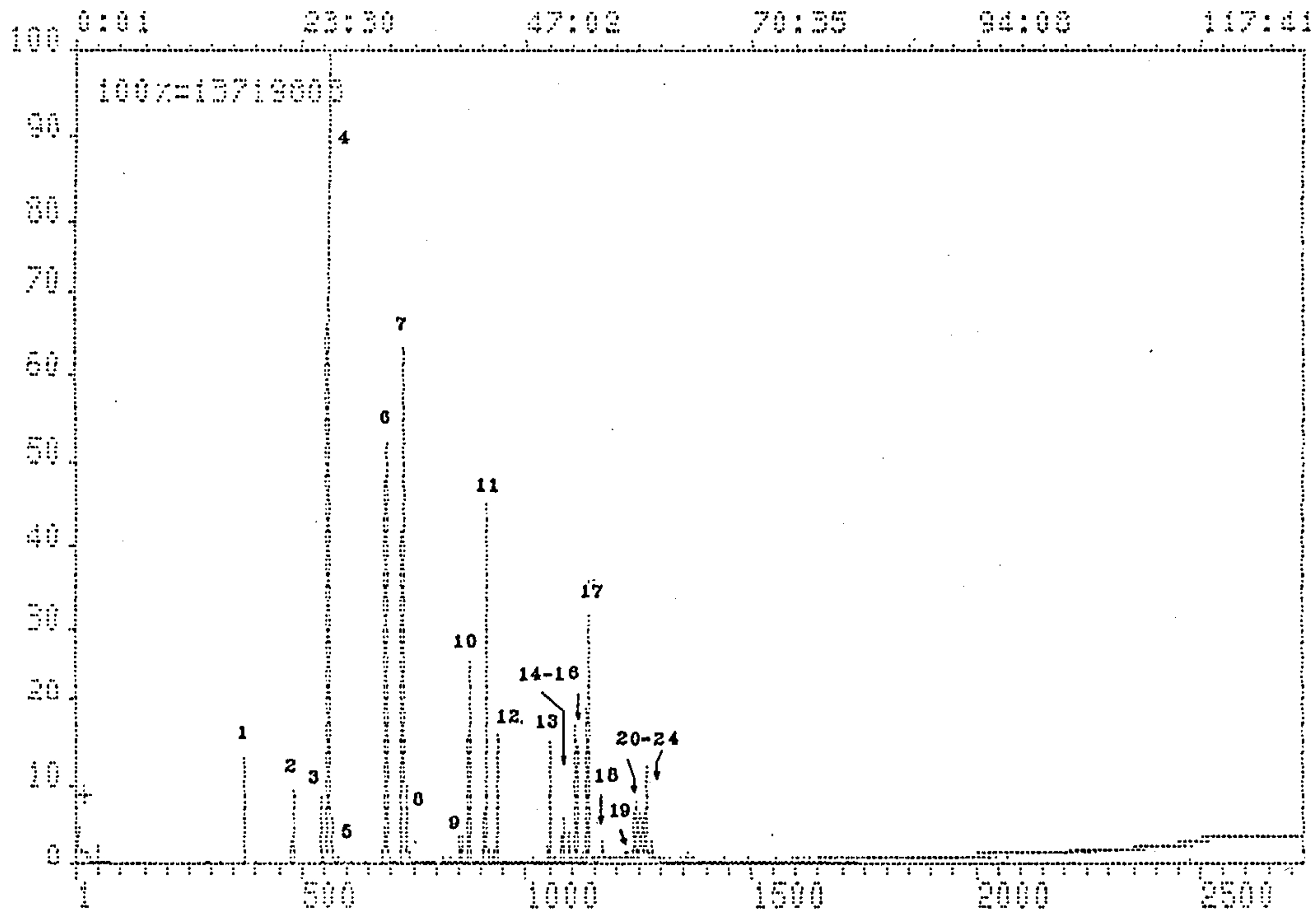


FIGURE 8.4. Total ion current trace obtained by EI-GC-MS from the essential oil of *Cymbopogon martinii* (Roxb.) Wats. Chromatographic conditions: as in Figure 8.3.

chromatogram (Figure 8.3) was accomplished (Table 8.5). Some peaks were tentatively identified on the basis that the mass spectrum resembles that of a reference mass spectrum/spectra. However, because of inevitable problems (see below), the total number of identified components was much less compared to those reported in the literature for other *C. martinii* essential oil samples [131-133].

A major problem in terpene analysis relates to the very large number of possible isomers within each group (mono-, sesqui-, etc.) of terpenes which makes it very difficult to obtain pure reference compounds [238] and the close similarity of many spectra. A reasonable match of the mass spectra often implies an identification, but sometimes it does not, since closely related compounds such as positional isomers can often give very similar mass spectra. Because of the limited number of reference standards and reference mass spectra, coupled with the lack of reference retention index data, considerable difficulty was encountered in the present work when trying to identify the higher molecular weight terpenes, particularly the sesquiterpenes and oxygenated sesquiterpenes.

Besides the lack of an adequate library of reference mass spectra for comparison, especially of terpenes, it was also noticed that differences occur sometimes in mass spectral fragmentation pattern of a compound depending on the operating conditions. This has been observed among some available reference mass spectra of some compounds, taken from different reference sources. These problems, together, imposed a considerable drawback on the mass spectral analysis. Nonetheless, the mass spectrometric method has proved to be a valuable assistant in the identification of the peaks. For instance, the presence of geranyl acetate and citronellyl acetate was elucidated from the mass spectral

TABLE 8.5. Identities of peaks in the GC-MS chromatograms of *Cymbopogon martinii* (Roxb.) Wats as determined by relative retention index and mass spectral comparisons.

GC-MS BP-1 peak numbers are as in Figure 8.4 and asterisk (*) indicates tentative identification.

GC-MS BP-1 peak no.	GC-MS BP-20 peak no.	Compound	Comparison with ref. mass spectra	RI comparison with:	
				Ref. compound	Lit. values
1	-	limonene	x(a,b,d,f)	x	x
2	3	linalool	x(a,b)	x	x
3	4	isopulegol	x(a)	x	x
4	2	citronellal	x(a,b,d)	x	x
6	13	citronellol	x(a,b)	x	x
7	16	geraniol	x(a,b)	x	x
8	8	geranial*	x(a,b)	x	x
9	-	eugenol	x(b)	x	x
10	6	citronellyl acetate	x(a,d)	-	x
11	11	geranyl acetate	x(c)	x	x
12	5	β-elemene	x(c)	-	x
13	9	guaiene*	x(c)	-	-
14	10	γ-muurolene*	x(e)	-	x
15	-	$M-204^+$	-	-	-
16	12	δ-cadinene	x(c,e)	-	x
17	18	$M-204^+$	-	-	-
18	-	$M-204^+$	-	-	-
19	-	$M-204^+$	-	-	-
20	19	β-selinene*	x(d)	-	x
21	-	$M-204^+$	-	-	-
22	-	$M-204^+$	-	-	-
23	-	$M-222^+$	-	-	-
24	-	$M-204^+$	-	-	-

Mass spectra references:
- a Ref. [238]
- b Ref. [239]
- c Ref. [240]
- d Ref. [241]
- e Ref. [242]
- e Ref. [243]

comparison studies, even though, for the latter, the retention index of the reference compound was unavailable for comparison.

One possible, but formidable solution to the above-mentioned problems in GLC and GCMS peak identification is by having a vast number of standards for the analysis. The standard compounds could be run under the same running conditions as the unknown sample, or the sample could be spiked with the pure compound to obtain unambiguous peak identification [243].

8.5 SFC SEPARATIONS OF ESSENTIAL OILS AND TERPENES

In order to examine the chromatographic behaviour of the terpenes and essential oils, SFC separations of selected terpenes: limonene, caryophyllene, geranial acetate, citronellol, and eugenol were carried out on an ODS-silica column with carbon dioxide as the mobile phase and flame ionization detection. At high pressures (hence densities) or high mobile phase flow rates the terpenes were generally eluted rapidly with poor resolution; therefore, most of the SFC separations were performed under the relatively low pressure and low eluent flow rate conditions. Under these conditions (e.g., 1660 psi, 60 °C) all the compounds were eluted in less than six minutes from a Spherisorb ODS-2 column (Figure 8.5a-e) with reasonably good peak shapes for the non-polar compounds. However, poor (tailing) peak shapes were observed for the separation of polar compounds containing hydroxyl groups (citronellol and eugenol). Similar effects have also been observed previously for the separations of polar compounds, such as benzoic acid and benzyl alcohol, under similar conditions (Chapter 5). This could probably be explained by the presence of specific polar-polar interactions between the solute

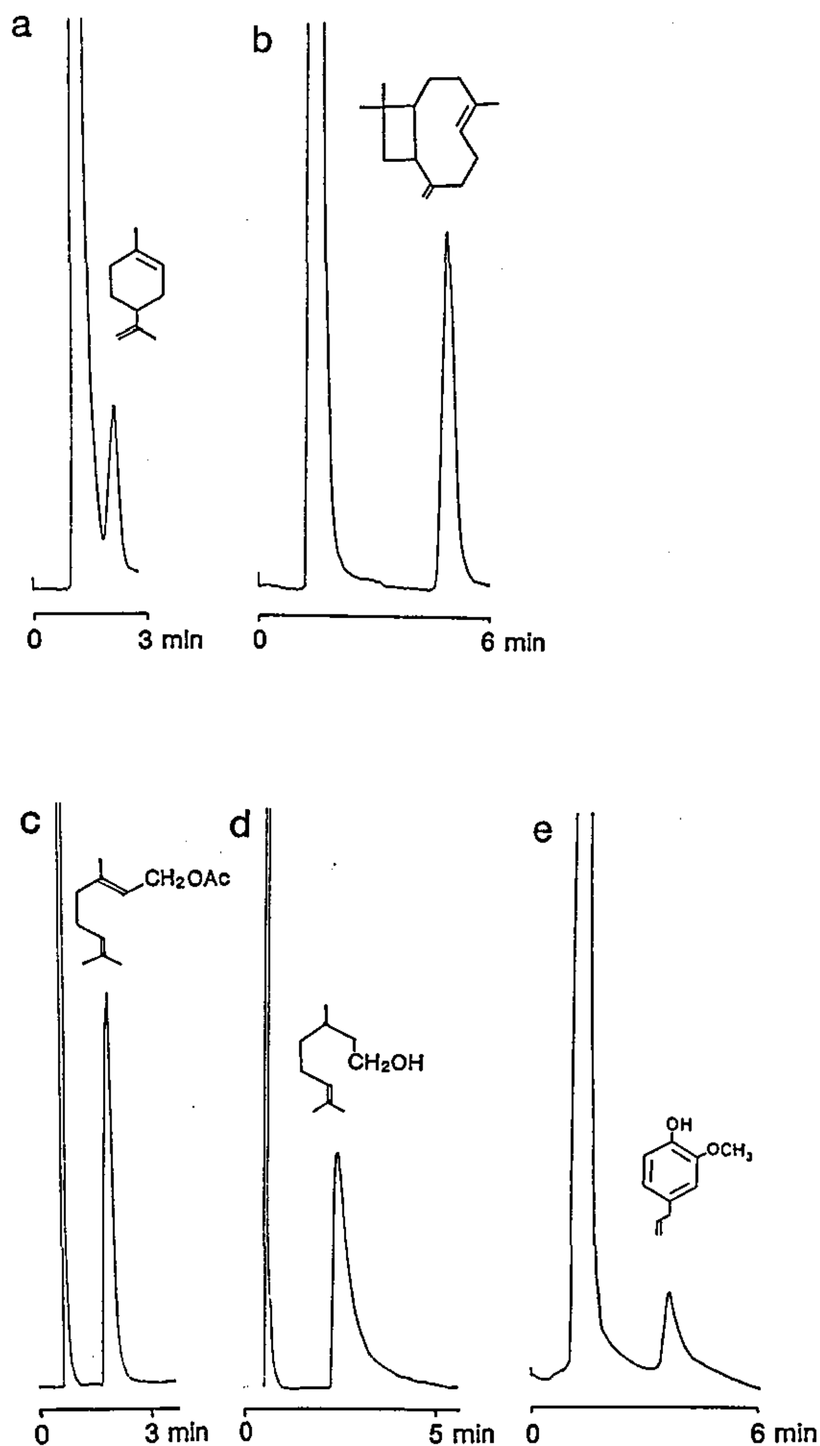


FIGURE 8.5. SFC separation of terpenes (a) linonene, (b) caryophyllene, (c) geranyl acetate, (d) citronellol, and (e) eugenol on ODS-silica column with carbon dioxide mobile phase. Column (200 x 3 mm i.d.), Spherisorb ODS-2 5 µm; mean column pressure ca. 1660 psi; 60 °C; flame ionization detection.

and unreacted residual silanol groups on the stationary phase and poor solubility of the substrate in the mobile phase.

Using the same column and under the same chromatographic conditions, SFC separations of the leaf essential oil of *C. martinii* were carried out (Figure 8.6). Several of the peaks were tentatively identified based on retention time comparisons with the reference compounds. However, most of the peaks were unresolved and besides exhibiting poor peak shapes, the polar compounds (the alcohols) were relatively more retained than aldehydes or hydrocarbons of comparable molecular weights. This has led to the suggestion that packed column SFC with carbon dioxide mobile phase can perhaps be useful for "group type separations".

It was also of interest to investigate the supercritical fluid chromatographic behaviour of the essential oil components on a PS-DVB stationary phase. Under the operating conditions (Figure 8.7), most of the peaks were not fully resolved and only two (citronellal and geraniol) were identified based on retention time comparisons. Although some of the peaks were broad, they generally showed comparatively less tailing than those obtained on an ODS-silica column (Figures 8.5 and 8.6).

The use of an organic modifier such as methanol in the carbon dioxide mobile phase has been shown to decrease the retention and improve the peak shapes particularly for polar compounds (Chapter 5). Because of the incompatibility of flame ionization detection with organic modifiers, spectroscopic detectors are normally needed in such cases. Since most of the terpenes have little or no chromophores and UV spectroscopic detector could not be used, the use of organic modifier in the mobile phase may not be very useful for SFC separations of essential oils. The use of modifier in the SFC separations of essential oils and terpenes has not been investigated in the present work.

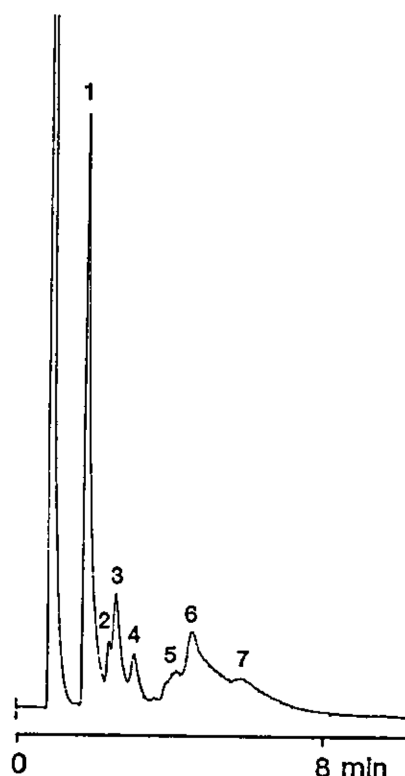


FIGURE 8.6. Separation of *Cymbopogon martinii* essential oil by SFC on ODS-silica column with carbon dioxide mobile phase. Column Spherisorb ODS-2 5 μm; mean column pressure 1660 psi; 60 °C; flame ionization detection. Tentative peak identification (based on retention time comparison): 1. citronellal, 2. limonene, 3. linalool, 4. citronellol, 5. geraniol.

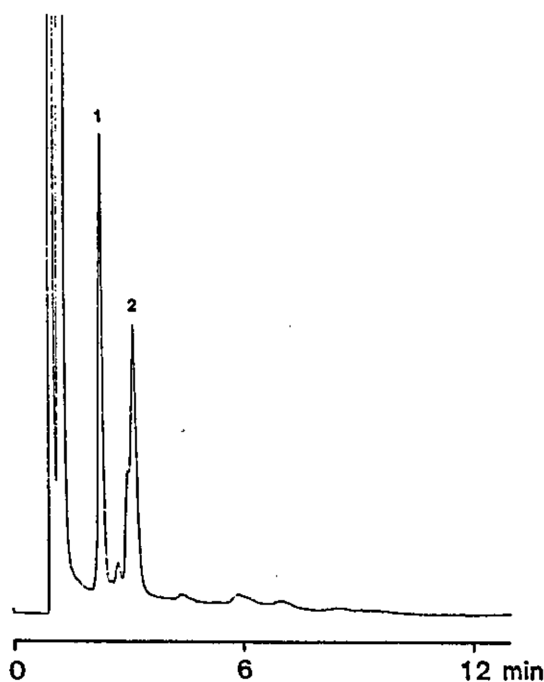


FIGURE 8.7. Isoconfertic SFC separation of *Cymbopogon martinii* essential oil on PS-DVB column with carbon dioxide mobile phase. Column PLRP-S 5 μm; mean column pressure 2260 psi; 60 °C; flame ionization detection. Tentative peak identification (based on retention time comparison): 1. citronellal, 2. geraniol.

8.6 COMPARISON OF SFC, GC, AND GC-MS METHODS IN THE ANALYSIS OF ESSENTIAL OILS AND TERPENES

Several inferences can be deduced from the observations made in the present study. Although the feasibility of the separation of terpenes and essential oil by packed column SFC has been demonstrated, the separations were generally characterized by broad peaks, and on an ODS-silica column, polar components showed tailing peaks. These have meant difficulties in peak identification by means of retention time, or retention index comparisons with the authentic reference compounds. The use of more efficient columns would probably be advantageous.

It is evident that GC and GC-MS techniques using capillary columns offer high resolving power suitable for the analysis of complex mixtures such as essential oils. Considering its limited efficiency and resolution, the use of packed column SFC in essential oil analysis would probably be most useful for fractionation of the essential oil sample in a semi-preparative scale. Because of the potentially higher efficiency and resolving power capability, capillary SFC would probably be the method of choice compared to packed column SFC as a potential viable alternative to GC for the separation of a complex mixture such as the essential oils.

It is well known that several essential oil constituents, such as terpineol and some of the sesquiterpenes, are artefacts produced during isolation or separation [205]. An on-line supercritical fluid extraction using carbon dioxide and injection of samples onto a chromatographic system could be advantageous and be an attractive solution to the problem. Such technique would offer the elimination of organic

solvents, elimination of higher temperature of steam distillation, and high separation efficiencies [244].

8.7 CONCLUSIONS

Samples of an essential oil and terpene reference standards have been examined using isothermal and temperature-programmed capillary GC. TPGC gave overall better resolution than isothermal GC when applied to essential oil analyses. The non-polar (BP-1) column gave overall better separations of the essential oil components compared to the polar (BP-20) column. From the two methods of identification, namely the Kovats' retention index and mass spectral comparisons using both non-polar and polar capillary columns, the following compounds have been determined to be present in the leaf essential oil of *Cymbopogon martinii* (Roxb.) Wats sample (steam distilled, February 1986): citronellal, geraniol, citronellol, limonene, linalool, eugenol, geranial, isopulegol, citronellyl acetate, geranyl acetate, δ-cadinene, and β-elemene. In addition, the presence of geranial, guaiene, γ-muurolene and β-selinene have also been tentatively identified.

The separation of the essential oil on packed-column SFC using carbon dioxide as the mobile phase was characterized by poor resolution of the component peaks. On an ODS-silica column, poor peak shapes were observed for polar terpenes which contain hydroxyl groups. It is also suggested that packed column SFC could be useful for semi-preparative fractionation for group-type separations. The use of a modifier such as methanol in the modifier would probably reduce these tailing effects but would be incompatible with the flame ionization detection.

CHAPTER 9

CONCLUSIONS AND SUGGESTIONS FOR FURTHER STUDIES

Successful results have been achieved in the present study of the selectivity and applications of SFC carried out using a home-made packed column instrument using different packing materials and with pure or modified carbon dioxide as the mobile phase. These will hopefully, contribute to a better understanding of this new technique, although, of course, there remain scopes available for further refinements. As a part of the study, it has also been shown that retention indices have a great potential for use in SFC as a means of recording retention which also enable selectivity changes to be clearly observed with changes in pressure, temperature, density, and mobile phase composition.

The supercritical fluid chromatograph was constructed based on a Pye-Unicam 104 gas chromatograph and a HPLC pump and it uses conventional HPLC packed columns and UV and flame ionization detectors. In some parts of the work, methanol and acetonitrile have been used as modifiers but they were found to be incompatible with flame ionization detector because of the strong background noise which resulted in poor detection sensitivity.

Preliminary experiments were carried out to gain familiarity with packed column SFC system and to establish suitable operating conditions for the separation of a number of types of analytes. The performance of the SFC system has been evaluated. Good short-term retention time and peak area reproducibilties of better than 0.76-1.56% RSD for propylbenzene, tetradecane, and butyrophenone were found. The long-term reproducibility of the system on a day to day basis was also good, with

RSDs in the range of 3.88 to 4.40%. The reproducibility results were comparable to those obtained by previous workers [76] and to those typically obtained from conventional HPLC systems, but they were inferior to those reported for capillary SFC [102,114].

A PS-DVB column has been shown to be suitable for SFC separations using unmodified and modified carbon dioxide mobile phases. The absence of specific solute-stationary phase interactions, corresponding to the silanol group effects found with silica-based columns, means that the PS-DVB column has advantages for the separation of polar solutes.

The possibilities of the application of retention indices in SFC have been investigated on PS-DVB, ODS-silica, and cyano-silica columns using different sets of homologues, n-alkanes, alkylarylketones, and alkylbenzenes. Emphasis was given to the use of alkylarylketones and n-alkanes as possible retention index scales. The former was more advantageous as it has a strong absorbing chromophore and hence is more suitable for use with UV detection in conjunction with the use of organic modifiers in the mobile phase.

The linearity of the correlations between log capacity factors and retention indices (carbon number x 100) was studied with variations in pressure, temperature, and mobile phase composition. Good correlations were obtained using the n-alkanes but significant deviations were observed for alkylarylketones, specifically for the first two members in series (acetophenone and propiophenone). However, good correlations for the relationship were observed if these two compounds were omitted from the statistical analysis.

As in HPLC, the use of retention indices have been shown to have advantages over capacity factors. However, unlike HPLC, retention indices in SFC are much more susceptible to selectivity changes caused

by variations in the operating parameters. These have been shown to be particularly evident with changes in organic modifier compositions and most noticeable with the more polar compounds such as carboxylic acids, amines, and alcohols.

The use of relative capacity factors has also been briefly examined in the analysis of barbiturates. This method of recording retention was found to be more reproducible than capacity factors or retention index methods but still showed some changes which are significantly greater than those typically found in HPLC.

Solute retention and resolution of the chromatographic separation in SFC can be controlled by a number of parameters. The technique has extra flexibility for the control of retention and resolution including temperature, pressure, density, as well as the nature and composition of the stationary and the mobile phases. However, the extra variables involved have proved to be more difficult to control which create additional problems in attempts to achieve good peak area and retention reproducibility.

In order to gain a better understanding of selectivity and solute retention behaviour in SFC, separations were carried out using selected model compounds which are characteristic of different interactions. Comparison of the chromatographic behaviour of these compounds were performed on ODS-silica, cyano-silica, and PS-DVB packing materials at different temperatures, pressures, and mobile phase compositions. In general, little change in selectivity with variations in the operating parameters was observed between compounds in a homologous series. However, selectivity differences were observed between compounds of different functionalities and very odd behaviour was seen for acetophenone on a cyano-silica column.

The results of the experiments suggest that increased pressure (density) can increase the selective solvation power of carbon dioxide mobile phase for non-aromatic, as compared to aromatic hydrocarbons. The results also showed that changes in temperature can also bring about different selectivities between compounds of different functionalities.

In order to examine the effect of modifiers in SFC, a series of experiments was carried out on the PS-DVB column with different percentages of methanol and acetonitrile. In each case, the addition of the modifiers into the carbon dioxide mobile phase decreased the retention of the compounds. However, with nominally identical concentrations, methanol was observed to be a stronger modifier than acetonitrile for the elution of polar compounds, benzyl alcohol, p-cresol, benzoic acid, and benzamide and gave better peak shapes.

Similar trends in retentions were observed for ODS-silica and cyano-silica stationary phases although generally the compounds were more retained on the PS-DVB column, suggesting that the selectivity was controlled mainly by the properties of the mobile phase.

The SFC system has been applied to the analysis of several drug compounds, barbiturates, benzodiazepines and terpenes in order to examine the usefulness of the technique. With no modifier in the carbon dioxide mobile phase, none of the barbiturates under investigation were eluted from an ODS-silica column but on a PS-DVB column, the barbiturates were eluted with reasonable analysis times. The separations of the barbiturates both on the PS-DVB and ODS-silica columns were possible when polar modifiers were used. A drastic decrease in retention was observed for all the barbiturates on the addition of small amounts of methanol in the mobile phase. Selectivity of the barbiturates on PS-DVB and ODS-silica columns have been compared and small but

significant variations were observed. However, the selectivity among the barbiturates did not change much with variations in modifier concentrations.

The supercritical fluid chromatography of benzodiazepines has been examined on PS-DVB, ODS-silica, and cyano-silica columns over a range of operating conditions. With pure carbon dioxide as the mobile phase, none of the compounds were eluted from ODS-silica and cyano-silica columns, and on a PS-DVB column, they were very retained and exhibited tailing peaks. With the use of methanol as the modifier in the mobile phase, all the benzodiazepines (except loprazolam) were readily eluted from the three columns with reasonable analysis times and considerable improvements in peak shapes. Some changes in selectivity among the benzodiazepines were observed with increasing methanol concentrations on PS-DVB and ODS-silica columns depending on the functionalities of the compounds. On the ODS-silica column, benzodiazepines containing hydroxyl groups were selectively more retained on the column at low methanol concentrations and their retention decreased relatively more with increasing methanol concentrations.

SFC separations of several terpenes and the essential oil from *Cymbopogon martinii* (Roxb.) Wats have been carried out on a PS-DVB column with carbon dioxide as the mobile phase and flame ionization detection. The results have been compared with those obtained in a separate set of experiments using GC and GC-MS methods. It was evident that the separation of the essential oil by GC methods gave better resolution and sensitivity and would still be the method of choice. A number of components of the essential oil have been identified by means of retention index and mass spectral comparisons.

Obviously, there is room for improvement in the present SFC system and vast areas worthy of further investigations and some of these aspects are briefly considered in this final section. A number of refinements can be made to the SFC system including in the detection system and column-to-FID detector interfacing to improve sensitivity. Other restrictors of different designs can be used and evaluated in a future work. Also, better ways could be pursued to improve the peak area and retention time reproducibilty of the system.

Further investigations can be carried out by extending the range of operating conditions of the temperature and/or pressure. Separations could also be performed on different column materials and using different mobile phase compositions. A number of common organic solvents such as formic acid give a low response in the FID [71] and they may be suitable for analysis of compounds with poor or non-existant absorbing chromophores such as aliphatic hydrocarbons and fatty acids.

Selective chromatographic detectors generally exhibit enhanced sensitivity to certain classes of compounds while simultaneously being unresponsive to other sample components that are less of interest to the analyst. This is particularly advantageous when trying to selectively detect single components in unresolved mixture. The use of selective detectors is worthy of further studies. For example, the nitrogen-phosphorous detector (NPD) with SFC would be advantageous for sensitive detection of many nitrogen-containing drug compounds such as the barbiturates and benzodiazepines.

Limited work has been carried out on the separation of the model compounds and the retention index standards on cyano-silica columns. Further work using this column is necessary to examine the retention behaviour of the compounds under greater range of operating conditions,

and also of interest, to investigate into the particularly anomalous retention behaviour of acetophenone on cyano-silica stationary phases in order to determine the unusual retention mechanism of this compound.

Literally, an endless number of potential applications of SFC awaits further studies. In the present work, no attempt has been made to demonstrate the application of the SFC system to the determinations of drug compounds in "real samples", such as in biological fluids, and this could be worth of further investigations. Finally, the combination of SFC with supercritical fluid extraction (SFE) could be investigated and such a system would allow direct quantitative and qualitative analyses of biological samples and various plant products.

REFERENCES

1. E. Klesper, A.H. Corwin, and D.A. Turner, J. Org. Chem., 1962, 27, 700.
2. R.M. Smith, (Ed.), "Supercritical Fluid Chromatography", RSC Chromatography Monographs, Royal Society of Chemistry, London, 1988.
3. T.G. Squires and M.E. Paulaitis, (Eds.), "Supercritical Fluids: Chemical Engineering Principles and Applications", ACS Symp. Ser., Vol. 329, American Chemical Society, Washington, D.C., 1987.
4. B.A. Carpentier and M.R. Sevenants, (Eds.), "Supercritical Fluid Extraction and Chromatography: Techniques and Applications", ACS Symp. Ser., Vol. 366, American Chemical Society, Washington, D.C., 1988.
5. K.E. Markides and M.L. Lee, (Compilers), "SFC Applications", Brigham Young University, Provo, 1988.
6. "SFaCts: A Comprehensive Bibliography of SFC and Related Articles", White Associates, Pittsburgh, 1987.
7. M.M. Sanagi and R.M. Smith, in Ref. 2, pp. 29-52.
8. P.A. Peaden and M.L. Lee, J. Chromatogr., 1982, 5 (Suppl. 2), 179.
9. D.R. Gere, Science, 1983, 222, 253.
10. D.W. Later, B.E. Richter, and M.R. Andersen, LC-GC, 1986, 4, 992.
11. M.L. Lee and K.E. Markides, J. High Resolut. Chromatogr. Chromatogr. Commun., 1986, 9, 652.
12. K.D. Bartle, I.K. Barker, A.A. Clifford, J.P. Kithinji, M.W. Raynor, and G.F. Shilstone, Anal. Proc., 1987, 24, 299.
13. M.L. Lee and K.E. Markides, Science, 1987, 235, 1342.
14. D.E. Games, A.J. Berry, I.C. Mylchreest, J.R. Perkins, and S. Pleasance, European Chromatogr. News, 1987, 1, 10.

15. P.J. Schoenmakers and L.G.M. Uunk, European Chromatogr. News, 1987, 1, 14.

16. P.J. Schoenmakers and F.C.C.J.G. Verhoeven, Trends Anal. Chem., 1987, 6, 10.

17. D.R. Gere, R.K. Houck, F. Pacholec, A. Athos, C.P. Rosselli, Fresenius Z. Anal. Chem., 1988, 330, 222.

18. P.J. Shoenmakers, "Optimization of Chromatographic Selectivity", J. Chromatogr. Libr., Vol. 35, Elsevier, Amsterdam, 1986.

19. R.M. Smith, Adv. Chromatogr., 1987, 26, 277.

20. H.T. Kalinoski, H.R. Udseth, B.W. Wright, and R.D. Smith, J. Chromatogr., 1987, 400, 307.

21. A.J. Berry, D.E. Games, I.C. Mylchreest, J.R. Perkins, and S. Pleasance, J. High Resolut. Chromatogr. Chromatogr. Commun., 1988, 11, 61.

22. J.D. Pinkston, G.D. Owens, L.J. Burkes, T.E. Delaney, D.S. Millington, and D.A. Maltby, Anal. Chem., 1988, 60, 962.

23. M.W. Raynor, K.D. Bartle, I.L. Davies, A. Wiliams, A.A. Clifford, J.M. Chalmers, and B.W. Cook, Anal. Chem., 1988, 60, 427.

24. P.R. Griffiths, S.L. Pentoney, Jr., A. Giorgetti, and K.H. Shafer, Anal. Chem., 1986, 58, 1349A.

25. D. Wieboldt and G. Adams, International Labmate Guide, 1987, p. 11.

26. M.V. Budahegyi, E.R. Lombosi, T.S. Lombosi, S.Y. Meszaros, Sz. Nyiredy, G. Tarjan, I. Timar, and J.M. Takacs, J. Chromatogr., Chromatogr. Rev., 1983, 271, 213.

27. J.K. Haken, Adv. Chromatogr., 1976, 14, 367.

28. R.M. Smith, J. Chromatogr., 1982, 236, 321.

29. J.A. Dean, (Ed.), "Lange's Handbook of Chemistry", 13th Ed., McGraw-Hill, New York, 1985.

30. U. van Wasen, T. Swaid, and G.M. Schneider, Angew. Chem. Int. Ed. Engl., 1980, 19, 575.

31. P.J. Schoenmakers and L.G.M. Uunk, Chromatographia, 1987, 24, 51.

32. K.D. Bartle, in Ref. 2., pp. 1-28.

33. F.P. Schmitz and E. Klesper, J. High Resolut. Chromatogr. Chromatogr. Commun., 1987, 10, 519.

34. C.R. Yonker, D.G. McMinn, B.W. Wright, and R.D. Smith, J. Chromatogr., 1987, 396, 19.

35. P. Mourier, P. Sassiat, M. Caude, and R. Rosset, J. Chromatogr., 1986, 353, 61.

36. D. Leyendecker, in Ref. 2, pp. 53-101.

37. U. van Wasen and G.M. Schneider, Chromatographia, 1975, 8, 274.

38. P.J. Schoenmakers and F.C.C.J.G. Verhoeven, J. Chromatogr., 1986, 352, 315.

39. A. Wilsch, Ber. Bunsenges. Phys. Chem., 1984, 88, 922.

40. D. Leyendecker, F.P. Schmitz, and E. Klesper, J. Chromatogr., 1984, 315, 19.

41. S.M. Fields and M.L. Lee, J. Chromatogr., 1985, 349, 305.

42. M. Proot, P. Sandra, and E. Geeraert, J. High Resolut. Chromatogr. Chromatogr. Commun., 1986, 9, 189.

43. C.R. Yonker and R.D. Smith, J. Chromatogr., 1986, 351, 211.

44. C.R. Yonker, B.W. Wright, R.C.. Petersen, and R.D. Smith, J. Phys. Chem., 1985, 89, 5526.

45. F. Bickmann and B. Wenclawiak, Fresenius Z. Anal. Chem., 1985, 320, 261.

46. S.T. Sie and G.W. Rijnders, Sep. Sci., 1967, 2, 729.

47. L. McLaren, M.N. Myers, and J.C. Giddings, Science, 1968, 159, 197.

48. M. Caude and R. Rosset, Analusis, 1986, 14, 310.

49. H.E. Schwartz, LC-GC, 1987, 5, 14.

50. P.J. Schoenmakers, in Ref. 2, pp. 102-136.

51. P.J. Schoenmakers, Laboratory News, July 10, 1987, p. 12.

52. J.R. Wheeler, M.E. McNally, J. Chromatogr., 1987, 410, 343.

53. M. Novotny, S.R. Springston, P.A. Peaden, J.C. Fjeldsted, and M.L. Lee, Anal. Chem., 1981, 53, 407A.

54. J&W Scientific, Catalog on High Resolution Chromatography Products, 1987/1988.

55. K.E. Markides and M.L. Lee, in "Advances in Capillary Chromatography", J.G. Nikelly (Ed.), Huethig, Heidelberg, 1986, pp. 19-34.

56. J.C. Kuei, B.J. Tarbet, W.P. Jackson, J.S. Bradshaw, K.E. Markides, and M.L. Lee, Chromatographia, 1985, 20, 25.

57. M.W. Ogden and H.M. McNair, J. High Resolut. Chromatogr. Chromatogr. Commun., 1985, 8, 816.

58. S. Rokushika, K.P. Naikwadi, A.L. Jadhav, and H. Hatano, J. High Resolut. Chromatogr. Chromatogr. Commun., 1985, 8, 480.

59. J.M. Levy and W.M. Ritchey, J. High Resolut. Chromatogr. Chromatogr. Commun., 1985, 8, 503.

60. J.V. Dawkins, in "Techniques in Liquid Chromatography", C.F. Simpson, (Ed.), Wiley, Chichester, 1982, pp. 337-366.

61. Hamilton, Bonaduz, Switzerland, Hamilton PRP-1 Literature, 1981; Jones Chromatography Catalogue, 1987.

62. Polymer Laboratories Ltd., PLRP-S Brochures, Church Stretton, Shropshire, 1987.

63. R. Board, D. McManigill, H. Weaver, and D.R. Gere, Hewlett-packard Co., Publication No. 43-5953-1647, 1982, pp. 17-20.

64. Ph. Morin, M. Caude, and R. Rosset, J. Chromatogr., 1987, 407, 87.

65. R.M. Smith and M.M. Sanagi, J. Pharm. Biomed. Applic., 1988, 6, in the press.

66. L.R. Snyder, J. Chromatogr. Sci., 1978, 16, 223.

67. L.G. Randall, Hewlett-Packard Co., Publication No. 43-5953-1722, 1983.

68. S.M. Fields, K.E. Markides, and M.L. Lee, J. Chromatogr., 1987, 406, 223.

69. B.W. Wright and R.D. Smith, J. Chromatogr., 1986, 355, 367.

70. A.L. Blilie and T. Greibrokk, Anal. Chem., 1985, 57, 2239.

71. J.M. Levy and W.M. Ritchey, J. Chromatogr. Sci., 1986, 24, 242.

72. J.B. Crowther and J.D. Henion, Anal. Chem., 1985, 57, 2711.

73. P. Mourier and R. Rosset, Analusis, 1985, 13, 299.

74. C.R. Yonker and R.D. Smith, J. Chromatogr., 1986, 361, 25.

75. A.L. Blilie and T. Greibrokk, J. Chromatogr., 1985, 349, 317.

76. B.W. Wright, H.T. Kalinoski, and R.D. Smith, Anal. Chem., 1985, 57, 2823.

77. C.R. Yonker and R.D. Smith, J. Phys. Chem., 1988, 92, 1664.

78. C.R. Yonker, R.W. Gale, and R.D. Smith, J. Phys. Chem., 1987, 91, 3333.

79. P.J. Schoenmakers, J. Chromatogr., 1984, 315, 1.

80. J.R. Wheeler and M.E. McNally, Fresenius Z. Anal. Chem., 1988, 330, 237.

81. C.H. Lochmuller and L.P. Mink, J. Chromatogr., 1987, 409, 55.

82. A.J. Berry, D.E. Games, and J.R. Perkins, Anal. Proc., 1986, 23, 451.

83. Brownlee Labs, Inc., Technical Notes No. 925, Santa Clara, 1986.

84. D.W. Later, D.J. Bornhop, E.D. Lee, J.D. Henion, and R.C. Wieboldt, LC-GC, 1987, 5, 804.

85. M. Novotny, J. High Resolut. Chromatogr. Chromatogr. Commun., 1986, 9, 137.

86. B.E. Richter, J. High Resolut. Chromatogr. Chromatogr. Commun., 1985, 8, 297.

87. O.G. Vitzthum, P. Werkhoff, and P. Hubert, J. Agric. Food Chem., 1975, 23, 999.

88. M.M. Sanagi and R.M. Smith, Anal Proc., 1987, 24, 304.

89. M.G. Rawdon, Anal. Chem., 1984, 56, 831.

90. D. Thiebaut, M. Caude, and R. Rosset, Analusis, 1987, 15, 528.

91. T.L. Chester, J. Chromatogr., 1984, 299, 424.

92. T.L. Chester, D.P Innis, and G.D. Owens, Anal. Chem., 1985, 57, 2243.

93. D.E. Games, A.J. Berry, I.C. Mylchreest, J.R. Perkins, and S. Pleasance, in Ref. 2, pp. 159-174.

94. J.W. Hellgeth, J.W. Jordan, L.T. Taylor, and M. Ashraf-Khorassani, J. Chromatogr. Sci., 1986, 24, 183.

95. K.H. Shafer, S.L. Pentoney, P.R. Griffiths, Anal. Chem., 1986, 58, 58.

96. J.C. Gluckman, D.C. Shelly, and M. Novotny, Anal. Chem., 1985, 57, 1546.

97. W.P. Jackson, K.E. Markides, and M.L. Lee, J. High Resolut. Chromatogr. Chromatogr. Commun., 1986, 9, 213.

98. K.E. Markides, E.D. Lee, R. Bolick, and M.L. Lee, Anal. Chem., 1986, 58, 740.

99. W.R. West and M.L Lee, J. High Resolut. Chromatogr. Chromatogr. Commun., 1986, 9, 161.

100. W. Gmuer, J.O. Bosset, and E. Plattner, Chromatographia, 1987, 23, 199.

101. T. Greibrokk, A.L. Blilie, E.J. Johansen, and E. Lundanes, Anal. Chem., 1984, 56, 2681.

102. N.L. Porter, B.E. Richter, D.J. Bornhop, D.W. Later, and F.H. Beyerlein, J. High Resolut. Chromatogr. Chromatogr. Commun., 1987, 10, 477.

103. K. Grob, J. High Resolut. Chromatogr. Chromatogr. Commun., 1983, 6, 178.

104. S.T. Sie, W. van Beersum, and G.W.A. Rijnders, Sep. Sci., 1966, 1, 459.

105. J.F.K. Huber, (Ed.), "Instrumentation for High Performance Liquid Chromatography", J. Chromatogr. Libr., Vol. 13, Elsevier, Amsterdam, 1978.

106. D.R. Gere, R. Board, and D. McManigill, Anal. Chem., 1982, 54, 736.

107. T.J. Bruno, LC Magazine, 1986, 4, 134.

108. R. Simpson, J.R. Gant, and P.R. Brown, J. Chromatogr., 1986, 371, 109.

109. T. Greibrokk, J. Doehl, A. Farbrot, and B. Iversen, J. Chromatogr., 1986, 371, 145.

110. P.A. Mourier, E. Eliot, M.H. Caude, R,H. Rosset, and A.G. Tambute, Anal. Chem., 1985, 57, 2819.

111. P.A. Peaden, J.C. Fjeldsted, M.L. Lee, S.R. Springston, and M. Novotny, Anal. Chem., 1982, 54, 1090.

112. J. Doehl, A. Farbrot, T. Greibrokk, and B. Iversen, J. Chromatogr., 1987, 392, 175.

113. M. Novotny and P. David, J. High Resolut. Chromatogr. Chromatogr. Commun., 1986, 9, 647.

114. H.E. Schwartz, P.J. Barthel, and S.E. Moring, J. High Resolut. Chromatogr. Chromatogr. Commun., 1987, 10, 668.

115. C.M. White, D.R. Gere, D. Boyer, F. Pacholec, and L.K. Wong, J. High Resolut. Chromatogr. Chromatogr. Commun., 1988, 11, 94.

116. E. Klesper, Fresenius Z. Anal. Chem., 1988, 330, 200.

117. F.P. Schmitz, D. Leyendecker, D. Leyendecker, and B. Gemmel, J. Chromatogr., 1987, 395, 111.

118. F.P. Schmitz and E. Klesper, J. Chromatogr., 1987, 388, 3.

119. R.E. Jentoft and T.H. Gouw, Anal. Chem., 1972, 44, 681.

120. F.J. van Lanten and L.D. Rothman, Anal. Chem., 1976, 48, 1430.

121. J.C. Fjeldsted, W.P. Jackson, P.A. Peaden, and M.L. Lee, J. Chromatogr. Sci., 1983, 21, 222.

122. R.D. Smith, E.G. Chapman, and B.W. Wright, Anal. Chem., 1985, 57, 2829.

123. A. Wilsch and G.M. Schneider, J. Chromatogr., 1986, 357, 239.

124. K.H. Linnemann, A. Wilsch, and G.M. Schneider, J. Chromatogr., 1986, 369, 39.

125. B. Wenclawiak, Fresenius Z. Anal. Chem., 1986, 323, 492.

126. B.W. Wenclawiak, Fresenius Z. Anal. Chem., 1988, 330, 218.

127. M. Novotny, W. Bertsch, and A. Zlatkis, J. Chromatogr., 1971, 61, 17.

128. M.P. Vukalovich and V.V. Altunin, "Thermophysical Properties of Carbon Dioxide", translated by D.S. Gaunt, Collet's, London, 1968.

129. S. Angus, B. Armstrong, and K.M. de Reuck, (Eds.), "IUPAC International Thermodynamic Tables of the Fluid State, Carbon Dioxide", Pergamon, Oxford, 1977.

130. P.J. Shoenmakers, P.E. Rothfusz, F.C.C.J.G. Verhoeven, J. Chromatogr., 1987, 395, 91.

131. M.V. Novotny, in "Microcolumn Separation", M.V. Novotny and D. Ishii, (Eds.), J. Chromatogr. Libr., Vol. 30, Elsevier, Amsterdam, 1985, pp. 105-120.

132. W. Asche, Chromatographia, 1978, 11, 411.

133. N.M. Karayannis and A.H. Corwin, J. Chromatogr. Sci., 1970, 8, 251.

134. T. Altares, Jr., J. Polym. Sci. Part B, 1970, 8, 761.

135. J.A. Nieman and L.B. Rogers, Sep. Sci. Technol., 1975, 10, 517.

136. J. Sevcik, "Detectors in Gas Chromatography", J. Chromatogr. Libr., Vol. 4, Elsevier, Amsterdam, 1976.

137. O.G. Vitzthum, P. Werkhoff, and P. Hubert, J. Food Sci., 1975, 40, 911.

138. J.C. Fjeldsted and M.L. Lee, Anal. Chem., 1984, 56, 619A.

139. M. Novotny, Anal. Chem., 1981, 53, 1924A.

140. M. Verzele and C. Dewaele, LC-GC, 1986, 4, 614.

141. J. Cousin and P.J. Arpino, J. Chromatogr., 1987, 398, 125.

142. P.J. Schoenmakers, F.C.C.J.G. Verhoeven, and H.M. van Denbogaert, J. Chromatogr., 1986, 371, 121.

143. S.J. Bale, Doctoral Thesis, Loughborough University of Technology, 1988.

144. M.L. Lee, F.J. Yang, and K.D. Bartle, "Open Tubular Column Gas Chromatography", Wiley, New York, 1984.

145. R.M. Smith, T.G. Hurdley, R. Gill, and A.C. Moffat, Chromatographia, 1984, 19, 401.

146. P.A. Mourier, M.H. Caude, R.H. Rosset, Chromatographia, 1987, 23, 21.

147. S.T. Sie and G.W.A. Rijnders, Sep. Sci., 1967, 2, 755.

148. R.E. Schirmer, (Ed.), "Modern Methods of Pharmaceutical Analysis", CRC Press, Boca Raton, 1982.

149. P. Sandra, F. David, F. Munari, G. Mapelli, and S. Trestianu, in Ref. 2, pp. 137-258.

150. D.K. Dandge, J.P. Heller, and K.V. Wilson, Ind. Eng. Chem. Prod. Res. Dev., 1985, 24, 162.

151. J.P. Chaytor, Food Chem., 1987, 23, 19.

152. J.H. Wolf and J. Korf, J. Chromatogr., 1988, 436, 437.

153. J.C. Berridge, "Techniques for the Automated Optimization of HPLC Separations", Wiley, Chichester, 1985.

154. E. Kovats, Helv. Chim. Acta, 1958, 41, 1915.

155. R.M. Smith, J. Chromatogr., 1982, 236, 313.

156. J.K. Barker and C.-Y. Ma, J. Chromatogr., 1979, 169, 107.

157. M. Bogusz and R. Aderjan, J. Chromatogr., 1988, 435, 43.

158. R.M. Smith and D.R. Garside, J. Chromatogr., 1987, 407, 19.

159. C.R. Yonker, R.W. Gale, and R.D. Smith, J. Chromatogr., 1987, 389, 433.

160. R.M. Smith and M.M Sanagi, Chromatographia, submitted for publication.

161. M.M. Sanagi and R.M. Smith, Anal. Proc., in the press.

162. R.M. Smith, J. Chromatogr., 1984, 291, 372.

163. G.M. Schneider, Ber. Bunsenges. Phys. Chem., 1984, 88, 841.

164. D. Leyendecker, F.P. Schmitz, D. Leyendecker, and E. Klesper, J. Chromatogr., 1985, 321, 273.

165. D.R. Gere, R. Board, and D. McManigill, Hewlett-Packard Co., Publication No. 43-5953-1647, 1982, pp. 7-15.

166. L. Rohrschneider, J. Chromatogr., 1966, 22, 6.

167. W.R. Supina and L.P. Rose, J. Chromatogr. Sci., 1970, 8, 214.

168. W.O. McReynolds, J. Chromatogr. Sci., 1970, 8, 685.

169. R.M. Smith, Anal. Chem., 1984, 56, 256.

170. V.G. Evans, M.Sc. Thesis, Loughborough University of Technology, 1988.

171. C.O Wilson, O. Gisvold, and R.F. Doerge, (Eds.), "Textbook of Organic Medicinal and Pharmaceutical Chemistry", Lippincott, Philadelphia, 7th Ed., 1977.

172. C. Ioannides, J. Chakraborty, and D.V. Parke, Chromatographia, 1974, 7, 351.

173. R.M. Smith, T.G. Hurdley, R. Gill, and A.C. Moffat, Chromatographia, 1984, 19, 407.

174. S.H.Y. Wong, (Ed.), "Therapeutic Drug Monitoring and Toxicology by Liquid Chromatography", Marcel Dekker, New York, 1985.

175. R.N. Gupta, J. Chromatogr. Biomed. Applic., 1985, 340, 139.

176. D.N. Pillai and S. Dilli, J. Chromatogr., 1981, 220, 253.

177. E.W. Cieplinski, Anal. Chem., 1963, 35, 256.

178. P. Sandra, M. van den Broeck, and M. Verzele, J. High Resolut. Chromatogr. Chromatogr. Commun., 1980, 3, 196.

179. A. Pryde and M.T. Gilbert, "Application of High Performance Liquid Chromatography", Chapman and Hall, London, 1979.

180. M.W. Anders and J.P. Latorre, Anal. Chem., 1970, 42, 1430.

181. M.C. Bowman and L.G. Rushing, J. Chromatogr., 1978, 16, 23.

182. U.R. Tjaden, J.C. Kraak, and J.F.K. Huber, J. Chromatogr. Biomed. Applic., 1977, 143, 183.

183. R. Gill, A.A.T. Lopes, and A.C. Moffat, J. Chromatogr., 1981, 226, 117.

184. J. Haginaka and J. Wakai, J. Chromatogr., 1987, 390, 421.

185. R.M. Smith, T.G. Hurdley, R. Gill, and A.C. Moffat, LC-GC, 1986, 4, 314.

186. R.M. Smith, T.G. Hurdley, R. Gill, and A.C. Moffat, Anal. Proc., 1985, 22, 331.

187. R.M. Smith, T.G. Hurdley, R. Gill, and A.C. Moffat, J. Chromatogr., 1986, 355, 75.

188. R.M. Smith, T.G. Hurdley, R. Gill, and D.M. Osselton, J. Chromatogr., 1987, 398, 73.

189. R. Gill, A.C. Moffat, R.M. Smith, and T.G. Hurdley, J. Chromatogr. Sci., 1986, 24, 153.

190. R. Gill, D.M Osselton, R.M. Smith, and T.G. Hurdley, J. Chromatogr., 1987, 386, 65.

191. M. Bogusz and R. Aderjan, J. Anal. Toxicol., 1988, 12, 67.

192. S. Garattini, E. Mussini, and L.O. Randall, "The Benzodiazepines", Raven Press, New York, 1973.

193. J.A.F. de Silva, J. Chromatogr. Biomed. Applic., 1985, 340, 3.

194. H. Heusler, J. Chromatogr. Biomed. Applic., 1985, 340, 273.

195. J.A.F. de Silva, J. Chromatogr., 1983, 273, 19.

196. R.N. Gupta, J. Chromatogr. Biomed. Applic., 1985, 340, 139.

197. A. Frigerio, K.M. Baker, and G. Belvedere, Anal. Chem., 1973, 45, 1846.

198. S. Gunawan and D.M. Treiman, Ther. Drug Monit., 1988, 10, 172.

199. A. Nahum and Cs. Horvath, J. Chromatogr., 1981, 203, 53.

200. R. Gill, S.P. Alexander, and A.C. Moffat, J. Chromatogr., 1982, 247, 39.

201. M.C. Pietrogrande, F. Dondi, G. Blo, P.A. Borea, and C. Bighi, J. Liq. Chromatogr., 1988, 11, 1313.

202. H. Schuetz and V. Wostenberger, J. Chromatogr., 1979, 169, 409.

203. Analytical Methods Committee (Essential Oil Sub-committee), Analyst, 1984, 109, 1339.

204. E. Guenther, "The Essential Oils", Vol. 1, van Nostrand, New York, 1948.

205. P. Sandra and C. Bicchi, (Eds.), "Capillary Gas Chromatography in Essential Oil Analysis", Huethig, Heidelberg, 1987.

206. E. Guenther, "The Essential Oils", Vol. 2, van Nostrand, New York, 1949.

207. R.M. Smith and J.M. Robinson, Phytochem, 1981, 20, 203.

208. A.R. Vinutha and E. von Rudloff, Can. J. Chem., 1968, 46, 3743.

209. T.S. Chamblee, B.C. Clark, T. Radford, and G.A. Iacobucci, J. Chromatogr., 1985, 330, 141.

210. Ph. Morin, M. Caude, H. Richard, and R. Rosset, J. Chromatogr., 1986, 363, 57.

211. R.M. Smith and S. Beck, J. Chromatogr.,1984, 291, 424.

212. G.P. Cartoni and F. Coccioli, Abstract of Papers: Poster Seccion II, 17th. Int. Symposium on Chromatography, Wien, Austria, September 25-30, 1988, p. 6.

213. M.H. Klowen and ter Heide, J. Chromatogr., 1962, 7, 297.

214. E. von Rudloff, Adv. Chromatogr., 1974, 10, pp. 173-230.

215. E. Guenther, G. Gilbertson, and R.I. Koenig, Anal. Chem., 1969, 41, 40R.

216. E. Guenther, G. Gilbertson, and R.I. Koenig, Anal. Chem., 1971, 43, 4R..

217. J.G. Nikelly, (Ed.), "Advances in Capillary Chromatography", Huethig, Heidelberg, 1986.

218. C. Bicchi, A. D'Amato, G.M. Nano, and C. Frattini, J. Chromatogr., 1983, 279, 409.

219. E. von Rudloff, Can. J. Chem., 1960, 38, 631.

220. E. von Rudloff, Can. J. Chem., 1968, 46, 679.

221. E.A. Day, P.H. Miller, Anal. Chem., 1962, 34, 869.

222. R.M. Silverstein, G.C. Bassler, and T.C. Morrill, "Spectrometric Identification of Organic Compounds", 3rd Ed., Wiley, New York, 1974.

223. G. Svehla, "Comprehensive Analytical Chemistry", Vol. 11, Elsevier, Amsterdam, 1981.

224. L.S. Ettre and W.H. McFadden, "Ancillary Techniques of Gas Chromatography", Wiley, New York, 1969.

225. W. McFadden, "Techniques of Combined Gas Chromatography/Mass Spectrometry", Wiley, New York, 1973.

226. W.L. Budde and J.W. Eichelberger, "Organic Analysis Using Gas Chromatography/ Mass Spectrometry", Ann Arbour Science, Ann Arbour, 1979.

227. G. Shomburg, H. Husman, and F.J. Weeke, J. Chromatogr., 1975, 112, 205.

228. J. Krupcik, P. Cellar, D. Repka, J. Garaj, and G. Guiochon, J. Chromatogr., 1986, 351, 111.

229. T. Wang and Y. Sun, J. Chromatogr., 1987, 390, 261.

230. E. Guenther, "The Essential Oils", Vol. 4, van Nostrand, New York, 1950.

231. R.P. Randriamiharisoa and E.M. Gaydou, J. Agric. Food Chem., 1987, 35, 62.

232. E.M. Gaydou and R.P. Randriamiharisoa, Phytochem., 1987, 26, 183.

233. A.T. Bottini, V. Dev, D.J. Garfagnoll, H. Hope, P. Joshi, H. Lohani, C.S. Mathela, and T.E. Nelson, Phytochem., 1987, 26, 2301.

234. L. Oliveros-Belardo, M.M. Sanagi, and R.M. Smith, Philippine J. Sci., 1988, 117(1), in the press.

235. Analytical Methods Committee (Essential Oil Sub-committee), Analyst, 1984, 109, 1343.

236. T. Sakai, H. Maarse, R.E. Kepner, W.G. Jennings, and W.M. Longhurst, J. Agr. Food Chem., 1967, 15, 1070.

237. N.H. Anderson and M.S. Falcone, J. Chromatogr., 1969, 44, 52.

238. A.A Swigar and M. Silversteine, "Monoterpenes", Aldrich, Milwaukee, 1981.

239. Y. Masada, "Analysis of Essential Oils by Gas Chromatography and Mass Spectrometry", Hirokawa Publishing Co., Tokyo, 1975.

240. M.G. Moshonas and E.D. Lund, Flavour Ind., June 1970, 375.

241. A. Cornu and R. Massot, "Compilation of Mass Spectral Data", Heyden, London, 1966.

242. E. Stenhegen, S. Abrahamsson, and F.W. McLafferty, "Atlas of Mass Spectra", Vol. 1-3, Interscience, New York, 1969.

243. L. Oliveros-Belardo, R.M. Smith, J.M Robinson, and V. Albano, J. Philippine Sci., 1986, 115, 1 .

244. M. Saito, T. Hondo, and Y. Yamauchi, in Ref. 2, pp. 203-231.

APPENDIX

PRESENTATIONS AND PUBLICATIONS

(A) Presentations

Parts of this work have been presented at the following meetings and symposia.

1. Joint UK/NL Symposium on Separations, University of Kent at Canterbury, March 30 - April 1, 1987.

2. Hewlett-Packard 1987 Analytical Symposium, Conference and Exhibition, Harrogate, June 23-25, 1987.

3. International Symposium on Pharmaceutical and Biomedical Analysis, Barcelona (Spain), September 23-25, 1987.

4. Royal Society of Chemistry (Chromatography and Electrophoresis Group) Young Chemists Meeting, Chepstow, June 16, 1988.

5. Royal Society of Chemistry (Analytical Division) R&D Topics Meeting Plymouth Polytechnic, July 18-19, 1988.

6. Seventeenth International Symposium on Chromatography, Hofburg, Wien (Austria), September 25-30 1988.

(B) Publications

The following articles have been based on parts of this thesis. Further papers are in preparation.

1. M.M Sanagi and R.M. Smith, in "Supercritical Fluid Chromatography", R.M. Smith (Ed.), RSC Chromatography Monographs, Royal Society of Chemistry, London, 1988, pp. 29-52.

2. M.M.Sanagi and R.M. Smith, Anal. Proc., 1987, 24, 304.

3. R.M. Smith and M.M. Sanagi, J. Pharm. Biomed. Anal., 1988, 6, in the press.

4. M.M. Sanagi and R.M. Smith, Anal. Proc., in the press.

5. R.M. Smith and M.M. Sanagi, Chromatographia, submitted for publication.

6. L. Oliveros-Belardo, M.M. Sanagi, and R.M. Smith, Philippine J. Sci, 1988, 117(1), in the press.