# STATIONARY PHASES FOR SUPERHEATED WATER CHROMATOGRAPHY

By

**SHIKHA SAHA (2002)**

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**Thesis Title: Stationary Phases for Superheated Water Chromatography**

**Shikha Saha**

**Date:**

## Thesis Abstract

This project focused on the comparison of conventional liquid chromatography and superheated water chromatography. It examined the differences in efficiency and retention of a range of different stationary phases.

Alkyl aryl ketones and eight aromatic compounds were separated on PBD-zirconia, Xterra™ RP 18, Luna ™ $C_{18}$ (2) and Oasis HLB columns using conventional LC and superheated water chromatography system. The retention indices were determined in the different eluents. On changing the organic component of the eluent from methanol to acetonitrile to superheated water considerable improvements were found in the peak shapes and column efficiencies on the PBD-zirconia and Oasis HLB ™ columns.

PS-DVB, PBD-zirconia and Xterra ™ RP 18 columns have been used in efficiency studies. It was found that simply elevating the column temperature did not increase the efficiency of a separation in superheated water chromatography. The efficiency depended on flow rate, injection volume and also mobile phase preheating system. Although high efficiencies were not achieved with superheated water on PS-DVB and Xterra ™ RP 18 columns, a higher efficiency was achieved on a PBD-zirconia column with superheated water than with 25-35% ACN at room temperature. The proposed theoretical increases in $u_{opt}$ were measured on three columns using superheated water as the mobile phase.

The application of the superheated water chromatographic method to the separation of the pungent constituents of ginger by superheated water chromatography-NMR coupling system was studied. The coupling of superheated water chromatography using deuterium oxide to NMR spectroscopy for the separation of dry ginger extract was successful, although the NMR sensitivity in on-line mode coupling system was low. However, four compounds were identified in the ginger extract by stop-flow mode on superheated water chromatography-UV-NMR detection system.

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# CHAPTER 1

## Introduction

Water was present on this planet long before the evolution of life. It forms a necessary constituent of the cells of all animal and plant tissues and life cannot exist, even for a limited period, in the absence of water. So, that we have the somewhat strange position that a naturally occurring inorganic liquid and gas are essential for the maintenance of organic life (1). However, until not so many years ago experimental studies of the physical and inorganic chemistry of solutions were restricted almost completely to aqueous systems. It was mainly Hildebrand and Scott (2) who, together with their many colleagues, drew the attention of the scientific and technological world to measure solvent polarity according the solubility parameters. They proposed water is the most polar solvent. According to the solubility parameters, water is a good solvent for polar and ionic compounds and very poor solvent for the vast majority of non-polar organic compounds at room temperature.

The inability of water at room temperature to solvate non-polar organic compounds is still not generally appreciated in organic and analytical chemistry. But since the ancient world, people have used hot water and steam to extract various drugs, remedies, etc. The properties of water at high temperature and pressures have been investigated both for technical and purely scientific reasons. Knowledge of the thermodynamic properties of water for example, has become increasingly important in designing power stations, in solving corrosion problems in these and similar units, and in growing synthetic crystals from hydrothermal solutions (3). But the use of pressurised hot water in analytical chemistry was rare.

Therefore, in this study, we will examine the use of superheated water as a mobile-phase in reversed-phase liquid chromatography, particularly the range of stationary phases that can be used for superheated water chromatography.

## 1.1: Reversed-phase Liquid Chromatography

In reversed-phase chromatography, the stationary phase is non-polar, and the mobile phase is polar. Reversed-phase liquid chromatography (RPLC) is a very popular separation and analytical technique employed in almost every chemistry laboratory worldwide (4). It is a versatile separation technique, from analytical separations of low molecular weight samples, to intermediate molecular weight biochemicals, such as peptides and oligonucleotides. The application of reversed-phased liquid chromatography is increasing day by day from pharmaceutical research, routine quality control to environmental and regulatory chemical analysis. Therefore, it is necessary to think about the separation desired and the best way to control it. This invariably means controlling the physical parameters of the chromatographic system to achieve the "best" separation; which in LC most often implies controlling the solvent strength and the flow characteristics of the mobile phase.

What would we list as ideal properties in a solvent for use as mobile phase in reversed phase liquid chromatography?

**Properties of an ideal mobile phase:**

- The ability to dissolve ionised, polar and non-polar analytes
- Non-flammable
- Non-toxic
- Readily available in a high and consistent state of purity
- No detection interferences, such as ultraviolet (UV) /Visible absorbance
- Low viscosity and high diffusion rate
- The ability to alter the solvent strength
- Inexpensive

However, a mixture of relatively polar organic solvents, such as methanol, acetonitrile, or tetrahydrofuran, with water or buffer is usually used as the mobile phase in reversed phase liquid chromatography (5). Therefore, the major disadvantage of RPLC is the need for organic solvents that are expensive, are potentially harmful to the operator and the laboratory environment and need to be disposed of safely (6). However, increasing the temperature has a dramatic effect on the properties of water (7). Therefore, superheated water can be a powerful solvent for reversed phase high performance liquid chromatography (RP-HPLC) overcoming many practical problems in separation and detection (because of the absence of organic solvent).

## 1.2: Definition of Superheated Water

The term-"superheated water"- refers to liquid water under pressure between its boiling point, 100 °C and its critical temperature, 374 °C. The alternative term "subcritical water" also refers to the state of water as a liquid between the critical point and boiling point (8-9). Superheated water is maintained in the liquid phase above its "normal" boiling point of 1 atmosphere of pressure (1.01325 bar) by the application of pressure.

At lower temperatures and through most of this temperature range, the pressure on the medium does not have much effect on its properties, provided it is high enough to maintain the water in the liquid phase (10). However, near the critical temperature, the medium is very compressible and it has some of the properties of a supercritical fluid and so the pressure would become very important.

The dielectric constant of water changes dramatically when its temperature rises (11), because of the breakdown in its hydrogen bonding structure with temperature. The high degree of association in the liquid causes its dielectric constant to be high at around 80 under ambient conditions, but as the temperature rises this value falls, **Figure (1.1)** (12).

As a consequence, superheated water can be a good solvent for organic compounds, particularly if they have some polar groups or are polarisable, like aromatic compounds (13). The solubility of an organic compound in superheated water is often many orders of magnitude higher than its solubility in water at ambient temperature due to the change in dielectric constant, described above.

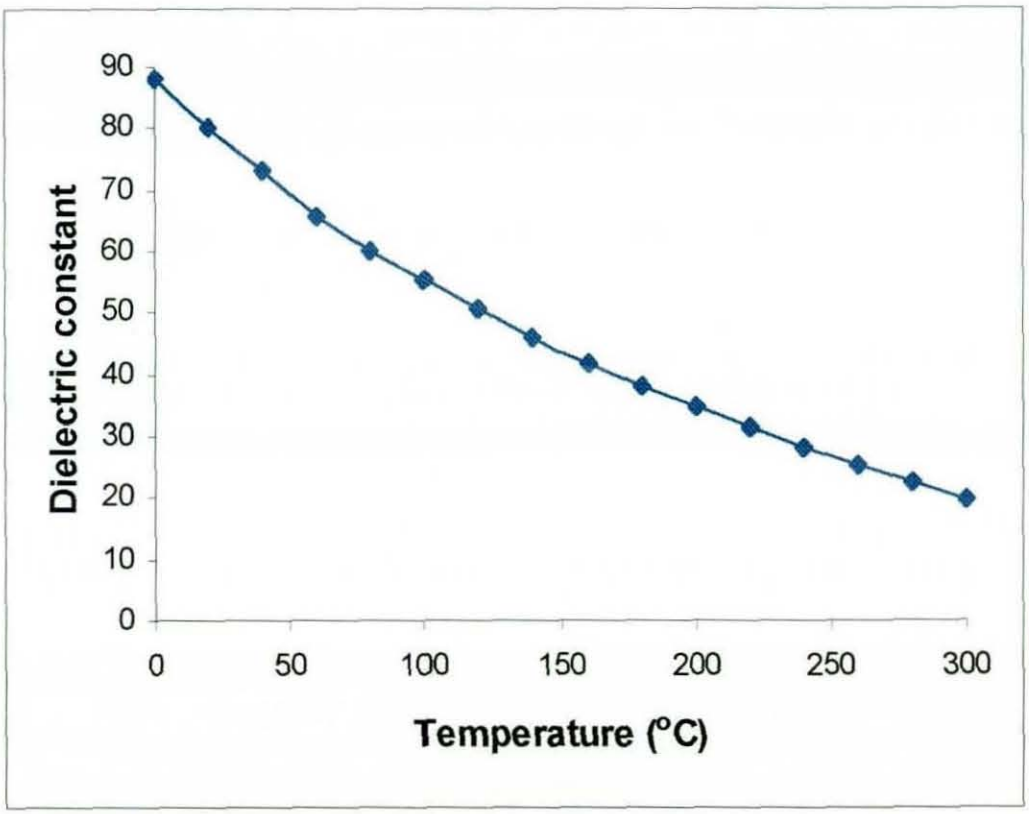



**Figure 1.1: The dielectric constant of liquid water as a function of temperature [Data obtained from ref. (12)].**

## 1.3: Background of Superheated Water and Superheated Water Chromatography

In recent years there has been a considerable interest in the adoption of clean technology to reduce the waste generated in all areas of the chemical industry. For example, by increasing the efficiency of synthetic processes, the

adoption of organic solvent-free applications of paints and the minimization of the usage of organic solvents in chemical analysis. Therefore, some chromatographers have looked for alternative solvents.

In part, this has led to considerable interest in the use of supercritical carbon dioxide as an eluent in SFC (14-15). Carbon dioxide satisfied many of our selection criteria and it appeared to be an attractive eluent, relatively inexpensive, non-toxic, environmentally friendly and with flexible elution strength.

However, it has failed to displace more conventional eluents. The technique can suffer from instrumental problems, because of the need to use high pressures. Another problem, it is relatively non-polar and functions primarily as a normal-phase eluent. In this mode it has competed successfully for the analysis of chiral analytes and for the separation of hydrocarbons and some non-polar polymers and oligomers (16). Other supercritical fluids that have also been examined as eluents include nitrous oxide, ammonia, Xenon and Freons. However, so far, none have gained acceptability, either being too reactive, too expensive or environmentally suspect.

Nowadays, pressured hot water is very well known in the physics, engineering and chemistry fields. Therefore, some research groups have drawn attention to the use of subcritical or supercritical water in fields such as in organic chemistry and extraction of organic compounds.

### 1.3.1: Uses of Subcritical and Supercritical Water

#### 1.3.1.1: Organic Chemistry

Supercritical water has been studied extensively as a medium for chemical waste treatment through hydrolysis, oxidation, and other chemical transformations (17-21). Few examples exist for the use of supercritical water as a reaction medium for preparative organic chemistry, even though its unique properties have long been recognised (22-23).

Kuhlmann and co-workers (17) required that all substances be water soluble in the temperature range from 200 to 300 °C at pressures generated solely by the expansion of the liquid medium (autogenic, approximately 250 to 110 psi). This focus complements extensive work by Siskin, Katritzky, and co-workers, (24), who generated a wealth of information concerning the reactivity of many organic compounds in hot water regardless of their solubilities, reaction pathway, or the complexity of products. Those studies emphasised the effects of the hot aqueous medium compared to the relatively unreactive hydrocarbon solvents that served as control solvent system for generating possible thermal radical pathways. M. Siskin and A. R. Katritzky (25) reported in another paper that water can act as an acidic or basic catalyst, that its reactivity can often be reinforced by autocatalysis from water-soluble reaction products. Additional positive aspects of the use of aqueous chemistry are its simplicity, low cost, and favourable environmental impact.

Most studies on reactivity in superheated water and supercritical water have focused on reactions in which hydrolysis, oxidation or thermolysis occur at enhanced rates either because of the high temperatures involved or because of the enhanced reactivity of the water. For example, the complete air oxidation to carbon dioxide, water and salts have been reported by workers interested in the destruction of toxic wastes (17-18, 26-29). The non-destructive reaction of a variety of organic molecules in superheated (200-300 °C, 75 bar) water (17, 24) has been reported by several groups. These conditions are quite vigorous compared to typical chemical reaction conditions.

### 1.3.1.2: Extraction

Recently, the use of supercritical and subcritical water for chemical extraction has become more widely used. Subcritical water has been used to extract organic compounds, such as organic pollutants (30-31), and hydrocarbons (32-33) from soil matrices including soil and waste.

Temperature has a primary effect on extraction efficiency (30, 33). A study by Hawthorne et al. (30) reported that decreasing the polarity of water by sequentially raising the extraction temperature from 50 to 250 °C (subcritical water) and finally to 400 °C (supercritical water if P>221 bar) allowed the extraction of polar organics (e.g., chlorinated phenols), low-polarity organics (e.g., PAHs) and non-polar organic compounds (alkanes).

A gradual decrease in the dielectric constant of water with increasing temperature under moderate pressure is paralleled by an increasing solubility of organic compounds (34-35), as well as higher molecular weight compounds (36). Miller et al. (34-35) revealed that the solubilities of PAH and pesticides increased 4-5 orders of magnitude on raising subcritical water from 298 K to 498 K. A study by the same research group show that at high temperature the percentage extraction of chlorinated biphenyls was consistent with their solubilities in water at ambient conditions, which depend on their molecular weights (30,32,36-37). For example, less chlorinated biphenyls are more water-soluble at ambient temperature, at 200 °C they were easier to extract than the highly chlorinated biphenyls (36). High molecular weight alkanes also fall into this category. The higher molecular weight alkanes (>C20), which are easily extracted using supercritical $CO_2$ at 300 °C could not be extracted by water. Nevertheless, those alkanes could be effectively and quantitatively removed by steam extraction (250 °C and 5 atm).

Crescenzi et al. (38) reported that water heated at a moderately high temperature (50, 90 and 120 °C) appears to be an efficient medium for extracting polar and medium polar compounds from solid matrices. Even at a low temperature (T < 100 °C), the temperature still has a great impact on the extraction efficiency of fungicides from agricultural products (39). Increasing temperature also increased the percentage removal of organic components from solid matrices (40-41), soil and waste (33).

Johnson et al. (33) studied absorption equilibria of organic carbon on soil samples using superheated water and steam at 150-250 °C. They described that liquid water removed more carbon from soil than steam, because of the many polar functional groups of the humic organic matter that readily associated with water.

Subcritical water extraction was compared to conventional extraction methods, such as Soxhlet extraction (38), ultrasonic extraction (37) and steam distillation. Again, the study by Hawthorne et al. (37) showed that the percentage removal of PAHs and aromatic amines from soils from rail-road beds and industrial sites, using a subcritical water extraction (250 °C, 15 and 60 min) on-line with solid-phase microextraction were in a good agreement with a 24 hr conventional Soxhlet extraction with dichloromethane. Whereas, Crescenzi et al. (38) found that subcritical water extraction was more efficient than Soxhlet extraction for non-acidic herbicides and also this extraction was more efficient than ultrasonic extraction for more polar acidic herbicides. Superheated water extraction of aroma compounds from rosemary plants gave higher yields than steam distillation, as reported by Basile et al. (42). By using water between 125 and 175 °C, rapid extract of oxygenated fragrance and flavour compounds could be achieved while leaving behind monoterpenes, higher hydrocarbons and lipids.

Young et al. (43) demonstrated an application of water only reversed phase LC by interfacing it with subcritical water extraction of organic compounds from soil matrices. They spiked benzene, ethylbenzene and naphthalene onto sand then extracted the analytes with SWE (subcritical water extraction) followed by chromatographic separation on a RP column. A sand sample contaminated with gasoline was also analysed using supercritical water extraction/ water-only reversed phase-LC. This extraction process also provided kinetic information about the rate of analyte extraction from the sand matrix.

Yang et al. (40) reported that subcritical extraction could be successfully coupled (off-line) to a HPLC system using a solid trap and two six-port LC injectors. Alkylbenzenes and polycyclic aromatic hydrocarbons were extracted from sand and contaminated soils and then analysed using this new coupling technique. They also reported that the extraction efficiency of PAHs was significantly improved by raising water temperature. This was especially true for high-molecular weight PAHs. The extraction pressure did not affect extraction and collection efficiency. The same authors also published a study (44) of an on-line coupled system of subcritical water extraction (SBWE) with HPLC, constructed with a solid trap and several shut-off valves. Several classes of organic compounds including chlorophenols, chloro- and methylanilines, caffeine, nitrotoluenes and PCBs were extracted and analysed by this on-line system. The peak shapes obtained after the coupling of SBWE-HPLC are similar to those of HPLC calibration result for all of the five classes studied. Therefore, only some peak broadening occurred using this on-line coupling technique. Compared to the off-line coupling of SBWE-HPLC reported previously (40), this on-line system is more convenient to operate.

A new method for the extraction-individual separation-determination of polycyclic aromatic hydrocarbons (PAHs) in soil was reported by Fernandez-Perez et al (45). The method is based on the integration of three steps: continuous subcritical extraction, solid-phase clean/preconcentration, and HPLC separation with post-column fluorimetric determination. Sodium dodecyl sulphate (SDS) was added to the water to favour the extractability of the low-polarity analytes. Soil samples spiked with the target PAHs were subjected to static-dynamic extraction with SDS-water at 50 bar, 150 °C, for 15 min of static extraction and 10 min dynamic extraction at a flow-rate of 3 ml/min.

## 1.4: Superheated Water Chromatography

In 1996 Smith and Burgess constructed a Superheated Water Chromatograph based on an HPLC system but placed the column in a gas liquid chromatography oven, with a temperature programmer controller (46). The detector and back-pressure regulator came from an SFC system but a later version of the instrument used a simple fixed restrictor and standard HPLC detector with a flow cell rated to 50 bars. A major advantage compared to SFC was that the pump head did not need to be cooled and only deionised water was needed. In addition no modifier pump was required.

In a previous study, it has been shown that silica is likely to dissolve at high temperature in an aqueous environment (47). However, alternative stationary phase materials are available that can accept higher temperatures. Therefore, Smith and Burgess started with a polystyrene-divinylbenzene (PS-DVB) column using methanol-water at room temperature and applied a small back-pressure of 10 bars. The retention of a group of phenols decreased as the temperature was raised in stages but could be restored by reducing the proportion of organic modifier. Eventually by 130 °C no modifier remained. Further raising of the temperature to 180 °C reduced the retentions further as predicted from the changes in the polarity of the superheated water.

They were able to demonstrate that a wide range of aromatic compounds with different functional groups could be separated under these conditions (48). The retentions were generally similar to those under reversed-phase conditions with small changes in selectivity (49). They then demonstrated that porous graphitic carbon (Hypercarb) would behave as in conventional liquid chromatography (49). An ODS silica column also performed well but showed limited stability and over a period of days the retention times decreased markedly and the column efficiency was lost (48-50). The recently introduced PBD-zirconia columns also gave good reversed-phase separations. For a number of these separations, van Deemter Curves were measured and these showed plate heights comparable to conventional HPLC but the efficiencies

dropped at higher flow-rates, with thermal equilibration possibly presenting a problem. They also found that higher temperatures, at which water behaved like an acetonitrile-water mixture, were needed to elute more hydrophobic compounds and the retention followed conventional reversed-phase LC. A plot of 1/T against retention factor was non-linear, suggesting that not only a conventional $\Delta H$ effect but dielectric constant also dominated the retention (49). Analytes eluted from a silica ODS column with lower temperature than from a PS-DVB column, this agreed with a conventional RP-mode that retention is weaker when using ODS than a PS-DVB column with the same eluent (51).

A potential advantage of superheated water was seen when different detectors were examined. With UV/Visible spectrometry there was no background signal even at 190 nm (46). Fluorescence spectroscopic detector could also be performed to detect riboflavin (52) and the low back pressures required in the system enable a conventional HPLC detector cell to be employed unlike the high-pressure systems usually required in SFC (50).

A potentially valuable mode of detection is possible because unlike conventional HPLC no modifier is present. The water eluent can, therefore, also be passed directly to a flame-ionization detector (49).

This approach was also demonstrated by Miller and Hawthorne, who reported the detection of phenol, alkanols and amino acids by flame-ionization detector (53-54). Other laboratories have also examined this detection method (55). However, some difficulties had been found in making a robust FID system.

More recently, Smith and Bone have developed a robust FID detector in this laboratory (56). They detected a wide range of compounds (carbohydrates, aliphatic, aromatic and inorganic compounds) with this detector. This type of detection is an exciting development as most previous attempts to produce a universal flame detector for liquid chromatography were unsuccessful. It offers an alternative, most robust and more sensitive universal HPLC

detector. Detection limits are in the low ppm range, better than those obtained by the universal RI detector.

Recently, Y. Yang et al. (57) reported the coupling of subcritical water separation with flame ionisation detection (FID) in the split mode technique. In order to keep the FID system stable during subcritical water separation, a Tee union was connected between the separation column and the FID system to split the water flow. They claimed that the FID system was very stable in this split mode even at total flow-rate as high as 1.24 ml/min but the sensitivity was reduced and the limits of detection ranged from 38 to 111 ng (306-925 ng/μl injection) with split ratios 1:10 to 1:17 (FID/waste bottle) for carbohydrates, amino acids, and carboxylic acids studied.

Superheated deuterium oxide has almost identical properties to water and can be used as an eluent for coupled LC-NMR-MS spectroscopy (58-59). Chienthavorn and co-workers could detect barbitone, amylobarbitone, heptabarbitone, paracetamol, caffeine, phenacetin, sulfonamides and also vitamins by LC-NMR-MS spectroscopy.

Therefore, superheated water chromatography offers conventional HPLC detection methods such as UV/Visible and fluorescence spectroscopy with minimal background interferences but also advantages with universal FID detection and easy NMR and mass spectrometry.

This method also offered a number of areas of potential interest to separation scientists. Separations are comparable to reverse-phase mode so are ideal for the separation of medium and high-polarity analytes, including pharmaceuticals (52), agrochemicals and vitamins (59) and a range of aromatic phenols, amides, amines and esters (48-50), carbohydrates (56), variety of alcohols, hydroxysubstituted benzene and amino acids (53). The pH of the eluent can also be readily controlled and the changes in the separation of the sulphonamides at pH 3, 7 and 11 have been examined (60). Clearly

other adjustments to the separation mechanism, such as ion-pair separations are also possible (59).

Teutenberg et al. (61) has eluted six anticancer drugs from a polystyrene-divinylbenzene (PS-DVB) column with buffered superheated water as the mobile phase. The temperature range was from ambient temperature up to 160 °C and the pH of the water was adjusted to 11.5 or 3.5 with phosphate buffer. During the whole investigation from ambient temperature to 160 °C, no degradation products were observed and also peak shapes were good and retention factors were satisfactory for all of the compounds that were investigated.

Pawlowski and Poole (62) studied the influence of temperature between 75-180 °C on the properties of a pressurised water mobile phase on a PLRP-S 100 column. In their study, 1% acetonitrile was used in pure water to maintain the peak shape of all solutes at low temperature and was claimed to have little effect on the solvation properties of water. A comparison with different RP-mobile phase systems showed that the elution strength of 1% acetonitrile-water at 180°C corresponded to 15-25% acetonitrile-water, 25-35% propanol-water, or 50-60% methanol-water. However, a decrease in retention by increasing temperature of 1% acetonitrile-water is different from that by increasing organic solvent composition in those three mobile phases. It was also noted that temperature programming with water as a mobile phase was less powerful than using a solvent composition gradient. They claimed that water at 75-180 °C, compared with acetonitrile and methanol; possess a relatively weak elution power in RP-HPLC.

The thermal stability of commercially available columns has been investigated.

McNeff et al. (63) have also compared five different columns on superheated water chromatography. They found that the polybutadiene-coated Zirconia

phase column was the most stable and was more efficient than other columns.

Carr et al. (64) used a PS-zirconia column for ultrafast liquid chromatography at high temperature. They separated phenol, 2-methylphenol, 4-ethylphenol, 2,4,6-triethylphenol, and 4-tert-butylphenol on this column using superheated water (120 °C) as the mobile phase within 30 sec with good efficiency.

Yang et al. (65) have examined three columns using subcritical water as the mobile phase. They tested alumina, C18 AB and PRP-1 columns for subcritical water separations at 100-200 °C. They found that the retention of both polar and non-polar analytes was the shortest on the alumina column, moderate on the C 18 AB column, and the longest on the PRP-1 column.

Wilson (66) separated a number of model drugs using superheated water on a range of stationary phases including polymer, graphitic carbon, zirconia and silica-based materials. He explained that in general polymer and carbon-based phases required the highest temperatures (in excess of 100 °C) in order to elute the analytes whilst silica-based materials could provide similar separations at elevated, but not necessarily superheated temperatures. The application of this type of chromatography to the detection of paracetamol in human urine was demonstrated.

Fields et al (67) separated testosterone and several related compounds on a porous zirconia, polybutadiene-coated column at temperatures up to 200 °C and compared this column with that of a porous silica, octadecylsilane-coated column and zirconia column under traditional reversed-phase conditions with an acetonitrile-water mobile phase at 40 °C. The selectivity differences observed for testosterone and related compounds show that the separation mechanisms are complementary and unique selectivity is obtained with the zirconia column under High Temperature Liquid Chromatography (HT-LC) conditions.

Kephart et al. (68) separated six benzene derivatives within 2 min on polybutadiene and elemental carbon modified zirconia-packing materials using superheated water with or without temperature gradients. They used an inexpensive capillary scale reversed phase liquid chromatography (LC) system.

## 1.5: Stationary Phases in High Temperature Liquid Chromatography

With all new chromatographic methods there is a certain necessity to establish the reproducibility and robustness of the system in question. Because there are concerns about the reactivity of superheated water (20), it needs to be an in-depth study into the long-term stability of the various column-packing materials currently available for RP-HPLC. In particular there is a real concern that silica-based packing materials are susceptible to some form of chemical attack from superheated water. At high temperatures in an aqueous environment silica is likely to dissolve (69). Perhaps the most suitable evaluation of column stability is the measurement of any changes in the retention factor for a given solute. An alternative method would be to observe possible changes in the column efficiency.

A previous study (49) reported that the mechanism of elution on superheated water chromatography remained reversed phase mode throughout, with polar solutes being eluted before non-polar ones. The explanation of retention in RP-HPLC can be explained by the hydrophobic theory. The differences in interactive energies between non-polar and polar solutes with the mobile phase and the differences in hydrophobic molecular surface areas of stationary phases are responsible for the functional group selectivity observed in RP-HPLC (70). Therefore, the properties and effect of temperature on various stationary phases are described below.

### 1.5.1: Effect of Temperature on Silica Based Columns

The development of high-quality reversed-phase packing materials led to an evaluation of the temperature effect of silica. In 1973 Knox and Vasari (71)

reported that the peak skewness was reduced and indeed almost eliminated on Permaphase ODS by running it at 60 °C. Their explanation for this effect was that the polymer layer of the ODS stationary phase became more swollen at higher temperatures (as suggested by the increased column resistance) and the partition isotherm became more linear through more structural flexibility as temperature increased.

Morel and Serpinet (72-73) examined the effect of temperature on ODS bonded silica by inverse gas chromatography. They suggested that the irreproducibilities of retention data and selectivities observed from one sample to another may for a large part be linked with changes in the physical state of the chemisorbed phase under the influence of temperature. This change was accounted for by a transition of the bonded $C_{18}$ phase. They proposed a melting of a solid, crystal-like phase to a liquid expanded phase.

The studies reported in these first two papers were not performed under real HPLC conditions. None of the packing materials were exposed to mobile phase. In a later paper Morel and Serpinet (74) included LC data for the retention of structurally diverse solutes on densely grafted $C_{22}$ silica. They included pure methanol and methanol-water mobile phases in their studies. The carbon load for the slightly longer $C_{22}$ chain bonded to the silica surface was 3.98 $\mu mol/m^2$. A slightly higher transition temperature was observed for the $C_{22}$ chain, 48 °C, as opposed to the $C_{18}$ chain, 27 °C. Under LC conditions with a pure methanol mobile phase a transition temperature of 46.5 °C was reported and with a methanol-water mobile phase containing up to 50 % water, the transition temperature was found to be the same as with neat methanol. The transition temperature was in the same range as with the inverse GC method. However, from about 55 % water in the mobile phase the transition temperature shifted from 46 °C to 53 to 54 °C and did not change further with higher concentrations of water. Although non-linear van't Hoff plots were obtained in all cases the actual transition temperature was dependent on the mobile phase composition hence on the solvation of the stationary phase in the mobile phase.

Jinno et al. (75) and Cole and Dorsey (76-77) investigated the effects of changing the mobile phase and the column temperature on the mechanism of retention. Jinno et al. compiled FT-IR data supporting the earlier findings by Morel and Serpinet (74) and Sander et al. (78) that changing the mobile phase caused changes in the ODS bonded phase conformation. From the solid state NMR data, it was evident that a change in temperature caused a drastic change in the stationary phase from solid-like to a liquid-like formation. Cole and Dorsey performed retention studies under reversed phase LC conditions and found non-linear van't Hoff behaviour. They supported the explanation suggested by Morel and Serpinet of a phase transition of reversed phase stationary phases. For densely packed $C_{18}$ stationary phases the transition temperature of 22 °C was reported. The phase transition was observed more pronounced on high bonding density columns than low-density columns (76, 79).

A recent study investigated the alkyl chain conformation of bonded $C_{18}$ stationary phases by Raman spectroscopy (80). The data presented showed the ability of the bonded alkyl chains to attain different degrees of conformational order dependent on temperature. Because of a greater heterogeneity of the environment the phase transition of the bonded alkylsilanes occurred over a much broader temperature range than with bulk alkylsilanes. Again it was noted that the mobile phase composition and the carbon load had an influence on the transition temperature.

In 1993 Wheeler et al. published a comprehensive review of the phenomenon of phase transition (81). They concluded that phase transition could be a function of virtually every chromatographic variable: bonded chain length, bonding density, bonding reaction (monomeric or polymeric), mobile phase and the choice of solute. Van't Hoff plots alone were in their opinion not an indication of change in phase structure as non-linearity could arise from other thermodynamic processes than stationary phase changes. Differential Scanning Calorimetry (DSC) experiments were generally not performed under chromatographic conditions. Those experiments were lacking the influence of

mobile phase and pressure on the stationary phase. Wheeler et al. recommended the term phase transition should be applied very loosely as there is not a sharp, distinct change in stationary phase structure but a diffuse change going from a solid-like to a liquid-like state. These transitions occurred over a rather diffuse temperature range. Molecular dynamics simulation was put forward as a means to understand the stationary phase structure and to successfully plan new experiments and interpret results.

Two years ago a study investigating the stationary phase structure under HPLC conditions has been published. Doyle et al. characterised a $C_{18}$ bonded phase by Raman spectroscopy. They investigated the effect of mobile phase composition (82) and temperature (83). The expected collapse of the $C_{18}$ chains with water as mobile phase could not be observed but a distinct temperature dependent change in stationary phase conformation was reported. The spectral features of the bonded ligands became increasingly more ordered as the temperature decreased from 45 to 2 °C. There was no sharp transition temperature rather a continuous change in structural flexibility with temperature. The temperature-induced ordering was observed for high and low bonding density. A non-linearity of the van't Hoff plot, which has been attributed to possible phase transition by other researchers, was only observed for high bonding density monomeric columns.

### 1.5.1.1: Disadvantages of Silica Based Columns

Silica would be the best packing for HPLC, were it not for many disadvantages; silica dissolves at alkaline pH values, the undesirable effects of silanols interaction, poor long term batch to batch reproducibility, sorbent instability at high temperature, low and irreproducible ionic capacity and variable retention resulting from poorly solvated sorbents (84). How rapidly this occurs depends on the nature of the stationary phase, the pH and the type of the buffer, the nature and content of the organic modifier in the mobile phase, and the temperature. Also, the time that it takes for the column to collapse may depend on the specific pore volume of the silica; packings with

a small pore volume (and therefore more massive skeleton) appear to be affected less than packings with a high pore volume. Furthermore, the type of bonded phase and the surface coverage play an important role in the hydrolytic stability of packing. So while column manufacturers usually recommend against using bonded-phase columns beyond pH 8, this is a very soft limit. Columns have been run successfully at more than pH 9 for extended periods of time at room temperature in mobile phases containing a large percentage of organic modifier in which the solubility of silica is reduced (84), on the other hand, columns have been found to collapse after only weeks, when phosphate buffers at pH 8 were used at elevated temperature.

Today the reversed-phase $C_{18}$ type bonded phases is most frequently used. Silica based columns remain the material of choice for superheated water chromatographers because they offer superior efficiency and their usage is well understood. A previous study in this laboratory (49) separated three phenols (phenol, m-methoxyphenol and m-cresol) with excellent peak shapes and efficiencies. The separation were reproducible over an 8-hours working day on Zorbax $C_{18}$ column using superheated water as mobile phase at 100 °C. But on opening the top of this column, it was found that at least two thirds of the packing material had disappeared, seemingly dissolved by the basic nature of the superheated water (85). It quickly became apparent that superheated water would not be suitable for any silica-based columns due to the accelerated dissolution of silica itself in basic conditions (69).

The mechanism for the dissolution of silica under basic conditions is as follows: firstly hydroxyl ions, $OH^-$, are chemisorbed onto the surface of the silica; this effectively increases the co-ordination of the silicon atom to more than four and subsequently weakens the oxygen bonds to the underlying silicon atoms (69). The silicon atom then goes into solution as the silicate ion, $Si(OH)^-_5$. If the pH of the water is much below 11, then the silicate ion hydrolyses to soluble silica, $Si(OH)_4$, and $OH^-$ ions and the process is repeated (**Figure1.2**).

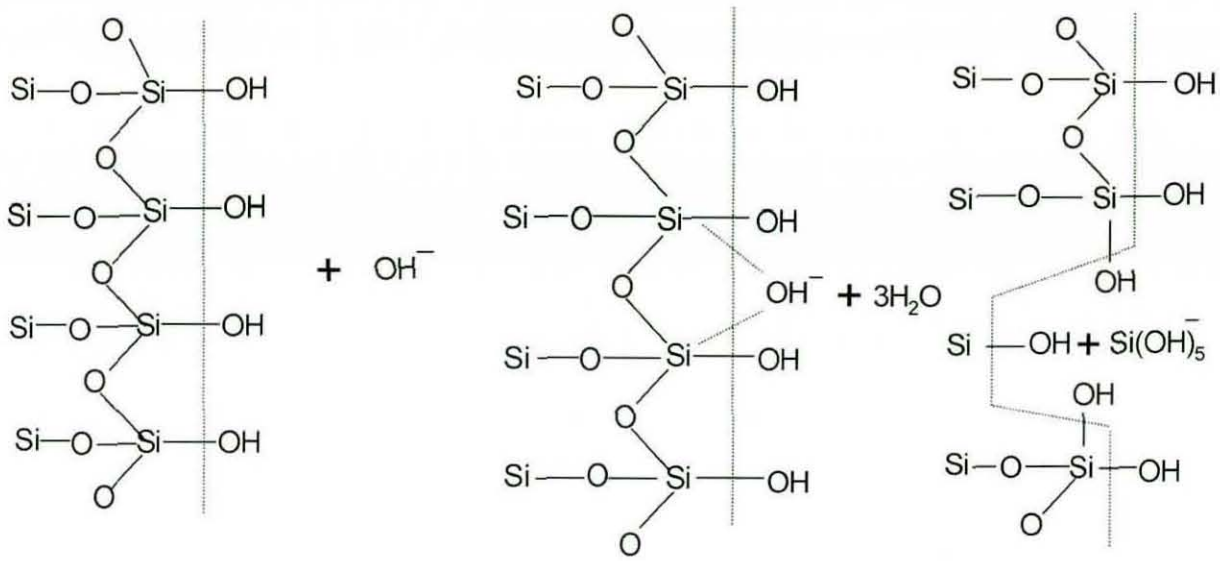


**Figure 1.2: The proposed mechanism of dissolution of silica in the presence of hydroxyl ions. The dotted line represents the interface between silica on the left and water on the right (69).**

### 1.5.1.2: Stable Silica Based Columns

Silica-based materials have not proved to be particularly robust when used for superheated water chromatography. However, providing that the chromatographic separation achieved is suitable, a short column lifetime could be tolerated for particular applications. Recently advances in column chemistry have been made that lead to column materials with enhanced thermal stability. Most recently Xterra ™ RP 18 and Luna ™ $C_{18}$ (2) two silica-based ($C_{18}$) columns have been introduced in HPLC. The molecular structure of a recently developed chromatographic support (Xterra ™ RP 18) is a mixture of organic and inorganic groups (86). This results in a fundamental improvement of the composition of the underlying chromatographic particle with the right organic/inorganic balance. With a high organic content, particles behave like a polymer, e.g. with low mechanical strength and low efficiency. With a high inorganic material, particles behave like silica, e.g. too many unbonded silanol groups are present introducing problems for bases and poor stability when used with aggressive mobile-phases. Because hydride particles

are partially organic (they have methylsiloxane units in place of one third of $SiO_2$ units), they yield bonded phases with increased surface coverage and reduced residual silanols (**Figure 1.3**). Significant improvements in peak shape and in capacity have been obtained in pH 1-12. Xterra columns also exhibit excellent water wettability, even in 100 % aqueous mobile phases. Therefore, the manufacturing company claimed that Xterra columns are stable over all the pH range and at high temperature (80 °C). The following reaction is involved in manufacturing of this column (**Figure 1.4**).

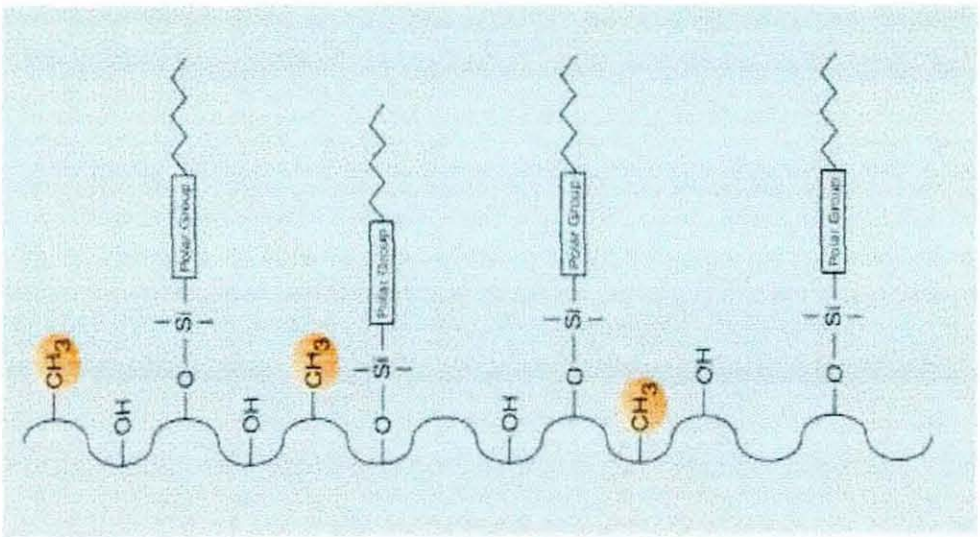



**Figure 1.3: Surface of Xterra ™ RP 18 column [Figure obtained from brochure of Waters].**

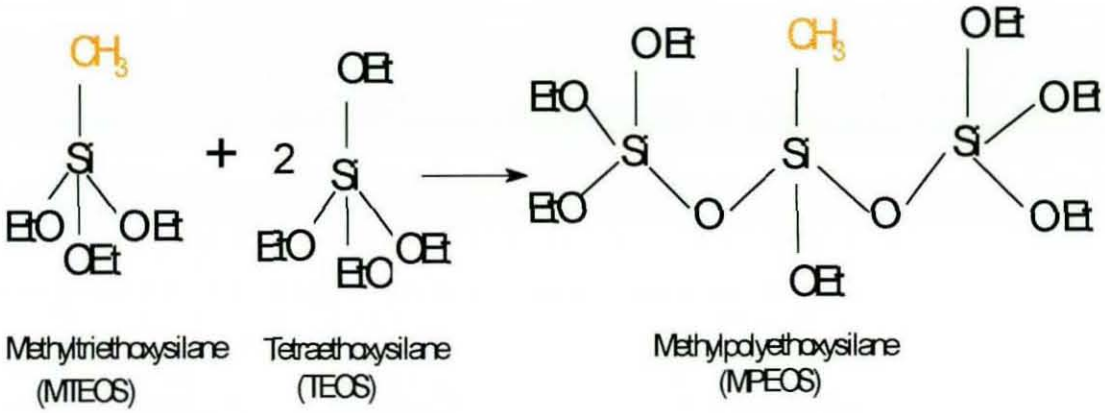



**Figure 1.4: Chemistry of Xterra column [Figure obtained from brochure of Waters].**

Wilson has used Xterra ™ RP18 columns in a superheated water chromatography system (66). He separated dimethylantipyrine; phenacetin, antipyrine, caffeine and paracetamol at 165 °C. These five test compounds were eluted on this column with excellent peak shape and resolution.

Luna ™ $C_{18}$ (2) columns have high density bonding with alkyl chains so that alkyl chains interact preferentially with each other, restricting the access to surface silanols (87). They can be pictured like "fence pickets" (**Figure 1.5**). Some authors have reported the synthesis process of densely monolayer-bonded silicas, using hydrogenosilanes (88), olefin hydrosilation or alkylsilanes as reagents (89). These bonded silicas are hydrolytically very stable. Selectivity is placed between the monolayer and polylayer bonded silicas, as a function of the temperature and the mobile phase. This process starts with pure silica, giving a stationary phase with a low tailing effect for basic solutes.

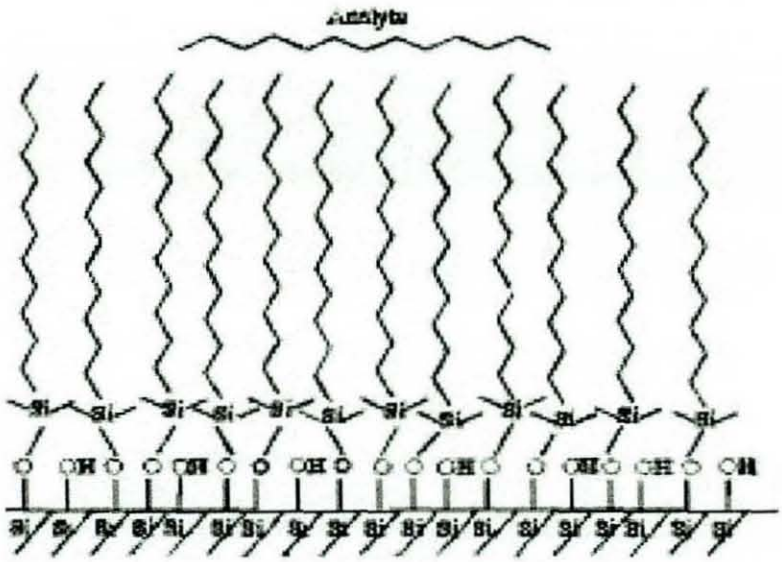


**Figure 1.5: Surface of Luna ™ C 18 (2) column [Figure obtained from ref. (88)].**

### 1.5.2: Hypercarb (Porous Graphitic Carbon)

Hypercarb is also commonly known as porous graphitic carbon (PGC) on account of its backbone formed from layered bands of graphite. Crystalline carbon was developed as a chromatographic support because of its resistance to organic solvents (90-91). A previous study in this laboratory (49) demonstrated that the PGC stationary phase is stable with a superheated water mobile phase over a long period of time (> 336 hours) at temperatures as high as 220 °C. The remarkable stability of this phase lies in its unique structure. The actual phase itself can be likened to graphite in that essentially, PGC is a crystalline material made up of sheets containing huge numbers of hexagonally arranged carbon atoms linked by the same conjugated 1.5 order bonds which are present in any large polynuclear aromatic hydrocarbon (92). The individual sheets of carbon atoms are about 3.5 angstroms apart and held together by dispersion interactions (**see Figure 1.6**).

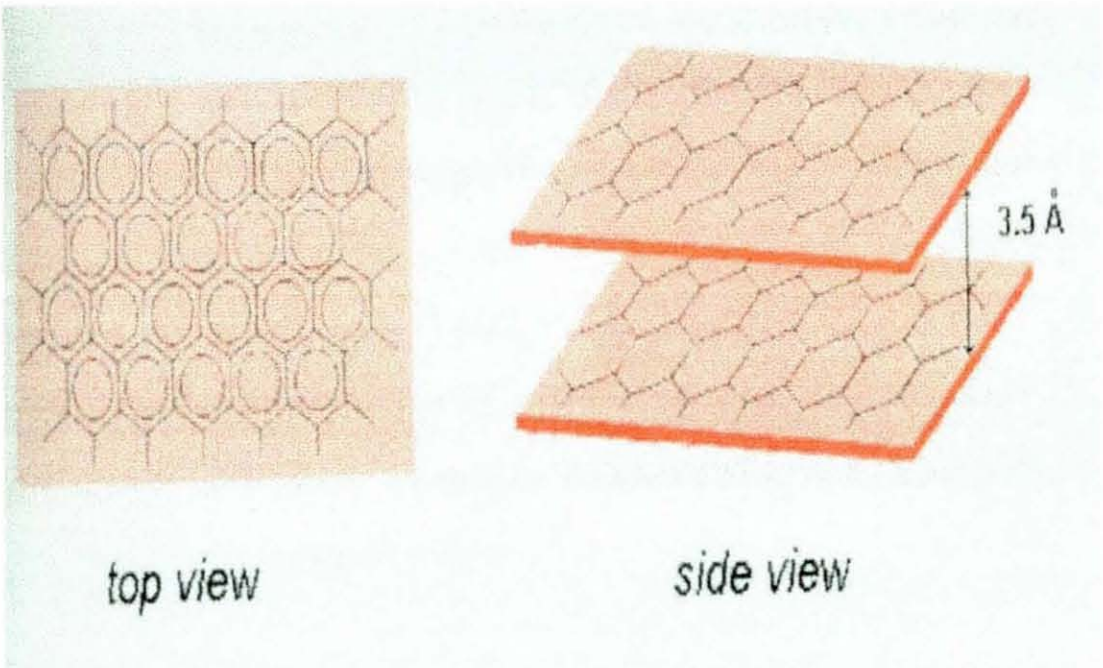



**Figure 1.6: Schematic structure of PGC [Figure obtained from ref. (49)].**

Because of the nature of this arrangement there are, in principle, no extraneous functional groups on the surface because the carbon atoms have all their valences satisfied. However, to produce a crystalline material with a high specific surface area suitable for use in HPLC, the graphite crystallites will have to be very small, in the order of 1-10 nm. This extremely small size means that surface defects will be present at the edges of the crystalline solid and carbon atoms with unsatisfied valences are likely to be occupied by –OH, =O and COOH groups (92). However, these functional groups will only make up a small part of the total bulk material. For example, a square array of graphite carbon atoms with a side of 10 nm will contain about 40,000 carbon atoms, of which about 160 will be at the edge of the sheet. The edge atoms will thus comprise only 0.4 % of the total carbon atoms. If the crystallites are about 30 layers thick, then their specific surface area will be of the order of 300 $m^2/g$, a value typical for HPLC materials.

The surface layers in two or three-dimensional graphite is almost identical with PGC. It is known that graphite itself is one of the most unreactive substances. Therefore, it is stable at high temperature and also is very stable to aggressive acidic and basic conditions. Indeed it is stable between 1-14 pH. The temperature stability of PGC should also be high. The fabrication of the material involves heating a base phenol-formaldehyde resin to 1000 °C in a nitrogen atmosphere so that it carbonises (93). Additional heating to 2000 °C is then applied to the material to close any unwanted micropores that proved detrimental to LC performance (94). Therefore, this type of material should also be stable with superheated water mobile phase. A similar material to the Hypercarb PGC phase has also been used in a high temperature environment. Yamaki et al (95) employed a Carbonex column from Biotech Research (Saitama, Japan) and analysed peptide samples at 160 °C temperature with no degradation of the column itself. However, these researchers used a conventional aqueous ACN mobile phase to achieve their separation.

Nazir et al. (96) used the PGC column to separate Cyclosporin A (a neutral cyclic peptide composed of 11-amino acids) and Cyclosporin U at high temperature. They also investigated the effects of different temperatures on the peak shape on this column. They showed the process of peak broadening to be kinetically controlled.

Barrett et al. (97) separated morphine, codeine and the related metabolites, normorphine, norcodeine, morphine-3-glucuronide (M3G), morphine-6-glucuronide (M6G), and morphine-3-O-sulphate on a PGC column at elevated temperature. They also studied the effects of temperature on the retention behaviour of morphine, M6G, and M3G on this column.

Pietrogrande et al. (98) reported the effect of column temperature on solute retention for 10 polychlorobiphenyls (PCBs) on PGC column with different degrees of planarity (degree of ortho position substitution). High temperature significantly improved separation efficiency (plate number significantly

increases): this effect was the dominant factor controlling chromatographic resolution, which improved with increasing temperature.

A previous study of our laboratory (49) separated a wide range of aromatic compounds on a PGC column using superheated water as mobile phase. The temperature effects were also studied in this project.

Wilson (66) successfully separated a range of drug compounds on a PGC column using superheated water as mobile phase.

Recently Y. Yang et al. (57) separated carbohydrates on a PGC column using subcritical water as the mobile phase.

### 1.5.3: Poly-Styrene-Divinylbenzene

A constant limitation in the use of bonded silica columns is that the eluent is restricted to the range pH 2.5 to 8 because of the instability of the silica gel matrix. This problem has led in recent years to considerable interest in the use of beads of crosslinked porous synthetic polymers as stationary phases. Originally organic polymers were used in chromatography as the basis of ion-exchange resins but most of these materials were too soft and were compressed or collapsed under the flow rates and pressures used in HPLC. Developments in polymer technology have now led to more rigid materials and smaller particle sizes. Most of the commercially available materials are based on crosslinked polystyrene-divinylbenzene (PS-DVB). Divinylbenzene- or styrene-divinylbenzene-based packing materials have been used since the 1960s for size exclusion chromatography of industrial polymers (99). They are prepared via radical polymerisation of monomers. The monomers used are, in principle, styrene and divinylbenzene isomers. The resulting network structure of PS-DVB is complex because all three divinylbenzene isomers (i.e. o-, m-, and p-) are used in their preparation. This network structure is made more complex as industrial-grade divinylbenzene also contains ethylvinylbenzene isomers.

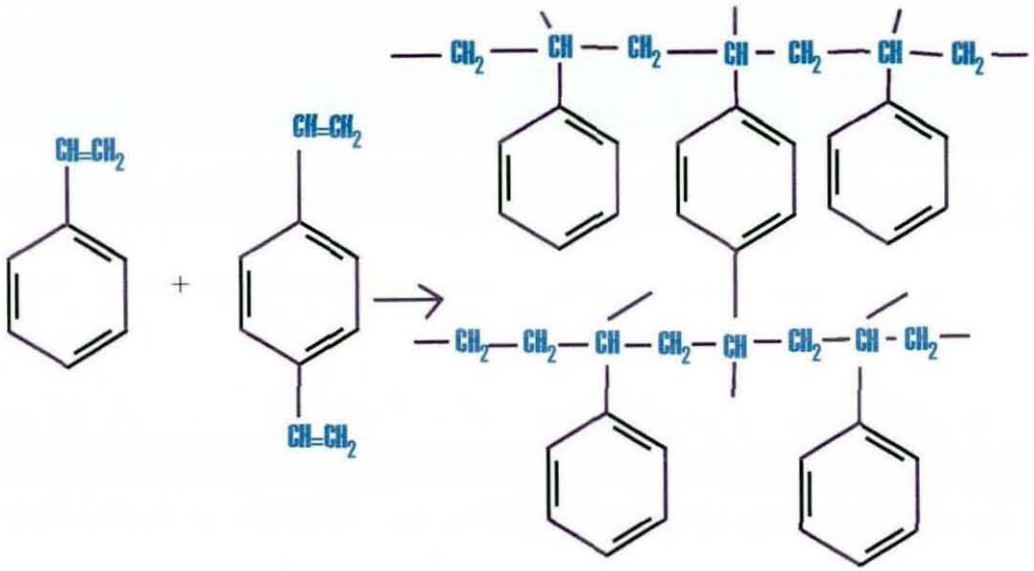


**Figure 1.7: Schematic structure of PS-DVB.**

The amount of divinylbenzene added in the reaction determines the degree of cross-linking and hence the pore structure. PS-DVB containing less than 6% divinylbenzene is not pressure stable and cannot be adequately used as HPLC materials. Semi-rigid PS-DVB with 8% divinylbenzene is stable up to approximately 60 bars. The polymers change the bed volume depending on solvent and ionic strength. Rigid PS-DVB gels are highly cross linked and are stable at pressure up to 350 bar, making them usable for RP-HPLC.

A further classification used to distinguish the various types of PS-DVB used for LC, is to describe them as being either macroreticulate or microreticulate. Microreticulate PS-DVB is less cross-linked. They are non-porous in the dry state and acquire a pore volume only by absorbing a solvent. Thus the pores are formed by the holes in the cross-linked polymer network. These packing are designed largely for the size-exclusion chromatography of small molecules and oligomeric analysis (100). They are generally not suitable for RPLC, as they are not mechanically stable at high pressure and swell and shrink with frequent solvent changes.

The pores of macroreticulate packing are not formed by a network, but by the spaces between spherules of "solid" polymer (101). In this aspect they are identical to inorganic packing materials and it is more appropriate to categorise these packings as macroporous. Macroporous packings have a permanent pore structure and internal surface that can be explored and measured by the same techniques as used for inorganic packings. Packings with a permanent pore structure are used in size-exclusion chromatography of larger molecules, but they also form the basis for packings used in retention chromatography. The pore size of the packing is determined by the size of the spherules that form its skeleton, and the size of the spherules, in turn, is determined by the preparation conditions of the polymer. The spaces between these microspheres are composed of macropores ($d_{pore}$ >50 nm) and mesopores (2 nm <$d_{pore}$ <50 nm); whereas the solid polymer matrix itself is microporous ($d_{pore} \leq$ 2 nm) (102). The micropores can be thought of as interstices between the polymer chains (103). Large particle (spherules) macroeticulate PS-DVB packing are used for size-exclusion chromatography (104), whereas smaller particle packings can be used for RP-HPLC (105-106).

The resulting matrix consists almost entirely of aliphatic and aromatic hydrocarbons (some radical initiator is always incorporated into the matrix). Therefore, it is highly hydrophobic and hydrolytically stable from pH 1-14. The dry powder is not wetted by water, that is to say the microporous structure is closed or unavailable when separations are carried out with mobile phases with high water content (103). In this aspect water does not swell the packing at all. However, organic solvents, even polar ones, such as methanol, wet the packing and swell the matrix, to improve a polymer's wettability a number of workers have modified PS-DVB by bonding hydrophilic groups to the surface (107-109). These materials subsequently swell very little when changing from aqueous to organic solvent mobile phases.

In a previous study, it was found that the polymer matrix is sensitive to the solvent used. Polar organic solvents, such as alcohols, seem to be particularly weak eluents (110). Smith and Garside compared the solvents THF, ACN and MeOH as modifiers on PLRP-S with respect to solvent strength and efficiency (111). MeOH was the weakest eluent; for example a (90:10) MeOH-$H_2O$ solution was only equivalent to a (70:30) ACN-$H_2O$ and (40:60) THF-$H_2O$ mobile phase. As comparison, on an ODS silica column a (40:60) THF-$H_2O$ mobile phase was found to be equivalent to (50:50) ACN-$H_2O$ and (70:30) MeOH-$H_2O$ (112). MeOH was also shown to give poor efficiencies with distorted tailing peaks. Other researchers have also found that MeOH is a weak solvent on PS-DVB whereas THF is anomalously strong (113-115). The reason for this observed anomaly is that polar solvents such as methanol have very little affinity with the totally hydrophobic polymer surface and are therefore not able to penetrate (swell) the pores of the polymer as effectively as non-polar solvents (116). It is postulated then that superheated water will be a weak mobile phase on this column material.

However, the use of polymer-based packing materials is increasing in RPLC, due to the need for packing materials that are more stable than silica ODS. The polymer-based stationary phases possess advantages in chemical stability, absence of silanol effects, and variety of surface structures. PS-DVB columns have been widely used for high temperature liquid chromatography. Smith and Burgess separated a wide range of aromatic compounds by a PS-DVB column on superheated water chromatography (49), Yang studied retention behaviour on a PS-DVB column at high temperature (65), Pawlowski used a PS-DVB column to determine solvation characteristics of pressurized hot water (62), and early studies demonstrated the separation of barbiturates, (52), analgesics (58), polar vitamins (59) and a range of aromatic phenols, amides, amines and esters (49) on a PS-DVB column for superheated water chromatography.

### 1.5.4: Oasis HLB ™ [Poly (divinylbenzene-co-N-vinylpyrrolidone (DVP/NP)

One limitation of both reversed-phase silica sorbents and many of the commercially available polymeric sorbent is that they must be conditioned with a wetting solvent and remain wetted before sample loading. Therefore some researchers looked for a hydrophilic-lipophilic balanced sorbent for solid-phase extraction. Researchers have taken several approaches to create a more hydrophilic surface for adsorption. Yang and Regnier (109) coated and cross-linked a hydrophilic monomer to a PS-DVB resin. Similarly Leonard and co-workers (117) cross-linked a polyvinyl alcohol to a PS-DVB surface. However, those coatings reduced the availability of the hydrophobic PS-DVB for reversed-phase interactions. Another approach used by Dumont and Fritz (118) was sulfonation of PS-DVB, which can generate a more hydrophilic surface. Retention can be affected by the extent of sulphonation,-above a certain level, analytes become less retained. One drawback to this approach is that the ion-exchange functionality provides an additional mode for adsorption, and this mode can complicate the SPE method development. Sun and Fritz (119) derivatized PS-DVB with acetyl or hydroxymethyl groups to provide a more hydrophilic surface.

Bouvier and co-workers synthesized a copolymer of N-vinylpyrrolidone and divinylbenzene to impart greater hydrophilic character, while still retaining analytes by reversed-phase interaction. The hydrophilic-lipophilic balanced resin, which they call HLB, contains a ratio of the two monomers optimised for *both hydrophilic wetting and lipophilic retention.* The chemistry of Oasis HLB is [poly (divinylbenzene-co-N-vinylpyrrolidone) (DVB/NP)] (**Figure 1.8).**

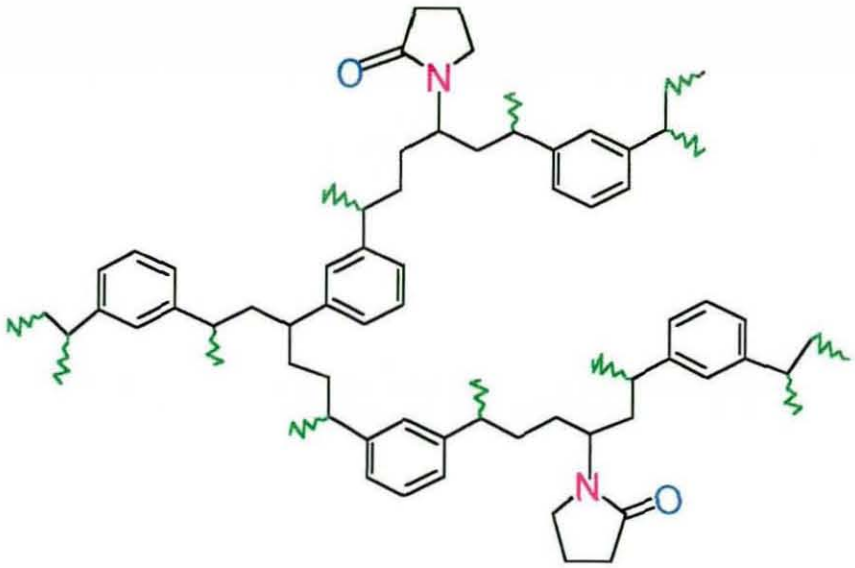



**Figure 1.8: [Poly (divinyl benzene-co-N-vinylpyrrolidone (DVB/NP).**

The pyrrolidone group in the hydrophilic-lipophilic balanced sorbent, which is a hydrogen-acceptor will form hydrogen bonds with hydrogen-donors. Analytes, such as theobromine without hydrogen donors group that are hydrogen acceptors have a less dramatic retention enhancement.

It is a novel hydrophilic-lipophilic balanced sorbent that does not require preconditioning. It is also stable at all pH range and high temperatures. Previous studies (120) demonstrated that it is stable at 200 °C. Another study had noticed that PS-DVB packing materials are more retentive and less efficient than silica-based columns on superheated water chromatography (49). However, Wilson (66) successfully used Oasis HLB™ column on superheated water chromatography system for separation of a range of drugs.

### 1.5.5: Polybutadiene- coated Zirconia

There are many disadvantages of both silica-based and polymer reversed-phase packing materials. The dual disadvantages of polymeric packing, reduced pressure capability and hindered mass transfer for small molecules, has limited their use to applications in reversed phase liquid chromatography. One of the major limitations of silica-based packing materials is the narrow range of pH, the alkyl-bonded phase is susceptible to hydrolysis, and silica dissolution occurs at high pH. Therefore some chromatographers were interested to develop other packing materials. Other inorganic oxides, especially alumina, zirconia and titania have also been investigated for their potential use as reversed-phase sorbents. Recently, spherical, porous, microparticulate zirconia and titania supports with excellent physical and mechanical properties have been prepared (121). However, there is no surface derivatization available for either oxide that compares to the silanization technique used in the preparation of the silica-based bonded phases (122). Simple organic derivatives of these oxides are hydrolytically unstable. While silanization of the oxide surfaces has been reported to result in packings with good pH stability, the same report also states that the hydrolytic stability of the zirconium and titanium siloxane bond is inferior to the pure siloxane bond. Chromatography on these derivatised supports is often poor with the result being tailing peak shapes. This is due to the active nature of the titania and zirconia surfaces, which are significantly different from either silica or alumina (123). However, other derivatization techniques have been developed that are suitable for application to any surface and therefore can be used for the modification of the surfaces of these three oxides.

The surfaces of titania and zirconia are significantly different from silica or alumina, but appear to be similar to each other, because they are both Group IVA elements, and thus it is seemed that they would show similar chromatographic properties. However, of the two inorganic oxides, zirconia perhaps shows more promise as a chromatographic stationary phase as its surface is less active than that of titania. Zirconia has been studied extensively by Carr and co-workers (124-126). Its surface comprises active

sites consisting of bridged oxides, bridged hydroxyls, and unlike silica, is strong Lewis acid (**see Figure 1.9**).

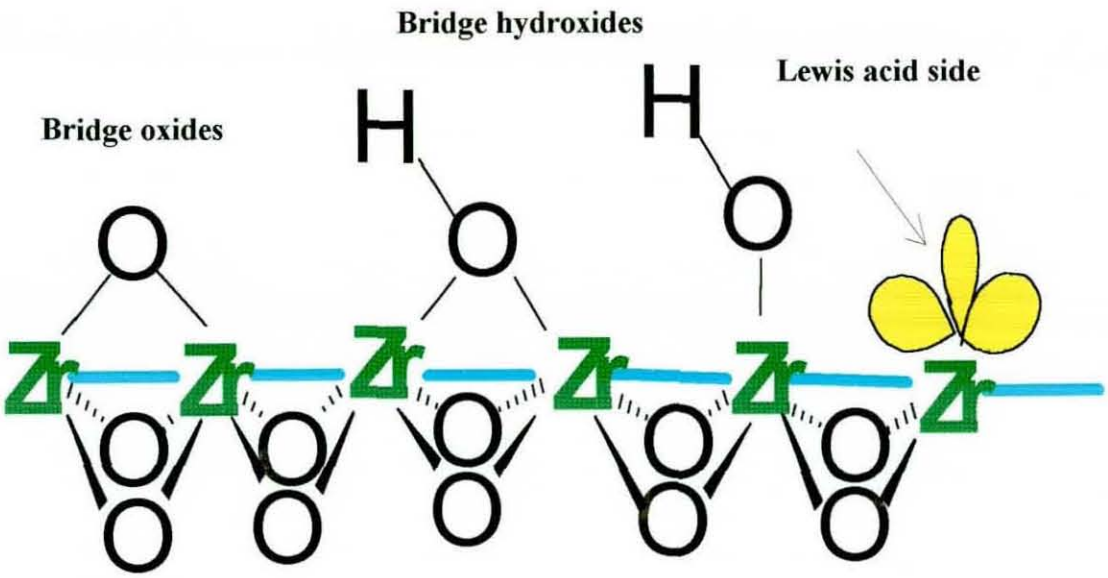



**Figure 1.9: Schematic illustration of the various active sites to be found on the surface of native zirconia.**

The Lewis acidity of the native zirconia surface is due to the presence of a co-ordinatively unsaturated zirconium (IV) species. The consequence is that hard Lewis bases such as hydroxide, phosphates, fluoride, or carboxylates (listed *according to affinity strength*) will interact strongly with the hard Lewis acid sites on the surface, resulting in poor peak shapes. These studies indicate that for the preparation of a well-behaved HPLC packing from zirconia it may be even more necessary to hide the surface than it is with silica. Nevertheless chromatography can be significantly improved by adding a competing Lewis base such as phosphate, or fluoride, or by raising the pH of the mobile phase (124).

Although titania and zirconia are both hydrolytically stable from pH 1-14, like alumina, the surface of zirconia can be either positively charged, neutral, or

negatively charged. Therefore, at low pH, zirconia acts as an anion exchanger, and at high pH it is a cation exchanger. In between, there is a balance of positively charged, negatively charged and neutral sites that varies as a function of the pH. However, for alumina, titania, and zirconia, there exists as yet no covalent bonding chemistry (123). Therefore, alternative surface modification techniques have been developed that do not rely on the attachment of the modifier to the surface. A coating can be simply insoluble in the intended mobile phases, or a crosslinked can be formed that stretches like a net around the skeleton of the particles. Both technique are, in principle, independent of the nature of the substrate and can be applied to all inorganic or polymeric packing.

The most widely used example of the second technique is based on a process developed by Schomburg (127). He coated a prepolymerized polybutadiene of controlled molecular weight onto the surface of a substrate and then completed the polymerisation using radical initiators at elevated temperature. The result is a hydrophobic surface, suitable for reversed-phase chromatography, that can be applied to silica, alumina, titania and zirconia. Reversed-phase packings with significantly improved pH stability have been obtained by this method (128). The range of polymer coatings that have been applied with this method has been extensively reviewed by Hanson et. al. (129). One drawback with these coated stationary phases is that the final thickness of the coating must be carefully controlled so that the mass transfer process is not hindered leading to lower column efficiencies.

The PBD-zirconia stationary phase was largely developed by Li and Carr (125, 130-131), and these preliminary papers had shown it to be stable to temperature up to 200 °C with aqueous-organic mobile phases. Carr's group had also shown PS-$ZrO_2$ to be stable in superheated water mobile phases at 120 °C (63). They found that high column temperatures can significantly decrease the analysis time and column back-pressures and can, in some instances improve chromatographic selectivity.

Recently Carr et al. (64) reported that, superheated water at 200 °C would separate phenol and four of its chloroderivatives on a zirconia column. They also compared five distinct columns that exhibit varying degrees of chemical and thermal stability, which showed that the polybutadiene-coated zirconia phase column was the most efficient and the PS-DVB polymeric column was the least efficient. In term of chromatographic selectivity for non-electrolytes, the polybutadiene-coated zirconia phase and polybutadiene-coated alumina columns were very similar. In contrast, the graphitised carbon-clad zirconia column showed the greatest difference in reversed-phase chemical selectivity of any of the columns tested compared with the $C_{18}$ silica column.

### 1.6: Efficiency

Efficiency is strongly influenced by the transport properties of the system: the eluent diffusivity and the viscosity of the mobile phase. Temperature has great effect on diffusivity and also on viscosity. Generally an increase in temperature will lead to a decrease in viscosity of a liquid according to the expression given below (132).

$$\eta = \alpha \exp(\beta/T) \qquad (1.1)$$

Where $\eta$ is the viscosity, and T is the absolute temperature. The constants $\alpha$ and $\beta$ are dependent upon the solvent used. The viscosity of the mobile phase plays a central role in the mass transfer properties of the chromatographic system. Lower viscosities not only reduced column back-pressures but also increase solute diffusivity in the mobile phase and the stationary phase. The temperature dependence of the elute diffusivity in the mobile phase can be represented by equation (1.2)

$$D_m = D_{m,25}\frac{T}{298}\frac{\eta_{25}}{\eta} \qquad (1.2)$$

Where $D_{m,25}$ and $\eta_{25}$ are the molecular diffusivity and the viscosity at 25 °C. This means that operation at elevated temperatures in LC will lead to a decrease in the mass transfer term in the van Deemter equation, that will ultimately lead to decreased plate heights and thus higher efficiencies.

### 1.6.1: The Reality of the Effect of Temperature on Efficiency in RP-HPLC

There are contradictory reports to whether or not efficiency is increase when temperature is increased. Schmit et al. reported in 1971 a gain in efficiency with increasing temperature and related the increased efficiency at higher temperatures to the drop in mobile phase viscosity and hence an increase in diffusion rate (133). They reported a twofold increase in efficiency, a similar increase in efficiency at ambient temperature was only achieved by increasing the column length four times. Knox and Varasi did not find a temperature-dependent increase in reduced efficiency (71). Therefore they reasoned that the increase in efficiency must result from a decrease in diffusion coefficient and was not induced by a change in properties of the packing material.

In 1987 Yang and Verzele (107) reported that the efficiency increases by increasing the column temperature. The slope of the H vs u curves increases with the number of hydroxyl groups, showing that the influence of temperature on efficiency is larger for compounds having higher hydroxyl group content.

Saner et al. (134) reported a loss in efficiency on a 5 μm $d_p$ RP18 column at a temperature of 60 °C. They attributed the loss in efficiency to a drop in diffusitivity within the stationary phase resulting in reduced mass transfer. In

the same study an increase in efficiency for 10 μm dp RP18 column was reported.

Warren and Bidlingmeyer reported against all expectations based on theory a loss in efficiency with increase temperature (135). They worked in the temperature range of 35-65 °C and used acenaphthene and dibutyl phthalate as solutes. Regardless of eluent and solute type and flow rate a higher temperature led to higher H values.

In the same year that Warren and Bidlingmeyer reported their results; Horvath and Anita (136) published a theoretical examination of the effect of temperature on the efficiency for separations of large molecules.

Their assumptions for their theoretical analysis were as such:

- The analytes have a **constant k** value of k = 3 throughout the temperature range
- The column **packing structure is invariant** with temperature
- The mobile phase shows the **properties of water** throughout the temperature range
- **Slow sorption kinetics** for large molecules are assumed

A variation of the van Deemter equation published in an earlier paper (137) was used for the calculation of plate height (**equ. 1.3**) and the reduced plate height (**equ. 1.4**) respectively:

Plate height $$H = Ad_p + B\frac{D_m}{u} + C\frac{d_p^{\,2}}{D_m} + \frac{Dd_p^{\,5/3}}{D_m^{\,2/3}}u^{2/3} + \frac{2ku}{(1+k)^2 k_d} \qquad (1.3)$$

Reduced plate height $$h = A + \frac{B}{v} + Cv + Dv^{2/3} + \frac{3D_m}{8k_d d^2{}_p}v \qquad (1.4)$$

A is the packing term, B the longitudinal diffusion, C the intraparticular mass transfer resistance, D the mass transfer resistance of a stagnant film of

mobile phase around the particle and the last term accounts for band spreading due to slow kinetics. Horvath and Antia (136) reasoned that the results from Schmit et al. (133) and Knox and Vasari (71) were evidence enough to claim that the terms A, B, C, and D in the equation for the reduced plate height were independent of temperature. However, the reduced velocity itself and the kinetic term are dependent on temperature. To theoretically examine the effect of temperature they proceeded by specifying the system variables. Under conditions where the column length, the inlet pressure and the particle size were set the dependent variable analysis time decreased and efficiency increased with temperature. With increasing the temperature under fixed conditions for the analysis time the pressure in the observed system decreased and the efficiency increased. All data was calculated for large molecular weight solutes with slow desorption kinetics and totally porous particles packed in a 3 cm column.

Horvath and Antia (136) proposed as a result of their theoretical investigation, an increase in efficiency with an increase in temperature because of enhanced diffusivity and sorption kinetics for large molecules at elevated temperatures.

In 1992 Erni et al. (138) reported an increase in efficiency at elevated temperatures for open tubular capillary LC. They found that the mobile phase had to be pre-heated to column temperature to achieve the best efficiency results. Like Horvath and Antia (136) they explained the increase in efficiency with improved solute diffusivity at elevated temperatures.

In 1993 Horvath and Chen (139) published experimental data supporting the results of the theoretical analysis by Horvath and Antia (136). They applied elevated temperatures to protein separations. It was claimed that the main advantages of high column temperatures derived from low mobile phase viscosity and the concomitantly increased solute diffusivity and drop in column back pressure and the accelerated sorption kinetics. Further elevated temperature brought an enhanced solubility for substances, which were not soluble under ordinary conditions.

Trones et al. (140) demonstrated that elevated temperature has a strong effect on the chromatographic peak shapes. Late eluting peaks were sharpened with better symmetry. The peak width was reduced by elevated temperatures, but the temperature effect on peak broadening was small compared to the effect on the retention time. Thus, elevated temperatures increased the eluting strength in such a way that it knocked out the other effects. Since the diffusion rates of heavy analytes are less influenced by temperature than small molecules, the benefits of HTLC on packed capillaries are demonstrated by peak shape, and analysis time, more than by plate numbers.

Molander et al. (141) reported the reduced plate height increased when elevating the temperature gradually from 50 to 175 °C. Contrary to the theoretical calculations by Horvath and Antia (136) they found a temperature dependence of the reduced plate height at the optimum flow velocity. The k values for the test solutes were not kept constant over the full temperature range.

In a recently published study, Yan et al. (64) found that the more retained the solute was, the more significant was the reduction of the C term at elevated temperatures. This indicated that the resistance to mass transfer was reduced at higher temperatures. They showed that for less retained solutes or if the mobile phase was not adjusted to keep the retention factor constant there was less improvement of the C term. On elevating the temperature, they found that the optimum linear velocity is moved to a higher velocity than at ambient temperatures. The conclusions from the study were that it is beneficial to operate a HPLC separation at high temperatures and flow rates to improve efficiency and shorten analysis time.

Li and Carr (142) reported that high temperature could improve the column efficiency by 30% mainly by increasing the diffusion rate in the stationary phase.

Carr et al. has produced a number of papers about the effect of high temperature on the efficiency of the separation on PBD-zirconia column (64, 142). They claimed that column efficiency at high flow rate is significantly improved by increasing the column temperature. They also claimed that they separated alkylphenones 50 times faster than at room temperature with good efficiency.

Pietrogrande et al. (98) reported that the separation efficiency of 10 standard PCBs on porous graphitic carbon significantly improved at higher temperatures, a mean increase of 15 % is achieved in plate number when the temperature increases by 20 °C.

### 1.7: Present Work

In light of these many potential uses of different packing materials on superheated water chromatography our own studies will look at a comparison of stationary phases on superheated water chromatography and conventional LC. Other studies also compared PS-DVB stationary phases in reversed-phase liquid chromatography using different organic mobile phases (111). Further, the efficiency of the superheated water chromatography will be compared to conventional RP-HPLC on different columns to see if there are any real increases.

A previous study demonstrated that the B-term (longitudinal term) of the van Deemter equation is detrimental to the efficiency of superheated water chromatography system, even at intermediate linear velocities. For the PLRP-S stationary phase there was a very marked decrease in the efficiency at high linear velocities with the H versus u plot appearing parabolic in shape. The peak shapes obtained for phenol at high velocity were severely distorted. Therefore the present study will repeat the same study on a different superheated water chromatographic system. Also efficiencies of another two columns will be studied in superheated water chromatography.

The successful on-line coupling of liquid chromatography (LC and SFC) to a number of widely used detectors, namely UV, and NMR lead to an interest in the feasibility of employing these coupled detection method to superheated water chromatography for separation of natural product.

In a preliminary study it was found that when a number of aryl alcohols were chromatographed by superheated water chromatography, it was found that an extra peak was present in each case. The present work will set to out to determine if these were degradation products produced by the superheated water conditions.

# CHAPTER 2

# Experimental

## 2.1: Chemicals

Benzyl alcohol, 3-phenylpropanol, 2-methylacetophenone, benzophenone, acetophenone, valerophenone, hexanophenone, p-cresol, methyl benzoate, anisole and phenol were obtained from Sigma-Aldrich (Aldrich-Chemical Co., Gillingham-Dorset, UK). Propiophenone, vanillin, dihydroferulic acid, ferulic acid, isoferulic acid and benzaldehyde were obtained from Lancaster Chemicals (Lancaster Chemicals, Morecambe, England). Nitrobenzene, toluene, acetonitrile (HPLC grade), methanol (HPLC grade) and THF (HPLC grade) were obtained from BDH Chemicals Ltd. (BDH, Poole, England). Butyrophenone was from Koch Light Lab. Ltd. (Koch Light Lab. Ltd., Colnbrook Bucks, England). N-Methylaniline was obtained from Hopkins and Williams Ltd. (Hopkins and Williams Ltd., Chadwell Heath Essex, England) and 2-phenylethanol was from Sigma Chemical Co. (Sigma, St. - Louis, MO, USA). Zingerone was obtained from Pfaltz and Bauer (Waterbury, USA).

## 2.2: Mobile Phase

Water used throughout this project, as the mobile phase was first triply deionised and treated through an Elga Maxima HPLC purification Unit (Elga Ltd., Wycombe Bucks, UK) at an output of 18.2 $M\Omega\ cm^{-1}$. This unit utilises a hydrophobic C-18 cartridge that removes unwanted organic compounds from the water. The solutions were prepared on a v/v basis.

## 2.3: Instrumentation

### 2.3.1: Superheated Water Chromatography

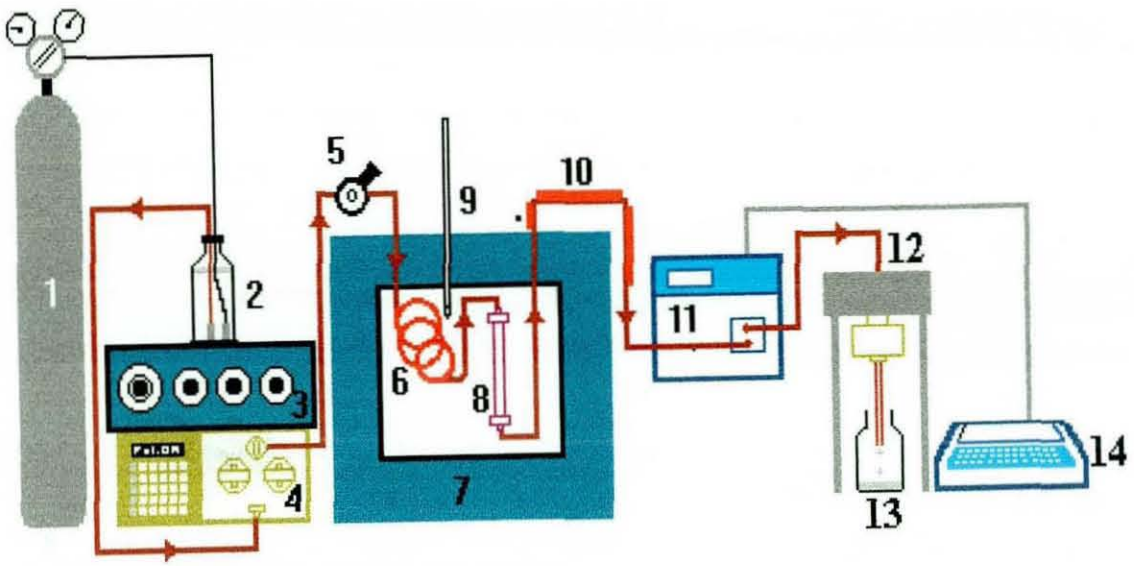



**Figure 2.1: Superheated Water Chromatography**

1. Helium cylinder, 2.Mobile phase reservoir, 3. Oven temperature programmer, 4. LC pump, 5. Injector loop, 6. Preheating coil,
7. Column oven, 8. Column, 9. Thermometer, 10. Cooling fins,
11. UV/Visible detector, 12. Back-pressure regulator, 13. Waste bottle,
14. Integrator.

The superheated water chromatography system comprised a HPLC system equipped with a GC oven. The superheated water chromatographic system **Figure (2.1)** comprised a LC-10AD Shimadzu pump (Shimadzu, Kyoto, Japan) (4), which delivered mobile phase from a reservoir (2) in a constant flow mode to a HPLC column (8) through a preheating coil (6) made by a 100 cm x 0.01 inch I.D. stainless steel tubing. The solvent was continually degassed with nitrogen (1). The sample was injected via a Rheodyne HPLC injector (Model 7125, Rheodyne, Cotati, USA) (5) (external to the oven) fitted with a 20 μl sample loop. The column and preheating coil were placed in a GC oven (Series 104, Pye Unicam, Cambridge, UK) (7) whose temperature was programmed using a programmer controller (Series 104, Pye Unicam, Cambridge) (3) and the temperature was read using a thermometer (9). A set of copper cooling fins (3 cm x 12 cm x 0.05mm) (10) was attached to the exit tubing to cool the mobile phase down to ambient temperature before the Jasco UV/visible detector (Model 870, Jasco, Kyoto, Japan) (11). A back pressure controller (Jasco 880/81) set at 35 kg $cm^{-2}$ or a 0.13 mm I. D. x 3 m length of PEEK tubing were used to maintain the pressure of superheated water in the column (12). The signal at 254 or 280 nm was collected by HP 3395 integrator (14) or a Star WS V5 Data Acquisition system (SW Single 317656686).

The above system was used for conventional LC eluents at room temperature (24-27 °C) as well as for superheated water chromatographic system.

Data acquisition was performed through an A/D converter (detector bunch rate 10 Hz and noise monitor length 6.4 see) and fed into the Star Chromatography Workstation (Varian, Surrey, England). This module determines the performance of a column from the following parameters: retention factor and column efficiency. These parameters are based on the following equations:

$$k = \frac{t_R - t_0}{t_0} \qquad (2.1)$$

$$N = 5.54\left(\frac{t_R}{w_h}\right)^2 \qquad (2.2)$$

Where $k$ = retention factor,

$t_0$ = dead time (retention time of unretained solute, breakthrough time),

$N$ = efficiency (number of theoretical plates),

$w_h$ = Peak width at 50% of peak height,

Efficiencies were calculated using the peak width at half height. Chromatograms were collected in triplicate for each flow rate studied and average was taken for the calculation of efficiency. The chromatograph was allowed to equilibrate for between 40 minutes to 60 minutes after a change in flow rate was made.

### 2.3.2: Calculation of Plate Height and Linear Velocity

The Plate height was calculated from the following equation (2.3)

$$H = \frac{L}{N} \qquad (2.3)$$

Where $N$ is the plate number and $L$ is the column length.

The mobile phase linear velocity was calculated from the following equation (2.4).

$$u = \frac{L}{t_0} \qquad (2.4)$$

Where $L$ is the length of the column and $t_o$ is the breakthrough time of an unretained solute.

### 2.3.3: Nuclear Magnetic Resonance Detector

In order to connect the NMR detector to the superheated water chromatographic system, a 0.25 mm I.D. x 3 m PEEK tubing (15) was connected from the UV detector to a Bruker DRX-500 NMR spectrometer (Bruker UK Ltd., Coventry, UK) (16-18) with a detector cell volume of 120 μl. The PEEK tubing also maintained pressure in the column during on-flow mode.

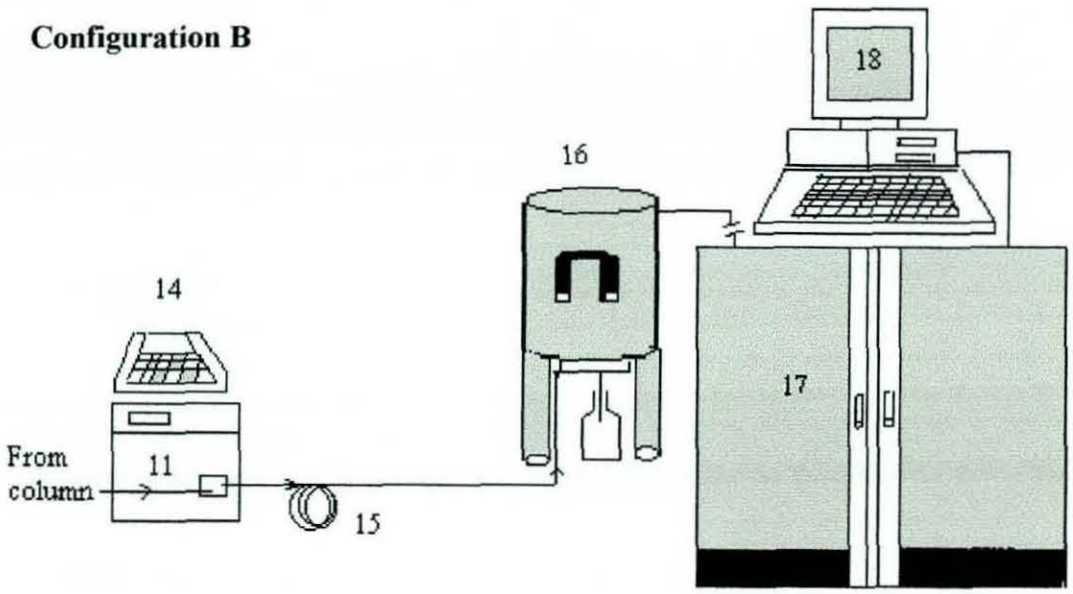



**Figure 2.2: An extension of the superheated water chromatographic system in figure 2.1.**

11. UV detector, 14.UV Integrator, 15. 3 m PEEK tubing, 16. Magnet, 17. Table, 18. NMR integrator.

The NMR spectra were measured at 500.13 MHz ($^1$H). Free induction decays (FID) were collected over a spectral width of 8278.15 Hz into 8194 data points using an acquisition of 0.49 s.

The NMR was also performed in the stopped-flow mode on selected peaks and the spectra were measured at 500.13 MHz ($^1$H). Free induction decays (FID) were collected over a spectral width of 8012.82 Hz into 16384 data points using an acquisition time of 1.022 s.

## 2.4: Chromatographic Columns

The following chromatographic columns were employed.

- 150 x 4.6 mm I.D., 5 μm PLRP-S (polystyrene divinylbenzene, PS-DVB), Polymer Laboratories, Shropshire, UK.

- 150 X 4.6 mm I.D., 3 μm Zirchrom-PBD (polybutadiene), Zirchrom Separations, Anoka, Minnesota, USA.

- 150 x 2.1 mm I.D., 3 μm Zirchrom-PBD (polybutadiene), Zirchrom Separations, Anoka, Minnesota, USA

- 150 x 4.6 mm I.D., 5 μm OASIS HLB$^{TM}$ [Poly (divinylbenzene-co-N-vinylpyrrolidone (DVB/NP)], Waters Corporation, Milford, Massachusetts, USA.

- 150 x 4.6 mm I.D., 5 μm Xterra $^{TM}$ RP 18 ($C_{18}$ bonded hydride particles), Waters Corporation, Milford, Massachusetts, USA.

- 100 x 2.1 mm I.D., 3 μm Luna $^{TM}$ C18 (2) column, Phenomenex, Queens Avenue, Hurdsfield Industrial, Estate, Macclesfield, Cheshire SK10 2BN, England.

- 100 x 4.6 mm I.D., 3 μm porous graphitic carbon (Hypercarb), Hypersil, Runcorn, Cheshire, UK.

## 2.5: Sample Preparation

The test solutions were prepared weighing out an appropriate amount of the solute and then adding water or aqueous organic solvent mix. The concentration most of the solutes were in the range 0.2-5 mg/ml. In the some of cases the samples were prepared by 20:80 v/v MeOH/water. For non-polar compounds, such as hexanophenone, the solutions were prepared in 70:30 v/v MeOH/water.

## 2.6: Purity Test

The purity of some compounds (benzyl alcohol, 2-phenylethanol, benzaldehyde and 2-phenylacetaldehyde) was tested by GC on an methyl-phenyl-silicone coated column (30 m x 0.32 mm I. D., 0.25 micron film thickness), Alltech Econocap, serial no., 2084-16). The detector was FID. The separation conditions were:

detector temperature = 180 °C
injector temperature = 180 °C
initial column Temperature = 40 °C (10 degree/min to final column temperature 99 °C)

## 2.7: UV Spectrophotometer

The comparison of UV absorbance between benzyl alcohol and benzaldehyde were performed by UV Spectrophotometer (Unicam 8700 series) at 254 nm.

## 2.8: Extraction of Ginger

The dry ginger powder was obtained from Sainsbury's supermarket, Loughborough, UK. Dry ginger powder (10.0 g) was weighed and then 20 ml methanol was added. The mixture was stirred overnight and subsequently filtered through a 10 cm column packed with silica gel (60-120 mesh), BDH Chemicals

Ltd. (BDH, Poole, England), followed by 20 ml methanol. The whole eluent was collected and evaporated to dryness under vacuum. The extract was separated by superheated water chromatography with UV and NMR detection. Selected fraction collected by loop collector (Bruker Peak Sampling Unit with 12 collection loops) and analysed in the stop flow mode

### 2.9: Extraction of Grains of Paradise

Grains of paradise seeds were a gift from Tropical Products Institute, London. Grains of paradise seeds (2g) were weighed and powdered by mortar, then 4 ml methanol was added. The mixture was stirred overnight and subsequently filtered through a 5 cm column packed with silica gel (60-120 mesh), BDH Chemicals Ltd. (BDH, Poole, England), followed by 10 ml methanol. The whole eluent was collected and evaporated to dryness under vacuum. The extract was separated by superheated water chromatography with UV detection.

### 2.10: Identification of Chromatographic Peaks by Mass Spectroscopy

Fractions were collected by loop collection in NMR study (**Figure 2.2**) and freeze dried. Fractions were injected on JEOL SX102 Mass Spectrometer. Fractions were introduced directly by means of an electrically heated probe inserted through a vacuum lock and mass spectra were measured in the electron impact mode (EI).

# CHAPTER 3

# Comparison of Conventional LC and Superheated Water Chromatography

## 3.1: Introduction

Superheated water (water under pressure above its boiling point) has been utilised successfully as a pure mobile phase for chromatography with a number of different columns, Spherisorb ODS1, C18 AB, Alumina, PRLP-S, PBD-zirconia (65), Xterra $^{TM}$ RP 18 and Hypercarb (66). The elution order followed conventional reversed-phase HPLC (48). Some chromatographers have compared superheated water with organic solvents. A previous study (49) in our laboratory demonstrated that superheated water at 240 °C is equivalent to a 40% ACN (v/v) aqueous mobile phase on a polystyrene column, superheated water at 200 °C is equivalent to 40% ACN (v/v), or 70% MeOH (v/v) on a PGC column and superheated water at 140 °C is equivalent to 25% ACN (v/v), and >30% MeOH (v/v) on PBD-zirconia column. Other studies by Yang et al. (65) reported that for aniline and phenol, the retention factors of subcritical water separations at 150 °C were similar to those achieved by using 43% methanol (v/v) in water or 40% acetonitrile (v/v) in water. When the water temperature was raised to 200 °C, the retention factors of the chlorinated phenols and anilines in subcritical water separation were similar to those in separations obtained by organic solvent/water mixtures with 68 or 69% organic solvents. The elution strength of subcritical water is weaker for benzene, toluene, ethylbenzene and xylene. Excluding benzene, the retention factors of water separation at 200 °C are still greater than those achieved by mixtures of 43% methanol in water or 40% acetonitrile in water. Pawlowski and Poole (62) showed that 1% acetonitrile-water at 180 °C corresponded to 15-25% acetonitrile-water, 25-35% propanol-water, or 50-60% methanol-water at 20 °C on a polystyrene column. However, so far there has been no systematic comparison study of conventional LC and superheated water chromatography on different columns.

The present study examines the retentions of alkyl aryl ketones and a number of test compounds on PBD-zirconia, Xterra ™ RP18, Luna $C_{18}$ (2) and Oasis HLB™ columns using different isoelutropic eluents and superheated water in order to determine the effect of the different eluents on the selectivity of the separation. A study has also been carried out of the effect of the mobile phases on the efficiency of the separation.

### 3.2: Results and discussion

To compare the chromatographic properties of conventional LC and superheated water chromatography the comparison of retention indices based on the alkyl aryl ketone scale proposed by, Kikta and Grushka (143) and Smith (144) were used. It has been shown that much of the variation in reported retentions in different laboratories can be eliminated if the results are calculated as retention index (RI) values instead of the conventional retention factors (*k*) (145).

Because retention indices are based on a common interpolation scale they are largely independent of the exact operating conditions and which can be reproduced in different laboratories. In contrast, capacity factors reflect changes both in selectivity and in the overall strength of the eluent.

Although widely applied in gas chromatography, the application of retention indices in high-performance liquid chromatography (HPLC) has been more limited. However, the indices of a wide range of different analytes have been reported using different scales of standards (146). As well as studies specifically aimed at the potential of retention indices for identification, mainly in toxicological drug analysis (147-148) and in the analysis of natural products (149), other studies have determined index values during studies of biological activity or structure-activity relationships. In addition, the retention indices of a wide range of analytes were reported during the retention prediction studies and during studies of the properties of stationary and mobile phases.

### 3.2.1: Retention Indices

The first retention index scale was introduced in 1958 by Kovats for use in gas-liquid chromatography (GLC) (150). Since then, alternative retention index scales have been proposed for GLC, HPLC, supercritical fluid chromatography (SFC) and recently for micellar capillary electrophoresis (CE). In most cases, the retention scale is defined as the carbon number of the standard x 100 (so that hexadecane, I = 1600) and the retention index values of analytes are determined by interpolation between the standards.

A number of homologous series of compounds have been studied as retention index standards for HPLC, each with advantages and disadvantages, principally related to their detectability or the compatibility of their retentions with the analytes of interest.

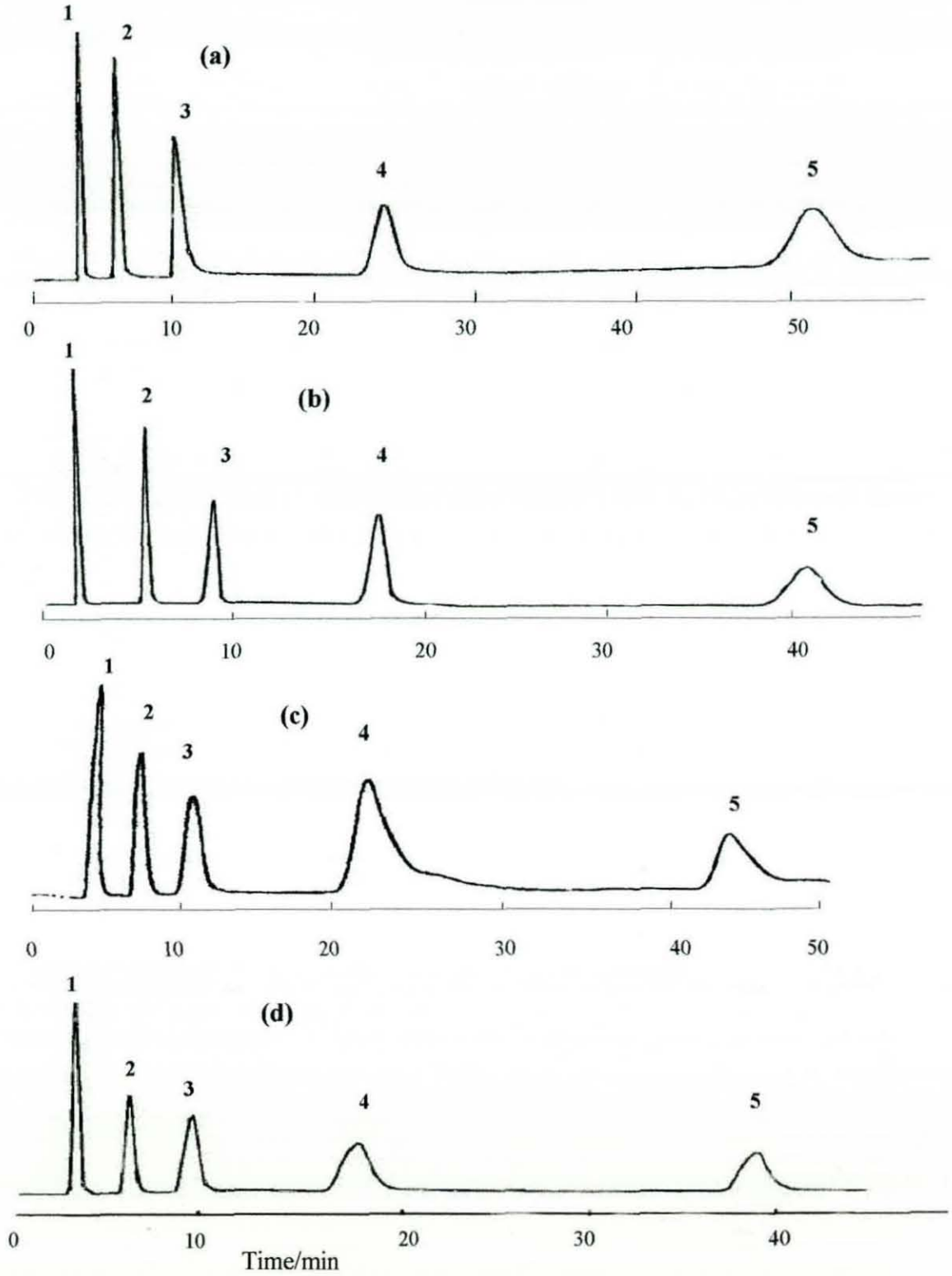
The alkyl aryl ketones (151) were identified as suitable retention index standards for a wide range of analytes, because of their ready detectability, easy availability and similar polarities, and hence retentions, to many aromatic analytes. Therefore, a homologous series of alkyl aryl ketones has used as standard in the present study.

Previous studies (151-154, 111) using the alkyl aryl ketones have examined isoelutropic eluents containing acetonitrile, tetrahydrofuran, or methanol for the study of different branded ODS silica and polystyrene columns. The aim of the study was to examine the effect on the retention times and retention indices of eight functional groups of aromatic compounds caused by changing eluents (including superheated water) for the analysis.

### 3.2.1.1: Studies on PBD-coated zirconia column

It is well known that temperature has a great effect on the retention, efficiency, and selectivity on reversed-phase liquid chromatography (137, 140). Most silica-based phases are thermally unstable and can only be used at temperatures marginally higher than ambient (155). Therefore, there has been a great deal of interest in alternative polymer-coated metal oxide supports for HPLC (128-129). Zirconia, due to its superb chemical and thermal stability, is one of the major alternatives to silica (126). Numerous studies have shown that zirconia-based materials can be used over a pH range from 0 to 14 (156) and that separations on PBD-$ZrO_2$ at 200 °C can be achieved in seconds (157). A few studies of the efficiency, selectivity, and thermodynamic properties of polybutadiene-coated zirconia (PBD-$ZrO_2$) at high temperature have appeared (130-131, 142, 157). Recently, phenol and four of its chloroderivatives (63), phenol, anilines, alkylbenzenes (65) and some drugs (67) have been successfully separated using polybutadiene-coated zirconia column with superheated water. Yang et al. (65) compared the separation of phenol and four chlorophenols using PBD-zirconia column with an acetonitrile-water mobile phase at 30 °C and 80 °C and a water-only mobile phase at 200 °C. Li and Carr (158-159) reported that PBD-zirconia column has a hydrophobic selectivity very comparable to that of an ODS phase. The sensitivities of both PBD and ODS phases towards a change in the mobile phase composition are similar, however, the PBD phase tended to be less sensitive to solute shape than was an ODS phase. Therefore, this column was chosen for the comparison of conventional LC and superheated water chromatography.

The first step was to separate the alkyl aryl ketones (acetophenone–hexanophenone) on a polybutadiene coated zirconia column using superheated water as mobile phase (140 °C) (**Figure 3.1a**).




**Figures 3.1 a-d: Chromatograms illustrating the separation of alkyl aryl ketones on PBD-Zirconia column 150 mm x 4.6 I. D. (a) Superheated water (140 °C), (b) 25% ACN (v/v), (c) 25% THF (v/v), (d) 40% MeOH (v/v). Peak identification (1) acetophenone, (2) propiophenone, (3) butyrophenone, (4) valerophenone, (5) hexanophenone.**

The retention factors of alkyl aryl ketones using superheated water were then matched with mobile phase with different ratios of methanol water (**Table 3.1**). The correlation graph (**Figure 3.2**) has shown that 40 % methanol (v/v) is correlated with superheated water (140 °C) on PBD-zirconia column. The retention factors of all the compounds decreased with increasing methanol proportion.

The next step was to match the retention factors of alkyl aryl ketones with other isoelutropic solvents (acetonitrile, or tetrahydrofuran). 25% acetonitrile/water (v/v), 25% THF/water (v/v), 40% methanol/water (v/v) and superheated water (140 °C). In each case there was a linear relationship between the logarithms of the retention factors of alkyl aryl ketones and their carbon number (log k = carbon number ketones x slope + constant) (**Figure 3.3**). In the previous studies (160), the first member (acetophenone) of alkyl aryl ketones homologous series showed some deviations from linearity on all the studied copolymers.

However, in this study it did not show any deviation. Therefore, the retention indices were calculated using the linear relationship for the retention factors of the alkyl aryl ketones from acetophenone to hexanophenone. In each case, the column void volume was determined using a solution of acetone. Other marker compounds have been proposed for the determination of column void volumes for reversed phase separations but all suffer from disadvantages (161).

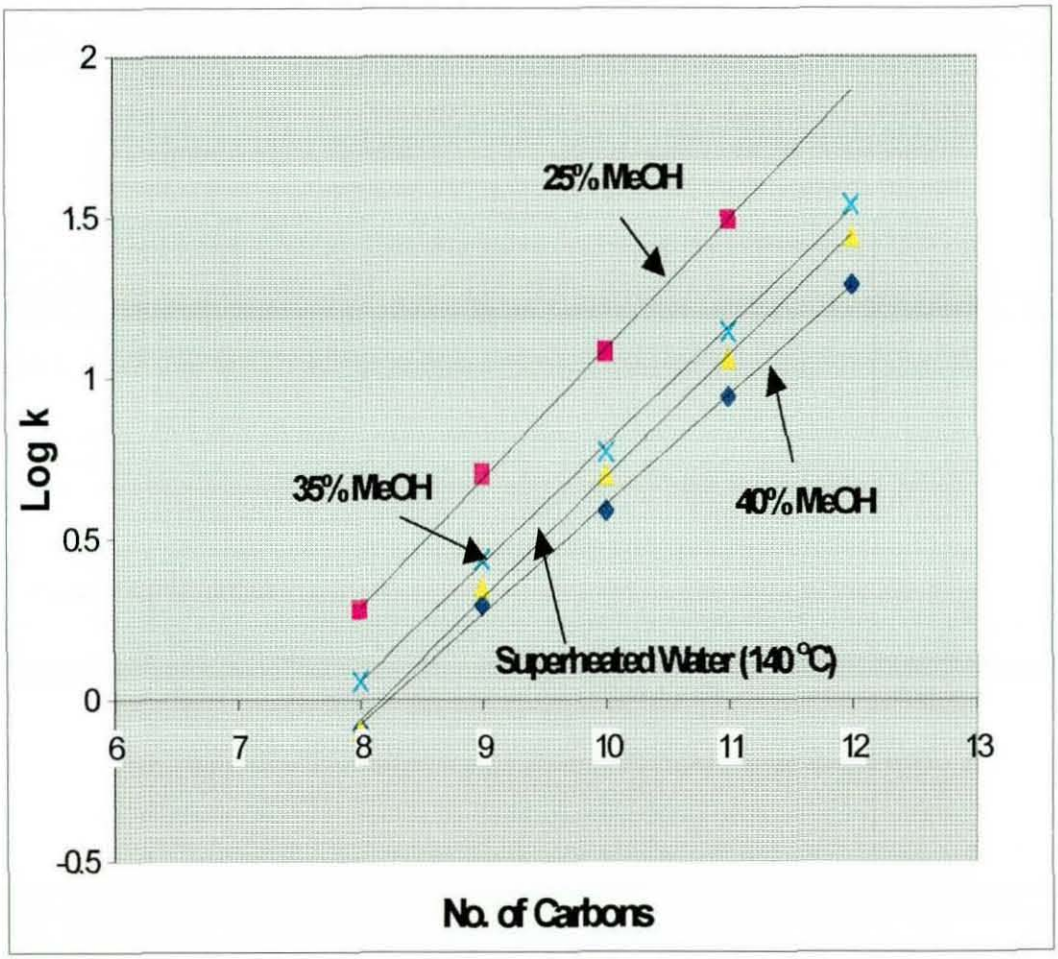



**Figure 3.2: Variation of log *k* with number of carbons for homologous series of alkyl aryl ketones in different ratios of methanol water on PBD-zirconia (150 x 4.6 mm I. D.) column.**

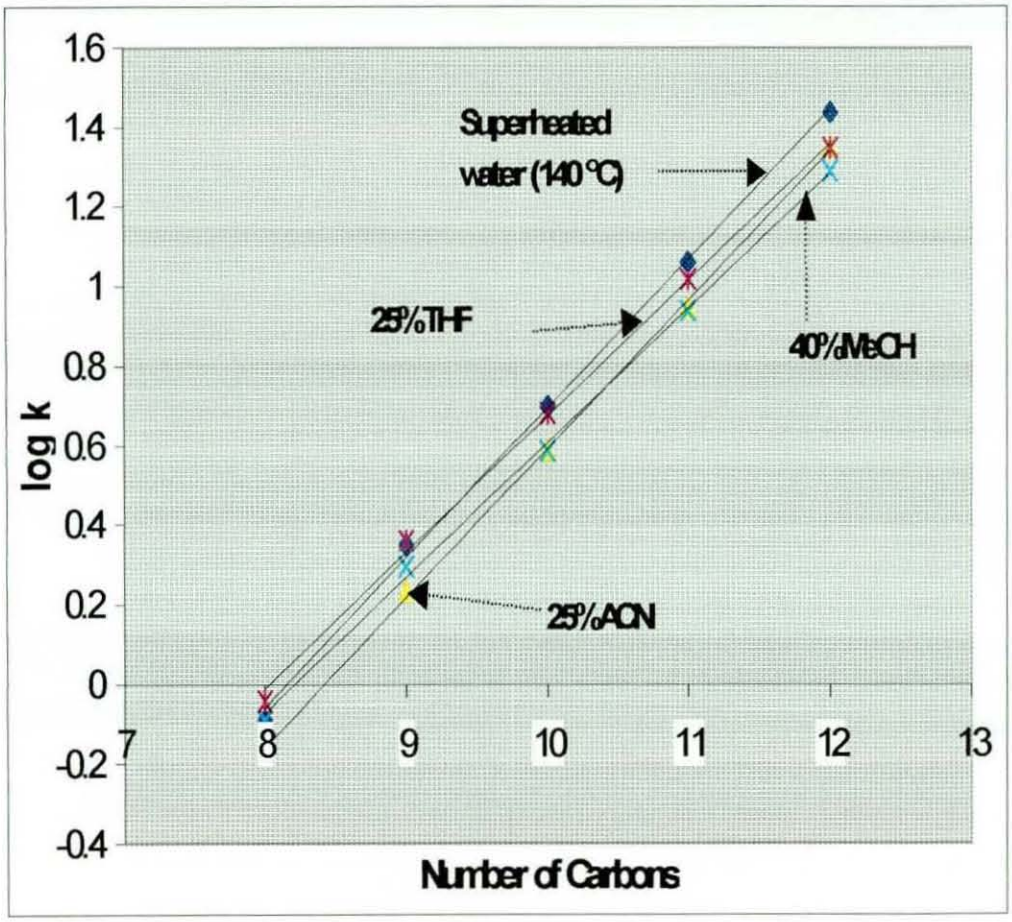



**Figure 3.3: Variation of log *k* with number of carbons for homologous series of alkyl aryl ketones in different isoelutropic eluents and superheated water (140 °C) on PBD-zirconia (150 x 4.6 mm I.D.) column.**

To compare the selectivity of PBD-zirconia column, a set of test compounds, (2-phenylethanol, benzaldehyde, p-cresol, nitrobenzene, n-methylaniline, methyl benzoate, anisole, and toluene) has been used to test the effect of electron-donation, electron-acceptance, dipole-dipole interaction and charge-transfer interaction in the different eluents.

**Table 3.1: Retention Times of homologous alkyl aryl ketones series and test compounds in different eluents on PBD-zirconia column**

| *Compound* | *Retention* | *Times* | | | | |
|---|---|---|---|---|---|---|
| | 25% MeOH (v/v) | 35% MeOH (v/v) | 40% MeOH (v/v) | 25% ACN (v/v) | 25% THF (v/v) | Super-heated Water (140°C) |
| **Alkyl aryl ketones** | | | | | | |
| Acetophenone | 4.90 | 3.82 | 3.21 | 3.18 | 3.46 | 3.35 |
| Propiophenone | 9.69 | 6.61 | 5.24 | 5.15 | 5.97 | 5.96 |
| Butyrophenone | 20.50 | 12.25 | 8.55 | 9.20 | 10.44 | 11.00 |
| Valerophenone | 51.613 | 26.56 | 17.08 | 18.60 | 20.65 | 23.07 |
| Hexanophenone | - | 63.37 | 36.42 | 44.64 | 42.14 | 52.85 |
| ***Test Compounds*** | | | | | | |
| 2-Phenylethanol | 3.63 | 3.00 | 2.53 | 2.62 | 2.63 | 2.60 |
| Benzaldehyde | 4.30 | 3.50 | 3.00 | 3.20 | 3.47 | 2.99 |
| p-Cresol | 5.86 | 4.38 | 3.49 | 3.66 | 4.56 | 3.22 |
| N-Methylaniline | 5.42 | 4.36 | 3.60 | 4.08 | 4.85 | 3.70 |
| Nitrobenzene | 7.94 | 5.90 | 4.93 | 5.19 | 6.73 | 4.05 |
| Methyl benzoate | 10.02 | 6.75 | 5.03 | 5.31 | 5.52 | 5.56 |
| Anisole | 10.35 | 7.34 | 6.11 | 6.49 | 7.50 | 5.79 |
| Toluene | 22.66 | 14.84 | 12.73 | 14.68 | 16.25 | 12.01 |
| **Void Marker** | | | | | | |
| Acetone | 1.78 | 1.78 | 1.76 | 1.89 | 1.81 | 1.84 |

In conventional LC, acetophenone, which has a nominal retention of 800, was found to have almost this value (792-801) on the PBD-zirchrom column and on the ODS silica-based column (151-154). In contrast, a much lower value (750-770) was previously found on a PS-DVB column (111). In this study, the retention index of each alkyl aryl ketone has shown similar values in different ratios of methanol or other eluents {(25% ACH (v/v), 25%THF (v/v) and 40% MeOH (v/v)} on the PBD- zirconia column (**Table 3.2**).

The retention indices of 2-phenylethanol decreased with increasing proportion of methanol and superheated water gave almost the same retention index as 40 % methanol (v/v). A noticeable difference is that the retention index of 2-phenylethanol was lower in 25% THF (v/v) than in superheated water (140 °C). It showed only a small variation between the four isoeluotropic eluents on this column (**Table 3.2**). In contrast, major differences in the retention indices were found between mobile phases on an ODS silica based column (151-154) and a very low retention index value (393) was found using 90% MeOH (v/v) on a polystyrene column (111).

p-Cresol showed the same retention indices in 25 % MeOH (v/v) and 35% MeOH (v/v) but 40% MeOH (v/v) gave lower value. The retention index of p-cresol using superheated water was close to the value with 40% MeOH (v/v).The PBD-zirconia column gave similar retention index values as different brands of ODS columns (151-154). But the polystyrene column gave lower retention index values (428) using 90% MeOH (v/v) (111) than the PDB-zirconia column using 40% (v/v) methanol, 25% (v/v) ACN, or 20% (v/v) THF.

However, PDB-zirconia and different brands of ODS silica-based columns showed almost the same retention behaviour with methanol. But the polar solvents such as methanol have little affinity with a hydrophobic polymer surface and therefore it cannot penetrate the pores of the polymer as effectively as a non-polar solvent (116). So, polystyrene divinyl-benzene columns showed lower retention index values using methanol/water as mobile phase.

**Table 3.2: Retention Indices of alkyl aryl ketones and test compounds on a PBD-zirconia Column**

| *Compound* | *Retention Indices* | | | | | |
|---|---|---|---|---|---|---|
| | 25% MeOH (v/v) | 35% MeOH (v/v) | 40% MeOH (v/v) | 25% ACN (v/v) | 25% THF (v/v) | Super-heated Water (140 °C) |
| **Alkyl aryl ketones** | | | | | | |
| Acetophenone | 800 | 801 | 796 | 798 | 792 | 793 |
| Propiophenone | 901 | 903 | 909 | 905 | 909 | 909 |
| Butyrophenone | 998 | 995 | 995 | 999 | 1002 | 1001 |
| Valerophenone | 1101 | 1097 | 1098 | 1095 | 1100 | 1098 |
| Hexanophenone | - | 1203 | 1202 | 1203 | 1197 | 1199 |
| **Test Compounds** | | | | | | |
| 2-Phenylethanol | 743 | 740 | 716 | 733 | 703 | 713 |
| Benzaldehyde | 777 | 781 | 778 | 799 | 792 | 763 |
| p-Cresol | 829 | 830 | 796 | 835 | 857 | 783 |
| N-Methylaniline | 817 | 829 | 828 | 859 | 870 | 827 |
| Nitrobenzene | 874 | 884 | 897 | 907 | 931 | 844 |
| Methyl benzoate | 906 | 907 | 901 | 911 | 895 | 897 |
| Anisole | 910 | 920 | 937 | 945 | 950 | 904 |
| Toluene | 1007 | 1021 | 1055 | 1063 | 1067 | 1013 |

The weakly basic compound N-methylaniline was found to have almost the same retention index in 35% (v/v) MeOH, 40% (v/v) MeOH and superheated water (140 °C). The behaviour of this compound in methanol/water, acetonitrile/water and tetrahydrofuran/water on a PBD-zirconia column is similar to the different brands of ODS silica-based columns (151-154).

Benzaldehyde and methyl benzoate were shown to have almost the same retention indices in 40% MeOH (v/v), 25% ACN (v/v), 25% THF (v/v) and superheated water (140 °C). Because the retention index value of methyl benzoate was found to be very consistent irrespective of the composition of the eluent, this compound appeared to reflect the same selectivity effects as the alkyl aryl ketones (144, 146). This consistency has also enabled this compound to be used previously as a secondary standard in estimating retention indices from published data of separations in methanol/water (162).

The non-polar compounds, nitrobenzene, anisole and toluene showed similar behaviour in the different eluents. Their retention indices on PBD-zirconia increased with increasing methanol ratio. These changes are similar to separations on PS-DVB column (111). Superheated water (140 °C) gave lower value than the other three eluents.

In an overall examination of **Table (3.2)**, we can seen that polar (hydroxyl group containing) test compounds have similar retention indices in 40% methanol and superheated water (140 °C) and non-polar test compounds (nitrobenzene, anisole and toluene) gave similar retention indices in 25% methanol and superheated water (140 °C).

The efficiency of each peak on PBD-zirconia was calculated based on the width at half height (**Table 3.3**). The values were found to be markedly

**Table 3.3: Efficiency of alkyl aryl ketones and test compounds on a PBD-zirconia column in different eluents**

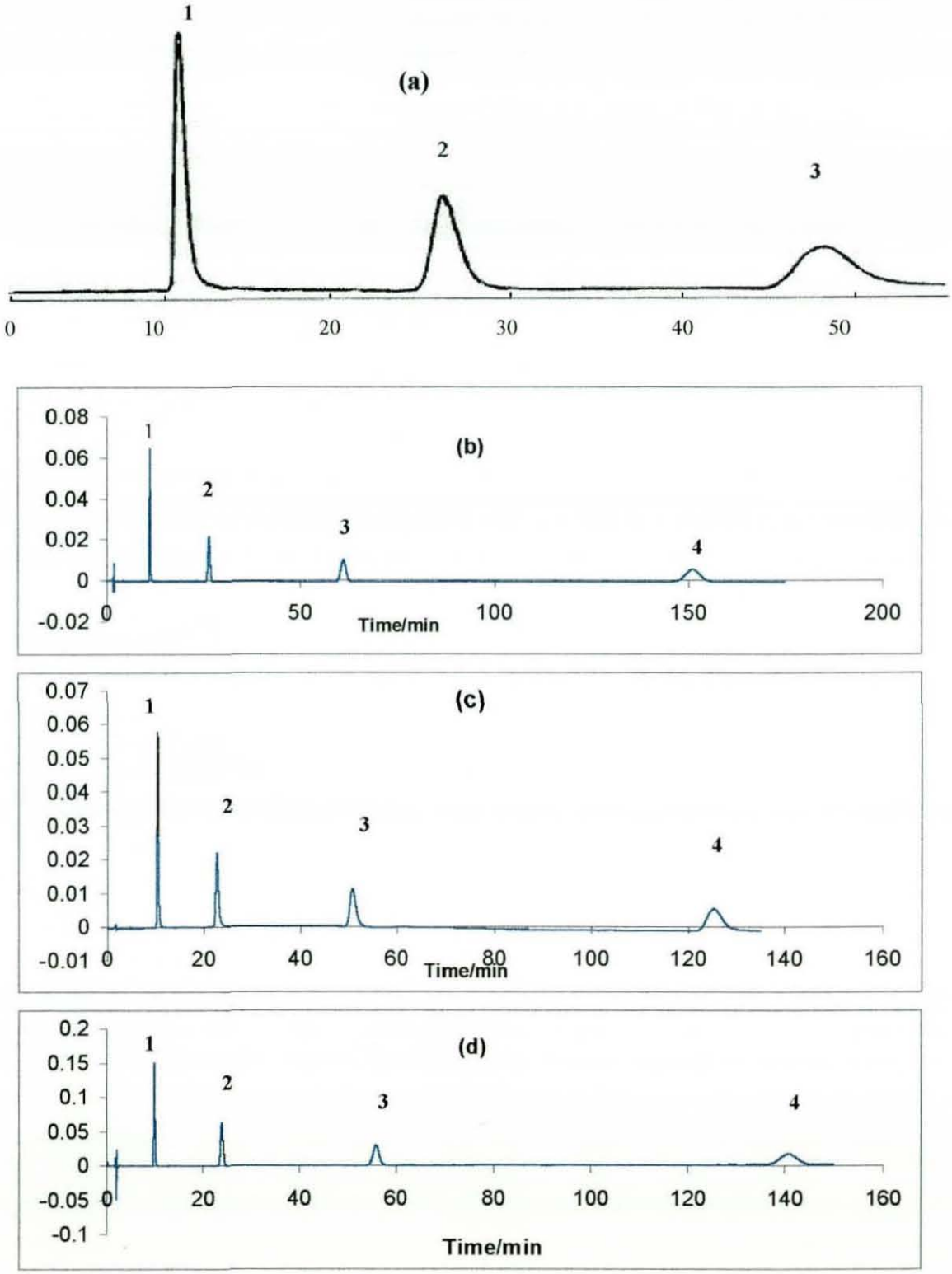
| *Compound* | *Efficiency* | | | | | |
|---|---|---|---|---|---|---|
| | 25% MeOH (v/v) | 35% MeOH (v/v) | 40% MeOH (v/v) | 25% ACN (v/v) | 25% THF (v/v) | Super-heated Water (140 °C) |
| **Alkyl aryl ketones** | | | | | | |
| Acetophenone | 2707 | 2389 | 2468 | 6744 | 2462 | 8426 |
| Propiophenone | 3098 | 2878 | 2811 | 9110 | 3829 | 11448 |
| Butyrophenone | 3428 | 3171 | 2974 | 10637 | 4732 | 10725 |
| Valerophenone | 3639 | 4286 | 3066 | 12916 | 3016 | 10576 |
| Hexanophenone | - | 3414 | 4236 | 13599 | 4585 | 11497 |
| **Test Compounds** | | | | | | |
| 2-Phenylethanol | 2107 | 1688 | 844 | 2034 | 68 | 3096 |
| Benzaldehyde | 2190 | 1963 | 1592 | 5944 | 1194 | 5726 |
| p-Cresol | 700 | 916 | 637 | 1045 | 1032 | 2695 |
| N-Methylaniline | 2425 | 2338 | 1772 | 7308 | 1330 | 7329 |
| Nitrobenzene | 3023 | 2855 | 1579 | 7605 | 1493 | 7648 |
| Methyl benzoate | 3138 | 3061 | 1220 | 7131 | 1803 | 10254 |
| Anisole | 3370 | 3503 | 1503 | 8468 | 1891 | 10300 |
| Toluene | 3800 | 4312 | 5044 | 11956 | 3819 | 10745 |

dependent on the organic component in the eluent or temperature of superheated water. In 25% THF–water (v/v) all compounds gave poor efficiencies, this behaviour is completely different from the polystyrene column (111). The separations in superheated water (140 °C) showed much better efficiencies and peak shapes than the other three eluents on PBD-zirconia column. Like the polystyrene column (111), PBD-zirconia column showed very poor efficiency for p-cresol and 2-phenylethanol.

### 3.2.1.2: Studies on an Xterra ™ RP 18 Silica Column

Silica based columns still remain the material of choice for chromatographers because they offer superior performance in terms of efficiency and their usage is well understood. Therefore, it would be advantageous to find a silica-based column that was more stable to hydrolytic attack at high temperatures and high/low pH. A number of column manufacturers are beginning to address this issue. During the course of this study, Waters introduced Xterra ™ RP 18 columns to the HPLC market. The chemistry of the Xterra column was described in **Chapter 1** (**Section 1.5.1.2**). It has been successfully used in superheated water chromatography for the separation of some drugs (66). Therefore, we selected this column for this study.

Three alkyl aryl ketones (acetophenone-butyrophenone) were separated on Xterra ™ RP18 column using superheated water (140 °C) as the mobile phase (**Figure 3.4a**). The retention time of butyrophenone was 51.88 min. We therefore did not include valerophenone and hexanophenone from the alkyl aryl ketones mixture as their retention would be excessive. The mixture of alkyl aryl ketones (acetophenone- valerophenone) was separated in aqueous methanol, acetonitrile and tetrahydrofuran as mobile phases and the composition was adjusted to match the retention factors to superheated water (140 °C) (**Figures 3.4b-d**). It was found that 25 % acetonitrile (v/v), 40 % methanol (v/v) and 20% THF (v/v) and superheated water (140 °C) gave




**Figures 3.4: a-d: Chromatograms illustrating the separation of alkyl aryl ketones on an Xterra ™ RP 18 column. (a) Superheated water (140 °C), (b) 25% ACN (v/v), (c) 40% MeOH (v/v), (d) 20% THF (v/v). Peak identification (1) acetophenone, (2) propiophenone, (3) butyrophenone, (4) valerophenone.**

comparable results (**Figure 3.5**). To test the overall retentivity of the Xterra ™ RP 18 packing material, eight aromatic compounds were eluted in superheated water (140 °C), 25 % acetonitrile (v/v), 40% methanol (v/v), and 20% THF (v/v) as mobile phases (**Table 3.4**). In each eluent, the column void volume was determined using a solution of aqueous sodium nitrate.

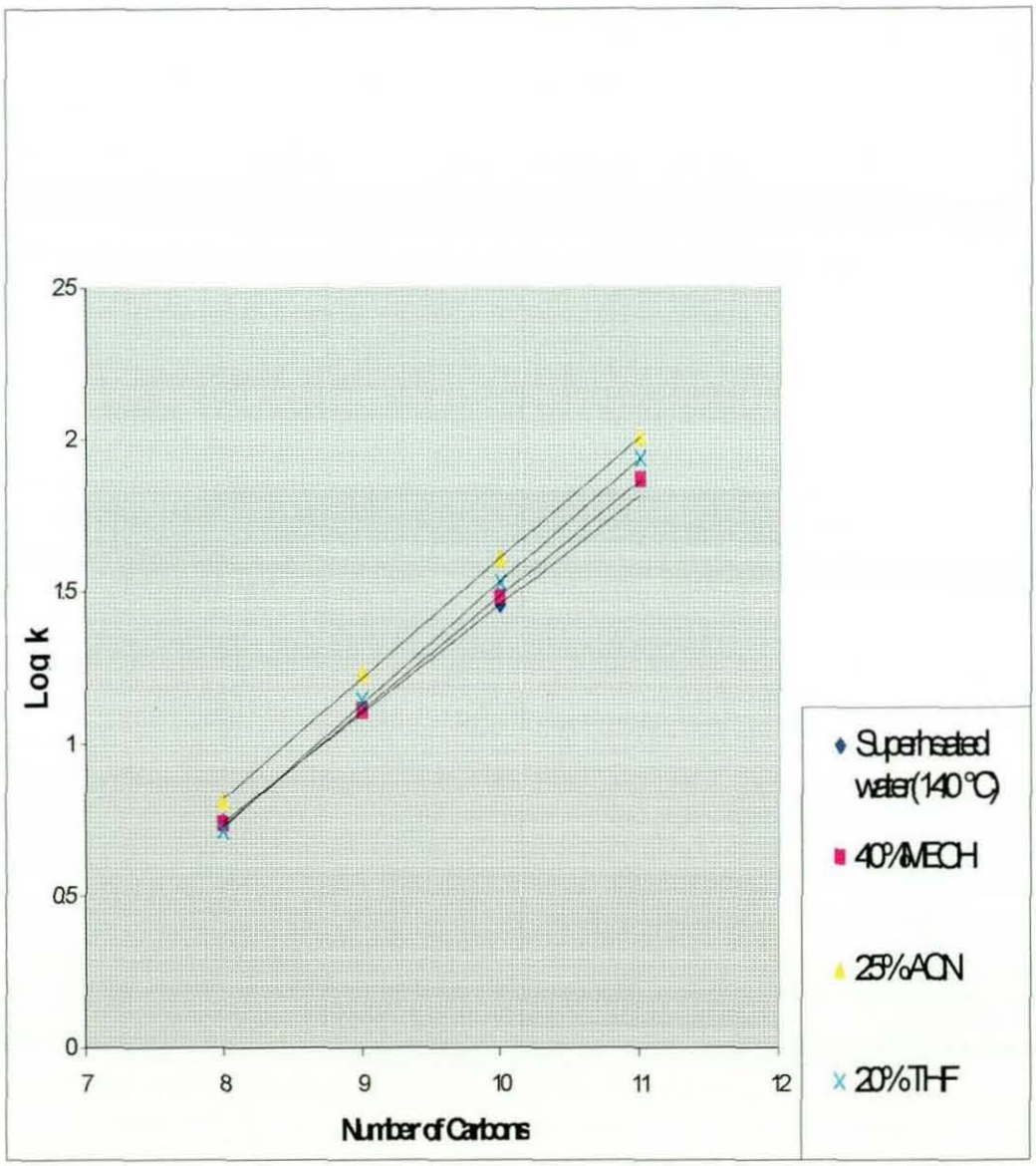



**Figure 3.5: Variation of log *k* with the number of carbons for *homologous series of alkyl aryl ketones in different isoelutropic eluents* and superheated water (140 °C) on an Xterra ™ RP 18 column.**

**Table 3.4: Retention factors of homologous alkyl aryl ketones and test compounds in different eluents on an Xterra ™ RP 18 column**

| *Compound* | *Retention Factors* | | | |
|---|---|---|---|---|
| | 40% MeOH (v/v) | 25% ACN (v/v) | 20% THF (v/v) | Superheated water (140 °C) |
| **Alkyl aryl ketones** | | | | |
| Acetophenone | 5.45 | 6.49 | 5.20 | 5.51 |
| Propiophenone | 12.99 | 16.83 | 13.96 | 12.88 |
| Butyrophenone | 30.01 | 40.33 | 33.87 | 28.65 |
| Valerophenone | 74.49 | 101.37 | 87.17 | - |
| **Test compounds** | | | | |
| 2-Phenylethanol | 1.65 | 3.21 | 1.39 | 1.69 |
| p-Cresol | 5.32 | 5.75 | 13.53 | 2.66 |
| Benzaldehyde | 4.10 | 5.34 | 4.92 | 3.09 |
| N-Methylaniline | 4.91 | 7.67 | 1.71 | 3.69 |
| Nitrobenzene | 7.40 | 11.52 | 16.09 | 4.09 |
| Anisole | 12.12 | 15.44 | 19.36 | 6.51 |
| Methyl benzoate | 14.15 | 14.49 | 14.53 | 9.22 |
| Toluene | 27.94 | 35.67 | 45.22 | 14.81 |
| **Void Marker** | | | | |
| Aq.Sodium nitrate | 1.60 | 1.55 | 1.60 | 1.75 |

A noticeable difference in the eluents was that methyl benzoate was eluted later than anisole in 40% MeOH (v/v) and superheated water (140 °C) (**Table 3.4**). In 25% ACN (v/v) it was eluted after nitrobenzene and in 20% THF (v/v) it was eluted faster than nitrobenzene.

Another remarkable difference was that N-methylaniline was eluted faster than benzaldehyde with a split peak in 20% THF (v/v). It can partially ionise in unbuffered 20 % THF (by dipole-dipole interaction) and often gave poor peak shapes and retention irreproducibility on reversed-phase chromatography.

The retention indices of the homologues and test compounds (2-phenylethanol, benzaldehyde, p-cresol, N-methylaniline, nitrobenzene, anisole, methyl benzoate and toluene) were calculated based on the linear relationship for the alkyl aryl ketones, scale (**Figure 3.5**) (**Table 3.5**).

The retention indices of 2-phenylethanol in 40% MeOH (v/v), 20% THF (v/v) and superheated water (140 °C) were similar on the Xterra ™ RP 18 column. The behaviour of these compounds was different from other ODS silica based columns studied in conventional LC with isoelutropic eluents {(70% MeOH (v/v), 50% ACN (v/v) and 40% THF (v/v)} (151-154). Because Smith reported that different brands of ODS silica gave lower retention indices in 50% ACN (v/v) than 70% MeOH (v/v) and also than 40% THF (v/v), but in the present study this compound showed much higher retention index in 25% ACN (v/v) on Xterra ™ RP 18 column than the other three eluents.

In the 20 % THF (v/v) eluent p-cresol showed a higher retention index (900) than the other three eluents. It was noticeable that p-cresol always showed higher retention indices in THF/water than in the other three eluents. The reason for this is yet unclear, but may be because of dipole-dipole interactions for THF/water, because methanol, superheated water and acetonitrile interact in different ways by proton-accepting, and proton-donating interactions. Smith (151-154) had reported similar behaviour on comparison

**Table 3.5: Retention indices of homologous alkyl aryl ketones and test compounds in different eluents on an Xterra ™ RP 18 column**

| *Compound* | *Retention* | *Indices* | | |
|---|---|---|---|---|
| | 40% MeOH (v/v) | 25% ACN (v/v) | 20% THF (v/v) | Superheated water (140 °C) |
| **Alkyl Aryl Ketones** | | | | |
| Acetophenone | 801 | 798 | 798 | 799 |
| Propiophenone | 900 | 903 | 902 | 902 |
| Butyrophenone | 998 | 999 | 999 | 999 |
| Valerophenone | 1101 | 1100 | 1100 | - |
| **Test Compounds** | | | | |
| 2-Phenylethanol | 664 | 721 | 657 | 655 |
| p-Cresol | 798 | 785 | 900 | 711 |
| Benzaldehyde | 768 | 777 | 792 | 729 |
| N-Methylaniline | 789 | 817 | 679 | 750 |
| Nitrobenzene | 836 | 861 | 919 | 763 |
| Anisole | 893 | 893 | 939 | 819 |
| Methyl benzoate | 910 | 886 | 908 | 861 |
| Toluene | 990 | 984 | 1029 | 919 |

studied on different bands of ODS-bonded silica in methanol/water, acetonitrile/water and tetrahydrofuran/water as the mobile phase.

The basic compound N-methylaniline was reported to give tailing peaks without buffer (163) on silica based columns because the interaction with acidic silanolic hydroxyl groups. But Xterra ™ RP 18 particles replace one third of the surface silanol groups with methyl groups (**Section 1.5.1.2**). The net result is that Xterra ™ RP 18 bonded phases has the highest, most homogenous coverage of $C_{18}$ reverse-phase material. With the 33% reduction in silanol concentration, it gave a significant improvement in the peak shape of N-methylaniline in superheated water (140 °C), 25% ACN (v/v), and 40% MeOH (v/v) (**Figure 3.6**). But the retention index of N-methyl aniline in 20%THF (v/v) was markedly lower than with other three eluents, this reason was described on page 67.

*Benzaldehyde and methyl benzoate showed similar behaviour* to that on other conventional ODS columns and also to the polystyrene column. The retention indices of these compounds gave similar retention index values in three organic mobile phases and slightly lower values in superheated water (140 °C) (**Table 3.5**).

The non-polar compounds nitrobenzene, anisole and toluene showed a similar behaviour in the different modifiers. In 20%THF (v/v) eluent they gave much higher retention indices (>100 increase) than in superheated water (140 °C). Nitrobenzene, anisole and toluene behaved as on other silica based columns and also on a polystyrene column (111).

The polar compounds 2-phenylethanol and p-cresol were eluted very well with good efficiencies on the Xterra ™ RP 18 column (**Table 3.6**). In contrast, *these compounds had shown* very poor efficiencies on the PBD-zirconia and PS-DVB columns (111) indicating marked differences in the properties of the stationary phases. The relative high efficiency may be because the surface concentration of the hydrocarbon moiety is relatively high on Xterra ™ RP 18,

as indicated by the 15.5% carbon content of the dry stationary phase. In octadecylsilyl silicas with a high carbon content, the pore surface of the sorbent is likely to be partially coated by an organosiloxane polymer film. The advantage of this molecular configuration is that there are silanol groups, at the surface, which facilitate wetting by the neat aqueous eluent. Thus, interfacial mass transfer resistance is minimized and therefore efficiency was increased. In contrast, hydroxyl-group-containing compounds have little affinity with aromatic ring of PS-PDB column. Therefore, the resulting efficiency is poorer on PS-DVB packing materials than Xterra ™ RP 18 column.

An unusual feature of the poor efficiency of the peaks of alkyl aryl ketones on superheated water (140 °C) on Xterra ™ RP 18 column is that the efficiency markedly decreased with increasing retention factor. This effect is not so marked for 20% THF-water (v/v). The reasons for this unusual behaviour with this column is not clear but could be related to poor mass transfer in the stationary phase. This suggests that at sufficiently high temperatures dissolution of the silica itself may occur, catalysed in basic conditions (69). After over 60 hours at 140 °C something drastic occurred within the column itself (the peak was splitting, which means that voids were forming between the column material and the column frit). On opening the column, it was found that at least two thirds of the packing material had disappeared, seemingly dissolved by the basic hydroxyl group of the superheated water (69). The mechanism of dissolution of silica was described in **Section 1.5.1.1**.

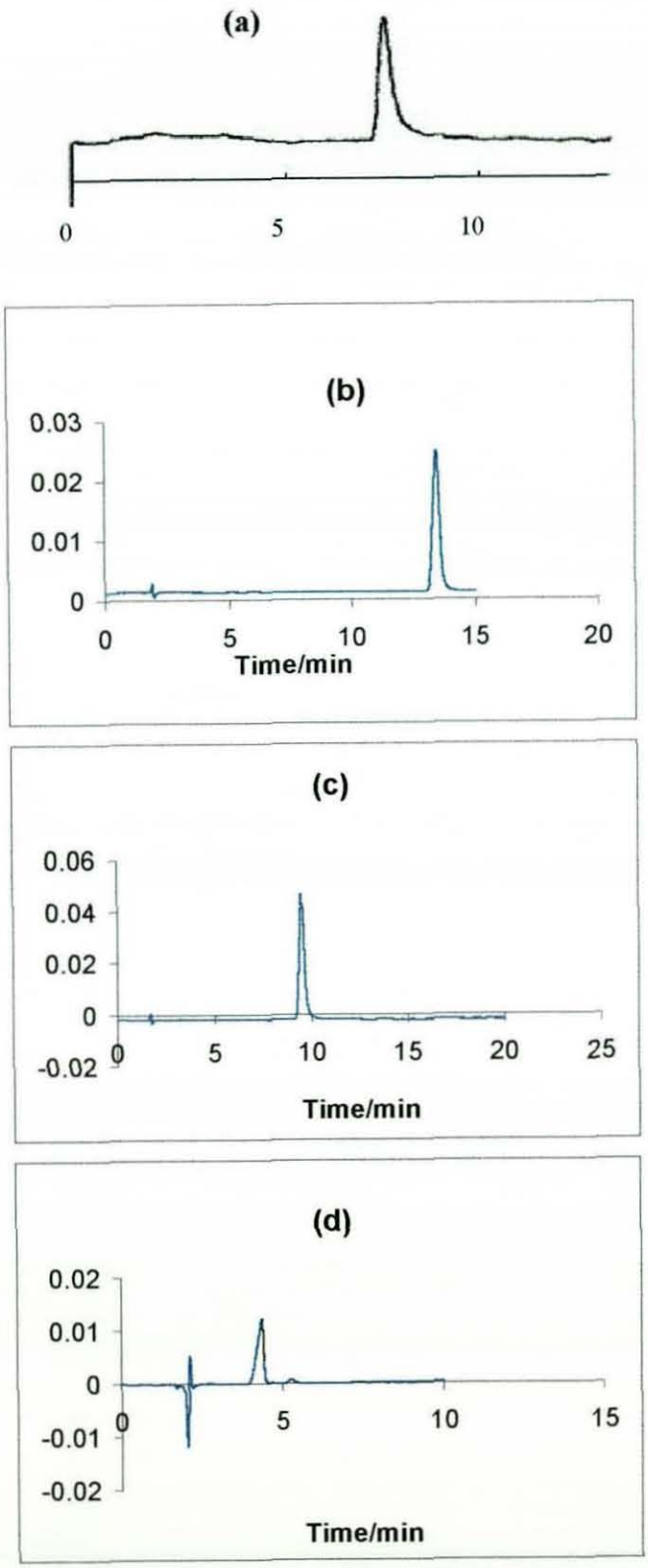



**Figure 3.6: Chromatograms of N-methylaniline on an Xterra ™ RP 18 column. (a) Superheated water (140 °C), (b) 25% ACN (v/v), (c) 40% MeOH (v/v), (d) 20% THF (v/v).**

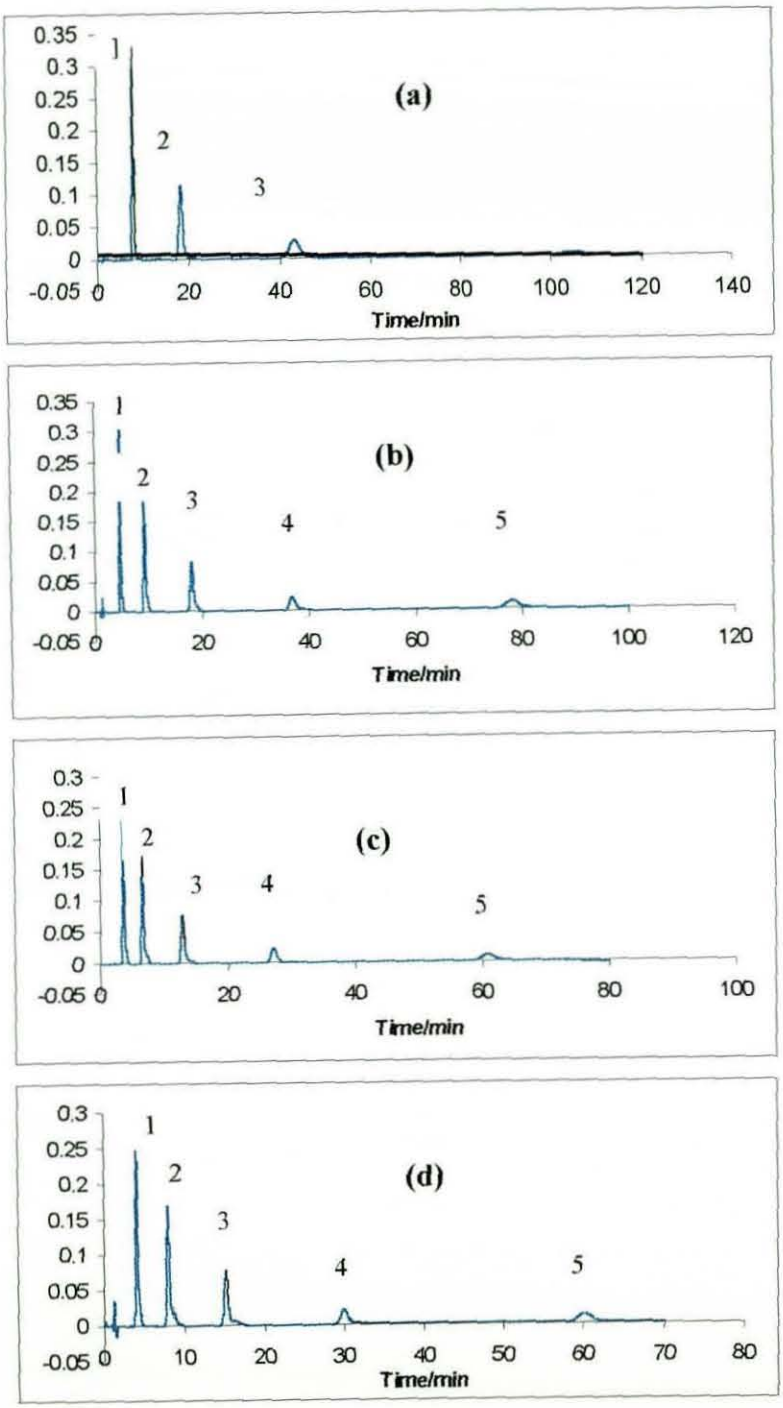
**Table 3.6: Efficiency of homologous alkyl aryl ketones and test compounds in different eluents on an Xterra ™ RP 18 column**

| *Compound* | *Efficiency* | | | |
|---|---|---|---|---|
| | 40% MeOH (v/v) | 25% ACN (v/v) | 20% THF (v/v) | Superheated water (140 °C) |
| **Alkyl Aryl Ketones** | | | | |
| Acetophenone | 6783 | 10935 | 9217 | 3400 |
| Propiophenone | 7705 | 10649 | 9690 | 2030 |
| Butyrophenone | 8023 | 9212 | 9875 | 1732 |
| Valerophenone | 7663 | 6951 | 9920 | - |
| **Test Compounds** | | | | |
| 2-Phenylethanol | 6744 | 9662 | 4598 | 3164 |
| p-Cresol | 7543 | 10312 | 8145 | 4068 |
| Benzaldehyde | 5430 | 10839 | 8189 | 2428 |
| N-Methylaniline | 6263 | 11102 | 2204 | 2211 |
| Nitrobenzene | 5062 | 10508 | 9336 | 2527 |
| Anisole | 5055 | 11208 | 9974 | 1424 |
| Methyl benzoate | 6022 | 10796 | 9328 | 2327 |
| Toluene | 3586 | 12880 | 9414 | 1040 |

### 3.2.1.3: Studies on a Luna ™ C $_{18}$ (2) column

A new type of high density bonded stationary phase, the Luna ™ $C_{18}$ (2) column recently appeared on the market from Phenomenex. The manufacturer claimed that the $C_{18}$ Luna's base silica is manufactured to extremely tight tolerances. Not only it is 99.999% ultra-pure, but also it has remarkable surface smoothness and sphericity of the particle. A particle free from imperfections provides more a consistent physical characteristic, thereby improving stability, bonding and column efficiencies. Luna columns employ a proprietary bonding technique that is claimed to provide extremely high surface coverage not only through high-bonding densities, but also controlled bonding uniformity. This high degree of uniformity creates a more inert "hydrophobic shield" around the underlying silica. The result is a more pH stable, and hydrolytically very stable, reproducible bonding with virtually no silanol activity. Phenomenex guaranteed Luna ™ $C_{18}$ (2) columns from pH 1.5 to 10.0 for 1000 hours stability. Because of these claims it was decided to test this column with the superheated water system and also conventional LC.

The homologous alkyl aryl ketones (acetophenone -hexanophenone) were separated in 50% MeOH (v/v)), 35% ACN (v/v), 30%THF (v/v) and superheated water (150 °C) on the Luna ™ $C_{18}$ (2) silica based column. They were well separated (**Figure 3.7**). Eight functional groups of aromatic compounds were eluted in each eluent. For each eluent composition the column void volume was determined using a solution of aqueous sodium nitrate. The retention factors of these compounds were determined (**Table 3.7**). The elution order of eight functional groups of aromatic compound was similar to the Xterra ™ RP 18 column in each eluent. Methyl benzoate and p-cresol gave almost the same retention time in 30% THF (v/v) on a Luna column.




**Figures 3.7: a-d: Chromatograms illustrating the separation of alkyl aryl ketones on a Luna ™ C 18 (2) column. (a) Superheated water (150 °C), (b) 35% ACN (v/v), (c) 50% MeOH (v/v), (d) 30% THF (v/v). Peak identification (1) acetophenone, (2) propiophenone, (3) butyrophenone, (4) valerophenone, (5) hexanophenone.**

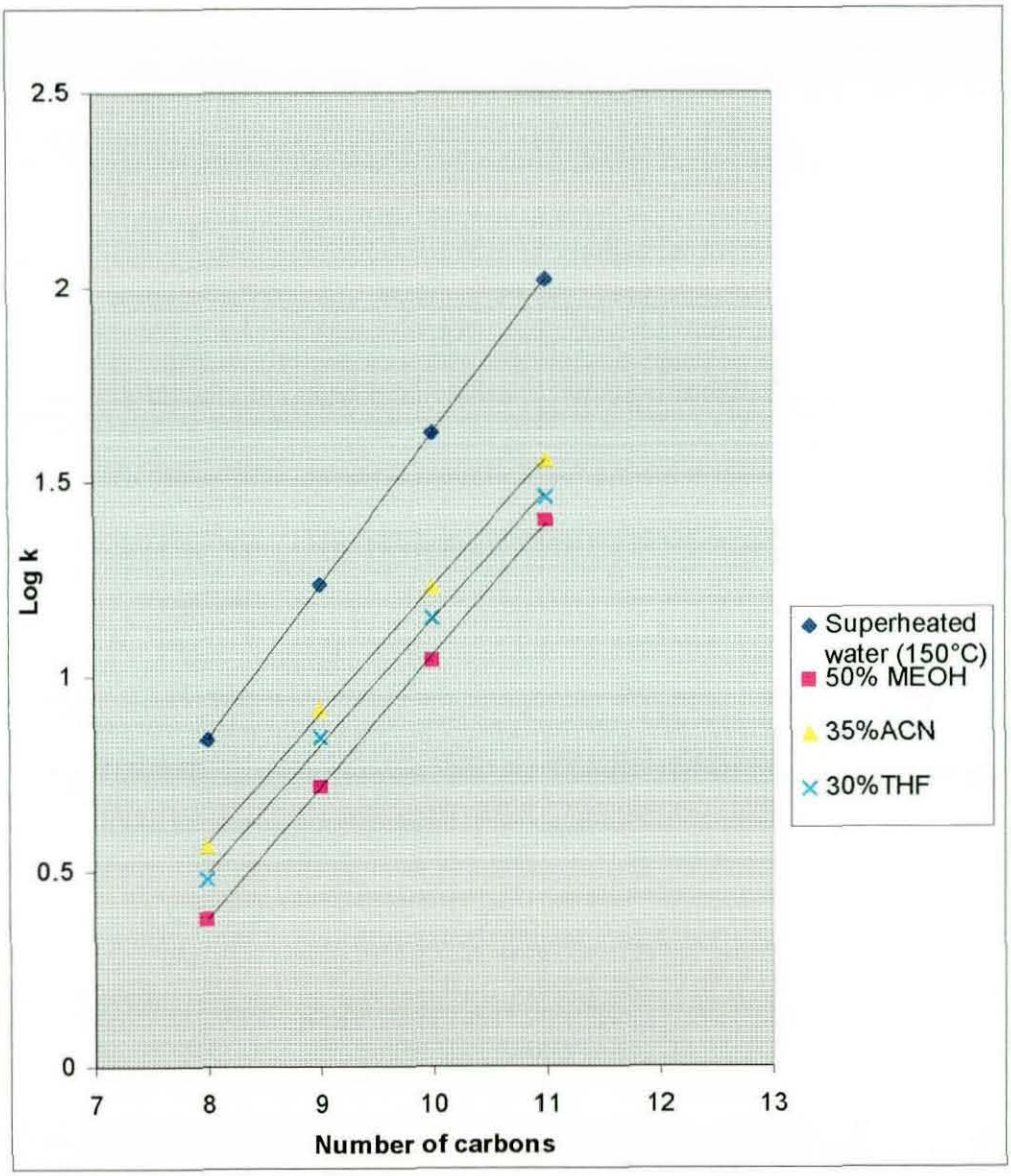



**Figure 3.8: Variation of log *k* with number of carbons for homologous series of alkyl aryl ketones in different isoelutropic eluents and superheated water (150 °C) on a Luna ™ C $_{18}$ (2) column.**

**Table 3.7: Retention Factors of homologous alkyl aryl ketones and test compounds in different eluents on a Luna silica ™ $C_{18}$ (2) column**

| *Compound* | *Retention* | *Factors* | | |
|---|---|---|---|---|
| | 50% MeOH | 35% ACN | 30% THF | Superheated water (150 °C) |
| **Alkyl aryl ketones** | | | | |
| Acetophenone | 2.40 | 3.69 | 3.05 | 6.89 |
| Propiophenone | 5.23 | 8.24 | 6.99 | 17.21 |
| Butyrophenone | 11.02 | 16.90 | 14.10 | 42.24 |
| Valerophenone | 24.86 | 35.80 | 28.89 | 104.23 |
| Hexanophenone | 58.11 | 77.27 | 59.05 | - |
| **Test compounds** | | | | |
| 2-Phenylethanol | 0.85 | 1.83 | 2.25 | 2.51 |
| p-Cresol | 2.46 | 3.36 | *6.51 | 3.78 |
| Benzaldehyde | 1.74 | 3.29 | 3.11 | 4.19 |
| N-Methylaniline | 2.26 | 4.89 | *2.81 | 5.12 |
| Nitrobenzene | 2.95 | 6.76 | 8.03 | 5.4 |
| Anisole | 4.59 | 8.30 | 9.11 | 9.32 |
| Methyl benzoate | 4.97 | 7.30 | *6.46 | 13.46 |
| Toluene | 10.94 | 17.63 | 21.11 | 21.26 |
| **Void marker** | | | | |
| Aq. Sodium nitrate | 1.11 | 1.00 | 1.00 | 1.00 |

* = The retention order had changed

**Table 3.8: Retention indices of homologous alkyl aryl ketones and test compounds in different eluents on a Luna silica ™ $C_{18}$ (2) column**

| *Compound* | *Retention* | *Indices* | | |
|---|---|---|---|---|
| | 50% MeOH (v/v) | 35% ACN (v/v) | 30% THF (v/v) | Superheated water (150 °C) |
| **Alkyl Aryl Ketones** | | | | |
| Acetophenone | 803 | 798 | 794 | 800 |
| Propiophenone | 901 | 904 | 907 | 901 |
| Butyrophenone | 994 | 999 | 1002 | 1000 |
| Valerophenone | 1098 | 1099 | 1100 | 1100 |
| Hexanophenone | 1204 | 1200 | 1197 | - |
| **Test Compounds** | | | | |
| 2-Phenylethanol | 672 | 705 | 652 | 688 |
| p-Cresol | 806 | 785 | 897 | 733 |
| Benzaldehyde | 762 | 783 | 796 | 745 |
| N-Methylaniline | 795 | 835 | 783 | 767 |
| Nitrobenzene | 829 | 878 | 926 | 773 |
| Anisole | 884 | 905 | 943 | 833 |
| Methyl benzoate | 894 | 888 | 896 | 874 |
| Toluene | 994 | 1005 | 1058 | 924 |

The retention indices were calculated in each eluent based on the linear relationship for the alkyl aryl ketones scales (**Figure 3.8**). The retention indices of alkyl aryl ketones and test compounds were then calculated (**Table 3.8**).

The polar compound, 2-phenylethanol had lower retention indices in 30% THF (v/v) than superheated water (150 °C) as on the PBD-zirconia column. In 50% MeOH (v/v), 2-phenylethanol has shown similar value on superheated water (150 °C). This behaviour is the same with the Xterra ™ RP 18 column and also other bonded ODS silica-based columns (151-154) but contrasts with a polystyrene column (111).

Benzaldehyde has shown very similar retention indices values in 50% MeOH (v/v) and superheated water (150 °C), but differs in 35% ACN (v/v) and 30% THF (v/v).

The basic compound N-methylaniline had similar retention indices in 30% THF (v/v) and superheated water (150 °C). Without a buffer solution this compound gave a tailing peak in ACN/water and THF/water because of silanophilic interactions.

Methyl benzoate showed almost the same retention indices in all eluents as on other ODS columns (151-154). The non-polar compounds nitrobenzene, anisole and toluene have shown similar effects to the Xterra ™ RP 18 column in different eluents and also on other ODS columns and the polystyrene column (111).

When the retention indices of the column test compounds were determined previously on different ODS columns, using THF-water, methanol-water and acetonitrile-water as the eluents, only small differences were observed between different makes of column packing material (151-154). In the present study, Xterra ™ RP 18 and Luna TM $C_{18}$ (2) packing material have shown a similar behaviour in THF-water, acetonitrile-water, methanol-water and

superheated water as the mobile phase. The retention order was slightly different in the four eluents on both columns. The same eluent gave the same retention order of eight test compounds on these columns.

The efficiency of each peak was calculated based on the width at half height (**Table 3.9**). The values were found to be markedly dependent on the organic component in the eluent and superheated water. It can be seen in methanol-water, all compounds gave poor efficiencies. In contrast, the separations in acetonitrile-water, and THF-water gave better efficiencies and peak shapes on a Luna $^{TM}$ $C_{18}$ (2) column. Superheated water showed the same behaviour for efficiency on a Luna $^{TM}$ $C_{18}$ (2) column as on an Xterra $^{TM}$ RP 18 column.

The stability of the Luna TM $C_{18}$ (2) column in aqueous solvent is different from an Xterra $^{TM}$ RP 18 column because after standing overnight with cold water in it, the retention times of alkyl aryl ketones had changed dramatically **(see Figure 3.9)**. They were suddenly much shorter. A similar effect has been observed in a previous study in our laboratory (49) on Spherisorb ODS1 materials. It is believed that this sudden loss in retention was caused by a collapse of the hydrophobic $C_{18}$ ligand, when left in cold water in the totally aqueous conditions as shown in (**Figure 3.10**). Reports of similar collapsing phenomenon have been reported in the literature with highly aqueous mobile phases (164-165).

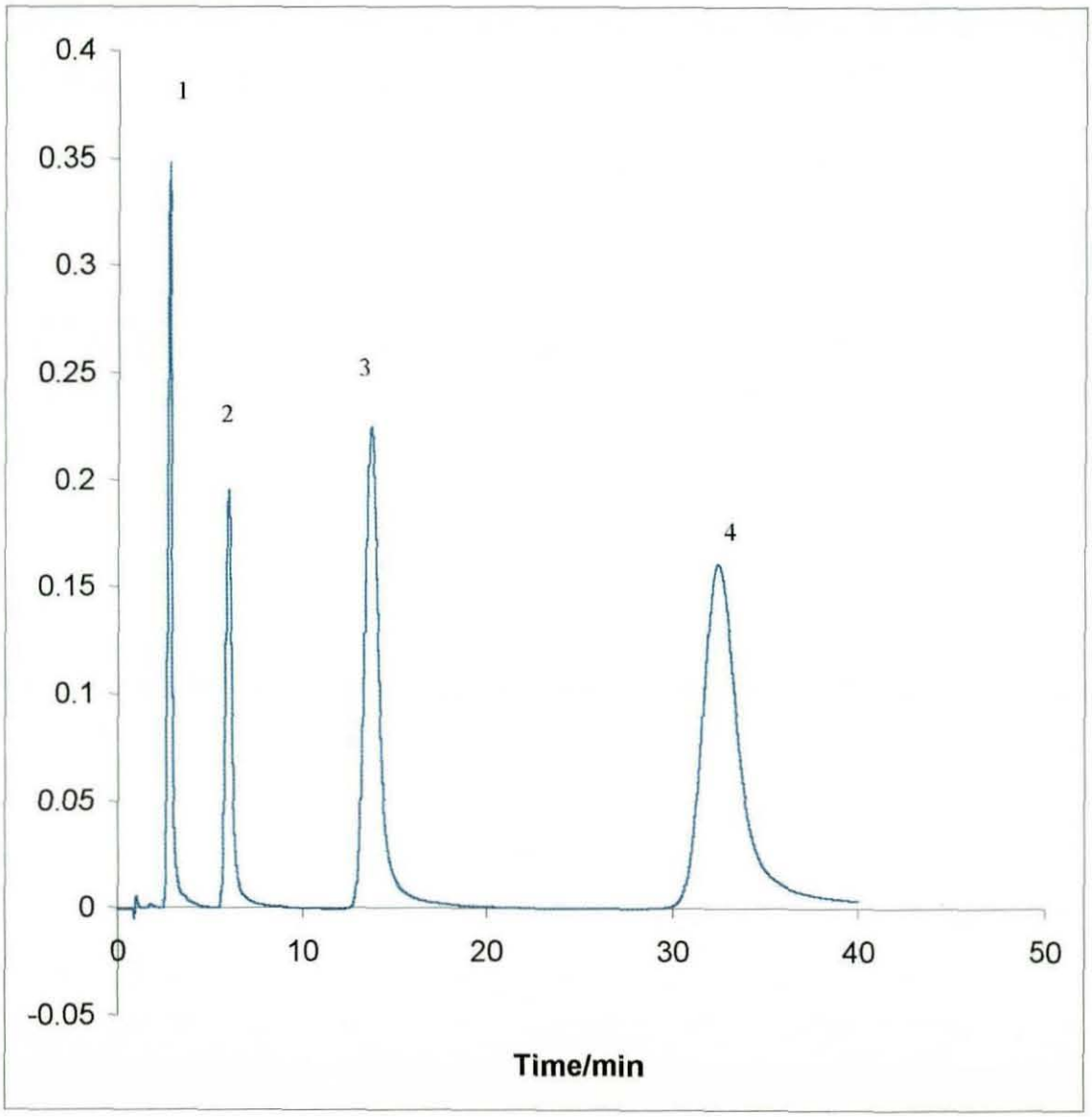



**Figure 3.9: Chromatogram of alkyl aryl ketones on a Luna Silica ™ $C_{18}$ (2) column using superheated water as mobile phase (150 °C) after overnight standing in cold water (1) acetophenone, (2) propiophenone, (3) butyrophenone, (4) valerophenone.**

**Table 3.9: Efficiency of homologous alkyl aryl ketones and test compounds in different eluents on a Luna ™ $C_{18}$ (2) column**

| ***Compound*** | ***Efficiency*** | | | |
|---|---|---|---|---|
| Compound | 50% MeOH (v/v) | 35% ACN (v/v) | 30% THF (v/v) | Superheated water (150 °C) |
| **Alkyl Aryl Ketones** | | | | |
| Acetophenone | 1019 | 1959 | 1239 | 2653 |
| Propiophenone | 2570 | 3512 | 3139 | 2227 |
| Butyrophenone | 4710 | 4266 | 4963 | 1658 |
| Valerophenone | 5756 | 4522 | 5726 | 1425 |
| Hexanophenone | 4892 | 4403 | 5420 | - |
| **Test Compounds** | | | | |
| 2-Phenylethanol | 370 | 945 | 768 | 1890 |
| p-Cresol | 1101 | 1586 | 2601 | 2135 |
| Benzaldehyde | 774 | 1805 | 1316 | 2163 |
| N-Methylaniline | 956 | 2269 | 840 | 2470 |
| Nitrobenzene | 1426 | 3307 | 3900 | 2310 |
| Anisole | 2209 | 3664 | 4291 | 2136 |
| Methyl benzoate | 2229 | 3376 | 2970 | 2242 |
| Toluene | 4612 | 4454 | 5556 | 1592 |

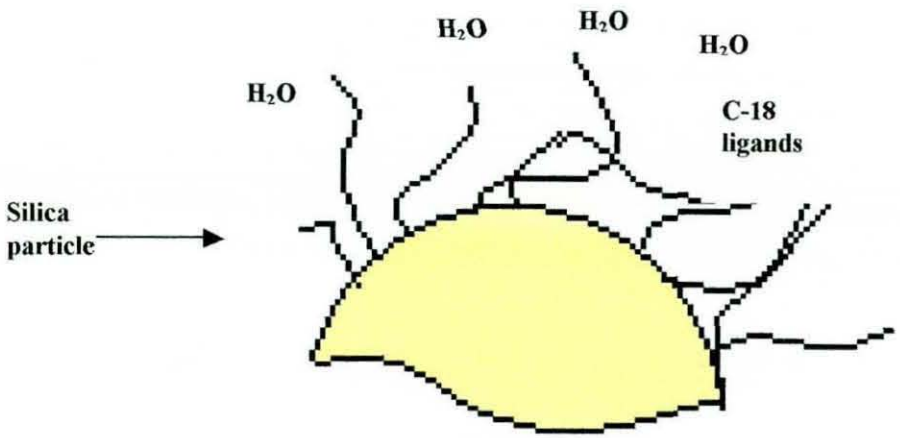



**Figure 3.10: Collapsing $C_{-18}$ bonded phase as a result of standing in a 100 % aqueous mobile phase.**

Another theory for the cause of this phenomenon is the large wetting angle (>90 °) of the totally aqueous mobile phase on the highly hydrophobic surface; as pressure is released, the mobile phase is driven out of the pores (**see Figure 3.11**). However, if an organic modifier is once again introduced to the mobile phase, these pores can once again be accessed and "wetting" of the surface can occur. If the organic modifier then reverts back to the totally aqueous mobile phase, there is a return to the original retention characteristics (166). To return the column to the original retentions, organic modifier was passed over-night through the column. But it did not come back to its original retention characteristics.

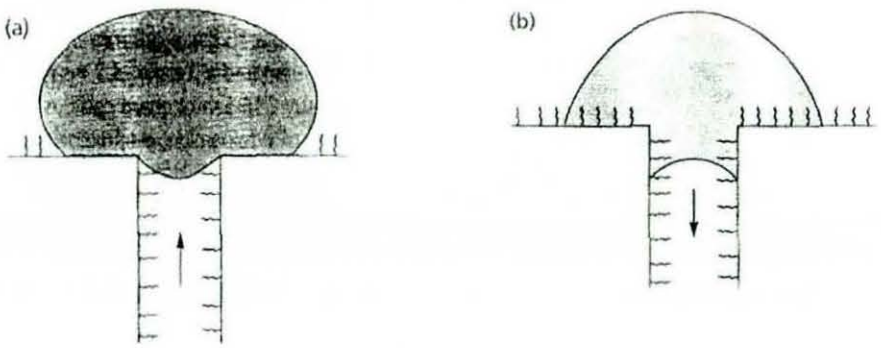



**Figure 3.11: Representation of a $C_{18}$ surface with (a) non-wetting solvent, such as water and (b) a wetting solvent, such as methanol.**

### 3.2.1.4: Studies on an OASIS HLB$^{TM}$ column

Many of the reported studies in the area of superheated water HPLC have been performed using polymer-based materials because of their temperature stability compared to silica-based phases. A new polymer, Waters Oasis HLB$^{TM}$ [Poly (divinylbenzene-co-N-vinylpyrrolidone (DVB/NP)], column was therefore examined. This polymeric material is more polar than PS-DVB and it might be expected to have a higher retention factor for polar analytes. As the sorbent has increased water wettability compared to PS-DVB, it should also provide better efficiency. This column has been used successfully in superheated water (210 °C) to trap extracts (120) and Wilson (66) separated some drugs on this column with superheated water chromatography. Therefore, we selected this column to compare conventional LC and superheated water chromatography.

First, alkyl aryl ketones were separated in 50% ACN (v/v) and 40% THF (v/v) on Oasis HLB$^{TM}$ [Poly (divinylbenzene-co-N-vinylpyrrolidone (DVB/NP)] column (**Figure 3.12**). In methanol/water acetophenone was eluted with a long retention time, but the peak was distorted and tailed. Therefore, methanol-water was avoided on Oasis HLM $^{TM}$ column. Acetophenone and propiophenone were also highly retained in superheated water (180 °C) (**Figure 3.12**). Therefore, butyrophenone to hexanophenone couldn't be separated on superheated water chromatography within a reasonable time.

The logarithms of the retention factors and carbon numbers were plotted for each of the homologous series **(Figure 3.13)**. For each eluent composition the column void volume was determined using a solution of uracil, because, Boweres and Pedigo (113) reported the use of uracil as non-retained markers on the PLPR-S column. They reported column void volumes of 1.74 ml with methanol-water (2:3), 1.43-144 ml for acetonitrile-water (3:2) and 1.41-1.51 ml for THF-water (3:2) eluents. In each case, there was very similar behaviour obtained in the present work for the corresponding superheated water, acetonitrile-water and THF-water using uracil solution. Test compounds (2-phenylethanol, benzaldehyde, p-cresol, N-methylaniline, methyl benzoate, nitrobenzene, anisole, and toluene) were eluted in 50% ACN (v/v) and 40% THF (v/v) (**Table 3.10**). The retention factors of alkyl aryl ketones and the test compounds were significantly higher in superheated water. This behaviour showed that superheated water (180 °C) was still relatively polar at the high temperatures compared to aqueous ACN or THF and was a weak solvent on this column. This is similar to the properties of methanol on a PS-DVB column. Because a divinylbenzene group is also present in Oasis HLB $^{TM}$ column packing material, the –OH group of superheated water may interact with the $\pi$-electron system of the benzene ring.

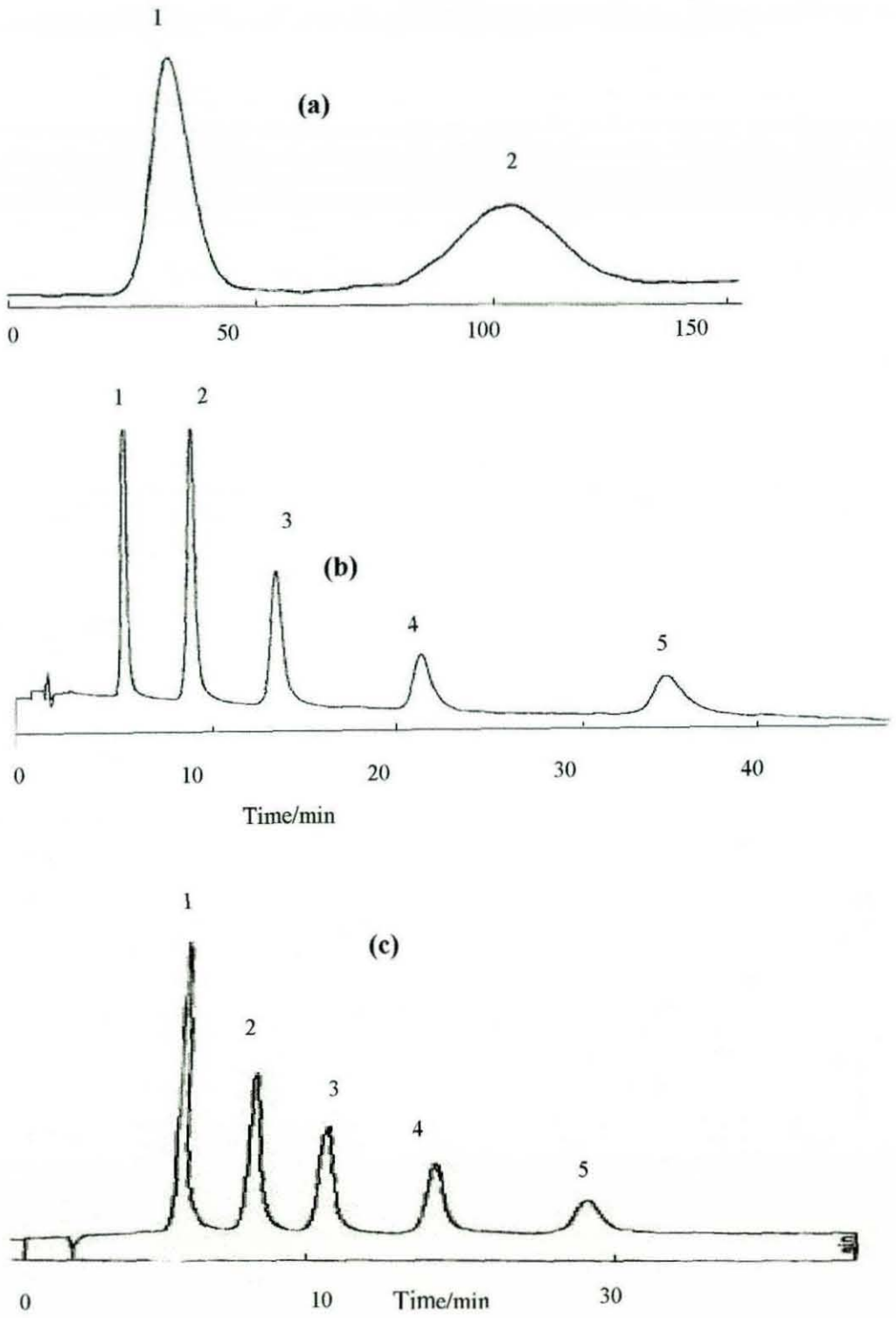



**Figure 3.12: Chromatograms illustrating the separation of alkyl aryl ketones on an Oasis HLB ™ column. (a) Superheated water (180 °C), (b) 50% ACN (v/v), (c) 40% THF (v/v). Peak identification (1) acetophenone, (2) propiophenone, (3) butyrophenone, (4) valerophenone, (5) hexanophenone.**

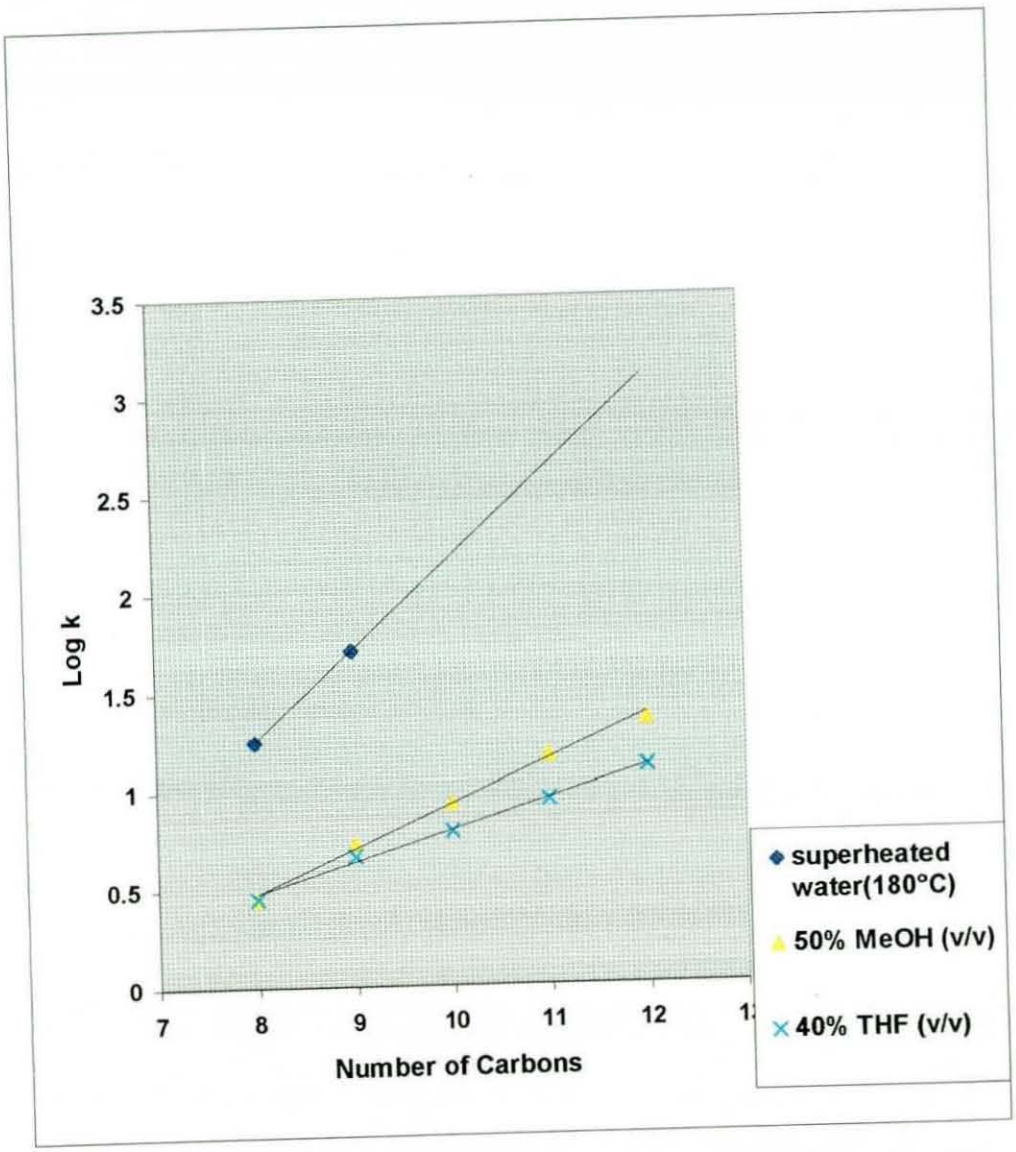



**Figure 3.13: Variation of log *k* with number of carbons for homologous series of alkyl aryl ketones in different eluents on an Oasis HLB™ column.**

**Table 3.10: Retention factors of homologous series of alkyl aryl ketones and Test compounds in different eluents on an Oasis HLB$^{TM}$ column**

| *Compounds* | *Retention Factors* | | |
|---|---|---|---|
| | 50% ACN (v/v) | 40% THF (v/v) | Superheated Water (180 °C) |
| **Alkyl aryl ketones** | | | |
| Acetophenone | 2.83 | 2.86 | 17.93 |
| Propiophenone | 5.35 | 4.66 | 50.81 |
| Butyrophenone | 8.49 | 6.17 | - |
| Valerophenone | 14.69 | 8.79 | - |
| Hexanophenone | 22.26 | 12.79 | - |
| **Test compounds** | | | |
| 2-Phenylethanol | 1.44 | 1.69 | 9.50 |
| Benzaldehyde | 3.15 | 3.01 | *11.33 |
| p-Cresol | 4.78 | *5.73 | 10.60 |
| Methyl benzoate | 5.06 | 4.21 | - |
| N-Methylaniline | 5.45 | 5.93 | 19.04 |
| Nitrobenzene | 5.66 | *5.79 | 19.76 |
| Anisole | 6.58 | 6.35 | 33.17 |
| Toluene | 10.60 | 9.16 | - |
| **Void Marker** | | | |
| Uracil | 1.85 | 1.60 | 2.00 |

* = The retention order had changed

The retention indices of the homologues and test compounds were calculated based on the linear relationship for the alkyl aryl ketones (**Table 3.11**). The retention indices of acetophenone were found to have much higher values (786-800) on the Oasis HLB$^{TM}$ column, than on a PS-DVB column (750-770) and also other columns containing porous copolymer of different chemical structure (167), indicating the different properties of the Oasis HLB $^{TM}$column from other polymer-base columns.

2-Phenylethanol has shown higher retention index value in superheated water than the other two eluents. Hydrogen donors, such as 2-phenylethanol, exhibit a greater retention on the hydrophilic-lipophilic balanced packing material because of the pyrrolidone groups in the polymer, which as a hydrogen acceptor will form hydrogen bonds with this compound. It can enhance retention in relatively polar eluents like superheated water (180 °C), because it has hydrogen donor properties.

p-Cresol gave much lower retention index values in superheated water (180 °C) than the other two eluents. This behaviour is analogous to that on ODS silica based columns and also PBD-Zirconia columns. In 50% ACN (v/v) this compound has shown a retention index of 890 on the Oasis column, in contrast, on the PS-DVB column it gave a much lower value (568) in 70% ACN (v/v). In 40% THF (v/v) p-cresol gave a retention index of 979 on the Oasis column, whereas on PS-DVB it gave a value of 922 in the same mobile phase (111).

Benzaldehyde was 53 units lower in superheated water than THF-water. Methyl benzoate did not elute in superheated water chromatography. It gave similar retention indices in 50% ACN (v/v) and 40% THF (v/v) as on the other polymer based column (111), because methyl benzoate is as relatively insensitive to changes of eluents as alkyl aryl ketones.

**Table 3.11: Retention indices of homologous series of alkyl aryl ketones and test compounds in different eluents on an Oasis HLB$^{TM}$ column**

| *Compound* | *Retention* | *Indices* | |
|---|---|---|---|
| | 50% ACN (v/v) | 40% THF (v/v) | Superheated water (180 °C) |
| **Alkyl Aryl ketones** | | | |
| Acetophenone | 788 | 786 | 800 |
| Propiophenone | 912 | 921 | 900 |
| Butyrophenone | 1002 | 998 | - |
| Valerophenone | 1109 | 1096 | - |
| Hexanophenone | 1190 | 1199 | - |
| **Test Compounds** | | | |
| 2-Phenylethanol | 657 | 643 | 741 |
| Benzaldehyde | 810 | 802 | 757 |
| p-Cresol | 890 | 979 | 750 |
| Methyl benzoate | 899 | 890 | - |
| N-Methylaniline | 917 | 985 | 807 |
| Nitrobenzene | 922 | 979 | 811 |
| Anisole | 953 | 1004 | 859 |
| Toluene | 1047 | 1106 | - |

Nitrobenzene, N-methylaniline and anisole have shown much lower retention indices in superheated water than the other two eluents on an Oasis HLB™ column. In 40% THF (v/v) mobile phase nitrobenzene has given a retention index of 979 on the Oasis HLM ™ column and 1070 on the PS-DVB column (111). In same mobile phase N-methylaniline gave a retention index of 985 on the Oasis HLB ™ column and 927 on the PS-DVB column (111). Toluene gave a lower retention index value in the same eluent on the Oasis HLB ™ column than on the PS-DVB column (111). This behaviour means that the property of the Oasis HLB ™ column is different from the property of the PS-DVB column.

Nitrobenzene, N-methylaniline and anisole showed similar behaviour on the Oasis HLB ™ column in acetonitrile-water, THF-water and superheated water, similar to ODS silica and also the PBD-zirconia column.

The Oasis HLB™ column showed much better efficiency in superheated water (180 °C) than in acetonitrile-water. The hydrophilic -N-pyrrolidone monomer group increases the water wettability of the polymer (168). Therefore in superheated water the mass transfer resistance is reduced and increases efficiency. In contrast, poor efficiency in 40% THF-water (v/v) was found on this column (**Table 3.12**). This behaviour is completely opposite that on the polystyrene columns (111). Acetonitrile/water showed better efficiency than THF on the Oasis HLB™ column.

**Table 3.12: Efficiency of homologous series of alkyl aryl ketones and Test compounds in different eluents on an Oasis HLB$^{TM}$ column**

| *Compound* | *Efficiency* | | |
|---|---|---|---|
| | 50% ACN (v/v) | 40% THF (v/v) | Superheated water (180 °C) |
| **Alkyl Aryl ketones** | | | |
| Acetophenone | 4286 | 322 | 4768 |
| Propiophenone | 5098 | 494 | 4982 |
| Butyrophenone | 10038 | 658 | - |
| Valerophenone | 1981 | 568 | - |
| Hexanophenone | 2348 | 509 | - |
| **Test Compounds** | | | |
| 2-Phenylethanol | 1612 | 47 | 3468 |
| Benzaldehyde | 1109 | 515 | 4301 |
| p-Cresol | 691 | 343 | 2248 |
| Methyl benzoate | 1713 | 340 | - |
| N-Methylaniline | 1032 | 335 | 4174 |
| Nitrobenzene | 1673 | 462 | 4763 |
| Anisole | 1297 | 361 | 4971 |
| Toluene | 1090 | 342 | - |

### 3.3: Summary

The standard ketones show a linear relationship between log *k* and carbon number in superheated water and also aqueous organic mobile phases on PBD-zirconia, Xterra ™ RP 18, Luna ™ $C_{18}$ (2) and Oasis HLB ™ column. 40% MeOH (v/v), and 25% ACN (v/v) are correlated with superheated water (140 °C) on PBD-zirconia column. 40% MeOH (v/v) and 25% ACN (v/v) are correlated with superheated water (140 °C) on Xterra TM RP 18 column. The behaviour of two silica-based columns (Xterra ™ RP 18 and Luna ™ $C_{18}$ (2)) is almost similar in superheated water and also aqueous organic solvents. The Oasis column is more retentive for polar and also non-polar compounds in superheated water (180 °C) and other eluents. It has been shown to be more retentive in comparison with PS-DVB column.

The Oasis stationary phase tended to give poor peak shapes with most of the solutes showing a large amount of tailing in 40% THF (v/v) solvent. The peak shapes on PBD-zirconia and Xterra ™ RP 18 columns were very good.

A notable finding was that superheated water offers a different selectivity for successful separations with better efficiency than traditional mixtures of water with organic solvents on PBD-zirconia and Oasis HLB ™ columns.

# CHAPTER 4

## Separation Efficiency in Conventional LC and Superheated Water Chromatography

### 4.1: Introduction

In previous studies (49, 65,66), PS-DVB, PGC, PBD-zirconia, Xterra ™ RP 18, ODS1, C18 AB, and alumina columns have been used successfully for separation of different compounds using superheated water as mobile phase. To the best of our knowledge the systematic study of the efficiencies of these columns in superheated water chromatography was rare.

In one previous study, Burgess (49) reported efficiency on PS-DVB and PGC columns in superheated water chromatography. He found that with the PS-DVB stationary phase there was a very marked decrease in the efficiency at high linear velocities with the H versus u plot appearing parabolic in shape. However, the peak shapes obtained for phenol at high velocity were severely distorted suggesting a poor equilibration between the incoming mobile phase and the temperature of the column.

Therefore, in this study, attention turned to reinvestigating the efficiency of the PS-DVB column and other columns in the superheated chromatography system. In particular the Xterra ™ RP 18 column was also investigated, because it was found that this particular silica based column is stable for a long time at high temperature.

### 4.2: van Deemter Equation

One of the most widely used models for the causes of band broadening on the column is based on the work of a Dutch group under van Deemter. In 1956 they derived an equation with three main components (169). Although, Giddings (132), and Knox et al. (170) have published papers about band-broadening theory, the van Deemter equation provided the most practical representation of a packed column. van Deemter described without difficulty

the results obtained with well-packed columns over the usual range of reduced velocities of interest in HPLC. For a normal packed bed with porous particles the van Deemter equation as a whole can be expressed as in equation (4.1).

$$H = 2\lambda d_p + \frac{2\lambda D_m}{u} + \left(\frac{f_1(k)d_p^{\,2}}{D_m} + \frac{f_2(k)d_f^2}{D_s}\right)u \qquad (4.1)$$

Where, $H$ is the plate height, $d_p$ is the diameter of the stationary phase particle, $D_m$ is the diffusion coefficient of the solute, $u$ is the linear velocity of the mobile phase, $k$ is the retention factor, $d_f$ is the effective film thickness of the stationary phase layer, $D_s$ is the diffusion coefficient in the stationary phases, and $f_1$ and $f_2$ are functions of the retention factor. From this equation we can see that the diffusion processes affect the plate height predominantly in the mobile phase. Because we are using a mobile phase at high temperature, it is necessary to consider the temperature effects on diffusion rate and hence on efficiency. Normally an increase in temperature increases diffusion rate, therefore using superheated water as mobile phase should lead to an increased chromatographic efficiency.

### 4.2.1: Diffusion and Temperature

Chromatography can be loosely described as a diffusion process; it is useful to describe the effect a high temperature mobile phase will have on solutes travelling within it. By their nature, solute molecules are bound into place by relatively strong intermolecular attractions. To be able to diffuse, solute molecules must gain sufficient energy to break free from these attractions. Thus diffusional transport is logically an activation process with usual exponential dependence on temperature:

$$D_m = D_0 \exp\left(-\frac{W_l}{\Re T}\right) \qquad (4.2)$$

Where, $W_l$ is the activation energy required for molecular displacement, $\mathcal{R}$ the gas constant, $T$ the absolute temperature, and $D_0$ a term with only a slight temperature variation. However, by using this equation it is difficult to quantify a reliable and accurate measure of $W_l$ *(132).* Because of this problem, working estimates of $W_l$ are usually made. One such estimate uses the correlation between the activation energies $(W_l)$ of $D_m$ and the viscous shear of a liquid $(1/\eta)$. This is helpful, since viscosity data is easier to obtain than measuring diffusivities directly. If $\eta$ is known at a series of temperatures, then a plot of log $(1/\eta)$ versus $1/T$ will yield a line of slope $W_l/\mathcal{R}$.

However, there are several different approaches available to estimate the diffusion coefficient in the mobile phase, but the one most frequently used in the HPLC literature is the Wilke-Chang equation. It is claimed to be accurate for small to medium-sized molecules within 10 %, which is sufficient for most purposes (132):

$$D(cm^2/s) = 7.4x10^{-8}\frac{\sqrt{\Psi MT}}{\eta V_s^{0.6}} \qquad (4.3)$$

Where $\psi$ is an association factor based on the solvent (2.6 for water, 1.9 for methanol, 1.5 for ethanol and 1.0 for the other non-associated solvents), $M$ is the molecular mass of the solvent, $T$ is the absolute temperature, $\eta$ is the solvent viscosity and $V_s$ is the molar volume of the solute (calculated from the molar mass (g $mol^{-1}$) divided by the density (g $cm^{-3}$).

### 4.2.2: Viscosity and Temperature

The temperature has a marked effect on the viscosity of the mobile phase, which governs two critical parameters in LC; the diffusion rates $(D_m)$ and the maximum permissible pressure drop across the column $(\Delta p)$. A lower viscosity

solvent will produce a lower pressure drop across the column than a solvent with a higher viscosity at a specific flow rate (equation *4.4*):

$$\Delta p = 1000 \frac{F \eta L}{\pi r_2 d_p^{\ 2}} \qquad (4.4)$$

Where $\Delta p$ is the pressure drop, $F$ is the flow rate, $\eta$ viscosity, $L$ column length, $r$ column radius and $d_p$ is the particle size. From this equation we can see that a low viscosity solvent will allow the use of longer columns and/or smaller particle sizes.

Viscosity usually increases with polarity, association and molecular weight. Thus at ambient temperature liquid water is relatively viscous ($\eta \sim 1$ mPa s at 25 °C) due to its extensive hydrogen bonding. However, the viscosity of water can be drastically lowered upon heating under pressure (pressure is needed to keep water as liquid form). Moszynski (171) measured the viscosity of superheated water and found that when water was heated to 200 °C, its viscosity was less than 20 % of its value at ambient temperatures.

Therefore, it is important to remember that the diffusion coefficient is proportional to temperature and inversely proportional to viscosity. It also means that temperature can improve diffusion by reducing the viscosity of the mobile phase itself.

Another approach is that reducing the viscosity can increase mass transfer of solute in mobile and stationary phase. Therefore, the use of high temperature, greatly decreases the *C* term and then allows the use of higher linear velocity, thereby enable a decreases in analysis time. The reason for this can be derived from differentiation of the van Deemter equation with respect to ($u$):

$$H = A + \frac{B}{u} + Cu \qquad (4.5)$$

And

Equating to zero, $dH/du = 0$ and consequently, $- B/u^2 + C = 0$

$$\frac{dH}{du} = -\frac{B}{u^2} + C$$

Thus,

$$u_{opt} = \sqrt{\frac{B}{C}} \qquad (4.6)$$

From this equation we can see that the $u_{opt}$ is directly proportional to the value of the diffusion coefficient in both the mobile phase and the stationary phase.

### 4.3: Effect of Column Material

In the present study, the influence of efficiency on PS-DVB, Xterra ™ RP 18 and PBD-zirconia columns were studied in a wide range of eluent flow rates at constant temperature and plots of *H and u* were constructed to determine *A*, *B*, and *C*, coefficients of the van Deemter equation.

#### 4.3.1: Polystyrene Column

For the construction of the van Deemter plots, phenol and p-cresol were chosen as the test solutes and a temperature of 190 °C was chosen. Phenol was chosen because we tried to reinvestigate Burgess's experimental work (49) and p-cresol as chosen so that retention factor 4.41 was long enough at 190 °C to reduce the influence of extra-column band broadening effects. Flow rates from 0.2 to 2.6 ml/min at intervals of 0.2 ml/min were chosen to give a representative spread of linear velocities. The retention factor of phenol was 1.72 and p-cresol was 4.41 at 1 ml/min flow rate using superheated water (190 °C) as the mobile phase. The retention factors should be independent of flow rate.

*H* versus *u* data for both samples was plotted (**Figures 4.1**). It is immediately observed that p-cresol clearly gave a lower efficiency than phenol in superheated water (190 °C) chromatography. In the present study, the highest efficiency for p-cresol was 1505 and for phenol was 2372 in superheated water chromatography at 190 °C. In a previous study, Dawkins and co-workers have examined the plate height (*H*) against flow-rate curves for phenol, toluene and diethyl phthalate on PLRP-S columns and found a typical van Deemter type curve using acetonitrile-water (90:10) as the eluent at ambient temperature (105). They found phenol gave better efficiency than toluene, although toluene showed a higher retention factor but they did not note a reason.

In another previous study, Nevejan and Verzele (103) proposed that separations carried out on PS-DVB column in high water content should close the micropores and thus make them unavailable to diffusion. Therefore, they concluded that, for all of the polystyrene phases examined, the low efficiency for larger apolar samples is due to the micropores. Small-size molecules, like phenones exhibit much better behaviour than larger molecules. The mass transfer was sufficiently fast for phenones to allow short analysis time.

In conventional LC Smith and Garside (111) showed that p-cresol had a much poorer efficiency in methanol-water than with other organic modifiers. Lloyd and et al. (172) reported the plate heights with methanol were two to three times poorer than with acetonitrile at the optimum flow-rate. Benson and co-workers (173) have suggested that better results could be obtained with methanol-water by using a $C_{18}$-bonded polymer material, in which there is only a surface interaction. Bowers and Pedigo concluded that hydroxyl groups have little affinity toward the polymer surface (113).

In the present study, in superheated water, the $u_{opt}$ *of* both compounds are reached at a linear velocity of about 0.12 cm/s whereas in the previous study (49) in superheated water (200 °C) with phenol $u_{opt}$ was about 0.16 cm/s and

the form of the van Deemter curve showed almost a parabolic shape whereas in this case it had a more conventional higher flow rate slope.

Mayr and Welsch (174) reported that the use of high velocities in high-speed HPLC resulted in high-pressure drops, which in turn lead to the generation of viscous heat within the column. The dissipation of viscous heat to the thermostated column wall produces temperature gradients within the column, which diminishes the achievable efficiency.

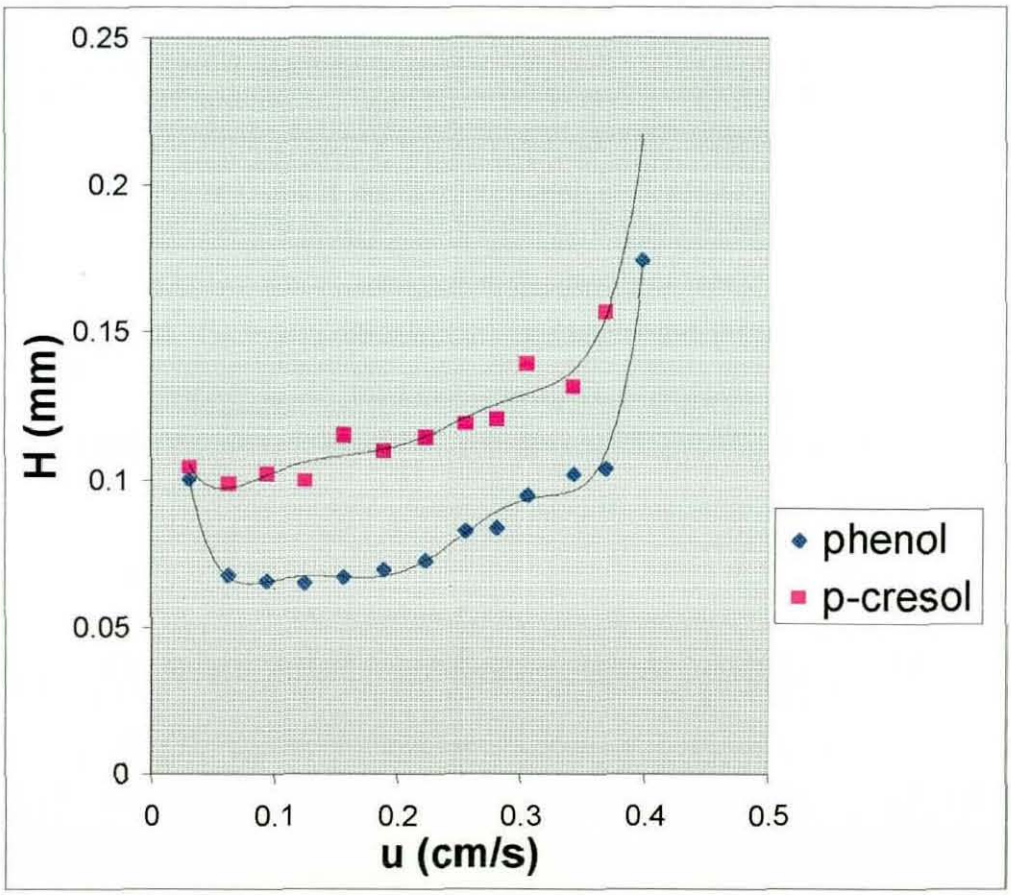



**Figure 4.1: Experimental H vs u plots obtained on the PLRP-S column for phenol and p-cresol using superheated water as the mobile phase.**

**Conditions: column: PS-DVB (150 x 4.6 mm I.D., 5 μm), eluent: water, oven temperature: 190 °C, detection: UV absorption wavelength 254 nm, injection volume 5 μl (1%) solution of phenol and p-cresol, retention factor of phenol was 1.72 and p-cresol was 4.14.**

To increase efficiency, the possible causes of band broadening effects in HTLC were investigated.

#### 4.3.1.1: Band Broadening Effects in Superheated Water Chromatography

There are a number of possible causes for the band broadening of peaks during chromatographic separations. If these can be identified then the operator can take steps to minimise their influence. Part of the spreading will take place on the column. In addition the injected sample peak can be broadened because of extra-column effects, particularly the presence of dead volumes in the injector, the detector, connecting tubing and column fittings. A dead volume may be formed at any point where the tubing/connection is wider creating a void so that stagnant areas occur, which are not swept by the flow of the eluent. The sample will eddy into these points and thus will be dispersed over a greater volume of the eluent. Dead volume can be very important in conventional LC because of the lower diffusion rates, which reduce mixing, and even small additional volumes of 10-20 μl can significantly reduce efficiencies. In a superheated water chromatography system, much longer tubing is used than conventional LC so making the situation worse. Any large pockets from injector to detector will increase plate height. Theoretically and also practically dead volume should be kept small. This is in keeping with good experimental technique.

Basically, the limitation and reduction of dead volumes are a matter of good mechanical design and should be inherent in the manufacture and design of the chromatograph. Apart from a number of simple factors, these problems are largely outside the direct control of the operator. Therefore, we changed a number of things from the previous setting.

**(a)** We first looked at the effects caused by connectors and ferrules. For different manufacturers of connection, the distance between the tip of the tubing and the tip of the ferrule can be different. This can lead to two problems (**Figure 4.2**): in the case that this distance is too long, the ferrule needs to be

completely reseated and we have some difficulty sealing the column (**Figure 4.2b**). In the opposite case (**Figure 4.2a**), some dead volume between the end of the tubing and the seat of the tubing inside the fitting, which results in band spreading and lowered column performance. Therefore, the ferrules in the chromatograph were reset. The efficiency increased slightly.

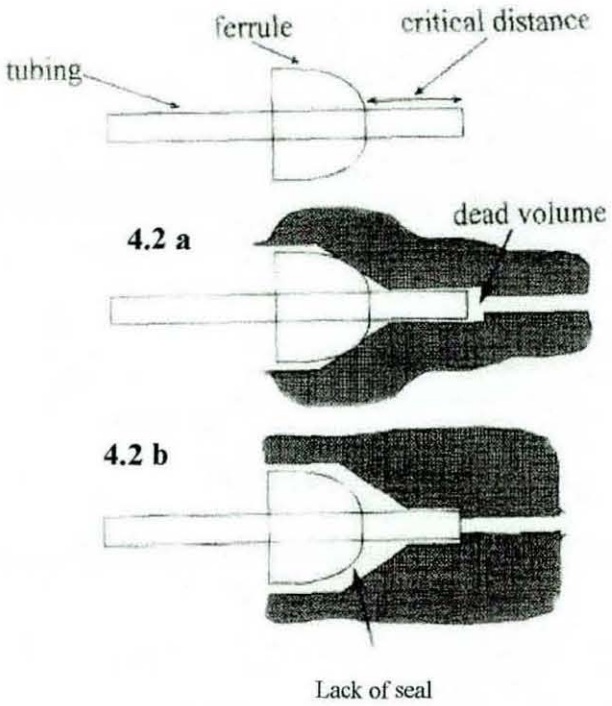



**Figure 4.2: Effect of the critical distance between the tip of the ferrule and the tip of the tubing (Figure obtained from ref. (84).**

**(b)** Any sample placed on to an LC column will have a finite volume, and the volume of the injected sample will contribute directly to efficiency of the final peak that results from the dispersion processes that take place in the column. It follows that the maximum volume of sample that can be placed on the column should be limited, or the column efficiency will be seriously reduced (175). Therefore, dilute sample (0.5% p-cresol solution) but small volume (1 μl) was injected. It gave better efficiency than 1% solution of p-cresol, injection volume 5 μl.

**(c)** When the UV detector is connected to a superheated water chromatographic system, the temperature should be controlled because the temperature of the mobile phase, entering the flow cell is often different from temperature of the flow cell in a UV absorbance compartment and a temperature alteration can cause a refractive index change (176). Therefore, five additional pieces (3 cm x 12 cm x 0.05 mm) of cooling foil were used compared with previously setting (**Section 2.3.1**) in tubing connection between the column and detector to cool the sample when it goes to the detector. But it had little effect on efficiency.

**(d)** Band broadening in the connecting tubing from injector to detector depends on the flow rate, the tubing length and its inner diameter, the temperature of the eluent, and the difference in temperature between the tubing wall and the centre of the tube. In superheated water chromatography a substantial length of tubing is needed to ensure that the eluent is heated before reaching the column. The radial temperature gradient generates a radial viscosity gradient this means that in the heating section the fluid near the wall has a lower viscosity than the fluid in the centre of the tube. This means that the linear velocity near the wall will be higher than it otherwise would be, thereby counteracting the Aris-Taylor dispersion phenomenon, which broadens the zone. Therefore, a narrower-bore and longer (100 cm x 0.01 in I.D.) connecting tube than previously (85 cm x 0.02 in I.D.) was used to link the injection valve and column. 70 cm of the tubing was loosely coiled in the oven to allow ready heating by the air circulation. A dilute sample (0.5 % p-cresol solution) 1 μl was injected. It gave the best efficiency (**Figure 4.3b**).

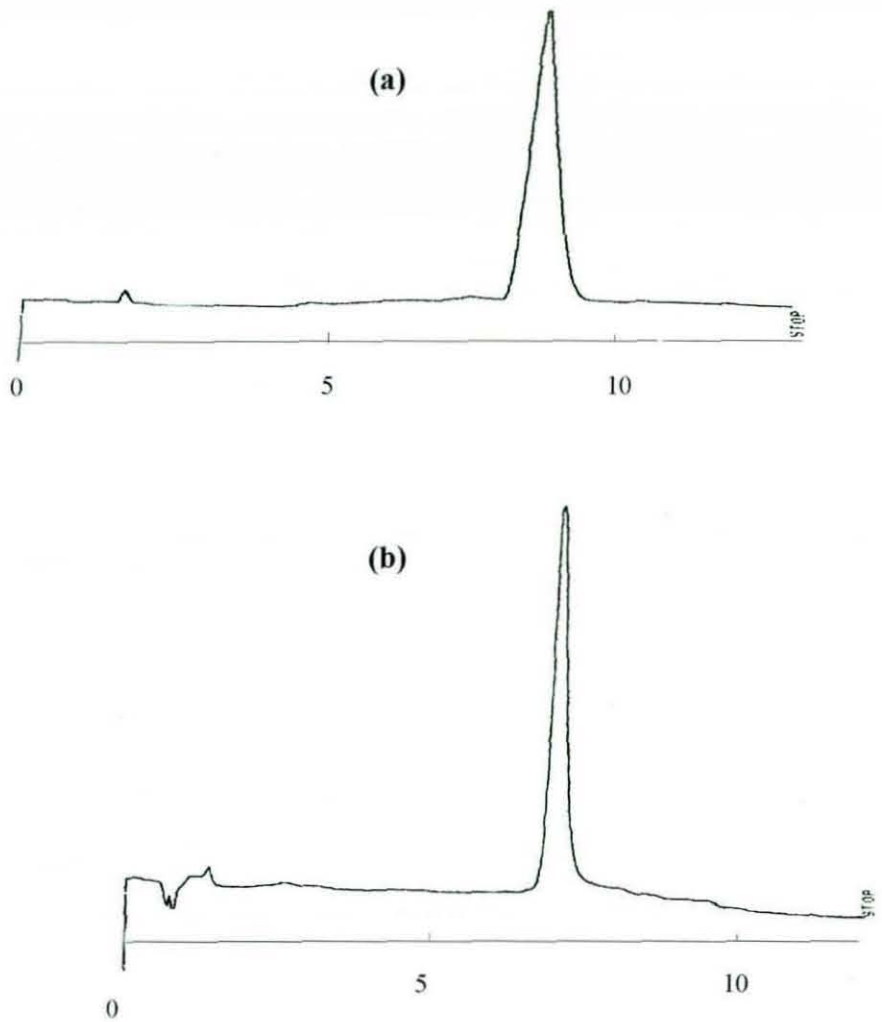



**Figure 4.3: Chromatograms of p-cresol with superheated water as the mobile phase (a) 85 cm (0.02 in I.D.) preheating tubing from injector to column (b) 100 cm (0.01 in I. D.) preheating tubing from injector to column.**

**Conditions: column: PS-DVB (150 x 4.6 mm I.D., 5 μm), eluent: water, oven temperature: 190 °C, detection: UV absorption wavelength 254 nm, injection volume: 1 μl (0.5%) solution of p-cresol, flow rate: 1 ml/min.**

Again, a van Deemter curve was made for p-cresol (**Figure 4.4**) to calculate *A*, *B* and *C* term of this curve. Here, dilute (0.5% p-cresol solution) and small volume (1 μl) sample was injected.

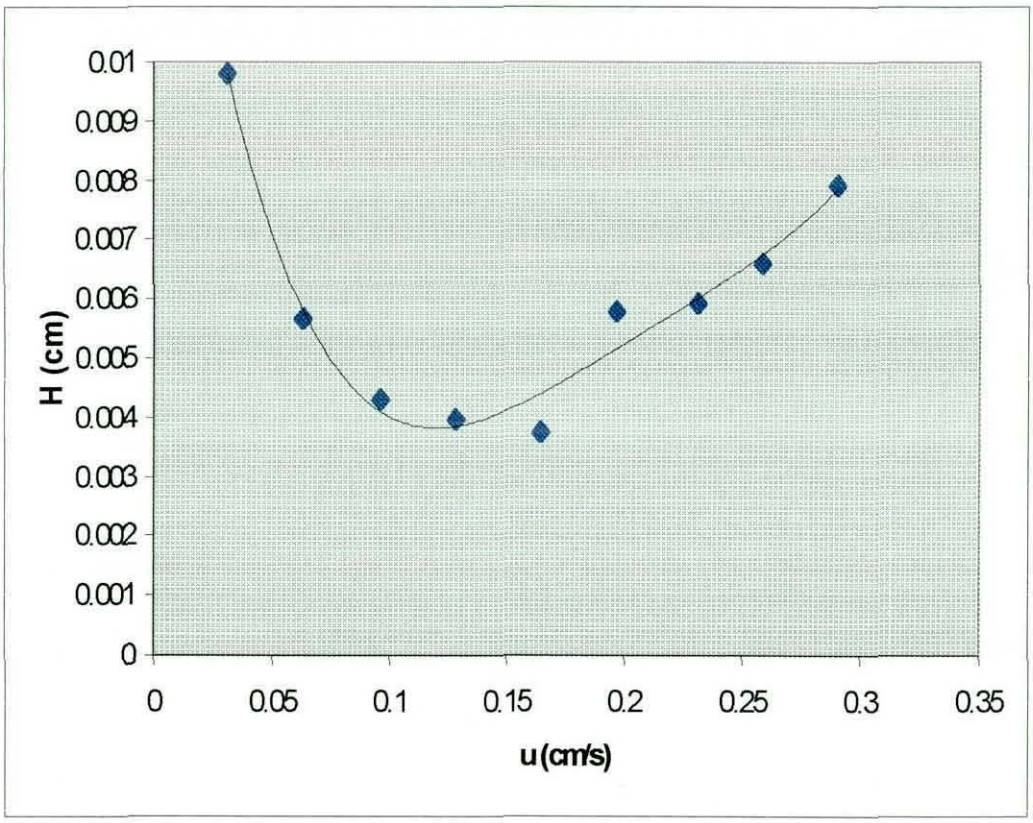



**Figure 4.4: Experimental H vs u plots obtained on PLRP-S column for p-cresol.**

**Conditions: column: PLRP-S (150 x 4.6 mm I.D., 5 μm), eluent: water, oven temperature: 190 °C, detection: UV wavelength 254 nm, injection volume: 1 μl (0.5%) solution of p-cresol.**

In this curve, the optimum efficiency was at 0.16 cm/s linear velocity (1 ml/min); it is three times higher than conventional LC (49) using 30 % acetonitrile/water as the mobile phase. In the present study, the peak shape was not distorted at the highest flow rate even at 2.0 ml/min (**Figure 4.5**).

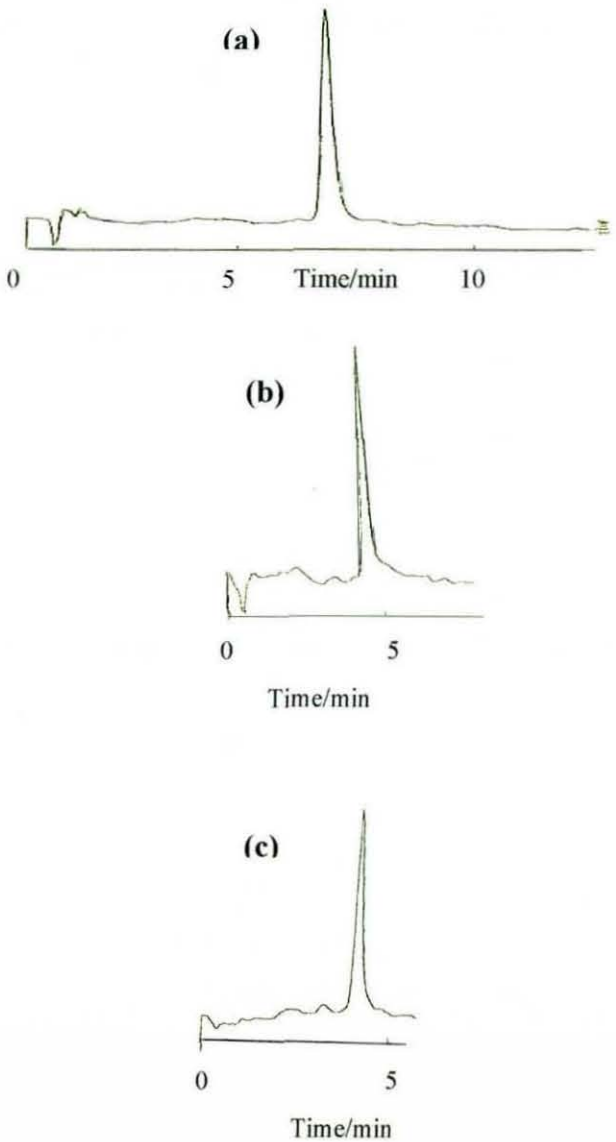



**Figure 4.5: Chromatograms of p-cresol on PLRP-S with superheated water as the mobile phase at three flow rates: (a) 1.2 ml/min, (b) 1.8 ml/min & (c) 2.0 ml/min.**

**Conditions: column: PLRP-S (150 x 4.6 mm I.D., 5 μm), eluent: water, oven temperature: 190 °C, detection: UV wavelength 254 nm, injection volume: 1 μl (0.5%) solution of p-cresol.**

At this point we can conclude that our longer preheated tubing (100 x 0.01 in I.D.) is more adequate to bring the mobile phase up to the intended temperature of 190 °C at flow rates up to 2.0 ml/min.

Burgess (49) also constructed a H versus u curve using 30% ACN (v/v) aqueous mobile phase at 40 °C. He calculated *A*, *B*, and *C* coefficients of van Deemter curve for phenol using the Excel spreadsheet program and solver function.

Using the same program the *A*, *B*, and *C* coefficients of the van Deemter equation were calculated in the present study using superheated water as the mobile phase (**Table 4.1**).

**Table 4.1: Computed values of van Deemter coefficients for superheated water (190 °C) for p-cresol (data obtained from present study) and 30 % ACN mobile phase for phenol {data obtained from previous study in our laboratory (49)}.**

| | A | B | C |
|---|---|---|---|
| | | Cm2/sec | sec |
| Superheated water (190 °C) | 0.00001 | 0.00030 | 0.02550 |
| 30/70 ACN/$H_2O$ w/w (40 °C) | 0.001242 | 0.00002 | 0.00912 |

When these computed coefficients are plotted with their respected relationship to u, a clear picture of their effect on H emerges (**Figure 4.6**).

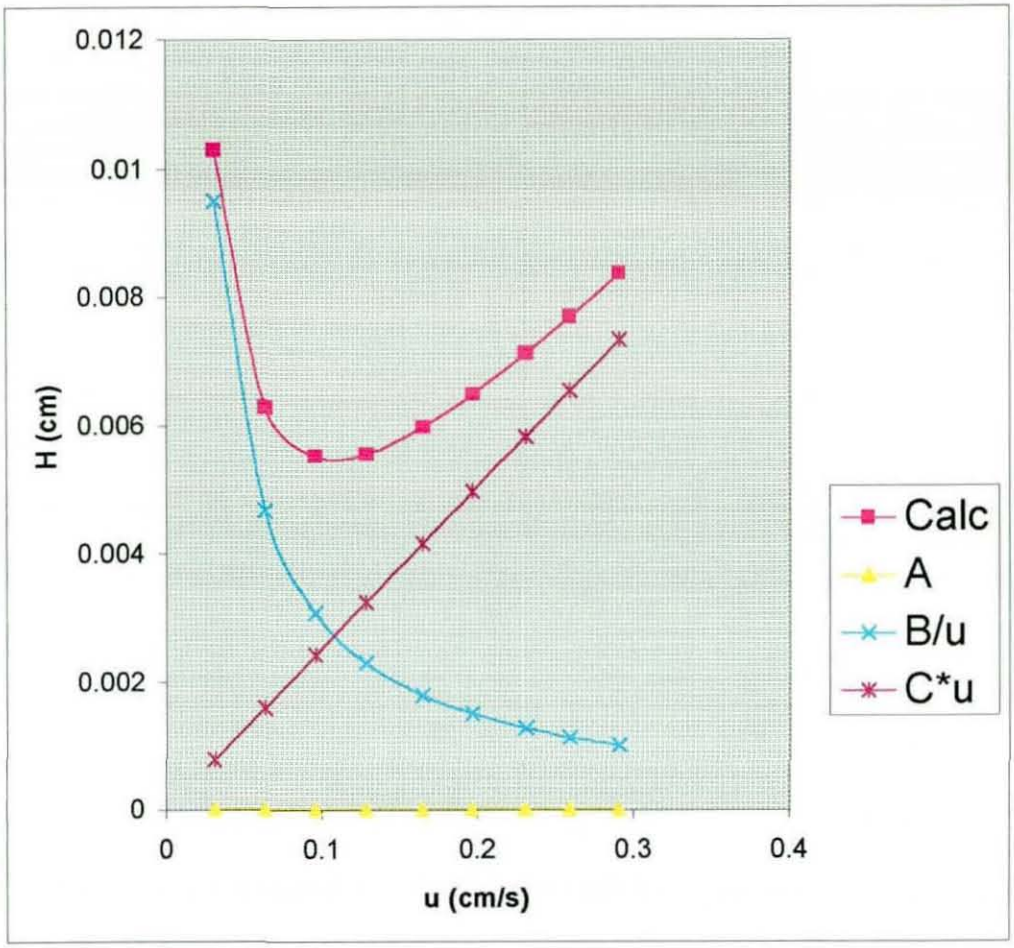



**Figure 4.6: Calculated H vs. u plots obtained on PLRP-S column (150 x 4.6 mm I.D.) for the values of van Deemter curve following a curve fitting procedure from present study.**

The plot for superheated water gives a clear picture of the effect of each individual term in the van Deemter equation. From this plot we can see that the influence of longitudinal diffusion (the B-term in the van Deemter equation) is the major contributor for the plate height observed for superheated water at lower linear velocity. The effect of the B-term with 30 % ACN is smaller than with superheated water (190 °C) (**Table 4.1**).

Two other noteworthy distinctions can be drawn from **Figure 4.6**. The coefficient of the C-term is higher in superheated water (190 °C) than in 30 % ACN (v/v) at 40 °C. Although theory suggests that the value for the C-term

should fall as diffusion in the mobile phase increases with temperature, this is not the case observed in this curve. In fact the 30 % (v/v) ACN mobile phase gave a smaller *C* coefficient than superheated water at 190 °C and thus the contribution to plate height was relatively smaller as the mobile phase velocity was increased. Knox and Vasari (71) reported that the lack of dependence of C-term upon temperature indicates that the activation energy for the rate-determining process is equilibration is close to that for diffusion in the mobile phase.

Finally, the difference in values computed for the A-term is significant. In superheated water the A-term was computed as being smaller than 30 % ACN (v/v) mobile phase (**see Table 4.1**). The reason for this finding is as yet unclear, but it could have something to do with improvements in the laminar flow and lateral mixing of solutes at high temperatures. The theory for this effect has been discussed in detail by Giddings (132). In another study Warren and Bidlingmeyer (135) reported an unanticipated effect of temperature on the *A* term of the van Deemter equation. They claimed a substantial reduction in the *A* term due to radial compression. They concluded that improvements in column efficiency for reversed-phase LC systems are not a guaranteed consequence of increased column temperature when temperature control systems are used.

Also some authors have previously reported that a negative impact of increased temperature on column efficiency of bonded phases (71, 140, 177-178). Poppe et al. (177-178) attributed the negative impact of temperature on *H* to radial thermal gradients that may exist for thermostated columns if the eluent temperature is not precisely equal to the temperature of the outer walls of the column.

In an overall examination of Table (**4.1**), we can see that the coefficient values of B and C are higher and A is lower in superheated water than in 30% ACN at 40 °C. Similar behaviour of the coefficient values of A, B, and C in superheated water reported by Burgess (49) on PGC column for phenol.

Recently Yang et al. (57) reported that because the UV detector is located outside the oven in superheated water chromatography systems, the temperature of the water eluent drops when it exits the oven thus a temperature gradient is established in the outlet tubing between the column and the detector and results in peak broadening.

### 4.3.2: PBD-zirconia columns

#### 4.3.2.1: Micro-bore PBD-zirconia Column (150 x 2.1mm)

PBD-zirconia columns are very stable at high temperature (156) and they have been successfully used in superheated water chromatography for separation of a wide range of aromatic compounds in **Section 3.2.1**. Therefore, we also examined that column to determine the van Deemter coefficient values in superheated water chromatography.

For the construction of an *H* versus *u* plot on the PBD-zirconia column, 2,4,6-trimethylphenol was chosen as the test compound, as it could be eluted at a more modest temperature (150 °C) to reduce stress on the column. The column diameter was 2.1 mm and flow rates from 0.1 to 1 ml/min at 0.1 ml/min increments were chosen to give a representative spread of linear velocities. At higher flow rates the back-pressured increased markedly.

With superheated water $u_{opt}$ is reached at a linear velocity of about 0.15 cm/s (**Figure 4.7**). However, under these conditions, the efficiency of this column was very poor, a maximum of 455 plates.

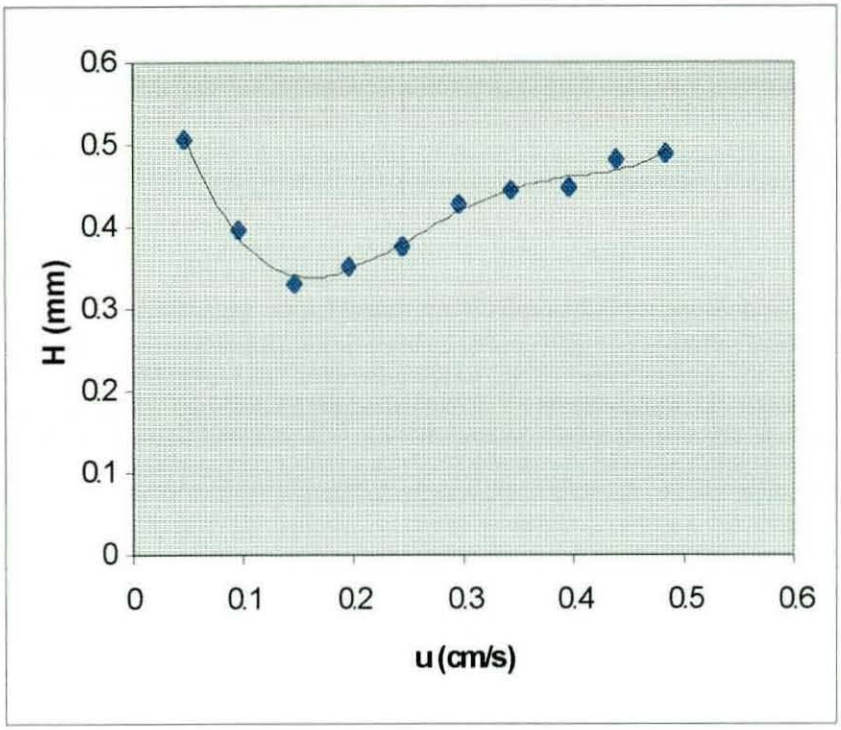



**Figure 4.7: Experimental H vs u obtained on PBD-zirconia column for 2,4,6-trimethylphenol.**

**Conditions: column: PBD-zirconia (150 x 2.1 mm I.D., 3 μm), eluent: water, oven temperature: 150 °C, detection: UV wavelength 254 nm, injection volume: 0.2 μl of 0.05% solution of 2,4,6-trimethylphenol.**

To increase efficiency, temperature was increased (190 °C) and the more highly retained ethylbenzene was injected and flow rate chosen from 0.05 to 1.0 ml/min. Efficiency was reproducible but unusual (**Figure 4.8**). This experiment was repeated but it gave almost the same result.

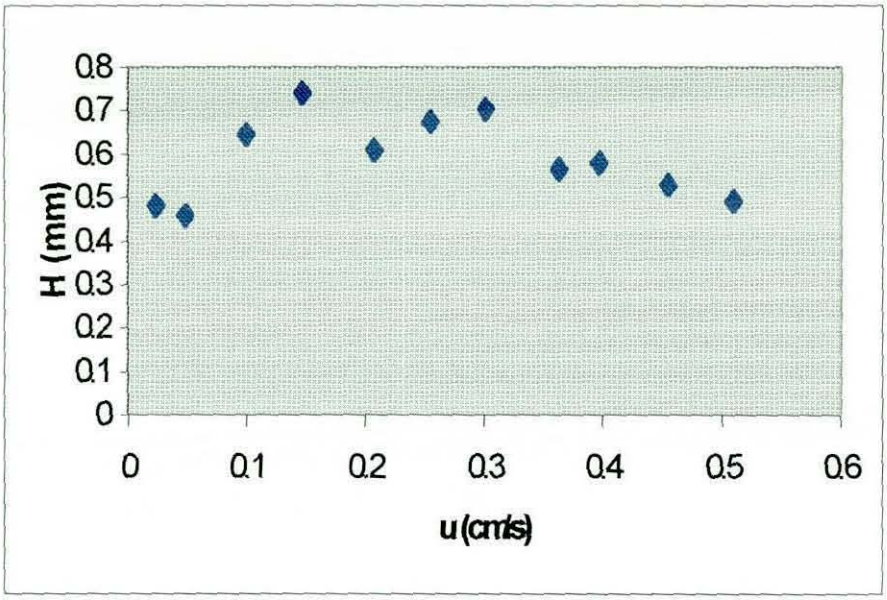



**Figure 4.8: Experimental H vs u plots obtained on PBD-zirconia column using superheated water (190 °C) as the mobile phase.**

**Conditions: column: PBD-zirconia (150 x 2.1 mm I.D., 3 μm), eluent: water, oven temperature: 150 °C, detection: UV wavelength 254 nm, injection volume: 0.2 μl of 0.05% solution of ethylbenzene.**

We therefore tested this column using a conventional HPLC and 25 % ACN (v/v) as mobile phase at room temperature and again it gave very poor efficiency in 25 % ACN (v/v) as mobile phase at room temperature. It appeared that although this material had been claimed to be stable up to 200 °C, this column had rapidly degraded and a void had formed. When the column was opened a gap was found, to a depth of about 3 mm.

#### 4.3.2.2: PBD-zirconia column (150 x 4.6 mm)

A new column was acquired and equilibrated with 50/50 acetonitrile/water as mobile phase in the same equipment (superheated water chromatography system) used for conventional LC and acetone was used for unretained

compound. Methyl benzoate, anisole and toluene were injected to determine column efficiency in conventional LC. The measured efficiencies were compared with the manufacturer's quality control report under the same separation conditions. The test compounds gave lower efficiencies (**Table 4.2**), but were acceptable as the superheated water chromatography system contained considerably more additional tubing (**Section 2.3.1**) than an optimised HPLC system.

**Table 4.2: Comparison of experimental values for retention and efficiency obtained on PBD-zirconia column with reported manufacturer's values.**

**Conditions: flow rate: 1 ml/min, mobile phase: 35 % ACN (v/v), detection: UV wavelength 254 nm, injection volume: 5 μL, Temperature: room temperature.**

| Analyte Name | Reported retention time (min) | Test retention time (min) | Reported efficiency (*N*) | Test efficiency (*N*) |
|---|---|---|---|---|
| Acetone | 1.80 | 1.85 | 8148 | 4114 |
| Methyl benzoate | 3.22 | 3.42 | 14454 | 8270 |
| Anisole | 3.64 | 4.00 | 16645 | 10250 |
| Toluene | 6.03 | 6.95 | 20690 | 15141 |

Anisole was chosen as the test solute for the superheated water separation as it could be eluted at a more modest temperature (140 °C) than was used previously to reduce stress on the column. Flow rates from 0.2 to 2.6 ml/min at 0.2 ml/min were chosen to give a representative spread of linear velocities and injection volume was chosen to be 5.0 μl, because the column diameter is higher than previous PBD-zirconia column. The efficiencies were plotted (**Figure 4.9**).

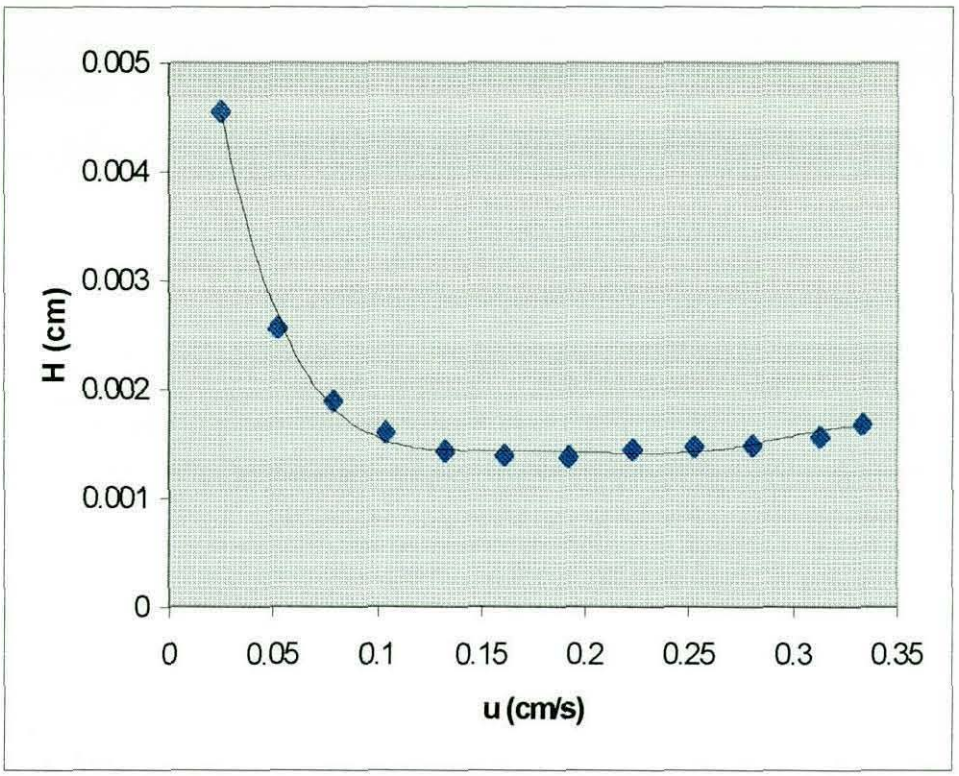



**Figure 4.9: Experimental H vs. Linear velocity plots obtained on PBD-zirconia column (150 x 4.6 mm I.D.) from present study.**

**Conditions: column: PBD-zirconia (150 x 4.6 mm I.D., 3 μm), eluent: water, oven temperature: 140 °C, detection: detection: UV absorption wavelength 254 nm, injection volume: 5.0 μl (0.045%) solution of anisole.**

The optimum linear velocity $u_{opt}$ is reached at about 0.16 cm/s and the highest efficiency was 10818 at a flow rate 1.2 ml/min. This was higher than the efficiency with 35% acetonitrile at room temperature (**Table 4.2**).

In other work Field et al. (67) reported the van Deemter curve for a PBD-zirconia column with superheated water chromatography. They used a 150 cm x 0.007 in I.D. preheating coil to provide heat transfer to the mobile phase prior to entering the column, and injected the higher mass compound testosterone at a temperature of 160 °C. The optimum velocity was around 1

ml/min and the optimum plate height was 7198 with a retention time of 17.5 min. They found that the efficiency decreased at higher flow-rates, and attributed this to increased thermal gradients. They did not describe a cooling system of mobile phase from column to detector. In contrast, our study used a 100 cm x 0.01 in I.D. preheating coil from the injector to column. The same tubing was used from column to detector, and 10 pieces (3 cm x 12 cm x 0.05 mm) of cooling foil was used to cool down the mobile phase before detector and a smaller molecule analyte anisole was injected. In our study, the plate height vs. flow-rate curve (**Figure 4.9**) indicates that the optimum velocity was around 1.2 ml/min and the plate height was higher (10818) than their study although retention time of anisole is smaller than testosterone.

In the present study, the coefficients values of *A, B, C* of the van Deemter curve were calculated **(Figure 4.10) (Table 4.3)**. The B coefficient in superheated water (140 °C) on the PBD-zirconia is greater than PS-zirconia column for 40 % ACN at 25 °C. Theory (169) predicts that the B term should increase as the column temperature is increased. The B term is only significant in the low velocity region and its consequences are nearly negligible at high operating velocities. Thus it will be advantageous to operate the columns at high temperatures and flow rates: this will improve the speed of separation.

**Table 4.3: Computed values of van Deemter coefficients for Superheated Water (140 °C) and 40 % ACN at 25 °C on zirconia column**

| Columns and Analytes | A | B<br>$Cm^2/sec$ | C<br>sec |
|---|---|---|---|
| On PBD-Zirconia (Analyte Anisole) | 0.00031 | 0.00011 | 0.00290 |
| On PS-Zirconia {Analyte acetophenone, data obtained from ref. (63)} | 0.0010 | 0.00003 | 0.00012 |

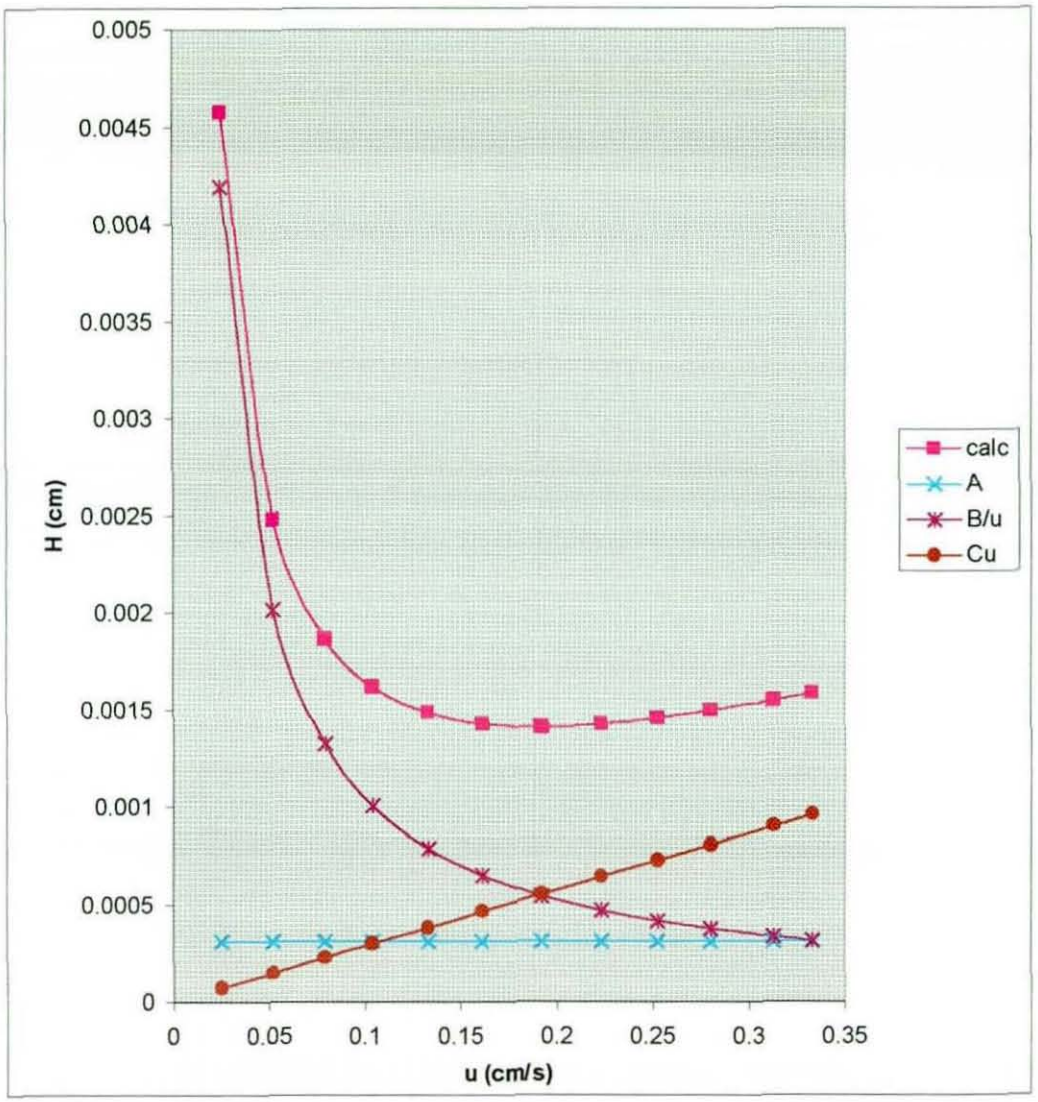



**Figure 4.10: Calculated H vs. u plots obtained on PBD-zirconia column (150 x 4.6 mm I.D.) for the value of van Deemter curve following a curve fitting procedure from present study.**

The C coefficient (**Table 4.3**) is higher in our study using superheated water than in the previous study (64) using the PS-zirconia column at ambient temperature with 40 % ACN (v/v). Carr et al. explained in another paper (142) that an increase in temperature would improve the diffusional rate in the stationary phase (and increase the rate constant ($k_d$) for the solute desorption process from the surface) and, therefore, decrease the C coefficient if the mass transfer resistance in the stationary phase was the dominant process on a PBD-zirconia column.

In superheated water on a PBD-zirconia column, the A-term was computed as being much smaller than in 40 % ACN mobile phase (**Table 4.3**) on a PS-zirconia column. But the A-term should be dictated more by the bed structure and be independent of temperature. The effect of temperature on the A coefficient is uncertain (132), however, high temperature should improve the laminar flow and lateral mixing of molecules among different flow channels. Thus, elevated temperature might improve the A term.

### 4.3.3: Xterra ™ RP 18 column (150 x 4.6 mm)

In Chapter 3, we examined the Xterra ™ RP 18 column for the separation of alkyl aryl ketones and eight aromatic compounds. Wilson (66) used the same column in superheated water chromatography for separation of a range of drug compounds. They concluded that Xterra ™ RP 18 column is more stable at high temperature and like the conventional C18 phase all five test compounds were eluted from the Xterra ™ RP 18 column, with excellent peak shape. Therefore, our attention has driven to construct van Deemter curve using this column in superheated water chromatography.

The effect of flow-rate on efficiency (**Figure 4.11**) for p-cresol on Xterra ™ RP 18 column (150 x 4.6 mm) was studied over the range of 0.2 to 2.2 ml/min. The column void time ($t_0$) was estimated by injecting sodium nitrate solution. A temperature of 130 °C was chosen because in the previous work at 140 °C it appeared that 60 hours running some of the silica had dissolved.

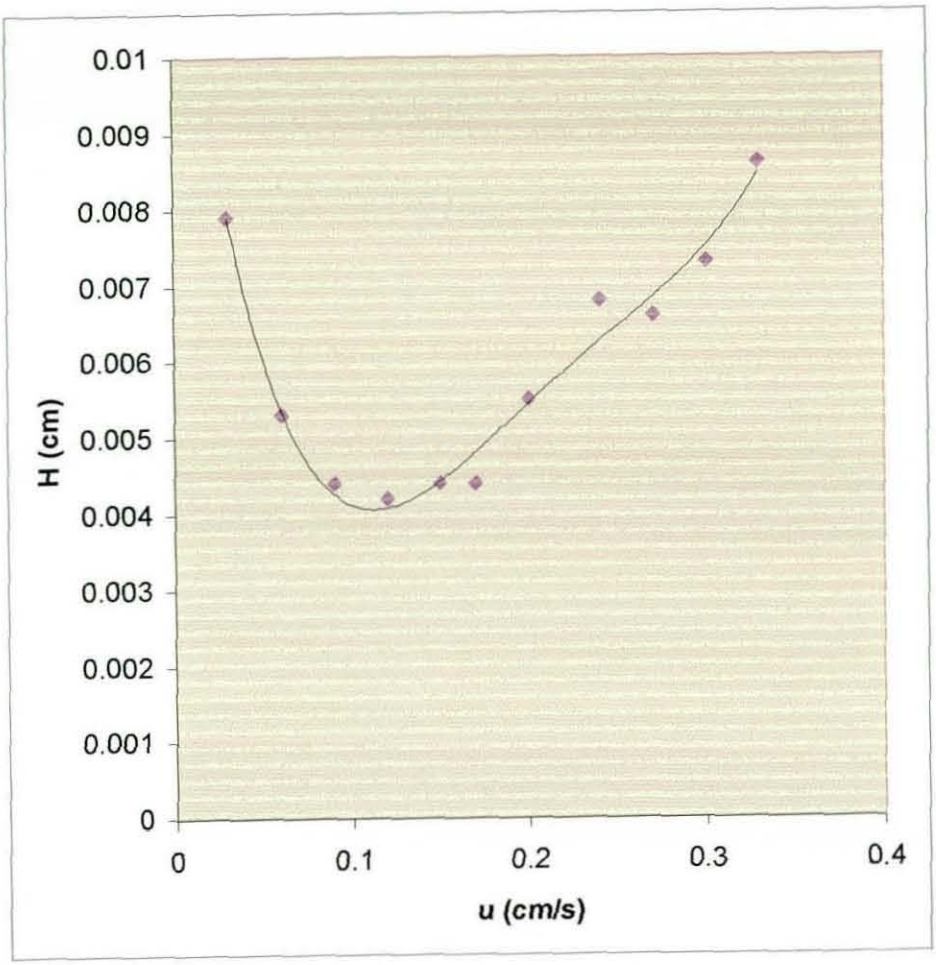



**Figure 4.11: Experimental H vs u plots obtained on Xterra ™ RP 18 silica based column (150 x 4.6 mm I.D.) from present study.**

**Conditions: column: Xterra ™ RP 18 (150 x 4.6 mm I.D., 5 μm), eluent: water, oven temperature: 130 °C, detection: UV wavelength 254 nm, injection volume: 10 μl 0.5% solution of p-cresol.**

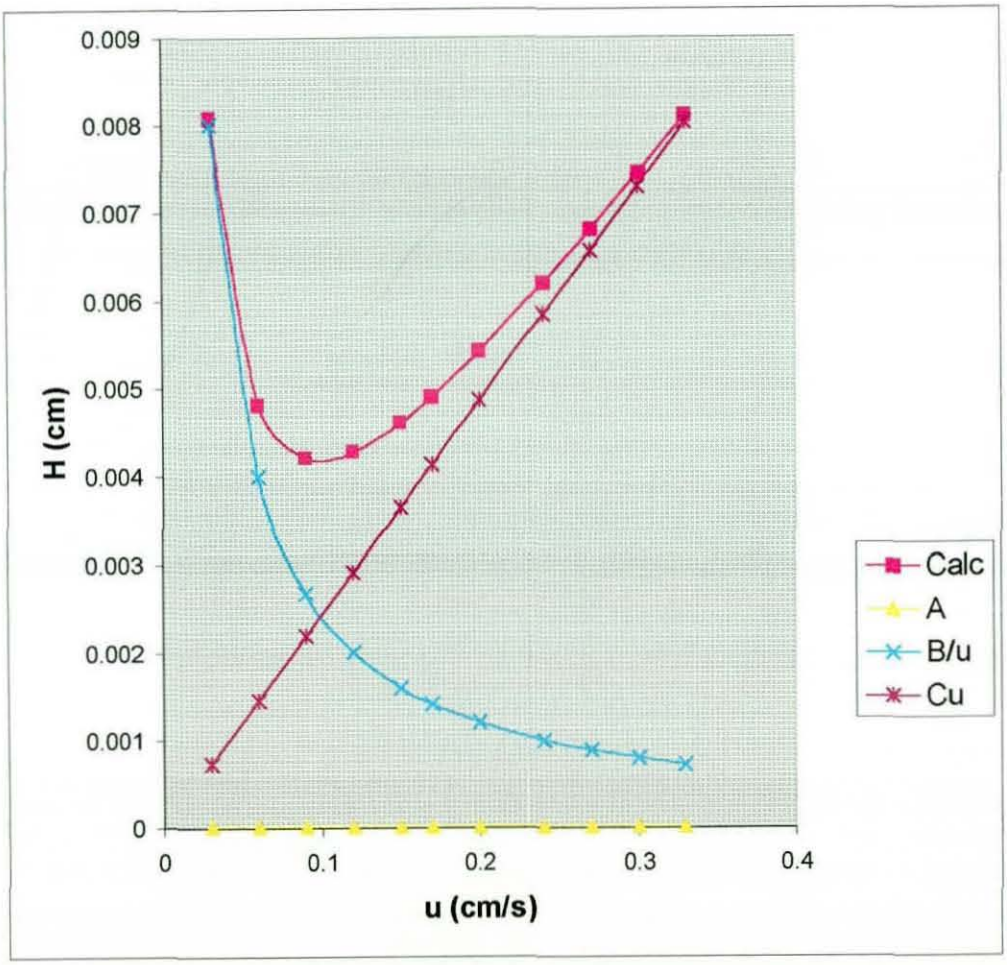



**Figure 4.12: Calculated H vs. u plots obtained on Xterra ™ RP 18 column (150 x 4.6 mm I.D.) for the value of van Deemter curve following a curve fitting procedure from present study.**

In this study, the efficiency vs. linear velocity (**Figure 4.11**) indicated that the optimum plate number was 3572 at a velocity around 0.12 cm/s. The minimum reduced plate height corresponded to about 0.04 mm. The efficiency decreased at higher flow-rates.

Computed values based on calculated A, B, and C-terms (**Table 4.4**) are plotted with respect to u (**Figure 4.12**). From these plots it is clear that the influence of longitudinal diffusion (the B-term in the van Deemter equation) continues to be a major contributor for superheated water.

**Table 4.4: Computed values of van Deemter coefficients for Superheated Water (130 °C) on Xterra ™ RP 18 column and 30 % ACN at 40 °C on polystyrene column.**

| Columns and Analytes | A | B<br>Cm2/sec | C<br>sec |
|---|---|---|---|
| On Xterra column (Analyte p-cresol) | 0.00001 | 0.00024 | 0.02433 |
| On polystyrene column {(Analyte phenol, data obtained from ref. (49)} | 0.00124 | 0.00002 | 0.00912 |

The value of the C-term is higher in superheated water on Xterra ™ RP 18 than 30 % ACN at 40 °C on polystyrene column. This result contradicts theory, because the diffusion coefficients increase as the temperature is increased and the C-term should therefore decrease. In superheated water the A-term on the Xterra ™ RP 18 column was lower than with 30 % ACN as mobile phase on a polystyrene column.

## 4.4: Summary

Efficiency of high temperature liquid chromatography (HTLC), with a superheated water mobile phase, depends on instrumental parameters of flow-rate, injection volume and mobile phase preheating. Simply elevating the column temperature does not increase the efficiency of a separation. In our detailed study of superheated water instrumental parameters, the preheating tubing of the mobile phase from injector to column, cold mobile phase before detector, small volume dilute sample and flow rate were found to have significant effects on the efficiency of the column.

A study of the band broadening in superheated water chromatography, found that the polystyrene column gave better efficiency. The efficiency of this column increased from N=1505 to 4000 after changing the preheating tubing from 85 cm (0.02 in. I.D.) to 100 cm (0.01 in. I.D.).

The values of the A-term are lower on three columns (polystyrene, Xterra ™ RP 18 and PBD-zirconia) in superheated water chromatography than conventional LC. The coefficient values of C and B-term are higher in superheated water chromatography on these columns than conventional LC.

Although high efficiency was not achieved with superheated water on polystyrene and Xterra ™ RP 18 columns, a higher efficiency was achieved on a PBD-zirconia column with superheated water than conventional LC with 35 % ACN at 1 ml/min flow rate at room temperature.

# CHAPTER 5

# Superheated Heavy Water as the Eluent for LC-NMR Analysis of Pungent Constituents of Ginger

## 5.1: Introduction

As the properties of heavy water (deuterium oxide) are very similar to those of water (59), it was considered that it could be employed as a superheated mobile phase and should provide an ideal eluent for coupling on-line and also off-line separations to NMR spectroscopy. Compared with deuterated organic solvents, it is comparatively cheap and is available in a high state of purity with no organic impurities. The absence of large signals, from the protons of the organic modifier in the HPLC solvents, should considerably simplify the NMR spectroscopic determination.

Smith et al. (179) have used superheated deuterium oxide as a mobile phase for the HPLC-NMR separation of barbiturates in both the on-line and stop-flow modes of detection (59) and the same research group also used superheated deuterium oxide for a HPLC-NMR and HPLC-NMR-MS for model drug compounds (58), such as barbiturates, some analgesics, and a kava extract. Wilson and colleagues have carried out a number of LC-NMR separations using this solvent (180-181).

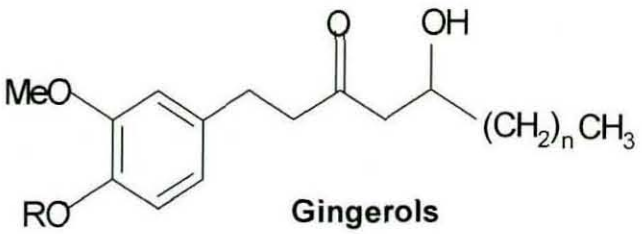
In the present study, we have examined the separation of extracts of ginger and grains of paradise extract using coupled superheated water chromatography with NMR using deuterium oxide as the mobile phase.

## 5.2: Analysis of Ginger Extracts

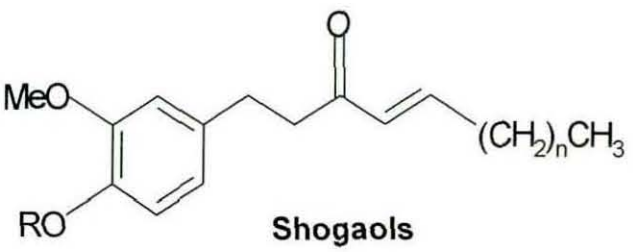
Ginger is the rhizome of ***Zingiber officinale Roscoe*** ***(Zingiberaceae)***, a plant cultivated in many tropical and subtropical countries. The part used is the pungent rhizome, commonly called 'root' both in fresh and dried forms. Major exporting countries are India, Australia, Fiji, Nigeria and China (182). As a spice, it is widely consumed in Asian cooking. In Chinese traditional

medicine it is used as a stomachic, antiemetic, antidiarrheal and cardiotonic, for the treatment of several gastrointestinal and respiratory diseases. Powdered ginger is also used for the treatment of motion sickness (183).

The constituents responsible for the pungent taste of ginger are a homologous series of phenolic ketones, the [4]-, [6]-, [8]-, [10]- and [12]-gingerols and shogaols (**Figure 5.1**).




**Gingerols**




**R=H** **Shogaols**

**Figure 5.1: Gingerols (n = 2, 4, 6, and 8 represent [4]-gingerol, [6]-gingerol, [8]-gingerol and [10]-gingerol respectively), shogaols (n = 2, 4, 6, and 8 represent [4]-shogaol, [6]-shogaol, [8]-shogaol and [10]-shogaol).**

The shogaols are derived from the corresponding gingerols during thermal processing or long-term storage (184). Shogaols are gingerol analogues with a 4, 5-double bond, resulting from elimination of the 5-hydroxy group. Several methods (GC-MS, TLC, HPLC, and HPLC-MS) (185 -187, 182) have been described for the analysis of the pungent constituents of ginger.

However, the main pungent constituents are thermally unstable and decompose under high temperature during GC and GC-MS analysis (188-189). HPLC has been used for ginger analysis (187); however, identification

may require retention time standards for identification of specific peaks. Obtaining these authentic compounds requires the expenditure of time and money. However, developments in the hyphenation of liquid chromatography and nuclear magnetic resonance spectroscopy (LC-NMR) offers the potential for unknown peaks to be identified rapidly and without the need for extensive purification and standard. Therefore, nowadays high-performance liquid chromatography-nuclear magnetic resonance (HPLC-NMR) and HPLC-MS can play an important role in natural product analysis (190). Analysis of pungent constituents of ginger by LC-NMR has not previous been reported.

LC-NMR is an important technique for the identification and structure elucidation of analytes from complex mixtures obtained from drug metabolism (191), environmental samples (192), and combinatorial synthetic methods. The principal complications that remain are often associated with strong signals in the NMR spectrum resulting from the proton-containing solvents used as conventional chromatographic eluents, which can either swamp the spectrum or result in interfering signals (193). In addition, even the purest conventional "HPLC-grade" mobile-phase constituents can also contain proton-containing impurities, which contribute additional interfering signals. The main signals can be suppressed by using a suitable pulse train, which can overlap with the signal resonance, but this can mean the loss of signals from a region of the analyte spectrum. Deuterated solvents, such as $D_3$-acetonitrile with minimal proton signals, can be used but these are costly. Alternatively a proton free solvent, such as supercritical fluid carbon dioxide, can be used in a normal phase mode (194). However, the spin-lattice relaxation time, $T_1$, is increased, resulting in a longer spectral acquisition time (194). The NMR flow cell must also be capable of operating at high pressures, up to 400 bars, and these must be maintained under stop-flow conditions and when pressure gradients are employed there are problems due to changes in the chemical shift with eluent density (195). Most importantly, SFC-NMR is essentially a normal-phase separation technique and is more restricted in its application than reversed-phase HPLC-NMR. Consequently, unmodified $CO_2$ is only really suitable for relatively nonpolar analytes, such as the phthalate esters (196) and vitamin A (194).

## 5.3: Separation of Ginger and Grains of Paradise Extract

In previous chapters, the PBD-zirconia column has been used successfully with superheated water chromatography for the separation of a wide range of aromatic compounds and also in the efficiency study. Therefore, initial studies tried to use PBD-column to separate a methanol extract of ginger and grains of paradise.

A separation (**Figures 5.2 and 5.3**) was obtained at 150 °C using superheated water as the eluent. However, the efficiencies of the separation of these compounds were very poor.

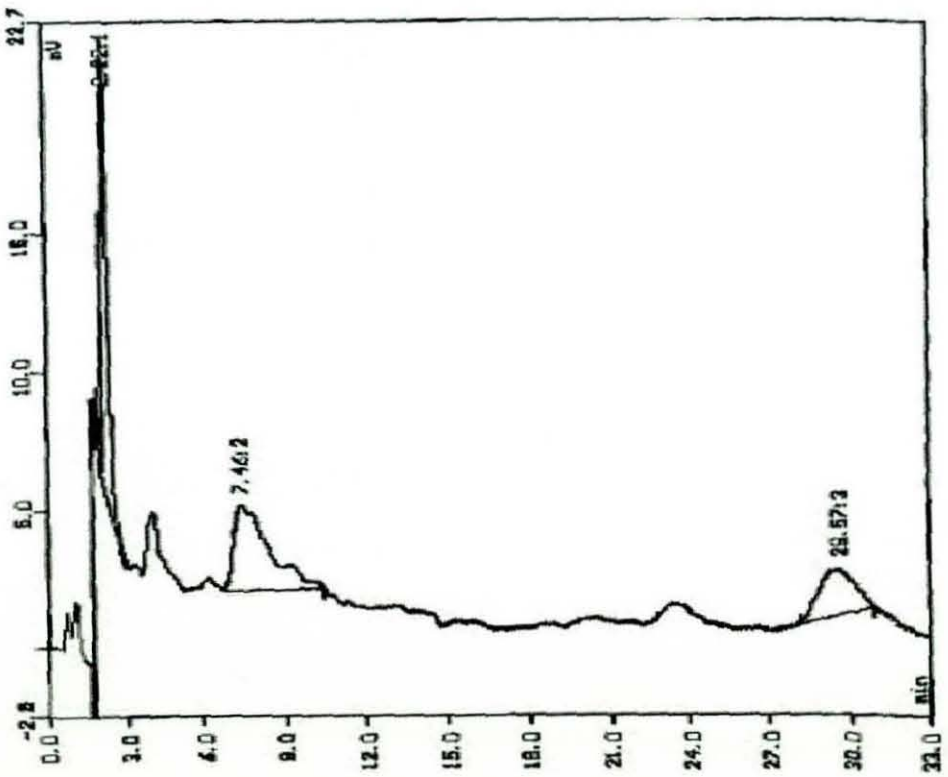



**Figure 5.2: Separation of ginger extract on a PBD-zirconia column using superheated water (150 °C) as the mobile phase.**

**Chromatographic conditions: column: PBD-zirconia (150 x 4.6 mm I. D.), mobile phase: superheated water (150 °C) at flow rate 1.0 ml/min, back-pressure: 35 kg/cm$^2$, detection: UV-absorption wavelength 280 nm, range: 0.02 amps/mV.**

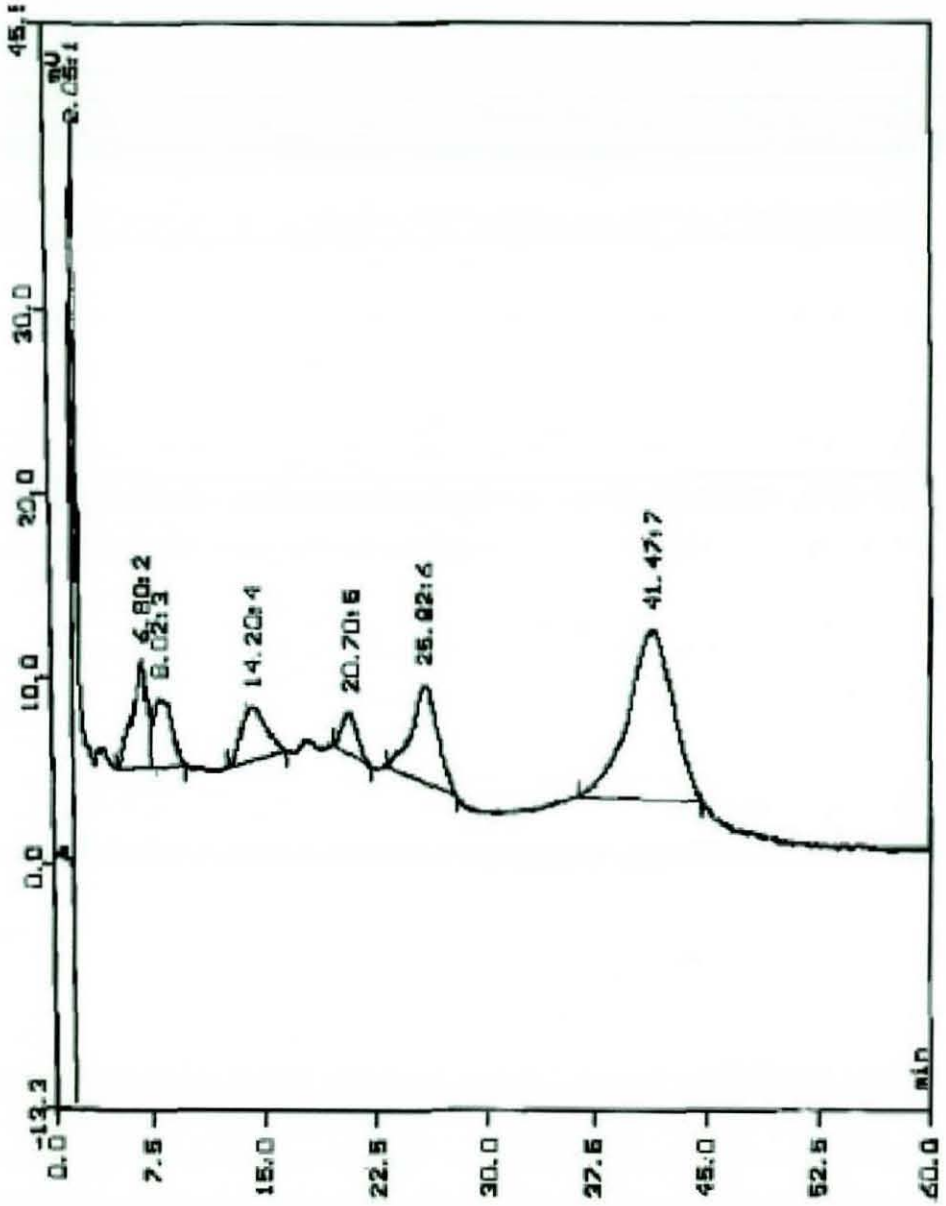



**Figure 5.3: Separation of grains of paradise extract on PBD-zirconia column using superheated water as the mobile phase.**

**Chromatographic conditions: as Figure 5.2.**

The same samples in conventional LC, on elution with 35:65 (v/v) acetonitrile-water also gave poor efficiency (**see Figure 5.4**). In contrast, earlier work had obtained good results with ODS silica columns (182,187).

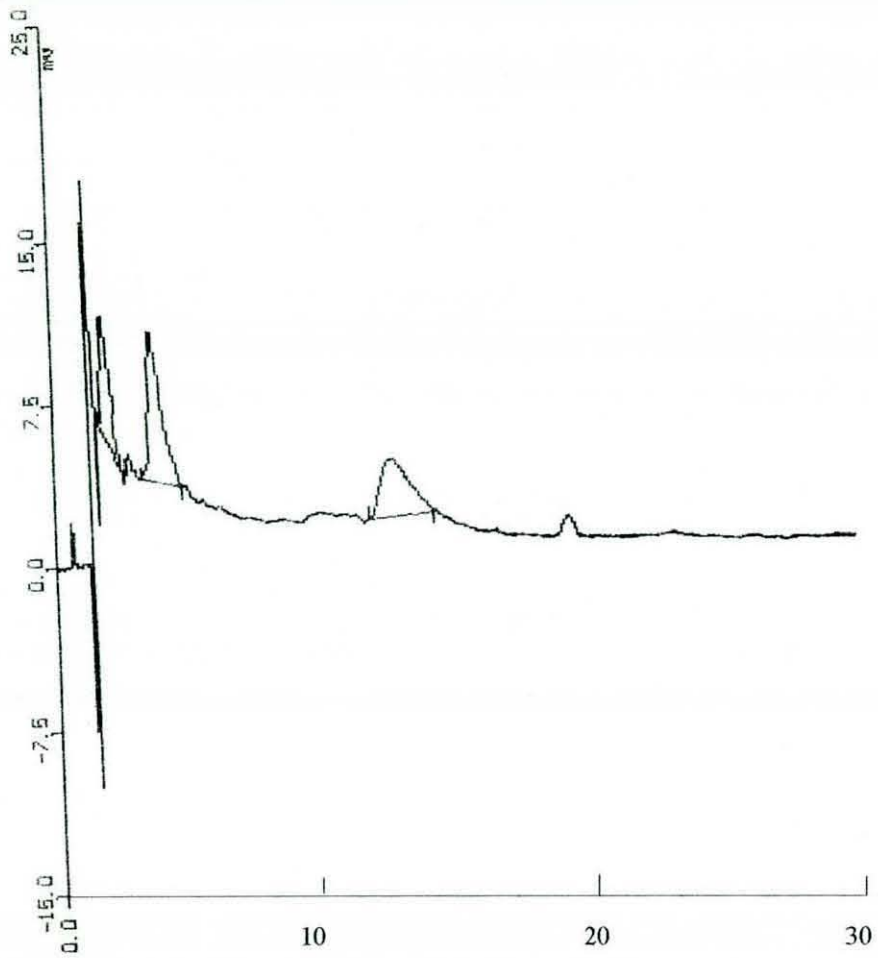



**Figure 5.4: Separation of ginger extract on a PBD-zirconia column using 35:65 ACN/water as the mobile phase at room temperature.**

**Chromatographic conditions: column: PBD-zirconia (150 x 4.6 mm I. D.), mobile phase: 35:65 ACN/water mixture at room temperature, flow rate: 1.0 ml/min, detection: UV-absorption: 280 nm, range: 0.02 amps/mV.**

However, these columns are thermally unstable at high temperature in hot water.

Recently, Xterra ™ RP 18 silica based columns have been introduced for HPLC. They are more stable at high temperatures (66) because the base is a mixture of inorganic and organic groups (replacing one third of the surface silanol groups with methyl groups). Therefore, an Xterra ™ RP 18 column was examined for the separation using superheated water as a mobile phase at different isocratic temperatures (140 °C, 130 °C, 120 °C, 110 °C and 100 °C). Some compounds did not separate very well in these separation conditions. Therefore, to imitate the gradient elution of RP-HPLC, a temperature programme was applied for better separation (59). By ramping the temperature of the oven from 50 °C to 130 °C at 4 °C $min^{-1}$, the extract was well separated **(Figure 5.5).**

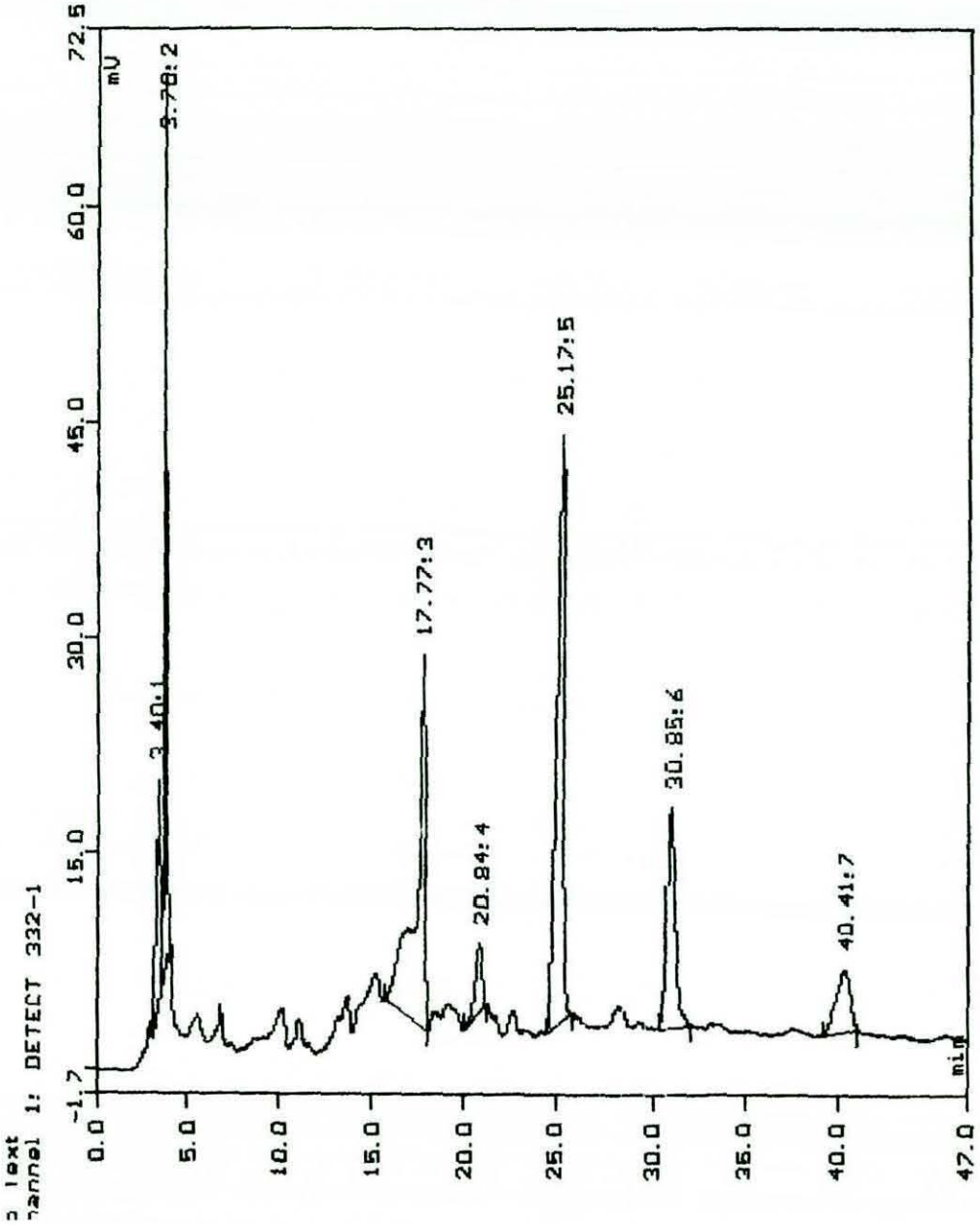



**Figure 5.5: Separation of ginger extract on Xterra ™ RP 18 column using superheated water (from 50 °C to 130 °C at 4 °C $min^{-1}$) as the mobile phase.**

**Separation conditions: column: Xterra ™ RP 18 column (150 X 4.6 mm I. D.), mobile phase: superheated water (from 50 °C to130 °C at 4 °C $min^{-1}$) at flow rate 0.5 ml/min, back-pressure: 35 $kg/cm^2$, detection: UV-absorption wavelength 280 nm, range: 0.02 amps/mV, injection volume: 30 μl.**

The same separation conditions were applied to the separate of an extraction of grains of paradise (**Figure 5.6**). The retention times of the peaks 17.5, 20.32, 24.74, 30.86 and 41.41 min corresponded to those in the ginger extract.

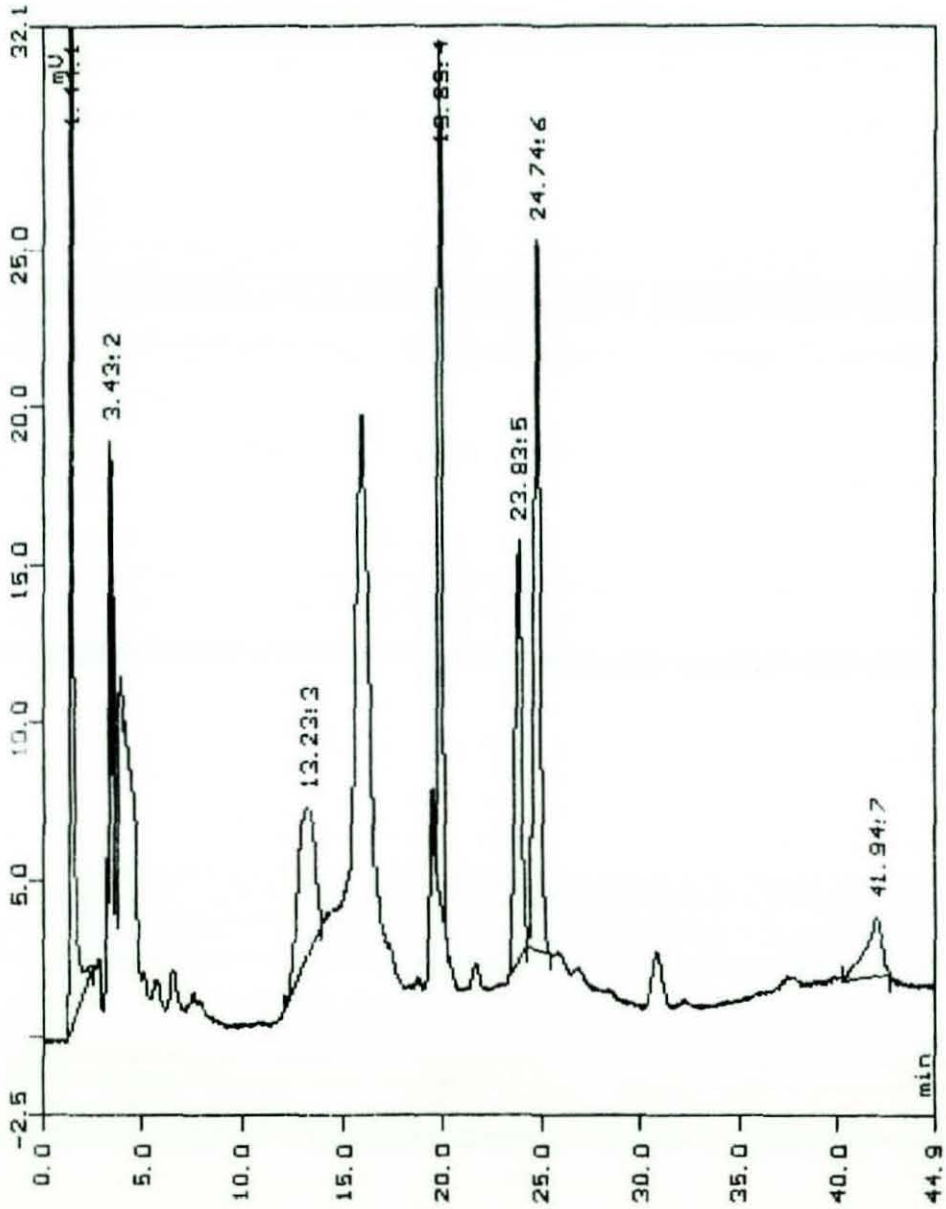



**Figure 5.6: Separation of grains of paradise extract on Xterra ™ RP 18 column using superheated water (from 50 °C to 130 °C at 4 °C $min^{-1}$).**

**Separation conditions: as Figure 5.5.**

### 5.3.1: Coupling Superheated Water Chromatography with NMR Spectroscopy

The separation was then transferred to a LC-NMR system using deuterium oxide as the eluent. Because of a need to position the LC away from the NMR magnet a 3-m polyethyl ether ketone (PEEK) narrow bore tube was used to couple the UV detector to the NMR flow cell. The length also created enough back-pressure to maintain a liquid state of heavy water in the chromatographic column so that a separate back-pressure regulator was not required.

Extracts were prepared at high concentrations because the sensitivity of NMR is moderately low. Firstly, an on-flow mode was used but this mode could identify only one compound (peak 2) as dihydroferulic acid (**Figure 5.7 and Figure 5.8**). Other peaks were visible but were insufficient for on-flow detection.

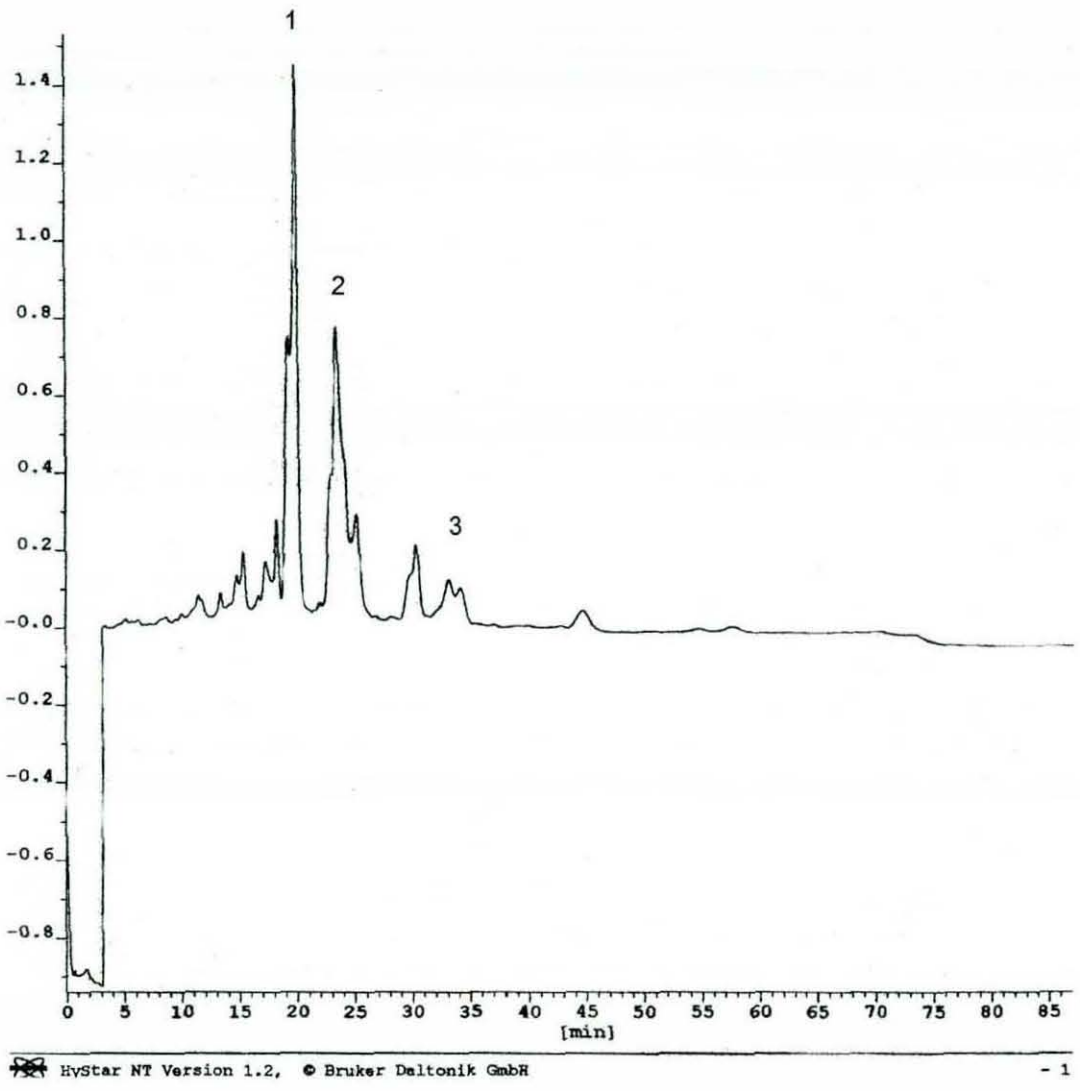



**Figure 5.7: First injection of ginger extract on Xterra ™ RP 18 column using superheated deuterium oxide as the mobile phase in superheated water chromatography-NMR system.**

**Separation conditions: column: Xterra ™ RP 18 column (150 x 4.6 mm I.D.), mobile phase: superheated deuterium oxide (from 50 °C to 130 °C at 4 °C $min^{-1}$) at flow rate 0.5 ml/min, detection: UV-absorption 280 nm, range: 0.02 amps/mV, injection volume: 100 μl.**

The spectrum (**Figure 5.8**) of peak 2 in the chromatogram could be identified as dihydroferulic acid, as it gave two doublets at 6.90, and 6.80 ppm and at 6.75 ppm doublet-doublet signal. These were assigned to the three protons of a 1,2,4-substituted aromatic ring. Two triplets at 2.60 and 2.82 ppm corresponded to $-CH_2$ and $-CH_2$ groups attached to an acid group ($-CH_2CH_2COOH$).

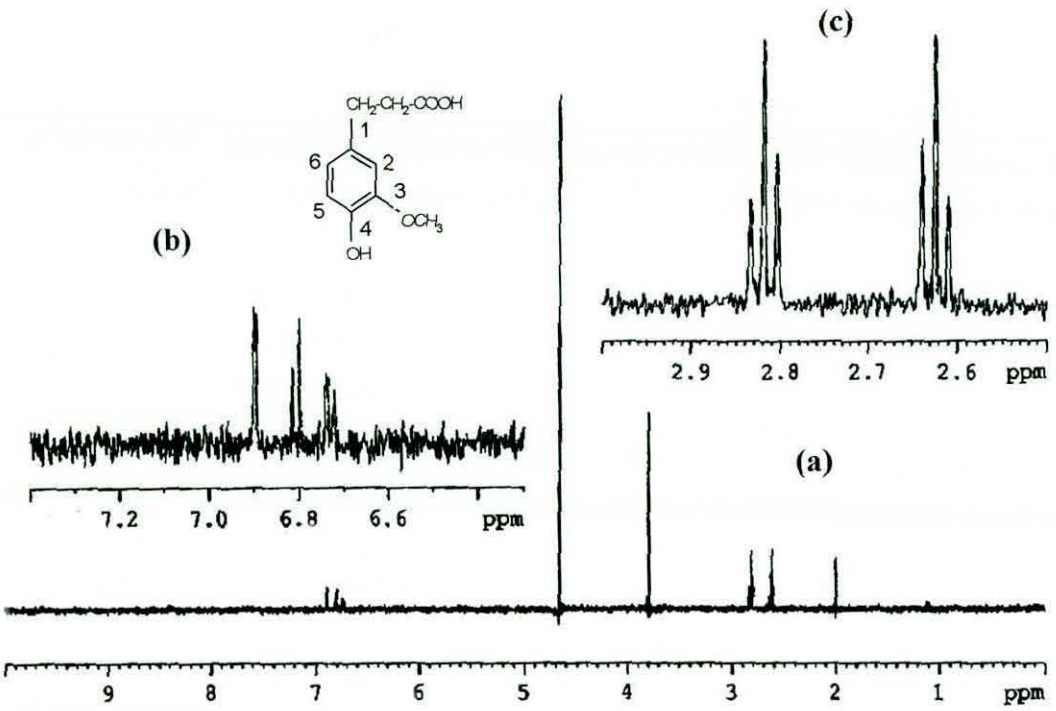



**Figure 5.8: Superheated Water Chromatography-NMR spectrum obtained using on-flow mode for peak 2.**

**(a) = A whole range spectrum between 1-10 ppm**

**(b) = An enlarged spectrum of (a) in a range of 6.3-7.4 ppm**

**(c) = An enlarged spectrum of (c) in a range of 2.5-3.0 ppm**

**Conditions: Magnetic field 500 MHz**

Although this spectrum appeared to be the same as that of dihydroferulic acid there were significant differences in the positions of the aromatic proton signals compared to those in a spectrum of dihydroferulic acid in deuterium oxide (**Figure 5.9a)**. Initially is was suspected that the isomeric compound with the hydroxyl and methoxyl group reversed might be present but when a sample was prepared by the hydrogenation of isoferulic acid the spectrum was again different and the C-2 proton was at a much higher field (**Figure 5.10**).

Because of a suggestion that the retention of dihydroferulic acid was dependent on the pH (**see later**) the effect of pH on the NMR spectrum was examined. Firstly the NMR spectrum of the standard was examined in acidic solution (HCl) (**Figure 5.9b**). In this case the C-2 proton was at slightly higher field and was less resolved from the C-5 doublet. If the pH was raised with bicarbonate solution in deuterium oxide the opposite shift was observed and the C-2 proton signal was now at a much lower field (**Figure 5.9c**) and closely resembled the spectrum obtained from the ginger extract (**Figure 5.8**). There was now a close correspondence across the full spectrum (**Figure 5.11**) with the standard. Suggesting that the sample from ginger was significantly ionised when examined in the on-line mode.

This study demonstrates an unusual effect of a pH change in NMR spectrum, which is not normally observed in the conventional deuterochloroform solvent. Although the ionisable carboxyl group is remote from the ring, a hydrogen bonding type interaction might be occurring between the carboxyl OH group and the C-3 methoxyl group that causes any ionisation to have a more significant influence on the C-2 proton compared to the C-5 and C-6 protons.

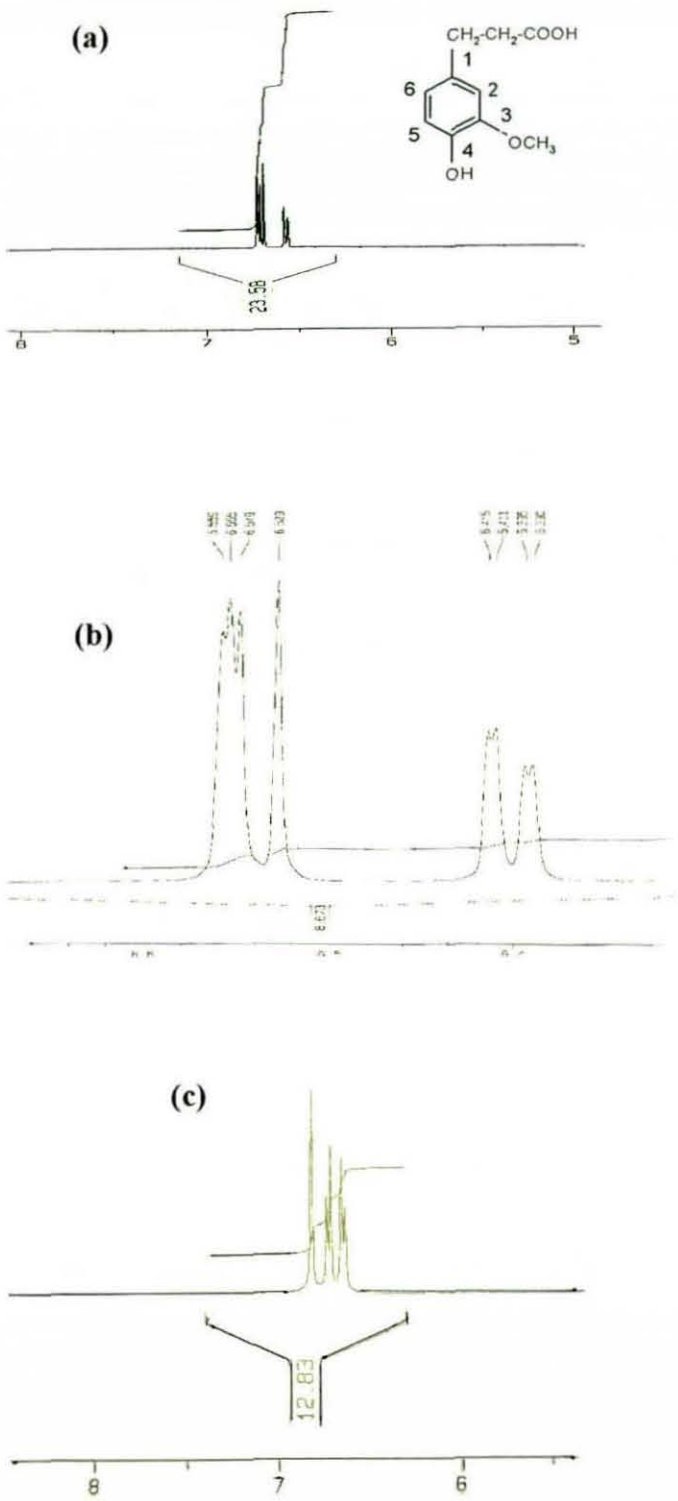



**Figure 5.9: Aromatic protons of dihydroferulic acid in (a) unbuffered condition, (b) acidic condition, and (c) basic condition.**

**Condition: Magnetic field 400 MHz**

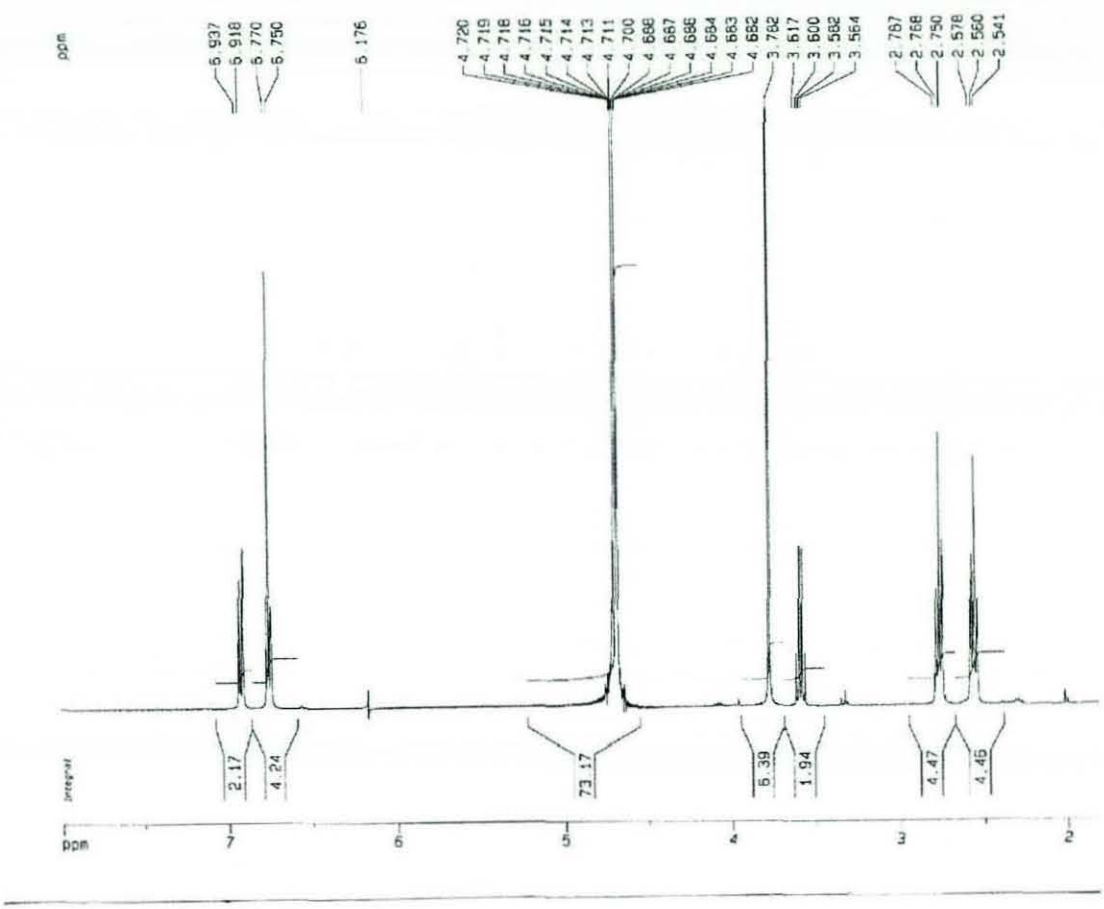



**Figure 5.10: NMR spectrum of saturated solution of isodihydroferulic acid in deuterium oxide.**

**Condition: Magnetic field 400 MHz**

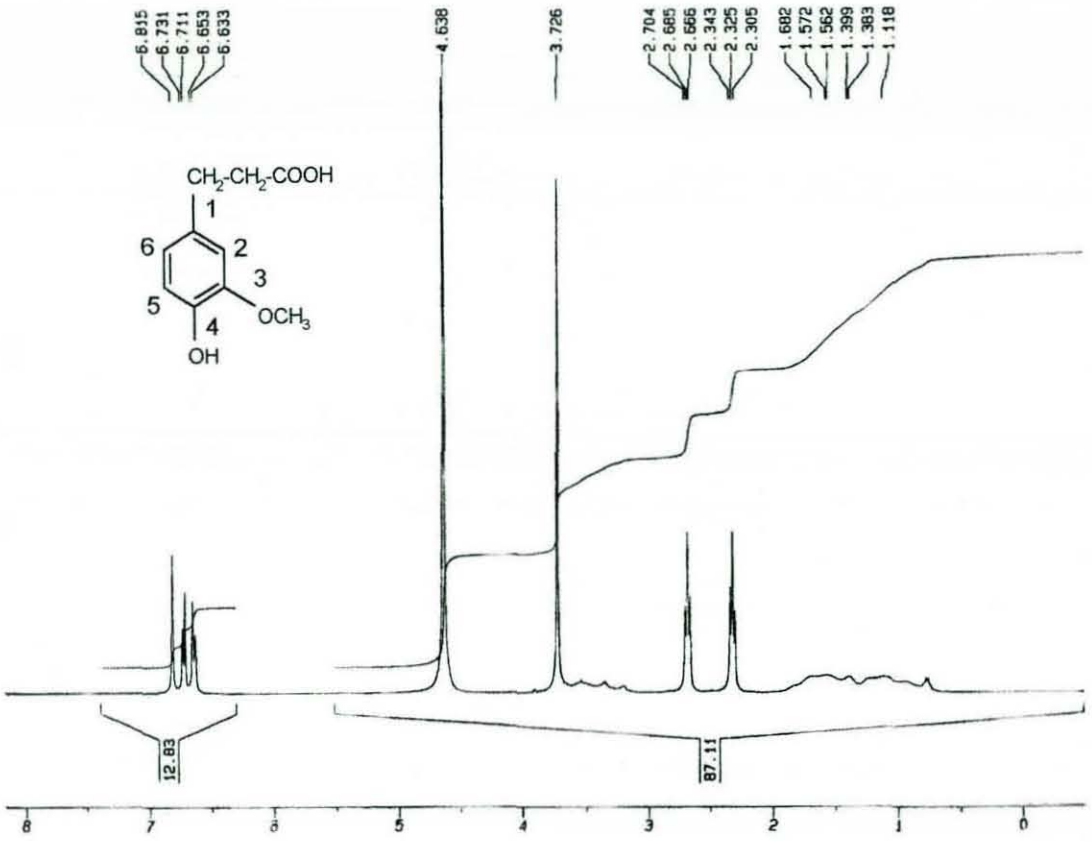



**Figure 5.11: NMR spectrum of saturated solution of a standard dihydroferulic acid in alkaline deuterium oxide.**

**Condition: Magnetic field 400 MHz**

To increase the sensitivity, the separation was repeated using the stop-flow mode by connecting the column to a Bruker Peak Sampling Unit with 12 collection loops. The UV detector was overloaded and peak shapes were distorted **(Figure 5.12).**

The three peaks (1-3) were collected in the loop collector. They were then transferred sequentially to the NMR and scanned in the off-line mode **(Figures 5.13, 5.15 and 5.16).**

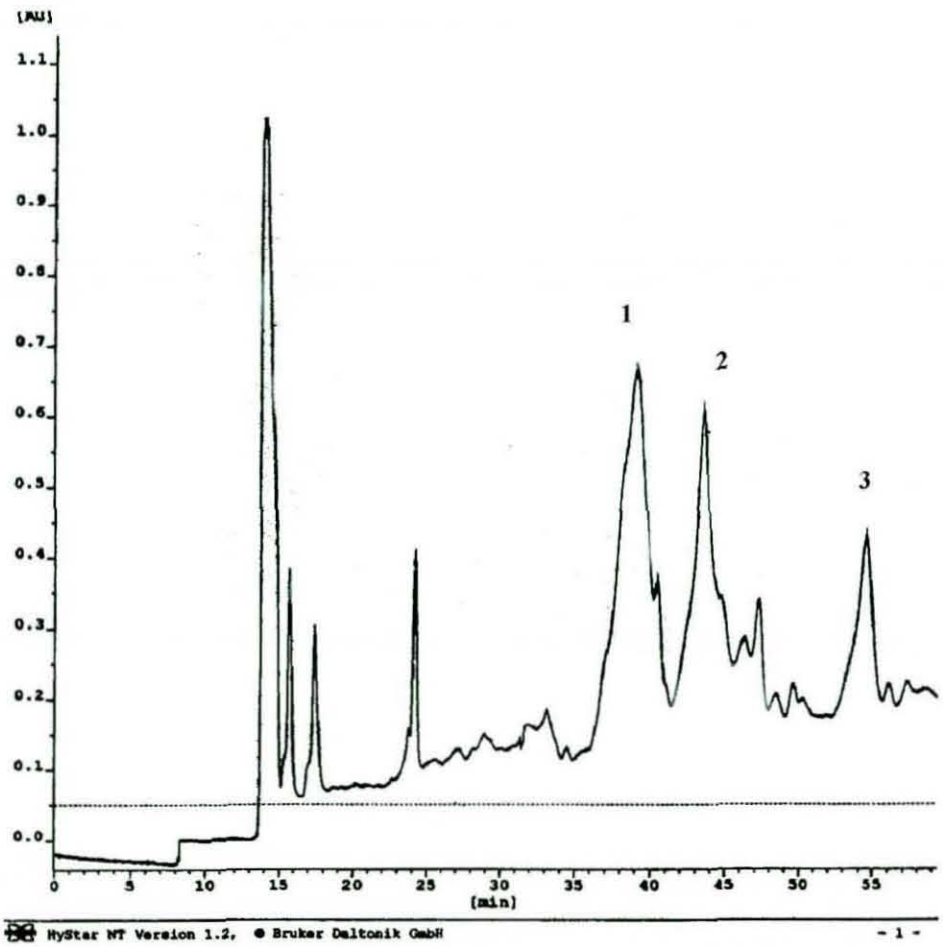



**Figure 5.12: Separation of high concentration ginger extract on Xterra™ RP 18 column using superheated water (from 50 °C to 130 °C at 4 °C min$^{-1}$) as the mobile phase.**

**Separation conditions: as Figure 5.7**

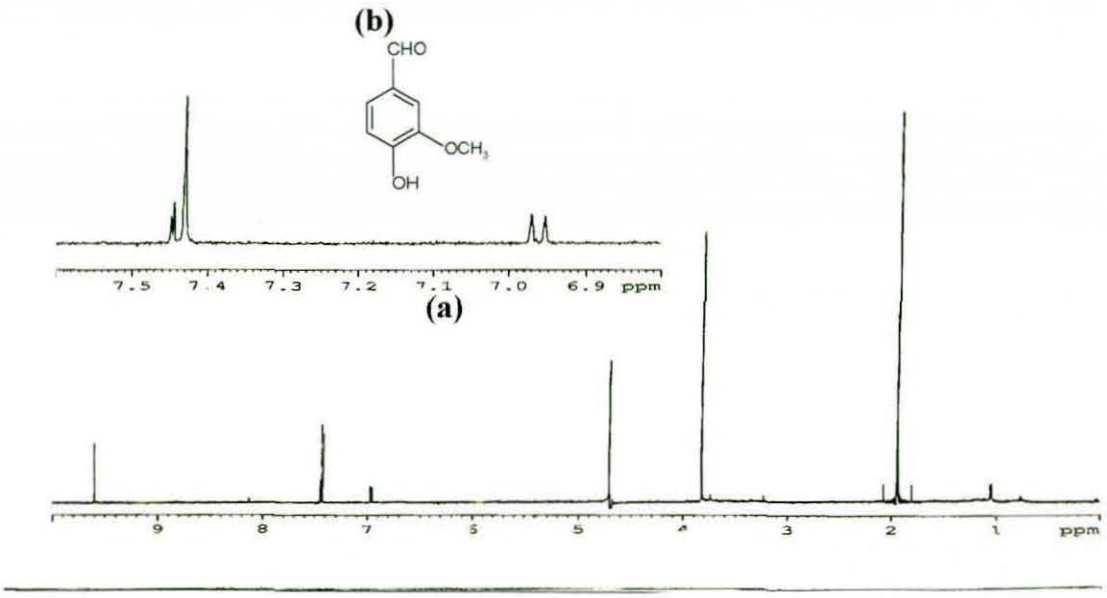



**Figure 5.13: Superheated water chromatography-NMR spectrum obtained using the stop-flow mode for the peak 1 in Figure 5.12.**

**(a) = A whole range spectrum between 1-10 ppm**

**(b) = An enlarged spectrum of (a) in a range of 6.8-7.6 ppm**

**Condition: Magnetic field 500 MHz**

From the NMR spectra in **Figures 5.13** and **5.15**, the first and second peaks from the chromatogram (**Figure 5.12)** were assigned to vanillin and dihydroferulic acid, respectively.

For example, the LC-NMR spectrum (**Figure 5.13**) of vanillin (peak 1 in **Figure 5.12)** gave two doublets (d, d) at 6.99 and 7.43 ppm and at 7.46 ppm doublet-doublet (dd), which represent aromatic ring protons. A singlet (3H) at 3.85 ppm, corresponded to a methoxy group (-$OCH_3$) protons and a singlet at 9.6 ppm, was assigned as an aldehyde proton. The NMR-spectrum **(Figure 5.14)** of a saturated solution of vanillin in deuterium oxide gave a matching standard spectrum.

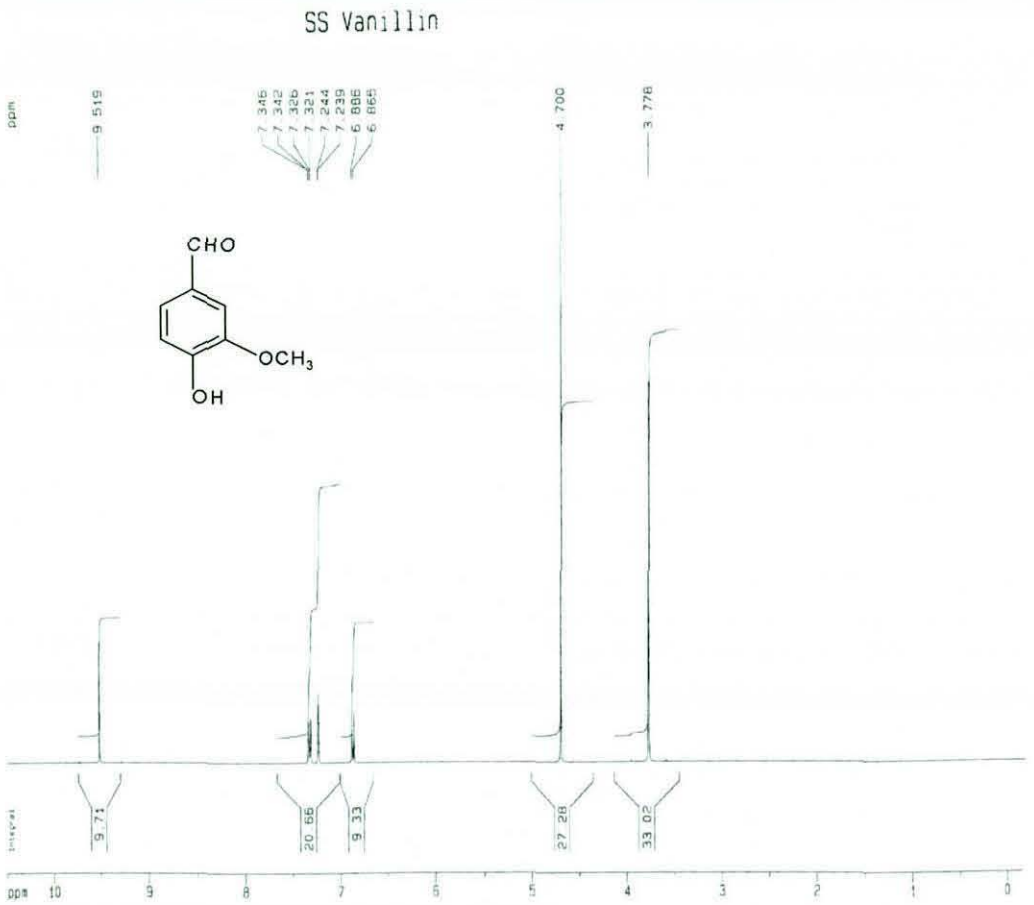



**Figure 5.14: NMR spectrum of a saturated solution of vanillin in deuterium oxide.**

**Condition: Magnetic field 400 MHz**

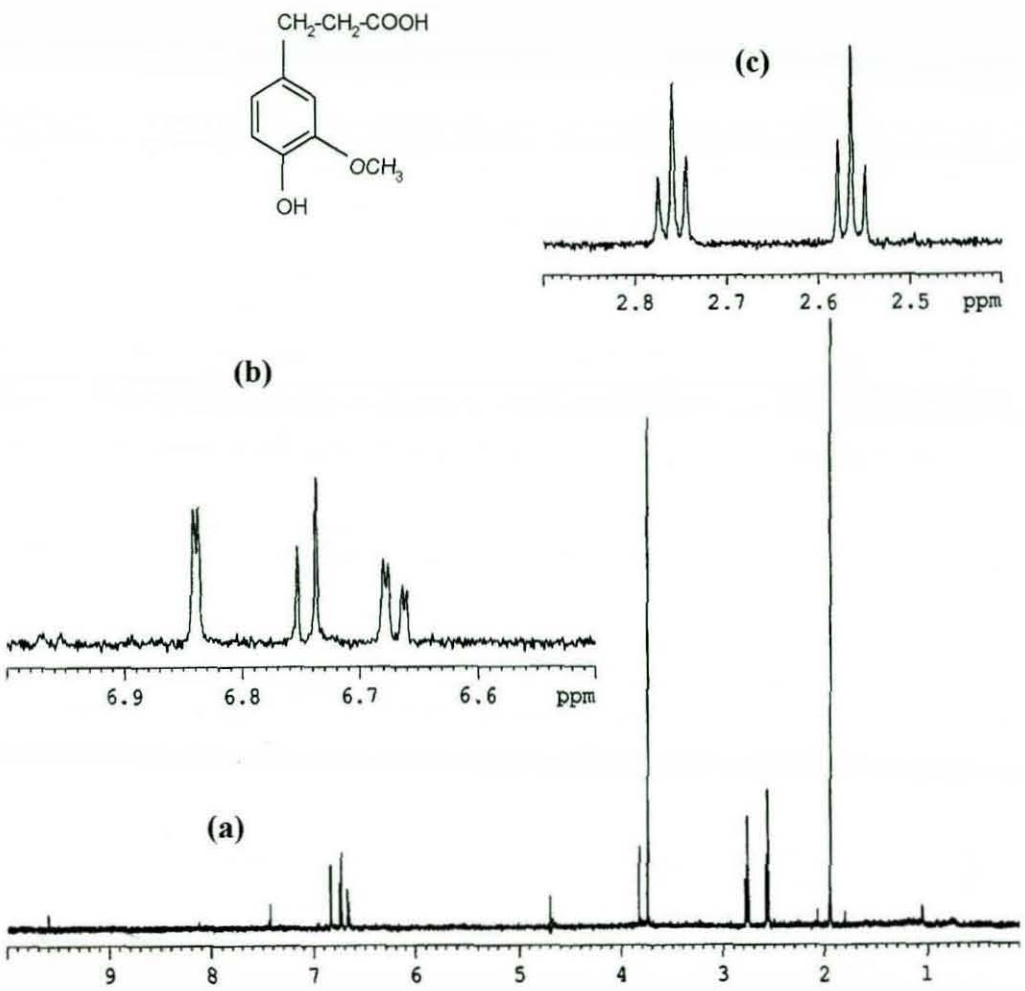



**Figure 5.15: Superheated Water Chromatography-NMR spectrum obtained using stop-flow mode for the peak 2 in (Figure 5.12).**

**(a) = A whole range spectrum between 1-10 ppm**

**(b) = An enlarged spectrum of (a) in a range of 6.5-7.0 ppm**

**(c) = An enlarged spectrum of (c) in a range of 2.4-2.9 ppm**

**Condition: Magnetic field 500 MHz**

The peak 2 from **figure 5.12** chromatogram gave the same NMR-spectrum as peak 2 in on-flow mode (**Figures 5.8 and 5.15**) and was assigned to dihydroferulic acid.

Peak 3 in the chromatogram (**Figure 5.12**) was assigned to a mixture of zingerone and ferulic acid in about a ratio of 10:7 from the NMR signals (**Figure 5.16**). The spectra of standard samples confirm the identification (**Figures 5.17 and 5.18**). For example, signals could be assigned to zingerone, which gave two doublets at 6.72, 6.80 ppm and at 6.63 ppm doublet-doublet (dd), for the three protons of an aromatic ring and a singlet (3H) at 3.7 ppm, which corresponded to $-OCH_3$ protons and two triplets at 2.70 and 2.80 ppm which corresponded to methylenes in a $-CH_2CH_2COCH_3$ and one singlet at 2.05 ppm, which was assigned to a methyl ketone of (- $CH_2CH_2COCH_3$), and agreed with an authentic spectrum (**Figure 5.17**)

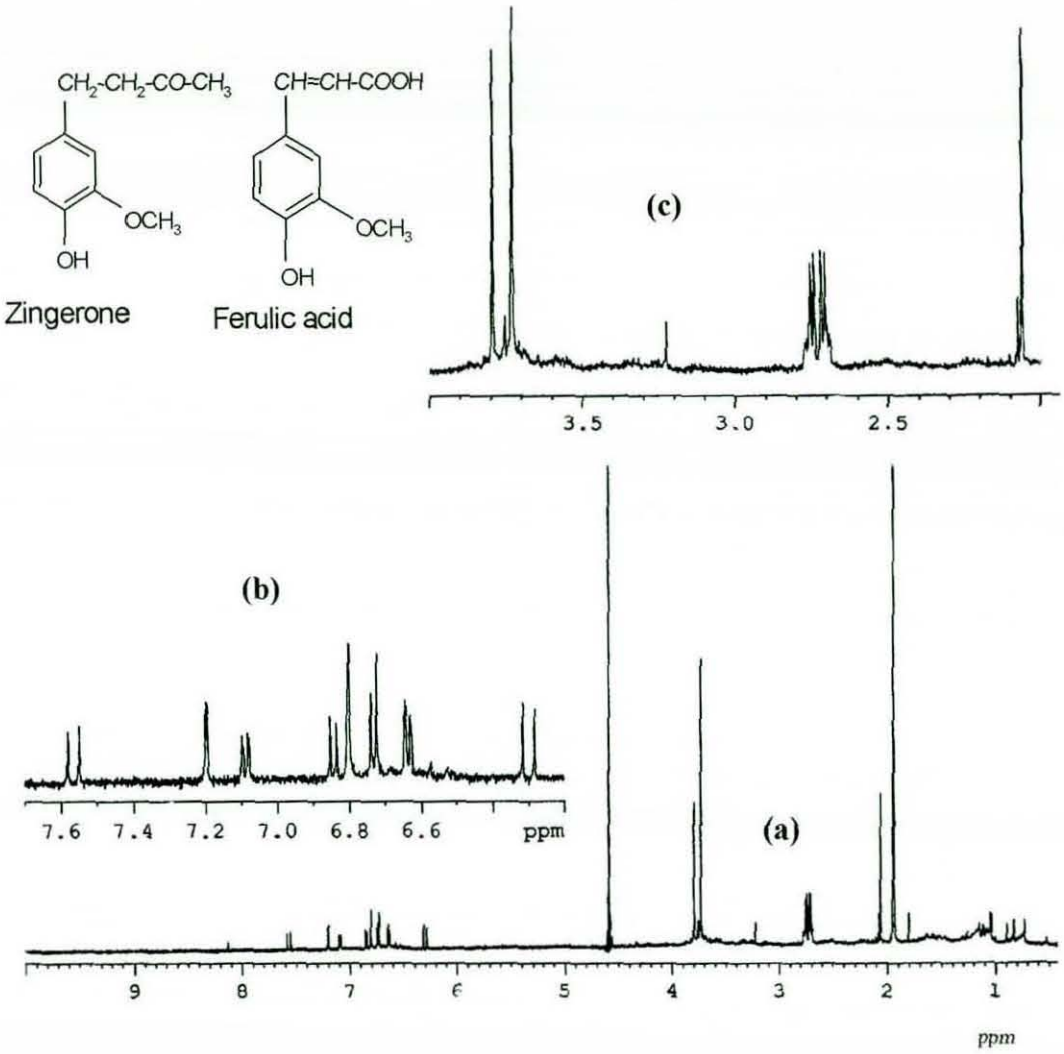



**Figure 5.16: Superheated Water Chromatography-NMR spectrum *obtained using* stop-flow mode for the peak 3 in Figure 5.12.**

**(a) = A whole range spectrum between 1-10 ppm**

**(b) = An enlarged spectrum of (a) in a range of 6.2-7.7 ppm**

**(c) = An enlarged spectrum of (a) in a range of 2.0-4.0 ppm**

**Condition: Magnetic field 500 MHz**

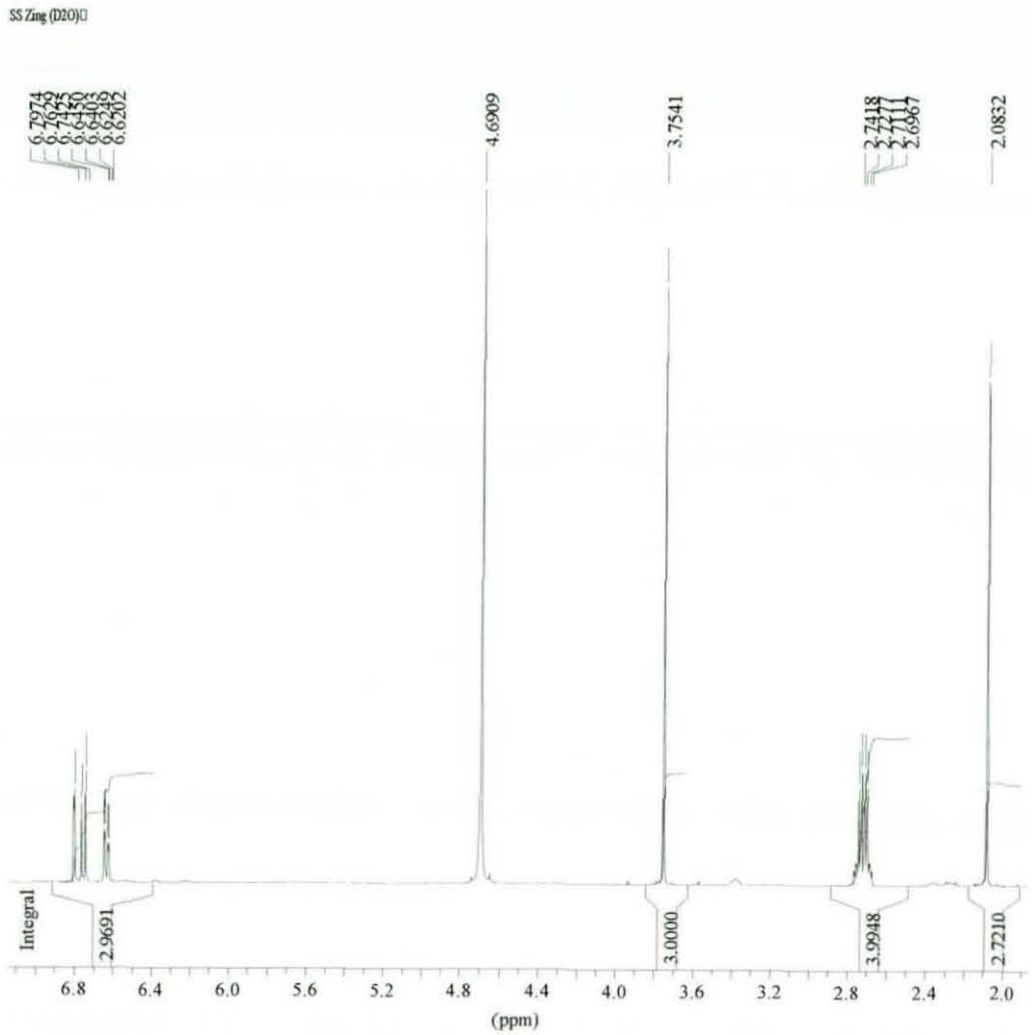



**Figure 5.17: NMR spectrum of saturated solution of a standard zingerone in deuterium oxide.**

**Condition: Magnetic field 400 MHz**

In the spectrum of peak 3 there were also weaker signals, which corresponded to ferulic acid. Two doublets at 7.58, 7.2 ppm and doublet-doublet (dd) at 7.1 ppm, which could be assigned to the three protons of an aromatic ring and two doublets at 6.85 and 6.65 ppm which corresponded to –CH=CH– group attached to an acid (-CH=CHCOOH) and a singlet (3H) at 3.8 ppm, which corresponded to a $–OCH_3$ protons. The spectrum agreed with the corresponding spectrum of the pure compound as a saturated solution in deuterium oxide **(Figure 5.18).** Ferulic acid is slightly soluble in water so 4.7 ppm signal was very strong compare other signals.

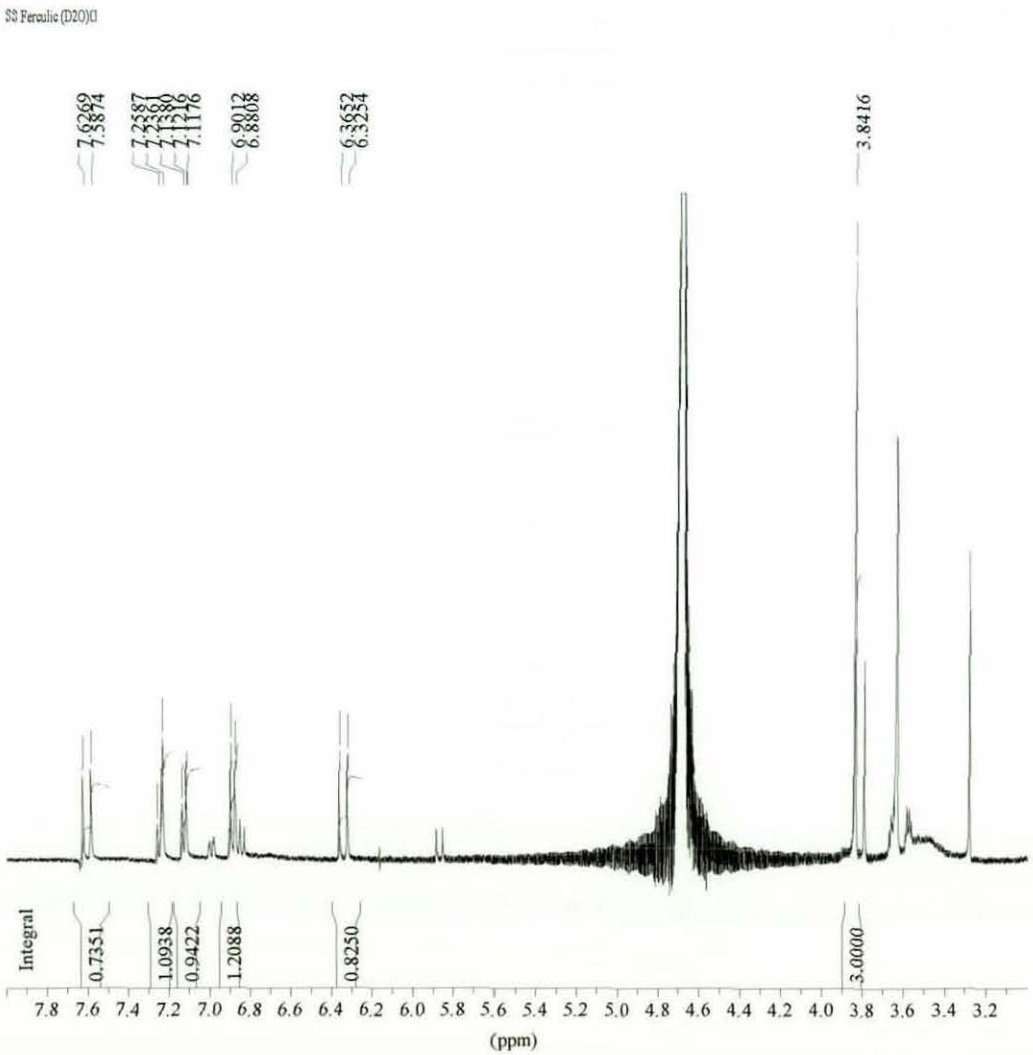
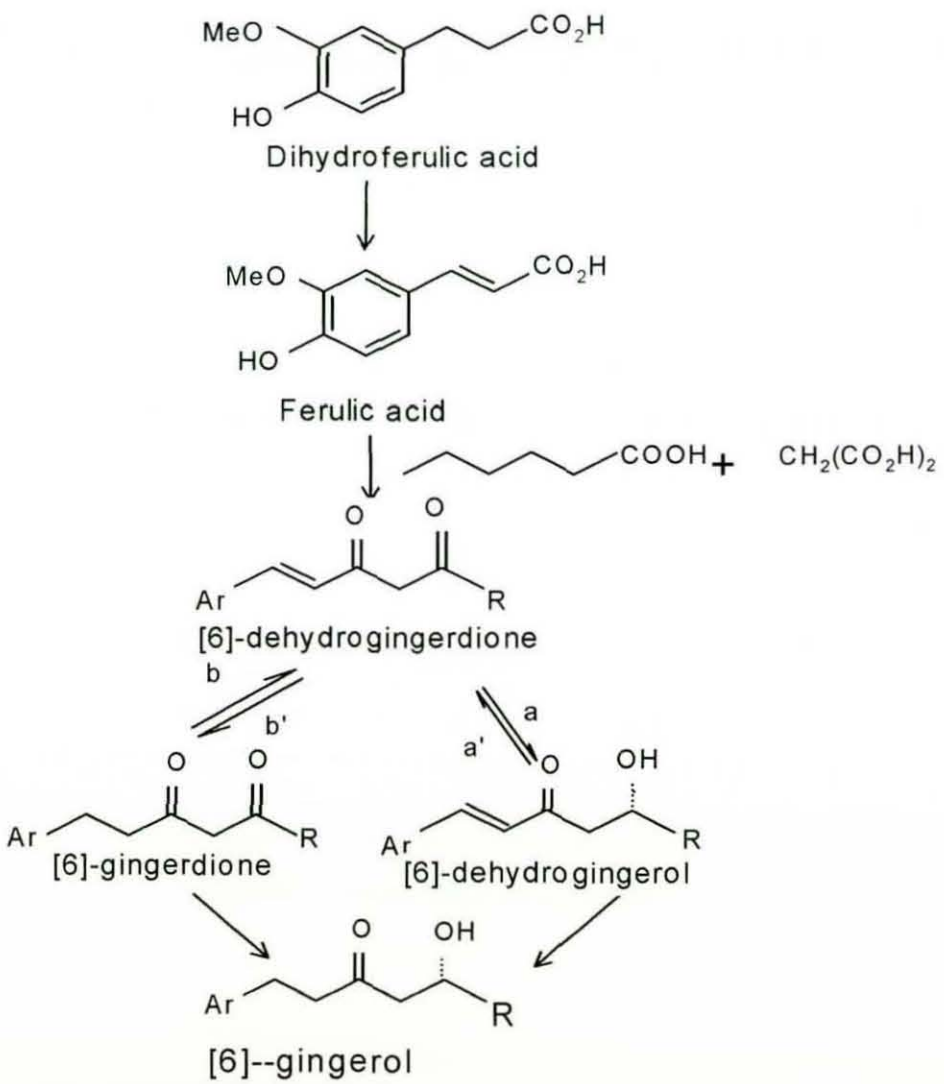



***Figure 5.18: NMR spectrum of saturated solution of a standard ferulic acid in deuterium oxide.***

**Condition: Magnetic field 400 MHz**

The origin of these components can be explained by considering the formation of the gingerols. Macleod and Whiting (197) have proposed that the biosynthesis of the gingerols involves the condensation of ferulic acid with malonic acid and with a short chain carboxylic acid such as hexanoic acid to give an intermediate [6]-dehydrogingerdione **(see Scheme 1).** Such a condensation requires [6]-dehydrogingerdione as an initial product; two reduction steps are then necessary to transform [6]-dehydrogingerdione to [6]-*gingerol*, *and either* [6]-dehydrogingerol or [6]-gingerdione must be the intermediates.




**Scheme 1**

Therefore, vanillin, dihydroferulic acid and ferulic acid are original constituents of gingerols. The NMR spectrum of the whole ginger extract (**Figure 5.19**) confirmed their structures of vanillin, dihydroferulic acid, and ferulic acid.

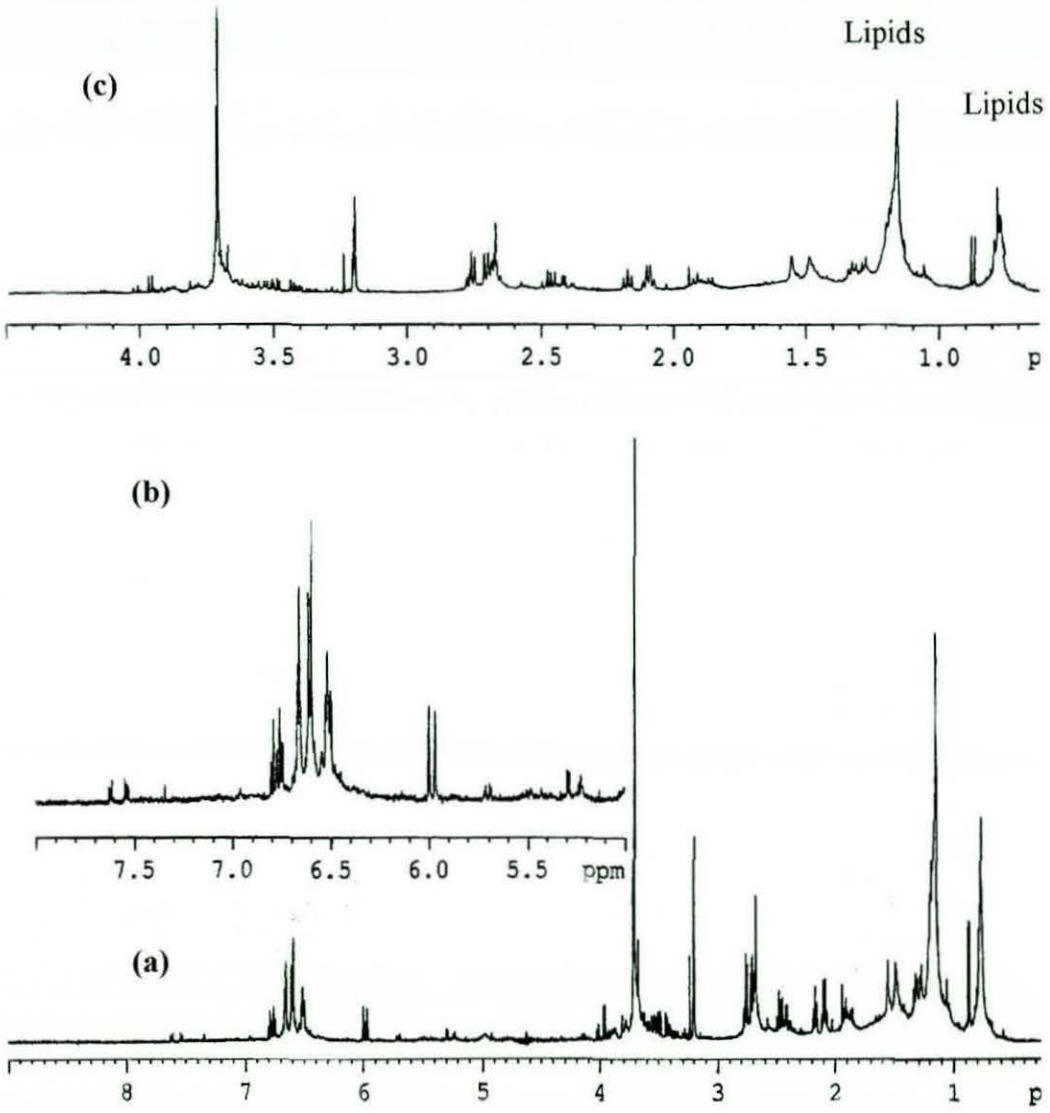



**Figure 5.19: NMR spectrum of whole ginger extract.**

**(a) = A whole range spectrum between 0-9 ppm**

**(b) = An enlarged spectrum of (a) in a range of 5.0-8.0 ppm**

***(c) = An enlarged spectrum of (a) in a range of 0.5-4.5 ppm***

**Condition: Magnetic field 500 MHz**

Unfortunately, we could not identify the constituents of grains of paradise, because the concentration was too weak **(see figure 5.20)**. This emphasises the inherent lack of absolute sensitivity in flow NMR, whereas conventional NMR can compensate by lengthening the data collection time and using more sampling scans, but this is not possible in a flow LC system.

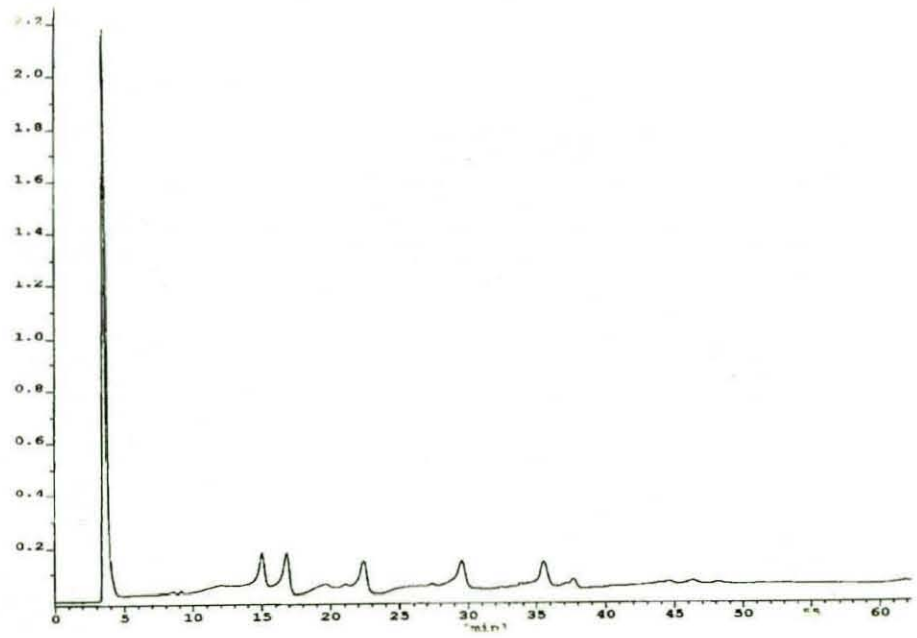



**Figure 5.20: Separation of grains of paradise extract on Xterra ™ RP 18 column on superheated water chromatography-UV-NMR system**

**Chromatographic conditions: As Figure 5.7.**

However, the signals of the expected main constituents, the gingerols and shogaols were apparently absent. Therefore, further investigations were needed to confirm the structure of vanillin, dihydroferulic acid, ferulic acid and gingerone.

### 5.3.2: Identification of the Chromatographic peaks by Mass Spectroscopy

The fractions from loop collection in NMR study (**Figure 5.12**) were freeze-dried. Fractions were introduced directly by means of electrically heated probe into a MS.

The identification of dihydroferulic acid and zingerone were confirmed by the mass spectra (**Figures 5.21 and 5.23**). In **figure (5.21)** the molecular ion ($M^+$) corresponded to 196 (relative abundance 31%) and a tropylium ion having one hydroxy and one methoxy substituent in the phenyl ring (m/z) 137 (relative abundance 100 %).

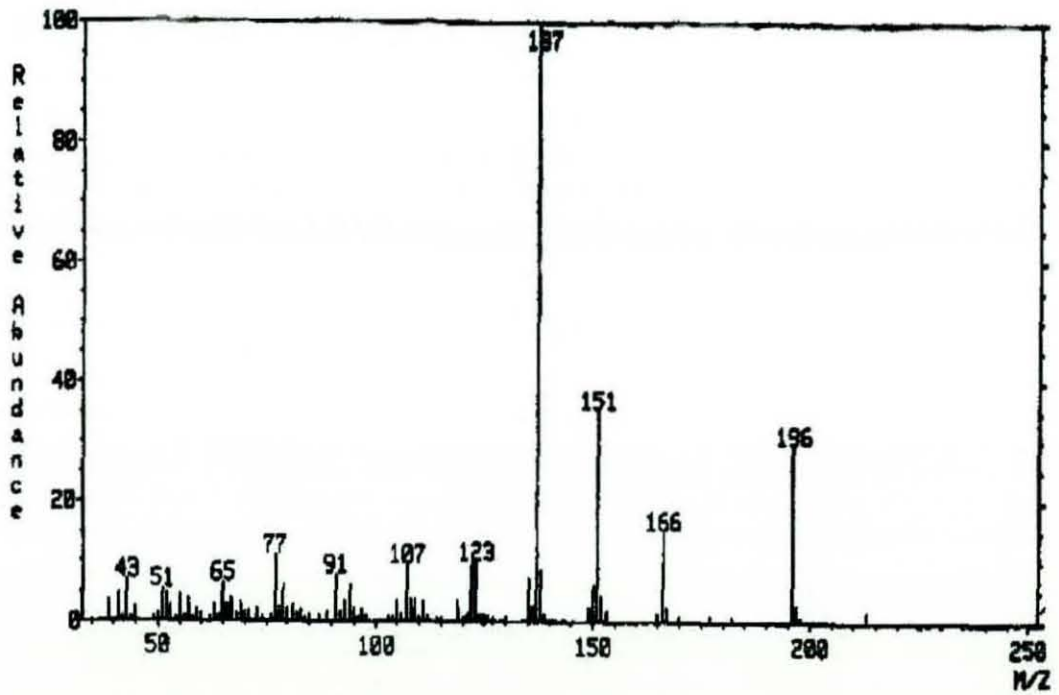



**Figure 5.21: MS spectrum obtained for the peak 2 in Figure 5.12 (From loop collection sample).**

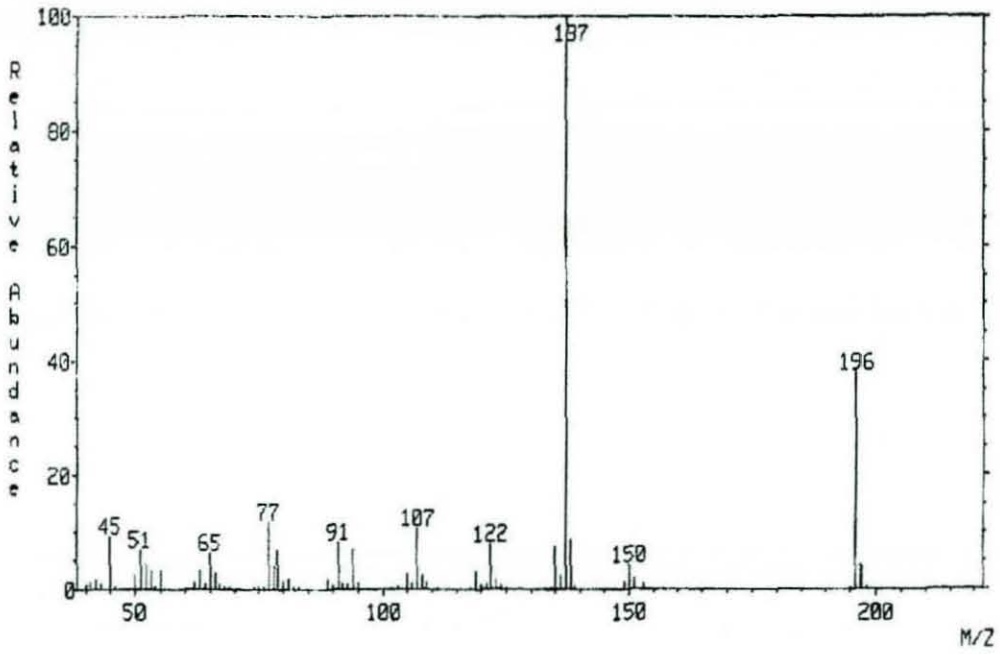



**Figure 5.22: Mass spectrum of standard sample of dihydroferulic acid.**

The mass spectrum of (**Figure 5.21**) closely resembled that of a standard sample of dihydroferulic acid (**Figure 5.22**).

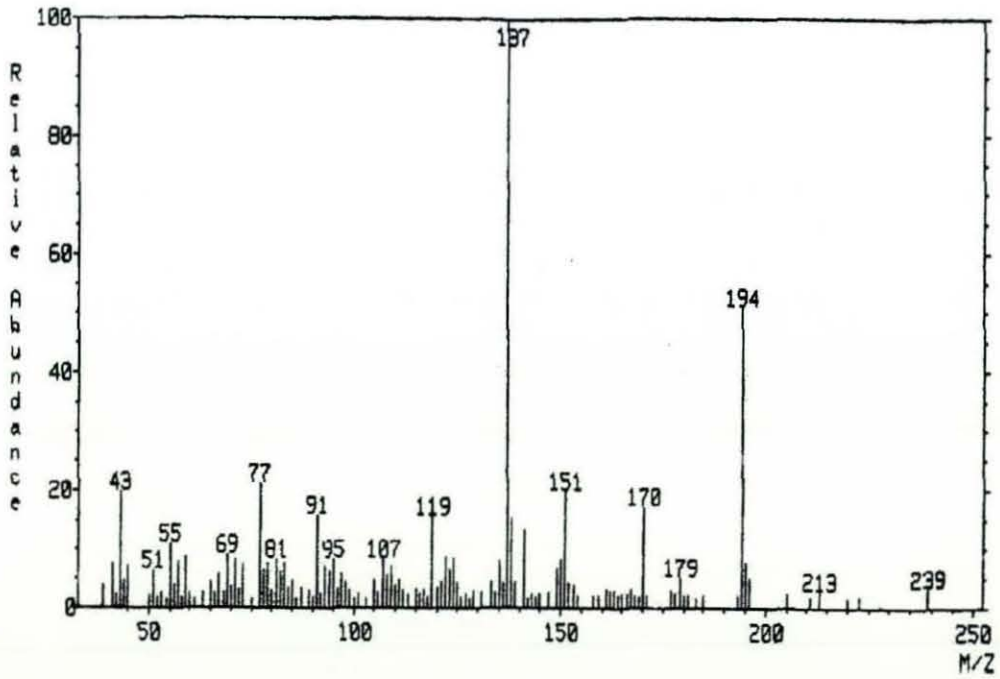



**Figure 5.23: MS spectrum obtained for the peak 3 in Figure 5.12 (From loop collection sample).**

The Mass spectrum (**Figure 5. 23**) of the peak 3 showed the molecular ion ($M^+$) of gingerone at m/z 194 (relative abundance 49%) and the same tropylium ion corresponded to (m/z) 137 (relative abundance 100 %).

The published mass spectrum (**Figure 5.24**) of zingerone matched our loop collection sample's mass spectrum (**Figure 5.23**).

Unfortunately, the MS spectra of peak 1 in **Figure 5.12** (Loop collection) did not match with the spectrum of vanillin. It could only be identified by superheated water chromatography-NMR spectroscopy.

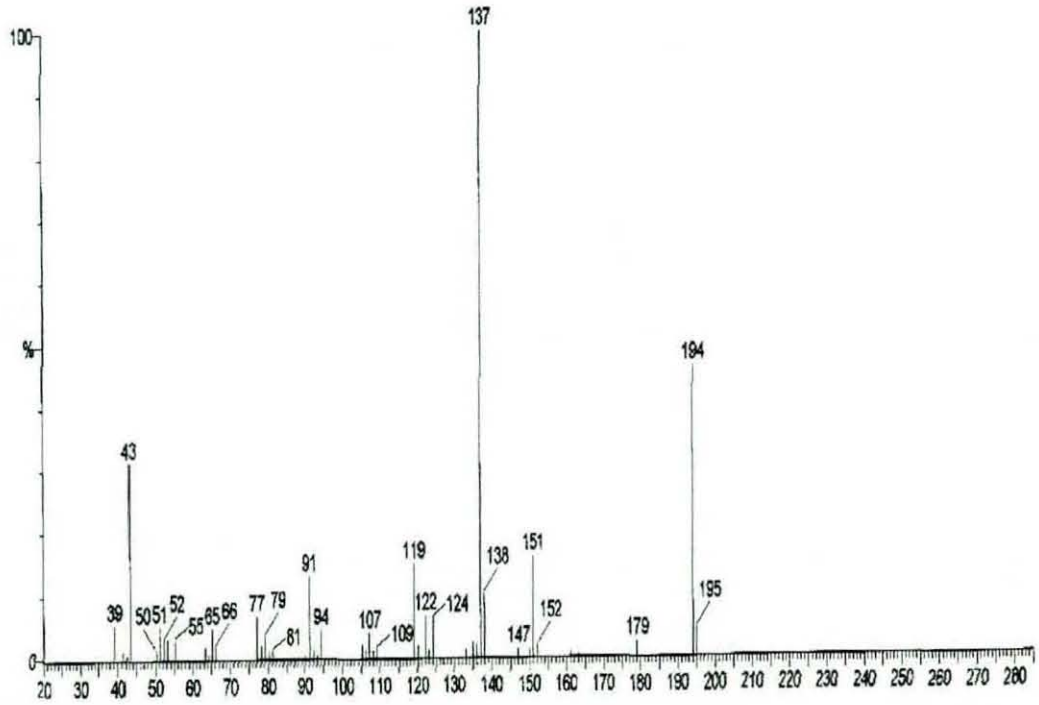



**Figure 5.24: Published MS spectrum of zingerone.**

So what have happened to the main constituents? Have the main compounds decomposed in superheated water chromatography?

### 5.3.3: Further Identifications of different fractions by Conventional LC and Superheated Water Chromatography

Zingerone, vanillin, dihydroferulic acid and ferulic acid are commercially available. Therefore, these compounds and the ginger extract were injected on an Xterra ™ RP 18 column using different ratios of acetonitrile-water (v/v) at room temperature. The constituent of ginger separated well using 10:90 (v/v) acetonitrile-water at room temperature. Vanillin, dihydroferulic acid, ferulic acid and zingerone were injected under the same conditions. The retention times of vanillin (4.32 min) and zingerone (9.81 min) were matched with peak 1 (4.30 min) and peak 4 (9.82 min) in ginger extract respectively. However, dihydroferulic acid and ferulic acid did not match, it was thought that this was because these compounds are partly ionised in normal conditions.

Further investigation was needed. Therefore, all standards and the ginger extract were injected in acidic condition (pH 3.5) 10 % ACN as the mobile phase. Then the retention times of vanillin (4. 30 min), dihydroferulic acid (5.59 min), ferulic acid (8.01 min) and zingerone (10.02 min) were matched with peaks 1 (4.20 min), 2 (5.64 min), 3 (8.02 min) and 4 (10.03 min) in ginger extract **(Figures 5.25, 5.26, and 5.27)**. In the chromatogram, the peak shapes were split, because the column had degraded and a void had formed.

Again all standards and ginger extract were injected in superheated water chromatography in temperature programming conditions (50-130 °C, ramping 4 °C per min.) on Xterra ™ RP 18 column. The retention time of vanillin and zingerone corresponded with peak 1 and peak 3, for example, (**Figure 5.28**).

The retention times of vanillin, dihydroferulic acid, and zingerone in acidic conditions (pH 3.5), were matched with peak (1), peak (2), and peak (3) in **Figure 5.28a**. Ferulic acid gave 28.05 min retention time retention time. Therefore, in loop collection ferulic acid and zingerone mixed together.

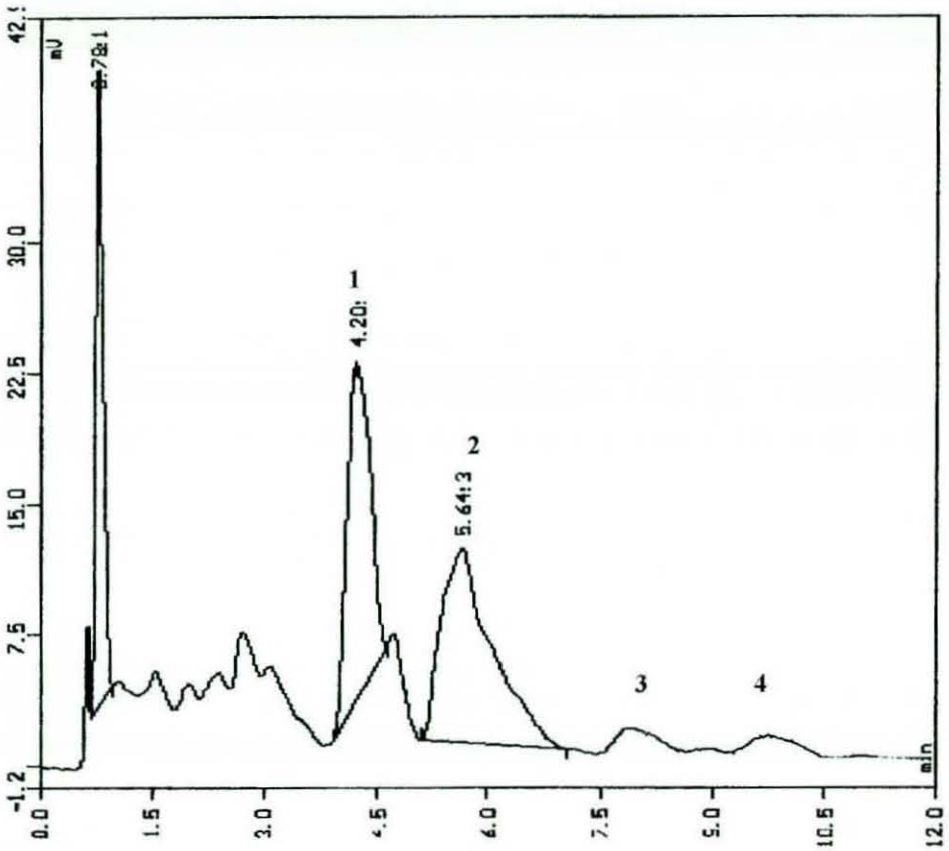



**Figure 5.25: Separation of ginger extract on Xterra ™ RP 18 column using 10:90 % acidic (pH 3.5) ACN/water as the mobile phase at room temperature.**

**Chromatographic conditions: column: Xterra ™ RP 18 (50 x 4.6 mm I. D.), injection volume: 5 μl, flow rate: 1.0 ml/min, detection: UV-absorption wavelength 280 nm and range 0.02 amps/mV.**

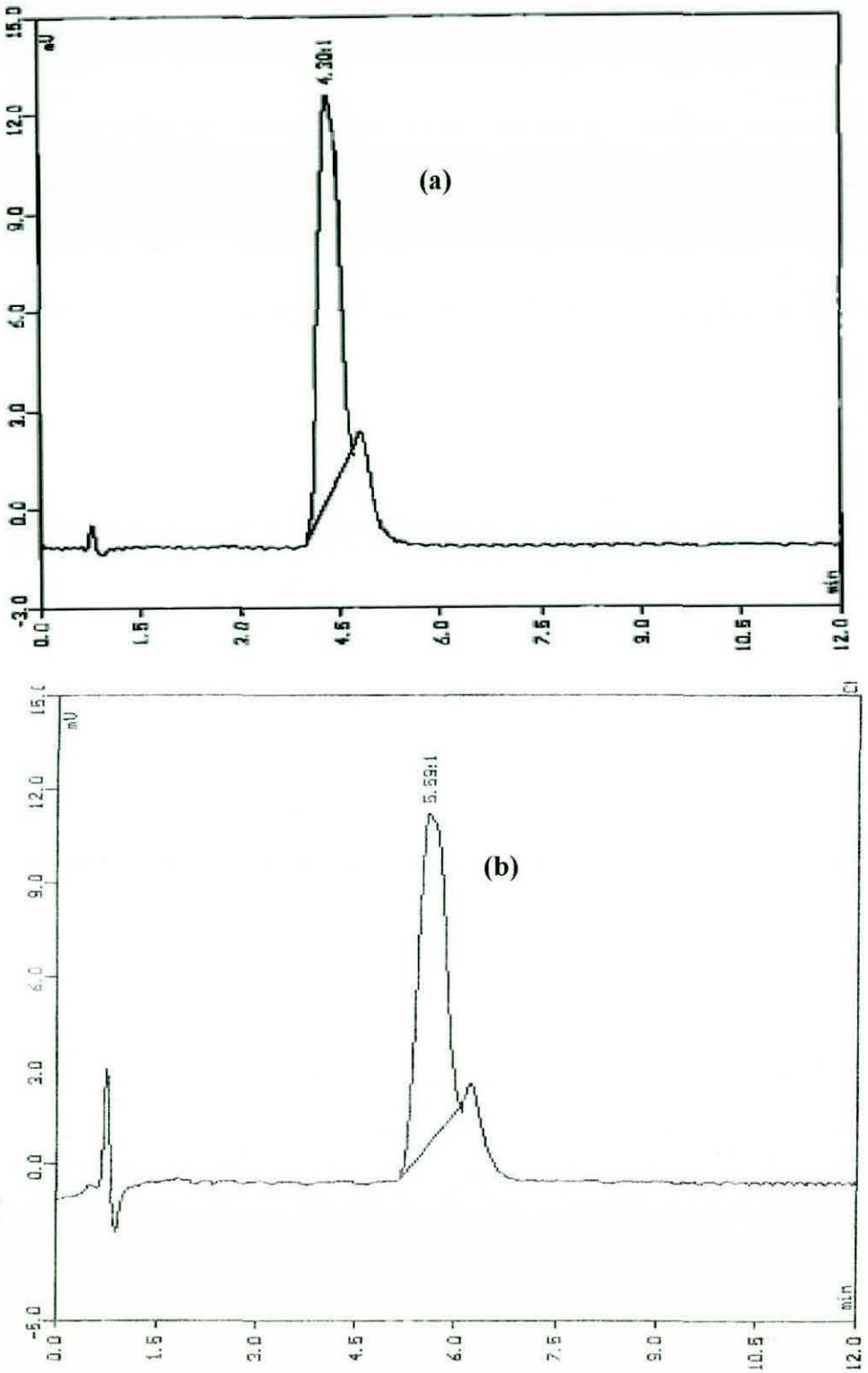



**Figure 5.26: Chromatograms of vanillin (a) and dihydroferulic acid (b) on Xterra ™ RP 18 column.**

***Separation conditions: as Figure 5.25***

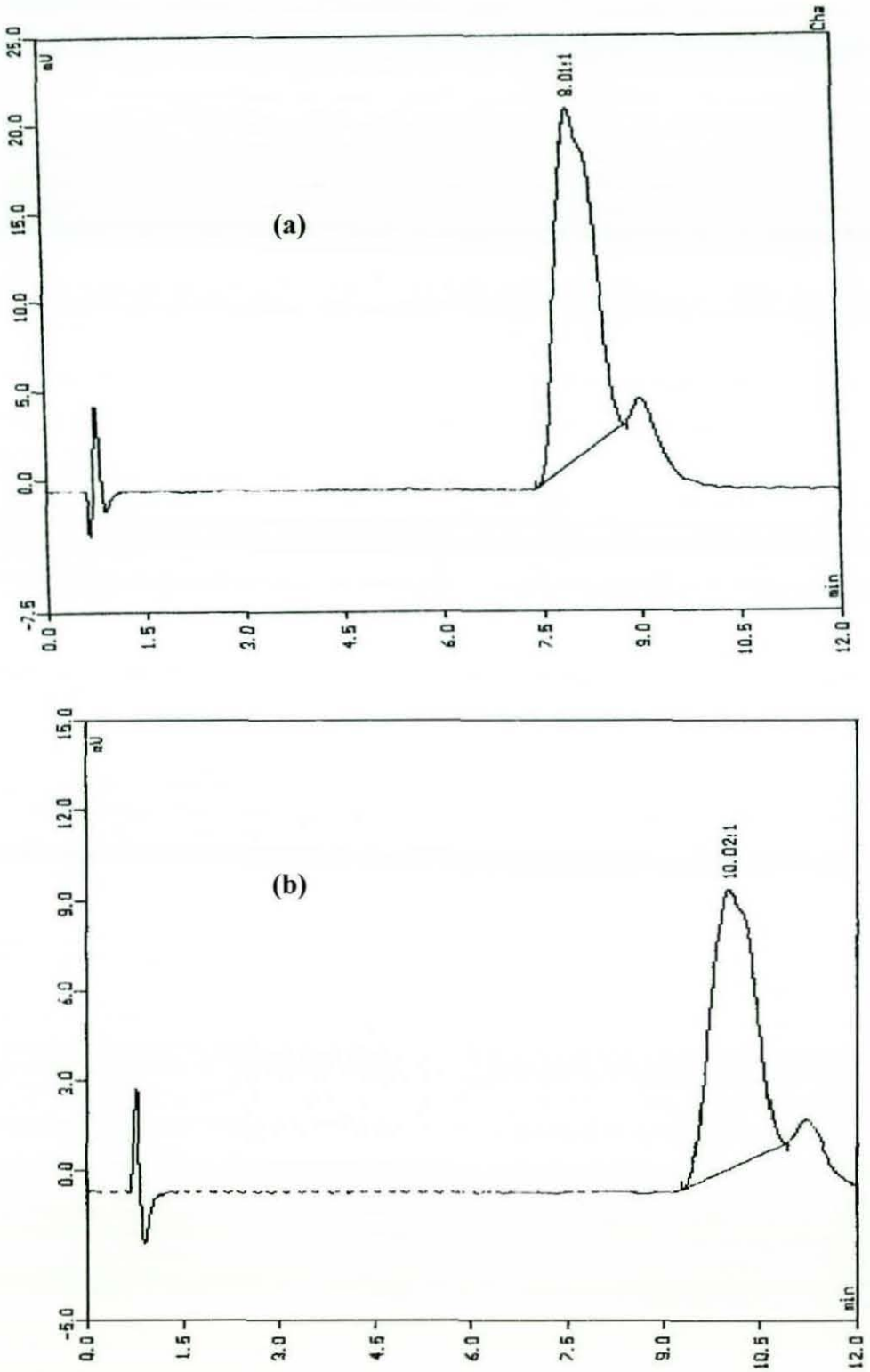



**Figure 5.27: Chromatograms of ferulic acid (a) and zingerone (b) on Xterra ™ RP 18 column.**

**Separation conditions: as Figure 5.25**

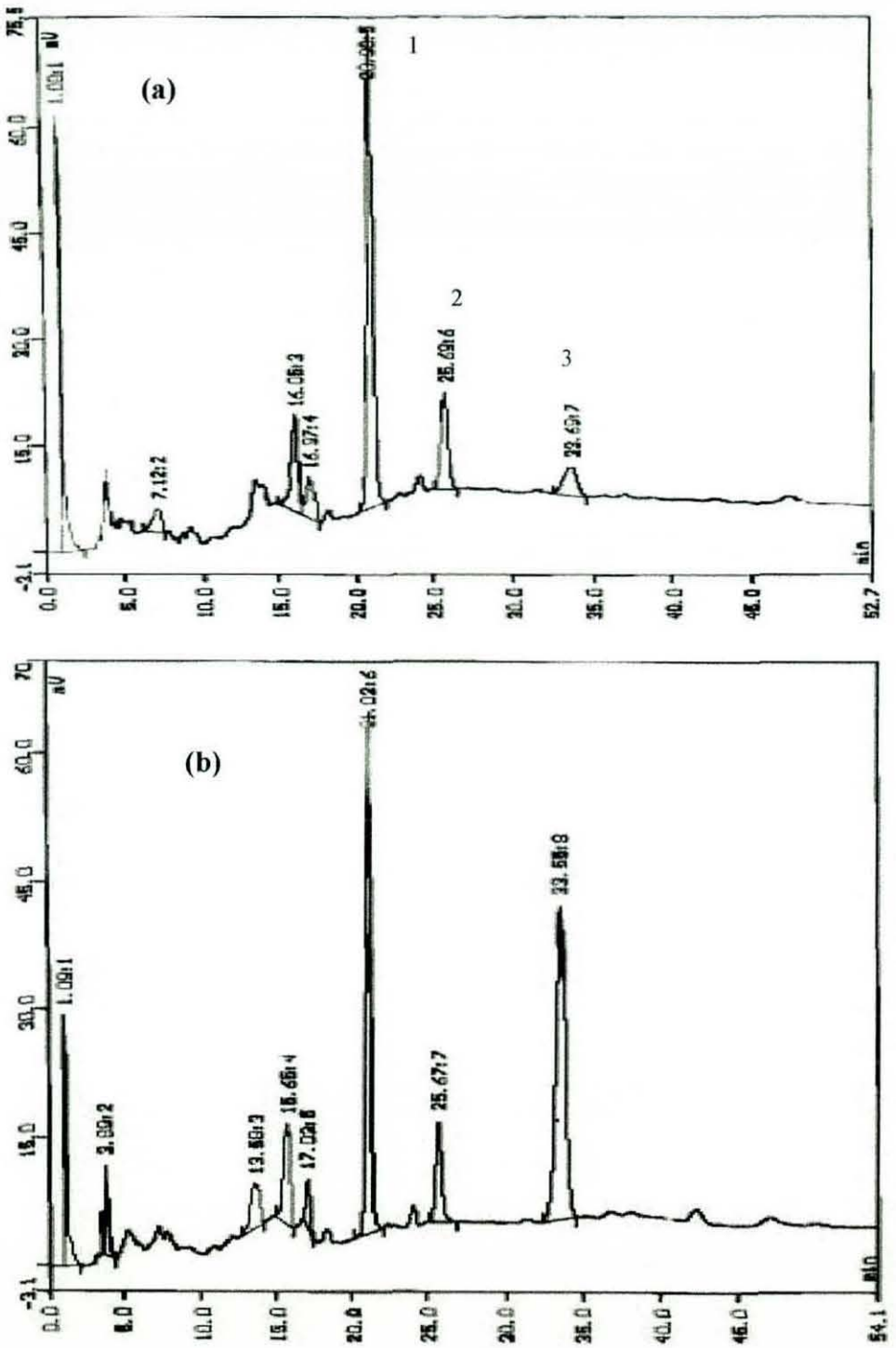



**Figure 5.28: Chromatograph for confirmation of zingerone (a) ginger extract alone (b) co-injection of zingerone and ginger extract.**

**Separation Conditions: as Figure 5.5**

Therefore, from our experiment, we can conclude that zingerone, vanillin, dihydroferulic acid, ferulic acid were present in ginger extract. They were not decomposition product formed during the superheated water chromatography from the gingerols. Other researchers have not published any papers about the separation of dihydroferulic acid and ferulic acid in ginger extract.

## 5.4: Summary

The compounds of lower molecular weight of ginger and grains of paradise extract were separated successfully by temperature programming on Xterra $^{TM}$ RP 18 column.

The coupling of superheated water chromatography to NMR spectroscopy was successful, although there was a little trouble with the difference in UV and NMR sensitivity. Vanillin, dihydroferulic acid, zingerone and ferulic acid were identified by superheated water chromatography-NMR system. Again dihydroferulic acid and zingerone were identified by mass spectroscopy from loop collection.

From a conventional LC study we can see that vanillin, dihydroferulic acid, ferulic acid and zingerone compounds were already present in ginger extract.

# CHAPTER 6

## Oxidation of alcohols in Superheated Water Chromatography

### 6.1: Introduction

Previous work by Burgess observed (49) two peaks for aromatic alcohols in superheated chromatography and suggested that either oxidation or degradation might be the cause of the extra peak. Therefore, we investigated the sources of the extra peaks because of concern that a reaction appeared to be occurring on-column by oxidation from oxygen in the sample or eluent.

The separation of a set of homologous aromatic alcohols, benzyl alcohol, 2-phenylethanol, 3-phenylpropanol, and the aldehydes, benzaldehyde and phenylacetaldehyde were studied on Hypercarb and on polystyrenedivinylbenzene columns using superheated water as the mobile phase.

### 6.2: Aromatic Alcohols on Hypercarb and Polystyrene divinylbenzene Columns

Benzyl alcohol was injected on a Hypercarb column was separated by superheated water chromatography. It gave two peaks at 5.72 min and 12.23 min with UV detection (**Figure 6.1**). The unexpected extra peak was suspected to be benzaldehye, a common oxidation product of benzyl alcohol. It was confirmed by injection of standard benzaldehyde solution, which gave the same retention time of 12.22 min.

However, a sample of 2-phenylethanol also gave two peaks at 5.83 min and 12.24 min (**Figure 6.2**) but 3-phenylpropanol showed only one peak at 10.14 min (**Figure 6.3**). Therefore phenylacetaldehyde was injected to confirm the identity of the second peak of 2-phenylethanol. Unfortunately phenylacetaldehyde gave two very similar peaks at 11.20 min and 16.5 min (**Figure 6.4**). The first peak of phenylacetaldehyde showed the same retention time as benzaldehyde. However, the peaks were not well resolved and it was decided to examine an alternative column.

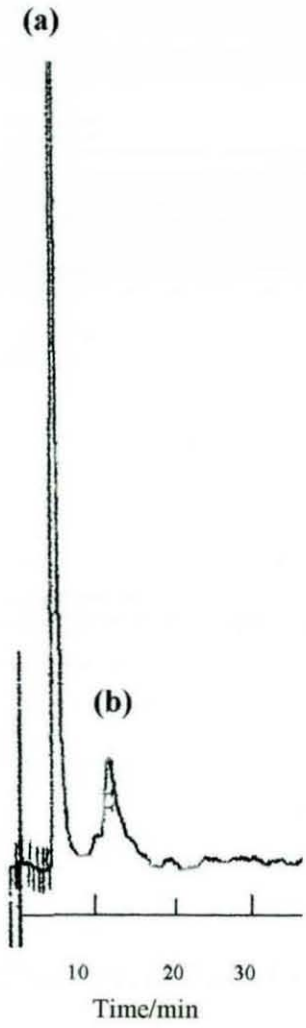



**Figure 6.1: Chromatograph of benzyl alcohol in superheated water chromatography, (a) benzyl alcohol (b) benzaldehyde.**

**Separation conditions: Column: Hypercarb (100 x 4.6 mm I.D.), Mobile phase: superheated water (190 °C), Flow rate: 1 ml/min, detection: UV absorption wavelength 254 nm.**

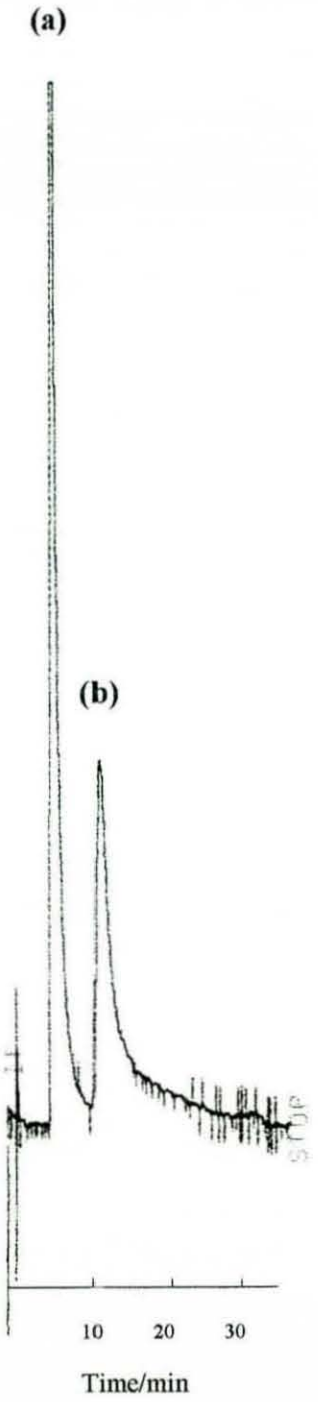



**Figure 6.2: Chromatograph of 2-phenylethanol in superheated water chromatography (a) 2-phenylethanol and (b) benzaldehyde.**

**Separation conditions: as Figure 6.1**

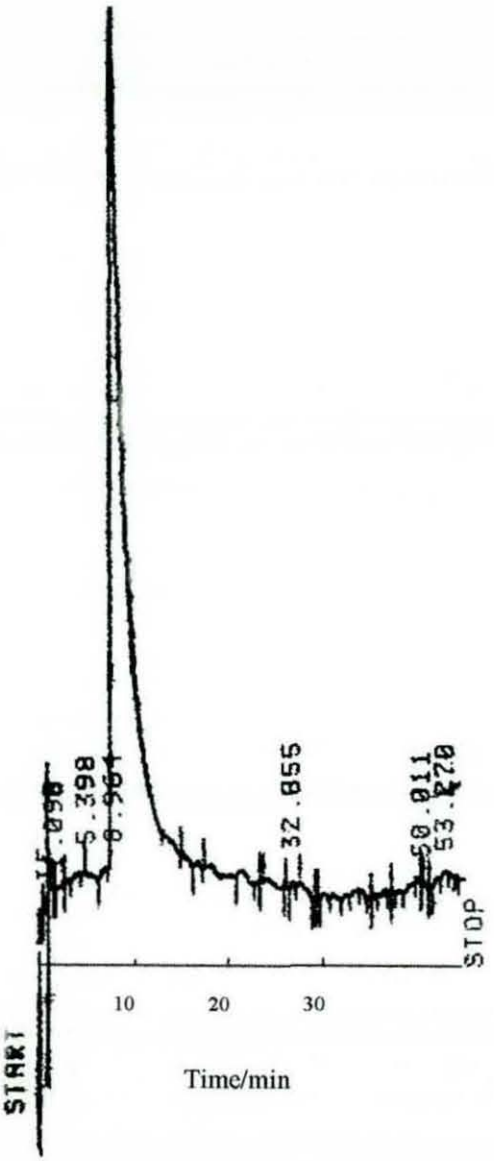



**Figure 6.3: Chromatograph of 3-phenylpropanol in superheated water chromatography.**

**Separation conditions: as Figure 6.1**


**Figure 6.4: Chromatograph of phenylacetaldehyde in superheated water chromatography, (a) benzaldehyde (b) phenylacetaldehyde.**

**Separation conditions: as Figure 6. 1**

The separations were then examined on a polystyrene divinyl-benzene column. Phenylacetaldehyde and benzaldehyde were well separated at 13.48 min and 16.12 min (**Figure 6.5**). The first peak from phenylacetaldehyde was confirmed to correspond to the benzaldehyde peak.

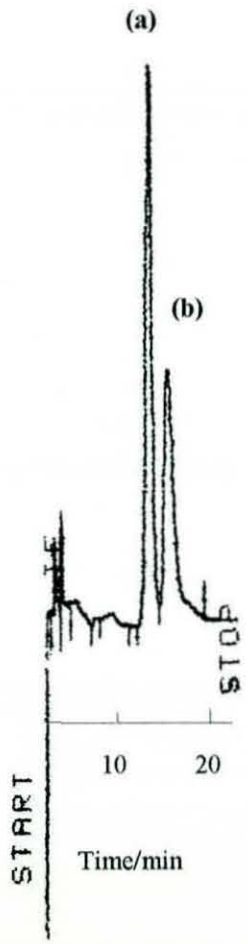



**Figure 6.5: Chromatograph of phenylacetaldehyde in superheated water chromatography, (a) benzaldehyde (b) phenylacetaldehyde.**

**Separation conditions: Column: PS-DVB (150 x 4.6 mm I.D.), Mobile phase: superheated water (190 °C), Flow rate: 1 ml/min, detection: UV absorption wavelength 254 nm.**

Thus benzyl alcohol, 2-phenylethanol and phenylacetaldehyde all gave benzaldehyde peaks. The proportion of the oxidation product increases with a decreased flow rate of mobile phase (**Table 6.1**).

**Table 6.1: Effect of flow rate on the relative proportion of Oxidation Product.**

| Compound | Flow rate ml/min | % Oxidation product |
|---|---|---|
| Benzyl alcohol | 0.4 | 0.010 |
| | 1.0 | 0.007 |
| | 2.0 | 0.003 |
| 2-Phenylethanol | 0.4 | 0.010 |
| | 1.0 | 0.006 |
| | 2.0 | 0.002 |
| Phenylacetaldehyde | 0.4 | 0.060 |
| | 1.0 | 0.020 |
| | 2.0 | 0.008 |

At low flow rates (0.4 ml/min) all compounds (benzal alcohol, 2-phenylethanol and phenylacetaldehyde) gave much higher oxidation peak than at higher flow rate (2.0 ml/min). This can be related to the longer residence time on the hot column. Therefore, oxidation was higher at lower flow rate of 0.2 ml/min. Because the benzaldehyde peak was sharp it suggested a rapid reaction on injection and that air in the sample itself might be a cause. Normally on superheated water chromatography, the mobile phase is deoxygenated with nitrogen. To reduce levels of benzaldehyde helium degassing also tried but little effect.

Helium gas is less soluble in water than oxygen. So helium gas was continually passed into the sample and mobile phase. After 1 hour degassing of sample and mobile phase the oxidation product from benzyl alcohol was reduced to 0.0007 % (**Figure 6.6**).

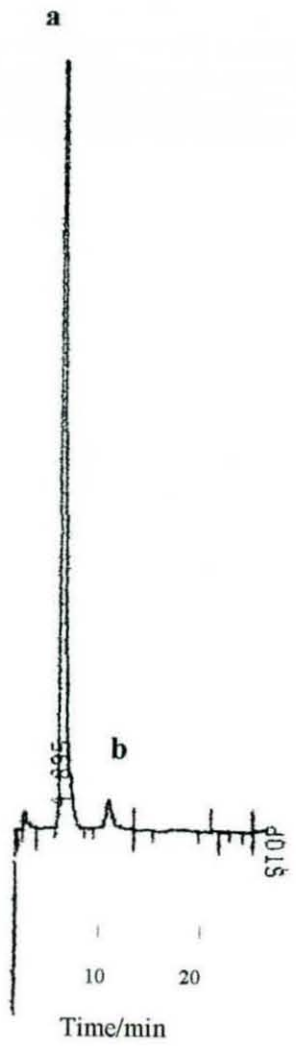



**Figure 6.6: Chromatograph of benzyl alcohol after degassing of mobile phase and also sample.**

**Separation conditions: as Figure 6.5**

Thus some reaction appeared to be occurring in the column but the extent of the reaction was low and the product was only detected because benzaldehyde is a stronger chromophore (molar extinction coefficient $\varepsilon_{max}$ 14000) than benzyl alcohol. So it has a much higher response in UV detection. Similar reactions appear to occur with the other alcohols but again at low levels.

1-Phenylethanol was injected to confirm that this was an oxidation effect. 1-Phenylethanol is a secondary alcohol, it should give an acetophenone peak on oxidation. It gave two peaks at 10.60 min and at 17.57 min (**Figure 6.7**). The unexpected extra peak was confirmed by injection of standard acetophenone solution, which gave the same retention time of 10.61 min.

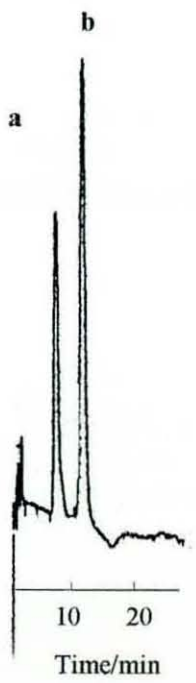



**Figure 6.7: Chromatograph of 1-phenylethanol in superheated water chromatography, (a) acetophenone and (b) 1-phenylethanol.**

**Separation conditions: as Figure 6.5.**

### 6.3: Mixed Analytes

A mixed sample of benzyl alcohol, 2-phenylethanol and 3-phenyl-1-propanol was injected. It gave four peaks (**Figure 6.8**).

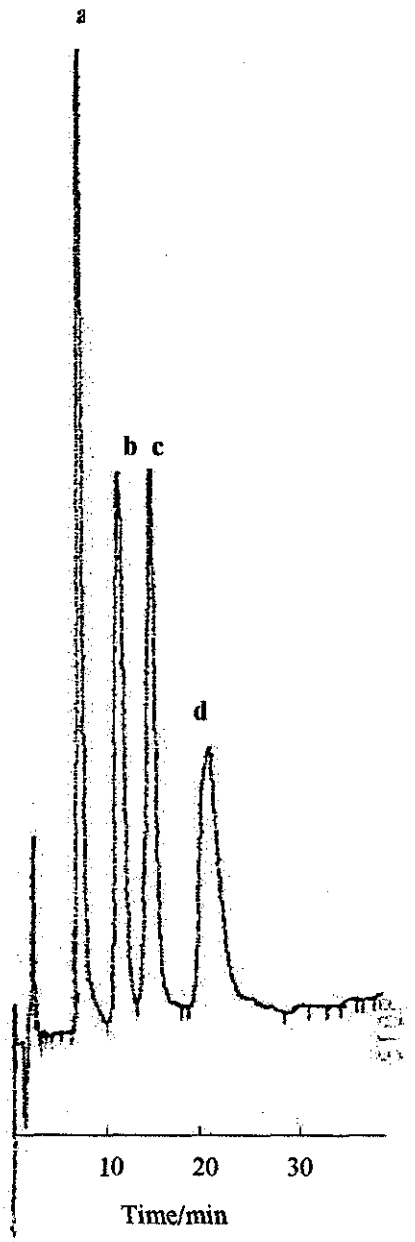



**Figure 6.8: Chromatograph of mixture of homologous alcohols in superheated water chromatography (a) benzyl alcohol, (b) 2-phenylethanol, (c) benzaldehyde and (d) 3-phenylpropanol.**

**Separation conditions: as Figure 6.5**

## 6.4: Verification of Superheated Water Method

To determine if the samples already contained benzaldehyde, benzyl alcohol, 2-phenylethanol and phenylacetaldehyde were analysed by GC on a column with FID **(Section 2.6)**. The result of GC analysis by peak integration was 0.4%, 0.05% and 0.04% benzaldehyde respectively.

Further, benzyl alcohol was studied in conventional HPLC with an organic solvent as the mobile phase at room temperature. Again it was confirmed that benzaldehyde was already present in benzyl alcohol and the present study is in agreement with other studies.

Rees et al. (198) determined benzaldehyde in benzyl alcohol by a spectrophotometric method and found 0.01285-0.01240% benzaldehyde. Pietra and et al. (199) have determined benzaldehyde traces in benzyl alcohol by two alternative procedures, based on reversed-phase HPLC and derivative UV spectrophotometry. They analysed commercial benzyl alcohol and the results obtained in HPLC at 254 nm (0.370-0.009%), at 282 nm (0.367-0.008%) and in UV method (0.460-0.025%) benzaldehyde.

## 6.5: Summary

The study confirmed that when benzyl alcohol was examined by superheated water chromatography a peak was obtained for benzaldehyde. However, benzaldehyde is an almost universal contamination in benzyl alcohol (198) and the level was similar to that detect by other chromatography methods. As the level could be reduced by degassing or shorter retention times, some reaction appeared to be occurring in the column, this result agreed with a recently published paper (25). Siskin and et al. reported that benzyl alcohol undergoes ~30% conversion to toluene, benzaldehyde and dibenzyl ether in water after 1 day at 250 °C. In the present study it is concluded that the extent of the reaction was very low and the product was only detected because of its much stronger chromophore.

A similar oxidation reaction appeared to occur with other homologous alcohols but again at very low level.

# CHAPTER 7

# Conclusions and Future work

## 7.1: Conclusions

Superheated water has been utilised successfully as a mobile phase for reversed-phase liquid chromatography for the separation of alkyl aryl ketones and a range of aromatic compounds on different stationary phases. It is an alternative to traditional reversed-phase mobile phases such as methanol, acetonitrile and THF mixture with water. The use of superheated water mobile phase for reversed phase chromatography has been shown to be a valuable alternative to traditional aqueous-organic mobile phases.

Superheated water has yielded higher efficiencies than an acetonitrile/water mixture on PBD-zirconia and Oasis HLB$^{TM}$ columns at 1 ml/min flow rate. The potential for increased chromatographic efficiency with a low viscosity solvent, such as superheated water was realised.

However, from van Deemter curves, we found that the values of the C-terms are higher in superheated water systems than in conventional LC on PS-DVB, PBD-zirconia and Xterra $^{TM}$ RP 18 columns. The optimum linear velocities of the van Deeemter curves were 3-4 times higher than in conventional LC and the values of the B-term are also higher in superheated water than in conventional LC on these columns. It is thought that efficiency does not depend only on temperature; it also depends on flow rate, injection volume and experimental set up.

In natural product separations, UV-NMR offered a successful coupled technique for the separation of a ginger extract. The sensitivity was improved by using the stop-flow mode and successfully detected four compounds, (vanillin, dihydroferulic acid, ferulic acid and zingerone).

From an oxidation study it was found that benzaldehyde was already present in benzyl alcohol. Some reaction appeared to be occurring in the column but the extent of the reaction was very low and the product was only detected

because of its much stronger chromophore. The level could be reduced by degassing or higher flow rate reducing the retention time.

## 7.2: Future Work

### 7.2.1: Suitable Stationary Phases

The most popular reversed-phase silica based columns are unstable in superheated water chromatography, so future work is needed into valuable alternatives. Some columns based on entirely different chemistry are stable. Four materials that have been investigated: PS-DVB, PGC, PBD-Zirconia and Oasis HLB ™ columns are stable for a long time in superheated water chromatography. Apart from these four packing materials there are a number of other column materials that are worthy of future investigation. For instance, two polymer materials: polyvinyl alcohol and polymers based on methyl methacrylate may well prove stable with superheated water. Modified polymers based on PS-DVB, for improved water compatibility, may be better suited for use on superheated water chromatography.

PS-zirconia, Carb-zirconia and other oxides based on either alumina or even titania could be tried. These two oxides along with zirconia are hydrolytically more stable than silica. This would be worthy of investigation to try separations normally performed on silica $C_{18}$ materials. An alumina column with bonded $C_{18}$ functionality and Carb-zirconia columns are now commercially available. A comparison study of conventional LC and superheated water chromatography on more columns like PGC, PS-zirconia, Carb-zirconia, modified polymers based on PS-DVB packing material and also other oxides based columns will give valuable information for reversed-phase liquid chromatography.

### 7.2.2: Efficiency Study

For an efficiency study using the van Deemter curve on Xterra ™ RP 18 and PBD-zirconia columns the same equipment should be used for conventional LC and superheated water. Van Deemter curves study on other packing materials like PS-zirconia, Carb-zirconia, modified based PS-DVB and also other oxide-based columns would be worthy of investigation in superheated water chromatography. The C-terms of the van Deemter curves on PBD-zirconia, polystyrene and Xterra ™ RP 18 columns in superheated water gave higher values than conventional LC in the present study, the reason why the mass transfer resistance increases in this system would be very a valuable investigation for efficiency study. In the present study we used an air thermostating system, perhaps a suitable liquid thermostating system can give improved efficiency.

In UV-superheated water chromatography system, the UV-detector is located outside the oven, therefore the temperature of the water eluents drops when it exits the oven on the way to the UV-detector. This temperature drop may potentially cause solute deposition in the pathway to the UV detector and result in peak broadening. If we can set the UV-detector inside the oven in superheated water chromatography system, no potential solute deposition can occur in the pathway of this system. Recently, the company Knauer (Berlin-Zehlendorf, Germany) has fabricated photometric detectors that have fibre optic capabilities built into their design. It can be possible then to set the detector cell in the oven in superheated water chromatography. Therefore, van Deemter curve study on PGC, PS-DVB, Xterra ™ RP 18, PBD-zirconia columns in this system using superheated water as the mobile will give very valuable information about temperature effects on efficiency.

### 7.2.3: Natural Product Separation

Application of superheated water chromatography-NMR system to unknown compounds in natural products identifications can be a positively role in

future. Grains of paradise extract were separated in present study, but the concentration of constituents was at a low level, therefore, we could not identify these compounds in superheated water chromatography-NMR system. It may be possible to identify these compounds by injecting high concentration of paradise extract.

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## PPESENTATIONS AND PAPER

**Regular Paper:**

- R. M. Smith, O. Chienthavorn, S. Saha, I. D. Wilson, B. Wright, & S. D. Taylor; Selective deuterium exchange during superheated heavy water chromatography-nuclear magnetic resonance spectroscopy-mass spectrometry of sulfonamides, Journal of Chromatography A, 886 (2000) 289-295, accepted 19 April 2000.

**Oral Presentation:**

- S. Saha & R. M. Smith; Comparison of Stationary phases in Conventional LC and Superheated Water Chromatography, A119, 23rd International Symposium on Chromatography, Olympia, London, 1st-5th October 2000.

**Poster Presentations:**

- S. Saha & R. M. Smith; Comparison of Conventional LC and Superheated Water Chromatography Poster, P.21, Analytical Research Forum, Incorporating Research and Development Topics, University of East Anglia, Norwich, UK, 16-18 July 2001.

- S. Saha & R. M. Smith; Stationary Phases in Conventional LC and Superheated Water Chromatography, Poster, A162, Young Researchers Meeting and Specialist Symposia, Umist, Manchester, UK, 16-17 April 2000.

- S. Saha & R. M. Smith; Separation using different stationary phases materials in superheated water chromatography, poster p.30, 36th Research and Development Topics in Analytical Chemistry, University of Greenwich, 12-14 April 1999.