RESISTIVELY HEATED COLUMNS FOR LIQUID CHROMATOGRAPHY

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A thesis presented for the degree of philosophy undertaken in the Department of Chemistry, Loughborough University, Loughborough

June 2010

Acknowledgements

I would like to thank the following people for their advice and continued support throughout this study.

Professor R.M. Smith for his support, discussion and expert guidance.

Dr John. K. Roberts for his advice and practical support and GSK R&D Ltd for their sponsorship of the research being carried out.

Phillip James director of CSI Ltd for his technical expertises and assistance.

Dave and Trevor for the benefit of their technical knowledge in all areas of analytical chemistry.

Lastly, but not least my mother Barbara Harvey-Towers who instilled self-belief.

Abstract

Interest in temperature as a modifier in HPLC separations has increased markedly recently due to miniaturisation, new stable hybrid stationary phases and the use of superheated water as an eluent. However, for decades temperature has generally been regarded as a parameter that should be kept constant in LC separations for retention reproducibility but there is now a mountain of journal papers supporting the use of this variable. Currently, the limit of implementation usually arises from the lack of LC ovens on the market capable of high temperature applications. This led to the development of a "*resistively heated*" LC oven which allowed rapid reproducible heating/cooling patterns of RP columns thus reducing equilibration times and realising high sample throughput. The main objective was to drive temperature programming to a new "*extreme*" by **rapid** column heating akin to GC rather than LC, the emphasis being to produce much sharper peaks very rapidly. It was hoped that temperature gradients could replace solvent gradients and extend the applicability of temperature-programming for hyphenation to other forms of detection. New column materials were sought and tested against high speed temperature gradients. This coupled with micro-column technology should reduce analysis time and appreciably limit the amount of solvent waste currently being generated by conventional LC techniques.

The column heating was achieved by two system components, the oven and the eluent pre-heater unit both based on resistive heating. Post-column cooling prior to detection minimised baseline disturbances imposed by a temperature gradient and enabled the use of detection modes such as MS, RI and ELSD as well as UV spectroscopy.

Chapter 1 Introduction

1.1 The Role of Temperature in Liquid Chromatography

In the late 70's early pioneers, such as Perchalski and Wilder[1], highlighted the improvement in high performance liquid chromatography (HPLC), such as increased resolving power, on increasing the temperature. However, for decades temperature has generally been regarded as a parameter that should be kept constant in LC separations for retention reproducibility. Although this area has been overlooked in the past there is now a mountain of journal papers supporting the use of this variable in HPLC and these were reviewed in 2003 by Greibrokk and Andersen[2].

Separations performed at elevated temperatures and on temperature programs have been noted to improve peak shape and peak capacity. Often efficiency is said to be greatly improved but this only contributes to a small part of the story. A comprehensive study to determine the effects of temperature on efficiency has yet to be explored. In any case it is normal practice to operate above the optimum window in a desire to speed up the analysis. The advantage is that in this region plate height is governed by mass transfer. Although the minimum plate height may not be improved compared to that observed at the lower temperature, when operating at a higher temperature, a flatter van Deemter curve results. Therefore, an increase in optimum velocity can speed up the separation without deterioration in peak shape[3]. This effect of temperature on peak shape has been modelled by Yang[4]. The model demonstrated that at low temperature the mass transfer term of the Van Deemter equation (see section 1.2.3) dominates the separation process whilst the longitudinal diffusion controls peak broadening. Wang *et al*.[5] found that they could optimise peak capacity for the separation of peptides by setting the gradient to the longest tolerable time and then increasing the temperature to the maximum set-point according to the oven manufacturers specification. These are important advances in chromatography as the study of proteins and genes are essential in supporting the growing biologics market.

The aim of the present work was to investigate the use of fast temperature gradients in HPLC and their potential role and benefits in pharmaceutical analysis. This study built on the increased interest in miniaturisation in LC, the availability of new stable hybrid stationary phases and the use of superheated water (SHW) as an eluent.

The common fears that have hindered the application of high temperature instrumentation are mainly stationary phase dissolution and analyte instability. Currently, the limit of implementation usually arises from the lack of LC ovens on the market capable of high temperature (HT) applications. GC (air-forced) ovens can be used in their absence but these are best-suited to heating low mass capillary columns. The overall resistance for heat transfer to a larger mass conventional LC column is too great. Serious consideration has to be given to the whole set-up otherwise any benefits derived from such an application can be masked. The benefits are many and are known to affect retention, peak shape and selectivity, solubility and diffusivity of the analyte leading potentially to higher mass loading[6]. This increase in analyte diffusivity at elevated temperature has been exploited in recent years for the rapid separation of biological macromolecules[7]. Analysis time is reduced as mobile phase viscosity decreases with increasing temperature allowing for higher throughput at elevated flow velocity without a high back pressure. Separations are improved as mass-transfer kinetics and thermodynamics are enhanced. The thermodynamic properties will also vary according to the stationary mobile phase system[8]. This usually leads to sharper peaks, providing any thermal mismatch is compensated for between the eluent and the column, which is one of our system set-up considerations. This can make the crucial difference when resolving critical pairs and the detection of trace components, which often sit on the front or tail of the main band. The temperature range of common interest is ambient to 60 °C (due to oven capability) with a few studies pursuing extreme temperatures of up to 200 °C[9-13]. The main driver towards superheated water as a mobile phase is to move towards greener chromatography and lower cost, which would be of great benefit to industry.

High-temperature methods have been used previously in limited applications, such as size exclusion and ion exchange chromatography, to improve column efficiency[14-17].

However, more recently vast improvements have been made in column packing materials and geometries leading to temperature control being increasingly investigated. Solvent gradients traditionally applied to resolve a mixture of components take too long to re-equilibrate between analyses and temperature programming offers a solution to lengthy cycle times. Miniaturisation is desirable with temperature gradients as there is less stationary phase volume to heat allowing for faster response times. A smaller bore column would increase mass sensitivity due to the reduced dilution effect. A small bore column is also reported to reduce radial thermal gradients associated with large bore columns, causing different flow velocities of the mobile phase leading to band broadening[18].

High temperature LC hyphenation to detectors that are solvent gradient sensitive is also possible but these do come with their own set of considerations. For example temperature fluctuations can lead to refractive index changes in the UV background stability of the mobile phase. The eluent is required to reach the detector at a constant temperature (below 80 °C otherwise this will damage the UV flow cell) and in a liquid state i.e. SHW applications need approximately 30 bar back-pressure (section 1.3.2, figure 8). Hyphenation to various other detectors should be treated individually as an eluent exiting a temperature program at a high temperature may benefit certain interfaces, such as MS. Other factors to consider are that the time for columns to equilibrate after heating and aqueous media may lead to decomposition of analytes and stationary phases at higher temperatures. However, although temperature based applications have disadvantages these are far outweighed by the advantages.

1.2 Temperature Effects in LC

1.2.1 Retention

Chromatography is a dynamic equilibrium process of the distribution of an analyte between the mobile and stationary phase. An increase in temperature can impact this

distribution due to increased mass transfer. The relationship between the two phases is described by a distribution constant:

$$K = \frac{C_S}{C_M}$$

Where :

K = partition constant

C_S = concentration of analyte in stationary phase

C_M = concentration of analyte in mobile phase

The distribution coefficient can be expressed in terms of free energy of a solute from one phase to another by the well established Arrhenius equation:

$$\Delta G^o = -RT \ln K$$

Where :

ΔG^o = Gibbs Free Energy

R = gas phase constant ($8.14\ KJ/mol^{-1}$)

T = absolute temperature

The standard energy of distribution can be further divided into different parts representing the energy sources:

$$\Delta G^o = \Delta H^o - T\Delta S^o$$

Where :

ΔH^o = enthalpy change for solute transfer

ΔS^o = entropy change for solute transfer

This temperature dependence of the retention factor, *k*, for a LC system is described by the Van't Hoff equation:

$$\Delta G = - RT \ln (k/\phi)$$

$$\ln k = \frac{-\Delta H^\circ}{RT} + \frac{\Delta S^\circ}{R} + \ln \phi$$

Where:

k = retention factor

ϕ = phase ratio

Both enthalpy and entropy terms are highly dependent on temperature. The enthalpy term represents chemical changes, such the solubility, diffusion and interaction of a solute between the two phases expressed as the heat transfer of the system. A decreasing negative value with increasing temperature represents an exothermic process that is enthalpically driven. For instance if we consider that the heat capacity change is less for polar interactions, i.e. less negative, then a C18 phase will have a large negative value as polar interactions are minimised having a reduced effect on retention. The entropy term represents the disorder of a system and the unavailability of a system's energy to do work. Therefore, a more positive value represents an endothermic process that is entropically driven. Bolliet and Poole[19] found that temperature variations can be expected to have a greater effect on retention and selectivity with mobile phases that contain low amounts of the organic modifier. This increases the interaction of the solute with the aqueous part of the mobile phase increasing system disorder. They found that to decrease the hydrogen-bond acidity of the mobile phase relative to the stationary phase resulted in a change in polar interactions. Thus, the band spacing of molecules was affected largely by size and hydrogen-bond basicity at high temperature.

Retention changes caused by temperature have been studied by a number of people on different stationary phases[20,21], including chiral stationary phases[22]. Hirata and Sumiya showed that for three solutes (C_{14}, C_{16} and C_{18}) $-\Delta H$ increased almost linearly with increasing number of carbon atoms with increasing temperature[23]. This was later translated further into a rapid method for determining the enthalpies and entropies for a series of compounds in HPLC proposed by Guillaume and Guinchard[24]. This has been used to study the retention mechanism of benzodiazepines[25] and has highlighted the need for high temperatures when separating difficult compounds[26]. Snyder found that if the

column temperature was increased by 1 °C then this will usually lead to a decrease in retention factor (k) by 1 to 2%[27]. Tran *et al.*[28] have used multivariate experimental designs to study the retention of different compounds of varying size and functionality at elevated temperature. This was combined with other parameters including % organic modifier, mobile phase pH, and stationary phase. The greatest effect was attributed to % organic but interestingly temperature was found to affect a range of molecules in different ways based on their functionality coupled with the solvent used. For example, for neutral compounds a 4-5 °C change in column temperature had the same effect as 1% change in acetonitrile concentration (5:1), whereas a ratio of 3:1 was observed for methanol. Acidic compounds also showed a smaller response in methanolic aqueous phases compared to acetonitrile aqueous phases. Basic compounds gave the highest effect and a 2:1 ratio was found for temperature versus % acetonitrile. Thus, temperature based applications should be advantageous in the pharmaceutical industry as most drug compounds are bases. The different thermodynamic responses of the compounds suggest that selectivity will also be affected where different functionality exists. Specificity can be improved and challenging separations resolved, such as in impurity profiling. A comparison of the change in solvent composition with a change in temperature has been documented by Bowermaster and McNair[29] who demonstrated that the two approaches complement each another.

The pH of the mobile phase is used extensively to speed up or slow down elution as it affects the physical parameters of a separation[30]. Temperature also affects the ionisation mechanisms, a rise in temperature speeds up the sorption kinetics and accelerates slow diffusion rates[31]. At elevated temperatures the pKa of a compound can be lowered in a buffered system, thus causing a change in the degree of ionisation and increasing lipophilicity. Buckenmaier *et al.*[32] studied bases at varying pH and found that retention actually increased at intermediate pH when coupled with elevated temperature. This was due the changing degree of protonation of the solute and thus acid/base equilibria. Thus, the use of temperature can also add another dimension as a variable to gain more control over selectivity and retention.

1.2.2 Selectivity and Peak Shape

The selectivity changes readily obtainable in LC enable it to compensate for the lack of narrow band widths found in GC. Since different compounds can have different enthalpy changes on elevating the temperature, their relative retention can also change causing larger selectivity differences. This is due to the different thermodynamic contributions of the entropy and enthalpy terms. The higher the entropy value i.e. the larger the molecule, the more it is expected to be affected by temperature in terms of selectivity e.g. proteins compared to small solutes (figure 1)[33,34].

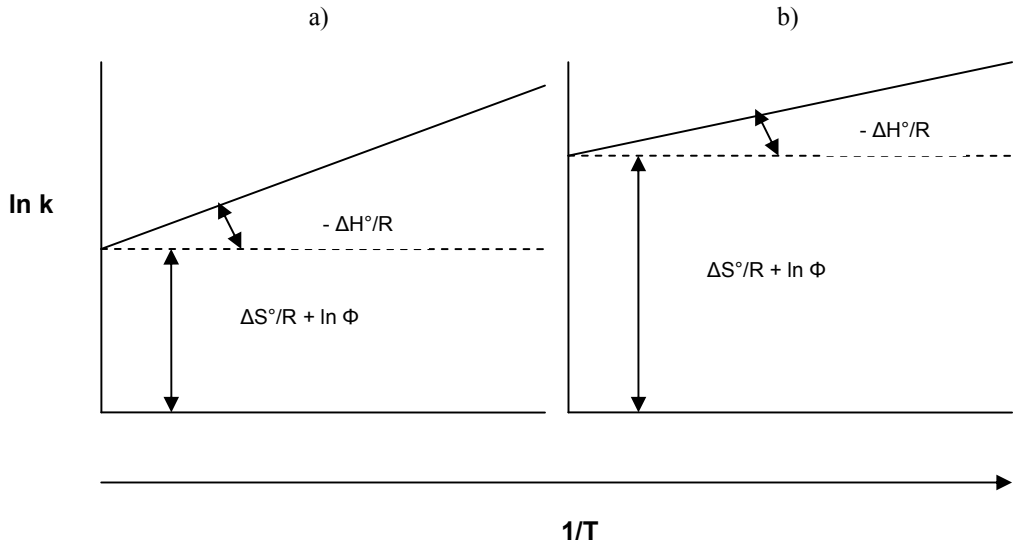


Figure 1: Van't Hoff plots demonstrating the varying degree of enthalpy and entropy contributions that affect selectivity due to increasing temperature: a) enthalpy driven separation and b) entropy driven separation

A solute with a large ΔH will be more affected by temperature changes than a solute with a small ΔH[35]. Small molecules tend to have enthalpy interactions in RPLC systems around –10 to –15 kJ mol^{-1}. Larger molecules usually have larger negative values and thus their selectivity is more likely to be affected at high temperatures. This increases the interaction of the solute with the aqueous part of the mobile phase increasing system disorder.

Li and Carr also demonstrated that for low molecular weight species the selectivity depended on the type of solute[36]. They investigated the elution order for compounds with increasing temperature using a PBD-coated zirconia stationary phase. They found that temperature had no effect on selectivity for members of a homologous series of poly aromatic hydrocarbons (PAHs). However, changes in elution order for condensed and less compact PAHs had been found much earlier by Chmielowiec and Sawatzky[37] at sub-ambient and elevated temperatures. Later Sentell *et al.*[38] reported selectivity differences for isomeric PAHs at sub-ambient temperatures, where improved shape recognition was observed. This was employed by Sander and Craft[39] for the improved separation of isomers. The enhanced selectivity was found to be due to the ordering of the individual alkyl chains of the stationary phase. The amount of kinks in the chains due to conformational disorder, were reduced by reducing the temperature. This causes an enhancement of homologous group selectivity for planar and linear solutes. Shape selectivity was less applicable to solutes with conformational freedom (e.g., alkanes). This influence of temperature was also investigated by Sander and Wise[40] on the selectivity for monomeric and polymeric C_{18} stationary phases. They concluded that at ambient temperature the polymeric phase exhibited better selectivity than at reduced temperature. Sander and Wise[41] also concluded that in general the separation quality for classes of compounds that exhibit well-defined (constrained) molecular structure, such as isomers, was improved at low temperature.

Simple mixtures that were found to have one major retention mechanism would not benefit from adjusting temperature. Complex mixtures that contained polar and ionisable groups, however, could be optimised by altering the temperature. Previous research conducted by Li and Carr[42], demonstrated that a change is seen for polar, partially-protonated species. The elution of tricyclic antidepressants demonstrated a marked changes for polar, partially protonated species. The selectivity of compounds with different functional groups, such as chlorophenols, was significantly affected by temperature with respect to selectivity. Mao and Carr[43] also found that the effect of changing the column temperature was analogous to changing the column length and that they could tune the selectivity in a thermally tuned tandem column concept using

columns with different selectivity's. This involves the coupling of two columns in series and independently controlling the temperature of each. The columns possessed different selectivity's (band spacing) so that peaks, which could not be separated on one column, could be separated on the other.

Vanhoenacker and Sandra[44] have perhaps delivered the most spectacular selectivity change with respect to a temperature on silica, zirconia and polystyrene/divinylbenzene based stationary phases with an acetonitrile/water mobile phase. They found a total reversal in elution order as shown in Figure 2 for octylphenol ethoxylate oligomers. They found that the chain length distribution was controlled by hydrophobic interaction.

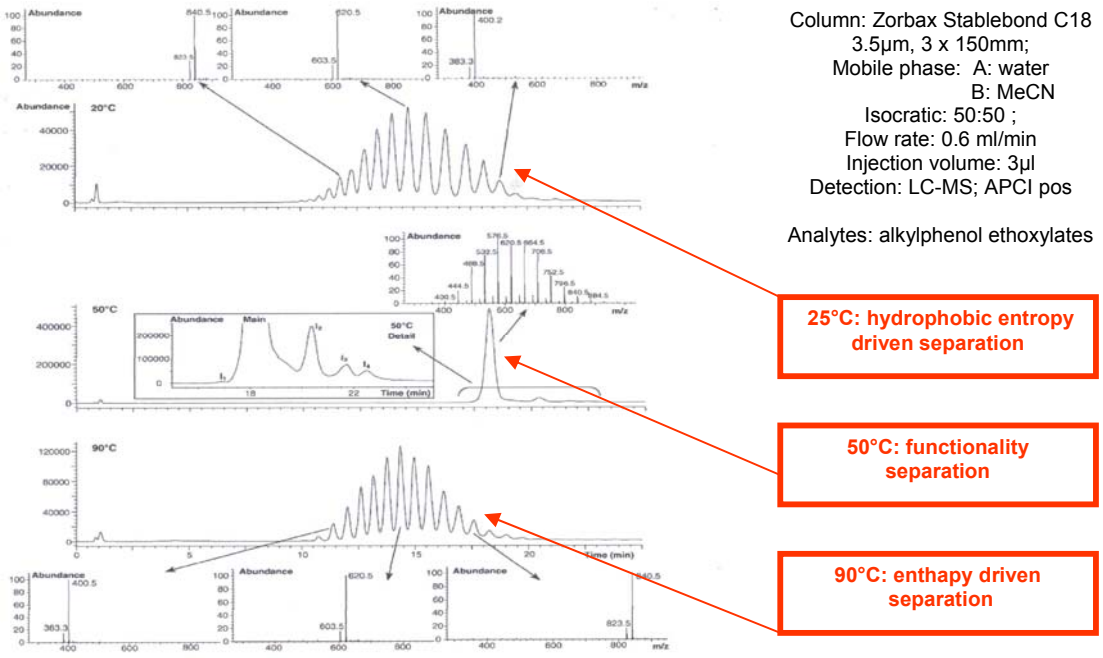
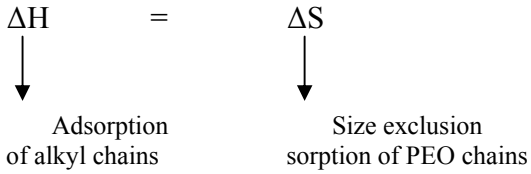
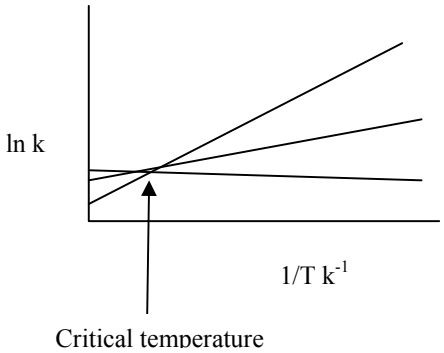


Figure 2: Reverse in Selectivity from 50 °C to 90 °C for Octylphenol ethoxylates Oligomers[44].

At 25°C: large less hydrophobic oligomers which were polymerised to a lesser extent eluted first. The longer the chain length the more polar, $\Delta H > 0$ therefore sorption of a polymer was unfavourable and was driven by $\Delta S > 0$.



At 50°C: a critical transition temperature was reached where the selectivity was reduced based on functionality of oligomers only i.e. ΔH was equal to ΔS and therefore the oligomers co-eluted



At 90°C: the chain length distribution reappeared with an inverse elution order denoting a change in retention mechanism where ΔH and ΔS are < 0 (with the exception of oligomer chains > 15).

This phenomenon was not found when the organic eluent was switched from acetonitrile to methanol or when graphitic columns were used regardless of the mobile phase type or column temperature. It appeared to be an enthalpy driven process and the exact cause has not been determined.

1.2.3 Column Efficiency

Unlike GC and SFC, LC suffers from relatively high back-pressures and low diffusion rates. An increase in temperature has been shown to lower the viscosity and hence the back-pressure and to give increased diffusion coefficients, which results in enhanced mass-transfer rates[45-49]. The reduced pressure drop also allows more flexibility in the choice of column variables when developing a method i.e. length, particle size, phase etc. and increased flow rates can result in a higher throughput.

The Van Deemter equation was originally applied to GC, and looked at the kinetics of a chromatographic separation. It deals with a proposal for determining band broadening in terms of three parameters; eddy diffusion (A term), molecular diffusion (B term) and mass transfer (C term):

$$H = A + B/u + Cu$$

Where:
u = linear flow velocity
H = height equivalent to a theoretical plate (the ratio of the column length to the plate number) H = L/N

L = column length

N = efficiency (number of theoretical plates (NTP) equivalent to United States Pharmacopeia (USP) plate count = 5.54 $(t_R/w_h)^2$, *where* t_R = peak retention time and w_h = width of peak at half height)

A smaller plate height corresponds to a more efficient column for a given length, therefore all of these terms (A, B and C) should be minimised. The plate height (H) has the added benefit that it increases more slowly with increasing flow rates at higher temperatures[50].

Column efficiency is hence dependent on flow rate. The alternative "Knox equation" describes the relationship between reduced plate height, h, and reduced velocity, υ:

$h = A\upsilon^{1/3} + B/\upsilon + C\upsilon$

In both of these equations:

A = the eddy diffusion is the availability of multi-paths which a molecule can take through a packed column and depends on the packing factor and particle size.

B = longitudinal diffusion in the mobile phase. For example at low flow rates (long run times) sample molecules can diffuse through the entire column, at higher flow rates the time for diffusion is shorter and the broadening is insignificant

C = mass transfer kinetics. For example at high flow rates the finite time for molecules to move in and out of the stationary phase and through the mobile phase results in large "lags", which contribute to band broadening

υ = reduced velocity = $\mu d_p/D_m$

Where:

d_p = particle size

D_m = mobile phase diffusion coefficient for sample in the mobile phase

This equation allows a comparison between different columns. This gives a dimensionless quantity where a small value for h implies higher plate numbers.

$h = H/d_p$

Where:

d_p = particle diameter

The A term in both equations is reflective of how well the column is packed, whereas the B term represents the diffusion of a molecule within the eluent in the column. The C term denotes the efficiency of the mass transfer between the mobile and stationary phases. As reported by Greibrokk and Andersen[51], it is expected that at elevated temperatures the laminar flow and laminar mixing of the analyte molecules will improve due to increased

diffusivity, thus the A term is likely to benefit in the Knox equation. The B term, is the longitudinal diffusion within the mobile phase and increases with increasing temperature and can become significant at low linear flows. Yung[52] summarised that a decrease of height equivalent theoretical plate count (HETP) had been observed up to 100 °C and that above this it would increase. He concluded that the mass transfer (C) was dominant at lower temperature and that the B term controlled peak broadening at higher temperatures. The C term is expected to reduce with increased temperature and to benefit the most from an increase in temperature. Naphthalene has been reported to have a faster diffusion rate when the temperature was increased from 24 to 80 °C in the mobile phase. This resulted in a 2.6 fold faster optimum linear velocity and a 2.5 fold decrease in the C term of the van Deemter equation. Smaller particle sizes or longer columns can be used due to the decrease in viscosity of the mobile phase (by 2.2 times) thus reducing back-pressure can allow for twice as many theoretical plates.

The use of elevated temperatures has been reported to particularly improve the peak shape of larger molecules, such as proteins[53-55]. Larger molecules are expected to be more affected by temperature than smaller molecules due to the thermodynamics of a separation. Increasing the column temperature enhances the kinetic and transport properties for the rapid analysis of biological macromolecules. Usually, proteins and peptides are analysed at sub-ambient temperature to preserve the integrity of the molecule. However, the growing need for rapid analyses due to the challenges concerning proteomics and metabonomics has necessitated the revival of elevated temperature in LC as an operational variable.

At temperatures of 200 °C long (50 µm id) open tubular liquid chromatography (OTLC) columns have yielded 1 million theoretical plates for a benzene peak[56,57]. Higher peak capacity was also reported by Lestremau *et al.*[58] when they applied high temperature to decrease the back-pressure, allowing the coupling of multiple conventional columns thus increasing the number of theoretical plates. Technology has now moved to ultra pressure LC systems with particle sizes of 1.7 µm[59,60]. This could form a powerful future

partnership with temperature producing ultra fast analysis with peak shape analogous to the sharp narrow bands indicative of GC.

1.2.3.1 Radial and Longitudinal Temperature Gradients

Viscosity heat dissipation can also affect a column's efficiency as a result of longitudinal and radial temperature gradients (figure 3). If the column is heated externally with no control over the eluent heating, a parabolic profile along the column packing bed is generated, whereas this profile should be flat in order to produce a sharp band.

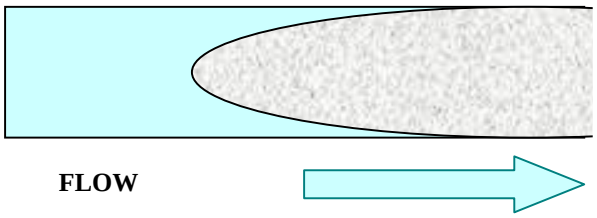


Figure 3: Parabolic flow profile demonstrating liquid travelling faster at the walls of a column compared to the centre when the column in heated from the outside only

Heating at the column walls lowers the viscosity of the eluent and can diminish the achievable efficiency from HTLC[61] due to band-spreading. This is commonly known as thermal mismatch broadening, and can be compensated for by pre-column eluent heating[53]. These effects can also be minimised by replacing conventional HPLC columns with narrow-bore/micro-bore columns.

1.2.3.2 Resolution, (R_s)

Since efficiency can be affected by temperature, this improves the width of peaks and can enhance resolution (R_s):

$$R_s = \left(\frac{t_{R2} - t_{R1}}{0.5(W_{b2} + W_{b1})} \right)$$

Where:

W_{b1} is the base width for the first eluting peak, and

W_{b2} is the base width for the second eluting peak

Resolution can also be expressed in terms of efficiency (N), selectivity (α) and retention factor *(k)* and again can all be improved with higher temperatures:

$$R_s = (1/4)N^{1/2}(\alpha - 1)[k/(1+k)]$$

efficiency **selectivity** **retention**

Where:

$k = (t_{R2} - t_{R1)} / t_0)$

These components can be optimised independently, *k* is influenced mainly by solvent strength where α is affected by chemical changes in the stationary or mobile phase. At higher temperatures the bands become sharper and an increasing temperature gradient can focus these bands in the same way as a solvent gradient. Bohm used an ambient and sub-ambient range of +30 °C to -7 °C to optimise the resolution of a number of isomers[62]. However, Berthod *et al.*[63] also looked at sub-ambient temperatures to resolve enantiomeric pairs and found that in a majority of cases an increase in temperature decreased enantiomeric resolution or it was lost completely. Van't Hoff plots showed that the separations were enthalpy-driven in NP and RP and for polar ionic mode (PIM) it could be either depending on the solute. However, structural changes can also be induced by higher temperatures. Figure 4 demonstrates an intra-column racemisation induced by temperature. At 25 °C and 35 °C a raised baseline between the oxazepam enantiomers indicates equilibrium between the two. Temperature can only be raised in the event of chiral separations when resolution factors are high to begin with and the phenomenon seen in figure 4 does not exist.

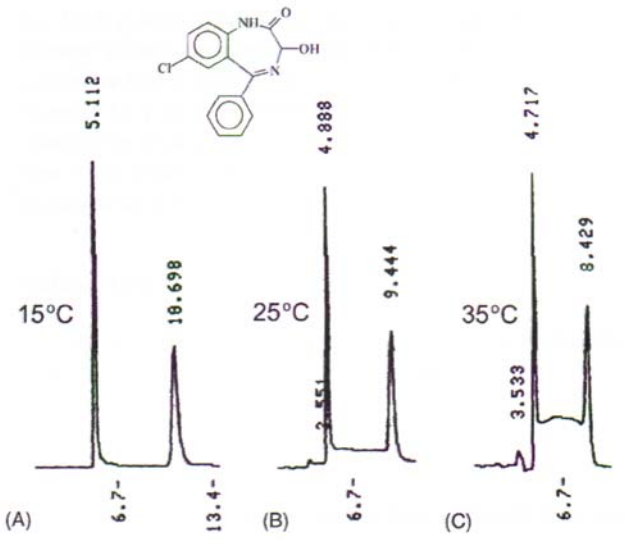


Figure 4: Intra-column enantiomer interconversion induced by temperature of oxazepam racemate on a Chirobiotic T column, PIM mobile phase: 100 % methanol with 0.2 % acetic acid and 0.2 % triethylamine (TEA), 0.9 ml/min[63].

1.2.3.3 Peak Asymmetry

The asymmetry of a peak is commonly measured at 10% of the peak height as detailed in figure 5[64,65]. Another definition of peak shape is the tailing factor defined in the USP as 2CB/AB at 5% of the peak height. Usually, asymmetry or tailing of ≤ 2.0 is acceptable, any greater than this and the quantitation and separation of peaks from interferences becomes difficult.

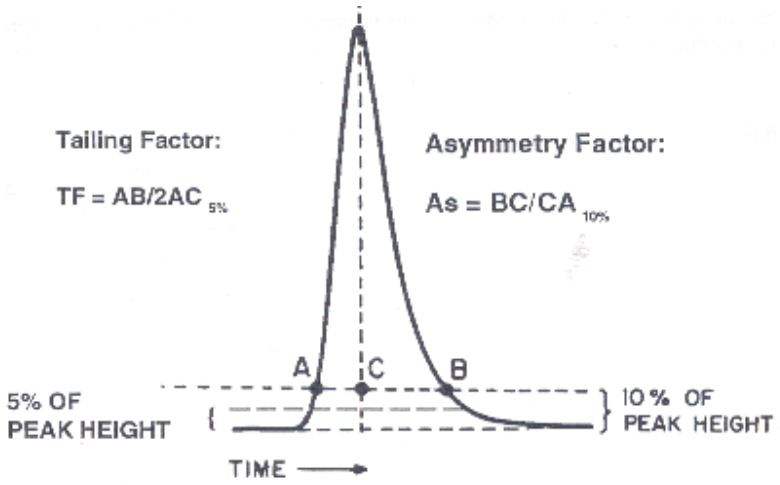


Figure 5: Calculation for peak asymmetry factor[66].

Peak asymmetry is an important parameter in method development as the front or tail of a peak can hide a number of smaller trace components, such as impurities and diastereoisomers, which may be present in small amounts but could be highly toxic. Peak shape is influenced by temperature as the acidity/basicity of the mobile phase and stationary phase changes[67,68].

1.2.4 Column Stability

The stability and lifetime of a given column depends largely on the type of stationary phase and conditions applied, such as the mobile phase pH and the type of buffer/organic modifier. Traditional silica based columns are manufactured by derivatising the surface of the silica support. Problems arise when applying the silane reagents as these groups are larger than the spacing between silanols thus resulting in incomplete coverage because of steric interference to reaction. This leaves the surface open to attack and possible dissolution or for interactions of the surface to occur with the mobile phase or analytes and over time the retention of the components of interest may change. Any remaining silanols, which have not been derivatised, are acidic and can cause peak tailing for basic compounds. Generally a pH range of 3 – 8 is acceptable when trying to avoid silica dissolution. At high temperature many conventional silica columns are less stable and

will readily dissolve at a pH below 3 or above 6. Hydrolysis occurs at siloxane (Si-O-Si) bond that binds the silane to the support and will be accelerated especially if coupled with highly aqueous mobile phases. This subsequently leads to a loss of silane bonded phases[69-71]. Stability has been improved for conventional silane phases by sterically protecting the silanol groups. A pH range of 1 – 12 is advertised for the the XBridge and XTerra columns (Waters) due to new hybrid particle technology. The XTerra used methyl substitution but this did not possess the same equivalent efficiency to the silica-based columns. An ethylene bridged hybrid was introduced in the XBridge column that demonstrated improved efficiency and mechanical strength lowering hydrolytic activity[72-76].

In addition columns have also been designed with enhanced capabilities[77] based on non-silica column materials, including; polybutadiene-coated (PBD) zirconia[78-84], graphite/carbon[85,86]. These covalently bonded silica substrates, polymer-coated inorganic substrates, and graphitic phases have been evaluated and classified according to their maximum temperature range[87-96]. They can be thermally stable up to 200 °C over extended pH ranges[97-100] and offer higher resistance to hydrolysis and increased thermal and mechanical stability than silica based columns. However, although many new types of stationary phase have proved to be isothermally stable, less is known about their temperature programming capability. It is a logical assumption to conclude that a temperature gradient would weaken a phase as it is ramped up and down the temperature scale repeatedly. For instance, Marin *et al.*[101] found a significant rise in baseline for zirconia based stationary phases on programming. Capillary stationary phases of silica, polydivinylbenzene and monolithic were reported by Andersen[102] to be stable after running more than 1,600 temperature programmes. This was possibly due to the efficient heating and cooling of the capillary column akin to GC, whereas traditional HPLC columns are heated and cooled much less efficiently for prolonged periods of time.

Teutenberg *et al.*[103] studied the temperature stability of several normal phase and RP columns using a water-only mobile phase. The temperature was adjusted to 120 °C for the bare silica stationary phases and to 185 °C for metal oxide and carbon stationary

phases. They showed that metal oxide stationary phases exhibited excellent thermal stability over the duration of the test period and are therefore suitable for high temperature LC applications.

1.2.5 Analyte stability

Many of today's active pharmaceutical ingredients are chosen on the premise that they are highly stable. They undergo forced degradation and extensive stability studies to prove that this is the case. However, the thermal stability of an analyte at elevated temperature is always one of the biggest concerns when choosing optimal separation conditions. On-column reactions can occur even in mild conditions where chilled auto-samplers and eluent additives try to minimise hydrolysis, oxidation and isomerisation. For example Horvath and co-workers found that residual iron acted as an on-column catalyst increasing the oxidation / reduction rate of substituted methoxyhydroquinones[104]. They also showed that proline dipeptides isomerize in the time scale of a chromatographic separation. Temperature; column length, pH and flow velocity could all be adjusted to decrease the resultant peak broadening. They determined that the critical parameter was the difference between enthalpy of transfer and the activation energy for the on-column reaction. Favourable conditions would mean that the analyte emerged faster from the column than it has had time to react. Thompson and Carr have laid down rejection criteria for an analyte exposed to high temperature[105]. They analysed a small number of basic drugs at high temperatures and high speed and determined that analyses at elevated temperature were as reliable as those done at ambient temperature. They also demonstrated that the residence time of a thermally unstable analyte was crucial to a reaction taking place and showed that complex molecules can be quantifiable at a temperature of more than 100 °C in aqueous media.

Even though dicumyl peroxide (DCP) is known to degrade thermally, when it was analysed by high temperature LC at 130 °C[106], a significant degree of decomposition only

occurred at low flow rates (1 – 2 ml/min) whereas at a high flow rate (4 ml/min) there was still no evidence of degradation due to its lower residence time on the column.

Teutenberg *et al.*[107] analysed three different HPLC methods for the quantification of thalidomide in tablets. They found that the stationary phase had a pronounced effect on the on-column degradation with thalidomide at high temperatures. Virtually no degradation occurs was observed when a polystyrene divinylbenzene column is used, whereas it was completely degraded at 180 °C when a carbon clad zirconium dioxide column was used.

1.2.6 Temperature Programmed Separations

The primary goals of a separation are to achieve high throughput of samples coupled with high efficiencies to satisfy the increased desire to analyse large numbers of small/trace quantities of complex samples. In GC temperature is used to achieve separation and temperature gradients are applied to speed up the analysis of samples with a wide range of volatilities. This technique relies on having a volatile and thermally stable compound, which limits the application of this method. Temperature is the easiest variable to control in GC and is controlled accurately to with ±1 % of the temperature set-point. The columns lend themselves well to the desired efficiency for heating and cooling. These are usually open-tubular columns, typical dimensions 30-300 m in length where the gas path is an open hole through the column centre. The tubing has an internal diameter of 0.1 - 0.5 mm and can accommodate flow rates of 1 – 10 ml/min.

In LC solvent strength gradients replaced temperature changes, extending the range of compounds accessible for separation. Temperature effects in HPLC are usually regarded as having a small response when compared to GC, since the enthalpy of transfer for a solute between the mobile and stationary phase is far lower for liquids than that found in gases. The regulation of column temperature was initially not common in HPLC, however, it is still an important parameter and most modern intruments use an isocratic oven (up to 80 °C) to achieve reproducibility. Recent improvements due to column

miniaturization, taking ideas from capillary GC to capillary LC has made temperature gradient applications more controllable and hence, much more viable. The lower thermal mass of capillary columns, where the dimensions are much smaller than conventional HPLC columns has reduced equilibration times and have led to a renewed interest in temperature gradient methods for LC. However, most chromatographers do not utilise temperature gradients for routine analysis. The reasons for this are instrumental and subsequently having confidence in the reproducibility of results.

The potential benefit of temperature gradient was realised in the 1960's by Hesse and Engelhart[108] whom demonstrated normal phase temperature programming. Bowermaster and McNair found the reproducibility of retention times to be 2%, which was comparable to that found for gradient elution. These temperature-programmed systems reduced analysis times, increased peak capacity and provide easy control of selectivity. Yan *et al.*[109] looked at high-temperature ultrafast LC (HTUFLC), and found in initial investigations that separations at elevated temperatures are more efficient, and provide up to fifty times faster separations and drastically reduces solvent consumption.

Combining coupling high-speed separation with temperature optimisation, eluent composition, gradient and stationary phase have all been investigated and reviewed[110-116].

1.2.6.1 Temperature-programming in capillary LC

Capillary LC has many advantages, which have been reviewed by Vissers *et al.*[117]. One advantage is the ability to analyse minute sample sizes due to reduced chromatographic dilution and it's compatibility for readily hyphenation to detectors, such as electrospray ionization time-of-flight mass spectroscopy (EI-TOF-MS) MS[118], or and micellar electrokinetic chromatography (MEKC) to improve selectivity[119].

The use of capillary columns also revived interest in temperature programming in LC[120-127].The low thermal mass of small bore columns ensures more effective heat dissipation eliminating problems associated with temperature gradients[128]. This requires the correct

configuration of chromatographic system to support this and has been covered in detail by Djordjevic and colleagues[129]. Band broadening is a problem if the void volume of the entire system is not held accountable and optimised. For instance a smaller flow cell volume is required (taking care to maintain path length) or on-column detection is used. However, temperature gradients are easy to control and facilitate the implementation of miniaturised LC systems. Unlike solvent gradients, temperature programming lends itself particularly well to capillary LC providing a means of generating a gradient elution to speed up late eluting components with minimal cycle times. Djordjevic *et al.*[130] went up to temperatures in excess of 100 °C with temperature programming on capillary columns (Figure 6) and further optimised with flow rate programming, which generated a thermal gradient across capillary columns.

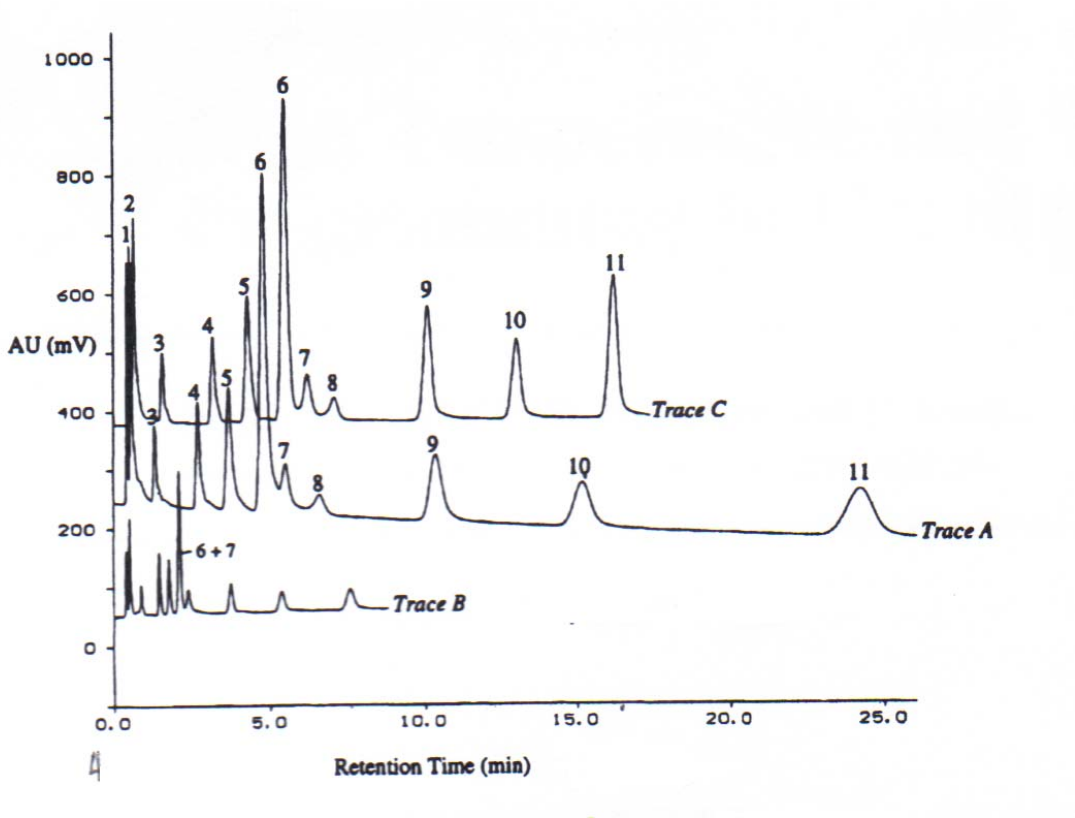


Figure 6: Separation of test mixture on a capillary column Hypersil ODS, 3 µm, 5 cm x 180 µm; acetonitrile/water 30:70 % v/v at 6 µL/min, 210 nm. Peaks: thiourea (1), benzyl alcohol (2), methyl benzoate (3), toluene (4), benzophenone (5), naphthalene (6), 1,4 dichlorobenzene (7) and 1,2,4,5 tetrachlorobenzene (8). Trace A = 48 °C; trace B = 98 °C and trace C = 48 – 138 °C at 6 °C/min[130].

This study demonstrated how elution strength can be controlled with a temperature gradient to obtain resolution of a complex mixture compared to isothermal analysis. However, the idea of a programmed analysis is to vary operating conditions during analysis so that the sample may be eluted under optimal conditions. Snyder made a theoretical comparison of the various techniques[131], showing that resolution per unit time decreases in the order: Solvent programming (best) > coupled columns > temperature-programming ≈ flow programming > normal elution.

Therefore temperature gradients can be successfully combined with solvent gradients[132] giving maximum flexibility during a separation. Andersen *et al.*[133] separated a highly complex sample of polyglycerol fatty acids using both a temperature gradient and a solvent gradient in capillary LC coupled to ELSD and ESI-TOF-MS (Figure 7). This compared the temperature gradient effect to a solvent gradient run and highlighted its advantages. The technical difficulties associated with a solvent gradient were overcome by the temperature gradient's ability to be enacted as soon as the run is initiated. The solvent gradient takes too long to take effect, which is evident in the reduced sensitivity of early eluting components. A temperature gradient is easy to control and apply to capillary columns and resulting baselines are much flatter enabling greater accuracy when quantifying.

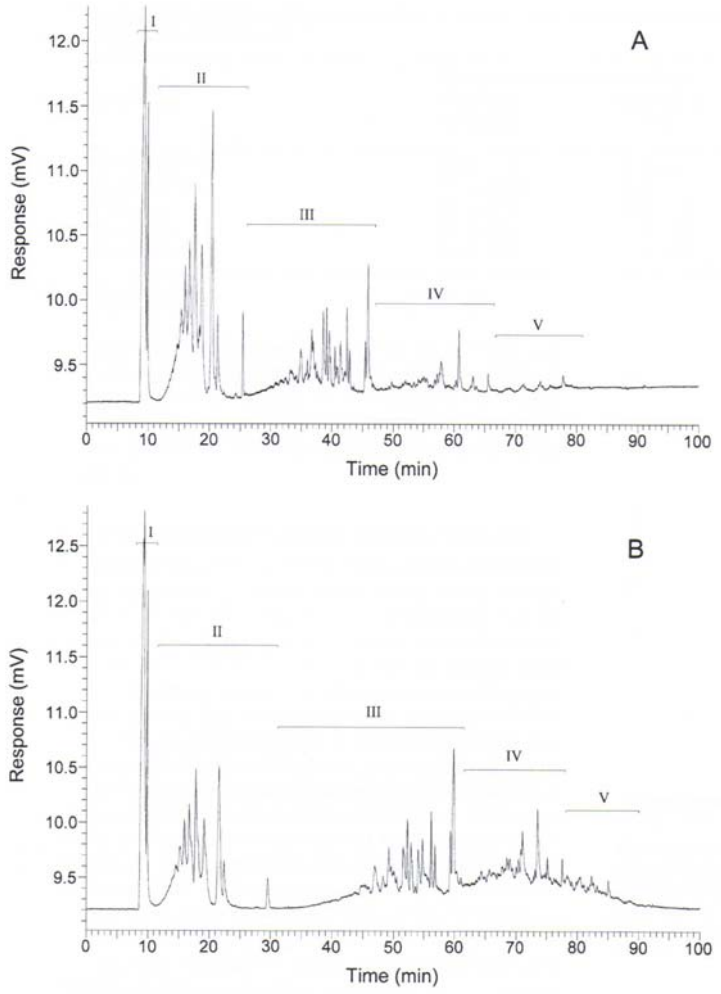


Figure 3. Separation of PGE using temperature and solvent gradient packed capillary LC-ELSD. Column: 0.32 mm ID × 60 cm Kromasil 3.5 C18-100. (A) mobile phase: acetone-acetonitrile (10:90, *v/v*), temperature gradient: 30°C for 10 min, then 1.5 K/min to 150°C. (B) solvent gradient: 100% acetonitrile for 10 min, then a linear gradient to 100% acetone at 70 min, column temperature: 50°C.

Figure 7: Temperature and solvent gradient packed capillary LC-ELSD chromatograms[134]

Capillary columns work well with a temperature program due to the peak compression effect described for GC. The peak compression factor occurs when each component focuses at its characteristic temperature (Figure 8). Column efficiency (H) is an important variable relevant to the pressure drop across a column. If this is large in long columns it can't be assumed that it is good for short columns, thus broadening as it moves down a column is determined by:

$$\sigma = \sqrt{Hx}$$

where

σ = band standard deviation

x = distance moved down the column

H = column efficiency

$$\left(\frac{d\sigma^2}{dt}\right)_{spreading} = \frac{Hu}{(k+1)}$$

where

t = time

k = retention factor

u = linear velocity

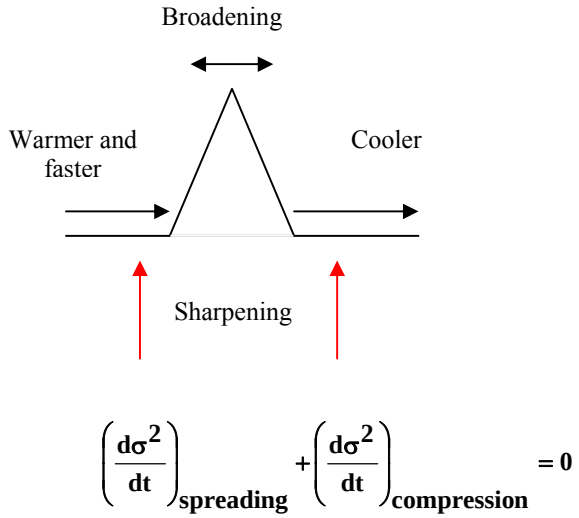


Figure 8: The process of band compression; the rear of the band moves faster than the head of the band, thus balances the normal band broadening process.

Increased sensitivity and peak capacity are good reasons to pursue capillary chromatography for LC. For example a 20 fold reduction in flow rate will result in a 20-fold increase in sensitivity as the analyte is contained in one twentieth of the mobile phase. If the sample mass is maintained when the sorbent bed is reduced 20-fold this will

also increase sensitivity. Efficiency and increased peak capacity is achieved as longer columns equate to more plates and better resolution.

1.3 Implementation of High Temperature Chromatography

The technology required for HTLC to work focuses on four areas: the column heater, column material, detector and the mobile phase.

1.3.1 Column heaters

A number of different heater types have been used and reviewed by Smith[134] these include block heaters, circulating air and immersion heaters. However, none of these approaches will probably provide a fast enough response to give the full benefits of temperature-programmed separation and the potential of resistively heated columns now used in GC will also be examined.

1.3.1.1 Metal Block LC Column Heaters

Static block heaters without air circulation are problematic due to the dependence of internal column temperature on the extent of contact between the column and oven material. The Agilent HP1100 demonstrates this principle, where a column is held in by metal-clips so that random hot spots are created that vary from user to user. Zhu *et al.* [135] observed that for method transferability a calibration was required as the nominal temperature setting of the thermostat can be in error by several degrees. This was also investigated by Spearman *et al.*[136] who measured effective column temperature by shape selectivity and hyphobicity and the effects of mobile phase temperature. In essence block column heaters are not capable of transferring heat rapidly, but can be modified depending on material type, for instance aluminium would heat at a faster rate than steel. The other limiting factor is the transfer of heat through the steel casing of conventional columns (4.6 mm I.D.). This would greatly lag behind the desired temperature, unassisted

by the volume of stationary phase. This would also be difficult to cool the column efficiently without possible hysteresis effects due to the high thermal mass of the block.

Agilent Technologies recently released the HP1200 series, which also works primarily as a block heater. However, its new thermostat goes some way to reducing the limitations mentioned (Figure 9) and is capable of producing runs up to 100 °C. The drawback is that it has isothermal ability only and still no temperature programming capability.

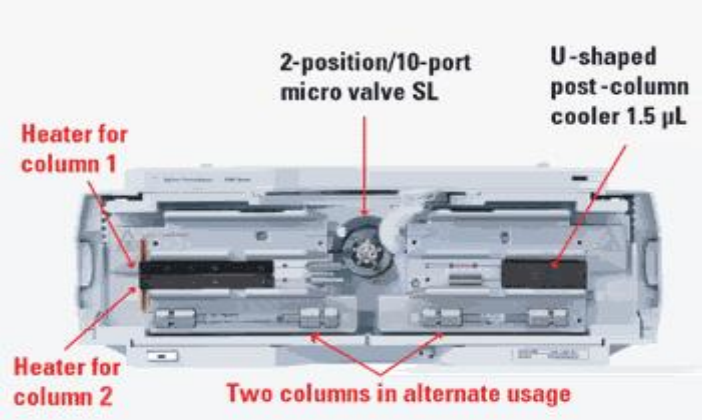


Figure 9: Agilent Technologies HP1200 series thermostat

Teutenberg *et al.*[137] in 2006 designed a heating system for temperature-programmed HPLC based on experimental measurements of eluent temperature inside a stainless steel capillary using a very thin thermocouple. This heating system can be operated at temperatures up to 225 °C and consists of a preheater, a tubular column heating oven and a cooling unit. They achieved fast cycle times after a temperature gradient by an internal silicone oil bath that cooled down the preheating and column heating unit. A mixture containing four steroids was separated at ambient conditions using a mobile phase of 25% acetonitrile: 75% de-ionized water and a mobile phase of pure de-ionized water at 185 °C using the specially designed heating system and a polybutadiene-coated zirconium dioxide, PBD column. Analysis time could be drastically reduced from 17 min at ambient conditions and a flow rate of 1 mL/min to only 1.2 min at 185 °C and a flow rate of 5 mL/min. They demonstrated that their system showed no signs of thermal mismatch through peak distortion. Temperature programming was also performed to

separate cytostatic and antibiotic drugs using only water as the mobile phase giving a fold reduction in Analysis time of 20 to 10 min.

1.3.1.2 Air Forced Oven

A GC type air forced oven is the usual method that has been employed to maintain a fixed eluent temperature in LC; a typical system is outlined in Figure 10 demonstrating the set-up. These are highly efficient at heating/cooling capillary columns (typically column length in the order of metres) and are thought to give better equilibration compared to block heaters. However, several limiting factors are associated with this technique when applying this to LC. There is an overall resistance to heat transfer to the eluent at varying velocity as aqueous eluents have a high heat capacities thus require more energy and high pressure drops when using long lengths of tubing to heat/cool the eluent.

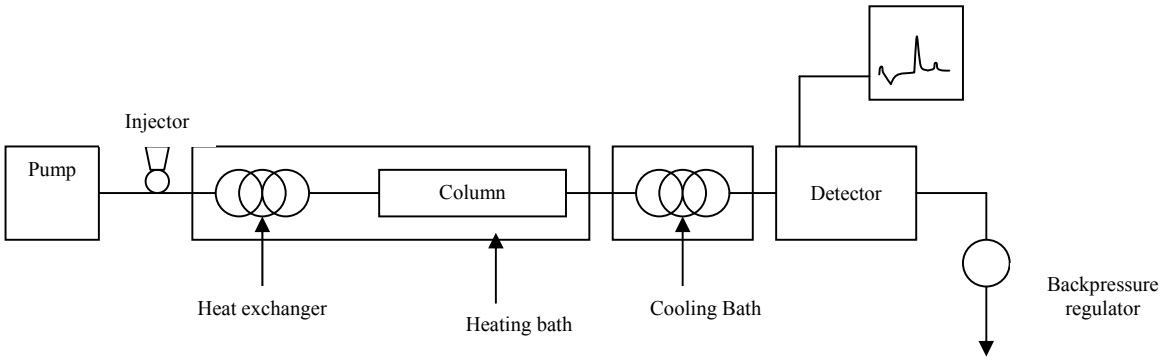


Figure 10: Block diagram of a simple high temperature HPLC configuration

These reasons make it difficult to alter the eluent temperature without introducing extra-column dead volume effects. An air-assisted oven requires long thermal equilibration tubing to overcome the inefficient heat transfer[138]. Air is a good insulator but not a good conductor and has a low heat capacity, which means it has a low capacity to store heat therefore, an air forced oven has a high overall resistance to heat transfer. The injector valve temperature is also important and can only be placed prior to the pre-heating tube, as they cannot withstand high temperature. Poppe and Kraak[139] highlighted that air

thermostating was insufficient to maintain column wall temperature along the length of the column. Transferability is compromised and air circulation rates hard to define without specifying an oven model number. A common problem is that the internal column temperature in all of these methods is not uniform across the length of the column. For instance Djordjevic *et al.*[140] found that eluent temperature increased significantly as it traversed the column. This can be due to poor heat distribution or the dependency on the flow rate variable that generates viscous heat formation as a result of the resistance of the column bed to the mobile phase flow. This can be compensated for provided that the parabolic flow profile associated with conventional columns (4.6 mm I.D.). Figure 11 contrasts different temperature of the incoming mobile phase and demonstrates that due to frictional heating (c), the incoming eluent temperature needs to be lower by approximately 5°C to 10 °C to see a gain in efficiency.

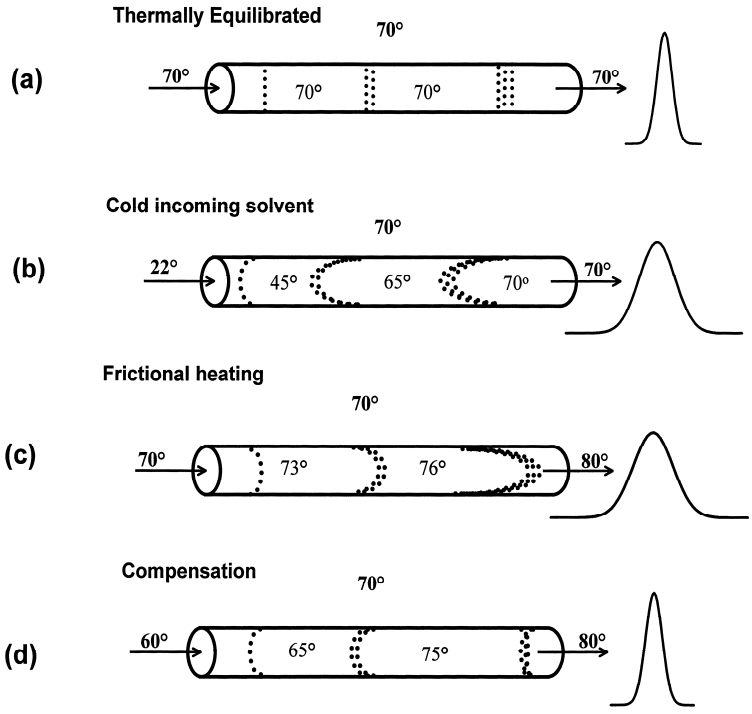


Figure 11: Band broadening due to thermal effects. (a) Ideal case, no thermal effects; (b) effect of incoming mobile phase that is at a lower temperature than the column; (c) effect of frictional heating; (d) combined effects of incoming mobile phase and frictional heating. An oven temperature of 70°C is assumed. Numbers shown inside column suggest plausible solvent temperatures at column centre[141].

A forced air LC oven with temperature programming has been released by *Selerity Technologies, Inc.* called the Polartherm Series 9000. Features such as mobile phase pre-heating and pre-cooling have been incorporated in to its design to enhance peak shape and resolution. However, the oven inherits the characteristics of this method of heating, where transfer is slow when performing a temperature program. This means that analysis times are only slightly reduced and the dramatic improvement in efficiency may not be seen.

1.3.1.3 Water/Oil Baths

Spearman *et al.*[142] investigated several methods of heating a LC column, which included a stirred water bath but obviously this has temperature limitations. These have a high capacity for heat transfer due to the large specific heat capacity of water (4.8 $kJg^{-1}K^{-1}$)[143]. Thermal contact is good and heat transfer should be homogeneous along the column. They found that this system was more stable than the previous methods outlined above but would be impractical to apply at high temperatures. The benefits of raising the temperature should yield higher efficiencies, however this was not found to be the case and poorer efficiency was noted even with adequate mobile phase preheating suggesting that by keeping the external wall at a fixed temperature might create cross column thermal gradients.

Thompson *et al.*[144] compared oil and air heat-transfer media to demonstrate that column efficiency is higher in a liquid due to faster heat transfer. This was highlighted by the oil bath requiring less than half the amount of tubing to pre-heat the mobile phase. Thompson demonstrated that the resistance to heat transfer in an air-assisted oven is 7 fold greater to that in an oil bath.

Although liquid-heating transfer media clearly have advantages compared to other thermostats they are clearly not the method of choice for fast gradients. The impracticality of such systems limits their industrial viability. In addition all of the methods added a large dead volume to a HPLC system masking the real potential.

1.3.1.4 Resistive Heating

An alternative method to those seen previously would be to resistively heat an LC column. This technique has previously been applied to the rapid and controlled heating of GC columns via a metal sleeve and has led to the application of fast temperature programming in GC[145-147]. For instance ThermoOrion "EZ Flash™" technology, which uses resistive heating was developed by Thermedics Detection Inc. in 1985, its first specific application of Flash-GC™ technology was in a machine called EGIS™ developed for the detection of explosives[148]. It has been found to increase sample throughput by a factor of 10 promoting the use of high-speed GCs for routine analysis and eliminates the limitations associated with the heating/cooling of a conventional high mass oven. Mastovska *et al.*[149] have shown that it improves peak shape, detectability of analytes (with higher signal to noise ratio), better retention time repeatability and very rapid re-equilibrium to initial conditions (i.e., cooling down). Bicchi *et al.*[150,151] showed that a group of essential oils of differing complexities could be separated reproducibly giving reliable component identification and quantitation. Analysis times were drastically reduced to between 40 seconds and 2 minutes compared to 20-60 minutes required by conventional oven heated GC.

The method of resistive heating is possible because temperature is related to the kinetic energy of molecules in a substance and can be measured by physical phenomena such as the change in electric resistance. Resistive heating works on the principle that if an adjustable resistance in a circuit exists, the current flow can be increased or decreased by altering this resistance. A temperature can be converted to a resistance programme and an electrical circuit applies an appropriate amount of power. Changes are then measured in resistance per unit time. This method is preferred because unlike heating via air streams (of poor to medium heat capacity) this has a fast response to temperature changes and adjustments.

One approach in LC has been to apply the heating element as a thin film directly to the outside wall of the column[152]. A controlled current ramp provided the thermal gradient that balanced the normal band broadening process.

Phillips and Jain[153] showed that a controlled current ramp provides a thermal gradient that balances the normal band broadening process. This work has been underpinned by the modelling of such marriages of aluminium and silica by Aviles *et al.*[154]. They have studied these bi-layered semiconductors and composite materials in detail important when predicting the temperature of these systems.

1.3.1.4.1 Resistive Heating and Mass Spectroscopy

Of particular interest in the proposed study is that resistive heating has shown promise when coupled to electron impact MS[208]. Dalluge *et al.* [155] coupled GC to a quadropole MS for generating heating ramps of 1200 °C/min and cools down from 300 – 50°C in approximately 30 seconds. Complex mixtures of natural products have been investigated by Carbonell *et al.* [156] using this technique and direct analysis and characterization of compounds from crude extracts of molecular masses of 500 to 1000 has been made possible. Hail also used resistively heated aluminium-clad capillary columns for GC-MS that were able to achieve rapid heating/cooling due to the nature of the materials used[157].

1.3.2 Mobile Phase Preheating and Analyte Focusing

Several studies outlined by Wolcott *et al.* [158] (Table 1) give an overview of the difficulties associated with heating columns.

Table 1: Column temperature control problems in liquid chromatography[158]

Problem	Comment
1. Temperature of column oven (thermostat)[159]	Oven set-point temperature may differ from actual temperature by several degrees and vary with position within the oven.
2. Cycling of oven temperature[160]	Oven temperature may vary with time
3. Equipment differences[160, 159]	Column temperature may differ from oven temperature to a degree that depends on the equipment.
4. Mobile phase preheating[159-162]	Mobile phase at column inlet is not at oven temperature.
5. Frictional heating of mobile phase[160,163]	Flow of mobile phase through a column generates heat, especially for a large pressure drop

Greater column efficiencies should be achievable this is often masked by inadequate temperature control of the eluent resulting in thermal mismatch that produces poor peak shapes. This usually occurs between the temperature of the incoming eluent and the column temperature leading to axial and radial temperature gradients across a column (Figure 12).

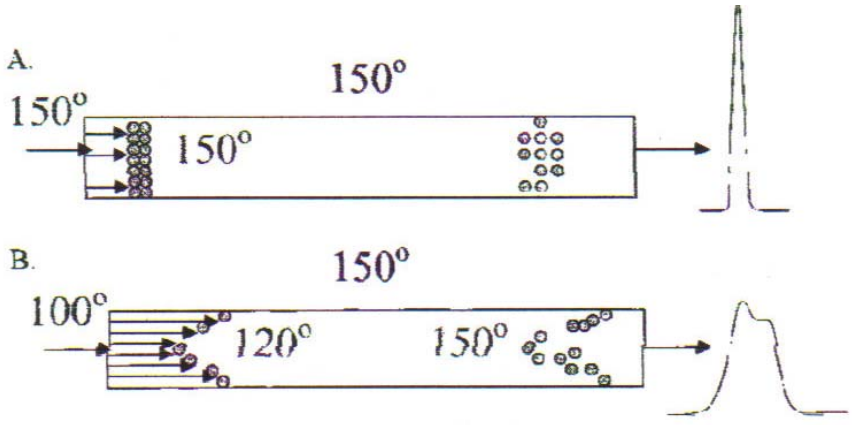


Figure 12: Schematic illustration of the effect of the eluent column temperature mismatch. (A) No – eluent column temperature mismatch leads to column and extra-column broadening. (B) The eluent is not fully thermally equilibrated. The cool eluent produces a radial gradient in retention factor and viscosity, thereby broadening the band and destroying peak shape and efficiency[164].

This agreed with Poppe and Kraak's[165] findings two decades earlier where thermal mismatch broadening was due to incomplete equilibration of the eluent in the pre-heater. However, thermal mismatch is not the dominant process for narrow bore columns as the broadening decreases as the column temperature is increased.

Capillary columns may be used with large volume injections (using an on-column focusing technique) coupled with temperature optimisation, when only limited sample volumes of low concentrations are available[166-169]. Holm *et al.*[170] investigated a novel column oven concept with a cold spot for large volume sample enrichment in high throughput temperature gradient capillary LC. A cold inlet zone was incorporated in the oven that was operated at sub-ambient temperatures. This suppressed elution resulting in the enrichment of large volumes of solutes. Schrenker[171] in the 80's designed a pre-heating device that would minimise band broadening due to a small volume that could match the temperature of the mobile phase to that of the column. This was found to improve peak shape at temperatures up to 80°C. More recently, Selerity Technologies[172] have produced a small mass/volume pre-heater. This enables a faster response during fast temperature gradient runs. The device can be programmed to a different temperature to that of the column and to perform a temperature gradient to follow an oven gradient. Previous attempts to house tubing in an air forced oven[173] or immersed in a liquid bath[174] had been unsuccessful.

1.3.3 Columns for High Temperature LC Separations

The column is the most important factor in the HPLC separation process but the column properties of a nominally equivalent column can vary widely between suppliers Some of the commonly used column materials have been examined for high temperatures applications and have been reviewed in recent years[175-180].

To develop a suitable rugged reproducible method for temperature applications it will be essential to source the most appropriate columns of mechanical and thermal stability. Considerations of the hardware as well as the phase type will influence heating rates and subsequently the stability.

1.3.3.1 Column diameter

The typical internal diameter (ID) for an analytical column is 3 - 4.6 mm. It has been found that for columns of an I.D. < 4.6 mm this was not critical in terms of peak shape when performing temperature-programming[181]. However, conventional columns (4.6 mm I.D.) at elevated temperatures suffer severely from thermal mismatch broadening (section 1.3.2).

Narrower columns 1-2 mm I.D. can also be used and have the advantage of a lower thermal mass and hence faster response to temperature.

Takeuchi[182] summarised the benefits of packed capillary columns in LC and highlighted the potential importance of their direct coupling to mass spectrometers. The monolithic capillary columns are also of interest when coupling direct to a LCMS system for an I.D. of 50-100 μm. Capillary columns also possess the low heat capacity desired in terms of applying elevated temperatures to LC. This reduces thermal gradient effects that would affect peak shape. Improvements to ultraviolet detection for high temperature OT-LC has led to the development of a Z-shape flow-cell[183].This has addressed the lack of sensitivity owing to reduced column load ability and limited light path-length.

Less frequently open tubular columns have been used in LC and are equivalent to those used in GC. The mobile phase runs through an open tube (fused silica, 5-25 μm I.D. in LC and 200-500 μm in GC) and the stationary phases may be physically coated, chemically-bonded, or cross-linked liquid phases directly on the wall. Nyholm and Markides[184] found that column lifetime under high-temperature conditions depended

strongly on the amount of cross-linking in a covalently bonded deactivated polymer layer.

Narrow bore and capillary columns give better sensitivity and perform well for detectors that require low flow rates (i.e., electrospray MS). Capillary LC is not without its problems, but if elevated temperature is incorporated in to a method the disadvantages could be diminished. For instance poor concentration sensitivity could be alleviated by analyte focusing by programming a heater to heat the mobile phase at a lower temperature (~10°C) so that of the column also may concentrate the amount of analyte available for detection.

1.3.4 Stationary phases for High Temperature Applications

Ideally a stationary support should consist of:

- ✓ Particles with a narrow size distribution and high surface area
- ✓ Pores must be able to accommodate the analyte
- ✓ Fast analyte mass transfer
- ✓ Thermal, mechanical and chemical stability

Current synthetic materials are available in a range of particle sizes and pore structure; however, the thermal stability aspect varies widely (section 1.2.4). In recent years new sources of stationary phase materials have been developed, investigated and reviewed[219].

The main concern for HT and superheated water applications has been the stability of the stationary phase supports. Burgess *et al.*[185] investigated materials, such as polystyrene-divinylbenzene and porous graphitic carbon, and demonstrated their use for a aromatic compounds, pharmaceuticals and vitamins whereas conventional ODS-silica phases were rapidly degraded.

1.3.4.1. Silica based phases

The traditional ODS bonded silica phases have generally poor thermal stability and are readily attacked by the mobile phase at more than 70-80 °C (see 1.2.4). More recently Waters Inc. have developed phases based on *"Hybrid Particle Technology"* resulting in the development of XTerra and then XBridge columns. A study by Mendez *et al.*[186] compared derivatized XTerra with underivatized XTerra. The XTerra MS C-18 gave no residual silanol acidity up to pH10 (pH in 60% methanol). This new breed of columns demonstrated stability over a wide pH range for Xterra and Symmetry Shield[187-189]. This technology was first highlighted by Unger *et al.*[190] that when two high purity monomers were combined one forms SiO_2 and the other $RSiO_{1.5}$. These groups are incorporated into the particles throughout their internal and surface structure. The added advantage of these hybrid particles is that they give enhanced peak shape for basic compounds. They also showed improved temperature stability where the latest addition to this type technology is the XBridge, which relies on ethylene bridging to give additional pH stability of a range of 1- 12[191].The XBridge material given its superior stability compared to other silica phases should be more amenable to high temperature

1.3.4.2 Polybutadiene-Coated metal oxides

Stationary phases utilizing metal oxides, such as alumina, titania and zirconia[192] have been stabilised using polybutadiene. Schomburg's group introduced polybutadiene (PBD) – coated alumina and led to much interest in non-silica based columns[193]. Zirconia proved to be most stable with higher pH stability and showed a catalytic inertness that is superior to other metal oxides[194]. They have been synthesized by Xiang *et al.*[195] and evaluated under high-pressure conditions at 100 °C and were found to be stable over 7000 column volumes. These types of columns have been thermodynamically characterised by Li and Carr[196] who demonstrated that (PBD)-coated zirconia is chemically and thermally stable. Thermal stability control has led to fast HPLC separations at elevated temperatures and high flow rates[197]. They also exhibited characteristics similar to the retention process of its silica counterpart, allows the incorporation of PBD zirconia phases into HPLC

methods. For polyaromatic hydrocarbons temperature played no role but for complex mixtures of polar and ionizable solutes optimizing the selectivity via temperature could be a valuable variable. The selectivity process for PBD-zirconia in RPLC separations depended on the solute type. However, differences were apparent and the PBD zirconia phase was less retentive than an ODS phase[198] but is comparable in hydrophobic selectivity and retention arises from a partition-like process. Li and Carr[199] found that the amount of polymer loaded affected the efficiency of this support and that low carbon loadings (<4%) had the least resistance to mass transfer.

A recent study by Andersen *et al.*[200] has looked at mesoporous PBD zirconia and temperature programming stability for capillary LC. With temperature programming not exceeding 100°C they found a rapid decrease in column efficiencies and retention factors. Hence a better packing technique was required to make this particular stationary support viable in high temperature capillary LC. More recent studies have shown that zirconia is prone to column bleed at high temperatures[201] in conventional columns. There is also evidence that dissolution can occur in phosphate and carbonate buffer mobile phases, especially for intermediate and high pH[202-204]. This leads to baseline shifts, hence the use of highly concentrated organic modifiers with silica columns in the mobile phase that adds extra stability. However, these are still not comparable in stability to that achieved with zirconia. The high pH stability that this phase affords led to investigations by Sun and Carr for large-scale protein separations[205,206]. However, the dominant mechanism of the zirconia phase is the Lewis acid/base interaction. The Lewis acid sites on the surface of zirconia accept electron pairs on the amino acid groups (linked by peptide bonds) this causes irreversible adsorption of the proteins. This was overcome by phosphate modified PBD-zirconia to counteract the Coulombic interactions between the negatively charged stationary phase and the positively charged mobile phase.

1.3.4.3 Monolithic columns

As well as packed columns, polymeric monolithic supports have gained much attention in the past years and have been the subject of extensive investigation[207-213]. Monoliths are

usually based on PS-DVB, acrylates or on methacrylates[214] and can be operated at much higher flow rates compared to the particulate phases leading to shorter run times. However, mass-transfer is critical for the analysis of larger molecules to avoid losses in efficiency. The separation of peptides for high-speed RPLC has been successful[215,216] and was due to an enhanced mass-transfer created by the convective flow through pores of diameters >600 Å.

It is unclear as to whether monolithic columns would be appropriate for high - temperature applications. Relatively few studies have examined the effects of temperature but polystyrene divinylbenzene (PS-DVB) monolithic capillaries have been reported to be stable for a temperature of 100 – 130°C for temperature-programming[217]. Monolithic ion-exchange columns have highlighted that temperature has a significant effect on the dynamic binding capacity. However, this is not without its problems, and the effect is complex and needs to be established for each system[218].

1.3.5 Mobile phases

Conventional aqueous organic mobile phases can be used in high temperature LC although the volatility of the organic modifier can require higher backpressure to remain as a single phase. As noted earlier, comparisons have been made been the composition and temperature effects.

Chester and Coym postulated that although over narrow temperature ranges there is a linear relationship between the retention factor and reciprocal absolute temperature, this does not always hold over wider temperature ranges[219]. They indicated that this may be caused by temperature-dependent phase ratio changes, and not necessarily changes in the transfer enthalpy. Temperature can also have an effect on stationary phase conformation[220] as well as the mobile phase itself. The different shadings in Figure 10 represent the three different “phases” involved in the chromatographic system. From top to bottom, the bulk mobile phase (a water-rich solution), the adsorbed mono- or multilayer of the organic modifier molecules (a phase rich in adsorbed organic modifier),

and the C18-bonded phase. The analyte (phenol or caffeine) is represented by small ovals. Deviations from linearity of the Van't Hoff relationship are either due to the retention mechanism and / or the stationary phase structure varying with temperature. Stationary phase ligands tend to be highly ordered at low temperature, and the chains are associated by dispersive interactions and form clumps of alkyl chains on the silica surface. At low concentrations of acetonitrile it has been hypothesized by Lochmuller and Hunnicutt[221] that acetonitrile becomes trapped in the pores of the silica by a collapsed chain structure in a highly aqueous environment, and effectively solvates at relatively low concentrations making it an ideal additive to preserve integrity of the stationary phase. The more that is added the bigger the association to other acetonitrile molecules and the less it forms associations with water. At high acetonitrile concentrations the water starts to interact with the stationary phase, it is thought by forming hydrogen bonds with residual silanol groups.

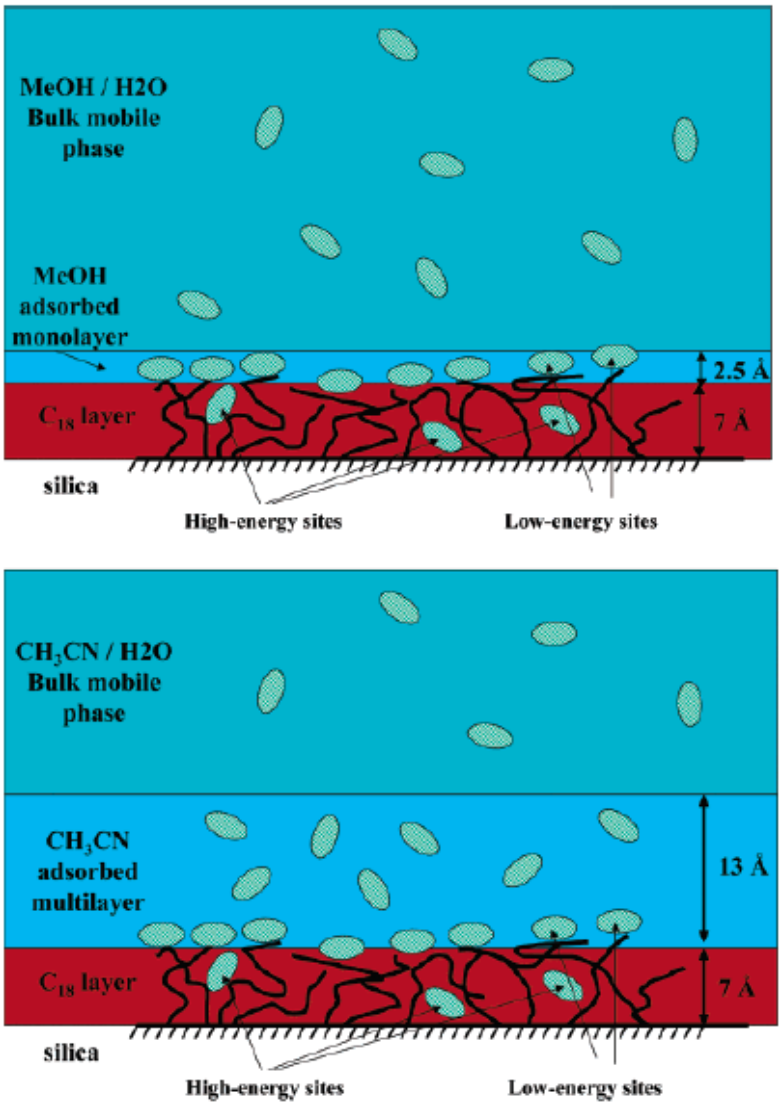


Figure 13: Schematic comparison of the adsorption mechanisms of a solute from the aqueous solutions of methanol (A) and acetonitrile (B) onto a RPLC material[222].

The absorption of small polar compounds depicted by Guiochon[222] (Figure 13) comprises of free or self-associated organic molecules, water- and organic solvent-associated molecules, and free (self-associated) water molecules. Water and methanol have more solvent micro heterogeneities, so they are unable to solvate the alkyl chains of the stationary phase unlike acetonitrile, which allows free movement. Wysocki[223] has explored what actually drives a separation and has described LC hydro organic mixtures to be binary on a macroscopic level but ternary on a microscopic level. This has been

substantiated by studies that have compared the methanol aqueous-based solvent system with an acetonitrile aqueous-based system. The acetonitrile / water phase yielded reduced analysis times and better separation quality of the compounds studied[224].

1.3.5.1 Superheated Water Separations

One development from high temperature separation is the use of superheated water (SHW) as a "green" eluent owing to the unique properties that water takes on at elevated temperatures. The water phase diagram (Figure 14) indicates a critical point where the water and gaseous phase are indistinguishable from each other. The properties of water between its boiling point and the critical point are very different from ambient water. For example, supercritical water is a poor solvent for electrolytes, which tend to form ion pairs. However, it is an excellent solvent for non-polar molecules, due to low polarity and poor hydrogen bonding so that many are completely miscible. The specific permittivity (dielectric constant) of water reduces from 80 at 25°C to 35 at 200 °C (Figure 15) [225,226], thus making hot water behave more like an organic solvent, such as acetonitrile. The retention thermodynamics at ambient temperature have been compared to SHW conditions by Coym and Dorsey[227]. They showed that the enthalpy of transfer for small, non-polar solutes from pure water to a non-polar stationary phase is much lower at ambient compared to elevated temperature and concluded that this is similar to that found for organic modifiers at ambient temperature. Therefore, the effects were to the change in solvation structure surrounding the solutes and that different solutes may "see" different thermodynamic phase ratios. In addition an attraction of utilising superheated water at temperatures of 100 to 250 °C as the solvent in HT-LC, is that only a small amount of back-pressure in required to keep the SHW phase in a liquid state[228-232].

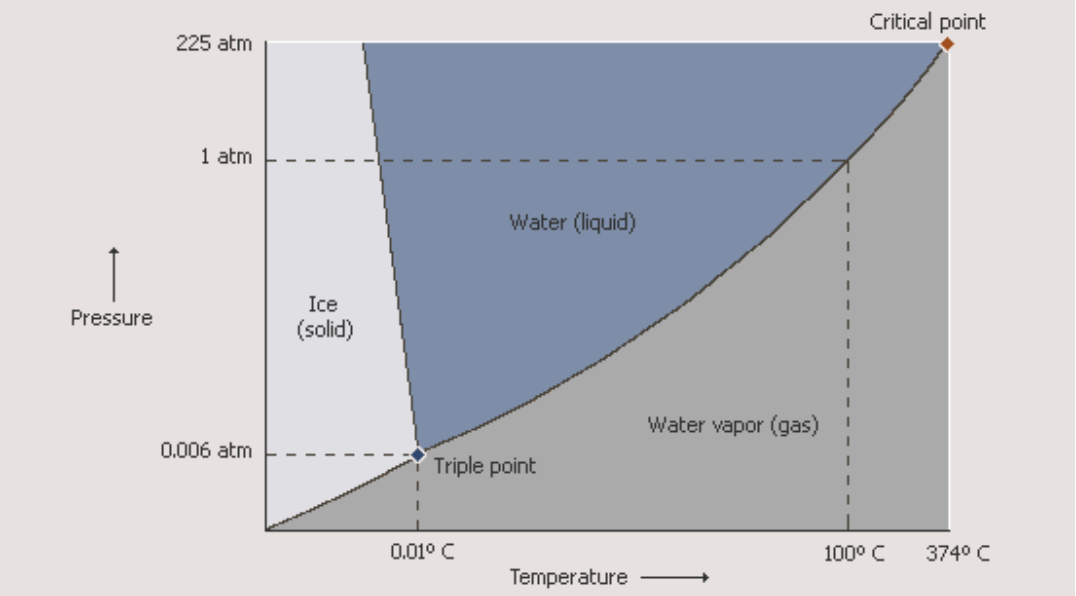


Figure 14: Water Phase diagram

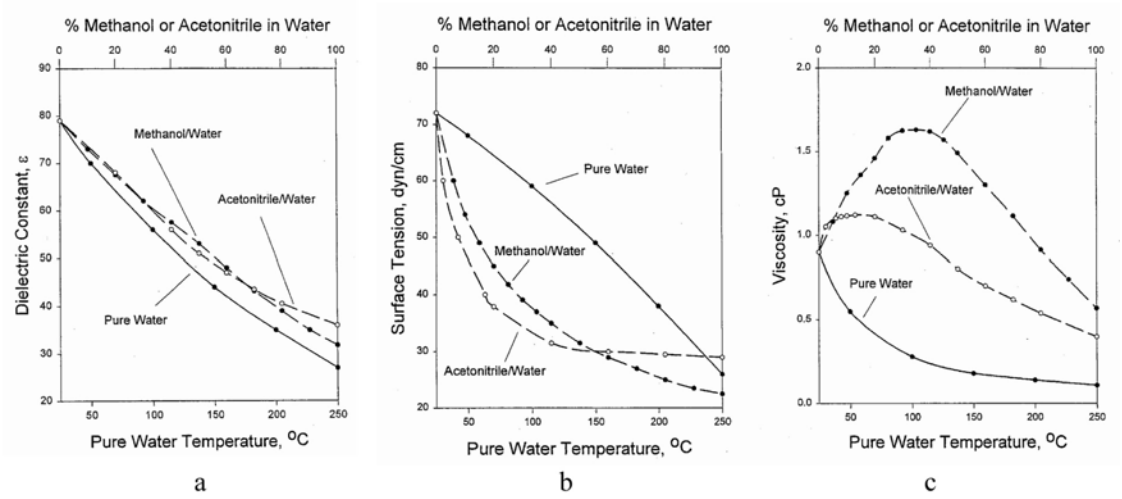


Figure 15: Dielectric constant (a), surface tension (b) and viscosity (c) of superheated water compared to that of methanol and acetonitrile as a function of temperature[232].

Water is no longer the weakest eluent in RP-HPLC provided that enough heat can be transferred to produce these physical effects. Importantly, it retains many advantages lost when traditional organic solvent are used as eluents, which can restrict the type of detector. For instance flammable, toxic and expensive solvents are eliminated and low

wavelength UV detection or the use of flame-based GC detectors[233] can be employed as a universal and sensitive alternative form of detection for LC. Ingelse *et al.*[234] have outlined improvements for the set-up of an HPLC-FID with temperature–programming capability. They demonstrated how sample enrichment could be obtained by large volume injection with low temperature enrichment resulting in good peak shapes and detection to 0.2 ppm. Smith *et al.*[235] have demonstrated the use of superheated heavy water with on-line HPLC-NMR and HPLC-NMR-MS detection of model drugs This combination enabled both NMR and MS data to be collected simultaneously of the same sample, with less spectral interference compared to conventional mobile phases.

Temperature programming has only been touched on in this area[236,237], but given the unique properties of water, such as its ability to store and release high amounts of energy more needs to be done to exploit its potential. Water has a large heat capacity, which is believed to be approximately 25 % greater at the surface of a stationary phase compared to the bulk property of water[238]. Provided the right instrumentation can be developed to apply temperature efficiently with a fast response time, the efficiency of separations could be enhanced and resolution improved.

1.4 Detection

Normally the detector in HPLC is regarded as being independent from the separation conditions; however, there are cases where the effect of the temperature of the column can alter the response.

1.4.1 UV/Visible Spectroscopy

Temperature fluctuations can lead to changes in UV transmittance of the mobile phase, however, these are subtle compared to the change in bulk properties of the mobile phase during a solvent gradient. This was demonstrated by Okada who used temperature programming combined with UV absorbance for the analysis of polyoxyethylene

oligomers[239]. Renn and Synovec also employed temperature programming with dual wavelength UV absorbance detection[240]. This gave a reduction in baseline shift compared to single wavelength. Therefore, one might expect to see a flatter baseline and a better signal to noise, S/N for a temperature programmed separation compared to a solvent gradient.

When using capillary columns the sensitivity of UV absorbance can be improved where traditionally on-column detection is used. Djordjevic *et al.*[241] used a z-shaped flow cell and compared it to an on-column detection technique where an optical fibre was used. The z-shaped flow cell proved to be 20 x more sensitive and was the method of choice where S/N was the most important aspect of a separation.

1.4.2 Mass Spectrometry

HTLC when coupled to MS can be of great benefit to the ion source. For instance in ESI the eluent is nebulised in the ion source via high voltage (applied to electrospray needle) and nebulising gas, followed by droplet desolvation at high temperature. In APCI the eluent exiting the column is vapourised by high temperature applied to a probe and a nebulising gas, followed by ionisation in the gas phase. This configuration may benefit more as the vapourisation occurs prior to ionisation in the gas phase. Thus, a hot eluent should increase the efficiency of a mass spectrometers interface, which is based on this vaporization, nebulisation and desolvation processes. Albert *et al.*[242] found that these processes were enhanced for ESI when heat was applied and a gain in sensitivity of 50 % was achievable. However, a solvent gradient can have a negative outcome. Buffers, modifiers, aqueous and organic content can all suppress ionisation and optimal conditions should be determined at the time of analysis. This influence changes with the mass to charge ratio (m/z), affecting the abundance of ions that one might see. This is due to the viscosity and surface tension of the solvent matrix being used and the nature of the desolvation process. A temperature program can resolve these issues by aiding the processes at the interface as discovered by Yoo and colleagues[243] whilst avoiding

suppression effects by the reduction in use of organic modifiers and buffers. Andersen *et al.*[244] (section 1.3.1 figure 7) demonstrated that a temperature program baseline for the separation of polyglycerol fatty acid esters of linear, branched, and cyclic isomers, was flatter when compared to a solvent gradient program. Relatively, sensitivity was increased with a temperature gradient connected to Micromass LCT TOF-MS (Z-spray API-ESI-POS capillary voltage 2.5kV, Sample + extraction cone +15 and +3V, respectively). The potential benefits of heat assisted chromatography are summarised in Figure 16:

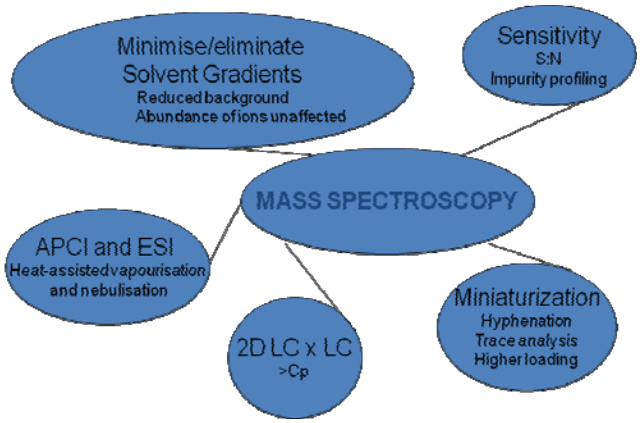


Figure 16: Summary of Potential Benefits of Heat-assisted Chromatography for MS

- APCI and ESI increased efficiency at interface via heat assisted vapourisation and nebulisation therefore increasing sensitivity
- Minimise / eliminate solvent gradients
  - Reduced background
  - Abundance of ions unaffected
- Increased sensitivity due to improved interface processes and increased mass transfer on-column
  - S/N
  - Impurity profiling
- Miniaturisation aided by better equilibrium times leading to:
  - Hyphenation

- Trace analysis
- Higher loading

- 2-Dimensional LC x LC lowered viscosity allows columns to be linked
  - Increased peak capacity

1.4.3 Refractive Index / Flame Ionisation Detector

Refractive index detection (RI) has been rarely explored for hyphenation to HTLC because of the inherent sensitivity of refractive indices to temperature. However, Chang *et al.*[245] fairly recently successfully applied a temperature program to the characterisation of synthetic polymers. Resolution and sample loading capacity and sensitivity were greater. Additionally, temperature provided a much finer control and reproducible retention control than solvent composition particularly important when determining molecular weight distribution.

Flame ionisation detector (FID), is particularly suitable for high temperature applications as it can handle water as the mobile phase[246] and give a good universal response to most analytes.

1.5 Present Study Aim and Objective

The present study set out to examine the application of "*resistive heating"* for the creation of rapid and reproducible temperature programming for conventional, narrow and capillary bore HPLC column. The main objective was to drive temperature programming to a new "*extreme*" by **rapid** column heating more akin to GC temperature ramp rates rather than LC, the emphasis to produce much sharper peaks at speed. The results should be of particular advantage for the pharmaceutical industry as rapid reproducible heating/cooling patterns of RP columns should reduce equilibration times and lead to higher sample throughput. It was also hoped that temperature gradients could replace solvent gradients and extend the applicability of temperature-programming for

hyphenation to other forms of detection. New column materials will be sought and tested against high speed temperature gradients. This coupled with micro-column technology should reduce analysis time and appreciably limit the amount of solvent waste currently being generated by conventional LC techniques.

The objectives of the study were therefore to examine:-

1. HPLC separations based on speed, efficiency and detection simplicity
2. High speed temperature gradient system demonstrating typical LC parameters, such as retention time stability and reproducibility
3. How column thermal mass affects the applicability of resistive heating
4. Temperature gradients for simple and complex mixtures
5. Conventional and new stable hybrid phases at high temperatures

Chapter 2 Experimental

2.1 Compounds Used in the Study

The following compounds (Figure 17) were chosen based on their stability indicated by previous work done in high temperature liquid chromatography[247-249] and were purchased from Sigma Aldrich

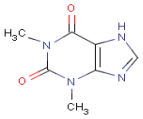
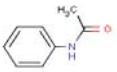
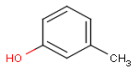
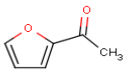
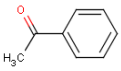
Uracil Mwt: 112.09 	Theophylline: Mwt 180.17 	Acetanilide: Mwt: 135.17 
m-Cresol: Mwt: 108.14 	Benzofuran: Mwt118.13 	2-Acetylfuran: Mwt 110.11 
Naphthalene: Mwt 112.09 	Alkyl acetophenone to octanophenone (7 phenones in total). Mwt(s): 120.15 to 204.31 	Caffeine: Mwt: 194.19 

Figure 17: Molecular Structures of Compounds used in the Study

2.2 GSK Specific Compounds

A number of developmental compounds and their impurities from GSK were also examined during the study.

2.3 Chemicals and solutions

2.3.1 Chemical Reagents

The solvents and organic modifiers used in this project including ammonia (specific gravity 0.88 – 0.89, HPLC grade), trifluoroacetic acid (TFA) and triethylamine (TEA) of certified grade were sourced from Fischer Chemicals with the exception of dimethylsulphoxide (DMSO), which was Hypurity grade from Romil.

De-ionised water was used throughout the project purified and Millipore filtered at 18.2Ω (MilliQ Integral System).

Ammonium hydrogen carbonate was obtained from Fischer Scientific.

2.3.2 Preparation of Buffer Solutions

These were prepared on a daily/weekly basis to avoid bacterial growth.

2.3.2.1 10 mM TFA pH 2.0

A 10 mM solution of TFA in deionised water was prepared and adjusted to pH 2.0 using sodium hydroxide (2M).

TFA, 0.6094g ± 0.02g was weighed into a suitable container and dissolved in 500 mL of deionised water. This was then adjusted to pH2.0 and degassed as appropriate.

2.3.3 Mobile Phases

Non-buffered mobile phases were given a month shelf life and were made up with the ratio of organic to aqueous content appropriate to the experiment.

2.3.4 Preparation of Test Mixtures

2.3.4.1 Temperature Gradient Test Components

A naphthalene solution was prepared in acetonitrile as described in Table 2 to apply a temperature gradient and compare ramp rates for an air-forced GC oven.

Table 2: Sample Preparation of Naphthalene

Compound	Weight (mg)	Volume (ml)	Target Concentration (mg/ml)
Naphthalene	20	25	0.8

2.3.4.2 Fast Temperature Gradient Test Mixture

This mixture in Table 3, mainly comprised of homologous phenones, was used as a generic test mix in GSK for the open access HPLCs. The test mix covered the whole polarity range and so the effects of rapid heating should be evident. Some overlap of early eluting components was also expected, so the aim was to separate and maintain resolution by producing very sharp peaks.

Table 3: Sample Preparation of a Phenone Test Mixture

Component	Quantity in 100ml of diluent (MeCN)	Component	Quantity in 100ml of diluent (MeCN)
Uracil	5mg	Benzofuran	10µl
Theophylline	10mg	Butyrophenone	20µl
2-Acetylfuran	10µl	Valerophenone	20µl
Acetanilide	10mg	Hexanophenone	20µl
m-Cresol	10µl	Heptanophenone	20µl
Acetophenone	20µl	Octanophenone	20µl
Propiophenone	20µl		

2.4 RPLC Columns Used in the Study

A comprehensive list of all of the columns (and the manufacturers names) used throughout the duration of the project are located in Appendix 1.

2.5 HPLC System Set-ups

2.5.1 Miniaturised HPLC System

A HPLC system was designed to minimise dead volume to achieve good resolution for narrow-bore/micro-bore separations. A Shimadzu LC-10AD (capable of low flow rates) with a low-pressure gradient delivery system provided the eluent pumping system. The eluent was sparged with helium gas and connected to a Rheodyne manual micro-injection valve, model 7410 and equipped with a 0.5 µl loop. A schematic representation of all of the components is given in Figure 18.

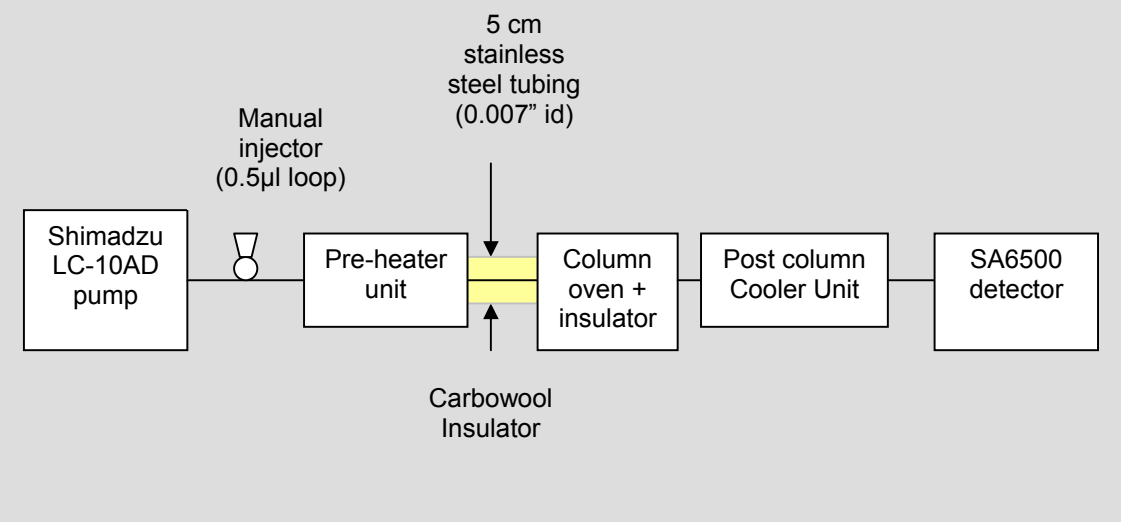


Figure 18: Schematic set-up of a miniaturised HPLC system

The injector was then connected to the CSI heating system covered in detail in chapter 4, where the tubing used from this point would be no greater than 0.007 in I.D. and the length was minimised where possible. Tubing lengths were also kept minimal to maximise heat transfer and minimise surface area to reduce heat losses from the pre-heater unit to the column. The eluent then flowed through the post-cooler unit prior to detection.

A Severn Analytical SA6500 UV/Vis absorbance detector was used equipped with a nominal cell volume of 1.2 µl and a 3mm path-length. The data was collected by Prime chromatographic software, triggered by a Rheodyne magnetic switching device.

2.5.2 HP1100 System with Hyphenation to Different Modes of Detection

This consisted of a HP1100 series (Agilent Technologies, Waldbronn, Germany) (Figure 19) with ChemStation with various detection modes: UV/Vis (high-pressure 2 µl volume 6 mm path-length flow cell), LC/MSD (SL), HP RI and a Sedex 75 ELSD (Sedere, France). The ELSD was connected to a different instrument of the same type.

Figure 19: Photograph of HP1100, LC/MSD SL and CSI heating system used at GSK R&D Ltd

2.5.2.1 Optimisation of the Agilent HP1100 LC System

Optimisation of the HP1100 system was carried out to reduce cycle times at low flow rates of 0.04 ml/min with a micro-bore column (1 mm I.D.). The following steps were carried out with reference to Agilents on-line technical notes to minimise dwell volume:

- Replacing all connecting tubing with Peek 0.0023 ” (natural)
- Removal of mixer and replacing with tubing supplied by Agilent for this purpose saves approximately 1 minute in delay time
- Writing an injector program to switch the auto-sampler into bypass positions after the sample has reached the column. This saves another 300 µl of system delay volume, which can have effect on dwell and cycle times due to low flow rate.

Order	Command	Time (min)
1	Draw	
2	Inject	
3	Wait	0.758
4	Valve bypass	
5	Wait	9 (run time – 1min)
6	Valve mainpass	

The third order was calculated using the following formula:

Wait time = 6 (injection volume + 5 µl) /flow rate

Where:

Injection volume was 0.5 µl

Flow rate was 40 µl/min

2.6 Oven Design and Heating Strategy

2.6.1 Overview of Cambridge Scientific Instruments Heating System

The Cambridge Scientific Instruments (CSI) oven is centred round achieving a constant temperature over the entire separation process. Hence the oven's performance and that of the individual units was assessed to look at the systems combined capability. This served to aid the development of the design based on physical measurements and the chromatography.

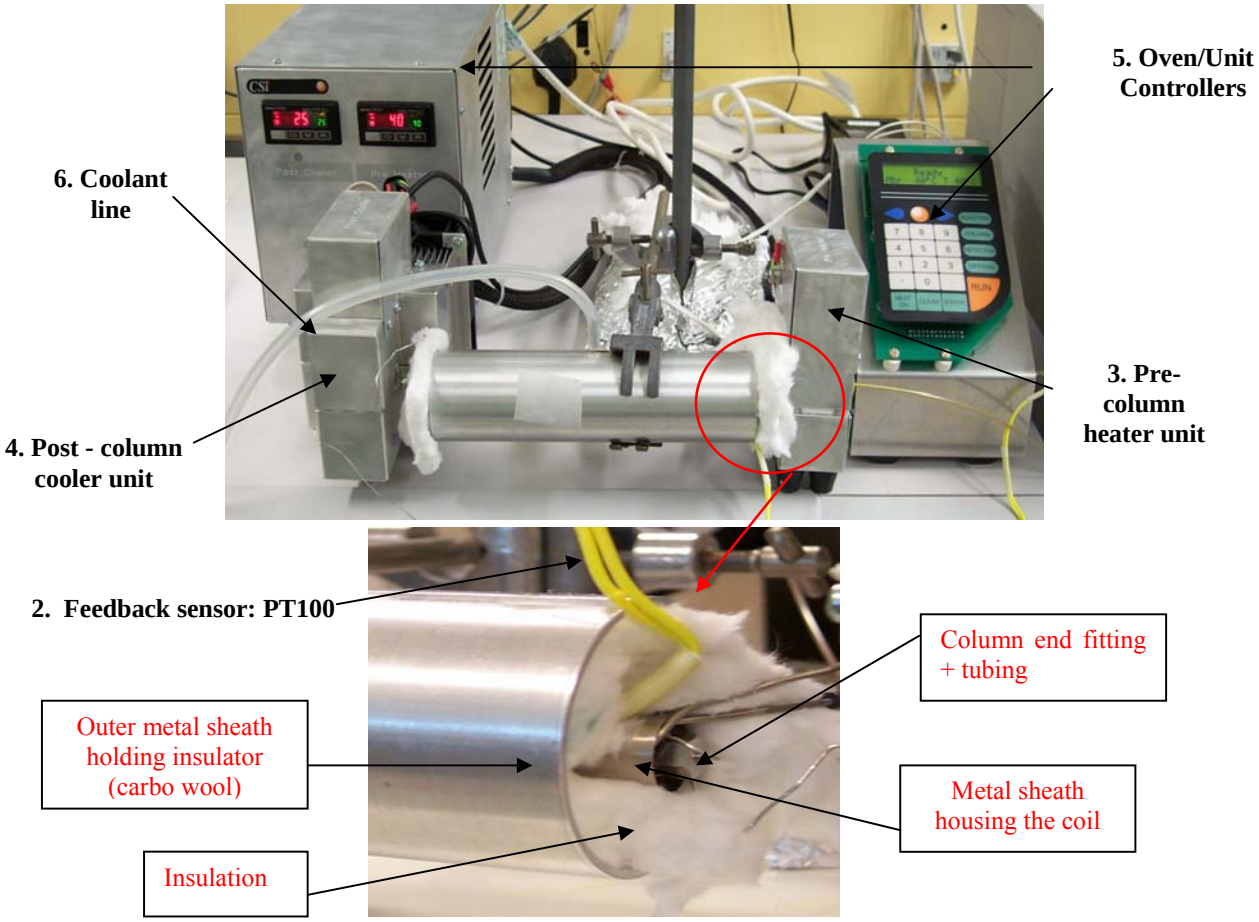


Figure 20: Cambridge Scientific Instruments (CSI) LC Heating System

The CSI LC heating system (Figure 20) consists of a column oven, assisted by a pre-column eluent heater and, a post-column eluent cooler.

2.6.2 LC Oven

The aim of the prototype design is to have good thermal contact to ensure maximum heat transfer to demonstrate the applicability of resistive heating for LC applications. This provided the rationale for placing the column in direct contact with a resistively heated coil Figure 21).

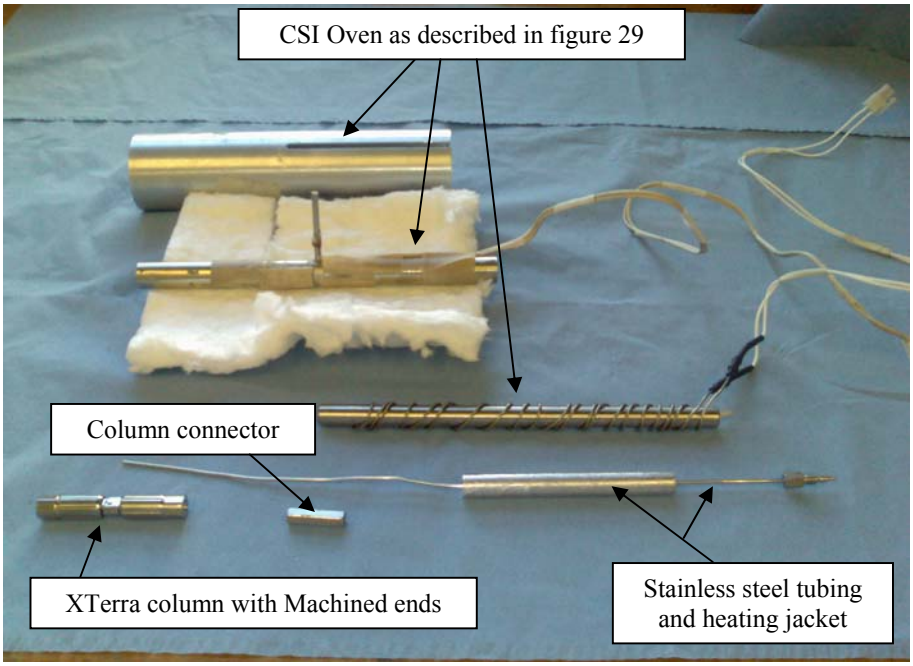


Figure 21: Picture of the CSI Oven broken down in to its individual components as detailed in Figure 23; including Stainless steel tubing and heating jacket used in conjunction with short columns: XTerra® Phenyl, 3.5 µm 4.6 x 50 mm

This works on the principle that if a current can be altered in a circuit then the resistance can also be changed, which can be directly related back to temperature. To accommodate a typical LC column, the outside diameter required, across the geometry of the column needs to be as close to 0.9 cm as possible. This resulted in columns being chosen on the basis of being able to withstand having their end fittings being ground down to meet this requirement. Where the column was very narrow in outer diameter a metal jacket is used to allow homogeneous heating both of which are illustrated in Figure 22.

Figure 22: Picture of (a) Merck Purospher® STAR RP18e, 3 µm 1 x150 mm with stainless steel heating jacket, (b) Merck column again with part of the heating jacket remove and (c) an XTerra® MS, 3.5 µm 4.6 x 100 mm column with the column ends machined compared to an original intact XTerra column.

2.6.2.1 Insulation

Carbowool (Carbo Ceramics Inc., McIntyre, GA, US) is used to insulate all of the CSI heating system where deemed appropriate. This includes the oven, the pre-heater and the post-cooler unit in the following sections. Insulating the oven is necessary to avoid heat loss to the surroundings and to ensure a well distributed heating profile.

2.6.2.2 Oven control

The oven is controlled by a feedback sensor that monitored the oven temperature allowing the electronics to make the necessary adjustments when this falls outside the intended target. This consisted of a platinum resistance thermometer (PT100), placed centrally on the metal sheath that houses the heating coil shown in Figure 23.

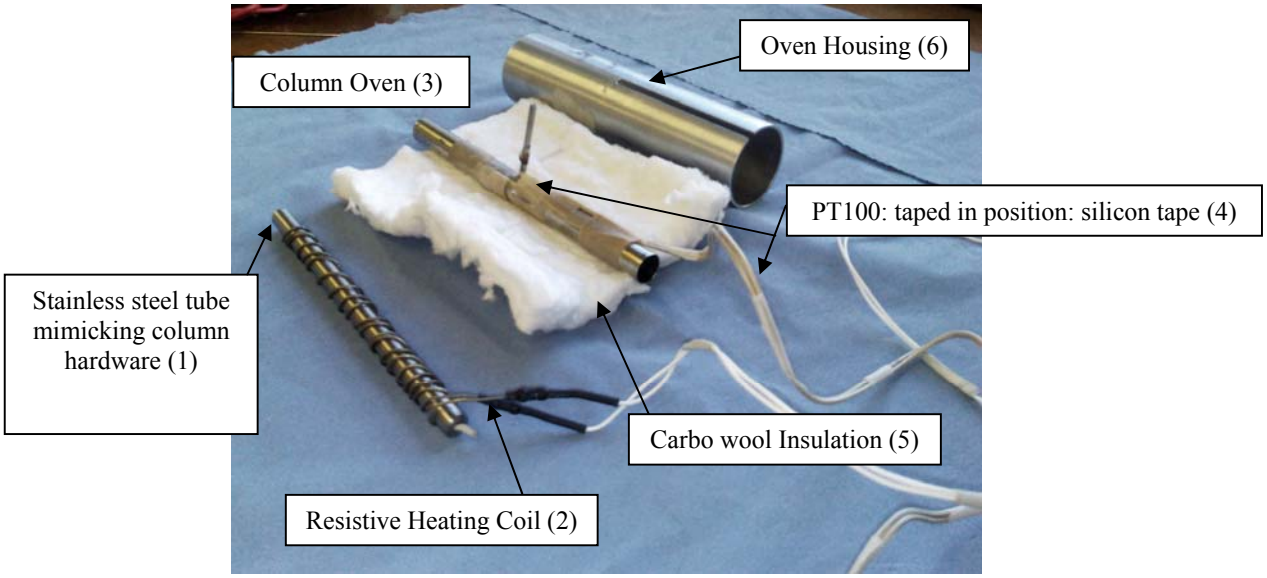


Figure 23: Picture of the CSI Oven broken down in to its individual components numbered in order from the centre outwards, therefore number 1 is placed inside number 2 etc.: (1)→(2)→(3)→(4)→(5)→(6)

Resistance temperature detectors (RTD's) contain a sensing element; this is an electrical resistor that changed resistance with temperature. This contained a wire coil that is concealed in a ceramic housing and ceramic cement. A PT100 sensor has a resistance of 100 ohms at 0°C and 138.4 ohms at 100°C and offers excellent accuracy over a wide temperature range. The temperature relationship to resistance is linear over a narrow temperature range therefore linearization is performed automatically by the software. In the development stage the data collected by the PT100 was recorded by TcmiDebug a software program supplied by CSI Ltd (Ely, Cambs) refer to section 2.6.4.

2.6.3 Eluent Pre-heater and Post-column Cooler Units

The eluent was heated (figure 30**)** over a short distance of 1.5 mm (0.8 mm id), as dead volumes needed to be kept to a minimum. This operated on the same principle as the resistively heated oven and was also governed by a PT100 sensor. The heater and sensor were positioned in relatively close proximity to one another in a large block of steel as

described in Figure 24, which aided heating providing stability and facilitating energy storage. This was then insulated and boxed in by a steel case.

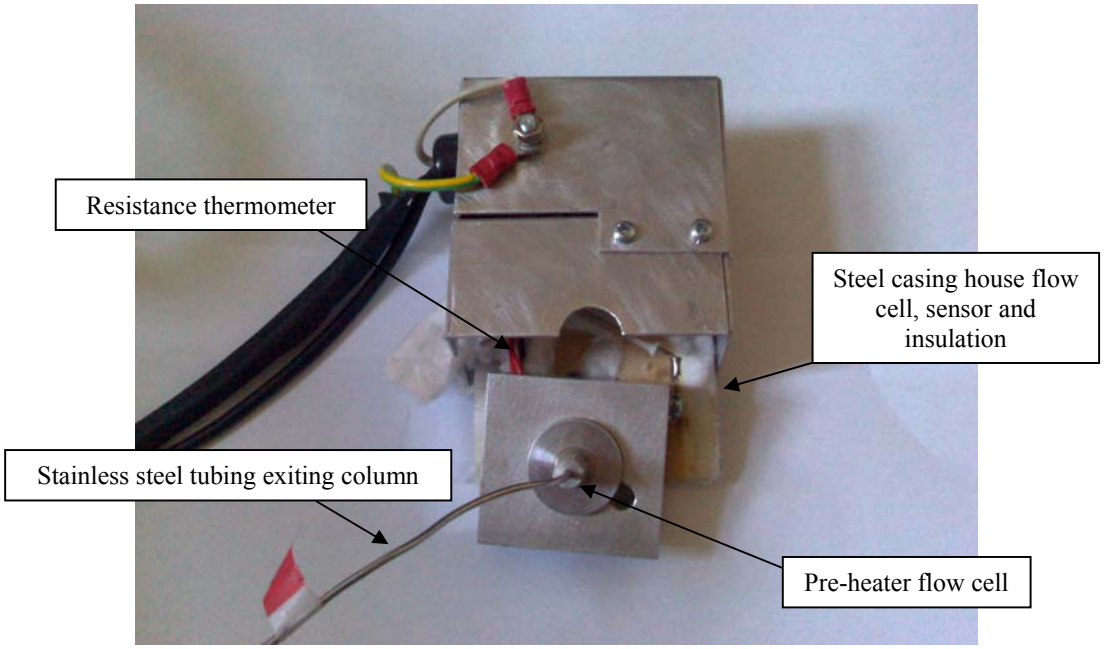


Figure 24: Picture of internal pre-heater

The pre-heater operated isothermally and was programmable for a range of ambient to 280°C. A temperature ramping version was not available due to a practical limitation and would have been desirable from a design perspective. The CSI programmable version of the controller available to the project was 32 volt not mains powered. A suitable heater could not be sourced in the 32 volt format.

The post-cooler unit (Figure 25) was an essential system component as this lowered the eluent temperature exiting the oven minimising detector drift due to temperature related refractive index changes. Internal flow volume again had been minimised to 0.75 µl, and two Peltier coolers acted as heat sinks dissipating heat away from the eluent. The eluent was cooled and the heat was directed away by a fan at the back of the post-cooler (figure 30).

Figure 25: Picture of the Post-column cooler flow path (side view) and of the Peltier and fan.

2.6.3.1 Control Unit

The eluent heater and cooler had their own controller separate to the oven based on a CSI GC oven controller. This allowed the operator to set an isothermal temperature in a range of 25 – 300 °C and 5 – 100 °C, respectively. All of the system components were insulated when heating or cooling takes place to minimise heat loss and give a uniformed response.

2.6.4 Software

TcmiDebug software program provided by CSI Ltd was developed to give a temperature response output of the oven for a given operation The software had a data acquisition rate of one point per sec This was used to assess the difference in heating rate of the oven compared to a column(see section 3.1).

2.6.5 Temperature Measurements

2.6.5.1 Thermal Sensors

The Pico ADC16 signal conditioner and Picolog software were used when independent temperature measurements were required for developmental purposes. Type K (glass fibre insulated) thermocouples were used to collect this data. A thermocouple contains two electrical conductors made from different materials, which are connected at one end. The end exposed to the temperature is called the measurement junction. This was a positive chromel wire and a negative Alumel wire, which have a temperature range of – 100 to 1370°C and good precision.

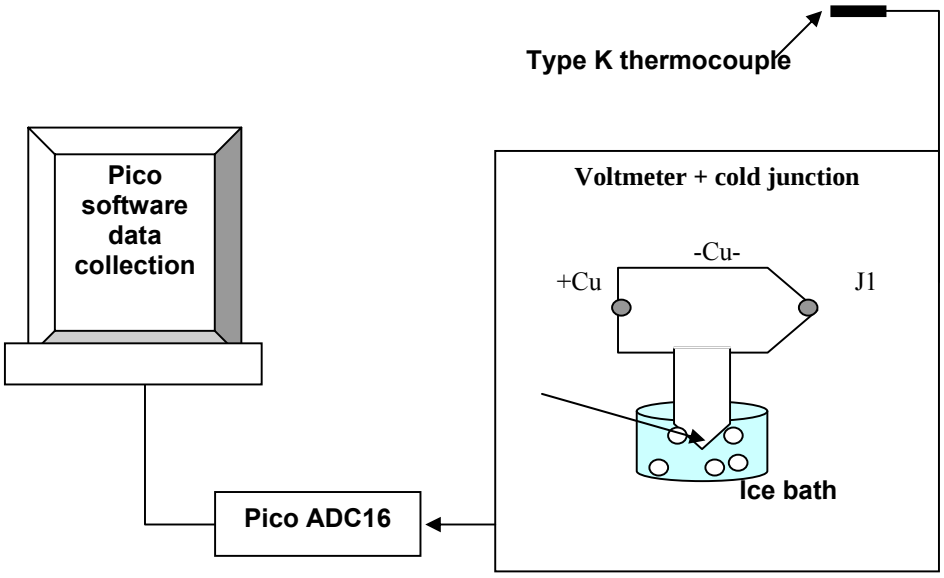


Figure 26: Schematic representation of a cold junction

The first thermocouple was connected to a different metal junction when it was connected to a Pico (ADC16) signal conditioner. This allowed the thermocouple output to be recorded by PicoLog software data management system (1997 – 2001). A second thermocouple was placed in the circuit to create a cold junction to offset bimetal junctions affected by ambient temperatures. The resultant voltmeter reading between the

two junctions is proportional to the temperature difference. Thus, if a second thermocouple was placed in an ice bath this creates a zero output and a true reading was found for the first thermocouple. This was tested at the start of each set of experiments by submerging both thermocouples in an ice bath to check for a zero milli-volt reading.

2.6.5.3 Fixed Power Ramps

The thermocouple was placed in the centre of column, at half-length, at approximately the same location as the PT100. The power was fixed as percentages of the maximum at 10, 20, 30 ..... 100 %. Each fixed power was performed for a temperature gradient of 50 to 200 °C. The oven was allowed to equilibrate at 50 °C and the data collection programs were started (Pico and TcmiDebug). The system parameters were set as described in table 4.

Table 4: Method Parameters for Fixed Power Ramps

Initial temperature	50°C	**Ramp 1**	10, 20, etc.
Sample time	-	**Upper temperature**	200°C
Purge time	-	**Nudge**	30
Initial	20 secs	**Damp**	120

The initial and final temperatures determined the gradient with an initial time to allow the system to stabilise. The sample and purge times became effective when an actual chromatographic separation was performed. These allowed for sample injection times and purge times between analyses.

The nudge (n) and damp (d) values were already set at arbitrary values to remain constant throughout the initial development stages of the oven. They performed a function that allows a controlled ramping rate. The nudge value was governed by the distance from the target temperature required, i.e., the further away, it was the higher the incremental power factor will be. The damping value was the rate of change of temperature. Therefore, the

closer to the target, the heating rate becomes slower to avoid over-reaching the desired temperature and to remain constant once at this point.

Chapter 3 CSI LC Heating System Development

The CSI heating system components had to be tested to assess their effectiveness for high temperature and temperature-programmed separations. Primary testing needed to be carried out initially on the oven. This was to look at:

- The temperature profiles along the length of a column to assess the homogeneity of the heating pattern at isothermal temperatures.
- The response of a column to heating on a temperature gradient i.e. ramping rates to demonstrate developmental areas for effective heat transfer from the oven unit to the column.
- Equilibration time i.e. the ability to re-equilibrate to initial conditions after a temperature gradient.

However, the column heating was to be achieved by two components, the oven and the eluent pre-column heater unit both based on resistive heating (Figure 27). This should allow very efficient heat transfer rates because of the heaters' close proximity/contact with the column.

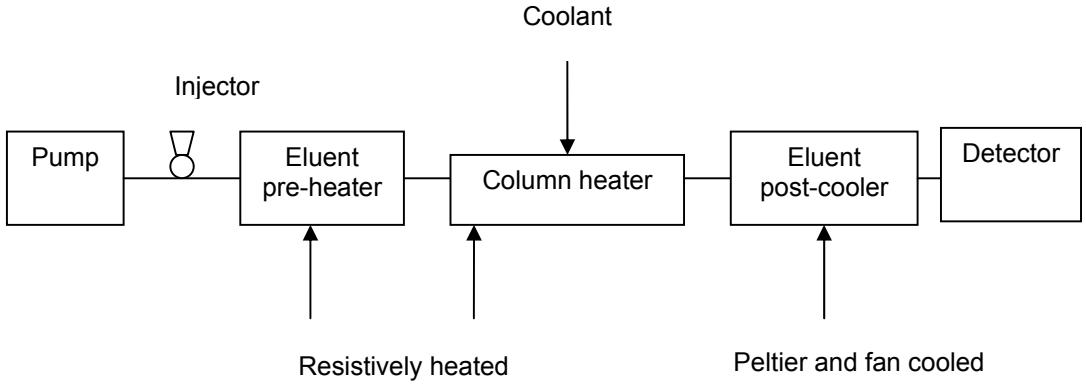


Figure 27: Schematic drawing of the proposed heating system

The pre-column eluent heater required assessment to ensure appropriate settings were applied for analysis to match the temperature of the eluent to that of the column. This also applied to the post-column eluent cooler unit that was required to deliver a suitable constant eluent temperature with respect to the detector and would include:

- Assessment of the rate of heating / cooling
- The variables that may influence the transfer of heat to the mobile phase i.e. flow rate or the loss of heat to the surroundings.
- Identification of developmental areas to ensure hyphenation to detectors is viable.

The viability of using the temperature variable is also dependent on other integral components to make it a practical system. The increase in cycle time resulting from heating the high thermal mass of a column will be reduced by investigating different coolants, such as air and liquid nitrogen. A target of $\leq$ 5 minutes is set to make this a practical alternative to using a solvent gradient. Post-column cooling prior to detection should minimise baseline disturbances imposed by a temperature gradient. Cooling prior to UV detection is an essential element to prevent damage to flow cells at 80°C+ as well as preventing refractive index changes. The latter could cause a drift in baseline in chromatography and will need to be eliminated for hyphenation to a RI detector. The eluent is to be cooled using Peltier technology allowing rapid cooling rates. This will make it ideal for hyphenation to other detection modes, such as MS, RI and ELSD.

The next step will be to demonstrate the benefits that such a system can bring to the separation goals of shorter analysis times and higher column efficiency without a loss in resolution. This method could then be applied to analysing multi-component mixtures (see Section 2.3.4.3) to determine if analysis times could be reduced significantly whilst maintaining/improving resolution. The detection of low level potentially toxic compounds is an area of interest for early phase development of pharmaceuticals where sensitivity is the main obstacle.

3.1 Oven Evaluation

3.1.2 Temperature Profile for a Simulated Column

3.1.2.1 Aluminium Column

The original intention was to design an oven that could heat a range of column geometries. An approximately 9 mm I.D. tube, which could hold a conventional 4.6 mm I.D. column was wound with the resistive heating coil. Aluminium jackets were made for columns that required a better contact with the heating coils, when the gap was significant. These would ensure as close a contact as possible with the heating coil giving maximum response to the heating profile. After initial trials using an aluminium column the positions of heating coils were adjusted and the performance of the oven with a stainless steel tube mimicking a 4.6 x 150 mm LC column was tested using sensors (thermocouples as described in section 2.6.2) inserted down the centre of the artificial column (Figure 28). The tip of the sensor was manipulated so that good surface contact would establish any hot or cold spots along the oven/column profile. Carbowool was used to insulate the oven and column sensor ensuring that the heat was dissipated more efficiently and reproducibly along the column. The temperature recorded by the thermocouple gave higher temperature readouts to that being read by the feedback control. This was thought to be due to the sensor positions (Figure 28). The feedback sensor was attached to the outside of the column oven and was maintained at a constant value due to the lagging insulating the oven.

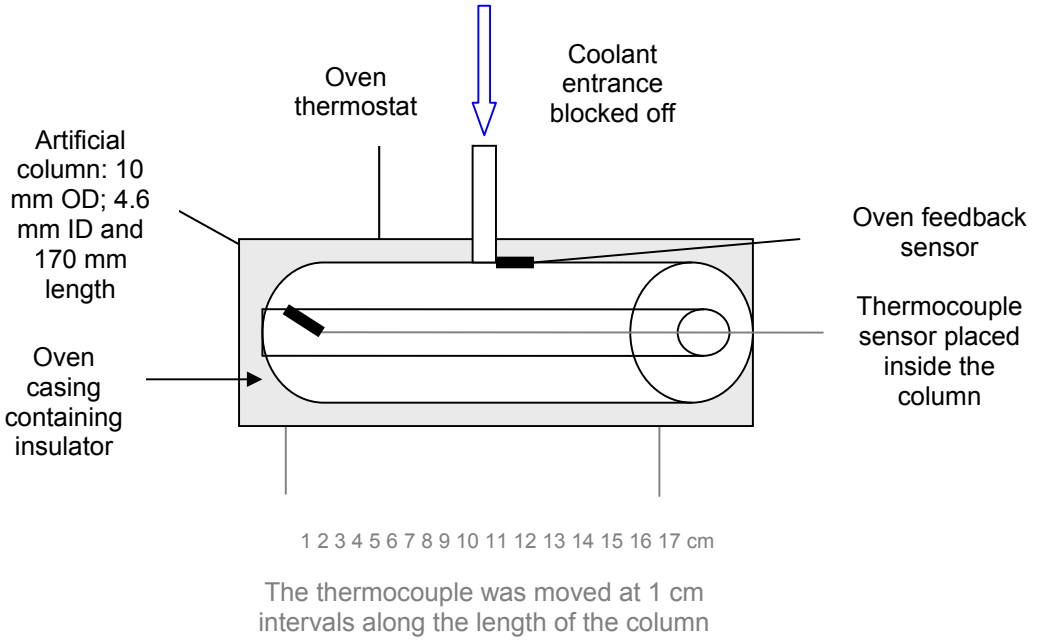


Figure 28: Schematic arrangement of sensor position for testing the temperature profile of the oven

A temperature difference of 24°C between the oven sensor located on the column oven wall and the thermocouple placed inside the artificial column inside the oven was recorded (Figure 29). The oven control for its point of contact again shows good control in terms of maintaining the target temperature of 200°C and an even distribution.

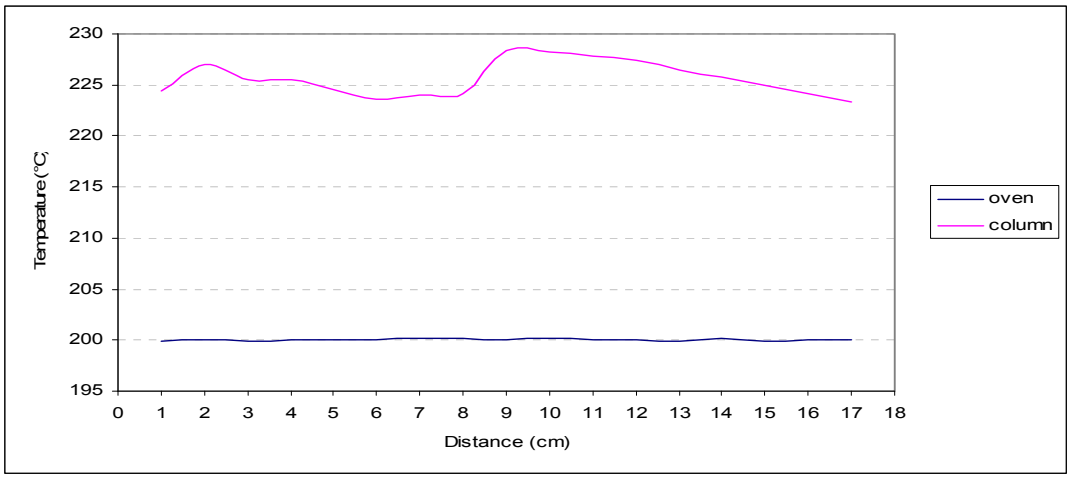


Figure 29: Insulated Thermal Profile for the Length of the Steel Column

3.1.3 Fixed Power Ramp Data for a Steel vs Aluminium Column

The responses of simulated columns made from different column materials were then examined. A type K thermocouple was inserted inside the artificial column at the mid-way point. Fixed power ramps were assessed to look at the efficiency of heat transfer for a steel and aluminium columns housed in an oven running a temperature program of 50 to 200 °C.. The steel data represented material common to column hardware, where the aluminium data demonstrated what could be attainable for materials directed at temperature programmed applications. The range of power input covered was 20 to 100 %. For moderate to high ramp rates, a hold time at the upper temperature of 200 °C was included to allow for any thermal lag between the internal column wall temperature and that of the oven set-point.

The data (Figure 30) showed that at a low ramp rate the heat transfer to the inside of the steel column wall was similar to that found for the aluminium column. There was a small thermal lag between the oven and the column temperature, which closed as it neared its target. This lag was greater for the steel compared to the aluminium and the gap grew as the rate (power) was increased (Figure 31).

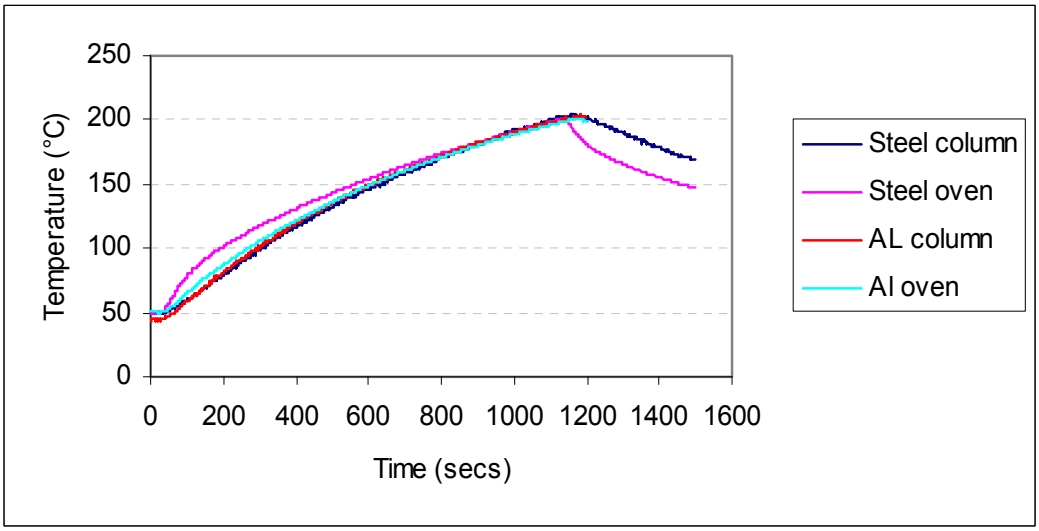


Figure 30: Oven and column thermal measurements taken using steel or aluminium simulated columns on a heating ramp at a fixed power of 20% (steel column refers to the simulated steel column).

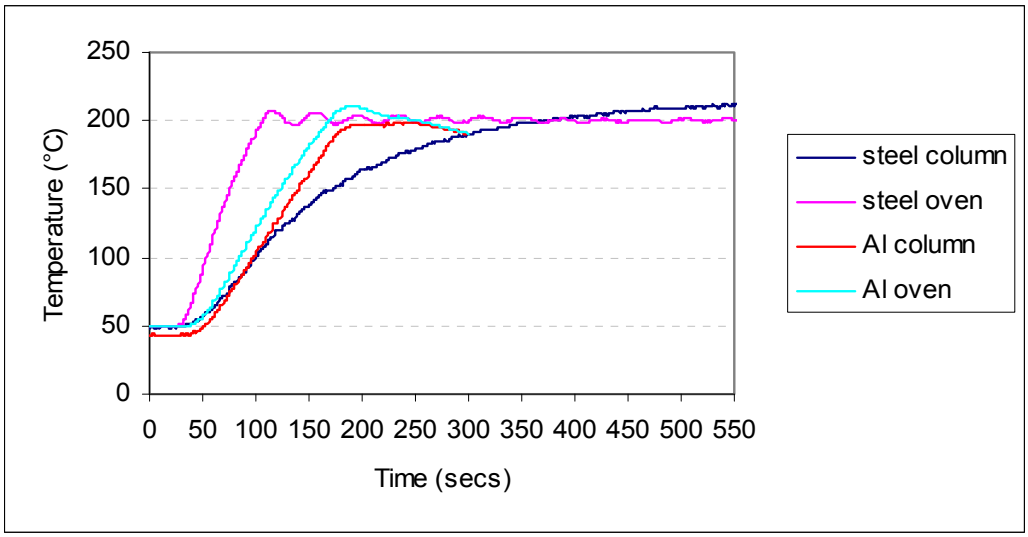


Figure 31: Oven and column thermal measurements taken using steel or aluminium simulated columns on a heating ramp at a fixed power of 100%

Aluminium is a better conductor than steel and as expected gave a faster response with the temperature programming.

The heating pattern for the steel column is summarised for the oven's range of input power (% full power) (Figures 32 and 33) and showed that the data bunches into two distinctive groups. From 50% power and up, a clear pattern emerges between the oven set-point compared to the temperature reading taken from the column's internal wall.

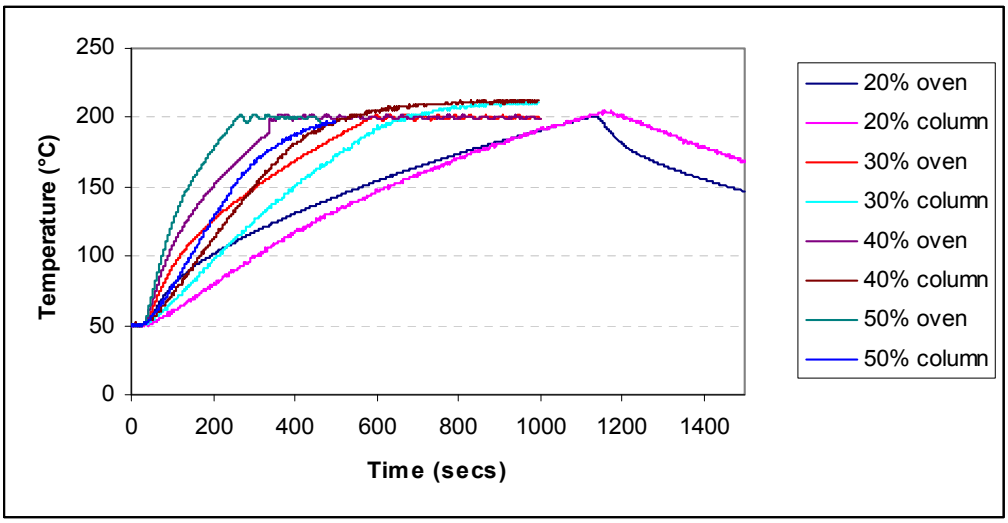


Figure 32: Oven and column thermal measurements taken using a steel simulated column on a heating ramp from 20 to 50 % power

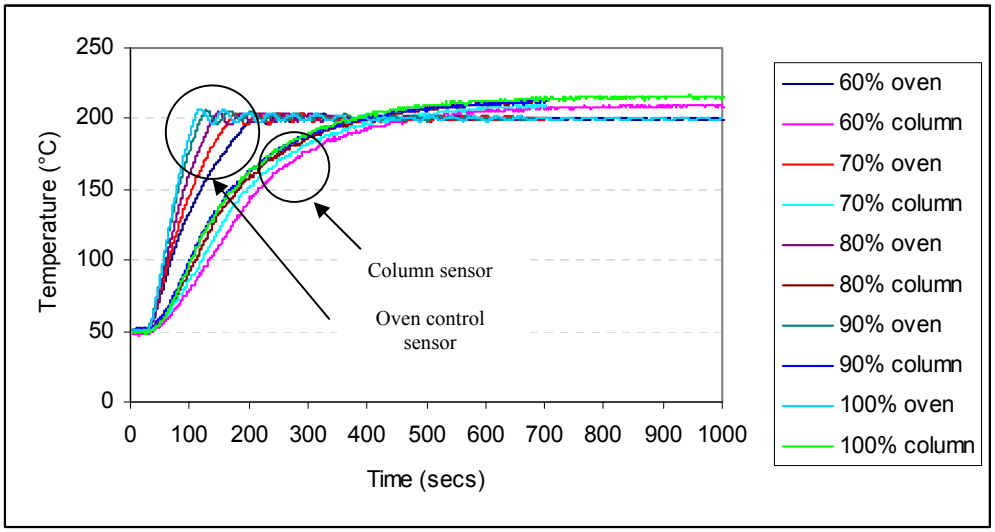


Figure 33: Oven and column thermal measurements taken using a steel simulated column on a heating ramp from 60 to 100% Power

The data (Table 5, Figure 34) was converted from heating rate expressed as percentage power to °C/min. This was determined by the temperature increase (150 °C) divided by the time it took to reach the upper temperature of the gradient. Also, tabulated are the temperature and time difference between the oven set-point compared to the inside wall temperature of the steel column.

Table 5: Thermal data showing the measured heating rate of a simulated column when placed inside the oven over a range of 20 – 100 % power

% Power	Oven Control Ramp Rate (°C/min)	Column (inside wall) Ramp Rate (°C/min)	Temperature Lag ($T_{oven} - T_{column}$) (°C)	Time Lag ($t_{oven} - t_{column}$) (min)
20	8	8	-1	- 0.2[1]
30	15	13	11	1.4
40	25	17	32	3
50	35	18	48	4.1
60	44	19	57	4.5
70	54	20	65	4.6
80	65	22	75	4.5
90	76	23	84	4.6
100	84	24	94	4.4

The thermal lag between the oven and the column was determined to be larger than expected. The oven temperature increases proportionally to the increase in power while the mass of the steel column slows down the rate at which the heat is transferred. There is not enough power to overcome the resistance of heat to mass transfer. A maximum ramp rate of 24 °C/min should therefore be possible for conventional columns (150 x 4.6 mm id). In a real case scenario this figure is likely to be even lower given the predicted lower thermal conductivity of the stationary phase, such as hybrid silica or a polymer. This is however, hoped to be compensate partly for this effect by eluent pre-heating, thus allowing internal heating to take place at the core of the separation.

[1] this is a minus value as the oven lagged slightly behind that of the column temperature

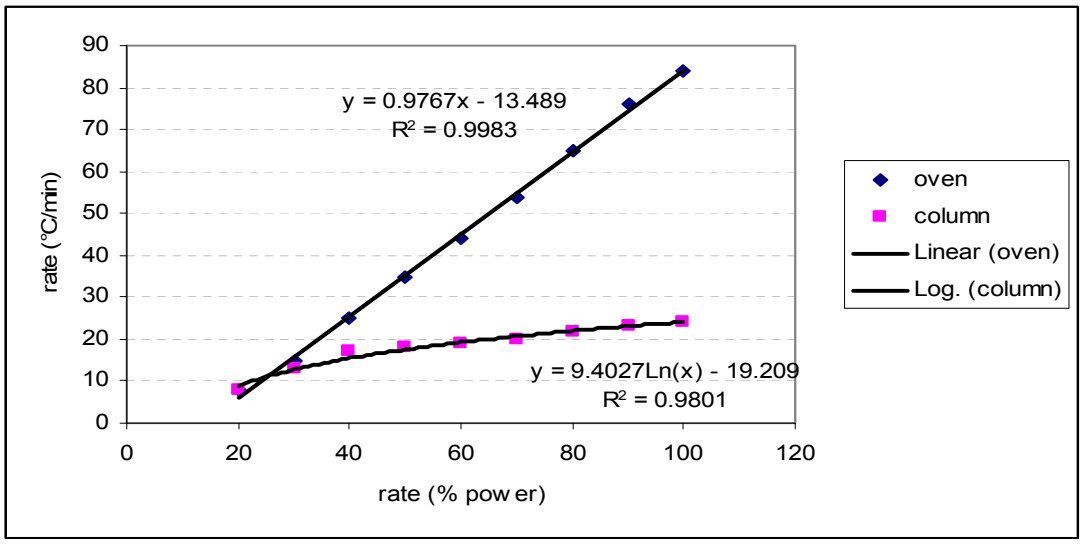


Figure 34: Plot of the heating rate for the oven compared to the inside of the simulated steel column wall.

The limitation of the response of the simulated steel column to a temperature program was higher than expected and the energy available at full power was determined to be 88 W. As the temperature increases the thermal resistance of the steel column mass also rises, thus the available energy could be overridden by this factor if there is not enough power. This results in a plateau effect, such as that seen in figure 44. A low wattage heater is an advantage in terms of running costs and the environment so the power for this unit would need to be increased to achieve the highest rates but is still substantially lower compared with a typical GC (1 KW) oven that uses 500 watts of its available power.

3.1.4 Energy Loss due to Column Cooling by the Eluent

An experiment was carried out to assess by how much the ambient eluent would cool the column in the absence of any eluent pre-heating. Water was pumped through a piece of steel tubing housed inside the oven and a fine thermocouple was inserted inside the exit to detect the water temperature. The results showed (Table 6) that the heat transfer was dependent on flow rate and that the heat transferred from the oven to the eluent was poor.

Table 6: Temperature of the Eluent exiting the oven at varying flow rate

Oven set-point (°C)	Thermal Measurements (°C)					
	0.04ml/min	0.2ml/min	0.5ml/min	1ml/min	1.5ml/min	2ml/min
40	28.4	31.7	28.4	24.7	23.7	23.5
60	29.9	42.1	36.6	32.9	29.9	26.4
80	36.1	53.3	48.1	39.6	34.1	32.7
100	40.1	62.2	63.0	50.5	44.8	41.3
120	49.8	74.4	68.7	56.3	45.3	42.3
150	73.9	66.2	77.9	63.2	50.5	46.8
200	91.3	72.2	78.1	75.9	66.2	52.8

3.2 Eluent Pre-column Heater

An eluent pre-column heater was fitted and its thermal input to the eluent was assessed to enable a more accurate approach when matching the temperature of the eluent to that of the column.

3.2.1 Temperature of Eluent Exiting a Column

The pre-column heating device was programmed to temperature set-points of 40 to 200 °C, for an eluent flow of 0.5 to 2.0 ml/min and the flow was passed though the insulated oven which was not heated. Measurements (Table 7) were then taken at the point that the eluent exited the oven. The isothermal heat transfer by pre-heating the eluent proved to be very efficient and more effective than heating the eluent through the oven especially, at lower temperatures. This is due to the huge resistance to heat loss by the mass of steel.

Table 7: Thermal data of the eluent exiting the insulated oven at varying flow rate with eluent pre-heating only

Pre-column heater set-point (°C)	Column Outlet (°C) at Different Flow Rates			
	0.5ml/min	1ml/min	1.5ml/min	2ml/min
40	34	34	35	34
100	32	44	56	57
150	58	65	80	81
200	90	99	97	105

The pre-heater is more effective than the oven at heating the eluent for varying flow velocity as demonstrated in the comparison plots (Figure 35). The data also shows larger deviations in terms of heat transfer for the oven and pre-heater observed at the lower and higher end of the temperature range.

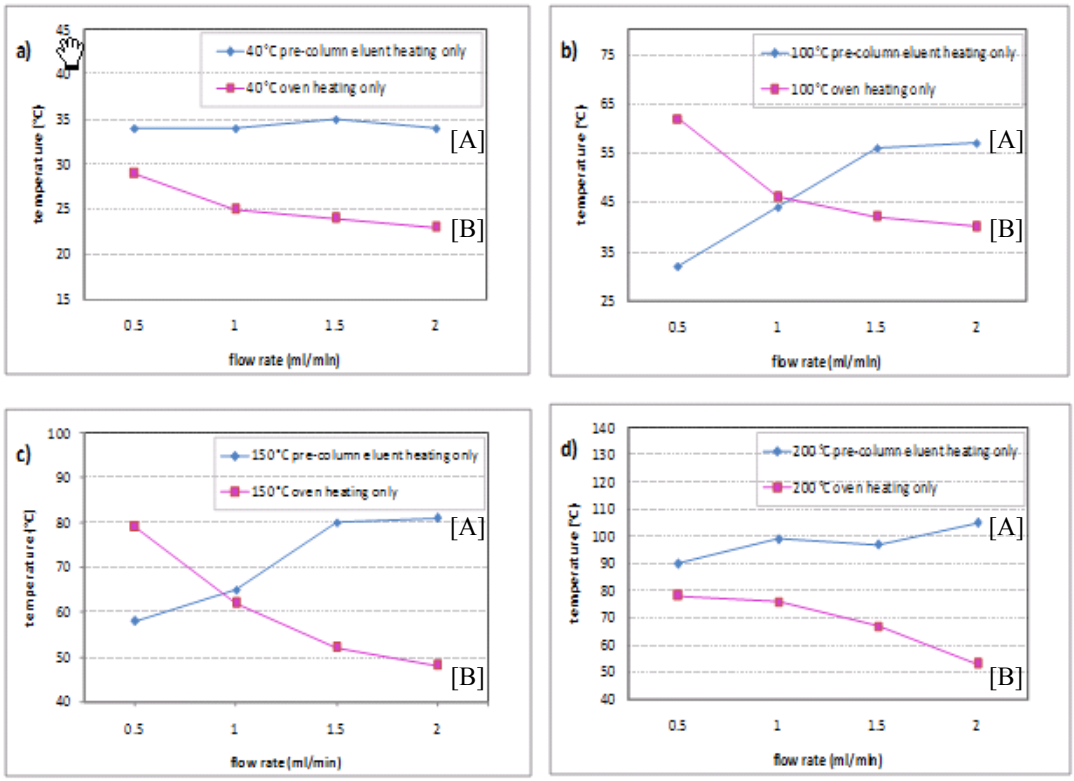


Figure 35: Thermal measurements made of the eluent exiting the insulated oven with [A] pre-column eluent heating only overlaid with [B] column oven heating only for a velocity range of 0.5 to 2 ml/min at a) 40 °C; b) 100 °C; c) 150 °C and d) 200 °C

3.2.2 Eluent Exiting the Pre-column Heater Unit

It was anticipated that unheated (or fixed temperature) eluent entering a column could have a cooling effect. This led to a set of experiments designed to assess the capacity of the pre-heater to heat the eluent. The transfer of heat to the eluent will depend on the time spent in contact with the heater. Thus, thermal measurements of the eluent were taken across a range of 40 to 200°C for flow rates of 0.04 to 1.5 ml/min. Initial observations (Table 8) suggested that at 130°C there is no further gain in heat despite increasing the pre-column heater set-point. However, at over 130°C the water was visibly turning to steam at the exit of the tubing because no back pressure was applied

Table 8: Pre-column heated eluent temperature measurements for varying flow rate

Pre-column heater set-point (^{o}C)	**Temperature Measurements (°C) at Different Flow Rates**						
	0.04 ml/min	**0.1 ml/min**	**0.2 ml/min**	**0.4 ml/min**	**0.8 ml/min**	**1 ml/min**	**1.5 ml/min**
40	32	34.6	35.3	34.7	33.1	32.5	31.4
70	46.8	57.1	59.7	58.8	54.7	52.7	49.1
100	62.4	78.5	84.6	83.2	75.2	72.7	66.5
130	97.6	98.2	98.1	97.5	97.2	97.1	87.5
160	96.7	98.9	97.9	97.8	97.6	97	96.5
200	95.8	101	99.7	98.8	98.8	98.6	98.1

The points above 130 °C up to 200°C were therefore extrapolated from linear functions derived from the data up to 130 C°. The completed thermal data set for the pre-heater (Table 9) provides an adequate description of its heating capability. The data was then used as a conversion chart to match future separation parameters in isothermal mode.

The data shows that as the temperature increased the greater the difference between the pre-heater target and the resulting eluent temperature. This inferred that although the temperature was still increasing, a limiting factor was that either the power or the surface contact area inside the pre-column heater was insufficient. At 40°C the lack of transfer does not exceed 10°C whereas at 200°C an average difference of 55°C was observed. Prevention of thermal mismatch is paramount as this will result in diminishing efficiency

and loss of resolution. Any benefits of applying temperature can easily be forsaken if the system components are not understood and optimised. This information could then be used to highlight necessary software changes or to alter the heating capacity of the unit.

Table 9: Temperature Calculated from Experimental and Predicted Data from the Pre-column heater to the Eluent

	Eluent Temperature (°C) at Different Flow Rate						
Pre-column heater set-point (^{o}C)	**0.04 ml/min**	**0.1 ml/min**	**0.2 ml/min**	**0.4 ml/min**	**0.8 ml/min**	**1 ml/min**	**1.5 ml/min**
40	32.0	34.6	35.3	34.7	33.1	32.5	31.4
70	46.8	57.1	59.7	58.8	54.7	52.7	49.1
100	62.4	78.5	84.6	83.2	75.2	72.7	66.5
130	97.6	98.2	98.1	97.5	97.2	97.1	87.5
160	112.9	120.2	122.8	121.8	118.2	117.2	105.1
200	141.2	148.5	151.2	150.2	146.6	145.7	129.8

The theoretical energy required to raise the temperature and the effective experimental energies were calculated (Table 10 and 11)

Table 10: Theoretical Energy required based on the different Eluent Temperature Exiting the Pre-column heater at different Flow Rates

Energy (kJ)							
Set-point (°C)	**0.04 ml/min**	**0.1 ml/min**	**0.2 ml/min**	**0.4 ml/min**	**0.8 ml/min**	**1 ml/min**	**1.5 ml/min**
40	0.04	0.10	0.21	0.42	0.84	1.05	1.57
70	0.13	0.31	0.63	1.25	2.51	3.14	4.70
100	0.21	0.52	1.05	2.09	4.18	5.23	7.84
130	0.29	0.73	1.46	2.93	5.85	7.32	10.97
160	0.38	0.94	1.88	3.76	7.52	9.41	14.11
200	0.49	1.22	2.44	4.88	9.75	12.19	18.29

Table 11: Energy Measurements Calculated for the effective transfer of energy from the Pre-column heater to the eluent based on the experimental or predicted measured change (from Table 14)

Energy (kJ)							
Set-point (^{o}C)	**0.04 ml/min**	**0.1 ml/min**	**0.2 ml/min**	**0.4 ml/min**	**0.8 ml/min**	**1 ml/min**	**1.5 ml/min**
40	0.02	0.07	0.14	0.27	0.45	0.52	0.67
70	0.06	0.22	0.48	0.94	1.66	1.93	2.52
100	0.10	0.37	0.83	1.62	2.80	3.32	4.33
130	0.20	0.51	1.02	2.02	4.02	5.02	6.54
160	0.25	0.66	1.36	2.70	5.19	6.43	8.37
200	0.33	0.85	1.76	3.49	6.77	8.42	10.96

The difference between these values suggested that over the range of velocities monitored, up to 60% of the available energy was not transferred.

The pre-heater device has the available capacity, in terms of its nominal power of 300 vA (equivalent to watts = 300 kJ/60 seconds) but an actual measurement gives 18 kJ/60 seconds at 200 °C for a flow rate of 1.5 ml/min. To deliver the target temperature change losses have to be minimised to the surroundings and/or the heat transfer improved. Although the heater was well insulated, the amount of material used to construct the flow path would take heat away from the target area. The heater was heating a block of steel (~68 g), which radiated heat in all directions and the sensor for the control was not placed close to the flow path.

The reason for using a large mass as this gives increased energy storage. However, from a practical aspect a low mass is preferential for rapid cooling and a disadvantage in gradient heating. The other option is to overheat the pre-column heater to a higher value than required in the column and this approach was chosen for practicality during the project. An alternative would be to increase the surface area of the flow path but this could increase the dead volume.

3.2.3 Modification of Pre-column Eluent Heater unit

In order to obtain a faster response to heating changes the block of steel used to surround the low volume heater was reduced from 68 grams to 32 grams. This should enable initial eluent temperature ramping tests to be performed. The effect on the exiting eluent was again measured (Table 12).

Table 12: Thermal Measurements for the Eluent Exiting the Modified Pre-column Eluent Heater unit for Varying Flow Rate

Pre-column heater set-point (°C)	**Thermal Measurement (°C) at Different Flow Rate (ml/min)**							
	0.04	**0.1**	**0.2**	**0.4**	**0.8**	**1**	**1.5**	**2**
40	39.4	39.4	34.6	34.6	31.4	29.4	24.2	21.0
70	63.7	61.2	62.2	60.2	54.0	51.8	46.1	43.6
100	94.0	94.0	96.7	94.8	87.3	80.6	76.9	67.4
130	109.2	103.9	103.9	104.4	103.9	103.5	94.8	84.6

The heat loss is now more prevalent at low temperatures and high flow rates, and although overall a small improvement was seen it could still be improved. The steel block also serves as a heat sink but because the issue is the response time, a low volume heater for a temperature gradient would serve the purpose more adequately.

3.3 Post-column Eluent Cooler

The purpose of the post-column cooler is to cool the eluent exiting the oven, in either isothermal or temperature-programming mode, to a constant temperature. The Peltier coolers should remove potential thermal gradients from the system prior to detection irrespective of the flow rate. The unit needs to be isothermally programmable and so can be set to match the requirement of the detector. The lowest acceptability criterion for this

parameter is the ability to use a refractive index detector. This led to a target fixed set-point of 25 °C.

3.3.1. Eluent Exiting Cooler Unit

The temperatures of the eluent exiting the post-cooler at different isothermal oven temperatures in the range of 50 – 200 °C at a flow rates over a range of 0.04 – 2.0 ml/min were measured (Table 13).

Table 13: Temperature of the eluent exiting the post-column cooler unit set to 25 °C for different flow rate at isothermal oven temperatures

	Temperature of Eluent Exiting Post-cooler for the oven Set-point:			
Flow Rate (ml/min)	**50°C**	**100°C**	**150°C**	**200°C**
0.04	24.2	20.0	23.0	29.4
0.2	24.7	20.0	47.6	44.6
0.5	24.7	29.4	78.9	100.5
1.0	27.7	41.8	69.2	101.5
2.0	28.4	50.3	69.2	98.7

The post-column cooler outlet, like the pre-heater, deviated further from the temperature set-point of the unit as the flow rate increased. At low velocities the potential heat loss to the surroundings is advantageous and the cooler unit can direct more heat through the Peltiers. The cooling ability was worse when the oven set-point exceeded 100 °C and a flow rate was greater than of 0.2 ml/min. With the exception of an oven setting of 50°C or at very low flow rate (microbore chromatography) the post-cooler is not removing enough heat prior to detection. From 0.5 to 2 ml/min the eluent cooler is only removing 57% of its target for an oven setting of 200°C. This leaves as problem as for UV detectors the temperature should not exceed 80 °C.

At elevated flow rates combined with high temperature the eluent is only cooled to a constant temperature approximately 50% of its initial value. If the eluent enters the detector at a constant rate and the temperature is within the limit of the detector, then this

is not a problem. However, if a multiple temperature profile reaches the detector during a temperature program this could have a detrimental effect on chromatography baseline.

Tubing from the post-column cooler to the detector will also lose heat to the surroundings. The difficulty for future applications will be when attempting to go to microbore separations, where dead volumes are more critical. However, at low flow velocities it should be less of a problem as the heat was dissipated away more effectively and equilibrium restored. For conventional LC column other measures were needed to cool the eluent to a constant temperature prior to it reaching the detector, including a longer tubing length aided by attaching copper fins to act as heat exchangers.

3.3.2 Baseline Disturbance Imposed by Thermal Gradients

A constant temperature reaching the detector is the key in any high temperature fast chromatography separation. The previous section suggested that the post-cooling device was not achieving this and therefore may also struggle to maintain adequate cooling during a changing temperature profile. The chromatography could also be a good indicator of the efficiency of the eluent cooler when applying a temperature gradient during an LC separation. This was tested on system 2, where tubing from the post-cooler to the UV detector could be minimised for a conventional column.

A temperature programmed run with and without the eluent cooling prior to detection (Figure 36) showed a shorter retention time for non-cooling. It seemed that the eluent post-column cooler could be having an effect on the oven.

Importantly the system with the cooler gave an improved baseline whereas with no post-column eluent cooling there was a continuous rise in baseline. This could be caused by temperature related pressure changes in the flow cell which is related to a change in refractive index. This was an important observation as a main benefit advocated by temperature programming compared to a solvent gradient is a flat baseline

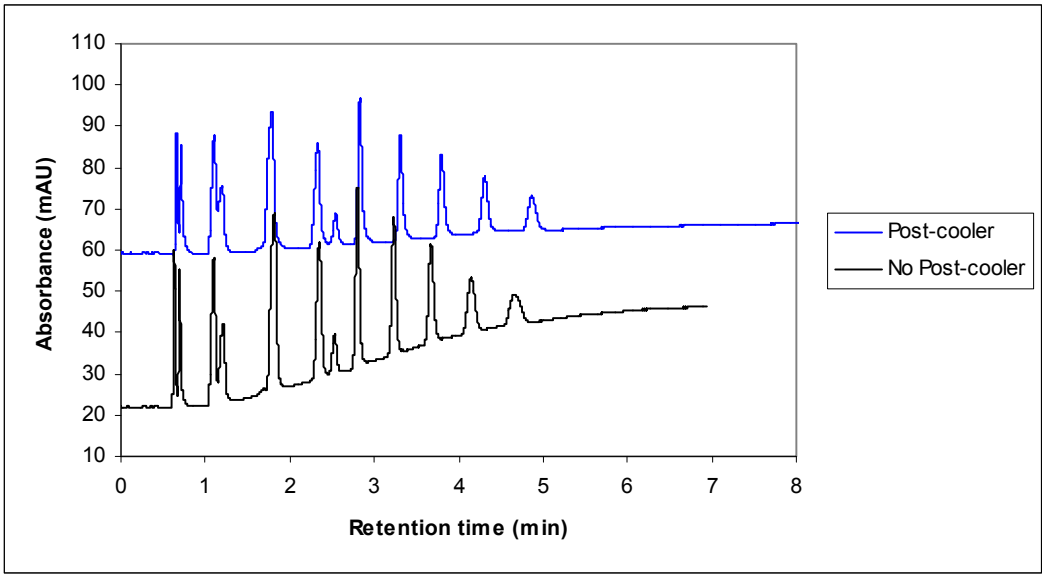
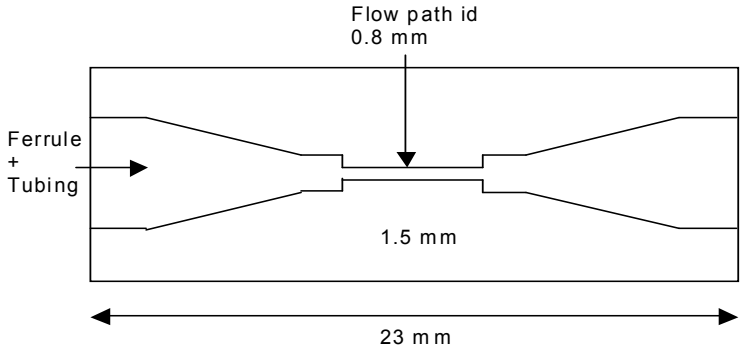


Figure 36: Temperature gradient run of 40 – 200°C (100 % power) at 25 % acetonitrile on an XTerra phenyl 3.5 µm, 4.6 x 50 mm at 1 ml/min; with and without post-column eluent cooling; detector UV at 254 nm (System 2)

3.3.2.1 Modification of Post-column cooler Unit

To achieve a flat baseline the cooling capacity was increased by the addition of two sinters to the flow path of the eluent cooler (Figure 37 a and b). These would in theory increase the surface contact area of the eluent, allowing more heat to be removed.

a) Original eluent post-cooler flow path design



b) Modified version of the eluent post-cooler

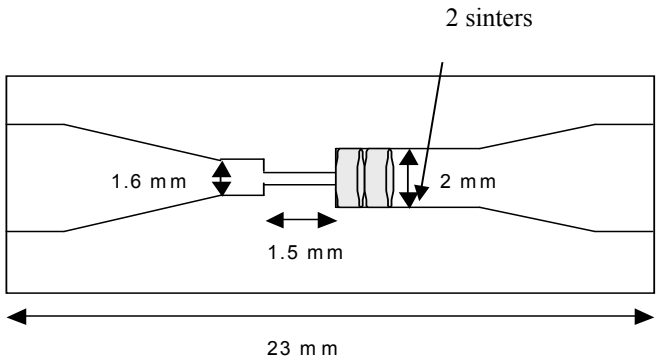


Figure 37: First modification of eluent post-cooler flow path

However, an error in the modification has effectively increased the dead volume of the unit as the sinters should ideally have matched the internal diameter of the flow path (1.6 mm), however, the larger sinter diameter causes a void

A modified version was found to have a detrimental effect peak efficiency of the heating system. This was evident in Figure 38 where integrating the Peltier eluent cooler into the system peak width increased compared to a run having 1 m length of stainless steel tubing with copper fins acting as heat exchangers instead.

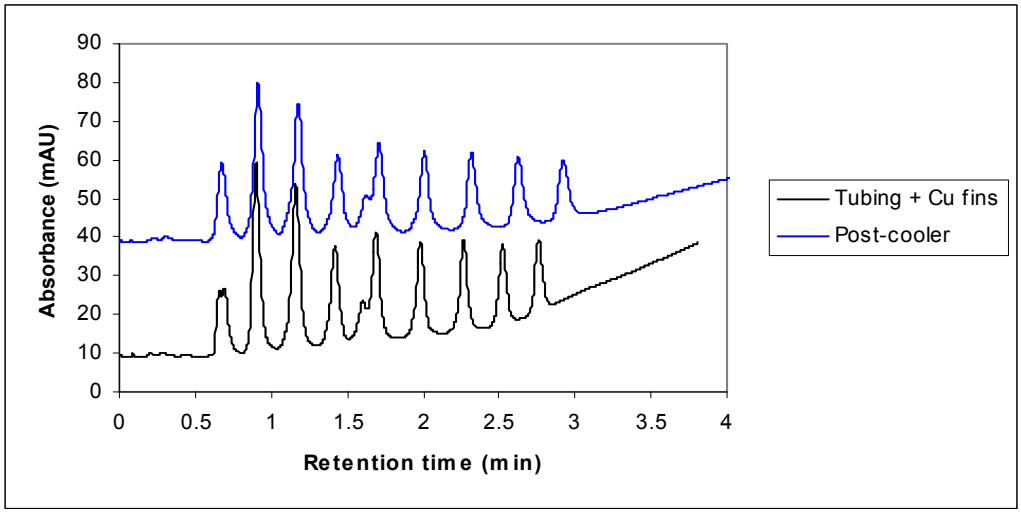


Figure 38: Phenone mixture run on a temperature gradient of 40 – 200°C (100 % power) at 40 % acetonitrile; with eluent cooling achieved via Tubing and with the CSI Peltier eluent cooler on an XTerra Phenyl, 3.5 µm 4.6 x 50 mm at 1 ml/min

The original Peltier cooler (version 1.1) was compared to a modified one (version 1.2, modified in the same way as the pre-heater unit). The baseline rises of the two runs were comparable (Figure 39) and thus, they appeared to deliver a similar cooling capacity.

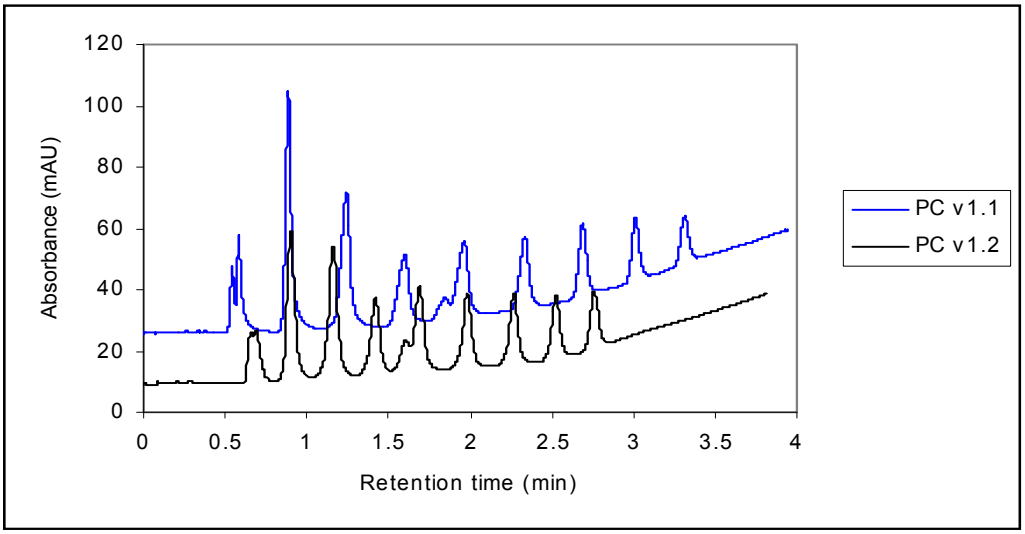


Figure 39: Phenone mixture run on a temperature gradient of 40 – 200°C (100 % power) at 40 % acetonitrile; overlay of CSI Peltier eluent cooler version 1.1 and 1.2 XTerra Phenyl, 3.5 µm 4.6 x 50 mm at 1 ml/min

The chromatography did not give an indication that the baseline noise was due to inadequate mobile phase cooling. Therefore, it was important to re-assess the modified version of the cooler by repeating thermal measurements over a short range of velocity and temperature. The results confirmed that the eluent had been more efficiently cooled to the set-point of 25 °C except at the upper end of 200 °C at 2 ml/min. However this still proved to be at an acceptable value for the detector and alternatives causes of the baseline drift were examined.

Column bleed was investigated as the potential cause although this type of hybrid phase (XTerra phenyl) has been found to be stable up to 150°C isothermally in other studies at Loughborough[261]. The absorbance reading on the detector set at a wavelength of 254 nm, increased significantly when the temperature approached (~150°C). Once the desired set-point was reached the baseline signal either declined as the column was cooled to the lower temperature or reached a plateau at the upper temperature of the gradient if a hold time was set. This indicated that a critical level at which something drastic occurred inside the column. For instance, peak retention times were slightly reduced upon repeat injections and more noticeably so over a number of weeks, an indication of loss of stationary phase. Columns will typically lose some stationary phase or organics used in the synthesis process, heat and highly aqueous conditions will speed up this action and strip ligands, such as the phenyl group. Therefore, samples of eluent were collected from the mobile phase at room temperature; and then from the waste exit to the HPLC system at 40 °C and 200 °C. The UV were examined and revealed an absorbance shift for both the 40°C and 200°C samples, suggesting some sort of bleed. At around 254 nm on the 200 °C run an aromatic chromophore was present This was found to be stronger when 95% aqueous conditions were used where a peak is now clearly visible at 254 nm (Figure 40).

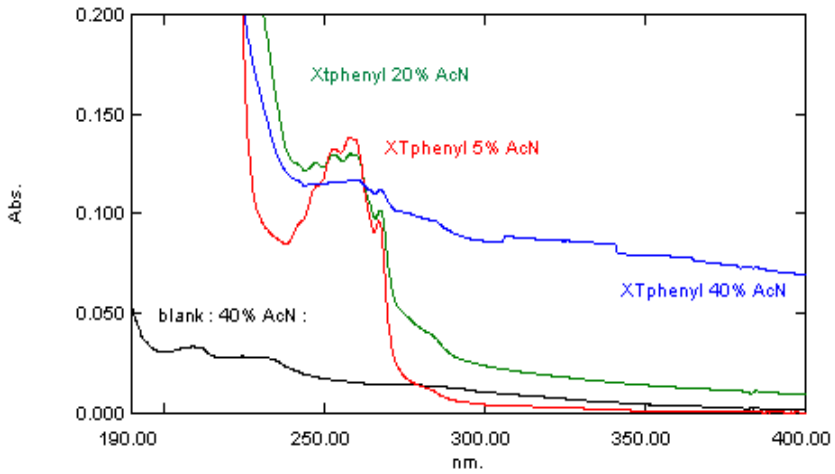


Figure 40: UV trace of mobile phase samples for varying acetonitrile concentration on a temperature-programmed run (blank sample of acetonitrile/water 40:60 v/v collected at 200 °C with a dead volume connector replacing the column) from an XTerra Phenyl, 3.5 µm 4.6 x 50 mm at 1 ml/min

These spectra would be consistent with stationary phase bleed as the column has phenyl groups attached to it surface. However, the UV trace suggests that another component is being detected at a lower wavelength. This was not observed in the run performed without a column, indicating another type of bleed which would not be detected in the baseline at 254 nm. A filter (2µm) was placed in-line post-column pre-detector, although as expected no effect was found on the resulting baseline under the temperature-programme but it was noticed that there was an increasing back pressure due to filter and the tubing blockages pointing towards loss of larger particles. A blank run (minus a column and sample) was overlaid with the post-cooling run on a temperature program and this revealed the desired flat baseline (Figure 41), indicating that the column was at the heart of the problem.

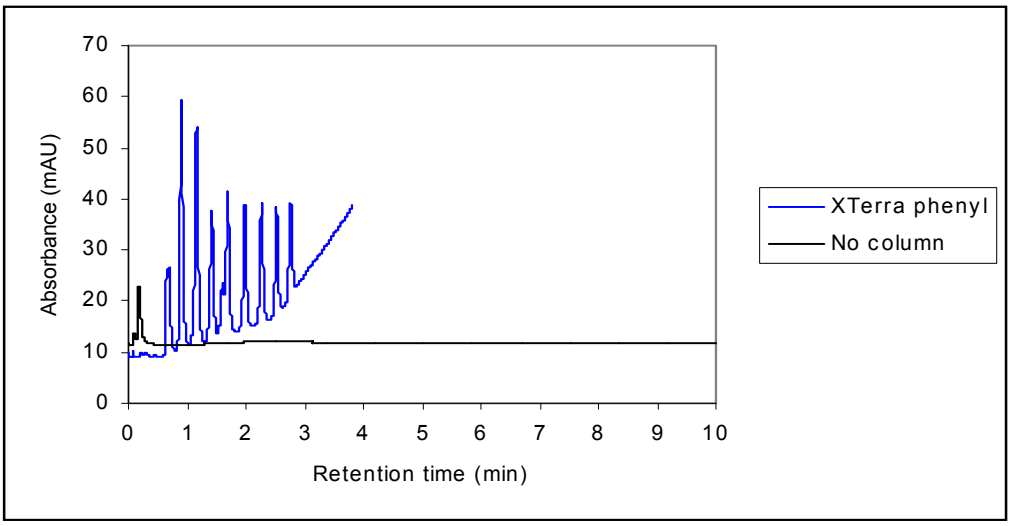


Figure 41: Temperature gradient run of 40 – 200°C (100 % power) at 40 % acetonitrile; overlay of CSI Peltier eluent cooled separation with a blank run and phenone mixture on XTerra Phenyl, 3.5 µm 4.6 x 50 mm

3.4 Oven Stability when coupled to the Eluent Pre-heater Unit

Simply increasing the temperature by only tens of degrees has shown significant benefits in terms of sharpness of peaks and resolution. So is there an even smarter approach when controlling temperature to dramatically improve these desirable chromatographic goals? The next question was what other factors enhance a temperature programmed separation? In GC the injector is heated to volatise the sample and so a hot injection produces sharp peaks. In LC a logical step might be to inject a cold sample onto the column. This could focus the band followed by rapid heating for fast elution.

Equally a hot injection may produce a sharp band followed by rapid elution resulting in ultrafast LC. This is a parameter that was explored but found to be problematic during its application. The intention was to set the incoming eluent temperature to the upper limit of the gradient, but this was found to be impractical as this would naturally in turn heat the column in the oven. Therefore, tests were carried out to establish the cut-off point at which the pre-heater unit would interfere with the starting conditions of the oven. With an XTerra MS 3.5 µm, 4.6 x 100 mm column and the PLRP-S 3 µm, 2.1 x 150 mm

column, the maximum threshold was found to be 70 °C at a flow of 1ml/min and 100 °C at a flow of 0.4 ml/min, respectively. At these settings the oven could still be stabilised at 40 °C. Thus a hot injection was not possible as the thermal data (section 3.2.1.2, Table 8) has already shown that the eluent temperature exiting the pre-heater device at these flow rates is approximately 10 °C above this target. If we take into account further heat losses through the steel tubing used in the set-up then the temperature of the mobile phase is matched approximately to the column.

This was a limiting factor when trying to determine the optimal conditions to reduce band broadening and by focussing the analytes, and requires future consideration. It also highlights the need for overheating the eluent upstream of the column when dealing with varying geometries and tubing set-up. The latter would need to be specified for this technique to be transferable.

3.4.1 Chromatographic Effects of Combining the Resistively Heated: Eluent Pre-heater with the Oven

3.4.1.1 Isothermal Separation

Thermal mismatch between the incoming eluent and the column is an important aspect of a separation if the temperature variable is to be fully exploited. Theoretically if the flow profile across the length of a column can be tuned then higher peak efficiency can be realised from a flat profile. Therefore, the next experiment was designed to test this theory by combining the pre-heater unit with the oven.

Naphthalene was injected isothermally at 40°C (oven temperature) on to an XTerra MS (C18) 3.5µm, 4.6 x 100 mm at 35 % acetonitrile concentration. This was injected with the post-column cooler and pre-column heater unit turned off and compared to two pre-column heater settings of 40 and 50°C (Figure 42). The post-cooler unit was held constant at 25°C.

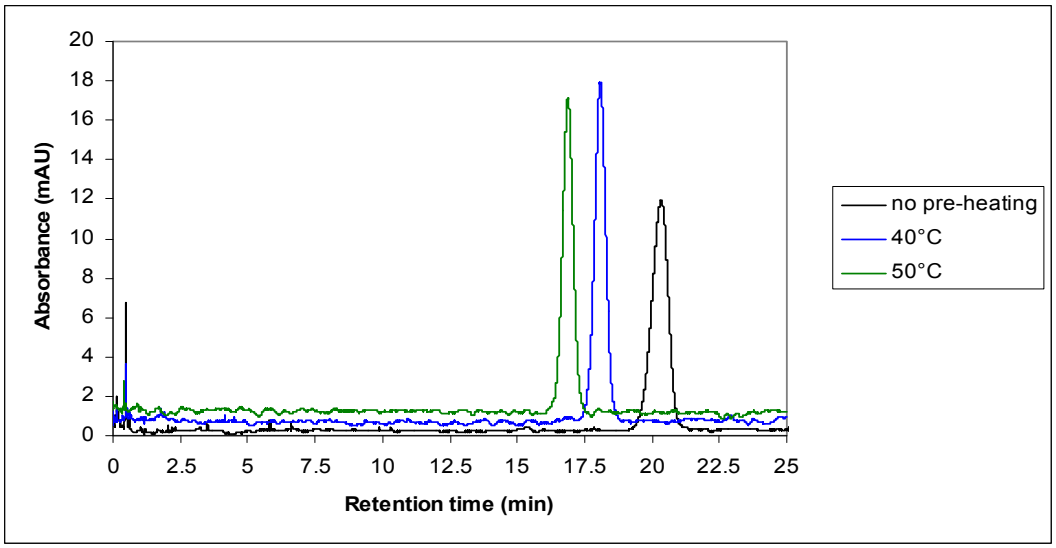


Figure 42: Chromatogram of isothermal runs with and without eluent heating at the column inlet for naphthalene at 40°C on a XTerra MS (C18) 3.5µm, 4.6 x 100 mm, 35 % acetonitrile concentration at 1 ml/min.

To find the maximum efficiency, the incoming eluent was set to 40°C, which was estimated to give an eluent with a slightly lower temperature (~33°C see earlier) than the column and increased efficiency by 3000 plates (Table 14).

Table 14: Results Table for 3 Repeat Injections of Naphthalene for Isothermal Conditions by Varying the Temperature of the Incoming Eluent

Mode	Temperature (°C)	Pressure (bar)	Retention time (min)	$W_{1/2}$ (sec)	NTP
No PH/PC	**-**	**126**	**20.18**	**37.9**	**5734**
PH	**40**	**115**	**18.08**	**27.4**	**8704**
PH	**50**	**109**	**17.27**	**26.4**	**8552**

At the pre-column heater set-point of 50°C the eluent is close (~39°C) to the column temperature of 40°C for a flow rate of 1 ml/min. A lower incoming eluent temperature may compensate for the heat of friction generated by the flow of viscous mobile phase through the packing. This could offset the low heat conductivity of the stationary phase

and once heat is evenly dissipated through the packing bed this is then less likely to be released.

This eluent pre-column heating study was repeated for different geometries of column as one would expect that as the column internal diameter decreases the thermal mismatch would also decrease. This is due to a lower volume of stationary phase having to be heated. Therefore, there will not be such a large temperature differential between the column wall and the centre of the stationary phase. This study was carried out on the Agilent HP1100 series (system 3) with a phenone mixture for a 1, 2.1 and 4.6 mm I.D. columns. The 4.6 mm I.D. run, as expected, benefited from eluent heating (Figure 43). The last peak to elute (octanophenone) showed a reduction in peak width by 19%, the efficiency increased by 27% and the peak height increased by 22%. However, peak fronting was evident, which could be due to a stronger injection solvent. This is where the front of the band is moving quicker than the rear of the band leading to a poor Gaussian distribution. It is caused where the injection solvent is stronger than the mobile phase and the molecules are moving at different speeds to each other.

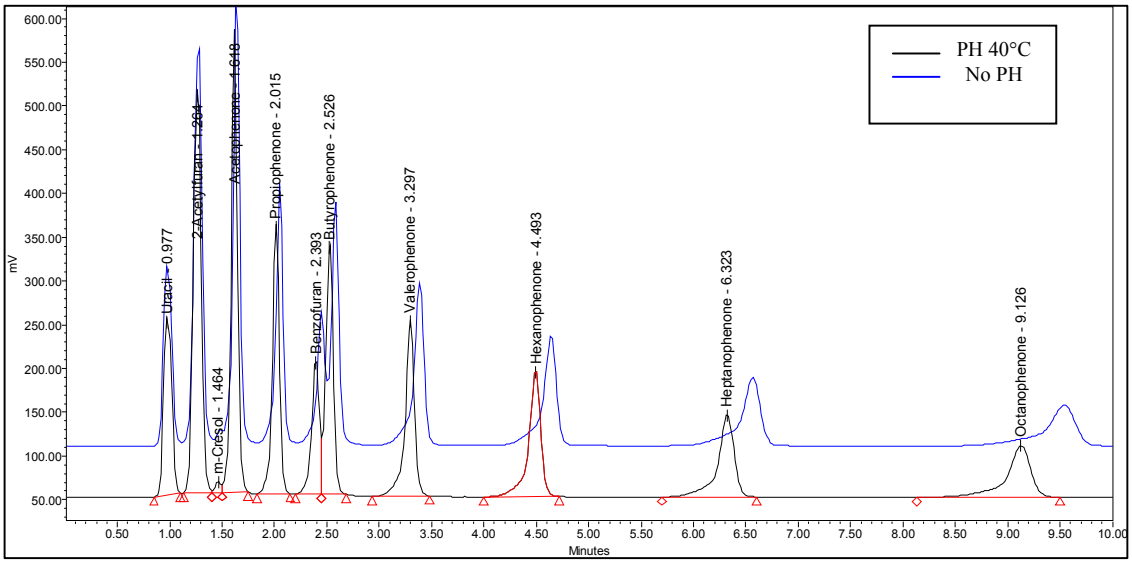


Figure 43: Phenone isothermal separation comparing the effects of pre-heating the eluent on an XTerra MS 3.5 µm, 4.6 x 100 mm, 40 °C at 1 ml/min

The two traces virtually overlaid each other for the 2.1 mm I.D. column (Figure 44). However, the 1 mm I.D. column at 40 °C suggests that the eluent heating was having an effect (Figure 45). This additional heating effect is contributing to the overall column temperature, drawing in late eluting peaks and reducing the retention factors. A possible reason for this could be due to the lag factor and may suggest that column heating could be achieved solely by eluent heating as this is clearly having the larger effect. The fronting seen in the last chromatograms is no longer evident indicating but this is not clearly understood and may be phase related. The smaller diameter columns were both purchased specifically for the project whereas the previous usage history of the XTerra column was unknown.

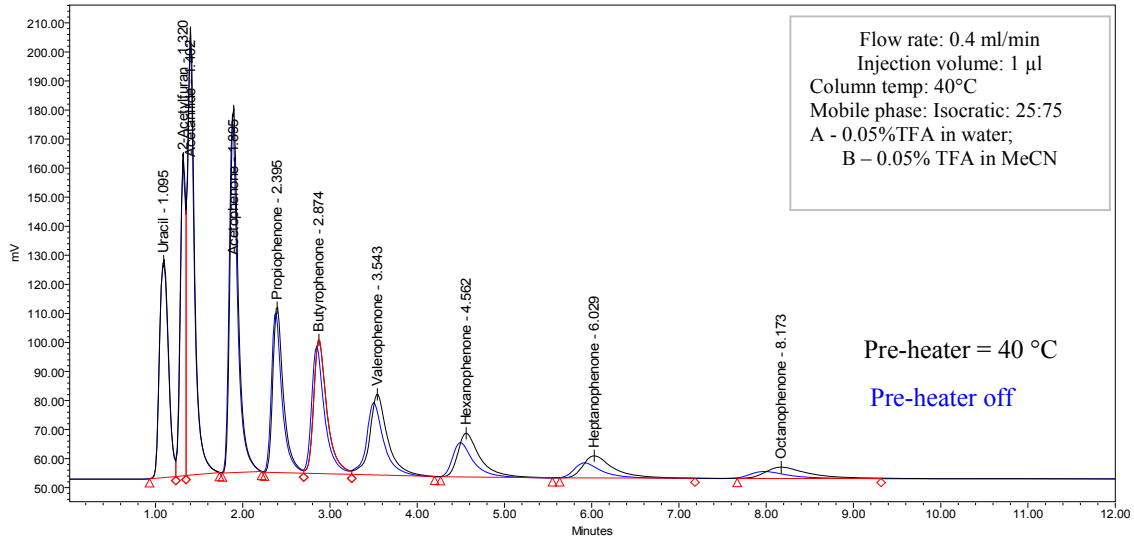


Figure 44: Phenone isothermal separation comparing the effects of pre-heating the eluent on a PLRP-S 3 µm, 2.1 x 150 mm at 40 °C with a) eluent pre-column heating at 40 °C and b) without eluent pre-column heating.

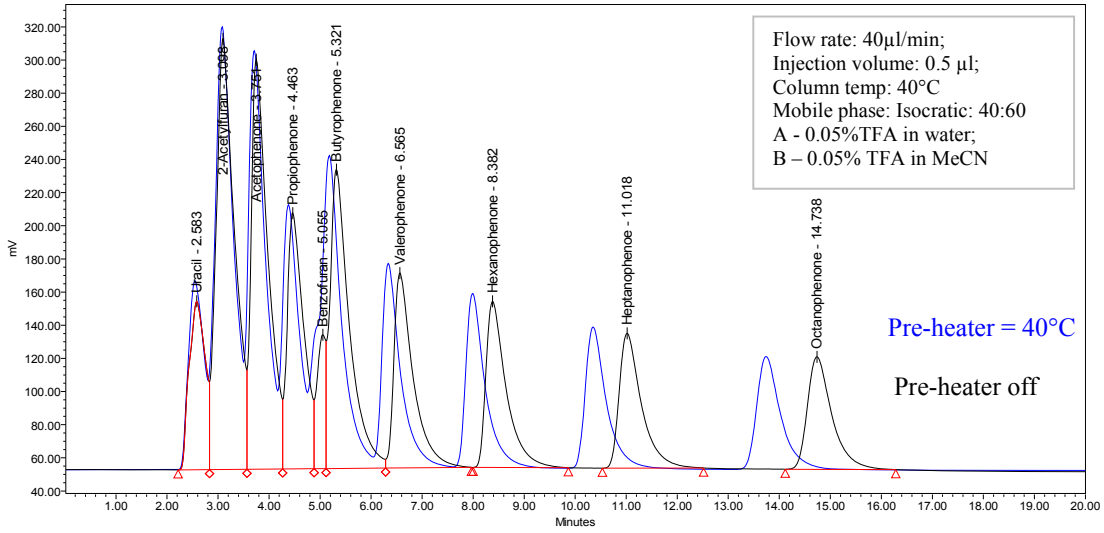


Figure 45: Phenone isothermal separation comparing the effects of pre-heating the eluent on a Merck Purospher® STAR 3 µm, 1 x 150 mm at 40 °C with a) eluent pre-column heating at 40 °C and b) without eluent pre-column heating

3.4.1.2 Temperature-programmed separations

A different concept of what may make a temperature program work compared to an isothermal separation was identified. The primary objective at a constant temperature was to lower the column inlet temperature by a few degrees to invert the parabolic flow profile. The new goal for a temperature programmed elution was to focus the analyte at the column inlet, either as a sharp band through a hot injection or trap it by using a cold injection. The pre-heater set-points of 70, 80 and 90 °C were translated to actual temperatures of 53, 59 and 66 °C, respectively at 1 ml/min. The temperature conditions were then run at an organic concentration of 35 % acetonitrile, the higher the aqueous content the better as water has a large heat capacity and thus should have a more pronounced effect. The pre-heater was turned off to achieve a cold injection (of 25 °C) on to the column for a temperature program run, but gave significant peak fronting for naphthalene (Figure 46). This could be due to solvent incompatibility but equally a broad peak shape could be attributed to thermal mismatch. The shape clearly improves when a

hot injection is performed at a pre-heater set-point of 70°C and was better still at 80 and 90 °C (Table 15).

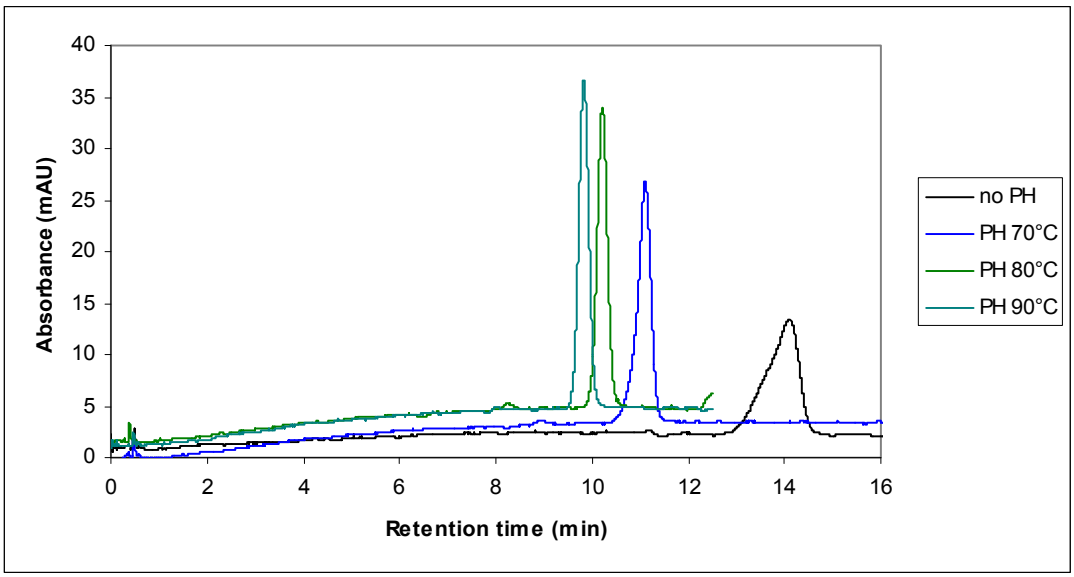


Figure 46: Temperature gradient run of 40 – 80 °C for naphthalene overlaid for varying eluent pre-heating (PH) settings of 70, 80 and 90°C on an XTerra MS 3.5µm 4.6 x 100 mm, 1 ml/min and 35 % acetonitrile

An important observation was that the peak fronting improved with a higher injection temperature, which confirmed that the fronting seen in both the isothermal and temperature programmed runs was temperature related. There is now, however, a difference between the two approaches with a cold injection giving a better isothermal run whereas a hot injection produced a better peak profile for the temperature program.

Table 15: Separation parameters for a temperature-programmed separation of naphthalene for varying incoming eluent temperature of unadjusted, 70, 80 and 90°C on an XTerra MS 3.5µm 4.6 x 100 mm, 1 ml/min and 35 % acetonitrile

Temperature gradient: 40 to 80°C at 10%P					
Mode	Pre-heater (PH) Temperature (°C)	Pressure (bar)	Retention time (min)	$W_{1/2}$ (sec)	NTP
No PH	**-**	**119**	**14.14**	**45.7**	**1914**
PH	**70**	**97**	**11.11**	**17.4**	**8118**
PH	**80**	**93**	**10.45**	**15.2**	**9580**
PH	**90**	**90**	**9.83**	**13.9**	**9944**

There is one drawback with this method and that is stabilisation of the oven, as detected previously, because when the inlet temperature exceeds the oven set-point the eluent starts to heat the column. At this point one has to ask what is improving the peak shape leading to narrower bands. Is this a focusing effect or an increased column temperature (reflected in the lower retention factors) producing a decreased peak width?

3.5 Agilent 1100 Series Thermostat vs. Resistive Heating System at Isothermal Conditions

The CSI heating system was expected to perform better than the HP1100 thermostat in isothermal mode, as the column is entirely enclosed and surrounded by resistively heated coils. Whereas with the heating block of the HP1100 the column has poor thermal contact and will usually be cooler than the pre-heated block.

The two oven performances were compared programmed isothermally at 40 °C (Figure 47) and then at 80 °C (Figure 48). A phenone mixture was injected on a Waters XTerra® MS column, 3.5 µm, 4.6 x 100 mm, mobile phase acetonitrile/water 40:60 v/v with 0.05 % trifluoroacetic acid at 1 ml/min.

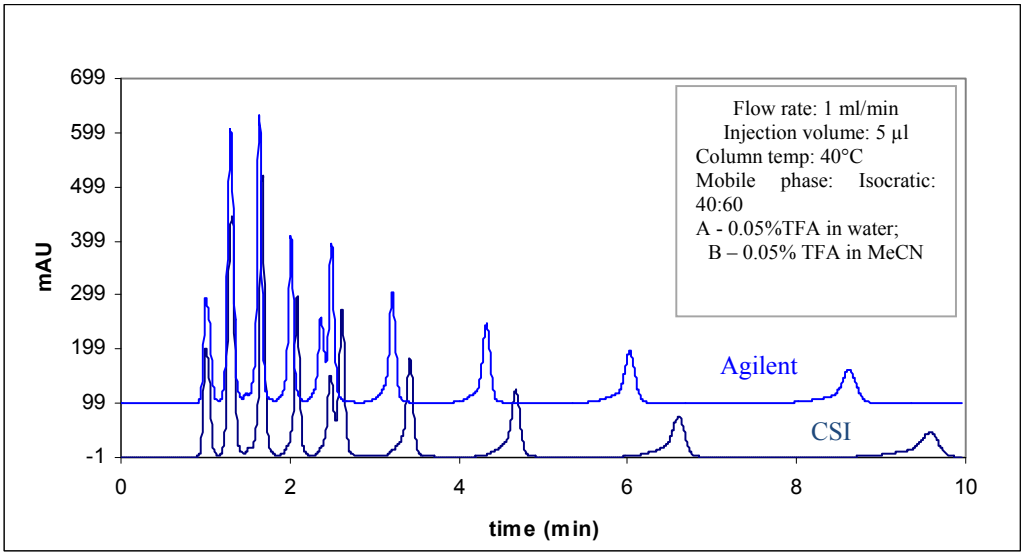


Figure 47: Phenone test mix on the Agilent HP1100 Series Thermostat vs CSI Heating System at 40°C on a Waters XTerra® MS Column, 3.5 µm 4.6 x 100 mm

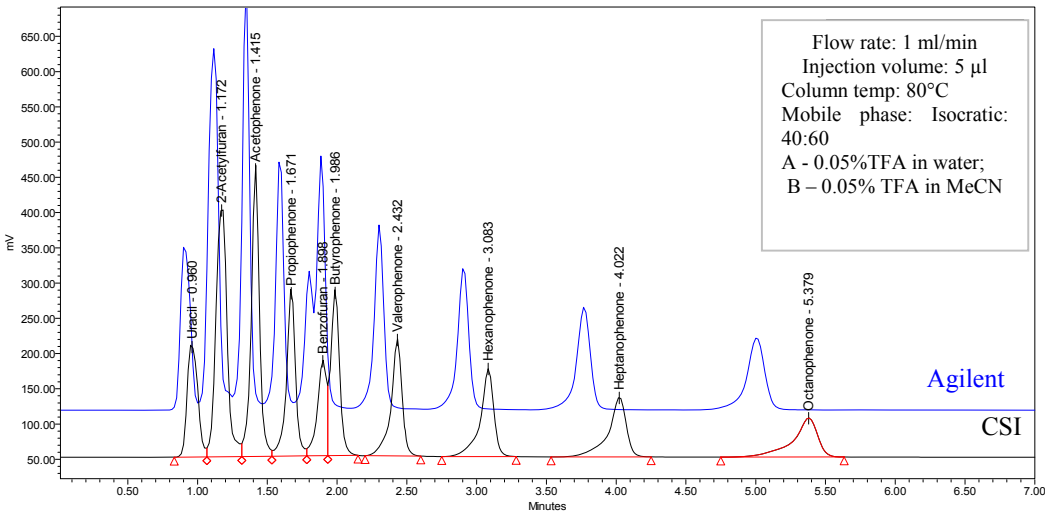


Figure 48: Phenone test mix on the Agilent HP1100 Series Thermostat vs CSI Heating System at 80°C on a Waters XTerra® MS Column, 3.5 µm 4.6 x 100 mm

Both systems gave good retention reproducibility of <1% RSD for 10 injections. The efficiency of the octanophenone, the last component to elute, was found to have increased by 20% at 40°C for the Agilent compared to the CSI heating unit. However, this could be due to the logistics of the instrument set-up. A 1 m length of tubing (0.007" id) was

necessary to connect the CSI heating system to the Agilent instrument. This can cause peak broadening and could explain the poorer result, to which heat losses could have contributed. Another element could be in the eluent pre-heating, as the Agilent pre-heats the eluent prior to the column inlet as the tubing is integral to the heating block. The mobile phase is equilibrated over a distance of approximately 20 cm and heats the column far more effectively than the column placed against the block creating hot and cold spots.

At 80°C the Agilent system was out-performing the CSI heating unit probably due to more effective heating eluent heating and reaching a higher column temperature as a consequence. Ideally the eluent would benefit from being heated over a longer distance or positioned much closer to the column inlet. A pre-heating device that could be inserted directly into a column or vice versa such as the Javelin columns (Thermo) would be advantageous as it has high temperature fittings. However, the Agilent oven is essentially isothermal and could not be programmed other than relatively slowly and the cool down time of the large metal blocks would be excessive.

3.6 Oven Cycle Times

The throughput of a temperature gradient system is dependent of the whole cycle time including the cool down and re-equilibration so these were studied.

3.6.1 The Effect of Eluent Heating on Re-equilibrium time

This data was collected on the Agilent system at GSK. Initial oven cycle times, between temperature gradient runs over the range 40 – 130 °C, were approximately 15 minutes, if the pre-heater unit was maintained at the initial temperature. However, equilibrium times would vary for the system set-up if the pre-heater setting was changed. To get an idea of this contribution the eluent was heated in 10 °C increments for a range of 40 – 100 °C (Table 16). The higher the temperature the longer it takes for the column to attain thermal

equilibrium after cooling. This is due to the pre-heating of the eluent influencing the column temperature, which is set to a lower temperature to the column. The obvious solution is to build a low mass pre-heater that can ramp up and down the temperature range to follow a temperature gradient and cool rapidly.

Table 16: Cycle times on increasing the inlet temperature with a flow rate of 0.4 ml/min on the PLRP-S 2.1 x 150 mm column over a cycle of 40 – 130 °C

Re-equilibrium temperature (°C)	Analysis time (min)	Re-equilibrium time (min)	Cycle time (min)
40 (initial)	7	15	22
50	7	20	27
60	7	25	32
70	7	25	32
80	7	28	35
90	7	29	36
100	7	39	46

3.6.2 System Cooling with Liquid Nitrogen

To reduce cycle time faster cooling is necessary and air cooled with liquid nitrogen was examined as a potential coolant. A cooled gas was passed into the resistive heating oven and flowed around the heating coils (Figure 49). The flow was controlled by the oven software, where a high pressure valve was switched to release the gas once the oven was in cooling mode. A column temperature sensor cut of the flow when the column was 10 °C below the start temperature (Figure 50). One difficulty was it took time to cool the insulated connecting tubing from the liquid nitrogen bath to the column.

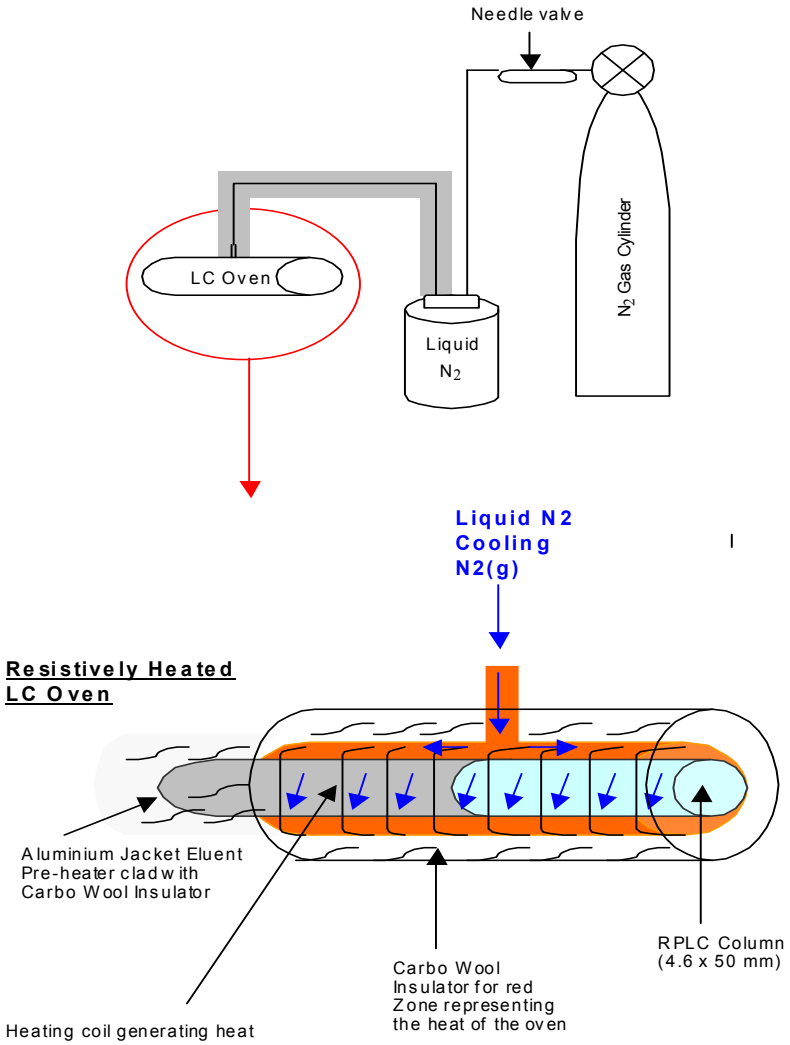


Figure 49: Schematic set-up of the oven's liquid nitrogen cooling system

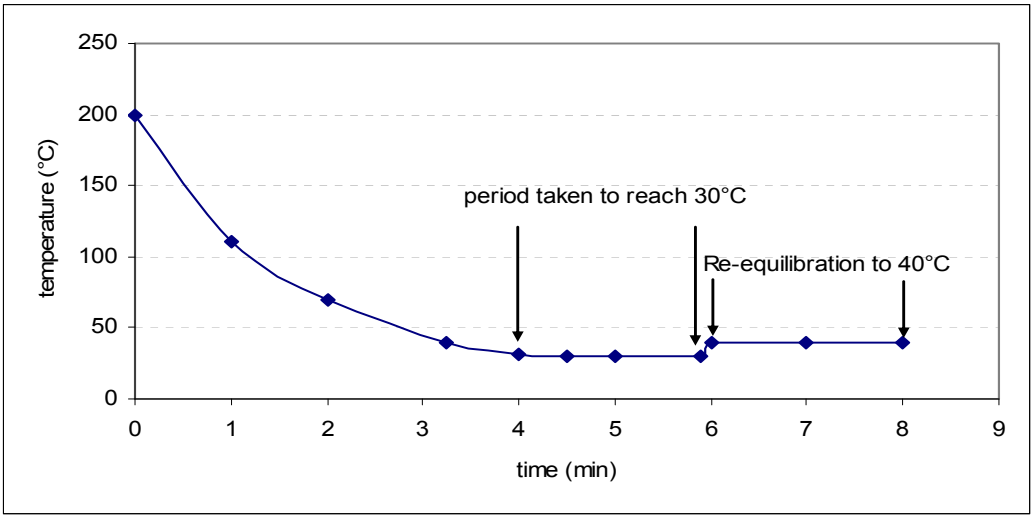


Figure 50: Temperature profile of the oven during cooling with liquid N_2 for a 4.6 x 150 mm I.D. column

The cooling time from 200°C to 40°C showed initial rapid cooling that tailed off within 4 minutes, giving an initial cooling rate of 40°C/min. The oven then was over-cooled for 2 minutes to reach 30°C. The re-equilibration time to obtain initial conditions took a further 2 minutes. This was due to the residual heat of the column, which took longer to cool than the oven. Thus, the overall rate of cooling based on a cycle time of 8 minutes was 20 °C/min. The best indicator to determine re-equilibration of the system was the pressure reading.

3.6.3 System Cooling with Air

An air flow was used to cool the oven with the HP1100 at GSK. The air line was capable of delivering many litres of air per minute, an obvious advantage when trying to cool rapidly. Initial runs with air cooling demonstrated similar results to those with liquid nitrogen where the latter part of the cooling curve was taking considerably longer than the beginning. This suggested that the sensor might be cooling more rapidly than the column and was causing the oven heater to come on prematurely and compete with the

cooling. The system was adjusted to that no heating was possible during the cool cycle and this reduced the cooling section of the cycle time to 5 min.

The pressure profile and refractive index detection trace was recorded by Chem Station for an assay run followed by a cooling stage on the Agilent HP1100 HPLC (Figure 51). This clearly relates the temperature program and cooling. The pressure drops from 120 to 54 bar during the assay run of 5 minutes and then rises to approximately 140 bar during cooling. The whole process gives a total cycle time of 10 minutes.

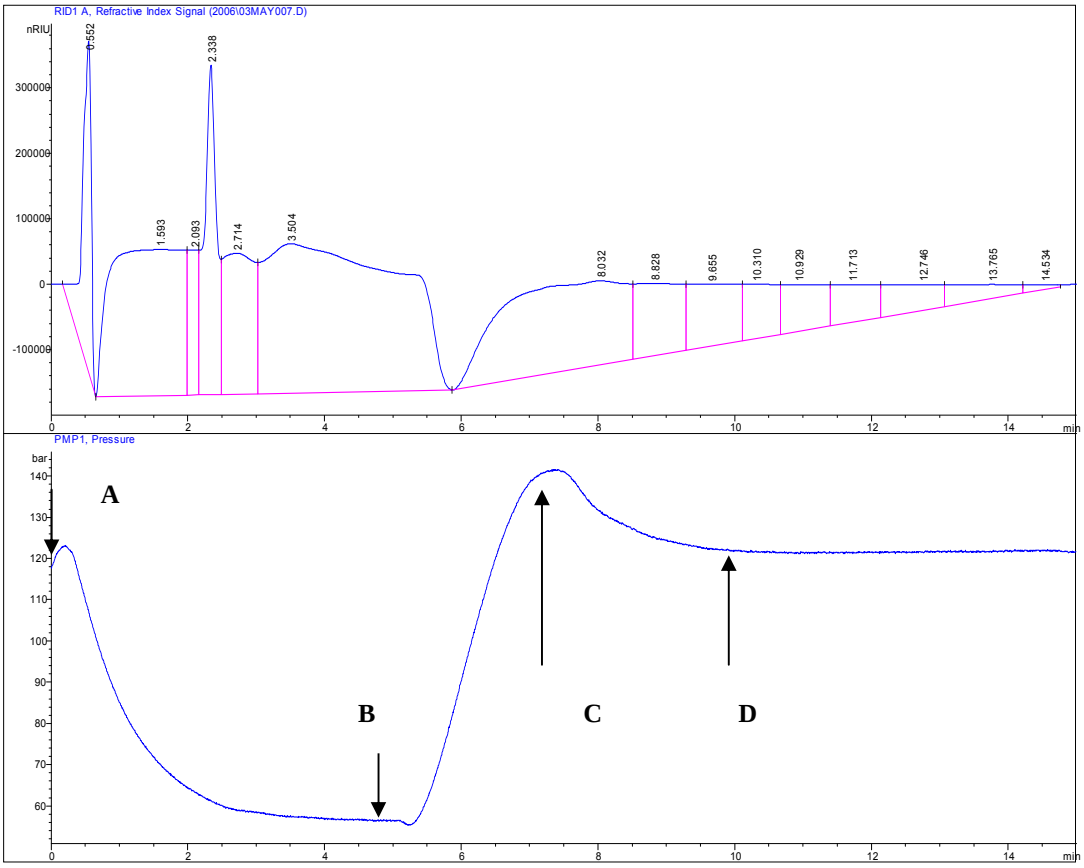


Figure 51: Cycle time approximated by the pressure profile for a 5 minute assay run with a 5 minute cooling time. A: temperature gradient start, B: temperature gradient end/ oven off/ air cooling on, C: cooling off/ heat on and D: equilibrated to initial start (40°C) overlaid with chromatogram for GSK compound SBA on an XBridge C8, 2 µm 3 x 30 mm column, 5 % acetonitrile / 95 % water at1 ml/min

3.7 Validation

A series of 6 repeated assay runs of a GSK development compound was performed on a temperature program of 40 – 200°C at 100% power with manually initiated cooling and equilibration. The signals overlaid each other (Figure 52) indicating that the cooling cycle of 5 minutes was reproducible. The retention time and area showed relative standard deviations < 0.3% (Table 17), below the limit set out in the United States Pharmacopeia criteria for validation purposes. If the process could be automated this would cut out time consuming manual operations and potentially reduced the cooling time further to less than 5 minutes.

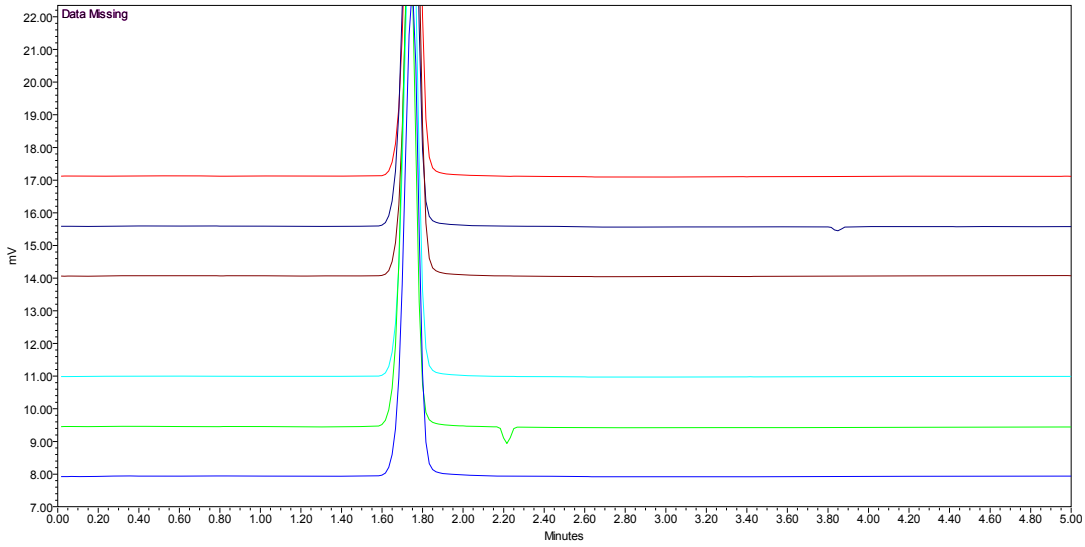


Figure 52: Chromatogram overlays for repeat injections of SBA for a temperature program of 40 – 200°C (100% power) after a cooling with air for 5 minutes Conditions on an XBridge C8 2µm, 3 x 30 mm column, 5 % acetonitrile / 95 % water at 1ml/min

Table 17: Retention time and area repeatability for GSK analyte for a cooling cycle of 5 minutes after a temperature program of 40 – 200°C (100% power)

Injection number	Retention time (min)	Area
1	1.755	74010
2	1.752	73942
3	1.754	73538
4	1.743	73547
5	1.753	73967
6	1.745	73925
7	1.747	73779
% RSD	**0.273**	**0.270**

The data has demonstrated that a cycle time of ≤ 5 minutes is possible with air-cooling down to ambient temperatures. This has been incorporated in to the validation assessment of the system for a temperature gradient method for assay. The results show that the criteria has been met well within the guidelines e.g. < 2.0 % RSDs. The stability of the heating system has been proven for its applicability to the analysis of pharmaceutical compounds on a temperature gradient.

Chapter 4. Temperature Gradient Separations

The long term objective of the study was to show that temperature-programming would improve speed, resolution, sensitivity and demonstrate equivalence to solvent gradients. The initial performance of the system was to be assessed using a range of phases and column geometries for a complex test mixture.

4.1 Improving the Speed of Temperature-programmed Chromatography with Different Phases and Varying Column Geometry

A phenone test mix was injected on three columns with different internal diameters (and phases): 4.6 mm, 2.1 mm and 1 mm. The aim was to reduce the analysis time by approximately 50% by the application of an appropriate temperature program. The resulting chromatograms were overlaid with an isothermal run performed prior to the temperature gradient at 40°C to enable relative differences to be assessed.

4.1.1 Chromatographic Parameters

System 3:	Agilent HP1100 LC system with the CSI oven
Column:	XTerra MS C18 3.5 µm, 4.6 x 100 mm
Mobile Phase:	Eluent A: 0.05% TFA in water
	Eluent B: 0.05% TFA in Acetonitrile
% B:	60
Temperature program:	40 to 130°C (20% power; 9°C/min)
Injection volume:	5 µl
Flow rate:	1 ml/min
Wavelength:	254 nm
Equilibrium time:	15 minutes allowed between temperature set-points

It was important to establish how the resistive heating system placed on a bench-top in a Pharma environment, such as GSK, would perform when connected to a conventional LC (in this case the Agilent 1100 series). Although, micro-bore columns have desirable attributes in temperature programming such as their low thermal masses, the emphasis in current practices are still very traditional in their approach, therefore it was important to show that temperature could have a positive effect on conventional 4.6 mm I.D. columns. A hybrid stationary phase column, XTerra MS C18, was selected for testing as this was a more robust example of a silica based column.

Later eluting peaks were speeded up and sensitivity increased by a factor of 2 on gradient elution (Figure 53). Therefore the initial coupling appears to be of benefit even with a conventional column, which takes a lot more heating, than a narrow/micro bore column. However, the temperature gradient did not appear to have had any effect on the compounds that are first to elute. This suggests that analyte focusing had not been achieved and that the amount of heat reaching the stationary phase may be sufficient but the effect was not sustained over the volume of phase/ length of the column. Peak shape was thought to be also affected by the test mixture diluent (100 % acetonitrile) and significant fronting was observed. A temperature gradient unlike a solvent gradient did not compensate for this effect at the nominal injection volume used.

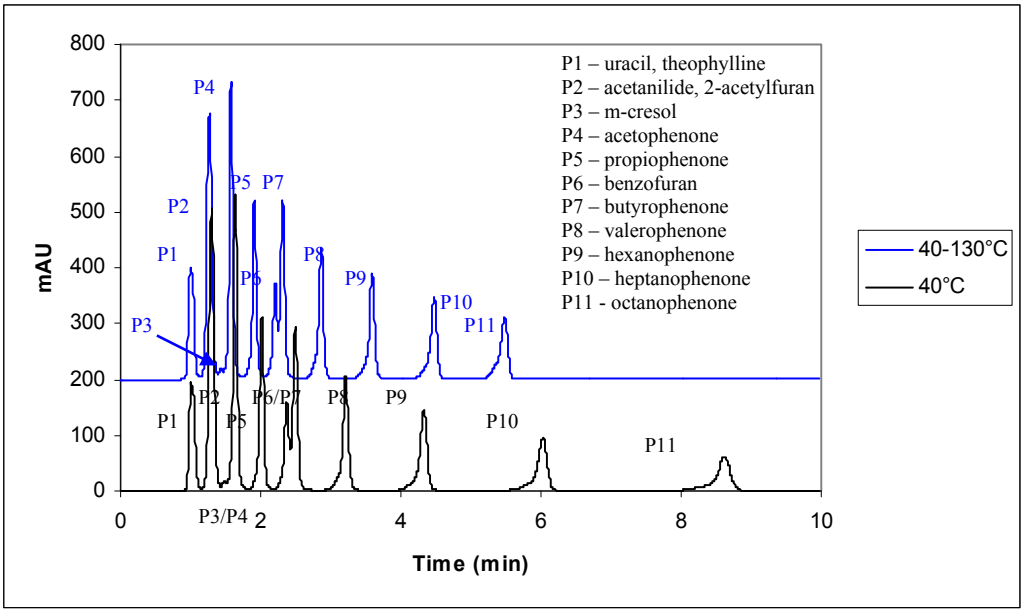


Figure 53: Temperature gradient of 40 – 130 °C overlaid with an isothermal 40°C run for a complex mixture of phenones, acidic and basic functionality on a conventional column XTerra MS C18 3.5 µm, 4.6 x 100 mm, 1 ml/min in 0.1 % TFA/60% acetonitrile/40% water

4.1.2 Narrow-bore polymer column

A PS-DVB polymeric stationary phase column was acquired in a narrow-bore format as this is stable up to 200°C and can be used with superheated water, if required. However, with this column the outer diameter (O.D.) was the same as the 4.6 mm I.D. column.

4.1.2.1 Chromatographic Parameters

System 3: Agilent HP1100 LC system with the CSI oven

Column: PLRP-S 3 µm, 2.1 x 150 mm

Mobile Phase: Eluent A: 0.05% TFA in water

Eluent B: 0.05% TFA in Acetonitrile

% B: 70

Temperature program: 50 to 200°C (30% power; 18°C/min)

Injection volume: 1µl

Flow rate: 0.4 ml/min

Wavelength: 254 nm

Equilibrium time: 15 minutes allowed between temperature set-points

The improvement demonstrated for the complex mixture was quite spectacular (Figure 54). The results are outlined in tables 18 and 19, which describe the overall performance.

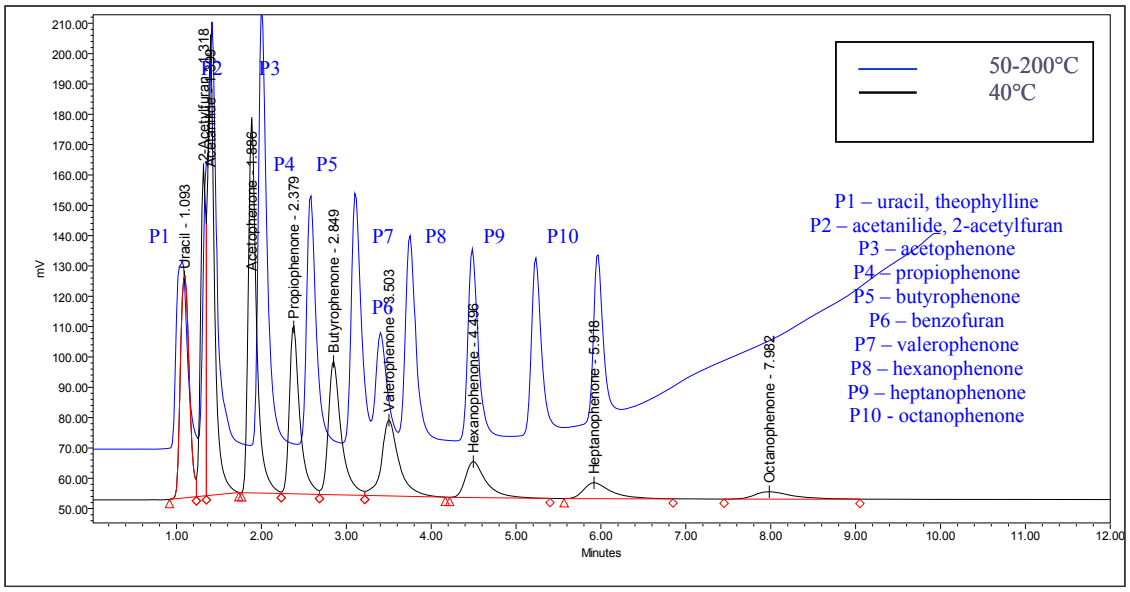


Figure 54: Temperature gradient of 50 – 200°C overlaid with an isothermal 40°C run for a complex mixture of phenones, acidic and basic functionality on a narrow-bore PLRP-S 3 µm, 2.1 x 150 mm column, 0.4 ml/min in 0.1 % TFA/70% acetonitrile/30% water

At 40°C late eluting peaks such as octanophenone are broad, making areas difficult to quantify accurately, a gradient run of 50 – 200°C has vastly improved conditions. Analysis time has been reduced by 21 % and sensitivity has been increased by 78 %, which has led to sharp bands on the polymer phase not evident on the hybrid silica columns. This implies that there is a marked difference in performance of the polymer column compared to the hybrid silica phases used.

Table 18: Chromatographic results of a phenone test mixture for an isothermal run of 40°C on a PLRP-S 3 µm, 2.1 x 150 mm column, 0.4 ml/min in 0.1 % TFA/70% acetonitrile/30% water, Agilent HP1100 system

Name	Retention Time	Area	Height	Width @ 50%
Uracil	1.090	644107	86185	0.121
2-Acetylfuran	1.327	-	-	-
Acetanilide	1.383	1613562	174872	0.149
Acetophenone	1.851	938619	131549	0.10
Propiophenone	2.314	542954	60890	0.124
Butyrophenone	2.756	620191	56244	0.151
Valerophenone	3.370	616735	44634	0.185
Hexanophenone	4.291	599882	34636	0.237
Heptanophenone	5.604	472660	21693	0.312
Octanophenone	7.505	373437	12854	0.429

Table 19: Chromatographic results of a phenone test mixture for a gradient run of 50 - 200°C on a PLRP-S 3 µm, 2.1 x 150 mm column, 0.4 ml/min in 0.1 % TFA/70% acetonitrile/30% water, Agilent HP1100 system

Name	Retention Time	Area	Height	Width @ 50%
Uracil	1.050	228550	58551	-
Theophylline	1.063	302697	59853	-
2-Acetylfuran	1.341	287913	94470	-
Acetanilide	1.410	954375	141363	-
Acetophenone	1.984	1009596	148044	0.10
Propiophenone	2.546	635602	83563	0.112
Benzofuran	3.065	667678	84029	0.120
Butyrophenone	3.362	329406	36019	0.138
Valerophenone	3.698	582719	69009	0.124
Hexanophenone	4.431	536417	64163	0.123
Heptanophenone	5.180	476892	59158	0.118
Octanophenone	5.914	473982	57691	0.115

The significant enhancement in chromatographic properties could be due to the kinetic transfer being speeded up rather than the thermodynamics of the absorption processes. Polymer columns have a higher diffusion rate in micropores at low temperature compared to silica-based columns. This in turn raises the point that phases specifically aimed at temperature-assisted chromatography are needed as the benefits could be immense

without the need for extreme temperatures. Isothermally heating a column to temperatures of 200°C affects the lifetime of a column to a much lesser extent than rapid heating in a short space of time.

The increase in baseline noise observed during a temperature programmed run was later found to be caused by the presence of TFA in the mobile phase, when this was removed, the baseline shift disappeared. This effect did not occur with the other types of phase used on the same system and an acid catalysed stripping of benzene groups from the polymer was suspected.

4.1.3 Micro-bore Column

Substantial benefits derived from miniaturizing systems have been established but the ease of applying associated techniques is usually the point at which the technology does not withstand criticism. However, temperature programs could solve both aspects as they are easy to apply and have the elution power of a solvent gradient.

The Merck Purospher 1 mm I.D. column was used in the next separation, which was expected to be only stable to about 80 – 90°C. Although this is not a high temperature column the heat transfer process should be demonstrative of the applicability of high temperature gradients for fast chromatography.

4.1.3.1 Chromatographic Parameters

System 3:	Agilent HP1100 LC system with the CSI oven
Column:	Merck Purospher® STAR E18 3µm, 1 x 150 mm
Mobile Phase:	Eluent A: 0.05% TFA in water
	Eluent B: 0.05% TFA in Acetonitrile
% B:	60

Temperature program: 40 – 60 - 80°C (10, 20% power; 4.5, 9 °C/min)

Injection volume: 0.5µl

Flow rate: 0.04ml/min

Wavelength: 254 nm

Equilibrium time: 15 minutes allowed between temperature set-points

A temperature difference of 40°C (for a 40 – 60 - 80°C ramp) led to sharper peaks and less tailing seen in the 40°C isothermal run (Figure 55). The response time for the 1 mm I.D. column has increased dramatically relative to the temperature increase compared to the wide bore columns. In the earlier studies with wider bore columns, the slower response attributed to the greater phase volume had only achieved peak sharpening for late eluting components. The faster response in this case allowed fine tuning of the chromatography and a two-stage ramp was applied to achieve greater resolving power, thus increasing peak capacity.

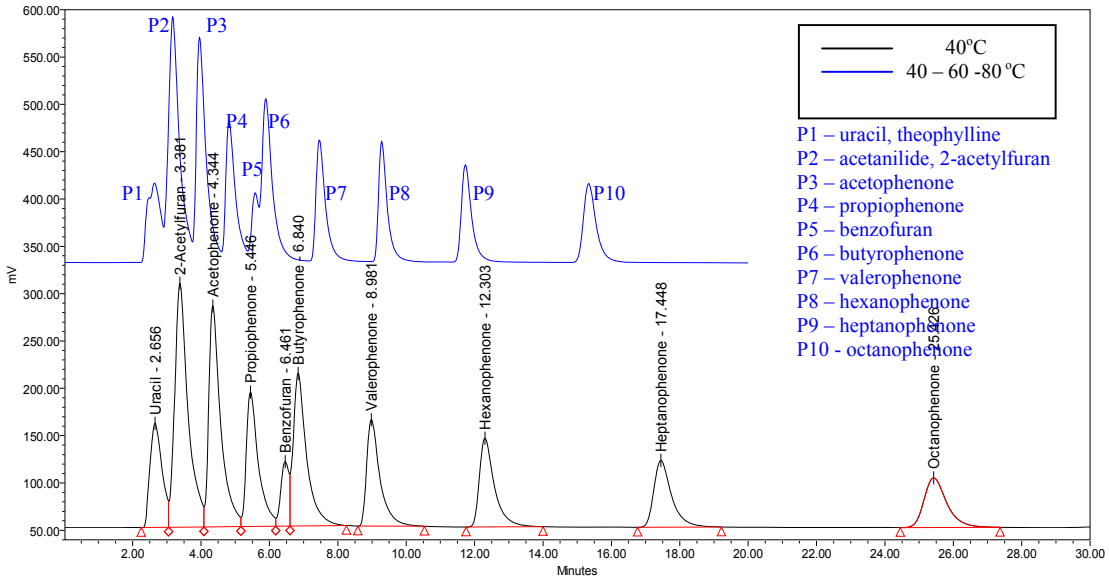


Figure 55: Temperature gradient of 40 – 60 (4.5°C/min) – 80 °C (9°C/min) overlaid with an isothermal 40 °C run for a complex mixture of phenones, acidic and basic functionality on a micro-bore Merck Purospher® STAR E18, 3.5 µm 1 x 150 mm column; 0.04 ml/min in 0.1 % TFA/60% acetonitrile/40% water

This is clear when a 40 °C run is compared to an isothermal 80 °C (Figure 56). Raising the temperature produced a shorter run time but the resolution suffered. A temperature gradient (Figure 54) behaved like a solvent gradient with potentially a much shorter response time and retained the resolution.

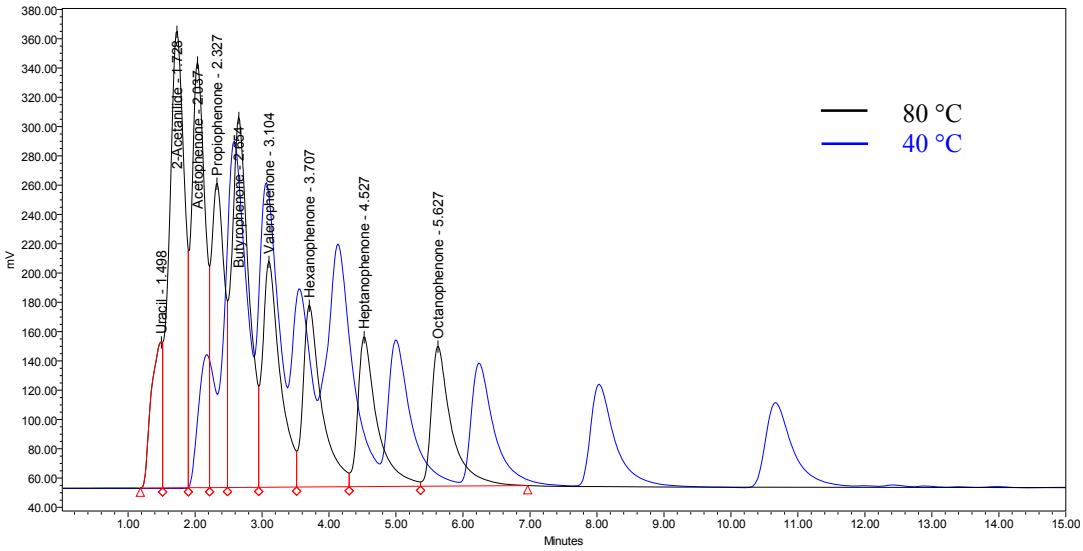


Figure 56: Isothermal Runs of 40 and 80 °C overlaid for a complex mixture of phenones, acidic and basic functionality on a micro-bore Merck Purospher® STAR E18, 3.5 µm 1 x 150 mm column; 0.04 ml/min in 0.1 % TFA/60% acetonitrile/40% water column

4.1.4 Data Comparison

The narrow-bore column, although not as impressive in terms of analysis time, demonstrated exceptional results with respect to peak shape improvement and sensitivity, an interesting property that was not reflected in the analysis time. Table 20 summarizes the achievements so far.

Table 20: Summary of Chromatographic Results for a complex mixture of phenones, acidic and basic functionality on a range of column geometry under temperature programmed conditions on a micro-bore Merck Purospher® STAR E18, 3.5 µm 1 x 150 mm column; 0.04 ml/min in 0.1 % TFA/60% acetonitrile/40% water column, a narrow-bore PLRP-S 3 µm, 2.1 x 150 mm column, 0.4 ml/min in 0.1 % TFA/70% acetonitrile/30% water, and on a conventional column XTerra MS C18 3.5 µm, 4.6 x 100 mm, 1 ml/min in 0.1 % TFA/60% acetonitrile/40% water.

		Octanophenone (%) increased (>) or decreased (<) relative to 40 °C runs		
Column Name and I.D.	**Temperature Gradient (°C)**	**Peak Height**	**W_h**	**Total Analysis Time**
Merck Purospher: 1mm	40 – 60 – 80	>37	<45	<40
PLRP-S: 2.1 mm	40 – 200	>78	<73	<21
XTerra MS C18: 4.6 mm	40 – 130	>47	<47	<41

The 1 mm I.D. column when compared to the other column internal diameters gave an impressive reduction in analysis time for a small increase in temperature across a gradient. To understand what was actually happening with the heat transfer process for each column, the pressure drop over the gradients was recorded and compared to isothermal runs as a guide to the actual temperature difference that was reached during the run time. The difference in the pressure readings recorded for each column run under isothermal separation conditions of 40 °C and 80 °C (Figure 57) were compared with the pressures recorded during a temperature gradient and the effect of each 1 °C were calculated (Table 21):

$$T_{80°C} - T_{40°C} = P_{40°C} - P_{80°C}$$

Table 21: Pressure Readings taken during Temperature Gradient Runs of 40 to 80 °C; 60% acetonitrile/40% water and at respective flow rates of 1 (XTerra MS C18 3.5 µm, 4.6 x 100 mm column), 0.4 (PLRP-S 3 µm, 2.1 x 150 mm column) and 0.04 ml/min (Merck Purospher® STAR E18, 3.5 µm 1 x 150 mm column)

Column I.D. (mm)	Initial Pressure (bar)	Final Pressure (bar)	$dP_{gradient}$ (bar) = 40°C	Calculated bar = 1°C
1	276	173	103	2.575
2.1	150	80	70	1.750
4.6	101	74	27	0.675

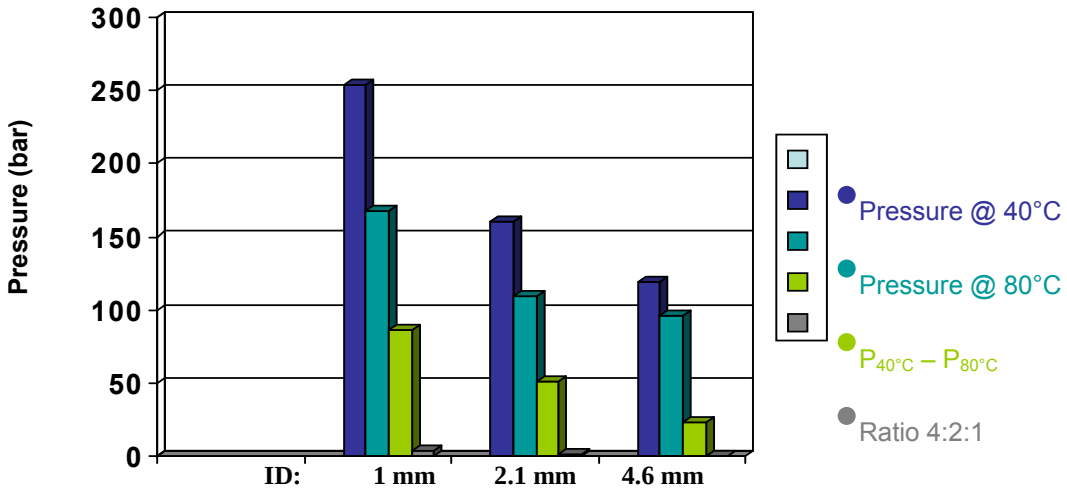


Figure 57: Bar Chart Representing Pressure Differences for Isothermal Runs of 40 and 80°C on Column Geometry of 1, 2.1 and 4.6 mm I.D. 60% acetonitrile/40% water and at respective flow rates of 1 (4.6 mm id), 0.4 (2.1 mm id) and 0.04 ml/min (1 mm id)

For an isothermal run it appeared that a difference in pressure drop going from 40 °C to 80 °C across the columns of 3:2:1 for a 1 mm, 2.1 mm and 4.6 mm id, respectively, was observed. This could be indicative of a more effective heat transfer process, which was 3 times more effective for a micro-bore column compared to the conventional 4.6 mm I.D. column. This is expected as the stationary phase surface area has been reduced and also as a consequence heat losses. This information was then used with the pressure differences seen over a temperature gradient run for each separation to calculate an

estimation of actual temperature reached during the ramps. Table 22 demonstrates the difference in heat transfer between column geometries. Actual temperatures reached showed that the micro-bore column is the only one to come close to its gradient target of 80°C. The narrow-bore column was the furthest away from its gradient target temperature of 200 °C followed by the conventional bore column (4.6 mm id).

Table 22: Estimation of **Actual** Temperatures Reached During Gradient Runs **(1)** 40 – 60 (4.5 °C/min) – 80 °C (9 °C/min) on a Merck Purospher® STAR E18, 3.5 µm 1 x 150 mm column; 0.04 ml/min in 0.1 % TFA/60% acetonitrile/40% water column **(2)** 50 - 200°C (18 °C/min) on a PLRP-S 3 µm, 2.1 x 150 mm column, 0.4 ml/min in 0.1 % TFA/70% acetonitrile/30% water **(3)** 40 – 130 °C (9 °C/min) XTerra MS C18 3.5 µm, 4.6 x 100 mm, 1 ml/min in 0.1 % TFA/60% acetonitrile/40% water

Gradient	**Column I.D. (mm)**	**Actual Pressure drop (bar)**	**Temperature calculated by P_{Actual} / $P_{1°C}$ (°C)**	**Column Temperature (°C) T_{calc} + $T_{initial}$**	**Column Ramp Rate (°C/min)[2]**
1	1	86	33	33 + 40 = **73**	2
2	2.1	51	29	29 + 50 = **79**	3
3	4.6	23.5	35	35 + 40 = **75**	5

The pressure profiles confirmed that for each separation a plateau was reached indicating that the target temperature has been reached, for example Figure 58 for the micro-bore column. However, the final value was dependent on the control sensor for the oven agreeing with inside column temperature and does not compensate for any lag. Hence, a shortfall of the temperature set-point is inevitable as the power input stops as a result of the oven sensor detecting that the target temperature has been reached. This makes elution on a gradient harder to estimate to improve overall peak shape. The stationary phase volume is critical to this balance in terms of transferring heat efficiently as shown by the 1 mm I.D. column. The phase volumes for a 1, 2.1 and 4.6 mm I.D. are 118, 520 and 1662 mm^3, respectively. Therefore, speculatively there appears to be another factor influencing this process with respect to the PLRP-S stationary phase that limits or resists

[2] Rate = Temperature rise (°C) / time took for pressure to stabilize

the thermal transfer of heat compared to the silica based columns. This corresponds to the unexpected small reduction in analysis time. However, its performance was strong for improving sensitivity and producing sharper bands indicating that a polymer column in a 1 mm I.D. format could yield surprising results for shallow temperature gradients. The chromatograms do tell us that this particular stationary phase is particularly well suited to high temperature work with further potential scope as an offset could be applied to compensate for the temperature lag.

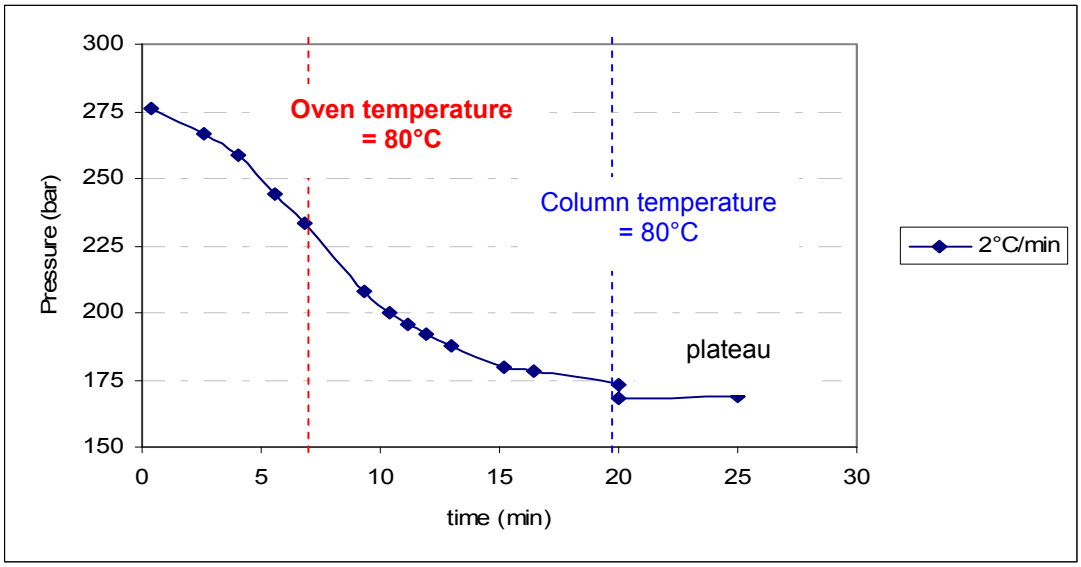


Figure 58: Graph Representing the Pressure Drop across a 1 mm I.D. column for a temperature program of 40 – 60 – 80°C

Another factor that can influence the rate at which the column is heated is the eluent flow rate, which could be transferring heat more effectively with a larger bore of column (and hence higher volume flow). This would have the necessary compensatory effect through pre-heating the eluent and via frictional heating.

Further experiments would be required to classify the temperature responses of columns with respect to the stationary phase materials etc. However, this could be difficult to carry out as other variables such as column hardware would also require standardizing to gain an accurate insight.

4.1.5 Equivalence to Solvent Gradients

There is demand in the pharmaceutical industry to have an alternative tool to optimise elution other than a solvent gradient. The hyphenation of solvent gradients to a number of various analytical detectors can cause inherent difficulties and is inapplicable in some instances, i.e. refractive index and other bulk property detectors. A temperature programme is at least an additional optimization tool and at the most a direct alternative.

4.1.5.1 Chromatographic Parameters

A study was therefore carried out to directly compare a gradient elution and temperature programme using conditions (Table 23) selected to give a similar outcome.

Table 23: Chromatographic parameters of temperature programs compared to solvent gradient elution

System:	Agilent HP1100 LC system with the CSI oven	
Column:	PRLP-S 3 µm 2.1 x 150 mm	XBridge C8 2 µm 4.6 x 30 mm
Mobile Phase: **Eluent A:** **Eluent B:**	 0.05 % TFA in water 0.05 % TFA in acetonitrile	 water acetonitrile
% B:	70 %	20 %
Gradient:	70 to 100 % B in 8.0 min	0 to 96 % B in 4.0 min
Temperature program:	50 to 200°C (30% power; 18°C/min)	40 – 200°C (40 °C/min)
Injection volume:	1 µl	10 µl
Flow rate:	0.4 ml/min	0.8 ml/min
Wavelength:	254 nm	254 nm

A starting condition of 70 % acetonitrile was set (Figure 59) for the solvent gradient and temperature program on the PS-DVB column. This demonstrated that although the temperature gradient was operating at an actual rate of 3°C/min compared to 4 %/min the temperature gradient had improved peak shape with a shorter analysis time.

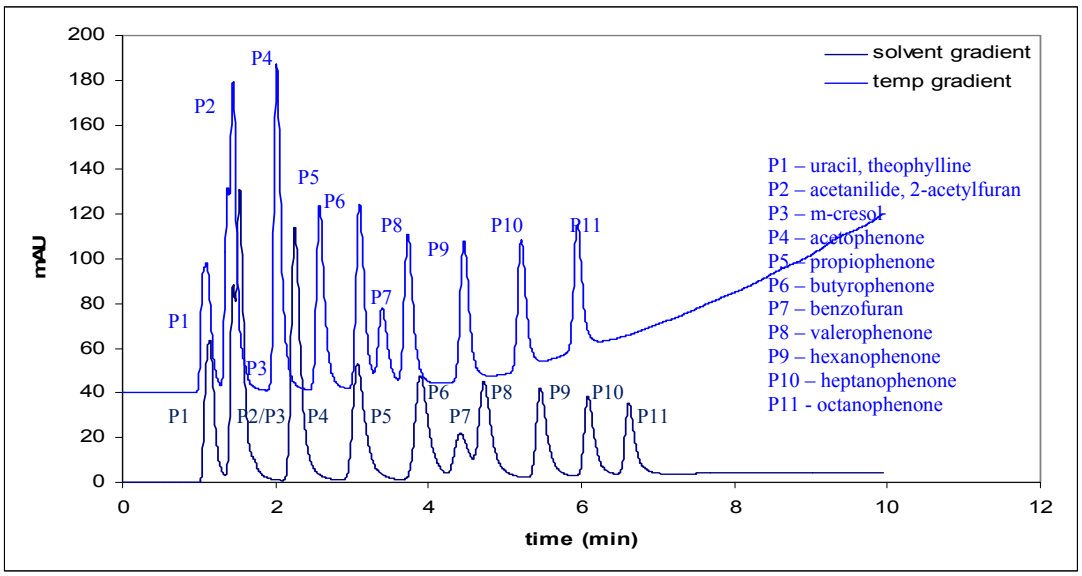


Figure 59: Temperature gradient (50 to 200°C, 18°C/min at 70% B) overlaid with a solvent gradient (70 to 100 %B in 8.0 min at 40 °C) for a complex mixture of phenones including acidic and basic functionalities on a narrow-bore PRLP-S 3 µm 2.1 x 150 mm column at 0.4 ml/min

If the peak capacity is to be improved the processes surrounding the attainment of good chromatography have to be tightened. A solvent gradient takes 2 – 4 minutes to take effect because of the dwell volume and internal column volume by which point peak capacity has already been limited, whereas the temperature could have an immediate effect on the separation.

Figure 60 shows the potential gain in peak capacity (Cp) if a temperature program is employed for a quick response, compared to a solvent gradient. The solvent gradient using an XBridge column was set to deliver a gradient of 0 to 100 % acetonitrile in 4 minutes and demonstrated a significant delay of approximately 2 minutes. At this point the gradient should be at 50 % organic and components should have started to elute under the same scheme as the temperature program at 0.8 minutes (20 %B). The temperature-programmed run indicates the potential for an increase in peak capacity due to the instant response to the temperature gradient. This enables a higher degree of separation for early

eluting components such as theophylline and 2-acetylfuran and denotes a selectivity change between this critical pair.

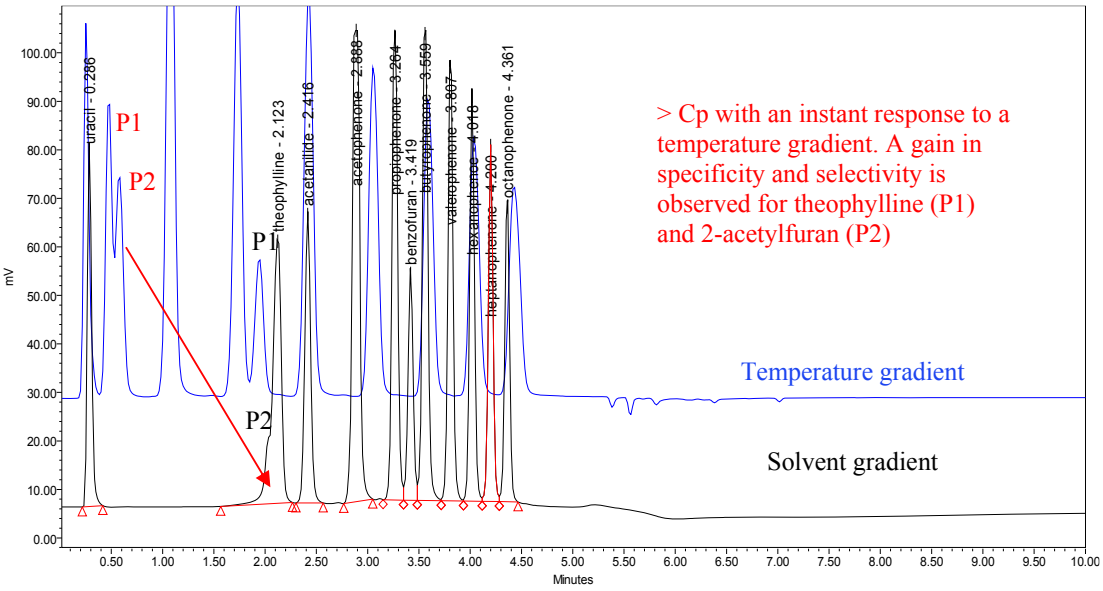


Figure 60: Temperature gradient (40 – 200°C, 40 °C/min at 20% B) overlaid with a solvent gradient (0 to 96 %B in 4.0 min at 40 °C) for a complex mixture of phenones, acidic and basic functionality on an XBridge^{TM} C8, 2 µm 4.6 x 30 mm at 0.8 ml/min

4.2 Analyte Focusing

4.2.1 Isothermal Separations by Pre-heating the eluent only with Resistive heating technology

It was demonstrated in the previous chapter that external heating of a column was more sustainable when the column length and volume were reduced. Peak shape was improved generally for these initial experiments with the biggest improvement seen towards the end of a run. However, a main focus of the project is to achieve analyte focusing at the beginning of the separation. Theoretically this could be achieved by performing a hot injection producing a sharp band at the beginning of the column. The practical implication is the need to heat the column rapidly at this point of the separation to maintain the sharp bands created at the focusing point. Pre-heating the eluent could provide the practical means in which to do so by providing a hot injection slug in the first few centimetres of the column delivering heat directly in to the stationary phase. As heat is then lost to the surroundings (more prevalent in a longer column i.e 150 mm) the sharp band will spread out as the parabolic flow profile inverts. This lead to a series of studies in which the column was not heated directly but only heated by the heat carried into the system by the eluent, which was heated in pre-heater. The column was lagged to reduce heat loss.

4.2.1.1 Experimental Conditions

System: Miniaturised HPLC system with the CSI pre-heater: Shimadzu LC-10AD pump, Rheodyne injection port model 7410 (0.5µl injection loop), CSI oven and CO_2 cooling with high-pressure switching valve, Connecting tubing 0.007" I.D. stainless steel, SA6500 detector (Severn Analytical, Cheshire) with 1.2 µl flow cell volume and 3 mm flow path, Prime data Logger and Software for data capture (set at 16Hz acquisition rate.)

Column:	XTerra phenyl 3.5 µm, 4.6 x 150 mm
Mobile Phase:	Acetonitrile / water
Organic range:	40 % and 30 %
Temperature range:	40 to 150°C
Injection volume:	5µl
Flow rate:	1 - 3 ml/min
Wavelength:	254 nm
Equilibrium time:	15 minutes allowed between temperature set-points unless cooling

An overall preheated eluent range of 40°C to 150°C was studied on an XTerra phenyl column. Examples at 40°C and 70°C of isothermal chromatograms were overlaid in Figure 61. This demonstrated a good peak profile with column heating via the eluent only at 40 °C, which was further improved when the column temperature was raised in conjunction with the flow rate. Initial results show that column heating via eluent heating only is an effective stand-alone technique for improving chromatography.

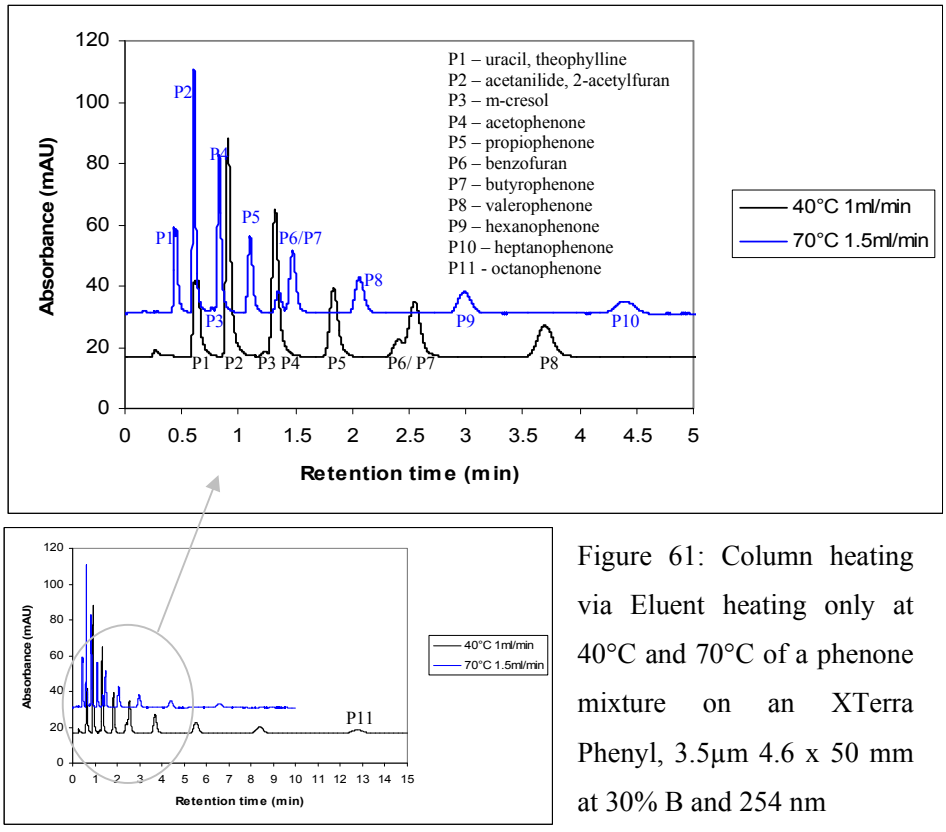


Figure 61: Column heating via Eluent heating only at 40°C and 70°C of a phenone mixture on an XTerra Phenyl, 3.5µm 4.6 x 50 mm at 30% B and 254 nm

4.2.1.2 Negative Temperature Gradient

Using a high flow rate when a column is being heated in an oven to high temperatures does not reduce the efficiency because the solute transfer is greatly increased between the mobile and stationary phases. However, if the eluent is being heated but not the column theoretically a negative temperature gradient must be traversing along the column. This should impinge on the flow profile as now the eluent travelling through the centre of the column is hotter than the walls initially and then starts to invert as heat is lost to the surroundings. The increased viscous heating on increasing the flow rate must be a contributory factor that compensates in some way to possible heat losses and therefore producing a flatter peak profile as the analyte exits the column.

The m-cresol peak is circled as this component sitting on the front of a peak does not demonstrate adequate resolution at 40 °C for a flow of 1 ml/min. However, at 70 °C and 1.5 ml/min the resolution is clearly improving (Figure 62). The desired focusing for early eluting components by hot injection is now taking effect by this technique of delivering heat to a column.

To show that the efficiency has been enhanced by increasing the flow rate at high temperature, a 70 °C run was performed for a flow rate range of 1 – 2 ml/min. This demonstrated that a new minimum was found on the van Deemter curve and the reduced plate value decreased with respect to increasing mobile phase velocity.

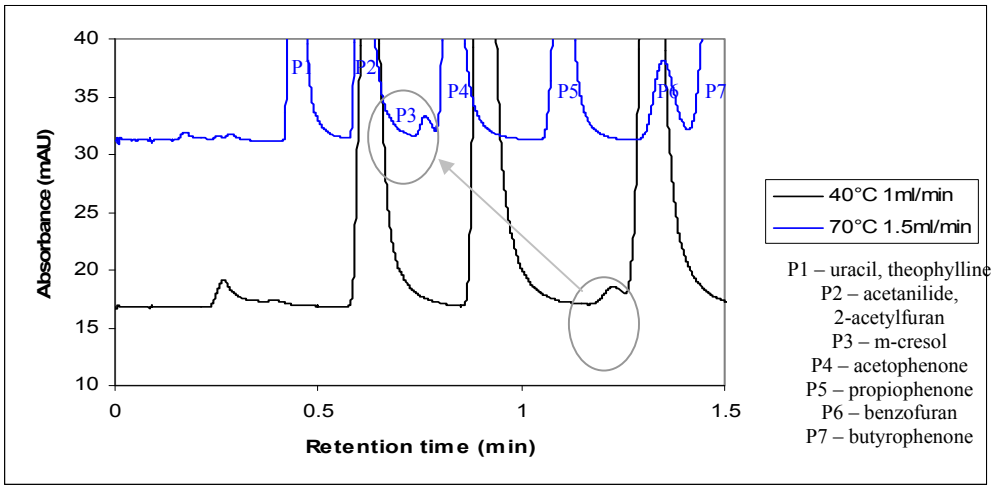


Figure 62: Analyte focusing effects for Column heating via Eluent heating only at 40 °C and 70 °C of a phenone mixture on an XTerra Phenyl, 3.5µm 4.6 x 50 mm at 30% B and 254 nm

4.2.1.3 Hot injection vs Cold injection

An injection was performed at a room temperature of 26°C, where the eluent was colder than the column at 31 °C, as frictional heating gave an additive effect. This was compared to hot incoming eluent heated prior to the column at 80°C, both at a flow rate of 1 ml/min. In the first instance it is assumed that the eluent is colder than the column which will incur some frictional heating. In the second instance the column although equilibrated with the incoming eluent for 15 minutes at 80 °C will suffer substantial heat losses to its surroundings that cannot be entirely compensated for by frictional heating at that point of the column length. A critical pair found to be co-eluting at approximately 2.7 minutes (for the cold injection) was benzofuran (P1) and butyrophenone (P2). These two components were separated by the hot injection with a resolution factor of 1.2 (Figure 63).

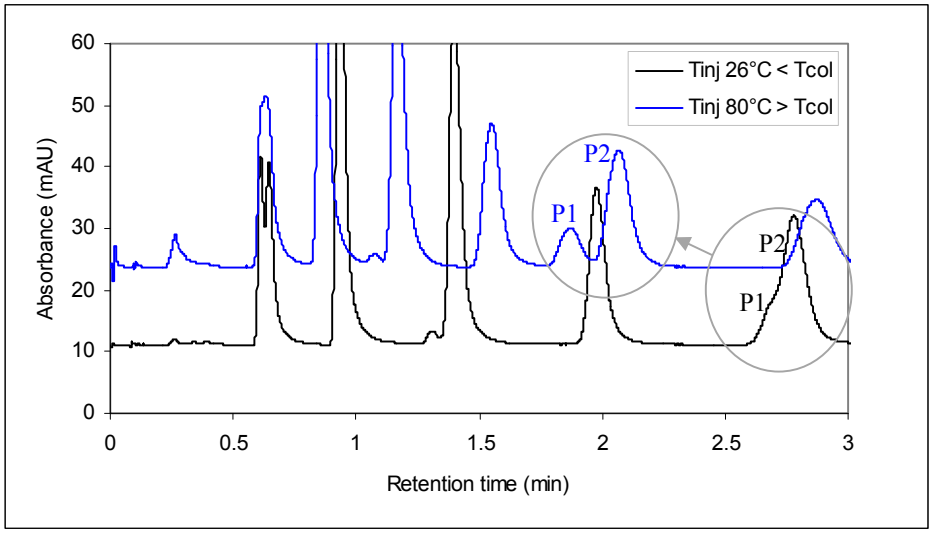


Figure 63: Hot eluent at 80°C via eluent heating only vs a cold eluent at 26°C for column heating (at ambient temperature of 31 °C) onto a heated column at 80 °C using an XTerra Phenyl, 3.5µm 4.6 x 50 mm with acetonitrile / water 40:60 % v/v at 254 nm

Despite the same flow rate early eluting peaks were sharper and better resolved at higher eluent temperature (relative to the dead volume).

4.2.1.4 Van Deemter effect due to Mobile Phase heating and Elevated Flow Rate

When the column is heated to a higher temperature the Van Deemter curve reaches a new optimum at a new optimum flow velocity. This is due to the enhanced mass transfer between mobile and stationary phase giving improved efficiency at an elevated flow rate. However, it is uncertain how the heating of the column via the mobile phase (only) would affect a separation. It was found over the range of 40°C to 150°C runs that the optimum flow velocity did increase with the higher temperature. The data tabulated for isothermal runs of 150°C for the lowest and highest flow velocity recorded in Table 24 and 25 showed that peak efficiency had improved by approximately 50% for all compounds on increasing the flow rate from 1 ml/min to 3 ml/min. This indicated that the ***'negative temperature'*** gradient effect induced across the column by only heating the eluent is compensated for by viscous heating. However, at high flow rates the time available for

longitudinal diffusion is limited reducing the B term even though at high temperature diffusion is increased but this reduces the C term. Therefore, this could be the effect between the B and C term causing a shift to a new optimum flow rate. The peaks are smaller at higher velocity due to dilution effects, which also means higher loading capacity is now possible.

Table 24: Separation of phenone mixture on an XTerra phenyl, 3.5µm 4.6 x 50 mm by eluent heating only at 150°C for 1 ml/min

Peak	RT	Area	Height	Width @ 0.50	Asym @ 0.05	NTP	HETP	Rs
Uracil	0.60	12308334	221553	3.1	NA	773	0.065	NA
2-acetylfuran	0.77	24800714	444679	2.7	NA	1570	0.032	2.0
Acetanilide	0.95	21532130	315719	3.6	NA	1383	0.036	2.0
Acetophenone	1.15	12069587	160053	4.6	NA	1235	0.04	1.7
Propiophenone	1.23	5123726	60476	NA	NA	NA	NA	NA
Benzofuran	1.4	13993834	136067	5.8	NA	1159	0.043	NA
Butyrophenone	1.76	10114162	81727	7.4	1.18	1127	0.044	1.9
Hexanophenone	2.29	8866804	54041	10	1.09	1048	0.048	2.1
Heptanophenone	3.03	7281809	32966	13.6	1.06	987	0.051	2.2
Octanophenone	4.08	6414736	20840	19.3	1.04	891	0.056	2.3

Table 25: Separation of phenone mixture on an XTerra phenyl, 3.5µm 4.6 x 50 mm by eluent heating only at 150°C for 3 ml/min

Peak	RT	Area	Height	Width @0.50	Asym @0.05	NTP	HETP	Rs
Uracil	0.28	4049242	195967	1.2	1.5862	1169	0.043	NA
Acetanilide	0.34	7877489	430927	1	1.5725	2426	0.021	2.1
m-cresol	0.39	183733	18540	NA	NA	NA	NA	NA
Acetophenone	0.42	6735935	306672	1.2	1.313	2339	0.021	NA
Propiophenone	0.5	4070580	159503	1.5	NA	2178	0.023	2.2
Benzofuran	0.55	1164202	46383	1.7	NA	2233	0.022	1.1
Butyrophenone	0.61	3973822	130059	1.8	1.0794	2248	0.022	1.2
Valerophenone	0.77	3473149	87348	2.4	1.2086	2145	0.023	2.7
Hexanophenone	1.02	2971202	57903	3.2	1.1248	2039	0.025	3.1
Heptanophenone	1.38	2133378	33357	4.1	1.0825	2214	0.023	3.5
Octanophenone	1.91	2073130	22130	5.7	0.999	2254	0.022	3.8

The van Deemter relationship is a hyperbolic function, which predicts that there is an optimum velocity at which there will be a minimum variance per unit column length and hence a maximum efficiency. The runs performed at 150°C for a velocity range of 1 – 3

ml/min did not reach a plateau HETP value. Therefore it was possible that the van Deemter curve had not reached an optimum in terms of peak efficiency with respect to flow rate at 150°C. The chromatogram (Figure 64) showed that the peaks were splitting when initially run at a high temperature and low flow rate. This is typical of thermal mismatch between the incoming eluent temperature and the column temperature.

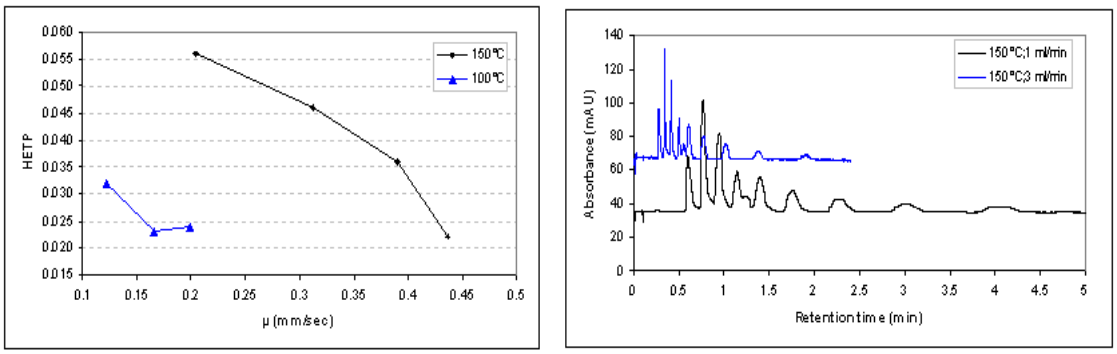


Figure 64: van Deemter plot and chromatogram demonstrating how separation efficiency of octanophenone improved for column heating via eluent heating only on an XTerra Phenyl, 3.5µm 4.6 x 50 mm at 150°C for a flow rate of 1 ml/min and 3 ml/min.

4.2.1.5 Selectivity Effects through Mobile Phase Heating

Selectivity could also be manipulated by altering only the temperature of the eluent to heat the column with the oven turned off. Figure 65 shows the separation of the phenone complex mixture at 1 ml/min.

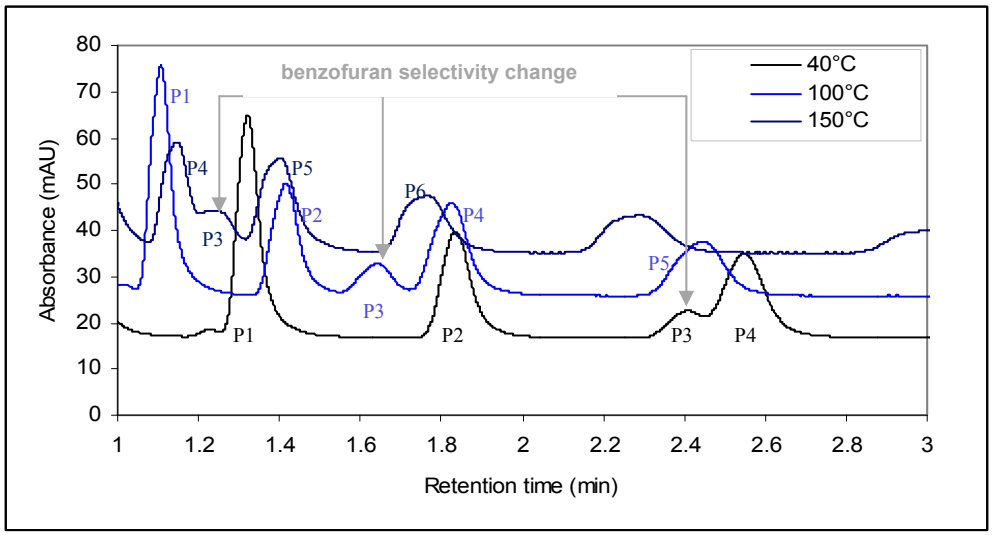


Figure 65: Selectivity changes for benzofuran at varying eluent temperature for 1 ml/min on an XTerra phenyl, 3.5µm 4.6 x 50 mm at 40, 100 and 150 °C with acetonitrile / water 40:60 % v/v at 254 nm

Benzofuran (P3) has undergone a selectivity change (change in relative retention order) denoted firstly by its co-elution with butyrophenone (P4) at 40 °C followed a reversal in retention order at 150 °C. This demonstrated selectivity change was another important aspect of high temperature work achieved by delivering enough heat to the heart of a separation in the first few centimetres of the column by manipulating the column inlet temperature only.

4.2.1.6 Higher aqueous conditions coupled with eluent heating

To resolve all of the components at 150 °C via eluent heating only (for a 2 ml/min flow rate) the organic content of the mobile phase was reduced to 30 % acetonitrile and compared with the run at 40 % acetonitrile (Figure 66).

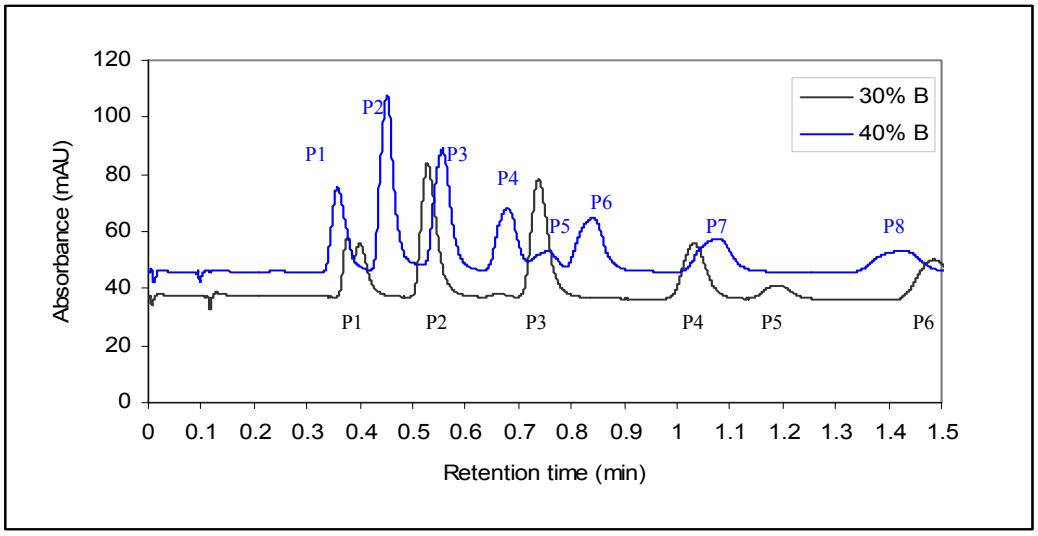


Figure 66: Expansion of chromatograms of 30 % and 40 % acetonitrile at 150°C eluent temperature for a flow rate of 2 ml/min on an XTerra phenyl, 3.5 µm 4.6 x 50 mm column at 254 nm

The method of injecting hot eluent showed improvements in efficiency of up to 40 % (Tables 26 and 27) from 0.6 – 2.3 minutes (m-cresol to valerophenone). Additional resolving power was gained and the speed of analysis reduced but late eluting components ideally required a heating method to sharpen their peak shape.

Table 26: Chromatographic data for a run at 40 % acetonitrile at 150°C eluent temperature for a flow rate of 2 ml/min on an XTerra phenyl, 3.5 µm 4.6 x 50 mm column

Peak	RT	Area	Height	Width @0.50	Asym @0.05	NTP	HETP	Rs
Uracil	0.36	6079402	199780	1.7	1.6	847	0.059	2.6
Acetanilide	0.45	12363913	415838	1.6	1.6	1575	0.032	2.0
Acetophenone	0.56	10364319	289795	2.0	NA	1494	0.033	2.1
Propiophenone	0.68	6144448	149134	2.5	NA	1523	0.033	1.9
Benzofuran	0.76	2355769	47493	NA	NA	NA	NA	NA
butyrophenone	0.84	6898739	127491	3.2	NA	1376	0.036	NA
Valerophenone	1.08	5274460	77266	4.1	1.0	1352	0.037	2.3
Hexanophenone	1.43	4565146	49997	5.6	1.0	1280	0.039	2.5
Heptanophenone	1.94	3924297	30197	8.0	0.9	1164	0.043	2.6
Octanophenone	2.67	3075502	16817	11.4	0.9	1097	0.046	2.4

Table 27: Chromatographic data for a run at 30 % acetonitrile at 150°C eluent temperature for a flow rate of 2 ml/min on an XTerra phenyl, 3.5 µm 4.6 x 50 mm column

Peak	RT	Area	Height	Width @0.50	Asym @0.05	NTP	HETP	Rs
Uracil	0.38	2430714	139665	NA	NA	NA	NA	NA
Theophylline	0.40	3290465	125227	NA	NA	NA	NA	NA
Acetanilide	0.53	10505989	312244	1.9	1.5	1499	0.033	NA
m-Cresol	0.66	241233	8420.8	1.8	1.1	2786	0.018	2.6
Acetophenone	0.74	10013170	277429	2.0	1.3	2589	0.019	1.4
Propiophenone	1.03	6274559	130447	2.8	1.2	2626	0.019	4.2
Benzofuran	1.19	1822843	32494	3.3	1.0	2598	0.019	1.8
Butyrophenone	1.48	6608509	95028	4.1	1.1	2567	0.019	2.8
Valerophenone	2.27	5476115	48152	6.7	1.1	2262	0.022	5.1
Hexanophenone	3.65	4640750	23453	12.2	0.9	1791	0.028	5.2
Heptanophenone	6.00	3827190	10643	22.4	0.8	1427	0.035	4.8
Octanophenone	10.0	3366653	5280	37.7	0.9	1399	0.036	4.7

A lower percentage of organic solvent coupled with elevated flow rate and with hot injections are all contributing to an improvement in separation quality and the goal of sharper peaks for faster LC separations. The next section looks at modifying the pre-heater to address the sharpening of late eluting bands.

4.2.1.7 Post pre-heater modification

The overall focus of the project was to obtain a sufficiently good response from a temperature gradient applied to a column to enable it to replace a solvent gradient. It was critical that the improvement in separation quality due to only heating the eluent was understood in the context of a temperature-programmed run. Therefore, it was also desirable to perform a temperature-programmed separation by only programme heating the eluent. The first practical problem was that the pre-column heater was not designed to perform a gradient program therefore two methods were tested for applicability. A 'step-wise gradient' involving a manually entered program of raising the temperature manually at set intervals i.e. 10°C/min was the first to be considered. However, the response was poor due to insufficient heat transfer in a short space of time, for each step

the heater equilibrated as the temperature reached the new level reducing the energy being delivered to the system.

The second method was a 'dynamic or ballistic gradient' where the column was equilibrated at initial temperature gradient conditions of 40 °C and the heater reset to an upper temperature of 200 °C immediately after injection so that the temperature rose rapidly without any feedback control. This method of heating the eluent at the column inlet gave a slow response relative to analyte retention and gave broad splitting peak shapes for components eluting after 5 minutes (Figure 67). The pre-heater was therefore modified (refer to section 3.2.3) to enable more heat to be delivered via the eluent to the column. This gave an improved peak shape (Figure 68) and the peaks were no longer splitting; however, there was insufficient improvement in chromatography to justify continuing this approach. The problem appeared to be that it was not possible to introduce sufficient energy into the column via the mobile phase with the current pre-column heater.

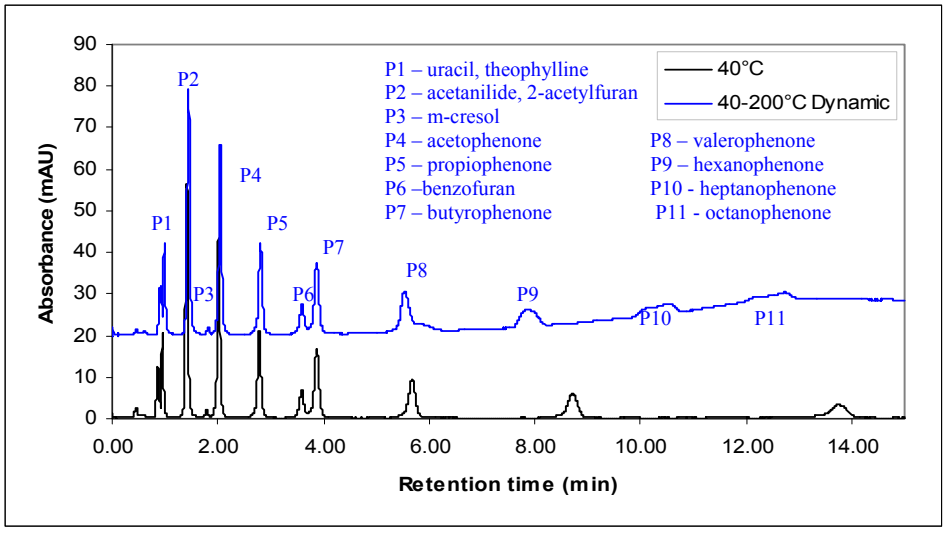


Figure 67: Dynamic gradient applied to the eluent by dynamic resistive heating from 40 – 200°C overlaid with a 40 °C run on an XTerra phenyl 3.5µm, 4.6 x 50 mm at 1ml/min with the resistive heated pre-heater unit

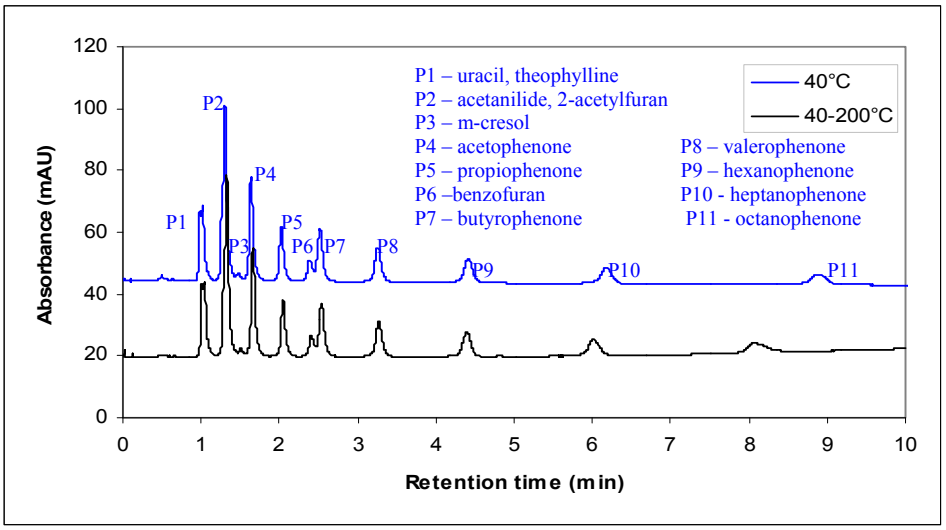


Figure 68: Dynamic gradient applied to the eluent by resistive heating from 40 – 200°C overlaid with a 40 °C run on an XTerra phenyl 3.5µm, 4.6 x 50 mm at 1ml/min with the modified resistive heated pre-heater unit

4.2.1.7.1 Summary

Both isothermal and temperature-programmed runs in this instance have yielded improvements whereas originally thermal mismatch was expected. The reduction in column length hence in plates has controversially provided greater resolving power by focusing analytes as a sharp band onto the column inlet by hot injection. This has led to better peak shape when combined with elevated flow rate. The peak shape of late eluting components was improved by column heating only, where early eluting components have been found to improve by eluent pre-column heating, only.

Producing a temperature gradient by ramping the eluent temperature prior to the column inlet without column heating demonstrated that by delivering heat more efficiently to the stationary phase (as the pre-heater modification did) the chromatography would improve. Therefore, the next stage was to apply eluent ramping by utilising the resistive heated oven. It has been demonstrated that heat can be delivered to a column in this way but the

next question was what conditions are disadvantageous when applying column heating inside an oven?

4.3 Pre-heating Effects with CSI Oven by Encompassing Connector Tubing Inside / Outside of the Oven (CSI Pre-heater absent)

High-temperature ultrafast liquid chromatography (HTUFC) has been achieved with short columns (4.6 x 50 mm) of 3 μm polystyrene-coated zirconia particles by Yan *et al.*[250] (section 1.4.7.3). They separated long chain alkylphenones in less than a minute on a purpose built system with runs up to 150 °C and 15 ml/min at 20% acetonitrile concentration. All of the peaks were resolved and analysis times decreased by a factor of 50 compared to room temperature (25 °C) at 4 ml/min. Their method of heating the column was to place it and heat exchangers into a silicone oil bath. This is a preferred method of heating compared to an air-assisted oven, as heat transfer is more efficient (section 1.4). This is of great importance when operating at high flow rates as experiments have already shown the dependence of heat transfer on this variable with respect to pre-heating the eluent. The CSI heating system gave better peak shapes at elevated flow rate when only the eluent was heated and the column insulated. The higher the temperature the higher the flow velocity required to give optimum separation conditions on a Van Deemter curve. At elevated temperature there is still scope for improvement by performing a separation under temperature programmed conditions. High temperature speeds up a separation, reducing analysis times but this is usually at the expense of resolution. A temperature program will allow control similar to that found with a solvent gradient with some practical implications. However, the CSI oven design is unlikely to provide the fast response required to program a conventional 4.6 mm I.D. column. Any gain in separation quality, as a result of a temperature gradient, may be sacrificed by parameters, such as column length and the difficulty in transferring heat effectively.

4.3.1 Rationale for the use of short columns

A conventional short column (due to the reduction in thermal mass) is easier to heat effectively. The reduced viscosity of the mobile phase at high temperature allows smaller particle sizes to be used, which are now available with the advent of UPLC. Although, high temperature separations are indeed fast, the resolution power dwindles and diminishes the higher the temperature increment. However, temperature gradients utilize dynamic elution, which enhances peak shape, and complex mixtures and their impurities may be resolved by eluting the components on a gradient. This is because of the focusing/re-focusing effect of the peaks as they traverse the column creating much sharper bands than those achieved isothermally. An inevitable problem is the temperature lag between the desired set-point and the actual rate at which the column can be ramped. In part it is hoped that this can be circumvented by a **short** XTerra column with a wide bore, which is not only compatible with heating more efficiently (as demonstrated in section 4.4.1) but also with the pharmaceutical companies, who are yet to embrace micro-bore technology due to instrumental constraints. Most of the separation occurs at the front of the column and therefore any initial gain in rapid heating is then lost if the peaks then travel large distances along the column. This was also important when ramping up the eluent to follow the temperature gradient as the eluent pre-heating was found to be crucial in achieving the right profile.

4.3.2 Chromatographic Conditions

Miniaturised System: Shimadzu LC-10AD pump, Rheodyne injection port model 7410 (0.5 µl injection loop), CSI oven **(only)** , SA6500 detector (Severn Analytical, Cheshire) with 1.2µl flow cell volume and 3 mm flow path, Prime data Logger and Software for data capture (set at 16Hz acq. rate

Column: XTerra Phenyl 3.5 µm, 4.6 x 50 mm

Isocratic Mobile Phase: Acetonitrile/water (40:60 % v/v)

Flow rate: 1 ml/min (unless otherwise stated)

Isothermal temperature: as stated on chromatogram

Temperature Ramp: 40 – 200°C (100%P), held at 200°C till end of run, wait time 3secs

Phenone Test Mixture: uracil (P1), theophylline (P2), 2–acetylfuran (P3), acetanilide (P4), m-cresol (P5), acetophenone (P6), propiophenone (P7), benzofuran (P8), butyrophenone (P9), valerophenone (P10), hexanophenone (P11), heptanophenone (P12) & octanophenone (P13).

4.3.3 Evidence of Thermal Mismatch when Eluent Temperature was not Equal to Column Temperature

The column was placed inside at the entrance of the oven and the tubing remained outside the heater. Initially a run was performed with no thermostat at ambient (23°C), this was then repeated with the oven set to 40°C and then 60°C to create the following conditions:

$T_{inj} = T_{oven}$: $T_{inj23°C}$: $T_{oven23°C}$ (ambient, column lagged inside the oven),
$T_{inj} < T_{oven}$: $T_{inj23°C}$: $T_{oven25°C}$ (oven set-point 40°C)
$T_{inj} << T_{oven}$: $T_{inj23°C}$: $T_{oven46°C}$ (oven set-point 60°C)

When the column conditions were set to a slightly lower injection temperature of 23 °C for a column temperature of 25 °C retention times and peak widths at half height were reduced compared to an oven temperature of 25 °C. This makes a significant difference in the resolving power as now peak 8 can be distinguished from peak 9 (Figure 69) although it should be noted that a selectivity change cannot be ruled out. If a large difference is programmed between the eluent (at 23 °C) and oven temperature (at 46 °C) peak shapes suffer where a probable cause may be thermal mismatch. Wolcott *et al.*[251] section 1.3.3) found that a 10 °C drop in eluent temperature compared to that of the column gave the best efficiency due a compensation effect. This effectively inversed the radial temperature gradient due partially to frictional heating inside the column and overall gave a flatter flow profile, thus reducing band broadening.

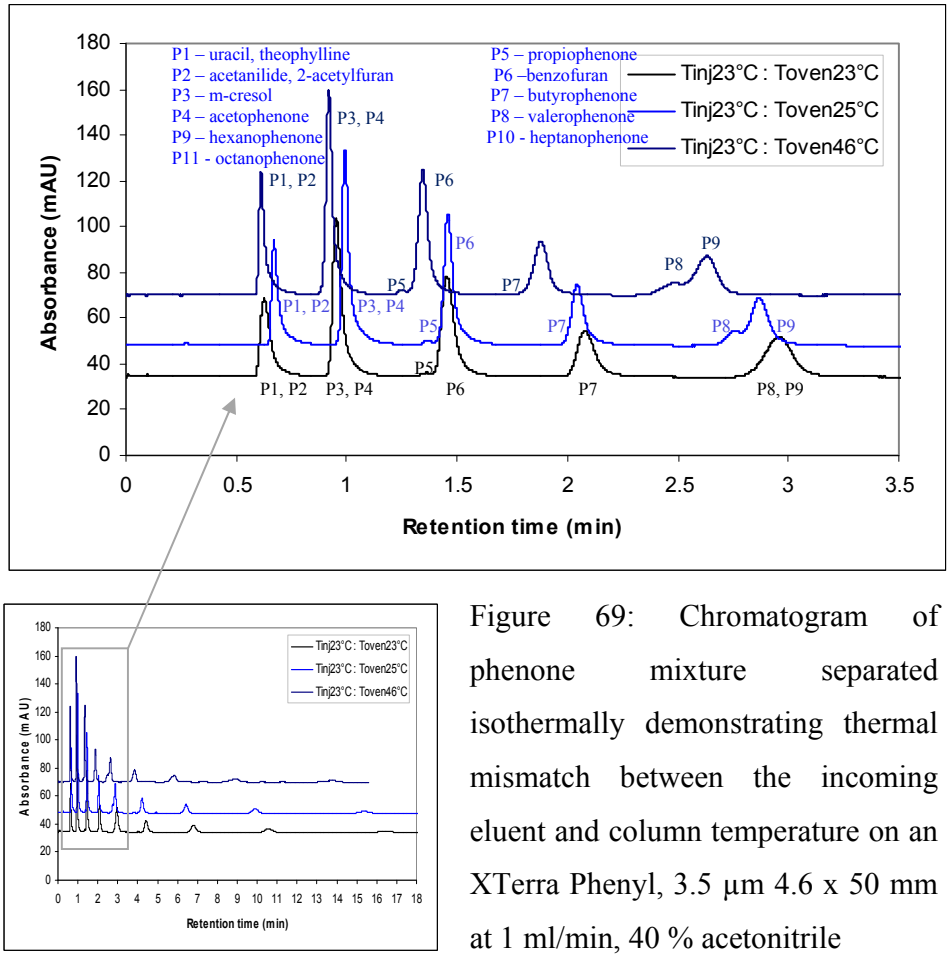


Figure 69: Chromatogram of phenone mixture separated isothermally demonstrating thermal mismatch between the incoming eluent and column temperature on an XTerra Phenyl, 3.5 µm 4.6 x 50 mm at 1 ml/min, 40 % acetonitrile

4.3.4 Position of pre-heating tubing

In order to examine the instrumental setup for preheating and determine the effect on the separation a number of different configurations were examined for the pre-heater tubing.

4.3.4.1 Isothermal Runs

This set-up was to look at the effects of pre-heating the eluent with tubing between the injector and the oven. The tubing was placed outside (1) and inside (2) the oven as depicted in Figure 70. The separation of the phenone mixture was performed at an oven temperature of 40°C to look at the effect on the chromatography. No separately powered pre-heater device was used.

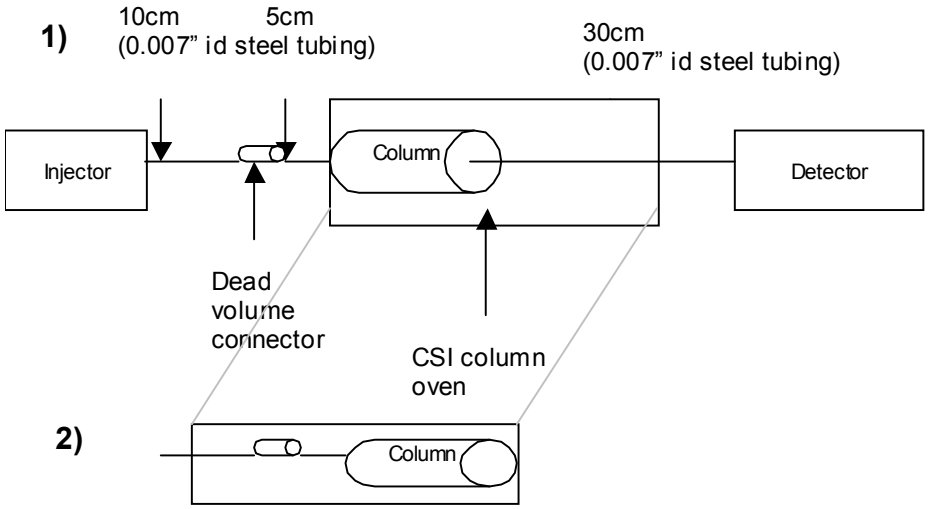


Figure 70: Schematic representation of the heating set-up: preheating tubing placed outside (1) and inside (2) the oven

The chromatography in Figure 71 demonstrated that when the tubing was placed inside the oven with the column compared to no heating where the tubing was outside, an improvement in resolution was observed for both m-cresol and benzofuran. A faster analysis time has resulted from an overall temperature increase.

The experiment outlined in section 1.3.3 suggested that injecting slightly colder gave a significant improvement in separation efficiency by inverting the flow profile along the column caused by frictional heating. In this instance this would also be the case where the column in slightly hotter than the incoming eluent.

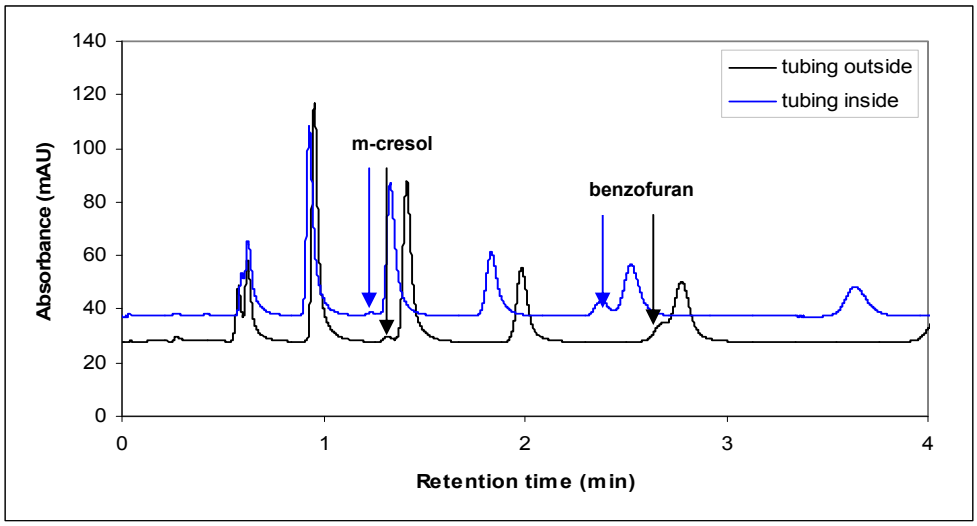


Figure 71: Chromatogram of Phenone Mixture Separated Isothermally at 40°C with and without oven eluent pre-heating via stainless steel tubing (0.07" id) on the XTerra phenyl 3.5 µm, 4.6 x 50 mm column at 40 % acetonitrile.

This result gives confidence for the argument that the eluent temperature should be several degrees lower than the column in order to compensate for other sources of heating within the stationary phase, such as friction. The second possibility is that we could be simply just seeing a selectivity difference due to a small temperature difference not a resolution improvement. This would explain the efficiency which improved in the last experiment.

4.3.5 Temperature Ramping of the Column only

The following experiment was performed to assess the effects of heating the column on a temperature ramp with the tubing outside the oven whilst running a temperature gradient (section 4.5.4.1). This was expected to yield poor results given the results of isothermal runs. The peak shapes deteriorated rapidly with retention time (Figure 72) and is typical of a thermal mismatch. Broad split peaks were evident similar to those found by Carr *et al.*[252] (section 1.3.3), where they demonstrated that a cool eluent produces a radial

gradient in retention factor and viscosity, thereby broadening the band and destroying peak shape.

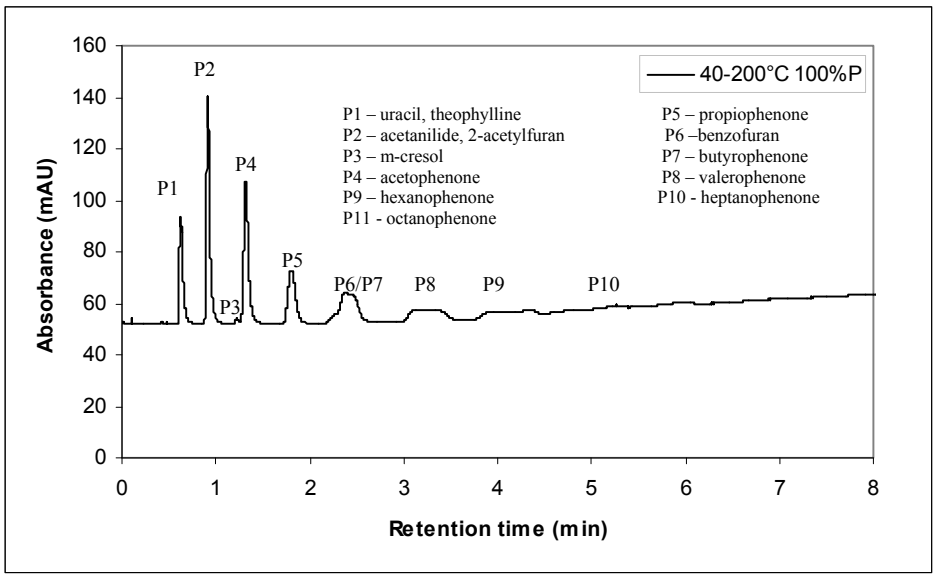


Figure 72: Chromatogram of phenone mixture separated on a temperature gradient of 40 – 200 °C at 40 °C/min with no eluent pre-heating on the XTerra phenyl 3.5 µm, 4.6 x 50 mm column at 40 % acetonitrile.

4.3.5.1 Temperature Programmed Runs

In contrast the following experiment was to assess the effects of pre-heating the mobile phase in a gradient by placing the preheating tubing within the oven whilst running a temperature gradient. Two temperature gradients of 40 – 150°C and 40 – 200°C were examined (Figure 73). A significant sharpening of the later eluting peaks was evident for the gradient runs compared to a 40 °C run, where the tubing is placed outside the oven (Figure 71). Heptanophenone (P11), hexanophenone (P12) and octanophenone (P13) showed a significant reduction in peak width and an increase in sensitivity.

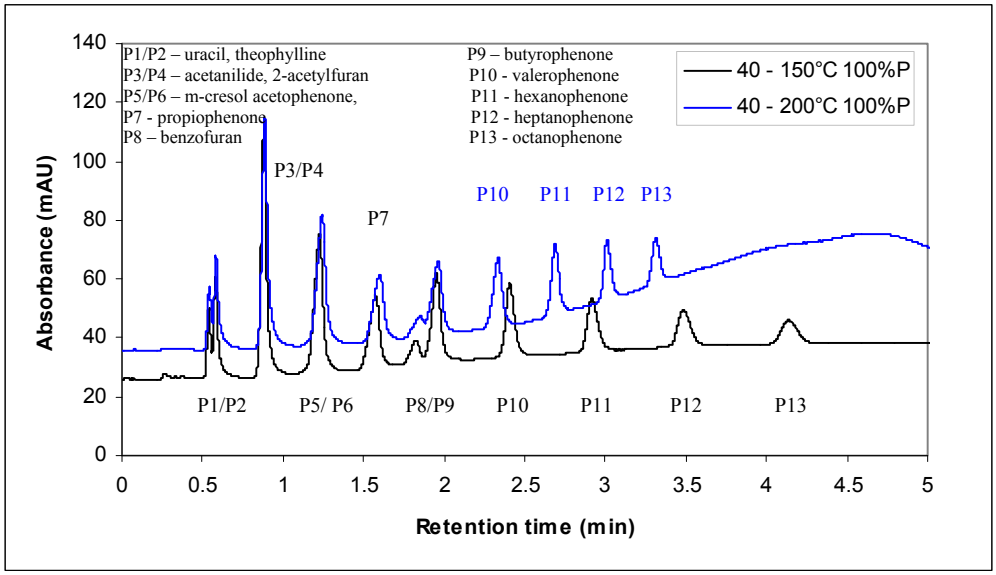


Figure 73: Chromatogram of phenone mixture separated on temperature gradients of 40 – 150°C and 40 – 200°C at 100% power with eluent pre-heating via tubing housed inside the oven to follow the ramp on the XTerra phenyl 3.5 µm, 4.6 x 50 mm column at 30 % acetonitrile.

4.3.6 Pre-heating the Eluent with Good Thermal Contact

As in the previous experiment the eluent was pre-heated inside the resistively heated oven but to improve the preheating, the inlet tubing was clad in an aluminium jacket to create good thermal contact with the heater coil (Figure 74).

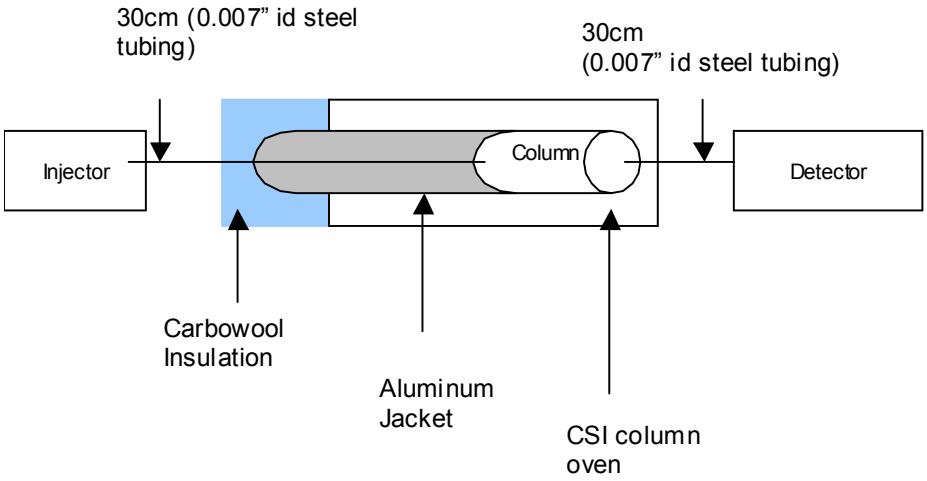


Figure 74: Schematic Diagram of Heating Set-up Incorporating an Aluminium Heating Jacket for Eluent Pre-heating

4.3.6.1 Isothermal Runs

This heating set-up was used to separate the phenone mixture isothermally at 40°C and 80°C (Figure 75). If the heating is homogeneous, the column efficiency should improve at 80 °C compared to 40 °C. However, in this case after m-cresol the peak shapes were notably worse for the later eluting peaks (Table 28) despite a significant reduction in analysis time.

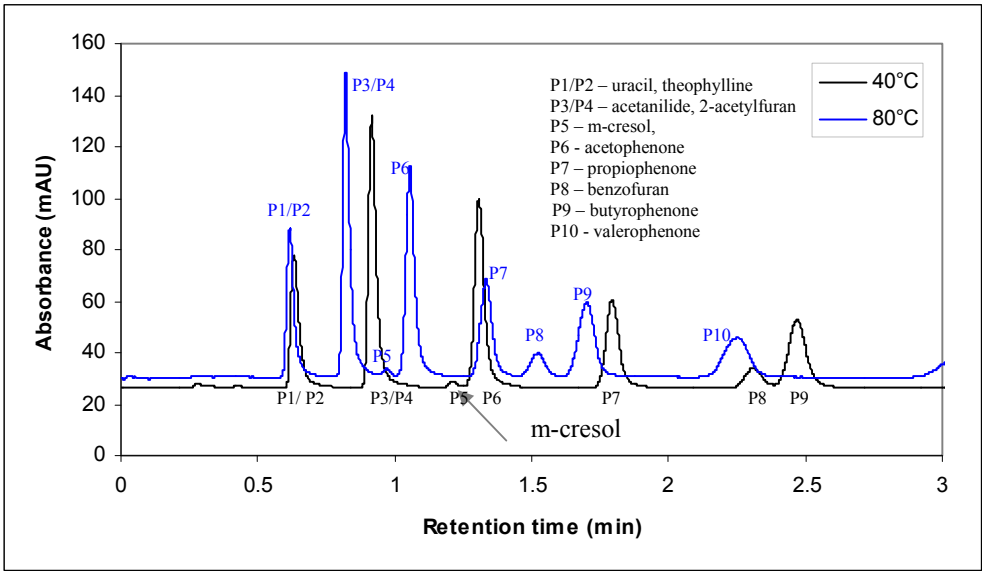


Figure 75: Chromatogram of phenone mixture separated isothermally at 40°C and 80°C at 40 % acetonitrile on an XTerra Phenyl 3.5 µm 4.6 x 50 mm with eluent pre-heating enhanced by an aluminium jacket housing the pre-heater tubing inside the oven at 1 ml/min

Table 28: Efficiency (NTP) for the later eluting components for isothermal runs of 40 °C and 80 °C (Figure 111), with eluent pre-heating aided by an aluminium jacket

Analyte	**NTP**	
	40°C	**80°C**
Hexanophenone	6060	2023
Heptanophenone	5356	1501
Octanophenone	4640	1162

This deterioration was indicative of thermal mismatch, which posed the question, was the eluent temperature actually matched to the column temperature? Previous experiments observed that for a length of tubing without a heating jacket housed inside the oven, the exit eluent temperatures for 40°C and 80°C at 1 ml/min were 25°C and 40°C, respectively, thus a cold injection would result. The current pre-heating set-up took into account close thermal contact between the tubing and heater and thus it was expected to

be more efficient than the pre-heater unit alone. This was assessed by measuring the temperature of the eluent exiting the column whose inlet tubing had been pre-heated by aluminium clad tubing (Figure 76). This was to improve the heat transfer via a good conductor metal and over a length of tubing to ensure equilibration was met.

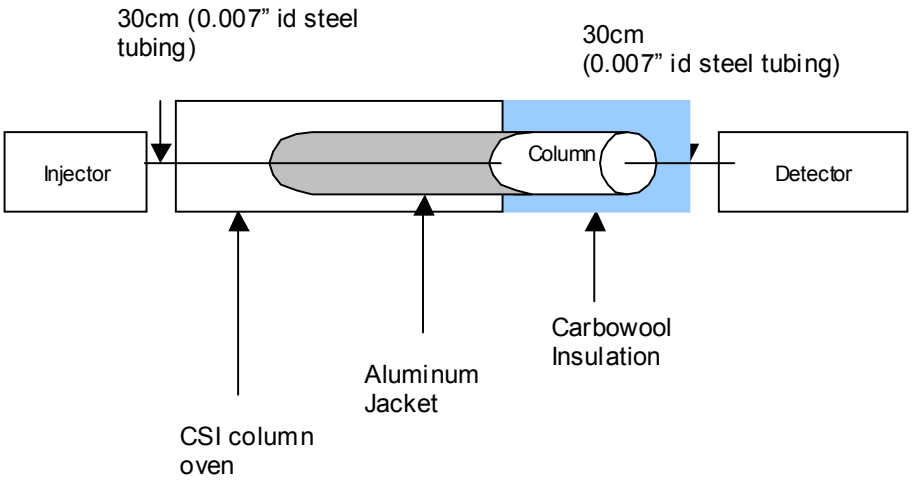


Figure 76: Schematic diagram of heating an insulated column via an eluent pre-heated tube clad in an aluminium jacket housed inside the resistively heated oven only

The temperatures recorded from separations using this configuration indicated that frictional heating was causing the temperature of the eluent at the column outlet to be higher to that of the pre-heated inlet prior to the column. This could exacerbate a parabolic profile if the eluent at the centre of the column is travelling faster to that at the walls. The stationary phase will lose heat to the surroundings as the steel surrounding the column conducts heat away as implied from the temperature measurement taken on the column half-way (Figure 77). This also explains why previously a slightly lower injection temperature was more favourable as it counteracted this detrimental flow profile.

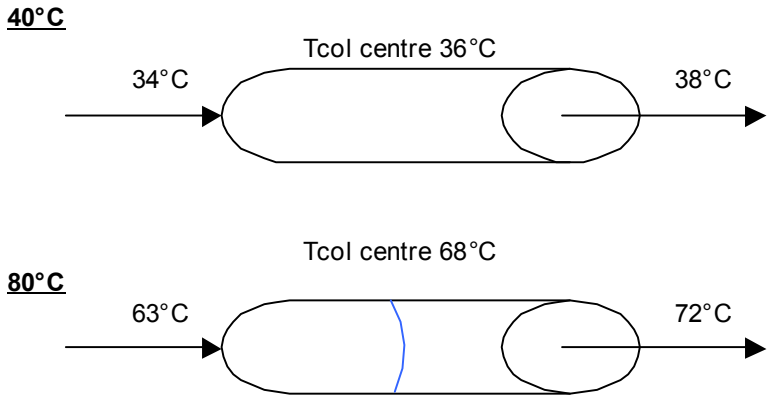


Figure 77: Heating profiles across the column for isothermal runs of 40°C and 80°C with the aluminium pre-heater[3] on an XTerra Phenyl 3.5 µm 4.6 x 50 mm at 1 ml/min

The difference in column inlet/outlet temperature is more significant the higher the oven temperature suggesting a bigger offset is required to balance the flow profile at 80 °C. Efficiency has dropped dramatically at 80 °C for all components by approximately 50 %.

4.3.6.2 Summary

Previous work involving column heating via 'resistive heating' of the eluent utilizing the pre-heater unit only (with column insulation) showed that isothermal separations still gave relatively good chromatography. This was not expected as a hot eluent entering a cold column would raise a thermal parabolic profile where the eluent at the centre of the column is less viscous and hence moving faster in relationship to the column walls. As this flow traverses the column one would expect it lose heat and effectively impose a negative temperature gradient across the column. However, if the column is now heated

[3] The Tcol centre is a temperature measurement taken on the surface of the insulated column mid-way and gives an approximation of the column temperature relative to the incoming eluent temperature.

there has to be some compensatory factor that offsets the resulting flow profile, such as a slightly lower injection temperature to that of the column found in the previous section.

The three different techniques for heating a column have been examined (Table 29) and show that all of the techniques are found to give a decrease in efficiency for late eluting components with an increase in temperature.

Table 29: Comparison of efficiency achieved with different column heating techniques for the 3 components to elute last from a complex mixture of phenones on an XTerra phenyl 3.5 µm 4.6 x 50 mm.

Peak	40°C PH	80°C PH	40°C Al PH	80°C Al PH	40°C Al PH + oven	80°C Al PH + oven
Hexanophenone	3277	2132	4975	2256	6060	2023
Heptanophenone	3489	2249	4607	2080	5356	1501
Octanophenone	3513	2159	4766	2079	4640	1162

Key: PH = resistively pre-heated eluent only; PH Al = pre-heated eluent only assisted by an aluminium sleeve for good thermal contact

At 40°C the best chromatographic performance in terms of efficiency was achieved by eluent and column heating inside the oven. This was followed by column heating by eluent heating inside the oven and then by the resistively heated pre-heater unit. In the reverse order the 80 °C data proved to be worse for the eluent and column heating combined inside the oven. The higher the temperature applied the greater the offset between column and eluent temperature. Therefore, the eluent entering the column should be lower to that of the column temperature for high temperature chromatography. This could be achieved by setting the incoming eluent temperature lower via programming the external eluent heater unit or separations could be carried out via eluent pre-heating only combined with elevated flow rate.

4.3.6.3 Temperature Programmed Runs

The insights gained from experiments on the different methods of pre-heating the eluent have shown that the flow profile can be balanced or compensated for by a huge gain in

efficiency. However, what works for an isothermal separation i.e. low injection temperature for an oven heated column will not necessarily enable a temperature programmed separation to achieve the best results. A temperature-programmed run is more complicated also in the sense that any gain in sharpness of peaks must be maintained throughout the run to make it a viable alternative technique to solvent gradients.

The heating set-up as shown in Figure 74 (Section 4.5.6) was used to separate the phenone mixture on a temperature gradient of 40°C to 200°C at 100 % power (Figure 78).

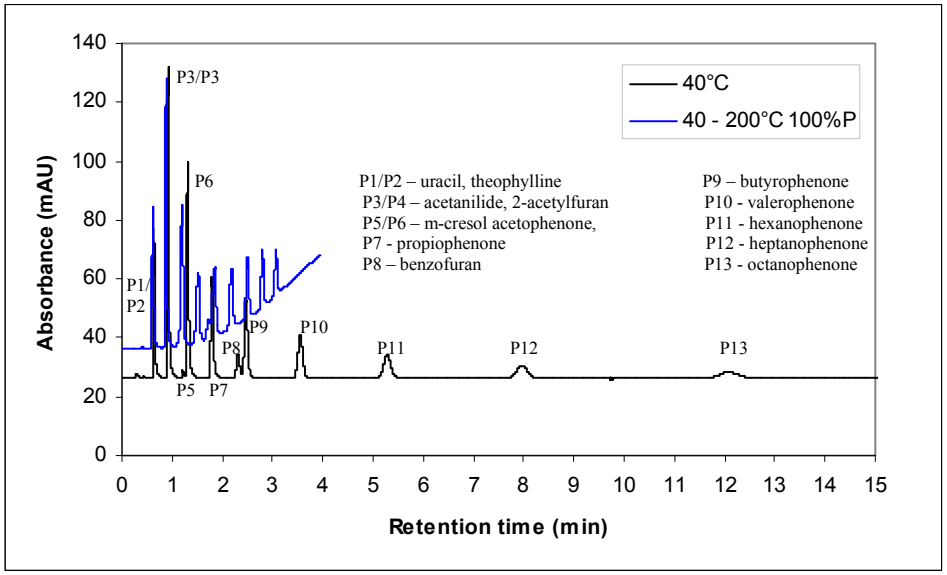


Figure 78: Chromatogram of phenone mixture separated isothermally at 40°C and on a temperature gradient run 40 – 200°C at 40 °C/min (100 % power) and 40 % acetonitrile with eluent pre-heating following the temperature gradient of the XTerra phenyl 3.5 µm 4.6 x 50 mm column.

Initial peak widths on a temperature ramp of 40 – 200°C at 40 °C/min compared to an isothermal run at 40 °C have fallen generally by approximately 50 %. The question was whether more could be achieved that would give even better results now that the right profile is being created. Is it the temperature gradient of the eluent that is giving the desired outcome or the heat being generated externally? The profile needs to be

determined as outlined above. This is a difficult procedure to perform so an approximation was obtained by measuring eluent temperature the eluent (water) exiting the column.

The temperature gradients for the water eluting from an aluminium clad pre-heater housed inside the oven and for a pre-heater coupled to a column were compared (Figure 79). The rates were 35 °C/min and 25 °C/min, respectively (Figure 80), therefore the rate at which the eluent was being heated when the pre-heater and column are combined drops by 10 °C/min. This indicates that the column walls and stationary phase are lagging behind in terms of heat being transferred in external heating. However, this combined with the faster heating of the mobile phase had an advantageous effect on the chromatography. It is probable that the two were displaying a compensation effect with the parabolic flow profile inverting and flattening giving characteristically sharp peaks. It would appear that a strong column focusing effect has compensated for this difference and that it may be possible to achieve even better sharper peaks. As the eluent heats up, a similar effect to that found with a solvent gradient was achieved where peaks were compressed giving rise to narrower bands.

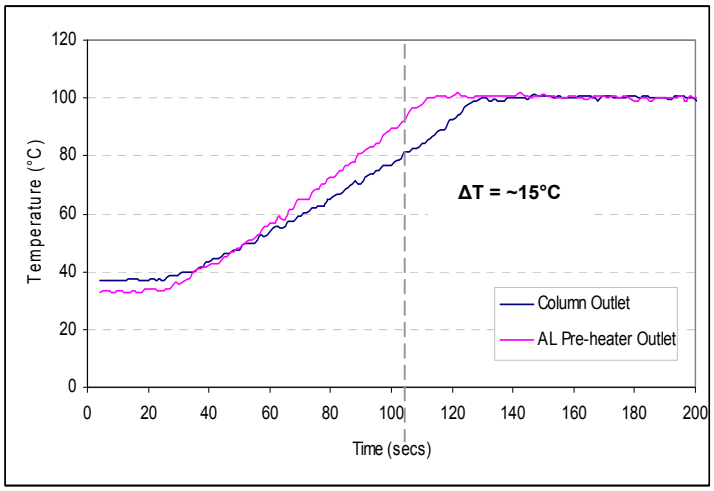


Figure 79: Individual thermal measurements taken of the eluent (water) pre-heated via (1) tubing contained in an aluminium sleeve inside the oven, and (2) tubing contained in an aluminium sleeve coupled to an XTerra phenyl 3.5 µm 4.6 x 50 mm inside the oven, both ramped from 40 – 200°C at 40 °C/min (100% Power) at 1 ml/min

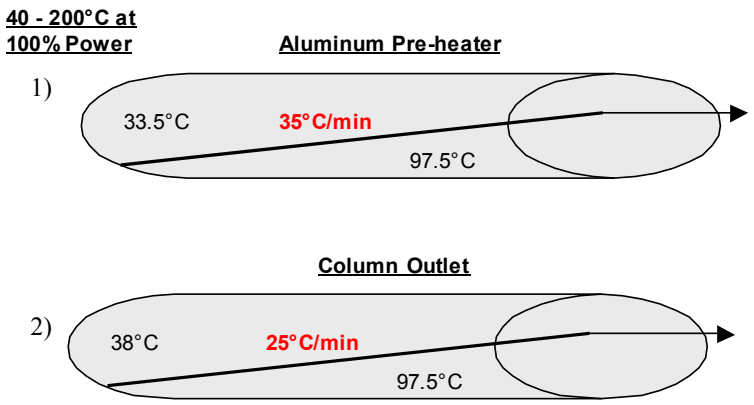


Figure 80: Heating profiles taken of the eluent (water) pre-heated via (1) tubing contained in an aluminium sleeve inside the oven, and (2) tubing contained in an aluminium sleeve coupled to an XTerra phenyl 3.5 µm 4.6 x 50 mm inside the oven, both ramped from 40 – 200°C at 40 °C/min (100% Power) at 1 ml/min

4.3.7 Temperature Programmed Runs at Different Eluent Compositions

Previous experiments have demonstrated that by lowering the organic component of the mobile phase combined with temperature can have an even greater impact on the chromatography. Therefore a set of experiments were performed to see if any what impact this would have on parameters, such as resolution and overall separation quality.

4.3.7.1 35 % Acetonitrile

The temperature gradient conditions in the last section were repeated but the organic concentration was lowered from 40 % to 35 % acetonitrile. As expected the peak profile started to show improvement for early eluting peaks (Figure 81). Resolution improved as the peaks were pulled apart by lowering the organic content of the mobile phase. The first two components, uracil and theophylline, which were previously co-eluting, are just starting to separate, this now gives 11 of the 13 potential components. Narrow bands were still obtained for late eluting peaks and the total analysis time is still below 4

minutes giving a 6 fold reduction. In conclusion lowering the organic content is having a positive effect on achieving good resolution.

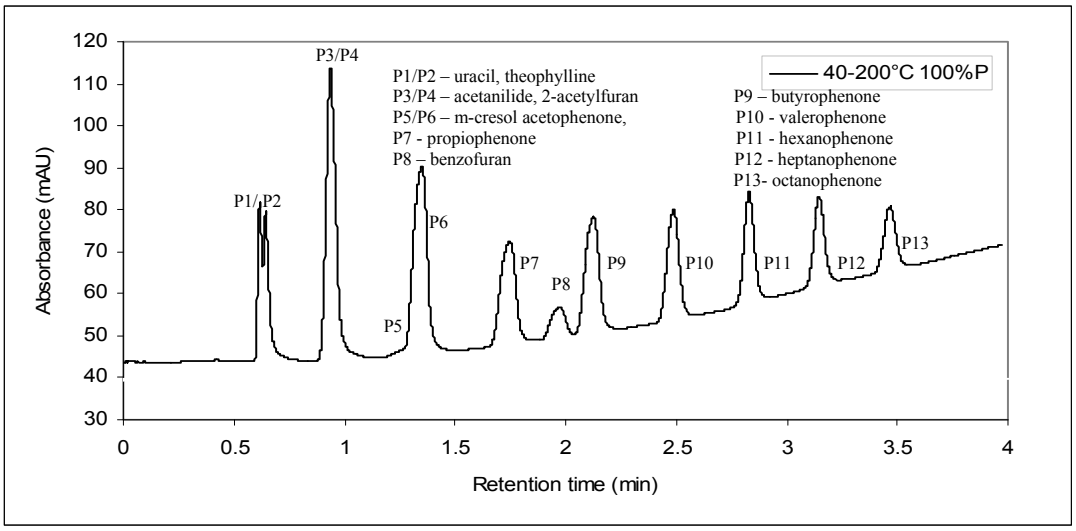


Figure 81: Chromatogram of phenone mixture separated isocratically at 35 % acetonitrile on a temperature gradient of 40 - 200°C at 100% power, with an aluminium jacket for good thermal contact during eluent pre-heating on an XTerra phenyl 3.5 µm 4.6 x 50 mm at 1 ml/min with detection at 254 nm

4.3.7.3 20 % Acetonitrile

The organic content of the mobile phase was lowered further to 20 % acetonitrile (Figure 82) and now all 13 components can be separated on the temperature gradient with a run time of only 6 minutes. The peaks shape was good for all of the peaks without compromising speed and resolution. Column focusing is crucial and peaks should be eluted on a temperature gradient to achieve maximum benefit from temperature-based applications. To achieve this, the ramp rate could be lowered or the upper temperature limit increased (stability of stationary phase permitting) to elute and focus all components on a gradient.

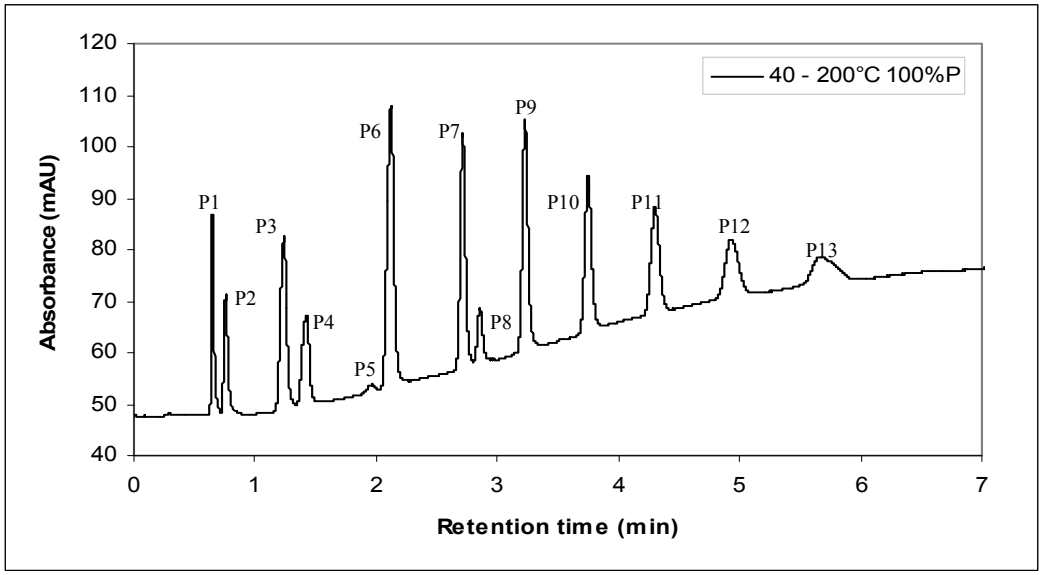


Figure 82: Chromatogram of phenone mixture separated isocratically at 20 % acetonitrile on a temperature gradient of 40 - 200°C at 100% power, with an aluminium jacket for good thermal contact during eluent pre-heating on an XTerra phenyl 3.5 µm 4.6 x 50 mm at 1 ml/min detection at 254 nm

It was found that the lower the organic concentration the more dramatic the effect when applying a temperature gradient. A vast improvement was seen for peak shape, once the temperature rapidly increases after the initial lag in heat transfer. This seems to be the key to maintaining and improving resolution whilst decreasing peak widths and reducing analysis time. A highly aqueous mobile will undergoes a more dramatic effect due to the marked drop in the dielectric constant of water at elevated temperature, being equivalent to that of acetonitrile at 200°C (section 1.3.2). In addition water has a high energy capacity and can utilize additional heat made available during a separation aiding mass transfer and the kinetics. Unlike traditional separations a 5 % decrease in organic concentration of the eluent no longer means an approximate 2 fold in analysis time when coupled with a high temperature gradient. It has been shown from runs performed at 40 % to 20 % organic concentration (sections 4.5.7.3 to1.4.5.6.3) that the loss was of 20 % in speed of analysis. In terms of criteria that would meet with fast chromatography this has less impact than the demonstrated gain in resolution, selectivity and peak shape.

The carbon footprint is also reduced as we are using a resistively heated oven that runs on approximately 40 kW unlike the conventional air-forced ovens that run at approximately 500 kW. A predictive model for the flow profile that can predict optimum parameters not unlike DryLab for temperature programming could provide a suggested optimum performance conditions required for a given set of parameters such as phase type, geometry, flow rate, temperature etc.

4.3.8 Generating a Temperature Gradient Run by Pre-heating the Mobile Phase.

Based on the experiments with the same column using the CSI pre-heater and no column heater (Section 4.4), which gave surprisingly good results, the inlet tubing was placed in the oven and the temperature was ramped but the column was only lagged (below Figure 83):

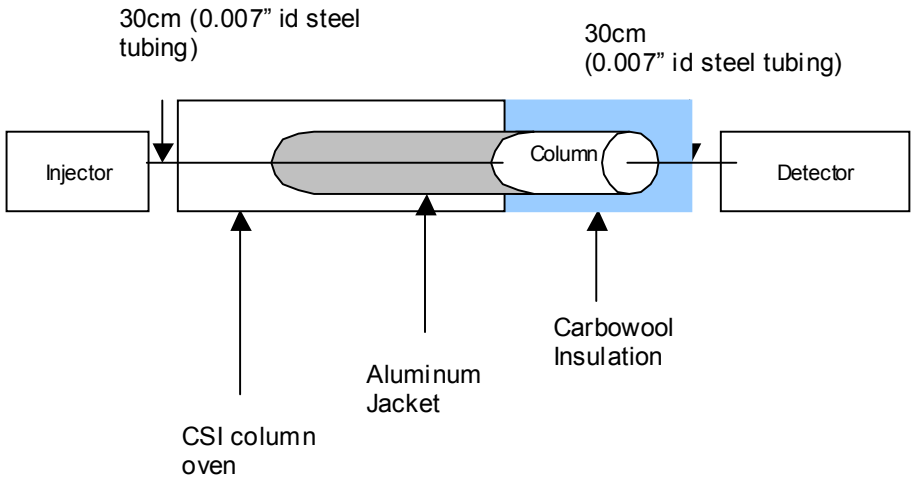


Figure 83: Schematic diagram of heating the column via eluent pre-heating

Using 40 % acetonitrile as the eluent the early eluting peaks (Figure 84) had reasonable peak shapes and were separated adequately, but the later eluting components were not

sharpened and thermal mismatch meant that peak shape deteriorated rapidly as the ramp progressed towards the target temperature.

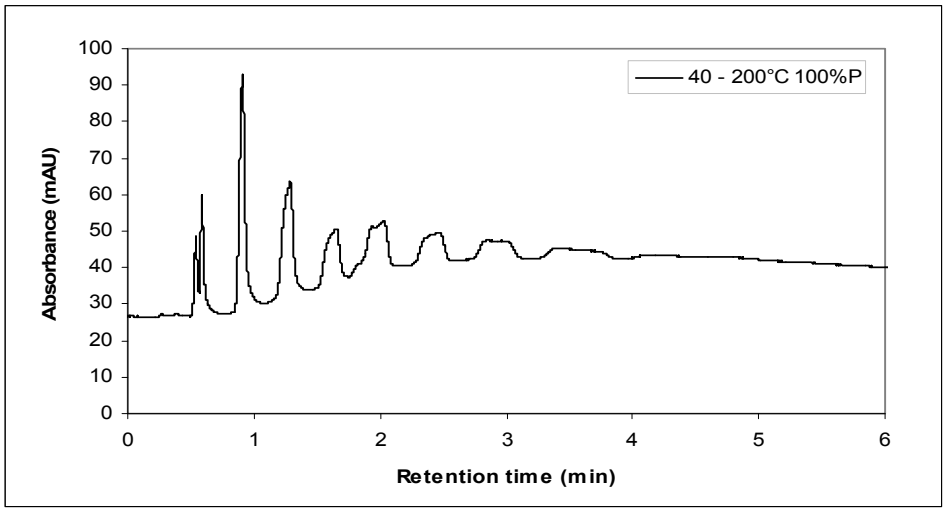


Figure 84: Chromatogram of phenone mixture separated by ramping up the temperature of the eluent only using the pre-heater from 40 - 200°C at 100% power on an XTerra Phenyl 3.5 µm, 4.6 x 50 mm column at 40 % acetonitrile and 1 ml/min.

This experiment was also tried with the column half way inside the oven to see if this would give adequate heating as most of the separation happens at this point. But clearly this is a much more complex process in terms of creating the right flow profile across the column. Although some later peaks were improved the chromatography was still poor, similar to that seen initially. Thus it was concluded that eluent and column ramping are essential to creating the right profile to obtain dramatic improvements for a temperature-programmed column.

4.3.9 Combination of Temperature Programming with Small Particle Size

An XBridge (C8) column was chosen to look at the effect of particle size on the basis of the initial success of the XTerra phenyl column under temperature ramping conditions of the eluent and column. The XBridge columns are an extension of the XTerra hybrid particle technology from Waters Corporation. The main difference now is that it has an

ethylene-bridged co-monomer backbone and more cross-linking than the XTerra methyl hybrid particle. Thus, it is more stable: thermally, chemically and mechanically. It is available in small particle sizes that should give better resolution allowing the length of the column to be reduced further to determine the effectiveness of heat transfer. It was therefore decided to extend the study of its capabilities under temperature gradient conditions using a short 30 mm column to minimise run times.

4.3.9.1 Chromatographic Conditions

System:	Miniaturised HPLC system with the CSI oven
Column:	XBridge C8 2 µm 4.6 x 30 mm
Mobile Phase:	Eluent A: Water
	Eluent B: Acetonitrile
% B:	50
Temperature program:	40 to 200 °C
Injection volume:	5 µl (0.5 µl loop)
Flow rate:	0.5 to 1.2 ml/min
Wavelength:	254 nm
Equilibrium time:	30 minutes allowed between temperature set-points

Initially, a run was performed at 40% acetonitrile at flow rates from 0.5 to 1.2 ml/min (Figure 85). This was to evaluate a typical optimal flow rate for the column as smaller particle sizes on a van Deemter plot should give better efficiencies at high flow rates. At elevated temperature this optimum flow rate should then increase.

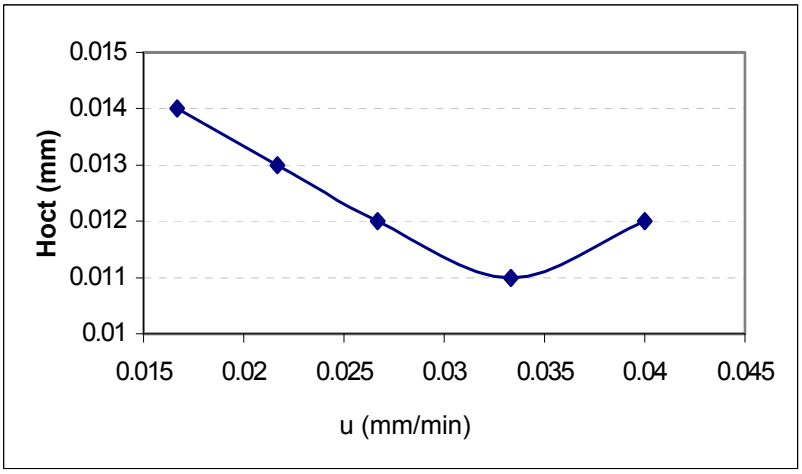


Figure 85: Van Deemter curve for octanophenone under conditions: 40% acetonitrile; 40°C on an XBridge™ C8 2 µm 3 x 30 mm from 0.5 to 1.2 ml/min at 254 nm

Resolution for early eluting components was not achieved compared to the XTerra column (50 mm length and 3.5 µm particle size) isothermally at 40 °C with 40 % or 30 % acetonitrile (Figure 86). Therefore, using a shorter column and reducing the particle size to 2 µm offered no advantage in terms of efficiency. The XTerra also had a phenyl group, which showed greater selectivity probably due its π stacking functionality. When the temperature of the run was increased for the 30% acetonitrile run there was evidence of thermal mismatch as the peak shapes for late eluting components were distorted. This was also found to be the case with the XTerra phenyl and clearly a lower inlet temperature is required to give a flat flow profile at the end of the column.

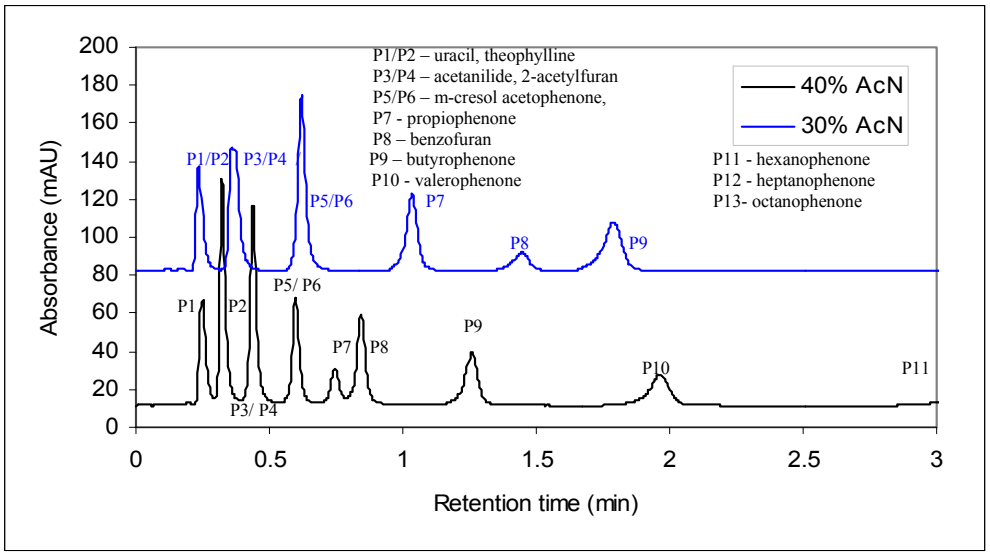


Figure 86: Isothermal runs at 40°C for 40% and 30% acetonitrile on an XBridge™ C8 2 µm 3 x 30mm column at 1 ml/min with detection at 254 nm.

A temperature program would have to be balanced by the incoming eluent temperature. The next parameter to test was a temperature gradient and whether this revealed a satisfactory profile. A temperature program was set from 40 to 100°C at 100% power (Figure 87). The chromatogram showed that significant thermal mismatch was affecting the late eluting components. This could be that the set-up for heating differed from the previous column as there was:

1. Less stationary phase therefore less heat was required,
2. The column outer diameter was representative of a 4.6 mm I.D. column, hence more stainless steel thus more resistance to heat transfer,
3. The tubing used to carry the eluent to the column was a lower I.D. (0.005”) and so is heating more effectively,
4. The column itself was shorter and so again less heat was required,
5. At low flows the rate of heat transfer would be much slower.

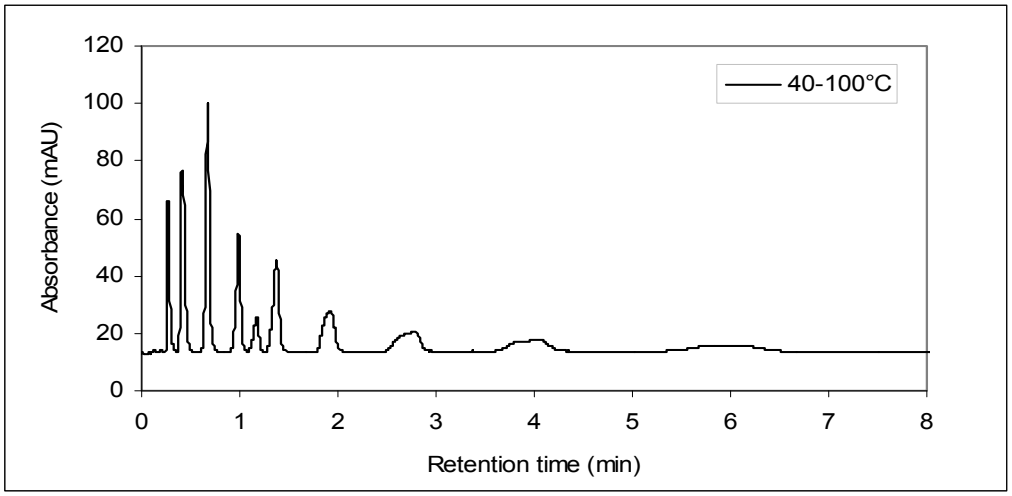
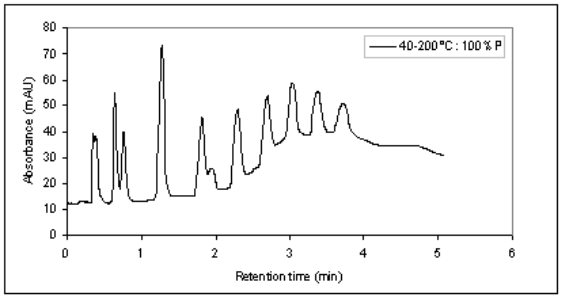
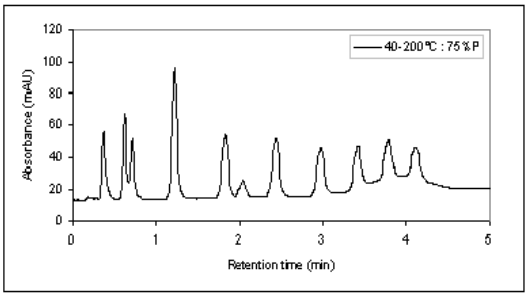


Figure 87: Temperature program run 40 to 100°C (100% power) at 30% acetonitrile on an Xbridge^{TM} C8 2 μm 3 x 30mm column at 1 ml/min with detection at 254 nm

If the programme was continued to an upper temperature of 200°C the thermal mismatch problem was overcome (Figure 88). Thus first and fourth suggestions that there was less stationary phase to heat were an unlikely cause but the need in the second suggestion that more heat was required for the heavier column hardware was possible.

The optimum conditions for chromatographic performance rate were found to depend on the column heating rate (Table 30) and power input and that if a ramp was maintained so that all the peaks were eluted during the gradient, the desired focusing effect could be achieved.





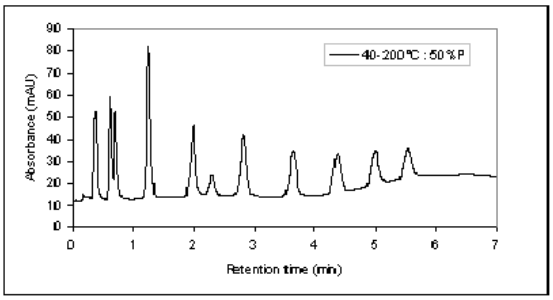


Figure 88: Temperature program run 40 to 100 °C for a Ramp Rate of 100, 75 and 50 % P (power) at 20% acetonitrile on an Xbridge™ C8 2 µm 3 x 30mm column at 1 ml/min

Table 30: Retention time (RT) and resolution (Rs), for a temperature program run 40 to 200°C with varying heating rates at 20% acetonitrile on an Xbridge™ C8 2 µm 3 x 30 mm column at 1 ml/min

	Ramping rate (% power)					
	100 %		75 %		50 %	
Peak	**RT**	**Rs**	**RT**	**Rs**	**RT**	**Rs**
Uracil	0.36	NA	0.37	NA	0.37	NA
Theophylline	0.39	NA	0.38	NA	0.38	NA
2-Acetylfuran	0.65	NA	0.62	3.1	0.62	NA
Acetanilide	0.77	1.5	0.71	1.2	0.71	1.3
Acetophenone	1.28	5.2	1.22	5.8	1.26	6.2
Propiophenone	1.82	4.0	1.83	5.4	2.00	6.0
Benzofuran	1.95	NA	2.04	1.5	2.30	2.0
Butyrophenone	2.30	NA	2.44	2.6	2.83	2.9
Valerophenone	2.70	2.3	2.98	3.3	3.65	4.1
Hexanophenone	3.04	1.9	3.42	2.7	4.37	3.4
Heptanophenone	3.37	1.7	3.79	2.2	5.00	2.8
Octanophenone	3.72	1.5	4.11	1.8	5.54	2.3

In conclusion, the short large I.D. column may compensate for the benefits of going towards longer narrow-bore columns.. This is important as we want to use a short column for rapid heating and short retention times.

4.3.9.2 Baseline Gradient Effects induced by Temperature Gradients

The ramping baseline found with this particular column was similar to that found with the XTerra phenyl, 3.5 µm, 3 x 30 mm column (section 3.3.2). The cause of this could lie in several explanations relating to column bleed. Firstly, already covered in the XTerra section there could have been a phase ratio change due to temperature, flow cell sensitivity and lastly, stationary phase loss. It was observed by Chester and Coym[263] that stationary phase ligands underwent an irreversible thermal transition with increasing temperature when in a pure water mobile phase. This reorganization releases residual organic solvent that has been trapped by the ligands at lower temperatures during organic solvent pre-treatments. Either way this particular effect was not apparent when performed on the Agilent HP1100 system (2 µl, 6 mm high pressure flow cell). The Linear detector was probably more sensitive to thermal gradients, as the inlet tubing was not wrapped around the flow cell to allow for thermal equilibration between the incoming eluent and the flow cell. The Agilent flow cell has a heat exchanger that houses the eluent tubing prior to the flow cell this heats the eluent and flow cell to 40 °C so eliminates slight fluctuations in temperature. Refractive index changes induced by altering the temperature might also be picked up by the Linear detector as the flow cell is not tapered. This type of disturbance tends to happen at the edge of the flow cell therefore later models are designed to combat this type of problem. Another consideration is that a reference wavelength monitors the background and lamp fluctuation noise and subtracts this in ChemStation. So the next check was to remove the reference signal to this to see if this was masking a rising baseline. The resulting chromatogram (Figure 89) clearly showed that a rising baseline was not an issue, thus pointed towards the flow cell in the Linear detector being sensitive to slight fluctuations in temperature. Column bleed would also have induced a rise in baseline. The issue was likely to be residual organics that had been trapped during phase synthesis and ligands that were being stripped once a temperature program was in operation. A continuous leaching of polar material should not give rise to actual peaks and but could give rise to a shift in baseline or baseline gradient.

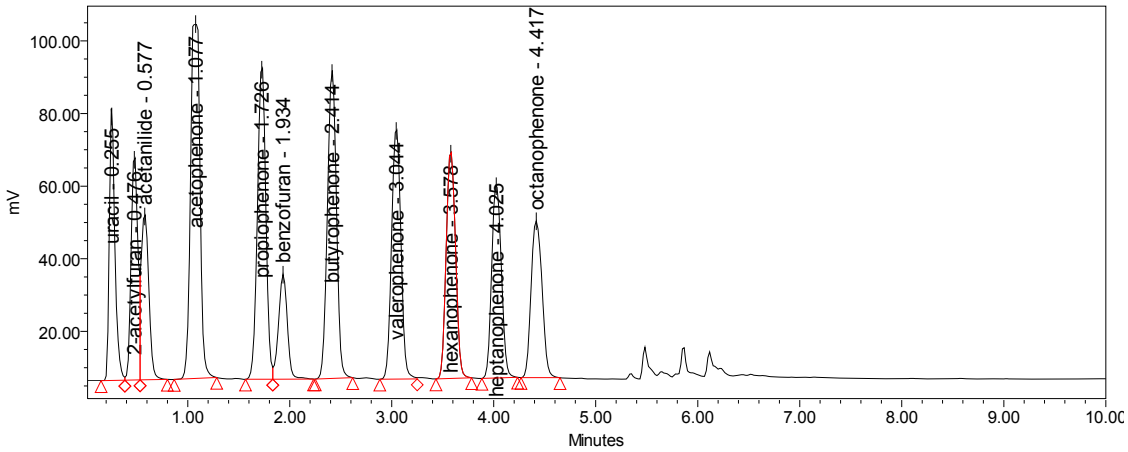


Figure 89: Temperature program run of 40 to 200°C (65% power) at 20% acetonitrile on an Xbridge™ C8 2 µm 3 x 30mm column on a HP1100, without the reference signal at 360 nm.

4.4 Temperature Gradients Hyphenated to Different Modes of Detection

The compatibilities of a number of different detectors with the temperature gradient elution were then examined to see if they were affected by the changing outlet temperature or if the baseline was shifted or became noisy.

4.4.1 Mass Spectroscopy

Both APCI and electrospray (ESI) ionisation interfaces were examined with a test mixture made up of a phenone mixture and basic and acidic functionalities consisting of: uracil, theophylline, acetanilide, 2-acetylfuran, m-cresol, acetophenone, propiophenone, benzofuran, butyrophenone, valerophenone, hexanophenone, heptanophenone and octanophenone.

4.4.1.1 Atmospheric Pressure Chemical Ionisation

4.4.1.1.1 Conditions

System 3:	Agilent HP1100 LC system with the CSI oven hyphenated to a SL MS
Column:	XTerra MS C18 3.5 µm, 4.6 x 100 mm
Mobile Phase:	Eluent A: 0.05% TFA in water Eluent B: 0.05% TFA in acetonitrile
% B:	60
Temperature program:	40 to 130°C (20% power; 9°C/min)
Injection volume:	5 µl
Flow rate:	1 ml/min
Wavelength:	254 nm
Equilibrium time:	15 minutes allowed between temperature set-points
Ionisation mode:	APCI
Polarity:	positive
Mode:	SIM ; fragmentation 70 eV
Spray chamber:	gas temperature 350 °C; drying gas 8.0 ml/min; nebulizer pressure 50 psig; capillary voltage 4000 V (positive) and 1500 V (negative), Corona 4.0 µA (positive) and 40 µA (negative)

The APCI technique was ideal for the analytes of interest (polar) and the conventional bore column was used as it was compatible with the flow rate and for this ionization technique. Although the peaks were swamped by the acetanilide signal (Figure 90) all of the masses were identified in SIM mode (selective ion monitoring) as demonstrated in Table 31. The baseline remained unchanged throughout the run and did not show the type of problems associated with solvent gradients caused by changes in the mobile phase viscosity and surface tension as a result of alterations in the composition of the eluent. A solvent comparable change would have been approximately 30% based on a 90°C increase in temperature.

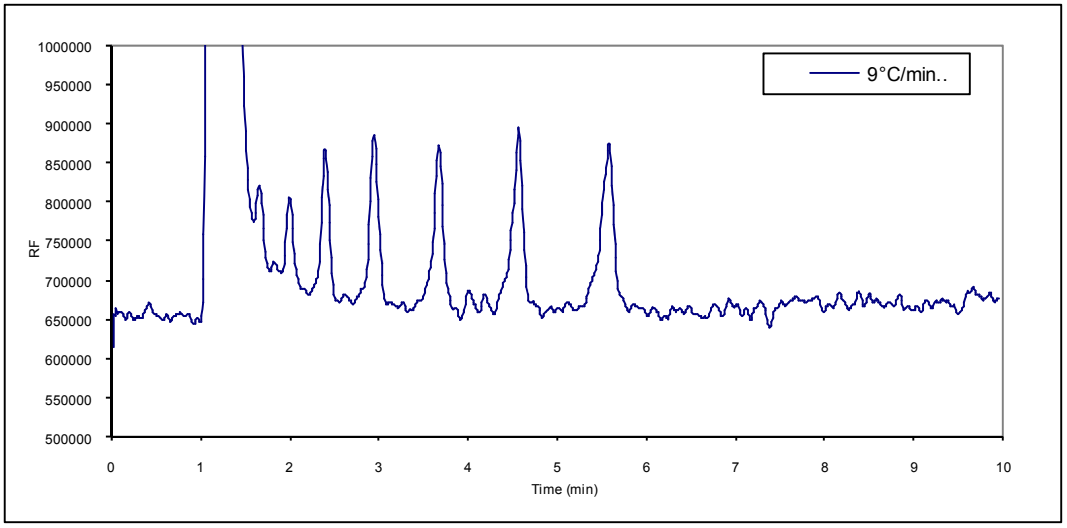


Figure 90: Mass spectroscopy trace of a phenone mixture during a temperature gradient run for 40 - 130 °C on an XTerra MS C18, 3.5 µm 4.6 x 100 mm at 1 ml/min, on an Agilent HP1100 /MS-SL with APCI source

Table 31: Masses Identified from MS Scan for a Temperature Gradient Run of 40 - 130°C on an XTerra MS C18, 3.5 µm 4.6 x 100 mm at 1 ml/min, on an Agilent HP1100 /MS-SL with APCI source

Component	Retention time (min)	Mass detected [M+1]
Uracil	1.00	113.1
Theophylline	1.00	181.1
2-Acetylfuran	1.67	111.1
Acetanilide	1.33	136.1
m-Cresol	1.67	109.2
Acetophenone	1.67	121.2
Propiophenone	2.01	135.2
Benzofuran	2.01	118.1
Butyrophenone	2.41	149.2
Valerophenone	2.95	163.2
Hexanphenone	3.68	177.3
Heptanophenone	4.58	191.3
Octanophenone	5.59	205.3

4.4.1.2 Atmospheric Pressure – Electro Spray Ionisation

4.4.1.2.1 Conditions

System 3:	Agilent HP1100 LC system with the CSI oven
Column:	Merck Purospher® STAR E18 3 µm, 1 x 150 mm id
Mobile Phase:	Eluent A: 0.05 % TFA in water
	Eluent B: 0.05 % TFA in acetonitrile
% B:	60
Temperature program:	40 – 60 – 80 °C (10, 20 % power; 4.5, 9 °C/min)
Injection volume:	0.5 µl
Flow rate:	0.04 ml/min
Wavelength:	254 nm
Equilibrium time:	15 minutes allowed between temperature set-points
Ionisation mode:	AP-ES
Polarity:	positive
Mode:	SIM; fragmentation 70 eV
Spray chamber:	gas temperature 350 °C; drying gas 8.0 ml/min; nebulizer pressure 50 psig; capillary voltage 4000 V (positive) and 1500 V (negative), Corona 4.0 µA (positive) and 40 µA (negative)

This mode was used as it was suitable for low flow rates with micro-bore chromatography; however, it was not the ionisation method of choice for this set of compounds. Only five of the thirteen masses in this temperature-programmed run were detected and identified (Figure 91). This was thought to be because the response of acetanilide suppressing the ionisation of other components. The baseline had improved notably compared to gradient elution and no sign of any detrimental effects due to ramping up the temperature were evident. The experiments carried out on system 3 required a long length of tubing between the post-cooler and the detector. This was contributing to the cooling of the eluent as steel tubing was used as opposed to PEEK. The eluent temperature had been measured at the exit of the post-cooler (section 3.2.2)

and the results showed that it was not adequately cooling the eluent prior to detection. However, this was relative to the amount of tubing as heat would have been dissipated proportional to the surface area, so it was assumed that the eluent had reached a constant temperature before detection.

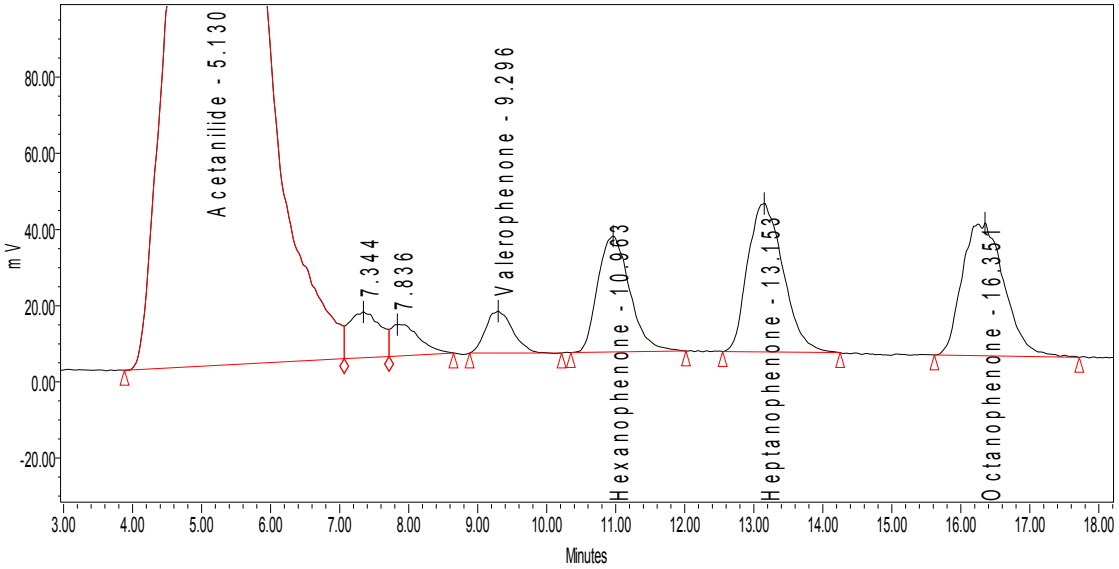


Figure 91: Mass Spectroscopy Trace of a Temperature Gradient Run for 40 – 60 - 80 °C on Merck RP18, 3 µm 1 x 150 mm

4.4.2 Impurity Profiling of SBA by LC-MS

The impurity profile for typical proprietary pharmaceutical SBA covered a wide range of polarity and the total analysis time was 45 minutes using an eluent gradient. The aim was to develop a temperature programme method to reduce this analysis time whilst maintaining resolution of all of the impurities. There were a large number of impurities associated with this compound (SAB - SBN) and some were not detectable by UV hence the use of the MS was necessary. The main difficulty was separating the impurities from the main assay peak, hence the rationale for trying a temperature program. The analysis time was also long and therefore may benefit from a temperature program. Previously they were separated under solvent gradient conditions. The aim was to inject the

compound initially to identify appropriate conditions followed by SBA, which included all known impurities spiked at a 0.1 % w/w level.

4.4.2.1 Equipment and Method Conditions to assess SBA

HPLC system:	Perkin-Elmer Integral 4000 or equivalent
Column:	HiChrom RPB, 5 micron particle size, 15 cm x 4.6 mm i.d.
Column temperature:	40°C
Wavelength:	265 nm
Sample volume:	10 µl
Sample loading:	8 µg
Mobile phase A:	0.15 M ammonium acetate, adjusted to pH 4.0 with trifluoroacetic acid
Mobile phase B:	Methanol
Elution:	Linear gradient from 25% B to 60% B over 40 minutes. Hold at 60% B for 5 minutes.
Flow rate:	1.0 $mL.min^{-1}$

4.4.2.2 Initial Conditions from thermal programmed elution

System:	Agilent HP1100 LC system with the CSI oven only
Column:	XBridge C8 2 µm, 4.6 x 30 mm
Mobile Phase:	Eluent A: water
	Eluent B: acetonitrile
% B:	50
Injection volume:	10 µl
SBA:	0.4 mg/ml (diluent MeCN/H_2O 50:50 % v/v)
Flow rate:	1 ml/min
Wavelength:	265 nm

Initial injections of SBA on the XBridge C8, 2 µm 4.6 x 30 mm gave very poor peak shape. The main band was spread over approximately 10 minutes (Figure 92). To reduce the band spreading 0.05 % formic acid was added to the mobile phase and the injection volume reduced to 1 µl. This method was also ran on a Luna, 3 µm, 3 x 30 mm column to establish if thermal ligand stripping from the column was responsible (Figure 91) The results showed that a relatively good peak shape was obtained on the Luna column under these conditions.

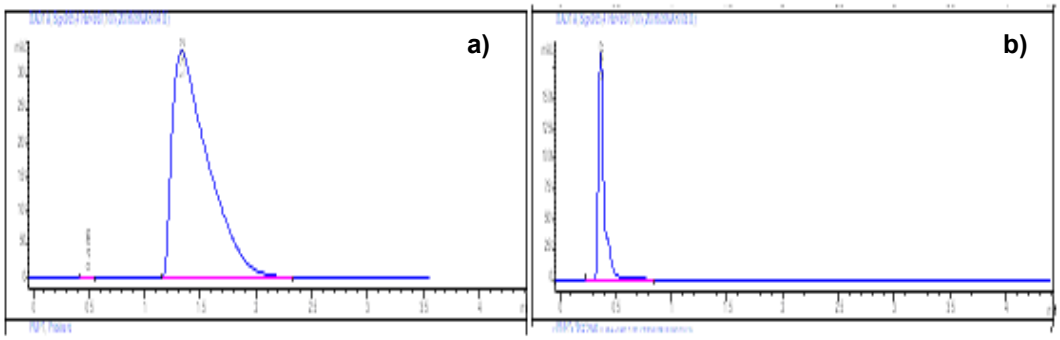


Figure 92: Chromatograms demonstrating peak shape of SBA on a) an XBridge column and b) a Luna column at 50 % acetonitrile at 1 ml/min and a column temperature of 40 °C at 265 nm.

SBA has been identified as being capable of chelating to metal impurities present on a column.

4.4.2.3 Chromatographic Conditions Identified for a Temperature Program

XBridge technology is such that these tailing effects would normally be minimized. However, due to ligand stripping as a result of temperature programming it was necessary to block the silanol activity by the addition of TEA.

System:	Agilent HP1100 LC system with the CSI oven only
Column:	XBridge C8 2 µm, 4.6 x 30 mm
Mobile Phase:	Eluent A: 0.1 % Formic acid + 0.1 % TEA in water
	Eluent B: 0.1 % Formic acid + 0.1 % TEA in acetonitrile

% B:	5 & 10
Temperature program:	40 – 200 °C at 40 °C/min
Injection volume:	5 µl
SBA:	0.08 mg/ml (diluent MeCN/H2O 50:50 % v/v)
Flow rate:	1 ml/min
Wavelength:	265 nm
Equilibrium time:	with cooling 5 minutes

The organic content of the mobile phase could also be reduced due to the short column length and two temperature gradients were examined (Figure 93).

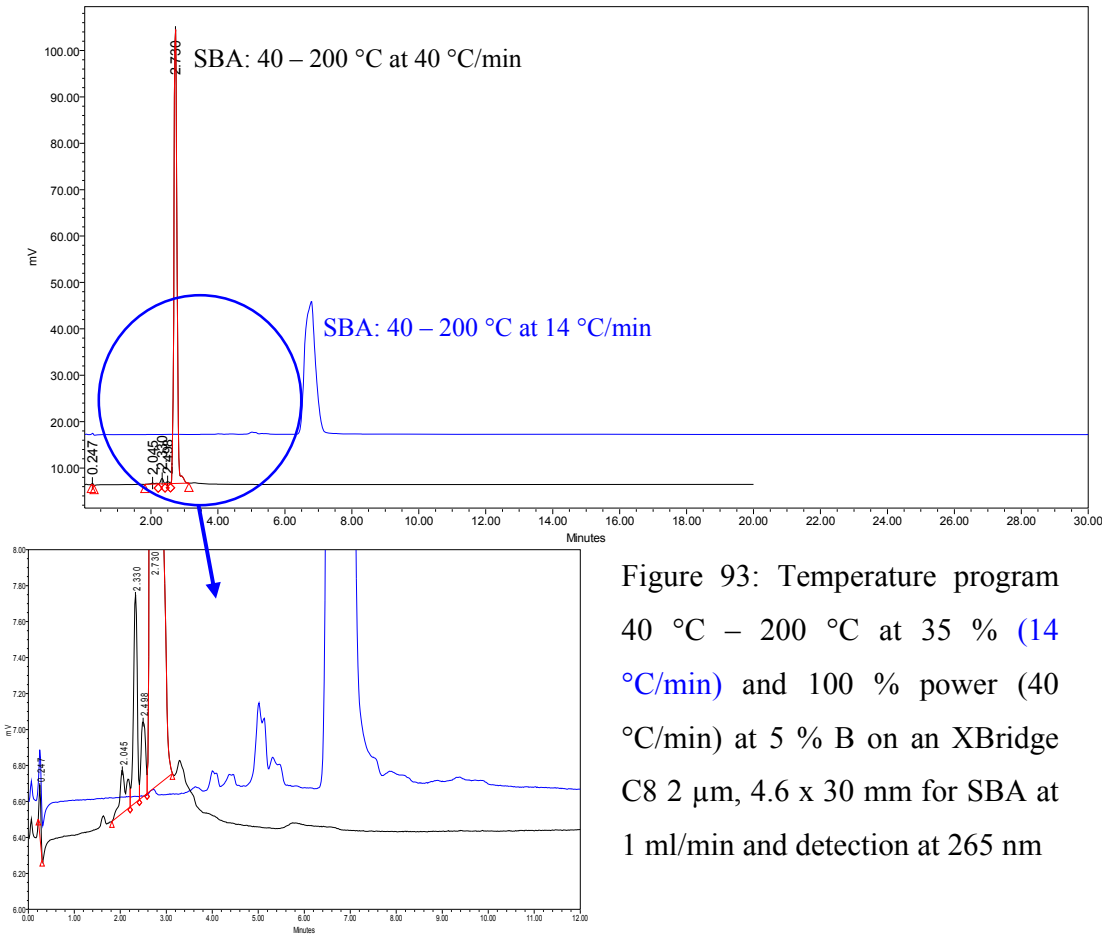


Figure 93: Temperature program 40 °C – 200 °C at 35 % (14 °C/min) and 100 % power (40 °C/min) at 5 % B on an XBridge C8 2 µm, 4.6 x 30 mm for SBA at 1 ml/min and detection at 265 nm

These showed the incredible increase in sensitivity and improvement in peak shape that could be achieved by applying a temperature program to highly aqueous conditions. The

isothermal band width of 2 min has been reduced to approximately 1 min and 0.5 min for 14 °C/min and 40 °C/min gradients, respectively, (the peaks differed in colour from the baseline due to the integration program used) for an eluent consisting of 95 % water.

4.4.2.4 Specificity

There were a lot of impurities associated with SBA which were individually injected during method development, including SBB, SBC SBD and tetra butyl ammonium bromide (TBAB), which were used as specificity indicators. SBE (a potential process impurity) was expected to have the highest retention and was injected as an end of run marker. UV and MS detection were combined to look at the impurity profile with the application of a temperature program to elute components under analyte focusing conditions (Figure 94). Potentially two critical pairs existed with respect to the main band (Figure 95), these included SBC and SBD with the main band SBA.

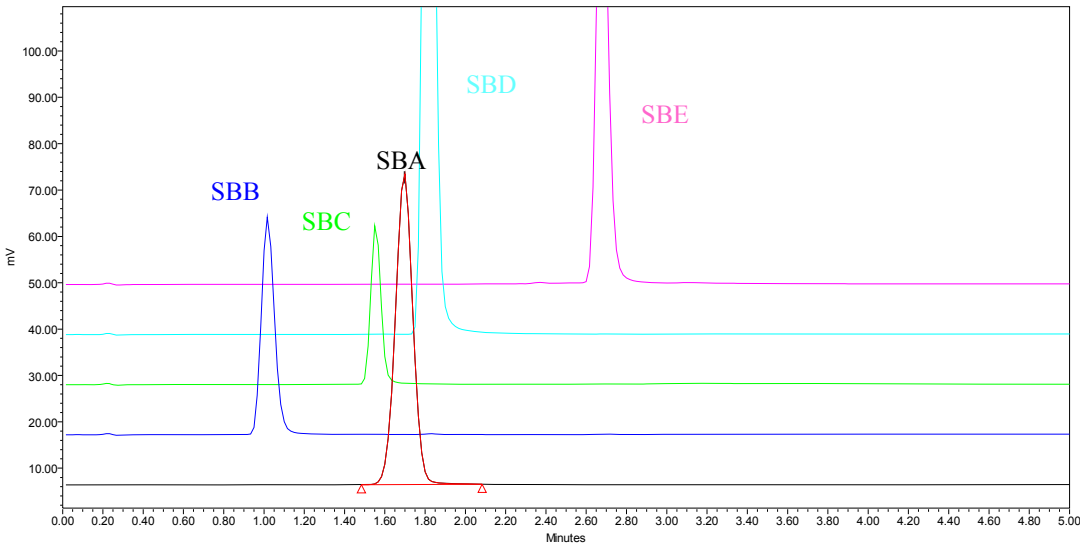


Figure 94: UV Overlay of Impurities SBB (RRT 0.6); SBC (RRT 0.9); SBD (RRT 1.1) and SBE (RRT 1.7) with main assay band SBA at 10 % B on an XBridge C8 2 µm, 4.6 x 30 mm at 1 ml/min and detection at 265 nm

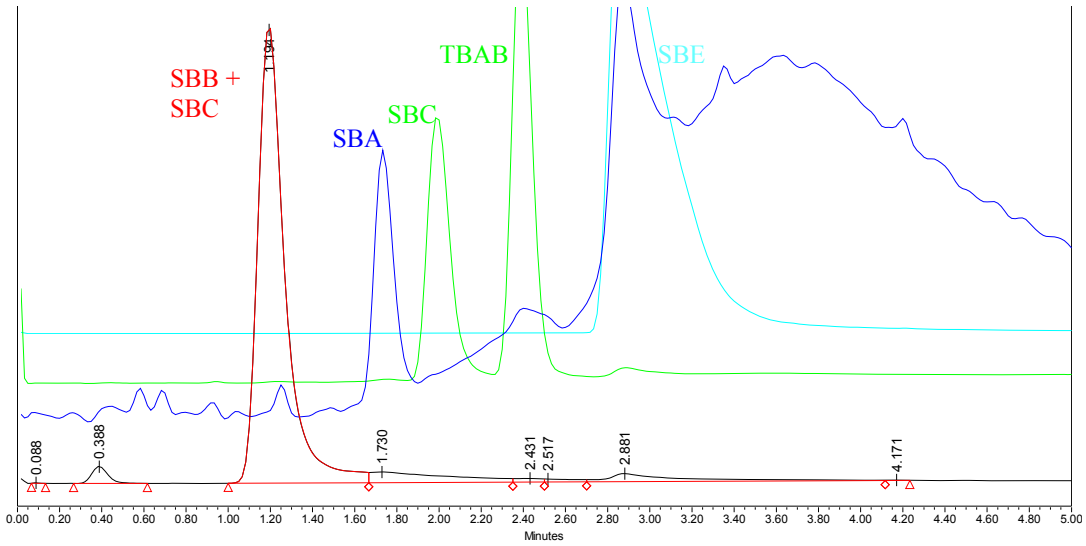


Figure 95: Normalized MS Trace Overlay of Impurities: SBB (RRT 0.6); SBC (RRT 0.9); SBD (RRT 1.0); TBAB (RRT 1.4) and SBE (RRT 1.7) with main assay band SBA at 10 % B on an XBridge C8 2 µm, 4.6 x 30 mm for SBA at 1 ml/min.

The mass spectroscopy traces for each component showed that each impurity had a good response but SBC may have its signal suppressed by its neighbours. The data has been normalized to show SBC, which had the smallest response. Masses detected/extracted corresponded to the ions found in Figure 94 (Table 32).

Table 32: Mass ions identified: SBB (RRT 0.6); SBC (RRT 0.9); SBD (RRT 1.0); TBAB (RRT 1.4), SBE (RRT 1.7) and main assay band SBA at 10 % B on an XBridge C8 2 µm, 4.6 x 30 mm for SBA at 1 ml/min.

RT (min)	**Observed M+1**	**Identified**
1.056	342	SBB
1.583	176	SBC
1.676	370	SBA(main band)
1.854	232	SBD
2.381	242	Tetra butyl ammonium bromide, TBAB (MS only)
2.864	513	SBE

Initial specificity checks for an isocratic phase of 10 % B demonstrated that the retention factors needed to be increased to separate the main assay peaks from some of the impurities. The most obvious way to do this was to change the concentration of the organic modifier and vary the temperature program conditions. A number of scouting gradients were run to determine how many peaks could be separated in a solution of SBA Figure 96) by varying the ramp rate of a temperature program for the lowest aqueous content of 5 % in the mobile phase. The scouting runs determined that sharper peaks were obtained denoting an increase in sensitivity. The next aim was to pull these peaks apart without losing this gain in sensitivity. The peaks sat close to the void volume solvent peak marker and effectively needed to have a longer retention coupled with a temperature gradient to maintain the benefits that have been derived from using the temperature variable.

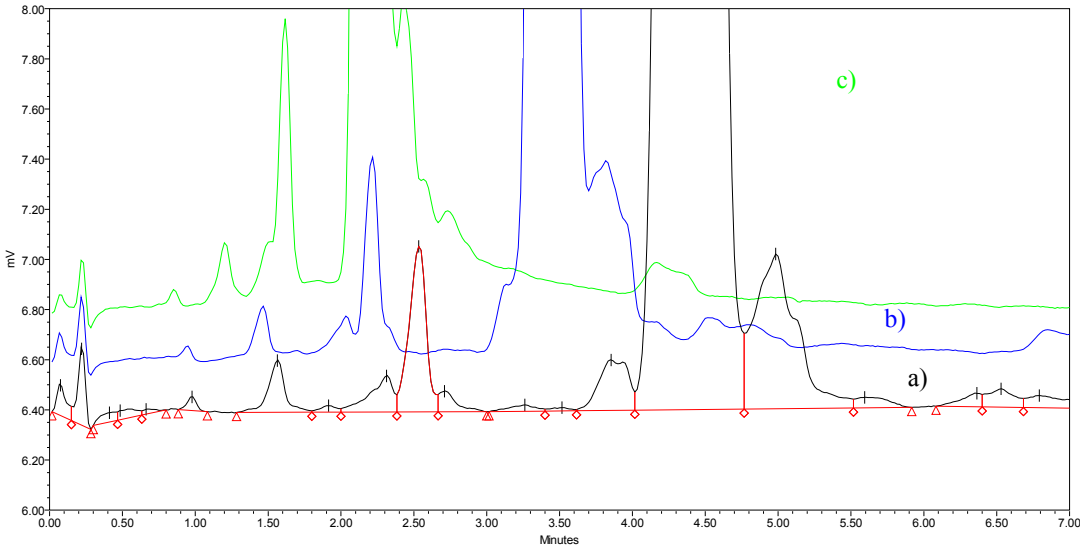


Figure 96: Chromatogram of a Temperature program 40 – 200 °C at a) 40 % power (16 °C/min), b) 50 % power (20 °C/min) and c) 100 % (40 °C/min), at 5 % B on an XBridge C8 2 µm, 4.6 x 30 mm for SBA for a 5 % acetonitrile eluent at 265 nm.

To improve resolution of the main impurities sitting on the front and tail of the peak a two stage temperature ramp was applied (Figure 97).

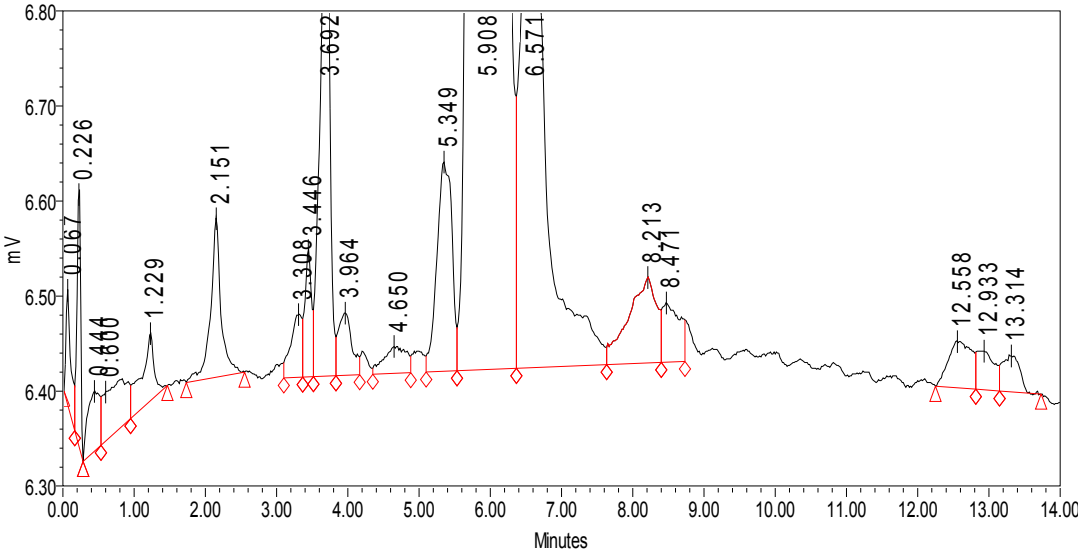


Figure 97: Chromatogram of a Temperature program: 5 %B 2 stage ramp: 30 – 50 (9°C/min / 18%P) – 200°C (18°C/min / 35%P) on an XBridge C8 2 µm, 4.6 x 30 mm for SBA (5 % acetonitrile diluent) at 265 nm.

The main band was starting to be resolved from its related impurities for the 5 % organic concentration on the two stage ramp where impurities SBB (RRT 0.6); SBC (RRT 0.9) and SBD (RRT 1.1) SBE (RRT 2.1) have all been identified (Table 33).

Table 33: Mass ions identified for a temperature program: 5%B 2 stage ramp: 30 – 50 (9°C/min / 18%P) – 200°C (18°C/min / 35%P) on an XBridge C8 2 µm, 4.6 x 30 mm for SBA (5 % acetonitrile diluent)

RT (min)	**Observed M+1**	**Identified**
0.341	232	SBD
2.934	513	SBF
3.668	328	SBC
4.120	342	SBB
6.190	370	SBA (main band)
6.703	386	SBG
6.924	540	SBH
8.251	242	Tetra butyl ammonium bromide, TBAB (MS only)

Conditions could be manipulated to give varying selectivity profiles, for instance when ran at 10 % acetonitrile for 2 staged temperature ramp peaks eluting first are sharpened and peaks eluting around the main band have a different elution order (Figure 98).

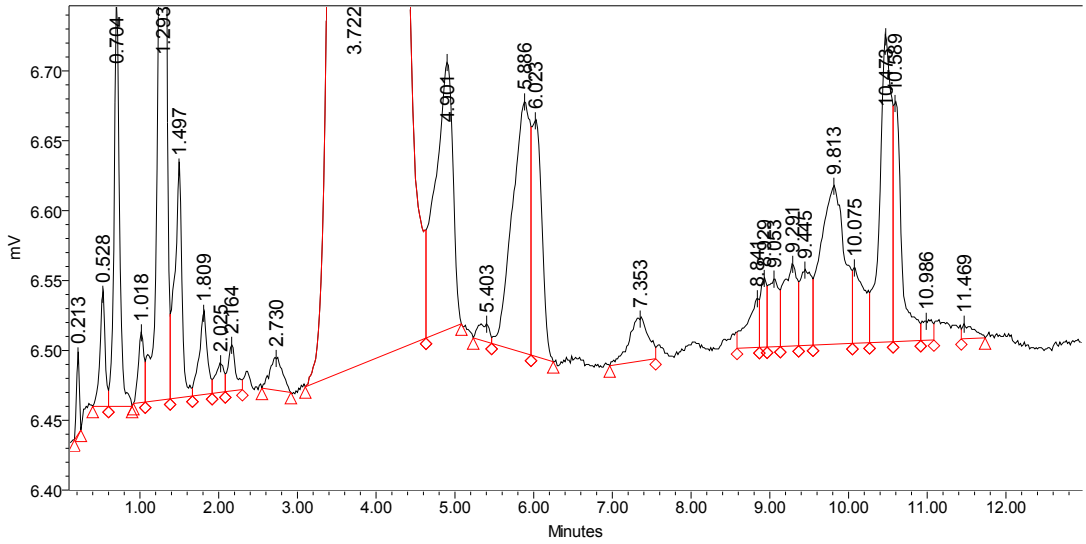


Figure 98: Chromatogram of a Temperature program: 10 %B 2 stage ramp: 30 – 100 (9°C/min / 18%P) – 200°C (40°C/min / 100%P) on an XBridge C8 2 µm, 4.6 x 30 mm for SBA (5 % acetonitrile diluent) at 265 nm.

4.4.2.5 Linearity

Data for linearity of the main band SBA related substances was obtained for the run performed with 10 % acetonitrile and yielded a correlation coefficient >0.95 for multiple R (Figure 99).

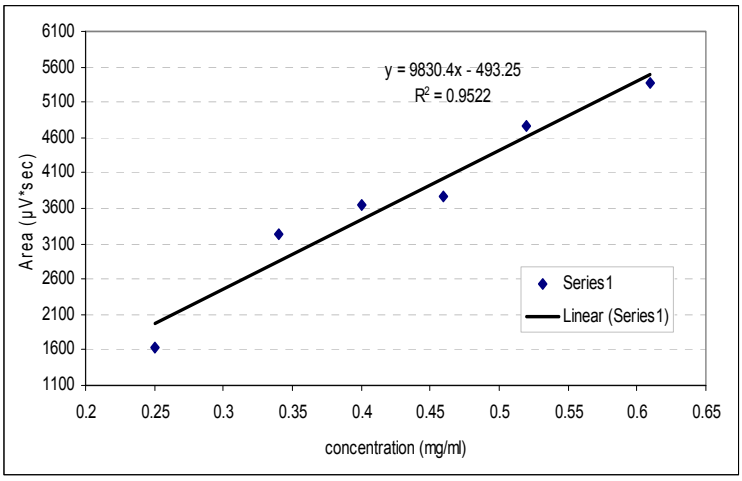


Figure 99: Linearity data for SBA related substances method performed at 10 % acetonitrile on a 2 stage ramp: 30 – 100 (9 °C/min) – 200 °C (40 °C/min) on an XBridge C8 2 µm, 4.6 x 30 mm at 265 nm.

This indicated that given a new column that had yet to suffer the phase loss encountered over the course of many temperature programmed runs, then acceptable linearity looked achievable. A lot was achieved with the specificity of the method by using a temperature programmed run instead of a solvent gradient. Peak capacity was increased with a short column demonstrating how increased selectivity and sensitivity were improved with temperature combined with superheated water. A change of the ligand i.e. C18 or phenyl group on the stationary phase could also improve resolution and could give greater selectivity.

4.4.2.6 Precision

Data was also obtained for precision on the 10 % acetonitrile run (40 – 200 °C at 40 °C/min). The SBA peak area and retention time for seven repeat injections on a temperature programmed gave < 2.0 % RSD's (Table 33).

Table 33: Precision data for SBA related substances method assayed as Figure 138.

Injection (20 mg/ml)	RT (min)	Area (µV*sec)
1	1.755	74010
2	1.752	73942
3	1.754	73538
4	1.743	73547
5	1.753	73967
6	1.745	73925
7	1.747	73779
RSD	**0.3**	**0.3**

4.4.2.7 Superheated Water Chromatography

The lower the organic proportion the better the resolving power of the temperature program; therefore several runs were performed with superheated water as the eluent. Changing the mobile phase to completely aqueous gave a change in selectivity whilst still maintaining a sharp peak for the main band under different gradient conditions (Figure 100).

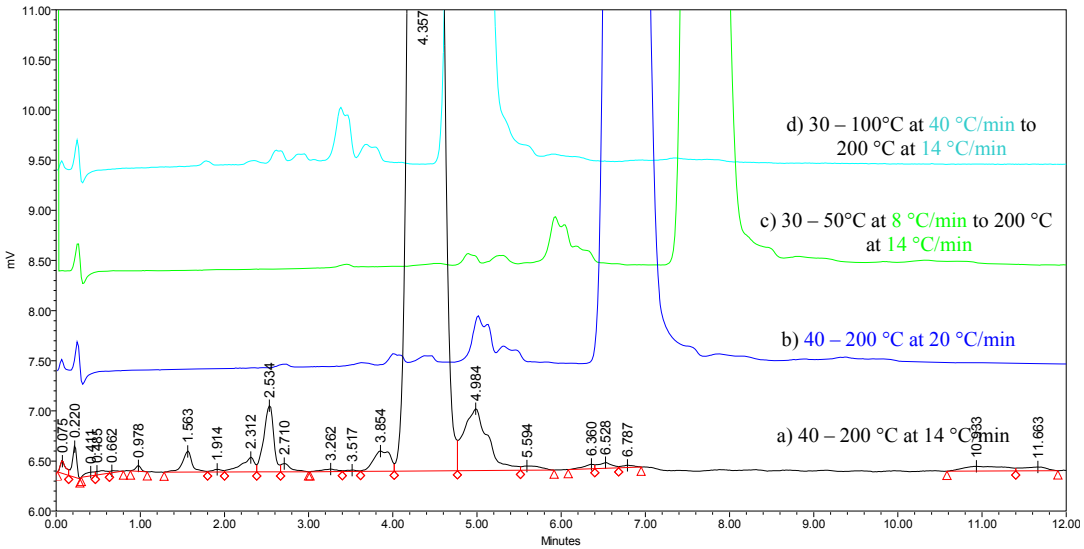


Figure 100: Overlaid chromatograms of a temperature programmed runs under superheated water conditions: a) 40 – 200 °C at 14 °C/min; b) 40 – 200 °C at 20 °C/min; c) 30 – 50°C at 8 °C/min to 200 °C at 14 °C/min and d) 30 – 100°C at 40 °C/min to 200 °C at 14 °C/min with 5 % B on an XBridge C8 2 µm, 4.6 x 30 mm for SBA with UV detection at 265 nm.

The chromatogram demonstrates how pure water can be used as the mobile phase under temperature programming conditions and a good peak shape obtained within a reasonable time frame. Peaks previously not resolved fully from the main band are now well removed; however, sensitivity of the UV response has been affected.

4.4.3 Refractive Index Detection

A refractive index detector was also linked in series to the HP1100 based at GSK. Initially the same conditions were used as in Section 4.6.2.2 for the separation of the test mixture but with RI detection the expectation was to see very little. However, surprisingly peaks were visible (Figure 101) and were baseline resolved indicating that this was a real application potential for temperature gradients.

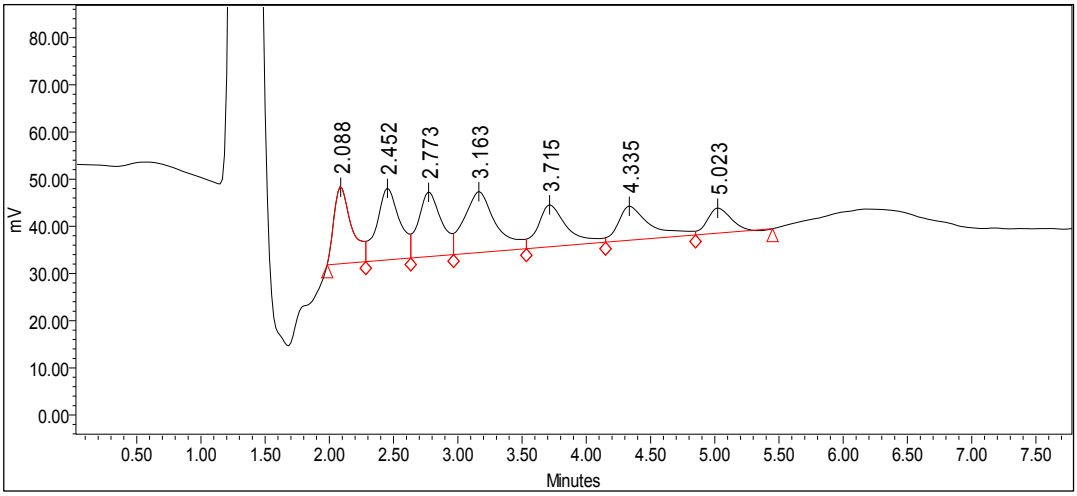


Figure 101: Separation of a phenone test mixture on a Temperature Gradient Run of 40 – 200 °C at 40 °C/min on a PLRP-S 2 x 150 mm 3 µm, with RI Detection at 70 % acetonitrile / 30 % water at 0.4 ml/min

The last 3 peaks in the chromatogram were identified by individual injections as hexanophenone, heptanophenone and octanophenone, respectively, of the phenone mixture. It is clear that the injection solvent is different to the eluent, which is

demonstrated by the huge solvent front at the start of the run. This highlights the problem with RI as it sees everything making it normally impossible to perform a solvent gradient run, thus limiting its application potential, whereas with a temperature gradient the eluent composition remains constant. This method was not optimised in terms of amounts injected and diluent / eluent compatibility and could probably be improved but demonstrated the feasibility of the approach. Refractive index detection is less sensitive than UV but this chromatogram has shown that detection is possible with a loading similar to that used in UV. The purpose was to show whether a temperature gradient would affect the baseline and subsequently the chromatography. This initial look gave the indication that it was worth pursuing with several objectives to consider.

4.4.3.1 Method Development for a Fast Temperature Program with RI Detection

Although RI detection is not as sensitive as UV it will detect compounds that do not have a chromophore or have a poor chromophore, such as the proprietary compound GSKA. This compound was used as a pilot to ascertain if acceptable chromatography could be obtained with RI detection under temperature programming conditions

4.4.3.1.1 Conditions

System:	Agilent HP1100 LC system with RI detector hyphenated to the CSI oven; eluent pre-heating carried out by incorporating tubing inside the oven with an aluminium jacket
RI attenuation:	500 x 010^3^{n}RIU
Column:	XBridge^{TM}, 2 µm 4.6 x 30 mm
Mobile Phase:	water / acetonitrile (pre-mixed)
Parameters varied:	% B and temperature programming conditions
Flow rate:	1.0 ml/min + flow rate programs
Injection volume:	10 µl
Amount of Active:	20 mg/ml

A blank injection was assessed for an extreme temperature ramp of 40 to 200 °C at 40 °C/min (100 % power) with 5% acetonitrile). This gave an undulating baseline (Figure 102) but compared to the normal method of assay, which was a solvent gradient ramp of 0 to 40 % B in 5 minutes still had promise (Figure 103).

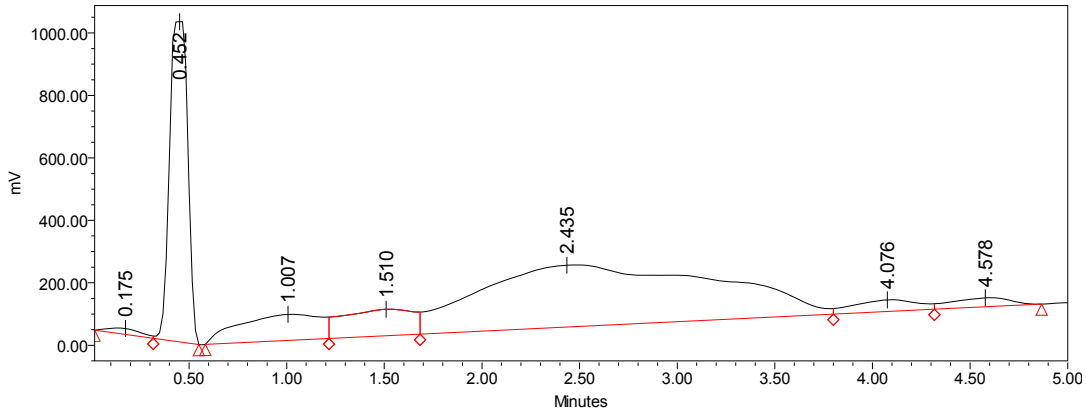


Figure 102: Chromatogram of a blank injection performed on a temperature gradient of 40 – 200°C at 100 % power XBridge™ 4.6 x 30 mm, 2 µm with 5 % acetonitrile/95 % water at 1 ml/min with refractive index detection

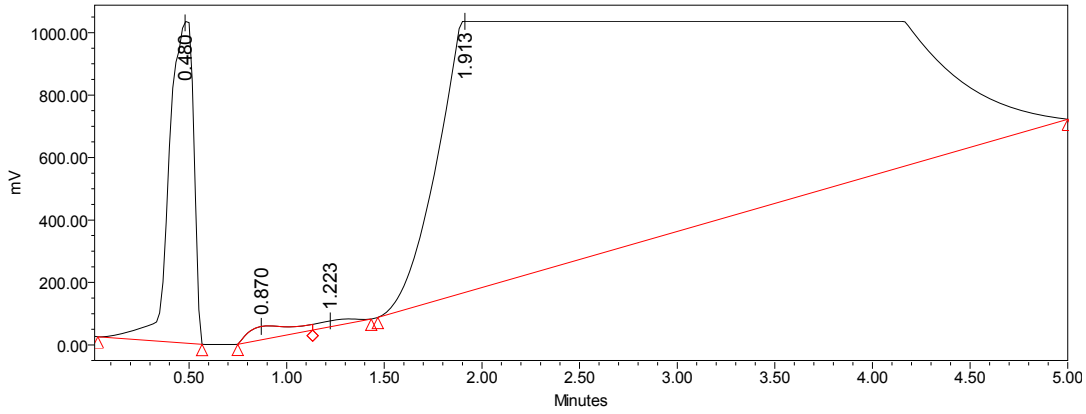


Figure 103: Chromatogram of a blank injection performed on a solvent gradient of 0 to 40 % B in 5 minutes at 40 °C on an XBridge™ 4.6 x 30 mm, 2 µm with 5 % acetonitrile/95 % water at 1 ml/min with refractive index detection

An initial run at 40% acetonitrile was performed as a benchmark. The acetonitrile content was then reduced until a run time of approximately 10 minutes was obtained at a concentration of 5 %. Temperature programs were then applied to the separation to

improve the peak shape and increase sensitivity. Several scouting runs overlaid in Figure 104 indicated that the higher the upper temperature and faster the ramping rate the sharper the peak profile.

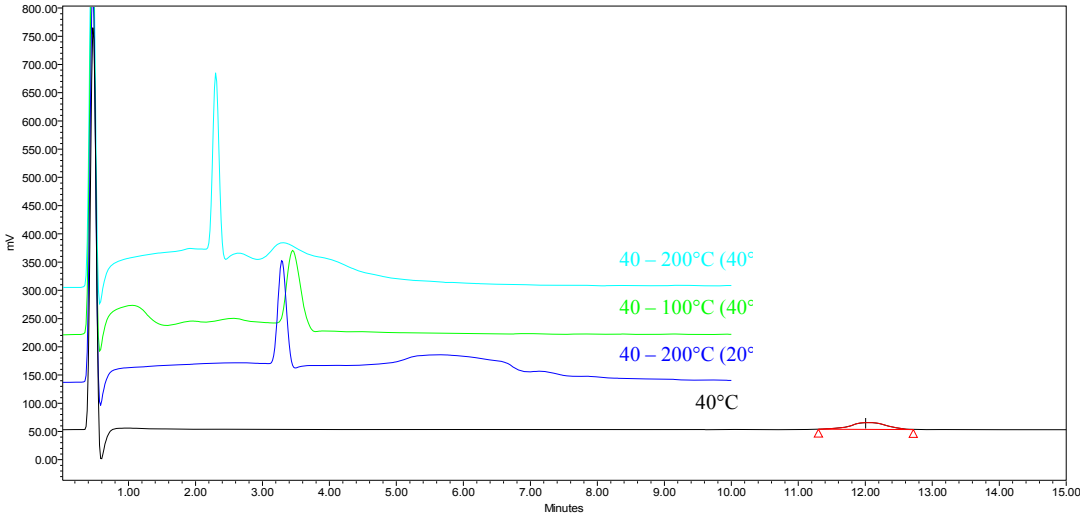


Figure 104: Temperature gradient scouting runs of GSKA: 40 – 200 °C (20 °C/min); 40 – 100 °C (40 °C/min) and 40 – 200 °C (40 °C/min) overlaid with a 40 °C run (red peak) on an XBridge^{TM} 2 µm 4.6 x 30 mm column, with 5 % acetonitrile/95 % water at 1 ml/min with refractive index detection

The chromatography parameters demonstrated how components were more easily quantified the sharper they became (Table 34). The symmetry and tailing factors were improved for all runs.

Table 34: Chromatographic data for temperature gradient scouting runs of GSKA on an XBridge^{TM} 2 µm 4.6 x 30 mm, with 5 % B at 1 ml/min with refractive index detection.

Temperature Program: 40 – 200 °C at 40 °C/min (100 % power)							
Name	Retention Time	Area	Height	Asym @ 10	USP Tailing	Symmetry Factor	Width @ 50%
GSKA	2.03	2099836	320772	1.1	1.04	1.04	0.10
Temperature Program: 40 – 100 °C at 40 °C/min (100 % power)							
Name	Retention Time	Area	Height	Asym @ 10	USP Tailing	Symmetry Factor	Width @ 50%
GSKA	3.45	1840391	134132	1.5	1.24	1.24	0.22
Temperature Program: 40 – 200 °C at 20 °C/min (50 % power)							
Name	Retention Time	Area	Height	Asym @ 10	USP Tailing	Symmetry Factor	Width @ 50%
GSKA	3.29	1668674	187350	1.1	1.05	1.05	0.14

The temperature program that gave the best compromise for optimum sensitivity and peak sharpness was the 40 – 200 °C at a ramp rate of 20 °C/min. Further work was required to maintain a good baseline throughout the run where two parameters were having an adverse effect. Firstly, GSKA is not very soluble in highly aqueous solvents therefore the sample solvent/diluent cannot be matched to the eluent. Secondly, a disturbance was encountered during the run that could be due to column bleed. The baseline at the upper end of the temperature gradient for 100°C compared to an upper limit of 200°C yielded a flatter baseline. This was a baseline problem encountered earlier with the Linear detector, whose baseline would start to rise due to temperature related RI changes of the eluent. The earlier difficulty was solved by using a column with higher retentivity such as the PLRP-S (a HT column) so that the organic content of the eluent could be increased. In this instance this was not possible as when tested the PS-DVB column produced poor peak profiles (in UV). The work was continued by further investigation of the underlying effects causing these disturbances with a view to optimizing the method for validation purposes.

4.4.3.2 Solubility of GSKA

The first problem to be addressed was the solubility; GSKA was not fully soluble in 5 % acetonitrile, which would match the diluent to the eluent. So it was dissolved in DMSO in varying concentrations with 5 % acetonitrile in Table 35. Dimethyl sulfoxide was used as it is a highly polar liquid used in other techniques, such as NMR spectroscopy, to dissolve most compounds. It is hoped that DMSO may improve the baseline whereas acetonitrile behaves as a biphasic mixture when added to water causing the huge front signal due to the diluent.

Table 35: Solubility data for GSKA

Concentration (mg/ml)	Acetonitrile (µl)	DMSO (µl)	H2O (µl)	Visual Observations
19.57	8 (ml)	-	12 (ml)	Fully dissolved
19.40	50	100	850	Not dissolved after 15 min sonication
20.70	50	200	750	Dissolved after 10 min sonication
19.75	50	450	500	Fully dissolved after 5 min sonication

The separations of these solutions were performed under the same temperature programme condition for all runs (Figure 105). A method had already been validated for a diluent of 40 % acetonitrile, thus this is deemed as adequate for solvation of the active pharmaceutical ingredient (API). However, although the DMSO appeared visually to have dissolved the API, the areas for the different solutions differed.

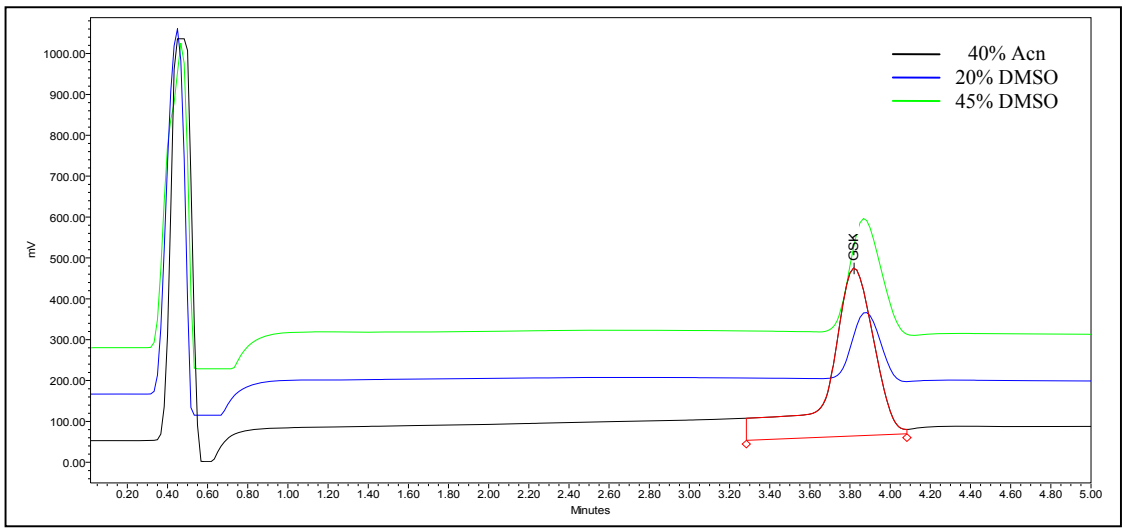


Figure 105: GSKA assay injections in different sample solvent combinations as outlined in table 53 at 5 % acetonitrile on an XBridge C8, 2 µm 3 x 30 mm at 1 ml/min with refractive index detection

The 40 % acetonitrile diluent demonstrates the problem with RI peaks as the plug of solvent has an effect on the tailing of the main assay peak. This characteristically steps down and the baseline is offset. Although the same effect is apparent with the DMSO diluent is not as pronounced allowing for more accurate quantitation. The areas for the peaks suggest that the API in 20 % DMSO was not fully soluble as the area is approximately half of that found for the 45% DMSO and 40% acetonitrile data. The 45% DMSO did compare favourably but there is still a difference of 32%, which may be attributable to the work-up. The change in baseline during the run was a major factor causing integration problems and subsequently validation of the temperature program method. The 40% acetonitrile assay peak had to be manually integrated to attain a reasonable peak area.

4.4.3.3 Investigation of the Ramp Rate of the Temperature Program

A likely cause of the baseline disturbances were RI changes related to a change in temperature so a blank solvent sample was injected on the 40 to 200 °C at 40°C/min to

determine if the baseline gave the same profile on a temperature gradient. The upper temperature limit was then altered to 150 °C to reduce suspected column bleed when at the upper temperature limit. The same profile was obtained slightly offset (Figure 105). Observations throughout the run implied that the baseline only became a problem when the oven had reached its target temperature and was trying to equilibrate. Thus, a change in ramp rate to 16 °C/min so that it did not reach its set-point during the 5 minutes needed for elution should ensure baseline regularity (Figure 106). This suggested that the baseline disturbance were related to temperature differences of a positive and negative nature. If there was constantly increasing temperature then the baseline remains flat.

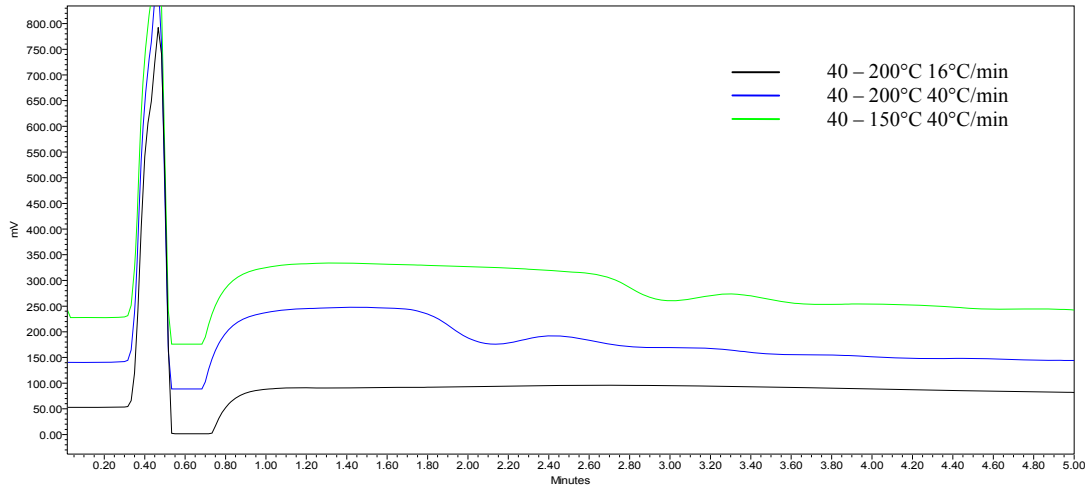


Figure 106: Blank injections for GSKA assay run to monitor baseline differences whilst varying temperature program parameters: 40 – 150 °C (40 °C/min); 40 – 200 °C (40 °C/min) and 40 – 200 °C (16 °C/min) at 5 % acetonitrile on an XBridge C8, 2 µm 3 x 30 mm at 1 ml/min with refractive index detection

The next task was to inject the analyte at 16 °C/min, which gave a flat baseline (Figure 107). This enabled an integration method to be used without the need for manual integration (Table 36).

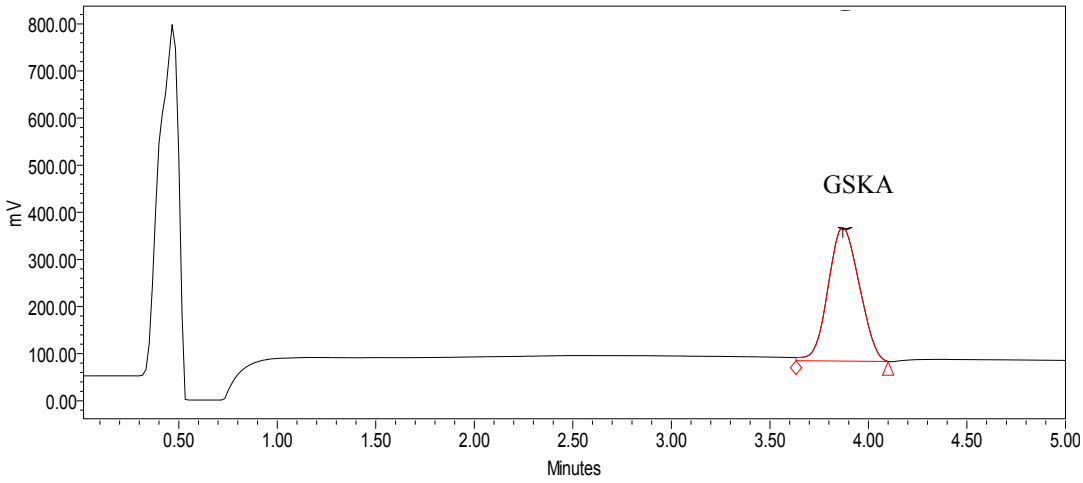


Figure 107: Separation of GSKA in MeCN/DMSO/H2O (5:45:50 % v/v/v) diluent on a temperature program: 40 – 200°C at 40 % power at 5 % acetonitrile on an XBridge C8, 2 µm 3 x 30 mm column at 1 ml/min with refractive index detection

Table 36: Chromatographic criteria for GSKA for a temperature program: 40 – 200°C 16°C/min (40 % power) in diluent MeCN/DMSO/H2O (5:45:50 % v/v/v) at 40 % acetonitrile on an XBridge C8, 2 µm 3 x 30 mm column at 1 ml/min with refractive index detection

Temperature Program: 40 – 200 °C at 16 °C/min (40 % power)							
Name	**Retention Time**	**Area**	**Height**	**Asym @ 10**	**USP Tailing**	**Symmetry Factor**	**Width @ 50%**
GSKA	3.87	3119667	284820	1.2	1.06	1.06	0.17

This separation was also examined using a 20 °C/min gradients but this gave the shift downwards shift in the baseline at the tail of the peak characteristic of RI. Although a sharper peak shape was achieved at a faster ramping rate it was found that a symmetrical peak was only obtainable with a shallow ramp. As suspected the perturbation in the baseline starts as the oven tries to maintain equilibrium at the upper gradient temperature limit. These subtle pressure changes are caused by the oven temperature fluctuating around the set-point of 200°C by ± 2.5°C (1.3%).

4.4.3.4 Flow rate Programming

Pressure changes within the system/oven set-up appeared to be responsible for baseline noise, which flagged up an opportunity to see if flow rate programming could be employed to improve the separation. Three flow rate programs (Table 37) were applied and the results are shown in Figure 108. A decreasing and increasing flow rate program was incorporated to challenge the stability of the baseline.

Table 37: Flow rate gradients used during a temperature program of 40 – 200°C at 40°C/min (100 % power) for 5 % acetonitrile on an XBridge C8 2µm 3 x 30 mm at 5 % acetonitrile at 1 ml/min

Gradient key	Time (min)	Flow rate (ml/min)
1	0.00	1.9
	2.00	0.8
2	0.00	0.8
	3.00	1.2
3	0.00	0.8
	2.00	2.0
4	0.00	1.0

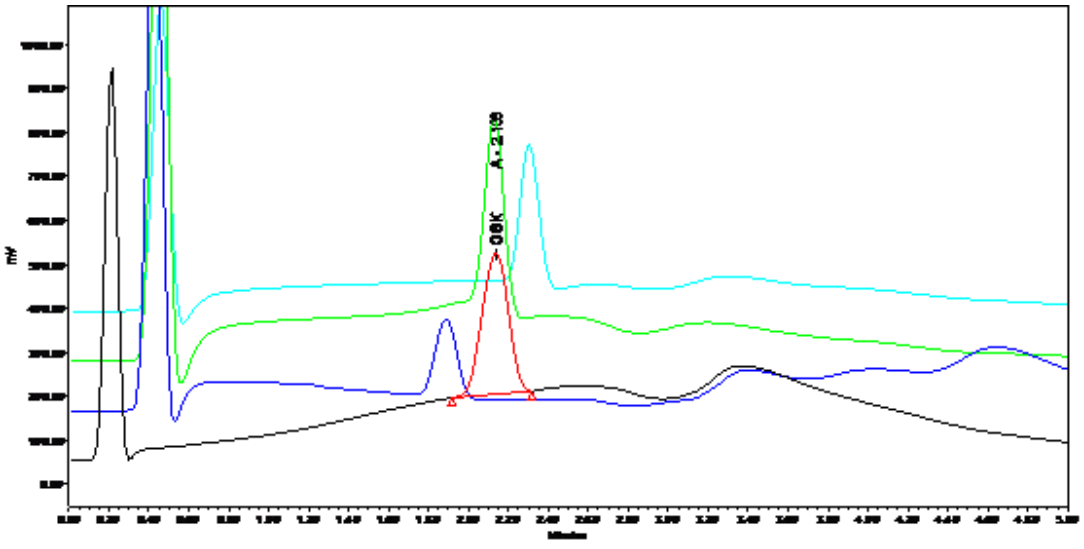


Figure 108: Separation of GSKA using temperature Gradient of 40-200, 40°C/min (100% power) with different flow rate program profiles (Table 37) at 5 % acetonitrile on an XBridge C8, 2 µm 3 x 30 mm at 1 ml/min with refractive index detection.

Surprisingly, a flow rate program (3) that more or less kept up with the temperature program improved the baseline. The baseline disturbances in the RI chromatogram appeared to occur when the greatest pressure changes were apparent. By increasing the flow as temperature increased, which reduced the viscosity of the mobile phase; there was a compensatory effect (Figure 109). As soon as the flow rate program stopped the baseline becomes more erratic. The only disadvantage of this method was peak sensitivity, increasing the flow led to on-column dilution effects reducing the peak area to approximately half but integration was more accurate. It was concluded that pressure effects were the cause of the post-peak baseline offset in RI detection. If a nearly constant pressure could be maintained then good baselines were obtainable without cycling effects.

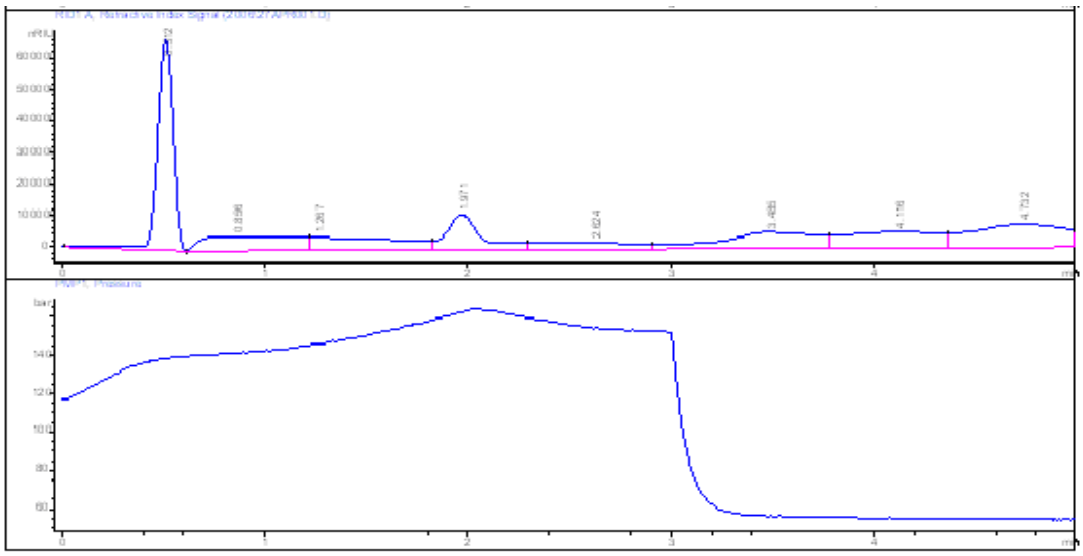


Figure 109: Pressure profile and RI chromatogram for flow rate program: 0.8 – 2.0 ml/min in 0.00 – 2.00 minutes, combined with temperature program: 40 – 200 °C, 40 °C/min (100 % power) at 5 % acetonitrile on an XBridge C8, 2 µm 3 x 30 mm column at 1 ml/min

4.4.3.5 Validation of RI Assay method under temperature program optimised conditions

The conditions chosen to validate the method are listed in Table 38:

Table 38: Optimised conditions for a validated method for the assay of GSKA

Diluent	MeCN/DMSO/H_2O (5:45:50 % v/v/v)
Eluent (pre-mixed)	MeCN/H_2O (5:95 % v/v)
Temperature program	40 – 200°C at 16°C/min (40 % power)
Flow rate program	0 – 3 min; 0.8 – 2.0 ml/min

The main compound and two impurities were then examined. However, there is insufficient resolution between the drug impurity GSKC and the main assay peak GSKA (Figure 110). This is due to column chemistry as a C8 phase has been used not due to the method.

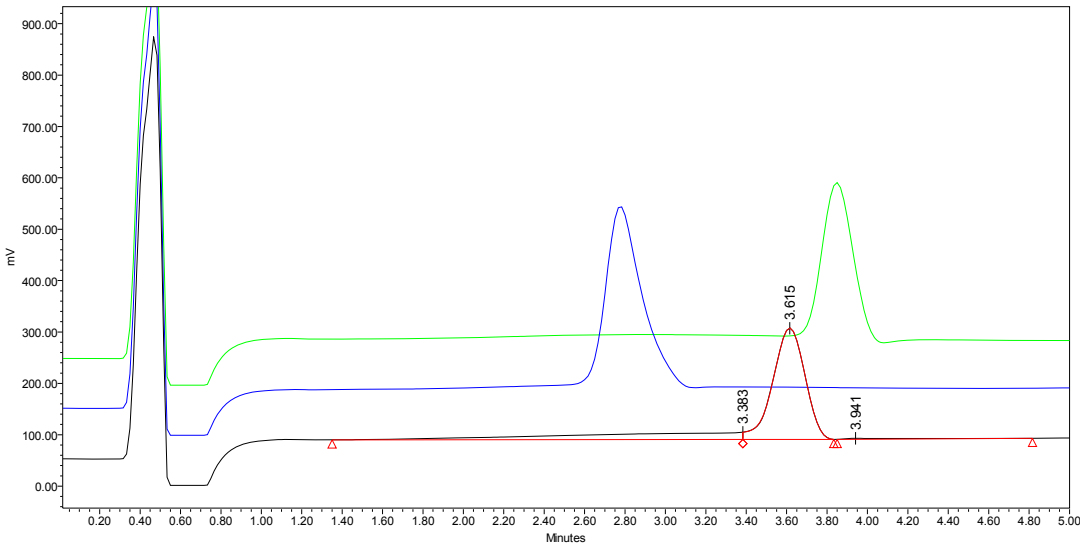


Figure 110: Overlay of Singly Injected Impurities GSKB and GSKC with GSKD for optimised conditions 40 – 200°C at 40°C/min (100 % power) on an XBridge C8 2µm 3 x 30 mm with 5 % acetonitrile at 1 ml/min (table 44) and refractive index detection

The detector response for GSKA was examined using the refractive index detection coupled with a temperature programme only and coupled with a flow programme (Table 39). The resulting linearity plots showed good correlations with R^2 values of 0.99 (Figure 111).

Table 39: Linearity Data for GSKA with RI Detection

	Temperature program (40 – 200 °C at 16 °C/min)	Temperature program (40 – 200 °C at 40 °C/min) + Flow rate program (0 – 3 min; 0.8 – 2.0 ml/min)
% nominal (20 mg/ml)	Area (µV*sec)	
80	3358211	1513477
90	3835993	1837271
100	4612444	2163498
110	5166787	2411288
120	6153068	2827787

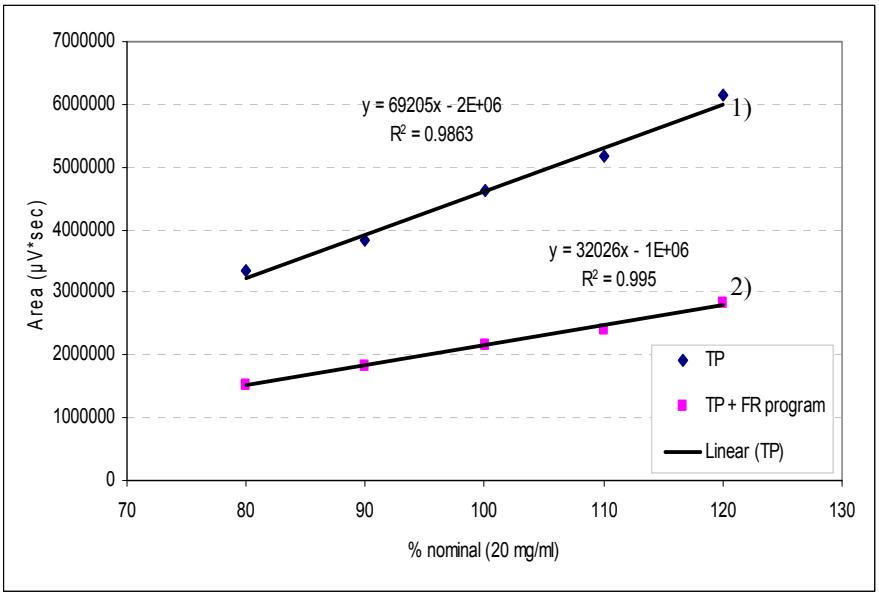


Figure 111: Linearity plots for GSKA for a temperature program 1) 40 – 200°C at 40°C/min (100 % power) and 2) the temperature program combined with a flow rate program: 0.8 – 2.0 ml/min in 0.00 – 2.00 minutes on an XBridge C8 2µm 3 x 30 mm with 5 % acetonitrile at 1 ml/min (table 44) and refractive index detection

4.4.3.5.1 Summary

Validation was not a problem with a temperature program provided the entire run was performed on a temperature gradient and the column was stable enough to withstand extreme conditions brought about by the very efficient heat transfer in this heating system. Unlike air-forced heating systems available for LC the resistive heating system

does not suffer the huge lag in heat transfer through the column wall and the eluent pre-heater as these have been incorporated inside the oven. The downside is that under temperature programming conditions the column is more likely to bleed compared to being ran isothermally but that is another area of technology that is catching up to the demands of the industry. Thus, this is an area that will gain direct benefit from utilising temperature programming as a means of assisting the elution capability for this type of detection, where chromophores are absent and analysis times cover a wide range.

4.4.4 Evaporative Light Scattering Detection

Another route often used for the determination of compounds lacking a chromophore is the light scattering detector and it was of interest to test if this could be coupled to temperature gradient elution. Caffeine, a component used to calibrate ELSD detectors (as it is non-volatile) was used to calibrate and optimize the detector settings for the ELSD. This component was then injected to determine if a temperature gradient was applicable / advantageous to this type of detection.

4.4.4.1 Chromatographic Conditions

System:	Agilent HP1100 LC system with ELSD detector hyphenated to the CSI oven
Detector settings:	N_2 pressure: 2.5 bar; temperature 40 °C; gain: 12
Column:	PLRP-S 3 µm 2.0 x 150 mm
Mobile Phase:	Eluent A: 0.05 % TFA in H_2O
	Eluent B: 0.05 % TFA in MeCN
% B:	15
Temperature Program:	40 – 130 °C (28 % power ~ 17 °C/min)
Flow rate:	0.4 ml/min
Injection volume:	10 µl
Amount of Active:	0.3 mg/ml (diluent: MeCN/H_2O 50:50 % v/v)

Time constraints meant that only a brief period of time was spent with the ELSD detector, however, this did yield some interesting results. For instance a temperature program did not affect the crystallization process for caffeine (in the evaporating eluent). The chromatogram obtained for temperature program against an isothermal temperature was superior in peak shape (Figure 112). Although subtle this was mainly due to the phase used, as the polymer phase behaved differently to a silica-based phase. This resulted in the temperature having a lessened effect on the retention of the compound. The baseline was unaffected by ramping up the temperature and in hindsight this would have been better without the post-cooler as the additional heat would probably have improved the desolvation process and the quality of the crystals produced. The temperature program proved its benefits and is an area worth pursuing and again temperature programming has demonstrated its flexibility and applicability to different LC (hyphenated) applications.

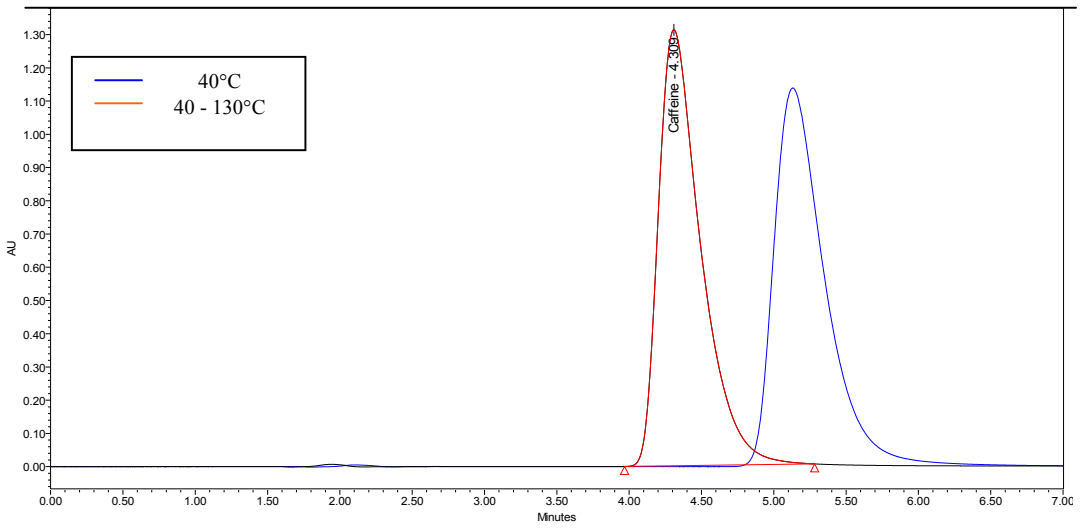


Figure 112: Temperature gradient run of 40 – 130 °C (17 °C/min) overlaid with a 40 °C run for Caffeine on a PLRP-S 3µm, 2.0 x 150 mm with 15 % B (0.1 % TFA in acetonitrile) at 0.4 ml/min with ELSD Detection.

Chapter 5. Conclusions and Future Work

5.1 Conclusions

The prototype Cambridge Scientific Instruments oven utilising "*resistive heating*" to heat a LC column both externally via the oven, and internally, via the eluent pre-column proved to be very effective. Actual ramping rates achieved of 40 °C/min were evidence that the heat was being successfully transferred with a response that yielded rapidly eluted and sharp peaks that were well resolved from each other. The column hardware that encased the stationary phase was found to have a big impact on the efficiency of this oven design. However, this was utilised to its best advantage by using short wide-bore (4.6 mm I.D.) columns. This combined with highly aqueous mobile phases enhanced energy transfer and allowed more heat to be pumped into the heart of the separation, the stationary phase. So much is reliant on this aspect and then the phase's ability stability both in mechanical and chemical strength. On a more basic level all of the separations irrespective of column geometry were improved in terms of peak efficiency and a reduction in analysis times. Therefore, the applicability of resistive heating was flexible in its support of current practices as well as easing the way towards miniaturisation.

The heating technique that was used (section 4.4) where the set-up of the system used the oven to heat both the eluent and the column out-stripped the other set-ups and the air-forced oven (GC columns ovens) approach used previously. The carbon footprint was reduced as we are using a resistively heated oven that runs on approximately 40 kW unlike the conventional air-forced ovens that run at approximately 500 kW. Solvent consumption was drastically reduced and in some cases eliminated.

Pre-heating the eluent pre-column showed that this could be used as a stand-alone technique for heating a column to enhance peak profiles. It was demonstrated that when combined with an elevated flow rate, the parabolic flow profile could be flattened by introducing additional heat by friction. This compensated for the temperature mismatch

between the incoming column eluent and the column temperature that was at ambient (with insulation). This also, supported the previous observations that the pre-heated eluent entering the column should be a few degrees lower to that of the column to compensate for the parabolic profile induced across the column phase by frictional heating.

The eluent pre-heater unit used for column heating by pre-heating the eluent only, led to a greater understanding of what is required to achieve an analyte focusing effect on a temperature gradient. This would be directly instrumental in winning the battle to generate faster separations, increase plates, and focus analytes creating much sharper peaks and providing improved resolution. The resolution test mixture used for the project was ideal in that it provided a real test bed that could demonstrate when outstanding capability was achieved. A temperature gradient assisted by an eluent that had also been subjected to a temperature gradient created this effect (section 4.5.6.3). When the organic content of the mobile phase was lowered further to 20 %, peak width across the run decreased and sharper peaks were produced. A more uniformed response was achieved and resolution > 1.2 for all critical pairs. Analytes when traversing the length of a long column i.e. 150 mm appeared to lose any focussing achieved in the first couple of centimetres of a column and need to be eluted at speed if these losses are not to be incurred. Peak compression plays its part but if the band profile is not optimised across the length of a column then any gain in terms narrower band widths is masked. This was why the short columns were important as they demonstrated what was believed to be achievable with temperature gradients.

Initially, the aim was to move towards micro-bore columns, and using aluminium column jackets for good thermal contact and efficient heat transfer. Taking into consideration the availability of HT phases with minimal column casing this proved to be a difficult objective to achieve. Although, the Merck Purospher (1mm I.D.) column was not a HTLC column, it did present the opportunity to use an aluminium jacket to aid heat transfer. The Waters XTerra columns (4.6 mm I.D.) were readily available and commonplace in pharmaceutical companies, such as GSK. Great things were expected

from the Polymer Laboratories PS-DVB column (2.1 mm I.D.) as it could be taken up to 200°C, enabling the flexibility in terms of moving towards superheated water separations. However, this phase seem to respond better to a gentle temperature gradient rather than a fast gradient of 40 – 200 °C at 40 °C/min. A short column (2.1 x 50 mm) was purchased for this purpose but baselines were so poor any further development was halted due to time constraints. This was thought to be the TFA content combined with temperature accelerating column bleed. Unfortunately, unlike the XTerra **phenyl** any column bleed induced by a temperature gradient gives rise to a highly UV active aromatic chromophore. Although, it is advertised to be stable to 200 °C a temperature gradient can weaken the stability of a phase where isothermally this would have no effect. Despite this, surprisingly sharper peaks were found for the longer column on a shallower temperature gradient (section 4.3.2). Interestingly, whilst peak width had reduced and sensitivity had improved for a 40 – 200 °C relative to a 40 °C run by ~80 % the overall analysis time had only been reduced by ~20 %. A polymer phase seemed to be having an additional contribution that is thermally responsive in a way not fully understood during this project. This could be a kinetic effect rather than a thermodynamic one and would require further investigation. This outlines the need for collaboration for a column to be designed purely with temperature-programmed separations in mind. Inevitably this would speed a temperature-programmed separation to its conclusion and make this a viable chromatography technique.

Hyphenation to other detector modes was successful and a temperature gradient was showed to be influential at assisting in various processes that resulted in impressive profiles that were not expected. For instance a MS trace was obtained in ESI mode for the phenone mixture on the Merck micro-bore column on a temperature program. The micro-bore column would normally be passed over due to long equilibration times. However, it causes less on-column dilution effects and results in an increase in sensitivity, highly beneficial in trace analysis. This demonstrated the applicability of temperature gradients in aiding the progression of micro-technology and omitting the use of solvent gradients. Temperature gradients have an obvious advantage as the viscosity and surface tension of the eluent is unchanged having less effect on ionisation and hence the abundance of ions

detected. Likewise, temperature programming has shown that it has the potential to be equivalent to solvent gradient elution, allowing for a separation with RI detection. This improved sensitivity and for a low sample loading produced peaks (section 4.6.3). A temperature gradient improved the baseline and with some further work (flow rate programming) removed the characteristic dip found on the tail of a peak in RI detection. Validation of a RI method for a GSK compound was achieved. An alternative detector ELSD was also trialled and again proved successful. This was with post-column cooling proving that this could replace a solvent gradient however, this is not required as the heat should assist in the vaporisation and crystallisation process. This detection was not exploited to its full potential as experiments feeding the hot eluent into the detector source were not investigated. A general point to make is that baselines were largely unaffected by a temperature gradient for UV, MS, RI and ELSD. Column bleed due to rigorous testing at extreme temperature proved to be the main cause of any baseline disturbance seen during the study.

5.2 Future Work

The prototype CSI Ltd LC column heating system proved that resistively heating a column was much more effective than conventional methods. The future focus should be to develop the superheated water capability and therefore exploit the analyte focusing aspect. Integral to the temperature programming capability of an LC column oven is the design of the eluent pre-heater. Clearly, from the experiments performed in the analyte focusing section (4.4) this needs to be designed to allow for a temperature programming functionality. This would certainly be desirable not only as an individual unit but also in combination with the oven as herein lays the instability factor (section 3.5). This would point towards a low mass heater or a zone controlled separately within the oven. New technologies could enable the development of a low mass pre-heater. For instance resistive heating via a thin conductive film coating is a technique that has already been used for fast temperature programming on open tubular capillary columns in gas chromatography (section 1.4.4). This could be applied to tubing to create a pre-heating device, which would fit the criteria of a low thermal mass allowing rapid heating/cooling

for an instant response. Equally, a LC column oven that had different controlled heating/cooling zones for the eluent pre- and post-column would give precise control providing rapid cooling is possible. Currently, the column is fed in to the oven, which is completely enclosed. If the oven design opened into two adjoining sections this would accommodate aluminium inserts moulded for all column types. However, this would not be the best approach as a standardised approach is needed to align a better outcome. Different materials present in different volumes/masses would complicate and compromise peaks separated on a temperature gradient. Column technology is one of the few hurdles that remain and challenge the applicability of temperature gradients as alternative to solvent gradients. Both, column phase and casing needs to be looked at more closely to establish the best materials fit for the purpose of efficient homogeneous heating. Temperature programming could then apply to wide and micro bore columns allowing for maximum flexibility. This should be followed by more investigation concerning the nature of the current phases available for HT applications. Collaboration with a suitable column company would be the most desirable way forward this way both of these elements could be addressed at the same time. Depending on the outcome this will also pre-determine oven design. For instance if a capillary column proved to be more stable this would have a big impact on the final construction.

This study showed that oven cooling patterns of less than 5 minutes can be achieved that enabled cycle times of 5 minutes. The future design of the oven should look further into using liquid nitrogen as it is possible that this could be reduced further and could introduce sub-ambient temperature capability for a complete LC oven solution. However, the work carried out in this study certainly clarifies that the bore of a column is not as important as the column casing when achieving sharp peaks. Given the difficulty securing appropriate columns, collaboration with a column company would be advisable so that a LC column oven could be designed to produce the best outcome in terms of chromatography and the cycle times. A bench-top LC-column oven is not so desirable given the inevitable difficulty in being able to accommodate all column hardware designs to transfer the heat necessary for impressive peak profiles and reduce analysis times.

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Appendix 1 Column List

Column number	Supplier/Company	Base material (functional group	Geometry (mm)	Particle size (d_p, μm)	pH range	Maximum temperature (°C)[4]
1	Phenomenex HyPURITY Hypersil	ODS	2.1 x 150	5	3 - 7	90
2	Waters Symmetry® Shield (Waters Corporation)	Silica (C8)	2.1 x 50	3.5	2 - 8	90
3	Phenomenex Gemini	Silica – organic layer (C18)	2 x 150	5	1 - 12	130 –150
4	Waters XTerra® RP18 (Waters Corporation)	Silica with methyl bonding	4.6 x 150	3.5	1 - 12	150
5	Waters XTerra® MS (Waters Corporation)	Silica with methyl bonding and endcapping (C18)	4.6 x 100	3.5	1 - 12	
6	Polymer Laboratories Limited, Shropshire, PLRP-S	Poly(styrene-divinylbenzene)	2.1 x 150, 4.6 x 50	3	1 – 14	200
7	Merck Purospher® STAR	Endcapped silica (RP18)	1 x 150mm	3	2 - 8	80
8	Waters XTerra® phenyl (Waters Corporation)	Silica with methyl bonding	4.6 x 50	3.5	1 - 12	150
9	Waters XBridge (Waters Corporation)	Ethylene-bridged Silica C8	3 x 30	2	1 - 12	150

[4] Not the manufacturers recommended range but from predicted stability

POSTGRADUATE RESEARCH TRAINING RECORD

Department-based training **Year** **2003 - 2006**

(including external training approved by the Department, but not by UK GRAD courses, Professional Development courses or other activities validated by Professional Development or supervisor)

Name of Student	**ID**	**FULL-TIME OR PART-TIME**
Fiona Harvey-Doyle	A388277	FT

Name(s) of Supervisor(s)	**Department**
R. M. Smith (Loughborough University), J. K. Roberts (GSK R&D Ltd) and P. A James (CSI Ltd)	Chemistry

Activity	**Time claimed in days**	**Date completed**
Destiny Memorial Lecture, London	1	20 Oct 04
UPLC Lectures (3M, Loughborough)	1	Oct 04
Laboratory demonstrator – post/under-graduates	12	Jun 03- Apr 06
Invigilating/Head Invigilator	3 (training)	17 Jan – 02 Feb 05
Project Meetings at GSK R&D Ltd, Stevenage	2	Feb 05, June 06
Telecoms with John Roberts, GSK R&D Ltd, Stevenage	2	Feb 04 – April 06
Project Meetings at Loughborough	1	17 Mar 05
Industrial Placements at GSK R&D Ltd, Stevenage	10 (total = 98)	April – Jun 05 May – Jul 06
EYP, Bedford	3	13 – 15 May 05

Presentation: GSK R&D Ltd. Stevenage	0.5	02 Jun 05
Presentation + Project meetings: CSI Ltd, Cambs.	2	09 Jun 05, Feb 06
HPLC 2005, Stockholm + Prize Winner – top 15 posters (total – 660)	5	27 Jun – 01 Jul
ARF, Plymouth: Poster Presentation	3	18 – 20 Jul 05
Supervision of Student Placement	4	25 – 28 Jul 05
Young European Chemist Conference, Brno 2005, Czech.	4	31 Aug – 03 Sep 05
PASG Autumn Conference, Coventry: Speaker	1	11 Oct 05
HTC-9, York: Poster Presentation	3	6 – 8 Feb 2006
Chromsoc Meeting: *Advances in selected key peripheral technologies*, Milton Keynes	1	24 May 06
Chromsoc Triad Meet: AstraZeneca, Charnwood, Loughborough	1	11 Oct 2006.