UNIVERSITY OF SOUTHAMPTON
FACULTY OF ENGINEERING, SCIENCE & MATHEMATICS

SUPERCRITICAL FLUID CHROMATOGRAPHY-MASS SPECTROMETRY.

The optimisation of ionisation and comparison of chemical space covered by high performance liquid chromatography and supercritical fluid chromatography, for compounds of pharmaceutical importance.

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A thesis submitted for the degree of Doctor of Philosophy

School of Chemistry

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ABSTRACT
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The Optimisation of ionisation and comparison of chemical space covered by HPLC and SFC, for compounds of pharmaceutical importance.

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High performance liquid chromatography (HPLC) is the most widely used separation technique within the pharmaceutical industry. Due to the growing need for high speed and high quality separations other techniques, such as supercritical fluid chromatography (SFC), are now being considered. The key advantage of SFC is minimal solvent waste, which is particularly important in preparative SFC, leading to fast sample recovery. Hence it is important to explore whether SFC, which promises to be cheaper and more environmentally friendly than conventional HPLC, can be applied more widely as a complementary method.

SFC coupled to mass spectrometry, with an electrospray ion source, was used to analyse diverse series of test compounds. The ionisation of samples in the absence of high voltage, *i.e.* ionisation voltages, was observed when a SFC was coupled to electrospray ionization (ESI) source. This novel ionisation process was further investigated and an attempt was made to explain this ionisation phenomenon and the improved sensitivity quantified. To probe this ionization mechanism, specific test compounds were analysed and data acquired with high voltages on (electrospray) or off (Novospray). Ammonium acetate and formic acid were introduced as buffer to see if Novospray ionization is thermo spray type or driven by charged residue model. The Novospray data was comparable or better than the classical ESI and atmospheric pressure chemical ionization (APCI) methods. The ionisation is neither thermospray type nor driven by charged residue model. Yet another possibility was sonic spray, where the ion intensity strongly depends on the gas flow velocity consentient with high pressure flow from SFC.

One of the objectives of this project has been to determine whether a generic set of rules can be applied to choosing the best technique for the separation and analysis of a given sample based on the chemistries of compounds involved. In an attempt to develop generic analytical and preparative methods, a diverse series of test compounds were analyzed on different stationary phase columns with use of a modifier, primarily methanol. To improve the chromatography on certain stationary phases additives have been used. For some compound types two peaks were observed upon injection, this appears to be linked to compound type and the injection procedure. Data attempting to explain this phenomenon is presented and in particular how the choice of injection solvent affects possible compound interactions with the stationary phase and peak shape.

Thus, a direct comparison of HPLC and SFC was undertaken with a diverse series of test compounds using the same conditions, to highlight the effectiveness of the two techniques in terms of speed and more importantly compound coverage. HPLC and SFC data are presented, comparing a generic analysis protocol with compound specific analyses. Preliminary findings showing the overlap between the two separation techniques is discussed and specific differences observed with the different column types used is outlined

Table of Contents

Abbreviations

2-EP	- 2-Ethyl pyridine
APCI	- Atmospheric pressure chemical ionisation
APPI	- Atmospheric pressure photo ionisation
BPI	- Base peak ion
CRM	- Charge residue model
CSP	- Chiral stationary phase
CV	- Cone voltage
DCM	- Dichloromethane
DEA	- Diethylamine
DMEA	- Dimethylethylamine
DMSO	- Dimethyl sulfoxide
ELSD	- Evaporative light scattering detector
EOR	- End of run
ES	- Electrospray
FAB	- Fast atom bombardment
FIA	- Flow injection analysis
FID	- Flame ionisation detector
GC	- Gas chromatography
HETP	- Height equivalent of theoretical plates
HPLC	- High performance liquid chromatography
HV	- High voltage
IEM	- Ion evaporation model
LC	- Liquid chromatography
Mol Wt	- Molecular weight
MS	- Mass spectrometry
PPU	- 2-Pyridyl propyl urea
SFC	- Supercritical Fluid Chromatography
SSI	- Sonic spray ionisation
TBA	- Tertiary-butylamine
TEA	- Triethylamine

TFA	- Trifluoroacetic acid
TIC	- Total ion chromatogram
t_R	- Retention time
UV	- Ultra violet
v/v	- Volume/volume

List of Figures

List of Tables

Declaration of Authorship

I, Mohini Thite, declare that this thesis entitled 'Supercritical fluid chromatography-mass spectrometry. The optimisation of ionisation and comparison of chemical space covered by high performance liquid chromatography and supercritical fluid chromatography, for compounds of pharmaceutical importance', and the work presented in it are both my own and have been generated by me as the result of my own original research. I confirm that:

- This work was done wholly and mainly while in candidature for a research degree at this University;
- No part of this thesis has been submitted for a degree or any other qualification at this university or any other institution;
- Where I have consulted the published work of others, this is always clearly attributed;
- Where I have quoted from the work of others, the source is always given. With the exception of such quotation, this thesis is entirely my own work;
- I have acknowledged all main sources of help.

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Chapter 1

Introduction

Drug discovery in the pharmaceutical industry is a complex and time consuming process involving hundreds of specialists. Typically, the process of design, synthesis, purification and testing of a drug candidate takes up to 10 to 15 years; a typical process cycle is shown in Figure 1.1. It is often a target-driven process, where the selected target is disease-relevant and, furthermore, suitable for drug development. Hence, along with the design and synthesis of molecules, identification, accurate and precise quantification at low levels also plays an important role in this industry. The accurate analysis of the drugs synthesised can take from six months to one year.

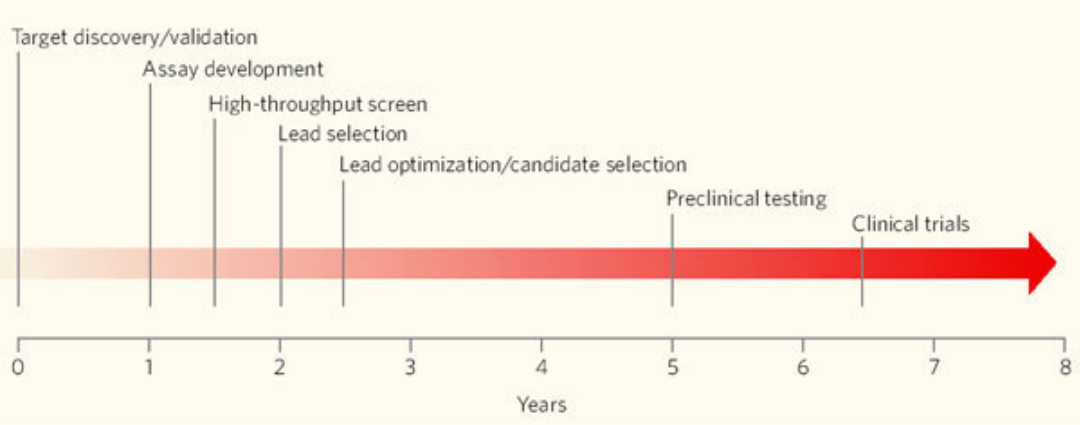


Figure 1.1: Drug-discovery timeline.[1]

Any time gained by faster analysis is crucial to the drug discovery process as it speeds the process of getting the drug to market. The success of achieving the final product is also linked to the accuracy of the analysis. Therefore, to avoid duplicating the analytical processes, it is important to be able to decide on the suitable technique, *e.g.* High Performance Liquid Chromatography or Supercritical Fluid Chromatography, for separation and identification of the target molecule. New drugs are generally more potent than earlier generations. As a

result the therapeutic dose is less and there is the requirement to detect smaller amounts of material. Thus, analytical techniques with low limits of detection are necessary. Further enantiometric purity is a requirement for many modern drugs as seen from the example of thalidomide drug which is discussed below.

The development of analytical methods that can separate and quantify enantiomers has gained more importance, since it became evident that enantiomers of pharmaceutical products may display different pharmacological behaviours; and that the desired biological activity of enantiomers is mostly restricted to one of the enantiomeric structures. Also the US Food and Drug Administration (FDA) have set more stringent guidelines for marketing chiral drugs. Often, one of the enantiomers is biologically active (eutomer) and the other (distomer) may exhibit unexpected adverse reactions, antagonistic activities, or toxic effects.[2] Thalidomide is one such known example from the past. The drug was marketed as a sedative with few side effects. It was also recommended as a remedy for morning sickness for pregnant women in the 1950s and early 1960s. Later it was discovered that thalidomide exists as two optical isomers. One enantiomer was effective against morning sickness and the other was teratogenic and caused birth defects. In the 1960s the effect of the drug on some of the painful symptoms of leprosy was discovered. In the 1980s, scientists once again became interested in the drug's complex properties and researchers began to explore its use in the treatment of a number of diseases, including cancer. Trials began in the 1990s and are now ongoing. It is now known that even when a stereoselective sample of thalidomide (only one of the optical isomers) is created, if administered, the pH in the body can cause racemisation. Thalidomide is now FDA approved drug for the treatment of leprosy and malignancy. But it is prescribed and received under strict and special process to avoid anymore children born with birth defects from the medication.

Other techniques such as ultra high performance liquid chromatography (U-HPLC or UPLC) have also been introduced as faster and robust analytical techniques. Since these are not easily scalable for preparative separations preparative SFC (Prep-SFC) is preferred. Prep-SFC was first introduced by Klesper *et al.*[3], where they mentioned the possibility to collect the separated compounds, but the

progress in development only started after Perrut[4] patented the large scale Prep-SFC. Some properties of supercritical fluids are particularly favourable for their use in Prep-SFC. These have good solvent properties, low viscosity and high diffusion coefficients and easy modulation of solvent properties by temperature and pressure adjustments.

Chromatography

The term ‘Chromatography’ covers a wide range of laboratory techniques used to separate sample mixtures into their individual components. Simply, it involves passing a mixture, dissolved in a "mobile phase" (which may be a gas, a liquid or a supercritical fluid) through a stationary phase, which can separate the compounds in the mixture and allow them to be isolated. It is known to be an important method for isolating and purifying compounds in the pharmaceutical industry. Chromatography was first mentioned by the Russian botanist Twsett, often referred to as the father of chromatography, in 1906. His work described the separation of plant pigments using column liquid chromatography.[5] Further developments in this field led to planar chromatography using paper as a plane support. This was soon superseded by thin layer chromatography (TLC). TLC is similar to paper chromatography except the plane stationary phase is a thin layer of adsorbent like silica gel on a flat, inert substrate instead of paper. Compared to paper, TLC has the advantage of better separations, and the choice between different adsorbents. Alongside, column chromatography was also developed with the introduction of liquid-liquid or partition chromatography. The accompanying theory came to be known as the plate theory.[6] Figure 1.2 given below is used to explain the terms used in chromatography.

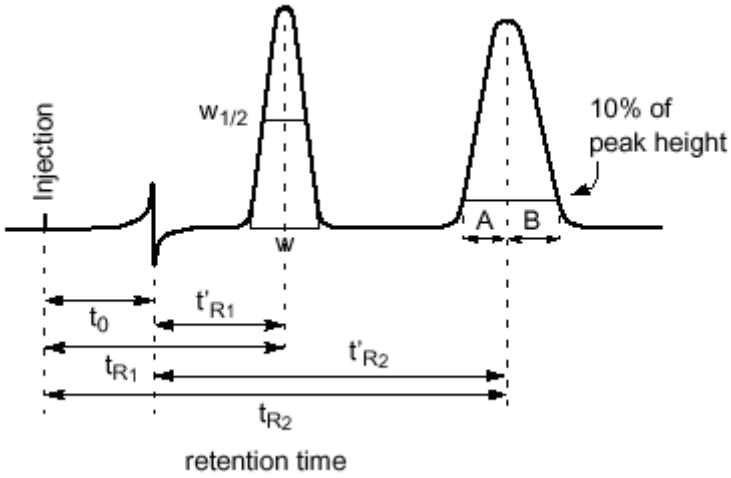


Figure 1.2: Basic chromatogram showing two analytes to explain the chromatographic terminology[7].

The retention times are as follows,

t_0 is the dead time of the column,

t_{R1} and t_{R2} are the retention times of analytes 1 and 2,

t'_{R1} and t'_{R2} are the net retention times of analytes 1 and 2.

The peak widths are as follows,

w is the peak width measured at the base of the peak,

$w_{1/2}$ is the peak width measured at half the peak height,

A is distance from the peak front to peak maximum at 10% of peak height,

B is distance from the peak maximum to peak end 10% of peak height,

Asymmetry, number of theoretical plates, HETP and resolution can be calculated from the above terms. Asymmetry (As) is the deviation from an ideal Gaussian peak shape. For a peak to be symmetrical the asymmetry factor should be as close to 1 as possible. This is calculated as follows,

$$As = \frac{B}{A} \tag{1.1}$$

Resolution (R) is the measure of separation of peaks. Resolution of the two analytes 1 and 2 is measured as follows,

$$R = \frac{2[t_{R1} - t_{R2}]}{[w_1 + w_2]} \tag{1.2}$$

In the separation processes, the mobile phase is continually flowing past the stationary phase; hence the solute does not spend sufficient time in the column for

equilibrium to be achieved. Therefore the column is divided into number of theoretical plates or cells of fixed height or length that will allow sufficient theoretical dwell time for the solute to equilibrate. The faster the equilibrium takes place the smaller will be the theoretical plates. Hence there will be more number of plates in a column. Thus the efficiency of the column depends on the number of theoretical plates (N).

$$N = 16\left(\frac{t_{R1}}{w}\right)^2 \quad \underline{\text{or}} \quad N = 5.54\left(\frac{t_{R1}}{w_{1/2}}\right)^2 \tag{1.3}$$

The height equivalent to a theoretical plate (HETP) (h) is the length in which the chromatographic equilibrium between mobile and stationary phase is established. Since n needs to be large, h should be as small as possible. The value of h is a criterion for the quality of the column. The h values depend on the particle size, the flow velocity, the mobile phase (viscosity) and especially on the quality of packing. The h value is calculated as follows,

$$h = \frac{L}{N} \tag{1.4}$$

where L is the length of the column.

Development of gas chromatography (GC) followed in the 1950s. Modern capillary GC gives good separation and narrow peaks due to rapid diffusion of the molecules in the gas phase. However, it is unsuitable for the analysis of thermally labile and non-volatile compounds. To achieve efficiency comparable to GC, high pressure was required for the LC column and thus HPLC originated describing high pressure LC which produced high performance separations.[6] Hence the term high performance liquid chromatography (HPLC) is also used. The most commonly used techniques in the pharmaceutical industry are HPLC and GC. The former can be readily used to analyze thermally labile compounds, but the separation is much less efficient because the diffusion is less in liquids than in gas.

HPLC is still the most widely used technique in the pharmaceutical industry. But, in order to achieve greater speed and accuracy in separation, further developments

have led to new techniques, namely ultra high pressure liquid chromatography (UHPLC) and supercritical fluid chromatography (SFC). SFC can be considered as a hybrid of gas and liquid chromatography. The difference between SFC and the other two methods is that SFC uses supercritical fluid as the mobile phase.

A supercritical fluid can be defined with the help of a phase diagram (Figure 1.3). Every substance above a certain temperature can no longer exist as a liquid no matter how much pressure is applied; likewise there is a pressure above which it can no longer exist as a gas no matter how high the temperature. These conditions are called the supercritical temperature and supercritical pressure respectively and are the defining boundaries on a phase diagram for a pure substance. A pure substance is considered to be in a supercritical state when its temperature and pressure are higher than its critical temperatures and pressures respectively.

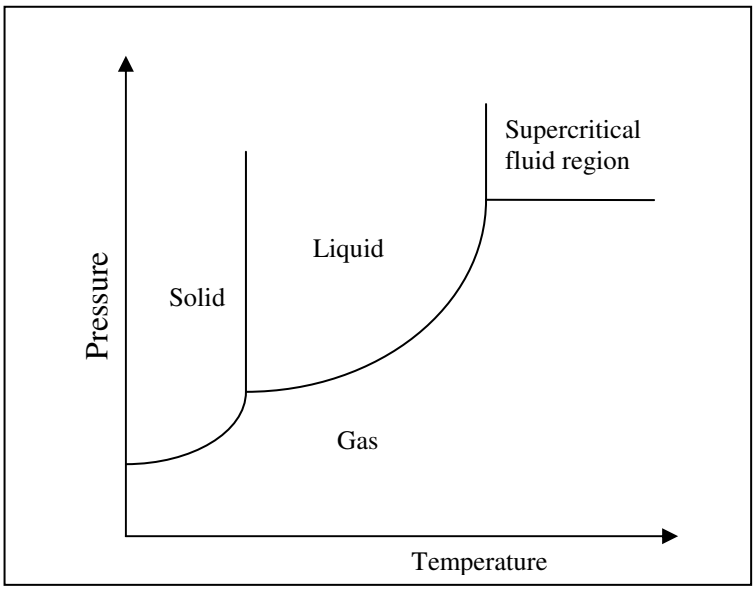


Figure 1.3: Phase diagram of a pure substance.

Supercritical fluids have good solvating properties and high diffusivity.[8] These properties of the supercritical fluid can make SFC up to ten times faster than HPLC, which is valuable for high throughput screening.[9] One of the most attractive features of SFC is the speed of method development. Column equilibration occurs much quicker for SFC compared with HPLC, and higher flow rates can be used without seriously compromising efficiency.[10] Most pharmaceutical laboratories face demands, like reduction in costs, which includes

maintaining equipment, solvent usage, and disposal of waste. Increased production and improved safety are the other requirements of these industries. The benefit of SFC is that it can be thought of as a subset of LC, utilizing similar equipments and methodologies. SFC has other advantages that are valuable to the pharmaceutical industry. Some of the advantages of SFC are fast/shorter method development analysis run times, higher efficiency and fast separations with high resolutions, rapid equilibration and high diffusivity rates resulting in faster column equilibrium. These properties of SFC lead to reduced cycle times along with minimal solvent waste, which is of importance in preparative SFC. This in turn results in fast sample recovery. It is also a financially less expensive and greener technology. SFC is becoming very popular in drug discovery due to the speed with which separations can be achieved. SFC has gained importance because it can analyse thermally labile and non-volatile compounds, not amenable to GC and with greater efficiency and detector compatibility than HPLC.

1.1 Mobile Phases for SFC

The use of supercritical fluid as the mobile phase in chromatography was first published in 1962 by Klesper *et al.*[11] In that study supercritical dichlorodifluoromethane and monochlorodifluoromethane were used as mobile phases to separate nickel etiporphyrin II from nickel mesoporphorin IX dimethyl ester. For a fluid to be an effective chromatographic mobile phase the solute must be soluble in it. Sie *et al.* extended the work on SFC to more polar solutes using iospropanol and Giddings used carbon dioxide. There are number of possible fluids that may be used in SFC as the mobile phase. Hydrofluorocarbons, supercritical ammonia, [12-14] sulfur dioxide and nitrous oxide have also been used as mobile phases. Although sulfur dioxide is polar, it is too corrosive. Due to general lack of success and safety reasons carbon dioxide (CO_2) has been a standard choice of the mobile phase. CO_2 has low interference with chromatographic techniques and suitable physical properties (non-flammable, low critical values) and low cost.[15] The critical temperature of CO_2 is 31°C and the critical pressure is 72 bar.[16] Supercritical CO_2 is a fluid with a polarity comparable to that of *n*-heptane. It is known from the supercritical fluid extraction processes, increasing the pressure increases the dissolving power of the fluid. Many

substances with single polar functional groups, *e.g.* formic acid, phenol, aniline are soluble in CO_2.[17] However, more complex, polyfunctional solutes tend to be much less soluble. These solutes are less likely to be eluted with CO_2. The only disadvantage of using CO_2 as mobile phase is its inability to elute very polar or ionic compounds. However this can often be overcome by addition of small amounts of a second liquid known as a modifier. Advantages of the use of supercritical CO_2 are that the cost for the disposal of solvent is reduced, and also it is easier to recover the compounds from it. This can be viewed as increasingly important to industries.

A modifier is generally an organic solvent that is completely miscible with CO_2 but can be any liquid including water. Up until the late 1980s it was believed that the addition of modifiers did not increase the polarity of carbon dioxide. Changes in retention were attributed to changes in the density of the fluid.[5] It is now known that polar solvents cause significant changes in retention of polar solutes, and that changes in the density of binary fluids cause smaller shifts in retention.[6] Ethanol, methanol, isopropanol, dichloromethane, tetrahydrofuran (THF), and acetonitrile are some of the examples of modifiers that have been used in SFC. However, methanol has been the most widely used. The addition of a modifier improves the solvent strength of the supercritical fluid and sometimes changes the selectivity of separation. It may also help improve separation efficiency by blocking some of the highly active sites on the stationary phase.[18] Systematic studies of the effect of modifiers on the retention of polar solutes using modified carbon dioxide and packed columns have been published.[19-21]

The use of modifier alone may not be sufficient to elute strong acids and bases. Hence further addition of organic acids and bases such as trifluoroacetic acid (TFA) and dimethylethylamine (DMEA) may be required to optimise the chromatography. These are called additives. The first use of additives to SFC eluent was reported in 1988.[22] Most strong organic bases do not elute or elute with poor peak shapes from packed columns such as –CN columns using pure CO_2. Addition of methanol caused the solutes to elute but with very poor peak shapes. The addition of basic additive to the mobile phase resulted in a dramatic improvement in peak shapes.[20] Most polyfunctional organic acids and hydroxy

acids do not elute or elute with poor peak shapes using packed columns and methanol modified CO_2. Numerous papers have been published studying the effectiveness of various additives in improving the peak shapes of acidic solutes.[23-26] In general, stronger acids make the best additives for suppressing tailing and improving peak shapes of acidic solutes. J. Zheng *et al.* established that cationic amine salts can be separated by SFC using an ionic additive in a methanol modified CO_2 mobile phase. An ion-pairing interaction between the positively charged analytes and the anionic part of the sulfonate additive was suggested.[27] The effects of modifiers and additives also depend on the type of stationary phase used.

1.2 Stationary Phases for SFC

A wide range of achiral and chiral stationary phases (CSP) can be used in SFC, most of which are silica-based, though polysaccharide-, zirconia-, polystyrene-, divinylbenzene[28,29] and porous graphitic carbon[30] based packing materials also exist. A number of these packing materials were first designed for HPLC, such as polysaccaride-based CSP Chiralcel OD (cellulose tris[3,5-dimethyl-phenylcarbamate]), Chiralcel OJ (cellulose tris[4-methylbenzoate]) and Chiralpak AD (amylose tris[3,5-dimethylphenylcarbamate]), which are all efficient in both SFC[2,31] and HPLC.[32] Structures of the stationary phases are shown in Figure 1.4.

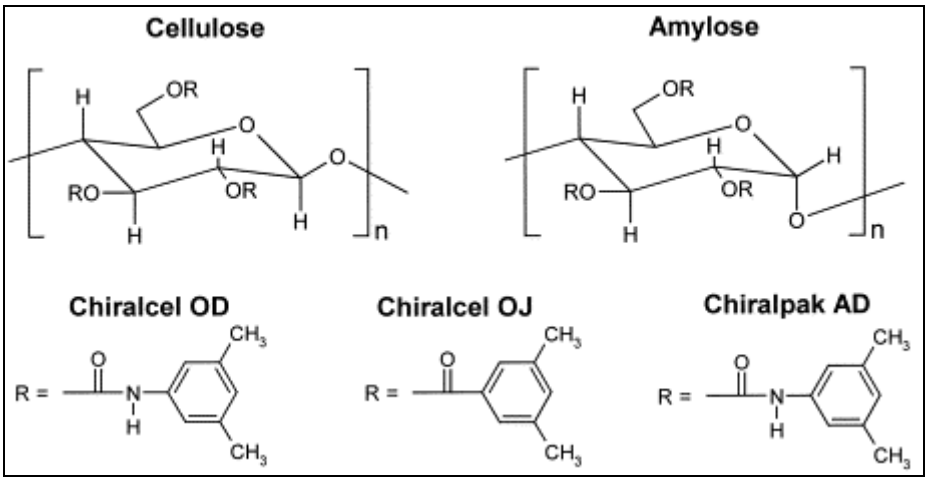


Figure 1.4: Structure of polysaccaride-based chiral stationary phases.[2]

In a chiral column, achiral silica gel (SiO_2) is converted into chiral stationary phase by a reaction with chiral molecule. As the mixture of enantiomers move along the column one enantiomer attaches to the stationary phase better than the other thus resulting in separation. There is a three point interaction with the carbonyl group aligning with the amino groups and the aromatic lining up with each other to form π-stacking interactions. The other enantiomer will not be able to have all three interactions because its groups are not aligned correctly and hence cannot associate itself to the stationary phase, thus eluting first.

Achiral SFC applications typically use polar stationary phases such as bare silica, amino and diol phases shown in Figure 1.5. Although these phases are adequate for many applications, there is still a need for additional polar phases. As a result, a series of amide, urea and pyridine phases have been developed to enhance the capability of SFC. Using spherical silica as the packing material provides low surface areas and therefore is beneficial for separations requiring low percentages of organic modifiers. Higher column efficiencies are obtained with spherical particles than with irregular particles because spherical particles can form more tightly packed beds thereby reducing the column void volume. Smaller particles offer more efficiency but produce higher back pressures. The amount of surface area, usually quoted in metres squared, which is available for bonding, and therefore available for analyte retention, is dependent on pore size. A larger pore size means bigger holes in the particle, leaving less surface area than would be the case with smaller holes. The higher the surface area, the higher is the carbon-load.

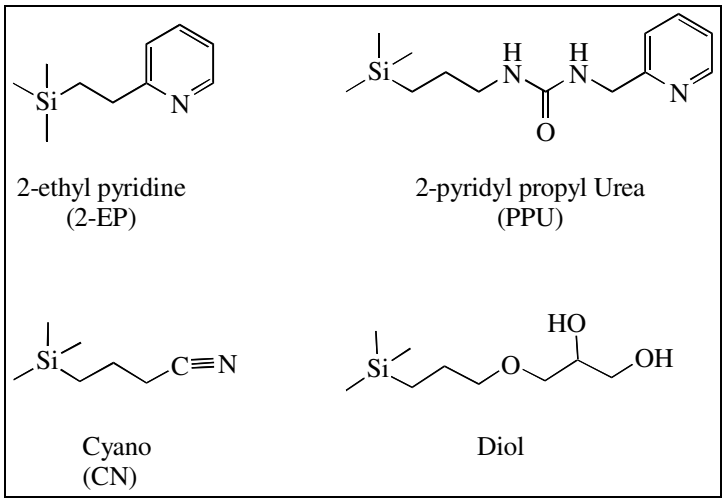


Figure 1.5: Structures of achiral stationary phases.

1.3 Instrumentation for SFC

Over several decades, inadequate instrumentation caused a number of misinterpretations and false directions in the development of SFC. A supercritical fluid chromatograph simply consists of a mobile phase container, an injector, a column contained in an oven, a restrictor and a detector. The mobile phase is initially pumped as a liquid and is brought into the supercritical phase by heating it above its supercritical temperature before it enters the chromatography column. The mobile phase is mixed in a mixing chamber, and then passes through an injection valve where the sample is introduced into the supercritical stream and then into the column. It is maintained supercritical as it passes through the column and into the detector by a pressure restrictor placed either at the end of the column or after the detector. Often the restrictor must be heated to prevent sample clogging. Figure 1.6 shows a typical instrumental set-up for SFC. Some of the components involved have been discussed briefly in this chapter.

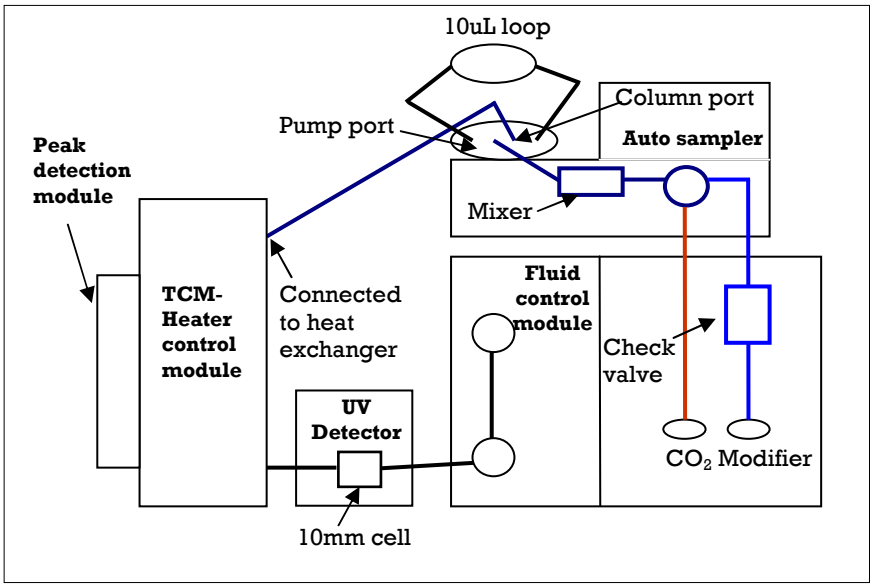


Figure 1.6: A typical SFC instrument.

1.3.1 Pumps for SFC

During the 1980s most SFC systems used syringe pumps, operated as pressure sources. Flow was passively controlled with a fixed restrictor mounted on the end of the column. The problem with this type of arrangement was the inability to control individual parameters, such as flow rate, solvent density, temperature and composition, and determining the effect of any one variable on retention.[17, 33] The use of syringe pumps with binary fluids, premixed binary mixtures were developed by gas supply companies. It is now known that the composition of the fluid withdrawn from premixed cylinder changes as the cylinder is used up. The MeOH-CO_2 composition delivered from a premixed cylinder changes by a factor of two from the first to the last use. As retention is approximately inversely proportional to methanol concentration, doubling the concentration halves the retention.[34, 35]

From 1979 to 1983, a group at Hewlett-Packard used a modified HPLC with two high pressure reciprocating pumps operated as flow sources. One pump delivered compressed fluid whilst the other was used to pump modifiers. A mechanical back pressure regulator controlled downstream pressure. Thus at different pressures and flows the combined pumps delivered different compositions, although the instrument set points remained different. Widmer and co-workers at Ciba Geigy,

in Basel published instrument details in 1980.[36-39] Modern packed column instruments use multiple high pressure reciprocating pumps operated as flow sources. The system pressure is independently controlled through the use of electronic back pressure regulators. Such a configuration allows accurate reproducible composition programming while retaining flow, pressure and temperature control.[5]

1.3.2 Columns for SFC

SFC has a wide range of capillary and packed columns suitable for analysis. Although special columns for SFC are now available, both normal and reversed phase HPLC columns can be used in SFC. Around 1980s Hewlett Packard introduced the instrument for packed column SFC. However, capillary columns were more popular during the time. Capillary columns are open tubular columns of narrow internal diameter made of fused silica with the stationary phase bonded to the wall of the column. These types of columns are most suited for high efficiency separations and complex samples. Packed columns emerged once again in the 1990's emphasising on more polar solutes from the pharmaceutical and agrochemical industries. The packed columns contain small, deactivated particles to which the stationary phase is chemically bound. The packed columns are useful for high speed separations requiring moderate column efficiency and for samples containing fewer components. Figure 1.7 shows the cross sections of both packed and capillary columns. The tubing for the packed columns is conventionally made of stainless steel. A third type of column called packed capillary columns show higher sample capacity and shorter analysis time in comparison to open-tubular capillary columns.

Retention on any column type is directly proportional to the phase ratio (β) of a column.[40]

$$\beta = \frac{(Void\ volume)}{(Stationary\ phase\ volume)} \tag{1.5}$$

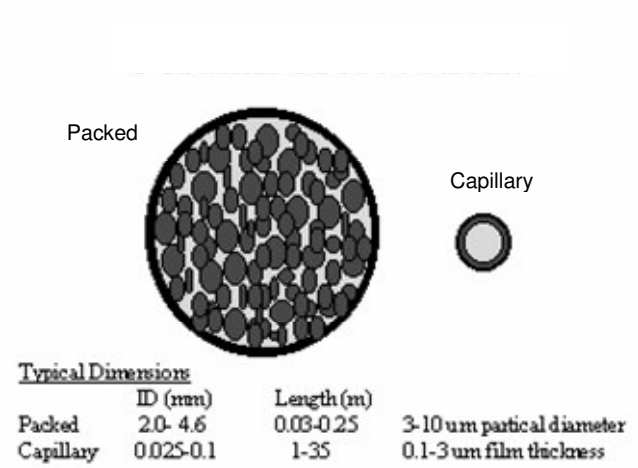


Figure 1.7: Column cross section of packed and capillary columns[15].

Typically, packed columns are 10 to 100 times more retentive than capillary columns as a result of their greater phase ratio.[41] The reason packed columns often require a modified mobile phase is due to their greater retention capacity. In an effort to retain the use of pure carbon dioxide, there have been attempts to either deactivate silica based packing materials or use polymer based packing.

1.3.3 Detectors for SFC

Chromatography is an important analytical technique which when equipped with sensitive detectors, is capable of performing qualitative and highly accurate quantitative analysis. The ultra-violet (UV) absorbance detector is by far the most popular LC detector which is currently available to the analyst. This is particularly true if multi-wavelength technology is included in this class of detectors. Its use is limited mainly because it does not give a uniform response to different analytes, *i.e.* it is not a universal detector. Although the UV detector has some definite limitations (particularly for the detection of non polar solutes that do not possess UV chromophores) it has the best combination of sensitivity, linearity, versatility and reliability of all the LC detectors developed until now. The refractive index (RI) detector is universal, but it is not as sensitive or stable and cannot be used with gradient elution. Mass spectrometry is now a popular LC detector.

SFC has the advantage of compatibility with both HPLC and GC detectors. However, it is important to consider the mobile phase composition, column type

and flow rate when selecting a detector for SFC. This is because for *e.g.* SFC polar modifiers will produce high background noise for flame ionisation detectors (FIDs), and certain detector components may not be compatible for the high pressures used in SFC. Some of the detectors interfaced with SFC are mass spectrometers, flame ionisation (FID), electron capture, evaporative light scattering (ELSD), nitrogen-phosphorus, and phosphorus sensitive detectors. In all, these detectors give a wide variety of sensitive selectivity. The practical universal detector of choice is MS as it provides structural information about the eluted compounds. When using capillary columns and CO_2 mobile phase, all GC detectors and many HPLC detectors can be used. The major advantage of capillary column SFC over HPLC is its compatibility with the FID. The combination of packed column SFC with MS or FID often requires splitting of the eluent from the SFC.

The most common detector used with SFC is the ultra-violet (UV) absorbance detector. In order to operate under high pressures, the flow cell in an UV detector needs modification. Most of the publications describing detection are that of spectroscopic and spectrometric detectors, which provide structural information about the eluted analytes. Harrasch and co workers showed that APCI was found to be superior to electrospray ionisation for the analysis of a mixture of four hydroxysteroids.[9] Garzotti and co-workers have described a novel feature of combining an ESI ion source with a TOF analyser without the need for an interface between the source and the SFC system which provided accurate mass positive ion electrospray mass spectra for a number of compounds.[2] In this project UV and MS were used as detectors and these will be discussed.

Ultra-violet detectors

There are two factors that control the detector sensitivity, the magnitude of the extinction coefficient of the solute being detected (which will depend on the wavelength of the UV light which is used and on the compound structure) and the path length of the light passing through the cell. It follows that the optimum detector cell design involves the determination of the cell length that will provide the maximum sensitivity and at the same time constrain detector dispersion to a

minimum so that there is minimum loss in resolution. There are three types of UV detectors. Fixed wavelength is the simplest version of UV detectors. It employs a UV light source, typically a low pressure mercury vapour lamp which provides several distinct lines of UV radiation with 254 nm being most intense. The radiation is then passed through a filter to remove other wavelength light. Due to the fact that not all compounds absorb at 254 nm variable wavelength detectors are used to allow the user, the option of choosing an appropriate wavelength. This was accomplished by adding a monochrometer. A deuterium lamp provides a continuous sequence of UV radiation producing a broad band of radiation from 190 up to 800 nm. This light is then separated into its component wavelengths by using a grating which is placed on a moveable platform. This allows the user to choose any wavelength from the spectrum at any one time. The photodiode array detectors are one step further than the variable wavelength detector as it allows the user to access all of the wavelengths simultaneously. Like in a variable wavelength detector, a continuum source is used and the entire spectrum of light is passed through the detector cell. A single detector is replaced by a multiple array of individual detectors called photodiodes. These are arranged on a single chip called the photodiode array. In this project a variable wavelength detector was used with the SFC instrument.

In recent years mass spectrometers have become increasingly popular as detectors for chromatographic systems. HPLC-MS is a commonly used technique in the pharmaceutical and other chemical industries. The coupling of HPLC to MS used to be a difficult process due to the transition of liquid phase ions at atmospheric pressure to gas phase ions in a vacuum. The difficulties involved introducing solutions to the vacuum system of the mass spectrometer. One solution is to apply heat to aid evaporation, but this can lead to the thermal degradation of the analyte. One other issue is the capacity of vacuum pumps to deal with a large gas load *i.e.* 1 mL of solvent creating approximately 1 L of vapour. In order to couple a liquid chromatograph to a mass spectrometer, the system must be capable of evaporating liquids into gases, be able to ionise the species present and be able to remove the large amounts of solvent whilst maintaining the required vacuum for the mass analysers. Early examples of LC-MS interfaces used were the moving belt, fast atom bombardment (FAB),[42] thermospray (TSP)[43] and particle beam (PB).[44]

These were subsequently followed in the late 1980s by atmospheric pressure ionisation (API) techniques; electrospray ionisation (ESI), atmospheric pressure chemical ionisation (APCI) and atmospheric pressure photoionisation (APPI). These interfaces simplified the coupling of HPLC to MS and are the most commonly used systems today.[45]

Supercritical fluid chromatography-mass spectrometry (SFC-MS) is fast becoming a recognised technique due to the growing need for high speed and high quality separations.[46] The interest in using MS with SFC has increased in the last decade following the advent of the atmospheric pressure ionisation sources.[47] SFC coupled with MS forms a more selective and informative technique when dealing with mixtures containing unknown compounds or those with poor UV absorption.[48] In most cases MS provides target compound detection, good sensitivity and in some cases structural information for identification of the unknown compounds. When SFC is interfaced with a mass sensitive detection, a powerful analytical tool for the analysis of difficult mixtures can be realised.[44] The key advantage of SFC is minimal solvent waste, which is particularly important in preparative SFC, leading to fast sample recovery. The coupling of SFC to MS was first reported by Randall and Wahrhaftig in 1978 using a molecular beam interface.[49] This realised a powerful analytical tool for the analysis of complex mixtures.[44] As mentioned above, CO_2 has become the standard choice of the mobile phase. Other advantages of CO_2 are its low cost, low carbon footprint low interference with chromatographic techniques and suitable physical properties (non-toxic, non-flammable, low critical values). Under supercritical conditions, CO_2 has the advantage of low viscosity, which allows a high flow rate without over-pressuring the system. Also, mass transfer is very high, thus improving column efficiency. When combined with MS, the CO_2 is more readily handled by the vacuum system due to its high volatility, and can enhance volatilisation at the inlet to the mass analyser. Several types of interface have been reported in the literature, some being modified from LC-MS systems, and some being specially developed for SFC-MS. The ionisation techniques used in such interfaces have been PB,[44] TSP,[50] FAB[42], APCI[51] and more recently ESI.[52] A novel arrangement in which SFC was directly coupled to a hybrid mass

spectrometer (Q-Tof 2) was reported; this provided accurate mass positive ion electrospray mass spectra for a number of compounds.[2, 8]

Quadrupole Mass Spectrometer

Quadrupole mass analysers are widely used in various areas of chemical analysis. In a quadrupole mass analyser the ions are separated on the basis of their mass to charge ratio. Only ions within a narrow mass region are allowed to pass through the device. The resolving properties of quadrupole result from an ionic intrinsic stability or instability within the device. The distance between the ion source and detector is about 15cms. The short distance combined with strong focussing properties makes the quadrupole analyser useful at high pressures and the capability of fast scanning, thus making it useful to be combined with GC and LC. Another feature of the quadrupole mass analyser is that it is mechanically simple, as it does not rely on the use of strong magnetic fields for their mass discrimination properties.[53]

A quadrupole consists of two pairs of metallic rods (Figure 1.8). The four rods are equidistant from the central axis. The adjacent rods have opposite potential. A combination of DC and RF voltages are applied to the rods. Each rod pair is successively positive and negative allowing ions to be both attracted and repelled from the central axis. A potential difference is applied at the entrance and exit of the quadrupole. This pushes the ions out of the quadrupole. Ions that undergo stable oscillations are separated according to their mass to charge ratio. Depending on the magnitude of the voltages only ions of certain mass are allowed to be detected. Other ions are deflected causing them to collide with the rods and then pass out of the analysing device.

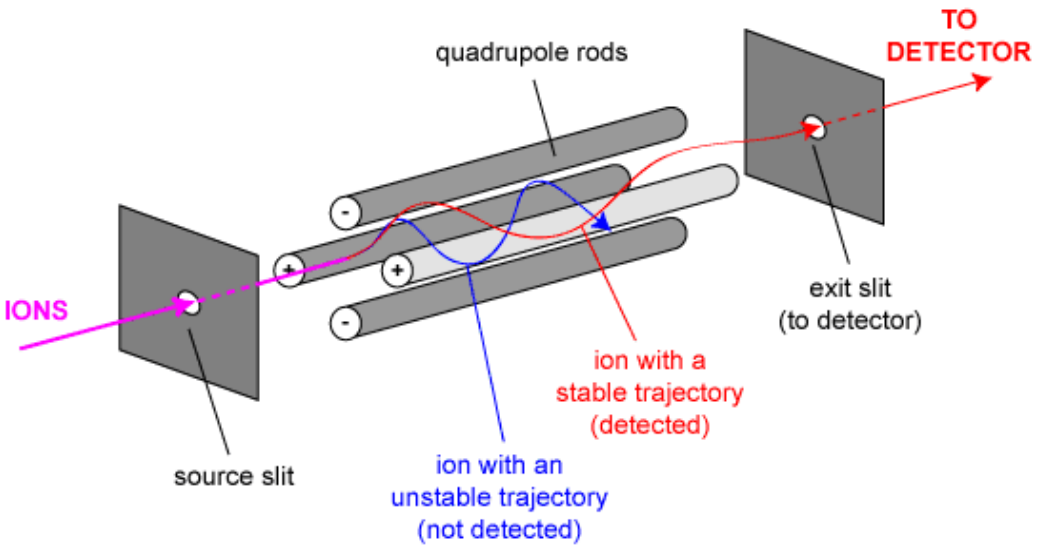


Figure 1.8: Schematic diagram of quadrupole mass spectrometer.[54]

1.4 Theory and coupling with SFC

Here in this project, a Berger Minigram SFC was coupled to a Platform LCZ. The Berger Minigram operates with the SFC Pronto software. A photograph of the SFC-MS set-up is shown in Figure 1.9. Further discussion on the system will be given in Chapter 2.

The SFC was interfaced with the MS by using of two Valco stainless steel T-pieces and a length of PEEK tubing (0.0025 inch I.D.) between the UV and MS. The first T-piece was inserted after the UV detector output and before the back pressure regulator. The other end of the PEEK tubing was connected at the second T- piece leading to the MS. The third arm of the second T-piece was connected to a make-up solvent pump. A Hewlett Packard HPLC pump was used as a make-up pump to deliver 0.1 mL min^{-1} of methanol. This post column modifier is essential for two reasons. Firstly, as CO_2 is not known to produce ions under any investigated conditions nor does it plays a direct role in ion formation, it is essential to add post column modifiers for example methanol into the CO_2 stream to aid generation of ions in the mass spectrometer. Secondly, adding a make-up fluid prevents the precipitation of solutes in the tubing.[16, 48]

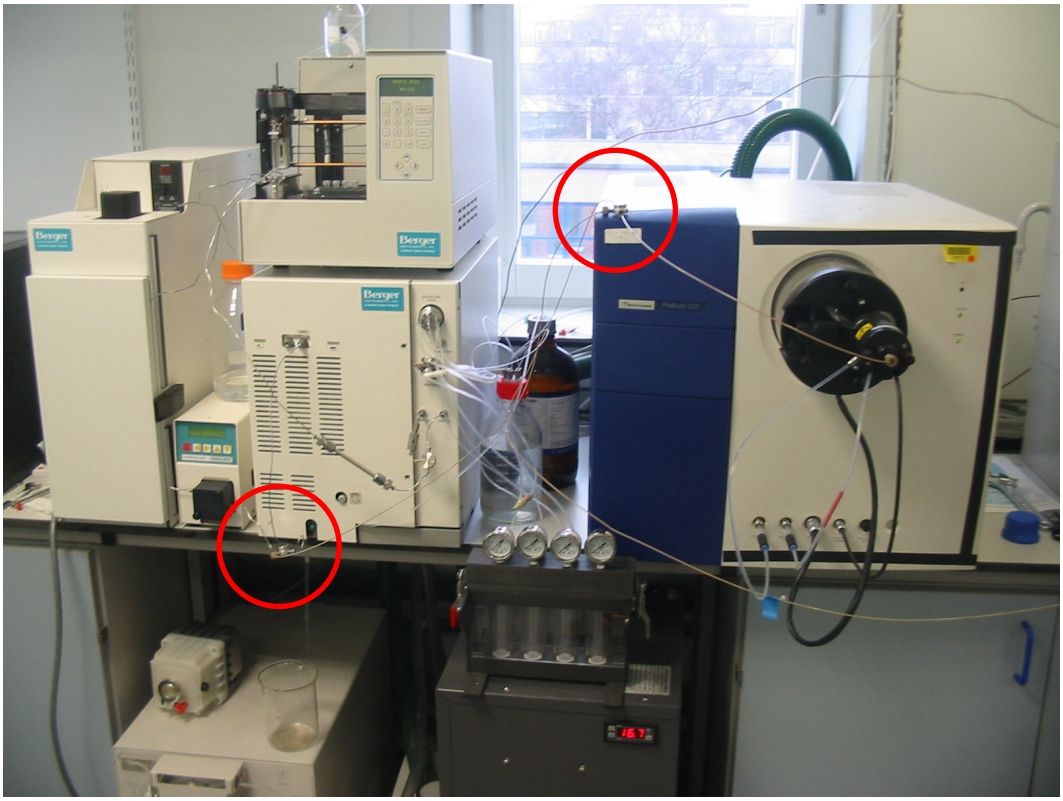


Figure 1.9: Photograph of the SFC-MS set-up showing the interface between SFC and MS.

Electrospray ionisation (ESI)

Electrospray ionisation is essentially a solution phase process. Electrospray takes place as a result of a strong electrical charge applied to the eluent as it emerges from the capillary. The sample is introduced in solution phase through a capillary tube to the end of which high electric field is applied. The solution is nebulised to form a spray of small droplets. Frequently, the sample nebulisation and subsequent droplet evaporation is assisted by the co-axial flow of warm nitrogen gas, *i.e.* pneumatically-assisted electrospray ionisation. This can be enhanced by introducing a gas near the end of the charged capillary. The spray from the capillary reaches strong electrostatic field at atmospheric pressure which results in production of fine aerosol of highly charged droplets. Evaporation of solvent from these droplets and droplet disintegration results in formation of ions. Ions may be singly or multiply charged (Figure 1.10).

The ionisation of the species follows one of the two paths depending on the polarity of the charge applied to the capillary

$$M + X \rightarrow [M + nX]^{n+}$$

$$M - X \rightarrow [M - nX]^{n-}$$

where X = H, Na, K, NH_4^+

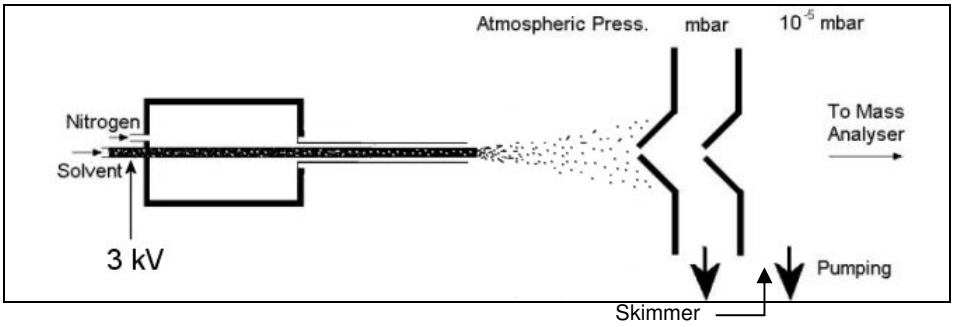


Figure 1.10: Diagram of electrospray ionisation source. (Modified from reference [55])

The source is simple in design and robust. Protonated or deprotonated molecules are produced depending upon the characteristics of the analyte and chosen polarity of mass spectral analysis. These characteristics have led to ESI being the most commonly used ionisation technique in the pharmaceutical industry.

Although a definitive mechanism for production of gas phase ions in ESI is not known, two models have been suggested (shown in Figure 1.11). Iribarne and Thompson[43, 56] put forward the ion evaporation model (IEM) where a single solvated analyte ion carrying some of the droplets charge is desorbed into the gas phase. This mechanism is said to be dominant for small molecules as these can be easily separated from the solvent molecules. The other mechanism is the charge residue model (CRM) proposed by Dole and co-workers.[57] This suggests that continuous evaporation and fission of the charged droplet occurs until it contains only one analyte molecule. The charge then transfers to the analyte. This model is said to be applicable when the solute molecule has linear dimensions significantly larger than the charged droplet that contains it. Hence it is proposed that gas phase ions of macromolecules are formed in this manner.[57, 58]

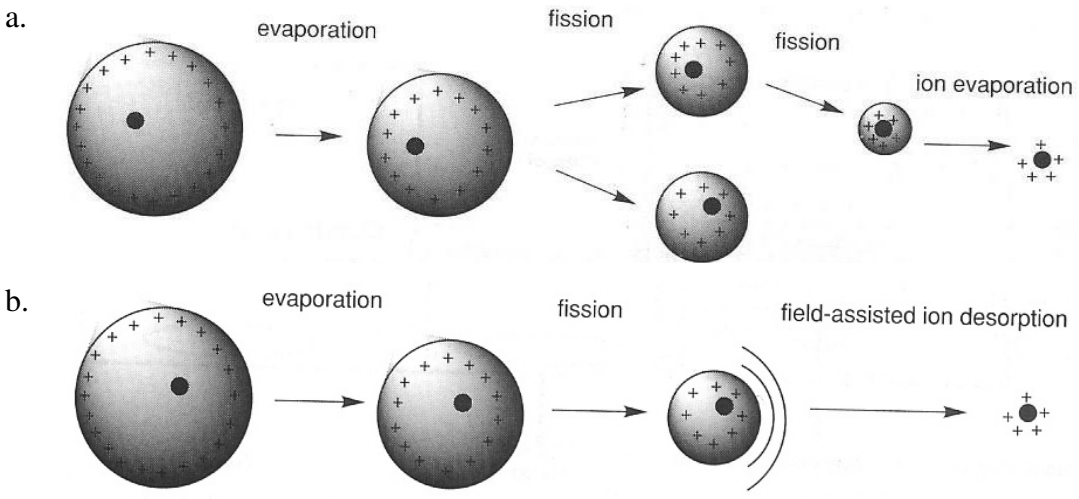


Figure 1.11: Schematic showing the (a) charged residue model and (b) ion evaporation model of electrospray ionisation .[59]

Atmospheric pressure chemical ionisation (APCI)

ESI and APCI are complementary techniques. APCI generally produces protonated or deprotonated molecular ions from the sample *via* a proton transfer (positive ion) or proton abstraction (negative ion) mechanism. The sample solution is vaporised in a heated chamber before entering the plasma consisting of solvent ions formed by a corona discharge within the atmospheric pressure ion source. The solvent vapour is then ionized by the electric field, either by removal (positive ion mode) or donation (negative ion mode) of an electron, which then results in the formation of reactive species. Ionisation occurs as a result of chemical reactions between the sample molecules and plasma ions. Proton transfer takes place between the solvent ions and the sample. For *e.g.* here methanol is present in the mobile phase.

Positive ion mode

$$MeOH \longrightarrow Me\overset{+\bullet}{O}H \longrightarrow MeOH_2^{+} + Me\overset{\bullet}{O}$$

Negative ion mode

$$MeOH \longrightarrow Me\overset{-\bullet}{O}H \longrightarrow MeO^{-} + Me\overset{\bullet}{O} + H_2$$

As the sample enters the plasma it then reacts with the reactive species to give protonated or deprotonated molecule.

Positive ion APCI follows

$$\text{Proton transfer: } MeOH_2^+ + M \rightarrow [M + H]^+ + MeOH$$

Negative ion APCI follows

$$\text{Proton abstraction: } MeO^- + M \rightarrow [M - H]^- + MeOH$$

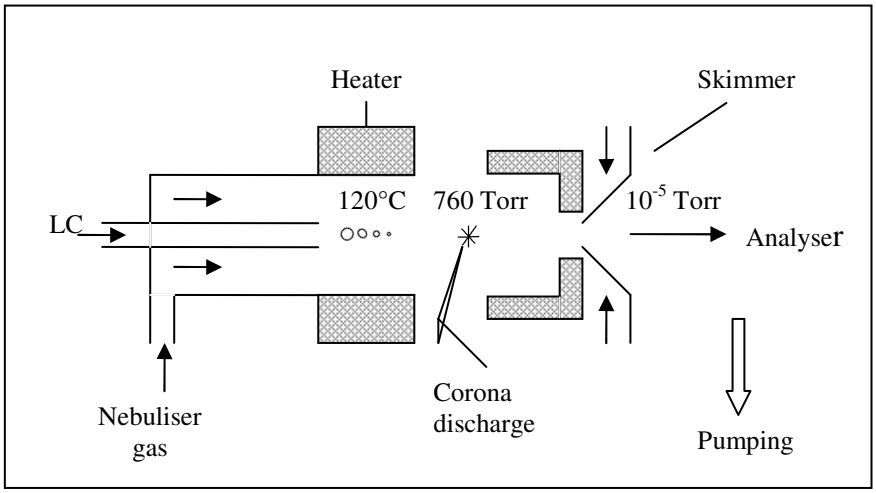


Figure 1.12: Diagram of APCI source. (Modified from reference[60])

APCI is a gas phase ionisation process. Thus the analyte must be stable in the gas phase. In APCI the sample solution is injected into a heated fused silica capillary which behaves as a vaporiser. The vaporisation is assisted by a nitrogen flow coaxial to the capillary (Figure 1.12). The analyte then ionises as it approaches the corona discharge needle. Solvent evaporation occurs first and then the ions are formed. The analyte is nebulised to very small droplets by evaporation of solvent using a very hot auxiliary gas. One of the problems experienced initially was the presence of still solvated analyte molecules *i.e.* the presence of clusters of analyte molecules with different numbers of solvent molecules. To obtain a de-clustering of these species different approaches have been proposed, among which non-reactive collisions with target gases and thermal treatments are those considered most effective and currently employed. In the case of quantitative analysis, particular care must be devoted to finding the best operating conditions for parameters such as the vaporising temperature and the solution flow. This assists

in achieving stable signals. Formation of the charged molecular species are based on the production of acidic or basic species in the gas phase which further react with a neutral molecule of analyte leading to $[M + H]^+$ or $[M - H]^-$ ions, respectively.

Thermospray ionisation (TSP)

In thermospray the chromatographic eluent is rapidly heated and the spray is formed under vacuum. The solution passes through the vacuum chamber as a supersonic beam. A fine droplet spray is generated which contains analyte and solvent molecules as well as ions. The ions in solution are extracted and accelerated towards the analyzer by a repeller electrode and a lens focusing system. The ions are desorbed from the droplets carrying one or several solvent molecules or dissolved compounds. Ions go directly from the liquid phase to the gas phase. To improve the ion extraction the droplets at the outlet of the capillary may be charged by a corona discharge, *i.e.* plasmaspray. The droplets stay on their supersonic path to the outlet, where they are pumped out continuously through an opening located in front of the supersonic beam. Large vapour volumes are thus avoided. In order to avoid freezing of the droplets under vacuum, the liquid must be heated during injection. Addition of a volatile buffer, *e.g.* ammonium acetate, enhances the ionisation process due to formation of $(M + NH_4)^+$ ions. Figure 1.13 shows the TSP source, first introduced by Blakely and Vestal,[43] was one of the early interfaces for an LC-MS system, which produced ions from an aqueous solution that had been sprayed directly in the mass spectrometry. Thermospray ionisation is achieved by passing a pressurised solution through a heated tube which partially vaporises the effluent to generate a spray before entering the ion source. The droplets gradually decrease in size by evaporation of neutral solvent molecules until the droplet reaches a size at which the charge repulsion forces overcome the cohesive forces of the droplet.

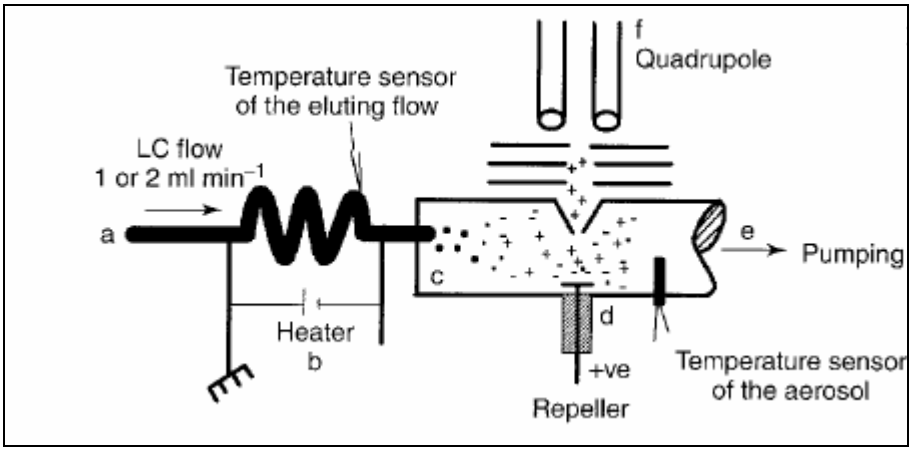


Figure 1.13: Diagram of thermospray ionisation source.[60]

Sonic spray ionisation (SSI)

SSI is a relatively novel API technique first developed by Hirabayashi and co-workers[61] in the 90s. It features an external ion source to a mass spectrometer similar to ESI. In this technique the column eluent is sprayed from a fused silica capillary with a sonic velocity gas flow co-axial to the capillary resulting in the formation of ions and charged droplets under atmospheric pressure. Figure 1.14 shows the schematic diagram for the sonic spray ionization interface. The ion intensity strongly depends on the gas flow velocity.[61] In sonic spray ionisation it is not necessary to apply heat or an electric field to the capillary of the ion source. A sonic spray ion source consists of two capillaries the inner fused silica capillary which introduces the analyte solution and the outer stainless steel capillary that provides a coaxial nitrogen gas flow to facilitate droplet nebulisation. Subsequently the ions are introduced through a sampling orifice into a vacuum region where the mass analysis can be performed. Since SSI is operated in API conditions without any electric potential applied, it is considered as a very soft ionisation method. Thus, fragmentation of analytes is minimised and the technique can be easily applied to thermally labile compounds.[62, 63] The maximum number of ions are produced by this method when the nebulisation gas is flowing at sonic velocity (gas velocity of Mach 1). When voltage is applied in SSI increased ion production and higher charge states are observed similar to that of ESI and this is termed as electro-sonic spray ionisation (ESSI). SSI requires

higher pressure and flow rates compared to ESI. The conditions of SFC system were then studied and, the results are explained in Chapter 2.

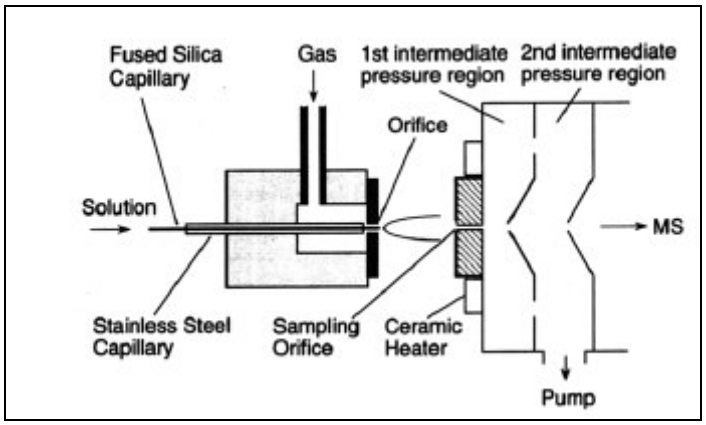


Figure 1.14: Schematic diagram of the sonic spray interface.[61]

An attempt to understand the ionisation mechanism in an SFC-MS system is shown in Chapter 2. Ionisations of compounds were observed in the absence of high voltage on an ESI source. The observations were also compared using an APCI source.

1.5 Applications of SFC

SFC is being widely used as a separation technique in several fields. It has been applied to a wide variety of materials including natural products, drugs, food, pesticides and herbicides, fossil fuels, explosives and propellants.[64] Listed below are some of the recent SFC applications areas.[65]

1.5.1 Food related applications

Several food related applications of SFC have been mentioned in the reviews[66-69] and these include analysis of pesticides and their metabolites in food stuffs, fatty acids and triacylglycerols and triglycerides *etc*. For example, SFC analysis was used to investigate the changes on heating of low-linolenic soybean oil and partially hydrogenated soybean oil.[70] Rezaei and Temelli used SFC to monitor the products of enzymatic reactions in supercritical CO_2.[71] In some other publications

SFC/UV was used to characterise polyphenolic compounds in an SFE extract of grape seed.[72] Recently SFC was used to isolate and quantitatively analyse minor components from palm oil.[73] Interests in the assessment of antioxidants in food have risen considerably. Although HPLC is popular in this field, use of SFC for analysis of phytochemical antioxidants was reported in the reviews by Tsao and Deng.[74]

Separation of antioxidants from rosemary essential oils using SFC is another example. In this case an attempt is made to analyse polar compounds without the use of added modifier. Columns were specially developed to tune the polarity of stationary phase by Ramirez *et al*. This would enable separation of polar compounds using pure CO_2. Two types of columns were developed, silica particles were coated with either SE (5% penyl, 95% methyl silone) or carbowax 20M (polyethylene glycol). Resolution of principal anti oxidant compounds was achieved at relatively high pressure and temperature.[75]

Han *et al*. reported the separation of tocopherols, vitamin E and similar antioxidants from palm oil.[76] I. Francois *et al*. have demonstrated the use of multidimensional chromatography for the analysis of fatty acids in fish oil. Silver ion SFC (SI-SFC) and RP-LC in first and second dimensions respectively was found to have increased orthogonality and peak capacity in comparison to RP-LC X 2RP-LC separation of phenacyl esters of fatty acids in fish oil extract. To obtain SI-SFC columns, two 25 cm acidic cation exchange columns were loaded with silver ions and then rinsed with ammonium acetate solution. The two columns were then coupled in series. For the second dimension a 5 cm C_{18} column was used[77].

1.5.2 Pharmaceuticals

The use of SFC has grown rapidly in the pharmaceutical industry both for chiral and achiral analysis. The majority of work in the recent years involves chiral chromatography. As a result of the increase in regulatory scrutiny of drugs, importance of enantiomer separation has also increased. SFC is also found to be useful in screening applications. Along with other techniques, the use of SFC in

the analysis of antibacterial and antirheumatism compounds found in traditional herbal medicines was reported by Wen *et al*.[78] Gyllenhaal showed that both chiral and achiral analyses can be performed on the same drug. This was demonstrated on a preoxysome proliferation receptor agonist drug. Using SFC both separation of enantiomers and separation of the active drug from process impurities was achieved.[79]

Achiral and Bioactive Compounds

Bromosulphone, which underwent on-column degradation during reversed-phase HPLC separation, was not only found stable under SFC conditions but the required separation of seven process related impurities from bromosulphone, was achieved in 5 minutes.[80] Gyllenhaal and Hulthe reported the direct injection of aqueous sample solutions of isosorbide-5-mononitrate by packed column SFC where Imdur tablets were dissolved in gastric media and directly injected. The mobile phase was CO_2 modified with 20% 2-propanol and the stationary phase was diol functionalized silica.[81] Aqueous injections are generally not used but in this application the stationary phase was sufficiently polar to allow such injections without significant breakthrough. A few publications discussing the use of SFC, supercritical fluid extraction (SFE) and related techniques in medical radioisotope processing and chemistry have also been reported.[82, 83]

In another instance, Gyllenhaal and Karlson separated metoprolol, a β-adrenoreceptor blocking drug from related amino alcohols using a porous graphite carbon column. Structures of these are shown in Figure 1.15. The analytes are structurally related and include impurities and metabolites. The study included different areas such as modifiers, additives and the influence of the additive concentration and column temperature on selectivity and retention. To obtain good symmetry of the metoprolol peak at high concentration, 200mM or more, of amine additive was used resulting in improved retention and column efficiency. The resolution between the meta and para metoprolol was found to vary with different mobile phase additives. The order additives was ammonia, followed by

N,*N*-dimethyloctylamine and lastly triethylamine. The effect of increasing the concentration of the amine additive on the retention or selectivity was negligible. The selectivity of the analytes changed with change in column temperature. Here the separation of closely related anlogues was also shown[84].

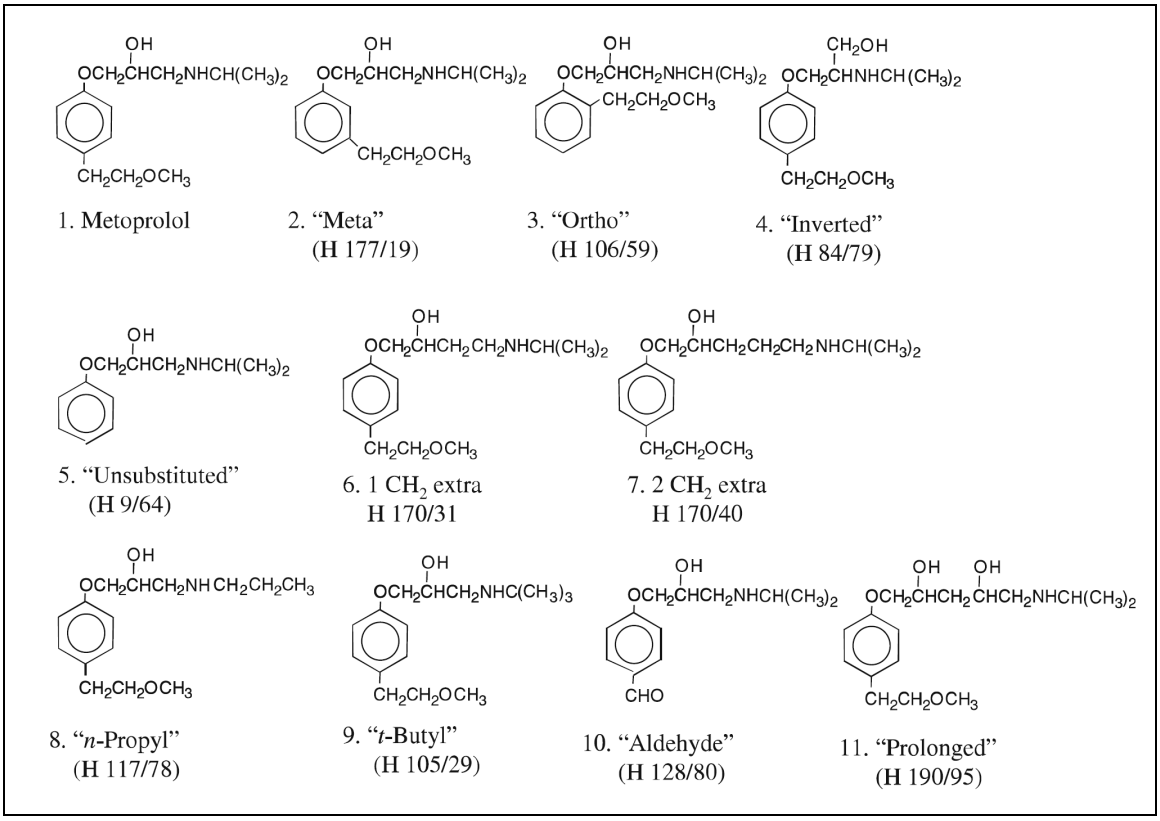


Figure 1.15: Structures of metoprolol and related amino alcohols.[84]

In the area of bioactive compounds, packed column SFC-UV was used for the determination of the potential carcinogen benzidine and its acetylated metabolite. The authors compared the method to the published HPLC-UV method and found SFC to be superior with respect to speed, organic solvent usage, sensitivity, specificity and accuracy.[85]

Chiral Applications

The separation of enantiomers is an important area of separation science. The vast majority of chiral separations by SFC have been performed using packed columns. Two new high performance amylase chiral columns, Chiralpak AS-H and Chiralpak AS-RH were designed to be compatible with SFC.[86] The effects of

two basic additives, isopropylamine and triethylamine, on chiral SFC separations using macrocyclic glycopeptide or derivatised polysaccharide columns are reported by Phinney and Sanders where they show that additives were required for elution of many of the analytes from the glycopeptide column and increasing the additive concentration resulted in a decrease in retention.[87] The additives had little effect on retention on the polysaccharide phase but resulted in better peak shapes and resolution.

Garzotti and Hamdan used SFC coupled to a hybrid mass spectrometer (Q-Tof2) equipped with electrospray ion source to separate and characterise a wide range of pharmaceutical racemates. Chiral SFC has been applied to a wide variety of chiral compounds, mostly in the analysis of pharmaceuticals. The speed of chiral SFC separations was compared to the conventional normal and reverse phase chiral HPLC. A comparative study of enantioselective separation of several anti-ulcer drugs using HPLC and SFC on a chiralpak AD column was carried out by L. Toribio *et al.*[88] The results showed that only two of the compounds, omeprazole and pantoprazole, could be resolved using HPLC whilst SFC allowed the enantiomeric separation of all four compounds studied with higher resolution and lower analysis times. Later on Toribo also separated racemic antifungal imidazoles with higher resolutions and analysis time as low as 10 minutes using a chirapak AD column.[89]

1.5.3 Natural Products

SFC was included in reviews of techniques for sesquiterpene and valepotriate analysis.[90] The selectivity of SFE at different pressures was highlighted by Kaplan *et al.*[91] Chromatographic resolution of the polyprenols with chain length and geometric isomer variations showed a marked improvement for SFC over conventional HPLC separations.[92] SFC was used to elute large peptides (at least 40mers) containing acidic and basic residues. A 2-EP column with ethanol modified CO_2 mobile phase and trifluoroacetic acid additive was used.[93] Desmortreux *et al.* have shown that SFC can remarkably reduce the analysis times

from 45 mins to 10 mins for the separation of furocoumarin in essential oil composition. A Discovery HS F5 column was used for the analysis with CO_2-EtOH 91:9 (v/v) modifiers.[94]

1.5.4 Fossil Fuels and Bio Fuels

SFC with UV detection can be used to determine conjugated dienes in petroleum products. The method used two coupled silica columns and pure CO_2 as the mobile phase.[95] Using SFC and two-dimensional SFC Lavison *et al.* analysed polar car lubricants. The specific detectors used were AED, FTIR or MS.[96] Further a hyphenation of SFC to APCI-MS is described for the analysis of polar car lubricant additives[97]. Various parameters such as temperature, ionisation additives, and gas flow rates have been detailed.

Cole *et al.* have developed a fast SFC method for the group separation of glycerol, FFA, FAME, the main target compounds for bio-diesel analysis. Using a C18 column the groups were separated within the analysis time of 35 mins which is a threefold and a fivefold increase in throughput compared to UPLC and HPLC respectively. Glycerol eluted first and FAME last.[98]

1.5.5 Synthetic oligomers, polymers, and polymer additives

Pure CO_2 was used as the mobile phase for the separations of ethoxylated and propoxylated oligomers and detected with both flame ionisation detectors and low wavelength UV absorbance detectors, both options were found to give equivalent results.[99] Separations of ethoxylated alkylphenols with coupled diol and CN packed columns produced the best results for SFC. Although SFC provided chromatographic resolution similar to HPLC, a shorter analysis time was achieved.[100] In another instance, a 1 m long packed column was used for high resolution SFC-MS separations of alkoxylated oligomers.[101]

Takahashi *et al*. have shown that the sensitivity of corona CAD is higher than that of ELSD for the analysis of synthetic polymer by SFC[102]. The analysis was performed using a silica gel column and CO_2 with MeOH-H_2O (9:1 v/v) as

modifier solvent using gradient conditions. The limit of detection was 10 times lower than ELSD.

1.6 Aims and Objectives of the project

SFC coupled to mass spectrometry, with an electrospray ion source, was used to analyse diverse series of test compounds. The simple coupling of the mass spectrometer to the SFC resulted in no significant loss in chromatographic integrity to the UV detected peaks - the MS detector affording identification of compounds with no or poor UV chromophores. The MS was particularly useful in identifying and confirm unknowns, peaks resulting from unusual chromatographic effects and also to track the presence of the organic acids or bases used as additives. The ionisation of samples in the absence of high voltage, *i.e.* ionisation voltages, was observed when a SFC was coupled to an ESI source. This novel ionisation process was further investigated.

Compounds showed increased sensitivity with the source high voltages turned off. The involved mechanism of ionisation for this has not been reported so far, nevertheless the increased sensitivity in the absence of high voltage can help in lowering the limit of detection for certain compounds. In this work an attempt was made to explain this ionisation phenomenon and the improved sensitivity quantified.

To probe this mode of ionisation specific test compounds *e.g.* diphenhydramine, terfenadine, oxybutynin and reserpine were analysed using standard ESI source and APCI source conditions. Structures of these test compounds are shown in Figure 1.16. In both cases data was acquired with the high voltage on (electrospray) or off (Novospray). To probe whether this is a thermospray type ionisation process, ammonium acetate was introduced as buffer. Similarly, formic acid was added to see if charged residue model was the driving force in this ionisation mechanism. The effect of organic solvent and composition was also explored.

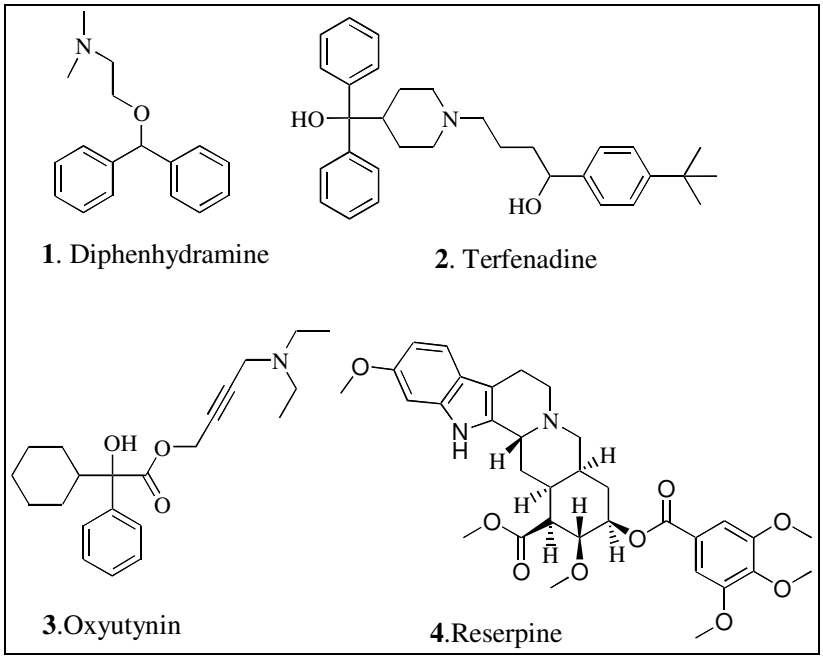


Figure 1.16: Structures of test compounds 1.Diphenhydramine, 2. Terfenadine, 3. Oxybutyin and 4. reserpine

For all the samples analysed by Novospray the resultant signal was comparable or better than the classical ESI and APCI methods. The addition of ammonium acetate did not assist in ionisation, on the contrary this resulted in reduced signal; suggesting that it is not a thermospray type of ionisation process. Similarly the addition of acid also resulted in reduced signal thus suggesting the molecule is not charged in solution as in charged residue model. The other explanation could be a variant of sonic spray. Here the ion intensity strongly depends on the gas flow velocity, consistent with the high pressure flow from the SFC.

Regardless of the method of ionisation, the most important point to note is enhanced ionisation with the high voltage turned off in SFC-MS system was observed, thus aiding low level detection. This also suggests that the majority of the ion current observed in API is independent of applied HV for ESI and APCI.

Another objective was to explore whether SFC, which promises to be cheaper and more environmentally friendly than conventional HPLC, can be applied more widely as a complementary method. Further in an attempt to develop generic analytical and preparative methods, a diverse series of test compounds were analyzed on different stationary phase columns with use of a modifier, primarily

methanol. To improve the chromatography on certain stationary phases additives have been used, *e.g.* simple organic acids, bases or ionic salts. This work shows a comparison of the different additives used and a comparison of different stationary phases. For some compound types two peaks were observed upon injection, this appears to be linked to compound type and the injection procedure. Data attempting to explain this phenomenon is presented and in particular how the choice of injection solvent affects possible compound interactions with the stationary phase and peak shape.

One of the objectives of this project has been to determine whether a generic set of rules can be applied to choosing the best technique for the separation and analysis of a given sample based on the chemistries of compounds involved. Thus, a direct comparison of HPLC and SFC was undertaken with a diverse series of test compounds using the same conditions, to highlight the effectiveness of the two techniques in terms of speed and more importantly compound coverage. The successful analysis of polar species by SFC was highlighted *i.e.* compounds that elute with the solvent front of the generic C_{18} methods used and ascertain which factors are important in determining the optimum technique for a general compound class.

HPLC and SFC data are presented, comparing a generic analysis protocol with compound specific analyses. Preliminary findings showing the overlap between the two separation techniques is discussed and specific differences observed with the different column types used is outlined.

Chapter 2

SFC – MS Ionisation in absence of high voltage: - Novospray

The coupling of HPLC to MS used to be a difficult process due to the transition of liquid phase ions at atmospheric pressure to gas phase ions in a vacuum. The difficulties would involve introducing solutions to the vacuum system of the mass spectrometer. One solution is to apply heat to aid evaporation, but this can lead to the thermal degradation of the analyte. One other issue is the capacity of vacuum pumps to deal with a large gas load *i.e.* 1 mL of solvent creating approximately 1 L of vapour. In order to couple a liquid chromatograph to a mass spectrometer, the interface must be capable of evaporating liquids into gases. It must then be able to ionise the species present and finally remove the large amounts of solvent whilst maintaining the required vacuum for the mass analysers. SFC-MS has been recognised as a favourable player due to the growing need for high speed and high quality separations. When combined with MS the CO_2 mobile phase of SFC is more easily handled by the vacuum system due to its high volatility. Further it can enhance volatilisation of the sample and residual solvent (modifier) at the inlet to the mass analyser. The coupling of SFC to MS has resulted in a powerful analytical tool for the analysis of complex mixtures.

In this project, an interesting observation of analytes ionising in the absence of high source voltage was noted when the SFC was interfaced to the MS *via* an electrospray interface. The SFC system was directly coupled to the mass spectrometer. A post column make-up flow of methanol was introduced prior to the MS interface for the following reasons. Firstly, after the restriction, the fluid density, and the solvating power of pure CO_2 is reduced. This may result in precipitation of the analytes before reaching the MS. Secondly the presence of a polar modifier is required to maintain ionisation of samples, as CO_2 is not known to assist ionisation.

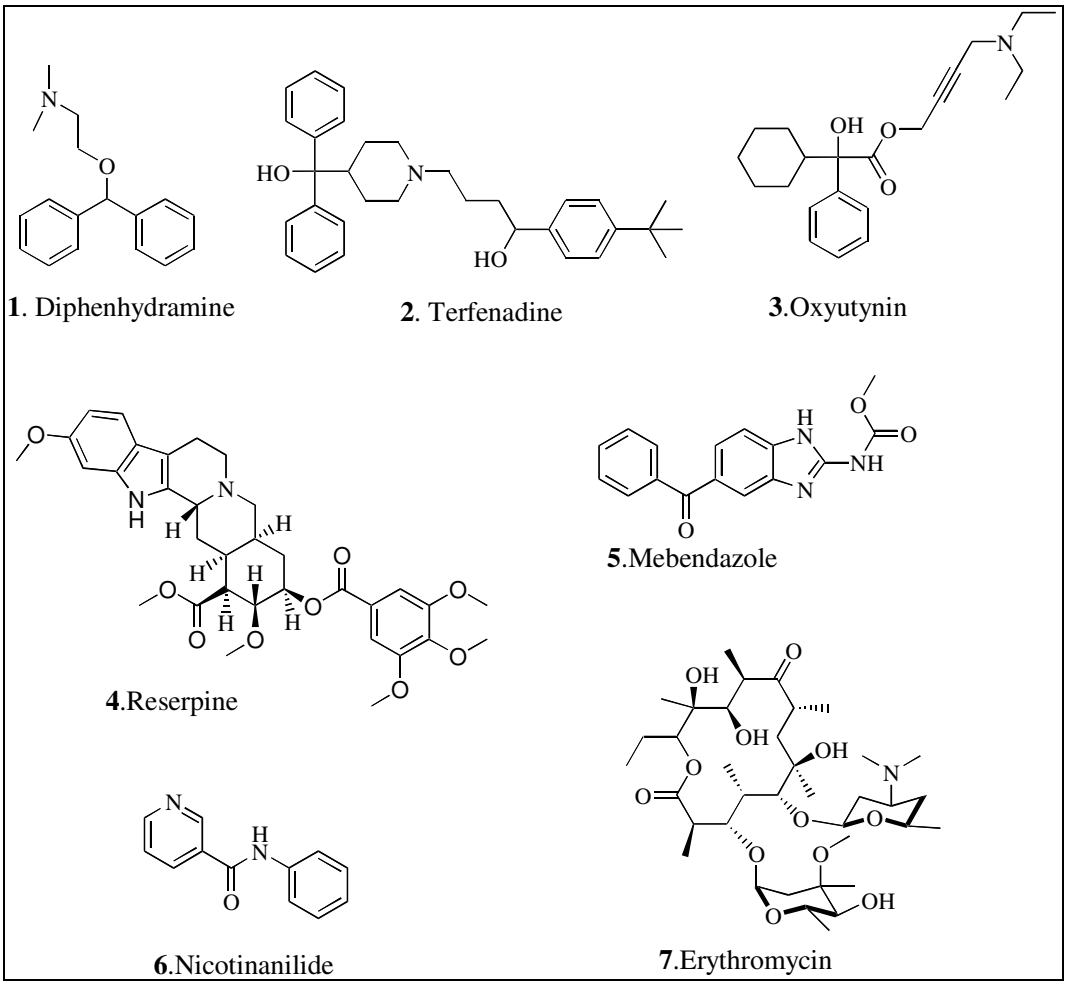


Figure 2.1: Structures of standard compounds used.

In this study, compounds were analysed on the SFC-MS set-up. Commonly a methanol make-up flow is introduced using an HPLC pump. For the purpose of ionisation studies the methanol make-up flow was replaced with acidified methanol to observe the effect of additional H^+ ions. Similarly the make-up flow was then replaced with ammoniated methanol to observe the effect of excessive NH_4^+ ions. Another condition was to avoid using any make-up flow from the HPLC. The results obtained showed that the post column make-up flow may not be necessary if sufficient amount of polar modifier is used in SFC, for *e.g.* where modifier percentage is 20% or more. To help probe the phenomenon of this ionisation process *i.e.* ionisation in the absence of high voltage, test compounds like diphenhydramine, terfenadine, oxybutynin and reserpine were chosen. Structures of the test compounds are shown in Figure 2.1. Their structural

differences covered the different ionisation properties of a pharmaceutical library. Thus allowing for the application of Novospray to be probed and understood.

The initial findings, with relation to ionisation in the absence of any high voltages applied to the ion source lead to the following discussion related to the actual source of ionisation. There are several solution based ionisation processes in literature, API techniques of ESI and APCI, other reported and used processes are TSP, PSP or sonic spray etc.

Table 2.1: Experimental conditions of make-up solvent, ion source high voltage and cone voltage

Make-up solvent	High voltage (kV)		Cone Voltage (V)	Desolvation temp (°C)	
	ESI	APCI		ESI	APCI
Pure MeOH MeOH + 0.1% NH_4OAc MeOH + 0.1% HCOOH No make-up	3.5	3.2	20	200	400
Pure MeOH MeOH + 0.1% NH_4OAc MeOH + 0.1% HCOOH No make-up	0	0	20	200	400

In order to ascertain if ionisation mechanism followed the electrospray charged residue model type, acid was added to the post column make-up solvent to facilitate the protonation of molecules, *i.e.* increase the population of $[M + H]^+$ ions in the system. Another option considered was that of thermospray type of ionisation process. Thermospray ionisation is enhanced through the presence of a volatile buffer such as ammonium acetate. This initially affords ammoniated molecules, $[M + NH_4]^+$, these can subsequently undergo collision within the ion source (often prompted by use of a repeller electrode) to produce the protonated molecule, $[M + H]^+$. Addition of ammonium acetate to the post column make-up solvent prior to mixing of the SFC eluent was investigated to see whether the novospray ionisation process was thermospray like. To probe whether thermospray like processes were involved, 0.1% NH_4OAc in methanol was used as make-up solvent. The presence of the volatile buffer would be expected to aid the ionisation process if it was related to thermospray. All experiments were

repeated using an APCI source configuration to investigate if the novospray ionisation process was dependent on the configuration of the source. To investigate whether novospray is related to or is a variant of sonic spray the variation of pressure, modifier percentage and the flow rate were studied.

2.1 High Voltage

During the coupling of SFC and MS, ionisation of samples in the absence of high voltage was observed. To investigate the ionisation mechanism involved, compounds 1 to 4 shown in Figure 2.1 were analysed, in triplicate, using the voltage conditions detailed in Table 2.1.

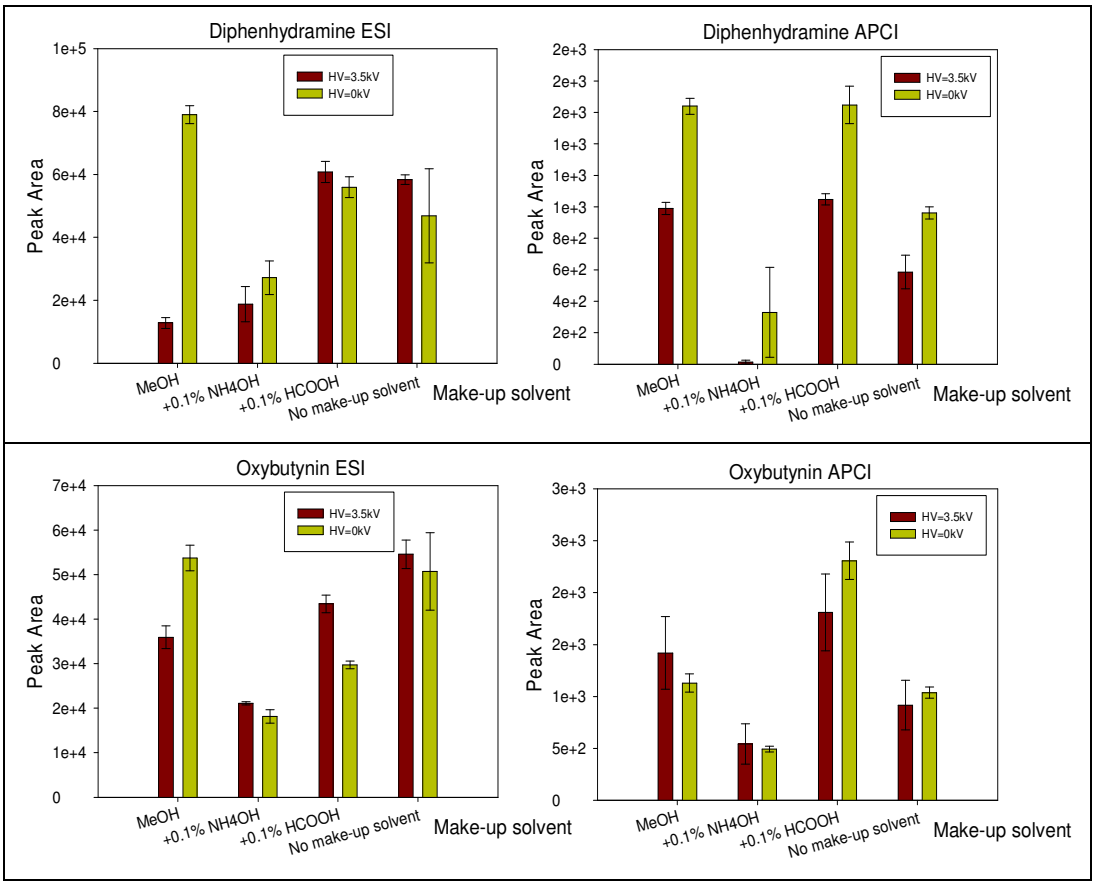


Figure 2.2: Plot of ionisation respose versus choice of make-up solvent using an ESI and an APCI source for (a) diphenhydramine and (b) oxybutynin. Number of replicates = 3

The results using the ESI source configuration are explained below. Figure 2.2 and Figure 2.3 show when pure methanol was used for make-up solvent for diphenhydramine, oxybutynin and reserpine, the novospray ionisation response is

greater than the ionisation response for the same sample under normal electrospray conditions. For *e.g.* Figure 2.4 shows the spectra of reserpine recorded under ESI and novospray conditions. In both cases the protonated molecule for reserpine can be observed. Although in the case of terfenadine the novospray response is less than normal electrospray response, ionisation is still observed at 0kV. This suggests that there is an underlying ionisation process involved that is not directly related to electrospray ionisation as described by either IEM or CRM. To investigate if the ionisation processes can be explained by the charge residue model (CRM) of ESI, the make-up solvent was then changed to 0.1% HCOOH in methanol. In CRM the ions are preformed in solution and the addition of acid would aid protonation, thus resulting in increased ionisation.

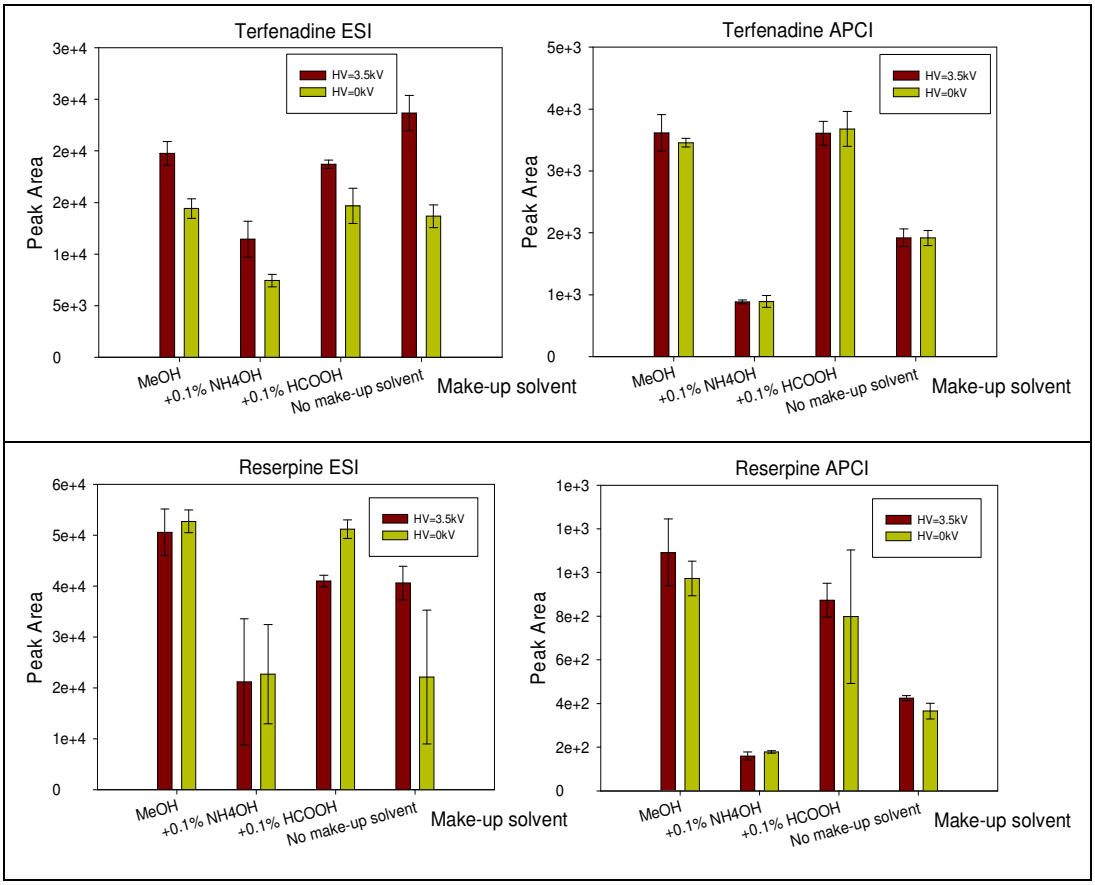


Figure 2.3: Plot of ionisation response versus choice of make-up solvent using an ESI and an APCI source for (a) terfenadine and (b) reserpine. Number of replicates = 3

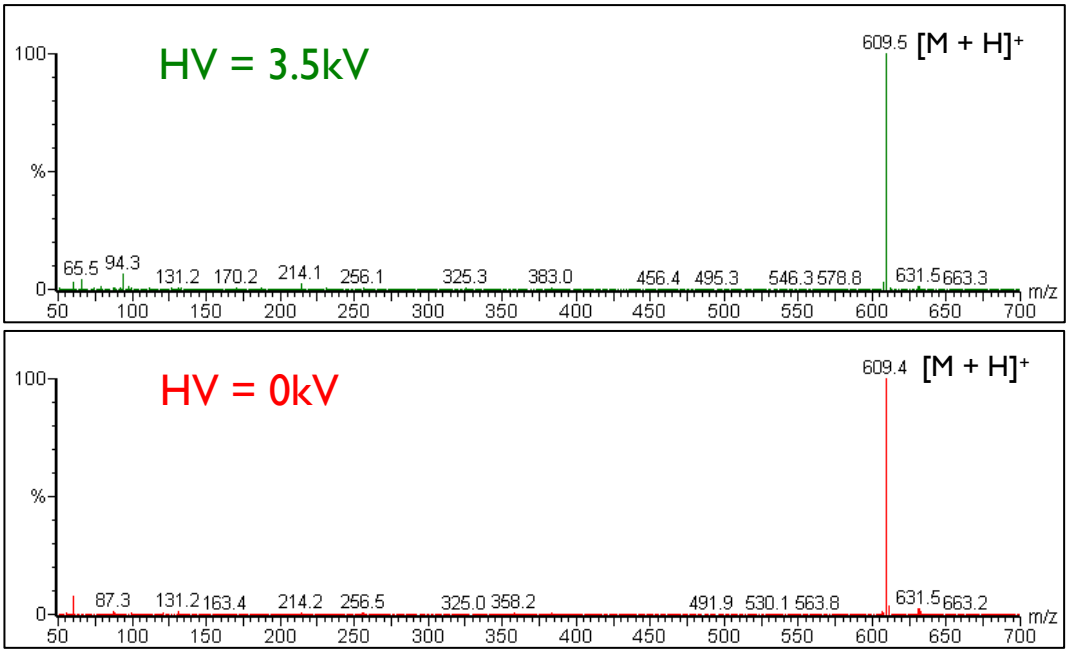


Figure 2.4: Mass Spectra of reserpine recorded at 3.5 kV and 0 kV showing the protonated molecule *m/z* = 609.

Diphenhydramine was also analysed under similar conditions. The sensitivity of diphenhydramine was noticeably increased using novospray conditions with pure methanol as make-up solvent. Diphenhydramine easily fragments to give *m/z* 167 under both normal ESI and novospray conditions. The peak areas for the reconstructed ion chromatogram (RIC) of *m/z* 256 and *m/z* 167 were plotted as shown in Figure 2.5. When pure methanol was used as the make-up solvent the ratio of *m/z* 256 to *m/z* 167 remained constant irrespective of whether the ESI high voltage was on or off, *i.e.* no more or no less fragmentation was observed for electrospray or novospray. Addition of NH_4OAc favoured fragmentation of diphenhydramine whereas the addition of acid did not.

All experiments were repeated using an APCI source configuration to investigate if the novospray ionisation process was dependent upon the configuration of the source. As shown in Figure 2.2 the results using APCI were found to be comparable to the results observed using the ESI source configuration. In both ESI and APCI the sensitivity dropped when NH_4OAc as introduced to the methanol make-up solvent and slight variation in sensitivity was observed on addition of 0.1% HCOOH to methanol make-up solvent. The sources differ in the

capillary types. The ESI source has a stainless steel capillary of 127 micron *i.d.* and the APCI source has a 100 micron *i.d.* fused silica capillary. The above results indicated that the ESI and APCI interface both exhibited ionisation under novospray conditions and that ionisation is independent of the capillary type. In each case the signal observed was comparable or better than normal ESI, thus suggesting that the observed mechanism of ionisation for novospray is not related to the high voltage applied to the droplet. Further, novospray is an underlying and major process within the electrospray ionisation process. Similarly, this process is underlying in APCI and may explain the regular observation of cationised molecules, *e.g.* $[M + Na]^+$ which cannot be formed as part of a gas phase ionisation process.

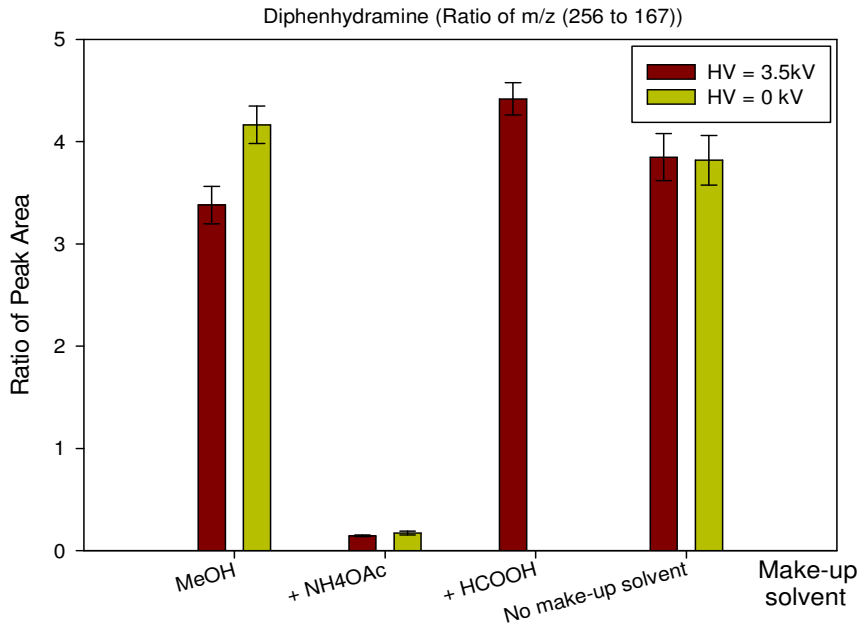


Figure 2.5: Ionisation response for Diphenhydramine with different make-up solvents for ratio of protonated molecule *m/z* = 256 to fragment ion *m/z* = 167. Number of replicates = 3

To understand the influence of applied voltage to the ionisation process, a study was carried out using four compounds (**3-6** shown in Figure 2.1) at different voltage steps between 0 and 5 kV. To avoid any residual charge the compounds were analysed starting from 0 kV and increased up to 5 kV, in increments of 0.5 kV. As seen from the results for oxybutynin in Figure 2.6 about 70% of the

ionisation is observed at 0 kV further suggesting that it is not electrospray type ionisation.

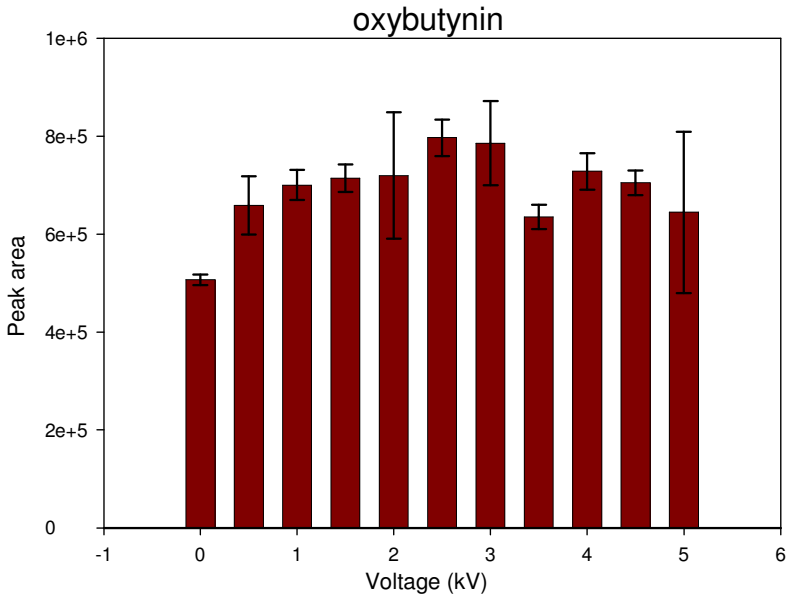


Figure 2.6: Dependence of ionisation response of ESI source high voltage for oxybutynin. Number of replicates = 3

2.2 Modifiers and Pressure conditions of SFC

In SFC the inability of non-polar CO_2 to elute very polar or ionic compounds is overcome by the addition of a small amount of a polar organic solvent. This organic solvent is referred to as the modifier when it is added directly to the mobile phase. The addition of a modifier improves the solvating properties of the supercritical fluid and sometimes enhances the selectivity of separation. It can also help improve separation efficiency by blocking some of the highly active sites on the stationary phase. Methanol is the most commonly used modifier in SFC as it produces high efficiency separation. Whilst other modifiers were investigated such as ethanol, propanol, all analyses reported here were undertaken using methanol modifier. The effect of changing the modifier on the novospray ionisation was considered. Therefore a less polar solvent, acetonitrile was chosen.

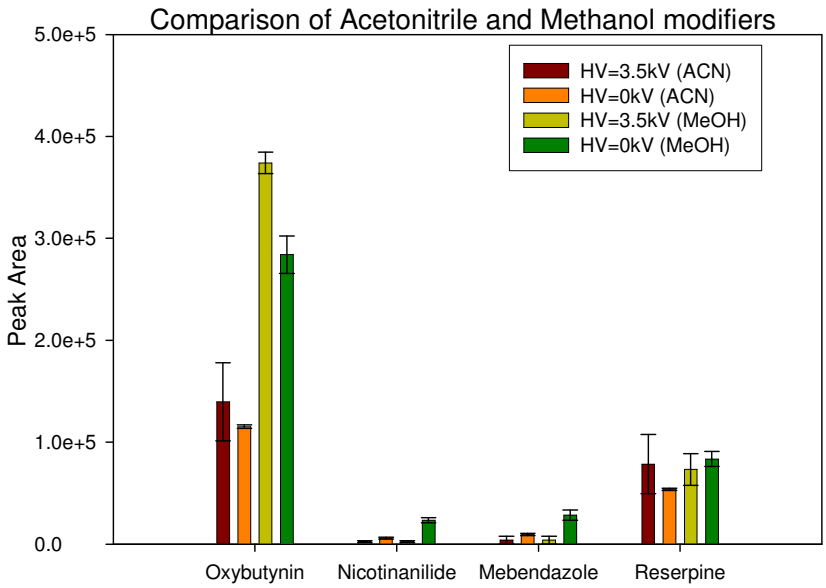


Figure 2.7: Comparison of ionisation response for MeOH and MeCN modifiers at ESI source HV = 0 kV and HV = 3.5 kV. Number of replicates = 3

A comparative FIA analysis was undertaken using acetonitrile in place of methanol. The results in Figure 2.7 show a drop in sensitivity when using acetonitrile as modifier particularly for nicotinanilide and mebendazole. This loss of sensitivity compared with the methanol experiments might be due to the acidic nature of methanol. The slightly acidic proton of the hydroxyl group may be assisting in protonation of the molecule, resulting in improved ionisation in comparison to the analyses in acetonitrile. Although the sensitivity drops, the plot for oxybutynin shows that the ratio of novospray ionisation to electrospray ionisation using acetonitrile is comparable to that with methanol modifier. This behaviour is also observed for the other compounds. The sensitivity is greater under novospray conditions with methanol make-up flow for compounds 4-6. This appears to show that the mobile phase composition of CO_2 and methanol is an important factor of the novospray ionisation. To confirm this, a mixture of three compounds (**2**, **3** and **7** shown in Figure 2.1) was analysed using FIA-ESI-MS with voltage conditions similar to novospray. The analytes ionised in the absence of high voltage on the FIA-ESI-MS system but with very low sensitivity. The enhanced ionisation in the SFC-MS system was thought to be related to the pressure, CO_2 flow rate and properties of the expanding supercritical/subcritical CO_2 which aid nebulisation of the eluent.

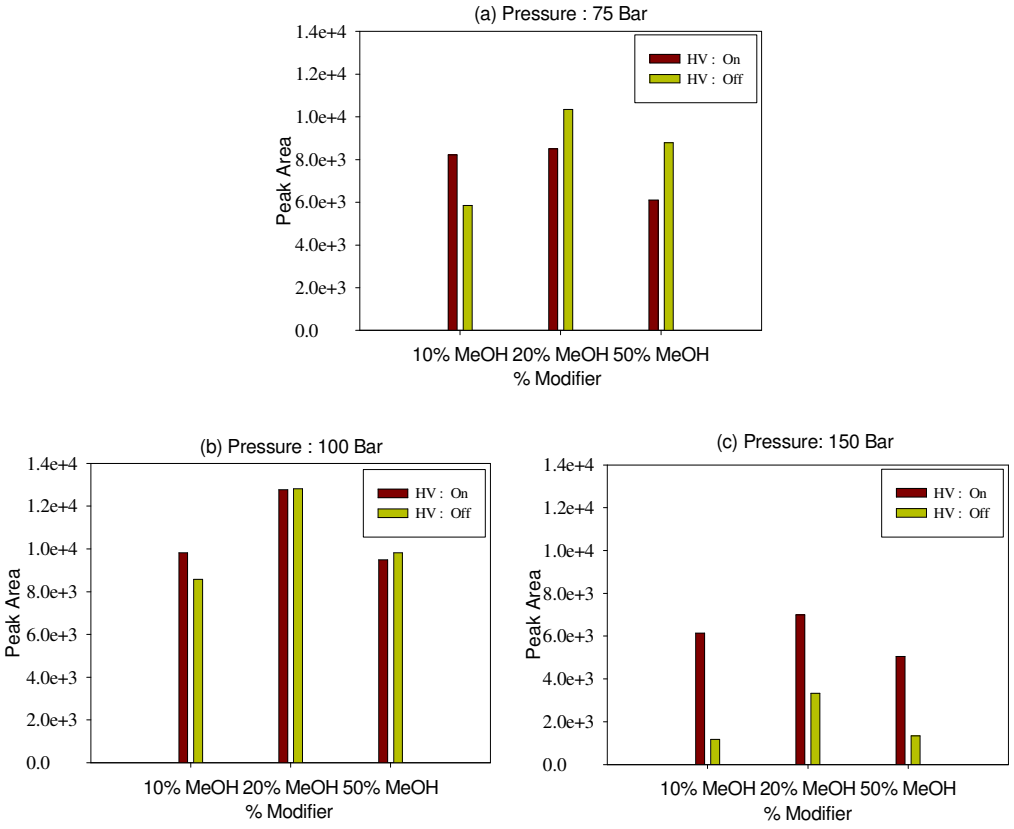


Figure 2.8: Dependence of ionisation response for flow injection analysis for the protonated molecule on pressure and modifier for a mixture of terfenadine, oxybutynin and erythromycin at pressure (a) 75 bar, (b) 100 bar, (c) 150 bar, and at 10, 20 and 50% modifier concetration.

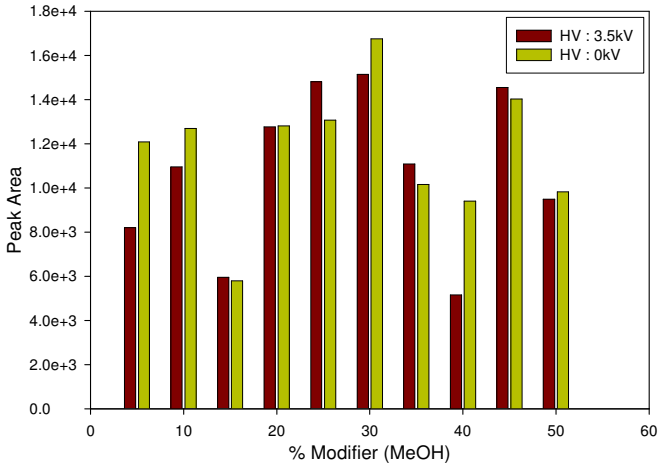


Figure 2.9: Dependence of ionisation response on percentage modifier for a mixture of terfenadine, oxybutynin and erythromycin at 100 bar pressure.

Another possibility considered to be the route to ionisation was sonic spray. Here the ion intensity strongly depends on the gas flow velocity.[61] Sonic spray ionisation was first developed by Hirabayashi and co-workers. In this technique

the eluent is sprayed from a fused silica capillary with gas flow coaxial to the capillary resulting in the formation of ions and charged droplets under atmospheric pressure. In sonic spray ionisation it is not necessary to apply heat or an electric field to the capillary of the ion source. To investigate whether novospray is related to or is a variant of sonic spray the variation of pressure, modifier percentage and the flow rate were studied. Initial experiments were performed using the same test mixture (compounds **2**, **3** and **8** shown in Figure 2.1) at 75, 100, and 150 bar and the mobile phase modifier percentage at 10, 20, and 50 organic using the SFC-MS system.

The plots showing the ionisation response for each of the conditions are in Figure 2.8. Reduced responses were observed at 75 bar in comparison to peak areas at 100 bar. Further at 75 bar pressure using 20% and 50% methanol modifier a noticeable increase in the novospray ionisation was observed in comparison to ESI. With the same modifier percentages at 150 bar pressure, a decrease in sensitivity for both ESI and novospray was observed. Also at 150 bar pressure ESI was the dominant process. In all cases novospray ionisation was observed. Since maximum sensitivity was achieved at 100 bar the effect of modifier was the studied over a percentage range in the mobile phase from 5 to 50 % (Figure 2.9). The optimum modifier condition for maximal ionisation was found to be 30 % at 100 bar pressure.

The initial work undertaken at three different pressure conditions was further extended over a range of pressure starting from 50 bar up to 150 bar with increments of 10 bar as shown in Figure 2.10. Once again four structurally different compounds (**2**, **4**, **5** and **6** shown in Figure 2.1) were analysed. All the standards showed similar trends with increases in pressure. Under novospray conditions the best sensitivity was obtained at around 90 -100 bar.

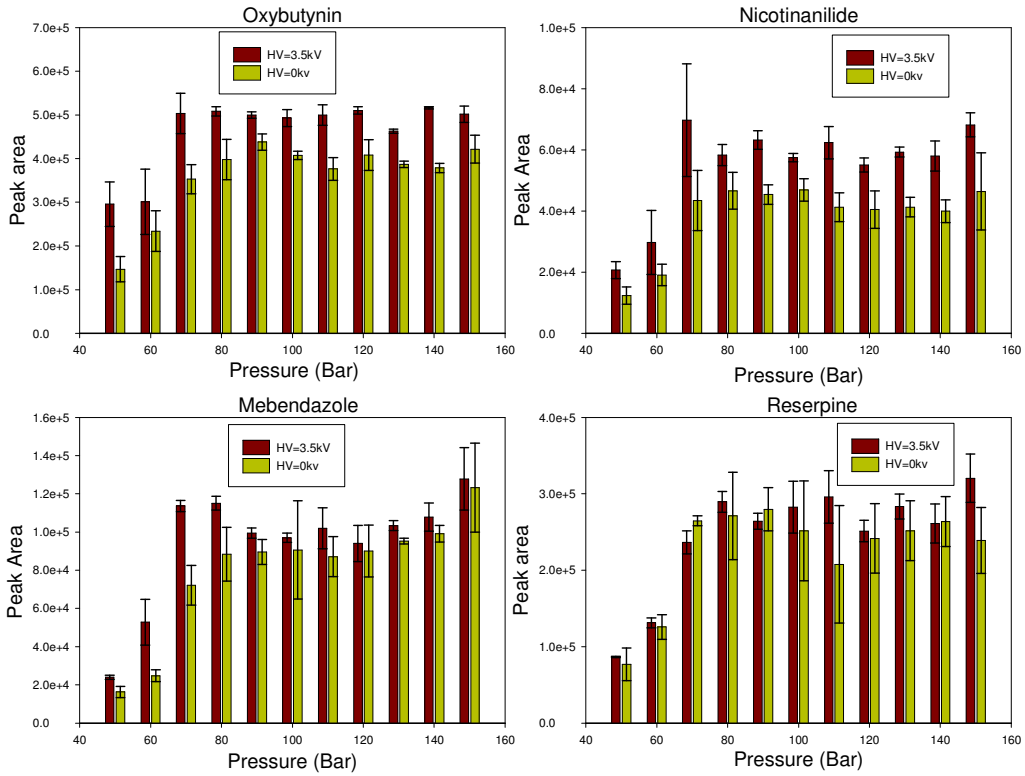


Figure 2.10: Dependence of ionisation response on pressure for (a) oxybutynin, (b) nicotinanilide, (c) mebendazole and (d) reserpine. Number of replicates = 3

2.3 SFC flow rate

The eluent flow rate is also one of the important parameters in SFC. Since novospray ionisation could be considered as a variation of sonic spray, varying the flow rate would affect the ionisation. The standards were analysed at different flow rates from the lowest possible 1.5 mL min^{-1} to 5.5 mL min^{-1} with increments of 0.5 mL min^{-1}. The plots of peak areas against the flow rate are shown in Figure 2.11. A similarity in trend under novospray conditions was observed for all standard compounds. Sensitivity of the analytes remains steady from 1.5 to 3.5 mL min^{-1} but decreases above a flow rate 4 mL min^{-1}. There could be two possible explanations for this observation. Firstly, as the flow rate is increased the amount of solvent is increased and this could result in an ineffective desolvation process. Secondly, as the flow rate is increased the size of the droplet leaving the tip of the API capillary could be larger; moving faster and so may take longer to evaporate before it attains the required size and desolvation state prior to entering the skimmer cone. This could be overcome by increasing the distance between the

end of the API capillary and the skimmer cone. This is demonstrated using mebendazole as shown in Figure 2.12. Here, increased sensitivity was observed on increasing the distance, thus indicating that the droplet size at higher flow rates has an impact on sensitivity. This may be due to the size of the droplet formed at higher flow rates being bigger at the default distance between the tip of the capillary. It was not possible with the instrument configuration used to adjust the distance sufficiently to optimise the desolvation process. These data suggesting that novospray has similarities to sonic spray.

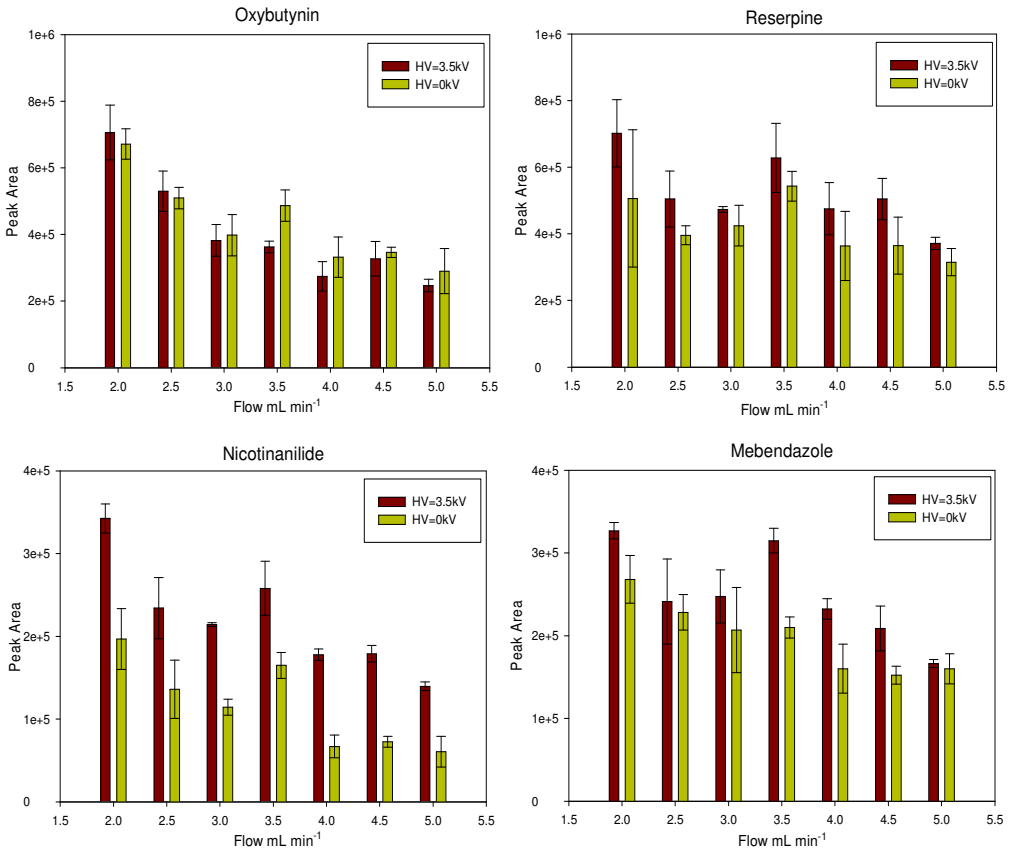


Figure 2.11: Dependence of the ionisation response on the SFC flow rate. (a) oxybutynin, (b) reserpine, (c) nicotinanilide and (d) mebendazole. Number of replicates = 3

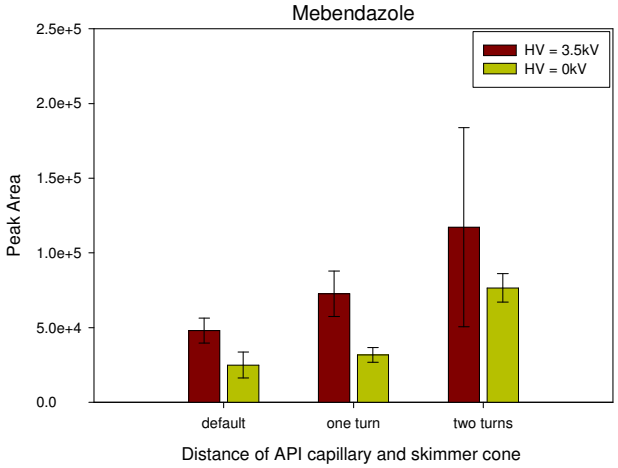


Figure 2.12: Plot showing increased ionisation on increasing the distance between the API capillary and the skimmer cone. Number of replicates = 3

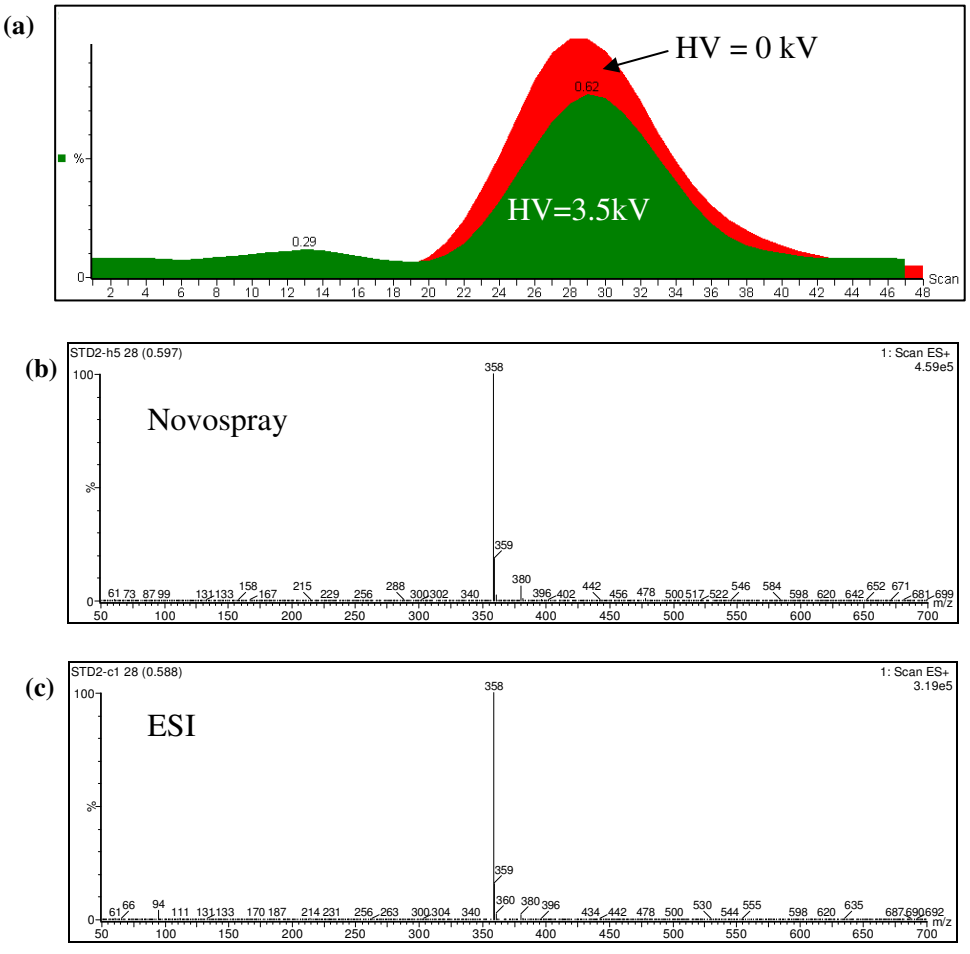


Figure 2.13: (a)Base peak ion chromatograms of flow injection analysis of oxybutynin showing increased sensitivity in the absence of high voltage; (b) mass spectrum recorded at 0 kV; (c) mass spectrum recorded at 3.5 kV.

From the above results a definitive ionisation mechanism could not be determined. A variant of sonic spray ionisation with charge residue model-type formation of analyte ions is a plausible mechanism, given the experimental data. Although the ionisation mechanism is not confirmed, the important point to note is that increased response can be achieved, in the absence of source HV, when using SFC-MS system with methanol modified CO_2 mobile phase. This ultimately could assist in lowering the limit of detection for such compounds as shown in Figure 2.13.

2.4 HPLC-ESI-MS and SFC-ESI-MS comparison

The novospray type of ionisation was first noticed when the SFC was coupled with MS and assumed to be specific to that specific instrument configuration. Hence to confirm that ionisation of compounds in the absence of high voltage is unique to SFC, a test mix which was a mixture of terfenadine, oxybutynin, erythromycin was introduced into the same mass spectrometer using an HPLC pump at a flow rate of 0.1 mL min^{-1}.

Interestingly a similar phenomenon, *i.e.* ionisation in the absence of ion source high voltages, was observed but the response was less than half when the HV was off compared to that observed for standard electrospray conditions. The experiment was then repeated at a flow rate of 1 mL min^{-1} and this time the peak areas were similar to that of 0.1 mL min^{-1} for both electrospray and novospray conditions. In comparison approximately 20% more sensitivity was observed with SFC-MS than HPLC-MS under novospray conditions. The SFC was operated using 20% methanol modifier at 100 bar pressure and with a flow of 3 mL min^{-1}. The sensitivity did not vary when the voltages were on or off in case of SFC. The chromatograms and spectra are shown in Figure 2.14 and Figure 2.15. For both SFC and HPLC a flow injection analysis was undertaken and the spectra for each were noted. In the spectrum, in case of HPLC terfenadine was the base peak whereas in case of SFC it was oxybutynin.

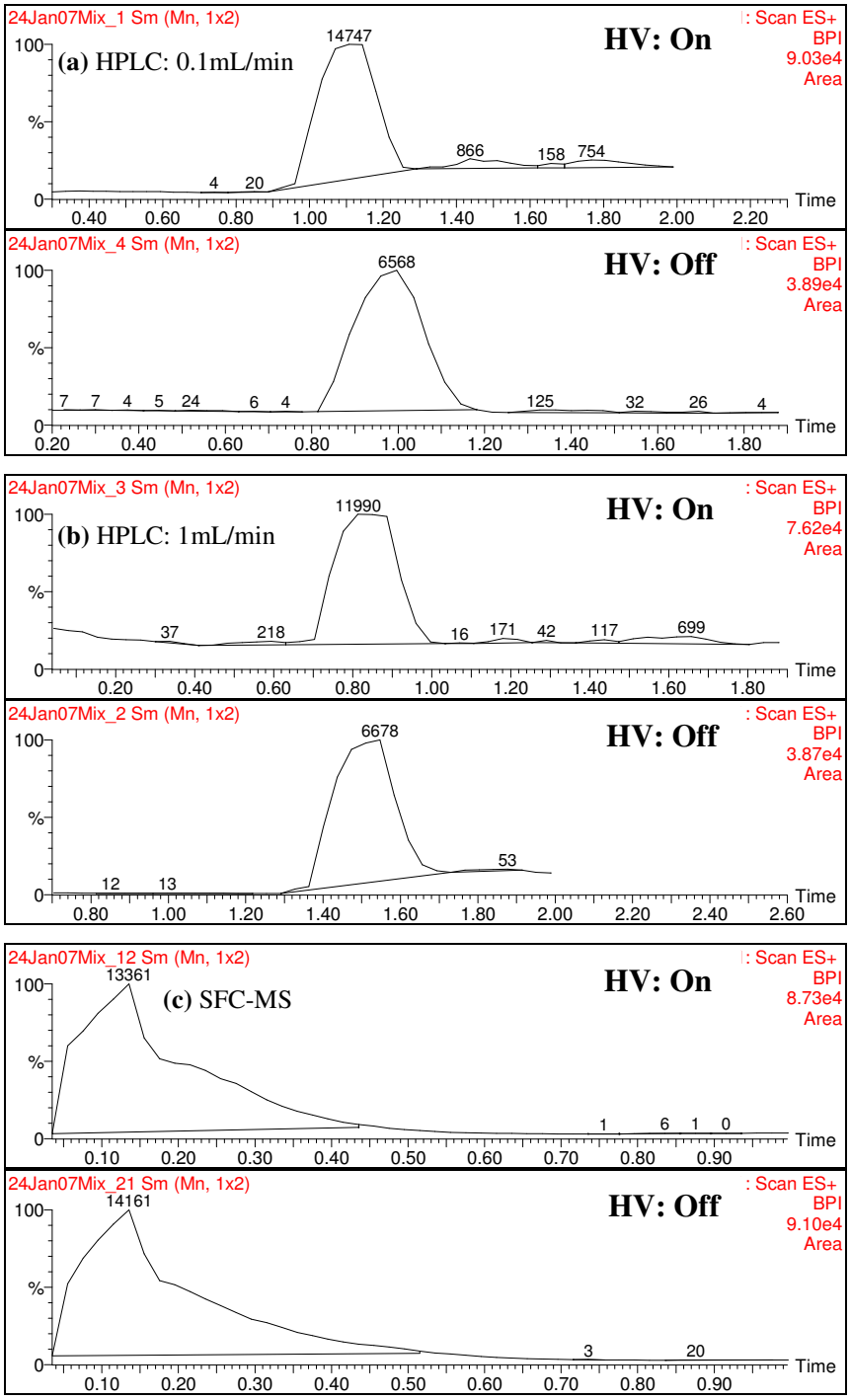


Figure 2.14: Base peak ion chromatograms of test mix (a) HPLC flow at 0.1 mL min^{-1} with HV on and off (b) HPLC flow at 1 mL min^{-1} with HV on and off (c) SFC-MS with HV on and off.

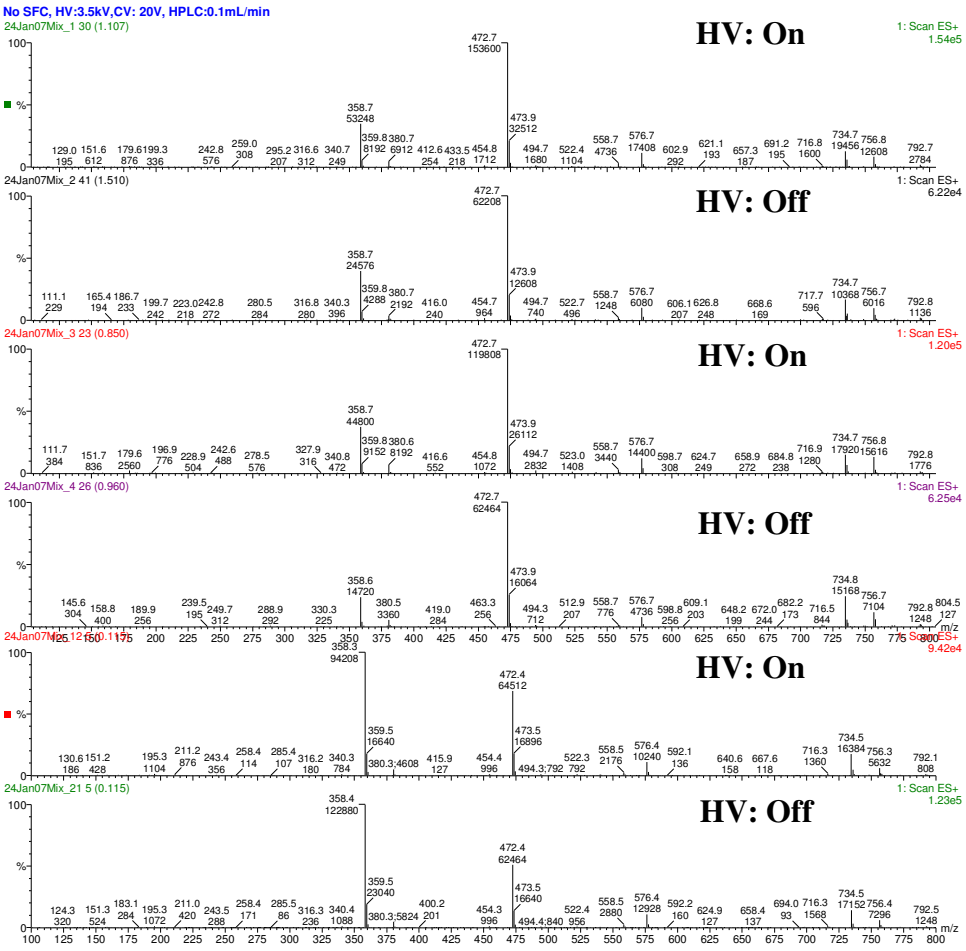


Figure 2.15: Spectra of test mix (Chromatograms shown in Figure 2.14) (a) HPLC flow at 0.1 mL min^{-1} with HV on and off (b) HPLC flow at 1 mL min^{-1} with HV on and off (c) SFC-MS with HV on and off. Sensitivity comparison of HPLC-ESI-MS and SFC-ESI-MS.

2.5 Experimental Section

Chemicals and reagents

HPLC grade methanol was purchased from Fisher Scientific (Loughborough, UK) and SFC grade CO_2 from BOC gases (Guildford, UK). Formic acid (HCOOH) (Riedel-de Haën Seelze, Germany) and ammonium acetate (NH_4OAc) (Sigma-Aldrich, Gillingham, UK) were used for the make-up solvent. Standard compounds diphenhydramine, oxybutynin chloride, terfenadine, reserpine and mebendazole (Sigma-Aldrich, Gillingham, UK), erythromycin (Fluka, Switzerland) and nicotinanilide (Lancaster, Morecambe, England) were used without further purification. Structures of the standards are given in Figure 2.1.

Instrumentation

A Minigram SFC system (Berger Instruments, Mettler-Toledo Autochem, Newark, DE, USA), equipped with a FCM-1100/1200 dual-pump fluid control module, a TCM-2250 heater control module, an ALS 3100/3150 autosampler and a Knauer k-2501 variable wavelength UV detector (Knauer, Berlin, Germany) were used in all experiments. The system was controlled and data acquisition was undertaken using PRONTO software (v1.5.305.15, Berger instruments). The eluent was split before the backpressure regulator using a zero dead volume stainless steel T-piece. 1524 mm length of 0.10 mm i.d. PEEK tubing was used to interface the SFC eluent to a Micromass Platform LCZ mass spectrometer, the latter fitted with a standard ESI Z-spray ion source. The mass spectrometer was controlled by MassLynx software (v3.5). Another zero dead volume stainless steel T-piece was used to connect a HP1050 HPLC system (HP, Agilent, Palo Alto, California) to deliver a make-up flow of solvent at 0.1 mL min^{-1} to the API interface. A schematic of the SFC-MS set-up is shown in Figure 2.16.

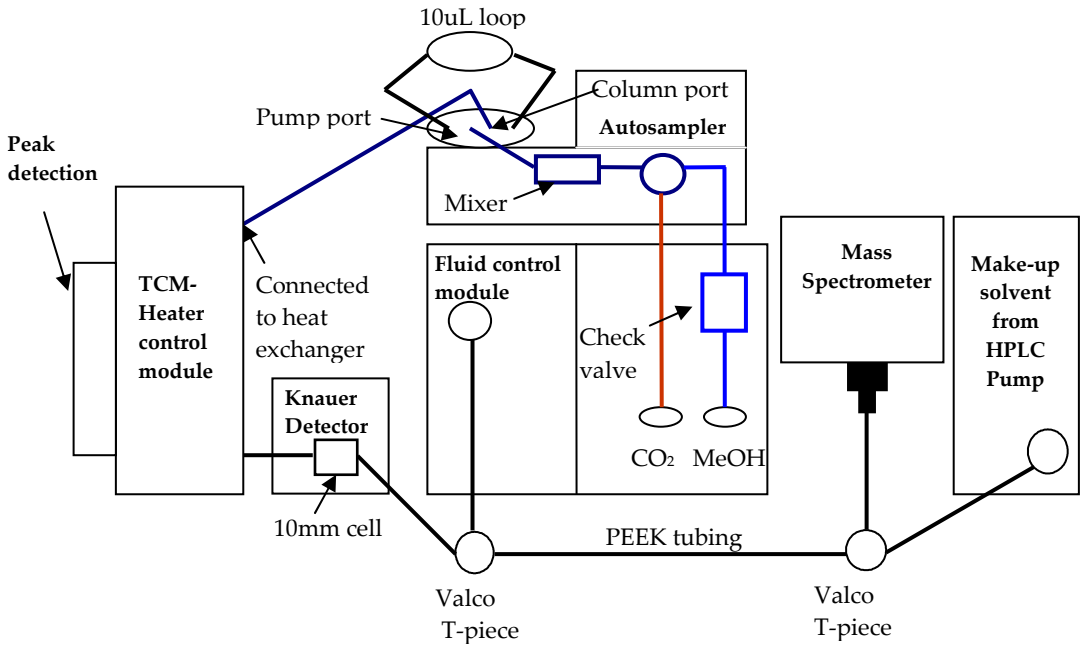


Figure 2.16: Schematic for SFC-MS.

Experimental conditions

Individual stock solutions of each compound were prepared in methanol at a concentration of 1 mg mL-1. New solutions were freshly prepared everyday with each component at a concentration of 0.1 mg mL-1 in the mixture. The analytes were analysed isocratically at modifier concentrations in the mobile phase between 5-50 % organic solvent. The pressure was set at 100 bar, oven 35 °C, flow rate 3 mL min-1 and a 2 μL injection volume used. All standards were analyzed using a 2-EP column, 50 mm x 4.6 mm, 5 μM, 60 Å pore size (Princeton Chromatography, Cranbury, NJ, USA). The UV detector was set at a wavelength of 220 nm. Both positive ion and negative ion mass spectra were recorded. The MS conditions are shown in

Table 2.2. The make-up flow consisted of (i) pure methanol, (ii) 0.1 % NH4OAc in methanol and (iii) 0.1 % formic acid in methanol. The make-up flow rate was delivered at 0.1 mL min^{-1}.

Table 2.2: Typically LCZ parameters used for ESI and APCI analyses.

Parameter	ESI	APCI
Cone voltage	20 V	20 V
High voltage	3.5 k V	3.2 kV
Ion energy	0.8 V	0.8 V
Multiplier	550 V	550 V
Analyzer vacuum	2.8 x 10^{-4}mPa	2.8 x 10^{-4} mPa
Desolvation gas flow	500 L h^{-1}	500 L h^{-1}
Mass range	*m/z* 100-800	*m/z* 100-800
Desolvation temperature	200°C	400°C
Source temperature	140°C	120°C

2.6 Conclusions

Compounds 1-7 (See Figure 2.1) were analysed using SFC-ESI-MS under normal electrospray conditions and with no high voltage. Ionisation was observed unexpectedly in the absence of high voltage. Analytes ionised in the absence of high voltage indicating the mechanism of ionisation in the SFC-MS system with supercritical CO_2 mobile phase does not exhibit typical electrospray ionisation. Three possible existing mechanisms were considered as contributing to this

ionisation. These were thermospray ionisation, charge residue model for electrospray ionisation and sonic spray ionisation.

The presence of NH_4OAc suppressed ionisation, suggesting that the thermospray type mechanism was unlikely. The addition of acid to the eluent to determine if the CRM type process existed showed very little variation in sensitivity; whereas a stepwise source high voltage study, over a range of 0 and 5 kV, indicated little variation in sensitivity suggesting the possibility of preformed ions in solution. This suggests that it may not be a complete CRM type process.

Novospray ionisation was found to be dependent on the pressure, modifier, and the flow rate of the SFC system but independent of the ESI ion source configuration since novospray ionisation was also observed when an APCI source configuration was used. The results obtained with the latter were comparable in both cases indicating either source is suitable to be interfaced with the SFC system.

A comparative study using acetonitrile and methanol as modifier suggests that the acidic nature of methanol may play a role in assisting in protonation of the molecule. This could again suggest a CRM-type of process. The mechanism of ionisation in the SFC-MS system when the API voltages are turned off was found to be similar to that of sonic spray ionisation consistent with the charge residue model. Another consideration might be that the ion formation is possibly due to collision of molecules in the free jet expansion region of the of the SFC eluent.

Chapter 3

SFC Method Development and comparison with HPLC

3.1 Introduction

The need for efficient high speed separations has resulted in further development of chromatographic systems. Amongst these chromatographic systems, some use higher pressures whilst others use improved stationary phase materials for columns.

Packed column SFC is another such development in which changing the mobile phase has resulted in efficient chromatographic separations. Packed column SFC has recently experienced tremendous growth as an analytical technique. It is a technique similar to HPLC that uses a supercritical fluid for the mobile phase. Retention of the analyte in SFC is a function of temperature, pressure, mobile phase density, mobile phase composition and the stationary phase. Furthermore, there may be the interaction with the packing material, *i.e.* silica, making it more complex than other chromatographic techniques. Many of these variables are inter-related and do not change in a predictable manner.

The aim of this project was to develop a near generic SFC method for compounds of pharmaceutical importance. The instrumental conditions of the SFC were varied to be able to achieve optimised conditions for the analyses of the given type of compounds. SFC is classed as a normal phase technique as pure CO_2 is non polar to solvate polar molecules binary mixtures ere used as the mobile phase to increase the polarity range. The solvent added to increase polarity is called the modifier. Although methanol is the most commonly used modifier in SFC the effects of other modifiers were studied by replacing methanol with ethanol, 2-propanol, and comparing the results.

A comparative study of the available stationary phases was undertaken to decide on the best suited column for the compound types investigated. These were 2-ethylpyridine (2-EP), cyanopropyl (CN), polypropyl urea (PPU), and diol. In all cases 20% methanol modified CO2 was used.

Additives are used in the mobile phase in small quantities to improve the chromatography of the analytes. In this case the additives were added to the mobile phase when analysing compounds using a CN column. Most compounds had sharp and narrow peaks on other columns *i.e.* 2-EP, hence the addition of additives did not show any noticeable improvement in chromatography. The retention time also did not change much either.

These results were assessed with regard to, *e.g.* speed of analysis, number of theoretical plates, resolution *etc.* The compounds analysed for the above study consisted of a series of discreet libraries, a training set of compounds, a diverse set that covered the chemical space of typical pharmaceutical compounds and a focused set. The latter consisted of compounds with similarity in structures, but with subtle differences at two positions on the basic structure, as seen in Figure 3.1. The structures of all the compounds analysed are shown in the Appendix 1-4.

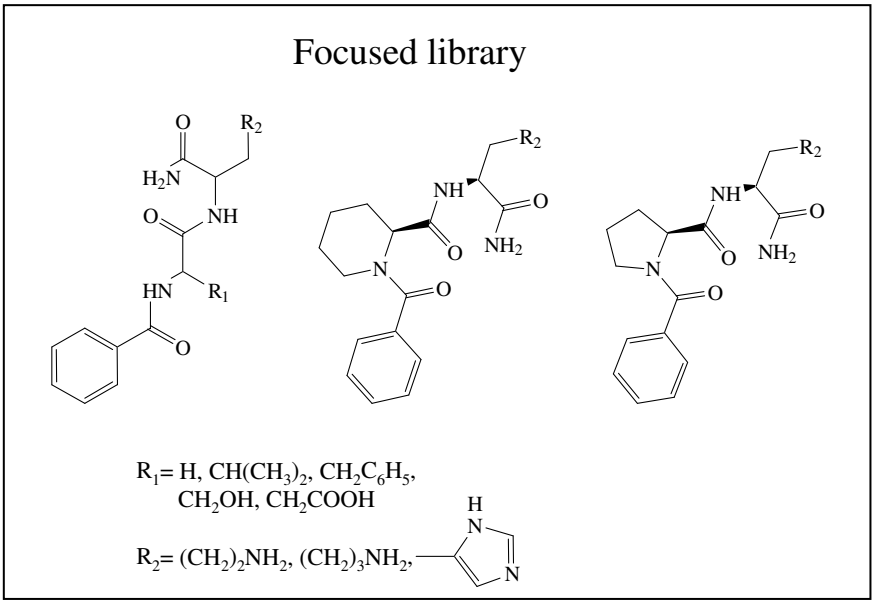


Figure 3.1: Basic structure of focused set of compounds.

The combined results from the studies of stationary phase, modifiers, additives resulted developing methods wherein most of the compounds being analysed with little or no difficulty. The efficiency N, asymmetry and HETP were calculated and compared for different modifiers, stationary phase and additives to enable determination of the most suitable parameter. However, a number of samples proved problematic, showing non ideal responses *e.g.* insolubility of analytes in methanol, peak splitting. An attempt to explain the reasons for the observations has been made. The possible solutions to tackle such variations are shown in this section.

A comparative study of SFC and HPLC was undertaken to understand the compound coverage of each technique; the speed of analysis was also considered. To be able to decide on the most suitable technique of analysis between HPLC and SFC, based on the chemical structures of the analytes, a comparative study was also undertaken. The overall results of a generic SFC method were compared with a generic HPLC method and the results are discussed towards the end of this chapter. A trend in retention times based on the structures and substructures of compounds was noted for a class of compounds. This trend was observed for the focused set. The substructure of the side chains was classified as aliphatic, aromatic. This trend could not be further explored as a result of the unavailability of compounds with similarity in structures.

3.2 Method development

The given sets of compounds were analysed using modified CO_2 for mobile phase with the following conditions. The outlet pressure was set at 100 bar, oven temperature 35^{o}C, flow rate 4 mL min^{-1} with a 4 µL injection volume. The pressure was altered between 80 bar and 150 bar and the temperature up to the instrument limit of 40ºC. These changes were made one at a time maintaining the other parameters at initial conditions. Changing the pressure or temperature did not affect the peak shapes but only slightly changed the retention times. Factors like modifiers, additives and stationary phases were explored to achieve an optimised method for compounds of pharmaceutical interests by SFC.

3.2.1 Modifiers

Pure CO_2 is known to be unsuitable for analysis of most pharmaceutical compounds on packed column SFC, due to its poor solvating ability. Hence the addition of small amounts of polar modifiers is necessary. Depending on the temperature and pressure of the chromatographic systems, mobile phase modifiers are known to affect the retention, selectivity and efficiency.[103] During the preliminary study of SFC, a training set of compounds (Structures shown in Appendix 2 on Pages112-114) was provided to familiarise the operator with the use of the SFC instrument. This set was used to choose the most suitable modifier. Methanol, ethanol, and propanol were used as the mobile phase modifiers under default instrument conditions and the results compared. Figure 3.2 shows the plot of retention times against compound numbers.

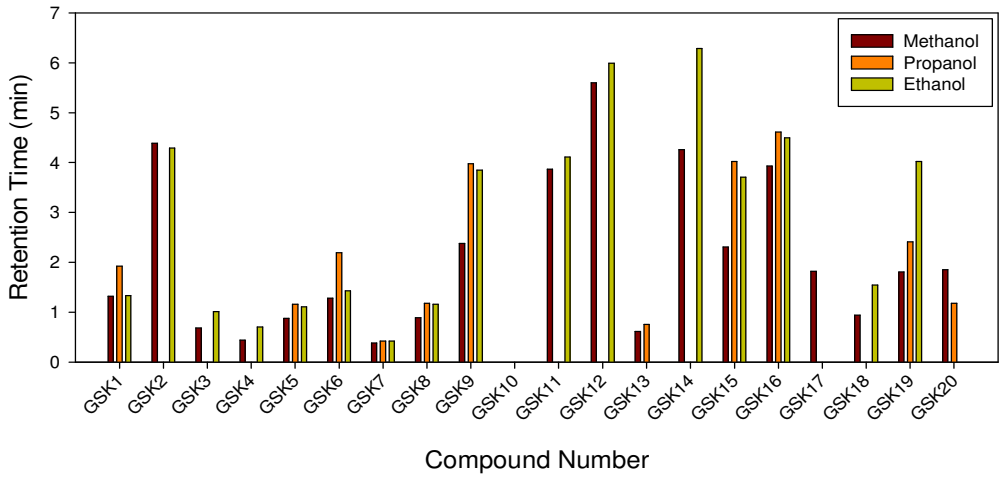


Figure 3.2: A comparison of retention times for the training set using modifiers methanol, 2-propanol and ethanol. In each case 20% modifier under isocratic conditions was used.

With 20% methanol (isocratic) most compounds eluted with sharp and narrow peak shapes with minimal tailing on 2-EP in comparison to PPU and CN columns. 20-25% compounds did not elute under these conditions with 2-propanol and ethanol. Most of these compounds were with acid and/or sulphonamide substructures. Compounds with amide substructures give broad peaks with ethanol. Examples of the chromatography for selected compounds are shown in Figure 3.3 and Figure 3.4.

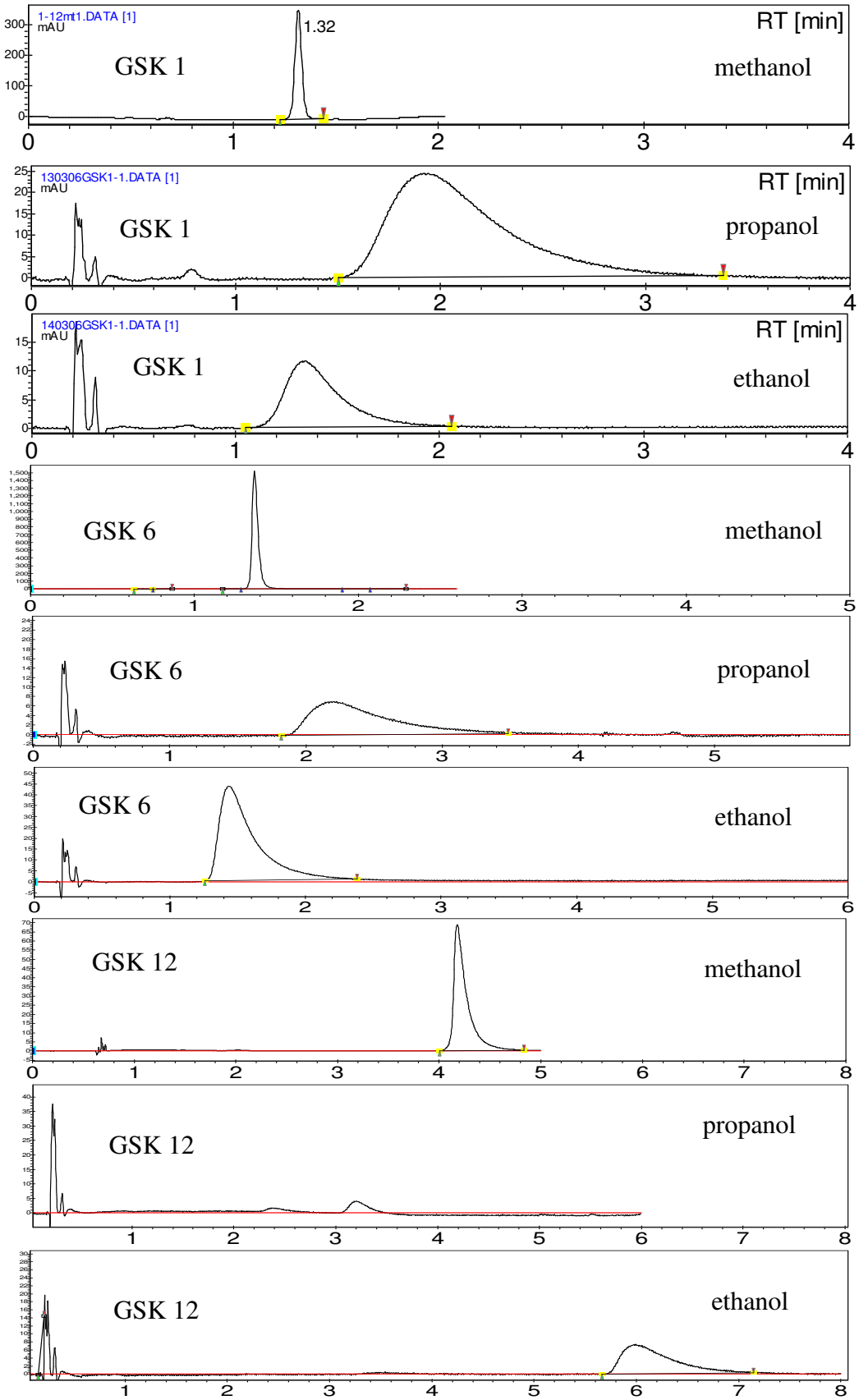


Figure 3.3: An example showing peak shapes for analyte GSK1, GSK6 and GSK12 with modifiers, methanol, ethanol, and 2-propanol.

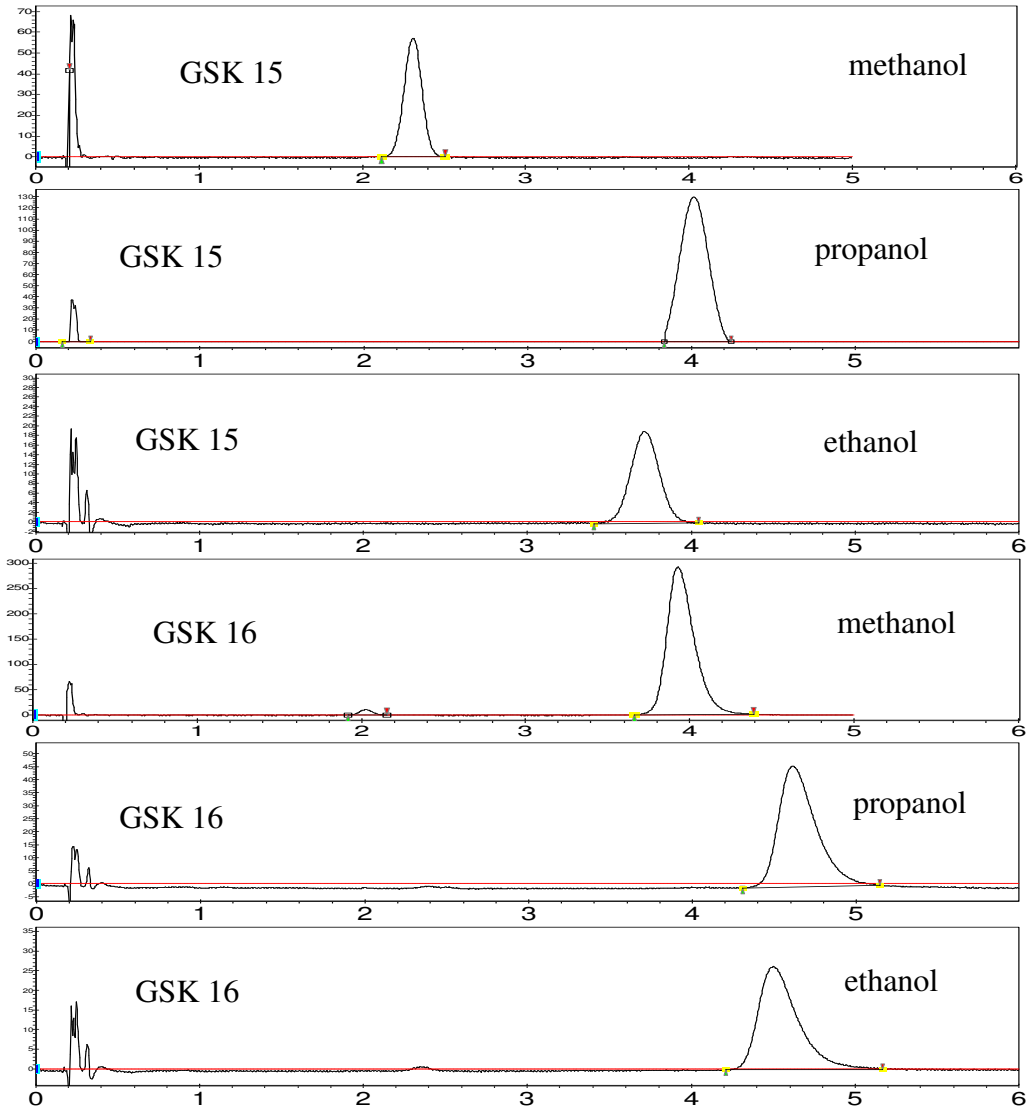


Figure 3.4: An example showing peak shapes for analyte GSK15 and GSK16 with modifiers, methanol, ethanol, and 2-propanol.

From the raw data the values for the retention factor k′, number of theoretical plates N, and height equivalent of theoretical plates HETP was calculated. These parameters for the training set are listed in Table 3.1 for selected compounds. Overall the modifiers show varied effectiveness. However, when methanol modifier is used most compounds show large N values. The scatter plot in Figure 3.5 confirms this finding.

Most small N value compounds also show larger HETP values. Figure 3.6 shows scatter plot of HETP values. But closer observation shows that the methanol modifier results in general smaller HETP values even for smaller N values.

Table 3.1: Values of k', N and HETP for selected compounds from Figure 3.2.

	methanol			2-propanol			ethanol		
	k'	**N**	**HETP**	**k'**	**N**	**HETP**	**k'**	**N**	**HETP**
GSK 1	1.10	5689	0.0439	8.60	41	1.2004	5.65	81	0.6150
GSK 5	3.40	1529	0.0327	4.80	1495	0.0334	4.55	1369	0.0365
GSK 6	5.40	28	1.7578	9.95	42	1.1875	6.15	87	0.5686
GSK 7	0.90	641	0.0779	1.10	441	0.1134	1.10	441	0.1134
GSK 8	3.45	1267	0.0395	4.90	1136	0.0440	4.80	1098	0.0455
GSK 9	10.90	1156	0.0433	18.90	532	0.0939	18.25	421	0.1186
GSK 11	18.35	95	0.5209	--	--	--	19.55	20	2.3843
GSK 13	2.05	1215	0.0412	2.75	900	0.0556	--	--	--
GSK 14	20.30	1909	0.0262	--	--	--	30.45	918	0.0544
GSK 15	10.55	1764	0.0283	19.10	2236	0.0224	17.55	1699	0.0294
GSK 16	18.65	2137	0.0234	22.05	1539	0.0325	21.50	1296	0.0386
GSK 19	8.05	1188	0.0421	11.05	241	0.2068	19.10	32	1.5597

The statistics of mean and standard deviation from these data tables can assist in determining the suitable modifier. For N values this statistics is given in

Table 3.2. Large mean value and small standard deviation of N is the ideal combination for a suitable modifier. The varied performance of modifiers comes clearly further from this Table. The highest mean, however, is seen for methanol modifier. Similarly for HETP values, the statistics is listed in Table 3.3. Here the ideal combination is of low mean and standard deviation. Methanol and 2-propanol show similar statistics with methanol showing marginally smaller mean value.

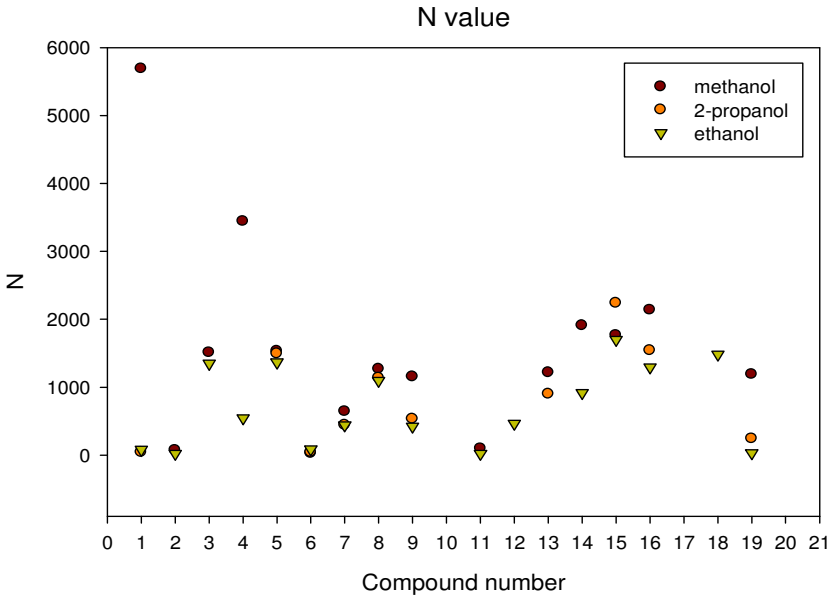


Figure 3.5: Scatter plot showing the spread of N values data for the modifiers methanol, 2-propanol and ethanol.

Table 3.2: Statistical data of N values of the peaks for modifiers methanol, 2-propanol and ethanol.

N	methanol	2-propanol	ethanol
Mean	1576	860	708
Std Dev	1442	733	599

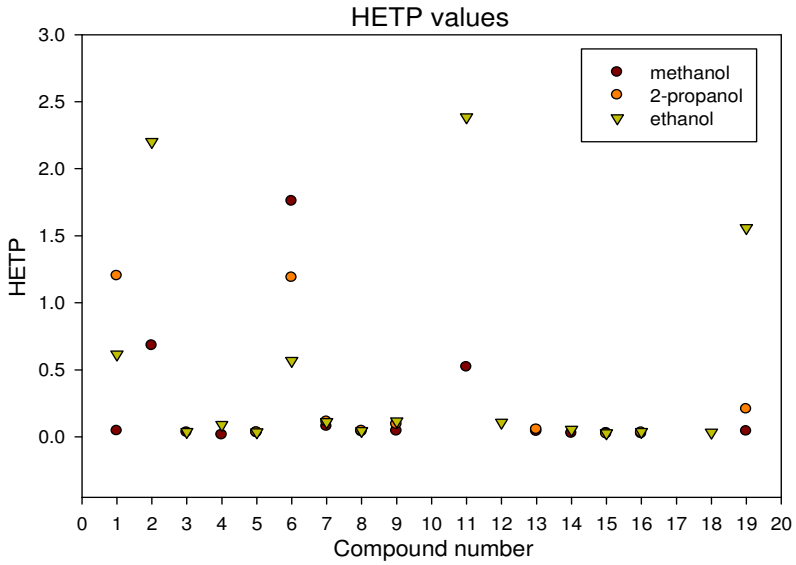


Figure 3.6: Scatter plot showing the spread of HETP data for the modifiers methanol, 2-propanol and ethanol.

Table 3.3: Statistical data of HETP values of the peaks for modifiers methanol, 2-propanol and ethanol.

HETP	methanol	2-propanol	ethanol
Mean	0.2271	0.2990	0.5022
Std Dev	0.4684	0.4749	0.8035

Another parameter that might help determination of better modifier is the scatter plot of peak asymmetry for the same compounds. This is shown in Figure 3.7. From the peak asymmetry data methanol modifier performs far better overall. The statistics for peak asymmetry are listed in Table 3.4. The ideal situation is combination of mean tending to unity and smaller standard deviation. Here as seen in scatter plot earlier, methanol performs much better than other two modifiers. As methanol performs better for large number compounds than others, it appears suitable as a universal modifier.

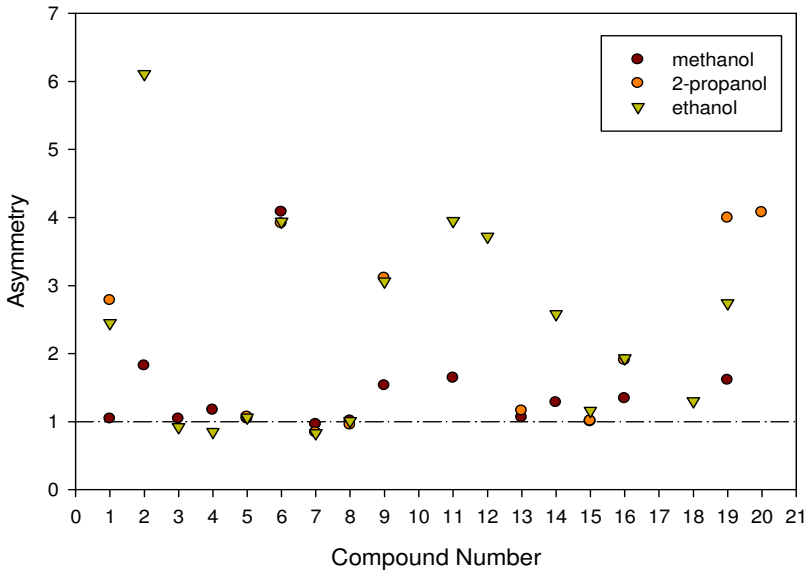


Figure 3.7: Scatter plot showing the spread of asymmetry data for the modifiers methanol, 2-propanol and ethanol.

Table 3.4: Statistical data of asymmetry of the peaks for modifiers methanol, 2-propanol and ethanol.

Asymmetry	methanol	2-propanol	ethanol
Mean	1.4413	2.2536	2.3506
Std Dev	0.7807	1.3429	1.5184

From the above results it can be concluded that methanol is the preferred choice of modifier for the elution of polar pharmaceutical compounds under the default temperature and pressure conditions in SFC. Methanol also has the favourable properties of low viscosity, high polarity with low boiling point and low surface tension hence helping in improving the ionisation efficiency and sensitivity for MS in comparison to ethanol and isopropanol. Overall the results indicate methanol as the suitable modifier for most compounds in this set. Hence methanol was chosen as the modifier. One other reason for the use of methanol is related to cost. Ethanol is about 10 times more expensive than methanol and hence not practical with the industries constantly trying to minimise costs.

3.2.2 Stationary Phases

The packing materials in SFC are very similar to that used in LC columns. Several studies have been reported for the selection of stationary phase based on the type of solute.[104-107] Here in this project the study was performed on using the five types of pSFC columns available. Initial experiments were undertaken were using columns 250 mm in length, 4.6 mm I.D., 6 μm particle sizes, and 60 Å pore size. The study was then extended to 50 mm in length, 4.6 mm I.D., 5 μm particle sizes, and 60 Å pore size. A mixture of analytes were analysed using a 250 mm column and a 50 mm column. The separation in peaks was not affected when the column length was changed but the retention of the analytes was reduced thus resulting in shorter run times. These results suggested that the chromatography often occurred early on in the column. Shorter retention of analytes not only speeds the entire process but also results in less use of solvents. Hence, smaller columns were used for the analyses. All the compounds were analysed using 50 mm columns with 2-EP, PPU, CN stationary phases. Initial experiments with diol and amino stationary phase resulted in the compounds not eluting, hence these were not used. The results obtained for the focused library (Structures shown in Appendix 1 on pages 110-112) are illustrated in Figure 3.8. This shows that for most compounds 2-EP gives the shortest retention times. All analyses were undertaken using 20% methanol modified, CO_2 mobile phase at a flow rate of 3 mL min^{-1}.

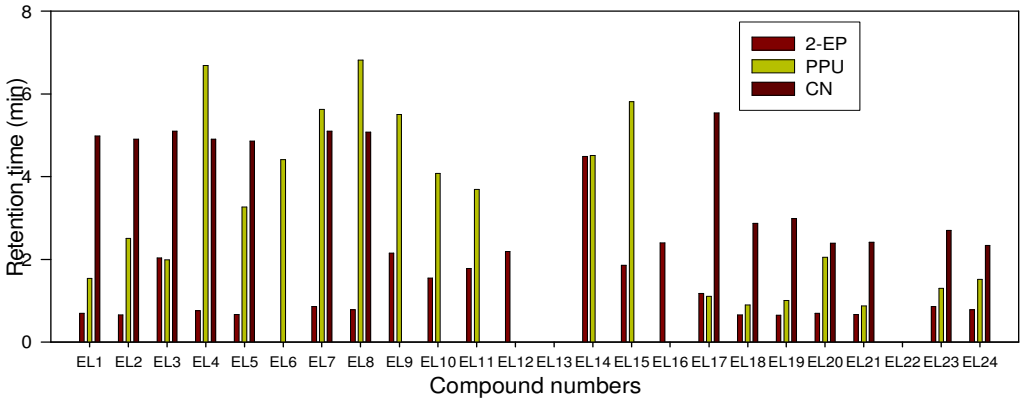


Figure 3.8: A comparison of retention times for the focused library (EL) for 2-EP, PPU, CN. Column length: 50mm

In Figure 3.8, 71 percent of compounds from the focused set eluted with shorter retention times with 2-EP column in comparison to the other two columns *i.e.* PPU and CN column.

Table 3.5: Values of k', N and HETP for with 2-EP, PPU and CN columns of selected compounds from Figure 3.8.

	2-EP			PPU			CN		
	k'	**N**	**HETP**	**k'**	**N**	**HETP**	**k'**	**N**	**HETP**
EL1	2.50	400.00	0.1250	6.70	60.80	0.8224	23.90	968.77	0.0516
EL2	2.30	144.00	0.3472	11.55	139.52	0.3584	23.55	2410.81	0.0207
EL4	2.80	641.78	0.0779	32.45	283.26	0.1765	23.55	971.86	0.0514
EL5	2.35	498.78	0.1002	15.35	445.07	0.1123	23.30	952.16	0.0525
EL7	3.30	150.94	0.3313	27.15	291.06	0.1718	24.50	1156.00	0.0433
EL8	2.90	243.36	0.2055	33.10	309.76	0.1614	24.40	977.28	0.0512
EL17	4.90	770.88	0.0649	4.55	291.62	0.1715	26.70	19.64	2.5455
EL18	2.30	144.00	0.3472	3.50	207.36	0.2411	13.35	18.77	2.6643
EL19	2.25	300.44	0.1664	4.05	564.76	0.0885	13.95	30.66	1.6309
EL20	2.50	544.44	0.0918	9.25	442.08	0.1131	10.95	30.19	1.6564
EL21	2.35	498.78	0.1002	3.40	632.16	0.0791	11.10	81.84	0.6109
EL23	3.30	150.94	0.3313	5.50	400.00	0.1250	12.50	62.15	0.8046
EL24	2.90	243.36	0.2055	6.60	507.08	0.0986	10.70	40.00	1.2501

The values of k′, N and HETP for a selected set of compounds are given in Table 3.5. The N values for 2-EP stationary phase are consistently large in comparison to PPU and CN. A large variation is seen for the PPU column. The scatter plot of N clearly shows this; CN based data is varying significantly providing good values for first few compounds and poor results for compounds P17-P24. This behaviour is clearly seen in the statistics listed in Table 3.6. The combination of lower standard deviation and reasonable mean value shows the good performance of 2-EP for the compound set. The HETP data, however, for most compounds appears to show similar performance for 2-EP and PPU (Table 3.5). The scatter plot in Figure 3.10 confirms this finding. The difference in column performance is clear from the statistics calculated (Table 3.7). The mean HETP values for PPU are similar to 2-EP, with 2-EP showing marginally better performance as seen from smaller standard deviation.

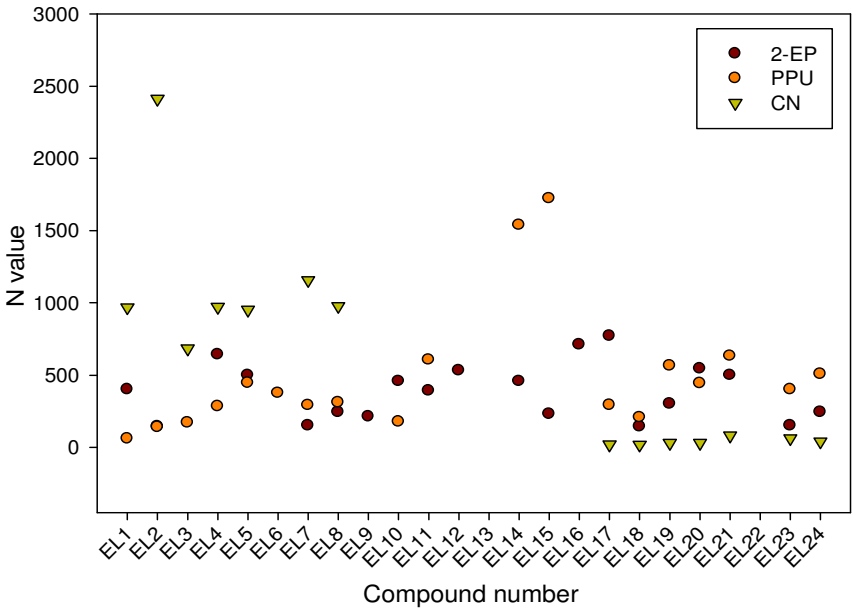


Figure 3.9: Scatter plot showing the spread of N value for the 2-EP, PPU, CN columns for focused set.

Table 3.6: Statistical data of N values of the peaks for stationary phases 2-EP, PPU, CN for focused set.

N	2-EP	PPU	CN
Mean	386	482	600
Std Dev	196	436	697

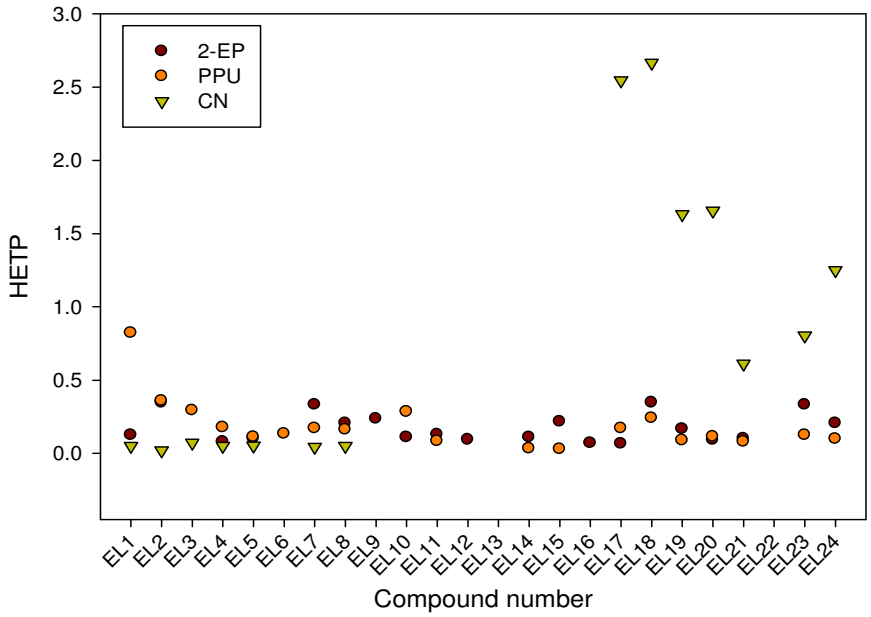


Figure 3.10: Scatter plot showing the spread of HETP for the 2-EP, PPU, CN columns for focused set.

Table 3.7: Statistical data of HETP of the peaks for stationary phases 2-EP, PPU, CN for focused set.

HETP	2-EP	PPU	CN
Mean	0.1729	0.1881	0.8219
Std Dev	0.0989	0.1771	0.9658

The scatter plot for the peak asymmetry values is shown in Figure 3.11. PPU appears to perform much better in this aspect, which is also confirmed by the statistics (Table 3.8); the mean value of PPU related data being very close to unity and showing smaller scatter compared to other options. The asymmetry data shows that the PPU column results in better chromatography for the peaks eluted. Overall 2-EP comes through as better performing column. Although the data indicates 2-EP as the column of choice, compounds with specific substructures resulted in peak splitting. The peak splitting phenomenon has been explained in section 3.3.2 of this chapter.

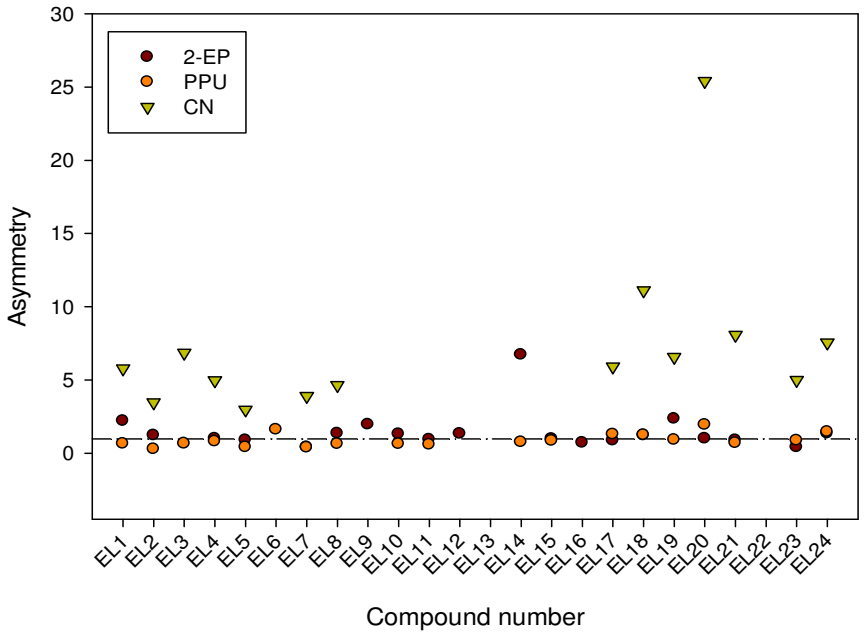


Figure 3.11: Scatter plot showing the spread of asymmetry data for the 2-EP, PPU, CN columns for focused set.

Table 3.8: Statistical data of asymmetry of the peaks for stationary phases 2-EP, PPU, CN for focused set.

Asymmetry	2-EP	PPU	CN
Mean	1.4535	0.8753	7.3021
Std Dev	1.3387	0.4413	5.6184

The study was further extended for the diverse library set of compounds (Structures shown in Appendix 3 on pages 114-117). Here as seen in Figure 3.12, for the diverse set of compounds, 87 percent of the compounds eluted with 2-EP. Forty seven percent of the compounds did not elute within the runtime of 10 minutes, under isocratic conditions of 20% methanol modifier, with CN columns. The peak shapes of the most of the compounds that did elute with the CN column were broad and tailing. The asymmetry values for most compounds analysed using CN column show tailing peaks. Similarly with PPU column several compounds were also found to have tailing peaks.

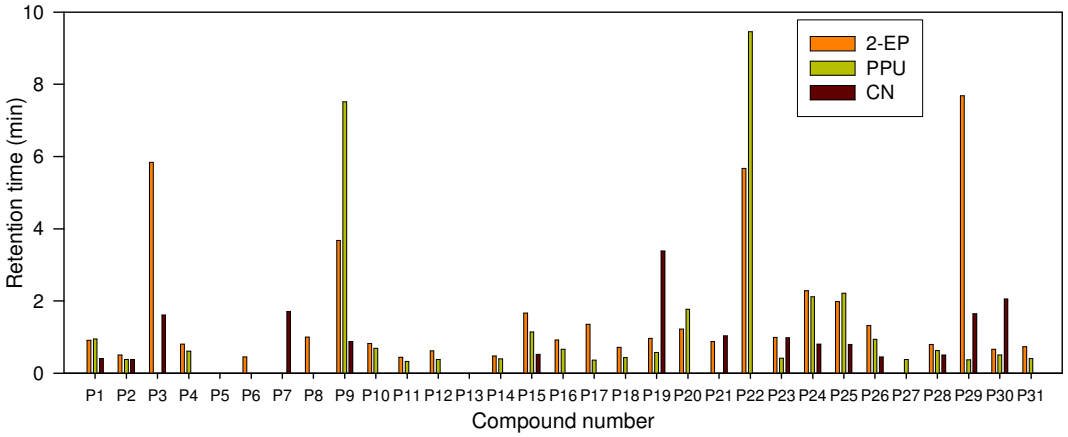


Figure 3.12: A comparison of retention times for the diverse set (P) for 2-EP, PPU, CN.

Table 3.9: Values for k', N, HETP for 2-EP, PPU and CN columns of selected compounds of diverse set from Figure 3.12.

	2-EP			PPU			CN		
Compound	**k′**	**N**	**HETP**	**k′**	**N**	**HETP**	**k′**	**N**	**HETP**
P1	3.55	1324.96	0.0377	3.75	1444.00	0.0346	1.05	747.11	0.0669
P9	17.4	1874.38	0.0267	36.60	2279.68	0.0219	3.40	860.44	0.0581
P15	7.35	1743.06	0.0287	4.70	1230.39	0.0406	1.60	256.00	0.1953
P19	3.85	520.91	0.0960	1.90	318.48	0.1570	15.9	51.47	0.9713
P23	3.95	542.61	0.0921	1.10	233.25	0.2144	3.90	141.10	0.3543
P25	8.95	1584.04	0.0316	10.10	1166.48	0.0429	3.00	605.91	0.0825
P29	37.4	2233.65	0.0224	0.85	18.94	2.6388	7.26	696.96	0.0717

The N and HETP values are listed in Table 3.9 for selected compounds of diverse set. The scatter plot of N values for all the compounds in the diverse set is shown in Figure 3.13. The varied N data makes it difficult to determine the performance of the columns. The statistics from Table 3.10, however, indicates marginally better performance of 2-EP column in comparison to PPU and CN columns. HETP data shown in scatter plot (Figure 3.14) indicates similarity in performance of 2-EP and CN columns. Although similar conclusions can be drawn from the statistics from HETP data (Table 3.11), CN column was eluting only half of the compounds in diverse set.

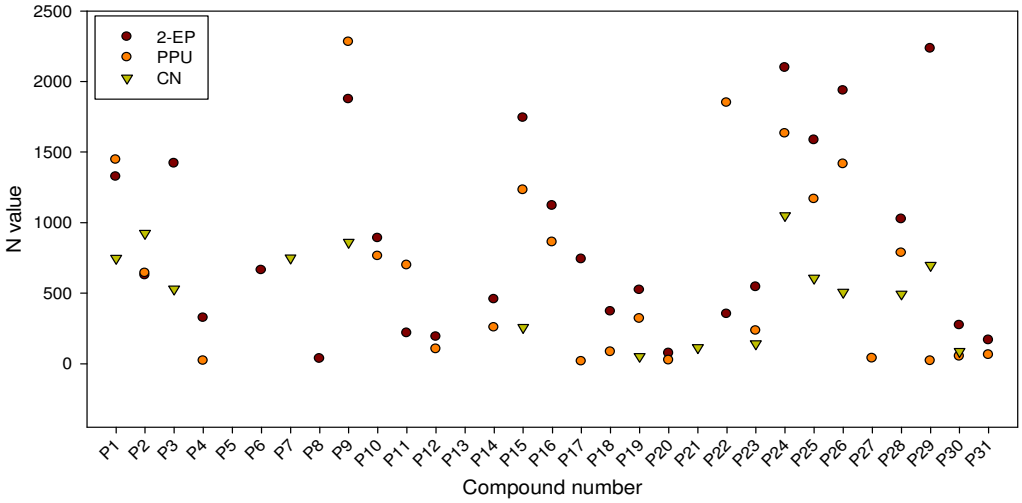


Figure 3.13: Scatter plot showing the spread of N for the 2-EP, PPU, CN columns for diverse set.

Table 3.10: Statistical data of N values for stationary phases 2-EP, PPU and CN.

N	2-EP	PPU	CN
Mean	876	666	521
Std deviation	687	681	326

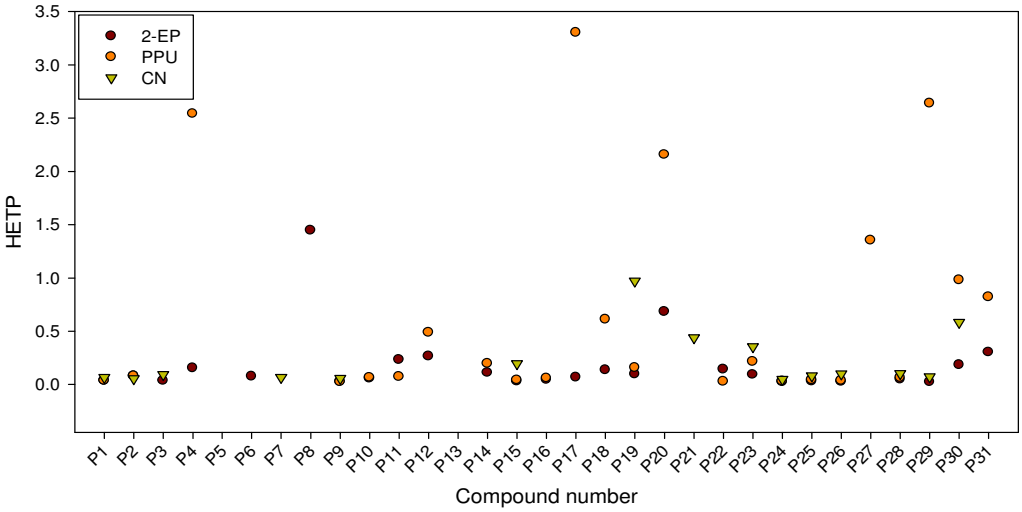


Figure 3.14: Scatter plot showing the spread of HETP for the 2-EP, PPU, CN columns for diverse set.

Table 3.11: Statistical data of HETP for stationary phases 2-EP, PPU and CN.

HETP	2-EP	PPU	CN
Mean	0.1709	0.6676	0.2191
Std deviation	0.2943	0.9885	0.2645

The peak asymmetry values (Table 3.12) indicate the good performance of 2-EP column which is further confirmed from the scatter plot in Figure 3.15 and the statistics from Table 3.13. The CN column also appears to show better results in peak asymmetry, however, with slightly larger standard deviation (larger scatter). Overall, for the diverse set 2-EP column shows good results in all the performance parameters analysed.

Table 3.12: Values of peak Asymmetry for selected compounds using 2-EP, PPU and CN columns.

	Asymmetry (As)		
Compound	**2-EP**	**PPU**	**CN**
P1	0.97	1.03	1.05
P9	1.09	1.10	0.85
P15	1.13	11.74	1.31
P19	1.70	1.72	1.89
P23	1.55	1.97	2.46
P25	0.94	0.99	0.46
P29	0.91	11.65	0.64

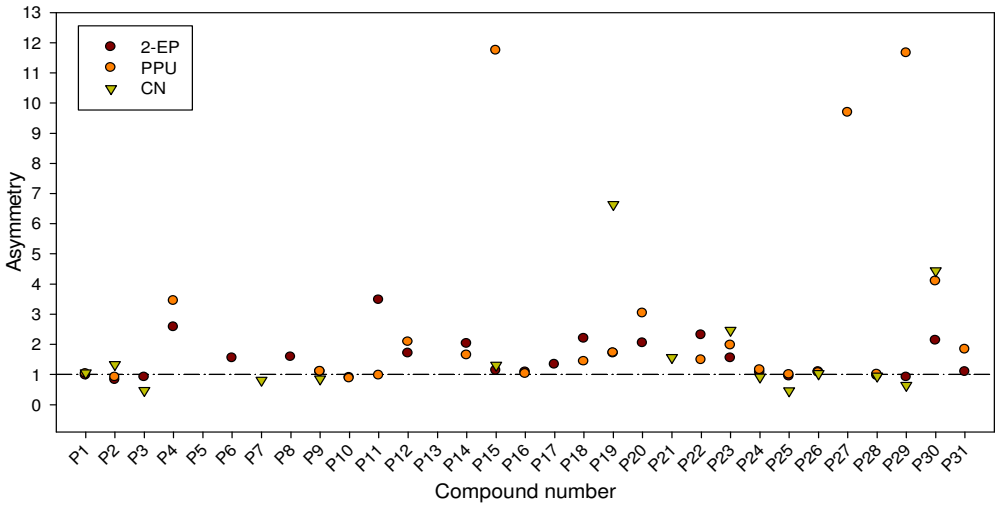


Figure 3.15: Scatter plot showing the spread of asymmetry data for the stationary phases 2-EP, PPU, CN for the diverse set.

Table 3.13: Statistical data of asymmetry of the peaks for stationary phases 2-EP, PPU and CN.

Asymmetry	2-EP	PPU	CN
Mean	1.5038	2.8647	1.6620
Std deviation	0.6525	3.3573	1.7009

3.2.3 Additives

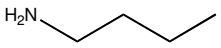
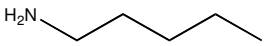
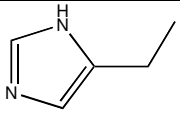
Pure modified mobile phase may not be sufficient to achieve the required chromatography. Distorted peak shapes and poor chromatography could be the result of certain functional groups of the analyte interacting with the residual silanol groups. Higher concentrations of strong modifiers may improve efficiency but may also result in decreased retention and sensitivity.[103] The need to improve peak shapes prompted the addition of small amounts of additives to the mobile phase. Polar additives are generally immiscible in supercritical fluids. Hence these are added to the modifier and then introduced as a single fluid. The presence of additive help improve the peak shapes by covering the active sites or by suppressing the ionisation or ion pair formation by solutes. Additives may also, change the polarity of the mobile or the stationary phase.[25] Depending on the nature of the analytes additives can be acidic or basic compounds. Basic additives

are used for compounds with basic functionality and acidic additives for compounds with acidic functionality.

In this project the additives were generally added to modifiers when using the CN columns. These were diethylamine (DEA), triethylamine (TEA), dimethylethylamine (DMEA), tertbutylamine (TBA) and ammonium acetate. The presence of additives did not affect the peak shapes or retention on the 2-EP column. The small differences in retention times were observed from the results of focused library for different additives used individually. For simplicity the focused library was divided into groups based on the sub structures as shown in

Table 3.14. This is further explained in detail in the experimental section on page 98.

Table 3.14: Differences in structures of the focused library

Group	Compound numbers	Substructure
A	EL1-EL8	
B	EL9-EL16	
C	EL17-EL24	

Each compound from the focused set was analysed using isocratic 20% methanol modifier with 0.1% v/v additive on CN column. A secondary amine, diethyl-amine, and two tertiary amines, triethylamine and dimethylethylamine, were used as additives on separate occasions. Figure 3.17 is a plot of retention times for all 24 compounds for secondary and tertiary amines. All the compounds with aliphatic side chain elute later in presence of a tertiary amine additive.

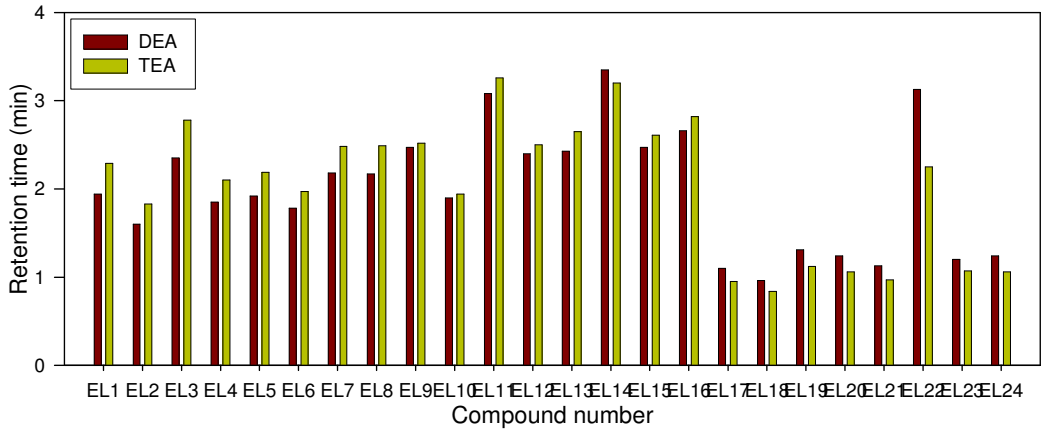


Figure 3.16: Plot showing trends of secondary and tertiary amine additive for specific compound substructure.

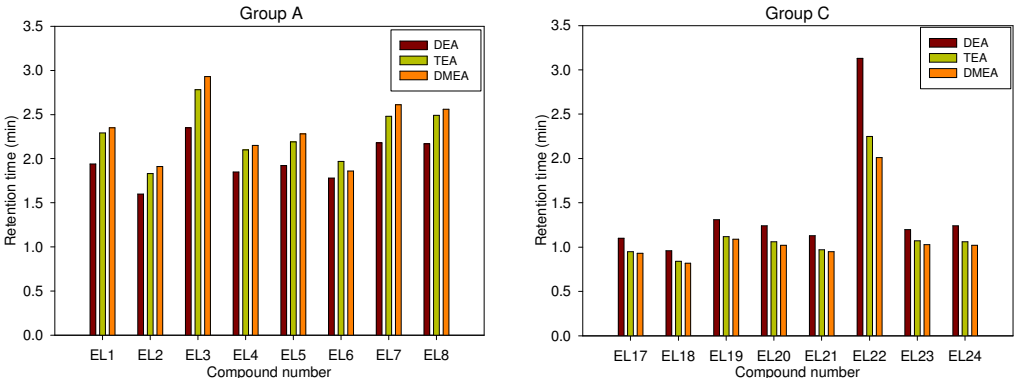


Figure 3.17: Trends Comparison of retention times of compounds using 0.1% DEA, TEA or DMEA as additive with 20% methanol modifier on 50mm CN column.

A similar comparison between two tertiary amines and a secondary amine is shown for group A, compounds with aliphatic side chains and group C, compounds with aromatic side chains, in Figure 3.17. In both cases the retention times do not vary much for tertiary amines DEA and TEA, suggesting that the identity of an amine had little or no effect on the retention times. But when compared to with the retention times using DMEA, which is also a tertiary amine as additive, this is not true as E9 to E16 elute earlier with DMEA as seen in Figure 3.18. These compounds were also found to be unstable in methanol, which may have an effect on the interaction of the solute with the stationary phase in the presence of DMEA.

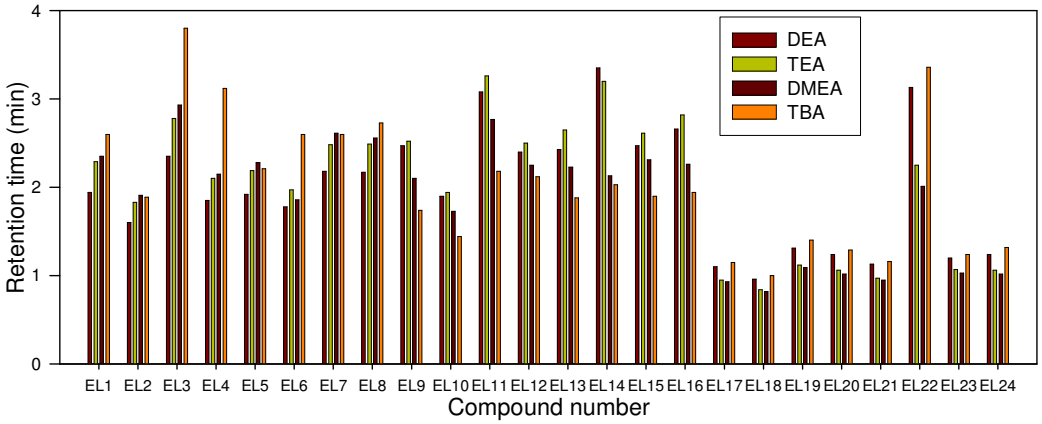


Figure 3.18: Trends and comparison of retention times using primary, secondary, and tertiary amine additive.

Following from the modifier and stationary phase studies k', N and HETP calculations were performed on the chromatograms of the additive study. These values for a selected list of compounds are shown in Table 3.15. The data on N values and HETP appears to show that DEA additive performing marginally better for most compounds. Similar is the case of HETP values.

For all the compounds scatter plots were generated for N and HETP values. Figure 3.19 shows variation N value for the compounds analysed; except for compound EL13, additive DEA shows consistently good performance. Other additives also show reasonably similar performance as seen from the figure; the N values for a compound are generally clustered together. The statistics of mean and standard deviation were also calculated to see if any more information can be obtained. These are listed in Table 3.16. The additive DEA appears to show slightly better performance than other two additives analysed. The HETP values are plotted in Figure 3.20; except for compound EL13 DEA shows good performance. The statistics for the HETP values are listed in Table 3.17. The performance of DEA is affected by one outlying value of EL-13 compound, which shows up as large standard deviation.

Table 3.15: Values for k', N, HETP for Additives DEA, TEA, DMEA for selected compounds from Figure 3.18.

	DEA			TEA			DMEA		
Compounds	**k'**	**N**	**HETP**	**k'**	**N**	**HETP**	**k'**	**N**	**HETP**
EL2	7.00	1024	0.0488	8.15	595	0.0840	8.55	744	0.0672
EL5	8.60	1337	0.0374	9.95	978	0.0511	10.40	924	0.0541
EL9	11.35	1015	0.0492	11.60	703	0.0711	9.50	689	0.0726
EL14	15.75	302	0.1651	15.00	163	0.3052	9.65	129	0.3874
EL19	5.55	1400	0.0357	4.60	1393	0.0359	4.45	1320	0.0379
EL22	13.90	238	0.2093	10.25	324	0.1543	9.05	139	0.3577

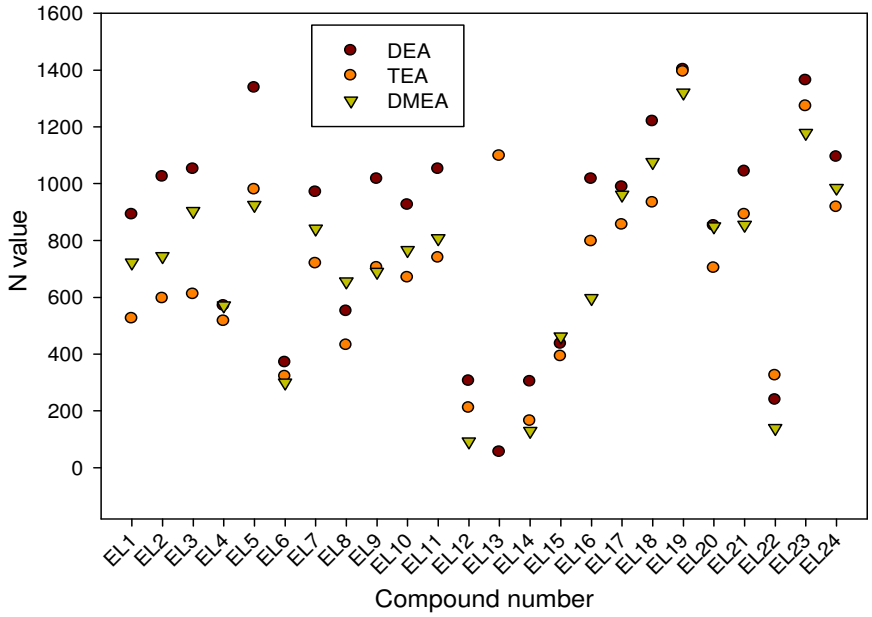


Figure 3.19: Scatter plot showing the spread of N values for the additives DEA, TEA, DMEA.

Table 3.16: Statistical data of N of the peaks for additives DEA, TEA, DMEA.

N	**DEA**	**TEA**	**DMEA**
Mean	836	698	720
Std deviation	385	314	325

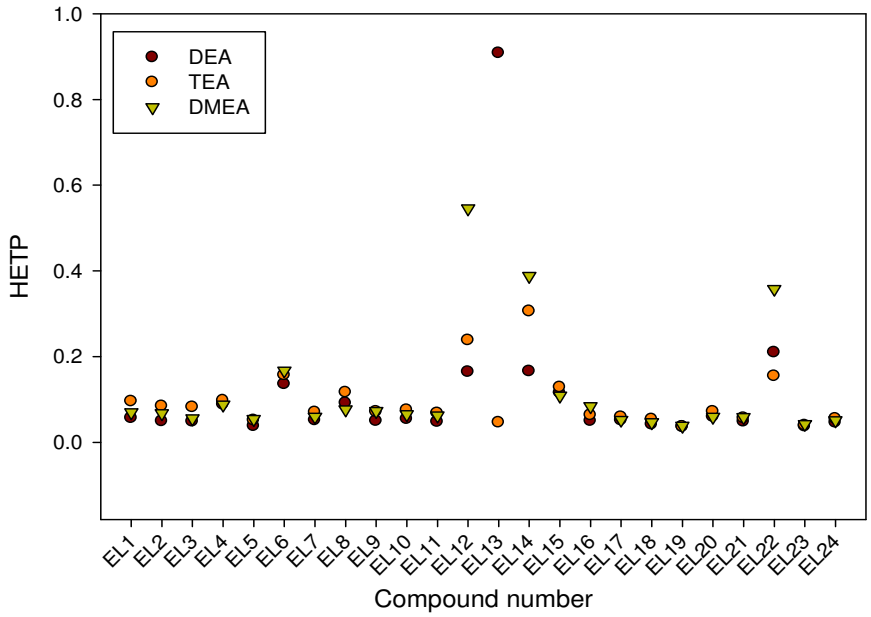


Figure 3.20: Scatter plot showing the spread of HETP values for the stationary phases DEA, TEA, DMEA.

Table 3.17: Statistical data of HETP of the peaks for additives DEA, TEA, DMEA.

HETP	DEA	TEA	DMEA
Mean	0.1097	0.0945	0.1159
Std deviation	0.1767	0.0642	0.1308

The peak asymmetry values are shown in Figure 3.21. The scattered values make it difficult to determine a single suitable additive. The statistics in Table 3.18 shows marginally better performance of DMEA. In overall context, however, the performance of additives is comparable. Although the performance of additives is comparable, as expected, the effect of additives is clear from peak asymmetry which is much improved in comparison to those without additive on the CN column. (Table 3.8 and Table 3.18).

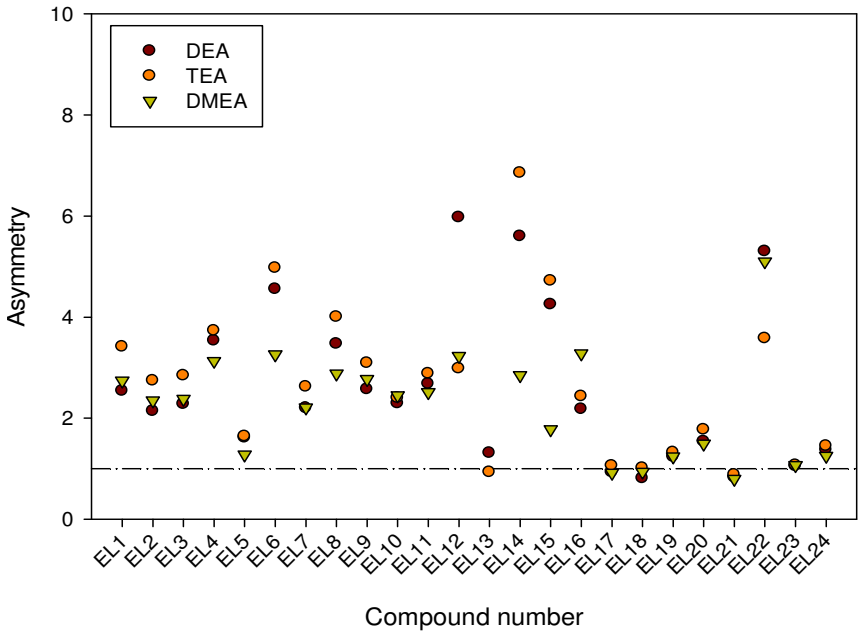


Figure 3.21: Scatter plot showing the spread of asymmetry data for the stationary phases DEA, TEA, DMEA.

Table 3.18: Statistical data of asymmetry data of the peaks for additives DEA, TEA, DMEA.

Asymmetry	DEA	TEA	DMEA
Mean	2.5933	2.6821	2.2570
Std deviation	1.5493	1.4896	1.0462

More recently volatile ammonium salts have been used as additives for basic compounds.[27, 108, 109] Ammonium salt additives were found to be compatible with mass spectrometric detection as they did not result in ion suppression as observed with acidic or basic additives. Figure 3.22 to Figure 3.24 below is one example showing the use of ammonium acetate as additive in comparison to using amine additive. Also shown is the chromatography in the absence of additive on the 250 mm CN column. This particular compound structure of which is shown in Figure 3.25, had mixed impurity. This could possibly be due the protecting group added during synthesis. In the absence of additive although the impurity peak separated from the compound peak, the compound peak was broad and tailing. The impurity peak appears around 8 min as seen in the mass spectrum. The addition of an amine additive improved the peak shape but also moved the retention of the impurity. This resulted in the two peaks eluting very close to each other making it

difficult to separate. Using ammonium acetate not only improved the peak shape but also resolved the impurity peak form the compound peak.

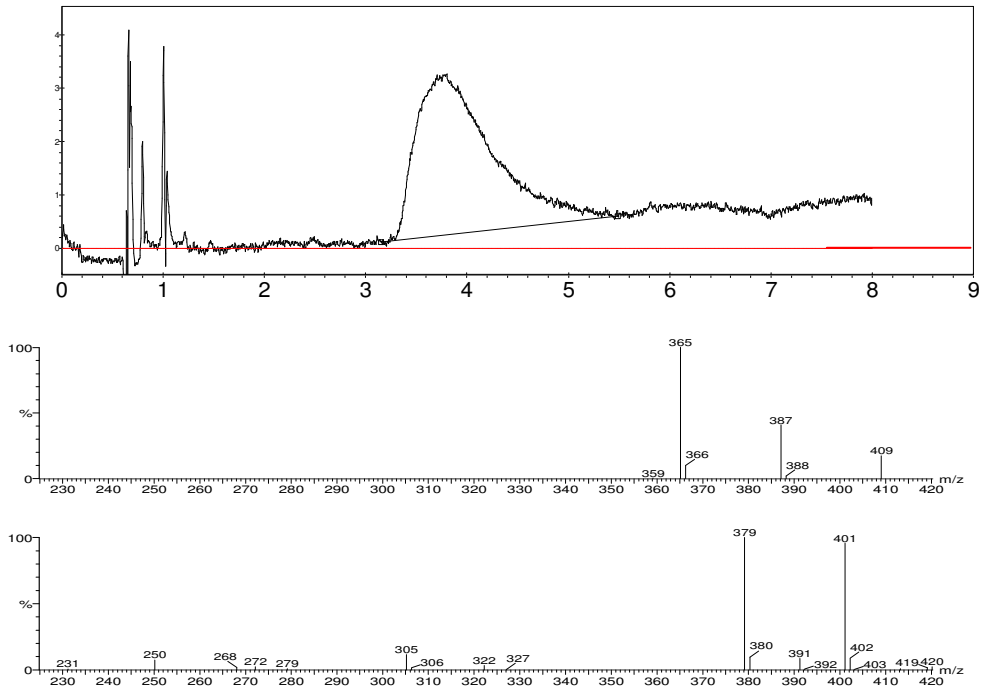


Figure 3.22: 250 mm CN column, 25% methanol, Flow: 4 mL min^{-1} with no additive, showing separation of impurity (*m/z* = 379) from compound peak (*m/z* = 365).

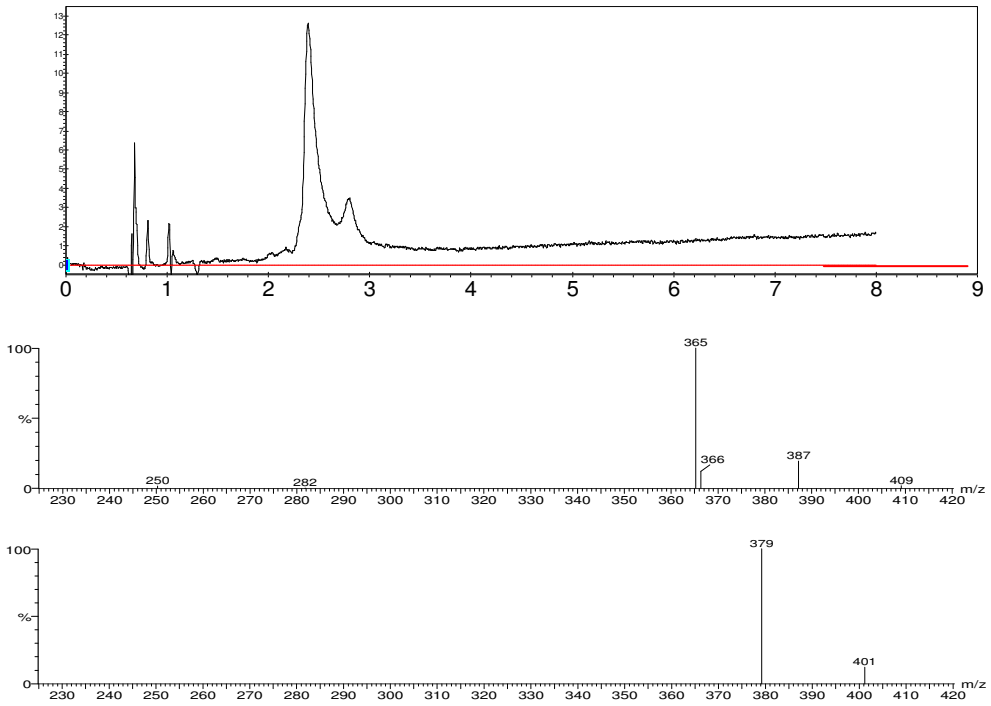


Figure 3.23: 250 mm CN column, 25% methanol, Flow: 4 mL min^{-1} with 0.1% DEA additive, showing separation of impurity (*m/z* = 379) from compound peak (*m/z* = 365).

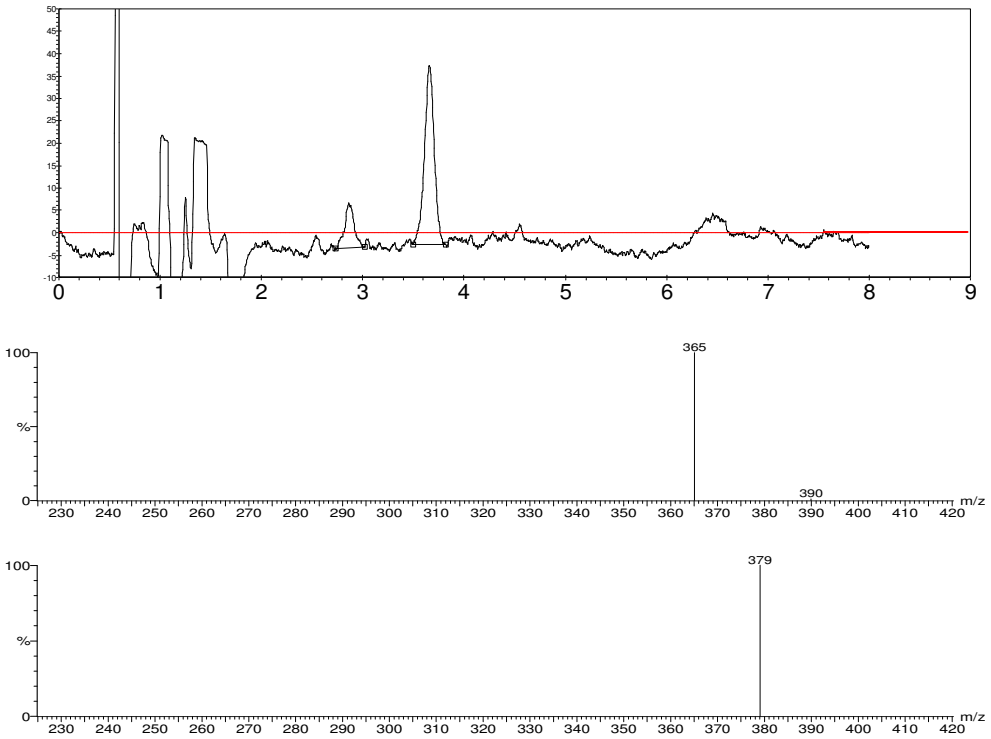
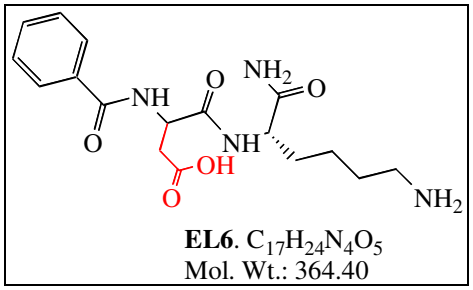


Figure 3.24: 250 mm CN column, 25% methanol, Flow: 4 mL min^{-1} with 0.6 mM ammonium acetate additive, showing separation of impurity (*m/z* = 379) from compound peak (*m/z* = 365).



EL6. $C_{17}H_{24}N_4O_5$
Mol. Wt.: 364.40

Figure 3.25: Structure of EL6.

Why use Isocratic chromatographic condition?

The training set of compounds were first analyzed with isocratic conditions and then analyzed with gradient conditions in an attempt to reduce the analysis time. Similar conditions were then used for the focused library of compounds. These conditions were found to be unsuitable for this set of compounds as the peaks showed poor chromatographic properties *i.e.* peak splitting. Figure 3.26 (a) is an example of the SFC chromatogram with 50mm 2-EP, 5-55% methanol gradient @ 10% min^{-1}, 3mL min^{-1} flow. Hence all the compounds were then analyzed with isocratic conditions as the peaks were now comparatively sharp and narrow as shown in Figure 3.26 (b) with 50mm 2-EP, 20% methanol @ 3 mL min^{-1} flow. Hence the isocratic condition of 20% methanol modifier was used in these studies.

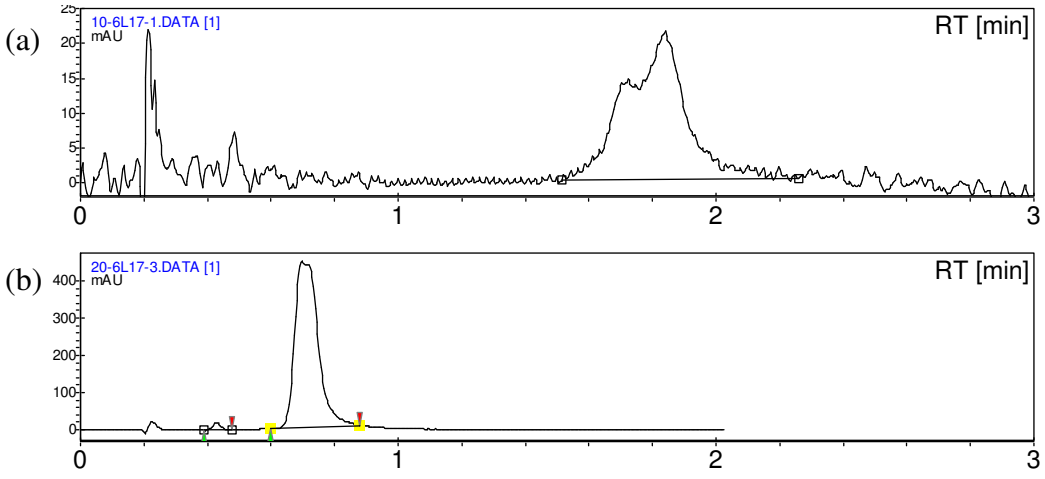


Figure 3.26: Compound EL17 analyzed with (a) gradient conditions (b) isocratic conditions.

Table 3.19: N and HETP values for Figure 3.26.

	$N=16(tr/w)^2$	HETP=L/N
(a)	318.7531	0.1569
(b)	400.0000	0.1250

Table 3.19 lists N and HETP values for the chromatograms shown in Figure 3.26. Not only helping reduce peak splitting, isocratic conditions appear to help improve N value and HETP value for the compound considered. Summarising the above results, two possible generic methods can be put forward. First using 50 mm 2-EP columns with 20% methanol modified CO_2 mobile phase at 3 mL min^{-1} flow rate, pressure 100bar and oven temperature of 40ºC. The second method uses conditions similar to the first with a 50 mm CN column. Further 0.1 M concentration of ammonium acetate additive is added to the 20% methanol modified mobile phase mobile. The presence of an amine additive may cause difficulty in preparative chromatography during isolation of target compound.

3.3 Difficulties encountered in SFC

In the given set of compounds some were insoluble in methanol which was initially considered to be major problem. A solution to this is explained in section 3.3.1. A group of compounds, with specific substructures cause peak splitting phenomenon or elute with two peaks. This is the major issue with this method and is discussed in detail in section 3.3.2.

3.3.1 Insolubility in methanol

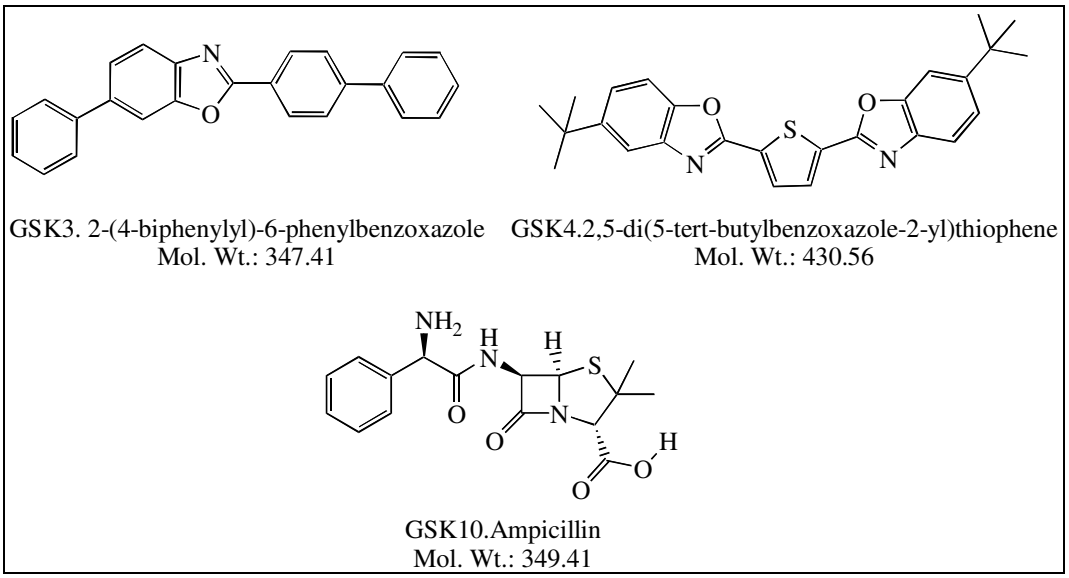


Figure 3.27: Structures of compound insoluble in methanol (Mix 1).

The insolubility of some compounds in methanol required a different approach to be investigated. The training set included three compounds that were insoluble in 100% methanol. See Figure 3.27. First attempt of dissolving these compounds in methanol by sonicating or warming the solutions was unsuccessful. Out of the three compounds two of them were soluble in DCM, this solution was further diluted with methanol and analysed by SFC.

Both of these compounds, prepared in DCM and methanol mixture, eluted from the 2-EP column with good peak shapes (Figure 3.28 a, c). The results were confirmed by MS data (Figure 3.28 b, d). This shows that compounds initially insoluble in methanol can still be analysed by SFC. Further on in this project several more such compounds were analysed using a mixture of DCM and methanol for sample preparation.

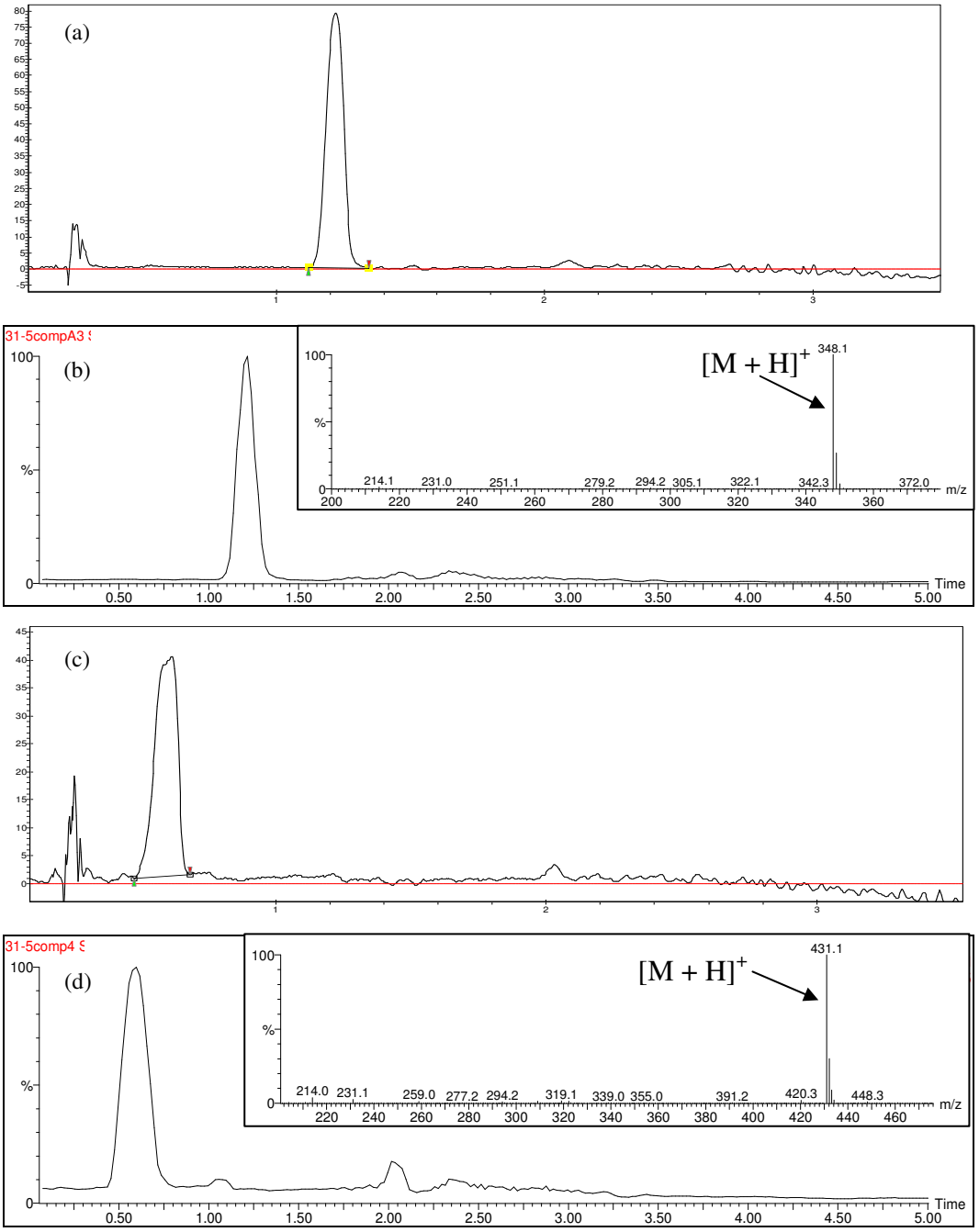


Figure 3.28: Chromatograms showing single peak when prepared using mixture of DCM and methanol (a) SFC chromatogram of GSK3, (b) MS data confirming GSK3, (c) SFC chromatogram for GSK4, (d) MS data confirming GSK4.

One of the compounds in the training set was Ampicillin (GSK10). It is known that Ampicillin can be analysed by liquid chromatography. Michael Margosis used reversed phase HPLC for the analysis of ampicillin to test the potency of trihydrate and anhydrous forms of ampicillin and its sodium salt. Acid phosphate acetonitrile was used for mobile phase[110]. Extracted and derivatised samples were analysed for ampicillin, two types of penicillin and amoxicillin by LC. Here a

reverse phased ODS column was used with acetonitrile, methanol and phosphate buffer as mobile phase. A Fluorescent detector was used for detection[111]. Kumar *et al.* developed a method to separate ampicillin and another drug cloxacillin from their degradation products using a reversed phase C18 column and acetonitrile phosphate buffer mobile phase[112].

Ampicillin was only soluble in a mixture of 50:50 acetonitrile/water. The stock solution was diluted with methanol and analysed by SFC-MS where the compound did not elute on any of the stationary phases under any of the experimental conditions used. Diluting the stock with a mixture of 50:50 acetonitrile/water made no difference to the elution, nor did using acetonitrile as the modifier. This lack of results could be due to the zwitterionic nature of the compound. Ampicillin was also not amenable to analysis with SFC-MS. This shows that not surprisingly the solubility of samples in the mobile phase is a critically important factor in SFC. The samples need to be either soluble or partially soluble and compatible with the mobile phase composition to some extent.

3.3.2 Peak Splitting

A drawback of using 50mm 2-EP column for analysis was observed. During the initial analysis of the training set two peaks were observed for compounds with specific substructure highlighted in red in Figure 3.31. Several trials were carried out using 50 mm columns, these columns having the same packing as the 250 mm column used. Initially the columns separated the mixtures but on continued use of the column peak splitting was observed. Overloading of the sample on the column was eliminated as diluting the solution had no effect. Other possibility of fault in the packing of stationary phase material was considered when the second column, of the same lot number, did not show any peak splitting. But, then this was disregarded as after a few injections the phenomenon was repeated. Example of the split peak is shown below in Figure 3.29**.** This phenomenon was noticed for compounds with specific substructure shown in red in Figure 3.31. Altering the modifier conditions, *i.e.* changing the percentage modifier, made no difference to

peak splitting and neither did reducing the flow rate from the default instrument setting of 4 mL min^{-1} to 2 mL min^{-1}. The problem with the peak splitting for this particular compound, shown in Figure 3.29 (b), was resolved by changing the flow rate from constant conditions to gradient conditions, 1 mL min^{-1} hold for 0.5 minutes, 4 mL min^{-1} @ 8 mL min^{-1} until the end of the analysis run time (Figure 3.30).

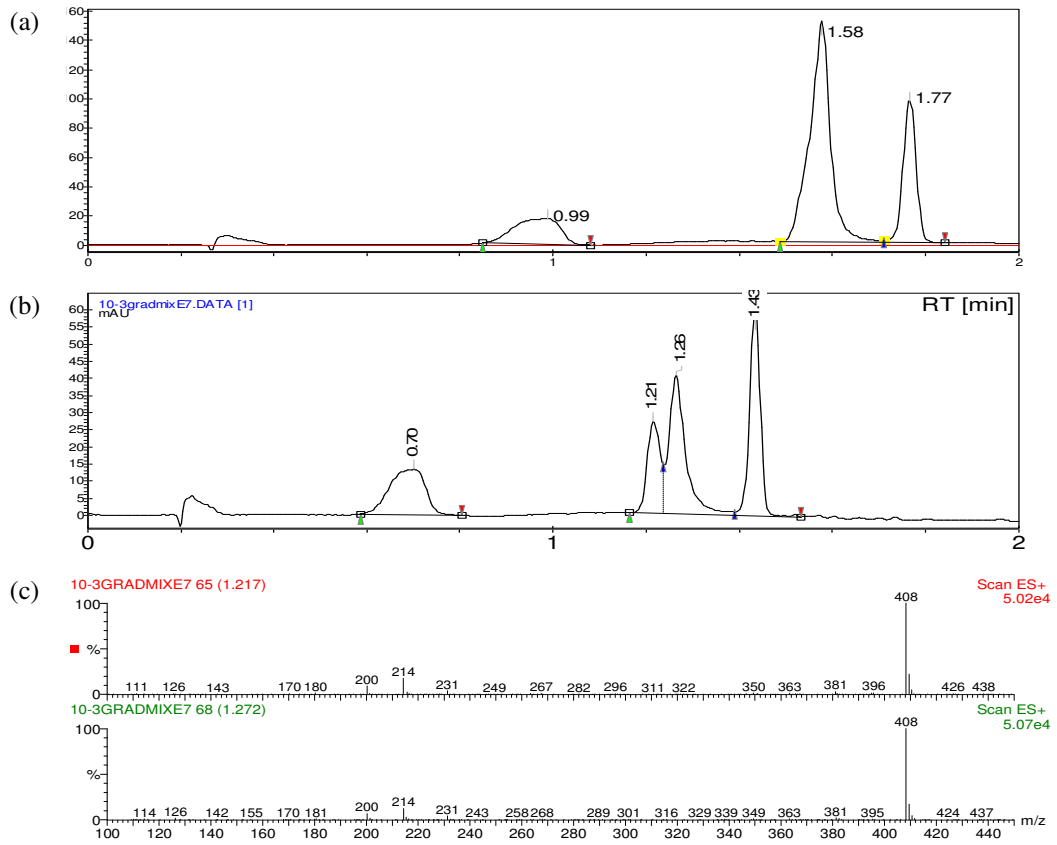


Figure 3.29: (a) Single peak of GSK6 with a new column is used (b) after a few analyses with exactly same conditions the peak splits into two. (c) MS data showing the two peaks in (b) have the same relative molecular mass.

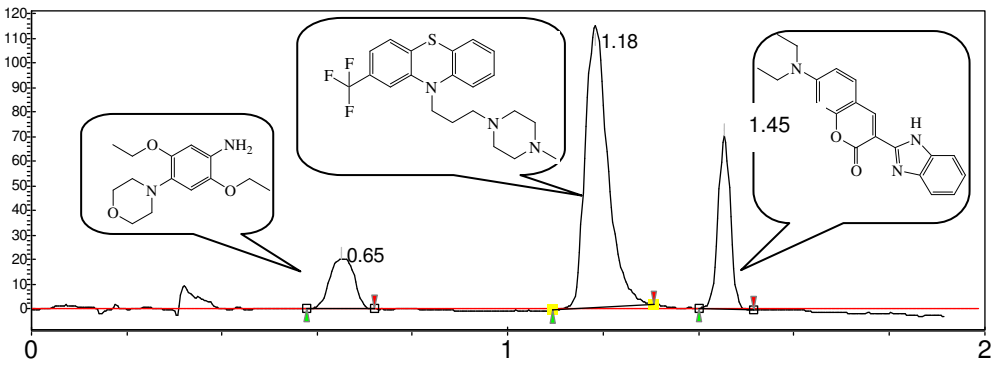


Figure 3.30: Chromatogram showing single peak of GSK6 SFC when analysed using gradient flow.

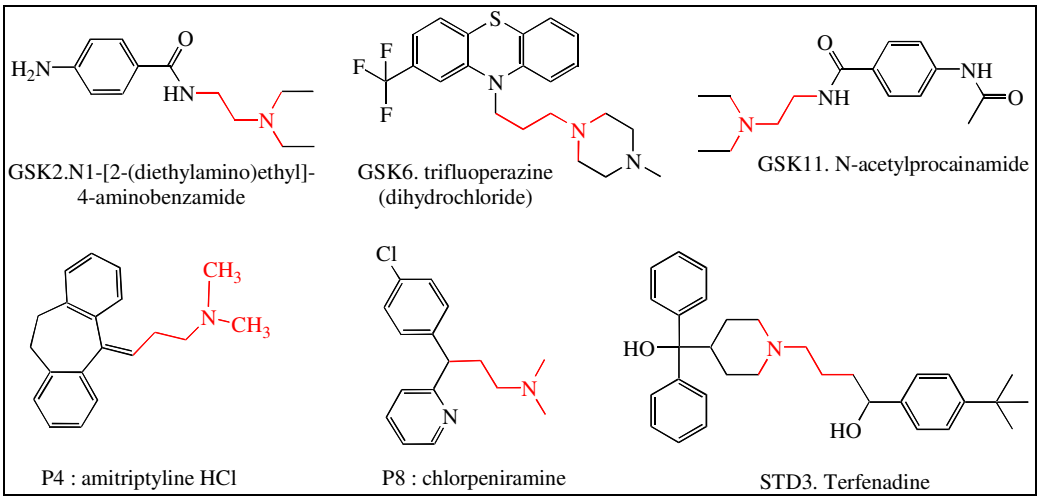


Figure 3.31: Structures of compounds exhibiting peak splitting. Similarity in structures is shown in red colour.

This was further explored and as a result of discussion possible reasons and explanations was put forward. These were to check for any isomers or stereoisomers, to explore the pH of the mobile phase, to try and separate the peaks and to test if the peaks are due to solvation of compound.

The compounds were analysed on a generic chiralpak AD-H column on the SFC. This resulted in a single peak. Since no conclusion could be drawn from the results, next the effect of the pH of the mobile phase was explored.

It is known that, in HPLC analytes show irregular peak shapes such as fronting, tailing or sometimes peak splitting when the pH of the mobile phase is ±2 units of pKa of the compound.[113] But as the mobile phase in SFC is CO_2-MeOH, an attempt to alter the pH of the CO_2-MeOH mobile phase was made by changing the modifier and additive concentrations. This did not result in any improvement.

Further an attempt to separate the peaks was made so as to characterise the two peaks. In order to separate them the instrument was switched over to mini-preparative mode from analytical mode. The difference between the two modes is the introduction of the sample into the chromatographic system. In an analytical mode the sample is injected into a premixed mobile phase of CO_2-MeOH,

whereas in preparative mode the sample gets injected in the modifier and then together introduced into a stream of CO_2. The schematic of both the plumbing systems are shown in Figure 3.32 and Figure 3.33.

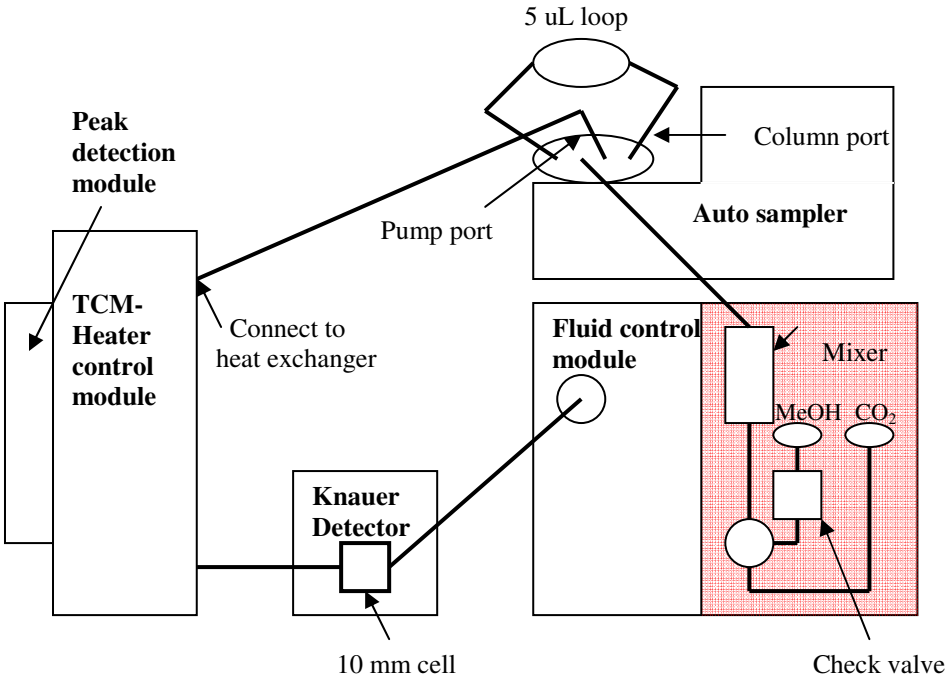


Figure 3.32: Schematic of the plumbing configuration for the operation of Berger Minigram system in Analytical mode.

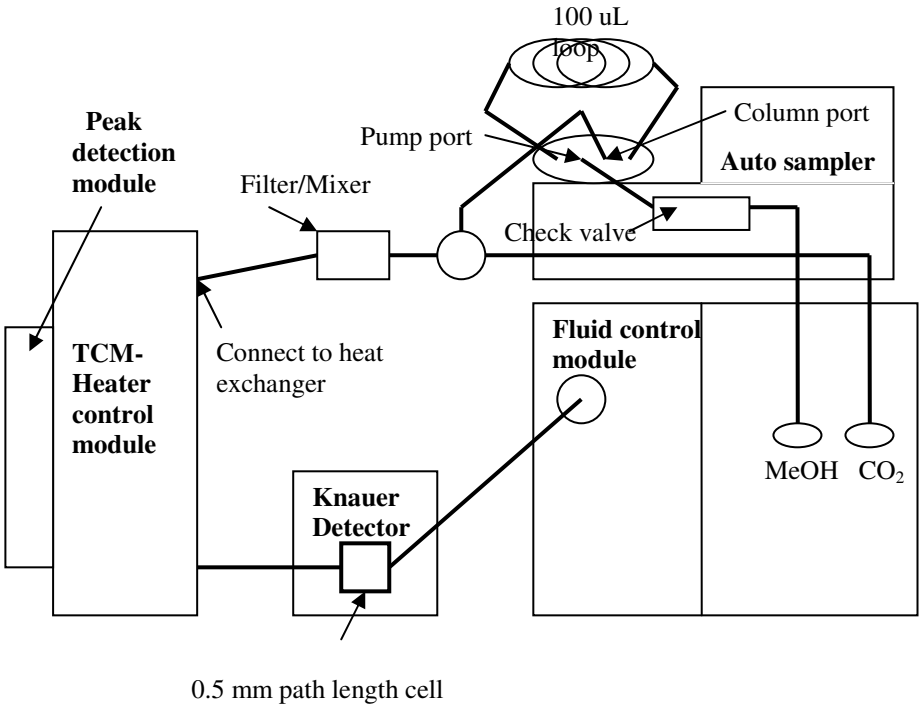


Figure 3.33: Schematic of the plumbing configuration for the operation of Berger Minigram system in Preparative mode.

The samples were prepared for the procainamide and GSK6 and analyzed using the preparative mode for sample injection, *i.e.* directly into the organic modifier prior to mixing with the supercritical CO_2. Initially the conditions used were same as that used for the analytical mode. Figure 3.34 and Figure 3.35 show the UV chromatograms and the corresponding MS data for procainamide when analyzed analytically and then in the preparative set-up. The chromatograms indicate that the compound gives two peaks in the analytical mode whereas a single sharp peak is observed in the preparative mode.

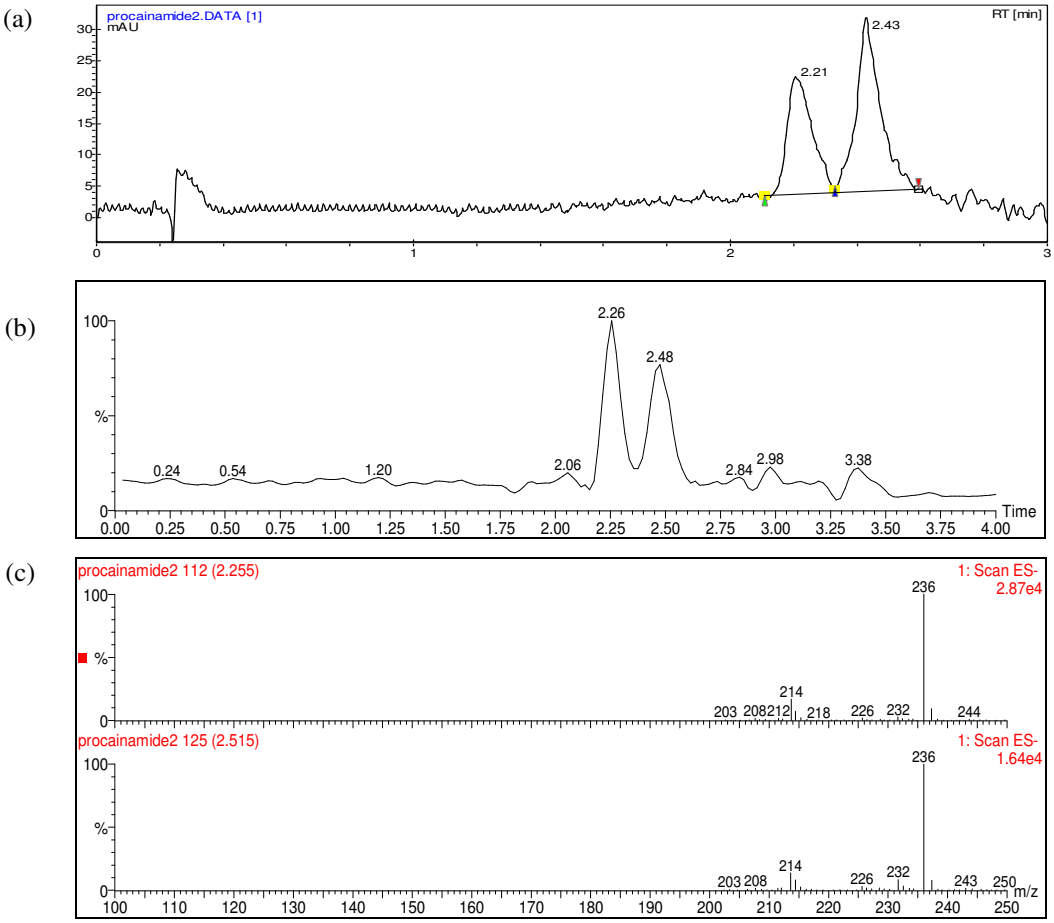


Figure 3.34: (a) UV chromatogram of procainamide showing peak splitting using analytical mode injection mode. Corresponding MS data showing; (b) RIC for procainamide (c) mass spectra for each split peak.

Similarly more compounds were analyzed in both modes but in each case the results were similar to that observed with procainamide, this confirming improvement of injection into the organic phase. Shown below in Figure 3.36 are

the UV chromatograms of GSK compound 6 for the two injection modes and again a single peak was observed in the preparative injection mode, using the same column and exactly the same chromatographic conditions.

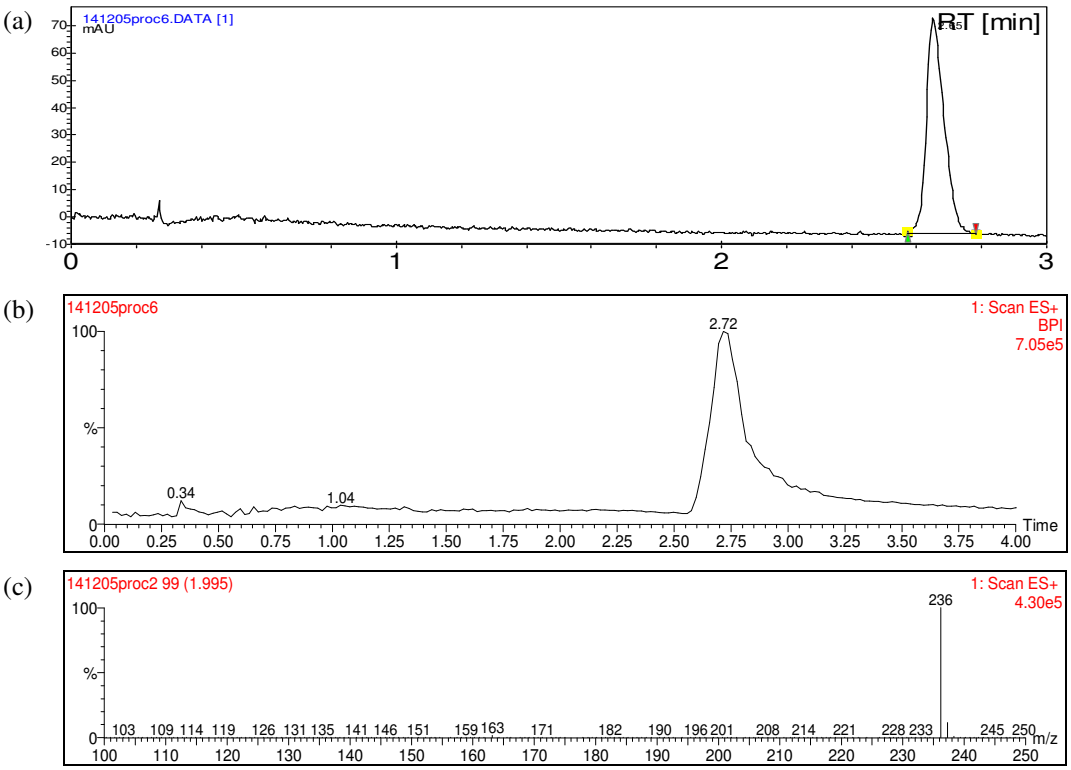


Figure 3.35: (a) UV chromatogram of procainamide showing single peak using preparative injection mode. Corresponding MS data showing (b) RIC for procainamide (c) mass spectra of the peak.

Analysis of the group of compounds, which resulted in peak splitting, with on 50 mm 2-EP column resulted in two peaks which were initially thought to be as peak splitting as the MS data showed identical ionisation for both peaks. This was further confirmed by in source CID-MS. Hence an attempt to obtain a single peak was made. This was carried out by injecting the sample at the lowest flow rate possible and then further ramping it to the required flow rate. Since this did not prove to be conclusive, further attempts to confirm the identity of the two peaks was made.

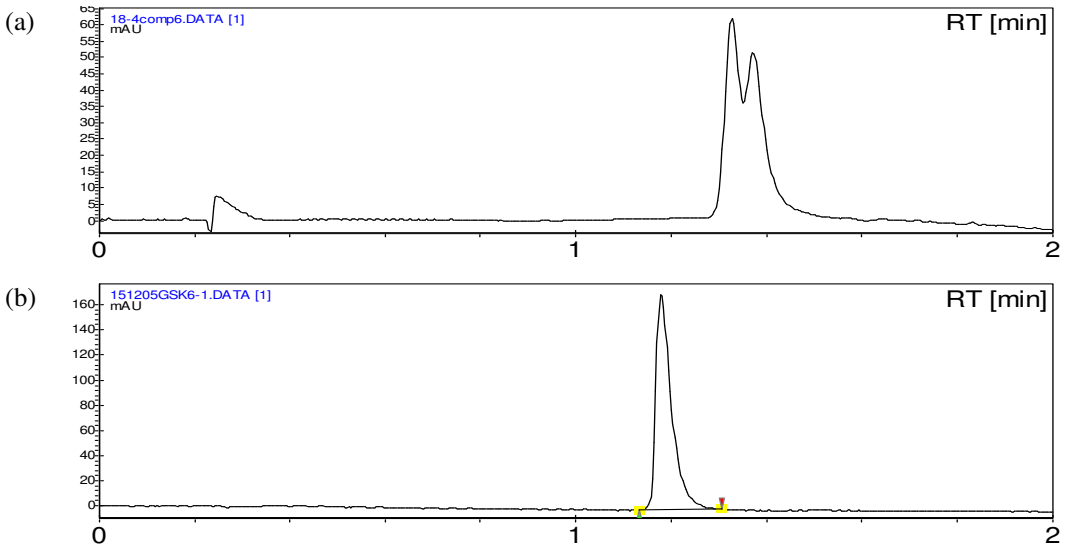


Figure 3.36: UV chromatograms for GSK compound 6 when analyzed in analytical and in preparative mode showing peak splitting and single peaks respectively.

Another possibility was that of one of the peak due to the solvation of the molecule with methanol. One of the samples was prepared in different solvents and then analyzed with two different modifiers. Figure 3.37 and Figure 3.38 show the chromatograms for each solvent and the modifiers used.

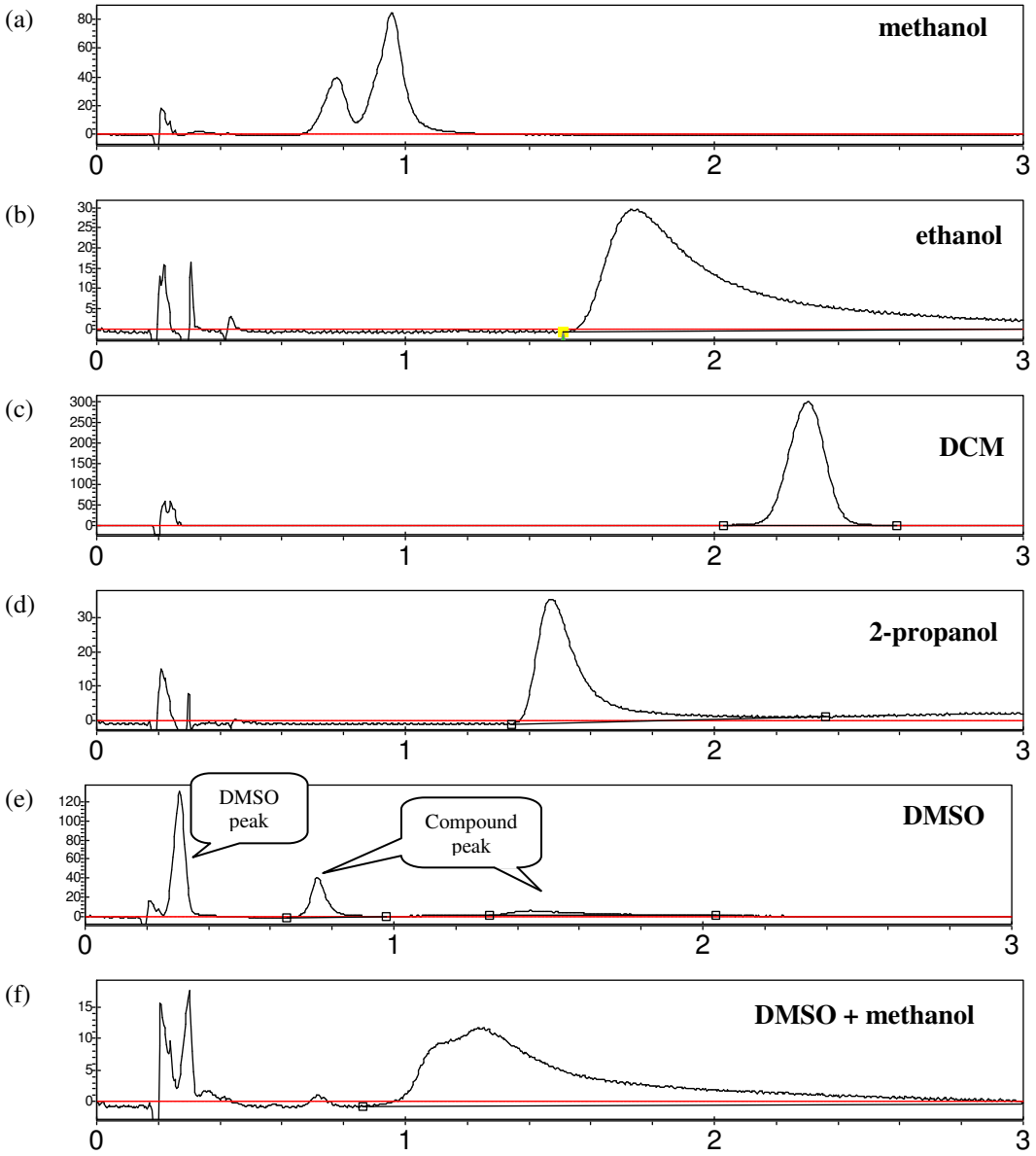


Figure 3.37: Using 20% methanol as modifier, chromatograms of procainamide sample prepared in different solvents (a) methanol, (b) ethanol, (c) DCM, (d) 2-propanol, (e) DMSO and (f) DMSO + methanol.

The peak splitting or two peaks were observed when a sample prepared in methanol was analyzed using methanol modifier. A similar effect was observed when DMSO was used for sample preparation. This suggested that methanol or DMSO form solvated species which cannot be differentiated in an electrospray ionisation process. Single tailing peaks were obtained using ethanol modifier. From the above chromatograms it was also noticed that the peaks were narrower and comparatively symmetrical (peak asymmetry = 0.95 and peak width

@ 10% = 0.23, pharmacopoeia standards) when the samples were prepared in DCM.

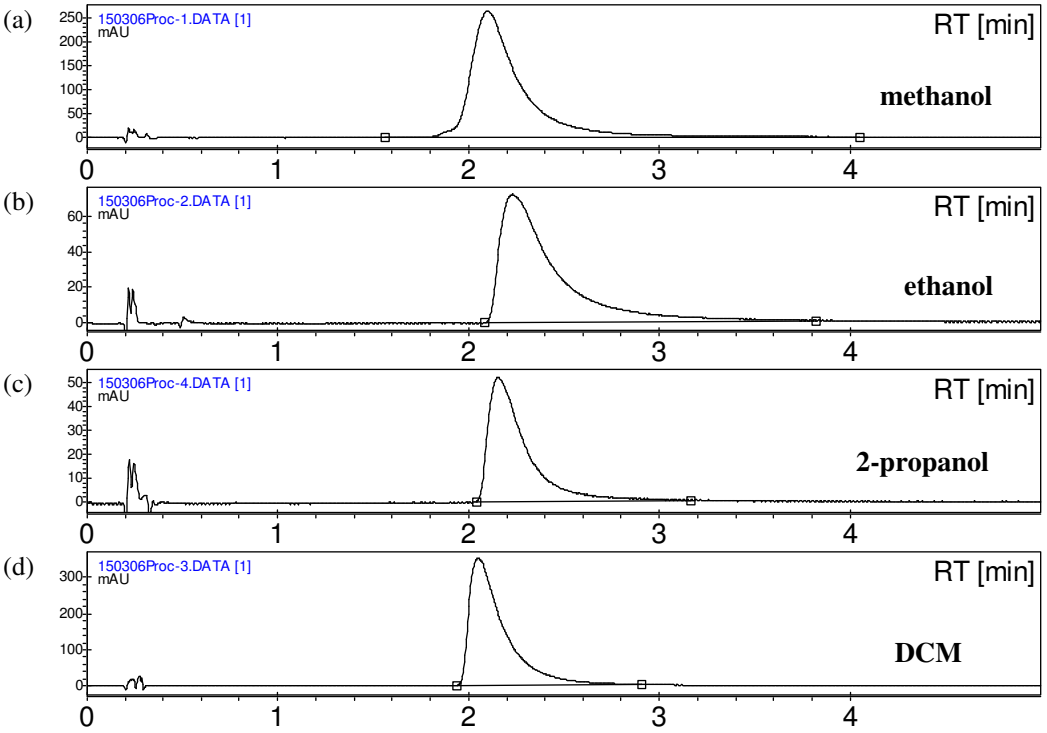


Figure 3.38: Using 20% Ethanol as modifier, chromatograms of procainamide sample prepared in different solvents (a) methanol, (b) ethanol, (c) 2-propanol and (d) DCM.

During the additive study, it was noticed that the peaks did not split on addition of 0.6 mM NH_4OAc to the mobile phase. This is illustrated in Figure 3.39. This may be due to the fact that addition of ammonium acetate would deactivate the silanol groups and therefore favour the second mechanism *i.e.* interaction with the positively charged nitrogen. The purpose of adding additive to the mobile phase is to reduce the interaction of the silanol groups with the analytes.[114]

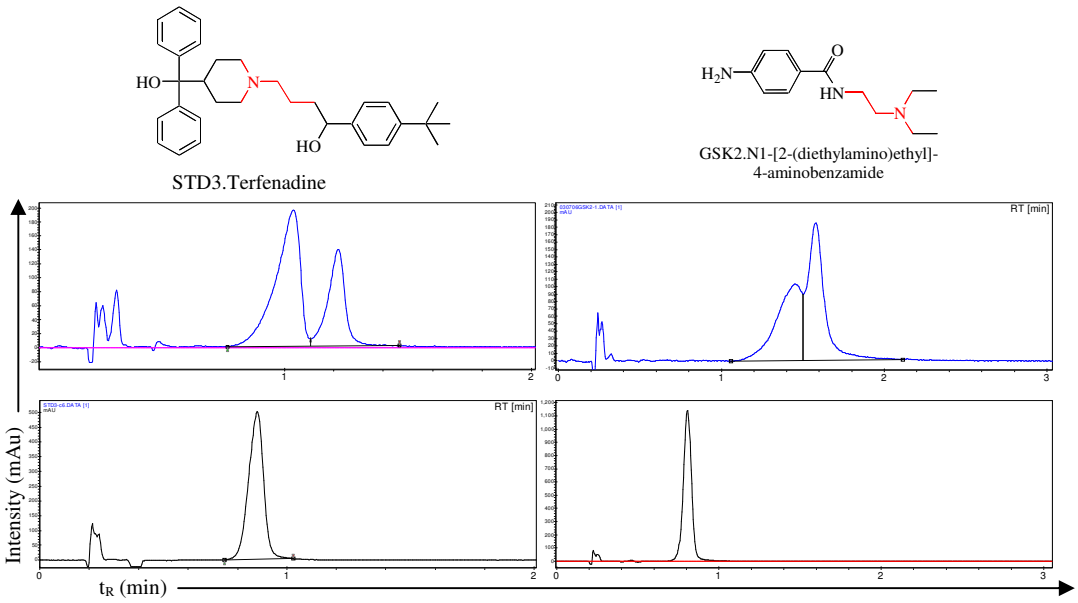
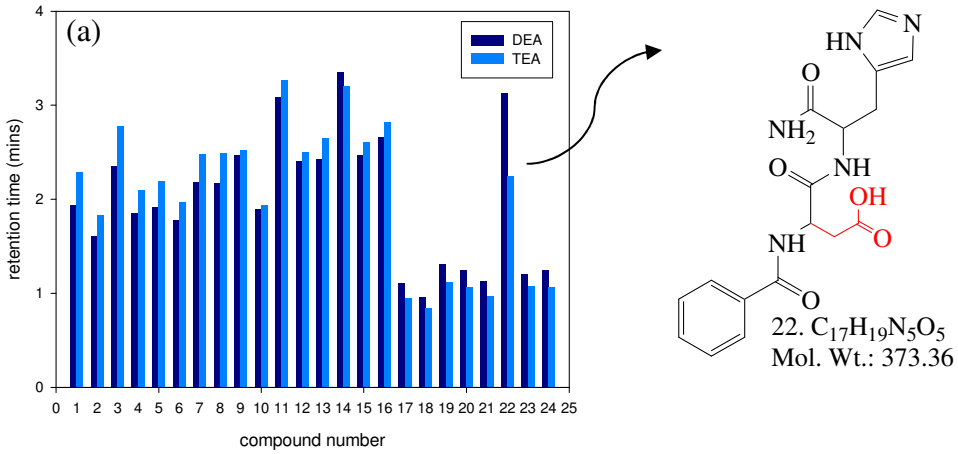


Figure 3.39: Chromatograms showing improved peak shapes for compounds terfenadine and GSK 2 on addition of NH_4OAc additive.

3.4 Possible Trend with the bpKa1 values

The data for log P, log D and pKa for the focused set provided with the compounds were used to find trends with the physico-chemical properties. The compounds were analysed using a CN column with 20% MeOH-CO_2 modifier and 0.1% amine additive. The retention times were then plotted in combination with physico-chemical properties in an attempt to find a trend. The bpKa1 values of the compounds may show a trend in relationship between compound type and elution from column.

The plots in Figure 3.40 showed that compounds **EL17** to **EL24**, *i.e.* group C of focused set, with imidazole substructure with lower bpKa1 elute early in comparison to the compounds with the aliphatic amine substructure. This suggests that may be a correlation with the bpKa1 values and retention times. EL22 is an exception though this may be due to the presence of the acid functionality on a side chain. More samples with similar structures need to be investigated to see whether this hypothesis is valid.



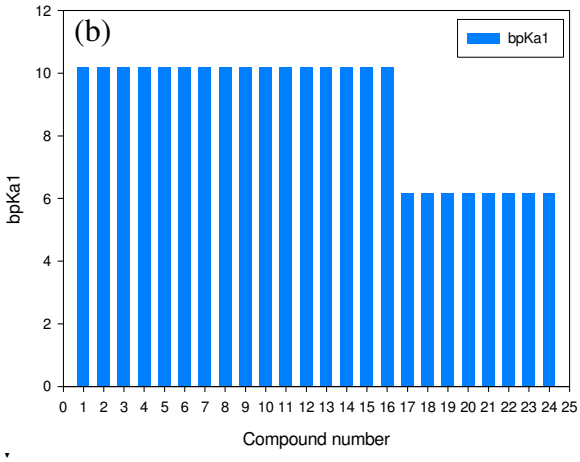


Figure 3.40:(a) Plot of compound number against retention time, (b) Plot of compound number against bpKa1 value.

3.5 Comparison of SFC and HPLC for the given set of compounds

Due to the advantages of SFC over HPLC, one of the aims of the project was to compare SFC and HPLC for compounds of pharmaceutical interests. The results from the SFC method development suggest the requirement of small amounts of additive to the CO_2 / MeOH mobile phase to improve the elution of compounds. Like wise the generic HPLC method used TFA. The study was undertaken by analysing the focused and the diverse set with the methods mentioned in the experimental section. For convenience the plots are split into four groups. The first plot consists of the focused library, Figure 3.41. Here for this class of compounds SFC was the preferred technique as the generic HPLC method

required 8min for the compounds to elute compared to 3.5 minutes with SFC. Moreover using HPLC the compounds **EL11** of **EL24** were not retained on C_{18} column and eluted with the solvent front. In contrast all the 24 compounds eluted with SFC on a 50 mm CN column, using an additive, within 3.5 minutes. Although the retention time is short the peaks are clearly separated from the solvent front as the injection peak. This shows that SFC gives greater compound coverage compared to HPLC for this class of compounds and is also significantly faster with comparable peak properties.

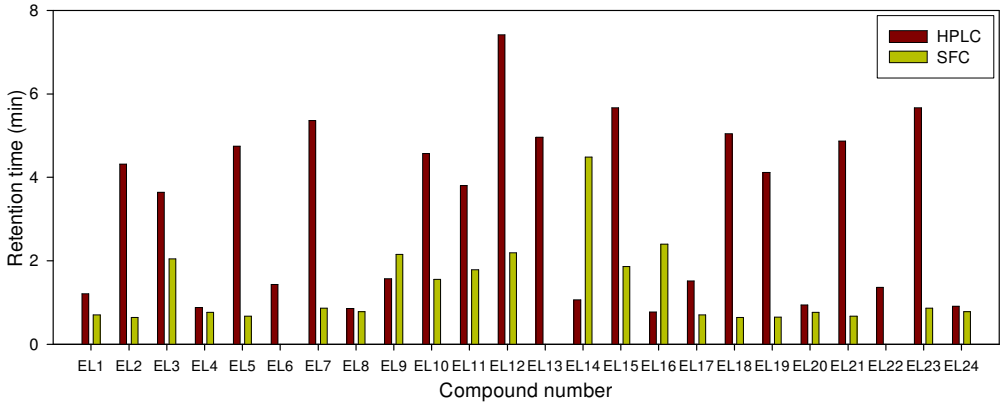


Figure 3.41: HPLC and SFC comparison for the focused library.

Similarly, the diverse set of compounds was also analysed on both SFC and HPLC and the results are given the figures below. Figure 3.42 and Figure 3.43 show that 83% compounds elute with 2-EP column on SFC in comparison to 95% by HPLC by generic C_{18} HPLC method. 4% of the compounds did not elute by HPLC or SFC. Two compounds elute with the solvent front by HPLC. Figure 3.44 shows that 87% compounds elute with 2-EP column on SFC in comparison to 97% by HPLC by generic C_{18} HPLC method. EL11 eluted only by SFC. 70% compounds elute within 2.5 min in comparison to 8 min by generic C_{18} HPLC method. Summarising the results with a Venn diagram in shown Figure 3.47, it can be concluded that both techniques are complementary to each other and that SFC gives greater coverage compared to HPLC.

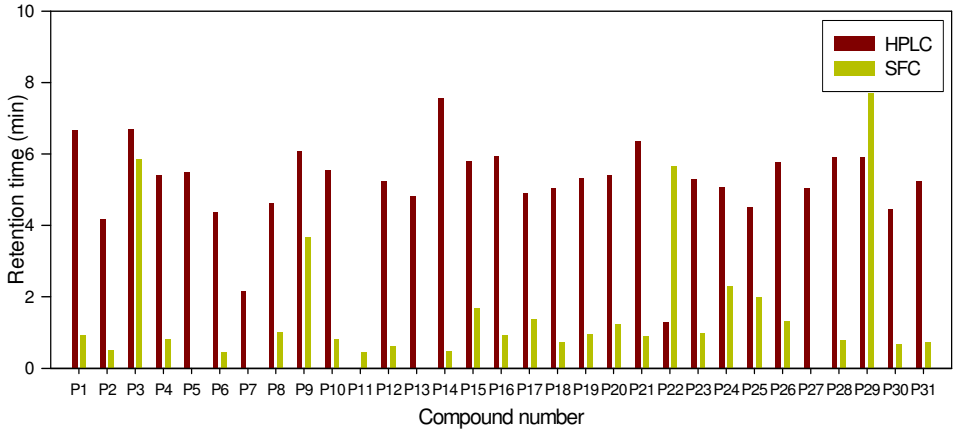


Figure 3.42: HPLC and SFC comparison for the diverse set I (Structures shown in Appendix 3, pages 114-117).

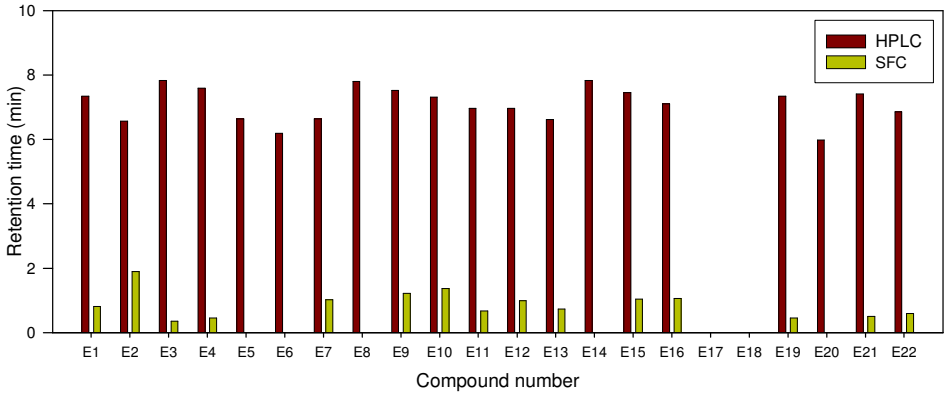


Figure 3.43: HPLC and SFC comparison for the diverse set II (Structures shown in Appendix 4, pages 117-119).

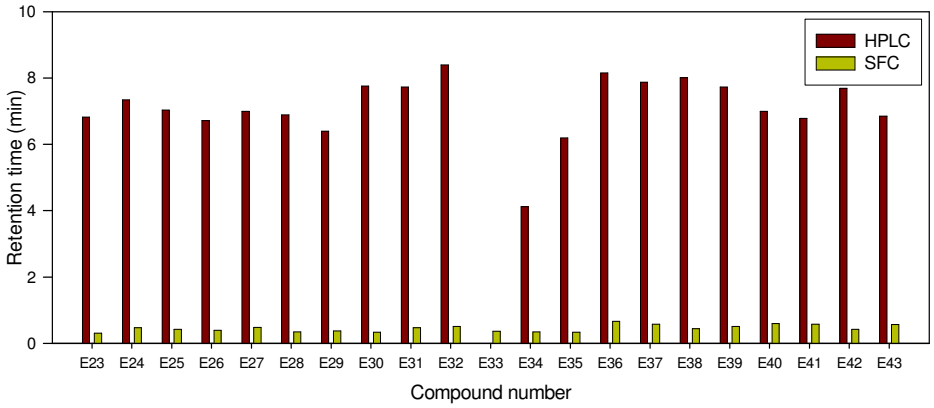


Figure 3.44: HPLC and SFC comparison for the diverse set II (Structures shown in Appendix 4, pages 119-121).

3.6 Experimental Section

Materials and Reagents

SFC grade CO_2 from BOC gases (Guildford, UK) was used for mobile phase. HPLC grade methanol from Fisher Scientific (Loughborough, UK) was used for modifier unless specified. TFA, Diethylamine (DEA), triethylamine (TEA), dimethylethylamine (DMEA) and ammonium hydroxide were used as additives were obtained from respectively. 2-propanol, ethanol and acetonitrile also used for mobile phase were all HPLC grade from Fisher Scientific (Loughborough, UK). DCM and DMSO were also used for sample preparation. These solvents were used for both sample preparation and as modifiers. A training set of 20 compounds was provided by GSK, and a second set of structurally related compounds, focused library, was provided by Eli Lilly. Compounds from Pfizer and Evotec were combined as the diverse set. Focused library from Eli Lilly explained in detail below.

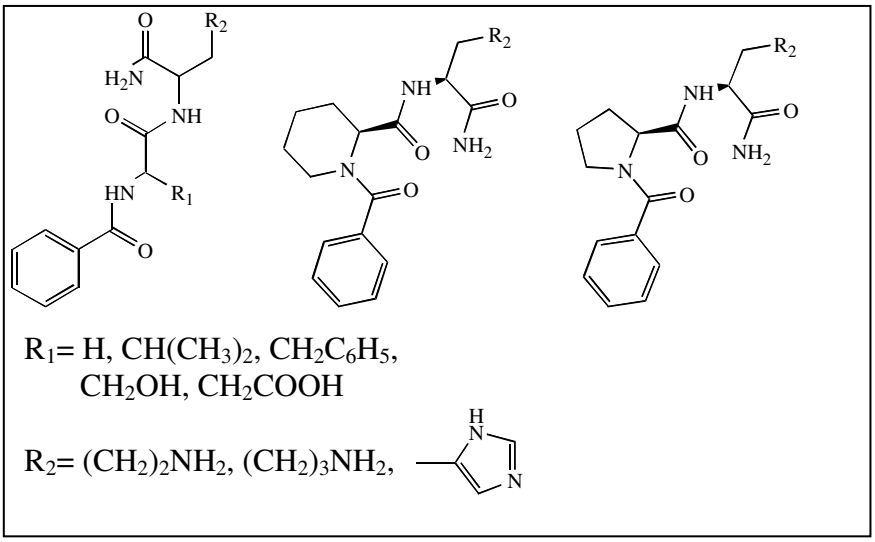


Figure 3.45: Explaining the structural similarity of the focused library.

Stationary phases

The stationary phases used are 2-ethyl pyridine (2-EP), 2-pyridyl propyl urea (PPU), cyano (CN) and Diol. The structures of these are shown in Figure 3.46. The 4.6 mm x 250 mm (6µ particle size) columns were provided by Mettler-Toledo AutoChem. This set did not include PPU. The 4.6 mm x 50 mm (5µ particle size) columns were provided by Princeton chromatography.

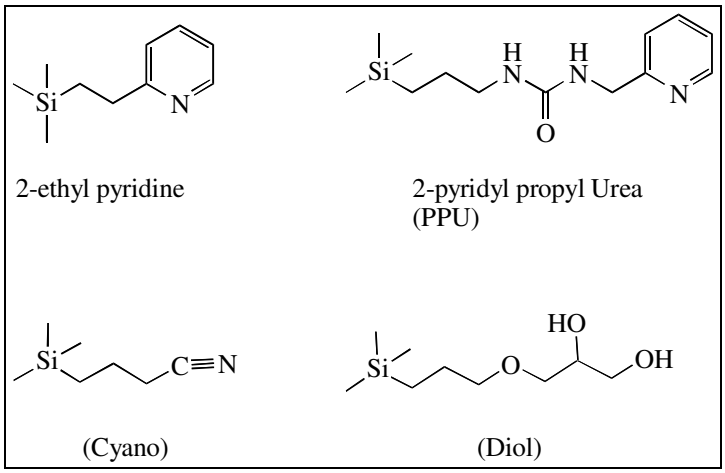


Figure 3.46: The structures of the stationary phases- 2-EP, PPU, CN and Diol.

Instrumentation

SFC analysis was performed on a Berger Minigram (Mettler-Toledo Autochem, Newark, DE, USA) system equipped with a FCM-1100/1200 dual pump fluid control module, a TCM-2250 heater control module, an ALS 3100/3150 autosampler and a Kauner k-2501 variable wavelength UV detector (Kauner, Berlin, Gemany). The UV detector is set at 250 nm for the GSK set and at 230 nm for the Eli Lilly set of compounds. The mass spectrometer coupled to SFC was initially a Platform II and was replaced by Micromass platform LCZ both donated by GSK. A HP1050 HPLC system (Agilent, Palo Alto, California) was used to deliver a make up flow of 0.1 mL min^{-1} of methanol to the MS. The SFC instrument is controlled by the SFC PRONTO software (v 1.5.305.15, Berger instruments) and the MS is controlled by MassLynx software (v 3.5). The HPLC-MS is an open access system Waters ZMD controlled by MassLynx software.

Chromatographic conditions

The experimental condition for each gradient and isocratic methods are given below. The samples were generally prepared in HPLC grade methanol unless specifically mentioned. Compounds insoluble in methanol were prepared in DCM and further diluted with methanol. One of them, GSK10 was prepared in a 50:50 mixture of acetonitrile and water. Compounds from Pfizer were supplied as 100 µg/mL solutions in 1:9 DMSO: MeOH solvent mixture. All samples analyzed on SFC-MS system are of approximate concentration 100 µg/mL.

Table 3.20: Chromatographic conditions for the GSK and Eli Lilly compounds (SFC).

Conditions	Gradient method	Isocratic method
Modifier	5-50% MeOH @ 20% min^{-1}	20% methanol
Additive	0.1% v/v of one of DEA, TEA, DMEA	0.1% v/v of one of DEA, TEA, DMEA, TFA
Flow	1.5 mL min^{-1} hold 0.5 min up to 4 @ 8 mL min^{-1}	3 mL min^{-1}
Pressure	100 bar	100 bar
Column oven temperature	35°C	35°C
Wavelength	254 nm	230 nm

Table 3.21: Chromatographic conditions for the SFC and HPLC.

Conditions	SFC	**HPLC:** XTerra™ Waters
Modifier	Isocratic 20% MeOH	5 minute gradient from 95% water to 100% MeOH at 1.25 mL min^{-1} hold 100% MeOH for 10 min
Column	250 mm CN	C_{18} 5 µm 3.0 mm x 50 mm
Additive	0.1% v/v of one of DEA, TEA, DMEA, TFA	
Flow	4 mL min^{-1}	1.5 mL min^{-1}
Pressure	100 bar	
Column oven temperature	35°C	35°C
Injection volume	2 µL	10 µL
Wavelength	230 nm	230 nm

3.7 Conclusions

In order to have a generic SFC method for pharmaceutical type of compounds methanol was the most appropriate modifier. Samples need to be soluble or compatible with the mobile phase composition. This was confirmed by replacing methanol with ethanol, 2-propanol, and comparing the results with other choices.

2-EP was found to be compatible with most compounds except for those with certain substructures. This problem can be fixed using DCM as solvent for sample preparation or ammonium acetate in the mobile phase. CN and diol columns also give good results only with addition of additive in the mobile phase. The use of amine additive may cause difficulty preparative chromatography.

The identity of an amine additive has little or no effect on the retention times. Ammonium acetate would be the preferred choice of additive as it is also MS compatible and easier to separate compared to amines.

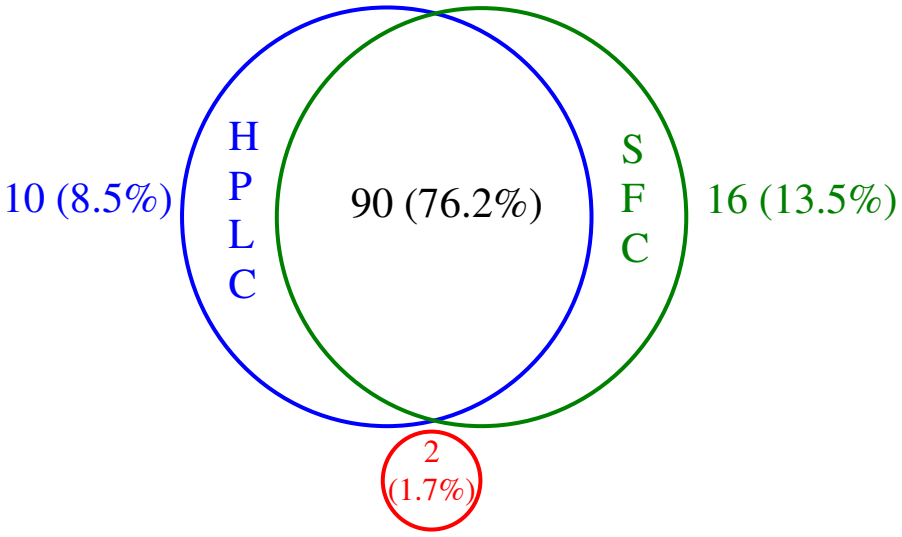


Figure 3.47: Venn diagram showing overlap of compound coverage by SFC and HPLC.

Finally the SFC and HPLC comparison results can be summarised with the Venn diagram shown in Figure 3.47. Around 76% of the total compounds analysed in the current project were effortlessly undertaken with both HPLC and SFC. A very

small percentage *i.e.* two compounds from the set eluted neither by HPLC nor by SFC. Out of the remaining 22% nearly 14% were analysed only by SFC. These results indicate that the two techniques are definitely complementary to each other. However, since the instrumentation of SFC is a modification of HPLC, when required it can be used like an LC by using 100% modifier as the mobile phase. This could cut down the costs of two different instruments.

Chapter 4

Conclusions and Future Work

Supercritical fluid chromatography is a normal phase technique orthogonal to the most commonly used high performance liquid chromatography. Supercritical fluids have liquid like diffusivity and gas like viscosity which speeds the separation process. Commonly used alcoholic mobile phase are also MS friendly. Detection using MS is more selective to most other detectors used. SFC was found to be compatible to all API techniques. In this project the two aspects of SFC were researched. One of them was the coupling of SFC to ESI-MS and the other was to optimize the SFC parameters in an attempt to achieve a generic method for the analysis of pharmaceutical type of compounds.

Samples ionized in the absence of high voltage, when SFC was coupled to ESI-MS. To understand the mechanism involved in this type of ionization, compounds 1-7 (see Figure 2.1) were analysed using SFC-ESI-MS under normal electrospray conditions and with high voltage switched off (Novo spray. Addition of ammonium acetate or acid to the make-up solvent did not enhance ionization, thus ruling out the possibility of thermospray type or charged residue model type of ionization mechanism. But with pure methanol as make-up solvent study of systematic increase in the high voltage from 0 to 5kV resulted in little variation in sensitivity suggesting the presence of pre-formed ions in solution similar to that of charge residue model (CRM).

Novospray was found to be dependent on the SFC parameters such as pressure, modifier and flow rate, but was independent of the API source. Novospray was also observed when ESI source was replaced with APCI source. Here novospray can be compared to sonic spray in which ionization is independent of heat or electricity. In sonic spray ionisation a coaxial nitrogen gas to the analyte solution facilitates droplet nebulisation. A comparative study of acetonitrile and methanol as modifiers suggests that the slight acidic nature of methanol may assist in

protonation on the molecule. Once again this leads to CRM type of mechanism. Based on the experimental study a definitive mechanism could not be determined, however, novospray may be described as a variant of sonic spray with a charge residue model-type formation of analyte ions is a likely mechanism. Another possibility is that the ions are formed due to collision of molecules in the free jet expansion of the SFC eluent. An important point to note is that increased sensitivity can be achieved, in the absence of source HV, when using SFC-MS system with methanol modified CO_2 mobile phase. This ultimately can assist in lowering the limit of detection for such compounds as shown in Figure 2.13 for oxybutynin.

In the second part of the project SFC parameters were explored in an attempt to develop a generic method for pharmaceutical type of compounds. The various parameters of SFC such as modifiers, column materials, additives were studied and the most suitable were chosen.

Methanol, ethanol and propanol were tried as modifiers for a set of training compounds and from the results obtained methanol was chosen as a suitable modifier. The modifier and pressure studies for the Novospray ionisation suggested 20 to 30% of modifier generally resulted in better ionisation. Also 100 bar pressure was found to be optimum.

Focused and diverse sets of compounds were analysed with different columns such as 2-EP, Cyano, Diol and PPU. 2-EP column was found to be compatible with most compounds giving shorter retention times with reasonably good peak shapes accept for analytes with certain substructures. These analytes exhibited peak splitting. Further experiments suggest that these analytes result in the formation of a solvated molecule resulting in a second peak. Use of DCM in the sample preparation stage eliminated peak spitting. Another solution was to introduce ammonium acetate as additive in the modifier. CN and Diol columns give good results on addition of additive to the modifier. This may be analytically acceptable, but the use of amine additive may cause difficulty preparative chromatography. The identity of an amine additive has little or no effect on the

retention times. Ammonium acetate would be the preferred choice of additive as it is also MS compatible and easier to separate compared to amines.

SFC and HPLC comparison results were summarised with the Venn diagram shown in Figure 3.47. Both techniques were found to be complimentary to each other with a large percentage of compounds compatible. A very small percentage *i.e.* two compounds from the set eluted neither by HPLC nor by SFC. Out of the remaining compounds, nearly 2/3rd of compounds could be analysed by SFC alone and the rest of the compounds by HPLC alone. However, since the instrumentation of SFC is a modification of HPLC, when required it can be used like an LC by using 100% modifier as the mobile phase. This could cut down the costs of two different instruments. SFC was found to give greater coverage compared to HPLC for the focused library and is significantly faster for most compounds.

Future Work

The work undertaken in this thesis used a limited set of compounds to understand the ionisation mechanism in the absence of high voltage. To explore whether this phenomenon is valid for wider range of compounds a further study with diverse set of compounds is required.

The experiments with the focused set showed co-relation between retention times and the pkb1 values of the compounds. Due to Eli Lilly withdrawing out of the project, the research could not be progressed in this area. There is scope for undertaking experiments to understand the relation between retention time and the physical properties.

Additives study using the focused set showed some correlation between the chemical structures and the nature of the additive used. Detailed study in this area with compounds sets very closely related in their chemical structures is required.

Lastly, both SFC and HPLC techniques were seen to perform well for most compounds analysed. More experimental work is required to clarify the

overlapping area based on chemical space or physical properties or combination of the two.

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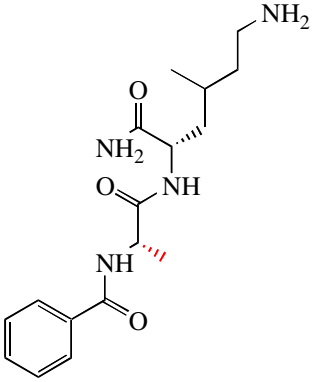
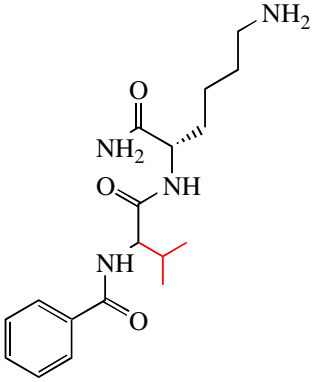
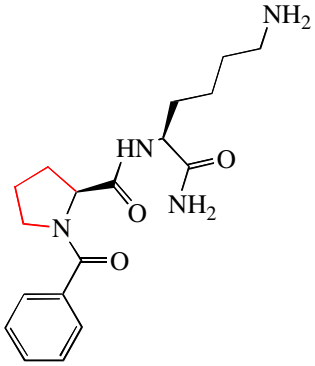
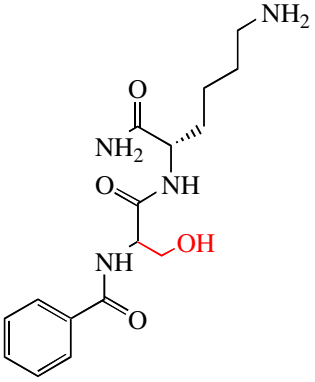
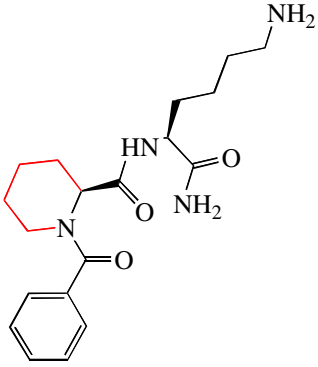
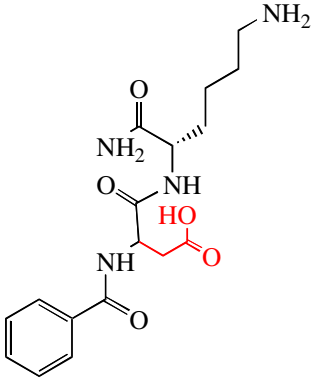
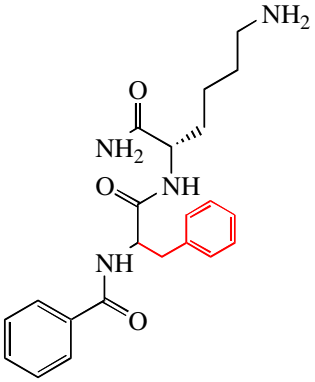
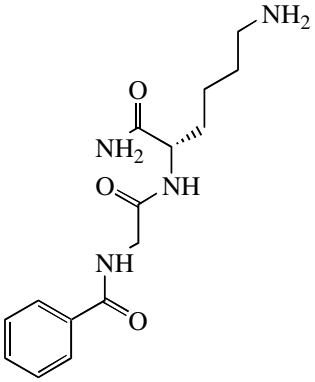
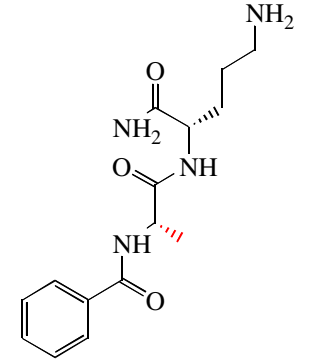
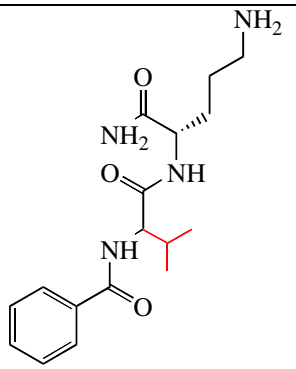
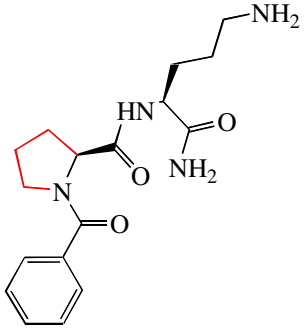
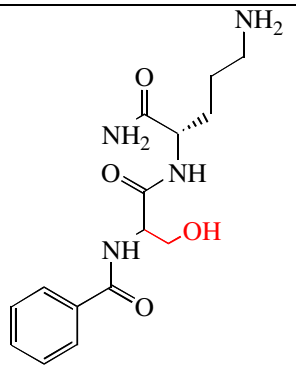
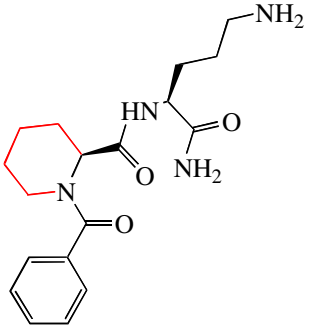
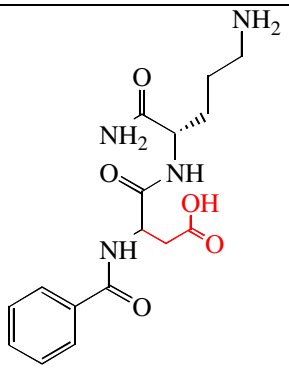
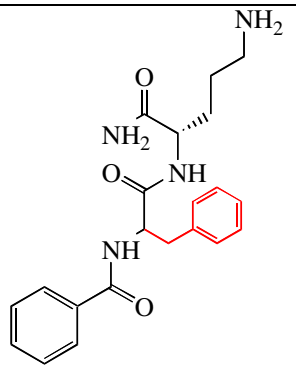
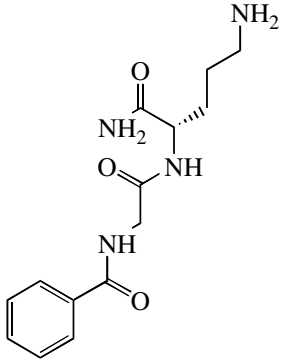
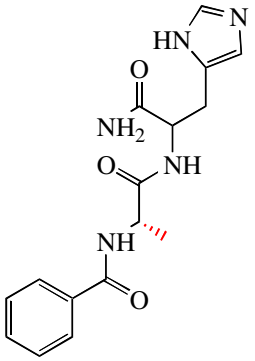
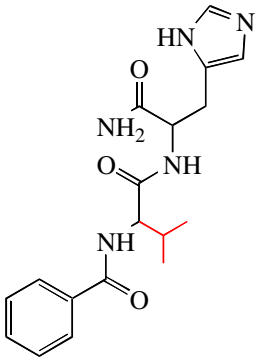
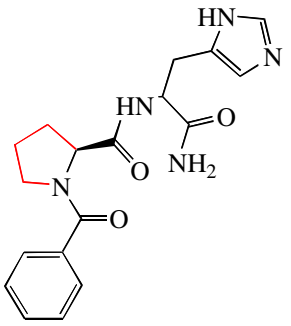
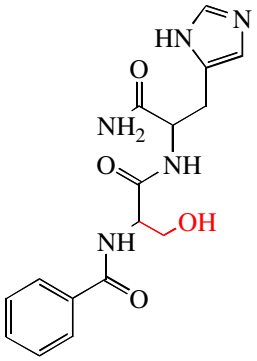
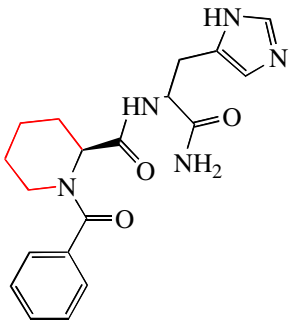
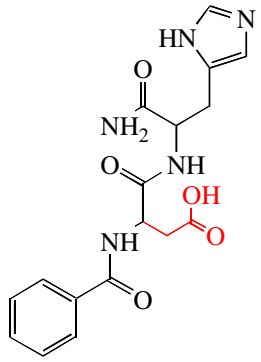
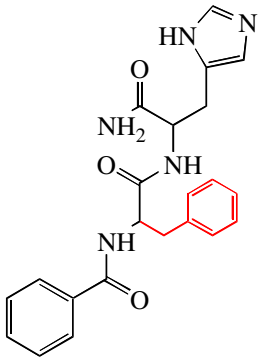
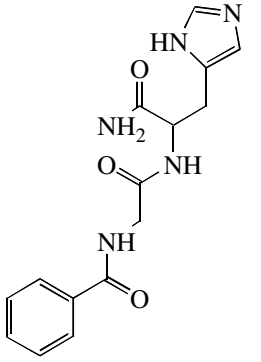
Appendix

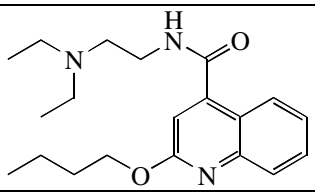
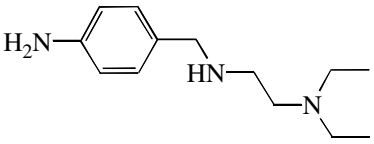
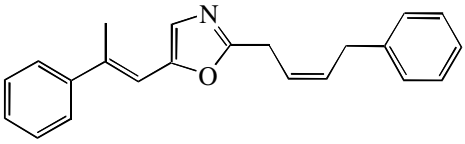
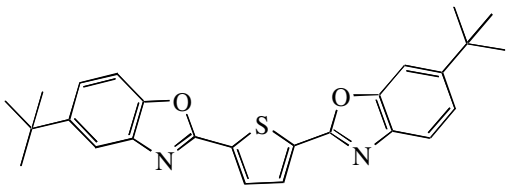
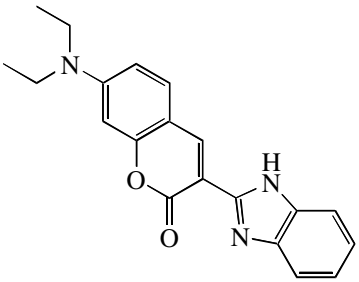
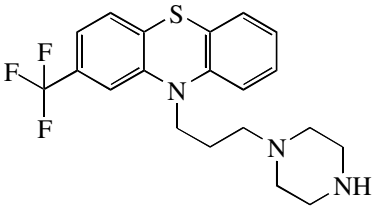
1. Focused Library

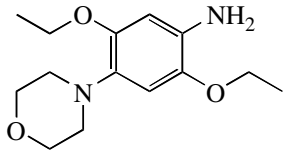
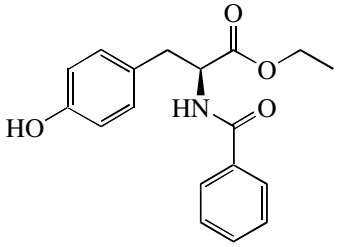
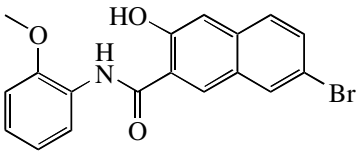
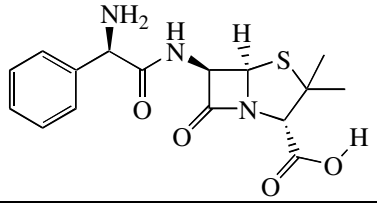
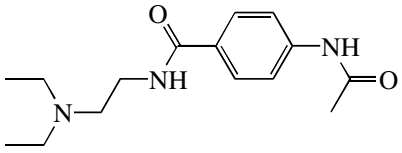
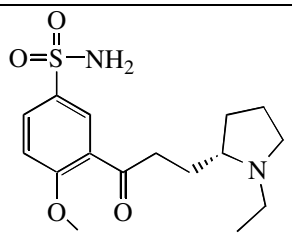
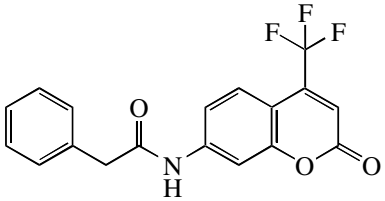
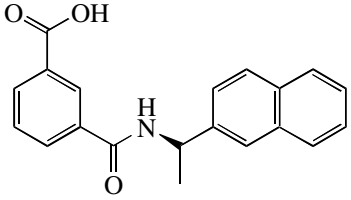
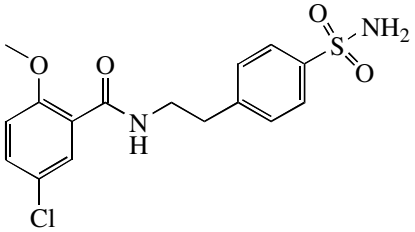
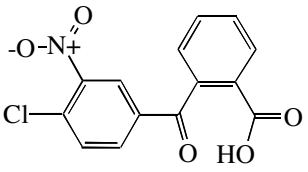
		
EL1. $C_{16}H_{24}N_4O_3$ Mol. Wt.: 320.39	**EL2**. $C_{18}H_{28}N_4O_3$ Mol. Wt.: 348.44	**EL3**. $C_{18}H_{26}N_4O_3$ Mol. Wt.: 346.42
		
EL4. $C_{16}H_{24}N_4O_4$ Mol. Wt.: 336.39	**EL5**. $C_{19}H_{28}N_4O_3$ Mol. Wt.: 360.45	**EL6**. $C_{17}H_{24}N_4O_5$ Mol. Wt.: 364.40
		
EL7. $C_{22}H_{28}N_4O_3$ Mol. Wt.: 396.48	**EL8**. $C_{15}H_{22}N_4O_3$ Mol. Wt.: 306.36	**EL9.** $C_{15}H_{22}N_4O_3$ Mol. Wt.: 306.36

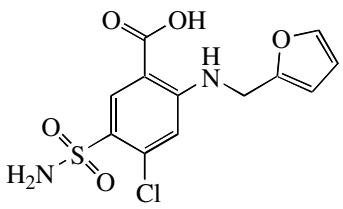
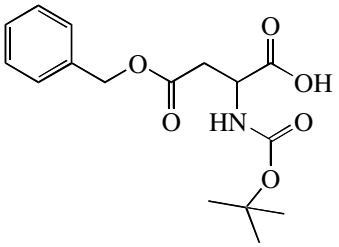
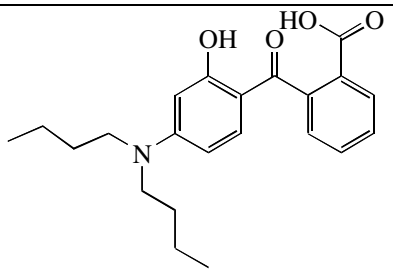
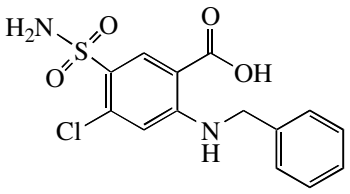
		
EL10. $C_{17}H_{26}N_4O_3$ Mol. Wt.: 334.41	**EL11**. $C_{17}H_{24}N_4O_3$ Mol. Wt.: 332.40	**EL12**. $C_{15}H_{22}N_4O_4$ Mol. Wt.: 322.36
		
EL13. $C_{18}H_{26}N_4O_3$ Mol. Wt.: 346.42	**EL14**. $C_{16}H_{22}N_4O_5$ Mol. Wt.: 350.37	**EL15**. $C_{21}H_{26}N_4O_3$ Mol. Wt.: 382.46
		
EL16. $C_{14}H_{20}N_4O_3$ Mol. Wt.: 292.33	**EL17**. $C_{16}H_{19}N_5O_3$ Mol. Wt.: 329.35	**EL18**. $C_{18}H_{23}N_5O_3$ Mol. Wt.: 357.41
		
EL19. $C_{18}H_{21}N_5O_3$ Mol. Wt.: 355.39	**EL20**. $C_{16}H_{19}N_5O_4$ Mol. Wt.: 345.35	**EL21**. $C_{19}H_{23}N_5O_3$ Mol. Wt.: 369.42

		
EL22. $C_{17}H_{19}N_5O_5$ Mol. Wt.: 373.36	**EL23**. $C_{22}H_{23}N_5O_3$ Mol. Wt.: 405.45	**EL24**. $C_{15}H_{17}N_5O_3$ Mol. Wt.: 315.33

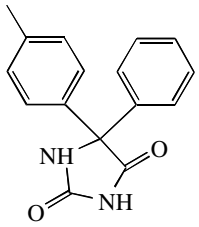
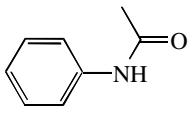
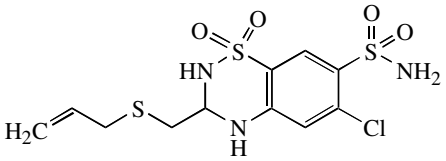
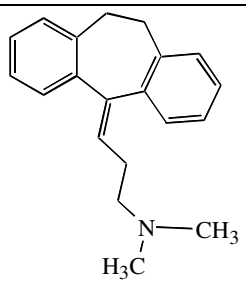
2. Training set

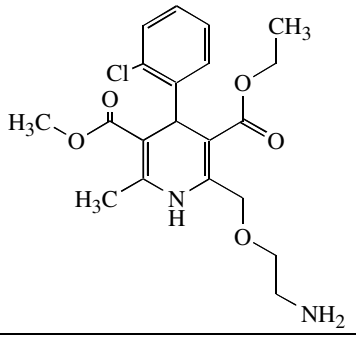
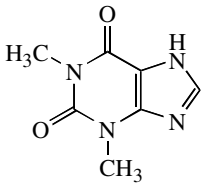
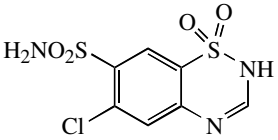
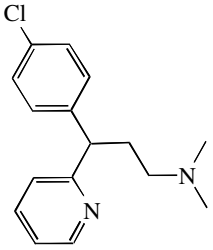
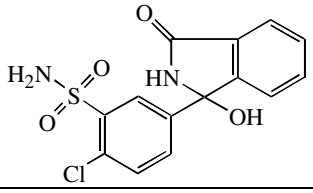
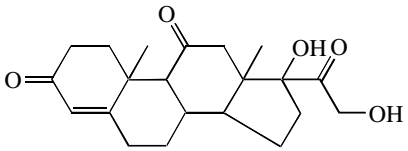
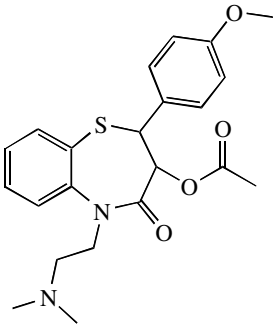
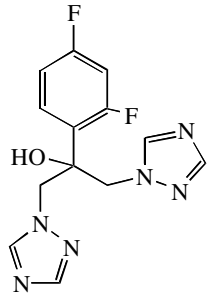
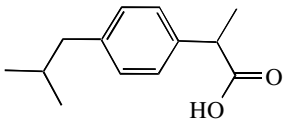
	
GSK1.Dibucaine Mol. Wt.: 343.46	**GSK2.**N1-[2-(diethylamino)ethyl]-4-aminobenzamide Mol. Wt.: 235.33
	
GSK3. 2-(4-iphenylyl)-6-phenylbenzoxazole Mol. Wt.: 347.41	**GSK4**.2,5-di(5-tert-butylbenzoxazole-2-yl) thiophene Mol. Wt.: 430.56
	
GSK5. 3-(2'-benzimidazolyl)-7-N,N-diethyl-aminocoumarin Mol. Wt.: 333.38	**GSK6.** Trifluoperazine (dihydrochloride) Mol. Wt.: 407.50

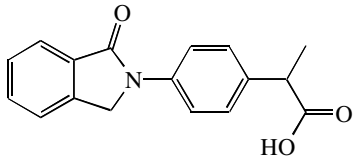
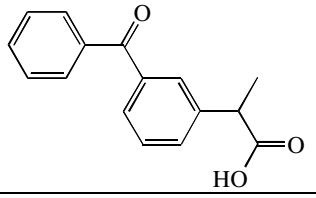
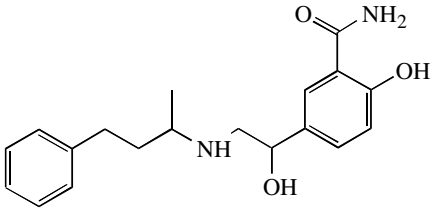
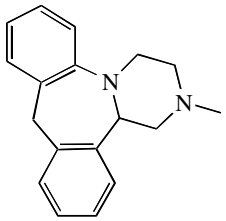
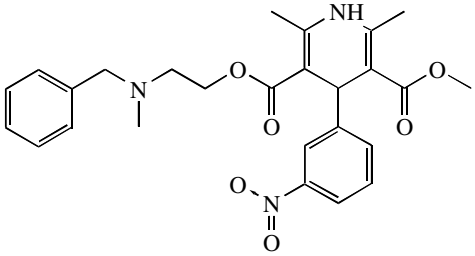
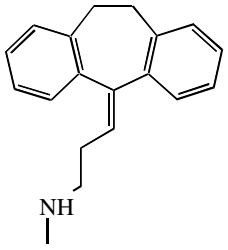
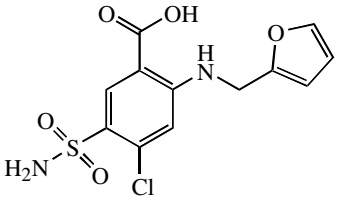
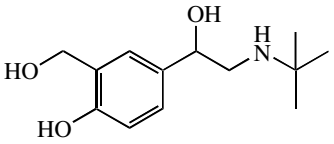
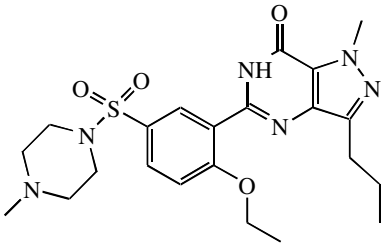
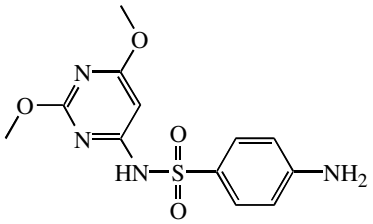
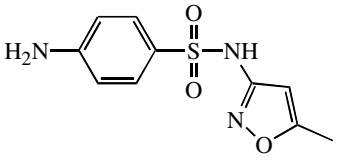
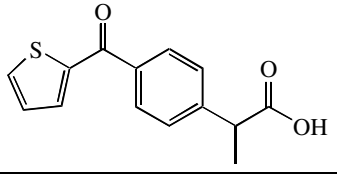
	
GSK7. 2,5-diethoxy- 4-morpholinoaniline(dihydrochloride) Mol. Wt.: 266.34	**GSK8**. N-benzoyl-L-Tyrosineethyl ester Mol. Wt.: 313.35
	
GSK9. Naphthol AS-BI Mol. Wt.: 372.21	**GSK10**.Ampicillin Mol. Wt.: 349.41
	
GSK11. N-acetylprocainamide Mol. Wt.: 277.36	**GSK12.** (S)-(-)-sulpiride Mol. Wt.: 340.44
	
GSK13. 7-(phenylacetamido)- 4-(trifluoro-methyl)coumarin Mol. Wt.: 347.29	**GSK14.** N-[(R)-1-(10-naphthyl)-ethyl]- phthalamic acid Mol. Wt.: 319.35
	
GSK15. 5-chloro-2-methoxy-N- [2-(4-sulphomoylphenyl)ethyl]benzamide Mol. Wt.: 368.84	**GSK16.** 2-(4-chloro-3-nitrobenzoyl) benzoic acid Mol. Wt.: 305.67

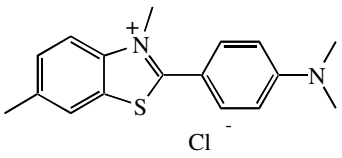
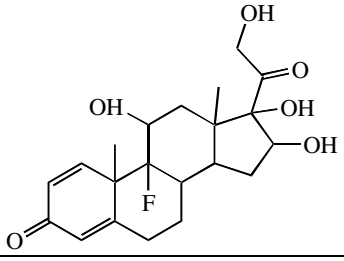
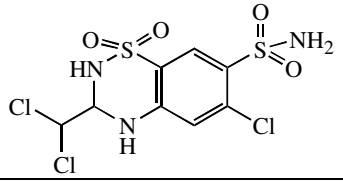
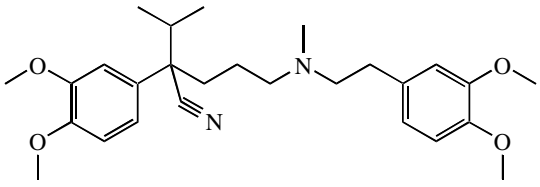
	
GSK17. Furosemide Mol. Wt.: 330.74	**GSK18.** Boc-Asp(oBzl)-OH Mol. Wt.: 323.34
	
GSK19. 2-[4-(dibutylamino)-2-hydroxybenzoyl] benzoic acid Mol. Wt.: 369.45	**GSK20.** N-benzyl-4-chloro-5-sulphomoylanthranilic acid Mol. Wt.: 340.78

3. Diverse Library I

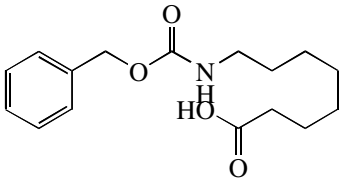
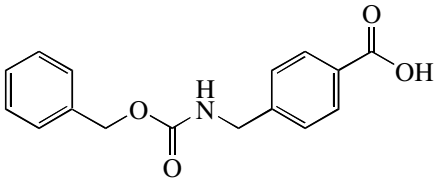
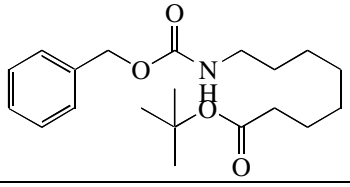
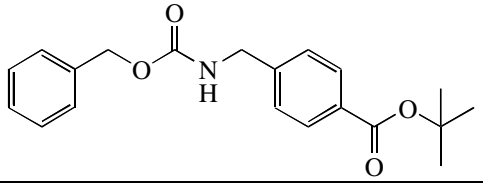
	
P1 : 5-(4-methylphenyl)-5-phenylhydantoin Mol. Wt.: 266.3	**P2** : Acetanilide Mol. Wt. : 135.2 (free base)
	
P3 : Althiazide Mol. Wt. : 383.9 (free base)	**P4** : Amitriptyline HCl Mol. Wt. : 277.4 (free base)

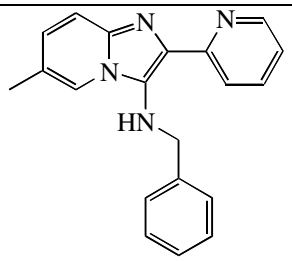
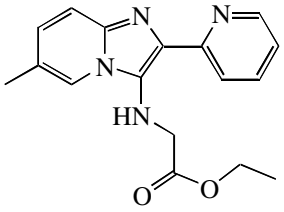
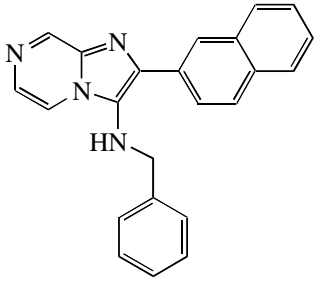
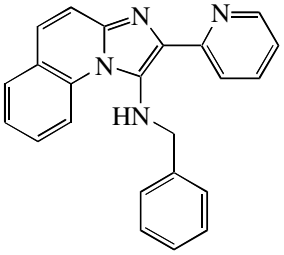
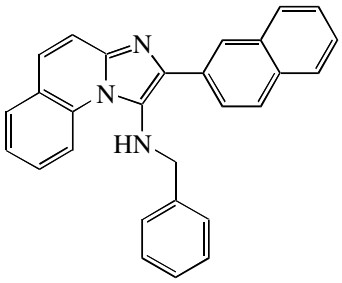
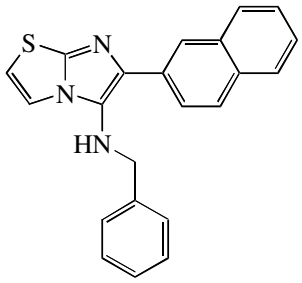
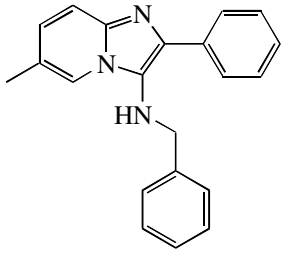
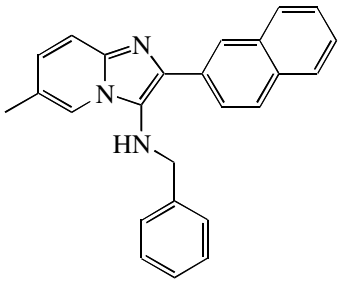
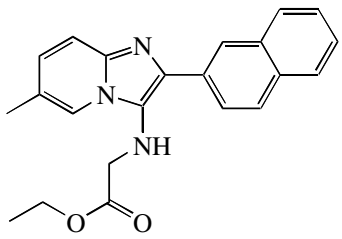
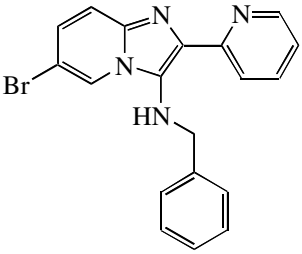
	
P5 : Amlodipine Mol. Wt. : 408.9 (free base)	**P6** : Caffeine Mol. Wt. : 194.2 (free base)
	
P7 : Chlorthiazide Mol. Wt.: 295.7	**P8** : Chlorpeniramine Mol. Wt.: 274.79
	
P9 : Chlorthalidone Mol. Wt.: 338.77	**P10** : Cortisone Mol. Wt.: 360.44
P11 : CP-052608 Mol. Wt. : 291.2	
P11 : CP-052608 Mol. Wt. : 291.2	**P12** : Diltiazem Mol. Wt.: 414.52
	
P13 : Fluconazole Mol. Wt.: 306.27	**P14** : Ibuprofen Mol. Wt.: 206.28

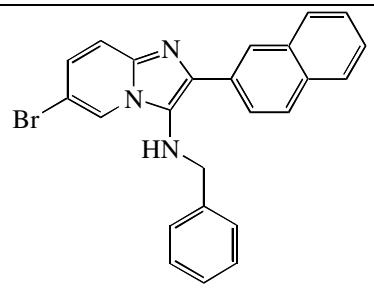
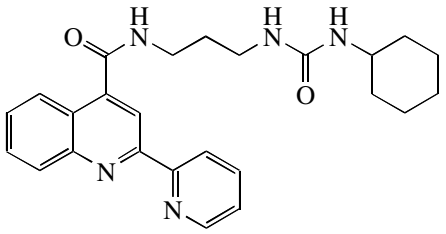
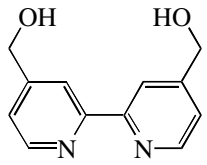
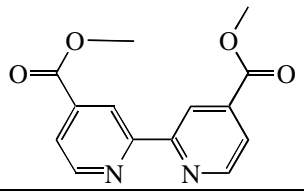
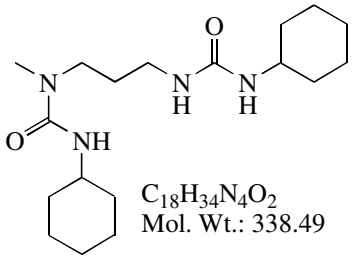
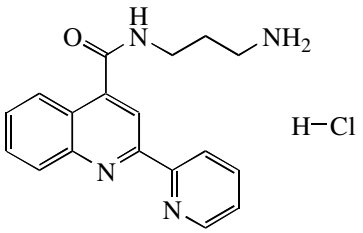
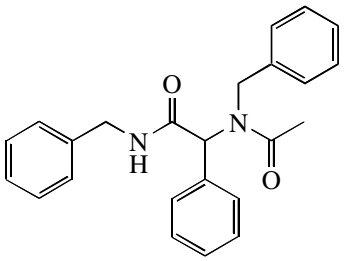
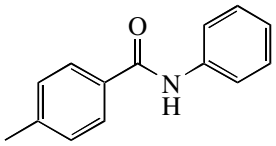
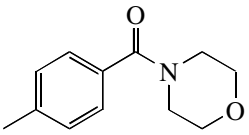
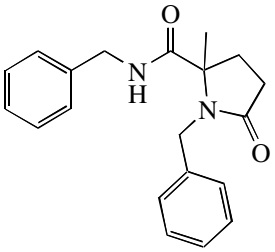
	
P15 : Indoprofen Mol. Wt. : 281.3	**P16** : Ketoprofen Mol. Wt.: 254.28
	
P17 : Labetalol Mol. Wt.: 328.41	**P18** : Mianserin Mol. Wt.: 264.36
	
P19 : Nicardipine Mol. Wt.: 480.53	**P20** : Nortryptyline Mol. Wt.: 263.38
	
P21 : Furosemide Mol. Wt.: 330.74	**P22** : Salbutamol Mol. Wt.: 239.31
	
P23 : Sildenafil Mol. Wt.: 474.58	**P24** : sulphadimethoxine Mol. Wt.: 310.33
	
P25 : sulphamethoxazole Mol. Wt.: 253.28	**P26** : Suprofen Mol. Wt.: 260.31

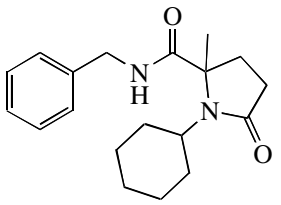
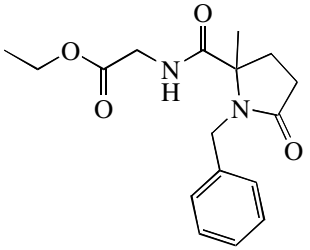
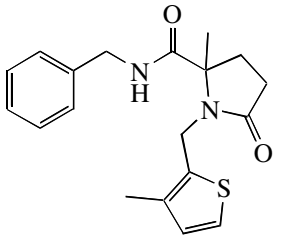
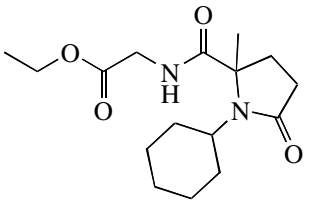
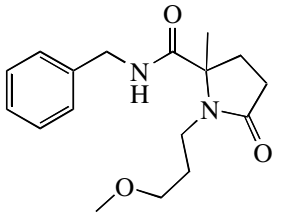
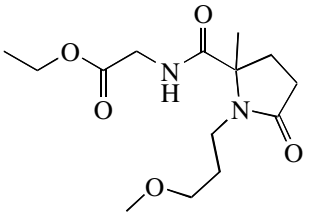
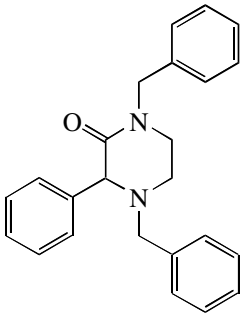
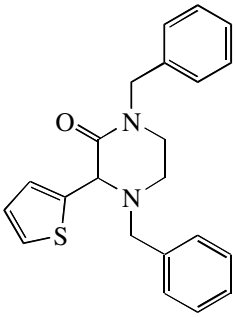
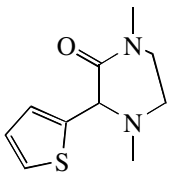
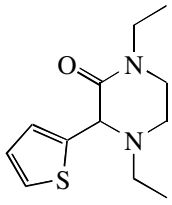
	
P27 : Thioflavin T Mol. Wt.: 283.41	**P28** : Triamcinolone Mol. Wt.: 394.43
	**P30** : UK 11147 Mol Wt.: 257.12
P29 : Trichloromethiazide Mol. Wt.: 380.66	**P30** : UK 11147 Mol Wt.: 257.12
	
P31 : Verapamil Mol. Wt.: 454.60	

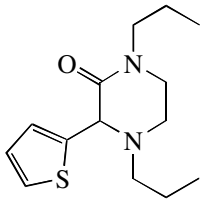
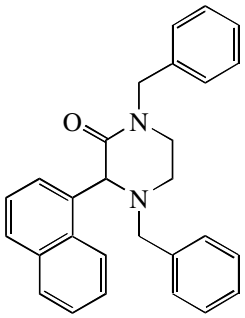
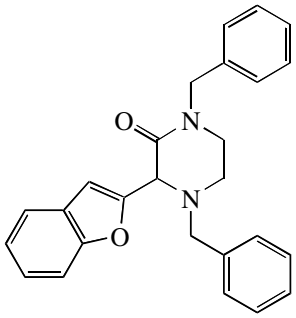
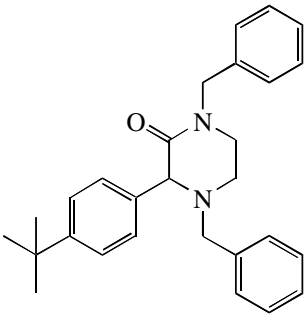
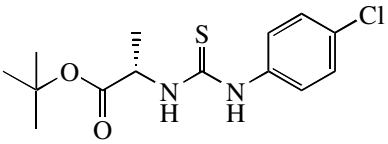
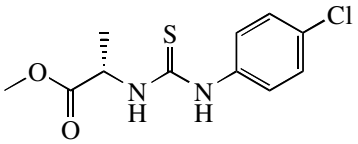
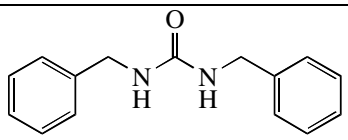
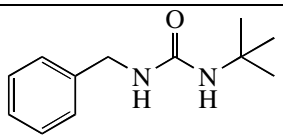
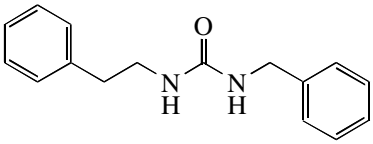
4. Diverse Library II

	
E1: 0773_00005 [A161] $C_{16}H_{23}NO_4$ Mol. Wt.: 293.36	**E2**: 0773_00006 [A162] $C_{16}H_{15}NO_4$ Mol. Wt.: 285.29
	
E3: 0773_00007 [A163] $C_{20}H_{31}NO_4$ Mol. Wt.: 349.46	**E4**: 0773_00008 [A164] $C_{20}H_{23}NO_4$ Mol. Wt.: 341.40

	
E5: 0773_00009 [A165] $C_{20}H_{18}N_4$ Mol. Wt.: 314.38	**E6**: 0773_00010 [A166] $C_{17}H_{18}N_4O_2$ Mol. Wt.: 310.35
	
E7: 0773_00011 [A167] $C_{23}H_{18}N_4$ Mol. Wt.: 350.42	**E8**: 0773_00012 [A168] $C_{23}H_{18}N_4$ Mol. Wt.: 350.42
	
E9: 0773_00013 [A169] $C_{28}H_{21}N_3$ Mol. Wt.: 399.49	**E10**: 0773_00014 [A170] $C_{22}H_{17}N_3S$ Mol. Wt.: 355.46
	
E11: 0773_00016 [A172] $C_{21}H_{19}N_3$ Mol. Wt.: 313.40	**E12**: 0773_00017 [A173] $C_{25}H_{21}N_3$ Mol. Wt.: 363.45
	
E13: 0773_00018 [A174] $C_{22}H_{21}N_3O_2$ Mol. Wt.: 359.42	**E14**: 0773_00019 [A175] $C_{19}H_{15}BrN_4$ Mol. Wt.: 379.25

	
E15: 0773_00020 [A176] $C_{24}H_{18}BrN_3$ Mol. Wt.: 428.32	**E16:** 0773_00038 [A178] $C_{25}H_{29}N_5O_2$ Mol. Wt.: 431.53
	
E17: 0773_00040 [A180] $C_{12}H_{12}N_2O_2$ Mol. Wt.: 216.24	**E18:** 0773_00041 [A181] $C_{14}H_{12}N_2O_4$ Mol. Wt.: 272.26
 $C_{18}H_{34}N_4O_2$ Mol. Wt.: 338.49	
E19: 0773_00043 [A183] $C_{18}H_{34}N_4O_2$ Mol. Wt.: 338.49	**E20:** 0773_00037 [A187] $C_{18}H_{19}ClN_4O$ Mol. Wt.: 342.82
	
E21: 0773_00052 [A188] $C_{24}H_{24}N_2O_2$ Mol. Wt.: 372.46	**E22:** 0773_00053 [A189] $C_{14}H_{13}NO$ Mol. Wt.: 211.26
	
E23: 0773_00054 [A190] $C_{12}H_{15}NO_2$ Mol. Wt.: 205.25	**E24:** 0773_00055 [A191] $C_{20}H_{22}N_2O_2$ Mol. Wt.: 322.40

	
E25: 0773_00056 [A192] $C_{19}H_{26}N_2O_2$ Mol. Wt.: 314.42	**E26:** 0773_00057 [A193] $C_{17}H_{22}N_2O_4$ Mol. Wt.: 318.37
	
E27: 0773_00058 [A194] $C_{19}H_{22}N_2O_2S$ Mol. Wt.: 342.46	**E28:** 0773_00059 [A195] $C_{16}H_{26}N_2O_4$ Mol. Wt.: 310.39
	
E29: 0773_00060 [A197] $C_{17}H_{24}N_2O_3$ Mol. Wt.: 304.38	**E30**: 0773_00061 [A198] $C_{14}H_{24}N_2O_5$ Mol. Wt.: 300.35
	
E31: 0773_00087 [A200] $C_{24}H_{24}N_2O$ Mol. Wt.: 356.46	**E32**: 0773_00088 [A201] $C_{22}H_{22}N_2OS$ Mol. Wt.: 362.49
	
E33: 0773_00089 [A202] $C_{10}H_{14}N_2OS$ Mol. Wt.: 210.30	**E34**: 0773_00090 [A203] $C_{12}H_{18}N_2OS$ Mol. Wt.: 238.35

	
E35: 0773_00091 [A204] $C_{14}H_{22}N_2OS$ Mol. Wt.: 266.40	**E36**: 0773_00092 [A205] $C_{28}H_{26}N_2O$ Mol. Wt.: 406.52
	
E37: 0773_00093 [A206] $C_{26}H_{24}N_2O_2$ Mol. Wt.: 396.48	**E38**: 0773_00094 [A207] $C_{28}H_{32}N_2O$ Mol. Wt.: 412.57
	
E39: 0773_00099 [A208] $C_{14}H_{19}ClN_2O_2S$ Mol. Wt.: 314.83	**E40:** 0773_00100 [A209] $C_{11}H_{13}ClN_2O_2S$ Mol. Wt.: 272.75
	
E41: 0773_00101 [A210] $C_{15}H_{16}N_2O$ Mol. Wt.: 240.30	**E42**: 0773_00102 [A211] $C_{12}H_{18}N_2O$ Mol. Wt.: 206.28
	
E43: 0773_00103 [A212] $C_{16}H_{18}N_2O$ Mol. Wt.: 254.33	

5. Published paper

Mohini A. Thite, Robert Boughtflower, Jeff Caldwell, Laure Hitzel, Clare Holyoak, Stephen J. Lane, Paul Oakley, Frank S. Pullen, Stefan Richardson and G. John Langley, *Ionisation in the absence of high voltage using supercritical fluid chromatography: a possible route to increased signal*, **Rapid Communications In Mass Spectrometry, 2008;** 22: 3673–3682.