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Chemistry

Applications and Implementation of Modern Ultrahigh-Performance Supercritical Fluid Chromatography-Mass Spectrometry

Volume 1 of 1

by

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Abstract

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Chromatography and mass spectrometry (MS) are powerful analytical techniques that have seen significant technological advancements over the last 20 years. These have facilitated and enhanced their use as routine tools in many scientific laboratories.

Since their introduction, the techniques have been mainly used in isolation with experts specialising in one field only. Recent developments in hyphenating (or coupling) the techniques mean that analysts need to be experts on both sides of the hyphen. *i.e.,* be competent in the fundamental properties of chromatographic separation and all aspects of mass spectrometry.

Modern technological advances in the field of ultra-high performance supercritical fluid chromatography (UHPSFC) mean that modern instrumentation is robust enough to be coupled to atmospheric pressure ionisation mass spectrometers. There is increased control of the pressure of the supercritical fluid and since it exhibits the properties of both a liquid and a gas, the optimum separation properties of both are utilised for chromatography. Sometimes referred to as convergence chromatography, the technology converges between liquid and gas chromatography. UHPSFC-MS is often considered as a replacement for normal phase chromatography as the mobile phase is non-polar, but by combining this solvent with a more polar co-solvent a larger range of compounds can be explored. This also means that SFC has the advantage of using the conventional reversed phase (RP) stationary phases such as the C_{18} to expand the range of compounds analysed.

UHPSFC-MS has proved to be a powerful technique in the following application areas:

- As a robust tool to be used in an open access environment to fill the analytical gap between RP UHPLC-MS and GC-MS for those unretained, or solvent incompatible samples
- To identify and quantify small organic acids and purines in sweat as early markers for the detection of bedsores
- As a rapid quality control screening and quantitation protocol for the active ingredients in home-made facemask testing solutions

Additional application areas include the fuel industry where it has been used as an alternative approach to analyse Fatty Acid Methyl Esters (FAMEs) in Aviation turbine fuel and to detect and quantify a new fuel marker (ACCUTRACE™ S10) in diesel fuel to combat fuel laundering. A further quantitation method was developed to analyse elemental sulfur in mineral oil to help understand sulfur-related power transformer failures.

Table of Contents

Table of Tables

Table of Figures

Table of Figures

Research Thesis: Declaration of Authorship

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Herniman, J. M.; Langley, G. J., Open Access UHPSFC/MS- an Additional Analytical Resource for an Academic Mass Spectrometry Facility. *Rapid Commun. Mass Spectrom*. **2016**, *30* (15), 1811-1817. (doi.org/ 10.1002/rcm.7660)

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Definitions and Abbreviations

ABPR	Automated Back Pressure Regulator
APCI	Atmospheric Pressure Chemical Ionisation
API	Atmospheric Pressure Ionisation
APPI	Atmospheric Pressure Photoionisation
CEM	Charge Ejection Model
CI	Chemical Ionisation
CID	Collison-induced Dissociation
CO_2	Carbon Dioxide
CRM	Charge Residue Model
CSH	Charged Surface Hybrid
DC	Direct Current
DoE	Design of Experiments
EI	Electron Ionisation
E_i	Ionisation Energy
EPSRC	Engineering and Physical Science Research Council
ESI	Electrospray Ionisation
eV	Electron Volts
FAB	Fast-atom Bombardment
FIA	Flow Injection Analysis
FSOT	Fused Silica Open Tubular
GC	Gas Chromatography
GC-MS	Gas Chromatography-Mass Spectrometry
HETP	Height Equivalent to a Theoretical Plate
HILIC	Hydrophobic Interaction Liquid Chromatography
HPLC	High Performance Liquid Chromatography
IEM	Ion Evaporation Model
ISM	Isocratic Solvent Manager
kV	Kilovolts
L	Litre
LC-MS	Liquid Chromatography-Mass Spectrometry
MALDI	Matrix-Assisted Laser Desorption/Ionisation

MCP	Microchannel Plates
mL	Millilitre
mm	Millimetre
MS	Mass Spectrometry
MS/MS	Tandem Mass Spectrometry
m/z	Mass-to-Charge Ratio
nm	Nanometres
NMR	Nuclear Magnetic Resonance
NP	Normal Phase
OA	Open Access
PDA	Photo Diode Array
RF	Radio Frequency
RP	Reversed Phase
sc	Supercritical
SFC	Supercritical Fluid Chromatography
SIM	Single Ion Monitoring
SIR	Single Ion Recording
SQD	Single Quadrupole Detector
TLC	Thin-layer Chromatography
TOF	Time-of-Flight
TQD	Tandem Quadrupole Detector
u	Linear Velocity of Mobile Phase
UHPLC	Ultrahigh Performance Liquid Chromatography
UHPSFC	Ultrahigh Performance Supercritical Fluid Chromatography
UPC^2	Ultrahigh Performance Convergence Chromatograph
UPLC	Ultra Performance Liquid Chromatography

Chapter 1 Commentary

The techniques of chromatography and mass spectrometry (MS) are used routinely as analytical tools in many scientific laboratories. The coupling (hyphenation) of the two techniques now provides a highly sensitive separation, detection, and quantification tool for researchers in many disciplines. This study introduces the background and common terminology related to each technique and presents a body of research work that specifically focusses on the coupling of ultrahigh-performance supercritical fluid chromatography (UHPSFC) to MS and its implementation into new applications areas. The UHPSFC instruments used in this study utilise carbon dioxide (CO_2) as the main mobile phase; to reach its supercritical state the critical temperature and pressure are more easily achieved than other supercritical fluids and it is miscible with a wide range of polar and non-polar solvents used to improve chromatographic performance. Once the CO_2 is in this supercritical state ($scCO_2$) it cannot be classified as a gas or a liquid because it exhibits properties of both. It has a density similar to that of water and the high diffusivity and low viscosity exhibited by the $scCO_2$ makes it an ideal mobile phase for modern chromatographic separations.

1.1 Introduction to Chromatography

Chromatography is the general term for techniques used in analytical chemistry to purify compounds or for the separation of multiple components in simple or complex mixtures. The term chromatography itself is derived from the Greek words χρῶμα *chroma* (colour) and γράφειν *graphein* (to write). It was first described by William Ramsey[1] and then the Russian botanist Mikhail Tswett in the 1900s. Published in 1906, it was the first paper to use the term chromatography and described the use of chromatographic apparatus to determine the variety and complexity of pigments in green chlorophyll using a calcium carbonate adsorbent[2].

Chromatography involves the use of an adsorbent material known as the stationary phase and a mobile phase that may be a liquid or a gas. Compounds are dissolved in a solvent and then passed over the stationary phase using a flow of this mobile phase. Some compounds interact with the stationary phase to a greater or lesser extent than other compounds and hence will migrate along this phase at different rates. This difference in the strength of the interaction of a compound with the stationary phase is termed its affinity. If a compound has a greater affinity with the stationary phase than other analytes, then it will be retained for longer and hence move more slowly.

1.2 Thin-Layer Chromatography

Thin-layer chromatography (TLC) is commonly used by chemists to follow reactions and as a simple technique to separate non-volatile compounds[3]. Here the stationary phase (usually

comprised of silica) is coated onto plates made of plastic, glass, or aluminium. The compound is spotted onto the plate and then a solvent (mobile phase) is drawn up the plate by capillary action and separation is achieved due to the different retention characteristics of the compounds.

1.3 Column Chromatography

An advancement of TLC is a method based on the use of a vertical column and is termed liquid or column chromatography. If a polar silica stationary phase is packed into the column, then a non-polar liquid mobile phase is added to the top of the column and flows downwards under gravity or positive external pressure. This method is termed normal phase (NP) chromatography. Conversely, if a non-polar stationary phase is packed into the column and the liquid mobile phase used is polar, then this is termed reversed phase (RP) chromatography. The term gas chromatography (GC) is used to describe chromatography where the mobile phase is an inert carrier gas such as hydrogen, helium, or nitrogen. In GC, liquid stationary phases were initially used, and these were packed into a glass or metal column using small inert spherical supports. Since the early 1980s capillary columns have been more commonly used, here a liquid/film coating is applied to the walls of a column. The column is usually comprised of fused silica tubing with a polyamide coating that enhances robustness and flexibility[4].

1.4 High Performance Liquid Chromatography

Chromatographic separations were enhanced when it was discovered that stationary phases comprising of small, porous, uniform spherical particles (5-10 μm) provided a larger surface area for the improved adsorption of compounds. High performance liquid chromatography (HPLC), previously known as high pressure liquid chromatography was developed when mechanical pumps were used to produce high pressures (up to 350 bar) to force the liquid through the columns rather than rely on gravity as used for traditional column chromatography. The technique was first developed by Horvarth and Lipsky in 1967 when they published a paper on fast liquid chromatography[5]. Here they developed their previous work by using a pump operating at higher pressures. Elution in HPLC may be by isocratic methods where there is a fixed ratio of solvents, or by gradient elution where the ratio of solvents used as the mobile phase is varied over the duration of the experiment, depending on the analytes and degree of separation required. Slight changes in the chemistry of the stationary phase can also improve the separation of different analytes.

1.5 Ultrahigh Performance Liquid Chromatography

Advances in column technology and manufacture led to the use of smaller particles sizes (2-5 µm) to improve resolution and efficiency. Analysis times became much shorter, making method development quicker and easier[6]. The first commercial UHPLC instrument was launched in 2004 by Waters (Wilmslow, UK), the Acquity UPLC[7]. Subsequently, instrumentation was further developed to deliver the higher pressures required (600-1400 bar)[8] when using sub 2 µm particle size columns and ultrahigh performance liquid chromatography (UHPLC) has now become a routine chromatographic technique.

1.6 Key Chromatographic Parameters

In all forms of chromatography, there are many important parameters that play a crucial role in optimising chromatographic separation. The most significant of these are described here.

1.6.1 Retention Factor

The retention factor (*k*) is a measure of the time an analyte resides in the stationary phase relative to the time it spends in the mobile phase. It is also commonly referred to as the partition ratio, capacity ratio, capacity factor or mass distribution ratio. It is used to determine a measurement of the retention of one analyte on the stationary phase and is used as a comparison with other analytes.

$$k = \frac{t_R - t_0}{t_0}$$

Equation 1 - Retention factor (*k*)

Where t_R = retention time of the analyte

t_0 = retention time of solvent front

If the *k* value is low, this means that the analyte shows little retention and if the *k* = 0 then this shows that the analyte is completely un-retained. Conversely if the retention number is high this means that the analyte is retained on the column[9].

1.6.2 The Separation Factor

The separation (or selectivity) factor (α) is used to determine the difference in capacity factors of two different retained analytes.

$$\alpha = \frac{k_2}{k_1}$$

Equation 2 - Separation factor (α)

The separation factor is always greater than or equal to 1, if the factor is 1 then the two analytes show identical retention and therefore co-elute[10].

1.6.3 Chromatographic Resolution

A further measure of the separation of two analyte peaks is calculated using chromatographic resolution (*R*). Here the peak width of each analyte is also taken into consideration along with the retention time of each eluting analyte peak. The width of each analyte peak is calculated using the peak width half height method since this removes any issues associated with poor peak shape at the base of the peak (fronting or tailing)[11].

$$R = 1.18 \frac{(t_{R2} - t_{R1})}{W_{h1} + W_{h2}}$$

Equation 3 - Chromatographic resolution (R)

Where t_{R1} = retention time of analyte peak 1

t_{R2} = retention time of analyte peak 2

W_{h1} = peak width at half height of analyte peak 1

W_{h2} = peak width at half height of analyte peak 2

Figure 1 shows that if *R* > 1.5 then the two analyte peaks are said to be baseline resolved and there is a gap between the two peaks, if the value for R is < 1.5 then there will be some overlap of the peaks and they will not be baseline separated.

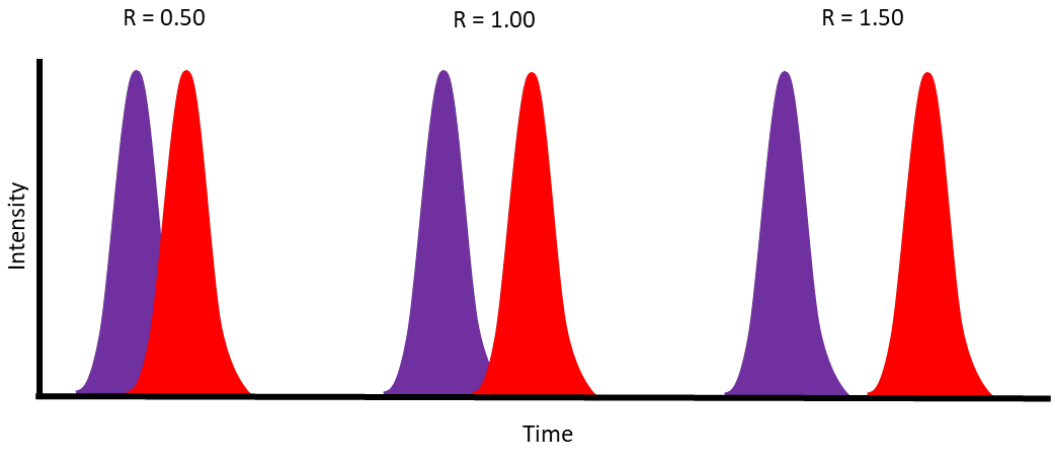

Figure 1 - Graphical representation of resolution *(R)* to illustrate 3 levels of chromatographic peak baseline separation (0.50 = no separation, 1.00 = partial separation and 1.50 = complete separation)

1.6.4 Theoretical Plates

The theoretical plate model assumes that a column is made up of several different layers called theoretical plates. Each theoretical plate is defined as the region in which equilibrium between phases occurs (Figure 2).

Separation is improved with increasing numbers of theoretical plates (*N*) and this number can be calculated using the following equation

$$N = 5.545 \left(\frac{t_R}{W_h}\right)^2$$

Equation 4 - Theoretical plates (*N*)

Where t_R = retention time of analyte

W_h = peak at half height of analyte

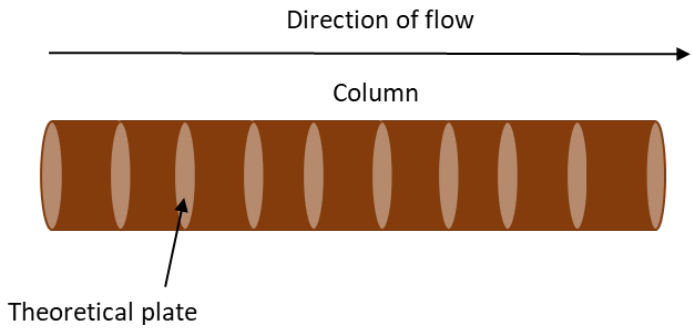

Figure 2 - Diagram to illustrate theoretical plates in a chromatographic column

The N value may be quoted for a whole column or per metre so that comparisons can be made between columns of differing length. If the number of theoretical plates is high, then more equilibrations are possible.

1.6.5 Height Equivalent to a Theoretical Plate

A further parameter that can be used to calculate column efficiency is height equivalent to a theoretical plate. (HETP)

$$HETP\ (H)\ =\ \frac{L}{N}$$

Equation 5 - Height Equivalent to a Theoretical Plate ($HETP$)

Where L = length of column

N = number of theoretical plates

If the HETP value is low, then the efficiency of the column is greater since the number of theoretical plates in a column is higher if the theoretical plate is shorter. Conversely if the HETP value is high then this indicates that there are fewer theoretical plates, and the column efficiency is much lower.

The problem of band broadening is significant in chromatography and is defined as the dispersion or widening of a peak as it passes through a column. van Deemter developed an equation that relates HETP to the linear velocity of the mobile phase (u) for packed columns and this is represented graphically in Figure 3[12].

$$HETP\ =\ A + \frac{B}{u} + \ Cu$$

Equation 6 - van Deemter equation

Where A = eddy diffusion

B = longitudinal diffusion

C = mass transfer

u = linear velocity of mobile phase

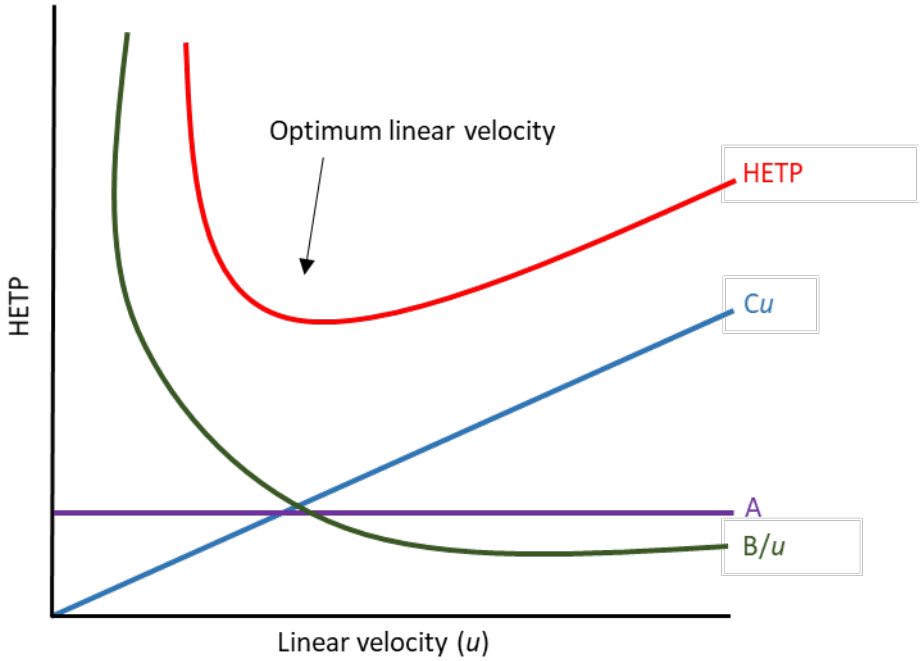

Figure 3 - van Deemter graph to illustrate the three terms (A, B and C) and their relationship to linear velocity (u)

1.6.5.1 Eddy Diffusion

Eddy diffusion (A) is specifically related to the pathways that the analyte takes as it moves through a packed column. Due to the nature of the packing material these paths can vary and hence band broadening can occur (Figure 4). The smaller and more homogeneous the packing material, the more efficient the pathway and hence band broadening is reduced. When using capillary columns such as in GC, then A term is not applicable and therefore Golay defined an equation where the A term is not included[13].

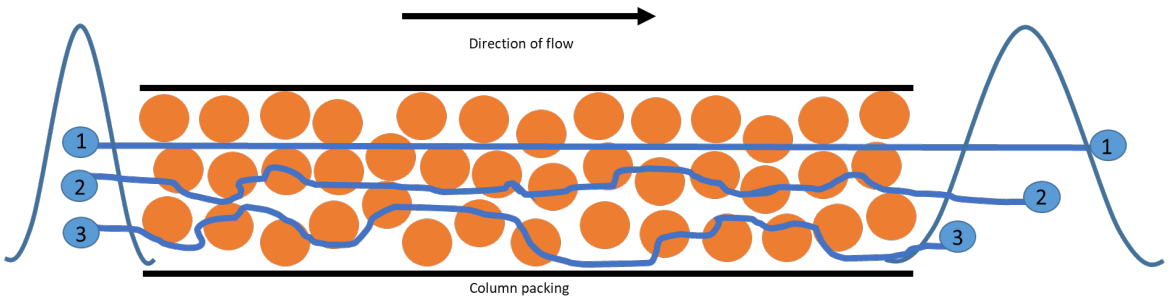

Figure 4 - Diagram to show how the pathways of an analyte differ as they travel through a packed column due to eddy diffusion

1.6.5.2 Longitudinal Diffusion

Longitudinal diffusion (B) is a term used to describe the diffusion of the analyte as it travels through the column in the mobile phase. On injection the tight band of analyte will be affected by diffusion as it travels through the column in the direction of flow, and this can cause band broadening. At the centre of the peak the concentration of the analyte is at is maximum, but towards the edges of the peak the concentration is reduced (Figure 5). The effect is more pronounced when the mobile phase flow rate is low and is less significant when flow rates are higher, and the internal diameter of the column is smaller. To minimise longitudinal diffusion, it is recommended that tubing and fittings are kept to a minimum and zero dead volume fittings are fitted correctly.

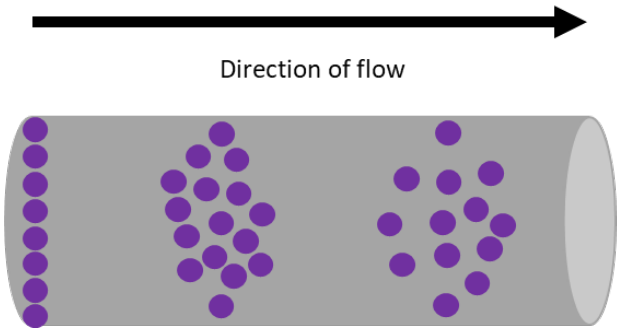

Figure 5 - Diagram to show band broadening of an analyte due to longitudinal diffusion

1.6.5.3 Mass Transfer

Mass Transfer (C) is a term used to describe the transfer of the analytes between the stationary phase and the mobile phase and is a way of determining the effect of the porous stationary phase. The porous column packing material provides a higher surface area over which interactions between the analyte and the stationary phase can take place. Within the pores, the mobile phase is stagnant and as the analyte moves through this stagnant mobile phase, some will diffuse into the pores only slightly whilst others will go much deeper (Figure 6). It is these differences that can cause band broadening and in order reduce this effect, lower linear velocities, smaller packing materials and increased column temperatures are preferred.

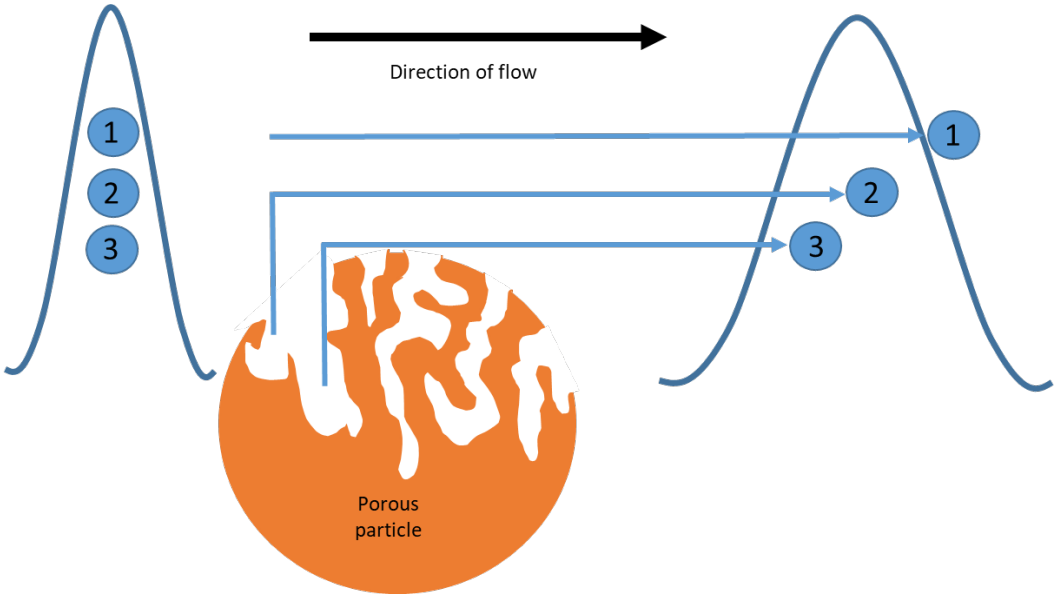

Figure 6 - Diagram to show the effect of porous stationary phase particles on the mass transfer of analyte

Originally sub 2 µm particle size column technology was widely employed with UHPLC but rarely used in UHPSFC. In one study, van Deemter curves were constructed for columns packed with sub 2 µm, 3.5 µm and 5 µm particle size stationary phases and comparisons were made between UHPLC and UHPSFC performance[14] (Figure 7).

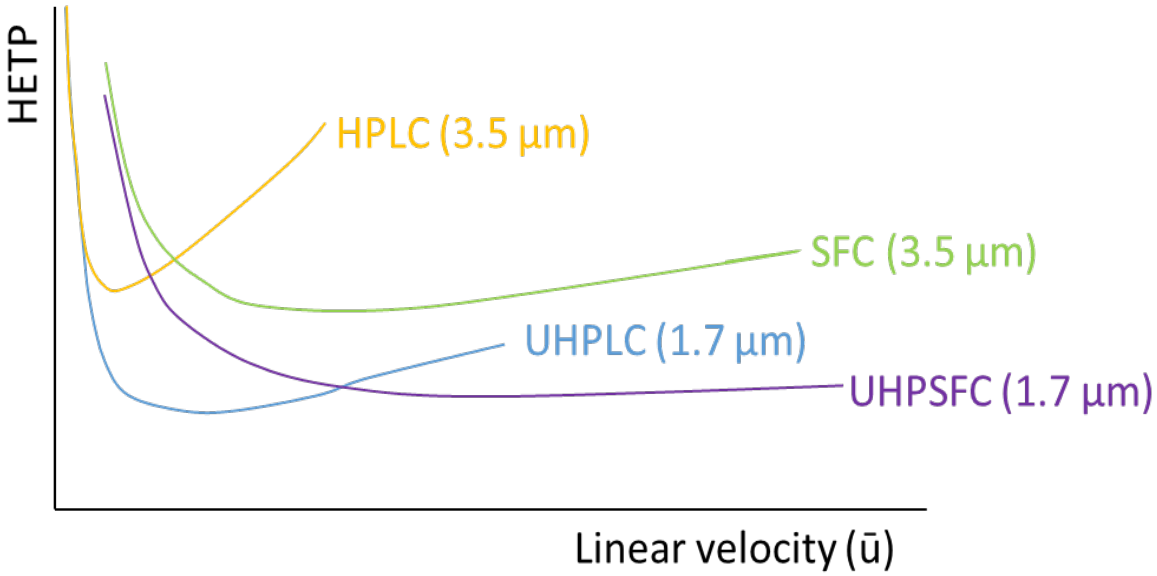

Figure 7 - van Deemter curve showing the comparison of HPLC, UHPLC, SFC and UHPLC with different column particle sizes adapted from[14]

Here it was reported that significant improvements in column efficiency were observed with the reduction in the particle size for UHPLC (from 3.5 µm to 1.7 µm) and the use of supercritical

conditions. The authors proposed that this was because of the reduction of the mobile phase viscosity and the improvement of the diffusion coefficient compared to liquid conditions[14]. However, they also noted that one of the major limitations of the Waters UPC^2 instrumentation was associated with the upper pressure limit of the system. The Southampton systems are currently set at an upper limit of ~400 bar although instrument improvements by Waters have increased this to ~ 600 bar for newer systems.

1.7 Modes of Chromatography

1.7.1 Normal Phase Chromatography

Normal phase (NP) chromatography is the name for the oldest from of chromatography and was developed before reversed phase chromatography. In NP chromatography, the stationary phase is more polar than the non-polar mobile phase (*e.g.,* toluene, ethyl acetate, hexane). The retention mechanism in NP chromatography is that of adsorption. Here the analyte adsorbs onto the stationary phase and the more polar the analyte, the longer the retention (the opposite of RP). NP chromatography is often used as an alternative to RP chromatography when analytes do not retain or over retain. It is also used when the analytes are not water soluble or are insoluble in other typical polar RP solvents (*e.g.*, methanol, acetonitrile). It is commonly used for isocratic elution and for large scale preparative chromatography. As shown in Figure 8, a bare silica support is the most common stationary phase in NP chromatography.

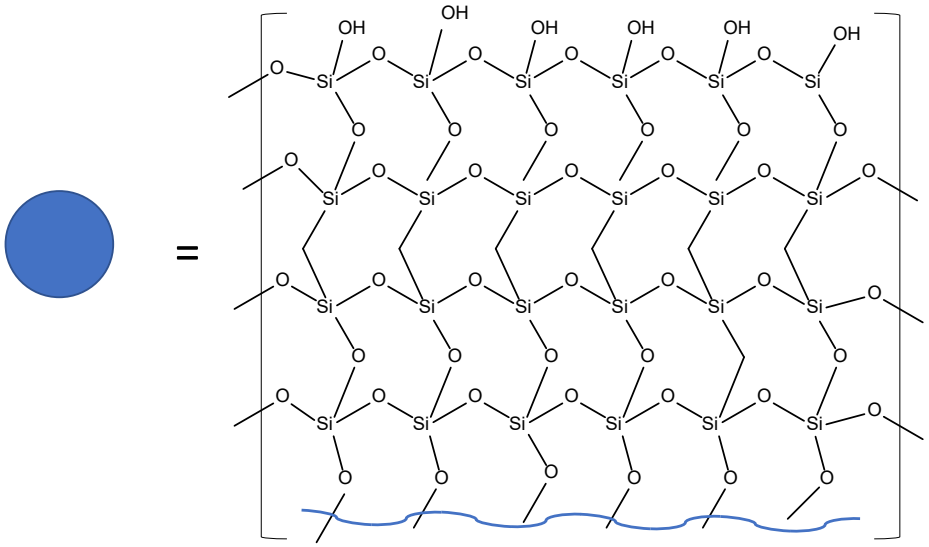

Figure 8 - Representation of bare silica column phase

Bonded phases such as amino, cyano or diol have recently been used in NP chromatography, particularly in analytical applications as they offer a greater level of selectivity with a wide range of polarities (Figure 9).

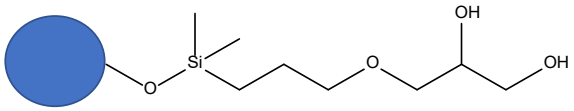

Figure 9 - Representation of bonded diol column phase

Since NP uses non-polar solvents, it is not ideally coupled to MS since the opportunities for atmospheric pressure ionisation are limited due to the lack of protic media.

Chiral chromatography is one of the most efficient and effective ways to separate enantiomers *e.g.,* chiral drugs[15]. In chiral chromatography the stationary phases are often comprised of chiral crown ethers[16], cyclodextrins[17], and more recently polysaccharides, *e.g*., cellulose and amylose[18]. Both NP and RP solvents can be used for chiral separations although NP chromatography is more commonly used for this application.

1.7.2 Reversed Phase Chromatography

RP chromatography is one of the most common forms of liquid chromatography. It uses polar mobile phases (*e.g*., methanol, acetonitrile, water) and is easily coupled to MS *via* an atmospheric pressure ionisation interface. In RP chromatography, the stationary phase is non-polar or less polar than the mobile phase. The term reversed phase is used as this technique was developed after NP chromatography. Typically, in RP chromatography, a silica particle is used as the solid support for a hydrophobic stationary coating. Different stationary phases will have varying degrees of hydrophobicity and those with higher hydrophobicity (*e.g.,* C_{18}) will increase the retention of non-polar analytes (Figure 10). Typical RP gradient elution methods are used to improve compound selectivity and peak shape. They comprise of two or more mobile phases that begin the gradient with low (or zero) organic and high water content at the start, increasing to a high organic and low (or zero) water content over time. Increasing the percentage of the organic mobile phase will alter the retention of analytes. Whilst adsorption was considered to be the main process used in chromatographic separation, Martin and Synge were the first authors to introduce partition chromatography in 1941. This was a new mechanism describing the chromatographic process that occurred with the use of a liquid stationary phase and a liquid mobile phase. Here, they used the mechanism to separate components in an acetylamino acid mixture using water as the stationary phase (impregnated into silica gel) and chloroform as the mobile phase. They packed the silica gel into a column and then poured in the chloroform from

the top of the column[19]. They were awarded the Nobel Prize in Chemistry in 1952 for their work resulting in the invention of partition chromatography[20].

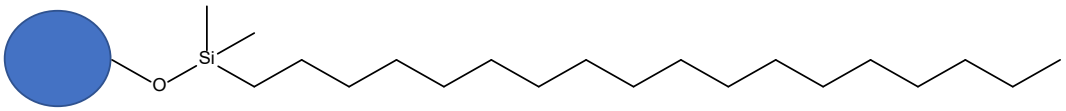

Figure 10 - Representation of C_{18} bonded phase

The hydrophobicity of an analyte is one of the main determining factors in its retention in RP chromatography. This is expressed in terms of log *P* which is the measure of the way an analyte partitions between two solvents (octanol and water). The higher the log *P* value (-1 to +1) then the more hydrophobic the analyte and the more likely it is to be retained.

$$Log\ P\ oct/wat\ =\ log\left(\frac{[solute]un-ionised\ octanol}{[solute]un-ionised\ water}\right)$$

Equation 7 - Logarithm of the partition coefficient P

Additives and buffers (*e.g.,* formic acid, ammonium acetate) or ion-pairing reagents may be added to the mobile phases to further influence the retention of analytes. Ion pairing reagents such as alklysulfonate, alkylsulfates or alkylammonium salts are sometimes used to improve the separation and alter the retention of ionic analytes in RP chromatography. The ion-pairing reagents maybe anionic or cationic, so that if one of the analytes contains the opposite charge to the ion-pair reagent, then its retention may be increased or conversely if an analyte has the same charge, then its retention may be reduced (Figure 11).

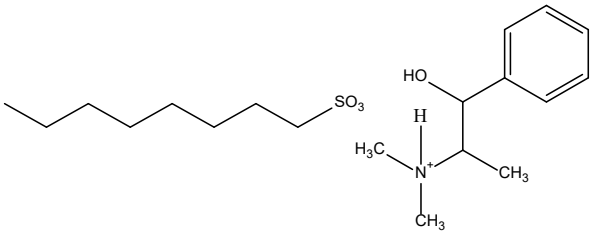

Figure 11 - Example of an ion pair complex (octanesulfonic acid and phenylpropanolamine)

In some cases, the use of an ion-pairing reagent is not necessary if the column stationary phase is charged. For example, the use of Waters charged surface hybrid (CSH) particles that incorporate a

low surface charge on the particle may be particularly effective for the separation of basic compounds when low pH, weak ionic strength mobile phases are used[21].

1.7.3 Hydrophilic Interaction Liquid Chromatography

Hydrophilic interaction liquid chromatography (HILIC) has been reported as an alternative to NP chromatography, particularly for polar compounds and the term was first used by Alpert in 1990[22]. HILIC employs polar stationary phases such as silica, amino or cyano but unlike NP, the mobile phases are also polar and hence HILIC is sometimes referred to as RP-RP chromatography. It can be used for the separation of polar compounds that may be poorly retained in RP chromatography and compounds that show good solubility in aqueous mobile phases. In gradient elution the initial mobile phases are low polarity, gradually increasing the percentage of polar aqueous content to elute the polar compounds[23]. The analytes will partition in order of increasing hydrophilicity between the aqueous enriched layer formed on the surface of the stationary phase and the organic mobile phase (acetonitrile for the majority of HILIC applications) (Figure 12). It is particularly useful for bioanalytical applications where drugs and metabolites may be of a polar structure. HILIC is ideally coupled to ESI sources due to the high levels of acetonitrile present. The higher organic content leads to more stable spray formation, quicker solvent evaporation and hence more efficient production of gas phase ions.

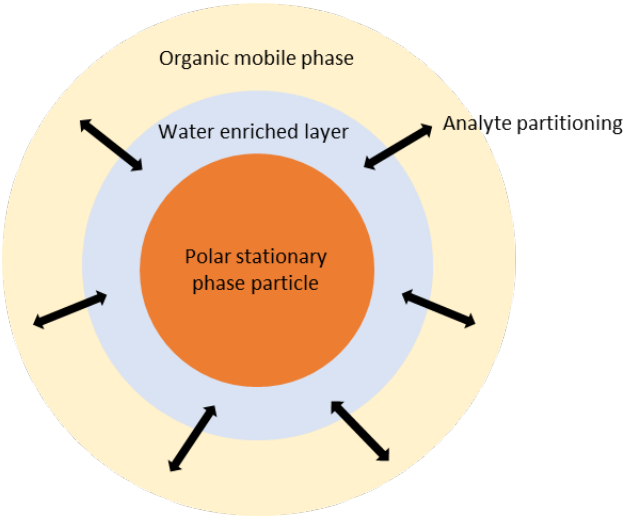

Figure 12 - Diagram of HILIC chromatography to show the analyte partitioning between the aqueous enriched layer formed on the stationary phase and the organic mobile phase

1.7.4 Supercritical Fluid Chromatography

Supercritical fluid chromatography (SFC) is known as a form of liquid chromatography, although here the mobile phase used is a supercritical fluid. A supercritical fluid is a substance that exists at

a pressure and temperature above its critical point (Figure 13). It possesses the properties of both a liquid and a gas and is considered as an intermediate between the two. It has the diffusivity and viscosity properties of a gas, (so that it can be used as a mobile phase at high flow rates) the density of a liquid and has a high solvating power (Table 1).

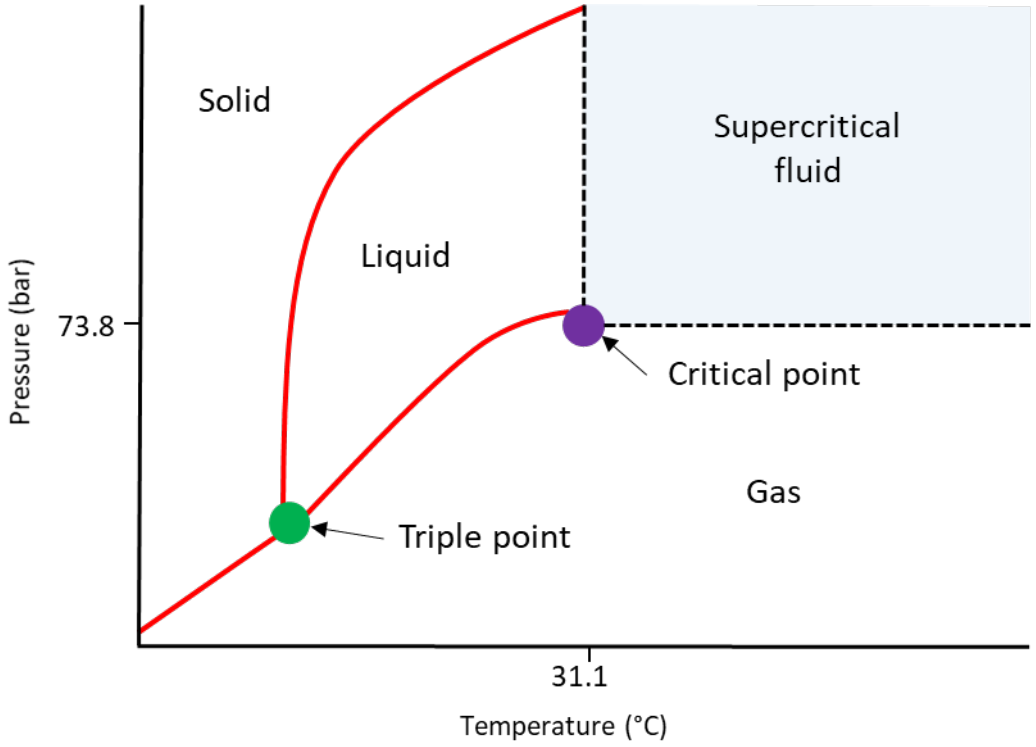

Figure 13 - Phase diagram for carbon dioxide

Supercritical carbon dioxide ($scCO_2$) is the most commonly used supercritical mobile phase in SFC since its critical points of 31.1 °C and 73.8 bar are more easily achieved than other supercritical fluids such as dichlorodifluoromethane and monochlorodifluoromethane[24,25]. Most of the CO_2 produced is either naturally occurring or derived from the effluent of ammonia plants[26] It is also a by-product from the fermentation of sugars in the beer and wine making processes[27]. Hence $scCO_2$ is readily available, inexpensive, and relatively safe to use[28-30].

	Density (g cm^3)	Diffusion (cm^2 s^{-1})	Viscosity ($g.cm^{-1}$ s^{-1})
Gas	10^{-3}	10^{-1}	10^{-4}
Supercritical fluid	10^{-1}-1	10^{-4}-10^{-3}	10^{-3} -10^{-4}
Liquid	1	<10^{-5}	10^{-2}

Table 1 - Properties of supercritical carbon dioxide

SFC was initially developed as “high pressure chromatography above critical temperatures” by Klesper *et al.* in 1963 using chlorofluoroalkanes as the mobile phase with a novel instrument designed and was constructed to separate a mixture of metal porphyrins[31]. Further approaches using $scCO_2$ were developed by Sie and Rijinders[32-36] and they were the first to use the term supercritical fluid chromatography. In 1968, Giddings introduced the term, unified chromatography, stating that one of the most interesting features of ultra-high-pressure gas chromatography would be its convergence with classical liquid chromatography[37].

In the early stages of SFC instrument development, capillary columns were used, and the instrumentation lacked robustness, often leading to excessive instrument down time and hence the technique was considered as niche since it was only used in a few laboratories with specialised personnel. Although it did not develop at the same rate as RP HPLC methods and instrumentation, SFC did continue to develop and became the optimum technique for chiral separations, mainly in the pharmaceutical industry, using modern packed columns[38].

In 2008 a world-wide shortage of acetonitrile led to the need for alternative and greener forms of chromatography, and this coincided with significant SFC technology advancements. Several manufacturers including Agilent, Berger (Mettler Toledo, Thar, Waters) and Shimadzu used existing UHPLC technology to develop equivalent UHPSFC instrumentation *e.g.,* the Agilent Aurora and 1260 Infinity series, the Waters UPC^2 and the Shimadzu Nexera[39,40].

One of the main challenges for SFC instrumentation is the need to regulate the pressure of the $scCO_2$ and this is accomplished using a back-pressure regulator. This back-pressure regulator is necessary to prevent the $scCO_2$ from expanding into the gas state. Back-pressure regulation may be achieved using either a static or active back pressure regulator. Static pressure utilises tubing or a capillary to apply a pressure to the supercritical fluid so that it maintains its supercritical status. The automated back pressure regulator (ABPR) used by the Waters UPC^2 offers a much finer control of the pressure and can be controlled using the instrument software. It is achieved through a combination of static and active back pressure control. The static BPR keep the pressure at a minimum level whilst the ABPR is used to enhance the control of the set-point defined by the user. Here at the University of Southampton, the School of Chemistry houses two Waters UPC^2 instruments coupled to Waters™ mass spectrometers and hence all work described further will be in reference to the configuration of this instrumentation (Figure 14).

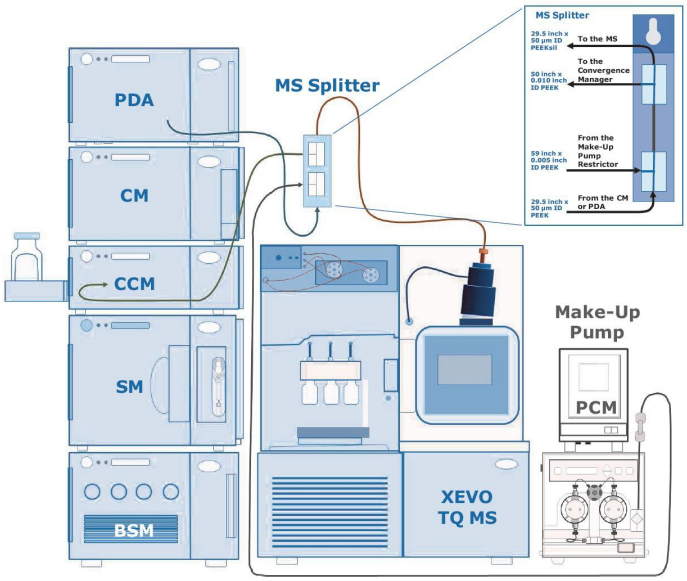

Figure 14 - Configuration of early Waters UPC^2 courtesy of Waters (where PDA is photo diode array detector, CM is column manager, CCM is convergence manager, SM is sample manager, BSM is binary solvent manager, XEVO TQ MS is mass spectrometer)

In many modern UHPSFC applications, 100% $scCO_2$ is rarely used in assays due to its weak polarity and polar compounds do not dissolve well in it. Polar organic co-solvents (also known as modifiers) are added to extend the breadth of application areas accessible by UHPSFC by modifying the polarity of the mobile phase, hence increasing its elution strength, and increasing the solubility for polar compounds. Methanol is the most used co-solvent and performance of the co-solvent can be further enhanced using additives, *e.g.,* ammonium acetate, ammonium formate, formic acid. UHPSFC can easily be coupled to a mass spectrometer using an API source.

A solvent splitter configuration is used in the Waters UPC^2 system and consists of a multiple connection splitter to allow for the introduction of a make-up solvent (Figure 15). The make-up solvent, such as methanol with formic acid, is now delivered from an integrated isocratic solvent manager (ISM) to the mass spectrometer *via* the first stage of the solvent splitter configuration. This is essential to enhance and stabilise the spray and aid ionisation of the analytes. The make-up solvent is combined with the column eluent flow (*via* an ultraviolet (UV) detector) and then flows to the second part of the splitter where the flow is split so that some flow is directed to the ABPR to control the density of the mobile phase, and some is directed to the MS (the exact split ratio here is unknown). The introduction of a make-up solvent is particularly crucial if separation is only achieved with low levels of co-solvent *e.g.,* below 5% and the composition can also selectively aid the formation of ion types in the API source[30].

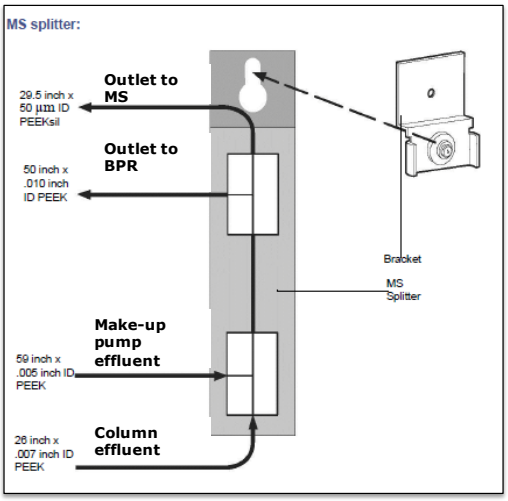

Figure 15 - Configuration of Waters UPC^2 splitter courtesy of Waters

1.8 Introduction to Mass Spectrometry

Mass spectrometry (MS) is a technique that measures the mass-to-charge ratio (*m/z*) of ions in the gas phase. In the 21st Century, mass spectrometry instrumentation has now become common place in many analytical laboratories due to significant technological and manufacturing innovations. This has also made the instrumentation more affordable and more easily coupled to chromatographic systems.

J. J. Thomson, who was awarded the Nobel prize for Chemistry in 1906, is considered as the pioneer of mass spectrometry following his discovery of the electron in 1897 [41,42] and the determination of its *m/z* ratio. He developed the earlier work of E. Goldstein who discovered canal rays in a discharge tube and demonstrated that these were positively charged particles known as protons[43]. F. W. Aston worked with Thomson and was also awarded the Nobel prize in Chemistry (1922) for his development of the mass spectrograph with which he separated the two isotopes of Neon (^{20}Ne and ^{22}Ne). A. J. Dempster was also one of the early pioneers of mass spectrometry and he developed the technique to discover the Uranium isotope ^{235}U[44].

A typical mass spectrometer consists of 5 major components: an inlet, an ion source, a mass analyser, a detector, and a data system. A vacuum is also present, and the generic representation of mass spectrometer is shown in Figure 16. There are many variations of each component part depending on the performance and manufacturer, although many do not differ greatly between instruments. The ion source and mass analyser are the two components that can differ the most as they play a crucial role in the analysis of different types of compounds. When coupling MS to UHPLC and UHPSFC systems, these chromatographic components become the inlet system.

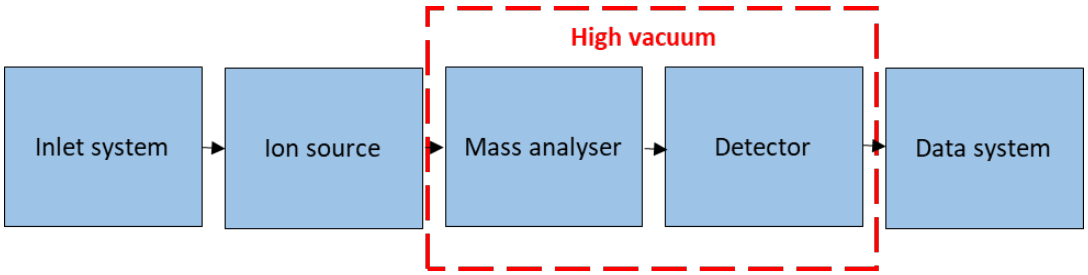

Figure 16 - Representation of a generic mass spectrometer

To be analysed by mass spectrometry, it is essential that a compound exists as gas-phase ions. There are many ways in which to ionise solids, liquids, and gases, and these can be grouped into two main types of ionisation technique. There are those that take place under vacuum (*e.g.,* electron ionisation (EI), chemical ionisation (CI)) and those that take place at atmospheric pressure (*e.g.,* electrospray ionisation (ESI), atmospheric pressure chemical ionisation (APCI), atmospheric pressure photoionisation (APPI) atmospheric pressure matrix-assisted laser desorption/ionisation time of flight (AP MALDI-TOF). The ionisation techniques used in this report are all undertaken at atmospheric pressure and hence will be described here.

1.9 Electrospray Ionisation

ESI was initially demonstrated by Dole and co-workers[45] who developed the production of gas-phase ions through an electrically charged capillary. However, it was the work of Fenn that became more widely acknowledged as he used the mass spectrometer to detect ions generated by positive and negative ion electrospray ionisation[46,47]. He initially showed that large molecules such as proteins produced multiply charged ions and the molecular weight of the protein could be determined with instruments that had an upper mass range as low as *m/*z 2000. His work was recognised by the award of the Nobel Prize for Chemistry in 2002 and shared this with Tanaka for his work on the analysis of large molecules using MALDI-TOF MS[48]. ESI is now believed to be the most common ionisation techniques for both small and large molecule analysis and it used in many diverse application areas.

During the ESI process, the analyte solution is passed through a capillary (flow rates of 2-20 uL/min may be induced *via* a syringe driver). A high voltage potential difference is created between the end of the metal capillary and a counter electrode (+2 to +5 kV for positive ionisation and -2 to -5 kV for negative ionisation). One alternative is to ground the capillary (0 kV) and apply a negative voltage to the counter electrode thus producing positive ions (applying the opposite polarity voltage to the counter electrode for negative ionisation). At the end of the capillary, charged droplets are produced through electrochemical processes. In positive ion ESI, this means

that positive ions move to the centre of the droplet (as they are repelled from the capillary walls), whilst negative ions are attracted to the capillary walls. The positive ions dominate the sprayed droplet and are hence attracted to the counter electrode. As the high voltage potential is increased, the droplets extend from the end of the capillary further and form a cone shaped meniscus known as the Taylor cone[49] (Figure 17).

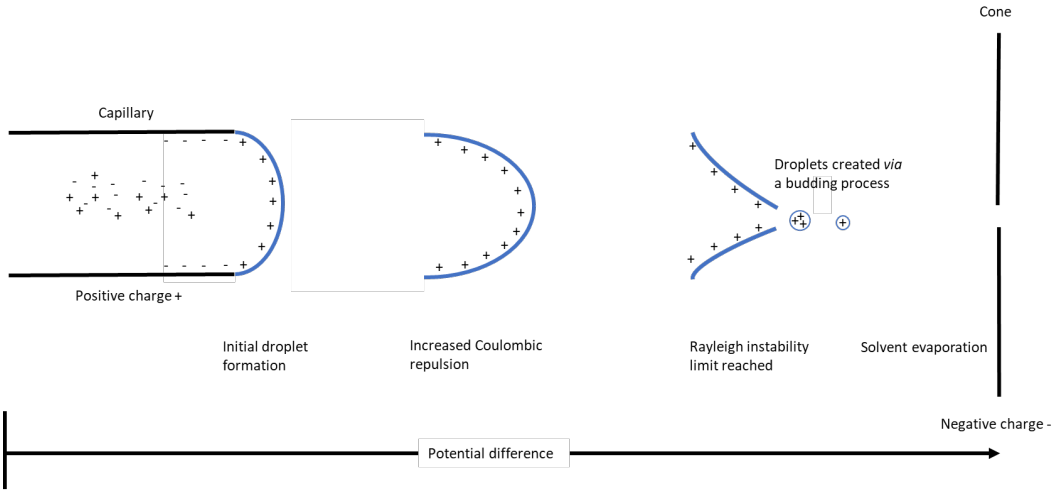

Figure 17 - Diagram to show the formation of the Taylor cone in ESI

As the solvent evaporates the charge density on the surface of the Taylor cone increases until the Rayleigh limit is reached. This is when the forces of Coulombic repulsion between like charges and the surface tension of the solution are equal[50]. When the Raleigh limit is exceeded and the surface tension of the droplet can no longer sustain the Coulombic forces, then Coulombic repulsions occur and the process of budding takes place. Smaller charged droplets are now produced and eventually after further desolvation events, ions are produced and transferred into the gas phase.

1.9.1 Nano-Electrospray Ionisation

Nano-electrospray ionisation (Nano-ESI) utilises very small amounts of sample and operates at very low flow rates. In nano-ESI, very small glass capillaries are used as the spray capillaries, these are made using either a mechanical or laser device to pull out one end of the glass capillary to leave a small hole of approximately 1 to 10 µm. The capillaries are coated with a conductive material and a small amount of the sample (1 to 5 µL) is loaded from the rear of the capillary. Neither a pump nor syringe driver is required to produce a flow as this is created due to the electrical fields applied (normally 500 to 1000 V). The needle can be placed very close to the orifice of the MS instrument and signal may be enhanced by a factor of 2 or 3[51].

1.9.2 Pneumatically-Assisted Electrospray Ionisation

Most modern instruments use pneumatically-assisted ESI, often referred to as ESI[52] where nitrogen is used as a nebuliser gas. This aids the desolvation process, reduces the influence of surface tension, making the charging of droplets more efficient, as well as directing the spray towards the mass analyser in a more parallel fashion (rather than the Taylor cone). This enables higher eluent flow rates to be used (0.1 - 1 mL/min) making it suitable for interfacing to HPLC/UHPLC or UHPSFC (Figure 18).

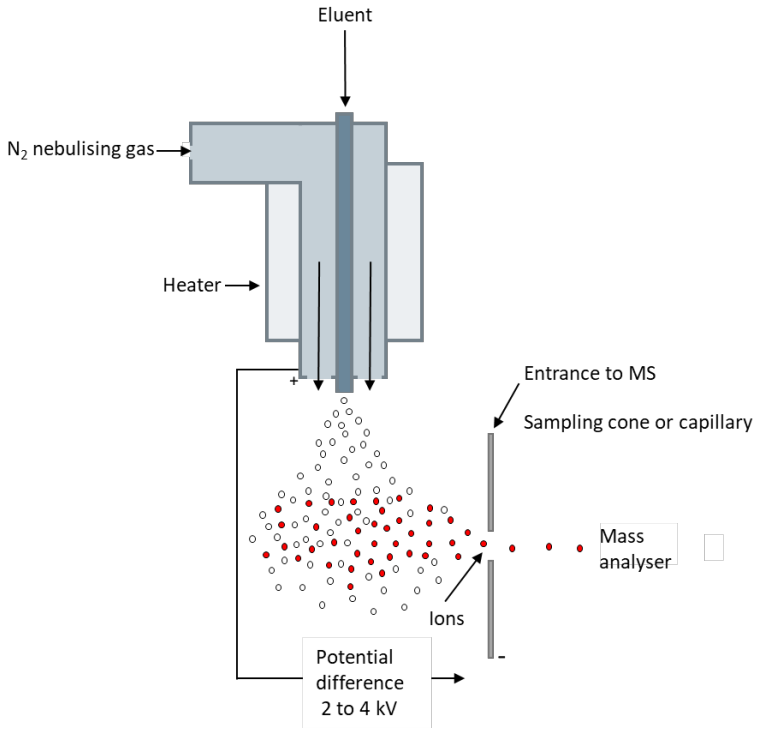

Figure 18 - Diagram of a pneumatically-assisted ESI source

One of the advantages of ESI is that it is known as a “soft” ionisation technique because there is little fragmentation of ions unlike the “hard” ionisation techniques such as EI where the energy transfer is so high that many fragment ions are observed, and the molecular ion is sometimes absent in the mass spectrum. Ions produced by ESI may either be formed by protonation or deprotonation depending on the structure of the compound and the ionisation technique used.

$$M + H^+ \Rightarrow [M + H]^+$$

Equation 8 - Protonation in positive ion ESI

Adducts ions are also commonly formed in ESI, particularly those formed using alkali metals such as ammonium, sodium, and potassium.

$$M + Na^+ \Rightarrow [M + Na]^+$$

Equation 9 - Cationisation in positive ion ESI

Since ESI measures the *m/z* of an ion, multiply charged species may also be produced (depending on the molecular weight of the compound) using protonation or cationisation in positive ion ESI or deprotonation in negative ion ESI.

$$[M + nX]^{n+}$$

Equation 10 - Multiple charging in positive ion ESI

Where M = analyte

X = charge

n = number of charged species

There are two primary mechanisms that have been proposed for the desolvation process and production of gas phase ions in ESI. The first is known as the charged residue model (CRM) and was proposed by Dole and co-workers[45]. Dole proposed that following the Coulombic explosions, droplets would be formed that would eventually contain just one single ion and these would then be transferred into the gas phase (Figure 19).

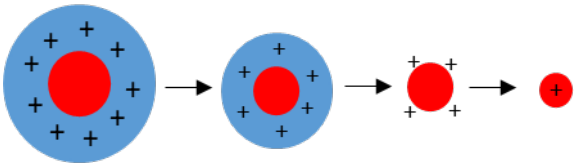

Figure 19 - Schematic representation of the charged residue model (CRM)

Iribarne and Thompson proposed an alternative model known as the ion evaporation model (IEM). Here the electric field at the surface of the droplets is sufficient to field desorb ions directly from the surface. The model proposes that when the droplets reach 10 nm, the electrostatic force is high enough to overcome the solvation forces[53] (Figure 20).

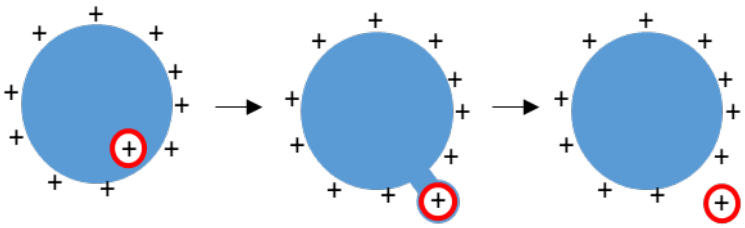

Figure 20 - Schematic representation of the ion evaporation model (IEM)

Although there is some uncertainty over which mechanism is preferred for which classes of compound, there is some evidence to suggest that the larger multiply charged ions are more likely to form using the CRM model and smaller singly charged ions are more likely to be formed using the IEM model [54,55]. It is probable that most ions are formed due to a combination of both these processes.

A third mechanism has more recently been proposed specifically for disordered polymers by Konermann in 2013. The chain ejection model (CEM) proposes that when unfolded proteins are ionised, they are driven to the droplet surface by hydrophobic and electrostatic factors, followed by gradual ejection *via* intermediates where droplets carry extended protein tails [56,57] (Figure 21).

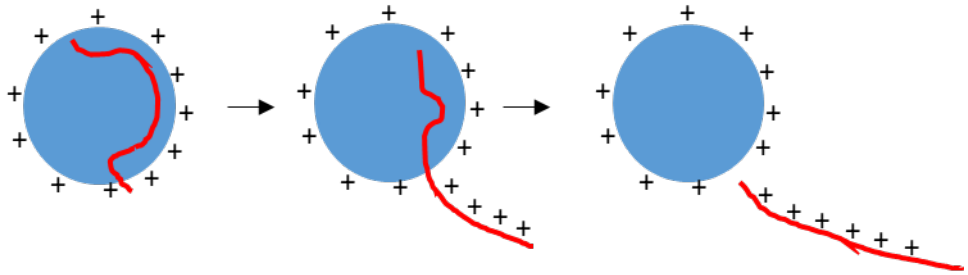

Figure 21 - Schematic representation of the chain ejection model (CEM)

ESI is a concentration dependent technique since the analyte signal responds to the relative amount (concentration) of the analyte within a certain volume or flow rate. Typical flow rates used in pneumatically-assisted ESI are in the region of 0.1 to 1 mL/min. If this flow rate is increased, the efficiency of the ionisation is lowered due to the limitations of droplet formation and the insufficient charge on the droplets leading to a reduced number of ions transferred to the gas phase[58]. This means that the ion signal is saturated and no matter how much the flow rate or concentration is increased the ion response remains level (Figure 22). If the eluent contains a high proportion of water (as is common with RP LC-MS) then solvent evaporation is also reduced.

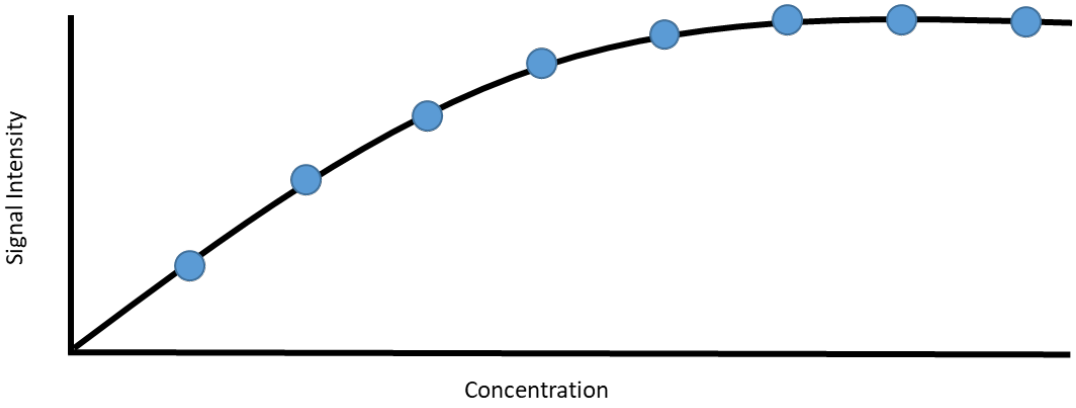

Figure 22 - Graphical representation of ESI signal saturation

1.10 Atmospheric Pressure Chemical Ionisation

APCI is another API technique and is used as an alternative technique to ESI for analytes that do not have basic or acid groups. The technique uses gas-phase ion-molecule reactions and is analogous to CI. It was first developed by Horning[59] in the early 1970s and in contrast to ESI the voltage is not applied to the capillary tip but to a corona discharge pin (Figure 23).

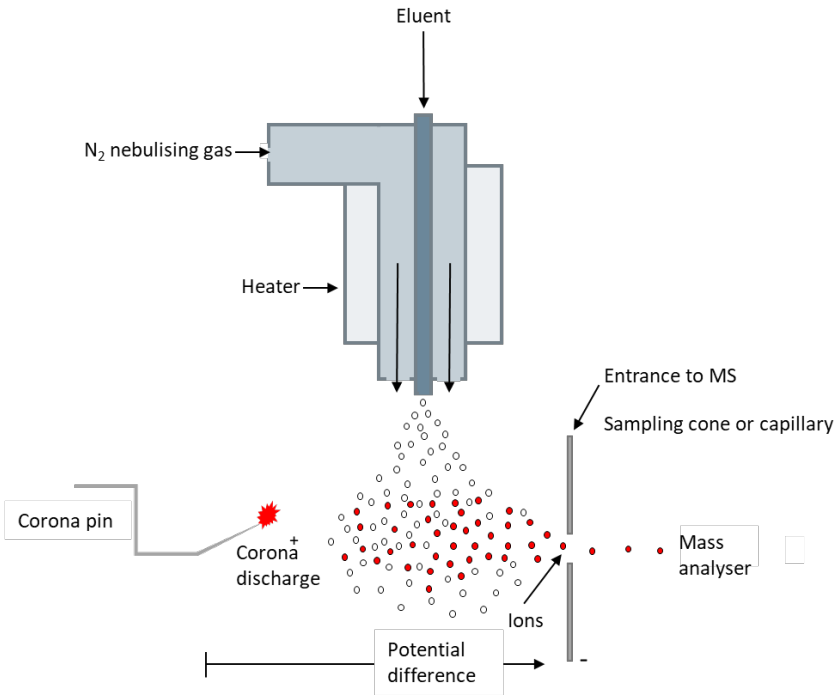

Figure 23 - Diagram of a pneumatically-assisted APCI source

High energy electrons from the corona discharge cause a cascade of ion/molecule reactions that can ultimately generate ions related to the analyte (Figure 24). Proton transfer or adduction of

reactant gas ions produces positive ions and proton abstraction or adduct formation produces negative ions[41].

$$N_2 + e^- \rightarrow N_2^{+\cdot} + 2e^-$$

$$N_2^{+\cdot} + 2N_2 \rightarrow N_4^{+\cdot} + N_2$$

$$N_4^{+\cdot} + H_2O \rightarrow H_2O^{+\cdot} + 2N_2$$

$$H_2O^{+\cdot} + H_2O \rightarrow H_3O^+ + OH^{\cdot}$$

$$H_3O^+ + H_2O + N_2 \rightarrow H^+(H_2O)_2 + N_2$$

$$H^+(H_2O)_{n-1} + H_2O + N_2 \rightarrow H^+(H_2O)_n + N_2$$

Figure 24 - Production of cluster ions in APCI due to the ionisation of N_2 molecules

APCI is a mass dependent technique since the analyte signal responds to the total amount of the analyte regardless of concentration or flow rate[58]. APCI accommodates much higher flow rates than ESI and hence if the concentration is increased, the analyte signal intensity increases proportionally (Figure 25). APCI is therefore ideally suited for the analysis of low volume samples at high concentration.

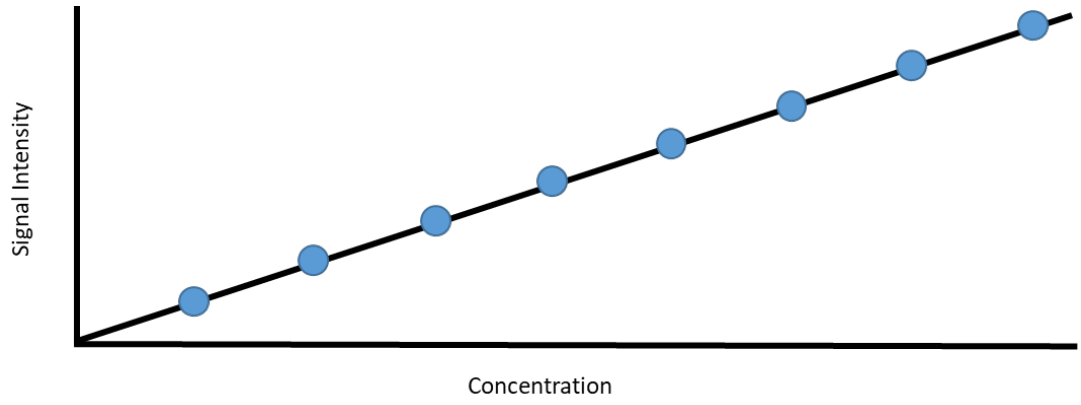

Figure 25 - Graphical representation of mass dependence in APCI

1.11 Atmospheric Pressure Photoionisation

Atmospheric pressure photo ionisation (APPI) is another mass dependent ionisation technique where gas phase ion/molecule reactions trigger ion formation in a similar way to APCI. It can also be used in conjunction with UHPLC and UHFSC and utilises the heated nebuliser used in the APCI source. Instead of using a corona discharge pin, a photoionisation lamp is used to emit photons (Figure 26).

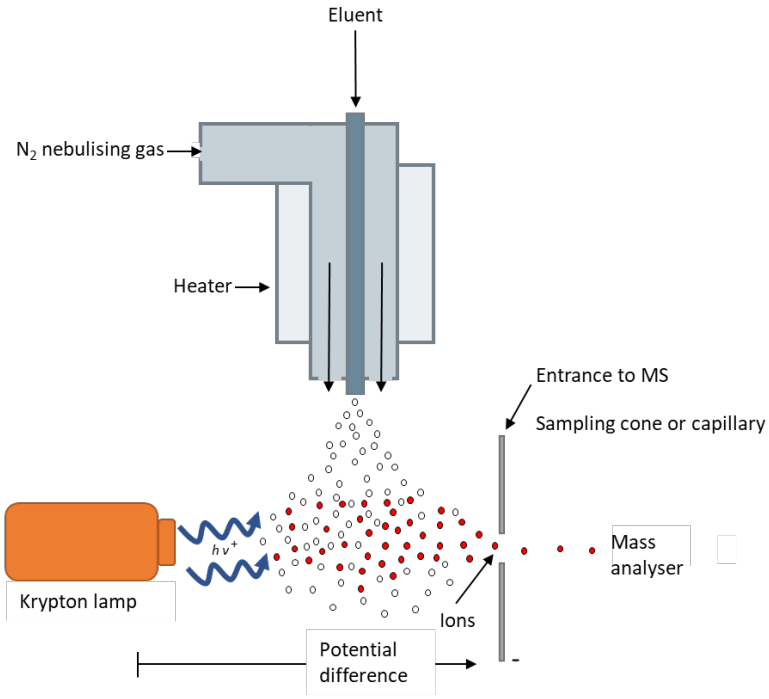

Figure 26 - Diagram of a pneumatically-assisted APPI source

It was first demonstrated by Robb and co-workers in 2000[60] before becoming commercially available in 2005 [61,62]. It is a particularly useful addition to ESI and APCI since it can ionise non-polar compounds. The most commonly used vacuum ultraviolet (VUV) lamp is the krypton lamp that emits photons at 10.6 eV energy. This is lower than the ionisation energy (E_i) of the solvents and gases used but higher than the E_i of the analyte. There are two mechanisms for ionisation in APPI

1.11.1 Direct Ionisation

If the E_i of the analyte is below 10 eV, then during the ionisation process, photons may be absorbed by the analyte molecules leading to electron ejection and formation of a radical cation.

$$M + h\nu \Rightarrow M^{+\cdot} + e^-$$

Equation 11 - Direct photoionisation of analyte (M)

Where $h\nu$ = energy of the photon

M = analyte

However, this is often characterised by low efficiency as the analytes are often at low concentration compared to the matrix or background ions that can also absorb photons. This often leads to fewer photons being available for the direct ionisation of the analyte. It is also common to observe protonated molecules *via* direct photoionisation due to the ionisation of solvent molecules followed by reactions between the radical cation and the solvent.

$$S + h\nu \Rightarrow S^{+\cdot} + e^{-}$$

Equation 12 - Direct photoionisation of solvent molecules (S)

Where $h\nu$ = energy of the photon

S = solvent

In APPI, further reactons may occur between the analyte radical cation ($M^{+\cdot}$) and the solvent molecules (S) to produce protonated molecules

$$M^{+\cdot} + S \Rightarrow [M + H]^{+} + [S - H]^{\cdot}$$

Equation 13 - Production of protonated molecule in APPI

1.11.2 Dopant-Assisted Ionisation

To enhance the APPI process a solvent dopant may be used in the process known as dopant-assisted APPI. The optimum dopants are those than have E_i values below 10 eV with the most common being toluene that has an E_i of 8.83 eV or acetone that has an E_i of 9.70 eV. The dopant will be easily photoionised to produce a radical cation *via* direct photoionisation.

$$D + h\nu \Rightarrow D^{+\cdot} + e^{-}$$

Equation 14 - Dopant-assisted ionisation in APPI (radical cation)

Where $h\nu$ = energy of the photon

D = dopant

If the analyte has an E_i below that of the dopant, then charge exchange can occur between the dopant and the analyte. (ionisation energy of D > M)

$$D^{+\cdot} + M \Rightarrow M^{+\cdot} + D$$

Equation 15 - Dopant-assisted ionisation in APPI (charge exchange)

Alternatively, the dopant radical cation may ionise a solvent molecule by proton transfer if the proton affinity of the solvent molecule is higher than that of the dopant. (proton affinity of S > D)

$$D^{+\cdot} + S \Rightarrow [S + H]^{+} + [D - H]^{\cdot}$$

Equation 16 - Dopant-assisted ionisation in APPI (proton transfer of dopant to solvent)

Protonated molecules may then be formed *via* proton transfer of the analyte if the proton affinity of the analyte is greater than that of the solvent. (proton affinity of M > S)

$$M + [S + H]^{+} \Rightarrow [M + H]^{+} + S$$

Equation 17 - Dopant-assisted ionisation in APPI (proton transfer of solvent to analyte)

The E_i and the proton affinity of all species present in the APPI ion source atmosphere can influence the ionisation mechanisms. In positive ion APPI, a variety of different ions, including $[M - H]^{+}$, $[M - H_2]^{+\cdot}$, $[M + H]^{+}$ and $M^{+\cdot}$ may be formed *via* reactions depending on the analyte and relative gas phase acidity or basicity of species present in the ionisation source[63].

Certain types of API MS instrument (including the Waters SQD 2 and TQD series) have the ability to acquire positive and negative ionisation data in one acquisition, although the number of data points across a chromatographic peak may diminish. This also occurs if two different ionisation techniques such as ESI and APCI are used at the same time and hence these methods should only be used in scoping exercises to determine the optimum ionisation technique for novel analytes.

1.12 Mass Analysers

The mass analyser is one of the most important parts of the mass spectrometer and separates ions formed in the ionisation source into their *m/z* value. There are many types of analysers commercially available, and each has its own advantages and limitations. The main types of

analysers associated with UHPLC and UHPSFC are low resolution quadrupoles and ion traps, and higher resolution TOF mass analysers and more recently Orbitraps.

1.12.1 Quadrupole Mass Analyser

The quadrupole mass analyser is one of the most used mass analysers in bench-top instruments. It was developed and commercialised by Finnigan (Shoulders and Storey) in the 1960s[64] following the earlier work of Paul and Steinwedel[65]. The quadrupole consists of four parallel rods that are arranged symmetrically to the Z axis (Figure 27).

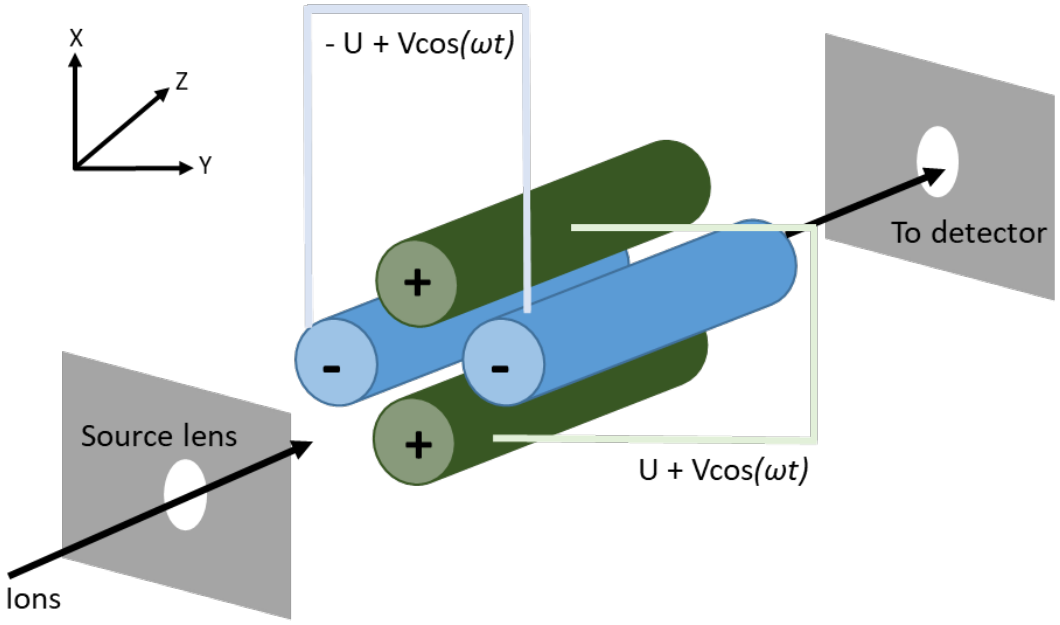

Figure 27 - Diagram of a cylindrical quadrupole mass analyser

Ideally the rods would be a hyperbolic shape, but circular rods are more commonly used, mainly due to lower manufacturing costs[66].Two of the four rods are electrically connected to each other with the same direct current (DC - *U*) and the other two rods are connected to each other with the opposite DC voltage. Superimposed on these DC voltages is also an oscillating radiofrequency (RF) voltage -*Vcos(ωt).* The polarity of the voltages on each pair of rods is alternated rapidly and it is this electric field that is used to separate the ions according to their *m/z* value. If a positive ion travels through the quadrupole, it will be attracted to the negatively charged rods (and repelled by the positively charged rods) and on polarity switching it will then be attracted to the previously positively charged rods (and repelled by the previously negatively charged rods). Rapidly repeating this switching of polarity causes the ion to travel with a spiralling trajectory. Only one ion is stable at any one time as it travels through the quadrupole, and this is governed by the equation

$$\Phi = [U + Vcos(\omega t)]\, x^2 - \frac{y^2}{r_0^2}$$

Equation 18 - Quadrupole equation

Where Φ = quadrupole potential

U = DC voltage

V = RF amplitude

$Vcos(\omega t)$ = RF voltages

x = distance from centre of quadrupole to rod in the x axis (horizontal)

y = distance from centre of quadrupole to rod in the y axis (vertical)

r_0 = radius of the quadrupole

A stable ion trajectory is obtained when the spacing of the rods (x and y) is less than radius of the quadrupole r_0.

To determine the stable trajectory of ions in a quadrupole, Mathieu derived an ion trajectory equation from Newton's second law of motion (F = ma)[67]. He determined that there were two factors critical to the stability of an ion, and these were a and q.

$$a_u = \frac{8zU}{mr_0^2\omega^2}$$

Equation 19 - Mathieu equation (a_u)

$$q_u = \frac{4zV}{mr_0^2\omega^2}$$

Equation 20 - Mathieu equation (q_u)

Where z = charge on an ion

m = mass of ion

ω= RF frequency

The two equations are often combined to give the following equation

$$\frac{a_u}{qu} = \frac{2U}{V}$$

Equation 21 - Combined Mathieu equation

When plotting a against q, Mathieu discovered that there were four regions where ions were stable in a quadrupole with varying RF and DC voltages. These regions were observed in the Mathieu stability diagrams (A-D) and the region labelled A represents the normal operating region for quadrupole analysers (termed the first stability or operating region) (Figures 28 and 29).

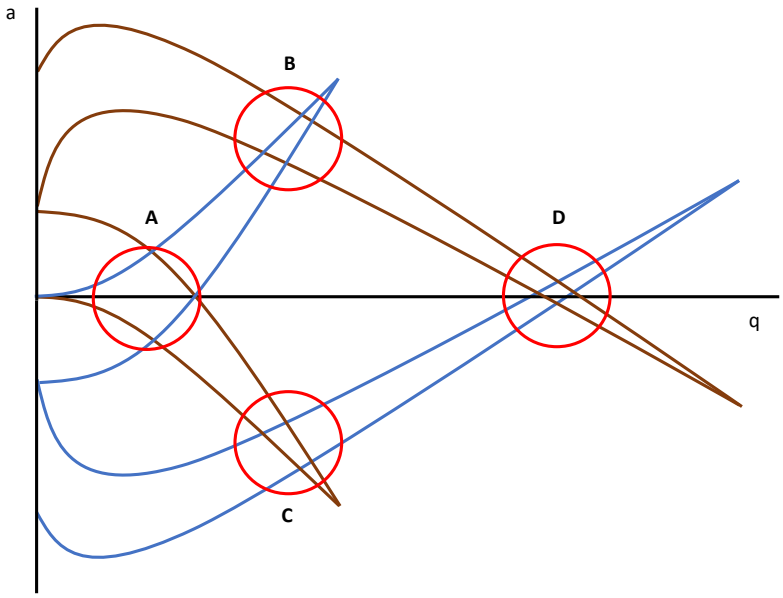

Figure 28 - Mathieu stability diagram illustrating four stability regions for a quadrupole mass analyser (A-D)

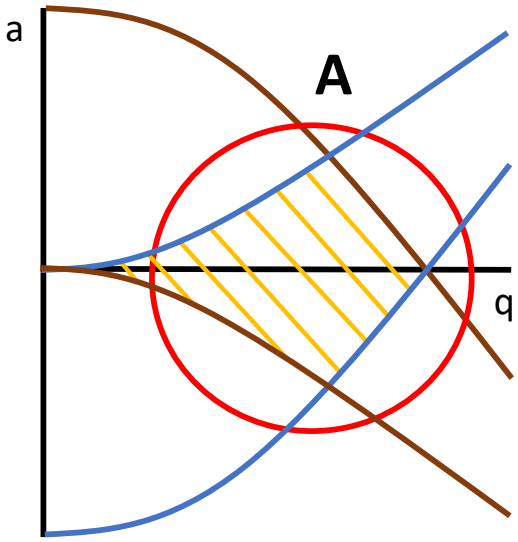

Figure 29 - Diagram to show the first stability region (A) and normal operating region for a quadrupole mass analyser

When operating the quadrupole, the ratio of the DC (U or a) to RF (V or q) voltages is varied in a linear fashion starting at the lowest voltage up to the highest voltage. This is known as the scan line. As the stability region for ions of similar mass will also be similar it is essential to ensure that only one *m/z* value is transmitted at any one time, the DC and RF voltages are selected so that the scan line passes close to the apex of each stability region (Figure 30). The slope of the scan line is known as the quadrupole gain and the intercept of the slope with the y-axis is known as the quadrupole offset (the original DC voltages with no RF voltages).

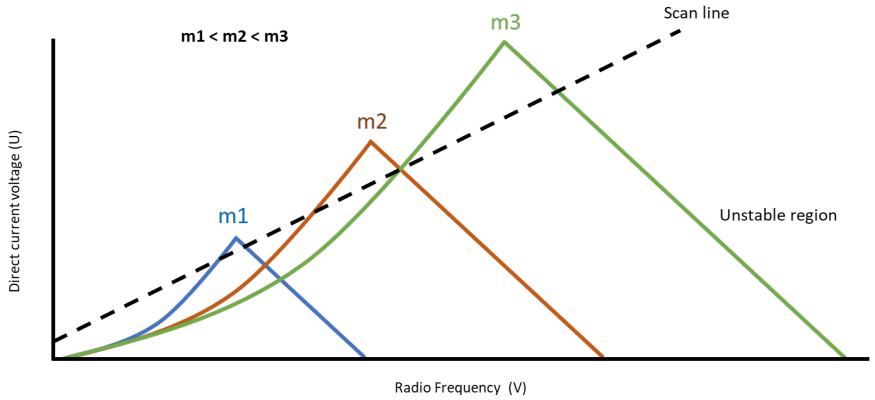

Figure 30 - Stability diagram plotting DC voltage (U) against RF voltage (V) for three ions (m1<m2 <m3) with the scan line also shown

To increase sensitivity in quadrupole analysers, the DC and RF voltages may be set so that only a specific *m/z* value is analysed with a much longer dwell time. This means that a greater number of scans will be collected for this specific *m/z* value. This decreases the level of background noise and increases the signal-to-noise ratio, and this is particularly useful in quantitative analysis where increased sensitivity is required. This method is known as single ion monitoring (SIM) although the deprecated term single ion recording (SIR) is still often used in literature.

1.13 Tandem Mass Spectrometry

Tandem mass spectrometry (MS/MS) may be divided into two categories. Tandem mass spectrometry in time occurs at the same time within the analyser, whereas tandem mass spectrometry in space occurs with the use of more than one analyser so that each step is separated physically. A tandem quadrupole analyser consists of three quadrupoles arranged in series. There are two quadrupoles labelled Q1 and Q3, these act as normal mass analysers (to select *m/z* values) whereas the middle quadrupole (Q2) acts as an ion bridge (RF only) or collision cell (Figure 31). A controlled amount of an inert gas such as nitrogen, helium or argon is

introduced to Q2 so that collisions may take place with enough energy being transferred to the precursor ion to create fragment ions.

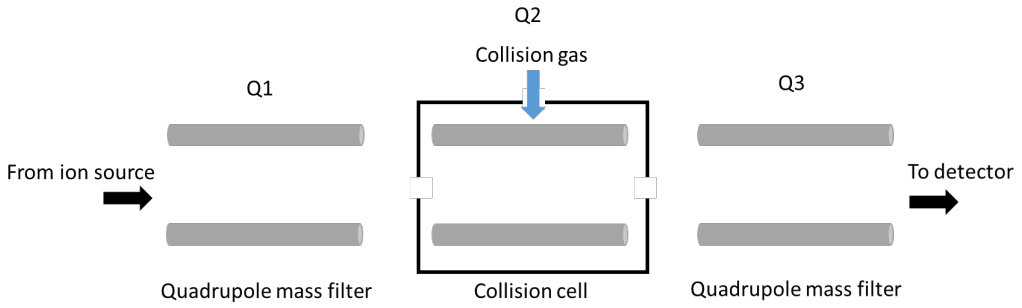

Figure 31 - Diagram of a generic tandem quadrupole mass analyser

A product ion scan is one of the simplest ways of obtaining structural information. The first quadrupole Q1 is set to SIM mode, and this only allows selected ions to pass into the collision cell, Q2. Here the ions will collide with the collision gas to produces fragment ions *via* collision-induced dissociation (CID). Any product ions formed will enter Q3, this will be set to scan over the *m/z* range required to detect all the products ions (at lower *m/z* for singly charged ions).

1.14 Detectors

There are several different types of detectors used in mass spectrometry including microchannel plates (MCPs) photomultiplier conversion dynodes and Faraday cups, however the most common is the electron multiplier and this is used in instruments such as the Waters SQD 2 and TQD. There are two different types of electron multiplier, the discrete dynode, and the continuous dynode and both are used to amplify the ion signal, increasing it by several orders of magnitude (up to 10^6-10^8). The discrete dynode consists of a series of dynodes or plates and the first plate is held at a negative potential (for positive ions). The electron hits the first dynode and accelerates towards the second dynode increasing the emission of electrons. The electric potential applied between each metal plate increases by about ~ 100 eV with each dynode. As the process is repeated and the electrons hit each dynode, a cascade of ions is produced. The continuous dynode electron multiplier is similar to the discrete dynode and is also known as the channeltron multiplier. The individual dynodes or plates are substituted with a tapered coiled glass tube covered with a thin film of semiconducting material. A high negative voltage is applied to the wider end of the funnel and the narrow end is at ground potential.

1.15 Coupling of Chromatography to Mass Spectrometry

Although stand-alone HPLC and SFC systems are extremely useful for the separation of a range of compound types, the coupling of chromatography to a detector such as a mass spectrometer offers significantly more information about the analytes including the *m/z* ratio and some structural characterisation. The mass spectrometer may be used to identify unknown peaks in a chromatogram (Figure 32) or conversely, the separation capability of the chromatography is utilised to confirm that ions observed in a mass spectrum originating from different compounds (Figure 33).

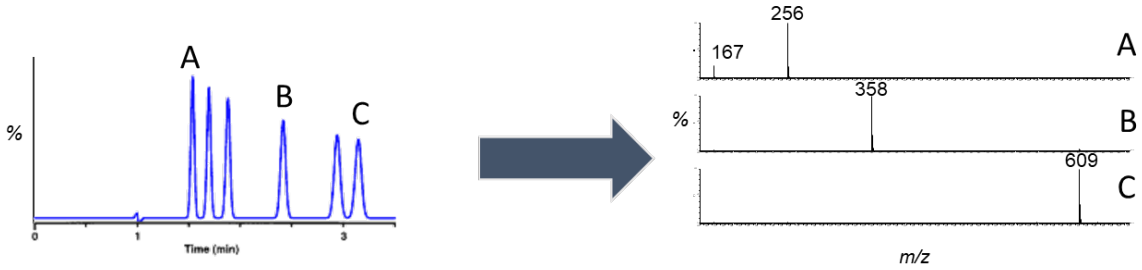

Figure 32 - Illustration to show unknown peaks in a chromatogram and identification using mass spectrometry

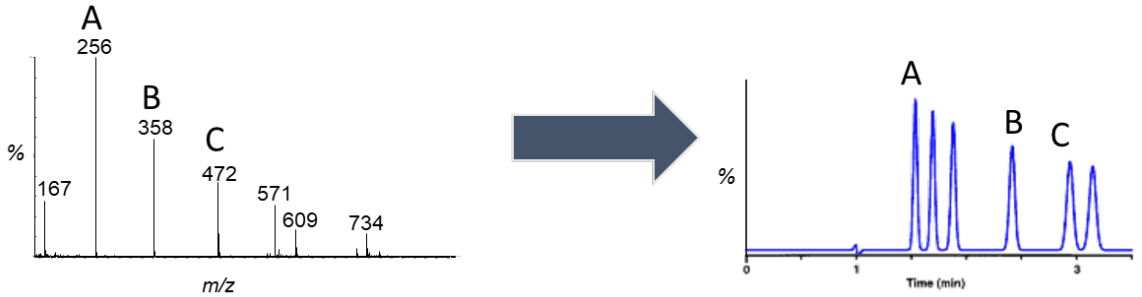

Figure 33 - Illustration to show multiple ions in a mass spectrum and separation into individual components using chromatography

The non-polar solvents mostly used in NP HPLC are incompatible with API techniques such as ESI or APCI since they are aprotic and hence ionisation is not easily achieved. In RP HPLC, the aqueous and mobile phases used for gradient elution are polar, making it easier to achieve ionisation of analytes, hence coupling to MS is more successful. Modifiers and buffers may also be added to the mobile phases to control pH and the use of ion-pairing reagents is common so that ionic compounds can also be separated. Although RP is ideal for the separation of many compound classes such as pharmaceuticals, peptides, and proteins, not all analytes of interest will ionise using one or more of the API techniques available (ESI, APCI or APPI). It is therefore particularly

important to ensure that, where possible, the most appropriate ionisation technique is used for the specific compounds to be analysed.

The important factor to consider when coupling a SFC or UHPSFC system to MS is the accurate control of the back pressure as this will affect the density of the supercritical fluid. As the pressure of the supercritical fluid increases, the density increases, and this will in turn lead to increased solvation and reduced retention. The Waters UPC^2 system utilises the post-column, pre-ABPR double splitter configuration (as described above in section 1.3.4). This ensures that the pressure of the CO_2 is maintained *via* the first split section (flow from the column and then the PDA) combined with the make-up solvent flow from the ISM. The combined flow is then split between the MS and the ABPR with approximately 1/10th of the programmed flow ending up at the MS. If the mass dependent APCI is utilised, this may be affected by the reduction in flow from the splitter.

1.16 Open Access Chromatography and Mass Spectrometry

Open access (OA) or "walk-up and use" chromatography and mass spectrometry has been widely available for synthetic chemists in the pharmaceutical industry since the 1990s[68-72]. The large volume of samples generated by these chemists made it an essential tool to complement the already successful but time intensive OA nuclear magnetic resonance (NMR) spectroscopy analysis. In the past, academia had been somewhat reluctant to follow this industrial open access model; the low volume of sample numbers and limited access to available funding often cited as reasons for not pursuing this high throughput approach. Further, most academic mass spectrometry facilities used EI as the ionisation technique of choice, followed by Fast-atom bombardment (FAB). This was governed by the nature of the samples produced and available technology. The addition of a GC-MS system for mixture analysis, a high-resolution instrument for accurate mass measurements and a single mass spectrometrist were typical of academic mass spectrometry facilities in the 1970s and 1980s[73]. The needs of the modern academic chemist have changed; the advent of combinatorial, high throughput chemistries in the early 2000s generated a significant increase in the quantities of samples requiring both chromatography and MS analysis with rapid turn-around times.

OA-MS at Southampton was implemented and has been available to research chemists since 1995, initially *via* a single quadrupole Platform II (Micromass, Manchester, UK) MS, believed to be the first open access API system in UK academia. Flow injection analysis (FIA), with 100% acetonitrile mobile phase, was favoured over RP chromatography at this stage since a wide variety of synthetic and natural compounds, plus inorganics and organometallic required analysis without the use of aqueous mobile phases. The emphasis was not just on fast turn-around times

but also on education, and users were encouraged to select an ionisation technique based on the structure of the analyte. If necessary, users buffered their own samples using an acidic or basic ionisation enhancer as appropriate. The system delivered real-time reaction monitoring and quick compound identification and the specialist staff provided all aspects of educational training in mass spectrometry and data interpretation. The success of this instrument was the forerunner to the addition of the Waters single quadrupole ZMD with an autosampler and chromatographic pump. This was provided by the I.C.C.S.P. (Industrial Consortium to Support Combinatorial and Solid Phase Synthesis) as an initiative to foster closer links between industry and universities in the UK. This included the training of undergraduate students in practical aspects of the use of modern mass spectrometry. At the same time, the School of Chemistry acquired two Trace 2000 GC-MS systems (ThermoFisher, San Jose, CA, USA) with funding from the Engineering and Physical Sciences Research Council (EPSRC) to offer rapid analysis for non-API compatible samples (e.g., small volatile organics) that could be analysed using EI or CI (ammonia reagent gas) with different column phases (polar and non-polar). Subsequently the ZMD was replaced with a Waters Acquity H-Class UHPLC-TQD MS system in 2013 with funding awarded by EPSRC support Core Chemistry activities within UK Chemistry departments. RP chromatography is now provided with a choice of six different columns (all 2.1 x 50 mm sub 2 µm particle size, including C_{18} and C_4 phases) in addition to a column bypass option. HPLC grade water (0.2% formic acid) and LC-MS grade acetonitrile (0.2% formic acid) are used as mobile phases with gradients typically starting between 0 and 20% acetonitrile increasing to 100% acetonitrile over 5 or 10 minutes.

Despite the success of the RP UHPLC-MS approach, many Southampton chemists work on molecules that are API compatible, *e.g.,* inorganic salts or catananes but are incompatible and/or reactive with typical RP solvents (*e.g.,* water, acetonitrile, methanol) and buffers (*e.g.,* formic acid). Further issues, such as early or late RP chromatographic elution, mean that they are incompatible with the OA RP-UHPLC-MS approach. This lack of chromatographic resolution and poor peak shape can lead to ion suppression issues due to presence of other non -retained compounds. NP chromatography maybe considered as an alternative methodology; however, ionisation is not easily achieved due to the aprotic solvents used.

SFC-MS was therefore considered as a surrogate NP OA option and having previously been used in Southampton Chemistry since 2004 by the Langley research group[74-76]. They initially worked in collaboration with Mettler Toledo as part of an industrial consortium named the EPSRC Combinatorial Centre of Excellence. The Berger Minigram™ was used as a tool to predict the retention behaviour of pharmaceutical compounds and investigate the performance of ammonium acetate as a co-solvent additive. Mettler Toledo was acquired by Thar and then Waters who developed robust analytical UHPSFC instrumentation, the Acquity UPC^2, allowing the technique to be used more widely for achiral separations as well as chiral separations. This

technology provided an alternative high throughput solution for chromatography and mass spectrometry for the types of compounds described above. Waters donated a collaborative SEED UPC^2 system, coupled to a single quadrupole mass spectrometer (SQD 2) to the Langley group in 2011 and this enabled the development of novel UHPSFC-MS assays for numerous applications. The School of Chemistry then purchased a subsequent UPC^2 system, this time coupled to a tandem quadrupole mass spectrometer (Waters TQD), and this was embedded into the Southampton Chemistry OA-MS facility.

1.17 Rationale for Submission of Publications

1.17.1 Publication 1: Open Access UHPSFC/MS – an Additional Analytical Resource for an Academic Mass Spectrometry Facility

The first paper submitted for this thesis encapsulates the role of UHPSFC-MS (UPC^2 TQD) in an academic environment and establishes the technique as a routine tool for the separation and MS analysis of a wide variety of compounds especially those that may have been "too difficult" to analyse using RP chromatography and MS due to the compatibility issues described above. Previous publications in this area have included the use of analytical SFC and MS for screening in a medicinal laboratory[77]. However, this is the first published paper on the use of UHPSFC-MS in a walk-up and use open access academic environment where it can be used by non-specialists. Other academic groups have recognised the increasing popularity of SFC and have called for the integration of SFC into the undergraduate curriculum so that is sits equally alongside LC and GC[78]. Several academic groups and commercial analytical laboratories have subsequently introduced open access SFC for chiral applications[79] and for purification of reaction mixtures in Discovery Chemistry[80]. Laboratory managers and analysts from industrial settings have also recently (2020) consulted with the Southampton group to implement OA-SFC-MS in their own facilities.

The most popular column used for RP chromatography is the C_{18} bonded silica and this is often used as a generic column for initial applications with a gradient of low to high organic mobile phase. There is no such equivalent column in SFC for achiral applications, although this generic column approach is not desired as this would compromise the selectivity and specificity offered by SFC. Rather a comprehensive column and modifier screening protocol is followed to determine optimum separation[30]. At Southampton Chemistry, applications are routinely screened using six of the most frequently used achiral columns 3.0 x 100 mm, < 2 µm, BEH, BEH 2-EP, HSS C_{18} SB, Torus 2-PIC, Torus DIOL, Torus 1-AA[38]. Four co-solvents (methanol, methanol/25 mM ammonium acetate, methanol/50 mM ammonium acetate/2% water, and methanol/1% formic acid) are selected for screening (Figure 34). Previous work by the Langley research group on the effects of retention mechanisms in SFC using ammonium acetate, demonstrated the advantages of this

additive by improving the peak shape and retention of many analytes[74]. The following instrumental conditions are routinely used: ABPR pressure 150 bar, column temperature 45°C, gradient 10 - 40% modifier over 3 minutes at an eluent flow rate of 1.5 mL/min. The make-up solvent is methanol (1% formic acid) and is delivered at a flow rate of 0.45 mL/min. Once the optimum conditions are determined, the designated method is then converted to an open access method and transferred to the open access interface, in this case RemoteAnalyzer, developed in conjunction with SpectralWorks.

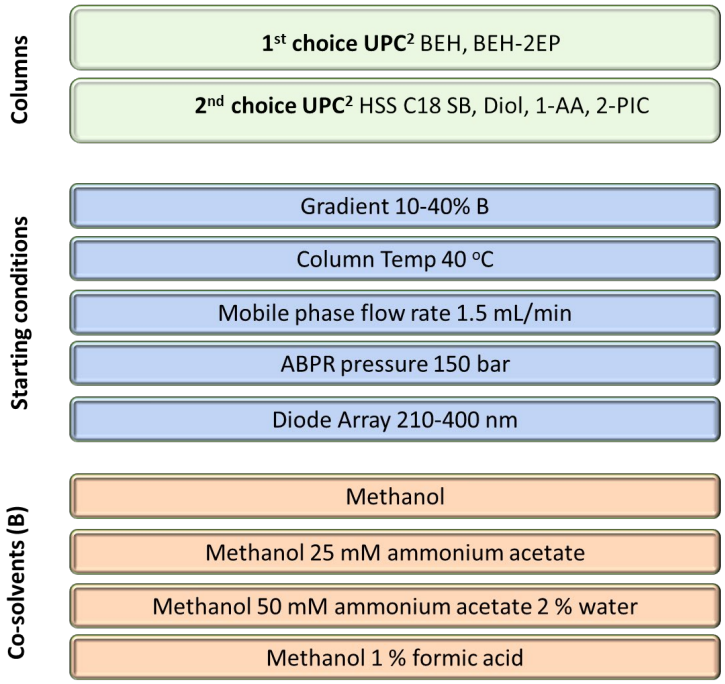

Figure 34 - Example of a typical University of Southampton UHPSFC-MS column, co-solvent method screen for new applications

Whilst much of the published research on SFC and UPSFC has concentrated on technological developments and instrumentation configuration, many researchers have also focussed on the fundamental aspects of the SFC process *e.g.,* the effects of adsorption of the mobile phase on peak shape[81-83], stationary phase optimisation[84-87] and column selectivity including the evaluation of superficially porous particles[88-90]. Prediction of retention behaviour is often required to improve and accelerate method development with the use of design of experiments (DoE) enhancing separation of complex mixtures and studies have been undertaken for both achiral[75,91.92] and chiral SFC[93,94]. The recent resurgence in SFC and UHPSFC due to advancements in modern, robust, instrumentation has resulted in easier hyphenation to MS, particularly those instruments with API sources. The different methods of hyphenation[95-97] and optimisation of the MS conditions[98] have been investigated by several groups. These technological advancements and improvements in

reliability have led to the increased use of UHPSFC-MS as an analytical tool in a wider variety of application areas where simple UV detection would not be sufficient for full characterisation.

1.17.2 Publication 2: Development of an Ultrahigh-Performance Supercritical Fluid Chromatography-Mass Spectrometry Assay to Analyse Potential Biomarkers in Sweat

The second paper submitted for this thesis is concerned with the development of UHPSFC-MS methods to detect and quantify purines and small organic acids that may be detected in complex matrices. The method was developed to provide quantitative analysis for collaborators at the School of Health Sciences (Professor Dan Bader and Dr Peter Worsley). Professor Bader had previously worked in the early 1990s on the analysis of metabolites (purines) in sweat using RP-HPLC. He subsequently utilised a 3 µm, 150 x 4.6 mm C_{18} column to separate individual purines. Each purine was detected using the ratio of 254:280 nm using a dual wavelength UV detector[99]. To aid the detection and separation of these purines, conversion to modern UHPLC methods proved difficult as the phosphate buffers used in the original method needed to be replaced with more volatile buffers in order to be coupled to API MS. Little or no retention was observed using the converted UHPLC-MS method and thus UHPSFC-MS methods were investigated, developed, and utilised to detect four different purines. A second UHPSFC-MS method was also developed to analyse metabolic concentrations of lactate and pyruvate in sweat and lactate in sebum. The samples were collected from volunteers using a mechanical loading device within a specialised environmental chamber at University Hospital, Southampton as described in the collaborative paper by Soetens[100]. The developed methods utilised SIM MS methods using positive ion ESI (purines) and negative ion ESI (lactate and pyruvate). The collaborative work by Soetens utilised the negative ion ESI SIM method to determine the expression levels of lactate and pyruvate reflecting the loading regimens applied to patients. This showed that the quantitation of these metabolites using UHPSFC-MS could potentially be used as early biomarkers for the detection of damaged skin tissue and hence lead to the early treatment of pressure ulcers and significant cost saving for the UK National Health Service (NHS)[101].

Whilst pharmaceutical analysis remains one of the most important application areas for SFC-MS and UHPSFC-MS[102-106], the scope has now widened to include other application areas such as natural products[107], bioanalysis[108-110], lipids[111-115], polymers[116], petroleum and biofuels[117-121] and more recently cosmetics[122-124] and cannabis products[125].

1.17.3 Publication 3: Development of Ultrahigh-Performance Liquid Chromatography- Mass Spectrometry and Ultrahigh-Performance Supercritical Fluid Chromatography-Mass Spectrometry Assays to Determine the Concentration of Bitrex™ and Sodium Saccharin in Homemade Facemask Fit Testing Solutions

The third paper has been submitted for this thesis as it describes the use of UHPSFC-MS and UHPLC-MS as analytical tools to quantify and detect the levels of Bitrex™ and sodium saccharin in homemade face mask fit testing solutions. As COVID-19 cases increased dramatically in the UK in early 2020, the supply of facemask fit testing solution rapidly became scarce. Chemists from the School of Chemistry at the University of Southampton started to make homemade fit testing solutions based on literature methods[126] to supply the local NHS Trusts and ambulance services with these facemask test solutions. UHPSFC-MS and UHPLC-MS methods were rapidly developed to quantify the different analytes and deliver a robust quality control process for the preparation and delivery of these solutions. Fast and easily transferable methods were developed with R^2 values consistently > 0.99. Solutions were made up in 5 L batches and then smaller 1 mL samples were removed, serially diluted, and tested prior to and after an autoclave sterilisation process giving repeatable measurements to within 10% of the expected concentration of the active ingredient. Batch variations were also monitored after the sterilisation process when the bulk solutions were transferred to smaller bottles for distribution. Here a 5 L batch was decanted into ~49-50 100 mL bottles and 1 mL samples were taken from a random selection of these bottles (a minimum of 10%) diluted and analysed in triplicate to provide the same qualitative and quantitative analysis.

These three primary author publications and other co-authored, collaborative UHPSFC-MS publications (appendix) have shown how the use of modern instrumentation and improved hyphenation to mass spectrometry can expand into an array of different application areas. UHPSFC-MS is now used as a routine technique for both chiral and achiral compounds in academia and industry.

1.18 Summary

The three publications selected for submission to this thesis encapsulate the advantages of modern UHPSFC-MS to different application areas. SFC has also been termed convergence chromatography since it has the ability to converge the separation of a much wider range of compounds with one system, RP and NP chromatography as well as chiral and achiral separations[127]. The work begins with the initial recognition of the power of SFC to provide solutions for researchers limited to RP-LC-MS and GC-MS only. The open access UHPSFC-MS provision is the perfect analytical tool to fill the analytical gap between RP-LC-MS and GC-MS for the separation and MS analysis of the wide range of mixtures and compound types that have solvent compatibility problems or suffer from poor retention (Figure 35). Working with colleagues across other disciplines also shows the breadth of the technique to offer alternative chromatography/MS solutions to help solve real-world problems.

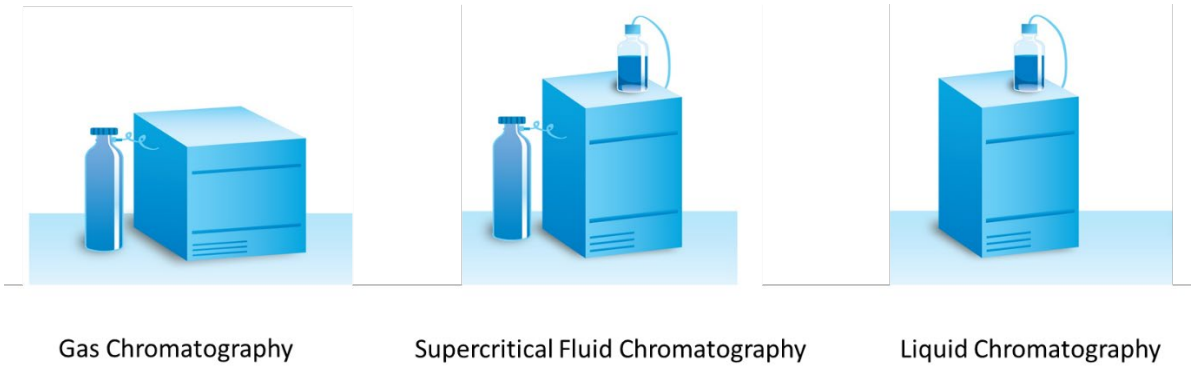

Figure 35 - Illustration to show the position of supercritical fluid chromatography between gas chromatography and liquid chromatography

1.19 List of Published Work Submitted for Thesis

1.19.1 Publication 1

Herniman, J. M.; Langley, G. J., Open Access UHPSFC/MS- an Additional Analytical Resource for an Academic Mass Spectrometry Facility. *Rapid Commun. Mass Spectrom*. **2016**, *30* (15), 1811-1817. (doi.org/10.1002/rcm.7660)

1.19.2 Publication 2

Herniman, J. M.; Worsley, P. R.; Greenhill, R.; Bader, D. L.; Langley, G.J., Development of an Ultrahigh-Performance Supercritical Fluid Chromatography-Mass Spectrometry Assay to Analyse Potential Biomarkers in Sweat. *J. Sep. Sci*. **2022**, *45* (2), 542-550. (doi.org/10.1002/jssc.202100261)

1.19.3 Publication 3

Herniman, J. M.; Langley, G. J., Development of Ultrahigh-Performance Liquid Chromatography-Mass Spectrometry and Ultrahigh-Performance Supercritical Fluid Chromatography-Mass Spectrometry Assays to Determine the Concentration of Bitrex™ and Sodium Saccharin in Homemade Facemask Fit Testing Solutions. *Rapid Commun. Mass Spectrom*. **2020**, *34* (16), e8848. (doi.org/10.1002.rcm.8858)

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Chapter 2 Publication 1: Open Access UHPSFC/MS – an Additional Analytical Resource for an Academic Mass Spectrometry Facility

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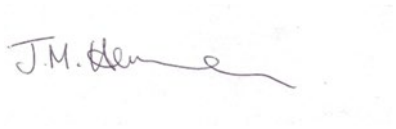
Author Contribution

Julie Herniman undertook the experimental work and prepared the manuscript. John Langley provided supervision and editing of manuscript.

Signature

Julie Herniman



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Chapter 2

Open Access UHPSFC/MS – an Additional Analytical Resource for an Academic Mass Spectrometry Facility

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RATIONALE: Many compounds submitted for analysis in Chemistry at the University of Southampton either do not retain, elute or ionise using open access RP-UHPLC/MS and required analysis *via* infusion. An ultra-high performance supercritical fluid chromatography mass spectrometry approach was implemented to afford high through-put analysis of these compounds with chromatographic separation.

METHODS: A UPC^2-TQD MS system has been incorporated into the open access MS provision within Chemistry at the University of Southampton, using an ESCi source (electrospray and atmospheric pressure chemical ionization) and an atmospheric pressure photoionization (APPI) source. Access to instrumentation is enabled *via* a web-based interface (RemoteAnalyzer™).

RESULTS: Compounds such as fluorosugars, fullerenes, phosphoramidites, porphyrins, and rotaxanes exhibiting properties incompatible with RP-UHPLC/MS have been analysed using automated chromatography and mass spectrometry methods. The speedy return of data enables research in these areas to progress unhindered by sample type. The provision of an electronic web format enables easy incorporation of chromatograms and mass spectra into electronic files and reports.

CONCLUSIONS: The implementation of UHPSFC/MS increases access to a wide range of chemistries incompatible with reversed-phase chromatography and polar solvents, enabling more than 90% of submitted samples to be analysed using an open access approach. Further, chromatographic separation is provided where previously flow injection or infusion analysis were the only options.

INTRODUCTION

The use of open access mass spectrometry (MS) has been widely available for synthetic chemists in the pharmaceutical industry since the 1990s[1-5]. The large volume of samples generated by these chemists made it an essential tool to complement the already successful but time intensive open access (OA) nuclear magnetic resonance (NMR) spectroscopy. In the past, academia had been somewhat reluctant to follow this industrial open access MS model; the low volume of sample numbers and limited access to available funding often cited as reasons for not pursuing this high throughput approach. Historically, most academic mass spectrometry facilities used electron ionization (EI) as the ionization technique of choice, this was governed by the nature of the samples produced and available technology. The addition of gas chromatography-mass spectrometry (GC/MS) systems for mixture analysis, a high resolution instrument for accurate mass measurements all operated by a single mass spectrometrist were typical of academic mass spectrometry facilities in the 1970s and 1980s[6]. This model expanded to soft ionization techniques, initially fast-atom bombardment (FAB) and then thermospray, increasing the sample types and numbers of analyses. In recent times, the needs of the modern academic chemist have changed; the advent of combinatorial, high throughput chemistries generated significant increase in the quantities of samples requiring MS analysis with rapid turn-around times demanded. However, these sample types and numbers exceeded the capability of most standard academic mass spectrometry facilities. The advent of atmospheric pressure ionization (API) techniques, *i.e.,* electrospray ionization (ESI) /atmospheric pressure chemical ionization (APCI) in the 1980s/90s, and their easy coupling to high performance liquid chromatography (HPLC) made them ideal tools for the open access environment[7]. These developments, together with other technological improvements, led to successful adoption of HPLC/API-MS into the pharmaceutical industry. Academic institutions eventually mimicked the adoption of a high throughput approach, with USA and UK universities now providing individual, departmental and campus-wide MS facilities[8].

University of Southampton, Chemistry, Mass Spectrometry

Following the successful introduction of the open access MS approach within the pharmaceutical industry, a single quadrupole Platform II (Waters™/Micromass, Manchester, UK) was installed at the University of Southampton, Chemistry in 1995, the first open access ESI/APCI system in UK academia. This complemented the EI, CI and FAB MS provision then afforded by the normal geometry, double focusing 70-250SE (VG Analytical, Manchester, UK). Flow injection analysis (FIA), with 100% acetonitrile mobile phase, was favoured over chromatography at this stage since a wide variety of synthetic compounds and natural products, including inorganics and organometallics, required analysis. This was in contrast to the methods established in the pharmaceutical industry where similar classes of compounds are analysed. Further, the option of

providing positive ionization and negative ionization data in a single analysis was purposely not provided. Users were encouraged to select the polarity of analysis based on the structure of their compound, rather than attempt a scattergun approach. If necessary, users buffered their samples using an acidic or basic ionization enhancer as appropriate. These educational protocols were imposed to enforce the link between compound structure and chemistry with ionization.

Initially, the system was available to over 150 members of the graduate school consisting of postdoctoral researchers and postgraduate students, providing 24-hour access to state-of–the-art instrumentation affording rapid sample analysis. This allowed for real-time reaction monitoring and quick compound identification in conjunction with all aspects of educational training in mass spectrometry and data interpretation.

Within five years, the total number of analyses on this single instrument reached over 20,000 samples per annum, including the analysis of larger molecules such as proteins and oligonucleotides. The success of this instrument was the forerunner to the addition of a single quadrupole ZMD, 600 HPLC pump and controller, 2700 sample manager, and 996 photodiode array detector (Waters™, Manchester, UK) in 2000. This was provided by the I.C.C.S.P. (Industrial Consortium to Support Combinatorial and Solid Phase Synthesis) as an initiative to foster closer links between industry and universities in the UK. This initiative included the training of undergraduate students in practical aspects of the use of modern mass spectrometry. Subsequently the ZMD was replaced with an Acquity H-Class UHPLC-TQD MS system (Waters™, Wilmslow, UK) in 2013 with funding provided by The Engineering and Physical Sciences Research Council (EPSRC) to support Core Chemistry activities within UK Chemistry departments. For reversed-phase chromatography, a choice of six columns (all 2.1 x 50 mm sub-2 µm particle size, including C_{18} and C_4 phases) is available in addition to a column bypass position to provide FIA. The mobile phases used are HPLC grade water (0.2% formic acid) and LC-MS grade acetonitrile (0.2% formic acid) with gradients starting between 0 and 20% acetonitrile increasing to 100% acetonitrile over 5 or 10 minutes. A dedicated open access MicrOTOF UHPLC/MS instrument (Bruker Daltonik GmbH, Bremen, Germany) is also used for the analysis of synthetic oligonucleotides (5 to 200 mers). The nature of the specific solvents required (triethyl ammonium acetate and hexafluoroisopropanol) demanding that this UHPLC/MS instrument is only exposed to these solvents.

Open access GC/MS (EI and ammonia CI) accommodates the analysis of small volatile organic compounds. Two Thermo Trace 2000 GC/MS (ThermoFisher, San Hose, CA, USA) instruments operate with temperature gradients ranging from 15 minutes to 1 hour duration depending on complexity of the sample. One instrument has a combined EI/CI source, to provide each ionization technique with identical column and gradient conditions, in this case a non-polar ZB5-MS capillary

column (Phenomenex, Macclesfield, UK) 30 m x 0.25 mm 0.25 µm. To facilitate the fundamental understanding of mass spectrometry, students are initially instructed to acquire EI data, only using CI to confirm the molecular weight of the compound if the molecular ion is absent from the EI mass spectrum. The second Trace GC/MS instrument has an EI only ion source (for improved sensitivity). In open access configuration a polar wax, HP-Innowax capillary column (Agilent J &W, Stockport UK) 30 m x 0.25 mm 0.25 µm is used as an orthogonal alternative to the non-polar column in the other open access GC/MS. Detailed one-to-one training is provided for users who need to create bespoke methods, analyse and process their own samples and data outside of the open access arena.

Implementation of UHPSFC/MS

Many samples submitted for analysis in Southampton have structures that are either ESI or APCI compatible, *e.g.,* inorganic salts or catananes but many of these compounds are incompatible and/or reactive with reversed-phase solvents (*e.g.,* water, acetonitrile, methanol *etc.*) and buffers (*e.g.,* formic acid). Further issues, such as early or late chromatographic elution, mean that they are incompatible with a high-throughput reversed-phase liquid chromatography approach. Normal phase chromatography maybe considered as an alternative methodology. However, the solvents used *e.g.,* hexane, chloroform are not compatible with ESI or APCI since they are aprotic solvents. If air is present in the ionization source (possibly due to a poor quality nitrogen gas supply) then there is also the risk of ignition due to the mix of flammable normal phase solvents by the APCI corona discharge. Supercritical fluid chromatography (SFC) can be considered as a normal phase chromatography option and has been used in the mass spectrometry research group at the University of Southampton, Chemistry since 2004. A Berger SFC™ Minigram (Mettler Toledo, Greifensee, Switzerland) was used to predict retention behaviour of pharmaceutical compounds and investigate the performance of ammonium acetate as additive to the modifier[9,10]. Experience and knowledge in this field of chromatography, particularly in the coupling to mass spectrometry, made the introduction of UHPSFC/MS to the open access facility an obvious approach.

The development of robust analytical UHPSFC instrumentation, the Acquity UPC^2 enabled the provision of a suitable high throughput solution for chromatography and mass spectrometry in the open access environment. Compounds that were previously un-retained, over-retained or incompatible with reversed-phase solvents can now be analysed *via* a high throughput approach. Low cost, food grade CO_2 (BOC Special Gases, Guildford UK) can be used for the supercritical (sc) mobile phase. Separation is achieved by changing the composition of this mobile phase, temperature and density and/or through the addition of an organic modifier (co-solvent), most commonly methanol, often in conjunction with an additive, to expand selectivity. A make-up

solvent (generally methanol with acid) is also essential when coupling SFC to a mass spectrometer. This helps stabilise the API spray and also aids ionization; this is particularly important if modifier is not required for separation, *i.e* 100% $scCO_2$ (Figure 1). This make-up solvent can also be used to produce specific ions *e.g.,* the addition of sodium ions to the make-up flow to produce sodiated molecules thus ensuring optimum sensitivity for quantitative analysis[11]. In an open access environment additional column ovens, with a range of six column phases, and four co-solvent channels provides optimal flexibility.

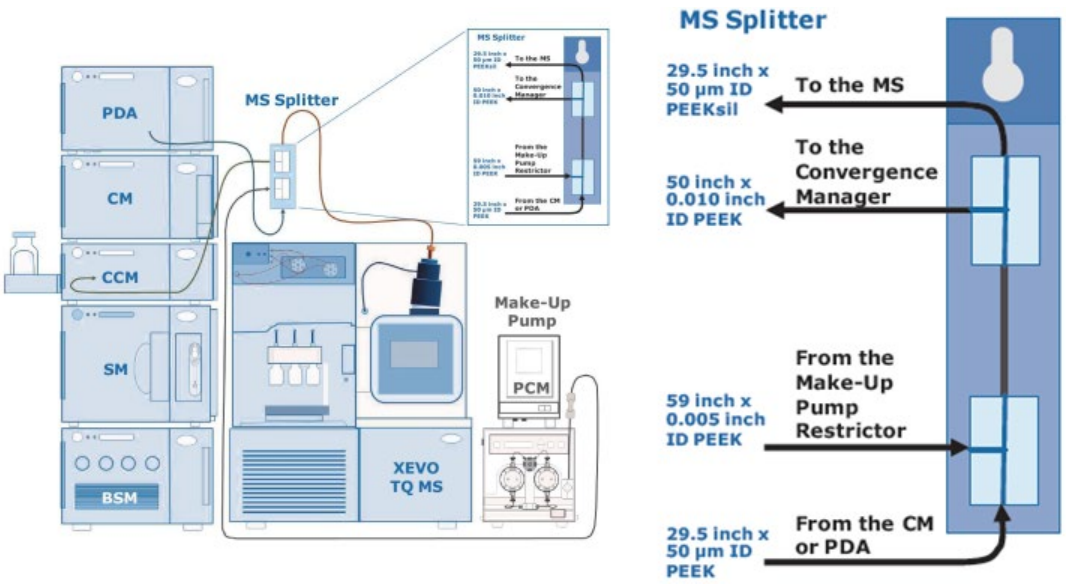

Figure 1 Schematic of UPC2-MS system with expanded splitter configuration inset (courtesy of Waters™

The most popular column used for reversed-phase chromatography is the C_{18} bonded silica and this is often used as a generic column for initial applications with a gradient of low to high organic solvent. The equivalent SFC column does not exist; moreover, this generic column approach is not desired as this would compromise the selectivity and specificity offered by SFC. Rather a comprehensive column and modifier screening protocol is followed to determine optimum separation[12]. At the University of Southampton Chemistry, applications are routinely screened using six columns, all 3.0 x 100 mm, < 2 µm, BEH, BEH EP, HSS C_{18} SB, Torus 2-PIC, Torus DIOL, Torus 1-AA (Waters™, Elstree, UK). Four modifiers (methanol, methanol/25 mM ammonium acetate, methanol/50 mM ammonium acetate/2% water, and methanol/1% formic acid) and the following instrumental conditions are routinely used: automated back pressure regulator (ABPR) pressure 150 bar, column temperature 45°C, gradient 10 - 40% modifier over 3 minutes at an eluent flow rate of 1.5 mL/min. The make-up solvent is methanol (1% formic acid) delivered at a flow rate of 0.45 mL/min *via* a stand-alone 515 HPLC pump. Once the optimum conditions are

determined, the designated method is then converted to an open access method and transferred to the open access interface (RemoteAnalyzer™).

The open access MS approach allows all users to identify their products, to monitor reactions and optimise their chemistries, and to screen their samples prior to subsequent submission for high resolution accurate mass measurement *via* infusion analysis.

RESULTS AND DISCUSSION

The following compounds are typical examples of sample types that were previously analysed manually *via* infusion API-MS. Each compound has been analysed *via* open access UHPSFC/MS.

Phosphoramidites are widely used in DNA or RNA synthesis. The dT phosphoramidite (Figure 2) was analysed using the generic reversed-phase UHPLC positive ion electrospray ionization MS method (BEH C_{18}, 2.1 x 50 mm 1.7 µm column). The mobile phase increases from 20 to 100% acetonitrile (0.2% formic acid) over 5 minutes. A single chromatographic peak is observed at T_R 2.30 minutes. The ion *m/z* 303 is the dimethoxytrityl fragment cation, and the ion at *m/z* 634 is consistent with the sodiated molecule for the H-phosphonate tautomer formed in the presence of formic acid and water. The same compound was analysed using an UHPSFC positive ion electrospray ionization MS method (HSS C_{18} column 3 x 100 mm 1.8 µm column), 10 to 40% methanol modifier gradient over 3 minutes. Ions at *m/z* 745 and *m/z* 303 are observed for two near-baseline resolved peaks at T_R 0.94 and 1.01 minutes. The ion *m/z* 745 observed at T_R 0.94 is consistent with the protonated molecule for the given structure (the same ion is observed in the chromatographic peak at T_R 1.01 minutes).

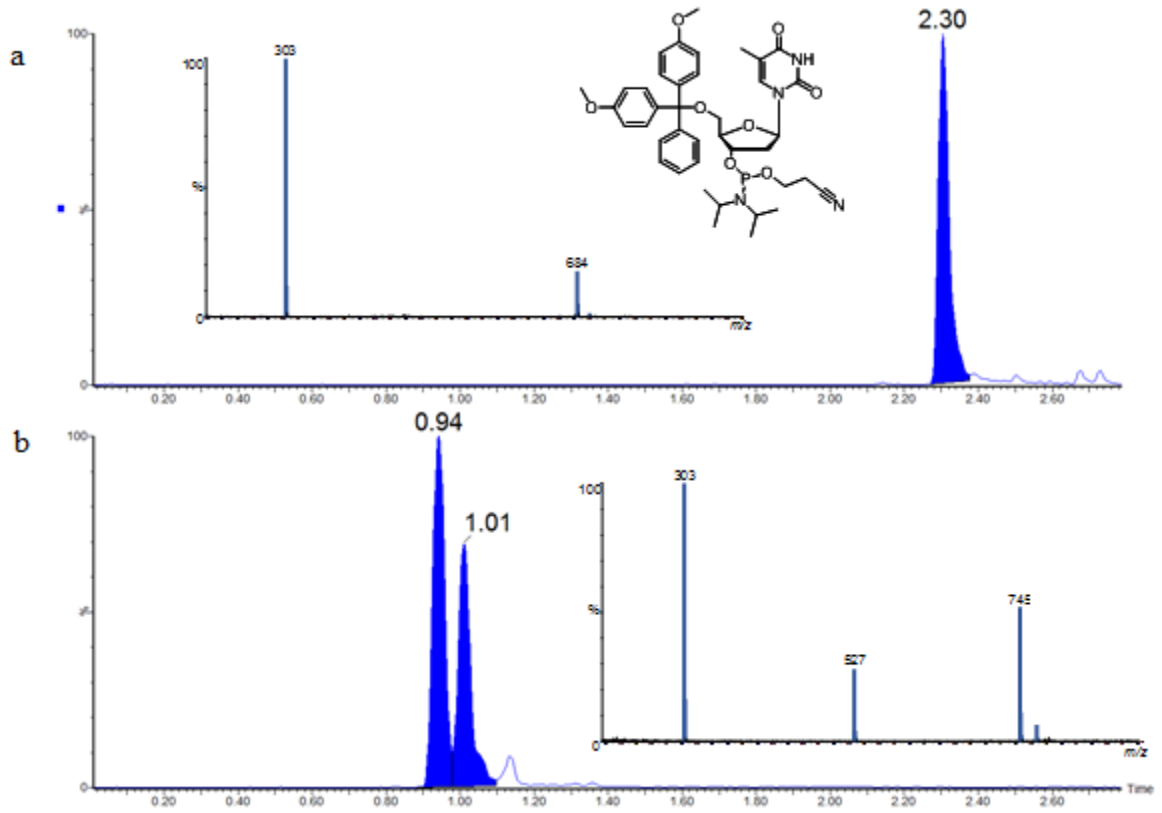

Figure 2. Positive ion electrospray ionization base peak ion current chromatograms for a phosphoramidite (M_r 744) analysed using a) reversed-phase UHPLC/MS and b) UHPSFC/MS.

Fluorinated carbohydrates are synthesised in order to investigate how fluorination can modify physical properties such as hydrogen bonding, lipophilicity and conformation. Most carbohydrates can be analysed with reversed-phase chromatography using positive and/or negative ion ESI. In the case of the fluorosugar (Figure 3), this was unretained using the generic reversed-phase UHPLC/MS method and also when the initial eluent composition was adjusted to 100% water. This sample was analysed using UHPSFC with a CSH Fluoro-Phenyl column 3 x 100 mm 1.7 µm, 10 to 40% methanol over 3 minutes and negative ion ESI. An ion at *m/z* 219 observed at T_R 1.24 minutes is consistent with the deprotonated molecule.

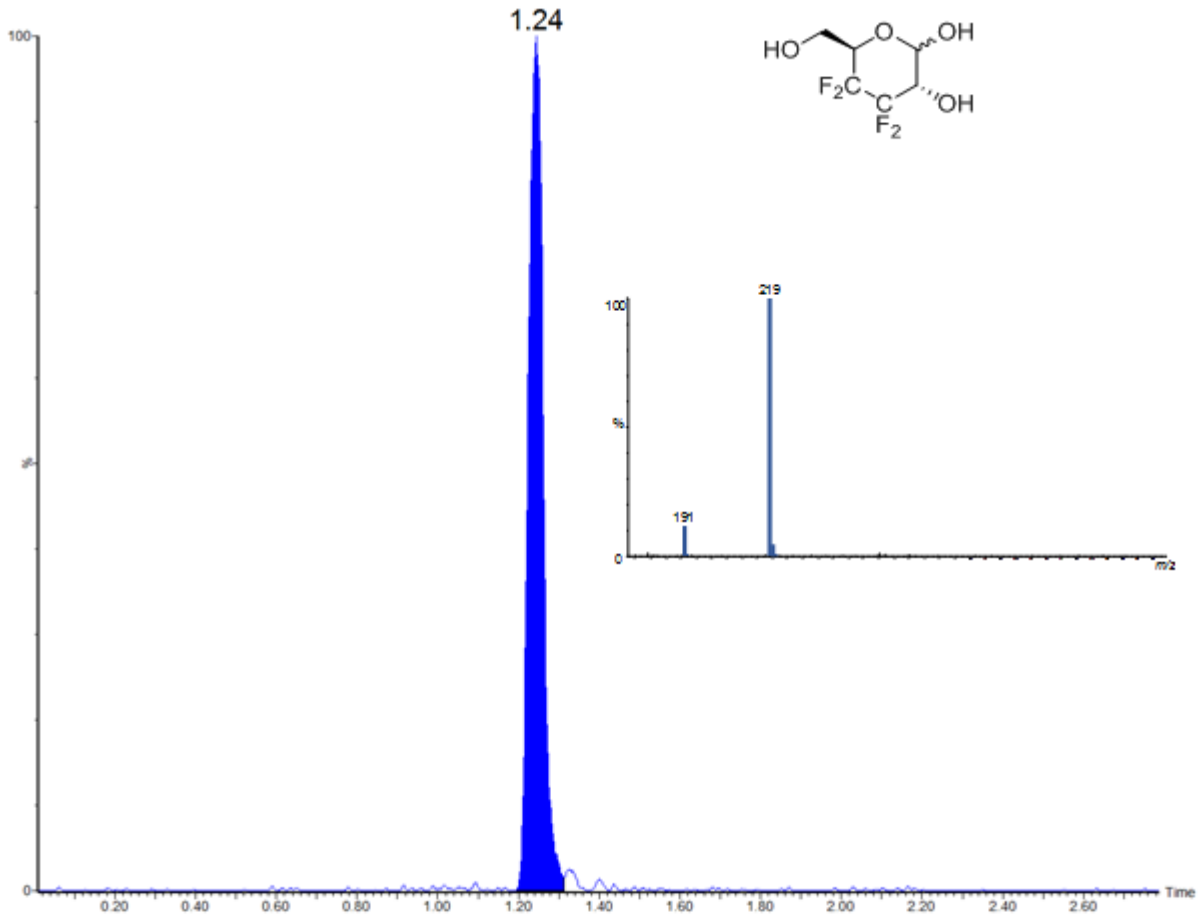

Figure 3. Negative ion electrospray ionization reconstructed ion current chromatogram for a fluorosugar (M_r 220) analysed using UHPSFC/MS.

Porphyrins are heterocyclic macrocyclic organic compounds. In Southampton, these compounds are incorporated into DNA sequences for applications in energy/electron transfer, DNA structure analysis and as lipid anchors. The porphyrin building block (Figure 4), incorporating the zinc (II) ion, is only soluble in 100% organic solvents and is not amenable to analysis by reversed-phase chromatography. This sample was analysed using UHPSFC with a BEH column 3 x 100 mm 1.7 µm, 10 to 40% methanol 25 mM ammonium acetate over 3 minutes and positive ion ESI. An ion at *m/z* 1039 observed at T_R 1.58 minutes was consistent with the protonated molecule with the distinctive zinc isotope pattern.

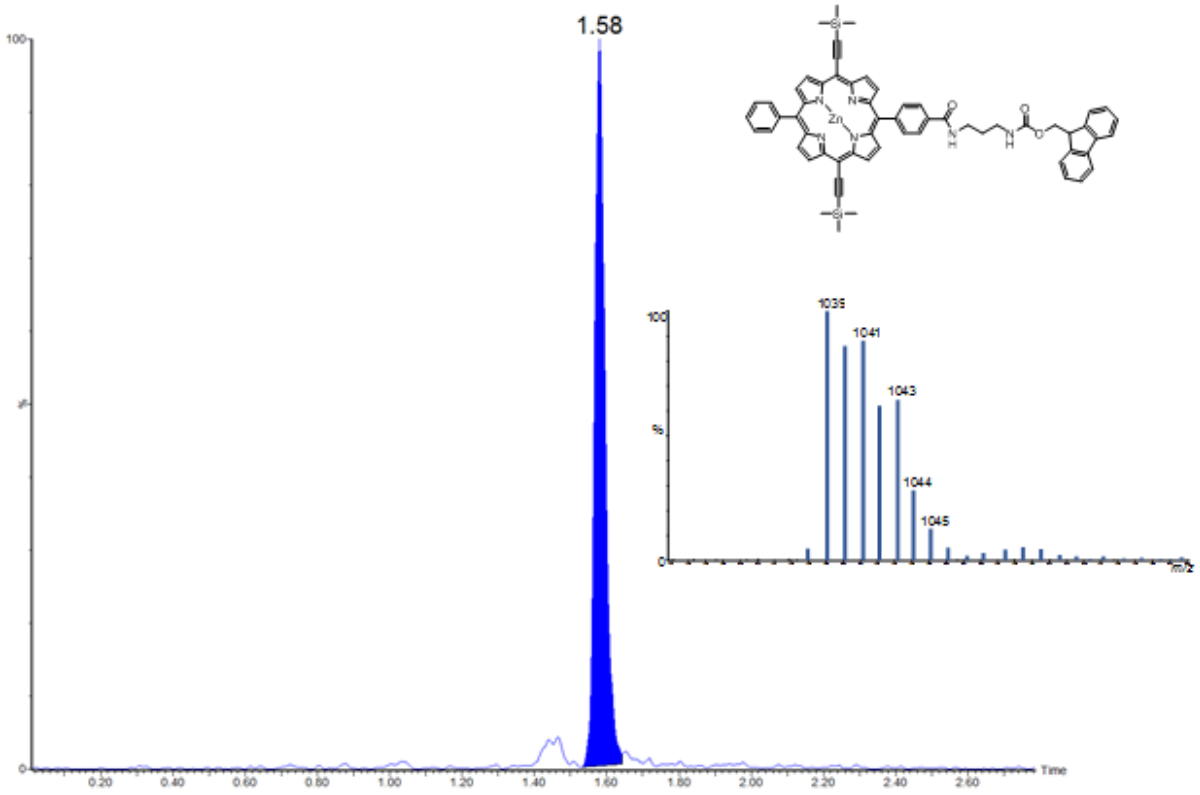

Figure 4. Positive ion electrospray ionization reconstructed ion current chromatogram for a zinc containing porphyrin (M_r 1038) analysed using UHPSFC/MS.

Rotaxanes consist of a linear species and a cyclic species bound together in a threaded structure by non-covalent forces. They are not water soluble and the optimum solvents for dissolution are acetonitrile and dichloromethane. Smaller rotaxanes can be analysed using reversed-phase chromatography with a BEH C18, 2.1 x 50 mm 1.7 µm column, 20 to 100% acetonitrile (0.2% formic acid over 5 minutes) and positive ion ESI. Larger rotaxanes (> 1000 Da) elute very late in the reversed-phase chromatograph when the gradient reaches 100% acetonitrile but with poor peak resolution. Extending the length of time at 100% organic improves this in some cases but as the size of the rotaxane increases then they fail to elute using these standard conditions. The rotaxane structure shown in Figure 5 did not elute using the extended reversed-phase gradient conditions but elutes at T_R 2.32 minutes under UHPSFC conditions using a HSS C_{18} SB column 3 x 100 mm 1.8 µm, 10 to 40% methanol over 3 minutes and positive ion ESI. The ion at *m/z* 1579 is consistent with the protonated molecule and the ion at *m/z* 790 is consistent with the doubly protonated molecule for the given structure.

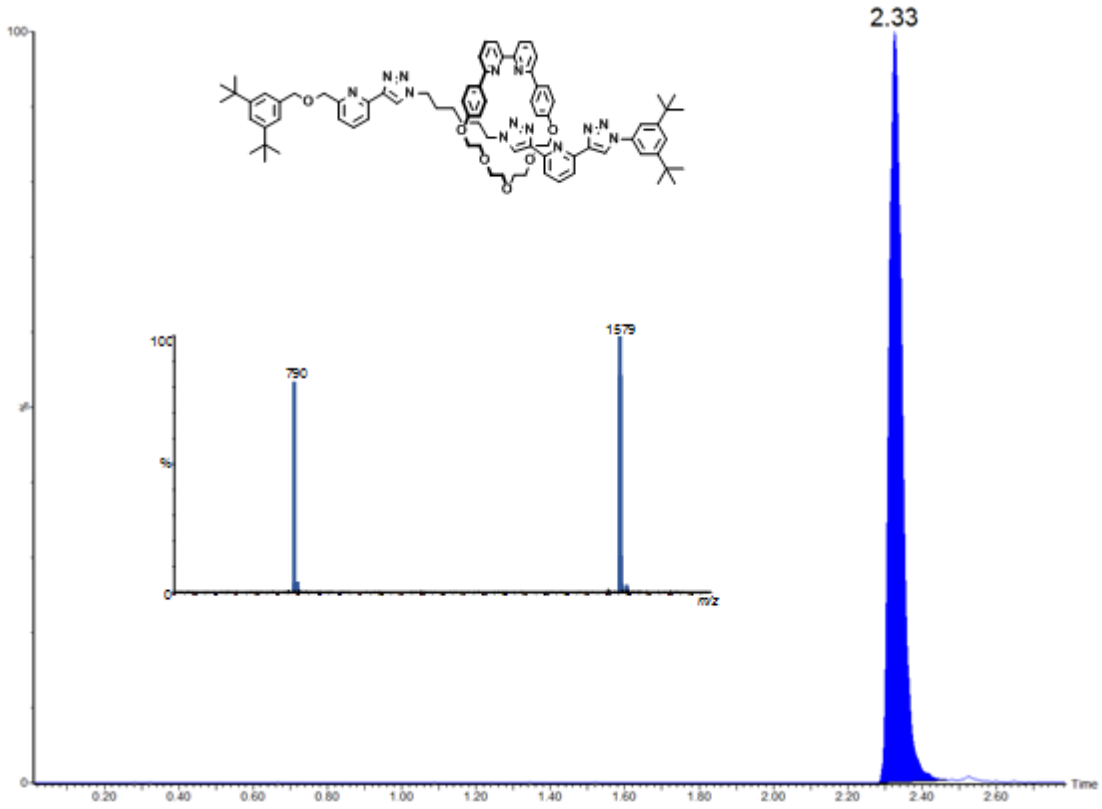

Figure 5. Positive ion electrospray ionization reconstructed ion current chromatogram for a rotaxane (M_r 1578) analysed using UHPSFC/MS.

The addition of an atmospheric pressure photoionization (APPI) source, that can easily be exchanged for the ESCi source on the TQD, has also broadened the range of chemical space that can be analysed with chromatography and mass spectrometry. APPI is a gas phase ionization technique and the source used here utilizes a 10.6 eV krypton discharge UV lamp (Syagen Technologies, Santa Ana, CA, USA) to emit photons. These photons are absorbed by the analyte molecules leading to electron ejection and the formation of radical cations [$M^{+\cdot}$]. If solvent molecules are present these can form a reagent ion that can produce the protonated molecule. The addition of a dopant (commonly toluene) is often used to promote the formation of the radical cation $D^{+\cdot}$ and analytes are ionised by electron transfer to produce D + $M^{+\cdot}$. Samples may be prepared in a solvent to act as the dopant or the dopant can easily be introduced *via* the external 515 HPLC make-up flow pump required to couple the UPC^2 to the mass spectrometer. Generic methods can easily be created and converted to open access methods, so that this ionization source can operate at specific times during the working week.

Molecular wires are organic conjugates that can act as semiconductors. These polyaromatic molecules are developed with terminal pyridine or nitrile groups to act as “alligator clips” to

secure the polyaromatic compound to a metal surface. Small conjugates < 500 Da are routinely analysed using EI GC/MS but larger systems are often too involatile or incompatible with GC/MS. Figure 6 shows structural isomers separated using UHPSFC with a HSS C_{18} column 3 x 100 mm 1.8 µm column, 10 to 40% toluene over 3 minutes and positive ion APPI. The samples are prepared in toluene and toluene is also used as the make-up solvent. An ion at *m/z* 664 observed at T_R 1.34 and 1.44 minutes is consistent with the radical cation [$M^{+\cdot}$] for each isomer.

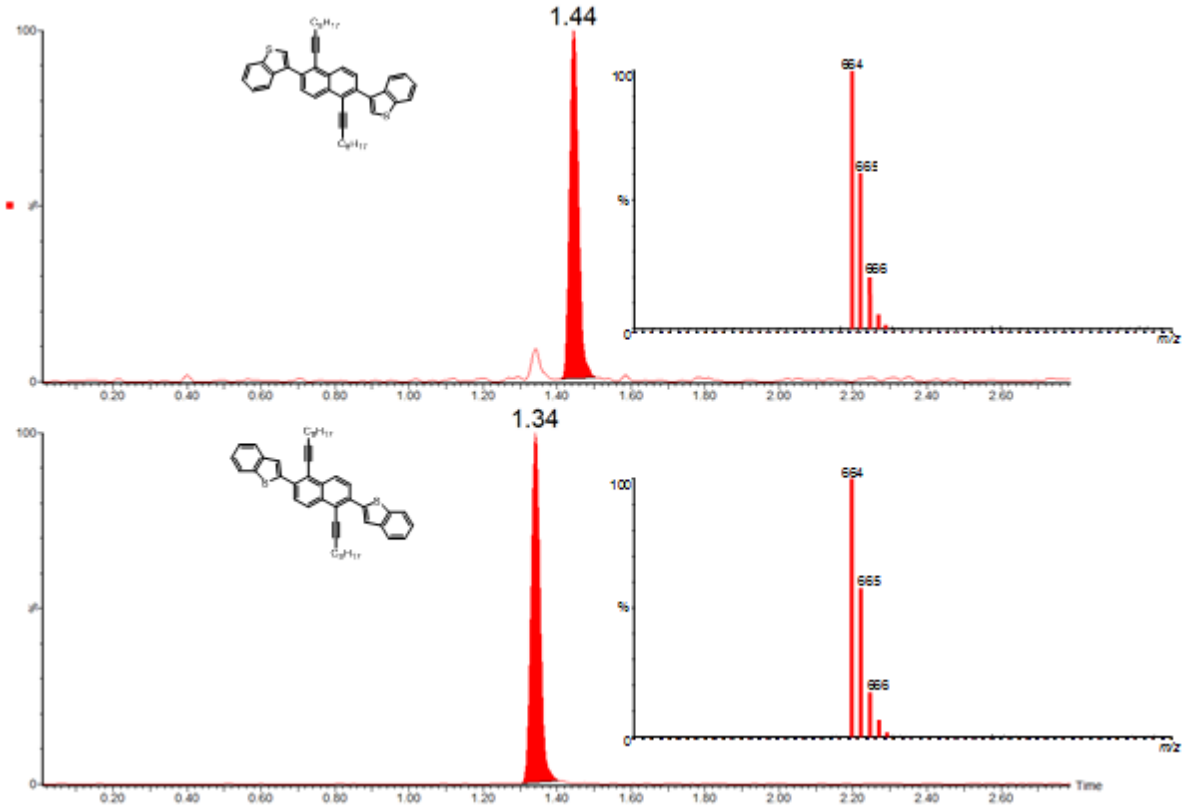

Figure 6. Positive ion atmospheric pressure photoionization reconstructed ion current chromatograms of molecular wires isomers (M_r 664) analysed using UHPSFC/MS.

Fullerenes are molecules comprised of at least 60 carbon atoms as a closed shell structure *e.g.,* C_{60} is spherical and C_{70} is ovoid. They have no functional groups making them difficult to ionise using conventional API mass spectrometry and are not soluble in conventional reversed-phase solvents. Figure 7 shows the UHPSFC analysis of C_{60} using a BEH EP 3 x 100 mm 1.7 µm column, 30 to 40% toluene over 3 minutes and positive ion APPI. An ion at *m/z* 720 observed at T_R 1.42 minutes is consistent with the radical cation [$M^{+\cdot}$] for C_{60}.

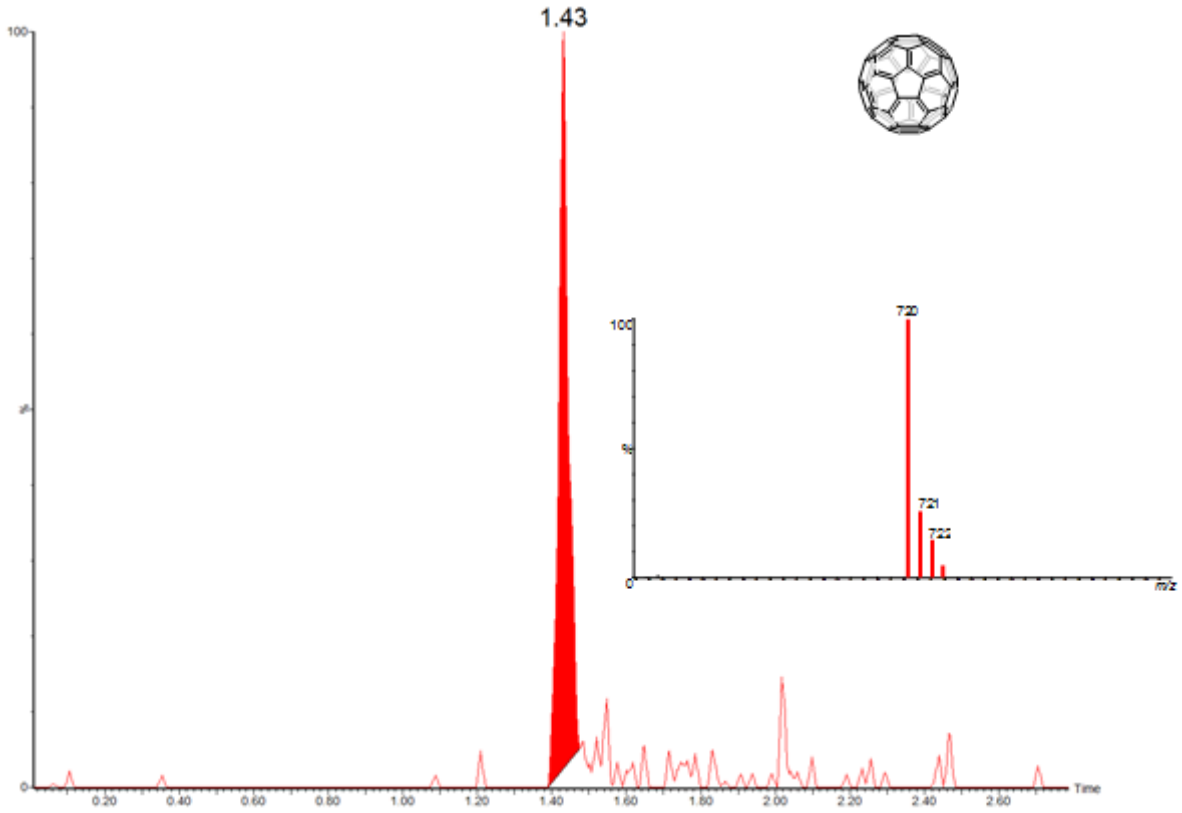

Figure 7. Positive ion atmospheric pressure photoionization reconstructed ion current chromatogram for fullerene C_{60} (M_r 720) analysed using UHPSFC/MS.

The driver for open access mass spectrometry is high throughput, automated sample turn-around time. The immediacy of the result allowing researchers to identify their chemistries and also use this approach for reaction monitoring. By moving to a centralised, vendor independent open access provision in 2006 (RemoteAnalyzer™, SpectralWorks Ltd., Runcorn, UK) this affords simplified training, uniformity of data reporting and improved data security. Now, the advent of robust and reliable SFC instrumentation has allowed easy integration of UHPSFC/MS into the open access MS suite. Hence allowing chemistries that were incompatible with RP-HPLC/MS and GC/MS access to the crucial high-throughput option.

Within RemoteAnalyzer™ each individual user has a unique, password protected account to facilitate access to all open access MS systems. The users prepare samples, bar code the sample vial and submit all sample information and choice of analysis *via* any networked PC, tablet or portable device. Once the barcode is scanned in the MS laboratory, the autosampler vial position is allocated and start of acquisition is confirmed *via* a touch screen monitor. On completion of analysis, the data are processed automatically, with the resultant data emailed to the user as a PDF file. The data may be viewed on-line and subsequently saved to electronic lab notebooks (ELNs) or other electronic files if required.

CONCLUSIONS

The implementation of UHPSFC/MS into the open access environment in Chemistry at the University of Southampton, means that more than 90% of samples submitted are analysed in an automated fashion *via* chromatography and mass spectrometry. UHPSFC increases access to a wide range of chemistries previously incompatible with reversed-phase chromatography and polar solvents. Further, chromatographic separation is available for specific research areas where previously the only options were flow injection or infusion analysis. In general, analysis time is less than five minutes, with users usually receiving their data within tens of minutes of sample submission.

Open access UHPSFC/MS allows these researchers to monitor their reactions in real-time and moves the routine analysis away from the MS staff, freeing staff time to focus on more challenging samples.

ACKNOWLEDGEMENTS

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Chapter 3 Publication 2: Development of an Ultrahigh-Performance Supercritical Fluid Chromatography-Mass Spectrometry Assay to Analyse Potential Biomarkers in Sweat

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Author Contribution

Peter Worsley and Dan Bader provided sweat samples and initiated the project. Julie Herniman and John Langley designed and undertook the experimental work, Rachel Greenhill helped with the experimental work as part of a summer project studentship. Julie Herniman prepared the manuscript, John Langley provided supervision and editing of manuscript with Peter Worsley and Dan Bader.

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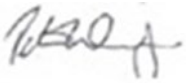
Peter Worsley	
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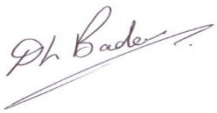
	Project supervisor
Dan Bader	
G. John Langley	

Chapter 3

Development of an Ultrahigh-Performance Supercritical Fluid Chromatography-Mass Spectrometry Assay to Analyse Potential Biomarkers in Sweat

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Running title: UHPSFC-MS of potential biomarkers in sweat

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Abbreviations: UHPSFC: ultrahigh-performance supercritical fluid chromatography; **$scCO_2$:** supercritical carbon dioxide; **SIM:** selected ion monitoring

Keywords: Acids, purines, sweat, ultrahigh-performance supercritical fluid chromatography-mass spectrometry

ABSTRACT

Liquid chromatography-mass spectrometry methods were required to afford the rapid separation and detection of purines and small organic acids. These compounds are found in sweat and sebum and are potential biomarkers for the early detection of pressures sores.

Two ultrahigh-performance supercritical fluid chromatography-mass spectrometry assays have been successfully developed for both classes of compounds. Separation for purines was achieved using a gradient of supercritical carbon dioxide and methanol with a 1-aminoanthracene sub 2 µm particle size column followed by positive ion electrospray ionization. Separation for organic acids was achieved using a gradient of supercritical carbon dioxide and methanol (50 mM ammonium acetate 2% water) with a Diol sub 2 µm particle size column followed by negative ion electrospray ionization.

Calibration curves were created in the absence of internal standards and R^2 values > 0.96 were achieved using single ion monitoring methods for the protonated purines and the deprotonated acids.

The two new assays afford rapid analytical methods for the separation and detection of potential biomarkers in human sweat leading to the early detection and prevention of pressure sores.

1. INTRODUCTION

A number of candidate biomarkers have been proposed to assess the metabolic state of loaded skin and soft tissues [1]. During mechanically induced tissue compromise, ischaemia is followed by a complex biochemical response when the blood supply is re-established, and this may result in additional injury to the tissue. Candidate metabolic biomarkers sampled non-invasively from sweat and sebum include lactate and pyruvate [2,3]. The corresponding markers of ischemia-reperfusion include purines (allantoin, hypoxanthine, inosine, uric acid, and xanthine), which may directly contribute to cell injury [4] when tissues are offloaded following a period of mechanical compromise.

Knight *et al.* reported that ischemia resulting from prolonged pressure affects the viability of body tissue, this pressure occludes the blood vessels within the tissue which may lead to pressure ulcers [3]. The delivery of oxygen and other key molecules is restricted, leading to tissue hypoxia that results in a disturbance between the balance of adenosine triphosphate (ATP) production and consumption, causing a breakdown of ATP and further metabolism, producing purines such as xanthine, hypoxanthine and inosine [4]. Many body fluids may be used as sources of these biomarkers, with sweat and sebum identified as potential non-invasive sources. There are several studies that report the relationship between metabolite concentrations in sweat and the loading and unloading of tissues [5-7]. Increased concentrations of selected purines in sweat have been observed in periods of mechanically induced ischemia compared with unloaded tissue and subsequent reperfusion [8,9]. Detection of the purine biomarkers, using modern analytical techniques would then allow for effective early stage prevention strategies. With prompt medical treatment, potential damage could be halted treatment times could be reduced, potentially saving millions of pounds for the UK National Health Service [10].

Anaerobic metabolites, such as lactate and pyruvate have also been identified as promising non-invasive biomarkers for indications of tissue ischemia. These metabolic waste products are produced through glycolysis and provide indicators for anaerobic cell metabolism. The lactate/pyruvate ratio (L/P) has previously been used as an indicator for several ischemic related medical conditions including acute liver failure, [11] myocardial injury [12] and traumatic brain injury [13]. Soetens *et al.* most recently demonstrated the use of these non-invasive inflammatory biomarkers as a potential indicator of pressure induced tissue damage using the analytical assay described in this paper to detect and quantify levels of lactate and pyruvate collected from sweat and sebum samples [14].

Previous studies to detect purine biomarkers have utilized separation techniques including RP-HPLC with UV detection [15,16]. Akula *et al.* used an isocratic mobile phase mixture (water/methanol/acetonitrile) and prepared rat tissue samples using H_2SO_4 and Tris buffer and

Bader *et al.* used a 5 mmol/L phosphate, 5mmol/L 1-heptanesulfonic acid pH 3.3 mobile phase with both methods utilizing C_{18} columns for separation. Purines have also been successfully separated using hydrophilic interaction chromatography (HILIC) [17]. Here a ZIC® HILIC column was utilized to fully resolve 12 purines in 60 minutes. However, none of these studies employed more modern small diameter columns nor were they coupled to mass spectrometry (MS) instruments. A more recent study in 2019 by Yuan *et al.* [18] developed a method for the quantification of 10 urinary purine metabolites using UHPLC-MS/MS with positive ion electrospray ionization. Separation was obtained using a 2 µm C_{18} bonded phase column developed specifically for 100% aqueous mobile phase applications.

To improve the identification of the individual purines, the challenge was to develop fast high throughput methods utilizing modern column technologies *i.e.* sub 2 µm particle size with ultrahigh-performance liquid chromatography (UHPLC) and hyphenated to MS. Replacement of the phosphate buffers to a more volatile buffer was required to allow the coupling to atmospheric pressure ionization (API) MS. Initial efforts to adapt HPLC-UV methods to UHPLC-MS, using a C_{18} column (1.7 µm 50 x 2.1 mm) resulted in little or no retention for the purine standards. Attempts to increase the retention of the purines using different column phases, mobile phases and mobile phase additives proved to be unsuccessful. Concurrently, UHPLC-MS assays were also attempted for the analysis of the small organic acids, lactic acid, and sodium pyruvate. Literature suggesting that successful assays could be achieved using reversed phase liquid chromatography with a high volume of aqueous in the mobile phase [19-22]. However, whilst ions corresponding to the deprotonated molecules of lactic acid and sodium pyruvate were detected using negative ion electrospray ionization MS, chromatographic co-elution of the two compounds occurred. Hence, the use of an alternative chromatographic technique, ultrahigh-performance supercritical fluid chromatography-mass spectrometry (UHPSFC-MS), was investigated to improve the separation, retention and identification of the purines and the small organic acids.

Like normal phase chromatography UHPSFC utilizes polar stationary phases and a non-polar supercritical carbon dioxide ($scCO_2$) mobile phase. Analytes that would normally elute early or be non-retained using reversed phase chromatography should be retained and elute later using UHPSFC. Polar organic co-solvents (modifiers) are often used in conjunction with $scCO_2$ in order extend the breadth of compounds accessible to UHPSFC by increasing the polarity of the mobile phase. Methanol is the most common co-solvent often enhanced by the addition of additives such as ammonium acetate, ammonium formate or formic acid [23].

The methodology was developed to provide fast quantitative assays for the analysis of sweat samples collected from volunteers subjected to a mechanical loading device within a specialised environmental chamber [24]. Low volume samples (10 to 40 µL) were collected for use in studies

to detect the early on-set of pressure sores, hence minimal sample preparation and no internal standards were used during the analysis.

2. MATERIALS AND METHODS

2.1. Chemicals

Methanol, water (LC-MS grade) and formic acid were purchased from Thermo Fisher Scientific (Loughborough, UK). Hypoxanthine, xanthine, allantoin, inosine (Figure 1) sodium pyruvate and lactic acid (Figure 2) were purchased from Sigma-Aldrich (Gillingham, UK) and used without further purification. Ammonium acetate, ammonium hydroxide and sodium chloride were also purchased from Sigma-Aldrich. Food grade carbon dioxide was purchased from BOC Special Gases (Manchester, UK).

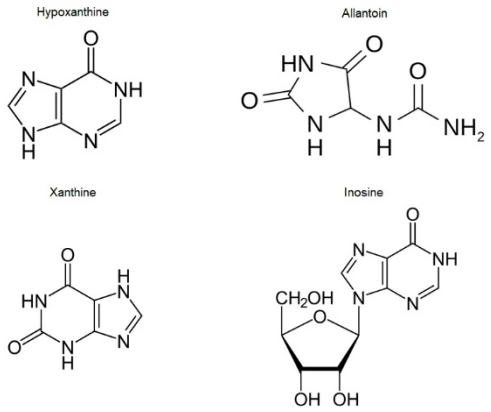

Figure 1 Structures of purines used in this study

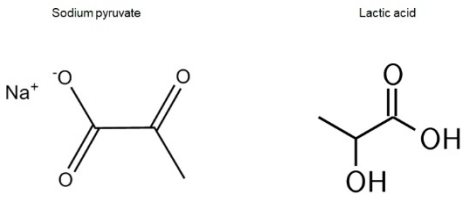

Figure 2 Structures of sodium pyruvate and lactic acid

2.2. Standard Preparation

All standard compounds were initially prepared at a concentration of 1000 ppm (1 mg/mL) and then diluted (apart from xanthine) into water using volumetric dilutions for analysis by UHPSFC-MS. Xanthine was diluted into 0.17 mM ammonium hydroxide for analysis. Standard calibration solutions were prepared for the purines containing nominally 0.1, 0.25, 0.5, 0.75, 1 and 10 ppm and 1, 10, 25, 50, 100, 200 ppm for sodium pyruvate and lactic acid.

2.3. Chromatography

Separation of the purines was achieved using an Acquity ultrahigh-performance convergence chromatograph (UPC$^{2®}$, Waters™, Wilmslow, UK.) with a Waters™ Torus 1-AA packed column, 1.7 µm particle size, 3 × 100 mm. The column was held at 50 °C in a column oven and 2.0 µL of each sample was injected. $scCO_2$ with methanol co-solvent were used for separation at a flow rate of 1.5 mL/min. The $scCO_2$ back pressure of the system was set to 150 bar. A gradient elution was performed starting at 10% co-solvent increasing to 30% co-solvent over 4.5 minutes and held at 30% for a further 0.5 minutes. A 1-minute isocratic pre-run was used for column equilibration.

Separation of the lactate and pyruvate was achieved using a Waters™ UPC^2 system with a Waters™ Torus Diol packed column, 1.7 µm particle size, 3 × 100 mm. The column was held at 40 °C in a column oven and 2.0 µL of each sample was injected. $scCO_2$ with methanol (50 mM ammonium acetate 2% water) co-solvent were used for separation at a flow rate of 1.5 mL/min. The $scCO_2$ back pressure of the system was set to 150 bar. A gradient elution was performed starting at 10% co-solvent increasing to 40% co-solvent over 2.5 minutes and held at 40% for a further 0.5 minutes. A 1-minute isocratic pre-run was used for column equilibration.

In cases where sample volume is limited (< 40 µL) and where multiple analyses are necessary for quantitation assays there is a need to reduce the injection volume. The Waters™ Acquity UPC$^{2®}$ system employs the Partial Loop with Needle Overfill (PLNO) method for sample injection. Here the system will also withdraw a small volume of solvent that is used to transfer the liquid from the vial to the injector loop and the default setting is 15 µL [25]. Whilst the injection volume may be set to 2 µL, this means that ~15 µL of sample is consumed for each injection. The PLNO volume can be manually adjusted and limited to a minimum total injection volume of 3 µL. Similar repeatability for this method compared to the standard PLNO method was observed for the standard test purines (data not shown) and therefore this approach was used where the volume of samples produced were limited.

2.5. Mass Spectrometry

Positive ion ESI mass spectra were recorded using a single quadrupole mass spectrometer (SQD 2) and ESI ionization source (Waters™, Wilmslow, UK) with the following conditions: capillary voltage 3.5 kV; cone voltage 20 V; extractor 3.0 V; source temperature 150°C; desolvation temperature 500°C; desolvation gas flow 650 L/h (nitrogen) and acquisition and data processing achieved using MassLynx™ version 4.1 Full scan mass spectra were acquired between *m/z* 150 - 1000 at a scan rate of 6 scans/second (scan time 0.15 s). An isocratic solvent manager (ISM) (Waters™, Wilmslow, UK) was used to introduce the make-up solvent, methanol (1 % formic acid) at a flow rate of 0.45 mL/min.

Selected ion monitoring (SIM) is the MS mode used in which the abundance of ions of one or more specific *m/z* values are recorded [26]. This method decreases the level of background noise and increases the signal-to-noise ratio resulting in improved sensitivity. Hence SIM was used to detect the protonated molecules $[M + H]^+$ for hypoxanthine, nominal *m/z* 137, xanthine, nominal *m/z* 153*,* allantoin nominal *m/z* 159 and inosine, nominal *m/z* 269 with an automated dwell time of 0.010 s.

Negative ion ESI mass spectra were recorded using a single quadrupole mass spectrometer (SQD 2) and ESI ionization source (Waters™, Wilmslow, UK) with the following conditions: capillary voltage 3.3 kV; cone voltage 20 V; extractor 3.0 V; source temperature 150°C; desolvation temperature 500°C; desolvation gas flow 650 L/h (nitrogen) and acquisition and data processing achieved using MassLynx™ version 4.1. Full scan mass spectra were acquired between *m/z* 150 - 1000 at a scan rate of 6 scans/second (scan time 0.15 s). An isocratic solvent manager (ISM) (Waters™, Manchester, UK) was used to introduce the make-up solvent, methanol (1% formic acid) at a flow rate of 0.45 mL/min.

SIM was used to detect the deprotonated molecules $[M - H]^-$ for pyruvate, nominal *m/z* 87 and lactate, nominal *m/z* 89 with an automated dwell time of 0.010 s.

3. RESULTS AND DISCUSSION

3.1. Method development

Initial UHPSFC-MS analyses of the standard compounds were undertaken using full scan mode. A typical combinatorial column and modifier screening protocol was used with the solvent gradient starting at 5% modifier increasing to 40% modifier over 5 minutes. Screening included the use of common UHPSFC column phases, BEH, BEH-EP and HSS C_{18} SB, 2 -PIC, DEA, 1-AA, Diol, and modifiers *e.g.* methanol, acetonitrile with and without the addition of additives. Optimisation was based on the retention, separation, and peak shape of each analyte together with the optimal ionization technique. This screening protocol highlighted that two different column phases are required, the 1-AA (aminoanthracene) is the optimal column phase to separate the purines and the Diol is the optimal column phase for separation of the acids. The chromatography conditions for each assay were further optimised by varying the $scCO_2$ back pressure (105 -200 bar) and column temperature (35- 50 °C) to further improve baseline separation and optimum peak shape. (Figures 3 and 4) Selection of mass spectrometry ionization and conditions were also optimised for each assay. SIM methods were used to improve the lower limit of detection (LLOD) for each individual compound. Once the optimum linear response region for each assay had been defined, the peak areas were used to create a 5-point calibration (samples analysed in triplicate). The LLOD and lower limit of quantification (LLOQ) were calculated for each individual compound.

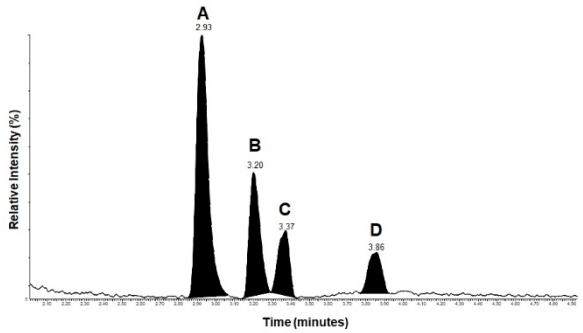

Figure 3 UHPSFC-MS base peak ion current chromatogram (BPICC) of four purines A) hypoxanthine B) xanthine C) allantoin and D) inosine

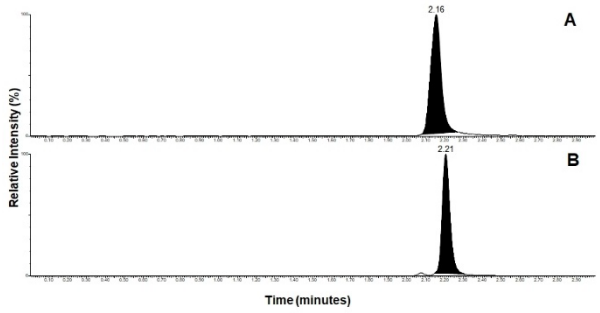

Figure 4 UHPSFC-MS single ion monitoring (SIM) chromatograms for A) sodium pyruvate and B) lactic acid

3.2. Lower Limit of Detection and Lower Limit of Quantification

The LLOD and LLOQ may be calculated using several different analytical methods, such as the signal-to-noise ratio, the standard deviation of the blank and the calibration plot at low concentration [27,28]. In this study, the LLOD and LLOQ were calculated using the S/N method using root mean squared (RMS) (S/N ≥ 3 and ≥ 10 respectively). Detection and quantification limits for the individual standard compounds were determined using the SIM chromatograms of the protonated molecules (purines) and deprotonated molecules (acids) without using an internal standard. The ion for each protonated molecule was used to produce LLODs of 0.1 ppm and LLOQs of 0.1 - 0.5 ppm (R^2 values 0.9637 – 0.9993). The ion for each deprotonated molecule was used to produce LLODs and LLOQs of < 0.1 ppm (R^2 values 0.9999 and 0.9997).

3.3. Application of methodology

To validate both assays for repeatability and assess the impact of the sweat matrix, the standard compounds were prepared in both water and a sweat surrogate comprised of water containing 1.15 mg/mL of sodium chloride. Data was collected for each assay from 5 replicates over 3 separate days. A mixture of the 4 purines was prepared so that each purine was present at a concentration of 10 ppm. The peak area calculated for each purine in water was compared to the

peak area of the same compound in the sweat surrogate. The calculated ratios (average 1.0-1.1) are shown in Table 1 with RSD values ranging from 6% to 18%.

Table 1. Ratio of peak areas (water: sweat surrogate) for each purine at a concentration of 10 ppm (15 replicates over 3 days)

Replicate	Hypoxanthine	Xanthine	Allantoin	Inosine
1	1.0	0.9	1.5	1.3
2	1.0	1.0	0.7	1.3
3	1.1	0.9	0.9	1.0
4	1.0	0.9	1.0	0.9
5	1.0	0.9	1.0	1.1
6	1.1	1.0	0.8	0.9
7	1.1	1.0	1.2	1.0
8	1.1	0.9	1.1	1.5
9	1.2	1.0	1.0	1.0
10	1.0	1.0	0.9	1.1
11	1.0	1.0	1.0	0.9
12	1.1	1.0	1.1	1.1
13	1.2	1.0	1.1	1.0
14	1.1	1.0	1.1	1.0
15	1.1	1.1	1.0	1.1
AVERAGE	**1.1**	**1.0**	**1.0**	**1.1**
% RSD	**6.2**	**6.0**	**18**	**14.4**

The same protocol was used for the lactic acid and sodium pyruvate assay. For this assay, the calculated peak area for each compound was used to calculate the average L/P ratio in water (2.8) and the sweat surrogate (2.8) at 50 ppm. The results are shown in Figure 5 with RSD values below 3% for each assay.

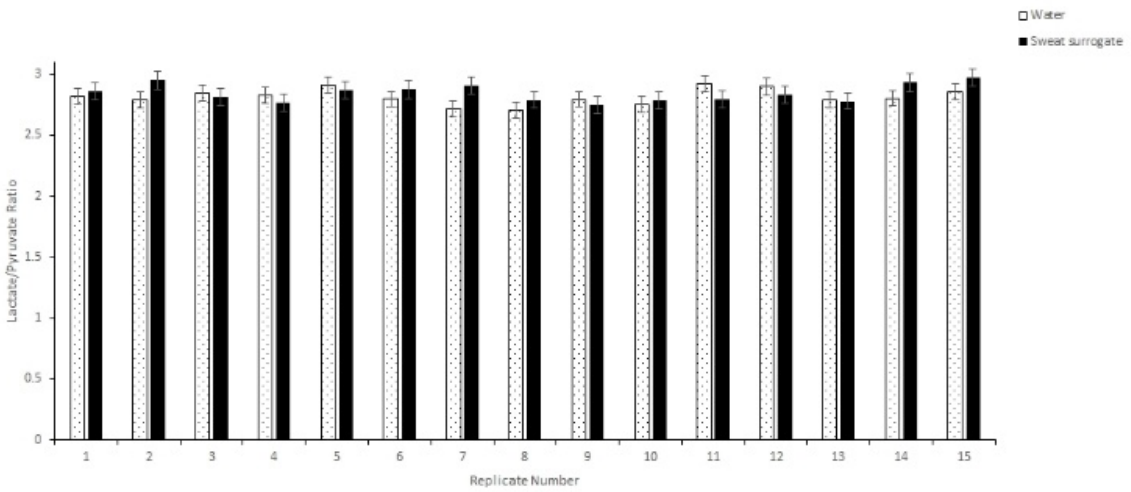

Figure 5 The L/P ratio calculated from a 50 ppm standard mix of lactic acid and sodium pyruvate prepared in water and a sweat surrogate of water containing 1.15 mg/mL of sodium chloride

Standard calibration curves for each compound, prepared separately in water and then the sweat surrogate solution were also compared and the data are shown in Figure 6 and 7.

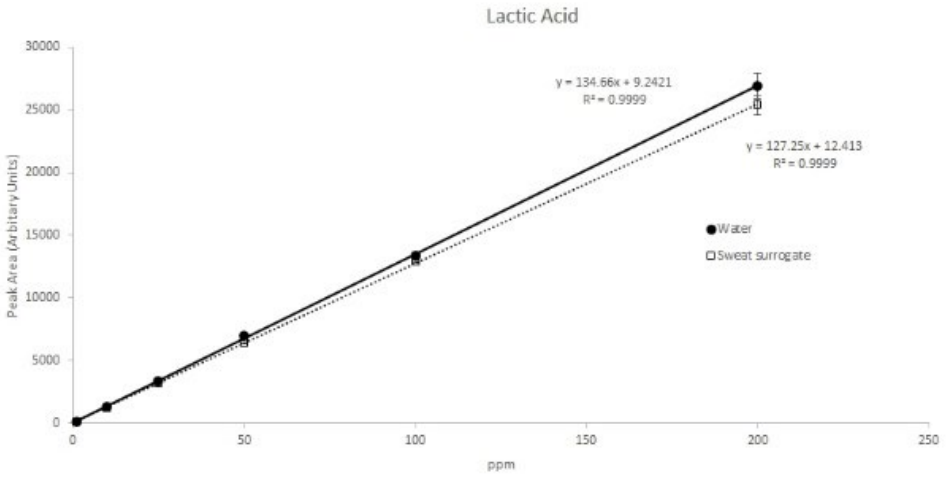

Figure 6 Lactic acid standard calibration curves using water and a sweat surrogate of water containing 1.15 mg/mL of sodium chloride

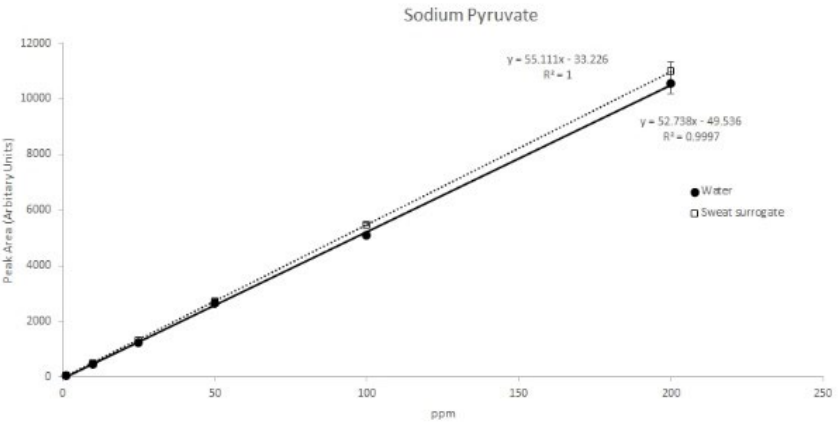

Figure 7 Sodium pyruvate standard calibration curves using water and a sweat surrogate of Waters™ containing 1.15 mg/mL of sodium chloride

These data also indicated that the presence of salt in sweat did not have deleterious effect on the peak shape, chromatographic performance, or lifetime of the column. Further validation of the assays described and the applicability to real samples was undertaken by collecting sweat from a small number of healthy volunteers. Sweat from the sacrum of the volunteers was collected following 1.5 hours in an environmentally controlled chamber with periods of no load, load, and release undertaken using a mechanical loading device as shown in Figure 8. The sweat was collected onto filter papers, frozen and stored at -80°C. Upon defrosting the filter papers were centrifuged for 10 minutes and the sweat collected. Those filter papers that yielded very small volumes of sweat were soaked in 100 µL of water and centrifuged for a second time. Whilst the volume collected for some participants was < 40 µL, adjustment of the PLNO sample injection method as previously described ensured the repeatability of each injection.

Figure 8 Picture of experimental setup in the heated environmental chamber with (A) the participant lying in the prone position with the left and right side of the sacrum subjected to axial loading with weighted indenters and (B) filter paper pads applied to obtain the sweat samples from the three measurements sites

Initial observations using the positive ion electrospray ionization assay showed that the concentration of the purines varied in all participant samples with xanthine giving the highest calculated concentration levels followed by allantoin and hypoxanthine. The levels of inosine were at or near the LLOD (0.1 ppm). Analyses of the same samples using the negative ion electrospray ionization assay indicated the concentrations of lactic acid exceeded the upper limit of the calibration curve (> 200 ppm) and hence the samples were diluted by a factor of 10 using LC-MS grade water. Analyses from these samples showed that the concentration of the lactic acid was approximately 10 times more than that of the sodium pyruvate and that levels of sodium

pyruvate decreased during the loading phase and continued to decrease during the unloading phase.

The high throughput negative ion electrospray ionization protocol was utilized in the recent study by Soetens *et al.*, to monitor and determine the metabolic concentrations of lactate and pyruvate in sweat and lactate in sebum [14]. The sweat and sebum samples were collected from healthy volunteers using the same mechanical loading device within the specialised environmental chamber at University Hospital, Southampton [2]. In this study, samples were collected every 20 minutes from different locations of the body including the forehead, forearm, and sacrum during periods of no load, load, and release. Initial lactate and pyruvate standard calibration curves were created using a concentration range of 1-2500 ppm with R^2 values of 0.9976 and 0.9970 respectively and these were used to quantify the concentration of each acid in the samples collected at every stage of the loading regime. The calculated L/P ratio increased at all the loading sites with levels of the metabolites increasing immediately after loading and recovering to the baseline after unloading. The expression levels of lactate and pyruvate also reflected the loading regimes applied to each participant. The study concluded that metabolites in both sweat and sebum are suitable biomarkers for the non-invasive monitoring of local ischaemia in periodically loaded skin and soft tissues.

4. CONCLUDING REMARKS

UHPLC-MS methods provided little or no retention for individual purines using conventional reversed phase solvents and columns. The new UHPSFC assay using SIM MS methods showed linearity of $R^2 > 0.96$ for each individual purine even in the absence of an internal standard. Adjusting the UPC^2 standard PLNO settings means that the assay can be used to detect purines that may be present in bodily fluids such as sweat, where samples volumes can be very low. The second assay was developed to detect and separate lactate and pyruvate to calculate the L/P ratio. This is particularly relevant since recent studies report that the L/P ratio calculated in sweat and sebum samples indicates that expression levels correspond to the applied temporal loading profiles and therefore may potentially be used as a biomarker for ischaemia. These two UHPSFC-MS methods afford robust, high throughput and quantitative assays for the separation and detection of metabolite biomarkers that show potential for the early diagnosis and prevention of pressure ulcers.

ACKNOWLEDGEMENTS

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CONFLICT OF INTEREST

The authors have declared no conflict of interest.

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SUPPORTING INFORMATION

The data that support the findings of this study are available from the corresponding author upon reasonable request

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Chapter 4 Publication 3: Development of Ultrahigh-Performance Liquid Chromatography- Mass Spectrometry and Ultrahigh-Performance Supercritical Fluid Chromatography-Mass Spectrometry Assays to Determine the Concentration of Bitrex™ and Sodium Saccharin in Homemade Facemask Fit Testing Solutions

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Author Contribution

Julie Herniman and John Langley designed and undertook the experimental work. Julie Herniman prepared the manuscript, John Langley provided supervision and editing of manuscript.

Signature

Julie Herniman

G. John Langley

Chapter 4

Development of Ultrahigh-Performance Liquid Chromatography- Mass Spectrometry and Ultrahigh-Performance Supercritical Fluid Chromatography-Mass Spectrometry Assays to Determine the Concentration of Bitrex™ and Sodium Saccharin in Homemade Facemask Fit Testing Solutions

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ABSTRACT

Rationale

Fast and easily transferable chromatography-mass spectrometry assays were required to detect and quantify the amount of Bitrex™ and sodium saccharin in homemade facemask fit testing solutions.

Methods

Bitrex™ solutions were analysed using reversed phase ultrahigh-performance liquid chromatography coupled with positive ion electrospray ionisation mass spectrometry (UHPLC-MS). Separation was achieved using a mobile phase gradient with an Acquity BEH C_{18} packed column. Sodium saccharin solutions were analysed using ultrahigh-performance supercritical fluid chromatography coupled with negative ion electrospray ionisation (UHPSFC-MS). Separation was achieved using isocratic elution with an Acquity UPC^2 Torus Diol packed column and a methanol (25 mM ammonium acetate) co-solvent.

Results

Calibration curves obtained using the ratio of the active compound an internal standard achieved $R^2 > 0.99$. Samples analysed prior to and after an autoclave sterilisation process and bottling give repeatable measurements within 10% of the expected concentration.

Conclusions

The two assays afford a fast robust and quantitative analytical method for the detection of the active components used to test the efficacy of the homemade facemask testing solutions.

1.INTRODUCTION

In extreme environments where airborne pollutants may be present *e.g.* toxic gases, fumes, vapours, and other harmful pollutants it is essential that personal protective equipment is worn and fitted correctly. In hospitals and care homes the use of respirators and face protection ensures that contaminants do not cause potential harm to the wearer. Masks work to protect individuals from spreading their own saliva and bodily fluids into the wider environment. Whilst a respirator face mask works to mitigate and filter out noxious and toxic chemicals/odours, vapours, and other harmful pollutants from the air. [1] All protective equipment must be fitted correctly and adjusted individually to the wearer's face. A Face Fit Test is normally undertaken prior to the respirator or facemask being worn and needs to be checked regularly. There are two tests as defined by Occupation Health and Standard Association (OSHA) in 29 CFR 1910.134. [2] The Qualitative Fit Test (QLFT) is a simple pass/fail test that measures the user's response to a test solution. The Quantitative Fit Test (QNFT) is an assessment of the adequacy of fit of the respirator by measuring the amount of leakage into the respirator. [3] Two different solutions are commonly used to assess disposable and reusable half-masks, denatonium benzoate otherwise known by the brand name Bitrex™ [4] and sodium saccharin. [5] The recent outbreak of COVID-19 in the UK has led to shortages of these commercial spray solutions as demand has significantly increased in-line with increased usage of masks and respirators. Chemists at the University of Southampton have produced homemade solutions [6] following a method adapted from Fakherpour *et al*. [7] and made to the British Standard BS ISO 16975-3:2017 [8] and the US standard set out by OHSA in 29 CFR 1910.134. [2]

An analytical quality control (QC) method was required to speedily assess the quantity of taste test compound in each prepared batch solution. This was required immediately after batch preparation and following an autoclave sterilisation (> 120 ° C) and subsequent bottling process. A minimum of 10 % of the bottles were tested prior to distribution.

The initial aim of the analytical QC methodology was to develop fast, easily transferable, fit for purpose tests that could be undertaken across many analytical laboratories. Reversed phase (RP) high or ultrahigh-performance liquid chromatography (UHPLC) is one of the most common instruments available to a QC laboratory, hence this separation technique coupled to mass spectrometry was initially attempted for the analysis of both solutions adapted from several published methods. [9-14]

Bitrex™ is a quaternary ammonium compound, therefore positive ion electrospray ionisation is required for the analysis and negative ion electrospray ionisation is required for the analysis of the sodium saccharin salt. (Figure 1)

A B

Figure 1. Chemical structures of A) Bitrex™ (Denatonium benzoate) and B) Sodium saccharin

Whilst reversed phase UHPLC-MS methods were successfully developed for both the Bitrex™ and sodium saccharin an alternative chromatographic technique, ultrahigh-performance supercritical fluid chromatography - mass spectrometry (UHPSFC-MS) was also investigated for both assays. This would maximise usage of available instrumentation at the University of Southampton, Chemistry Mass Spectrometry Laboratory. The two QC assays could then run in parallel and both assays could also be undertaken on either instrument. Here the optimum chromatography and mass spectrometry methods are reported for each assay (RP-UHPLC-MS for Bitrex™ and UHPSFC-MS for sodium saccharin). In its simplest form UHPSFC can be considered as a surrogate normal phase chromatography technique. Supercritical carbon dioxide ($scCO_2$) is the primary mobile phase used and is a supercritical fluid above its critical point of 31.1 °C and 73.8 bar where it has properties intermediate between those of a gas and a liquid. [15-18] For many reasons $scCO_2$ is the most commonly used mobile phase; its critical point is easily obtainable, it is readily available, inexpensive, considered green and relatively safe to use. [19]

UHPSFC can be easily coupled to a mass spectrometer using an atmospheric pressure ionisation source with a flow splitter. A make-up solvent, such as methanol with formic acid, is delivered to the mass spectrometer *via* a splitter configuration to promote ionisation and ensure a stable spray.

2. EXPERIMENTAL

2.1 Chemicals

Acetonitrile, methanol, water (LC-MS grade) and formic acid were purchased from Thermo Fisher Scientific (Loughborough, UK). Denatonium benzoate, oxybutynin chloride, vanillic acid and ammonium acetate were purchased from Sigma-Aldrich (Gillingham, UK). Sodium saccharin was obtained from Vimto Soft Drinks-Nichols plc (Newton-Le-Willows, UK) and Nutraceutical Group Europe (Redhill, UK) and used without further purification. Food grade carbon dioxide was purchased from BOC Special Gases (Manchester, UK).

2.2 Stock Solutions

The standard compounds (Bitrex™ and sodium saccharin) were prepared volumetrically at a concentration of 10 mg made up to 10 mL (methanol) to give stock solutions of 1mg/mL.

2.3 Internal Standard Preparation

The internal standard compounds (oxybutynin chloride and vanillic acid) were initially prepared at a concentration of 1mg/mL in methanol and then diluted using volumetric dilution to the appropriate concentration.

2.4 Calibration Preparation

Standard calibration solutions were prepared volumetrically for Bitrex™ containing nominally 20, 50, 100, 150 and 200 ng/mL with oxybutynin chloride as the internal standard at 100 ng/L. Standard calibration curves were prepared for sodium saccharin containing nominally 5, 10, 15, 20, 32.5 and 50 µg/mL with vanillic acid as the internal standard at 10 µg/mL. The concentration of the standards was selected to ensure that the calibration curves correspond to the linear ionisation response region of each instrument.

2.5 Sample Preparation

Each batch of Bitrex™ and sodium saccharin is prepared at two concentrations, one for SENSITIVITY (to test the response of an individual) and one TEST solution to test the facemask once it has been fitted to the individual. 1 mL of each batch solution is removed from the bulk and prepared for QC analysis. The Bitrex™ SENSITIVITY solution is prepared at a concentration of 135 mg/mL and this is serially diluted using methanol containing an internal standard oxybutynin chloride. The first step is a 1:1 dilution using the internal standard at 200 ng/mL followed by dilutions using the internal standard at 100 ng/mL to a concentration of 67.5 ng/mL. (x 2000 dilution). The TEST solution is at 1.69 g/L and this is serially diluted using methanol containing the same internal standard to a concentration of 84.7 ng/mL. (x 20,000 dilution) The saccharin SENSITIVITY solution is prepared at a concentration of 8.3 mg/mL and is serially diluted using methanol containing an internal standard vanillic acid. The first step is a 1:1 dilution using the internal standard at 20 µg/mL followed by dilutions using the internal standard at 10 µg/mL to a concentration of 10.4 µg/mL. (x 800 dilution) The TEST solution is at 536 mg/mL and is serially diluted using methanol containing the same internal standard to a concentration of 26.8 ug/mL. (x 20,000 dilution)

2.6 Chromatography

Separations were performed for the Bitrex™ samples using an Acquity ultrahigh- performance liquid chromatograph (Waters™, Manchester, UK.) with a Waters™ BEH C_{18} column, 1.7 µm particle size, 2 × 50 mm. The column was held at 50 °C in a column oven and 2.0 µL of each sample

was injected. Solvent A, water 0.2 % formic acid and solvent B, acetonitrile 0.2 % formic were used for separation at a flow rate of 0.6 mL/min. A gradient elution was performed using the method in Table 1 and a 1-minute isocratic pre-run was used for column equilibration.

Separations were performed for the sodium saccharin samples using an Acquity ultrahigh-performance convergence chromatograph (UPC^2, Waters™, Manchester, UK.) with a Waters™ Torus Diol packed column, 1.7 µm particle size, 3 × 100 mm. The column was held at 40 °C in a column oven and 2.0 µL of each sample was injected. $scCO_2$ with methanol (25 mM ammonium acetate) co-solvent were used for separation at a flow rate of 1.5 mL/min. The $scCO_2$ back pressure of the system was set to 150 bar. An isocratic elution was performed using co-solvent B at 40% for 1.5 minutes.

Time **min.**	**Solvent A** **%**	**Solvent B** **%**
0.00	80	20
1.30	0	100
1.35	0	100
1.50	80	20

Table 1. UHPLC gradient for the analysis of Bitrex™

2.7 Mass Spectrometry

Positive ion ESI mass spectra were recorded using a tandem quadrupole mass spectrometer (TQD) and ESCi multi-mode ionisation source (Waters™, Manchester, UK) with the following conditions: capillary voltage 2.5 kV; cone voltage 20 V; extractor 3.0 V; source temperature 150°C; desolvation temperature 600°C; desolvation gas flow 600 L/h (nitrogen) and acquisition and data processing was achieved using MassLynx™ version 4.1 and TargetLynx. Selected ion monitoring (SIM) was used to detect $[M]^+$ for Bitrex™, nominal *m/z* 325, and $[M + H]^+$ for the internal standard oxybutynin chloride nominal mass *m/z* 358 with a dwell time of 0.088 s.

Negative ion ESI mass spectra were recorded using a single quadrupole mass spectrometer (SQD 2) and ESCi multi-mode ionisation source (Waters™, Manchester, UK) with the following conditions: capillary voltage 2.5 kV; cone voltage 20 V; extractor 3.0 V; source temperature 150°C; desolvation temperature 500°C; desolvation gas flow 650 L/h (nitrogen) and acquisition and data processing achieved using MassLynx™ version 4.1 and TargetLynx. An isocratic solvent manager

(ISM) (Waters™, Manchester, UK) was used to introduce the make-up solvent, methanol (50 µM ammonium acetate) at a flow rate of 0.45 mL/min.

Selected ion monitoring (SIM) was used to detect the deprotonated molecules $[M - H]^-$ for saccharin, nominal *m/z* 182, and the internal standard vanillic acid, nominal *m/z* 167 with a dwell time of 0.163 s.

3. RESULTS AND DISCUSSION

3.1 Method development

In the first instance, commonly available reversed phase UHPLC-MS assays requiring minimal modification and customisation were used to facilitate the QC analysis for both compounds. Generic gradients were used, starting at 5% solvent B increasing to 100% solvent B over 5 minutes with a standard BEH C_{18} column. The first task was to identify a suitable internal standard for each assay. Oxybutynin chloride was selected for the Bitrex™ assay since this was readily available in the laboratory and is routinely used as a component of the system suitability test used for RP UHPLC-MS (Figure 2A).

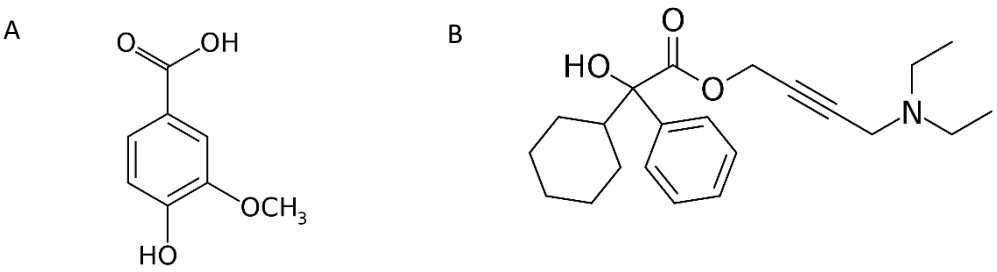

Figure 2. Chemical structures of internal standards A) Oxybutynin chloride and B) Vanillic acid

It has a similar retention time to the Bitrex™ and the positive ion electrospray ionisation efficiency closely matched to that of Bitrex™. To accelerate the analysis, the generic gradient was optimised to 20% solvent B increasing to 100% solvent B in 1.5 minutes with a 1-minute isocratic pre-run (Figure 3).

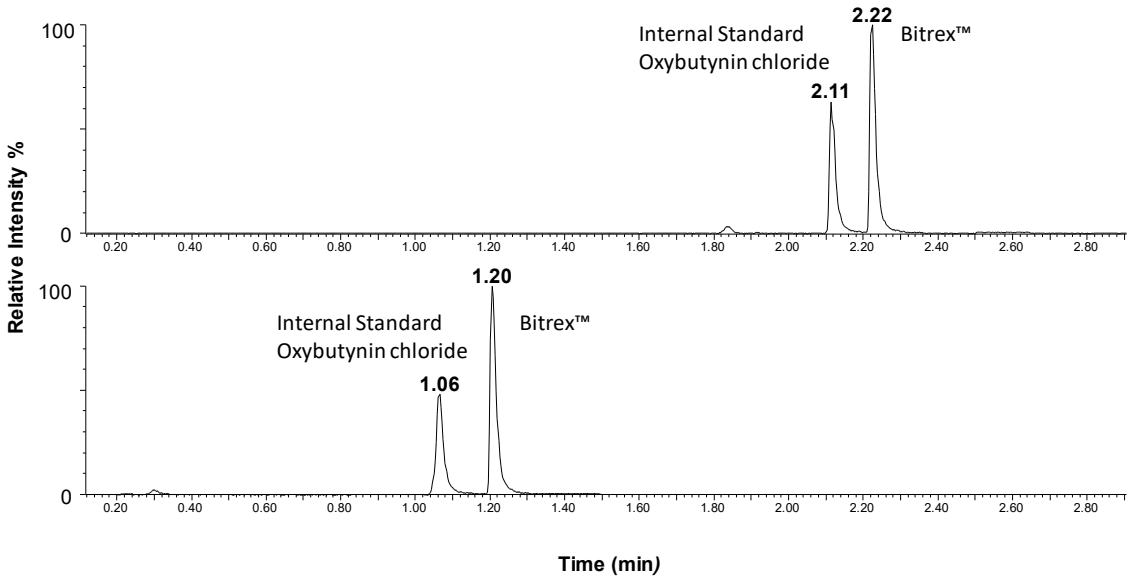

Figure 3. Reversed Phase UHPLC-MS SIR chromatograms for Oxybutynin chloride and Bitrex™ using A) 5-minute gradient and B) 1.5-minute gradient

The linear response for the Bitrex™ using this assay was defined using solutions of standard Bitrex™ ranging from 1 ng/mL to 1000 ng/mL. Single ion monitoring (SIM) for the molecule was used to improve peak profile and ensure that the methods could be transferred to other analysers. Once the linear response region has been defined, calibrations solutions were prepared (20-200 ng/mL) containing the internal standard at a concentration within the middle of the curve (100 ng/mL). A 5-point calibration curve was constructed automatically with TargetLynx using the ratio of the Bitrex™ to the internal standard, to give R^2 values of > 0.99. (Figure 4)

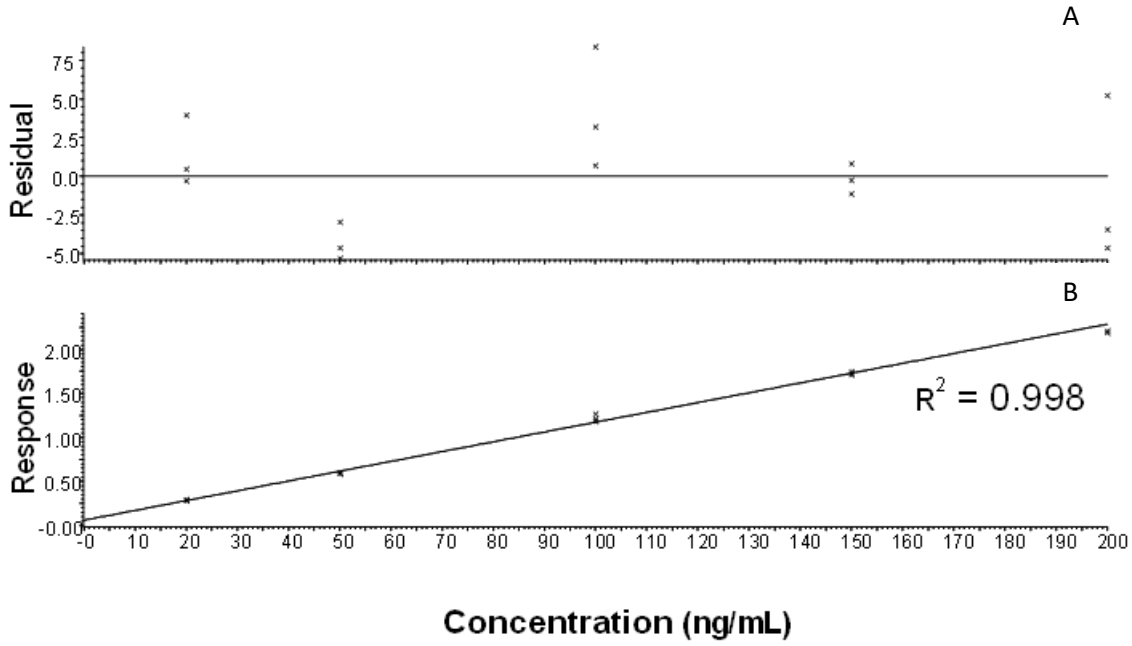

Figure 4. Calibration measurements for Bitrex™ using oxybutynin chloride as the internal standard A) residuals and B) calibration curve produced using TargetLynx.

The same assay was attempted for the analysis of the sodium saccharin using negative ion electrospray ionisation, however the peak shape for sodium saccharin was initially poor. This was somewhat improved by changing to water as the internal standard diluent to give a fit for purpose assay. Saccharin analysis was developed using an in-house UHPSFC-MS assay, 10% co-solvent (methanol 25 mM ammonium acetate) increasing to 40% solvent co-solvent over 5 minutes using a Torus Diol column. Vanillic acid was selected as the internal standard for this assay since this was readily available in the laboratory, the negative ion electrospray ionisation closely matched to that of sodium saccharin and gave similar good chromatographic peak shape (Figure 2B). To accelerate the analysis, the assay was optimised using an isocratic 40% co-solvent method for 1.5 minutes (Figure 5) hence removing the need for a pre-run column equilibration method. The linear response for the sodium saccharin using this assay was defined using solutions of standard sodium saccharin ranging from 1 µg/mL to 1000 µg/mL using SIM for the deprotonated molecule, to improve peak profile. Once the linear response region has been defined, calibrations solutions were prepared (5-50 µg/mL) containing the internal standard at a concentration in the middle of the curve (20 µg/mL). A 5-point calibration curve was constructed automatically with TargetLynx using the ratio of the sodium saccharin to the internal standard, to give R^2 values of > 0.99.

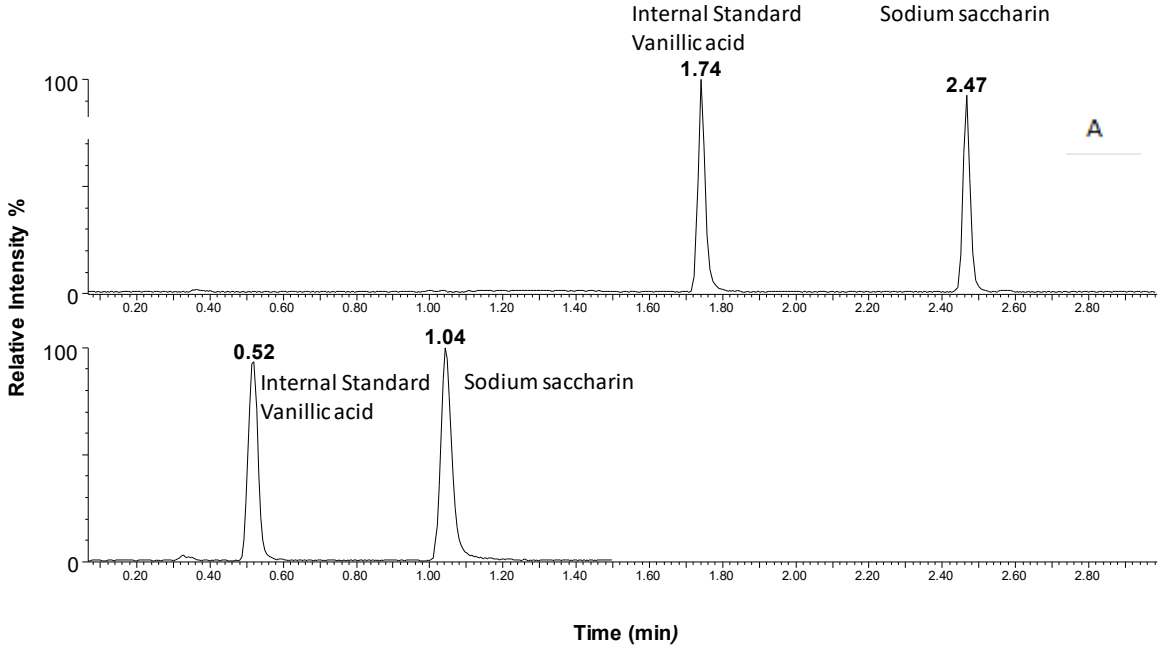

Figure 5. UHPSFC-MS SIR chromatograms for Vanillic acid and Sodium saccharin using A) 3-minute gradient and B) isocratic elution

3.2 Application of methodology

Following preparation of the bulk solutions by the University of Southampton chemists, 1 mL of solution is removed ready for QC analysis. A calibration curve is created with the standards over the linear response range of the instrument prior to analysis and this must give an R^2 value > 0.99 before proceeding. If the R^2 value is < 0.99 then the calibration curve must be repeated and adjusted if necessary. Each solution is diluted with the pre-prepared internal standard solution in methanol to the appropriate concentration (Bitrex™, 67.5 and 84.75 ng/mL SENSE and TEST respectively, sodium saccharin 10.4 and 26.8 µg/mL SENSE and TEST respectively). These pre-autoclave solutions are then measured in triplicate ensuring a solvent blank is analysed between each sample to monitor and prevent carry over. The data are plotted against the calibration curve using TargetLynx and the results recorded. If the required concentration is calculated to be within10-12% of the required concentration, then the batch solution can proceed to the bottling and autoclave stage. Following the autoclave stage, a minimum of 10% of bottles are randomly selected and 1 mL of solution is removed for QC analysis. These solutions are diluted to the appropriate concentration using the internal standard solution in methanol. These samples are analysed in triplicate ensuring a solvent blank in between each analysis to monitor and prevent carry over. The data are plotted against the calibration curve using TargetLynx and the results recorded. If the required concentration is calculated to be within 10% of the required concentration, then the bottled solutions can be released for distribution. (Tables 2 and 3)

Bitrex™ Batch	Bulk Prepared Concentration g/L	Diluted Concentration ng/mL	Pre-Autoclave Measured ng/mL	Pre-Autoclave Calculated g/L	Post-Autoclave Measured ng/mL	Post-Autoclave Measured g/L
TEST	1.69	84.7	75.5±0.4	1.51	75.7±5.5	1.51
SENSE	0.135	67.5	67.2±0.9	0.134	62.9±4.2	0.126

Table 2. Quality control measurements and calculations for one batch of Bitrex™ TEST and SENSITIVITY (5 L)

Sodium saccharin Batch	Bulk Prepared Concentration g/L	Diluted Concentration µg/mL	Pre-Autoclave Measured µg/mL	Pre-Autoclave Calculated g/L	Post-Autoclave Measured µg/mL	Post-Autoclave Measured g/L
TEST	536*	26.8	26.6±1.1	532	25.0±2.0	501
SENSE	0.008	10.4	9.3±0.7	0.007	10.7±0.4	0.008

*calculated applying theoretical adjustment of solute volume change

Table 3. Quality control measurements and calculations for one batch of sodium saccharin TEST and SENSITIVITY (5 L)

CONCLUSIONS

The reversed phase UHPLC-MS and UHPSFC-MS assays developed here deliver fast, robust and quantitative for the analysis of homemade Bitrex™ and sodium saccharin solutions. Calibration curves acquired using SIM methods over the linear response range for each instrument give R^2 values > 0.99. If UHPSFC-MS is not available, then an alternative reversed phase UHPLC-MS method could be utilized providing calibration and samples solution are made up using water rather than methanol as a solvent.

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Appendix A Other Peer Reviewed Publications

A.1 Relevant UHPSFC-MS Publications (4)

1. Garcia, S. B.; Herniman, J.; Birkin, P.; Pilgrim, J.; Lewin, P.; Wilson, G.; Langley, G. J.; Brown R. C. D., Quantitative UHPSFC-MS Analysis of Elemental Sulfur in Mineral Oil *via* Derivatisation with Triphenylphosphine: Application to Corrosive Sulfur-Related Power Transformer Failure. *Analyst* **2020**, *145*, 4782-4786. (doi.org/10.1039/d0an00602e)
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22. Black, C.; Poile, C.; Langley, J.; Herniman, J., The Use of Pencil Lead as a Matrix for Matrix-assisted Laser Desorption/Ionisation (MALDI) Calibration and Analysis of Actinides and Small Molecules. *Rapid Commun. Mass Spectrom*. **2006**, *20*, 1053-1060.
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24. Herniman, J. M.; Bristow, T. W. T.; O'Connor; G.; Jarvis; J.; Langley, G. J., Improved Precision and Accuracy for High Performance Liquid Chromatography-Fourier Transform Ion Cyclotron Resonance Mass Spectrometry Exact Mass Measurement of Small Molecules from the Simultaneous and Controlled Introduction of Internal Calibrants *via* a Second Electrospray Nebuliser. *Rapid Commun. Mass Spectrom*. **2004**, *18,* 3035-3040.
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Appendix B Conference Presentations

B.1 SFC and UHPSFC-MS Conference Presentations (24)

Poster unless stated

1. Cancho Gonzalez, S.; Ferguson, P.; Bailes, S.; Herniman, J. M.; Langley G. J., Investigation of UHPSFC-MS as a Tool to Understand Challenging Polymers used in the Pharmaceutical Industry. 39th Meeting of the British Mass Spectrometry Society; Manchester; September 2019.
2. Panagiotopoulos, A.; Barker, J.; Reid, J.; Herniman, J. M.; Langley G. J., Application of UHPSFC-MS in Petroleomics: Analysis of Gasoline Gum Content. 39th Meeting of the British Mass Spectrometry Society; Manchester; September 2019.
3. Herniman, J. M.; Franco, P.; Langley, G. J.; Attard, G.; Roda A., The Analysis of Archaeological Lipids using UHPSFC-MS. 12th International SFC Green Chemistry Meeting; Strasbourg; France; October 2018.
4. Herniman, J. M.; Franco, P.; Langley, G. J.; Attard, G.; Roda A., The Analysis of Archaeological Lipids using UHPSFC-MS. 66th ASMS Conference on Mass Spectrometry; San Diego; USA; June 2018.
5. Langley, G. J.; Herniman, J. M.; Wilmot, E.; Barker, J., Exploiting API; UHRMS and UHPSFC-MS to Unlock Complexity to Detect and Understand Fuel Additives. 66th ASMS Conference on Mass Spectrometry; San Diego; USA; June 2018.
6. Herniman, J. M.; Langley G. J., The Analysis of Steroidal Compounds using UHPSFC-MS Hyphenated Techniques in Chromatography 15; Cardiff; Wales; January 2018. **ORAL**
7. Lingaityte, D.; Carr, R.; Herniman, J. M.; Langley G. J., UHPSFC-APPI-MS of Complex MDI Oligomers. Hyphenated Techniques in Chromatography 15; Cardiff; Wales; January 2018.
8. Herniman, J. M.; Langley, G. J., The Analysis of Steroidal Compounds using UHPSFC-MS; 11th International SFC Green Chemistry Meeting; Washington; USA; October 2017. **ORAL**
9. Herniman, J. M.; Langley, G. J.; Attard, G.; Franco, P.; Patel K., Coupling UHPSFC to MS for the Analysis of Steroidal Compounds. 37th Meeting of the British Mass Spectrometry Society; Manchester; September 2017.
10. Herniman, J. M.; Langley, G. J.; Carr, R., Coupling UHPSFC to APPI-MS to Analyse Methylene Diphenyl Diisocyanates. 10th International SFC Green Chemistry Meeting; Vienna; Austria; October 2016.

11. Langley, G. J.; Herniman, J. M.; Elia, E.; Sprake, E.; Jenkins, T.; Durnie, W., Ionisation Enhancement Through use of a Supercritical Fluid and a Novel Ionisation Technique. 21st International Mass Spectrometry Conference; Toronto; Canada; August 2016.
12. Herniman, J. M.; Langley, G. J.; Jenkins, T., Expanded Analytical Capability Through the Use of Open Access UHPSFC-APPI-MS. 21st International Mass Spectrometry Conference; Toronto; Canada; August 2016.
13. Langley. G. J.; Herniman, J. M.; Elia. E.; Sprake. E.; Jenkins. T.; Durnie, W., Ionisation Enhancement Through Use of a Supercritical Fluid and a Novel Ionisation Technique. 64th ASMS Conference on Mass Spectrometry; San Antonio; USA; June 2016.
14. Herniman, J. M.; Langley G. J.; Carr R. The Characterisation of Polymeric Methylene Diphenyl Diisocyanates using UHPSFC and FT-ICR APPI-MS. 64th ASMS Conference on Mass Spectrometry; San Antonio; USA; June 2016.
15. Herniman, J. M.; Greenhill, R.; Worsley, P.; Bader, D.; Jenkins, T.; Langley G. J., The Analysis of Sweat Biomarkers using UHPSFC-MS. 35th Meeting of the British Mass Spectrometry Society; Birmingham; September 2015.
16. Herniman, J. M.; Greenhill, R.; Worsley, P.; Bader, D.; Jenkins, T.; Langley G. J., The Analysis of Sweat Biomarkers using UHPSFC-MS. 63rd ASMS Conference on Mass Spectrometry; St. Louis; USA; June 2015.
17. Herniman, J. M.; Jenkins, T.; Langley, G. J., Expanded Capability Through the Application of UPC^2-APPI MS. 8th International SFC Green Chemistry Meeting; Basel; Switzerland; October 2014.
18. Herniman, J. M.; Langley, G.J., Utilising UPC^2-MS in a Chemistry Environment. 20th International Mass Spectrometry Conference; Geneva; Switzerland; August 2014.
19. Langley, G. J.; Herniman, J. M.; Ratsameepakai, W.; Fitt, M.; Jenkins T., Qualitative and Quantitative Application of UPC^2-MS to Biofuels Screening. 20th International Mass Spectrometry Conference; Geneva; Switzerland; August 2014.
20. Herniman, J. M.; Langley G. J., Utilising UPC^2 and MS for the Analysis of Biofuels. Hyphenated Techniques in Chromatography 13; Bruges; Belgium; January 2014. **ORAL**
21. Ratsameepakai, W.; Herniman, J. M.; Jenkins T.; Langley G. J., Ultra-Performance Convergence Chromatography-Electrospray Ionisation/Mass Spectrometry (UPC^2-ESI/MS) for Analysis of Trace Fatty Acid Methyl Esters (FAMEs) in Aviation Fuels. Hyphenated Techniques in Chromatography 13; Bruges; Belgium; January 2014.
22. Herniman, J. M.; Langley, G. J., Utilising UPC^2 and MS in a Chemistry Environment. 33rd Meeting of the British Mass Spectrometry Society; Eastbourne; September 2013. **ORAL**
23. Herniman, J. M.; Langley, G.J.; Potter, M.; Newland, S.; Raja, R.; Jenkins, T., Analysis of Catalytic Conversion of Biomass Feedstocks and Products by UPC^2-MS. 61st ASMS Conference on Mass Spectrometry; Minneapolis; USA; June 2013.

24. Herniman, J. M; Racine, T.; Langley, G. J., SFC-MS Screening of Sugars from Renewable Feedstocks for n-Butanol Production. 60th ASMS Conference on Mass Spectrometry; Vancouver; Canada; June 2012.

B.2 Other Conference Presentations (38)

Poster unless stated

1. Herniman, J. M.; Langley G. J., Is my Decaffeinated Coffee Caffeine-Free? Engaging Chemistry Undergraduates with Mass Spectrometry and Chromatography. Hyphenated Techniques in Chromatography 16; Ghent; Belgium; January 2020.
2. Herniman, J. M.; Langley, G. J; Bloodworth, S.; Whitby, R. J.; Sitinova G., Mass Spectrometry Analysis of Fullerenes and Endohedral Fullerenes. 39th Meeting of the British Mass Spectrometry Society; Manchester; September 2019. **ORAL**
3. Herniman, J. M.; Langley, G. J.; Bloodworth, S.; Whitby, R. J.; Sitinova G., APPI-MS Analysis of Endohedral Fullerenes. 67th ASMS Conference on Mass Spectrometry; Atlanta; USA; June 2019. **ORAL**
4. Herniman, J. M.; Langley, G. J.; Elves, P.; Hopley, C., Can ASAP Provide a Quantitative Screen for Rapid Detection of Drugs in Saliva? 21st International Mass Spectrometry Conference; Toronto; Canada; August 2016.
5. Ratsameepakai, W.; Wicking, C.; Herniman, J. M.; Whitmarsh, S.; Lambert, S.; Langley, G.J., Hyphenation of on-line EC/ESI-Q-TOF/MS for the Generation and Identification of FAME Oxidation Products. 34th Meeting of the British Mass Spectrometry Society; Eastbourne; September 2013.
6. Ratsameepakai, W.; Wicking, C.; Herniman, J. M.; Lambert S.; Langley, G.J., Hyphenation of On-line EC-ESI-TOF-MS: An Analytical Method for Monitoring FAME Oxidation. Warwick Mass Spectrometry 80/60 Conference; Warwick; UK; December 2012.
7. Herniman, J. M.; Langley G. J., The Implementation of High Throughput Screening of Modified Synthetic Oligonucleotides. 59th ASMS Conference on Mass Spectrometry; Denver; USA; May 2011.
8. Herniman, J. M.; Langley G. J., An Automated Approach for Routine Analysis of Oligonucleotides. 31st Meeting of the British Mass Spectrometry Society; Cardiff; September 2010. **ORAL**
9. Herniman, J. M.; Langley G. J., A Single Solution to Walk-Up Mass Spectrometry Access and Data Presentation. 58th ASMS Conference on Mass Spectrometry; Salt Lake City; USA; May 2010.

10. Herniman, J. M.; Langley G. J., Mass Spectrometry and Undergraduate Chemistry: Development; Simple Access; Time Efficient and Subliminal Training. 18th International Mass Spectrometry Conference; Bremen; Germany; September 2009.
11. Langley, G. J.; Herniman, J. M.; Wicking, C. C.; Lynch, T., Biodiesel: Profiling; Stability and MS Solutions. 57th ASMS Conference on Mass Spectrometry; Philadelphia; USA; June 2009.
12. Herniman, J. M.; Langley G. J.; Lynch T., MALDI-TOF Screening of Aged Biofuels. 57th ASMS Conference on Mass Spectrometry; Philadelphia; USA; June 2009.
13. Langley, G. J.; Herniman, J. M.; Gower M.; Lamond S.; McMahon P.; Lynch T.; Preston H., Biofuels and the Analytical Challenge. 30th Meeting of the British Mass Spectrometry Society; York; September 2008.
14. Wronska, L. V. M; Langley, G. J.; Herniman, J. M.; O'Connor, G., An Uncertain Future for Accurate Mass Measurements? 30th Meeting of the British Mass Spectrometry Society; York; September 2008.
15. Langley, G. J.; Herniman, J. M.; Gower, M.; Lamond, S.; McMahon, P.; Lynch, T.; Preston H., First Generation Biofuels: Simple Mixtures - Complex Analytics. 56th ASMS Conference on Mass Spectrometry; Denver; USA; June 2008.
16. Herniman, J. M.; Langley G. J., First Investigations of Quantitative Pencil-Assisted Laser Desorption/Ionisation (PALDI). 56th ASMS Conference on Mass Spectrometry; Denver; USA; June 2008.
17. Wronska, L. V. M; Langley, G. J.; Herniman, J. M.; O'Connor G., A Study of the Factors & Protocols that Influence Accurate Mass Measurement by FT-ICR MS to Improve the Confidence of Assignment of Elemental Composition. 29th Meeting of the British Mass Spectrometry Society; Edinburgh; September 2007.
18. Herniman, J. M.; Langley, G. J.; Mellor, J.; Klagkou, K., The Analysis of Traditional Herbal Plants from Eritrea, East Africa using GC-MS, HPLC-ESI-MS and HPLC-ESI-MS/MS. 55th ASMS Conference on Mass Spectrometry; Indianapolis; USA; June 2007.
19. Langley, G. J.; Herniman, J. M.; Townell M. S.; Black C.; Poile C., Further Investigations into the Use of Pencil Lead as a MALDI-TOFMS Matrix. 17th International Mass Spectrometry Conference; Prague; Czech Republic; September 2006.
20. Herniman, J. M.; Langley, G. J.; Bristow, T.; O'Connor G., Improved Confidence in the Exact Mass Measurement of Small Molecules. 17th International Mass Spectrometry Conference; Prague; Czech Republic; September 2006.
21. Hopley, C.; Bristow, T.; Lubben, A.; Simpson, A.; Bull; E.; Klagkou, K.; Herniman, J. M.; Langley, J., Reproducibility of Product Ion MS Spectra: Towards a Universal Product Ion MS Library. 17th International Mass Spectrometry Conference; Prague; Czech Republic; September 2006.

22. Langley, G. J.; Herniman, J. M.; Townell, M. S.; Black C.; Poile C., Further Investigations into the Use of Pencil Lead as a MALDI-TOFMS Matrix. 54th ASMS Conference on Mass Spectrometry; Seattle; USA; June 2006.
23. Langley, G. J.; Bristow, T. W. T.; Herniman, J. M.; O'Connor, G., The Validation of Exact Mass Measurements for Small Molecules using FT-ICRMS for Improved Confidence in the Selection of Elemental Formulae. 5th North American FT-ICR MS Meeting. Key West; Florida U.S.A; April 2005.
24. Hopley, C.; Bristow, T.; Lubben, A.; Simpson, A.; Bull, E.; Klagkou, K.; Herniman, J. M.; Langley, J., Towards a Universal MS/MS library. 28th Meeting of the British Mass Spectrometry Society; York; September 2005.
25. Campbell, S. J.; Herniman, J. M.; Langley G. J., Simplified Multi-Vendor Open Access Mass Spectrometry *via* an Easy to Use Web Interface. 28th Meeting of the British Mass Spectrometry Society; York; September 2005.
26. Herniman, J. M.; Bristow, T. W. T; Jarvis; J.; Langley, G. J.; O'Connor; G., The Use of a Second ESI Sprayer for the Separate and Controlled Introduction of a Calibration Mixture for FT-MS accurate Mass Measurement. 27th Meeting of the British Mass Spectrometry Society; Derby; September 2004.
27. Herniman, J. M.; Bristow T. W. T.; Langley G. J. O'Connor G., A Study of Factors Influencing Mass Accuracy and Precision Using FT-MS. 27th Meeting of the British Mass Spectrometry Society; Derby; September 2004.
28. Langley, G. J.; Herniman, J. M., Initial Experiences of FT-MS with HPLC. HTC-8; Bruges; January 2004.
29. Herniman, J. M.; Langley, G. J., An Open Access Fourier Transform Mass Spectrometry (FTMS) approach to address a High Throughput Analytical Bottleneck in Academia. 16th International Mass Spectrometry Conference; Edinburgh; September 2003.
30. Langley, G. J.; Herniman, J. M., High-Throughput Synthesis and Analysis: Routine and Research Perspectives. LabAutomation; Palm Springs; 2003.
31. Ball, R. J.; Langley, G. J.; Brown, T.; Herniman, J. M., Identification of Single Nucleotide Polymorphisms using Mass Tagged Hybridisation Probes. 26th Meeting of the British Mass Spectrometry Society; Loughborough; September 2002.
32. Herniman, J. M.; Langley, G. J., Automated Flow Injection Analysis (FIA) for High Resolution Mass Spectrometry (HRMS): First Steps towards Open Access Fourier Transform Mass Spectrometry. 26th Meeting of the British Mass Spectrometry Society; Loughborough; September 2002.

33. Herniman, J. M.; Langley G. J.; Lawlor S. E.; Mellor J. M., The Use of ES-MS to Effectively Determine the Metal Binding Affinities and Selectivities of a Tetramine Series. 25th Meeting of the British Mass Spectrometry Society; Southampton; September 2001.
34. Herniman, J. M.; Langley G. J.; Pullen F. S., Open Access Mass Spectrometry: Its Role in Academia. 25th Meeting of the British Mass Spectrometry Society; Southampton; September 2001.
35. Lander, C. P.; Langley, G. J.; Brown, T.; Herniman, J. M.; Brown, L. J.; McKeen, C., Mass Tagged Hybridisation Probes for Gene Mutation Analysis. 15th International Mass Spectrometry Conference; Barcelona; August 2000.
36. Herniman, J. M.; Langley G. J., Analysis of Organometallic Species by MALDI-TOFMS. 15th International Mass Spectrometry Conference; Barcelona; August 2000.
37. Langley, G. J.; Herniman, J. M.; Davies, N. L.; Brown T., Improved Detection and Resolution using a Novel In-situ Sample Treatment to aid DNA Screening by MALDI-TOFMS. 24th Meeting of the British Mass Spectrometry Society; Reading; September 1999.
38. Langley, G. J.; Herniman, J. M.; Davies, N. L.; Clarke, G. T.; Brown T., A Comparison between two methods for the sequencing of Oligonucleotides by MALDI-TOFMS. 24th Meeting of the British Mass Spectrometry Society; Reading; September 1999.

B.3 Tutorial Lecture

1. Herniman, J. M.; Langley, G. J., Coupling LC to MS. Hyphenated Techniques in Chromatography 15; Cardiff; Wales; January 2018. **ORAL**