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 samples 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 C18 and C4 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.

The most popular column used for reversed-phase chromatography is the C18 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 C18 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 C18, 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* 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 C18 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).

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.

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 negative 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.

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 C18 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.

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 C18 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.

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}.

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.

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LIST OF FIGURES

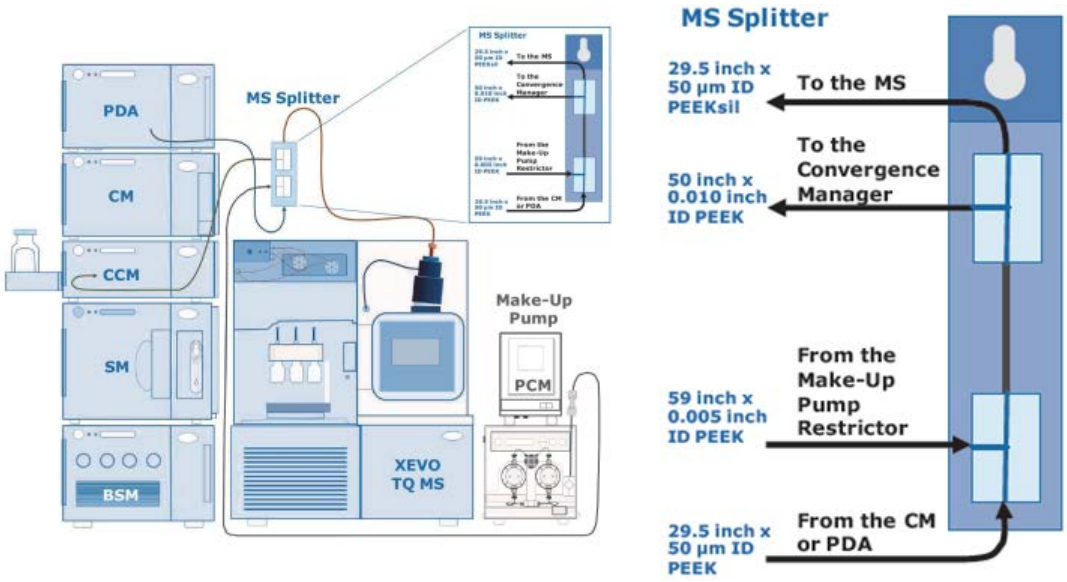

Figure 1. Schematic of UPC^2-MS system with expanded splitter configuration inset courtesy of Waters.

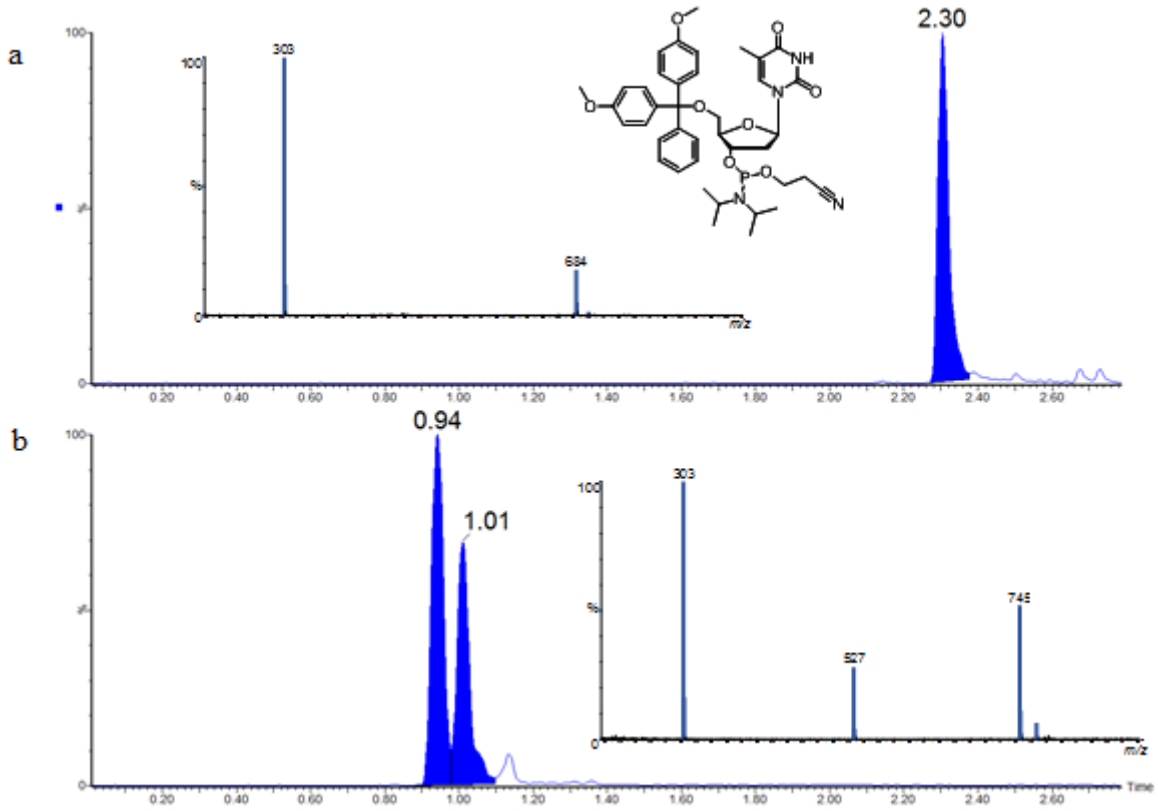

Figure 2. Positive ion electrospray ionization base peak ion current chromatograms for a phosphoramidite (M_r 744) analysed using a) reversed-phase UHPLC/MS and b) UHPSFC/MS.

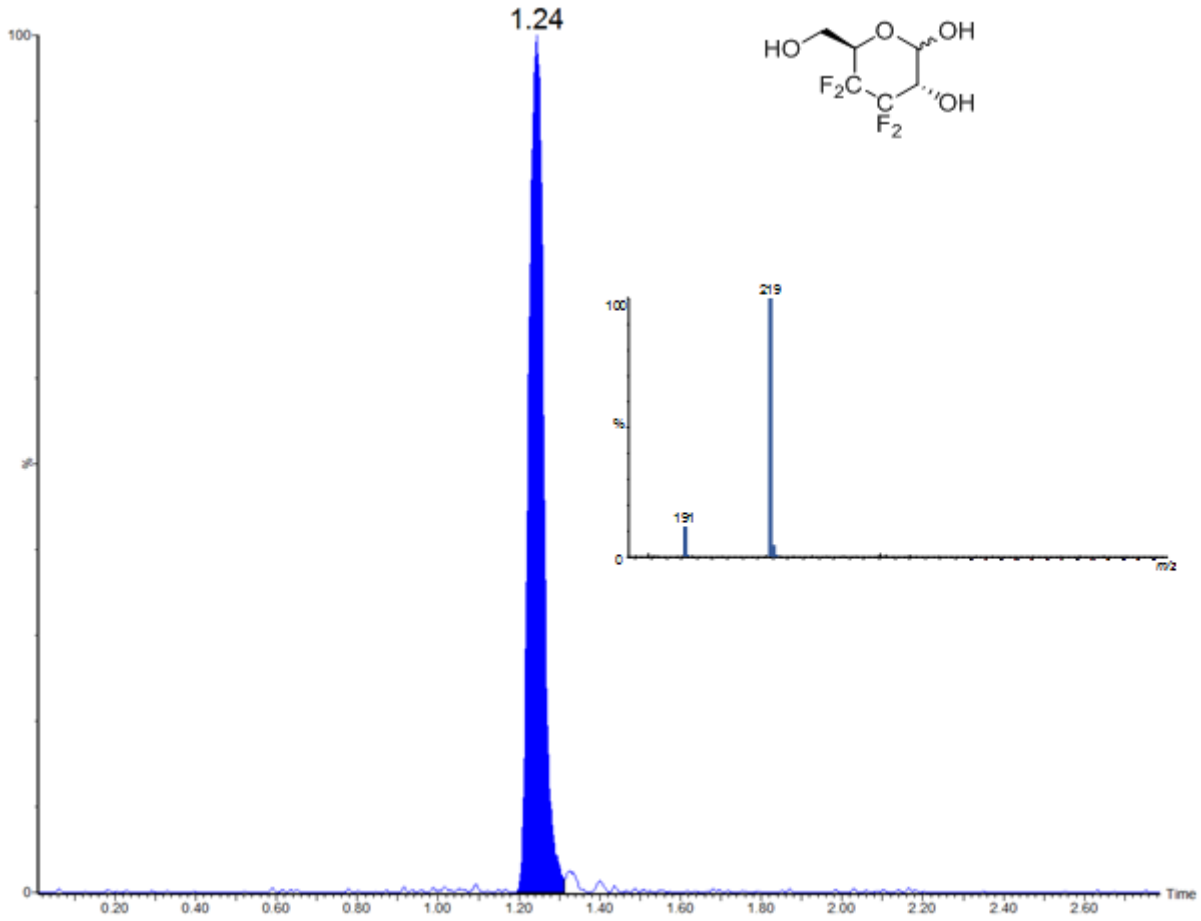

Figure 3. Negative ion electrospray ionization reconstructed ion current chromatogram for a fluorosugar (M_r 220) analysed using UHPSFC/MS.

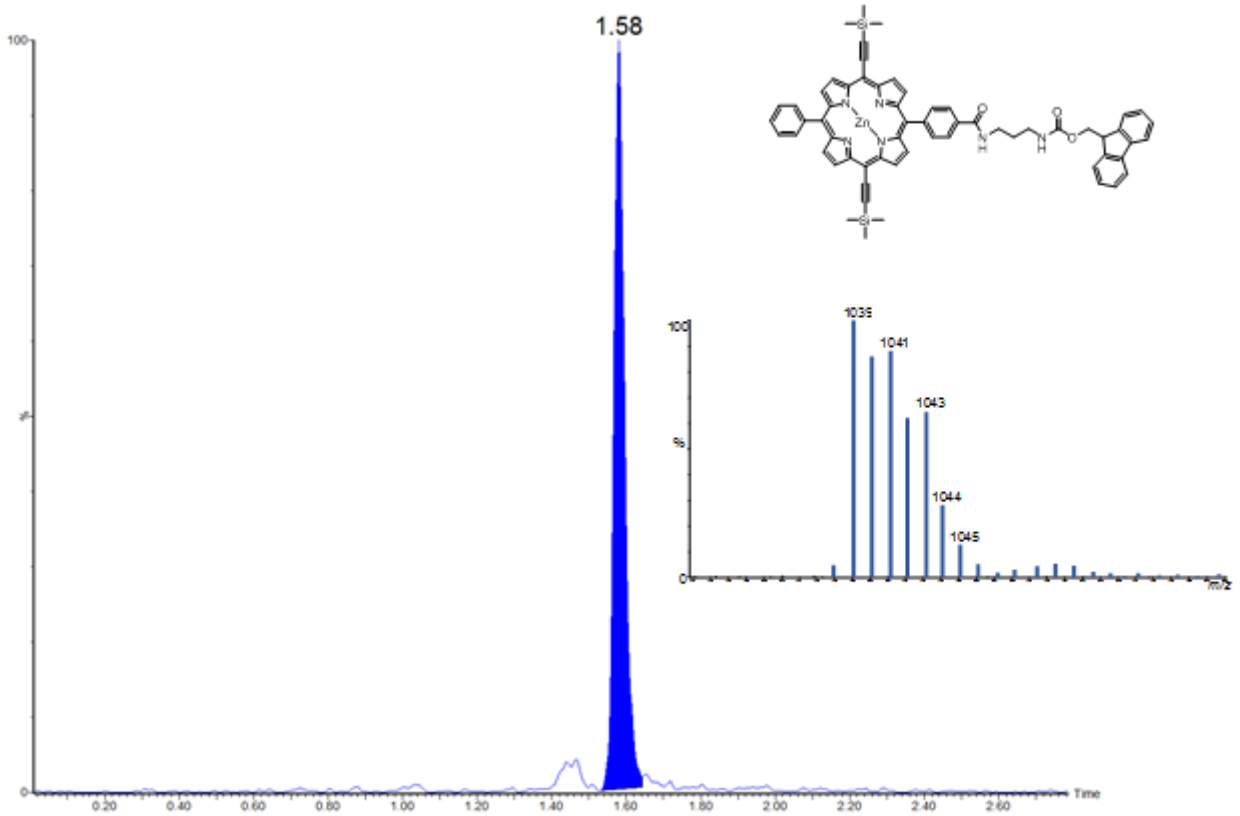

Figure 4. Positive ion electrospray ionization reconstructed ion current chromatogram for a zinc containing porphyrin (M_r 1038) analysed using UHPSFC/MS.

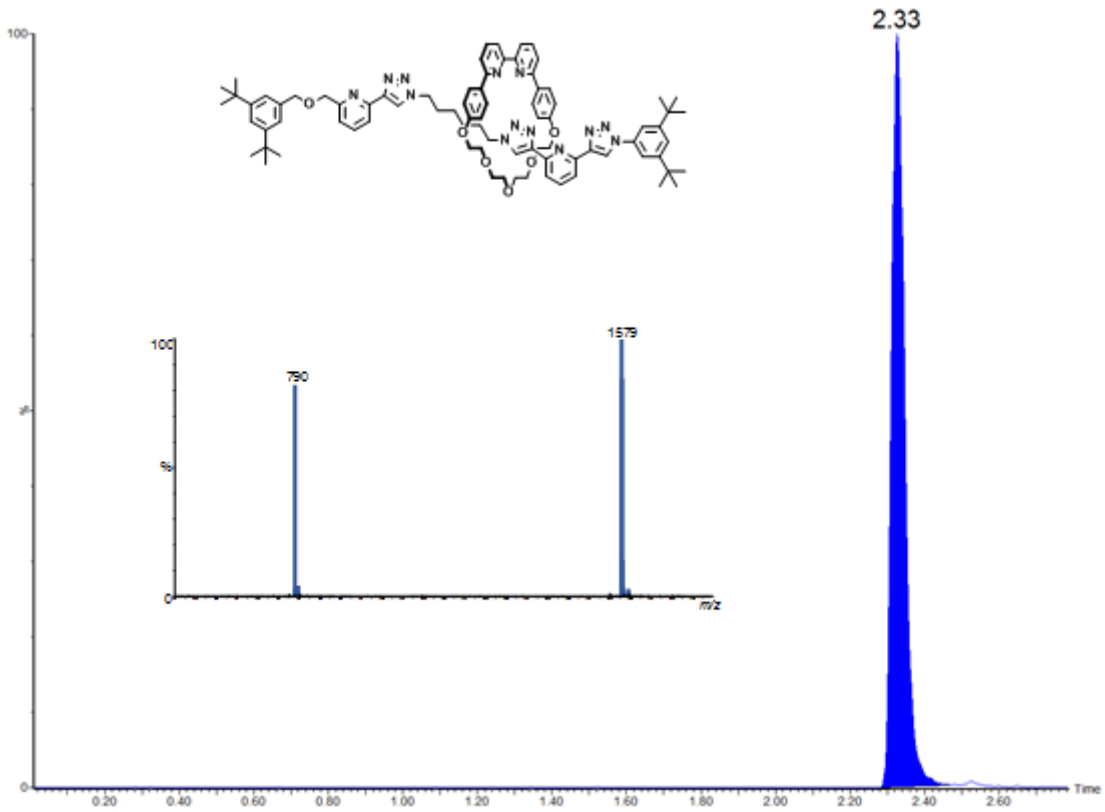

Figure 5. Positive ion electrospray ionization reconstructed ion current chromatogram for a rotaxane (M_r 1578) analysed using UHPSFC/MS.

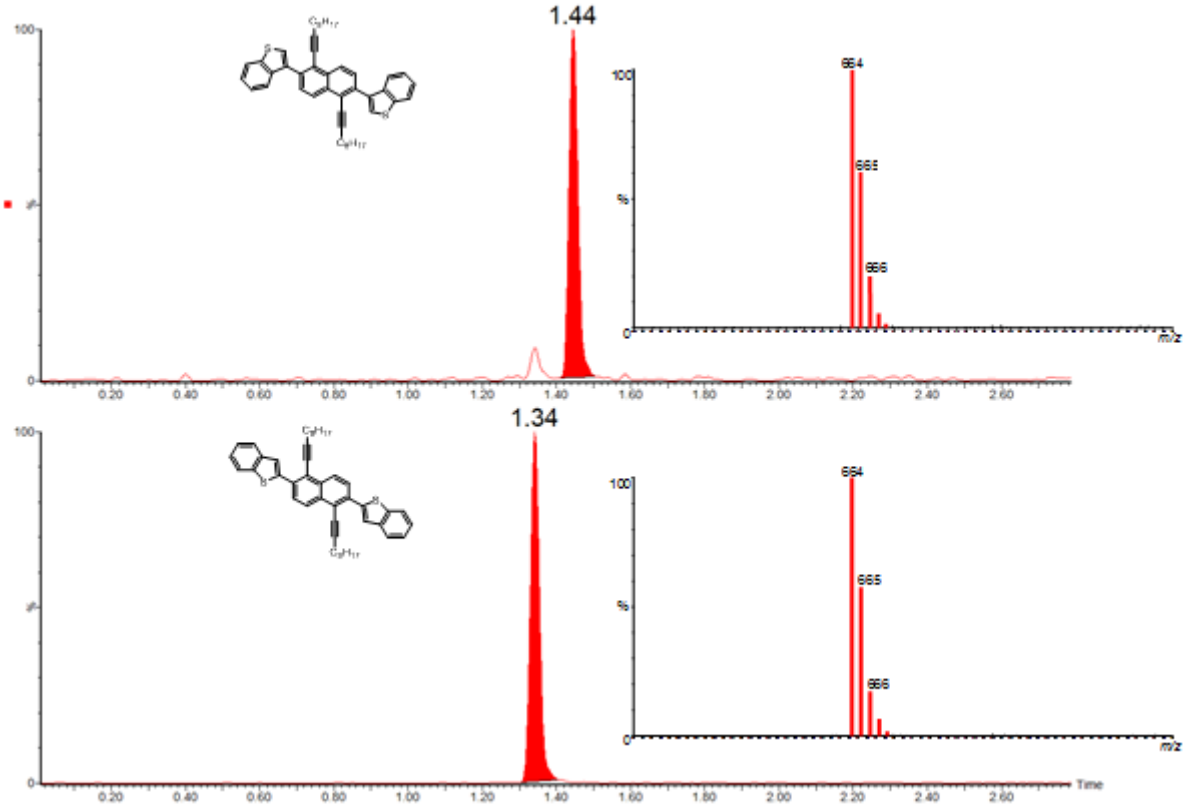

Figure 6. Positive ion atmospheric pressure photoionization reconstructed ion current chromatograms of molecular wires isomers (M_r 664) analysed using UHPSFC/MS.

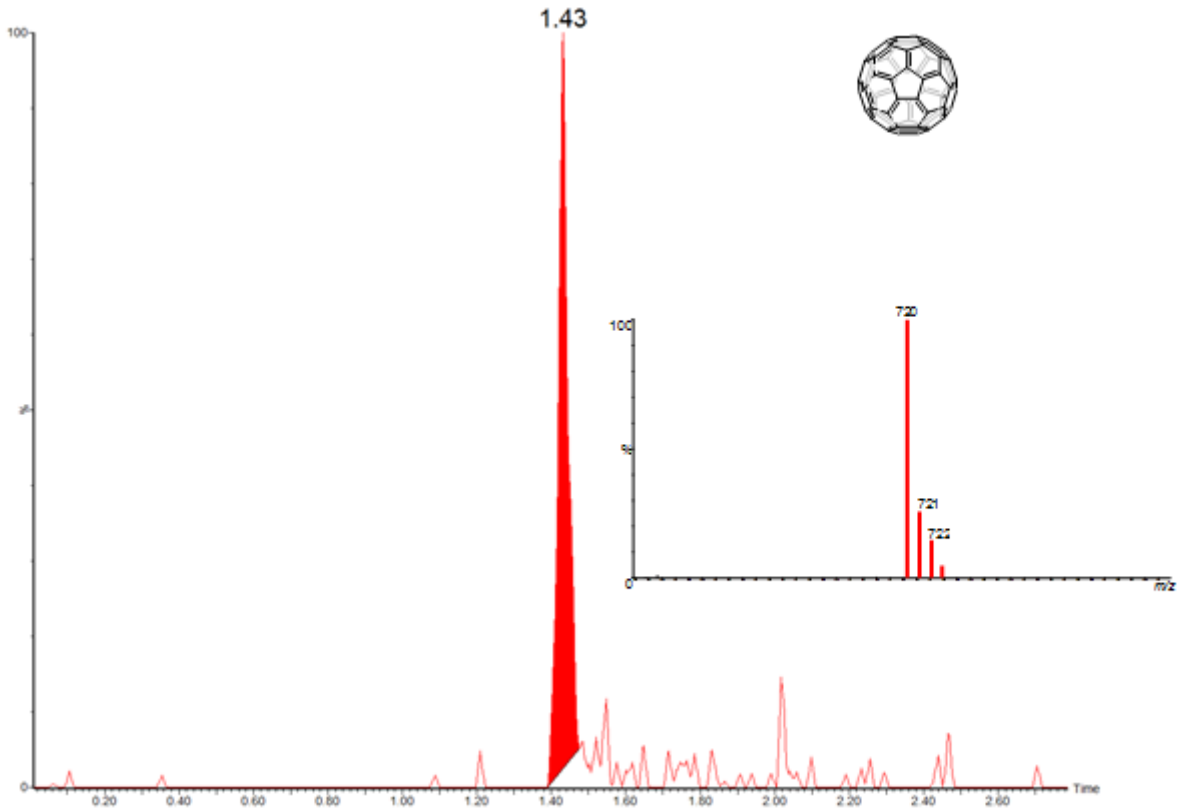

Figure 7. Positive ion atmospheric pressure photoionization reconstructed ion current chromatogram for fullerene C_{60} (M_r 720) analysed using UHPSFC/MS.