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AN OVERVIEW ON HIGH PERFORMANCE LIQUIDCHROMATOGRAPHY- MASS SPECTROSCOPY

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ABSTRACT

Ultra high performance liquid chromatography\ms is an extremely versatile instrumental technique. As the name suggested the instrumentation comprises a high performance liquid chromatography attached, via suitable interface, to a mass spectrometer. The primary advantage UHPLC/MS has over GC/MS is that it is capable of analyzing a much wider range of components. Compounds that are thermally liable, exhibit high polarity or have a high molecular mass may all be analyzed using UHPLC/MS even proteins may be routinely analyzed. Ultra high performance liquid chromatography (UHPLC) performs separations 5 to 10 times faster than conventional HPLC by employing sub-2 µm diameter particles. The 1-2 second peak widths and relatively high separation efficiency of UHPLC are more competitive with capillary GC, making UHPLC-MS an attractive alternative method for illicit drug analysis. The columns packed with porous sub-2µm particles and the extension of the upper pressure limit of HPLC instrumentation to 1300bar (ultra-high pressure liquid chromatography, UHPLC) has opened new frontiers in resolution and speed of analysis. However, certain constraints appear when coupling UHPLC technology with mass spectrometry (MS). Firs Chromatography (HPLC) directly coupled to mass spectrometry (MS) was in routine use in drug metabolism laboratories. It can give Enhanced selectivity and sensitivity, and rapid, generic analysis.

Keywords: Ultra performance, Spectroscopy, Sensitivity, Ionization, Analiser.

INTRODUCTION

As the name suggested the instrumentation comprises a high performance liquid chromatography attached, via suitable interface, to a mass spectrometer. The primary advantage UHPLC/MS has over GC/MS is that it is capable of analyzing a much wider range of components. Compounds that are thermally liable, exhibit high polarity or have a high molecular mass may all be analyzed using UHPLC/MS even proteins may be routinely analyzed.

Gas chromatography-mass spectrometry (GC-MS) is commonly employed for the separation and identification of drugs and metabolites in forensic toxicology, using electron impact (EI) or chemical ionization (CI) [1]. This methodology has become a “gold standard” in terms of admissibility and defensibility in court because of its good sensitivity, excellent selectivity and a high degree of standardization [2]. However, laborious and time consuming procedures and sample clean ups are mandatory in most cases. LC/MS methods eliminate the need to derivatize and often simplify sample preparation. However, long run times and low separation efficiency limit the utility of conventional HPLC. Ultra high performance liquid chromatography (UHPLC) performs separations 5 to 10 times faster than conventional HPLC by employing sub-2 µm diameter particles. The 1-2 second peak widths and relatively high separation efficiency of UHPLC are more competitive with capillary GC, making UHPLC-MS an attractive alternative method for illicit drug analysis. Chromatography (HPLC) directly coupled to mass spectrometry (MS) was in routine use in drug metabolism laboratories for these types of studies (5–12). Enhanced selectivity and sensitivity, and rapid, generic gradients made LC–MS the predominate technology for both quantitative and qualitative analyses.

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However, with the ever increasing numbers and diversity of compounds entering development, and the complex nature of the biological matrices being analyzed, new analytical procedures and technology were required to keep pace with the testing demands.

UPLC-MS

The most significant limitation is related to the narrow peaks that are produced by UHPLC that require a fast duty cycle, which is only available on the latest generations of MS devices. Thus, certain analyzers are more readily compatible with UHPLC (e.g., QqQ or TOF/MS) than others (e.g., ion trap or FT-MS). Second, due to the reduction of the column volume, extra-column band broadening can become significant, leading to a reduction in the kinetic performance of the UHPLC-MS configuration. Third, as the mobile phase linear velocity is higher in UHPLC, the electrospray ionization source must also be able to provide high sensitivity at flow rates of up to 1mL/min. Despite these limitations, the UHPLC-MS/MS platform has successfully been employed over the last decade for various types of applications, including those related to bioanalysis, drug metabolism, multi-residue screening, metabolomics, biopharmaceuticals and polar compounds

- Sensitivity is the disadvantage of using proton nuclear magnetic resonance analysis(HNMR) for this purpose yet it is the only technique which produces signals directly correlating with the amount of analytes in the sample. Thus, the most commonly chromatographic or electrophoretic techniques in combination with different detectors are employed for this purpose.
- Due to extremely small sample volumes capillary electrophoresis often faces sensitivity as well.
- Gas chromatography is unsuitable for non volatile and thermally fragile molecules (derivatisation of analytes is required) [3].

PRINCIPLE

As the name suggested the instrumentation comprises a high performance liquid chromatography attached, via suitable interface, to a mass spectrometer. The primary advantage UHPLC/MS has over GC/MS is that it is capable of analyzing a much wider range of components. Compounds that are thermally liable, exhibit high polarity or have a high molecular mass may all be analyzed using UHPLC/MS even proteins may be routinely analyzed. Solutions derived from samples of interest are injected onto an UHPLC column that comprises a narrow stainless steel tube(usually 150mm length and 2mm internal diameter, or smaller) packed with fine, chemically modified silica particles. Compounds are separated on the basis of their relative interaction with the chemical coating of these particles (stationary phase) and the solvent eluting through the column (mobile phase). Components eluting from the chromatographic column are then introduced to the mass spectrometer via a specialized interface. The two most common interfaces used for UHPLC/MS are the electrospray ionization and the atmospheric pressure chemical ionization interfaces. The various other approaches in interfacing UHPLC with mass spectrometry are thermospray method, mono disperse aerosol-generation interface and moving-Bell interface.

RESOLUTION AND SENSITIVITY OF THE UPLC MS SYSTEM

This extra resolution is particularly important when analyzing isobaric compounds such as these de alkylated metabolites. By incorporating a more efficient UPLC separation into the MS there is less ion suppression from competing compounds in the source and therefore more discreet ionization of the metabolites. Without the resolution generated by UPLC it would be possible to falsely assign the structure of a metabolite or miss a potential toxic moiety [4].

The extra sensitivity produced by the UPLC system ensures more low concentration metabolites will be detected, helping to prevent potentially toxic compounds from progressing further into the drug discovery process. This added sensitivity is extremely important when performing MS–MS experiments as it can make the difference between obtaining an interpretable spectrum or not.

Sensitivity, selectivity, and analysis time (sample throughput) are also some of the challenges analysts face when analyzing environmental samples such as soil and water. Explosives residues in soil or environmental waters are of both forensic and environmental interest. These types of assays prove challenging because of the selectivity needed to resolve positional isomers. Typical HPLC analyses require viscous, buffered mobile phases operated at high temperatures, and analysis times exceeding 30 min. the separation of a complex mixture of explosive compounds in less than seven minutes, with a much simpler, more robust mobile phase than that commonly

used in HPLC assays. The simpler non buffered mobile phase also is ideal for MS detection if desired [5].

THEORY

Over the past few years, there has been tremendous interest in approaches to speed up and/or increase the resolving power of the analytical separation process, particularly with the development of columns packed with porous sub-2µm particles used in very high pressure conditions (namely UHPLC, for ultra high pressure liquid chromatography). Many laboratories want to transition some of their conventional HPLC methods to fast UHPLC methods, but their lack of experience sometimes acts as a deterrent to trying. In this white paper, we share our experience in HPLC-to-UHPLC method transfer in the form of a tutorial introductory guide to help those who want to try this new and exciting UHPLC methodology to improve their productivity [6].

It is well known in liquid chromatography that the use of small particle size results in higher plate numbers, as well as faster separations. These effects are due to the fact that i) the chromatographic efficiency, N, is directly proportional to the ratio of column length and particle diameter, L/dp and ii) the mobile phase linear velocity, u, is inversely proportional to the particle diameter, dp. for high throughput separations it is indeed possible to maintain an equivalent efficiency between a 150mm column packed with 5µm particles and a 50mm column packed with sub-2µm particles, while the analysis time is divided by 9- fold. It is also theoretically possible to maintain the analysis time equivalent between a conventional HPLC columns and a 450mm column packed with sub-2µm, but with an efficiency enhancement by 9-fold with the latter [7].

However, the particle size reduction also generates a high backpressure (> 400 bar) not compatible with conventional instrumentation. Therefore, to benefit from the full potential of columns packed with small particles, it is recommended to work with a chromatographic system that withstands pressures up to 600 and even 1000 bar. By comparing the intrinsic performance of such packing size with other existing techniques, such as monoliths, fused-core technology or high temperature liquid chromatography with conventional particle size, it is shown here that UHPLC, with a maximal pressure of 1000 bar is a very attractive strategy (i.e. approach that generates the lowest analysis time for a given efficiency) in the range 1,000 to 80,000 plates. Only the monolithic approach performs better than UHPLC for efficiencies higher than 80,000 plates (such efficiency is however often beyond the needs of a conventional LC analysis) [8].

UHPLC vs. HPLC

A comparison between Ultra High Performance Liquid Chromatography and High Peformance Liquid Chromatography is shown in the table below:

Attribute	**HPLC**	**UHPLC**
Pressure	6000 Psi	100,000 Psi
Particle Size	5µm	1.7µm
Flow Rate	Milliliters per minute	Microliters per minute
Max Resolution	Relatively low	Relatively high

The lower bead size is the true reason for UHPLC increased flow rate and resolution. This can be shown mathematically using deemter's equation: $H = A + B/\mu + C\mu$. H being the plate height and µ being the particle size.The A constant remains constant independent of flow rate (it is referred to as the 'Eddy diffusion term'). The B constant is the diffusion coefficient, and C is the "analyte mass transfer" coefficient. As µ decreases, the A and C values of needed for a similar H value decrease, allowing for higher resolution. This also reduces the effect of the C value on the H value, yielding faster separations for similar resolutions. Note UHPLC out classes HPLC in all aspects, and is expected to replace HPLC in the near future [9].

INSTRUMENTATION

Mass spectrometers work by ionizing molecules and then sorting identifying the ions according to their mass to charge(m/z) ratios. Two key components in this process are ion source which generates the ions, and the mass analyzer, which sorts the ions.

INJECTORS

Samples are injected into the UHPLC via an injection port. The injection port of an UHPLC commonly consists of an injection valve and the sample loopLoop volumes can range between 10 µl to over 500 µl. In modern UHPLC systems, the sample injection is typically automated.

Stopped-flow Injection

It is a method whereby the pump is turned off allowing the injection port to attain atmospheric pressure. The syringe containing the sample is then injected into the valve in the usual manner, and the pump is turned on.

For syringe type and reciprocation pumps, flow in the column can be brought to zero and rapidly resumed by diverting the mobile phase by means of a three-way valve placed in front of the injector. This method can be used up to very high pressures.

PUMPS

There are several types of pumps available for use with UHPLC analysis, they are: Reciprocating Piston Pumps, Syringe Type Pumps, and Constant Pressure Pumps.

Advanced Nano UHPLC Pump

The Advance LC pump is a modular system incorporating one, two, or three pumps, with optional integrated valving for multidimensional or parallel HTS analyses. Each pump module has an integrated flow controller and are capable of providing precise fluid delivery from 1 nL/min to 50,000 nL/minute at pressures up to 10,000 psi (70 MPa) with no hardware change. Pumps can be changed by easy slide-in for accessible maintenance or increased application capability by plug-and-play into the pump housing. The Advance pumps feature constant flow with intelligent piston refill such that the column never loses pressure or flow, extending the life of the column and spray tip [10].

Advanced nano UHPLC pump

Reciprocating Piston Pumps consist of a small motor driven piston which moves rapidly back and forth in a hydraulic chamber that may vary from 35-400 µL in volume. On the back stroke, the separation column valve is closed, and the piston pulls in solvent from the mobile phase reservoir. On the forward stroke, the pump pushes solvent out to the column from the reservoir. A wide range of flow rates can be attained by altering the piston stroke volume during each cycle, or by altering the stroke frequency. Dual and triple head pumps consist of identical piston-chamber units which operate at 180 or 120 degrees out of phase. This type of pump system is significantly smoother because one pump is filling while the other is in the delivery cycle.

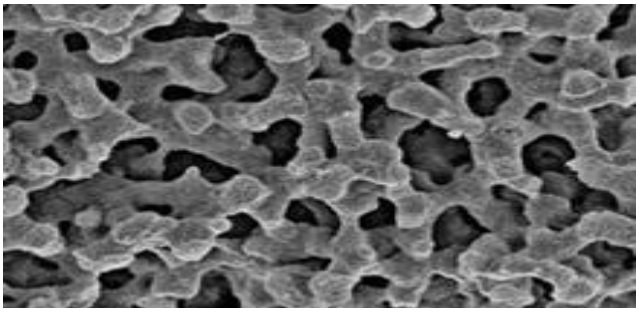
Syringe Type Pumps are most suitable for small bore columns because this pump delivers only a finite volume of mobile phase before it has to be refilled. These pumps have a volume between 250 to 500 ml. The pump operates by a motorized lead screw that delivers mobile phase to the column at a constant rate. The rate of solvent delivery is controlled by changing the voltage on the motor.

In **Constant Pressure Pumps** the mobile phase is driven through the column with the use of pressure from a gas cylinder. A low-pressure gas source is needed to generate high liquid pressures. The valving arrangement allows the rapid refill of the solvent chamber whose capacity is about 70 mL. This provides continuous mobile phase flow rates [11].

COLUMNS

One of the main criticisms made by early UHPLC users has been the reduced lifetime of columns packed with sub-2µm, compared to conventional columns. It is true that UHPLC columns are always exposed to very high pressures, but the packing pressure has been increased proportionally.

MONOLITHIC COLUMNS



Monoliths are rod-shaped continuous bed silica or polymeric materials, which offer an alternative to conventional particle-packed columns for analytical and preparative liquid chromatography. Silica monoliths are more easily prepared in comparison with their polymeric counterparts and provide larger surface areas for small molecule separations. The main advantages of monoliths are their superior performance for fast separations with low pressure at high flow rates and favorable mass transfer efficiency.

- Reduce run times by more than 50 %
- Extremely high efficiencies with low backpressures
- "Dilute-and-shoot" biological samples eliminating time consuming sample preparation
- Available in C18, C8, and Si phases

Provides excellent efficiencies and low backpressures under a variety of conditions. Whether the goal is to baseline resolve a large mixture of analytes, or to complete a separation in less than 2 minutes, Onyx is an excellent choice. Silica monolithic columns rarely exceed 100 bar, even at 9 mL/min, while particle-based columns reach backpressure limits at much lower flow rates.

CORE SHELL CILICA COLLUMNS

The Key Components of Core Enhanced Technology are Tight Control of Particle Diameter. Enhanced selection process keeps particle size distribution to a minimum and produces high efficiency columns. Solid Core Particles 4 µm diameter particles with a solid core generate very high efficiencies with conventional UHPLC methods Automated Packing Process Enhanced automated procedures ensure that all columns are packed with the highest quality Advanced Bonding Technology Optimized

phase bonding creates a series of high coverage, robust phases.

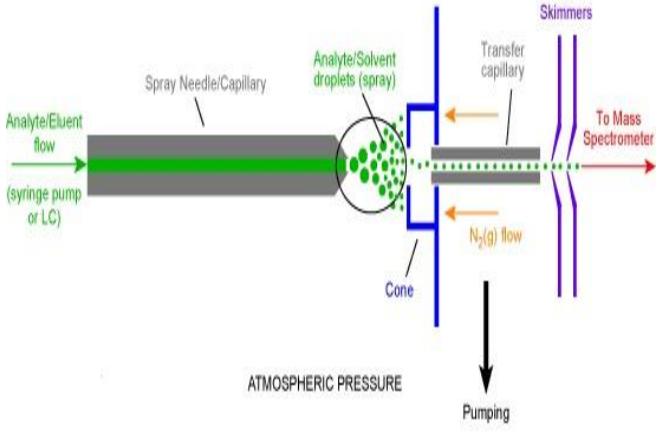
• Compatible with conventional HPLC methods. No need to change methods or invest in new equipment. High resolution. Separate difficult to resolve peaks. Sharp, tall peak shape Lower limits of detection – detect trace levels of analytes. Reproducible chromatography Confidence in your results.Long column lifetime [12].

IONIZATION

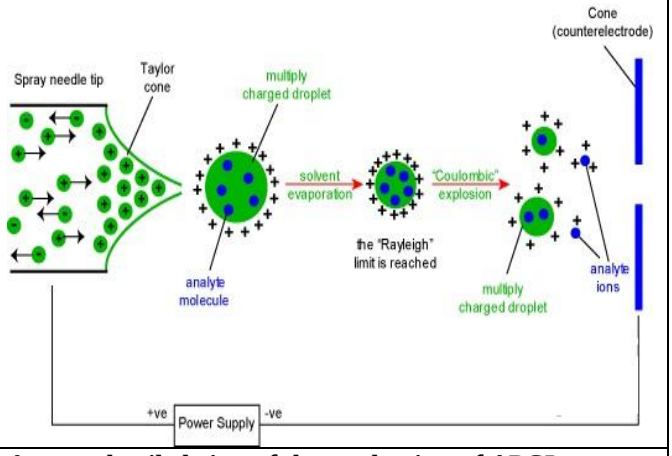
ELECTROSPRAY IONISATION

In electrospray ionization the analyte is introduced to the source at flow rates typically of the order of 1µl min^{-1}. The analyte solution flow passes through the electrospray needle that has a high potential difference (with respect to the counter electrode) applied to it (typically in the range from 2.5 to 4 kV). This forces the spraying of charged droplets from the needle with a surface charge of the same polarity to the charge on the needle. The droplets are repelled from the needle towards the source sampling cone on the counter electrode (shown in blue). As the droplets traverse the space between the needle tip and the cone, solvent evaporation occurs. This is circled on the Fig.1 and enlarged upon in Fig.2. As the solvent evaporation occurs, the droplet shrinks until it reaches the point that the surface tension can no longer sustain the charge (the Rayleigh limit) at which point a "Coulombic explosion" occurs and the droplet is ripped apart. This produces smaller droplets that can repeat the process as well as naked charged analyte molecules. These charged analyte molecules (they are not strictly ions) can be singly or multiply charged. This is a very soft method of ionization as very little residual energy is retained by the analyte upon ionization. It is the generation of multiply charged molecules that enables high molecular weight components such as proteins to be analyzed since the mass range of the mass spectrometer is greatly increased since it actually measures the *mass to charge ratio* rather than mass *per se*. The major disadvantage of the technique is that very little (usually no) fragmentation is produced although this may be overcome through the use of tandem mass spectrometric techniques such as MS/MS or MS [13].

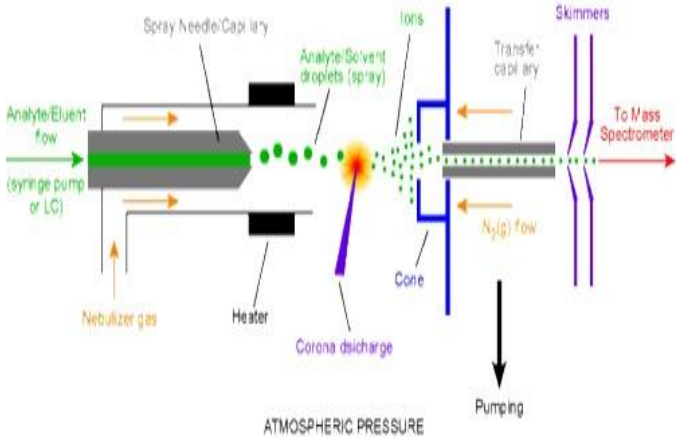
A schematic of an ESI interface



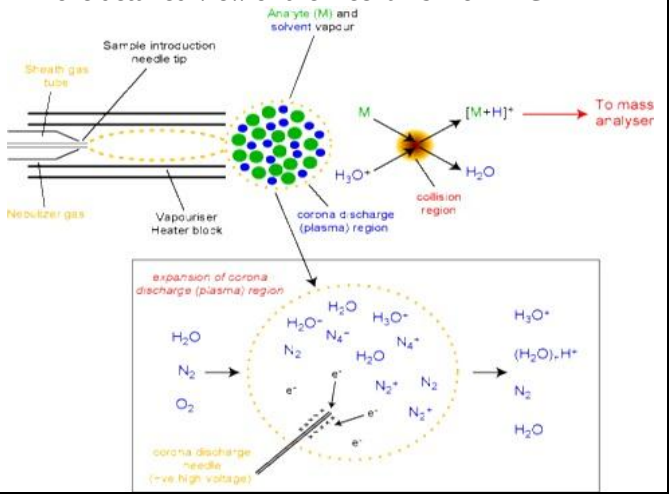
A schematic of the mechanism of ion formation



A schematic representation of the components of an APCI source



A more detailed view of the mechanism of APCI



TMOSPHERIC PRESSURE CHEMICAL IONISATION

Atmospheric pressure chemical ionization (APCI) is an analogous ionization method to chemical ionization (CI). The significant difference is that APCI occurs at atmospheric pressure and has its primary applications in the areas of ionization of low mass compounds (APCI is not suitable for the analysis of thermally labile compounds). The general source set-up (see Fig. 3) shares a strong resemblance to ESI. Where APCI differs to ESI, is in the way ionization occurs. In ESI, ionization is bought about through the potential difference between the spray needle and the cone along with rapid but gentle desolvation. In APCI, the analyte solution is introduced into a pneumatic nebulizer and desolvated in a heated quartz tube before interacting with the corona discharge creating ions. The corona discharge replaces the electron filament in CI - the atmospheric pressure would quickly "burn out" any filaments - and produces primary N2°+ and N4°+ by electron ionization. These primary ions collide with the vaporized solvent molecules to form secondary reactant gas ions - e.g. H_3O^+ and $(H_2O)_n^{H+}$ (see Fig. 4). These reactant gas ions then undergo repeated collisions with the analyte resulting in the formation of analyte ions. The high frequency of collisions results in a high ionization efficiency and thermalisation of the analyte ions. This results in spectra of predominantly molecular species and adduct ions with very little fragmentation. Once the ions are formed, they enter the pumping and focusing stage in much the same as ESI [14].

MASS ANALYSERS

Quadrapole

The quadrupole mass analyzer is one type of mass analyzer used in mass spectrometry. As the name implies, it consists of four cylindrical rods, set parallel to each other.[1]In a quadrupole mass spectrometer (acronym *QMS*) the quadrupole is the component of the instrument responsible for filtering sample ions, based on their mass-to-charge ratio(*m/z*). Ions are separated in a quadrupole based on the stability of their trajectories in the oscillating electric fields that are applied to the rods Principle of operation [15].

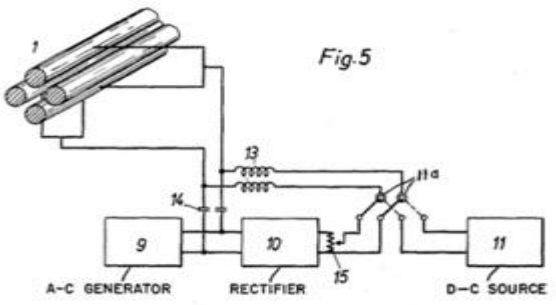
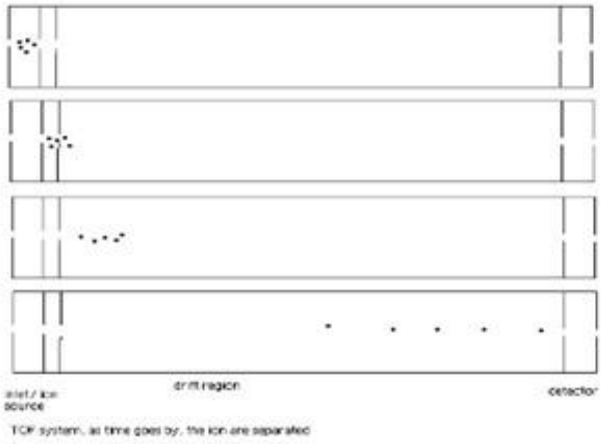


Fig.5

The quadrupole consists of four parallel metal rods. Each opposing rod pair is connected together electrically, and a radio frequency (RF) voltage is applied between one pair of rods and the other. A direct current voltage is then superimposed on the RF voltage. Ions travel down the quadrupole between the rods. Only ions of a certain mass-to-charge ratio will reach the detector for a given ratio of voltages: other ions have unstable trajectories and will collide with the rods. This permits selection of an ion with a particular *m/z* or allows the operator to scan for a range of*m/z*-values by continuously varying the applied voltage [1]. Mathematically this can be modeled with the help of the Hill differential equation [3]. Ideally, the rods are hyperbolic. Cylindrical rods with a specific ratio of rod diameter-to-spacing provide an easier-to-manufacture adequate approximation to hyperbolas. Small variations in the ratio have large effects on resolution and peak shape. Different manufacturers choose slightly different ratios to fine-tune operating characteristics in context of anticipated application requirements. In recent decades some manufacturers have produced quadrupole mass spectrometers with true hyperbolic rods.

TOF (TIME OF FLIGHT) MASS ANALYZER

TOF Analyzers separate ions by time without the use of an electric or magnetic field. In a crude sense, TOF is similar to chromatography, except there is no stationary/ mobile phase, instead the separation is based on the kinetic energy and velocity of the ions.



Ions of the same charges have equal kinetic energies; kinetic energy of the ion in the flight tube is equal to the kinetic energy of the ion as it leaves the ion source:

$$KE = mv^2/2 = zV$$

The time of flight, or time it takes for the ion to travel the length of the flight tube is:

$$T_f = L(\text{length of tube})/v(\text{velocity of ion})$$

Substituting the equation for kinetic energy in equation for time of flight:

$$T_f = L(m/z)^{1/2}(1/2V)^{1/2}? \text{ (Constant)}*(m/z)^{1/2}$$

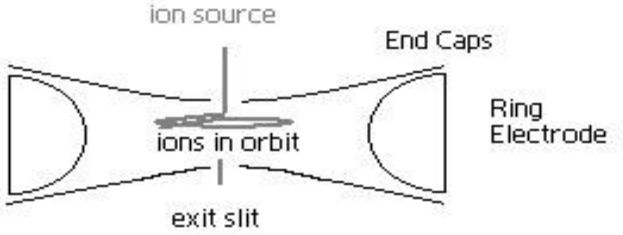
During the analysis, L, length of tube, V, Voltage from the ion source, are all held constant, which can be used to say that time of flight is directly proportional to the root of the mass to charge ratio.

Unfortunately, at higher masses, resolution is difficult because flight time is longer. Also at high masses, not all of the ions of the same m/z values reach their ideal TOF velocities. To fix this problem, often a reflectron is added to the analyzer. The reflectron consists of a series of ring electrodes of very high voltage placed at the end of the flight tube. When an ion travels into the reflectron, it is reflected in the opposite direction due to the high voltage.

The reflectron increases resolution by narrowing the broadband range of flight times for a single m/z value. Faster ions travel further into the reflectrons, and slower ions travel less into the reflector. This way both slow and fast ions, of the same m/z value, reach the detector at the same time rather then at different times, narrowing the bandwidth for the output signal [15].

QUADRUPOLE ION TRAP MASS ANALYZERS

This analyzer employs similar principles as the quadrupole analyzer mentioned above, it uses an electric field for the separation of the ions by mass to charge ratios. The analyzer is made with a ring electrode of a specific voltage and grounded end cap electrodes. The ions enter the area between the electrodes through one of the end caps. After entry, the electric field in the cavity due to the electrodes causes the ions of certain m/z values to orbit in the space. As the radio frequency voltage increases, heavier mass ion orbits become more stabilized and the light mass ions become less stabilized, causing them to collide with the wall, and eliminating the possibility of traveling to and being detected by the detector [16].



The quadrupole ion trap usually runs a mass selective ejection, where selectively it ejects the trapped ions in order of increasing mass by gradually increasing the applied radio frequency voltage.

ION CYCLOTRON RESONANCE (ICR)

ICR is an ion trap that uses a magnetic field in order to trap ions into an orbit inside of it. In this analyzer there is no separation that occurs rather all the ions of a particular range are trapped inside, and an applied external electric field helps to generate a signal. As mentioned earlier, when a moving charge enters a magnetic field, it experiences a centripetal force making the ion orbit. Again the force on the ion due to the magnetic field is equal to the centripetal force on the ion.

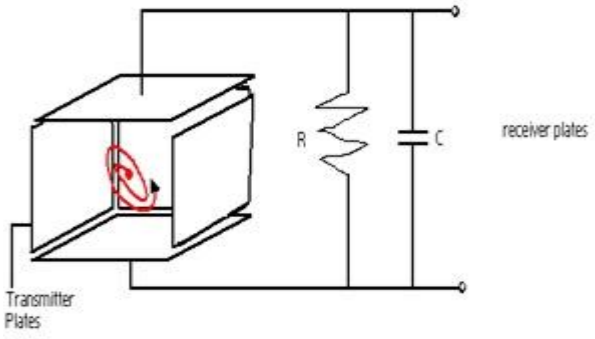
$$zvB=mv^2/r$$

Angular velocity of the ion perpendicular to the magnetic field can be substituted here $w_c=v/r$

$$zB=mw_c$$

$$w_c=zB/m$$

Frequency of the orbit depends on the charge and mass of the ions, not the velocity. If the magnetic field is held constant, the charge to mass ratio of each ion can be determined by measuring the angular velocity w_c. The relationship is that, at high w_c, there is low m/z value, and at low w_c, there is a high m/z value. Charges of opposite signs have the same angular velocity, the only difference is that they orbit in the opposite direction [16].



In order to generate an electric signal from the trapped ions, a vary electric field is applied to the ion trap $E=E_o\cos(w_ct)$. When the w_c in the electric field matches the w_c of a certain ion, the ion absorbs energy making the velocity and orbiting radius of the ion increase. In this high energy orbit, as the ion oscillates between two plates, electrons accumulate at one of the plates over the other inducing an oscillating current, or current image. The current is directly proportional to the number of ions in the cell at a certain frequency.

In a Fourier Transform ICR, all of the ions within the cell are excited simultaneously so that the current image is coupled with the image of all of the individual ion frequencies. A Fourier transform is used to differential the summed signals to produce the desired results [17].

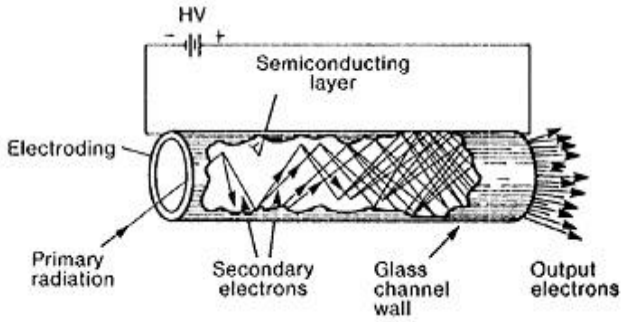
DETECTORS

ELECTRON MULTIPLIER

The fragments that survive the journey through the analyzer shoot into the electron multiplier - the detector of the UHPLC-MS. The electron multiplier detects every ion of the selected mass that passes through the quadrupole analyzer.

Electron multipliers use a process known as **secondary electron emission**. When the ions hit a surface, it causes the electrons in the outermost area of the

atom to be released, which are known as **secondary electrons**. The number of secondary electrons released depends on several factors, such as the type of particle, the angle at which it strikes the surface, and the energy and characteristics of the surface struck.



The GCMS uses a **continuous dynode** electron multiplier, also known as a **channel electron multiplier**. It is comprised of "the channel," a hollow, cornucopia-shaped tube made of semi-conductive glass. Semi-conductive glass is glass that has a limited ability to conduct (or transmit) electricity. On the UHPLC-MS, lead silicate glass is used.

When the ions hit the inner surface, secondary electrons are emitted. These electrons are then accelerated through an electric field, which is generated by applying the proper voltage to the surface of the tube. The electric field forces the emitted electrons to hit the wall, and these electrons, like the ion, also cause electrons to be emitted. This process continues until there are enough electrons to emitted to create a measurable current. Because the electrons are depleted by the process, the tube wall needs time to "recover." The period of recovery time is known as **dead time [18]**.

APPLICATIONS

UHPLC/MS has several advantages over regular HPLC systems

- Significantly higher sensitivity
- Molecular weight determination
- Information on chemical structure
- Identification of analytes
- Analytes resolved from co-eluting contaminants
- Accurate background free quantitation

Optimize a UHPLC/MS method with respect to stationary phase, mobile phase, and detector settings to achieve picogram level quantitation of fourteen drugs and metabolites employing a 12 minutes separation.

•Ppb (ng/mL) level sensitivity and accuracy were achieved by this method

•The developed method is appropriate for QA/QC of the active pharmaceutical ingredient and the identification and quantification of pseudoephedrine and/or other components in illicit drug samples.

•This methodology offers an efficient tool to determine the source and manufacture pathway of drugs seized in the illicit market.

PHARMACEUTICAL APPLICATIONS

1. Identification and quantization of antibodies, their conjugates and complexes
2. For analyzing biotin in pharmaceutical formulations—a comparative study.
3. UHPLC-MSto characterise the purity and safety of biotechnology drugs.
4. Determination of matrine and oxymatrine in *Sophorasubprostata* by UHPLC-MS
5. Analysis and confirmation of synthetic anorexics in adulterated traditional Chinese medicine.
6. Application of to analysis of carbohydrates by UHPLC-MS, especially those in glycoproteins .
7. Fractionation and Analysis of Phospholipids
8. A reproducible, simple and sensitive of UHPLC-MS method for simultaneous determination capreomycin, ofloxacin and pasiniazide in urine
9. Separation and quantitation of azimilide and its putative metabolites by UHPLC-MS.
10. UHPLC-MS method for determination of ibuprofen enantiomers in human serum and urine .
11. Development and validation of a plasma assay for acyclovir using UHPLC-MS with sample stacking .
12. Quantitative determination of insulin entrapment efficiency in triblock copolymer nanoparticles by high-performance liquid chromatography coupled with MS [19].

PROTEOMIC ANALYSIS

Proteomics concerns the characterization of the full complement of proteins in a specific organism, tissue or cell type at a given time. In short, proteomics is a field that involves the identification, characterization and quantification of proteins. This challenge can be to analyze 10 000-30 000 protein variants expressed in a mammalian cell and characterize all of them at a given time. In an ideal situation the protein characterization would include cellular localization and sequence analysis as well as identification of post-translational modifications and binding partners.

HERBAL DRUG ANALYSIS

The analysis of secondary metabolites in plants and other plant origin substances is a challenging task because of their chemical diversity , usually low abundance and variability even within the same species. It is estimated that 100000-200000 metabolites occur in the plant kingdom and other traditional drugs needs highly selective and sensitive methods will be suitable for controlling their composition of quality, uhplc\ms is very accurate method for analysis of herbal drugs. Acids, alkaloids, coumarins, phenols, flavanoids, quinines, xanthones, terpenes and others can be analysed by UHPLC\MS [20].

AMINOACID CHARACTERIZATION

A rapid (1.5 min versus 20-90 min for standard methods) HPLC-MS assay for separating 19 amino acids was developed for quantifying levels of free amino acids in plant tissue. This assay was used to determine the free amino acid content in the seeds of 10,000 randomly mutagenized Arabidopsis lines, and 322 Arabidopsis lines with increased levels of one or more amino acids were identified. The heritability of the mutant phenotype was confirmed for 43 lines with increased seed levels of the aspartate-derived amino acids Ile, Lys, Thr, or Met. Genetic mapping and DNA sequencing identified a mutation in an Arabidopsis threonine aldolase (AT1G08630, EC 4.1.2.5) as the cause of increased seed Thr levels in one mutant. The assay that was developed for this project has broad applicability to Arabidopsis and other plant species.

METABONAMICS

Metabonomics is a term used to describe the non-targeted "global" analysis of tissues and biofluids for low molecular mass organic endogenous metabolites. Using the metabolic fingerprints, thus obtained, it is often possible to distinguish between strains of animal, disease states or to detect pharmacological or toxic effects obtained following the administration of, e.g., candidate drugs. In combination with genomics, transcriptomics and proteomics, metabonomic analysis is being increasingly used in the discovery and development of new medicines. Much, therefore, depends on the ability of the analytical technique employed to detect often-subtle differences in the complex mixtures found for biofluids, such as urine and plasma, or in tissue extracts [21].

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BIO ANALYSIS AND BIO EQUALANCE STUDIES

For Pharmacokinetic, bio equivalence and toxicity studies , the quantitative analysis of a drug in biological samples is n important part of drug development process and this is carried out by uplc\ms. The drugs having low molecular weight are during both preclinical and clinical studies , as several biological matrics are used for quantitative bioanalysis, the most common being used are the blood, plasma, and urine [22].

FUTURE PROSPECTS

The development of reliable UHPLC-MS systems has been of great benefit in areas such as drug bio-analysis. The application of this technology to metabolite fingerprinting for metabonomic analysis was inevitable given the great potential of this type of technology. Our initial studies, and those reported in the literature to date and reviewed above, do indeed demonstrate considerable promise for HPLC76.

Further the indications are that the use of capillary and very high pressure HPLC methods will improve the coverage of the metabonome provided by HPLC-MS as a result of reduced ion suppression. The widespread availability of high resolution HPLC-MS/MS systems will inevitably result in a great increase in the use of this type of analytical approach for obtaining metabolite fingerprints. This will be particularly the case as an appreciation of both the potential for metabonomicsto answer important biological questions becomes more widely known and the relative cheapness of the technology compared to, e.g., genomics and proteomics comes to be appreciated.

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