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An Introduction to Liquid Chromatography–Mass Spectrometry Instrumentation Applied in Plant Metabolomic Analyses†

J. William Allwood[a]* and Royston Goodacre[a,b]

ABSTRACT: Over the past decade the application of non-targeted high-throughput metabolomic analysis within the plant sciences has gained ever increasing interest and has truly established itself as a valuable tool for plant functional genomics and studies of plant biochemical composition. Whilst proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy is particularly appropriate for the analysis of bulk metabolites and gas chromatography mass spectrometry (GC-MS) to the analysis of volatile organic compounds (VOC's) and derivatised primary metabolites, liquid chromatography (LC)-MS is highly applicable to the analysis of a wide range of semi-polar compounds including many secondary metabolites of interest to plant researchers and nutritionists. In view of the recent developments in the separation sciences, leading to the advent of ultra high performance liquid chromatography (UHPLC) and MS based technology showing the ever improving resolution of metabolite species and precision of mass measurements (sub-ppm accuracy now being achievable), this review sets out to introduce the background and update the reader upon LC, high performance (HP)LC and UHPLC, as well as the large range of MS instruments that are being applied in current plant metabolomic studies. As well as covering the theory behind modern day LC-MS, the review also discusses the most relevant metabolomics applications for the wide range of MS instruments that are currently being applied to LC. Copyright © 2009 John Wiley & Sons, Ltd.

Keywords: plant; metabolomics; high performance liquid chromatography (HPLC); ultra high performance (UHP)LC; mass spectrometry (MS)

Untargeted Plant Metabolomics and the Potential of Liquid Chromatography Mass Spectrometry

Metabolomics has proven to be a valuable tool for the comprehensive profiling of plant derived samples for the study of plant systems (Fiehn *et al*., 2000; Ratcliffe and Shachar-Hill, 2001; Catchpole *et al*., 2005; Krishnan *et al*., 2005; von Roepenack-Lahaye *et al*., 2004; Moco *et al*., 2006) and natural products research (Dixon and Sumner, 2003; Ketchum *et al*., 2003; Pauli *et al*., 2005), especially when combined with chemometric data analysis approaches (Goodacre *et al*., 2004; Jonsson *et al*., 2004; van der Greef and Smilde, 2005; Goodacre, 2007). Plants provide a great challenge in metabolomics due to the rich chemical diversity of metabolites that they possess across a huge range of concentrations; estimates of 100000–200000 metabolites have been made for the plant kingdom (Oksman-Caldentey and Inzé, 2004). Currently no single analytical method or combination of analytical methods (i.e. chromatography combined with mass spectrometry (MS) or nuclear magnetic resonance (NMR) spectroscopy is capable of detecting all metabolites within a given biological sample (Sumner *et al*., 2003; Hall, 2006; Allwood *et al*., 2008). Over the past decade many methods for the high-throughput metabolomic analysis of plant derived samples have been established for gas chromatography combined with MS (GC-MS; Fiehn *et al*., 2000; Fernie *et al*., 2004; Lisec *et al*., 2006), direct injection mass spectrometry (DIMS; Goodacre *et al*., 2003; Catchpole *et al*., 2005; Allwood *et al*., 2006), liquid chromatography MS (LC-MS; Tolstikov *et al*., 2003; Jander *et al*., 2004; von Roepenack-Lahaye *et al*., 2004; Vorst *et al*., 2005; Moco *et al*., 2006; Rischer *et al*., 2006; De Vos *et al*., 2007), capillary electrophoresis MS (CE-MS; Sato *et al*., 2004), and ^{1}H-NMR (Ratcliffe and Shachar-Hill, 2001; Le Gall *et al*., 2003; Ward *et al*., 2003; Choi *et al*., 2004, 2006).

^{1}H-NMR has proven to be an appropriate tool for untargeted plant metabolomics, especially where studies focus upon samples that contain highly abundant bulk metabolite species, for example sugars in fruits (Biais *et al*., 2009). ^{1}H-NMR has the advantage that it produces signals that correlate directly and linearly with compound abundance (Lewis *et al*., 2007), although for many studies it lacks the sensitivity required to obtain information on low-level metabolites that can alternatively be

* Correspondence to: J. W. Allwood, School of Chemistry, Manchester Interdisciplinary Biocentre, University of Manchester, 131 Princess Street, Manchester M1 7DN, UK. E-mail: William.Allwood@manchester.ac.uk

[a] School of Chemistry, Manchester Interdisciplinary Biocentre, University of Manchester, 131 Princess Street, Manchester M1 7DN, UK

[b] Manchester Centre for Integrative Systems Biology (MCISB), Manchester Interdisciplinary Biocentre, University of Manchester, 131 Princess Street, Manchester, M1 7DN, UK

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provided by MS based approaches. DIMS provides a rapid (~2 min per sample) metabolite fingerprint (Goodacre *et al*., 2003; Allwood *et al*., 2006), which has proven to be valuable for a first-round screen of material (indicating robust sample growth, collection and extraction methods) prior to more expensive and time-consuming chromatography combined with MS approaches (Catchpole *et al*., 2005). DIMS is also gaining in popularity with the advent of high-resolution and -precision Fourier transform ion cyclotron resonance (FT-ICR)-MS instruments (Aharoni *et al*., 2002; Hirai *et al*., 2004; Southam *et al*., 2007), although without the application of chromatography, DIMS alone cannot resolve isobaric metabolites. GC-MS has proven to be a robust and reproducible tool for the study of volatile organic compounds (VOCs; Li *et al*., 2006, 2008; Zhang and Li, 2007; Riazanskaia *et al*., 2008) as well as a range of largely primary metabolites made amenable to GC-MS detection via the addition of volatile trimethylsyl (TMS) groups by *N*-methyl-*N*-trifluoroacetamide (MSTFA) based derivatisation (Fiehn *et al*., 2000; Fernie *et al*., 2004; Lisec *et al*., 2006; Biais *et al*., 2009; Allwood *et al*. 2009). LC-MS alternatively does not necessitate the prior derivatisation of samples to make metabolite groups of interest amenable to detection and has been shown to be appropriate for the detection of a wide range of metabolite classes (De Vos *et al*., 2007).

It is predicted that LC-MS-based approaches will be of particular importance for non-targeted plant metabolomics, largely owing to the plant kingdoms' rich metabolite diversity. Plant metabolism embodies a huge range of semi-polar compounds, including many key groups of secondary metabolites, which are best separated and detected by LC-MS approaches (Tolstikov *et al*., 2003; von Roepenack-Lahaye *et al*., 2004; Dixon *et al*., 2006; Hall, 2006; Moco *et al*., 2006; De Vos *et al*., 2007). MS-based techniques which are applicable to LC are also undergoing continual development, showing ever improving levels of mass resolution and precision and thus aiding metabolite detection and identification greatly (Kind and Fiehn, 2007; Draper *et al*., 2009). Plant-derived secondary metabolites have already been shown to be of phenotypic and physiological importance, for example, protective properties in disease or stress scenarios (Allwood *et al*., 2006, 2008; Jansen *et al*., 2009), antioxidant properties, colour and flavour characteristics (Beekwider *et al*., 2005; Dixon *et al*., 2006). Such quality traits of plant materials are commonly related to a delicate balance of various mixtures of metabolites from diverse related and unrelated metabolite groups as opposed to a small number of key biomarker metabolites. As a result, untargeted LC-MS-based metabolomics approaches will be of huge value to better understand the complexity of plant metabolism, to detect and identify which metabolic components contribute the most highly to key phenotypic and physiological traits, and how the biosynthesis of such components is controlled (DeVos *et al*., 2007). This review, largely aimed towards scientists new to or unfamiliar with the plant metabolomics field and/or analytical chemistry, aims to give an introduction to, the background of LC, high-performance (HP)LC and ultra-high-performance (UHP)LC, as well as the large range of MS instruments that may be applied to LC for metabolomic analysis.

An Introduction to Liquid Chromatography

Liquid chromatography (LC) defined in its simplest terms can be regarded as the separation of components of a mixture based upon the rates at which they elute from a stationary phase typically over a mobile phase gradient. Differing affinities of the mixtures components for the stationary and mobile phases leads to their separation, since certain components will be more attracted to the mobile phase and will elute quickly whilst others will be retained by the stationary phase for longer and therefore will elute more slowly, i.e. have a later retention time (RT). The simplest forms of LC are based upon the separation of a sample's components either on paper with a solvent, known as paper chromatography, or on a thin layer of stationary phase coated onto a slide which is placed in solvent—thin-layer chromatography (TLC). Column chromatography-based LC, a much more powerful technique than the planar paper chromatography and TLC methods, and with a greater sample capacity, was first credited to Mikhail S. Tswett, a Russian-born botanist who in the early 1900s successfully separated plant pigments from a complex extract using a basic calcium carbonate and alumina-packed glass column stationary phase and liquid solvent mobile phase (Ettre and Sakodynskii, 1992).

For column chromatography, flow of the mobile phase solvent is achieved in several ways. Gravity or a vacuum may be used where columns are not designed to withstand high pressures, as with the open glass columns employed by Tswett. Such columns employ large-diameter solid-phase particles (>50 μm), so that there is less resistance to flow and less chance of building up pressure (Novakova *et al*., 2006). One modern day application of this technique is solid-phase extraction (SPE), where typically a plastic cartridge is packed with particles which a liquid sample is passed through under vacuum-assisted flow. Different SPE packing materials have differing affinities towards certain classes or polarities of compound, so that SPE cartridges are available for the removal of specific small molecules from samples, for example SPE for the removal of free sugars (Suzuki *et al*., 2002). By employing smaller solid-phase particle sizes (<10 μm), improved chromatographic separation can be achieved due to the overall greater solid-phase surface area, improving compound retention (Novakova *et al*., 2006); however, due to smaller particles having a greater flow resistance, there is a need for greater pressures to drive the liquid mobile phase solvent through the column. Thus high pressure pumps and columns packed within metal casing are employed to create and withstand the high pressures necessary for the technique known as high-performance (HP) LC (Horváth *et al*., 1981; Snyder *et al*., 1997; Meyer, 2004).

Column chromatography in its simplest form may employ a mobile phase of constant single composition in a procedure termed isocratic elution. A disadvantage of isocratic elution is that peak width increases with RT according to the equation for the number of theoretical plates, thus leading to peak broadening and flattening for late eluting peaks. In a later development known as gradient elution, the mobile phase composition is altered during the chromatographic separation process. For example, a polar elution gradient may begin with 5% acetonitrile or methanol in water and end with 95% acetonitrile or methanol in water; a nonpolar gradient may employ solvents such as toluene (Snyder *et al*., 1997). In reverse-phase HPLC employing typical silica-C_{18} based columns, the two mobile phase components are referred to as solvent 'A' and 'B', 'A' typically being a highly aqueous solvent which elutes sample components from the stationary phase slowly, while 'B' is typically a highly organic solvent which rapidly elutes sample components off the column's stationary phase. By starting the elution with 100% A and 0% B, then step-wise increasing the percentage of B over time until 100% B is reached, an elution gradient is achieved.

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The increasing organic composition of the mobile phase displaces the stationary phase's retention of the late eluting sample components, thus preventing peak broadening and flattening, as well as reducing peak tailing effects (Snyder *et al*., 1997; Meyer, 2004). The elution gradient steps and time periods between them can be optimised for specific plant matrices so that the maximum number of components can be resolved within a reasonable analysis time that lends itself to high sample throughput.

There are many different variations of LC which are largely dependent on the stationary phase chemistry. The simplest forms of LC thus far discussed, paper chromatography and TLC, are categorised as partition chromatography methods, here a solvent is retained on or within the stationary phase, thus sample components are retained by equilibrium between the liquid stationary and liquid mobile phases (Hao *et al*., 2008). Hydrophilic interaction chromatography (HILIC) is a modern example of normal-phase partition chromatography applied in HPLC; here polar analytes diffuse into a bonded polar stationary phase (being more polar than the mobile phase) the interaction between which depends upon an analyte polarity dictated by the various functional groups that the analyte possesses (Hao *et al*., 2008; Cubbon *et al*., 2009). By starting the gradient elution with a highly organic mobile phase solvent, the non-polar metabolites elute rapidly, whereas the retention of polar metabolites is maximised. Thus, HILIC is particularly appropriate for the analysis of polar metabolites but until recently has been largely redundant in metabolomics due to a lack of column stationary phase stability and robustness; however, with the recent improvements being made in column chemistry stability, the development of robust HILIC analytical methods for HPLC and UHPLC is currently an ongoing focus (Hao *et al*., 2008; Cubbon *et al*., 2009).

Adsorption chromatography was another early development in LC and as the name suggests involves the adsorption of a samples components or metabolites directly onto a solid stationary phase, the retention of which again is related to the metabolite and stationary phase polarities (Horváth *et al*., 1981; Snyder *et al*., 1997; Meyer, 2004). Adsorption chromatography typically employs silica packed columns, the silica stationary phase particles possess R groups which can be polar (e.g. amine or nitrile groups) or nonpolar hydrocarbons (e.g. C_8H_{17} or $C_{18}H_{37}$). Adsorption chromatography is further divided into two subcategories: (i) normal-phase liquid chromatography (NP-LC), which employs polar stationary phase materials and so by starting the gradient elution with a non-polar mobile phase solvent, the non-polar metabolites elute first; (ii) reversed-phase liquid chromatography (RP-LC), which employs non-polar stationary phase materials and so by starting the gradient elution with a highly polar aqueous mobile phase solvent, the most polar metabolites elute first; by increasing the mobile phase organic composition with solvents such as methanol or acetonitrile, the retention of non-polar compounds to the column is weakened (i.e. separation on the basis of hydrophobicity) and thus they elute at later RTs (Snyder *et al*., 1997; Meyer, 2004). RP-LC has been and still is the most commonly employed method in metabolomics due to the high stability offered by the silica-C_8H_{17}/$C_{18}H_{37}$ stationary phase materials, robust analytical methodologies have also been developed by several plant research groups (Tolstikov *et al*., 2003; von Roepenack-Lahaye *et al*., 2004; Rischer *et al*., 2006; De Vos *et al*., 2007); therefore this review will focus upon the analysis of semi-polar plant metabolites with RP-LC.

High-performance Liquid Chromatography, Increasing Its Efficiency, and the Development of Ultra-high-performance Liquid Chromatography

The technique known as HPLC was first coined by C. Horváth in 1970, who by combining high pressure pumps (~500 psi) and strengthened packed columns, created the high pressures and strengths of column necessary for the technique then known as high-pressure LC. Tremendous leaps in technology were seen over the following decade with new instruments being produced that could develop ~6000 psi of pressure and which directly incorporated injector systems, detectors and columns. By combining improved instruments with improved column chemistries (smaller particles and higher pressures), improved separations were achieved, therefore the technique was re-coined as high-performance liquid chromatography, thus preserving the original HPLC acronym (Horváth *et al*., 1981). HPLC is now one of the most widely used tools in analytical chemistry, having been applied to the analysis of a huge range of samples from across science and industry. Almost any compound that dissolves in liquid can be analysed, even in trace concentrations as low as parts per trillion (Snyder *et al*., 1997; Meyer, 2004).

The components of a basic HPLC system are shown in Fig. 1A. The HPLC consists of solvent reservoirs, one for each of the mobile phase eluents (solvent A and B), as well as further reservoirs for autosampler syringe and line washes. A high-pressure pump forms the solvent delivery system, which generates and meters a specified flow rate (typically millimetres per min). A solvent partitioning valve allows the mixing of solvents A and B at specified ratios and time gaps, thus permitting the operator to programme the HPLC to perform gradient elution steps (Meyer, 2004). To commence the HPLC cycle of a single sample analysis, the HPLC system first introduces 100% solvent A (e.g. 5% aqueous acetonitrile acidified for control of pH with 0.1% formic acid); an autosampler injector system introduces the sample to the mobile phase stream and is carried to the HPLC column (Fig. 1B). High-pressure tubing and fittings are used to interconnect the pump, autosampler injector system, column, and detector(s), thus forming a conduit for the sample, mobile phase, and separated sample components. In the case of RP-HPLC, the column is packed typically with silica–$C_{18}H_{37}$ particles to act as a non-polar retaining stationary phase. At an appropriate time point during the HPLC separation (which is sample matrix-dependent and must be optimised to allow for the elution of the maximum number of polar sample components), solvent B (e.g. 95% aqueous acetonitrile acidified with 0.1% formic acid) is introduced to the mobile phase in increasing ratios in an incremental manner, thus the increasing organic content of the solvent leads to the elution of the retained non-polar compounds from the stationary phase (Fig. 1C). The elution gradient continues until 100% solvent B is reached; this mobile phase composition is maintained for a final hold period. Throughout the chromatographic run a detector is recording the signal of the eluted sample components, the signal is amplified and delivered to a computer where it is processed and displayed as a chromatogram. In the case of hyphenated HPLC approaches, the eluting sample components are further delivered to a linked detector such as a mass spectrometer for in-depth characterisation. There are a huge range of various detectors that may be linked to HPLC, some of which will be discussed later; HPLC can also be linked to

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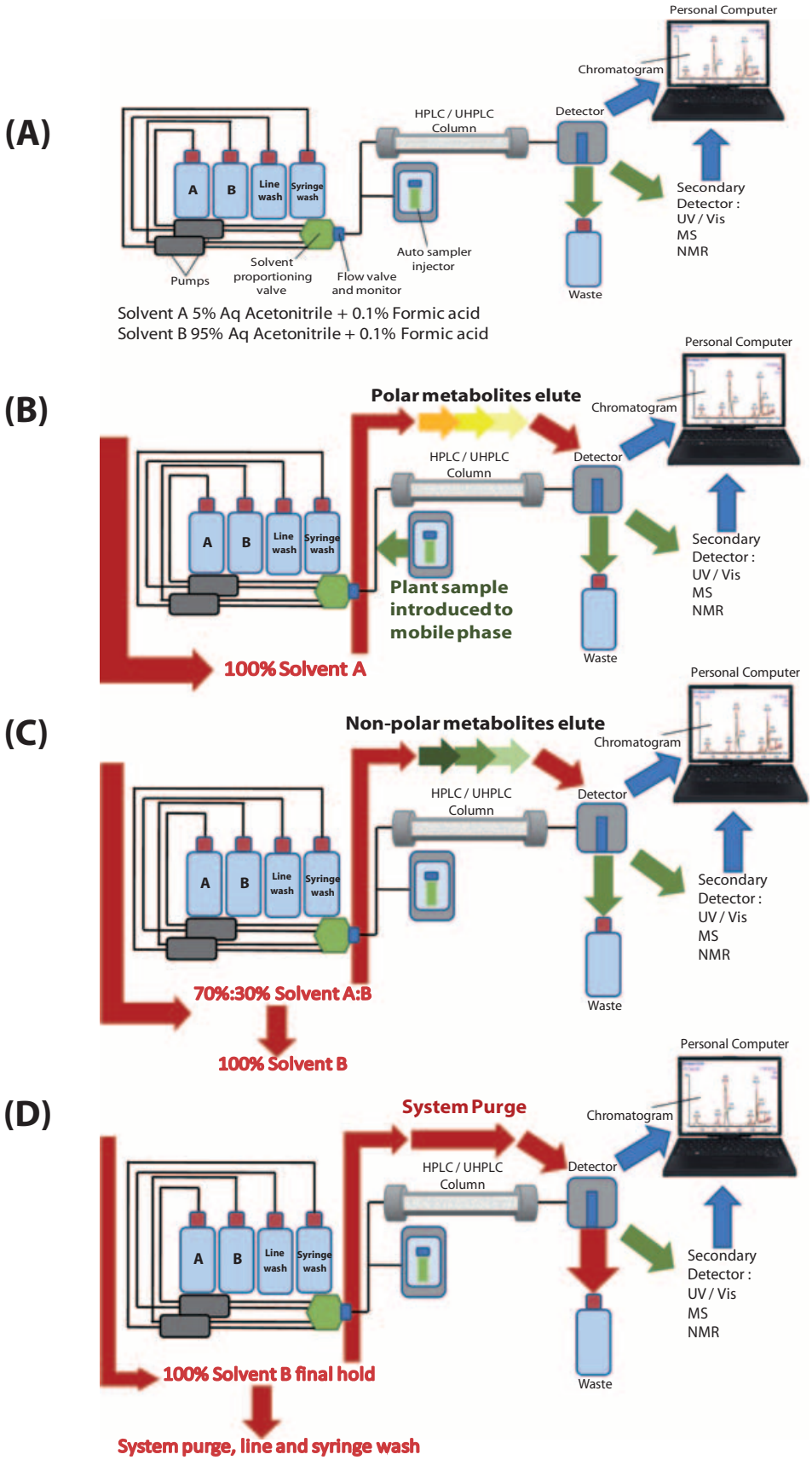

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Figure 1. An illustration of high- and ultra-high-performance liquid chromatography instrumentation and the analysis of a single sample with reverse phase separation by hydrophobicity. (A) A simplified illustration of high-performance liquid chromatography instrumentation. (B) Initially 100% solvent A (5% aqueous acetonitrile acidified with 0.1% formic acid) is introduced to the system; the sample is injected into the mobile phase flow; solvent B (95% aqueous acetonitrile acidified with 0.1% formic acid) is introduced to the mobile phase until a ratio of 70:30% solvent A:B is reached. Over this period polar components of the sample elute from the column and the signal is detected amplified and sent to a personal computer. (C) Solvent B continues to be introduced to the mobile phase until the composition reaches 100% solvent B. Over this period the non-polar sample components, initially retained within the column when the mobile phase was highly aqueous, start to elute with the increasing strength of the organic solvent B. Once the mobile phase reaches a composition of 100% solvent B there is a final hold period of typically several minutes, after which the system purges itself with clean solvents to remove all traces of the sample. The lines, column and autosampler components of the HPLC system are all purged and left clean, the mobile phase solvents are returned to starting equilibrium and the system is ready for the introduction of the next sample.

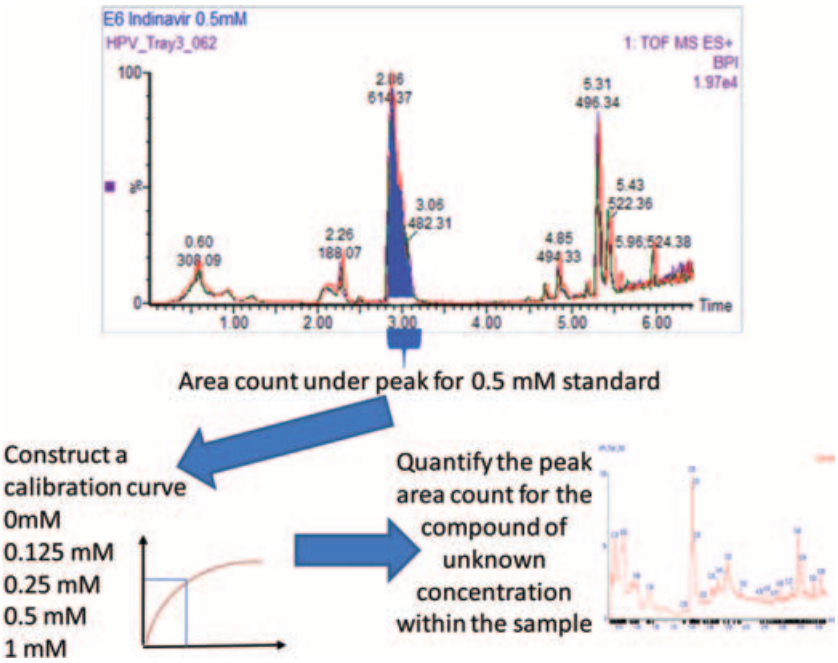

Figure 2. An illustration of compound identification and quantification by high-performance liquid chromatography.

fraction collection systems for purified sample preparation. At the end of the HPLC cycle the system purges itself with clean flow solvents, undertakes the syringe and line washes and equilibrates the starting mobile phase composition (Fig. 1D), thus returning the system to a background state clear of the previously analysed sample and ready to commence the cycle for the analysis of the next sample. The excess flow and wash solvents created throughout the HPLC cycle are delivered to a waste solvent reservoir. Providing that a compound within a sample can be cleanly chromatographically resolved and does not have overlapping peaks from other sample components, it can be isolated and potentially identified. This is achieved by matching the RT of the compound within the sample to the RT that a pure standard of the same compound elutes at under the same chromatographic conditions. By constructing a calibration curve based upon the detected chromatographic peak areas for the pure compound serially diluted across a concentration series, the unknown concentration of the compound within the sample can be quantified by its detected peak area (Fig. 2) (Kimura and Rodriguez-Amaya, 2002; Allwood *et al*., 2006). However, for the analysis of complex metabolomics samples there are many co-eluting peaks which can prevent identification and quantification in this manner, thus MS is employed to perform these tasks (Tolstikov *et al*., 2003).

Thus far this section has largely focused upon the separation of compounds based upon three factors: the polarity of the sample compounds that are being analysed; the composition of the mobile phase; and the chemical properties of the stationary phase. However, there are several other parameters which all influence the efficiency of HPLC separations and were utilised in the development of UHPLC. The first of these parameters to be considered is column length; providing that a column bed is uniformly packed and stable, the chromatographic separation efficiency is determined by the particle size and column length. Longer lengths of column enhance chromatographic separation, but at the expense of increased analysis times and system back pressure (Romanyshyn and Tiller, 2001). Smaller particle sizes lead to an increased surface area, thus improving compound retention and providing improved chromatographic separation at the expense of an increased pressure being required to drive the mobile phase (Nguyen *et al*., 2006; Novakova *et al*., 2006). The chromatographic separation efficiency is commonly expressed as a theoretical plate value, with greater efficiencies having greater theoretical plate values. The height equivalent to the theoretical plate (HETP) shows an inverse relationship to the theoretical plate value; thus as particle size decreases or column length increases, the column separation efficiency increases and the HETP decreases (Fig. 3). Unfortunately, decreases in column

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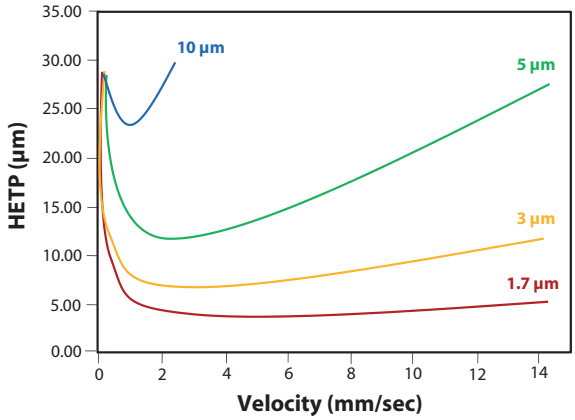

Figure 3. The relationship between column stationary phase particle size, flow velocity and the height equivalent to the theoretical plate (HETP) value as an expression of chromatographic separation efficiency. The lower the HETP, the greater the efficiency of chromatographic separation. As linear velocity increases the efficiency of conventional 3–10 µm stationary phase particles reaches an optimum and decreases. However, sub-2 µm particles reach a greater efficiency and maintain it under increasing linear velocity.

particle size result in greater system back pressures, as does increases in column length along with increased solvent consumption and sample analysis times (Romanyshyn and Tiller, 2001; Nguyen *et al*., 2006). In modern HPLC and UHPLC sub-2 µm particles are commonly employed to maximise the chromatographic resolution power along with short column lengths to reduce sample analysis times, backpressure, and solvent consumption (Novakova *et al*., 2006). The column's internal diameter (i.d.) influences the selectivity of separation and detection sensitivity in gradient elution as well as determining the volume of sample that can be loaded. Large-i.d. columns (10 mm) are typically applied in industry and analytical-scale columns (i.d. 4.6 mm) for standard HPLC applications; narrow-bore columns (i.d. 1–2 mm) are commonly used in LC-MS applications since they offer greater sensitivity than the larger-i.d. columns, although they restrict the volume of sample that can be loaded (Snyder *et al*., 1997; Meyer, 2004). The final significant parameter is the pump pressure, which drives the mobile phase flow velocity. Generally as flow velocity increases, the efficiency of chromatographic separation decreases, as does the analysis time, since the sample is being driven through the column more rapidly (Nguyen *et al*., 2006). This trend generally applies to columns that employ particle sizes of 3 µm and larger, however with sub-2 µm particles separation efficiency is maintained with increasing mobile phase flow velocity (Fig. 3). By combining sub-2 µm particle short length columns and HPLC instrumentation capable of generating and withstanding constant pressures up to 15000 psi and thus establishing highly stable mobile phase flow rates, the newly coined technique UHPLC was created (Novakova *et al*., 2006). Over conventional HPLC, UHPLC gives enhanced chromatographic separation, permitting resolution of chemical species not possible with HPLC and with faster analysis times and reduced use and waste of solvents. A further parameter that can help reduce analysis times that is now being applied to UHPLC is operation at high temperatures (Gika *et al*., 2008). Both the column and mobile phase temperatures can be increased, which reduces mobile phase viscosity allowing operation at higher flow rates. For plant metabolomic analysis this may well cause problems due to the low thermal stability of both sample metabolites and stationary phase silica.

An Introduction to Mass Spectrometry Instrumentation

Mass spectrometry is just one of many varieties of detector that can be linked to HPLC or UHPLC. Alternative routes of detection that are commonly employed include visible radiation methods such as measuring light scattering or refractive index and absorbance methods such as UV or fluorescence spectroscopy and photodiode array (PDA) detection (Snyder *et al*., 1997; Meyer, 2004). These latter methods can be extremely useful for detecting certain classes of compound that either absorb UV or fluoresce (e.g. kaempferols, quercetins, spermidines); indeed PDA detectors have frequently been linked online to HPLC or UHPLC prior to MS in plant metabolomics studies to provide a further dimension to aid in the characterisation of the detected analyte species (HPLC/UHPLC-PDA-MS; Vorst *et al*., 2005; Moco *et al*., 2006; De Vos *et al*., 2007; Hanhineva *et al*., 2008). However, to acquire the volume of information from an analyte required for full structural elucidation, it is a necessity to employ MS and/or NMR spectroscopy detectors. A MS in simple terms functions to detect the mass-to-charge ratio (*m/z*) and abundance of the various analytes generated during ionisation of a sample extract or chromatographic fraction. Ionisation is a key step since ions are far more easily manipulated than neutral molecules. Unfortunately differing analytes vary in their ionisation efficiencies, i.e. the proportion of metabolite in solution converted to ions in the gas phase. Once ionised the mass analyser detects the ion abundance and *m/z*, which can be related back to the analytes absolute molecular weight. A data system (run on a personal computer) is responsible for the storage of the paired *m/z* and abundance values as well as their processing and display in a mass spectral format. The three principal components found in all varieties of MS are an ionisation source, a mass analyser and a detector; all three components are maintained under vacuum to optimise the transmission of ions to the analyser and detector (Niessen, 1998; Gross, 2004). The following sections will discuss the ionisation methods, mass analysers and detectors commonly applied in current LC-MS based metabolomics.

Sample introduction and ionisation methods employed in liquid chromatography–mass spectrometry-based metabolomics

Prior to ionisation, the sample must first be introduced to the ionisation source. A probe is commonly employed in both LC- and GC-MS analyses. The sample is introduced either by manual direct infusion (DI) or via HPLC or UHPLC followed by DI, following which the sample is delivered to the probe via a capillary; the probe functions to translocate the liquid sample from the capillary to the needle of the ion source. Two ionisation sources are generally employed in LC-MS based metabolomics: electrospray ionisation (ESI) is the most commonly employed (Tolstikov *et al*., 2003; Jander *et al*., 2004; von Roepenack-Lahaye *et al*., 2004; Vorst *et al*., 2005; Moco *et al*., 2006; Rischer *et al*., 2006; De Vos *et al*., 2007; Hanhineva *et al*., 2008) and is particularly well suited to the ionisation of a wide range of metabolites including, drug compounds (Chen *et al*., 2007), amino and organic acids

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(Tolstikov and Fiehn, 2002), sugars and sugar alcohols (Tolstikov *et al*., 2003; Moco *et al*., 2006), sterols, steroids, phospholipids and fatty acids (Allwood *et al*., 2006); however atmospheric pressure chemical ionisation (APCI) is also an appropriate method, especially for the ionisation of non-polar metabolite species such as phospholipids, fatty acids, sterols, steroids, certain esters and essential oils, one classic example in plant studies being the analysis of lipid soluble carotenoids (Dachtler *et al*., 2001; Schweiggert *et al*., 2005).

Essentially the hardware requirements for APCI (Fig. 4A) and ESI (Fig. 4B) are very similar; the sample introduction needles are near identical with the inner needle having a central channel for translocation of sample and outer channels for the delivery of nebulisation and desolvation gases. The arrangement of sample and extraction cones is also near identical, the core differences relating to the ionisation method each technique employs: in ESI the sample is ionised by application of a high electric charge to the sample needle, whereas in APCI the sample is ionised by application of a high charge to a corona pin after the sample has exited (been sprayed) from the sample needle. APCI is achieved by passing the sample in solution through the fused sample needle. As it emerges from the needle it enters a heated tube at atmospheric pressure where it is nebulised by a flow of typically nitrogen gas and vaporised (Fig. 4A). Ionisation of the vaporised sample is achieved by applying a high charge (typically ~5 kV) to the corona pin located at the exit of the heated tube. The corona discharge around the pin causes the vaporised solvent molecules to ionise and form a reagent gas plasma; in turn the vaporised sample molecules are ionised by proton transfer either forming $[M + H]^+$ or $[M - H]^-$ species (Zaikin and Halket, 2006). ESI is also achieved (Fig. 4B) by passing the sample in solution through the capillary to the fused sample needle. The needle has a high charge (typically ~4 kV) directly applied as the sample emerges (is sprayed) from the needle in to the heated tube. The application of the high voltage to the needle results in charge transfer within the sample solution, leading to the formation of ions from the sample molecules (Gaskell, 1997). The sample is then nebulised, again typically with nitrogen gas, prior to vaporisation via solvent evaporation within the heated tube. Once vaporised ions are formed, either by ESI or APCI, they must be extracted from the ionisation source to the mass analyser and detector. In the case of Waters ionisation sources (Fig. 4A and B), they are extracted from the heater tube through the sample cone that is maintained under vacuum to aid the extraction. Once ions have entered the sample cone they are likewise further extracted under a greater vacuum pressure (maintained by turbo pumping) through the extraction cone. Once the ions have entered the extraction cone they are focused into a fine stream using a radio frequency (RF) hexapole lens. The ion stream is then channelled to the mass spectrometer which again is maintained under vacuum pressure. Alternative manufacturers such as Thermo-Finnigan employ different hardware arrangements.

ESI can be applied in both positive and negative ionisation polarities, depending on the capillary voltage that is applied (typically ~+3 kV and ~−2 kV, respectively). In ESI+ mode, common adducts include protonated $[M + H]^+$, sodiated $[M + Na]^+$, and potasiated $[M + K]^+$. In ESI– mode the most common ions are deprotonated $[M - H]^-$, although when chlorine is present within the sample metabolites, negatively charged chlorine adducts are also observed. Many further complex and diverse adduct species can be observed in both ionisation modes. Naturally occurring ^{13}C isotope-containing compounds and double charging of the adduct species (e.g. a Na adduct where the Na is doubly charged) further increase the data complexity (Brown *et al*., 2009), although the use of high-purity solvents and formic acid can help to reduce the number of species observed for each analyte. In comparison to alternative ionisation techniques, ESI has several key advantages, including ionisation across a large mass range appropriate for proteome and metabolome analysis, good sensitivity, soft ionisation (i.e. does not 'over' fragment target analytes) and high adaptability (Gaskell, 1997). It is largely the mass range that can be covered by ESI that has made it one of the most widespread ionisation techniques in use, since it is applicable to both metabolite and peptide/protein

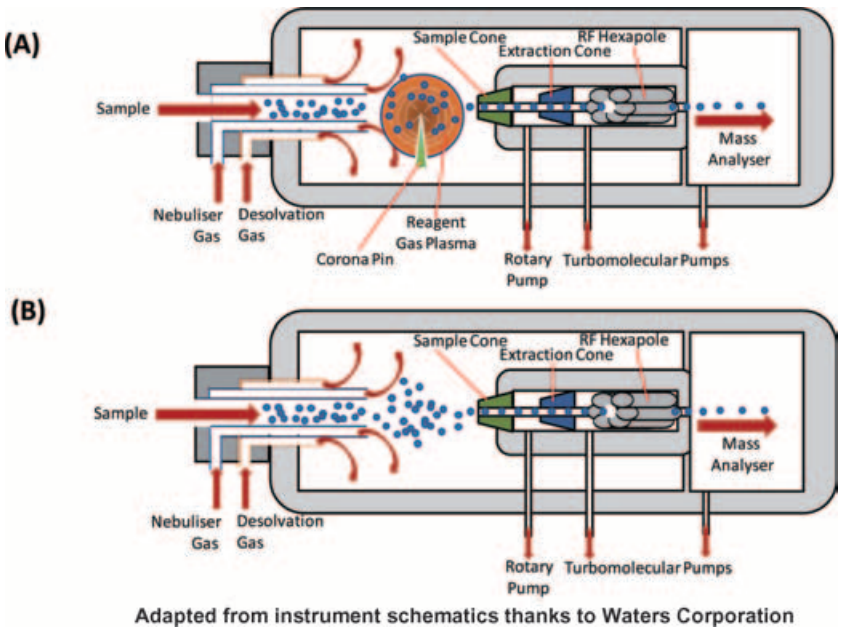

Figure 4. Sample ionisation by the methods of (A) atmospheric pressure chemical ionisation (APCI) and (B) electrospray ionisation (ESI).

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analysis. However, ESI also has several disadvantages, including adduct formation, quenching, low tolerance towards salts and suppression of the ionisation of one metabolite species due to the presence of a high concentration of a different species (i.e. matrix effects), both leading to ionisation suppression (King *et al*., 2000) and failure to detect certain metabolite classes/species.

The Quadrupole (Q) and Triple Quadrupole (QQQ) mass analysers

Quadrupoles were one of the first varieties of mass analyser employed in metabolomics studies, being applied to both LC-MS (Tolstikov and Fiehn, 2002) and GC-MS (Fiehn *et al*., 2000). The single quadrupole consists of four parallel metal rods. Each opposing rod pair is connected electrically and an RF voltage is applied between one pair of rods and the other (Fig. 5A). A direct current (DC) voltage is then superimposed on the RF voltage. An identical DC voltage is applied to each of the four rods; the +/– rods are positioned 180° out of phase, and thus a quadrupolar field is created when the DC is applied. This quadrupolar field allows only mass ions within a certain mass range to pass through; this may be limited to the ions present within a single nominal mass unit range, or by continuously varying the DC voltage the quadrupole can scan across a large user specified mass range. The passage of a specific mass ion through the quadrupole is dependent on the ratio of RF to DC voltage. Mass ions outside the specified range hit the rods and are discharged. Positive mass ions are attracted to negative rods while the converse is observed for negative mass ions. The quadrupole can thus be regarded as a mass filter which functions to allow the passage of only a narrow range of mass ions to the detector at any one time (Niessen, 1998; Gross, 2004).

A latter development in quadrupole-based mass analysers was the triple quadrupole or QQQ, which permitted specialised analyses such as product ion monitoring. Here, three quadrupole cells are arranged linearly to one another (Fig. 5B): the first quadrupole, like the classic single quadrupole analyser, functions to filter specified mass ions or ranges of mass ions; a second non RF quadrupole serves as a collision cell, the collision cell passes a stream of collision gas (e.g. N, Ar, He) through the trapped sample mass ion or ions at a pressure of ~10^{-3} Torr and collision energy of ~30 eV. Collisions between the mass ion and gas result in the generation of fragments, or product ions, from the original precursor ion in a process termed collision-induced decomposition (CID); the third and final quadrupole serves to filter and allow the detection of the daughter fragments produced within the collision cell. Therefore the QQQ mass analyser represents a form of MS/MS and is regarded as a tandem-MS in space analyser, which is suited to targeted MS analysis of intact ions and MS/MS structural analysis of a metabolite by product ion monitoring, thus aiding annotation and characterisation greatly (Niessen, 1998; Gross, 2004; Kopka *et al*., 2004; Catchpole *et al*., 2005). Product ion monitoring is not the only function mode that can be performed with a QQQ; there are also precursor ion monitoring where a product ion is selected in the second analyser and the precursor masses are scanned in the first analyser (Gross, 2004); neutral loss monitoring where the first analyser scans all mass ions and the second analyser is given an offset, based upon the neutral mass of interest, to identify mass ions that have lost that specific neutral moiety (Brügger *et al*., 1996); and selected reaction monitoring (SRM) where both mass analysers are set to scan for a specific precursor and product mass ion transition pair, which can help to improve sensitivity over conventional product ion monitoring (Kopka *et al*., 2004).

The time of flight, reflectron time of flight and quadrupole-time of flight mass analysers

Time-of-flight mass spectrometry (TOF/MS) was first devised in the 1940s with high-resolution instruments becoming available in the 1950s (Willey and McLaren, 1955). However, the use of TOF/MS for routine biochemical analysis did not become popular until the 1990s, largely due to the emergence of ionisation technologies such as ESI and matrix assisted laser desorption ionisation

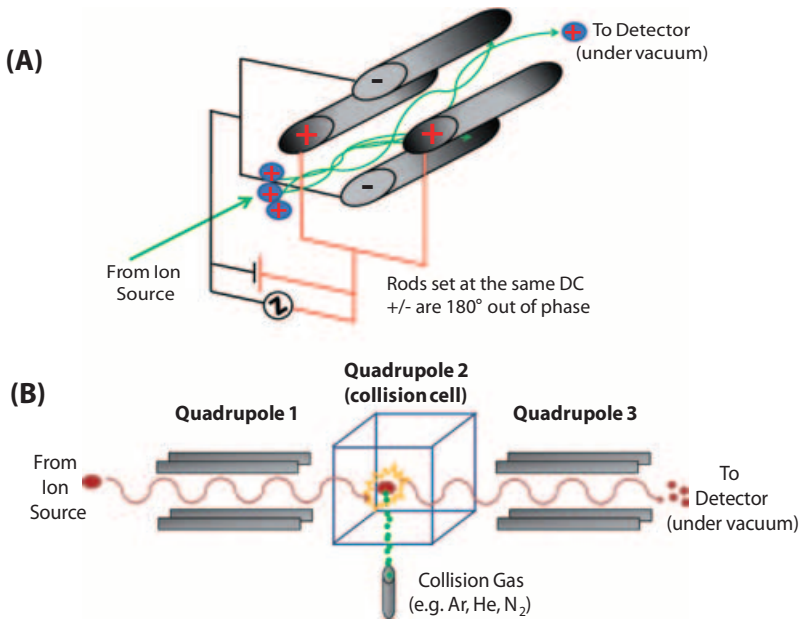

Figure 5. Instrument schematics to illustrate the quadrupole (A) and the triple quadrupole (QQQ) arrangement (B).

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(MALDI), which were amenable to the analysis of large biomolecules, as well as the rapid ongoing developments in digital electronics and computing permitting high-speed data handling (Guilhaus *et al*., 1997). Despite this late rise in the popularity of TOF/MS, it had been predicted as early as the 1980s that there would be a massive revival in its use due to its speeds of detection being on the microsecond time scale, making it ideally suited for application to high speed chromatography (Holland *et al*., 1983).

The concept of TOF/MS is based upon the theory that the mass of the ion is related to its flight velocity along the flight tube and therefore the arrival time at the detector of a mass ion is proportional to its mass, the mass ions reach the detector in order of increasing *m/z* (Guilhaus *et al*., 1997). Early TOF/MS instruments are classed as linear instruments (Fig. 6A); here the ion source is positioned linearly to the source of the TOF/MS and the mass ions pass linearly from the source to the detector at the opposite end of the flight tube. It was later found that the precision of linear TOF/MS could be enhanced by a factor of >10 when the ion source was positioned orthogonally to the TOF/MS (i.e. orthogonal acceleration (OA)TOF/MS; Fig. 6B). The improved precision of OATOF/MS was ascribed to higher efficiency ion gating between the ionisation source and TOF/MS, correction of ion spatial and velocity dispersions and an improved maximum duty factor at the high mass end of the spectra (Dawson and Guilhaus, 1989). The precision of linear and OATOF/MS instruments can be further improved by increasing the length of the flight tube; since the distance the ions travel is greater, there will be a greater time and space separation of mass ions of similar *m/z* (Dawson and Guilhaus, 1989; Guilhaus *et al*., 1997). However, on occasion mass ions of the same *m/z* but with differing flight velocities are detected resulting in mass ion peak broadening (due to one mass ion reaching the detector in a shorter flight time than the other, i.e. caused by dispersions of the initial position or velocity of the mass ions in the accelerators pusher region). This problem can be avoided by employing a reflectron [(Re)TOF/MS; Fig. 6C and D]; the reflectron (also known as the ion mirror) typically consists of a series of rings that function to create a retarding electrostatic field. The mass ions penetrate and are held within the electrostatic field. Higher energy mass ions with greater flight velocities penetrate the electrostatic field further and are retained for longer, whereas low energy mass ions of the same *m/z* do not

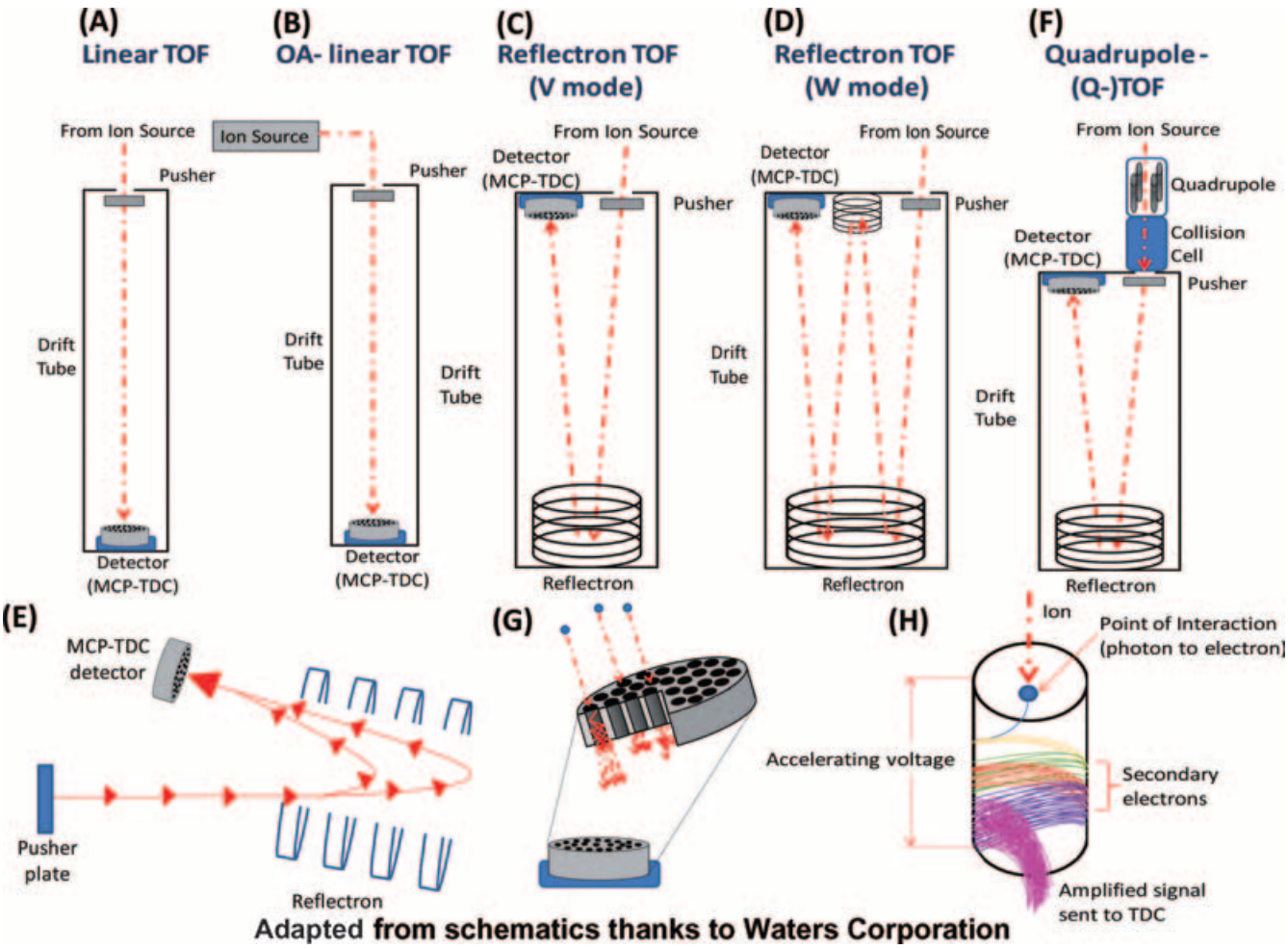

Figure 6. Instrument schematics to illustrate time of flight (TOF), reflectron time of flight (ReTOF) and quadrupole time of flight (Q-TOF) mass spectrometry arrangements, as well as the micro-channel plate (MCP) detector and signal amplification. (A) Linear time of flight mass spectrometry; (B) orthogonal acceleration linear time of flight mass spectrometry ($_{OA}$TOF/MS); (C) reflectron time of flight mass spectrometry—V mode arrangement; (D) reflectron time of flight mass spectrometry—W mode arrangement; (E) illustration as to how the reflectron equalises differential ion velocities for ions of the same mass-to-charge (*m/z*); (F) quadrupole reflectron time of flight mass spectrometry; (G) the MCP; (H) an MCP pore illustrating signal amplification via generation of secondary electrons. TDC: time-to-digital converter.

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penetrate so far into the electrostatic field and are retained for shorter time periods. Thus, the arrival time of the low and high energy mass ions of the same *m/z* at the detector are equalised (Fig. 6E) and peak broadening is prevented which results in greatly enhanced mass spectral resolution (Guilhaus *et al*., 1997; Gross, 2004). Early TOF/MS instruments were based upon the classic V mode arrangement (Fig. 6C); by positioning a second reflectron at the opposing end of the TOF flight tube, the W mode arrangement (Fig. 6D) was made possible, further enhancing mass resolution not only due to equalising ion velocities through two reflectrons but also the longer path length that the ions travel between the ionisation source and detector. Current ReTOF/MS instruments are typically capable of operation in both the V and W modes.

TOF/MS provides both good sensitivity and resolution for the profiling of intact precursor ions generated from metabolites by ESI and perhaps represents the most appropriate MS instrument to apply LC separations to for this objective. ESI is a soft ionisation method resulting in minimal in-source fragmentation (i.e. fragmentation of precursor ions within the ionisation source, leading to the formation of numerous product ions) especially when low capillary, extraction and sample cone voltages are employed (Gaskell, 1997). To make ESI-TOF/MS applicable to tandem-MS analyses it was combined with the quadrupole mass analyser, thus forming the instrument known as a quadrupole-time of flight (Q-TOF/MS; Fig. 6F). The Q-TOF/MS consists of a quadrupole to filter the mass ion or mass ion range of interest, linked to a collision cell for CID where the filtered precursor ion is fragmented via a stream of collision gas, further linked to a TOF/MS or ReTOF/MS for the detection of the fragmented product ions (Gross, 2004). The ability to undertake product ion monitoring or SEM with tandem-MS using Q-TOF (Allwood *et al*., 2006), or QQQ (Catchpole *et al*., 2005) as previously described is key for the unambiguous identification of metabolites. Ion trap-based instrumentation can also be used to achieve this objective although it be based upon the separation of ions in time rather than space (as for Q-TOF and QQQ analysers). Ion trap instruments will be discussed later. Q-TOF and QQQ can be programmed to perform two functions within a single sample analysis, the first function being scanning of intact ions (i.e. MS), and the second function being scanning of product ions generated by CID (i.e. MS/MS) within the collision cell or by applying very high energies to the ESI resulting in in-source fragmentation (Moco *et al*., 2006; De Vos *et al*., 2007; Hanhineva *et al*., 2008). Recent developments in Q-TOF/MS have led to instruments with extremely high levels of resolution and mass accuracy, making them particularly appropriate for combined MS profiling and MS/MS analysis within a single experimental run. Within certain instruments, the Waters Synapt HDMS being one example, an ion mobility spectrometry (IMS) cell is incorporated between the quadrupole and TOF/MS. IMS separates mass ions based upon shape and size rather than charge, thus mass ions of equal *m/z* or even isomers which cannot be separated by LC or within the quadrupole can potentially be separated by their drift within the IMS (Kanu *et al*., 2008).

The micro-channel plate and time-to-digital converter-based detector, and construction of a mass spectrum

After the sample has been ionised within the source and has passed through the mass analyser, it has reached the final element of the mass spectral instrument, the detector. Detectors typically record either a current produced or a charge induced when an ion passes close to or strikes the detector's surface (Gross, 2004). Typical detectors are based upon electron multipliers, although many modern day Q/MS, QQQ/MS, TOF/MS and Q-TOF/MS instruments employ the micro-channel plate (MCP) linked to a time-to-digital converter (TDC). The MCP is typically made up of a highly resistive material of ~2 mm thickness which has an array of small (~10 μm) micro-channels passing through it (Fig. 6G). A strong electric field is created within the MCP; when a mass ion strikes the detector, it results in the conversion of the particle or photon signal to an electron signal. As the electron passes through the pore striking its surfaces, it results in the production of secondary electron cascades; the more cascades that occur, the greater the signal amplification becomes (Fig. 6H) (Matsuura *et al*., 1985). The electron signal is next passed to a data managing system which further amplifies the signal on its way to the TDC. The task of the TDC is to count the amplified signal information it receives into time bins across a mass spectral scan. A typical mass spectral scan for a 4 GHz TDC consists of 20 time bins, which produce an approximately 50 μs scan window (Matsuura *et al*., 1985; Dubois *et al*., 1999). Each time the accelerator region pulses the ions, the TDC is initiated (Fig. 7A) and starts counting the ions into separate bins and records their time interval (Fig. 7B). Once the ions within the mass analyser have been detected, they must be expelled in order to purge the system prior to the next scan event, this is achieved by maintaining the analyser under vacuum pressure generated by turbo pumping. A single chromatographic peak will have many mass spectral scans of data collected across it. By summing the mass spectral scans across a peak and displaying the summed data on a plot of *m/z* versus abundance/intensity, a mass spectrum representative of the mass ions within the chromatographic peak is produced (Fig. 7C).

Trap-based mass spectrometry

The key aim of an ion trap mass spectrometer is to isolate ions of low abundance while maintaining that they are trapped, thus building an ever increasing population of that specific ion until the population is great enough to permit MS or MS^n-based analyses (Gross, 2004; Kang *et al*., 2007). MS^n, also known as sequential MS, is similar to the MS/MS analyses described earlier for QQQ and Q-TOF instruments; however rather than just performing two MS events in space, due to being able to trap and then re-trap mass ions, many sequential MS events can be achieved in time. Therefore an ion trap-based instrument can detect a precursor ions *m/z* prior to CID and analysis of the fragment product ions *m/z*, prior to repeat trapping and CID of the various fragment product ions. This process can continually be repeated in a sequential manner (i.e. 'MS to the ^{n}') each time gathering further more in-depth structural information (Kang *et al*., 2007). Ion trap MS instruments are particularly appropriate for qualitative analysis, especially of low abundance mass ions due to their high trapping potential (Li *et al*., 2009), although they are not so appropriate for quantitative analysis due to having to consider the trapping times applied to the different ion species in order to back-calculate to their true concentration. A typical ion trap analyser consists of two hyperbolic metal electrodes with their foci facing each other and a hyperbolic ring electrode positioned half-way between the hyperbolic electrodes (Fig. 8). The mass ions are trapped in the space between these three electrodes by AC (oscillating, non-static) and DC (non-oscillating, static) electric

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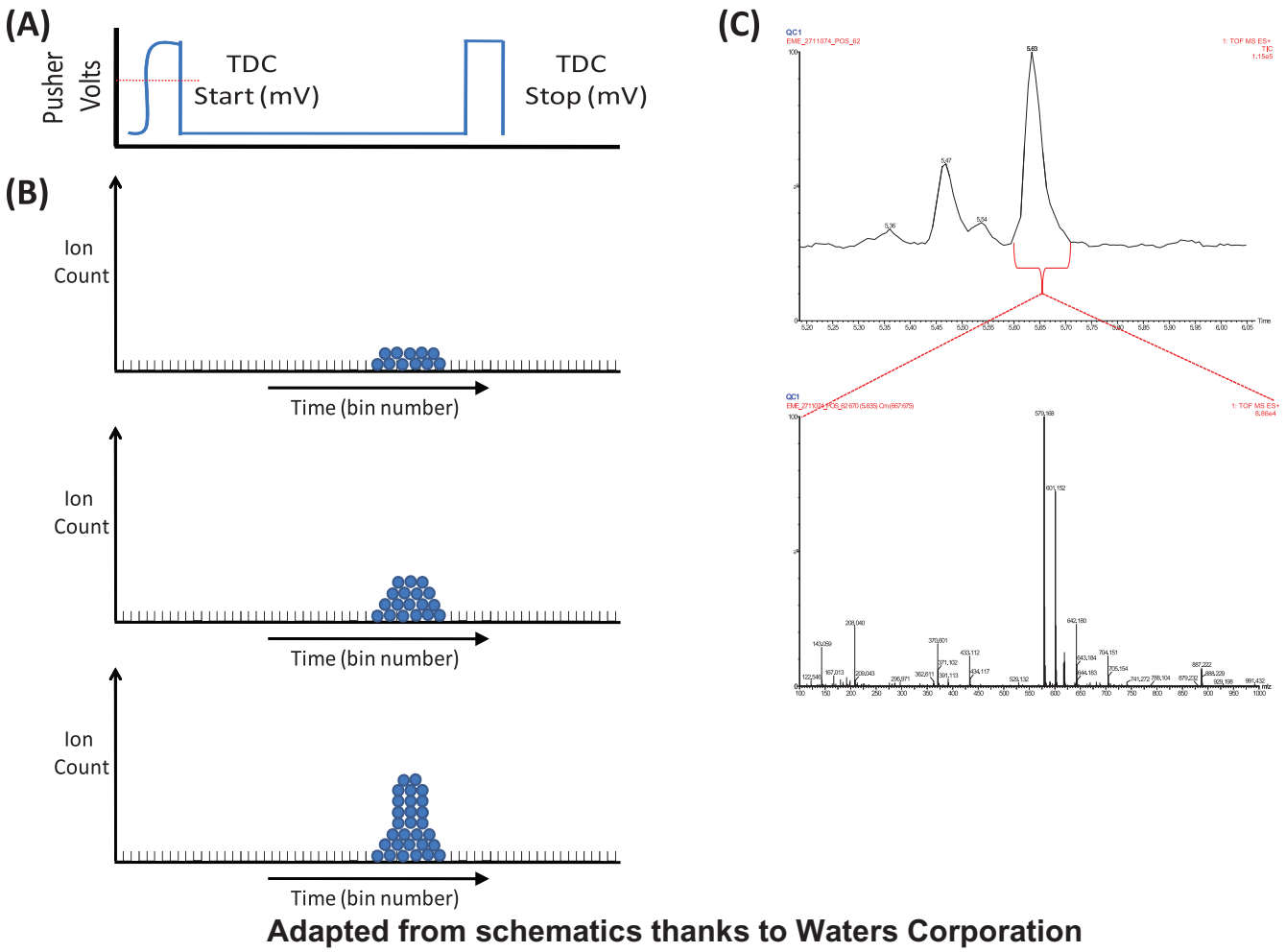

Figure 7. Construction of a mass spectrum. (A) Data acquisition across a single mass spectral scan; (B) generation of a mass spectrum by summing all mass spectral scans across a chromatographic peak. TDC: time-to-digital converter.

fields. The AC RF voltage oscillates between the two hyperbolic metal end cap electrodes, if ion excitation is desired the driving AC voltage is applied to the ring electrode. This results in the formation of a plume of trapped ions which oscillate between forming a thin stretched plume and a short squashed plume between the ring and end cap electrodes (Xia *et al*., 2003). An example of possibly the most commonly employed ion trap that is applied to LC in metabolomic analyses is the Thermo-Finnigan LTQ™ (Linear Trap Quadrupole; Fig. 8A). Linear ion traps such as the LTQ use a set of quadrupole rods to confine ions radially and a static electrical potential on end electrodes to confine the ions axially. The linear form of the trap can be employed simply as a low-sensitivity ion trap MS which employs an electron multiplier detector (Jackson *et al*., 2008); however it may also be applied as a selective mass filter prior to upstream analysis with a mass analyser that gives higher sensitivity and resolution, for example the Thermo hybrid LTQ-Fourier transform ion cyclotron resonance (FT-ICR)-MS (Southam *et al*., 2007) and Thermo LTQ-Orbitrap XL MS systems (Koulman *et al*., 2009).

The LTQ is an example of a linear trap, over the past decade however much focus has switched from linear traps to 3D traps which offer superior levels of resolution and mass precision (De Hoffmann and Stroobant, 2007). There are three varieties of 3D ion trap, the standard 3D ion trap known as a Paul trap, the combined electrode and magnetic 3D trap known as the Penning trap [e.g. Fourier transform ion cyclotron resonance (FT-ICR)-MS], and the Kingdon trap that was later utilised in the development of the Thermo Orbitrap system. The FT-ICR-MS instrument is one of great future potential since it currently offers the highest levels of resolution (>1000000 for non-complex samples) and mass precision (typically within 1–2 ppm). The instrument's dynamic range is also good (~5000) (Barrow *et al*., 2005; Brown *et al*., 2005). However, due to the low scan speeds employed by FT-ICR-MS, typically 1 scan event per second, it is more appropriate for the direct infusion of samples rather than to apply *a priori* LC. The Thermo Hybrid LTQ-Orbitrap MS system (Fig. 8B) employs much higher scan speeds and thus conversely to FT-ICR-MS is equally appropriate for the application of either *a priori* LC or direct infusion. The Orbitrap, despite the higher scan speeds employed, still produces high levels of resolution (~100000) and mass accuracy (1–2 ppm), as well as maintaining a good dynamic range across 3–4 orders of magnitude (Hu *et al*., 2005; Markarov *et al*., 2006).

Both the FT-ICR-MS and the Thermo Orbitrap function to induce a cyclotron motion within the trapped ion populations. FT-ICR-MS achieves this via magnetism which naturally induces a cycling (cyclotron) motion of ions which is proportional to their *m/z* (refer to Fleming's right-hand rule for the motion of an ion in a magnetic field). An RF signal is applied to the ICR cell through

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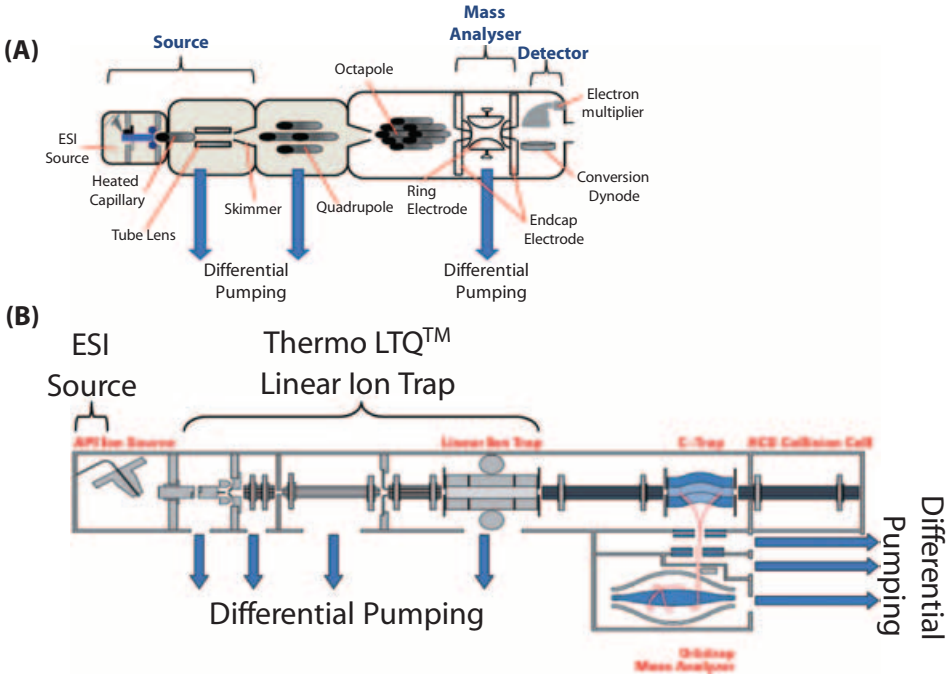

Figure 8. Instrument schematics to illustrate the Thermo-Finnigan linear trap quadrupole (LTQ™) and LTQ hybrid Orbitrap mass spectrometry system.

two opposing acceleration electrodes, which induces the cyclotron of ions into a greater orbit. As the ions orbit they pass two linked detector plate electrodes which record the weak AC image current generated by the passing mass ions (Barrow *et al*., 2005). The signal is then amplified; this recorded time domain image current is further Fourier transformed, thus producing a mass domain signal that is summed to form a mass spectra. The Orbitrap also induces mass ions into a cyclotron motion; however, this is achieved by trapping ions between an outer electrode plate and an inner electrode spindle as opposed to within a magnetic field. Here, the ions are injected tangentially into an electric field between the electrodes and trapped because their electrostatic attraction to the inner electrode is balanced by centrifugal forces. Therefore, ions of a specific *m/z* (dictated by the strength of electric field applied to the trap) move in rings around and oscillate back and forth along the central spindle. The frequency of these harmonic oscillations is independent of the ion velocity and is inversely proportional to the square root of the *m/z*, again by employing detection electrodes the time domain image current is detected, amplified and Fourier transformed to produce a mass domain signal and eventual mass spectra (Hu *et al*., 2005; Markarov *et al*., 2006). Both FT-ICR-MS and the Thermo Orbitrap detect the mass ions in a non-destructive manner and thus the ion population is maintained within the trap; again to purge the system of mass ions they are expelled under vacuum pressure maintained by turbo pumping. It is likely that applications of LC to Thermo Orbitrap MS will gain pace over the coming years especially for the identification of metabolites via MS^n.

Applications of the various mass spectrometry analysers within metabolomics

Each of the various MS-based analysers discussed within this section have differing advantages and disadvantages which make them more applicable to specific areas within metabolite profiling research. For example, certain MS analysers are more suited to quantitative MS profiling, whereas alternative analysers are more suited to metabolite identification due to possession of MS/MS or MS^n capabilities. The ionisation source that is employed effectively has the greatest bearing as to the range of metabolite groups detected. ESI is capable of ionising large biomolecules such as peptides and proteins as well as small molecules when compared with APCI. APCI generally has a greater ability to ionise non-polar compounds and so if a study was particularly targeted towards phospholipids and fatty acids (Allwood *et al*., 2006), plant sterols or oil soluble pigments such as carotenoids (Dachtler *et al*., 2001; Schweiggert *et al*., 2005), APCI would be the most appropriate ionisation method. Alternatively, ESI is more suited to the ionisation of more polar compounds, for example, amino acids (Tolstikov and Fiehn, 2002), sugars and sugar alcohols (Tolstikov *et al*., 2003; Moco *et al*., 2006), glucosides and flavanoids (De Vos *et al*., 2007; Hanhineva *et al*., 2008). Certain groups of compounds, such as the organic acids, ionise well with both APCI and ESI (Tolstikov and Fiehn, 2002), although certain specific organic acids are only amenable to ionisation via one method or the other, depending upon their polarity. To obtain information on as wide a range of plant metabolites as possible, both ESI and APCI should be employed in both positive and negative ionisation modes; however this can result in a lot of data redundancy due to metabolites which are ionised and detected via both methods under one or both polarities. For general studies of plant metabolism, if only one ionisation source was to be employed, then ESI would be the favoured method, since it offers overall greater coverage of polar metabolites and yet still permits the ionisation of non-polar species such as sterols and phospholipids.

Once the ionisation source/sources has/have been selected, an appropriate MS analyser must next be considered (Table 1). For

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Table 1. Applications of various mass spectrometry analysers within metabolomics

	Application of HPLC/UHPLC	MS quantitative metabolite profiling	MS/MS or MS^n metabolite identification
Quad	+	+	–
TOF	++	++	–
Q-TOF	++	+	+
QQQ	+	++	+
Ion trap	+	–	++
FT-ICR-MS	–	+	++

quantitative profiling of plant metabolites it is best to avoid ion trap-based instruments since in order to derive quantitative values the trapping times for each detected metabolite must be considered when calculating the quantity. Quadrupole-based MS analysers are particularly appropriate for quantitative profiling, although since the rejuvenation of TOF/MS analysers they have become more redundant since TOF/MS has greater scan speeds, dynamic range and sensitivity, thus allowing quantitative profiling over a wider range of metabolite concentrations and the detection of low concentration metabolites below the sensitivity limit of quadrupole analysers. For the analysis of extremely low concentration metabolites, an ion trap based instrument would be the most appropriate since they permit the trapping of specific ions so that a large population of that ion species can be collected and further analysed. Ion trapping can be performed with conventional ion trap analysers as well as more recent developments such as the FT-ICR-MS and LTQ-Orbitrap; however it must be considered that, due to the low scan speeds employed by FT-ICR-MS, it is not as appropriate for the application of HPLC or UHPLC as the LTQ-Orbitrap or conventional ion trap analysers. For the identification of metabolites, an analyser that possesses MS/MS or MS^n capabilities is a necessity. Q-TOF, QQQ and conventional ion traps have commonly been employed to perform MS/MS within plant metabolomics for the identification of a broad range of phytochemical species; however, compared with the more recent developments of FT-ICR-MS and LTQ-Orbitrap MS analysers that have MS^n capabilities for enhanced levels of structural analysis, the conventional MS/MS = based techniques lack mass accuracy. The combination of extremely high levels of mass accuracy and resolution as well as the applicability to HPLC or UHPLC and high levels of user-friendliness make the LTQ-Orbitrap MS analyser one of the current instruments of choice for metabolite identification. Interest in Q-TOF instruments has also increased since the development of higher sensitivity analysers such as the Waters Synapt HDMS. The main advantage of Q-TOF/MS over trap-based instruments is that they permit both accurate quantitative profiling via TOF/MS without the need to take into account ion trapping times, as well as allowing for the filtering of specific ranges of mass ions within the quadrupole and CID for structural analysis via fragmentation. Modern Q-TOF/MS instruments also allow the user to collect two functions of data within a single run; thus for each chromatographic peak both MS quantitative profiling data and MS/MS fragmentation data for metabolite identification can be collected, therefore saving time, costs and instrument resources (since only one instrument is employed for MS and MS/MS analyses rather than two independent analysers). A summary of the above-mentioned appropriate applications for each of the discussed MS analysers is presented in Table 1.

Concluding Remarks

In conclusion it is hoped that this review first has highlighted the potential value that LC-MS has in the analysis of semi polar compounds of plant origin, and second will serve as a helpful introduction or recap to scientists from outside of the metabolomics or analytical chemistry fields on LC theory, the development and hardware associated with HPLC and UHPLC analyses, and the range of mass spectrometry instrumentation that may be applied to LC-based analyses.

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