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Advances in ultrasensitive mass spectrometry of organic molecules

Mathivathani Kandiah[a] and Pawel L. Urban*[ab]

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Ultrasensitive mass spectrometric analysis of organic molecules is important for various branches of chemistry, and other fields including physics, earth and environmental sciences, archaeology, biomedicine, and materials science. It finds applications – as an enabling tool – in systems biology, biological imaging, clinical analysis, and forensics. Although there are a number of technical obstacles associated with the analysis of samples by mass spectrometry at ultratrace level (for example analyte losses during sample preparation, insufficient sensitivity, ion suppression), several noteworthy developments have been made over the years. They include: sensitive ion sources, loss-free interfaces, ion optics components, efficient mass analyzers and detectors, as well as ''smart'' sample preparation strategies. Some of the mass spectrometric methods published to date can achieve sensitivity which is by several orders of magnitude higher than that of alternative approaches. Femto- and attomole level limits of detection are nowadays common, while zepto- and yoctomole level limits of detection have also been reported. We envision that the ultrasensitive mass spectrometric assays will soon contribute to new discoveries in bioscience and other areas.

1. Introduction

Scientists necessitate sensitive analytical methods which could provide reliable data on the low-abundance analytes present at nano- to yoctomole level.[1] Mass spectrometry (MS) has become recognised as one of the most valuable tools available for structural characterisation of organic molecules.[2] Undoubtedly, the high interest in applying mass spectrometry is due to the development of efficient ion sources and mass analyzers, which can fulfil the sensitivity requirements of many experimental studies. Identification of analytes in volume-limited or dilute samples can nowadays be accomplished using many of the available mass spectrometric methods. Ultrasensitive MS-based protocols already benefit various areas of chemistry and biochemistry, including proteomics, lipidomics, metabolomics, drug discovery, and single-cell analysis.

There is a common view that sensitivity in mass spectrometry is just a matter of instrument design and development, and, therefore, it is not addressed by individual users of MS; here, the authors argue that this view is not necessarily accurate. Although many aspects of sensitivity improvement are beyond the capabilities of an average chemistry or biochemistry laboratory, there is much that can be done to convert a sensitive mass spectrometer into an ultrasensitive instrument. However, in order to accomplish mass spectrometric analysis at the ultratrace level, it is necessary to address and optimize various stages of the analysis process (*cf.* Fig. 1). In fact, different factors limit sensitivity in MS; they can roughly be classified into three groups: (i) sample-related (*e.g.* sample matrix interference), (ii) ion source-related (*e.g.* ionization bias, dispersion), and (iii) mass analyzer-related (*e.g.* poor transmission of ions to the detector, detection). As will become clear in the following sections, despite many improvements on the instrumental side, sample preparation is critical for ultrasensitive MS analyses.

When discussing various experimental approaches in mass spectrometry, the difference between the concepts of ''mass sensitivity'' and ''concentration sensitivity'' shall be clarified. Mass sensitivity refers to the ability of an instrument (or a method used with it) to detect minute amounts of analytes within limited amounts of samples. The concentration

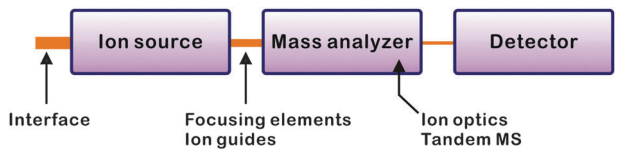

Fig. 1 Main components of mass spectrometer.

[a] *Department of Applied Chemistry, National Chiao Tung University, Hsinchu 300, Taiwan. E-mail: plurban@nctu.edu.tw*

[b] *Institute of Molecular Science, National Chiao Tung University, Hsinchu 300, Taiwan*

sensitivity refers to the ability of an instrument (or a method) to detect low concentrations of analytes in samples which are not limited by volume. These two concepts partly overlap; however, they are useful to describe the performance of various instruments, ionization techniques, and sample preparation methods designed for the analysis of low abundance molecules.

We believe that a thorough understanding of various modes of MS will let the users take full benefit of this powerful technique. For more insight on the basic principles of the mass spectrometry technology, the readers are referred to the textbooks and specialized reviews.[2–6] In the present review, we are aiming to highlight the important contributions in ultrasensitive MS-aided detection of organic molecules. We will discuss the methods of interfacing upstream sample preparation steps with mass spectrometry, so as to ascertain the minimum loss of sensitivity. We acknowledge all the brilliant work on ultrasensitive mass spectrometry published by many researchers over the years, and apologize those authors whose valuable reports were not included in the reference list.

2. Ionization techniques for high-sensitivity mass spectrometry

The ion source is the part of mass spectrometer which ionizes analyte molecules (*cf.* Fig. 1). The resulting gas-phase ions are subsequently transported in an electric field to the mass analyzer. The selection of an ionization technique is the key factor that determines the types of samples which can be analyzed. It is also critical to achieve a high sensitivity. Table 1 lists the ion sources that are commonly used in mass spectrometry along with the authors' assessment of sensitivity and standing as standard techniques, while Table 2 provides examples of high sensitivity determinations conducted using different MS-aided methods.

The main factors that affect the choice of ionization mode are polarity, molecular weight and thermal stability of the compounds which are analyzed, as well as the state in which the sample is delivered (solid, liquid, or gas), and the type of sample matrix. It should be noted that careful control of ionization conditions can provide a significant increase in sensitivity, upper mass limit, and it can reduce the level of chemical noise. Once an ion source is selected, further optimization often includes tuning the preset voltage values, which are applied to the electrodes of the ion source, and the electrodes at the orifice of the mass spectrometer (analyzer module). If the performance is not as good as expected, then steps should be taken to retrieve any losses in sensitivity or resolution. Regular maintenance is normally needed to achieve high performance and stable operation. The performance of an ion source will also depend on the purity of solvents or gases used,

Mathivathani Kandiah

Mathivathani Kandiah conducted her doctoral studies in the School of Chemistry at the University of Bristol, UK, under the guidance of Prof. Neil Connelly; and she obtained her PhD degree in organometallic chemistry. Then she moved to Canada to work on a postdoctoral project related to the development of hydrogen-storage materials in the University of New Brunswick, Fredericton. Dr Kandiah returned to the UK to work as a postdoctoral assistant in the University of Bath, which was followed by a postdoctoral stay in the University of Oslo, Norway. There she pursued her interest in metal organic frameworks. Since April 2012, Dr Kandiah has been working as a postdoctoral fellow in the group of Prof. Pawel Urban in the National Chiao Tung University, Taiwan. Her research is focused on the analysis of extracellular metabolites by novel mass spectrometric tools.

Pawel L. Urban

Pawel Urban obtained his higher education in the College of Interfaculty Individual Studies in Mathematics and Natural Sciences (MISMaP), University of Warsaw, Poland. After a short stay in the University of Alcala, Spain, he embarked on doctoral studies in the Department of Chemistry at the University of York, UK, where he developed methods for monitoring biocatalytic processes in microscale. Following an assistantship in the Faculty of Biology, University of Warsaw; Dr Urban pursued metabolomics of single eukaryotic cells, working as a postdoctoral fellow at the ETH Zurich, Switzerland. His main contribution from that period is related to the development of micro-arrays for mass spectrometry (MAMS) for applications in single-cell analysis. Afterwards, as a postdoctoral fellow in the Department of Applied Chemistry at the National Chiao Tung University, Taiwan, he continued research on mass spectrometric analysis of microscale samples. Since August 2011, he has been appointed in the NCTU as assistant professor. His research interests include time-resolved mass spectrometry, mass spectrometric analysis of microscale samples, isotopic labelling of metabolites, and studies of spatiotemporal dynamics in chemistry and biochemistry using novel analytical approaches.

Table 1 Sensitivity of mass spectrometric detection of organic molecules using popular ion sources

Ion source	Concentration sensitivity	Mass sensitivity	Standard technique	Remarks
EI	++	+++	+++	Normally used with GC. In-source fragmentation.
CI	++	+++	++	Normally used with GC.
ESI	+++	++	+++	Soft; hyphenated with various separation techniques.
nanoESI	++	+++	++	Suitable for low flow-rate systems.
APCI	++	++	+	Suitable for analysing non-polar molecules.
MALDI	++	+++	+++	Matrix interference, matrix-related selectivity.
LDI	+	++	++	In-source fragmentation, limited applicability.
SIMS	++	+++	+	Significant in-source fragmentation.

Table 2 Selected examples of high sensitivity analyses conducted using different mass spectrometry systems

Ion source	Mass analyzer	Setup/conditions	Analytes tested	Amount detected[b]	References
EI	Quadrupole	GC-MS	Nucleobases of DNA	Sub-fmol level	Byun *et al.*,12
CI	Magnetic sector	GC-MS	DNA adducts	5 fmol	Heppel *et al.*,15
NanoESI	FT-ICR[a]	—	Proteins	30 zmol	Belov *et al.*,108
NanoESI	Orbital trap	Segmented flow	Leucine-enkephalin	amol level	Pei *et al.*,38
NanoESI	Time-of-flight (TOF)	Microfluidic nanoESI	Leucine-enkephalin	80 zmol	Sun *et al.*,34
APCI	Triple quadrupole	LC-MS	Posaconazole	~700 fmol	Shen *et al.*,46
MALDI	TOF	MALDI target, picoliter vials, 2,5-dihydroxybenzoic acid	Bradykinin, cytochrome *c*	amol level	Jespersen *et al.*,59
MALDI	TOF	MALDI target 9-aminoacridine	Metabolites in yeast	amol level	Amantonico *et al.*,123
SIMS	TOF	Nanostructured silicon	Peptide	1 fmol	Northen *et al.*,82
SALDI	TOF	Diamond nanowires	Verapamil	200 zmol	Coffinier *et. al.*,78
-DIOS	TOF	Nanostructured silicon	Des-Arg9-bradykinin	800 ymol	Trauger *et al.*,81
-NIMS	TOF	Nanostructured silicon with ''initiator''	Verapamil	700 ymol	Northen *et al.*,82
-LISMA	TOF	Silicon microcolumn arrays	Peptides	fmol level	Chen *et al.*,85
-NAPA	TOF	Nanospot array with chromium layer	Verapamil	800 zmol	Walker *et al.*,86

[a] FT-ICR: Fourier transform ion cyclotron resonance mass spectrometry. [b] Approximate mole levels.

and the performance of the on-line systems (*e.g.* chromatographs) connected to the device.

2.1 Electron ionization

Electron ionization (EI) – also known as electron impact – is the oldest and best-characterized of all the current ionization techniques.[7] In the EI source, a beam of 70 eV electrons passes through a gas-phase sample. An electron that collides with a neutral analyte molecule can knock off another electron, resulting in a radical cation.[8] To improve the efficiency of ionization, a weak magnetic field can be applied parallel to the moving direction of the electrons in order to restrict the electrons in a narrow helical trajectory. The ionization process can either produce a molecular ion which will have the same molecular mass and elemental composition, or it can produce fragment ions.[9] Positive ions are formed due to loss of electrons while negative ions are produced due to addition of electrons to the analyte molecules. Decreasing the electron energy can reduce fragmentation, but it also reduces the number of ions, thus affecting sensitivity.[10] EI is widely used in MS to characterize structure, and measure molecular masses of gaseous and volatile organic compounds with the molecular weight typically below 1000 Da.[11] The advantage of this technique is that it is simple to use, and it provides fingerprint mass spectra of organic compounds. The analyzed molecules can readily be identified by careful analysis and interpretation of EI mass spectra as well as using commercially available libraries.

As an example, Byun *et al.*[12] demonstrated highly sensitive detection of dimethyl-*tert*-butylsilyl derivatives of nucleobases of DNA using gas chromatography (GC) coupled with a mass spectrometer equipped with the EI source. Spectra represented a single major fragment ion that was readily monitored by EI-MS. A number of factors contribute to the sensitivity of GC-EI-MS. In this particular case, sensitivity increased by 10-fold when DNA bases were converted to trimethylsilyl (Me_3Si) or dimethyl-*tert*-butylsilyl, with the use of *N*-methyl-*N*-(*tert*-butyldimethylsilyl)trifluoroacetamide, rather than the more commonly used bis(trimethylsilyl)trifluoroacetamide. It was possible to detect halogenated DNA adducts in the low femtomole range by GC-EI-MS in the full scanning mode. Limits of detection (LODs) obtained in the selected ion monitoring mode were 10 times lower than in the case of non-derivatized nucleobases. The dynamic range between 2 fmol and 20 pmol demonstrate that GC-MS with EI (positive-ion mode) can enable quantitative analyses of small amounts of halogenated nucleobases.[12]

2.2 Chemical ionization

Chemical ionization (CI) – introduced in 1966 by Munson and Field[13] – is a particularly useful technique when no molecular ions can be observed in EI mass spectra. In such cases, it enables determination of the mass-to-charge (m/z) ratios of intact ions corresponding to the analytes of interest. CI is a lower energy process than EI; this results in a considerable

reduction of fragmentation processes. However, CI still requires volatilisation of analytes, so thermal degradation of the analyte can still lead to fragment ions being observed.[13] Since the ionization in CI is the result of one or several competing chemical reactions,[3,14] the sensitivity strongly depends on the conditions of the experiment. In addition to primary electron energy and electron current, the reagent gas, the reagent-gas pressure, and the ion-source temperature have to be stated with the sensitivity data to make valid comparisons.[3]

For example, Heppel *et al.*[15] presented an ultrasensitive and highly specific method for adduct determination in milligram amounts of biopsy samples by using capillary GC coupled to a high-resolution mass spectrometer operated in the negative chemical ionization (NCI) mode. Ionization was conducted using ammonia as the reagent gas at a pressure of 2.73 kPa while the ion source temperature was kept at 115 °C. Electron energy and emission current were set to 255 eV and 1.00 mA, respectively. The analysis targeted 4-hydroxy-1-(3-pyridyl)-1-butanone (HPB)-releasing DNA adducts which were formed by metabolic activation of the tobacco-specific nitrosamines 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and *N*′-nitrosonornicotine (NNN). Both NNK and NNN are considered carcinogenic to humans by the International Agency for Research on Cancer.[16] The GC-NCI-MS method has an LOD of 4.6 fmol HPB, and a limit of quantification (LOQ) of 14.9 fmol HPB.[15] It enables determination of DNA adducts in less than 10 mg of tissue, while other analytical methods for determination of HPB-releasing DNA adducts require 0.3–2.0 g of human tissues samples (*e.g.* from lung or oesophagus). Therefore, it has the potential to facilitate studies of HPB-releasing DNA adducts in oesophageal biopsy samples from patients with different stages of oesophageal cancer.[15]

2.3 Electrospray ionization

Electrospray ionization (ESI) – developed by John Fenn[17] – is one of the common atmospheric pressure ionization techniques, and it has become widely used in the analyses of polar organic molecules, including biopolymers[18] ranging from less than 100 Da to more than 10^6 Da in molecular mass. Formation of gas-phase ions in ESI-MS typically relies on applying an external electric potential to the ESI emitter.[17,19,20] One of the main advantages of ESI-MS is the capability of analyzing extremely small volumes of samples.[21] In general, a dilute analyte solution (typically, <1 mM, dissolved in a polar volatile solvent) is injected by a mechanical syringe pump through a stainless steel capillary (typically, o.d $\sim$0.2 mm, i.d. $\sim$0.1 mm) at a low flow rate (typically, 1–20 μL min^{-1}).[22] A high voltage (typically, 2–6 kV) is applied to the metal capillary relative to the surrounding source-sampling cone. The resulting electric field causes the dispersion of the sample solution into an aerosol of highly charged electrospray droplets.[22] A coaxial sheath gas flow (dry N_2) around the capillary ensures sufficient nebulization of the sample solution, and stabilizes the spray. Improving nebulization efficiency can lower detection limit by one order of magnitude.[23]

Regarding the mechanism of ion formation in ESI, it is believed that low molecular weight analytes follow the so-called ''ion evaporation model'', whilst large globular species undergo ionization according to the alternative ''charged residue model''.[24] In line with the ion-evaporation model, as the droplet reaches a certain radius, the field strength at the surface of the droplet becomes large enough to assist the field desorption of solvated ions.[25,26] The ionization is ''soft'' since little or no fragmentation occurs. Consequently, weak non-covalent interactions can be preserved in the gas phase.[27]

Electrosprays operated at low flow rates generate much smaller initial droplets, which ensure improved ionization efficiency. Thus, in 1994 Wilm and Mann miniaturized Fenn's ESI technique obtaining superior sensitivity.[28,29] They demonstrated that a capillary flow of $\sim$25 nL min^{-1} can sustain an electrospray at the tip of a capillary emitter. The technique was later renamed nano-electrospray (nanospray, nanoESI).[29] NanoESI produces droplets approximately 10 times smaller than the droplets obtained with the traditional ESI, rendering the desolvation process more efficient.[24] This variant of ESI (using a lower potential difference between the emitter and the sampling orifice; 1–1.5 kV; *cf.* 2–6 kV in ESI) is even less destructive to non-covalent interactions.[30,31] The lower flow rates mean that less sample material is required for analysis allowing for the interrogation of ''precious'' samples, and give the potential for multiple high-throughput measurements.[32] In nanoESI, application of an electric potential to the upstream part of the flow line can be sufficient to create an electrospray at the outlet of the capillary channel.[33] The operation at low flow rates partly contributes to the superior mass sensitivity of this technique. Thus, the technique is very suitable for analysis of volume-limited samples, including pico- and nanolitre volume sample plugs transported along capillary tubing at low flow rates, or analyte zones separated by capillary techniques, such as nanoflow liquid chromatography (nanoLC) or capillary electrophoresis (CE).

Although the usage of ESI and nanoESI can ensure a high sensitivity; as a general rule, the sensitivity deteriorates with the presence of non-volatile buffers and other additives, which should be avoided as much as possible. Therefore, these ion sources are usually coupled with separation techniques (see sections 5.3.2 and 5.3.3), or appropriate sample purification procedures are executed prior to analysis. Nonetheless, since ESI and nanoESI usually operate at atmospheric pressure, many of the sprayed analyte molecules do not enter the small orifice of mass spectrometer, which lowers the sensitivity of those techniques.

NanoESI emitters can be made of materials used in the preparation of microfluidic chips, enabling detection of peptides at 1 nM concentrations.[34] An impressive mass detection limit of $\sim$80 zmol was demonstrated in that study. In another embodiment, Smith and co-workers demonstrated a membrane-based emitter for coupling microfluidics with MS.[35] The commercial product ''TriVersa NanoMate'' comprises microarrays with a multitude of nanoESI emitters, which can provide stable spray, and eliminate sample-to-sample carryover.[36]

ESI and nanoESI sources can readily be coupled with on-line sampling systems, which can facilitate the analysis of volume-limited samples. Notably, droplets or plugs within multiphase microfluidic systems have rapidly gained interest as a way to manipulate samples and conduct chemical reactions on the femtolitre to microlitre scale. Recently, a method to perform ESI-MS of a stream of segmented flow has been reported.[37] In this method, a stream of aqueous droplets, segmented by immiscible oil, was periodically sampled by using electrical pulses applied to transfer the droplet into an aqueous stream that was directed to an ESI source. The study showed the feasibility of online droplet analysis; however, LOD for bradykinin was ~500 μM. The relatively high concentration LOD was – at least in part – due to dilution of droplets once transferred to the aqueous stream, and a relatively high flow rate used (~3 μL min^{-1}). Further on, Pei *et al.*[38] have observed that nanolitre plugs of sample separated by air or oil can be analyzed by ESI-MS when pumped directly into a fused silica nanoESI emitter tip at a relatively low flow rate (Fig. 2). They showed that direct ESI-MS analysis of samples in a segmented flow stream can be performed with little carry-over, good sensitivity, no dilution, and high speed. High sample analysis rate (0.8 Hz) was achieved by pumping 13 nL samples separated by 3 mm long air gaps in a 75 μm inner diameter tube. The detection limit estimated for leucine-enkephalin was ~1 nM; which – after conversion to the absolute amount of analyte (attomole range) – labels this method as mass-sensitive. Importantly, sample consumption was efficient since all the sample material, which was withdrawn from the sample wells, was subsequently sent to the mass spectrometer. Another important advantage of this approach is that the duty cycle of the MS is high since the time spent on the rinsing between sample injections can be minimized.

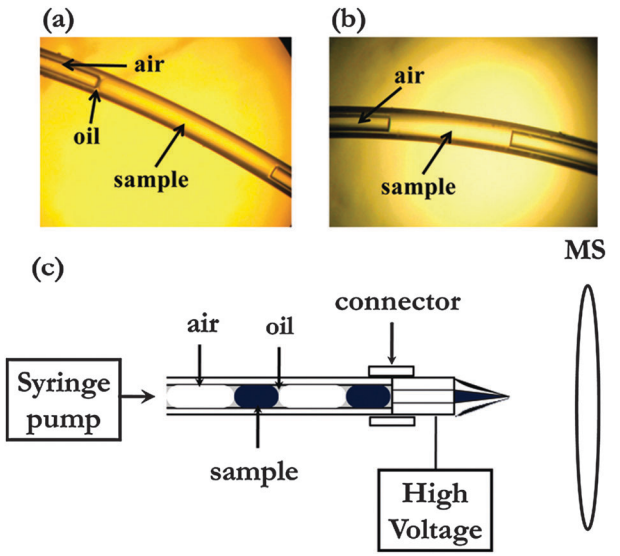

Fig. 2 Sample introduction for MS by segmented flow. (a) Photograph of a 3 mm long (50 nL) plug stored in a 150 μm i.d. Teflon tube. The plug was created by withdrawing sample and air alternately into the tube prefilled with Fluorinert FC-40. (b) Same as part a, except the tube was prefilled with air instead of oil. (c) Overview of the scheme for analyzing a train of plugs stored in the Teflon tube. A voltage of 2 kV is applied at the spray tip. The connector is a Teflon tube that fits snugly over the cartridge tube and emitter tip. Reprinted with permission from ref. 38. Copyright 2009, American Chemical Society.

2.4 Atmospheric pressure chemical ionization

Atmospheric-pressure chemical ionization (APCI) is a form of chemical ionization in which ions created from nitrogen, water vapor, or solvents – due to the action of corona discharge – are used to ionize the analytes in the atmospheric pressure region.[39–43] Depending on the analyte electronegativity and polarity, protonated or deprotonated molecules, as well as radical cations or anions are formed. Similarly to ESI, this ionization method is often coupled with high performance liquid chromatography (HPLC), in which case the mobile phase – containing eluting analytes – is sprayed at high flow rates of nitrogen, and the aerosol spray is subjected to a corona discharge to create ions.[44] However, a remarkable advantage of APCI (relative to ESI) is the possibility to ionize non-polar analytes. In general, APCI is a less ''soft'' ionization technique than ESI, *i.e.* it generates more fragment ions relative to the parent ion,[45] which can inevitably affect sensitivity in the case of more fragile molecules.

For example, Shen *et al.*[46] validated a sensitive liquid chromatography (LC) and atmospheric pressure chemical ionization mass spectrometry method for the determination of posaconazole in human plasma. They chose APCI rather than ESI since APCI is less prone to ion suppression,[47] and the human plasma matrix contains a lot of potential suppressants. The oral dosage of posaconazole is 800 mg per day in divided doses. Based on earlier toxicokinetic data and allometric scaling, the analytical method needed to be refined to measure concentrations as low as 5 ng mL^{-1} in order to accurately characterize the human plasma concentration-time profile. This LC-MS/MS method was suitable for quantifying posaconazole over a dynamic range of 5–5000 ng mL^{-1}. It has then been used in a number of studies aimed at characterizing the pharmacokinetics of posaconazole.[46]

2.5 Matrix-assisted laser desorption/ionization

Matrix-assisted laser desorption/ionization (MALDI) has been a popular ionization technique since its invention by Hillenkamp and co-workers[48] in 1985. MALDI produces fewer multiply charged ions than ESI, and it can be considered a two step process. First, desorption is initiated by a UV laser beam. Matrix material heavily absorbs UV laser light, leading to the ablation of the upper layer (~micron) of the matrix material. A hot plume produced during the ablation contains many species: neutral and ionized matrix molecules, protonated and deprotonated matrix molecules, matrix clusters, and nanodroplets.[49] The second step is the ionization. It is believed to take place in the hot plume where some of the ablated species participate in ionization of analyte molecules.[50] For an in-depth review on the MALDI mechanism see, for example, the articles by Knochenmuss and co-workers.[51,52]

A big advantage of MALDI-MS is that it enables detection of medium- to high mass ions with high sensitivity. MALDI is useful in numerous organic analyses. For example, it can readily be applied in analysis of polycyclic aromatic hydrocarbons (PAHs),[53] metabolites,[54] glycans,[55] lipids,[56] peptides and proteins.[57] Limits of detection in the femtomole range, and occasionally in the attomole range, are achieved for many of these analytes.

Sensitivity of MALDI-MS heavily depends on the preparation of samples, sample/matrix layer, and the selection of matrix type. These steps must be optimized by taking into account prior knowledge, and by ''trial and error''. Preparing a very thin matrix layer and applying the sample on top of it, so as to enable its exposure to the laser light, can provide LODs in even the low attomole (10^{-18} M) range.[58] Small matrix spots using nanolitre volumes of matrix and analyte solution, combined with purification, concentration, and application procedures have also gained high sensitivity in MALDI-MS. By reducing sample volumes from a few μL down to 250 pL, and simultaneous reduction of the sample spot area from a few mm^2 down to 0.01 mm^2, low attomole detection limits could be obtained for bradykinin and cytochrome *c*.[59] The detection limit for a single-shot mass spectrum of bradykinin was estimated to be as low as 250 zeptomoles.[59] Li *et al.*[60] presented a microspot MALDI approach for highly sensitive detection of small-volume samples. The idea of microspot MALDI is to reduce the sample presentation surface with respect to the laser desorption site and ion acceptance volume in the mass spectrometer to improve the sampling efficiency. The high sensitivity provided by the microspot MALDI approach was demonstrated with the substance P (oxidized form) using α-cyano-4-hydroxycinnamic acid (CHCA) as the matrix. An amount of 0.97 amol (195 pL of a 5 nM solution) of the sample was loaded onto the CHCA layer. When the sample dried, very small (~1 μm) crystals formed. The signal-to-noise ratio was 16, and the mass resolution sufficient to observe the isotope peaks. This method provided attomole-level sensitivity towards peptides, whilst detection limits obtained for proteins, such as myoglobin, were in the order of tens of attomoles.

Several groups practice preparation of ultra-thin matrix/analyte layers in order to analyze peptides and proteins by MALDI-MS.[61,62] The ultra-thin layer method involves the preparation of a layer of matrix crystals (for example, CHCA) on the sample plate, which provides favourable conditions for the co-crystallization of a matrix/analyte mixture.[63] The ultra-thin layer method has advantages over other sample deposition approaches (*e.g.* ''dried droplet'') due to its greater tolerance to impurities such as salts and detergents, better resolution, and higher spatial uniformity. For instance, Fenyo *et al.*[63] have used it for analyzing a large number of proteins having a wide range of properties, including those with molecular masses as high as 380 kDa. As noted, the described procedure consistently produced high-quality spectra, and it was sensitive, robust, and easy to implement.[63]

During the ionization process the matrix compound generates fragment and cluster ions which have low molecular weight (typically, <600 Da). These ions can significantly mask the signal of any analytes with a similar mass-to-charge (*m*/*z*) ratio, hence limiting the sensitivity when analyzing small organic molecules such as pharmaceutical compounds, and metabolites.[64] To overcome this problem, several strategies can be implemented. The simplest way is to switch to an alternative MALDI matrix which would generate a different set of matrix cluster ions. To this point, new ion-less matrices with little or no spectral interference are being sought.[65] However, different MALDI matrices have different ionization properties, and will not always ensure efficient ionization of the target analytes. Recently, Cheng *et al.* used the special properties of 9-aminoacridine (9-AA) as MALDI matrix for quantitative analyses of acidic metabolites, as well as glycero(phospho)lipids. When using this matrix, various problems that are usually associated with the analysis of lipids by MALDI-MS (*e.g.* the presence of high background resulting from ionization of matrix or matrix clusters, severe post-source decay, multiple adducts, and lipid aggregation) have been addressed. The proposed method possessed a linear dynamic range of over 1000-fold and a detection limit in the high attomole level.[66]

2.6 Organic-matrix-free laser desorption/ionization

In the late 1980s, Tanaka used an inorganic matrix to facilitate ionization of analytes: he dissolved the sample in a suspension of cobalt nanoparticles in glycerol.[67] This ''soft ionization'' method earned its inventor the Nobel Prize in Chemistry in 2002. Although Tanaka's method is no longer used in its original form, a few groups currently use organic-matrix-free laser desorption/ionization techniques. The surface-assisted laser desorption/ionization (SALDI) was introduced by the group of Sunner;[68] they used an inorganic graphite matrix to ionize small quantities of organic molecules. As discussed in section 2.5, MALDI is nowadays an essential mass spectrometric technique for the analysis and characterization of biomolecules.[56] On the other hand, small biomolecules are known to play an important role in regulating cellular functions, or are relevant to biomarker discovery and disease diagnosis.[69] However, it is difficult to detect low molecular weight compounds (<600 Da) by MALDI-MS due to the spectral overlap with matrix/contaminant signals. Therefore, the methods based on SALDI-MS greatly facilitate analysis of small molecules.[67,70,71] SALDI (or organic-matrix-free LDI) techniques rely on the implementation of inorganic substrates, yielding low background signals, and thus providing high ionization efficiency of small molecules.[67] Some of the successful organic-matrix-free LDI surfaces include germanium nanodots,[72] gold and silver nanoparticles,[73] platinum nanoflowers,[74] boron nitride, zinc oxide, silicon nanowires and porous alumina.[75] Carbon-based interfaces such as carbon-like graphite, carbon nanotubes, fullerenes, or amorphous carbon have also been employed as substrates for the detection of small macromolecules such as synthetic polymers and biomolecules.[76,77] Recently, researchers reported on the use of diamond nanowires to analyze peptides and small molecules with a high sensitivity.[78] As noted, fabrication of the boron-doped diamond

nanowire substrate does not require elaborate processing steps, such as mask deposition, or template removal. The functionalized boron-doped diamond nanowires provided a detection limit for Verapamil of 200 zmol μL^{-1}.

In 1999, another LDI technique was invented; it was named desorption/ionization on porous silicon (DIOS).[79] Using the same type of mass spectrometer as for MALDI, mass spectra with no or little chemical background in the low m/z range were obtained.[80] Later on, Trauger *et al.*[81] demonstrated that it is possible to improve the sensitivity of the DIOS-MS technique dramatically by modifying the surface of the DIOS chip with aid of silylation chemistry. Surface modification with appropriate hydrophobic silanes allows analytes to adsorb to the surface *via* hydrophobic interactions. This facilitates sample cleanup by spotting the sample onto the modified DIOS target and removing the liquid phase containing the interferences. The high-sensitivity DIOS-MS experiments were performed on a MALDI-TOF mass spectrometer. A spectrum of 800 ymol of des-Arg^9-bradykinin was recorded (Fig. 3).[81] In 2007, Northen *et al.*[82] introduced nanostructure-initiator mass spectrometry (NIMS), and demonstrated detection of a multiply charged protein (50 nmol of β-lactoglobulin), a bovine serum albumine (BSA) tryptic digest (500 amol), Verapamil (700 ymol), and an endogenous metabolite 1-palmitoyllysophosphatidylcholine (50 amol) (Fig. 4).[82] Although this technique does not use a "matrix" *sensu stricto* (in the way as MALDI does), NIMS takes advantage of an "initiator" substance, which assists desorption and/or ionization of analyte molecules. Despite the promising features,[83,84] NIMS has not been widely used since its invention. This is probably due to the fact that NIMS chips are not available commercially, and their fabrication requires usage of expensive silicon wafers and toxic chemicals, such as hydrofluoric acid. It should also be pointed out that – to our knowledge – the yoctomole sensitivity of DIOS and NIMS has not yet widely been documented in independent reports. However, the impressive results obtained by the inventors of these techniques suggest that the ionization efficiencies must have been extremely high (possibly, two-digit percentage values), so that the very few ions – reaching the detector – could produce measurable signals.

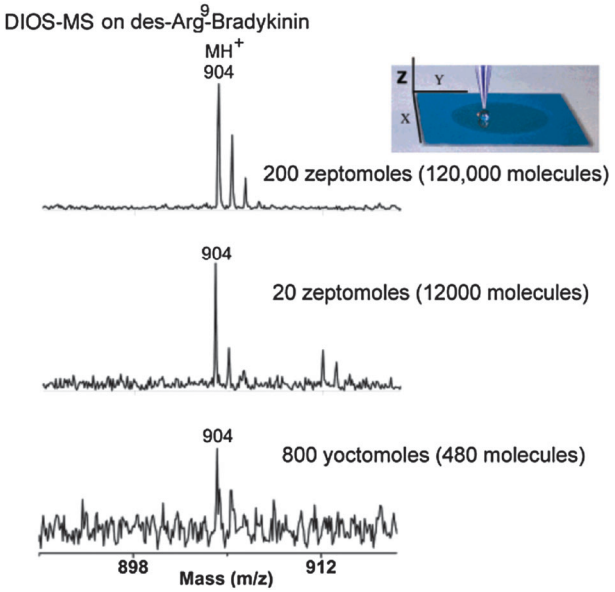

Fig. 3 DIOS mass spectra obtained for 200 zmol, 20 zmol, and 800 ymol for des-Arg9-bradykinin using the perfluorophenyl silylated modified chip. Reprinted with permission from ref. 81. Copyright 2004, American Chemical Society.

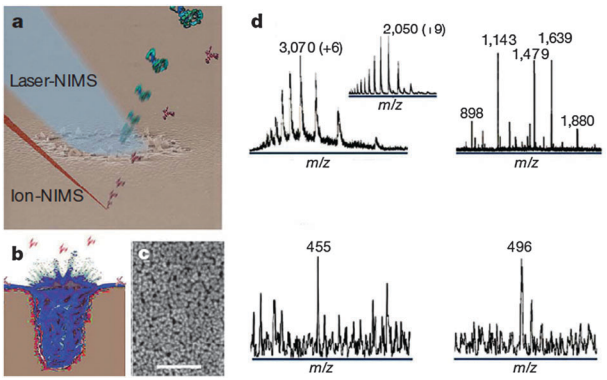

Fig. 4 Nanostructure-initiator mass spectrometry. (a) Illustration superimposed on an SEM image of a NIMS surface after irradiation with a single laser shot (blue), revealing localized surface distortion and destruction. By comparison, ion irradiation (red) allows a much higher lateral resolution. (b) Illustration of possible mechanism in which surface irradiation results in the vaporization or fragmentation of initiator (blue) trapped in a surface pore, triggering analyte desorption/ionization. (c) SEM image revealing that the NIMS surface is composed of 10 nm pores. Scale bar, 100 nm. (d) Laser irradiation (wavelength 337 nm) of a NIMS surface. Upper left panel: detection of a multiply charged protein (50 nmol of β-lactoglobulin) in a similar manner to ESI (inset). Upper right panel: detection of a BSA tryptic digest (500 amol). Lower left panel: detection of the calcium antagonist Verapamil (700 ymol). Lower right panel: detection of the endogenous metabolite 1-palmitoyllysophosphatidylcholine (50 amol). The initiator was bis(tridecafluoro-1,1,2,2-tetrahydrooctyl)tetramethyldisiloxane; 0.5 μL drops were used. Reprinted by permission from Macmillan Publishers Ltd: *Nature*,[82] copyright 2007.

Other studies also illustrate the utility of organic-matrix-free LDI-MS. Chen and Vertes[85] developed laser-induced silicon microcolumn arrays (LISMA) as organic-matrix-free substrates for soft laser desorption/ionization mass spectrometry of small molecules. LISMA exhibits low femtomole detection limits, and it is capable of desorbing/ionizing peptides of up to 6000 Da molecular mass.[85] Walker *et al.*[86] explored nanopost arrays (NAPA) in combination with LDI-MS. The high ionization efficiencies enabled detection of ultratrace amounts of analytes (*e.g.* ~800 zmol of Verapamil) within a dynamic range spanning up to four orders of magnitude. Due to the clean nanofabrication process, and the lack of matrix material, minimal background interferences were present in the low-mass range. They showed that LDI from NAPA can enable analysis of a broad class of small molecules including pharmaceuticals, natural products, metabolites, and explosives. They also showed that multiple metabolite species could be detected in single yeast cells deposited on the NAPA chip (see also section 7.6).

The terminology in the area of organic-matrix-free LDI-MS appears unclear and inconsistent. Several new names have

been coined in the recent years. Boundaries between many of these ionization concepts are somewhat blurred, and some very similar techniques received rather disparate names or acronyms. Therefore, the authors advocate the use of ''organic-matrix-free LDI'' or ''SALDI'' as generic names.

2.7 Secondary ion mass spectrometry

In secondary ion mass spectrometry (SIMS), the sample surface is collided by high-energy (0.5–20 keV) primary ions in vacuum. The energy of the primary ions is transferred to the surface atoms by collisions. Secondary ions, neutral atoms, clusters of atoms and molecular fragments are emitted from the sample surface, followed by mass spectrometric analysis, usually conducted using a time-of-flight analyzer.[87] SIMS allows ultrasensitive trace-element analysis – capable of detecting impurity elements present in a surface layer at concentrations of less than 1 ppm, and bulk concentrations of impurities of around 1 ppb in favourable cases.[87] Although the LOD of fibrinogen was found to be $\sim$0.1 ng cm^{-2},[88] SIMS has not widely been used in the analysis of large biomolecules, because secondary ion fragments complicate identification.[89]

The idea of NIMS – already mentioned in section 2.6 – has also been implemented in the SIMS format.[82] The high lateral resolution (about 150 nm), reduced fragmentation, and sensitivity of ion-NIMS allow the direct characterization of peptide microarrays. The technique enables label-free characterization of arrays, and it might enable the analysis of biomolecules bound to arrays from complex mixtures such as serum. Using ion-NIMS for high-resolution label-free analysis of a peptide array resulted in mass spectra and mass images obtained at 1 fmol of peptide, which is a 1000-fold enhancement in sensitivity over other TOF-SIMS strategies for intact biomolecules. This improvement in ion sensitivity can be attributed to a decrease in fragmentation, typical of TOF-SIMS seen in identical samples spotted on a control surface.[82]

2.8 Other ion sources

Other ion sources are perhaps less popular but should not be left unnoticed when discussing the sensitivity issues in mass spectrometry. For example, Hsieh *et al.*[90] proposed that a short tapered capillary can be utilized as a nanolitre-volume sampling tool and sample emitter for generation of gas-phase ions in front of the mass spectrometer, without the need for using an additional electric power supply, a gas supply, laser, or a syringe pump. Unlike in the conventional ESI/nanoESI setups, the spray emitter was not connected to any defined electrical potential.[90] A wide range of molecules could be analyzed in pure solutions and complex matrices (cell extract, urine, and plant tissue) with no or minimum sample preparation. The concentration LOD for bradykinin was $\sim$1 nM (based on the S/N = 6 criterion), which corresponds to a mass LOD of $\sim$5 amol.[90] This analytical strategy can be considered a simplified version of nanoESI. Pagnotti *et al.*[91] showed the use of solvent-assisted inlet ionization in conjunction with an orbital ion trap. The fused-silica capillary (for sample delivery) was introduced directly to the heated ion-transfer tube of the MS. Detection limits below parts per trillion were obtained for several small molecules, including arginine, ciprofloxacin, and acetaminophen. Attomoles of bovine insulin produced multiply charged ions. This suggests that the inlet ionization may surpass nanoESI in sensitivity.[91] However, one can expect that the implementation of this method might lead to excessive contamination of the mass spectrometer.

In other work, Schiewek *et al.*[92] applied atmospheric pressure laser ionization (APLI) as an ionization method for coupling LC with MS. This enabled ionization of non-polar aromatic compounds *via* near-resonant two-photon excitation. This technique permits the qualitative and quantitative determination of aromatic compounds of polycyclic aromatic hydrocarbons (PAHs), alkylated PAHs, and hetero-PAHs in an ultralow concentration range. An outstanding mass sensitivity is demonstrated for chrysene (LOD, 22 amol). Another ionization technique, known as atmospheric pressure photoionization (APPI), was used in analysis of microbial respiratory ubiquinones and menaquinones: LODs in the order of fmol μL^{-1} were achieved.[93]

An interesting ion source for ultrasensitive MS analysis – named ''resonance ionization with multi-mirror system photon accumulation'' (RIMMPA) – has been presented by Suzuki *et al.*[94] The RIMMPA device features multi-mirror system (MMS), which can store the photon beam with the high efficiency in order to irradiate the injected gaseous sample multiple times. This way, a superior ionization efficiency is achieved, enabling the analysis of ultratrace amounts of substances.[94] Interestingly, 1 ppt benzene in helium gas could be detected using RIMMPA connected to a time-of-flight mass analyzer. The authors claimed that it was not possible to test concentrations below 1 ppt because the dilution gas itself contained contaminant benzene on the $\sim$1 ppt level. As noted by the authors of that study, the applications of the RIMMPA-MS platform encompass on-site/real-time analysis of air pollutants such as dioxins and endocrine disruptors.[94]

In order to achieve a high sensitivity in detection of biomolecules, an indirect detection strategy can also be implemented. This is well illustrated in the new technique termed ''mass cytometry''.[95] It takes advantage of inductively coupled plasma (ICP) mass spectrometry. ICP is a type of plasma source in which the energy is supplied by electric currents which are produced by electromagnetic induction, that is, by time-varying magnetic fields.[96] ICP-MS is normally used to analyze elemental composition with high sensitivity. Cells which had been stained with stable isotope tags were sprayed as single-cell droplets into inductively coupled argon plasma at $\sim$5500 K.[97] Such a high temperature vaporizes each cell and induces ionization of its atomic constituents. The resulting elemental ions were then sampled by a TOF mass spectrometer for detection and quantification. The mass cytometer itself is a specific configuration of an ICP-TOF mass spectrometer, adapted for the analysis of up to 1000 cells per second (Fig. 5).[97] Bendall *et al.*[97] used the mass cytometry concept to examine healthy human bone marrow, measuring 34 parameters simultaneously in single cells (binding of 31 antibodies, viability, DNA content, and

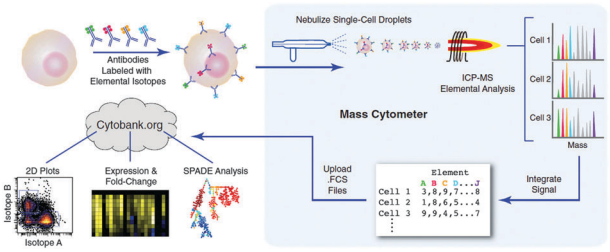

Fig. 5 Mass cytometry profiling of immune cell response patterns. Workflow summary of mass cytometry analysis. Cells are stained with epitope-specific antibodies conjugated to transition element isotope reporters, each with a different mass. Cells are nebulized into single-cell droplets, and an elemental mass spectrum is acquired for each. The integrated elemental reporter signals for each cell can then be analyzed by using traditional flow cytometry methods as well as more advanced approaches such as heat maps of induced phosphorylation and tree plots. From ref. 97. Reprinted with permission from AAAS.

relative cell size). The signalling behaviour of cell subsets spanning a defined hematopoietic hierarchy was monitored with 18 simultaneous markers of functional signalling states perturbed by a set of *ex vivo* stimuli and inhibitors.[97]

In addition, a number of so-called "ambient ionization techniques"[98–100] have also been introduced during the past few years. For instance, mass spectrometric analysis using the flowing atmospheric-pressure afterglow (FAPA) achieves the limits of detection below 100 fmol.[101] Application of the low-temperature plasma (LTP) ion source enabled detection of trinitro toluene (TNT) at sub-picogram level.[102] Other ambient techniques include but are not limited to: desorption electrospray ionization (DESI), extractive electrospray ionization (EESI), electrosonic spray ionization (ESSI), and direct analysis in real time (DART). They are typically used with little or no sample pretreatment, and advertised as simple tools for mass spectrometric analysis of a variety of samples. However, the utility of some of these techniques in ultrasensitive MS is yet to be shown, and readers who want to learn more about these approaches are directed to specialized literature.

3. Ion transfer and sensitivity issues

Following ionization, and before entering the mass analyzer compartment, gas-phase ions are transferred through ion guides and ion optics elements. In fact, major losses of analyte molecules occur during ion transport from the atmospheric pressure region to the first stage of the mass spectrometer. The reduction or elimination of these losses has been a major challenge.[21] Atmospheric pressure ESI can be very efficient when analyzing dilute samples delivered to the ESI emitter at low flow rates. However, when using atmospheric pressure ion sources, the introduction of an ion stream to the mass analyzer is complicated with the inherent difference of pressures. Certainly, the loss of sensitivity at this stage of ion transfer will influence the performance of conventional electrospray ion sources operating at atmospheric pressure, as well as the so-called "ambient ionization techniques" (see section 2.8). One possible remedy is, for example, electrospray ionization in sub-ambient pressure; such a configuration has been demonstrated to ionize a relatively large share of analyte molecules present in the sample solution.[103] Although this demonstration is very promising, using the commercially available systems, it is nowadays more common and convenient to perform ESI at atmospheric pressure.

The design of the intermediate stage between the ion source and the mass analyzer is relatively simple in the instruments using vacuum ion sources, for example MALDI-TOF-MS instruments. In this case, the ions delivered by the ion source as a plume need to be extracted (for example, using the so-called "delayed extraction"), focused, and transferred to the flight tube. Thus, the transfer of gas-phase ions from the vacuum MALDI ion source to the mass analyzer is somewhat less problematic than in the case of atmospheric-pressure ion sources. These characteristics can contribute to the outstanding mass sensitivities of MALDI-MS and LDI-MS methods.

Ions formed in an atmospheric-pressure ion source (such as ESI) typically enter the mass spectrometer through a metalized ion transfer capillary or a metal tube. The so-called "skimmer" is an element mounted between the ion source and the mass analyzer – usually in line with other ion-guide components. It lets the gas-phase ions into the compartment maintained under vacuum. Ions are guided from the orifice of the mass spectrometer towards the skimmer by applying different electric potentials to the ion optics components. The presence of these intermediate stages additionally reduces the influence of gases in the atmosphere on the vacuum in the mass analyzer. Due to the reduction of pressure in the ion line, collisions of ions with gas molecules are diminished, which minimizes losses of analyte ions and increases transmission efficiency. Additional devices can be set in order to prevent ion losses, or to minimize the influence of non-analyte species. Octopoles are commonly used to focus the ion stream en route to the mass analyzer. Tuning RF potential on such an ion guide enables the removal of low-mass chemical noise that would affect sensitivity as well as mass resolution and dynamic range.[104]

Another strategy involves the use of ion funnels (Fig. 6).[105] Originally, ion funnels were developed to efficiently capture ions in the expanding gas jet of an electrospray ionization interface and radially focus them in front of the MS orifice.[106] The device is composed of a series of ring electrodes with different inner diameter. Electric potentials are connected to each of the electrodes, so that the ions are focused due to the presence of a non-homogeneous electric field.[107] Several-fold sensitivity improvements can be observed in various implementations of ion funnels.[106] Belov *et al.*[108] used an ion funnel in the ESI interface of the FT-ICR-MS system, and determined an LOD of 30 zmol for proteins with molecular weights 8–20 kDa, proving an excellent mass sensitivity of the approach. A recently constructed device has demonstrated a substantial improvement in the ion transport efficiency through the first vacuum stage of a mass spectrometer.[109] Ion funnels are currently incorporated into commercial mass spectrometers.[110] Readers interested in

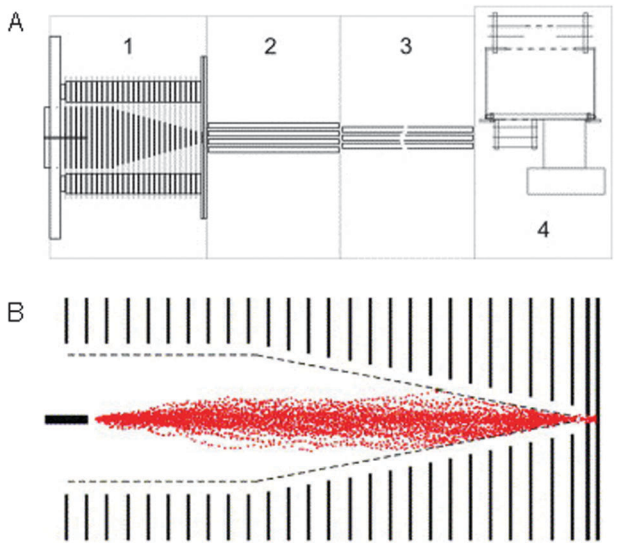

Fig. 6 Design of a mass spectrometer equipped with ion funnel. (A) Block diagram of the instrument. Ions are generated by an electrospray source, collected in the ion funnel (1), transferred through an octopole (2) and then hexapole (3), and, finally, analyzed by a TOF-MS (4). Differential pumping regions are labelled 1–4 in accordance with the description in the text. (B) Results for 50 simulated ion trajectories through the modified ion funnel. Fig. 1 and 4, R. R. Julian, S. R. Mabbett, and M. F. Jarrold, Ion Funnels for the Masses: Experiments and Simulations with a Simplified Ion Funnel, *J. Am. Soc. Mass. Spectrom.*, 2005, **16**, 1708–1712, copyright 2005, published by Elsevier Inc.[107] Reprinted with kind permission from Springer Science and Business Media.

the ion-funnel technology are referred to an expert review by Smith and co-authors.[106]

Other strategies are used by various instrument manufacturers to reduce ion background in mass spectra. For example, the so-called "S-lenses" – which, to a certain degree, may be considered an adaptation of ion funnels – are applied to capture and transfer ions to mass analyzer.[111] The so-called "pre-quads" are used to remove undesirable contamination before the main quadrupole in a GC-quadrupole-MS instrument.[112] Another commercial technology called "StepWave" – incorporating a stacked ring ion guide – also enables increasing ion transmission from the ion source to the mass analyzer.[113] Certainly, the existence of such devices may enhance ion transmission and reduce the background noise, and – in this way – increase the signal-to-noise ratio of the analyte peaks. Regrettably, these devices constitute integral parts of the new instruments of specific brands, and cannot normally be incorporated into older instruments to boost their sensitivity.

It is noteworthy that cleaning of the ion guide elements may occasionally help to restore the original sensitivity of an instrument. Dirty ion source elements lead to lower sensitivity due to charging and other electric field distortions to the ion beam path. Thus, contamination of ion transfer capillaries/tubes, octopoles, and ion funnels will also lower sensitivity of the instrument. Therefore, it is imperative to clean these elements according to the manufacturer's guidelines, and with a frequency that depends on the usage of the instrument. Disassembly of the ion source and ion guides in modern instruments is relatively easy, and various patents exist that provide technology to facilitate removal of the ion source elements without major hassle.

4. Mass analyzers and detectors

After gas-phase ions are created and focused, the next step is to introduce them into the mass analyzer. This part plays the key role in the separation of ions generated in the ion source, and it guides the separated ions towards the detector. As with ion sources, there are several kinds of analyzers; most of them can be divided into two groups:[2] beam analyzers and trapping analyzers. In the former type, the ions leave the ion source in a beam, and pass (through the analyzing field) to the detector. For example, magnetic sectors and quadrupole instruments operate in this way. In the latter type, ions are trapped in the analyzing field, prior to or during the detection. Examples include ion traps, orbital ion traps, and FT-ICR mass analyzers.[2]

The key parameters of the mass scan of an analyzer – such as voltages at the electrodes – influence the sensitivity, and need to be tuned if high sensitivity is the target. The main figures of merit of a mass spectrometer – including sensitivity, mass resolution, and scan speed – are often connected with one another, and it is up to the experimenter to tune the parameters to obtain satisfactory figures of merit to tackle an analytical problem. For example, in ion traps, it is possible to enhance sensitivity by accumulating a larger amount of analyte ions before the ejection of the accumulated ions to the detector. In this case, higher signal intensity can be obtained at the expense of scan rate, which becomes lower. This strategy allows one to improve concentration sensitivity, *i.e.* detection of very dilute analytes in samples which are not limited by volume. High sensitivity is obtained due to the outstanding trapping efficiency of modern ion traps. It should also be noted that high-resolution mass spectrometers have a more complex design than the basic ones. Therefore, while using such instruments, there is a concern of analyte loss between ion source and analyzer/detector. On the other hand, good signal-to-noise ratios can be achieved with such systems because the analyte peaks can readily be mass-separated from background peaks. As outlined above, many methods for ultrasensitive detection of organic molecules involve the use of TOF analyzers. For example, when used in combination with MALDI or LDI sources, TOF analyzers provide optimum conditions for detecting analytes in volume-limited samples, such as single-cell lysates.

Popular triple quadrupole mass analyzers find numerous applications in routine biochemical analysis. In the multiple reaction monitoring (MRM) mode, ions are selected in the first quadrupole, fragmented in the second quadrupole – acting as a collision cell – and the fragmentation products analyzed in the third quadrupole. MRM, as well as the similar approach called selected reaction monitoring (SRM), effectively filter interfering species, thus contributing to improved sensitivity and selectivity. Using a liquid chromatography system coupled with a triple quadrupole MS, Sun *et al.*[114] achieved the concentration limit of quantification (LOQ) of raltegravir of 1 pg mL^{-1}.

A 0.5 pg mL^{-1} LOQ of lansoprazole was achieved in another study using a LC-MS/MS system operated in the MRM mode.[107] Using capillary isotachophoresis/capillary zone electrophoresis coupled with a triple quadrupole mass spectrometer operated in the SRM mode, Wang *et al.*[115] were able to detect 50 amol (total amount) of a target peptide in the presence of bovine serum albumin digest.

Various types of detectors could be encountered in commercial mass spectrometers, including scintillation screens, electron multipliers, Faraday cups, microchannel plates (MCPs), or Daly detectors. Classical detectors, such as electron multipliers, come with improved designs, for example featuring a greater number of dynodes. Various types of mass analyzers are usually accompanied by different types of detectors, for example ion traps are often equipped with electron multiplier tubes while TOF instruments often incorporate MCPs. While the user of a commercial instrument does not usually modify the detection system to enhance sensitivity, it is imperative that service maintenance is conducted regularly by a qualified engineer. For example, prolonged use of an MCP while analyzing matrix-rich samples will lead to deterioration of its sensitivity. In such cases, replacement of the sensor may be required. During the past few years, progress has also been made towards the development of high mass detection systems for TOF analyzers. One commercial device designed for high mass detection[116] enables sensitive analysis of large biomolecules, for example proteins and intact protein complexes with satisfactory mass resolution. The design of this detector is proprietary, and involves using a conversion dynode at an increased voltage while maintaining a minimum distance between the conversion dynode and the front of the electron multiplier; which shortens flight time of the secondary ions produced within the detector, allowing for higher time resolution and sensitivity.[117] The process takes place in the distal end of the TOF tube in high vacuum.

5. Strategies to boost sensitivity

There are a number of ways to increase sensitivity of the existing MS-based methods. Analytes can be concentrated before analysis, either off-line or on-line. A reduction of ion suppression – and the inherent increase in sensitivity – can be achieved by desalting the analyzed samples. Separation of analytes by chromatography and electrophoresis reduces ion suppression and interferences between different compounds present in the sample. This not only increases sensitivity but also facilitates conducting quantitative analyses. Eventually, analytes can selectively be amplified by using smart strategies involving antibodies, aptamers, and enzymes.

5.1 Analyte preconcentration

Sample preparation is at the core of analytical chemistry, and interested readers are referred to the comprehensive monograph by J. Pawliszyn.[118] Undoubtedly, many mass spectrometrists will also admit that proper sample preparation, is the key to performing sensitive determinations and obtaining high-quality results. When analyzing dilute solutes, standard sample preconcentration techniques can be used to enhance concentration sensitivity. Analytes can be extracted from large volumes of solutions into small volumes of stationary phases in solid-phase extraction (SPE) columns. However, using SPE columns before MS analysis incurs the issue of compatibility of the elution buffers with MS; if the elution buffer contains salts at high concentration, ion suppression may occur decreasing the sensitivity. For this and other reasons, SPE is often used as a general sample preconcentration technique before analysis on hyphenated systems, including LC-MS and GC-MS[119] An alternative to SPE is liquid/liquid extraction (*cf.* ref. 120). The miniaturized version of SPE, solid phase microextraction (SPME),[121] is especially compatible with GC-MS and LC-MS systems, and it can readily be integrated with sampling directly from the environment or biological fluids. (For references on SPME, see the literature database at http://www.spme.uwaterloo.ca/.[122])

Less generic preconcentration strategies are intrinsic to MALDI-MS. In this case, sample confinement is critical for attaining high mass sensitivity.[123] Sample preparation for MALDI-MS can easily be integrated with preconcentration of analytes. As described elsewhere,[124] on-target preconcentration of analytes can occur either passively or actively. The popular commercial product ''AnchorChip''[125] is a conductive target coated with a hydrophobic layer.[126] The coating is depleted in the sample recipient sites. Once a small volume of sample is pipetted into a recipient site, the droplet shrinks due to the evaporation of solvent, and the sample precipitates, and is co-crystallized with MALDI matrix within the confined area delimited by the surrounding coating layer. For example, signal enhancement was investigated in MALDI-MS by extracting peptides by reverse micelle-forming amphiphilic homopolymers.[127] Detection of these peptides in the presence of such polymers can significantly enhance ion signals. The signal enhancement is caused by coalescence of polymer-peptide conjugates into ''hotspots'' on the MALDI target.[127] With the use of an ''AnchorChip'' MALDI target, the hotspot formation could be exploited for ultrasensitive MALDI-MS analyses of peptides and peptide mixtures.[127] A similar strategy has been implemented in numerous embodiments, either commercial or experimental (Fig. 7). Different wettability of the sample posts and the surrounding area is not always the only factor involved in the capture/concentration of analytes. Samples can actively be captured on the target plate due to affinity interactions. Phosphopeptides specifically bind to zirconia (ZrO_2)[128] and titania (TiO_2);[129] therefore, particles of these materials are occasionally

Fig. 7 Common embodiments of the on-target pre-concentration of samples before the analysis by MALDI-MS or LDI-MS. The samples can be loaded into microwells on the MALDI target, deposited onto a hydrophobic target, deposited into hydrophilic sites surrounded by the hydrophobic area, or incubated with a molecular recognition surface on the MALDI target.

used to capture phosphopeptides, and eliminate interfering species. In fact, the minimization of the sample presentation area can also be achieved by using spotters, for example those based on piezoelectric effect[123] or induction-based microfluidics.[130] For more information on these and other analyte enrichment strategies used in MALDI-MS and phosphoproteomics, the reader is directed to comprehensive reviews.[124,131]

5.2 Reduction of ion suppression

The simplest way to reduce suppression of analyte signals due to the sample matrix is to dilute the sample. The benefit of lowering ion suppression outweighs the inherent dilution of the analyte. However, this simple strategy cannot always be applied, especially when analyzing low-abundance analytes present in complex matrices (*e.g.* urine, serum). Another popular approach for concentrating, desalting and fractionating picomole amounts of peptides, proteins, or oligonucleotides prior to analysing them by MALDI or ESI is using a ''ZipTip''.[132] Each tip can only be used to concentrate and desalt a sample once, which is performed in about a minute. Similarly to preconcentration, desalting of samples can readily be integrated with the spotting of liquid samples on the surface of functional MALDI plates (for a review, see Urban *et al.*[124]). Functional MALDI plates are a very efficient tool for performing numerous sample preparation steps prior to sensitive analysis by MALDI-MS. Commercial products are available, while others are in the experimental stage.

5.3 Hyphenated separation systems

In addition to the ample choice of ionization techniques and mass analyzers, there exist various ways of introducing samples into the ion source, which accommodate various types of samples under investigation. For example, single-analyte samples can readily be injected directly into an ion source such as ESI while complex mixtures need to be separated, for example, by gas chromatography (GC), liquid chromatography (LC), or capillary electrophoresis (CE) coupled to the mass spectrometer. In fact, separation of the sample components prior to MS can be considered a more elaborate way of reducing ion suppression and interferences during ionization, as compared with the facile methods mentioned in section 5.2. This is in contrast with UV-Vis absorption detection coupled with separation techniques, in which case the key goal of the separation step is to improve analytical selectivity. On the other hand, the MS detection is an analytical technique characterized with very good selectivity; however, it often suffers from ion suppression if no separation or sample purification is conducted. In fact, the interest in coupling mass spectrometers with chromatographs and other on-line systems has been increasing over the past two decades. Some of the hyphenated systems have already become standard tools in the pharmaceutical industry. For example, GC-EI-MS and LC-ESI-MS systems are commonly used in purity testing of pharmaceutical preparations, doping control, and in clinical assays.

5.3.1 Gas chromatography–mass spectrometry. The combined technique of gas chromatography–mass spectrometry (GC-MS) provides a powerful and routine tool for separation, identification and quantification of compounds in complex mixtures. For example, a sensitive GC-MS operated in EI selected ion monitoring mode was developed and validated for the simultaneous measurement of 3,4-methylenedioxy-*N*-ethylamphetamine, 3,4-ethylenedioxymethamphetamine, and its metabolites 4-hydroxy-3-methoxyamphetamine, 3,4-methylenedioxyamphetamine, and 4-hydroxy-3-methoxyamphetamine in human urine. The LOD was in the order of 10 μg L^{-1}.[133]

Sensitivity improvement can be achieved by increasing signal or decreasing noise, or by a combination of both. Many components of the GC-MS system can contribute to background, or chemical, noise. In addition, noise can originate from the sample itself, from the injection technique or from the type of chromatographic column. Electronic and detector noise are primarily dependent upon instrument design and manufacturing. Significant noise can also emanate from the electrical power source. Alternative ionization techniques (chemical ionization, negative ion chemical ionization) can enhance sensitivity. Lowering the background signal originating from the GC column, and improving the chromatographic resolution, can improve GC sensitivity. As the quantity of a specific analyte to be detected is decreased, the effects of minor interferences on the ability to detect the specific analyte become an increasingly significant problem. Chromatographers also face the challenge of assuring complete sample volatilization and transfer of sample to the GC. Use of appropriate injection-port operating parameters and maintenance procedures ensure optimum transfer of sample to the GC column.

As an example, Ballesteros *et al.*[134] analyzed phthalate esters, alkylphenols, bisphenol A, and their chlorinated derivatives in wastewater samples by solid-phase extraction with LiChrolut RP-18 cartridges followed by GC-MS. Quantification limits found were between 20 ng L^{-1} for 4-nonylphenol and 400 ng L^{-1} for benzylbutyl phthalate while inter- and intra-day variability was under 5% in all cases. In another study, single-drop microextraction (SDME) followed by GC-MS was used to determine the dimethoate, methyl parathion, ethion (organophosphates) and permethrin (pyrethroid) pesticides in water samples.[135] For all pesticides, the method showed the limits of detection in a range between 0.05 and 0.38 μg L^{-1}. These demonstrate a high sensitivity of the developed GC-MS method and the capability for detecting and quantifying low levels of pesticides in water samples.

Higher sensitivity also can be achieved by two-dimensional chromatography. It is done by injecting the effluent from one column into a second column. Separations in the two columns should be orthogonal (for example, using different stationary phases), so that the analytes, which are not resolved in a one-dimensional system, can be resolved in the two-dimensional system. 2D peak deconvolution can be applied. GC × GC offers high peak capacity, sensitivity, and resolution. In addition, it generates structured two-dimensional chromatograms, which facilitate the identification of compound classes.[136] Ryona *et al.*[137] described a protocol for analysis of the herbaceous-smelling 3-alkyl-2-methoxypyrazines (MP) in berries that relate

to the MP concentrations of red wines. The 2D separation enabled reduction of interferences originating from the complex sample matrix. The LODs were in the low ng kg^{-1} level.[137] GC × GC has also been shown to be an effective approach for the characterization of petroleum-based fuels such as gasoline and diesel.[138,139]

5.3.2 Liquid chromatography–mass spectrometry. The decision on what form of LC to couple with MS is largely dependent on the application of interest. In many LC-MS applications, the most common separation system is the reversed phase (RP). LC columns come in a number of formats, most of which can directly be interfaced to an ESI source. Conventional LC columns (i.d. 3.0–4.6 mm), narrow-bore LC columns (i.d. 1–2 mm), capillary LC columns (i.d. 150–800 μm), and nanoLC columns (i.d. 20–100 μm) can all be interfaced directly to ESI ion sources.[140] Since the ionization efficiency is high at low flow rates (compatible with nanoESI), using narrow capillary columns at low flow rates, in conjunction with a nanoESI source, may lead to obtaining superior mass sensitivities towards the injected analytes. Such analysis systems are well exploited by the proteomics community, and are also finding numerous applications in metabolomics and lipidomics.[141] Regarding metabolomic analyses – in particular, when handling μL-volume samples prior to the analysis by LC-MS – a major problem is imposed by insufficient ability to trap polar molecules. On the other hand, in proteomics, ''pre-columns'' are commonly used to trap peptides injected in μL-volumes. In metabolomic applications, there is a trend to move from high performance liquid chromatography (HPLC) to ultra performance liquid chromatography (UPLC), executed at elevated pressures, and using columns packed with small particles (<2 μm).[142] UPLC offers short chromatography run times without a loss of chromatographic performance. In fact, 10× faster analyses and increased sample throughput can be achieved by using shorter columns without reducing efficiency.[143,144]

The sensitivity of LC-MS coupling *via* ESI is approximately inversely related to the LC flow rate.[145] Greater sensitivity is achieved with the lower flow rates provided by very narrow diameter LC columns until the point where ionization efficiency becomes limited by the number of analyte species available. If greater mass sensitivity is desired, nanoLC can be implemented. For example, Haskins *et al.*[146] used a 25 μm i.d. capillary LC column with ion trap MS/MS to enable identification of peptides at the ~60 amol level (a detection limit of 4 amol was estimated). In previous studies with capillary LC coupled with FT-ICR-MS, Shen *et al.*[147] achieved high separation efficiencies (peptide peak capacities of $>10^3$) using nanoLC with 15 μm i.d. columns (flow rates of ~20 nL min^{-1}). They used replaceable ESI emitters and micro solid-phase extraction (microSPE) to effectively introduce small mass samples without loss of separation efficiency. The on-line coupling of microSPE enabled ~400-fold faster sample loading flow rates compared to the LC mobile-phase flow rate (*e.g.* 8 μm min^{-1} sample loading for the 15 μm i.d. nanoLC column), allowing much faster introduction of large sample volumes.[148,149] When coupled to FT-ICR-MS instruments, proteomics studies have been conducted showing low-zeptomole level sensitivity when analysing proteolytic peptides.[149]

The recent discovery of 5-hydroxymethylcytosine (5hmC) in embryonic stem cells and post-mitotic neurons has exerted the need for quantitative measurements of both 5-methylcytosine (5mC) and 5hmC in the same sample. Thuc *et al.*[150] developed a method using liquid chromatography electrospray ionization tandem mass spectrometry with multiple reaction monitoring (UPLC-ESI-MS/MS-(MRM)) to simultaneously measure levels of 5mC and 5hmC in digested genomic DNA. Their data exhibit high reproducibility and limits of detection of approximately 0.5 fmol per sample. Only 50 ng of digested genomic DNA is required to measure the presence of 0.1% 5hmC in DNA from mouse embryonic stem cells. This method is fast, robust, and accurate, and it is more sensitive than the current 5hmC quantitation methods such as end labelling with thin layer chromatography and radiolabelling by glycosylation.[150]

5.3.3 Capillary electrophoresis–mass spectrometry. The coupling of capillary electrophoresis (CE) with a mass spectrometer was first demonstrated by Smith and co-workers.[151] Since then, a number of embodiments have been presented.[152–157] In a nutshell, nanolitre-volume plugs of samples are separated in the electric field present in hair-thin capillaries (i.d. 20–100 μm) and the separated zones reach the outlet of the capillary, which is connected to an ion source. Due to the fact that the volumes of the sections containing separated analytes are very small (nanolitres), the CE-MS systems exemplify mass spectrometric detection with excellent mass sensitivity. The best sensitivities were achieved with a sheathless ESI-MS interface.[158] For example, a CE-MS method that enables metabolomic profiling of single cells and sub-cellular structures was described by Lapainis *et al.*[159] using CE coupled to ESI-TOF mass spectrometry. Concentration LODs for a number of cell-to-cell signalling molecules were in the low nanomolar range (<50 nM). However, considering the minute sample volumes, the mass LODs are estimated to fall within attomole range. Single-cell electropherograms obtained using this method were reproducible, and a large number of metabolites were detected.[159]

More and more efforts have been made for the ultra-sensitive quantification of peptides and proteins, because many important peptides and proteins are present at ultra-low levels. Changes of their abundance may reflect a perturbation to the biological system. Recently, Yang *et al.*[160] reported a novel ultra-sensitive method for the simultaneous quantification of three β-casomorphins (β-CMs; are a group of exogenous opioid peptides derived from the hydrolysis of β-casein) based on the Eu^{3+} diethylenetriamine-*N,N,N′,N″,N″*-pentaacetic acid labelling and capillary electrophoresis with on-line inductively coupled plasma mass spectrometry detection (CE-ICP-MS). Three β-CMs were baseline separated and detected within 15 min with a detection limit in the low attomole range.[160] Therefore, this method offers the possibility of simultaneous detection of low-abundance proteins.

5.4 Derivatisation

Yet another way of boosting sensitivity and selectivity of mass spectrometric analyses is derivatisation of analytes. Derivatisation is also carried out in order to enhance separation of analytes on chromatographic columns. Many non-volatile compounds can be analyzed by GC-MS or LC-MS following prior derivatization.[161,162]

Dai *et al.*[163] developed a method for analysis of steroid hormones by LC-MS. An easily protonated stable isotope tag was introduced to a hydroxyl-containing steroid hormone with a synthesized derivatization reagent, deuterium 4-(dimethylamino)-benzoic acid (d_4-DMBA). Using this approach, it was possible to detect 24 steroid hormones at sub-ng mL^{-1} levels (down to 5 pg mL^{-1} for estrone and 16α-hydroxy estrone, corresponding to 0.1 pg). In fact, various derivatising reagents – *e.g.* hydroxylamine, dansyl chloride, HCl-butanol, or 4-fluoro-7-nitro-2,1,3-benzoxadiazole – can be used to derivatise different classes of compounds before analysis by LC-MS.[161]

5.5 Amplification

Establishing amplification methods for different classes of analytes can bring advantages to research in chemistry and biology.[164]

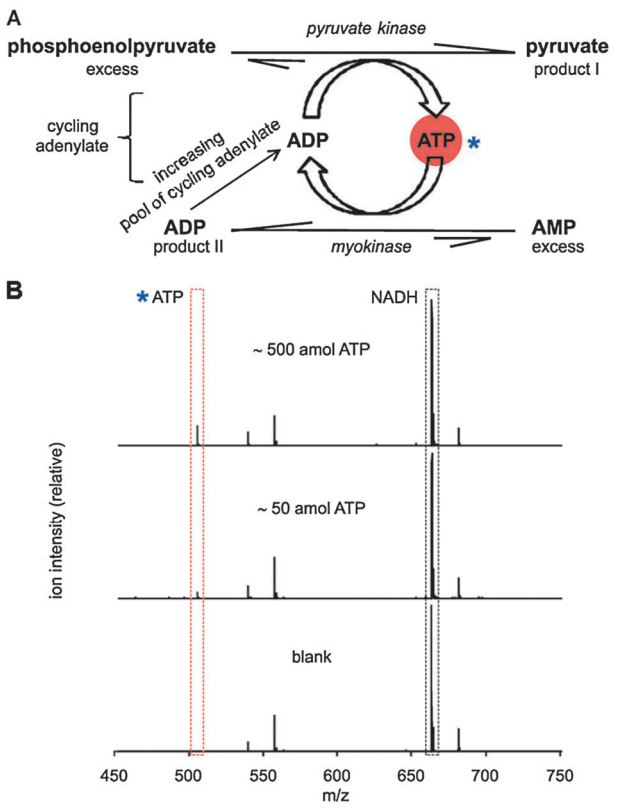

Fig. 8 Enzymatic amplification conducted on MALDI plate. (A) Reaction scheme; (B) MALDI-MS spectra obtained after the *in situ* molecular amplification. The peak of ATP appears already with ~50 amol of ATP (an amount significantly below the limit of detection of the direct MALDI-MS measurement). All spectra have been scaled to the peak of nicotinamide adenine dinucleotide (NADH), used as an internal standard. MALDI matrix: 9 mg mL^{-1} 9-aminoacridine in acetone; negative ion mode. MALDI scan: 81 sampling points × 2 laser shots.[165] Reproduced by the permission of The Royal Society of Chemistry.

Using a cycling enzymatic reaction (Fig. 8A), it is feasible to detect primary metabolites of interest with superior sensitivity.[165] The MS signal is enhanced by up to three orders of magnitude in the course of an *in situ* enzymatic amplification (Fig. 8B). The method relies on two different enzymes to cycle two forms of the analyte in order to increase the amount and the signal response. Attomole sensitivity for adenosine nucleotides was achieved using this method without the need for focusing samples on the target plate. However, this method requires the use of enzymes, which are costly, and require special handling and storage; therefore, it has not become widely used. In other work, Lee *et al.*[166] showed that by using on-target signal amplification with the aid of microparticles it is possible to exceed the sensitivity of direct detection of target biomolecules by several orders of magnitude. An analogous "mass barcoding" method for the detection of DNA has also been presented.[167] However, we believe that the amplification methods coupled with MS have limited applicability, and they can only find applications while solving specific analytical problems.

6. Coupling microfluidics with MS

The ability to carry out chemical reactions at a sub-attomole scale is desirable. Analytical techniques have made strides towards sub-attomole detection limits, yet reactors capable of the preparation of chemical species on an ultra-small scale are not readily available, are expensive, or do not allow effective control of chemical reactions.[168,169] This seems to be largely due to the complexity of the methods used for microreactor fabrication.[170] In the case of sub-nanolitre-volume reactors, top-down reactor fabrication using microfluidics and lab-on-a-chip technologies have emerged.[171] Microfluidics encompasses development and application of micro-miniaturized devices with chambers and channels for the containment and flow of fluids which deals with volumes of fluid on the order of nanolitres (10^{-9} L) or picolitres (10^{-12} L).[172,173] Microfluidic devices combined with nanoESI provide ultra-low LODs.[34,174,175]

In one recent example, Ramsey and co-workers[176] used a microchip CE platform integrating an electrosprayer to analyze red blood cells, which are easy to lyse and contain relatively large (femtomole) quantities of the detected haemoglobin subunits. Following a brief CE separation, the contents were ionized on the electrosprayer and detected by MS. The platform was capable of detecting ~12 cells min^{-1}.[156] In our opinion, this may be a significant advancement in terms of analysis throughput, as compared with manual or semi-automated cell handling.[177] Although ESI-MS has successfully been integrated with microfluidic formats in an on-line fashion, its integration with microdroplet microfluidics has remained a challenge. The direct MS analysis of microdroplets is problematic for several reasons. The main difficulty is the presence of the carrier fluid, which is often composed of fluorous or mineral oils as well as significant amounts of surfactant. Such immiscible fluids interfere with the ESI process by sequestering charge carriers and preventing the formation of a stable Taylor cone.[178]

However, recently this problem was tackled by Kennedy *et al.*[179] Their invention shows that nanolitre plugs of sample separated by air or oil can be analyzed by electrospray ionization mass spectrometry when pumped directly into a fused silica nanospray emitter nozzle (see also section 2.3). Along these lines, Fidalgo *et al.*[37] showed that they can record mass spectra of compounds encapsulated in microdroplets, identify droplets based on their components, and combine fluorescence screening with MS analysis.

Kelly *et al.*[175] have developed dilution-free analysis from picolitre droplets by nano-ESI-MS (Fig. 9). Such devices, capable of automatically transferring the contents of droplets to an aqueous stream for analysis by nano-ESI-MS using integrated electrospray emitters, make the achievement of high sensitivity possible.[164] Treatment of clinical samples can also be accomplished using digital microfluidic devices based on the "electrowetting on dielectric" (EWOD) concept. Extractions can be carried out directly on the chip, and the treated samples sent to MS. This approach finds application in neonatal screens, in which case a very small quantity of blood is available for analysis.[180] While the microfluidics-MS technology is in an early stage of development, further improvements are expected to accommodate their use in the areas such as single-cell analysis.

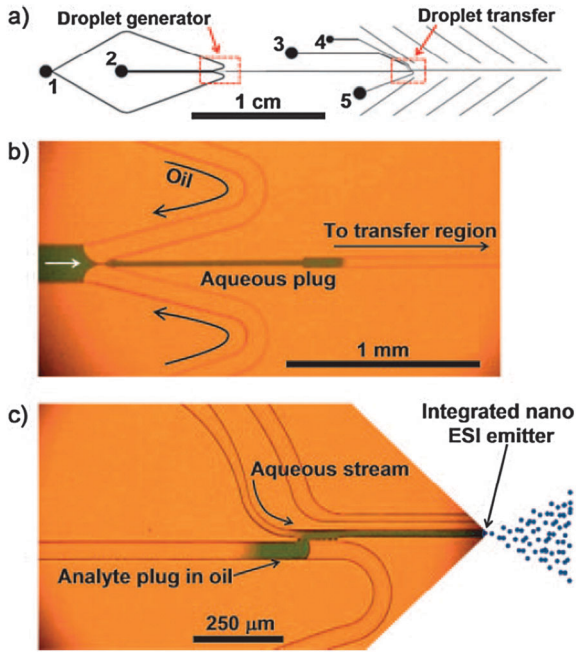

Fig. 9 Device design. (a) Representation of the device. Oil and the analyte-containing aqueous solution are supplied through ports 1 and 2, respectively. The aqueous carrier solution into which droplets are transferred is infused through port 3. High voltage (*ca.* +3 kV) to drive the electrospray is supplied at the stainless-steel needle of the syringe providing carrier solution to port 3. Port 4 supplies electrically conductive solution through a channel for an in-Taylor-cone liquid junction to enable electrophoretic separations, and was not used for these experiments. Port 5 is the waste reservoir for the oil. The angled lines that are not connected to the fluidic circuitry served as guides for accurate cutting of the PDMS devices to form nano-ESI emitters, enabling variable distances from the droplet transfer region to the emitter to be easily obtained. (b) Droplet generator. (c) Droplet transfer region. The interface between the aqueous and oil channels is comprised of 6 cylindrical columns, each 15 μm in diameter, leaving 3 μm wide apertures in between. Channel widths are 50 μm for the droplet/oil channel, and 20 μm for the aqueous stream leading to the ESI emitter. The channel above the aqueous stream in (c) was connected to port 4 and was not used for this work. Reproduced by the permission from ref. 175, copyright 2009, John Wiley and Sons.

7. Areas of application of ultrasensitive mass spectrometry

In the following sections, we will provide examples of areas where ultrasensitive MS has played (or will play) an enabling role while arriving at scientifically sound conclusions. This list of examples is not comprehensive in any way.

7.1 Organic residue contaminants

The analysis of organic contaminants is of great importance in environmental chemistry and biology. For example, recent advances and developments in ultra high performance liquid chromatography (UHPLC) systems have resulted in an unprecedented increase of sensitivity.[181] This imposed the requirements that reagents and solvents used have to be of highest purity. Water plays a critical role in reversed phase UHPLC separations and analyses; therefore, contamination has to be at the lowest possible level.[182] Organic contaminants in water and solvents used to prepare the aqueous mobile phases may accumulate in the chromatographic column, and cause problems such as high background noise, drifting baselines, appearance of ghost peaks, and – in this way – impair sensitivity. Water used in ultratrace analysis needs to be of high ionic purity to avoid formation of metal adducts. For example, in an application note from Agilent Technologies, the authors presented a study of organic contamination in high purity water using UHPLC with diode array and MS detection.[181] Ultrasensitive MS methods are necessary to assay the level of contaminants in "high purity" solvents for analysis. It is difficult to purify some popular organic solvents, such as acetonitrile or acetone efficiently. Therefore, it is common to find solvent contaminant-related peaks in the mass spectra obtained by direct infusion ESI-MS, or even LC-MS if the solvent used for the preparation of the mobile phase is not "perfectly pure". In fact, many common background peaks present in mass spectra – for example, those related to plasticizers – have been identified.[183]

7.2 Environmental organic contaminants

One of the indirect consequences of the Industrial Revolution is the significant contamination of the natural environment. Numerous analytical techniques have aimed to detect these contaminants; especially, mass spectrometry provides unprecedented selectivity and sensitivity, enabling detection of trace contaminants. GC-MS is sensitive enough to detect pollutants such as polychlorinated biphenyls (PCBs). PCBs are found in surface water in concentrations on the ng per dm^3 (ng L^{-1} or 10^{-9} g L^{-1}) scale, although preconcentration of analytes is sometimes required. It is important to detect PCBs because

they are not easily degraded in nature and build up in the food chain. Some seals have been found to have several per cents of PCBs in their fat tissue.[184] Alda *et al.*[185] have published LC-MS methods for the determination of alkylphenolic surfactants, steroid sex hormones and drugs in the aquatic environment. These pollutants are of particular concern due to the volume of these substances being used and their activity as endocrine disruptors or as causative agents of bacterial resistance, as is the case of antibiotics. High sensitivity (concentration LODs in the range of $\sim$5 ng L^{-1}–0.05 μg L^{-1}) was achieved due to the sample pretreatment by the preparation of selective supports, especially immunosorbents, for the solid-phase extraction and purification of the environmental samples.[185] It should be pointed out that – in the applications related to environmental analysis – samples are not normally volume-limited but the concentrations of the pollutants to detect are often very small (ppb level and less). In fact, the concentration sensitivity is often more relevant than the mass sensitivity in the case of environmental analyses.

7.3 Pharmaceutical analysis

Lappin *et al.*[186] presented an ultrasensitive detection technique for use in microdosing studies. In a microdosing study, sub-pharmacologically active doses of drug are given to human volunteers at an early stage of development in order to obtain preliminary pharmacokinetic data. The very low doses of drugs administered to subjects ($\leq$100 μg) lead to very low concentrations of drugs appearing in the bodily fluids and, therefore, highly sensitive analytical techniques are required. There are three such analytical technologies currently used in microdosing studies: LC-MS/MS, positron emission tomography (PET), and accelerator mass spectrometry (AMS). Both PET and AMS employ radioisotopic tracers. PET is an imaging technique and AMS is an extremely sensitive isotope ratio method, able to measure drug concentrations in the ag mL^{-1} range. On the other hand, LC-MS/MS does not require the presence of an isotopic tracer, and its sensitivity is in the pg mL^{-1} range.[187]

Because of the complex nature of clinical samples (*e.g.* blood, urine), pharmacokinetic studies are often aided by mass spectrometry. High sensitivity of MS is beneficial here because low quantities of drug metabolites can be detected. The most common instrumentation used in this application is LC-MS with a triple quadrupole mass spectrometer (see section 4). ICP-MS can be used to determine metalloorganic drugs and their metabolites. Brouwers *et al.*[188,189] presented a highly sensitive rapid method for the determination of platinum originating from the anticancer agents carboplatin and oxaliplatin, and ruthenium originating from the investigational anti-cancer drug NAMI-A,[190] in human plasma ultrafiltrate. In fact, cancer is a disease of great concern because it is the second main cause of death in the world.[191] Cures for most cancer pathologies have not yet been found, and an accurate and early diagnosis is essential for successful treatment. Therefore, research on tumour biomarkers has noticeably increased in recent years. One of the techniques applied to the investigation of tumour biomarkers is CE-MS,[192] which can enable sensitive detection of low-abundance biomarkers in minute volumes of clinical (*e.g.* biopsy) samples.

7.4 Law enforcement

MS is also a powerful technique for forensic investigations. Latent fingerprints (LFPs) potentially contain more forensic information than the simple identification of the subject; they may contain evidence of contact with explosives or substances of abuse.[193] Chemical information can also be useful in resolving overlapping LFPs from different individuals. Recently, Ifa *et al.*[193] used desorption electrospray ionization (DESI) mass spectrometry in an imaging mode to record compound-specific chemical fingerprints. The chemicals identified by their *m*/*z* ratios can be confirmed by their fragmentation patterns by using tandem mass spectrometry (MS/MS). This application of the DESI-MS method has become popular beyond academia, and it has been featured in the *CSI: Miami* episode ''Power Trip''. Ultratrace analysis also finds application in identifying illicit practices, such as doping in sports and adulteration of foodstuffs. For example, residues of anabolic steroids in meat can be detected and quantified over a range of 5–100 ng kg^{-1} by GC coupled with quadrupole MS.[194] In this case, the sample preparation included solid-phase extraction, liquid-liquid partitioning, and derivatisation.

7.5 Systems biology

One of the areas that greatly benefits from novel mass spectrometry tools is undoubtedly proteomics. Nowadays, ESI-MS and MALDI-MS are indispensable tools in the field of proteomics.[195] Due to the capability to provide molecular identification and structural information by accurate mass measurement, ESI-MS and MALDI-MS can provide a depth of information that other techniques (also employed in proteomics; *e.g.* two-dimensional gel electrophoresis, two-hybrid analysis, and protein microarrays) cannot provide.[196] One of the emerging fields is the utilization of ESI-MS for detection and diagnosis of early stages of diseases.[197] In such applications, success of the analysis is often dependent on detection and quantitation of molecular biomarkers in bodily fluids.[198] However, a major problem is the low abundance of many biomarkers.[199] Biological samples also exhibit the inherent complexity. The concentration span of the proteins present in bodily fluids far exceeds the dynamic range of current mass spectrometers.[200,201] Consequently, analytes of potential interest may escape detection.[202] Phosphorylation plays a vital role in cell regulation and diseases such as cancer.[203] Low abundances of phosphopeptides have the requirement to isolate and concentrate such analytes prior to MS.[131] A depletion of highly abundant proteins, or enrichment of specific target proteins, using various pre-fractionation technologies, have become increasingly important.[202] Alongside, new MS techniques that provide improved sensitivity and lower limits of detection from small amounts of samples in complex matrices are being sought.

Whilst proteomics deals with proteins and peptides, metabolomics is concerned with a comprehensive analysis of low-molecular-weight compounds in a biological system.

LC-MS methods enable quantification of various metabolites and finding novel metabolic routes.[204] Similarly to proteins, the concentrations of various metabolites also span over several orders of magnitudes. Often, the most biologically interesting metabolites are those present at low concentrations,[205] which again points to the need for implementing highly sensitive mass spectrometric methods. For a more comprehensive overview of proteomics, lipidomics and metabolomics, and the role of mass spectrometry in these fields, the readers are referred to the topical reviews.[141,169,206–208]

7.6 Single-cell analysis

The ultimate goal in biochemical analysis is the analysis of single cells. Initially, fluorescence and electrochemical detection were the obvious choices for single-cell analysis due to their high sensitivity. However, in order to analyze tens of cellular metabolites in single cells, a more powerful approach was required. Ultrasensitive mass spectrometric techniques were the obvious choice. Standard techniques, such as GC-MS and LC-MS, already enable analysis of large cells such as frog oocytes.[209,210] Analysis of much smaller cells requires the implementation of other techniques. The main groups of mass spectrometric methods currently implemented in single-cell analysis encompass the usage of ESI and its derivations, as well as MALDI.

The group of Masujima developed a method for analysing single mammalian cells by nanoESI-MS.[177] Since sampling from cytoplasm of live cells was conducted under a microscope, they termed this method ''live single-cell video-mass spectrometry''. More recently, they used a similar approach to analyse single cells in plant tissues.[211,212] Another group devised a method for ''on-demand printing'' of living cells, and used it to deposit cells in microarrays for subsequent processing by robot-controlled liquid microextraction coupled with chip-based nanoelectospray mass spectrometry.[213] A noteworthy approach – abbreviated as LAESI, and popularized by Vertes' group – involves the combination of electrospray ionization and laser desorption; it was successfully used in the analysis of cells in onion bulb and other plants.[214] Later on, this technique was applied in conjunction with microdissection to conduct chemical analysis of metabolites localized in subcellular compartments.[215]

Li *et al.*[60] showed that loading and analyzing a small-volume mammalian cell is feasible using the microspot MALDI technique. To demonstrate application of this method in single-cell analysis, red blood cells were chosen as a model system. In fact, several laboratories (for example, the groups of Svatoš, Sweedler, Yeung, and Zenobi) have actively popularized the use of (MA)LDI-MS in the analysis of single cells of various species during the past few years. Amantonico *et al.*[123] have developed an ultrasensitive MALDI-MS method – using 9-aminoacridine as MALDI matrix – which is capable of detecting a wide set of endogenous metabolites with a sensitivity that allows the chemical analysis of single yeast cells.[123] The method encompasses creating sample spots which have diameters matching the diameter of the UV laser beam used in the MALDI process. According to a similar strategy, Urban *et al.*[216] further developed functional high-density micro-arrays for mass spectrometry to enable rapid picolitre-volume aliquoting and ultrasensitive analysis of microscale samples, for example, single cells. As pointed out, being able to irradiate the entire micrometre-scale sample deposit with the MALDI laser beam offers a considerable advantage to ultratrace MS analysis, since no sample is wasted. The entire amount of the analyte present in each recipient site may be desorbed within a short period of time. Detection limits for three metabolites were as low as ~500 zeptomoles, which should warrant detection of sub-millimolar concentrations of these compounds present in the average-sized yeast cells (5–10 μm) (Fig. 10).[216] More recently, Walker *et al.*[86] implemented an organic-matrix-free LDI method (NAPA), which is also applicable in the analysis of single yeast cells. In the future, one may anticipate further improvement of single-cell MS methods to be able to detect metabolites extracted from single bacteria (*e.g. Escherichia coli*), which are used as standard model organisms in biology. No matter which ionization technique is chosen, sample preparation is of paramount importance in single-cell mass spectrometry.

The emerging MS imaging methods, such as LDI and MALDI imaging, (*e.g.* ref. 217–222) also allow one to minimize sample pooling effects. Therefore, it is possible to detect highly localized analytes within the microscale structures of biological samples such as tissues. In one pioneering work, Hölscher *et al.* described LDI imaging of single plant cells.[223] Sub-cellular MALDI imaging of relatively small cells has also been demonstrated.[224,225] Interestingly, not only a native metabolite but also its isotopologues could be imaged; this can enable temporal and spatial tracking of metabolic fluxes.[224] Mass spectrometric imaging based on SIMS has even better capabilities in

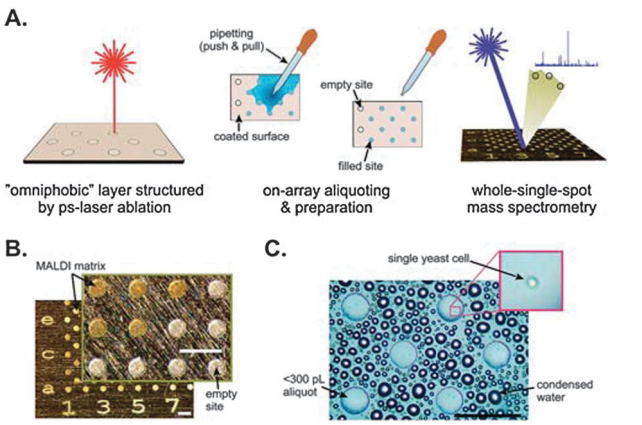

Fig. 10 Micro-arrays for mass spectrometry: (A) General workflow for fabrication and application of MAMS. (B) Homogeneous crystallization of a MALDI matrix (9-aminoacridine) aliquoted on a non-transparent MAMS with a dense arrangement of recipient sites (inset rotated by 45°). The matrix solution is applied with a standard micropipette (''push and pull'' method). (C) A transparent MAMS (based on indium tin oxide glass) useful for analysis of small cells and particles. Yeast cells (< 10 μm) distributed among ~100 μm recipients; high ambient humidity (generated with an ultrasonic humidifier). Single cells are present in some of the recipients filled with culture medium (*cf.* inset), while no cells are present on the adjacent coated area. Water condensation can be seen between the recipient sites. Scale bars: 300 μm. Reproduced by the permission of The Royal Society of Chemistry.[216]

terms of mapping individual cells,[226–229] thus bringing obvious benefits to cell biologists. Apart from mass spectrometric imaging, another way of eliminating the sample pooling when studying tissue samples can be the application of laser microdissection; however, this adds to the costs of sample preparation, and may not provide a high analytical throughput.

For a comprehensive coverage of recent advances in single-cell mass spectrometry, the readers are encouraged to check recent reviews.[230–232]

7.7 Monitoring chemical reactions at micro- and nano-scale

Ultrasensitive MS methodology can also contribute to monitoring chemical reactions in the micro- and nanoscale. Due to the minute volumes of reaction mixtures, mass sensitivity is of great relevance in these cases. For example, Hatakeyama *et al.*[233] took advantage of segmented flow to perform chemical reactions in microdroplets on the milligram scale and transfer them onto a MALDI plate for offline analysis.

In another report, an easy-to-perform and versatile method enabling chemical reactions in femto- to attolitre volumes on a scale down to 1000 molecules (zeptomole range) was presented by Anzenbacher *et al.*[234] The method is based on the deposition of a rectangular grid of two nanofibre types, each nanofibre being doped with a different reagent (Fig. 11A). Heat or solvent vapour-welding of the softened polymer nanofibres resulted in mixing of the contents of the fibres at the intersection, thereby establishing a mixed junction (Fig. 11B). The reaction products were analyzed directly within the nanofibre junctions by fluorescence measurements, and by mass spectrometry. In one experiment, the diazo-coupling reaction of resorcinol and 4-nitrobenzenediazonium tetrafluoroborate in 1:1 ratio in attolitre (10^{-18}) volume yielded products of multiple substitution (Fig. 11C); when the nanofibres were deposited directly onto a MALDI-MS target, as expected all three diazo-coupling products could be detected (Fig. 11C–E).[234] Certainly, multiple junctions had to be scanned by the MALDI laser beam; therefore, matching of the sensitivities of MALDI-MS and fluorescence detection cannot be confirmed in this case.

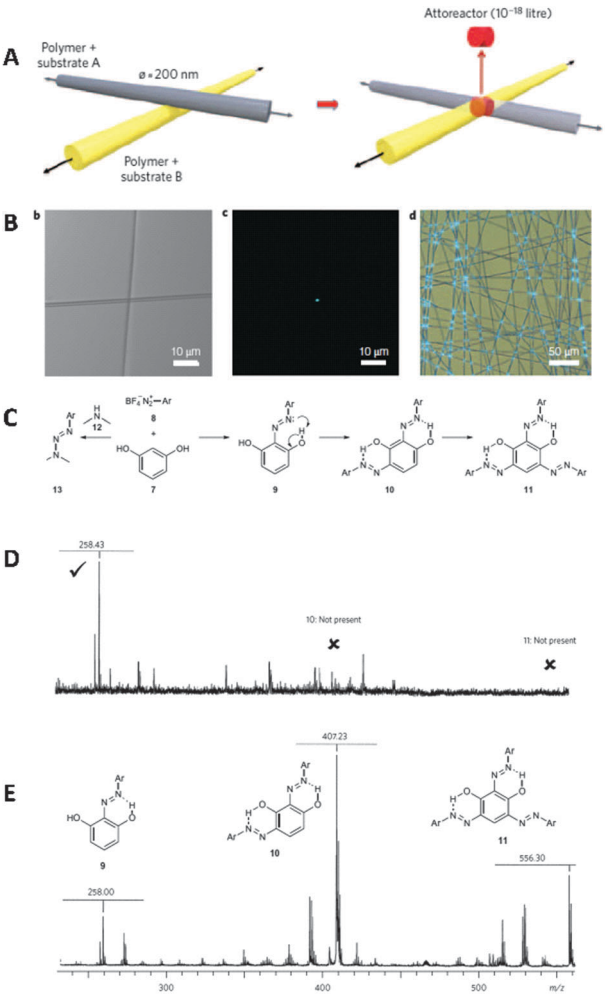

Fig. 11 The principle of attolitre reactor formation. (A) Polymer nanofibres doped with reagents are overlaid to form a rectangular grid-like mat. Fusing the nanofibres at their intersections leads to mixing of their contents in the junctions to create an attoreactor containing zeptomole amounts of reagents and products. (B) Formation of reaction products at the junction of crossed polymer nanofibres. (b) A single attoreactor comprising 500–1000 molecules (bright field + ultraviolet image) separated by a wide margin allows harvesting of individual reactors. (c) The same attoreactor on ultraviolet light excitation. (d) Attoreactors deposited in a random mat. (C) The diffusion-limited nature of reactions in an attoreactor. As a result of hydrogen-bonding-mediated activation, resorcinol (**7**) reacts with 4-nitrobenzenediazonium tetrafluoroborate (**8**) to yield multiple products of electrophilic substitution. (D) In an attoreactor, where the reaction is diffusion-limited rather than activation-limited, only the monosubstituted product (**9**) is observed. (E) When the same reaction is performed in solution, disubstituted (**10**) and trisubstituted (**11**) products are also formed. All products **9–11** may be detected directly within the polymer matrix using MALDI-MS. Adapted by permission from Macmillan Publishers Ltd: *Nature*,[234] copyright 2009.

7.8 Astrobiology

The 20th century is linked to numerous achievements in the exploration of the universe. There have also been attempts to send analytical instrumentation into outer space. For example, the Viking program in the 1970s included the analysis of Martian soil samples by GC-MS. It was hard to interpret the negative results obtained from the missions. The negative result could be due to the fact that the Viking GC-MS instrument was equipped with pyrolysis-based extraction of organics, and some scholars suggested that liquid-based extraction would be more appropriate for the search of organic molecules in celestial bodies in future missions.[235,236] Currently, several groups on different continents are working on the miniaturization of mass spectrometers and simple sample preparation schemes. We expect that once prototypes of sensitive and robust miniature mass spectrometers are fabricated, some of them may leave the Earth's atmosphere to provide an insight on the chemical composition of various celestial bodies including Mars. MS will certainly be the prime technique to tackle the question: "Are biomolecules present on Mars?"

Another branch of research in astrobiology is the investigation of extremophile bacteria which live in unusual environments on the Earth, and which therefore are good models for organisms that could be found in other planets. For example, Wolfe-Simon *et al.*[237] described a strain of bacteria (GFAJ-1)

isolated from Mono Lake in California (USA), which – as suggested – did not require phosphorus for growth. They concluded that phosphorus is replaced with arsenic. However, this work was criticized for not providing sufficient evidence that arsenic really substitutes phosphorus in phosphorus-rich compounds such as nucleic acids. Eventually, using sensitive LC-MS methods, no evidence was found that the bacteria replace phosphorus with arsenic.[238] Arsenic could not be found in the DNA of these bacteria in significant amounts. This story clearly shows that sensitive mass spectrometric methods are required to prove or disprove unexpected findings related to astrobiology.

8. Concluding remarks

Mass spectrometry provides a versatile set of tools with excellent figures of merit. It is capable of measuring many classes of organic molecules in various biological specimens and artificial samples. The rapid increase in the performance of ion sources, mass analyzers, and detectors has led to a variety of ultrasensitive MS methods. It is possible to detect very small quantities of molecules present in microscale samples. However, due to the limitations imposed by the currently used ion sources, ion-handling devices, and mass analyzers, we cannot achieve a method that would enable detection of all kinds of molecules. In fact, ionization efficiencies of various analytes differ considerably. Even so, some of the mass spectrometric methods published so far can achieve sensitivities towards the target analytes which are by several orders of magnitude greater than those of alternative approaches. Some of the reported methods provide outstanding mass sensitivity while the others excel in detection of dilute components. Femto- and attomole level mass limits of detection are already common, whereas zepto- and yoctomole level limits of detection have recently been reported. For the users of commercial mass spectrometers, sample preparation seems to be the most straightforward way to improve sensitivity of mass spectrometric methods. Ultrasensitive MS has an enabling potential, and it already brings significant advantages to research in chemistry and biochemistry. For example, metabolite and protein composition of single biological cells can be characterized, or low-abundance disease biomarkers can be detected in biofluid samples. We envision that ultrasensitive mass spectrometry will soon contribute to new discoveries in bioscience and other areas.

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