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Progress in liquid chromatography–mass spectrometry instrumentation and its impact on high-throughput screening

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Abstract

In the past 10 years, liquid chromatography–mass spectrometry (LC–MS) has rapidly matured to become a very powerful and useful analytical tool that is widely applied in many areas of chemistry, pharmaceutical sciences and biochemistry. In this paper, recent instrumental developments in LC–MS-related interfacing, ionization and mass analysis are reviewed from the perspective of the application of LC–MS in high-throughput screening of combinatorial libraries and the related high-throughput quantitative bioanalysis in early drug-discovery studies, such as early adsorption, distribution, metabolism and excretion studies.

Keywords: Reviews; Instrumentation, LC–MS; Liquid chromatography–mass spectrometry; Interfaces, LC–MS; Ionization techniques; Mass analysis; High-throughput screening

Contents

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1. Introduction

In the past 10 years, research efforts in the field of liquid chromatography–mass spectrometry (LC–MS) have changed considerably. Investigation into the coupling of LC and MS began in the early 1970s. In the first 20 years, most of the attention had to be given to solving interface problems and building new technology. However, most workers with LC–MS today are only concerned with application of the commercially available techniques in their field of interest. Technological problems in interfacing appear to be solved, and from the wide variety of interfaces developed over the years basically only two remain, i.e. electrospray ionization (ESI) and atmospheric-pressure chemical ionization (APCI), which are both atmospheric-pressure ionization (API) techniques. With ESI and APCI, LC–MS has been implemented in analytical strategies in many application areas, e.g. in environmental analysis, drug development within the pharmaceutical industry, veterinary drug residue analysis, characterization of natural products, and the characterization of biomolecules like peptides, proteins, oligosaccharides, lipids and oligonucleotides [1].

In this paper, the developments in LC–MS instrumentation and methodology are reviewed from the special perspective of high-throughput screening (HTS). At present, HTS appears to be an acronym applied to inaccurately indicate various activities related to early stages in drug discovery and development within the pharmaceutical industry. It involves the rapid characterization of combinatorial libraries, in terms of molecular mass, where it provides information supplementary to the bioactivity screening, done separately in most cases. However, high-throughput metabolite screening and rapid quantitative (generic) bioanalysis in relation to early-ADME (adsorption, distribution, metabolism and excretion) studies are frequently mentioned in this respect as well.

The paper starts by summarizing and reviewing the current state-of-the-art in instrumentation for LC–MS, with respect to interfacing, analyte ionization, and mass analysis. Next, open-access LC–MS approaches as well as HTS of combinatorial libraries are discussed. HTS may benefit from further instrumental developments in LC–MS interfacing, especially with respect to multichannel and/or chip-based electrospray inlets. After paying attention to various methods of further enhancing HTS, finally, the focus is directed at high-throughput quantitative bioanalysis as part of early-ADME studies in drug discovery. The review is not meant to be comprehensive, but aims at indicating important trends and illustrating these with some typical examples.

2. Developments in interfacing for LC–MS

A wide variety of API source designs are available from the various instrument manufacturers. An API

source consists of five parts: (i) the liquid introduction device, (ii) the actual atmospheric-pressure ion-source region, where the ions are generated by ESI ionization, APCI, or other means, (iii) the ion-sampling aperture, (iv) the atmospheric-pressure to high-vacuum interface: the transition region, and (v) the ion-optical system, where the ions generated in the source are analyte-enriched and transported towards the high-vacuum mass analyser. A schematic diagram of an API source is shown in Fig. 1. By exchanging the right-hand side, easy change-over between ESI and APCI is possible.

2.1. General principle of operation

The operational principle of most API interfaces and general ion-source designs is as follows [1]. The column effluent from the LC (or any other liquid stream) is nebulized into an atmospheric-pressure ion-source region. Nebulization is performed (i) pneumatically, i.e. in heated-nebulizer APCI, (ii) by means of the action of a strong electrical field, i.e. in ESI, or (iii) by a combination of both, i.e. in ion-spray or pneumatically-assisted ESI. Ions are produced from the evaporating droplets, either by gas-phase ion–molecule reactions, or by the desorption, evaporation or soft desolvation of ions from these droplets. The ions generated, together with solvent vapour and nitrogen bath gas, are sampled by a ion-sampling aperture into a first pumping stage. The mixture of gas, solvent vapour and ions supersonically expands into this low-pressure region. The core of the expansion is sampled by a skimmer into a second pumping stage, containing ion focussing and transfer devices to optimally transport the ions in a suitable manner to the mass analyser. From the vacuum point-of-view, it is not important whether a high flow-rate or a low flow-rate of liquid is nebulized, because the sampling orifice acts as a fixed restriction between the atmospheric-pressure region and the first pumping stage. From the mass-spectrometric point-of-view, it is also unimportant whether the ions are generated by ESI or APCI, although (slightly) different tuning of voltages in the ion optics might be needed due to some differences in the ion kinetic energies.

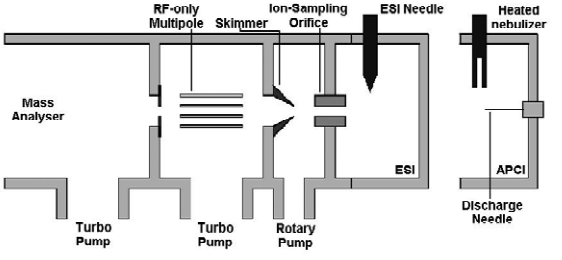

Fig. 1. Schematic diagram of an API source for LC–MS. Exchange of the right-hand side allows change-over between ESI and APCI.

Because a variety of instrument manufacturers offer LC–MS systems, a variety of specific source designs are available. Some important features of these designs are summarized below.

2.2. Electrospray interfacing and ionization

In most commercial (pneumatically-assisted) ESI systems, a coaxial ESI needle is used, consisting of either a stainless-steel or a fused-silica inner capillary used to introduce the sample and a stainless-steel outer capillary used to introduce the nebulizing nitrogen gas. The liquid nebulization is due to the combined action of the application of a high electric field resulting from a 3–5-kV potential difference between the ESI needle and a surrounding counter electrode, and the pneumatic nebulization. The solvent emerging from the needle breaks into fine threads which subsequently disintegrate into small droplets. These charged droplets shrink due to liquid evaporation. As a result, the distance between the charges on the surface becomes smaller and smaller, leading to a field-induced electrohydrodynamic droplet disintegration or Coulomb explosion. This results in the formation of highly-charged microdroplets. From the microdroplets, ions appear into the gas phase, either due to emission or desorption of preformed ions from the droplet surface (ion-evaporation model) or due to soft desolvation of preformed ions (charge-residue model) [2,3]. This sequence of events leading to the formation of gas-phase ion in electrospray ionization is depicted in Fig. 2.

An important development in ESI ionization and interfacing is the production of low flow-rate (<1 μl/min) ESI devices. The main objectives of this are either the reduction of the sample consumption during protein characterization by ESI-MS or the

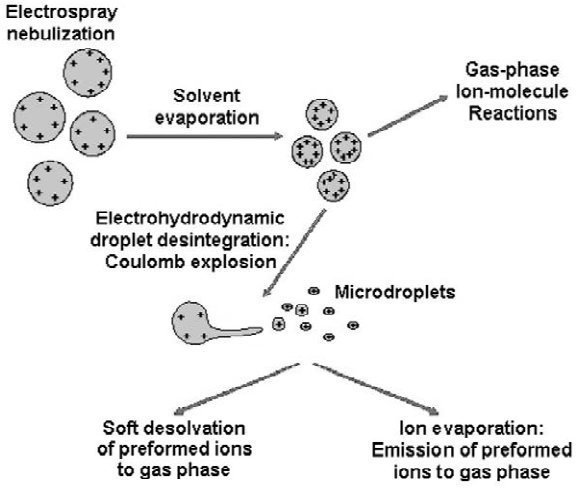

Fig. 2. Sequence of events leading to ion formation in electrospray ionization.

on-line coupling of low flow-rate techniques like capillary electrophoresis (CE) and nanocapillary LC. Initially, micro-ESI devices were described, such as 5–20-μm-I.D. etched fused-silica capillaries to introduce flow-rates smaller than 0.25 μl/min [4]. The first nanoelectrospray (nanoESI) needle device, produced by drawing heat-softened 0.5-mm-I.D. glass capillaries into 1–3-μm-I.D. glass tips, was demonstrated by Wilm and Mann [5,6]. The tips can be used to generate flow-rates as low as 25–50 nl/min. In this set-up, the needle is filled with ~1 μl of the protein solution to be investigated. ESI operation may be stable for as long as 1 h, allowing the performance of a large series of MS and MS–MS experiments on various sample constituents. The needles are positioned close to and in front of the ion-sampling orifice. The voltage required to achieve a stable nanoESI is typically less than 1 kV, which is significantly lower than that in high-flow ESI where voltages between 3 and 5 kV are applied.

NanoESI has become a very important technique in protein analysis. Optimization of the nanoESI needle designs has been the objective of extensive research. Important issues are establishing the electrical contact to the needle and the durability of metal-coated capillary tips. The stability of various sheathless nanoESI emitters was evaluated by Nilsson et al. [7]. The limited stability of most metallized nanoESI tips was found to be related to electrochemical reactions during ESI operation. The coating may be damaged as a result of gas formation due to water electrolysis at the tip and/or oxidized and stripped off the surface.

2.3. Heated-nebulizer APCI interfacing and ionization

In a heated-nebulizer APCI interface, the column effluent is nebulized into a heated vaporizer tube (350–500°C), where solvent evaporation is almost completed. The gas-vapour mixture enters an atmospheric-pressure ion source, where analyte ionization is initiated by a corona discharge needle. The solvent vapour acts as reagent gas.

2.4. General API source design topics

Initially, the spray probes were positioned on-axis or only slightly off-axis with the ion-sampling orifice. The major disadvantage of this set-up is that any particulate or non-volatile material in the spray may start clogging the ion-sampling orifice. A number of measures were proposed to avoid such contamination problems, such as a nitrogen curtain gas which flows between the orifice plate and the curtain plate and emerges as a countercurrent gas stream into the ion source [8,9], a countercurrent heated gas flow around the entrance tip of the ion-sampling glass capillary or cone, a so-called ‘pepperpot’ or cross-flow device positioned in front of the ion-sampling orifice, which promotes desolvation of the droplets and traps non-volatile materials, and a small solvent stream along the tip of the ion-sampling cone. However, the most successful source modification to reduce contamination and clogging of the ion-sampling aperture is the orthogonal positioning of the spray probe [10], which is currently applied in most commercial systems for LC–MS.

Over the years, there has been some debate on the need to apply heat to the ESI source to assist the evaporation of the LC solvent. Thermally-assisted solvent evaporation is especially important at higher flow-rates. In some source designs, a heated countercurrent or concurrent gas is applied to assist solvent evaporation. In a so-called turboionspray source, heated gas is applied orthogonally or in a V-shape to

the liquid introduction probe. In yet another API source design, a heated transfer capillary is used.

Four types of ion-sampling orifices are in use in the various commercially-available API systems: (i) a flat ion-sampling orifice, (ii) an ion-sampling cone, (iii) a 0.5-mm-I.D. glass capillary with metallized ends, and (iv) a 0.5-mm-I.D. heated stainless-steel capillary.

A three-stage differentially-pumped vacuum system is most frequently applied. Dual-inlet turbomolecular pumps are frequently used in small benchtop systems. In designing the vacuum system, a compromise must be struck between sampling the largest possible number of ions from the atmospheric-pressure ion source, i.e. by increasing the size of the ion-sampling orifice, and the pumping efficiency, especially at the second vacuum stage.

2.5. RF-only multipole devices in the transition region

The second pumping stage of the transition region contains an ion optical device, which should transfer as many ions as possible towards the mass analyser, while simultaneously enabling efficient vacuum pumping in this region. Initially, a series of three flat lenses, as commonly used in EI/CI sources, was used. Subsequently, it was demonstrated that better ion transmission in this region could be achieved by replacing the lens stack by an RF-only multipole device (with either four, six or eight rods). The effect of the pressure on the ion transmission in an RF-only quadrupole device was discussed in detail by Douglas and French [11]. The higher transmission at higher pressure is attributed to collisional focussing, which is a mass-dependent rather than an m/z-dependent process. An important feature of such an RF-only device is the possibility of transporting ions within the quadrupole field over a relatively long distance without large losses, enabling efficient pumping by a large turbomolecular pump in this region.

Generally, the RF-only multipole devices are used for ion transport and focussing only, i.e. to transport all ions emerging through the skimmer towards the mass analyser. The use of API sources in combination with quadrupole ion traps has stimulated additional research on the potential of RF-only multipoles. Due to space-charge effects in an quadrupole ion trap, unit-mass resolution and good mass accuracy can only be achieved when a limited number of ions (typically 10^4 ions) is stored. Ion-current dependent ion injection times are applied routinely on commercial ion traps to avoid problems in this respect. In analytical practice, this translates to a competition between analyte ions on the one hand and solvent background and matrix interference ions on the other for storage in the trap. By applying waveforms to the end caps of the ion trap, unwanted ions can be removed from the trap during ion injection. Alternatively, the use of the RF-only multipole as a high-pass mass filter may reduce low-mass interfering ions entering the ion trap, as was investigated by Voyksner and Lee [12]. In addition, the RF-only multipole can actually be used to store ions, prior to their pulsed introduction into the ion trap. In this way, a ten-fold improvement in the detection limit was demonstrated in the quantitative analysis of the betalactam antibiotic ceftiofur in milk. Similarly, the use of a RF-only quadrupole ion guide in combination with a quadrupole ion trap for ion-storage and mass filtering was described [13]. The charge capacity limitation of an RF-only multipole device was investigated by Tolmachev et al. [14]. It was found that the charge capacity limits are only determined by the number of poles and the RF voltage, and are independent of the RF frequency, the radius, and ion mass and charge. A larger number of poles allows more charge to be stored for a given dissociation energy threshold. The RF-only quadrupole has a somewhat smaller charge capacity than the higher-order RF multipoles, but shows a better confinement of the charge close to the axis [14]. These results are also relevant for the two-dimensional linear quadrupole ion trap, discussed below.

As an alternative to the RF-only multipole, electrodynamic ion funnel interfaces were described [15,16]. A stacked-ring RF-only ion-transmission device (‘ion tunnel’) was recently introduced [17]. The device consists of a series of constant-aperture and equally-spaced ring electrodes. An RF voltage is applied with 180° phase shift to adjacent plates, generating a field that constrains the ions to the centre of the device. The ion tunnel devices can replace the RF-only multipole in intermediate vacuum chambers.

2.6. In-source CID

An important function of the transition region is the desolvation or declustering of solvated ions by ion–molecule collisions, especially in the region between the ion-sampling aperture and the skimmer. A small potential difference between the nozzle and the skimmer is applied to enhance the declustering effect [18]. By further increasing the potential difference, collisional activation of the ions can be achieved, resulting in an increase of the internal energy of the ions. This may lead to fragmentation of the ions by collision-induced dissociation (CID) [19,20]. A nice application of this so-called in-source CID was described by Bitsch et al. [21] in the structural analysis of taxol-related compounds. Taxoid side chain fragments, generated by means of in-source CID, were further structurally characterized by means of CID in the collision cell of a triple-quadrupole instrument. As an example, the in-source CID spectrum of taxol is shown in Fig. 3a. The base peak in the spectrum, the side-chain fragment at m/z 286, is selected for CID in the triple-quadrupole instrument. The resulting product-ion mass spectrum is shown in Fig. 3b. Although in-source CID certainly is a useful technique, especially when used for pure compounds or with efficient separation methods, the method also has clear limitations. By means of in-source CID all ions present are dissociated, while in CID in the collision cell of an MS–MS instrument selection of a particular precursor m/z is performed first, which leads to significant improvements in signal-to-noise ratios, not achievable by in-source CID. The use of in-source CID has been proposed for general unknown screening in toxicological and/or forensic applications in combination with a spectral library. Adequate calibration of the instrument performance in terms of in-source CID is especially important then. Tuning compounds for low, medium and high nozzle-skimmer potential differences have been proposed [22–25].

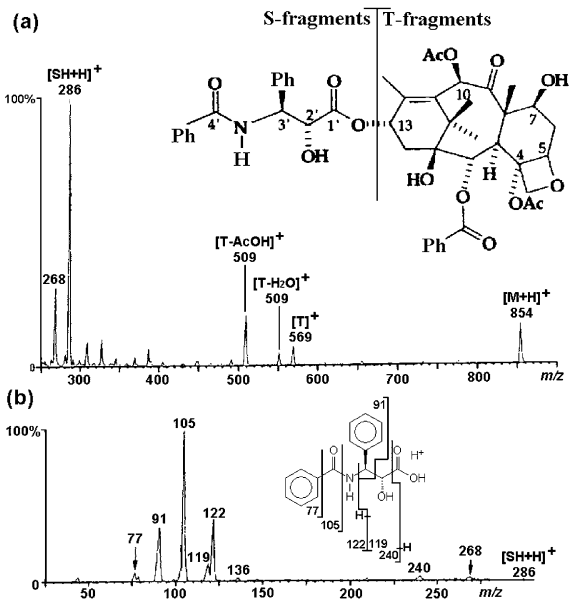

Fig. 3. Application of in-source CID in structure elucidation. (a) ESI in-source CID spectrum of taxol. (b) Product-ion MS–MS spectrum of the side-chain fragment at m/z 286. Adapted from Ref. [21], ©1993, John Wiley and Sons, Ltd. Reprinted with permission.

2.7. Alternative API interfacing and ionization techniques

Although ESI and APCI are applied in the majority of applications, a number of alternative atmospheric-pressure ionization techniques have been proposed.

As part of the Hitachi family of atmospheric-pressure LC–MS interfaces [26], Hirabayashi et al. [27,28] described the sonic-spray ionization interface. It is based on the production of charged droplets from 30 μl/min of liquid in a pneumatic nebulizer using sonic gas velocities (3 l/min nitrogen, Mach 1). The nebulization takes place in an atmospheric-pressure ion source. No heating is applied to the sprayer.

Explosive vaporization and mist formation occurs when a 100-μl/min aqueous effluent at the tip of a 0.1-mm-I.D. stainless-steel capillary is irradiated by a 10.6-μm infrared laser, as already demonstrated by Blakley et al. [29] in the development of the thermospray interface. Simultaneous application of a 3–4-kV voltage to the capillary results in strong signals of single- and multiple-charge ions with intensities more than one order of magnitude higher than obtained from ESI ionization. This new interfacing and ionization mode is named laser spray [30].

A relatively widely-available alternative ionization

technique is atmospheric-pressure photoionization (APPI) [31]. In APPI, the ionization is similar to APCI, except that the ionization process is initiated by photons from a discharge lamp rather than a corona discharge electrode. The technique is promising for relatively non-polar analytes, as demonstrated in some recent applications [32–36].

3. Developments in mass analysers for LC–MS

The huge interest in the application of LC–MS techniques significantly stimulated the developments and improvements in mass analyser technology. Some topics are discussed here.

3.1. (Triple) quadrupole mass analysers

The quadrupole mass filter still is the most widely used mass analyser. A triple-quadrupole instrument in selected-reaction monitoring (SRM) mode is the instrument-of-choice in routine and high-throughput quantitative bioanalysis. Improvements in quadrupole manufacturing processes and RF power supply stabilities enabled the production of a commercial system with enhanced mass resolution without significant losses in ion transmission [37,38]. Mass spectra have been demonstrated with 0.1 Da full width at half maximum (FWHM) instead of the usual 0.6 Da, typical for unit-mass resolution. For mass-defective compounds, this improvement in resolution enables a more selective quantitative determination without significant losses in signal-to-noise ratio [38].

Other triple-quadrupole related innovations concern the collision cell. A high-pressure linear-acceleration collision cell (LINAC) has been proposed. It provides enhanced ion transmission as well as a significant reduction of the cross-talk between components, for which the same product ion was selected in SRM, in ultra-fast multi-component quantitative bioanalysis. In a LINAC, the rod distance at the ion-entrance side is larger than that at the ion-exit side. By applying an axial DC voltage over the rods, the ions are accelerated through the LINAC [39]. Improved product-ion transmission is also achieved by the use of ion tunnels, consisting of a series of stacked rings to which an RF voltage is applied with 180° phase shift between adjacent plates [17]. Such devices have been applied as collision cells as well.

3.2. Quadrupole ion trap mass analysers

Three-dimensional quadrupole ion traps have found wide application, especially in structure elucidation studies. This is mainly due to the possibility of performing multiple stages of MS–MS and the powerful implementation of data-dependent operation in the instrument control software [40]. Relevant innovations related to three-dimensional quadrupole ion traps are the development of a MALDI source for an ion-trap instrument [41–43] and more recently an atmospheric-pressure MALDI source [44], the use of infrared multiphoton photodissociation (IRMPD) to produce fragment ions over a large mass range [45–47], and the study of the fragmentation of alkali adducts ions and other metal complexation products by ion-trap MS–MS^n [48,49].

A very recent innovation is the commercial availability of linear two-dimensional ion traps [50]. The linear ion trap is found to be less prone to space charging effects, enabling a higher number of ions to be accumulated, which results in enhanced sensitivity. In the commercial instrument, the linear ion trap is the third quadrupole in a triple-quadrupole arrangement. In that set-up, it can be used to accumulate product ions generated by CID in a LINAC collision cell, providing enhanced sensitivity and lack of low-mass cut-off. Further stages of MS–MS can be performed in the linear ion-trap, which then has features similar to the three-dimensional ion-trap. Applications demonstrating the features and advantages of linear ion trap are not yet available in the literature.

3.3. Time-of-flight mass analysers

While the principle of time-of-flight (TOF) MS has been well established for many years, significant breakthroughs in TOF technology and application were made in the 1990s [51], as a result of the emergence of MALDI as an ionization technique and progress in computer technology. In particular, the mass resolution of TOF analysers has been significantly improved. Resolution up to 20 000 (based on the FWHM definition) can now be achieved,

resulting in mass accuracies of better than 15 ppm for routine reflectron instruments in combination with MALDI and better than 5 ppm for orthogonal-acceleration TOF-MS (oaTOF) in combination with ESI. As a result, TOF-MS has to a large extent replaced high-resolution double-focussing magnetic sector instruments for LC–MS applications. However, it must be kept in mind that the TOF-MS is an integrating instrument. The instrument performs very well in full-scan mode, but cannot be efficiently applied in selected-ion monitoring (SIM) mode. High-resolution MS in SIM mode continues to be a major application area of a double-focussing mass spectrometer.

In order to perform MS–MS with a time-of-flight instrument, one has to combine the TOF with another mass analyser to form a so-called hybrid instrument. Combinations with a quadrupole, an ion trap, and a double-focussing sector have been described. The most successful, or at least most widely applied, among these hybrids is the quadrupole–time-of-flight (Q–TOF) instrument [52–54]. The Q–TOF provides accurate-mass determination for product ions generated in MS–MS. This can be of great help in understanding fragmentation in MS–MS, as can be illustrated with the fragmentation of pirimicarb (Fig. 4, based on data from Ref. [55]). The loss of 44 and 57 Da from the protonated molecule can only be accounted for by assuming rearrangements of the dimethylamino and a methyl group, respectively, to the heterocyclic aromatic ring.

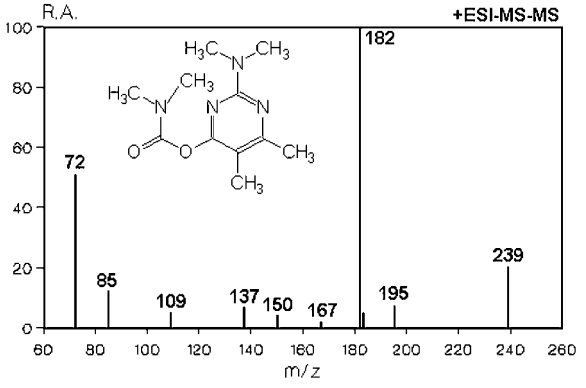

Fig. 4. Q–TOF product-ion MS–MS spectrum of pirimicarb. The fragments at m/z 195 and 182 are discussed in more detail in the text (see also Table 1). Data based on Ref. [55].

Table 1
Experimental and calculated elemental compositions for a number of fragment ions in the Q–TOF product-ion MS–MS spectrum of pirimicarb

Composition	m/z_{Calc}	m/z_{Exp}	Δ (mDa)
C_3H_6NO	72.0449	72.0465	1.6
$C_9H_{16}N_3O$	182.1293	182.1306	1.3
$C_{10}H_{19}N_4$	195.161	195.1627	1.7
$C_{11}H_{19}N_4O_2$	239.1508	239.1501	−0.7

For further explanation, see text.

From the accurate masses, determined in a Q–TOF MS–MS experiment (Table 1), the losses of CO_2 and $H_3C–N=C=O$, respectively, can be proved, which provides solid evidence for the two rearrangement reactions indicated.

A TOF–TOF tandem instrument has been described as well, but is currently only available for application in combination with MALDI [56].

3.4. Fourier-transform ion-cyclotron resonance instruments

For many years, Fourier-transform ion-cyclotron resonance mass spectrometry (FT-ICR-MS) has been a tool used in fundamental studies of gas-phase ion–molecule reactions, i.e. studies on organic chemistry of isolated molecules in the gas phase. Due to its high-resolution and MS–MS capabilities, the application of FT-ICR-MS in combination with ESI ionization for large biomacromolecules has been extensively investigated [57,58]. At present, FT-ICR-MS can be considered an important tool in the analysis of biomacromolecules as well as in applications where high resolution and/or high mass accuracy are important. The technique is not as widespread as quadrupole, ion-trap, or TOF technologies, but is certainly gaining territory, also due to the introduction of user-friendly instruments by manufacturers.

Some milestones and innovations in FT-ICR-MS in the analysis of large biomolecules are: the selected accumulation of ions of non-covalent complexes [59] based on quadrupolar excitation of the ions in the FT-ICR cell, the mass analysis of very large biomolecules, such as the coliphage T4 DNA ion with a molecular weight of 10^8 Da [60], and on-line CE–MS in the analysis of single cells [61].

Similar to a quadrupole ion-trap, FT-ICR-MS has capabilities for multiple-stage MS–MS experiments. Targeted ions can be selectively trapped in the FT-ICR cell by the application of RF pulses to eliminate unwanted ions. Subsequently, a variety of ion excitation methods to induce fragmentation can be applied, e.g. collision-induced dissociation (CID), laser photodissociation or infrared multiphoton dissociation (IRMPD) [62], surface-induced dissociation, sustained off-resonance irradiation (SORI) [63], black-body infrared radiative dissociation (BIRD) [64], and electron-capture dissociation [65]. Elaborate discussion of these techniques and the results obtained is beyond the scope of the present paper.

4. LC–MS in high-throughput screening of combinatorial libraries

Drug development consists of four distinct stages: (i) drug discovery, (ii) preclinical development, (iii) clinical development, and (iv) manufacturing [105]. Significant efforts have been put into accelerating each of these stages in order to reduce the time between discovery and introduction on to the market of new drugs. One of the consequences of this has been huge demand for high-throughput analytical methods, applicable especially in the early stages of drug development. This would enable the testing of a larger number of possible lead compounds in a discovery phase.

In recent years, developments in combinatorial chemistry has led to changes in drug discovery approaches [66,67]. The rationale in the application of combinatorial synthesis is to accelerate the discovery of molecules showing affinity against a target such as an enzyme or receptor through the simultaneous synthesis of a large number of structurally-related analogues. In addition to discovery of relevant new chemical entities as lead compounds, combinatorial synthesis can also play an important role in lead optimization, i.e. the synthesis of related compounds to evaluate and optimize structural features responsible for the required biological activity as well as to evaluate and reduce the side-effects. Combinatorial libraries are generated containing as many as hundreds or thousands of compounds, which must be screened for biological activity using a high-throughput screening method and for which occurrence, structure and purity must be assessed. MS and especially LC–MS have been found to be a very powerful tool in the analytical support of such activities. The role of MS and LC–MS in characterization of combinatorial chemistry libraries has been reviewed by several authors [68–73]. Early studies include the direct analysis of bead surface associated materials [74] and characterization of a peptide library using NMR, MS, MS–MS and capillary electrophoresis [75].

4.1. Open-access LC–MS for synthetic chemists

An important opening towards the application of LC–MS in relation to combinatorial synthesis was the introduction of open-access LC–MS methods, which convert an LC–MS instrument into a walk-up ‘black box’ for synthetic chemists in need of rapid confirmation of the proper progress of their synthesis by molecular mass determination of their products. A remote computer serves as a log-in to the system. After entering the sample identification code and selecting from a menu the type of LC–MS experiments to be performed, the computer indicates the position(s) in the autosampler rack to be used for the sample. The sample is run automatically, e.g. by a fast wide-range gradient LC–MS run in both positive-ion and negative-ion mode and at both a high and a low in-source CID potential. The resulting spectra are placed onto the LIMS network or sent to the chemist by electronic mail. This approach, pioneered Hayward et al. [76], was optimized by Pullen and coworkers [77–79] at Pfizer Central Research. Initially, an automated column-bypass thermospray MS system was used, but later this was related to an ESI-MS system [79]. The success of the approach within Pfizer Central Research is illustrated by the fact that in 1995 a staff of two took care of seven open-access instruments, providing structural confirmation on over 120 000 newly synthesized compounds. Seven more specialized MS specialists used five other instruments to generate ~10 000 mass spectra for compounds requiring more elaborate attention [78]. Dedicated open-access software modules are currently offered by most instrument manufacturers to be used in combination with mainly single-quadrupole and oaTOF instruments.

Open-access LC–MS systems are widely applied within the pharmaceutical industry by synthetic organic chemists. After the proof of principle, obviously little data on this have been published. Sauvagnat et al. [80] reported on step-by-step monitoring of a liquid-phase organic synthesis, using an open-access type of system. Open-access MS was reviewed by Spreen and Schaffter [81]. The most recent progress in this area is the implementation of on-line LC–NMR within the complete strategy [82].

4.2. High-throughput MS characterization

Commercial software, developed by instrument manufacturers for open-access operation, was subsequently adapted to enable unattended data acquisition and automated data processing for large series of samples from autosamplers supporting the 96-well microtiter plate format, which is the sample format of choice in combinatorial synthesis. Initially, mainly Gilson 215 or 233 XL autosamplers were used, but other systems have meanwhile become available from other instrument manufacturers.

Commercial software allows rapid and unattended analysis of large series of samples (up to 60 samples per hour in column-bypass or flow-injection mode, and up to 15 samples per hour in gradient LC–MS mode). It provides a user-interface, a so-called databrowser, which allows rapid decisions on whether the expected products are present in the various wells of the 96-well microtiter plates. Based on the expected molecular mass of the compounds in the various wells, the instrument decides whether the compound is present or not from data in both positive-ion and negative-ion ionization, taking into account the various cationized and anionized ions that may be produced during ESI or APCI analysis. This results in a reduction of the data into a form which can be quickly accessed. The reduced data can rapidly viewed in the browser, based on flagged sample positions in a representation of the wells of the microtiter plate (green colour if the expected compound was detected, and red if not). The mass spectrum for each of these well positions can then be viewed by selecting the well by clicking with the mouse. An example of the typical screen layout of such a databrowser is shown in Fig. 5.

While this software was mainly developed and distributed by manufacturers of MS instrumentation, related approaches were also developed within pharmaceutical industries. Görlach and Richmond and coworkers [83–86] from Novartis (Switzerland) described in-house developed software (RackViewer, written in Visual Basic) enabling automated high-throughput flow-injection analysis of combinatorial chemistry samples. An important issue in this work is purity assessment, without performing LC separation. The purity of each sample is calculated from the summed ion currents of ions related to the expected compound divided by the total ion current. The purity of each sample is visualized by different colours in the results layout of the RackViewer program. Data input concerning the compounds to be expected as well as reports of results are networked to chemists at the company's research facilities in various countries. In this way, over 70 000 samples were analysed in a 2-year period [86]. Issues related to the logistics of secure sending of many samples from remote laboratories to a central laboratory, where analysis is performed, required serious attention. In these high-throughput flow-injection analyses, sample carry-over is a serious concern [86–88]. Estimation of sample carry-over was implemented into the RackViewer colour-rendered data processing as well. Initially, median carry-over was estimated to be 0.88% [86]. With the growing number of samples presented for analysis, a further reduction in the sample measurement duty cycle was required, while maintaining the carry-over below 1%. Using recently introduced high-speed autosamplers, the duty cycle could be reduced from 168 [125] to 44 s [88]. With further optimization of syringe and loop wash steps, the median inter-sample carry-over could at best be reduced to 0.01%.

Shortening of the sample measurement duty cycle was described by Wang et al. [89] using a multiprobe autosampler for flow-injection analysis of eight samples within 1 min. The system permits eight samples to be drawn up and transferred to an injector loop simultaneously. Each of the eight injector valves are in sequential order switched from load to inject. In this way, the subsequently injected samples are resolved in time during their MS analysis.

Purity assessment of combinatorial libraries is an issue addressed by others as well, e.g. with respect to synthetic peptides [90], aryl ether compounds [91],

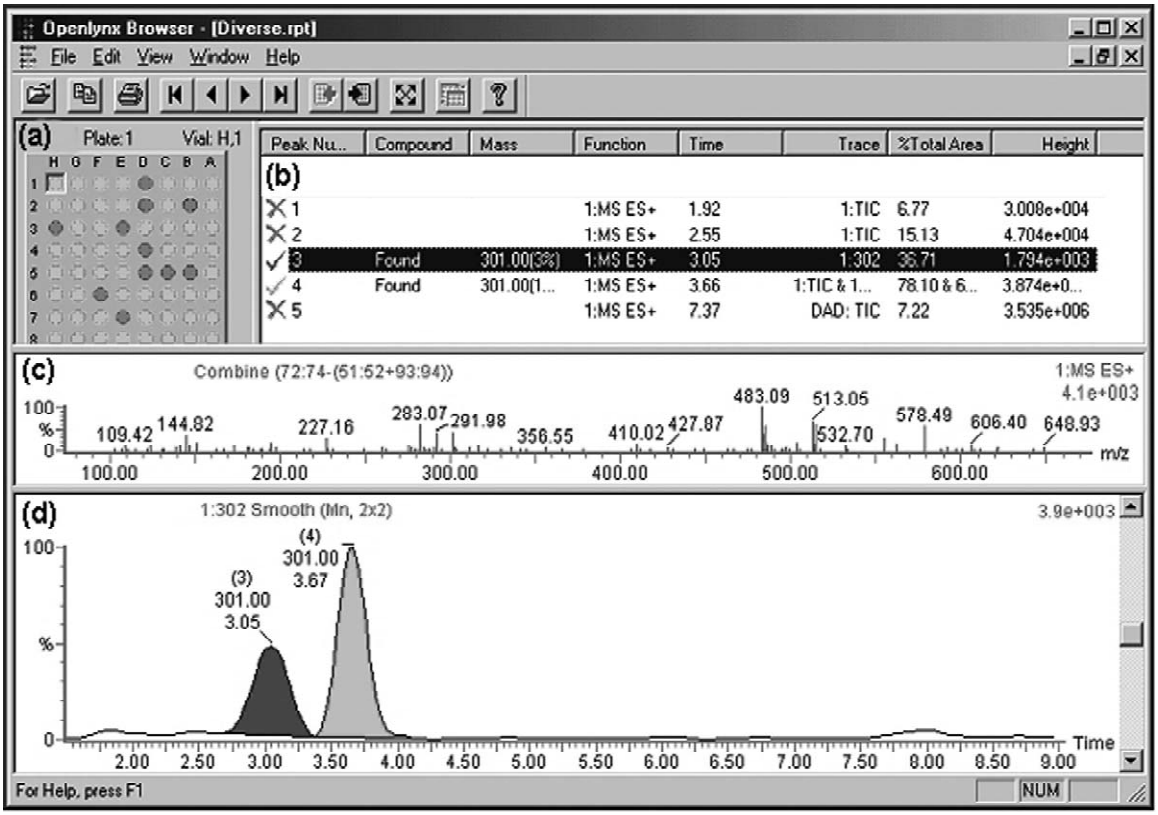

Fig. 5. Typical screen layout of a databrowser (Micromass OpenLynx©) enabling a rapid overview as well as easy access to the data acquired in the analysis of samples from a 96-well plate. The screen shows (a) a representation of the 96-well plate with colour-coded samples (green is found, red is not found), (b) the peak list for the selected well on the plate (position H1 in this particular case), (c) a back-ground subtracted spectrum of the component selected in the peak list, and (d) the smoothed total-ion chromatogram acquired for the selected sample.

and small organic compounds [92]. In the latter case, the applicability of a chemiluminescent nitrogen detector was evaluated as well. The importance of purity assessment in the characterization of combinatorial libraries has led to the use of alternative detectors next to MS, such as UV-photodiode-array (PDA) and especially evaporative light scattering detection [93]. Structural characterization and purity assessment of compound libraries obtained by combinatorial parallel synthesis using LC–APCI–MS and MS–MS, UV–Vis PDA, and NMR has been reported by Duléry et al. [94]. Initial purification of combinatorial libraries by automated high-throughput liquid–liquid extraction (LLE) has been proposed by Peng et al. [95].

Automated data processing, interpretation and reporting via electronic mail for high-throughput open-access LC–MS was also described by Siegel and colleagues [96,97] for both low-resolution quadrupole MS and high-resolution FT-ICR-MS. One of the important issues in fully-automated data processing is the prediction of the ions related to the compound of interest which will occur in the mass spectra. Both ESI and APCI are prone to adduct formation, leading to the possible occurrence of a variety of other ionic species next to the protonated or deprotonated molecule. Prediction of mass spectra of combinatorial libraries may be performed using the MASP program [98]. Correlation of measured and predicted ESI mass spectra for combinatorial mixtures was investigated by Yates et al. [99]. Useful listings of frequently occurring adducts ions as well

as artefact peaks have been reported by Tong et al. [96].

High-throughput screening of combinatorial libraries results in large amounts of data. The databrowsers and automated processing are of considerable help in this respect. However, for some applications, other additional tools and/or strategies can be helpful in processing the data. The use of stable-isotope labelling has been proposed to encode libraries in order to help identify the active compounds. The unique isotopic pattern can subsequently facilitate further pharmacokinetic analysis [100,101]. SIM has been applied to visualize and identify a family of isobaric peptides in larger peptide libraries (over 10^5 components) [102].

4.3. High-throughput screening of natural products

Natural product extracts have been found to provide a valuable source of molecular diversity, complementary to that provided by traditional or combinatorial organic synthesis. A large fraction of anticancer agents, for instance, are either natural products or synthetic molecules based on natural compounds. High-throughput screening methods applied to natural products have been reviewed by Strege [103]. Natural product extracts may include alkaloids from plants and some bacteria, amino acids, proteins and antibiotics from microbes, pigments from microbes and plants, pyrimidines and purines from microbes, steroids from marine animals, plants, and fungal sources, and terpenes, carbohydrates and fats from all sources, including terrestrial animals. The inherent diversity of natural product extracts has not only stimulated the evaluation of their biological activity, but also presented significant challenges for separation and detection to enable rapid characterization. Reversed-phase LC in combination with PDA and ESI-MS are most important in this respect. For the more polar compounds, the use of hydrophilic interaction chromatography has been evaluated as well [104]. More recently, the use of on-line LC–NMR has been promoted for this purpose [105].

Some applications in this field comprise the screening of annonaceous acetogenins in bioactive plant extracts [106], the evaluation of Q–TOF and multiple stage ion-trap MS–MS for the dereplication of flavonoids and related compounds in crude plant extracts [107], high-throughput screening of tocopherols [108], and the characterization of the asterosaponin fraction of the starfish *Asterias rubens* using a combination of matrix-dispersion SPE and direct on-line LC–NMR–MS–MS [109].

5. Enhancing sample throughput by LC–MS interface modifications

As soon as one starts to move on the path of enhancing sample throughput, often the demand for further increases in throughput appears. In some laboratories, the nature of the compounds analysed demands both ESI and APCI analyses to be performed, in order to have the widest possible applicability range. A so-called ESCI source has recently been proposed for such cases. The system enables scan-wise switching between ESI and APCI during LC–MS acquisition [110,111].

APPI, briefly discussed in Section 2.7, is found to significantly extend the applicability range towards less polar compounds. An example of this is illustrated for the analysis of polycyclic aromatic hydrocarbons by reversed-phase and normal-phase LC in combination with APCI and APPI (Fig. 6). APPI is capable of detecting a larger number of compounds in the synthetic multi-component mixtures tested [112].

Next to these approaches, multichannel electrospray inlets have been proposed. Another development of interest in this respect is the current research on the use of microfabricated microfluidic and chip-based electrospray devices.

5.1. Multichannel electrospray inlets

Multichannel ESI inlets have been developed for a number of reasons. They either divide the effluent from one LC system over several parallel ESI needles or introduce different solvent streams via separate needles into one source housing and one mass spectrometer. With respect to high-throughput screening, the latter approach is obviously more important.

A dual-sprayer ESI interface, enabling parallel LC–MS for characterization and purification of

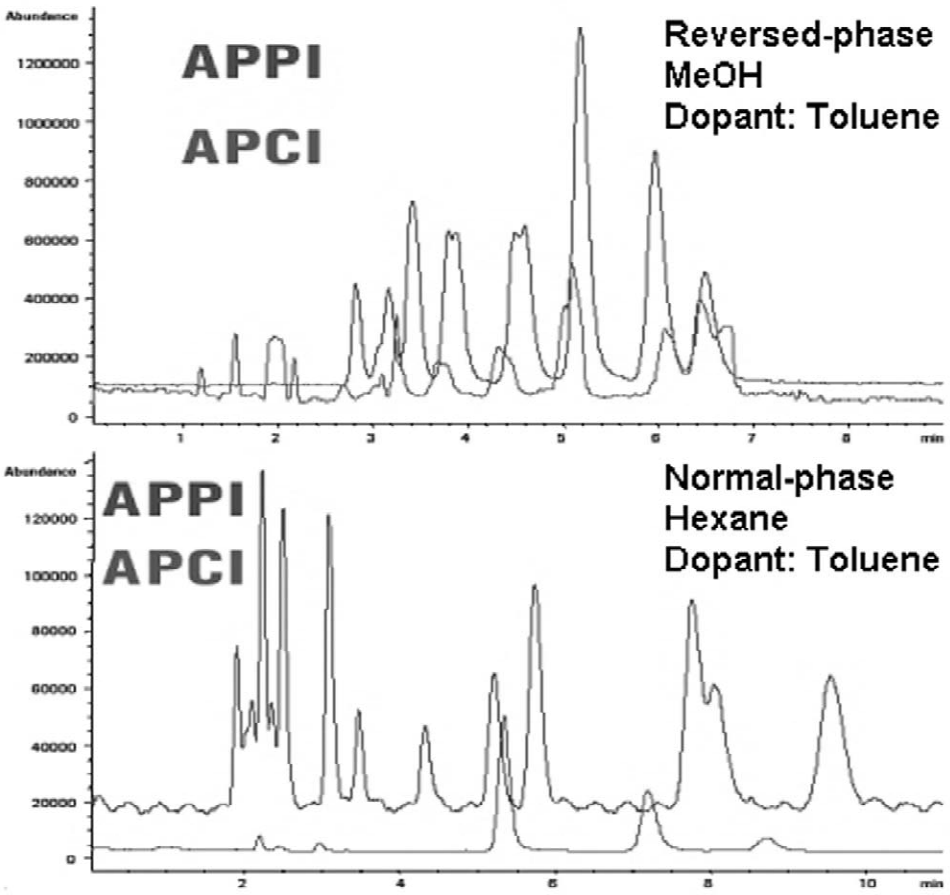

Fig. 6. Comparison of the performance of APCI and APPI in the analysis of a standard mixture of polycyclic aromatic hydrocarbons. Data from Ref. [112].

combinatorial libraries, was described by Zeng and Kassel [113]. Four- and eight-channel parallel introduction from four or eight LC systems into a multiplexed ESI source was introduced by Micromass in 1999. The continuous ESI nebulization from all sprayers is sampled successively using a rotating aperture, driven by a variable-speed step motor. Each sprayer is sampled for typically 0.1 s each 0.5–1 s. Initially, this device, the so-called MUX, schematically drawn in Fig. 7, was implemented on time-of-flight instruments, enabling fast data-acquisition. Such a four-channel system was applied by de Biasi et al. [114] to perform high-throughput accurate molecular-mass determination of some drugs and their synthetic byproducts. The data acquired from each sprayer are collected in separate datafiles; the multiple-sprayer device is 'indexed'.

A non-indexed dual-sprayer device was developed and applied in high-throughput quantitative bioanalysis [115]. The ions generated from both sprayers within the same ion source are sampled through one orifice. Therefore, different non-isobaric compounds must be introduced through the two inlets in order to obtain useful results. A system containing multiple sprayers in combination with multiple ion-sampling orifices on a time-of-flight instrument has been described by Jiang and Moini [116] for high-throughput accurate-mass determination. A multichannel device with an array of ESI tips was described for the high-throughput nanoESI analysis of series of samples contained in a 96-well

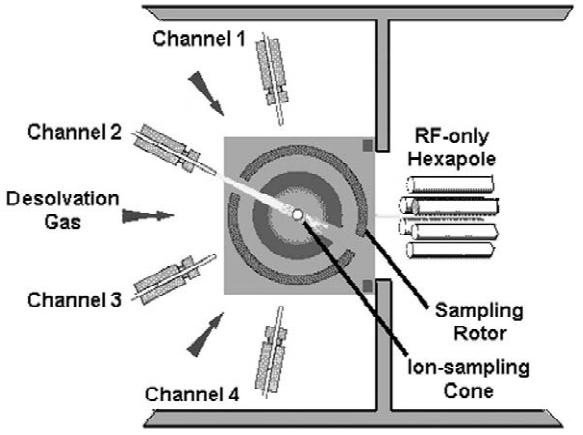

Fig. 7. Schematic diagram of a four-channel indexed multiplexed ESI source, commercially available as MUX© from Micromass (Manchester, UK).

microtitre plate. The analysis of 96 peptide samples in only 480 s was demonstrated [117].

Dual-sprayer devices were also described for co-introduction of a reference compound to act as a lock-mass for accurate-mass determination, essentially based on the same principle as the MUX-source described above, i.e. using the rotating aperture [118,119].

5.2. Microfabricated microfluidic and chip-based electrospray devices

Microchip-based separation techniques are expected to play an important role in future high-throughput screening strategies. Given the importance of MS as an analytical tool in potential application areas of microchip analytical technology, on-line microchip–MS via nanoESI interfacing is under investigation [120–122]. Potential advantages of on-line microchip–MS coupling comprise the reduction of sample consumption and of sample losses due to handling, and the potential for multiplexing. Technology for on-line microchip–MS features either spraying directly from an exposed channel at the side of the chip, or from a nanoESI emitter attached to the microchip.

In the first experiments reported, a multiple-channel glass microchip was developed by the group of Karger [123]. Sample solutions were sequentially sprayed at 100–200 nl/min from a glass microchip containing series of parallel 60-μm-wide, 25-μm-deep and 35–50-mm-long channels, produced by photolithography and wet chemical etching. Each channel contains an individual ESI electrode. The microchip is mounted on an XYZ-manipulator.

Single 60-μm-wide and 10-μm-deep channels on glass microchips were produced by Ramsey and Ramsey [124]. The sample solution is delivered to the spray opening at the side of the chip by electro-osmotic pumping. By attachment of commercially available tapered nanoESI tips to such a single-channel microchip, a flow-rate reduction from ~100 to 20–30 nl/min was achieved [125]. A further improvement involves the implementation of electrically-permeable glass membrane between the ESI electrode and the microfluidic capillary. This design also enable on-chip electrophoretic separation [126].

Figeys et al. [127,128] introduced on-line microchip–MS using an ESI emitter coupled to the microchip via a liquid-junction coupling [129]. The advantage of this approach is that optimum ESI conditions are achieved more readily from a separate and optimized needle. The sample is electrokinetically delivered to the emitter. Initially, these microchips were fabricated from glass, but a microchip made of poly(dimethylsiloxane) (PDMS) was described as well [130]. These 30×50-mm PDMS devices with a 70-μm-deep and 75-μm-wide channel were connected to an ESI needle through a 200–300-mm 75-μm-I.D. fused-silica transfer capillary.

Subsequently, microchip–MS devices were reported enabling not only sample introduction, but also sample handling, e.g. on-chip tryptic digestion [131,132], dialysis [133], or CE separation [134].

Further developments in microchip production for on-line coupling to MS comprise the choice of new materials and the production of multichannel devices. A microchip produced on a silicon chip and containing parylene polymer layers to generate a system of chambers, filters, channels, and hollow needle structures as ESI emitters is described by Licklider et al. [135]. PDMS microchips, featuring multichannel ESI directly from the tip of the microchip, were described by Kim and Knapp [136]. A star-shaped poly(methyl methacrylate) chip, containing eight 375-μm-wide, 300-μm-deep and 12.5-mm-long channels, for sequential ESI analysis, was described by Yuan and Shiea [137].

Although these microchip-based multichannel electrospray devices have not yet been routinely applied in HTS of combinatorial libraries, interesting developments are going on this field. Significant further developments in this research area as well as possibly routine applications are expected to be reported in the years to come.

6. Enhancing the potential and power of high-throughput screening

The HTS described so far in this review is based on rapid nominal molecular-mass determination using a single-quadrupole mass analyser. The power of HTS may be enhanced by a variety of more advanced approaches. Other dimensions may be opened by the application of mass spectrometers capable of more accurate mass determination, such as oaTOF and FT-ICR-MS. The application of LC–MS in HTS is mainly directed as ensuring the identity (and/or purity) of the target compounds subjected to bioactivity screening. Combining bioactivity screening and mass spectrometry might be an interesting hyphenated approach.

6.1. Accurate mass in characterization of combinatorial libraries

While initially high-throughput characterization of combinatorial libraries was developed for and performed with single quadrupole instruments, providing unit-mass resolution and nominal molecular mass determination, the potential of accurate mass determination for such applications was soon recognized [138,139]. High-resolution data acquisition can be achieved using oaTOF, Q–TOF, and FT-ICR-MS instruments.

As regards oaTOF instruments, one of the main issues is a further enhancement of the sample throughput resulting from the faster data acquisition applicable on oaTOF systems (typically 10 spectra/s) [140,141]. The potential of oaTOF in this respect was fully exploited by the introduction of multiplexed LC–MS interfaces featuring four or eight multichannel parallel ESI inlets, the so-called MUX [114,117]. An example of the use of oaTOF is the simultaneous accurate-mass determination of compounds on single beads, the decoding of dansylated orthogonal tags pertaining to compounds, and accurate isotopic difference target analysis using one single generic LC–MS method [142].

The potential of the extremely high resolution achievable with FT-ICR-MS in characterization of complex mixtures, such as combinatorial libraries, was readily recognized. Good examples are the characterization of a 19-component octapeptide library to differentiate between Lys and Gln [143], or of a peptide library containing over 10 000 compounds at a resolution of 130 000 [144]. More recent examples of the use of FT-ICR-MS in combinatorial chemistry were described by others [145–148]. In one report, the significance of monoisotopic and carbon-13 isobars for the identification of a 19-component dodecapeptide library was studied [148].

An interesting approach to the identification of compounds in combinatorial libraries using multistage accurate-mass determination using FT-ICR-MS was proposed by Wu [149]. Accurate-mass determination of precursor and product ions in multistage MS–MS is applied in order to obtain a unique elemental composition of a 517-Da compound containing C, H, N, O, S, and F. Next to the experimental data, the approach requires input of the elements used during the synthesis, as well as some constraints for some unusual elements like fluorine. Alternatively, a mass accuracy better than 0.02 ppm would be required in a direct single-stage accurate-mass measurement.

6.2. Implementing bioactivity screening

The on-line combination of mass spectrometric characterization and biological screening based on ligand–receptor or antigen–antibody interactions can be attractive for some applications.

An early example of such an approach is on-line affinity CE–MS [150]. The receptor is present in the electrophoresis buffer and the ligand library is injected as the sample. Ligands that show strong binding to the receptor are retained and thus separated from compounds that do not interact. The on-line MS detection allows direct characterization of the interacting ligands.

The use of on-line immunoaffinity extraction in combination with coupled-column LC–MS–MS was

demonstrated for the characterization of benzodiazepine libraries [151]. The benzodiazepine library was injected onto a Protein G column loaded with benzodiazepine antibodies. By lowering the pH of the mobile phase, the benzodiazepine–antibody complex is stripped off the column and eluted to a restricted-access material, where the benzodiazepines are separated from the antibodies. The compounds of interest are finally separated on a C_8 reversed-phase column and identified by MS–MS.

On-line monitoring of the glutathione-S-transferase catalysed reaction between 1-chloro-2,4-dinitrobenzene and a H-γ-Glu-Cys-Xxx-OH library using ESI on FT-ICR-MS was described by Wigger et al. [152] in order to demonstrate the feasibility of assessment of enzyme-substrate specificity by MS methods.

An on-line combination of ultrafiltration and ESI-MS was proposed as a means to assist in the identification of lead compounds in the rapid screening of combinatorial libraries [153,154]. The procedure consists of three steps: (i) the ligands from a library mixture are bound to a macromolecular receptor, (ii) the ligand–receptor complexes are purified by ultrafiltration, i.e. unbound ligands are washed away, and (iii) the complexes are dissociated with methanol to elute the ligands into an ESI-MS system for MS characterization. The method was first applied to binding of warfarin, salicylate, furosemide and thyroxine to human serum albumin and of *erythro*-9-(2-hydroxy-3-nonyl)adenine to calf intestine adenosine deaminase. The versatility of this approach was also demonstrated by Wieboldt et al. [155], who applied ultrafiltration to benzodiazepine–antibody complexes and the subsequent characterization of the most active benzodiazepines by LC–MS–MS. On-line pulsed ultrafiltration–MS has recently been reviewed [156].

These immunoaffinity methods rely on ligand–receptor or antigen–antibody interactions in a separation column. Further characterization of the ligand or antigen must be performed in a separate step in the MS. In bioaffinity characterization MS, as proposed by the group of Smith [157], various steps are combined into the measurement cell of an FT-ICR-MS instrument. The affinity target and ligand library are electrosprayed directly from solution, and the resulting ion population is trapped in the FT-ICR cell and subjected to a number of consecutive measurement steps. The non-covalent receptor–ligand complexes are first identified in the mass spectrum and isolated by selected-ion accumulation. The accumulated ions are dissociated to release and trap the ligands that show significant affinity to the receptor studied. These ligands can then be further characterized using MS–MS related approaches. This concept avoids the need for time-consuming steps related to the use of solid supports. Competitive binding of various inhibitors to carbonic anhydrase II has been investigated along this line [158,159].

On-line monitoring of biospecific interactions in a homogeneous biochemical assay using ESI-MS has been reported by Hogenboom et al. [160]. Rather than measuring protein–ligand complexes or dissociated ligands directly, a reported ligand is applied to indirectly determine the interaction between bioactive ligands and their receptor target. A scheme of the continuous-flow system is shown in Fig. 8. First, the ligand to be investigated, i.e. biotinylated compounds or digoxin, is injected into a continuous-flow system and allowed to react with the receptor protein, i.e. streptavidin or anti-digoxigenin. Next, a reporter ligand is added to saturate the remaining free binding sites of the affinity protein. Finally, the concentration of the free reporter ligand is determined by ESI-MS. Along these lines, continuous-flow biochemical assays can be tailored and monitored using MS, providing the required sensitivity without the use of fluorescence or radioactive label-

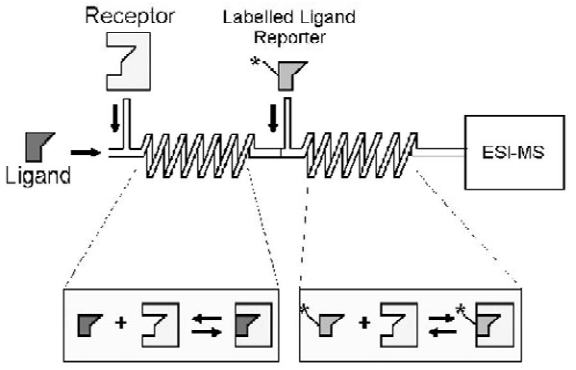

Fig. 8. Scheme of the continuous-flow system applied for on-line monitoring of ligand–receptor interactions in a homogeneous biochemical assay. Adapted from Ref. [160].

ling as well as the specificity to identify unknown ligands in a slightly different set-up.

6.3. Automated MS-directed preparative purification of combinatorial libraries

MS characterization of combinatorial libraries generated by parallel synthesis often reveals that the compounds generated in this way are not sufficiently pure for successful biological screening. Therefore, there is a need for high-throughput preparative purification procedures. Initial purification of combinatorial libraries by automated high-throughput liquid–liquid extraction (LLE) was proposed by Peng et al. [95]. An automated analytical/preparative LC–MS system was proposed for this purpose by the group of Kassel [161]. Samples from parallel synthesis are analysed by rapid analytical LC–MS using a 5–10-min gradient on a C_{18} column. Purity assessment is part of the automated post-acquisition data processing. Any sample falling below the set purity threshold, e.g. 90%, is subjected to automated on-line preparative LC–MS, where the triggering of the fraction collection is based on the real-time MS signal. In this way, unattended purification at milligram level was achieved for several compounds from libraries. A further development [113] of this approach is the use of parallel columns, two for analytical and two for preparative LC, enabling a sample throughput of 200 samples in analytical mode during daytime and 200 samples in preparative mode overnight. A dual-inlet ESI source is applied in this set-up.

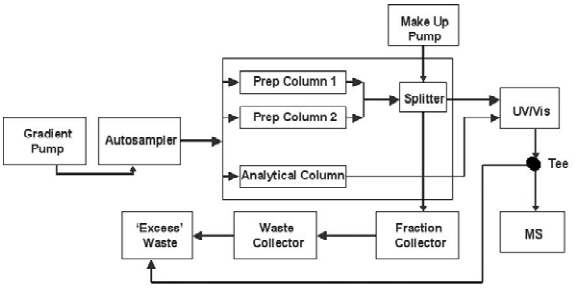

Fig. 9. Schematic diagram of the set-up for MS-directed fraction collection in preparative-LC available from Waters. The system allows rapid switching between two different preparative columns as well as between analytical and preparative LC.

Preparative purification based on MS-directed fraction collection has subsequently been described by others [162–164]. The same approach can obviously also be applied for the purification of drug metabolites [165,166] or process impurities in drug substances [167]. A schematic diagram of one of the commercially available set-ups for MS-directed fraction collection in preparative-LC, capable of rapidly switching between analytical and preparative LC, is shown in Fig. 9.

7. High-throughput quantitative bioanalysis in early drug discovery

The first phases in drug development, i.e. discovery of new chemical entities and lead optimization, metabolic and toxicological screening, and early-ADME (adsorption, distribution, metabolism and excretion) studies, are becoming more and more integrated in modern drug discovery strategies [66]. As a result, severe demands are put on high-throughput quantitative bioanalysis and metabolic profiling. It is virtually impossible to comprehensively review the current developments in this area, partly because most of the research efforts are not published for propriety reasons. This section is aimed at providing a glimpse of the ongoing developments with special focus on technological developments related to LC–MS.

7.1. High-throughput sample pretreatment methods

In most cases, automated off-line sample pretreatment methods are preferred in high-throughput quantitative bioanalysis. This is partly due to the risk of column clogging due to proteins present in the biological samples. Furthermore, per sample the time needed to perform the sample pretreatment step, e.g. by liquid–liquid extraction (LLE) or solid-phase extraction (SPE), may be longer than the time needed for fast LC–MS–MS analysis. This was for instance the case in the quantitative bioanalysis of the 5-HT receptor agonist 311C90 and its desmethyl metabolite in human plasma, developed by Allanson et al. [168], where the SPE procedure on the Gilson ASPEC took ~8 min, while the analysis time in LC–MS–MS was less than 4 min. When the sample

pretreatment is the limiting step, off-line sample pretreatment is more cost effective. The off-line procedure will not limit the sample throughput on the very expensive MS–MS instrument.

Obviously, research has also been directed at decreasing the time needed for sample pretreatment. One of the early examples of batch-wise off-line SPE in 96-well plate format is the Porvair Microlute™ micro-preparation SPE system, developed by Kaye et al. [169]. It consists of 96 SPE cartridges bundled together and mounted on a block that fits the standard 96-well microtitre plate. The Microlute™ is used in combination with a Multiprobe robotic sample processor. Alternatively, the use of Empore disks for SPE in a '96-well' format was also described [170]. With such a device, 96 samples can be extracted in only 10 min. Off-line SPE in 96-well plate format has found frequent application, using either SPE cartridges or Empore disks [171–173].

Parallel liquid–liquid extraction (LLE) in a 96-well plate format in LC–MS has been pioneered by Steinborner and Henion [174,175] in the quantitative bioanalysis of methotrexate and its major metabolite in human plasma. Subsequently, 96-well plate LLE has been applied by others [176].

Obviously, less time-consuming and demanding sample pretreatment methods have been evaluated as well. Direct plasma injection for multi-component analysis in pharmacokinetic screening was studied by Wu et al. [177] and protein precipitation in a 96-well plate format by Watt et al. [178]. However, these crude methods often result in samples prone to ion suppression by matrix effects in ESI-MS (Section 7.3).

The use of a number of alternative sample pretreatment methods has been demonstrated. On-line immunoaffinity chromatography for quantitative bioanalysis was proposed by Rule and Henion [179] for propanolol and lysergic acid diethylamide (LSD) and by Creaser et al. [180] for steroids. The use of restricted-access precolumns [181] was investigated as well. Restricted-access column materials combine the size-exclusion of proteins and the simultaneous enrichment by SPE of analytes that interact with hydrophobic groups at the inner surface of the packing and allow the direct injection of plasma samples. The application of a restricted-access precolumn in combination with LC–MS was for instance reported by van der Hoeven et al. [182] and by Hsieh et al. [183].

Another versatile approach is the so-called turbulent-flow LC [184,185]. Very high flow-rates are used through columns packed with special large-particle-size materials (50 μm I.D.). The turbulent flow profile at these high flow-rates reduces the peak broadening and provides more efficient eddy diffusion, resulting in an enhanced mass transfer between mobile and stationary phase. Although the use of turbulent-flow LC–MS has not yet been frequently reported, the technique is successfully applied within several laboratories in the pharmaceutical industry. Turbulent-flow LC is performed either on one column, combining sample pretreatment and separation on a single column, or on two columns, i.e. one for sample pretreatment and one, packed with smaller particles, for separation.

7.2. Fast chromatography for high-throughput analysis

Next to enhancing the sample throughput in sample pretreatment, the analysis time in chromatographic separations has also been greatly reduced. In most cases, this is achieved by reducing the column length: instead of the 100–250-mm-long columns conventionally applied in combination with UV and PDA detectors, 20–50-mm-long columns (particle size 3–5 μm I.D.) are routinely applied in quantitative bioanalysis using LC–MS–MS. The higher selectivity of the MS operated in SRM mode is exploited to compensate for the loss in chromatography resolution resulting from the column length reduction. Applications of fast LC–MS for quantitative bioanalysis are reported by, for instance, Romanyshyn et al. [186] and Bakhtiar et al. [187]. Various chromatographic aspects of high-throughput LC–MS are discussed by Pereira et al. [188] and by Law and Temesi [189]. The latter addressed mobile-phase compatibility aspects as well [190].

LC instrumentation has also been adapted by various LC instrument manufacturers to enable high-speed gradients, e.g. 10–90% acetonitrile in aqueous buffer within 3–5 min, combined with fast, high-flow column regeneration. The use of 1-min fast gradients is reported by Hsieh et al. [191] for

quantitative screening of drug discovery compounds in monkey plasma.

Another trend is the use of generic methods in order to avoid the need to perform time-consuming method development [189,192–194]. This is especially important in quantitative bioanalysis supporting early drug discovery phases, because a wide variety of analytes with generally a turn-over time in the laboratory of only a few days at the most has to be analysed. Once appropriate lead-optimized compounds appear to be found, more detailed studies including proper chromatographic method development are performed.

New column materials are also explored for high-throughput quantitative analysis, with the monolithic compounds attracting most attention at the moment [195]. The use of monolithic columns for fast LC in quantitative bioanalysis using LC–MS has been reported by several authors [196–200]. In one of these papers [200], turbulent-flow LC in a first column is combined with fast LC on a monolithic second column. Another important application area of the monolithic column is capillary electrochromatography (CEC) [201], which has been a promising technique for some years now, but which has not realised its real breakthrough yet.

Mixed-mode chromatographic columns are also frequently investigated in relation to high-throughput quantitative analysis [202–204]. These mixed mode materials exhibit a combination of hydrophilic, hydrophobic and/or ion-exchange properties.

Coupled-column approaches, featuring either the transfer of a heart-cut of a fraction of the eluate of a first column to the second column, or on-line SPE–LC, are continuously attracting attention, although on-line SPE–LC is less popular in the pharmaceutical field than in environmental analysis. Some examples are the use of SPE–LC for high-throughput preclinical pharmacokinetic studies, as reported by Goa et al. [205], and the use of two parallel Oasis-HLB SPE columns connected to one C_{18} column to enable direct injection of diluted rat plasma, as reported by Xia et al. [206].

Analytical strategies based on multi-component analysis is another way of increasing the sample throughput in quantitative bioanalysis or metabolic profiling. Korfmacher et al. [207] reported the use of two parallel LC systems connected to one LC–MS system via a tee-piece just in front of the interface probe. Different samples, e.g. containing ten compounds with different precursor-ion m/z, are injected onto the two columns and the columns are eluted simultaneously. The first part of the chromatogram is directed to waste.

7.3. Ion suppression by matrix effects in high-throughput quantitative analysis

One of the major concerns in high-throughput quantitative bioanalysis is the occurrence of ion suppression (or enhancement) by matrix effects. Although already well-known by many workers, one of the first accounts of matrix effect was reported in 1996 by Buhrman et al. [208] in the method development for the quantitative bioanalysis of the platelet-activating factor receptor antagonist SR 27417 in human plasma. They demonstrated significant response losses for the target compound in comparing three sample pretreatment methods. In each case, additional peaks showed up in the full-scan mass spectra, which could be held responsible for the ion suppression effect. However, in practice, ion suppression is frequently observed without any changes in the full-scan spectra. An excellent demonstration and discussion of matrix effects has been given by Matuszewski et al. [209,210] in their paper on the method development for the bioanalysis of finasteride in plasma. In principle, ion suppression or enhancement does not have to be a problem, as long as sufficient quantitation limits are still feasible. However, the ion suppression by matrix effects frequently leads to poor results in both accuracy and precision, due to the apparent irreproducible character of the process.

The mechanism of matrix effects was also investigated by the same group [211,212]. By the use of a dual-inlet ESI system, they demonstrated that the matrix effects primarily is a liquid-phase process. The effects were also found to be related to the amount of non-volatiles in the sample. Non-volatiles appear to reduce the ion evaporation of preformed analyte ions from solution. Evidence for this was shown by comparing the amount of analyte precipitating at the curtain plate of a Sciex API source upon introducing the analyte either in a protein-precipitated sample or in mobile phase.

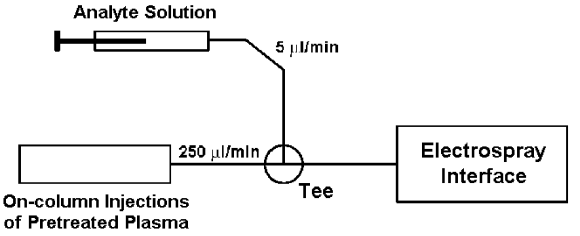

Fig. 10. Set-up for the evaluation of sample pretreatment method with respect to ion suppression by matrix effects in method development for high-throughput quantitative bioanalysis. Adapted from Ref. [211].

Inadequate sample pretreatment has been demonstrated to be an important source of poor reproducibility due to ion suppression and matrix effects [209,210]. A method to evaluate sample pretreatment methods in terms of ion suppression, based on post-column mixing of the analyte of interest into the eluate of a column to which the pretreated sample is injected (Fig. 10), has been proposed by Bonfiglio et al. [211] and is frequently applied in practice [191]. An application of this method is described by Nelson and Dolan [213] in solving a problem in the analysis of a drug, its major metabolite and an analogue internal standard (Fig. 11). Upon transfer of the method from one LC–MS system to another, a matrix effect was observed for the parent compound. After evaluating the effect of the steepness of the gradient program, mobile-phase composition and the column choice, it was found that the second LC system had a significantly larger dead volume in the gradient mixing system. After reducing this internal volume, the problem was said to be solved [213]. However, from the severe ion suppression and enhancement observed in the trace generated by the post-column mixing method, one may conclude that an additional improvement of the sample pretreatment method would be beneficial in this case as well.

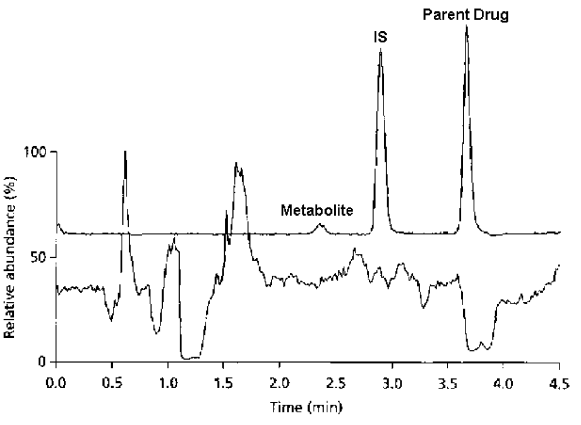

Fig. 11. Evaluation of ion suppression via the T-in method (cf. Fig. 10). The chromatogram of the sample (parent drug, metabolite and internal standard) is shown at the top. Trace of continuous infusion of the parent drug is shown at the bottom. Adapted from Ref. [213].

Conclusions concerning matrix effects should be handled with some care. In some cases, it appears that any poor results in quantitative bioanalysis are attributed to matrix effects. Matuszewski et al. [210] outlined a number of experiment to investigate whether a matrix effect is actually present. The occurrence of matrix effect does not discharge us from the need to perform proper problem solving in method development for bioanalysis.

8. Conclusions

The field of high-throughput screening and analysis using LC–MS is a fast moving field, with many new and interesting developments appearing. The important role played by LC–MS definitively indicates that LC–MS has become a mature, reliable and robust analytical technique. While impressive progress in LC–MS applications and instrumentation has been achieved in the past 10 years, the concepts and basic technologies of interfacing the liquid-phase separation technique to MS and analyte ionization have not changed significantly. New developments in that respect have not had a similar impact on the LC–MS field as did the earlier introduction of electrospray.

Whether this review provides a proper overview of current state-of-the-art in high-throughput screening of combinatorial libraries and high-throughput analysis in early drug discovery, is difficult to decide from an overview of the public literature. Many developments in this area are hidden behind the walls of the pharmaceutical industries for propriety reasons. Reviewing developments in a fast moving field comes with the risk that the paper is already to some extent obsolete when it is published. It continues to be a fascinating application area of LC–MS.

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