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Review

State-of-the-art in liquid chromatography–mass spectrometry

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Abstract

Impressive progress has been made in the technology and application of combined liquid chromatography–mass spectrometry (LC–MS) in the past decennium. From a technique, that could only be used by a specialist, it has developed into a routinely applicable technique. LC–MS has become the method-of-choice of analytical support in many stages of drug development within pharmaceutical industries and has found its way into environmental, biochemical and other laboratories. This paper provides a perspective on the current technology, principles and applications of LC–MS. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Reviews; Mass spectrometry; Liquid chromatography–mass spectrometry; Ionization mechanisms; Atmospheric pressure ionization methods

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1. Introduction

Combined liquid chromatography–mass spectrometry (LC–MS, [1]) can be considered as being one of the most important techniques of the last decade of twentieth century. The extreme speed of development and its acceptance and high spread, especially considering its price tag, is astonishing. LC–MS has become the method-of-choice for analytical support in many stages of drug development within the pharmaceutical industry. In addition, it plays an important role in environmental analysis,

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while LC–MS instrumentation is also heavily used in biochemical and biotechnological applications, as well as in many other fields of application.

In their recent book on LC–MS, Willoughby and co-workers [2] analyzed the development of LC–MS as well as its acceptance as an analytical technique. As pictured in Fig. 1, at present we are still in the early stage of acceptance and use of LC–MS in real-world applications. While new developments in instrumentation are slowing down, significant progress is made in the implementation and application of LC–MS in many different fields. This stage puts different demands on the development of LC–MS as an analytical technique, as is for instance indicated by the currently growing interest in software development, especially for more efficient data processing after LC–MS analysis.

LC–MS interfacing and its applications have frequently been reviewed. Recently, the advances in the instrumentation in LC–MS were reviewed, with a special emphasis on the development of interfaces for atmospheric-pressure ionization (API) [3]. The current paper provides an overview of current technology with an emphasis to commercially available technology. At present, most of the instrumental developments are taking place within the instrumental manufacturers laboratories and workshops, although to a high extent such developments are steered and initiated by the demands from users, especially the pharmaceutical industries. Their efforts and research and economical interests in the implementation of LC–MS in the various stage of drug development demands more advanced instrumentation as well as software support.

Next to reviewing the state-of-the-art in instrumentation, the current status of LC–MS as an analytical tool is evaluated. What are the possibilities and limitations of current instrumentation and technology? What are topics which still require considerable research interest and/or development? What to

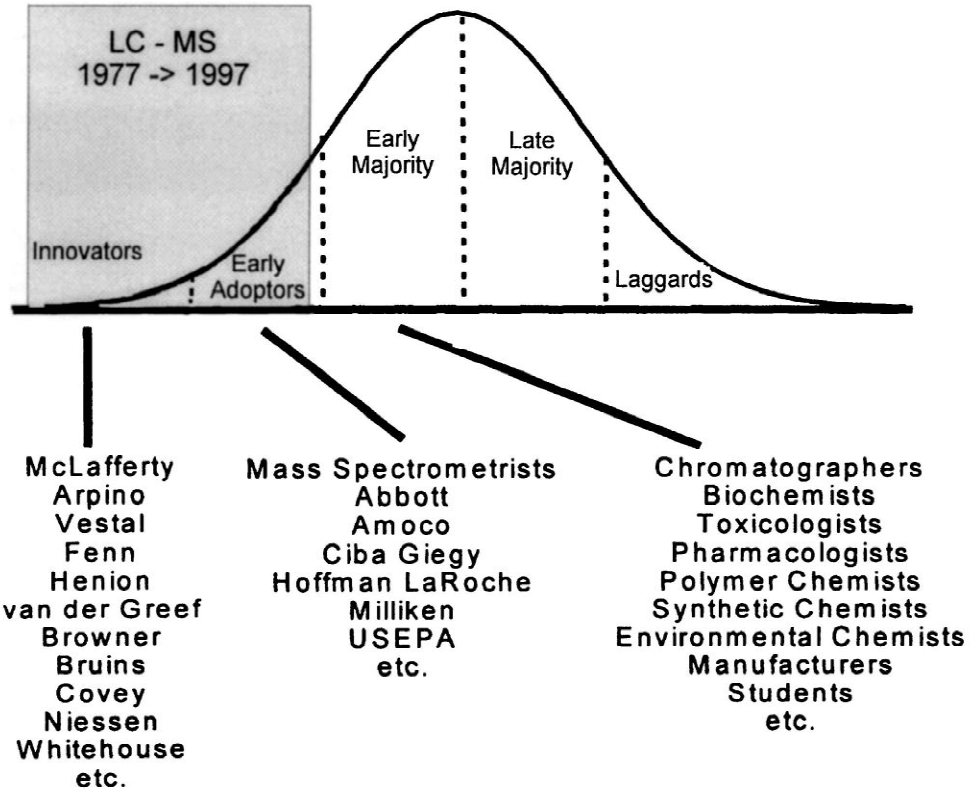

Fig. 1. Model for the distribution of people applying LC–MS (based on ref. [3]).

expect from LC–MS in the first decade of the next century? In addition, attention is paid to some of the most important applications of LC–MS, perhaps in order to allow the reader to answer questions like: Is the current huge interest in LC–MS a transitory hype? Why so many laboratories are fascinated by the potential of LC–MS in their developing applications?

2. General view on API instrumentation

An API interface/source consists of five parts (cf. Fig. 2): (1) the liquid introduction device or spray probe, (2) the actual atmospheric-pressure ion source region, where the ions are generated by means of electrospray ionization (ESI), atmospheric-pressure chemical ionization (APCI), or by other means, (3) an ion sampling aperture, (4) an atmospheric-pressure to vacuum interface, and (5) an ion optical system, where the ions are subsequently transported into the mass analyzer. API interfaces are available from all major mass spectrometer manufacturers [4–13].

The operational principle of an API interface and ion source for LC–MS is as follows. The column effluent from the LC is nebulized into an atmospheric-pressure ion source region. Nebulization is either performed pneumatically, i.e., in heated-nebulizer APCI, by means of the action of a strong electrical field, i.e., in ESI, or by a combination of both, i.e., in pneumatically assisted ESI ('ionspray' [14]). In addition, ultrasonically assisted electrospray nebulization has also been described [15]. From the aerosol, gas-phase ions are generated by mechanisms discussed in more detail below. These ions, together with solvent vapor and nitrogen bath gas, are sampled by an ion sampling device into a first pumping stage. The mixture of gas, solvent vapour and ions is supersonically expanded into this low-pressure region (10–100 Pa). The core of the expansion, containing the ions and other (neutral) material of higher molecular mass, is sampled by a skimmer into a second pumping stage (pressure 0.1–1 Pa), containing an ion focussing and transfer device to optimally transport and focus the ions in a suitable manner to the mass analyzer region (pressure $<10^{-3}$ Pa). In most systems, the ion transfer device consists of an RF-only quadrupole, hexapole or octapole. 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 actually acts as a fixed restriction between the atmospheric-pressure region and the first pumping stage. From the ionization point-of-view, it is also not important how the ions are generated, i.e., 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. In addition, ESI-generated ions generally contain less internal energy than the ions generated via APCI.

Two major mechanisms contribute to the ionization of the analytes: gas-phase ion–molecule reactions and a process generally called ion evapora-

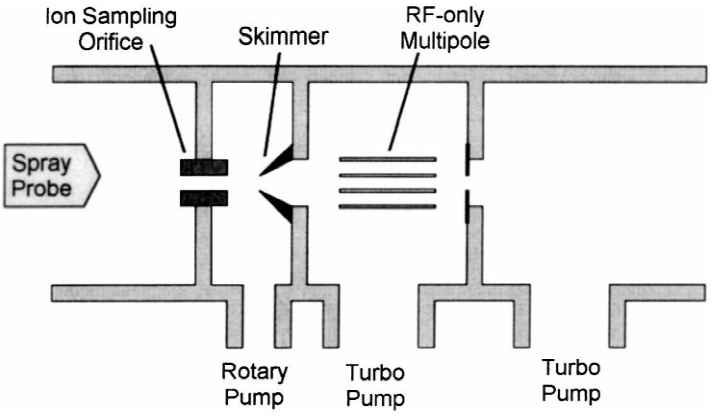

Fig. 2. General scheme of an atmospheric-pressure interface and ion source.

tion. Ionization mechanisms are dealt with in more detail below.

3. Recent advances in interface technology

The historical development of API interfaces for LC–MS was reviewed in detail elsewhere [3]. A variety of API interfaces is now commercially available from a number of instrument manufacturers. The interfaces are not discussed in detail here. Important design characteristics of the most widely applied interfaces are summarized in Table 1. Other manufacturers apply slightly different concepts, i.e., different combinations of the devices described in the table. All API interfaces operate in the way described above. However, in evaluating the available interfaces, a number of interesting trends can be observed.

First, ESI interfacing has developed into two more-or-less distinct approaches. In most LC–MS applications, one aims at introducing the highest possible flow-rate to the interface. While early ESI interfaces showed best performance at 5–10 μl/min, present pneumatically-assisted ESI interfaces are optimized for flow-rates between 50 and 200 μl/min. Although instrument manufacturers indicate that their ESI interfaces can be applied with flow-rates up to 1 ml/min (or even higher), such a high flow-rate is hardly used in practice and also not recommended. In practice, the response is not adversely influenced by using a solvent splitter between column outlet and ESI probe to reduce the flow-rate of 1 ml/min from the column to ca. 100 μl/min fed into the interface probe.

It is generally believed that under API conditions, the mass spectrometer acts as a concentration-sensitive detector, i.e., the response of the detector should be independent of the flow-rate [16–19]. It is in fact difficult to prove experimentally, whether this is

Table 1
Design characteristics of the most widely applied LC–MS interfaces

Interface type	Design characteristics	Ref.
Initial design	On-axis ESI probe with three concentric tubes: sample, sheath and gas. Countercurrent drying gas. Ion sampling via glass capillary with metallized ends. Lens stack.	
Heated capillary	On-axis pneumatically-assisted ESI probe with three concentric tubes: sample, sheath and gas. No drying gas. Ion sampling via heated stainless-steel capillary. RF-only quadrupole or octapole.	[12]
Turboionspray	Pneumatically-assisted ESI probe at 45° and heated gas at 90°. Countercurrent curtain gas. Ion sampling via orifice. High-pressure RF-only quadrupole.	[10]
Z-spray	Orthogonal pneumatically-assisted ESI probe. Concurrent desolvation gas. Ion sampling via conical orifice and orthogonal skimmer. RF-only hexapole.	[9,13]
Orthogonal	Orthogonal pneumatically-assisted ESI probe. Countercurrent drying gas. Ion sampling via glass capillary with metallized ends. RF-only octapole.	[4–6]
aQa	Orthogonal pneumatically-assisted ESI probe. No drying gas. Ion sampling via conical orifice (washed with solvent) and orthogonal skimmer. Flow focussing. RF-only hexapole.	[12]

actually true. A proper experimental design for such an experiment is difficult, because it is difficult to achieve a constant mass-flow in the analyte peak while changing the solvent flow-rate. The behaviour of the detection system as a concentration-sensitive device can be considered to result from the splitting at the sampling orifice, where only a fixed part of the source volume can be sampled (cf. Fig. 3). In addition, the concentration of ions in the spray plume generated in the electrospray nebulization is limited by space charging [20]. Attempts to improve the ion sampling by electrostatic means are bound not to succeed as they adversely influence the potential required to produce an efficient electrospray nebulization.

In sample limited cases, nano-ESI interfaces are applied which can efficiently be operated at sub-μl/min flow-rates [21,22]. Two types of nano-ESI interfaces should be distinguished: 'static' and 'dynamic' interfaces. Static nano-ESI devices are especially applied in the field of protein characterization [21,22]: a narrowbore nano-ESI needle is filled with the protein solution and positioned on a probe. The probe is positioned in the API source and the protein solution is sprayed at liquid flow-rate in the range of 10–100 nl/min. In this way, about one hour of mass spectrometric experiments can be performed with only about 1 μl of sample. The optimum design of the nano-ESI needle is studied [23,24]. Dynamic nano-ESI devices are applied in combination with capillary electrophoresis, nano-LC, microcapillary LC, and capillary electrochromatography. Typical flow-rates are in the range of 30–1000 nl/min. There is considerable development in the design and optimization of dynamic nano-ESI devices [25–28]. Nano-ESI interfaces and supplies are commercially available from major instrument manufacturers [4–13], but also from two specialized companies [29,30].

In the high flow-rate ESI interfacing, a clear trend is the application of heat in the source, in order to stimulate the evaporation of the mostly aqueous droplets. Heated nitrogen gas is applied to enhance droplet desolvation, where the gas is introduced either in countercurrent flow, in concurrent flow (in Z-spray interfaces [9,13]), or perpendicular to the spray (in turboionspray interfaces [10]). Relatively high gas temperatures must be applied to achieve sufficient heat transfer to the evaporating droplets.

Another trend in interface development is related to the need to avoid source contamination during the routine analysis of large series of biological samples. These generally contain significant amounts of non-volatile material (salts, proteins, etc.), which will contaminate the ion source and especially the ion-sampling cone. Off-axis electrospray nebulization instead of on-axis with the sampling orifice appears to be an important progress in that respect. Ionspray

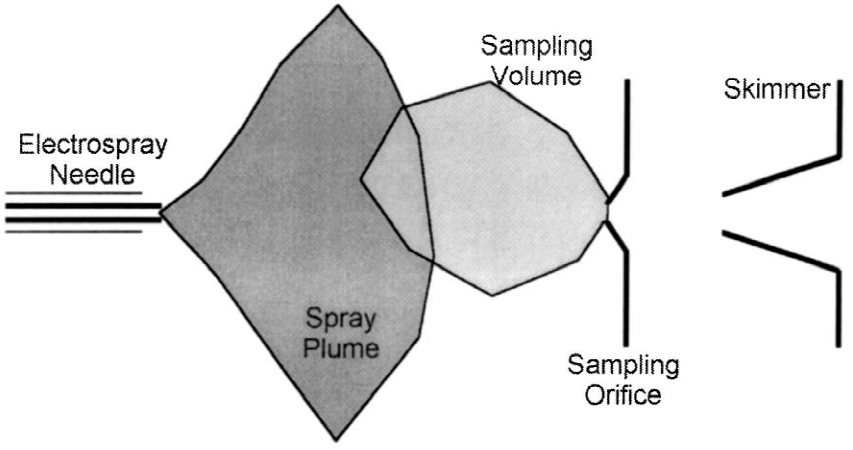

Fig. 3. Splitting at ion-sampling orifice: the sampling orifices acts as a splitter which enables the introduction of only a limited part of the ions generated in atmospheric-pressure ionization.

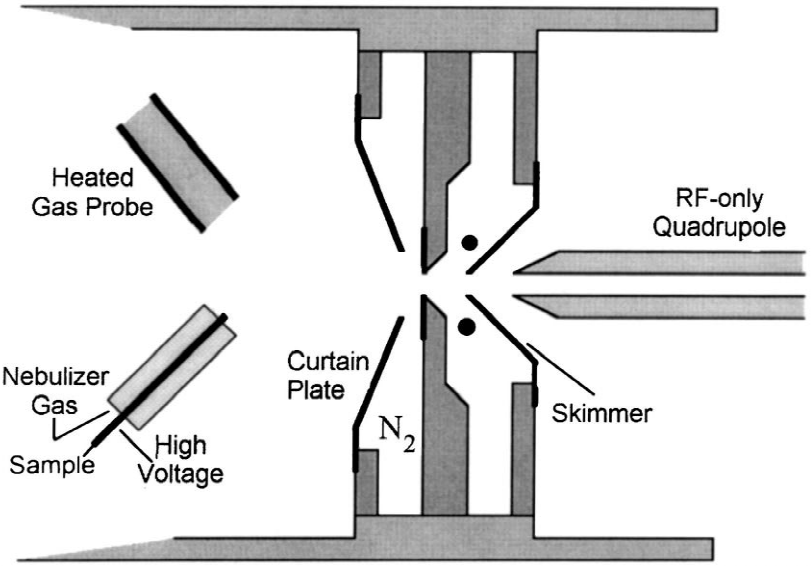

Fig. 4. Schematic diagram of a turboionspray source (based on information from the manufacturer [10]).

and turboionspray interfaces [10] are generally used off-axis, positioned at 30–45° relative to the axis (see Fig. 4). In addition, the use of a curtain gas flowing around the sampling cone greatly reduces the contamination of the cone and orifice.

In most interface designs (c.f. Table 1), an orthogonal spray probe is applied [4–6,9,12,13]. An example of an orthogonal ESI source is shown in Fig. 5. Some manufacturers use orthogonal spray probes in combination with additional devices to avoid ion source contamination, i.e., in the Z-spray [9,13] and in the aQa source [12]. In the Z-spray source (see Fig. 6, [9]), the electrospray nebulization is performed orthogonally to the sampling cone. Ions are extracted orthogonally from the spray into the sampling cone, while large droplets and nonvolatile material are collected onto a baffle plate. Subsequently, the ions are extracted orthogonally from the expansion behind the sampling cone into the high vacuum of the mass spectrometer. The aQa source (see Fig. 7, [12]) is described as a self-cleaning source: a constant low flow-rate of solvent is delivered to the edge of the ion sampling orifice to prevent the build-up of nonvolatile material. The LC effluent is sprayed orthogonally to the sampling cone. After passing through the entrance cone, the gas flow is disrupted and ions are send to the skimmer. No tuning of voltages of ion source devices is needed in the aQa source.

Obviously, all manufacturers continuously pursue improved performance of their interfaces by optimizing the design, e.g., with respect to the shape of the ion focusing parts or with respect to pumping at the ion source.

4. Ionization mechanisms

In the discussions on the ionization mechanisms active in atmospheric-pressure ionization (API), generally a clear distinction is made between ESI and APCI. The ionization in an ESI interface is considered primarily a liquid-phase ionization technique: preformed ions in solution are desorbed or evaporated to the gas phase and can subsequently be mass analyzed. The ionization in APCI is considered to be primarily based on gas-phase ion–molecule reactions between analyte molecules and a solvent-based reagent gas, generated by a series of ion–molecule reactions initiated by electrons from the corona discharge needle. Whether this distinction is actually valid, may be questioned, especially in the analysis of small compounds.

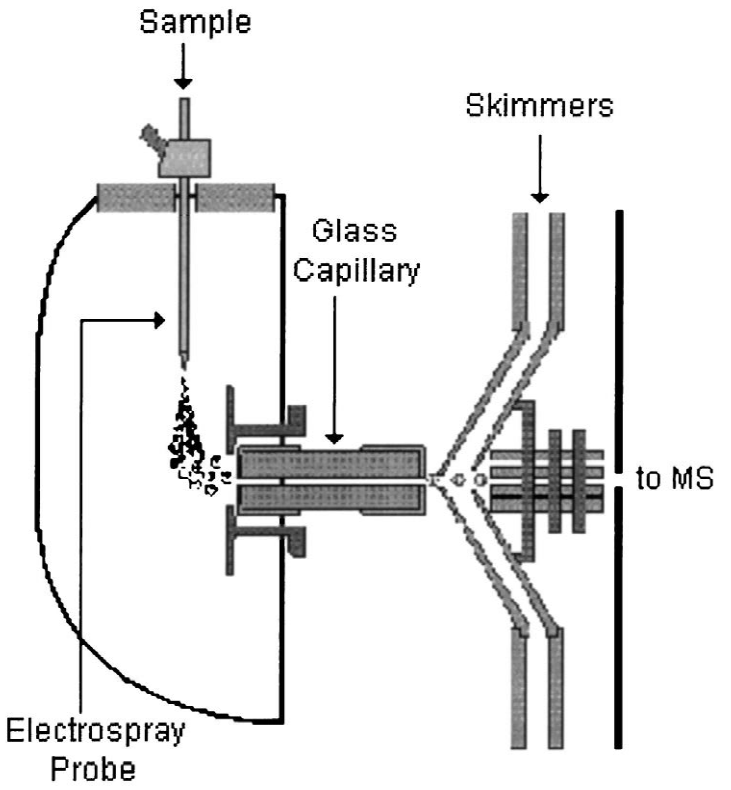

Fig. 5. Schematic diagram of an orthogonal electrospray source (based on information from the manufacturer [5,6]).

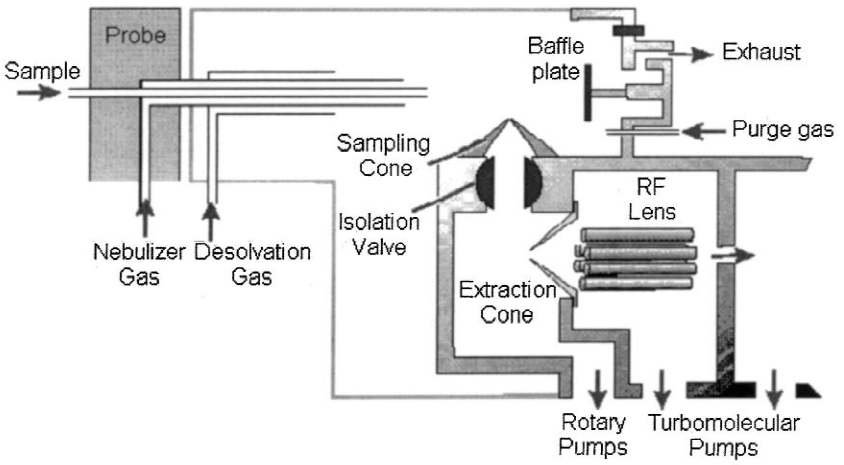

Fig. 6. Schematic diagram of a Z-spray ion source (based on information from the manufacturer [9,13]).

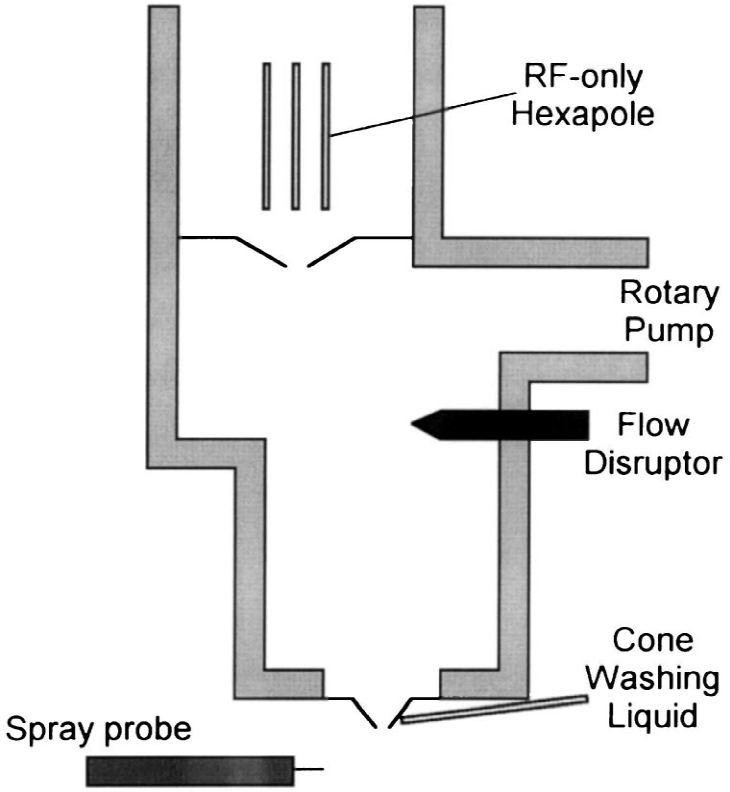

Fig. 7. Schematic diagram of an aQa ion source (based on information from the manufacturer [12]).

4.1. Liquid-phase processes in analyte ionization

Nebulization of the column effluent is a common feature of the current API interfaces. It was demonstrated by Dedieu et al. [31] in 1982, using a direct liquid introduction interface, that the nebulization plays a major role in the soft transfer of analytes from the liquid phase to the gas phase prior to chemical ionization. Highly labile molecules like the vitamin B_{12} could be transferred from the liquid phase to the gas phase in this way. This process, indicated as soft desolvation, is active in all interfaces based on liquid nebulization, and is certainly important in heated-nebulizer APCI systems. In fact, the underlying concept resembles the ionization mechanism proposed by Dole et al. [32,33] for ESI: by spraying a solution of preformed protein ions, small droplets will be generated, from which gas-phase protein ions will emerge due to charge-preserving desolvation of the droplets. This ESI mechanism is currently known as the charge-residue model.

Subsequently, both in the discussion on the mechanism of the thermospray buffer ionization by Vestal et al. [34–36] and in the discussion on the ESI phenomena observed in the early experiments of Yamashita and Fenn [37–39], the ion-evaporation concept introduced by Iribarne and Thomson [40–43] was considered to be the most adequate model.

Both the ion-evaporation and the charge residue model [44,45] start from the nebulization of the liquid stream into small droplets. Due to statistical random sampling of the positive and negative (buffer) ions present in solution [46], positively and negatively charged droplets are generated. In thermospray and heated nebulizer interfaces, both positively and negatively charged droplets are generated. In electrospray nebulization, droplets of only one polarity are generated, because the droplets of opposite

polarity are discharged prior to their formation at the needle surface, as a result of electrochemical nature of the electrospray process [44,47].

The charged droplets enter into a series of processes. Charge-preserving solvent evaporation results in a decreasing droplet size, which is accompanied by an increasing electric field at the droplet surface. At a certain stage, the coulomb repulsion between the surface charges exceeds the cohesive forces due to surface tension and the droplet will explode. This Coulomb explosion was photographed using a shadowgraph technique by Gomez and Tang [48]. In this way, it was demonstrated that the droplet fission take place from a surface deformation; a droplet-jet is formed at the surface, as schematically drawn in Fig. 8. Microdroplets carrying a relatively high number of charges are emitted. The processes of charge-preserving solvent evaporation and electrohydrodynamic droplet disintegration may proceed until sufficiently small droplets are produced for the final step.

It is in this final step where the ion-evaporation model and the charge-residue model differ. Iribarne and Thomson [32–35] argued that ions may be directly emitted ('evaporated') into the gas phase when the droplets are sufficiently small and charged. The charge-residue model assumes that these small droplets will actually contain only one preformed analyte molecule, which is transferred to the gas phase by subsequent solvent evaporation from the droplet. Whatever mechanism is the correct one – and it has even been argued that different mechanisms are valid for small and large molecules [49] –, both mechanisms indicate the importance of preformed ions in solution, e.g., by (de)protonation in the liquid phase. The pH of the solution is thus considered an important parameter. However, the pH in the liquid will change during the droplet evaporation as a result of the differences in volatility of the compounds applied to set the pH, as discussed for instance by Appfel et al. [50] and Gatlin and Tureček [51].

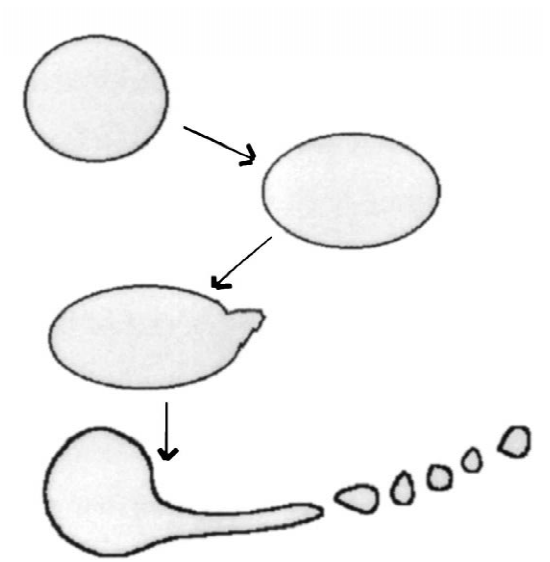

Fig. 8. Representation of the Coulomb explosion, leading to the production of small and highly-charged microdroplets.

4.2. Gas-phase process in analyte ionization

Not only analyte ions will ion evaporate from the droplets, but the buffer ions will do so as well. Therefore, next to the protonated analyte ions, buffer ions like the ammonium ion will be present in the gas phase. In the discussions of the ESI mechanism, like previously in the discussion of the thermospray ionization mechanism [52], the importance of gas-phase ion–molecule reactions is often neglected or ignored. Protonated analyte molecules may also be formed by gas-phase reactions between the protonated ammonia from the buffer and a neutral analyte ion, transferred to the gas phase by the soft desolvation process described above. In addition, the ions generated in the gas phase undergo series of collisions to neutral solvent and nitrogen molecules in the atmospheric-pressure ion source. In combination with acceleration of the ions, collisionally induced dissociation (CID) of the ions may occur. But in general, the multiple collisions may alter the composition of the ionic distribution initially generated by the ESI process. The potential difference between nozzle and skimmer may, for instance, alter the charge state distribution of multiple-charged protein ions [53,54].

The gas-phase ion–molecule reactions described above are conventional chemical ionization (CI) reactions [55]. CI is the prime ionization mechanism in APCI. The process is initiated by electrons from the corona discharge [56]. The electrons ionize the nitrogen in the source and the nitrogen molecular ion

reacts with the solvent molecules. Protonated solvent species are formed. The most abundant species in the reagent gas is due to the solvent constituent with the highest proton affinity (in positive-ion mode) or the lowest gas-phase acidity (in negative-ion mode). When the proton affinity of the analyte exceeds that of the reagent gas, the protonated analyte will be generated. Similarly, the deprotonated analyte will be generated when the gas-phase acidity of the reagent gas exceeds that of the analyte. In addition, adduct ions are generated when the proton affinities or gas-phase acidities of analyte and reagent gas are similar.

These gas-phase ion–molecule reactions are effective in the gas–vapor mixture generated in the ESI ion source as well. Reactions between ion-evaporated buffer ions, e.g., protonated ammonia, and analytes result in protonated analyte molecules. From the mass spectrum, it is impossible to determine whether the protonated molecule was formed in the liquid phase or in the gas phase. For many small molecules, the ESI response can be predicted from CI rules.

Similarly, ion-evaporation-like processes may be important in heated nebulizer APCI, i.e., for some compounds good response can be achieved in the corona discharge-off mode in APCI. In method development for quantitative analysis, it is worthwhile to investigate the response in APCI discharge-off mode as well.

In conclusion, both liquid-phase and gas-phase ionization processes should be taken into account in describing the ionization in both ESI and APCI. The actual ion yield is the resultant of mixed mechanisms, especially in the analysis of small molecules. In the analysis of large molecules like proteins, the major effect of gas-phase reaction is often gas-phase reneutralization. Gas-phase reactions of electrosprayed protein ions were studied by Ogorzalek-Loo et al. [57,58].

Another important issue, especially in relation to ESI, is the influence of the other solvent constituents and the matrix. In the Coulomb explosion, preferentially the ions at the droplet surface are transferred to the microdroplets. This explains why the analyte response may be greatly reduced by the presence of surfactants in the mobile phase [59]. High concentrations of other ionic species reduce the analyte response as a result of competitive effects in ion evaporation. In real matrices, often a significant reduction of the analyte response is observed without a clear indication on what compound is responsible for the ion suppression. In general, no response is observed from the competing species. This type of matrix effects often requires considerable attention in the development of quantitative bioanalysis. A nice and well documented example is the development of a bioanalytical method for the drug finasteride, as described by Matuszewski et al. [60]. On-column injections of standard solutions indicate a five-fold better signal-to-noise ratio in ESI than in APCI. However, due to a severe and not-reproducible matrix effect in ESI, the R.S.D. of the peak area of the internal standard is unacceptably high, both for repetitive injections of one particular plasma sample and between plasma samples from different patients. In order to avoid these matrix effects, the method was developed for APCI.

5. Advances in MS instrumentation

Almost equally important to optimization of API interfaces is the progress in MS instrumentation. The computer-controlled operation of modern instrumentation helps in achieving optimum performance. In addition, new developments in the instrumentation help to improve the overall performance of LC–MS.

The majority of LC–MS applications are still run on single and triple quadrupole instruments. In the past years, the performance of triple quadrupole (QqQ) instruments for MS–MS is improved by replacing the RF-only quadrupole collision cell by RF-only hexapole [9] and octapole [12] collision cells. The latter provide an improved product ion collection and transmission. This is also pursued with the modified quadrupole collision cell described by Mansoori et al. [61] and the linear accelerating high-pressure collision cell (LINAC, [10]). The LINAC also allows shorter dwell times in selective reaction monitoring (SRM), thereby allowing to speed up the analysis or to monitor more SRM transitions during one (fast) chromatographic run.

API on an ion-trap mass spectrometers appears to be a very successful combination, which is available from two manufacturers [5,12]. While in low-level

quantitative analysis SRM on a QqQ instrument has to be preferred [62,63], the API ion-trap instrument especially proves its power in qualitative analysis, where the multiple stages of MS–MS can be applied to achieve structure elucidation of the unknown. Data-dependent acquisition further enhances the performance in structure elucidation of minor components in a mixture [64,65].

Orthogonal-acceleration reflectron time-of-flight (oa-TOF) instruments, available from several manufacturers [4,9,10], combine the ability to perform accurate mass determination with an excellent full-scan sensitivity. Mass accuracies of better than 20 ppm without and better than 5 ppm with an internal lock mass can be achieved routinely. For compounds with an M_r below 1000 Da, this accuracy provides an excellent confirmation of identity based on calculated elemental compositions. An oa-TOF instrument is an integrating rather than a scanning system. In practice, the 'all-ion-detection' capability of the oa-TOF system provides a 20–100-fold improvement in sensitivity, compared to a scanning QqQ system. Obviously, the QqQ instrument operated in SRM mode will provide better sensitivity, but only at the expense of the information content.

Given these features of an oa-TOF analyzer, a logical combination is the hybrid of a quadrupole front-end and an oa-TOF back-end for MS–MS. This so-called Q–TOF, with unsurpassed performance in terms of specificity and sensitivity, was first introduced and built by one manufacturer [9,66,67], while recently a similar Qq–TOF instrument was introduced by another [10,68]. The most interesting feature of the Q–TOF hybrid is its ability to perform accurate mass determination at excellent sensitivity after conventional low-energy CID in a hexapole collision cell. This greatly facilitates identification of unknowns, not only in the field of protein chemistry, where the instrument was originally built for, but also in studies related to impurities, degradation products and metabolites of drugs.

Another powerful hybrid instrument is the ion-trap storage – reflectron TOF instrument (IT–reTOF), as developed by the group of Lubman [69,70]. Initially, the ion-trap part of the instrument was primarily applied to achieve a pulsed ion introduction to the reTOF with an improved duty cycle. Currently, the potential of the ion trap in (multiple stages of) MS–MS prior to TOF mass analysis is explored as well [71].

Finally, multiply-charging of proteins by ESI has also stimulated the use of Fourier-transform ion-cyclotron resonance mass spectrometers (FT–ICR–MS) [72,73]. Again, the enhanced resolution is an important feature. The high-resolution operation in combination with various dissociation techniques, such as CID, sustained off-resonance irradiation, infrared multiphoton dissociation, and surface induced dissociation, enables the use of ESI FT–ICR–MS for advanced structure elucidation of proteins. In addition, reaction chemistry and gas-phase confirmation studies can be performed [72,73].

Among these new and exciting developments, the role of ESI on magnetic sector instruments has diminished. The main reason for the use magnetic sector instrument appears to be the use of an array-type of detector to enhance sensitivity. However, for many applications a single TOF instrument is a viable alternative, being easier to operate and with a better price/performance characteristic. The need for high-energy CID in peptide sequencing appears to be overcome to a large extent by the use of protein databases [74,75].

6. LC–MS applications in perspective

At present, the three major application areas of LC–MS technology are in the pharmaceutical, environmental and biochemical fields. The applications in the field of drug development and testing can be considered as an important driving force in the current development of LC–MS technology.

6.1. Pharmaceutical applications

LC–MS technology is applied in virtually every stage of drug development. The three main issues in this field are sensitivity, selectivity and speed. Compared to UV and UV-photodiode array (PDA) detection, the LC–MS combination provides enhanced confirmation of identity and often enhanced selectivity. In addition, the ease-of-operation and the achieved level of automation make LC–MS an attractive tool in drug development.

The role of LC–MS already starts in the drug

discovery stage, irrespective whether this is performed by conventional 'intelligent' synthesis or by combinatorial chemistry. Three powerful software tools have been developed for LC–MS to support in drug discovery. These software tools are available from all major instrument manufacturers.

The first and most generally applicable tool is Open Access [76,77]. This tool transforms the API-MS instrument into a walk-up 'black-box' for synthetic chemist in need for a rapid confirmation of the good progress of their synthesis by molecular mass determination of their product. A remote computer serves as a log-in to the system. After entering the sample identification code and the type of LC–MS experiments to be performed, the computer indicates the position in the autosampler rack to be used. The sample is run automatically, e.g., 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. Recently, open-access LC–MS systems are extended by the implementation of on-line LC–NMR as well [78].

To a growing extent, drug discovery is based on synthesis by combinatorial chemistry procedures. The combinatorial libraries need to be screened for biological activity, while a rapid characterization of identity is also required. LC–MS technology is frequently applied for the latter step [79]. The analytical system consists of an *x–y* autosampler, enabling sample introduction from a 96-well plate, connected to a column-bypass API-MS [80] or fast LC–MS system [81]. Single quadrupole systems are often used for this purpose, although the use of oa-TOF instruments is of growing importance; the accurate mass determination allows a better confirmation of identity. The software allows the rapid analysis of large series of samples at a high sample throughput, i.e., up to 60 samples per hour in column-bypass mode [82]. On-line UV–PDA as well as other LC detectors may be used to establish the compound purity [83]. The data-processing software provides a user interface, often called a data browser, which allows rapid answers on whether or not the expected products are present. In reaching this decision, data from both positive-ion and negative-ion acquisition are used, taking into account the various adduct ions that might be generated. The reduced data can be viewed by means of the browser: in a graphical representation of the 96-well plate the confirmed samples are colored green, while the others are colored red. More advanced color schemes have been described as well [84]. In some applications, the screening for biological activity and the mass spectral characterization is combined into one system [85], e.g., by the application of on-line bioaffinity columns [86] or integrated biodetection systems based on antigen–antibody or ligand–receptor interaction [87]. The latter systems are commercially available as tailor-made solutions to a particular application [88].

The third tool combines the rapid screening of combinatorial libraries or of series of extracts from natural products to preparative scale purification of biologically active compounds. The fractionation is controlled by the response of the compound of interest in the LC–MS. Initially, these systems were developed using conventional LC columns, but preparative-scale LC columns are applied now as well [89–91].

While the structure confirmation in open-access and combinatorial-chemistry strategies is primarily based on molecular mass of the intact compound, more elaborate structure elucidation is required in subsequent stages of drug development, i.e., the stages related to impurity screening, identification of drug metabolites, and the search for degradation products in drug substances and drug formulations. Screening strategies based on precursor-ion and neutral-loss scans in MS–MS are frequently applied to search for structurally-related compounds. Based on the product-ion mass spectrum of the parent compound and educated guesses of possible products, selective neutral-losses and/or possible common product-ions are selected. An example of the use of these complementary strategies in the impurity profiling of butorphanol tartrate is described by Volk et al. [92]. Similar MS–MS strategies can be applied in drug metabolism studies as well. Common neutral losses in the identification of Phase-II metabolites are the losses of 80 and 176 Da, for aryl-*O*-sulphate and *O*-glucuronic acid conjugates, respectively. An example is described by Brownshill et al. [93]. The advantages of accurate mass determination in these studies is also clearly recognized. FT–ICR–MS [94] and the Q–TOF hybrid [95] are applied for this

purpose. Another interesting approach, circumventing the need for on-line LC separation, is the use of nano-ESI: a small volume of the sample solution is introduced via nano-ESI. The mass spectrum is searched for minor impurities and product-ion MS–MS spectra are subsequently generated. The applications of multiply MS–MS stages, as available on ion-trap MS systems, can be of great help in structure elucidation, as demonstrated by Tiller et al. [96] in a glyburide metabolism study. Another emerging trend is the on-line combination of LC–NMR and LC–MS in metabolite studies, using either (triple) quadrupole [97–99] or ion-trap [100] MS systems.

6.2. Quantitative bioanalysis in pharmaceutical applications

Quantitative bioanalysis is the most important application area of LC–MS, in terms of number of instruments applied and the number of analyses performed. Quantitative bioanalysis is required to support preclinical and clinical drug testing and to provide pharmacokinetic and pharmacodynamic data. Automated unattended operation of the LC–MS instrumentation is required. Fast and routine LC–MS analysis also demands fast and automated sample pretreatment strategies and advanced data-processing software.

The keys to the success of LC–MS in quantitative bioanalysis are: (a) typical detection limits in the pg and in favorable cases even sub-pg range, (b) excellent selectivity against possibly interfering compounds in the biological matrix, especially when operated in SRM mode, (c) enhanced confidence of identity of the compound(s) analyzed, and (d) the ability to use the ideal internal standards: isotopically labelled compounds. LC–MS–MS is often as easy to operate as LC–UV–PDA, but provides better selectivity. As a result, LC–MS–MS has become the method-of-choice in quantitative bioanalysis within pharmaceutical industries. For proprietary reasons, unfortunately, there are not many reports on successful LC–MS applications in quantitative bioanalysis available in the public literature.

The higher selectivity achievable due to the use of SRM procedures is often immediately given away by decreasing the quality of the sample pretreatment and/or the chromatography. The rationale of this is an increase in the sample throughput. However, this often puts serious demands on the sample pretreatment methods. As an example, Knebel et al. [101] described the fast bioanalysis of saquinavir, a selective inhibitor of HIV proteinases, using an off-line solid-phase extraction (SPE) using a Gilson ASPEC followed by fast LC–APCI-MS–MS on 30×4.6 mm I.D. columns with a run time of only 1.5 min. In the overall sample throughput, the off-line sample pretreatment appeared to be the rate-limiting step. In the case of saquinavir, the sample throughput was ~100 samples per analyst per day.

This indicates the clear need for speeding up the sample pretreatment. One of most widely applied approaches is the use of parallel SPE procedures on short SPE columns or Empore disks, mounted in a 96-well plate format [102]. Allanson et al. [103], for instance, achieved a 4–7-fold improvement in sample throughput in the sample pretreatment by replacing SPE on individual samples by a robotic SPE sample pretreatment in 96-well plate format. Similar approaches were described by others [104,105]. While off-line sample pretreatment seems to be preferred by most researchers, on-line strategies, e.g., based on Prospekt SPE instrumentation [106], are described as well. An example is the rapid determination of pranlukast and metabolites in human plasma [107].

Serious matrix problems may be experienced in quantitative bioanalysis, especially in ESI. Signal suppression due to unknown matrix interferences is often observed. Changes in the sample pretreatment procedures may be successful in solving the problem, but in some cases changing-over to APCI, when applicable, appears to be the only feasible solution. A well-documented example of signal suppression was described by Matuszewski et al. [60].

6.3. Environmental applications

Environmental analysis is another important application area of LC–MS [108]. The strategies required are often different from those in pharmaceutical applications due to different aims of the analysis. In pharmaceutical applications, the analysis is directed to particularly one or only a few target compounds. SRM provides additional confirmation of identity

and selectivity. In environmental analysis, often a multiresidue screening is required. The analysis is directed at many different compounds from various compound classes. Restrictions to a more limited number of compounds or compound classes are made for practical reasons. For regulatory purposes, generally a broad screening is required.

A clear example of the dilemma in developing analytical strategies in environmental analysis is the screening of surface and ground waters prior to their use in the production of drinking water. According to European regulations, the determination of any individual pesticide at the level of 0.1 μg/l is demanded. Both quantitation and identification is required at this level. Detection of pesticides at these levels can only be achieved by the use of preconcentrating sample pretreatment, e.g., off-line or on-line liquid–liquid extraction or SPE, in combination with selective ion monitoring or SRM. Such a procedure is obviously not a general screening procedure. Increasing the sample volume often results in the preconcentration of not only the analytes of interest, but also of interferences. The latter hamper full-scan analysis. In addition, the various compound classes show quite different responses in ESI or APCI, e.g., some compounds should be analyzed in positive-ion mode, while others only provide sufficient response in negative-ion mode. For optimum SRM performance, optimization of the tuning parameters of ion source and collision cell is required, basically for each individual compound or compound class. The problems increase even further when not only pesticides but other environmental microcontaminants like surfactants, (azo) dyes, and perhaps even drug residues [109], have to be taken into account.

A typical approach in environmental analysis is an integrated system called SAMOS [110], which enables the automated, unattended analysis of filtered 100 ml surface water samples by means of on-line SPE on a short cartridge column, and subsequent gradient-HPLC analysis with UV–PDA detection. Compounds with sufficient UV activity can be quantified. In addition, using a UV spectral library, provisional identification of compound or compound class is possible, at least when the UV spectrum acquired is not too much distorted by the presence of humic acids. Any compound detected by a SAMOS system, applied in the field, must be identified by MS, i.e., often LC–MS. In this step, a more target-compound oriented approach can be applied because often the compound class of the unknown could be determined by UV–PDA. SAMOS-like systems can be on-line coupled to an LC–MS interface, as for instance demonstrated by the research groups of Brinkman [111,112] and Barceló [113,114]. In recent years, the volume to be applied in sample pretreatment could be reduced, e.g., from 100 down to 10 ml, as a result of the improved performance of the LC–MS instrumentation. Another approach is to use single short columns, applied for both preconcentrating sample pretreatment and minimum separation of the target compounds, as demonstrated by Hogenboom et al. [115]. The use of the so-called RFD mode, which enables CID of all ions produced in the ion source above a certain cut-off mass, on a TSQ-70 QqQ instrument [12] has been described by Kienhuis et al. [116]. Off-line SPE strategies combined with flow-injection analysis MS–MS have been described by Geerdink et al. [117].

Recently, the advantages of the enhanced full-scan sensitivity of an oa–TOF instrument were demonstrated in the quantitation and identification of pesticides in environmental surface water [118]. On-line SPE–LC–MS on the oa–TOF was performed to determine accurate masses of the pesticides. Calculated elemental compositions were searched against a pesticide database for identification.

Next to strategies directed at the analysis of pesticides and related compounds, the environmental analysis of various other contaminants has attracted significant attention as well, e.g., surfactants [119], organotin compounds [120], and dyes [121].

6.4. Biochemical and miscellaneous applications

ESI is frequently applied in the various stages of the characterization of peptides and proteins: molecular mass determination, amino acid sequencing, determination of nature and position of chemical and post-translational modifications of proteins, investigation in protein tertiary and quaternary conformation, and the study of noncovalent associates. In most cases, no on-line separation is applied, but the sample solution is introduced directly via the ESI or

nano-ESI interface. Impressive results have been achieved in this area [122–124].

On-line LC–MS for peptide and protein characterization has also been described, especially in relation to peptide sequencing and characterization of secondary protein structure, e.g., post-translational modifications. The determination of the *N*-glycosylation sites in recombinant Human Factor VIII protein by reversed-phase LC–ESI–MS [125] may serve as an example in this area. A microcapillary column-switching system to be applied in combination with LC–MS has been described by van der Heeft et al. [126] for the direct identification of peptides present in major histocompatibility complex class I molecules.

Similarly, the application of LC–MS for the characterization of oligosaccharides [127,128] and oligonucleotides [129,130] has also been described.

LC–MS is applied in many other fields as well, e.g., in the study of natural products [131–133], endogenous compounds like acylcarnitines and arachidonic acid metabolites [134], and DNA adducts [135,136]. LC–MS has become a routinely applicable technique. It is rapidly entering the chromatography laboratories to act as an LC detector in a variety of analyses. As such, LC–MS is appreciated for its sensitivity and selectivity, its specificity and the information obtained, e.g., on molecular mass of the analyte. The operation of an LC–MS system is no longer reserved to a MS specialist.

7. Discussion and conclusions

The previous sections indicate the huge progress that has been made in LC–MS. Fascinating applications are indicated, which clearly prove that LC–MS has developed into a mature technique. While it is often not difficult to achieve an appreciation of the benefits and advantages of LC–MS, the limitations of the current technology appear to be somewhat neglected. Clearly, current LC–MS instrumentation can solve problems that could not be solved (as efficiently) a couple of years ago. Nevertheless, LC–MS requires a different appreciation of MS as an analytical tool than does GC–MS. This is evaluated below in relation to peak finding and analyte identification.

Unlike in GC–MS, the analyte peaks have to be searched for against a relatively high background of solvent-related ion current in LC–MS. While a total-ion chromatogram (TIC) in GC–MS often reveals the presence of a number of compounds, even at low injected amounts, this is not necessarily true in LC–MS. As such, low-level multiresidue screening and/or the search for unknowns at low concentrations is a difficult topic in LC–MS. Due to the high background ion current, hardly any peaks show up in the TIC. By generating reconstructed mass chromatograms, these hidden peaks can be detected, but searching for peaks over a wide mass range might be a tedious and time-consuming procedure. The use of base-peak chromatograms can be helpful in this respect, but the implementation of the base-peak chromatograms in most commercial MS software packages is rather poor: in most cases the m/z range to be searched for base peaks cannot be specified. Even more powerful chemometric approaches might be available for peak recognition in a TIC obtained from LC–MS.

Conventionally, MS is considered as an important tool in the identification of (totally) unknown analytes. For GC–MS, clear successes in this respect have been achieved. The excessive fragmentation in electron ionization, eventually in combination with high-resolution MS, can be applied to identify an unknown. The additional use of computer library searching helps in identification because the acquired mass spectrum shows sufficient agreement with one of the library entries, or the entries found pinpoint towards specific structural characteristics in the unknown.

Identification by means of LC–MS technology of unknowns is more difficult. LC–MS is an excellent technique to confirm the identity of a target compound, even when the target compound is a member of a relatively large group of (related) compounds. Either in-source CID or preferentially product-ion MS–MS spectra can be applied for this purpose. Accurate mass determination by means of an oa-TOF–MS can be useful as well. However, the fragmentation of protonated molecules in low-energy CID often leads to only a limited number of fragments. This will often not allow unambiguous identification of the unknown, because the information in the MS–MS spectrum is insufficient. In addition, the

interpretation of the MS–MS spectrum is often hampered by the lack of insight in the fragmentation rules. While fragmentation of the odd-electron radical cation generated in electron ionization is well understood, this is not true for the fragmentation of the even-electron protonated molecule. The available knowledge is also not available in a systematic way.

Because of the influence of the experimental conditions on the appearance of the MS–MS or in-source CID spectra, it is not possible to produce MS–MS libraries, which are applicable for a variety of instruments from different manufacturers. This statement is based on the assumption that such a library should be searched by similar algorithms as used for the conventional electron ionization libraries, i.e., by taking into account both the m/z value and the relative intensity. Building a library where the m/z values of the fragment peaks is considered of prime important appears to be a potential alternative [137].

The softer and step-wise fragmentation achieved in CID in an ion trap often is a very helpful tool in structure elucidation. The high number of ion-trap systems currently installed perhaps enables the building of spectral libraries. However, such a library will only be applicable with ion traps, because MS–MS spectra from QqQ instrument in most cases are significantly different. The stepwise fragmentation might be applied to study fragmentation mechanisms of protonated molecules. However, the general applicability of this knowledge may be questionable because the apparent differences in the CID process. This is exemplified by the ability to fragment sodiated molecules in an ion trap, while this is generally not possible in a QqQ instrument.

The current interest in the use of oa-TOF, Q–TOF and FT–ICR–MS instruments in structure elucidation is mainly based on the assumption, that accurate mass determination of the (product) ions facilitates the interpretation of the fragments.

As indicated above, the identification of a total unknown is not required in most of the current applications of LC–MS. Generally, target-compound like strategies are applied. In these areas, the performance of current LC–MS instrumentation is generally satisfactory. Improvements in software facilitates the rapid optimization of experimental parameters, the automated unattended data acquisition as well as the data processing. Excellent integrated and user-friendly software tools are available for the evaluation of quantitative analyse by LC–MS. Dedicated software packages are available to operate LC–MS in specific applications, e.g., open-access, screening of combinatorial libraries, peptide sequencing via (Internet) database searching, neonatal screening of blood of newborn children for metabolic disorders, and MS-controlled fractionation in preparative LC. These tools assist in establishing LC–MS as a useful tool in the respective application areas, as they effectively give access to the parameters important to a specific application and guide through the complete analytical process.

These type of applications will dominate the use of LC–MS in the first decade on the next century. Powerful applications, e.g., quantitative bioanalysis, screening and confirmation of target compounds, and to a limited extent identification of partially unknowns, will continue to give an important impetus to the application and development of LC–MS. Although there are some indications of emerging new interface technology, e.g., sonic spray [138,139], laser spray [140], and continuous-flow and aerosol matrix-assisted laser desorption/ionization [141,142], ESI and APCI will continue to be the most important interfacing and ionization approaches. Improvements in the instrumentation and especially in the software are to be expected, in order to accommodate LC–MS to perform specific tasks within particular applications. And for the time to come, applications within the pharmaceutical industry continue to be the most important application area for LC–MS.

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