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Review

Impact of mass spectrometry on combinatorial chemistry

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Abstract

In the past few years, the emergence of combinatorial chemistry has drawn increasing attention and a great deal of analytical research has been centered around this new methodology. These new methods capable of producing vast numbers of samples, which are in many cases highly complex, demand fast and reliable analytical techniques able to provide high quality information concerning sample compositions. Mass spectrometry (MS) is the method of choice to face these analytical challenges. In particular, the introduction of electrospray ionization (ESI and matrix assisted laser desorption/ionization (MALDI)) have been the driving forces for many of the recent innovations, not only within the fields of the biosciences, but also in combinatorial chemistry. These ionization techniques are extremely versatile for the characterization of both single compound collections and compound mixture collections. The high-throughput capabilities, as well as many possible couplings with separation techniques (HPLC, CE) have been thus facilitated. However, mass spectrometry is not only limited to use as an instrument for synthesis control, but also plays an increasing role in the identification of active compounds from complex libraries. Recently, new initiatives for library analysis and screening have arisen from the application of the latest developments in mass spectrometry, Fourier transform ion cyclotron resonance (FTICR).

Keywords: Reviews; Mass spectrometry

Contents

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1. Introduction

There is an ever increasing demand for new drugs as pharmaceutical agents on the world market. This demand cannot be overestimated and to face this need, enormous efforts are being made in pharmaceutical research. The search for, and the subsequent optimization of lead structures, are the initial steps in the development of new therapeutic agents. The ensuing synthesis and analysis of structural analogues and the final screening in bioassays are still traditional steps. The rational design of drugs, via computer-assisted molecular modeling, despite enormous progress in the computational sector, may not have fulfilled the expectations given to this method.

However, a totally different and randomized approach is offered by combinatorial chemistry. The driving force behind the rapid development of combinatorial chemistry came initially from solid-phase peptide and oligonucleotide synthesis. Initially, with the introduction of this new methodology, large combinatorial libraries of peptides and oligonucleotides were synthesized and screened. Due to poor bioavailability of many biopolymers, interest in synthesis has shifted rapidly towards peptidomimetics and small organic molecules ($<$700 Da) which are the principal target molecules. The combinatorial synthesis can be performed in solution or on solid-support, whereas the solid-phase synthesis is of predominant use. The large number of scientific contributions being reviewed reflects the attention that is paid to the field of combinatorial chemistry [1–7].

The enormous acceleration in synthesis using the various combinatorial approaches has led to the production of vast sample amounts and has required a major change in viewpoint. Formerly, the rate-limiting step in medicinal chemistry was the synthesis and this has now become the pacemaker, requiring a concommitant adjustment in the various analytical and screening methods. Shortly after the emergence of combinatorial chemistry, analysts were forced to re-evaluate the versatility of analytical methods for the combinatorial approach e.g. IR, NMR, MS and these have been reviewed in several contributions [8–10]. From the diverse analytical techniques at our disposal, mass spectrometry seems to be the most versatile tool to cope with the analytical demands of combinatorial chemistry.

Shortly before combinatorial chemistry was developed, the electrospray ionization (ESI) [11,12] and matrix assisted laser desorption/ionization (MALDI) [13,14] techniques for MS emerged as a result of which ESI-quadrupole and/or MALDI-TOF (time-of-flight) instruments can now be found in many analytical laboratories. For this reason, unless otherwise specified, in the following report reference to ESI or MALDI implies in a quadrupole or TOF configuration, respectively. A major breakthrough was achieved with these techniques, which revolutionized peptide, oligonucleotide and protein analysis. Some years later the FTICR-analyzer technique [15] is set to provide another quantum step in mass spectrometric analysis. With these techniques a new age in mass spectrometric analysis began, particularly with respect to gentle ionization of sensitive samples, sensitivity, resolution, high-throughput and ease of handling.

As combinatorial chemistry emerged, these methods were ready to meet these new demands. As mentioned above, the major focus of combinatorial synthesis are small compounds which are mainly polar. The suitability of ESI and MALDI, the preferred techniques amongst the various ionization methods for the analysis of combinatorial compounds is shown schematically in Fig. 1. In this article we want to indicate how mass spectrometric analysis can solve some of the problems posed by the rapid advancements in combinatorial synthesis.

The two main approaches of combinatorial synthesis are the parallel synthesis of single compounds and the synthesis of mixtures or so-called libraries, normally containing anywhere between fifteen to several million compounds. The preference of one approach over the other however, depends on the

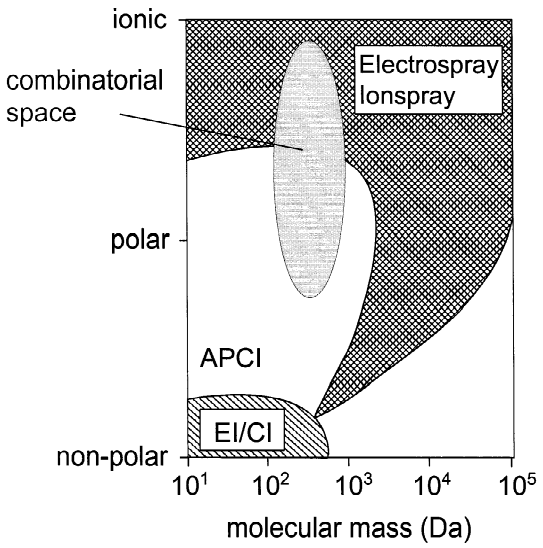

Fig. 1. Schematic diagram of the range of application of mass spectrometric ionization methods for combinatorial chemistry with regard to molecular mass and molecule properties. The circle encloses the area of combinatorial space comprising small oligomeric and non-oligomeric molecules.

various screening approaches and, in particular, on the different targets. It is quite obvious that the library size plays an important role for the subsequent mass spectrometric analysis of the compounds.

The initial step of combinatorial synthesis is to establish the synthesis protocol of a particular scaffold molecule as shown in the flow sheet in Fig. 2.

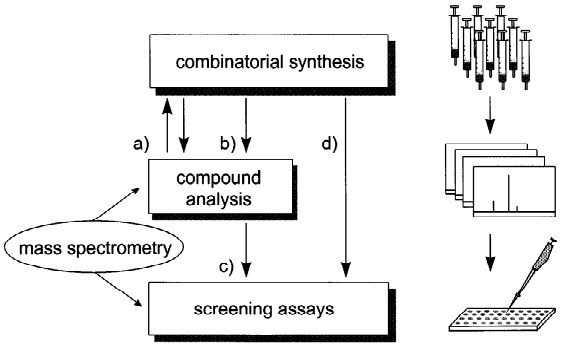

Fig. 2. Impact of mass spectrometry on combinatorial chemistry. Synthesis control of (a) model compounds (validation stage). (b) High-throughput analysis of compound collections in single compound or library format. Mass spectrometric compound screening with (c) or without (d) previous sample characterization.

Such a feasibility study is firstly established in several cycles to optimize reaction conditions and applicability with some model compounds. After this procedure has been successfully completed, either the single compound or library synthesis approach is adopted, in order to produce large numbers of differently substituted compounds. During both of the phases, mass spectrometry is able to provide high quality information for both synthesis validation and routine control of large numbers of samples. Techniques which enable the coupling of mass spectrometry with HPLC and CE provide an additional dimension in the characterization of compound collections or libraries. An exact compound and library analysis enables the reliable gathering of results in the following library screening using different bioassays. It is of particular importance to not only characterize the desired products, but also to identify by-products, which may eventually exhibit biological activity. Moreover, in the last few years additional methods using mass spectrometry as a screening method to identify active compounds directly from organic compound mixtures have become available. Using this approach, the library compounds can either be tested after cleavage in standard bioassay format or on-bead. In the following review the suitability of mass spectrometry in meeting the demands for the analysis of combinatorial compound collections and recent developments in these rapidly growing fields are discussed.

2. Small oligomeric compounds and libraries

Some of the first contributions to the literature concerned the analysis of peptides using ESI mass spectrometry are related to peptide libraries. Thus, these peptide libraries served as the first representative model systems for mass spectrometric analysis of compound collections. However, libraries consisting of oligomeric structures such as peptides are somewhat limited in structural diversity due to repetitive binding of similar building blocks. We will not discuss any further, the routine characterization of single peptides or oligonucleotides, which is a less specific problem for the analysis of combinatorially generated compounds and therefore an item of more common mass spectrometric analysis.

2.1. Peptides

In 1993 Metzger et al. [16,17] presented the first mass spectrometric investigations of combinatorial libraries using, as an example, synthetic peptides. In that report several 48-component mixtures and a nonapeptide library theoretically containing 24 576 components were investigated with ESI-MS as a means of synthesis quality control. In addition, the HPLC-MS coupling technique was successfully introduced in order to distinguish between isobaric peptides and the use of a computer program for the calculation of theoretical mass spectra was also discussed. As the line shape of the ESI mass spectrum of the library in Fig. 3 shows, the density of the compounds exhibiting the same nominal molecular mass does not allow any resolution of distinct molecular masses. However, these programs for the calculation of the theoretical mass distribution provide qualitative information about the library synthesis (fingerprint spectrum) [18].

In a subsequent report [19] these ESI investigations were extended in a full analysis of a 48-compound library using tandem MS experiments. Here it was elegantly shown that daughter ion-, parent ion- and neutral loss experiments are useful tools not only for the identification of library compounds, but also for the detection of by-products and side reactions. Moreover, it was demonstrated that for structurally similar peptides, signal intensities reflect the relative concentrations present in a mixture, thus allowing semiquantitative estimations of sample composition. Deviations from this observation became apparent for by-products and for libraries present in different charge states as influenced by the choice of ionization conditions.

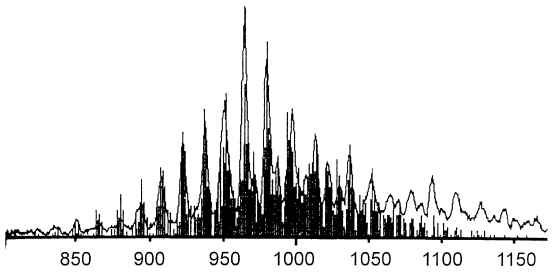

Fig. 3. Experimental and calculated (vertical bars) mass spectrum of the octapeptide library SXXXFEKL containing theoretically $19^3=6859$ components. (Reprinted with permission from Ref. [18]. ©1997 Springer-Verlag.)

The evaluation of libraries by LC–ESI-MS using selected-ion monitoring (SIM) has also been shown to be an efficient method for a more detailed analysis of larger libraries [20], i.e. libraries containing a large number of constituents. Thus, out of a mixture of about 10^5 peptides, the presence of a peptide with the highest possible molecular mass as well as randomly chosen peptides was verified. In particular, the peptides with the highest and lowest molecular masses in a library are normally present in low amounts, and are normally lost within the background noise. However, by this procedure the quality of a library can be tested more precisely using sample surveys.

A more general contribution o peptide library analysis from Boutin et al. [21] deals with FAB-MS, NMR and HPCE. Although suppression effects [22] are major disadvantages of the older FAB-ionization technique [23], similar results can be obtained in comparison with ES-ionization.

Initial attempts to address the problems of MS analysis of combinatorial libraries with the TOF-technique were also carried out using secondary ion mass spectrometry (SIMS) [24]. The potential sensitivity of the technique, which is in the low femtomole range, was demonstrated after vapor phase clipping of a tripeptide from a single bead with trifluoroacetic acid. Fragmentation allowed sequence assignment of the peptide. The vapor phase clipping is a commonly established method for single bead MALDI analyses. Resin beads are prepared for mass spectrometric investigation by exposure to trifluoroacetic acid vapor and the subsequent addition of matrix for cocrystallization on the MALDI target carrier.

The versatility of MALDI for single bead analysis by direct monitoring of peptides (~1000 Da) was demonstrated by Egner et al. with a linear TOF analyzer [25]. Moreover, different linkers and matrices were tested for this application [26]. Single bead structure determination of a covalently bound peptoid was performed by Zambias et al. with

MALDI-TOF using a reflectron device, thus providing an improved resolution [27]. In addition, a direct readout of structural information from fragment ions was obtained with post source decay (PSD).

In contrast, Geysen and co-workers developed photosensitive linkers as an alternative to the cleavage of the compound from the bead with trifluoroacetic acid [28]. After UV-irradiation of resin beads in a methanol/water mixture, samples are directed to subsequent ESI-MS measurement. A further minimization in sample handling results from this technique, particularly for MALDI analysis. After transfer of an untreated single bead into the ionization device, only a single laser pulse is required for the desorption and ionization of a resin-bound peptide [29].

The utility of FTICR mass spectrometry for the exact mass determination and characterization of a library has already been shown at an early stage of the development of this technique using ES-ionization. High resolution measurements enabled the assignment of molecules with isobaric nominal masses and by-product identification of a 19 component peptide library [30]. At the same time Nawrocki et al. [31] clearly demonstrated the assessment of diversity and degeneracy with this technique. As test libraries, a tripeptide and two hexapeptide libraries were investigated. The extremely high resolving power was demonstrated for the example of the tripeptide library ($>$350 compounds) and, for an expanded region of a mass spectrum, closely spaced signals could be unambigously assigned to the corresponding synthetic peptides. In the case of mass redundancies (isobaric amino acids or inverse peptide sequence) peak areas of the signals seemed to be proportional to the degeneracy of the peptides.

With respect to qualitative aspects, library synthesis employing solid supports is dependent on the synthetic approach utilized. The split–mix method is based on the partitioning of the resin, the subsequent reaction in different reaction vessels and a remixing of the resin, whereas in the premix-method the educt components are premixed and then directly submitted to react competitively with the resin. As expected, the comparison of the split–mix method versus the premix-method in peptide synthesis reveals more reliable results for the split–mix method [32]. However, the split–mix method forces inherent restrictions on the complexity of the library [33,34]. Evident problems in MS analysis arise for larger randomized peptide libraries containing more than 100 compounds. When considering difficult sequences such as hexaglycine or hexaalanine, it may be questionable if these peptides are really present in a hexapeptide library synthesized by standard synthesis protocol. The same problems arise for a number of similar peptides e.g. AGGGGG, GAGGGG, etc. With respect to the limited time which can be invested in order to characterize a large library, the low resolving analyzer techniques in most cases only provide rough, fingerprinting-like qualitative information. For time reasons, an accurate library analysis e.g. with selected-ion monitoring can only be used for the characterization of some more or less arbitrarily chosen peptides.

2.2. Oligonucleotides

Unlike peptide libraries, the literature on mass spectrometric analysis of combinatorially synthesized oligonucleotides is limited. Attempts to characterize oligonucleotide libraries containing 9–27 compounds with ESI-MS were presented by Pommerantz et al. [35]. Direct analysis of oligonucleotides is similar to peptides but they are recorded in the negative ion mode due to the negatively charged nature of the phosphate backbone. However, mostly multiple charge states are obtained, which have to be transformed to a molecular mass scale for a more transparent overview. CID-experiments (collision-induced dissociation) of the multiple charged signals in negative ion mode were employed to gain structural information from the fragment ions. Although these experiments were relatively successful even for isobaric oligonucleotides, the complexity of spectra is extremely high and increases with the number of sequence isomers of the same molecular mass. The verification of oligonucleotide structure exploiting failure synthesis products [36] may be an alternative which could be transferred to combinatorial synthesis of oligonucleotides and their modified derivatives using MALDI.

3. Small non-oligomeric compounds and libraries

It has to be taken into account, that oligomeric compounds include only a part of the whole diversity space within combinatorial chemistry. The limited number of coupling procedures and the resulting compounds also restrict the problem area for mass spectrometric analysis. For non-oligomeric structures, the number of usable reagents and reaction types generates a much greater variety of core or scaffold structures and requires more flexible analytical methods. Thus, the applicability of mass spectrometry for an extended analysis of a greater variety of compounds has to be demonstrated.

3.1. Direct analysis of non-oligomeric compound libraries

The direct ESI-MS characterization of crude combinatorial libraries for a further screening was investigated by Carell et al. [37]. The identification success-rate of a 55 compound library was about 93%. Although the solution synthesis of the substituted xanthene derivatives is closely related to peptide chemistry, this article clearly addresses some additional requirements for small compound analysis in comparison to mass spectrometric characterization of peptides.

The same core molecule was used for more extensive investigations in a following contribution also using ES ionization [38]. Since direct mixture analysis of 10^4–10^5 compounds is not a realistic enterprise to ensure the presence of all library compounds by MS analysis, an alternative was applied using the full and accurate analysis of small representative sublibraries (containing approximately 50 compounds). From the analysis of these sublibraries, conclusions can be drawn which aid the development of improved synthetic procedures and finally the estimation of the complexity and composition of the original library. The use of positive and negative ion scan mode as well as tandem experiments was emphasized and provides an almost complete characterization of a library. As has been demonstrated in several examples, it must be emphasized that the negative ion mode is equally important for compound and library characterization. This fact is often overlooked and not always been considered during peptide analysis.

The single bead analysis approach with MALDI has also been transferred to small compound libraries (200–600 Da) and also uses the vapor-phase clipping technique [39]. This report again shows the high sensitivity of MALDI, but clearness was hampered by the presence of unknown structures in the systems investigated. Although the linear TOF analyzer is not well suited for low mass ranges, because of inherent problems with resolving power and disturbing matrix effects, the contribution demonstrates the possibility of a general use of MALDI for the analysis of combinatorial compounds. The development of reflectron devices [40] and delayed extraction (DE) [41] makes MALDI's use for small compound analysis much more attractive.

As a consequence of the greater structural variety of small organic compound libraries, an appropriate ionization of the molecules is not necessarily apparent. One elegant strategy to enhance ionization of poorly- or non-ionizable molecules is the introduction of ionization tags [42]. A photocleavable linker enables direct monitoring detecting the tagged molecules with MALDI. In addition the tag provides a mass shift which overcomes signal overlap with matrix molecules. Due to different cleavage conditions of the tagged and the untagged molecules, there is no interference of the tagging linker with the bioassay. This approach also seems to be applicable with ESI-MS.

A direct comparison of ESI, MALDI and SIMS-TOF which demonstrates the use of all three methods for single bead analysis was performed by Brummel et al. [43]. As a model compound a resin bound angiotensin II antagonist was chosen, which was synthesized via a Knoevenagel condensation. For all three methods fragments were consulted to unequivocally resolve possible structural unambiguities. Furthermore, it was shown that all three mass spectrometric techniques possess appropriate sensitivity for combinatorially synthesized compounds with respect to a single bead (40-μm in diameter) which contained about 50–200 pmol of compound depending on the resin-loading. As emphasized the particular power of ESI provides the potential for high-throughput analysis by automated flow injection. In contrast, TOF-SIMS, due to its spatial

resolution, can resolve the components of one bead from other library members in a mixture of beads.

Likewise, FTICR using ES-ionization has been investigated for the characterization of small compound libraries using the example of the above-mentioned xanthene library [44]. Currently, work is underway to study the feasability of high accuracy measurements with FTICR-MS to determine the elemental composition of a compound, which provides further valuable information for compound characterization. However, as discussed by Beimann [45], a severe problem which still needs to be overcome is that the required mass accuracy to define an elemental composition increases exponentially with the mass of the analyte. As an example, even for a mass accuracy of about 1 ppm, dependent on the technical device, there are still 16–19 elemental compositions possible for masses between 500 and 600 Da, respectively, assuming that only the elements C, H, O, N and S are involved in this calculation. A solution may be the so-called multistage accurate mass spectrometry (MAMS) technique using four stages of MS^n experiments to obtain the unique elemental composition [46]. Thus, the achievable accuracy is sufficient to determine the elemental composition of the fragments with lower molecular mass. In a reconstructive process starting with fragments from the MS^5 to MS^2 experiments the elemental composition is calculated. This procedure was successfully demonstrated for a compound with a molecular mass of 517 Da within a library containing 25 650 compounds.

3.2. Coupling of separation techniques with mass spectrometry

An additional dimension to library analysis is introduced through coupling with common separation techniques, for example: LC and CE. However, these couplings are only suited for spray ionization techniques such as ESI or atmospheric pressure chemical ionization (APCI) and more or less exclude the use of the MALDI technique.

An accurate characterization of a sample combinatorial library was given by Haap et al. [47]. Two arylether libraries (10 and 45 compounds) were investigated using LC–ESI-MS and related tandem experiments. Additionally, UV-detection was employed using a single detection wavelength. A comparison of both MS- and UV-detection was found to give different information about the library composition, i.e. some library compounds evaded the detection in one or the other detection mode. This example clearly showed that the exclusive use of one detection method may not be sufficient for a reliable library characterization. Another result of these library investigations is the occurrence of fragments for small molecules even under the gentle electrospray ionization conditions. As further shown, the 2D-plot presentation (full presentation of all occurring masses versus the time axis) of HPLC-MS analysis facilitates the library evaluation and offers a complete overview over all occurring mass signals. Due to an increasing complexity of the chromatograms complicating the evaluation process, even the use of LC–MS showed significant limitations for libraries containing more than 50 compounds.

The alternative resolving capacity of CE offers a meaningful alternative to LC for the characterization of larger libraries. A disubstituted xanthene library containing 171 compounds was used to demonstrate the power of CE–ESI-MS-coupling [48]. As shown in earlier contributions [37,38], this approach to investigate representative sublibraries led to the identification of 160 compounds (>90% of all library compounds). With respect to 124 derivatives with an overlapping molecular mass, only eight additional analyses using MS/MS experiments were necessary to identify the remaining molecules.

Less commonly used analytical techniques can also be successfully used for the analysis of libraries generated by combinatorial chemistry. This was shown by the group of Kurth [49,50] who utilized GC–MS to study three compound sublibraries of β-mercaptoketones detected as the corresponding formate esters. As demonstrated, the high resolving power of gas chromatography and a high content of structural information due to molecule fragments resulting from EI/CI-ionization are major advantages of this method. Comparable to the work of Ohlmeyer et al. [51] using GC as a decoding tool in combinatorial synthesis, sensitivity of this method has been shown to be suitable for single bead analysis. So far, in library analysis the GC–MS technique never achieved the wide-spread importance of LC-ESI techniques. One reason may be that chemists

coming from peptide chemistry and now working in the field of combinatorial chemistry are more familiar with ESI and MALDI. Another reason however may be that the majority of the molecules prepared in combinatorial chemistry exhibit polar properties e.g. heterocycles, whereas the application range of GC is restricted to volatile compounds of limited molecular mass. Fig. 4 shows a direct comparison of the LC–ESI-MS and GC–EI-MS analyses of a pyrrol library synthesized on Rink-Amide-resin [52]. Using LC-MS all 17 compounds could be detected, whereas with GC–MS only 15 compounds have been identified. The different signal intensities of the reconstructed ion chromatograms indicate the different ionization yields of the compounds. However, in combinatorial solid-phase syntheses the most commonly used resin types are e.g. Wang- or trityl-resins to which carboxy functionalities are attached. This limits the applicability of direct GC-analysis and necessitates prior derivatization. Nevertheless, as the use of GC–MS for herbicide research [53] and others

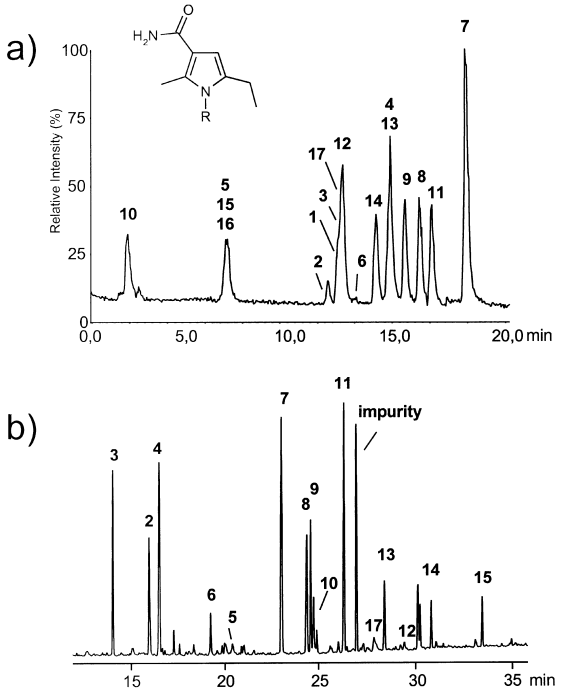

Fig. 4. Library analysis of a combinatorial pyrrol-library [52] containing 17 compounds in the mass range of 152–325 Da (*R*=variable substituent). Chromatographic traces of: (a) HPLC–ESI-MS analysis and (b) GC–EI-MS analysis.

[54] suggests that further advancements in library characterization may be expected to result from this technique in the future.

The occurrence of impurities after cleavage from polystyrene-based polymeric carriers in solid-phase synthesis hamper routine product analysis and may have considerable influence on the screening assay [55]. Particularly, in mass spectrometric analysis, these impurities may compete with poorly ionizable products and drastically disturb a proper compound analysis. Most of these impurities are so far not characterized, however they presumably arise from various linker and resin components. Moreover, the impurity profile is not consistent and depends on the commercial supplier and the charge. In any case it is advisable to carry out blank syntheses followed by careful analysis of the resulting background, which may accompany a library production. The observation of such effects was made in several groups and should not be underestimated in compound analysis.

The continued development of combinatorial synthesis will lead to the synthesis of ever more complex lead structures. This includes the synthesis of stereoisomers. The LC–ESI-MS analysis of an isoxazolidine library [56] emphasizes some upcoming problems. Taking the example of the isoxazolidines, the library size increases by a factor of four due to the formation of endo/exo-isomers and stereoisomers. The endo/exo-isomers could be separated in a LC–MS experiment, whilst a differentiation of enantiomers was enabled after chemical conversion to the corresponding diastereomers with a chiral derivatization agent. On the other hand, the chiral chromatographical techniques as well as chiral CE may offer significant improvements for the separation of enantiomeric combinatorial compounds. Presumably, the combinatorial synthesis of the next compound generations will shift to the synthesis of more complex compounds including those formed during enantioselective reactions.

4. Spectra prediction programs

Computer programs have also been developed to compare mass spectra with theoretical mass distribution in order to validate a successful library synthesis [18]. Such programs for peptide libraries

implement the calculation of mass distribution on the basis of both proteinogenic and user-defined unusual amino acids. Moreover, specific protecting groups and known side reactions can be included in the calculation process. The degree of by-product formation in a theoretical spectrum can be simulated by the operator to approximate the observed mass distribution. The theoretical mass spectra are presented as line spectra ranging in applicability for a library size from as few as 19 compounds to fully randomized pentadecapeptide libraries.

A program with extended capabilities for mass spectra prediction was developed by Steinbeck et al. [57] for peptides and small compound libraries of moderate size. The program, tailored for use with MALDI spectra, allows the design of libraries from user-defined building blocks in order to generate minimal peak overlap and thus avoids ambiguities in mass spectrometric assignment. Diversity parameters, e.g. size and hydrophobicity, can also be chosen. A routine generates several libraries and their corresponding mass spectra which can be selected by the operator for the most favorable building block composition. In several reports similar types of prediction programs are often employed for a comparison with the observed mass spectra. Considerable agreement between the calculated and measured mass spectra can however be achieved with permanently charged molecules, as has been shown for a tertiary amine library [58]. The high ionization efficiencies for all components of such a library result in a minimum of signal supression effects. Moreover, in a more detailed contribution theoretical considerations have been made to probe combinatorial library diversity using peptide libraries as a model [59].

5. Compound screening with mass spectrometry

The applicability of mass spectrometry to the analysis of combinatorial compound collections is not only limited to the bare analysis of synthetic products as a means of quality control, but also provides a valuable tool for the screening and the identification of active compounds. The most significant approaches are the on-bead high-throughput screening (HTS) of compounds as well as solution based assays. During on-bead screening, active compounds are identified by several bead encoding strategies [60,61]. For the identification of HTS hits from single beads, mass spectrometric techniques are of particular value as described in the following section.

5.1. Single bead characterization techniques

Quite a number of tagging strategies are based on the synthesis of encoding molecules, e.g. peptides [62], oligonucleotides [63] in parallel to the target molecules using sequencing techniques for bead decoding. Inert haloarene tags can be identified with high sensitivity ($<$0.1 pmol) using electron-capture gas chromatography detection [51]. Other approaches include radiofrequency tags or the use of dyes. However, in addition to the increased time and cost for a particular synthesis, the tagging molecules themselves may interfere with the screening results. An alternative is the direct identification and characterization e.g. of peptides from positively tested beads with MALDI, as has been demonstrated by Youngquist et al. [64] using the termination synthesis approach. Detailed evaluation of acetylated and non-acetylated pentapeptide libraries and sublibraries has also been carried out using this approach [65]. The termination synthesis method (admixing of small amounts of capping reagent during the coupling steps) enabled the direct sequence determination of a selected peptide after cleavage from a bead. Sensitivity is excellent (5% of 5 pmol per bead), although resolution due to the stage of technical development of the linear MALDI-TOF was restricted. Mass spectrometric investigations showed that a high percentage of sequences can be properly identified in a direct way. Unusual or isobaric amino acids are distinguished using different capping reagents during the synthesis steps. The usefulness of this approach was demonstrated for the identification of binding motifs of peptides to streptavidin and an HIV-neutralizing antibody. The same method has also been used for the screening of peptides for the Lyme disease-related antibody H9724 [66,67].

Another approach, similar to that of Youngquist deals with methionine scanning [68]. During peptide synthesis small amounts of methionine are included at every position of the peptide sequence. For bead

characterization CNBr-cleavage at the methionine is performed and different chain lengths are obtained. Thus, resulting mass differences in the subsequent mixture analysis with ESI or MALDI-MS reflect the peptide sequence. The same principle is performed for peptides with thiolester building blocks which are cleavable with hydroxylamine [69]. Generally these self-encoding techniques are applicable to any other oligomer synthesis such as oligonucleotides.

5.2. Affinity selection and screening mass spectrometry

The use of affinity selection mass spectrometry as a second application for the identification of active compounds with MS is becoming more and more important. The proponents of solution based assays criticize altered screening conditions as a major argument against on-bead screening. Initial attempts to identify affinity ligands with mass spectrometry were presented by Zuckermann and co-workers with antibodies [70]. After gel-filtration of the assay mixture, the antibody-peptide complexes were dissociated, HPLC-purified and analyzed off-line by mass spectrometry. Other contributions deal with biosensor-monitored binding of peptides to immobilized antibodies, a subsequent release and mass spectrometric identification [71]. For all these examples ESI-MS has been predominantly used. ESI-MS has been used as well to probe novel enzymatic reactions such as oxidative decarboxylation [72] and protein kinase substrate specifity of synthetic peptide libraries [73]. In the latter report the exploitation of diagnostic ions with negative on-stepped collision mass spectrometry led to the detection of phosphorylated peptides. However, as the use of the peptide antibiotic vancomycin as a model receptor shows not only proteins may be involved in the screening of combinatorial peptide libraries [74]. In order to compare the affinity of dipeptides for vancomycin, a library was used to examine the binding properties of specific dipeptide sequences (Fig. 5). Clear effects are observed in CE with vancomycin, resulting in a retarded migration of the binding peptides in comparison to non-binding peptides. In a subsequent contribution, the separation and identification of vancomycin-binding peptides

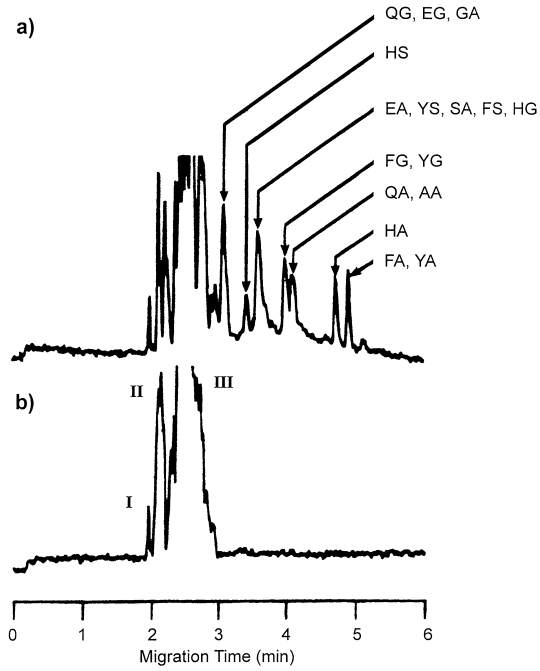

Fig. 5. UV trace of an ACE analysis of a Fmoc-DDXX library (100 peptides). (a) with vancomycin (b) without vancomycin. Compounds are identified with ESI mass spectrometry. (Reprinted with permission from Ref. [75]. ©1996 American Chemical Society.)

from mixtures containing up to 351 compounds using CE–ESI-MS analysis was reported [75].

Different concepts for the recovery and the analysis with mass spectrometry of active compounds have been presented in literature. One strategy is the on-column immobilization of the receptor: the immobilized receptor is incubated with the library, washed and, bound ligands are released. Their identity can be determined by mass spectrometric analysis [76]. Twenty benzodiazepines have been likewise tested for binding to an on-column immobilized antibody. A column switch protocol for the whole assay procedure reduced sample handling to a minimum. The protocol includes the release of bound ligands, loading and separation on a RP-column and the on-line detection and characterization of the ligands with ESI-MS and ESI-MS-MS. The system was found to be highly flexible with

respect to the transfer to other columns and assay systems indicating a potential for a fully automated screening assay.

The on-column immobilization of the receptor protein has been further used to determine binding ligand-motifs extracted from a 361 component peptide library [77]. In this study peptides were released from the column-bound phosphatidylinositol-3-kinase domain with pH gradient elution. Structures were elucidated using direct ESI-MS and ESI-MS-MS characterization without previous RP-column separation.

However as with resin-bound compounds, the immobilization of the receptor or the ligand may change the affinity characteristics and result in misleading screening results. One alternative is to employ solution-based assays using size exclusion chromatography or ultrafiltration membranes for the recovery of the receptor–ligand complexes. Library screening is performed by purifying the ligand–protein complex over a size exclusion column and subsequent dissociation. The analysis with mass spectrometry has been shown to be applicable for large libraries, in particular the discovery of a single lead compound from a 600 compound library (Fig. 6) [78]. Carried out in parallel, the binding experiments were performed with and without the presence of a known competitor to monitor binding efficiency. After lead identification, a structure refinement of the library was performed. The following assay of the sublibrary containing 100 compounds led to one compound with better binding properties than the identified lead compound.

Gel-filtration has been used for the recovery of the protein–ligand complex of a composed drug library with the subsequent compound identification involving both CE and LC–ESI-MS [79]. However, special care was required during the purification process, because a substantial loss of protein was observed after gel-filtration.

The approach to use pulsed ultrafiltration mass spectrometry, introduced by van Breemen et al. [80], appears to be highly promising. The principle of this method was clearly shown with the example of adenosine desaminase screening, probing a 20 compound library of different adenosine analogues at the same time. After incubation of the enzyme with the library, the mixture was injected into an ultrafiltration chamber. Non-binding library components were rinsed out, while the ligand–receptor complex was held back by the ultrafiltration membrane. A change of the elution conditions caused a release of ligands

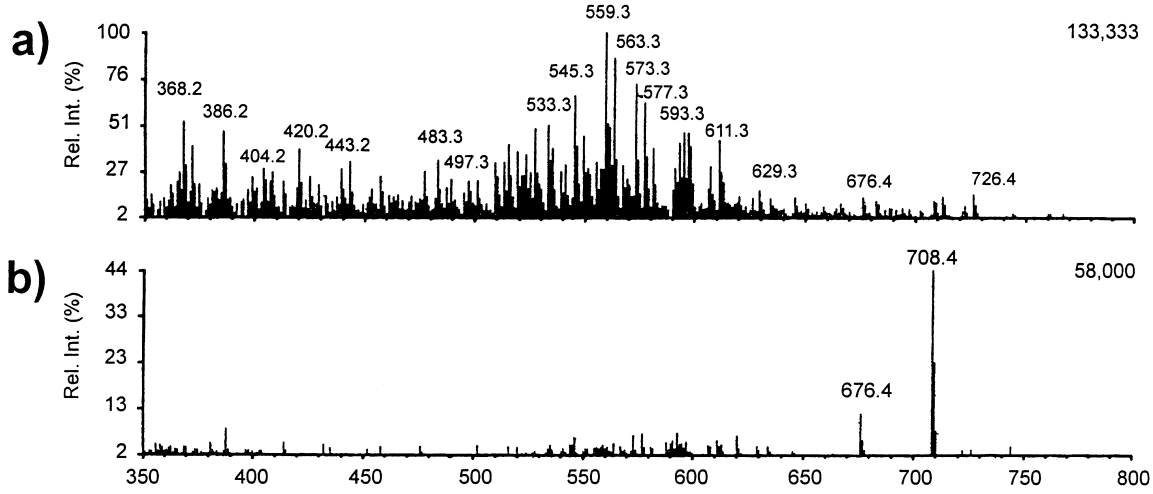

Fig. 6. Electrospray mass spectra showing (a) combinatorial library containing approximately 600 compounds, (b) compounds identified after affinity selection. (Reprinted with permission from Ref. [78]. ©1997 Plenum Publishing Corp.)

and subsequent detection with mass spectrometry was enabled. However, shorter release times would be necessary for this method applicable to a high-throughput screening.

In the example of the Caco-2 model, six arbitrary diverse compound libraries were screened for intestinal absorption properties by Taylor et al. [81]. The membrane permeable peptoids (24 compounds/library) were detected using LC–ESI-MS in order to study the absorption velocity of potential orally-applicable drugs.

Exploring automated LC–ESI-MS couplings, two minute sample interval screening of single glycosyltransferase inhibitors has been performed [82]. In contrast to the above-mentioned method of van Breemen, the detection of an inhibitor is based on the lack of the respective inhibitor signal in the total ion current whereby the method exploits the comparatively long release times of the ligand–receptor complexes (Fig. 7). In the experiment, the incubated ligand–receptor mixture is quenched with methanol. Although the addition of methanol changed the solution conditions, the release of bound inhibitor was found to be slow. Thus only weak or non-binding compounds in solution are detected with mass spectrometry. Employing different inhibitor concentrations, IC_{50} values were determined on the basis of the ion intensities. This was also adapted to the assay of small libraries and provided comparable results.

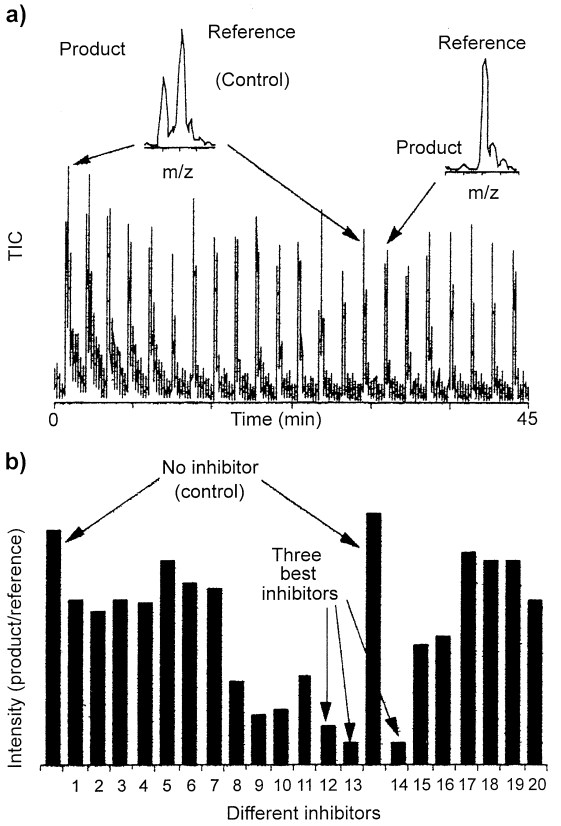

Fig. 7. High-throughput screening with mass spectrometry. (a) Total ion chromatogram of 22 reactions of different inhibitors measured at two minute intervals. (b) Bargraph of the ratio of the intensities of the product and reference ions. (Reprinted with permission from Ref. [82]. ©1997 Current Biology.)

The literature reviewed above shows that ESI in combination with a quadrupole analyzer is the dominant technique for the mass spectrometric assay of substrate libraries. However, MALDI can also give valuable information for the monitoring of selection experiments. Delayed extraction technique and the use of a reflectron improve the reliability of the mass spectrometric results. The contribution of Berlin et al. [83] deals with the applicability of MALDI in probing compound libraries (containing between 2 and 35 compounds) in three different assay systems. Quantitative results could also be obtained when, in the experiments, calibration curves were employed.

Attempts to evaluate the use of FTICR with ESI for library screening are already at a preliminary stage. A model experiment for screening of a peptide substrate library for glutathione transferase specificity was reported, although high-resolution features were not fully explored [84]. Using the trapping and gentle desolvation possibilities of the ICR-cell, protein–ligand complexes could be investigated in order to characterize competitive binding of libraries [85]. As a model protein carbonic anhydrase (CA-II) was probed using carboxybenzenesulfonamide peptides. The trapped protein–ligand complexes were dissociated with collision gas and the observed ion intensities of the inhibitors in the low mass range were correlated to the relative binding affinities (Fig. 8). The high-resolution capability was brought into play to distinguish inhibitors having similar molecular masses. The work was subsequently extended from 18 to about 300 inhibitor compound libraries

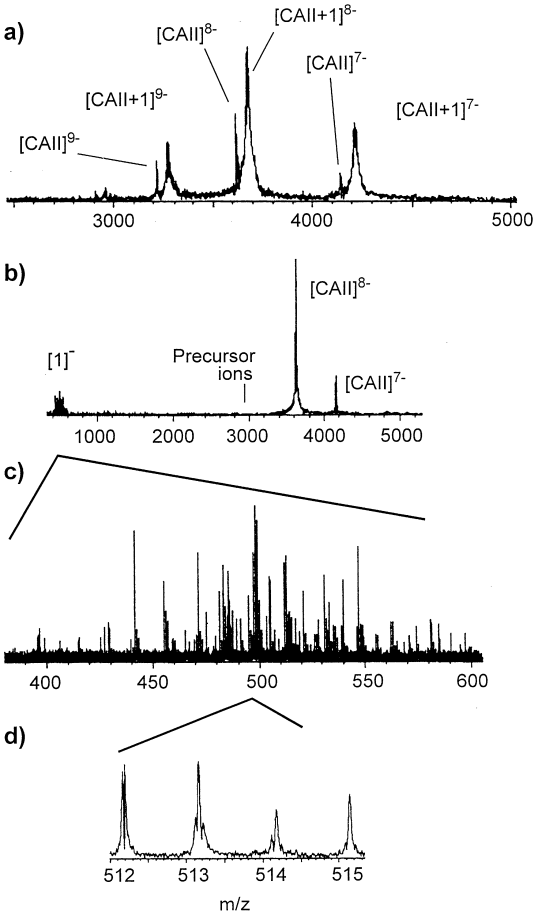

Fig. 8. Bioaffinity characterization mass spectrometry with ESI-FTICR. (a) Mass spectrum of the protein–ligand complexes of a 289-component library and the protein CAII. (b) Complete dissociation of the isolated complex ions of $[CAII+1]^{9-}$. (c) and (d) Expanded views of the singly charged inhibitors $[1]^{1-}$. (Reproduced by permission of The Royal Society of Chemistry from Ref. [87]. ©1997.)

comparing the binding affinities of L- and D-amino acids [86].

The FTICR-technique seems to be a reliable method for the observation of such interactions, because of slow gentle desolvation in comparison to conventional analyzer instrumentation. However, as has also been discussed [87], several assumptions must be made (e.g. transferance of solution-phase stabilities to the gas phase) and these have to be critically considered before widening the application range.

6. High-throughput analysis

For the full integration and wide ranging application, particularly in the pharmaceutical industry, any MS technique must also be capable of adaptation to fully automated high-throughput methods. The current trend toward characterization of vast sample numbers has to be considered as a major task for the future analysis with mass spectrometry. Luckily, ESI, APCI and MALDI analyses are generally well suited to addressing this high sample throughput problem.

In regard to this high-throughput requirement, a useful contribution was presented by Hegy et al. [88] using ESI and APCI interfacing techniques applicable in industrial laboratories. An immediate data exchange between the laboratories was achieved using local networks. The concept includes incoming data spread sheets direct in the chemists office, the automated sample measurement and automated data processing. Data processing of the sample files implements background noise subtraction and averaging of significant spectra. Carry-over effects of persistent contaminations are checked and subsequently reported to the customer. Both carry-over-effects of compounds (Fig. 9), as well as persistent contaminations, falsify the mass spectra of subsequent samples and therefore interfere severely with

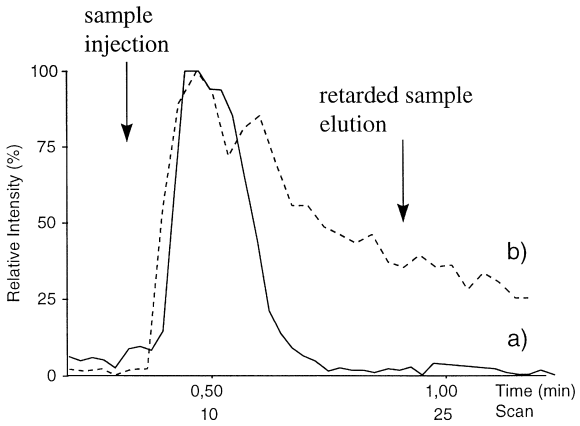

Fig. 9. Total ion chromatogram (TIC) of high-throughput measurements at two minute intervals, (a) Usual shape of the TIC for a measured sample. (b) Shape of the TIC of a retarded eluting compound causing a carry-over effect disturbing subsequent samples.

the evaluation of mass spectrometric results. Suggestions to operate mass spectrometric analysis in an open access mode [89] to proceed more rapidly in synthesis are still under debate. Although these systems are prevented from manipulations from the user the continuous sample analysis should be kept in the hands of analysts.

At the same time, the data-controlled automation of LC–MS and LC–MS-MS analysis offers possibilities for high-throughput sequencing of peptides from mixtures [90]. The transference of this approach to oligonucleotide libraries and to small compound collections seems to be attractive. However, one has to be aware of the vast amounts of additional data which will be accumulated when performing MS-MS experiments. Application for high-throughput tasks is only conceivable for software-supported spectra interpretation. However, even peptide spectra interpretation programs cannot yet resolve all ambiguities in the sequence determination.

In comparison with other ionization techniques, the combination of electrospray ionization and the quadrupole analyzer technique is the most versatile tool for high-throughput sample analysis at the present time, particularly with regard to sample preparation, handling and abilities for LC– and CE–MS characterization of libraries.

Attempts for high-throughput analysis using MALDI-TOF [91] have begun, although there still exist some drawbacks regarding sample preparation, matrix-effects and local resolution as a result of the ionization process. Nevertheless, for oligonucleotides, for example, it has been shown that autosampler arrays provide highly rapid and reproducible results [92].

The FTICR-technique followed suit with the evaluation of automated MALDI measurements capable for 20 samples per hour including high accuracy measurements in MS and MS-MS mode [93]. The accuracy was in the 5 ppm range and activation energy was programmed to be held constant. An internal mass reference using MITS (multiple ionization techniques simultaneously) technique [94] avoids issues of ion discrimination.

A future directed application of extended significance will be the use of ESI-MS for automated sample purification. As shown earlier, a fully automated high-throughput HPLC system was developed for the purification of combinatorially generated single compounds [95]. The efficiency of this approach is increased using mass spectrometric detection. One strategy comprises the use of analytical LC–MS runs in combination with UV-detection [96]. The analytical data are used to guide the subsequent preparative compound purification by UV-detection alone. As shown in this report, the system is applicable to different column types (reversed-phase, normal-phase, enantioselective columns). The other strategy provides a direct coupling of the mass spectrometer to a preparative HPLC column [97]. Compounds of a purity level below a pre-set threshold in analytical HPLC analysis are subjected to this process suitable for semipreparative and preparative quantities. The mass spectrometer and fraction-collector are controlled by a computer and fraction collection is programmed to the selection of definite compound masses. High flow-rates and short columns allow rapid separations providing the purification of approximately 100 samples per day. Moderate prices of bench-top single quadrupole mass spectrometers make future application of these systems attractive.

In a recent report, the high-throughput problem was once more discussed [98], with the authors focusing on the high throughput analysis of libraries synthesized in parallel rather than mixtures. Some representative examples clearly demonstrate that, due to the relative inherent shortcomings of MS and HPLC alone, an exclusive employment of only one of these methods is not practicable. Coincident with our own experiences, signal suppression in the case of coeluting compounds or in the presence of easily ionizable contaminants are severe problems in ESI analysis an a complementary use of both, MS and HPLC is needed.

7. Conclusions

The number and quality of analytical contributions clearly show a highly advanced stage for the characterization of libraries with mass spectrometry. The main techniques employed for the analytical demands impressed by the vast synthesis possibilities opened by combinatorial chemistry are ESI and MALDI in combination with quadrupole or time-of-flight analyzers. Other techniques such as FAB or

plasma desorption mass spectrometry [99] have not gained such a common application. The versatility of the APCI technique may have been underestimated despite the fact that it is a commonly used method in pharmaceutical industry.

As shown, a reliable direct library analysis is possible for all components of a small compound mixture. However, with growing library size the probability of peak overlap and signal suppression of weakly ionizable compounds increases. The high resolving power of the FTICR-technique extends the use for direct measurements to a larger library size, preferably with ESI. Moreover, interfacing techniques suitable for separation methods like HPLC or CE, offer a further dimension, extending the applicability of these techniques for library characterization. GC–MS techniques will be helpful in solving special problems, particularly for apolar libraries not easily measureable by ESI or APCI.

For the characterization of libraries as well as for single compound analysis the UV absorbance detection method provides additional information about the compound purity. Particularly, with regard to suppression effects of more easily ionizable impurities, real sample composition may be wrongly interpreted in the absence of multiple detection techniques. The evaporative light scattering detector (ELSD) has also been proposed as an alternative detector to give absorption-independent information [100].

The future developments in high accuracy measurement and high-throughput capability are important if FTICR is to become a valuable technique for structure identification in combinatorial chemistry. The potential of this rather costly method has not yet been fully explored. However, it should not be necessary to transfer trivial tasks from the smaller and perhaps more agile quadrupole analyzers to the FTICR-technique. There will be still some time needed to assign the FTICR-technique its place in the analysis of combinatorially generated compounds.

The implementation of computer-directed automated sample throughput in combination with LC–MS capabilities smoothes the way directly from synthesis to the bioassay of the compounds. Although impressive results can be obtained in identifying a few compounds from vast libraries for characterization or screening purposes, more attention needs to be paid to the high-throughout analysis and purification as well as for screening purposes of single compounds using mass spectrometry. Highly sophisticated equipment or complicated screening procedures are not attractive to the pharmaceutical industry where they are time consuming and rigid in their applicability.

With a view to the future, hyphenated technologies such as a LC–NMR-MS have also been presented as a means to support structure elucidation of combinatorial libraries [101]. Although such technologies are still in a developmental stage, it remains to be seen if such large and rigid systems may give routine impact on combinatorial chemistry. Present problems include the tuning of the mass spectrometer near to the NMR magnet, a significant loss of sensitivity and the obligatory use of deuterated solvents. In addition to current variety of on-bead and off-bead NMR techniques, recent efforts have focussed on high-throughput NMR analysis [102]. However, to MS techniques the use of NMR will be limited to some representative compounds selected out of a compound library.

To summarize, mass spectrometry provides a rapid and reliable analysis with high sensitivity and accuracy for a wide range of structurally different compounds including structure elucidation of possible by-products within a very short time. Therefore, it is the method of choice for the analysis and screening of high numbers of combinatorially generated single compounds and compound collections.

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