

# FTICR-Mass Spectrometry for High-Resolution Analysis in Combinatorial Chemistry

Dietmar G. Schmid, Philipp Grosche, Holger Bandel, Günther Jung

*Institute for Organic Chemistry, University of Tübingen, Auf der Morgenstelle 18, 72076 Tübingen, Germany; telephone: + 49 7071/29-75350, -76925 fax: + 49 7071/29-5560; e-mail: guenther.jung@uni-tuebingen.de*

*Received 3 September 2000; accepted 20 October 2000*

**Abstract:** The diversity of compound collections required for finding lead structures in pharmaceutical research can be provided by means of combinatorial organic chemistry. The resultant enormous number of single compounds but also of compound mixtures represents a challenge for the analyst. With the introduction of Fourier transform ion cyclotron resonance mass spectrometry (FTICR-MS or FT-MS), a new and, as yet, not widespread mass spectrometric technique (a means of analysis of such compound libraries with a very high mass resolution) high mass accuracy and high sensitivity has become available. Moreover, in combination with electrospray ionization (ESI), not only high-throughput measurements via flow-injection analysis (FIA) but also coupling with separation techniques such as high-performance liquid chromatography (HPLC) or capillary electrophoresis (CE) is possible. Structural verification by way of decomposing ions ( $MS^n$ ;  $n \geq 2$ ) using a variety of different dissociation techniques can be performed by FTICR-MS. This is the first review specifically covering applications of FTICR-MS in the field of combinatorial chemistry. © 2001 John Wiley & Sons, Inc. *Biotechnol Bioeng (Comb Chem)* **71**:149–161, 2000/2001. **Keywords:** FTICR-MS; combinatorial chemistry; library; mass spectrometry; single bead analysis; review

## INTRODUCTION

Combinatorial chemistry and high-throughput synthesis<sup>1–4</sup> have been established as key technologies in the lead discovery and lead optimization process. Combi-

natorial chemistry, both on solid phase<sup>5,6</sup> and in solution<sup>7</sup> enables the synthesis of either single compounds and compound collections in the form of mixtures. The rapid and meaningful analysis of these compound collections is required because well characterized compounds are preferred for biological tests in high-throughput screening.<sup>1</sup>

Electrospray ionization mass spectrometry (ESI-MS) and high-performance liquid chromatography electrospray ionization mass spectrometry (HPLC-ESI-MS) have previously been shown to give reliable information on molecular mass even for extremely complex peptide libraries.<sup>8,9</sup> Presently, mass spectrometry offers the best potential for researching complex compound collections produced by combinatorial synthesis.<sup>10,11</sup> In the wide field of MS methods, FTICR-MS is especially able to characterize single compounds in complex mixtures with high sensitivity because of its very high mass resolving power and mass accuracy. In combination with an autosampler this method is suitable for high-throughput analysis. For this reason FTICR-MS is able to widen the analytical bottleneck and to keep pace with high-throughput synthesis and high-throughput screening.

## PRINCIPLES IN FTICR-MS

### Introduction

In the wide field of mass spectrometry, FTICR-MS attracts attention because of its combination of high- to ultrahigh mass resolution and very high mass accu-

*Correspondence to:* Günther Jung

racy. This versatile technique is not only applicable in organic, inorganic, and physical chemistry but also in biotechnological research.<sup>12</sup> Although, in principle, any internal or external on-line or off-line source can be utilized for sample ionization, the combination with electrospray ionization (ESI) or matrix-assisted laser desorption/ionization (MALDI)<sup>13,14</sup> is usually encountered. It is only recently that a new MALDI source has been developed especially for FTICR-MS.<sup>15</sup> Automated sample introduction to facilitate high-throughput analysis is simple with the on-line technique of ESI<sup>16</sup> but is also possible with the off-line MALDI ionization method.<sup>17</sup> The capability of ESI to ionize compounds at atmospheric pressure makes it amenable to coupling with high-performance liquid chromatography (HPLC)<sup>16,18,19</sup> and capillary electrophoresis (CE).<sup>20–22</sup> The analyzer cell<sup>23</sup> can be used as reaction chamber for ion/molecule reactions<sup>24</sup> and multiple-step ion fragmentations may be performed for structural analysis. To this end, several techniques are available: infrared multiphoton dissociation (IRMPD)<sup>25,26</sup> surface-induced dissociation (SID),<sup>27,28</sup> blackbody infrared dissociation (BIRD),<sup>29</sup> ultraviolet photodissociation (UVPD),<sup>30</sup> collision-induced or collision-activated dissociation (CID or CAD)<sup>31</sup> and electron capture dissociation (ECD).<sup>32</sup>

Since its introduction in 1974 by Comisarow and Marshall,<sup>33</sup> interest in FTICR-MS has increased steadily and consequently, the number of instruments installed worldwide. Though the principle of ICR-MS is not new,<sup>34</sup> it is only since the advent of the ionization techniques of ESI and MALDI<sup>13,14</sup> that it has drawn the attention of peptide and protein analysts. Among the numerous reviews published, Marshall et al.<sup>23</sup> and Amster<sup>35</sup> give a good overview and introduction in this technique. Further, there are several books which deal especially with FTICR-MS.<sup>36–38</sup>

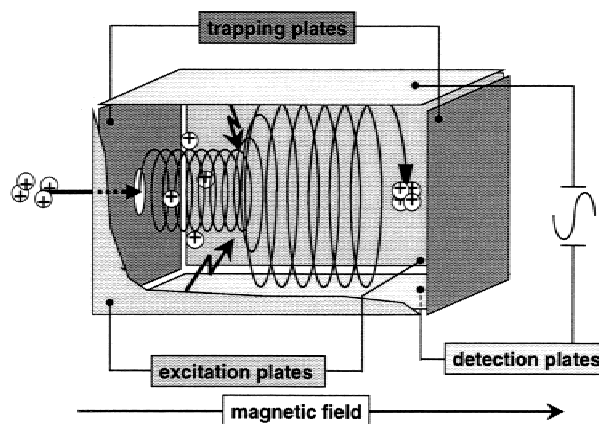
## Apparatus

Considering the components of this technique, it is clear that not only the analytical features of very high resolution and very high mass accuracy that distinguish FTICR-MS from other kinds of mass spectrometers. For the operation of FTICR-MS, the system is equipped with three main components: a superconductive magnet, an ultrahigh vacuum system, and an analyzer cell.<sup>23</sup>

To force the ions in their corresponding cyclotron motion a magnetic field is necessary, provided either by a permanent magnet, an electromagnet, or a superconductive magnet. The latter is generally used be-

cause permanent magnets and electromagnets only produce weak magnetic field strengths. With increasing magnetic field strength however, the performance of the FTICR-MS instrument improves.<sup>39</sup> Thus, the mass resolution is directly proportional to the magnetic field strength and the highest mass to charge ratio ( $m/q$ ) that can be determined in the analyzer cell increases with the square of the magnetic field. This has led to the use of increasingly strong superconductive magnets and instruments employing magnets with field strengths of 11.5 T (Tesla)<sup>40</sup> or even 20 T<sup>41</sup> have been constructed. Generally, superconductive magnets with magnetic field strengths of 3.0 T, 4.7 T, 7.0 T, or 9.4 T are used. Liquid helium is required to maintain the coil at a temperature of 4.2 Kelvin. Ancillary equipment such as rotating pumps, computers, etc., have to be protected against the strong magnetic field. The magnet can be shielded either passively with iron plates or actively by applying a compensatory magnetic field.

The heart of FTICR-MS is the analyzer cell. Here the ions are trapped, exposed to the magnetic field, forced into their cyclotron motion, analyzed, and detected. The use of a cell to trap ions enhances the possible time of detection and thus sensitivity and resolution. The cell is located in the homogeneous region of the magnetic field. In contrast to NMR (nuclear magnetic resonance) spectroscopy, the magnet is mounted horizontally and has a wide bore to accommodate the analyzer cell. The function of the analyzer cell is best demonstrated with a cubic cell<sup>42</sup> (Fig. 1) although numerous other different designs (cylindrical, orthorhombic, etc.) have been built.<sup>23</sup> Three opposing pairs of plates forming the cube are positioned such that one pair (trapping plates) lies perpendicular and the other two pairs (excitation and detection plates) parallel to the magnetic field lines. In



**Figure 1.** Scheme of a cubic analyzer cell. The cyclotron motion and the excitation process is shown schematically.

one of the trapping plates is an orifice through which ions can enter the cell (Fig. 1). Ions are trapped and stored for up to hours in the cell by applying a small voltage to the trapping plates (usually +/- 1–2 Volts, depending on the polarity of the ions). At the same time the excitation plates and the detection plates are held at ground potential. The function of the remaining two plates are described below.

The third component is the ultrahigh vacuum system. Although all types of mass spectrometer require vacuum, FTICR-MS is most sensitive in this respect. As mentioned above, ions can be stored for long periods in the analyzer cell. Long dwell times of ions in the cell are only possible in a very high vacuum because residual gas molecules from air disturb the motion of the ions and so shorten the time for analysis and detection. To perform ultrahigh mass resolution analysis, a pressure of  $10^{-9}$  to  $10^{-10}$  mbars is necessary in FTICR-mass spectrometry. Such a vacuum is provided by turbo molecular pumps or cryogenic pumps. In the case of ESI, in which solutes are ionized under atmospheric pressure, several pump stages are required to overcome the enormous pressure difference from ion introduction to ion detection.

### Ion Motion

Ions describe three discrete forms of motion in the analyzer cell: trapping motion, cyclotron motion, and magnetron motion (Fig. 2). After injection into the cell, ions undergo harmonic oscillations in the electric field between the trapping plates (trapping motion). Simultaneously, ions in the analyzer cell are exposed to the strong magnetic field and undergo stable cyclic

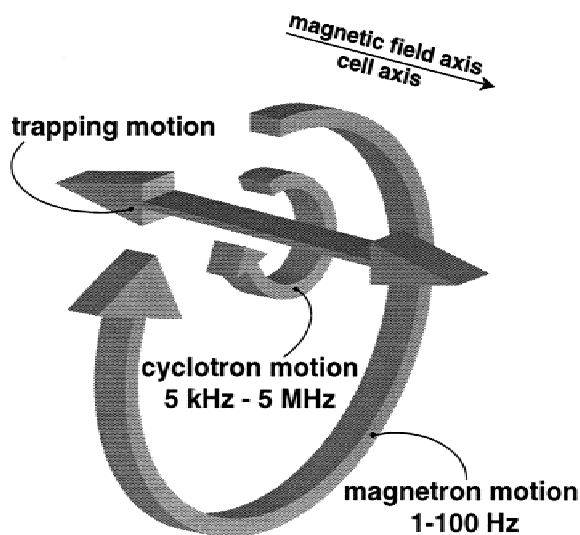


Figure 2. Scheme of the three ion motions in the analyzer cell.

motion in a plane perpendicularly to the magnetic field, the so-called cyclotron motion. Movement of ions parallel to the magnetic field is not influenced by this field. Each ion rotates with its typical frequency in respect to its mass-to-charge-ratio ( $m/q$ ), the so-called cyclotron frequency. This frequency lies in the range of several kilohertz to several megahertz. The third motion, called magnetron motion, which is centered around the cell axis, arises from the combination of the magnetic and the electric field. The combination of these three motions leads to a complex three-dimensional movement of ions in the analyzer cell.

### Cyclotron Motion

The cyclic motion of ions due to a strong magnetic field is the basis of FTICR-MS. Here we explain in a simplified manner the principle of the cyclotron motion.

In a magnetic field  $B$ , ions of charge  $q$  and velocity  $v$  experience the Lorentz force  $F_L$ , [Eq. (1)] perpendicular to both the ions velocity and the magnetic field lines.

$$F_L = q \cdot v \otimes B \quad (1)$$

The Lorentz force is directed inward and is counterbalanced by the centrifugal force  $F_Z$ , which is directed outward (Fig. 3) and defined by the ion mass  $m$ , the ions velocity  $v_{xy}$  in the  $x$ - $y$ -plane and the orbital radius  $r$  [Eq. (2)].

$$F_Z = \frac{m \cdot v_{xy}^2}{r} \quad (2)$$

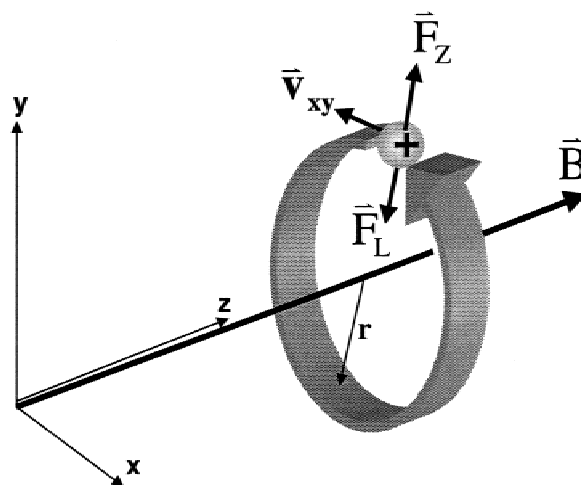


Figure 3. Cyclotron motion of ions in the magnetic field induced by the counterbalance of the centrifugal force  $F_Z$  and Lorentz force  $F_L$ .

Thereby, positive-charged ions rotate counterclockwise and negative ions rotate clockwise (viewed in the direction of the magnetic field line  $B$ ) (Fig. 3). By equating Equations (1) and (2) and introducing the frequency component  $\nu_c$  via the rectangular velocity  $\omega$ , shown in Equation (3),

$$\omega = \frac{v_{xy}^2}{r} = 2\pi\nu_c \quad (3)$$

the so called cyclotron Equation (4) is obtained, which describes the relationship between the ions mass-to-charge-ratio  $m/q$  and the cyclotron frequency  $\nu_c$ :

$$\nu_c = \frac{q \cdot B}{2 \cdot \pi \cdot m} \quad (4)$$

Cyclotron motion is characterized by a cyclotron frequency  $\nu_c$  and each ion of a certain  $m/q$  is thus characterized by its typical cyclotron frequency. It should, however, be pointed out that the measured frequency is not strictly identical with the cyclotron frequency  $\nu_c$  but in reality equal to the difference between the cyclotron and magnetron frequency, the latter being dependent upon the trapping potential. This discrepancy can affect the mass accuracy of a measurement.

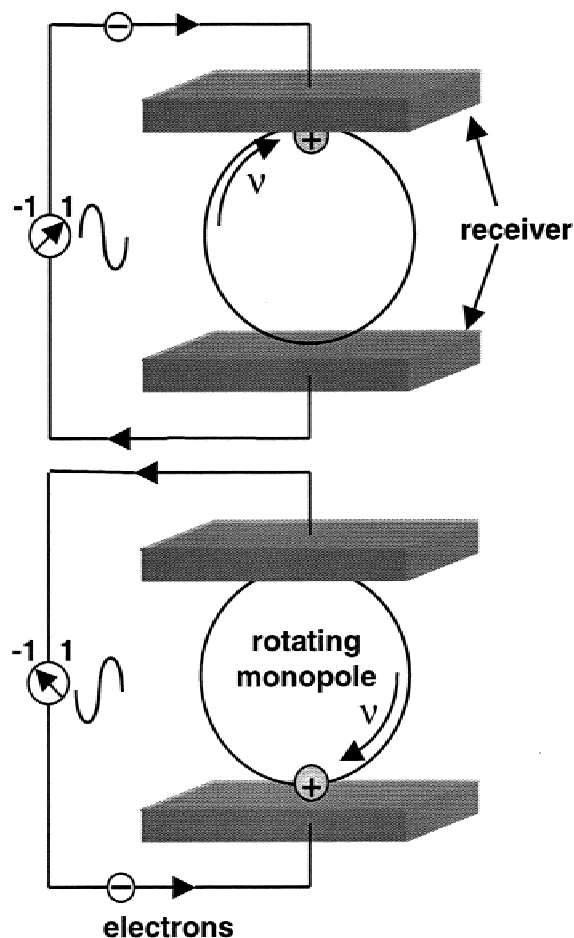
Ions with different  $m/q$  can be analyzed, detected, and separated by way of their different cyclotron frequencies. Because  $\nu_c$  is inversely proportional to  $m/q$ , the smaller the mass-to-charge-ratio, the higher the corresponding cyclotron frequency. The cyclotron frequency depends only upon the ion's charge  $q$ , its mass  $m$  and the magnetic field  $B$ , which, in general, is constant. In this respect FTICR-MS is unique compared with other mass spectrometry techniques. Because the velocity of the ions plays no role, translational energy focusing as encountered in all other types of mass spectrometer, is not necessary. In addition, the path length described by ions during rotation within a measurement can amount to several kilometers, in contrast to a magnetic sector field instrument, where the ion pathlength is a maximum of a few meters long.<sup>35</sup>

### Signal Generation

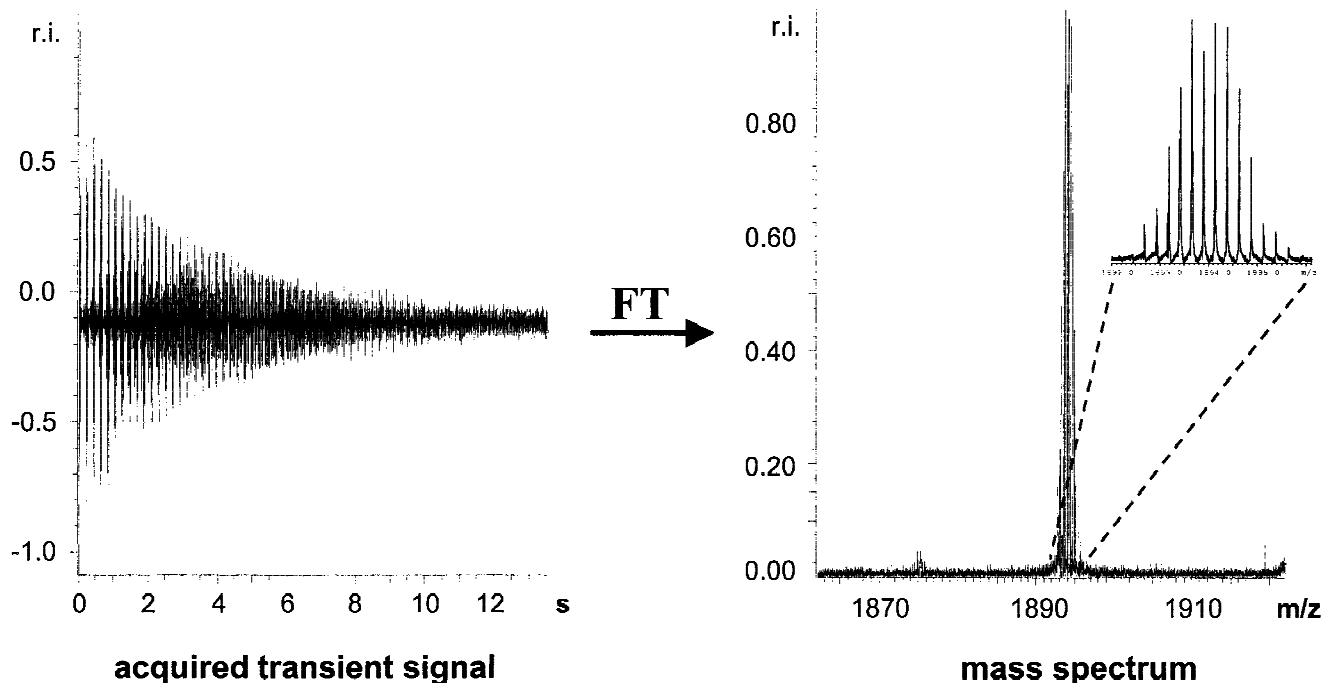
After introduction, the ions are stored in the analyzer cell using the trapping plates (Fig. 1). Simultaneously, they start their cyclotron motion because of the magnetic field. Under these initial conditions the ions are distributed statistically in the cell. Ions with the same  $m/q$  have the same cyclotron frequency but different energies and consequently different velocities in their orbits and different radii. To obtain a measurable sig-

nal, the ions must be excited by applying a sinusoidal voltage with the excitation plates (transmitter), which lie parallel to the magnetic field<sup>43</sup> (Fig. 1). The energetically distributed ions of the same  $m/q$  are coherently excited as in "ion packets," in which at least 100 combined ions of one  $m/q$  are necessary to generate a measurable signal.<sup>44</sup> The coherence of an "ion-packet" is only stable over a long period if the magnetic field is sufficiently homogenous and a pressure of  $10^{-9}$  mbar or lower prevails in the cell. Otherwise the "ion-packets" would be decomposed by collisions with residual air molecules. For the ion-detection principle Comisarow<sup>45</sup> suggests the model of a rotating monopole (Fig. 4).

Always when one "ion-packet" passes near the detection plates (Fig. 1) an "image-current" is induced in the two plates by attraction (positive-ion detection) or repulsion (negative-ion detection). The result is an alternating voltage which fades away with time (transient). In this electric magnetic wave (time-domain) the cyclotron frequencies of all ions are contained (multichannel principle). By accumulation of  $n$  tran-



**Figure 4.** Signal generation induced by the rotating monopole. As a result, an "image-current" is produced.



**Figure 5.** Obtained time-domain signal (transient) and its conversion into a mass spectrum using the algorithm Fourier transformation (FT).

sients, the signal-to-noise-ratio can be improved by the factor of  $n^{1/2}$ . This complex signal is then converted from the time-domain to the frequency-domain by Fourier transformation (FT). Employing Equation (4) and with constant magnetic field, the frequency spectrum can be converted into a mass spectrum (Fig. 5).

### Resolution

In general, maximum attainable resolution is desired in all analytical techniques to enhance the signal capacity for a given detection range. Fourier transform ion cyclotron resonance mass spectrometry is capable of extremely high mass resolution, much higher than other mass spectrometric techniques.

There are two modes of detection. The most common, the broadband mode, allows detection over a wide  $m/q$  range. Mass resolutions of up to 200,000 (FWHM, full width at half maximum) can be achieved for low values of  $m/q$ . Still higher mass resolution-up to millions-can be achieved in the heterodyne detection mode (narrowband detection, mixer mode).

This very high resolution is a consequence of the simplicity of the cyclotron equation [Eq. (4)], which has been explained above. As shown in equation (5), the resolution  $R_{FWHM}$  increases both with increasing transient duration  $T$  and with increasing cyclotron frequency  $\nu_C$ .

$$R_{FWHM} = \frac{\nu_C \cdot T}{2} \quad (5)$$

Because  $\nu_C$  is inversely proportional to  $m/q$  [Eq. (4)], the resolution  $R_{FWHM}$  also varies inversely with mass-to-charge-ratio. Using the heterodyne mode, a narrow range around the sample frequency can be measured with a long transient duration  $T$  and thus still higher resolutions than in the commonly used broadband mode can be obtained.<sup>23,35</sup>

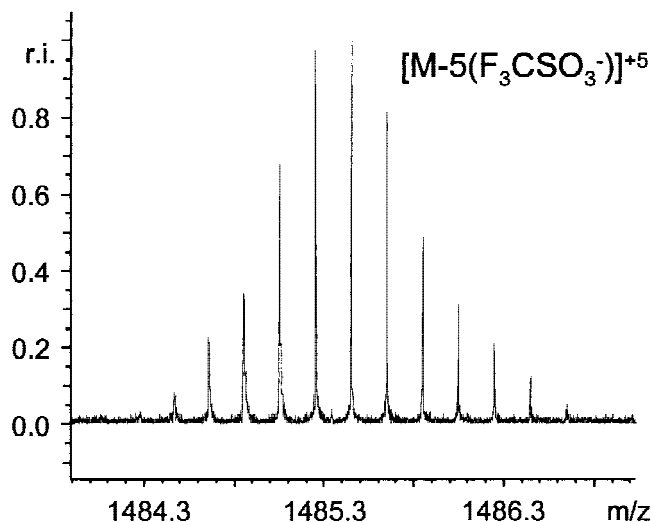
Using ESI for ionization, a highly symmetrical and high-molecular-weight perylene bisimide platinum complex<sup>46</sup> was measured in the heterodyne mode with a mass resolution of up to 600,000 (FWHM). In Figure 6 the resolved isotopic pattern of the fivefold charged ion  $[M-5(F_3CSO_3^-)]^{5+}$  is shown.

Even the isotopic pattern of multiply-charged polypeptides or proteins of up to several kDa can be resolved. The isotopic distribution gives information about the charge state and consequently about the molecular mass of the sample.

### Mass Accuracy

In addition to the very high mass resolution, FTICR-MS is able to determine ions with a very high mass accuracy. The difference between the experimental mass  $m_{exp}$  and calculated mass  $m_{calc}$  is generally extremely small. Mass accuracy is usually defined as shown in Equation (6).

$$\text{mass accuracy (ppm)} = \frac{m_{calc} - m_{exp}}{m_{calc}} \cdot 10^6 \quad (6)$$



**Figure 6.** Ultrahigh mass resolution spectrum of a highly symmetric and high-molecular-weight perylene bisimide platinum complex. The  $[M-5(F_3CSO_3^-)]^{5+}$  ion could be measured up to a resolution of 600,000 (FWHM).

Thus, the mass accuracy of different masses over the whole  $m/q$ -range can be compared.

Accurate mass determination is important for confident verification of compound identity. With FTICR-MS the elemental composition of small molecules up to 500 Da can generally be reliably determined. Because the number of possible molecular formulae increases exponentially with mass and with increasing number of different elements, nonambiguous determination of the elemental composition may require a mass accuracy of 1 ppm and better. This feature is of importance, for example, for the analysis of natural products. Thus, the identity of the synthetic antibiotic tetramic acid reutericyclin<sup>47</sup> with the natural product from a *Lactobacillus sp.* could be exactly verified. For the product a mass  $([M + H]^+)$  of 350.232683 Da was found. Comparison with the calculated mass (350.232585 Da) of natural reutericyclin leads to a mass accuracy of 0.28 ppm. Because of this the elemental composition  $C_{20}H_{31}NO_4$  could be determined unambiguously. Fourier transform ion cyclotron resonance mass spectrometry is able to measure with such a high mass accuracy that even the mass of the missing electron has to be considered when calculating the ion mass of a positively charged ion. If the mass of the electron were not subtracted, the calculated mass of reutericyclin would be 350.233134 Da leading to a relative mass error of  $-1.29$  ppm, which is 1.57 ppm higher than the accuracy of 0.28 ppm mentioned above.

In addition to inhomogeneities in the electric field due to the trapping voltage and space-charge interac-

tions, the mass accuracy also depends partially on the resolution. Other possible interfering factors such as magnetic field inhomogeneity<sup>48</sup> or magnetic drift<sup>49</sup> do not reduce mass accuracy when using superconductive magnets.

## FTICR-MS IN COMBINATORIAL CHEMISTRY

To date relatively few articles dealing with combinatorial chemistry in combination with FTICR-MS have been published. These articles, however, do exemplify why FTICR-MS is a valuable analytical technique in combinatorial chemistry.

### Investigation of Compound Collections (Nonpeptides and Peptides) Produced by Combinatorial Chemistry

With its capability to measure with a very high mass resolution and mass accuracy, FTICR-MS is predestined for the investigation of complex mixtures such as those produced in combinatorial chemistry. Jung and co-workers validated the synthesis of a library of heterocyclic compounds synthesized by the split-mix-method. In a three-step procedure,<sup>50</sup> six sublibraries containing 24 trisubstituted pyrazole carboxylic acids each were synthesized. They were analyzed both by FTICR-MS and micro-HPLC-FTICR-MS. Figure 7 shows the ESI-FTICR-MS-spectrum of a sublibrary that was obtained using 4-fluorophenylhydrazine·HCl as phenylhydrazine component. All compounds were found with a relative mass error of better than 2 ppm.

The high resolution of the method is highly advantageous for this purpose. Compounds with very similar masses can be detected as well-resolved signals. As an example, part of the spectrum is shown (Fig. 8). Two molecules that differ by only 0.1 amu yielded well-separated signals using broadband detection.

A limitation for the number of compounds in a mixture that can be measured without previous separation is due to different ionization probabilities that can cause signal suppression. Also isomeric compounds (identical elementary composition) cannot be detected individually. In this case HPLC-FTICR-MS is the method of choice. The results of a micro-HPLC-FTICR-MS measurement on an analogous sublibrary, obtained by reaction with phenylhydrazine·HCl are presented as a contour plot (Fig. 9). Here, signal suppression could be avoided. On the right side of the figure, a magnified section shows, among other compounds, four brominated compounds (marked with an asterisk) identifiable by means of the typical isotopic distribution. Using FTICR-MS we recently analyzed

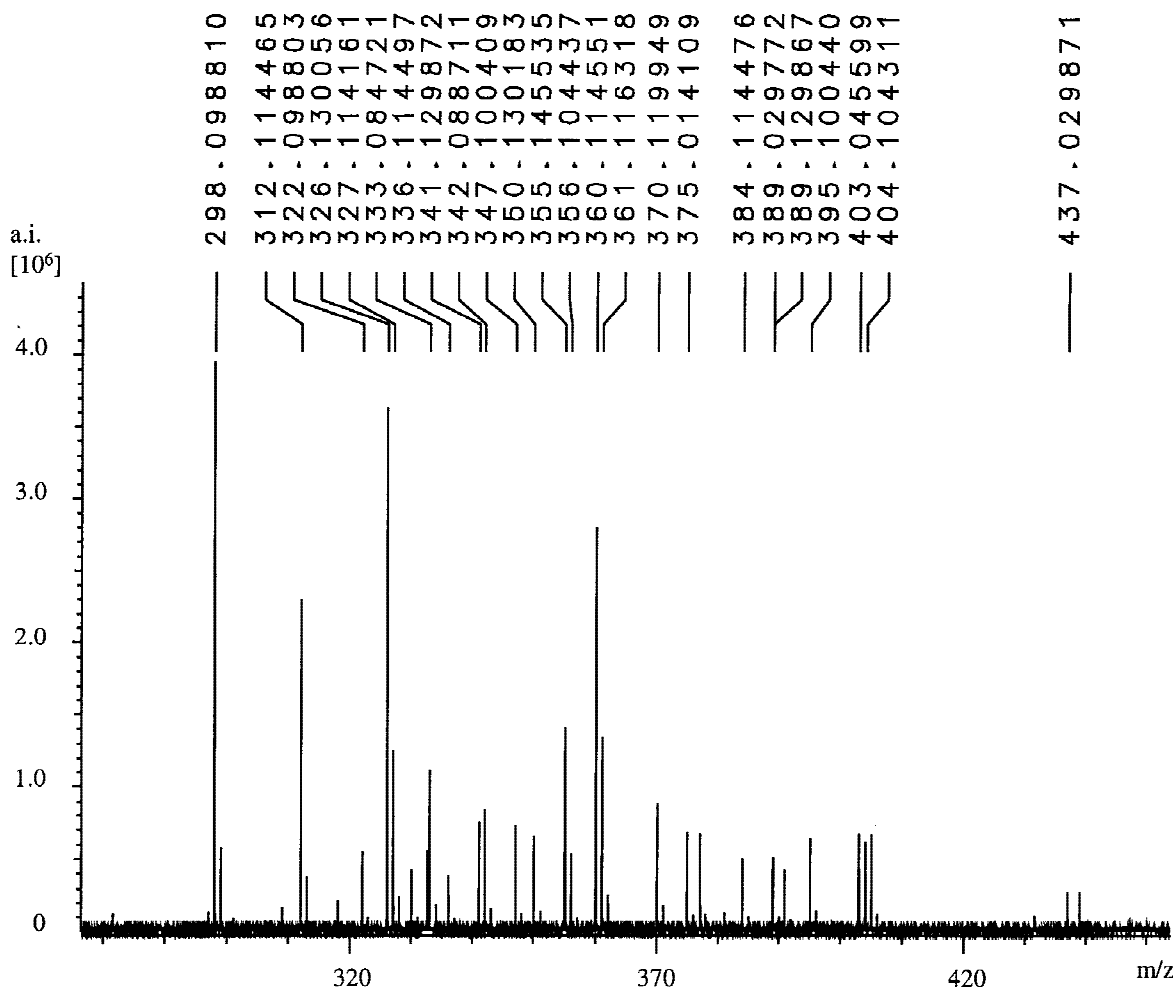


Figure 7. ESI-FTICR-MS of a sublibrary of 24 pyrazole carboxylic acids, all of them were detected.

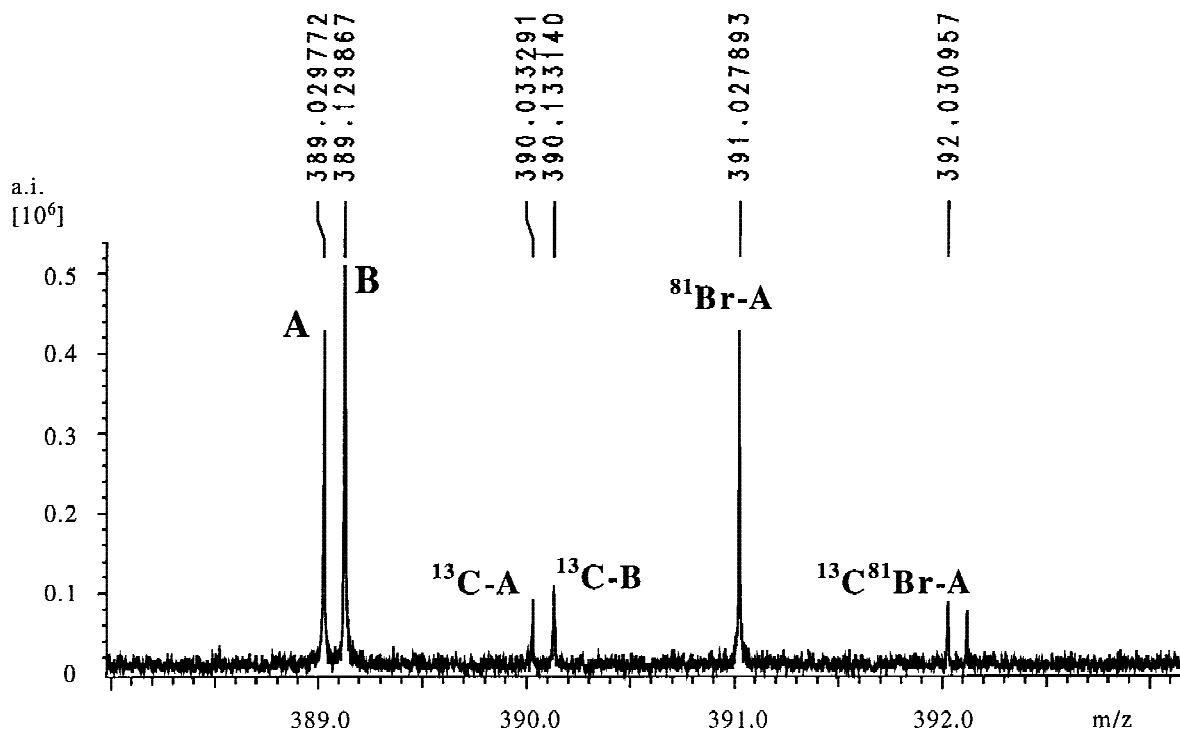
libraries of pyrazolic esters formed by esterification with an alkylating polymer.<sup>19</sup>

Walk et al.<sup>16</sup> investigated the elemental composition of 14 compounds in a sublibrary forming part of a split-mix library of 140 different pyrrole amides synthesized on Rink amide AM resin.<sup>51</sup> The sample consumption was 1.4 pmol per compound, the average mass resolution 38,000 (FWHM) and the average relative mass error 1.05 ppm. The same sublibrary was also investigated using HPLC-FTICR-MS. The number of plausible elemental formulae was generally one of two, at a maximum three, when the elements considered in the calculation were restricted to C, H, N, O, and S. Such elemental composition studies were also used for the synthesis control of a library of *N*-substituted  $\alpha$ -Aminonitriles.<sup>52</sup> Despite the very high mass accuracy of FTICR-MS, the number of possible elemental compositions increases with increasing mass and with the number of elements potentially present.

This can be overcome by performing a “basket in a

basket” method based on a multistage accurate mass spectrometric (MAMS) technique as suggested by Wu.<sup>53</sup> Here, a compound from combinatorial synthesis was used whereby the complexity of this library was 25,650. In accordance with the known starting materials of the synthesis, only the elements C, H, N, O, and S were considered. Using the technique MAMS the molecule was decomposed in four fragmentation steps. The small masses of the fragments and consequently their unique elemental composition permitted the reconstruction of a single possible elemental composition of the original compound. However due to inherent problems, such highly complex mixtures are of less and less of interest for test purposes.

The first determination of compounds from dynamic combinatorial libraries (DCL) using FTICR-MS with regard to a fragmentation of the compounds (FTICR-MS/MS) was carried out by Poulsen et al.<sup>54</sup> In DCLs, the library composition is not static but rather can change by interactions of library members via covalent or noncovalent mechanisms according to



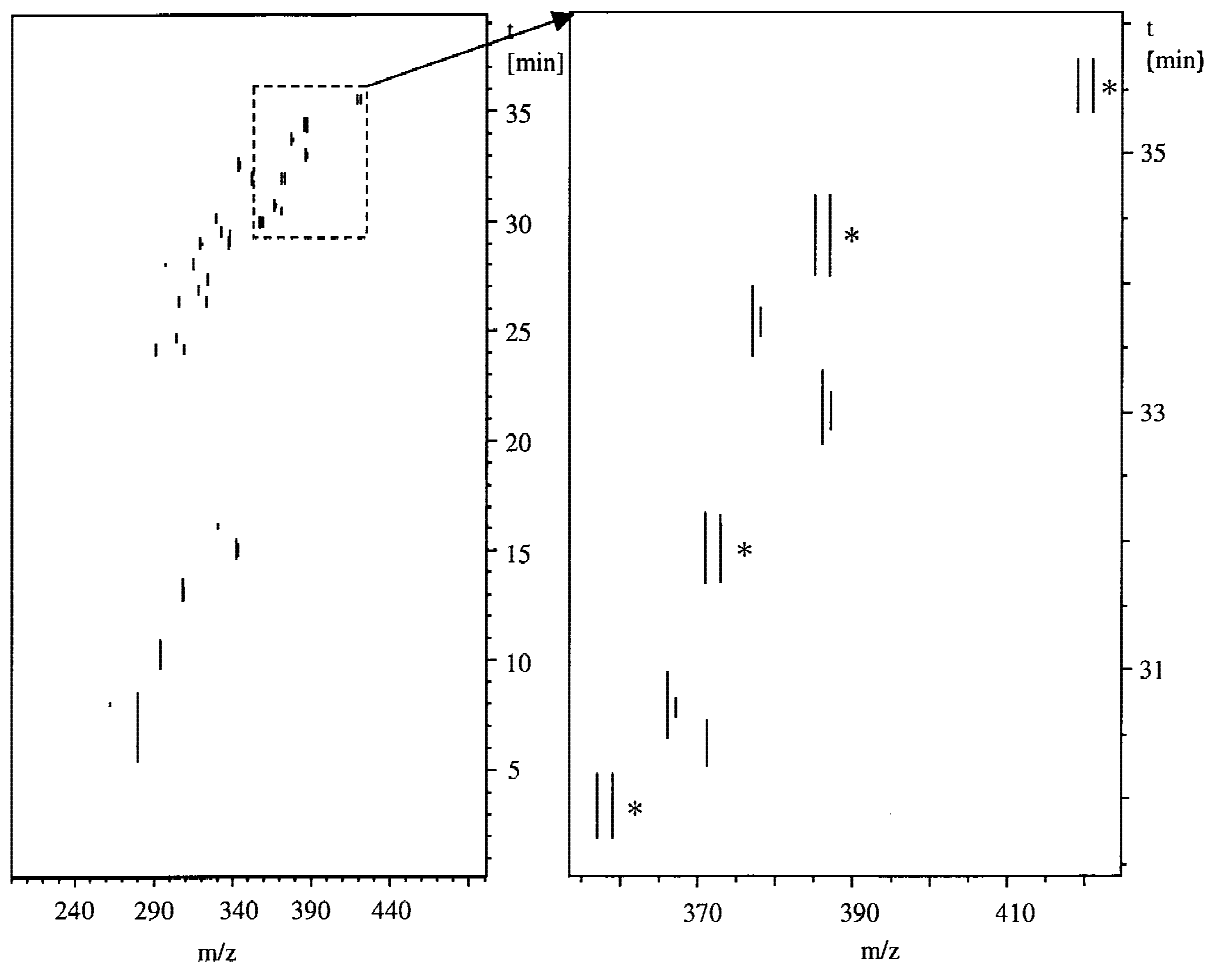
**Figure 8.** Magnification of part of Figure 7. Two adjacent signals are separated by using ESI-FTICR-MS. Compound A shows the isotopic pattern which is typical for presence of one bromine atom.

the selection pressure exerted by the individual library members.<sup>56</sup> This method, which mimics the situation in natural systems, still poses several problems.<sup>57</sup> For example, the number of types of chemical reactions for the necessary equilibrium modulation due to the reversibility of the reaction is very limited. Poulsen et al.<sup>54</sup> used monomers derived from amino acids and carrying a dimethoxy acetal and a hydrazide group. Under acidic conditions these monomers form pseudo-peptide macrocyclic hydrazones (Scheme 1). DCLs prepared from one monomer contained up to 16 cyclic oligomers while a mixture of two monomers led to 130 products. The heptamer was decomposed using collision-induced dissociation (CID) leading to the loss of 1 to 6 monomer units from the parent ion. The CID was performed with very little energy, so that no loss of H<sub>2</sub>O, NH<sub>3</sub>, or CO was observed. Masses were determined with a relative mass error of better than 10 ppm. In addition, the existence of sequence isomers in DCLs generated by two monomers was confirmed by FTICR-MS/MS. Later on, this group investigated DCLs of macrocyclic disulfides in water by also using FTICR-MS.<sup>55</sup>

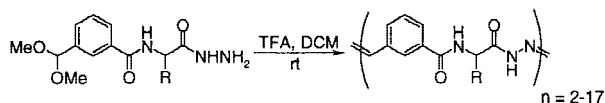
Fang and co-workers<sup>58</sup> investigated libraries of 36, 78, and 120 compounds derived from a xanthene as core molecule and 8, 12, and 15 amino acid building blocks with regard to purity and completeness. The mass spectra of the libraries were determined by direct

infusion with pneumatically supported ESI in both the positive- and the negative-ion mode. A decrease in the mass accuracy and resolution was observed with increasing complexity of the library. As a result, 29 components (81%) could be identified in the small library, 54 (70%) in the library of medium complexity. Because of the presence of several sets of isomeric compounds in the most complex library, only 103 signals were to be expected from the 120 compounds. Of these 103 signals, 68% could be assigned. Here, relative mass errors of 10 ppm and better were obtained. Previous and later investigations of this group confirmed the capabilities of FTICR-MS for combinatorial library characterization.<sup>59–61</sup>

Winger and Campana<sup>62</sup> investigated a library of the octapeptide [SIIN-X-EKL], where X can represent any of the 19 natural amino acids (excluding cysteine), using positive-ion ESI combined with FTICR-MS. The analysis of this library of low complexity with FTICR-MS makes sense because the amino acids lysine and glutamine differ in mass by only 0.036 mass units. The two relevant components could be resolved with FTICR-MS without prior HPLC separation. In addition, it was possible to resolve the first <sup>13</sup>C isotopic signals of the K- and Q-substituted octapeptides from the monoisotopic signal of the glutamic acid (E) analogue. It was also found that the methionine containing peptide was oxidized during processing and/or



**Figure 9.** Contour plot of a sublibrary of 24 pyrazole carboxylic acids obtained by performing HPLC-FTICR-MS. The signals of the four brominated compounds are marked with an asterisk.



**Scheme 1.** Generation of DCLs from amino acids derived monomers equipped with both hydrazide and aldehyde (protected as a dimethoxy acetal) functional groups. R represents the amino acid side-chain.

handling, leading to a nominal molecular mass identical to that of the phenylalanine analogue. All the results were confirmed by coupling liquid chromatography with a quadrupole MS as detector. Later on, Winger and Castoro<sup>63</sup> performed a coupling from HPLC to FTICR-MS and a fragmentation (FTICR-MS/MS) to study a mixture containing eight cyclopeptides.

In the same year Nawrocki et al.<sup>64</sup> studied the degeneracy and diversity of selected small-peptide combinatorial libraries containing  $10^2$  to  $10^4$  compounds using FTICR-MS. Experimental mass spectra were compared with simulated results to pinpoint where discrimination during synthesis had led to incomplete li-

braries. Even at the relatively low resolution obtained with the common broadband detection ( $R_{FWHM} = 20,000$ ), an extremely detailed analysis of the library H-Xxx-Xxx-Lys-OH and a semiquantitative data analysis for a library of  $10^4$  compounds was possible. Using heterodyne detection and a stronger magnetic field improved separation of adjacent signals of a more complex hexapeptide library ( $R_{FWHM} = 120,000$ ).

Ramjit et al.<sup>65</sup> identified the components of a library containing 19 dodecapeptides simultaneously within the mass windows of 1380-1385 and 1395-1400 Da using direct infusion ultrahigh mass resolution ESI-FTICR-MS without a prior separation. Within these detection windows an average resolution of 275,000 (FWHM) was achieved, allowing the resolution of monoisotopic signals from the  $^{13}\text{C}$  signals of other constituents. For example the monoisotopic signal of the asparagine analogue, which differs only by 0.0189 mass units from the  $^{13}\text{C}$  signal of the aspartate analogue, could be resolved by measuring with a relative mass error of better than 2.8 ppm.

## Automation (High-Throughput Analysis)

With the pressure to save time and costs, interest in automation of analysis (high-throughput analysis) and verification of the data is prevalent especially in the pharmaceutical industry. Particularly the on-line ion sources ESI and APCI (atmospheric pressure chemical ionization) but also the off-line source MALDI are amenable to coupling with an autosampler. ESI and APCI also can be used to monitor the eluent of an automated HPLC. Though these techniques are frequently used with other types of mass spectrometer FTICR-MS is the first choice (albeit the most expensive) because of its outstandingly high mass resolution and mass accuracy.

Walk et al.<sup>16</sup> was one of the first investigators to perform high-throughput analysis with FTICR-MS by coupling an automatic sample injector with ESI as ionizing interface. The sampling rate was one measurement every 2 minutes leading to a throughput of 300 samples per night. The subsequent data processing was also automatically performed.

Similarly, Bandel et al.<sup>66</sup> screened single compounds produced by the split and combine strategy following cleavage from a single bead (single-bead analysis). As an example, a pyrrolidine library of 18 compounds was synthesized on monodisperse Wang resin. Single-resin beads were separated by a micro-manipulator and transferred into HPLC vials. After cleavage from the resin the heterocyclic products were analyzed automatically by ESI-FTICR mass spectrometry. The very low sample consumption of approxi-

mately 15 pmol left sufficient product for subsequent biological testing. Samples were analyzed with an average resolution of 100,000 and an average mass accuracy of 2.63 ppm. The whole procedure is shown in Figure 10.

All separated beads were examined, the masses of all 18 compounds were found and the elemental composition calculated automatically. The position of each compound in the vial rack was assigned. With the resolution used, the calculation programme creates up to four possible formulae for each compound. The correct formula was determined by common exclusion rules. A further example for the high resolution of the FTICR-MS in the automated mode is given in Figure 11. The zoomed spectrum shows the typical isotopic pattern caused by two bromine atoms.

Tutko et al.<sup>17</sup> also used automated FTICR-MS and additionally FTICR-MS/MS analysis to determine a 20-compound library using the off-line ion source MALDI. The samples were deposited on an auto-indexed multiple sample (AIMS) disk which is rotatable. To enhance the accuracy of mass determination, multiple ionization techniques simultaneously (MITS) were used. Here, the mass reference ions were generated by electron ionization (EI), while the analyte ions were generated by MALDI. Thus, no prior mixture of the reference and sample compounds is necessary. The 40 analyses for the 20 compounds (MS and MS/MS) could be performed within 1 hour. A relative mass error of better than 5 ppm (fragments better than 8 ppm) was obtained.

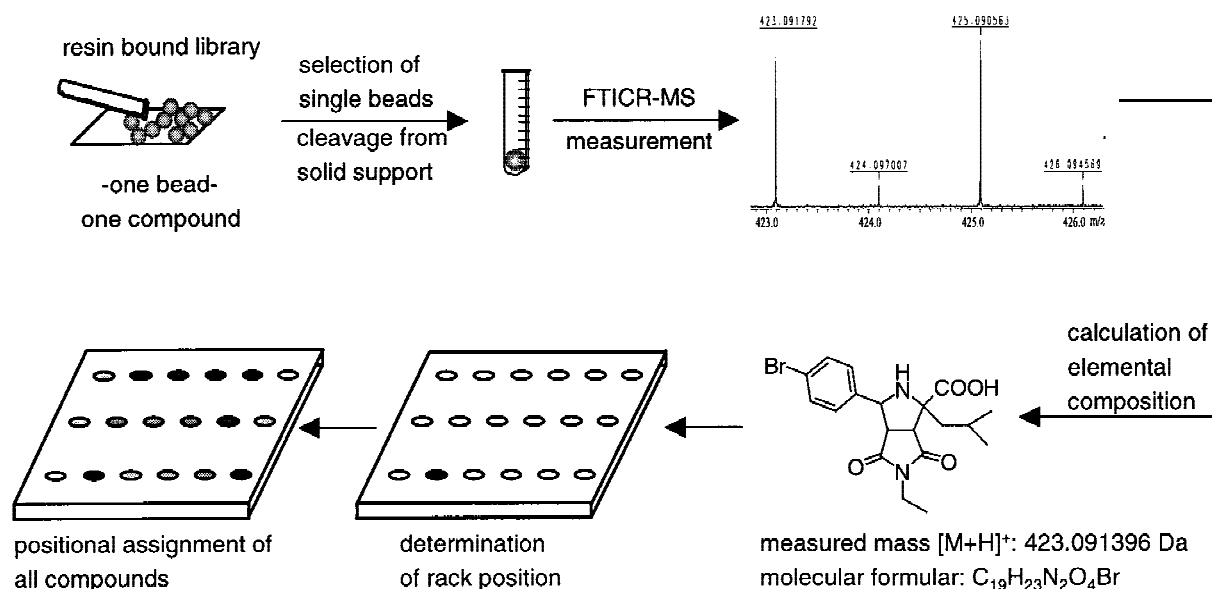
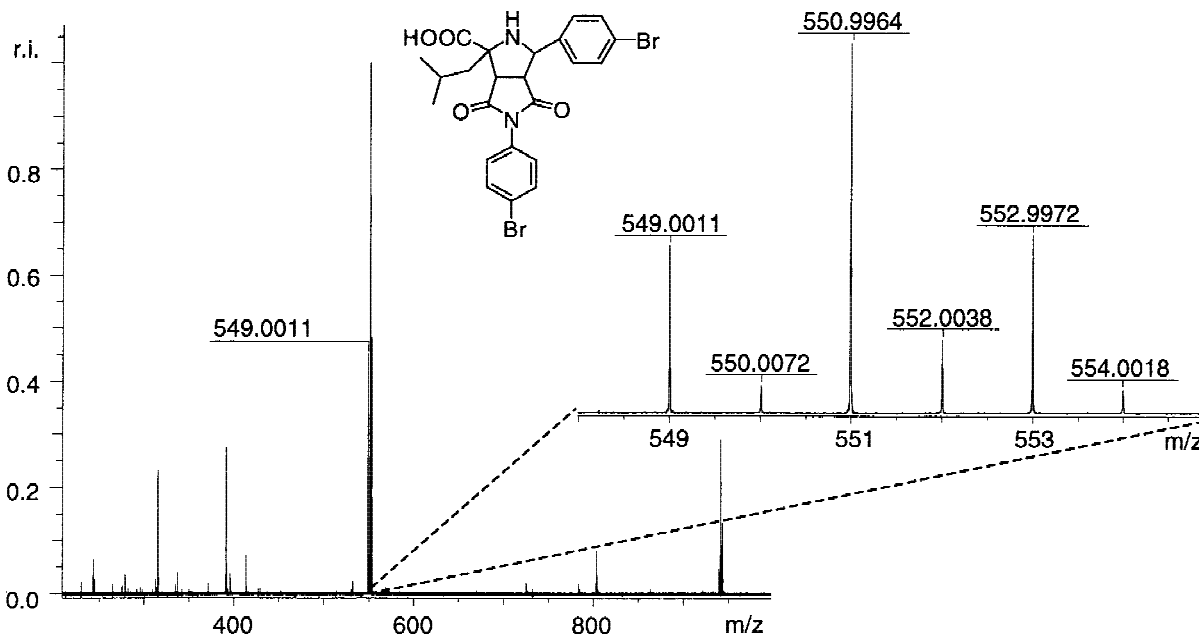


Figure 10. Single-bead analysis using FTICR-MS and assignment of the vial places.



**Figure 11.** Single-bead analysis using FTICR-MS with a resolution of 100,000; upper spectra: zoomed range from  $m/z$  548–554. Left spectra: overview from  $m/z$  100–1000; sample consumption: 15 pmol; acquisition time: 2 min; elemental composition:  $C_{23}H_{23}N_2O_4Br_2$ .

### Screening with FTICR-MS

In the fields of biological, biochemical, biotechnological and drug research, the investigation of substrate specificity toward enzymes is still a challenge.<sup>67</sup> Proof of the presence of noncovalent complexes in the gas phase using FTICR-MS was named bio-affinity characterization mass spectrometry (BACMS).<sup>68</sup> A critical point is the comparability of the stability of specific noncovalently associated macromolecular complexes in the gas phase, as measured in ESI-MS with that in solution.<sup>69,70</sup> The advantage is that no radiolabeling, fluorescent tagging, or single-compound resynthesis is required.

Wigger et al.<sup>70</sup> first used FTICR-MS to determine substrate specificity of glutathione-S-transferase (GST) in a “two-dimensional” combinatorial experiment. The reaction between the electrophilic 1-chloro-2,4-dinitrobenzene (CDNB) and components of the synthesized library H- $\gamma$ -Glu-Cys-Xxx-OH, catalyzed by GST, was demonstrated by the mass shift after incubation. As result GST was found to be > 95% specific to the peptide with Xxx = Gly, the natural substrate glutathione.

The formation of noncovalent protein complexes with mixtures of ligands was investigated by Cheng et al.<sup>72</sup> using ESI-FTICR-MS. Here, the competitive binding to bovine carbonic anhydrase II (BCAII) of two inhibitors consisting of 7 and 18 acylated tripeptides with carboxybenzenesulfonamide was studied.

To overcome problems in the direct differentiation of some BCAII-complexes because of mass overlap, the components were identified by means of gas-phase dissociation of the complexes using tandem mass spectrometry (MS/MS). After dissociation, all inhibitors were identified as negative ions. The relative intensities were found to be similar to those obtained from spectra of the intact complexes and correlated with the relative binding affinities of the ligands to BCAII in solution. Three inhibitors containing the amino acids glutamine, lysine and glutamate had very similar masses. Nonetheless, FTICR-MS was able to resolve the corresponding signals.

This work was expanded later using L-amino acids and D-amino acids leading to libraries with a complexity of 289 or 256 compounds, respectively.<sup>73</sup> It was found that the addition of hydrophobic groups at the *para* position of benzenesulfonamide leads to an increase in the binding constants to carbonic anhydrase (CAII). Further, the chirality of the amino acids appears to influence the binding affinity of the inhibitors, such that inhibitors with L-amino acids in their side chain interact with a higher efficiency with the active site of CAII than the inhibitors with D-amino acids.

Hofstadler et al.<sup>74</sup> utilized FTICR-MS for the simultaneous screening of multiple targets against ligand mixtures. The binding of five aminoglycosides to 2'-methoxy analogues of RNA, representing the prokaryotic (16S) and the eukaryotic (18S) rRNA A-site was

studied. For a better separation of the signals, the 18S analogue was modified with a polyethylene glycol mass tag. Only complexes of the ligands to the prokaryotic RNA model were observed. The high resolution allows the precise mass determination of the ligand by measuring the difference in  $m/z$  between the RNA target and the RNA-ligand complex. This becomes especially important when larger libraries of ligands are tested.

## CONCLUSIONS

Fourier transform ion cyclotron resonance mass spectrometry is used in combinatorial chemistry for several purposes. Due to the high mass resolution the method is valuable for the analysis of compound mixtures without previous separation as well as for coupling to separation techniques. The high mass accuracy enables the determination of the elemental composition of both single compounds and compounds in mixtures. A further important property is the low sample consumption, which was exploited, for example, in single-bead analysis. The possibility of automating all these operations makes the method suitable for high-throughput analysis in combinatorial chemistry. Further, this technique is used for  $MS^n$ -experiments and also for screening.

With the increasing recognition of these capabilities in the fields of combinatorial chemistry and structural elucidation, the number of FTICR-MS instruments is growing, with more than 320 having already been installed world wide.<sup>75</sup>

We thank R. Jertz for helpful discussions. We also thank G.J. Nicholson for reading the manuscript.

## References

- Jung, G. *Combinatorial Chemistry: Synthesis, Analysis, Screening*, Wiley-VCH, Weinheim, 1999.
- Jung, G. *Combinatorial Peptide and Nonpeptide Libraries: A Handbook*. Wiley-VCH, Weinheim, 1996.
- Balkenhohl, F.; von dem Busche-Hünnefeld, C.; Lansky, A.; Zechel, C. *Angew Chem Int Ed Engl* 1996, 35, 2289–2337.
- Früchtel, J.S.; Jung, G. *Angew Chem Int Ed Engl* 1996, 35, 17–42.
- Zaragoza Dörwald, F. Wiley-VCH, Weinheim, 2000.
- Booth, S.E.; Dreef-Tromp, C.M.; Hermkens, P.H.H.; de Man, J.A.P.A.; Ottenheijm, H.C.J. In *Combinatorial Chemistry: Synthesis, Analysis, Screening*; Jung, G., Ed.; Verlag Wiley-VCH, Weinheim, 1999; pp. 35–70.
- Austel, V. In *Combinatorial Chemistry: Synthesis, Analysis, Screening*; Jung, G., Ed.; Verlag Wiley-VCH, Weinheim, 1999; pp. 77–119.
- Metzger, J.W.; Wiesmüller, K.-H.; Gnau, V.; Brünjes, J.; Jung, G. *Angew Chem Int Ed Engl* 1993, 32, 894–896.
- Stevanovic, S.; Wiesmüller, K.-H.; Metzger, J.; Beck-Sickinger, A.G.; Jung, G. *Bioorg Med Chem Lett* 1993, 3, 431–436.
- Enjalbal, C.; Martinez, J.; Aubagnac, J.-L. *Mass Spectrom Rev* 1993, 19, 139–161.
- Süßmuth, R.D.; Jung, G.J. *Chrom B* 1999, 725, 49–65.
- Stockton, G.W.; Millen, W.G.; Meek, J.T. In *FT-ICR/MS—Analytical Applications of Fourier Transform Ion Cyclotron Resonance Mass Spectrometry*; Asamoto, B., Ed.; VCH Publishers Ltd., Cambridge, 1991; pp. 273–291.
- McIver, J.; Robert, T.; Li, Y.; Hunter, R.L. *Intl J Mass Spectrom Ion Proc* 1994, 132, L1–7.
- Buchanan, M.V.; Hettich, R.L. *Anal Chem* 1993, 65, 245A–259A.
- Baykut, G.; Jertz, R.; Witt, M. *Rapid Commun Mass Spectrom* 2000, 14, 1238–1247.
- Walk, T.B.; Trautwein, A.W.; Richter, H.; Jung, G. *Angew Chem Int Ed Engl* 1999, 38, 1763–1765.
- Tutko, D.C.; Henry, K.D.; Winger, B.E.; Stout, H.; Hemling, M. *Rapid Commun Mass Spectrom* 1998, 12, 335–338.
- Haskins, N.; Eckers, C.; Organ, A.; Dunk, M.; Winger, B.E. *Rapid Commun Mass Spectrom* 1995, 9, 1027–1030.
- Rademann, J.; Smerdka, J.; Jung, G.; Grosche, P.; Schmid, D.G. *Angew Chem Int Ed Engl* 2001, 40, 381–389.
- Hofstadler, S.A.; Swaneck, F.D.; Gale, D.C.; Ewing, A.; Smith, R.D. *Anal Chem* 1995, 67, 1477–1480.
- Hofstadler, S.A.; Wahl, J.H.; Bakthiar, R.; Anderson, G.A.; Bruce, J.E.; Smith, R.D. *J Am Mass Spectrom* 1994, 5(10), 894–895.
- Hofstadler, S.A.; Wahl, J.H.; Bruce, J.E.; Smith, R.D. *J Am Chem Soc* 1993, 115, 6983–6984.
- Marshall, A.G.; Hendrickson, C.L.; Jackson, G.S. *Mass Spectrom Rev* 1998, 17, 1–35.
- Nibbering, N.M.M. *Acc Chem Res* 1990, 23, 279–285.
- Tonner, D.S.; McMahan, T.B. *Anal Chem* 1997, 69(23), 4735–4740.
- Little, D.P.; Speir, J.P.; Senko, M.W.; O’Cornnor, P.B.; McLafferty, F.W. *Anal Chem* 1994, 66, 2809–2815.
- Zhong, W.Q.; Nikolaev, E.N.; Futrell, J.H.; Wysocki, V.H. *Anal Chem* 1997, 69, 2496–2503.
- Williams, E.R.; Henry, K.D.; McLafferty, F.W.; Shabanowitz, J.; Hunt, D.F. *J Am Soc Mass Spectrom* 1990, 1, 413–416.
- Dunbar, R.C.; McMahan, T.B. *Science* 1998, 279, 194–197.
- Bowers, W.D.; Delbert, S.-S.; Hunter, R.L.; McIver, R.T. Jr. *J Am Chem Soc* 1984, 106, 7288–7289.
- Cody, R.B.; Burnier, R.C.; Freiser, B.S. *Anal Chem* 1982, 54, 96–101.
- Axelsson, J.; Palmblad, M.; Hakansson, K.; Hakansson, P. *Rapid Commun Mass Spectrom* 1999, 13(6), 474–477.
- Comisarow, M.B.; Marshall, A.G. *Chem Phys Lett* 1974, 25, 282–283.
- Sommer, H.; Thomas, H.A.; Hipple, J.A. *Phys Rev* 1949, 76, 1877.
- Amster, J. *J Mass Spectrom* 1996, 31, 1325–1337.
- Asamoto, B. *FT-ICR/MS—Analytical Applications of Fourier Transform Ion Cyclotron Resonance Mass Spectrometry*, VCH Publishers Ltd, Cambridge, 1991.
- Marshall, A.G.; Verdun F.R. *Fourier Transforms in NMR, Optical and Mass Spectrometry, A User’s Handbook*, Elsevier, Amsterdam, 1990.
- Buchanan, M.V. *Fourier Transform Mass Spectrometry: Evo-*

- lution, Innovation and Applications, American Chemical Society, Washington DC, 1987.
39. Marshall, A.G.; Guan, S.H. *Rapid Commun Mass Spectrom* 1996, 10, 1819–1823.
  40. Gorshkov, M.V.; Tolic, L.P.; Udseth, H.R.; Anderson, G.A.; Huang, B.M.; Bruce, J.E.; Prior, D.C.; Hofstadler, S.A.; Tang, L.A.; Chen, L.Z.; Willett, J.A.; Rockwood, A.L.; Sherman, M.S.; Smith, R.D. *J Am Soc Mass Spectrom* 1998, 9, 692–700.
  41. Hendrickson, C.L.; Drader, J.J.; Laude, D.A.; Guan, S.H.; Marshall, A.G. *Rapid Commun Mass Spectrom* 1996, 10, 1829–1832.
  42. Comisarow, M.B. *Int J Mass Spectrom Ion Phys* 1981, 37, 251–257.
  43. Schweikhard, L.; Marshall, A.G. *J Am Soc Mass Spectrom* 1993, 4, 433–452.
  44. Dunbar, R.C.; Asamoto, B. In *FT-ICR/MS—Analytical Applications of Fourier Transform Ion Cyclotron Resonance Mass Spectrometry*; Asamoto, B., Ed.; VCH Publishers Ltd, Cambridge, 1991; pp. 29–81.
  45. Comisarow, M.B. *J Chem Phys* 1978, 69, 4097–4104.
  46. Würthner, F.; Sautter, A.; Schmid, D.G.; Weber, P.J.A. *Chem Eur J*, 2001, 7, 894–902.
  47. Marquardt, U.; Schmid, D.G.; Jung, G. *Synlett* 2000, 8, 1131–1132.
  48. Schuch, D.; Chung, K.-M.; Hartmann, H. *Int J Mass Spectrom Ion Proc* 1984, 56, 109–121.
  49. White, R.L.; Onyiriuka, E.C.; Wilkins, C.L. *Anal Chem* 1983, 55, 339–343.
  50. Grosche, P.; Hölzel, A.; Walk, T.B.; Trautwein, A.W.; Jung, G. *Synthesis* 1999, 11, 1961–1970.
  51. Trautwein, A.W.; Stüßmuth, R.D.; Jung, G. *Bioorg Med Chem Lett* 1998, 8, 2381–2384.
  52. Probst, K.; Schmid, D.G.; Jung, G. *Proceedings of the 6<sup>th</sup> International Symposium on Innovation and Perspectives in Solid Phase Synthesis and Combinatorial Chemistry Libraries*, 1999, 339–342.
  53. Wu, Q. *Anal Chem* 1998, 70, 865–872.
  54. Poulsen, S.A.; Gates, P.J.; Cousins, G.R.L.; Sanders, J.K.M. *Rapid Commun Mass Spectrom* 2000, 14, 44–48.
  55. Otto, S.; Furlan, R.L.E.; Sanders, J.K.M. *J Am Chem Soc* 2000, 122, 12063–12064.
  56. Brady, P.A.; Sanders, J.K.M. *Chem Soc Rev* 1997, 26, 327–336.
  57. Ganesan, A. *Angew Chem Int Ed Engl* 1998, 37 (20), 2828–2831.
  58. Fang, A.S.; Vouros, P.; Stacey, C.C.; Kruppa, G.H.; Laukien, F.H.; Wintner, E.A.; Carell, T.; Rebek, J. Jr. *Comb Chem & Highthroughput Screening* 1998, 1, 23–33.
  59. Ross III, C.W.; Bilodeau, M.T.; Kruppa, G.H.; Ramjit, H.G. *Proceedings of the 46th ASMS Conference on Mass Spectrometry and Allied Topics*, 1998, p. 1038.
  60. Ross III, C.W.; Young, M.B.; Patrick, D.R.; Ramjit, H.G. *Proceedings of the 45th ASMS Conference on Mass Spectrometry and Allied Topics*, 1997, p. 1257.
  61. Spier, J.P.; Ross III, C.W.; Kruppa, G.H.; Laukien, F.H.; Ramjit, H.G. *Proceedings of the 44th ASMS Conference on Mass Spectrometry and Allied Topics*, 1996, p. 1035.
  62. Winger, B.E.; Campana, J.E. *Rapid Commun Mass Spectrom* 1996, 10, 1811–1813.
  63. Winger, B.E.; Castoro, J.A. *Proceedings of the 44th ASMS Conference on Mass Spectrometry and Allied Topics*, 1996, p. 1029.
  64. Nawrocki, J.P.; Wigger, M.; Watson, C.H.; Hayes, T.W.; Senko, M.W.; Benner, S.A.; Eyler, J.R. *Rapid Commun Mass Spectrom* 1996, 10, 1860–1864.
  65. Ramjit, H.G.; Kruppa, G.H.; Spier, J.P.; Ross III, C.W.; Garsky, V.M. *Rapid Commun Mass Spectrom* 2000, 14, 1368–1376.
  66. Bandel, H.; Schmid, D.G.; Walk, T.B.; Jung, G. 1999. *Proceedings of the 6th International Symposium on Innovation and Perspectives in Solid Phase Synthesis and Combinatorial Chemical Libraries*, 233–236.
  67. Benner, S.A.; Ellington, A.D. *Bioorg Chem Front* 1990, 1, 1–70.
  68. Bruce, J.E.; Anderson, G.A.; Chen, R.; Cheng, X.; Gale, D.C.; Hofstadler, S.A.; Schwartz, B.L.; Smith, R.D. *Rapid Commun Mass Spectrom* 1995, 9, 644–650.
  69. Smith, R.D.; Bruce, J.E.; Wu, Q.Y.; Lei, Q.P. *Chem Soc Rev* 1997, 26, 191–202.
  70. Loo, J.A. *Mass Spectrom Rev* 1997, 16, 1–23.
  71. Wigger, M.; Nawrocki, J.P.; Watson, C.H.; Eyler, J.R.; Benner, S.A. *Rapid Commun Mass Spectrom* 1997, 11, 1749–1752.
  72. Cheng, X.; Chen, R.; Bruce, J.E.; Schwartz, B.L.; Anderson, G.A.; Hofstadler, S.A.; Gale, D.C.; Smith, R.D. *J Am Chem Soc* 1995, 117, 8859–8860.
  73. Gao, J.; Cheng, X.; Chen, R.; Sigal, G.B.; Bruce, J.E.; Schwartz, B.L.; Hofstadler, S.A.; Anderson, G.A.; Smith, R.D.; Whitesides, G.M. *J Med Chem* 1996, 39, 1949–1955.
  74. Hofstadler, S.A.; Sannes-Lowery, K.A.; Crooke, S.T.; Ecker, D.J.; Sasmor, H.; Manalili, S.; Griffey, R.H. *Anal Chem* 1999, 71, 3436–3440.
  75. Marshall, A.G. Private communication, September, 2000, National High Magnetic Field Laboratory, Florida State University.