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HPLC-MS with an Ion Trap Mass Spectrometer

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Abstract. Ion traps have become an important and powerful tool in many fields of analytical chemistry. The immanent sensitivity of the device and the ability to perform MS^n opens a wide variety of experimental setups even at very low sample concentration levels. Modern and intelligent instrument control is of particular importance. The spread of ion trap mass spectrometry will greatly enhance the amount of data generated by LC-MS laboratories. The ability to handle and screen the flood of information puts high demands on companies and research facilities to keep pace with these developments.

1. Introduction

The ion trap mass spectrometer was developed in the fifties by *Paul* and *Steinwedel* [1] at the University of Bonn. *Paul* and his colleagues have recognized the principle of using strong focussing fields for mass analysis, and *Berkling* described the operation of a quadrupole ion trap in his thesis in 1956 [2]. In *Paul*'s and *Berkling*'s early work, the mode of operation of their ion trap was the mass-selective stability mode similar to the operation of quadrupole mass spectrometers [3]. This mode limits mass range and resolution of the instrument and is not suitable for most analytical applications. The break-through of ion traps in analytical mass spectrometers was the introduction of the mass-selective instability scan by *George Stafford* and his co-workers at *Finnigan MAT* in 1983 and the discovery that the performance of an ion trap is greatly improved by the use of a helium damping gas of *ca.* 10^{-3} mbar within the trap [4].

The development of atmospheric-pressure ionization (API) techniques, electrospray ionization (ESI), and atmospheric-pressure chemical ionization (APCI) for the coupling of liquid chromatography (LC) and capillary electrophoresis (CE) with mass spectrometry made the ion trap mass spectrometer one of the most popular instruments [5–7][22].

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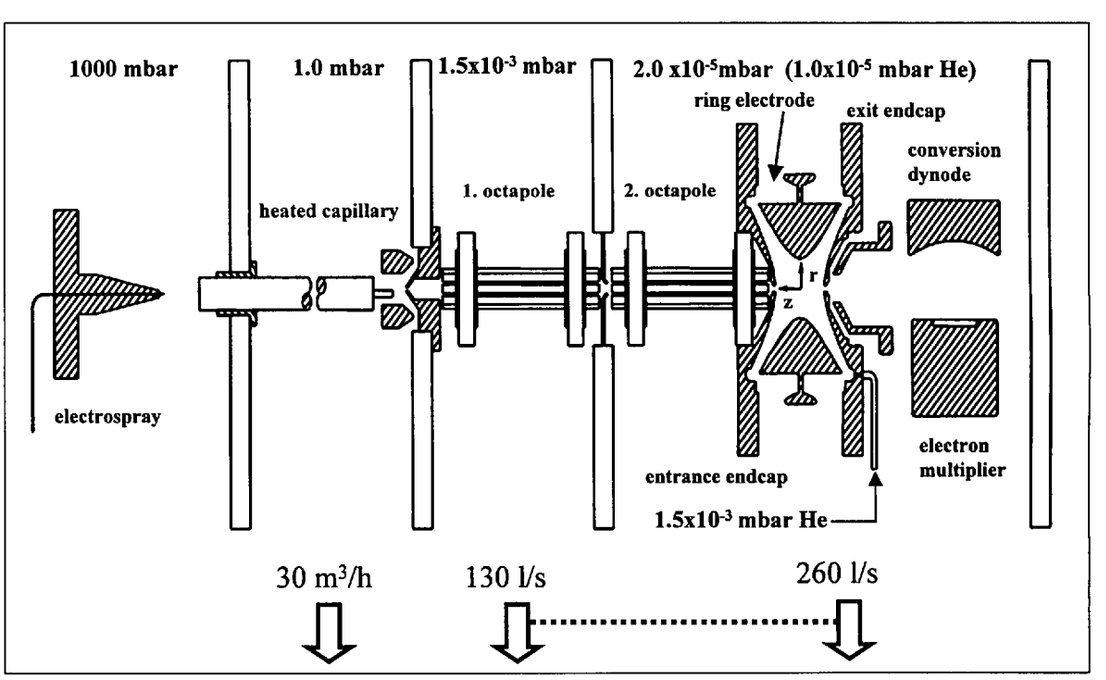

Fig. 1. *Schematic drawing of the LCQ ion trap mass spectrometer including the electrospray source and interface in front of the ion trap*

2. Ion Trap Theory

2.1. *Ion Trap Design*

The quadrupole ion trap consists of a ring electrode and two endcap electrodes. An AC-voltage (fundamental RF) of constant frequency and variable amplitude is applied to the ring electrode. When the proper RF-voltage is applied to the central ring electrode relative to the endcap electrodes, a three-dimensional, rotationally symmetric quadrupole field is generated. An ideal quadrupole field is created when

$$r_0^2 = 2z_0^2$$

where r_0 is the internal radius of the ring electrode and $2z_0$ is the closest distance between the two endcap electrodes. Externally generated ions are injected into the ion trap through a hole in the entrance endcap electrode. Due to the helium buffer gas (10^{-3} mbar) inside the trap, the ions are cooled down quickly by collisions and trapped within a volume of approximately 1 cm^3 in the center of the trap. For mass analysis, the ions are scanned out through a hole inside the exit endcap electrode. A schematic of an API ion trap mass spectrometer (LCQ ion trap design, *Finnigan*, San Jose) is shown in *Fig. 1*. In this example, ions are generated using electrospray ionization and desolvated using a heated stainless-steel capillary. A tube lens at the exit of the heated capillary focuses the ions onto the orifice of the skimmer electrode. Two RF-only octapoles serve as an ion guide and transport the beam to the ion trap, where they are injected at a kinetic energy of a few electron volts. Typical operation pressures in the different parts of the device are shown in *Fig. 1*.

The detection system of the mass spectrometer consists of a conversion dynode and a continuous dynode electron multiplier. The ions are attracted towards the conversion dynode where secondary ions are emitted and accelerated into the direction of the multiplier.

2.2. *Stability Diagram*

Ion motion within a quadrupole ion-trap mass spectrometer is described by the *Mathieu* equation. The stability diagram displays the solution of the equation of motion in a parameterized form. The coordinates of the diagram are the parameters a_u and q_u ($u = z, r$) [8],

$$a_z = -16eU/m(r_0^2+2z_0^2)\Omega^2$$
$$a_r = 8eU/m(r_0^2+2z_0^2)\Omega^2$$
$$q_z = 8eV/m(r_0^2+2z_0^2)\Omega^2$$
$$q_r = -4eU/m(r_0^2+2z_0^2)\Omega^2$$

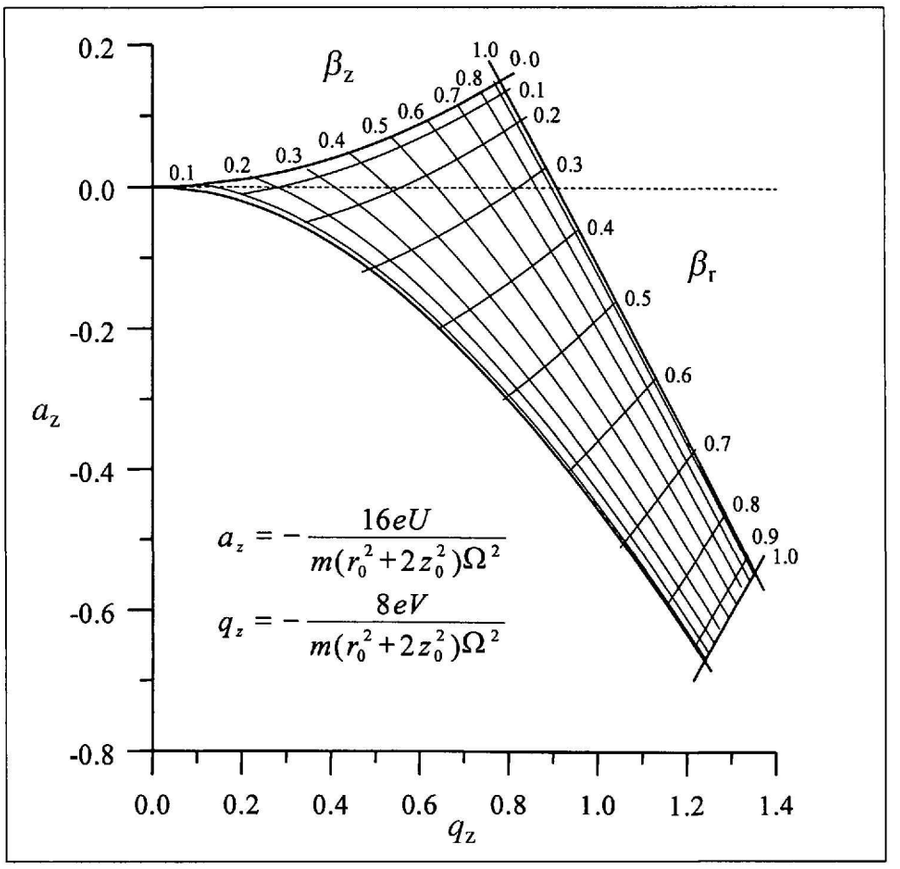

Fig. 2. *Parameterized stability diagram using the* Mathieu *parameters* a_z *and* q_z

where U is the direct current voltage and V the amplitude of the radio frequency, the RF-voltage, and Ω is the angular frequency of the RF-voltage, m the mass of the ion and e the electric charge. The most important part of the stability diagram for ion-trap operation is shown in *Fig. 2*. Stable ion trajectories are characterized by a_z,q_z values falling inside the indicated stability region. When the ion is trapped, it must be stable in both the r and z direction. The parameters β_z and β_r characterize the secular frequencies of the ions in the z and r direction. In a pure quadrupole field, these frequencies are independent from each other.

All modern commercial ion traps have a stretched geometry, in which the distance between the two endcap electrodes has been elongated. Due to these changes in geometry, the potential $\phi_{r,z}$ within the ion trap and therefore the stability diagram is affected by the presence of higher-order multipoles [6]; a truncation of the ion trap

$$\phi_{r,z} = C_0^0 + C_1^0 z + C_2^0(1/2r^2-z^2) +$$
$$C_3^0 z(3/2r^2-z^2) + C_4^0(3/8r^4-3r^2z^2+z^4) + \ldots$$

The coefficients C_n^0 correspond to the respective multipole components ($n = 0$, monopole; $n = 1$, dipole; $n = 2$, quadrupole), and for the ideal quadrupole ion trap only C_0^0 and C_2^0 are unequal to zero.

The ion motion in a three-dimensional ion trap can be described as a figure-of-eight or *Lissajous* curves and is composed of two fundamental frequencies $\omega_{r,0}$ and $\omega_{z,0}$ of the secular motion. The contribution of higher-order fields to the ion motion can be neglected. The secular frequencies can be described by

$$\omega_{u,n} = (n+1/2\beta_u)\Omega \qquad 0 \le n < \infty$$
and
$$\omega_{u,n} = -(n+1/2\beta_u)\Omega \qquad -\infty < n < 0$$
where
$$\beta_u \approx (a_u + q_u^2/2)^{1/2} \qquad \omega_u = \beta_u \Omega/2$$

for $q_r < 0.2$ and $q_z < 0.4$.

The iso-β-lines inside the stability diagram are shown in *Fig. 2*. On these lines, ions which fulfil the above equation have the same secular frequency even though their m/z values are different.

2.3. *Scan Modes*

Before ions can be mass-analyzed, they have to be trapped for a certain time. The performance of the ion trap mass spectrometer is strongly dependent on the number of stored ions. If the number of

ions inside the ion trap is too high, ion/molecule reactions may occur. A large number of ions may also generate space-charge effects, modifying the applied field. Mass assignment and resolution may be affected. To solve these problems, *Stafford* and co-workers [11] introduced a regulation mechanism, called automatic gain control (AGC). On the LCQ, for example, a fast prescan of approximately 50 ms measures the total number of ions generated by the API source. The total ion intensity is then used to adjust the gating time. If the ion current is high, the gating time is lowered, thus limiting the number of ions injected into the trap. Alternatively, if only few ions are present, the gating time is increased and more ions will be injected into the trap enhancing the sensitivity. AGC guarantees that the number of ions is always in the optimal range, even if the total ion current changes rapidly like in LC or CE.

After trapping, the ions are mass-analyzed using the mass-selective instability scan. A supplementary frequency applied to the endcap electrodes increases the performance of ion ejection. In the stability diagram, the q_z-axis intersects the $\beta_z = 1$ boundary at $q_z = 0.908$ which is q_{max} in the mass-selective instability mode. During resonant ejection or axial modulation, the ions will be scanned out at a slightly lower q_z-value [9]. In an RF-only ion trap, an ion oscillates in the quadrupole field with a characteristic secular frequency that is determined by the mass-to-charge ratio, the frequency and the amplitude of the applied alternating-current voltage. At the q_{eject}-point, the secular motion enters into resonance with the field applied to the endcap electrodes. When the ions reach the q_{eject}-value, they suddenly absorb energy and leave the ion trap which results in an improved resolution. In addition, the mass range is increased when the ions are ejected at a lower q_{eject}-value according to the following equation [10]:

$$[m/z]_{max} = 8eV_{max}/q_{eject}(r_0^2+2z_0^2)\Omega^2$$

For ion isolation, a broad band of frequencies is applied to the endcap electrodes with a notch at the resonance frequency of the ion of interest. In this way, all ions will be removed except one small mass range. The ion-isolation step is used for the selected-ion monitoring mode (SIM) and for the MS^n mode. The scan function of the RF-amplitude for an MS and MS/MS experiment is shown in *Fig. 3*. The scan function describes the timely events that will occur in one analytical scan. First, the ions are injected into the ion trap where they are cooled down by collisions with helium. When the trap is filled with a specified number of ions, applying a broad band of frequencies removing all other ions isolates the parent ion. The next step is the excitation of ions, which is done by applying an AC-voltage to the endcap electrodes. The additional AC-voltage brings the parent ions into resonance, resulting in absorption of kinetic energy. Kinetic energy is converted into internal energy by collisions with He. If the ion's internal energy exceeds the barrier for fragmentation, collision-induced dissociation occurs. The last step is the ejection of ions to the detector. A high-amplitude AC-voltage will cause resonance ejection, while a low-amplitude AC-voltage will cause resonance excitation.

For ion fragmentation, the q_z-value of the parent ion plays an important role for the trapping-potential well created within the electrode assembly of the quadrupole ion trap. The pseudopotential-well depth in the z-direction can be described by the following equation [12].

$$\bar{D}_z = eV_0^2/4mz_0^2\Omega^2 = mz_0^2\Omega q_z^2/16e$$

The potential depth depends on the mass and the q_z-value of the ion. On the LCQ, the parent ion in MS/MS mode

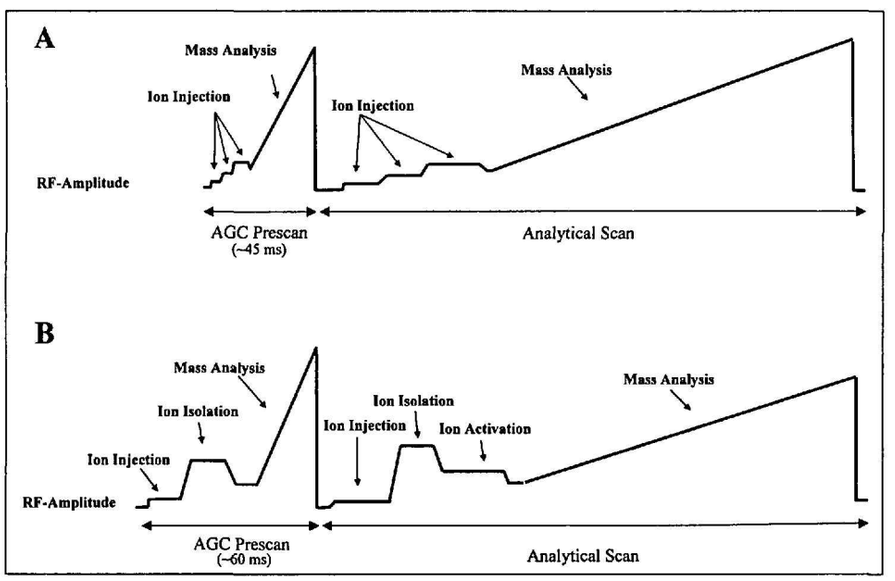

Fig. 3. *RF-Amplitude scan function on the LCQ ion trap mass spectrometer,* A) *MS full scan,* B) *MS/MS full scan*

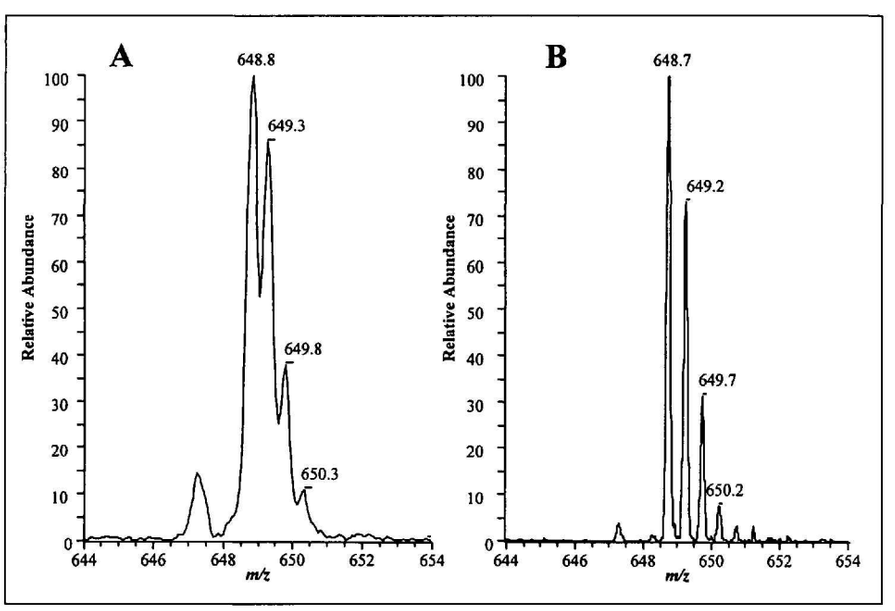

Fig. 4. *Mass spectra of the doubly charged ion of the peptide MRFA at* A) *unit resolution* (5500 Da/s) *and* B) *increased resolution* (550 Da/s)

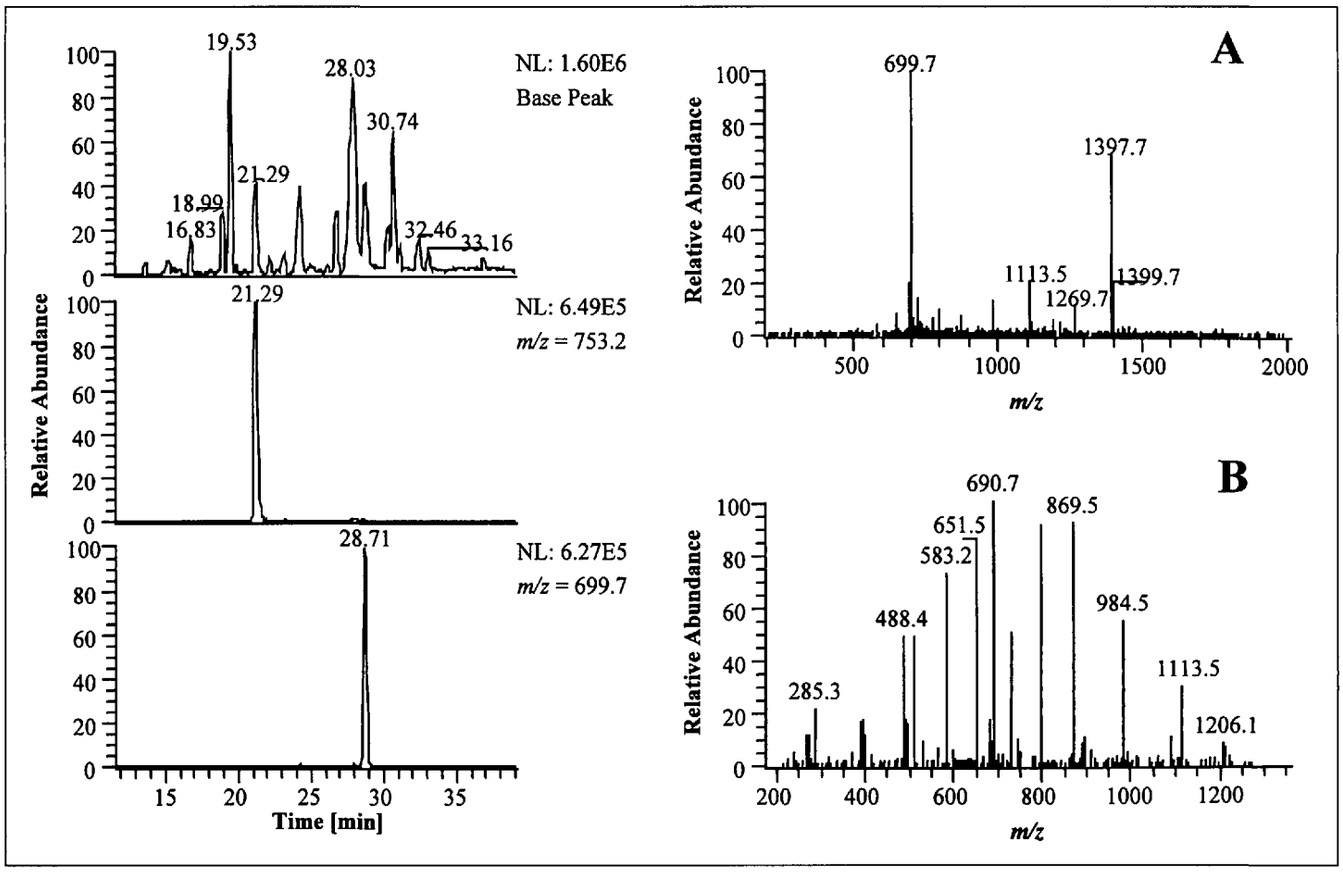

Fig. 5. *LC-MS Chromatogram of a protein digest,* A) *MS spectrum of the compound at 28.71 min,* B) *MS/MS spectrum of the doubly charged peptide ion QGVEDAFYTLVR*

normally has a q_z-value of 0.25. If the activation energy for the dissociation process is relatively high, most ions can be ejected from the ion trap before they become fragmented. Therefore, the q_z-value can be increased to deepen the potential well and to avoid the loss of the ion before it is fragmented.

The ion-trap mass spectrometer normally operates at unit resolution. The mass resolution of an ion-trap mass spectrometer depends, beside other parameters, on the time used for ion ejection. If the scan speed is reduced, an increase in resolution can be obtained (*Fig. 4*).

Fig. 4 illustrates the influence of scan speed on mass resolution. The mass spectra of the doubly charged ion of the peptide MRFA is shown on the left side using a scan speed of 5500 Da/s (unit resolution). On the right side, the acquisition was done using a scan speed of 550 Da/s. The lower scan rate of 550 Da/s enables the unequivocal determination of the charge state of multiply charged ions (up to a charge state of 5) simply by the mass difference of a peak relative to its isotope.

3. Applications

Some applications from different fields illustrate the power of ion-trap mass spectrometry in analytical chemistry. The sensitivity and the ability to perform MS^n are important for peptide sequencing, or structure elucidation of carbohydrates and many other compounds.

3.1. *Peptide and Protein Identification*

In proteomics, the identification of proteins or protein modifications like phosphorylation or glycosylation is of key importance. Mass spectrometry, especially ion-trap mass spectrometry plays a major role in these experiments, due to the high sensitivity offered in MS and MS/MS [13][14]. The use of micro- and nano-HPLC columns further improves sensitivity, and the identifications of proteins in the femtomole range is almost routine. Among the different strategies to identify proteins by mass spectrometry [16], the *Sequest* search program introduced by *Eng* and *Yates* [17][20] has developed into a very powerful tool. It makes use of the fact that a protein can easily be identified even from very large databases (protein or DNA databases, *e.g.*, swissprot), if a partial sequence of the protein is know. The *Sequest* algorithm uses the MS/MS spectrum of a peptide of the enzymatically-digested protein to identify the partial sequence. In *Fig. 5*, a product-ion spectrum of the doubly charged peptide ion QGVEDAFYTLVR from a tryptic digest of Ras protein is shown.

The fragmentation of the doubly charged ion yields a series of y- and b-ions [15] which is characteristic for the sequence of this peptide. By making use of the peptide mass and its MS/MS spectrum, *Sequest* is able to identify the protein fully automatically, even without the need to interpret the CID spectrum of the peptide.

3.2. *Structure Elucidation of Digitonin*

Saponins are a large and widely distributed group of plant substances. They are mostly glycosylated and, depending on the nature of their aglycon, they are divided into triterpene-, steroid-, and steroid-alkaloid saponins. Many saponins have antibiotic activity, mainly against lower fungi. In *Fig. 6*, two MS^n spectra of digitonin are shown (negative-ion mode).

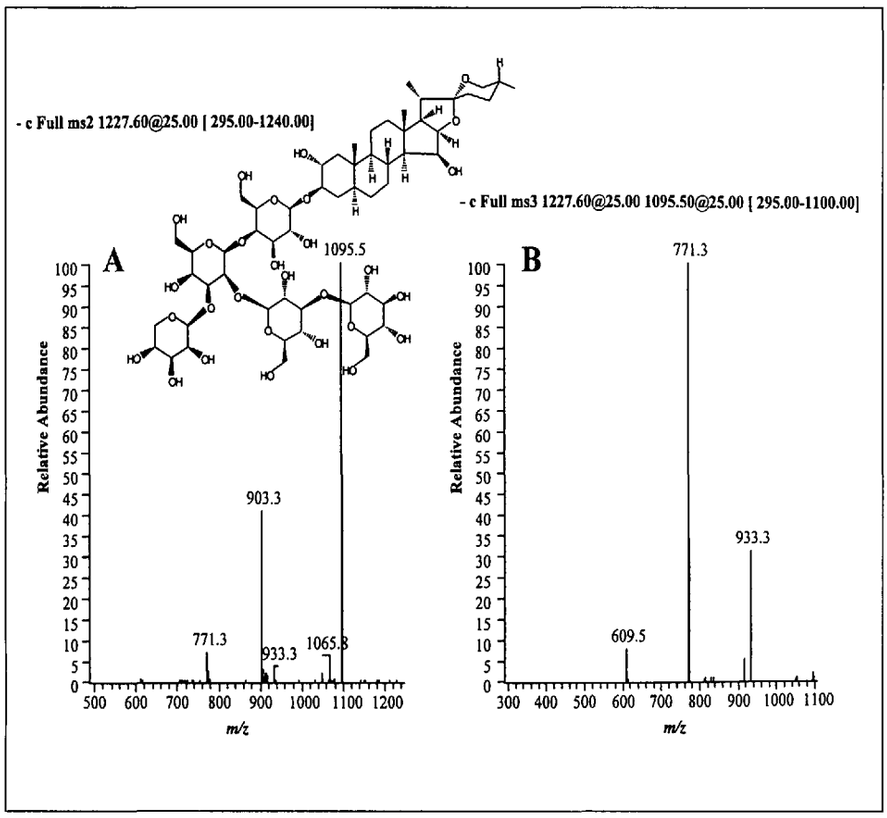

Fig. 6. *MS^n Spectra of digitonin,* A) *MS/MS of* m/z *1127.6 and* B) *MS^3 of 1095.5.* A relative collision energy of 25% was used in all experiments.

In the MS^n spectra, the successive loss of the sugar moiety can be observed. The aglycon itself is much more stable and does not decay under normal fragmentation conditions. In some cases, the use of a higher q_z-value for the parent ion in the MS^n experiment is helpful to fragment even such stable ions.

3.3. *LC-MS/MS of Tetracyclins*

Tetracyclins were first discovered in the late 1940s; they are highly effective, broad-spectrum, bacteriostatic antibiotics. Tetracyclins are effective against a wide variety of gram-positive and gram-negative bacteria, rickettsia, and the large virus-like agents that cause trachoma and related diseases. They are partly effective against some biologically more complex protozoan parasites. Tetracyclins are administered orally, usually for acne or urinary tract infections. In *Fig. 7*, a LC-MS/MS analysis of a mixture of four tetracyclins is shown (positive-ion mode).

Three of the compounds in this example show the same molecular weight. Differentiation between two of them can be achieved, however, by MS/MS due to the completely different ion intensities for the

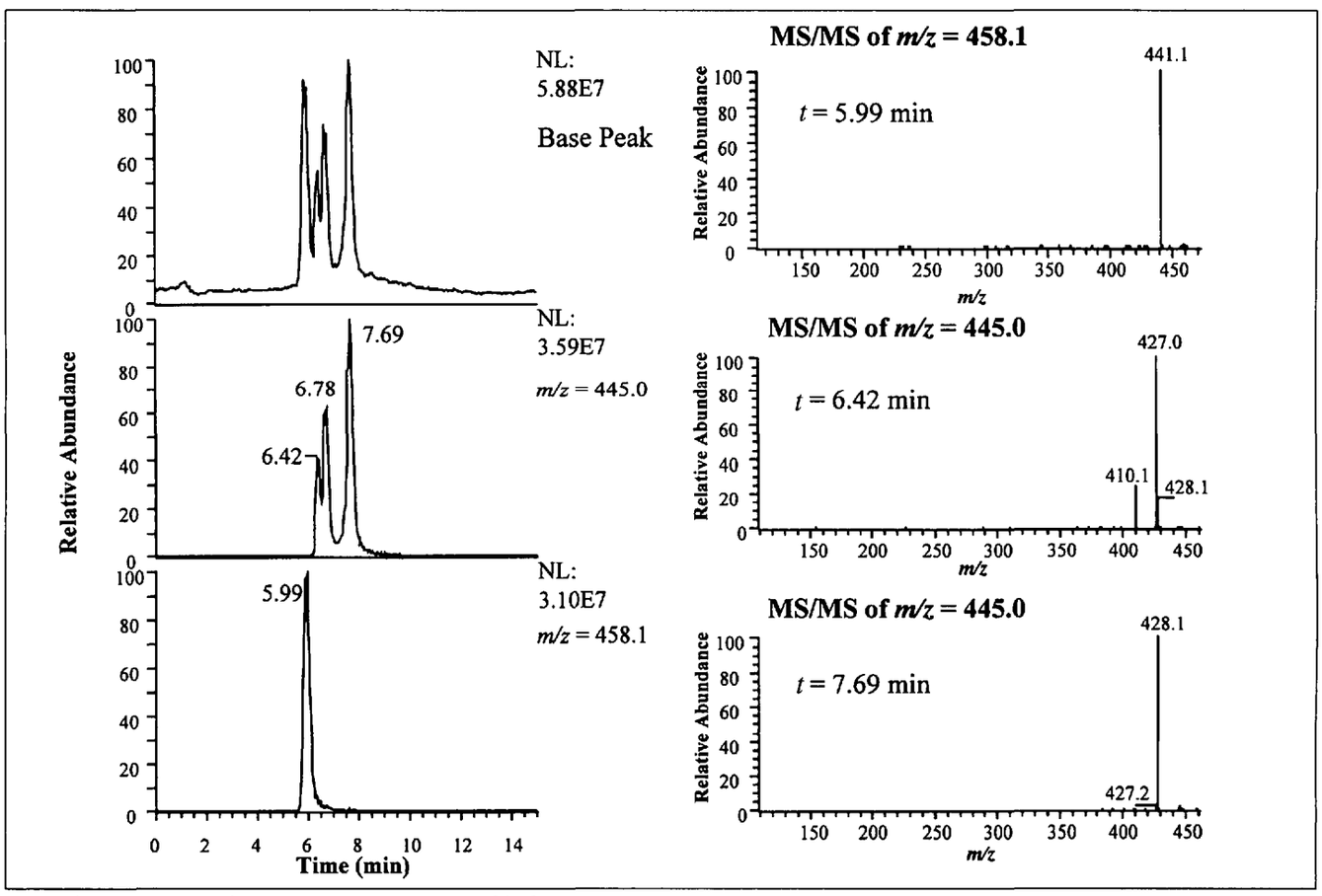

Fig. 7. *Chromatogram of a tetracyclin mixture and MS/MS spectra*

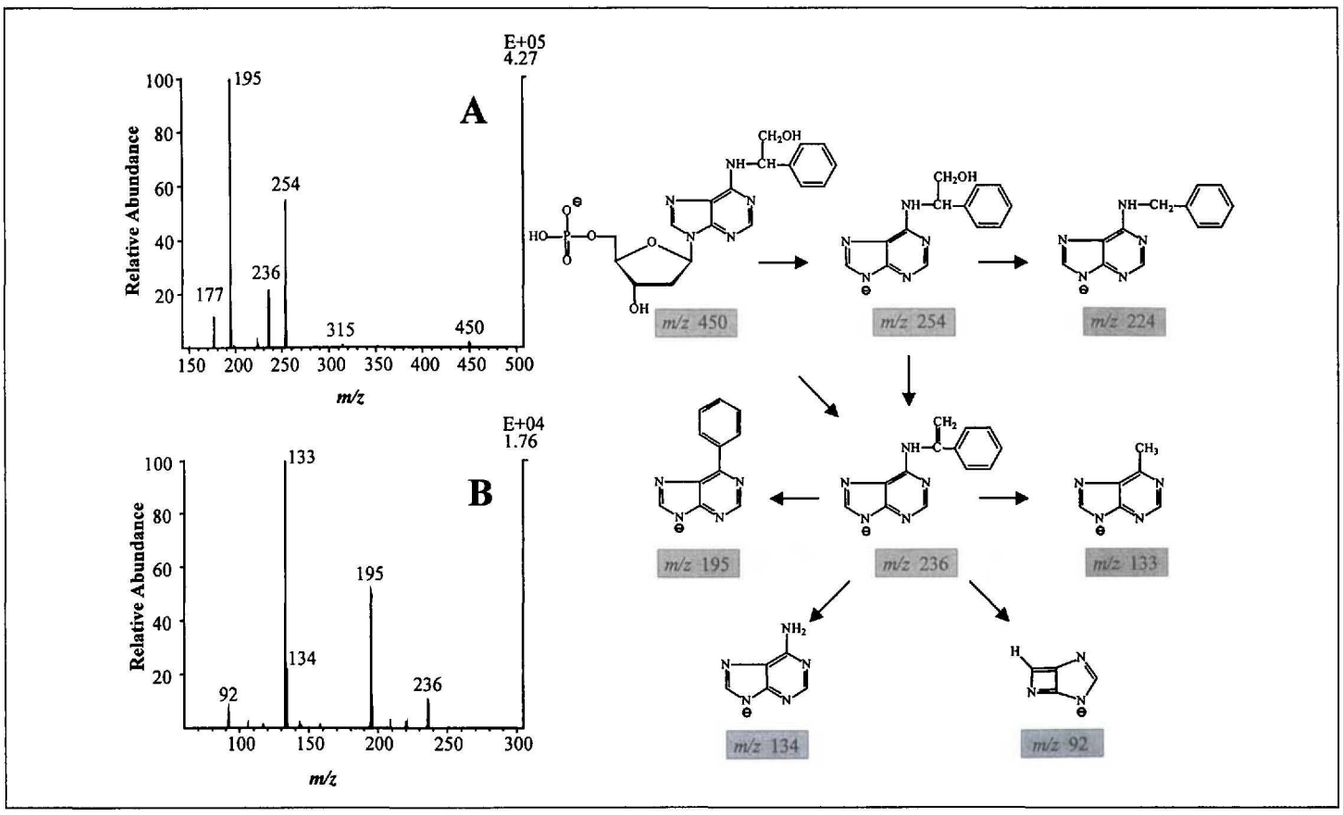

Fig. 8. *MS^n Spectra of adenosine-5'-monophosphate styrene oxide adduct* (α-SO-N^6-d(pA)), A) *MS/MS of the ion* m/z *450*, B) *MS^3 of the Ion* m/z *236*

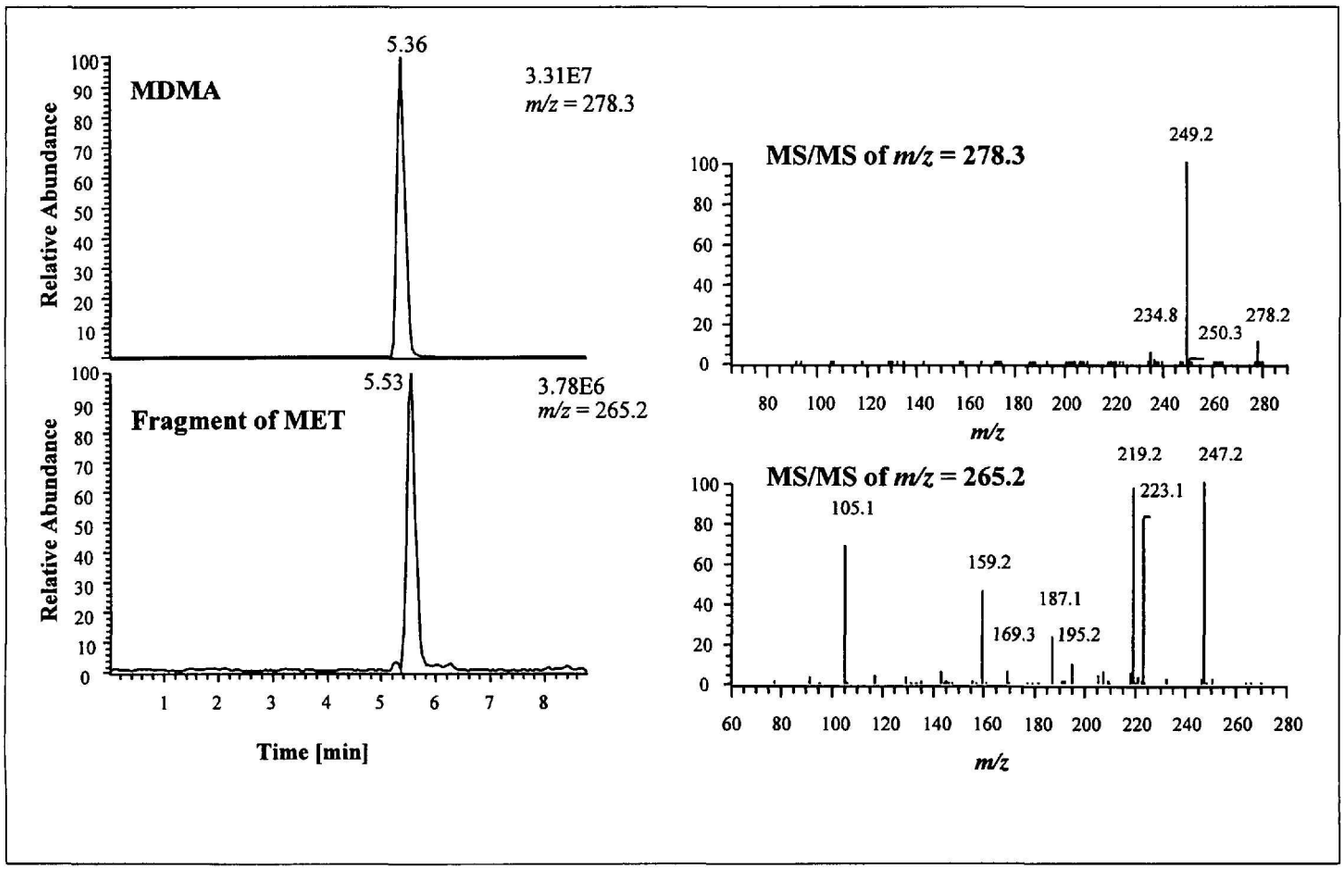

Fig. 9. *Electropherogram and MS/MS spectra of 3,4-methylenedioxymethamphetamine* (MDMA, *m/z* 278.3) *and of a fragment of methadone (MET, m/z 265.2)*

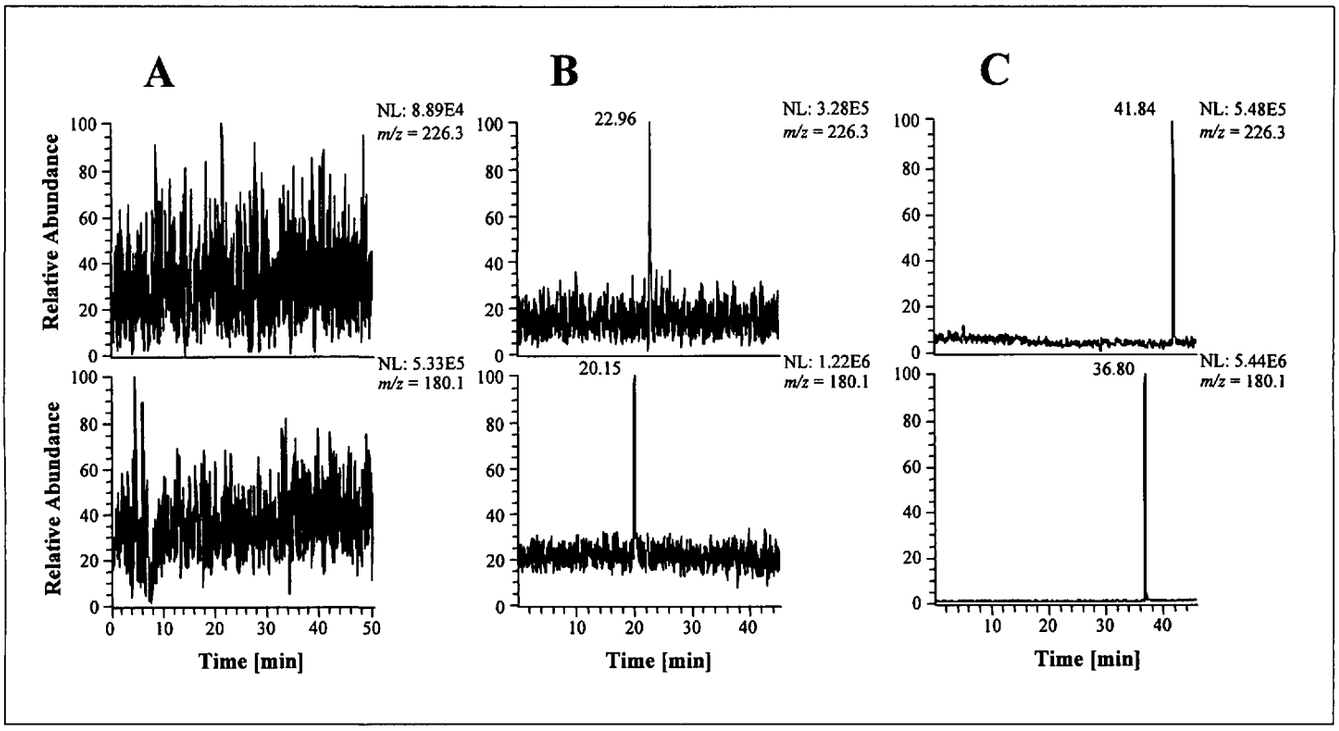

Fig. 10. *Electropherograms of amphetamine:* A) *CE-MS,* B) *transient ITP-CE/MS,* C) *counterflow ITP-CE/MS*

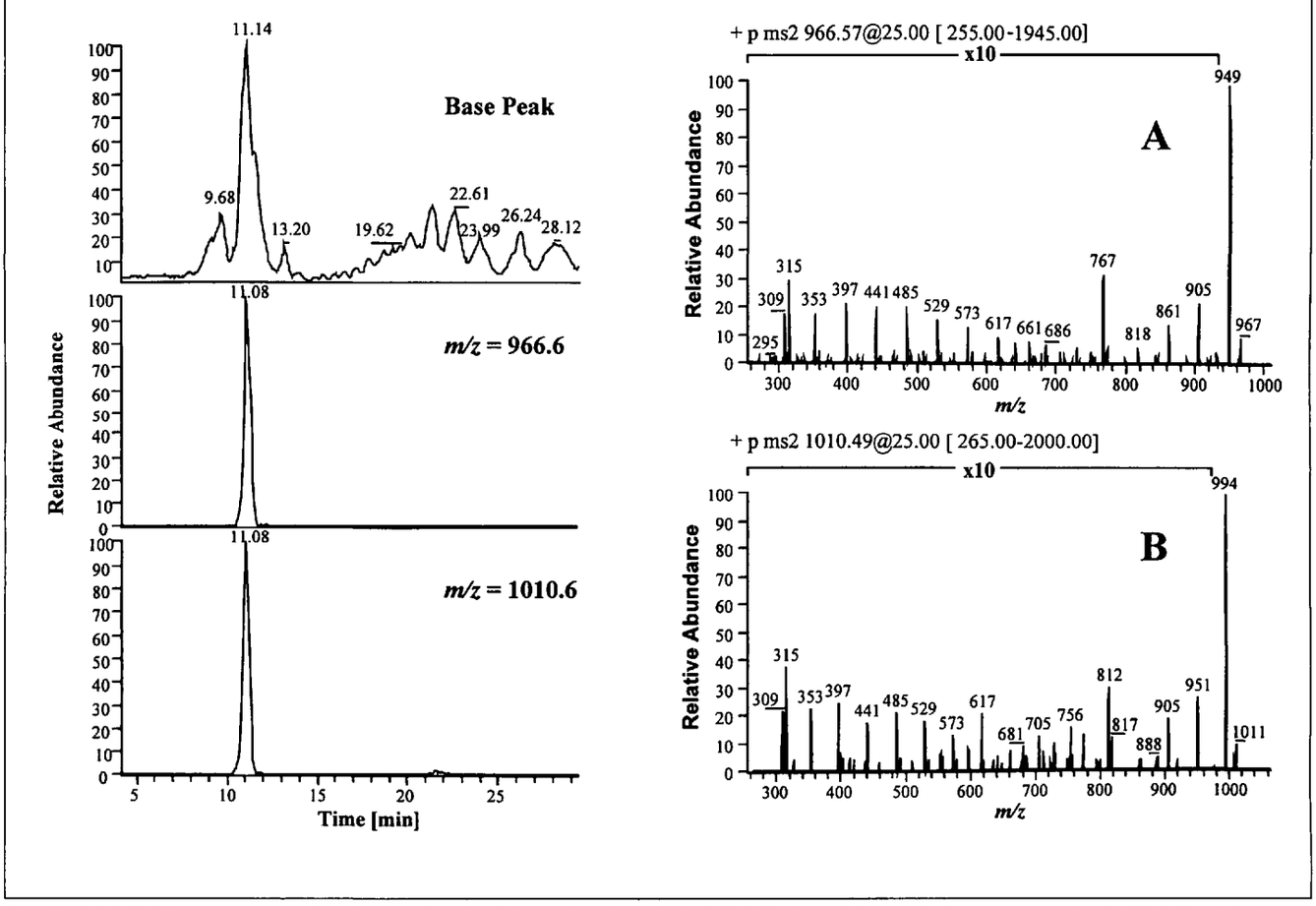

Fig. 11. *LC/MS Chromatogram of a mixture of polyethylene glycols and the MS/MS data of the coeluting compounds,* A) *MS/MS of the ion at* m/z *996.6,* B) *MS/MS of the ion at* m/z *1010.6*

$[M+H]^+-18$ (loss of water) and $[M+H]^+-17$ (loss of ammonia). The compounds at 6.42 min and 6.78 min gave the same MS/MS spectra. Further information about the structure can be achieved by MS^3 or by the use of wide-band activation [21].

3.4. *Structure Elucidation of Modified Nucleotides*

The ability to measure chemically induced DNA damage in specific tissues is important in risk assessment, because alteration of DNA appears to be a key step in the initiation of carcinogenesis. MS^n can successfully be used in the structure elucidation of a styrene oxide adduct of adenosine-5'-monophosphate that is formed in the reaction of DNA with the xenobiotic styrene oxide [18]. The DNA was digested to the respective nucleotides using the exonuclease Nuclease P1. For the investigation of the modification site, successive fragmentation in up to MS^4 experiments is sometimes necessary.

The fragmentation pathway of (α-SO-N^6-d(pA)) is shown in *Fig. 8*, and the data from the MS^3 experiment unambiguously yields the site of modification (negative-ion mode).

3.5. *CE/MS of Amphetamines*

Amphetamines are a class of synthetic drugs that are strong stimulants of the central nervous system. They are used for increasing alertness, reducing hunger, and for the treatment of some forms of depression and the sleep disorder called narcolepsy. They found increasing nonmedical use as 'pep pills' or 'uppers' for staying awake. They also gained popularity as recreational drugs, since large doses produce exhilaration like that from cocaine but more prolonged.

Amphetamine-heroin mixtures, called 'speedballs' after earlier cocaine-heroin mixtures, gave rise to the name 'speed' for injections of amphetamine alone. Later manifestations of amphetamine abuse include 'ecstasy', a combination of methamphetamine with a synthetic psychedelic drug (MDMA), and a smokable form of crystalline methamphetamine called 'ice'. The latter is extremely potent and has been the cause of long-lasting psychological damage and severe lung and kidney disorders.

In *Fig. 9*, the CE-MS electropherogram of a urine extract is shown (positive-ion mode). The amphetamines are identified by collision-induced fragmentation [19].

3.6. *ITP-CE/MS of Amphetamines*

The sensitivity in CE/MS can be enhanced using on-line preconcentration techniques like isotachophoresis (ITP). ITP makes use of the strong focussing effects of the field within the CE capillary, if buffers of different conductivity are employed. In ITP-CE, the capillary is filled with a leading buffer, a relatively broad zone of sample solution, and a terminating buffer. The leading buffer has the highest conductivity, the terminating buffer has the lowest conductivity, and the conductivity of the sample is in between. When the ITP step is finished (*e.g.*, 15 kV for 15 s) the terminating buffer, is exchanged against the leading buffer and normal CE is performed (transient ITP-CE/MS). In counterflow ITP-CE/MS, an additional step is performed, which repositions the sample region at the inlet side of the CE capillary. Counterflow ITP-CE yields better resolution due the increase in effective capillary lengths. Therefore, the sample volume can be increased. In *Fig. 10*, a comparison between CE/MS, transient ITP-CE/MS, and counterflow ITP-CE/MS is given. At the used concentration level, simple CE without on-line sample concentration gave no detectable signal, whereas with ITP, the amphetamines can be unequivocally identified. Use of counterflow ITP-CE/MS even improves this result, and better resolution and lower detection limits can be achieved. Due to the increase in effective capillary lengths, the analysis time is increased.

3.7. *Special Applications*

In modern ion traps, an LC/MS analysis including MS^n can be performed in an automated fashion without any prior knowledge about the sample composition. The experiment has been described as data-dependent™ analysis [18] and is usually activated by a threshold intensity of a mass of an ion. If the intensity of an ion (any ion within the API spectrum, or an ion from a predefined list) exceeds the threshold, the data system switches from full-scan mode to MS/MS and collects the CID spectrum of the compound. As soon as the ion intensities falls below the threshold, the system switches back to normal full-scan conditions.

Concerning the LCQ ion-trap mass spectrometer, an improvement in automation has been introduced recently. A method called dynamic exclusion™ allows to automatically perform MS/MS of coeluting compounds even if no separation is achieved. In *Fig. 11*, a LC/MS chromatogram of different polyethylene glycols is shown (positive-ion mode). The principle of data-dependent analysis and of dynamic exclusion enabled the automated acquisition of hundreds of MS/MS spectra within one analytical run. The new features are extremely powerful and will stimulate research in many fields of application, like metabolite profiling. In combination with other improvements, like normalized collision energy [21] and wide-band excitation [21], MS/MS libraries are already within the reach of a routine LC-MS laboratory and will become an important tool in compound characterization.

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