CHAPTER 10
STRUCTURE ELUCIDATION OF UNKNOWN POLLUTANTS IN ENVIRONMENTAL SAMPLES BY COUPLING OF HPLC TO NMR AND MS

Karsten Levens1, Alfred Preiss1 and Manfred Spraul2
1Fraunhofer-Institute of Toxicology and Medical Research, Nikolai-Fuchs-Str. 1, D-30625 Hannover, Germany,
2Bruker BioSpin GmbH, Silberstreifen, D-76287 Rheinstetten, Germany

ABSTRACT
HPLC coupled simultaneously to NMR and MS is particularly suited to the identification of unknown polar, non-volatile compounds in complex environmental samples, i.e. to non-target analysis of such samples, if pollutants are present in the ppb range. Both methods give complementary structural information. Optimum information on unknowns is gained if both techniques are coupled on-line.
This review discusses the fundamental aspects of on-line HPLC-NMR, HPLC-MS and HPLC-NMR-MS and compares the advantages and disadvantages of these methods for target and non-target analysis with emphasis on their potential for the structure elucidation of unknowns. The application of these hyphenated techniques to environmental samples is illustrated with contaminated ground water from a former ammunition plant, leachate from industrial waste disposal sites, waste water from a textile company and soil polluted with polycyclic aromatic hydrocarbons as examples.
1 INTRODUCTION
The pollution of the environment by a large variety of chemicals represents a particular
challenge to the analytical chemist. This is especially the case for hazardous waste sites
where both soil and ground water may be contaminated by complex mixtures of organic
chemicals, comprising compounds with very different polarities present in a wide range
of concentrations. Industrial waste water or leachates from industrial waste disposal
sites are other examples where pollutants are present as complex mixtures.
Identification of the constituents is difficult as not only the original compounds released
into the environment are present in these samples. Chemical, photochemical and
microbiological transformation processes may have led to additional and unexpected
products.

Monitoring of such environmental samples is usually restricted to known or
suspected compounds or compound classes ("target analysis") for which specific
methods have been developed and optimised. Identification of unknown compounds
("non-target analysis") is much more difficult. Here, sample extraction and separation
must take into account the very different (and a priori unknown) physical and chemical
properties of the individual organic compounds, while the instrumental method must
have high separation efficiency and provide optimum structural information. For
compounds of high to medium volatility which are thermally stable, gas
chromatography coupled to mass spectrometry (GC-MS) under electron impact
conditions is the method of choice as this method combines the high separation
efficiency of the GC with the structural specificity of MS. Large MS libraries are
available for compound identification and the fragmentation mechanisms of the odd
electron molecular ions formed under electron impact conditions are well documented.
Moreover, quantification is readily possible, in particular if isotopically labelled internal
standards are used, which compensate both for reduced recovery during extraction and
variation in the instrumental response.

Polar, non-volatile or thermally labile compounds in environmental samples
are more difficult to identify. For GC or GC-MS analysis, these compounds can be
derivatized to enhance their volatility. This approach, although time-consuming is used
frequently for target analysis, but can hardly be used if the compound to be analysed is
completely unknown.

Although high performance liquid chromatography (HPLC) is well suited to
the separation of polar or thermally labile compounds this method is hardly suited to the
structure elucidation of unknowns, even if a photodiode array detector is used.
Moreover, the separation efficiency of HPLC is significantly poorer than that of GC.
Finally, detection by photo diode array is only possible if a chromophoric group is
present in the molecule.

More structural information is available if HPLC is coupled to MS which is
now widely used for the analysis of polar, non-volatile compounds in environmental
samples [1-3].

While this technique is ideally suited to the analysis of known or suspected
compounds (even if a chromophoric group is lacking), the structural elucidation of
unknowns by this approach remains difficult, as discussed below.

Although nuclear magnetic resonance (NMR) has been well-known as a
powerful technique for structural elucidation for many decades, this technique was
generally considered to be too insensitive for the analysis of organic compounds in
environmental samples.
However, with the advance of modern high-field NMR instruments and cryoprobes, the sensitivity of this method was significantly improved. Moreover, as a result of the very narrow NMR signals the "resolution of information" provided by this method is good [4] and was continuously increased by developing instruments of higher magnetic field strength (see below). It was shown by us that NMR (without any chromatographic separation) can be applied to a direct mixture analysis of environmental samples [4-6]. However, the resolution of analytical information may be further enhanced if the NMR spectrometer is on-line coupled to chromatographic systems, for instance to HPLC [7,8]. Since the commercial introduction of HPLC-NMR systems, this hyphenated technique has been mainly applied to the structural elucidation of metabolites in drug development studies [9,29,36] and the identification of natural compounds in plant extracts [10,30,32]. We have demonstrated the application of this technique to the analysis of environmental samples [11-14,31]. However, the optimum structural information is obtained if both HPLC-MS and HPLC-NMR are used together to identify unknowns in complex mixtures. This is demonstrated in this review using the analysis of contaminated ground water in the vicinity of former ammunition production sites, leachates from industrial waste disposal sites, industrial waste water and soil contaminated with polycyclic aromatic hydrocarbon as examples.

In the following, first the HPLC-NMR and the HPLC-MS techniques are presented and their advantages and disadvantages, in particular for structure elucidation of unknowns, is discussed. In part 4 follows a discussion of on-line HPLC-NMR-MS while the application of these techniques to environmental samples is reported in part 5.

2 HPLC-NMR
There are two basic requirements for the coupling of HPLC to NMR [9,27,28]:

a) The sample tubes in conventional probes have to be replaced by a flow through cell. Today this is usually a U-shaped glass with an i.d. of 1.7 - 4 mm inserted vertically into the magnet with resulting detection volumes of 30 - 200 µl (the detection volume should not be larger than the HPLC peak volume, which can be approximated by 2 x the peak width at half-height times the flow rate). The RF transmitting and receiving coils are directly fixed to the outside of the glass cell to obtain an optimum "filling factor" (this is the ratio of the sample volume to the volume inside the detector coil) for maximum sensitivity. The flow cell should be designed in such a way that a laminar flow of the HPLC eluent is achieved to maintain the chromatographic resolution. With such an arrangement, an adequate spectral resolution can be achieved.

b) When HPLC is coupled to NMR using proton-carrying solvents the resonance signals of the mobile phase are by far the most intense signals in this spectrum. With spectrometer systems currently available, a direct detection of weak analyte signals in the presence of strong solvent signals is not efficient due to the limited dynamic range of the digitizer and the receiver. In principle, this problem could be overcome by using fully deuterated solvents. Indeed this approach is used if solid phase trapping of the analyte is employed as described below. However, in normal HPLC-NMR experiments, such as on-flow runs, the use of fully deuterated solvents is often too expensive. Today, very efficient methods for suppression of the solvent signals exist, which are based on conventional presaturation techniques or newer pulse sequences such as WET (water suppression enhanced through T1 effects). However, multiple solvent suppression leaves a larger observable window if D2O is used in the mobile phase instead of H2O, where the costs of D2O are
relatively modest. Multiple suppression is done using phase modulated shaped pulses, that are combined with the WET or presaturation based sequences. To improve the observable spectral window, it is also possible to run the spectra with carbon decoupling, to get rid of carbon satellites of the solvent signals.

2.1 Coupling modes
Coupling of HPLC to NMR can be achieved in four modes [9,15]:

a) In the "stopped flow" mode, the entire chromatographic process is halted after a defined transfer time, after which the centre of the HPLC peak, that is detected using another spectroscopic method like UV, will be located in the NMR measuring cell. Now one- or two-dimensional experiments can be performed for several hours. If the flow is halted for less than 2 h, peak broadening due to diffusion will be refocussed on the column again and therefore the subsequent HPLC peaks will not be affected, while at longer times peak broadening may occur. Isocratic separation should be avoided as this will also lead to peak broadening of the later eluting peaks.

b) To overcome this problem and to save HPLC operation time, various fractions of the chromatogram or parts of the chromatographic peaks can successively be transferred into loops without interruption of the HPLC run. The Bruker Biospin company developed such a loop trapping unit (termed BPSU) with 12 or 36 loops which is under complete computer control. Peaks stored in the loops are subsequently transferred to the NMR cell for measurement [17]. LC-peak shapes are retained in the loops, with the 36 loop system the loops can additionally be cooled to minimize diffusion, which is very small anyhow due to the thin capillaries.

c) Alternatively, the analytes within a HPLC peak may be trapped by solid phase extraction (SPE). In contrast to normal on-line – SPE - HPLC in which the SPE cartridge is arranged in front of the HPLC column for sample clean-up and pre-concentration, in HPLC-NMR such SPE cartridges have recently been arranged after the column for an intermediate trapping of the analyte. This is achieved by post column addition of water which focuses the analyte at the beginning of reversed phase SPE cartridge. By repetitive trapping of a HPLC peak (e.g. by trapping the peak four times) a substantial enrichment of the analyte is achieved. In the next step the cartridge is dried by nitrogen gas to remove the remaining water and all other solvents. Subsequently pure deuterated solvents (such as deuterated acetonitrile) are used to flush the enriched analytes from the cartridge into the NMR probe. This results in a very narrow elution band (elution volume ~ 25 µL) and a significant increase of the signal to noise (S/N) ratio for the NMR which depends on the eluting peak volume with and without peak trapping, the probe volume and the peak broadening during transfer (see also Fig. 1). The Bruker company uses a modified commercial SPE unit with trays of 192 cartridges for this peak trapping. Again the SPE unit is under computer control.

d) In the "on-flow" mode, the eluent is continuously transferred to the NMR measuring cell. While the mobile phase with analytes flows through the cell, the spectrometer records 1D spectra. These spectra are stored and may be subsequently presented as a function of the (chromatographic) time analogous to HPLC-DAD, GC-MS or HPLC-MS, where UV or mass spectra are continuously acquired and stored and a UV spectrum or a mass spectrum may be represented as a function of time at fixed intervals. Alternatively, the total HPLC-NMR experiment can be represented as a
pseudo-two-dimensional HPLC-NMR chromatogram or contour plot, where the chemical shift values are plotted on one axis, the retention times on the second axis of the graph and the intensities of the signals are represented by contour lines (s. below).

2.2.1 Sensitivity

As compared to HPLC-MS with electrospray (ESI) or atmospheric pressure chemical ionisation (APCI) HPLC-NMR is a much less sensitive (approx. by a three orders of magnitude) which excludes the application of this technique to ultra trace analysis. The signal to noise ratio of an NMR peak is given by equation (1)

\[
S/N \sim \frac{\omega \cdot B_0}{\sqrt{k \cdot T \cdot (R_S + R_{WHIRL}) \cdot \Delta f}} \cdot S_I
\]

(1)

where
\( \omega \) is the Lamor frequency, \( B_0 \) = the magnetic field, \( S_I \) = the coil sensitivity, \( k \) = coupling factor, \( T \) = absolute temperature, \( R_S \) = serial resistance, \( \Delta f \) = receiver band width.

Figure 1. Sensitivity increase in HPLC-NMR using SPE trapping and a cryoprobe
It is apparent from equation (1) that the signal to noise ratio, S/N, is directly proportional to the magnetic field strength and inversely proportional to the square root of the absolute temperature. Thus the S/N ratio and therefore the sensitivity of the NMR instrument can be improved by increasing the field strength. Consequently superconducting magnets with steadily increasing field strengths (expressed in MHz) were developed in the last years, recently 900 MHz magnets were introduced. Such large magnets are not only very expensive and require a significant space for the entire instrument to be installed. If no additional measures are taken they also have a wide ranging stray field which makes it impossible to couple other instruments nearby. Thus in recent years magnets with actively shielded magnet and field strength of up to 800 MHz were developed which permit to arrange the HPLC instrument < 2 m apart from the magnet without interference. These shielded magnets are a further prerequisite for a successful HPLC-NMR coupling.

Equation (1) demonstrates that there is an alternative way to increase the sensitivity, i.e. to lower the temperature of the coil of the probe. Cryoprobes have been developed in recent years by the Bruker company and are now also available as flow through probes for HPLC-NMR applications for instruments up to 600 MHz [17]. With such cryoprobes an increase in the S/R ratio of up to a factor of 8 has been observed. The use of cryoprobes (in which the coils are cooled down to 15 °C) and repetitive SPE trapping are (as compared to an increase of the field strength) without doubt the less expensive ways to increase the overall sensitivity of the HPLC-NMR system. The impact of the various recent modifications and improvements of HPLC-NMR systems on the sensitivity is illustrated in Fig. 1 using a 500 MHz instrument and a quercitine glycoside as example[17]. In this figure the S/N ratio for a loop collection is compared with a single SPE and a fourfold SPE trapping and finally with a fourfold SPE trapping and the additional use of a cryoprobe where the latter arrangement leads to an increase in the S/N ratio by a factor of 70 as compared to simple loop collection.

Thus with these improvements in sensitivity analytes which are in the measuring cell in the low to medium nanogram range can now be measured.

2.3 Structure elucidation

With HPLC/\textsuperscript{1}H NMR experiments, chemical shift values, peak multiplicities and coupling constants and retention times can be used for structural elucidation. Complete chromatographic separation, which is often difficult with complex environmental samples, is not a prerequisite. As a result of the very narrow resonance signals, in many cases several distinct compounds within one NMR spectrum can be identified simultaneously. In particular, coelution of two compounds generally does not present a problem. For sensitivity reasons, HPLC-NMR measurements will often be restricted to \textsuperscript{1}H and \textsuperscript{19}F NMR. In particular, direct HPLC/\textsuperscript{13}C NMR will probably not be realised in the near future, but several correlation spectra such as heteronuclear multiple quantum coherence (HMQC), heteronuclear single quantum coherence (HSQC), 2 D total correlation spectroscopy (TOCSY) and 2 D nuclear Overhauser effect spectroscopy (NOESY) have been successfully acquired in the past to obtain additional structural information [10,27].
2.4 Quantification

It is often overlooked that NMR is well-suited to quantification, which is particularly important in environmental analysis. Quantification of a mixture is based on the following equation:

\[
\frac{c_s}{c_a} = \frac{n_s}{n_a} \frac{F_a \cdot M_a}{F_s \cdot M_s}
\]

where

c stands for the concentration and the subscript s and a stand for the internal standard and analyte, respectively. n represents the number of protons generating the NMR signal selected for quantification, F the area of the NMR signal and M the molecular weight of the compound.

For quantification an internal standard has to be added to the sample. It is of advantage that quantification is possible without acquiring calibration data. Quantification is even possible when no reference compounds are available.

In HPLC-NMR quantification is carried out in the on-flow mode as described previously [16].

2.5 Advantages and disadvantages of HPLC-NMR in environmental analysis

HPLC-NMR uses the high structural information of the NMR technique which is of particular advantage in non-target analysis. Due to the low analyte concentration in environmental samples and the limited sensitivity of NMR discussed above, HPLC-NMR is often restricted to $^1$H NMR and the full potential of two-dimensional NMR techniques not always available. Thus, identification of unknowns will not be possible in every case. The technique is particularly powerful for the differentiation of isomers, while the differentiation between functional groups leading to similar chemical shift values of neighbouring protons (e.g. COOH versus NO$_2$ group) is not always easy.

Of particular importance is the high "resolution of information" available for small molecules. This "resolution of information" has been defined for NMR as the ratio of the expected chemical shift range to the width of the resonance signals (in Hz [4]) and may be as large as 3000. (Note that the presence of several resonance signals even for small molecule reduces this number). If, for chromatographic methods, such a "resolution of information" is defined as the ratio of the maximum retention time to the peak width, it becomes apparent that e.g. for HPLC this "resolution of information" is much poorer than for NMR (e.g. 100).

As a result of the coupling of the two independent analytical methods, the chromatographic resolution of all analytes in a complex environmental sample is not a necessary prerequisite for their identification by HPLC-NMR. This is demonstrated in Fig. 2 which shows the results of an HPLC-NMR experiment of an artificial mixture of aromatic acids (which have been identified or were suspected in the leachate of an industrial waste disposal site). The figure represents the $^1$H NMR spectra as a function of the retention time and shows that the high "resolution of information" of the NMR methods allows the identification of several analytes in one NMR spectrum, while all analytes are only resolved on the chemical shift axis if NMR is coupled to HPLC (even with modest resolution).
Compounds with a wide range of polarities may be analysed by HPLC-NMR, including thermally labile and non-volatile compounds which are not amenable to GC-MS. The identification of this class of non-volatile compounds in environmental samples in particular, has been difficult up to now.

In contrast to GC-MS and HPLC-MS, HPLC-NMR is a non-destructive method. Thus, the sample can be retrieved after analysis and then analysed by other methods.

It is of further advantage that in $^1$H NMR the detection response of an analyte directly reflects its concentration (i.e. for a given molecular mass the signal intensity expressed as peak area only depends on the concentration of the compound and the number of equivalent protons), while with HPLC-MS, the detection response depends on the ionisation efficiency, i.e. the chemical properties of the analytes (see below).

Finally, as mentioned above, quantification of analytes is possible in the on-flow

Figure 2. Dispersion of the NMR information by the chromatographic process (by courtesy of Elsevier).
mode without reference compounds and without calibration.

A main disadvantage of HPLC-NMR is its modest sensitivity, which in general is three orders of magnitude lower than that of HPLC-MS. Thus, ultratrace analysis in environmental samples will not be possible without sample pretreatment. Moreover, if analytes are present at low concentrations in the sample (i.e. in the upper ppt range) the technique is time-consuming as several 1000 FIDs have to be accumulated in an overnight run to obtain an acceptable signal-to-noise (S/N) ratio. Here the above mentioned method of multiple SPE trapping will be of particular advantage. It is of further disadvantage that solvent impurities are often of the order of the analyte signals and that the solvent suppression often influences (reduces) the intensity of signals in the neighbourhood of the solvent signals. Furthermore, analyte signals which are superimposed with solvent signals are suppressed. The use of fully deuterated solvents in combination with SPE trapping as described above will become of particular value in these instances. Finally, the equipment creates higher costs. Thus, in general, HPLC-NMR will not be used for routine analysis of environmental samples. Rather, the method gives a first complete survey on the pollutants present in an environmental sample (in the ppb and possibly in the future in the upper ppt range) and permits the identification of unknowns. On the basis of these HPLC-NMR results, simpler and less expensive methods may be developed in a second step.

3 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY COUPLED TO MASS SPECTROMETRY (HPLC-MS)

A large variety of interfaces for the coupling of HPLC to MS have been developed over three decades [1]. While interfacing these two techniques, two major problems had to be overcome:
- the vacuum system of the MS had to handle the large amounts of solvents eluting from the HPLC;
- new methods for the ionisation of non-volatile, polar compounds (present in solution) had to be developed.

The vacuum problem was solved on the one hand by introducing the mobile phase of the HPLC as a spray into the MS ion source where only a small fraction of this solvent enters the MS analyser, and on the other hand by the development of sophisticated differential pumping systems which permit the handling of pressure differences of 8 orders of magnitude (11 orders of magnitude in the case of Fourier transform electrospray mass spectrometry). Moreover, new ionisation methods have been developed which differ in the mechanism for ion formation, but are all based again on the introduction of the HPLC eluent into the MS ion source as a spray of fine droplets.

With the introduction of ion sources operating at atmospheric pressure and the invention of atmospheric pressure chemical ionisation (APCI) [18,19], atmospheric pressure photoionisation (APPI) [33] and electrospray ionisation (ESI) [20,21], robust and dedicated HPLC-MS instruments came onto the market which originally were mainly used in the bioanalytical area, but in recent years became also widespread in environmental analysis. [3]

In this chapter, the ionisation methods and mechanisms and the instrumental details of HPLC-MS are not discussed. Rather, the general approach of applying HPLC-MS to target and in particular to non-target analysis in complex environmental samples as well as the advantages and disadvantages of this technique (in particular as compared to HPLC-NMR) will be discussed.
3.1 Target analysis by HPLC-MS
If compounds are too polar to pass a GC (for GC-MS determination), HPLC-MS is the method of choice for target analysis or identification of suspected compounds (for instance, metabolites formed by biotic or abiotic degradation can be considered as examples of suspected compounds, as the common routes for such degradation reactions are known at least for some compound classes). HPLC-MS is by far more selective and in most instances also more sensitive than HPLC (UV). In most cases, the (quasi)molecular ion and the retention time are used for compound confirmation. For complex environmental samples this approach may not be sufficiently specific. The specificity can be enhanced by optimised clean-up procedures and, in particular, by the use of collision-induced fragmentation in a tandem mass spectrometer. This approach, termed mass spectrometry/mass spectrometry (MS/MS) [22], not only leads to the formation of (structure-specific) fragments, but reduces interference from coeluting components and the matrix. Specific scanning procedures, such as the neutral loss, allow the identification of common functional groups within a class of compounds. Tandem mass spectrometry cannot only be realised in space (i.e. by two consecutive quadrupoles) but also in time (i.e. by inducing fragmentation in an ion trap), [22].

3.2 Quantification by HPLC-MS
In contrast to GC-MS (with electron impact ionisation) and in particular to HPLC-NMR the peak area in the HPLC-MS chromatogram of a complex mixture (e.g. an environmental sample) provides no direct information on the concentration of the compound in the sample since, under APCI and ESI conditions, ionisation efficiencies of compounds may differ substantially. In the case of APCI the gas phase basicity and acidity determines both the type of (quasi)molecular ion formed and its abundance. Thus if two compounds of different gas phase basicities coelute in the HPLC-MS chromatogram one compound may be largely suppressed.

Quantification of a compound by HPLC-MS with electrospray or (to a lesser extent) atmospheric pressure chemical ionisation in real life samples is further complicated by the fact that matrix constituents often lead to an ion suppression or enhancement. Thus matrix calibration is a necessary prerequisite for quantification. The availability of isotope labelled internal standards for quantification by HPLC-MS is highly desirable.
In environmental analysis the matrix effect on the quantification of analytes is low in water samples, but may be pronounced in soil samples.

3.3 Non-target analysis by HPLC-MS
The structure elucidation of unknowns (non-target analysis) by HPLC-MS in complex mixtures such as environmental samples is by far more difficult and often not successful if based on mass spectrometry alone. Compared to capillary GC-MS the chromatographic resolution in HPLC (and thus HPLC-MS) is significantly poorer. Thus in complex environmental matrices, coelution is a common problem.
In the following the main steps in the interpretation of the mass spectrum of an unknown compound are summarised:
3.3.1 The (quasi)molecular ion.

The before mentioned coelution may make it difficult to identify the (quasi)molecular ion, which is usually the first step in the structure elucidation of unknowns. The above discussed ionisation techniques mainly used in HPLC-MS (i.e. thermospray (TSP), ESI, APCI or APPI) usually yield even electron [M + H]^+ and/or [M - H]^− ions although in the negative ionisation mode the odd electron [M ]^− radical ion is also sometimes observed. Except for very basic compound or compounds which are ionic over the entire pH range many compounds can be ionised under positive and negative ion formation conditions (although with different yield) and the search for these complimentary [M + H]^+ and [M - H]^− ions is important in identifying the (quasi)molecular ion. If however only positive (quasi)molecular ions are formed under the above mentioned ionisation conditions this is a strong indication that the compound is very basic, i.e. it probably contains one or several nitrogen atoms, while the exclusive observation of negative ions may be considered as a first indication of the presence of an acidic compound.

In addition of (quasi)molecular ions formed by protonation or deprotonation of the molecule adduct ions such ammonium or alkali ion attachment i.e. [M + NH₄]^+, [M + Na]^+ or [M + K]^+ ions under positive ion formation or [M + COOH]− ions under negative ion formation in the presence of formic acid or ammonium formate as buffer are formed under the above mentioned ionisation conditions. These adduct ions on the one hand complicate the interpretation of the mass spectrum, while on the other hand they can be used to corroborate the assignment of the (quasi)molecular ion. Finally the occurrence of cluster ions such as [2M + H]^+, [3M + H]^+ or [C_{n+1}A_n]^+ ions (under positive ion formation conditions), where C is a cation and A an anion, may further complicate the interpretation of the mass spectrum. Cluster ions are usually loosely bound complexes which decompose under collision induced conditions (see below) to form the ionised monomer. This fragmentation can be used to identify cluster ions. In addition the dependence of the abundance of the cluster ion on the analyte concentration may be used to differentiate between molecular ions and cluster ions, as the latter increase more rapidly with the analyte concentration than the former.

The nitrogen rule may be used to obtain information on the presence or absence of nitrogen atoms. While the m/z value of [M + H]^+ ions of compounds containing C, H, O, S or halogens are always odd, the presence of an [M + H]^+ of even m/z value proofs that the compound has 1, 3, 5, …nitrogen atoms.

High resolution. Although the (quasi)molecular ions of an unknown compound provides the valuable information on the molecular weight (which is not available from NMR spectra), no information on its structure is provided except if high resolution data are available. Thus for a successful structure elucidation by mass spectrometry in non target analysis at least high resolution MS of the (quasi)molecular ion, but if possible also on the fragment ions is highly desirable. Apart from the rather expensive Fourier transform ion cyclotron resonance mass spectrometers which provide these data, electrospray time-of-flight TOF mass spectrometers are on the market which allow such high resolution data to be generated for the (quasi)molecular ion, while high resolution of collision induced fragment ions is possible using a quadrupol/time-of-flight (Q-TOF) instrument. High resolution of the (quasi)molecular ion (e.g. a resolution of M/ΔM = 10 000 - 20 000) allows the elemental composition of the (quasi)molecular ion to be determined which is a often a necessary prerequisite for a successful structure elucidation by MS.
If the elemental composition of the (quasi)molecular ion is known the “rings plus double bonds” rule can be applied to obtain further information on the structure. For compounds of the general formula $\text{C}_x\text{H}_y\text{N}_z\text{O}_n$ the sum of the rings plus double bonds, $\text{RD}$, for an even electron $[\text{M} + \text{H}]^+$ is given as $\text{RD} = x - \frac{1}{2} y + \frac{1}{2} z + 1$.

**Isotope peaks.** Isotope peaks ($^{37}\text{Cl}$, $^{81}\text{Br}$, $^{34}\text{S}$) are of particular value for the structure elucidation of unknowns as they allow the number of halogen or sulfur atoms to be determined, while the $^{13}\text{C}$ peak permits to determine the number of carbon atoms approximately (at least in negative ion ESI or APCI spectra), if high resolution data are not available.

**Retention times.** Also chromatographic data such as the retention time may be used advantageously to obtain structural information as in reversed phase HPLC compounds elute in the order of decreasing polarity, i.e. early eluting compounds must have polar groups and vice versa.

### 3.3.2 The fragment ions

In APCI spectra of labile compounds fragments are sometimes observed as a result of the thermal stress prior to and during the ionisation. The fragments in APCI may result both from a thermal fragmentation prior to ionisation and from a true mass spectrometric fragmentation, i.e. a dissociation of the thermally excited (quasi)molecular ion. Both types of fragments can be used for the identification of the structural features in unknowns. In contrast ESI spectra are often devoid of any fragment ions. Here fragmentation can be induced by collisional activation with a neutral gas such as helium or argon. Such collisional activation can already be induced in the skimmer region of a two dimensional or three dimensional quadrupole mass spectrometer (i.e. a mass filter or an ion trap mass spectrometer) by collisions of the analyte ions with residual gas atoms. If complex mixtures, such as environmental samples are to be analysed such skimmer fragments in a normal mass spectrum are of little value as they can be confused with (quasi)molecular ions of coeluting compounds. Much better suited for the structure elucidation of unknowns are tandem (MS/MS) mass spectrometers in which mass separation of the precursor ions, e.g. the (quasi)molecular ions in a mixture, and collisional activation and dissociation (in a collision cell or in special collision regions) are separated in space (such as in triple quadrupole or Q-TOF instruments) or in time (such as in ion trap instruments). In ion trap mass spectrometers, mass separation of the precursor ions and collisional activation + dissociation can be achieved in several consecutive steps, leading to MS², MS³..., MSⁿ spectra. Such MSⁿ spectra are particularly suited for the structure elucidation of unknowns as they allow to differentiate between primary, secondary, tertiary… fragmentation.

Nevertheless the interpretation of the collision induced fragments generated from the $[\text{M} + \text{H}]^+$ and $[\text{M} - \text{H}]^-$ ions of compounds ionised under electrospray or APCI conditions in a HPLC-MS instrument is less straight forward than the interpretation of fragments generated under electron ionisation conditions for several reasons:

(a) After electron impact odd electron molecular ions, e.g. $[\text{M}]^-$ ions, after ESI or APCI even electron ions such as $[\text{M} + \text{H}]^+$ and $[\text{M} - \text{H}]^-$ or the above mentioned adduct ions are formed which differ in their fragmentation behaviour. While the fragmentation of the main compound classes after electron impact ionisation, i.e. the fragmentation of odd electron molecular ions, is well documented in numerous publications and several books, much less is known about the fragmentation of even electron ions.
(b) The fragmentation of the even electron ions generated under ESI or APCI conditions is in general described by the “even electron rule” [34]: an even electron quasi molecular ion decomposes predominantly to form an even electron ion and an even electron neutral (i.e. a neutral molecule), thus forming both thermochemically stable ions and neutrals. (Note that many exceptions to this even electron rule are known). This fragmentation of an even electron ion is in general accompanied by a rearrangement reaction in the transition state. In contrast, an odd electron radical molecular ion, as formed by electron impact, may decompose via direct bond rupture to form an even electron ion and a neutral radical or via a rearrangement reaction to form an odd electron fragment ion and a neutral molecule which explains i.a. why odd and even electron ions differ in their fragmentation.

(c) Under electron impact condition several electron volts may be transferred as internal energy onto the molecular ion, which becomes highly excited. At these high internal energies structure specific direct bond cleavages prevail. In contrast much lower energies are transferred during the low energy collisions in a tandem quadrupole instrument and even lower energies during excitation in an ion trap. Thus both as a result of the “even electron rule” and the on average much lower excitation energies during collisional activation in a linear quadrupole or an ion trap mass spectrometer, rearrangement reactions (with less structure information than direct bond cleavages) prevail. Thus the fragments generated by collisional activation of the even electron (quasi)molecular ions formed during HPLC-MS under ESI or APCI conditions provide less structure information than electron impact mass spectra generated e.g. in a GC-MS instrument.

(d) MS libraries which contain > 100 000 MS spectra are available for electron impact spectra, but hardly for collision induced fragments from the [M + H]+ ions formed by electrospray or APCI.

Neutral losses. The observed neutral losses play an important rule in the interpretation of the mass spectra of unknowns, which not only holds for the electron impact mass spectra of odd electron molecular ions, but in particular for the collision induced fragments generated from even electron ions, as the formation of an even electron fragment ion is always accompanied with the loss of a neutral molecule, while the loss of neutral radicals is only rarely observed.

Table 1 summarises typical neutral losses observed during collision induced fragmentation of even electron ions. Some of these neutral losses such as loss of water are relatively non specific and thus of limited use for the structure elucidation. Moreover, several neutral losses, such as loss of CO and C₂H₄, have the same nominal
TABLE 1.
Neutral Losses during collision induced fragmentation of even electron ions

<table>
<thead>
<tr>
<th>Mass</th>
<th>Neutral Loss</th>
<th>Ionisation</th>
<th>Compound Class</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>H</td>
<td>-</td>
<td>+ anthraquinones (-), arom. diamines</td>
</tr>
<tr>
<td>2</td>
<td>H₂</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>CH₃</td>
<td></td>
<td>methyl subst. arom. ring, N- methyl</td>
</tr>
<tr>
<td>16</td>
<td>CH₄</td>
<td></td>
<td>ethylanthraquinones</td>
</tr>
<tr>
<td>17</td>
<td>NH₃</td>
<td></td>
<td>amines, arom. amines, ammonium adducts</td>
</tr>
<tr>
<td></td>
<td>OH</td>
<td></td>
<td>N-oxide, phenols, anthraquinones</td>
</tr>
<tr>
<td>18</td>
<td>H₂O</td>
<td>+</td>
<td>aliph. alcohols, ketones, anthraquinones, carbonic acids, N-oxides, sulfoxides</td>
</tr>
<tr>
<td></td>
<td>H₂F</td>
<td></td>
<td>fluorides</td>
</tr>
<tr>
<td>26</td>
<td>CN</td>
<td></td>
<td>subst. aromatic amines</td>
</tr>
<tr>
<td>27</td>
<td>HCN</td>
<td>+</td>
<td>(subst). aromatic amines</td>
</tr>
<tr>
<td>28</td>
<td>C₂H₄</td>
<td>+</td>
<td>ethyl groups bound to heteroatom or arom. ring</td>
</tr>
<tr>
<td></td>
<td>CO</td>
<td>+</td>
<td>ketones, anthraquinones, phenols, esters</td>
</tr>
<tr>
<td>29</td>
<td>C₂H₅, HCO</td>
<td></td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>CH₂=O</td>
<td></td>
<td>alkyl subst. ethers</td>
</tr>
<tr>
<td></td>
<td>NO</td>
<td></td>
<td>nitroaromatics</td>
</tr>
<tr>
<td>32</td>
<td>S</td>
<td></td>
<td>sulfides</td>
</tr>
<tr>
<td></td>
<td>CH₃OH</td>
<td></td>
<td>methyl esters</td>
</tr>
<tr>
<td>34</td>
<td>H₂S</td>
<td></td>
<td>sulfur compounds</td>
</tr>
<tr>
<td>35</td>
<td>Cl</td>
<td></td>
<td>aromatic chlorides</td>
</tr>
<tr>
<td>36</td>
<td>HCl</td>
<td></td>
<td>aliph., (aromatic) chlorides</td>
</tr>
<tr>
<td>42</td>
<td>CH₂N₂ (carbodiimide)</td>
<td>+</td>
<td>triazines</td>
</tr>
<tr>
<td></td>
<td>C₃H₆</td>
<td>+</td>
<td>N - propyl</td>
</tr>
<tr>
<td></td>
<td>CH₂=C=O</td>
<td>+</td>
<td>acetyl-deriv., N-acetyl-conjugates</td>
</tr>
<tr>
<td>43</td>
<td>HN=C=O</td>
<td>(+)</td>
<td>hydroxytriazines</td>
</tr>
<tr>
<td>44</td>
<td>C₂H₄O</td>
<td></td>
<td></td>
</tr>
<tr>
<td>44</td>
<td>CO₂</td>
<td>(+)</td>
<td>carbonic acids, carbamates</td>
</tr>
<tr>
<td>46</td>
<td>HCOOH</td>
<td></td>
<td>carboxylic acids</td>
</tr>
<tr>
<td></td>
<td>NO₂</td>
<td></td>
<td>nitroaromatics</td>
</tr>
<tr>
<td></td>
<td>CH₃OCH₃</td>
<td></td>
<td>methylesters</td>
</tr>
<tr>
<td></td>
<td>C₂H₅OH</td>
<td></td>
<td>ethylesters</td>
</tr>
<tr>
<td></td>
<td>C₄H₈</td>
<td>+</td>
<td>N - butyl</td>
</tr>
<tr>
<td>58</td>
<td>C₂H₂O₂ (glyoxale)</td>
<td>-</td>
<td>phenoxy acids</td>
</tr>
<tr>
<td>60</td>
<td>CH₃COOH</td>
<td></td>
<td>acetates</td>
</tr>
<tr>
<td>60</td>
<td>HCOOCH₃</td>
<td></td>
<td>methylesters</td>
</tr>
<tr>
<td></td>
<td>(methylformiate)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>S=C=O</td>
<td></td>
<td></td>
</tr>
<tr>
<td>64</td>
<td>SO₂</td>
<td>(+)</td>
<td>sulfonates, sulfates</td>
</tr>
<tr>
<td>64</td>
<td>CHClO (formyl chloride)</td>
<td>-</td>
<td>chlor. anthraquinones</td>
</tr>
<tr>
<td>70</td>
<td>C₃H₆N₂ (ethylcarbodiimide)</td>
<td>+</td>
<td>triazines</td>
</tr>
<tr>
<td>Mass</td>
<td>Chemical Name</td>
<td>Functional Group</td>
<td></td>
</tr>
<tr>
<td>-------</td>
<td>--------------------------------</td>
<td>---------------------------</td>
<td></td>
</tr>
<tr>
<td>72</td>
<td>C₃H₄O₂ (acrylic acid)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>75</td>
<td>glycine</td>
<td>glutathion conjugates</td>
<td></td>
</tr>
<tr>
<td>79</td>
<td>Br</td>
<td>aromatic bromides</td>
<td></td>
</tr>
<tr>
<td>80</td>
<td>HBr</td>
<td>bromides</td>
<td></td>
</tr>
<tr>
<td>82</td>
<td>SO₃</td>
<td>sulfonates, sulfate esters</td>
<td></td>
</tr>
<tr>
<td>92</td>
<td>toluene</td>
<td>benzyl derivatives</td>
<td></td>
</tr>
<tr>
<td>129</td>
<td>glutamic acid</td>
<td>glutathion conjugates</td>
<td></td>
</tr>
<tr>
<td>162</td>
<td>anhydroglucose</td>
<td>glucose conjugates</td>
<td></td>
</tr>
<tr>
<td>176</td>
<td>anhydroglucuronic acid</td>
<td>glucuronides</td>
<td></td>
</tr>
<tr>
<td>194</td>
<td>glucuronic acid</td>
<td>acyl-, benzylglucuronides</td>
<td></td>
</tr>
<tr>
<td>248</td>
<td>anhydromalonyl glucose</td>
<td>glycosymlmalonyl-conjugates</td>
<td></td>
</tr>
</tbody>
</table>

mass and can only be differentiated if high resolution data of the molecular and the fragment ions are available.

**Small fragment ions.** In addition to neutral losses small fragment ions may be of general diagnostic value. Thus a positive fragment ion at m/z 43 (CH₃CO⁺) is indicative of an acetylation of the compound, while a negative ion at m/z 46 (NO₂⁻) demonstrated the presence of a nitroaromatic compound. For the structure elucidation of unknowns it is of disadvantage that the widespread used ion trap mass spectrometers suppress these small fragment ions.

### 3.3.3 Fragmentation pathways of even electron ions

**Protonation site.** In order to establish the fragmentation pathway of a (positive) even electron (quasi)molecular ion some information of the protonation site should be available. Based on known proton affinity data of different compound classes one can derive that the ease of protonation of a molecule increases in the following order: sulfonic acids < carboxylic acids < aromatic hydrocarbons < ethers, ketones < alcohols, mercaptanes < phosphines < secondary amines < primary amines. Thus in compounds with both an amino and a hydroxyl functionality electrospray or atmospheric chemical ionisation leads preferentially to a protonation of the amino group.

**Fragmentation rules.** For the interpretation of electron impact mass spectra of odd electron molecular ions general fragmentation rules have been established while such rules are largely missing for collision induced fragmentation of the even electron (quasi)molecular ions usually formed in HPLC-MS which renders the structure elucidation of unknowns more difficult.

**Isomeric compounds.** The mass spectra of isomers are often very similar. This holds in particular for isomers differing in the substitution of the aromatic ring. Thus, in general mass spectrometry does not allow to differentiate between meta and para substituted compounds, while this information is readily available from ¹H-NMR.

Summarising mass spectrometry provides valuable information on the structure of unknown compounds, but a complete and unambiguous structure elucidation will only be possible in favourable cases.
3.4 Comparison of HPLC-NMR and HPLC-MS for the structure elucidation of unknowns in environmental samples

Mass spectrometry allows the determination of the molecular weight and (if high resolution data are available) the elemental composition of an unknown, which is not possible by nuclear magnetic resonance spectroscopy. Moreover functional groups (e.g. chlorine, bromine, COOH, NO$_2$, SO$_3$H) are readily detected either by the isotope pattern or by characteristic neutral losses while the chemical shift values in $^1$H-NMR reflect the presence of such functional groups only indirectly. On the other hand the $^1$H-NMR spectra give more detailed information on the structure of an unknown than mass spectra. E.g. the peak area of an NMR signal at a given concentration directly reflects the number of equivalent protons. In contrast to MS the substitution pattern of an aromatic ring can be well determined. Structure elucidation is supported by several correlation spectra such as heteronuclear multiple quantum coherence (HMQC), heteronuclear single quantum coherence (HSQC), 2D total correlation spectroscopy (TOCSY) and 2D nuclear Overhauser effect spectroscopy (NOESY), which have been successfully acquired in the past by HPLC-NMR to obtain additional structural information [10,27,35,36].

Summarising, HPLC-MS and HPLC-NMR provide complementary information on the structure of unknowns and thus should be used in combination.

4 On-line HPLC-NMR-MS

Such simultaneous coupling of HPLC to NMR and MS (HPLC-NMR-MS) was already reported several years ago [23,24] and commercial instruments are available. In this commercial version both units are controlled by a common software. The schematics of this set-up is shown in Fig. 3. The HPLC eluent (possibly before passing the peak sampling unit, BPSU, or before a trapping in a solid phase extraction unit, see above) is split. As the mass spectrometer (operating under ESI or APCI conditions) is far more sensitive than the NMR instrument only 5% of the eluent is transferred to the MS and the remaining 95% to the NMR instrument. As shown in the figure two syringe pumps may be connected to the system via a T-piece which allows to add an additional buffer or water to the eluent prior to the MS in order to (a) improve the ionisation conditions, (b) for D/H - back exchange experiments or (c) to dilute the sample for multiple MS experiments such as alternative positive and negative ionisation or MS$^n$ studies. For such sample dilution a storage loop is added to the system via an nine port valve. This valve also permits connection of the HPLC to other applications while the HPLC-NMR is busy.

As the NMR and the MS data in a on-line HPLC-NMR-MS experiment originate from the same HPLC run the above mentioned ambiguities in separate HPLC-NMR and HPLC-MS experiments no longer exist. Moreover, structure elucidation is performed with much more time and sample efficiency than with separate systems.

The additional MS not only enhances the power of the system for structure elucidation of unknowns, it also permits the triggering of NMR experiments (such as stopped flow runs) much better than the UV detector of the HPLC. This holds in particular if there are components with low UV absorption in the sample. In target analysis the NMR can be triggered by the (quasi)molecular and by one or several fragment ions. Moreover, if in a HPLC run a peak overlapping occurs monitoring of the (quasi)molecular ions of the two overlapping components may be used to trigger the NMR experiment only at a retention time at which the peak of the target compound is pure.
**H/D back exchange.** As discussed above, solvent suppression in the NMR instrument is facilitated if D$_2$O is used as one solvent in the mobile phase of the HPLC. This leads to a partial or complete D/H exchange of all acidic protons in the analyte and a shift of the m/z value of the (quasi)molecular ion which corresponds to the number of acidic protons (including to the proton used for protonation of the analyte under ESI and APCI conditions). If in a second experiment the dilutors in Fig. 3 are used for a H/D back exchange the difference in the m/z value of the (quasi)molecular ion before and after H/D back exchange can be used to identify the number of acidic protons which is a valuable additional structural information. Furthermore, in favourable cases these H/D back exchange experiments allow to differentiation between isobaric fragments. Thus in the MS/MS spectrum of a secondary methoxyphenylalkylamine we observed the neutral loss of 31 Da, which was originally assigned as loss of a CH$_3$O radical. However after H/D exchange a loss of 33 instead of 31 Da was observed proving that (deuterated) methylamine and not a methoxy radical was eliminated during collision induced dissociation (which is in agreement with the “even electron rule” described above). In principle such H/D exchange experiments are of course possible with any mass spectrometer, but such experiments are particularly obvious in a HPLC-NMR-MS system.

Figure 3. Interface for on-line of HPLC-NMR-MS coupling (BPSU = peak sampling unit)
5 APPLICATION OF HPLC-NMR AND HPLC-MS TO THE ANALYSIS OF ENVIRONMENTAL SAMPLES

Four examples will be selected to illustrate the potential of the combined application of HPLC-NMR and HPLC-MS to the non-target analysis of environmental samples:

- Contaminated ground water from a former ammunition plant,
- leachate water from an industrial waste disposal site,
- waste water from a textile company,
- polycyclic aromatic hydrocarbons in soil.

The extraction of the aqueous sample will not be discussed in detail. In general, extraction was performed by solid phase extraction (SPE) using special polystyrene-divinylbenzene copolymers. If acidic organic compounds are to be extracted, the sample must be acidified. In several instances, SPE extracts were lyophilised prior to analysis. Mass spectrometric detection was achieved mainly using atmospheric pressure chemical ionisation (APCI) or electrospray ionisation (ESI) both in the positive and negative ion mode, but also by thermospray ionisation (TSP). A quadrupole or an ion trap was used as mass analyser. The ion trap allowed multiple MS/MS experiments (MS^n) to be performed, which proved particularly valuable for structural elucidation. HPLC-NMR measurements were carried out both in the on-flow or stopped-flow mode on a 600 MHz NMR spectrometer.

5.1 Ammunition hazardous waste sites

As a result of extensive production of ammunition before and during World-War II, a large number of hazardous waste sites exist in Germany, where both soil and water are polluted by explosives and their transformation products. In Germany as many as 3400 potentially contaminated sites have been identified [25]. To carry out a risk assessment of these sites, a large number of soil and water samples were analysed, where the analysis was usually restricted to target compounds such as explosives expected or found on the site, but also to several by-products and neutral transformation products formed by photo- or biodegradation of these precursors. Studies by us and others [4-6, 11-12, 26] have revealed that aqueous samples from such ammunition hazardous waste sites may also contain a variety of acidic compounds which have been overlooked in previous investigations during target analysis. Contaminated ground water was analysed by us using direct NMR and HPLC-NMR and the tentative structures elucidated by these techniques confirmed by HPLC-MS [6,11,12].
Figure 4: Pseudo-two-dimensional $^1$H-NMR chromatogram (contour plot, aromatic protons) of a ground water sample contaminated with explosives and their degradation products (a: full contour plot; b: extracted contour plot of target compounds, for further details see ref. 11)
Fig. 4 shows a part of the pseudo-two-dimensional NMR chromatogram (contour plot) of a ground water sample contaminated with explosives and their degradation products. A non target analysis of the sample by $^1$H HPLC-NMR leads to the pseudo-two-dimensional NMR chromatogram (contour plot) shown in Fig. 4a. If one extracts from this contour plot only those components included in normal target analysis by HPLC one arrives at the contour plot shown in Fig. 4b. Comparison of the two plots demonstrates that many (in particular polar) components are missed during target analysis. A variety of these new components have been identified by us as illustrated in Fig. 4a using both HPLC-NMR and HPLC-MS (although many unknowns still exist).

5.2 Leachate from industrial landfills
Leachates from industrial waste disposal sites may frequently contain a broad mixture of different chemicals. These compounds may be the final products, precursors or intermediates of the process, or else impurities and by-products obtained in a way that is often difficult to predict and, as a consequence, difficult to control. The leachate may contain pollutants originally contained in the waste; furthermore, transformation occurs during the ageing of the waste and, as a consequence, the leachate may collect contaminants produced inside the landfill. Transformation products are often more polar than precursors. Leachate water from two industrial landfills was characterised by us using HPLC-NMR and HPLC-MS and thermospray HPLC-MS where particular

![HPLC (UV) chromatogram of a leachate sample from an industrial waste disposal site](image)

Figure 5: HPLC (UV) chromatogram of a leachate sample from an industrial waste disposal site: 1: 4-chlorobenzenesulfonic acid; 2: phthalic acid; 3: terephthalic acid; 4: isophthalic acid; 5: phenylacetic acid; 6: benzoic acid; $\alpha$-chlorobenzoic acid, 8: $\alpha$-hydroxybenzoic acid; 9: 3-phenylpropionic acid; 10: 3-chlorobenzoic acid; 11: 4-chlorobenzoic acid (by courtesy of Elsevier)
emphasis was paid to acidic compounds [13] while the more volatile compounds were analysed by GC-MS, not reported here [13]. To remove the excess of neutral and basic analytes, the water sample was first made basic and pre-extracted with methylene chloride. After acidification, the aqueous phase was then extracted by SPE. The sample was first analysed by HPLC with diode array detection (PDA) as shown in Fig. 5 where two chromatograms at different detection wavelengths are shown. From these chromatograms it becomes obvious that the separation efficiency of the HPLC method was insufficient for this type of application. Compound identification by the UV spectrum alone was not possible. Furthermore, UV-inactive compounds such as aliphatic carboxylic acids or sulfates

Figure 6: Time slices of the NMR chromatogram of the leachate sample shown in Fig. 5 (for chemical shift value see ref. 13) (By courtesy of Elsevier)
cannot be detected. Therefore, the LC-PDA method is not suitable for the analysis of such complex mixtures. In the next step the sample was analysed by thermospray HPLC-MS both in the negative and positive ion mode. Using this approach, 11 organic acids could be identified in the sample as reported elsewhere [13]. The identification was corroborated by comparison with reference compounds and by HPLC-NMR (see below).

As expected, the TSP mass spectra show abundant quasi-molecular ions and cluster ions in both ionisation modes. Under negative ion conditions, [M-H]⁻ and cluster ions formed by the buffer ammonium formate, i.e. [M+HCOO]⁻, are predominantly formed. The weak acids phenylacetic acid and phenylpropionic acid show more abundant positive than negative ions under TSP conditions, where the [M+NH₄]⁺ ion dominates the spectrum. These ions are also intense in the spectra of the isomeric phthalic acids and benzoic acid, although with these compounds more negative than positive ions are formed.

Finally, HPLC-NMR experiments were carried out. Fig. 6 shows various time slices of the NMR chromatogram. The spectra are rather simple and can be analysed without use of further techniques. Eight organic acids could be identified in the NMR chromatogram on the basis of their retention times and by comparison with the chemical shift values of the reference compounds [13]. Note again that the NMR spectra shown in Fig. 6 consist of several compounds. HPLC chromatography simplifies the NMR spectra to such an extent that the NMR spectra of the individual compounds do not show interference from other components.

However, with this technique too, several early and late eluting compounds could still not be identified (see for instance row 14 - 17).

5.3 Effluent from a textile company
Industrial waste water is an important potential source for the pollution of the aquatic environment. It is assumed that usually only ~20% of the organic constituents in the waste water of a chemical plant are known, where the unknown components are considered to be mainly polar, non-volatile compounds. For the identification of these polar compounds, the combined use of both HPLC-NMR and HPLC-MS is particularly promising, as illustrated by us with the analysis of untreated waste water from a textile company as an example [14]. The HPLC (UV) chromatogram in Fig. 7 gives an overview of the early eluting components of this waste water sample (which was extracted by methylene chloride).

The NMR spectra of the various peaks were acquired using the stopped-flow method. Some NMR spectra are shown in Fig. 8 while the complete NMR data and the structure assignments are summarised in Table 3. The interpretation of the NMR spectra has been reported in detail previously [14]. Here, only the NMR spectrum of peak 7 in Fig. 8 will be discussed as an example. The ¹H NMR spectrum of this peak shows in the chemical shift region of the aromatic protons, two multiplets (two protons each) centered at 7.77 and 8.27 ppm typical for the ring A protons (H 5,8 and H 6,7, respectively) of an anthraquinone skeleton (see Scheme 1).
Figure 7: Partial HPLC(UV) chromatogram of a waste water sample from a textile company (by courtesy of the American Chemical Society)

subspectrum at 7.47 and 7.51 ppm, respectively, correspond to the protons of the C ring of the anthraquinone skeleton. They indicate that the substituents at positions 1 and 4 of the ring differ. Furthermore, a singlet in the aliphatic region at 3.11 ppm is within the expected shift range of the N-bonded methyl group, while the two triplets at lower fields (3.59 and 3.80 ppm) are in the expected shift range of N- and 0-bonded methylene groups, respectively. Thus, the compound corresponding to peak 7 was tentatively identified as disperse blue 3.

This assignment was confirmed by mass spectrometry where HPLC-MS was performed using atmospheric pressure chemical ionisation both in the positive and negative ion mode. The mass spectral data of this and other compounds in the sample are also summarised in Table 3 while the MS² to MS⁵ spectra of the collision-induced fragments were recorded in an ion trap. Not only the quasi-molecular ions \([M+H]^+\) at \(m/z\) 297 and \([M]^-\) at \(m/z\) 296, but also the fragments are consistent with the proposed structure (see ref. 14). The final confirmation of the structure assignment was achieved by comparing the NMR and MS spectra of peak 7 with those of the reference compounds.

The fragmentation of this compound [14] highlights the above statement that the fragmentation rules of odd electron ions as known from electron impact mass spectrometry do not apply to the even electron \([M+H]^+\) ions formed by APCI-HPLC-MS. While ionized amines (such as disperse blue 3) should show an abundant cleavage of the \(\alpha\)-C-C bond, cleavage of the N-C bond is observed in the MS² spectrum of the \([M+H]^+\) ion. This cleavage leads to an odd electron ion which, in the following fragmentation step, (MS³), shows the well-known \(\alpha\)-cleavage.

As summarised in Table 2, several anthraquinone-type dyes and their by-products, a
Figure 8. HPLC-NMR analysis of the waste water sample from a textile company. The $^1$H NMR spectra of the peaks in the chromatogram of Fig. 7 and some reference spectra are shown (by courtesy of the American Chemical Society).

fluorescent brightener, a by-product from polyester production and auxiliaries such as anionic and non-ionic surfactants and their degradation products were also identified in this waste water sample. With the exception of nonylphenol and nonylphenolmonoethoxylates, the compounds listed in Table 2 could not be identified by GC-MS.
TABLE 2.
Summary of the NMR and MS data of the waste water sample from a textile company (including peak assignment and structure of the identified compounds)

<table>
<thead>
<tr>
<th>compound</th>
<th>structure</th>
<th>NMR data (δ-values, signal multiplicities)</th>
<th>MS data (APCI, neg./ pos. mode)</th>
</tr>
</thead>
</table>
| 1        | ![Structure 1] | C_{11}H_{23} | 311 [M-H]^-  
            ↓ 183 (-C_{10}H_{20}) |
| 2        | ![Structure 2] | C_{12}H_{26} | 325 [M-H]^+  
            ↓ 183 (-C_{11}H_{22}) |
| 3        | ![Structure 3] | C_{13}H_{27} | 339 [M-H]^+  
            ↓ 183 (-C_{11}H_{22}) |
| 4        | ![Structure 4] | H_{6}, H_{6}: 7.49 (s)  
            H_{6}, H_{6}: 8.27 (m)  
            H_{6}, H_{6}: 7.78 (m)  
            -NHCH_{2}CH_{2}OH: 3.53 (t)  
            -NHCH_{2}CH_{2}OH: 3.80 (t) | 326 [M]^+ / 327 [M+H]^+  
            ↓ 282 (-C_{2}H_{4}OH)  
            ↓ 251 (-CH_{2}OH) |
| 5        | ![Structure 5] | H_{6}, H_{6}: 7.25 (d), 7.37 (d)  
            H_{6}, H_{6}: 8.25 (m)  
            H_{6}, H_{6}: 7.78 (m)  
            -NHCH_{2}CH_{2}OH: 3.56 (t)  
            -NHCH_{2}CH_{2}OH: 3.79 (t) | 282 [M]^+ / 283 [M+H]^+  
            ↓ 238 (-C_{2}H_{4}OH) |
| 6        | ![Structure 6] | H_{6}, H_{6}: 7.12 (s)  
            H_{6}, H_{6}: 8.32 (m)  
            H_{6}, H_{6}: 7.79 (m) | / 239 [M+H]^+  
            ↓ 221 (-H_{2}O) |
| 7        | ![Structure 7] | H_{6}, H_{6}: 7.47 (d), 7.51 (d)  
            H_{6}, H_{6}: 8.27 (m)  
            H_{6}, H_{6}: 7.77 (m)  
            -NHCH_{3}: 3.11 (s)  
            -NHCH_{2}CH_{2}OH: 3.59 (t)  
            -NHCH_{2}CH_{2}OH: 3.80 (t) | 296 [M]^+ / 297 [M+H]^+  
            ↓ 282 (-CH_{3})  
            ↓ 251 (-CH_{2}OH)  
            ↓ 223 (-H_{2}CN) |
### TABLE 2
(continued)

<table>
<thead>
<tr>
<th>compound</th>
<th>structure</th>
<th>NMR data</th>
<th>MS data</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>δ-values, signal multiplicities[a]</td>
<td>APCI, neg./pos. mode</td>
</tr>
</tbody>
</table>
| 8        | ![Structure 8](image1.png) | H<sub>2</sub>, H<sub>4</sub>: 7.26 (d), 7.34 (d)  
H<sub>6</sub>, H<sub>6</sub>: 8.27 (m)  
H<sub>8</sub>, H<sub>8</sub>: 7.77 (m)  
-NHCH<sub>3</sub>: 3.09 (s) | / 253 [M+H]<sup>+</sup>  
↓ | 238 (-CH<sub>3</sub>) |
| 9        | ![Structure 9](image2.png) | H<sub>2</sub>: 5.93 (q), H<sub>4</sub>: 6.52 (d)  
H<sub>2</sub>: 6.73 (dd), H<sub>2</sub>: 7.52 (d)  
-CH<sub>2</sub>: 2.35 (d)  
-N(CH<sub>2</sub>CH<sub>3</sub>): 3.43 (q),  
-N(CH<sub>2</sub>CH<sub>3</sub>): 1.15 (t) | / 232 [M+H]<sup>+</sup>  
↓ | 204 (-C<sub>2</sub>H<sub>4</sub>), 203 (-C<sub>3</sub>H<sub>3</sub>)  
↓ | 178 (-C<sub>2</sub>H<sub>4</sub> or CO), 160 (-CO<sub>2</sub>) |
| 10       | ![Structure 10](image3.png) | H<sub>2</sub>, H<sub>4</sub>: 7.47 (s)  
H<sub>8</sub>, H<sub>8</sub>: 8.28 (m)  
H<sub>8</sub>, H<sub>8</sub>: 7.76 (m)  
-NHCH<sub>3</sub>: 3.10 (s) | 268 [M]<sup>+</sup> / 267 [M+H]<sup>+</sup>  
↓ | 252 (-CH<sub>3</sub>)  
↓ | 235 (-OH), 237 (-CH<sub>3</sub>) |
| 11       | ![Structure 11](image4.png) | aromat. H: 8.07 (s)  
aliph. H: 4.66 (s) | 578 [M]<sup>+</sup> / 577 [M+H]<sup>+</sup>  
↓ | 385 (-C<sub>2</sub>H<sub>4</sub>CO<sub>2</sub>C<sub>4</sub>H<sub>4</sub>CO<sub>2</sub>) +  
193 (-2C<sub>2</sub>H<sub>4</sub>CO<sub>2</sub>C<sub>4</sub>H<sub>4</sub>CO<sub>2</sub>)  
↓ | 149 (-CO<sub>2</sub>) |
| 12       | ![Structure 12](image5.png) | H<sub>8</sub>, H<sub>8</sub>: 6.70 – 6.76 (m)  
H<sub>8</sub>, H<sub>8</sub>: 7.10 – 7.22 (m)  
C<sub>9</sub>H<sub>19</sub>: 0.50 – 1.80 | 219 [M-H]<sup>+</sup> |
| 13       | ![Structure 13](image6.png) | H<sub>8</sub>, H<sub>8</sub>: 6.82 – 6.90 (m)  
H<sub>8</sub>, H<sub>8</sub>: 7.20 – 7.32 (m)  
C<sub>9</sub>H<sub>19</sub>: 0.50 – 1.80  
-OCH<sub>3</sub>CH<sub>2</sub>OH: 4.01 (t)  
-OCH<sub>3</sub>CH<sub>2</sub>OH: 3.81 (t) | 353 – 969 [M+H]<sup>+</sup>  
X = 1-15 |
| 14       | ![Structure 14](image7.png) | H<sub>2</sub>: 6.88 – 8.89 (m)  
H<sub>2</sub>: 7.22 – 7.32 (m)  
C<sub>9</sub>H<sub>19</sub>: 0.50 – 1.80  
a: 3.52 (s)  
b: 3.83 (s)  
X: 3.60 (s)  
c: 3.80 (s)  
d: 4.10 (s) |
5.4 Polycyclic aromatic hydrocarbons in soil

The next example illustrates the potential of the HPLC-NMR method for the analysis of PAHs [37]. Polycyclic aromatic hydrocarbons are frequently found in environmental samples. In general, only the 16 most frequent PAHs listed in the EPA method 610 are analysed. But even for these compounds often the selectivity of the chromatographic methods is not high enough to separate them from other non-EPA PAHs and/or matrix components occurring in real samples. Furthermore, for the identification of unknown PAHs and PAH metabolites NMR data are of great importance since many isobaric PAHs exist and the position of functional groups in PAH metabolites can only be determined by NMR. On the other hand, the \(^1\)H NMR spectra of PAH mixtures show a multitude of signals in the chemical shift region of aromatic protons associated with strong signal overlappings. Therefore, an unambiguous identification of individual PAHs present in the sample without chromatography is in many cases not possible, even if 2D techniques such as COSY and TOCSY are applied. Furthermore, quantification of individual PAHs by NMR requires isolated signals in the spectrum of the mixture, a condition which is often not fulfilled.

![Figure 9](image)

Figure 9. 600 MHz \(^1\)H NMR spectrum (low field region) of the 16 EPA PAH mixture in CH\(_3\)CN/D\(_2\)O (bottom). Extracted rows (rows 9 and 10 were added) of the HPLC-NMR chromatogram of the same PAH mixture (top) (by courtesy of Springer Verlag)

This is demonstrated in Fig. 9 which shows in the lower part a 600 MHz \(^1\)H NMR spectrum of a standard mixture of the 16 EPA PAHs recorded without any chromatography. There are numerous signal overlappings. Only for half of the compounds isolated signals exist for quantification. However, this crowded NMR spectrum can be resolved using the HPLC-NMR technique. This is demonstrated in the upper part of the Figure 9 which shows extracted rows (rows 9 and 10 were added) of the
Figure 10. HPLC-NMR chromatogram of PAH mixtures (early eluting PAHs (CH$_3$CN/D$_2$O, 60/40 v/v), flow 0.05 ml min$^{-1}$, 40 scans/row); left: the standard solution of EPA PAHs; right: soil extract of the BAM reference material PAK/S/UE-080. a: early eluting PAHs (CH$_3$CN/D$_2$O, 60/40 v/v), flow 0.05 ml min$^{-1}$, 40 scans/row); b: late eluting PAHs (CH$_3$CN/D$_2$O, 90/10 v/v), flow 0.05 ml min$^{-1}$, 56 scans/row), (by courtesy of Springer Verlag)
HPLC-NMR chromatogram of the same PAH mixture (see also below). The two isomeric PAHs chrysene and benz(a)anthracene, which partially coelute under the applied chromatographic conditions, appear well resolved in the NMR dimension. HPLC methods for the separation of PAHs are usually gradient methods, but isocratic elution allows the automatic analysis of samples using spectra data bases.

Therefore, a method was developed that consists of two isocratic HPLC runs, one for the early eluting PAHs and one for the late eluting PAHs. Figure 10a shows the on-flow NMR chromatograms of the first eight EPA PAHs (naphthalene to pyrene; standard solution (left), soil extract (right)) while in Fig. 10b the corresponding HPLC-NMR chromatograms of the late eluting PAHs benz(a)anthracene to indeno[1,2,3-cd]pyrene are represented. (The concentration of the PAHs in the reference soil is given in Ref. 37. It ranges from 0.2 mg kg\textsuperscript{-1} for naphthalene to 14 mg/kg for fluoranthene). Most of these

Figure 11. HPLC-NMR chromatogram of the late eluting PAHs of a 16 EPA PAHs/8 non-EPA PAHs mixture (CH\textsubscript{3}CN/D\textsubscript{2}O, 90/10 v/v), flow 0.05 ml min\textsuperscript{-1}, 56 scans row\textsuperscript{-1}) and extracted rows (rows 12 and 13 added). Abbreviations: BaA = benz[a]anthracene, Chr = chrysene, BbF = benzo(b)fluoranthene, BjF = benzo(j)fluoranthene, Bep = benzo[e]pyrene, Per = perylene, DbacA = dibenz[a,c]anthracene, BkF = benzo[k]fluoranthene, Bap = benzo[a]pyrene (by courtesy of Springer Verlag)
PAHs are sufficiently separated in the chromatographic axes. Even in cases where coelution occurs (naphthalene/acenaphthene and chrysene/ benz[a]anthracene), at least one undisturbed signal for each compound exist in the NMR dimension that can be used for quantification.

As can be seen from Fig. 11, this is also true if further non-EPA PAHs are added to the mixture. For instance, between 100 and 125 min, not only the EPA PAH benzo[b]fluoranthene elutes, but also the non-EPA PAHs benzo[e]pyrene, benzo[j]fluoranthene, and perylene. Again at least one separated signal per compound can be observed in the NMR dimension suitable for quantification.

The HPLC-NMR method for the determination of PAHs has a relatively low sensitivity (LODs are between 2-10 µg absolute). Thus, LODs of 0.2-0.7 mg kg⁻¹ can be achieved if 50 g of a soil sample is extracted, the extract is reduced to a final volume of 1 ml, and 250 µl are injected. Apart from its low sensitivity the method offers a high selectivity and unique structural information for the identification of unknown compounds. Within the limits of sensitivity this method is therefore suited for the evaluation of other analytical techniques and reference materials.

REFERENCES


