Structure Elucidation of Unknown Pollutants in Environmental Samples by Coupling of HPLC to NMR and MS

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Problem

Pollution of the environment by many chemicals
Contaminants present as complex mixture
Pollutants vary largely in their polarity
Besides many known chemicals for which standardized analytical methods exist unknown compounds
Structure elucidation

Target versus Non-Target Analysis

Target analysis
- Compound known, partially known or suspected
(Suspected compounds: Metabolites, polymers and bio-polymers)
- Physico-chemical properties known (important for sample preparation)
- Often analytical methods known and standardized

Non-target analysis
- Compound unknown
- Sample preparation and structure elucidation difficult,
  (in particular if compounds are polar → discussed here)

Methods for Structure Elucidation of Polar Compounds in Mixtures

Hyphenated techniques
- HPLC - NMR
- HPLC – MS
- HPLC – NMR – MS

Sample preparation often on-line
HPLC-NMR: Instrumental Aspects

Two basic requirements:

- **Probe:**
  Conventional probe replaced by flow-through cell (U-shaped, 2 – 4 mm i.d., 30 – 200 µl)
  Transmitting and receiving coils directly fixed on cell

- **Solvent suppression:**
  Proton carrying solvents result in strong solvent signals
  - Use of fully deuterated solvents (expensive, except D₂O)
  - Suppression of solvent signals by special pulse sequences (e.g. WET pulse)

NMR Techniques

NMR spectroscopy relatively insensitive

Therefore in HPLC – NMR mainly

- ¹H NMR
- No ¹³C NMR
- Several correlation spectra possible
  - Hetero multiple quantum coherence (HMQC)
  - 2D - Hetero single quantum coherence (HSQC)
  - Total correlation spectroscopy (TOCSY)
  - 2D – Nuclear Overhauser effect (NOESY)

HPLC-NMR: Coupling Modes

**Stopped-flow mode**

- HPLC – run stopped at peak height up to two hours for one- or two dimensional NMR experiments
- Accumulation of up to 1000 pulses for better sensitivity
- Triggered by UV detector or mass spectrometer
- Disadvantage: Long HPLC-runs

**Peak sampling**

- Various fractions (slices) of the chromatogram successively stored in loops without interruption of HPLC - run
- Peaks sampling units (PSU) with up to 36 loops available
- Peaks stored in loops subsequently transferred to NMR

**On-flow mode**

- HPLC eluent continuously transferred to NMR while spectrometer records continuously ¹H NMR spectra
- Spectra are stored and may be presented as a function of time (as in GC/MS or HPLC/MS)
- Alternatively HPLC – NMR experiment can be plotted in a three dimensional way as a function of the intensity and time (contour plot):
  - One axis: chemical shift
  - Second axis: Retention time
  - Intensity: contour lines

HPLC-NMR: Coupling Modes

**HPLC – solid phase extraction – NMR (HPLC-SPE-NMR)**

- To avoid the use of (never perfect) solvent suppression and to enhance the overall sensitivity, the on-line HPLC-SPE-NMR approach has recently been introduced
- Automatic SPE unit (192 cartridges) arranged after HPLC
- SPE not used for sample enrichment, but for peak trapping
- Post column addition of water for peak focussing
- By repetitive HPLC runs the same peak can be trapped several times (e.g. four times)
- After drying of cartridge the sample is flushed from the SPE cartridge into the NMR cell using e.g. a small volume (25 µl) of fully deuterated acetonitrile
  - Narrow elution band
  - Significant increase in sensitivity

HPLC-NMR: Sensitivity

¹H NMR is ~ 1000 times less sensitive than HPLC-MS.

Signal to noise ratio S/N:

\[
S/N = \frac{S_0}{\sqrt{k + T(R_b + R_{sample}) + \Delta f}}
\]

\(T = \text{absol. Temperature, } B_b = \text{field strength, } \omega = \text{Larmor frequency}\)

Three approaches to increase the sensitivity:

- Repetitive peak trapping using SPE (already mentioned)
- Higher field strength
- Cryoprobes
HPLC-NMR: Sensitivity

Field strength:
- S/N proportional to field strength
- Increase from 600 to 900 MHz increases the S/N ratio by only a factor of 1 ½, but the costs by a factor of 7.
- Decrease of the temperature of the probe coils to 15°C increases the S/N ratio by a factor of up to 8.

Using four repetitive SPE trappings and a cryoprobe leads to an increase of the S/N ratio by a factor of up to 70.

Today absolute quantities of an analyte (in the NMR cell) of ~10 ng can be detected.

HPLC-NMR: Quantification

NMR well suited for quantification

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

- \( c_s \) = sample, \( a \) = analyte, \( M_s \) = molecular mass, \( F_s \) = area of NMR signal, number of protons generating NMR signal.
- Important in environmental analysis
- Absolute method
- Internal standard has to be added, reference compounds not required
- Quantification in on-flow mode.

HPLC-NMR: Structure Elucidation

Based on:

(a) One dimensional 1H NMR-spectroscopy
- Chemical shift values
- Peak multiplicities
- Coupling constants

(b) Two dimensional NMR (correlation spectra)
- Hetero multiple quantum coherence (HMQC)
- 2D Hetero single quantum coherence (HSQC)
- Total correlation spectroscopy (TOCSY)
- 2D Nuclear Overhauser effect (NOESY)

(c) Retention times

HPLC-NMR of 10 Acids: Enhanced Specificity

- Complete chromatographic resolution not necessary
- Very narrow NMR signals (2 – 5 Hz)
- Identification of several distinct compounds in one NMR spectrum
- Thus: coelution no problem
- NMR: High resolution of information
- Coupling of HPLC to NMR increases specificity
HPLC-NMR: Advantages and disadvantages

Advantages:
- High structural information
- Particularly powerful for differentiation between isomers
- High resolution of information
  → ratio of expected range of chemical shift values to width of resonance signal (~3000 as compared to HPLC alone: ~100)
- Amenable to compounds with wide range of polarities
- Non-destructive (sample may be retrieved after analysis)
- Quantification without reference compounds
  → Response directly reflects concentration

Disadvantages:
- Modest sensitivity
  (e.g. water sample: low ppb)
- Ultratrace analysis (pg range) not possible
- Time consuming (overnight run with accumulation of ~1000 FIDs)
- Interference from solvent signals (in spite of solvent suppression)
- Equipment expensive
- $^{13}$C NMR in general not possible
- Identification of functional groups may be difficult (if similar chemical shift values)
- Not suited for routine analysis of environmental samples
- HPLC/NMR provides first complete survey on pollutants in sample

HPLC/MS: Instrumental Aspects

Originally two major problems:
- Vacuum: ion source at atmospheric pressure, analyser (quadrupole) at $10^{-5}$ mbar
  Solution: Better pumps, differential pumping
- Ionisation methods for polar compounds
  - Thermospray (TSP) (now obsolete)
  - Atmospheric pressure chemical ionisation (APCI)
  - Atmospheric pressure photoionisation (APPI)
  - Electrospray ionisation (ESI)
  Different ionisation mechanism in common: HPLC eluent introduced as spray into ion source

HPLC-MS: Target Analysis

• HPLC-MS method of choice for identification of known or suspected polar compounds → target analysis
• Suspected compounds in the environment → e.g. biotic or abiotic degradation products
• Identification by retention time, molecular ion and/or selected fragment ions (MS/MS)
• HPLC/MS/MS: very specific
  - significant reduction of chemical noise (matrix)
  - very sensitive (low pg level)

HPLC-MS: Quantification

Two major problems:
1. With APCI or ESI response in mixture does not reflect amount of analyte (in contrast to NMR)
   - Response (peak area) depends on ionisation efficiency
   - Co-elution of two compounds: suppression of one component possible
2. Matrix may lead to a significant ion suppression or ion enhancement
   - In environmental samples pronounced for soil and plant samples
  Solution: Matrix calibration
  - Isotopically labelled internal standards

HPLC-MS: Non-target analysis

Structure elucidation of unknown compound by interpretation of its mass spectrum (MS)

based on
- (Quasi) molecular ion
- Fragment ions
Identification of (Quasi) molecular ion
- HPLC-MS with TSP, ESI, APCI, APPI mainly leads to protonated, [M + H]+, and deprotonated ions [M - H]-
  - even electron ions (odd mass if C,H,O,S,Cl, even mass if 1, 3, 5, N atoms)
- In complex mixtures identification of (quasi) molecular ion not always simple
  Confirmation by:
  - Complementary [M + H]+ and [M - H]- ions
  Complicated by:
  - Coelution
  - Cluster ions: [2M+H]+

Structure Elucidation: The (Quasi) Molecular Ion
Structural information from (quasi) molecular ion:
- Molecular weight
- Nitrogen rule: Presence of nitrogen atoms
- If high resolution available:
  - Elemental composition
    - Ring + double bond rule
  - Isotope peaks: 32Cl, 81Br, 34S, 13C (only with negative ion spectra)
In addition in HPLC-MS: retention time
High resolution very important

Structure Elucidation: Fragment Ions
- ESI, APCI hardly any fragmentation
- Collision induced dissociation (with neutral target gas: Ar, He)
- Separation of ionisation and fragmentation in tandem mass spectrometers (MS/MS)
  - Separation in space:
    - Triple quadrupole (MS3 only low resolution)
    - Quadrupole (Q) – time of flight (TOF): Q-TOF (MS3 high resolution)
  - Separation in time
    - Ion trap (MSn, only low resolution)
  - Separation in space and time
    - Linear ion trap, (MSn, medium resolution)

Electron impact:
- Formation of odd electron molecular ions, [M]+.
  - Fragmentation leads to
    - Odd electron fragment ions + neutral molecules (rearrangement)
    - Even electron fragment ions + neutral radicals (direct bond cleav.)

Structure Elucidation: Fragment Ions
Electrospray, APCI, APPI, TSP
- Formation of even electron (quasi) molecular ions, [M + H]+
  - Fragmentation leads to
    - Even electron fragment ions + neutral molecules (rearrangement)
    - Stable products ➔ Even electron rule (Exceptions)

HPLC-MS: Interpretation of Mass Spectra
Fragmentation of Even Electron [M + H]+ ions:
- Hardly any MS libraries
- Fragmentation rules not established
- High resolution data desirable
Interpretation of MS spectra of [M + H]+ ions:
- (Quasi) molecular ions
- Neutral losses
- Small fragment ions
- Fragmentation rules (?) : Even electron rule

Interpretation of Mass Spectra, Neutral Losses
60 CH3COOH  acetic acids
60 HOOCC(CH3)  (methylformate)  methylesters
54 SO2  thioesters
54 SO3  thioesters
70 CH3CN (acetonitrile)  +  chlor. anthraquinones
72 CH2O (formyl chloride)  +  triazines
75 glycolic acid  +  glyoxal derivatives
79 Br  -  glutathione conjugates
80 Br2  +  bromides
92 glycolic acid  +  glutathione conjugates
129 gluconic acid  +  glucose conjugates
162 anhydroglucose  +  acetate conjugates
176 anhydroglucuronic acid  +  glucuronides
194 glucuronic acid  +  glucuronides
- acyl-benzylglucuronides
Protonation Site

Protonation site determined by proton affinity of functional group:
- Decreasing proton affinity:
  - Primary amines
  - Second amines
  - Phosphines
  - Mercaptanes
  - Alcohols
  - Ethers, ketones
  - Aromatic hydrocarbons
  - Carboxylic acids
  - Sulfonic acids

Comparison of HPLC-NMR and HPLC-MS for Structure Elucidation of Unknown Compounds

HPLC - 1H NMR:
- Detailed structural information
- Easy differentiation between isomers
- Chemical shift values do not always reflect functional group
  - e.g. Nitrobenzene (NO2) δH-2 = 8.19
  - Benzoic acid (COOH) δH-2 = 8.13
- No molecular weight information
- Signal area reflects concentration
- In addition 2D experiments possible
- Unambiguous structure elucidation of unknowns not always possible

HPLC - MS:
- Limited structural information
- Differentiation between isomers often not possible
  - (meta- and para-substituted compounds give identical MS spectra)
- MS often reflects functional group
  - e.g. Nitrobenzene: (loss of NO)
  - Benzoic acid: (loss of COOH)
- Molecular weight information
- Signal area does not reflects concentration

HPLC-NMR and HPLC-MS provide complimentary information

HPLC-NMR-MS: Instrumental Aspects

Coupling easy:
- HPLC eluent split (e.g. 95% introduced into NMR, 5% into MS)
- After splitter T-piece for additional introduction of buffer, solvent, water via syringe pump
- Additional loop to store complete HPLC peak for consecutive MS experiments (neg./pos. ionisation, MS^n)

Advantages of simultaneous coupling of HPLC to NMR and MS:
- Additional MS provides complimentary structural information
- Less ambiguities than with separate HPLC-NMR and HPLC-MS experiments
- MS triggers NMR experiments
HPLC-NMR-MS

H/D exchange:
- In HPLC-NMR usually D$_2$O is used as one solvent of mobile phase
  - exchange of all acidic protons by deuterium
  - increase of molecular mass by number of exchanged protons

D/H back-exchange allows:
- Determination of number of acidic protons
- Additional structure elucidation of neutral loss and fragments

Example: Loss of 31 Da from methoxylated methylamines
without H/D exchange:                      with H/D exchange:
  - loss of CH$_3$O (31 Da) or          - loss of CH$_3$OH (31 Da)
  - loss of CH$_3$NH$_2$ (31 Da)        - loss of CH$_3$ND$_2$ (33 Da)

Application of HPLC-NMR-MS to Environmental Samples

Method restricted to environmental samples with relatively high pollution level

Examples:
- Contaminated ground water from a former ammunition plant (water)
- Leachate from industrial landfills (water)
- Waste water from a textile company (water)
- Polycyclic aromatic hydrocarbons in soil (soil)

Application of HPLC/NMR and HPLC/MS for the Structure Elucidation of Unknowns in Environmental Samples

Main application areas of HPLC-NMR-MS
- Metabolism studies: drugs, pesticides
- Natural compounds in plants
- Environmental samples

Experimental Details

Aqueous samples
- Extraction
  - Solid phase extraction (Lichrolut EN)
  - Lyophilisation
- Mass Spectrometry: Thermospray, Electrospray, APCI, Ion trap
- NMR: Bruker 600 MHz

Soil sample

Ground Water below a Former Ammunition Plant

Leachate from Industrial Landfill
Polycyclic Aromatic Hydrocarbons in Soil
(early eluting components)

Reference compounds

Soil

Polycyclic Aromatic Hydrocarbons in Soil:
Late Eluting Components

Reference compounds

Soil

Polycyclic Aromatic Hydrocarbons in Soil
(reference compounds, including 8 non-EPA PAHs)