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Focal Point: Hyphenated Mass Spectrometry

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A. Liquid Chromatography – Mass Spectrometry

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The identification of unknown compounds is essential in a wide variety of research areas and especially so in environmental analysis. Combining liquid chromatography with tandem mass spectrometry today allows the identification of substances that were not amenable to classical techniques. Environmental toxicologists and chemists increasingly focus on chemicals that can potentially cause adverse effects in organisms and humans. Since effect concentrations are often very low, but also because the matrix can affect the sensitivity, selective enrichment has become a popular tool for sample clean-up and lowering the limit of detection. Advances in environmental analysis, including techniques based on molecular recognition for selective enrichment or effect-oriented research were presented in this session.

Keywords: Estrogenic compounds · Liquid chromatography · Mass spectrometry · Selective enrichment · Tandem MS · Time of flight MS

Structure Elucidation of Unknown Compounds by LC/MS

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It is generally accepted that structure elucidation by LC/MS is much more demanding compared to GC/MS. It requires MS/MS or multiple mass spectrometry (MS^n) to compensate for the very limited fragmentation of the pseudo molecular ions formed by the applied soft ionization processes (electrospray (ESI) and atmospheric pressure ionization (APCI)). Even then, often only so-called plausibility interpretations are carried out (verification of an assumed structure by the fragmentation pattern of the registered mass spectra). This is the usual procedure if additional information is available (synthesis route, metabolism of a known compound, further compound properties and/or partial structures).

What about structure elucidation when little or no further information is available (real unknowns)? The following three situations can be differentiated:

- The structure has the same carbon skeleton but a different substitution.
- The compound has a partially similar structure (*e.g.* obtained by derivatization).
- The structure is completely unknown.

The first case is the easiest one if MS^n is applied in an ion trap (*e.g.* fragmentation of the pseudo-molecular ion, keeping an abundant fragment and repeated fragmentation *etc*. in this way forming sequences of daughter ion spectra). The collision-induced dissociation in an ion trap allows control of the transferred energy, so that often functional groups are cleaved-off first (MS/MS) followed by the fragmentation of the carbon skeleton ($MS^{3–5}$). An example is the structure elucidation of trichothecenes. MS^3 spectra contain a typical fingerprint of the basic carbon structure, while MS^2 spectra and specific ion–molecule adducts give information about substitution [1]. In a similar way information about structure could be gained for aconitum alkaloids [2] or epoxy oligomers leached from coatings of food cans [3].

The second case is more demanding as the following example shows. Derivatization of carbonyls in air to 2,4-dinitrophenylhydrazones yields in a first round the information that the compound is a carbonyl as well as its mass. The increasing number of isomers of $>C_5$ carbonyls requires further knowledge (type of carbonyl, chain length, degree of saturation, branching). Therefore, the relation between fragmentation and structure was studied with reference compounds, some of them isotope-labeled. This procedure allowed a fragmentation scheme to be established for carbonyls and even the elucidation of rather complex molecules including isomers by MS^n only [4].

The third case is most demanding and requires a classical interpretation of the MS to MS^n spectra including:

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- Isotope information to identify heteroatoms and number of C-atoms (sufficiently precise up to *ca*. C_{30}).
- Ionization behavior by ESI and APCI: Difficult or no ionization with ESI indicates the lack of 'ionizable' groups such as -NH_2 or -OH.
- Information about substitution by loss of functional groups.
- Comparison of the highest mass in the ESI and APCI mass spectra. Differences indicate the presence of larger groups (*e.g.* sugar moieties cleaved off under ESI).
- MS^n fragmentation behavior indicates the stability of the carbon skeleton. Aromatic structures are more difficult to cleave.

In favorable cases, a structure can be elucidated or reduced to a few alternatives for smaller molecules (up to 300 u). In the case of the lefthand structure in Fig. 1, the following information was obtained: 10 C-atoms, no heteroatoms with prominent isotope clusters, aromatic structure (difficult to fragment in the trap), at least two NO_2 groups, presence of an ethyl group, probably a tertiary amine (only $[M]^-$ and not $[M-H]^-$). This led to two alternative structures due to the uncertain number of N atoms including the correct one (N,N-diethyl-2,4-dinitroaniline). The interpretation of the mass spectra of the righthand, rather large molecule was less successful due to limited information. Problems in fragmenting the molecule in the ion trap further indicated a (partially) aromatic structure. Much easier ionization by ESI(+) than ESI(–) or APCI(+/–) pointed to amino groups as substructure and an even number of N atoms. Further information was: *ca.* 32 C, presence of methoxy groups (-32 u; CH_3OH) and anisole groups (-116 u; methoxybenzene). The structure of the compound contained all identified elements, but further details could not be elucidated. In any case, classical EI/MS would not have given more information. In conclusion, structure elucidation with LC/MS is more demanding but not impossible as these two examples show.

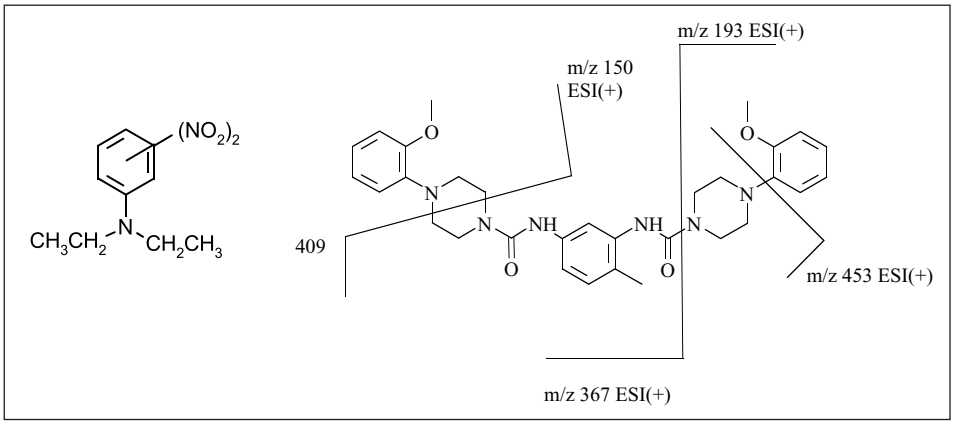

Fig. 1. Training molecule for the MS interpretation of totally unknown structures.

Advances in the Application of High Performance Mass Spectrometry to Problems in Environmental Toxicology and Chemistry

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The recent development of high-resolution, orthogonal acceleration time-of-flight mass spectrometers (oaTOF) and quadrupole-oaTOF hybrids (QTOF) has enabled routine accurate mass measurement online with HPLC separations, providing a valuable tool for qualitative analysis of environmentally relevant polar compounds in complex mixtures. Applications of accurate mass HPLC-oaTOF analysis to the qualitative and quantitative analysis of target and non-target polar contaminants in environmental samples such as wastewater and surface water were discussed. Strategies for identification of non-target compounds by accurate mass MS, such as pattern recognition (Fig. 2), constrained database searching, and high-resolution MS/MS have been presented. In addition, advantages of HPLC-oaTOF for routine, high-fidelity analysis of target compounds were discussed. Example applications, including high-sensitivity determination of emerging contaminants (such as pharmaceuticals and environmental endocrine disrupters) in aqueous environmental samples by HPLC-oaTOF and structural elucidation of endogenous surfactants in marine worm di-

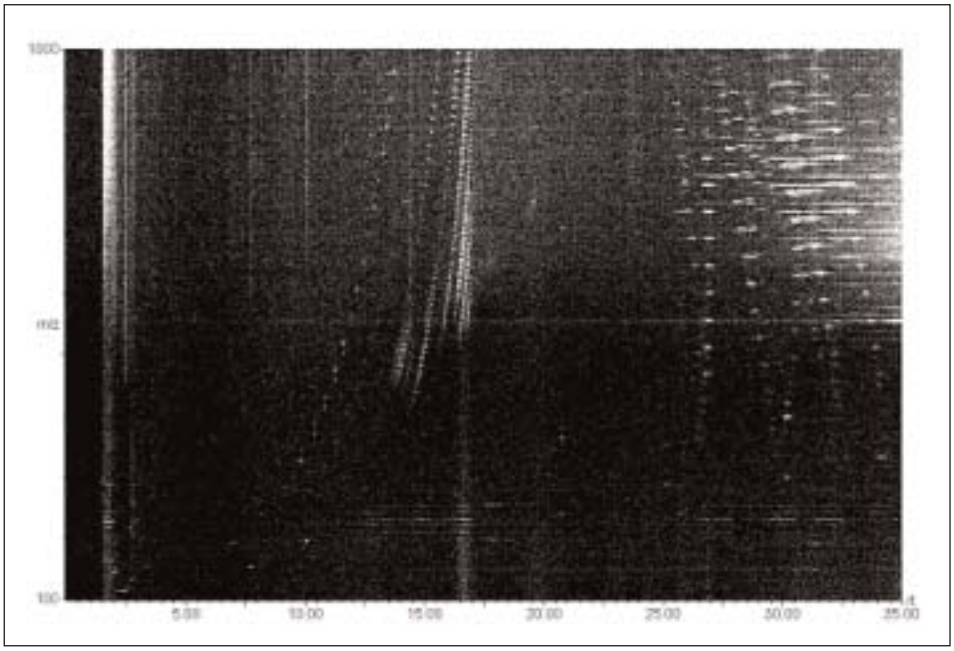

Fig. 2. Map display of m/z vs retention time for a wastewater influent analyzed by HPLC-TOFMS. Polymeric components are clearly visible as evenly spaced pixel clusters on the map. Accurate mass analysis confirmed the identity of polyethylene glycols and polyethoxylated surfactants.

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gestive fluids by QTOF have been presented. In addition, a perspective on the future development of robust analytical systems, based on high-resolution HPLC, accurate mass oaTOF, and MS/MS for routine qualitative and quantitative analysis of polar organic contaminants in complex mixtures was provided. Finally, the utility of QTOF mass spectrometry in proteome analysis has been explored, with special regard to the application of proteomics in environmental toxicology.

Sample Pre-Treatment Based on Molecular Recognition for More Efficient LC/MS Environmental Analysis

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Despite the advance in the development of highly sensitive analytical instrumentation for the final determination of analytes in complex matrices, a pre-treatment step is still required to extract and concentrate the analytes of interest. For aqueous environmental samples, solid-phase extraction (SPE) is nowadays widely accepted. However, classical SPE sorbents such as n-alkylsilicas, polymers and carbons suffer from a lack of selectivity. The co-extraction of other analytes and matrix interferences occurs when analytes are at a trace level and interferences at a higher concentration.

Tailor-made selectivity for a group of structurally related contaminants can be obtained using sorbents involving molecular recognition mechanisms.

A first biological approach consists in developing antibodies against a target molecule, which are further immobilized onto silica to form a so-called immunosorbent. The main parameters affecting extraction recoveries are presented. These immunosorbents have been used to selectively extract groups of structurally related contaminants (pesticides, phenols, anilines, PAHs, azodyes…) from complex matrices (waters, industrial effluents, soil, sediments, sludges, foodstuff…). Highly selective immunoextraction allows more rapid analysis and provides clean extracts for MS identification. Some applications combining on-line immunoextraction, microliquid chromatography and mass spectrometry were presented.

A second approach consists in the development of molecularly-imprinted polymers (MIP). Their selectivity is based on hydrogen interactions between the analytes and the MIP. Their direct use in aqueous samples involves two steps. Some examples illustrated the high selectivity that can be achieved after optimization of the extraction conditions.

Effect-Oriented Analysis for the Determination of Estrogenic Compounds

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Endocrine disrupting chemicals (EDCs) interfere with the endogenous hormonal system of humans and animals and may potentially cause adverse effects on human health and wildlife [1]. One class of EDCs are estrogenic substances. In the environment estrogenic compounds not only occur as residues of natural sexual hormones of humans and animals but also as constituents of plants and fungi (phytoestrogens, mycoestrogens). Also pharmaceutical substances and xenobiotics can act like estrogens. The wide variety of potentially estrogenic compounds makes it difficult to predict the estrogenicity of xenobiotics on a structural basis [2]. Thus, monitoring strategies cannot be based on simple analysis of environmental samples for known or suspected structures. In addition, an analytical system should be able to detect estrogenic compounds which are as yet unknown. Effect-oriented analysis is able to solve this problem by combining biomolecular recognition processes with chemical analysis. Therefore instrumental chemical analysis is reduced to relevant samples, giving information on relevant effective substances. This concept, which is known as bioresponse-linked instrumental analysis [3], was applied to the analysis of estrogens and xenoestrogens.

To prove this concept an enzyme-linked receptor assay (ELRA) for the detection of estrogenic substances was applied as described previously [4]. In brief the ELRA is a microwell-based receptor-binding assay using human estrogen receptor α (ERα). The ERα is produced in a recombinant yeast expression system. Measurements are carried out in 96-well microwell-plates. In the first incubation step, an estradiol-BSA conjugate is absorbed to the walls of the microwells. In the second (competition) step, an estradiol solution of defined concentration is added together with the ERα. After receptor binding, a biotinylated mouse anti-estrogen receptor antibody is added. A streptavidin-peroxidase-biotin enhancement system is applied. By using the luminescent substrate luminol, a significant improvement of the detection limit for estradiol (20 ng/l) could be achieved recently (Fig. 3).

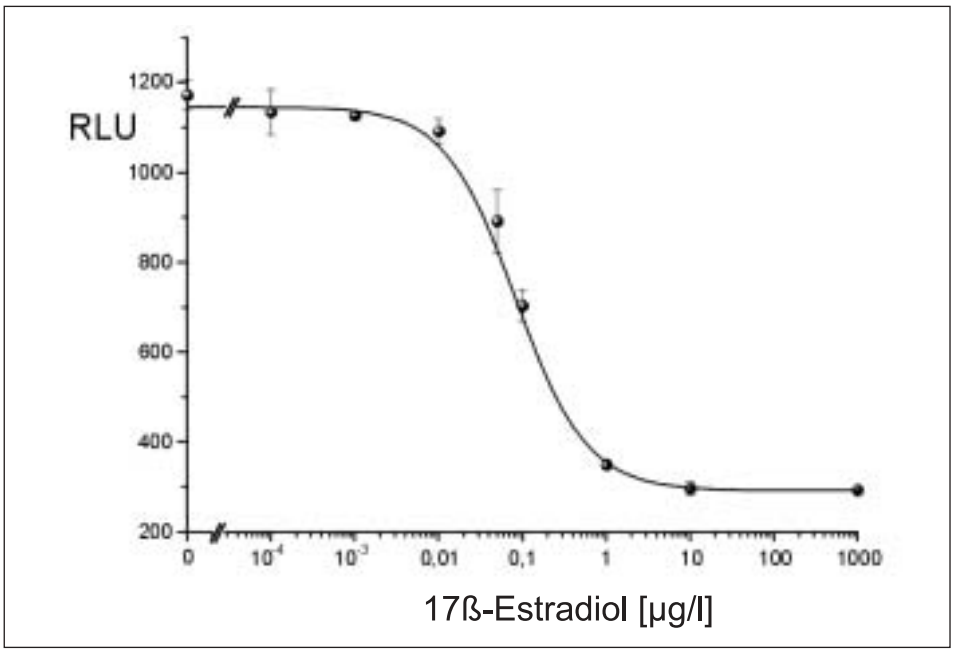

Fig. 3. ELRA calibration curve, using the luminescent substrate luminol. The detection limit is 0.02 µg/l

The ELRA approach has proved to be robust and highly suited for the detection of natural and synthetic estrogens as well as xenoestrogens in field studies (Fig. 4).

For the purpose of structural evaluation of effective substances, positively tested samples can be further characterized by liquid chromatography/tandem mass spectrometry (LC/MS/MS). For a tighter coupling of biomolecular recognition and structural evaluation methods, receptor affinity enrichment was directly linked to LC/MS/MS. This combination exploits the affinity of the ERα to bind bioeffective ligands. For this purpose ERα is immobilised on a receptor affinity column and samples are passed through the column. After washing and elution of relevant compounds, structural evaluation and quantification can be carried out by LC/MS/MS.

Bioresponse-linked instrumental analysis is a suitable tool for the detection and evaluation of estrogenic compounds in environmental samples. However, it is not possible to gain insight into action mechanisms in a target organism. This means that it remains unclear whether different estrogenic compounds all cause the same effect or whether the variety of estrogenic compounds also triggers a variety of effects.

For this reason other approaches have to be applied, which give information on effect mechanisms in living organisms. As estrogenic compounds act by altering the gene expression in different tissues, one possibility is to examine gene expression patterns. This can be done in different ways. For the mRNA-level, DNA-microarray techniques can be applied. For the protein level 2D-gelelectrophoresis combined with mass spectrometry is used. To carry out gene expression experiments organisms *e.g.* zebrafish are exposed to xenobiotic substances and the resulting alterations of the gene expression pattern in different tissues are mapped. DNA microarray techniques are based on the hybridization of two complementary nucleic acid strands. The probe consists of an oligonucleotide with the sequence of a target gene which is immobilized on a suitable surface such as a glass slide. The mRNA from a target tissue is reverse-transcribed to cDNA. In this process a fluorescent dye is incorporated into the cDNA. The cDNA is then hybridized to the probe. After read-out with a fluorimeter the gene expression pattern can be analyzed.

On the protein level gene expression patterns can be determined by 2D-gel electrophoresis giving information on the proteins expressed at the time the sample is collected. After picking differentially expressed spots, identification of the protein can be carried out by mass spectrometry *e.g.* MALDI-TOF. Using the massive parallel approaches of DNA-microarrays and/or 2D-gelelectrophoresis, a global view of the gene response in an organism can be gained, allowing questions on effect mechanisms of different estrogenic compounds to be answered. This approach can be expanded to other classes of xenobiotics and active substances opening the field for environmental genomics and proteomics.

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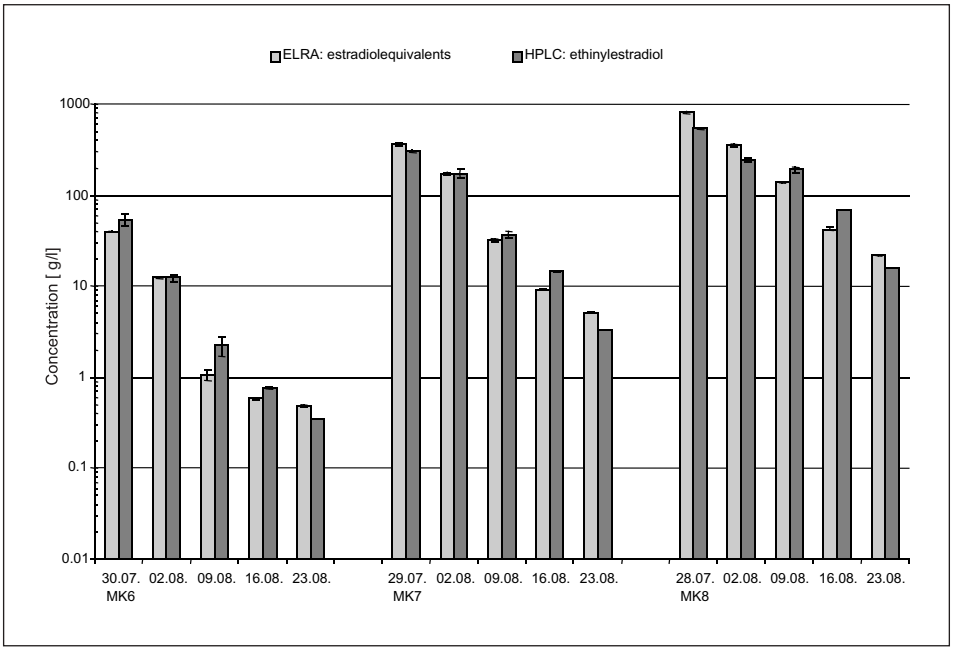

Fig. 4. ELRA applicability for real water samples. The results show the measurement of spiked lake water from a mesocosm experiment. Reference analytics were carried out by HPLC (the mesocosm experiment and HPLC measurements were performed by the Institute of Ecological Chemistry, GSF, Neuherberg, Germany). MK6, MK7, MK8: mesocosm

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B. Isotope Gas Chromatography – Mass Spectrometry

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The highly precise measurement of isotope ratios has a long tradition in earth sciences. The elements of most interest are carbon, oxygen, and hydrogen. More recently, the direct coupling of gas chromatography with the determination of isotope ratios for individual compounds was developed. This analytical method was first applied in organic geochemistry and yielded important results. Later the same technique allowed the evaluation of sources and processes in food analyses and contaminated environments.

Keywords: Flavors · GC/MS · Geochemistry · Halogenated solvents · Isotopes · Oil spills · Polycyclic aromatic hydrocarbons

Application of Carbon Isotope GC-MS in Organic Chemistry

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Until a decade ago, only careful structural characterization of biological markers occurring in geological samples was used as a major way of understanding their origin. The development of gas chromatography coupled with carbon isotope ratio monitoring mass spectrometry (GC-irmMS) has added a new dimension for the investigation of the sources of organic matter in ancient and present-day sedimentary deposits. Indeed, by this method, stable carbon isotope ratios ($^{13}C/^{12}C$) can be measured on individual molecules, the value of this ratio depending upon the source of carbon and the biosynthetic pathways by which the biological compounds have been formed.

This approach has been successfully applied to the study of hopanoid triterpenes formed by aerobic methanotrophic bacteria feeding on ^{13}C-depleted methane or for deciphering the carotenoids specific for phototrophic purple and green sulfur bacteria in actual and ancient ecosystems.

Furthermore, investigations of sedimentary deposits formed at methane seeps have recently disclosed the occurrence of anoxygenic bacterial methanotrophy, mediated by a consortium of microorganisms. Structural and isotopic studies show evidence that archaea are playing a key role in this newly discovered process which may be important for the understanding of the global carbon cycle.

Finally, ^{13}C-labelled compounds can also be used in order to trace metabolic pathways in soils or recent sedimentary deposits. In this respect, incubation of labeled phytol in sulfur-rich sediments from an anoxic lake shows evidence after a short period of time for the occurrence of biogeochemical processes such as reduction, condensation or defunctionalization.

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Flavor Authenticity Studies. Progress in Multi-Element HRGC-IRMS Techniques

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Flavorings – Does the Content Correspond to the Declaration?

The quality of food is essentially determined by its aroma which is an important part of the flavor, *i.e.* the complex sensory impression of odor, taste, and texture that appears during eating. Constituents of flavors are the volatile, chemically exactly defined flavor compounds. About 8000 flavor substances have been found in food to date; in future, 2000 will be integrated in a positive list by the EU. Among them, approximately 300 are of particular relevance for the industry.

All constituents of industrially produced flavors are governed by legislation and codes. In a 1998 directive, the EU defined six classes of flavoring substances, *i.e.* (i) natural compounds; (ii) nature-identical compounds; (iii) artificial compounds; (iv) flavor extracts; (v) processed flavors; and (vi) smoked flavors. If we focus our interest on the first two groups, we will define: *Natural flavor compounds* are substances that are obtained only from suitable natural raw materials or plant or animal origin by means of physical processes, such as extraction or distillation, or through microbiological or enzymatic, *i.e.* biotechnological processes. *Nature-identical flavor compounds* are substances that are obtained *via* chemical synthesis or through isolation from natural products by means of chemical processes. The chemical structure of nature-identical flavor compounds is identical to that of the corresponding natural ones. In order to be considered nature-identical, the flavor compound has to have been identified in plant or animal material traditionally consumed by humans as food.

Flavor compounds from these two categories differ in their market values. Between nature-identical substances and natural ones average price differences ranging from 1:10 to 1:100 exist. As a consequence, there is a certain temptation to increase the profit by unlawfully giving false declarations, *e.g.* declaring a nature-identical flavor compound as natural. Both the industry and the consumer are confronted with this situation, the first when buying raw materials to be added to a food, the latter when selecting the flavored product at the supermarket.

How can the question be answered whether the content corresponds to the declaration? Principally, differences of selected parameters arising between nature and laboratory chemistry are analytically evaluated. Firstly, the well-known selectivity of nature is used to biosynthesize preferably one of the enantiomers of a chiral compound, while chemical synthesis – except for asymmetric modifications – leads to an enantiomeric ratio of 50:50. Gas chromatographic (HRGC) techniques are established to exactly determine the enantiomeric ratio of flavor compounds [1], but they are limit-

ed to the small amount of chiral flavor substances. Secondly, the determination of the ratio of stable isotopes is the method of choice to obtain more comprehensive information.

Determination of Global Isotope Ratios in Organic Compounds by Mass Spectrometry

The exact determination of the extremely small differences in isotope abundances can be realized by a precise quantitative mass spectrometric method, *i.e.* the isotope ratio mass spectrometry (IRMS) applicable to simple gases [2][3]. These have to be produced from the organic compounds by combustion (C) or/and pyrolysis (P). The measuring gases needed are CO_2 formed in the C-mode as well as hydrogen gas and CO, the latter two generated in the P-mode.

The prerequisite for IRMS measurements of complex mixtures of flavor compounds in food is their separation and purification. Separation into pure compounds and IRMS analysis can be performed by online combination of HRGC with IRMS (Fig. 5). The integrated interface consists of two different reactor types, one to realize the combustion of carbon into CO_2 (for $^{13}C/^{12}C$ analysis) and two others to pyrolyze the compounds, forming H_2 and CO as measuring gases for $^{2}H/^{1}H$ and $^{18}O/^{16}O$ determinations, respectively (Fig. 6).

Whereas on-line $^{13}C/^{12}C$ ratio determinations have already been performed previously [2][3], the technical prerequisites for on-line HRGC-P-IRMS measurements have been made available only recently [4–6]. Thus, the additional information about the $^{2}H/^{1}H$ and $^{18}O/^{16}O$ ratios of industrially attractive flavor compounds opens the way for multi-element approaches. Among the 'key' flavor substances are, for instance, the group of C_6 compounds ((*E*)-2-hexenal, (*E*)-2-hexenol, (*Z*)-3-hexenol), benzaldehyde, and citral. They are responsible for 'green-grassy' odors desired in many fruit flavors, cherry flavor and lemon-type flavors, respectively. Based on comprehensive linearity studies combined with comparisons of EA-P- and HRGC-P-IRMS data we have demonstrated recently the additional value of on-line $^{2}H/^{1}H$ and $^{18}O/^{16}O$ measurements for the authenticity assessment of these flavor compounds [7–10]. As a representative example, in Fig. 7 the correlation of $\delta^{2}H_{VSMOW}$ and $\delta^{18}O_{VSMOW}$ values of the 'leaf alcohol' (*Z*)-3-hexenol is outlined. As shown from the graph, the two categories of synthetic (nature-identical) and natural (*Z*)-3-hexenol are distinctly differentiated.

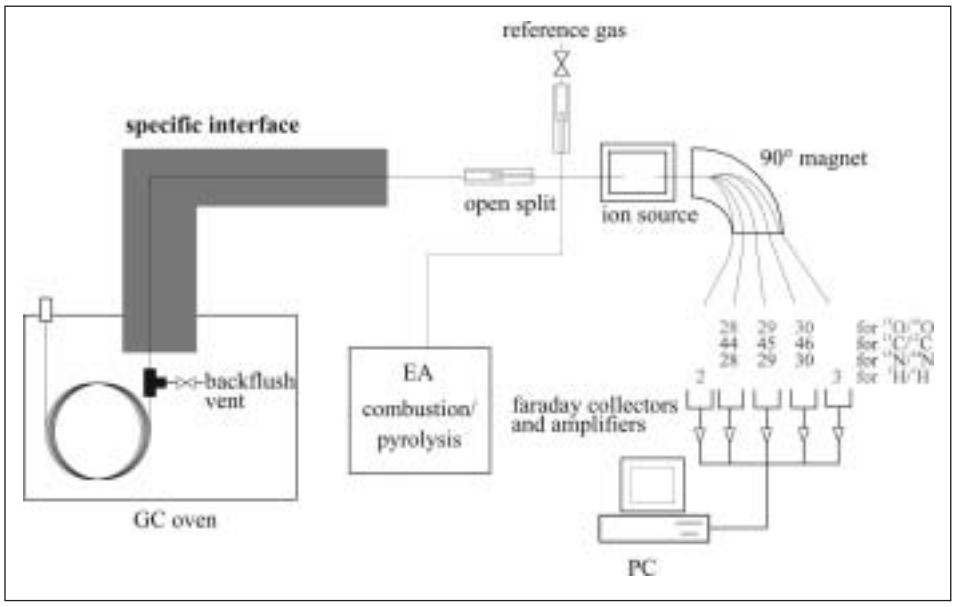

Fig. 5. HRGC-C/P-IRMS coupling for the authentication analysis of aroma compounds in complex flavor mixtures.

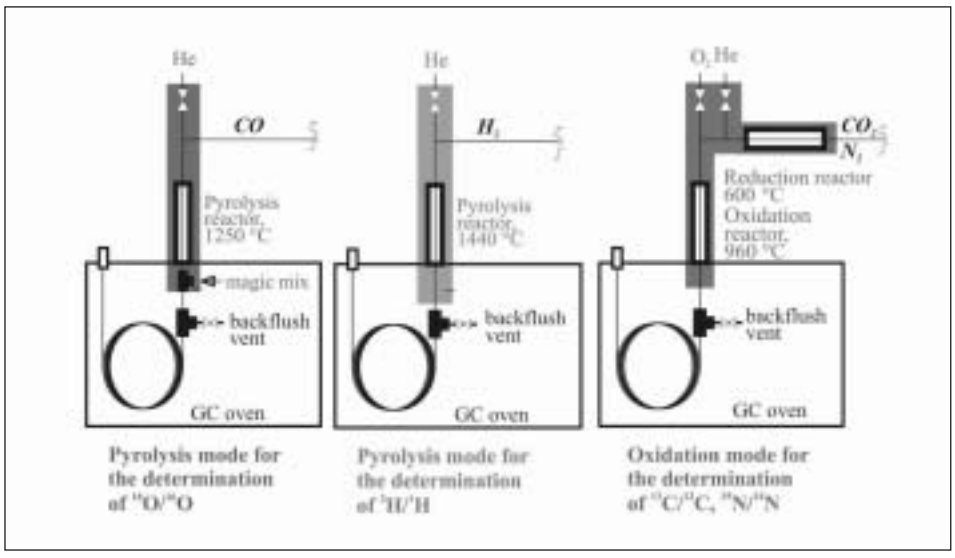

Fig. 6. On-line combustion (C) and pyrolysis (P) interfaces to transfer organic compounds into measurable gases, *i.e.* CO_2 for $^{13}C/^{12}C$ measurement and hydrogen/CO for $^{2}H/^{1}H$ and $^{18}O/^{16}O$ determinations

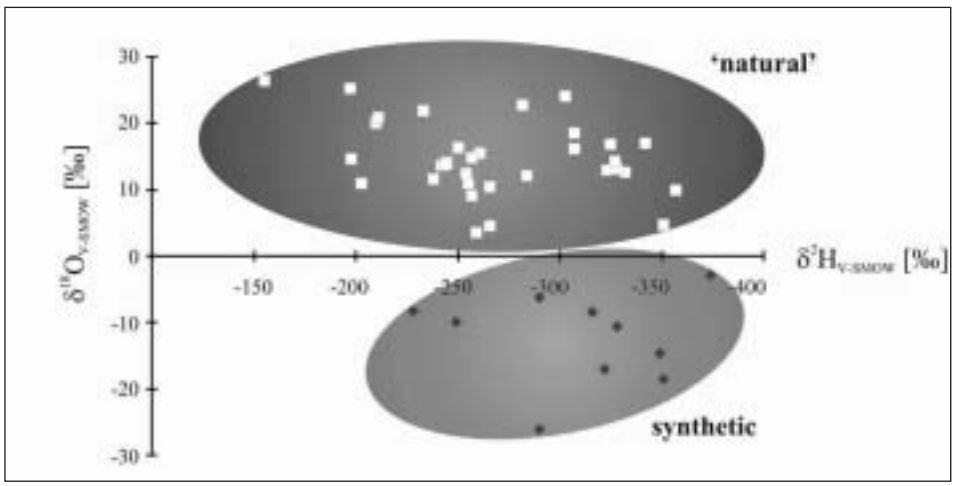

Fig. 7. Correlation of $\delta^{2}H_{VSMOW}$ and $\delta^{18}O_{VSMOW}$ values [‰] of (*Z*)-3-hexenol, samples from natural and synthetic origin.

From these and current running studies a number of conclusions can be drawn concerning the (i) general and (ii) element-specific requirements for HRGC-C/P-IRMS measurements. They comprise for (i) the availability of authentic reference material, statistically relevant sample numbers, the exclusion of isotope discrimination in the course of sample preparation and chromatographic steps, as well as continuous checks of system stability using certified standards. As to (ii), HRGC-C-IRMS measurements of $^{13}C/^{12}C$ ratios can be routinely performed; the on-line $^{2}H/^{1}H$ determination is a highly promising technique provided that the dynamic linearity is checked carefully and, depending on the structure of the target molecule, potential isotope exchange is excluded. HRGC-P-IRMS measurements of $^{18}O/^{16}O$ ratios still suffers from the empirical pyrolysis technique and the need to use tertiary standardization. In addition, similarly to $^{2}H/^{1}H$ determinations, check of dynamic linearity and potential isotope exchange is required. Considering these requirements, HRGC-P-IRMS is an helpful tool in the authenticity assessment, in particular, as predictions about the global $\delta^{18}O_{VSMOW}$ and $\delta^{2}H_{VSMOW}$ values of natural compounds on the basis of their biogenesis will become increasingly available [11][12].

Acknowledgements

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^{13}C Analysis of PAH: A New Dimension in Source Assessment Studies

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1. Introduction

Among various chemical contaminants, the pollution caused by polycyclic aromatic hydrocarbons (PAHs) has led in recent years to numerous studies on the origin, distribution and fate of these compounds in the environment. Polycyclic aromatic hydrocarbons are ubiquitous organic pollutants because of their chemical stability and the multiplicity of their sources. It is currently assumed that they are mainly of pyrolytic origin. Besides this origin, PAHs are introduced in the environment through contamination by crude oils, coal tar or various refinery products (petrogenic origin). They can also derived from biogenic precursors like terpenes, pigments and steroids (diagenetic origin).

It is important to study the biogeochemical cycles of PAHs as these compounds show mutagenic and carcinogenic properties. Nevertheless, the elucidation of PAH source and behavior can be problematic. Indeed these studies are based on PAH molecular fingerprints or concentration ratios [1–3]. Physical, chemical and microbial transformations can alter the characteristics of many sources during transport and deposition [4][5]. In this respect, qualitative and even more quantitative source assessment of PAHs using solely molecular considerations can then be hazardous in some cases, especially for weathered samples. Alternative methods are thus very desirable. In that prospect, GC-C-IRMS (gas chramatography/combustion/isotope ratio mass spectrometry) can be a complementary technique of source assessment. In this context, the use of molecular carbon isotopic composition ($^{12}C/^{13}C$), which depends on the carbon source used for the synthesis of the compound, on the biosynthesis pathway and on the environmental conditions [5] (geographical origin, temperature…), should allow PAH source assignment to be improved [6]. Due to the analytical improvements of the last decade, it is now possible to access easily the isotopic ratios of individual compounds in a complex mixture by the use of gas chromatography/isotope ratio mass spectrometry (GC/IRMS) [7].

The measurement by GC-C-IRMS of isotopic composition of individual PAHs in conjunction with a molecular approach can add a new dimension to biogeochemical studies and provides the geochemist with a powerful tool to study the fate and the origin of the organic matter.

In this work we presented this technique and the specific problems related to the analysis of PAHs by GC/IRMS (sample preparation, oxidation of PAHs, chromatographic resolution). In order to evaluate the potentialities of this isotopic PAH source apportionment strategy, the isotopic composition of phenanthrene and methylphenanthrenes was monitored during an *in situ* oil spill simulation experiment. In this study, a known quantity of petroleum was intentionally introduced at the top of the sedimentary column. In such samples both natural and petrogenic PAHs are present. The aim of this study was to quantify petrogenic PAHs present in the samples during a natural biorestoration period. Another application deals with the Erika oil spill. The origin of PAHs in oil residues and oiled bird feathers sampled along the Atlantic coast of France after the Erika oil spill has also been studied.

2. Experimental

2.1. *In situ Bioremediation Experiment [8]*

In the cove of Carteau, close to the mouth of the Rhone River (Mediterranean coast of France) 10 cm diameter and 25 cm long PVC tubes were placed in order to delimit a biotope and biocenose fraction. A 1 cm thick freezed slice of sieved sediment from the experimental site was placed on the top of each corer. Some of those slices were contaminated with petroleum (blended Arabian light distilled at 250 °C, 40 g of BAL 250 per kg of dry sediment) and some others were free of petroleum, giving respectively contaminated cores and control ones. Three contaminated and control cores were sampled every six months. Each core was cut in 2 cm thick slices. In this study, isotopic compositions of phenanthrene and methylphenanthrenes in the petroleum used for the contamination (BAL 250), in the 2 to 4 cm layer of a reference core and three contaminated cores sampled every six months, from 6 months up to 36 months, have been measured.

2.2. *Erika Oil Spill [9]*

A sample of the heavy fuel oil loaded by the Erika tanker at the Flandres refinery (Dunkerque, France) and a sample collected from an oil slick released by the Erika tanker were provided by the CEDRE (French institute for water pollution, Brest, France). Oil residues sampled on the Atlantic Coast after the oil spill were also analyzed. Oiled bird feathers collected on the Southern Atlantic Shoreline were provided by the Teich Ornithological Park (Gironde, France).

2.3. *Sample Preparation*

The freeze-dried 2 cm slices of sediment were sieved at 2 mm and then extracted using microwave assisted extraction (10 min, 30W) [10]. Methylene chloride (Scharlau, HPLC grade) was the extraction solvent. The sample was filtered and the total organic extract was reduced to a small volume using a rotary evaporator. The extracts were then purified on a florisil column to eliminate polar compounds. In order to be able to measure isotopic compositions of methylphenanthrenes it was necessary to separate them from methyldibenzothiophenes as those two classes of compounds coelute during gas chromatographic separation using a classical apolar capillary column. The phenanthrenic fraction was individualized by high-pressure liquid chromatography separation on aminosilane phase (Spherisorb, 5 µm, 25 cm, 4.6 mm ID) with pentane (Scharlau, HPLC grade) as eluent. The integrity of the phenanthrenic fraction was controlled by checking for the absence of phenanthrene and methylphenanthrenes in both the dibenzothiophenic and tetra- aromatic fraction by gas chromatography/mass spectrometry (GC/MS) analysis. The absence of compounds coeluting with phenanthrenic compounds was controlled by full scan GC/MS analysis. The entire protocol has been validated [11].

2.4. *GC/IRMS Analysis*

Isotopic analyses of individual PAHs were carried out using an HP 5890 Series II Plus gas chromatograph interfaced *via* a CuO furnace (940 °C) and a hygroscopic membrane (nafion) to a Delta Plus isotopic ratio mass spectrometer from Finigan corporation. Injections were performed in the splitless mode. The injector temperature was maintained at 270 °C. The GC temperature program was from 50 °C to 180 °C (2 min) at 10 °C.min^{-1}, to 230 °C at 2 °C.min^{-1} and to 290 °C at 10 °C.min^{-1}. The carrier gas was helium (flow rate: 1.35 ml.min^{-1}). The capillary column used was a SGE BPX5: 60 m × 0.22 mm ID × 0.25 µm film thickness. For calculation purposes CO_2 reference gas was automatically introduced into the isotopic ratio mass spectrometer in a series of pulses at the beginning and the end of each analyses. The reproducibility of the individual isotopic measurements, determined through repeated analysis of 2-methylphenanthrene standard, was ± 0.25‰. Precision reported in this study is based on multiple analyses (at least two analyses) of each sample.

3. Results and Discussion

3.1. *Analytical Developments*

The sample preparation protocol developed in order to determine the isotopic composition of PAHs [11] allowed both PAH fractions to be obtained in which compounds do not suffer from coelutions and the importance of the unresolved complex mixture to be reduced which otherwise contributes to the background signal during GC-IRMS analysis. This analytical procedure has allowed the measurement of the stable carbon isotopic composition of polycyclic aromatic hydrocarbons in petroleum and sediment with accuracy and good reproducibility (Fig. 8).

3.2. *In situ Experiment*

As the stable carbon isotopic composition of the petrogenic methylphenanthrenes added and those of the methylphenanthrenes present in the sediment of the experimental site were significantly different due to variation in origin it has been possible to specifically study the behavior of petrogenic PAHs during an oil spill simulation experiment. After 36 months of experiment, all the petrogenic methylphenanthrenes were removed whereas the ones that were present in the sediment before the contamination were not affected by the processes responsible for the remediation of the site (Fig. 9). This origin-dependent fate might be due to the fact that native PAHs are more strongly adsorbed to the sedimentary particles and are less available for processes such as dissolution and biodegradation. Using molecular stable

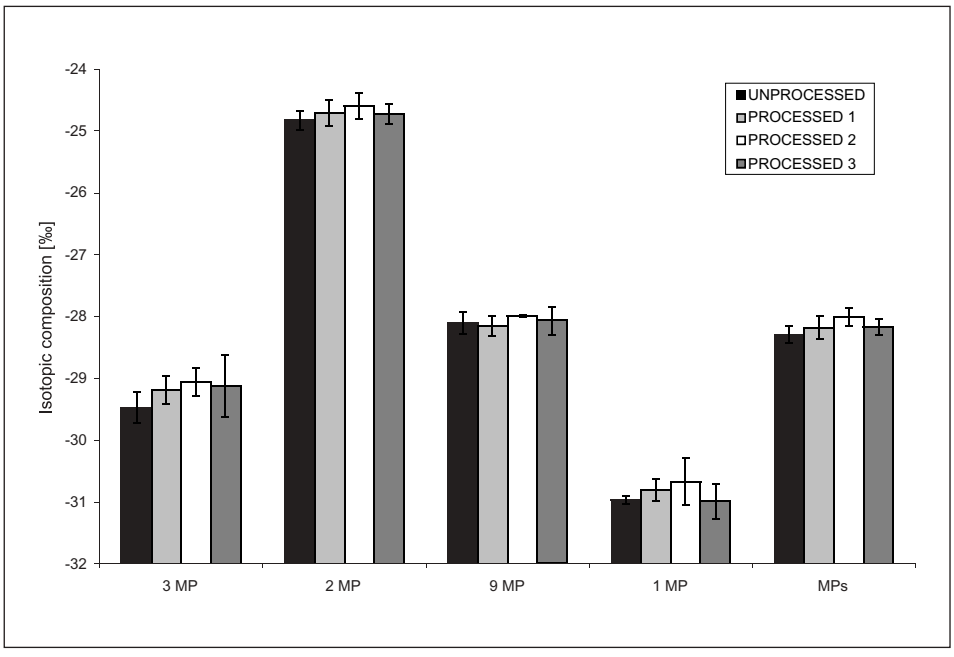

Fig. 8. Validation of the analytical procedure on standard methylphenanthrenes. The values represented are the mean of three analyses for each assay.

carbon isotopic composition of methylphenanthrenes the preferential degradation of the 2- methyphenanthrene isomer, observed all along this experiment, was also detected [8].

3.3. *Erika Oil Spill*

The molecular isotopic compositions of phenanthrene compounds contained in oil from the Erika tanker, in the oil residues collected on the north part of the Atlantic Coast, in some oiled bird feathers and in the oil residues collected in the Arcachon Bay area are compared in Fig. 10. The isotopic compositions of the sums 3+2-MP and 9+1-MP determined by integrating the two peaks representing each compound together (3- and 2-MP on one hand and 9- and 1-MP on the other hand, respectively) were then measured. Samples collected on the north part of the Atlantic Coast and all bird feathers are well correlated and show phenanthrenic isotopic profiles close to that of the Erika oil. This is additional proof for the link between these samples and the Erika oil spill. However, all the samples collected in the Arcachon Bay area exhibit isotopic compositions significantly enriched in ^{13}C compared to the Erika oil. This shows, as with the previous evidence, that they are not linked to the Erika oil spill. Oil residues sampled on the Pereire beach and the big tar balls collected on the Salie beach exhibit similar isotopic profiles whereas samples collected on the Crohot beach and the small tar ball collected on the Salie beach exhibit significant individual isotopic composition discrepancies showing that they come from independent sources.

As the molecular isotopic composition of hydrocarbons has been previously shown to not be affected during degradation of oil [12–14], its use should be helpful for the identification of weathered residues. Molecular distributions would be specifically modified in such samples.

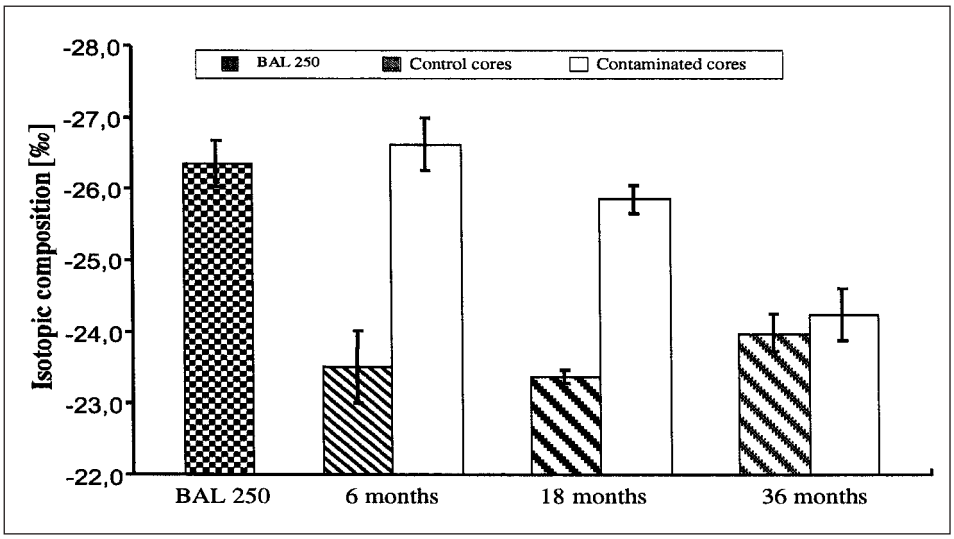

Fig. 9. Isotopic composition of the methylphenanthrenes present in the BAL 250 and in the two to four centimeter layer of the control and contaminated cores sampled after 6, 18 and 36 months of the experiment.

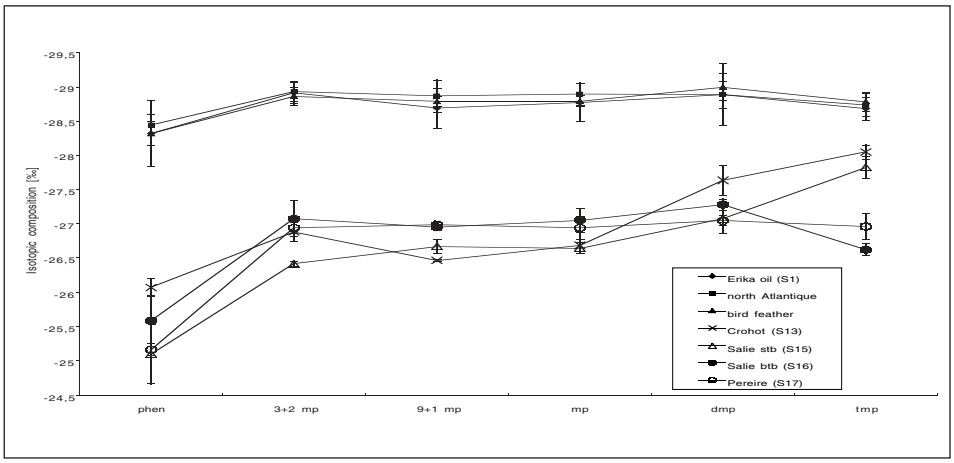

Fig. 10. Phenanthrene compound isotopic composition of oil residues and oiled bird feathers collected along the Atlantic Coast of France compared with Erika oil isotopic composition. S1: mean of three analyses. North Atlantic: Average between the mean values (n = 3) of 7 residues and S11. Bird feathers: Average between the mean values (n = 3) of 8 samples

Acknowledgments

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Compound-Specific Stable Isotope Analysis of Organic Groundwater Contaminants: Environmental Applications and Analytical Challenges

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Background

Compound-specific isotope analysis (CSIA) is a very promising approach to help determine both *in-situ* transformation processes of pollutants in contaminated aquifers as well as sources of groundwater contaminants. Various laboratory studies have demonstrated the potential of this tool in contaminant hydrology [1–3]. To date, the use of CSIA in field studies is, however, confined to near-source zones of groundwater contamination with high pollutant concentrations. The method detection limits in CSIA are in the order of 150 µg/l for chlorinated solvents using solid phase microextraction as preconcentration method [4]. Hence the method sensitivity is currently insufficient to investigate tail and fringe zones of contaminant plumes, which would be most helpful to assess natural attenuation processes in the field.

Method Evaluation, Improvement of Sensitivity and Field Application

We evaluated different injection and concentration techniques in combination with isotope ratio mass spectrometry. In order to improve the method detection limits of CSIA, we applied solid-phase microextraction (SPME) and online purge and trap (P&T) preconcentration techniques and investigated their effects on the isotopic composition of the analytes. Isotopic fractionation effects of the various processes involved in SPME and/or P&T (*i.e.* evaporation, sorption, desorption, and condensation of the analytes) have been evaluated for a series of analytes. The effects were found to be compound-specific but showed a high reproducibility. The detection limits for δ^{13}C-determinations of volatile organic compounds could be drastically lowered with the developed P+T-GC/IRMS method to < 5 µg/l (Fig. 11).

Applications

P&T-GC/IRMS was successfully applied to study the fate of halogenated solvents in a contaminant plume down gradient of a municipal landfill. Contrary to previous evaluations based on concentration data alone, our P&T-GC/IRMS data strongly suggest the absence of *in-situ* degradation of trichloroethene (TCE) despite the presence of *cis*-dichloroethene, a known metabolite of TCE.

The SPME-technique allowed the determination of δ^{13}C-values of MTBE and its degradation product tert-butyl alcohol in a highly contaminated aquifer in Brazil. The results suggest that the contaminated aquifer has two different zones, one with isotope fractionation linked to MTBE-degradation and a second where the observed concentration decrease is mainly due to dilution or other non-fractionating processes. Furthermore the stable isotope data allow the identification of different spill zones at the site.

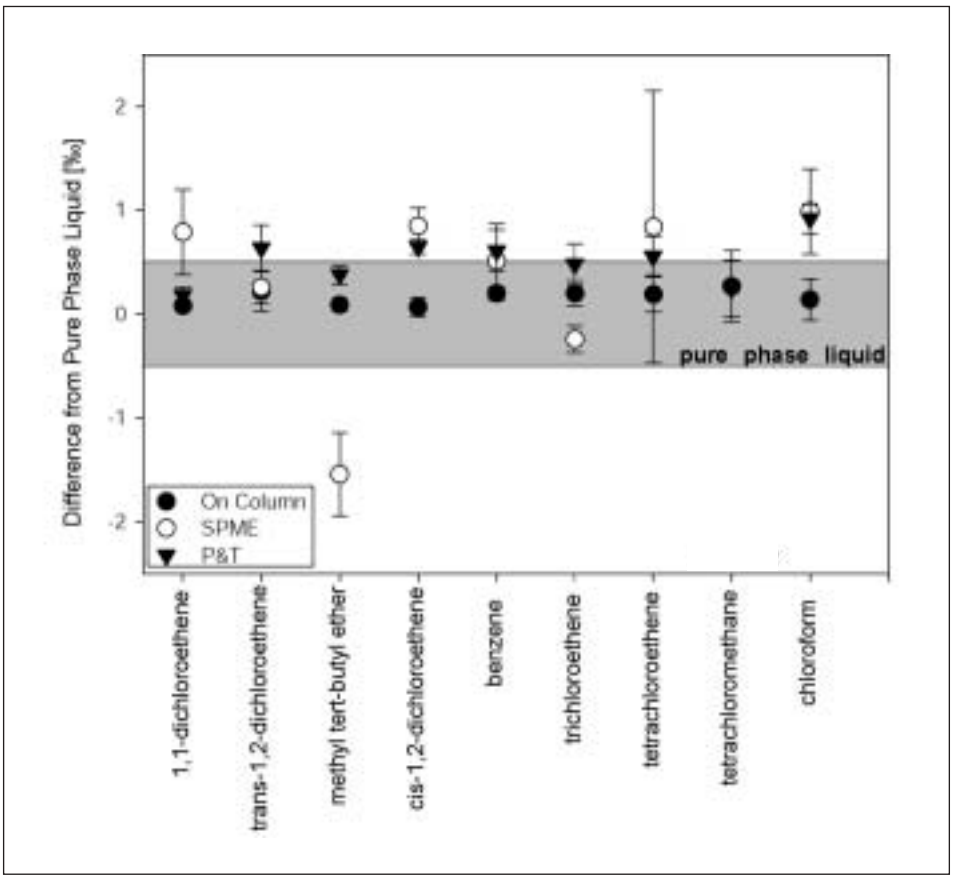

Fig. 11. Accuracy and reproducibility of $^{13}C/^{12}C$-determination for cold on column injection (•), solid-phase microextraction (o) and purge and trap (▼). The horizontal bar corresponds to a δ^{13}C-measurement within a +0.5‰ interval of the EA-IRMS measurements.

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