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Organic Trace Analysis – Challenges – Unsolved Problems

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The session 'Organic Trace Analysis – Challenges – Unsolved Problems' tried to give a 'state-of-the-art' view of some of the fundamental problems in organic trace analysis:

- How to resolve very complex mixtures into single compounds and to deal with the resulting flood of information.
- How to improve resolution of a demanding separation and to speed up analysis time.
- How to extract efficiently volatile polar compounds from water; a hardly solvable problem.

In the following, leading experts in these fields give a synopsis progress report and outlook about some pieces of the complex puzzle of possible solutions, namely the role and status of separation columns in high performance liquid chromatography, the possibilities and limitations of electrochromatography, the on-line combination of sample preconcentration/clean-up with separation techniques, the modular on-line coupling of two GC columns and the enrichment of polar compounds in water by a LiCl-precolumn and high-resolution gas chromatography.

Keywords: Analytical chemistry · Polar compounds · Selectivity · Separation techniques · State-of-the-art · Trace analysis

New Directions In HPLC Column Technology For Rapid And Selective Separations

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Major improvements in HPLC columns have occurred over the past decade. Wide-pore silica packings have dominated the HPLC marketplace for biomolecular separations. They allow diffusion of large molecules into the pores for improved solute–stationary phase interactions. The modern porous packing materials are mostly based on silica gel with siloxane chemically bonded phases. Significant advances have been made in reproducibly bonding various functional groups to perform ion exchange, reversed phase, hydrophobic interaction, affinity, and size exclusion separations. New silica-based and hybrid phases allow columns to be operated in the high pH domain (pH ~12) with all the advantages of rigid HPLC packings. For example, Fig. 1 shows stability test data of a new type of bidentate bonded phase that extends the upper pH range of silica beyond its normal limits [1]. Conventional bonded phase silica gel-based columns fail due to the dissolution of the underlying silica gel and the more stable polymer columns lack adequate efficiency.

In addition to silica-based columns capable of operating at high pH with good efficiency, new bonded-phase chemistries can also extend column stability in the low pH zone. Bonded phases with steric protecting groups prevent acid-catalyzed hydrolysis of the siloxane (Si-O-Si) and allow the use of these packings below pH 1 without loss of bonded phase. New bonded phases with polar functional groups embedded into the alkyl chain allow the use of reversed-phase packings with low percentages of organic modifier or even 100% water in the HPLC mobile phase without 'phase collapse' and provide selectivity differences compared to conventional alkyl bonded phases [2].

Short conventional analytical columns packed with 3 and 3.5 µm porous microparticulate particles have resulted in faster separations and solvent savings but provide similar resolution to longer columns packed with the more conventional 5 µm particles. Such 'rapid resolution' or 'fast LC' columns have been driven by the needs of LC/MS, combinatorial chemistry and high-speed quality control applications.

New high performance phases such as superficially porous packings have provided high-speed columns that allow separations in a matter of seconds to minutes (Fig. 2). Because of decreased diffusion path lengths in the thin porous shell, they are useful for high-speed separation of large biomolecules whose slower mass transfer limits separation speed in traditional porous

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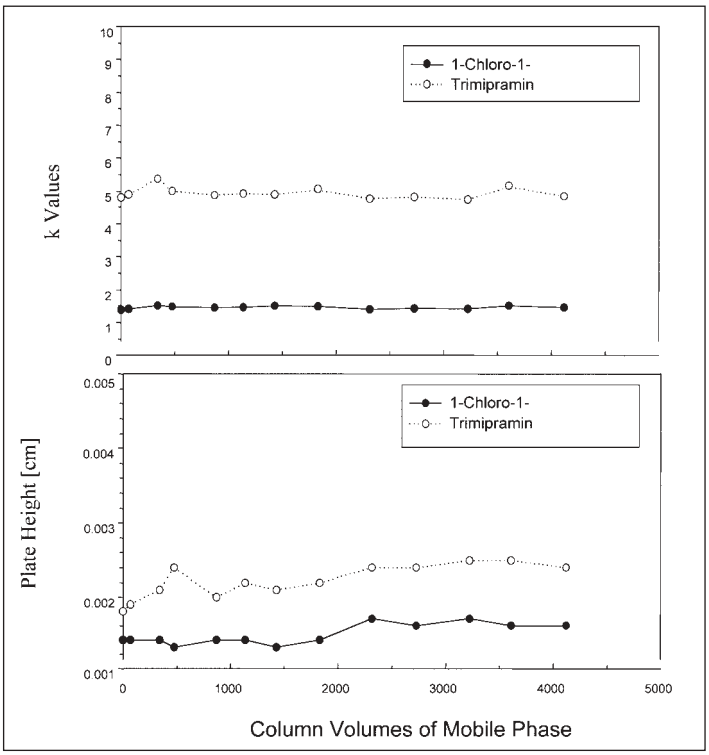

Fig. 1. Bidentate C18 HPLC column stability test at high pH. Column: Zorbax Extend-C18 (Agilent Technologies, Wilmington, DE), 4.6 mm i.d. × 150 mm, 5 mm particle size. Mobile phase: 80% methanol; 20% 20mM ammonium hydroxide, pH 10.5. Flow rate: 1.5 ml/min. Solutes: 1-chloro-1-nitrobenzene (neutral compound) and Trimipramine (basic compound). The mobile phase continually passed through column. Samples were injected, capacity factors (k) and column efficiency determined at given intervals (one column volume ~ 2.5 ml).

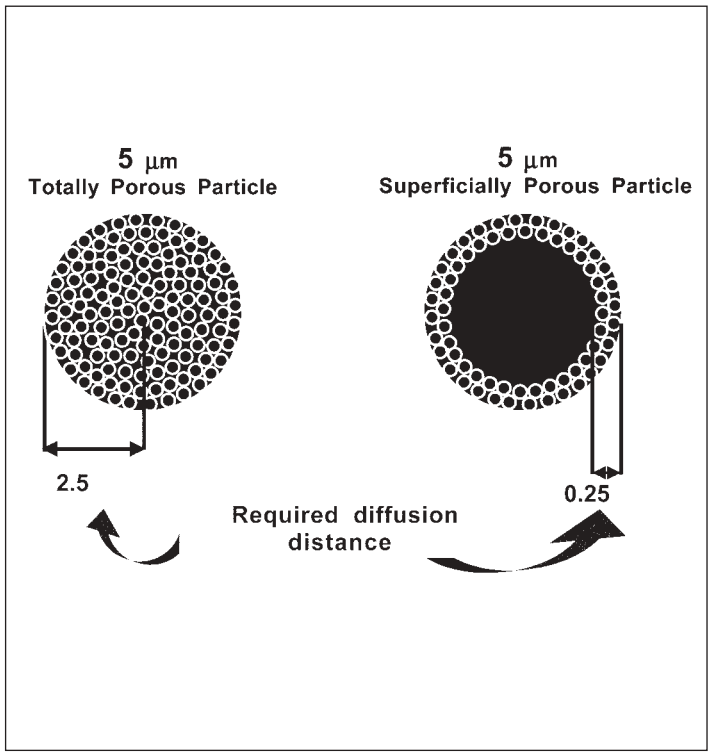

Fig. 2. Comparison of the diffusion distances in a totally porous silica particle and a superficially porous particle. In the superficially porous particle macromolecules that diffuse slowly in solution have a shorter diffusion distance to traverse and therefore diffuse faster into and out of the stationary phase.

packings. Monolithic columns that are cast into rods rather than packed, are particularly appealing due to their good efficiency at a lower operating pressure than microparticulate packings. Monoliths are available in both silica gel and polymeric versions. The use of polymeric perfusion packings is an alternative approach that has been successfully applied to many biopurifications.

Recently, column developments are being spurred by the need for more rapid separations of and for purer biological macromolecules. Proteomics studies of protein digests often result in the need to identify and quantitate tens of thousands of trace peptides using multidimensional LC followed by tandem mass spectrometry (MS) using nanocolumns with internal diameters less than 100 µm. The future looks bright for continued developments in column technology particularly for new biocompatible columns, capillary electrochromatography, and LC-on-chip using monolith technology [3].

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The Potential of Electrochromatographic Separation Methods in Environmental Analysis

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High-resolution gas chromatography (HRGC) and high-performance liquid chromatography (HPLC) are the separation methods of choice in environmental analysis. However, HPLC fails to separate very complex mixtures due to a lack of resolution. The outstanding success of capillary columns in HRGC in achieving high efficiencies could not be transferred to HPLC due to the small diffusion coefficients in liquids and the parabolic flow profile produced by pressure driven flows resulting in a substantial band broadening, if capillary diameters are not extremely small (<10 µm) [1], which requires demanding technology for injection, detection, and equipment.

Capillary electrophoretic and electrochromatographic techniques are more and more considered as alternative liquid phase separation methods for charged and neutral analytes. Compared to HPLC, they offer higher efficiency and different selectivities. Electrochromatographic separation methods benefit from the plug-like electroosmotic flow which is generated in open or packed fused silica capillaries when an electric field is applied. In addition, the electroosmotic flow velocity is almost always independent of the capillary diameter or the channel diameter within a packing structure.

In capillary electrochromatography (CEC), 50–100 µm i.d. capillaries are used packed with real stationary phases such RP materials [2][3]. The mobile phase is electroosmotically moved by applying an electric field over the capillary length. Unfortunately, the performance of CEC with respect to resolution and analysis time does not match theoretical expectations in most cases. In CEC, it is difficult to generate a constant electroosmotic flow because of

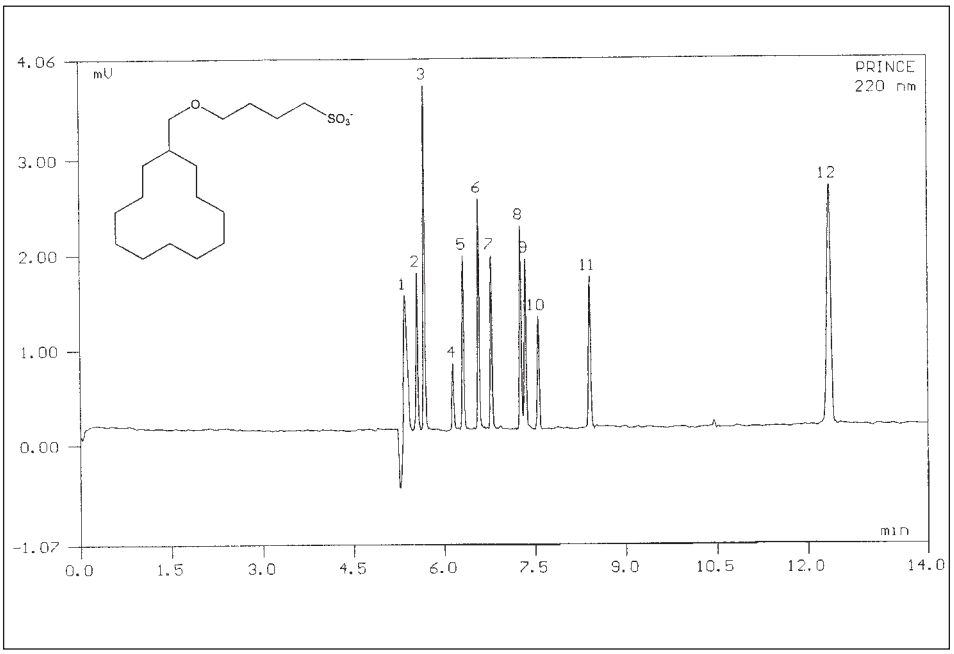

Fig. 3. MEKC separation of some explosives and related compounds. Fused silica capillary, 75 cm long, (L_{eff} 47 cm), 50 mm i.d.; buffer (20 mM borate, pH 9.4 + 25 mM 4-cyclododecylmethoxybutane-1-sodium-sulfonate) and CH_3OH, 90+10 (v/v); field, 400 V/cm; detection: UV, 220 nm. Compounds (plate number in brackets): (1) thiourea; (2) 2,4-diaminotoluene (165 000); (3) aniline (165 000); (4) 1,4-dinitrobenzene (160 000); (5) 1,3-dinitro-benzene (200 000); (6) 2,4,6-trinitrotoluene (240 000); (7) 1,2-dinitro-benzene (200 000); (8) 2-nitro-4-amino-toluene (200 000); (9) 2,4-dinitrotoluene (220 000); (10) 2,6-dinitrotoluene (215 000); (11) 2,3-dinitrotoluene (215 000); (12) 2,4-dinitro-6-methylphenol (195 000).

changing surface characteristics within the capillary column. In addition, local velocity differences in the sections of the packing, the frits and the open-tubular detection capillary can lead to intersegmental pressures and flow profile distortions, which decrease efficiency. Formation of bubbles is another problem which can impede routine use of CEC. Considerable methodical progress seems to be necessary before CEC becomes feasible for environmental analysis.

On the other hand, (micellar) electrokinetic chromatography ((M)EKC) has already developed into a matured method [4]. Here, a surfactant, the so-called 'pseudostationary phase', dissolved in the buffer solution serves as retentive medium. Very high plate numbers can be generated in EKC, since the analytes have to pass very small diffusion distances in order to interact with the surfactant. Longitudinal diffusion is the main contribution to plate height in EKC. Temperature effects are minimized by keeping the capillary diameter below 75 μm. MEKC separations are superior to HPLC separations especially for components with small k-values as shown in Fig. 3 for a mixtures of explosives.

Micellar electrokinetic chromatography has also some drawbacks such as a limited migration time window and organic modifier content of the buffer in order to maintain the micelle stability, and a restricted selectivity when using sodium dodecylsulfate as separation additive. Other separation additives were introduced such as cyclodextrines, calixarenes, proteins or charge-transfer interacting additives in order to provide alternative selectivities. However, many of these additives are UV-active or disturb mass-spectrometric detection. These problems could be overcome by a partial filling technique (PF-MEKC) in combination with a modulation of the migration times by moderate counter pressures applied at the cathodic side of the capillary [5]. Unfortunately, the partial filling technique affects separation efficiency both by boundaries between the background and separation buffer zone and the superposition of electroosmotic and hydrodynamic flow profiles. Adaptation of ion strengths can minimize negative effects of zone boundaries.

The poor sensitivity of common UV detection is another major drawback of EKC. Due to inner capillary diameters below 100 μm, the optical path length is very small. It can be improved in an elegant way by applying stacking and sweeping techniques [6]. Especially for environmental samples, a concentration of the analyte zone is necessary. Briefly, the stacking process is based on focusing effects on boundaries that separate regions with different ionic strengths. In MEKC neutral analytes can be swept by a stacked micelle zone that enters the analyte zone. Depending on the injection method, sensitivity can be increased from at least tenfold to 10^5 in peak height.

In conclusion, EKC is an interesting alternative and addition to HPLC in environmental analysis. It is easy to perform, offers fast and efficient separations and allows different in-capillary analyte enrichment possibilities without technical expense. These features will bring EKC a growing acceptance in environmental analysis.

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New Strategies for Analysis of Polar Organic Ultra Trace Components in Water

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Gas chromatographic trace analysis of volatile micro-pollutants in water has been and still is a true challenge for the gas chromatographer. A tremendous effort has been invested in developing techniques for trace analysis in water samples, and, today, numerous methods are available. Essentially, these techniques can be classified as either direct or indirect. An excellent review on the subject has been presented by Mol *et al.* [1]. However, most of the proposed techniques deal with non-polar analytes. Such compounds have little affinity for the water matrix, and therefore, affinity-based indirect methods like solid phase micro extrac-

tion (SPME) using silicone rubber as the extraction medium are a simple and reliable approach.

For volatile polar trace components, few of the methods available are really suitable. Affinity principles can only be utilized successfully if they compete with the analyte–matrix affinity. This can be very difficult to achieve *e.g.* in the case of water and methanol. In principle, large volume direct injection of water is an attractive concept, but numerous artifacts (early fractionation, peak splitting, non-wetting of retention gaps *etc.*) make this approach impractical or inappropriate. Two-dimensional chromatography is a partially feasible route, but requires a more complex system [2]. Additionally, contaminants from the Tenax adsorbent may disturb the analysis at low level analyte concentrations.

We have developed a new technique for the trace analysis of volatile polar compounds in water samples. The technique utilizes a simple gas chromatographic precolumn combined with a backflush system in a configuration described earlier [3]. This system is now commercially available (R&B Scientific AB, 147 40 Tumba, Sweden). The aqueous sample is introduced without any preceding clean-up or extraction stage by direct injection onto a short precolumn packed with lithium chloride. A capillary column coupled in series is used for the final separation. Initially, the lithium chloride is present in a dry form. It retains most of the water from the sample due to its hygroscopic properties provided that the temperature of the precolumn is not too high.

Non-polar components including fairly high boiling components (>C_{10}) show practically no retention. Even very polar compounds like methanol or THF are barely retained, since the water affinity of LiCl is much stronger than the retentive power of the water for the organic components. After breakthrough of the organic compounds into the capillary column, the water absorbed by the salt is removed in the back flush mode by elevating the temperature of the precolumn during the ongoing separation on the capillary column. Then, the precolumn is brought to its original starting temperature, and the system is ready for the next run.

The procedure can easily be automated. Quantitative transfer of some volatile polar test solutes from the LiCl precolumn was possible for injections of at least 120 µl water. The pre-separation was only 3.5 min. The technique proved to be suitable for trace analysis of both volatile polar (*e.g.* alcohols, ketones) as well as non-polar compounds with limits of detection around 50–200 ppt utilizing a flame ionization detector. Since the precolumn only contains inorganic material, conditioning at high temperature is possible, which minimizes elution of ghost peaks from the precolumn. This is an important aspect in ultra trace analysis.

It should be possible to extend the injection volume further. Furthermore, combination with other (selective) detectors such as mass spectrometry should allow to lower detection limits to low ppt or even less.

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Combining Forces: Rapid Sample Preparation and Sophisticated Detection/Identification

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Multidimensional separation techniques increasingly attract attention as tools to detect and identify trace-level constituents at low ng/g concentration levels in a variety of environmental, biological, food and other samples. Two branches have emerged, coupled-column techniques and more important 'hyphenation', the on-line combination of a separation technique and a spectroscopic detector providing structural information. Many powerful procedures can be developed by combining solid-phase extraction (SPE), dialysis or ultrafiltration for sample preparation, LC or GC for separation, and DAD UV, fluorescence, FTIR, AED (atomic emission detector) or, most frequently, MS or MS/MS for detection plus identification.

Inclusion of the sample preparation step in the analysis (on-line or at-line) allows automation and the design of integrated analytical systems for screening and monitoring purposes down to the ng level and with modest sample sizes. The latter aspect enables a more efficient sample treatment and a reduction of organic solvent consumption. Furthermore, the nowadays often 'too good' analyte detectability can be used partly to reduce the sample size. Typical examples of integrated systems are SPE-LC-MS, SPE-LC-DAD/UV-MS, LC-GC-MS and SPE-GC-tandem MS. In GC, the combined use of AED and MS detection has also been shown to be most rewarding. It is important to note here that the continued improvement of mass spectrometric instruments is the main cause of the dramatically improved overall performance of many analytical procedures. It is the added selectivity and sensitivity on the back end of the analytical set-up, which allows one to re-optimize the front-end of the system in terms of more information, smaller samples and/or faster analyses.

Still, even with the high discriminatory power of hyphenation (notably when MS/MS is used) sample preparation remains an essential aspect of many procedures and, when a targeted approach is required, immunoaffinity-SPE or, a novel alternative (!), immunofiltration serves a good purpose. One interesting aspect of using systems which combine immuno selectivity and a MS step is that confirmation of analyte identity and identification are carried out in the same run, and for all target analytes. Of course, it may be argued here that ultrafiltration and immunofiltration are usually performed by means of off-line sample treatment, which typically takes about 45 min. This is true, but one should also consider that these techniques allow up to 24 samples to be treated simultaneously. In other words, the real preparation time per sample is on the order of a few minutes only. Today, the approach is increasingly being used for the trace-level analysis of veterinary drugs in a variety of food samples. And, to quote one example of the performance that can be achieved, 1 ml milk samples then suffice to obtain detection limits of around 1 ng/ml for drugs such as sulphonamides.

Another approach that can be used when the number of analytes of interest is limited, is to replace the SPE-LC part of the analytical set-up with a single short column (SSC) of the same length as a SPE-cartridge (1–2 cm), but packed with sorbents of 5–7 µm particle size of LC quality. In recent years, the success of SSC-MS/MS has repeatedly been demonstrated. The very short run times of 5 min or less are one main advantage enabling the monitoring of studies of rapid analyte decomposition studies in real time. Another benefit compared to SPE-MS/MS procedures, is that the analyte retention on an SSC provides further information on analyte identity (not possible with SPE). Due to the increasing demand of high-throughput screening, SSC-based approaches will be a topic of future research.

In the general context of sample preparation, one should also be aware of the preference many workers still have for off-line compared to on-line procedures. The disadvantages of the former (more manipulation, 'open' systems, only minor aliquots of the sample injected) are obvious, and their adverse effects on the outcome of an analysis should not be underestimated. Nowadays, both off-line and at-line procedures can be combined with using large-volume injection (LVI) such as LVI-GC or LVI-LC. If one assumes that final sample extracts often have a volume of 0.5–1 ml, and that up to 100–200 µl can be introduced routinely, detection limits are no longer a restraining factor. For example, several studies have demonstrated that 100 ml water samples allow the detection of compounds at a level of ca. 1 ng/ml. This means an LVI analysis from the very start! Food and environmental analysis are two application areas in which these techniques are increasingly being used.

Another area of much current interest, next to conventional coupled-column systems such as LC-LC and GC-GC with their well-known heart-cut approach, is their much more powerful comprehensive counterpart – specifically GCxGC, a technique which has made tremendous progress in recent years. A, generally non-polar, first-dimension column of conventional size, *i.e.* about 25–30 m length, is coupled to a much shorter (0.5–1 m length), polar or shape-selective second-dimension column *via* an interface called a modulator. In the modulator, each 1–3 s wide effluent fraction from the first GC column is trapped, refocused and, next, injected into the second column. To enable a second-dimension separation of, typically, 3–6 s, the first-dimension temperature programming rate is usually somewhat less steep than usual in conventional one-dimensional GC. Therefore, run times of the first dimension separation are 45–120 min. Because of the (on-line and real-time!) second-dimension separation of GCxGC, the total run time is equal to that of the first dimension and, next to this dramatic advantage, information is obtained regarding the entire sample rather than for one or a few selected heart-cuts only. Moreover, related (classes of) compounds show up as 'ordered structures' in the final two-dimensional GCxGC chromatogram. This provides an interesting means for the provisional identification of unknown compounds, and is a powerful tool in fingerprinting and group-type analyses which are the goal in *e.g.* many petrochemical analyses.

Because of the very rapid second-dimension separation, fast detection requires special attention. Initially, only flame ionization and (micro) electron-capture detectors were able to meet the stringent demands of GCxGC. Very recently, the introduction of time-of-flight (ToF) MS has significantly enhanced the power of the technique. Next to peak recognition, rapid and sophisticated identification is now possible by powerful deconvoluting algorithms. Consequently, today GCxGC-ToF MS is the preferred technique for the analysis of complex mixtures such as petroleum products, cigarette smoke, food extracts and air. Among compound classes of current interest are polychlorinated biphenyls, toxaphenes, other complex mixtures of organohalogens, fatty acid methyl esters and flavors and fragrances. Moreover, peaks can be located in the two-dimensional GCxGC plane with similar precision as peaks in normal GC, and quantification causes no special problems.

The main disadvantage is still that no proper software is available to handle the huge amount of data generated by a single GCxGC run. In some cases up to 10,000 signals were detected, of them about 80% being unknowns. This is the real challenge concerning implementation of the technique in routine operations.

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