Volume 32 Number 3, 161–228

LC GC
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solutions for separation scientists
Volume 32, Number 3, March 2014

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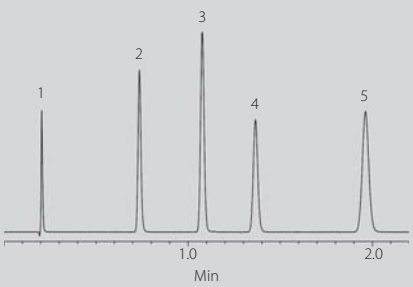
1. Uracil
2. Diazepam
3. Toluene
4. Naphthalene
5. Biphenyl

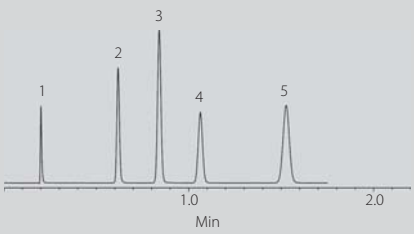
**Titan C18, 1.9 µm**

N= 14,710
N/m = 294,200
h= 1.75
σ= 1.05
Pressure*= 4,100psi



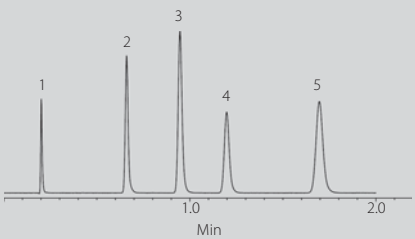

**Competitor A C18, 1.8 µm**

N= 9,260
N/m= 185,200
h= 3.18
σ= 1.02
Pressure*= 4,650psi




**Competitor B C18, 1.7 µm**

N= 9,783
N/m= 195,700
h= 2.84
σ= 1.16
Pressure*= 4,900psi

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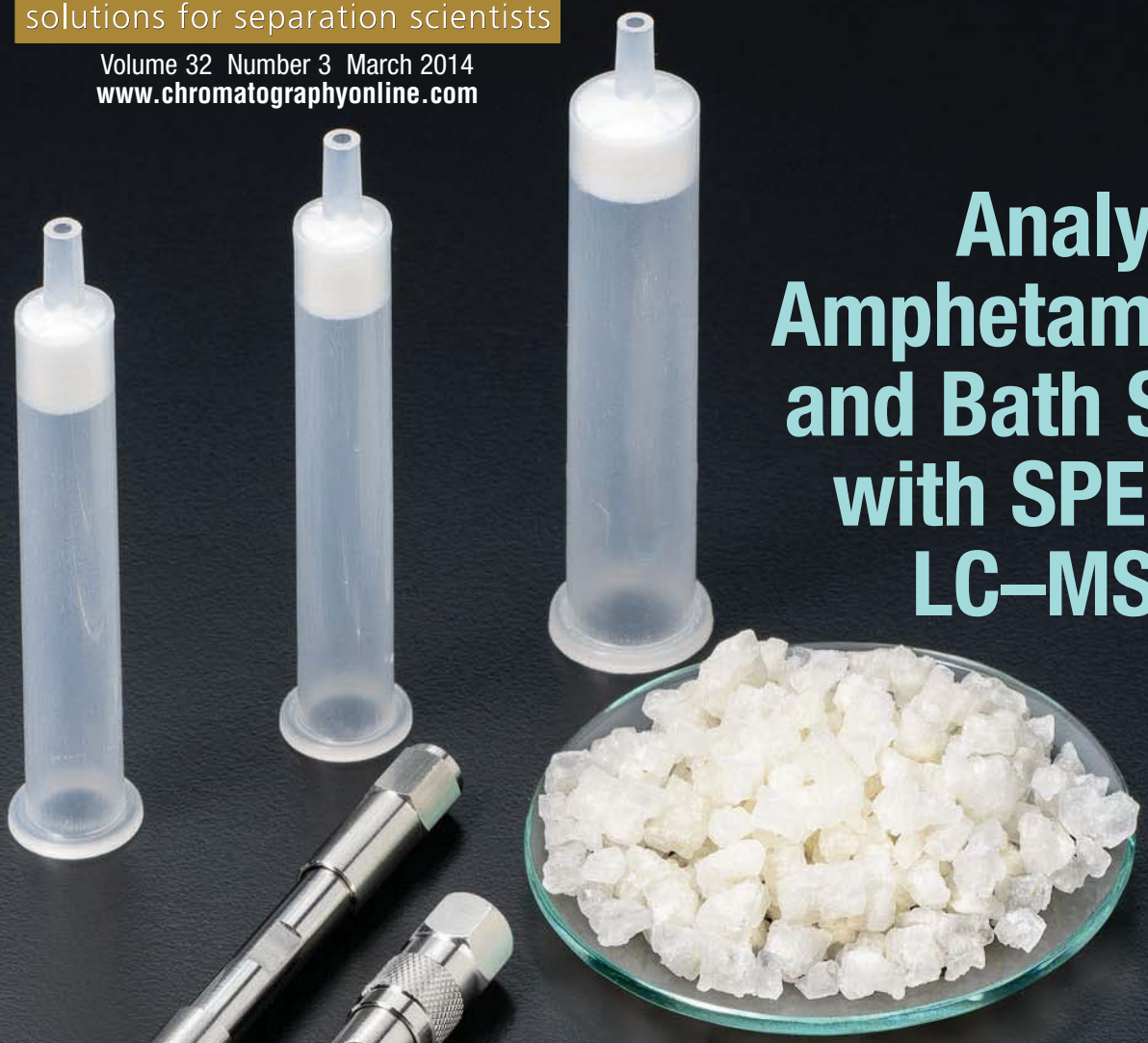

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## In-Capillary Sample Concentration in CE

## LC Method Scaling with Gradient Separations

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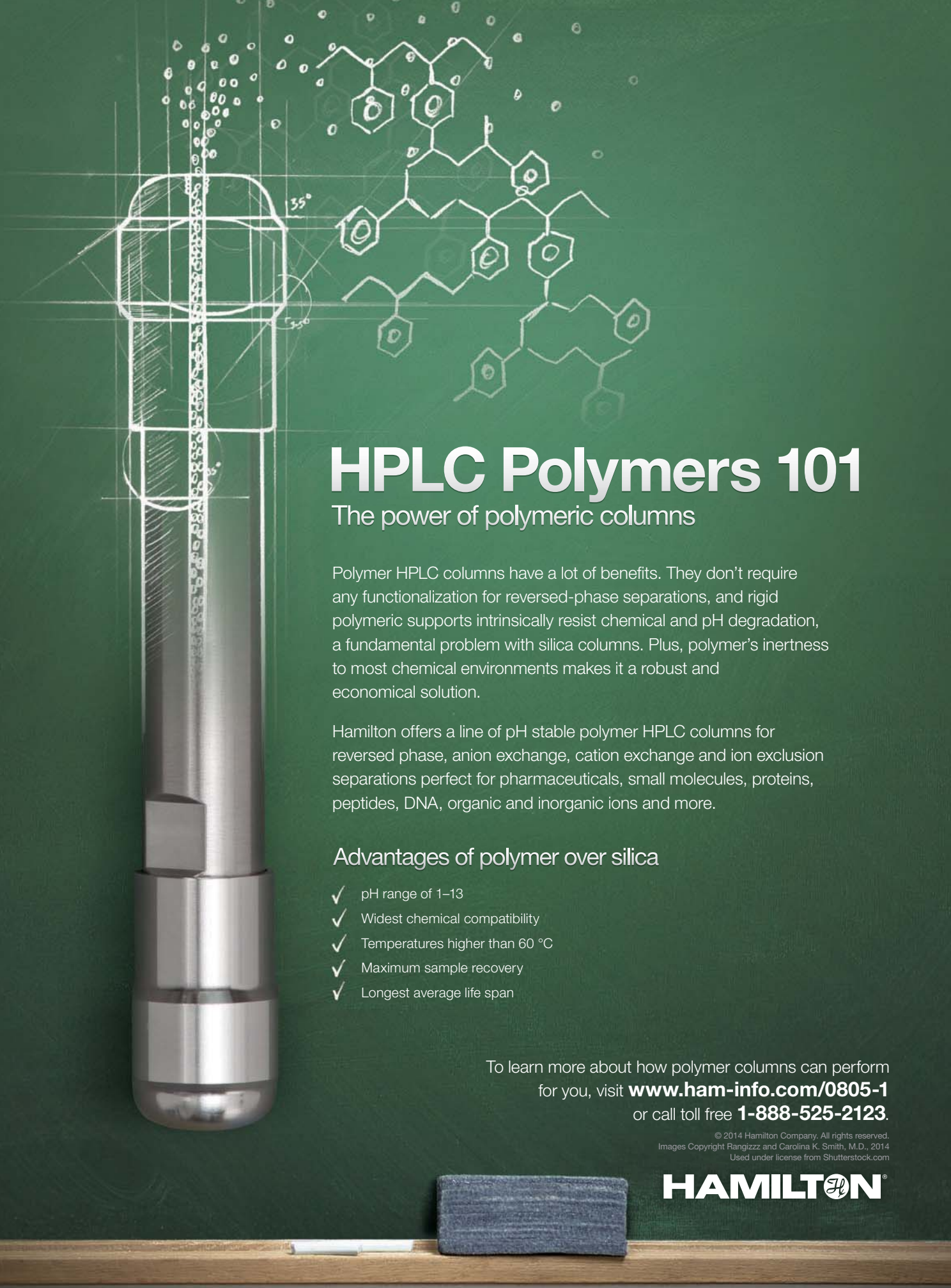

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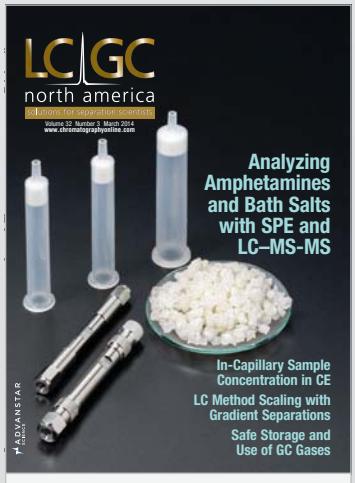


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# PEAKS of Interest

## Daniel W. Armstrong to Receive ACS Award in Separation Science and Technology 2014

Daniel W. Armstrong, a professor of chemistry and biochemistry at the University of Texas Arlington in Arlington, Texas, is to receive the 2014 American Chemical Society Award in Separations Science and Technology. This is the second time this year that the ACS has recognized the work of Armstrong — in August last year he was selected to join the ACS Class of Fellows 2013.

The award, sponsored by Waters Corporation, recognizes "the development of novel applications with major impacts and/or the practical implementations of modern advancements in the field of separation science and technology." It will be presented to Armstrong on March 17 at the ACS national conference in Dallas, Texas.

Armstrong is thought of as a leader in his field and is considered to be the "father" of pseudo-phase separations — a type of chromatography that lowers costs, volatility, and toxicity while providing higher selectivity than other analytical methods. His commercialized innovations are applied across the drug development, environmental analysis, and petrochemical industries.

In his career, Armstrong has developed more than 30 different chromatography columns — a gas chromatography column that he developed is now part of the Rosetta mission of the European Space Agency exploring the composition of comets in space.

## Purdue Research Foundation Invests in Two Start-up Companies

The Purdue Research Foundation (West Lafayette, Indiana) has awarded $150,000 in investment to two companies that have licensed Purdue University Innovations: Telos Discovery Systems Inc., and bioVidria Inc. The investment is from the Emerging Innovations Fund, which was set up in 2008 to accelerate the commercialization of early stage technologies in the Purdue community.

The company bioVidria Inc., received $75,000. It is based on the work of Mary J. Wirth, the W. Brooks Fortune Distinguished Professor of Analytical Chemistry in the Department of Chemistry. The company focuses on developing new materials for chromatography, specifically protein analysis, for the pharmaceutical and agricultural industries.

Telos Discovery Systems Inc., also received $75,000. The company is a boutique equipment manufacturer that supports basic and discovery medical research. The company is based on the work of Joseph Garner, a former Purdue associate professor in the Department of Animal Sciences.

Elizabeth Hart-Wells, assistant vice president and director of the Purdue Office of Technology Commercialization said: "The fund makes seed investments and early-stage investments in the form of convertible debt and/or warrants, with a goal of accelerating the commercialization of early-stage innovations." ■



## IS THERE AN APP FOR THAT?

### HIGHLIGHTS OF APPS FOR SEPARATION SCIENCE

4G 100% 11:59 AM

**APP NAME:** HPLC Troubleshooting Guide

**OFFERED BY:** Thermo Scientific

**PLATFORMS:** Android, iPhone (optimized for iPhone 5), iPad, iPod Touch

**WHAT IT DOES:** This app diagnoses various potential issues with HPLC analysis. Solutions range from general nature issues that are common to standard instrument setups from various manufacturers to troubleshooting special technologies such as nano LC and fluorescence, charged aerosol, and electrochemical detection. The app also offers interactive functions to connect to Thermo Scientific social media, recommend the app to a friend, and contact the company for further support. By tapping the Share function, troubleshooting solutions can be shared among teams.

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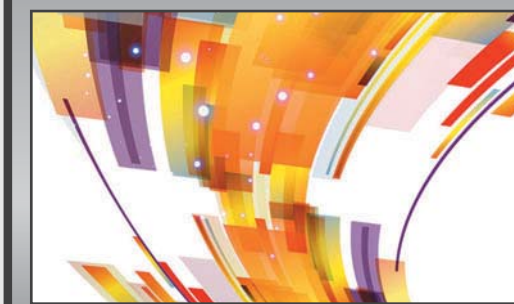

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# COLUMN WATCH

## In-Capillary Sample Concentration in CE

**In this installment, guest authors and capillary electrophoresis (CE) experts Breadmore and Sänger-van de Griend bring a practical perspective to enhancing the sensitivity of CE. They compare stacking and sweeping injection methods and provide practical suggestions about how to enhance sensitivity for charged and neutral compounds.**

***Michael C. Breadmore and Cari E. Sänger-van de Griend*** *are the guest authors this month.*
***Ronald E. Majors*** *is the editor of Column Watch.*

One of the most frequently cited concerns in capillary electrophoresis (CE) is that the concentration limits of detection are inferior to what can be achieved with liquid chromatography (LC) (1). Extensive research has been carried out to overcome this limitation and now there are many different ways to increase sensitivity by controlling the way the sample is injected, how it is prepared, and how the targets are separated. After all, the concentration in the sample vial does not need to be the same as the concentration needed for detection if we can concentrate on-line. Early researchers in the field diluted samples in the electrolyte (parallel to dissolving or diluting in eluent in LC); however, doing so dilutes the target analytes, further reducing sensitivity. A better approach is to use the sample straight, which means that the composition of the sample plug is different from the composition of the electrolyte. Although intuitively this may not seem like a smart idea, depending on the exact analyte and conditions there may be a significant difference in their migration behavior in the sample zone compared to their migration behavior in the electrolyte. Very early researchers realized that this approach could enhance sensitivity. A brief search of the literature yields many excellent research papers and reviews (1–5), with different kinds of concentration mechanisms occurring because of different analyte, matrix, and electrolyte properties. Table I gives an overview of the most frequently used concentration techniques; as can be seen there are quite a number of them. It is worthwhile noting that these are the most common single concentration approaches used. It is possible to combine multiple mechanisms to produce even more powerful approaches; however, these are more complex. Most reviews discuss the various techniques from the mechanistic point of view. Here, we'd like to consider these options from a practical point of view and ask the question: I have XYZ in my sample; how do I improve my sensitivity? After all, in real life one usually cannot pick all conditions and sample matrices to suit a theoretically useful technique; instead, one has to work from the situation at hand.

### Velocity Differences

First of all, the fundamental premise of concentration in CE comes back to creating a different velocity of the analytes of interest in the sample zone compared to their velocity in the background electrolyte (BGE) (6). There are many different techniques based on a change in velocity. Each technique has its own abbreviation, but the only difference may be a change in direction of the electroosmotic flow (EOF). These techniques fall into two groups: stacking techniques and sweeping techniques. If the velocity in the sample zone is higher than in the BGE (Figure 1b), the technique is generally called *stacking* (7). When stacking, the analytes quickly migrate through the sample zone to the electrolyte boundary where they slow down and "stack" together on the boundary with an increase in concentration and, thus, sensitivity (Figure 1b). In so-called *sweeping* techniques, in contrast, the velocity in the sample zone is slower than the velocity in the electrolyte. As the boundary between the electrolyte and sample moves through the sample matrix, analytes are concentrated around this

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| Table I: In-capillary sample concentration in CE | |
|---|---|
| Stacking | The analyte velocity in the sample zone is faster than the analyte velocity in the BGE |
| **Electric Field Strength Induced Velocity Differences** | |
| Field-amplified sample stacking (FASS) | Sample has low conductivity (<1/10 BGE), so the local field strength is high and local velocities are high. The analytes slow down and are stacked when entering the BGE. |
| Field-amplified sample injection (FASI) | Sample in a low-conductivity matrix is injected electrokinetically. The local field strength is high, increasing the amount injected. If the EOF can be suppressed, long injection times are feasible. Injection is discriminative. |
| FASI with solvent plug | Same as FASI, but a solvent plug is injected with the sample for low conductivity and high local field strength. |
| Large volume sample stacking (LVSS) | A large sample plug is injected. The sample matrix is continuously removed by applying a reversed voltage during stacking. |
| Large volume sample stacking with an EOF pump (LVSEP) | A large sample plug is injected. The sample matrix is continuously removed by the reversed or suppressed EOF in the sample zone (compared to the EOF in the BGE). |
| Micellar electrokinetic chromatography (MEKC) stacking | Low concentration of pseudo-stationary phase (PSP) moves rapidly through low conductivity sample (<1/10 BGE). PSP with analyte stacks at boundary between sample and electrolyte. |
| **pH-Induced Velocity Differences** | |
| Dynamic pH-junction | The analytes have a higher mobility in the sample zone and will be stacked at the boundary with the BGE |
| pH-mediated FASS | The sample is injected between a strong base and strong acid. When the voltage is applied, the $OH^-$ and $H^+$ migrate through the sample zone and neutralize, thus reducing the conductivity. Stacking can then take place. |
| **Sweeping** | |
| Sweeping | The analyte velocity in the sample zone is slower than the analyte velocity in the BGE. Sample without PSP is injected onto a system with a PSP BGE. The analytes are concentrated at the front of the PSP zone moving through the sample zone. Conductivity of sample equivalent to BGE. |
| Micelle to solvent stacking (MSS) and analyte focusing by micelle collapse (AFMC) | The sample is mixed with micelles, the BGE is micelle-free. The micelles collapse when meeting the solvent or the BGE. The analytes are concentrated at the boundary. |
| **Isotachophoresis** | |
| Transient isotachophoresis (tITP) | The sample is injected between a higher mobility co-ion (leading electrolyte) and a lower mobility terminating electrolyte. When the voltage is applied, the analytes sort in order of mobilities. The concentrations in the analyte zones depend on the concentration of the LE (Kohlrausch regulation function). |
| **In-Capillary SPE** | |
| In-capillary solid-phase extraction (SPE) | A small SPE column is manufactured inside the capillary. Analyte is extracted through conventional chromatographic principles and eluted in solvent for electrophoretic analysis. Easily automated, attractive for low-volume samples. |
| **Liquid-Phase Microextraction** | |
| Single-drop microextraction (SDME) | Analytes are extracted into a small organic drop suspended at the tip of the capillary. Contents of the drop are then analyzed by electrophoresis. Can be performed in commercial instrumentation with no modifications. |
| In-vial supported liquid membrane (SLM) | Organic liquid is held in a thin membrane. Analytes are extracted from sample through organic phase into aqueous acceptor. Can be performed in-vial. |
| In-vial electrokinetic microextraction (EME) | Similar to in-vial SLM, except a voltage is applied across the membrane to enhance transport of charged species through the organic phase. |

moving boundary and are considered to be "swept" together with an increase in concentration and, thus, sensitivity (Figure 1c) (8).

## The Easiest Way: FASS

So if we want to create a concentrating effect inside the capillary, we have to ensure that the analyte has a difference in velocity between the sample zone and BGE. In an electric field, the velocity of an analyte is directly proportional to its electrophoretic mobility, which is governed by charge, size, and the electric field strength. This means that we need to know the analyte's physical-chemical properties. The first question in the decision process (see Figure 2) is as follows: Is the analyte charged? (And yes, whether the species is anionic or cationic is important, but only for determining the conditions to use when implementing the method; this does not matter when choosing the ideal approach for concentration.) If the analyte is neutral, then you need to use affinity approaches to achieve concentration, which are discussed later on.

The second question is as follows: Does the sample matrix have a lower conductivity than the BGE? If yes, then *field-amplified sample stacking* (FASS) may well work (Figure 1b). This is the simplest approach because the lower conductivity of the sample zone automatically means that the field strength in the sample zone is higher than in the BGE. If the mobility of the analyte remains the same, then stacking will occur as a higher field strength results in a higher velocity without any other active manipulation (there is nothing you can do about it except to remove the conductivity difference). This is the reason that it is preferred to have the sample dissolved or diluted in something that has a lower conductivity than the BGE, including diluted BGE, pure water, or organic solvents. Ethanol, dimethyl sulfoxide (DMSO), and acetone can give even better results

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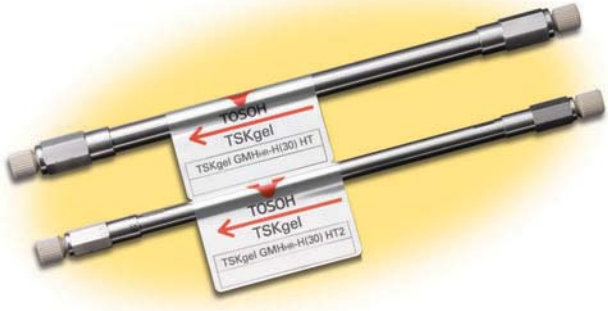


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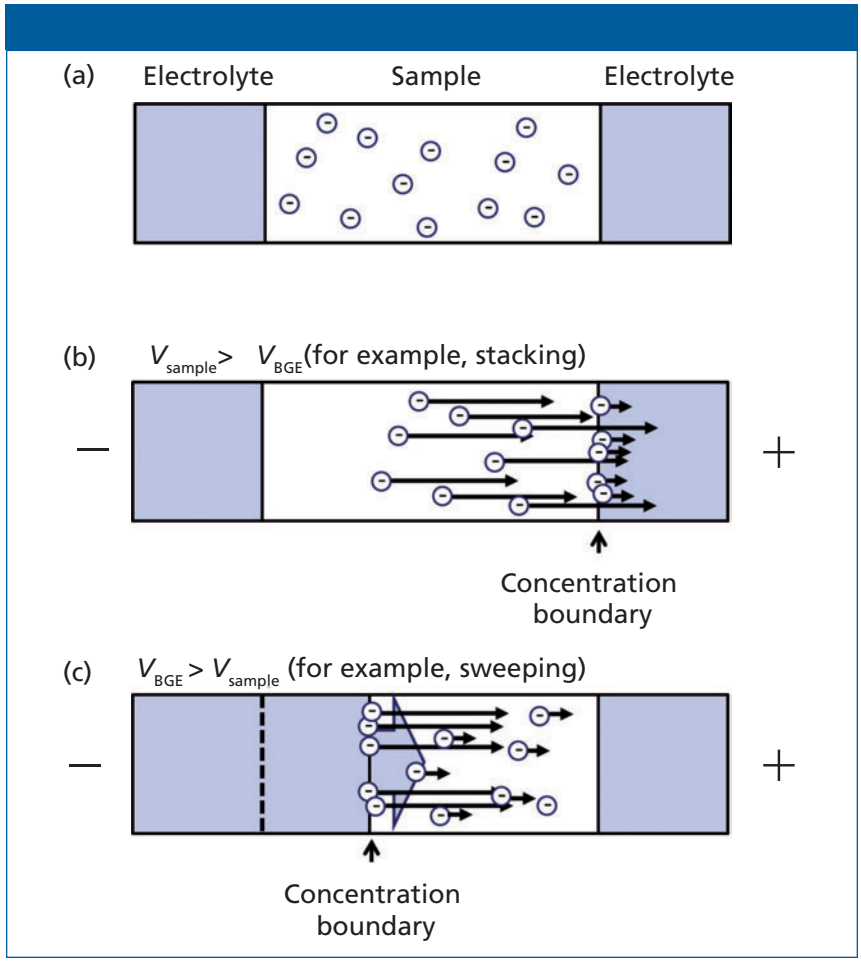


**Figure 1:** Concentrating in CE. Creating velocity differences of the analyte in the sample zone versus the velocity of the analyte in the BGE. (a) Initial situation after injection and before applying the voltage. (b) Stacking, the analyte has a higher velocity in the sample zone than in the BGE. (c) Sweeping, the analyte has a lower velocity in the sample zone than in the BGE.

## How Much Is Injected?

How much sample you should inject in CE depends on your injection method.

In hydrodynamic injection, the injection volume is determined by the following equation:

$$V = \frac{\Delta p\, d^4\, \pi\, t}{128\, \eta L}$$

The volume ($V$) of sample injected depends on the injection time ($t$) and pressure difference ($\Delta p$), the viscosity ($\eta$), the capillary diameter ($d$), and total capillary length ($L$).

In electrokinetic injection, the injection volume is determined by the following equation:

$$Q = \frac{(\mu_e + \mu_{eo})\, E \pi d^2 C t}{4}$$

The amount ($Q$) injected of a certain analyte depends on the analyte's effective mobility ($\mu_e + \mu_{eo}$) and concentration ($C$), the capillary diameter ($d$), the applied electric field ($E$), and the injection time ($t$). Electrokinetic injection is a selective or discriminative injection mode as analytes with different mobilities are injected in different amounts.

than pure water (9). As a consequence of the stacking effect, a larger volume than the usually mentioned 1% of the capillary volume can be injected. As a rule of thumb, injection volumes up to 5% of the total capillary volume are feasible with FASS, with enhancements in sensitivity typically less than about 50 achieved. Larger volumes do not produce further improvements because of mismatches in EOF, which causes hydrodynamic dispersion, a loss of efficiency (10,11), and decreased reliability because of the occurrence of bubbles.

### When a Higher Concentration Is Needed

The sidebar "How Much Is Injected" provides some help in calculating the injected volumes or amounts. Sometimes a greater increase in concentration is required than can be achieved in FASS. This is easiest if the sample matrix has a controlled and constant composition. In such a case, *field-amplified sample injection* (FASI) (12) (also called *field-enhanced sample injection* [FESI]) and *large-volume sample stacking* (LVSS) can be easily used. The only difference between FASS and FASI is the injection — for FASI, electrokinetic injection is used. Because the amount injected is strongly dependent on the local field strength, the sample matrix conductivity should be constant. A variable sample matrix composition will result in variable amounts injected, which is why for all practical purposes FASI requires a controlled sample composition. The same is true for LVSS for similar reasons. In LVSS, the sample matrix is removed either through polarity switching (denoted *LVSS*) (10) or EOF pumping (denoted *large-volume sample stacking with an EOF pump* [*LVSEP*]) (13).

If the matrix varies, local field strengths vary and timing becomes an issue, resulting in variable concentration effects and migration times. To some extent these problems can be compensated for with an internal standard, but method performance (detection limit, repeatability, and so on) will be compromised so it is better to address the cause of the problem, rather than doing a patch-up job with duct tape and silly putty.

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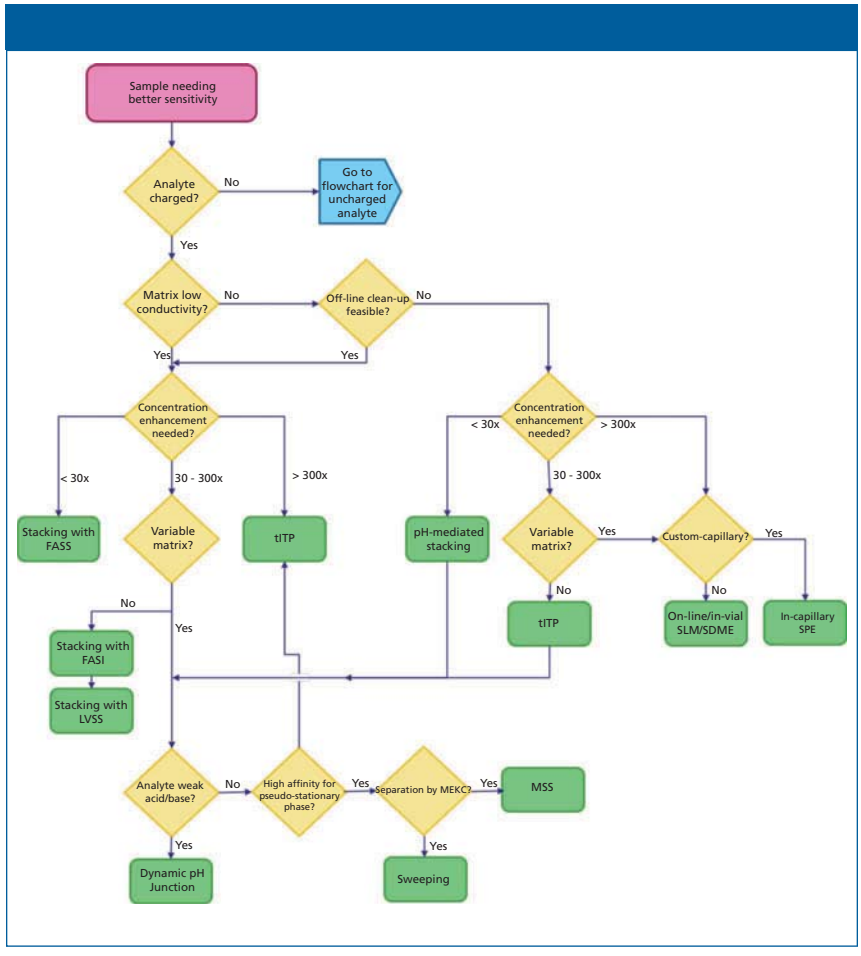


**Figure 2:** In-capillary sample concentration decision tree for charged analytes.

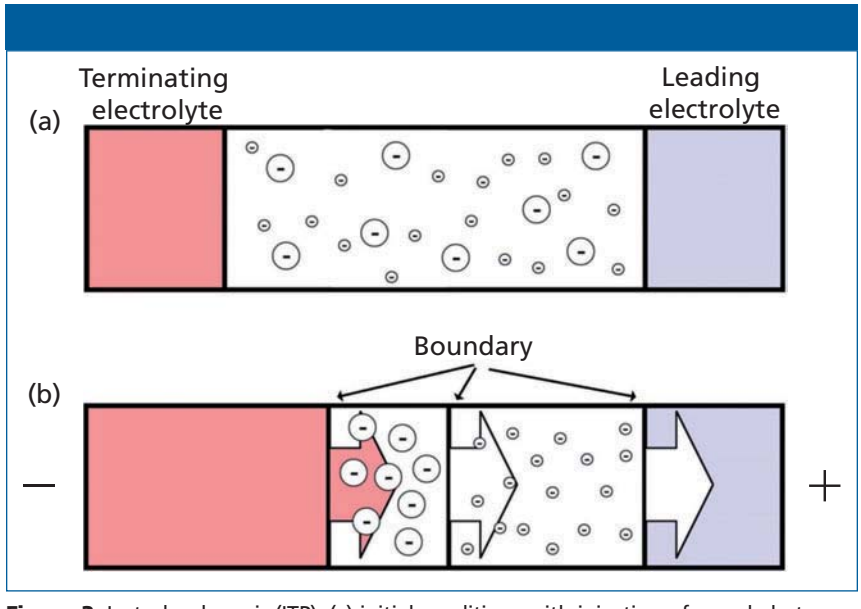


**Figure 3:** Isotachophoresis (ITP), (a) initial conditions with injection of sample between the leading and terminating electrolytes, (b) isotachophoretic zones at steady state.

If the matrix composition is variable, we first look again at the analyte: Is it a weak base or acid? If yes, stacking with a *dynamic pH junction* (6,14), also called a *moving neutralization boundary* (15), is feasible. Here, the BGE and sample composition are selected such that there is a difference in charge, because a charge difference will also result in different velocities between the sample and BGE zone. It is important to note that it is not necessary to completely reverse the charge; a simple change from charged to neutral may be sufficient if the system is designed correctly. If the answer is no, sweeping techniques may be appropriate. In sweeping, analyte affinity for a pseudo-stationary phase (PSP) is key. When the PSP migrates through the sample zone, analyte molecules are concentrated at the front of the PSP as it moves through the sample zone (Figure 1c) (8). If the PSP is a part of the BGE, such as in micellar electrokinetic chromatography (MEKC), it is called *sweeping*. If the PSP is in the sample and the BGE is PSP-free, then when the micelles migrate into a PSP-free zone, the concentration of the surfactant reduces to below the critical micelle concentration and the micelles collapse, releasing the analytes. This was first introduced for neutral species as *analyte focusing by micelle collapse* (AFMC), but has been applied to charged species with organic solvents to collapse the micelles and is then called *micelle to solvent stacking* (MSS). It is important to note that the mechanism of sweeping and MSS still function with a variable matrix, but there may be some resultant variability in the final separation. Nevertheless, similar to isotachophoresis (ITP), discussed below, these approaches are very useful for samples with a variable matrix.

## High-Conductivity Sample Matrix

If the sample matrix has a high conductivity, it is important to consider off-line cleanup. From the discussion above and from the decision tree (Figure 2), it can be seen that with a lower conducting sample matrix, many approaches can be used for successful sensitivity enhancement. If the sample matrix has a high level of conductivity but off-line cleanup is not feasible, there are some options available. These are based on creating velocity differences by sweeping or MSS, by manipulating the charge by pH-mediated techniques (such as pH-mediated FASS or dynamic pH junction), or by ITP.

Of these, *pH-mediated FASS* is an interesting and unique approach that induces a FASS effect in an indirect manner (16). In this approach, sample is injected electrokinetically, and for anions, the electrolyte would contain a weak base, like ammonium, such that during injection the anions migrate into a zone where the counterion is ammonium (not sodium as it may have been in the sample). After injection of the sample, there is a short electrokinetic injection of base (or acid for cations). The base migrates into the sample zone (hydroxide and hydronium have a high mobility) and titrates the buffer counterion into water, thereby producing neutral species and reducing the conductivity of the sample zone. With this change in conductivity, there is now a higher electric field over the sample and FASS can occur. Although this is a very clever approach, it is not easy to optimize the conditions to achieve the outcome required.

### Transient ITP

Perhaps the most attractive approach for charged species in samples with a high conductivity matrix is to use *isotachophoresis* (ITP) (Figure 3). ITP is one of the oldest forms of CE and probably the most intimidating — and also poorly understood by many experienced practioners of CE. In ITP, the sample is sandwiched between a leading electrolyte and a terminating electrolyte. The leading electrolyte has an ion with the same charge as the analyte but with a higher mobility. The terminating electrolyte has an ion with the same charge as the analyte but a lower mobility. The sample analytes sort in migration order and are concentrated to obtain the same conductivity in each analyte zone. The mobility and concentration of the leading electrolyte determine the concentrating effect on the analytes, according to the Kohlrausch regulation function, and the mobility window between the leading and terminating ions provides a zone in which the analyte range can be selectively tuned. The unique mechanism of ITP makes it the great equalizer. Macrocomponents are diluted and trace components are concentrated, making ITP exceptionally useful as a stacking technique.

The simplest way to use ITP is to perform transient ITP (tITP) (17). Here, a short ITP stage is induced before the CE separation. Transient ITP can be done by injecting small volumes of leading and terminating electrolytes on either side of the sample; alternatively, either the leading or termination co-ion comes from the BGE (not both) and the other comes from the sample or from an additionally injected plug. Transient ITP is particularly suited to salty samples — especially those that contain lots of sodium chloride as the chloride is a very high mobility ion that can function as a leader for almost all anions. Sodium is a mid-range mobility cation so it cannot be used for all cations, but it typically has a higher mobility than many organic cations. It is also worth briefly mentioning *pseudo-ITP* (pITP), in which the terminating zone is created not from a low mobility ion, but from a solvent added to the sample in which there is a lower conductivity (18). This approach arose out of work

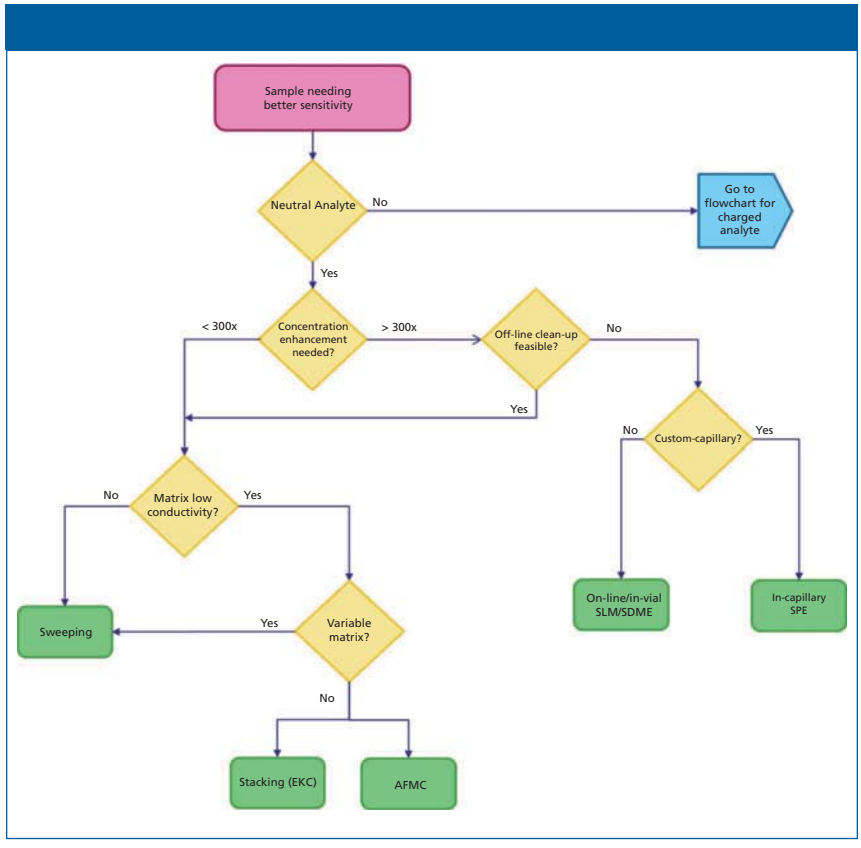


**Figure 4:** In-capillary sample concentration decision tree for neutral analytes.

where the supernatant of biological samples after deproteination with acetonitrile was injected into the CE, and it is therefore a very useful technique for plasma and serum samples because they require very simple sample processing.

## The Last Resort: In-Capillary and In-Vial Extraction

If stacking and sweeping approaches do not work, then the only real alternative is to consider the use of an on-line or in-capillary extraction. Such extractions have traditionally been performed by making a small chromatographic bed (19) at the tip of the capillary (most easily done with larger-internal-diameter tubing and particles that have a larger diameter than the internal diameter of the separation capillary) and allowing analytes to be extracted chromatographically before electrophoretic separation. The technique resembles on-line *solid-phase extraction* (SPE) and includes possibilities for immunoaffinity extraction as well (20). While this is a potentially very attractive approach, there are a number of limitations including analysis time, repeatability, and the lack of commercially available capillaries. Therefore, it is really only useful for researchers.

The most common off-line alternative to SPE is *liquid–liquid extraction* (LLE) (21), which has only very recently been reported coupled directly with CE through developments in *single-drop microextraction* (SDME). Small individual drops of organic and organic–aqueous drops can be created on the tip of the capillary in a commercial CE unit (22). This allows for easy automation, with very good analytical performance; however, this is not a straightforward approach at this point in time because a slight back pressure is required to stabilize the drop. A more attractive approach is the implementation of the technique in a vial. This can be done with a water-immiscible ionic liquid (23), and excellent results have been obtained from urine and serum; this is an area very much still in its infancy, and it is unclear yet whether this technique will be attractive. The most promising approach to appear recently is the development of in-vial *supported liquid membranes* (SLM) (24) and *electromembrane extraction* (EME) (25). Still very much in the research phase, these techniques involve creating a custom vial in which the sample is placed below a membrane impregnated with a suitable extraction solvent with an aqueous acceptor phase placed on top of the membrane. When placed in a commercial CE system, the capillary samples directly from the surface of the membrane, which considerably decreases the time required to reach localized equilibrium (26). This approach has been demonstrated for inorganic ions and a few drugs from various biological fluids. If it can be transitioned into a commercial vial, it will significantly aid the ability of CE to easily handle complex and high conductivity samples.

## How to Deal with Neutral Species

The options for uncharged analytes are fewer than those for charged analytes. For uncharged analytes, all in-capillary approaches are based on some kind of affinity interaction (27). This is because neutral species are uncharged and do not have their own electrophoretic mobility and, thus, cannot be concentrated by approaches developed for charged compounds. In contrast, many of the approaches developed for neutral analytes can also be applied to charged analytes and some were already discussed in detail above. Figure 4 shows the decision tree for uncharged analytes.

The first approaches to concentrate neutral species were based on stacking: By using a low conductivity sample and affinity between the analyte and a PSP, it is possible to rapidly transport analyte molecules from the sample zone to the sample or electrolyte boundary where they concentrate. There is an abundance of slightly different approaches here depending on the direction of the micelle and the polarity, but they all rely on stacking (not sweeping) for concentration (27).

Today, however, the method of choice for concentrating neutral analytes is sweeping as this can provide greater enhancements than stacking and is applicable to samples with a low, high, or variable sample matrix (8,28). The challenge with sweeping is that for good enhancement a high affinity with the PSP is required (the zone length

after sweeping is related to the affinity interaction), but this approach is bad for separation as all of the analytes will migrate with the PSP. Therefore, sweeping requires careful optimization to ensure that sufficient sensitivity enhancements can be obtained. Sweeping is also attractive because it is applicable to high conductivity samples — the mechanism of concentration does not depend on the conductivity of the sample. In fact, when sweeping is combined with electrokinetic injection, multiple capillary volumes of sample can be injected by the EOF with the analytes concentrated and held at the front of the micelle zone (29). This approach can provide very significant enhancements, but the trade-off is time — typically, very long injections (approaching an hour) are required.

### The Chicken or the Egg — Which Part of Your Method to Consider First

The typical approach used by most workers in the field is to develop separation conditions to resolve the target analytes from matrix components, and to then consider whether or not any on-line concentration techniques can be used. The justification here is twofold; it doesn't really matter what sensitivity you have if you can't separate the analytes (with increased prevalence of mass spectrometers this may become arguable) and you can always resort to off-line treatment of your sample to improve the sensitivity. This latter point in particular is worth some consideration because it is not easy, for example, to concentrate by more than 100 — this is the equivalent of taking 10 mL of sample, and extracting the analytes into a volume of 100 µL. A volume of less than 100 µL can be difficult to handle and introduces other errors. Stacking and sweeping approaches are getting closer to being able to electrokinetically extract from 100 µL into 1–5 nL (100,000-fold), but this is only possible for certain sample types and is only possible if the separation method is compatible with these approaches.

You don't need a chicken or an egg — you need both. Therefore, it would be prudent to consider the requirements of sensitivity during method development and ensure that the developed method is compatible with at least some of the concentration approaches. For example, if you know that you will need nanogram-per-liter detection limits or lower, then that really does place restrictions on the concentration approaches that can be used and implicates which methods you need to use. This, in turn, defines the electrolyte compositions that may be more ideal and compatible.

### Other Practical Limitations: pH, pI, Stability, and Analyte Size

In the discussion of sensitivity enhancement so far, we have not indicated whether any technique is typically more or less suitable for a certain class of molecules, other than via physicochemical properties like weak acid and bases, charged or uncharged analytes, and high or low affinity for a pseudo-stationary phase. This is deliberate because it is the physicochemical properties that govern which method is suitable. A dynamic pH junction (essentially a step pH gradient) is useful for proteins with an isoelectric point (pI) between the pH of the sample and the electrolyte, not because of size,

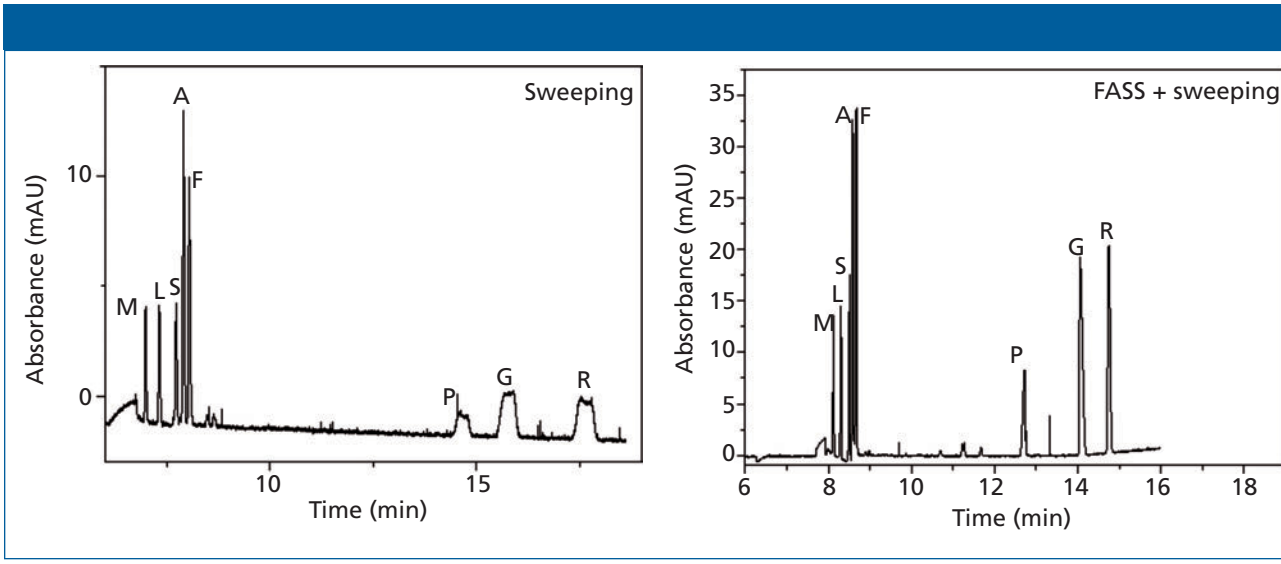


**Figure 5:** Sweeping and FASS plus sweeping of standard mixture of hypolipidemic drugs. Capillary: 78 cm × 50 µm LPA-coated capillary; BGE: 20 mM ammonium bicarbonate (pH 8.50) containing 50 mM SDS; voltage: -28 kV. Sweeping: hydrodynamic injection of 10 µg/mL drug mixture in ammonium bicarbonate with conductivity similar to BGE at 50 mbar for 40 s. FASS plus sweeping: hydrodynamic injection of 1 µg/mL drug mixture in Milli-Q water at 50 mbar for 80 s. Peaks: A = atorvastatin, F = fluvastatin, G = gemfibrozil, L = lovastatin, M = mevastatin, P = pravastatin, R = rosuvastatin, S = simvastatin. Adapted from reference 30 with permission from Elsevier.

| Good CE Injection Practice |
|---|
| Injection times should be<br>• As short as possible<br>• But at least 3 s to compensate for injection pressure or voltage variability |
| Injection plug length less than 1–2% of the total capillary length |
| Constant, precise temperature for capillary and vials |
| Constant level at outlet vial during injection, no waste vial |
| Sample vials same levels |
| Background electrolyte (BGE) inlet and outlet vials same levels |
| Dip capillary inlet in clean water or BGE vial after injection |
| Inject BGE or water plug after sample injection |
| Burn off polyimide capillary ends (not for certain coated capillaries) |
| Cut the capillary ends straight |
| Sample matrix of samples and standards should match |
| When using an internal standard:<br>• Reduce injection volume variability<br>• Reduce injected amount variability<br>• Correct for migration time variability |
| When using a stacking technique:<br>• Dilute or dissolve sample in water or diluted BGE<br>• Use literature for inspiration to gain sensitivity |
| When using electrokinetic injection:<br>• Selective<br>• Only one injection per vial<br>• Variable ionic strengths influence injected amount<br>• Amount injected related to $d^2$ and $L$ |
| When using hydrodynamic injection:<br>• Generally more precise than electrokinetic injection<br>• Not appropriate for viscous applications<br>• Multiple injections per vial feasible<br>• Volume injected related to $d^4$ and $L$ |

but because of their pI values. Of course there are other practical limitations that need to be considered for all molecules, but have some greater relevance for larger molecules. Knowledge about other parameters such as stability or adsorption should always be included in the decision process. Limiting for all concentration efforts is the solubility of the analyte — low solubility situations should be avoided (such as proteins at their pI) unless a solubility-enhancing solvent or compound is added. The sidebar "Good CE Injection Practice" lists some other practical tips to improve method performance.

### What's Good for the Goose, Isn't Always Good for the Gander

As can be understood from the discussions above, opportunities for sensitivity enhancement through injection are strongly influenced by other method parameters, such as the BGE composition. A higher BGE concentration, for example, makes it easier to increase the conductivity difference with the sample, but this causes higher currents, which may lead to greater method instability. Although very high sensitivity enhancements have been published with stacking techniques, in the end it always comes down to compromising between robustness and resolution (that is, separation plus efficiency) versus sensitivity. Depending on the required use one has to make choices and take the consequences.

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## Stacking or Sweeping — Or Both?

The right in-capillary sample concentration method to use with CE depends on the properties of the analytes. With a target set of analytes with diverse properties, finding a suitable method can be complex. An example of this is shown in Figure 5. This is a mixture of eight hypolipidemics with a diverse range of properties including mobility, p$K_a$, and lipophilicity. This latter property is a problem because pravastatin, gemfibrozil, and rosuvastatin show poor interaction with the PSP and thus are not swept well. The solution in this case was to perform sweeping under FASS conditions — sweeping the high lipophilic analytes and stacking the low lipophilic ones. The enhancements are modest (40–80-fold) but this nevertheless shows the potential of using multiple mechanisms simultaneously to improve analyte coverage. This is different than using sequential approaches such as dynamic pH junction–sweeping, which uses one method to concentrate and then a second method to sharpen the peaks further and improve efficiency. These combined approaches are more complex and beyond the scope of the article, but there really is no limit to the combinations of methods that can be used.

## The Final Word

The right approach to sample concentration for CE is one that will meet your needs. Determining this isn't a simple matter, and in many cases there may be more than one possible approach that would be suitable. Literature can be useful to identify what may be more appropriate, but ultimately it will come down to experimenting until the appropriate conditions are found. The best advice is to start with a simple approach and only add complexity if it is absolutely needed.

## Acknowledgment

The sidebar "Good CE Injection Practice" was reproduced with permission, Cari Sänger, CESolutions 5. Injection precision and sensitivity, Separation Science, http://www.sepscience.com/Techniques/CE/1352-/CE-Solutions-5-Injection-Precision-and-Sensitivity.

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**Ronald E. Majors** *"Column Watch" Editor Ronald E. Majors is an analytical consultant and is a member of LCGC's editorial advisory board. Direct correspondence about this column to lcgcedit@lcgcmag.com.*

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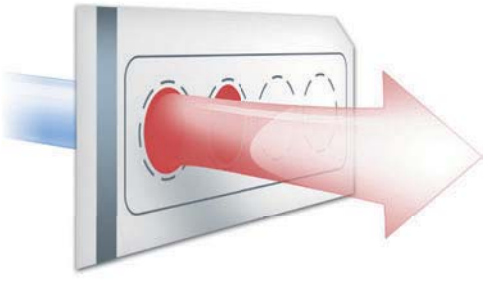


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# LC TROUBLESHOOTING

# LC Method Scaling, Part II: Gradient Separations

**If scaling isocratic separations is so simple, why is gradient scaling so confusing?**

***John W. Dolan***
*LC Troubleshooting Editor*

In last month's "LC Troubleshooting" installment (1), we looked at how to scale isocratic separations when the column size or packing particle size is changed. The process is quite simple. First, find a column with approximately the same plate number, then adjust the flow rate to give an acceptable pressure. The most common problems that result from mistakes in this scaling process give somewhat lower resolution than is expected or higher pressures. With gradients, unexpected consequences may occur from the changes that may be relatively unimportant in isocratic methods. In this month's discussion, we turn our attention to the proper scaling of gradient methods.

## Resolution and Plate Number

Last month (1) we looked at the fundamental resolution equation for isocratic conditions:

$$R_s = \tfrac{1}{4}N^{0.5}(\alpha - 1)(k/[1+k]) \qquad [1]$$

where $R_s$ is resolution, $N$ is the column plate number, $\alpha$ is the separation factor, and $k$ is the retention factor. A similar equation can be stated for gradient separations:

$$R_s = \tfrac{1}{4}N^{*0.5}(\alpha^* - 1)(k^*/[1+k^*]) \qquad [2]$$

where $N^*$ is the effective plate number under gradient conditions, $\alpha^*$ is the gradient separation factor, and $k^*$ is the gradient retention factor. As with isocratic conditions, we must be careful to keep from changing the chemistry of the system by keeping the same brand and series of column packing, the same mobile phase, and the same column temperature. We'll see below that we have some additional factors to be careful of with gradients. If we keep these things constant, $k^*$ and $\alpha^*$ (the ratio of $k^*$ values for two adjacent peaks) should remain constant. If $\alpha^*$ is unchanged, we will obtain the same resolution if we keep the same column plate number.

The column plate number cannot be measured easily under gradient conditions, so we measure it under isocratic conditions. Because the plate number is a characteristic of the column, the use of isocratic conditions is not a problem. We use the same approach we used with isocratic conditions to select a column with an equivalent plate number so that we maintain the same resolution with the scaled method. We saw that we could obtain approximately the same plate number if we kept the column length-to-particle-diameter ratio constant within a range of +50% to -25%. Thus, we can determine the desired column length from

$$L_2 = L_1 d_{p2}/d_{p1} \qquad [3]$$

where $L_1$ and $L_2$ are the column lengths, and $d_{p1}$ and $d_{p2}$ are the particle diameters of the original and new column, respectively. So far, nothing is different between the isocratic and gradient scaling process.

## Gradient *k** Is the Key

With isocratic separation, we saw that the flow rate had no influence on the retention factor, $k$, and only a minor influence on the plate number, $N$. So, although we often start the isocratic scaling process by keeping the linear velocity of the mobile phase the same,

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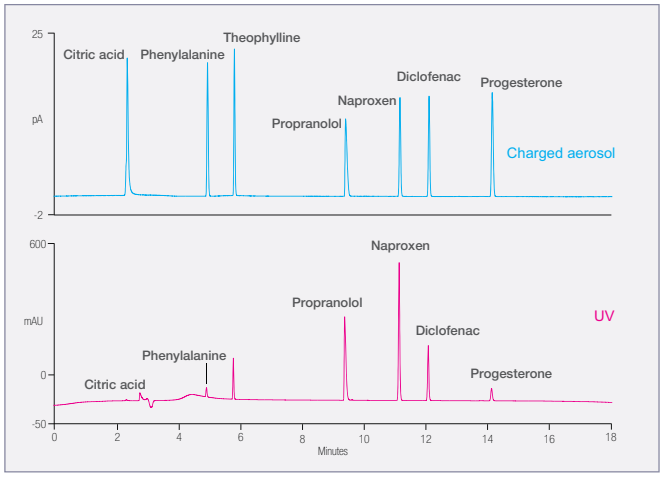


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from a practical standpoint the flow rate is adjusted to maintain an acceptable pressure. With gradients, however, a change in the flow rate without some other compensating change can generate problems. This is because $k^*$ is affected by flow rate:

$$k^* = (t_G F)/(1.15 V_m \Delta\Phi S) \quad [4]$$

where $t_G$ is the gradient time (minutes), $F$ is the flow rate (milliliters per minute), $V_m$ is the column volume (milliliters), $\Delta\Phi$ is the gradient range (for example, 5–95% = 0.9), and $S$ is a constant related to the molecular weight and is characteristic of each analyte. For the present discussion on scaling separations, we will not be changing the gradient range or the analytes, so $\Delta\Phi$ and $S$ will be constant and can be dropped for a simpler relationship:

$$k^* \approx (t_G F)/V_m \quad [5]$$

With isocratic separations, the separation factor, $\alpha$, is an expression of the peak spacing of two peaks with $k$ values of $k_1$ and $k_2$:

$$\alpha = k_2/k_1 \quad [6]$$

As long as we do not change the chemistry of the system (mobile phase composition or column chemistry) or the column temperature, $\alpha$ will remain constant, as will the peak spacing. Isocratic $k$ is calculated as follows:

$$k = (t_R - t_0)/t_0 \quad [7]$$

where $t_R$ is the retention time and $t_0$ is the column dead time. If $t_R$ is changed by nonchemical or temperature means, $t_0$ will change in parallel, so $k$ stays constant. This is why we can change the column length, the column diameter, or the flow rate and not affect $k$ in isocratic separation. If $k$ is constant, $\alpha$ will also remain constant. On the other hand, if we do something that changes $k$, we expect that $\alpha$ will change because it is rare for a variable that changes $k$ for one compound to change $k$ in exactly the same manner for all the other compounds in the separation. Thus, a change in the mobile phase or column temperature usually results in a change in peak spacing — in fact, we count on this effect as a tool for moving peaks relative to each other during method development.

A similar requirement of keeping $\alpha^*$ constant holds for gradient separations, where $\alpha^*$ is expressed as follows:

$$\alpha^* = k^*_2/k^*_1 \quad [8]$$

As with isocratic separation, chemical or temperature changes are not likely to change $k^*_1$ and $k^*_2$ in the same way, so these variables must be kept constant with gradients too. But with gradients, the added complication is that the variables on the right-hand side of equation 5 must also be kept constant, or they should be changed in a manner that allows $\alpha^*$ to stay constant. Next, let's consider how this requirement influences the scaling of gradient methods.

## Scaling a Simple Gradient

We'll start with a simple linear gradient of 5–95% acetonitrile over 20 min at 1 mL/min with a 150 mm × 4.6 mm, 5-µm particle diameter ($d_p$) column. Let's say that we want to get the same separation under ultrahigh-pressure liquid chromatography (UHPLC) conditions on a 1.8-µm $d_p$ column with an internal diameter of 2.1 mm.

The first step is to find a column with approximately the same plate number. We can use equation 3 to help us. The new column length ($L_2$) should be (150 mm × 1.8 µm)/5 µm = 54 mm long. Because 54-mm columns aren't available, we can use any length between 41 mm (-25%) and 81 mm (+50%) and be within our -25% to +50% guidelines. I would probably choose a 50 mm × 2.1 mm column in the present case.

As with isocratic separations, with gradients it usually is a good idea to start with a flow rate that gives us the same linear velocity. We use the same technique we discussed last month:

$$F_2 = F_1(d_{c2}/d_{c1})^2 \quad [9]$$

where $F$ is the flow rate and $d_c$ is the column internal diameter for columns 1 and 2. In the present case, the new flow rate should be 1 mL/min × (2.1 mm/4.6 mm)$^2$ = 0.208 mL/min; we'll round this to 0.2 mL/min for convenience. So, our new method will run on a 50 mm × 2.1 mm, 1.8-µm $d_p$ column at 0.2 mL/min.

Let's check to see if we are done. Because we want $k^*$ to remain constant, we can restate equation 5 as follows:

$$(t_{G1}F_1)/V_{m1} = (t_{G2}F_2)/V_{m2} \quad [10]$$

We can calculate each half of equation 10 for the proposed conditions and see how close we are. For the column volume, we'll use the approximation of ($L d_c^2$), because these are the only two variables that change between the columns. For the original conditions, (20 × 1)/(150 × 4.6$^2$) = 0.00630, and for the proposed conditions, (20 × 0.2)/(50 × 2.1$^2$) = 0.0181. The value for the new conditions is approximately three times too large. Because we've already defined the column size and the flow rate, the only variable we can adjust to fix this problem is the gradient time. If we reduce the gradient time to 7 min, the calculated value drops to 0.0635, which is close enough. So the gradient equivalent to the original conditions is a 7-min gradient at 0.2 mL/min on a 50 mm × 2.1 mm, 1.8-µm $d_p$ column. This means that the switch from the high performance liquid chromatography (HPLC) to the UHPLC column reduces the run time by approximately threefold (20 min to 7 min).

But if we're running under UHPLC conditions, we can operate at higher pressures and because we're using sub-2-µm particles, flow rate will not affect $N$, so a higher flow rate may help to further speed the separation. We use the same equation to calculate the system pressure as we did last month for isocratic conditions:

$$P_2 = P_1(L_2/L_1)(d_{c1}/d_{c2})^2(d_{p1}/d_{p2})^2(F_2/F_1) \quad [11]$$

Where $P_1$ and $P_2$ are the original and new pressures, respectively; all the other variables were defined above. Let's assume that the original method generated a 2000 psi (140 bar) back pressure. For the proposed conditions, $P_2$ = 2000 × (50/150) × (4.6/2.1)$^2$

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$\times$ $(5/1.8)^2$ $\times$ $(0.2/1)$ $\approx$ 4935 psi. For UHPLC, we probably can tolerate twice this pressure, and the easiest way to do this is to double the flow rate so that a new pressure of ~9875 psi would be expected. Wait! We've changed the flow rate, so equation 5 tells us that this will change $k^*$, and we may find a change in peak spacing — not something we want. So we need to make another compensating change using equation 5 as a guideline. If we double $F$, we can reduce $t_G$ by the same factor of two and $k^*$ will stay constant. Now our method would be a 3.5-min gradient at 0.4 mL/min on a 50 mm $\times$ 2.1 mm, 1.8-µm $d_p$ column. This would reduce the original run time by approximately sixfold (20 min to 3.5 min).

## What About Segmented Gradients?

The example above used a simple, one-segment linear gradient. Let's add a bit of complication and see how a two-segment gradient is handled. We'll use a 5–50% gradient in 10 min followed by a 50–95% gradient in 5 min, the other conditions (column and flow rate) are the same as before. Let's scale this to the same 2.1-mm i.d., 1.8-µm $d_p$ column. The same column, flow rate, and pressure scaling results will be obtained: scale to a 50 mm $\times$ 2.1 mm column operated at 0.2 mL/min resulting in a back pressure of ~4935 psi.

When it comes to scaling the remaining gradient conditions, we proceed in the same manner as with a single-segment gradient, but we need to treat each segment as a separate gradient. Thus, we have two gradients to consider: 5–50%/10 min and 50–95%/5 min. Because we're not changing the gradient range, we can scale the gradient times so that equation 10 is balanced. For the first segment, the original conditions give $(10 \times 1)/(150 \times 4.6^2) = 0.00315$ and the proposed conditions give $(10 \times 0.2)/(50 \times 2.1^2) = 0.00907$, approximately three times too large. A change in the gradient time to 3.5 min gives a value of 0.00317. For the second segment, our original gives $(5 \times 1)/(150 \times 4.6^2) = 0.00158$. We would expect the same gradient time ratio adjustment to hold for the second segment as for the first, so we can use it in our trial calculation. So we'll use $(3.5/10) \times 5$ min = 1.75 min, rounded to 1.7 min for convenience: $(1.7 \times 0.2)/(50 \times 2.1^2) = 0.00154$, which is close enough. Now our new method will be 5–50% in 3.5 min followed by 50–95% in 1.7 min at 0.2 mL/min on a 50 mm $\times$ 2.1 mm, 1.8-µm $d_p$ column.

If we want to increase the flow rate to accommodate the higher pressure capability of a UHPLC system, we use the same technique as discussed above with equation 11, but remember to adjust the gradient time of each segment by a factor of two using equation 5 if you change the flow rate by a factor of two.

## Don't Forget to Adjust the Injection Volume

One thing I didn't mention in last month's discussion of isocratic scaling is the potential need to adjust the injection volume when the column size is changed. The same rules hold for both isocratic and gradient conditions. As a general rule, the injection volume should be adjusted in proportion to the column volume. We can use the following relationship as a guideline:

$$V_{inj2} = V_{inj1}(L_2 d_{c2}^2)/(L_1 d_{c1}^2) \quad [12]$$

Where $V_{inj1}$ and $V_{inj2}$ are the injection volumes of the original and new conditions, respectively. If our original method used a 25-µL injection, the new method should use an injection of $(25 \times 50 \times 2.1^2)/(150 \times 4.6^2) = 1.75$ µL, which we can round to 2 µL.

Often the injection volume for the original method may not have been optimized, so although equation 12 gives the proper scaling, I suggest using an empirical test to determine the injection volume of the new method. I would try the calculated volume plus additional injections at twice and half the recommended volume. In the present case, I would try injections of 1, 2, and 4 (or 5) µL and observe the chromatogram. If there is no unacceptable degradation of the chromatogram, especially in the first part of the run, it is likely that the injection volume can be tolerated. I like to have a safety margin in my conditions, so the injection volume should not be so large that it is right on the edge of failure. This can be achieved by allowing a safety margin of 50–100%. For example, if in the above example, 5 µL still looked OK, try 8 or 10 µL. If these injection volumes are OK, 5 µL is safe.

## Further Cautions

As you can see from the discussion above, when the number of segments in the gradient increases, the work in calculating new conditions increases because each gradient segment must be scaled individually. What happens when there is a curved (convex or concave) gradient? I recommend strongly against using this type of gradient, because they are hard to reproduce from one instrument to another, and very difficult to scale. I don't know of any way to scale such gradients, because they are essentially composed of an infinite number of very small segments with different slopes. So, if you have an existing curved gradient that needs to be scaled, use the single-segment rules and hope that the new gradient works.

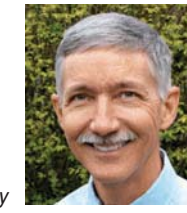
Those of you who are familiar with gradients will notice that I ignored the instrument dwell volume (gradient delay volume) in this discussion. The new dwell volume requirements can be calculated if the dwell time (the time to wash out the dwell volume) is considered as a gradient segment. The complication is that the dwell volume is seldom adjustable. Usually when you are moving a method from a conventional column to a small-volume or UHPLC column, you also will be moving from a conventional LC system to a UHPLC or a newer LC system, either of which is likely to have a reduced dwell volume. Dwell volume differences are most likely to affect the peak spacing for peaks eluted early in the gradient, so watch for such changes when the method is scaled. You may need to adjust the initial conditions to compensate for such changes. Dwell volume, of course, is not important in isocratic methods.

Finally, as with the isocratic examples last month, gradient calculations are tedious. You can simplify the process by using one of the on-line calculators to do the work for you. Some of these also calculate scaling of the injection volume and take dwell volume into account. Search the internet for "HPLC method transfer calculator" and you'll find several to choose from. I suggest that you try several and settle on the one that seems like the easiest for you.

## References

(1) J.W. Dolan, *LCGC North Am.* **32**(2), 98–102 (2014).

**John W. Dolan**



*"LC Troubleshooting" Editor John Dolan has been writing "LC Troubleshooting" for* LCGC *for more than 30 years. One of the industry's most respected professionals, John is currently the Vice President of and a principal instructor for LC Resources in Walnut Creek, California. He is also a member of* LCGC*'s editorial advisory board. Direct correspondence about this column via e-mail to John.Dolan@LCResources.com*

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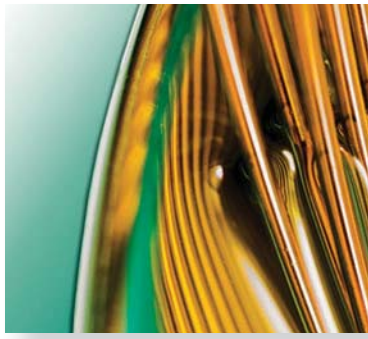

GC CONNECTIONS

# The Storage and Use of Gases for Gas Chromatography

**This month's column discusses the safe storage and use of calibration, carrier, and detector gas cylinders for small or large organizations.**

***John V. Hinshaw***
*GC Connections Editor*

Carrier and detector gases, when not generated directly on-site, are transported to laboratory facilities in gas cylinders and stored there until they are used. A recent "GC Connections" installment (1) explored the sources, both natural and synthetic, of carrier and detector gases for gas chromatography (GC). When put into service, secured gas cylinders or laboratory generators are connected to pressure- and flow-regulating devices that establish appropriate flow and pressure conditions for delivery to one or more gas chromatographs. The delivery system may be a simple length of tubing or it may be a more complex manifolded arrangement with intermediate regulation and valving. Ideally, the gas stream passes through a final purification stage positioned close to each chromatograph.

Much of the equipment associated with gas supplies in the laboratory ensures the purity and control of carrier and detector gases; the rest provides necessary safety measures for day-to-day encounters with flammable or asphyxiant gases that come in heavy high-pressure containers. Both the correct equipment and the procedures associated with gas deployment are critical for achieving the safest work environment and the best possible results.

In the United States, both the federal and state government authorities promulgate regulations and guidelines for the safe handling and use of gases according to their delivery system and chemical nature. In 29 *CFR* 1910.101, the United States Occupational Health and Safety Administration (OSHA) incorporates standards issued by the Compressed Gas Association (CGA) (2). Several organizations have made their policies and procedures for compressed gas handling available on-line; among these, the State University of New York (SUNY) campus at Stony Brook Compressed Gas Safety Guide (3) is one that is easily accessible and useful for typical chromatography laboratory scenarios.

Industrial gas consumers — including both companies and their employees — are responsible for the safe storage and use of gas cylinders and their contents from the moment the tanks come off a delivery truck until the gas supplier collects the empties for return. During this period of responsibility, certain procedures and equipment are used to mitigate the hazards and get the best use out of the cylinders' contents. In addition, companies should enact specific training programs to qualify employees to handle compressed gases safely and effectively. The contents of this article are intended as general professional advice and are not meant to reduce or replace any requirements, procedures, or regulations imposed by companies or their local authorities.

## Gas Cylinders

Gas cylinders are ubiquitous in chromatography laboratories. Their presence in nearly all chromatography work environments, combined with the rarity of accidental injury or property damage, engenders a sometimes too-relaxed attitude toward them on the part of laboratory workers and managers alike. Other hazards, such as burns from hot GC inlets or punctures suffered while cutting fused-silica columns, are much more common and can be mitigated by following easily understood and simple procedures. It is safe to say that the gas cylinders in a chromatography laboratory present an array of hazards that, short of the presence of toxic chemicals, comprise the greatest personal and property safety challenges chromatographers are likely to encounter.

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## Cylinder Hazards

Gas cylinder users do not always follow safe procedures. I often see cylinders that are not properly restrained or lack valve-protection caps that should be installed when the cylinders are not in use. Sometimes I observe incorrect transfer of cylinders from one location to another. For example, years ago at a university a gas delivery worker tilted two cylinders 15° from vertical, one with each gloved hand, and proceeded to roll them on their bottom edges down a hallway and into a laboratory. The cylinders did not fall, but I wonder how many times they had fallen during his tenure in that job and what kind of damage had been caused.

And how about moving tanks with a regulator attached and the main valve open, or without a protective valve cap? Not a good idea, but I've seen it done more than once. So, why not handle and use compressed gas cylinders without following the prescribed safe procedures? There are lots of reasons not to do so, and they can be grouped into three categories: physical hazards, energy hazards, and chemical hazards.

### Physical Hazards

Gas cylinders are massive. A 49-L capacity steel cylinder, such as what is commonly used for helium or nitrogen carrier gas, weighs about 63 kg (138 lb) and is physically unstable in its normal vertical position. Such a mass of steel can do considerable damage to limbs and toes that happen to be in the way as it falls.



### Energy Hazards

In addition to being bulky, massive, and unstable, compressed gas cylinders constrain a considerable amount of potential energy as pressurized gas. Should that pressure be released suddenly, a cylinder can become a spinning projectile that can penetrate walls and potentially inflict deadly harm. In an earlier "GC Connections" installment (4), I calculated that a full helium cylinder could attain a velocity of more than 108 km/h (67 mph) in a few seconds if the gas were released along the cylinder's main axis. The result depends on how quickly the gas is released, but by an estimate that is enough energy to do a lot of damage.

### Chemical Hazards

In the case of hydrogen, a gas cylinder represents considerable combustible chemical energy as well. Hydrogen concentrations can reach the lower explosive limit (LEL) of only 4% in air in any enclosed space into which sufficient quantities are released.

Cylinders that contain gases other than air also pose an asphyxiation hazard that results from the potential displacement of oxygen should the gas be released more rapidly than ambient air can be replaced by ventilation.

Some laboratories also use cryogenic liquefied gases such as liquid nitrogen or carbon dioxide. The proper use and storage of cryogenic liquids is a separate topic about which the interested reader can learn more from the references at the end of this article. Specific personnel protective equipment such as face masks, gloves, and boots should always be used when handling cryogenic liquids.

## Built-In Protection

Gas cylinders for the laboratory include some built-in safety measures. They are protected against over-pressurization caused by heating or inadvertent over-filling by a burst or rupture disk in the cylinder valve stem. Safety burst disks will open at pressures greater than the normal operating pressure for the cylinder, but less than the cylinder's test pressure. For a laboratory carrier-gas tank, the disk will open when the tank pressure exceeds about 225 MPa (3200 psi). This pressure is greater than what would be attained should a completely full cylinder be stored at up to 60 °C; the maximum recommended storage temperature for nonreactive gases is around 50 °C. A ruptured disk will release the contents of the cylinder safely (and loudly). A so-affected cylinder must be returned to the supplier for inspection and refurbishing.

## Managing Cylinders

Now let's examine how to manage and handle gas cylinders safely. A cylinder passes through three stages at a laboratory site: receipt and storage, in-use, and ready to return. These stages usually represent three separate locations within a facility; the first and last have nearly identical requirements, while the in-use stage is different.

Regardless of the stage of a cylinder's lifetime, store inert laboratory gases separately from reactive gases, including hydrogen fuel gas or any other gases used for other purposes. Hydrogen has some special venting and storage requirements that are detailed in the standards; keeping different types of gases separate helps avoid mix-ups.

**Receipt and Storage**

Receiving personnel should check the labeled contents of all cylinders upon delivery, as well as the overall appearance and condition of the cylinders. Any exceptions should be brought to the attention of the supplier as soon as possible. Suppliers don't deliver damaged or incorrect cylinders intentionally, but a quick check is in order. Ideally, the date of the most recent hydrostatic pressure test should be verified; however, this information can be difficult to ascertain from the markings on a cylinder. Cylinder pressure can be verified if the valve assembly includes an indicating regulator; otherwise it's better to wait until a cylinder is put into service before checking the fill pressure.

Gas cylinder contents generally are color-coded on the cylinder, but the color scheme is not consistent from supplier to supplier. Thus, it is not possible to rely on the color of a cylinder for identification. An attached label or tag will give the necessary information. If there is no tag or label, then the cylinder must be returned immediately to the supplier — or another supplier if the original supplier is unknown — for proper disposition.

Gas cylinders must be moved and stored properly from receipt until they are returned to the supplier. Outdoor and indoor storage are both possible. Indoor storage is more convenient if space is available, while outdoor storage should provide protection from weather and dirt. Cylinders must be kept dry in all cases to avoid corrosion.

Suitable cylinder storage locations include any area with limited access where they can be properly restrained. For small organizations the laboratory itself is often the best spot. Storage in a public hallway is not desirable, of course, while too-limited access might cause a problem should quick access be required in an emergency. Each location where gas cylinders are used is unique and, if at all possible, the question of proper cylinder locations should be considered carefully.

Often, and especially in situations where a larger number of cylinders are used, there may be more than one location for cylinder storage. For example, some companies store full cylinders in a location near a loading dock while keeping in-use cylinders in the laboratory or in a shared area that feeds multiple locations.

**In-Use**

Cylinders are classified as in-use if a regulator is attached or the protective valve cap is removed, whether or not gas is flowing at any particular time. With the protective cap removed or with a regulator attached, the high-pressure cylinder valve is exposed and represents an increased hazard. A cylinder that falls on its side with the cap or protective collar in place is unlikely to decompress explosively, whereas one with an exposed valve is much more likely to do so. Therefore, the in-use category requires very careful attention to cylinder restraints.

**Ready to Return**

Used cylinders ready for return should be kept in a dedicated empty-cylinder area that is convenient for their removal from the building, or at least the empties should be marked clearly as such. Once empty or otherwise no longer needed, cylinders fall back into a category with the same requirements as full cylinders waiting to be put in use — the protective valve cap must be installed; otherwise the valve could be damaged. Even nonobvious damage to the valve can

be a problem for the supplier when the cylinder is refilled.

If possible, gas cylinders should not be emptied completely before they are returned, since doing so may expose the inside of the cylinder to ambient air and moisture. The worst case is for an empty cylinder to be stored outside with an open valve. It will be difficult for the supplier to completely purge and clean the cylinder before the next filling. A residual pressure of 175–700 kPa (about 25–100 psi) is suitable.

## Moving Gas Cylinders

The image of two large cylinders being rolled by hand on their edges down a hallway remains with me more than 30 years after I witnessed it. The right way to move cylinders is of course with a cylinder cart or hand-truck. These are available in single- and dual-cylinder models with a variety of configurations. The better designs include static-dissipating wheels, which are much appreciated when moving hydrogen cylinders. Never move a cylinder with the regulator attached or the valve cover missing. And never use a gas cart as a permanent cylinder stand. Some tilting and rolling is required to maneuver a cylinder onto the cart, so caution is appropriate. Wear gloves and protective goggles and footwear.

If a cylinder starts to fall from upright toward prone, do not attempt to catch it. Jump back quickly out of the way. Return a fallen or otherwise physically stressed cylinder to the supplier immediately. A fall can introduce weaknesses in the cylinder that may make it potentially unsafe for continued use.

## How to Restrain Gas Cylinders

Proper restraint of gas cylinders is extremely important for their safe use. When set into position, cylinders must be prevented from tipping over, especially when in use with regulators attached. Gas cylinder restraints provide protection from accidental tippage that could compromise attached gas lines or, in the extreme case, cause a regulator or the tank valve to break or detach.

Several types of restraining systems are available, including single- or dual-tank straps that attach to a fixed object or a wall; individual free-standing cylinder floor stands; small multicylinder corrals with a restraining chain; and completely enclosed and lockable cylinder cages.

Do not place cylinders on their sides: moving them down to the floor or back to the upright position is a lifting hazard at the least, and the movement greatly increases the chances for dropping the cylinder.

### Tank Straps

These are available in several forms. They all feature an attachable strap or brace that encircles one or two cylinders approximately 1 m above the floor. The chain, woven belt, or metal strap is attached with a snap, buckle, or bolt so that the cylinder is prevented from falling over or moving out of position. Tank straps should be affixed permanently to an immovable object such as a wall or a laboratory bench that is bolted to the wall or floor. Some straps feature a clamp that attaches temporarily to a benchtop and allows the strap to be relocated easily. Although this type does not provide strict immobility it may be sufficient in some locations, but only if the bench is secured.

### Floor Stands

Floor stands are single-cylinder stands that clamp or strap onto a cylinder and provide a larger footprint than the cylinder itself. They provide improved stability but are not immune to tipping or movement, so they are recommended only for temporary cylinder placement.

### Cylinder Corrals

A cylinder corral is affixed permanently to the floor or wall. The corral admits two or three cylinders across and two or three deep, and the cylinders are restrained by a chain that attaches onto several hooks welded to the corral. This arrangement is suitable for stored or in-use cylinders where single tank straps are not enough — for example, in a gas analysis laboratory where multiple calibration gas cylinders plus carrier gas, hydrogen, and air are required. A cylinder corral is the most flexible arrangement and requires the least amount of linear wall space for multiple cylinders.

### Cage Enclosures

A cylinder cage or locker is a large box with heavy mesh walls and usually a floor as well. The door is firmly secured with a lockable bolt. Cylinder cabinets are similar, but they have solid walls. A cylinder cage holds up to 16 or more laboratory gas tanks, but only for storage purposes as it is not a good practice to snake gas lines through the cage mesh. It is the most secure method of cylinder storage. Cages or cabinets are also the most expensive storage method, but they are well worth the investment in safety and security for organizations that have to manage a large number of cylinders.

## Using Laboratory Gases

After verifying gas cylinders and their content upon receipt, safely moving and securing them in place, and organizing them by their current use, the next step is to attach the appropriate gas regulators, tubing, filters, and fittings to bring the high-purity gas supplies up to one or more gas chromatographs. The next "GC Connections" installment will discuss these topics and more, as we continue to follow the multiple gas paths through a GC system.

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For more information on this topic, please visit www.chromatographyonline.com/hinshaw

# Analysis of Amphetamines and Synthetic Cathinones in Hair Samples Using LC–Tandem Mass Spectrometry and Solid-Phase Extraction

**In this study, the procedure for analyzing amphetamines and synthetic cathinones (also known as bath salts drugs) in hair samples using a mixed-mode solid-phase extraction (SPE) is described. Samples of hair were digested with a dilute solution of base (containing internal standards), neutralized, and diluted with an aqueous phosphate buffer (pH 6). Each sample was applied to a conditioned SPE column, after which the sorbent was rinsed with deionized water, acetic acid, and methanol. After drying, the analytes were eluted and collected from the SPE column with 3 mL of an elution solvent consisting of methylene chloride–isopropanol–ammonium hydroxide. To the eluate, 200 µL of mobile phase was added and the samples were evaporated to the mobile phase for analysis by liquid chromatography–tandem mass spectrometry (LC–MS-MS). Chromatography was performed in gradient mode using a C18 column and a mobile phase consisting of acetonitrile and 0.1% aqueous formic acid. The total run time for each analysis was under 5 min.**

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Amphetamine (α-methylphenethylamine) (Figure 1) belongs to a class of compounds known as *sympathiomimetic amines* (1). This class of drugs includes the illicit drugs methamphetamine, methylenedioxyamphetamine (MDA), and methylenedioxymethamphetamine (MDMA), as well as ephedrine and pseudoephedrine, which can be found in over-the-counter medications. Amphetamine is administered as a prescription medication (for example, Adderall [Shire Pharmaceuticals]) for treating medical issues such as narcolepsy, obesity, or hypotension (2), whereas methamphetamine, MDA, and MDMA are considered controlled substances — that is, pharmaceuticals with little or no medical use.

Synthetic cathinones are derived structurally from the parent compound (Figure 2) and have become noticeable in the scientific literature in recent times because of the fatalities arising from administration (3–6). The drugs are commonly referred to as "bath salts" because they were originally packaged with names such as "Ivory Wave" and marketed as "not for human consumption" or "research chemicals." These drugs are now scheduled in the same way as other controlled substances.

The popularity of amphetamines is because of their euphoria effect and ease of synthesis. Their use or abuse is generally verified by the analysis of biological samples, such as urine, blood, oral fluid, or hair. Of these samples, hair is a biological matrix that has been used as an alternative to urine or blood for drug testing because it allows noninvasive sampling and can document the use of the drugs over a longer period of time than blood or urine (7). In this study, amphetamine in the form of Adderall was determined in the hair of a subject along with several other amphetamines and a range of synthetic cathinones.

In terms of metabolism in the human system, amphetamine is inactivated during

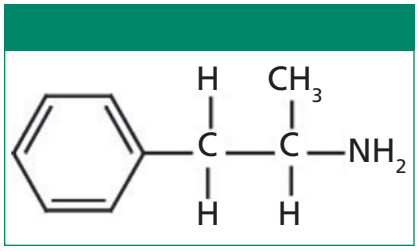


**Figure 1:** Structure of amphetamine.

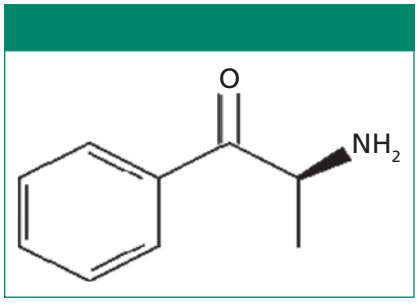


**Figure 2:** Structure of cathinone.

this process, undergoing deamination to form phenylacetone, which is converted to benzoic acid and excreted in a conjugated form (2). A small amount of the parent is oxidized to norephedrine, which is also metabolized to the parahydroxylated forms of this compound, all of which is pharmacologically active and are thought to contribute to the effects of the drug (8,9). The therapeutic, toxic, and fatal concentrations of amphetamines in samples such as blood and urine are well documented (2,10), but not so much for the synthetic cathinones because of the recent nature of their abuse. Most of the published studies on hair analysis have been performed in the area of work place drug testing and drugs or driving cases (11,12), not postmortem studies.

Gas chromatography coupled to mass spectrometry (GC–MS) has been reported as a technique for quantifying amphetamines in hair (13). GC–MS analysis of the amphetamines requires derivatization using compounds such as heptafluorobutyric anhydride (HFAA) or pentafluoropropionic anhydride (PFAA) (14,15). Because amphetamine exists as a *d–l* isomeric pair, some laboratories have used chiral modification to separate the isomers in samples such as hair (16). The ratio of the isomeric forms may indicate whether or not the amphetamine has been taken legally. GC–MS analysis of the cathinones used similar fluoroacyl derivatives (3). Liquid chromatography coupled to tandem mass spectrometry (LC–MS-MS) is gaining popularity for analysis in this matrix (17,18). The use of solid-phase extraction (SPE) described in this article uses the LC mobile phase as a keeper solvent for amphetamine, reducing its volatility. In previous methods the addition of methanolic hydrochloric acid or a solvent such as dimethylformamide has been reported, and the solvent was evaporated to dryness (19). The addition of the mobile phase presents the LC–MS-MS with a more amenable analytical solvent. SPE has been reported in the analysis of hair samples previously (20–22), but not using this type of keeper solution format.

## Experimental

### Chemicals and Reagents

Amphetamine, amphetamine-$d_5$, methamphetamine, methamphetamine-$d_5$, MDA, MDA-$d_5$, MDMA, MDMA-$d_5$, butylone, ethylone, flephedrone, mephedrone, mephedrone-$d_5$, methylone, methedrone, methcathinone (4-MEC), methylenedioxypyravalerone (MDPV), and pyravalerone were obtained from Lipomed as 1-mg/mL methanolic solutions. Acetonitrile, acetic

**Table I: Tandem mass spectrometry conditions**

| Compound | Q1 | Q3 | $t_R$ (min) | DP (V) | CE (V) | CXP (V) | CE (V) |
|---|---|---|---|---|---|---|---|
| Amphetamine (1) | 136.1 | 91.0 | 1.30 | 51 | 10 | 23 | 16 |
| Amphetamine (2) | 136.1 | 65.0 | 1.30 | 51 | 10 | 13 | 8 |
| Amphetamine-$d_5$ (2) | 141.1 | 124.0 | 1.30 | 51 | 10 | 25 | 4 |
| Amphetamine-$d_5$ (2) | 141.1 | 93.0 | 1.30 | 51 | 10 | 21 | 6 |
| Methamphetamine (1) | 150.1 | 91.0 | 1.62 | 61 | 10 | 29 | 16 |
| Methamphetamine (2) | 150.1 | 119.1 | 1.62 | 61 | 10 | 17 | 8 |
| Methamphetamine-$d_5$ (1) | 155.2 | 92.1 | 1.62 | 46 | 10 | 57 | 10 |
| Methamphetamine-$d_5$ (2) | 155.2 | 121.2 | 1.62 | 46 | 10 | 25 | 8 |
| MDA (1) | 180.2 | 163.1 | 1.51 | 46 | 10 | 15 | 8 |
| MDA (2) | 180.2 | 105.1 | 1.51 | 46 | 10 | 33 | 8 |
| MDA-$d_5$ (1) | 185.2 | 168.1 | 1.51 | 46 | 10 | 33 | 6 |
| MDA-$d_5$ (2) | 185.2 | 110.1 | 1.51 | 66 | 10 | 55 | 14 |
| MDMA (1) | 194.2 | 163.1 | 1.73 | 76 | 10 | 19 | 12 |
| MDMA (2) | 194.2 | 105.1 | 1.73 | 76 | 12 | 33 | 18 |
| MDMA-$d_5$ (1) | 199.2 | 165.1 | 1.73 | 66 | 10 | 19 | 12 |
| MDMA-$d_5$ (2) | 199.2 | 106.8 | 1.73 | 66 | 10 | 19 | 12 |
| Mephedrone-$d_3$ (1) | 181.2 | 123.0 | 1.94 | 66 | 10 | 35 | 6 |
| Mephedrone-$d_3$ (2) | 181.2 | 163.0 | 1.94 | 46 | 10 | 17 | 10 |
| Methedrone (1) | 194.1 | 176.1 | 1.66 | 56 | 10 | 29 | 12 |
| Methedrone (2) | 194.1 | 161.1 | 1.66 | 56 | 10 | 39 | 10 |
| Butylone (1) | 222.1 | 174.2 | 1.56 | 35 | 5 | 22 | 26 |
| Butylone (2) | 222.1 | 204.2 | 1.56 | 35 | 5 | 22 | 34 |
| Ethylone (1) | 222.1 | 174.2 | 1.86 | 35 | 5 | 22 | 26 |
| Ethylone (2) | 222.1 | 174.2 | 1.86 | 35 | 5 | 22 | 34 |
| Flephedrone (1) | 182.1 | 164.2 | 1.22 | 37 | 5 | 18 | 19 |
| Flephedrone (2) | 182.1 | 149.1 | 1.22 | 37 | 5 | 18 | 30 |
| Mephedrone (1) | 178.1 | 145.1 | 1.94 | 36 | 4 | 18 | 28 |
| Mephedrone (2) | 178.1 | 160.1 | 1.94 | 36 | 4 | 18 | 18 |
| Methylone (1) | 208.1 | 160.1 | 1.15 | 66 | 10 | 27 | 10 |
| Methylone (2) | 208.1 | 132.1 | 1.15 | 66 | 10 | 19 | 12 |
| 4-MEC (1) | 192.1 | 174.2 | 2.07 | 66 | 10 | 19 | 12 |
| 4-MEC (2) | 192.1 | 144.1 | 2.07 | 66 | 10 | 41 | 10 |
| MDPV (1) | 276.2 | 135.1 | 2.32 | 101 | 10 | 37 | 12 |
| MDPV (2) | 276.2 | 126.1 | 2.32 | 101 | 10 | 31 | 16 |
| Pyravalerone (1) | 246.2 | 105.1 | 2.55 | 56 | 10 | 33 | 188 |
| Pyravalerone (2) | 246.2 | 175.2 | 2.55 | 56 | 10 | 59 | 16 |

DP = declustering potential; EP = exit potential; CXP = collision cell exit potential; CE =collision energy; $t_R$ = retention time (in minutes).

acid (glacial), concentrated ammonium hydroxide solution (32% by volume), formic acid, isopropanol, methanol, and methylene chloride were obtained from Fisher Scientific. The SPE columns (CSDAU206) were obtained from UCT Inc. Deionized (DI) water was laboratory grade, and it was generated in the Massachusetts State Police Crime Laboratory (MSPCL). All chemicals were of ACS grade.

Acetic acid was prepared as a 0.1 M solution by diluting glacial acetic acid (5.8 mL to 500 mL) and then increasing the volume to 1 L by adding DI water and mixing well. Formic acid was prepared as a 0.1%

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**Table II: Precision results for control hair samples shown as percent recovery**

| Compound | 2 ng/mg | 8 ng/mg |
|---|---|---|
| Amphetamine | 100 ± 5 | 102 ± 7 |
| Methamphetamine | 95 ± 5 | 101 ± 7 |
| MDA | 110 ± 15 | 103 ± 11 |
| MDMA | 105 ± 5 | 102 ± 10 |
| Butylone | 120 ± 20 | 106 ± 12 |
| Ethylone | 90 ± 15 | 108 ± 13 |
| Flephedrone | 85 ± 20 | 103 ± 8 |
| Mephedrone | 120 ± 15 | 106 ± 10 |
| Methylone | 115 ± 20 | 98 ± 10 |
| Methedrone | 106 ± 7 | 101 ± 13 |
| 4-MEC | 90 ± 15 | 103 ± 12 |
| MDPV | 95 ± 10 | 97 ± 11 |
| Pyravalerone | 115 ± 10 | 103 ± 10 |

(v/v) solution by adding 1 mL of the acid to 900 mL of DI water and diluting it to 1 L (mobile-phase A). Acetonitrile containing 0.1% formic acid (v/v) was prepared by adding 1 mL of formic acid to 900 mL of acetonitrile and diluting to 1 L (mobile-phase B). Aqueous sodium hydroxide was prepared as a 0.1 M solution by adding 4 g of the solid to 500 mL of DI water and dissolving before diluting to 1 L. Phosphate buffer (pH 6, 0.1 M) was purchased from Fisher Scientific as a ready-to-use solution.

### Chromatographic Analysis

Analysis was performed using an API 3200 Q-Trap instrument supplied by Applied Biosystems. The chromatographic system consisted of a Shimadzu CBM 20 A controller, two Shimadzu LC 20 AD pumps (including a degasser), a Shimadzu SIL 20 AC autosampler, and a Shimadzu CTO AC oven (set at 10 °C). The instrument was fitted with a 50 mm × 2 mm, 5-µm $d_p$ Cadenza-C18 column from Imtakt USA (formerly Silvertone Sciences) that was attached to a 5 mm × 2 mm Cadenza-C18 guard column obtained from the same supplier. The LC column oven was maintained at 40 °C throughout the analyses. The injection volume was 10 µL. The mobile phase was delivered at a flow rate of 0.5 mL/min. The mobile-phase gradient program started at 5% mobile-phase B, rose to 90% B in 4.0 min, and then returned to 5.0% B. The instrument was readied for reinjection after 5.1 min.

The mass spectrometry was performed using positive multiple reaction monitoring (MRM). The mass spectrometer conditions for each of the amphetamines and synthetic cathinones are shown in Table I. Tandem MS was performed using the following conditions: curtain gas setting, 15; collision gas setting, medium; ion spray voltage setting, 5000 V; temperature setting, 650 °C; ion source gas 1 setting, 50; ion source gas 2 setting, 50. The tandem mass spectrometer conditions are shown in Table I. The analytical data were collected using Analyst software version 1.5.2 supplied by Applied Biosystems.

### Sample Preparation for Analysis

*Calibrators and Controls*

A solution of amphetamines and synthetic cathinones (amphetamine, methamphetamine, MDA, MDMA, butylone, ethylone, flephedrone, mephedrone, methylone, methedrone, methcathinone [4-MEC], methylenedioxypyravalerone [MDPV], and pyravalerone) was prepared at a concentration of 1 µg/mL by the dilution of 10 µL of stock solution with acetonitrile to 10 mL in a volumetric flask. A 1-µg/mL solution of the internal standards (amphetamine-$d_5$, methamphetamine-$d_5$, MDMA-$d_5$, MDA-$d_5$, and mephedrone-$d_3$) was prepared by the diluting 100 µL of the stock solution (100 µg/mL) to 10 mL with acetonitrile in a volumetric flask. The choice of internal standard was based on the fact that deuterated analogues of amphetamines and synthetic cathinones would not only chromatograph in a very similar mode to the drugs themselves, but also would be extracted via SPE as efficiently as amphetamine and the synthetic cathinones and would not likely be observed in the samples under analysis.

Calibrators were prepared by the addition of 1.0, 2.5, 5.0, 10.0, 25.0, 50.0, and 100.0 ng of amphetamines and synthetic cathinones into 10-mg samples of drug-free hair samples. The hair samples had been previously decontaminated by washing two times with methylene chloride (10 mL) and two times with DI water (10 mL) before being air dried for 24 h. Each wash step was analyzed for the presence of drugs and found to be negative. Next, 50 ng of the internal standard was added to these samples. Control samples were prepared by the addition of 2 ng and 8 ng of amphetamine and synthetic cathinones to drug-free hair samples in addition to 50 ng of the internal standard. All determinations were performed in duplicate. A negative control sample was prepared by the addition of only the internal standard (50 ng) to a sample of drug-free hair samples (10 mg). Calibrators, control samples, and test samples were treated in an identical mode with regard to sample extraction.

To assess the performance of the procedure, calibration curves were constructed twice daily over five consecutive days using the spiked controls, and from these data intraday and interday values were obtained.

### Sample Extraction

A 1-mL volume of 0.1 M aqueous sodium hydroxide solution was added to each sample (calibrator, control, and test) in a clean glass tube (75 mm × 12 mm) that was then capped. The tubes and contents were digested for 30 min at room temperature. Then, 4 mL of 0.1 M phosphate buffer (pH 6) was added to the solution, which was mixed and centrifuged at 3000 rpm for 10 min before it was applied to the SPE column.

### Solid-Phase Extraction

SPE columns were conditioned by the sequential addition of 3 mL of methanol, 3 mL of DI water, and 1 mL of 0.1 M phosphate buffer (pH 6). Each liquid was allowed to percolate through the sorbent using gravity without allowing the sorbent to dry out between steps.

Following the passage of the methanol, DI water, and 0.1 M phosphate buffer (pH 6) through the SPE columns, each diluted sample (that is, calibrator, control, and case item) was loaded on to an individually marked SPE tube and allowed to pass through the sorbent using gravitational flow. The columns were then washed with sequential additions of 3 mL of DI water, 1 mL of 0.1 M acetic acid, and 3 mL of methanol. The SPE columns were then dried by applying a vacuum to the SPE manifold at 15 in. of mercury pressure using an electric vacuum pump.

The analytes were eluted from the SPE columns by the addition of 3 mL of a 78:20:2 methylene chloride–isopropanol–ammonium hydroxide solution. This solution was prepared daily by adding 2 mL of concentrated ammonium hydroxide solution to 20 mL of isopropanol and mixing well. Then, 78 mL of methylene chloride was added to this solution and

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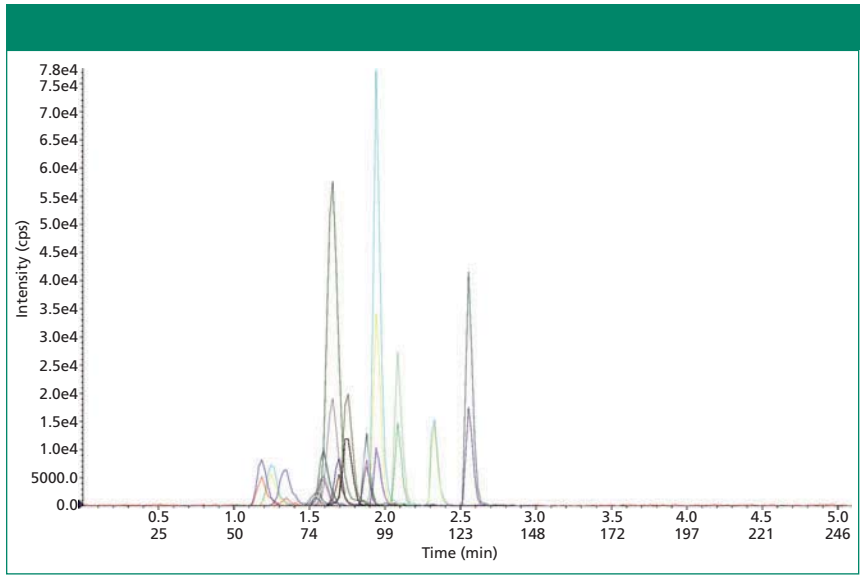


**Figure 3:** Chromatogram of hair analysis containing amphetamines and synthetic cathinones at the LOQ (0.1 ng/mg). See Table I for compound identification.

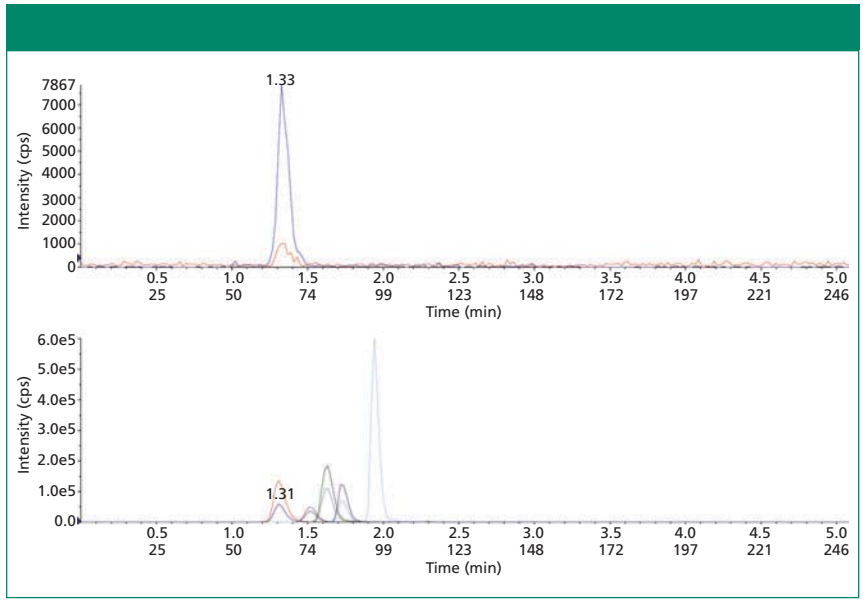


**Figure 4:** Chromatogram of genuine hair demonstrating the presence of amphetamine only (upper trace). Lower trace indicates internal standards. See Table I for compound identification.

the resultant solution was transferred to a clean screw-top bottle for use. A screw-top bottle ensures that the basicity of the solution remains high by eliminating any loss of ammonia from the bottle. The elution solvent was allowed to flow through the SPE sorbent with the aid of gravity and collected in separate glass tubes (75 mm × 12 mm). Glass tubes were chosen because they are standard laboratory materials within this toxicology laboratory.

Following elution, 200 µL of a solution containing 95% of mobile-phase A and 5% of mobile-phase B was added to each sample tube. The tubes were vortex mixed for approximately 1 min before the eluates were evaporated to the mobile phase using a gentle stream of nitrogen at 35 °C. After further vortex mixing, the samples were transferred to an autosampler vial (2 mL) containing a low-volume insert (250 µL) and the vial was capped for analysis.

## Matrix Effects

Studies of the matrix effects were performed according to a previously published procedure (23). In this part of the study, aliquots of amphetamine and synthetic cathinones (covering the linear range) were introduced into 200 µL of a solution containing 95% of mobile-phase A and 5% mobile-phase B. Each elution solution was evaporated to remove the organic component until only the mobile phase remained and then they were analyzed by LC–MS-MS (analysis A). Concurrently, a set of hair samples were subjected to the SPE process; after elution of the analytes from the SPE columns, the elution solvent was spiked with amphetamine, and 200 µL of mobile phase (95:5 A–B) was added, and then the solution was evaporated to the mobile phase (analysis B). A second set of hair samples was spiked with amphetamine and synthetic cathinones and processed via the SPE method. After elution, 200 µL of mobile phase was added and after vortex mixing, the solution was evaporated to the mobile phase (analysis C). The data (peak areas) for analyses A, B, and C were collected by the data analysis software. By comparing the peak areas of analysis B with those of A, an assessment of matrix effects was made. The comparison of peak areas for C with B provided data for the recoveries.

Amphetamine and synthetic cathinone solution (concentration: 50 ng/mg) was infused into the tandem mass spectrometer using the on-board syringe pump (controlled by the data analysis software) via a Hamilton syringe (model 1001TLL, 1 mL volume, supplied by Fisher Scientific) at a flow rate of 5 µL/min. At the same time as the amphetamine solution was flowing into the mass spectrometer, a 10-µL aliquot of the SPE-extracted hair matrix (samples of hair confirmed to contain no drug material) was injected using the autosampler syringe on the Shimadzu liquid chromatograph. The liquid chromatograph and mass spectrometer were arranged so that samples from the liquid chromatograph were mixed into the flow of amphetamine and synthetic cathinones via a three-port tee section before the total flow entered the mass spectrometer. Any suppression effects on the amphetamine could be monitored at the MRM step for the noted drugs.

## Selectivity

When analyzing samples of hair extracts via SPE and LC–MS-MS it is essential to ensure that the interfering effects of

other drug compounds can be eliminated. In this procedure, samples of hair extracts were spiked with 49 drugs at a concentration equivalent to 100 ng/mg of hair sample: (bupropion, lidocaine, methadone, amitriptyline, nortriptyline, thioridazine, trazodone, mesoridazine, meperidine, diphenhydramine, phenyltoloxamine, imipramine, desipramine, benztropine, trimethoprim, diltiazem, haloperidol, strychnine, morphine, codeine, 6-acetylmorphine, oxycodone, oxymorphone, hydrocodone, noroxycodone, hydromorphone, diazepam, nordiazepam, oxazepam, temazepam, alprazolam, α-hydroxyalprazolam, lorazepam, triazolam, α-hydroxytriazolam, flunitrazepam, 7-amino-flunitrazepam, chlordiazepoxide, midazolam, α-hydroxymidazolam, flurazepam, desalkyl-flurazepam, cocaine, ecgonine methyl ester, ecgonine ethyl ester, benzoylecgonine, cocaethylene, clonazepam, and 7-aminoclonazepam) and extracted according to the SPE method. It was observed that the interfering effect of these compounds was not found to be significant.

## Results and Discussion

### Recovery

The mean recovery of amphetamine and synthetic cathinones from drug-free hair samples was determined to be 95% (±2%). This is an excellent indicator for the efficiency of the extraction procedure of amphetamines and synthetic cathinones using hair as a matrix. This procedure was performed twice daily over a period of five days.

### Imprecision of Analysis

The results of the analysis of the spiked control samples of hair (2 ng/mg and 8 ng/mg, respectively) are shown in Table II. Analysis of the control samples was performed at the same time as the calibration curves were constructed — that is, over a period of five days. Control samples were prepared by adding the amphetamine and synthetic cathinone solution to the hair sample (10 mg) in the digestion mixture and treating as per the test samples.

Intraday and interday variation for the analysis of amphetamines and synthetic cathinones was found to be less than 7% and less than 10%, respectively. Ion suppression studies revealed that suppression of the monitored ions was less than 5%. This method was found to be linear ($r^2 > 0.995$) over the dynamic range 0.1–10 ng/mg.

### Limit of Detection and Limit of Quantification

The *limit of detection* (LOD) of a particular method can be defined as the level at which the signal-to-noise ratio for the particular analyte is greater than or equal to 3:1. The *limit of quantification* (LOQ) for the method is the level at which the signal-to-noise ratio for a particular analyte is greater than or equal to 10:1. In this study, LOD values were determined empirically by analyzing extracted samples of drug-free hair fortified with amphetamines and synthetic cathinones by LC–MS-MS according to the SPE method. This was performed until the lowest level at which each of the respective analytes just failed the signal-to-noise ratio of 3:1. This was observed as 0.05 ng/mg. In terms of LOQ, samples of drug-free hair samples were spiked with amphetamines and synthetic cathinones at

concentrations below 10 ng/mg and extracted according to the SPE procedure until the analytes just failed a signal-to-noise ratio of 10:1; this value was found to be 0.1 ng/mg. This is better than the recommendations by the Society of Hair Testing (SoHT) of 0.2 ng/mg (24). Representative chromatograms at LOQ and genuine hair sample are shown in Figures 3 and 4.

### Solid-Phase Extraction

Because hair is a solid matrix, the sample requires digestion to produce a liquid that is able to flow through the SPE column. As the digest is basic, buffering to pH 6 permits both efficient flow and optimal sorbing of the drugs onto the SPE sorbent. In using a mixed-mode (C8 and strong cation-exchange chemistries), the sample can be cleaned up via aqueous acid and methanol washes, which leaves the drugs in a much cleaner state than when they were originally applied to the SPE column. This effect is noted in the low matrix effects and ion suppression values.

In this new methodology, the SPE eluates are not evaporated to dryness as in typical SPE procedures, but are evaporated to a solution of mobile phase. Without the addition of methanolic hydrochloric acid to reduce their volatility, these drugs are known to be lost during this evaporation step. Using the mobile phase as a keeper solvent permits the volatile amphetamines and synthetic cathinones to be kept in solution and results in high recovery values by eliminating the loss during the evaporation.

### Tandem MS

In this methodology, LC–MS-MS has been successfully applied to the extraction and analysis of amphetamine and synthetic cathinones rather than GC–MS in which a multistep derivatization procedure (that is, reaction with a fluoroacyl reagent such as heptafluorobutyric anhydride), evaporation, and reconstitution in a volatile solvent is required not only to quantify, but also to confirm the identity of the amphetamine or synthetic cathinone. By using LC–MS-MS with specific MRM values, amphetamine and synthetic cathinones can be targeted, confirmed, and quantified in hair samples without the use of derivatization. Coupling this procedure with a quick LC method offers analysts the ability of determine concentrations of the drug within a short turnaround time.

### Concentrations of Amphetamines and Synthetic Cathinones in Genuine Hair Sample

It was observed that the donor hair sample contained only amphetamine (confirmed by a prescription of Adderall). The concentration was found to be 1.2 ng/mg. The synthetic cathinones (butylone, ethylone, flephedrone, mephedrone,methylone, methedrone, methcathinone [4-MEC], methylenedioxypyravalerone [MDPV], and pyravalerone) were not observed in the sample and neither were methamphetamine, MDA, or MDMA. Although studies were not performed on different colored hair samples, the recoveries of the drugs on donated hair samples (gray) were notably high.

## Conclusion

The use of hair samples is recognized as an alternative specimen of choice for the forensic and clinical analysis of drugs. Analysts

around the world are going to be asked to test it on a routine basis. With this in mind, this SPE and LC–MS-MS procedure will offer these facilities the ability to perform a quick and efficient analysis of amphetamine and synthetic cathinones in small samples of hair. The novel use of the mobile phase as the keeper solvent serves to improve efficiency and maintain high recovery values.

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# Application of Pyrolysis–Gas Chromatography–Mass Spectrometry for the Identification of Polymeric Materials

**The analytical pyrolysis technique hyphenated to gas chromatography–mass spectrometry (GC–MS) has extended the range of possible tools for the characterization of synthetic polymers and copolymers. Pyrolysis involves thermal fragmentation of the analytical sample at temperatures of 500–1400 °C. In the presence of an inert gas, reproducible decomposition products characteristic for the original polymer or copolymer sample are formed. The pyrolysis products are chromatographically separated using a fused-silica capillary column and are subsequently identified by interpretation of the obtained mass spectra or by using mass spectra libraries. The analytical technique eliminates the need for pretreatment by performing analyses directly on the solid or liquid polymer sample. In this article, application examples of analytical pyrolysis hyphenated to GC–MS for the identification of different polymeric materials in the plastic and automotive industry, dentistry, and occupational safety are demonstrated. For the first time, results of identification of commercial light-curing dental filling material and a car wrapping foil by pyrolysis–GC–MS are presented.**

***Peter Kusch*, Gerd Knupp*, Wolfgang Fink*, Dorothee Schroeder-Obst*, Volker Obst†, and Johannes Steinhaus****

**Hochschule Bonn-Rhein-Sieg, University of Applied Sciences, Department of Applied Natural Sciences, Rheinbach, Germany; †Volker Obst, Dr. Obst Technische Werkstoffe GmbH, Rheinbach, Germany. Direct correspondence to: Peter.Kusch@h-brs.de*

Structural analysis and the study of degradation properties are important to understand and improve performance characteristics of synthetic polymers and copolymers in many industrial applications. Traditional analytical techniques used for characterization of polymers and copolymers such as thermal analysis and Fourier transform infrared (FT–IR) spectroscopy have limitations or are not sufficiently sensitive (1). Pyrolysis techniques hyphenated to gas chromatography–mass spectrometry (GC–MS) have extended the range of possible tools for the characterization of synthetic polymers and copolymers. Under controlled conditions, at elevated temperatures (500–1400 °C) in the presence of an inert gas, reproducible decomposition products characteristic for the original polymer or copolymer sample are formed. The pyrolysis products are chromatographically separated using a fused-silica capillary column and subsequently identified by interpretation of the obtained mass spectra or by using mass spectra libraries (such as the National Institute of Standards and Technology [NIST] or Wiley). Pyrolysis methods eliminate the need for pretreatment by performing analyses directly on the solid polymer or copolymer sample (1). (Please note that this article was presented at the XVII European Conference on Analytical Chemistry, which was held in Warsaw, Poland, on August 25–29, 2013).

Most of the thermal degradation results from free radical reactions

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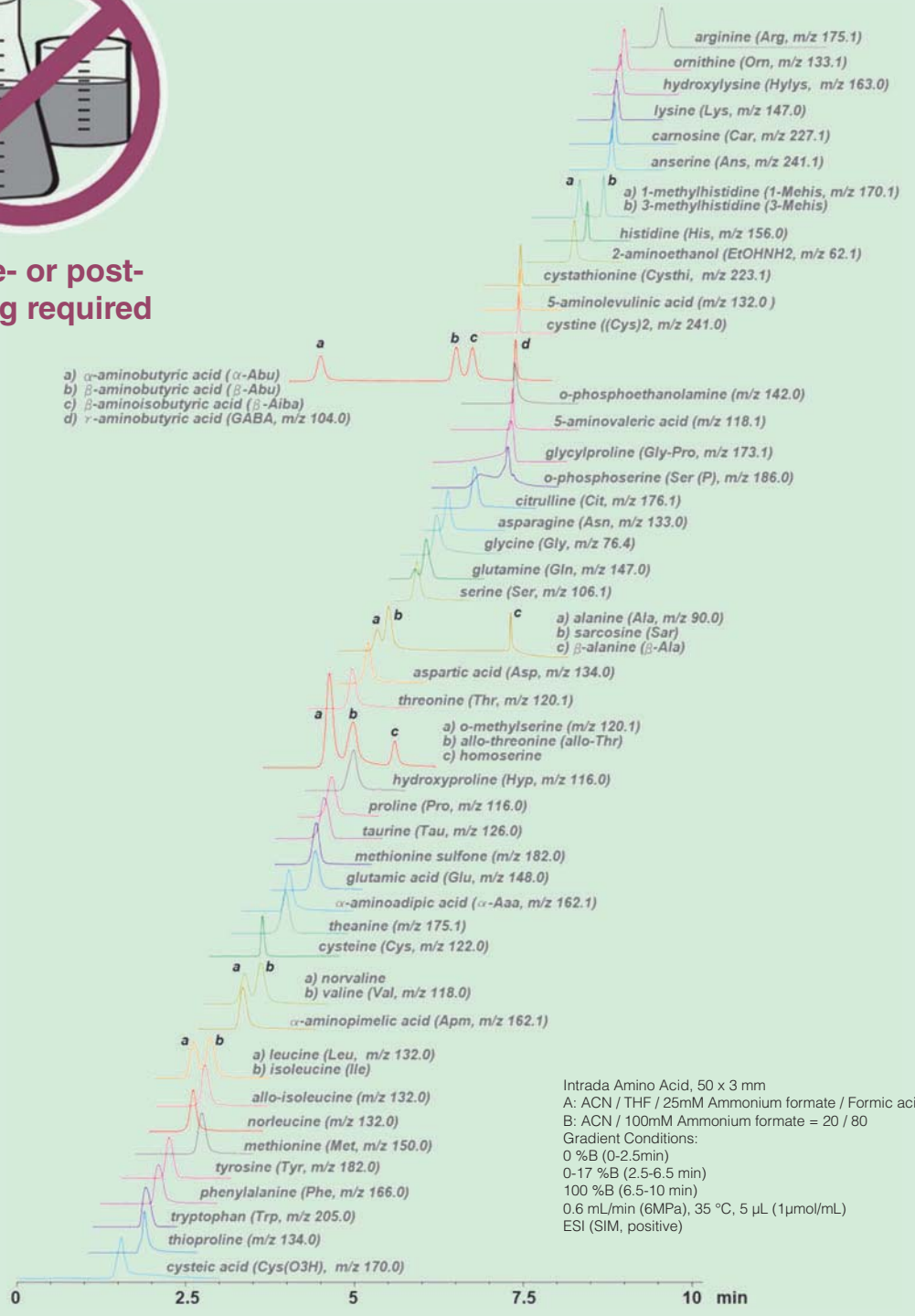


Intrada Amino Acid, 50 x 3 mm
A: ACN / THF / 25mM Ammonium formate / Formic acid = 9 / 75 / 16 / 0.3
B: ACN / 100mM Ammonium formate = 20 / 80
Gradient Conditions:
0 %B (0-2.5min)
0-17 %B (2.5-6.5 min)
100 %B (6.5-10 min)
0.6 mL/min (6MPa), 35 °C, 5 µL (1µmol/mL)
ESI (SIM, positive)

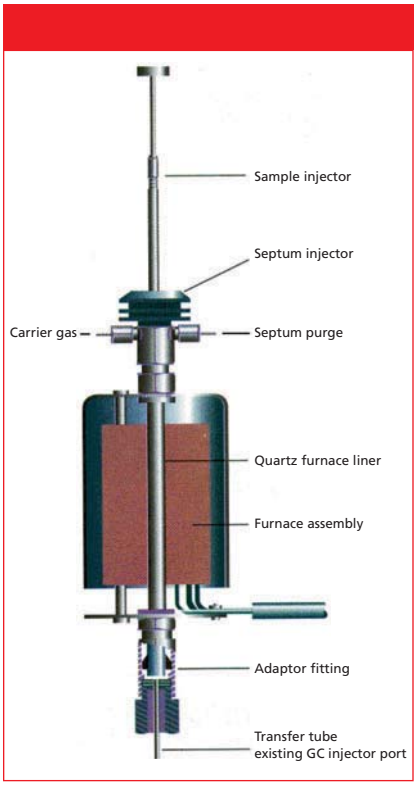


**Figure 1:** Schematic view of the furnace pyrolyzer used in this study.

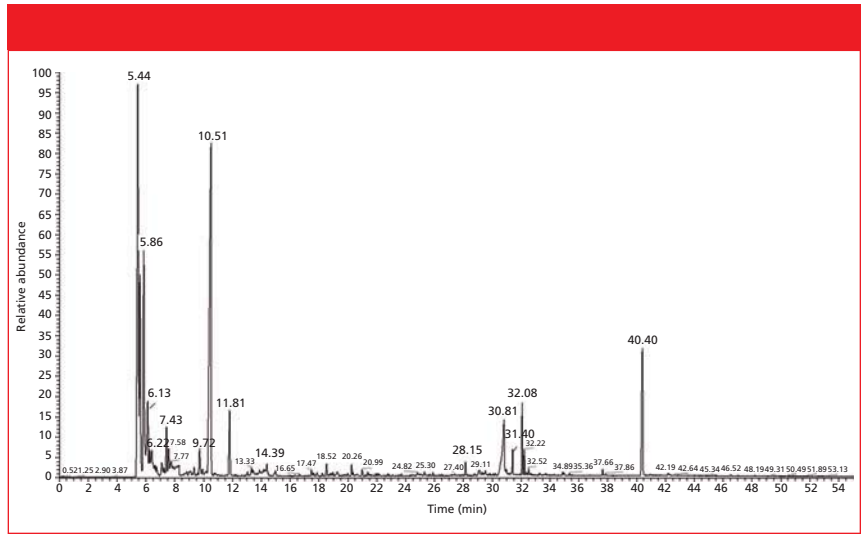


**Figure 2:** Pyrolysis–GC–MS chromatogram of plastic particles from industrial filter fins at 700 °C obtained with apparatus 1. Fused-silica GC capillary column: 60 m × 0.25 mm, 0.25-µm $d_f$ Elite-5ms. GC conditions: programmed column temperature: 60 °C for 1 min, then 60–100 °C at 2.5 °C/min and then 100–280 °C at 10 °C/min (20 min hold at 280 °C); split–splitless injector temperature: 250 °C; split flow: 50 cm³/min; helium programmed pressure: 70 kPa for 1 min, then 70–110 kPa at 1 kPa/min (hold at 110 kPa to the end of analysis). For peak identification, see Table I.

**Table I: Pyrolysis products and identified materials in plastic particles from industrial filter fins**

| Retention Time $t_R$ (min) | Pyrolysis Product at 700 °C | Matching Factor | Identified Material |
|---|---|---|---|
| 5.44 | Propylene | 820 | Polypropylene glycol |
| 5.58 | 1-Butene/1,3-butadiene | 840 | Styrene–butadiene rubber (SBR) |
| 5.86 | Acetone | 850 | Polypropylene glycol |
| 6.13 | Pentadiene | 885 | SBR |
| 7.43 | Benzene | 954 | SBR |
| 9.72 | Toluene | 863 | SBR |
| 10.51 | Cyclopentanone | 933 | Poly(hexamethylene adipamide) (nylon 6-6) |
| 11.81 | 2-Cyclopenten-1-one | 906 | Poly(hexamethylene adipamide) (nylon 6-6) |
| 14.39 | Styrene | 851 | SBR |
| 28.15 | 4-Isopropylphenol | 944 | Polycarbonate or bisphenol A epoxy resin |
| 40.40 | *N*-Phenyl-1-naphthalen-amine | 948 | Antioxidant |

initiated by bond breaking and depends on the relative strengths of the bonds that hold the molecules together. A large molecule will break apart and rearrange in a characteristic way (2–4). If the energy transfer to the sample is controlled by temperature, heating rate, and time, the fragmentation pattern is reproducible and characteristic for the original polymer or copolymer. Another sample of the same composition, heated at the same rate to the same temperature for the same period of time, will produce the same decomposition products. Therefore, the essential requirements of the apparatus in analytical pyrolysis are reproducibility of the final pyrolysis temperature, rapid temperature rise, and accurate temperature control. Depending on the heating mechanism, pyrolysis systems have been classified into two groups: the continuous-mode pyrolyzer (furnace pyrolyzer) and pulse-mode pyrolyzer (flash pyrolyzer, such as the heated filament, Curie-point, and laser pyrolyzer). The pyrolysis unit is directly connected to the injector port of a gas chromatograph. A flow of an inert carrier gas, such as helium, flushes the pyrolyzates into the fused-silica capillary column. Figure 1 shows the schematic view of the furnace pyrolyzer used in our investigation. The detection technique of the separated compounds is typically MS, but other GC detectors have also been used depending on the intentions of the analysis (1,4).

The applications of analytical pyrolysis–GC–MS range from research and development of new materials, quality control, characterization and competitor product evaluation, medicine, biology and biotechnology, geology, airspace, and environmental analysis to forensic purposes or conservation and restoration of cultural heritage. These applications cover analysis and identification of polymers, copolymers, and additives in components of automobiles, tires, packaging materials, textile fibers, coatings, half-finished products for electronics, paints or varnishes,

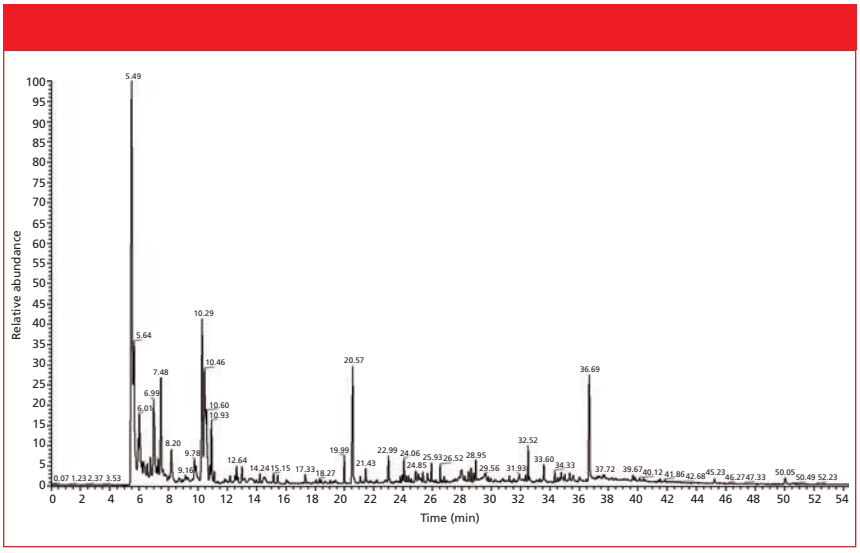


**Figure 3:** Pyrolysis–GC–MS chromatogram of a car wrapping material at 600 °C obtained with apparatus 1. Fused-silica GC capillary column 60 m × 0.25 mm, 0.25-µm $d_f$ Elite-5ms. GC conditions: programmed column temperature: 60 °C for 1 min, then 60–100 °C at 2.5 °C/min and then 100–280 °C at 10 °C/min (20 min hold at 280 °C); split–splitless injector temperature: 250 °C; split flow: 50 cm³/min; helium programmed pressure: 70 kPa for 1 min, then 70–110 kPa at 1 kPa/min (hold at 110 kPa to the end of analysis). For peak identification, see Table II.

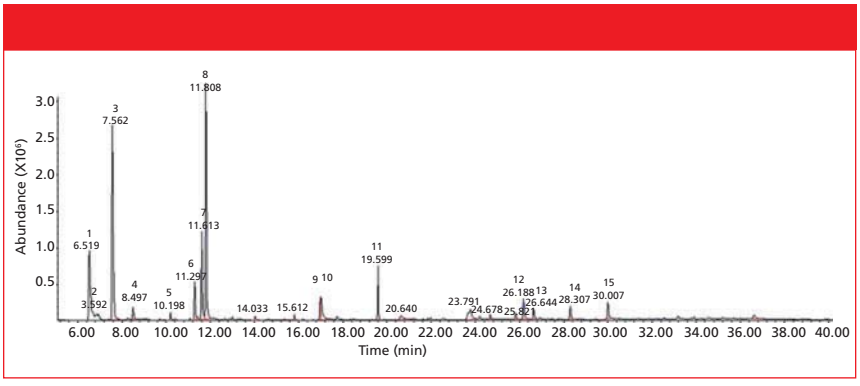


**Figure 4:** Pyrolysis–GC–MS chromatogram of polyaramid fibers at 900 °C obtained with apparatus 2. Fused-silica GC capillary column: 59 m × 0.25 mm, 0.25-µm $d_f$ DB-5ms. GC conditions: programmed column temperature: 75 °C for 1 min, then 75–280 °C at 7 °C/min (hold to the end of analysis); programmed pressure of helium carrier gas: 122.2 kPa for 1 min, then 122.2–212.9 kPa at 7 kPa/min (hold at 212.9 kPa to the end of analysis). For peak identification, see Table III.

lacquers, leather, paper or wood products, food, pharmaceuticals, surfactants, and fragrances.

Our earlier publications (1,5–12) presented the analysis and identification of degradation products of commercially available synthetic polymers and copolymers by using analytical pyrolysis hyphenated to gas chromatography with flame ionization detection (GC–FID) and GC–MS. In this work, new examples of applications of this analytical technique for the identification of different polymeric materials are demonstrated.

## Experimental

### Samples

Plastic particles from industrial filter fins, a car wrapping foil, unknown fibers, and commercial light-curing dental filling material were used in the investigation.

### Instrumentation and Analytical Conditions

Approximately 100–200 µg of solid sample was cut out with a scalpel and inserted without any further preparation into the bore of the pyrolysis solids-injector and then placed with the plunger on the quartz wool of the quartz tube of the furnace pyrolyzer Pyrojector II (SGE Analytical Science). Three spots on each sample were analyzed in duplicate. The pyrolyzer was operated at a constant temperature of 550, 600, 700, or 900 °C. The pressure of helium carrier gas at the inlet to the furnace was 95 kPa.

Pyrolysis–GC–MS measurements were made using two apparatus. In the first apparatus (1), the pyrolyzer was connected to a Trace 2000 gas chromatograph (ThermoQuest, CE Instruments) with a quadrupole mass spectrometer Voyager (ThermoQuest, Finnigan, MassLab Group) operated in electron ionization (EI) mode. A 60 m × 0.25 mm, 0.25-µm Elite-5ms fused-silica GC capillary column (PerkinElmer Instruments) was used. The GC conditions were as follows: programmed column temperature: 60 °C for 1 min, then 60–100 °C at 2.5 °C/min, 100–280 °C at 10 °C/min (20-min hold at 280 °C). The temperature of the split–splitless injector was 250 °C and the split flow was 50 cm³/min. Helium, grade 5.0 (Westfalen AG), was used as a carrier gas. The helium programmed pressure was 70 kPa for 1 min, then 70–110 kPa at 1 kPa/min (hold at 110 kPa to the end of analysis) was used. The transfer line temperature was 280 °C. The MS EI ion source temperature was kept at 250 °C. The ionization occurred with a kinetic energy of the impacting electrons of 70 eV. The current emission of the rhenium filament was 150 µA. The MS detector voltage was 350 V. Mass spectra and reconstructed chromatograms (total ion current [TIC]) were obtained by automatic scanning in the mass range *m/z* 35–450 u. Pyrolysis–GC–MS data were processed with the Xcalibur software (ThermoQuest) and the NIST 05 mass spectra library.

In the second apparatus (2), the pyrolyzer was connected to a 7890A gas chromatograph with a series 5975C quadrupole mass spectrometer (Agilent Technologies Inc.) operated in EI mode. A 59 m × 0.25 mm, 0.25-µm $d_f$ DB-5ms fused-silica GC capillary column (J&W Scientific) was used. Helium, grade 5.0 (Westfalen AG), was used as a carrier gas. The GC conditions were as follows: programmed

**Table II: Pyrolysis products and identified materials in car wrapping foil**

| Retention time $t_R$ (min) | Pyrolysis product at 700 °C | Matching factor | Identified material |
|---|---|---|---|
| 5.49 | Hydrogen chloride | 945 | Poly(vinyl chloride) (PVC) |
| 5.58 | Methyl chloride | 800 | PVC |
| 5.64 | 1-Butene | 938 | PVC |
| 6.01 | 1,3-Pentadiene | 921 | PVC |
| 6.99 | Tetrahydrofuran | 769 | Solvent |
| 7.29 | 1,4-Cyclohexadiene | 923 | PVC |
| 7.48 | Benzene | 945 | PVC |
| 8.20 | Methyl methacrylate | 750 | Poly(methyl methacrylate) (PMMA) |
| 9.78 | Toluene | 904 | PVC |
| 10.29 | 2-Ethyl-1-hexene | 890 | Bis(2-ethylhexyl) phthalate (plasticizer) |
| 10.46 | Cyclopentanone | 912 | Poly(hexamethylene adipamide) (nylon 6-6) |
| 10.53 | 1-Octene | 907 | PVC |
| 14.47 | Styrene | 934 | PVC |
| 17.33 | 2-Ethylhexanal | 856 | Bis(2-ethylhexyl) phthalate (plasticizer) |
| 20.57 | 2-Ethyl-1-hexanol | 916 | Bis(2-ethylhexyl) phthalate (plasticizer) |
| 21.07 | *o*-Methylstyrene | 888 | PVC |
| 21.43 | Indene | 870 | PVC |
| 22.99 | *p-tert*-Butyltoluene | 856 | 2,6-Bis-(1,1-dimethylethyl)-4-methylphenol (BHT) (antioxidant) (?) |
| 25.93 | Naphthalene | 920 | PVC |
| 28.46 | 2-Methylnaphthalene | 862 | PVC |
| 28.63 | Phthalic anhydride | 906 | Bis(2-ethylhexyl) phthalate (plasticizer) |
| 28.79 | 1-Methylnaphthalene | 875 | PVC |
| 36.69 | 3,3-Diphenylacrylonitrile | 937 | Adhesive layer |

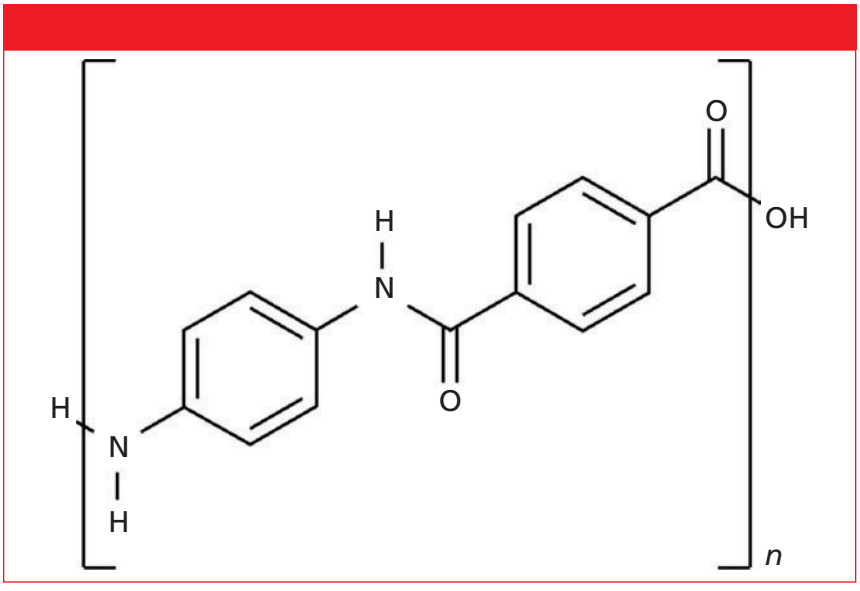


**Figure 5:** Chemical structure of poly(*p*-phenylene terephthalamide) (polyaramid).

column temperature: 60 °C for 1 min, then 60–280 °C at 7 °C/min (hold at 280 °C to the end of analysis); programmed helium pressure: 122.2 kPa for 1 min, then 122.2–212.9 kPa at 7 kPa/min (hold at 212.9 kPa to the end of analysis). Second set of GC conditions: programmed column temperature: 75 °C for 1 min, then 75–280 °C at 7 °C/min (hold at 280 °C to the end of analysis); programmed helium pressure: 122.2 kPa for 1 min, then 122.2–212.9 kPa at 7 kPa/min (hold at 212.9 kPa to the end of analysis).

The temperature of the split–splitless injector was 250 °C and the split ratio was 50:1. The transfer line temperature was 280 °C. The MS EI ion source temperature was kept at 230 °C. The ionization occurred with a kinetic energy of the impacting electrons of 70 eV. The quadrupole temperature was 150 °C. Mass spectra and reconstructed chromatograms (total ion current) were obtained by automatic scanning in the mass range *m/z* 35–750 u. Pyrolysis–GC–MS data were processed with the ChemStation software (Agilent Technologies) and the NIST 05 mass spectra library.

## Results and Discussion

### Pyrolysis–GC–MS of Plastic Particles from Industrial Filter Fins

A sample of plastic particles from industrial filter fins was pyrolyzed at 700 °C to identify its composition. Figure 2 shows the obtained pyrolysis–GC–MS chromatogram of the sample. Based on the decomposition products summarized in Table I, the plastic particles were identified as a mixture of poly(hexamethylene adipamide) (nylon 6-6) and polypropylene glycol with a small amount of styrene–butadiene-rubber (SBR). The peaks of propylene and acetone indicate the presence of polypropylene glycol. The main decomposition product of nylon 6-6 is cyclopentanone (retention time [$t_R$] = 10.51 min). Other peaks in Figure 2, like butene/1,3-butadiene ($t_R$ = 5.58 min), benzene ($t_R$ = 7.43 min), toluene ($t_R$ = 9.72 min), and styrene ($t_R$ = 14.39 min) are typical pyrolysis products of SBR (1,2,5,6,18). The small peak of 4-isopropylphenol ($t_R$ = 28.15 min) may be a clue to the presence of polycarbonate or bisphenol A epoxy resin (5,6). All of the pyrolysis products and the materials

identified from pyrolysis products in filter fins are summarized in Table I.

### Pyrolysis–GC–MS of a Car Wrapping Foil

The next object of identification was a car wrapping foil pyrolyzed at 600 °C. Figure 3 shows the obtained pyrolysis–GC–MS chromatogram of the car wrapping foil. Based on the decomposition products summarized in Table II, the plastic material was identified as a mixture of flexible poly(vinyl chloride) (PVC) with bis(2-ethylhexyl) phthalate (BEHP) plasticizer and poly(hexamethylene adipamide) (nylon 6-6). The chromatogram in Figure 3 shows the typical pyrolysis products of PVC, like hydrogen chloride ($t_R$ = 5.49 min), benzene ($t_R$ = 7.48 min), and naphthalene ($t_R$ = 25.93 min). This is the result of the formation of double bonds by the elimination of hydrogen chloride from the poly(vinyl chloride) macromolecules, followed by the breaking of the carbon chain with or without cyclization reaction (2). The detected cyclopentanone ($t_R$ = 10.46 min) is generally known as characteristic pyrolysis product of nylon 6-6 (2,3,6). Methyl methacrylate ($t_R$ = 8.20 min) identified in pyrolyzate is formed from poly(methyl methacrylate) (6) and most likely comes from the adhesive film. Thus, the identified 3,3-diphenylacrylonitrile ($t_R$ = 36.69 min) may be from the adhesive layer of the foil.

The thermal decomposition of the plasticizer bis(2-ethylhexyl) phthalate identified in car wrapping foil leads to the formation at 600 °C of 2-ethyl-1-hexene ($t_R$ = 10.29 min), 2-ethylhexanal ($t_R$ = 17.33 min), 2-ethyl-1-hexanol ($t_R$ = 20.57 min), and phthalic anhydride ($t_R$ = 28.63 min) (1,7). In the car wrapping material, the rest of the tetrahydrofuran solvent ($t_R$ = 6.99 min) was also detected. Table II shows the identified ingredients of the pyrolyzed car wrapping foil.

### Identification of Unknown Plastic Fibers

A sample of unknown plastic fibers was pyrolyzed at 700 °C and 900 °C, respectively, to identify its composition. Figure 4 shows the pyrolysis–GC–MS chromatogram of the sample pyrolyzed at 900 °C. Based on the decomposition products summarized in Table III, the fibers were identified as polyaramid [poly(*p*-phenylene terephthalamide)] (Figure 5). The main identified degradation products of polyaramid at 900 °C are benzene ($t_R$ = 7.56), aniline ($t_R$ = 11.61 min), and benzonitrile ($t_R$ = 11.81 min). Currently, polyaramid fibers have only been characterized in a few publications using thermal analysis (thermogravimetry, derivative thermogravimetry, and differential thermal analysis), infrared spectroscopy techniques (13–15), and pyrolysis–GC–MS (2,16–19).

Polyaramid fibers are a class of heat-resistant, strong synthetic fibers. They are used in aerospace and military applications for ballistic-rated body armor, fabric, ballistic composites, and fire fighters protective clothing as well as in bicycle tires and as an asbestos substitute.

### Identification of Commercial Light-Curing Dental Filling Material

A number of dental filling materials are presently available for tooth restorations.

**Table III: Pyrolysis products of polyaramid fibers**

| Peak Number | Retention Time $t_R$ (min) | Pyrolysis Product at 900 °C | Matching Factor |
|---|---|---|---|
| 1 | 6.52 | Carbon dioxide | 957 |
| 2 | 6.70 | Acrylonitrile | 896 |
| 3 | 7.56 | Benzene | 968 |
| 4 | 8.50 | Toluene | 918 |
| 5 | 10.20 | Styrene | 924 |
| 6 | 11.30 | Isocyanatobenzene | 947 |
| 7 | 11.61 | Aniline | 959 |
| 8 | 11.81 | Benzonitrile | 967 |
| 9 | 16.98 | 1,2-Benzodinitrile | 959 |
| 10 | 17.02 | 1,4-Benzodiamine | 905 |
| 11 | 19.60 | Biphenyl | 957 |
| 12 | 26.19 | Acridine | 931 |
| 13 | 26.64 | 1,1´-Biphenyl-4-amine | 960 |
| 14 | 28.31 | Carbazole | 928 |
| 15 | 30.01 | *N*-Phenylbenzamide | 916 |

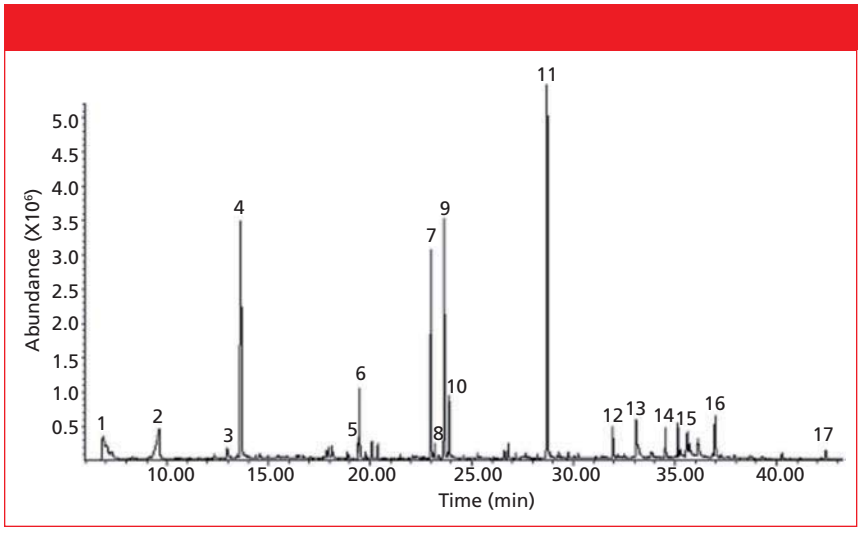


**Figure 6:** Pyrolysis–GC–MS chromatogram of commercial light-curing dental filling material at 550 °C obtained with apparatus 2. Fused-silica GC capillary column: 59 m × 0.25 mm, 0.25-µm $d_f$ DB-5ms. GC conditions: programmed column temperature: 60 °C for 1 min, then 60–280 °C at 7 °C/min (hold at 280 °C to the end of analysis); programmed helium pressure: 122.2 kPa for 1 min, then 122.2–212.9 kPa at 7 kPa/min (hold at 212.9 kPa to the end of analysis). For peak identification, see Table IV.

The four main groups of these materials, which dentists have used for about 35 years, are the conventional glass-ionomer cements, resin-based composites, resin-modified glass-ionomer cements, and polyacid-modified resinous composites (20). Light-curing glass-ionomer cements contain polyacrylic acid, chemically or photo-curing monomers (multifunctional methacrylates, like triethylene glycol dimethacrylate or 2-hydroxyethyl methacrylate), an ion-leaching glass, and additives (initiators, inhibitors, stabilizers, and others) (20). Resin-modified glass-ionomer cements are now widely used in dentistry as direct filling materials, liners, bases, luting cements, and fissure sealants (21). These materials mainly consist of polymer matrix and glass-ionomer parts. The polymer matrix is based on a monomer system and different multifunctional methacrylates with additives (21). Methacrylic monomers, such as bisphenol A glycidyl methacrylate (Bis-GMA), urethane dimethacrylate (UDMA), triethylene glycol dimethacrylate (TEGDMA), and 2-hydroxyethyl methacrylate (HEMA), are the main components of resin-based dental filling materials. The presence of additives such as initiators, activators, inhibitors, and plasticizers in uncured dental material mixture is necessary (21).

Figure 6 shows the total ion current pyrolysis–GC–MS chromatogram of commercial light-curing dental filling material pyrolyzed at 550 °C. The pyrolysis products identified by using mass spectra library NIST 05 are summarized in Table IV. The carbon dioxide ($t_R$ = 6.85 min) identified in pyrolyzate is formed from polyacrylic acid (2,18). The identified substances 2-hydroxyethyl methacrylate (HEMA) ($t_R$ = 13.65 min), ethylene glycol dimethacrylate (EGDMA) ($t_R$ = 19.48 min), and triethylene glycol dimethacrylate (TEDMA) ($t_R$ = 28.72 min) are known as standard composites of dental filling materials (1). Other compounds in Table IV, such as bisphenol A ($t_R$ = 33.10 min) or bisphenol A diglycidyl ether ($t_R$ = 42.42 min), are probably formed by thermal degradation of bisphenol A diglycidyl mono- or dimethacrylates. The presence of the additives, such as the antioxidant 2,6-bis(1,1-dimethylethyl)-4-methylphenol (BHT) ($t_R$ = 23.17 min) or the UV-absorber drometrizol ($t_R$ = 31.95 min) was also confirmed. The triphenylantimony ($t_R$ = 34.55 min) identified in pyrolyzate is used as catalyst in the UV-induced polymerization (1).

## Conclusion

Analytical pyrolysis–GC–MS has been proven as a valuable technique for the analysis and identification of organic polymeric materials in the plastic and rubber industry. For the first time pyrolysis–GC–MS was used for the identification of commercial light-curing dental filling material and for the identification of a car wrapping foil. This technique allows the direct analysis of very small sample amounts (5–200 µg) without the need for time-consuming sample preparation.

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**Table IV: Pyrolysis products of commercial light-curing dental filling material**

| Peak Number | Retention Time $t_R$ (min) | Pyrolysis Product at 550 °C | Matching Factor |
|---|---|---|---|
| 1 | 6.85 | Carbon dioxide | 999 |
| 2 | 9.62 | Methacrylic acid | 936 |
| 3 | 12.96 | Phenol | 909 |
| 4 | 13.65 | 2-Hydroxyethyl methacrylate (HEMA) | 918 |
| 5 | 19.40 | 4-Isopropenylphenol | 928 |
| 6 | 19.48 | Ethylene glycol dimethacrylate (EGDMA) | 915 |
| 7 | 23.00 | Not identified | |
| 8 | 23.17 | 2,6-Bis-(1,1-dimethylethyl)-4-methylphenol (BHT) | 923 |
| 9 | 23.65 | Not identified | |
| 10 | 23.89 | Not identified | |
| 11 | 28.72 | Triethylene glycol dimethacrylate (TEDMA) | 958 |
| 12 | 31.95 | Drometrizol (Tinuvin-P) | 938 |
| 13 | 33.10 | 4,4´-Dihydroxy-2,2-diphenylpropane (bisphenol A) | 924 |
| 14 | 34.55 | Triphenylantimony | 911 |
| 15 | 35.25 | Tetraethylene glycol dimethacrylate | 791 |
| 16 | 36.98 | Not identified | |
| 17 | 42.42 | Bisphenol A diglycidyl ether (BADGE) | 839 |

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*Direct correspondence to: Peter.Kusch@h-brs.de* ■

# PRODUCTS & RESOURCES

## MS system

The ionKey/MS system from Waters is designed for applications such as bioanalyses, pharmacokinetics studies, food safety analyses, and environmental analyses. According to the company, the system is intended for use with the company's Acquity UPLC M-Class system and Xevo TQS mass spectrometer.

**Waters Corporation,**
Milford, MA.
www.waters.com/ionkey

## SEC system

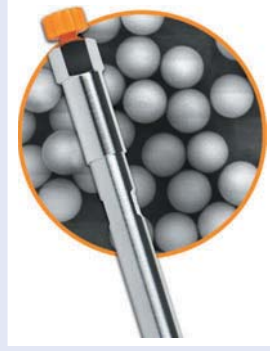
Agilent's 1260 Infinity Multi-Detector Bio SEC Solution system is designed for the analysis of proteins and biotherapeutics. According to the company, the instrument includes a photodiode-array detector and a light-scattering detector, bioinert flowpaths, and intuitive software to determine accurate molecular weight, size, and aggregate presence with high reproducibility.

**Agilent Technologies,**
Santa Clara, CA.
www.agilent.com/chem/infinity-bio-sec

## HPLC columns

Diamond-based Flare HPLC columns from Diamond Analytics are designed to expand the existing range of analytical capabilities in separation. According to the company, the columns are stable at pH 11.3 and 120 °C and can be regenerated for repeat use.

**Diamond Analytics,**
Orem, UT.
www.diamond-analytics.com



## HPLC and UHPLC columns product bulletin

A product bulletin from Advanced Materials Technology contains information about the company's BioClass reversed-phase HPLC and UHPLC columns. According to the company, the eight-page bulletin includes nine figure that illustrate the benefits of the columns. The bulletin also includes product specifications and part numbers.

**Advanced Materials Technology, Inc.,**
Wilmington, DE.
www.advanced-materials-tech.com


## GC autosampler

The Flex Series GC autosampler from EST Analytical is designed for both end users and OEM applications. According to the company, the autosampler has liquid injection capability with an upgrade path to headspace or SPME analysis.

**EST Analytical,**
Fairfield, OH.
www.estanalytical.com

## GC system

The TRACE 1300 gas chromatography system from Thermo Scientific is designed to conserve helium, permit use of multiple detectors simultaneously, automate sampling gas workflows, and perform flame photometric detection. According to the company, new options include a helium-saver module, a high-capacity auxiliary oven, a dedicated flame photometric detector, and a gas sampling valve module. **Thermo Fisher Scientific,** Santa Clara, CA.
www.thermoscientific.com/trace1300

## Thermal desorption autosampler

The 7500 TD autosampler from CDS Analytical is designed as an extension of the company's 7400 purge-and-trap autosampler. According to the company, the autosampler has the ability to change between TD tubes for air and VOA vials for soil and water analysis.

**CDS Analytical,**
Oxford, PA.
www.cdsanalytical.com

## GC valves

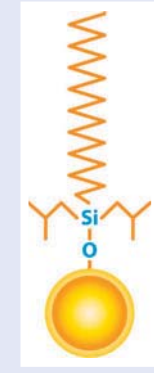
Analytical Flow Products GC valves from Norgren are designed to prevent cross-contamination and excessive wear in challenging applications. According to the company, a purge groove design improves data quality, extends instrument life, increases environmental safety, and decreases downtime.

**Norgren,**
Littleton, CO.
www.norgren.com

## Gas generator

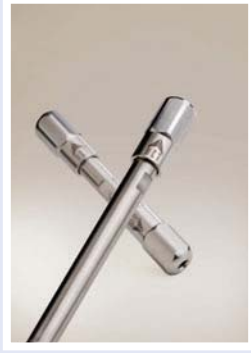
The Corona Nitrogen 1010 gas generator from Peak Scientific is designed to supply pure nitrogen to Thermo Fisher Scientific's Corona Veo charged aerosol detector. According to the company, the generator has been fully tested and approved.
**Peak Scientific,**
Billerica, MA.
www.peakscientific.com

## LC columns




Restek's Raptor ARC-18 superficially porous particle LC columns are designed for use with LC–MS-MS systems. According to the company, the columns can be used in low pH (≤2.0) conditions and provide consistent retention, peak shape, and response for charged bases, neutral acids, and small polar compounds.
**Restek Corporation,**
Bellefonte, PA.
www.restek.com

## HPLC columns

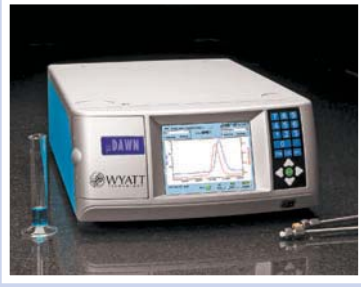


Selectra HPLC columns from UCT include a pentafluorophenylpropyl phase, a polyaromatic phase, and a C18 phase. According to the company, the columns are produced using high purity, type B spherical silica, the material is fully endcapped, and columns are available with either 3-µm or 5-µm particles.
**UCT, LLC,**
Bristol, PA.
www.unitedchem.com

## GPC instrument

Tosoh Bioscience's Eco-SEC high-temperature GPC system is designed to be used at temperatures ranging from 40 °C to 220 °C. According to the company, the dual-pump system includes a dual-flow refractive index detector, a column oven, system temperature control, an autosampler, a sample prep station, and control software.
**Tosoh Bioscience, LLC,** King of Prussia, PA.
www.tosohbioscience.com

## SEC–MALS detector




The mDAWN multiangle light scattering (MALS) detector from Wyatt Technology is designed to be coupled to any UHPLC system to determine absolute molecular weights and sizes of polymers, peptides, and proteins or other biopolymers directly, without using column calibration or reference standards. According to the company, the instrument's flow cell volume is less than 10 mL.
**Wyatt Technology Corp.,** Santa Barbara, CA.
www.wyatt.com

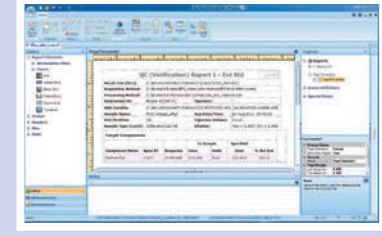
## Photodiode-array detector

Shimadzu's SPD-M30A photodiode-array detector is designed for a variety of HPLC and UHPLC conditions and reportedly can be used for a range of analyses without replacing its capillary cell. According to the company, the detector's capillary cell allows the peak from the principal component and a 0.005% infinitesimal peak to be quantified simultaneously.
**Shimadzu Scientific Instruments,**
Columbia, MD.
www.ssi.shimadzu.com

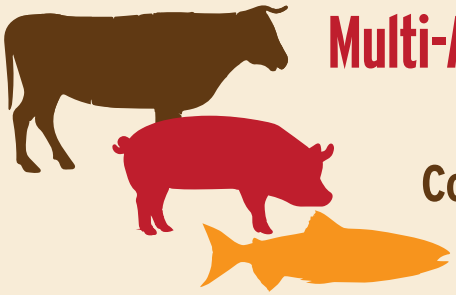
## LC columns

ACE UltraCore solid-core LC columns from Advanced Chromatography Technologies are designed to provide a low column back pressure and are available in SuperC18 and SuperPhenyHexyl bonding. According to the company, both phases feature proprietary encapsulated bonding technology for peak shape and phase stability across a pH range of 1.5 to 11.0.
**Advanced Chromatography Technologies Ltd,**
Aberdeen, Scotland. www.ace-hplc.com

## Report development and generation software



Bruker Dash Reporting software, from Bruker Chemical and Applied Markets is designed to provide customized reporting that centers on Dash Designer, a purpose-built standalone application that allows users to position and closely format report elements, and preview reports with relevant data. According to the company, individual elements can be sorted, filtered, resized, and formatted with common editing operations and advanced functions.
**Bruker Corporation,** Fremont, CA. www.bruker.com



# Multi-Antibiotic Residue Detection in Food:

## An Improved Method for Screening and Confirmation Testing, in Accordance with EU Commission Decision 2002/657/EC

## ON-DEMAND WEBCAST

**Register Free at: www.chromatographyonline.com/detection**

### EVENT OVERVIEW:

In the complex field of testing food for veterinary drug residues, innovative known/unknown screening techniques using high-resolution accurate mass (HRAM) LC-MS offer unique opportunities and provide improved cost efficiency. Compared to conventional screening methods such as inhibitory tests or other biotests, HRAM can cover a large set of targeted substances, including transformation or metabolic products, with high selectivity and sensitivity.

Tune into this web seminar to learn about a newly developed HRAM-LC-MS/MS method for screening, quantitation, and confirmation of more than 100 antibiotic, anthelmintic, and antiparasitic residues in a single run. The method has been fully validated in accordance with 2002/657/EC guidelines and can be applied for analysis of veterinary drug residues in samples of animal origin, such as meat or fish.

### Key Learning Objectives:

- Discover how high-resolution accurate mass spectrometry helps to overcome traditional challenges of MS/MS based methods
- Learn how to improve your lab throughput and save costs by performing screening, quantitation, and confirmation of residues in a single run
- Learn how to improve confidence in your data by eliminating false positives and negatives

For questions, contact Kristen Moore at kmoore@advanstar.com

**PRESENTER**

**Nelli Jochim, PhD**
**Mass Spectrometry Specialist**
**Eurofins WEJ Contaminants GmbH**

**MODERATOR**

**Laura Bush**
**Editorial Director**
**LC/GC**

### Who Should Attend:

- Routine laboratory managers and technical specialists working in residue analysis
- Analytical professionals from regulatory and official control authorities
- Analytical chemists interested in new developments in accurate mass spectrometry

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LC|GC

## Food safety analysis virtual conference

EMD Millipore's Lab Solutions Virtual Conference on Food Safety Analysis reportedly includes three webinars: "ISO Standardization in Food Microbiology and Quality of Media," "The Importance of Water Quality in Food Analyses," and "Monolith Chromolith Columns – an Ideal Tool for the Analysis of Food Samples with Complex 'Dirty' Matrices." Registration for on-demand viewing is available at https://engage.vevent.com/index.jsp?eid=2484&seid=425

**EMD Millipore,** Billerica, MA. www.millipore.com

## Custom HPLC columns

Custom HPLC columns from Hamilton are designed to allow any of the company's stationary phases to be packed into almost any hardware format. According to the company, the HPLC stationary phases are available in varying particle sizes and in most column hardware dimensions.

**Hamilton, Company,**
Reno, NV.
www.hamiltoncompany.com

## Quick-connect system

The Opti-Lynx II system from Optimize Technologies is designed as a combination of quick-connect holders with a selection of packed-bed cartridges. According to the company, accessing and changing the insert takes a quarter turn and the connection is rated up to 6000 psi.

**Optimize Technologies,**
Oregon City, OR.
www.optimizetech.com

## Sorbents

Supelco's Supel QuE Z-Sep, Z-Sep/C18, and Z-Sep+ sorbents are designed to provide robust LC–MS and GC–MS methods for a variety of analytes in difficult matrices. According to the company, the Z-Sep family of sorbents is available in 2-mL and 12-mL tub formats for QuEChERS.

**Supelco/Sigma-Aldrich,**
Bellefonte, PA.
www.sigma-aldrich.com/zsep

## Sample cleanup workstation

The Freestyle workstation from Pickering Laboratories is designed for automated sample cleanup work flow. The instrument is based on a suspended rack design, with an XY robot arm for liquid handling. The workstation reportedly is able to handle multiple flask shapes with volumes ranging from 1 mL to 1 L, and the instrument's software enables users to program multiple sample parameters and to prepare graphical reports and audit logs. SPE, GPC, and evaporation and solvent exchange modules are available.

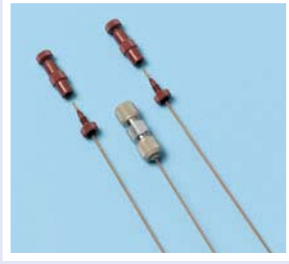
**Pickering Laboratories, Inc.,**
Mountain View, CA. www.pickeringlabs.com

## Hydrogen gas generators

High-pressure hydrogen generators from Parker Hannifin are designed to produce up to 1300 cc/min of 99.99999+% pure hydrogen and eliminate cylinders of hydrogen fuel gases and helium carrier gases. According to the company, a single generator can support up to 20 instruments with fuel and carrier gas.

**Parker Hannifin,**
Haverhill, MA.
www.parker.com/fns/balstonlabgas-generators

## Capillary tubing



Polymicro's flexible fused-silica capillary tubing is designed with an outer diameter of 1/32 in. According to the company, the tubing mates with existing 1/32-in. fittings and is available in a range of internal diameters from 50 µm to 500 µm.

**Polymicro Technologies,**
Phoenix, AZ.
www.polymicro.com

## Peptide mapping columns

AdvanceBio peptide mapping columns from Neta Scientific are designed for resolution and identification of amino acid modifications in primary structure. According to the company, the columns feature a 120-Å pore size with superficially porous 2.7-µm particles.

**Neta Scientific,**
Hainesport, NJ.
www.netascientific.com

# The Use of Solid Core Technology in the Pharmaceutical Environment

## LIVE WEBCAST: Tuesday, March 25, 8:00 am PDT/ 11:00 am EDT/ 3:00 pm GMT

**Register free at www.chromatographyonline.com/core_technology**

### EVENT OVERVIEW:

Since the introduction of solid core technology, there has been great interest within the separation science field to look at ways in which this innovative technology can be employed in a variety of different industries. One such area is the pharmaceutical arena, where there is a broad range of sample types as well as requirements throughout the process of developing new chemical entities. This presentation will look at how the technology of solid core can be readily adapted to cope with the various challenges associated with the pharmaceutical sector, looking at various sample matrices and various molecular entities, from small molecules to large biomolecules.

The presentation will seek to advise users on appropriate column selection, with an insight into how varying the solid core to porous layer can allow the user to optimize separation performance by reducing extra band broadening. Data will also be presented that demonstrates how this technology is inherently more robust than standard fully porous systems when analyzing biological extracts, routinely used in DMPK departments, resulting in longer column lifetimes.

*Presented by*

LC|GC

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Thermo SCIENTIFIC

### Key Learning Objectives:

- How to chose an appropriate solid core column dependent on the application
- How solid core morphology can be optimized for small and large molecule analysis
- How solid core technology can extend column lifetimes

### Who Should Attend:

- Anybody involved in analyzing pharmaceutical compounds

### Presenters

**Dafydd Milton**
Product Manager,
LC & LC-MS Columns
Chromatography
Consumables
Thermo Fisher Scientific

**Tony Edge**
R&D Principal
Chromatography
Consumables
Thermo Fisher Scientific

### Moderator

**Laura Bush**
Editorial Director
*LCGC*

**For questions, contact Kristen Moore at kmoore@advanstar.com**

# AD INDEX

## H P L C INSTRUMENTS & SUPPLIES

THE ESSENTIALS

*Excerpts from LCGC's professional development platform, CHROMacademy.com*

# Mixed-Mode HPLC Separations: What, Why, and How

Although reversed-phase columns (such as C18) are used in a wide range of applications, they often fail to retain highly polar (or charged) analytes, and offer limited selectivity.

Hydrophilic-interaction chromatography (HILIC) is often used to analyze polar analytes, but it encounters challenges such as increased use of organic solvents, poor solubility of analytes in highly organic media, retention affects created by the sample matrix, and a limitation to the extent to which the retention of hydrophobic analytes can be controlled.

Ion-exchange chromatography can be used to retain charged molecules, but it fails to retain neutral analytes and can show poor selectivity for analytes of the same charge.

A current estimate of the usage of these techniques is reversed phase ~60+%; HILIC or normal phase ~18%; ion exchange ~5%.

The use of mixed-mode stationary phases can offer a solution to some of the problems outlined above and their properties and applications can be summarized as follows:

- Mixed-mode chromatography is a method that uses more than one separation mode; mainly reversed-phase combined with ion-exchange interactions, which allows the retention and separation of both polar and nonpolar analytes in a single analysis.
- The biggest benefit of this approach is that selectivity can be optimized by adjusting mobile-phase ionic strength, pH, or organic solvent. As a result, the selectivity can be finely tuned for the separation of compounds with widely different physico-chemical properties. For example, drug molecules and their counterions may be separated in a single analysis.
- Mixed-mode chromatography requires no ion-pairing agents in the mobile phase for separating highly hydrophilic charged analytes, which simplifies the mobile phase and is compatible with mass spectrometry (MS).

A general schematic for the various approaches to producing mixed-mode stationary phases is shown in Figure 1.

The mixed-beads approach in Figure 1 is a blend of two different stationary phases into a single column: reversed phase (in black) and ion exchange (in red). Issues with this approach include concerns regarding the batch-to-batch reproducibility and homogeneity of the resulting high performance liquid chromatography (HPLC) columns.

The mixed-ligand approach to mixed-mode stationary phase production was a second-generation product, but again, ensuring the absolutely reproducibility and homogeneity of the surface is problematic.

The embedded and tipped ligands are a third-generation approach to mixed-mode phases and produce homogeneous and reproducible mixed-mode phases. The position of the ion-exchange group within the bonded phase ligand dictates the relative strength of the hydrophobic and electrostatic interactions and the embedded ligand can be described as an ion-exchange–modified reversed-phase column and the tipped ligand as a reversed-phase–modified ion-exchange column.

The zwitterionic ligand is a fourth-generation approach to mixed-mode separations in which both anionic and cationic functional groups are included in each ligand and the strength and position of the ion-exchange moieties can be altered to affect the selectivity of each ligand towards various analytes.

In mixed-mode separations, the hydrophobic moieties of analyte molecules are retained by interaction with the hydrophobic parts of the bonded ligand, and the charged groups are either retained or repelled by the ion-exchange groups on the stationary phase surface. There are several ways to control retention and selectivity with mixed-mode phases, as one might imagine.

The amount of organic modifier can be altered to affect changes in retention and selectivity of the separation, primarily by changing the hydrophobicity of the eluent, as would be typical for a reversed-phase separation.

Eluent pH can also be used to alter selectivity and retention, and the pH will affect not only the degree of ionization of the analyte molecule, but also the extent of the surface charge if weak anion- or cation-exchange ligands are used. The ionic strength of the eluent can be used to alter the separation characteristics, and ionic-strength gradients are often used to increase the elution strength of the eluent with respect to the electrostatic interactions between analytes and the stationary phase surface.

The nature of mixed-mode stationary phases offer several possibilities to "tune" the selectivity of a separation and as such offer an important tool in the analytical armory for anyone dealing with highly polar analytes or analytes whose physico-chemical properties differ widely.

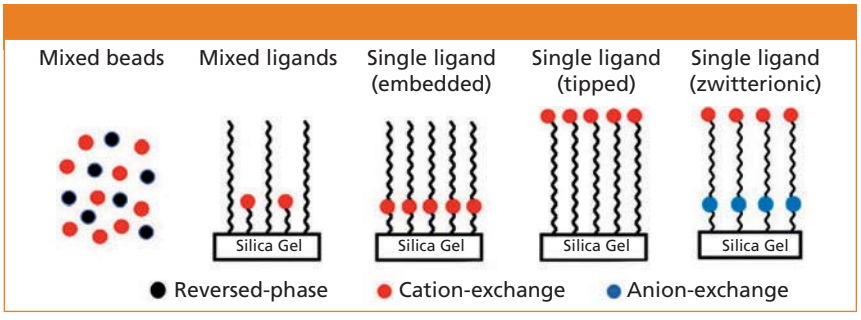


**Figure 1:** Schematic representation of the various approaches to producing mixed-mode stationary phases.

**More Online:**

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