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A review of practical applications

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Cover:
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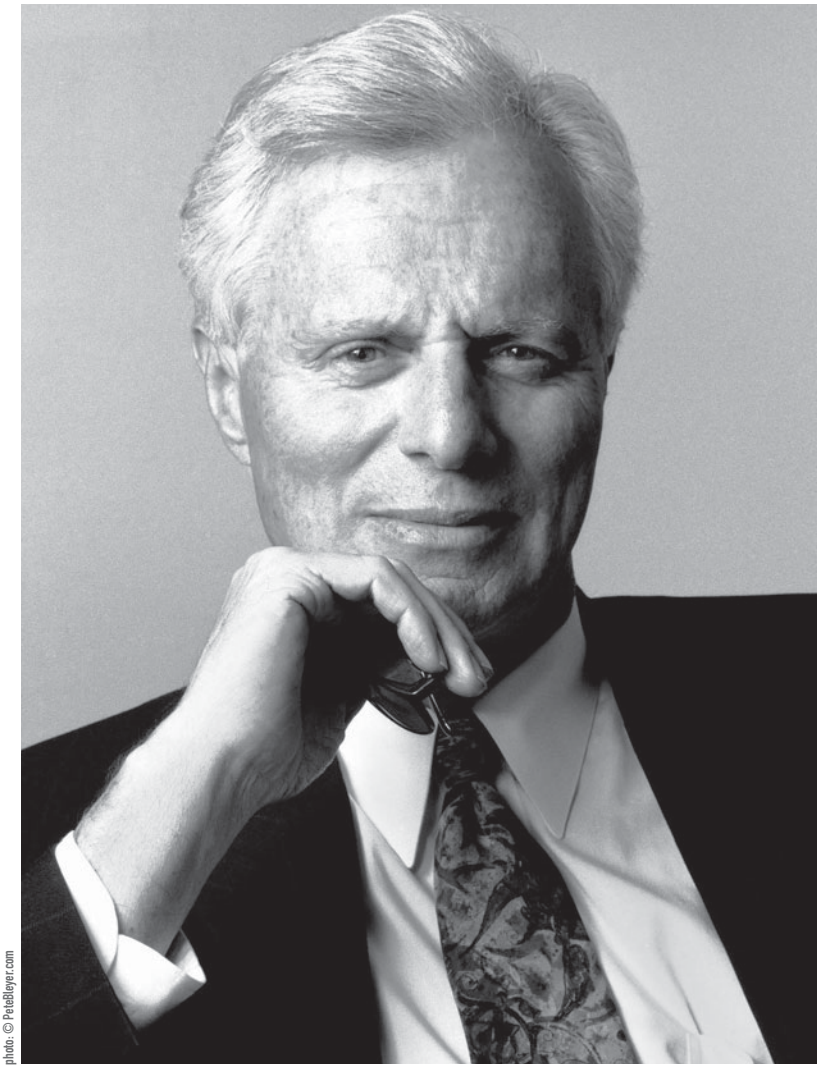

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Practical Applications of **Asymmetrical Flow Field-Flow Fractionation (AF4): A Review**

Mats Leeman[1], Matilda Ulmius Storm[1], and Lars Nilsson[2] [1]SOLVE Research and Consultancy AB, Lund, Sweden, [2]Lund Center for Field-Flow Fractionation, Department of Food Technology, Engineering and Nutrition, Lund University, Sweden.

Characterization of macromolecules and colloids is an area of considerable interest. Asymmetrical flow field-flow fractionation (AF4) has become a well-established method, but many potential users possess limited knowledge of its capabilities, or how it can provide additional information or serve as validation to the traditional analytical techniques. This review article highlights several practical applications where AF4 should be given special consideration, and discusses benefits and drawbacks of the different methods.

It is almost 50 years since the late Professor Giddings (1) invented field-flow fractionation (FFF) — a family of separation techniques. A number of different sub-techniques exist including centrifugal FFF (CFFF), thermal FFF (TFFF), and electrical FFF (EFFF), each with its own appropriate application area. However, the most widely spread and commonly used sub-technique is asymmetrical flow field-flow fractionation (AF4) (see Table 1). From the first reports of AF4 in the 1980s (2) the technique has evolved to become a well-established method for size separation of macromolecules and particles in academia. The technique has also found its way into the industry, largely because of the availability of commercial systems in the latter half of the 1990s.

However, many potential users have still not heard about AF4 or refrain from using it because they have little experience with the technique. With the advent of improved instrumentation and an ever-increasing number of articles to help to select separation parameters, the relevance of AF4 is being re-evaluated. In this review article we highlight applications where AF4 is especially well suited in comparison to other common techniques.

KEY POINTS

- AF4 can be a valuable tool when analyzing complex samples, which might be difficult with other techniques. For example large- and shear-sensitive macromolecules or compounds that interact with stationary phases.
- Five case studies: protein aggregation, complex nanoparticle size distribution, large polysaccharides, liposomes, and branched polymers are discussed.
- AF4 should be considered as a complement to other commonly used techniques because it can give additional or complementary information or serve as validation.

Detection of Protein Aggregates

Studies of protein aggregation are common in both academia and industry. Protein aggregation can be of fundamental interest to understand the characteristics of a protein, but is also important to assess during product development and optimization, or to study the stability of the finished formulation. Size-exclusion chromatography (SEC) is a well-proven and excellent method for size separation of macromolecules, and is often used for size characterization of proteins including aggregation studies. However, it has been reported that SEC has limitations

Table 1: About AF4: More detailed descriptions on how asymmetrical flow field-flow fractionation (AF4) works can be found in other *LCGC* articles (3,4) and in several books on the subject (5,6). In short, AF4 is a size separation technique for macromolecules, aggregates, particles, and other colloids that are dispersed in a solvent. The separation takes place in an open channel (no stationary phase) in which one wall consists of a semi-permeable ultra-filtration membrane. The open channel provides a low surface area, low shear forces, and the ability to separate very large-sized components. In combination with the on-line coupling of light scattering detection AF4 give information on molar mass and size on the separated fractions directly without calibration standards. The parameters depending on detection are listed below.

Detection	Parameters
AF4	Hydrodynamic size distribution
AF4–MALS–dRI	As above + molar mass and r.m.s. radius distribution*, conformation, apparent density
AF4–DLS	Hydrodynamic size distribution (from both AF4 retention time and DLS)
SEC	Size relative standards
SEC–MALS–dRI	Molar mass and r.m.s. radius distribution*, conformation
DLS	Hydrodynamic size distribution

*For molecules larger than ~20 nm

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Figure 1: Separation of heat stressed IgG by (a) AF4–UV–MALS–dRI and (b) SEC–UV–MALS–dRI.

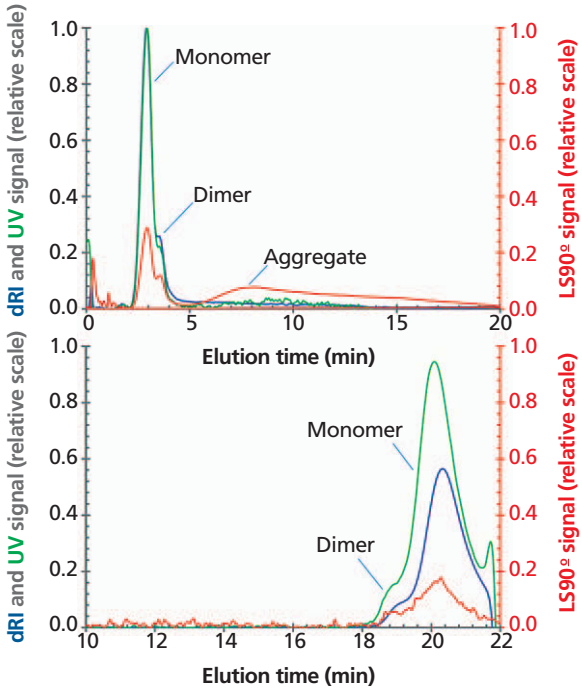
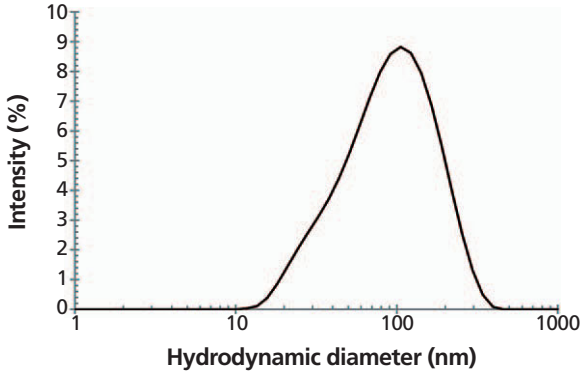


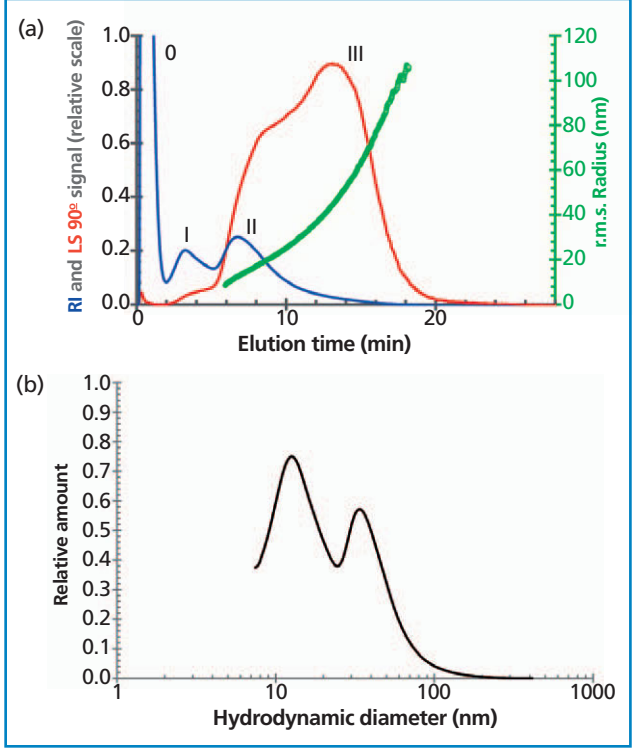
Figure 2: Size distribution of nanoparticles using DLS.



for the protein aggregates, indicating that the method is not always well suited for the separation and detection of higher aggregates (7,8,9), which can result in low recovery (10).

An example of differences that can be noticed in the results from SEC and AF4 analyses of an aggregated protein is presented in Figure 1. The sample is a solution of IgG (molecular weight ~150 kDa) that is well-known to aggregate at elevated temperatures. For this illustration the solution was heated to ~65 °C for 5 min to promote the formation of aggregates. Elution of the protein monomer and dimer from AF4 was detected on both the differential refractive index (dRI) and multi-angle light scattering (MALS) detectors. The light scattering detector, which is very sensitive to high molar mass compounds, also picked up the elution of much higher aggregates (not detected in the IgG solution before heat-stress). The amount of these

Figure 3: Size characterization of nanoparticles using (a) AF4 and (b) particle hydrodynamic diameter distribution obtained from the AF4 elution times, for comparison with Figure 2. (a) The blue trace is the dRI and red trace is the MALS 90° fractograms. Right axis is the root-mean-square (r.m.s.) radius of the eluted fractions (green circles) determined directly by MALS. Different size populations are indicated by I, II, and III. The early dRI peak (marked with 0) shows the elution of salts in the sample solution.



aggregates is low compared to the monomer and dimer, representing less than 10% of the total protein. In contrast to the AF4 results, no higher aggregates were detected in the analysis by SEC. This is presumably a result of either shear degradation of the higher aggregates or that the aggregates do not elute from the column.

The SEC column had a nominal pore-size of 1000 Å, a nominal protein molecular weight separation range of 50–7500 kDa, and was operated at the relatively low flow rate of 0.5 mL/min (7.8 mm i.d. column). Hence, the protein and potential aggregates are expected to be well within column specifications. Nevertheless, the higher protein aggregates were not eluted from the column. Optimization of elution conditions such as the mobile phase ionic strength and other additives (10 mM sodium nitrate in water was used) and testing of a variety of columns with different pore-sizes and chemistries could improve the situation. However, it is not certain that one would go through such an optimization procedure unless there is an indication that the current SEC method may fail to detect the aggregates. Great care should therefore be taken when estimating protein aggregation based on data from one method alone; the use of a complementary technique can be valuable for validation reasons.

Size Distribution of Nanoparticles

Nanoparticles are becoming more and more closely studied. One property of high interest is the particle

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Figure 4: AF4–MALS fractogram showing molar mass (M, obtained from MALS–RI) and r.m.s. radius vs. elution time for maize amylopectin obtained from dissolution in water at 140 °C (autoclave). Adapted and reproduced with permission from *Analytical and Bioanalytical Chemistry* **407**(15), D. Perez-Rea *et al.*, *Development and evaluation of methods for starch dissolution using asymmetrical flow field-flow fractionation. Part I: Dissolution of amylopectin*, 4315–4326 (2015) © Springer.

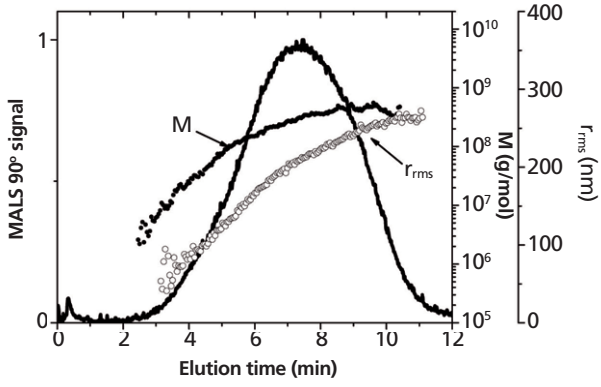
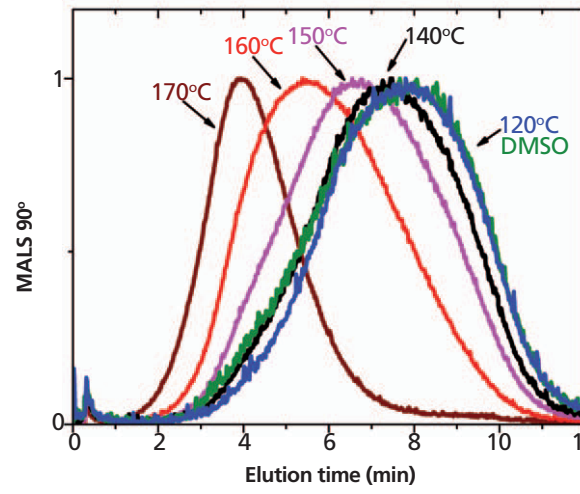


Figure 5: The effect of dissolution conditions (120–170°C in water in an autoclave and in DMSO at 100 °C) on the elution time in AF4–MALS of maize amylopectin. AF4 analyses were performed under aqueous conditions. Adapted and reproduced with permission from *Analytical and Bioanalytical Chemistry* **407**(15), D. Perez-Rea *et al.*, *Development and evaluation of methods for starch dissolution using asymmetrical flow field-flow fractionation. Part I: Dissolution of amylopectin*, 4315–4326 (2015) © Springer.



size, which is commonly analyzed with dynamic light scattering (DLS). This technique measures the fluctuations in scattered light from a sample (such as a particle dispersion) over time and provides information on the hydrodynamic diameter. DLS has several benefits such as compatibility with most solvents and is a relatively fast technique with low workload for the user. However, in some cases, it is difficult to obtain reliable size distribution data (11) on complex samples such as particles with multi-modal (12) or broad size distributions (13), or when measuring small particles at low concentrations. This can be explained by the fact that an inherent property of DLS is that the measurements are performed on unfractionated samples, making the data processing and evaluation more complicated for samples with broad size distributions. For small particles, the limitation lies in the relatively low scattering intensity (the intensity of scattered light increases with particle size by the power of 6), which becomes especially challenging if the concentration is low.

The following example is from a recent project in which we participated where DLS was used to characterize the size of nanoparticles. Our DLS measurement gave an average hydrodynamic size of 73 nm and a polydispersity index of 0.25, which was in excellent agreement with the data given by the supplier; 72 nm and 0.25, respectively (also determined by DLS). Looking at the intensity graph from the DLS (Figure 2) it is obvious that the size distribution is broad (ranging from 10–400 nm) but there was otherwise no indication of anything remarkable. Possibly there is an indication of a shoulder on the size distribution curve at approximately 20–30 nm.

In contrast, AF4 analysis of the same sample gave a more detailed picture of the size distribution (Figure 3). The use of MALS-dRI detection gave molar mass and r.m.s. radius (a measure of [a molecules or particles] size weighted by the mass distribution around its centre of mass) information directly of the eluted fractions (independent of the separation and no standards necessary). The analysis detected three size populations on the refractive index and light scattering detectors. The first population (I) eluted from 2–5 min, the second (II) from 5–10 min, and the last (III) was visible mainly in the MALS-signal from 11–18 min. The size populations were incompletely resolved, partly as a result of band broadening, but a major factor is likely overlapping size distributions. This particle was produced in a two-step process, with a solid core that was coated. The core had a size of 5–10 nm as determined with transmission electron microscopy (TEM) prior to coating (likely population I in Figure 3). The second size population (II) probably corresponds to successfully coated particles and the third size population (III) to aggregated particles.

DLS is a rapid tool, excellent for fast screenings of well defined, low dispersity samples. However, this data illustrates the difficulties involved in detecting different size populations in a complex sample using DLS and is one example when it could be suitable to characterize the sample with AF4. The use of a separation technique also offers the potential for an additional dimension because the size fractions can be collected and analyzed by other techniques to further characterize the sample.

Molar Mass and Size Determination of Large Polysaccharides

Polysaccharides are a class of compounds that are used in a wide variety of applications. Some of the polysaccharides, such as starch and cellulose, can be very large and are often used for rheological modification of products. However, the large sizes can also result in challenging size distribution analysis (14,15). Often only average properties are reported, or indirect measurements of the size such as viscosity. However, with AF4 it is possible to analyze very large polysaccharides (16,17,18,19)

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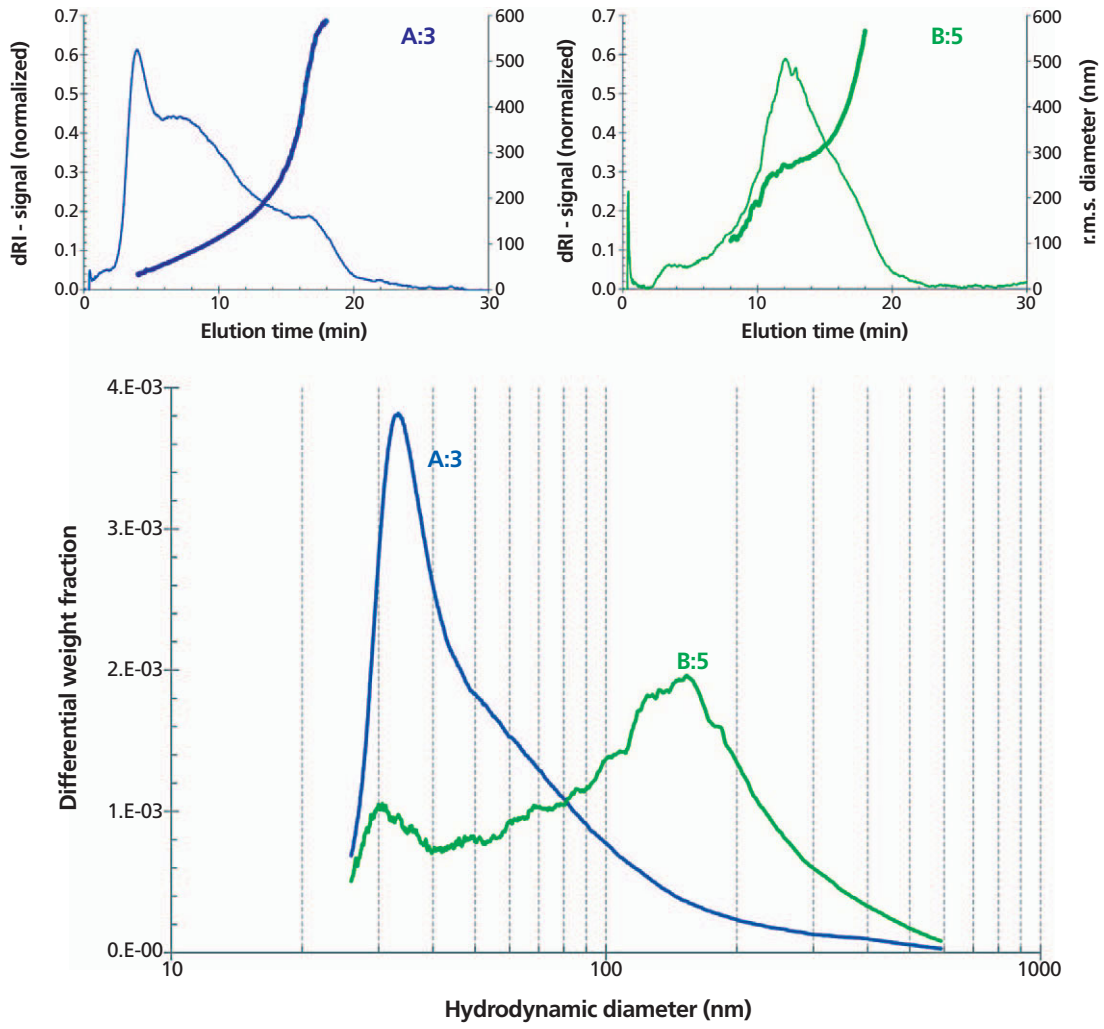
Figure 6: AF4 analysis of liposomes prepared by procedure A (top left graph) and B (top right graph), respectively, taken at different points in the respective procedure. Also plotted is the hydrodynamic diameter distribution of the liposomes (lower graph, log scale, obtained from the AF4 elution times).



as well as supramolecular aggregates (20,21,22). Size analysis can be important because the molar mass distribution can be affected during functionalization or by processing conditions (23,24,25,26,27,28).

The analysis of amylopectin (one of the main constituents of starch) by AF4 is presented in Figure 4. Amylopectin is a branched polysaccharide of very large size and molar mass. Molar mass ranges from approximately ten million up to several hundred millions g/mol and r.m.s. radius ranges up to ~250 nm corresponding to a hydrodynamic diameter of 400–500 nm, which emphasizes the large size range over which AF4 can operate.

In this case AF4 was used to monitor dissolution conditions. Amylopectin is not easily dissolved in water, and the dissolution conditions can have a pronounced effect on the properties of the final amylopectin solution. In this example the dissolution temperature and its impact on the size distribution was investigated and compared to dissolution in dimethyl sulphoxide (DMSO) (Figure 5). The AF4 analysis clearly revealed the impact of high temperature dissolution conditions on the amylopectin (degradation) and provided guidance on how to choose the amylopectin processing conditions for the particular application.

Using SEC for such large compounds as in the example above would definitely be challenging. The size of the amylopectin would likely be excluded from the pores of the stationary phase. Furthermore, even if the pores are sufficiently large enough to allow the amylopectin to enter, the flow rate has to be kept very low to allow the sample time to diffuse in and out of the pores. In addition, shear degradation of amylopectin readily occurs as has been shown by Cave *et al.* (29).

Size Analysis of Vesicles/Liposomes

Liposomes as carriers for pharmaceutically active compounds have attracted both academic and commercial interest for several decades. One parameter that really matters in drug applications of liposomes is the size distribution because it has a considerable impact on the clearance of the liposomes from the blood stream (30,31,32).

AF4 is a technique very well suited for liposome characterization (33,34,35,36,37,38). The open channel reduces the risk of shear-induced changes to the sample and has a very low surface area (typically in the order of 10^{-3}–10^{-2} m^2) compared to packed columns (at least 1–10 m^2/g stationary phase). An example of an AF4 analysis of liposomes is presented in Figure 6. The liposome size distribution was investigated after different processing steps for different liposome preparation procedures.

The AF4 separation demonstrated good recovery (98%) and was able to separate the relatively disperse systems, with liposome distributions spanning from ~25 to ~500 nm depending on production protocol. The use of a separation method also allowed different liposome size fractions to be collected after the AF4, and analyzed for their compositions by, for example, liquid chromatography–mass spectrometry (LC–MS).

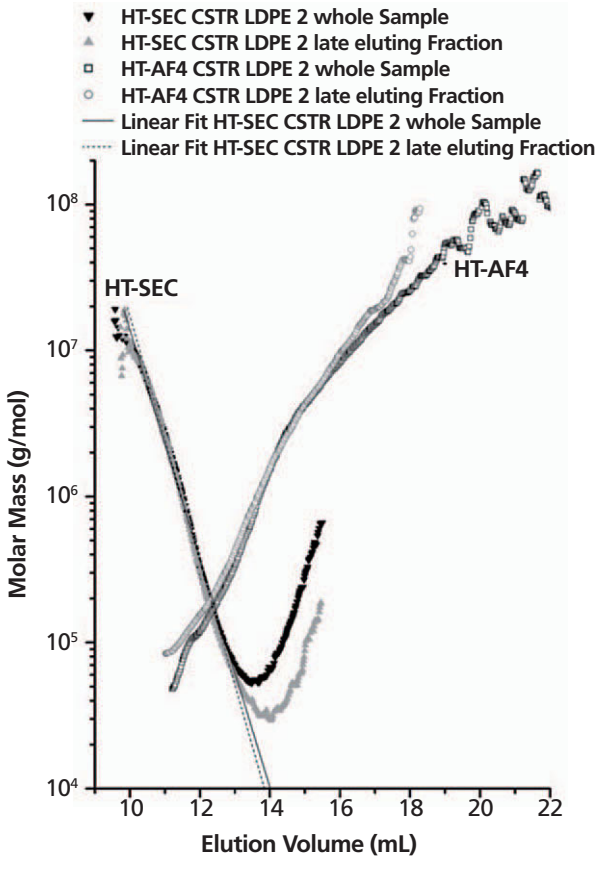
The most commonly used techniques to analyze size and size distribution of liposomes are DLS and SEC (39). DLS suffers from the same limitations as was mentioned in the nanoparticle example above (difficulty to obtain accurate size distribution data for broad or complex size distribution), and this has also been noted for liposomes (35). SEC differs from DLS in that it give a physical separation of the differently sized liposomes, enabling direct measurement of the size distribution (40,41,42,43). However, lipid adsorption to the SEC column is often reported (41,43). To counter this, it is sometimes recommended to saturate (41) the column by several injections of lipid before the actual separation can be performed. Obviously, saturating the stationary phase prior to separation might affect column performance and retention properties. Therefore, using AF4 in such cases might be a better option.

Separation of Branched Polymers

Branched polymers are a widely used class of macromolecules. The amount of branching as well as the molar mass is of considerable interest since it can have a profound affect on the properties of the polymer in the application. Both parameters can be investigated by SEC when connected to viscosity and light scattering detectors. However, it is not uncommon to see reports with anomalies in the elution behaviour when analyzing branched polymers (44,45,46,47,48,49,50,51), making the evaluation challenging or even impossible. Several investigations point at the difficulties of separating branched high molar mass polymers. One suggested explanation has been anchoring of the branches in the SEC stationary phase pores (47).

For polymers of this complex character, AF4 is an attractive alternative. One illustrative example of using AF4 for separation of branched polymers can be seen in Figure 7 (including a comparison with SEC). The absence of a packing material in AF4 significantly reduces the risk for shear-induced degradation or branches interacting with a stationary phase.

Figure 7: Analysis of polyethylene by AF4 and SEC showing molar mass as a function of elution volume. Note the inversion of the elution order from SEC at 14–16 mL elution volume range. Adapted and reproduced with permission from *Journal of Chromatography A* **1218**(27), D. T. Otte, T. Klein, R. Brüll, T. Macko, and H. Pasch, *Study of the abnormal late co-elution phenomenon of low density polyethylene in size exclusion chromatography using high temperature size exclusion chromatography and high temperature asymmetrical flow field-flow fractionation*, 4240–4248 (2011) © Elsevier.



When Not to Use AFFF — and What Not to Expect

Compounds smaller in size than the cut-off of the membrane used are not possible to analyze. The most commonly used membranes have a nominal molecular weight cut-off of 5 kDa, or 10 kDa, which gives an indication of the practical lower limit of AF4. SEC can be expected to exhibit better efficiency or fractionating power than field-flow fractionation (FFF) for low-molecular-weight compounds (~ 10^4 g/mol range and less) (52,53,54).

If the amount of sample is very limited, the use of AF4 might not be possible. The amount injected (in terms of mass) is comparable to what is used in SEC, usually 10–100 µg depending on detection. However, one benefit of AF4 is that if the sample concentration is low, larger volumes can be analyzed (thus reaching the required detection) without affecting resolution thanks to the initial focusing step when the analytes are relaxed in the channel.

The length of an AF4 separation varies depending on sample complexity. In general, total AF4 analysis time (injection, focusing, and elution) is in the 15–60 min range. Add to this the time for data evaluation, which can be very fast for a simple yes/no answer or take considerably more time if data from multiple detectors are to be evaluated and sample characteristics in terms of conformation/branching/density are desired. Therefore, answers are not obtained as fast as with DLS; however, AF4 offers considerably shorter run times compared to analytical ultracentrifugation.

While AF4 does separate according to size, which in turn is related to mass, do not expect mass spectrometry-like data with isotope resolution. The resolution is simply not of that magnitude.

Conclusion

We have offered some examples where AF4 should be considered in comparison to other commonly used techniques, either because it can provide additional or complementary information or can serve as validation of other analytical techniques. Speaking in general terms, AF4 should be considered when the analytical task involves:

- Aggregation-prone samples or when investigating aggregation and aggregate properties;
- Broad or complex size distributions;
- Large macromolecules;
- Shear sensitive samples;
- Compounds that are difficult to analyze because of interactions with stationary phases.

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Detective Work, Part 2: Physical Problems with the Column

John W. Dolan, LC Troubleshooting Editor.

Particulate matter from the sample or the system can cause havoc in the chromatogram.

In last month's "LC Troubleshooting" (1) we looked at the major causes and symptoms associated with failure of liquid chromatography (LC) columns. These are summarized in Table 1. The most common problems associated with physical failure of the column are blockage and void formation, which are expressed as symptoms of increased pressure and peak tailing, as well as a reduction in the column plate number. We examine these problems in the present discussion. Problems that are primarily associated with chemical changes in the column are usually caused by adsorption of sample components on the column surface or chemical attack on the column stationary phase. These commonly show up as changes in peak spacing and retention time. Chemical changes will be the subject of next month's discussion.

Look for Changes

Perhaps the most common mode of column failure is indicated by an increase in column back pressure. Many times this increase is accompanied by increased peak tailing. Obviously, these symptoms are noticeable only when compared to the normal performance of the system.

Factors that determine the column back pressure include the column itself — length, diameter, and column packing particle size — as well as the mobile-phase composition, temperature, and flow rate. These are the primary contributions for traditional LC systems. With ultrahigh-pressure LC (UHPLC), the system plumbing can also contribute significantly to the back pressure because pumping 0.5–1 mL/min of mobile phase through 0.0625-mm i.d. tubing generates a significant amount of friction compared to the 0.125–0.175 mm i.d. tubing used in conventional LC systems. Normal values of system back pressure will vary between methods, so it is a good idea to note the normal pressure for each method. System suitability acceptance parameters often do not include a value for pressure, but pressure is easy to note when the method is set up for each batch of samples. Experience will tell you what is normal for each method with a new column. Over the lifetime of a column, the pressure may increase by 25% or more, and for most columns, pressure itself is not detrimental to normal performance. An exception is for some UHPLC methods where significant increases in pressure can cause changes in peak spacing (2,3).

Most sample peaks exhibit some tailing; it is rare that all peaks exhibit the symmetric Gaussian shape of an ideal chromatographic peak. Usually peak tailing is tracked as part of the system suitability test by measuring the tailing factor, *TF*, or the asymmetry factor, A_s, as illustrated in Figure 1. *TF* and A_s give approximately the same tailing values for $TF \approx A_s < 2$. *TF* is the standard measurement in the pharmaceutical industry; A_s is often used in other applications. As mentioned above, most chromatographic peaks tail a bit, and $TF \approx A_s \leq 1.5$ is usually acceptable and allows good peak quantification. When $TF \approx A_s > 2$, corrective action is recommended for most methods.

What Does a Pressure Increase Mean?

It is normal for the column back pressure to rise over the lifetime of the column. Real samples that originate from a biological source, the environment, or a manufactured product often contain nonsoluble particulate matter. Depending on the extent of sample pretreatment, some of these particles can be injected and collect on the frit at the inlet of the column. Components subject to wear within the LC system itself, such as pump seals and injection valve seals, can also wear and shed particulate debris over time. As these particles collect on the column inlet frit, the column back pressure will gradually rise. The tolerance for increased pressure will depend on the method, but an increase of 25%, for example, from 200 bar to 250 bar, is usually tolerable as long as it does not exceed the upper pressure limit of the system (generally 400 bar for a conventional LC, 600–1000 bar or more for UHPLC). If the tolerated rise happens over the normal lifetime of the column (500 to >2000 injections is common), there is no need for corrective action. If the pressure rise is faster, such as over a single batch of samples or <500 injections, you should be able to increase the useful lifetime of the column by reducing the particulate load of the sample. Be sure to use reference conditions for a new column under normal operation, or you may find yourself performing unnecessary maintenance. Under gradient operation with water (or buffer) as the A-solvent and methanol as the B-solvent, different mixtures of water and methanol will change the pressure during a run. For example, relative to 100% water (pressure = 100), the mid-gradient pressure will rise by ~50% (~150 relative) and drop to half (~50 relative) at 100% methanol.

The simplest way to reduce the amount of particulate matter that reaches the column inlet frit is to add an in-line filter before the column. Such filters are inexpensive (relative to the cost of a column) and are available from most chromatography supplies vendors. The

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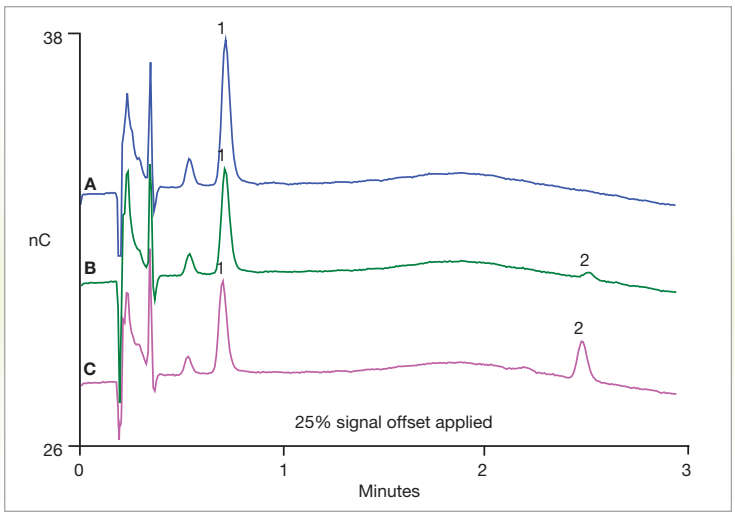

Peaks:	A	B	C
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2. Neu5Gc	-	0.17	0.93

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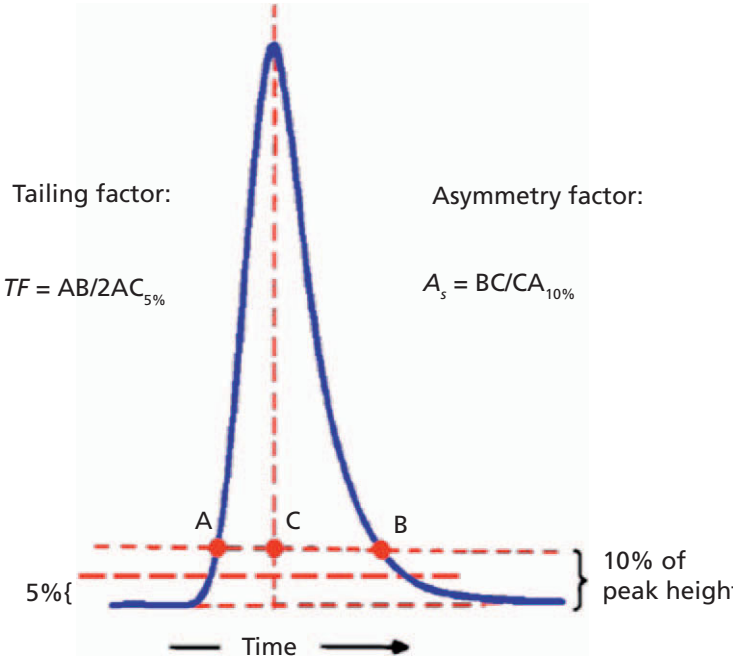
Table 1: Diagnosing column problems.

Symptom	Cause				
	Pressure	Tailing	Plates	Selectivity	Retention
Blockage	XX	XX	X		
Voids		XX	XX		
Adsorbed sample			X	X	XX
Chemical attack		X	X	XX	X

X – commonly observed correlation

XX – strongly correlated

Figure 1: Illustration of the calculation of the tailing factor and asymmetry factor for a chromatographic peak.



filters with user-replaceable frits are the most economic choice. Select an 0.5-µm porosity filter if you use a column with ≥3-µm-diameter particles; for smaller particles, an 0.2-µm porosity frit is recommended. These recommended frits have porosity equal to or smaller than the frit at the column inlet, so they will trap particles that would otherwise block the column. You can monitor the column pressure during method equilibration and decide if the observed pressure indicates that it is time to change the frit. Alternatively, if the pressure rise over time is predictable, you may schedule replacement of the in-line filter on a calendar or injection-count basis.

An alternative to the in-line filter is to use a guard column, or precolumn, upstream from the column. A guard column will stop particulate matter from the sample or system and provide some chemical protection of the column, as well. The downside of a guard column is that it can cost one-fourth (or more) of the price of a new column. If you opt to use a guard column, I suggest using an in-line filter upstream of the guard column to keep it from becoming blocked prematurely.

If the pressure rise over time is more rapid than is convenient to mitigate by regular in-line frit replacement, additional sample pretreatment may be necessary. You may find that filtration of the sample through a membrane filter (0.5- or 0.2-µm porosity) may be sufficient. Many times, centrifugation of the samples in a benchtop centrifuge provides similar particle removal. The use of solid-phase extraction (SPE) or liquid–liquid extraction (LLE) will remove additional sample contaminants, but it may not be appropriate if you need to analyze for impurities or degradants in the sample.

If particulate matter has accumulated on the frit at the inlet of the column, reverse-flushing the column can be a successful technique to remove particles, but don't put too much faith in this procedure, because it is only successful approximately one-third of the time. To reverse-flush the column, simply reverse the column direction and pump 10–20 column volumes of mobile phase (15–30 mL for a 150 mm × 4.6 mm column) through the column directly to waste. The column can then be left in the reverse direction or returned to normal flow. Before you attempt reverse-flushing, check to be sure that your column will tolerate reverse flow. Most columns that contain 5-µm-diameter particles can be reverse-flushed; other column configurations may or may not be appropriate for reverse-flushing, depending on the frit configuration. Check with the column manufacturer if you are not sure if reverse-flushing is allowed. If you find that reverse-flushing is necessary, I advise that you then modify the system to add an in-line filter to stop the particles before they get to the column or guard column frit.

What About Peak Tailing?

As most columns age, peak tailing will increase. In this discussion, we'll be concerned only about the case in which peak tailing increases for all peaks in the chromatogram. (If tailing increases for only one or a few peaks in the chromatogram, it is an indication of a change in column or mobile phase chemistry, and will not be discussed here.) If all of the peaks tail, front, split, or otherwise show the same shape defect, the problem occurred before any separation took place. Examples of tailing or misshapen peaks can be seen at the top of Figure 2.

The most common cause of tailing of all the peaks is partial blockage of the frit at the inlet of the column. At the lower left of Figure 2 is a cartoon of the cross-section at the top of the column. The particles are held within the column by a frit at the inlet (and one at the outlet, not shown). The injected sample is represented by the arrows, each of which contains a mixture of the unseparated sample components. Under normal operation, these sample streams (arrows) all arrive at the head of the column at the same time, then the separation begins. Under these circumstances, all sample molecules start the separation process at the same time, so each sample band is homogeneous. The cartoon at the

bottom right of Figure 2 is a representation of the same column inlet, but with a frit that is partially blocked by some sample debris. Now most of the sample streams reach the column at the same time and proceed as normal. However, some of the sample has to divert around the blockage, which causes it to arrive later than the other streams. This stream starts late, and the sample molecules all lag behind their normal counterparts. Because this delay occurs before any separation takes place, all peaks suffer the same defect. Another way to think of this delay is in an analogy to a 100-m dash in a track meet. If all the runners have the same capability and all the starting blocks are lined up properly, all the competitors will reach the finish at the same time — this situation is analogous to the column inlet under normal circumstances. If, on the other hand, three of eight lanes have the starting blocks shifted back 5 m, those athletes will run the same speed as their competitors, but will lag behind by 5 m, creating a "tail" on the grouping of runners — just as the sample is slightly delayed at the column inlet with a partially blocked frit. Because the nature of the disturbance in the flow at the frit may vary, peak defects may vary as well, and can include peak fronting or tailing, double peaks, or peak splitting with a similar appearance for all peaks in the chromatogram.

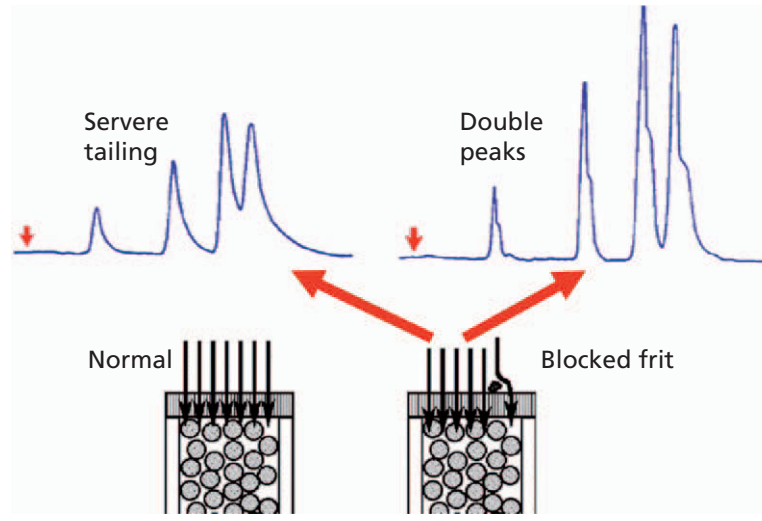
A partially blocked frit may or may not be accompanied by an increase in back pressure, depending on the magnitude of the blockage. The only way to correct a partially blocked frit is to reverse-flush the column, as was described earlier. Before the use of current column packing techniques, it was possible to remove the frit at the head of the column and replace it with a new one, but frit removal is very likely to ruin modern columns, so it is not recommended. If a partially blocked frit is suspected, it is best to prevent this blockage by using an in-line filter or a guard column to keep particulate matter from reaching the column. You should should evaluate the appropriateness of improving sample pretreatment.

Other Observations

Another problem that can occur at the inlet of the column is the creation of a void or other disturbance in the column packing bed. This problem may be caused by chemical attack on the particles or severe mechanical abuse; in the past when columns weren't packed as well, settling of the packing material was another cause of fronting. Column voids because of chemical attack will be discussed in a future instalment of "LC Troubleshooting". Historically, some workers filled in a column void with used packing material, but this approach is not a viable technique with modern columns. Void formation cannot be corrected, and the column should be replaced.

If you look at the symptoms and causes in Table 1, you'll see that a reduction in the column plate number, *N*, is also seen when physical problems of blockage or column voids are observed. Any defect in peak shape will increase its width, thus lowering the plate number. Because plate number reduction is symptomatic of all modes of column failure, it is not a discriminating diagnostic tool. However, if the plate number is tracked as part of system suitability, it can alert you to column problems in general so you can look more carefully for a specific problem.

Figure 2: Examples of tailing or misshapen peaks and their probable cause. See text for details.



Summary

We've considered the most common cause of increased column back pressure and peak tailing for all peaks in the chromatogram: the accumulation of particulate matter on the column inlet frit. The source of the particles is most likely the sample, although deterioration of pump seals and injection valve rotors can also generate particulate matter. If the column is not protected otherwise, the particles collect on the column inlet frit. Sometimes the problem can be corrected by reverse-flushing the column, but reverse-flushing is often not successful and may not be allowed for some column configurations. A better approach is to replace the column and then take measures to avoid recurrence of the problem. These measures may be as simple as installing an inline filter to remove particles before they reach the column. Guard columns are another option. Changes in sample pretreatment can eliminate the problem entirely. Centrifugation of the samples may be sufficient pretreatment in many cases; in others, SPE, LLE, or other sample preparation steps may be necessary. In next month's "LC Troubleshooting" we'll consider problems associated with chemical changes to the column.

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Future Needs of **HPLC and UHPLC Column Technology**

Ronald E. Majors, Column Watch Editor.

In his final "Column Watch" instalment, Ron Majors looks into his crystal ball and discusses future needs in the areas of high performance liquid chromatography (HPLC) and ultrahigh-pressure liquid chromatography (UHPLC) column technology and related instrumentation. He looks at where current technology may be heading and makes a prediction that monolith-based columns may still have a rightful place in the HPLC and UHPLC laboratory. This article concludes Majors' legendary tenure as a monthly columnist for *LCGC*.

Next month, high performance liquid chromatography (HPLC) will celebrate its Golden Jubilee — 50 years of solving separation and analysis problems in just about every realm of science. Since the beginning, there have been major advances in the technology from the separation columns to the instrumentation to the data analysis and reporting. Large-particle packings of 50-µm diameter have given way to microparticles with diameters smaller than 2 µm, HPLC systems have gone from constant gas pressure pumps operating at 1000 psi to pumps capable of 20,000 psi pressure, detectors have progressed from simple 254-nm UV detectors to several hundred thousand dollar high-resolution mass spectrometers, and data output has moved from strip-chart recorders to high-speed computers with the ability to handle complex chromatograms. Constant improvements have been made along the way and should continue into the future. Bear in mind that chromatographers are, for the most part, rather conservative individuals who are not necessarily adopting improvements as soon as they are shown in the research laboratory. In fact, I have observed that it takes nearly a decade for a new column technology to become commonplace in the chromatography laboratory, not just because of conservative chemists but also partly the result of requirements for regulated methods, instrumentation keeping up with the column technology, and the time it requires to take column manufacturers to make the investment to transfer the technology from the research laboratory to the manufacturing floor.

Where Are HPLC and UHPLC Columns Heading?

For nearly 33 years in *LC* and *LCGC* magazine, I have been reporting on developments in HPLC column technology. I would now like to look into my crystal ball and attempt to focus on areas where further improvements in column technology may be needed. Continued investment in column technology is bound to continue since the HPLC and ultrahigh-pressure liquid chromatography (UHPLC) columns market has been very strong; otherwise there wouldn't be around 200 companies involved in some aspect of the business. Columns are considered a consumable. An average instrument uses seven columns per year (1) so as the number of instruments grows, so does the columns market. The overall market for columns (analytical, preparative, capillary–nano, packings, and column accessories) is now estimated to be just over a billion dollars with an overall growth of 3.5%, but a higher growth in the UHPLC segment (2).

As pointed out earlier, tremendous strides have been made in particle and stationary-phase technology over the years. However, user demands in industry for productivity and sensitivity improvements continue to push further development in columns that are more efficient, faster, and more inert. These needs stretch from the research laboratory and through all phases of development up to manufacturing and quality control.

Recently, the 2.6–2.7 µm superficially porous particle (SPP) columns have established themselves as the favoured column type for new method development compared to the sub-2-µm totally porous particle (TPP) columns. The SPP columns provide lower pressures, higher or equivalent efficiency, and nearly the same loadability (3). In last year's Pittcon report (4), SPP column introductions

A Special Thank You

This column marks the last instalment of Ron Majors' authorship of "Column Watch". The editors of *LCGC* wish to thank Ron for all of his contributions to the magazine and special supplements over the past three-plus decades. We invite our readers to check out the November supplement in tribute to Ron's vast legacy at *LCGC* titled "The Best of Column Watch and Sample Prep Perspectives: A Farewell to Ron Majors" (www.chromatographyonline.com/Ron-Majors-Tribute). Thank you, Ron!

Looking for a reliable and cost effective method to do large-volume liquid extractions?

SOLID-PHASE EXTRACTION (SPE) is a technique that is used to isolate and concentrate compounds dissolved or suspended in liquid mixtures based on their physical and chemical properties. Analytical laboratories use SPE to concentrate and purify samples for analysis. SPE can be used to isolate analytes of interest from a wide variety of matrices, including urine, blood, beverage, and water samples. Depending on the amount of sample mixture to process, SPE can be separated into large or small volume techniques.

Current analytical methods that may require SPE preparation include GC, GC-MS, LC, and LC-MS, and cover the following sample types:

- Pesticides (OCPs, OPPs, diquats, and urea ionic pesticides)
- Pollutants (phenols, PCBs, nitrosamines, and dioxins)
- Personal care products (pharmaceuticals, steroids, and endocrine disruptors)
- Total petroleum hydrocarbons (DRO)
- Explosive residues
- Beverages and flavor components

AUTOMATION OF LIQUID EXTRACTIONS provides a lower cost of analysis by reducing the amount of analyst time spent on the extraction. More than 50% of the sample preparation cost for a typical vacuum manifold extraction is from operator labor. Automated SPE delivers unattended operation and increased reproducibility, providing a significant reduction in the cost of analysis. Figure 1 shows a comparison of savings on solvent and labor between using the Thermo Scientific™ Dionex™ AutoTrace™ 280 Solid-Phase Extraction instrument, vacuum box cartridges SPE, and vacuum box disk SPE. It is clear that the automated technique saves a much higher percentage of the sample cost, thereby increasing the profitability of the laboratory.

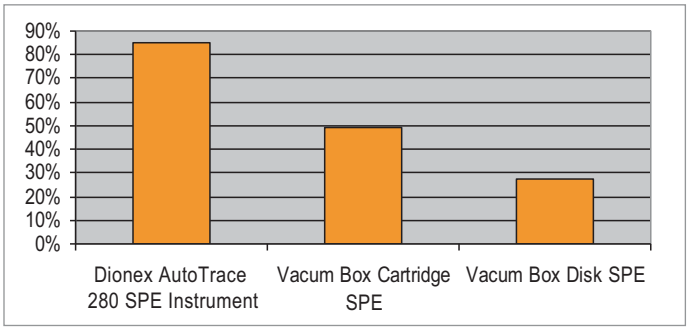


Figure 1: Percent savings comparing automated solvent extraction and liquid-liquid techniques such as a separation funnel

The Dionex AutoTrace 280 SPE instrument is a powerful, high-throughput solution dedicated specifically for automating SPE. It automates cartridge or disk conditioning, sample loading, rinsing, and eluting steps using a closed and vented, positive pressure system for large-volume aqueous solution extractions (20 mL to 20 L).

THE DIONEX AUTOTRACE 280 SPE INSTRUMENT processes up to six samples in parallel, with a choice of up to five reagents, while segregating aqueous and solvent waste lines for efficient, and cost effective waste handling. This instrument is designed to dramatically reduce solvent consumption and sample handling, while allowing automation across a wide range of methods approved by regulatory agencies.

In addition to the time and solvent savings, automation also increases percent of recovery and reproducibility. These increases are attributed to automated control, and consistent flow of sample, SPE solvents and reagents.

Table 1: Positive pressure constant flow greatly improves analytical precision

Pesticide Recovery Study N=6	Dionex AutoTrace 280 SPE Instrument		Vacuum manifold SPE	
Compound	Recovery %	RSD	Recovery %	RSD
Atrazine	88	1.8	54	12.2
Propazine	91	1.5	80	7.3
Alachlor	99	3.4	96	4.1
Metachlor	99	4.3	96	2.9

Automation of SPE reduces solvent usage, eliminates excess glassware, and saves labor by automating the manual processes of maintaining a reservoir of liquid and controlling the flow of organic solvents through the SPE cartridge. Providing a constant flow of liquids through the SPE cartridges decreases the analytical costs and increases productivity from unattended operation.

Visit thermoscientific.com/autotrace to learn how you can automate your SPE analysis and save your laboratory time, solvent, and labor.

The Dionex AutoTrace 280 SPE instrument

Thermo Scientific™ Dionex™ SolEx™ SPE Cartridges are designed for fast, easy pre-concentration of contaminants prior to analysis. The Dionex SolEx SPE cartridges are offered in a variety of chemistries and sizes to fit your needs.

Thermo SCIENTIFIC

Figure 1: Comparison of pore structure and diffusion paths of SPPs prepared by different processes: Particles made by (a) the multilayer coacervation method and (b) the PMT method. (Courtesy of Bill Barber, Agilent Technologies).

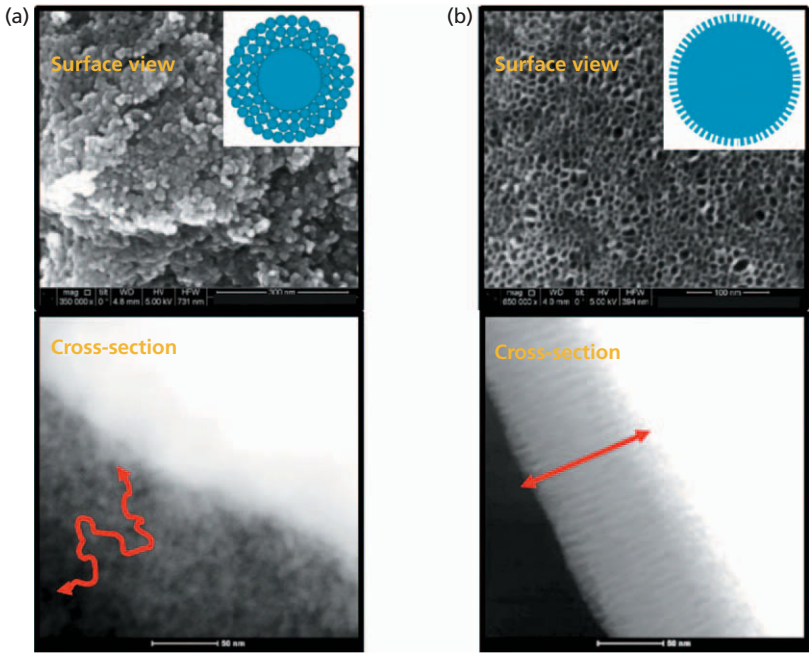
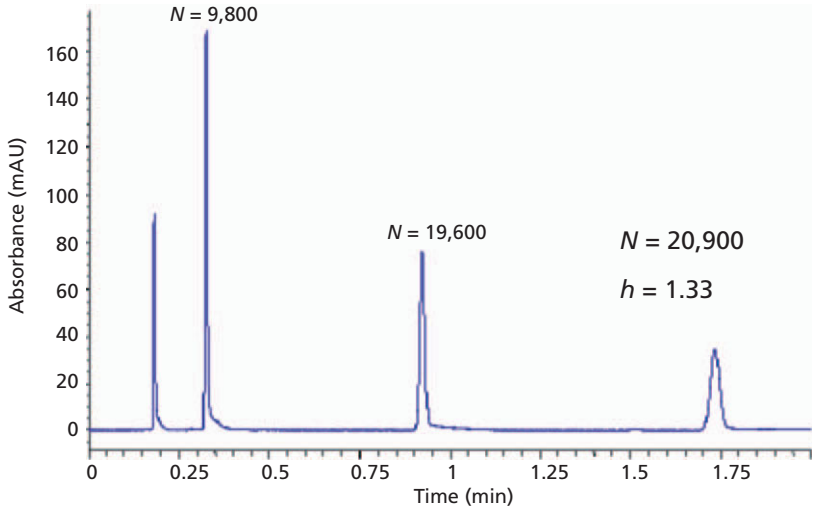


Figure 2: Chromatographic results obtained using a 1.8-µm PMT SPP column. Column dimensions: 50 mm × 4.6 mm; mobile phase: 55:45 (v/v) acetonitrile–water; flow rate: 2.0 mL/min. (Courtesy of Bill Barber, Agilent Technologies).



outnumbered TPP introductions 10:1. It has been shown that methods developed on older porous particle columns can be switched to these newer column types with minor adjustments. Regulatory bodies are already blessing these new column types. The new breed of SPP columns may actually break the 10-year cycle in adoption delay!

Over the next few years, expectations are that column manufacturers will continue to exploit this technology by filling out their SPP offerings with stationary phases that chromatographers apply to their everyday separations as well as for specialized separations, such as chiral compounds and biological pharmaceuticals. Sub-2-µm SPPs are already available and more are envisioned in the future. Larger particle SPPs in the 4–5 µm range are displacing established methods based on the popular 5-µm TPP columns. Wide-pore SPP columns have been developed for the efficient separations of large biomolecules.

Next month, high performance liquid chromatography (HPLC) will celebrate its Golden Jubilee — 50 years of solving separation and analysis problems in just about every realm of science.

Even with all of the current improvements in column particle technology, work is continuing on further improvements in SPPs. Current techniques for making SPPs are based on a multilayer process or a coacervation process (5), and most commercial SPPs use one of these two processes. There are alternatives being investigated that result in improved TPP and SPP materials by allowing the formation of more-uniform mesopores compared to present packings. One pore-formation process is termed *micelle-templating*; in this process, mesoporous silicas are synthesized in the presence of cationic or nonionic surfactants to form highly uniform pores (6,7). A second process for making superficially porous micelle-templated particles is by *direct synthesis* (8,9). A third process termed *micelle-templating with pseudomorphic transformation* (PMT) has been used to make SPPs that also have a uniform porous structure (10). Without getting into the details of the actual patented process, Figure 1 provides the resultant difference in structures between current SPPs and one micelle-templated SPP produced using the PMT process. As you can see from this figure, the uniform pores are normal to the surface, contain a thinner shell with a high surface area, and result in a smooth surface with a uniform particle size. As can be seen in Figure 2, a 1.8-µm PMT SPP column shows excellent efficiency, roughly a 50% increase compared to TPPs of the same particle size, column dimensions, and chromatographic conditions.

Table 1: Common methods to compare column types.

Resolution equation (does not include pressure)
Van Deemter equation (does not include pressure)
Knox equation (includes pressure, time, and efficiency)
Separation impedance (includes pressure and efficiency)
Poppe plot (includes time, efficiency, and pressure)
Kinetic plot (includes pressure, efficiency,th1215 and time)
Note: The first five methods are applicable to isocratic cases only, but kinetic plots extend to gradient cases.

Further investigations into TPPs are continuing. With all the discussion about improvements in SPP column performance based on the narrow particle size distribution of SPPs and how it affects the van Deemter *A* term, Supelco chemists looked back at sub-2-µm TPPs with equally narrow particle size distributions and noted an increase in chromatography efficiency by this change alone (11). Despite all the advances in SPP technology, there are still many more TPP stationary phases covering the particle size range from 1.5 µm to 10 µm so users presently have a wider choice of columns to use for analytical to preparative scaleup.

Don't Count Monolithic Columns Out Just Yet

Monolithic columns have been around for a couple of decades. They have the advantages of being a single particle in a column with large macropores through which solvent can easily flow and with mesopores where the stationary phase can interact with the solutes coming through the column. The macropores give very low pressure drops and most of the commercial monoliths can be used with conventional 400 bar HPLC systems. Silica monoliths were introduced in 1998 and commercialized in 2000. Because of intellectual property concerns, they were mainly commercialized by one company and thus there was little incentive for other manufacturers to work on improving the technology. Now that patent protection is going away, there may be more interest in pursuing this technology. These monolithic columns, both silica- and polymer-based, still have great promise if researchers can figure out how to improve the efficiency without great increases in back pressure, make them in longer lengths needed for difficult separations, and contain them in a suitable housing to withstand the high pressures for very long columns. Silica-based monolith columns have low pressure drops, even lower than SPP columns with the same column efficiency. The first generation of silica monoliths had the efficiency of a 3.5–4 µm silica. The second generation has the efficiency equivalent to 2.0–2.3 µm silica albeit at a higher pressure drop (>2× higher for the same chromatographic conditions) because of the change in the macropore–mesopore domain ratio. For many years, only bare silica and C8 and C18 bonded phases were available, but now a few more stationary phases have become commercially available. Capillary, analytical (2.0- and 4.6-mm i.d.), and preparative 25-mm i.d. columns are available.

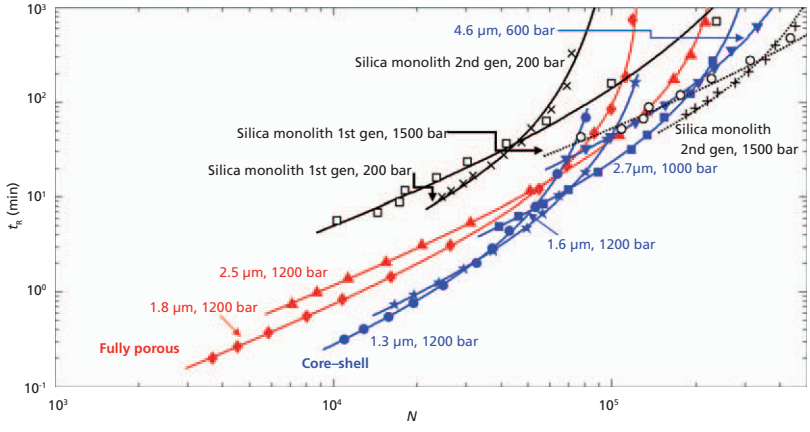
Polymeric monoliths, which are less covered by patent issues, could be quite attractive since their wider pH operating range gives them some advantages compared to silica monoliths, but the efficiencies of research polymeric columns still don't live up to the commercial silica monoliths. A significant advance in polymeric monoliths has been the ongoing research work in their application to smaller molecules. In the past, polymeric monoliths were considered to be suitable for large biomolecules only. Novel approaches to make polymeric monoliths more appealing haven't resulted in commercial products that can be used by practicing chromatographers. Monoliths may become the favoured approach for laboratory-on-a-chip systems since they can be synthesized *in situ* inside the narrow channels where efficient packing of particles has proven exceedingly difficult.

When comparing the performance of various types of HPLC and UHPLC columns (see later section), if monolith columns can be improved to fulfill the three criteria mentioned above, in the long run, they may prove to be the best approach for difficult separations needing many theoretical plates but with longer analysis times. The containment issue with silica monoliths will be a continuing challenge as long as the silica rods have to be made outside of a high-pressure column enclosure. Perhaps a return to the old radially compressed column concept would be one way to provide higher pressure and longer monolith columns.

Instrumentation Improvements Needed to Support Further Column Development

Instruments have been trying to keep up with column developments. Obviously, the life cycle for instrument development is much longer than what is required for

Figure 3: Comparison of kinetic performance (residence time of analyte eluted at 10 times t_0 versus maximally achievable plate number at the given permissible pressure) for a series of state-of-the-art fully porous particles (red data), core–shell particles (blue data), and silica monolith columns (black data) used in isocratic reversed-phase LC analysis of small molecules using acetonitrile–water mobile phases. The dashed black data lines represent the hypothetical performance of the silica monoliths if they could withstand a pressure of 1500 bar. (Adapted with permission from references 14 and 15.)



new packings and columns. An area where it is well recognized as a hindrance to exploiting further improvements in column efficiency is the instrument contribution to band dispersion attributed to the HPLC and UHPLC instruments and their column-instrument interface designs. There is no doubt in my mind that instruments will see further improvement in lowering band dispersion to handle smaller SPPs. What is needed is a closer integration of the column hardware and instrument connections such that dead volumes may be almost nil, much like what has been achieved in some nano and chip instruments. The area of frit and endfitting design needs attention since the column packing where the separation actually takes place should be located at or near the injector device and the detector measurement device. This may necessitate a new column design that not only cuts down on this extracolumn volume, but also can withstand the higher pressures anticipated with smaller SPPs. Such designs are within the capabilities of engineers at the instrument companies but may necessitate the development of proprietary interfaces that may rule out the ability of the end user to select a column of their choice. Getting universal agreement among the instrument companies on a standard zero-dead volume interface would probably be next to impossible given the competitive environment that currently exists within the community. Perhaps some sort of cassette system without the typical compression endfittings, similar to what has been used in some commercial chip-based column configurations, might be used advantageously for closer coupling and easy, rapid column replacement.

As far as instrument pressure capability, UHPLC systems can be built to go to even higher pressures since pumps capable of thousands of bar (tens of thousands of psi) are already available for industrial use and chromatography engineers would have to adapt some of the same operating principles to achieve pulseless flow control in the microlitre to millilitre per minute range at pressures up to 100,000 psi. If SPP columns continue to dominate in the future, there may not be a need to greatly exceed today's pressure limits. However, in chromatography pressure is always a useful commodity.

Miniaturization of Columns and Instruments

Small internal diameter columns in the capillary area (0.1–0.3 mm i.d). and nano area (less than 0.1-mm i.d.) are readily available, but their long-term column stability relative to larger bore analytical columns has been questioned and column efficiency is not as good as typical large-bore analytical columns. A lot of these problems have to do with the lack of adequate packing techniques for small internal diameter columns, in general. More attention should be paid to this aspect of column technology.

Bear in mind that chromatographers are, for the most part, rather conservative individuals who are not necessarily adopting improvements as soon as they are shown in the research laboratory.

Further miniaturization of standard UHPLC instrumentation is possible. Microfluidics has already proven to be an alternative approach to perform analytical separations. Such downsizing of the LC experiment would certainly require a major redesign in the column and instrumentation. The use of miniaturized instruments would result in significant solvent, bench-space, and sample savings, and with mass spectrometry (MS) would allow even better interfacing. Chip-based LC systems have been investigated extensively, and a limited number of instruments have already been introduced to the market. However, the adoption rate for commercial instruments has been rather slow and, compared to regular analytical columns, in microfluidics column efficiencies are not as high as expected. Nevertheless, the significant advantages provided by miniaturized LC systems may spur further commercial development beyond the academic environment. One of the bright spots in column technology adaptable to microfluidics-based systems is the potential for the synthesis of *in situ* stationary phases via monolith formation. The packing of microparticles within narrow channels is difficult, and one of the

reasons for low column efficiency in microfluidics-based column systems.

How to Compare HPLC Column Performances

How does one know if a particular column is the best that can be chosen? Table 1 shows the variety of common methods used to compare the performance of HPLC and UHPLC columns. Some of them are well known, but the ones that provide the best comparison take into account efficiency, analysis time, and pressure drop — three of the main chromatographic parameters that users are interested in for their analysis. The Poppe plot favoured for a number of years has been refined and is now supplemented by the kinetic plot. Developed by Professor Gert Desmet and colleagues at the Free University of Brussels in Belgium (12), the kinetic plot can be constructed by converting van Deemter plots to those based on a t_R versus N plot, where t_R is solute residence time (here defined at 10 times the column void time, t_0), and N is the number of theoretical plates. The kinetic plot can compare the performance of different length columns and different stationary phases and compare the analysis times needed to achieve a certain level of efficiency at a maximum instrument pressure. For example, with the kinetic plot one can look at practical constraints on column length and particle size to choose an optimum configuration for the needed efficiency or analysis time.

Desmet and his colleagues Cabooter and Broeckhoven have done an actual experimental comparison of three types of popular columns using the simplified kinetic plots shown in the busy Figure 3 (13) based on data from references 14 and 15. The figure shows the following column types: fully porous particles (red), core–shell SPP (blue), and silica monoliths, first and second generation (black). In addition, they added hypothetical silica monoliths (first and second generation) that would be capable of withstanding a pressure of 1500 bar (black data in the upper right). Note that current silica monoliths are operational at pressures as high as 200 bar because of the polyether ether ketone (PEEK) housing that encompasses the silica rod.

The lower left side of the kinetic plot is where one would focus to find the best column for a simple separation requiring a few thousand theoretical plates and an analysis time of a few minutes at most. The upper right hand side of the plot would be best if the user has a very complex sample and needs a lot of theoretical plates and has time on their hands to realize these large number of plates. According to the plot in Figure 3, in the simple case, the 1.3-µm core–shell SPP run at a pressure of 1200 bar would win out because it provides the greatest number of plates in the shortest time. However, a TPP of 1.8-µm diameter operating at 1200 bar would give the SPP a run for its money timewise, but only provide a fraction of the theoretical plates for the separation. The present silica monoliths shown in black in the top middle of the plot do not provide very good kinetic performance since they are limited to a pressure of

200 bar. However, as shown by the black lines in the upper right of the plot, because of their lower pressure drops and good efficiency, if a new generation (hypothetical right now) of silica monolith could be developed to stand up to high pressure (1500 bar) and be able to provide a high number of theoretical plates by an increased column length, they would win out for complex, multicomponent separations and would have a rightful place in the chromatography laboratory. Thus, the work is cut out for the monolith proponents to come up with that new phase and an appropriate column hardware.

Other Areas of Needed Improvements in Column Technology

Biochromatography Columns: As biopharmaceuticals such as monoclonal antibodies and peptide-based compounds continue to make inroads in the drug market, columns capable of providing high recovery separations of biologically-derived compounds, oligonucleotides, and biosimilars, both neat and in biological fluids, will be in big demand. Column manufacturers are already responding with biocompatible columns that provide more selective separations with higher recovery. Oligonucleotide purity requires columns that separate a wide range of oligomers, sometimes at high pH, so chromatographers in that field are always on the lookout for high efficiency, high-pH-tolerant columns. Biochromatographers will need to have columns that cover most of the HPLC modes including reversed-phase, ion-exchange, aqueous size exclusion, affinity, normal-phase, hydrophilic interaction, and hydrophobic interaction chromatography. The main requirement, of course, is that the pore size of biochromatography packings must be large enough to accommodate the largest biomolecules encountered.

Column Specifications and Features: Columns that are more inert and provide symmetrical peak shape will always be in demand. In the past couple of decades, stationary phases have come a long way and there are seldom complaints heard about nonreproducibility. Column packing is still considered by many to be a black art. Many laboratories have tried to study optimized packing conditions, but as particle sizes and particle chemistry change and column diameters become smaller, column performance has not been a linear transition. To realize equivalent performance with conventional analytical, narrow-bore, capillary, and nano columns, a more systematic study on column packing requirements will be needed.

Continued investment in column technology is bound to continue since the HPLC and ultrahigh-pressure liquid chromatography (UHPLC) columns market has been very strong.

Approaches to increase and predict chromatographic resolution with improved stationary phases that show better control of selectivity for critical separations will be needed in the future. Small changes in selectivity provide the biggest changes in overall resolution — much bigger than particle size effects alone, which affect only column efficiency. Decreases in particle size only give moderate increases in resolution (that is, $R \approx N^{1/2}$) . Most of the work in the past 25 years has been focused on improving efficiency, with many stationary phases based on commercially available silane reagents.

Although column lifetimes, even at higher pH values, are much longer nowadays than in yesteryear, many users (especially in the pharmaceutical environment) consider columns expendable. When dealing with precious, high-activity, high-value pharmaceuticals, compound purity and accuracy of analysis are of the utmost importance and a column that has been used for thousands of injections may have some degree of contamination that may affect retention and peak shape as well as compound purity, which is not worth risking in quantitative analysis. Some laboratories actually take perfectly good columns out of service that have reached 1000 analytical injections. Similar procedures are used for preparative columns that cost much more than analytical columns because of the increased amount of packing.

Supercritical Fluid Chromatography Columns: With its orthogonal separation power, supercritical fluid chromatography (SFC) has made a comeback in the rapid analysis of small pharmaceutical compounds. Initially, SFC made its contributions in the preparative arena for chiral drugs, but now it has been applied to more general small molecule applications. For some separations, SFC can be superior to HPLC and UHPLC, especially in the speed of analysis. The phases used for SFC (for example, ethyl pyridines, pyridyl amide, and DEAP) are different to those used for LC, so additional polar phases are required to exploit this technology. Further systematic studies on new phases by SFC column suppliers would add to the knowledge of reliably selecting the best stationary phase for a given separation (16).

Columns for Two-Dimensional LC: In the research community, two-dimensional (2D) LC has been gaining momentum when extremely difficult separations are encountered or when every compound in a complex sample needs to be separated (LC×LC). Here, truly orthogonal stationary phases are desired; so phases that are specifically designed for multidimensional separations could be on the horizon. Fast columns in the second dimension will be in popular demand and specialty phases based on SPP or monolithic technology may be needed to fill the gap. The 2D technique has not been accepted yet for routine pharmaceutical assays, but the day may come when more complete characterizations required by regulatory bodies may require this degree of separation power. Major instrument companies are already assembling multidimensional instruments to respond to this potential marketplace.

Stationary-Phase Chemistries: Reversed-phase chromatography has dominated HPLC column usage for the past four decades. Undoubtedly there are enough reversed-phase

chromatography columns around to meet the needs of the entire chromatography community. Yet each year, tens of new reversed-phase chromatography columns are introduced because the market seems to be big enough to absorb some of these columns. Other modes do not get as much attention, but hydrophilic interaction chromatography (HILIC) has been growing from year to year. Many chromatographers continue to use bare silica for HILIC, even though there are a number of other HILIC phases available that provide unique selectivities. The mechanism for HILIC separations has become better understood. However, only a few new types of HILIC stationary phases have recently become commercially available to take advantage of this increased understanding. Since the HILIC mode in becoming more popular for polar analytes, unretained or slightly retained by reversed-phase chromatography, the market for HILIC columns has been growing. Likely, more specialized HILIC phases will be forthcoming. As more HILIC phases are introduced, a systematic study on how to choose the best HILIC column for the job at hand would be a welcome addition to the LC community.

The ion exchange–ion chromatography columns area has become a polymer only market. At Pittcon over the last several years, new silica-based ion-exchange columns have become almost nonexistent. The silica-based columns cannot stand up to the rigorous conditions used by ion-exchange separations, such as high ionic strength mobile phases, high pH, and high temperatures. Only a single ion-exchange polymeric monolith has been introduced in the last 5 years. It is anticipated that as monoliths become more established, ion-exchange chromatography could benefit from low-pressure-drop, high-efficiency monolith columns.

In the size-exclusion chromatography (SEC) area, particle sizes have seen a decrease giving rise to higher speed separations, but SEC is a technique where a sufficiently large stationary-phase volume is required to provide a large molecule weight operating range so making smaller internal diameter columns is probably not the answer. Column packings with noninteractive surface chemistries are always in demand, and in the organic SEC area (gel permeation chromatography), high-temperature-stable columns that stand up to somewhat exotic mobile phases are needed. Phases and column hardware for very high molecular weight polymers are required, mainly to reduce the high elongation strain rates that give rise to shear degradation. Very large pore packings with narrow particle size distributions for these type of polymers are usually very fragile, and it would be a real contribution to this field to finally solve the fragility problem.

Conclusion

It has been my pleasure for 33 years to write about columns and sample preparation for *LC* and *LCGC*. I hope that the magazine continues to thrive and provide useful and practical information on all aspects of chromatography to you, its loyal readers.

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Micropacked GC

Shinwa Chemical Industries has introdcued Micropacked-ST columns, which are loaded with Shincarbon-ST. Micropacked-ST columns are reportedly suitable for analyses of permanent gases and can simultaneously analyze multiple permanent gases including CO and CO_2. They can be used with capillary GC and GC–MS systems. Futhermore the columns are not affected by gaseous samples containing water vapour (water is not eluted as a peak).

http://shinwa-cpc.co.jp/en/

Shinwa Chemical Industries Ltd, Kyoto, Japan.

UHPLC method development kits

Advanced Chromatography Technologies has introduced ACE (U)HPLC Method Development Kits (providing up to 3 columns for the cost of a single column). These kits are designed to maximize selectivity, offering a powerful approach to UHPLC/HPLC method development. Porous, solid-core, and 300 Å bioanalytical column kits are available, in a wide range of formats including 0.5 and 1.0 mm microbore i.d.

www.ace-hplc.com

Advanced Chromatography Technologies Limited, Aberdeen, Scotland.

Dynamic headspace

The Gerstel DHS Large (DHS L) system enables the analysis of larger more representative samples placed in inert containers with volumes of up to 1 L. A single sample extension of the Gerstel DHS system and an autosampler for up to 11 samples are available. According to the company, the system offers improved detection limits and headspace analysis ruggedness.

http://www.gerstel.co.uk/en/

Gerstel GmbH & Co. KG, Mülheim an der Ruhr, Germany.

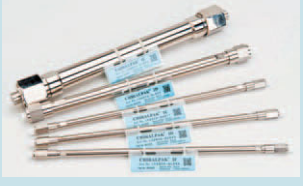
MALS detector

The mDAWN is, according to the company, the world's first multi-angle light scattering (MALS) detector that can be coupled to any UHPLC system to determine absolute molecular weights and sizes of polymers, peptides, and proteins or other biopolymers directly, without resorting to column calibration or reference standards. The WyattQELS Dynamic Light Scattering (DLS) module, which measures hydrodynamic radii "on-the-fly", reportedly expands the versatility of the mDAWN.

www.wyatt.com

Wyatt Technology, Santa Barbara, California, USA.

Chiral columns



The immobilized chiral column series from Daicel uses a combination of unique and trusted selectors. Immobilizing the polymers onto the silica ensures that they are robust, insoluble, and long lasting. According to the company, the range of six selectors ensures that these columns can separate racemates of most pharmaceutical, agrochemical, and fragrance compounds. Chiralpak IA, IB, IC, ID, IE, and IF are available for HPLC, SFC, UHPLC, flash, and TLC.

www.chiraltech.com

Chiral Technologies Europe, Cedex, France.

GC–MS

The Agilent 5877B GC/MSD with high efficiency ion source (HES) gives you 10× improvement in capabilities, according to the company. This single-quadrupole GC–MS offers two distinct advantages: 10× greater sensitivity (detection limits as low as 1.5 fg IDL) and 10× less sample required. Less time can now reportedly be spent on performing sample prep and maintenance, while also saving on shipping costs.

www.agilent.com/chem/5977b

Agilent Technologies, Inc., Santa Clara, USA.

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www.GasTrap.com • GasTrap, Almancil, Portugal.

Gas generators

Calibration gas generators, used with their respective VICI permeation devices, generate known concentrations from low ppb to mid-ppm of various compounds. Applications include air pollution monitoring, industrial hygiene surveys, odour surveys, and analyses in chemical, petrochemical, paper, power, and related industries. The new models are all CE certified and can be controlled by a convenient touch pad and Mass Flow Controllers.

http://www.vici.com/support/manuals/dyna235.pdf/
VICI AG International, Schenkon, Switzerland.

HPLC column brochure

Machery-Nagel has published a brochure titled "Nucleosil — the original", which reportedly features information on manufacturer-packed Nucleosil columns that HPLC users will find valuable. The robust spherical silica phases that are available with different modifications (C18 or C8, base-deactivated C18 HD or C18 AB, polar CN, NH_2 or SiOH, and many more) were originally developed in Germany and are still manufactured there exclusively by Machery-Nagel. The company report that Nucleosil is still one of the most used HPLC silicas in quality control.

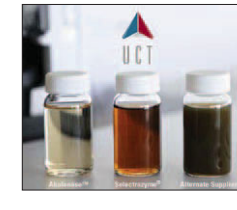
www.mn-net.com
Machery-Nagel GmbH & Co. KG, Düren, Germany.

Fast LC–MS system

Shimadzu has released the Nexera MX ultra-fast multianalyte LC–MS system. The system combines the high-speed analytical performance of the Shimadzu LC–MS systems with the SIL-30ACMP autosampler, and is designed with a unique flow line structure and high-speed sample injection capability. According to the company, this combination dramatically improves analytical efficiency. The system also includes the LabSolutions Insight software, providing assistance with quantitative analysis of large volumes of data.

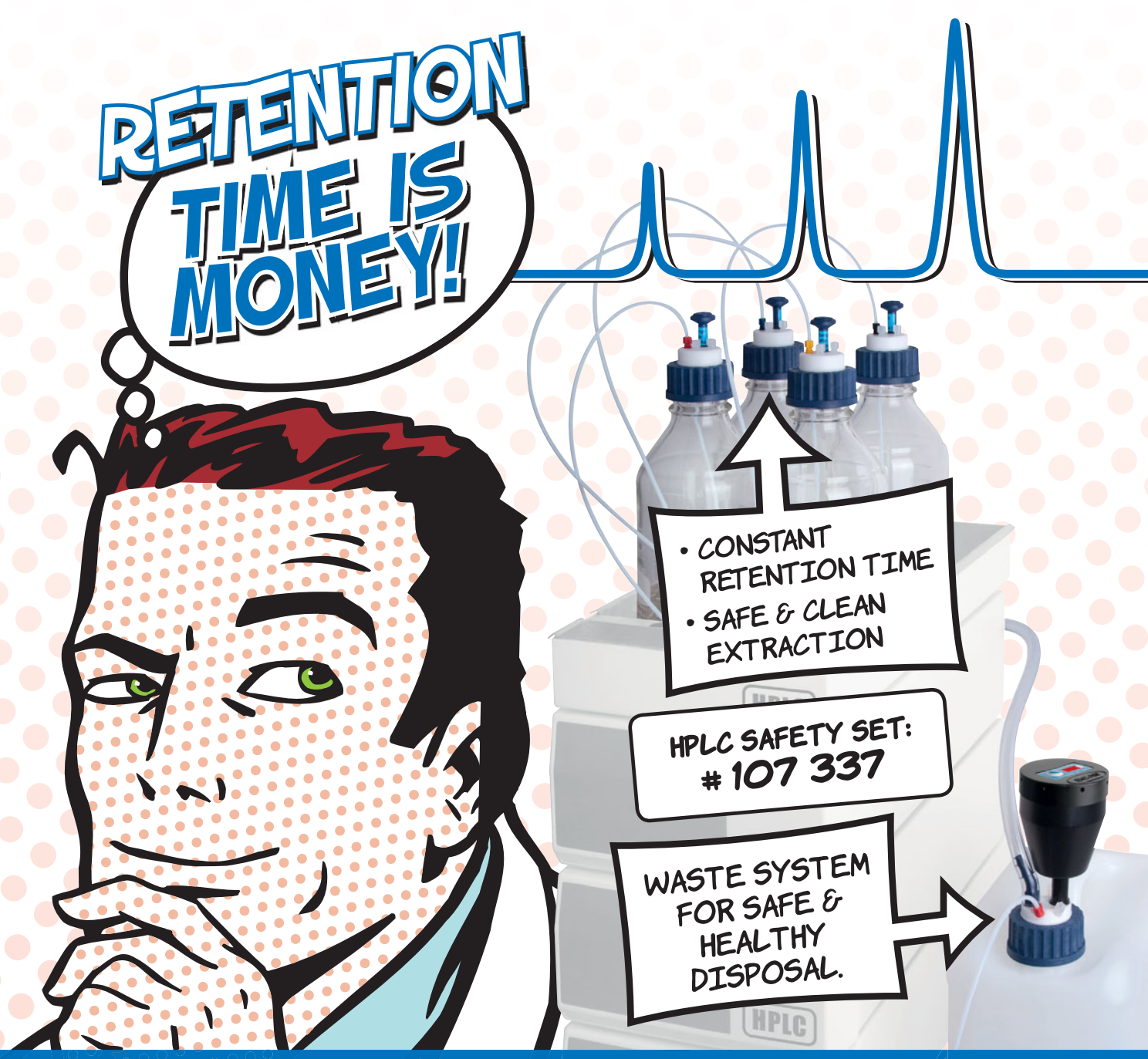
www.shimadzu.eu
Shimadzu Europa GmbH, Duisburg, Germany.

Enzyme hydrolysis



UCT has launched the Abalonase and Abalonse+, purified Beta-glucuronidase formulas to expand its enzyme hydrolysis line. According to the company, this product provides half the units of activity with the same conversion rate as a traditional abalone-derived enzyme. Within minutes of application, ultra-pure abalonase can deconjugate the major metabolites of interest including benzodiazepines, opioids, cannabinoids (naturally occurring and synthetic), and steroids. For the deconjugation of both glucuronidated and sulphated metabolites, abalonse+ is enriched with 4 arylsulphatase ensuring complete hydrolysis in both human- and animal-based urine samples. Purified Beta-glucuronidase formula is available in 10, 25, 50, and 100 mL volumes.

www.unitedchem.com
UCT, Inc, Bristol, PA, USA.



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18th International Symposium on Field- and Flow-Based Separations (FFF2016)

The **18th International Symposium on Field- and Flow-Based Separations (FFF2016)** will be held at **Hotel Elbflorenz, Dresden, Germany,** from **22–26 May 2016**.

The FFF symposia focus on the application of field-flow fractionation and related techniques in advanced material science. The series was launched in 1989 and since then it has been held every two years in different places around the world. The meeting series is of interest to scientists and engineers concerned with separation and characterization of all types of polymers and particles in liquid media. Previous meetings have been successful and have covered a range of molecules/materials to be investigated as well as instrumentation development and theory.

The topics of the coming symposium are related to field flow separations:

- Nanomaterials and Related Technology
- Synthetic and Natural Macromolecules
- Cells, Viruses, and Bioparticles
- Proteins and Drug Delivery
- Environment and Toxicology
- Food and Agriculture
- Theory and Instrumentation Development
- Industrial Application of FFF.

The following renowned experts have confirmed their participation: Helmut Coelfen (Konstanz, Germany); Martin Brandl (Odense, Denmark); Wei Gao (Midland, USA); Julien Gigault (Bordeaux, France); Oleg Iliev (Kaiserslautern, Germany); Vince Hackley (Gaithersburg, MD, USA); Michael Maskos (Mainz, Germany); Lars Nilsson (Lund, Sweden); Antje Potthast (Vienna, Austria); Ulrich S. Schubert (Jena, Germany); Kim Williams (Golden, CO, USA); and Francoise Winnik (Montreal, Canada).

The programme will be completed by contributed lectures and posters; each poster will be introduced by a short presentation of approximately three minutes. Companies will be displaying relevant equipment and services.

Abstracts for contributions must be submitted on-line at **www.fff2016.de** by **15 January 2016** (oral presentations) or **15 February 2016** (posters).

The meeting will be preceded by two one-day workshops that will be free of charge for students and for participants of the **FFF2016** symposium.

Saturday 21 May 2016 (venue: IPF): Basics of FFF Field-flow fractionation: Exciting perspectives for (bio-)polymer and nanoparticle separations presented by Wim Kok (Amsterdam, The Netherlands) and Harald Pasch (Stellenbosch, South Africa).

Sunday 22 May 2016 (venue: IPF): FFF: A powerful tool for characterization of biomolecules, bioparticles, and biomaterials presented by Serge Battu (Limoges, France) and Myeong Hee Moon (Seoul, Korea).

Aside from the interesting scientific programme, participants can expect a nice meeting in a pleasant venue with enough time for intensive discussion in an inspiring atmosphere. The venue, Hotel Elbflorenz, is situated close to the famous Baroque city centre of Dresden.

Do not miss the chance to explore the potential and latest findings of field-flow fractionation as well as one of Germany's most beautiful towns!

Chairperson: Albena Lederer
Tel: +49 351 4658 282
E-mail: lederer@ipfdd.de
Website: www.fff2016.de

27–29 January 2016
4th International Symposium on Hyphenated Techniques in Chromatography and Separation Technology (HTC-14)
Ghent, Belgium
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Website: www.htc-conference.com

6–10 March 2016
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Georgia World Congress Center, Atlanta, Georgia, USA
E-mail: info@pittcon.org
Website: http://pittcon.org

20–21 April 2016
XI International Conference ION CHROMATOGRAPHY 2016
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Contact: Rajmund Michalski
E-mail: rajmund.michalski@ipis.zabrze.pl
Website: www.ipis.zabrze.pl

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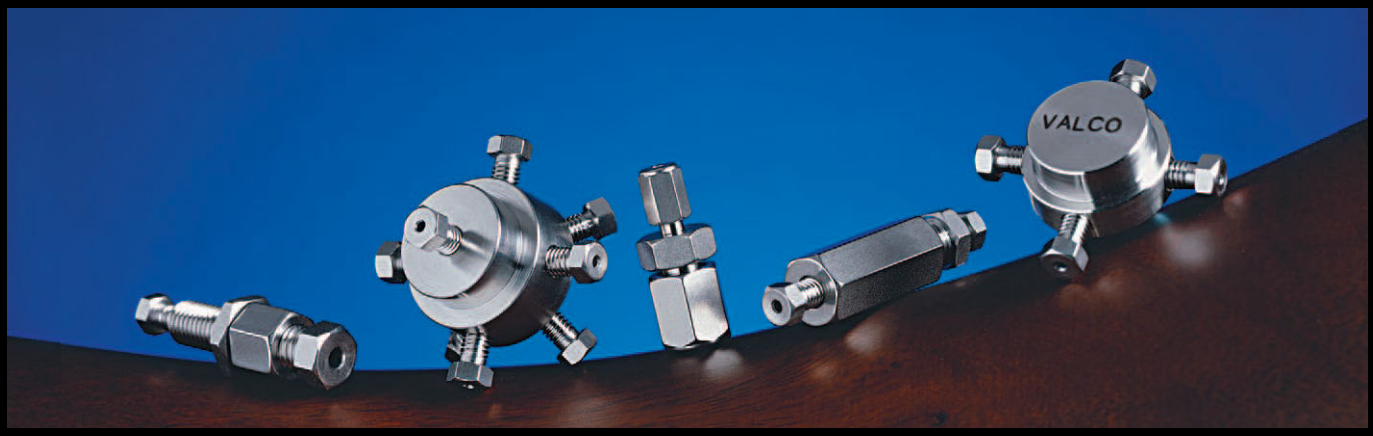

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Selected highlights of digital content from *LCGC Europe* and *LCGC North America*:

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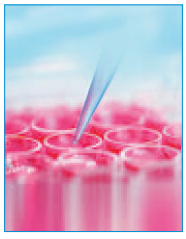
Biopharmaceutical Special Issue October 2015

LCGC supplement focusing on advances within biopharmaceutical analysis. The supplement highlights the challenges in biopharmaceutical analysis and within the pharmaceutical industry, and details the research shift from small to large molecules.

Read Here: http://goo.gl/SkKzw1



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ADVANCES IN BIO-SEPARATIONS REVIEW

Meeting review of a two-day symposium, organized by The Chromatographic Society, focusing on the characterization of biopharmaceutical molecules at AstraZeneca's MedImmune site in Cambridge, UK.

Read Here: http://goo.gl/TIBOIW

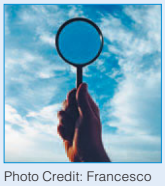
Photo Credit: Rafe Swan/ Getty Images

FEATURED INTERVIEW

Environmental Forensics in the Workplace

David Megson from Ryerson University in Toronto, Canada, spoke to *The Column* about recent developments and his team's work in assessing human exposure to PCBs and chiral enantiomer fractions (EFs) in the workplace.

Read Here: http://goo.gl/rm6DTg



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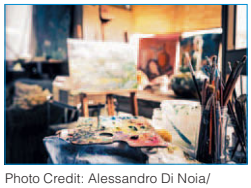
NEWS

Analyzing Edvard Munch

Researchers from the University of Pisa, Italy, have devised a new method to characterize complex oil mixtures in paintings using high performance liquid chromatography–electrospray ionization-quadrupole-time of flight mass spectrometry (HPLC–ESI-QTOF-MS).

Read Here: http://goo.gl/R4pofc



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INTERVIEW

The Rise of Hydrophilic Interaction Chromatography (HILIC)

Hydrophilic interaction chromatography (HILIC) is becoming more popular. Dr Tohru Ikegami from the Department of Biomolecular Engineering, Kyoto Institute of Technology in Kyoto, Japan, reveals more about the evolution of HILIC, important new developments, and tips on how to get the most from this technique.

Read Here: http://goo.gl/yFrhTh

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This article presents baseline and separation problems and identifies their causes from the everyday pictures we see on data systems. A fundamental skill that every chromatographer should learn.

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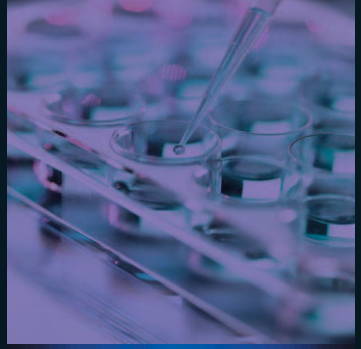
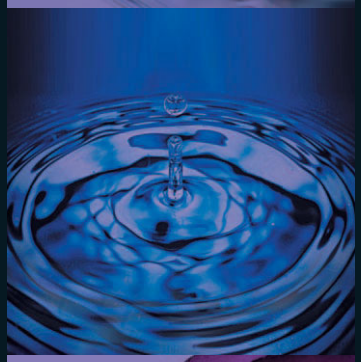
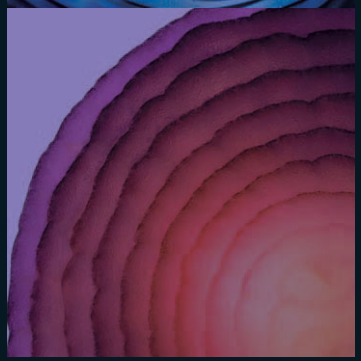
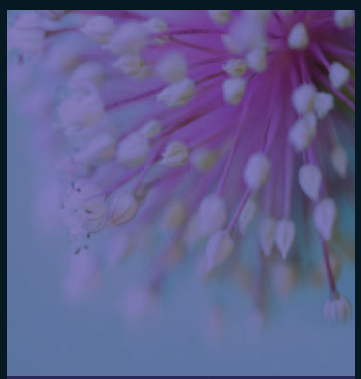
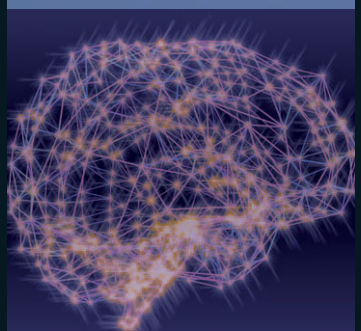






THE APPLICATIONS BOOK





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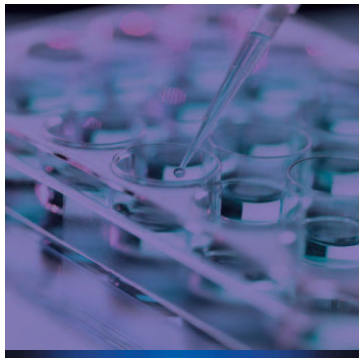

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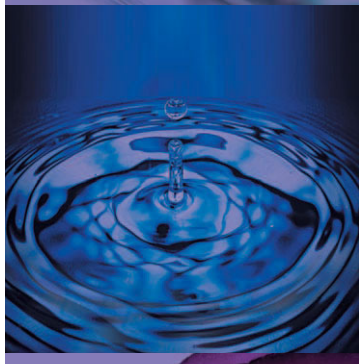

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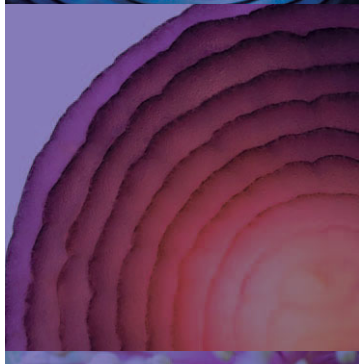

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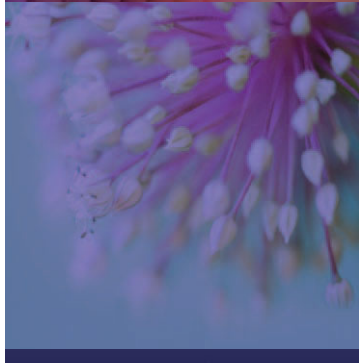

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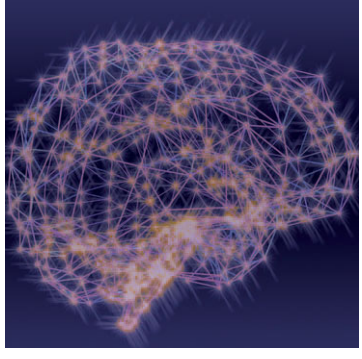

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THE APPLICATIONS BOOK

Environmental

Food and Beverage

Chromate in Toys, Leather, and Potable Water

Metrohm AG

Generally speaking, chromate — Cr(VI) — is classified as allergenic, carcinogenic, and extremely toxic and is subject to strict monitoring. Cr(VI) can occur in various concentrations in different areas, for example, drinking water, toys, or textile and leather products. Metrohm has developed ion chromatographic determination methods for determining Cr(VI) in various concentration ranges (ng/L to mg/L) with inline sample preparation techniques for various matrices. The method can be almost entirely automated. In the following application note, we present the analysis of hexavalent chromium in toys, leather, and drinking water.

Chromate in Toys According to DIN EN 71-3

Children are exposed to heavy metals from a wide variety of sources. Cr(VI) in particular represents a potential hazard because it is absorbed from food and drinking water, from the air, from textiles, and from toys. Analytical determination of the Cr(VI) content in toys is described in the European Standard DIN EN-71-3-2013 (Safety of toys Part 3 — Migration of certain elements), as well as in the EU directive 2009/48/EC. The limit values that apply to Cr(VI) according to the EU directive are listed in Table 1.

According to DIN EN 71 Part 3, the user is free to choose whichever analysis method he or she prefers, as long it is validated. Metrohm has developed an ion chromatography method for this application that uses Inline Preconcentration and Inline Matrix Elimination. The method can be almost entirely automated.

Sample Digestion Using Synthetic Gastric Juice

Chromium(VI) is extracted from the toy material at body temperature using hydrochloric acid. This type of sample digestion simulates how gastric juice dissolves out the harmful substance from swallowed toy material. The sample that is obtained using this method is then manually neutralized and diluted. Dilution is required because the high ion concentration present in the sample — which is caused by hydrochloric acid extraction and subsequent neutralization — does not permit preconcentration of chromium(VI).

Automation Improves Convenience and Safety

All the remaining steps are automated. To start with, the entire sample flow path is equilibrated using sample. A Dosino then feeds a defined sample volume onto the preconcentration column (Figure 1). The Dosino can accurately control the injection volume, and this is what forms the basis of reliable determination, especially when dealing with low analyte concentrations. Equally important for the sensitivity, however, is the post-column reaction of Cr(VI) with 1,5-diphenylcarabazide that results in the formation of VIS active complexes. As illustrated in Figure 2, the method presented accurately determines hexavalent chromium in the single digit ppt

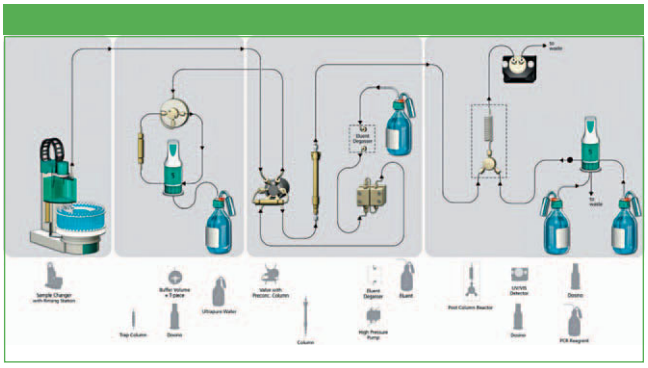

Figure 1: Diagram of the IC system for the determination of chromate in toy samples. Following manual extraction, dilution, and neutralization of the samples, the fully automated analysis process takes place according to the measurement setup shown above. Dosing the sample and post-column reagent using Dosinos rather than pumps offers a number of benefits, because it allows not only exceptionally precise control of the sample volume, but also post-column reagent dosing that is synchronized with the column flow — as well as automatic switching between the post-column reagent and rinsing solution. The setup can be easily modified for analyzing leather or drinking water samples.

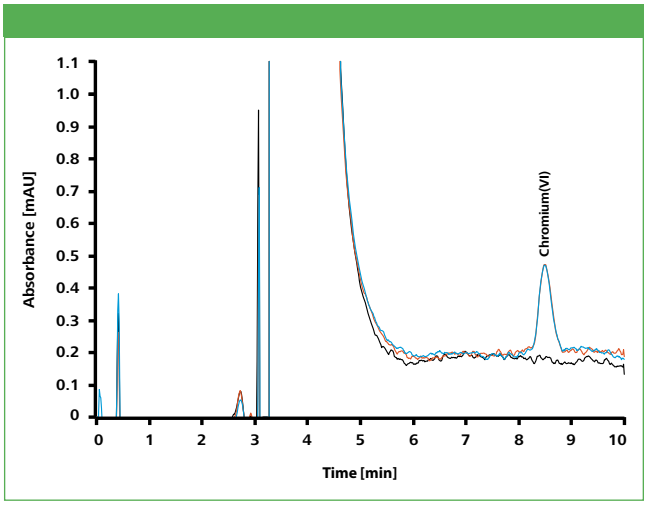

Figure 2: Two determinations of a standard solution containing 0.04 µg/L chromium(VI) in a matrix corresponding to that of a neutralized migration solution — this contains HCl, Na_2CO_3, $NaHCO_3$, and $(NH_4)_2SO_4$ (red and blue). The reference curve (black) shows the chromatogram of ultrapure water.

Table 1: The three types of toy materials and their migration limits for Cr(VI) according to EU directive 2009/48/EC, plus some examples.

Toy material group	Dry, brittle, powder-like, or pliable toy materials	Liquid or sticky toy materials	Scraped-off toy materials
Examples	Coloured pencil leads, chalk, wax crayons, modelling clay	Finger paint, varnish, liquid ink in pens, soap bubble solution, glue sticks	Varnish coatings, polymers and similar, paper, cardboard, glass, ceramics, metallic materials, wood, leather
Migration limit	0.02 mg/kg	0.005 mg/kg	0.2 mg/kg

range, easily complying with the 10 ppt limit set by the EU directive 2009/48/EC.

Chromate in Leather According to DIN EN ISO 17075

The Cr(VI) that may form during the chrome tanning process is regarded as allergenic and carcinogenic. Causes of the Cr(VI) load in leather include, among other things, contamination of the Cr(III) salts by Cr(VI) compounds. In addition, excess Cr(III) salts that are not bound to the collagen in the skin of the leather may oxidize to form chromium(VI) under certain conditions.

The Cr(VI) content in leather is determined in accordance with DIN EN ISO 17075:2007. Strongly coloured leather extracts disrupt the determination of chromium(VI), and require filtration of the extracts. Metrohm has developed an ion chromatography method for this application using Inline Dialysis for automatic sample preparation and UV–vis detection following post-column reaction. Before injection of the sample on the separation column, the high-molecular compounds of the sample matrix are separated from Cr(VI) with Inline Dialysis. In the second step, chromatographic separation takes place on an anion exchanger before post-column reaction with 1,5-diphenylcarbazide, as already described in the toy section.

Advantages of the method in comparison with the photometric method with solid-phase extraction include:

- **Cost savings**
  The moderate price of the membrane for Inline Dialysis keeps operating costs very low in comparison with solid-phase extraction.
- **Time savings**
  The automatic sample preparation of the next sample is already taking place with Inline Dialysis during the running time of the chromatogram of each sample.
- **Increased detection sensitivity**
  Detection sensitivity can be enhanced considerably in comparison with the conventional photometric method thanks to the sensitivity of the UV–vis detector after post-column reaction.

Chromate in Mineral and Drinking Water According to EPA 218.7

Cr(VI) is a highly toxic contaminant of drinking water. Even the smallest quantities are hazardous to human health.

Even though the RoHS Directive has severely restricted the use of Cr(VI) compounds in Europe since 1 July 2006, chromate nonetheless remains a widespread pollutant in the environment.

Some pigments formerly used in dyes also contained Cr(VI) compounds. These chromate pollutants appear in the environment when decontamination is done improperly. The carcinogenic Cr(VI) can then find its way into the soil, and over time also into our water.

The EU limit value for total chromium, which corresponds to both the WHO limit value and to the German Drinking Water Ordinance, is still set at 50 µg/L. There are ongoing discussions as to whether this limit value should be considerably lowered.

Metrohm has developed an exceptionally sensitive ion chromatography method for the detection of Cr(VI), with a limit of determination of less than 0.02 µg/L chromate (Figure 3). The application is also in compliance with the limit value stipulated in EPA Standard 218.7.

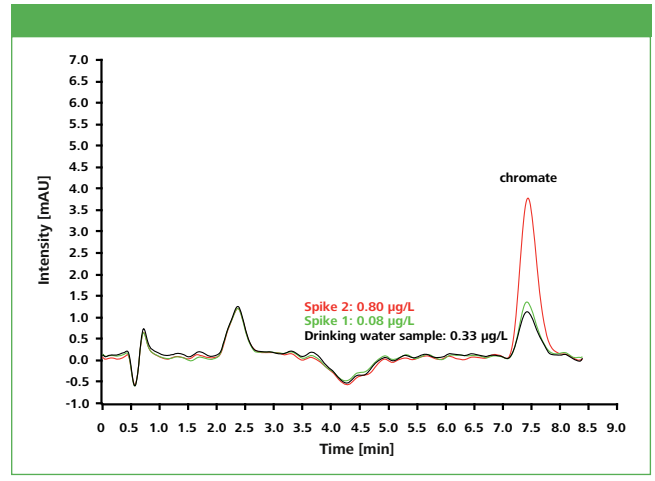

Figure 3: Chromate determination in a drinking water sample — once unspiked and once spiked with 0.08 µg/L and 0.8 µg/L Cr(VI). Recoveries were between 99.8 and 99.9%. Column: Metrosep A Supp 5 - 150/4.0; eluent: 12.8 mmol/L Na_2CO_3, 4 mmol/L $NaHCO_3$, 2.5 g/L $(NH_4)_2SO_4$, 0.7 mL/min.

If required, the sample can be prepared for injection using Inline Ultrafiltration in an automated sequence. As described earlier, detection takes place by post-column reaction with 1,5-diphenylcarbazide and subsequent detection at 540 nm. The procedure is controlled using the intelligent ion chromatography software MagIC Net.

References

(1) EU Directive 2009/48/EC.
(2) DIN EN 71-3:2013-07: Safety of toys — Part 3: Migration of certain elements.
(3) U.S. Environmental Protection Agency, Method 218.7, Version 1.0, Cincinnati, Ohio, USA (2011).
(4) Metrohm Whitepaper WP-001EN: Chromium(VI) determination in children's toys.
(5) Metrohm Application Note AN-U-015: Chromium(VI) in a leather extract.
(6) Metrohm Application Note AN-U-057: Chromate in drinking water by ion chromatography with PCR and UV–vis detection, according to EPA Method 218.7.

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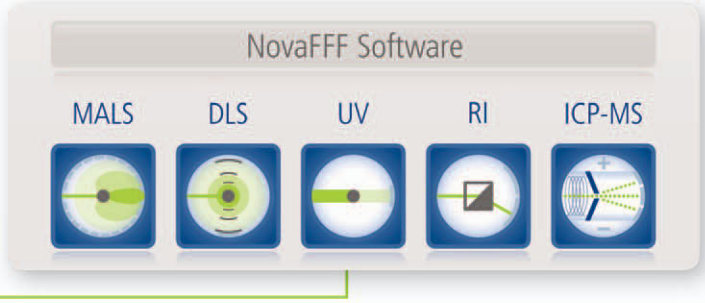

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Determination of Pesticide Residues in Blueberries by AOAC QuEChERS Approach and Dispersive SPE Cleanup with a Novel Sorbent ChloroFiltr®

Xiaoyan Wang, UCT, LLC

This application outlines a simple, fast, and cost-effective method for the determination of 34 multi-class pesticides, including one of the most problematic pesticides, pymetrozine in blueberries. The acetate buffered AOAC QuEChERS protocol demonstrated higher extraction efficiency for pymetrozine than the other two QuEChERS protocols (the EN citrate buffered or the original unbuffered), and thus was selected for the extraction of pesticide residues in blueberries. UCT's Aqueous C18 HPLC column was used for analyte analysis, which demonstrated enhanced retention for several polar pesticides, such as methamidophos and acephate.

Table 1: Extraction/analytical materials.

ECMSSA50CT-MP	Enviro-Clean® Mylar pouch containing 6 g $MgSO_4$ and 1.5 g NaOAc-50 mL centrifuge tubes included
CUMPSGGC182CT	Enviro-Clean® 2 mL dSPE tube with 150 mg $MgSO_4$, 50 mg PSA, 50 mg C18 and 50 mg ChloroFiltr®
SLAQ100ID21-3UM	Selectra® Aqueous C18 HPLC column, 100 × 2.1 mm, 3-µm

QuEChERS Procedure

a) Weigh 15 ± 0.3 g of homogenized blueberry sample into 50-mL centrifuge tubes.
b) Add triphenyl phosphate (TPP) as internal standard (IS) (optional), and appropriate amounts of spiking solution to fortified samples.
c) Add 15 mL of MeCN with 1% HAc. Cap and shake for 1 min at 1000 strokes/min using a Spex 2010 Geno-Grinder.
d) Add salts (6 g $MgSO_4$ and 1.5 g NaOAc) from pouch **(ECMSSA50-CT-MP)** to the 50-mL tube and vortex for 10 s to break up salt agglomerates.
e) Cap and shake for 1 min at 1000 strokes/min using the Geno-Grinder.
f) Centrifuge at 3000 rcf for 5 min.

dSPE Cleanup

g) Transfer 1 mL of the supernatant to a 2-mL dSPE tube containing 150 mg $MgSO_4$, 50 mg of PSA, 50 mg C18, and 50 mg ChloroFiltr® **(CUMPSGGC182CT)**.
h) Shake 1 min at 1000 strokes/min using the Geno-Grinder.
i) Centrifuge at 3000 rcf for 5 min.
j) Transfer 0.2 mL of the cleaned extract into a 2-mL auto-sampler vial; add 0.2 mL of DI water, and vortex for 30 s.

Instrumental

LC–MS–MS: Thermo Scientific Dionex Ultimate 3000® LC System/ Thermo Scientific TSQ Vantage tandem MS
Column: 100 × 2.1 mm, 3-µm UCT Selectra® aQ C18 LC column
Guard column: 10 × 2.0 mm, 3-µm UCT Selectra® aQ C18 guard column
Injection volume: 2 µL
Mobile phase A: 10 mM ammonium acetate in DI water
Mobile phase B: 0.1% formic acid in methanol
Column flow rate: 0.30 mL/min

Results

Excellent recoveries ranging from 81.3% to 108.7% were obtained for the determination of 34 multi-class pesticides in blueberries. Dispersive SPE cleanup using just PSA or a PSA/C18 combination was ineffective in removing all the pigments in the blueberry extract. The addition of GCB or UCT's patented ChloroFiltr® in the dSPE tube resulted in a much cleaner extract; however, GCB was detrimental to the recoveries for several planar pesticides including pymetrozine, carbendazim, thiabendazole, and cyprodinil. The recovery data of GCB versus ChloroFiltr® is shown below.

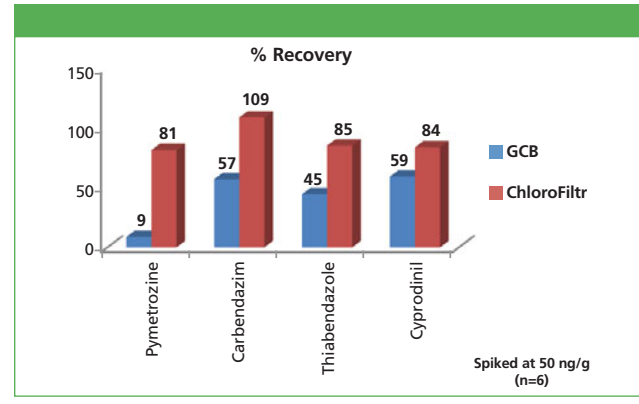

Figure 1: Recovery data of GCB versus ChloroFiltr®.

Conclusion

This application demonstrated the successful extraction of 34 multi-class pesticides in blueberries using UCT's QuEChERS extraction kit **(ECMSSA50CT-MP)** and dispersive SPE cleanup products **(CUMPSGGC182CT)** in conjunction with the Selectra® aQ C18 HPLC column **(SLAQ100ID21-3UM).**

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Quantification of Purine Alkaloids and Catechins in Green and Black Tea Using Comprehensive 2D-LC

Sonja Krieger, Agilent Technologies, Inc.

This application note discusses the comprehensive 2D-LC analysis of green and black tea. The purine alkaloids caffeine and theobromine, as well as the catechins catechin, epicatechin, and epigallocatechin gallate, are quantified.

Tea, produced from the tea plant *Camellia sinensis,* is one of the most widely consumed beverages worldwide and is characterized by a highly complex composition. Depending on the processing methods of the leaves, three forms of tea are obtained; green tea, oolong, and black tea. The predominant polyphenols in green tea are catechins, whereas in the production of black tea, the monomeric catechins undergo oxidative polymerization (1–4). Quantification of the purine alkaloids caffeine and theobromine, and the tea catechins catechin, epicatechin, and epigallocatechin gallate, enables a comparison of green and black tea.

Experimental Conditions

Comprehensive 2D-LC analysis was achieved with the Agilent 1290 Infinity II 2D-LC Solution. In the first dimension, an Agilent ZORBAX Eclipse Plus C18 column (2.1 × 100 mm, 3.5-µm) was used with a gradient of water and methanol, each with 0.05% trifluoroacetic acid and at a flow rate of 0.1 mL/min. The second dimension separation used an Agilent Poroshell 120 Bonus-RP column (3.0 × 50 mm, 2.7-µm) with shifted gradients of water and acetonitrile, each with 0.05% trifluoroacetic acid, at a flow rate of 2.5 mL/min. Modulation was achieved using the Agilent 2-position/4-port-duo valve, equipped with two 60 µL loops. A modulation time of 21 s was employed. Detection was performed at 280 nm.

Results

Deploying reversed-phase LC in the first and second dimension, a comprehensive 2D-LC method for the analysis of purine alkaloids and catechins in green and black tea was developed. Figure 1a shows that only the two-dimensional setup enabled complete separation of the purine alkaloids and catechins. In the first-dimension separation, caffeine and epigallocatechin gallate coeluted but were resolved in the second-dimension separation. Deploying only the second-dimension separation, catechin and epicatechin would coelute. The precision of retention times and peak volumes was determined by multiple injection (n = 10) of purine alkaloids and catechins. In the second dimension, the retention time precision was always below 2.5%, and the peak volume precision was always below 1%. Excellent linearity was achieved for all purine alkaloids and catechins. Ten different samples of green and black tea were analyzed, and purine alkaloids and catechins were quantified. Theophylline could not be detected in any of the tea samples.

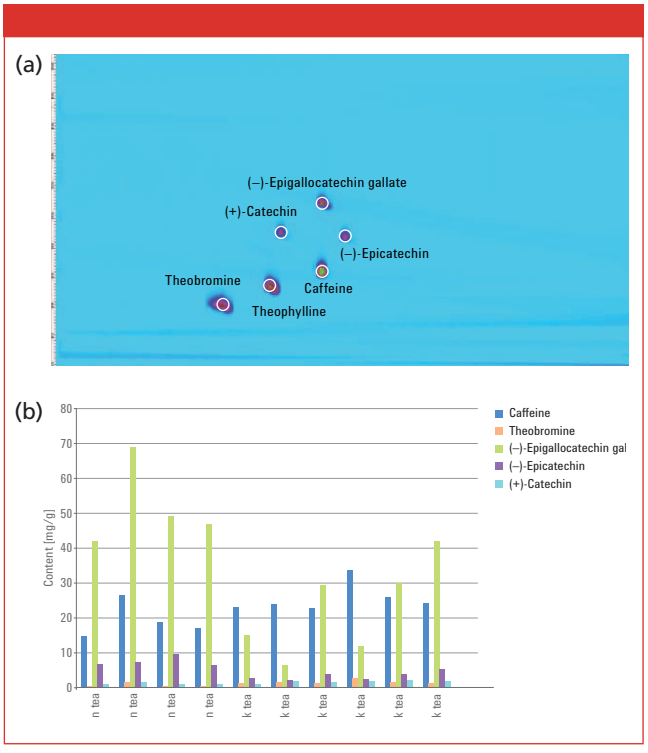

Figure 1: (a) Comprehensive 2D-LC separation of purine alkaloids and catechins; (b) quantification results of purine alkaloids and catechins in green and black tea.

Figure 1b shows the quantification results for purine alkaloids and catechins in green and black tea. As expected, the green tea samples generally contained higher amounts of the catechins epigallocatechin gallate and epicatechin than the black tea samples.

Conclusions

Comprehensive 2D-LC enables the analysis and quantification of purine alkaloids and catechins in green and black tea. As expected, green tea contained higher amounts of the catechins epigallocatechin gallate and epicatechin than black tea.

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Sensitive Analysis of the Lactose Content of Lactose-Free Labelled Products Using HPAEC-PAD

Mareike Margraf and Kate Monks, Knauer

The market for lactose-free products is growing rapidly and constantly and Europe is a worldwide leader in the lactose-free market. Between 2012 and 2016 the sales of lactose-free products are expected to increase by 75% (1,2). Studies have stated that customers who are lactose intolerant, or believe they are, will pay a big premium for the right product (1). From these statements it becomes obvious that a huge demand for lactose-free products exists in the industry. A high performance liquid chromatography (HPLC) method that easily reaches the required limits of detection (LOD) by using high performance anion-exchange chromatography coupled to pulsed amperometric detection (HPAEC-PAD) on a Knauer AZURA® analytical HPLC system coupled to the DECADE II electrochemical detector is presented. Besides lactose, sucrose and glucose were also analyzed to prove the method´s ability to differentiate several sugars.

Generally, lactose intolerance is the inability to digest lactose caused by the deficiency in the enzyme lactase, which hydrolyzes lactose into glucose and galactose. More than 65% of the world´s population loses the ability to completely digest lactose after infancy in what is called primary or late onset lactose intolerance (3). Reduction in lactase activity is rarely total but decreases to 10–30% of the initial level between the ages 5 and 20 (4). In addition, secondary and developmental lactose intolerance can occur. It can be stated that lactose intolerance is an important subject worldwide.

Although in European countries like Sweden and Finland lactose tolerance levels of 74% and 82% are widespread, the market for lactose-free products is growing and the regulations are getting more strict (5).

In many European countries, the limit of lactose in lactose-free labelled products has recently been decreased from 100 to 10 mg/100 g product(6). This makes an HPLC method with low detection limits inevitable for the quality control of these products. Special methods and systems are needed because classical determination of sugars in food products is not sensitive enough in this special case. The lactose content of food products can generally be determined in several ways. Validated methods do exist for enzymatic essays, polarimetry, gravimetry, differential pH, and HPLC.

Today, HPLC is the method of choice when sugar contents in dairy products have to be analyzed because it is a highly specific method with the ability to differentiate other sugars. HPLC on an ion exclusion column coupled to RI detection is the typical method used. However, in the special case of lactose analysis in lactose-free products, this method is nowhere near sensitive enough. Therefore, special methods have to be applied to reach the wanted low detection limit of 10 mg/100 g sample.

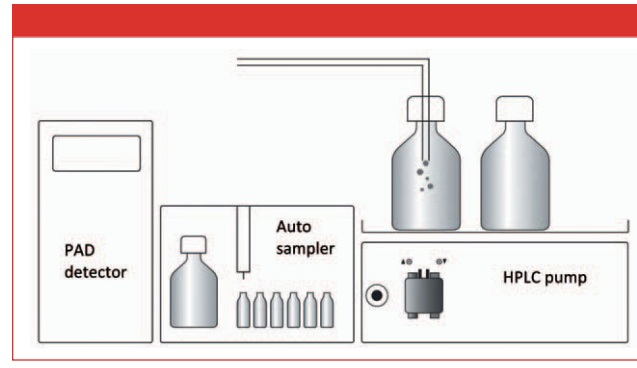

Figure 1: System for the sensitive analysis of lactose.

Experimental Sample Preparation

Samples from different non-dairy food products were extracted using various extraction protocols, filtered, and injected into the HPLC system.

Experimental Preparation of Standard Solution

Standards of lactose, sucrose, and glucose were weighed in and dissolved in deionized water. They were afterwards diluted in deionized water to reach low concentrations down to less than 100 µg/L lactose. Food labelled as lactose free must contain less than 10 mg lactose in 100 g food product. When sample prep is taken into consideration, the target LOD becomes < 10 mg/L.

Method Parameters

The HPLC analysis was performed using a Knauer AZURA Analytical HPLC system consisting of an isocratic high pressure pump P 6.1L in the metal-free ceramic edition, an autosampler 3950, and the DECADE II electrochemical detector. The mobile phase was continuously sparged with helium to keep it inert. A schematic drawing of the system can be seen in Figure 1. The applied anion exchange column is stored in the tempered section of the DECADE II detector.

The system was flushed and allowed to equilibrate overnight because the applied method is very sensitive to any changes. A sufficient equilibration time is recommended especially when the system is running this method for the first time.

Method Parameters

Column:	250 × 4.6 mm, 7-µm RCX-10 PEEK hardware
Column order no.:	25EE158HML
Eluent A:	30 mM NaOH continuously sparged with helium
Gradient:	Isocratic
Flow rate:	2 mL/min
Injection volume:	50 µL
Temperature:	30 °C (column and flow cell)
System pressure:	approximately 870 bar
Detection:	ECD (electrochemical detection)
	E cell: E1, E2, E3: 0.05, 0.75, -0.80 volts
	ts, t1, t2, t3: 0.06, 0.5, 0.13, 0.12 s
	I-cell: 300–500 nA

Results

Figure 2 shows an overlay of four chromatograms measured with the described method. It is obvious that a separation of the three applied sugars is possible. In addition, really low lactose concentrations could still be detected.

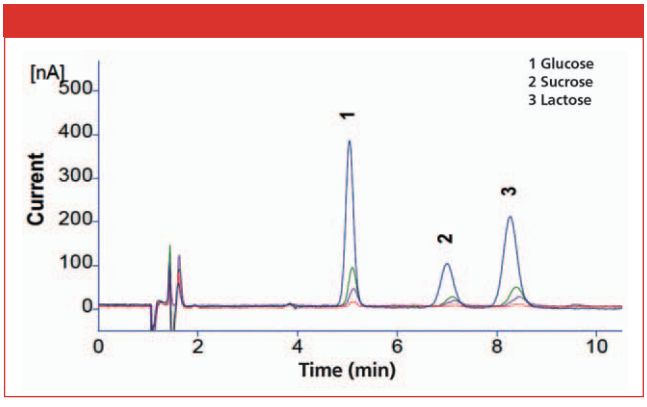

Figure 2: Chromatograms of a standard solution at different concentrations: Blue: 1090.0 µg/L, green: 218.0 µg/L, violet: 109.0 µg/L, red: 21.8 µg/L.

To figure out the limit of detection and the limit of quantification of the method, standard dilutions from around 1000 down to 21 µg/L were injected. Using the resulting peak heights, the parameters could be calculated. Figure 3 shows the calibration curve and the method performance parameters.

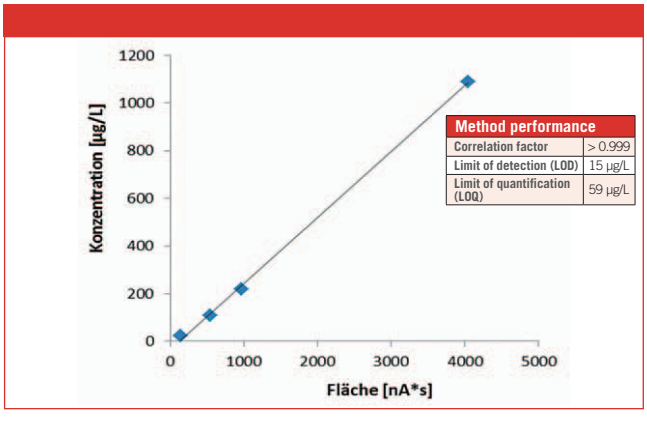

Figure 3: Calibration curve for lactose concentrations in the range of 21.8 µg/L to 1090.0 µg/L and results for method performance.

The following analysis of four samples from typical German food proved that most of them were lactose-free and therefore allowed to use this label, even with the new lactose limits given by the EU. Figures 4 to 7 show the chromatograms of the sugar standard (blue) overlaid with the samples (red) from Hähnchenspieß (chicken skewers), Leberkäse (meatloaf), Paprikalyoner (sausage), and Nürnberger (sausage). Only in one case could a significantly high lactose peak be found. Using the presented method, 3 out of 4 samples could be declared as lactose-free; one sample is not allowed to be called a lactose-free product.

In addition the chromatograms show that few disturbing peaks were detected. This is caused by the specialized detection method that is sensitive to sugar analysis and does not show many of the samples impurities.

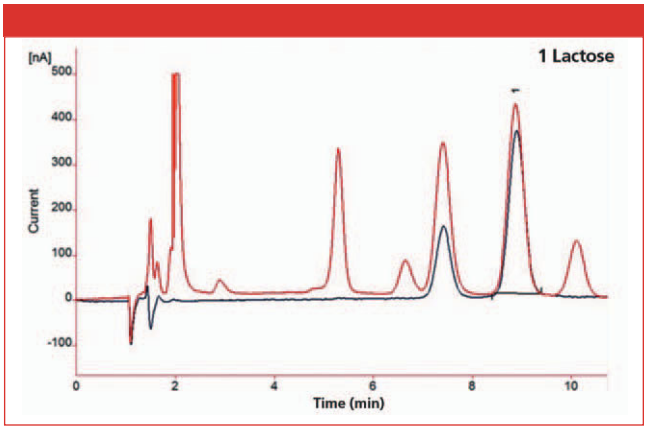

Figure 4: Chromatogram of "Hähnchenspieß" chicken skewers (red) with an overlay of the sugar standard (blue).

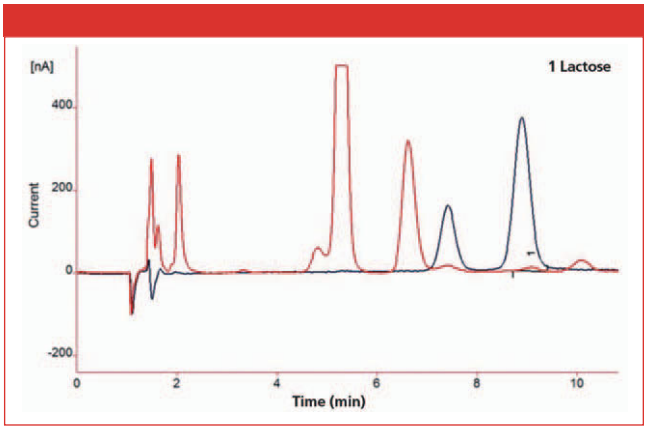

Figure 5: Chromatogram of "Paprikalyoner" sausage (red) with an overlay of the sugar standard (blue).

Using the calibration, the lactose contents in the samples were determined. Table 1 shows the results.

Three out of four samples can be declared as lactose-free according to the definition.

If there is a need in the future to detect even lower concentrations, this method allows for optimization by less

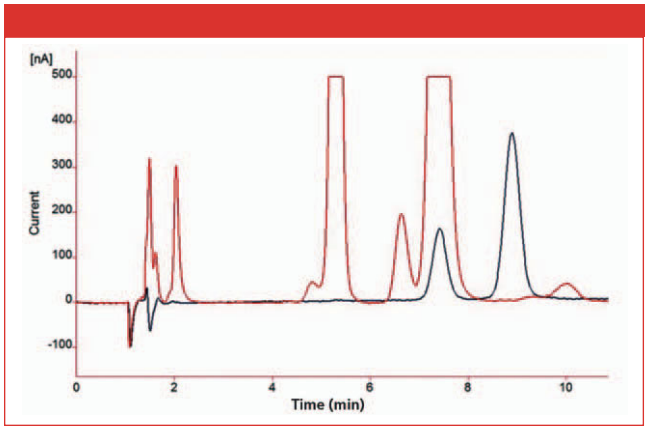

Figure 6: Chromatogram of "Leberkäse" meatloaf (red) with an overlay of the sugar standard (blue).

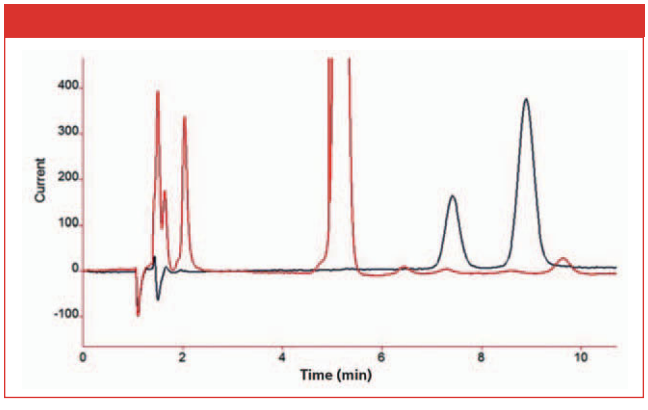

Figure 7: Chromatogram of "Nürnberger" sausage (red) with an overlay of the sugar standard (blue).

Table 1: Determination of lactose contents in the real samples.

Sample	Peak area	Calculated concentration* (µg/L)	Lactose content in 100 g product (mg)	Result**
Paprikalyoner	209	23	9	Lactose-free
Leberkäse	NF	< 15	< 6	Lactose-free
Hähnchenspieße	9342	25544	10217	Not Lactose-free
Nürnberger	NF	< 15	< 6	Lactose-free

* dilution of the sample already included
**lactose-free means that the sample contains less than 10 mg lactose per 100 g product

dilution of the samples. This becomes possible by the very specific detection method where nearly no interfering matrix peaks are seen.

Conclusion

The presented method of HPAEC-PAD on a Knauer HPLC system was well-suited to determine low limits of sugars in food products. The detection principle was quite specific for sugars and only showed very little interference by matrix peaks. Using the AZURA analytical system combined with the DECADE II electrochemical detector, the analysis of lactose in lactose-free labelled products can be performed in a robust and reproducible manner. This system reaches the detection limits defined by the EU and can therefore be used in food control.

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Comprehensive Analysis of Raw Foodstuffs Using Dynamic Headspace Sampling with Thermal Desorption–GC–MS Analysis

Caroline Widdowson, Hannah Calder, and David Barden, Markes International

Herbs are widely used in many food products, but substantial variations in aroma can result from differences in growing conditions or preparation of the plant material, which can affect product quality.

In this application note we show the wide range of aroma chemicals that can be detected in the headspace of basil leaves using a micro-chamber sampling device with analysis by thermal desorption (TD) and gas chromatography–mass spectrometry (GC–MS).

Micro-Chamber/Thermal Extractor

Of the numerous TD-compatible sampling instruments, the Micro-Chamber/Thermal Extractor™ (μ-CTE™) from Markes International is one of the most versatile. It is a compact, stand-alone unit comprising cylindrical chambers suitable for sampling chemical emissions from larger samples, or from materials that are not entirely homogeneous.

Operation is simple — materials are placed in one of the chambers, and the headspace vapours are dynamically extracted onto a 3½-inch × ¼-inch sorbent-packed TD tube by a flow of heated air or gas. This tube is then placed into the thermal desorber and analyzed as described below. Sampling times are short (typically < 60 min), and the instrument can analyze up to four or six samples at once, depending on the model chosen.

Thermal Desorption

Thermal desorption (TD) uses heat and a flow of inert gas to desorb volatile and semi-volatile organic compounds (VOCs and SVOCs) from sorbents or sample materials. Extracted vapours are swept onto an electrically-cooled focusing trap, which is then rapidly heated to inject them into a gas chromatograph (GC).

TD offers many advantages over conventional solvent-based sample preparation methods such as liquid extraction. These include wider analyte range (from acetylene to n-C_{44} and reactive species on one platform), quantitative re-collection of split flows for repeat analysis and simple method validation, and enhanced sensitivity.

In this study, the TD-100™ automated cryogen-free thermal desorber from Markes International was employed, which has capacity for 100 industry-standard tubes.

Analysis of Fresh Basil Leaves

Figure 1 shows the results obtained by dynamic headspace sampling of fresh basil leaves with analysis by TD–GC–MS. As well as the rapidity with which the entire vapour profile can be collected using the Micro-Chamber/Thermal Extractor, the inertness and adjustable flow-path temperature of Markes' TD systems ensure reliable analysis of a wide range of analytes, including reactive or difficult-to-analyze species such as sulphur species and certain monoterpenoids.

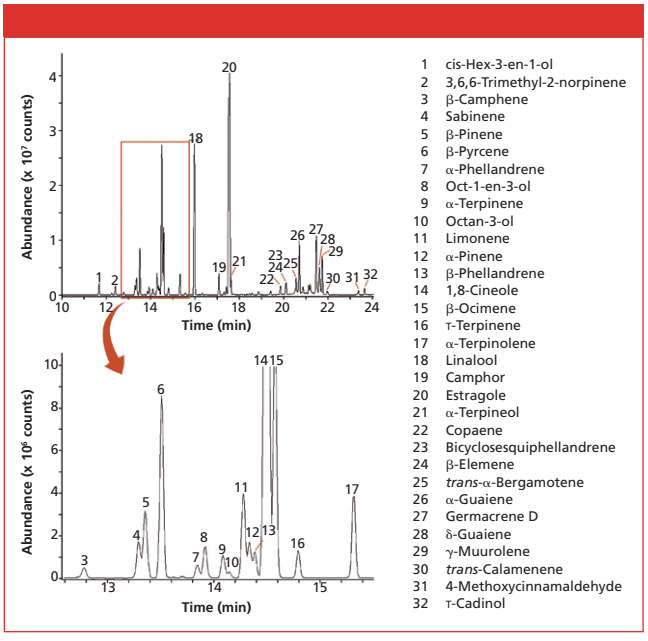

Figure 1: Dynamic headspace sampling of fresh basil leaves, with analysis by TD–GC–MS. The inset highlights some of the lower-level compounds identified.

The information obtained in this case illustrates the power of TD and associated sampling techniques to provide quick yet comprehensive analyses of foodstuffs, for improved understanding of aroma profiles and product quality.

Typical Analytical Conditions

Sample: 5 g pre-packaged fresh basil leaves.

Dynamic headspace (Micro-Chamber/Thermal Extractor): Flow rate: 50 mL/min for 20 min. Chamber temperature: 40 °C.

TD (TD-100): Tube (Tenax TA): Desorbed at 280 °C (10 min). Trap (Tenax TA): Analytes trapped at 20 °C, desorbed at 290 °C (3 min). Split ratio: Inlet 2:1, Outlet: 16:1.

Analysis: Single-quadrupole GC–MS operated in full-scan mode (*m/z* 45–600).

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Quick and Convenient Comparison of Curry Powders Using Direct Thermal Desorption with GC–MS Analysis

Caroline Widdowson, Gareth Roberts, and David Barden, Markes International

Herbs and spices are used in many food preparations, and identifying the differences between samples is of particular interest to manufacturers, both for ongoing quality control and to compare their products against competitors. However, the volatile organic compound (VOC) profiles of such samples often differ in the relative abundance of key components, and these differences can be difficult to assess by traditional methods such as solvent extraction, equilibrium headspace, or solid-phase microextraction (SPME).

In this application note we show the value of direct thermal desorption (TD) with analysis by gas chromatography–mass spectrometry (GC–MS) for assessing aroma profiles from small samples of curry powder.

Thermal Desorption

TD uses heat and a flow of inert gas to desorb VOCs and semi-volatile organic compounds (SVOCs) from sorbents or sample materials. Extracted vapours are swept onto an electrically-cooled focusing trap, which is then rapidly heated to inject them into a gas chromatograph (GC).

TD offers many advantages over conventional solvent-based sample preparation methods such as liquid extraction. These include wider analyte range (from acetylene to n-C_{44} and reactive species on one platform), quantitative re-collection of split flows for repeat analysis and simple method validation, and enhanced sensitivity.

In this study, the TD-100™ automated cryogen-free thermal desorber from Markes International was employed, which has capacity for 100 industry-standard tubes.

Direct Desorption

Of the numerous TD-compatible sampling procedures, direct thermal desorption is the most straightforward and cost-effective for small quantities of relatively homogeneous, finely-divided materials — for example, therapeutic drugs, packaging materials, resins, spices, ointments/creams, polymers, water-based paints, and edible fats.

The material is simply weighed into an empty 3½-inch × ¼-inch TD tube, and heated directly within a thermal desorption instrument, followed by direct injection into the GC system. In this way, sample preparation is essentially reduced to zero, and the associated risk of introducing errors is eliminated.

Analysis of Curry powder

To illustrate the usefulness of direct desorption, Figure 1 shows the results obtained by direct desorption and TD–GC–MS analysis of two brands of curry powder. The range of analytes is very similar, but there are substantial differences in relative abundance. In particular, the cheaper brand (top) shows much higher quantities of linalool (#11), camphor (#12), and estragole (#13) compared to a mid-range brand (bottom), but lower concentrations of cuminaldehyde (#14) and caryophyllene (#19).

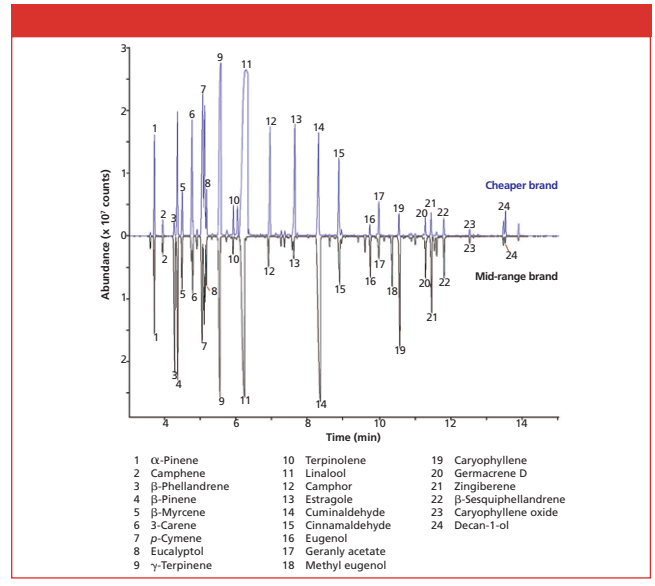

Figure 1: Direct desorption of two brands of curry powder, with analysis by TD–GC–MS.

This analysis exemplifies how direct desorption can enable quick, robust analysis of multiple samples, which make it of considerable value to food analysts for quality control and product comparisons.

Typical Analytical Conditions

Sample: Curry powder (~50 mg), placed in an empty TD tube.

TD (TD-100): Tube: Desorbed at 50 °C (3 min). Trap (Material emissions): Analytes trapped at 10 °C, desorbed at 280 °C (5 min). Split ratio: Outlet 25:1.

Analysis: Single-quadrupole GC–MS operated in full-scan mode (*m/z* 45–600).

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Protecting Consumers Against Pesticides: Development of a Fast and Simple LC–MS Pesticide Screening Method

Dr Gesa Johanna Schad, Shimadzu Europa GmbH

Although pesticides protect crops and plants, they are known to be harmful and of toxicological significance. In order to ensure the safety of the food chain and to protect consumers, fast and reliable methods for the qualification and quantification of residual chemicals, contaminants, and pesticides in food and feed are essential. This article describes the method development and optimization of a fast and simple high performance liquid chromatography–mass spectrometry (HPLC–MS) assay for the separation, qualification, and quantification of common pesticides. A quick and accurate method scouting workflow was achieved by using a special Method Scouting Solution control software and a robust, high-pressure resistant column switching system to switch mobile phases and columns automatically.

Introduction

Pesticides, as their name says, are developed to kill "pests" and are used in agriculture to protect plants and crops from detrimental influences. They can be separated into different groups such as herbicides, insecticides, bactericide, insect repellent, animal repellent, or fungicides; all are known to be harmful and of toxicological significance. Pesticides are suspected to damage the nervous system, the hormonal system, and DNA, or to cause cancer.

Annually, more than 200,000 tons of pesticides are applied in the European Union. In 2012, the world market had a volume of sales of 36.3 billion Euros. Since the 1950s, the amount of pesticides has multiplied.

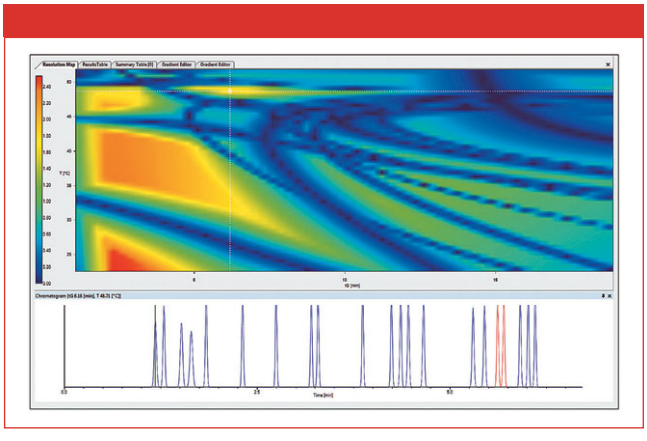

Figure 1: Colour-coded DryLab resolution map for UHPLC method development.

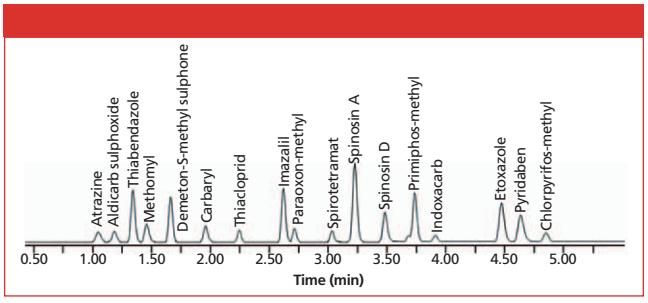

Figure 2: Typical chromatogram of the UHPLC analysis of pesticides as predicted by DryLab®.

Mineral fertilizers and pesticides are dispersed freely on fields and plantations and their residues or degradation products remain in our fruits, vegetables, and cereals. Besides the risks to farmers applying the chemical substances, pesticides are particularly dangerous to babies, children, and expectant mothers, as well as the average consumer.

In order to protect consumers, fast and reliable methods for the qualification and quantification of residual chemicals, contaminants, and pesticides in food and feed are essential to ensure the safety of the food chain.

Efficient HPLC Method Development

This article describes the method development and optimization of a fast and simple HPLC–MS assay for the separation, qualification, and quantification of common pesticides.

For the development of a fast and reliable LC–MS screening method for residual pesticides, a Shimadzu Nexera X2 Method Scouting System was used, consisting of two quaternary solvent pumps (LC-30AD), a 20 µL solvent mixer, an autosampler (SIL-30AC), and a column oven (CTO-20AC) including a six column switching valve (FCV-34AH). The system was also equipped with a Shimadzu LCMS-2020 single quadrupole mass spectrometer via an ESI source. The different mobile and stationary phases used for method scouting for the separation of 20 common pesticides are displayed in Table 1.

Method scouting was performed in an overnight sequence using gradient runs from 10 to 98% B in 6 min at 40 °C with combinations of aqueous and organic mobile phases on the five columns specified in Table 1. The most promising mobile phase/stationary phase combination was then used for computer simulation using DryLab method optimization software to identify the optimum separation conditions with respect to gradient slope and oven temperature.

Table 1: Mobile and stationary phases used in method scouting.

Solvent	Column
Mobile phase A	
A1: 0.1% Formic acid	C18 column, 100 × 2.1 mm
A2: 10 nM $HCOONH_4$ in H_2O	C18 column + aromatic selectivity, 100 × 2.1 mm
Mobile phase B	Pentafluorophenyl bonded phase, 100 × 2.1 mm
B1: Acetonitrile	Polar embedded C18 column, 100 × 2.1 mm
B2: Methanol	Reversed phase cyano column, 100 × 2.1 mm

Results

A total of 40 different chromatographic conditions were evaluated for ideal separation and detection conditions. Best peak intensity, peak shape, and separation were obtained with a mobile phase combination of 0.1% formic acid and acetonitrile on the pentafluorophenyl bonded stationary phase. These conditions were used to create a two-dimensional DryLab model using 5 and 15 min gradient runs at 25 °C and 50 °C as input data. These experiments resulted in a colour-coded resolution map for simple identification of the optimum separation conditions (Figure 1).

The software predicted an optimum separation with a minimum resolution of the critical peak pair of 1.6 in a gradient run from 10 to 75%B in 3 min at a flow rate of 0.5 mL/min at 35 °C.

Conclusion

The Nexera X2 method scouting system in combination with computer simulation software is an ideal tool for quick and efficient development of reliable, fast UHPLC methods. A robust, selective, and sensitive separation of 17 commonly used pesticides in 5 min was established successfully within two working days. The automated workflow starting with a method scouting experiment and further optimization using a computer simulation software package saved time and offered visualization of the design space in a resolution map to establish the most robust separation method. The use of highly sensitive and selective LC–MS detection was feasible using the ESI source and facilitated peak assignment.

Figure 3: Application Handbook "Food, Beverages, Agriculture".

The new Shimadzu Application Handbook "Food, Beverages, Agriculture" (Figure 3) combines real life applications and most advanced technologies and solutions on consumer and product safety. All major analytical instrumentation methods are covered, such as chromatography, mass spectrometry, spectroscopy, life science lab instruments, sum parameter (TOC/TN), and materials testing & inspection. Over 200 pages, "Food, Beverages, Agriculture" covers 58 real-life applications related to hot subjects such as food scandals, which have recently alarmed consumers all around the world. The book is free of charge and can be downloaded on http://www.shimadzu.eu/food-beverages-agriculture or by using the QR code.

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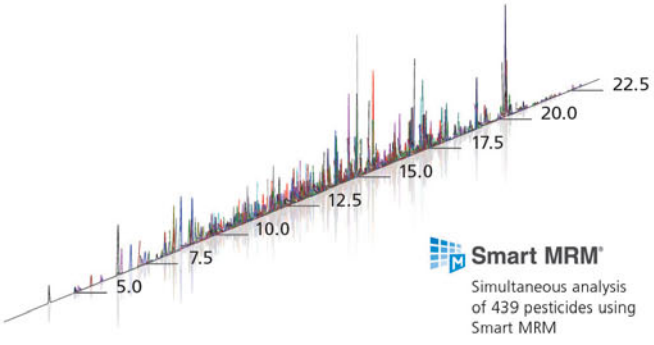

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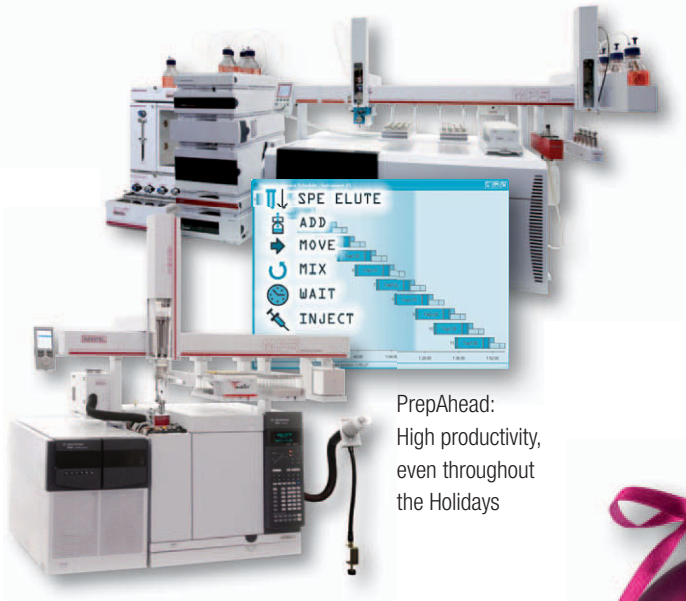

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