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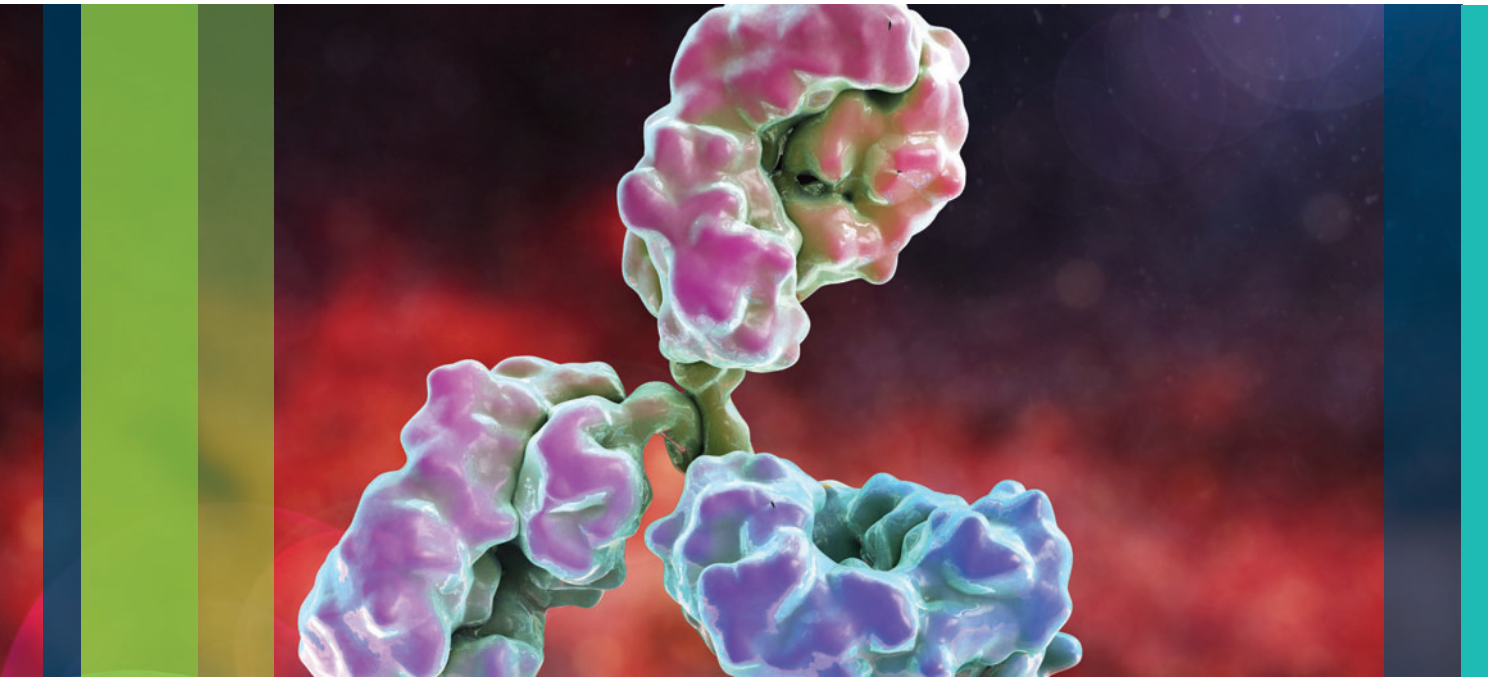

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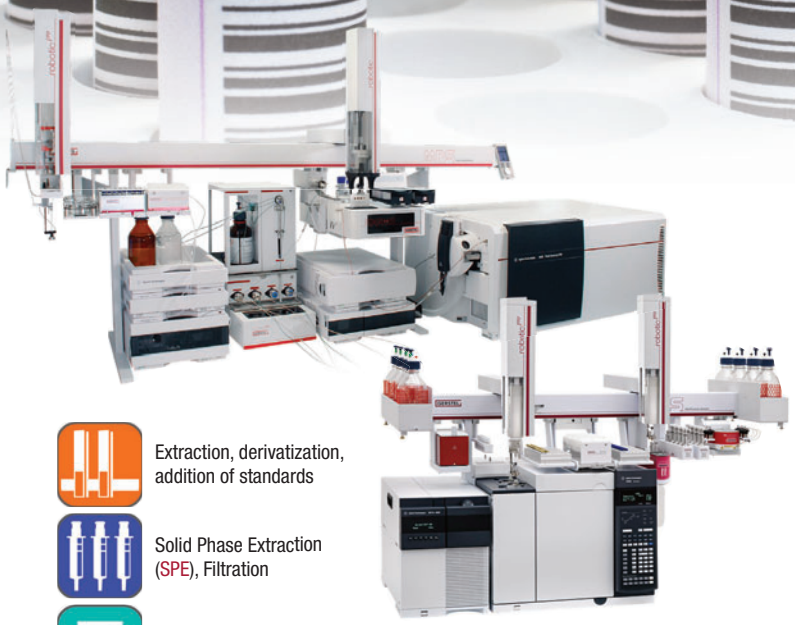

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COLUMN WATCH

Measurement of Interstitial Space Dispersion in Packed Bed Columns: Comparison of Superficially Porous and Fully Porous Particles

The difference in packing quality of superficially porous and fully porous columns has been measured using the total pore blocking technique. This technique physically eliminates all mass transfer band broadening contributions, and hence provides the purest possible measure of the packing quality of a column. The measurements confirm earlier assertions made in the literature about the generally better packing quality of superficially porous columns over fully porous particle based columns.

Kim Vanderlinden, Ken Broeckhoven, and Gert Desmet

Producing reduced plate height curve minima that are typically on the order of 0.5 units lower than possible with fully porous particles (FPPs), the (re-)introduction of superficially porous particles (SPPs) around the year 2007 has absolutely revolutionized the efficiency of HPLC columns (1–6). Part of this higher efficiency (roughly some 40 to 50%) can be attributed to the fact that SPPs exhibit a lower longitudinal diffusion band broadening (presence of the core partially blocks the longitudinal diffusion, and most SPPs have a lower internal porosity, that also tends to slow down diffusion) as well as a lower stationary phase mass transfer resistance (presence of the core minimizes internal diffusion distances). The latter, however, typically accounts for some 5 to 10% of the observed gain, which is considerably smaller than claimed in the advertisements of some SPP manufacturers.

The remaining 40 to 55% of the gain, therefore, necessarily needs to be attributed to the fact that SPP columns display a significantly lower eddy-dispersion (so-called *A*-term band broadening, describing the packing heterogeneity). The reason underlying this prior, unexpected effect is a problem that has not been fully resolved up to now. There have been speculations about differences in surface roughness, leading to differences in packing quality (3). Another proposed explanation was that SPPs have a markedly narrower particle size distribution than FP particles, which can, in turn, be expected to lead to more uniform packings. This explanation has also been the subject of considerable controversy (3,7,8). Nevertheless, the fact that the introduction of FPPs with a narrower PSD also lead to a marked decrease in *h*-values has now recently brought new evidence to support this hypothesis (9).

Traditionally, the eddy-dispersion is determined by subtracting known (or estimated) values of the longitudinal diffusion and the mobile and stationary phase mass transfer resistances (so-called C_m- and C_s- term; see equations 2 and 3) from the measured plate height. However, the model for the mobile phase mass transfer resistance is still under debate, and the observed eddy-dispersion plate heights tend to depend on the retention factor and the intraparticle dispersion of the analytes, complicating the direct comparison of SPP and FPP columns.

In the present contribution, the mass transfer resistance contributions are physically switched off using the so-called *total pore blocking* (TPB) technique to render the mesoporous space of the particles completely inaccessible for the injected analytes (10,11). As a consequence, the mass transfer processes are blocked, and the only remaining source of band broadening (apart from the longitudinal diffusion) is the heterogeneity of the interstitial space.

In brief, the TPB method works as follows: First, the column is flushed with a solvent, such as isopropanol, that is fully miscible with both hydrophobic and hydrophilic liquids. Subsequently, the column is filled with a strong hydrophobic solvent, such as decane, which can fully replace the isopropanol in both the interstitial space as well as in the particle mesopores (Figure 1a). Next, the decane is pushed out of the interstitial space again, using a hydrophilic buffer that is immiscible with the decane. Due to the high affinity of the decane for the C18 layer inside the mesopores, the decane cannot be removed from the particle's interior (Figure 1b). Injecting now a strongly hydrophilic marker (such as potassium iodide), this marker will not be able to penetrate the particles, mimicking (at least for the marker) a completely nonporous column.

The TPB technique was originally introduced to make accurate measurements

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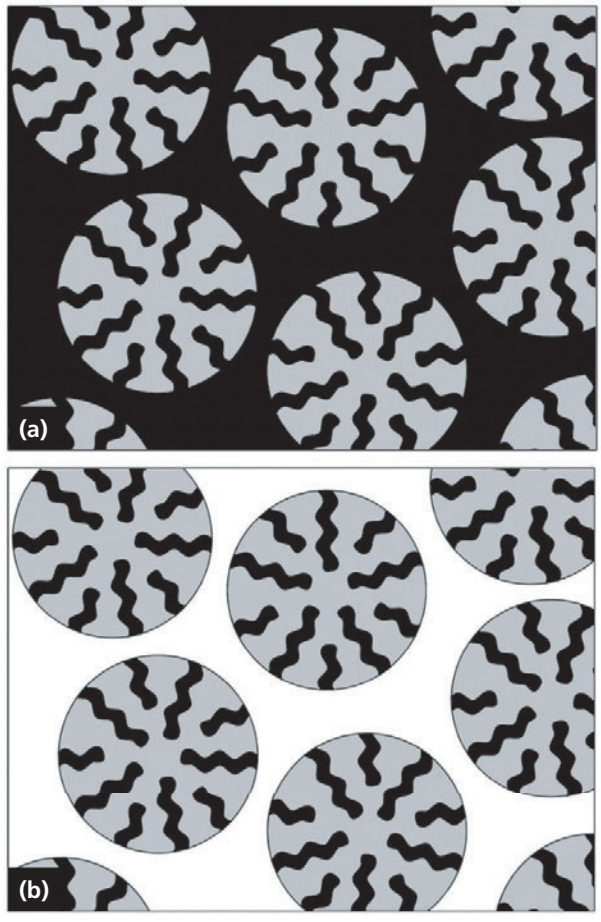


FIGURE 1: Schematic representation of the total pore blocking principle showing the column composition (a) after the column is completely filled with decane, and (b) after all the decane is chased out of the interstitial space by flushing the column with an immiscible hydrophilic buffer.

of the external porosity (ε) of packed columns (10,11). Conventionally, this is measured using so-called *inverse size exclusion chromatography* (ISEC). Whereas the polystyrene standards used in ISEC have difficulties accessing the smallest spaces of the interstitial volume, and thus require an extrapolation to assess the full extent of the interstitial space, the TPB method works with small molecule tracers that can explore the entire space.

Since its introduction, TPB has been used frequently by others (9,12–16). Here, we report on the TPB measurement to investigate differences in interstitial space dispersion between FPPs and SPPs. This was done on a set of 4.6 mm x 150 mm columns packed with 5 µm particles. These dimensions were purposely selected to minimize contributions from extracolumn sources, because the peaks under TPB conditions are eluted at zero retention (as a matter of fact, they are even eluted before the t_0-marker of the unblocked column), and therefore are very narrow.

Experimental

All experimental work was performed on an Agilent 1290 Infinity UHPLC system with a 1260 DAD, equipped with an 80 or 500 nL detector cell (Agilent Technologies). All columns used in this study were 4.6 mm x 150 mm columns, packed with either FPPs or SPPs with a diameter of 5 µm and C18-derivatized. Excel (FP_A) and Ultracore (SP_A) columns (ACE), as well as Luna (FP_B) and Kinetex (SP_B) columns (Phenomenex) were compared. Van Deemter measurements on the unblocked columns were performed at flowrates ranging from 0.05 to 2 mL/min, with a mobile phase of 50 *v/v*% (Ultracore) or 49 *v/v*% (Excel) acetonitrile in water, to obtain the same retention factor for butyrophenone. A mixture of potassium iodide, acetophenone, propiophenone, and butyrophenone (Sigma-Aldrich), all dissolved to a concentration of 100 µg/mL in the mobile phase solvent, was used as the sample to study the column under retained (open-pore) conditions. In order to block the columns, they were flushed with IPA (Biosolve B.V.) at a flowrate of 0.2 mL/min for 60 min, subsequently filled with decane (Acros Organics) at a flowrate of 0.2 mL/min for approximately 100 column volumes, and finally flushed with a 10 mM ammonium acetate (Sigma-Aldrich) buffer (pH = 3) at a flowrate of 0.4 mL/min (10). Retention times under blocked pore conditions were measured by injecting 500 µg/mL of potassium iodide dissolved in the buffer every 5 min during this final flushing step. The same buffer and potassium iodide sample were used to measure the van Deemter curves on the blocked columns. All measurements were performed at 30 °C, at a wavelength of 254 nm and a frequency of 40 Hz, and the injection volume was set to 1 µL. Peak widths were measured at half height and 4.4% height (5σ).

Reduced van Deemter plots were calculated using the nominal value of the particle size as mentioned by the manufacturer ($h = H/d_p$). The calculated values of the true particle size were in very good agreement with the values provided by the vendor for the particle batches. The particle size estimated based on the experimentally measured permeability and porosity measured on the different columns varied at most 10% from their nominal size. The columns of the same type from the same vendor had a difference in particle size less than 3%, based on the experimentally measured permeability and porosity. Furthermore, since only a relative comparison of column performance between columns of the same vendors are discussed, the true value of the particle has no impact on the conclusions. For the diffusion coefficients, experimentally measured values reported in (17) were used, for the given

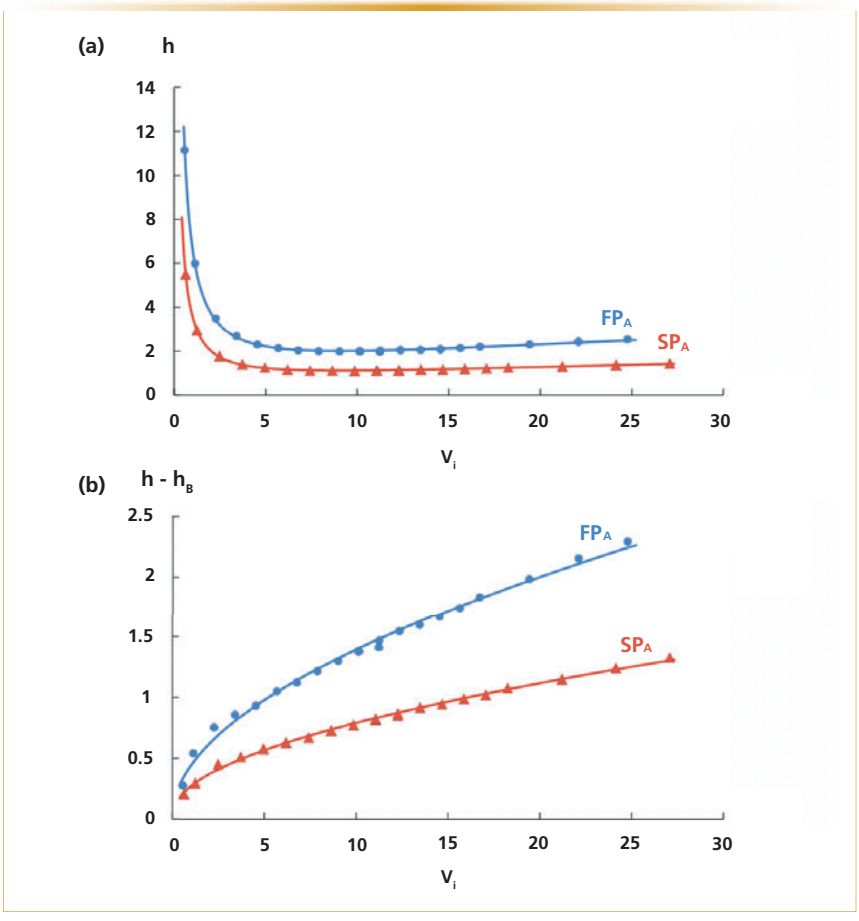


FIGURE 2: (a) Reduced plate height plot, and (b) *B*-term subtracted reduced plate height plot for acetophenone (k' = 1.6) on the FP_A and SP_A columns before blocking. Curve fits according to equation 1. FP_A: blue dots and SP_A: red triangles.

TABLE I: Fitted parameter values for (equation 1) of all columns. For the blocked columns the *C*-term was set at $C = 0$ as mentioned in the text, indicated with an asterisk.

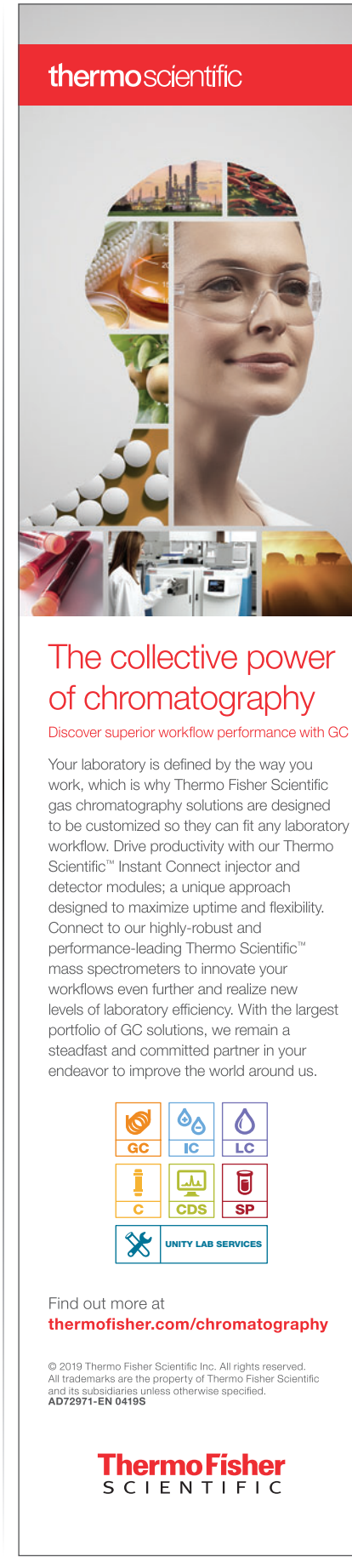
Name	A	B	C	n
FP_A	0.43	6.23	0.0094	0.48
SP_A	0.26	3.31	0.0070	0.44
Blocked FP_A	1.01	1.16	0*	0.32
Blocked SP_A	1.18	1.03	0*	0.14
Blocked FP_B	0.82	1.03	0*	0.30
Blocked SP_B	0.58	1.24	0*	0.24

component and employed mobile phase composition ($\nu_i = u_i \cdot d_p / D_m$), with D_m equal to 1.2 x 10^{-9} m^2/s for acetophenone, and 2.1 x 10^{-9} m^2/s for potassium iodide.

Results and Discussion

Unblocked Pore Dispersion

First, the columns were tested under retained component conditions (with normally accessible mesopores). The resulting reduced van Deemter curves for acetophenone are shown in Figure 2a. The curves display the typically observed difference between both particle types: Whereas the FPP column produces a minimal reduced plate height of h_{min} = 2.1 (indicative of a well-packed column [18]), the SPP column produces a significantly lower minimum (h_{min} = 1.2). These minima are obtained at an optimal velocity around



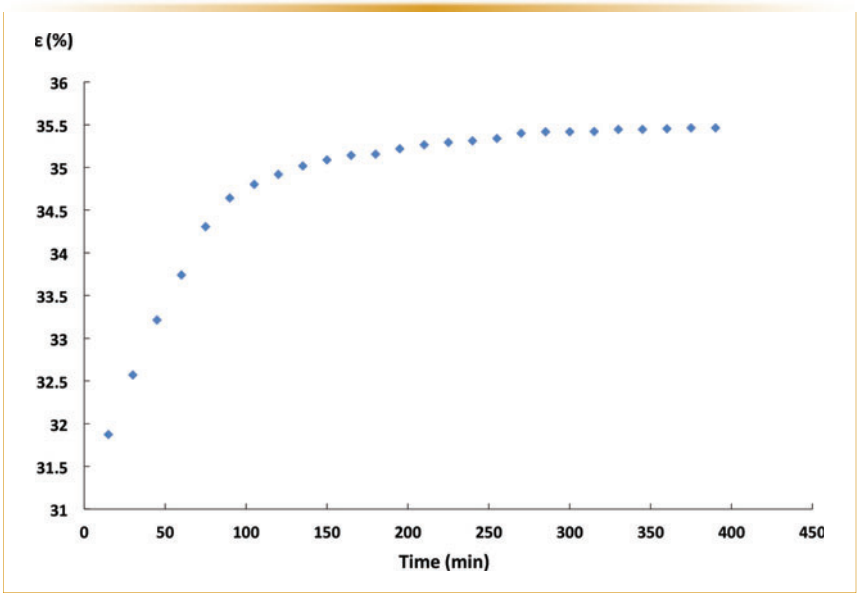


FIGURE 3: Plot of the external porosity versus time during the flushing step of the total pore blocking method of the SP_B column.

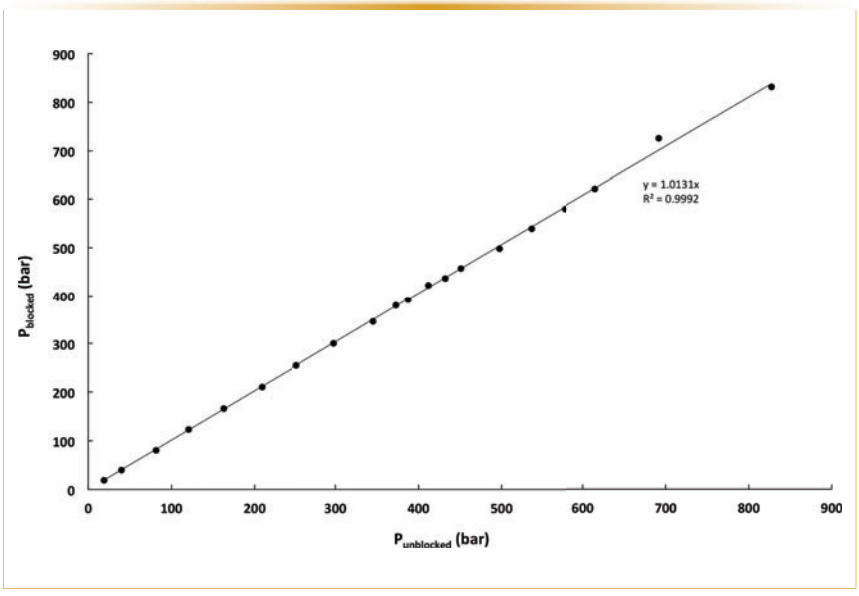


FIGURE 4: Plots of the measured pressures after blocking the column versus the measured pressures before blocking of the column for the SP_A column.

ν_{opt} = 9 for both the FPP and the SPP column. A similar difference (on the order of 0.8 to 1 reduced plate height units) is observed for the other, more strongly retained test analytes (data not shown). For the stronger retained compounds, the absolute h_{min} values are also shifted to a larger value because of the larger *B*-term contribution typically observed for components with a higher *k*. For the same reason, the optimal velocity is shifted to a somewhat larger value with increasing retention.

The data were fitted using the free-exponent Knox-model (19), producing a good fit (R^2 = 0.98–0.999):

$$h = Av_i^n + B/v_i + Cv_i \quad [1]$$

This model has been preferred over the more frequently used fixed-exponent Knox model (where *n* is fixed at *n* = 0.333). The original data set on which the classic *n* = 0.333 value was based was rather limited, while later work showed the exponent itself contains relevant information on the packing and can vary considerably (cf. range between *n* = 0.5 and *n* = 1 in [19]). The resulting fitting parameters are given in Table I. During the fitting, the *C*-term constant was fixed at the C_s constant value found from equation 2, discussed further on. In agreement with its larger overall *h* values, the *A*-, *B*-, and *C*-term constants of the FPP column are clearly higher than those found for the SPP column. The *n*-exponent (order of *n* = 0.45 to *n* = 0.5) is typical for the behavior of a retained analyte in a packed bed column (19).

Figure 2b shows what remains of the observed band broadening when subtracting the contribution of the longitudinal diffusion. Because of the subtraction, all curves now tend to zero when ν_i tends to zero. As can be noted, the difference between the SPP and the FPP column is about 0.57 reduced plate height units at the ν_i = 9 (h minimum of the two particle types in Figure 2a). This shows that roughly 35% of the difference between the FPP and the SPP column can be attributed to the difference in the *B*-term. As explained in (20–22), the significantly lower *B*-term band broadening of superficially porous particles is due to both the presence of the core as well as that the fabrication processes of the majority of the vendors leads to a mesoporous shell layer with a relatively low internal porosity.

Using the fitted value of the *B*-term to deduce the diffusion coefficient (D_{pz}) inside the mesoporous zone of the particles using the effective medium theory (20), we respectively obtained a value of D_{pz}= 7.8 x 10^{-10} m^2/s (fully porous) and D_{pz}= 4.2 x 10^{-10} m^2/s (superficially porous). These values indicate the true transport rate inside the mesoporous material of the SPPs is significantly smaller (close to a factor of 2) than in the FPPs This has been observed in the past (20,23), and has been explained by differences in synthesis procedures and a concomitant difference in internal porosity. With these values, we

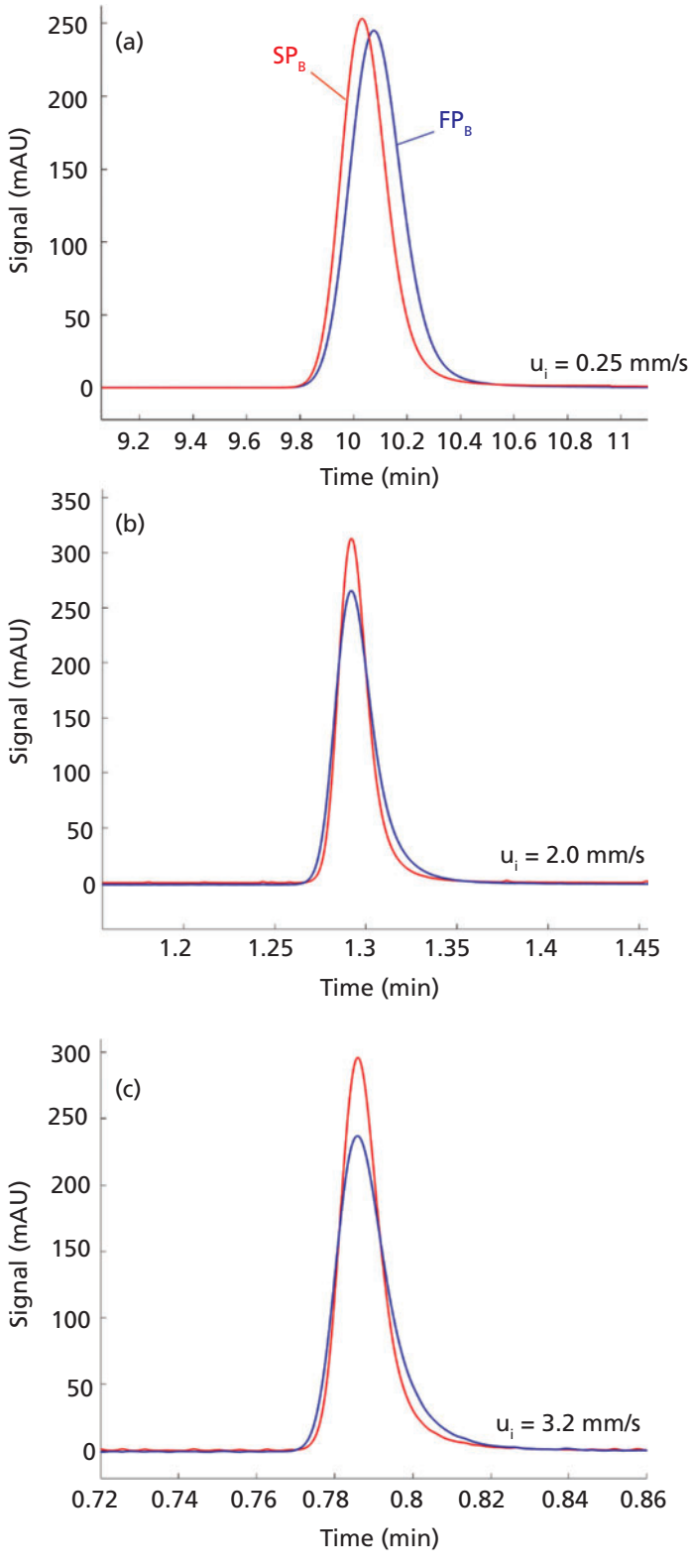


FIGURE 5: Overlay of the chromatogram peaks for KI of the blocked columns at three different interstitial velocities (a) u_i = 0.25 mm/s, (b) u_i = 2.0 mm/s, and (c) u_i = 3.2 mm/s. Blocked FP_B in blue and blocked SP_B in red.

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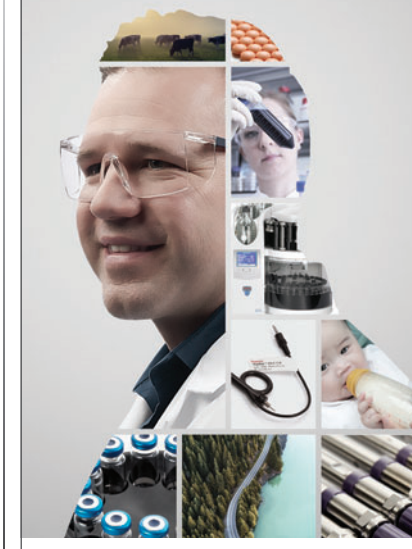

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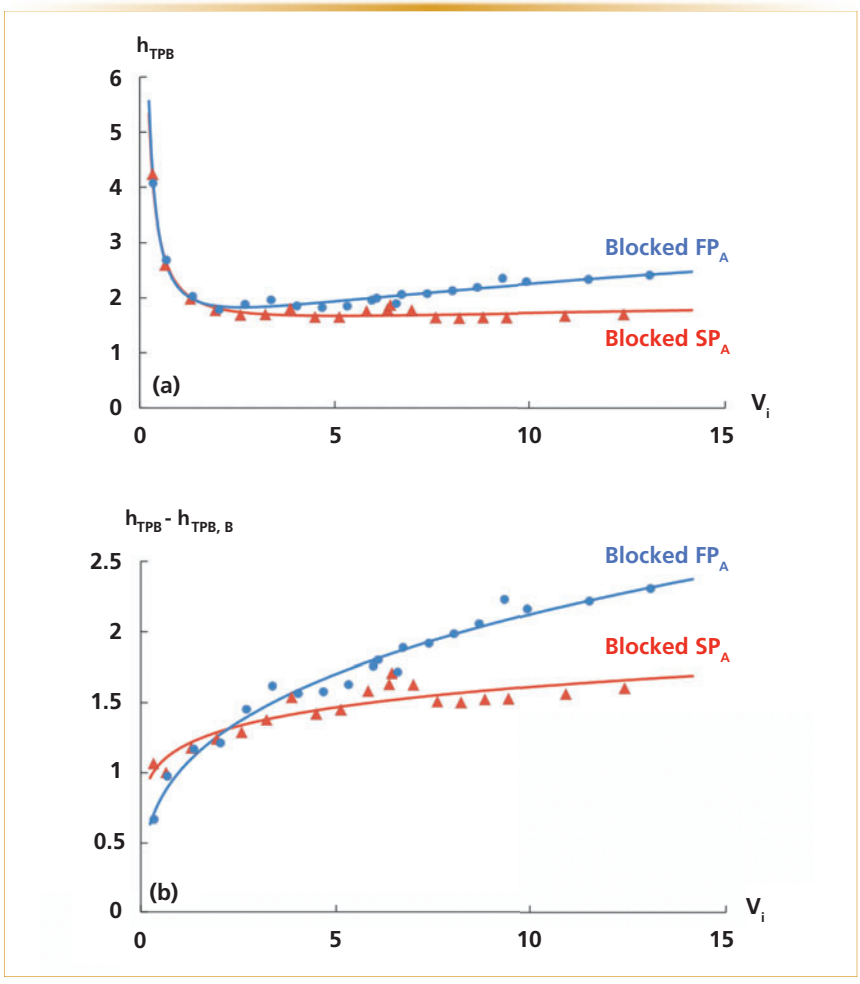

FIGURE 6: (a) Reduced plate height plot, and (b) B-term subtracted reduced plate height plot for *Kovats index* on the FP_A and SP_A columns after blocking. Curve fits according to equation 1. Blocked FP_A: blue dots and blocked SP_A: red triangles.

can now calculate the expected contribution of the intraparticle mass transfer resistance (h_{Cs}) (so-called C_s-term band broadening):

$$h_{Cs} = \frac{2}{\alpha}\frac{1}{Sh_{part}}\frac{k''}{(1+k'')^2}\frac{D_m}{D_{pz}}v_i \qquad [2]$$

with α, the shape factor, equal to 6 for packed bed spheres, k'' the zone retention factor ($k'' = (1 + k')\,(\varepsilon_T/\varepsilon) - 1$), ε and ε_T the external and total porosity, respectively, and Sh_{part} given by equation (T-35) in Andrés and associates (24).

From equation 2, it can be calculated that the difference in h_{Cs} around the optimal velocities ($\nu_i = 9$) is only on the order of about 0.025 reduced plate height units. (only about 3% of the difference in h_{min} observed in Figure 2a can be attributed to the presence of the core). Obviously, this effect is much smaller than the initial claims made by some manufacturers.

A similar exercise can be done to estimate the expected mobile zone mass transfer resistance (h_{Cm}) (so-called C_m-term band broadening):

$$h_{Cm} = \frac{2}{\alpha}\frac{1}{Sh_m}\frac{k''^2}{(1+k'')^2}\frac{\varepsilon}{1-\varepsilon}v_i \qquad [3]$$

with Sh_m given by equation 11 in Deridder and associates (25). With the known k''-value(s), and using $\alpha = 6$ for spherical particles and the ε-values determined below (see discussion of Figure 3), it can be calculated that the difference in h_{Cm} around $\nu_i = 9$ is on the order of about 0.02 reduced plate height units, corresponding to some 2% of the difference observed in Figure 2a.

Blocked Pore Dispersion

The above analysis implies the remaining difference must be due to differences in eddy-dispersion (such as due to differences in packing quality and interstitial space heterogeneity). To investigate and measure this difference in the absence of any intraparticle contribution (to have the purest possible measure of the dispersion in the interstitial space), the columns were subsequently blocked following the procedure established in (10). During the flushing step needed to make the gradual transition between condition 1 and condition 2 in Figure 1, the relative retention volume of the KI marker ($F{\cdot}t_i/V_G = \varepsilon$ with V_G *the* geometrical volume of the open column) is continuously monitored until this value reaches its plateau value (see Figure 3, for example). Reaching the plateau is indicative of the state (Figure 1b) wherein the interstitial space is completely cleared of the decane originally occupying it (Figure 1a). This event is typically also marked by the fact that the detector trace becomes flat and stable again, after a period of strong disturbances caused by small amounts of decane passing the detector.

A final control to verify whether the entire interstitial space is cleared of decane is made by verifying that the pressure needed to send a given flow rate F through the column in the blocked state is equal to that required in the unblocked state. If this is the case, it can be guaranteed the interstitial space is in both cases the same (10). This can be understood by writing the well-known Kozeny-Carman equation in the following form (26), showing that, for a given F, the measured pressure drop only depends on the external porosity, and is independent of the intraparticle porosity:

$$\Delta P = \frac{180}{d_p^2}\frac{(1-\varepsilon)^2}{\varepsilon^2}\frac{F}{A\cdot\varepsilon}\mu\cdot L \qquad [4]$$

wherein d_p is the particle diameter, ε the external porosity, F the flow rate, A the cross-sectional area, μ the viscosity, and L the column length.

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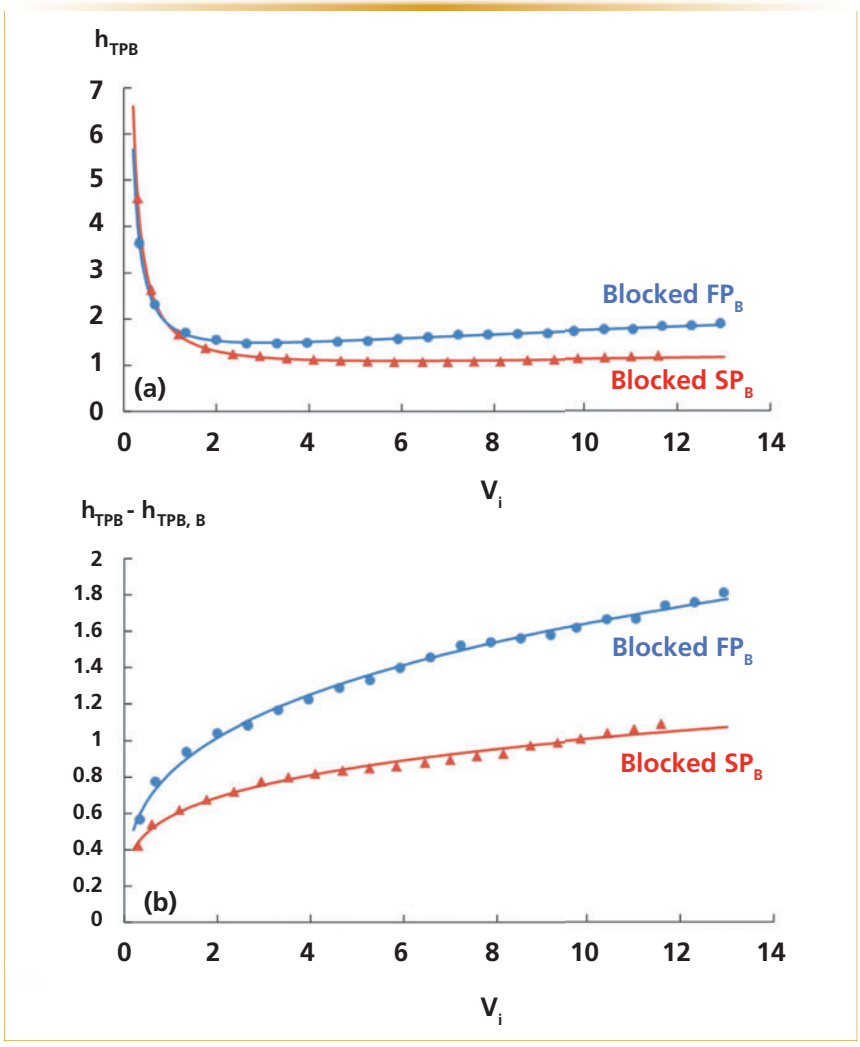

FIGURE 7: (a) Reduced plate height plot, and (b) *B*-term subtracted plate height plot for Kovats index on the FP_B and SP_B columns after blocking. Curve fits according to equation 1. Blocked FP_B: blue dots and blocked SP_B: red triangles.

If part of the interstitial space were still occupied by a remaining fraction of decane, this would reduce the accessible interstitial space, and the mobile phase flow would experience a smaller external porosity ε. As can be noted from equation 4, showing a strong dependency between ΔP and ε (close to a ε^{-5} dependency), even a small decrease in ε can already be expected to lead to a significant increase of the required inlet pressure. Figure 4 shows one of the pressure tests conducted in the plateau phase of Figure 3 to verify this. As can be noted, the measured pressures before and after blocking the column all lie close to the bisecting line, thus indicating the above criterion is satisfied, and indicating the interstitial space under blocked conditions is indeed identical to the actual interstitial space observed in the open-pore conditions.

Figure 5 shows some of the potassium iodide tracer peaks eluting from the column in the plateau phase, such as under perfect blocking conditions. The peaks eluting from the FPP column are clearly broader than those eluting from the SPP column over the entire range of different flow rates, indicating the former suffers from a significantly larger packing heterogeneity than the latter. The difference also clearly increases with increasing flow rate. This is mainly due to the fact that, at the lower flow rates, a significant part of the band broadening is due to longitudinal diffusion, which is to a very large extent is independent of the degree of packing heterogeneity (see also small differences between *B*-term values in Table I).

The observations in Figure 5 are more quantitatively and comprehensively represented in the reduced van Deemter plots (h_{TPB} versus υ_i) in Figure 6. These show a clear difference (of around 0.2 to 0.3 reduced plate height units) between the FPP and the SPP column also shown in Figure 2. The contribution of the extra-column dispersion can be estimated to be on the order of 5 μL^2; for example, about 5% at $\upsilon_i = \upsilon_{opt}$, and around 8% at the highest υ_i. The curves appears to cross for low υ_i, but this should not be emphasized too much, as it was recently shown that even small errors on the *B*-term constant result in large deviations in the estimated h-h_B values at low velocity ($\upsilon_i < 2$) (27).

An important observation from Figure 6 is that the h_{TPB}-values are of the same order (and even larger in the SPP case) than the *h*-values observed in Figure 2a. This implies the dispersion measured using the total pore blocking technique cannot simply be considered as a measurement of the eddy-dispersion contribution prevailing under normal open-pore conditions; therefore, it cannot be seen as a measurement of the first term of equation 1. This lack of correspondence is due to the fact that the total pore blocking technique is measuring the dispersion under different boundary conditions at the particle outer surface than in the open-pore case (no diffusion towards and through the particles in blocked pore case, equal diffusion fluxes and chemical potentials in the open-pore case). However, this does not imply the measured differences under total pore blocking conditions are not relevant. On the contrary, it is precisely the absence of any interference with the intraparticle and transparticle diffusion that makes the h_{TPB}-values the purest possible measurement of the packing homogeneity of a column.

The same comparison was also conducted for another set of FPP and SPP particle columns from a different vendor. In this case, two columns were tested per particle

type and the curves represented in Figure 7 show the average of the two columns.

The h_{TPB}-curves in Figures 7a and 7b have also been fitted with equation 1. This was done by keeping the *C*-term constant at $C = 0$ to represent the absence of any mass transfer to and in the particles. One interesting observation from the fitting parameters shown in Table I is that the difference between the *B*-term constants of the SPP and FPP particles has dropped below the significance level. This is again in agreement with the fact that longitudinal diffusion is known to be virtually independent of the packing heterogeneity. The actual *B*-values are somewhat smaller than the theoretically expected value around 1.5, but this can be attributed to the lack of a sufficient number of data points in the low υ_i-range. Another observation from Table I is that the *n*-values are significantly smaller in the blocked pore conditions than in the open pore conditions. A lower *n* indicates a larger contribution of velocity biases that are terminated by changes in the velocity field compared to those terminated by lateral diffusion. This makes perfect sense, given the blocked particles are now reducing the possibilities for transparticle diffusion equilibration.

Conclusions

The total pore blocking technique can be used to measure the interstitial space dispersion in the absence of any inter-, intra- or transparticle mass transfer contribution. Subsequently also removing the *B*-term contribution, the plots in Figures 6b and 7b clearly show that fully porous particle columns tend to have a higher interstitial space dispersion (for example, packing heterogeneity) than superficially porous particle columns. This was confirmed for two different vendors. The difference in dispersion, expressed in reduced plate height units, increases with increasing velocity. Around the minimum of the retained component van Deemter curve ($\upsilon_{opt} = 9$), the difference is on the order of 0.6 to 0.8 reduced plate height units, depending on the column manufacturer.

Given the very small peak volumes eluted under pore blocking conditions, the measurements are limited to columns with a large volume packed with large particles. The latter is also needed to prevent high pressures that would make the blocking agent leak or contract.

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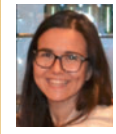

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LC TROUBLESHOOTING

Mind the Diluent: Effects of Sample Diluent on Analyte Recovery in Reversed-Phase and HILIC Separations

The sample solvent can have a big impact on peak shape in both reversed-phase and HILIC separations, especially when large volumes are injected. Diluting the sample with weak solvent can be an effective solution to mitigate this problem, but we have to be careful to not lose analytes of interest to precipitation or phase separation.

Dwight R. Stoll and Anne E. Mack

In the August 2019 installment of "LC Troubleshooting," I wrote about one of the hot topics that was discussed by several speakers at the The HPLC Meeting in Milan, Italy, in June—coupling of ion-exchange separations to mass spectrometric detection (1). This month, I'd like to discuss another topic addressed at the meeting. Anne Mack gave a talk that highlighted some things to consider when deciding whether to use reversed-phase or hydrophilic interaction (HILIC) liquid chromatography, especially when some of the components of the sample mixture at hand are hydrophilic. These considerations included the relative retention of the analytes of interest under reversed-phase and HILIC conditions, the effect of the sample diluent and injection volume and peak shape, and the effect of the sample diluent on apparent recovery of the analyte. The recovery aspect nicely complements the "LC Troubleshooting" article I wrote in January 2019 on the effects of the diluent on peak shape (2), so I've asked Anne to join me in writing this month's installment of "LC Troubleshooting."

Dwight Stoll

Liquid chromatography (LC) is an incredibly versatile analytical tool, enabling quantitative and qualitative analysis of diverse molecule types, ranging from highly hydrophilic and water soluble (for example, inorganic metal ions) to highly lipophilic molecules that are soluble in organic solvents (for example, lipids). The reversed-phase mode of separation is arguably the most versatile single mode of LC separation, and can provide retention and separation of analytes covering a wide range of water solubility. In an extreme case, it could separate molecules as hydrophilic as small organic acids (like succinic acid [3]), and as lipophilic as fat soluble vitamins (like vitamin A [4]). However, a practical problem we run into quickly is that molecules as different in properties as these will not be highly soluble in the same sample solvent. In the worst case, choosing an inappropriate sample solvent or diluent will lead to inaccurate results, particularly for quantitation, because the analytes of interest will not be soluble in the sample solvent, and the sample that is injected into the LC instrument will not be representative of the analytes actually present in the sample vial.

The problem described here is not new by any means. But, as we push the limits of analytical methods in the reach for more speed, simplicity, and sensitivity, we inevitably run into conditions that will "break" the method, leading to inaccurate results. Therefore, we have to be careful not to break our methods, and a deeper understanding of where the limits lie and the basis for them puts us in a better position to be successful in the long run.

Review: Effects of Sample Diluent and Injection Volume on Peak Shape

As users of LC, it is common to encounter situations where the samples presented to us from some prior process or step contain a sample solvent that is quite different from the mobile phase associated with the LC method used for the analysis of the sample. For example, when using solid-phase extraction (SPE) to preconcentrate low-concentration analytes from water samples, the last step in the SPE process typically involves elution of the analytes of interest from the SPE adsorbent using an organic solvent like methanol. Frequently, such extracts are subsequently analyzed by reversed-phase LC, with solvent gradient elution that starts with a water-rich mobile phase. On the other hand, we might be interested in analysis of the water-soluble components from a urine sample. In this case, the sample solvent is water, while the HILIC separation will start with a mobile phase containing a high fraction of acetonitrile on the order of 90%. These mismatches between the solvent composition of the sample and the mobile phase of the LC method can lead to trouble. In January of this year, we wrote about how the combination of the volume of sample

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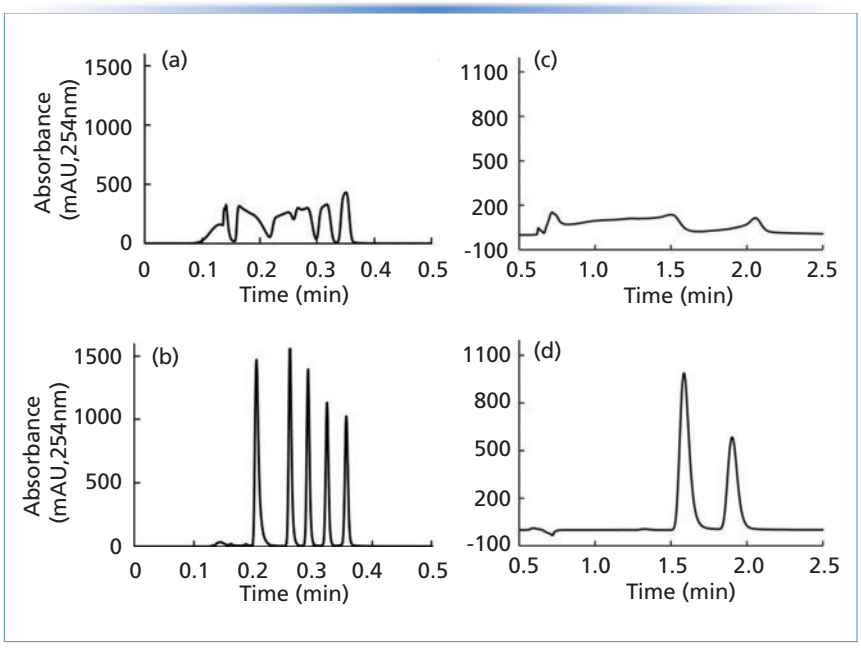

FIGURE 1: Examples of the effect of sample solvent on reversed-phase-LC (a,b) and HILIC (c,d) separations. The analyte mixture for the reversed-phase-LC separation was a series of alkylphenones, and a solvent gradient was used for elution starting at 50% acetonitrile. The analytes for the HILIC separation were cytidine and guanosine, and the mobile phase was isocratic with 90:10 acetonotrile:water buffer. Complete chromatographic conditions are given in reference (2). Figures are (a) sample solvent 70:30 acetonitrile:water, (b) sample solvent 30:70 acetonitrile:water, (c) sample solvent 0:100 acetonitrile:water, and (d) sample solvent 95:5 acetonitrile:water.

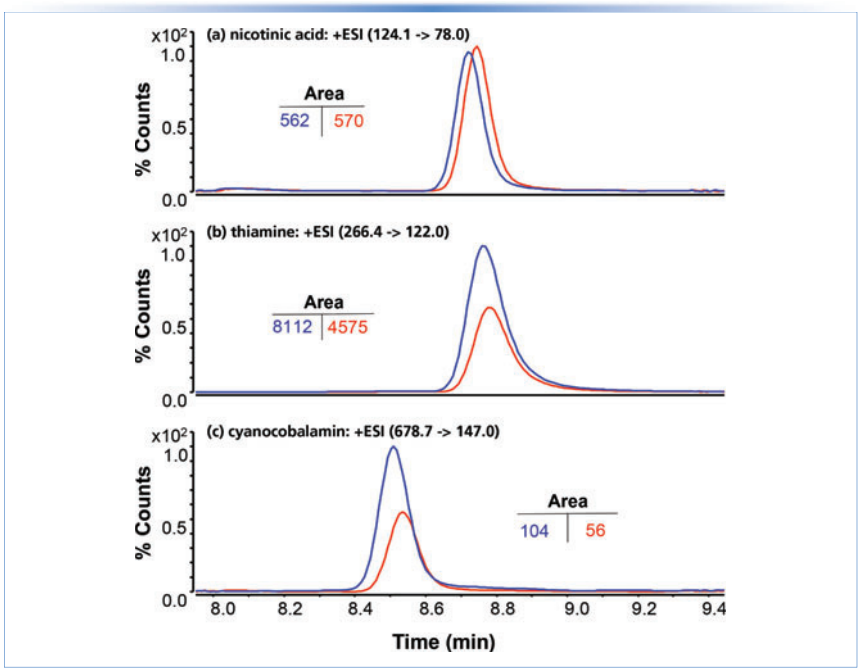

FIGURE 2: Comparison of peak areas obtained from HILIC separations for injected from samples prepared in either water (blue) or 90:10 acetonitrile:water (red). Chromatographic conditions: Solvent A: water, Solvent B: acetonitrile; gradient elution from 95–65% B in 10 min., with 10 mM ammonium acetate (pH unadjusted) throughout; Temperature, 25 °C; Flow rate, 0.5 mL/min.; Column, 100 mm x 2.1 mm i.d. Poroshell 120 HILIC-OH5 (2.7-µm); Injection volume, 0.5 µL; Detection by triple quadrupole mass spectrometry.

that is injected and the sample solvent composition can have a dramatic effect on peak shape. As a reminder of the data discussed in that installment, Figure 1 shows examples of bad results obtained under reversed-phase and HILIC conditions, and how much better separations can be obtained simply changing the sample solvent. In the case of the reversed-phase separation, terrible peak shape is observed (Figure 1a) for some simple alkylphenones when the sample solvent contains 20% more acetonitrile than the starting mobile phase used for the solvent gradient elution program. However, simply changing the sample solvent to contain 20% less acetonitrile than the starting mobile phase in the gradient leads to a much nicer separation (Figure 1b). Similar effects can be observed in HILIC separations as well. Figure 1c shows the terrible peak shapes that are observed when a completely aqueous sample is injected into a HILIC column when the mobile phase contains 85% acetonitrile. However, this separation can also be improved dramatically by simply changing the sample solvent composition to contain 95% acetonitrile, as shown in Figure 1d.

Recognizing the importance of the sample solvent composition, particularly when the injection volume is large relative to the column volume, several different groups have developed a number of approaches to address this issue. The simplest approach is to adjust the sample solvent composition offline—that is, by addition of "weak solvent" (for example, water in reversed-phase, or acetonitrile in HILIC separations) as part of the sample preparation process prior to the LC separation. For example, in the case of the SPE extract in methanol described above, one could simply dilute this extract with some amount of water prior to analysis.

Other approaches described over the years include:

- Online dilution of the sample with weak solvent in the autosampler needle; this is sometimes referred to as "sandwich injection" (5).
- Online dilution of the sample with weak solvent by deliberately adding a mixer between the sample injection point and the column inlet (6).

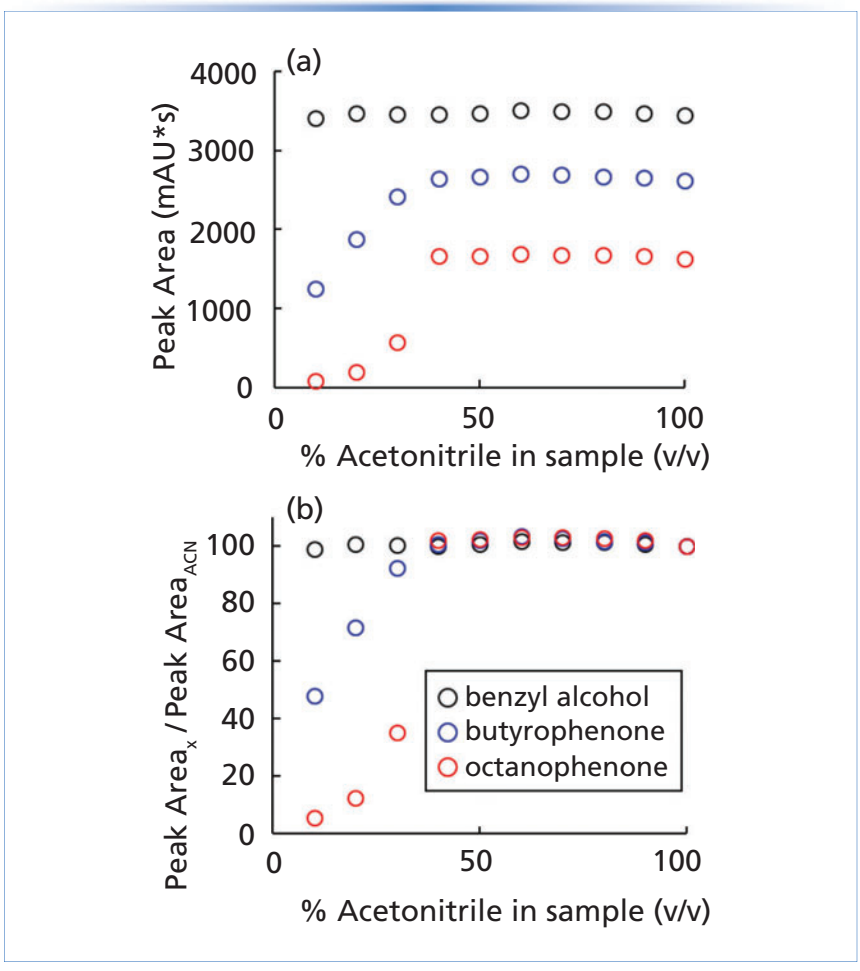

FIGURE 3: Effect of sample solvent on peak area observed for neutral compounds of varying lipophilicity under reversed-phase-LC conditions. Peak areas are plotted as (a) absolute values or as (b) a percentage of the area observed when the sample solvent is 100% acetonitrile. Chromatographic conditions: Solvent A: water, Solvent B: acetonitrile; Gradient: 10-100-100% B for 0-3.5-4.0 min; Temperature, 40 °C; Flow rate, 1.0 mL/min.; Column, 50 mm x 4.6 mm i.d. Zorbax SB-C18 (5-µm); Injection volume, 1.0 µL; Detection by ultraviolet (UV) absorption at 210 nm. The balance of the sample solvent was water, and the concentration of each analyte was 1 mg/mL.

- Online dilution of the sample with weak solvent during injection into the LC column—this is sometimes referred to as *at-column dilution*, and requires an auxiliary pump to deliver the diluent (7).
- Online dilution of the sample with weak solvent during injection by splitting the mobile phase flow path to achieve inline mixing of the sample and diluent (8).

While these approaches have been used with conventional one-dimensional LC (1D-LC), this issue is also very important in 2D-LC, and a variety of approaches have also been developed to address the problem in the context of 2D-LC specifically (9–12).

As with most challenges in chromatography, there is no perfect solution to this sample solvent matrix issue, and all of these approaches have advantages and disadvantages. Common to all of them, however, is the question, "How much dilution is enough?" This is obviously a very practical question that must be confronted in method development. Unfortunately, there is no universal answer that is suitable for all types of separation and analyte. Most often, the answer for a particular situation is determined by experiment, and typically the primary focus of such experiments is the effect of the sample solvent on peak shape. In this installment we address a secondary, but still important concern—analyte recovery.

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Effect of Sample Diluent on Analyte Recovery: HILIC Separations

As discussed above, one straightforward approach to address the effect of the sample solvent mismatch relative to the mobile phase is to simply dilute the sample with weak solvent, mix, and inject. However, we have to be careful that when the diluent is added the sample solution remains homogeneous. Two mechanisms that can result in a heterogeneous solution are: 1) precipitation of some analytes or matrix components such that solids and liquids are present in the sample; and 2) phase separation of some analytes or matrix components such that two or more liquid phases are present after adding the diluent. Both of these outcomes are undesirable, because the material sampled from these heterogeneous solutions will not be representative of the entire sample, and will lead to inaccurate quantitative results. For samples that contain analytes having similar physico-chemical properties, these outcomes can easily be avoided by doing a few scouting experiments to see when or if precipitation or phase separation occurs. However, avoiding these outcomes can be more challenging when the components of the sample are more diverse in terms of their solubilities in the sample solvent, particularly when the diluent is added.

Figure 2 shows an example of this problem in the context of use of a HILIC method for the analysis of a sample containing water soluble vitamins. For nicotinic acid (Figure 2a), the observed peak area is nominally same whether the sample is prepared in a matrix of 100% water or 90:10 acetonitrile/water. However, for thiamine and cyanocobalamin (Figures 2b and 2c, respectively), the observed peak areas in the 90:10 acetonitrile/water matrix are roughly 50% of the area observed when the sample is prepared in 100% water and all other conditions are the same. This suggests that thiamine and cyanocobalamin are not fully soluble in 90:10 acetonitrile/water, and some of the analyte precipitates from the sample matrix before it is sampled for analysis. In a case like this, the effect of the diluent on analyte recovery must be studied, and taken into consideration when deciding what sample solvent composition will be used for the final method.

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Effect of Sample Diluent on Analyte Recovery: Reversed-Phase Separations

The sample diluent can adversely affect analyte recovery in reversed-phase-LC separations as well. To illustrate this effect, we prepared an analyte mixture of benzylalcohol, butyrophenone, and octanophenone—all at 1 mg/mL—in different sample solvents ranging from 10:90 acetonitrile/water to 100% acetonitrile. The predicted water solubilities for these compounds are 15, 0.34, and 0.0037 mg/mL, respectively (www.chemicalize.com). Based on this, we would expect to see consistent peak areas over the full range of sample solvent compositions for benzyl alcohol. On the other hand, we would expect to see consistent peak areas for the lipophilic butyro- and octanophenones in the samples with high levels of acetonitrile, but lower areas in the water-rich samples, because of the low water solubilities of these molecules. Figure 3a shows the absolute peak areas measured for the three compounds at each solvent composition; Figure 3b shows the same data, but normalized to the area observed with the sample prepared in 100% acetonitrile. As expected, we see consistent peak areas for benzyl alcohol across the entire range of sample solvents. Note that the areas for intermediate sample solvent mixtures are slightly greater than 100%. This is most likely due to the volume contraction of acetonitrile-water mixtures, given the way the samples were prepared (for example, for the 50% acetonitrile sample, 500 µL of water was added to 500 µL of acetonitrile, and this results in a mixture that has a volume of about 970 µL). However, for butyro- and octanophenone, we see that consistent peak areas are observed down to 40% acetonitrile, but then there is a precipitous decrease in the peak area, with the decrease more dramatic for octanophenone than for butyrophenone. This results from the formation of two phases in the samples with 30% acetonitrile or less because of the very low water solubilities of these compounds. Given that their densities are lower than that of water, there is probably a top layer of the solution in the HPLC vial that is enriched in these compounds, but not sampled by the autosampler needle, which samples from well below the liquid surface.

Closing Thoughts

In the everyday practice of LC, it is common to encounter samples where the sample solvent composition is very different from the mobile phase used in the LC separation. When injecting volumes of these samples that are large relative to the volume of the column itself, this can lead to poor peak shape. Several approaches have been developed to overcome this challenge, most of which involve

dilution of the sample with weak solvent prior to or during the injection step. While this can be an effective remedy for poor peak shapes, we must be careful to avoid analyte precipitation or phase separation of the sample as a result of adding too much of the weak solvent diluent. The level of diluent that is considered too much is compound dependent, but can be determined experimentally through simple screening experiments.

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GC CONNECTIONS

Essential GC Accessories

Most manufacturers ship gas chromatographs with a small collection of consumable parts and accessories, such as extra ferrules, inlet liners, or septa, and a few instrument-specific tools. In earlier times, some convenience items might have been included as well, like a bubble flow meter or a small set of tools, but now it's rare to find such things in the shipping boxes. This edition of "GC Connections" lists a number of essential items that should be on hand in every GC laboratory, their function, and how to use them effectively.

John V. Hinshaw

Every profession has its specialized tools. Those used in chromatography often are just as specialized as those used in computer repair or automotive work. Many of the tools and accessories that gas chromatographers keep on hand for installing, maintaining, and repairing their chromatographs are also found in plumbers', carpenters', and home toolkits. Wrenches, screwdrivers, pliers, and metal tubing cutters are some easily recognized examples. Other items such as dental instruments or paper correction fluid are familiar, but their use in the laboratory environment may not be immediately obvious. Still others, like a column flow meter, septum nut wrench, or a specialized fused-silica column cutter, aren't found outside the laboratory at all.

Here then is an updated list of today's tools and accessories, with some information on their use and significance. One or more specialty manufacturers offer many of the chromatography-specific items; just look at their catalogs or on-line offerings. I scanned through several, and gleaned some new items that I have included here.

Source of Gas for Measuring Unretained Peak Times

A butane lighter is a convenient source of hydrocarbon gas for measuring an approximate unretained peak time. Butane is effectively unretained at temperatures above 75 °C on liquid-phase coated columns with phase ratios above 50. Columns at low temperatures or with lower phase ratios (thick stationary films) may retain butane, and separate the traces of ethane and propane present in the butane fuel. Use the earliest observable peak for the best estimate of unretained peak time. Natural gas is mostly methane; if your laboratory has a supply of natural gas (mine doesn't), it makes a good substitute for a lighter, and is less retained than butane—just be sure to turn off the gas once you've filled a syringe with it. A lecture bottle of methane with a suitable pressure regulator is another excellent source of the unretained substance. Concentrations in the low percent range work well. At least one chromatography supplier offers a prefilled methane bottle with a low-pressure regulator for this purpose.

Hydrocarbons won't work for unretained peak time measurements with an electron-capture detector (ECD), because it doesn't respond. Instead, try loading the syringe with a puff from a pressurized can of dust remover such as Dust-Off, which contains 100% 1,1-difluoroethane. Other halocarbons will work as well; check the label to be sure there is an ECD-sensitive compound present.

Hydrogen or helium—whichever is not the same as the carrier gas—makes good unretained peak markers for porous polymer or molecular sieve columns that retain hydrocarbons strongly, plus these two gases should be readily available in most GC laboratories. Flame ionization detection (FID) will not respond to hydrogen or helium, but other detection methods such as thermal conductivity (TCD), pulsed discharge (PDD), or helium ionization (HID), the latter with hydrogen as the unretained substance, should respond well.

Remember to use the same inlet pressure and oven temperature to remeasure unretained peak times for any specific column; that is, one with the same dimensions, film thickness, and stationary phase for comparative purposes.

Caps, Capillary Column

Polymer caps for capillary columns establish an airtight seal for storage, so that air, moisture, and airborne contaminants cannot enter. I have used septa for this purpose, but there's a risk of breaking the column and puncturing a finger instead; this only happened to me once. Another approach is to seal the end of the column by melting it. This is not recommended, because it requires a hydrogen torch to achieve a high enough temperature, and it will cause stationary phase combustion by-products to migrate a significant distance into the column.

Icon Image: Joe Zugcic/Zugcic Photographers, Inc.

Correction Fluid

Use white correction fluid to mark the measured position on a column that corresponds to the correct column penetration depth into an inlet or detector. After inserting the column into the nut and ferrule and making a fresh cut on the column end, measure the depth and apply a small dab of correction fluid. For data integrity reasons, some regulated laboratories' policies don't permit correction fluid on site, although in the fully electronic laboratory this is moot. A septum into which a slot has been cut may be slid onto the column below the nut and ferrule to act as a positioning aid. Remove the septum before heating the column oven. A positioning gauge (see "Ruler" later in this column) is a good alternative. I also have used a black permanent marker for this purpose; just be careful not to get it on your fingers or clothing.

Cutters, Fused-Silica Column

The best fused-silica column cutting tool is the one that holds the column in an adjustable chuck, and cuts with a diamond chip as the operator rotates a thumb wheel. This tool also has a magnifying glass on the opposite end for inspecting the fresh cut for squareness and lack of burrs or hanging polyimide coating. A pen-like tool with a sapphire tip or ceramic scoring wafers or scribes that make a sharp cut on the column so that it may be broken cleanly in two are the best inexpensive alternatives. In any case, a fresh cut should be made and inspected just before placing the column into the inlet or detector, after sliding on the nut and ferrule.

Cutters, Diagonal

Diagonal cutters are used only for cutting electrical wires. They should never be used as a substitute for a tubing cutter. Don't even think about threatening your fused-silica column with one!

Cutters, Tubing

A large plumber's tubing cutter for 1/4-inch metal tubing, and a smaller one for 1/8-inch tubing, are used extensively during instrument installation and gas-supply setup. The correct 1/8-inch size cutters are available from some chromatography supply companies; don't try to use the mini cutters found in home supply stores—they're only good for those weekend plumbing projects. Keep a supply of new cutter blades on hand. These disks wear out rapidly, and a dull blade will distort the tubing diameter, and make it difficult to slide on the back and front ferrules. Power cutters with high-speed rotary abrasive cutting wheels are also available. These are mandatory for cutting 1/16-inch tubing for low dead-volume gas valving applications, and they work well for any tubing up to 1/4-inch diameter. After cutting and squaring the tubing (see "Tubing Reamer" and "Deburring Tools" later in this article), be sure to clean out the ends with a solvent, so that loose particles cannot get into the instrument components, or interfere with the passage of peaks through column ends.

Dental Mirror

A plastic dental mirror with a front-silvered surface makes it easy to examine the underside of an inlet fitting in the oven, or to check other inaccessible areas for loose or missing parts. The mirror also can be used to detect the flame in a flame ionization or flame photometric detector by observing condensation of emitted water vapor on the cool mirror surface. A shiny wrench is a good substitute for the mirror in this case.

Dental Pick

A dental pick is very handy for removing septa from septum nuts, and debris such as bits of graphite ferrule from fittings.

Eyedropper (Plastic)

I have a box of plastic eyedrops and they are in frequent demand. I use them to place small drops of isopropanol onto fittings for leak checks. Some types have rough volume indications more like a

pipette, and I have used them to make a crude dilution of qualitative test mixtures when accuracy wasn't required.

Files, Needle

An assortment of needle files can be used to pick out ferrules from fittings, as well as to remove burrs and shape the ends of metal tubing before it is connected to a fitting. Don't forget to clean off all traces of metal before connecting.

Flexible Magnetic Pickup

A flexible 2-foot magnetic pickup comes in handy when you drop a small part inside the instrument. Another similar tool has a three-jawed "claw" operated by a plunger, and it will pick up non-magnetic items, too.

Flowmeter, Electronic

An electronic flowmeter is an expensive investment, but I believe that it will pay for itself many times over with improved accuracy and precision over bargain-priced bubble flowmeters. I prefer the type of electronic meter that senses flow directly, and that allows the operator to select the type of gas in use, such as air, helium, or hydrogen. The option to calculate split ratios from the measured split vent and column flows is a handy feature, but many GCs now include this capability in their software.

Flowmeters, Bubble

If you use bubble flowmeters, keep two sizes on hand. The large size is good for measuring FID air or inlet split vent flows up to several hundred milliliters per minute. The smaller size is better for packed-column or hydrogen flame-gas flows in the 10 to 50 mL/min range. Don't try to use a bubble flowmeter to measure capillary column flows below 10 mL/min. The carrier gas will diffuse out of the bubble, and you will get a low reading. Measure the unretained peak time instead, and calculate the flow rate from it. Note that this calculated flow rate or the rate displayed by electronic pressure control will only be as accurate as the column dimensions the operator uses.

Flow Measuring Adapter

Manufacturer-specific flow adapters allow a flow meter to connect to the exact dimensions of a detector's exit. Short pieces of flexible tubing in various diameters are handy for connecting a flow meter to different size tubing (see "Tubing, Plastic and Rubber" later in this article).

Glass Wool Insertion/Removal Tool

This item is useful for those who must install glass wool in inlet liners, or for the hardy few who pack their own columns and use glass wool to hold in the packing. These days I find little use for it, because I use prepacked deactivated inlet liners instead.

Gloves, Lint-free

Polymeric nitrile or vinyl lint-free gloves are essential for column installation and detector maintenance, especially for high-sensitivity detectors such as mass spectrometric, electron capture, and helium ionization types. Handling the column inlet without gloves opens the door to contamination from finger oils and dirt, while cleaning the rods of a quadrupole mass-selective detector (MSD) without gloves is asking for trouble. I recall a particular MSD that exhibited poor sensitivity. A tear-down revealed a huge thumb print on the photomultiplier tube window, perhaps deposited there by a technician who had recently finished lunch.

Hexagonal Key Set

I keep several sets of hexagonal key wrenches and drivers in the toolbox. I have sets of the traditional right-angle Allen keys in both imperial and metric sizes, as well as a collection of hex screwdrivers of both types. Although used less often, a set of star wrenches is indispensable as well. These also come in spline and security variations, some of which I've accumulated over the years. Many of these are used in laboratory instruments instead of the more traditional Phillips screw head, so they are essential for performing maintenance.

High-Temperature String

High-temperature string is a useful item for use in a GC oven to restring capillary columns, attach column connectors to column cages, or hold the column in the GC oven. The string is made of materials that can withstand temperatures of up to 400 °C, or higher.

Inlet Liner Removal Tool

A tapered high-temperature silicone rubber tool on a metal holder does a good job of grabbing glass inlet liners and removing them without cracking or chipping the liner top. Most GC instrument manufacturers will supply specific tools and instructions for a particular inlet option.

Leak Detector, Electronic

An electronic leak detector is expensive, but it indispensable for finding small leaks around hot fittings or inlets on which a liquid cannot be used (see "Leak-Checking Solution" later in the article). The most sensitive type of leak detector uses a small pump to pull air from a probe through a thermal-conductivity cell. The presence of carrier gas or hydrogen changes the thermal conductivity and causes a change in the detector's readout compared to a reference air flow. Sensitivity for nitrogen carrier is limited. I also have a small handheld battery-powered leak detector that has a series of light-emitting diodes, which indicate the detected leak rate. This detector is great for carrying around in a laboratory like mine with lots of instruments and little clear bench space.

Leak-Checking Solution

In my toolbox, the only acceptable leak-checking solution is a small bottle of pure isopropanol with an eyedropper. Other solutions may contain material such as surfactants that can leak into the gas-supply lines or columns and cause ghost peaks or other contamination.

Magnifier

A small magnifier is used to examine freshly-made column or tubing cuts for burrs or uneven edges.

Manufacturer-Specific Tools

For each GC system, there are always some specialized tools. These are used,

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for example, to open up a split–splitless inlet, or to remove the inlet liner. Perhaps a special wrench is required for FID system flame jet replacement. Whatever the case, keep all such tools with their instrument; you will need them eventually. Some of the chromatography suppliers offer their own versions of these tools, which often are more useful than the freebie ones that come with the instruments.

Maintenance Kits

Kits consisting of a collection of the most often replaced parts and supplies are a good starting point with a new instrument. Available for inlets and detectors, these packages will get used almost immediately upon commissioning. After a while, I find it convenient to re-order only those items that are used frequently instead of purchasing additional kits. The kits commonly include inlet liners, ferrules, flame jets, gauges, cleaning items, adapters, and common tools for their removal and replacement.

Mini Flashlight

A mini-flashlight is very handy for inspecting the interior of inlets and detectors for obstructions, as well as for illuminating the oven interior. I prefer the type with the bulb on a flexible gooseneck. A small short LED flashlight, the kind that you might get as giveaways at conference booths, also makes an excellent GC oven light. No one yet has built a GC oven with a light that comes on when the door is opened. A flashlight also makes a good dropped small part finder, to locate ferrules, for example. Just place the flashlight horizontally on the laboratory floor, sweep it around, and look for the shadow.

Paintbrush

An artist's paintbrush with handle is handy to clean out debris from small areas inside detectors or inlets. Just watch that a fiber from the brush does not dislodge. It can also be used to apply leak-checking solution to fittings, although I don't recommended this practice, due to potential contamination of the gas stream with the leak-checking solution.

Paper Clips

Jumbo-size paper clips with smooth sides are convenient for blocking off inlet or detector fittings for testing purposes. Unbend the clip and attach it to the fitting with a nut and 1-mm i.d. graphite-vespel ferrule. With the column connection blocked off, you can pressure-check an inlet. A detector check can be run in this manner without column influences on noise or stability.

Pin Vise and Drills

A small pin vise and a set of drills can be used in an emergency to drill out a used ferrule, or to enlarge one that is too small to fit a column. Sometimes the small drills can help to remove a ferrule that is stuck in a fitting, or to remove debris from inside fittings or tubing ends.

Pliers

I keep some small needlenose pliers, a pair of larger multigrip pliers, and one pair of locking pliers in my toolkit. The larger gripping pliers are useful for holding a straight length of 1/8- or 1/4-inch metal tubing while cutting it, although I take care not to grip the tubing anywhere near a location where a connection is to be made, because the scratches from the pliers would make it impossible to get a good seal.

Press-Fit Connectors

Glass press-fit connectors make it easy to repair a broken column temporarily (until a replacement can be installed). These are available in many sizes to connect fused-silica tubing of the same or different diameter. They also connect a column with a retention gap. One manufacturer offers a vacuum–melting device that makes near-perfect connections.

Pressure Gauge, Inlet

I have a conventional 0–60 psig pressure gauge with a syringe needle attached that I can insert into an inlet through the septum. Once in a while, I need to check the inlet pressure this way, instead of relying on the instrument's gauges or electronic pressure readouts.

PTFE Tape

PTFE tape is used sparingly on tanks and interconnecting fittings where threads form the seal. Use two layers of tape, not more, and wrap them around the threads in the direction the nut tightens, so that the tape will be drawn into the fitting instead of getting pushed out. PTFE tape is never used in swage-type ferrule-sealed fittings, where it will only cause a leak, nor is it used at the high-pressure supply cylinder connection. Several types of this tape are available; be sure to select the right one.

Rinsing Reservoir for Capillary Columns

A rinsing reservoir may be useful for reviving bonded-phase capillary columns contaminated by soluble but nonvolatile sample residues. Solvent rinsing is intended for use only after bake-outs and trimming of about 0.5 m of the inlet end plus the exit portion that enters the detector heated zone have failed to restore performance. Use only the solvents that the column manufacturer recommends, and rinse only from the detector end to the inlet end to avoid depositing contaminants deeper into the column. Check and revalidate column performance after rinsing.

Ruler

A small metal ruler measures the correct column penetration depth into an inlet or detector. Don't use a plastic ruler, because it might melt in contact with heated inlets or detectors. For convenience, make marks on the ruler that correspond to the correct inlet and detector depths. Several manufacturers offer capillary column installation gauges with the appropriate markings.

Scissors

A good sharp pair of scissors comes in handy for opening packages of ferrules, or for making paper stars out of waste paper that's waiting to be recycled while watching for peaks to be eluted. Scissors are never to be used to cut fused-silica columns (but you can believe that I've seen someone try it).

Screwdrivers, Phillips-Head

I have three Phillips-head screwdrivers in the toolbox: large, medium, and small. The small one is part of a set of jeweler's screwdrivers with rotating handles. These are general purpose items that any laboratory should have on hand.

Screwdrivers, Slotted-Head

I also keep three slotted-head screwdrivers. The small one is useful for securing electrical connections to screw-type terminals.

Septum Puller

This is a specialized tool for removing septa that become stuck due to heat-adherence to the inside of the septum area of an inlet. I have also used a dental pick for this purpose. Avoid using a flat-bladed screwdriver or a knife, because these may scratch the metal surfaces that seal to the septum.

Static Pad

A static pad is a grounded, conductive plastic sheet onto which it is safe to place electronic components that must be protected from damaging electrostatic discharge. Any circuit boards removed from an instrument should be placed on a grounded static pad, or in a static-proof bag.

Static Wrist Wrap

A grounded static wrist strap prevents the technician from imparting a potentially harmful static discharge into instrumentation or components. Always wear one when working inside an instrument or removing components, and in all cases be quite sure that the instrument power has been removed while the instrument itself remains grounded.

Stopwatch, Digital

A digital stopwatch times bubbles in a bubble flowmeter, and also times an unre-

tained peak. It's often more convenient to use a stopwatch when setting up an instrument than to operate the chromatography data system for each test injection. Select a stopwatch with readout to 0.01 second. Some GC systems include a stopwatch function on the display that includes flow, split ratio, and linear velocity calculations. These days, I just use my smart phone's stopwatch and timer functions, and then its calculator to find flow rates or average linear velocities. Good phone applications are available with additional chromatography functions.

Swabs

Recently I have used polyester swabs, mostly intended for cleanroom use, for cleaning out inlets and detector bodies. Take care that the solvents used are compatible with the swab material. Methylene chloride, for example, will dissolve polyester readily. I don't like to use cotton-tipped swabs on a wooden stick. Not only will the cotton release fibers, but the wood may release higher molecular weight compounds into cleaning solvents.

Syringe

I keep two manual syringes for setup purposes. One 10-µL gas-tight syringe is for injecting methane or butane to measure the unretained peak time and ascertain that the flame is lit and carrier gas is flowing. The other is for making liquid test-mixture injections as part of a column checkout. Sample syringes are kept separately.

Syringe-Cleaning Wires

Syringe cleaning wires may be used in an emergency to clear septum particles or other debris from syringe needles. I recommend discarding stubborn contaminated syringes; take steps to keep the syringe clean instead.

Text Mixtures

Column and detector test mixtures verify column performance and detector sensitivity. Keep a fresh vial of each type on hand, and put them in the chemical refrigerator if the label so directs. Column test mixtures are available for polar and for non-polar capillary columns, and there are test mixtures for each detector type. Some manufacturers provide a detector test mix that combines components for testing several different detectors. Once opened, test mixtures can be kept for a while in septum-sealed vials. Their lifetime is limited, because of gradual evaporation. If you keep test mix in a vial, remove the vial cap rather than puncture the septum when withdrawing liquid for injection. Some laboratories find it more convenient to keep dilute test mixtures on hand, because these are more easily disposed of than the concentrated mixes. Many laboratories have their own qualification and validation standards, of course, but the manufacturer's mixtures allow easy comparison to the factory test results, and may be used for a factory-level checkout.

Tube Blender

I use this simple tool to make controlled bends of copper or stainless steel tubing for connecting the supply tanks to the filters, and then to the back of the instrument. It makes for a clean and organized looking installation. Tube benders come in sizes to fit standard tubing diameters.

Tube Reaming and Deburring Tools

These tools are used to remove burrs and irregularities from metal tubing after cutting. They are available for the standard tubing diameters, and I highly recommend using them in order to ensure leak-free connections. As always, be sure to clean off any loose metal particles or shavings.

Tubing, Plastic and Rubber

I keep several pieces of black and clear silicone rubber tubing on hand for connecting my flow meter to column ends, split vents, and other flow sources. The narrower pieces of tubing fit inside the wider ones so that I can adapt the flow meter fitting to a variety of connections. I have some silicone tubing that seals nicely to 1/16-inch tubing, and then slides into the flow meter's 1/8-inch inner diameter inlet tubing. We ordered a small spool of this silicone tubing, because it was always disappearing into other locations in the building. Of course, I never use plastic or rubber tubing for any gas at elevated pressure, or for permanent supply or internal connections.

Tweezers and Hemostats

A pair of tweezers can hold small nuts or ferrules without risking contamination with skin oils or a burn from hot items. Some tweezers have a convenient locking feature that frees one hand for other tasks, as will a spring-loaded hemostat. Rubber tips help hold fragile capillary columns or inlet liners.

Vial, Autosampler

I keep a spare autosampler vial to check for carrier gas flow during column installation. Fill the vial halfway with distilled water, and then insert the column outlet after connecting to the inlet but before connecting to the detector. After turning on carrier gas pressure, the presence of bubbles shows positive carrier gas flow. Never heat a column until you are sure there is positive flow through it.

Vial Crimper

Vial crimpers attach aluminum crimp-top seals to autosampler vials. Several crimp-top sizes are commonly used for GC: 8-mm for 0.8-mL vials, 11-mm for 1.5 or 2.0-mL vials, and 20-mm for 5-mL and 20-mL headspace vials. Hand crimpers are the least expensive, and some are available with interchangeable jaws that accommodate different vial sizes. Automated bench-top crimpers are less mobile, but the jaws can be interchanged quickly, and they are best for laboratories with high sample throughput. I prefer the type with rechargeable batteries.

Vial Decapper

Vial decappers perform the opposite function of a crimper; they remove crimp-top seals from vials. Decappers come in the same sizes as the crimpers, and resemble a pair of pliers. Some caution is required in use, so as not to break the neck of the vial. Once caps are removed, the contents may be properly disposed. Some laboratories reuse sample vials, but I recommend a fresh vial for each sample if at all possible.

Wipes, Laboratory

I wet laboratory wipes with some isopropanol, and then clean any debris or oil off the ends of capillary columns before inserting them into inlets or detectors. They also are handy for tipping a drop of test mix off a syringe needle, if disposed of properly. Paper towels don't work as well; they may leave fibers behind, and they may deposit a chemical residue. I recently have started to use particle-free cleanroom type wipes premoistened with a mixture of 70% isopropanol and 30% deionized water.

Wire Brushes

Wire brushes can dislodge particles and debris from detector parts and some sealing surfaces. Be careful not to score polished metal surfaces, or damage ceramics. It is better to replace a severely dirty FID flame jet or collector than to clean it forcibly. The manufacturer's guidelines for cleaning will have ways to assess the reusability of these parts.

Wrenches, Adjustable

I have one large 18-inch long adjustable wrench that looks like it belongs in an automotive garage. This is used exclusively for attaching or removing pressure regulators on gas tanks. I also have a smaller 6-inch long adjustable wrench that I use occasionally, if someone else has walked off with the exact open-ended wrench size I need.

Wrenches, Open-Ended

I have an assortment of open-end wrenches in inch sizes, as well as a metric set. I keep two or three with the following sizes: 1/4-, 5/16-, 3/8-, 7/16-, 1/2-, and 9/16-inch, as well as 11/16-, 3/4-, and 1-inch, although these latter sizes are used only rarely. Chromatographers located outside the United States will find the standard metric wrenches to be a bit more useful. I apply two wrenches at once to prevent counter-rotation while tightening or loosening fittings.

Conclusion

Chromatographers, like all craftspeople, use a variety of tools to practice their craft. In a pinch, tools that are somewhat inappropriate can be used to make do, but the rapidity and ease with which the right tool gets the job done make it well worth the expense of obtaining what's needed for the job.

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Data Integrity Focus, Part VIII: What is Good Documentation Practice (GDocP)?

EU GMP Chapter 4 and *United States Pharmacopoeia* (USP) Chapter *<1029>* on Good Documentation Guidelines are two sources for good documentation practice (GDocP). Are they similar, or are they complementary, and should we consider any other sources of advice?

R.D. McDowall

In the first seven parts of "Data Integrity Focus," we have focused on a few elements of data integrity (1–7). In this part, we will look at an essential requirement for data integrity: good documentation practice (GDocP). As it states in the principle of EU **Good Manufacturing Practice** (GMP) Chapter 4 on documentation:

> *Good documentation constitutes an essential part of the quality assurance system and is key to operating in compliance with GMP requirements (8).*

This statement can also be applied to good laboratory practice (GLP), or any other quality system, such as ISO 17025 or ISO 9001. Although this is a great statement, what does it mean in practice? What is good documentation, as opposed to bad documentation? In this part, we will look at the regulations and sources of advice on this key subject for data integrity.

The principle of Chapter 4 describes the main types of documents that are possible in a regulated organization (8):

> *There are two primary types of documentation used to manage and record GMP compliance: instructions (directions, requirements) and records/reports.*

As shown in Figure 1, the execution of an instruction results in the generation of records or a report. Underneath both instructions and records or reports, the types of document are described in more detail. It is the existence of accurate and reliable records that demonstrate that instructions have been followed, and hence the work is compliance with GMP regulations.

Chapter 4 (8) goes into more detail about records and reports:

- *Records: Provide evidence of various actions taken to demonstrate compliance with instructions, e.g. activities, events, investigations, and in the case of manufactured batches a history of each batch of product, including its distribution.*
- *Certificates of Analysis: Provide a summary of testing results on samples of products or materials together with the evaluation for compliance to a stated specification.*
- *Reports: Document the conduct of particular exercises, projects or investigations, together with results, conclusions and recommendations.*

Therefore, from Chapter 4 (8), we can draw up some basic requirements for good documentation practices for any quality system:

- *Say what you do:* Have an instruction to document a repetitive task such as an analytical method or an SOP.
- *Do what you say:* Follow the instruction and if there are any deviations from the procedure document them.
- *Document it:* Generate a record to show that the instruction was followed.

This chapter (8) also notes that "the term 'written' means recorded, or documented on media from which data may be rendered in a human readable form." In Chapter 4 speak, a document (instruction or records) does not exist only on paper; it can be any medium, as long as it can be converted into a readable format.

Chapter 4 also has a section called "Good Documentation Practices," that include clauses 4.7 to 4.9 as shown in Table I. As you can see, the requirements are remarkably short and brief, and need to be interpreted by each regulated organization and laboratory. The focus of the regulatory requirements in Table I is generally on paper, and there is no specific mention of a computerized system, because the applicable regulations are found in EU GMP Annex 11 (9). There are other sections in Chapter 4 on the generation and control of documentation (clauses 4.1–4.6), records retention (clauses 4.10–4.12), and the expected types of documents (clauses 4.13–4.32) (8). This is a good source of information on what is expected in GMP documentation compared with the 21 *CFR* 211, the US GMP regulations (10).

Given the focus of this article is GDocP, you can see that there are not many "Attributable, Legible, Contemporaneous, Original, Accurate" (ALCOA) principles shown in Table I. Clause 4.7 equates to legible, 4.8 to contemporaneous, and 4.9. in part to accurate. We could conclude that Chapter 4 does not comply with the ALCOA principles! That is why Chapter 4, along with Annex 11, is being updated to enhance data integrity requirements (11).

ALCOA and ALCOA+ Principles

Having mentioned ALCOA in the previous paragraph, I need to explain what

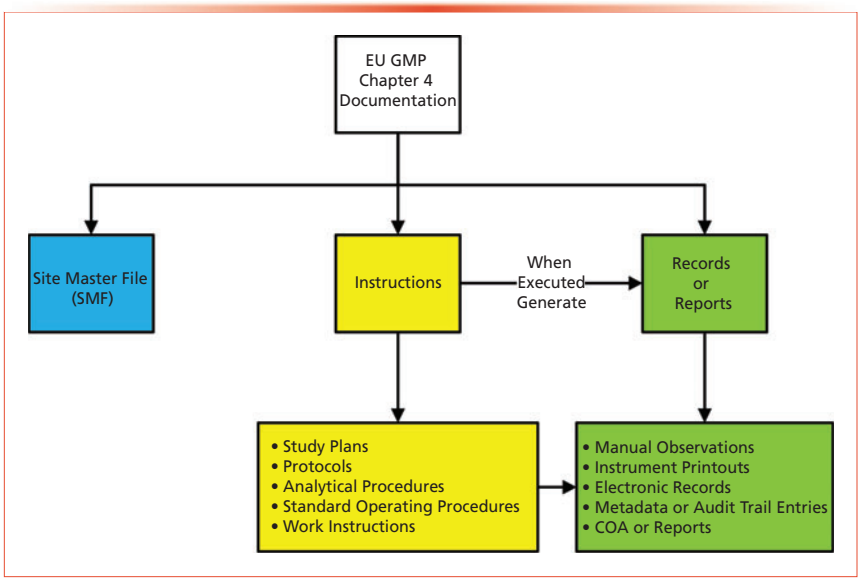

FIGURE 1: Principles of good documentation practice from EU GMP, Chapter 4 (8).

TABLE I: EU GMP Chapter 4 Requirements for good documentation practices (8).

Clause	GDocP Requirement
4.7	Handwritten entries should be made in clear, legible, indelible way.
4.8	Records should be made or completed at the time each action is taken and in such a way that all significant activities concerning the manufacture of medicinal products are traceable.
4.9	Any alteration made to the entry on a document should be signed and dated; the alteration should permit the reading of the original information. Where appropriate, the reason for the alteration should be recorded.

this means for those that do not know. ALCOA was developed by an FDA GLP inspector as an acronym for five requirements for data quality and is now used for data integrity. The four additional criteria were added by the European Medicines Agency on a GCP guidance on electronic source data (12) and called ALCOA+. The nine criteria are:

- Attributable: Who acquired the data or performed an action and when?
- Legible: Can you read and understand the entry or data?
- Contemporaneous: Is work is documented at the time of the activity?
- Original: Is there a printout or observation, or a certified copy thereof?
- Accurate: Are there no errors or editing without documented amendments?
- Complete: Are all data, including any repeat or reanalysis performed on the sample, available?
- Consistent: Are all elements of a chromatographic analysis, such as the sequence of events follow on and dates or time stamped in expected sequence, done in the same way over time, especially so as to be fair or accurate?
- Enduring: Are all data recorded in official media (either paper or a computerised system) , and not on the back of envelopes, cigarette packets, post-it notes, body parts or the sleeves of a laboratory coat?
- Available: Are all data easily retrieved for review, audit or inspection over the lifetime of the record?

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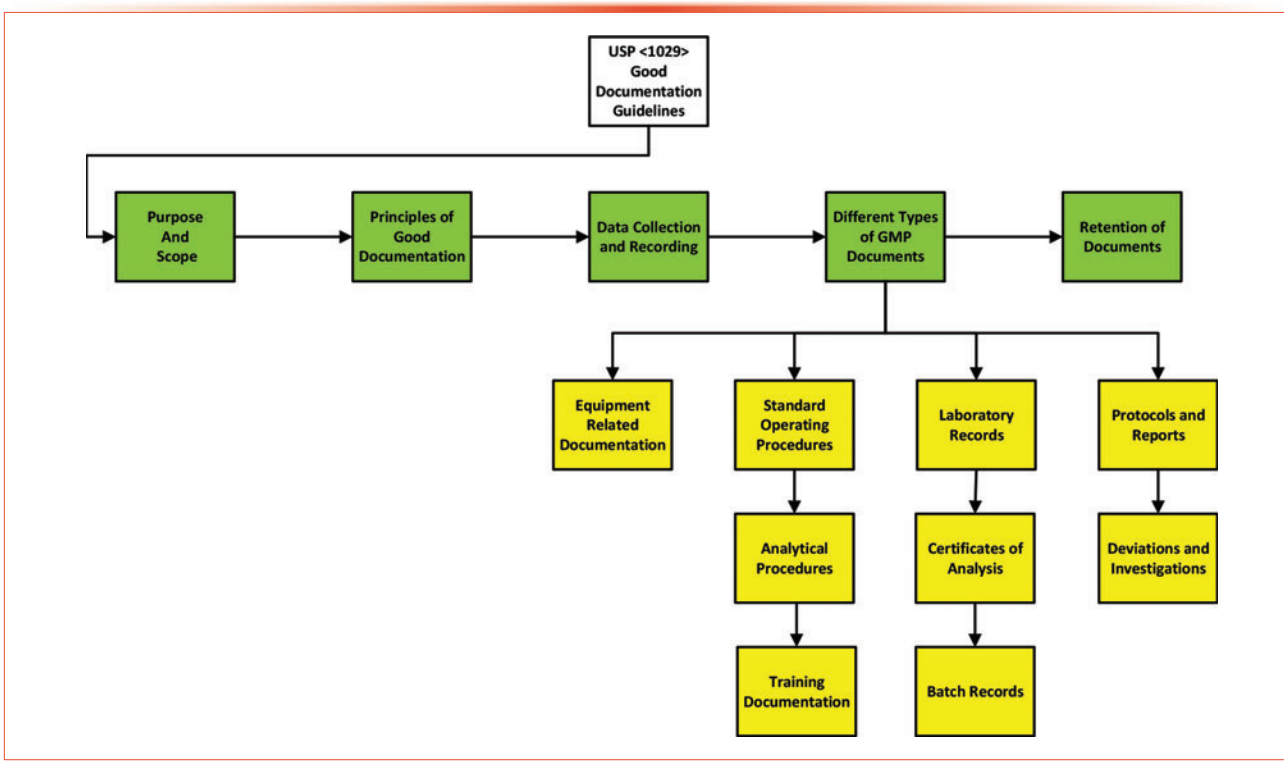

FIGURE 2: Structure of USP Chapter <1029> on good documentation guidelines (19).

TABLE II: Definitions of ALCOA Criteria from WHO Guidance (15)

ALCOA Criterion	Definition
Attributable	*Attributable* means information is captured in the record so that it is uniquely identified as having been executed by the originator of the data (a person or computer system).
Legible, traceable and permanent	The terms *legible*, *traceable* and *permanent* refer to the requirements that data are readable, understandable and allow a clear picture of the sequencing of steps or events in the record so that all GXP activities conducted can be fully reconstructed by people reviewing these records at any point during the record retention period set by the applicable GXP.
Contemporaneous	Contemporaneous data are data recorded at the time they are generated or observed.
Original	Original data include the first or source capture of data or information and all subsequent data required to fully reconstruct the conduct of the GXP activity. The GXP requirements for original data include the following: • original data should be reviewed; • original data or true and verified copies that preserve the content and meaning of the original data should be retained; • as such, original records should be complete, enduring, and readily retrievable and readable throughout the records retention period.
Accurate	The term *accurate* means data are correct, truthful, complete, valid, and reliable. For both paper and electronic records, achieving the goal of accurate data requires adequate procedures, processes, systems, and controls that comprise the quality management system. The quality management system should be appropriate to the scope of its activities and risk-based.

USP <1029> Good Documentation Guidelines

The draft of *USP* Chapter <1029> on Good Documentation Guidelines was issued in 2014 for public comment. The timing of this is significant, as it was issued before the publication of the tsunami of regulatory and industry guidance on data integrity (13–18) since the start of 2015. The structure of the final version of the general chapter released in 2018 (19) is shown in Figure 2. The high-level structure looks fine, but the listing order of the different types of GMP documents looks as if it has been drawn at random from a grab bag. Figure 2 attempts to put an order to the document, but it is not the same order as in the published version of *USP* <1029> (19).

The focus of our discussion here is on the scope, principles of good documentation and data collection, and reporting sections. Also, you must bear in mind the numbering of the *USP* general chapters: Those between <1> and <999> are mandatory, whereas those between <1000> and <1999> are informational or strong guidance. Hence *USP* <1029> is an informational general chapter.

Overall there are some good points in this general chapter, such as:

- This is the only guidance to mention how to document decimals and also refers to the rounding rules in the USP General Notices, an essential element of data integrity.
- The date format suggested is European: day, month, and year; an excellent approach to harmonization.
- There is a section on second person review of data, but there are also some gaps as can be seen below.

Let's look at some of the gaps in *USP*<1029>:

- There is no specific mention at all of the ALCOA+ criteria for data integrity. "Legible" and "accurate" are mentioned specifically, and "attributable" and "contemporaneous" are mentioned indirectly. This, in my view, is a failure; as Food and Drug Administration (FDA), World Health Organization (WHO), Pharmaceutical Inspection Cooperation Scheme (PIC/S), and Medicines and Healthcare Products Regulatory Agency (MHRA) guidances (14–16,20) all mention ALCOA or ALOCA+ specifically, why does *USP* <1029> not use the term? The general chapter omits the ALCOA+ criteria complete and consistent in the principles of good documentation, although it does mention *complete* in the discussion of purpose and in second person review.
- In the whole document, there is no mention of hybrid systems. Paper and electronic records are covered, but there is no explicit mention of hybrid systems. According to the WHO, hybrid systems are to be discouraged and should be replaced (15). According to me, hybrid systems are the worst possible option, because a laboratory must manage and synchronize signed paper printouts with the electronic records in the computer system (21).
- There is the possibility that predefined correction codes can be used for changes to data, but it does not mention that a procedure needs to define and maintain these codes.
- There is no discussion of the review of audit trails in hybrid and electronic systems
- Substitution of a true, complete, or verified copy of a record implies that the original can be discarded, because there is no explicit requirement for the original to be available for the second person review to check the copying process.
- Although it states that controls should be in place to protect record integrity, *USP* <1029> does not differentiate between procedural and technical controls. For computerized systems, technical controls are preferred over procedural controls; this needs to be made clearer in the chapter.
- There is no specific mention of the need to control blank forms used in laboratory analysis, which is a regulatory expectation in a number of guidance documents (14,16,20). Also, there is a bullet point in *USP* <1029> that states that notebooks, data sheets, and worksheets should be traceable, but this is not specific enough, and can be confusing as to its meaning. A major regulatory concern is the use of blank forms; why is this not mentioned explicitly? For a detailed discussion on the implications of using master templates and blank forms, please read the "Questions of Quality" column by Burgess and McDowall (22), or a recent publication (21).
- Although *USP* <1029> mentions that computerized systems should comply with the requirements of 21 *CFR* 11 on electronic records and electronic signatures (23), there is a paragraph at the end of data collection and recording section that should state that print outs from computerized systems should be linked to the data and metadata the generated them.
- The term *raw data* is not defined, and is used incorrectly. *Raw data* applies to all data and records collected during a GMP activity, not just initial observations. Please read several of my articles on this subject for further explanation (24,25), including Part 4 of this series (4).

All in all, these gaps make the value of *USP* <1029> doubtful. Therefore, we need to find better information. This comes from the WHO (15) and PIC/S (16) guidance documents, that we will consider now.

WHO Guidance on Good Data and Record Management Practices

The guide that offers the most comprehensive discussion of ALCOA and good documentation practices for both paper and electronic records is the WHO guidance (15). Appendix 1, com-

prising nearly half of the whole document, is entitled "Expectations," and examples of special risk management considerations for the implementation of ALCOA or ALCOA+ principles in paper-based and electronic systems are provided. For each of the five ALCOA requirements (attributable, legible, contemporaneous, original and accurate), there is a:

- definition of the term
- table with the expectations for paper and electronic records
- discussion of any special risk factors to consider.

This is an excellent guidance document, and if a reader wants to understand the meaning and scope of ALCOA, this is the place to start. The definitions for the five ALCOA criteria are given in Table II, and expand the ones outlined earlier in this article.

The document jumps through hoops as it attempts to expand ALCOA to be ALCOA+, but still keep the five main criteria, as it expands *legible* to include traceable and permanent (the latter equivalent to enduring).

Good Practices for Data Management and Integrity (PIC/S PI-041)

The PIC/S guidance document PI-041 is still a draft, with the third version issued at the end of November 2018 for comment (16), and therefore the content can change when the final version is issued. This is a very comprehensive guidance document, and to understand the good documentation and record keeping practices there are the following sections:

- Section 8 covers paper records and the regulatory requirements for this medium.
- Section 9 considers the data integrity requirements for computerized systems. Hybrid system requirements are specifically mentioned in section 9.8.

Despite its draft status and the possibility of change following industry comments, it is worth reading to get a comprehensive view of data integrity. When the final version is issued, PI-041 will be one of the best regulatory data integrity guidance documents.

Remember that this document has the designation *PI*, meaning PIC/S Internal, meaning it was written by inspectors for inspectors; this document will be used in inspections. The good news (for inspectors) is that there are 52 inspectorate members with four countries who have applied for membership. You can run, but you cannot hide from the data integrity inspectors!

Summary

Good documentation practices (GDocP) have been discussed from the regulations in EU GMP Chapter 4 through USP <1029> and the data integrity guidances from the World Heath Organization and Pharmaceutical Inspection Cooperation Scheme. The latter two provide very good guidance and interpretation of the ALCOA+ principles for both paper and electronic systems including hybrid ones. Understanding GDocP before an inspector calls is a key requirement for ensuring data integrity.

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Frontiers of Sampling: Design of High Surface Area Thin-Film Samplers for On-site Environmental Analysis

In on-site environmental applications, representative sampling and proper replication are essential to properly characterize a given site. For these reasons, recent work in thin-film solid-phase microextraction (TF-SPME), an extension of fiber-based SPME, has focused on the development of unique holders and customized samplers that have been tailored for distinct sampling environments. Here, we explore the latest developments and designs of high-surface-area thin-film samplers for on-site environmental analysis using TF-SPME.

Jonathan Grandy, Maryam Lashgari, Varoon Singh, and Janusz Pawliszyn

We all know the saying, "Garbage in equals garbage out." With the proliferation of ultrahigh-pressure liquid chromatography (LC) systems and ever more sensitive mass spectrometry detectors, this adage that is more commonly thrown to the wayside in favor of simpler sample introduction approaches such as dilute-and-shoot. It can be easy to forget that where sampling and sample preparation precede instrumental analysis, any errors experienced during these crucial steps will affect the results, possibly leading an analyst to draw a false conclusion regarding his or her work. This holds especially true for on-site environmental applications, where representative sampling and proper replication are essential to satisfactorily characterize a given site (1,2). For these reasons, recent work in thin-film solid phase microextraction (TF-SPME), an extension of fiber based SPME, has focused on the development of unique holders and customized samplers that have been tailored for distinct sampling environments (3,4). These include more rigid carbon fabric supported membranes to give physical stability for high river flows and agitation (5), a six-replicate TF-SPME membrane holder that attaches to a specialized sampling case or standard power drill (6,7), in-bottle TF-SPME samplers for surface water grab sampling (6), broad spectrum sorbents for the simultaneous extraction of polar and nonpolar analytes (7), time-weighted average (TWA) membrane holders for long duration TWA river sampling (8), and even highly durable thin-film-coated bolts for the sampling of extreme environments (9,10). These devices are all shown in Figure 1.

Much like fiber-based SPME, TF-SPME employs the fundamentals of thermodynamic equilibrium to drive the passive diffusion of small organic molecules, defined by the diffusion coefficient (D_s), across a static boundary layer of thickness (δ) that separates the free flowing sample matrix of analyte concentration (C_s) from the open sorbent bed. However, unlike an SPME fiber, thin-film devices are designed to have a total surface area (A) that is much larger than the sorbent thickness, resulting in extraction rates (dn/dt) that are orders of magnitude faster (11,12). This relationship is described mathematically in equation 1 below. Increased extraction kinetics are important to maximize the sensitivity of on-site environmental sampling approaches where one may be constrained to only a few minutes to collect each sample.

$$\frac{dn}{dt} = Cs\left(\frac{Ds\,A}{\delta}\right) \qquad [1]$$

Appropriately, it was shown experimentally that when short, 15-min pre-equilibrium extractions were performed from water that had been spiked with various pesticides, a 40 mm x 4.8 mm (L x W) carbon fabric supported divinylbenzene/polydimethylsiloxane (DVB/PDMS) TF-SPME membrane could extract 21.2, 19.8, 18.5, 18,4, 26.8, and 23.7 times the amount of 2,4 dichlorophenol, 2,4,6 trichlorophenol, phorate D10, fonofos, chloropyrifos, and parathion than a comparable 65 µm DVB/PDMS SPME fiber respectively (5). This result was inline with what was expected fundamentally (equation 1), as the aforementioned TF-SPME membranes had a surface area that was approximately 25 times greater than that of the corresponding SPME fiber. Moreover, when directly compared to standardized liquid–liquid extraction, adopting a slightly modified US-EPA 8270 methodology, these sensitive membranes were shown to give MLOD values that were anywhere from 4 to 200 times lower than those achievable using LLE at a Standards Council of Canada accredited laboratory, while maintaining a similar level of accuracy in a double-blind split comparison (13). In fact, this benchtop TF-SPME methodology also required far less sample with all analytes being extracted simultaneously by directly immersing the membrane on a cotter pin into 30 mL of spiked surface water at a pH of 2.5, with 10% NaCl and a stir rate of 900 rpm. This was in sharp contrast to the LLE approach, which required three sequential 50 mL dichloromethane extractions from 800 mL of spiked surface water at acidic, neutral, and basic conditions. With these results in mind, similarly designed membrane holders, shown in Figure 2, were included with the recent commercial release (Gerstel US) of the carbon mesh supported TF-SPME membrane (14).

To further improve the applicability of TF-SPME related methods, subsequent research focussed on the development of more novel TF-SPME holders to allow for unique sampling opportunities. In a recently published study, Piri-Moghadam and associates compared an in-bottle TF-SPME methodology to on-site sampling approaches with and without hand portable gas chromatography-mass spectrometry (GC–MS) instrumentation (6). This in-bottle approach was devised in a manner such that the sampling process was not a major departure from conventional grab sampling. When applying the bottle sampling approach, a technician simply needs to place the TF-SPME–equipped PTFE adapter, shown in Figure 1b, and spike an appropriate internal standard into the 800 mL glass bottle when collecting a surface water sample. Following sampling, these bottles were then

allowed to sit on an orbital shaker for three days pending analysis by use of a Gerstel thermal desorption unit and cooling injection system (TDU-CIS4) equipped Agilent 6890-5973N GC–MS instrument. As shown in Table I, this in-bottle approach gave the lowest limits of quantitation observed in the study with MLOQ values typically ranging between 1–10 ppt for most analytes. A more novel approach also explored the use of the custom six-replicate TF-SPME membrane holder shown in Figure 1a to perform on-site extraction directly from river water. These membranes were then either analyzed directly on-site using a hand-portable Tridion-9 GC–MS instrument, or transported back to the laboratory for desorption and analysis using the aforementioned TDU-CIS4 equipped Agilent 6890-5973n benchtop GC–MS instrument (6). Although the targeted, banned pesticides could not be detected at appreciable levels from Ontario Rivers during the study, a comparison of the MLOQ values for a selection of these compounds indicated that the entirely on-site TF-SPME methodology could provide quantitative limits similar to those of conventional benchtop LLE and SPME. The comparison of these methods and associated MLODs are listed in Table I.

Following the optimization and validation of these carbon mesh supported TF-SPME membranes and their various specialized holders, research efforts were shifted toward the development of more sensitive sorbents, particularly those with a broader affinity for polar analytes. Appropriately, hydrophilic lipophilic balance (HLB) particles, based on a co-polymerization of N-vinylpyrrolidone onto a divinylbenzene backbone, made for a logical choice to compare to the now validated DVB/PDMS carbon mesh supported membranes (7). In this study, three types of HLB particles, differing in their particle and pore size, were compared to the established DVB-based thin-film membranes. From the three HLB particles tested, one came from a commercial source, while the other two were synthesized in-house using either a precipitation, or suspension polymerization methodology. All particles were suspended in PDMS, spread onto the carbon fabric support and cut to 20 mm x 4.8 mm to fit the TDU-CIS4 system as before.

It was observed that the commercial HLB particles, those prepared by suspension polymerization, and the DVB particles had similar specific surface areas (727–800 m^2/g), but demonstrated greatly different extraction capabilities for the targeted McReynolds mixture. To better understand this result, complete characterization of the particles using Fourier-transform infrared spectroscopy, elemental analysis and Brunauer-Emmett-Teller analysis were performed, indicating that despite having a similar proportion of the N-vinylpyrrolidone moiety, the diameter of the pores and total pore volume for the sorbents were substantially different. HLB particles synthesized by suspension polymerization and obtained from commercial source had pore sizes of 80 and 71

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A Q&A

Next-Gen Multi-Angle Light Scattering

Daniel Some, PhD
Principal Scientist
Wyatt Technology

New HPLC/UHPLC product line offers more robust measurements and increased uptime.

In March 2019, Wyatt Technology Corporation launched its next-generation of online multi-angle light scattering (MALS), refractive index, and differential viscometry detectors for high performance liquid chromatography (HPLC) and ultrahigh-pressure liquid chromatography (UHPLC) systems. LCGC recently asked Dan Some, PhD, Principal Scientist at Wyatt Technology, about the advancements made in Wyatt's product line for absolute macromolecular characterization.

LCGC: Can you explain what is size-exclusion chromatography (SEC)-MALS and why it is of interest to protein and polymer scientists?

Some: SEC-MALS couples online multi-angle light scattering detection and other online detectors (such as refractive index and differential viscometry) to size-exclusion chromatography. With this technique, the only purpose of the SEC column is to separate the different molecules from each other. The actual characterization of the molecules takes place solely within the detectors, which allows absolute characterization to be performed. This method does not depend on the retention time within the column, the conformation of the molecule, or a molecule's interactions with the column. Thus, in SEC-MALS we do not encounter the errors of typical analytical SEC where reference molecules are run even though they might (and often do) behave differently on the column than your molecules.

This technique allows us to analyze monodispersed molecules, such as proteins, or polydispersed macromolecules, such as heterogeneous polymers, to determine their molecular weight, size, conformation, and branching ratio. The oligomeric state of proteins in native solution can be determined, resulting in a much better understanding of the essential biophysical properties of the macromolecules than can be obtained from analytical SEC.

LCGC: What would you say is new and improved in Wyatt's DAWN, Optilab, and ViscoStar products launched in March 2019?

Some: In March, we launched a re-envisioned product line of the DAWN, Optilab, and ViscoStar online detectors for SEC with multi-angle light scattering. While these detectors offer the same industry-leading sensitivity, range of measurements, and other features

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> "Across all these products—DAWN, Optilab, and ViscoStar—the key added value is enhanced productivity arising from the new Smart Services Platform. The platform includes the System Ready Monitor and System Health Indicators, ensuring users do not waste runs due to sub-optimal chromatography conditions."

that our customers are used to for maximum characterization of their macromolecules, the new products have a sexy, new modern look and feel. For example, there is a large capacitive touchscreen that allows users to interact more intuitively with the instrument and access the information that they need from the front panel. The instruments also have improvements in serviceability and maintainability, achieved by making them more modular. In fact, individual modules can be swapped out on-site. In addition, CheckPlus software performs a full diagnosis and sends those diagnostics to an engineer at Wyatt for a more in-depth look. Depending on what the engineer decides, a technician can come on-site and swap out the modules with very little downtime.

LCGC: What are some of the newest innovations in the DAWN line, which has been Wyatt's flagship product for 37 years?

Some: In previous generations, we worked on improving the technical specifications, getting higher sensitivity, expanding the range of measurements, and adding user interface improvements. Key in the new generation of DAWN detectors is the built-in intelligence that assists users in knowing when their SEC-MALS system is ready for optimal measurements, when the noise level is low enough, and when the system is fully equilibrated. In addition, swappable flow cells allow for a new flow cell to be swapped in without the need for laser alignment. Opto-mechanics are more robust, and modifications to the optical design further reduce stray light. Dedicated slots for the WyattQELS dynamic light scattering module have been added so that, rather than sacrificing one of the MALS angles as with the previous models, WyattQELS gets its own slot, and the software automatically identifies into which angle the user has placed the WyattQELS optical fiber.

LCGC: What do you see as the main value to customers in the updated product line?

Some: Across all these products—DAWN, Optilab, and ViscoStar—the key added value is enhanced productivity arising from the new Smart Services Platform. The platform includes the System Ready Monitor and System Health Indicators, ensuring users do not waste runs due to sub-optimal chromatography conditions. The platform's self-diagnostics and CheckPlus instrument log application permit remote evaluation by our service team, and full on-site repair service.

LCGC: What can Wyatt offer to those who use UHPLC?

Some: microDAWN is the multi-angle light scattering online product for use with UHPLC. microOptilab is the refractive index detector UHPLC, and microViscoStar is the differential viscometer for UHPLC. Users can get the complete range of characterization of molecular weight, size, and conformation, with all the benefits of UHPLC, which means faster runs, lower sample consumption, lower mobile phase consumption, and enhanced productivity.

LCGC: Where can readers go to learn more about SEC-MALS technology and applications?

Some: The best place to start is our website, which is www.wyatt.com, and there we have information about the theory of SEC-MALS, light scattering, and other technologies. Folks can learn about the various solutions that the instruments offer, the applications they provide, the different types of analytes that can be analyzed, the industries served, and the products' features and benefits. There is also an extensive library of webinars that can be viewed to learn more.

With a long history in scientific instrumentation, Wyatt Technology is a leader in light scattering instruments, accessories, software and services for determining the properties of macromolecules and nanoparticles in solution.

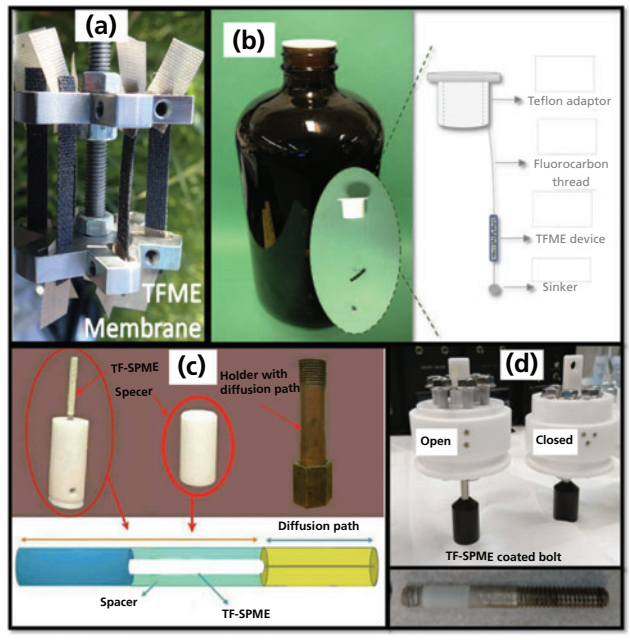

FIGURE 1: Compilation of various thin-film solid phase microextraction (TF-SPME) sampler formats including: (a) carbon mesh supported TF-SPME membrane on six-replicate drill sampler (7), (b) TF-SPME in-bottle grab sampler (6), (c) TF-SPME time weighted average sampler with hydrophilic lipophilic balance-particle-polyacrylonitrile (HLB-PAN) coated thin film blade (8), (d) TF-SPME coated bolt sampler in sampling (open) and storage (closed) position with HLB–PAN coated bolts (10).

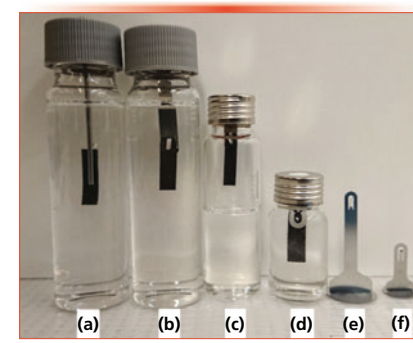

FIGURE 2: In-vial thin-film solid phase microextraction (TF-SPME) membrane holders including: (a) prototype cotter pin TF-SPME holder pushed through septa used for inter-laboratory study (13), (b) comparable commercial membrane holder for direct immersion extraction from 40 mL vial, (c) TF-SPME holder set-up for 10 mL headspace extraction (14), (d) TF-SPME holder set-up for 10 mL direct immersion extractions, (e) TF-SPME holder for 40 mL vial, (f) TF-SPME holder for 10 and 20 mL vials.

angstroms (Å) and pore volumes of 1.30 and 0.64 mL/g, respectively, making them generally mesoporous. In the meantime, the DVB particles had the largest pores, with a diameter of 400 Å and volume 1.54 mL/g. Finally the smallest pores were observed in those particles prepared by precipitation polymerization with an average pore diameter of 13 Å and volume of 0.20 mL/g. Surprisingly, despite having the smallest pore volume, the HLB particles synthesized by precipitation polymerization gave the best results, extracting nearly double the amount of the polar standards than the DVB/PDMS membranes (Table II). This result was attributed to the fact that the precipitation polymerized HLB particles had a generally microporous structure stemming from the use of acetonitrile as a porogen. Moreover, the smaller 1.33 µm particle diameter could have also played an important role considering the spread distribution and presence of a higher number of particles on the membranes. Unsurprisingly, as shown in Table II, it was found that all of the HLB sorbents tested did in fact outperform the established DVB particles for all of the McReynolds standards tested. This increase in the affinity for semipolar analytes can be attributed to the presence of the N-vinylpyrrolidone group. These groups provide sites for hydrogen bonding, and allow the hydrophilic functionalities to interact more strongly with the sorbent. Although not as pronounced, it was somewhat surprising to also see slightly increased performance offered by the HLB particles for the octane standard added to the McReynolds mixture. With a LogP value of 5.01, one would expect octane to be primarily extracted by the nonpolar interactions offered by the divinylbenzene. It is also important to note that instrumental runs were fully randomized during the experiment, ruling out the possibility of detector drift during experimentation. Speculatively, it is possible that this result could also be explained by the much smaller pore diameter present in all of the HLB materials tested.

Interestingly, it was also found that the physical characteristics of the carbon mesh support allowed for an innovative calibration approach for converting raw instrument signal to tangible amounts such as nanograms. This calibration approach, called *on-membrane liquid injection*, allowed for liquid standard to be introduced to the GC–MS instrument, without having to remove the TDU-CIS4 system or install a liquid injection syringe onto the autosampler. Instead, it was possible to manually spike a few microliters of a methanolic McReynolds standard directly onto the edge of the TF-SPME membrane, that then wicked the solution into the carbon mesh support. Once analyzed, it was found that an exceptional correlation to response ($R^2 \geq 0.996$) for all analytes could be achieved while most of the volatile methanol solvent was purged from the CIS4 during analyte reconstitution, never reaching the GC column. Moreover, it was also found that once extracted onto the TF-SPME membranes and stored within the Gerstel thermal desorption tubes on the autosampler rack, all of the McReynolds standards were stable on-membrane for up to 24 h. In fact, only the photosensitive pyridine was shown to deplete when this test was further lengthened to 120 h.

Having shown favorable results for polar VOC analysis, these HLB/PDMS membranes were then applied for an untargeted, on-site determination of chlorination byproducts from a private hot tub. For

TABLE I: Comparison of reported method limits of quantitation for selected pesticides and pesticide precursors extracted from spiked surface water using varied sample preparation and instrumental approaches (concentration reported in parts per trillion, 10^{-12}).

Sample Preparation Approach	LLE*, US EPA 8270 (13,15)	DVB-PDMS SPME Fiber (15)	DVB-PDMS TF-SPME (13)	DVB-PDMS TF-SPME In-bottle Sampling (6)	DVB-PDMS TF-SPME Portable GC–MS (5)
Instrument used	Agilent 7890A-5975C	Agilent 7890A-5975C	Agilent 6890-5973N	Agilent 6890-5973N	PerkinElmer Tridion-9 portable GC–MS
Extraction time (min)	N/A	30	60	4320 (3 d)	15
Sample volume (mL)	800	15.5	30	800	300
2,4-DCP	500	100	50	N/T	100
2,4,6-TCP	500	200	25	10	100
2,3,4,6-TeCP	500	200	10	3	N/T
Phorate	500	250	10	N/T	100
Atrazine	500	500	25	10	1000
Atrazine	1000	50	25	N/T	1000
Me-Parathion	1000	200	250	100	N/T
Chlorpyrifos	1000	200	100	10	500

DCP = dichlorophenyl; TCP = trichlorophenyl; ME = methyl; N/T = not tested
Note: Table is not inclusive of all analytes investigated in each study, only those common to all/most studies
* Based on reporting limit, roughly equivalent to MLOQ for more details please read (15)

TABLE II: Comparison of the relative extraction capabilities of the tested sorbent particles. Extractions were performed at equilibrium from a McReynolds standard headspace generating vial for 10 min at 55 °C. Results presented as factor of signal improvement when compared to the established divinylbenzene-polydimethylsiloxane (DVB-PDMS).

TF-SPME Chemistry	DVB-PDMS	HLB-PDMS (Suspension)	HLB-PDMS (Commercial)	HLB-PDMS (Precipitation)
Particle size (µm)	5	30-60	5	1.33
SSA (m^2/g)	750	800	727	335
Pore size (Å)	400	71	80	13
Pore volume (mL/g)	1.54	0.64	1.30	0.20
Benzene	1.00	1.41	1.52	1.77
2-Pentanone	1.00	1.36	1.55	2.16
1-Nitropropane	1.00	1.28	1.55	1.86
Pyridine	1.00	1.15	1.32	1.72
1-Pentanol	1.00	1.36	1.65	1.99
Octane	1.00	1.11	1.14	1.30

this proof of concept application the HLB/PDMS thin films prepared using the precipitation polymerized HLB were cut to 40 mm x 4.8 mm (L × W) for analysis on the Tridion-9 portable GC–MS system. As shown in Figure 3, sampling was accomplished by placing four membranes onto the six-replicate TF-SPME holder, which was connected to a sampling case (PAS Technologies GmbH), allowing for extractions to be performed at 2000 rpm for 10 min from the 39.5 °C, pH 7.2–7.4 hot-tub water. Immediately after sampling, the membranes were removed from the drill, and dabbed dry with a Kimwipe before being placed in the 3.5-in. thermal desorption tubes. These tubes were then inserted into the SPS-3 module and desorbed at 250 °C using 35 mL/min of helium to transfer the desorbed analytes to a 19 gauge Tenax/Carboxen needle trap device (NTD). This NTD could then be placed directly into the Tridion-9 portable GC–MS instrument for desorption and analysis of the compounds transferred from the TF-SPME membranes. In total, six different chlorination by products were identified from the hot tub water. This identification was performed by matching with the NIST 2011 mass spectral database, followed by preliminary verification by use of a standard n-alkanes linear retention index plot. These compounds included chloroform, bromodichloromethane, dichloroacetonitrile, chlorobenzene, benzonitrile, and benzyl chloride, all of which were known disinfectant by-products.

Finally, two of these compounds were quantified using a matrix-matched external calibration curve at five concentration levels (n = 3), ranging from 25 to 250 µg/L for chloroform, and 125 to 1000 µg/L for dichloroacetonitrile. Calibration was performed in 3 L of matrix-matched water which yielded R^2 values over 0.99, indicating very good correlation to response for the TF-SPME equipped portable GC–MS instrument. The chloroform and dichloroacetonitrile were found to be present at a concentration of 270 µg/L and 79 µg/L, having RSDs of 13% and 5%, respectively (n = 4). It is worth noting that the World Health Organization (WHO) considers chloroform levels up to 300 µg/L and dichloroacetonitrile levels approaching 20 µg/L to be safe for bathing and drinking (16).

Of course, TF-SPME devices are not exclusive to GC-based applications. There has been a great deal of work performed on HPLC-amenable thin-film blades, as shown in the TWA holder of Figure 1c. These blades are generally manufactured using a more porous and permeable polymeric binder, such as polyacrylonitrile (PAN), to facilitate solvent desorption of the suspended sorbent particles. In this study, performed by Ahmadi and associates (8), the aforementioned TWA-TF-SPME-HPLC amenable sampler was shown as an environmentally friendly means to extract various biocides and ultraviolet (UV) blocking agents of mixed polarity from aqueous environments. These thin-film blades were prepared with either HLB/PAN or C18/

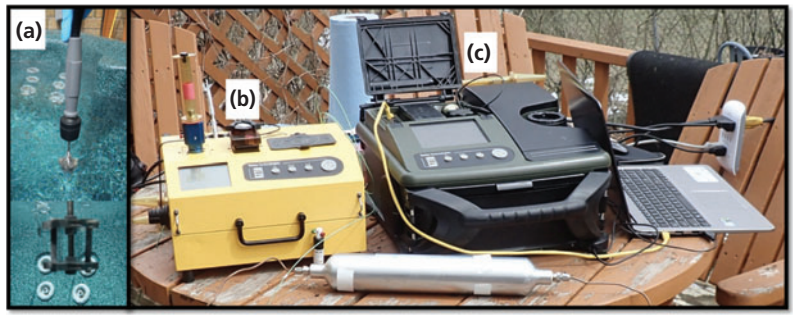

FIGURE 3: On-site hot tub sampling set-up showing: (a) the flexible cable drive attachment of the thin-film solid phase microextraction (TF-SPME) sampling head for direct analysis of chlorination byproducts from a hot tub, (b) the SPS-3 portable desorption module undergoing TF-SPME desorption, and, (c) the Tridion-9 portable GC–MS. Sampling was performed at 2000 rpm and the hot tub was measured to be 39.5 °C with a pH of 7.2–7.4.

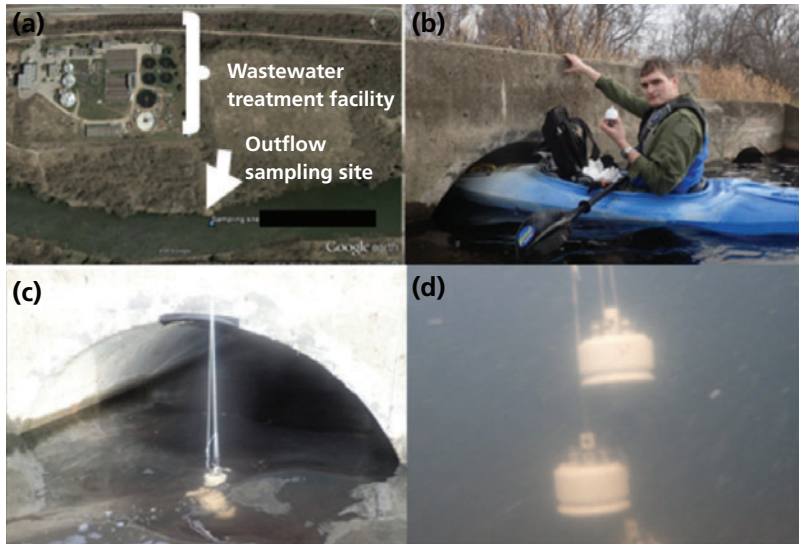

FIGURE 4: Coated bolt thin-film solid phase microextraction (TF-SPME) sampler deployment via kayak at an undisclosed wastewater treatment facility in Ontario, Canada.

PAN. As shown in Figure 1c, the TWA sampler was constructed such that the thin film blade was mounted onto a chemically inert polytetrafluoroethylene (PTFE) holder, and then covered by another PTFE spacer tube to reduce the dead-volume within the device. This assembly was then inserted into an anti-biofouling copper tube with a predrilled diffusion path with a length (*Z*) of 10 mm, and cross sectional area (*A*) of 0.49 mm^2. When combined with the known static diffusion coefficients (*D*) of a given analyte, the sampling time (*t*) and the total amount extracted over the sampling period (*n*) these values can be combined to create equation 2 and calculate the time weighted average concentration (C_{avg}). After validating the accuracy of the TWA assembly and that analyte uptake remained linear for 70 days using an in-lab simulated river system, the samplers were deployed on wastewater affected portions of the Grand River, Ontario, for a period of 90 d. Although not all of the targeted benzophenone compounds were above the MLOQs of the TWA methodology, the time weighted average concentrations of Benzophenone-4 and 2-phenylbenzimidazole-5-sulphonic acid (PBSA) were determined to be 5.4 parts per billion (ppb) and 4.0 ppb over the 90-d sampling period. This was found to be in good agreement with corresponding grab sampling data giving respective concentrations of 4.5 and 2.3 ppb in June when the TWA devices were deployed and, at levels of 6.6 ppb and 4.9 ppb when the TWA samplers were collected in August.

$$C_{avg} = \left(\frac{nz}{ADt}\right) \quad [2]$$

Moving forward, the principle of thin-film devices does not need to be restricted to flat planar shapes, such as the above-mentioned fabric supported membranes and thin-film blades. As facilitated by solvent desorption, adsorbent thin film coatings can be applied to solid supports of varying size and shape. Fittingly, in a very recent and innovative study performed by the Pawliszyn Research Group, a new design of TF-SPME was explored by applying a thin, 25 µm, coating onto 6 mm diameter cylindrical bolts. Shown in Figure 1d, these bolts were then used to develop a robust, sealable TF-SPME sampler for the on-site sampling and extraction of a wide range of untargeted pollutants in environmental water samples. This design was tailored to allow for the extraction of a broad spectrum of mixed polarity compounds from extreme environmental waters while offering a means to stabilize the extracted compounds on the sorbent coatings for extended periods in ambient conditions. In order to properly test these devices, a real world proof of concept was performed for the analysis of untargeted pollutants in water samples from five different rivers in China and Canada (10).

Coated bolts were prepared by using dip coating to deposit an HLB/PAN sorbent within a 1.2 cm long recession on the stainless steel bolts. As can be seen in Table I, among all of the currently discussed TF-SPME morphologies, the coated bolt layout provides a considerably large surface area, larger than that of any of the other HPLC amenable devices. This large 241 mm^2 surface area is needed to achieve adequate sensitivity during the relatively short sampling times expected for on-site sampling. With these factors in mind, one could expect a signal improvement by a factor of

TABLE III: Comparative physical dimensions of coated SPME fibers, TF-SPME membranes, TF-SPME blades, and the coated-bolt sampler.

	Coated Diameter (mm)	Coating Thickness (µm)	Coating Length (cm)	Coating Volume (mm^3)	Coating Surface Area (mm^2)
GC DVB-PDMS fiber*	0.270	65	2.0	0.440	15.0
Benchtop GC TF-SPME membrane	4.80**	400***	2.0	38.4	192
Portable GC TF-SPME membrane	4.80**	400***	4.0	76.8	384
HPLC SPME fiber*	0.270	45	1.5	0.39	11.1
TFME blade (17)	2.55**	120	2.0	12.2	102
Coated bolt (recessed)	6.40	25	1.2	6.20	241

* As per characteristics of commercially available (Millipore-Sigma) SPME fiber probes
** Coated width of blade-membrane
*** Coating permeates the carbon fabric support

22 times over a comparable HLB/PAN SPME fiber when short pre-equilibrium extractions are performed.

To confirm that the sampler was able to stabilize extracted compounds for long periods of time, the effects of storage time and temperature were evaluated. This was accomplished by deploying three coated bolt samplers at the outflow pipe of an undisclosed wastewater treatment facility for approximately 1 h via kayak (Figure 4). Upon retrieving the samplers, they were sealed and transported back to the lab where four of the 18 bolts were removed, and immediately desorbed by use of solvent for analysis on a high-resolution HPLC-orbital ion trap-MS instrument. The remaining 14 bolts were then stored in one of three ways before desorption and analysis including: 3 d at room temperature, 12 d at room temperature, and 12 d within a -80 °C freezer. The results of this test showed no significant differences in the quantity and quality of the extracted chemicals following any of the storage conditions, thus confirming the device's suitability for use at sampling sites that are far away from the laboratory facilities (10).

A full application of these samplers was performed by sampling four different rivers in China, including the Pearl River in the city of Guangzhou, the Yangtze River in Wuhan, a tributary of the Yangtze River in Shanghai, and the Xinghai River in Dalian (10). After returning to Canada, these samplers were desorbed and analyzed using high-resolution HPLC-orbital ion trap-MS, followed by multivariate statistical processing, to tentatively select significant features from each of the sampling sites. The Toxin and Toxin-Target Database was used as a reference for toxins and environmental contaminants. Eventually, more than 80 tentative compounds with widely varying hydrophobicity's from $-2.43 < \log P < 11.9$ were determined. These compounds included a variety of drugs, metabolites, miscellaneous toxins, and pesticides, which were extracted by the sampler from these different rivers. It is important to note, however, that although relative HPLC retention times and adduct annotation were used to further improve the reliability of the results, these identifications were still tentative in nature. Regardless, the logP values of these tentatively identified compounds indicate that these devices were in fact capable of extracting and stabilizing a wide variety of mixed polarity compounds from real environmental samples.

Conclusions

Solid-phase microextraction can come in many shapes and forms, with recent research indicating the high surface area thin-film morphologies are likely the ideal choice in performing environmental analysis, whether in the laboratory or on-site. Moreover, the design of a given TF-SPME sampler and holder can greatly enhance usability, sensitivity, and reliability, if these are properly designed for a given application, whether this be the six-replicate TF-SPME holder which allows for on-site agitation, broad polarity HLB-based sorbent for GC and LC amenable thin films, and even a sealable coated bolt sampler that can stabilize extracts for weeks when refrigeration and dry ice are not available. These deployments show just how important sampler design and sample preparation are for improving the sensitivity and reliability of results generated from our ever improving analytical instrumentation.

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PRODUCTS

Dynamic headspace module

The 7000C dynamic headspace module from CDS Analytical is designed with a plug-and-play connectivity to the CTC PAL robotic systems. According to the company, the sampler features their industry standard foam sensor, a newly designed wet trap, and exchangeable sample pneumatics systems. **CDS Analytical,** Oxford, PA. www.cdsanalytical.com/purge-trap-7000c

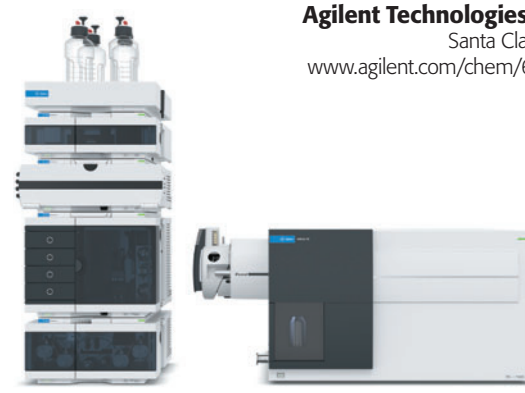
Ready-to-use vials

Vials, 96-well plates, closures, and services are available from DWK Life Sciences. According to the company, the products provide a range of sample-handling solutions for analysts working with chromatography and scintillation-counting applications. **DWK Life Sciences,** Millville, NJ. www.dwk.com/backtoquality

6495C Triple Quadrupole LC/MS

The highest performance LC/TQ available with Agilent's third generation iFunnel design, ideally suited for peptide quantitation as well as multiresidue applications that require ppt sensitivity. The combination of utmost sensitivity, extended mass range, and ease of maintenance with VacShield technology, and the power and flexibility of MassHunter, makes this the system of choice for demanding applications.

Agilent Technologies, Inc., Santa Clara, CA. www.agilent.com/chem/6495C



Agilent Technologies

Automated SIFT-MS

The thermal desorption– selected ion flow tube–mass spectrometry (TD-SIFT–MS) autosampler is designed as a collaboration between Gerstel and Syft Technologies to provide automated platforms for SIFT-MS analyses in routine and R&D laboratories. According to the company, using the autosampler can improve repeatability and reproducibility compared to using manual analysis, eliminate operator error, and provide part-per-trillion sensitivity and run times under 5 min. **Gerstel GmbH & Co. KG,** Linthicum, MD. www.gerstelus.com

Automated high-throughput thermal desorption system

Markes International's UNITY–ULTRA-xr Pro thermal desorber for high-throughput thermal desorption–gas chromatography–mass spectrometry (TD-GC–MS) is designed for the analysis of volatile and semi-volatile organic compounds. According to the company, the system combines sorbent tube automation with sample recollection capability for sample security. **Markes International,** Llantrisant, UK. http://chem.markes.com/ULTRA-Pro

LC–MS Q-TOF analysis interface

The Micro-ESI 9030 system from Shimadzu Scientific is designed as an interface that is compatible with its Nexera Mikros Microflow liquid chromatography–mass spectrometry (LC–MS) system to enable micro flowrate analysis with the LCMS-9030 quadrupole time-of-flight (Q-TOF) system. According to the company, the interface detects target components with sensitivity by optimizing the electrospray ionization (ESI) probe position relative to the sample injection port for low flowrates. **Shimadzu Scientific Instruments,** Columbia, MD. www.ssi.shimadzu.com

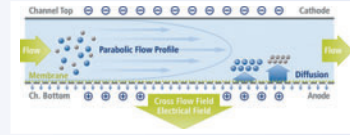
Direct-sampling ion source

Thermo Fisher Scientific's VeriSpray PaperSpray direct-sampling ion source is designed as a high-throughput direct sampling system for decreasing reliance on a complex chromatographic separation step for a range of routine mass spectrometry-based applications. According to the company, the ion source is suitable for drug analyte testing and drugs of abuse analysis. **Thermo Fisher Scientific,** San Jose, CA. www.thermofisher.com

Biphenyl phases

Macherey-Nagel's columns are packed with biphenylpropyl silica gel Nucleodur π^2 and the core-shell phase Nucleoshell biphenyl. According to the company, biphenyl phases provide an enhanced retention for aromatic and unsaturated substances. **Macherey-Nagel,** Bethlehem, PA. www.mn-net.com

Field-flow fractionation system



The Postnova EAF2000 simultaneous electrical and asymmetrical flow field-flow fractionation system is designed to enhance separation and characterization of biopharmaceuticals, environmental compounds, and nanomaterials. According to the company, electrical and cross flow fields are applied simultaneously, enabling separations by particle size and particle charge based on electrophoretic mobility for characterizing complex proteins, antibodies, and viruses, as well as environmental and charged nanoparticles or polymers. **Postnova Analytics GmbH,** Landberg, Germany. www.postnova.com

Hydrogen lab server

Proton OnSite's hydrogen lab server is designed with a solid electrolyte proton exchange membrane, electricity, and deionized water to produce up to 18.8 standard liters per min (equivalent to four cylinders) of ultra-high purity hydrogen gas per day. According to the company, the lab server senses demand and adjusts production accordingly. **Proton OnSite,** Wallingford, CT. www.protononsite.com

CBDV reference standards

Restek's cannabidivarin (CBDV) stand-alone reference standard is designed for quantitation of compounds for potency and strain identification. According to the company, the standard is suitable for creating multipoint (five-point minimum suggested) calibration curves for liquid chromatography–tandem mass spectrometry (LC–MS/MS) and gas chromatography–tandem mass spectrometry (GC–MS/MS). **Restek Corporation,** Bellefonte, PA. www.restek.com/cannabis

Ad Index

THE ESSENTIALS

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

An Introduction to Glycan Analysis

Glycosylation is the most common monoclonal antibody (mAb) post-translational modification. It can affect the safety, half-life, and efficacy of mAbs. For example, fucosylation has a direct, negative impact on the overall efficacy of a mAb, by means of a reduction in its ability to perform antibody-dependent cellular cytotoxicity (ADCC), whereas the addition of N-acetylneuramic acid (NANA) generates acidic residues, and typically leads to an immunogenic response.

Glycosylation occurs in two types, O- and N-linked. O-linked glycans are attached through an oxygen atom on a serine (Ser) or threonine (Thr) residue. This type of glycosylation is rare in human or murine immunoglobulin G (IgG) proteins. N-glycosylation can occur whenever the following consensus sequences occur: Asn-XXX-Ser or Asn-XXX-Thr (where XXX is any amino acid, except for proline [Pro]). The N-glycosylation site in mAbs is on Asn-297 in the fragment crystallizable (Fc) region. Glycan structure in mAbs can be highly heterogeneous, varying widely between different cell lines, clones, and production conditions. Therefore, it is important to be able to accurately characterize mAb glycoforms, including during biosimilar development.

Reversed-Phase HPLC Analysis

At the peptide level, reversed-phase high performance liquid chromatography (HPLC) analysis of the differing glycoforms produces a chromatogram in which the glycoforms are represented as several unresolved peaks, rather than a single peak. Further examination of this peak cluster allows fine details relating to the glycosylation profile to be extracted. It is worth noting that, even though reversed-phase LC is not an ideal technique for glycan separation, its high resolving power permits partial resolution of some isomers, and, at the peptide level, a near full approximation of the glycoprofile can be observed. As expected, an increase in the number of polar glycan units reduces retention in reversed-phase LC.

HILIC Analysis

Hydrophilic-interaction chromatography (HILIC) methodology is particularly applicable to the analysis of glycans, due to their highly polar nature. Typically, glycans are enzymatically released from the mAb, labeled with a fluorotag (2-aminobenzamide (2-AB) or procainamide), and then analyzed via a HILIC separation followed by fluorescence detection. It should be noted that 2-AB and procainamide are both chromotags and fluorotags, indicating that they can be used in conjunction with ultraviolet (UV) and fluorescence detection. However, fluorescence detection adds the benefit of increased sensitivity, due to fewer compounds fluorescing. When employing mass spectrometric (MS) detection, as is often the case during development and characterization, one may be tempted to forego the labeling step, as the unlabeled glycans readily ionize in negative mode electrospray ionization via the numerous terminal hydroxyl groups. However, performing the labeling is always recommended, as the unlabeled, or free, glycan can exist in two anomeric forms, an alpha and a beta, and this can lead to peak splitting. The benefits of labeling glycans is not merely from a detection perspective; the labeled glycans are also more easily retained and separated.

MS Analysis

Mass spectrometry is a powerful tool for the characterization of glycoproteins, and is often used in conjunction with other methodologies to allow full characterization. Glycosylation can be studied at both the intact protein and peptide levels, with MS methods employed depending on the information required and the known properties of the molecules. Intact antibodies or antibody fragments can be rapidly screened using electrospray ionization-quadrupole time-of-flight mass spectrometry (ESI-QTOF) instruments. The high resolution and extended mass range of these instruments allow glycan distribution, relative quantitation of specific glycoforms, and the degree of glycosylation to be assessed. Detection of major glycoforms is routinely performed; however, analysis of minor glycoforms is still challenging at the intact protein level.

Analysis of glycosylation at the peptide level provides information such as glycosylation site and site-specific distribution of different glycoforms when multiple glycosylation sites are present. This approach also allows analysis of peptide sequences and other post-translational modifications. If glycosylation occurs at other sites other than the Fc region site, specific glycoforms can be analyzed at the glycopeptide level. Antigen-binding fragments (Fab) and Fc-specific glycan profiles can also be analyzed at the antibody fragment or released glycan level.

ESI and matrix-assisted laser desorption–ionization (MALDI)-MS are both employed for profiling and structural characterization of glycans. A common phenomenon in MALDI is the loss of sialic acids; however, this can be avoided by permethylation or carboxyl group derivatization. ESI-MS of glycans is either carried out using an infusion or coupled to a separation technique, such as HILIC or capillary zone electrophoresis (CZE).

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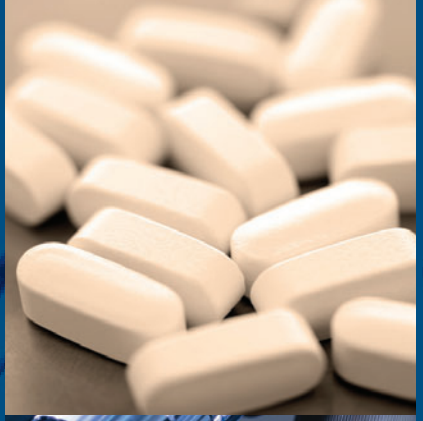

THE APPLICATION NOTEBOOK

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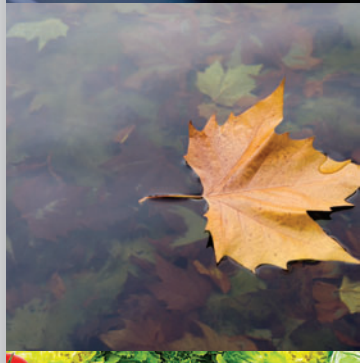
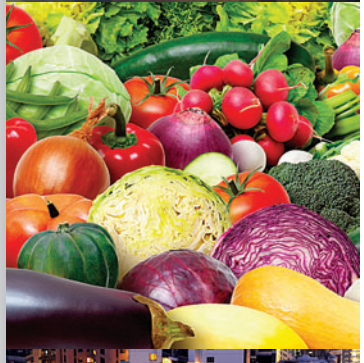
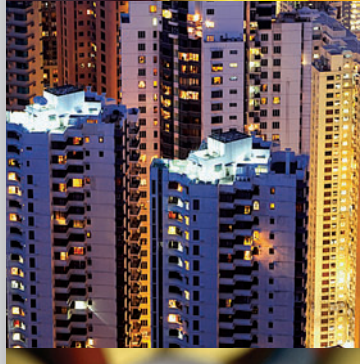
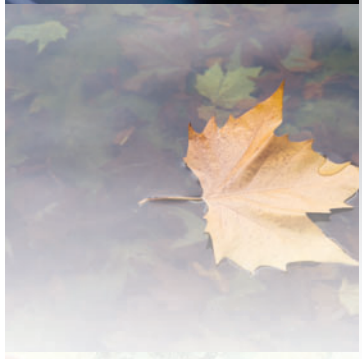
THE APPLICATION NOTEBOOK


Environmental

Food and Beverage

General



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Separation of Nanoplastics and Determination of Their Surface Charge by Electrical Asymmetrical Flow Field-Flow Fractionation

Postnova Analytics

Plastic micro- and nanoparticles are increasingly in the headlines, particularly when discussing marine pollution, but also with regard to their potential impact on human health. In this application note, we present data on separation of polystyrene nanoplastics, and demonstrate how electrical asymmetrical flow field-flow fractionation (EAF4) can be used for simultaneous size separation and particle surface charge measurement.

Plastic micro- and nanoparticles are increasingly in the headlines, particularly when discussing marine pollution (1), but also with regard to their potential impact on human health (2). Typically formed by the weathering and breakdown of plastic materials in the environment, nanoplastics are challenging to separate and characterize by commonly used techniques such as dynamic light scattering or size exclusion chromatography. In this application note, we present data on separation of polystyrene nanoplastics, and demonstrate how electrical asymmetrical flow field-flow fractionation (EAF4) can be used for simultaneous size separation and particle surface charge measurement. A schematic for the EAF4 channel and its separation principle is shown in Figure 1.

Experimental Details and Results

A mixture of two polystyrene latex particles (nominal diameters of 61 nm and 125 nm, respectively) was used as a proxy for a polydisperse nanoplastics system. This mixture was separated by EAF4 using four different electrical field conditions, enabling measurement of the electrophoretic mobility and thus the surface zeta potential of both particles. In addition, multi-angle light scattering (MALS) was used as a detector to simultaneously collect information about the size of both particles.

Figure 2 displays two EAF4-MALS fractograms. In the first fractogram (blue graph), separation was achieved solely by the cross

Table I: Radii of gyration for both polystyrene latex particles derived from EAF4-MALS with and without application of electrical field (0 mA and 1.45 mA, respectively).

	Nominal diameter, TEM (nm)	Radius of gyration, MALS (nm)	
		at 0 mA	at 1.45 mA
Polystyrene latex nanoplastics mixture	61 ± 4	24.0 ± 0.1	24.2 ± 0.3
	125 ± 3	46.4 ± 0.1	46.6 ± 0.1

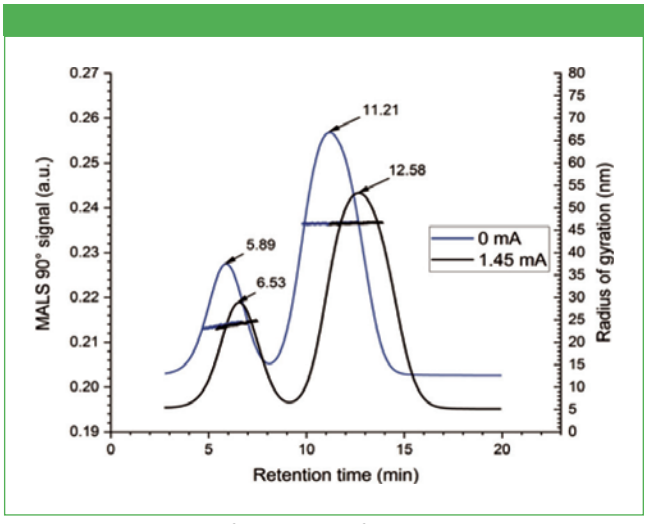

Figure 2: EAF4-MALS fractograms of the investigated nanoplastics particle mixture with and without application of an electrical field (black and blue graph, respectively). The blue and black dotted lines display the radii of gyration obtained from MALS indicating no influence of the electrical fi eld on the particle size.

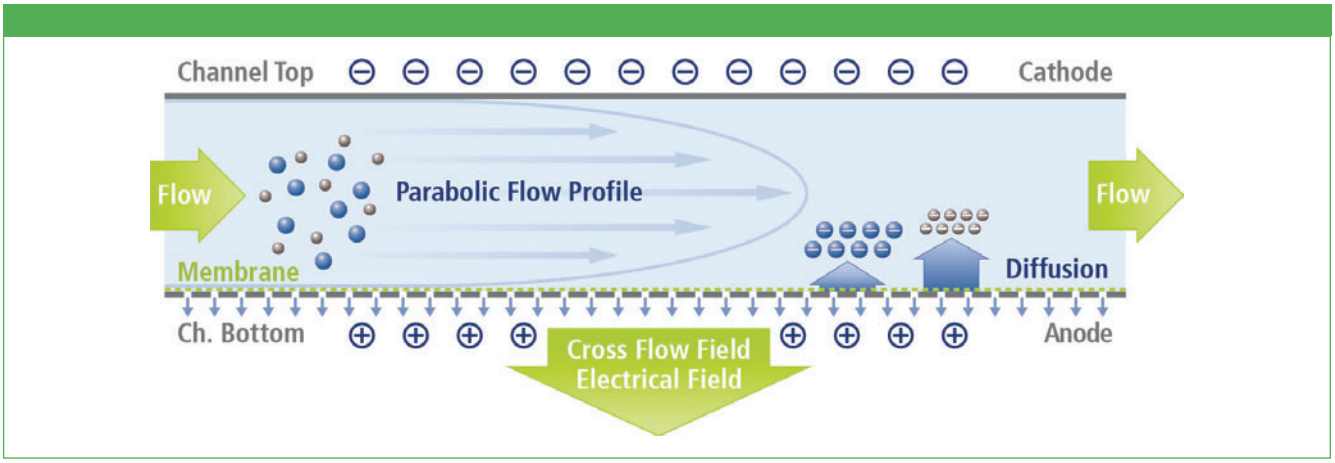

Figure 1: Schematic representation of an electrical asymmetrical flow field-flow fractionation channel.

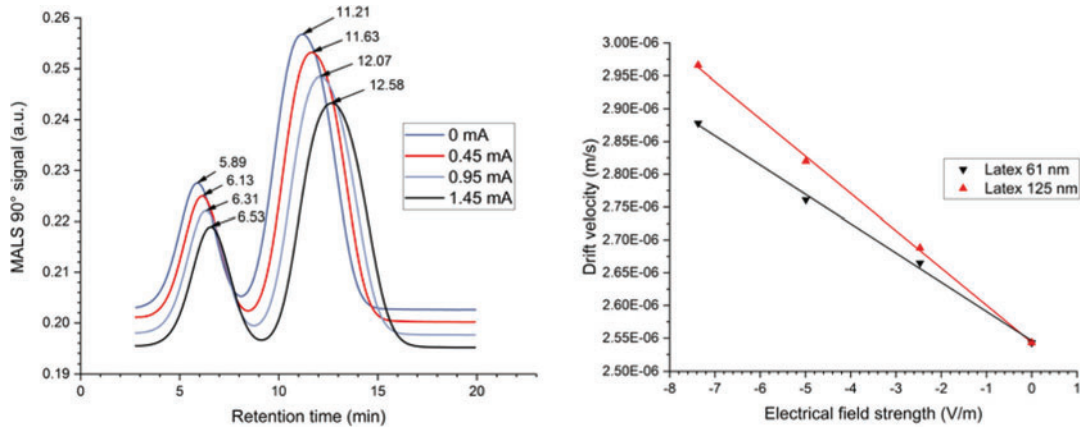

Figure 3: EAF4-MALS fractograms of the investigated nanoplastics particle mixture obtained for four different electrical field strengths (left). Differential velocity versus electrical field strength plot to determine the electrophoretic mobility of the two nanoplastic particles in the mixture (right).

Table II: Overview of the electrophoretic mobilities and zeta potentials of the two investigated nanoplastic particles calculated from EAF4 measurements. Zeta potentials are calculated using the Smoluchowski approximation and compared with data obtained from bulk zeta potential measurements.

	Nominal diameter, TEM (nm)	Electrophoretic mobility, EAF4 (1E-8 m^2 V^{-1} s^{-1})	Zeta potential, EAF4 (mV)	Zeta potential, bulk measurement (mV)
Polystyrene latex nanoplastics mixture	61 ± 4	-4.31 ± 0.06	-55.2 ± 0.8	-62.1 ± 1.0
	125 ± 3	-5.11 ± 0.23	-65.5 ± 3.0	

flow field without application of an electrical field (0 mA), while, in the second fractogram (black graph), an additional electrical field (1.45 mA) was applied. It can be clearly seen that the electrical field induced a measurable shift in the retention time due to the surface charge of both particles. At the same time, the measured size of both particles (radius of gyration, Rg, blue and black dotted line) remained unaffected, highlighting that there is no influence of the electrical field on the stability of the particle mixture (Table I).

In order to derive reliable data about the electrophoretic mobiliy and zeta potential of a sample, repeated EAF4 measurements under similar cross flow conditions, but different electrical fields, needed to be performed.

Figure 3 displays the EAF4-MALS fractograms of the investigated nanoplastics mixture obtained under four different electrical field strengths. By measuring the shift in retention time and relating it to the applied electrical field, the electrophoretic mobility and zeta potential of the particles can be calculated.

Comparing the EAF4 results with data obtained from bulk zeta potential measurements clearly highlight the advantage of EAF4 for polydisperse samples, particularly when sample constituents exhibit different surface charges (Table II).

Conclusion

The EAF4 system allows both size and surface charge separation, enabling determination of size or molecular weight distribution and electrophoretic mobility and zeta potential in one single instrument. As applications for nanoplastics analysis increase, high-resolution separation techniques will be required for these likely polydisperse distributions. Different polymer materials may have different electrophoretic mobility, leading to the need for a characterization tool such as EAF4 to provide size and charge information for complex samples.

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Fractionation of Aliphatic and Aromatic Hydrocarbons in Petroleum Extracts

Dr. Xiaoyan Wang, UCT, LLC

The composition of petroleum is a complex mixture of hundreds of different hydrocarbon compounds. The resultant makeup of hydrocarbons released into the environment is variable, and dependent on the conditions to which it is subsequently exposed. The fractionation of the total extractable petroleum hydrocarbons (EPH) is necessary to determine the concentration of the aliphatic versus aromatic compounds. This application note describes the use of unbonded silica gel solid phase extraction (SPE) cartridges to fractionate the C9-C18 aliphatic hydrocarbons (n-nonane to n-octadecane), C19-C36 aliphatic hydrocarbons (n-nonadecane to hexatriacontane), and the C11-C22 aromatic hydrocarbons (naphthalene to benzo(ghi)perylene).

Extraction/Analytical Materials	
XRSIHT15M25	ENVIRO-CLEAN® EPH fractionation Silica 5000 mg/25mL (Gravity Flow) capped at both ends to ensure silica integrity
VMF016GL	16 position glass block manifold
VMF02116	Pack of 16 stopcocks
VMF02125	12 position large volume collection rack

Procedure

1. Cartridge Conditioning

a) Remove the caps from both ends of the silica gel SPE cartridges and attach the SPE cartridges to the stopcocks on a 16-position glass block manifold. Add 10 mL of n-hexane immediately into the cartridges to prevent silica gel from adsorbing the ambient moisture and allow to pass through via gravity.

b) Add 2 more aliquots of 10 mL hexane. Close the stopcock to stop the flow once the hexane level reaches the top frit. Do not allow the top frit or the silica gel sorbent to go dry.

2. Sample Loading

a) Add 1 mL EPH standard prepared in hexane or sample extract (exchanged into 1 mL hexane) to the SPE cartridges; 0.5 mL hexane rinse can be used to ensure quantitative transfer.

b) Let samples pass by gravity until the level reaches the top frit, then close the stopcock.

3. Elution

a) Insert the 12-position collection rack with 40-mL VOA glass vials into the manifold.

b) Elute the aliphatic fraction with 20* mL of n-hexane (2 ×10 mL) by gravity; collect and label the eluates as "aliphatic."

c) Remove the vials with aliphatic fractions from the collection rack, and insert new VOA vials to collect the aromatic fractions.

d) Elute the aromatic fraction with 20 mL of DCM (2 × 10 mL) by gravity; collect and label the eluates as "aromatic."

e) Concentrate the eluates to 1 mL or a higher volume if the desired detection limits can be achieved and analyze the two fractions separately by gas chromatography–mass spectrometry.

*The volume of n-hexane should be optimized so that only the aliphatic hydrocarbons are eluted without breakthrough of the aromatic hydrocarbons into the aliphatic fraction (the naphthalene and 2-methyl naphthalene breakthrough should be <5%). The optimum hexane volume may vary from lab to lab, depending on the moisture content in the lab environment.

Results

Breakthrough of Aromatic Hydrocarbons into the Aliphatic Fraction

Aromatic Hydrocarbons	Breakthrough %
Naphthalene	0.1
2-Methylnaphthalene	0.0

Demonstration of Fractionation Efficiency—Aliphatic and Aromatic Fractions

Hydrocarbons	Recovery %	RSD % (n = 4)
Aliphatic C9 - C18	91.0	2.1
Aliphatic C19 -C36	94.6	2.3
Aromatic C11 - C22	90.7	2.8

Conclusion

Excellent analytical performance has been obtained using UCT's EPH silica gel SPE cartridges (heat treated, large particle size) for the fractionation of aliphatic and aromatic hydrocarbons. Recoveries were ranged from 90.7 to 94.6% for three EPH fractions. The naphthalene and 2-methyl naphthalene breakthrough was < 1%, also meeting the required <5% breakthrough of the aromatics into the aliphatic fraction.

UCT, LLC
2731 Bartram Road, Bristol, PA 19007, USA
tel: (800) 385-3153; Email: methods@unitedchem.com
Website: www.unitedchem.com

Separation of Selected Components of Ylang-Ylang Oil

Adam L. Moore, PhD, Hamilton Company

Essential oils have been a staple of the food, fragrance, and health markets for many years. Owing to their unique composition, essential oils can be used for a variety of applications, including antiparasitics, antivirals, bactericides, fungicides, insecticides, and repellants, as well as food flavoring and mood enhancers. Market growth, coupled with limited production of naturally occurring products, has led a large segment of industrial essential oil suppliers to turn to the addition of synthetic additives. The production of an essential oil through synthetic routes (arrived at through the use of petroleum products) has been adopted for two main reasons; the price of natural oils, and for consistency of products (natural oils can vary in the percentage of individual components from year-to-year). Unfortunately, the addition of these synthetic adjuncts can give rise to a number of health concerns that are generally associated with petroleum products, such as allergic reactions, migraines, asthma attacks, nausea, eczema, and various other sensitivities.

In this study of an essential oil, we have developed a high performance liquid chromatography (HPLC) method for the separation of six well-known markers for the determination of purity of ylang-ylang oil. This method utilizes one of the benefits of polymeric based columns: the ability to consistently operate in alkaline pH without sacrificing performance. In this method, we have chosen the typical markers for an extracted essential oil utilizing the Hamilton PRP-C18 column.

Column Information	
Packing Material	PRP-C18, 5 µm
Part Number	79676
Chromatographic Conditions	
Gradient	0–5 min, 60% B / 5.01–9 min, 60–100% B / 9.01–14 min, 100% B
Temperature	30 °C
Injection Volume	5 µL
Detection	UV at 214
Dimensions	150 × 4.6 mm
Eluent A	30 mM Et_2NH (pH = 12)
Eluent B	Acetonitrile
Flow Rate	2.0 mL/min

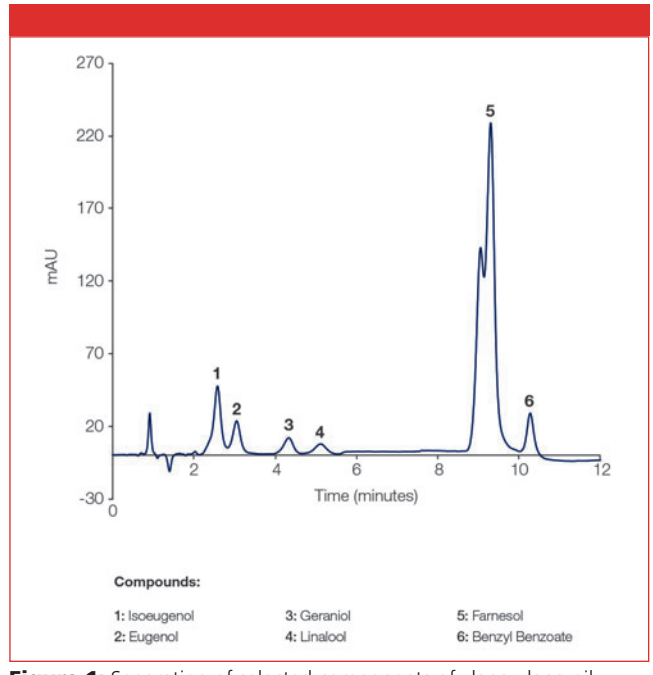

Figure 1: Separation of selected components of ylang-ylang oil

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Website: www.hamiltoncompany.com

Determination of Triphenylmethane Dyes from Aquaculture Samples

Hans Rainer Wollseifen, Torsten Kretschmer, Johannes Brand, and Detlef Lambrecht, Macherey-Nagel GmbH & Co. KG

This application note describes the determination of the triphenylmethane dyes Malachite Green and Crystal Violet and their metabolites from the aquaculture samples brown trout, shrimp, and tuna using dispersive SPE (dSPE) with CHROMABOND® QuEChERS mixes for sample clean-up. The eluates from dSPE cleaning are finally analyzed by HPLC–MS/MS using the biphenyl phase NUCLEODUR® π².

Motivated by various potential health benefits, human feeding behavior is changing, leading to an increasing demand for aquatic products. Farming aquatic species is becoming an important way of producing large amounts of aquatic products all over the world. One challenge of farming aquatic species is the control of infectious diseases.

Triphenylmethane dyes (TPM) are organic compounds originally used as textile and paper dyes and have been found to be effective as antibacterial, antifungal, and antiparasitic agents in fisheries. They accumulate in fish and metabolize to the equivalent, colorless leuco-forms, which are also known as *mutagenic* (1). To protect human health, triphenylmethane dyes have been banned in many countries according to the recommendations of national and international related agencies. For example, the European Union has implemented a minimum required performance limit (MRPL) for the sum of Malachite Green and Leucomalachite Green of 2 µg/kg (2).

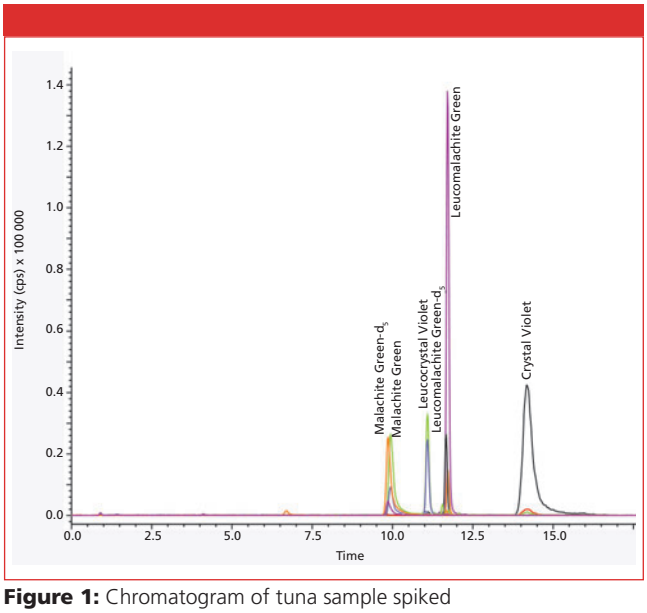

Figure 1: Chromatogram of tuna sample spiked with 1.0 µg/kg sample for each analyte.

Table 1: MRM transitions for the analysis of triphenylmethane dyes

Analyte	$[M+H]^+$	Q_1 (Quantifier)	Q_2 (Qualifier)
Crystal Violet	372.2	356.3	251.1
Leucocrystal Violet	373.9	358.0	238.2
Leucomalachite Green	330.9	316.2	239.1
Leucomalachite Green-d_5	335.9	321.1	243.1
Malachite Green	328.9	313.1	207.9
Malachite Green-d_5	333.9	318.3	213.1

QuEChERS (Quick, Easy, Cheap, Effective, Rugged, Safe) methodology is known as a common sample preparation technique in modern analysis of pesticides in food, and offers a quick and cost-efficient determination of different analytes in strongly matrix-contaminated samples by GC–MS and LC–MS (3).

Biphenyl-modified HPLC phases feature a better retention of aromatic analytes compared to classical C_{18} phases due to additional π-π interactions.

Dispersive Solid-Phase Extraction (4)

Extraction:

- Weigh out 5 g of homogenized sample into a 50 mL brown centrifuge tube
- Add 5 µL of internal standard solution (β = 1 µg/mL Malachite Green-d_5 and Leucomalachite Green-d_5 each in acetonitrile)
- Add 25 µL of standard solution (β = 200 ng/mL Malachite Green, Leucomalachite Green, Crystal Violet, Leucocrystal Violet each in acetonitrile) for determining recovery rate
- Add 5 mL water and shake
- Add 10 mL 1% acetic acid in acetonitrile and shake
- Add the CHROMABOND QuEChERS extraction Mix II (Macherey-Nagel REF 730971)
- Shake vigorously for 30 s and cool the mixture down in an ice bath
- Centrifuge the mixture at 4500 rpm for 5 min at 4 °C

Clean-up:

- Put 5 mL acetonitrile supernatant in a 15 mL brown centrifuge tube
- Add the CHROMABOND QuEChERS clean-up Mix III (Macherey-Nagel REF 730972) (for samples with high fat content add 1 mL hexane)
- Shake vigorously for 30 s
- Centrifuge the mixture at 4500 rpm for 5 min at 4 °C
- Dilute the extract 1:1 with an aqueous solution of 5 mmol/L

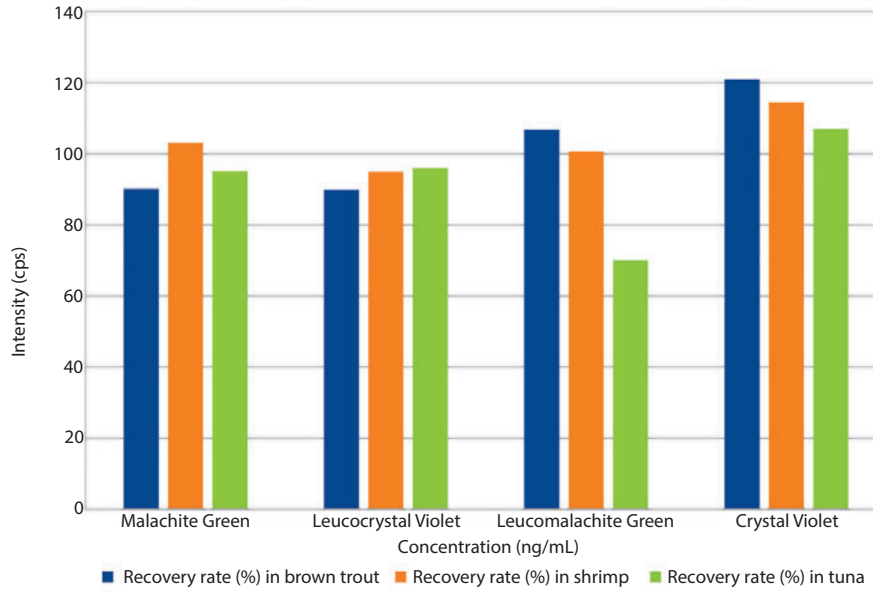

Figure 2: Recovery rates for presented dispersive solid-phase extraction method.

ammonium acetate + 1 mL/L formic acid and filter through a syringe filter (CHROMAFIL® Xtra PET-20/13, Macherey-Nagel REF 729208)

Subsequent Analysis: HPLC–MS/MS (5)

HPLC column: EC 100/3 NUCLEODUR π^2, 3 µm (Macherey-Nagel REF 760636.30)
Eluent A: 5 mmol/L ammonium acetate + 0.1% formic acid in water
Eluent B: acetonitrile
Gradient: 20–85% B in 10 min, 85% B for 5 min, 85–20% B in 1 min, 20% B for 5 min
Flow rate: 0.4 mL/min
Temperature: 25 °C
Injection volume: 5 µL
MS/MS detection: LCMS 8050 (Shimadzu); ion source: ESI, positive ionization mode; scan type: MRM; interface heater on; interface current: 4000 V; interface temperature: 300 °C; DL temperature: 250 °C; nebulizing gas flow: 3.00 L/min; heating gas on; heating gas flow: 10.00 L/min; heat block: 400 °C; drying gas on; drying gas flow: 10.00 L/min

Results

The identification and quantification of TPM dyes in the cleaned sample extracts of brown trout, shrimp, and tuna were successfully performed by ESI mass spectrometry. Calibration curves were in the concentration range between 0.5 ng/mL and 50 ng/mL for each analyte. Figure 1 shows the chromatogram of QuEChERS extract from the tuna sample spiked with 1 µg/kg with high signal response and less matrix interferences.

By using the QuEChERS methodology it was possible to recover more than 70% of TPM dyes from the different matrices (see Figure 2). The use of brown centrifuge tubes protects the analytes from light-induced degradation and leads to better sensitivities and recovery rates.

Conclusion

In summary, the presented application describes a quick and convenient method for the determination of TPM dyes from aquatic samples.

References

(1) BfR Expert Opinion No. 007/2008, 24 August 2007.
(2) Official Journal of the European Communities L 221, 17 August 2002, 8–36.
(3) M. Anastassiades, S.J. Lehotay, D. Stajnbaher, and F.J. Schenck, *J. AOAC Int.* **86**, 412–431 (2003).
(4) SPE Application Nos. 306560, 306570 and 306580, Macherey-Nagel, available from www.mn-net.com/apps.
(5) HPLC Application No. 128430, Macherey-Nagel, available from www.mn-net.com/apps.

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LC–MS/MS Analysis of Mycotoxins in Peanut Powder in 5.5 Min

Restek Corporation

- Fast analysis for higher sample throughput
- Excellent separation improves accuracy for 12 regulated mycotoxins
- Quick and easy sample preparation (dilute-filter-shoot)

Certain fungi that can grow on agricultural products produce toxic metabolites known as mycotoxins. Modern food processing procedures cannot completely remove these compounds if they are present, so strict monitoring protocols have been established. Although a universal method for the analysis of mycotoxins would allow highly efficient screening, it is very challenging to develop such a method, due to differences in physiochemical properties of mycotoxins, extraction efficiencies, and matrix effects. Zhang and associates published a multi-lab study (1) aimed at providing labs with an analytical procedure that could be broadly applied to the analysis of a variety of mycotoxins in many different matrices. Using that work as inspiration, we developed the following LC–MS/MS method that resolves 12 FDA regulated mycotoxins within the pressure limits of traditional HPLC instruments.

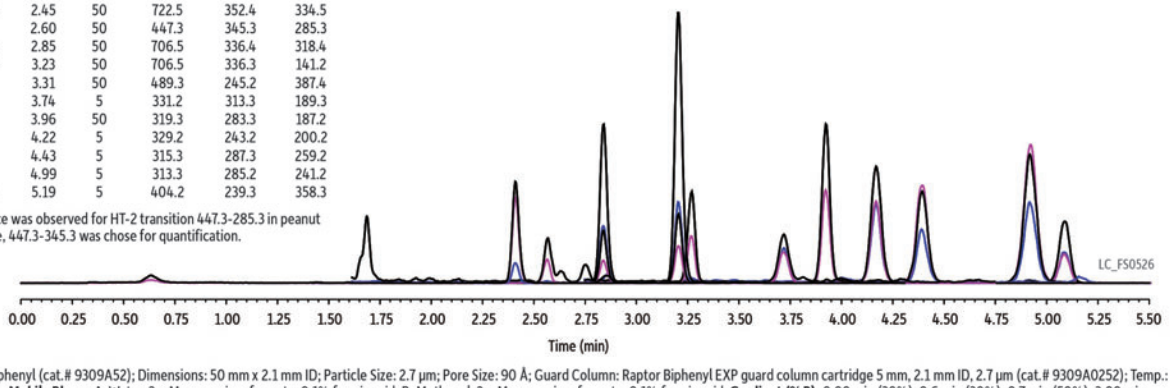
In this example, mycotoxins were analyzed in a peanut powder matrix. The use of a relatively short column format, the selectivity of the Biphenyl stationary phase, and the efficiency of 2.7-µm Raptor superficially porous particles provided excellent separations in a fast 5.5-min analysis (total cycle time of 7 min). A coeluting matrix compound that shared the most abundant MRM transition for mycotoxin HT-2 (447.3-285.3) was observed, so a less abundant transition (447.3-345.3) was selected for quantitation. To increase sensitivity, an ammonium buffer was used to promote better ionization of mycotoxins. The Raptor Biphenyl column worked very well for the 12 mycotoxins studied in the cited work, but for longer compound lists containing isobaric mycotoxins with similar structures, the Raptor FluoroPhenyl phase may be necessary to provide adequate chromatographic resolution. The selectivity of the Raptor FluoroPhenyl column is demonstrated in an analysis of 20 mycotoxins that can be found by visiting www.restek.com and entering LC_FS0511 in the search.

This method showed excellent precision and accuracy for the 12 FDA regulated mycotoxins that were evaluated during a validation study that covered a variety of matrices (including multiple sources of cornmeal and brown rice flour, in addition to the peanut powder example shown here).

Restek would like to thank Dr. Zhang for his technical support during this project.

Peaks	tR (min)	Conc. (ng/g)	Precursor Ion	Product Ion 1	Product Ion 2
1. Deoxynivalenol	0.62	50	297.3	249.3	231.2
2. Fumonisin B1	2.45	50	722.5	352.4	334.5
3. HT-2	2.60	50	447.3	345.3	285.3
4. Fumonisin B3	2.85	50	706.5	336.4	318.4
5. Fumonisin B2	3.23	50	706.5	336.3	141.2
6. T2	3.31	50	489.3	245.2	387.4
7. Aflatoxin G2	3.74	5	331.2	313.3	189.3
8. Zearalenone	3.96	50	319.3	283.3	187.2
9. Aflatoxin G1	4.22	5	329.2	243.2	200.2
10. Aflatoxin B2	4.43	5	315.3	287.3	259.2
11. Aflatoxin B1	4.99	5	313.3	285.2	241.2
12. Ochratoxin A	5.19	5	404.2	239.3	358.3

Matrix interference was observed for HT-2 transition 447.3-285.3 in peanut powder. Therefore, 447.3-345.3 was chose for quantification.



Column: Raptor Biphenyl (cat.# 9309A52); Dimensions: 50 mm x 2.1 mm ID; Particle Size: 2.7 µm; Pore Size: 90 Å; Guard Column: Raptor Biphenyl EXP guard column cartridge 5 mm, 2.1 mm ID, 2.7 µm (cat.# 9309A0252); Temp.: 40 °C; Inj. Vol.: 5 µL; **Mobile Phase:** A: Water, 2 mM ammonium formate, 0.1% formic acid; B: Methanol, 2 mM ammonium formate, 0.1% formic acid; **Gradient (%B):** 0.00 min (30%), 0.6 min (30%); 0.7 min (50%); 3.00 min (70%); 4.5 min (75%); 5.0 min (90%); 5.2 min (90%); 5.21 min (75%); 6.00 min (75%); 6.01 min (30%); 7.00 min (30%); **Flow:** 0.5 mL/min; **Detector:** MS/MS; Ion Mode: ESI+; Mode: MRM; **Instrument**: UHPLC; **Notes:** Weighed 1.00 gram of peanut powder in a 50 mL centrifuge tube and added 2.00 mL of water. Vortexed at 3000 rpm for 5 min followed by the addition of 4.0 mL of extraction solvent (50:50 water:acetonitrile, v/v). The tube was then vortexed at 3000 rpm for 5 min followed by centrifugation for 15 min at 4200 rpm. 475 µL of the supernatant was filtered through a Thomson SINGLE StEP Nano filter vial (0.2 µm, cat.# 25882). The sample was then fortified with 25 µL of a standard solution prepared in water at 1000 ng/mL (100 ng/mL for aflatoxins and ochratoxin A) as part of the matrix-matched calibration curve. Vortexed at 3000 rpm for 1 min prior to analysis.

Raptor Biphenyl LC Columns (USP L11)

Length	2.1 mm cat.#	3.0 mm cat.#	4.6 mm cat.#
1.8 µm Columns			
30 mm	9309232	—	—
50 mm	9309252	930925E	—
100 mm	9309212	930921E	—
150 mm	9309262	—	—
2.7 µm Columns			
30 mm	9309A32	9309A3E	9309A35
50 mm	9309A52	9309A5E	9309A55
100 mm	9309A12	9309A1E	9309A15
150 mm	9309A62	9309A6E	9309A65
5 µm Columns			
30 mm	—	930953E	—
50 mm	9309552	930955E	9309555
100 mm	9309512	930951E	9309515
150 mm	9309562	930956E	9309565
250 mm	—	—	9309575

Reference

(1) K. Zhang, M.R. Schaab, G. Southwood, E.R. Tor, L.S. Aston, W. Song, B. Eitzer, S. Majumdar, T. Lapainis, H. Mai, K. Tran, A. El-Demerdash, V. Vega, Y. Cai, J.W. Wong, A.J. Krynitsky, T.H. Begley, *J Agr Food Chem,* **65**(33) 7138–7152 (2017). https://www.ncbi.nlm.nih.gov/pubmed/27983809.

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Shodex Rapid SUGAR Series: Fast Fermentation Monitoring and Sourdough Cultures

Alex Schrum, Ron Benson, Marlon Rettis, and Leah Sullivan, Showa Denko America, Inc.

Living organisms (yeast and bacteria) were not identified as the agents that produced or spoiled common food items until the 19th century. Baking, especially of bread, was among the first industries to be transformed by the emerging scientific understanding of fermentation. The introduction of commercialized yeast in the early 20th century steadily reduced the time needed to bake bread both at home and on the production line, making way for modern packaged breads (1). However, traditional fermentation techniques, including sourdough breads, have resurged in popularity. Typical sourdough cultures are created from yeast and bacteria populations naturally present in the air and on many surfaces, providing sourdough loaves with their characteristic rise and "tangy" flavor by converting fermentable sugars into CO_2 and flavor compounds like lactic acid. In this application note, a "homemade" sourdough starter culture was analyzed to determine how key fermentation products changed over the course of 24 h with the new Rapid SUGAR SH1011 8C column.

The Shodex™ Rapid SUGAR SH1011 8C column contains a styrene divinylbenzene base material ligand exchange column and is designed for rapid fermentation monitoring, allowing for the analysis of saccharides, organic acids, and ethanol in less than 5 min. The method uses simple aqueous conditions and RID detection, ideal for large sample workflows and QC environments.

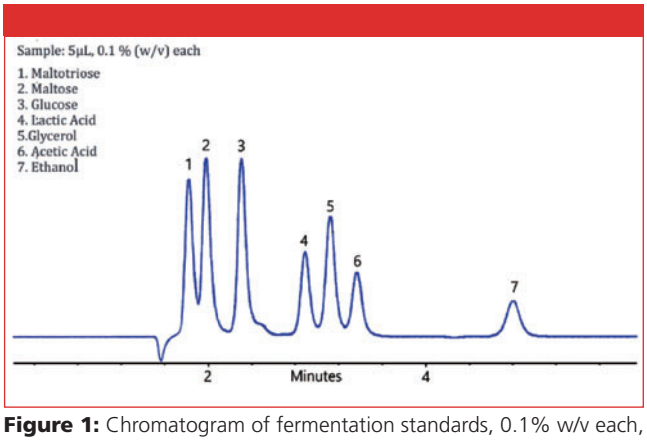

Figure 1: Chromatogram of fermentation standards, 0.1% w/v each, 5 µL injection

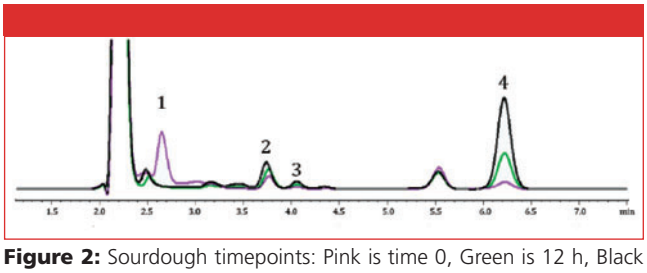

Figure 2: Sourdough timepoints: Pink is time 0, Green is 12 h, Black is 24 h. Peak 1: glucose and sample matrix. Peak 2: lactic acid. Peak 3: glycerol. Peak 4: ethanol

Experimental

Seven common fermentation compounds (maltotriose, maltose, glucose, lactic acid, acetic acid, glycerol, and ethanol) were used as standards.

The sourdough starter culture was adapted from King Arthur (2) with the procedure repeated daily for two weeks.

Sample Preparation

Approximately 10 g of sample was homogenized with 90 mL distilled water (maximal speed, 30 s). Five milliliters of 1 mol/L $HClO_4$ solution was added to a 10 mL aliquot of the homogenate. The mixture was centrifuged for 15 min at 4000 g at 15 1C, the supernatant was neutralized (pH 7.0) with 2 mol/L KOH and the volume was adjusted to 25 mL with distilled water. After 30 min precipitation on ice, the solution was filtered on 0.45 mm cellulose filter, adapted from reference (3).

Samples were taken starting on Day 15 at three time points:

Sample 1: Immediately after mixing in flour and water, labelled time 0

Sample 2: 12 h after time 0

Sample 3: Day 16, approximately 24 after time 0

Shodex SUGAR SH1011 8C (8.0 mm I.D. × 100 mm, 6 µm) was used with a Shodex RI-501 detector. The eluent conditions were: 1 mM $H2SO_4$ aq. The column was kept at 75 °C and the flow rate was 1.0 mL/min. The chromatography was performed on a Shimadzu Prominence I series LC 2030.

Results and Discussion

This simple method using 1 mM sulfuric acid as the eluent demonstrated a successful simulated analysis of fermentation byproducts in less than 5 min, including the column equilibration time. For the analysis of the sourdough starter culture a guard column was used: (Shodex™ SUGAR SH-G (6.0 × 50 mm id, 10 µm) (Figure 2).

A method for rapid fermentation monitoring was developed using the Shodex SUGAR SH1011 8C column. Fermentation sample measurement completes in 6 min. The column proved to be robust when used with complex sample matrixes like sourdough cultures. The results show that the SH1011 8C can be used to monitor fermentation products like organic acids and ethanol.

References

(1) J. Marx, J.H. Litchfield, *A Revolution in Biotechnology,* (Cambridge University Press), **71,** (1989).

(2) *The King Arthur Flour Baker's Companion: The All-Purpose Baking Cookbook* (The Countryman Press, New York, New York, 2012).

(3) D. Lefebvre, V. Gabriel, Y. Vayssier, and C. Fontagné-Faucher, *Food Sci. Tech* **35**, 407–414 (2002).

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Analysis of Mycotoxins in Hemp and Hemp-Containing Edible Products

Maria Ofitserova, PhD, and Sareeta Nerkar, PhD,
Pickering Laboratories, Inc.

Pickering Laboratories developed an easy and sensitive method to analyze aflatoxins B1, B2, G1, G2, and ochratoxin A in hemp and hemp-containing edible products. Mycotoxins are isolated using immunoaffinity clean-up columns and analyzed with fluorescence detection. To increase sensitivity of aflatoxins B1 and G1, an in-line photochemical reactor (UVE™) is installed before the detector. This method utilizes standard HPLC equipment and allows laboratories to easily determine these mycotoxins at low ppb levels.

Method

Isolation of aflatoxins B1, B2, G1, G2, and ochratoxin A

Blend 1 g of finely ground sample with extraction solution (10 mL of methanol/water 80:20) using a handheld homogenizer. Centrifuge for 10 min. Mix 2 mL of the extract with 10 mL of PBS buffer (containing 2% Tween 20). Clean the extracts using AflaOchra HPLC immunoaffinity column (Vicam).

Analytical Conditions

Analytical Column: Mycotox™ (Pickering), C18, 4.6 × 250 mm
HPLC eluent: sodium phosphate buffer (Cat #1700–1108), methanol, acetonitrile
Flow rate: 1 mL/min
Post-column photochemical reactor: UVE (Cat # 10519 (240V), Cat # 10742 (120 V) FLD: Excitation 36 nm, Emission 430 nm for Aflatoxins Excitation 333 nm, Emission 477 nm for Ochratoxin A

HPLC gradient			
Time	1700–1108 (%)	Methanol (%)	Acetonitrile (%)
0	57	28	15
13	57	28	15
16	30	45	25
25	30	45	25
Equilibration time: 10 min			

Calibration

The 5-point calibration curves were built in the ranges of 0.325–3.25 ppb for B1, 0.088–0.882 ppb for B2, 0.310–3.099 ppb for G1, 0.099–0.996 ppb for G2, and 1–10 ppb for ochratoxin A. Correlation coefficient was R^2 >0.999 for all toxins.

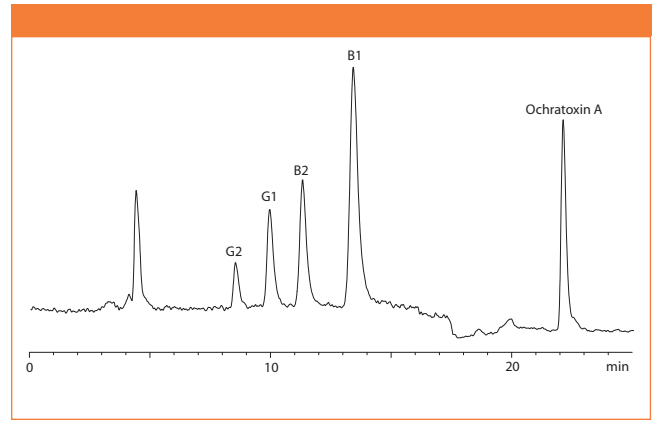

Figure 1: Chromatogram of hemp pre-roll spiked with 6.5 ng/g of aflatoxin B1; 1.8 ng/g of aflatoxin B2; 6.1 ng/g of aflatoxin G1; 1.9 ng/g of aflatoxins G2; and 20.1 ng/g of ochratoxin A.

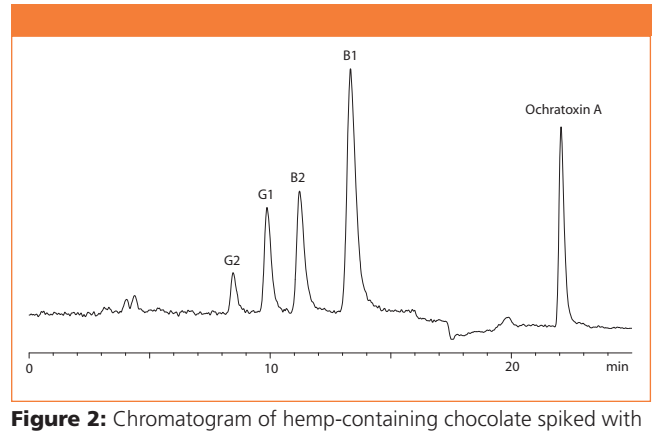

Figure 2: Chromatogram of hemp-containing chocolate spiked with 6.5 ng/g of aflatoxin B1; 1.8 ng/g of aflatoxin B2; 6.1 ng/g of aflatoxin G1; 1.9 ng/g of aflatoxins G2; and 20.1 ng/g of ochratoxin A.

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Website: www.pickeringlabs.com

Table I: Recoveries

		CBD-containing tincture		CBD-containing Chocolate		CBD oil		Hemp Pre-rolls		Full-spectrum CBD chews	
Toxins	Spike levels, ng/g	Recoveries, %	RSDr, %	Recoveries, %	RSDr, %	Recoveries, %	RSDr, %	Recoveries, %	RSDr, %	Recoveries, %	RSDr, %
Aflatoxin G2	1.932	91.85	2.54	78.89	1.42	88.67	7.32	84.66	10.82	93.90	6.85
Aflatoxin G1	6.198	92.50	7.86	85.89	1.21	96.35	1.24	93.56	7.05	97.93	2.42
Aflatoxin B2	1.764	92.99	5.30	88.73	2.88	99.31	1.84	94.28	6.77	101.63	1.72
Aflatoxin B1	6.498	84.73	3.51	87.88	0.56	91.62	3.08	85.78	7.50	91.97	1.99
Ochratoxin A	20.16	92.31	4.50	91.86	4.17	89.75	2.26	85.68	5.08	86.97	5.27

Complete Solution for TCA Cycle Organic Acid Analysis

Robert Puryear*, Piotr Macech*, and Itaru Yazawa†,
*Imtakt USA, Portland, OR, †Imtakt Corp., Kyoto, Japan

The TCA cycle organic acids come in a wide variety, including mono-, di-, and tri- carboxylic acids. They are notoriously difficult to analyze, especially on a single stationary phase. Our new Intrada Organic Acid column was designed not only for these compounds, but for nearly all organic acids.

TCA cycle organic acids (OA) can be important biomarkers in metabolomic studies of disease. They can be mono-, di-, or tri-carboxylic acids, which presents significant challenges to chromatographic analysis. OAs are polar, making them difficult to retain on traditional reversed phase columns without ion-pairing or derivatization. They can also contain multiple pKa values, where variations in the charge states of your analytes may cause poor peak shapes.

Recognizing these challenges, we developed them most complete solution for OA analysis, with a novel stationary phase called Intrada Organic Acid. This proprietary multi-mode column optimizes the use of multiple stationary phase structures specifically targeting OAs, delivering the most complete LC-MS-based solution on the market today.

Experimental Conditions

Running conditions are shown within the figures. MS detection was used in all cases.

Result and Discussion

Figure 1 shows simultaneous analysis of the OAs in the TCA cycle. Retention of these polar compounds was excellent with times ranging from 2.3 to 5.4 min, without using ion-pairing or derivatization. Peak shapes are nearly ideal for all analytes, but oxaloacetate does show some minor tailing. This is most likely due to the highly unstable nature of this compound, which spontaneously decarboxylates.

In Figure 2, the mobile phase was optimized for OAs with multiple pKa values. For this specific challenge, we were able to achieve single sharp peaks, utilizing a combination of normal phase mode in neutral conditions and ion-exchange. We did not observe any peak splitting, shouldering, or tailing, which are common problems for polycarboxylic acids. Therefore, under certain circumstances, normal phase conditions might be preferred.

Conclusion

Organic acids comprise a staggeringly broad group, ranging from the simple carboxylic acids to complex polycarboxylic acids, where multiple acidic residues and pKA values present significant chromatographic woes. As a group, OAs can be as challenging in their analysis as the varieties that are analyzed today, but our Intrada Organic Acid can be used for wide array of OAs, on a single column. The specially developed multi-mode stationary phases utilized in this column's design provide the broadest capability for the analysis of OAs under MS-friendly conditions on the market today. We have shown that its versatility in the use of many different mobile phase conditions enables optimization to meet a wide range of needs. Never before has such a solution been available to chromatographers. Whether you are analyzing simple carboxylic acids or complex multivalent species with multiple pKa values, Intrada Organic Acid could be a single column solution for all your OA analysis needs. Please feel free to reach out to one of our column experts to learn how this revolutionary column could be used in your laboratory.

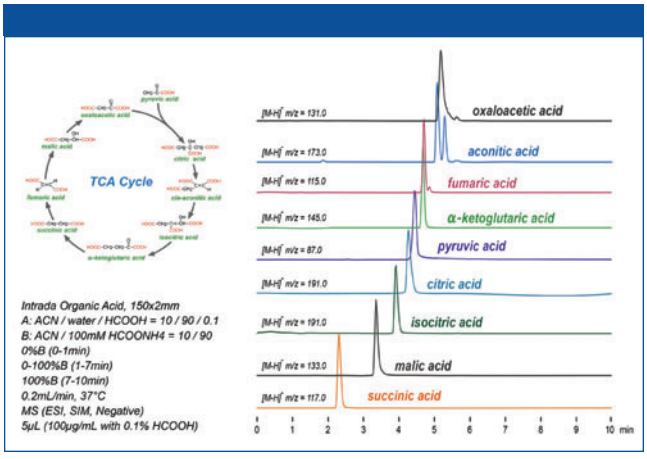

Figure 1: Analysis for all TCA cycle organic acids

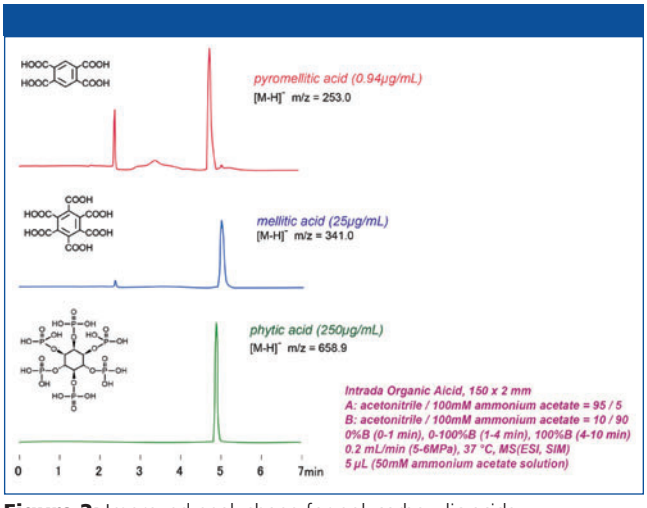

Figure 2: Improved peak shape for polycarboxylic acids

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Website: ImtaktUSA.com

Transient Protein Self-Association Determined by SEC-MALS

M. Burman and O. Schön, Glaxo-SmithKline

Domain antibodies (dAbs) are the smallest functional binding units of antibodies, corresponding to the variable regions of either the heavy (VH) or light (VL) chains of human antibodies. Different dAbs can show different degrees of self-association, which drives the need for a cheap, quick—yet robust—technique to study self-association behavior in drug discovery. This study outlines the use of size exclusion chromatography with multi-angle light scattering (SEC-MALS) to determine self-association properties, and validates the work by comparison with analytical ultracentrifugation (AUC).

Materials and Methods

A Shimazdu LC-20AD Prominence HPLC was used in series with miniDAWN® and Optilab® for this study. The columns used were TSK-GEL3000SW$_{XL}$ and Superdex S200. Data were collected and analyzed in ASTRA® software to determine weight-average molar mass at each concentration.

The dAb_1 sample was expressed, purified, and dialyzed into PBS, pH 7.4 and submitted to SEC-MALS and AUC. For MALS, the sample was concentrated to 2.4, 7.0, 75, and 130 mg/mL and followed by appropriate dilution to obtain nine different concentrations which were run on SEC-MALS (Table I).

Stock Conc. (mg/mL)	Sequence Monomer Mw (kDa)	Sequence Monomer M_w (kDa)	
		Concentration mid-peak (mg/mL)	Determined M_w
0.1	No11.634	0.015	11.5
0.5		0.048	11.26
1		0.092	11.9
2		0.185	12.85

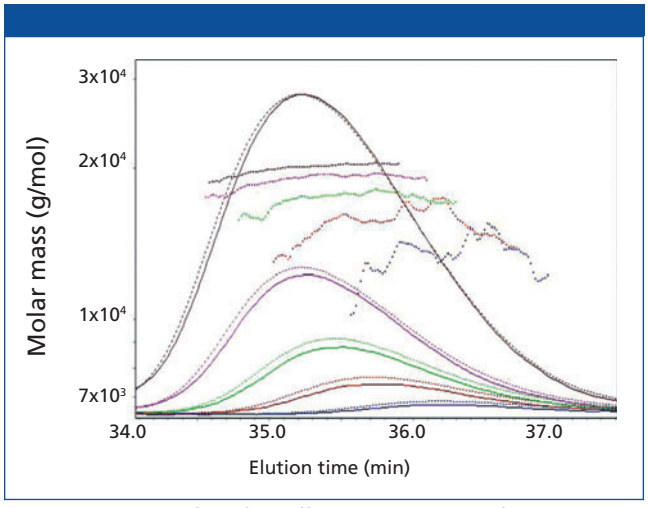

Figure 1: Overlay of the five different sample results from SEC-MALS highlight-ing the a "Determined M_w." Samples with stock concentrations 5 mg/mL, 15 mg/mL, 30 mg/mL, 60 mg/mL, and 120 mg/mL, representing the smallest to biggest peaks respectively.

Table I: List of the samples injected, Mw and concentration as determined by SEC-MALS. A plot of M_w against log (concentration) is used to determine K_d

5		0.369	13.54
15		1.03	15.59
30		2.11	17.35
60		4.08	18.92
120		9.05	19.92

The equilibrium dissociation constant for dimerization $K_{2,1}$ was determined by plotting a graph of M_w versus log-concentration obtained from SEC-MALS mid peak.

Results and Discussion

Figure 1 shows the overlay of dRI and light scattering signals for five injections corresponding to stock concentrations from 5 mg/mL to 120 mg/mL (additional injections at lower concentration were also performed, per Table I). Dilution on the size-exclusion columns decreases the peak concentration by approximately seven-fold, and other areas of the peak have respectively lower concentrations.

Due to dynamic monomer-dimer equilibrium, the molar mass determined by MALS is not uniform across the peak. For best accuracy, the calculations are performed using the concentration and molar mass values determined at the maximum of each peak. The $K_{2,1}$ values for dAb_1 from SEC-MALS was calculated as 300 µM, comparing well to 391 µM from the AUC experiment.

While AUC is often considered the gold standard for the determination of self-association in solution, scientists are looking for alternatives due to AUC's high costs, low throughput and expertise requirements. Here, SEC-MALS has shown excellent agreement with AUC results and can be used as a cheaper, easier and quicker alternative.

Another light-scattering method that is suitable for characterization of transient self-association beyond monomer-dimer equilibrium is composition-gradient multi-angle light scattering (CG-MALS). With similar low cost and ease of use, CG-MALS provides extended analysis of self- and hetero-association, similar to equilibrium sedimentation by AUC.

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Characterization of a Novel Antibody Drug Conjugate Mimic by Size-exclusion and Hydrophobic Interaction Chromatography

Tosoh Bioscience

Empowered antibodies, such as antibody drug conjugates (ADCs), continue to be investigated as biotherapeutic drug candidates. ADCs combine the tumor specificity and targeting capability of monoclonal antibodies (mAbs) with the cytotoxicity of potent small molecule drugs into hybrid molecules that are promising anticancer therapeutics. These molecules are comprised of three components: a monoclonal antibody, a stable linker, and a cytotoxic small molecule drug. For the cysteine-linked ADC mimic used in this study, a dansyl fluorophore (~668 Da) is covalently bonded to an IgG_1 mAb (150 kDa) via a LC-SMCC crosslinker (Figure 1). This procedure results in a mixture of drug-loaded antibody species with 0 to 8 drugs (Figure 2).

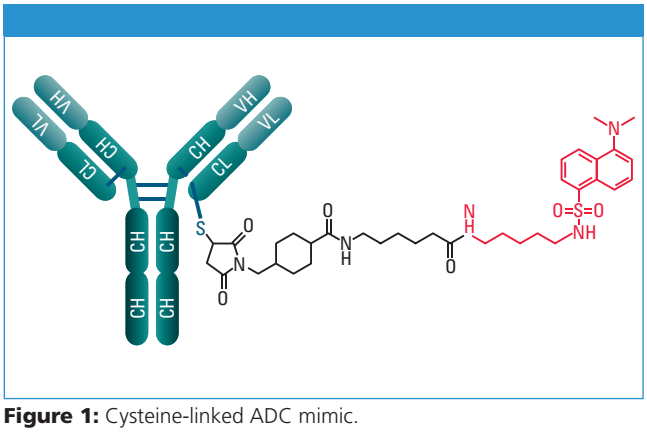

Figure 1: Cysteine-linked ADC mimic.

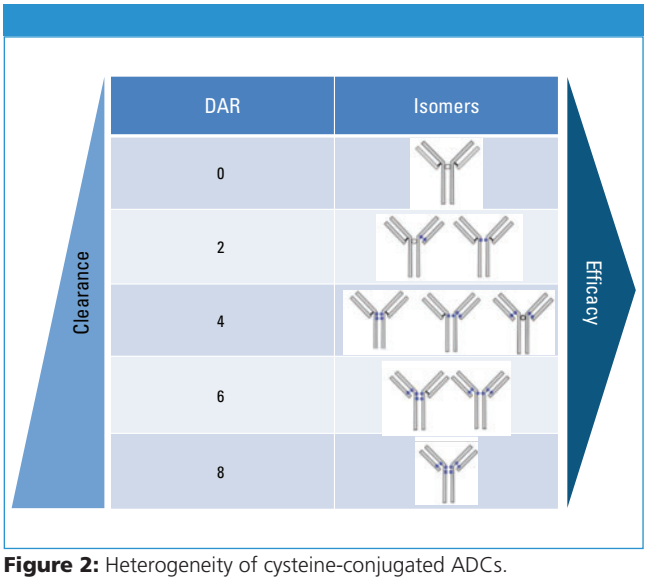

Figure 2: Heterogeneity of cysteine-conjugated ADCs.

ADC conjugation plays a role in both drug efficacy as well as clearance, and must be well understood during drug development. Size-exclusion chromatography (SEC) and hydrophobic interaction chromatography (HIC) are two commonly employed techniques used to characterize the drug-to-antibody ratio (DAR) under native, physiological conditions. In this application note, the ADC mimic was analyzed by size-exclusion chromatography/mass spectrometry (SEC/MS) using a TSKgel® SuperSW3000 column and by HIC using a TSKgel Butyl-NPR column. Coupling these chromatographic techniques allowed elucidation and verification of the DAR profile for this model biomolecule.

Experimental HPLC Conditions

SEC/MS Conditions

Column:	TSKgel SuperSW3000, 4 µm, 2 mm ID × 30 cm
Mobile phase:	100 mmol/L ammonium acetate, pH 7.0
Gradient:	Isocratic
Flow rate:	0.07 mL/min
Detection:	ESI–MS
Temperature:	35 °C
Injection vol.:	1.0 µL
Sample:	ADC mimic, 100 µg/mL (MilliporeSigma™), 100 mmol/L ammonium acetate, pH 7.0
MS mode:	Scanning, *m/z* 1000–8000

HIC/UV Conditions

Column:	TSKgel Butyl-NPR, 2.5 µm, 4.6 mm ID × 10 cm
Mobile phase:	A. 50 mmol/L potassium phosphate, 1.5 mol/L ammonium sulfate, pH 7.0 plus 5% (v/v) isopropyl alcohol B. 50 mmol/L potassium phosphate, pH 7.0 plus 20% (v/v) isopropyl alcohol
Gradient:	0% B to 100% B in 50 min
Flow rate:	1.0 mL/min
Detection:	UV @ 215 nm
Temperature:	35 °C
Injection vol.:	5.0 µL
Samples:	ADC mimic, 100 µg/mL (MilliporeSigma), 100 mmol/L ammonium acetate, pH 7.0

Results and Discussion

The ADC mimic was injected onto a TSKgel SuperSW3000 SEC column coupled to a mass spectrometer in order to examine the DAR profile. Figure 3 shows the deconvoluted mass spectrum of the ADC mimic. Main peaks can be seen at *m/z* 143,799; 145,135; 146,474; 147,812; and 149,147. The difference in molecular weight between each main peak is 1336 Da, corresponding to the molecular weight of two dansyl fluorophore molecules. The average DAR was found to be 3.9.

The DAR profile was then confirmed by HIC using a TSKgel Butyl-NPR column and ultraviolet (UV) detection. As more drug is conjugated to the mAb vehicle, the ADC becomes more hydrophobic and is retained longer by the HIC stationary phase, allowing resolution of the different drug loaded species. Figure 4 shows the DAR profile of the ADC mimic. The chromatogram shows well resolved peaks ranging from a DAR of 0 to 8.

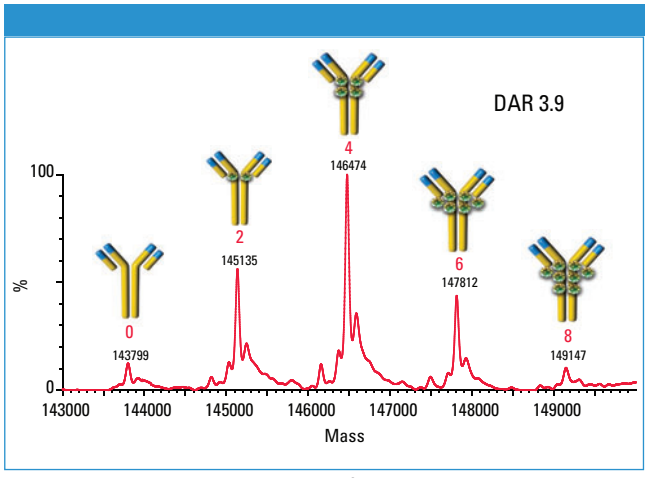

Figure 3: Native SEC/MS spectrum of the ADC mimic.

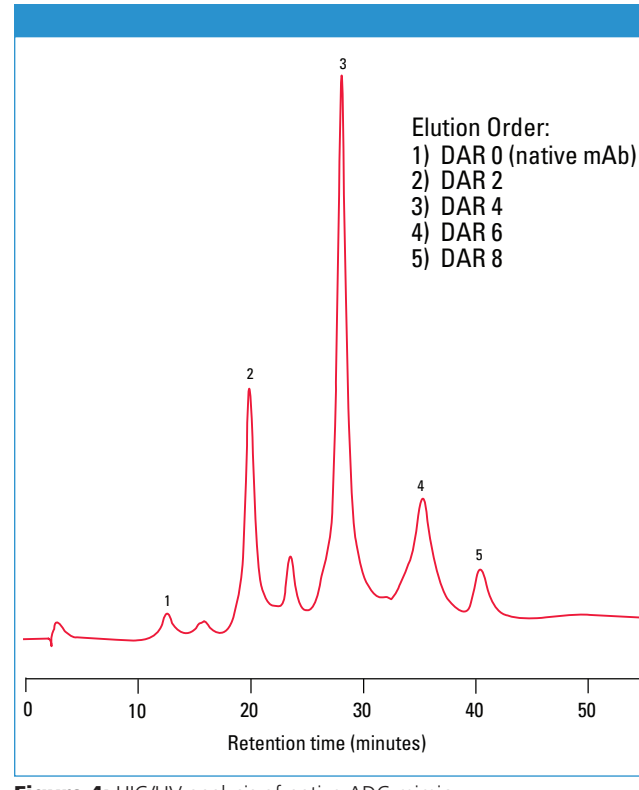

Figure 4: HIC/UV analysis of native ADC mimic.

Conclusions

SEC/MS and HIC/UV can be effectively used to characterize the DAR profile of ADCs. The mobile phase ensured a non-denaturing, MS compatible condition that was successfully used with the TSKgel SuperSW3000 SEC column to elucidate the molecular weight of the ADC species present in the drug mimic by high resolution ESI-MS detection; SEC/MS analysis indicated that the average DAR was 3.9. HIC/UV using a TSKgel Butyl-NPR column further confirmed the DAR profile by probing the hydrophobic character of the various antibody-payload combinations present in the sample. An average DAR of 3.9 was verified via HIC/UV analysis.

Data Contributed by MilliporeSigma

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Quantitative Analysis of Copolymers Using a Pyroprobe

Karen Sam, CDS Analytical

This application note demonstrates quantitative analysis of poly(styrene-isoprene) copolymers, including relative standard deviations (RSDs), and a calibration curve using a CDS Model 6150 Pyroprobe.

Analytical pyrolysis is a powerful tool for the qualitative analysis of polymers. The analysis usually starts from a simple pyrogram match to an existing pyrolysis database to identify the polymer chemical structure. If multiple polymers are identified, a quantitative method is often adopted by comparing peak areas of the pyrolysis products to determine each polymer ratio. In this application, styrene-isoprene block copolymers, which are large-volume, low-priced commercial thermoplastic elastomers, are analyzed by following this approach.

Experimental Conditions

Block copolymer standards styrene-isoprene at 14%, 17%, and 22% (styrene to copolymer weight ratio) were obtained from Sigma Aldrich. Solutions of each copolymer standard were prepared in tetrahydrofuran to 1 mg/mL. A 5µL aliquot of each weight % copolymer solution was added to a Drop-In-Sample Chamber (DISC) tube, then pyrolyzed to a setpoint of 600 °C using a CDS 6150 Pyroprobe.

Pyroprobe

Setpoint:	600 °C 30 s
DISC Interface:	300 °C
Transfer Line:	300 °C
Valve Oven:	300 °C

GC–MS

Column:	5% phenyl (30 m × 0.25 mm)
Carrier:	Helium 1.25 mL/min, 75:1 split
Oven:	40 °C for 2 min, 10 °C/min to 300 °C
Ion Source:	230 °C
Mass Range:	35–600 amu

Results

Figure 1 shows pyrograms of poly(styrene-isoprene) copolymers containing 14, 17, and 22 weight % styrene. When pyrolyzed, polystyrene is principally broken down to monomer (Peak 2 in Figure 1) and trimer (Peak 4 in Figure 1). As the styrene weight increases in the copolymer, so does the area of the peaks from polystyrene (Peaks 2 and 4) in relation to the peaks from polyisoprene (Peaks 1 and 3).

Considering the signal-to-noise ratio and the simplicity of algorithm, the highest peaks from styrene monomer (Peak 2) and isoprene dimer (Peak 3) were chosen for quantitative analysis. Area ratios of these two peaks were plotted against the weight percent of styrene in each of the standards in Figure 2, which shows a linear calibration with a R^2>0.99.

Table I: Seven runs of styrene monomer to isoprene dimer ratios of 17% styrene-isoprene copolymer

Styrene: Limonene Area Ratio	
Rep 1	0.85
Rep 2	0.86
Rep 3	0.87
Rep 4	0.87
Rep 5	0.86
Rep 6	0.84
Rep 7	0.87
RSD	**1.13%**

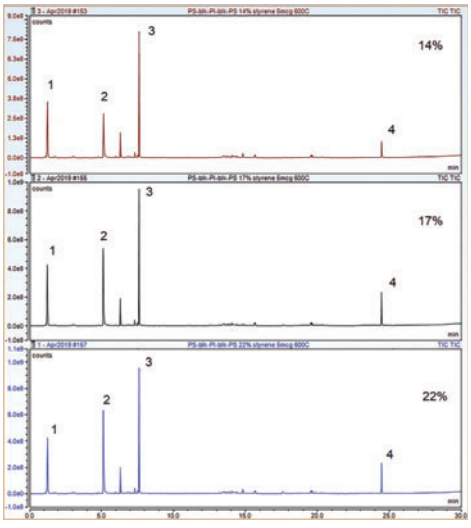

Figure 1: Poly(styrene-isoprene) copolymer pyrogram with 14% (top), 17% (middle), and 22%(bottom) styrene. Peak 1: Isoprene monomer, Peak 2: Styrene monomer, Peak 3: Isoprene dimer, Peak 4: Styrene trimer

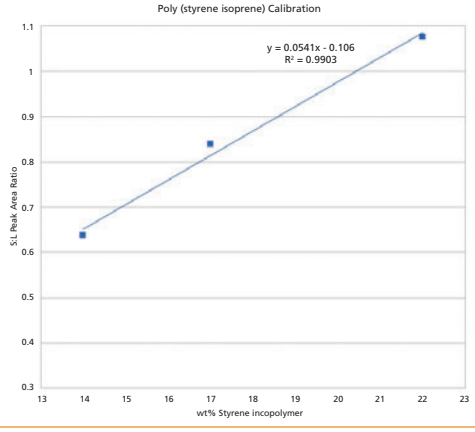

Figure 2: Styrene monomer to isoprene dimer ratio vs. styrene weight % in copolymer

The reproducibility study was also carried out from seven sample runs on the 17% styrene standard. An RSD of 1.13% is obtained in Table I.

Conclusions

The linearity and RSDs demonstrate that the latest version of the Pyroprobe from CDS is adept at the quantitative analysis of copolymers.

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