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TSKgel[®] Protein A-5PW Affinity Columns

Rugged and Robust Columns for High Throughput Analysis



20 μL of CHO cell supernatant spiked with polyclonal antibody (0.5 mg/mL)

Gradient conditions

Flow rate (mL/min)	Binding buffer (min)	Elution buffer (min)	Binding buffer (min)
4.0	0-0.25	0.25-0.55	0.55-1.00
3.0	0-0.33	0.33-0.73	0.73-1.33
2.0	0-0.50	0.50-1.10	1.10-2.00
1.0	0-1.00	1.00-2.20	2.20-4.00

• TSKgel Protein A-5PW column shows similar recovery of IgG at flow rates up to 4.0 mL/min.

• Less than 1 minute analysis was possible at 4.0 mL/min with reproducible peak profile.

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TSKgel Protein A-5PW Columns

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- With hundreds of injections of crude feedstock, the TSKgel Protein A-5PW column did not show any significant change of peak profile.
- The column maintains peak area consistency with % RSD of 1.7 after 1710 injections.

TSKgel Protein A-5PW Column Characteristics

Particle size 20 μm
Pore size100 nm
Ligand Recombinant Protein A, hexamer of C domain
pH stability 2-12
Exclusion limit 1,000 kDa

TSKgel Protein A-5PW Ordering Information

Part number	Description	ID (mm)	Length (cm)
23483	TSKgel Protein A-5PW	4.6	3.5

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SUPPLEMENT TO



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Recent Developments in LC Column Technology

A supplement to LCGC North America

June 2018

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Beyond Particle Technology
Current and Future Chromatographic Columns: Is One Column Enough to Rule Them All?. 9 K. Broeckhoven, D. Cabooter, S. Eeltink, W. De Malsche, F. Matheuse, and G. Desmet The packed particle bed format still rules LC columns, but advances continue in monoliths. Meanwhile, newer formats are on the horizon, including microfabricated columns and 3D-printed columns. This article provides a critical review of all these technologies and demonstrates how further development of chromatographic columns will be of paramount importance in the future.
Designing Vacuum-Jacketed User-Friendly Columns for Maximum Resolution Under Extreme UHPLC and SFC Conditions
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The Use of HILIC Zwitterionic Phase Superficially Porous Particles for Metabolomics Analysis 30 Jordy J. Hsiao, Andrew P. Kennedy, Genevieve C. Van de Bittner, and Ta-Chen Wei This article highlights the use of a new HILIC zwitterionic phase on superficially porous particles. A study on the use of a novel mobile-phase additive to achieve superior peak shape and isomer separation is also discussed, as well as improved LC–MS detection capabilities for metabolomics analysis.
Liquid Chromatography's Complementary Role to Gas Chromatography in Cannabis Testing. 36 Justin Steimling and Ty Kahler

Liquid chromatography (LC) is proving to be a valuable complementary technique to gas chromatography (GC) in cannabis testing for the analysis of cannabinoids, mycotoxins, and pesticides.

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FROM the GUEST EDITOR

Beyond Particle Technology



David S. Bell LCGC "Column Watch" Editor

elcome to the 2018 edition of *Recent Developments in LC Column Technology*. Several significant advances have transpired in the field during the past decade. Two of the more significant technologies, sub-2-µm particles and superficially porous particles (SPP), have taken a firm hold on modern liquid chromatography practice. Each of these developments were initially met with both excitement and their share of skepticism. Both emotions drove extensive research and ultimately adoption of the ideas. Today, both technologies are routinely used in many industries around the world, but where do we go from here? This supplement was assembled to provide examples of the ongoing research that is building upon recent particle technology developments.

Ken Broeckhoven and colleagues discuss the current status of the most recently accepted advances in liquid chromatography (LC) technology. Further, the authors interpret the present state-of-the-art developments such as three-dimensional (3D) printing and microfabricated pillar arrays, noting that further fundamental research toward improvements are expected to continue.

There are often downsides when adopting new technologies. Thermal effects in ultrahigh-pressure liquid chromatography (UHPLC), for example, exhibit a negative impact on chromatographic performance. Fabrice Gritti describes a userfriendly column hardware design for use in both UHPLC and supercritical fluid chromatography (SFC) that promises to eliminate radial temperature gradients responsible for peak distortion.

In an effort to expand the utility of modern particle designs, Jason Anspach and colleagues examine the use of biocompatible column hardware aimed at minimizing undesired adsorption of target analytes. The authors compare PEEK and titania hardware to traditional stainless steel column designs in terms of analyte adsorption, pressure tolerance, chromatographic performance, and reproducibility.

Jordy Hsaio and colleagues discuss the expansion of particle technology in terms of novel surface chemistry developments. The group reports on the use of an SPP-based zwitterionic stationary phase for hydrophilic-interaction chromatography (HILIC) applications. This article is a good example of how the base particle technologies are impacting more than just traditional reversedphase separations.

Lastly, new areas of application interest sometimes develop concurrently with new technology developments. Justin Steimling provides an excellent example using the cannabis industry. Because of limited legacy regulations and methods pertaining to various characterization needs, the cannabis industry is poised to take full advantage of modern particle technologies.

Advances in particle technologies are driving subsequent developments in hardware design and surface chemistry. Collectively, these developments are providing a positive influence on both mainstream and alternative modes of chromatography. With state-of-the-art technologies such as microfabricated pillar arrays and 3D printing on the horizon, advances that are even more significant may not be far away. Enjoy! The vast majority of separations in liquid chromatography (LC) still use the typical packed particle bed format, most commonly with fully or superficially porous particles in particle sizes as low as 1.3 μ m. As an alternative, monolithic columns have been the topic of many studies, but they are currently used only in some niche applications. Research

into perfectly ordered microfabricated columns has shown tremendous possibility for these high performance columns for use in nano-LC, but their development is still ongoing. The possibilities that emerging three-dimensional (3D) printing technology offers make it theoretically possible to develop any imaginable structure with high precision, but the technology is currently limited. This article provides a critical review of all these technologies and demonstrates how further development of chromatographic columns will be of paramount importance in the future.

Current and Future Chromatographic Columns: Is One Column Enough to Rule Them All?



mage credit: Joe Zugcic, Zugcic Photographers.

he improvements in instrument and column performance in liquid chromatography (LC) over the past 15-20 years have resulted in an almost 10-fold reduction in analysis time and threefold increase in separation efficiency. Nevertheless, the complexity of the samples emerging in life sciences (proteomics, metabolomics, lipidomics, and so forth), containing 10,000 or more analytes in a wide range of concentrations and physicochemical properties (including size, polarity, and ionization state) is so vast that it is impossible to even dream of ever achieving full resolution using one-dimensional chromatography with the present stateof-the-art instrumentation and columns. The rise of two-dimensional (2D) separation techniques, in combination with modern tandem mass spectrometry (MS/ MS) systems, vastly increases the overall resolving power that can be achieved (1-4). Nevertheless, even 2D separations are ultimately limited by the efficiency of the chromatographic column in the individual dimensions. In addition to the need for enhanced resolution, a further increase in separation speed for the second-dimension column is in high demand as well because this would allow the sampling rate in the first dimension to increase, better preserving its resolution.

The further development of faster and more efficient LC chromatographic columns thus remains of paramount importance in the years and decades to come. In addition, the chromatographic instrumentation will need to follow these improvements and changes in the performance and format of chromatographic columns (5).

Overall, the main factors that determine the separation power and speed of any system are given by the Knox and Saleem equation (6,7), determining the time, *t*, needed to achieve a given number of theoretical plates, *N*, under fully optimized kinetic conditions and for a given maximum pressure, ΔP_{max} (which can be either column or instrument limited), and mobile phase viscosity, η :

$$t = \frac{\eta}{\Delta P_{max}} \cdot E \cdot N^2$$
 [1]

with $E = h^2_{\min} \bullet \phi = H^2_{\min} / K_v$ [2]

with H_{\min} and b_{\min} the minimum absolute and reduced plate height, respectively, and K_{v} and ϕ the hydraulic permeability and

K. Broeckhoven, D. Cabooter, S. Eeltink, W. De Malsche, F. Matheuse, and G. Desmet



Figure 1: (a) Scanning electron microscope (SEM) image of monodisperse particles in a random sphere packing showing the inherent packing heterogeneities. (b) Artist's impression of a packed bed column made using additive layer manufacturing, with monodisperse particles assembled layer by layer to form a 3D printed particle column.



Figure 2: SEM images of a (a) silica monolithic column, (b) silica monolithic layer deposited on REP column (24), (c) polymer monolithic column, (d) silica monolithic layer deposited on capillary column for use in open-tubular LC (23), (e) silica monolithic column synthesized in pillar array column (25), and (f) 3D printed monolithic column. Figures adapted from references 23–25 with permission.

the flow resistance of the bed, respectively. The importance of the instrumentation (pressure limit, extracolumn dispersion) and the effect of mobile phase viscosity (LC, gas chromatography [GC], and supercritical fluid chromatography [SFC]) in this equation were discussed earlier (5,8). The focus of the present contribution is on the factors grouped in the so-called separation impedance E (6,7), determining the column quality. E is a dimensionless number and hence is independent of the size of the support. It only depends on its shape. In fact, the *E* number represents the ability of a given chromatographic support shape to transport the mobile phase through the column with a minimum of dispersion and pressure losses. The smaller the value of *E*, the smaller these losses, and hence the shorter the time needed to achieve a given

N. By far the most important geometrical factor determining the value of E is the external porosity ε , essentially because of its effect on the flow resistance. Computational fluid dynamics simulations on idealized monolithic support structures with varying ε have revealed that, while the domain-size based h does not vary much when ε increases, ϕ can easily decrease with an order of magnitude when the structure becomes more open and, for example, increases from $\varepsilon = 40\%$ (equal to the porosity of the packed bed of spheres) to say ε = 85% (9). As a consequence, also E and the associated separation time (equation 1) can be expected to drop by an order of magnitude. For an increase in $\varepsilon = 40\%$ to $\varepsilon =$ 60%, ϕ is about three times smaller, offering threefold faster separations than with a packed bed. However, to benefit from

the shorter analysis times via the reduction of E with increasing porosity, this increase should be accompanied by a significant decrease of the support size. If not, the optimal N value for which equation 1 holds increases as well, leading to longer analysis times. Finding ways to simultaneously increase ε and decrease the size of the support elements, while maintaining a good structural homogeneity, mechanical strength, and sufficient retention surface, is the key to realizing a paradigm shift in the speed and performance of LC columns.

Packed-Bed Columns

The vast majority of chromatographic columns sold nowadays are filled with fully or superficially porous particles (10). These columns show excellent reproducibility in both performance and selectivity and are available from capillary up to (semi-)preparative scale in a wide range of lengths, packed with different particle sizes and stationary phase chemistries. Whereas $h_{\min} = 2$ was long considered the practically achievable lower limit for column efficiency for analytical columns (2.1-4.6 mm i.d.), the new generation of superficially porous particles (SPPs) allows us to achieve h_{\min} values as low as 1.4. Spurred by these developments, attempts have been made to produce fully porous particle batches with a reduced particle size distribution, such that nowadays h_{\min} values as low as 1.7 can be achieved with fully porous particles (11-14). It should be noted that in capillary formats, \boldsymbol{b}_{\min} values down to $h_{\min} = 1$ have been demonstrated in research laboratories (15).

The fact that particles in a packed bed need to be in contact with each other to obtain a stable and pressure resistant bed means that the external porosity ε of a randomly packed bed is always around 36–40%. As a consequence, the flow resistance ϕ_0 of packed beds is difficult to alter or optimize and usually lies between 600 and 800 (16,17). In a first approximation, ϕ_0 can be calculated according to Kozeny-Karman's law (based on the u_0 velocity of an unretained t_0 -marker):

$$\phi_0 = 180 \cdot \frac{(1 - \varepsilon)^2 \cdot \varepsilon_T}{\varepsilon^3}$$
[3]

Since ϕ_0 not only depends on ε but also on the total porosity ε_T , a clear reduction of the flow resistance is obtained when switching from fully porous to superficially or nonporous particles (lower total porosity $\varepsilon_{\rm T}$ than fully porous particles). However, the effect is rather small and difficult to exploit because a reduction of the porous zone fraction of the particles reduces the sample loadability, causing efficiency loss when a large sample mass is injected (18).

The only way to further improve the kinetic performance of packed-bed columns would thus be a further reduction in h_{\min} . The latter can be highly effective, given the quadratic variation of E with h_{\min} . As a result of this quadratic dependency, the seemingly modest reduction of h_{\min} by some 20–30% that is typically observed when moving from fully porous to superficially porous corresponds to a very significant twofold reduction in analysis time. Using typical values for h_{\min} and ϕ_0 for superficially porous (h_{\min} = 1.5, ϕ_0 = 600) and fully porous (h_{\min} = 2, $\phi_0 = 800$) particle columns, *E* values are around 1350 and 3200, respectively. These values are in good agreement with experimental results (17,19).

Further improvements in packing heterogeneity, reducing the so-called eddy dispersion contribution (A term), are thus of high interest. As recently discussed by Gritti and colleagues, perfectly ordered packed beds (A term = 0) are expected to yield b_{\min} values equal to 0.9, 0.7, or 0.5 for fully, superficially, and nonporous particle columns, respectively, because it is impossible to eliminate longitudinal diffusion and mass transfer resistance contributions (13). Finding ways to further suppress the eddy dispersion while sticking to the traditional slurry packing methods seems to be rather difficult (Figure 1a), if not impossible, given the many efforts already devoted to the problem in the past decades (13). It seems that radically novel packing methods are needed. One approach, currently under investigation in our group, would be the use of additive layer manufacturing, where ordered layers of monodisperse silica particles are assembled layer by layer to form a threedimensionally (3D) printed particle column (Figure 1b). However, this concept is still far from reality. Besides the cost efficiency, one critical aspect is the pressure stability of these beds under the very high operating pressures (up to 1500 bar) nowadays available in commercial ultrahigh-pressure liquid chromatography (UHPLC) equipment.

An approach to lower the h_{\min} of packed bed columns that appears closer to reality (given the existence of an experimental proof delivered by Wei and colleagues [20]) is the production of core–shell particles wherein the mesopores are oriented purely radially instead of forming a randomly connected network. Although this difference seems only a small change, it has such a strong effect on the *B* term that it can be expected to lead to a further reduction of 0.5 reduced *h* units compared to the conventional core–shell particle performance (20,21).

Monolithic Columns

Instead of stacks of individual particles as in packed bed columns, monolithic columns consist of a continuous porous skeleton with large through-pores (Figure 2). In the early 1950s, the potential of this column format was discussed by Nobel Prize Laureates Martin and Synge (22). The in situ synthesis of monolithic materials has several advantages, including the absence of frits to retain particles in the column and a facilitated development of miniaturized column formats, such as capillaries (Figures 2a and 2c)





Figure 3: High-resolution LC–MS/MS analysis of a tryptic digest of *E. coli* obtained on a 1-m long monolithic column. Adapted with permission from reference 39.



Figure 4: Kinetic plots of analysis time (t_R) versus plate count (N) for benzophenone and for first-generation monoliths (\diamond and Δ) and second-generation monoliths ($\blacksquare \blacklozenge$ • \blacktriangle) evaluated at 200 bar. Open, blue symbols refer to first generation monoliths, closed, black symbols refer to second generation monoliths. The red curves (x) are obtained for a core-shell column (100 mm x 2.0 mm, $d_p = 2.7 \mu$ m) operated at a maximum pressure of 600 bar. The mobile phase was adapted on all columns to obtain k =8.7 for benzophenone. Adapted with permission from reference 55.

and microfluidic chips (Figure 2e). Also the use of thin monolithic layers to obtain a retentive porous layer for use in opentubular (Figure 2d) or pillar-array devices (Figure 2b) has been demonstrated (23– 25). In principle, monolithic stationary phases have the potential to outperform packed columns. Whereas the efficiency of packed columns is related to the particle size while the total porosity and thus flow resistance is fixed, the use of porogenic solvents in the preparation of monolithic materials facilitates the optimization of the globule size or skeleton (almost) independently of ε . The advantage that ε can be made very large (values up to ε = 86% have been reported for use in LC [26]), makes them intrinsically much better suited to obtain small *E* values and a correspondingly improved kinetic performance. Silica-based capillary monolithic columns, for example, have been shown to produce *E* values as low as 300 (27). This quantum leap in *E* is entirely because of the lower flow resistance of monolithic columns (in turn a direct consequence of their higher external porosity ε), because monolithic columns can at best (that is,

when they are produced with similar degrees of eddy dispersion) be expected to produce about the same (domain size-based) h_{\min} value as packed bed columns (see the small effect of ε on h_{\min} in Figure 10 of reference 9).

However, an advantageous shape and a concomitantly low E number is not everything. The absolute size of the support also matters. Here the rule is very simple: instead of creating a large external porosity by increasing the size of the through-pores, the latter should be kept constant (or even made smaller) to keep the same mobile to stationary zone diffusion distances. The only way to achieve the required high external porosity then consists of shrinking the size of the structural elements. However, this approach brings about a number of problems that seem so difficult to solve that they currently impede the success of monolithic columns. By far, the most tenacious problem in this respect is the so-called small domain size limit (28,29). This problem originates from the fact that each monolith synthesis process inevitably displays a local variability on the size and position of the produced solid zone elements, which are at best absolute in size. This variability implies that the general heterogeneity of the structure will increase when smaller feature sizes are being pursued, putting a fundamental limit on the possible feature size reduction of monolithic columns.

Polymer Monolithic Columns

Whereas early forms of (gel-like) polymer monolithic materials collapsed when pressure was applied, rigid polymer-based monolithic materials (Figure 2c) that are compatible with high-pressure operation have been available since the 1990s (30,31). The two most prominent classes of materials are the poly(styrene-co-divinylbenzene)based materials and monolithic entities based on methacrylate ester-based precursors. Polymerization mixtures are typically prepared from mono- and oligovinylic monomers and an initiator in the presence of an inert diluent, called porogen. The porogen, typically a binary solvent mixture, is selected based on its ability to dissolve the monomers, yielding a homogenous solution. During the course of the polymerization reaction microgel particles are formed, following interparticle reactions via pendant vinyl groups leading to

the formation of microgel clusters (32). Ultimately, a microscopic porous network is formed, and a phase separation occurs. Details of how the reaction conditions affect the size of the microglobules and resulting macropore structure can be found in the literature (33,34). To advance the kinetic performance of monoliths, Vaast and colleagues described the development of nanostructured highporosity monolithic supports allowing for sub-minute peptide separations (35). Furthermore, Vaast linked the effects of macropore and microglobule size, and structure homogeneity, to the separation performance measured in gradient elution, both in terms of peak capacity and gradient plate height (35).

Polymer-monolithic stationary phases have emerged as an attractive alternative for packed columns in the field of biomolecule separations, and their potential has been demonstrated for a wide range of biomolecules (36,37). In reversed-phase gradient mode, ultrafast separations (<1-min gradients) of intact proteins have been realized in both large internal diameter columns and using capillary column formats (35). Using a 250-mm-long capillary monolithic column and applying a 2-h gradient, intact proteins, including protein isoforms arising from various amino-acid modifications, were resolved yielding a maximum peak capacity of 650 (38). Figure 3 shows the separation of an E. coli digest using a 1-m monolithic column yielding a peak capacity in excess of 1000 (39). To further extend the kinetic performance and applicability of monolithic columns, different innovative approaches are currently being explored, such as composite cryopolymers (40) or the incorporation of nanoparticles to extend monoliths with only reversed-phase functionalities to ion exchange (41). These nanoparticles might also act as structure directing agents to improve kinetic performance.

Although excellent results can be obtained for the separation of larger biomolecules, the plate numbers achieved for small molecules on polymer-monolithic columns are typically one order of magnitude lower than those obtained on classical packed columns. Whereas the C18 layer on modified silica particles is extremely thin and hence the diffusion distance is short, it has been speculated that small molecules can penetrate into the polymer globules of monolithic materials, and excessive dispersion is a result of "surface diffusion" (42).

Silica Monolithic Columns

Silica monoliths (Figure 2a) are produced via a sol-gel process wherein alkoxysilanes are hydrolyzed and then polycondensed in the presence of a water-soluble porogen (43,44). Siloxane oligomers formed during successive condensation reactions link together to form a gel network. Spinodal decomposition occurs and phase separation takes place between the silica-rich and solvent-rich phase, forming the future silica skeletons and through-pores, respectively. Similar to polymer monoliths, the phase separation and the pore size of the gel are controlled by varying the concentration of the porogen. The stiffness and strength of the gel are increased by aging in a siloxane solution, and mesopores are formed by adding ammonium to the aging solution. Finally, the gel is dried and clad with polyether ether ketone (PEEK) to obtain a silica monolith suitable for chromatographic purposes. This column housing, however, limits the maximum operating pressure in analytical scale columns to high performance liquid chromatography (HPLC)-like operating pressures (generally below 400 bar), while

it has recently been shown that the silica monolithic skeleton itself can withstand pressures up to at least 800 bar (45).

For analytical scale monoliths (2.1-4.6 mm i.d.), through-pore sizes are typically $d_{\rm rp} = 1-2 \ \mu m$ and high external porosities (ε > 60%) are obtained. Because of their intrinsic high permeability, silica monoliths can be operated at high linear velocities, or in long (coupled) columns, resulting in extremely high efficiencies (46). The small size of the silica skeletons (typically $d_{skel} = 1-2 \ \mu m$) results in efficiencies comparable to those obtained in columns packed with 5-µm particles, especially when operated at high flow rates (47). However, because of the poor radial homogeneitywhich can be related to their fabrication process, concomitant high eddy dispersion, and their limited pressure resistance-silica monoliths (48) have not been able to compete with the particlepacked columns (sub-2 µm or sub-3 µm core-shell) that were developed around the same time (49-51). To improve their performance, efforts have been made to improve the radial homogeneity while at the same time reducing their feature sizes by adjusting the preparation process (for example, concentration and porogen type). This improvement has resulted in the introduction of the so-called second generation of silica monoliths (27,52,53). Because of their improved radial homogeneity and reduced skeleton (d_{skel}) < 1 μ m) and through-pore sizes ($d_{\rm tp}$ = 1.1–1.2 μ m), $H_{\rm min}$ values are much lower compared to the first generation, and comparable to what can be obtained in 3-3.5 µm particle packed columns (54). The downside of these reduced feature sizes is that the permeability of the monolithic column decreases accordingly, from $K_{v0} = 4.7 \text{ x } 10^{-14} \text{ m}^2$ and $K_{v0} = 4.0 \text{ x } 10^{-13} \text{ m}^2$ for the first gen-





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	35-350 µg	100 µg	100 µL	50-100 μL
	17-170 µg	50 µg	50 µL	25-50 µL
)	35-350 µg	100 µg	100 µL	50-100 µL
d	17-350 µg	50 & 100 µg	50 & 100 µL	25-100 µL
al	200-500 µg			
	Sample	Packed	Void	Min. Elution
	Capacity	Volume	Volume	Volume
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)	35-350 µg	100 µg	100 µL	50-100 µL

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Figure 5: (a) Silicon wafer featuring several pillar-array columns, with enlarged views of (b) turn structures at the end of each channel to increase total column length, (c) cylindrical pillars making up the chromatographic bed, (d) anodized pillars to increase retentive surface (67), (e) detail of porous shell (67), (f) channel inlet flow distributor, (g) alternative bed structure with radially elongated pillars (65). Figures 5d–5e were adapted from reference 67 with permission from the Royal Society of Chemistry and Figure 5g was adapted from reference 65, with permission.

eration (26,27,55) to significantly smaller values $K_{v0} = 2.0 \text{ x } 10^{-14} \text{ m}^2$ for the second generation (55). According to Deridder and colleagues, the permeability of a silica monolith is directly related to the square of its skeleton size, while a more complex relation between permeability and external porosity exists, depending on the geometry of the monolith (56). Considering that external porosity values measured for first- and second-generation monoliths are largely the same, the decreased permeability of the second-generation monoliths must therefore mainly be attributed to the reduced skeleton and through-pore sizes (55). Nevertheless, the lower permeability of the second-generation monoliths is still well above those measured for sub-3-µm particle columns.

Comparing silica monoliths with packed-bed columns, similar E values (at the lower end of the range) as for fully porous particles columns are found, with *E* = 2200–4600 and 2200–3400 for the first and second generation, respectively (55). To compare the separation power for a given separation problem, the kinetic plot method is a useful alternative to the impedance because it represents the maximum plate count obtainable in a certain analysis time. Figure 4 compares the kinetic performance for first and second generation monoliths with $\Delta P_{\text{max}} = 200$ bar. It is clear that second-generation monoliths perform better (1.5-2.5x faster for a certain N)than the first generation monoliths for N< 50.000. For more challenging separations (N > 50.000), the first-generation monoliths perform better because their large permeability allows them to be used in longer columns without compromising the separation speed (55). Comparing their performance with a 2.7-µm superficially porous particle packed column, operated at its own $\Delta P_{\text{max}} = 600$ bar, shows that the SPP-based column outperforms both generations of monoliths over the entire range of practically relevant plate counts. A similar conclusion can be drawn when comparing silica monoliths with sub-2-µm particle columns with ΔP_{max} = 1000–1500 bar (57). Further improvements in the structural size and homogeneity of the silica monoliths by improving their production process, together with the development of higher pressure-resistant material to clad the monolithic columns, are required before the monolithic columns can become competitive with the current state of the art in particle-packed column technology.

In capillary formats, the radial variation in external porosity (and hence in flow resistance) caused by the inevitable post-synthesis shrinking process is much smaller than in analytical bore columns. Producing silica monoliths with a domain size of about 2 μ m in 100-µm columns, H_{\min} values as low as 4.1-4.4 µm have been demonstrated (58). However, these values are still around two times larger than the lowest H_{\min} ever reported for packed bed columns (H_{\min} = 2 µm when using 1.3-µm core-shell particles) (59). This discrepancy shows that a further significant decrease of the domain size of silica monolithic columns is still needed.

Microfabricated Columns

Pillar-array columns were introduced in 1998 by Fred Regnier and coworkers as an ordered alternative to disordered chromatographic packings (60,61). Because the packing was originally intended for capillary electrochromatography separations requiring nonconducting substrates, the first experiments were carried out using columns produced in fused silica and polydimethylsiloxane (PDMS). Because these substrates do not easily allow fabrication of pillars with sidewall slopes close to 90°, the substrates had to be replaced with silicon before the predicted absence of eddy dispersion in the perfectly ordered structures were indeed reflected in the measured van Deemter curves. The first reversedphase separations on silicon micropillar arrays were reported in 2007 (62), showing plate heights as low as 4 µm for retained components in a nonporous pillar bed. These initial results were obtained by measuring the band broadening in the center of the beds-that is, by excluding the sidewall region where the flow resistance of the bed was different from that in the rest of the bed. Using computational fluid dynamics simulations this problem could be solved and appropriate designs for the sidewall region were proposed (63). One particularly useful solution were radially elongated pillars (REP, Figure 5g) having a lateral-toaxial aspect ratio larger than 10 (64-66). The use of (at least a number of rows of) such REP structures in the flow distributors (Figure 5f) at the inlet and outlet sections of the bed also proved to be essential to interface the columns with the outer world (67). These distributors were also key



Figure 6: Example of a 3D-printed tetrahadron skeleton model type bed (zoom: see Figure 2f).

to producing sufficiently long columns on the (relatively limited) surface of a silicon wafer, connecting different channel tracks using low dispersion turns (Figure 5b). An interesting alternative approach was implemented by Isokawa and colleagues (68), who designed a dedicated curve and pillar bed with varying density to minimize dispersion, while at the same time increasing permeability in the turn zone.

In 2017, the first generation of pillararray columns was introduced, consisting of a 2-m-long pillar bed with 5-µm-diameter pillars, spaced 2.5 µm from each other (width 315 µm, depth 18 µm, porous layer of 200 nm) (Figures 5a-5c). This column has the permeability of a 10- μ m packed bed (that is, 2.7 x 10⁻¹³ m²), while it produces plate heights comparable with 3-4 µm porous spherical beads (that is, $5-7 \mu m$) (67). The corresponding E number is on the order of 50-100-that is, more than an order of magnitude less than in packed bed columns. The pressure tolerance of more than 400 bar would even allow one to construct columns with lengths of more than 10 m, producing more than 1.5 million plates under retained conditions (small molecules like phenones) in about 12 h. A lot of effort was put in the conformal integration of porous layers into the chips to increase the specific surface of the nonporous pillars. Two methods that additionally allow tuning of the pore geometry

have been developed to this end and have been applied for 2.5-µm pillar spacings (24,69). With electrochemical anodization (silicon) pores are grown inside the silicon pillar (Figures 5d and 5e), leaving the contour of the pillar unaltered (69), whereas with the sol-gel deposition technique a porous glass layer is grown on the pillar (Figure 2b), thereby increasing the size of the pillar (24). For some applications, such as ion-pair reversed-phase chromatography of nucleotides (70) or hydrodynamic chromatography (71), it is actually preferred that the pillars are nonporous (Figure 5b) (72). In this case, the plate count roughly doubles (73). These columns have an equivalent cylindrical diameter of around 80 µm, which is a typical targeted diameter to achieve optimal flow rates for electrospray ionization (ESI)-MS detection. These column features make the first generation of commercial pillar-array columns extremely suited to achieve peak capacities of >1000 in the nano-LC flow rate range.

Since this first generation still uses relatively large pillars (5 μ m), great improvements in both speed and efficiency can be expected when new generations will be produced with pillar diameter and spacing of the same order as the current sub-2- μ m particles used in packed bed columns. Technologically this approach is feasible, given that the Bosch etching technology has the potential to achieve even submicrometer resolution (63). Sidewall effects might appear again, but they could be countered by using REP structures that are insensitive to this effect (65).

3D-Printed Columns

In the last decade, almost every field in scientific research has started to use 3D printing or additive manufacturing. Compared to traditional manufacturing technologies, 3D printing offers the possibility to use the full three-dimensional fabrication potential to freely tune and fabricate any favorable geometry. For applications in chromatography, 3D printing especially holds the promise of providing a way to produce perfectly ordered structures, thus allowing one to eliminate any eddy-dispersion contributions. Similar to microfabricated columns, the external porosity and thus the flow resistance can freely be tuned within the range where mechanically stable structures can be printed. Combining 3D printing with computational fluid dynamics simulations can lead to the design of "fully optimized" stationary phases having limited diffusion and a low resistance to mass transfer. Another asset of 3D printing is the fast manufacturing of prototypes, making rapid iterative device development possible. The use of additive manufacturing should not be limited to stationary phases, because complete chromatographic columns (containing stationary phase, column wall, flow inlet, and frits) can be fabricated simultaneously. Combining 3D-printed columns with printed valves, micropumps, connectors, and other microfluidic parts (74), one can even dream of producing a complete 3D-printed chromatographic system on a chip.

The emerging possibilities of 3D printing have already reached the field of HPLC as could be seen at the latest HPLC conferences. In 2016, Brett Paul and coworkers showed the possibilities to fabricate 3D metal printed chromatographic columns, which were functionalized in situ with a thermally polymerized monolith (75). Instead of using 3D printing to obtain a scaffold on which a stationary phase is deposited, the whole column, including walls, can be printed in one step, as shown by Fee and colleagues (76). They exploited the full 3D potential of additive manufacturing, printing both octahedral beads in a simple cubic configuration (apothems of

113.6 \pm 1.9 μ m), and monolith hexagonal channels, both in parallel and herringbone arrangements (apothems of 148.2 ± 2.0 µm). In 2017, Fee's group printed a broad range of particle shapes including tetrahedral, octahedral, stellar octangular, triangular bipyramid, and truncated icosahedra particles, in different geometric arrangements as simple cubic, body-centered cubic, and face-centered cubic (77). However, for 3D printing to become the new standard of column manufacturing, some significant disadvantages need to be overcome. The most important hurdle is the resolution or minimal printed feature size, which is about 25-100 µm for the most widespread technologies, such as extrusion-based printing (fused deposition modeling), stereolithography (stereolithographic apparatus, digital light processing), and powder-based printing (selective laser melting, selective laser sintering, inkjet-based printers) (74). State-of-the art packed bed column technologies with, for example, sub-2-µm particles, have feature sizes (flow-through pores) on the order of 500 nm and below. Currently, only one additive manufacturing technology exists that offers competitive resolutions, namely two-photon polymerization printing (2PP). Nevertheless, other 3D-print technologies with lower resolution can be applied for the manufacturing of preparative columns, not being so demanding toward micrometerscale feature sizes. A 2PP printer operates by emitting near-infrared (NIR) femtosecond pulses of photons into a photopolymerizable resin. Two photons need to be simultaneously absorbed to initiate radical polymerization, a process so rare that it only takes place in the focal spot size of the laser, leading to extremely small voxel (volume-pixel) sizes. With minimal feature sizes smaller than 50 nm (78), stationary phases with characteristic distances of 1 µm can thus be manufactured with high accuracy (see Figures 2f and 6). A drawback of 2PP, however, is the trade-off between high resolution and printing time or volumes. Whereas new structures can be quickly prototyped in manifold with all other 3D-print technologies, the speed of production with 2PP still has a long way to go. The manufacturing of a structure as shown in Figure 2f ($\varepsilon = 80\%$, edges of 1 µm, through-pores of 1.5 µm) for a 1-cmlong column with a width of 100 µm and

depth of 10 μ m (equivalent to a 40- μ m i.d. capillary) takes almost 1 day to print. It needs to be mentioned that printing time is highly influenced by the spatial dimensions, structure, material choice, laser objective, writing direction, and many other parameters. In addition, 2PP is much more expensive than the more widespread technologies.

Another important hurdle is the material choice, as the final obtained structure needs to be temperature and solvent resistant (such as no swelling). In addition, these materials will have to be functionalized to obtain an appropriate retentive surface chemistry (for example, C8, C18, phenyl, amide), to such an extent that no undesired interactions are possible with the starting material. Because typical 2PP printed materials have no inherent porosity, either the printed structure will need to be modified to create mesopores or a porous layer will need to be deposited on the surface of the printed structure to obtain a sufficiently high retention surface.

Conclusions

Despite the many research efforts on novel column technologies, packed columns are still the first choice in liquid chromatography. Monolithic columns are used in rather niche applications or for their flexible applicability and possible in situ generation in complex geometries. Further improvements in the production process of the silica-gel monoliths should aim at reducing the domain size further while maintaining or further improving the homogeneity before they can ever become competitive with the current state-of-the-art particlepacked columns. The development of a suitable ultrahigh-pressure column housing is another issue. Polymeric monolithic columns show great potential for the separation of large molecules, including biomolecules, but have inherent disadvantages for small-molecule separations. The first generation of microfabricated pillar arrays shows promising results for separations that require high resolving power, but reduced feature sizes are required to further enhance separation speed and efficiency. Three-dimensional printing shows a tremendous intrinsic potential for the fabrication of chromatographic columns, but the current printing hardware either

does not allow one to obtain the desired submicrometer resolution or the printing time is too long, limiting the technology at this moment to either preparative scale separations or the development of chipscale devices. These novel developments clearly show that we have not yet reached the end of column development and that further improvements can be expected in upcoming decades.

References

- D.R. Stoll and T.D. Maloney, *LCGC North* Am. 35, 680–687 (2017).
- (2) D.R. Stoll, K. Shoykhet, P. Petersson, and S. Buckenmaier, *Anal. Chem.* **89**, 9260–9267 (2017).
- (3) M. Pursch and S. Buckenmaier, *Anal. Chem.* 87, 5310–5317 (2015).
- (4) K. Sandra, M. Steenbeke, I. Vandenheede, G. Vanhoenacker, and P. Sandra, *J. Chromatogr.* A 1523, 283–292 (2017).
- (5) K. Broeckhoven, J. De Vos, and G. Desmet, *LCGC Eur.* **30**, 618–625 (2017).
- (6) M. Golay, in *Gas Chromatography* D.H. Desty, Ed. (Butterworths, London, 1959), p. 36.
- (7) J.H. Knox and M. Saleem, *J. Chromatogr. Sci.* 7, 614 (1969).
- (8) S. Jespers, K. Broeckhoven, and G. Desmet, *LCGC Eur.* **30**, 284–291 (2017).
- (9) J. Billen and G. Desmet, J. Chromatogr. A 1168, 73–99 (2007).
- (10) R.E. Majors, LCGC North Am. 30, 20–34 (2012).
- (11) O.H. Ismail, M. Catani, L. Pasti, A. Cavazzini, A. Ciogli, C. Villani, D. Kotoni, F. Gasparrini, and D.S. Bell, *J. Chromatogr. A* 1454, 86–92 (2016).
- (12) M. Catani, O.H. Ismail, A. Cavazzini, A. Ciogli, C. Villani, L. Pasti, C. Bergantin, D. Cabooter, G. Desmet, F. Gasparrini, and D.S. Bell, *J. Chromatogr. A* **1454**, 78–85 (2016).
- (13) F. Gritti and M.F. Wahab, *LCGC North Am.* 36, 82–98 (2018).
- (14) R. Henry, LCGC North Am. 32, 12–19 (2014).
- (15) J.M. Godinho, A.E. Reising, U. Tallarek, and J.W. Jorgenson, *J. Chromatogr. A* 1462, 165–169 (2016).
- (16) D. Cabooter, J. Billen, H. Terryn, F. Lynen, P. Sandra, and G. Desmet, *J. Chromatogr. A* **1178**, 108–117 (2008).
- (17) D. Cabooter, A. Fanigliulo, G. Bellazzi, B. Allieri, A. Rottigni, and G. Desmet, *J. Chromatogr. A* **1217**, 7074–7081 (2010).
- (18) D.V. McCalley, J. Chromatogr. A 1218, 2887– 2897 (2011).

- (19) Y. Vanderheyden, D. Cabooter, G. Desmet, and K. Broeckhoven, *J. Chromatogr. A* **1312**, 80–86 (2013).
- (20) T.-C. Wei, A. Mack, W. Chen, J. Liu, M. Dittmann, X. Wang, and W.E. Barber, *J. Chromatogr. A* 1440, 55–65 (2016).
- (21) S. Deridder, M. Catani, A. Cavazzini, and G. Desmet, *J. Chromatogr. A* 1456, 137–144 (2016).
- (22) A. Tiselius, Disc. Faraday Soc. 7, 7-11 (1949).
- (23) T. Hara, S. Futagami, S. Eeltink, W. De Malsche, G. V. Baron, and G. Desmet, *Anal. Chem.* 88, 10158–10166 (2016).
- (24) S. Futagami, T. Hara., H. Ottevaere, G.V. Baron, and W. De Malsche, *J. Chromatogr. A* 1523, 234–241 (2017).
- (25) F. Detobel, H. Eghbali, S. De Bruyne, H. Terryn, H. Gardeniers, and G. Desmet, *J. Chromatogr. A* **1216**, 7360–7367 (2009).
- (26) N. Tanaka, H. Kobayashi, K. Nakanishi, H. Minakuchi, and N. Ishizuka, *Anal. Chem.* 73, 420A–429A (2001).
- (27) H. Minakuchi, K. Nkanishi, N. Soga, N. Ishizuka, and N. Tanaka, *J. Chromatogr. A* 797, 121–131 (1998).
- (28) J. Billen, P. Gzil, G.V. Baron, and G. Desmet, J. Chromatogr. A 1077, 28–36 (2005).
- (29) J. Billen, P. Gzil, and G. Desmet, *Anal. Chem.* 78, 6191–6201 (2006).
- (30) F. Svec and J.M.J. Fréchet, Anal. Chem. 64, 820–822 (1992).
- (31) Q.C. Wang, F. Svec, and J.M.J Fréchet, *Anal. Chem.* 65, 2243–2248 (1993).
- (32) S. Wouters, T. Hauffman, M.C. Mittelmeijer-Hazeleger, G. Rothenberg, G. Desmet, G.V. Baron, and S. Eeltink, *J. Sep. Sci.* **39**, 4492– 4501 (2016).
- (33) C. Viklund, F. Svec, J.M.J. Fréchet, and K. Irgum, *Chem. Mater.* 8, 744–750 (1996).
- (34) E.C. Peters, F. Svec, J.M.J. Fréchet, C. Viklund, and K. Irgum, *Macromolecules* 32, 6377–6379 (1999).
- (35) A. Vaast, H. Terryn, F. Svec, and S. Eeltink, J. Chromatogr. A 1374, 171–179 (2014).
- (36) S. Eeltink, S. Wouters, J.L. Dores-Sousa, and F. Svec, *J. Chromatogr. A* 1498, 8–21 (2017).
- (37) A. Premstaller, H. Oberacher, and C.G. Huber, *Anal. Chem.* **72**, 4386–4393 (2000).
- (38) S. Eeltink, B. Wouters, G. Desmet, M. Ursem, D. Blinco, G.D. Kemp, and A. Treumann, *J. Chromatogr. A* **1218**, 5504–5511 (2011).
- (39) S. Eeltink, S. Dolman, F. Detobel, R. Swart, M. Ursem, and P.J. Schoenmakers, *J. Chromatogr. A* **1217**, 6610–6615 (2010).
- (40) R.D. Arrua and E.F. Hilder, *RSC Adv.* 5, 71131–71138 (2015).
- (41) R.D. Arrua, A. Nordborg, P.R. Haddad, andE.F. Hilder, J. Chromatogr. A 1273, 26–33 (2)

013).

- (42) I. Nischang, I. Teasdale, and O. Bruggeman, J. Chromatogr. A 1217, 7514–7522 (2010).
- (43) K. Nakanishi and N. Soga, J. Non-Cryst. Sol. 139, 1–13 (1992).
- (44) K. Nakanishi and N. Soga, J. Non-Cryst. Sol. 139, 14–24 (1992).
- (45) T. Hara, S. Eeltink, and G. Desmet, J. Chromatogr. A 1446, 164–169 (2016).
- (46) K. Miyamoto, T. Hara, H. Kobayashi, H. Morisaka, D. Tokuda, K. Horie, K. Koduki, S. Makino, O. Núñez, C. Yang, T. Kawabe, T. Ikegami, H. Takubo, Y. Ishihama, and N. Tanaka, *Anal. Chem.* **80**, 8741 (2008).
- (47) D.V. McCalley, J. Chromatogr. A 965, 51–64 (2002).
- (48) F. Gritti and G. Guiochon, J. Chromatogr. A 1218, 5216–5227 (2011).
- (49) D.T.-T. Nguyen, D. Guillarme, S. Heinisch, M.-P. Barrioulet, J.-L. Rocca, S. Rudaz, and J.-L. Veuthey, *J. Chromatogr. A* **1167**, 76 (2007).
- (50) R.E. Majors, LCGC North Am. 23, 1248 (2005).
- (51) J.E. MacNair, K.C. Lewis, and J.W. Jorgenson, *Anal. Chem.* **69**, 983 (1997).
- (52) K. Morisato, S. Miyazaki, M. Ohira, M. Furuno, M. Nyudo, H. Terashima, and K. Nakanishi, *J. Chromatogr. A* **1216**, 7384– 7387 (2009).
- (53) S. Altmaier and K. Cabrera, J. Sep. Sci. 31, 2551–2559 (2008).
- (54) F. Gritti and G. Guiochon, J. Chromatogr A 1225, 79–90 (2012).
- (55) D. Cabooter, K. Broeckhoven, R. Sterken, A. Vanmessen, I. Vandendael, K. Nakanishi, S. Deridder, and G. Desmet, *J. Chromatogr. A* 1325, 72–82 (2014).
- (56) S. Deridder, S. Eeltink, and G. Desmet, *J. Sep. Sci.* 34, 2038–2046 (2011).
- (57) R.E. Majors, LCGC North Am. 33, 886–887 (2015).
- (58) T. Hara, G. Desmet, G.V. Baron, H. Minakuchi, and S. Eeltink, *J. Chromatogr. A* 1442, 42–52 (2016).
- (59) S. Fekete and D. Guillarme, J. Chromatogr. A 1308, 104–113 (2013).
- (60) B. He, N. Tait, and F.E. Regnier, *Anal. Chem.* 70, 3790–3797 (1998).
- (61) F.E. Regnier, J. High Resolut. Chromatogr. 23, 19–26 (2000).
- (62) W. De Malsche, H. Eghbali, D. Clicq, J. Vangelooven, H. Gardeniers, and G. Desmet, *Anal. Chem.* **79**, 5915–5926 (2007).
- (63) J. Op De Beeck, W. De Malsche, S.T. Tezcan,
 P. De Moor, C. Van Hoof, and G. Desmet, *J. Chromatogr. A* 1239, 35–48 (2012).
- (64) J. Op De Beeck, M. Callewaert, H. Otte-

vaere, H. Gardeniers, G. Desmet, and W. De Malsche, *Anal. Chem.* **85**, 5207–5212 (2013).

- (65) J. Op De Beeck, M. Callewaert, H. Ottevaere, H. Gardeniers, G. Desmet, and W. De Malsche, *J. Chromatogr. A* 1367, 118–122 (2014).
- (66) G. Desmet, M. Callewaert, H. Ottevaere, and W. De Malsche, *Anal. Chem.* 87, 7382–7388 (2015).
- (67) M. Callewaert, J. Op De Beeck, K. Maeno, S. Sukas, H. Thienpont, H. Ottevaere, H. Gardeniers, G. Desmet, and W. De Malsche, *Analyst* 139, 618–625 (2014).
- (68) M. Isokawa, K. Takatsuki, Y.T. Song, K. Shih, K. Nakanishi, Z.M. Xie, D.H. Yoon, T. Sekiguchi, T. Funatsu, S. Shoji, and M. Tsunoda, *Anal. Chem.* 88, 6485–6491 (2016).
- (69) W. De Malsche, D. Clicq, V. Verdoold, P. Gzil, G. Desmet, and H. Gardeniers, *Lab Chip* 7, 1705–1711 (2007).
- (70) W. De Malsche, L. Zhang, J. Op De Beeck, J. Vangelooven, B. Majeed, and G. Desmet, *J. Sep. Sci.* 33, 3613–3618 (2010).
- (71) J. Op De Beeck, W. De Malsche, P. De Moor, and G. Desmet, *J. Sep. Sci.* **35**, 1877–1883 (2012).
- (72) W. De Malsche, S. De Bruyne, J. Op De Beeck, P. Sandra, H. Gardeniers, G. Desmet, and F. Lynen, *J. Chromatogr. A* **1230**, 41–47 (2012).
- (73) W. De Malsche, J. Op De Beeck, S. De Bruyne, H. Gardeniers, and G. Desmet, *Anal. Chem.* 84, 1214–1219 (2012).
- (74) S. Waheed, J.M. Cabot, N.P. Macdonald, T. Lewis, R.M. Guijt, B. Paull, and M.C. Breadmore, *Lab Chip* 16, 1993–2013 (2016).
- (75) S. Sandron, B. Heery, V. Gupta, D.A. Collins, E.P. Nesterenko, P.N. Nesterenko, M. Talebi, S. Beirne, F. Thompson, G.G. Wallace, D. Brabazon, F. Regan, and B. Paull, *Analyst* 139, 6343–6347 (2014).
- (76) C. Fee, S. Nawada, S.D. Dimartino, J. Chromatogr. A. 1333, 18–24 (2014).
- (77) S. Nawada, S. Dimartino, and C. Fee, *Chem. Eng. Sci.* 164, 90–98 (2017).
- (78) M. Emons, K. Obata, T. Binhammer, A. Ovsianikov, B.N. Chichkov, and U. Morgner, *Opt. Mater. Express* 2, 942 (2012).

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Designing Vacuum-Jacketed User-Friendly Columns for Maximum Resolution Under Extreme UHPLC and SFC Conditions



Thermal effects occurring in ultrahigh-pressure liquid chromatography (UHPLC) and low-density supercritical fluid chromatography (SFC) have a negative impact on chromatographic performance, because peaks broaden and get distorted. A solution to this problem is to thermally insulate the chromatographic column. A strict adiabatic environment can be achieved by embedding the whole chromatographic column in a large vacuum chamber, itself connected to a turbomolecular pump that delivers a high vacuum. From a practical viewpoint, this prototype research apparatus is costly, its assembly is time-consuming, and it is complex. Consequently, it is not adapted to routine analyses in standard analytical laboratories. Therefore, a new approach, using vacuum-jacketed column technology, has been developed to cope with these practical limitations. The applications and advantages of this new technology relative to standard columns are presented and discussed for both UHPLC and SFC separations operated under extreme conditions.

he high resolution power of chromatographic columns is lost when operating under extreme experimental conditions because of undesirable thermal effects. These situations occur when applying very high pressures and high flow rates, which occurs in ultrahigh-pressure liquid chromatography (UHPLC), with significant viscous heating (1), or when using low-density mobile phases at high temperature and low pressure, which occurs in supercritical fluid chromatography (SFC), with Joule-Thomson decompression cooling (2). In both cases, under a steady state temperature regime, heat is continuously exchanged between the column and its immediate external environment (3,4). As a result, temperature profiles are no longer uniform along and across the packed bed. Longitudinal temperature gradients affect essentially the retention of the analytes while radial temperature gradients negatively affect column performance in terms of efficiency and peak capacity (5,6). To get rid of the negative

influence of thermal effects on the resolution power of a chromatographic column, the ideal solution consists of thermally insulating the whole body of the column. In this situation, the heat flux at the column wall would then be stopped and the amplitude of the radial temperature gradients would be reduced to zero, ensuring the most uniform flow velocity profile across the bed and maximum column performance. This solution was recently achieved by using high-vacuum technology (turbomolecular pumps) at 10⁻⁵ Torr (~1 mPa or 10⁻³ bar), by placing the whole column in a large (60 mm i.d.) vacuum chamber, and by covering the entire exchange surface (external and internal stainless steel walls of the column and chamber, respectively) with a thin film of aluminum (7-10).

Such an apparatus is costly, timeconsuming, and complex, however, because of the need for a turbomolecular pump (to deliver air pressure as low as 10^{-5} Torr), an oil vacuum pump (to initiate a low vacuum), pressure gauges



Figure 1: Illustration of the impact on column efficiency *N* of air pressure (decreasing from right to left from 1 atm down to 10^{-5} Torr) applied around a 100 mm x 2.1 mm column packed with 1.8-µm HSS-C₁₈ particles using a research prototype LC system. Mobile phase: 70:30 (v/v) acetonitrile–water; flow rate: 0.7 mL/min; pressure drop: 13,000 psi; temperature: ambient (room) Note the average 40% relative increase in column efficiency for all the compounds irrespective of their retention factor (from –0.29 to 2.64), revealing a more uniform flow profile across the column diameter relative to standard columns.



Figure 2: Development of vacuum-jacketed columns after reducing the size of the stainless steel housing chamber embedding the chromatographic column. Top: large i.d. (60 mm) housing chamber. Middle: small i.d. (12 mm) vacuum tee. Bottom: extension of the small i.d. vacuum tee to the very end of the column. Note that strict adiabatic conditions (100% maximum efficiency) are not fully achieved with the small i.d. vacuum tees; only 86% and 88% of the maximum expected efficiency, is achieved, respectively.

(to accurately control air pressure and detect air or eluent leaks), an externally controlled eluent preheater (to set the inlet temperature), a large stainless steel vacuum chamber (to insulate both column and active preheater), and a long time (overnight) to establish a steady high vacuum. As a result, even though this research apparatus delivers a perfect adiabatic environment to the column (7–10), it remains somewhat impractical for routine analyses in UHPLC and SFC.

Therefore, in this article, a new approach to this problem is discussed, involving the step-by-step development of a column hardware technology (called vacuum-jacketed column technology) that provides a strict adiabatic environment to the column. In particular, it is shown that the size of the large vacuum chamber can be significantly reduced and that a highvacuum pump is not necessary to keep the column fully thermally insulated. Applications in UHPLC and lowdensity SFC are presented to illustrate the advantages of this approach under extreme operating conditions. It is also discussed how this new column technology can help users to bridge the gap between gas chromatography (GC)- and LC-like separations with a single instrument.

How to Achieve a Strict Adiabatic Environment

As mentioned above, the prototype research assembly (the solution that consists of thermally insulating the whole body of the column) consists of an oil vacuum pump (to generate a low-vacuum), a turbomolecular vacuum pump (to deliver a high-vacuum around 10⁻⁵ Torr), six pressure gauges operating in specific pressure ranges to accurately control air pressure, two pressure control readouts, two large (6 cm i.d.) stainless steel housing chambers embedding the eluent preheater and the chromatographic column, and a standard LC or SFC system. The injection valve and the inlet port of the detection cell of the chromatographic system are directly connected to the eluent preheater and to the column outlet, respectively. The strict adiabaticity of the system is controlled by accurately measuring eluent pressures and eluent temperatures at both the outlet and inlet of the column (8). The chromatographic advantage of this vacuum system is shown in Figure 1, which plots the efficiency of a 100 mm x 2.1 mm column packed with 2-µm fully porous particles as a function of the air pressure at the column surface. The pressure drop



Figure 3: Development of vacuum-jacketed columns after delivering strict adiabatic conditions (100% maximum efficiency) and discarding the turbo-molecular pump. Top: partial thermal insulation of the column in a small i.d. vacuum tee extended to the very end of the column. Middle: full thermal insulation of the column placed in the small i.d. vacuum tee after reducing the endfitting mass and replacing the metal side flanges with PEEK side flanges. Bottom: picture taken with an infrared camera of the fully thermally insulated column in the absence of a high-vacuum pump. Comparison to the same type of column without thermal insulation.



Figure 4: Effect of air pressure (750 Torr, 5 Torr, and 10^{-5} Torr or adiabatic conditions) applied around a chromatographic column on the van Deemter plot of a small analyte (*n*-hexanophenone). Column: 100 mm x 2.1 mm, 1.8-µm fully porous HSS-C₁₈ particles; mobile phase: 70:30 (v/v) acetonitrile–water; temperature: ambient (room); flow rate: 0.1, 0.2, 0.3, 0.4, 0.5, and 0.6 mL/min. Note the increasing reduction in reduced plate height, *h*, with increasing flow rate and thermal effects.

was fixed at 13,000 psi, the eluent is a mixture of acetonitrile and water (70:30, v/v) and six small molecules (uracil and 5 *n*-alkanophenones) were injected at room temperature. From atmospheric pressure

(760 Torr) down to 10^{-5} Torr, a relative gain in column efficiency of ~35% is observed, which remains the same irrespective of the retention factor of the analyte (from -0.29 for uracil to 2.64 for

n-hexanophenone). An air pressure level of 10⁻⁴ Torr is required for a 6 cm i.d. housing chamber. It is noteworthy that efficiency levels off between 10⁻¹ and 10² Torr because, for such pressures, the mean free path of air molecules is always negligible with respect to the distance separating the column and the inner wall of the housing chamber. As a result, the thermal conductivity of air remain unchanged (0.03 W/m/K) regardless of the applied air pressure between 10⁻¹ and 10² Torr. The overall efficiency gain is directly explained by the elimination of heat transport through air (heat is mostly carried by natural air convection and diffusion; a smaller portion of heat transport results from electromagnetic radiation) leading to more uniform temperature and flow velocity profiles across the packed bed than those experienced under standard conditions (7). In other words, long-range eddy dispersion in the column is minimized by keeping the temperature profile uniform across the column inner diameter. Maximum column performance is then guaranteed even under extreme operating conditions that cause undesirable thermal effects.

Designing Column Hardware to Deliver a Strict Adiabatic Environment

The advantage of the research device described above is to provide a strict adiabatic environment for the column. However, such a device is highly impractical for routine analyses because of its cost, complexity, and excessive time required (about a full day) to prepare the complete system and to establish a steady high-vacuum (10⁻⁵ Torr) in the large volume housing chambers. Instead, vacuum-jacketed columns may be an alternative.

To prepare vacuum-jacketed columns, the first development step is to reduce the size of the 60-mm i.d. housing chamber (see top picture in Figure 2, labeled with orange text) that systematically delivers 100% of the maximum column performance. The middle picture in Figure 2 (prototype device phase 1, labeled with blue text) shows the same column as in the top picture but partially embedded (the inlet and outlet metal endfittings are somewhat exposed to the still air of the laboratory) in a narrower (12 mm i.d.) stainless steel tee tube. The dis-



Figure 5: Left: Effect of air pressure (from 1 atm down to 10^{-5} Torr or adiabatic conditions) applied around a chromatographic column on the gradient peak capacity measured for three different flow rates (0.30, 0.65, and 0.95 mL/min). Sample: peptides from enolase digest. Column: 100 mm x 3.0 mm packed with 1.7 µm BEH-C₁₈ particles; gradient: 5–40% acetonitrile in water (0.1% trifluoroacetic acid). The ratio of the gradient time to the hold-up time is kept constant at 15 for all flow rates. Right: Effect of air pressure (760 Torr, 1 Torr, and 10^{-5} Torr or strict adiabatic conditions) on the peak width of a selected peptide (retention time 7.5 min) at the highest flow rate of 0.95 mL/min. Note the decrease in peak width with decreasing air pressure.



Figure 6: Isobaric separation of volatile *n*-alkanes (from C₅ to C₂₀) and 2,2,4-trimethylpentane under extreme operating conditions. Column: 150 mm x 3.0 mm packed with 1.8-µm fully porous HSS-SB-C₁₈ particles; mobile phase: pure carbon dioxide; flow rate: 3.0 mL/min; column outlet pressure: 1500 psi; inlet eluent temperature: 105 °C; detection: flame ionization; injection volume: 0.2 µL. Top: standard column. Bottom: Same column type, but with vacuum-jacketing. Note the higher resolution power for the vacuum-jacketed column.

tance between the column wall and the cylindrical chamber is then decreased from 27 mm to only 3 mm, eliminating heat transport by natural air convection. However, the column efficiency measured after decreasing the air pressure from 1 atm to 10^{-5} Torr is only 86% of the maximum column efficiency mea-

sured when the column is fully embedded in the large vacuum chamber. It is obvious that the hot outlet metal column endfitting is steadily exchanging heat with the external environment regardless of the air pressure in the narrow vacuum tee. Evidently, the phase 2 vacuum-jacketed column is not operating under strict adiabatic conditions.

Therefore, a new phase 2 prototype device was designed. It is shown in the bottom picture of Figure 2 (labeled with red text). In comparison to the phase 1 prototype device, both column endfittings are now fully embedded in the cylindrical housing chamber by placing two side flanges at the very ends of the column. Surprisingly, the column efficiency measured at an air pressure of 10⁻⁵ Torr (adiabatic conditions) does not increase much with respect to that observed for phase 1 device (88% versus 86% of the maximum column performance). Heat is still circulating from the hot, bulky, metal outlet endfitting to the metal side flanges (by metal-to-metal contact and heat diffusion) and from the side flanges to the external environment (by natural laboratory air convection). The side flanges act as an undesirable heat sink and, again, the column is not operating under strict adiabatic conditions. Therefore, a phase 3 prototype device was designed, as shown in the middle picture of Figure 3 (labeled in dark green text). Two significant modifications relative to the phase 2 prototype device have been made. First, most of the metallic mass of the column endfittings has been removed (it was grinded off by a hard rotating axis) to minimize the thermal mass of the column ends. Second, the metallic flanges were replaced with two polyether ether ketone (PEEK) side flanges, which act as thermal barriers between the metal endfittings and the external environment. Eventually, under high-vacuum conditions (10⁻⁵ Torr), the measured efficiency of the column embedded in the phase 3 prototype device exactly matches the maximum efficiency expected. The key points toward the fabrication of vacuum-jacketed columns is then to ensure that the volume of the outlet metal endfitting is reduced while maintaining mechanical



Figure 7: Isobaric separation of three volatile terpenes extracted from recreational herbs (limonene, α -humulene, and β -caryophyllene). The sample was dissolved in carbon disulfide (nearly flame ionization detection transparent). The same extreme experimental conditions as in Figure 6, except the eluent temperature is set at 90 °C. Left: Standard column. Right: Same column type, but with vacuum jacketing. Note the higher resolution power observed for the vacuum-jacketed column.



Figure 8: Simultaneous separation of the volatile (from C_5 to C_{20}) and nonvolatile (from C_{21} to C_{36}) fractions of a complex sample by applying a single SFC run using either a standard (top) or the same but vacuum-jacketed column (bottom). Column: 150 mm x 3.0 mm packed with 1.8-µm fully porous HSS-SB- C_{18} particles; detection: flam ionization; injection volume: 0.2 µL. The separation of the most volatile compounds required extreme isobaric conditions (pure carbon dioxide, low column outlet pressure of 1500 psi, high eluent temperature 90 °C), and the elution of the heavy fraction was carried out under gradient conditions by linearly increasing the column outlet pressure from 1500 psi to 3500 psi over 5 min. The pressure gradient starts after a 2-min run.

stability for the packed column, and that the residual heat transferred from the small metal endfitting to the external environment is reduced to zero (as a result of the insulating PEEK flanges). The second most relevant step toward the realization of vacuum-jacketed columns is to permanently remove the lowand high-vacuum pumps used to reduce air pressure down to 10⁻⁵ Torr. This task was eventually achieved but, for the sake of intellectual property protection, the techniques and the description of the final vacuum-jacketed column are not released in this article. However, the resulting chromatographic properties are reported in the application sections below. The bottom picture in Figure 3 simply compares the experimental temperature profiles observed with an infrared camera along the external surface area of the standard and vacuumjacketed columns. These data confirm that heat cannot be exchanged with the external environment along the entire length of the vacuum-jacketed column, thus ensuring complete thermal insulation of the packed bed.

UHPLC Applications

The advantage of vacuum-jacketed columns in UHPLC is first illustrated in Figure 4. The figure shows the impact of the applied air pressure surrounding the external surface area of the column on the experimental van Deemter curves of a small molecule (*n*-hexanophenone). When vacuum-jacketed columns are used (10⁻⁵ Torr, blue line), a reduced plate height of only 2.0 can be observed even under extreme conditions of viscous heating (13,000 psi pressure drop, 0.7 mL/min flow rate or 10.5 W/m). In contrast, a reduced plate height as large as 2.9 is actually observed when standard columns are used (750 Torr or atmospheric pressure, green line). Remarkably, the true minimum reduced plate height is expected to be smaller than 2.0 in the absence of radial temperature gradients at a reduced velocity larger than 12 (the minimum reduced plate height is just 2.6 with standard columns at a reduced velocity of 8). Thus, the apparent C term observed in separations conducted on standard columns has nothing to do with slow solid-liquid mass transfer resistance: It is fully explained by the large trans-column heterogeneity of the flow velocity (eddy dispersion, the *A* term) caused by the existence of a nonuniform temperature profile across the column when operating under extreme UHPLC conditions. Obviously, at the lowest applied flow rates or when viscous heating power is smaller than about a few watts per meter, the observed reduced plate height remains the same whether vacuum-jacketed or standard columns are used.

The left graph in Figure 5 demonstrates that vacuum-jacketed columns significantly improve gradient performance relative to that of standard columns. The observed gain in peak capacity is close to +20% at 1 mL/min and 13,000 psi pressure drop. Similarly to what occurs under isocratic conditions, the higher the applied flow rate is, the larger the relative gain in gradient peak capacity, as it increases from +10% (0.3 mL/min) to +15% (0.65 mL/ min) and to +20% (0.95 mL/min). The right graph in Figure 5 illustrates this result by looking directly at the gradient peak width: At 0.95 mL/min, the half-height peak width of a selected compound eluted at 7.5 min decreases from 1.10 s to 0.8 s when reducing the pressure from 760 to 10⁻⁵ Torr. This result confirms the gain observed for the peak capacity.

SFC Applications

Figure 6 compares the SFC chromatograms of 12 light alkanes recorded with a standard (top) and a vacuum-jacketed (bottom) chromatographic column under the same extreme operating conditions (pure carbon dioxide, 1500 psi outlet pressure, 105 °C) leading to severe eluent cooling during its decompression along the column and to a dramatic loss in chromatographic performance. It is noteworthy that the peak shapes of the most strongly retained compounds are severely distorted while the resolution of the most volatile C5-C8 compounds is incomplete for standard columns. In contrast, when the newly designed vacuumjacketed column is used, the peaks of the most strongly retained alkanes return to quasi-Gaussian shapes and baseline resolution of the most volatile compounds is achieved. Figure 7 shows another practical application, the separation of volatile terpenes (present in medicinal herbs), which requires very challenging SFC conditions (pure carbon dioxide, 1500 psi outlet pressure, 90 °C inlet temperature) to generate sufficient retention: The peak shape of the two most-retained terpenes (α-humulene and β-caryophylene) is clearly improved and the unknown impurity that is eluted between them can now be fully resolved when using the vacuum-jacketed column. Finally, Figure 8 demonstrates that vacuum-jacketed columns can be advantageous for the simultaneous separation of very volatile $(C_5-C_8$, present in gasoline) and nonvolatile (C_{21} - C_{36} , in paraffin) *n*-alkanes. It is then no longer necessary to use two distinct systems to separate both volatile and nonvolatile components (a GC system to analyze the volatile fraction, and an LC system for the analysis of the nonvolatile fraction). Extreme operating conditions (pure carbon dioxide, 90 °C, and 1500 psi outlet pressure) leading to undesirable thermal effects are first required to resolve the most volatile compounds. Then, a transition from these extreme temperature-pressure conditions to lessproblematic SFC conditions (higher pressure and higher content of organic modifier) is programmed: A smooth outlet pressure gradient is applied from 1500 psi to 3500 psi in 5 min to elute the heaviest compounds up to C₃₆. Similarly, a gradient of the organic modifier content can be programmed to elute the heaviest compounds. The resolution of both the volatile and nonvolatile fractions is improved when using vacuum-jacketed columns relative to standard columns. The use of such columns makes it possible to bridge the gap between GC and LC analyses by transitioning smoothly from low-density SFC to usual SFC conditions.

Conclusion

This work has demonstrated that the undesirable thermal effects of extreme UHPLC and SFC operating conditions can be circumvented by placing the chromatographic column in a strict adiabatic environment. A vacuum-jacketed column technology has been developed to thermally insulate the packed bed and to maintain temperature and flow velocity profiles as uniform as possible across the column diameter. This approach ensures maximum column performance regardless of the experimental conditions. The vacuum-jacketed column can be installed on any LC or SFC system without the need for a high-vacuum turbomolecular pump or large vacuum housing chambers. It is advantageously used when specifically operating under extreme experimental conditions, such as with high-speed UHPLC gradients at pressures as high as 1 kbar for faster resolution of peptide mixtures (protein digests) and low-density SFC separations of very volatile compounds traditionally analyzed by GC. This approach also opens new opportunities to bridge the gap between GC- and LC-like analyses. Lowdensity SFC conditions (safely applied for the analysis of volatile compounds) can be smoothly returned to usual SFC conditions (based on smooth gradients of either outlet pressure or organic modifier content), and this approach enables the analysis of both volatile and nonvolatile fractions present in the same sample mixture in a single run.

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References

- H.-J. Lin and Sc. Horvath, *Chem. Eng. Sci.* 36, 47–55 (1981).
- (2) D. Poe and J. Schroden, J. Chromatogr. A 1216, 7915–7926 (2009).
- (3) F. Gritti and G. Guiochon, *Anal. Chem.* 80, 5009–5020 (2008).
- (4) F. Gritti and G. Guiochon, *Anal. Chem.* 80, 5009–5020 (2008).
- (5) J. Kostka, F. Gritti, G. Guiochon, and K. Kaczmarski, J. Chromatogr. A 1217, 4704– 4712 (2010).
- (6) K. Kaczmarski, F. Gritti, J. Kostka, and G. Guiochon, *J. Chromatogr. A* 1216, 6575–6586 (2009).
- (7) F. Gritti, M. Gilar, and J. Jarrell, J. Chromatogr. A, 1444, 86–98 (2016).
- (8) F. Gritti, M. Gilar, and J. Jarrell, J. Chromatogr. A 1456, 226–234 (2016).
- (9) F. Gritti, M. Fogwill, M. Gilar, and J. Jarrell, J. Chromatogr. A 1468, 217–227 (2016).
- (10) F. Gritti, M. Fogwill, M. Gilar, and J. Jarrell, J. Chromatogr. A 1472, 107–116 (2016).

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Bioinert Versus Biocompatible: The Benefits of Different Column Materials in Liquid Chromatography Separations



For separations of biological molecules, there is concern about potentially irreversible adsorption of analyte molecules onto wetted surfaces in high performance liquid chromatography (HPLC) or ultrahigh-pressure LC (UHPLC) instruments and columns. Solutions to such concerns involve using materials referred to as being *bioinert* or *biocompatible*, which traditionally have been made from polyether ether ketone (PEEK). With the emergence of UHPLC, however, materials such as titanium and MP35N alloys are often preferred over PEEK because of their greater ability to withstand high pressures. In this study, we compare the performance of plastic and metal materials for UHPLC column construction. We evaluate the performance of these materials in terms of inertness, column chromatographic performance, and reproducibility to highlight the benefits and drawbacks for biological separations in reversed-phase, size-exclusion, and ion-exchange LC.

nalytical liquid chromatography (LC) methods for protein characterization typically are quantitative. In size-exclusion chromatography (SEC), for example, highmolecular-weight aggregate, or the amount of irreversibly agglomerated protein, is quantitated by percentage of peak area relative to monomer. Because aggregate can potentially be immunogenic, percent monomer is commonly considered a "critical quality attribute" and is monitored throughout the drug development and life-cycle process. Cation-exchange chromatography is also quantitative because it assesses the charge heterogeneity of proteins by analyzing the peak areas of both acidic and basic variants. Understanding the isoelectric point (pI) is especially important for monoclonal antibodies (mAbs) because any changes in the pI will affect clearance and pharmacokinetics of the mAb. Any post-translational modifications (PTMs) to the antibody must be characterized and accounted for, which SEC and cationexchange chromatography are also used to characterize. In both SEC and

cation-exchange chromatography, not only are resolution and selectivity critical to method success, but ensuring that peak areas are consistent is also of critical importance. As such, minimizing nonspecific adsorption of proteins is critical for ensuring quality data and method robustness.

Nonspecific adsorption of proteins is typically addressed by performing several "priming" injections before beginning an analytical LC run. A common practice upon receipt of a new column is to perform consecutive injections, typically 10–100 μ g depending on the column dimensions, to adsorb proteins to "active sites" on the chromatographic hardware or media. This involves an inert protein, such as bovine serum albumin (BSA) or other small proteins that are commonly used for blocking steps in other biochemical analytical techniques.

This approach can work reasonably well provided that the protein used for priming injections does indeed cover all the active sites. However, because the mechanism of adsorption is often uncharacterized and poorly under-

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stood, a protein such as BSA may not have the same priming effect as the mAb high-molecular-weight aggregate, which often have very unique adsorption characteristics. This possible difference especially becomes a problem with analytical techniques such as SEC because the primary purpose for running SEC is to quantitate the aggregate. If the priming was ineffective, there is a strong possibility that the percentage of monomer is misreported.

Further complicating "priming" is the inconsistency of the mass load required to prime a column. This inconsistency can be observed even when the same batch of chromatographic media is packed into two separate columns. This result strongly indicates that stainless steel surfaces are the primary culprit for nonspecific protein adsorption. Even with the emergence of so-called "bioinert" or "biocompatible" systems, there is still a chance that nonspecific adsorption can occur, thus affecting quantitation and robustness of the analytical method.

In this work, we examine the priming effects observed in reversed-phase, size exclusion, and weak cation-exchange separation modes for columns packed into stainless steel, polyether ether ketone (PEEK), and titanium (Ti) column hardware systems. We also look into other column properties brought about by these different hardware systems, such as column inner diameter reproducibility, column pressure ratings, retention times, and frit flow resistances.

Experimental

All columns were packed in house using the materials described in the discussion section. For comparisons between different hardware systems all columns were packed identically unless otherwise noted.

SEC separations were performed on an Agilent 1100 series high performance liquid chromatography (HPLC) system with a G1329A autosampler, a G1316A column oven, a G1314A ultraviolet (UV) detector, and a G1312A binary pump. The mobile phase was 100 mM sodium phosphate in water. The mobile phase was filtered with a 0.22- μm filter (Phenomenex) before use. The sample was 5-mg/mL γ -globulin and 0.25-mg/mL ovalbumin (Sigma Aldrich) in mobile phase. The samples were filtered with a 0.45- μ m syringe filter (Phenomenex) before use. All separations were performed at a flow rate of 0.3 mL/min with a 2-µL injection and detection at 280 nm.

The weak cation-exchange phase with carboxylic functionality was packed into different hardware configurations (150 mm x 4.6 mm), and the protein separations were performed on a Thermo Fisher Scientific ICS 5000 HPLC system with a dual pump, a temperature controlled AS-AP autosampler, a temperature-controlled column compartment, and a variable-wavelength detector. Chromatography and data analysis was controlled by Chromeleon software version 7.2.6.

For the weak cation-exchange separations of proteins, a protein mixture containing 0.5 mg/mL of each cytochrome c (bovine), ribonuclease A (bovine), and lysozyme (chicken egg) in water was used. The injection volume was 7 µL. Mobile-phase A was 20 mM sodium phosphate, pH 6.5,

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Figure 1: Overlays of injection 1, 5, 10, 15, and 20 are shown for size-exclusion separations of γ -globulin and ovalbumin on (a) a stainless steel column with stainless steel frits, (b) a bio titanium column with bio titanium frits, and (c) a PEEK-lined stainless steel column system with PEEK frits.

and mobile-hase B was 20 mM sodium phosphate and 1.0 M sodium chloride, pH 6.5. The separations were performed in gradient elution mode where the gradient was 0-100% B in 25 min with 12 min equilibration. The flow rate was 1 mL/min. UV detection was performed at 214 nm.

Monoclonal antibody separations were performed using a Waters H Class bio instrument with a bioquaternary pump, thermostated biosampler manager, thermostated column compartment, photodiode-array detector, and fluorescent detector. The Empower 3 chromatographic data system was used for data collection and analysis. Fluorescence was measured using an excitation wavelength of 280 nm and emission at 360 nm.

Monoclonal antibody separation reproducibility was tested using pH gradients made from a commercial pH-gradient buffer (Thermo Fisher Scientific). Also, $10 \times$ buffers (eluent C; pH 5.6) and (eluent D; pH 10.2) were diluted to $1 \times$ with purifed water (Millipore) before use. A linear gradient of 0–100% D in 20 min was used. The mAb concentration was 2.5 mg/mL and 3 μ L of sample was injected.

Frit permeability studies were performed by measuring the pressure generated when flowing isopropanol through the frit at 40 mL/min. The pressure that was generated by the system tubing and column hardware minus the frit was subtracted from each frit measurement. Column inner diameter measurements were performed using a pin gauge system (Vermont Gauge).

Reversed-phase separations were performed with columns packed with a 2.6-µm core-shell C18 material. The column internal diameter was measured for each column to ensure they were identical. The columns were each nominally 150 mm x 4.6 mm with the actual measured inner diameter of 4.58 mm. Separations were performed in gradient mode with mobile-phase A being 0.1% formic acid in water and mobile-phase B being 0.1% formic acid in acetonitrile. The gradient was 5-35% B in 10 min. The separations were performed on an Agilent 1260 system with a binary pump, wellplate autosampler, thermostated column compartment, and diode-array detector. UV detection was performed at 214 nm, and the column was thermostated at 40 °C. A 1-µL injection of a sample of 0.15-mg/mL bradykinin, dynorphin A, angiotensin II, Met enkephalin, and Leu enkephalin in 0.1% formic acid in water was used for this analysis.

Results and Discussion

One of the primary reasons to avoid using non-bioinert materials in chromatography equipment and column hardware is the need to preload, also known as *priming*, the column or system. Typically, this priming problem manifests itself as increasing peak areas as a function of injection. In some extreme cases, analytes will be totally adsorbed onto the column or system regardless of the amount of material injected. We investigated the num-



Figure 2: Plots of peak area versus injection number for a SEC separation of γ -globulin and ovalbumin on column packed in PEEK, bio titanium, and stainless steel column hardware.



Figure 3: Weak cation-exchange separation of cytochrome c (bovine), ribonuclease A (bovine), and lysozyme (chicken egg) on columns packed in (a) PEEK, (b) stainless steel, and (c) titanium column hardware. Absorbance was measured (UV). Peaks: 1 = cyto-chrome c, 2 = ribonuclease A, 3 = lysozyme.

ber of injections that were required to reach a steady-state peak area in a sizeexclusion separation of γ -globulin and ovalbumin. We investigated the same SEC material packed into a stainless steel column with stainless steel frits, a bio titanium column with bio titanium frits, and a PEEK-lined stainless steel column system with PEEK frits. Figure 1 shows overlays of injections 1, 5, 10, 15, and 20 for each of these three columns. Figure 2 shows a plot of peak area versus injection number for these three columns. The stainless steel column took 20 injections to reach a steady state in terms of peak area, while the bio titanium column took five injections to reach maximum peak area. The PEEK-lined column showed a slight decrease in peak area for the first five injections and then reached a steady state. We currently do not have a concrete explanation for this observation, but it has been observed on multiple columns. From the injection overlays shown in Figure 1 there is a definite advantage in terms of column priming for the titanium columns versus that of stainless steel (five injections versus 20 injections).

Adsorption and priming can occur in different separation modes other than just size exclusion. Another important separation mode in the characterization of mAbs is ion-exchange separation. In Figure 3, the separation of a three-protein mixture on a weak cation-exchange mode is shown for a titanium column with titanium frits (Figure 3a), a stainless steel column with stainless steel frits (Figure 3b), and PEEK-lined column with PEEK frits (Figure 3c). We can see in Figure 3 (data is shown in Table I) that the selectivity of the separation is unaffected by the column hardware material. There is, however, a large difference in the peak areas obtained from the same material packed in these different column hardware materials. Table I shows the relative peak area for the chromatograms shown in Figure 3. For the columns packed in stainless steel, the relative total areas are reduced by 54% for cytochrome c (peak 1), 22% for ribonuclease A (peak 2), and 37% for lysozyme (peak 3) when compared to PEEK-lined stainless steel columns. Weak cation-exchange material packed with titanium hardware gave between 87-94% of the peak area that was observed in the PEEK hardware column. The improvements in peak area recovery for the titanium column in comparison to stainless steel also translate to mAb separations. Figure 4 shows injection to injection reproducibility of a mAb separation done on a column packed in titanium hardware.

Because PEEK columns are made via a mold process, the manufacturing process is inherently susceptible to variability in the column dimensions. In Table II, the column internal diameter



Figure 4: 180 sequential injections of a 2.5-mg/mL monoclonal antibody onto a weak cation-exchange column packed in titanium column hardware.



Figure 5: Plot of the back pressure generated by one column frit made of different media filtration grades of stainless steel in comparison to a 0.5-µm media grade PEEK frit at 40 mL/min with 100% isopropanol mobile phase.

Table I: Peak area recoveries of three proteins separated on a weak cation exchange columns packed into stainless steel, titanium, and PEEK column hardware, respectively					
	Peak	Retention Time (min)	Area	% Area of Expected	
	Cytochrome c	6.5	32.3	90%	
Titanium	Ribonuclease	6.9	34.2	94%	
	Lysozyme	8.6	49.3	87%	
	Cytochrome c	6.6	16.7	47%	
Stainless steel	Ribonuclease	7.0	28.4	78%	
	Lysozyme	8.8	35.4	63%	
	Cytochrome c	6.6	35.8	100%	
PEEK	Ribonuclease	7.0	36.3	100%	
	Lysozyme	8.7	56.5	100%	

measurements are shown for 12 different columns made from PEEK, stainless steel, and titanium. The nominal dimension for all columns was 150 mm x 4.6 mm. The column internal diameter relative standard deviation (RSD) for the PEEK columns was 1.31% whereas it was less than 0.5% for both the stainless steel and the titanium hardware. This result will translate into a retention time RSD of 2.6% whereas it is less than 0.6% for the stainless steel and titanium columns. The PEEK manufacturing process also affects the frits. PEEK frits are less permeable at the same filtration rating than their metal counterparts. In Figure 5, the back pressure generated by different frit sizes is shown. The size of the media grade was obtained via a bubble point measurement and was provided to us by the respective frit manufacturers. To obtain the back pressure readings, we attached the frits to an empty HPLC column and subsequently ran isopropanol through them at 40 mL/ min using a preparative HPLC pump. We subtracted the back pressure generated by the pump, empty column, and pump tubing at the same flow rate from the readings with the frit, to be able to determine the back pressure just caused by the frit. The high flow rate was chosen to increase the signal and thereby reduce the noise in the measurement. We can see from Figure 5 that the flow resistance of a 0.5-µm PEEK frit was higher than even that of 0.2-µm stainless steel frit. This flow resistance not only adds to the overall back pressure of a packed column, but it leads to problems with fouling when injecting dirty samples or samples with borderline solubility issues.

Figure 6 shows reversed-phase separations of a mixture of five peptides obtained using columns packed in PEEK, stainless steel, and titanium column hardware. The retention times and peak area recoveries were the same on all three columns. The column tubes used for these columns were measured for their inner diameter and tubes with the same inner diameter were chosen. This approach allows us to directly compare the impact of frit permeability on the back pressure generated by these col-



Figure 6: Reversed-phase separation of a mixture of five peptides on columns packed in (a) PEEK, (b) stainless steel, and (c) titanium hardware.

Table II: Column inner diameter measurements of 12 different PEEK, stainless steel, and titanium columns for 12 different column tubing samples of each					
	PEEK	Stainless Steel	Titanium		
	4.56	4.58	4.58		
	4.44	4.58	4.58		
	4.62	4.58	4.58		
	4.64	4.58	4.58		
	4.56	4.58	4.58		
	4.6	4.56	4.6		
	4.64	4.58	4.62		
	4.6	4.58	4.58		
	4.68	4.58	4.58		
	4.62	4.58	4.6		
	4.58	4.58	4.6		
	4.62	4.56	4.6		
Average	4.60	4.58	4.59		
% RSD	1.31%	0.17%	0.29%		
Retention time % RSD	2.60%	0.34%	0.59%		

umns during these separations. In Figure 5, it was demonstrated that 0.5-µm PEEK frits gave significantly higher back pressures than their stainless steel counterparts of the same media grade. This high back pressure was also observed in the reversed-phase separation, where the PEEK column generated a back pressure of 227 bar and the stainless steel and titanium columns had back pressures of 209 and 207 bar, respectively.

Conclusions

Biological samples can exhibit adsorption to traditional HPLC and ultrahigh-pressure liquid chromatography (UHPLC) column hardware, especially when the column hardware is constructed out of

stainless steel. This adsorption typically manifests itself via low initial peak areas, requiring several injections to obtain a steady state in the peak area. One alternative to this priming approach is to construct the column using plastic, usually PEEK, materials. However, these materials suffer from lower pressure tolerance, higher column-to-column variation in inner diameter, and higher flow resistance in the frits. In this study, we saw that columns packed in titanium hardware greatly reduced the sample priming and adsorption affects to levels near to, or identical to, PEEK columns. The metal construction of the titanium columns allows for inner diameter reproducibilties, pressure capabilities, and frit flow resistances that are similar to those of traditional stainless steel column hardware. In general, the use of titanium hardware seems to be an excellent compromise between the inertness of PEEK and the mechanical properties of traditional stainless steel.

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The Use of HILIC Zwitterionic Phase Superficially Porous Particles for Metabolomics Analysis

Jordy J. Hsiao, Andrew P. Kennedy, Genevieve C. Van de Bittner, and Ta-Chen Wei Recently, superficially porous particles (SPPs) have generated significant interest because of the enhanced separation efficiency achieved at lower back pressure compared to that obtained using fully porous particles of the same particle size. Although many reversed-phase chemistries are available on SPPs, the chemistries for hydrophilic-interaction chromatography (HILIC) are limited. This article highlights the use of a new HILIC zwitterionic phase on superficially porous particles. A study on the use of a novel mobile-phase additive to achieve superior peak shape and isomer separation is also discussed, as well as improved liquid chromatography–mass spectrometry (LC–MS) detection capabilities for metabolomics analysis.

eversed-phase chromatography is the most popular high performance liquid chromatography (HPLC) method for the purification and analysis of a wide variety of analytes (1). However, analysis of polar metabolites such as amino acids, nucleotides, and organic acids by reversed-phase chemistries can be difficult because of low retention by the stationary phase (2). To promote the retention of polar analytes on reversed-phase columns, derivatization methods or ion-pairing reagents can be used (3,4), but there are disadvantages of these approaches that have been described previously (5,6). In contrast, hydrophilic-interaction chromatography (HILIC) serves as an alternative approach to analyze hydrophilic and polar analytes (7,8). Here, we developed a new HILIC chemistry on superficially porous particles (SPPs) that withstands high-pH mobile-phase solvents and chromatographically separates underivatized carbohydrates, amino acids, and metabolites. The wide operating pH range of the new HILIC column allows chromatographers to test a larger pH range on a single column to determine the optimal

chromatographic performance for their targeted analytes. Moreover, high-pH mobile-phase solvents can limit the interactions between stainless steel surfaces and phosphorylated analytes (9), which is thought to be a major contributor to the difficulties associated with the analysis of phosphorylated and carboxylated metabolites (10,11). Furthermore, a detailed study carried out to investigate the effect of bioinert hardware and a novel mobile-phase additive that deactivates metals in the sample flow path are presented.

Experimental Column Stability Study

For the high-pH test, solvent A was made by mixing 125 mL of 30% ammonium hydroxide in 875 mL of water. The pH was measured at around 11 in aqueous buffer. Solvent B was made by adding 4 L of acetonitrile into the mobile-phase container. HILIC was performed using a 100 mm x 2.1 mm Poroshell 120 HILIC-Z column (Agilent Technologies). The flow rate was 0.40 mL/min and column temperature was set at 30 °C. A sample volume of 1.0 μ L was injected onto the column



Figure 1: Lifetime analysis with sugars were conducted at (a) high pH and (b) high temperature. Methods parameters are (a) 60:40 acetonitrile–3.75% ammonium hydroxide at pH 11, 30 °C (b) 90:10 acetonitrile–100 mM ammonium acetate at pH 7.0, 80 °C; 0.4 mL/min; (a,b) ELSD: 60 °C, 3.5 bar.



Figure 2: LC–MS analysis of 18 underivatized amino acid mixture on a 100 mm x 2.1 mm HILIC-Z column. Peaks: 1 = Phe, 2 = Leu, 3 = Ile, 4 = Met, 5 = Tyr, 6 = Val, 7 = Pro, 8 = Ala, 9 = Thr, 10 = Cys, 11 = Gly, 12 = Ser, 13 = Glu, 14 = Asp, 15 = His, 16 = Arg, 17 = Lys, 18 = 2-Cys (dimer).

every 80 column volume push at 40:60 (v/v) A:B. The settings for evaporative light scattering detection (ELSD) were 60 °C, 3.5 bar, and 30 Hz. For the high-temperature test, the flow rate was 0.40 mL/min and the column temperature was set at 80 °C. A sample volume of 1.0 μ L was injected onto the column for every 80 column volume push at 10:90 (v/v) 100 mM ammonium acetate at pH 7.0 acetonitrile. The settings for ELSD were 60 °C, 3.5 bar, and 30 Hz.

Liquid Chromatography– Mass Spectrometry (LC–MS) Analysis of Amino Acids

Amino acid analysis was performed using a 100 mm x 2.1 mm Poroshell 120 HILIC-Z column (Agilent Technologies). First, a stock solution of 200 mM ammonium formate (adjusted to pH 3.0 with formic acid) in water was prepared. Solvent A was made by mixing 100 mL of the stock solution and 900 mL of water. Solvent B was made by mixing 100 mL of the stock solution with 900 mL of acetonitrile, resulting in mobile-phase A and B both having an ionic concentration of 20 mM. The flow rate was 0.80 mL/min and the column temperature was set at 30 °C. An 18 amino acid sample mixture in 0.1 M hydrochloric acid (2.5 mM amino acids, except cysteine at 1.25 mM) was diluted 1000-fold with starting condition mobile phase, and 0.10 µL of sample volume was injected onto the column for each experiment. The gradient elution profile was from 100% to 70% B for 12 min and the column was equilibrated with 100% B for 4 min before subsequent analysis. A multiple reaction monitoring (MRM) method was set up to acquire data on a 6470 QQQ system (Agilent Technologies).

LC-MS Analysis of Metabolites

Stock solutions of the analytes were made in Milli-Q purified water at 5 mg/mL. Sample solutions were made by diluting the stock to 1 ng/ μ L (ppm) in 80:20 acetonitrile-water. HILIC was performed using a 50 mm or 150 mm x 2.1 mm Poroshell 120 HILIC-Z column in both stainless steel and PEEK-lined stainless steel hardware (Agilent Technologies). Stock solutions of 100 mM ammonium acetate (adjusted to pH 9.0 with ammonium hydroxide) in water were first made. Solvent A was prepared by mixing 100 mL of the stock solution and 900 mL of water, which yields a final concentration of 10 mM ammonium acetate (pH 9.0) in water. Solvent B was made by mixing 100 mL of the stock solution with 900 mL of acetonitrile, which yields a final concentration of 10 mM ammonium acetate (pH 9.0) in 90% acetonitrile. A deactivator additive (Agilent Technologies) was spiked into indicated solvents at a final 5 µM concentration for analysis. The flow rate was 0.25 mL/min and the column temperature was set at 25 °C. A sample volume of 0.2-3 µL was injected onto the column for each experiment. After loading of the sample solution, the column was conditioned with 90% solvent B for 2 min before the gradient with solvent A was applied. The gradient elution profile was from 90% to 60% B for 10 min followed by washing with 60% B for 3 min. The column was equilibrated with 90% B for 8 min before subsequent analysis. Full MS (MS1) data were acquired with a mass range of 50-1000 m/z and an acquisition rate of 1 spectrum/s on a 6545 Q-TOF system (Agilent Technologies). An MRM method was also set up to acquire data on a 6490 iFunnel QQQ system (Agilent Technologies). The instruments were operated in negative mode for metabolite analysis except for polyamines, which was analyzed in positive mode.

Cell Culture Study

K562 leukemia cells were cultured in suspension in Roswell Park Memorial

Table I: Amino acid retention time (min) reproducibility study								
	Injection				Standard	0/ 000		
	1	2	3	4	5	wiean	Deviation	%KSD
Phe	1.94	1.95	1.93	1.94	1.94	1.94	0.00	0.26
Leu	2.14	2.15	2.14	2.15	2.15	2.15	0.00	0.21
lle	2.33	2.33	2.32	2.33	2.33	2.33	0.00	0.19
Met	2.55	2.56	2.55	2.55	2.55	2.55	0.00	0.16
Tyr	2.89	2.90	2.89	2.90	2.90	2.89	0.00	0.13
Val	3.01	3.02	3.01	3.01	3.02	3.01	0.00	0.16
Pro	3.13	3.14	3.13	3.13	3.14	3.13	0.01	0.16
Ala	3.70	3.70	3.69	3.70	3.70	3.70	0.00	0.11
Thr	3.82	3.82	3.81	3.82	3.82	3.82	0.00	0.10
Cys	NA	NA	NA	NA	NA	NA	NA	NA
Gly	4.06	4.07	4.06	4.06	4.06	4.06	0.00	0.07
Ser	4.26	4.26	4.25	4.26	4.26	4.26	0.00	0.05
Glu	4.54	5.54	4.53	5.54	4.54	4.54	0.00	0.05
Asp	5.11	5.11	5.10	5.10	5.11	5.11	0.00	0.05
His	5.78	5.78	5.78	5.78	5.78	5.78	0.00	0.01
Arg	6.12	6.12	6.12	6.12	6.12	6.12	0.00	0.02
Lys	6.60	6.60	6.60	6.60	6.60	6.60	0.00	0.02
Cys dimer	6.69	6.69	6.69	6.69	6.69	6.69	0.00	0.02
1.0 min equilibration at 0.80 mL/min = 1.2 mL. At 1 column volume = 0.24 mL, =~5 column volumes								

Institute (RPMI) 1640 medium supplemented with 10% fetal bovine serum. A portion of the cells and media collected immediately (day 0) or six days later (day 6) were centrifuged at 250g for 5 min to pellet the cells. The collected growth media (100 μ L) was mixed with 400 μ L of 50% acetonitrile and centrifuged at 10,000g for 5 min. A 0.5- μ L volume of the supernatant was subjected to HILIC–LC–MS analysis.

Results and Discussion Stability at High pH or High Temperature for Carbohydrates

The 2.7-µm Poroshell 120 HILIC-Z particles synthesized with a proprietary hybrid protected zwitterionic bonding are resistant to silica dissolution at high-pH conditions. The lifetime of the HILIC-Z column was tested under conditions suitable for carbohydrate analysis, wherein high-pH or hightemperature methods are commonly used to reduce double peaks from anomeric configurations. It was previously reported that several HILIC columns were highly unstable and even seriously damaged after exposure to basic condition (pH >10) (12). As shown in Figure 1a, the retention time of raffinose remained stable after passing over 6000

column volumes of high-pH mobilephase buffer (pH 11) in 60% acetonitrile through the column at 30 °C. In contrast, although the combination of neutral pH and 80 °C offers faster separations and lower back pressure, this approach resulted in noticeable retention time loss for the carbohydrates (Figure 1b). The retention time loss indicated degradation of column chemistry under high-temperature analytical conditions. Nevertheless, the retention time of most sugars dropped less than 10% over 10,000 column volumes, while narrow peak widths and peak shapes were maintained.

Separation of

Underivatized Amino Acids

Historically, amino acids are analyzed by gas chromatography (GC) or cationexchange or reversed-phase LC with ultraviolet (UV) or MS detection. However, the use of derivatization agents is not optimal for LC–MS because it often reduces MS sensitivity and adds complexity to the spectra. Although the polarity of amino acids makes analysis by reversed-phase LC difficult, it makes them a perfect candidate for analysis with the combination of HILIC coupled to MS.

The separation of underivatized amino

acids using the HILIC column with lowpH mobile-phase solvents and MS detection in positive analysis mode resulted in the best MS sensitivity and chromatographic performance. As shown in Figure 2, the HILIC column separated a mixture of 18 underivatized amino acids in 12 min while providing excellent peak shape and resolution. This analysis includes the complete separation of leucine and isoleucine isomers under LC–MS-friendly conditions.

The perceived limitations of HILIC are extremely long column reequilibration times and inconsistent retention time reproducibility, which prevent extensive use of the technique. However, it has been demonstrated that a consistent reequilibration time results in reproducible analyte retention times; furthermore, analyte retention time variability is most often observed with extended reequilibration times with basic analytes on a bare silica stationary phase (13). With the HILIC column and a reequilibration of five column volumes, we achieved consistent amino acid retention times (Table I).

Effect of Mobile-Phase pH on Chromatographic Performance

A distinct advantage of the HILIC particles is the stationary phases's abil-



Figure 3: Citrate and isocitrate isomers were resolved using neutral-pH mobile-phase buffer: (a) citrate, (b) isocitrate, (c) citrate/isocitrate. Samples were analyzed with ammonium acetate mobile phase buffers at the indicated pH values using an 80–70% B gradient in 10 min.



Figure 4: (a) Polyamines and (b) phosphorylated nucleotides were analyzed with mobile-phase solvents at the indicated pH values to determine the optimal analytical conditions.

ity to withstand a wide operating pH range (pH 3–11) (Figure 1). In general, acidic mobile phases favor positive mode ionization, whereas basic mobile phases favor negative mode ionization (14). The wide operating pH feature was useful for resolving difficult to separate structural isomers such as citrate and isocitrate. As shown in Figure 3, citrate and isocitrate are coeluted when analyzed with mobile-phase buffers at pH 9.0. However, when the pH of the mobile-phase solvent was acidified to pH 6.8, citrate and isocitrate were resolved.

The versatility of the stationary phases's operating pH also allows a wide range of metabolites to be evaluated. For example, polyamines such as putrescine, spermidine, and spermine were found to yield a better signal and peak shape at lower pH (3.5) compared to neutral pH (pH 6.8) conditions (Figure 4a). In contrast, phosphorylated nucleotides such as adenosine monophosphate (AMP), adenosine diphosphate (ADP), and adenosine triphosphate (ATP) were found to have higher signal and better peak shape under high-pH (9.0) conditions compared to lower-pH (3.5) conditions (Figure 4b). The nucleotide results are supported by a previously published report that basic solutions (pH >8.5) limit interactions between phosphorylated analytes and metal, which often coats the inner surface of LC columns (9). Choosing the best mobile-phase buffer conditions will undoubtedly be the first critical decision to yield optimal chromatography results for users.

Mobile-Phase Additives Enhance the Chromatographic Performance of Metal-Sensitive Metabolites

Severe peak tailing of phosphorylated and carboxylated metabolites is a well-documented issue in LC-MS analysis (10,11). The poor peak shapes are thought to be influenced by trace metal leaching from the chromatographic hardware or direct interaction of the analytes with metal oxides along the sample flow path (10). Metal chelators, such as ethylenediaminetetraacetic acid (EDTA), have previously been used to deactivate the LC system, or have been spiked into mobilephase solvents or samples to improve the chromatographic performance of metal-sensitive analytes (10,11). However, these metal chelators are highly ionizable and cause ion suppression of target analytes (11). Thus, we sought



Figure 5: 1-µL injections of (a) ATP or (b) malate at indicated concentrations were analyzed with HILIC-Z particles packed in either stainless steel or PEEK-lined column hardware. The efficacy of the deactivator additive was demonstrated to enhance the chromatographic performance of analytes with multiple phosphate and carboxylate groups.

to identify a mobile-phase additive that would be as effective at chelating metal ions as EDTA, but without the ion suppression effects. Here, we discovered a novel mobile-phase additive that could significantly improve the peak shape and signal strength of metal-sensitive metabolites (Figure 5). Moreover, switching column hardware from stainless steel to PEEKlined improved ATP and malate's peak shape and signal intensity (Figure 5). Because of the high-pH tolerance of the HILIC column (Figure 1), analyzing the phosphorylated compounds under high-pH conditions also further limits the metal-analyte interactions (9,15). Thus, the combined use of the

HILIC stationary phase in PEEKlined column hardware, the high-pH mobile phase, and the deactivator additive yields excellent chromatographic performance for metal-sensitive analytes.

HILIC LC-MS Analysis of Cell Culture Media

To test our optimized HILIC LC-MS method in a real-world application, an experiment was designed to monitor nutrient consumption and metabolic waste product secretion in cell culture media after six days (Figure 6). As expected, lactate accumulation was observed in the growth media as a metabolic waste product (Figure 6a, column 1). Moreover, other organic acids associated with the TCA cycle (that is, malate, α-ketoglutarate [α-KG], glutamate, and citrate) were excreted and found to accumulate in the cell culture media (Figure 6a, columns 3-6). In contrast, glucose levels decreased over time as the cells consumed the sugar to fuel their cellular metabolism until the glucose was either completely depleted or below the limit of detection (Figure 6a, column 2). Amino acid levels also decreased over time, which correlated with the consumption of the nutrients from the growth media by the cells (Figures 6b and 6c). Interestingly, the cysteine dimer, cystine, was detected in the cell culture media but not monomeric cysteine (Figure 6c, column 7). Further investigation into the formulation of the culture media revealed that cystine is supplemented in the culture media and not the cysteine monomer. These results demonstrated that in a single HILIC LC-MS run, a wide range of metabolites including organic acids and amino acids could be profiled and monitored from mammalian cell culture media.

Summary

We have developed a HILIC zwitterionic phase superficially porous particle (SPP) that is chemically stable over a pH range of 3-11. The wide operating pH range enables chromatographers to study a broad range of metabolites including underivatized carbohydrates, amino acids, organic acids, polyamines, phosphorylated nucleotides, and sugar phosphates. Importantly, the HILIC chemistry was shown to successfully resolve difficult to separate structural isomers such as leucine and isoleucine and citrate and isocitrate.

For many metabolites commonly studied in the TCA cycle and glycolysis pathway, we found that high-pH mobile-phase buffers greatly improved the signal and peak shape of these metal-sensitive analytes. The use of bioinert hardware helps decrease the amount of contact between metal and targeted metabolites, and thus results in better peak shape and sensitivity. Moreover, we discovered a mobile-



Figure 6: 100 μ L of growth media was mixed with 400 μ L of 50% acetonitrile. The sample was centrifuged at 10,000*g* for 5 min and 0.5 μ L of the sample was subjected to LC–MS analysis. The sample was analyzed on a 150 mm x 2.1 mm PEEK-lined HILIC-Z column with 10 mM ammonium acetate buffer, pH 9, and 5 μ M of the deactivator additive. The connection capillaries were PEEK-lined with a 1290 binary pump coupled to a 6545 Q-TOF system. Runs were repeated in triplicate and the experiment was conducted in negative analysis mode.

phase additive that limited the metalanalyte interaction in the sample flow path and enhanced the chromatographic performance of these metalsensitive metabolites. This method ultimately facilitated the monitoring of cell culture feedstock (that is, glucose and amino acids) and metabolic waste (such as organic acids) in a complex sample matrix within a single LC-MS analytical run.

References

- R.E. Majors, LCGC North Am. 28(4), 8-17 (2010).
- (2) V.V. Tolstikov and O. Fiehn, Anal Biochem. 301(2), 298–307 (2002).
- (3) A. Masuda and N. Dohmae, *Biosci Trends*.
 5(6), 231–238 (2011).

- (4) P. Kiefer, N. Delmotte, and J.A. Vorholt, *Anal. Chem.* 83(3), 850–855 (2011).
- (5) T.O. Metz et al., Biomark. Med. 1(1), 159– 185 (2007).
- (6) J.W. Dolan, LCGC Eur. 21, 258-263 (2008).
- (7) P. Appelblad, T. Jonsson, W. Jiang, and K. Irgum, *J. Sep. Sci.* 31(9), 1529–1536 (2008).
- (8) K. Spagou, H. Tsoukali, N. Raikos, H. Gika, I.D. Wilson, and G. Theodoridis, *J. Sep. Sci.* **33**(6–7), 716–727 (2010).
- (9) R. Tuytten et al., J. Chromatogr. A 1104(1-2), 209-221 (2006).
- (10) K.T. Myint, T. Uehara, K. Aoshima, and Y. Oda, *Anal. Chem.* 81(18), 7766–7772 (2009).
- (11) J.J. Pesek, M.T. Matyska, and S.M. Fischer, J. Sep. Sci. 34(24), 3509–3516 (2011).

- (12) D. Kotoni, I. D'Acquarica, A. Ciogli, C. Villani, D. Capitani, and F. Gasparrini, J. Chromatogr. A 1232, 196–211 (2012).
- (13) D.L. Shollenberger and D.S. Bell, *LCGC Eur.* 29(12), 687–692 (2016).
- (14) W.J Long, A.E Mack, X. Wang, and W.E. Barber, *LCGC North Am.* **33**(4), 31–39 (2015).
- (15) J.C. Heaton and D.V. McCalley, J. Chromatogr. A 1427, 37–44 (2016).

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Liquid Chromatography's Complementary Role to Gas Chromatography in Cannabis Testing



The absence of consensus methods for cannabis testing is a challenging, but refreshing opportunity for analytical chemists in the field because it enables the incorporation of the newest technologies and best practices without the restrictions imposed by legacy approaches that often impede method development in other industries. Liquid chromatography (LC) is proving to be a valuable complementary technique to gas chromatography (GC) in cannabis testing for the analysis of cannabinoids, mycotoxins, and pesticides. The industry is emerging during a time when superficially porous particles (SPPs) and ultrahigh-pressure liquid chromatography (UHPLC) have become market standards. This article discusses the adoption of LC technology and its role in cannabis testing.

ith the legalization of recreational marijuana in the state of California, cannabis and cannabis-infused products are facing a new level of scrutiny. California regulations require that cannabis and cannabisinfused products are tested for cannabinoids, pesticides, mycotoxins, residual solvents, terpenes (verification of label claims), heavy metals, microbiological contaminants, filth and foreign material, moisture content, and water activity (1). All of these requirements have created a new level of demand for analytical techniques that are capable of meeting regulatory requirements. Gas chromatography (GC) and liquid chromatography (LC) have largely filled the needs of analytical chemists in what has become almost equal utilization of both techniques. LC has taken a predominant role in the analysis of cannabinoids, mycotoxins, and pesticides, and GC has set industry standards for the analysis of residual solvents, terpenes, and the remaining pesticides that are not amenable to LC-tandem mass spectrometry (MS/MS).

Due in large part to the federal illegality of cannabis in the United States, strict method requirements imposed in other industries have not been applied to can-

nabis testing. Instead, the results provided by each laboratory rely on methods developed in-house, typically following acceptance criteria used by the food industry when state requirements are unavailable. The absence of consensus methods and strict method requirements has allowed analytical chemists to explore the more recent technological advances in liquid chromatography that improve the speed, sensitivity, and cost of existing analytical methods. Herein, the use of recent advances in LC technology for the analysis of cannabinoids, mycotoxins, and pesticides as they pertain to the cannabis industry is presented.

Cannabinoids

The term *cannabinoids* refers to a class of compounds that have the capability to act on cannabinoid receptors in the brain. The term *phytocannabinoid* is specific to those compounds found in cannabis and other plants. Within the cannabis industry, these two terms are used interchangeably. Cannabinoids are of great interest for their therapeutic value because cannabis has been indicated for the treatment of pain, glaucoma, nausea, depression, and neuralgia. Cannabinoids have been classified into the following 11 main types:



Figure 1: The separation of 16 cannabinoids by HPLC–UV. Column: 150 mm x 4.6 mm, 2.7-µm Raptor ARC-18; mobile-phase A: water, 0.1% formic acid (v/v), 5 mM ammonium formate; mobile-phase B: acetonitrile, 0.1% formic acid (v/v); elution: isocratic at 75% B over 9 min; flow rate: 1.5 mL/min; injection volume: 5 µL; oven temperature: 30 °C; detection: UV absorbance at 228 nm. Peaks: 1 = cannabidivarinic acid (CBDVA), 2 = cannabidivarin (CBDV), 3 = cannabidiolic acid (CBDA), 4 = cannabigerolic acid (CBGA), 5 = cannabigerol (CBG), 6 = cannabidiol (CBD), 7 = tetrahydrocannabivarin (THCV), 8 = tetrahydrocannabivarinic acid (THCVA), 9 = cannabinol (CBN), 10 = cannabinolic acid (CBNA), 11 = Δ^9 -tetrahydrocannabinol (Δ^9 -THC), 12 = Δ^8 -tetrahydrocannabivarin nabinol (Δ^8 -THC), 13 = cannabicyclol (CBL), 14 = cannabichromene (CBC), 15 = Δ^9 -tetrahydrocannabinolic acid (CBCA).

(–)-delta-9-*trans*-tetrahydrocannabinbol (Δ^9 -THC), (–)-delta-8-*trans*-tetrahydrocannabinbol (Δ^8 -THC), cannabigerol (CBG), cannabichromene (CBC), cannabidiol (CBD), cannabinodiol (CBND), cannabielsoin (CBE), cannabicyclol (CBL), cannabinol (CBN), cannabitriol (CBT), and miscellaneous-type cannabinoids. The cannabinoid profile of a cannabis strain is of great interest because the medicinal and psychotropic value can vary significantly between cultivars (2).

The analysis of cannabinoids was initially dominated by GC-based techniques until it was discovered that the hot injection port of a GC results in the incomplete decarboxylation of acidic cannabinoids. Although GC-based techniques are still used for the analysis of cannabinoids, derivatization before injection must be performed to protect the carboxylic acid functional groups (3,4). Because of the additional sample preparation steps required for GC analysis, LC-based techniques are preferred for the determination of cannabinoids in testing laboratories. Ultraviolet (UV) detection is most frequently paired with LC analysis based on low initial cost, ease of use, and robustness.

As states began to legalize medical and recreational marijuana, analytical laboratories turned to existing publications for guidance on method development. C18based stationary phases quickly found traction in potency testing because their associated hydrophobic interactions and shape-selective characteristics enabled the separation of cannabinoids. Coincidentally, as the demand for testing increased, column manufacturers began releasing applications on superficially porous particles (SPPs). The increased speed and improved resolution with the use of SPPs compared to traditional fully porous particles (FPPs) of the same particle size met the needs of the cannabis testing industry. By pairing C18-based stationary phases with SPPs, the speed and resolution capabilities of cannabinoid separations have been significantly improved. The baseline separation of 16 cannabinoids by LC–UV with a 9-min cycle time has been reported (Figure 1) (5).

Beyond SPPs compatible with 400-bar instrumentation, sub-2-µm SPPs have been introduced by a number of LC column manufacturers. The decreased particle size requires instrumentation to be capable of handling system pressures of 1000 bar or more. Additionally, minimal extracolumn volumes in the valves, connecting tubing, and flow cell are required for acceptable separation performance because of their contributions to dispersion. Low-volume flow cells (approximately 1 µL) are of particular importance because of the large contribution to band broadening that occurs in standard flow cells (6). Effective pairing of sub-2-µm SPPs with appropriate ultrahigh-pressure liquid chromatography (UHPLC) instrumentation enables faster separations with sharper peaks compared to the use of larger particles with traditional systems (Figure 2) (7). These characteristics decrease the amount of mobile phase solvent required per analysis, facilitate higher detection sensitivity, and improve productivity.

Although LC-UV has found its place in routine cannabis testing, the complexity of the matrix can complicate analysis. Namely, more than 100 cannabinoids have been isolated from C. sativa (2), which makes UV detection impractical because of the limited spectral deconvolution abilities and LC column resolving capabilities. For these reasons, the use of tandem mass spectrometric techniques have been proposed to overcome peak capacity limits. The use of a triple-quadrupole MS/MS system in multiple reaction monitoring (MRM) mode makes baseline resolution unnecessary, provided that the compounds or matrix interferences are not isobaric with the cannabinoids of interest. Unfortunately, the high cost of instrumentation and maintenance coupled with the need for more advanced personnel training generally prohibits the adoption of LC-MS/ MS for routine analysis. If these barriers to entry were overcome, MS techniques could be used for the analysis of new cultivars to identify potential sources of UV interference that may impact routine testing.

Beyond high performance liquid chromatography (HPLC) and UHPLC,



Figure 2: The separation of 16 cannabinoids by UHPLC–UV. Column: 100 mm x 3.0 mm, 1.8- μ m Raptor ARC-18; mobile-phase A: water, 0.1% formic acid (v/v), 5 mM ammonium formate; mobile-phase B: acetonitrile, 0.1% formic acid (v/v); elution: isocratic at 75% B over 4 min; flow rate = 1.0 mL/min; injection volume: 1 μ L; oven temperature: 30 °C; detection: UV absorbance at 228 nm. Peaks: 1 = CBDVA, 2 = CBDV, 3 = CBDA, 4 = CBGA, 5 = CBG, 6 = CBD, 7 = THCV, 8 = THCVA, 9 = CBN, 10 = CBNA, 11 = 9-THC, 12 = Δ^{8} -THC, 13 = CBL, 14 = CBC, 15 = THCA, 16 = CBCA.

ultrahigh-performance supercritical fluid chromatography (UHPSFC) has found utility in cannabinoid analysis because of advances in instrumentation. As with UHPLC, the combination of sub-2-µm particle columns and photodiode arraymass spectrometry (PDA-MS) detection enables efficient and rapid separations. UHPSFC uses compressed carbon dioxide in combination with both polar and nonpolar cosolvents to separate a wide array of compounds. Classic polar phases such as bare silica, cyano, and amino are typically used for polar solutes. For low polarity solutes, reversed-phase columns such as C18, C8, C4, and biphenyl are sometimes used. In the past few years, a number of stationary phases have been developed specifically for SFC and include ethylpyridines as well as several proprietary phases. The retention order in SFC is roughly the opposite of that in reversed-phase LC, which makes the techniques highly orthogonal. The complementary nature of SFC and LC make them powerful techniques when combined, allowing a full characterization capable of identifying coelutions. Using

UHPSFC–PDA, the baseline separation of 11 cannabinoids in less than 10 min can be achieved, making SFC another powerful analytical tool compatible with routine testing laboratories (8).

Additionally, enantioselective UHPSFC (eUHPSFC) methods have been developed using an inverted chirality columns approach to determine the enantiomeric excess of (-)- Δ^9 -THC (9). Most endogenous cannabinoids in the plant are chiral and predominantly occur as a single-enantiomer (10). The compatibility of enantiomers with cannabinoid receptors can lead to interesting effects, where one isomer is psychotropically active and the other devoid of any psychotropic effects. The characterization of the enantiomers of the cannabinoids could have profound effects in medical research where a positive patient outcome is desired without undesirable psychotropic effects. Although eUHPSFC is not applicable to routine testing laboratories, the technique would be critical to the pharmaceutical industry where the efficacy of single-molecule cannabinoid formulations could be highly dependent on stereochemistry.

To screen for the more than 500 components in cannabis (2), two-dimensional (2D)-LC has also been shown to have utility. In comparison to one-dimensional (1D)-LC or LCxLC (heart-cutting), comprehensive 2D-LC is capable of providing much more information about sample composition. Comprehensive 2D-LC most often pairs two orthogonal stationary phases to separate complex mixtures or resolve difficult coelutions observed in 1D-LC. The power of 2D-LC becomes more apparent as the complexity of the sample increases. The 1D-LC approaches cannot reach the same effective peak capacities within practical time constraints. Online 2D-LC requires fractions of the ¹D effluent to be transferred to the ²D column using transfer volumes larger than the peak volumes to avoid undersampling. This technique requires that the second dimension separation be performed in the order of seconds. The main challenge associated with comprehensive 2D-LC is the impact of solvent strength mismatch. For example, if the effluent of the first dimension is stronger than the mobile phases used in the second dimension, significant changes in retention, selectivity, and peak shape may result. Approaches to address peak focusing include such techniques as online dilution of the ¹D effluent, temperature modulation, and the use of trapping media (11).

2D-LC can also be applied in an offline configuration, where the ¹D fractions are collected and reinjected. Using offline mode, instruments designed specifically for 2D-LC are not required and the user is no longer obligated to employ rapid ²D separations. The offline mode makes the technique more accessible providing that appropriate software is available. In the cannabis industry, this technique could be applied to generate detailed chemical fingerprints for each cultivar, which could prove to be an invaluable technique for strain identification. This technique has already found utility in the screening of cannabinoids in industrial-grade hemp (12). Although online 2D-LC has various approaches to mitigate solvent mismatches, the offline technique affords the ability to perform full evaporation on the collected ¹D fractions followed by reconstitution to tailor the diluent specifically for the ²D analysis.

Currently, requirements for potency testing vary from state to state. Many



Figure 3: Chemical structures of aflatoxins and ochratoxin A: (1) aflatoxin B1, (2) aflatoxin B2, (3) aflatoxin G1, (4) aflatoxin G2, and (5) ochratoxin A.



Figure 4: Example system configuration for the trap-and-elute technique. The addition of valve L allows for the rapid transition between routine, single dimension analysis and a trap-and-elute configuration.

jurisdictions only require that products be tested for total THC and total CBD. California requires that the cannabinoid profile of samples are evaluated for THC, tetrahydrocannabinolic acid (THCA), CBD, cannabidiolic acid (CBDA), CBG, and CBN (1). As more states begin to legalize medical and recreational marijuana, additional cannabinoids may become regulated. Furthermore, regulatory requirements are not the only driving factor for improved cannabinoid profiling. Expanding medical research could place significant value on minor cannabinoids. By updating legacy methods with improved analytical instrumentation, column technology, detection, and analytical techniques, the cannabis testing industry will not only be able to exceed current requirements, but also meet the needs of future research.

Mycotoxins

Mycotoxins, secondary metabolites produced by fungi, are among the major contaminants in agricultural products that can cause disease and death in humans and other animals. Techniques for controlling mycotoxins are largely preventative. Careful control of temperature and humidity prevents mycotoxin-producing fungi from flourishing (13). Cannabis is particularly susceptible to the growth of molds and fungi during post-harvest curing, when moisture content must be well-controlled. Unlike cannabinoids that have only recently become the subject of regulations, the health concerns associated with exposure to mycotoxins through the ingestion of contaminated foods and feeds have prompted the U.S. Food and Drug Administration (FDA) to enforce strict regulatory limits since 1985. A number of official methods have been published for their analysis such as AOAC method number 2008.2 (14). This method measures aflatoxin B1, B2, G1, G2, and ochratoxin A in ginseng and ginger (14).

Much like cannabinoid testing, requirements for mycotoxin testing in cannabis vary from state to state. Not all states require testing for mycotoxins, but those that do require testing for the same five: aflatoxin B1, B2, G1, G2, and ochratoxin A (Figure 3). Acceptable levels of mycotoxins in samples are currently set at <20 µg/kg for total aflatoxins (summation of B1, B2, G1, and G2) and <20 µg/kg for ochratoxin A in the state of California (1). The detection of mycotoxins is possible by various analytical methods: GC-MS with preinjection derivatization, HPLC in combination with post-column reaction and fluorescence detection, enzyme-linked immunosorbent assay (ELISA), or LC-MS/MS. LC-MS/ MS is considered the gold standard since derivatization is not required, some mycotoxins lack fluorescence, and ELISA may be susceptible to false positives.

In the analysis of mycotoxins, using immunoaffinity columns (IACs) to reduce matrix effects and eliminate potential sources of interference for LC-MS/MS analysis is common. These columns contain monoclonal antibodies that are cross reactive towards specific mycotoxins resulting in highly selective sample cleanup. Connection of these columns in tandem has been shown to be a cost-effective solution for multimycotoxin analysis by LC-MS/MS. IACs that target the mycotoxins of interest in cannabis testing are commercially available. When not used, significant matrix interferences have been shown to be eluted near target mycotoxins, which resulted in an adverse effect on measured ion ratios (15).

Although IACs are effective for sample cleanup, online sample preparation techniques are attractive from the standpoint of increased throughput. To the best of our



Figure 5: Chemical structure of a highly polar pesticide requiring analysis in the state of California per proposed regulations: daminozide.

knowledge, no methods have been published that pair IACs to an online workflow. Beyond IAC cleanup, multiple heart-cutting 2D-LC has been shown to be a viable technique for the analysis of aflatoxin B1, B2, G1, G2, and ochratoxin A in smokeless tobacco (16). This technique could be modified to find applicability in routine cannabis testing laboratories. Trap columns, in particular, could play a vital role in bringing the workflow online (Figure 4).

Pesticides

Akin to mycotoxins, multiresidue pesticide methods have been established in food safety testing for many years. MS/MS is the gold standard for detection, and both LC and GC have their places because of sample complexity, compound amenability, and technique orthogonality. LC-MS/ MS is a much more versatile and universal technique compared to GC-MS/MS, but organochlorine pesticide residues, for example, are notoriously difficult to ionize by electrospray LC-MS/MS (Table I). Exploiting the overlap in pesticide residues that are amenable to both techniques is a powerful strategy for confirmation analysis. Regulatory requirements for pesticide testing in cannabis vary significantly from state to state, not only in the number of residues monitored, but also in allowable tolerances. These differences heavily influence the sample preparation, instrumentation, and techniques performed in each state. In California, 66 pesticide residues have been proposed for regulation with levels of detection as low as 10 ppb (1). This is the largest panel of pesticide residues and the lowest detection threshold requirements proposed for state regulation to date.

Not only is sensitivity a concern for multiresidue pesticide analysis, but also the retention and selectivity of pesticides that have different charge states, functional groups, and varying amounts of hydrophobicity.

Table I: California's proposed threshold values of pesticides not amenable to ESI- LC–MS/MS						
Analyte	Edible Cannabis Products (ppm)	Dried Cannabis Flowers (ppm)	All Other Processed Cannabis (ppm)			
Acequinocyl	0.27	0.10	0.02			
Captan	1.00	0.70	0.05			
Chlorfenapyr	0.01	0.01	0.01			
Cyfluthrin	0.01	0.01	0.01			
Cypermethrin	1.00	1.00	0.50			
Dichlorvos	0.02	0.02	0.02			

Highly polar residues such as daminozide (Figure 5) are difficult to retain using reversed-phase mechanisms. HPLC stationary phases with polar functionalities have been developed for the retention of polar pesticides, but peak shapes for different classes of residues could be adversely affected and result in an overall loss in sensitivity. Two interesting strategies that have been used for multiresidue pesticide analysis both employ the use of two analytical columns with orthogonal stationary phases, but are not true comprehensive 2D-LC techniques. The first technique requires two UHPLC systems to allow for the simultaneous injection of sample onto two separate, orthogonal stationary phases, after which the effluent is combined before detection by MS/MS (17). The second technique uses the combination of a hydrophilic-interaction chromatography (HILIC) column, a reversed-phase trap column, and a reversed-phase analytical column. Pesticides not retained on the HILIC column are diverted to the reversed-phase trap column at the beginning of the run. After the initial fraction is trapped, the valve switches to MS detection, which allows the completion of the HILIC analysis. The trap is then backflushed onto the reversed-phase analytical column for the analysis of morenonpolar pesticides (18).

Although the chromatographic analysis of pesticide residues in solvent can be challenging on its own because of a wide range of polarities, complex matrices add a new level of difficulty. Cannabis itself contains cannabinoids, terpenes, fatty acids, sugars, flavonoids, and pigments (2), but the matrix is further complicated when added to cannabis-infused products. This matrix complexity presents a huge challenge during method development since every new product type that is received for testing may require a unique sample preparation. There are numerous products that are commercially available for the many facets of sample cleanup, but a universal method such as homogenization followed by solvent extraction is highly desired.

Looking beyond sample preparation alone, chromatography could be the enabling technology that allows for a universal method. Simply "diluting out" matrix interferences has been proposed as a viable means of effective sample preparation (19). This approach requires both state-of-the-art mass spectrometers and careful consideration of the chromatography. One possible solution to improve the sensitivity with electrospray is to downscale the size of the liquid separation to improve the ionization efficiency associated with reduced flow rates. The application of nanoflow liquid chromatography has successfully been applied to veterinary drugs in food samples of animal origin using a 100-fold dilution factor, which resulted in the complete removal of matrix effects (20). Removing all influences from matrix allows for the use of solvent-based calibration curves, which is both convenient and cost-saving. Advances in nano LC columns (<0.1 mm i.d.) have resulted in performance, robustness, and ease of use comparable to those of standard HPLC columns (21).

In addition to modified LC–MS/MSbased techniques, SFC–MS/MS is an alternative, orthogonal approach that has also had success for the quantification of pesticides. A powerful aspect of this approach is the ability to analyze a wider range of polarities than currently available by GC– MS/MS or LC–MS/MS (22).

Summary

The need for cannabis testing has increased rapidly over the past several years, requiring testing laboratories to swiftly adapt to increased demand and develop methodologies that meet or exceed the expanding (Continued on page 42)



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requirements of state regulations. Recent LC technology has already found its utility in the field through the use of SPPs for the analysis of cannabinoids. SFC is also emerging as a technique that could address analysis needs for both cannabinoid profiling and pesticide testing. The successful application of hyphenated techniques for the analysis of pesticides and mycotoxins in the food industry could serve as a foundation for the utilization of technology advancements in cannabis testing. The use of multidimensional chromatography for cannabis has already begun to appear in literature and is an encouraging sign for the rapid adoption of new technology in this budding industry.

References

- California Code of Regulations, "Chapter 5. Testing Laboratories," *Bureau of Marijuana Control Proposed Text of Regulations* (CA Code of Regulations, Title 16, Div. 42).
- (2) M. ElSohly and W. Gul, in *Handbook of Cannabis*, 1st ed., R.J. Pertwee, Ed. (Oxford University Press, Oxford, United Kingdom, 2014), pp. 3–22.
- (3) F.E. Dussy, C. Hamberg, M. Luginbuhl, T. Scherzmann, and T.A. Briellmann, *Forensic Sci. Int.* 149, 3–10 (2005).

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- (4) T.J. Raharjo and R. Verpoorte, *Phytochem. Anal.* 15, 79–94 (2004).
- (5) "16 Cannabinoids on Raptor ARC-18 2.7 μm by LC-UV," http://www.restek.com/chromatogram/view/LC_GN0578 (accessed Mar 23, 2018).
- (6) J. De Vos, K. Broeckhoven, and S. Eeltink, *Anal. Chem.* 88, 262–278 (2016).
- (7) "16 Cannabinoids on Raptor ARC-18 1.8 μm by LC-UV," http://www.restek.com/chromatogram/view/LC_GN0579 (accessed Mar 23, 2018).
- (8) M. Wang, Y. Wang, B. Avula, M.M. Radwan, A.S. Wanas, Z. Mehmedic, J. van Antwerp, M.A. ElSohly, and I.A. Khan, *J. Forensic Sci.* 62, 602–611 (2017).
- (9) G. Mazzoccanti, O.H. Ismail, I D'Acquarica, C. Villani, C. Manzo, M. Wilcox, and A. Cavazzini, *Chem. Commun.* 53, 12262– 12265 (2017).
- (10) J.M. Finefield, D.H. Sherman, M. Kreitman, and R.M. Williams, *Angew. Chem. Int. Ed.* **51**, 4802–4836 (2012).
- (11) D.R. Stoll and P.W. Carr, Anal. Chem. 89, 519–531 (2017).
- (12) J. Pandohee, B.J. Holland, B. Li, T. Tsuzuki, P.G. Stevenson, N.W. Barnett, J.R. Pearson, O.A.H. Jones, and X.A. Conlan, *J. Sep. Sci.* 38, 2024–2032 (2015).
- (13) J.W. Bennett and M. Klich, *Clin. Microbiol. Rev.* 16, 497–516 (2003).
- (14) AOAC Official Method 2008.02, Aflatoxins

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> B1, B2, G1, and G2 and Ochratoxin A in Ginseng and Ginger. Multitoxin Immunoaffinity Column Cleanup and Liquid Chromatographic Quantitation (First Action 2008, Final Action 2013).

- (15) J. Wilcox, C. Donnelly, D. Leeman, and E. Marley, J. Chromatogr. A 1400, 91–97 (2015).
- (16) D. Qi, T. Fei, H. Liu, H. Yao, D. Wu, and B. Liu, *J. Agric. Food Chem.* **65**, 9923–9929 (2017).
- (17) J. Robles-Molina, B. Gilbert-López, J.F. García-Reyes, and A. Molina-Díaz, J. Chromatogr. A 1517, 108–116 (2017).
- (18) S. Kittlaus, J. Schimanke, G. Kempe, and K. Speer, J. Chromatogr. A 1283, 98–109 (2013).
- (19) H. Stahnke, S. Kittlaus, G. Kempe, and L. Alder, J. Anal. Chem. 84, 1474–1482 (2012).
- (20) J. Alcántara-Durán, D. Moreno-González, B. Gilbert-López, A. Molina-Díaz, and J.F. García-Reyes, *Food Chem.* 245, 29–38 (2018).
- (21) L. Rieux, E.J. Sneekes, and R. Swart, *LCGC North Am.* 29, 926–934 (2011).
- (22) D. Baker, A. Barnes, C. Titman, J. Horner, and N. Loftus, "Application of SFC-MS/MS for the Quantification of Highly Polar Pesticides in a Range of Food Samples," https:// www.shimadzu.com/an/literature/lcms/ ego117080.html (accessed Mar 27, 2018).

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