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State-of-the-Art Separations With Convergence Chromatography

Supercritical fluid chromatography (SFC) has many potential advantages, including rapid separation of chiral analytes and separation by hydrocarbon class (i.e., saturates, olefins, and aromatics). Yet, historically, SFC earned the reputation of being experimentally difficult. This has been addressed with a new instrument that seeks to blend the distinctions in the physical state of the mobile phase (gas, supercritical fluid, or liquid) to offer convergence chromatography, which unifies them all in practice as well as concept.

The ACQUITY UPC²™ (UltraPerformance Convergence Chromatography™ System) from **Waters** (Milford, MA) was introduced at Pittcon® 2012, where it received the Pittcon Editors' Gold Award. Many greeted the introduction with skepticism since, for decades, SFC had failed to live up to expectations, but **Waters** was adamant that, at last, they had gotten it right.

Now, one year later, Dr. Davy Guillarme of the School of Pharmaceutical Sciences at the University of Geneva, University of Lausanne in Switzerland, shares some of his experiences using UPCC.

RLS: Please tell us about your experience with SFC prior to UPCC.

DG: I had years of experience with HPLC and UHPLC (ultra-high-performance liquid chromatography). UHPLC was a disruptive new technology that greatly improved the productivity of LC, especially in the pharmaceutical industry. About two years ago, I decided to investigate SFC in more detail.

At the beginning, I found SFC was experimentally difficult and frustrating. Experience with HPLC and UPLC was not helpful with SFC. Some people from **Waters** told me the new UPCC technology could address and solve the problems. I was skeptical, but since SFC was not working well, I decided to try it: I was indeed positively surprised by the levels of performance that could be achieved with UPCC, compared to SFC.

RLS: What did you find?

DG: UPCC was really an improvement over conventional SFC. The reliability and precision of pumping system and backpressure regulation, required for controlling the delivery of mixtures of CO_2 and organic modifier, were drastically enhanced. With the engineering issues behind us, we looked into developing methods and found that we needed to start with the basics of stationary and mobile phase chemistry. Indeed, the differences between LC and UPCC are significant, and new paradigms are needed. LC experience did not intuitively transfer to UPCC.

RLS: Tell us about the differences.

DG: First, most SFC/UPCC experiments are conducted under the normal-phase mode (polar stationary phase and apolar mobile phase), while reversed-phase liquid chromatography (RPLC)/UHPLC are reversed-phase approaches. When developing an RPLC method for the analysis of small molecule drugs, one optimizes the separation by focusing mostly on the mobile phase (pH, organic modifier, gradient profile). One seldom really tests dozens of columns, although there are accessories designed to automatically scout the suitability of RPLC columns with different bonding, % carbon, endcapping, pore diameter, polar embedded groups, etc. With UPCC, a fixed set of mobile phase conditions (150 bar, 40 °C, generic gradient from 5 to 40% MeOH in CO_2) is preferentially selected. Then one scouts the separation for different columns. The differences between stationary phases are indeed much larger than are available by tweaking the mobile phase.

Next, the mobile phase has to be optimized according to the nature of the analyzed compound. We found it necessary to divide our analytes into two groups by considering their physicochemical properties. The neutral and acidic compounds are not problematic and can be analyzed under SFC/UPCC conditions using almost any mobile and stationary phases. On the other hand, the basic ones were much more critical and deserve special attention. This is due to the mobile phase acidity (apparent pH of 5) related to the transient formation of methylcarbonic acid originating from the reaction of methanol and CO_2. To improve the peak shape of basic drugs, particularly those with a pKa higher than 8, it is necessary to change the nature of the stationary phase and work preferentially with a column bonded with a basic group, such as ethylpyridine. On the other hand, it is also possible to use additives (e.g., 20 mM ammonium hydroxide) within the mobile phase.

RLS: Fascinating! How well does it work?

DG: In our first study,[1] we performed a systematic evaluation of basic drugs using UPCC. For this purpose, we evaluated five commercially available ethylpyridine columns for separation of a library of 92 basic drugs (basic pKa between 0 and 11). We particularly

focused on peak shape and asymmetry between 0.8 and 1.4, which we found to be useful to improve peak capacity and quantitation. Among the five columns tested, two ethylpyridine phases gave symmetrical peaks for 77% and 69% of the drug library, without pH control. We hypothesized that the poor peak shapes were caused by mixed-mode separations, possibly due to strong hydrogen bonding or ion exchange.

Alternatively, we also evaluated **Waters** bare hybrid silica phases containing ethylene bridged hybrid (BEH) particles, which are stable in a wide pH range. Adding 20 mM ammonium hydroxide to the mobile phase improved the % symmetrical peaks from 44% to 81%. In contrast, when the eluent was changed to 20 mM formic acid in methanol/CO_2, only 10% of the analytes gave an acceptable peak shape. We did not try this with conventional silica columns since they were expected to have reduced column lifetime at high pH.

Quantitative analysis

RLS: Quantitation is a major need for most assays. Utility is usually measured by the ability to produce actionable data. How does UPCC compare to RPLC, ion exchange, and other modes for quantitative analysis?

DG: We are working to develop quantitative assays. These require measuring the various factors affecting retention time and detector response, etc. UPCC is different than LC, so we are working systematically to understand the cause and effect of these differences.

UPCC has not been on the market for long enough to have a history of performance of validated methods. We expect that we will discover and then solve issues.

Method development

RLS: Method development in UPCC begins with comparing a range of columns under sort of standardized conditions. Do you have apparatus to do the scouting automatically overnight?

DG: This is indeed an important point. For the moment, the current UPCC instrument available in our laboratory allows the screening of only two different stationary phases, in an automated way, using switching valves. It is possible to increase the number of oven modules in a UPCC system and to screen up to six stationary phases automatically, but such a configuration could become quite expensive. In my opinion, during the early stages of achiral method development in UPCC, it would be ideal to screen five different stationary phases automatically. To meet this requirement, the current UPCC instrumentation still has to evolve.

RLS: If one changes columns manually, how long does it take to get a stable baseline?

DG: The time required to change and tighten the column is estimated at around 2 min in SFC/UPCC, equivalent to the time required to change a UHPLC/HPLC column. On the other hand, the linear velocity is significantly higher in SFC/UPCC compared to HPLC/UHPLC and, thus, the equilibration time required to achieve a stable baseline is also reduced. On average, the optimal mobile phase flow rate, 2–3 min, is sufficient before injecting the first sample.

Mobile phase density

RLS: Mobile phase density is a key parameter in solubility in SFC and also SFE. Density is dependent on pressure. The pressure needed for elution of a column packed with 5-µm-diameter particles is not high, but what do you find when one decreases the particle size to "sub-twos"?

DG: Yes, most of our work on peak shape[1] was done with columns packed with with 5-µm-diameter particles. Thus, pressure drop was insignificant. However, we also studied the effect of decreasing the particle size from 5 to 1.7 µm for a bare silica phase in our second study.[2] The change in column pressure along the column packed with 1.7-µm particles can produce adiabatic cooling due to the expansion. This causes possible changes in retention, selectivity, and kinetic performance. Based on our experience, the kinetic performance remains acceptable when using columns packed with sub-2-µm particles in SFC, but it is true that retention is affected (lower retention on 1.7-µm vs 5-µm particles) and sometimes also selectivity. This has to be taken into account when dealing with method development.

Backpressure

RLS: Typically in HPLC, as columns age or process many samples, the backpressure increases gradually. Is this seen in UPCC? Does this change the retention and resolution?

DG: The answer depends upon the type of analytes, matrix, mobile phase, and the column backpressure as well as temperature. In our laboratory, we have good expertise in UHPLC and we have observed that the UPCC column possesses similar lifetime to UHPLC phases (on average, we are able to perform around 1000 injections, before performance loss). Similarly to what can be observed in UHPLC, the column backpressure is increased and the retentions, as well as peak shapes, are affected when the column is near its death.

High-resolution analysis

RLS: You report that you have obtained separations of pharmaceuticals with a peak capacity of 250 in 40 min. Please tell us more.

In addition to the ability of UPCC to perform fast analysis (in the subminute range), we have also demonstrated that this strategy could be useful to reach very high resolution. This is of prime interest when analyzing complex samples. We have coupled in series two columns of 10 cm packed with sub-2-µm particles and, using a 40-min gradient run, a peak capacity of 250 was attained. In other

words, based on the achieved peak widths, it could theoretically be possible to separate 250 peaks in 40 min, with a resolution of 1.

RLS: When UPCC was introduced, there was little mention of analyte classes that would not be suitable. What classes do you anticipate are not suitable?

DG: UPCC is really normal-phase chromatography. Successful analytes usually are smaller molecules with low to intermediate polarity. A time-proven generalization requires solubility in chloroform. This precluded salts and hydrophilic compounds such as carbohydrates, including DNA. Normal-phase columns can irreversibly bind with large biomolecules, including proteins and peptides larger than about 500 Da. UPCC has a relatively small "sweet spot." But this is where the action is—in small molecules, particularly pharmaceuticals.

RLS: You commented that the maximum flow rate (4 mL/min) and P_{max} of the UPCC systems prevented taking advantage of the faster flow rates that SFC potentially offers. How serious do you see this limitation?

You also mentioned that the sample injector's 50-sec cycle time was slower than some separations. Do you have other items that engineers should address?

DG: Because of the low viscosity of supercritical CO_2, it is possible to use columns packed with sub-2-µm particles in SFC, close to their optimal linear velocity (>10 mm/sec). However, when mixing CO_2 with organic modifier to extend the range of UPCC applications, the mobile phase viscosity drastically increases and it becomes impossible to work at the optimal velocity because the upper pressure of the system is too restrictive—fixed at 400 bar. On the other hand, 4 mL/min could indeed be a limitation when using 4.6-mm-i.d. phases. Except for injector cycle time, the system volume can be reduced to make the UPCC system compatible with 2.1-mm-i.d. columns, with limited impact on peak broadening.

Two-dimensional separation

RLS: In one of your papers, you mentioned the possibility of two-dimensional separations including UHPLC × UPCC and UPCC × UPCC. Can you expand on these please?

DG: Because the separation mechanisms are rather orthogonal between UHPLC and UPCC, the two techniques could be successfully implemented in a 2-D setup, with the goal of increasing the resolving power. However, because the selectivities achieved with two columns of different chemistries (e.g., bare silica, ethylpyridine, cyano, diol, fluorophenyl, C18, etc.) in UPCC are also quite different, it could be beneficial to evaluate comprehensive UPCC × UPCC. This could limit the problems related to sample diluent incompatibility.

Evolution of SFC

RLS: Clearly, your experience with UPCC has changed your attitude toward SFC. What advice would you give to others who are contemplating embarking on a similar journey?

DG: For those who ever tested SFC in the past, I feel that SFC has evolved, particularly with the introduction of this UPCC technology. The performance achieved today in UPCC is close to what can be achieved in UHPLC in terms of robustness, reliability, and sensitivity.

For those who never tested SFC before, don't be afraid of this technique—the instrumentation and basics of separations look very similar to HPLC, but there are several advantages, such as orthogonal retention mechanism, greener approach, and ability to perform chiral and achiral separation with the same mobile phase.

References

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