An Executive Summary

Orthogonal Chromatographic Techniques for the Quantitative Analysis of Potential Mutagenic Impurities

Jennifer Simeone
Senior Scientist, Separations Technologies
Waters Corporation

How the use of convergence chromatography enables the quantitation of highly polar impurities at the very low levels required by regulatory agencies.

Introduction to Convergence Chromatography

Convergence chromatography is a name sometimes given to supercritical fluid chromatography (SFC) to highlight the convergence of liquid and gas states in the mobile phase. This convergence results in a single technique that can deliver the benefits offered by both liquid chromatography (LC) and gas chromatography (GC), while avoiding some of their limitations. In GC systems, the temperature-driven separation is highly efficient, but selectivity is limited by the available column chemistries and the types of carrier gases. LC instruments, and in particular, its high pressure and ultrahigh pressure versions (HPLC and UPLC), are also highly efficient but limited by the available instrumentation, the suitability of the solvents used, and the particle size of the stationary phase.

Conversely, in convergence chromatography, separation efficiency can be controlled by manipulating the density of the mobile phase, and by varying the solvent gradient—a two-pronged approach that enhances the flexibility of this technique (see **Figure 1**). The mobile phase is mainly comprised of compressed CO_2 and it may contain a suitable co-solvent. Because of CO_2's very low density and high diffusion, separation efficiency is very high. In addition, the range of choices of stationary phases and co-solvents is much broader than that for LC systems, which results in a larger selectivity space available for method development. As an added benefit, utilizing a polar stationary phase provides the same selectivity possibilities as normal-phase LC instruments, thus offering a high degree of orthogonality to reversed-phase LC (RPLC) systems.

A further benefit of convergence chromatography is that many of the solvents that can be used with these instruments are compatible with the most popular detection techniques, such as photodiode array (PDA), evaporative light scattering (ELSD), and mass spectrometry (MS). Because of the similarity to normal phase LC, convergence chromatography is ideally suited for the analysis of chiral compounds, or any other structurally similar compounds, without the use of toxic solvents. In general, convergence chromatography can be used to analyze any compound typically analyzed by normal-phase LC systems, hydrophilic interaction chromatography (HILIC), and GC instruments, if they are soluble in organic solvents.

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Other advantages provided by convergence chromatography, in addition to its diverse applicability, include avoiding the need for the derivatization often needed in GC systems, and allowing for the direct injection of high organic extracts. Finally, it requires much less use of hazardous solvents and generates less hazardous waste.

Improvements Over SFC

Current perception is that the difficulty of managing supercritical fluids, with or without modifiers, results in a general lack of robustness in SFC methods. Shifting retention times from injection to injection, and a lack of accuracy in delivering low percentages of co-solvent are common concerns. These perceptions were likely caused by the use of repurposed HPLC or GC instruments for SFC analysis. These instruments were often restricted to full loop injection if quantitative analysis is required, since their partial loop injection mode periodically suffers from poor accuracy and precision.

Furthermore, the reliability of pumping systems, sample introduction devices, and back pressure regulators left a lot to be desired. Baseline noise in earlier SFC instruments was high and sensitivity was poor because of the lack of algorithms designed to handle compressed CO_2. Moreover, the available detectors were not designed to accommodate the refractive index of compressed CO_2.

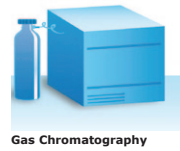
Ultra-Performance Convergence Chromatography System

These problems are specifically addressed and minimized in a new system—the ACQUITY UltraPerformance Convergence Chromatography (UPC²®) System from Waters®—designed specifically for use with supercritical/compressed CO_2 (see **Figure 2**). For starters, the pump design includes an integrated device that recompresses and chills the CO_2 to provide precise control of its density, which is essential to the separation in SFC. The CO_2 and co-solvent pumps have separate control algorithms, which results in greatly enhanced ability to accurately and precisely blend compositions, even at co-solvent levels as low as 1%.

A novel dual injection valve design for the sample manager, which includes an auxiliary injection valve, makes accurate and precise partial loop injections possible while maintaining the physical state of CO_2.

The optical detectors for the UPC² were redesigned to minimize baseline noise when using CO_2. In addition, the

Figure 1: How convergence chromatography works.

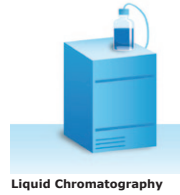


Gas Chromatography

GC

Separation achieved by a temperature gradient

- **High efficiency [N]**
  - Virtually no limitation on column length
- **Limited selectivity [α]**
  - Limited stationary phase options



Liquid Chromatography

LC

Separation achieved by a solvent gradient

- **High efficiency [N]**
  - Limited to pressure drop across column
- **Moderate selectivity [α]**
  - Different modes: reversed-phase, normal-phase, SEC, IEX, affinity, ion pair, HILIC, GPC…etc.

Convergence Chromatography

CC

Separation achieved by density/solvent gradient

- **High efficiency [N]**
  - Very low viscosity enables longer columns and smaller particles
- **High selectivity [α]**
  - Wide variety of stationary phase and mobile phase co-solvent and modifier options

Figure 2: Evolution of SFC Instrumentation: ACQUITY UPC².

- Built upon proven UPLC® Technology, but each component designed specifically for use with compressed CO_2
  - **Pump**: Integrated CO_2 chilling device for exceptional density control, independently cooled pump heads, separate control algorithms for compressed CO_2 and organic co-solvent
  - **Sample manager**: Auxiliary injection valve to maintain pressure of system and physical state of CO_2 , specially designed rotor and stator to accommodate supercritical CO_2
  - **PDA**: high strength silica lens improves low UV energy, thermal management of optics bench, low dispersion stainless steel TaperSlit flow cell with increased pressure rating
  - **Convergence manager**: Innovative two-stage dynamic and static back pressure regulator for improved density control, contains active back pressure regulator [ABPR] to maintain desired CO_2 pressure, Heated static cartridge BPR

flow cells are capable of withstanding the high pressures needed post-column and post-detector to maintain the density of the CO_2 or CO_2 co-solvent blends.

To better control the mobile phase density, the UPC² system uses an innovative dual-stage regulator that controls dynamic and static back pressure to achieve improved density control. The static cartridge is heated to mitigate valve freezing during the CO_2 liquid to gas phase change.

The historical views of unreliability or lack of robustness in SFC instrumentation are negated by the UPC² system. Several large pharmaceutical companies, contract organizations, and manufacturing sites are using modern SFC technology for critical analyses. These include published articles on the use of SFC for good manufacturing practices (GMP) for improved selectivity and shorter run times; the separation of fulvestrant isomers; product testing for illegal dyes; the analysis of impurities in Vitamin D3; and a fully validated stability confirmation method for an injectable drug, to name a few.

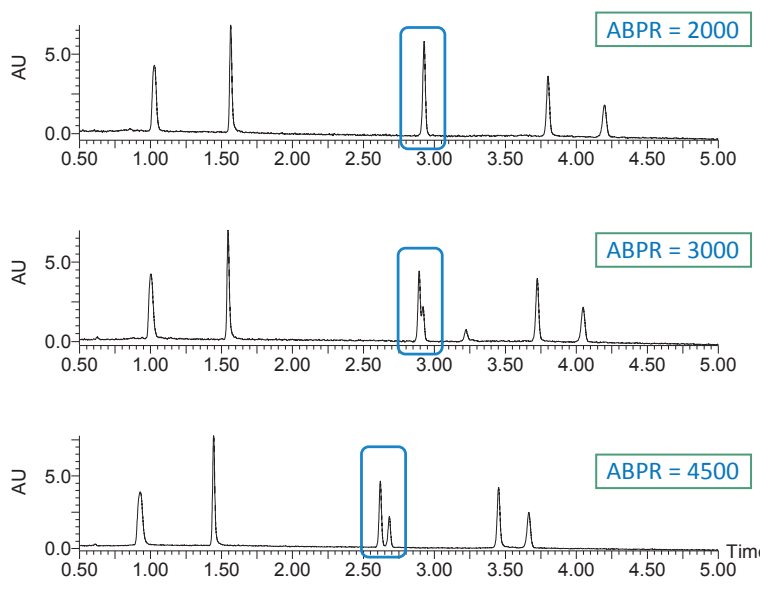
Comparison to RPLC

In RPLC systems, polar compounds elute early in the aqueous gradient, while less polar compounds are more retained and thus elute later. RPLC analysis is typically limited to the analysis of achiral compounds, and it often uses additives to improve peak shape and manipulate selectivity. Normal-phase LC, which uses non-polar solvents and a polar stationary phase is often used for separation of chiral compounds. Convergence chromatography combines the best of both techniques. Similar to NPLC, convergence chromatography it uses a less polar solvent (CO_2), but can use either non-polar or polar stationary phases which expands the selectivity space where CC can be used. Additionally,convergence chromatography uses pressure to effect separation and selectivity. Convergence chromatography is able to separate chiral and achiral compounds without having to change solvents, as would be needed when going from a RPLC instrument to a normal-phase LC instrument. Furthermore, the low viscosity and high diffusion of supercritical CO_2 enables highly efficient separations in a short analysis time.

To illustrate the differences between convergence chromatography and RPLC, a separation of six pharmaceutical compounds was done under typical conditions for RPLC and convergence chromatography. The separation with the UPC² system, using a CO_2-methanol gradient, is noticeably faster than the RPLC system, which used water–acetonitrile. The UPC² system also delivers narrow peak widths for all six compounds. RPLC was only able to separate five compounds, as two of them co-eluted due to their structural similarities (see **Figure 3**), while all six compounds were separated by using convergence chromatography.

Figure 3: Achieving separation using pressure.

- Retention and Selectivity are directly linked to the pressure and density of the CO_2/Co-Solvent blend
- Modifying the ABPR setting is an additional method parameter to control/change selectivity



Torus Technology

- 3x100mm Torus DEA column maintained at 30°C
- Mixture of 6 pharmaceutical compounds
- Only ABPR settings have been varied

- What initially appears to be only 5 compounds is actually 6 compounds
- Full separation achieved at a higher ABPR setting

Case Study: Ondansetron

Impurities are always present in drug products, either as byproducts of the synthesis and manufacturing processes, or as a result of degradation. Regulatory agencies, such as the International Conference on Harmonization, set strict upper limits for the amounts of impurities that can be present in any drug. Potential mutagenic impurities, also referred to as genotoxic impurities, fall under a special guidance issued in 2014, which sets even lower limits because of their potential for carcinogenicity.

Ondansetron (branded as Zofran) is an anti-nausea medication often prescribed to chemotherapy patients. The United States Pharmacopeia (USP) lists five compounds as impurities found in ondansetron, which include imidazole (Impurity E) and 2-methylimidazole (Impurity F). As seen in **Figure 4**, Impurities E and F are very small, polar compounds. Their allowable limit would be 2,000 ppm, but when considered as potential mutagens, their limit (based on the dosage level of the drug and the total number of days the dosage is taken) is 417 ppm, in reference to the active pharmaceutical ingredient (API). This amount is approximately 50 ng/mL of Impurity E or F in a solution of 125 µg/mL of API

The need to quantify these low levels of impurities rules out UV detection, as it does not provide sufficient sensitivity. Tandem-quadrupole mass spectrometry can provide the required sensitivity, but its use is hindered by the use of ion-pairing reagents required in the USP monograph. The monograph uses ion-pairing reagents to enable retention of the two small, highly polar impurities imidazole and 2-methyl imidazole, therefore it will be necessary to develop a method that doesn't require the use of ion-pairing reagents. This leaves two possibilities: developing LC methods in which the impurities are retained, or using an orthogonal technique, such as convergence chromatography, which is readily compatible with MS instruments.

The polar impurities proved to be too much of a challenge for a RPLC system (see **Figure 5**). In spite of trying several column chemistries, along with mobile-phase pH variations to better separate the API from all impurities, the polar molecules were not retained and eluted in the void volume of the column, which is undesirable.

The next step was to try HILIC, which is a technique often used for the retention of highly polar compounds. In this case, the starting place was a literature method developed for 2-methylimidazole. The method was modified to facilitate better separation between the API and impurities and ultimately delivered good retention for Impurities E and F, but two other impurities (C&D) now eluted in the void. In spite of this, the method was used to quantify Impurities E and F at levels of 120–4,000 ppm, and yielded good signal-to-noise ratios for the

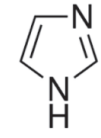
Figure 4: USP monograph – impurity limits.

Compound Name	Relative Retention Time	Relative Response Factor	Limit (%)
Ondansetron related compound C	about 0.32	1.2	0.2
Ondansetron related compound D*	about 0.34	—	0.1
Imidazole	about 0.49	0.3	0.2
2-methylimidazole	about 0.54	0.4	0.2
Ondansetron	1.0	—	—
Ondansetron related compound A	about 1.10	0.8	0.2
Unknown	—	1.0	0.1
Total	—	—	0.5

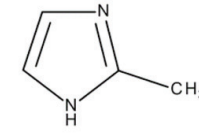
*Quantified in the test for *Limit of ondansetron related compound D.*

Imp A, C, E & F = 0.2% = 2000ppm

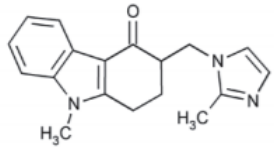
Imp D = 0.1% = 1000ppm



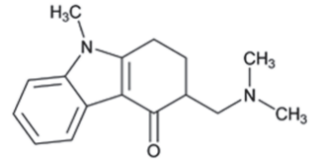
Impurity E (imidazole)



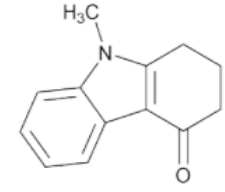
Impurity F (2-methyl imidazole)



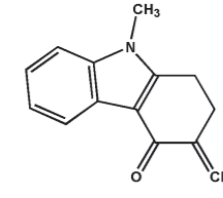
Ondansetron



Impurity A



Impurity C



Impurity D

USP40–NF35, Page 5441

Figure 5: RPLC method development: scouting conditions.

- Mobile Phases used: either 0.1% formic acid or 0.1% NH_4OH in Water / ACN
- Columns Tested:

  ACQUITY UPLC CSH Fluoro-phenyl (2.1 x 100 mm, 1.7µm)
  ACQUITY UPLC BEH Amide (2.1 x 150 mm, 1.7µm)
  ACQUITY UPLC HSS T3 (2.1 x 100 mm, 1.8µm)
  ACQUITY UPLC HSS Cyano (3 x 100 mm, 1.7µm)

- Initial results show no retention of Imp E or Imp F under all conditions tested

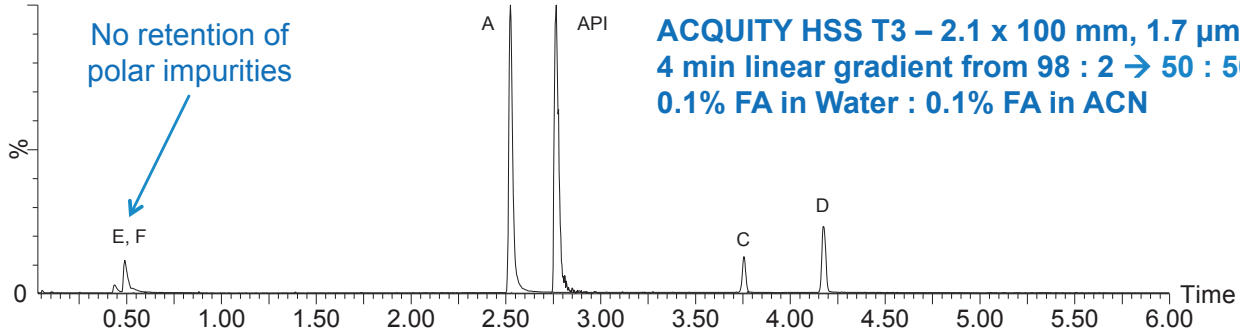
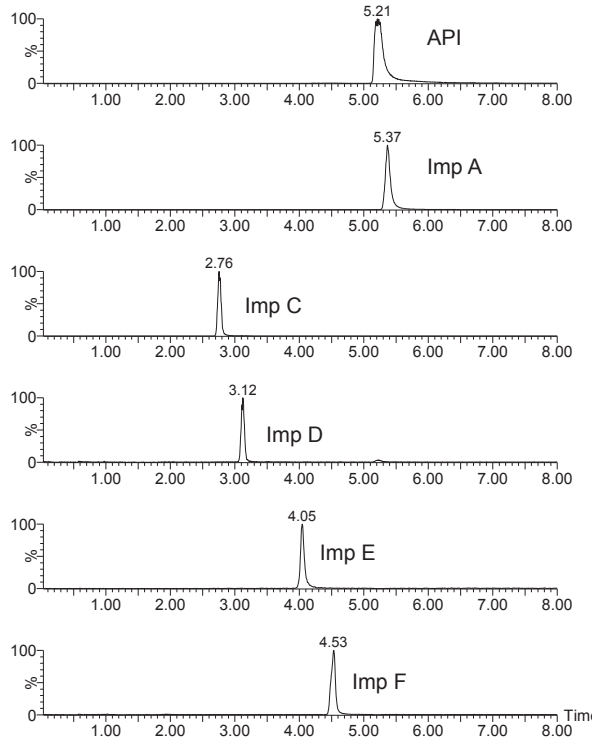


Figure 6: Final UPC² methodology.



Optimized Method Conditions:

Column: Waters ACQUITY UPC² Torus 2-PIC, 1.7 µm, 3.0 x 100 mm
Column Temperature: 30°C
Mobile phase A: CO_2
Mobile phase B: 0.2% (v/v) NH_4OH in Methanol
Flow Rate: 1.00 mL/min
ABPR: 2000 psi
Injection Volume: 2.0 µL
Weak Needle Wash / Seal Wash: Isopropanol
Strong Needle wash: Methanol
LC Gradient:

Time	Flow	%A	%B	Curve
Initial	1.00	95	5	--
6.00	1.00	85	15	6
7.50	1.00	85	15	1
9.00	1.00	95	5	1

Xevo TQ-S micro with ISM for Makeup Flow Conditions:
Ionization Mode: ESI+
Dwell Time: 24 msec
Probe Temperature: 650 °C
Cone Voltage: 15V
Capillary Voltage: 0.4 kV
MRM conditions:
Ondansetron - 294.1 > 170.1; CE = 6
Impurity A - 257.2 > 58; CE = 15
Impurity C - 200.1 > 144.1; CE = 25
Impurity D - 212.1 > 184.1; CE = 18
Impurity E - 69.1 > 42; CE = 15
Impurity F - 83.1 > 41; CE = 16

Makeup Flow Solvent: 0.2% (v/v) NH_4OH in Methanol
Makeup Flow Rate: 0.500 mL/min

limits of quantification (LOQs). However, the remaining impurities required the development of another method for their quantitation. In this case, a generic RPLC method using formic acid and water was developed for this purpose. For this method, good linearity was established between 120 and 4,000 ppm and the LOQs met the required levelBut, this approach requires two separate methods and two separate sample preparations.

Developing a single method for this analysis, however, was possible using convergence chromatography. Using CO_2 and methanol as the mobile phase, and ammonium hydroxide as an additive, resulted in excellent peak shapes and fast separation of all the impurities in ondansetron. The two polar impurities are well retained on all the columns that were examined. All impurities were well separated from the API (see **Figure 6**).

The final method developed for ondansetron uses a UPC² Torus 2-PIC column, with methanol and ammonium hydroxide as co-solvent at a flow rate of 1 mL/min. The range of quantitation is 120–4,000 ppm, with very good signal-to-noise ratios and good coefficients of determination (R-squared) values for the calibration curves. The overall analysis time is shorter than that for RPLC or HILIC systems. In addition, all impurities can be analyzed in a single method.

Conclusions

The latest improvements in pumping systems, sample introduction devices, and back pressure regulators incorporated into the UPC² system corrected many of the perceived pitfalls of earlier convergence chromatography (SFC) systems. Because of its orthogonality to RPLC, convergence chromatography can successfully be used to develop a single test method in cases where two or more LC methods may be needed. In addition, for separations where UV detection may not provide the level of detection needed, convergence chromatography can also be used with mass spectrometry to meet the lower sensitivity levels required.