

Effects of Aqueous Sample Content and Aqueous Co-Solvent Composition on UPC² Separation Performance

Christopher Knappy, Norman W. Smith, Arundhuti Sen, Paul D. Rainville, Robert S. Plumb

King's College London, Waters Corporation



For research use only. Not for use in diagnostic procedures.

Abstract

In this application note, we demonstrate the application of UPC² for the analysis of polar analytes in aqueous matrices.

Benefits

- Analysis of polar metabolites
- Throughput
- Aqueous sample compatibility
- Resolution

UltraPerformance Convergence Chromatography (UPC²) can be used for the direct analysis of polar metabolites from aqueous biological samples, such as urine, or from extracted serum samples. Analytical methods can be rapidly developed from a simple set of starting conditions of column flow-rate, temperature, and pressure. Modifying the co-solvent by the addition of small amounts of water buffer and acid provides rapid high resolution analysis with rapid re-equilibration times.

Introduction

Liquid chromatography (LC) coupled with accurate mass, mass-spectrometry has become the technique of choice for the screening of biological fluid such as urine and plasma for small molecule biomarkers. While reversed-phase LC can successfully analyze moderately polar analyte, lipids, and non-polar steroidal metabolites, the analysis of highly polar analytes still represents a challenge. The ability to analyze these biological samples with minimal pre-treatment is critical as the introduction of multiple sample preparation steps can result in a reduction in analytical precision and/or loss of analyte species due to poor solvent solubility. Hydrophilic interaction liquid chromatography (HILIC) has shown some success for the analysis of highly polar compounds, however the long re-equilibration times and low tolerance to highly aqueous sample loading limits its utility. Here we explore the application of UPC² for the analysis of polar analytes in aqueous matrices.

Experimental

Caffeine was employed as a model polar compound in order to investigate the choice of injection parameters for the analysis of polar compounds by UPC²,² as it maintains a constant charge across a wide pH range (pKa = 14.2) and is both PDA and ES+ active. A standard solution was prepared at a concentration of 0.1 mg/mL⁻¹ in 9:1 heptane:isopropanol. To investigate the feasibility of aqueous sample injection using UPC²,² 0.1 mg/mL⁻¹ solutions of caffeine were prepared using water, methanol (MeOH), ethanol (EtOH), isopropanol (IPA), acetonitrile (ACN), acetone (Me₂CO), and 9:1 heptane:IPA as solvents. Water miscible organic solvents were chosen in order to appraise their potential as diluents for aqueous solutions during the same set of experiments. Each working solution was analyzed on the ACQUITY UPC² System with PDA Detector, with injection volumes ranging from 1–7 μL.

The chromatography was performed on an ACQUITY UPC² System, comprising of an ACQUITY UPC² Binary Solvent Manager, ACQUITY UPC² Sample Manager, Automatic Back-Pressure Regulator (ABPR), and an ACQUITY Photo Diode Array (PDA) Detector. The separation was performed on ACQUITY UPC² BEH Column (1.7 μm, 3.0 x 100 mm) and eluted with an isocratic mobile phase comprising of 95:5 (v/v) CO₂:MeOH, operated at 2.5 mL/min.⁻¹ The density of the subcritical fluid was regulated via a column temperature of 55 °C and automatic backpressure regulator (ABPR) pressure setting of 2,000 psi. The column effluent was monitored by PDA at 273 nm.

LC conditions

LC system:	ACQUITY UPC ²
Detection:	UV 273 nm
Column:	ACQUITY UPC ² BEH Column (1.7 μ m, 3.0 x 100 mm)
Column temp.:	55 °C
Sample temp.:	Room temperature
Injection volume:	1 μ L/min
Flow rate:	2.5 mL/min ⁻¹
Mobile phase A:	CO ₂
Mobile phase B:	Methanol isocratic mobile phase comprising of 95:5 (v/v) CO ₂ :MeOH

Results and Discussion

The ACQUITY UPC² System produced a highly efficient peak with a mean N value of 18,910 +/- 193 being recorded. An example chromatogram is given in Figure 1. This illustrates that both the ACQUITY UPC² System and chromatography column were performing correctly.

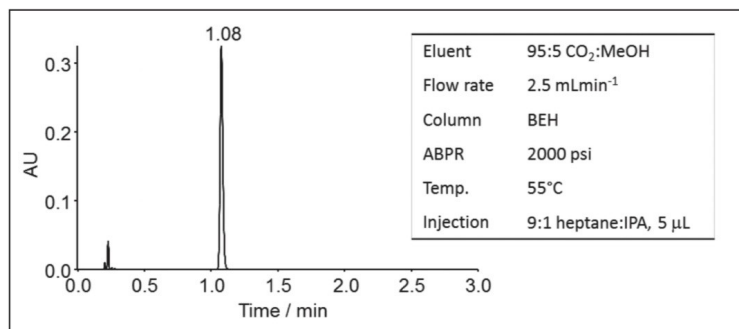


Figure 1. ACQUITY UPC² System with PDA Detector chromatogram (273 nm) of caffeine via the precision test method.

Effect of injection solvent on UPC² efficiency

Very few studies have managed to successfully introduce analytes for SFC as solutions in water without severe disruption to chromatographic peak shapes.¹⁻³ The effect of different injection solvents was investigated over the injection range of 1-7μL. The polarity ranged from the very polar water and methanol through to the aprotic solvent acetone to the very non-polar heptane/IPA mix. The effect of the injection solvent was evaluated by comparing the efficiency of the observed peak to that of the 9:1 heptane:isopropanol injection solvent and expressed as N_{rel} , where a value >1 represents an improvement in performance and a value <1 represents a reduction in chromatographic performance. The data obtained is presented below in Figure 2.

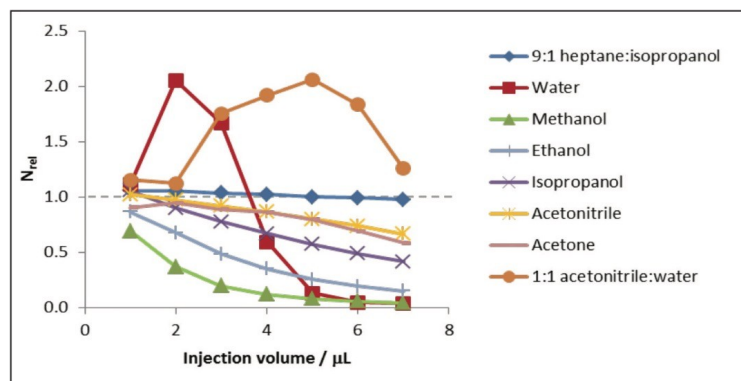


Figure 2. Relative UPC² efficiencies for caffeine in various solvents as a function of injection volume.

Heptane:IPA gave the smallest changes in N_{rel} with changing injection volume (1.06-0.98), and this is due to the close similarity in polarity to the effluent mobile phase. The water miscible organic solvents gave the poorest efficiencies, progressively deteriorating as solvent polarity increased (i.e. in general, N_{rel} ACN > Me₂CO > IPA > EtOH > MeOH). The sequence presumably reflects the increasingly poor mismatch in polarity to the eluent. The organic solvents generally showed an anticipated steady decrease in N_{rel} from 1 to 7 μL injection as a result of the increasing size of the injection plug. Of the water miscible solvents, acetonitrile and acetone showed the smallest changes in N_{rel} (1.02–0.67 and 0.91–0.58, respectively), and methanol the greatest change (0.70–0.04).

Water behaved somewhat unexpectedly as an injection solvent. The maximum efficiency ($N_{rel} = 2.05$) was significantly higher than for heptane:IPA, despite the poorer mismatch in polarity to the eluent. This maximum efficiency occurred at 2 μL as opposed to 1 μL injection volume, which was the observed optimal injection volume for all of the other pure solvents. The efficiency N_{rel} quickly reduced with injection volumes greater than 2 μL , with peak splitting observed in aqueous injection volumes of 5 μL or greater. Interestingly the use of an acetonitrile:water as the injection solvent showed and increase in N_{rel} increased up to nearly 6 μL . Although the use of stronger elution injection solvents resulted in marginally earlier elution than heptane:IPA, reductions in retention times were less than 10%. Accordingly, injection in water was not only tolerated in UPC², but effected a reduction in peak broadening at some injection volumes. Of the water miscible organic solvents, acetonitrile showed greatest promise as a potential diluent for aqueous solutions.

Several factors could account for the beneficial effect of water on the UPC² efficiency for the analysis of caffeine, including improved mass transfer with the polar BEH phase or focusing (reduced longitudinal diffusion) of the elution band. To better assess the influence of water on the chromatographic system as a whole, further investigations were made via modification of both the injection solvent and the co-solvent with water.

Further investigation of the effect of water on UPC² efficiency

To assess the chromatographic benefits of water as an injection solvent irrespective of how it was introduced into the ACQUITY UPC² System, a 0.1 mg/mL⁻¹ caffeine solution in the various solvents was analyzed by the ACQUITY UPC² System with PDA Detector, using a modification to the method in which 95:5 MeOH:H₂O was used as the co-solvent instead of pure methanol. The derived data is shown below in Figure 3. The results show that for injection volumes of less than 2 μL, the observed efficiencies for water injections were higher than those obtained for all of the organic injection solvents.

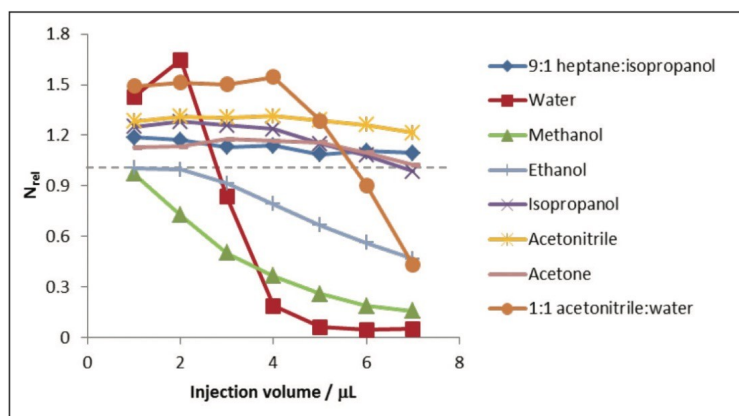


Figure 3. Relative UPC² efficiencies for caffeine in various solvents as a function of injection volume, achieved using 95:5 MeOH:H₂O as co-solvent.

Interestingly, acetonitrile replaced heptane:IPA as the most tolerant injection solvent, giving the smallest changes in N_{rel} with increasing injection volume (1.37–1.27). Injection in water still yielded the optimal efficiencies, although with smaller magnitudes (maximum N_{rel} = 1.72), but these were shifted towards lower injection volumes compared to the non-water containing co-solvent data. The results suggest that water is inherently beneficial to the chromatographic analysis of caffeine whether introduced via the co-solvent or injection solvent.

Effect of the proportion and volume of water injected on UPC² performance

The reduction in UPC² efficiency observed in this study at higher aqueous injection volumes suggests a limit to the amount of water that can be injected without deleteriously affecting the chromatography. To determine the dependence of chromatographic performance on the volumetric injection of aqueous solutions, a 0.1 mg/mL⁻¹ solution of caffeine was prepared in 1:1 acetonitrile:water and analyzed using an ACQUITY UPC² System with PDA Detector, either with MeOH or 95:5 MeOH:H₂O as co-solvents. In each case, the maximum efficiency was similar to that obtained via injection of pure water but occurred at approximately double the injection volume,

suggesting the volume of the aqueous component was the critical factor, not the total injection volume.

In order to identify the maximum amount of water tolerated by UPC², caffeine solutions comprising 0, 10, 33, 50, 67, 90, and 100% water were made by diluting a 1 mg/mL⁻¹ aqueous caffeine stock with acetonitrile to give a total volume of 1 mL. Each solution was then analyzed by using an ACQUITY UPC² System with PDA Detecor, using methanol or 95:5 methanol : water as the co-solvent, with the injection volume varied from 1–7 μ L.

Plots of N_{rel} against the volume of water injected are given in Figure 4. The data shows that a maximum in chromatographic performance occurs when the injection volume contains approximately 2 μ L of water. It can be seen that injections with greater volumes of water produced significantly poorer efficiencies irrespective of the co-solvents. By contrast, injection of lesser volumes of water were highly detrimental to the chromatographic efficiency in MeOH co-solvent, but only moderately detrimental with the MeOH:H₂O (95:5) co-solvent. The results suggest that the total volume of water injected should be kept ≤ 2 μ L for UPC², irrespective of the composition of the injection solvent.

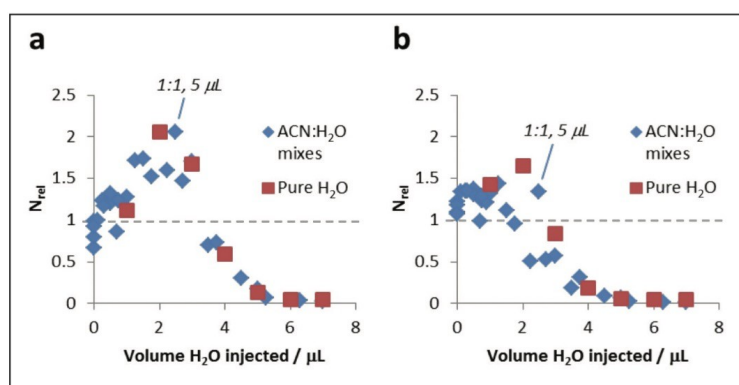


Figure 4. Relative UPC² efficiencies for caffeine in pure H₂O and in ACN:H₂O solutions, plotted as a function of the total volume of water injected, with a) methanol and b) 95:5 MeOH:H₂O as co-solvent.

Effect of system parameters on UPC² efficiency

Along with the injection conditions, changes to the ACQUITY UPC² System operating parameters could also enhance, diminish or destroy the beneficial effects of water on the chromatographic performance of UPC² for polar analytes. To determine the optimal operating parameters and robustness to changes in these conditions the proportion of co-solvent in the eluent (2–10% methanol), the eluent flow rate (1.0–3.0 mL/min⁻¹), the ABPR pressure (1,500–3,000 psi), and the column temperature (35–75 °C) were independently varied in order to screen for any influences on

system efficiency (N_{rel}). For these experiments, 1:1 acetonitrile:water was employed as the injection solvent and a 5 μL injection volume was chosen as previous data suggested that these conditions gave optimal or near-optimal efficiencies with either MeOH or MeOH:H₂O co-solvents. A 5 μL injection of caffeine in 9:1 heptane:IPA was employed to determine N_{rel} .

From a careful interrogation of the data shown in Figure 5 it can be determined that the parameters which gave optimal efficiencies were largely the same for both aqueous and control solutions, with the notable exception of those for column temperature, which optimized at 55 °C and 35 °C, respectively. For both injection solvents, N_{rel} values increased with increasing ABPR pressure up to 3000 psi. For each of the system parameters, N_{rel} was far more sensitive to systematic change when the aqueous injection solvent was employed rather than heptane:IPA (9:1). This data suggest that particular care should be taken to optimize the parameters when using aqueous solutions, as small changes can severely diminish the chromatographic efficiency.

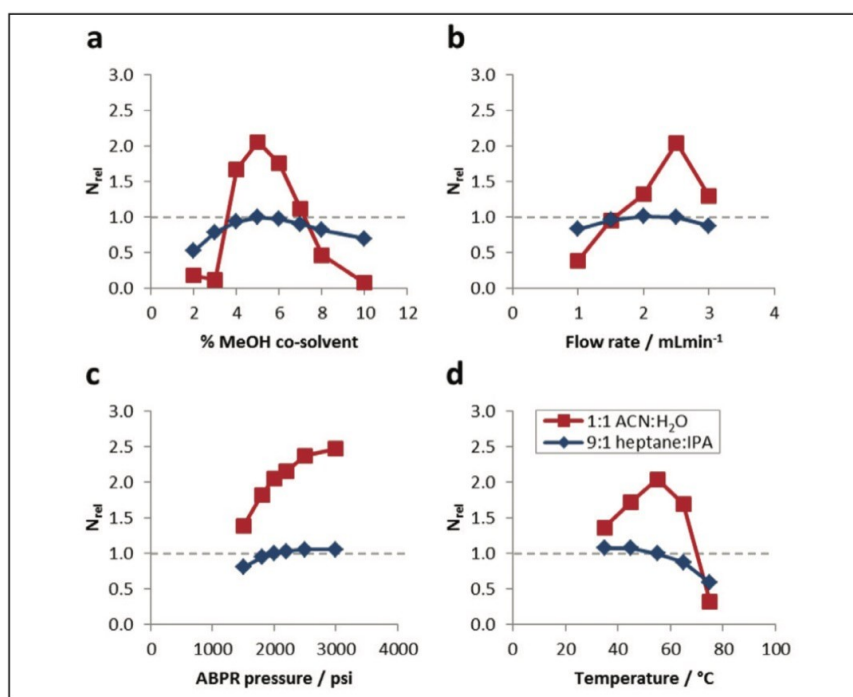


Figure 5. Relative UPC² efficiencies for caffeine in aqueous (■) and control (◆) solutions, achieved via modifications to a) % MeOH co-solvent composition, b) eluent flow rate, c) ABPR pressure, and d) column temperature.

Conclusion

- UPC² can be employed for the analysis of polar analytes in aqueous media if the injection volume and co-solvent conditions are carefully controlled.
- Enhanced UPC² efficiency was observed when water was incorporated in the co-solvent at low concentrations.
- The UPC² chromatographic efficiency of polar analytes increased linearly with pressure, and increased with temperature up to 50 °C, a flow-rate/pressure combination of 2 mL/min and 3,000 psi yielded a good starting point for method development.

References

1. Gyllenhaal O. Packed column supercritical fluid chromatography of a peroxisome proliferator-activating receptor agonist drug: Achiral and chiral purity of substance, formulation assay and its enantiomeric purity. *J Chrom A*. 2004;1042:173–80.
2. Mukherjee PS and Cook SE. A feasibility study on direct assay of an aqueous formulation by chiral supercritical fluid chromatography (SFC). *J Pharm Biomed Anal*. 2006;41:1287–92.
3. Mukherjee PS. Validation of direct assay of an aqueous formulation of a drug compound AZY by chiral supercritical fluid chromatography (SFC). *J Pharm Biomed Anal*. 2007;43:464–70.
4. Liu Y, Li X, Yang C, Tai S, Zhang X, Liu G. UPLC-MS/MS Method for Simultaneous Determination of caffeine, Tolbutamide, Metoprolol, and Dapsone in Rat Plasma and its Application to Cytochrome P450 Activity Study in Rats. *J Chrom Sci*. 2013;51:26–32.
5. Taylor LT. Packed column supercritical fluid chromatography of hydrophilic analytes via water-rich modifiers. *J Pharm Biomed Anal*. 2012;43:464–70.

Featured Products

[ACQUITY UPC2 System](#)

[ACQUITY UPLC PDA Detector](#)

720005505, September 2015

©2019 Waters Corporation. All Rights Reserved.