[UPLC AND UPLC/MS APPLICATION NOTEBOOK]

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Today, more than ever, laboratory-dependent organizations like yours are striving to understand where to best focus their efforts and assets. Part of that task involves the consideration of forward-looking technology platforms to meet the needs of an ever-evolving business climate. The Waters® ACQUITY UPLC® System was created especially for those who are seeking proven, reliable technology that simultaneously improves laboratory productivity, efficiency, and throughput.

With thousands of installed systems and columns worldwide, ACQUITY UPLC reflects today's laboratory requirements for a versatile system that decreases sample run times by a factor of 10, uses up to 95 percent less solvent, significantly enhances chromatographic and MS performance, and saves laboratory space and energy. Demonstrated by more than 400 peer-reviewed papers, 300 application notes, and dramatic process improvements, many companies and institutions around the world have standardized on UPLC® Technology for measurable scientific and business benefits.

With ACQUITY UPLC as the undisputed technology leader for five years running, follow-on or "me-too" technologies continue to emerge. As a result, the LC landscape is increasingly muddled, specifically around comparisons of UPLC to UHPLC. UHPLC or other high-pressure LC systems simply take HPLC to higher pressure limits and provide increased speed, but dispersion and other factors often compromise the separation at the expense of data quality. UPLC was crafted with a decidedly different approach, optimizing performance through a total ground-up design, including advanced column packing materials, innovations in fluidics and detection, and the comprehensive understanding of their interaction as a system.

Waters continues to expand the application range of UPLC Technology for even more chromatographers by embodying the many advantages of sub-two micron particles in industry-leading, fit-for-purpose innovations such as:

- nanoACQUITY UPLC® and TRIZAIC UPLC™ systems, the first microfluidic LC platforms to optimize the scientific advantages of UPLC for some of the most challenging sample-limited applications
- PATROL™ UPLC Process Analyzer, a real-time PAT system that detects and quantifies complex multi-component manufacturing samples and final product directly on the production floor for maximum production efficiency
- ACQUITY UPLC Columns, in more than 100 combinations of configurations and chemistries for every analytical task

In light of current economic conditions, laboratory transformations and investments are taking place with careful and comprehensive understanding of existing operational capabilities and requirements, as well as demonstration of return on investment. Consequently, we continue to see unprecedented levels of UPLC adoption as the future-proof LC technology of choice.

As you continue to transform your laboratory into one of your organization's greatest assets, we'll stay committed to always providing the next advancements in separation and MS sciences to help you get there.

Art Caputo

Executive Vice President and President, Waters Division
Waters Corporation

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COMPARISON OF A FAST HPLC METHOD ACROSS MULTIPLE LC SYSTEMS

Tanya Jenkins
Waters Corporation, Milford, MA, U.S.

INTRODUCTION

Since the introduction of the Waters® ACQUITY UPLC® System, many vendors have introduced modified high-performance liquid chromatography (HPLC) systems designed for fast LC or ultra-high-pressure liquid chromatography (UHPLC). These systems, which can yield satisfactory chromatography at an analytical scale (4.6-mm I.D.), where system volume and system bandspread have less of an impact on peak width, struggle significantly with microbore chromatography (2.1-mm I.D.). These low-volume separations require a system designed to maximize the separation efficiency to provide greater quality information for the user.

Liquid chromatography system vendors will claim improvements in resolution and sample throughput by migrating traditional HPLC methods to analytical-scale fast LC. Migrating a method from HPLC to fast LC is an attractive solution for businesses looking for ways to reduce the cost of analysis per sample and increase profitability; however the transition to an analytical-scale fast LC method only yields a small percentage of the solvent savings compared to converting the method to a microbore-scale fast LC method. Solvent consumption can be further reduced by nearly five times or 80% with a 2.1-mm I.D. column, compared to a 4.6-mm I.D. column of the same length, resulting in a significantly greater cost reduction per sample. In addition to the ACQUITY UPLC System being the world's only UltraPerformance LC® system, it is also ideally suited for fast LC or compressed chromatography (separation beyond the optimal linear velocity to maximize speed at reduced resolution), since it is optimized out-of-the-box for low dispersion chromatography.

This application note compares the performance of multiple LC systems for separation of a series of anesthetics on a microbore Intelligent Speed™ (IS™) Column. Although significant benefits can be realized by fast LC on the ACQUITY UPLC System, the greatest benefits are achieved by UPLC® separations. A comparison of multiple vendors for UHPLC performance is discussed in Waters Application Note, *Comparison of a UPLC Method across Multiple UHPLC Systems, no. 720003166EN*.

EXPERIMENTAL

The method used to compare the six LC systems was as follows:

Sample:	Anesthetic mix at 50 µg/mL in water
Column:	IS XBridge™ C_{18}, 2.5 µm 2.1 x 20 mm
Injection volume:	2 µL
Temp.:	50 °C
Flow rate:	600 µL/min
Mobile phase A:	10-mM ammonium bicarbonate pH 10
Mobile phase B:	Acetonitrile
Gradient:	25% to 75% B over 2 min (with re-equilibration as required for each system)
Detection wavelength:	220 nm
Data rate/filtering:	Optimized for equivalency on each system
Needle wash:	70:15:15 acetonitrile/isopropanol/water Default wash parameters for each system
Run time:	2.5 min
Data:	All Data were processed with Empower™ 2 Software

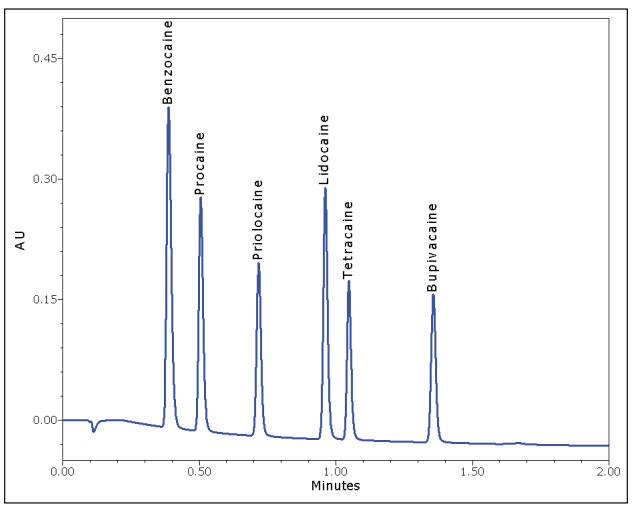

Figure 1. Separation of six anesthetics by fast LC on the ACQUITY UPLC System.

The same column, mobile-phase lot, and wash solvents were used on all the systems. Instruments were configured according to the manufacturer's recommendations for low system delay volume. When possible, the shortest piece of 0.0025 in. I.D. tubing was used before and after the column. Depending upon the system, this included installing a microbore flowcell, reduced volume mixers, reduced volume tubing, bypass of pump components, and utilizing the bypass mode in the injector to further reduce gradient delay. Figure 1 shows the separation of the anesthetic mix on the ACQUITY UPLC System. No system modifications were necessary for the ACQUITY UPLC System since the stock configuration is optimized for ultra-low dispersion for both UPLC and HPLC applications, whether analytical or microbore scale.

RESULTS AND DISCUSSION

For all the separation parameters assessed, the ACQUITY UPLC System, which was designed for high sensitivity and minimal band spread, easily outperformed all of the other LC systems for fast microbore LC. Figure 2 compares the separation for each of the LC systems with a fixed y-axis. Note that all of the other systems in the comparison had reduced sensitivity compared to the ACQUITY UPLC System. This was a result of the shorter pathlength of the microbore flow cells used to reduce the extra column band spread, and additional system dispersion. The other vendors' systems were not designed to be compatible with high-resolution, low-volume separations. If the y-axis is normalized, as shown in Figure 3, the effect of the increased system dispersion and the higher gradient delay is dramatically highlighted. The separation on the ACQUITY UPLC System had significantly narrower peak widths than all of the other systems. Additionally, the early eluting peaks in the chromatograms had significantly greater peak widths than the later eluting peaks on most of the other systems. This demonstrates the impact of both extra-column band spread from the injector and increased gradient delay volumes.

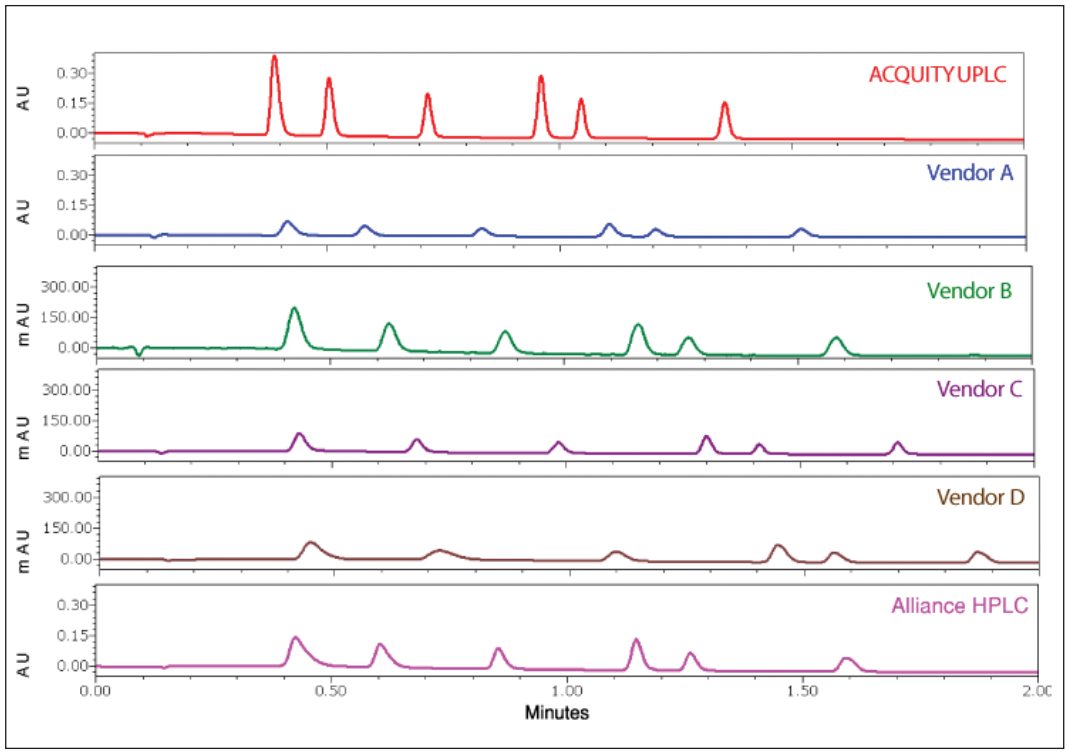

Figure 2. Comparison of the anesthetics separation of six different vendors' LC systems. The y-axis is fixed to demonstrate the impact of system dispersion and detector path length reduction of peak height.

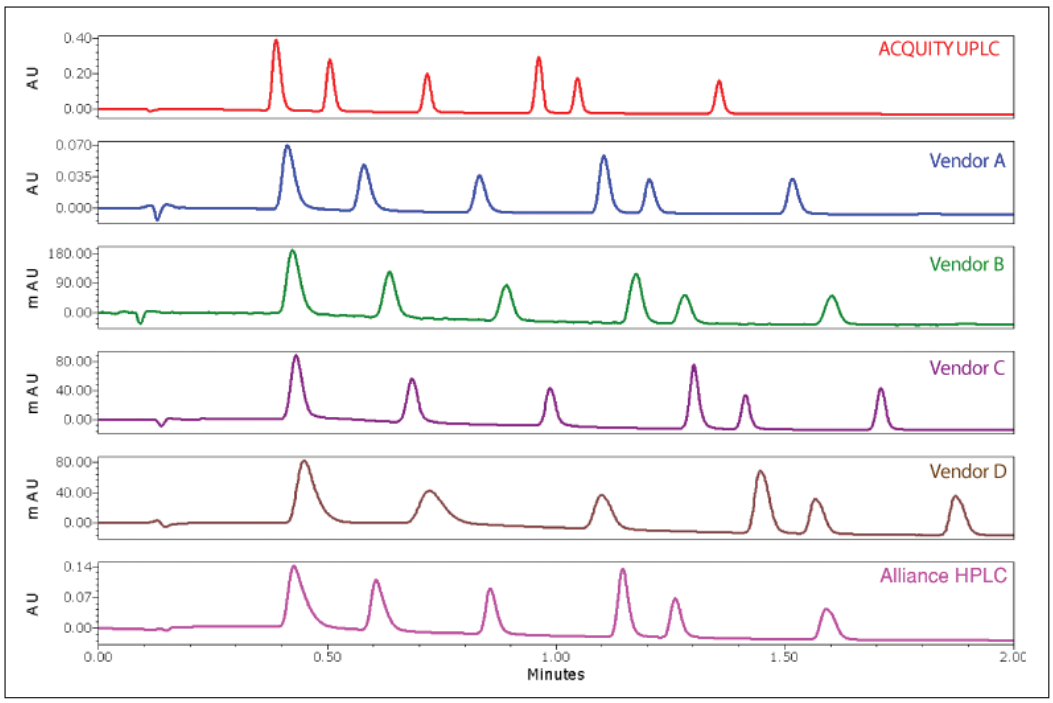

Figure 3. Comparison of the anesthetic separation on the six different LC systems. The y-axis is normalized to demonstrate the impact of the system volume and system band spread of the peak shape.

System	Peak capacity*	1/2 Height peak capacity	Peak width ratio first/last†	Elution time of last peak (s)
ACQUITY UPLC	51	115	1.02	81.4
Vendor A	33	76	1.36	91.0
Vendor B	29	64	1.08	96.4
Vendor C	35	83	1.33	102.6
Vendor D	22	48	1.45	112.3
Alliance HPLC	29	66	1.35	95.5

Table 1. Comparison of critical separation parameters impacted by system volume and band spread.
**The number of peaks that can be separated during the gradient time (2 min) is based upon peak width at 4.4%. This value is based upon the average peak width of all six peaks in the separation.*
†A ratio of 1.00 indicates the peaks widths of the first and last peaks are equivalent, and therefore the system dispersion is minimal. As the ratio increases, it indicates increasing system dispersion, which impacts the more polar components of the separation.

A summary of the critical separation and peak parameters that were assessed is shown in Table 1. The peak capacity of a separation is defined as the number of peaks that can be resolved during the gradient time. This is typically reported at 4.4% of the peak height (5 Sigma), which is indicative of resolved peaks. Some LC/MS literature will calculate peak capacity at 1/2 peak height (which does not indicate the resolving power of a LC/UV separation); therefore, this value has also been included for comparison. The peak width ratio compares the width of the most polar and least polar components in the separation. If the system dispersion and gradient delay are minimal, these values should approach 1, indicating an efficient gradient separation.

The elution time of the last peak is also an indication of the system volume as it requires the strongest part of the gradient to elute it off the column, and will define the final run time. When these values are plotted graphically, as shown in Figures 4 to 6, the performance benefits of the ACQUITY UPLC System (which was designed for high-resolution, low-volume separations) is dramatically realized. From these values, an assessment of the performance of each of the LC systems compared to the ACQUITY UPLC System can be made.

The system from Vendor A required hardware changes to the pump and detector, as well as an injection loop bypass function in the instrument method to reduce the system volume. Even with these changes, the impact of the gradient delay volume was apparent. The peak capacity was 34% lower than the ACQUITY UPLC System, resulting in significantly less resolving power. The peak width ratio of the first and last peaks indicated dispersion in the more polar components, which typically results from the initial isocratic hold imparted by the increased gradient delay and extra-column band spread from the injector or column pre-heating assembly. The increased gradient delay also caused longer elution times and therefore required a longer chromatographic run time. Additionally, Vendor A's higher system volume required longer re-equilibration times, resulting in even longer injection-to-injection cycle times.

The system from Vendor B did not require any hardware changes according to its system literature. The resulting separation had a peak capacity of 29, which was 43% lower than the ACQUITY UPLC System. Despite the significantly lower peak capacity, the peak width ratio indicated that the first and last peaks had very similar dispersion characteristics, with a ratio near 1. This would indicate that the greatest contribution to extra-column band spread in this system is post-column, likely in the flow cell and its internal connection tubing. Additionally, the elution time of the last peak indicates that the gradient delay of this system is higher than that of the ACQUITY UPLC System.

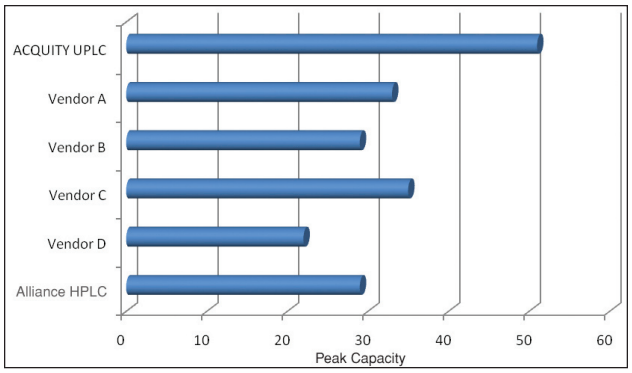

Figure 4. Comparison of peak capacity at 4.4% peak height highlights the increased resolving power of the ACQUITY UPLC System.

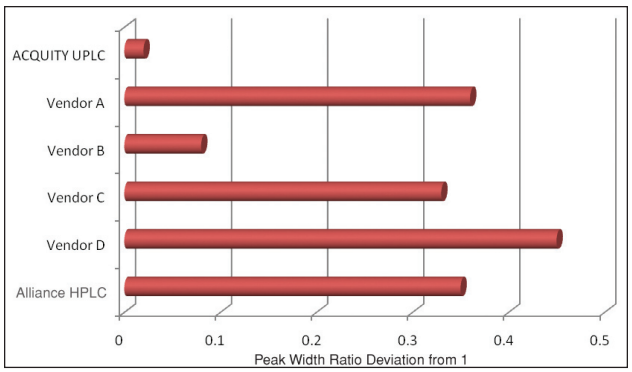

Figure 5. Comparison of the first-to-last peak width ratio deviation from 1 (ideal) demonstrates the impact of band spread on early eluting peaks.

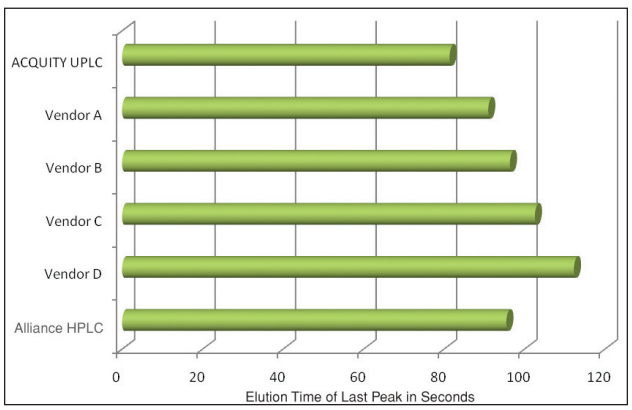

Figure 6. Comparison of the elution times of the last peak in the separation demonstrates the impact of system volume on the chromatographic run time.

The system from Vendor C required hardware changes to the pump, autosampler, and detector, as well as a bypass function in the instrument method to reduce gradient delay. Even with all these system changes, the resulting separation had a peak capacity that was 31% lower than the ACQUITY UPLC System. The peak width ratio indicated that there was dispersion in the system that resulted from either the isocratic hold imparted by the gradient delay, or from the injector/pre-heating assembly. Also, the late elution time of the last peak indicates contributions to the run time from the gradient delay, even though significant system modifications and bypass mode had been implemented.

The system from Vendor D recommended only a microbore flow cell and a low-volume tube from the injector to the column to reduce system dispersion. There were no options available for the reduction of the gradient delay volume (either hardware or software). This system had the lowest overall performance for this comparison. The peak capacity was 57% lower than the ACQUITY UPLC System. The peak width ratio was the highest of all the systems compared, and the elution time of the last peak was 38% longer than the ACQUITY UPLC System. Although this system had some components (microbore flow cell and low-volume tube) available to make it more compatible with fast microbore LC, it was definitely not intended to be used in this capacity.

As a point of comparison, the Waters Alliance® HPLC System was also included. To make the system compatible with fast microbore LC, the microbore flow cell (Part no. 205000400) was installed. The system pre-column volume setting in the instrument method was configured for 650 µL to reduce the gradient delay volume. The resulting peak capacity was in line with the values that were achieved with the other vendors' systems that were designed for fast LC and UHPLC. Additionally, the peak width ratios and the elution times achieved with the other vendors' systems were in line with those of the Alliance HPLC System, not those of the ACQUITY UPLC System, indicating that these systems truly perform within the realm of HPLC rather than that of UPLC.

CONCLUSION

For this fast LC method, the ACQUITY UPLC System delivered a separation that had the greatest peak capacity, highest sensitivity, and fastest analysis time.

This application note demonstrates the impact of LC design differences upon the overall quality of the LC separation, and the subsequent quality of the results generated by the laboratory. Careful consideration should be applied to the selection criteria of new LC systems to ensure laboratory workflow is not compromised by the perceived "need for speed." Today's ideal LC platform has an intrinsically low dispersion volume and other fluidic design considerations that allow it to reliably and accurately run conventional LC separations, fast LC separations, and sub-2-µm LC.

The 2004 introduction of the Waters ACQUITY UPLC System had these objectives in mind, and represents the best choice for laboratories that want to leverage UPLC's unique attributes to improve their workflow, and positively impact the bottom line of their businesses.

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COMPARISON OF A UPLC METHOD ACROSS MULTIPLE UHPLC SYSTEMS

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INTRODUCTION

In 2004, Waters introduced the ACQUITY UPLC® System. Since this launch, many liquid chromatography (LC) vendors have introduced modified high-performance liquid chromatography (HPLC) systems designed for ultra-high-pressure liquid chromatography (UHPLC). Although these systems may provide satisfactory performance for analytical-scale compressed chromatography (4.6-mm I.D.), they struggle significantly to provide high-resolution chromatography with sub-2-µm microbore columns (2.1-mm I.D.), which require a system designed to maximize the separation efficiency.

Typically, vendors of modified HPLC systems will claim improvements in sample throughput and a reduction in solvent consumption by migrating traditional HPLC methods to analytical-scale UHPLC methods, rather than discuss resolution. However, the transition to an analytical-scale UHPLC method yields only a small percentage of solvent savings compared to converting the method to a microbore-UPLC® method. Solvent consumption can be further reduced by nearly 5X or 80% with a 2.1-mm I.D. column compared to a 4.6-mm I.D. column of the same length.

Additionally, the improvements in the separation quality generated by a low-dispersion UPLC System provides the user with higher quality information than that possible with HPLC systems modified for UHPLC. The ACQUITY UPLC System is the world's only system that is optimized out-of-the-box to deliver high-resolution, low-volume liquid chromatography.

This application note compares the performance of multiple vendors' UHPLC systems for the separation of a series of anesthetics using an ACQUITY UPLC sub-2-µm column. It demonstrates that the performance of a modified HPLC system does not equal that of a holistically-designed UPLC System for achieving the highest separation efficiency, best sensitivity, and fastest analysis time.

EXPERIMENTAL

The method used to compare the four LC systems is as follows:

Sample:	Anesthetic mix at 50 µg/mL in water
Column:	ACQUITY UPLC BEH C_{18}, 1.7 µm 2.1 x 30 mm
Injection volume:	2 µL
Temp.:	50 °C
Flow rate:	1.0 mL/min
Mobile phase A:	10 mM ammonium bicarbonate pH 10
Mobile phase B:	Acetonitrile
Gradient:	25% to 75% B over 1 min (with re-equilibration as needed for each system)
Detection wavelength:	220 nm
Data rate/filtering:	Optimized for equivalency on each system
Needle wash:	70:15:15 acetonitrile/isopropanol/water Default wash parameters for each system
Run time:	1.5 min
Data:	All data were processed with Empower™ 2 Software

The same column, lot of mobile phase, and wash solvents were used on all the systems. Instruments were configured according to the manufacturer's recommendations for low system delay volume and, when possible, the shortest piece of 0.0025 in. I.D. tubing was used before and after the column to minimize peak dispersion. Depending upon the system, this included installing a microbore flow cell, reduced-volume mixers, reduced-volume tubing, bypassing pump components, and utilizing the bypass mode in the injector to further reduce gradient delay.

As a baseline for this comparison, Figure 1 shows the separation of the anesthetic mix on the ACQUITY UPLC System. No system modifications were necessary for the ACQUITY UPLC System since the stock configuration is optimized for high-resolution, low-dispersion UPLC analyses.

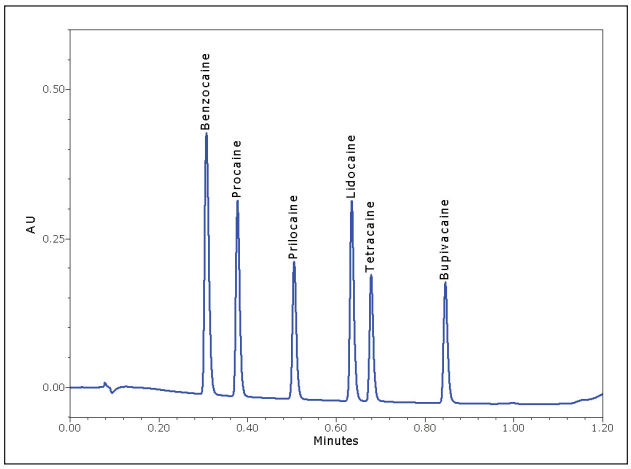

Figure 1. Separation of six anesthetics by UPLC on the ACQUITY UPLC System.

RESULTS AND DISCUSSION

For all the separation parameters assessed, the ACQUITY UPLC System, which was designed for high sensitivity and minimal band spread, easily outperformed all of the UHPLC systems.

Figure 2 compares the separation on each of the UHPLC systems with a fixed y-axis. Note that all of the other systems in the comparison had experienced reduced sensitivity compared to the ACQUITY UPLC System. This is a result of the shorter path length of the microbore flow cells used to reduce the extra-column band spread. It is evident that these systems were not designed to be compatible with high-resolution, low-volume separations, but rather modified in an attempt to compete with the ACQUITY UPLC System. If the y-axis is normalized, as shown in Figure 3, the effect of the increased system dispersion and the higher gradient delay is highlighted. The separation on the ACQUITY UPLC System has narrower peak widths than those on all of the other systems. Additionally, the early eluting peaks in the chromatograms have significantly greater peak widths than the later eluting peaks on most of the other systems, demonstrating the impact of both extra-column band spread from the injector, and the increased gradient delay volumes.

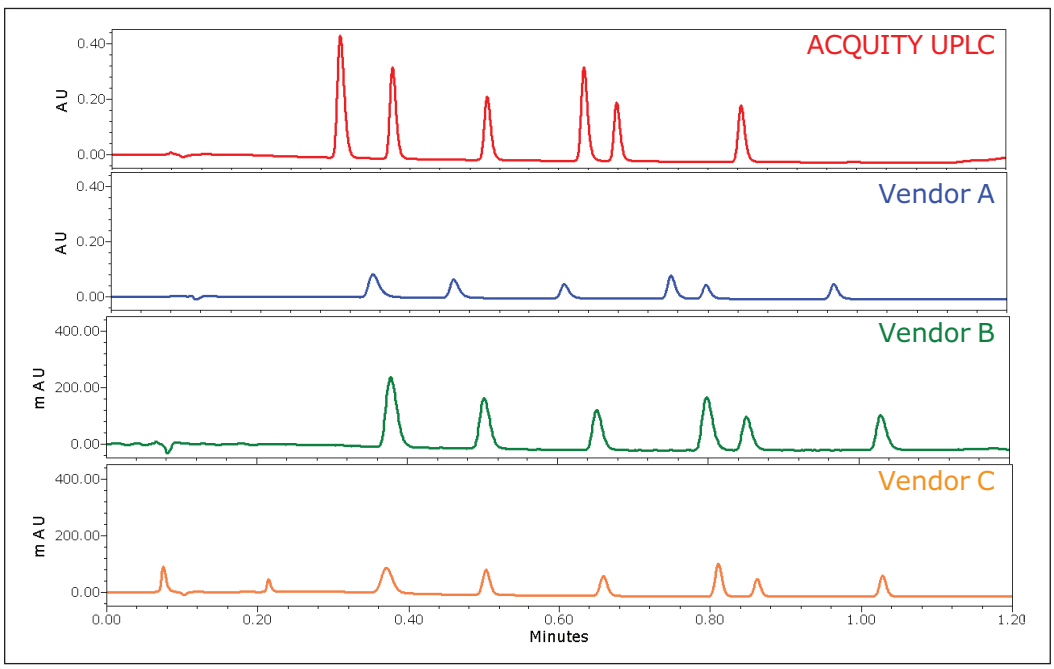

Figure 2. Comparison of separation of the anesthetic mixture using four different vendors' UHPLC Systems. The y-axis is fixed to demonstrate the impact of system dispersion and detector path length education on peak height.

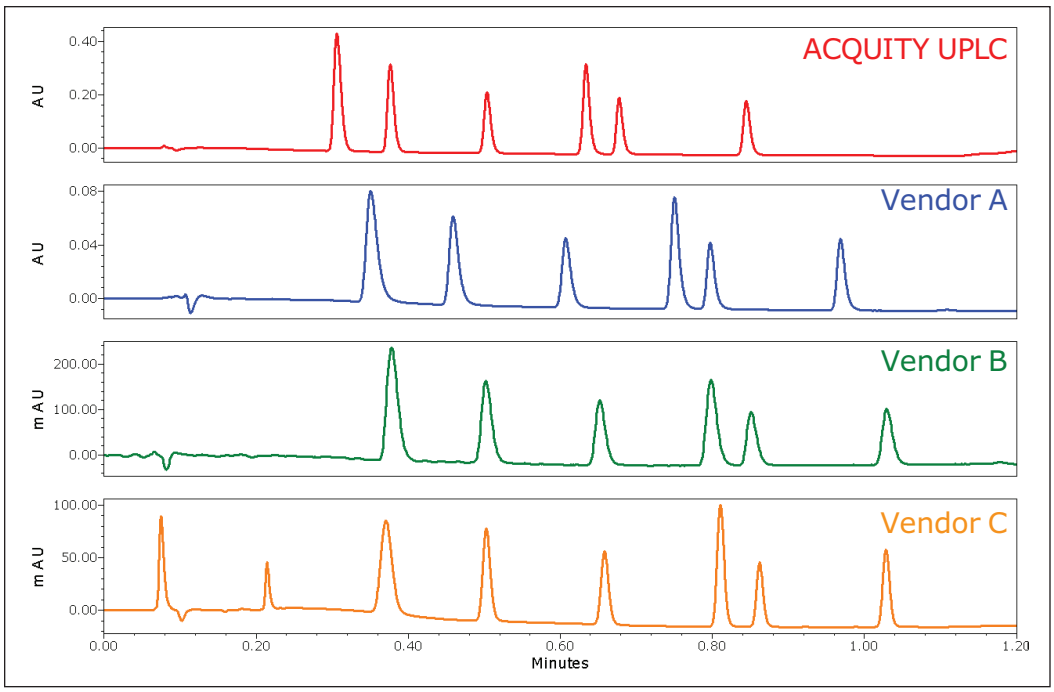

Figure 3. Comparison of the anesthetic separation on four different UHPLC systems. The y-axis is normalized to demonstrate the impact of the system volume and system band spread on the peak shape.

System capacity*	Peak capacity*	1/2 Height peak capacity	Peak width ratio first/last†	Elution time of last peak (s)
ACQUITY UPLC	46	107	1.00	50.7
Vendor A	33	81	1.38	58.2
Vendor B	31	67	1.14	62.2
Vendor C	39	85	1.62	61.7

Table 1. Comparison of critical separation parameters impacted by system volume and band spread.
**The number of peaks that can be separated during the gradient time (1 minute) is based upon peak width at 4.4%. This value based upon the average peak width of all six peaks in the separation.*
†A ratio of 1.00 indicates that the peak widths of the first and last peaks are equivalent and therefore, system dispersion is minimal. As the ratio increases, it indicates increasing system dispersion, which impacts the more polar components of the separation.

A summary of the critical separation and peak parameters that were assessed is shown in Table 1. The peak capacity of a separation is defined as the number of peaks that can be resolved during the gradient time. This is typically reported at 4.4% of the peak height (5 Sigma), which is indicative of resolved peaks. Some LC/MS literature will calculate peak capacity at ½ peak height. While it does not practically indicate the resolving power of a LC/UV separation, it has also been included for comparison. The peak width ratio compares the width of the most polar and least polar components in the separation. If the system dispersion and gradient delays are minimal, these values should approach 1, indicating an efficient gradient separation. The elution time of the last peak is also an indication of the system volume, as it requires the strongest part of the gradient to elute it off the column, and will define the final run time. When these values are plotted graphically (Figures 4 to 6), the performance benefits of the ACQUITY UPLC System are obvious. From these

values, an assessment of the performance of each of the UHPLC systems compared to the ACQUITY UPLC System can be made.

The UHPLC system from Vendor A required hardware changes to the pump and detector, as well as an injection loop bypass function in the instrument method in order to reduce system volume. Even with these significant changes, the impact of high system volume on the gradient delay is apparent. The peak capacity for this separation using Vendor A's UHPLC system was 28% lower than the ACQUITY UPLC System. The lower peak capacity results in significantly reduced chromatographic resolution, which impacts the quality of information available to the user. Another indication that the system dispersion was too high for quality chromatographic results is the peak width ratio of the first and last peaks. This value indicates there is dispersion in the more polar components that typically results from the initial isocratic hold caused by the increased gradient delay, and/or extra-column band spread from the injector or column pre-heating assembly. The larger system volume and increased gradient delay resulted in longer elution times for each of the components compared to the ACQUITY UPLC System, and therefore required longer chromatographic run times. Additionally, a higher system volume will require longer system re-equilibration times resulting in even longer injection-to-injection cycle times.

The UHPLC system from Vendor B did not require any hardware changes according to the system literature. The resulting separation had a peak capacity of 31, which was 33% lower than the ACQUITY UPLC System. The peak width ratio for the system from Vendor B appears to be the best of the three UHPLC vendors. However, when combined with the lowest peak capacity, this indicates that the first and last peak have very similar and high dispersion characteristics. This implies that the greatest contribution to extra-column band spread in this system is post-column, likely in the flow cell and its internal connection tubing. The elution time of the last peak indicates that the gradient delay on this UHPLC system was longer than the ACQUITY UPLC System and therefore the system volume is significantly higher.

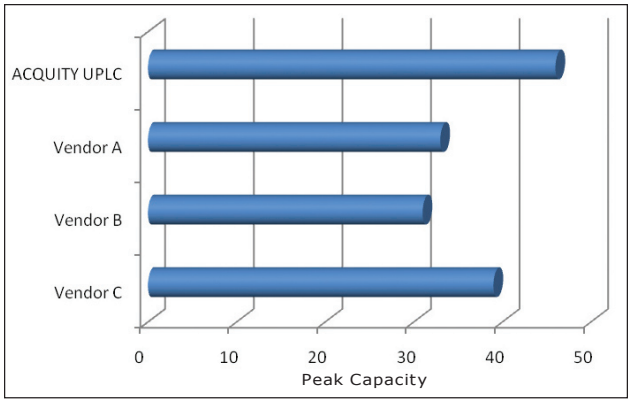

Figure 4. Comparison of peak capacity at 4.4% peak height highlights the increased resolving power of the ACQUITY UPLC System.

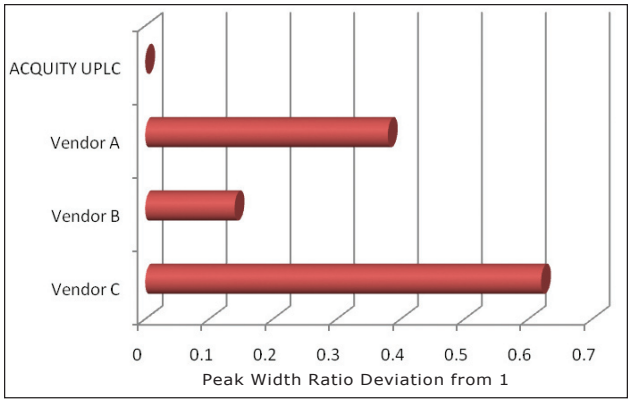

Figure 5. Comparison of the first-to-last peak width ratio deviation from 1 (ideal) demonstrates the impact of band spread on early eluting peaks.

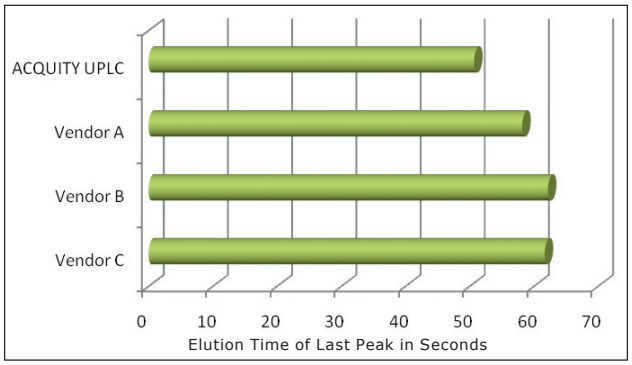

Figure 6. Comparison of the elution times of the last peak in the separation demonstrates the impact of system volume on the chromatographic run time.

The system from Vendor C required hardware changes to the pump, autosampler, and detector, as well as the bypass function in the instrument method to reduce gradient delay. Even with all these system changes, the resulting separation had a peak capacity that was 15% lower than that observed on the ACQUITY UPLC System. The first-to-last peak width ratio indicated that there was significant dispersion in the system that was the result of either the isocratic hold caused by the gradient delay, or from the injector/pre-heater assembly. The late elution time of the last peak indicates contributions to the run time from the gradient delay even though significant system modifications and the injection bypass mode had been implemented to reduce the system volume. The injector bypass function added a system peak at 0.22 min, which could easily be mistaken as an unknown peak in the sample.

CONCLUSION

This application note demonstrates the importance of a holistically-designed system for UPLC analysis. Although a sub-2-µm particle column provides high-resolution separations, a low-dispersion system is required to maximize the benefits of its resolving power. The design differences of LC systems can significantly impact resolution, sensitivity, sample throughput, and can ultimately impact the quality of the results generated in the laboratory.

For this UHPLC separation, the ACQUITY UPLC System easily outperformed all of the UHPLC vendors' systems with the greatest peak capacity, highest sensitivity, and fastest analysis time.

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July 2009 720003166EN LB-LCGC

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ACQUITY UPLC SYSTEM-TO-SYSTEM REPRODUCIBILITY FOR PEPTIDE MAPPING

Azita Kaffashan and Thomas E. Wheat
Waters Corporation, Milford, MA, U.S.

INTRODUCTION

Recombinant proteins and monoclonal antibodies are developed for therapeutic purposes. Peptide mapping is used to confirm the primary structure of a protein, identify post-translational modification (PTMs), and analyze potential impurities. Any difference in structure of a protein should be reflected in a change in retention time for the peptide containing the modification. The relative amounts of the peptide with and without a particular modification are used to measure the fraction of the protein in the particular sample that carries that modification. Changes in area proportions correspond to the fraction of the protein molecules in the sample having a particular modification.

UltraPerformance LC® (UPLC®) shows greater resolution and higher sensitivity for peptide mapping as compared to HPLC. To achieve maximum resolution, all elements of the analysis, including the instrument, column, solvents, and sample must be optimized to work together as a complete system. Using the UPLC® Peptide Analysis Solution, ACQUITY UPLC has been shown to give consistent chromatographic separations and reproducible quantitation for peptide mapping.[1]

When a completely satisfactory peptide map has been developed, it will be used on multiple systems within a department, as well as transferred to another department, laboratory, or CRO. Reproducibility of retention time and relative area must be consistent from one ACQUITY UPLC System to another.

In this application note, we demonstrate reproducibility of the peptide map of a protein digest run on three identical ACQUITY UPLC Systems. Retention time, area, and relative area reproducibility of selected peaks were evaluated.

EXPERIMENTAL

Experimental design

Three identical ACQUITY UPLC Systems (ACQUITY UPLC 1, 2, and 3) were configured according to the instructions found in the UPLC Peptide Analysis Application Solution.[2] Briefly, a core ACQUITY UPLC System, consisting of an ACQUITY UPLC Binary Solvent Manger, ACQUITY UPLC Sample Manager with Column Heater Module, and ACQUITY UPLC TUV Detector, was modified to be compatible with peptide analysis. A high-sensitivity peptide mixer was installed on the pump. The autosampler was equipped with a 20-µL loop, and a 15-µL PEEK/Sil ACQUITY UPLC peptide needle. One Waters® Peptide Separation Technology Column was used on all three systems. A shallow gradient of 0.5%/column volume was selected as typical of peptide mapping gradients. ACQUITY UPLC Systems 1, 2, and 3 were run on days 1, 2, and 3 respectively. Six consecutive runs were completed on each system before moving the column to the next system. The mobile phase was prepared fresh on day 1 and divided among the instruments. Waters MassPREP™ Enolase Digestion Standard was reconstituted with sample buffer to 10 pmol/µL on day 1. Aliquots of 100 µL were frozen in a -80 °C freezer. On day 1, a fresh, unfrozen aliquot was loaded on ACQUITY UPLC System 1. A frozen aliquot was defrosted and loaded on ACQUITY UPLC Systems 2 and 3 just before the start of the injections. Data were processed using Empower™ 2 Software. The peaks in the chromatograms were integrated using the ApexTrack™ integration alogorithm. The first injection of each day was a system blank run.

Materials and methods

Samples:	Waters MassPREP Enolase Digestion Standard (3 vials of 1 nmol tryptic digest of protein, 8 pmol/ µL)
Sample buffer:	0.2% TFA in 95:5 water/acetonitrile (100 µL per vial of digestion standard)
LC system:	Waters ACQUITY UPLC, configured for peptide analysis (Details in experimental design section)

Column:	Waters Peptide Separation Technology ACQUITY UPLC BEH 300 C_{18}, 1.7 µm 2.1 X 100 mm
Flow rate:	200 µL/min
Mobile phase A:	0.020% TFA in water
Mobile phase B:	0.018% TFA in acetonitrile

Gradient:	Time (min)	%A	%B	Curve
	0.0	98	2	NA
	5.0	98	2	6
	206.0	40	60	6
	206.1	10	90	6
	208.1	10	90	6
	208.2	98	2	6
	234.2	98	2	6

Column temp:	40 °C
Injection volume:	8 µL of 10 pmol/µL of reconstituted MassPREP Enolase Digestion Standard
Mode:	Partial Loop
Weak wash:	600 µL of 95:5 H_2O/ACN 0.2% TFA
Strong wash:	200 µL of 20:80 Mobile phase A/mobile phase B
Sample temp:	4 °C
Detection:	Wavelength: 214 nm Sampling rate: 10 pts/sec Filter time constant: Normal

RESULTS AND DISCUSSION

The peptide map of the MassPREP Enolase Digestion Standard is shown in Figure 1. Empower 2 Software using ApexTrack integration was used to integrate all chromatograms. The software-generated integrated chromatogram showed over 300 peaks, of which three were compared in this study. Early-eluting (peak A), middle-eluting (peak B), and later-eluting (peak C) were selected as representative peaks in the chromatogram.

Figure 2 shows the overlay of five consecutive runs of the MassPREP Enolase Digestion Standard for the ACQUITY UPLC System 1 run on day 1. There is no observable shift in retention time that compromises the identification of a peak. For all peaks, retention time reproducibility within a single system is better than 0.3% RSD.

Inter-system reproducibility is shown by the overlay of the chromatograms of the MassPREP Enolase Digestion Standard peptide map from injection 3 on ACQUITY UPLC Systems 1, 2, and 3 run over three days in Figure 3. The peaks detected from the three systems were identified and counted without any manual manipulation. The same number of peaks was found in all chromatograms. Additionally, peaks A, B, and C were correctly identified in all chromatograms.

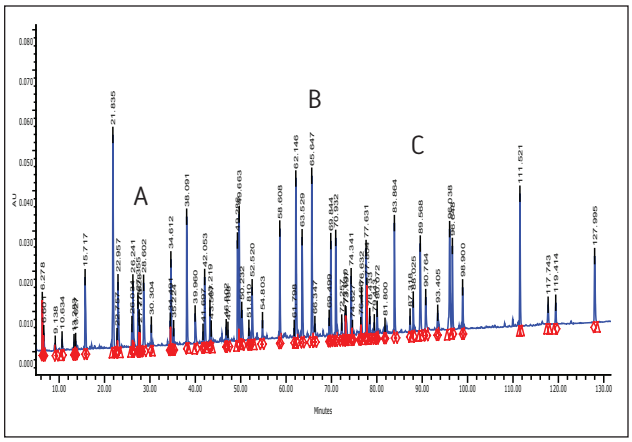

Figure 1. UV chromatogram of the peptide map of MassPREP Enolase Digestion Standard.

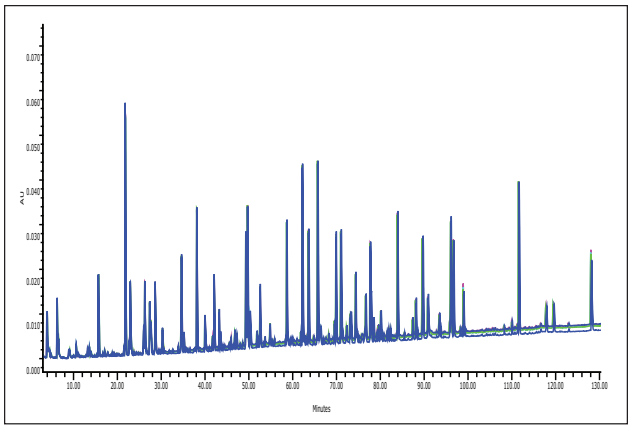

Figure 2. Overlay of five consecutive runs of the MassPREP Enolase Digestion Standard peptide map on ACQUITY UPLC 1.

Table 1 compares the average and standard deviation for retention time for each system, and all of the runs for the three selected representative peaks. As expected, there was more retention time variability in the inter-system runs, compared to runs within a single system. The standard deviation for all of the runs across the three marker peaks was better than 0.20 minutes. As with the runs within a system, there was no shift in retention time for the inter-system runs to compromise the identification of a peak.

The expanded view of a pair of closely resolved peptides that includes peak A is shown in Figure 4. The shape of the peaks and the valley between them is sensitive to all aspects of the separation including flow rate, gradient, and temperature. The consistency of this separation is a measure of the similarity among the three ACQUITY UPLC Systems.

Many factors contribute to judging quantitative behavior. Different peptides have distinctive properties resulting in more or less variability in area. Optimization of the diluents and injection modes will influence the reproducibility of the peak areas. Table 2 compares the average and percent relative standard deviation of the peak area for the three marker peaks using all of the runs from each of the three ACQUITY UPLC Systems. The peak area %RSD for the three peaks within a system is better than 3.3%. The peak area %RSD for all runs is between 5.0% and 6.3%.

For quantitative characterization of a protein sample, the amount of the modified structure is often reported as a percentage of the native structure. Area ratios are a useful measure of the reliability of quantification across three systems. The area ratios shown in Table 3 for the three marker peaks are essentially identical.

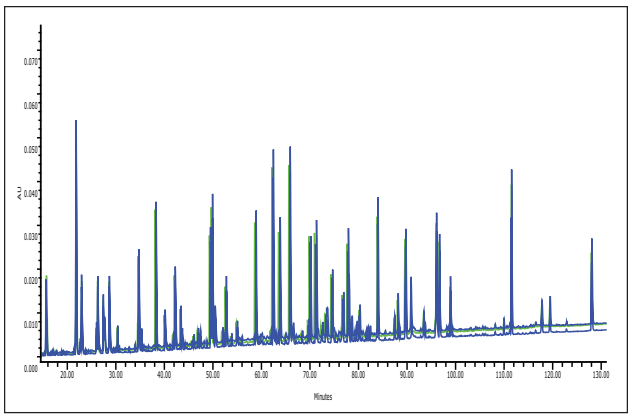

Figure 3. Overlay of injection 3 of the peptide map of the MassPREP Enolase Digestion Standard on ACQUITY UPLC Systems 1, 2, and 3.

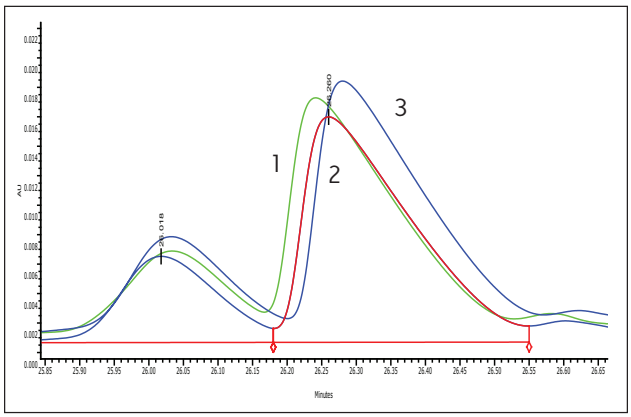

Figure 4. Overlay of injection 3 of peak A on ACQUITY UPLC Systems 1, 2, and 3.

Retention time	Mean	Peak A Standard deviation	Mean	Peak B Standard deviation	Mean	Peak C Standard deviation
ACQUITY UPLC System 1	26.242	0.007	65.686	0.048	87.377	0.068
ACQUITY UPLC System 2	26.284	0.005	66.007	0.120	87.522	0.077
ACQUITY UPLC System 3	26.266	0.006	65.867	0.026	87.474	0.045
All	26.264	0.019	65.900	0.185	87.458	0.088

Table 1. Retention time average and standard deviation of peaks A, B, and C for ACQUITY UPLC 1, 2, 3 and all runs.

Area	Peak A		Peak B		Peak C	
	Mean	%RSD	Mean	%RSD	Mean	%RSD
ACQUITY UPLC System 1	172802	0.799	430452	0.670	40855	2.140
ACQUITY UPLC System 2	187035	0.999	467935	1.284	44650	2.186
ACQUITY UPLC System 3	165696	0.384	415678	0.752	39062	3.267
All	175178	5.294	438021	5.278	41522	6.292

Table 2. Area average and %RSD of peaks A, B, and C for ACQUITY UPLC Systems 1, 2, 3, and all runs.

	ACQUITY UPLC System 1	ACQUITY UPLC System 2	ACQUITY UPLC System 3
Ratio			
A/B	0.401	0.400	0.399
C/A	0.236	0.239	0.236
C/B	0.095	0.095	0.094

Table 3. Area ratio of peaks A, B, and C for ACQUITY UPLC Systems 1, 2, and 3.

CONCLUSION

Peptide mapping is used to confirm the primary structure of a protein, identify post-translational modification (PTMs), and analyze potential impurities. ACQUITY UPLC peptide mapping provides the high resolution required by these applications. A peptide mapping method can be developed and consistently observed on a single system. With careful attention to detail, the same separation can be transferred to additional ACQUITY UPLC Systems. The quantitative reliability within such a transfer is more than satisfactory for relative quantitation. The total system solution including the instrument, column, and solvents is essential to achieving these results. Protein characterization laboratories can develop fully-defined peptide maps on the ACQUITY UPLC System. The peptide mapping method can be transferred to another department, laboratory, or CRO using the same instrument and column chemistry.

References

1. Wheat TE, Lu Z, Gillece-Castro B, Mazzeo JR. Quantitative Aspects of UPLC Peptide Mapping, Waters Application Note no. 720001839EN.
2. UPLC Peptide Analysis Application Solution Kit no. 715001381.

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METHOD FOR HEART-CUT ANALYSIS USING NANOACQUITY UPLC WITH 2D TECHNOLOGY FOR PROTEOMIC SAMPLES

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INTRODUCTION

A biomarker is measured as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to therapeutic intervention. Many times, a putative biomarker is a protein or peptide that is expressed at a relatively low level compared to the surrounding proteome. The constitutive or housekeeping proteins are present in concentrations that are orders of magnitude above the protein of interest, which makes identification and quantitation difficult. In order to validate a candidate biomarker, many samples need to be analyzed to prove that the same analytes are reproducibly identified and are changing in a statistically significant manner due to a perturbation.

Two-dimensional (2D) chromatography is often used to separate peptides from proteomic samples in a biomarker discovery workflow. 2D chromatography of tryptic peptides has traditionally been performed using strong cation exchange (SCX) followed by reversed-phase (RP) chromatography, due to the orthogonal nature of the separation mechanisms of these two techniques. The major disadvantage of this approach is that peptides are often split across first dimension SCX fractions due to the relatively low resolution of peptides on SCX material.

A highly reproducible method for performing online 2D chromatography with mass spectrometry has been developed where peptides are separated by RP chromatography at high pH in the first dimension, followed by an orthogonal separation at low pH in the second dimension. An online dilution of the effluent was performed after the first dimension to ensure no peptides were lost during trapping prior to the second dimension. For targeted biomarker validation, running an entire 2D experiment would be time consuming given the limited number of target molecules that might need to be monitored and the number samples in a typical validation experiment. A preferred approach is to elute the targeted peptides in one fraction in a heart-cut manner. This application note will illustrate the application of online high/low pH RP/RP chromatography for heart-cut analysis.

EXPERIMENTAL

LC/MS conditions

LC/MS system:	nanoACQUITY UPLC® 2D/ SYNAPT™ HDMS™
First dimension (Figure 1A):	
Column:	XBridge™ 300 µm x 5 cm C_{18} 5.0 µm
Gradient formation:	Discontinuous step gradient at 2 µL/min
Eluent A:	20 mM ammonium formate pH 10.0
Eluent B:	Acetonitrile
Online dilution flow rate:	20 µL/min aqueous
Second dimension (Figure 1B):	
Column:	75 µm x 15 cm BEH C_{18} 1.7 µm
Trapping column:	Symmetry® 180 µm x 2 cm C_{18} 5.0 µm
Gradient:	5% to 40% B for 90 min at 300 nL/min
Eluent A:	0.1% formic acid in water
Eluent B:	0.1% formic acid in acetonitrile

Sample preparation and loading: Waters MassPREP™ *E. coli* digestion standard (2.4 µg) with either MassPREP Protein Digestion Standard Mix 1 or Mix 2 was analyzed. In each case, three replicate injections were performed.

Online dilution with RP/RP: To maximize sample recovery on the second-dimension trap column from the organic-containing fractions, an aqueous flow was delivered with the second dimension pump, and mixed with the eluted fraction prior to trapping, as shown in Figure 1B.

Heart-cut method: Peptides from unwanted fractions were washed away by loading the sample at an ACN concentration just below the fraction of interest. During dilution, the second dimension pump delivered a high ACN concentration to prevent peptides sticking onto the trap column. To retain the desired fraction, aqueous conditions were utilized on the second dimension dilution pump.

Data acquisition and processing: LC-MS^E data from the individual chromatograms were processed separately and subsequently merged into one file prior to database searching with ProteinLynx Global Server™ Software (PLGS 2.4 with Identity^E Informatics). An *E. coli* database with an equal number of random sequences concatenated onto it was used for the searches to limit the false positive rate to less than 4%.

RESULTS AND DISCUSSION

2D separations

The 2D separation of an *E. coli* tryptic digest containing Mix 1 is shown in Figure 2. Organic concentrations for each step were selected to ensure nearly equivalent peptide load and MS intensity for each second-dimension run. Due to the nature of RP gradients, the majority of peptides were only identified in one fraction (86.2% for Mix 1 in *E. coli*, and 87.3% for Mix 2 in *E. coli*). Figure 3 depicts the number of *E. coli* proteins identified in two of three replicate 2D experiments. The data point in red is 50 fmol ADH, to show the level of the spiked-in proteins relative to the background. In three replicate analyses, the average relative standard deviation of the protein fmol amount was 15%.

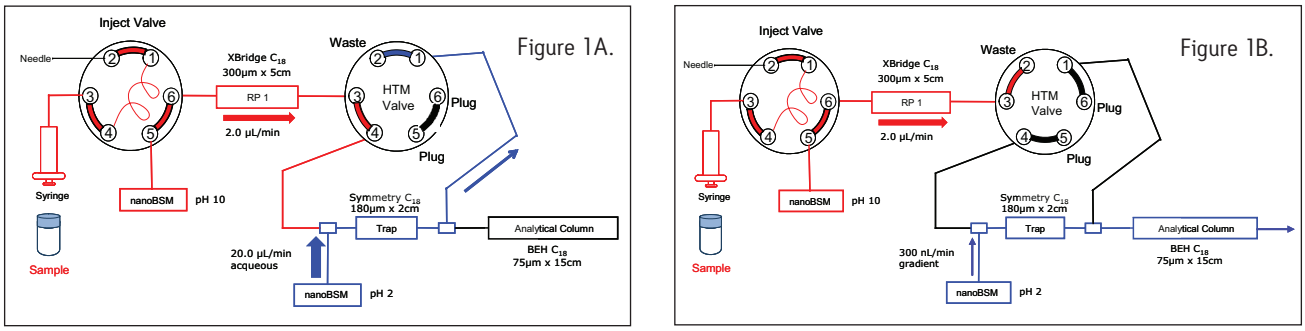

Figure 1. Fluidic layout for the 2D-nanoACQUITY UPLC System for high/low pH RP/RP analysis with online dilution. Two gradient pumps (nanoBSM) were utilized for (A) sample injection and fractionation, as well as (B) analytical gradient delivery.

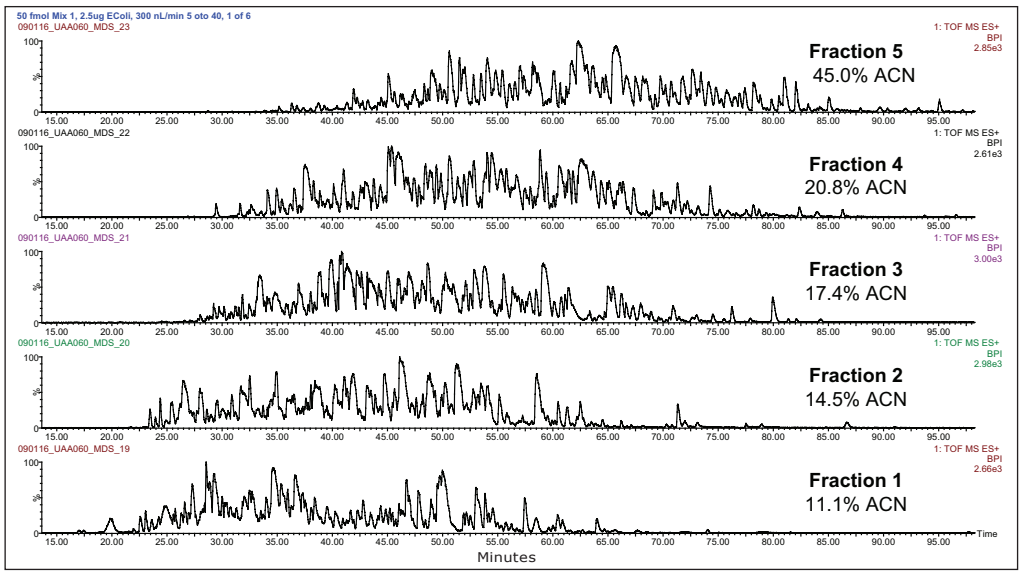

Figure 2. Chromatograms for five fraction 2D-analysis of Mix 1 in E. coli.

Heart-cut separations

Figure 4 shows the replicate analyses of the heart-cut analysis of the *E. coli* sample containing Mix 1, where peptides were eluted with 20.8% ACN from the first-dimension column (the equivalent of fraction 4). The elution profile of the second-dimension is the same as in the full 2D-analysis, with peptides eluting from 25 to 85 minutes.

Qualitative reproducibility

The 2D method was run in triplicate on both Mix 1 and 2 spiked into *E. coli*. It was found that 607 and 593 proteins (87% of the total number of identified proteins) replicated in two of the three analyses of each of the samples, respectively. The comparison of the identified peptides from the fourth fraction of the 2D-experiment with the heart-cut experiment is shown in Figure 5. In order to ensure that the peptides of interest are found in the heart-cut fractions, the acetonitrile steps can be widened slightly (0.5% on each side of the step) without significantly altering the separation efficiency.

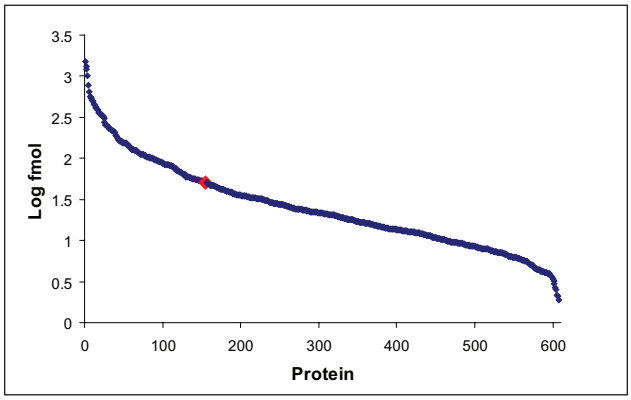

Figure 3. Absolute amount[2] of proteins detected from a 2D 5-fraction analysis of Mix 1 spiked in E. coli.

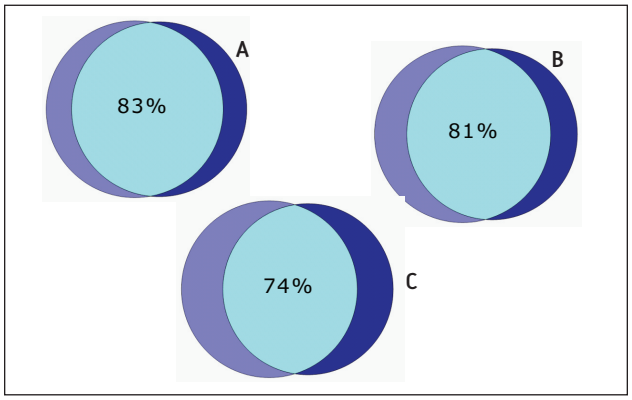

Figure 5. Venn diagrams showing (A) the overlap of highly confident peptides identified in fraction 4 from replicate 2D experiments, (B) the overlap of peptides identified from replicate heart-cuts, and (C) the overlap of peptides identified from the two different methods.

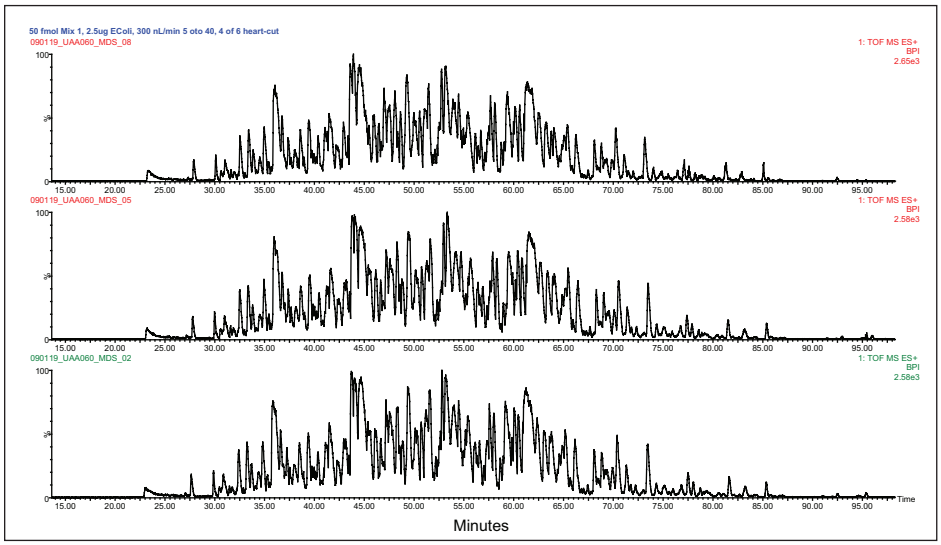

Figure 4. Replicate heart-cut analyses of fraction 4 of Mix 1 in E. coli.

Quantitative reproducibility

Peak areas of peptides from digested protein standards were measured to test the ability of the system to perform label-free quantitation, especially with a heart-cut analysis of only one fraction. Figures 6 and 7 show the results from this analysis. The average measured protein ratios were 7.30, 0.52, 1.00, and 2.20 for the 2D analysis; and 6.70, 0.53, 1.00, and 1.90 for the heart-cut analysis for BSA, glycogen phosphorylase (Phos B), alcohol dehydrogenase (ADH), and enolase, respectively. The measured ratios were within 6.3% and 7.7% of the expected theoretical values on average for the 2D and heart-cut methods. The peak areas were very consistent between the two methods with more peptides and greater intensity for common peptides identified in the 2D-method, as expected.

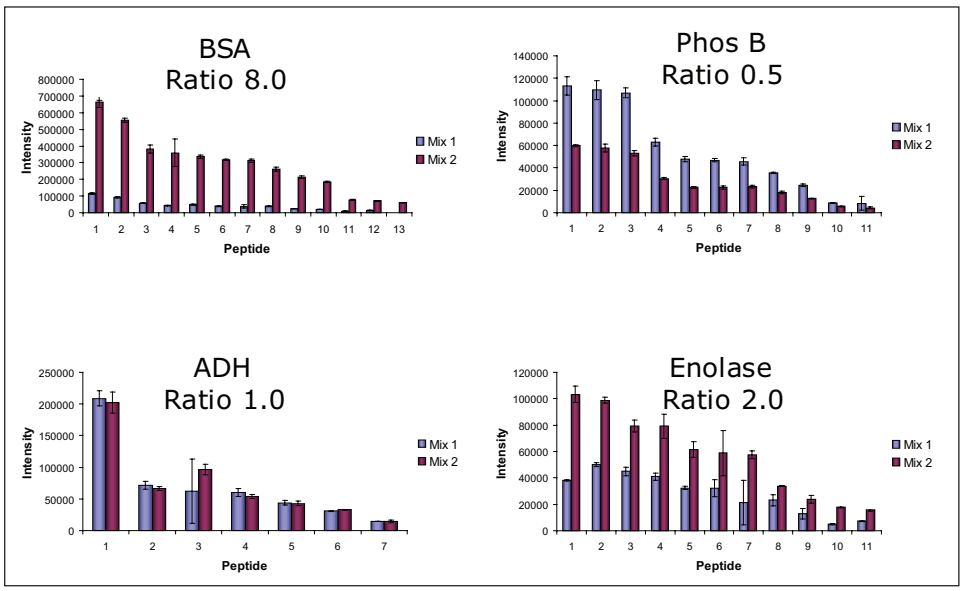

Figure 6. Intensity of peptides to the four standards in Mixes 1 and 2 found in the 5 fraction 2D-LC method. The theoretical protein ratios are shown.

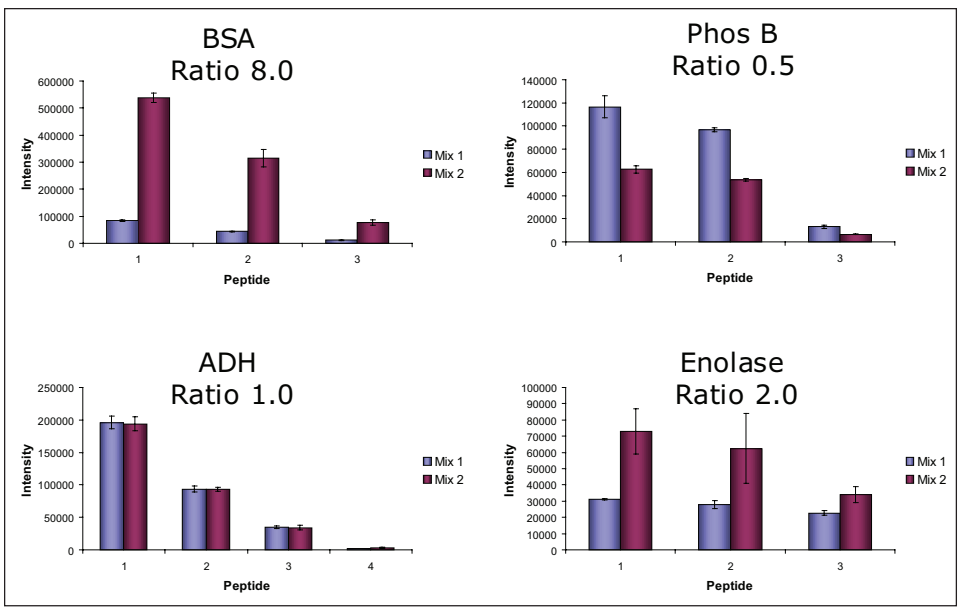

Figure 7. Intensity of peptides to the four protein standards in Mixes 1 and 2 found in the fraction 4 from the first dimension using the heart-cut method. The theoretical protein ratios are shown.

CONCLUSION

A method for heart-cut analysis has been implemented on the nanoACQUITY UPLC System and can yield the same qualitative and quantitative information that is obtained in entire 2D analyses, in a fraction of the time. The 2D experiment shown here (two samples with five fractions run in triplicate) took 60 hours of instrument time, while the heart-cut equivalent took 15 hours to complete. Since the majority of peptides in 2D RP/RP analyses were only found in one fraction and they were reproducibly found in the same fraction in replicate analyses, this technique is well-suited to subsequent heart-cut analyses for targeted proteomics and biomarker verification studies. The results from the analysis of standard proteins spiked into *E. coli* show that label-free relative quantitation works well with both methods.

References

1. M. Gilar, *et.al.*, J. Sep. Sci. 2005, 28, 1694-1703.
2. J. Silva, *et.al.*, Mol. Cell Proteomics, 2006, 144-156.

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DETERMINATION AND CONFIRMATION OF PRIORITY PESTICIDE RESIDUES IN BABY FOOD

James Morphet and Peter Hancock
Waters Corporation, Manchester, UK

AIM

To utilize the power of the Waters® UltraPerformance Liquid Chromatography (UPLC®) System combined with the fast MS acquisition rates of the Xevo™ TQ Mass Spectrometer for rapid determination and confirmation of pesticide residues in baby food.

INTRODUCTION

As the population of the world grows, it becomes increasingly important to produce enough food to satisfy the needs of its inhabitants. This escalation in consumer demand has led farmers to use increasing amounts of pesticides to improve their yields and make their operations more cost-effective. This growth in the use of pesticides, coupled with poor agricultural practices and illegal use, can pose significant risks to human health through the presence of pesticide and metabolite residues in food products.

Most countries have strict regulations that govern pesticides. Legislation imposes Maximum Residue Limits[1] (MRLs) for pesticide residues in food products requiring analytical techniques that are sensitive, selective, and robust. The EU baby food directive, 2003/13/EC[2] covers a list of both GC amenable and LC amenable pesticides. These prohibited pesticides have a maximum level in baby food that should not exceed 0.003 mg/kg, or not greater than a limit between 0.004-0.008 mg/kg. This is considered to be among the strictest legislation in the world.

The need to meet mandated detection limits, develop generic sample preparation techniques for complex matrices, and the desire to increase sample throughput are the main challenges that face food safety testing laboratories today. The use of a single multi-residue method per instrument dramatically improves return on investment in a laboratory by removing the need to change method parameters. This is often the case in labs that analyze a wide variety of commodities with various lists of legislated pesticides.

The following application note describes a solution for the rapid analysis of pesticides in fruit- and meat-based baby food extracts that is able to exceed both current European and worldwide legislation.

EXPERIMENTAL

A DisQuE™ (QuEChERS style dispersive solid-phase extraction) extraction was utilized for this multi-residue method as described below:

Extraction procedure[3]

1. Add 15 g of homogenized baby food to the 50 mL DisQuE extraction tube. Add 15 mL of 1% acetic acid in acetonitrile.
2. Shake vigorously for 1 minute and centrifuge > 1500 rcf for 1 minute.
3. Transfer 1 mL of the acetonitrile extract into the 2 mL DisQuE cleanup tube.
4. Shake for 30 seconds and centrifuge >1500 rcf for 1 minute.
5. Transfer 100 µL of final extract into an autosampler vial. Dilute with 900 µL water, mix, and inject.

LC conditions

LC system:	ACQUITY UPLC® System	
Column:	ACQUITY UPLC BEH C_{18} 2.1 x 50 mm, 1.7 µm	
Column temp.:	40 °C	
Sample temp.:	4 °C	
Flow rate:	0.7 mL/min	
Mobile phase A:	Water + 0.1% formic acid	
Mobile phase B:	Methanol + 0.1% formic acid	
Gradient:	0.00 min	99% A
	5.00 min	1% A
	6.00 min	1% A
	6.10 min	99% A
	8.00 min	99% A
Weak needle wash:	Water + 0.1% formic acid	
Strong needle wash:	Methanol + 0.1% formic acid	
Total run time:	8 min	
Injection volume:	50 µL, full loop injection	

MS conditions

MS system:	Xevo TQ MS
Ionization mode:	ESI +
Capillary voltage:	0.6 kV
Desolvation gas:	Nitrogen, 1000 L/Hr, 400 °C
Cone gas:	Nitrogen, 25 L/Hr
Source temp.:	120 °C
Acquisition:	Multiple Reaction Monitoring (MRM)
Collision gas:	Argon at 3.5 x 10^{-3} mBar

Refer to Appendix 1 at the end of this document for further MS parameters.

Quanpedia: Database and method creation tool

Method creation has been streamlined with the use of Quanpedia,™ a searchable database for quantitative LC/MS method information that can be updated with user information.

Each entry in Quanpedia is populated with information that associates the compound name with details of optimal SIR/MRM acquisition methods, acceptable confirmatory ion ratios, appropriate LC methods, and expected peak retention times. For fast-paced, multi-user environments that require quality results the first time, Quanpedia offers a simple and convenient way to rapidly create complete LC/MS data and acquisition methods.

Acquisition and processing methods

These data were acquired using Waters MassLynx™ Software v. 4.1. Incorporated into MassLynx, the IntelliStart™ Software automates optimization of MS parameters for the sample and also monitors the health of the MS system, which reduces the time for operator-intensive troubleshooting and upkeep.

Figure 1. Quanpedia is a simple and easy-to-use method creation tool and MS database.

RESULTS AND DISCUSSION

The analysis of the priority pesticide residues in baby food was achieved by combining the ACQUITY UPLC System with the Xevo TQ MS System – UltraPerformance LC with tandem quadrupole mass spectrometry (UPLC/MS/MS) operated in MRM mode. This tandem quadrupole mass spectrometer offers a highly specific and selective detection technique that has become the technique of choice within the laboratory.[4]

The selectivity given using a tandem quadrupole mass spectrometer (Xevo TQ MS System) shows an advantage over a single quadrupole instrument as it allows co-eluting compounds to be identified and quantified with confidence. Figure 2 shows fensulfothion sulfone and terbufos sulfone that co-elute at 3.32 minutes. All dwell times were optimized to give approximately 12 data points across each peak.

These data were processed using TargetLynx™ Application Manager. This quantification package from MassLynx Software enables automated data processing and reporting for quantitative data, which incorporate a range of confirmatory checks that identify samples that fall outside user-specified or regulatory thresholds.

QCMonitor: Automating your quality control

The QCMonitor™ is an automated tool that provides real-time quantitative data quality monitoring to determine whether injections meet tolerances specified by the user. QCMonitor will automatically decide if subsequent samples should be injected, or if more detailed checks are required to ensure the best use of valuable laboratory resources. Injections that fail to meet the acceptance criteria set for calibration curves, QC samples, and blanks can be re-injected or, in worst case scenarios, the batch can

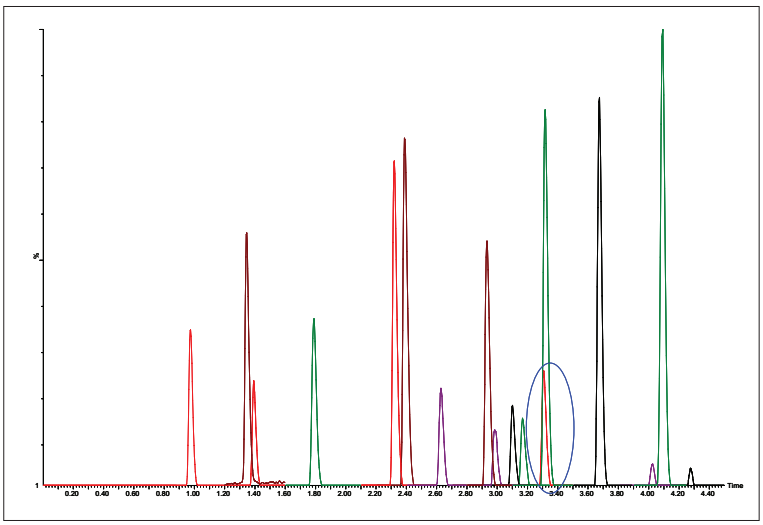

Figure 2. Chromatogram showing all 17 pesticide residues in one injection at 1 ng/mL in water.

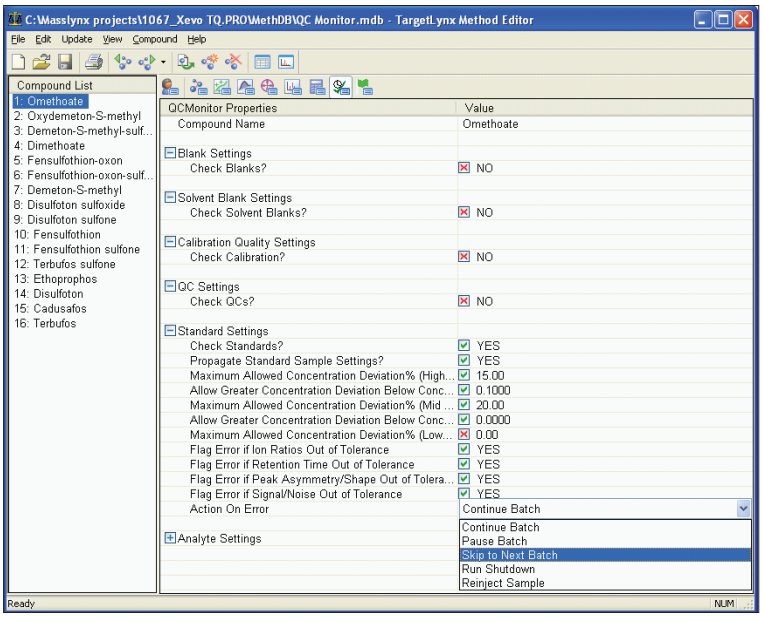

Figure 3. The features of the TargetLynx Software Method Editor detail the parameters that can be modified within QCMonitor. Highlighted are the settings for the calibration standards, the limits that must be obeyed, and the type of actions that can be applied if the injection fails to meet the set criteria.

be skipped over, allowing for other revenue generating batches to be injected. This is especially important during overnight slots when the instrumentation is left unattended. An email facility is also available to send messages if and when deviations occur, alerting chemists to problems as soon as they arise.

Diagnosis of whether co-extracted interferants are the source of QCMonitor flags is also made easier by the ability to acquire high-sensitivity full scan data simultaneously with MRM, adding a new dimension to QC for LC/MS/MS analyses. QCMonitor can be found in the TargetLynx Software Method Editor, as shown in Figure 3.

A calibration curve was prepared in matrix matched standards and injected. Excellent linearity was achieved using a weighting factor of 1/x with a high coefficient of determination. This is shown in Figure 4.

With TargetLynx Software as standard, the process of quantification is simpler than ever. The results browser and report generator clearly indicate when samples contain residues that are above minimum reporting levels.

The advantage of using the ACQUITY UPLC System with the Xevo TQ MS System is that ion ratio confirmation is also possible. Figures 4 and 5 show an ion ratio for disulfoton sulfone that fails to meet the criteria required. The TargetLynx Software Method Editor can be easily manipulated by the user to set pass and fail criteria for each compound with regard to ion ratio. The ability of each injection to meet these criteria is then shown with a red or a white box. This injection shows a concentration of 0.7 ng/mL (0.007 mg/kg for the sample), but the ratio between the areas of the primary and secondary MRM transition traces do not lie within the limits set by the chemist. Within the EU, ion ratio confirmation is important for pesticide analysis as documented in SANCO/2007/3131.[5] When the mouse is positioned over this red box, a further message is displayed detailing the problem. In this instance the actual ion ratio does not lie within the limits specified by the chemist. This feature automatically performs these calculations, which allows chemists to use their time more cost-effectively and improve laboratory workflow.

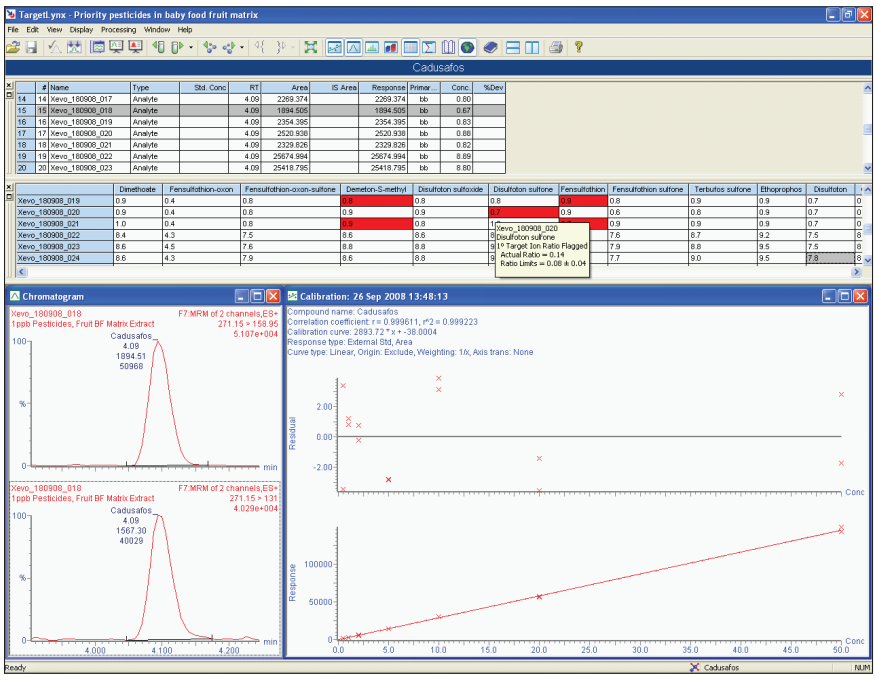

Figure 4. TargetLynx Software browser view showing cadusafos in fruit-based baby food extract at 0.001 mg/kg. Also highlighted are the red boxes which show ion ratios that are out of tolerance.

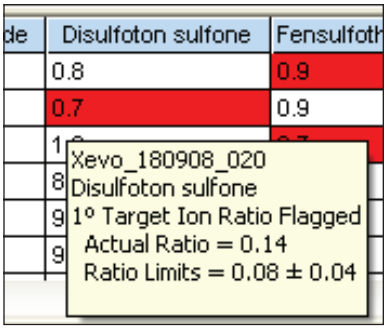

Figure 5. Closeup view showing the type of user message TargetLynx Software produces to flag an out-of-range ion ratio result.

TrendPlot: Monitor long-term system health

The TrendPlot™ tool provides confirmation that the Xevo TQ MS System results generated by your laboratory are consistently of the highest quality. It is possible to choose specific injections by charting both short-term intra-batch and long-term inter-batch trends in your analytical performance. In this example, ethoprophos samples have been plotted with the outlier easily seen in Figure 6.

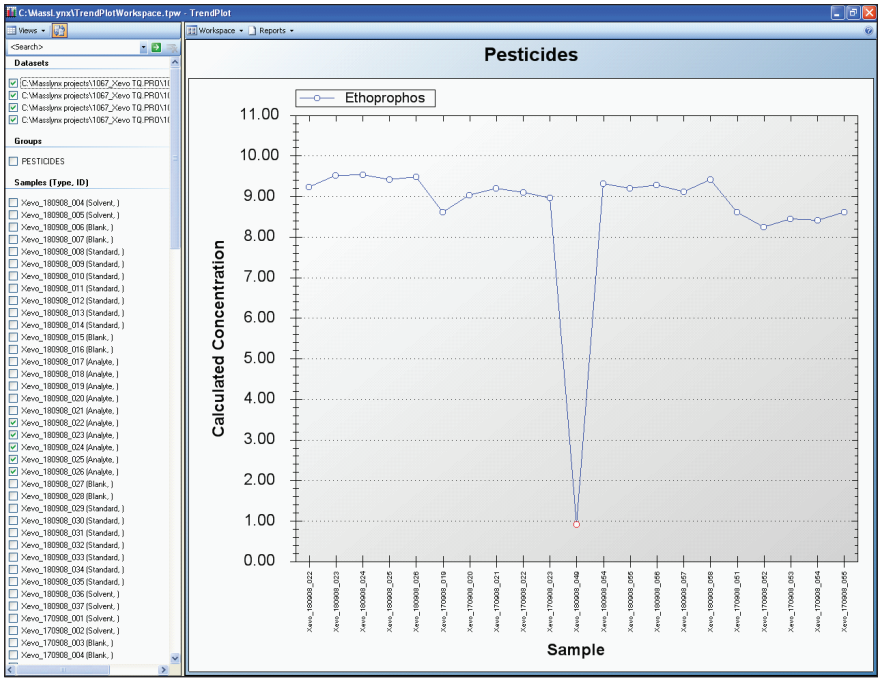

Figure 6. TrendPlot Tool shows outliers within a batch or performance of the instrument when data from several batches is added together.

CONCLUSION

A rapid multi-residue method was developed for the determination and confirmation of LC amenable priority pesticides. The analysis of pesticides in fruit- and meat-based baby food extracts exceeds current worldwide legislated limits.

Improved efficiency and increased sample throughput was realized through the combination of powerful UPLC and fast MS acquisition technologies. ACQUITY UPLC combined with the Xevo TQ MS offers:

- Enhanced chromatographic resolution and short analysis times.
- Incorporation of confirmatory MRM traces.
- Complies with legislative regulations such as SANCO.
- IntelliStart technology that is designed to reduce the burden of complicated operation, training new users, time-intensive troubleshooting, and upkeep.
- The compact features of the ACQUITY UPLC and Xevo TQ MS Systems will give any laboratory an advantage as it gives high-end performance with a benchtop footprint.
- Automated system setup and quality control system checks for simple access.

The benefits of this Waters UPLC/MS/MS solution for a revenue conscious laboratory can be realized through increased efficiency by analytical time savings, a decreased need for sample retesting, and increased lab productivity. Cost savings can be made by lowering the use of lab consumables with the environmental impact of solvent usage also being reduced.

The sensitivity achieved for a large number of pesticide residues in real food matrices indicates this UPLC/MS/MS method is the ideal basis for rapid analysis of pesticides in a wide range of food samples.

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4. Leandro CC, Hancock P, Fussell RJ, Keely BJ. J. Chrom A. 2007; 1144:161.
5. Method Validation and Quality Control Procedures for Pesticide Residues in Food and Feed. Document No SANCO/2007/3131.

Appendix 1. Xevo TQ MS parameters

Pesticide	RT	MRM transitions	Dwell time (s)	Cone voltage (V)	Collision energy (eV)
Omethoate	0.97	214>183 214>155	0.08	16	12 15
Oxydemeton-S-methyl	1.35	247>169 247>109	0.04	18	14 28
Demeton-S-methyl sulfone	1.39	263>169 263>121	0.04	20	16 16
Dimethoate	1.79	230>125 230>171	0.10	12	20 14
Fensulfothion-oxon	2.32	293>237 293>265	0.04	22	18 13
Fensulfothion-oxon-sulfone	2.39	309>253 309>175	0.04	19	15 25
Demeton-S-methyl	2.63	231>89 231>61	0.10	12	12 22
Disulfoton sulfoxide	2.93	291>185 291>97	0.04	15	13 32
Disulfoton sulfone	2.98	307>97 307>115	0.02	16	28 23
Fensulfothion	3.10	309>281 309>157	0.02	25	14 24
Fensulfothion sulfone	3.17	325>269 325>297	0.02	19	15 11
Terbufos sulfone	3.30	321>171 321>115	0.03	19	11 28
Terbufos sulfoxide	3.32	305>187 305>131	0.03	10	11 27
Ethoprophos	3.68	243>131 243>173	0.10	18	19 14
Disulfoton	4.03	275>89 275>61	0.08	14	10 32
Cadusafos	4.09	271>159 271>131	0.02	16	14 22
Terbufos	4.28	289>103 289>233	0.06	12	9 5

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ONLINE UPLC METHOD FOR THE SUPPORT OF CLEANING VALIDATION AND THE ROUTINE MONITORING OF CLEANING PROCEDURES

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INTRODUCTION

During the manufacture of active pharmaceutical ingredients (APIs), the formulation of drug substances, and therapeutic fill and finish, the removal of residues from manufacturing equipment is performed by a series of cleaning procedures. It is imperative that the production equipment be properly cleaned in order to avoid cross-contamination of drug products.[1-3] The efficiency of the cleaning procedures must be demonstrated through cleaning validation. This involves demonstrating that residual API, starting material, intermediates, and impurities have been removed from the production equipment.

During the cleaning procedure development and validation process, it is important to evaluate the effectiveness of each cleaning step in the overall process to adequately understand at what point the equipment becomes clean. It is also important to confirm that an unclean piece of equipment yields an unacceptable result.

Once the cleaning method has been validated, routine equipment cleaning should be monitored. Typically samples (either swabs or wash solvents) are taken to an off-line quality control (QC) laboratory for analysis. The time it takes to receive results from the off-line laboratory can range from hours to days. During this time, the production equipment must sit idle. If laboratory results are positive for API residues, the cleaning process and subsequent off-line QC testing must be repeated, increasing the amount of time the manufacturing equipment sits idle.

An analytical method is required that can simultaneously monitor all of the components present on the production equipment at the required safety levels. The acceptance criteria for API residues vary according to the potency of a drug substance. In general, most processes aim to have a low safety limit in the 10 ppb to 1 ppm range (10 ng/mL to 1 µg/mL). In order to achieve these limits, sensitive analytical techniques are required.[4]

This application note describes a fast, online, UltraPerformance Liquid Chromatography (UPLC®) method that monitors wash solvents directly from a sampling point on the manufacturing equipment. By monitoring wash solvents online, the point at which the API has been removed from the production equipment can be determined. This can reduce the volume of wash solvent required, particularly on equipment that is used for multiple APIs and where a cleaning procedure was developed against the "worst case." By gaining a better understanding of the cleaning procedure and reducing the dependency on off-line QC results, the time that the equipment must be taken off-line for cleaning and verification can be substantially reduced.

The results from the online method are compared to those obtained by testing swabs and wash solvents at an off-line UPLC system. The PATROL™ UPLC Process Analyzer Online System (Figure 1), which includes specialized, integrated hardware and software, was designed to be utilized in a manufacturing environment and provides near real-time analysis of inprocess samples, both online and atline.

Figure 1. The PATROL UPLC Process Analyzer System.

EXPERIMENTAL

Reaction conditions

Cleaning was performed on reaction vessels used for the conversion of acetylsalicylic acid (ASA) to salicylic acid.[5] A solution of 0.3 g/L ASA in water was prepared in a 1-L reaction vessel. Nitric acid (10 mL) was added to the reactor, which was placed in a heated bath at 75 °C. After 2 hours the temperature was reduced to 7 °C, and after 2 additional hours the reactor was removed from the bath. The reactor was then emptied in preparation of cleaning.

Cleaning procedure

The final cleaning procedure included three wash steps using 100 mL of 50:50 water/methanol to clean the inside of the reactor, and two wash steps to clean the exit port of the reactor using 200 mL of the same solvent. Wash solvents after each step were sampled and analyzed to monitor the cleaning progress. Swabs were used to assess the reactor cleanliness throughout the procedure and also after the final cleaning step to ensure levels were below acceptable limits.

Quantitative methodology

Calibration curves for the starting material and final product were based upon four standards at levels ranging from 10 ng/mL to 50 µg/mL, depending on which step in the cleaning process was being assessed. The linear range was determined by analyzing 12 standards across the entire concentration range. The limit of detection (LOD) was defined as s/n=3 and the limit of quantification (LOQ) was defined as s/n=10.

Chromatographic conditions

LC systems:	Waters PATROL UPLC Process Analyzer Online System Waters ACQUITY UPLC® System (for off-line comparisons)
Column:	ACQUITY UPLC HSS T3, 2.1 x 50 mm, 1.8 µm
Column temp.:	50 °C
Flow rate:	1.0 mL/min
Mobile phase:	75:25 Water/acetonitrile + 0.1% formic acid
Injection volume:	1 µL
Needle wash:	70:15:15 Acetonitrile/isopropanol/water
Wavelength:	230 nm
Data rate:	10 Hz
Time constant:	0.2 s (normal)
Run time:	1 minute

RESULTS AND DISCUSSION

Chromatographic method

A fast isocratic method was developed for online monitoring of the wash solvents. The final method had a 60-second run time with an inject-to-inject cycle time of 160 seconds, resulting in near real-time analysis. The method provided excellent resolution of the starting material, final product, and the two critical process impurities. An example of the chromatography for a standard and the first reactor wash step are shown in Figure 2.

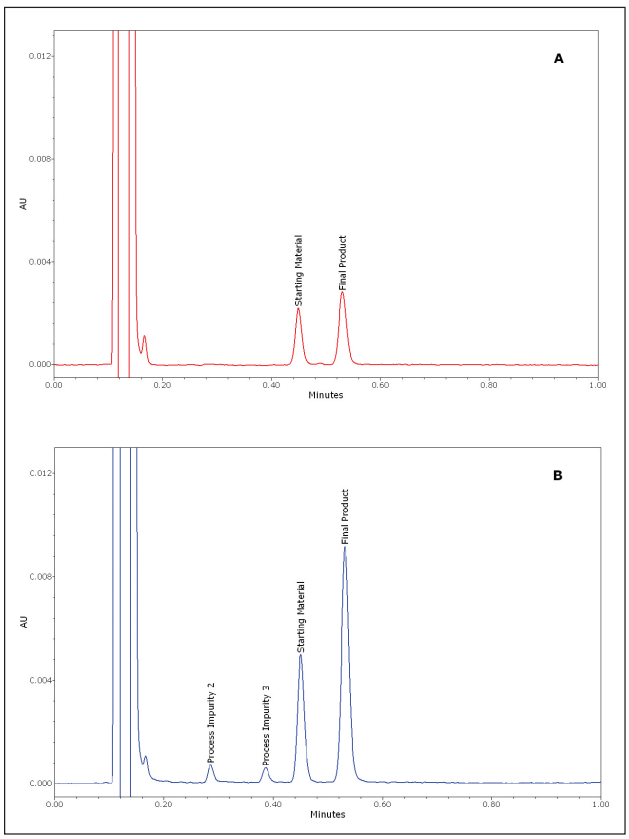

Figure 2. Example chromatograms for a standard (A); and the first wash step (B) containing starting material, final product, and two process impurities.

Limits of detection/quantification and linear range

To ensure that the method met sensitivity requirements and that the linear range was sufficient to quantify across the required range, a calibration curve was generated from 10 ng/mL to 50 µg/mL. The calibration curve used a 1/x weighting to ensure good quantification at low concentration levels. Exceptional linearity was observed with R^2 values in excess of 0.999 for the curve, which extended across more than three orders of magnitude (Figure 3). The final method had excellent limits of detection, as low as 24 ng/mL (Table 1). LOD and LOQ were determined by plotting amount versus s/n for the low level standards. For each analysis, only 1 µL was injected on column, indicating the method was sensitive enough to detect levels as low as 24 pg on column.

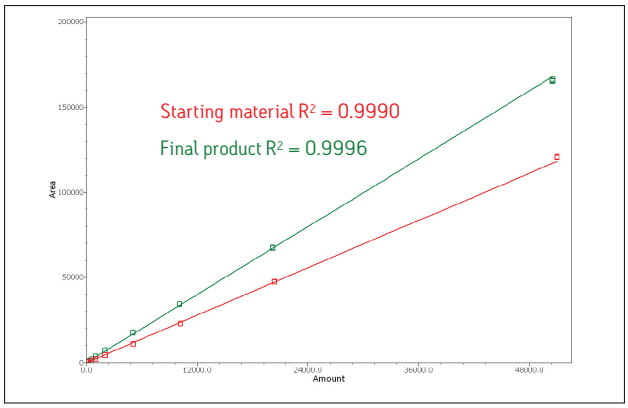

Figure 3. Calibration curves for the starting material and final product (10 ng/mL to 50 µg/mL).

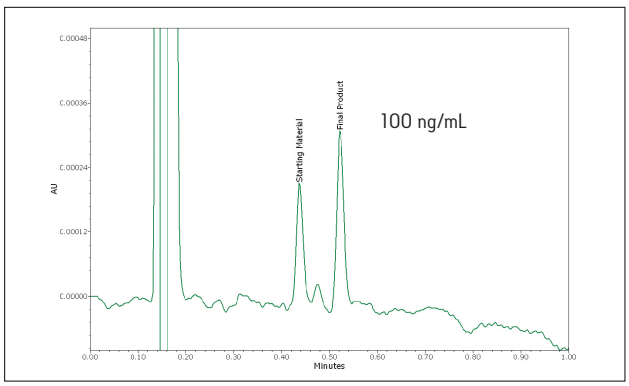

Figure 4. Standard injection near LOQ.

Compound	LOD (s/n = 3)	LOQ (s/n = 10)
Starting Material	31 ng/mL	102 ng/mL
Final Product	24 ng/mL	80 ng/mL

Table 1. LOD and LOQ of the reaction components.

Assessing online monitoring by UPLC

To demonstrate the viability of using the PATROL UPLC Process Analyzer Online System for the support of cleaning validation and the routine monitoring of cleaning procedures, equivalency to off-line results must be determined.

A cleaning protocol for the reactor was developed and residual levels were assessed after each step by both online and off-line analysis. The final cleaning procedure consisted of three wash steps inside the reactor (protocol A) and two wash steps at the outlet (protocol B). The residual levels determined by tests at each step are listed in Table 2. It is important to note that if the final product was detected by off-line analysis (wash solvents or swabs), it was also detected by online monitoring.

Additionally, if the online results indicated the equipment was clean, the subsequent off-line analyses (wash solvents and swabs) also indicated cleanliness. The PATROL UPLC System was an extremely useful tool in developing the cleaning protocol, as the level of contamination could quickly and easily be determined at each cleaning step.

Sample	Wash A1	Wash A2	Wash A3	Wash B1	Wash B2
Online 1	1767	28	—	46	—
Off-line 1	1416	22	—	47	—
Swab 1	172	—	—	—	—
Online 2	1807	29	—	94	—
Off-line 2	1443	19	—	83	—
Swab 2	71	—	—	6	—

Table 2. Levels of final product in the wash solvents during the cleaning protocol development. Results from the online method were in agreement with off-line results (both swab and wash solvent). Test performed in duplicate. Levels in ng/mL.

Once the final cleaning procedure was developed, the repeatability of the PATROL UPLC System to routinely monitor the cleaning process was assessed. The reactor was cleaned four times, and the results of online and off-line monitoring were consistent for determining the presence of both the starting material and final product (Table 3). The final results indicate that if residue was not detected in the A wash steps, the inside of the reactor was clean; and if residue was not detected in the B wash steps, the outlet of the reactor was clean (as confirmed by swab analysis).

STARTING MATERIAL IN WASH SOLVENTS (ng/mL)						
Trial	Sample	Wash A1	Wash A2	Wash A3	Wash B1	Wash B2
Trial #1	Online	1124	48	—	1131	—
Trial #1	Off-line	1098	56	—	1045	—
Trial #2	Online	2164	24	—	73	—
Trial #2	Off-line	2023	24	—	67	—
Trial #3	Online	1726	38	—	61	—
Trial #3	Off-line	1676	45	—	60	—
Trial #4	Online	855	—	—	128	—
Trial #4	Off-line	816	—	—	118	—

FINAL PRODUCT IN WASH SOLVENTS (ng/mL)						
Trial	Sample	Wash A1	Wash A2	Wash A3	Wash B1	Wash B2
Trial #1	Online	1580	27	—	60	—
Trial #1	Off-line	1632	31	—	56	—
Trial #2	Online	1647	19	—	40	—
Trial #2	Off-line	1647	21	—	40	—
Trial #3	Online	1658	29	—	50	—
Trial #3	Off-line	1678	32	—	51	—
Trial #4	Online	1619	15	—	127	—
Trial #4	Off-line	1587	17	—	131	—

Table 3. Levels of starting material and final product (ng/mL) as determined online by the PATROL UPLC System and an off-line method. All corresponding swabs after the final wash step were also clear.

Benefits of online monitoring by UPLC

Routine online monitoring of the cleaning procedures for manufacturing equipment is more effective than traditional off-line tests. A reactor used for multiple APIs can be cleaned in-place and analyzed to ensure it meets specifications rather than over-washing to "worst-case," which utilizes excess solvent and time. It also eliminates the risk of equipment failing repetitive cycles of off-line QC testing and sitting idle while the cleaning procedures are repeated.

TIME FOR ANALYSIS		
Online	Near real-time analysis	Typically < 4 minutes
Off-line	Analysis time includes laboratory activities	2 hours to days

SOLVENT CONSUMPTION		
Online	Clean until clean	Wash only as long as necessary, no extra solvent consumption
Off-line	Clean to worst-case	Consumes excess solvent

EQUIPMENT DOWN TIME		
Online	Clean until clean	Minimizes time to clean equipment
Off-line	Clean to worst-case	Excess down time; if samples fail QC test, cleaning/testing cycle must be repeated

CONCLUSION

- The results obtained by online monitoring with the PATROL UPLC System were consistent with those determined by off-line analysis.
- The PATROL UPLC System was able to monitor low ng/mL levels required to support cleaning validation.
- The large linear dynamic range of the PATROL UPLC System provides the means to monitor reactions at high concentrations and monitor the low levels required for cleaning procedures on the same instrument.
- The PATROL UPLC Process Analyzer Online System provides a highly effective solution to support cleaning validation and the routine monitoring of wash solvents from the cleaning of manufacturing instrumentation.
- Use of the PATROL UPLC System for online monitoring reduces manufacturing equipment down-time for cleaning procedures.

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