A FLUORESCENCE DETECTOR FOR ULTRA PERFORMANCE LC®

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Tanya Jenkins, Patricia McConville, Andrew Aubin, Mark Benvenuti, Jeannine Jordan
Waters Corporation, 34 Maple Street, Milford, MA 01757, USA

INTRODUCTION

UltraPerformance LC® is a separation technique based upon sub 2 micron particles and the LC system designed to take advantage of the separation potential of these particles. There has been increasing interest in expanding UPLC® to include high sensitivity detection techniques such as fluorescence. However, the narrow peak widths achieved with UPLC can pose a problem to conventional LC detection systems. The ACQUITY UPLC® FLR detector design (Figure 1) incorporates a low volume flow cell which serves to reduce the extra column band broadening which can negatively impact chromatographic resolution. The ACQUITY UPLC FLR detector is also capable of collecting at data rates up to 80Hz to ensure optimum definition of the very narrow peaks generated by the UPLC technique. With these requirements met, HPLC methods can easily be transferred to the ACQUITY UPLC FLR system while maintaining high levels of sensitivity.

In addition to meeting UPLC requirements, the ACQUITY UPLC FLR employs tools to aid in detection method development. The scan features and 3D capabilities available on the ACQUITY UPLC FLR can help reduce the time needed to develop detection methods.

Figure 1. ACQUITY UPLC FLR Detector

OPTIMIZING FLUORESCENCE DETECTION FOR UPLC

Hardware Considerations

A typical HPLC fluorescence detector can have upwards of 8 µL of volume or greater, which results in significant band spread for UPLC. The volume of the ACQUITY UPLC FLR flow cell was reduced to 2 µL to minimize these effects. However, reducing the volume of a flow cell can have detrimental effects on sensitivity since this translates into a reduction in pathlength and inlet slit. The axially illuminated flow cell of the ACQUITY UPLC FLR (Figure 2) focuses the excitation energy to enhance fluorescence and increase sensitivity. In addition, a high energy Xe-Hg lamp increases the intensity of the source light to promote high sensitivity.

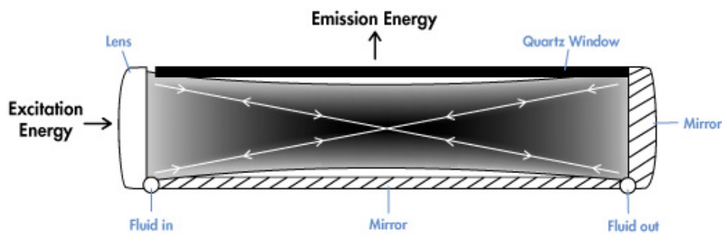

Figure 2. ACQUITY UPLC FLR axially illuminated flow cell

Method Considerations

Fluorescence detection is a highly sensitive technique. It is important when developing and optimizing fluorescence detection methods not to sacrifice this sensitivity. The ACQUITY UPLC FLR offers independent optimization of data rate and filter time constants to optimize sensitivity and resolution. When selecting a data rate, it is important to maintain 25-50 points across a chromatographic peak to achieve reproducible and accurate quantification. The ACQUITY UPLC FLR is capable of collecting at data rates up to 80Hz (Figure 3) to ensure good reproducibility for even very narrow peaks. For the fast separation of three PAHs, good quantitation is achieved at data rates above 10Hz (Table 1). The filter time constant can then be optimized to achieve the desired resolution and sensitivity without adding noise (Figure 4).

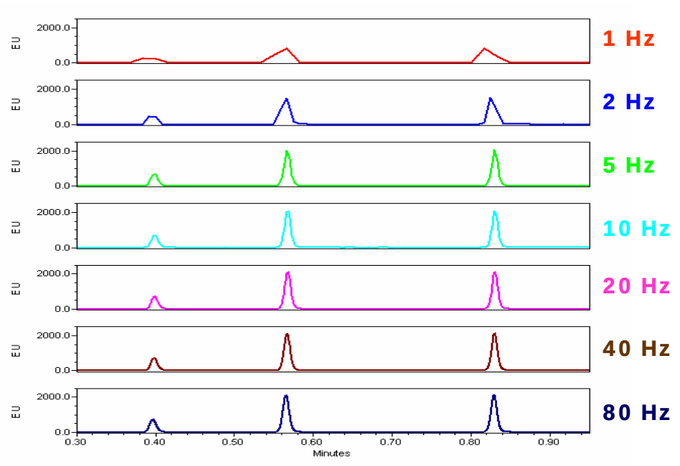

Figure 3. Separation of Naphthalene (700ng/mL), Anthracene (200ng/mL), and Benzo(a)pyrene (100ng/mL) at various data rates. Method Conditions: Column: 2.1 x 50mm ACQUITY UPLC BEH C_{18}, 1.7µm; Injection Volume - 2µL; Flow Rate - 1 mL/min; Solvent A - Water; Solvent B - Acetonitrile; Gradient - 45% to 95% B over 45s, hold at 95% for 15s; Temperature - 45°C; Detection program - 270/325, 0.5min 240/380, 0.7min 295/410, Filter Time Constant - 0.1sec.

Data Rate	Points Across Peak	Peak Area %RSD	Peak Height %RSD
1	4	1.78	6.72
2	6	0.44	6.69
5	13	0.70	1.04
10	24	0.34	0.44
20	48	0.30	0.32
40	96	0.26	0.17
80	193	0.14	0.20

Table 1. Reproducibility and points across the peak as a function of data rate for Benzo(a)pyrene (Peak 3). For conditions see Figure 3.

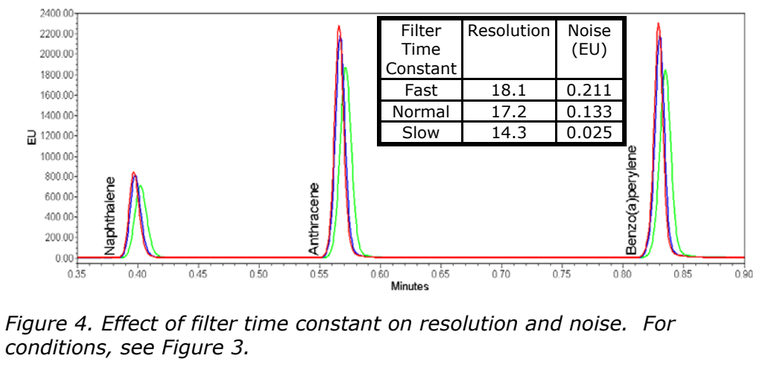

Figure 4. Effect of filter time constant on resolution and noise. For conditions, see Figure 3.

DEVELOPING UPLC FLUORESCENCE METHODS

Natural fluorophores typically have very different excitation and emission maxima. To develop an optimal fluorescence detection method, these excitation and emission wavelengths must be determined. The ACQUITY UPLC FLR can be used as a benchtop fluorimeter to determine these values. The ACQUITY Console provides a scan wavelength function which can determine these values to produce an optimized 2D detection method. The 3D Scan mode in Empower with 'on the fly' spectral data is another method to determine these optimal wavelengths.

A Method for Water Soluble B Vitamins

A fast UPLC fluorescence method for vitamins B2 and B6 was developed. The excitation and emission maxima were determined using the ACQUITY Console (Figure 5). The absorbance maxima of B2 (Riboflavin) and B6 (Pyroxidine, Pyridoxamine, and Pyridoxal) were used as the fixed excitation wavelengths for the emission scans. Once the emission maxima had been determined the excitation range could be scanned. Using these values an optimized 2D method was created and a UPLC separation was achieved in 90 seconds (Figure 6). The data rate and filter time constant were optimized to yield the best resolution and signal-to-noise ratios. Limits of detection were determined using 7 low level standards and verified with replicate injections.

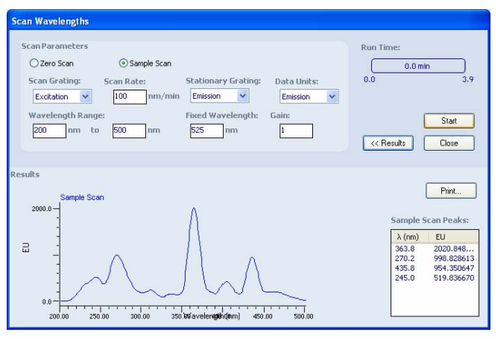

Figure 5. ACQUITY Console scan wavelength feature for determining the optimal excitation and emission wavelengths of Vitamin B2.

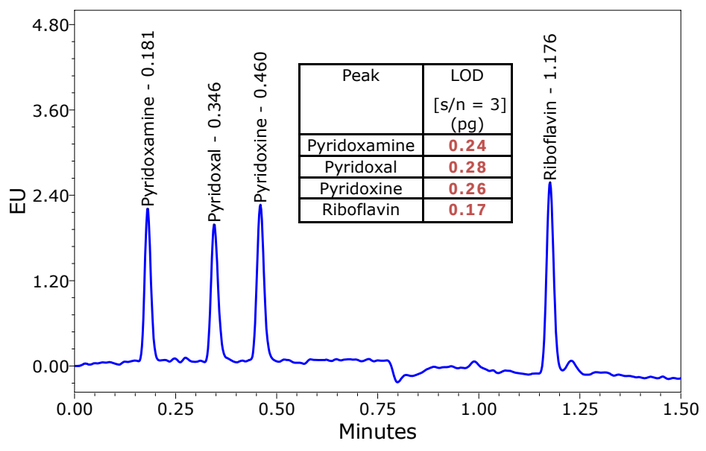

Figure 6. Separation of water soluble vitamins B2 and B6 (500ng/mL). Method Conditions: Column 2.1x50mm ACQUITY UPLC HSS T3 1.8µm; Injection Volume: 2µL; Flow Rate 0.75mL/min; Solvent A - 0.1% Formic Acid in Water; Solvent B - 0.1% Formic Acid in Acetonitrile; Gradient: Hold 1% B for 24s, linear to 15% B to 36s, hold 15% B to 90s; Temperature 40° C; Detection: 0.0 - 0.6min 295ex/385em, 0.6 - 1.5min 365ex/525em; Data Rate - 10Hz; Filter Time Constant - 0.4.

A Method for Polynuclear Aromatic Hydrocarbons

For samples with many components, such as PAHs, it is time consuming to scan each individual standard. A better solution is to use the 3D scan function in Empower™. This feature scans either the excitation or emission range to determine the wavelength maxima (Figure 7). In as few as two injections, the optimal wavelengths for each component can be determined. The EPA PAH 610-QC standard was run in scan mode and the optimized wavelength pairs were determined for the detection method and are listed in Table 2. The UPLC fluorescence method for PAHs yielded excellent repeatability with an average of 0.05% RSD for retention time and 0.45% for peak area (Figure 8).

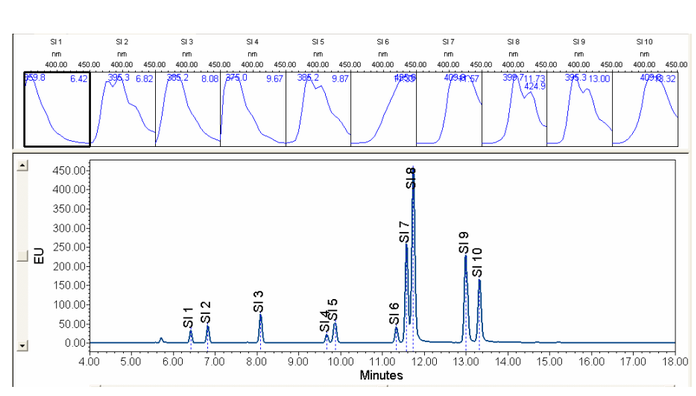

Figure 7. Spectrum index plot generated using the ACQUITY UPLC FLR 3D scan mode for detection method development of PAHS.

Time (min)	Excitation Wavelength (nm)	Emission Wavelength (nm)
0.00	270	325
3.50	295	315
5.50	240	380
6.30	235	460
6.65	310	385
7.40	295	380
8.50	295	410
10.00	295	400
10.60	295	495

Table 2. 2D Detector program generated from data collected using 3D Scan Mode

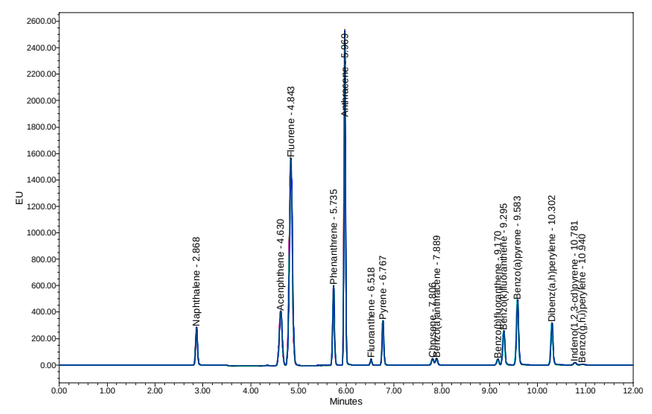

Figure 8. UPLC separation of PAHs (overlay of 6 replicates). Method Conditions: Sample - 610-QC 100:1 Dilution in Acetonitrile; Column: 2.1 x 150mm ACQUITY UPLC BEH RP18 1.7µm; Injection Volume - 2µL; Flow Rate - 600 µL/min; Solvent A - Water; Solvent B - Acetonitrile; Gradient - hold 50% B for 4.5min, linear to 67%B to 5.5min, hold 67% B to 7.5min, linear to 77% B to 9.5 min, hold 77% B to 12.0min; Temperature - 45°C; Data Rate - 10Hz; Filter Time Constant - 0.1.

TRANSFERRING FLUORESCENCE METHODS FROM HPLC TO UPLC

Methods can be easily transferred from HPLC to UPLC. However, it is important when transferring methods that certain criteria still be met such as resolution and sensitivity requirements. Many tools are available to assist in the transfer of methods from column selectivity charts to the ACQUITY UPLC calculator which will automatically scale the method conditions.

A Method for Drugs of Abuse

A legacy isocratic HPLC method for MDMA and its metabolite MDA was scaled to a UPLC method on the ACQUITY UPLC FLR System. The original HPLC and new UPLC separations are displayed in Figures 9-10. The detection parameters were adjusted for the narrower peak widths on the UPLC separation. A reduction in run time of 87.5% was achieved with equivalent sensitivity for the same mass on column (Table 3). As with the original HPLC method, the peaks were baseline resolved. This method was then further optimized by converting it to a gradient method and reducing the run time to 30s, a 97.5% reduction over the original HPLC method. An increase in sensitivity was observed over the original HPLC method while still maintaining baseline resolution.

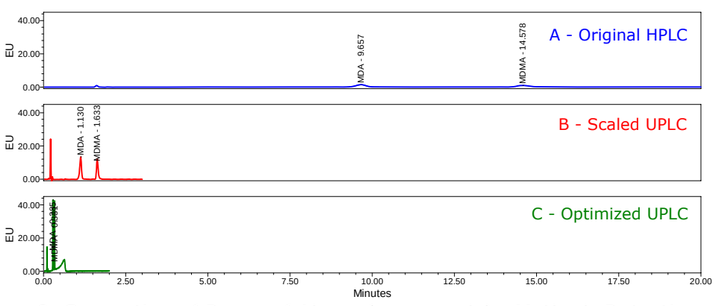

Figure 9. Separation of Drugs of Abuse (same scale). Method: Detection - 280ex/320em; Solvent A - 10mM Ammonium Bicarbonate; Solvent B - Acetonitrile; Injection Volume - 5µL.

Method	Run Time (min)	Resolution	Peak Width ½ Height (sec)	Data Rate (Hz)	Time Constant (sec)	Signal-to-Noise
HPLC	20.0	7.9	20.3	1	2.0 (Normal)	104
Scaled UPLC	2.5	5.5	3.0	5	0.4 (Normal)	110
Optimized UPLC	0.5	3.4	0.43	20	0.05 (Fast)	185

Table 3. Improvements in run time and sensitivity when the method for drugs of abuse is transferred from HPLC to UPLC for an equivalent mass on column.

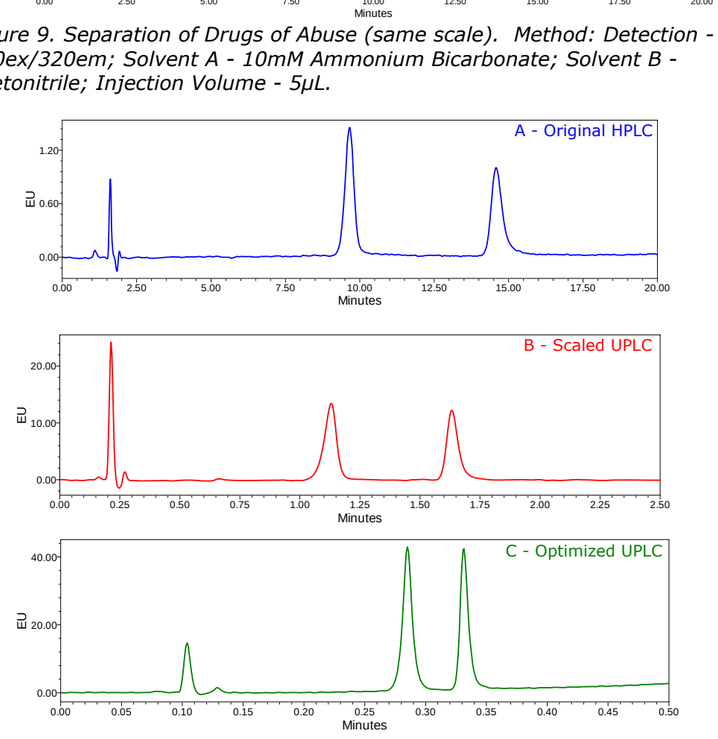

Figure 10. Separation of Drugs of Abuse (expanded view)

LIMIT OF DETECTION

The limit of detection (LOD) of a fluorescence detector is often characterized using anthracene. In order to accurately quantitate this value, a method must be developed which elutes the peak away from the void volume. The LOD was determined by injecting a series of standards near the limit of detection. Once the LOD was calculated, replicate injections were performed to verify the result. The limit of detection for anthracene, on the ACQUITY UPLC FLR, was determined to be 3.2 fg on column.

*Figure 11. Low level anthracene standard (12 fg on column). Method: Column- 2.1 x 50mm, ACQUITY UPLC BEH RP18, 1.7µm; Mobile Phase - 30/70 Water/*Acetonitrile; Flow Rate - 600µL/min; *Temperature - 40°C, Injection Volume - 2.3µL Full Loop; Detection - 247ex/399em; Gain - 100; Data Rate - 5Hz Slow*

CONCLUSIONS

- **The ACQUITY UPLC FLR was designed to meet the challenges of UPLC detection**
- **Fast data rates and independent optimization of filter constants yield the best sensitivity and resolution on the ACQUITY UPLC FLR**
- **Scanning and 3D capabilities reduce the time needed to develop fluorescence detection methods**
- **Transferring an HPLC fluorescence method to UPLC yielded faster run times without loss in sensitivity**
- **High levels of sensitivity, to meet the challenges of everyday applications, are achieved**

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