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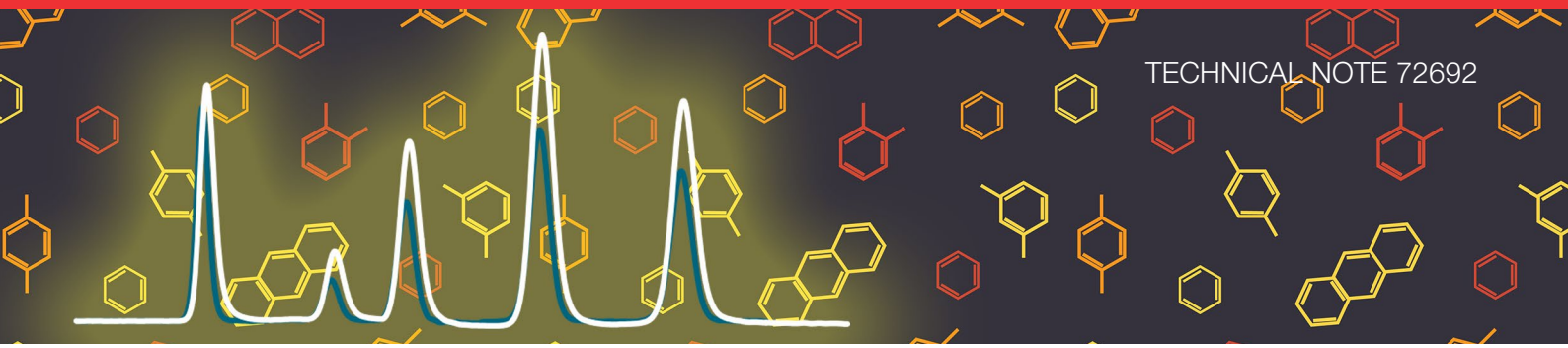

TECHNICAL NOTE 72692

Combining fluorescence detection with UHPLC: an overview of the technical requirements

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Keywords
Fluorescence detection, UHPLC, UltiMate 3000

Goal

Provide an overview of requirements for fluorescence detection in combination with UHPLC and describe how the UltiMate 3000 design matches these.

Introduction

Analysts are more frequently transferring HPLC applications to UHPLC methods to increase lab productivity and/or the method resolution. Performing separations under UHPLC conditions, however, intensifies the technical requirements for both the separation column and the UHPLC instrument. Pumps and autosamplers must handle the operating pressures generated by UHPLC columns with sub-3 µm or sub-2 µm particles. UHPLC-compatible UV detectors have high data collection rates to accurately and precisely integrate narrow analyte bands. In addition, they must operate with low-volume flow cells to resolve small-volume UHPLC analyte bands.

The requirements for a UHPLC-compatible fluorescence detector (FLD) are even more demanding. Obviously, these detectors also need to have high data collection rates and small-volume flow cells. They must also be able to provide sufficient sensitivity, as fluorescence detection is typically used for trace analysis. In multi-compound separations of complex matrices, such as polyaromatic hydrocarbon (PAH) analysis in food and beverages, the coupling of UHPLC with fluorescence detection has the potential of both reducing the run time and increasing the chromatographic resolution to improve discrimination of co-eluted interferences.[1] For best support of these applications, the detector must also be capable of changing the excitation and emission wavelengths quickly enough to complete the change even between marginally baseline-resolved peaks.[2] This technical note discusses these requirements and demonstrates how they are met using the Thermo Scientific™ UltiMate™ 3000 fluorescence detectors.

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Background

A common strategy in method transfer from HPLC to UHPLC is to maintain the resolving power of the application by using shorter columns packed with smaller particles.[3] One consequence of these shorter separations is that analyte peak widths and volumes are reduced.

Smaller peak volumes require optimized detector flow cells. These cells must provide lowest peak dispersion as a consequence of the minimized volume, and an optimized flow profile within the cell. Generally, extracolumn band broadening will be insignificant if the flow cell volume is no larger than approximately 10% of the (smallest) peak volume.[4,5] In Figure 1A, a flow cell volume of ≤15 µL does the job. A suitable flow cell volume for the UHPLC analyte band is ≤2.7 µL (Figure 1B).

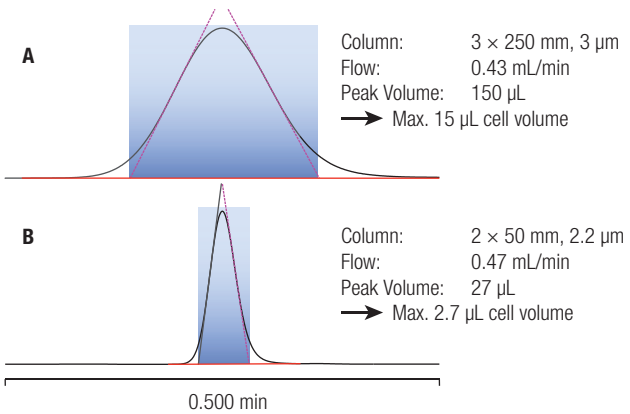


Figure 1. Flow cell volumes of A) a conventional HPLC peak and B) a UHPLC peak. UHPLC separations produce small peak volumes and therefore require small-volume detector flow cells.

Following Beer-Lambert's law, absorption is proportional to the light path in a UV flow cell and therefore to the signal intensity of a UV chromatogram. UV flow cells can be designed to combine a relatively long light path with a small detection volume, conserving a significant part of the detection sensitivity with UHPLC separations.

In fluorescence detection, the signal intensity is approximately proportional to the illuminated flow cell volume, as the intensity of the emitted light is proportional to the amount of excited analyte. In addition, the noise increases with smaller flow cells. UHPLC separations with fluorescence detection therefore typically do not achieve the same trace detection performance as conventional HPLC applications. A highly sensitive FLD designed for UHPLC requirements helps to achieve both UHPLC separations and sufficient limits of detection for most applications.

Conventional FLDs succeed in switching detection wavelengths between conventional HPLC analyte peaks with different excitation and emission requirements. When these detectors were developed, wavelength switching times of several seconds did not affect results. UHPLC conditions significantly shorten the available time window for the required grating movements. The Experimental section below will demonstrate that the UltiMate 3000 FLD detectors (FLD-3100 and FLD-3400RS) are designed to meet this requirement.

Experimental

Equipment

Thermo Scientific™ UltiMate™ 3000 Quaternary Rapid Separation System consisting of the following modules:

- SR-3000 Solvent Rack
- LPG-3400RS Quaternary Pump
- WPS-3000RS Wellplate Sampler
- TCC-3000RS Thermostatted Column Compartment
- VWD-3400RS Variable Wavelength UV-vis Detector with semi-micro flow cell (2.5 µL)
- FLD-3400RS Fluorescence Detector with Dual-PMT; analytical (8 µL) and micro (2 µL) flow cell
- Third-party FLD with analytical flow cell

All modules were connected with 0.005 in. (0.13 mm) i.d. Thermo Scientific™ Viper™ fingertight fittings.

LC conditions

Eluent A:	Water
Eluent B:	Acetonitrile
Column 1:	Thermo Scientific™ Acclaim™ 120 C18, 3 µm, 3 × 75 mm (P/N 066273)
Flow Rate:	1.1 mL/min
Column 2:	Thermo Scientific™ Acclaim™ 120 C18, 3 µm, 2.1 × 50 mm (P/N 068981)
Flow Rate:	0.90 mL/min
Inj. Volume:	1 µL
Samples:	Uracil, naphthalene, biphenyl, fluorene, anthracene, fluoranthene
Analyte Conc.:	8–114 pg/µL (fluorescence detection) and 0.4–5.7 ng/µL (UV detection in acetonitrile/methanol/water 2/1/1 (v/v/v)

Column Temp.:	40 °C
Lamp Mode:	High Power
PMT Used:	1 (FLD-3400RS), third-party detector with only one PMT installed
UV Wavelength:	251 nm

Wavelength Switching Time Experiments

Data Collection Rate:	100 Hz
Response Time:	0.02 Hz

Optimized Flow Cell Design Experiments

Data Collection Rate:	20 Hz
Response Time:	0.4 Hz

Table 1. Event table for experiments using column 1

Time, Column 1 (min)	%B	Excitation (nm)	Emission (nm)	Sensitivity Setting of FLD-3400RS	Variable Emission Filter (nm)
0	70	220	325	2	280
1.12		246	360	6	
1.30		225	315	6	
1.40	95				
1.45		244	400	5	370
1.60	95				
1.65	70	237	460	5	435
3.50	70				

Table 2. Event table for experiments using column 2

Time, Column 2 (min)	%B	Excitation (nm)	Emission (nm)	Sensitivity Setting of FLD-3400RS	Variable Emission Filter (nm)
0	65	220	325	2	280
0.58		246	360	6	
0.72		225	315	6	
0.86		244	400	5	370
1.01		237	460	5	435
1.10	95				
1.20	95				
1.30	65				
3.50	65				

Wavelength switching time

A simple five component sample was separated on a 3 × 75 mm, 3 µm column. This column was selected as it provides both quick separations and compatibility with analytical flow cells. Figure 2 shows two chromatograms for the same run. The fluorescence detector was in series after the UV detector with a 2.5 µL flow cell, leading to a slight time shift of the peaks in the fluorescence chromatogram. The peaks in chromatogram B are slightly broadened due to the additional extracolumn volume (semi-micro UV cell and additional connection capillary) and the use of an analytical flow cell in the FLD.

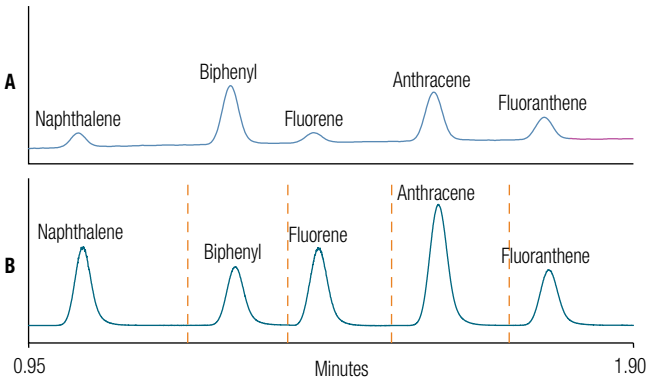


Figure 2. A) UV and B) fluorescence chromatograms of the same sample: All analytes are clearly baseline resolved. The detectors were coupled sequentially, with the UV before the fluorescence. Dotted lines in chromatogram B indicate wavelengths switches.

Figure 3 zooms to baseline level at 1.70 min, between anthracene and fluoranthene. Although the resolution is R_s = 3.2, the time of constant baseline without slope from any of the peaks is extremely short. In fact, even with this resolution, there will always be a slight influence on peak area integration. The goal is therefore to minimize this influence by using short and precise wavelength switching times. The Rapid Separation FLD features a unique variable emission filter. This unit automatically selects the optimum emission filter for the given wavelength pair to achieve the best stray-light suppression. Other vendors' detectors use a fixed emission filter with limited stray-light suppression capabilities. While it is important to have another tool for method optimization, obviously this additional switching process must also be finished before the data collection can continue.

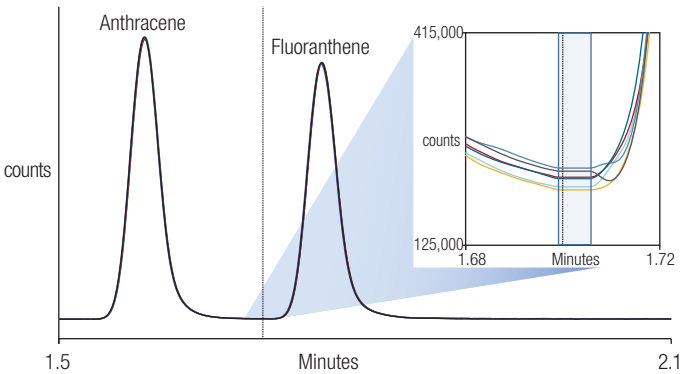
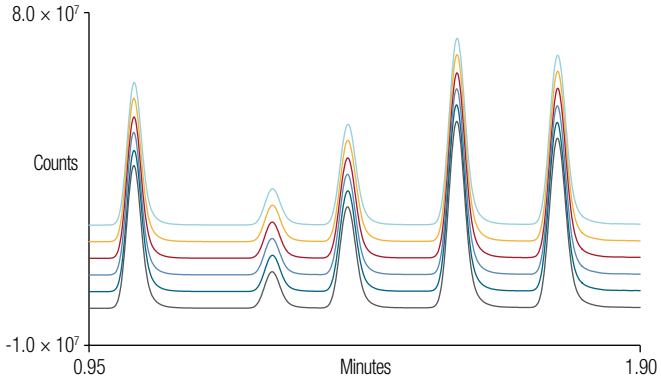


Figure 3. Overlay of six consecutive standard injections with a zoom to baseline level at 1.70 min. Wavelength switching times are extremely short and precise.

As demonstrated in Figure 3, the Rapid Separation FLD can switch wavelength and emission filter as quickly as 0.4 s (relative standard variation [RSD] only 0.5%). This result was obtained thanks to fast and precise mechanical drives and controlled by Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS) software version 6.8 Service Release 11 or higher and version 7.1 Service Release 1 or higher.

Figure 4 focuses on retention time and peak area precision. The displayed chromatograms were obtained with a response time of 0.02 s, the shortest possible setting. The response time (or a comparable parameter in a different detector control) is an electronic filter that defines how quickly the detector responds to a change of the signal and how much averaging is done. A typical recommendation is to set the response time to 25% of the peak width at half height for the best combination in signal-to-noise ratio (S/N) and resolution. A response time of 0.02 s was selected to precisely measure the switching time. However, too short of a response time unnecessarily increases signal noise, which typically has a negative impact on peak integration precision. Too short of a response time also makes setting accurate integration limits more difficult, and decreases the precision of peak area detection. Despite this setting and the influence of the wavelength switches, the area RSD for all peaks is only approximately 0.5%. The retention time precision is outstanding, with RSDs between 0.02% and 0.03%. These results are mainly a consequence of the highly precise pump flow and gradient proportioning of the quaternary RSLC pump, but also of the seamless interplay between the different system components and the Chromeleon CDS software control.



Analyte	Retention Time (min)	RSD Retention Time (%)	Area (counts*min)	RSD area (%)
Naphthalene	1.028	0.03	1.21E+06	0.55
Biphenyl	1.266	0.04	3.31E+05	0.54
Fluorene	1.396	0.02	9.38E+05	0.47
Anthracene	1.584	0.02	1.74E+06	0.43
Fluoranthene	1.758	0.04	1.61E+06	0.56

Figure 4. All analyte peaks show excellent retention time and area precisions despite the wavelength switching processes between them.

Optimized flow cell design

One requirement for UHPLC is to reduce extracolumn volumes to a minimum. An optimized flow path ensures that the chromatographic efficiency of small-particle columns is measured by the detector. Figure 5 compares two sub-2 min separations obtained using a 3 × 75 mm, 3 µm fast LC column and the FLD-3000 Series detector equipped with either the standard (8 µL, red trace) or the micro (2 µL, blue trace) flow cell.

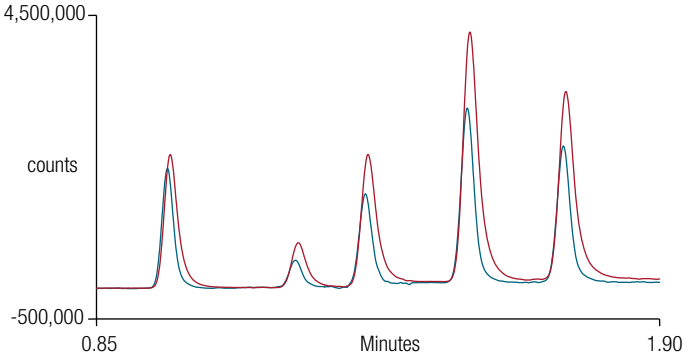


Figure 5. Overlay of two separations obtained on a 3 × 75 mm, 3 µm column with an analytical flow cell (red) and a micro flow cell (blue). The micro flow cell achieves better resolution, the analytical flow cell provides better S/N.

All peaks obtained with the analytical flow cell show a slight increase of retention time, peak width, and asymmetry caused by the larger extracolumn volume of this cell. However, this comparison also shows that peak heights increase by 10–60% due to the larger cell volume.

The column is operated at a flow rate of 1.1 mL/min and provides peak volumes between 45 µL and 51 µL for the micro flow cell and 52 µL to 58 µL for the analytical flow cell. With the micro flow cell, peaks are always 6 µL to 8 µL smaller (Table 3). Despite the rule of thumb that the flow cell volume should not be larger than 1/10th of the peak volume, the 8 µL analytical cell provides good chromatographic efficiency and clearly separates all peaks to baseline.

Table 3. Peak volumes obtained with analytical and micro flow cells

	Peak Volumes (µL)				
Flow Cell	Naphthalene	Biphenyl	Fluorene	Anthracene	Fluoranthene
Standard	52	56	58	58	58
Micro	45	50	50	50	51
Difference	7	6	8	8	7

Table 4 provides chromatographic performance data based on naphthalene and biphenyl peaks of six consecutive standard injections. The table includes the resolution Rs (1,2) between naphthalene and biphenyl and the following results for naphthalene (peak 1): signal-to-noise (S/N), theoretical plates (TP), and asymmetry (As) according to the European Pharmacopeia (EP) method requirements. Note that although TP are only defined for isocratic separations, a relative comparison based on two gradient separations when the analyte retention time is equivalent is considered valid.

Table 4 demonstrates that the micro flow cell supports a better separation with more resolution, more TPs, and less asymmetry. However, the S/N performance with the analytical flow cell is 3.1× better than with the micro flow cell. Therefore, the analytical flow cell is the best choice to achieve lowest detection limits with this application.

Table 4. Comparison of analytical and micro flow cell results based on the naphthalene and biphenyl peaks separated on a 3 × 75 mm, 3 µm column

	Analytical Flow Cell	Micro Flow Cell
Rs (EP) (1,2)	4.94	**5.61**
S/N, peak 1	**1107**	360
TP (EP), peak 1	7282	**9457**
As (EP), peak 1	1.41	**1.22**

The analytical flow cell is even compatible with lower-volume UHPLC columns. Figure 6 displays an overlay of two separations performed on a 2.1 × 50 mm, 3 µm column. The red chromatogram was obtained using an analytical flow cell, the blue with a micro flow cell. Separation time was below 1.2 min. Although peak volumes were only 27 µL to 46 µL (measured with the micro flow cell) and therefore even less in line with the 1/10th rule for the flow cell volume, a good separation was still obtained with the analytical flow cell. In fact the chromatogram is very similar to that of the separation on the 3 × 75 mm column: The analytical cell causes a slight reduction in chromatographic performance but provides better peak heights. Wavelength switches are easily performed because all peaks are clearly baseline resolved.

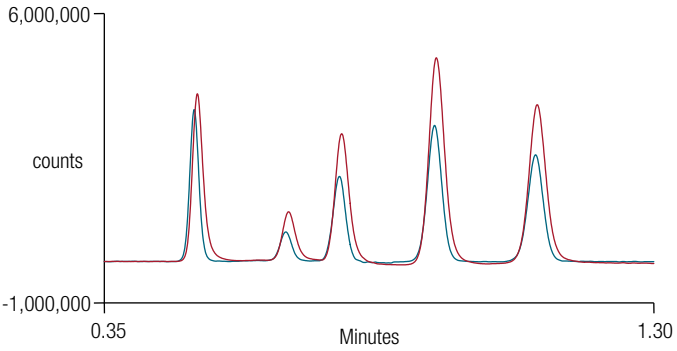


Figure 6. Overlay of two separations obtained on a 2.1 × 50 mm, 3 µm column; detected with a 2 µL micro flow cell (blue) and an 8 µL analytical flow cell (red). The micro flow cell achieves the best resolution, the analytical flow cell provides the best S/N.

Table 5 compares some key performance data of these separations. With the micro flow cell, the resolution slightly increases by 0.6. This is comparable with the results in Table 4 (resolution increases by 0.67). Theoretical plates increase by 31% (Table 4: 30%). With both flow cells, asymmetry is low and the micro flow cell is close to an ideal peak with As=1.05. The difference between the As factors is 0.2 and, therefore, again in line with the results of Table 4 (ΔAs=0.19).

With the 3 × 75 mm, 3 µm column, the analytical flow cell provides a 3.1× better S/N performance than the micro flow cell. This factor significantly changes with the smaller column format used here. The S/N with the analytical flow cell is now only 1.4× better than with the micro flow cell. This demonstrates that, with decreasing peak volumes (for instance, by operating short 2 mm columns and/or smaller particles), the difference in S/N performance between the two cell variants decreases.

Table 5. Comparison of analytical and micro flow cell results based on the naphthalene and biphenyl peaks separated on a 2.1 × 50 mm, 3 µm column

	Analytical Flow Cell	Micro Flow Cell
Rs (1,2)	4.14	**4.74**
S/N, peak 1	**2267**	1578
TP (EP), peak 1	3557	**4653**
As, peak 1	1.25	**1.05**

Conclusions

- The FLD-3000 Series fluorescence detectors (FLD-3400RS and FLD-3100) are designed for optimum UHPLC support.
- These detectors achieve very fast wavelength switching times for UHPLC separations of compounds with varying excitation and emission wavelengths, e.g. polycyclic aromatic hydrocarbons (PAH).
- The unique variable emission filter of the FLD-3400RS also switches in a fraction of a second and provides optimum stray light suppression even with ultrafast separations.
- Two flow cells are available for UltiMate 3000 fluorescence detectors:
  - 8 µL analytical flow cell for the best signal-to-noise ratio from conventional to 2 mm i.d. UHPLC columns
  - 2 µL micro flow cell for best efficiency and resolution with 2 mm i.d. UHPLC columns

References

1. Plaza-Bolaños, P.; Garrido Frenich, A.; Martínez Vidal, J.L. *J. Chromatogr., A* **2010,** *1217,* 6303–6326.
2. Steiner, F.; Martin, M.M.; Franz, H. PAH in Tea—Ultra-fast HPLC and Fluorescence Detection at Trace Levels. *lab&more,* **2010,** No. 2, 34–36.
3. Thermo Fisher Scientific. *A Universal Tool for Method Transfer From HPLC to UHPLC,* TN 70828, 2018. https://assets.thermofisher.com/TFS-Assets/CMD/Application-Notes/TN-75-HPLC-UHPLC-Universal-Tool-Method-Transfer-TN70828-EN.pdf
4. Poole, C.F. *The Essence of Chromatography;* Elsevier Science: Amsterdam, The Netherlands, 2003; pp 44–46.
5. Hanai, T.T. *HPLC: A Practical Guide;* The Royal Society of Chemistry: Cambridge, U.K., 1999; pp 25–26.

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