Benefits of High-Speed Wavelength Switching in UHPLC Methods Using Fluorescence Detection

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INTRODUCTION

Ultrahigh-performance chromatographic separations are increasingly accepted as a key approach to getting more analytic information in less time, even for complex sample mixtures. However, there are some challenges; not only is the chromatography system required to handle higher pressures (600 bar or more), the detection systems must keep pace with the increasing speed of the analysis. Whereas UV detectors can generally cope with this, fluorescence detectors are more difficult to optimize. This is especially true with regards to wavelength switching; this technique is used to achieve maximum sensitivity with fluorescence detection by switching the excitation and emission wavelengths to best match the spectral properties of the separated analytes. In practice, it implies a certain delay during data acquisition due to the mechanical inertia of the equipment.

As a consequence, marginally resolved and narrow analyte bands with different wavelength setting requirements could not be detected by fluorescence in the past. The total switching time was a summation of both wavelength switching times and the delay caused by the detector response time. To overcome these limitations and provide ultrafast separations in combination with fluorescence detection, a new generation of detectors encompassing simultaneous, ultrafast switching of the optics and fluidics was required with the following criteria:

- High data collection rates and small volume flow cells
- Ultrahigh sensitivity (fluorescence detection is typically used for trace analysis)
- Ultrafast changing of the excitation and emission wavelengths to account for correct integration, even with barely baseline-resolved peak groups

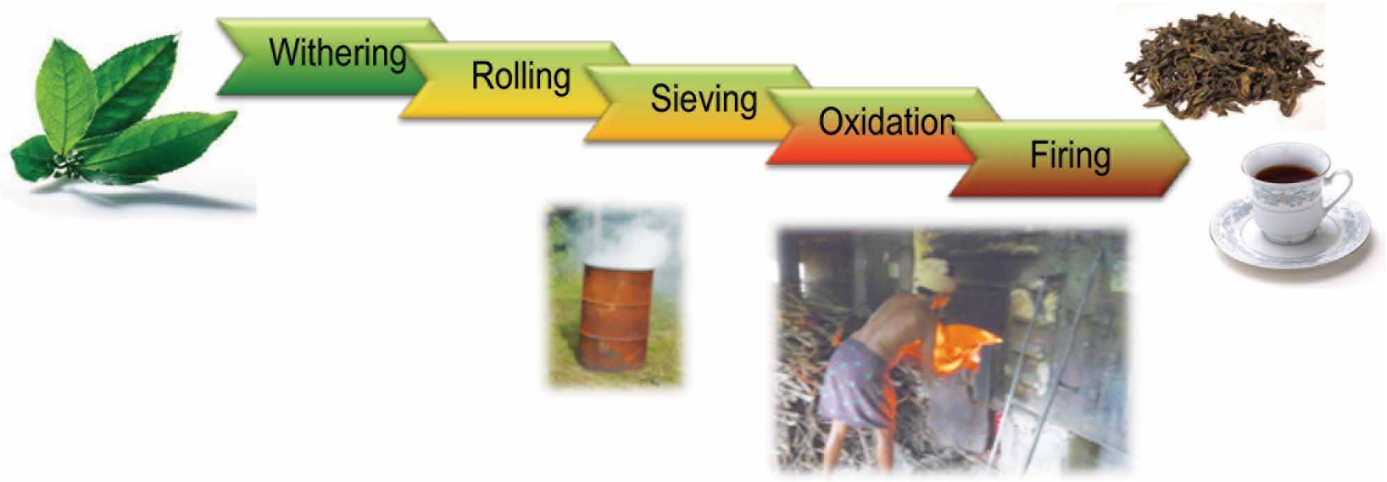

Figure 1. Tea processing and potential polyaromatic hydrocarbon (PAH) contamination sources.

A challenging field of application is the determination of polyaromatic hydrocarbon (PAH) contamination in foodstuffs, e.g., tea samples. After water, tea is the most widely consumed beverage in the world. While tea has the reputation of being healthy due to many pharmaceutically active ingredients and antioxidants, tea leaves also act as a perfect enrichment matrix for environmental pollutants. In particular, PAHs are a critical class of contaminants requiring careful and sensitive monitoring. Green, black, and oolong tea are all processed using a multistep procedure (Figure 1) that exposes them to manifold sources for PAHs. Using the high-performance Thermo Scientific Dionex FLD-3100/3400RS Fluorescence Detector for UHPLC determination of PAHs in different tea samples achieves run times of less than 6 min, thus ensuring high sample throughput.

ULTRAFAST WAVELENGTH SWITCHING

To illustrate the high-speed switching capabilities of the Dionex FLD-3100/3400RS detectors, a simple five-component sample consisting of uracil, naphthalene, biphenyl, anthracene, and fluoranthene was separated using a 3 × 75 mm, 3 µm column (see Table 1 for gradient and detection parameter switching).

System

Thermo Scientific Dionex UltiMate® 3000 Quaternary Analytical LC System consisting of the following modules:

- SR-3000 Solvent Rack
- LPG-3400RS Quaternary Rapid Separation (RS) Pump
- WPS-3000RS RS Well Plate Sampler
- TCC-3000RS Thermostatted Column Compartment
- FLD-3400RS Fluorescence Detector
- Third-party FLD with analytical flow cell

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

LC Conditions

Eluent A:	Water
Eluent B:	Acetonitrile
Column:	Thermo Scientific Dionex Acclaim® 120 C18, 3 × 75 mm, 3 µm
Flow:	1.1 mL/min
Inj. Volume:	1 µL
Column Temp.:	40 °C
Data Collection Rate:	100 Hz
Response Time:	0.02 s
Lamp Mode:	High power
Photomultiplier Tube:	1 (FLD-3400RS), third-party detector with only one PMT installed
Analyte Concentrations:	8–114 pg/µL in water/methanol/acetonitrile 1/1/2 (v/v/v)

Table 1. Gradient Elution and Separation Conditions of the Five-PAH Mix

Time (min)	% B	Excitation (nm)	Emission (nm)	Sensitivity Setting of FLD-3400RS	Variable Emission Filter (nm)
0	70	220	325	3	280
1.20		246	360	6	
1.34		225	315	6	
1.40	95				
1.50		244	400	5	370
1.60	95				
1.70	70	237	460	6	435
3.50	70				

Figure 2 shows the separation of the five-compound test mix under UHPLC conditions, and demonstrates clear baseline resolution of all analytes. Dotted lines indicate the time setpoint for the wavelength switching process. This means not only moving the grating for excitation and emission wavelength, but also simultaneously setting two additional parameters. The Dionex rapid separation FLD features a unique variable high-pass emission filter. This unit automatically selects the optimum emission filter for the given wavelength pair to achieve the best stray light suppression. Simultaneously, a change of the photomultiplier sensitivity level may be required. Figure 2 indicates that often all four parameters must be changed.

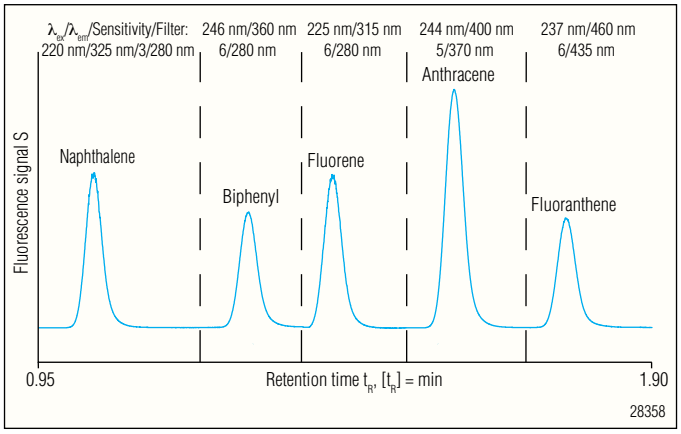

Figure 2. Fluorescence chromatogram of the test sample. Dotted lines indicate wavelength switches.

Figure 3 shows an enlargement of the baseline at 1.70 min, between anthracene and fluoranthene. While 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, therefore, is to minimize this influence using short, precise wavelength switching times. For Figure 3, the time required by the Dionex rapid separation FLD for the wavelengths and emission filter switching is as short as 0.405 s (relative standard deviation [RSD] 0.5%).

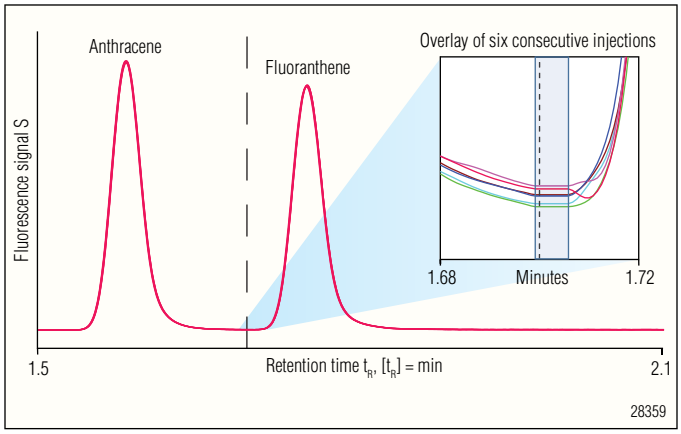

Figure 3. Overlay of six consecutive standard injections with a section of the baseline enlarged at 1.70 min.

Figure 4 focuses on the precision of the retention time and the area, with an area RSD for all peaks of only ~0.5%. The retention time precision is outstanding, with RSDs between 0.02 and 0.03%; this is a result of the precise pump flow and gradient proportioning of the Quaternary RSLC Pump and the seamless interplay between the different system components and the Thermo Scientific Dionex Chromeleon® Chromatography Data System (CDS) software control.

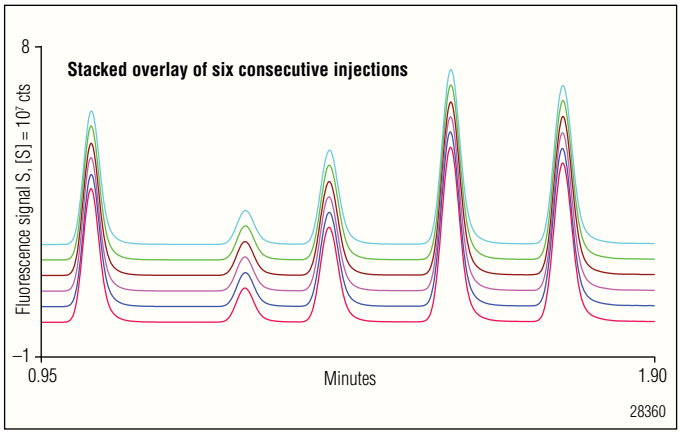

Figure 4. All analyte peaks show excellent retention time and area precisions, despite the wavelength switching processes between them.

Other vendors' detectors are not optimized to support these short switching times. Figure 5 displays a chromatogram obtained from a state-of-the-art third-party FLD, using the same LC front-end, parameter timing, and tubing as for those shown Figures 2 through 4.

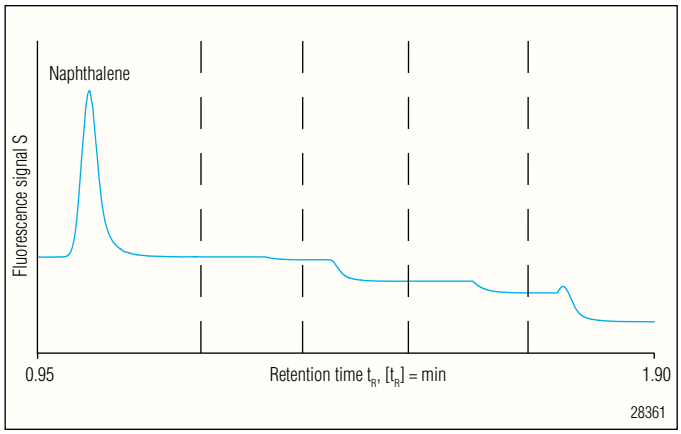

Figure 5. Chromatogram obtained using identical settings and method parameters, but with a third-party detector. Wavelength switching times are too long to detect the consecutive peaks.

Only the naphthalene peak is detected because the wavelength switching between the peaks requires so much time that the next peak completely or partly migrates through the flow cell before the detector is ready to acquire the emission again.

Figure 6 directly compares the output of the Dionex FLD (black) and the third-party FLD (blue). The tinted area represents the switching time of the third-party detector. Both detectors switch wavelengths at the same time; however, biphenyl is detected with the Dionex FLD, while the competitive instrument does not reach the new detection parameters before most of the peak has alreadypassed through the flow cell. The third-party detector requires 6.1 s for this switch, whereas the Dionex FLD accomplishes this in only 0.4 s.

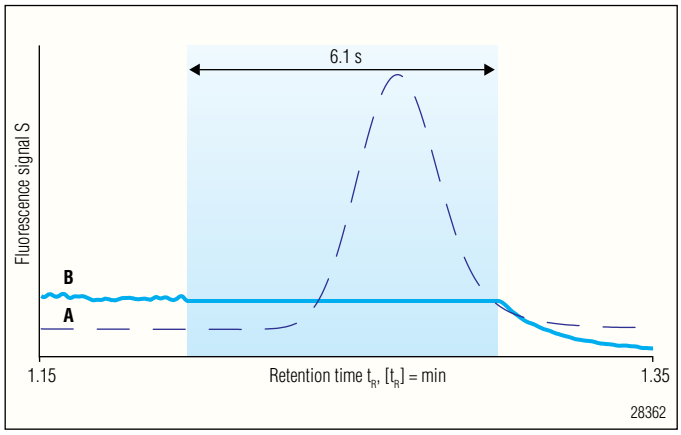

Figure 6. Overlay of data acquisition with Dionex FLD (A) and third-party detector (B). The peak cannot be detected by the third-party detector because it elutes during its wavelength switching time (tinted area).

It is possible to modify the switching times of the other detector using trial and error, so that peaks can be displayed (Figure 7). This chromatogram looks good at first glance, but a zoom to baseline level shows that peaks are not completely displayed. Small variations in retention and slight shifts in the duration of the wavelength switching process adversely affect the peak integration limits and, therefore, peak area precision.

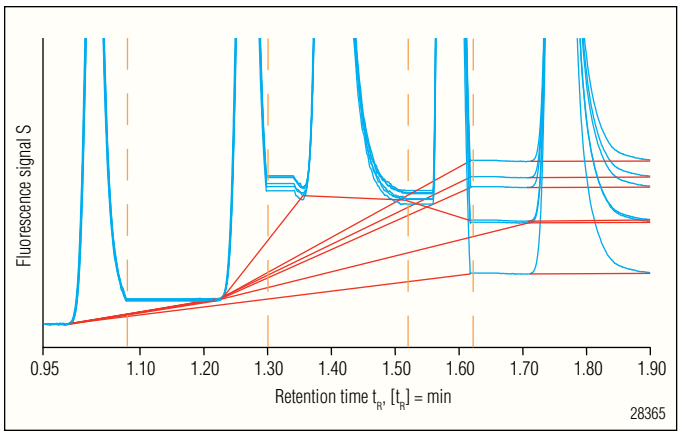

Figure 7. Overlay of six consecutive fluorescence chromatograms obtained with the third-party FLD. Dotted lines indicate wavelength switching events. Wavelength switching times that are too long for the given resolution result in imprecise peak integration.

The peak area precision of peaks 2 through 4 is poor using the third-party detector, with RSDs of 4.2 to 7.8% (Table 2). Due to the superior wavelength switching process of the Dionex FLD, RSDs of ~0.5% are achieved for all peaks. Long-term effects—such as slightly varying eluent compositions or decreasing separation efficiency—are also better compensated and do not affect area precision as much as with the other detector.

Table 2. Comparison of Peak Precisions with Third-Party and Dionex FLD

Analyte	Area Precision with Third-Party FLD (% RSD)	Area Precision with Dionex FLD (% RSD)
Naphthalene	0.87	0.55
Biphenyl	7.8	0.54
Fluorene	7.4	0.47
Anthracene	4.2	0.43
Fluoranthene	0.22	0.56

APPLICATION TO TEA SAMPLES

The high-throughput determination of PAHs in foodstuffs is a significant challenge because the analysis must be precise, and requires trace-level analysis in the low ppb range. This can be achieved both with GC and HPLC methods; the latter has the advantage of offering faster separations with run times of less than 10 min; while the lowest limits of detection (LOD) can be achieved using fluorescence detection. A high-resolution separation of the 17 fluorescent PAHs mentioned in the Environmental Protection Agency (EPA) Method 610 standard was developed using a dedicated C18 PAH stationary phase from Macherey-Nagel. The method parameters are listed in Table 3.

System

UltiMate 3000 Binary Rapid Separation LC System including the following modules:

- SRD-3400 Solvent Rack
- HPG-3400RS Binary RS Pump with Solvent Sector Valves
- WPS-3000TRS Well Plate Sampler
- TCC-3000RS Thermostatted Column Compartment
- FLD-3400RS Fluorescence Detector with Dual-PMT and analytical flow cell (8 µL)

All modules are connected with 0.005 in. (0.13 mm) i.d. Viper fingertight fittings.

LC Conditions

Eluent A:	Water
Eluent B:	Acetonitrile
Column:	MN EC 100/3 Nucleodur® C18 PAH, 3 × 100 mm, 3 µm
Flow Rate:	2.0 mL/min
Inj. Volume:	2 µL
Column Temp.:	30 °C
Data Collection Rate:	100 Hz
Response Time:	0.02 s
Lamp Mode:	Standard
Photomultiplier Tube:	1 (FLD-3400RS)

Table 3. Gradient Elution and Separation Conditions of the EPA Method 610 PAH Mix (17 Fluorescent PAHs)				
Time (min)	**% B**	**Excitation (nm)**	**Emission (nm)**	**Variable Emission Filter (nm)**
0	45	255	315	280
0.500	45			
2.560		244	360	280
2.750		244	400	370
2.970		237	460	435
3.150		237	385	370
3.400		277	376	280
3.762	90			
4.050		255	420	370
4.275	95			
4.800		300	415	370
5.264		250	495	435
5.288	95			
5.523	45			
5.650		225	315	280
6.169	45	237	460	435

Figure 8 shows the separation of the EPA Method 610 standard with a PAH concentration of 10 ng/mL each. All analytes are baseline separated in < 6 min; in particular, the highly critical 4- and 5-membered ring compounds (11–17) are well separated and demostrate excellent baseline resolution.

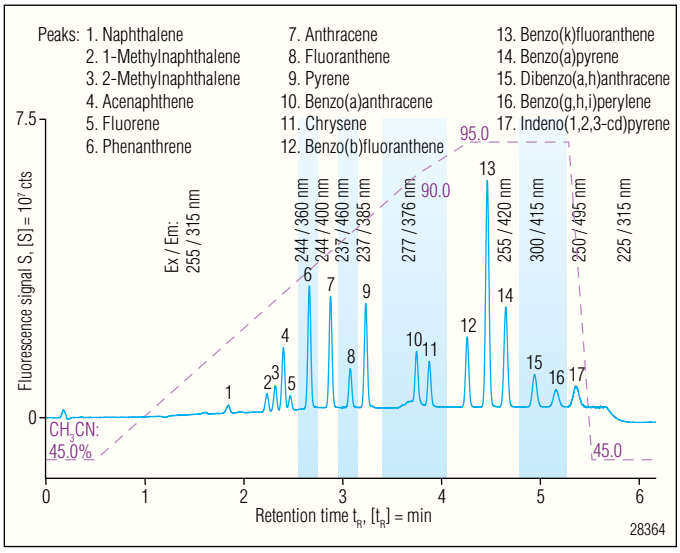

Figure 8. Separation of the 17 fluorescent PAHs of the EPA Method 610 standard mix; tints mark the dedicated detection parameters.

This separation has been applied to the analysis of PAHs in tea samples. The dried tea leaves typically require a solid–liquid extraction (SLE) for sample preparation and cleanup. In this case, an ultrasound-supported SLE with acetonitrile/acetone followed by filtration of the extract was performed prior to injection. Because this procedure is not assumed to be quantitative, the true amount of PAH is expected to be even higher than detected in these experiments.

Figure 9 depicts the analysis of four different tea solid-liquid extracts of varying type and processing mode. Several undiluted tea liquors have were also investigated; one (trace C) is shown for comparative purposes. Note: no low-condensed aromatics are present; only three-membered or higher-condensed rings are detected, which is of critical importance because these analytes have a very significant hazard potential. Phenanthrene, fluoranthene, and pyrene are present in almost all samples in remarkable concentrations of >10 ng/mL. However, the injection of undiluted teas (trace C as representative) reveals no measurable PAH content in the final beverage; hot water is an ineffective solvent for the extraction of PAHs present in raw tea leaves in significant amounts.

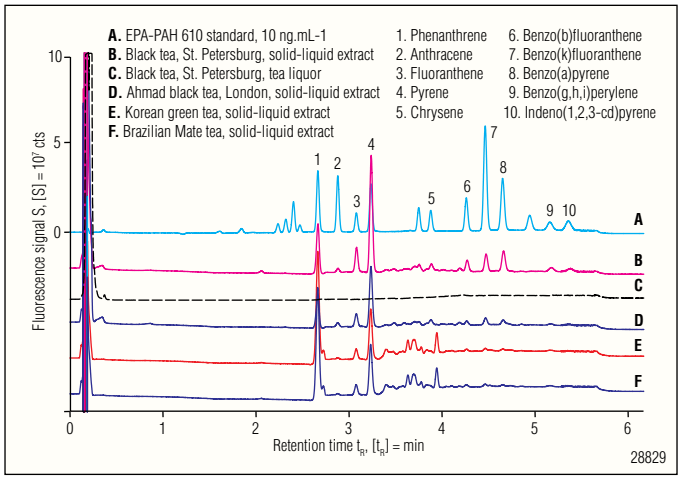

Figure 9. Separation of PAHs in tea samples.

CONCLUSIONS

- Dionex fluorescence detectors (FLD-3100/3400RS) are designed and ideally suited for UHPLC performance.
- These detectors achieve the fastest wavelength switching times in liquid chromatography. Other vendors' fluorescence detectors require significantly longer time for switching, which can lead to missing peaks or imprecise peak integration.
- The unique variable emission filter of the Dionex FLD-3400RS switches in a fraction of a second and provides optimum stray light suppression, even with ultrafast separations.
- In combination with the high-speed Dionex FLD-3100/3400RS detectors, a UHPLC separation method for the analysis of the 17 fluorescent EPA-610 standard PAHs in less than 6 min can be established, providing PAH determination in tea samples at low ppb range.

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2. Steiner, F.; Martin, M. M.; Franz, H. PAH in Tea—Ultra-Fast HPLC and Fluorescence Detection at Trace Levels. *labor&more* **2010,** 34–36. http://www.laborundmore.de/archive/764121/PAH-in-Tea.html (accessed Feb 14, 2011).

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