Application Handbook 70302

Fluorescence Method Development Handbook

Holger Franz and Verena Jendreizik;
Thermo Fisher Scientific, Germering, Germany

Key Words
UltiMate 3000 System, Vanquish System, Fluorescence, Synchronous, Excitation, Emission Scans, Variable Emission Filter, Sensitivity, Xenon Flash Lamp, Eluent Quality Test

Fluorescence spectroscopy is the most sensitive optical detection technique used with high-performance liquid chromatography (HPLC). Fluorescence of a molecule can occur naturally or can be achieved by labeling the molecule with a fluorescent tag in a derivatization reaction. A classic application of such a derivatization is for amino acid analysis. The chemical modification can be performed in an automated way prior to the chromatographic separation (precolumn)[1] or after the separation (postcolumn).[2]

In the flow cell of a fluorescence detector (FLD), the active molecule is exposed to light of a defined wavelength originating from a high energy light source, typically a xenon lamp. Within a few nanoseconds (ns), the excited analyte relaxes and emits the energy at a less energetic, longer wavelength.

A wavelength-selective FLD usually utilizes a photomultiplier positioned at an angle of 90° to the light source and detects the light that is emitted from the fluorescing compounds.

In contrast to UV-vis detectors, a fluorescence detector measures a very weak light signal rather than the difference between light intensities (absorbance). A related challenge is to separate high-intensity excitation radiation from low-intensity emission light; a related benefit of fluorescence detection compared to UV detection is its ability to discriminate an analyte from interferences or background peaks.[3] Generally speaking, fluorescence detection has different requirements compared to the almost ubiquitous UV detection.

This handbook demonstrates how to optimize fluorescence detection using the Thermo Scientific™ Dionex™ UltiMate™ 3000 FLD-3000 or the Thermo Scientific™ Vanquish™ Fluorescence Detector series (FL Detectors). This document guides the analyst through the different method development and optimization steps, recommends the most suitable procedures, and explains optical effects that might be observed. The handbook does not discuss special requirements when combining fluorescence detection with UHPLC.[4]

Conditions

Column:	Thermo Scientific™ Acclaim™ RSLC 120, C18, 2.2 µm, 3.0 × 100 mm (P/N 071604)
Standard:	PAH-Mix 221, Dr. Ehrenstorfer GmbH, P/N XS20950221AL, Lot 91124AL 12/2011 including naphthalene, anthracene, fluoranthene, pyrene and dibenzo(*a,h*)anthracene; 100 ng/µL in CH_3CN, Dilution 1:10 (standard 1) or 1:100 (standard 2) injection volumes 1 µL or 10 µL
Eluent A:	Water, Fisher Scientific Optima™ LC/MS (P/N W6-212)
Eluent B:	Acetonitrile, Fisher Scientific Optima LC/MS (P/N A955-212)

Gradient:	Time (min)	% Acetonitrile
	0.0	75
	2.0	75
	4.0	95
	5.0	95
	5.5	75
	7.5	75

Column Temp.:	45 °C
Fluorescence Detector Flow Cell Temp.:	45 °C
Flow Rate:	1 mL/min
Inj. Volume:	1 µL or 10 µL

Experimental

Initial Steps and Overview

As for all scientific work, method development for UHPLC with fluorescence detection starts with a literature review. This typically provides valuable information on the columns, eluents, and gradients used by other researchers. One must consider if the analyte fluoresces, and if so, what are the published excitation and emission wavelengths, and what is the UV absorption spectrum? An absorption spectrum is a good starting point for selecting the excitation wavelength of an analyte because the spectrum indicates which energy is absorbed to excite an electron to a higher quantum state. Ultraviolet-active substances transform electromagnetic radiation into heat; fluorescent compounds emit light of longer wavelength during the relaxation process.

In preparation for the initial experiments, make sure that the mobile phase is compatible with highly sensitive fluorescence measurements. Incompatible solvents may create background fluorescence or stray light. This reduces the dynamic range of the detector, directly contributes to baseline noise, and therefore adversely affects the limit of detection (LOD). Solvent suppliers typically specify if solvents are suitable for fluorescence spectroscopy.

Figure 1 provides an overview of the method development steps discussed in this document.

Initial Experiments

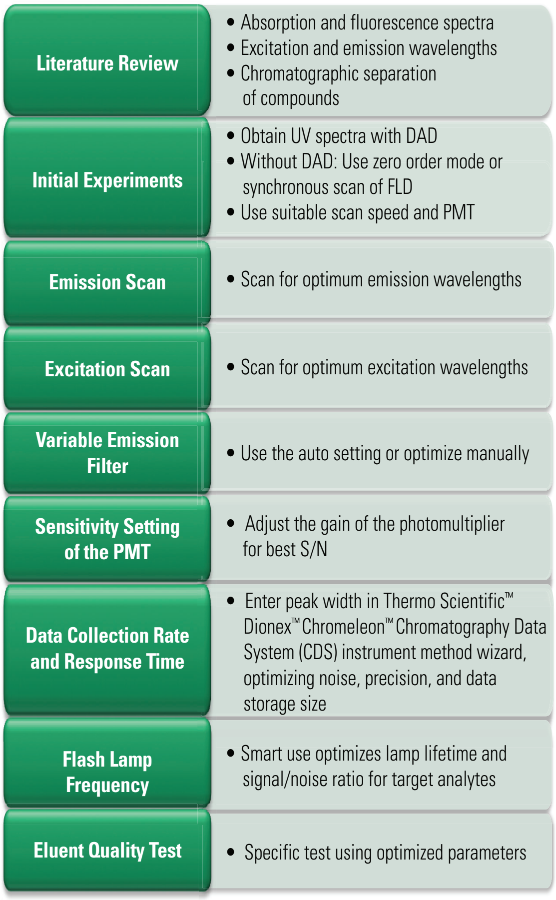
A suitable UHPLC system may use an FLD as the only detector, or an FLD in combination with a diode array detector (DAD). This combination is not required for routine use, but is useful for method development. The following sections explain how to work efficiently with both system configurations.

System with DAD and FLD

The first step is to optimize the chromatographic separation. The easiest way is to use a DAD, which can be considered a universal detector for fluorophores, because the absorption spectrum for the molecule is typically similar to the excitation spectrum. Fluorophores can be detected by UV-vis detectors and at the same time the obtained spectra indicate how to excite them. Note that the applied standard must be typically 50–100 times more concentrated than for the later fluorescence detection.

This handbook does not focus on optimization of the separation but uses constant separation conditions with respect to column selection and gradient profile. All work is performed using a mixture of five polycyclic aromatic hydrocarbons (PAHs). The PAHs are easy to separate and detect at higher concentrations with a DAD, as shown in Figure 2. Note that the four inital peaks are eluted isocratically; the gradient is applied to speed the elution of the fifth peak.

The DAD now provides easy access to UV spectral



Literature Review	• Absorption and fluorescence spectra • Excitation and emission wavelengths • Chromatographic separation of compounds
Initial Experiments	• Obtain UV spectra with DAD • Without DAD: Use zero order mode or synchronous scan of FLD • Use suitable scan speed and PMT
Emission Scan	• Scan for optimum emission wavelengths
Excitation Scan	• Scan for optimum excitation wavelengths
Variable Emission Filter	• Use the auto setting or optimize manually
Sensitivity Setting of the PMT	• Adjust the gain of the photomultiplier for best S/N
Data Collection Rate and Response Time	• Enter peak width in Thermo Scientific™ Dionex™ Chromeleon™ Chromatography Data System (CDS) instrument method wizard, optimizing noise, precision, and data storage size
Flash Lamp Frequency	• Smart use optimizes lamp lifetime and signal/noise ratio for target analytes
Eluent Quality Test	• Specific test using optimized parameters

Figure 1. Overview of fluorescence method development steps discussed in this document.

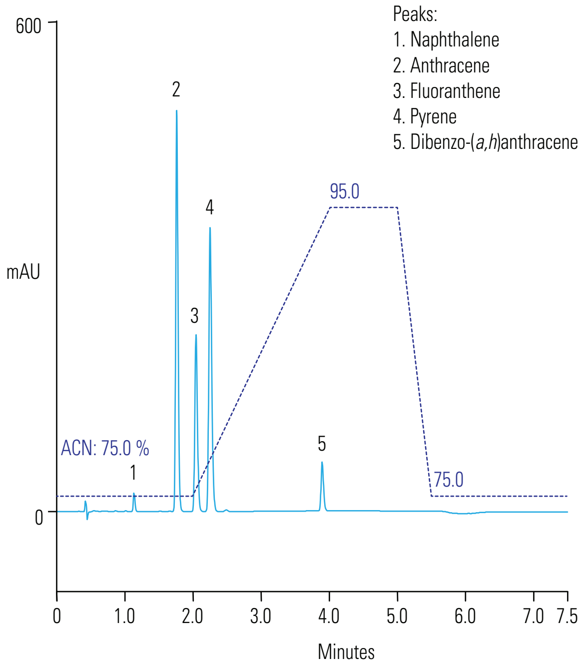


Figure 2. Separation of five PAHs with UV detection at 240 nm, standard 1, 10 µL injection.

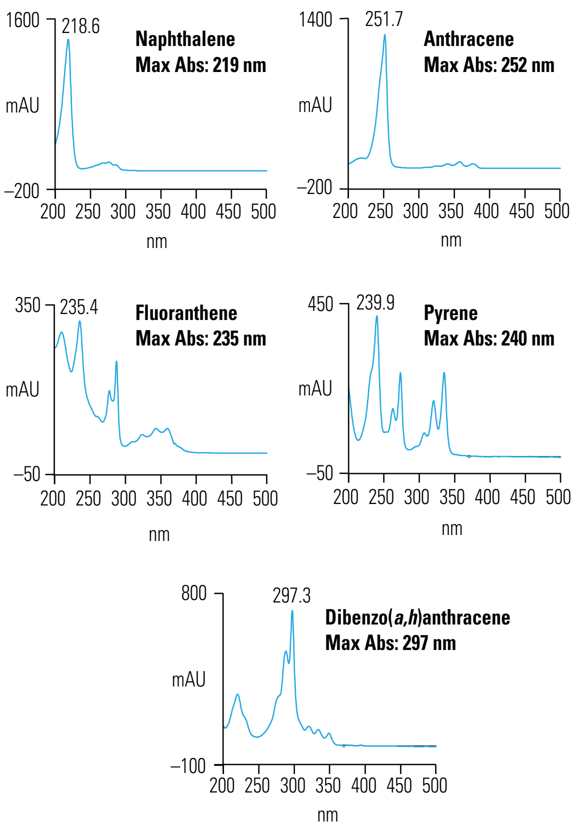


Figure 3. The UV absorption spectra of the five analytes, which also indicate the excitation maximum of each.

information for each compound. Figure 3 shows all five spectra (obtained at peak apex) and determines the wavelength of the absorption maximum. As the absorption maximum is almost identical with the excitation maximum of the analyte, the excitation wavelength is typically chosen close to the absorption maximum.

System with FLD Only

The FL Detectors support two operation modes that are recommended for initial detection when little is known about the nature of the analytes and their retention times. Note that all the following FLD experiments were performed with the standard photomultiplier tube (PMT) that can be used in the emission wavelength range of 220–650 nm because all compounds of the test application fall within this range. The FL detectors are also available in a unique Dual-PMT configuration. This configuration adds a second PMT, which expands the emission wavelength range up to 900 nm. The benefit of the Dual-PMT is that the wavelength range can be expanded without sacrificing sensitivity in the UV range, as commonly occurs with other FL detectors (Figure 4).

The Dual-PMT can be ordered with the detector or as an upgrade. If analytes are present that require detection in the visible or near-infrared range, use the extended wavelength range of the Dual-PMT.

Zero Order Mode

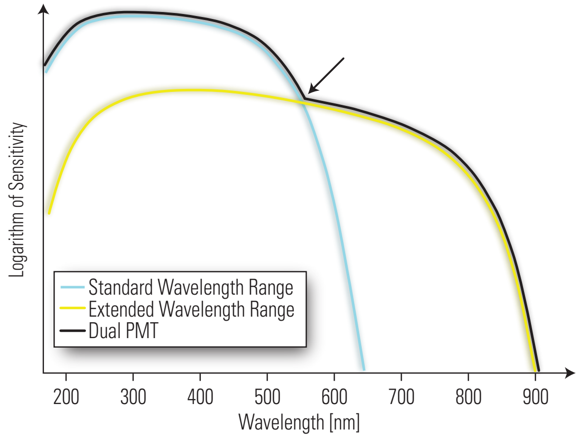


Figure 4. The Dual-PMT option of UltiMate 3000 and Vanquish FL detectors are a unique solution for providing optimum sensitivity across all wavelengths.

In zero order mode, the grating of the emission monochromator reflects the entire emission spectrum of the sample onto the PMT, rather than only a single wavelength. The excitation monochromator is set to a single wavelength, as usual. Based on literature reviews and other UV-absorption documentation, the excitation light must have a wavelength somewhere near the excitation maximum of the target analytes to detect peaks in zero order mode. For instance, PAHs can be excited at 220–250 nm, although the optimum excitation wavelength may be quite different.

Figure 5 compares two chromatograms obtained in zero order mode with (A) excitation at 220 nm and (B) excitation at 250 nm. The responses for the five peaks in chromatogram (A) are similar, whereas chromatogram (B) is dominated by the size of the second peak, anthracene. This compound has its excitation maximum at 250 nm; however, all other peaks still can be detected. At both 220 and 250 nm, all analytes can be excited and their retention times can be determined and optimized. Zero order mode allows simultaneous detection of all emitted light, independent of the emission spectrum.

Synchronous Scans

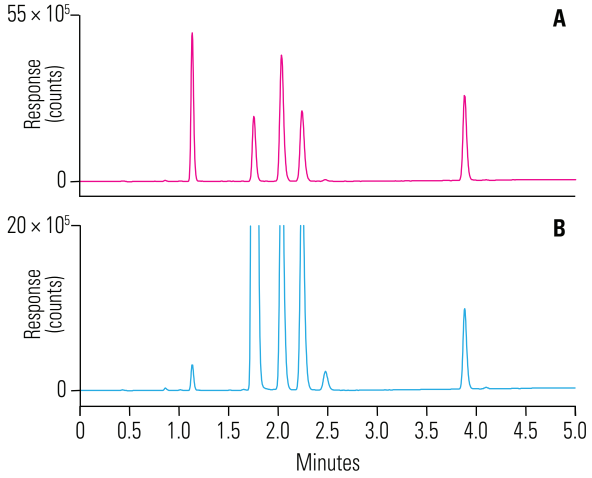


Figure 5. Different excitation wavelengths can be used for detection in zero order mode, as long as the analytes can be excited at all. Depending on the characteristics of the analytes, the response varies.

The synchronous scan is a tool that provides fluorescence response as long as any combination of excitation and offset results in a measurable light emission. This mode is even more generic than the zero order mode because knowledge about the excitation range of the analyte is not required. Use this mode in the early chromatographic development stage: for instance, to optimize the chromatographic resolution. It is not a tool for discovering the most suitable excitation or emission wavelengths.

A user-defined excitation wavelength range is scanned, whereas the emission wavelength is synchronously scanned with a fixed distance to the excitation wavelength. This scan is repeated over the entire data acquisition time, resulting in a DAD-like 3D data field called FL field. The offset must be reasonably large, such as 30-100 nm, because emitted light typically has less energy than the absorbed light (Stokes shift). This is another approach to determine the retention times of the target analytes. In addition, rough excitation and emission wavelengths pairs can be obtained; however, optimum excitation and emission wavelengths can only be determined by performing individual excitation and emission scans.

Figure 6 shows how a synchronous FL field is displayed in Chromeleon software. In a synchronous FL field, the y-axis represents the excitation wavelength. The emission wavelength is Em=Ex+offset. In the example shown in Figure 6, the offset is 30 nm. Using this plot, wavelength–specific chromatograms can be extracted.

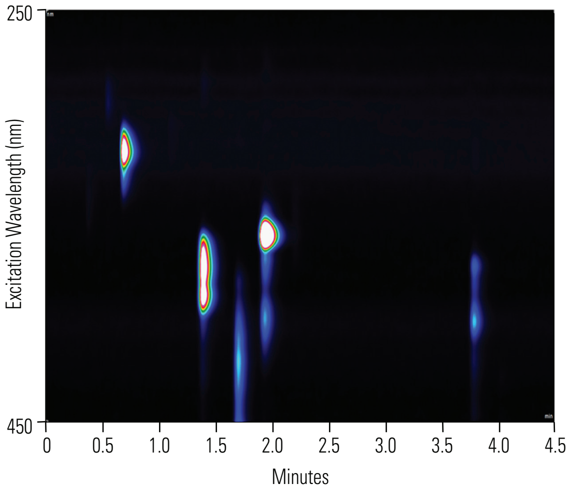


Figure 6. Contour plot of a synchronous FL field with 30 nm offset between excitation and emission wavelengths.

Figure 7 shows an example of a single synchronous scan.

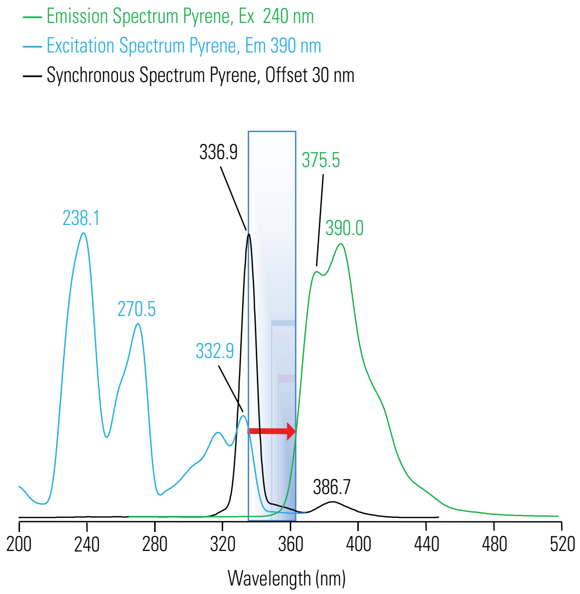


Figure 7. Synchronous scan of pyrene at 30 nm offset (black), overlaid with the respective excitation (blue) and emission spectra (green). The synchronous scan maximum represents the excitation wavelength at which the best response is obtained with the given offset.

The black synchronous spectrum is a result of the combination of the excitation and the emission spectra also provided in Figure 7. The synchronous spectrum maximum at 337 nm represents the most suitable excitation and emission wavelength combination at the given offset of 30 nm. The excitation wavelength of 337 nm is close to pyrene's excitation maximum at 333 nm. The emission wavelength of 367 nm (337 nm + 30 nm) provides a fair light intensity.

A shift to a shorter wavelength—for instance, to 333 nm—improves the excitation but decreases the emission response. A slight shift toward 345 nm does the opposite: excitation reduces as emission improves. Given the 30 nm offset, an excitation at the excitation maximum of 238 nm would result in almost zero response.

For all scans, the selection of a suitable scan speed is important. In Chromeleon CDS software, the scan can be set to four different speeds: slow, medium, fast, and superfast. Slow provides the lowest noise because it efficiently combines small wavelength steps with a significant number of data point repetitions at each wavelength selection. This speed is ideal for monitoring the quality of a mobile phase, but it is typically too slow to be used for scanning peaks.

Figure 8 identifies the recommended scan speed based on

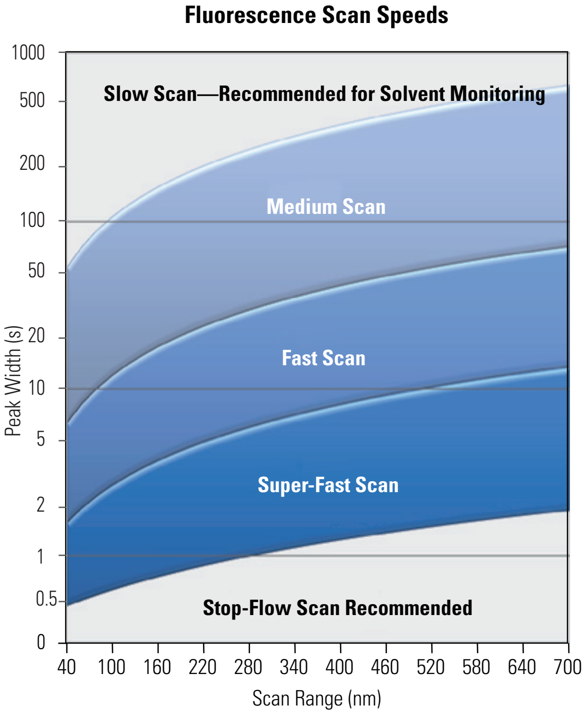


Figure 8. The scan speed can be selected based on expected peak width and scan range. Even UHPLC peaks are easily supported. Note that the y-axis uses a logarithmic scale.

the peak width and the scan range. If the peak width/scan range coordinates are within a certain scan speed range, a few repeated scans per peak are guaranteed, which is enough to detect a peak and measure related spectra.

Therefore, the majority of UHPLC and HPLC peaks, even at sub-2 second peak widths, can be detected by the fast scan speed.

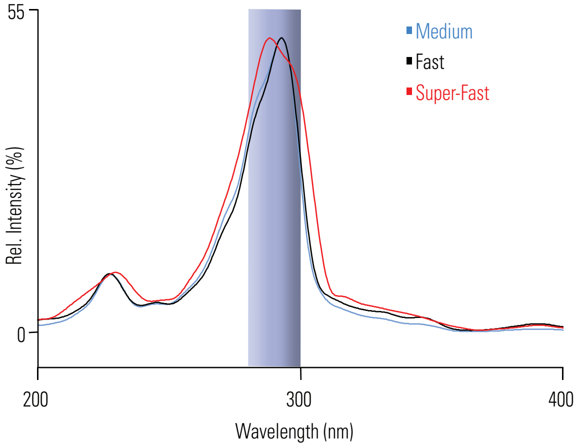


Figure 9. Overlay of dibenzo(a,h)anthracene excitation spectra obtained with medium (blue), fast (black), and superfast scan speed (red). Medium and fast spectra are similar; the superfast spectrum is not as precise but supports scanning of very narrow peaks.

Varying scan speeds will impact data quality. Figure 9 compares dibenzo(*a,h*)anthracene excitation spectra obtained at different speeds. The very similar medium and fast excitation spectra vary to some extent from the superfast spectrum. Medium and fast scan acquisitions differ by a wavelength step difference of only 0.3 nm. Their main differentiation is the number of flashes obtained per wavelength step, influencing noise.

For the sake of speed, the superfast scan provides only 1/10 of the wavelength steps of the fast scan. The lack of wavelength steps explains why the shape of the superfast excitation spectrum in Figure 5 is similar but not very close to the spectra obtained at slower scan speeds. The excitation maxima, however, only vary by 5 nm. This difference is close enough to the correct maximum, considering the 20 nm spectral resolution of the FL Detectors. The superfast scan is therefore suited for an initial screening of very narrow peaks obtained in ultrafast separations. If very narrow peaks require superfast scans, it is possible to repeat the experiment at fast scan speed and a reduced wavelength range of 40–60 nm around the maximum.

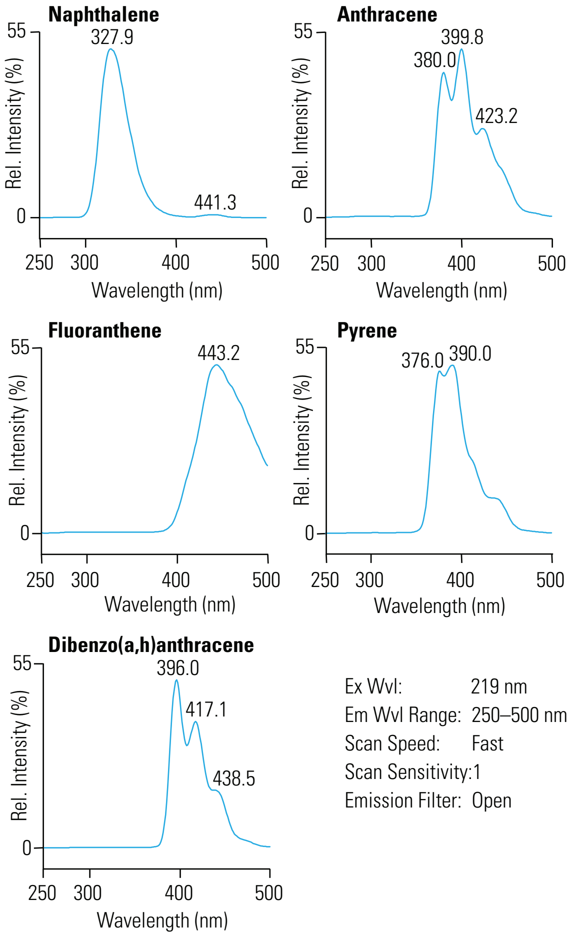
In general, the fast scan speed is a good default setting. It is suitable to scan typical peak widths of both conventional HPLC and UHPLC separations. By selecting a smaller wavelength range, the scan rate and therefore the support of narrower analyte bands can be further improved.

Optimizing the Emission Wavelength

Once the chromatographic method development is performed with the help of a UV detector, the zero order mode, or the synchronous scans of the FL Detectors, the next step is to optimize the detection parameters of the fluorescence detector to achieve the best signal-to-noise (S/N) ratio. The first tool for this optimization process is the emission scan feature.

Both, the UltiMate 3000 and Vanquish fluorescence detectors support the acquisition of permanent emission scans. During the scan, the excitation remains constant at a defined wavelength. Across the entire acquisition time, the emission mono-chromator repeatedly scans a user-defined emission wavelength range. Similar to the synchronous scan, this results in a DAD-like 3D field, called emission field. However, the synchronous scan is a more generic approach because an excitation wavelength does not have to be defined. Instead, only a reasonable Stokes shift is entered (also see: Synchronous Scans).

Figure 10 shows emission spectra extracted from a single separation. The data were obtained by setting the emission



Ex Wvl: 219 nm
Em Wvl Range: 250–500 nm
Scan Speed: Fast
Scan Sensitivity: 1
Emission Filter: Open

Figure 10. Emission spectra obtained from a single chromatographic run with emission FL Field acquisition. The optimum emission wavelength for each compound is at the absolute maximum of the emission spectrum.

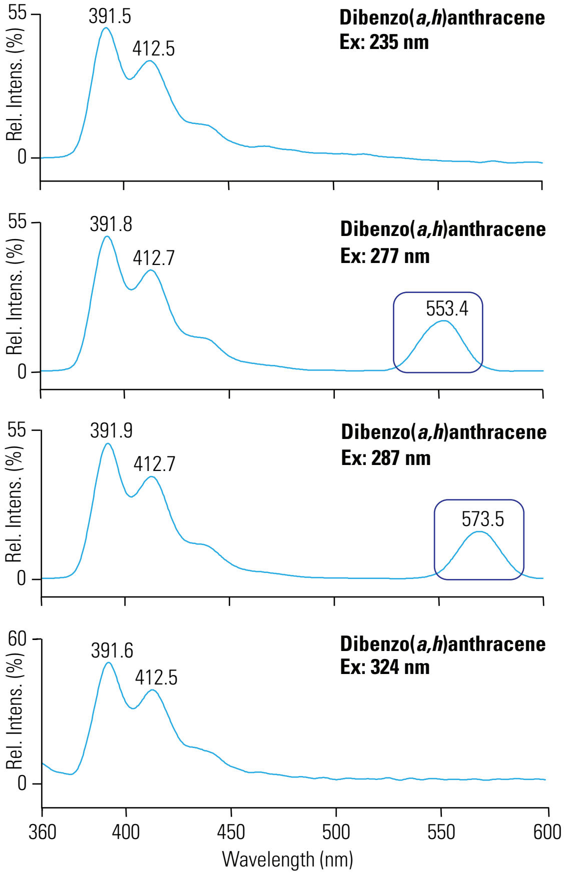


Figure 11. Dibenzo(a,h)anthracene emission spectra extracted from four different emission fields. Each spectrum was obtained with a different fixed excitation wavelength. The relative emission spectra are not influenced by the excitation wavelength selection, except for the highlighted areas which are a consequence of second order diffraction light.

scan speed to fast and the constant excitation wavelength to 219 nm, because the naphthalene UV spectrum in Figure 3 suggested that 219 nm is a good excitation wavelength for this compound. However, it is likely that exciting at 219 nm is not ideal for the other analytes. When compounds show quite different excitation spectra, it is questionable whether the excitation wavelength chosen has an impact on the resulting emission spectra.

Figure 11 shows four emission spectra of dibenzo(*a*,*h*)-anthracene obtained from four different emission FL Field acquisitions. The emission wavelength range remained constant, the excitation wavelength was alternated between 235 nm, 277 nm, 287 nm, and 324 nm. All spectra look qualitatively the same, except for the peaks indicated by the blue rectangles. These peaks are a result of second order diffraction light of the applied excitation wavelengths. Light diffraction at a grating occurs as a first, second, or higher order process. These diffraction orders frequently overlap. Second order diffraction light, for example, is always visible at twice the excitation wavelength; so when exciting at 287 nm, second order light is visible at 574 nm.

In an optical detector, higher order diffraction light is typically suppressed by optical long-pass filters. The Vanquish Fluorescence Detector F and the UltiMate 3000 FLD-3400RS feature a unique variable emission filter that automatically selects a suitable emission cut-off filter element.

The ideal filter cut-off wavelength is between the excitation and the emission wavelengths. In the case of

Figure 11, the filter was set to 280 nm for all runs to provoke the occurence of second order diffraction light. For excitation at 277 nm, the second order effect is visible because the filter does not cut off sharply at 280 nm and has a certain transition range. This allows light of 277 nm to partially pass. The ideal emission filter depends on the excitation and the emission wavelength. The nominal filter wavelength should be between the excitation and the emission wavelength. Therefore, for excitation wavelengths of 277 nm or 287 nm, the selection of the next higher emission filter (370 nm) would suppress higher order effects. The emission range must be adjusted so that it does not overlap with the excitation. During scans, light of higher diffraction orders typically can be recognized by the band that is present during the complete separation (Figure 12).

The FLD-3100 fluorescence detector uses a fixed 280 nm emission filter, similar to other FLD detectors. With this configuration, second order light from excitation wave-lengths around 280 nm and higher cannot be suppressed.

Returning to the emission field example of dibenzo(*a*,*h*) anthracene, Figure 13 shows an excitation spectrum of this molecule. The excitation maximum is at 293 nm. Below this wavelength, excitation decreases but remains present. It is therefore possible to excite dibenzo*(a,h)*anthracene across the range of 220–300 nm. Typically, the obtained emission intensity will vary with the selected excitation wavelength, but the qualitative appearance of the emission spectrum remains unchanged.

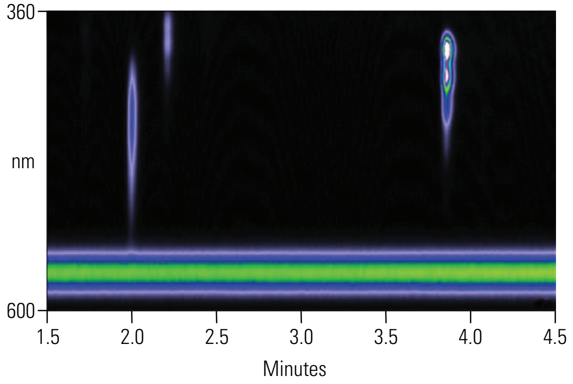


Figure 12. An emission FL Field with an excitation wavelength of 287 nm. Second order stray light is visible as a permanent band at 554 nm.

In summary, optimum emission wavelengths can easily be extracted from emission FL Fields. The Vanquish FLD-F and the UltiMate 3000 FLD-3400RS detector are most efficient in suppressing higher order diffraction light because they automatically select the best suitable emission filter. The emission spectrum is typically not affected by the excitation wavelength (also known as Kasha's rule).[5] Analysts using the UltiMate 3000 or Vanquish FL Detectors can therefore typically extract optimum emission wavelengths for all analytes from a single emission FL Field.

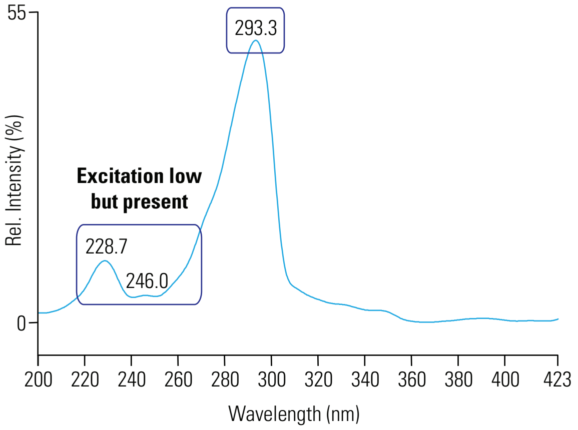


Figure 13. Excitation spectrum of dibenzo(*a,h*)anthracene. The molecule can be excited best at 293 nm but also in the range down to approximately 220 nm.

Optimizing the Excitation Wavelength

The second part of the wavelength optimization is the identification of suitable excitation wavelengths. The easiest way to do this is to acquire an excitation FL Field. During the FL Field acquisition, the emission wavelength remains constant. Across the entire acquisition time, the excitation monochromator repeatedly scans a user-defined excitation wavelength range. Similar to synchronous and emission FL Fields, this results in a DAD-like data field.

The detector logic requires constant settings of the scan range and the emission wavelength across the entire chromatogram. For a separation of quite different analytes, as in the example, there are options to run an FL Field sample with the optimized emission wavelength for each compound, or to use a generic emission wavelength across all analytes (i.e., a single run for all peaks). This leads to a question similar to that raised for the emission FL Field: does the selection of the constant emission wavelength influence the shape of the observed excitation spectrum?

Figure 14 shows an emission spectrum of dibenzo(*a*,*h*)-anthracene. With UltiMate 3000 and Vanquish FL

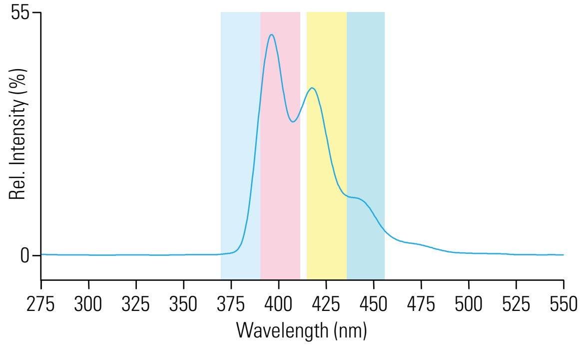


Figure 14. Emission spectrum of dibenzo(*a,h*)anthracene. The colored boxes indicate the bandwidths of four different emission wavelengths. These were used as constant emission wavelengths for the excitation spectra in Figure 15.

Detectors, the spectral emission bandwidth is 20 nm, indicated by the width of the colored boxes in the figure. The positions of the boxes represent the effective bandwidths of four emission wavelengths which were used in an excitation scan experiment (Figure 15). Figure 14 demonstrates that the wavelengths lie well within the emission spectrum of dibenzo(*a,h*)anthracene. As long as this is the case, the selection of the constant emission wavelength does not affect the excitation spectrum.

Figure 15 shows four excitation spectra of dibenzo(*a,h*)-anthracene (200–360 nm) extracted from four excitation FL Field acquisitions. Each spectrum was paired with a different emission wavelength, as indicated in Figure 14. All relative intensity spectra are similar, so as long as the constant emission wavelength is part of the emission spectrum of an analyte, it can be considered valid. The intensity of the spectrum varies depending on the efficiency of the analyte's emission at the selected emission (Em) wavelength. This, however, has no impact on the identification of the best suitable excitation wavelength. Depending on the nature of the analytes in a separation, a single excitation scan may be sufficient. If the emission spectra differ to the extent that there is hardly any overlapping emission range, more than one excitation scan with varying scan ranges and emission wavelengths may be required. The same may be true if excitation and emission spectra show considerable overlap (as in the case of anthracene).

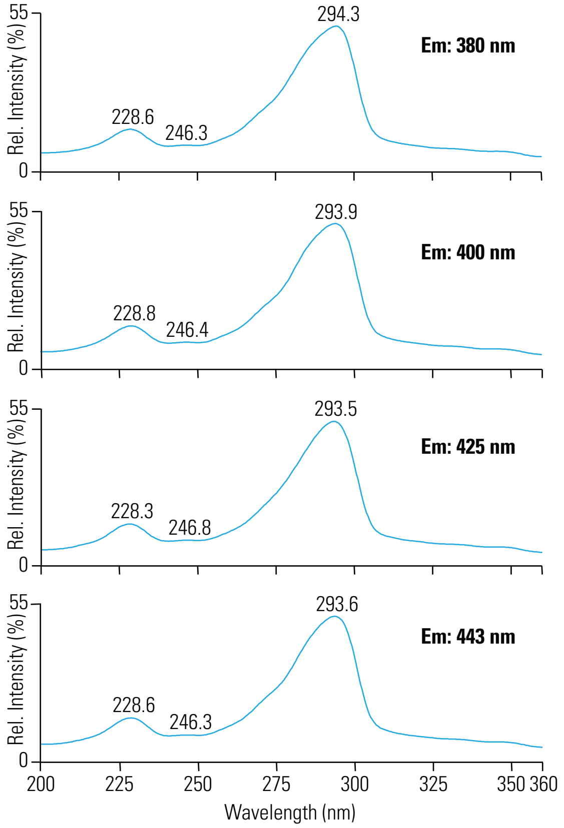


Figure 15. Excitation spectra of dibenzo(*a,h*)anthracene pairing with different emission wavelengths: 380 nm, 400 nm, 425 nm, and 443 nm. In all cases, the resulting relative excitation spectra are similar. The relative intensity Ex spectrum is independent of the Em wavelength as long as the compound emits light of the selected wavelength.

The excitation spectra in Figure 15 were obtained with the scan signal type **Standard.** A scan signal type determined if the recorded signal will be corrected regarding the xenon lamp spectrum (**ExCorrected**) or not (**Standard**). **Standard** provides the best S/N ratio for the detector type used and is therefore the default setting. **ExCorrected** provides best comparability with spectra in the literature.[6]

Optimizing the Emission Filter

The Variable Emission Filter (VEF), also referred to as the Filter Wheel, is a unique tool to suppress stray light and higher order effects. The VEF is located before the emission monochromator (Figure 16).

The VEF holds an empty position and four different long-pass filters (280 nm, 370 nm, 435 nm, and 530 nm), which block light below the respective wavelength. Within a fraction of a second, a fast-turning motor moves the selected filter into the light path. The filter setting can be changed at any time during the separation, so each analyte can be detected under optimized filter conditions. The UltiMate 3000 FLD-3100 fluorescence detector operates with a constant 280 nm emission filter, as is

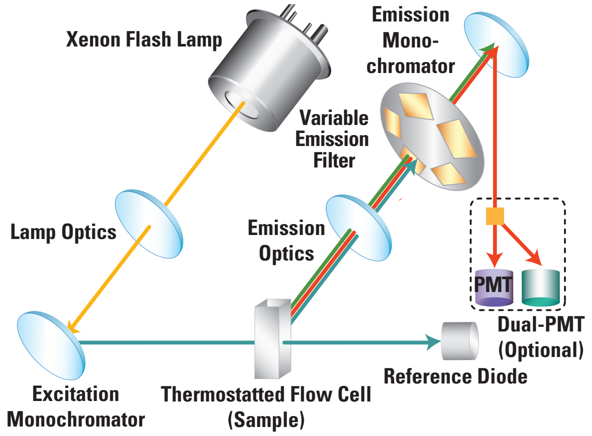


Figure 16. Schematic of the optical design of the Vanquish and UltiMate 3000 FL Detectors. The VEF is located before the emission monochromator.

common for FLD detectors. When applying the auto setting of the VEF, the built-in logic of the detector selects the best filter for the vast majority of analytes and wavelength combinations. Figure 17 provides an example. The graph shows the relationship between filter setting and the S/N ratio for two analytes. The filter that achieves the highest S/N is labeled with the related ratio. In both cases, the 370 nm filter provides the best result. This filter also would have been selected by the Auto setting.

Note that the 435 nm cut-off filter still allows a certain part of the light to pass, although the emission wavelengths of fluoranthene and pyrene are below the cut-off filter wavelength (443 nm and 390 nm). The reason for this is the 20 nm bandwidth of the emission monochromator and the transmission characteristics of the filter. At the specified cut-off wavelength, 50% of the light can pass. As indicated in Figure 18, the usable emission wavelength range starts at 15 nm below the nominal filter wavelength (i.e., for a 280 nm filter, at 265 nm).

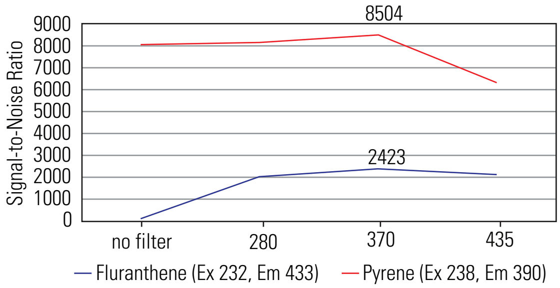


Figure 17. Signal-to-noise ratio for two analytes with different cut-off filter settings. The best result is obtained with the 370 nm filter, which would also be selected by the Auto setting.

In summary, the Vanquish F and FLD-3400RS FL Detectors automatically pick the best suitable filter for a selected wavelength pair. This process reduces stray light and therefore improves S/N by design.

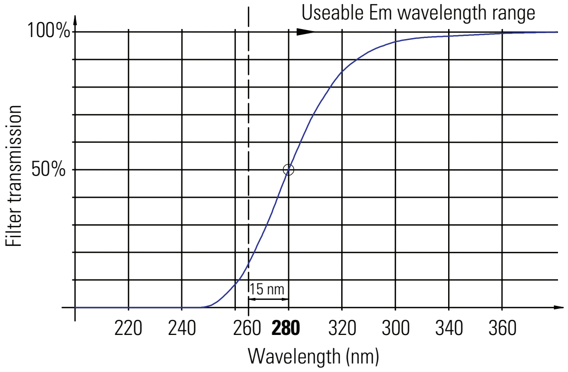


Figure 18. At the specified cut-off wavelength of the filter, 50% of the light is transmitted. The usable emission wavelength range starts at 15 nm below the nominal filter wavelength.

Optimizing the Sensitivity Setting

All commercial FLDs for use with HPLC use a PMT for measuring the intensity of the emitted light. It is therefore important to understand the capabilities and limitations of this device. A PMT can be regarded as a current source that generates a current proportional to the light intensity to which it is exposed.

A PMT consists of a photocathode, several dynodes, and an anode. When photons hit the photocathode, electrons are ejected from its surface. Between the photocathode and the first dynode is an electrostatic potential difference that accelerates these electrons towards the dynode. Based on the phenomenon of secondary emission, this dynode emits a larger number of electrons. A following cascade of dynodes functions as an electron multiplier. Depending on the voltage difference between the dynodes, fewer electrons are emitted at each incident (i.e., the gain of the chain of dynodes can be adjusted by the applied voltage). Finally, a current pulse arrives at the anode.[7]

Depending on the wavelength of the detected light and its intensity, it is useful to tune the voltage applied to the PMT. Too large a voltage can cause an electron overflow or even damage the light-sensitive photocathode. Too low a voltage can result in increased noise and therefore in a reduced S/N ratio. The FL Detector property for adjusting the gain of the PMT is called **Sensitivity**. At any time of data acquisition, this setting can be altered between 1 and 8; 1 represents the lowest and 8 the highest settable voltage (i.e., the sensitivity of the PMT to light). Typically, the best limit of detection can be obtained with a setting of 5 or 6.

The intensity of the emission light (measured by the PMT) is normalized with the intensity of excitation light passing through the sample measured by the reference diode (Figure 16). Therefore, the values (counts) that the emission channel displays cannot be used for optimizing the sensitivity. Instead, a different procedure is required to tune the sensitivity setting for the best S/N ratio. The schematic of this procedure is depicted in Figure 19.

At this point of the method development, elution windows

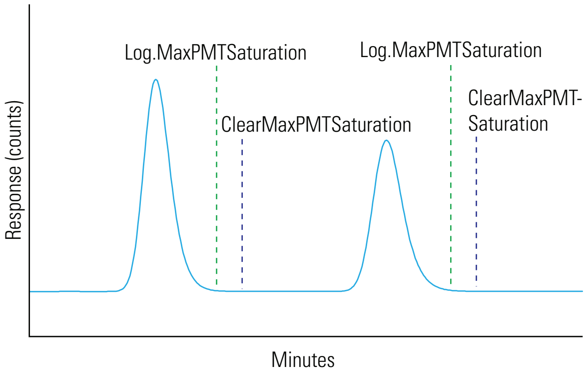


Figure 19. *MaxPMTSaturation* is a useful parameter to optimize the sensitivity of the PMT.

of peaks of interest are known. Between the elution of the peaks, a pair of commands can be placed to determine the PMT saturation, i.e. the ratio between light actually received and the maximum allowed light level.
To optimize this setting, use a sample or standard with highest expected concentration.

The parameter **MaxPMTSaturation** indicates the highest saturation of the PMT signal measured since the last reset. Logging this value after the elution of a peak with the command **log.MaxPMTSaturation** will create an entry in the sample/injection audit trail. Before the next eluting peak, reset the PMT saturation with the **ClearMaxPMTSaturation** command. Repeat the process after each peak.

The saturation of the PMT is ideal when it is between 30–80% (Table 1). If the saturation is below 30%, increase the sensitivity so that it reaches the optimum range. A one-step increase of the sensitivity increases the peak height by a factor of approximately 2. When the PMT is 100% saturated, the detector automatically reduces the sensitivity setting. This ensures that the PMT is not damaged. However, any peak which causes the PMT overflow cannot be used for quantitative analysis.

Note that a PMT overflow at sensitivity 1 does not lead to a reduced sensitivity, but reports an error in the audit trail, and potentially leads to a non-optimal peak shape and probably incorrect quantification of the peak.
Thus, for a successful quantification, reinject the sample and reduce the sensitivity setting as suggested by the Chromeleon CDS software sample audit trail, or dilute the sample if sensitivity 1 is still too sensitive.

Table 1. Recommended actions to optimize the sensitivity setting.

Value	Action to Optimize
<30%	Increase the sensitivity.
30%–80%	The sensitivity value is optimal.
80%–99%	The sensitivity should be reduced by one step to avoid undesired saturation when concentration varies.
≥100%	The detector reduces the sensitivity automatically (autoranging).

Note that it is not mandatory to optimize the sensitivity; it is possible to operate the PMT at a lower-than-optimal setting.

Figure 20 provides an example of how much the signal height, noise, resulting S/N, and PMT saturation change, depending on the sensitivity setting. When changing from 1 to 5, the noise increases from 2501 to 10,759 counts, equating to a factor of 4 (calculated with equation 1). At the same time, the peak height increases by a factor of almost 17. As a consequence, the S/N ratio improves by a factor of 4. Sensitivity 5 provides the best S/N ratio; however, it is only slightly better than sensitivity 4. So sensitivity 4 may be the better choice because it provides a larger dynamic range; it can handle unknowns with concentrations 2× higher than those of sensitivity 5.

Equation 1: $$S/N = 2 \cdot \frac{\mathit{Peak\ Height}}{\mathit{Noise}}$$

Sensitivity	Height dibenzo(*a,h*)anthracene (counts)	Noise (counts)	S/N	PMT Saturation (%)
1	3.2×10^6	2501	2537	4
2	7.0×10^6	1754	7929	9
3	15.1×10^6	3518	8576	19
4	28.0×10^6	5749	9755	36
5	53.8×10^6	10759	9998	69
6	PMT overflow			

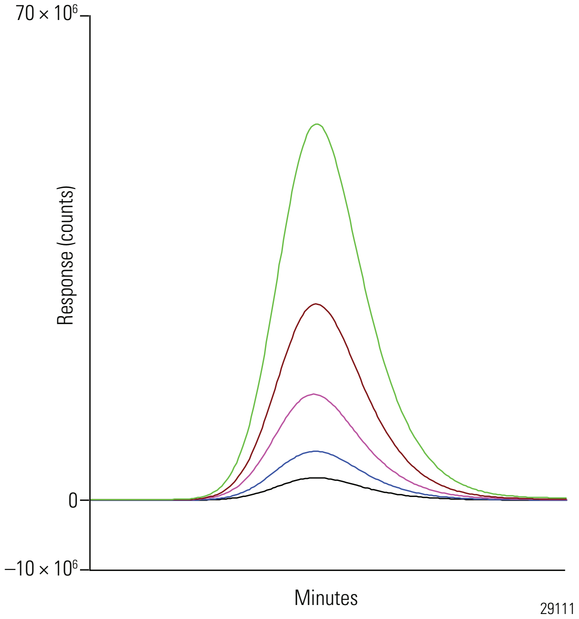


Figure 20. Effect of the sensitivity setting on the peak height, noise, and S/N ratio of dibenzo(*a,h*)anthracene. The S/N improves by a factor of 4 from sensitivity 1 to sensitivity 5. At sensitivity 6, the PMT overflows (standard 2, 1 µL injection).

In summary, the sensitivity setting allows tuning of the PMT for the best S/N ratio. If different sensitivities provide the same S/N ratio, it is beneficial to use the lower setting because it supports higher concentrated samples. The optimization can easily be done with the command pair of **MaxPMTSaturation** and **ClearMaxPMTSaturation.** If a PMT overflows in routine operation, the detector automatically reduces the sensitivity setting. Rerun the same sample with the adjusted sensitivity in order to quantify the peak that previously caused the PMT overload.

Data Collection Rate and Response Time

Data collection rate (DCR) and response time are settings which influence data storage size and S/N ratio.

The DCR is the number of data points per second generated by the detector electronics. For good precision during quantitative analysis, a peak should be defined by ≥20–30 data points. If the data collection rate is too low, the start and end points of peaks will not be determined accurately. If the data collection rate is too high, data files may occupy excessive disk space and post-run analyses may require more processing time.

The DCR is typically paired with a mathematical data filter called response time (or simply response or rise time). The response time is a measure of how quickly the detector responds to a change in signal. It is defined as the time it takes the detector's output signal to rise from 10% to 90% of its final value. The input value is postulated to directly jump from 0% to 100% (Figure 21).

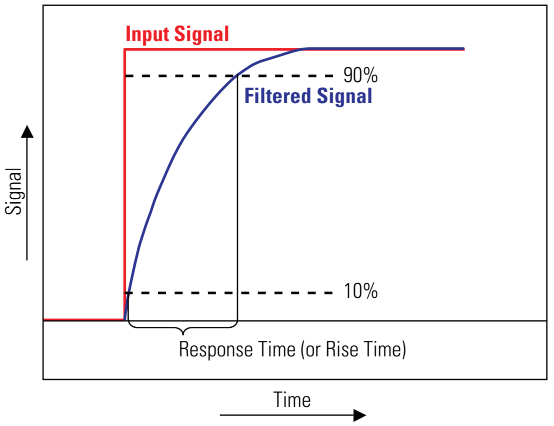
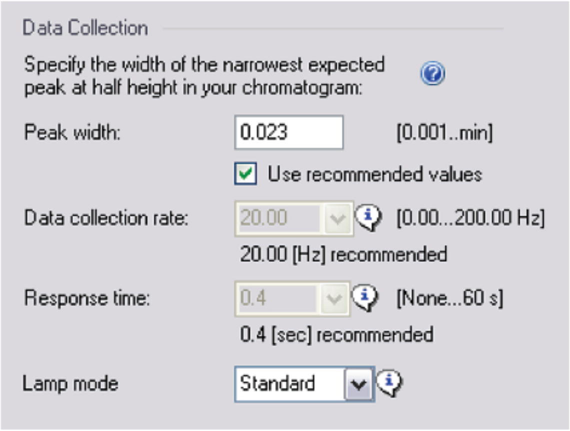


Figure 21. The response time measures the time until the detector responds to a change in signal. It is defined as the time it takes the detector's output signal to rise from 10% to 90% of its final value following an instantaneous change of the input signal.


Data Collection

Specify the width of the narrowest expected peak at half height in your chromatogram:

Peak width: 0.023 [0.001..min]

☑ Use recommended values

Data collection rate: 20.00 [0.00...200.00 Hz]

20.00 [Hz] recommended

Response time: 0.4 [None...60 s]

0.4 [sec] recommended

Lamp mode Standard

Figure 22. The Chromeleon CDS instrument method wizard calculates suitable DCR and response times based on the width of the narrowest peak of interest (shown for the UltiMate 3000 system).

Initial experiments should be performed with relatively high data collection rates and short response times. Once separations are optimized, the widths of relevant peaks can be extracted from the chromatogram. As in Chromeleon CDS software version 6, Chromeleon CDS software version 7 provides guidance on the selection of data collection rates and response times. Calculated based on the peak width, Chromeleon CDS software makes sure that a sufficient number of data points across a peak are obtained and optimizes the related response time. To access this guidance, open the instrument method (Chromeleon CDS software version 7), click on the FLD symbol on the left, and select the Property Page Channel Settings. In the Data Collection section, the peak width can be entered (Figure 22). Select DCR and response times as suggested by the software. Selecting appropriate data collection settings is therefore an easy task.

Effect of Changing the Xenon Flash Lamp Frequency

The FL Detectors use a xenon flash lamp as a light source. The expected lifetime of this lamp is related to the lamp operation time and the flash frequency used. Xenon flash lamp operation time is similar to data acquisition time because the lamp only requires a few seconds to warm up. Continuous xenon and mercury lamps need time to reach a thermal equilibrium and typically must be switched on ≥1 h prior to the first injections and usually remain on between samples. Leaving the lamp on obviously reduces the number of samples analyzed during the lifetime of a continuous lamp. A flash lamp with a lifetime of 4000 h can therefore process far more than 4 times the number of samples of a 1000 h continuous lamp.

The xenon flash lamps of the FL Detectors offer three different flash frequencies: HighPower (300 Hz), Standard (100 Hz), and LongLife (20 Hz). A higher flash frequency improves the S/N ratio; a lower flash frequency improves the lifetime of the lamp. Operating the lamp in LongLife mode results in >15,000 h of chromatography. Note that the lifetime of the lamp is defined as such that the specifications of the Raman S/N (an internal measurement to judge lamp health) will still be achieved. Thus, at the end of the lamp lifetime, the sensitivity of the detector is expected to reach a limit, but the lamp may be used longer without any problem.

Next, consider the influence of the lamp mode on both the analyte peak and the baseline. Figure 23 shows an overlay of three dibenzo(*a,h*)anthracene peaks detected using HighPower, Standard, and LongLife modes. All other settings remained constant. The lamp mode change does not affect the peak height or area, but the baseline noise increases with a lower flash frequency.

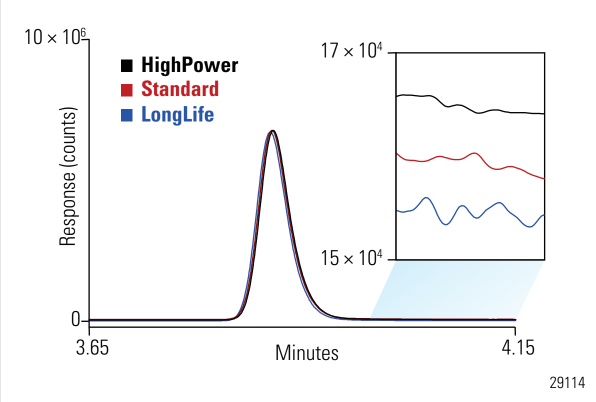


Figure 23. Changing the flash lamp frequency has an impact on the baseline noise, but the peak height remains constant. The lamp mode therefore only influences the noise and respective S/N (standard 2, 1 µL).

The related S/N ratios are shown in Figure 24. A rough estimate of the noise change can be calculated using Equation 2.

Equation 2: $$Noise\ (F2) = \sqrt{\frac{F_1}{F_2}} \times Noise\ (F1)$$

With

F1= Previous flash lamp frequency

F2= Planned flash lamp frequency

Changing the lamp mode from HighPower (300 Hz) to Standard (100 Hz) therefore increases the baseline noise by approximately √3 which is a factor of ~1.7. In the example, the noise changes by a factor of 1.53. From Standard (100 Hz) to LongLife (20 Hz), the predicted factor is 2.24. The factor calculated from real results is 1.7. In summary, the equation is not precise, but allows estimation of the effect of the lamp frequency change.

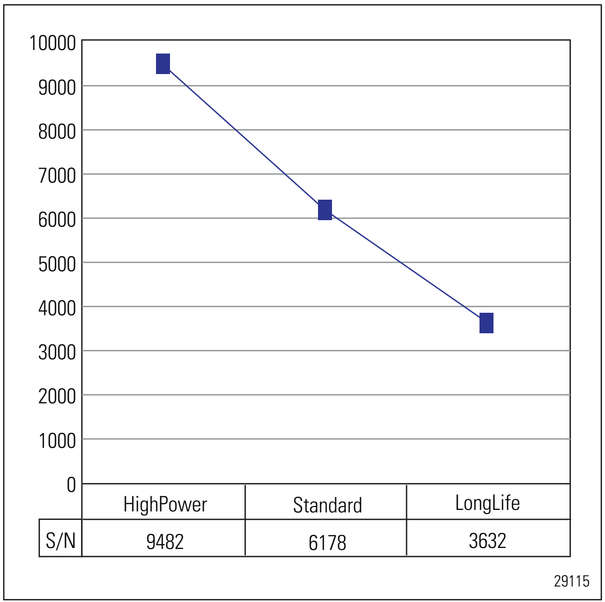


Figure 24. Signal-to-noise ratio for dibenzo(*a,h*)anthracene obtained with HighPower, Standard, and LongLife lamp modes.

Smart Use of the Xenon Lamp Mode

The flash lamps of the UltiMate 3000 and Vanquish FL Detectors are so flexible that the lamp mode can even be changed during a chromatographic run, an easy way to optimize methods.

A chromatogram typically has regions in which no peaks of interest elute. In gradient separations, this includes the time before the elution of the first peak and the wash/re-equilibration time at the end of the run. A flash lamp could even be switched off during this time, but the variable lamp modes of the FL Detectors allow an even smarter way to achieve both the best S/N ratio and an increased lifetime of the light source. During chromatographic idle times, the lamp mode can be switched to LongLife; during peak elution, the mode can be changed to Standard or HighPower.

Figure 25 applies this approach to the separation used throughout this document. Total run time, including re-equilibration, is 7.5 min. The authors applied LongLife mode from 0–0.75 min and from 4.25–7.5 min. In total, the LongLife mode was active for 4 min. During the remaining 3.5 min of the chromatogram, the lamp mode was set to HighPower. The number of flashes decreased by 50%, compared to if HighPower had been used across the entire chromatogram. Thus, this approach leads to 100% longer lifetime of the flash lamp without any sacrifice to the obtained data quality. Data acquisition is always on, and during idle times, the baseline is monitored at reasonable sensitivity.

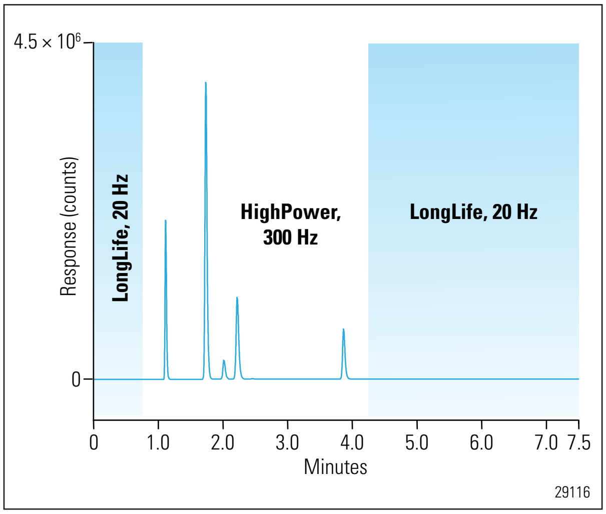


Figure 25. Smart use of the lamp modes further extends the lifetime of the xenon flash lamp (standard 2, 1 µL) injection.

Summary of Optimizations

Several optimized settings were obtained for the separation of the PAH standard. The data collection rate and the response time are settings which are applied across the entire chromatogram:

Data collection rate:	20 Hz
Response time:	0.4 s

Other settings are optimized based on the characteristics of a fluorophore such as excitation and emission wave-length, sensitivity setting, and the emission filter. The lamp mode is also varied to apply HighPower mode during peak elution and LongLife mode to sections of the chromatogram with no peaks of interest. Table 2 summarizes these timed settings. Sensitivities were evaluated for 1 µL injections of standard 2.

Table 2. Timed settings across the chromatogram.

Time (min)	Lamp Mode	Wavelengths Optimized For	Excitation (nm)	Emission (nm)	Sensitivity	Filter
0.00	LongLife	Naphthtalene	219	330	6	280
0.75	HighPower					
1.50		Anthracene	247	400	3	370
1.85		Fluoranthene	232	443	6	370
2.13		Pyrene	238	39	4	370
2.60		Dibenzo(*a*,*h*)anthracene	294	396	4	370
4.25	LongLife					

Test of Eluent Quality

This chapter demonstrates a way to test the eluent quality, particularly when switching to a new lot or even a new supplier.

As mentioned at the beginning of the Experimental section, it is important to use solvents of compatible quality for fluorescence detection. Noncompatible solvents may create background fluorescence that reduces the dynamic range of the detector, directly contributes to baseline noise, and therefore adversely affects the limit of detection (LOD).

It is not only important to buy a solvent product that is nominally fluorescence compatible, but it is also useful to make a relative comparison between different suppliers. In addition, a brief test when switching to a new batch or a new supplier gives confidence that the solvent quality is suitable.

Results may differ with storage time of the water, so freshly purified lab water may be the best choice.

In a typical run, the signal is set to zero by an autozero command. The disadvantage of this procedure is that the level of background fluorescence created by the solvent cannot be evaluated; for any solvent quality, the baseline starts at zero counts. Use the ClearAutoZero command to release the baseline. A certain level of stray light is always present, so the baseline will rise to a higher level. The worse the eluent quality, the higher the observed baseline level will be. Figure 26 shows an overlay of three experiments with water from three different sources, obtained with a backpressure capillary instead of a column. A backpressure of a few bar ensures that the pump logic operates smoothly. As the stray light level is influenced by the sensitivity setting, it must be kept constant during comparison experiments. Here, the sensitivity was set to 5 throughout the run.

At 0 min, the run starts with the excitation and emission wavelengths for the first analyte, naphthalene. At 0.5 min, the baseline is released with the ClearAutoZero command. Deionized water reaches the highest background fluorescence with ~400,000 counts under these conditions. Lab water and the LC/MS grade water are of similar quality at ~100,000 counts.

At 1 min, wavelengths are switched to the ones used for detecting the second analyte, anthracene, followed by an AutoZero. As a consequence, the baseline returns to zero counts response. At 1.5 min, the baseline is released again, resulting in increased baseline levels. This process is repeated for all the wavelength/filter combinations.

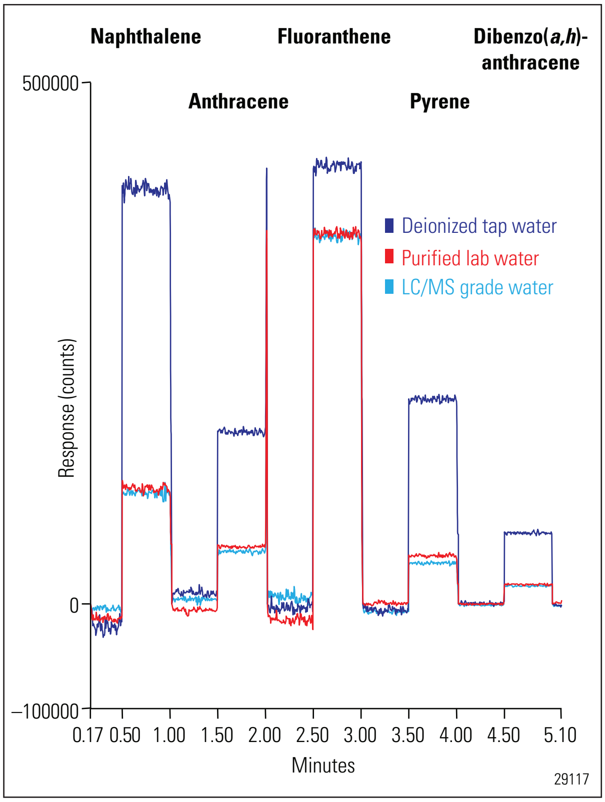


Figure 26. Three different water qualities are tested for their background fluorescence at analyte-relevant detection parameters. This quick test makes eluent quality monitoring an effective yet easy task.

This test ensures that the quality of the solvent is not just checked at a single wavelength combination that might not even be relevant for a given analysis. All relevant parameters are tested quickly and can easily be compared when overlaying them with earlier results or other eluent products.

Note that the peak at 2 min is a consequence of the wavelength switch to fluoranthene detection conditions. For a short time, the background fluorescence already reaches the levels that are observed between 2.5 and 3 min. The combination of the wavelength switch and AutoZero produces a signal that resembles a very narrow peak.

More guidance on how to find out if mobile phase quality is sufficient for application specific UV-Vis, fluorescence, and charged aerosol detection requirements are provided.[8]

Conclusion

- Vanquish and UltiMate 3000 fluorescence detectors are designed for highest performance and method development flexibility.
- Zero order mode and Synchronous FL Fields are effective tools for initial experiments and do not require a diode array detector.
- Emission and excitation FL Fields minimize the effort to determine the most suitable wavelength pair.
- The variable emission filter and the sensitivity setting of the photomultiplier can significantly improve the S/N ratio (Vanquish F and UltiMate 3000 FLD-3400RS FL Detectors).
- Chromeleon software calculates optimal data collection rates and response times based on the narrowest peak width for the best S/N ratio and data storage size.
- This study demonstrates the effect of different xenon lamp flash frequencies on noise and shows how to combine the modes within a chromatogram to achieve the best detection limits while further increasing lamp lifetime.
- A simple yet application-tailored test for evaluating the eluent quality is explained.

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