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) or after the separation (postcolumn).

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. 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 Fluorescence Detector series. 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. For additional information, see References 4 and 5. A number of application notes using the FLD-3000RS series fluorescence detector are also available. See References 6 to 10 for details.

**Conditions**

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**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 column, 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 initial peaks are eluted isocratically; the gradient is applied to speed the elution of the fifth peak.

![Figure 1. Overview of fluorescence method development steps discussed in this document.](image1.png)

![Figure 2. Separation of five PAHs with UV detection at 240 nm, standard 1, 10 µL injection.](image2.png)
The DAD now provides easy access to UV spectral 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 FLD-3000 series fluorescence 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 FLD-3000 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 FLD 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**

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. This excitation wavelength must be within the wavelength range of the target analytes’ absorbance spectra. Literature reviews may help with this if no UV spectrum can be obtained. 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, as long as the analytes can be excited at all. Depending on the characteristics of the analytes, the response varies.

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

![Figure 3](image)

![Figure 4](image)

*Figure 4. The Dual-PMT option of the FLD-3000 detector is a unique solution for providing optimum sensitivity across all wavelengths.*

![Figure 5](image)

*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.*
3D Synchro Scans

The 3D synchro 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 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 7 shows an example of a single synchro scan. The black synchro spectrum is a result of the combination of the excitation and the emission spectra also provided in Figure 7. The synchro 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 3D scans, the selection of a suitable scan speed is important. In Chromeleon 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 the peak width and scan range. Even UHPLC peaks are easily supported. Note that the y-axis uses a logarithmic scale.

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

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 FLD-3000 series. 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 3D synchro scans of the FLD-3000 detector series, 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 3D emission scan feature.

The FLD-3000 Series supports the acquisition of permanent 3D emission scans. During the scan, the excitation remains constant at a defined wavelength. Across the entire acquisition time, the emission monochromator repeatedly scans a user-defined emission wavelength range. Similar to the synchro 3D scan, this results in a DAD-like 3D field. However, the synchro 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: 3D Synchro Scans).
Figure 11 shows four emission spectra of dibenzo(a,h)anthracene obtained in four different 3D emission scans. 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 FLD-3400RS fluorescence detector features a unique variable emission filter that automatically selects a suitable emission cut-off filter element.

Figure 10 shows emission scans extracted from a single separation. The data were obtained by setting the emission 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.
Returning to the 3D emission scan 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.

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 occurrence 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 3D 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 wavelengths around 280 nm and higher cannot be suppressed.

In summary, optimum emission wavelengths can easily be extracted from 3D emission scans. The FLD-3400RS detector is most efficient in suppressing higher order diffraction light because it automatically selects the best suitable emission filter. The emission spectrum is typically not affected by the excitation wavelength (also known as Kasha’s rule). Analysts using the FLD-3000 detector series can therefore typically extract optimum emission wavelengths for all analytes from a single 3D emission scan.

### 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 permanent 3D excitation scans. During the scan, the emission wavelength remains constant. Across the entire acquisition time, the excitation monochromator repeatedly scans a user-defined excitation wavelength range. Similar to synchro and emission 3D scans, this results in a DAD-like 3D 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 a 3D scan 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 scan: does the selection of the constant emission wavelength influence the shape of the observed excitation spectrum?
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.

Figure 15 shows four excitation spectra of dibenzo(a,h)-anthracene (200–360 nm) extracted from four 3D excitation scans. 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 3D excitation scan may be sufficient. If the emission spectra differ to the extent that there is hardly any overlapping emission range, more than one 3D 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).

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.

**Optimizing the Emission Filter**

The Variable Emission Filter (VEF), also referred to as the Filter Wheel, of the FLD-3400RS fluorescence detector is a unique tool to suppress stray light and higher order effects. The VEF is located before the emission monochromator (Figure 16).
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 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.

In summary, the FLD-3400RS fluorescence detector automatically picks the best suitable filter for a selected wavelength pair. This process reduces stray light and therefore improves S/N by design.

The FLD-3100 fluorescence detector operates with a constant 280 nm emission filter, as is 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.

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.

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. \(^{12}\)

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 FLD-3000 series 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.

Figure 19. MaxPMTSaturation is a useful parameter to optimize the sensitivity of the PMT.
At this point of the method development, elution windows 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.

Table 1. Recommended actions to optimize the sensitivity setting.

<table>
<thead>
<tr>
<th>Value</th>
<th>Action to Optimize</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;30%</td>
<td>Increase the sensitivity.</td>
</tr>
<tr>
<td>30%–80%</td>
<td>The sensitivity value is optimal.</td>
</tr>
<tr>
<td>80%–99%</td>
<td>The sensitivity should be reduced by one step to avoid undesired saturation when concentration varies.</td>
</tr>
<tr>
<td>≥100%</td>
<td>The detector reduces the sensitivity automatically (autoranging).</td>
</tr>
</tbody>
</table>

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 software sample audit trail, or dilute the sample if sensitivity 1 is still too sensitive.

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: \[ S/N = 2^\left(\frac{\text{Peak Height}}{\text{Noise}}\right) \]

<table>
<thead>
<tr>
<th>Sensitivity</th>
<th>Height dibenzo(a,h)anthracene (counts)</th>
<th>Noise (counts)</th>
<th>S/N</th>
<th>PMT Saturation (%)</th>
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<td>PMT overflow</td>
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</table>

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).
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 software version 6, Chromeleon software version 7 provides guidance on the selection of data collection rates and response times. Calculated based on the peak width, Chromeleon 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 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.

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. Run 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 at least 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.

**Figure 22.** The Chromeleon instrument method wizard calculates suitable DCR and response times based on the width of the narrowest peak of interest.
Effect of Changing the Xenon Flash Lamp Frequency

The FLD-3000 detector series uses 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 FLD-3000 detector series xenon flash lamp offers 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.

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

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

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 \(\sqrt{3}\) 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.
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 wavelengths, 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.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Lamp Mode</th>
<th>Wavelengths Optimized For</th>
<th>Excitation (nm)</th>
<th>Emission (nm)</th>
<th>Sensitivity</th>
<th>Filter</th>
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<td>0.00</td>
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<td>Naphthalene</td>
<td>219</td>
<td>330</td>
<td>6</td>
<td>280</td>
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<td>HighPower</td>
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<tr>
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<td></td>
<td>Anthracene</td>
<td>247</td>
<td>400</td>
<td>3</td>
<td>370</td>
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<td>1.85</td>
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<td>Fluoranthene</td>
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<td>370</td>
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<td>Pyrene</td>
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<td>39</td>
<td>4</td>
<td>370</td>
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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. With the FLD-3000 detector series, 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. 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.

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.
Conclusion

- UltiMate 3000 FLD-3100 and FLD-3400RS fluorescence detectors are designed for highest performance and method development flexibility.
- Zero order mode and 3D synchro scans are effective tools for initial experiments and do not require a diode array detector.
- 3D emission and 3D excitation scans 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 (FLD-3400RS only).
- 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.

References


