Technical Note 166

Fast and Easy Optimization of Detection Wavelengths with Vanquish Fluorescence Detectors

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Key Words
HPLC, UHPLC, Fluorescence, Glycan Analysis, Excitation and Emission Wavelengths, Sensitivity Optimization

Goal
The goal of this technical note is to explain why the optimal fluorescence wavelength pair can vary between different fluorescence detector models and to demonstrate how users can easily verify and optimize fluorescence detection wavelengths with the Thermo Scientific™ Vanquish™ Fluorescence Detector.

Introduction
The selection of the excitation (Ex) and emission (Em) wavelength pair is essential for optimizing the sensitivity of a fluorescence detection assay. Many high-performance liquid chromatography (HPLC) analysts rely on published settings for their experiments. These settings can vary and therefore should be verified during method implementation. For example, different Ex/Em wavelength pairs for 2-aminobenzamide (2-AB)-labeled glycans including 330/420 nm[1], 320/420 nm[2], 320-360/420 nm[3], and 250/428 nm[4] can be found. One explanation for these differences is that the most sensitive wavelength pair is not only defined by the sample but also by the fluorescence detector design itself. The optimum excitation wavelength is a product of both the excitation spectrum of the analyte and the light intensity spectrum of the detector lamp, which may vary for different detectors.

This technical note explains how users can easily optimize the wavelength pair to obtain the best detection using the combination of a Vanquish Fluorescence Detector and Thermo Scientific™ Dionex™ Chromeleon™ Chromatography Data System (CDS) software.

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Experimental

Instrumentation

Thermo Scientific Vanquish Flex UHPLC system, equipped with:

- System Base (P/N VF-S01-A)
- Quaternary Pump F (P/N VF-P20-A)
- Split Sampler FT with 25 µL sample loop (P/N VF-A10-A)
- Column Compartment H (P/N VH-C10-A) with active pre-heater (P/N 6732.0110)
- Fluorescence Detector F (P/N VH-D51-A) equipped with 2 µL flow cell (P/N 6079.4330)

Chromatographic Conditions

Column:	Thermo Scientific™ Accucore™ 150 Amide HILIC, 2.1 × 150 mm, 2.6 µm (P/N 16726-152130)	
Mixer volume:	350 µL + 50 µL	
Mobile Phase A:	Acetonitrile	
Mobile Phase B:	Ammonium formate, 50 mM, pH 4.4	
Gradient:	Time (min)	%B
	0	20
	30	42
	30.5	50
	32	50
	32.5	20
	60	20
Flow rate:	0.40 mL/min	
Temperature:	Column compartment:	50 °C
	Detector flow cell:	45 °C
Detection Parameters:	Excitation wavelength:	320 nm (literature) and 250 nm
	Emission wavelength:	420 nm (literature) and 430 nm
	Lamp mode:	HighPower
	Sensitivity:	8
	Data collection rate:	5 Hz
	Response time:	1 s
Samples:	1 nmol/mL 2-AB-labeled *N*-glycan IgG library Sample was dissolved with appropriate amounts of 82:18 acetonitrile/ammonium formate 50 mM pH 4.4	

Data Processing

Chromeleon Chromatography Data System, version 7.2 SR 3

Results and Discussion

Figure 1 indicates typical light intensity distributions of continuous and xenon flash lamps. These lamps are common light sources in fluorescence detectors. Xenon flash lamps typically have a higher light intensity in the range of 200–300 nm where many analytes can be excited. The Vanquish Fluorescence Detector uses a xenon flash lamp.

Continuous lamps used in some other fluorescence detectors provide more light intensity at longer wavelengths. For best signal-to-noise (S/N) performance of the detector in use, it is typically beneficial to excite the analyte at a wavelength range where a lot of light is available. Depending on the type of lamp and the characteristics of the optical design of the fluorescence detector, this may lead to changes in the characteristics of the original excitation spectrum of the analyte.

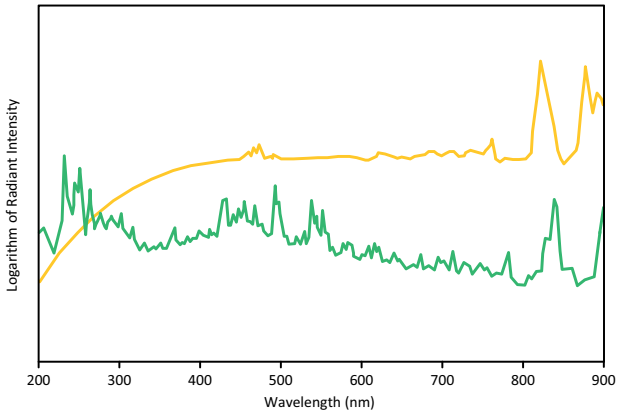

Figure 1. Typical light intensity distributions of continuous (orange) and flash (green) xenon lamps.

Chromeleon CDS supports easy acquisition of repeated scans over the entire data acquisition time, resulting in a diode array detector-like three-dimensional (3D) data field. It is possible to scan for the optimal excitation wavelength in a defined wavelength range with fixed emission wavelength (Scan mode: Excitation) or, vice versa, to scan for the optimal emission wavelength with fixed excitation wavelength (Scan mode: Emission). A third option is a synchronous scan where both the excitation and the emission wavelength vary with a fixed offset (Scan mode: Synchronous). The resulting data fields are called the FL Field. The FL Field Acquisition is easily established with the optimized user interface for the Vanquish Fluorescence Detector in Chromeleon CDS. Commands are sorted in different categories: **Easy** for routine operations, **Advanced** for more sophisticated parameter settings, and **FL Field** for all commands related to FL Field Acquisition. Help texts are related to all relevant commands.

As shown in Figure 2, two modes are available for the acquisition of excitation FL Fields. **ExCorrected** divides by the xenon lamp spectrum. The resulting spectrum is therefore analyte-specific and should be similar to published spectra. **Standard** does not divide by the lamp spectrum. Operating at a maximum of a spectrum acquired in standard mode will typically result in an improved signal-to-noise ratio. The FL Field Acquisition can be started like a single wavelength channel.

Two examples of output plots are shown in Figure 3: the contour plot and the 3D view. Chromeleon CDS software provides easy-to-use tools to further investigate any spectrum obtained at any time during the separation.

Reference 5 provides more details on this and gives guidance on how easy it is to optimize methods with Thermo Scientific fluorescence detectors.

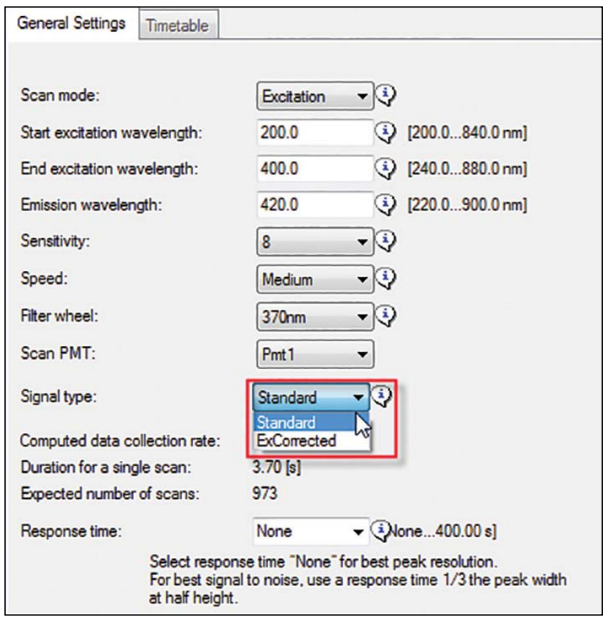

Figure 2. Screenshot of the Chromeleon CDS software spectra user interface.

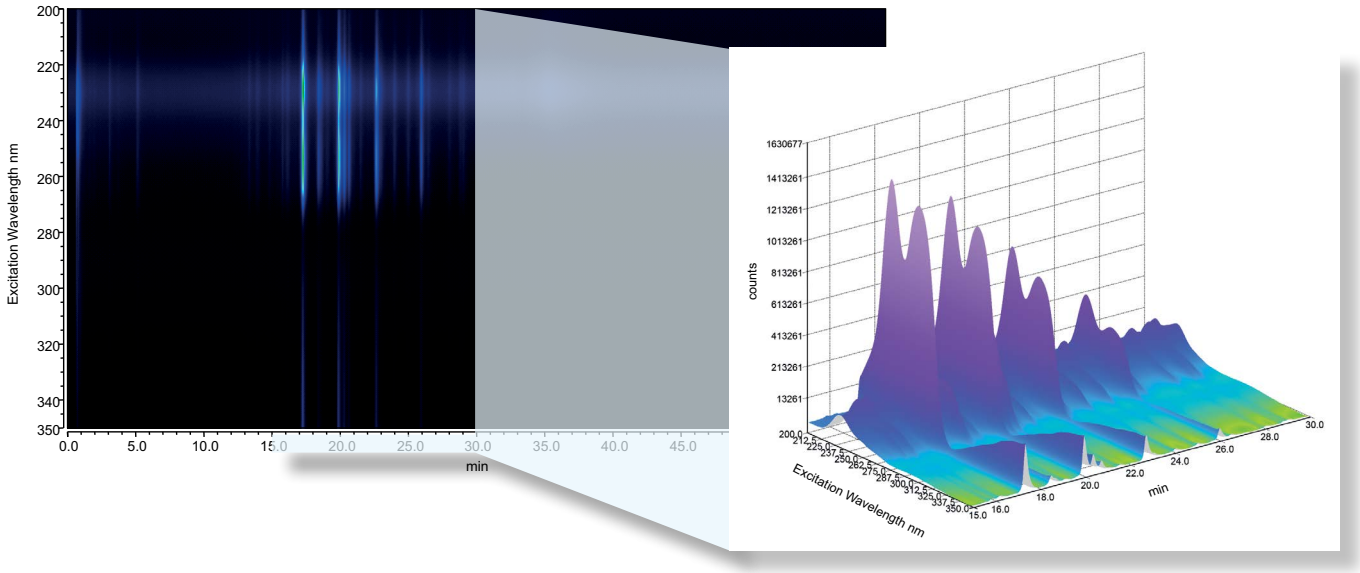

Figure 3. Contour plot of an Ex FL Field Acquisition, Ex/Em 200–350/420 nm, Standard mode (left) and 3D view zoom into FL Field from 15–30 min (right) for the separation of 2-AB-labeled Immunoglobulin G glycans.

Figure 4 shows two excitation spectra of a peak from the separation of 2-AB-labeled *N*-glycan Immunoglobulin G, extracted of the FL Field at 17.3 min. The orange spectrum has been acquired in **ExCorrected** mode. The green spectrum has been acquired in **Standard** mode.

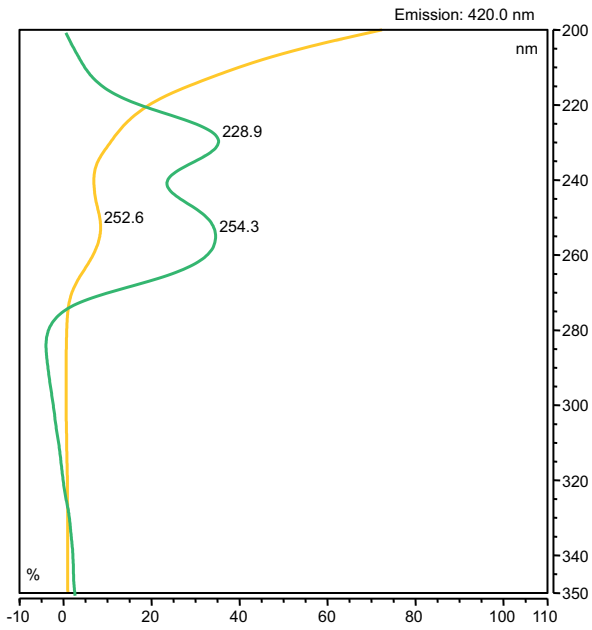

Figure 4. Excitation spectra extracted from an FL Field Acquisition of a 2-AB-labeled *N*-glycan Immunoglobulin G separation in Standard mode (green) and ExCorrected (orange). During FL Field Acquisition, the emission wavelength is constant at 420 nm.

When comparing the two spectra, it becomes obvious that they are significantly different:

- The standard spectrum provides a second maximum at 228 nm. This is present over the entire acquisition time and is therefore likely to be related to fluorescence of the mobile phase.
- The maximum at 252.6 nm is slightly shifted towards longer wavelengths: 254.3 nm
- The high response between 220 nm und 200 nm with ExCorrected acquisition does not translate into a high signal-to-noise ratio. Obviously, the light intensity is higher at wavelengths > 220 nm.

An additional Emission FL Field Acquisition (not shown) identified that 430 nm is the best-suited Em wavelength setting. Note that the selection of the Em wavelength typically has no impact on the qualitative assessment of the Ex spectrum.[5]

Figure 5 overlays two chromatograms, one with a literature wavelength pair Ex/Em 320/420 nm and the other with an optimized wavelength pair Ex/Em 250/430 nm. The peaks obtained with the optimized wavelength pair are approximately seven times higher. The noise also increases, but not as much, resulting in an average signal-to-noise ratio improvement of 2.4. The increased noise might be a consequence of increased background fluorescence introduced by the mobile phase.[6] Figure 5 also shows additional benefits from this extra sensitivity as many small, previously unseen peaks are now visible.

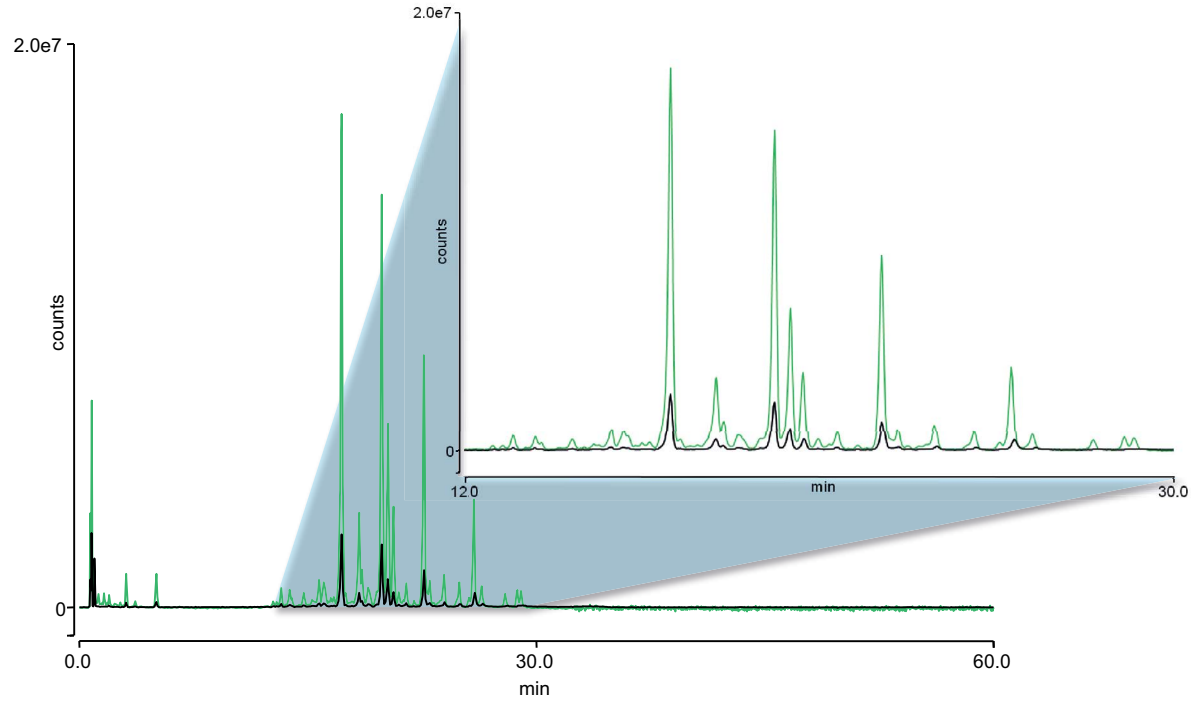

Figure 5. Separation of 5 pmol 2-AB-labeled *N*-glycan Immunoglobulin G detected with literature wavelengths Ex/Em 320/420 nm (black), and optimized wavelengths Ex/Em 250/430 nm (green). With the latter wavelength pair, peaks are seven times higher, the average S/N is improved by factor 2.4.

Conclusion

The most sensitive wavelength pair for a fluorescence detection assay depends on both the analyte and the detector characteristics. The Vanquish Fluorescence Detector in combination with Chromeleon CDS Software supports easy verification and optimization of wavelength settings for improved detection sensitivity.

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