APPLICATION NOTE

Fluorescence Spectroscopy

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Validation of the Performance of the PerkinElmer FL 6500 and FL 8500 Fluorescence Spectrometers for Pharmaceutical and Industrial Analysis

Introduction

For certain analyses, fluorescence is the method of choice due to its superior sensitivity compared to UV/Visible absorption spectrophotometry. Fluorescence has the potential to be more sensitive – provide that the material under investigation has a high quantum efficiency at the wavelengths being used. It is also more selective as each molecule is defined in terms of the excitation and emission wavelengths and so the instrument can be "tuned" to detect that compound.

Fluorescence is a relative technique – this means that a quantitative measurement requires a comparative measurement against a material of known concentration. This can be a single standard or it may be a series of standards. The advantage of using multiple standards is that it is possible to verify that the analysis is being conducted over the linear range and that inner filtering (self-absorption by the sample) is not occurring. The observed fluorescence intensity (often expressed as RFU – relative fluorescence units) will also depend on how the instrument is set up in terms of slits, detector gain (voltage), lamp power and so on. Different instruments (even built by the same manufacturer and using a seemingly identical optical system) will give different results and the absolute RFU values between models from different manufacturers are no indication of the quality of the instrument as each manufacturer divides up the ordinate scale differently.

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Validation of Fluorescence Spectrometers

In the light of the considerations of the technique as previously discussed, this makes validation of the performance more challenging compared to other, more absolute, techniques such as absorption spectroscopy. Laboratories – particularly those involved in contract analysis – are working to get accreditation (eg UKAS or ISO 17025) and so need to be able to demonstrate that their instruments have been validated and are fully working for the analysis in question. Unfortunately, the pharmaceutical market (one of the biggest drivers for validation) is a small subset of a relatively (compared to absorption) market and so the range of materials and methods for testing instruments is more limited. There is a good and obvious reason why this should be the case. The sample under investigation must either fluoresce natively or be reacted (derivatized) with something that will produce a fluorescent species (eg the reaction of non-fluorescent histamine with OPA to produce a highly fluorescent complex). There also needs to be consideration of any absorbing compounds that would mask or quench the fluorescence. This, therefore, restricts the applicability of fluorescence for general QA/QC analysis.

The US Pharmacopoeia method USP<853> (which is part of USP 40)[1] gives some guidelines for measuring performance of fluorescence spectrometers. The Spectrum FL software used for the FL6500 and FL8500 has a validation module which can be used to test key specifications.

Spectrum FL Validation Module

Spectrum FL is supplied with a validation module to enable key specifications to be tested. These are:

- Raman band peak position
- Raman band sensitivity
- Emission wavelength accuracy
- Emission wavelength reproducibility
- Excitation wavelength accuracy
- Excitation wavelength reproducibility
- Stray Light

The module is very intuitive and guides the user through the process. Individual tests can be selected so that there is no requirement to run all the checks each time

The validation module is shown in Figure 1.

Measuring Sensitivity

Sensitivity is determined by measuring the signal to noise of a sample of pure water by measuring the Raman Peak by fixing the excitation at 350 nm and scanning the peak at around 397 nm using 10 nm slits and then measuring the noise at the peak wavelengths.

For convenience, the water standards are sold in sealed cuvettes (in order to help prevent microbiological growth inside the cuvette over time) and the quality of the water (in terms of the electrical conductivity) may or may not be certified. Similarly, some standards carry a serial number whilst others do no not.

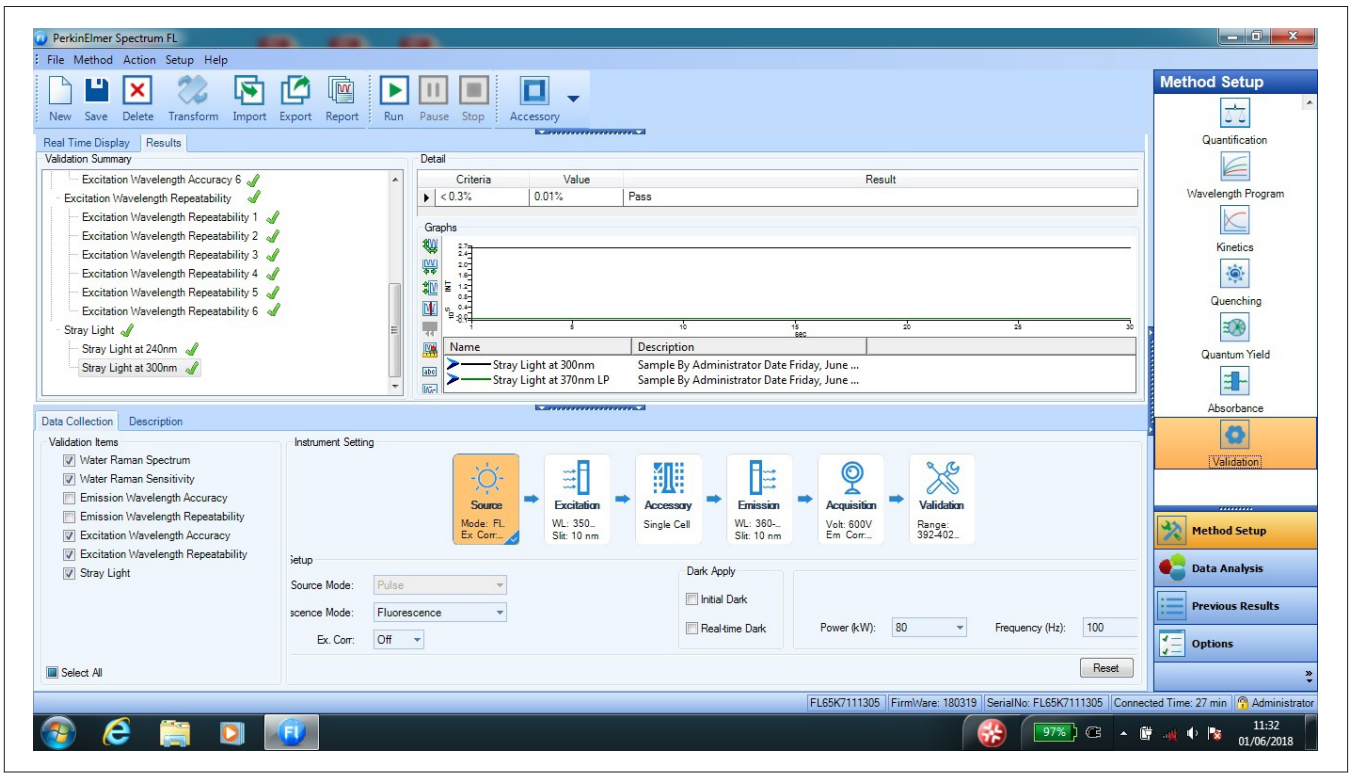

Figure 1. Spectrum FL Validation Module.

Figure 2. Sealed Water Standard (L2251293).

Measuring Emission Wavelength Accuracy and Reproducibility

The most common way to measure instrument sensitivity is to use a sealed water cell and check the position of Rayleigh-Tyndall and Raman Peak positions. This is a very simple approach but it is the least rigorous as the observed peak positions depend on the positions of both monochromators and it is possible that they are both incorrect. Nevertheless it does serve as a useful quick check in any diagnosis of instrument wavelength accuracy.

The best approach is to use a calibrated lamp with a line emission (such as mercury (Hg) or the Hg/argon lamp) The Hg/Argon lamp gives additional lines at the red end of the range. The main advantages here are that the line sources are inviolate (ie they never move) and, as they are line sources, they are not affected by any errors due to poor emission correction – particularly at wavelengths above 650 nm which can result in apparent peak shifts of non-line emissions due to the detector cutting off the peak and causing an apparent peak shift. For a broad peak, this could cause a shift of 50 nm or more.

The main drawback of using a lamp is one of cost relative to calibrated standards but it is the best methodology available for the purpose.

Measuring Excitation Wavelength Accuracy and Reproducibility

Once the emission wavelength has been calibrated successfully, it is a relatively simple matter to calibrate the excitation wavelength. This is done by inserting a PTFE diffuser (supplied with the instrument) into the sample position and driving the emission monochromator to a series of wavelengths and scanning the excitation monochromator through each of these.

The point where the two monochromators are equal should result in a strong Rayleigh-Tindall scatter peak. This position should correspond with the fixed emission wavelength (allowing for the specified wavelength accuracy of the monochromators).

Measuring Stray Light

Stray light can be broadly defined as light which is present in the beam of wavelengths other than those chosen by the user. One of the biggest potential sources of stray light is white light which is generated by the intense xenon source (either pulsed or continuous) which "seeps" past the excitation monochromator and manifests itself as stray light. This can cause spectral artefacts such as small shoulders or similar features on spectra.

A triangular cell (Part Number L2251366) filled with concentrated Rhodamine 101 is used as a stray light filter and the stray light is measured at 240 nm and 300 nm.

Set of Fluorescent Blocks

We offer a set of fluorescent materials. These are not standards, as such, and are not certificated but can still be useful in terms of checking that the instrument is working correctly.

The blocks are referenced in USP<853> and so their use is ermitted as part of a validation regime in pharmaceutical labs that fall under the aegis of the USP. These blocks are made from polished PMMA and are doped with low levels of various compounds such as anthracene, p-terphenyl, ovalene, rhodamine and europium (the latter being useful for time-resolved delayed fluorescence and phosphorescence measurements).

Figure 3. Set of Six Fluorescent Blocks (Part Number 52019600).

Future Trends

Several groups are investigating alternative materials for calibrating fluorescence spectrophotometers. The German BAM Laboratory[2] is investigating the use of quantum dots (nanoparticles of materials such as cadmium telluride, CdTe, which are engineered to a specific size and so will, therefore, have a precise emission wavelength).

References

1. US Pharmacopoeia, USP<853> (Part of USP 40).
2. Resch-Genger, Ute (ed.) Standardization and Quality Assurance in Fluorescence Measurements 1: Techniques, Springer Science and Business Media (2008).

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