

Reliable Fluorescence Detection at 366 nm for HPTLC Applications

For decades, mercury vapor lamps have been the standard excitation source for fluorescence densitometry. While providing strong excitation energy, mercury (Hg) lamps are associated with several practical limitations, including warm-up requirements and environmental considerations related to mercury handling and disposal. To address these challenges, CAMAG has developed a new LED-based excitation system for the TLC Scanner 4. The LED source provides mercury-free operation, immediate readiness, reduced maintenance requirements, and long operational lifetime while remaining fully integrated within established Scanner 4 workflows and *visionCATS* software.

Because LED and mercury lamps generate ultraviolet radiation through fundamentally different emission mechanisms, equivalent analytical performance cannot be assumed automatically. Although the monochromator is calibrated to 366 nm for both excitation sources, the effective excitation characteristics are not identical because the Hg lamp and LED exhibit different intensity distributions around this wavelength. In the TLC Scanner 4, the emitted light passes through the monochromator before reaching the plate. Consequently, the relevant difference between Hg and LED excitation is not simply the overall emission spectrum of the light source, but the intensity distribution within the wavelength range reaching the plate. The LED and Hg lamp exhibit slightly different intensity distributions around 366 nm, which may result in analyte-dependent differences in fluorescence response. As the fluorescence response depends on several properties of individual analytes, systematic evaluation is required before transferring the method between the two excitation technologies.

In this technical note, the performance of the LED-equipped CAMAG TLC Scanner 4 was evaluated using representative fluorescence-active compounds and a complex plant extract. Precision, functional equivalence, calibration characteristics, detection sensitivity, and quantitative performance were assessed and compared with a conventional Hg-equipped TLC Scanner 4.

Key features of the CAMAG LED TLC Scanner 4

- Mercury-free LED excitation at 366 nm
- Immediate operational readiness (no warm-up time)
- Long lamp lifetime
- High stability of excitation intensity over the operational LED lifetime
- Comparable precision to Hg excitation across the investigated analytes in this note
- Functional equivalence demonstrated for multiple investigated fluorescence-active analytes
- Compatible with existing TLC Scanner 4 workflows
- Seamless integration with *visionCATS* software (4.2 SP or higher)
- An upgrade kit is available to convert existing TLC Scanner 4 systems equipped with an Hg lamp to LED excitation.

Methods

Evaluated analytes

A representative panel of fluorescent compounds covering different structural classes was selected together with a complex botanical matrix:

- Coumarins: Umbelliferone, Esculin
- Furanocoumarins: Psoralen
- Thioxanthenes: Thioxanthone
- Phenolic acid: Ferulic acid
- Plant extract: *Cinnamomum cassia* bark extract

Chromatography

Stationary phase	HPTLC Si 60 F ₂₅₄ , 20 × 10 cm (Supelco)
Sample application	8.0 mm bands with CAMAG ATS 4, 15 tracks, track distance 11.4 mm, distance from left edge 20.0 mm, distance from lower edge 8.0 mm.
Developing solvent	The Complementary Developing Solvents (CDS) previously described in [1] were used. The low-polar developing solvent ethyl acetate, toluene 10:90 (V/V) was used for developing psoralen, thioxanthone, and <i>Cinnamomum cassia</i> extract, while the medium-polar developing solvent cyclopentyl methyl ether, tetrahydrofuran, water, formic acid 40:24:1:1 (V/V) was used for separating umbelliferone, esculin, and ferulic acid.
Development	Development was performed in the CAMAG ADC 2/3. Plate activation (10 min) was applied where specified by the respective method. The chamber was saturated for 20 min using a saturation pad.
Developing distance	Standard 70.0 mm from the lower edge.
Plate drying	Drying for 5 min in the ADC 2/3.
Densitometry	Plates were scanned in fluorescence mode using the TLC Scanner 4 with either a mercury lamp or an LED excitation source. In both configurations, the excitation light passes through the monochromator before reaching the plate. For both Hg and LED excitation, the wavelength is selected using the monochromator, which is calibrated to 366 nm. The effective excitation characteristics reaching the plate are therefore determined by the light source in combination with the optical system and instrument settings. Hg and LED excitation nevertheless exhibit slightly different intensity distributions within this wavelength range. Fluorescence was detected using the >400 nm filter. Measurements were performed using a scan speed of 20 mm/s, a slit dimension of 5.0 × 0.20 mm, and a data resolution of 100 µm per step. All densitometric acquisition parameters, with the exception of the excitation source, were kept identical for both LED and Hg measurements to ensure direct comparability of the results.

Analytical Performance Evaluation

1. Representative Fluorescence Profiles of a Real Sample

To illustrate the practical impact of replacing Hg excitation with LED illumination, a representative sample of *Cinnamomum cassia* extract was analyzed under identical chromatographic and densitometric conditions using both excitation sources. As shown in Figure 1, the overall chromatographic fingerprint remains highly comparable between the two excitation sources. The major fluorescent zones appear at identical positions and exhibit similar peak shapes and relative intensity distributions.

While the overall fingerprint, peak positions, and peak shapes remain comparable, small differences in absolute fluorescence intensity may occur between the two excitation sources.

Peak Profiles of *Cinnamomum cassia* Extract

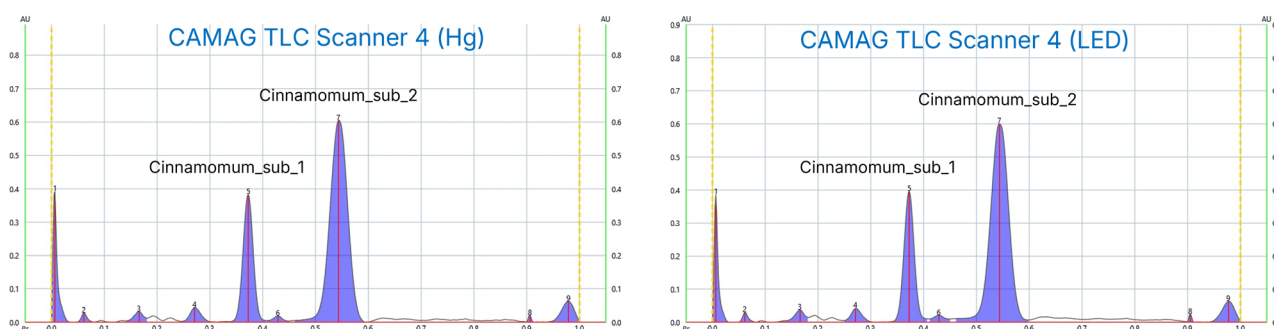


Figure 1 Representative peak profile from scan densitometry at 366 nm / >400 nm obtained from *Cinnamomum cassia* extract under Hg and LED excitation.

2. Overall Signal Comparison

For a direct comparison between LED and Hg excitation, sample tracks were scanned under both illumination sources for the five representative analytes and the two major fluorescent zones detected in *Cinnamomum cassia* extract. For each analyte or selected fluorescent zone, 18 paired peak-area measurements were obtained from three independent plates. Each pair consisted of the measurement of the same chromatographic zone under Hg and LED excitation.

Figure 2 presents the overall comparison, with each point representing a single paired measurement obtained under both excitation conditions. The close clustering of data points around the identity line demonstrates a high degree of agreement between the two excitation sources across multiple analyte classes and a broad range of fluorescence intensities.

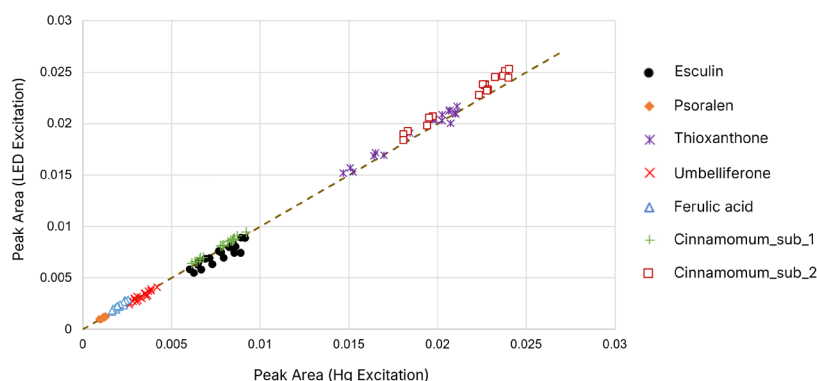


Figure 2 Overall comparison of Hg and LED fluorescence responses. Each point represents an individual densitometric peak area measurement obtained under both excitation conditions. Different colors and marker shapes correspond to the investigated analytes.

3. Precision

Precision was evaluated using the coefficient of variation (CV) and two-sided F-test. The F-test was used to assess whether the variances obtained under LED and Hg excitation differed significantly, providing a statistical complement to the descriptive comparison of CV values.

As detailed in Table 1, the majority of CV ratios were close to unity (0.86–1.12), although ferulic acid exhibited a lower ratio of 0.67. Despite this variation, the corresponding F-test indicated no statistically significant difference in variance.

Table 1 Multi-analyte comparison of LED and Hg excitation sources. Mean relative signal difference (%), CV Ratio LED/Hg and F-test *p*-value obtained for representative fluorescent compounds and two major fluorescent zones in *Cinnamomum cassia* extract.

Analyte	Mean relative signal difference (%)	CV Ratio (LED/Hg)	F-test <i>p</i> -value
Umbelliferone	-3.57%	1.12	0.7575
Esculin	-6.13%	1.08	0.9521
Psoralen	-1.65%	0.86	0.5063
Thioxanthone	+2.02%	0.96	0.9311
Ferulic Acid	+11.24%	0.67	0.3846
Cinnamomum_sub_1	+3.36%	0.99	0.9304
Cinnamomum_sub_2	+4.07%	1.01	0.8266

4. Quantitative Performance: Thioxanthone Case Study

To evaluate the impact of LED excitation on quantitative performance, thioxanthone was selected as a model analyte. Calibration curves (Figure 3) were generated from 20 to 50 ng per spot using three independent replicate series (*n* = 3). Regression slope, correlation coefficient, limit of detection (LOD), and limit of quantitation (LOQ) were compared between Hg and LED excitation conditions. The LOD and LOQ were calculated according to USP General Chapter <1225>, Validation of Compendial Procedures, using the standard deviation of the response (σ) and the slope of the calibration curve (*S*), according to $LOD = 3.3\sigma/S$ and $LOQ = 10\sigma/S$.

The average regression slope ratio (LED/Hg) was 1.026 (Table 2), corresponding to a small and highly consistent signal increase of approximately 2.6% under LED excitation. This finding is consistent with the +2.02% mean relative signal difference observed in the paired comparison.

Similarly, sensitivity parameters remained highly comparable. Mean LED/Hg ratios of 1.066 were obtained for both LOD and LOQ, indicating slightly higher detection and quantitation limits under LED excitation. However, the magnitude of the difference was small, and the overall detection capability remained comparable between the two excitation sources.

Together with the comparable calibration slopes, these results indicate comparable quantitative performance under the investigated conditions.

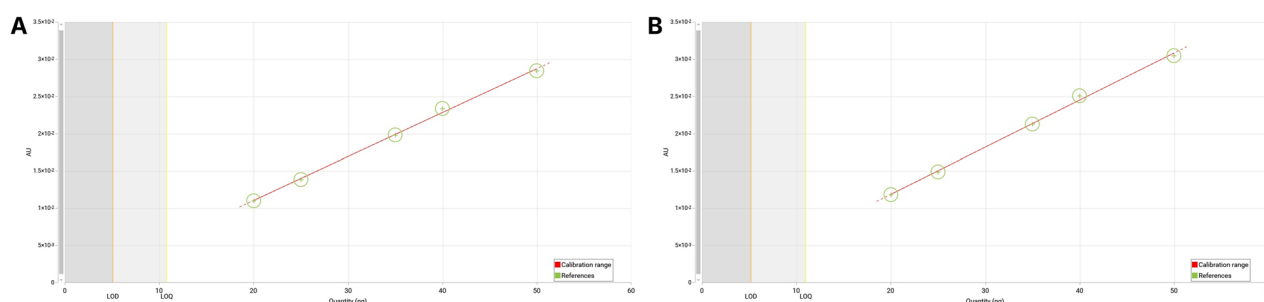


Figure 3 Calibration curve of thioxanthone under Hg (A) and LED (B) excitation.

Table 2 Comparison of quantitative performance parameters obtained using LED and Hg excitation. Mean ratios (LED/Hg) were calculated for calibration slope, limit of detection (LOD), and limit of quantitation (LOQ) using thioxanthone as a model analyte.

Calibration Parameter	Mean Ratio (LED/Hg)	Mean Shift (%)
Regression Curve Slope	1.026	+2.56%
Limit of Detection (LOD)	1.066	+6.63%
Limit of Quantitation (LOQ)	1.066	+6.63%

5. Functional Equivalence of LED and Hg Excitation

Functional equivalence was assessed using the same 18 paired measurements collected for the overall signal comparison and precision evaluation. For each paired measurement, a track-wise normalized difference was calculated according to:

$$\text{Percentage Difference (\%)} = (Area_{LED} - Area_{Hg}) / Area_{Hg} \times 100$$

This normalization ensures that differences are evaluated relative to the Hg baseline, allowing direct interpretation in terms of relative signal deviation. Functional equivalence between the two excitation sources was assessed using the Two One-Sided Tests (TOST). An equivalence margin of $\pm 10\%$ was predefined based on the acceptance criterion used for instrument performance comparison. Functional equivalence was concluded when the entire 90% confidence interval (CI90%) remained within these predefined acceptance limits.

Comparable peak shape, signal distribution, and chromatographic response were observed for the studied analytes; representative profiles are shown in Figure 4.

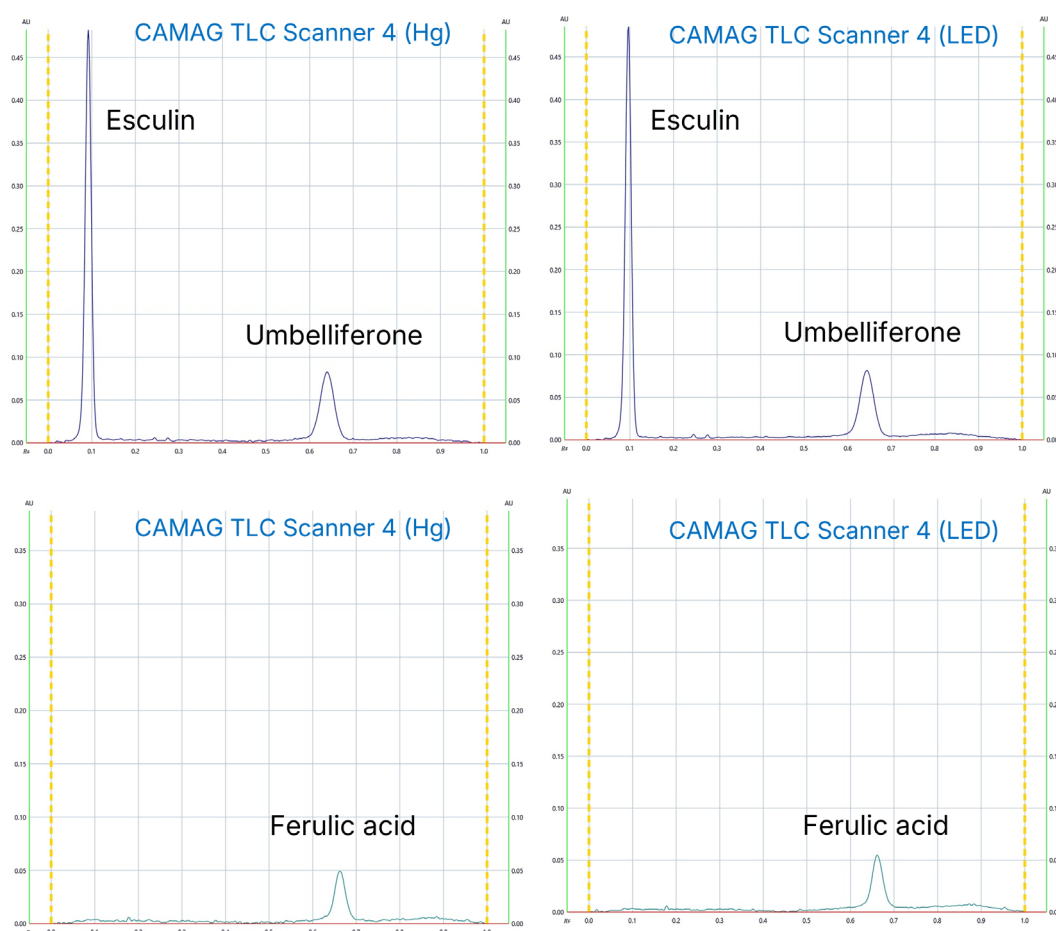


Figure 4 Densitometric profiles for esculin, umbelliferone, and ferulic acid obtained under Hg and LED excitation.

Despite the comparable precision and quantitative performance, comparison of fluorescence responses revealed that functional equivalence was analyte-dependent. Figure 5 summarizes the CI90 ranges associated with the relative signal differences obtained for all investigated analytes. Ferulic acid was the only investigated analyte that exceeded the predefined equivalence criterion. A systematic signal increase of 11.24% was observed under LED excitation, resulting in a confidence interval extending beyond the +10% acceptance limit. This observation does not necessarily indicate a performance issue of the LED scanner. Rather, it may reflect the interaction between analyte-specific absorption characteristics and differences in the effective excitation intensity distribution around 366 nm. This illustrates why identical nominal excitation wavelengths do not necessarily produce identical fluorescence responses for every compound.

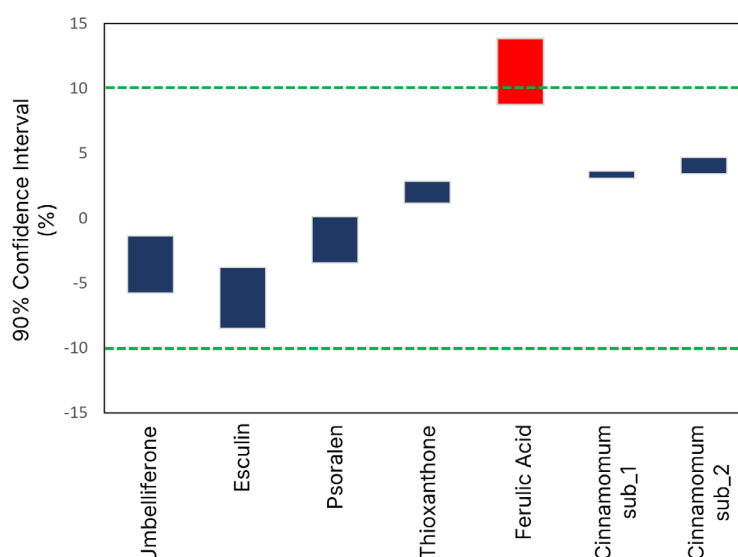


Figure 5 Functional equivalence of LED and Hg excitation sources. Mean relative signal differences and corresponding 90% confidence intervals obtained for the investigated analytes. The green dashed lines indicate the predefined equivalence limits ($\pm 10\%$).

Considerations for Method Transfer and Historical Data Comparability

The results indicate that comparable analytical performance does not necessarily imply identical absolute fluorescence response between Hg and LED excitation. For qualitative applications, differences in absolute signal intensity may have limited practical impact provided that the relevant zones remain reliably detectable. For quantitative applications, calibration performance should be verified when transferring an existing method from Hg to LED excitation.

Historical quantitative results or calibration models obtained using Hg excitation should not automatically be considered directly interchangeable with results obtained using LED excitation. Where direct comparison with historical Hg data is required, method-specific verification is recommended.

Conclusion

The LED-equipped CAMAG TLC Scanner 4 provides a mercury-free alternative for fluorescence densitometry at 366 nm while maintaining compatibility with established CAMAG TLC Scanner 4 workflows and software operation. The system offers immediate operational readiness, long service life, and eliminates the environmental and handling concerns associated with mercury-containing lamps.

A multi-analyte evaluation covering coumarins, furanocoumarins, thioxanthone, phenolic acids, and a complex plant extract showed comparable precision, with no statistically significant difference in variance detected between LED and Hg excitation. Evaluation of calibration performance using thioxanthone further demonstrated comparable quantitative behavior under LED and Hg excitation. Comparable calibration slopes, LODs, and LOQs were obtained for both illumination sources, supporting the applicability of the LED scanner for quantitative HPTLC analysis under the investigated conditions.

Functional equivalence was found to be analyte-dependent. Umbelliferone, esculin, psoralen, thioxanthone, and two major fluorescent zones from *Cinnamomum cassia* extract fulfilled the predefined $\pm 10\%$ equivalence criterion. Ferulic acid exhibited a systematic signal enhancement under LED excitation and exceeded the acceptance limits, indicating that the impact of transferring methods from Hg to LED excitation may be analyte- and application-dependent. For qualitative fluorescence detection, such signal differences may have limited practical impact. For quantitative applications, however, verification of calibration performance following transfer from Hg to LED excitation is recommended.

Overall, LED excitation does not necessarily reproduce exactly the absolute fluorescence signal generated by Hg excitation, but it provides comparable analytical performance for the investigated applications while offering significant operational and sustainability advantages. Verification of method transferability remains recommended, particularly for quantitative applications.

Reference

[1] Do TKT, Reich E. Complementary developing solvents for simpler and more powerful HPTLC fingerprinting. J Planar Chromatogr - Mod TLC. 2022 Aug;35(4):313-322. doi: [10.1007/s00764-022-00185-1](https://doi.org/10.1007/s00764-022-00185-1).