

Digitally Enhanced Thin-Layer Chromatography: An Inexpensive, New Technique for Qualitative and Quantitative Analysis

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Thin-layer chromatography (TLC) is often used as a *qualitative* method. It can determine the number of components in a mixture, verify the identity of substances, monitor the progress of a reaction, or ascertain the effectiveness of a purification. Since TLC is so easy and practical, many chemists frequently use TLC methods in their labs.

Quantitative TLC analysis is typically the province of HPTLC (high-performance TLC). HPTLC plates have finer particles than regular TLC plates, and consequently they are better suited for quantitative analysis, although they are more costly. HPTLC samples are applied by mechanical applicators (dosimeters) and analyzed with automated equipment, such as a TLC scanner.

Unfortunately, the equipment for quantitative TLC analysis is expensive—CAMAG's TLC Scanner 3 costs about \$30,000 with the software needed for analytical work. The documentation system, DigiStore 2, from CAMAG costs \$17,500 but needs Videoscan software (\$6,300) to do analysis (1). This equipment fills a specific niche in the market for production-oriented systems (mainly large companies that need automated equipment for processes such as food analysis); however, it is not practical for small companies and labs that also do analysis. High schools and colleges that cannot afford such equipment are limited to regular TLC, thus their students do not acquire experience with quantitative methods.

This study shows that if digital photography is combined with regular TLC ("digitally enhanced" or DE-TLC), it can perform highly improved qualitative analysis as well as make accurate quantitative analysis possible for a much lower cost than commercial equipment (a quality digital camera costs about \$500, and the software is free).

Researchers have used a CCD camera to do quantitative TLC analysis (2–4), while another researcher used a scanner with visible light to do TLC analysis (5). All of the research projects used HPTLC plates except Johnson (5). Four of the research projects used fluorescent HPTLC plates (2, 3, 6, 7). In general, the use of expensive equipment, such as TLC scanners and costly video cameras, has been the norm for quantitative analysis.

DE-TLC is the first technique to use a digital camera and software analysis to separate red, green, and blue brightness values in an image of a TLC plate to create multispectral scans. It is like a "poor man's" spectrophotometer, and it is easy to utilize. DE-TLC uses commonly available fluorescent TLC plates (*not* HPTLC plates), a digital camera with manual exposure control, and conventional TLC equipment. DE-TLC

can be used at any institution, but it is especially appropriate for high schools and colleges that do not have the budget to pay for more expensive equipment for TLC analysis.

Materials

Quantitative analysis focused mainly on nicotinamide (Fluka, Switzerland, 98.5%). Vanillin (Spectrum Chemicals, min 97.0%) was also tested. DE-TLC's qualitative and quantitative analysis should work with any sample you would analyze with fluorescent TLC plates. A Nikon D1x 6-megapixel digital camera was used to photograph the EMD Silica Gel 60 F254 fluorescent TLC plates. A 254-nm UV lamp (Mineral Light UVS-11) was used to illuminate the plates.

Procedure

TLC Procedure

The sample was weighed and dissolved in a measured quantity of appropriate solvent (water dissolved the nicotinamide, ethanol dissolved the vanillin). So that each spot contained a known quantity of material, the solution was spotted with one microliter microcaps (Drummond cat #1-000-0010) on a fluorescent TLC plate and developed in a TLC chamber using the appropriate mobile phase. For nicotinamide the mobile phase was water, ethyl acetate, and ethanol in the ratio of 2:2:1; for vanillin, it was ethanol and water, in the ratio of 1:1. After the solvent had moved far enough up the plate, the plate was removed and air dried. Spotting the TLC plate with the microcaps can take some practice. It is easy to damage the plate when spotting. Round spots are extremely important to get good quantitative results.

A digital camera with manual exposure settings and a 254-nm UV lamp were attached to a stand so that they were the same distance away from the TLC plates for each picture (Figure 1). It is essential to have a camera with manual exposure settings to make sure that all pictures have exactly the same exposure. If the camera has a zoom lens, the lens must keep the same focal length in addition to the same aperture. It is also important to have the room as dark as possible so that the room light does not alter the exposure. All pictures were taken at a lens aperture of f5.6, a film speed of ISO 400 and a 1 s exposure time. These settings will vary depending upon how far the camera and UV lamp are from the TLC plate.

The UV lamp was warmed up for five minutes before taking pictures. The fluorescent TLC plate was placed under

the UV lamp and camera. The camera was focused on the plate, with the entire TLC plate in the field of view. A picture was taken of the TLC plate and downloaded to a computer.

TLC Analyzer Procedure

To make digital image analysis easier, TLC Analyzer, a computer program that scans a digital picture and returns the values needed, was written.¹ Essentially, TLC Analyzer is a simulated TLC scanner. TLC scanners pan across an HPTLC plate with a beam of light emitted through an adjustable slit. In contrast, a digital image is made up of many rows and columns of “dots” called pixels. Thus, a digital image is essentially a matrix of numbers, and TLC Analyzer “virtually” pans across the matrix, combining moving averages to create a graph.

To use TLC Analyzer, the user first loads the digital image file. The image appears on screen with a readout of the x and y axes in pixels (Figure 2). The user then specifies the “Scan Row” (y coordinate of the desired scan), as well as the “Left Margin” and “Right Margin”. Instead of specifying a slit size, one specifies the “Dropper Size”, reminiscent of the eyedropper found in most photo-editing programs. The eyedropper size determines the number of pixels TLC Analyzer averages together to form a single point in the resulting line graph. The eyedropper size ranges from 1×1 to 25×25 pixels. All of the brightness values within the dropper size are averaged. The user presses “Plot Graph” to analyze the image.

TLC Analyzer shows the path of the scan on the image and creates graphs of the red, green, and blue components (a multispectral scan) as well as the black and white image density (a densitogram). It also finds the maximum and minimum values for these same variables. In addition, it calculates the optical density (used to create calibration curves) for the maximum black and white and monochromatic density values. Optionally, it exports all of the underlying numeric values to a spreadsheet file for further analysis.

Hazards

There are no significant hazards in this experiment. As with most chemicals, none should be breathed in, and any skin that comes in contact with them should be washed thoroughly. Care should be taken to protect students' eyes and hands from the UV lamp by using UV absorbing goggles and gloves respectively.

Discussion

The discussion will focus on four aspects of this research: (i) the principles of what occurs on the TLC plate under UV illumination, (ii) the tighter procedures necessary to turn what is traditionally a qualitative technique into one that is also quantitative, (iii) the technology of digital images, including the transformations necessary to undo image processing done within a digital camera to accommodate the nonlinear nature of human vision, and (iv) a description of multispectral scans, densitograms, and calibration curves as produced by either TLC Analyzer or photo-editing programs.



Figure 1. Setup for digital photography of a TLC plate. The camera and UV lamp were attached to a stand.

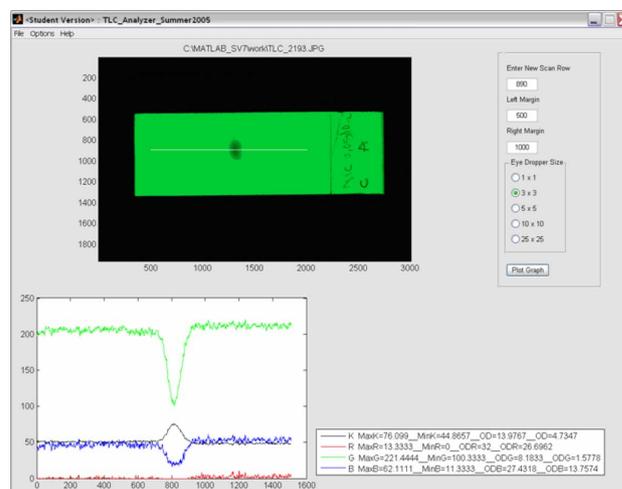


Figure 2. A screen shot of the public domain program created for this research, TLC Analyzer.

The TLC Plate under UV Illumination

To understand the results of DE-TLC, it is important to consider what happens to a fluorescent TLC plate subjected to UV light. The UV lamp emits 254-nm light that can either hit the silica gel on the plate or the sample on its surface. The silica gel will fluoresce green light, while the sample will absorb the light, blocking it from reaching the

silica gel. For this to occur, the sample must have a high extinction coefficient (the quantity of light absorbed and scattered) near 254 nm (8). The light reaching the silica substrate is reduced as the amount of sample in the spot increases, thus reducing the substrate fluorescence in this location. Some chemical samples will themselves fluoresce like the silica gel on the plate, but at different wavelengths (for more information, see the Supplemental Material^W).

Error Avoidance for Quantitative TLC

TLC has never been thought of as a quantitative technique. When using TLC for quantitative work, the researcher must exercise extra care in a number of areas. For quantitative work, keeping the UV lamp and camera the same distance from the TLC plate is crucial. An expensive stand is not necessary to hold the camera and UV light (duct-tape and wire were used to place the UV lamp on the stand). When taking pictures, the spot(s) on the TLC plate should be in the center of the image to avoid light falloff on the edges of the image. During the first five minutes that a UV lamp is turned on, the lamp becomes increasingly brighter; thus, it should be warmed up before use.

The microcaps used had a $\pm 1\%$ error, but this error is only applicable if the microcaps are filled and emptied correctly. When filling the microcap, make sure the microcap is completely full. When spotting the plate, the microcap must be emptied entirely. The spot should be completely dry before placing the TLC plate into the TLC chamber. Round spots are important for quantitative analysis. To ensure rounder spots, a TLC chamber with a flat bottom should be used.

TLC Analyzer works with TIFF and JPEG images. Since JPEG images are compressed with a "lossy" algorithm, they will have different brightness values that are not the same as the original image's values. Each time a JPEG image is saved, it is compressed again, consequently, it is important *not* to resave JPEG images multiple times when using images for quantitative work—save them as TIFFs instead. These techniques take practice, so results may not be perfect the first few times. For a detailed error analysis, see the Supplemental Material.^W

Digital Images

Pixels store color information in three numbers that represent the quantities of red, green, and blue. When all of the numbers are at their maximum value, the pixel is white. When all of the numbers are at their minimum value, the pixel is black. Each of the numbers can be thought of as the brightness of that color, with a range of 0 to 255 (9, 10).

A digital camera captures these brightness values using an array of light-sensitive pixels on a semiconductor chip. The camera lens focuses on the semiconductor chip when taking a picture. Since pixels are monochrome, color filters are used to record a picture's color. The filter array has rows with an alternating pattern of red, green, and blue filters to generate color brightness values. While intensity measures the brightness of a pixel, density measures the darkness. We will follow the example of Adobe Photoshop software and measure image density, D , as a percentage,

$$D = 1 - \frac{n}{255} \quad (1)$$

where n is any red, green, blue, or black and white brightness (intensity) value. Equation 1 is a general equation for density and will be used to calculate more specific densities.

Most photo-editing programs calculate the black and white intensity, $I_{B\&W}$, of an image by the following equation originally formulated by the NTSC for color television signals (11):

$$I_{B\&W} = 0.2989(\text{Red}) + 0.5870(\text{Green}) + 0.1140(\text{Blue}) \quad (2)$$

The variable K is used to designate black and white density. To calculate K , $I_{B\&W}$ is substituted into eq 1 as n . If K is 100%, the pixel being measured is completely black. If K is 0%, the pixel is entirely white. The coefficients on the brightness values for black and white intensity correspond to the human eye's sensitivity to brightness—the eye is most sensitive to green and less sensitive to red and blue (11). Consumer digital cameras and film scanners do a great deal of processing to the raw image sensor output to make it appear normal to the human eye. While the camera's image sensor has linear output, the image that is ultimately formed has logarithmic output.

By repeatedly taking a photo of a uniformly illuminated scene, it was empirically determined that for the camera used, K rose by twenty every time the brightness in the image was decreased by two. This pattern is probably close to other cameras, but it is easy to check; while photographing a white paper, decrease the aperture of the lens by half multiple times and measure what happens to the black and white density, K . An equation was created for this recalibrated "optical density". Black and white density is made up of the RGB brightness values, so optical density can also be calculated for monochromatic densities.

$$\text{Optical Density} = 2.0 \frac{D}{20} \quad (3)$$

Notice how the base is a 2 and the density D (for R, G, B densities and K) is divided by 20, which directly relates to the fact that the density rises by twenty when the light in the image is divided by two.

Multi-Spectral Scans, Densitograms, and Calibration Curves

A multispectral scan shows the reflected and fluorescent light from a TLC plate at different wavelengths. Conveniently, a digital camera captures a range of wavelengths by storing each pixel with separate RGB (red, green, blue) brightness values. Recording and plotting the RGB values of an image of a TLC plate allows the researcher to see how the colors in the plate fluctuate while traveling across it. Notably, DE-TLC is sensitive to colors that are otherwise invisible to the naked eye. If two compounds have similar R_f values, a multispectral scan might be able to differentiate the two compounds, especially if the chemicals display different fluorescence.

TLC Analyzer automatically produces multispectral scans from an image; however, multispectral scans can also be produced using almost any image-editing software by simply reading the pixel brightness values using the eyedropper tool

Table 1. Repeatability of DE TLC with Nicotinamide

Spot	Peak Optical Density			
	2.57×10^{-9} moles/spot	5.12×10^{-9} moles/spot	1.02×10^{-8} moles/spot	2.05×10^{-8} moles/spot
1	6.36	6.77	8.00	9.55
2	6.73	6.92	8.28	9.48
3	5.96	6.82	8.72	9.55
4	6.23	6.89	8.94	9.48
5	6.46	—	8.04	9.24
6	6.73	—	8.08	10.02
Mean	6.41	6.85	8.35	9.55
RSD (%)	4.63	0.95	4.72	2.67

then plotting those values in a graphics program. In fact, DE-TLC was developed using an image-editing program, but TLC Analyzer saves a great deal of time.

Densitograms are created by taking brightness values from a multispectral scan, transforming them in terms of density using eq 1, then plotting the density values against their distance from the origin on the plate. Black and white densitograms (*K*) help analyze the pixel values of a TLC plate; allowing a chemist to see any brightness trends across the plate. Seeing these trends can help differentiate between chemicals spotted on a TLC plate. A group of densitograms can also be used to make a calibration curve for a chemical. Both the black and white density of an image (*K*) and the monochromatic densities were used to create densitograms in this research.

Most calibration curves in this research were produced by plotting the peak black and white optical density (calculated from the peak *K* value using eq 3) for different concentrations of the sample. Recently, this research investigated plotting the peak optical density of either the green or blue brightness values (calculated from their respective peak densities). TLC Analyzer automatically outputs the peak optical density calculated in this manner. For nicotinamide, this research showed that the green monochromatic calibration curves are actually better than the corresponding black and white density curves. Calibration curves can be used to help find an unknown concentration of a chemical. It should be noted that the calibration curves do not go through the origin; in fact, they do not even go through 1 ($OD = 2^0$, eq 3). The zero point for optical density (y intercept) is equal to the optical density of the background fluorescence on the TLC plate.

Qualitative Analysis Results

Multispectral scans with luminol and riboflavin are shown in Figure 3. The red brightness values rise when going over the riboflavin spot because riboflavin fluoresces red under UV light. Similarly, the blue brightness values rise when going over the luminol spot because luminol fluoresces blue under UV light. By using multispectral scans effectively, fluorescence can be seen in a fluorescent chemical's spot. There is also a black and white densitogram in both multispectral scans. Thus, qualitative TLC can be enhanced using DE-TLC and multispectral scans. In comparison, a CAMAG TLC

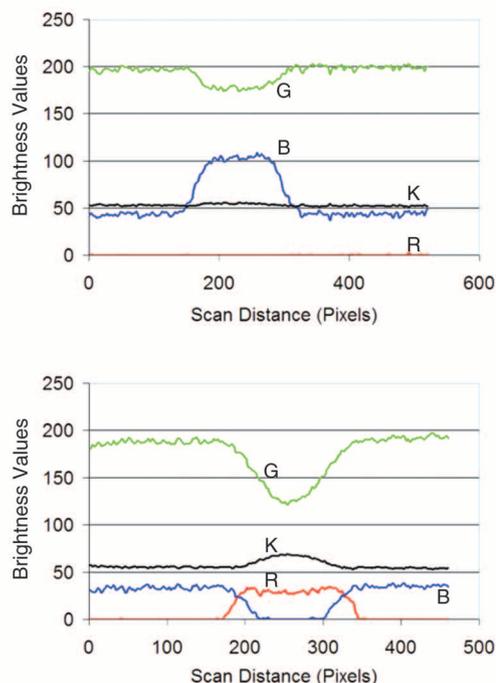


Figure 3. Multi-spectral scans produced by TLC Analyzer for (top) luminol and (bottom) riboflavin.

Scanner 3 used for HPTLC can produce a multispectral scan with up to 31 wavelengths from UV to IR (12). While DE-TLC provides only three visible wavelengths, when using TLC Analyzer it can produce multispectral scans almost instantly. A TLC scanner can take many minutes to produce a scan depending on a number of variables.

Quantitative Analysis Results

Many trials were completed before the sources of error were found and corrected in quantitative TLC. Ultimately, good results were obtained with this simple and inexpensive procedure.

Detection Limits

At small amounts (around 1.28×10^{-9} mol, 1.56×10^{-7} g, in a nicotinamide sample on the plate), DE-TLC can detect a spot, but the procedure is not sensitive enough to distinguish between the minute amounts. This is similar to the detection limits cited by Petrovic (2) for video densitometry, with scanning densitometry 2–3 times lower. For a given chemical the detection limit depends on the λ_{\max} (the wavelength of light absorbed most efficiently) and the extinction coefficient (13).

At high amounts (higher than 8.19×10^{-8} mol, 1.00×10^{-5} g, in a nicotinamide sample on the plate), the optical density starts to saturate. DE-TLC cannot be used effectively, perhaps because the chemical spreads out instead of piling on top of itself. It is possible that DE-TLC could be used at higher amounts if the areas of the peaks in the densitograms

Table 2. DE TLC Linearity (Black and White Density)

Chemical	Limit of Detection		Range		R^2
	10^{-9} moles/spot	10^{-7} grams/spot	10^{-9} moles/spot	10^{-7} grams/spot	
Nicotinamide	1.28	1.56	2.56 – 81.9	3.13 – 100	0.987
Vanillin	< 8.22	< 12.5	8.22 – 131	12.5 – 200	0.971

NOTE: Six trials were combined for nicotinamide.

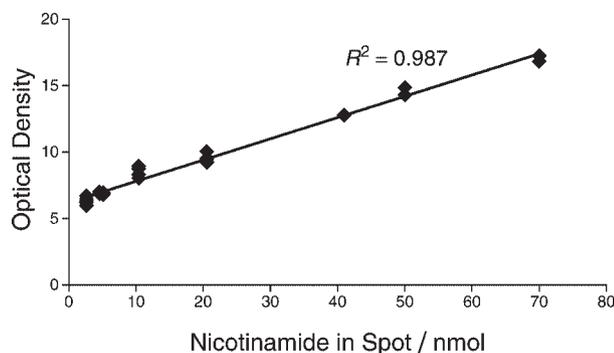


Figure 4. Black and white calibration curve for nicotinamide composed of multiple trials.

(or width of the spot versus quantity) were plotted to make a calibration curve, but this idea has not been studied in detail; it is outside the scope of this article.

Repeatability

The RSD values for DE-TLC were from 0.95% to 4.72% for different concentrations of nicotinamide (Table 1). In comparison, Vovk (4) had RSD values of 3.6% when measuring reflectance with a CCD camera and 1.6% when measuring reflectance with the CAMAG Video Documentation System. For the CAMAG TLC Scanner II, the RSD values were from 0.1% to 2.6%. Simon (3) had RSD values that ranged from 1.37% to 2.87% with a CCD camera. When using a CAMAG Video Documentation System, Petrovic (2) had RSD values from 3.54% to 5.39% and RSD values of 0.61% to 2.05% when using scanning densitometry. Thus, DE-TLC repeatability is acceptable and comparable to other, more expensive video techniques that use HPTLC plates. However, a TLC scanner or densitometer has the best repeatability.

Linearity

A calibration curve for nicotinamide composed of a collection of black and white optical densities from six different trials is shown in Figure 4. The graph has a concentration range of 27:1. Linearity was measured using the correlation coefficient (R^2), which equaled 0.987.

Representative examples of DE-TLC linearity with R^2 values ranging from 0.971 to 0.987 are shown in Table 2. These high R^2 values show that the Beer-Lambert law is a good approximation for a fluorescent TLC plate with a UV absorbing sample. Even with preliminary data, vanillin

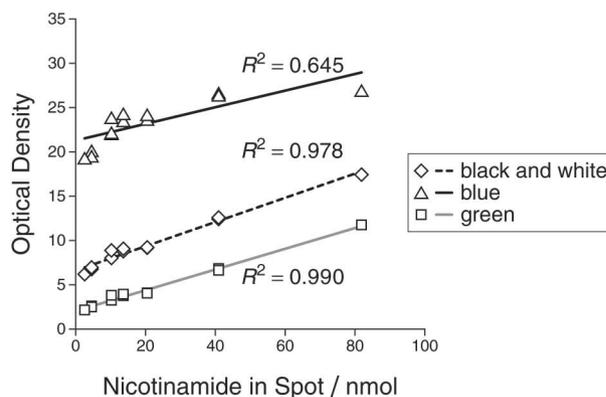


Figure 5. Monochromatic and black and white calibration curves for nicotinamide composed of three trials.

showed a high correlation coefficient. In summary, the optical density of the spot has a linear relationship to the amount of sample in the spot over a fairly wide range. For comparison, HPTLC researchers typically report R^2 greater than 0.99 (2–7).

Black and white calibration curves are composed of an “average” of the red, green, and blue components. Sometimes one monochromatic curve is better than another monochromatic curve, or even the composite (black and white) curve. For example, for nicotinamide the green monochromatic calibration curve has a higher R^2 than the blue curve's. Since nicotinamide fluoresces blue light, it is hypothesized that as the concentration increases, both the green and blue light from the plate decreases in intensity linearly. But the fluorescent blue light from the nicotinamide sample increases with concentration, so the blue brightness values do not decrease as linearly as the green values, giving the blue monochromatic curve a low R^2 value (Figure 5).

Conclusion

Future Study

Even though DE-TLC is already a valuable technique, there are some areas where it can be improved upon. The range of accuracy of DE-TLC could be extended. Peak area (or width of spot versus quantity) should be considered in conjunction with density for possible improvement of calibration curve linearity and range. Since DE-TLC can detect fluorescence in a sample, fluorescent enhancement techniques (14) might increase the sensitivity of DE-TLC. Finally, a larger variety of chemicals needs to be investigated to examine how concentration versus intensity is different for each chemical.

Closing Comments

This study has demonstrated that a digital camera with manual exposure and regular fluorescent TLC plates can be used to do quantitative and qualitative analysis with accuracy that is surprising with such inexpensive equipment. Qualitative TLC analysis is improved with DE-TLC. TLC Analyzer software lets the researcher quickly create multispectral scans that provide useful information, such as fluorescence detection, to help identify chemicals on the TLC plate.

The high linearity ($R^2 \approx 0.97\text{--}0.98$) shows that the Beer-Lambert law is a good approximation for a fluorescent TLC plate with a UV absorbing sample. Over a wide range, the optical density of the spot has a linear relationship to the amount of sample in the spot. Linearity combined with good repeatability (RSD < 5%) and detection limits approaching those of HPTLC mean that DE-TLC can also be used for quantitative analysis. TLC Analyzer software automates most of the required number crunching.

DE-TLC is low-cost and easy to use and thus can be utilized in a similar manner as a TLC scanner in any science lab, including college and high school labs. Although digitally enhanced TLC cannot take the place of a TLC scanner, it is a great alternative for people who cannot afford more costly equipment.

Acknowledgments

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Supplemental Material

A detailed error analysis is available in this issue of *JCE Online*.

Note

1. TLC Analyzer is a program that analyzes digital images of TLC plates and generates multispectral scans, densitograms, and calibration curves. To learn more and/or to download a copy, visit this Web site: http://www.sciencebuddies.org/science-research-papers/tlc_analyzer.shtml (accessed Mar 2007).

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