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

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Introduction

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, a plethora of chemists frequently use TLC methods in their labs.

Quantitative TLC analysis is typically the province of HPTLC (High-Performance TLC). HPTLC plates have much finer particles than regular TLC plates, and consequently they are better suited for quantitative analysis, although they are more costly. HPTLC samples are typically applied by mechanical applicators (dosimeters) and analyzed with automated equipment, such as a TLC scanner (*1*).

Unfortunately, the equipment for quantitative TLC analysis is very expensive—a TLC scanner costs approximately $30,000. High schools and colleges that cannot afford such expensive equipment are limited to regular TLC, thus their students do not acquire experience with quantitative methods.

I demonstrate 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 quantitative analysis possible. In this novel procedure, a fluorescent TLC plate is illuminated with UV light and a picture of the plate is taken with a digital camera. Then using either TLC Analyzer, the public domain software I wrote, or common photo-editing software, one can

quickly produce multi-spectral scans, densitograms, and calibration curves, output previously available only from more expensive equipment or complex procedures.

Other researchers have used a CCD camera to do quantitative TLC analysis (*2, 3, 4*). One other 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 researchers used expensive equipment, such as TLC scanners and costly video cameras.

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 multi-spectral scans. It is like a "poor man's" spectrophotometer, and it is very 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 anywhere, 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.

Procedure

TLC Procedure

The sample was weighed out and dissolved in a measured quantity of appropriate solvent. So that each spot contained a known quantity of material, the solution was then spotted with one microliter microcaps (Drummond cat #1-000-0010) on an EMD Silica Gel 60 F254 fluorescent TLC plate and developed in a TLC chamber using the appropriate mobile phase. For nicotinamide (Fluka, Switzerland, 98.5%) the mobile phase was water, ethyl acetate, and ethanol in the ratio of 2:2:1. 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 (Nikon D1X) and a 254 nanometer UV lamp (Mineral Light UVS·11) were attached to a stand so that they were the same distance away from the TLC plates for each picture taken (see Figure 1). It is essential to have a camera with manual exposure settings to make sure that all pictures have the exact same exposure so they can be compared to each other. 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 doesn't alter the exposure. All pictures were taken at a lens aperture of f5.6, a film speed of ISO 400, and a one second exposure time. These settings will vary depending upon how far the camera and UV lamp are from the TLC plate. It is a good idea to do a few tests with different exposures to see which exposure is best for a particular setup.

Figure 1: Setup for digital photography of a TLC plate. The camera and UV lamp were attached to a stand to insure that they were always the same distance from the 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 camera's 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, I wrote TLC Analyzer, a computer program that scans a digital picture and returns the values needed. 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. (See 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 1x1 to 5x5 pixels. All of the brightness values within the dropper size are averaged. The user presses "Plot Graph" to analyze the image.

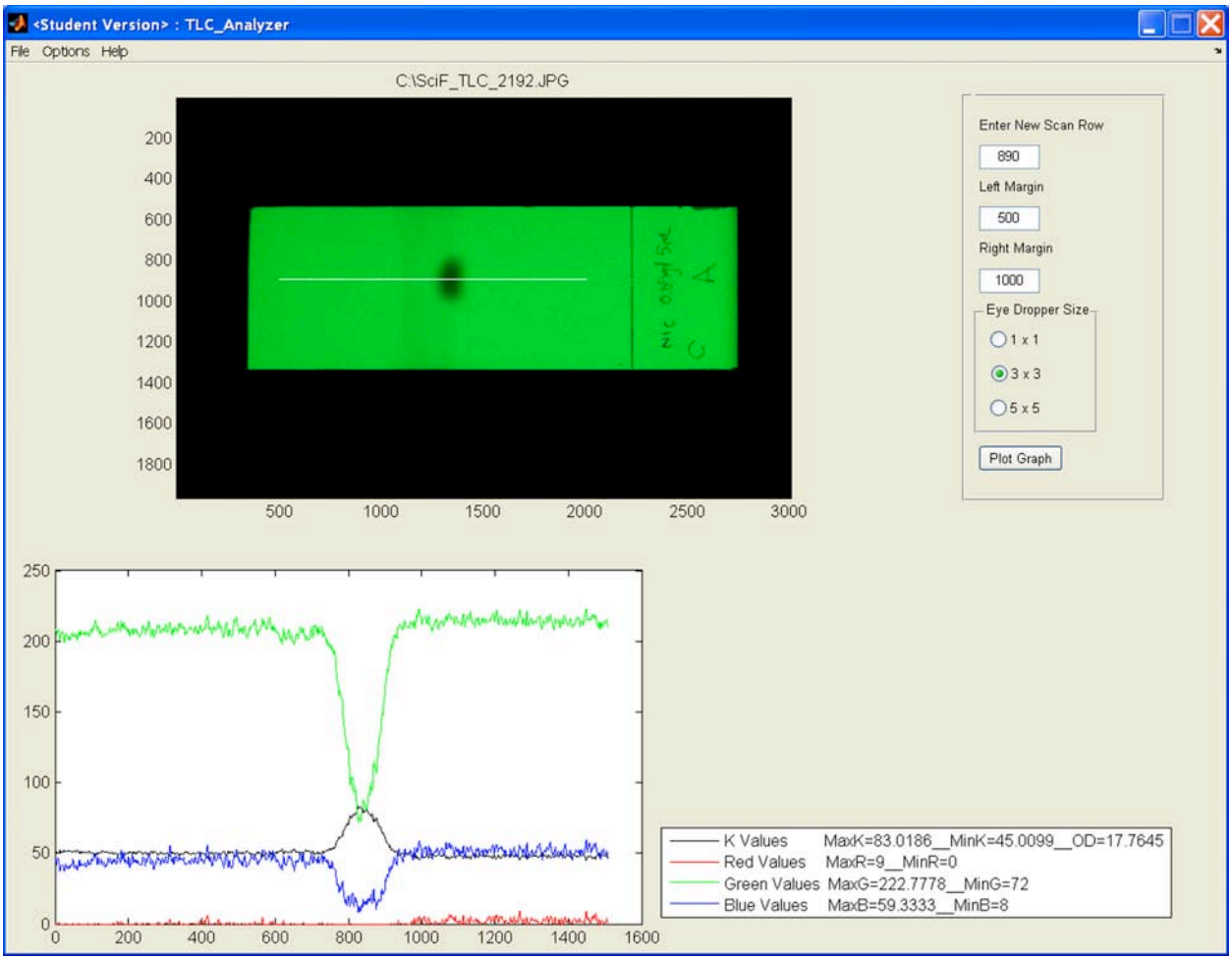

Figure 2. A screen shot of the public domain program I created, TLC Analyzer. The user loads a digital image and then chooses what row(s) in the picture to scan (shown at row 890). The left and right margins are then chosen (shown at 500 and 1000 pixels, respectively). The "Eye Dropper Size" determines the number of pixels TLC Analyzer averages together to form a single point in the resulting line graph. The white line across the image shows what rows and columns TLC Analyzer is "scanning." The RGB and K values are then plotted to make a multi-spectral scan. TLC Analyzer is much easier to use and quicker than a photo-editing program.

TLC Analyzer shows the path of the scan on the image and creates graphs of the red, green, and blue components (a multi-spectral 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 density value. Optionally, it exports all of the underlying numeric values to a spreadsheet file for further analysis.

Discussion

The discussion will focus on four aspects of this research: (1) the principles of what occurs on the TLC plate under UV illumination, (2) the tighter procedures necessary to turn what is traditionally a qualitative technique into one that is also quantitative, (3) 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 (4) a description of multi-spectral scans, densitograms, and calibration curves as produced by either TLC Analyzer or photo-editing programs.

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 generally absorb the light and block the light from reaching the silica gel. Of course, in order for the sample to absorb UV light and block it from the silica gel, the sample must have a high extinction coefficient (the amount of light absorbed and scattered) near 254 nm (*8*). Some chemical samples will themselves fluoresce like the silica gel on the plate, but at different wavelengths. The amount of the sample in the spot determines how much of the silica gel that the UV light is able to reach. If the concentration of a sample on the plate is relatively high, the chemical's spot will be dark under UV light. The UV light can also go between some of the molecules in the sample, hitting the silica gel. Thus, if the concentration of a sample is relatively low, the spot will appear greenish gray under UV light. In summary, the fluorescent green light that is seen within the sample's spot occurs when the UV light goes between spaces in the chemical and excites the silica gel on the plate. (See Figure 3.)

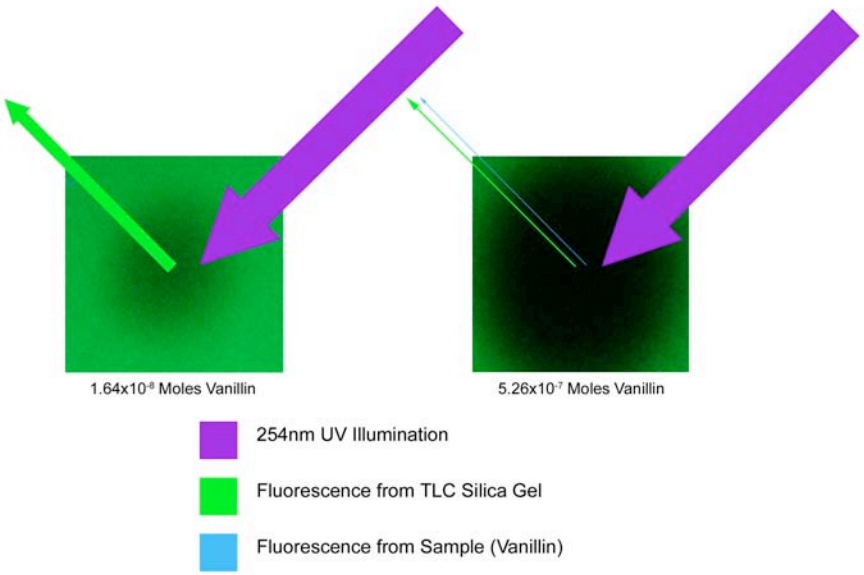

Figure 3. The lower the concentration of the sample on a TLC plate, the more 254 nm UV light that can go between molecules of the sample to reach the green fluorescing compound in the silica gel on the plate. As the concentration of the sample increases, less and less UV light reaches the silica gel and the spot becomes darker (unless it is an extremely bright fluorescent compound in its own right).

If the chemical's spot does not change significantly in size as the concentration increases, the Beer-Lambert law (Eq 1) should apply to fluorescent TLC slides with a UV absorbing sample. The Beer-Lambert law does not account for temperature, wavelength, or interactions between molecules, so the law typically applies to dilute solutions (*9*). However, on a fluorescent TLC plate, a sample's density should approximately have a linear relationship with its concentration.

$$A = \varepsilon bc \qquad (1)$$

where, A is the absorbance, epsilon (ε) is the extinction coefficient of the chemical, b is the absorption path length, or how thick the specimen is, and c is the concentration of the specimen.

A variation in the Beer-Lambert law can also describe the fluorescence of the sample—if, of course, the chemical is fluorescent. Adsorption is a key factor in fluorescence because the more light energy the molecule is able to absorb, the more intense its fluorescence. This variation

in the Beer-Lambert law shows the relationship between the concentration of the chemical and the intensity of the chemical's fluorescence:

$$I = 2.3Q\varepsilon bcP_o \quad (2)$$

where *I* is the intensity of the fluorescence of the chemical, *Q* is the quantum efficiency of the chemical—how efficient an electron in a molecule of that chemical absorbs a photon of light, epsilon (ε) is the extinction coefficient of the chemical, *b* is the absorption path length of the chemical, *c* is the concentration of the chemical, and P_o is the power of the exciting light (*8*).

If P_o and *b* are held constant, a simple relationship can be seen in the equation:

$$I = Qc \quad (3)$$

By this variation, the concentration of the chemical is proportional to its intensity once excited (*9*). On a TLC plate, the amount of sample in the spot is proportional to the concentration of the sample, and the intensity of the spot under UV light should be proportional to the amount in the spot.

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 very crucial. Moving the lamp a centimeter could change the pixel brightness values 10%-20%. No expensive stand is necessary to hold the camera and UV light (I used duct-tape and wire to place my UV lamp on the stand).

Camera lenses have light falloff in the corners of the image frame (*10*). 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. Light falloff can easily change the brightness on the edges of the image by one f-stop, a factor of two in brightness.

During the first five minutes that a UV lamp is turned on, the lamp becomes increasingly brighter. Therefore, it is important to warm up the UV lamp for five minutes before taking any pictures. If the lamp is not warmed up, variations of the image's brightness values can be 10% or more.

The microcaps I used had a +/-1% error, but this error is only applicable if the microcaps are filled and emptied correctly. When filling up the microcap, make sure the microcap is completely full. When spotting the plate, the microcap must be emptied entirely. This ensures that the amount of chemical on each TLC plate is as accurate as possible. Do not push on the plate when spotting, just touch the plate and squeeze the dropper on top of the microcap. Make sure that the spot is completely dry before placing the TLC plate into the TLC chamber, otherwise the solvent will run into the wet spot and proceed more quickly up the plate from the spot's original location, and more slowly up the plate on the edges.

A chemical's spots need to be a consistent shape to do quantitative TLC analysis. Round spots are the best way to insure consistency because it is much easier to produce a round shape multiple times than the same exact crescent shape, for example. There are a few strategies to get good spots (*11*, *12*). A TLC chamber with a flat bottom needs to be used, otherwise the solvent will rise like a crescent instead of straight. If a standard TLC chamber is not available, a jar with a flat bottom can be used. Sometimes, even when using a chamber with a flat bottom, the solvent also forms a type of crescent when it moves up the TLC plate. Switching solvents may help or changing the solvent ratio may help produce more consistent spots.

Most digital cameras give the user the chance to choose the file type for storing pictures. 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. Depending upon how much the image is compressed, the brightness values can

vary from 1%-2% to even more when compared to the values in the original image. 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.

If these instructions are followed, quantitative TLC analysis should be relatively easy. These techniques do take practice, however, so do not get discouraged if the results are not perfect the first few times.

Digital Images

Pixels store color information in three numbers that represent the amounts of red, green, and blue. When all of the three numbers are at their maximum value, the pixel is white. When all of them are at their minimum value, the pixel is black. Each of the three numbers can be thought of as the brightness of that color, with a range of 0 to 255 (*13*, *14*).

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 an alternating pattern of red and green filter rows and green and blue filter rows (*15*).

For quantitative work, black and white brightness values are needed in addition to color values. Most photo-editing programs calculate the black and white intensity of an image by the following equation originally formulated by the NTSC for color television signals (*16*):

$$\text{Intensity} = 0.2989*\text{Red} + 0.5870*\text{Green} + 0.1140*\text{Blue} \quad (4)$$

While intensity measures the brightness of a pixel, black and white density measures the darkness. We will follow the example of Adobe Photoshop software and measure image density (K) as a percentage.

$$K = 1 - (0.2989*\text{Red} + 0.5870*\text{Green} + 0.1140*\text{Blue}) / 255 \quad (5)$$

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 (*16*). Consumer digital cameras and film scanners do a great deal of processing to the raw image sensor output in order to make it appear normal to the human eye. Digital photography is filled with accommodations like this. While the camera's image sensor has linear output, the image that is ultimately formed has logarithmic output. The transformation applied can be quite complex, especially for extremely dark and bright pixel values.

By repeatedly taking a photo of a uniformly illuminated scene, I empirically determined that for the camera I 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.

Since K rose by 20 when the light in an image was halved, I was able to create an equation for optical density[1]:

$$\text{Optical Density} = 2.0^{K/20} \tag{6}$$

Notice how the base is a 2 and the number K is divided by is 20, which directly relates to the fact that K rises by twenty when the light in the image is divided by two.

Multi-spectral Scans, Densitograms, and Calibration Curves

A multi-spectral 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

[1] One can verify this by taking a scene and photographing it multiple times, each time halving the amount of light that enters the camera.

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 multi-spectral scan might be able to differentiate the two compounds, especially if the chemicals display different fluorescence.

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

Although densitograms are created in the same way as a multi-spectral scan, they analyze the black and white 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. The black and white density of an image (K) was used to create densitograms in this research.

All calibration curves in this research were produced by plotting the peak optical density (calculated from the peak K value) for each of the different concentrations of the sample. TLC Analyzer automatically outputs the peak optical density calculated in this manner. The calibration curve can then be used to help find an unknown concentration of a chemical.

Results: Qualitative Analysis

Figure 4 shows a multi-spectral scan of a TLC plate. Three different samples were put on the plate: The two on either side of the plate are the starting materials in a synthesis, while the spots in the middle are from a sample of the reactants after a few minutes of reaction time. There appear to be only two spots, and thus two chemicals from the sample of the reaction. The two

spots correspond to the two starting chemicals. However, there is another spot hidden by the starting chemical veratraldehyde because the product and veratraldehyde have very similar R_f values. The product happens to be fluorescent, and by looking at a multi-spectral scan of the plate, the two spots can be differentiated. The blue in the image rises *after* the K values for veratraldehyde do. If the source of the blue came from the veratraldehyde, the blue brightness values should start rising right when the scan goes over the veratraldehyde spot. Figure 4 also contains a densitogram used for creating a calibration curve.

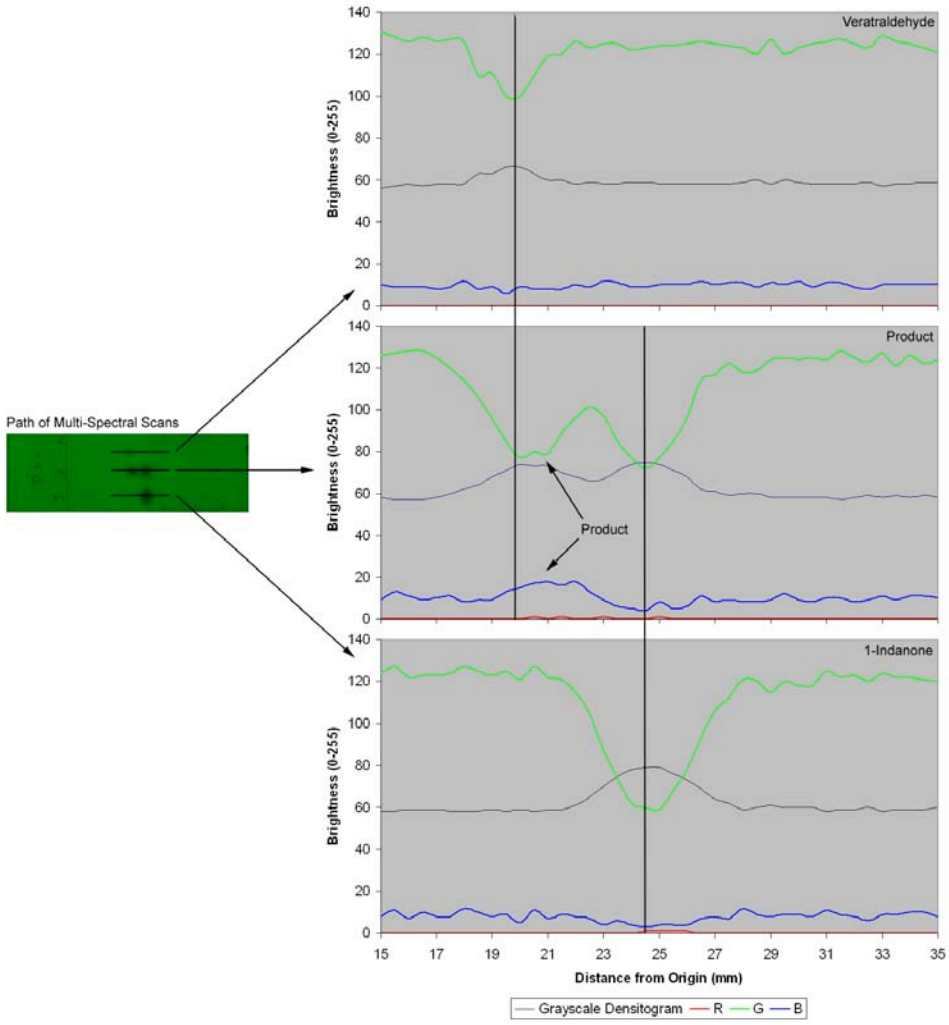

Figure 4. Multi-spectral scan of a TLC plate created by plotting brightness values from Adobe Photoshop. Three different samples were put on the plate: the two on either side of the plate are the starting materials in a synthesis, while the spots in the middle are from a sample of the reactants after a few minutes of reaction time. The product is hidden by one of the starting materials (Veratraldehyde) because they have very similar R_f values. The product is fluorescent because it is a relatively rigid molecule, and by looking at a multi-spectral scan of the plate, the two spots can be differentiated.

Figure 5 shows another example of multi-spectral scans with luminol and riboflavin. 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 multi-spectral scans effectively, fluorescence can be seen in a fluorescent chemical's spot.

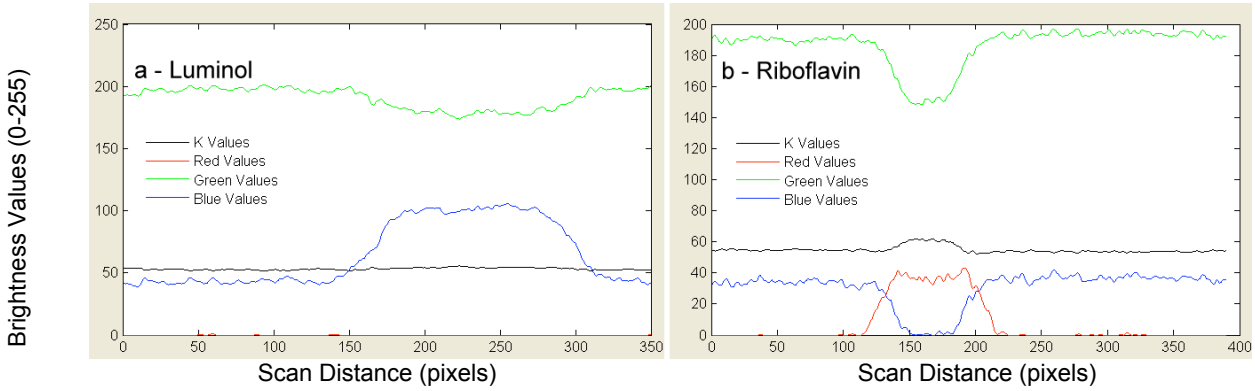

Figure 5. Multi-spectral scans produced by TLC Analyzer for (a) luminol and (b) riboflavin.

Thus, qualitative TLC can be enhanced using DE TLC and multi-spectral scans.

In comparison, a CAMAG TLC Scanner 3 used for HPTLC can produce a multi-spectral scan with up to 31 wavelengths from UV to IR (*17*). While DE TLC provides only three visible wavelengths, when using TLC Analyzer it can produce multi-spectral scans almost instantly. A TLC scanner can take many minutes to produce a scan depending on a number of variables.

Results: Quantitative Analysis

Many trials were completed before I was finally able to find and correct the many sources of error in quantitative TLC (see Discussion). Ultimately, the results were quite good for such a simple and inexpensive procedure.

Detection Limits

At very small amounts (around 1.28 x 10^{-09} moles—1.56 x 10^{-07} grams—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. Of course, for a given chemical the detection limit depends on the lambda max (the wavelength of light absorbed most efficiently) and the extinction coefficient (*8*).

At high amounts (higher than 8.19×10^{-08} moles—1.00×10^{-05} grams— 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 were plotted to make a calibration curve, but I have not studied this idea in detail.

Repeatability

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%

The RSD values for DE TLC were from 0.95% to 4.72% for different concentrations of nicotinamide (see 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

Figure 6 is a calibration curve for nicotinamide comprised of a collection of optical densities from six different trials. The graph has a concentration range of 32:1. Linearity was measured using the correlation coefficient (R^2) which equaled 0.983.

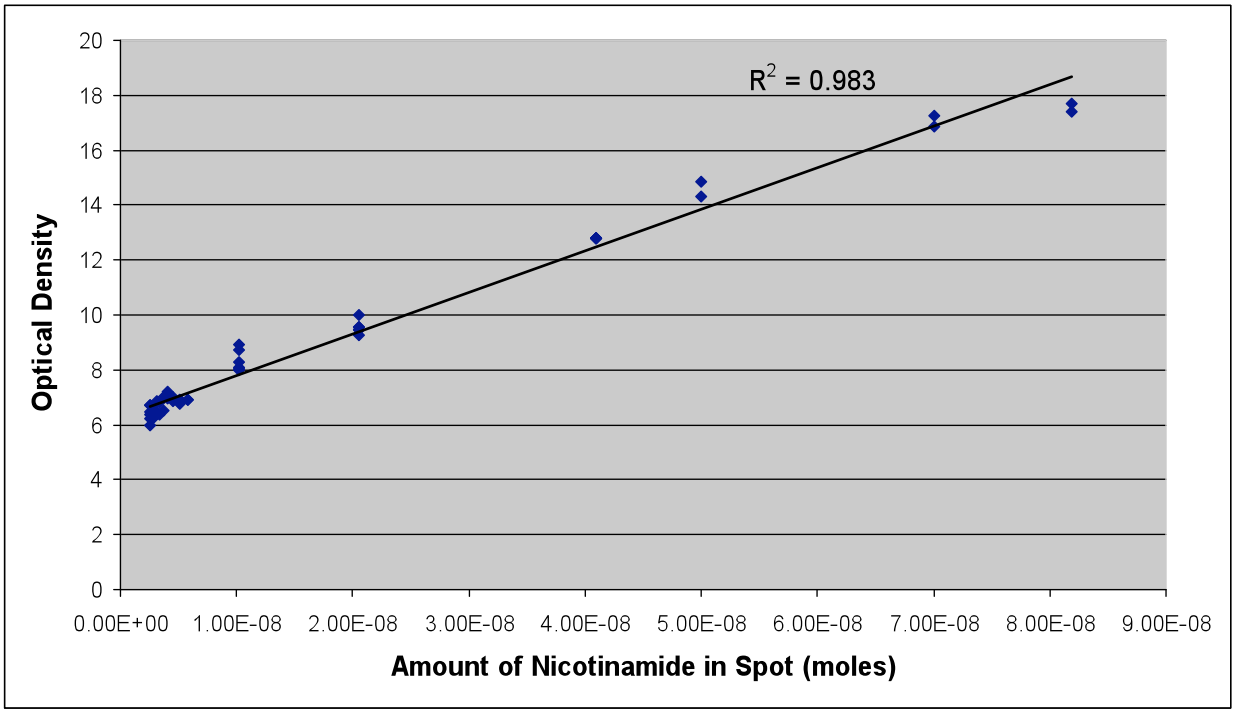

Figure 6. Calibration curve for nicotinamide comprised of multiple trials.

Table 2 shows representative examples of DE TLC linearity with R^2 values ranging from 0.971 to 0.991. 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. 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.

Table 2: DE TLC Linearity

Chemical	Limit of Detection (10^{-09} moles/spot)	Limit of Detection (10^{-07} grams/spot)	Range (10^{-09} moles/spot)	Range (10^{-07} grams/spot)	Correlation Coefficient (R^2)
Nicotinamide (Six Trials Combined)	1.28	1.56	2.56 - 81.9	3.13 - 100	0.983
Nicotinamide (Best Trial)	1.28	1.56	2.56 - 81.9	3.13 - 100	0.991
Vanillin	< 8.22	< 12.5	8.22 - 131	12.5 - 200	0.971

For comparison, HPTLC researchers typically report R^2 greater than 0.99 (*2, 3, 4, 5, 6, 7*).

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 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 (*11*) might increase the sensitivity of DE TLC. In this research, K has been calculated in the traditional manner with coefficients appropriate for human vision (see Eq 5). Different coefficients may offer better results for DE TLC. Finally, I would like to test a much larger variety of chemicals and examine how concentration vs. intensity is different for each chemical.

Closing Comments

I have 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 greatly improved with DE TLC. TLC Analyzer software lets the researcher quickly create multi-spectral scans that provide useful information to help identify chemicals on the TLC plate. For example, some spots have similar R_f values and overlap each other; they may not separate with a different mobile phase or solvent ratio. DE TLC can be used to detect fluorescence invisible to the naked eye, which can help distinguish between different chemicals with similar R_f values.

The high linearity ($R^2 \approx 0.97 – 0.98$) shows that the Beer-Lambert law is a good approximation for a fluorescent TLC plate with a UV absorbing sample. In summary, 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 like 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.

Acknowledgements

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