Standalone Software

Analysis: Quantitative Thin Layer Chromatography

- demonstrate the separation and quantification of target compounds.

Learning Objectives:

1. Become familiar with the image processing and analysis of developed and dried TLC plates containing fluorescent and non-fluorescent samples.
2. Understand how to obtain a calibration curve that serves for the determination of an unknown sample.
3. Relate critical parameters in the image acquisition and analysis to accuracy in the sample quantification.

Theory:

TLC is a well established procedure by which components of a mixture can be separated.[1,2] Careful image analysis of the TLC plate can further allow for quantification of these components.[1] At sufficiently low concentrations, the intensity of the absorbance or fluorescence of compounds varies linearly with their concentration. This enables the construction of a calibration curve based on the intensities of known concentrations for a given target compound. The measured intensity of an unknown sample can then be directly related to its concentration. The qTLC software uses an image of the TLC plate to evaluate each band on the plate in 2D and provides the integrated intensity.

Procedure:

Image Acquisition

The quality of the image used in the qTLC software can significantly affect the results. The main cause of error related to image quality is image saturation. Saturation occurs when the light intensity exceeds the ability of the sensor to count photons, hence additional photons are no longer counted. The qTLC software will help to identify image saturation after the user uploads the image. The following are some guidelines for optimizing the image acquisition process.

- Taking images with an embedded camera of a smartphone in 'automatic' mode is possible but not ideal. This is related to the large dynamic range of the TLC plate image. In particular when imaging fluorescent samples, the brightest regions of the image (the bands where most of the fluorescent compound is located) can become saturated and unusable for quantification purposes. If available on the smartphone or imaging device, it is preferable to use 'manual' exposure settings. If applicable, it should be ensured that the exposure settings are calculated using 'spot' or 'crop' metering (rather than 'full-frame' metering).
- In order to maximize the amount of light passing through the lens of the camera, the F-stop (or F number) should be set to its smallest value. This warrants that the aperture of the camera is fully-open.
- If possible to mount the phone in a stable position (e.g. on a tripod or lab clamp stand), images can be taken with long exposures (i.e. $> 1/30$ second) without inducing movement blur. If this is not possible, as a rule of thumb, ensure the exposure time / shutter speed is 1/30 second or shorter.
- High ISO values increase the sensitivity of the image sensor to incoming light at the cost of increased image noise. Therefore, it is preferable to use the smallest ISO value that still results in an acceptable shutter speed. It is acceptable to set the ISO of the image to 'auto'.
- Finally, it can be challenging to get the correct focus position using autofocus when the TLC plate is in the dark box under fluorescent conditions. Therefore, it is advisable to set the focus position under non-fluorescent conditions by maximising the sharpness of the pencil-drawn elution line at the top of the TLC plate.

qTLC Software

- Once the program has been opened, use the 'Upload Image' button to find the required image and load it into the software.
- Rotate the image for upright orientation (if required).

- For non-fluorescent samples, 'Invert' the colors to ensure the bands are bright on a dark background. For ease of use, adjust the 'Max' and 'Min' contrast sliders so that all the bands on the TLC plate can be observed. This will not affect the results.
- Crop the image using the 'Crop' button to exclude any areas that are not part of the TLC plate. Ideally, the cropping would select only the region containing the bands.
- A background correction must be applied. This helps to mitigate uneven illumination across the TLC plate. To perform this background correction, select the 'Background' button and draw a rectangle around the largest band on the plate. The background is then adjusted automatically.
- The lanes and bands on the TLC plate need to be identified; select the 'Lane and Band Selection' button on the software and draw a rectangle around the first lane. This opens a new figure displaying the selected region. Select each band in that particular lane. A red box will appear around the region selected for each band. The lane and band selection process must be repeated for each lane.
- To re-select a band, use the 'Reselect Lane and Band' button. This opens a dialogue box asking which lane and band should be reselected. Once this information has been provided, reselect the chosen band and a blue box will appear around the reselected area.
- Once satisfied with the lane and band selection, click the 'Next' button for band analysis.
- At this point the minimum peak prominence (MinPeakProm) and Divisor values will need to be adjusted to optimise analysis of the TLC plate. The default values are 1 and 2 respectively. To adjust these values, enter a number and use the 'Update' button. The image of the TLC plate with the red overlay will update to reflect the changes. The red overlay shows the region that the program defines as the physical boundaries of the band itself. All bands should have red regions covering them. The size of the red region can vary from band to band based on the level of intensity.
- Once suitable MinPeakProm and Divisor values have been selected, click 'Calculate' to obtain a table of the integrated areas for each band. Additionally, the image of the TLC plate with the red overlay of the integrated areas can be saved by clicking on 'Save Image'.
- Use the 'Help' menu on the toolbar to learn more about each step.

Minimum Peak Prominence: The peak prominence relates to how much a peak (or signal) stands out when compared to the background noise or other signals.[3] In the qTLC software, the minimum peak prominence (MinPeakProm) value defines how much the signal must rise about the surrounding noise. If the MinPeakProm is too high, no peaks will be identified and the signal will be ignored by the software but if it is too low, noise will significantly impact the signal. Therefore, it is important to find a good balance for the MinPeakProm.

Divisor: The divisor determines how far down from the maximum of the signal the qTLC software will integrate. For example, if the divisor value is 2, the software will integrate down to half the height of the signal.

Discussion Questions

1. Using the results from the qTLC software, construct the calibration curve. What is the R^2 value for the calibration curve? How might it be improved?
2. Using the given concentration, What is the percent error for the calculated unknown concentration?
3. Discuss the Beer-Lambert law and how it pertains to this analysis method. Describe an experiment in which the linearity of the sample could be determined.
4. Highlight the major sources of error and how they might be mitigated.

References

1. Spangenberg, B., Poole, C. F. & Weins, C. *Quantitative Thin-Layer Chromatography* (Springer, Heidelberg, 2011).
2. Hansen, S., Pedersen-Bjergaard, S. & Rasmussen, K. Thin-Layer Chromatography. In *Introduction to Pharmaceutical Chemical Analysis*, 163–172 (John Wiley & Sons, Ltd, Chichester, UK, 2011).
3. Prominence - MATLAB & Simulink - MathWorks, United Kingdom. https://uk.mathworks.com/help/signal/ug/prominence.html (accessed Sep 2018).