A Toolkit to Quantify Target Compounds in Thin Layer Chromatography Experiments

Stuart Ibsen, Niamh Mac Fhionnlaoich, Luis Serrano, Alaric Taylor, Runzhang Qi, Stefan Guldin

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File list (6)

- Educational_TLC_paper_v10.pdf (4.44 MiB)
- SI Educational TLC Paper v10.pdf (15.21 MiB)
- qTLC_v3_macOS_sepRT.zip (31.22 MiB)
- qTLC_v3_macOS_standalone.zip (1.17 GiB)
- qTLC_v3_Windows_sepRT.zip (237.06 MiB)
- qTLC_v3_Windows_standalone.zip (1.70 GiB)
A Toolkit to Quantify Target Compounds in Thin Layer Chromatography Experiments

Niamh Mac Fhionnlaoich,†,‡ Stuart Ibsen,†,‡ Luis A. Serrano,† Alaric Taylor,†
Runzhang Qi,† and Stefan Guldin*,†

†Department of Chemical Engineering, University College London, Torrington Place,
London, WC1E 7JE, UK
‡Contributed equally to this work

E-mail: s.guldin@ucl.ac.uk

Abstract

Thin layer chromatography (TLC) is one of the basic analytical procedures in chemistry and allows the demonstration of various chemical principles in an educational setting. An often-overlooked aspect of TLC is the capability to quantify isolated target compounds in an unknown sample. Here, we present a suitable route to implement quantitative analysis in a lesson plan. We freely provide both a stand-alone software and an online webapp that allow students to obtain quantitative information from a developed TLC plate and present two suitable experiments, namely the absorbance-based quantification of the colorant Sudan IV and the fluorescence-based quantification of Rhodamine 6G, a fluorophore widely used in biotechnology. Students conduct TLC experiments following established protocols, then take pictures of their TLC plates with mobile phones and subsequently quantify the amount of different compounds in the separate bands they observe.
Keywords

Audience: General Public; High School / Introductory Chemistry; First-Year Undergraduate / General; Second-Year Undergraduate; Upper-Division Undergraduate; Graduate Education / Research; Continuing Education. Domain: Analytical Chemistry; Demonstrations; Environmental Chemistry; Laboratory Instruction; Public Understanding / Outreach. Pedagogy: Computer-Based Learning; Hands-On Learning / Manipulatives; Inquiry-Based / Discovery Learning; Problem Solving / Decision Making. Topic: Laboratory Computing / Interfacing; Laboratory Equipment / Apparatus; Microscale Lab; Quantitative Analysis; Student-Centered Learning; Thin Layer Chromatography

Introduction

Thin layer chromatography (TLC) is a well-known chemical analysis procedure which is inexpensive and simple to perform. The technique involves spotting a sample solution onto a TLC plate, the stationary phase, and placing the plate into a vessel containing the mobile phase. Capillary forces draw the mobile phase through the plate, which drives the chromatographic separation of the individual non-volatile components of the sample mixture. Once the TLC plate is dried, individual bands may be observed in absorbance and/or fluorescence.

TLC has been thoroughly developed for use in an educational setting. Dickson et al. proposed a series of simple laboratory experiments designed to teach students the fundamentals of TLC, such as the roles of the retardation factor, hydrogen bonding, and polarity. Six suitable separations of mixtures of organic compounds and inorganic ions were developed by Brinkman and De Vries. Olensen and Hopson presented an experiment, whereby students used principles of TLC to identify the composition of an unknown black ink. Ma and Yeung designed an experiment for the separation of components from beverages. Building on this practical example, Torres y Torres et al. proposed extraction and analysis of caffeine from beverages using TLC. Recently, Sjurnes et al. outlined an experiment to teach normal and
reverse phase TLC using an example of green leaf extracts.\textsuperscript{8}

The quantification of target compounds is typically not included in these lesson plans due

to a lack of inexpensive and easy-to-use tools that are compatible with TLC and designed for

an educational setting. To this end, Valverde \textit{et al.} introduced a route to quantify the pho-
tosynthetic pigments in green beans by using a flatbed scanner.\textsuperscript{9} While this work provides

an interesting approach to densitometric quantification, the integration of flatbed scanners

into suitable work stations and safe experimental conduct remains challenging. Furthermore,
the method lacks compatibility with fluorimetry, and staining of target compounds is

only available for some of the colourless compounds.\textsuperscript{10} An alternative route to obtain im-

ages suitable for quantitative analysis is digital photography. Hess introduced an approach

compatible with classroom settings and provided an appropriate algorithm.\textsuperscript{11} Further de-
velopments were aimed towards research applications. Fichou \textit{et al.} recently presented a

web-based application for quantitative TLC analysis (rTLC).\textsuperscript{12} Meanwhile, ImageJ provides

a tool for electrophoretic gels which can easily be applied to TLCs.\textsuperscript{13,14} While these works

and the accompanied software represent an important contribution to quantitative read-out

and promote their accessibility, limitations remain, especially for educational settings. In

all three of the above methods, the quantification routine reduces the two dimensional (2D)
densitogram to an one dimensional (1D) array for calculation purposes. This loss of dimen-
sional information limits the achievable accuracy and promotes user errors. Furthermore,

no accommodation for uneven illumination is made by the software and any thresholding is

applied on a local basis, which will negatively affect the consistency of the analysis across
the TLC plate. Both the rTLC and ImageJ software require a consistent lane width and
the rTLC further demands regular lane spacing; this is difficult to achieve in an educational
setting where users are less experienced with TLC sample preparation.

Here, we present an intuitive and user-friendly analytic platform that enables students
to reliably quantify the compounds they are studying. Alongside, we supply specifically
designed and freely available software for Windows and macOS operating systems and an
online webapp for all mainstream browsers.\textsuperscript{15} These programs are developed to work with images obtained by non-specialized equipment, which can be as simple as a mobile phone. Uniquely, these tools identify overexposed pixels and allow for correction of uneven illumination. The image analysis routines enable students to build their own calibration curve using known dilutions of a stock solution and to quantify the target compound in an unknown sample concentration. By placing the calibration curve and the ‘unknown sample’ on the same TLC plate, more accurate results can be obtained. The tools require the user to select the bands of interest and then automatically determine their physical dimensions and intensities in 2D. This removes user error/bias from the process of quantification. Furthermore, these tools requires no assumption on lane width or spacing. We present two example experiments and provide step-by-step guides for successful implementation. The first experiment relates to the colourant Sudan IV for an absorbance-based quantification with relevance in analytical environmental chemistry. In a second experiment, Rhodamine 6G, a fluorophore widely used in biotechnology, is quantified by fluorimetric means. While these two experiments demonstrate the capabilities of the tools, the analytic approach is generic and can be applied to a wide variety of other TLC experiments.

**Experimental**

**Materials**

Acetic acid (99.8 %), methanol (MeOH, HPLC grade), Rhodamine 6G (99 %) (CAS number 989-38-8) and Sudan IV (CAS number 85-83-6) were purchased from Sigma Aldrich and used without further purification. Non-fluorescent, aluminium-backed, normal phase TLC plates (TLC silica gel 60) were obtained from Merck.

**TLC Plate Preparation**

The TLC separation may be performed using any of the well-established TLC lesson
Figure 1: **TLC plate preparation.** a) A serial dilution of the target compound is prepared and spotted onto the plate along with the ‘unknown sample’. b) The TLC plate is developed in a chamber containing the mobile phase to elute the sample. c) Once the plate has dried, absorbing compounds can be directly imaged with a mobile phone or other digital camera. For non-absorbing but fluorescent samples, a black box containing the respective excitation light may be used.

plans already available in the literature. An appropriate schematic protocol is shown in Figure 1.

Herein, TLC plates were carefully cut in rectangles of 5 cm x 5 cm (width x height). Other dimensions may be used depending on instructor preferences and available resources (e.g. 5 cm x 10 cm (width x height)). A horizontal line was drawn with a pencil 1.0 cm above the bottom of the plate to define the spotting level and 0.5 cm below the top of the TLC plate to indicate an upper limit for elution. At least three different concentrations of the target compound were spotted on the plate in order to produce a calibration curve. An equal volume of the ‘unknown sample’ solution was then applied to the right of the calibration points. After elution in a dedicated chamber, the TLC plates were dried on a flat surface at room temperature for at least 5 minutes and subsequently imaged.

In Experiment 1, suitability for absorbance-based quantification was demonstrated with the compound Sudan IV. Concentrations of 50, 25 and 10 μM in MeOH were prepared for the standard curve and 35 μM for the ‘unknown sample’, also in MeOH (errors associated with these values are discussed in the Supporting Information (SI)). A volume of 5 μL was used for each spot using a pipette. Note that the consistent volume dosing is of high importance for the quantification. An equally suitable method involves the use of capillary tubes, which
are weighed before and after filling to determine the quantity used.\textsuperscript{9,11} The experiments were performed on a standard TLC plate with a mobile phase of 1 vol\% acetic acid in MeOH. The absorbance of Sudan IV was found to be linear up to 50\textmu M (see the SI, Figure S7).

In Experiment 2, the compatibility of the platform for fluorescence-based quantification was validated with the compound Rhodamine 6G. Concentrations of 10, 5, and 2\textmu M in MeOH were prepared for the standard curve and 6\textmu M for the ‘unknown sample’. Volumes of 5\textmu L were applied for each spot. Standard TLC plates were used with a mobile phase of 1 vol\% acetic acid in MeOH. The fluorescent signal of Rhodamine 6G was found to be linear up to 10\textmu M (see SI, Figure S8).

We refer to the SI for a sample student handout on TLC experimentation that contains learning objectives, theory, materials & hazards, experimental methods and discussion questions.

\textit{Quantitative Image Analysis}

Quantitative image analysis of TLC plates may be conducted following the detailed tutorial presented in the Supporting Information. In short, once the elution and subsequent drying was completed, imaging was carried out with a mobile phone camera. For the quantification of the fluorescent compound Rhodamine 6G, the plate was placed in a black box with a UV lamp at 254nm. These units contain a UV filter in the observation window to allow the investigation of the UV-excited fluorescent signal while reducing exposure to the UV light. In this example, the image was taken with a mobile phone through the eye-piece of the black box as shown in Figure 1c.

The image was subsequently uploaded to the quantitative TLC (qTLC) desktop software. A demonstration of the qTLC software analysis can be found in Figure 2. For absorbance-based, \textit{i.e.} non-fluorescent quantification, the image colours were manually inverted such that the bands appeared bright on a dark background. This allowed the program to quantify non-fluorescent compounds in the same manner as fluorescent compounds. The user then
cropped the image around the region containing the bands and specified the number of lanes and bands per lane for the given sample. Subsequently, the user coarsely identified the lanes by drawing rectangles and then subdivided this region into the individual bands. The qTLC program then determined the signal over the background based on the minimum peak prominence value provided (‘MinPeakProm’ in the desktop software and ‘Min Peak Prominence’ in the webapp). The upper and lower boundaries of the band were established by the ‘Divisor’ value. The recognized area was subsequently shown as a red overlay on the image of the TLC plate. The procedure was repeated for each band and the results were obtained by using the ‘Calculate’ option in the qTLC software with the same ‘MinPeakProm’ and ‘Divisor’ values. Note, in the webapp both the recognized area and the result are shown simultaneously. The data that was retrieved for the reference dilutions then serves to construct a calibration curve for the quantification of the ‘unknown sample’.

We refer to the SI for a sample student handout on quantitative image analysis that contains learning objectives, theory, details on the analysis procedure incl. image acquisition, use of qTLC software, signal integration with ‘MinPeakProm’ and ‘Divisor’ and suitable discussion questions.

Hazards

Sudan IV can cause eye, skin and respiratory irritation. Rhodamine 6G is very hazardous if ingested; it can cause eye, skin and respiratory irritation and is hazardous if inhaled. Both acetic acid and methanol (MeOH) are flammable liquids and vapours. Acetic acid is skin corrosive. MeOH is toxic if ingested, inhaled, or absorbed through skin contact and can cause damage to organs. Silica from the TLC plates may cause mild eye and skin irritation and may cause irritation to the respiratory system. Users should consult the respective Material Safety Data Sheets of all chemicals, which are available from the supplier. Students must
Figure 2: **Overview of software implementation.** a) The image of the TLC plate is uploaded into the software. b) The image colors are inverted and a background correction is applied. c) The lane and band numbers are specified and lane and band selection is performed. d) The selected regions for each band appear as a red box on the image. e) The ‘MinPeakProm’ and ‘Divisor’ values are selected and the corresponding red overlay is displayed on the image. f) The final results for the integrated bands are collected for data analysis.
wear eye protection, gloves and lab coats and work in a fume hood. Care should be taken to minimize the production of and contact with the silica dust produced whilst handling TLC plates.

**Results & Discussions**

*qTLC Analysis and Results*

Once the band intensities are quantified, they are used to determine the concentration of the ‘unknown sample’ in the example experiments. The integrated intensity of each band is therefore plotted against the known concentration of each sample. A linear trend is fitted to obtain a standard curve, and this is used to determine the concentration of the ‘unknown sample’ based on its integrated intensity. Including the calibration curve on the same plate as the ‘unknown sample’ is an important step in this process in order to mitigate possible systematic experimental errors as well as plate-to-plate variability. Variations between TLC plates, stock solutions, mobile phases or elution handling could otherwise detrimentally affect the quantification. We note that the calibration spots require to obey a linear trend and the intensity of the ‘unknown sample’ needs to lie within the linear regime. Otherwise, further dilutions are required for reliable interpolation, see the SI (Figures S7 and S8).

We demonstrate absorbance-based quantification for Sudan IV. This compound, alongside Sudan I, II, and III, is an azo-dye widely used for staining biological samples and as colorant in plastics, oils, emulsions, other hydrocarbon products and industrial solvents. In Figure 3a, a sample digital photograph of the TLC plate is shown after spotting three known concentrations of the compound (spot 1-3) alongside the ‘unknown sample’ (spot S), elution and subsequent drying. The qTLC software was then used to quantitatively analyze the bands. The obtained intensity plotted against the respective concentration is shown in Figure 3b. A linear fit was applied to the obtained calibration data to produce a standard curve. The concentration of the ‘unknown sample’ was subsequently determined by interpolation
Figure 3: **Absorbance-based quantification of Sudan IV.** a) Example digital photograph of the TLC plate after spotting, elution and drying. b) Plot of the integrated band intensities the three calibration points with corresponding standard curve. The obtained linear trend ($R^2 = 0.99998$) allowed the determination of the concentration in an 'unknown sample' based on the integrated intensity, in this example 34.1 μM compared to the actual concentration 35.0 μM (2.6% error) based on the obtained integrated peak intensity. In this example, an experimentally derived concentration of 34.1 μM was found for the 'unknown sample', which compared to an actual concentration 35.0 μM (2.6% error). Across nine runs the average absolute error was found to be 6.5 ± 3.7%.

Rhodamine 6G is a common organic dye and serves here as example for fluorescence-based quantification. The compound is used in many applications ranging from labelling of biological materials to dye lasers, solar cells, paper products and textiles.\textsuperscript{16,17} In Figure 4a, an example digital photograph of the developed TLC plate is shown at an excitation wavelength of 254 nm with three known concentrations of the compound alongside the 'unknown sample'. In Figure 4b results are plotted for the integration of the bands using the qTLC software. Based on band integration and a linear calibration curve, the concentration of the 'unknown sample' was determined to be 6.2 μM, which compared to an expected value of 6.0 μM (3.8% error). Over nine runs, the average absolute error was found to be 7.0±3.6%. 

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Figure 4: **Fluorescence-based quantification of Rhodamine 6G.** a) Example digital photograph of TLC plate with three known concentrations of Rhodamine 6G and the ‘unknown sample’. Imaging was carried out in a black box with excitation wavelength 254 nm. b) Plot of the integrated band intensities with standard curve based on three calibration spots \( R^2 = 0.99967 \) and interpolation of the concentration for the ‘unknown sample’.

**Digital Images**

The quality of the image can affect the reliability of the final results obtained by the qTLC software. The software integrates the bands based on the intensity of the signal and so color is neglected. An RGB image is automatically converted to grayscale based on a weighted sum of the RGB components.\(^\text{18}\) Effectively, the image is reduced to a 2D matrix, where the intensity of light recorded by each pixel is represented by a value in the matrix. As the bands are identified by the user, the matrix is subdivided. During the integration process, the software moves across the sub-matrix analysing a column of data one pixel wide at a time. As such, a higher pixel density can improve the analysis. Another consideration is the bit depth of the image. A grayscale image with a bit depth of 8 can have intensity values in the range of 0 to 255 while a bit depth of 16 may exhibit values in the range of 0 to 65535. An image with a higher bit depth will typically have a higher degree of accuracy in the analysis. Our quantification routine by the qTLC software accounts for the two dimensional area in which the spot is located in addition to the pixel intensity. Therefore, an image taken
by a standard smart phone is typically more than sufficient for reliable quantification.

\textit{Error Management}

There are a number of ways errors can be introduced into these experiments. As the determination of the concentration of the ‘unknown sample’ is dependent on the volume and concentrations of the sample curve, any volumetric error in making these solutions or spotting them onto the TLC plate will affect the final results. Students should ensure they plan their dilutions carefully and ensure the volume handling is carried out as accurately as possible. If the spotting is accomplished with a pipette, the students need to ensure that the same pipetting protocol is used for each spot (e.g. wetting the pipette first before every spot). If capillary tubes are employed for the spotting, the calculated weight of the actual sample volume can then be used to correct the integrated intensity of each spot.

The image analysis of the TLC plate is another potential source of error. While the qTLC software is able to perform a background correction, results will be best if the plate is illuminated as evenly as possible. Any stray shadows from the camera or other apparatus may affect the results. Furthermore, extraneous material on the TLC plate may negatively affect the results. Students should take care to avoid stray marks or material (such as dust, silica particulates, or droplets from the sample) on the TLC plate. Lastly, the quantification process looks at the relative integrated intensities to determine the concentration of an ‘unknown sample’. As such, an image in which the bands are saturated will have less accuracy.

Please see the SI for more comprehensive discussion on potential sources of errors including image acquisition guidelines, information on how saturation can impact the results, signal linearity vs. sample concentration and image analysis using ‘MinPeakProm’ and ‘Divisor’.

\textit{Separation of Mixtures}
For more advanced lesson plans, mixtures of different compounds or more complex samples can be used. The same quantification process shown here can be applied by analyzing bands from individual components, allowing the students to quantify each component separately from one another and even identifying which compounds are present in an unknown sample. We note that separate calibration curves may be obtained for several target compounds by co-addition in the dilution experiments. Adequate elution and band separation needs to be verified in this case, and the mobile phase composition adjusted where required.

Conclusions

In conclusion, we present an intuitive and user-friendly analytic platform that allows students to reliably quantify the compounds they are studying by TLC using a standard mobile phone and a simple computer interface. The two experiments reported herein demonstrate that our free and purpose-built software qTLC is capable of providing reliable quantitative data for both absorbance-based and fluorescence-based TLC read-outs. The experimental requirements are easily implemented in an educational laboratory setting and will allow students to not only observe the separation of the compounds being studied but carry out quantitative analysis.

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Supporting Information Available

The following files are available free of charge.

- **Supporting Information**: .pdf file with qTLC software tutorial; details on Minimum Peak Prominence, Divisor, signal integration process and background subtraction; image acquisition guidelines; choice of concentration range; overview of experimental errors.
- **Student Handout - TLC prep**: .pdf file with instructions for students on experimental TLC.
- **Student Handout - Analysis**: .pdf file with instructions for students on image analysis and data acquisition.
- **qTLC_v3_Windows_standalone**: .exe file for the standalone installation of the qTLC software on Windows operating systems - size: 1.83GB.
- **qTLC_v3_Windows_sepRT**: .app file for the installation of the qTLC software on Windows operating systems with separate MATLAB Runtime installation (free) - size: 33MB.
- **qTLC_v3_Windows_sepRT_readme**: .txt file for the installation of the qTLC software on Windows operating systems with separate MATLAB Runtime installation.
- **qTLC_v3_macOS_standalone**: .app file for the standalone installation of the qTLC software on macOS operating systems - size: 1.25GB.
- **qTLC_v3_macOS_sepRT**: .app file for the installation of the qTLC software on macOS operating systems with separate MATLAB Runtime installation (free) - size: 250MB.
- **qTLC_v3_macOS_sepRT_readme**: .txt file for the installation of the qTLC software on macOS operating systems with separate MATLAB Runtime installation.
- **Software Component Parts**: .zip folder with all Matlab component for customization and further development of qTLC software.
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Niamh Mac Fhionnlaoich,† Stuart Ibsen,†† Luis A. Serrano,1 Alaric Taylor,1 Runzhang Qi,1 Stefan Guldin†*  

qTLC Software Tutorial (see Figure S1)  

1. Once the program is launched, the user should use the ‘Upload Image’ button to find the required image and load it into the software. If regions of the image are saturated, a new figure will pop up indicating the saturated regions, see Figure S2. In case any of the bands appear significantly saturated, imaging of the plate should be repeated following the instructions in the image acquisition guidelines to reduce or eliminate saturation.  

2. The user should rotate the image for upright orientation.  

3. For non-fluorescent samples, the user should ‘Invert’ the colors to ensure the bands are bright on a dark background.  

4. For ease of use, the user can 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.  

5. The image must be cropped 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.  

6. Next, a background correction must be applied. This helps to mitigate uneven illumination across the TLC plate. To perform this background correction, the user must select the ‘Background’ button and draw a rectangle around the largest band on the plate. The background is then adjusted automatically.  

7. The lanes and bands on the TLC plate need to be identified. The user must select the ‘Lane and Band Selection’ button on the software and will then be able to draw a rectangle around the first lane. This opens a new figure displaying the selected region. The user is then asked to select each band in that particular lane. A red box appears around the region selected for each band.  

8. The lane and band selection process is then repeated for each lane.  

9. If required, the user may re-select a band via 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, the user can reselect the chosen band and a blue box will appear around the reselected area.  

10. When the user is satisfied with the lane and band selection, the ‘Next’ button brings them to the section of the software for band analysis. At this point the user can adjust the minimum peak prominence (MinPeakProm) and Divisor values. The default values are 1 and 2 respectively. Adjustments may be done by entering a number and using 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 their entire area. The size of the red region may vary from band to band based on the level of intensity (Supplementary Figure S1i).  

11. Once suitable MinPeakProm and Divisor values have been identified, the user can 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’.  

12. At any time the user may use the ‘Help’ menu on the toolbar to learn more about each step.

† Authors contributed equally to this work. †Department of Chemical Engineering, University College London, Torrington Place, London, WC1E 7JE, UK. †† Now at: Department of Biomedical Engineering, Oregon Health and Science University School of Medicine, 3303 SW Bond Avenue, Portland, OR 97239, USA. *e-mail: S.Guldin@ucl.ac.uk
Figure S1 | qTLC software, step-by-step illustration. a) The software interface when starting up. b) Load an image and auto-contrast to highlight the bands. c) Invert the colours and rotate the image as needed. d) Crop the image. e) Apply the background correction. f) The contrast can be adjusted to visualize the bands. g) Specify the lane and band numbers, begin the lane and band selection process by selecting the first lane and then the first band in that lane. h) Repeat for each lane and band. i) Ensure all bands have been selected properly by reviewing the red outlines. j) Reselect a band as required. k) Check the blue outline of the reselected band encompass the band properly. Click ‘Next’ when done. l) Check if the red overlay covers the bands well. m) Adjust the ‘Divisor’ and ‘MinPeakProm’ as needed. n) Obtain the intensity data by clicking ‘Calculate’ and save the image with the ‘Save Image’ button. o) Plot the results with any data analysis software and fit a linear trend line. Determine the concentration of the ‘unknown sample’ by interpolation via the obtained intensity.
Figure S2 | Image Saturation Upon uploading an image, if the image is saturated, the software will open a new figure with the saturated regions highlighted in purple.

qTLC Webapp Tutorial (see Figure S3)
The qTLC webapp is available at www.qTLC.app.¹

1. The user should rotate the image for upright orientation before uploading to the webapp. Once the program is launched, the user should use the ‘Choose Image’ button to select the required image and load it into the webapp. If regions of the image are saturated, a dialogue will pop up indicating the saturated regions, see FigureS4. In case any of the bands appear significantly saturated, imaging of the plate should be repeated following the instructions in the image acquisition guidelines to reduce or eliminate saturation.

2. For non-fluorescent samples, the user should ‘Invert’ the colors to ensure the bands are bright on a dark background.

3. For ease of use, the user can adjust the image so that all the bands on the TLC plate can be observed. This will not affect the results. At the end of this step, click ‘Next’.

4. Then the user should crop the image by adjusting the red box to exclude any areas that are not part of the TLC plate. Ideally, the cropping would select only the region containing the bands. Then the user should click ‘Next’.

5. Next, a background correction must be applied. This helps to mitigate uneven illumination across the TLC plate. To perform this background correction, the user must adjust the red box to just cover the largest band on the plate. When this is completed, the user should click ‘Next’ button to apply the correction.

6. After background correction, the bands may be no longer clear. The user should change the image again in order to make the bands visible. Then the user should click ‘Next’ to confirm adjustment.

7. The lanes and bands on the TLC plate need to be identified. In this page, The user must select the ‘Add’ button and will then be able to adjust the red box to cover the lane to be selected. Afterwards, the user should click ‘Select’ to confirm the lane selection. The user will need to adjust the red box to cover a band and click ‘Select’ to save. The user must repeat band selection until all bands in a lane are selected and click ‘Next’.

8. The user may add a lane by clicking ‘Add’ button again. The selection process is then repeated for the each lane.

9. Once all lanes and bands are selected, the ‘Next’ button brings the user to the section of the webapp for band analysis. At this point the user can adjust the minimum peak prominence (‘Min Peak Prominence’) and ‘Divisor’ values, updated by clicking ‘Calculate’. The results are shown in a table on the left hand side. On the right hand side, there is an image of the TLC plate with the red overlay showing the region that the program defines as the calculation boundaries of each band. All bands should have red regions covering their entire area. The size of the red region may vary from band to band based on the level of intensity (Supplementary Figure S3n).
**Figure S3 | qTLC webapp, step-by-step illustration.** a) The webapp interface when starting up. b) Load an image and adjust it to highlight the bands. c) Invert the colours as needed. d) Crop the image. e) Apply the background correction. f) The image can be adjusted to visualize the bands. g) Add a new lane h) Select lane region i) Select the first band. j) Select each one until all bands are selected. k) Add a new lane if needed l) Once all lanes and bands are selected, click ‘Next’ m) Click ‘Calculate’ to obtain results. n) Adjust the ‘Divisor’ and ‘Min Peak Prominence’ as needed and click ‘Calculate’ to update results. o) Plot the results with any data analysis software and fit a linear trend line. Determine the concentration of the ‘unknown sample’ by interpolation via the obtained intensity.
Figure S4 | Image Saturation Upon uploading an image, if the image is saturated, the webapp will open a new figure with the saturated regions highlighted in red.

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.\(^2\) In the qTLC software, the user selects each band individually, so the minimum peak prominence (MinPeakProm) value defines how much the signal must rise above the surrounding noise. In Figure S5a, a vertical cross section of a single band obtained from Experiment 1 was extracted and plotted. The peaks identified by the qTLC software are shown for MinPeakProm values of 1 and 5, respectively. It is evident that a value of 1 for the MinPeakProm identifies noise as signal to be integrated. A value of 5 excludes the noise and only identifies one peak as signal for Sudan IV. In contrast, a MinPeakProm of too high value results in no peak identification and, thus, the signal will be ignored by the software. Therefore, it is important to find a good balance for the MinPeakProm. The qTLC software is adaptable, so the user is able to identify a reasonable value simply by observing the red overlay in the image on the qTLC GUI. If too much noise is picked up, the user can increase the MinPeakProm. If signal is being ignored, the MinPeakProm may be reduced.

Divisor

The Divisor determines a relative intensity baseline, i.e. how far down from the maximum of the signal the qTLC software will integrate. As an example, for a Divisor value of 2, the software will integrate down to half the height of the signal. This is demonstrated in Figure S5b. The user can use the red overlay of the image to help decide a suitable value. If the red overlay exceeds far beyond the outline of the band, the Divisor value is likely too large, if the overlay does not cover the band, it is likely too small.

Integration Process

During the integration process, the region containing the band (selected by the user) is divided into vertical slices, each one pixel wide. Figure S5 is an example of one of these slices. For each slice, the MinPeakProm determines if there is a signal to be integrated or only noise, which will be neglected. Once the MinPeakProm has identified whether the respective slice contains signal, the software then uses the Divisor value to determine how far down from the maximum of the signal the integration should be carried out. Therefore, the effect of the MinPeakProm value will usually be most strongly observed on the left and right hand side of the band where the signal is weaker. The effect of the divisor is most prominent on the upper and lower borders of the band where the integrated region will grow or shrink with larger or smaller values of the Divisor, respectively.

Background Subtraction

A background correction routine is required as illumination across the TLC plate is not uniform. In order to compensate for this inhomogeneity, we extract the background illumination profile from the captured image and subsequently
subtract this from the original image. To implement this, the user must select the largest band which will create a morphological structuring element in the shape of a disk. The diameter of this disk corresponds to the diagonal of the rectangle drawn around the band by the user.\(^3\) The background is identified as any feature that is larger than the defined morphological structuring element.\(^4\) This process creates a good approximation of the background. It should be noted that this procedure will not remove any stray marks that are smaller than the morphological structuring element.

**Image Acquisition Guidelines**

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. As such, the integrated intensities as a function of concentration will no longer be linear. In fact, this loss of linearity can occur before saturation as shown in Figure S6. This can be a significant problem when imaging fluorescing compounds but is not an issue for absorbing compounds. The qTLC software will help to identify image saturation after the user uploads the image. A new window will open showing the provided image with a purple overlay indicating the saturated regions as seen in Figure S2. We provide the following 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 (< 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.
Figure S6 | Effect of Image Saturation on Error  A TLC plate from Experiment 2 was imaged at increasing exposure times. a) The percentage of saturated pixels was compared to the exposure time. b) The $R^2$ value was determined for the calibration curve and plotted against the percentage of saturated pixels. c) The error was plotted against the $R^2$.

Image saturation can have significant effects on the calculated concentration of the ‘unknown sample’. A TLC plate, prepared using the method in Experiment 2, was imaged at increasing exposure time while maintaining a constant ISO and F-stop. The resulting images were analysed using the qTLC software and the percentage of saturated pixels used in the integration of the band intensities was recorded (Figure S6a). The $R^2$ value was determined for the calibration curve for each image. This was found to decrease with increasing exposure time and saturated pixels. The calibration curve loses linearity as the image nears and becomes saturated (Figure S6b). Finally, the error is compared to the $R^2$ for $R^2$ values above 0.9, as expected, the error was observed to increase with decreasing $R^2$ values. The $R^2$ cut-off value of 0.9 was chosen as values below this corresponded to calibration curves which were very non-linear, as such the methodology used to determine the concentration of the ‘unknown sample’ was no longer valid.

Linearity of Sudan IV & Rhodamine 6G
The presented approach for quantification relies on a linear relationship between the concentration of target compounds and the obtained intensity by 2D integration of the respective band on the TLC plate. It is therefore important to identify a concentration range that obeys this requirement. For comparison, the linearity of both model compounds, i.e. Sudan IV (quantification by absorbance) and Rhodamine 6G (quantification by fluorescence), was studied in solution as well as on a TLC plate using the qTLC software.

For Sudan IV, concentrations between 10 and 200 μM were investigated. In Figure S7a, the absorbance maximum is shown of a respective liquid sample (in MeOH) as obtained by a fibre-based optical transmission set-up with a tungsten-halogen light source (Thorlabs, SLS201), a temperature-controlled cuvette holder (Quantum Northwest, QPod 2e) and a high-sensitivity spectrometer (Ocean Optics, QE Pro). The same concentrations were then spotted onto a TLC plate and eluted following the protocol in Experiment 1. The results from the qTLC software analysis are plotted in Figure S7b. In both scenarios, a closely linear trend was found up to concentrations of 50 μM. We note that the non-zero Y-intercept is related to a residual background absorbance of the TLC plate which does not affect the accuracy of the interpolation-based quantification.

For Rhodamine 6G concentrations between 2 and 100 μM were prepared and measured using both fluorimetry and the qTLC software (Figure S8). For the fluorimetry measurements, samples were placed in a quartz cuvette (3 mL) and characterized with a spectrophotometer (Shimadzu RF-600). The samples were excited with a wavelength of 254 nm, and the peak fluorescence was recorded and plotted in Figure S8a. The same concentrations were then spotted onto a non-fluorescent TLC plate and eluted following the protocol in Experiment 2. The resulting bands were imaged under UV light (at 254 nm) and the integrated intensities were determined using the qTLC software, see Figure S8b. A good linear trend was found in both scenarios up to a concentrations of 10 μM.

Experimental Error
The errors associated to the dilutions by pipetting in Experiments 1 and 2 were determined as follows. The uncertainty for each of the pipettes used was determined using a micro-balance and water. Volumes of 10, 50 and 100% of the nominal value were pipetted into a vessel on the microbalance. The pipette was pre-wet and brought in contact with
Figure S7 | Absorbance vs. concentration - linearity for Sudan IV. a) Absorbance measurements of respective liquid samples in MeOH. b) Integrated intensity of each concentration on the TLC plate as determined by the qTLC software.

Figure S8 | Fluorescence vs. concentration - linearity for Rhodamine 6G. a) Emission of respective liquid samples in MeOH. b) The qTLC software was used to determine the integrated intensities of each concentration.

the vessel wall after filling and emptying. The resulting weight was recorded and converted to volume. This process was repeated 10x for each pipette used. Once this uncertainty was calculated, the propagation of this uncertainty was determined for each dilution. For Experiment 1 (SudanIV in MeOH), the uncertainty related to pipetting was 50±0.35, 25±0.18 and 10±0.71 μM for the standard curve and 35±0.25 for the ‘unknown sample’. For Experiment 2 (Rhodamine 6G in MeOH), the uncertainty related to pipetting was 10±0.067, 5±0.033, and 2±0.013 μM and 6±0.040 μM for the ‘unknown sample’.

A systematic screening of the effect of MinPeakProm and Divisor on the error associated with the calculation of the ‘unknown sample’ resulted in the error maps presented in Figure S9. A similar trend is shown for both Sudan IV and Rhodamine 6G (Figure S9 a and b, respectively). In both cases, an optimal region of MinPeakProm and Divisor values is depicted in blue which relates to an error below 5%. Both plots show regions of increased error at concurrent low Divisor values and high MinPeakProm values. This is because these conditions result in a significant portion of the signal from the bands being discarded by the software. At concurrent high values of Divisor and low values of MinPeakProm the pronounced error is due to the inclusion of too much noise in the analysis.

Another important aspect to note is the range of values appropriate for the non-fluorescent sample (Sudan IV) is much lower than for the fluorescent sample (Rhodamine 6G). For fluorescent-based quantification, the intensity of the signal far exceeds the signal obtained by absorbance for non-fluorescent samples, thus the signal to noise ratio is much greater. Therefore, higher Divisor and MinPeakProm values can be tolerated before signal is excluded from the analysis.
Figure S9 | Effect of MinPeakProm and Divisor on the error

a) Error map for Sudan IV. The area denoted by the shaded region corresponds to MinPeakProm and Divisor values that yielded an $R^2$ value below 0.85 for the calibration curve. b) Error map for Rhodamine 6G. The $R^2$ was not found to drop below 0.95 in the presented parameter space.

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| File Name                        | Size       | Actions                             |
|---------------------------------|------------|-------------------------------------|
| qTLC_v3_macOS_sepRT.zip          | 31.22 MiB  | view on ChemRxiv ▶ download file    |
| qTLC_v3_macOS_standalone.zip     | 1.17 GiB   | view on ChemRxiv ▶ download file    |
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