

Quantitative measurements on wetted thin layer chromatography plates using a charge coupled device camera

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Abstract

This paper presents the first study of imaging of spots on thin-layer chromatographic plates whilst still wet with solvent. Imaging and quantification of Sudan II after development with dichloromethane was carried out in both reflectance and transmission modes, using a charge coupled device (CCD) camera. The relationship between peak area and sample loading was established at low sample loading, and found to be linear over an order of magnitude for both wet and dry modes with r^2 -values >0.99 . All data processing was carried out using the Beer–Lambert equation. Curvature at high loadings in the plots of integrated absorbance as a function of sample loading was accounted for using an empirical expression designed for use with the Kubelka–Munk treatment and apparent absorbance of the stationary phase due to scattering. Results are consistent with an effective pathlength significantly longer than the thickness of the sorbent layer. The limit of detection on a dry plate (0.5 ng) was found to be lower than on a wetted plate (2 ng). Precision was found to be 1–4% RSD intra-plate and 8–14% RSD inter-plate. Results are compared with quantification of the same analyte on dried plates.

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1. Introduction

TLC is a fast, cheap and efficient means of separating many classes of compounds. It holds several advantages over other chromatographic techniques, including that of separating in space rather than time (so the whole sample is evaluated), the ability to carry out parallel separations, and simultaneous separation and clean up [1]. This makes it the method of choice for many applications, e.g. pharmaceutical.

Scanning densitometry is the standard tool for evaluating flat bed separations [2]. However, this technique is slow (it can take up to twenty minutes to scan a plate) and cannot be used for on-line applications. Charge coupled devices (CCDs) [3,4] are two-dimensional detectors containing an

array of sensors that can image an area in seconds; this makes them ideal for the imaging of TLC plates both off line and in real-time. The output from each sensor pixel on the CCD is a voltage, which is proportional to the intensity of light falling on the sensor and the exposure time. This series of voltages is digitised and transferred to a computer for storage and data processing. CCD cameras have been used to evaluate TLC plates after drying in several modes, including transmission [5], reflectance [6] and fluorescence in conjunction with post-run derivatisation [7]. Results using CCD in reflectance mode have been compared with those of scanning densitometry; sensitivities were shown to be similar, but the acquisition times using CCD detection were faster [8–10]. Relative standard deviation (RSD) values were measured in one study to be $<5\%$, and r^2 -values were >0.99 [11]. Linearity has been demonstrated to be over an order of magnitude, with limit of detection in the range 10–50 ng [12,13].

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In previous work TLC plates have been imaged dry, after the solvent has been evaporated. The objective of this paper is to find out if it is possible to get quantitative results from a wetted plate using a CCD camera in either reflectance or transmission modes. The longer-term aim is to develop a real-time method of TLC plate evaluation during chromatographic development.

2. Experimental

2.1. Chemicals

Sudan II (structure shown in Fig. 1) was supplied by Aldrich Chemicals (Poole, UK). The solvents used were HPLC grade hexane, acetonitrile, *p*-xylene and dichloromethane from Fisher Scientific (Loughborough, UK). A stock solution of Sudan II was prepared by dissolving an accurately weighed amount of dye in hexane. Standards were made by dilution of this stock solution.

2.2. Chromatography

Thin layer chromatography was performed on 5 cm × 10 cm Silica gel 60 F₂₅₄ plates from Merck (Darmstadt, Germany), layer thickness 200 μm. Samples were applied as spots approximately 1 cm apart and 0.5 cm from the plate edge by means of 1 μl glass capillaries, supplied by Camlab (Cambridge, UK). Development was performed at room temperature in a 10 cm × 10 cm horizontal chamber (Camag, Switzerland). A two-step development was chosen, with solvent focusing using acetonitrile before the development proper with dichloromethane.

In the first set of experiments dried plates were imaged. After spotting, the spots were focused with acetonitrile to a distance of approximately 1 cm from the bottom of the plate. The plate was then allowed to dry and an image taken. Subsequently the focused bands were developed for a further 4–5 cm with dichloromethane, after which the plate was allowed to dry again before imaging in either reflectance or transmission.

In the second set of experiments, the plates were imaged whilst wet with solvent, both before and during chromatography. The blank plate was initially developed up to a predetermined distance (marked on the plate), and an image taken. The plate was allowed to dry and then spotted, focused and dried again. The plate was then developed for a second time and another image taken either in reflectance or transmission,

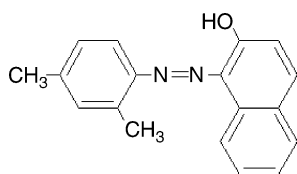


Fig. 1. Structure of Sudan II dye.

when the solvent front was at the same position as in the first image.

2.3. Imaging and processing

Images were taken using an Astromed TE4/A CCD camera (Cambridge, UK) operated to run with maximum dynamic range (5×10^5 electrons per pixel) and at maximum read-out rate (80 kHz). Image acquisition was controlled using the associated Imager 2 software. The CCD was fitted with a camera lens (50 mm focal length) from Nikon (Kingston upon Thames, UK) and filters from Edmund Optics (York, UK). A green additive filter (X-52-534) and a blue additive filter (X-52-531) were used.

The wet reflectance experiments were carried out at f 2.8 and 150 ms exposure using the green filter, and at f 2.8 and 200 ms exposure using the blue filter. The wet transmission experiments were carried out at f 8 and 100 ms exposure using the green filter, and at f 4 and 500 ms exposure using the blue filter. Spectral characteristics of the filters, dye and CCD response are given in Fig. 2.

In reflectance mode, the fluorescent strip lights in the laboratory were used to illuminate the plates. In transmission mode, plates were backlit by an 11 W, 250 V fluorescent lamp from CPC (Preston, UK). Centre wavelengths of the emission bands of the phosphor coating of the tubes were measured for both light sources using a calibrated graded interference filter, and found to be 436, 492, 531, 576, 604 and 629 nm. Of these bands, those at 531 and 576 nm overlap with the green filter, whilst those at 436 and 492 nm overlap with the blue filter.

Two images were taken in each experiment, one before and one after chromatography. In the wet plate experiments all images were taken through the glass lid of the tank with the plate still inside. In the transmission experiments any stray light was blanked out between light box and camera using a cover. A schematic diagram of the apparatus is shown in

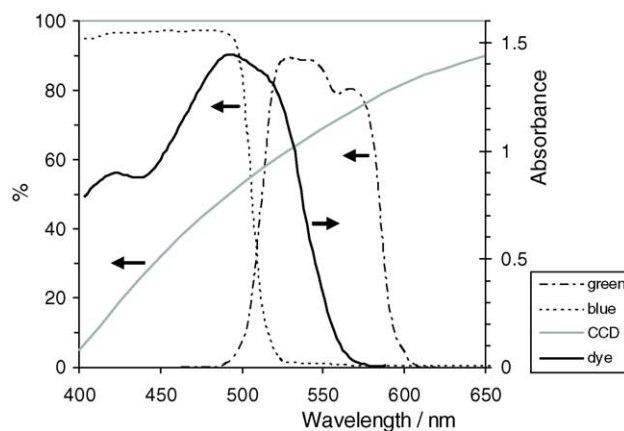


Fig. 2. Transmittance for the green and blue additive filters, relative sensitivity of CCD (%), and absorption spectrum of Sudan II at 25 mg l⁻¹ in dichloromethane in a 1 cm path length cell.

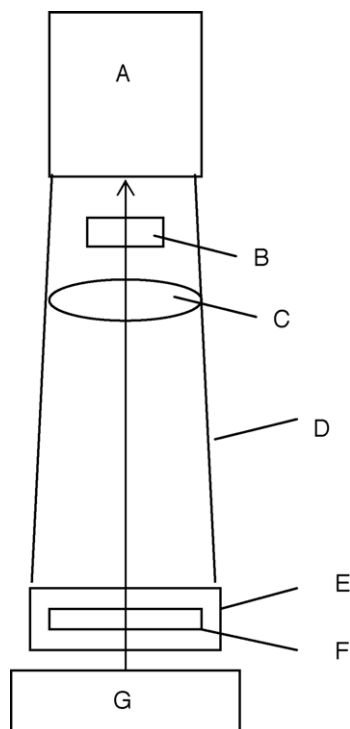


Fig. 3. Schematic diagram of apparatus to image wet plates in transmission: A, CCD camera, B filter, C lens, D cover, E developing tank, F TLC plate, G light box. The system is enclosed between the top of the developing tank and the CCD to prevent any stray light from entering. For reflectance experiments, the apparatus is the same apart from the removal of the light box and cover.

Fig. 3. All images had a blank image (that is one taken with the shutter closed) subtracted.

Signal referencing was accomplished using an in-house program which ratios the signal of each corresponding pixel in two images, and takes the logarithm of this ratio. Further data processing was carried out using Scion Image Beta 4.02 software from Scion Corporation (MD, USA) and PeakFit version 4 from Systat Software UK (London, UK).

3. Results and discussion

3.1. Beer–Lambert analysis and data processing

To process the data obtained from the CCD camera the Beer–Lambert law was used:

$$\log \left(\frac{I_0}{I} \right) = A = \varepsilon bc \quad (1)$$

where A is absorbance, ε is the absorption coefficient, b is the path length of light through the sample and c is the concentration of the analyte. The quantity of analyte imaged by one pixel is $q_i = a_i t c_i$, where a_i is the area imaged by pixel i and t is the layer thickness. Substituting for c_i and summing over

a whole imaged analyte spot gives

$$q = \frac{1}{\varepsilon} \frac{t}{b} \sum_i A_i a_i = \frac{1}{\varepsilon_{\text{app}}} \sum_i A_i a_i \quad (2)$$

This equation introduces the term apparent absorption coefficient, ε_{app} , which is larger than ε by a factor b/t due to the increased effective pathlength found in the scattering medium of the TLC layer. The procedure used was to first calculate the absorbance for each pixel using the values for I_0 , obtained from the image taken before chromatography, and I from the image taken after chromatography. These absorbance values are then summed to give a signal for the spot, with units AU m^2 , that is proportional to the total analyte quantity in the spot.

An example of the signal referencing process is given in Fig. 4 where four spots of 50, 25, 10 and 5 ng loading have been run. The first picture (a) is the raw image direct from the CCD. The second picture (b) shows the data after ratioing with our in-house program in order to correct for fixed pattern effects and also to convert from signal level into absorbance using Eq. (1). The fixed pattern contribution is due to the inhomogeneity of light across the plate and also due to irregularities in the plate surface. This is then integrated in the y direction using Scion Image to produce the peaks seen in the next step plotted as a function of distance in the x direction (c). These peaks are integrated a second time using PeakFit to give results, which can be plotted as a calibration (d). Very good linearity is observed in the calibration, with correlation coefficient of 0.9989. This validates use of the Beer–Lambert approach over this range of sample loading.

The Beer–Lambert law is normally used to describe absorption in non-scattering media, and since the silica gel of a TLC plate is highly scattering this is not the ideal model. The theory of Kubelka and Munk [14] has been extensively used to describe the propagation of light in intensely scattering media and in particular to quantitative analysis in TLC [15–17]. The equation:

$$A_{\text{B-L}} = 2 \exp(-2A_0)(A + 0.4A^2) \quad (3)$$

was presented by Goldman and Goodall as a semi-empirical equation approximating the relation between the absorbance expected from the Beer–Lambert law ($A_{\text{B-L}}$) and apparent absorbance measured in transmission, with A_0 the apparent absorbance of the chromatographic adsorbent or layer [15]. In the case where the sorbent layer does not absorb light at the wavelengths used, A_0 is a measure of its scattering power; the equation holds for $0.7 < A_0 < 1.3$ [15], a range that should include the scattering power of TLC plates. The increased effective pathlength gives an overall enhanced response by the factor $1/[2 \exp(-2A_0)]$ and the curvature, away from the linear Beer–Lambert behaviour, is accounted for by $(A + 0.4A^2)$ [15]. In the approach used by Treiber [16], the constants in the equations derived from Kubelka–Munk theory are obtained empirically.

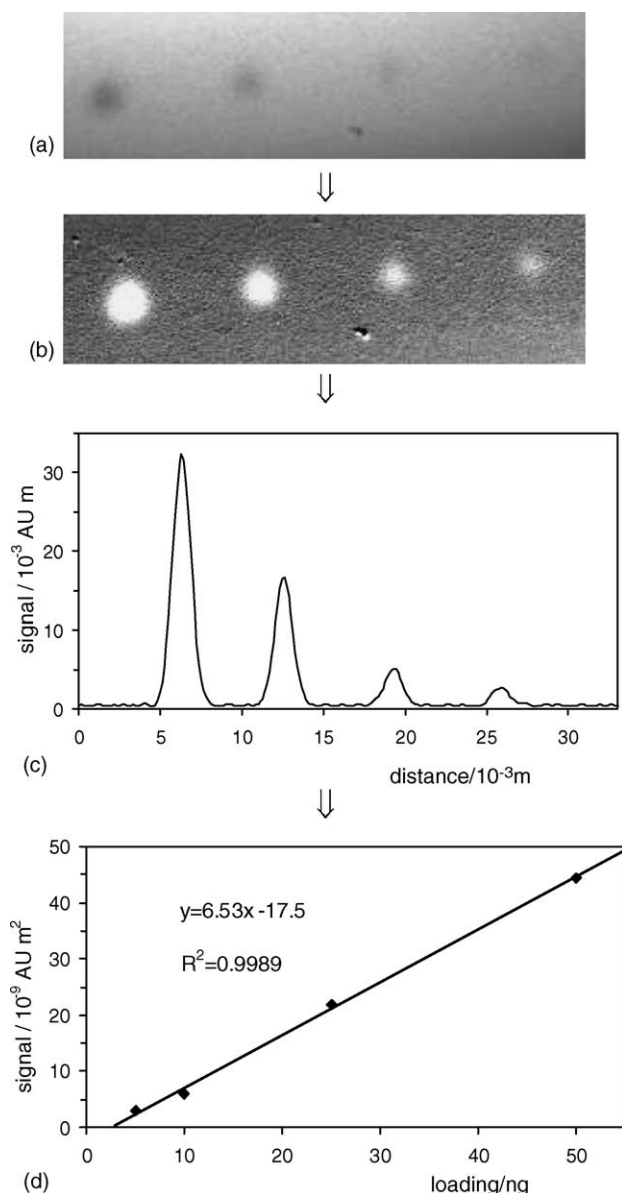


Fig. 4. Signal referencing and processing for a calibration of Sudan II imaged on wet plates in reflectance with blue filter—50, 25, 10 and 5 ng spots: (a) raw image; (b) after correction for fixed pattern noise; (c) after integration in one direction using Scion Image and (d) calibration plot obtained by integration of (c) using PeakFit.

Eq. (3) was applied to the data shown in Fig. 5, which covers loadings between 10 and 100 ng, a wider range than that in Fig. 4. Using A_0 as an empirical variable, the best fit (solid line) to the observed data was obtained with a value for A_0 of 1.2. This lies within the range where the authors proposed that the equation is valid.

The value obtained for A_0 by measuring the reduction in light transmission caused by the presence of the TLC layer is expected to be highly dependent on the nature of the illumination [18], with higher values obtained when a collimated source is used. Measurements of A_0 were made with and without slits to investigate this. Values for A_0 of ~ 0.1 were

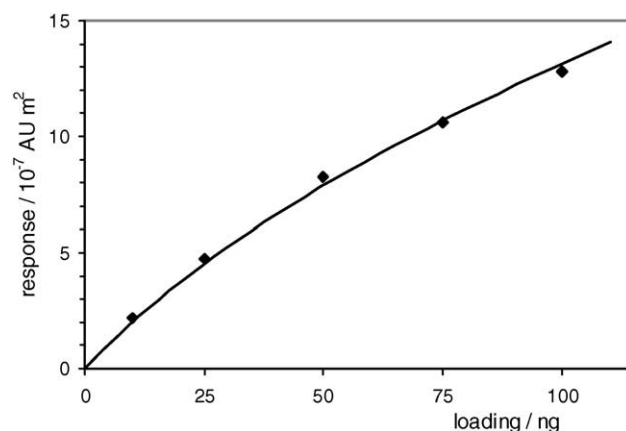


Fig. 5. A five-point calibration of Sudan II imaged on wet plates in transmission using the blue additive filter: 10; 25; 50; 75 and 100 ng spots.

obtained for wet plates with the highly diffused source in the arrangement of Fig. 3, and values of ~ 1.6 were obtained when a mask with a 1 mm width slit was placed on top of the light box and under the wet plate. This application of the Kubelka–Munk theory accounts for both the slope and curvature of the data shown in Fig. 5, with a value for A_0 that is within the range measured experimentally. It is also consistent with the observation in the transmission experiments of a smaller absolute loading range and increased apparent absorbance coefficient for the low loading region that gives an approximately linear response under the conditions of increased scattering in dry plates.

Results obtained using sample loadings in the range up to 50 ng and using the Beer–Lambert equation are shown Table 1 for transmission on wet plates, Table 2 for reflectance on wet plates, and Table 3 for transmission on dry plates. Correlation coefficients were greater than 0.995 for linear regression. Intercepts in all calibrations are within experimental error close to zero, but the fact that all intercepts are systematically positive in the transmission data (Table 1) and negative in the reflectance calibrations (Table 2), rather than being randomly distributed around zero, is consistent with the linear

Table 1
Calibrations for Sudan II in transmission on wet plates: 5; 10; 25 and 50 ng spots

Calibration	Filter	Gradient (AU m ² g ⁻¹)	Intercept (AU m ²)	r^2
1	Blue	25.7 ± 1.5	56 ± 42	0.9996
2	Blue	29.8 ± 2.3	30 ± 66	0.9993
3	Blue	27.5 ± 2.0	31 ± 56	0.9994
4	Blue	32.2 ± 1.4	43 ± 40	0.9998
Average		28.8 ± 2.8	40 ± 12	
5	Green	19.4 ± 1.6	7 ± 44	0.9993
6	Green	23.5 ± 0.4	65 ± 12	0.9999
7	Green	18.9 ± 1.5	33 ± 43	0.9993
8	Green	22.7 ± 2.1	36 ± 59	0.9991
Average		21.1 ± 2.3	35 ± 24	

Table 2
Calibrations for Sudan II in reflectance on wet plates: 5; 10; 25 and 50 ng spots

Calibration	Filter	Gradient (AU m ² g ⁻¹)	Intercept (AU m ²)	r ²
1	Blue	7.41 ± 1.28	16 ± 36	0.9994
2	Blue	7.01 ± 1.18	12 ± 34	0.9981
3	Blue	8.75 ± 1.09	21 ± 31	0.9993
4	Blue	6.53 ± 0.65	17 ± 18	0.9989
Average		7.42 ± 0.95	16 ± 4	
5	Green	4.37 ± 0.35	8 ± 25	0.9996
6	Green	4.49 ± 0.72	11 ± 20	0.9991
7	Green	3.90 ± 0.52	2 ± 15	0.9994
8	Green	5.87 ± 0.82	6 ± 23	0.9987
Average		4.66 ± 0.85	6 ± 4	

Beer–Lambert fitting being an approximation to reality, as discussed above.

The gradients in Tables 1–3 are the apparent absorption coefficients, ϵ_{app} (Eq. (2)), and as such they can be compared directly with values for absorption coefficients measured in solution. Measurements for Sudan II in DCM were carried out in three ways: (i) using UV–vis spectrophotometry with a standard 1 cm cell; (ii) using the CCD camera in conjunction with the blue filter, and sample in a 0.5 mm pathlength cell positioned horizontally in place of the TLC plate in Fig. 3; (iii) as in (ii), but with a narrow-band blue filter centred at 400 nm. All methods gave comparable values of ϵ : 3.4; 4.0 and 4.1 AU m² g⁻¹ for (i) (at 400 nm), (ii) and (iii), respectively. This shows that the optical configuration in the present work gives the expected value for absorbance in the absence of any scattering. The value of ϵ_{app} measured for Sudan II in transmittance through the DCM-wetted TLC plate, 28.8 AU m² g⁻¹, is a factor ~ 7 higher than ϵ . This increase in value for ϵ_{app} is consistent with a longer effective pathlength through the scattering medium on the TLC plates, and the effective pathlength in the TLC experiments being considerably greater than the thickness of the sorbent layer. A value of 47.6 AU m² g⁻¹ for ϵ_{app} was obtained for Sudan II in transmission on the dry plate with the blue filter, which

Table 3
Calibrations for Sudan II in transmission on dry plates: 1; 2; 5 and 10 ng spots

Calibration	Filter	Gradient (AU m ² g ⁻¹)	Intercept (AU m ²)	r ²
1	Blue	49.6 ± 5.0	-8.8 ± 11	0.9989
2	Blue	49.2 ± 4.0	-0.7 ± 23	0.9993
3	Blue	46.1 ± 5.1	6 ± 29	0.9987
4	Blue	45.3 ± 2.9	-1.9 ± 16	0.9996
Average		47.6 ± 2.2	-1.3 ± 6.1	
5	Green	22.0 ± 4.6	-1 ± 26	0.9954
6	Green	24.1 ± 0.7	-3 ± 4	0.9999
7	Green	23.9 ± 4.7	7 ± 26	0.9959
8	Green	22.4 ± 3.5	-11 ± 20	0.9973
Average		23.1 ± 1.1	-2 ± 7	

is a factor 1.5 greater than the value on the wet plate and a factor 12 greater than ϵ in solution. This is consistent with a further increase in the effective pathlength under the condition of greater scattering in the absence of solvent. The fact that the emission band at 576 nm of the light source used is transmitted by the green filter but is not absorbed by the dye (see Fig. 2) in part accounts for the lower ϵ_{app} observed for the green filter measurements.

3.2. Limit of detection and reproducibility

Four point calibrations were used; this is because of the limited space available on the 5 cm × 10 cm plates. As discussed in Section 3.1, the linear range was approximately an order of magnitude, between 1–10 ng on dry plates and 5–50 ng on wet plates. The relationship between signal and sample loading was found to become non-linear at higher loading; above about 10 ng dry and 50 ng wet (Fig. 5).

The limit of detection (LOD) was determined using the signal to noise ratio. A typical figure giving raw data is shown in Fig. 6. The limit of detection is approximately 2 ng on a wetted plate. A loading of 0.5 ng can be easily detected on a dried plate. These figures are significantly better than LODs of 10–50 ng previously reported using reflectance with CCD cameras and dry plates [12,13]. The LOD is higher on a wet than on a dry plate for two reasons. Firstly, there is increased noise due to the presence of solvent, making signal referencing less reproducible. Secondly, the effective absorption coefficient is lower, most probably due to shorter effective pathlength as discussed in the previous section.

Precision of chromatography and detection was assessed by means of the RSD values between spots of the same loading (Tables 4–7). Note that all RSD values are for the full analytical process from spotting through to evaluation. Precision was generally good between spots on wet plates in transmission and reflectance (Tables 4–6) and also in transmission on dry plates (Table 7), with RSD values in the range 1–4%. These are comparable with results previously reported using CCD detection on dry plates and RSDs less than 5% [11]. The results were rather poorer between plates,

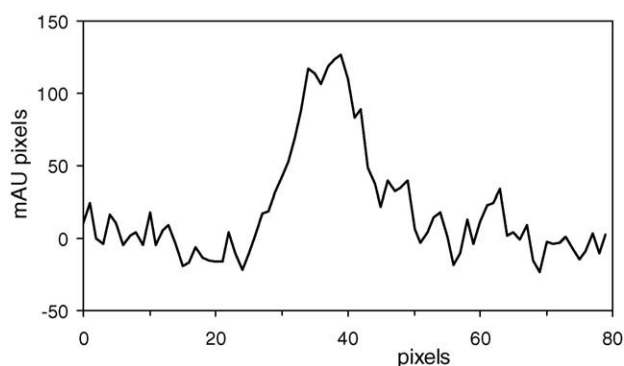


Fig. 6. Raw data for a 5 ng peak imaged on a wet plate in reflectance with the green filter. The peak-to-peak S/N ratio is approximately 2:1 without any smoothing.

Table 4
Reproducibility in quantification of peak area of Sudan II after chromatographic development, using transmission on wet plates with the blue additive filter ($n = 4$)

Plate	Loading (ng)	Average signal (AU m ²)	RSD (%)
1	10	$(2.95 \pm 0.060) \times 10^{-7}$	2.1
2	10	$(3.05 \pm 0.082) \times 10^{-7}$	2.7
3	10	$(2.33 \pm 0.060) \times 10^{-7}$	2.6
4	10	$(2.36 \pm 0.057) \times 10^{-7}$	2.4
Average		$(2.67 \pm 0.32) \times 10^{-7}$	14
5	50	$(1.32 \pm 0.027) \times 10^{-6}$	2.5
6	50	$(1.09 \pm 0.027) \times 10^{-6}$	2.1
7	50	$(1.16 \pm 0.021) \times 10^{-6}$	1.8
8	50	$(1.21 \pm 0.031) \times 10^{-6}$	2.6
Average		$(1.19 \pm 0.086) \times 10^{-6}$	7

Table 5
Reproducibility of Sudan II in transmission on wet plates with the green additive filter ($n = 4$)

Plate	Loading (ng)	Average signal (AU m ²)	RSD (%)
1	10	$(1.86 \pm 0.035) \times 10^{-7}$	1.9
2	10	$(2.33 \pm 0.032) \times 10^{-7}$	1.4
3	10	$(2.18 \pm 0.058) \times 10^{-7}$	2.7
4	10	$(2.23 \pm 0.038) \times 10^{-7}$	1.7
Average		$(2.15 \pm 0.17) \times 10^{-7}$	8
5	50	$(1.05 \pm 0.038) \times 10^{-6}$	3.7
6	50	$(1.04 \pm 0.032) \times 10^{-6}$	3.1
7	50	$(9.88 \pm 0.28) \times 10^{-7}$	2.9
8	50	$(8.61 \pm 0.21) \times 10^{-7}$	2.5
Average		$(9.84 \pm 0.87) \times 10^{-7}$	9

Table 6
Reproducibility of 50 ng spots of Sudan II in reflectance on wet plates ($n = 4$)

Plate	No of spots	Filter	Average signal (AU m ²)	RSD (%)
1	4	Green	$(2.58 \pm 0.075) \times 10^{-7}$	3.0
2	5	Green	$(3.02 \pm 0.11) \times 10^{-7}$	3.2
3	4	Green	$(3.66 \pm 0.097) \times 10^{-7}$	2.7
4	4	Green	$(3.04 \pm 0.11) \times 10^{-7}$	3.7
Average			$(3.08 \pm 0.44) \times 10^{-7}$	14
5	4	Blue	$(3.89 \pm 0.090) \times 10^{-7}$	2.4
6	5	Blue	$(4.58 \pm 0.061) \times 10^{-7}$	1.2
7	5	Blue	$(4.86 \pm 0.091) \times 10^{-7}$	1.7
Average			$(4.44 \pm 0.50) \times 10^{-7}$	11

Table 7
Reproducibility in transmission on dry plates ($n = 5$)

Plate	Loading (ng)	Filter	Average signal (AU m ²)	RSD (%)
1	5	Blue	$(2.07 \pm 0.045) \times 10^{-7}$	2.2
2	5	Blue	$(2.31 \pm 0.040) \times 10^{-7}$	1.7
3	10	Green	$(2.20 \pm 0.070) \times 10^{-7}$	3.2

as expected [19], with RSD values in the range 8–14% for the inter-plate averages of the intra-plate mean values.

4. Conclusions

It has been shown that quantitative TLC can be carried out with CCD imaging in both transmission and reflectance modes on wetted plates. All data processing was carried out using the Beer–Lambert equation. Curvature at high loadings in the plots of integrated absorbance as a function of sample loading was accounted for using an empirical expression designed for use with the Kubelka–Munk treatment and apparent absorbance of the chromatographic sorbent layer due to scattering. Results are consistent with an effective pathlength significantly longer than the thickness of the sorbent layer. The limit of detection was 2 ng and the linear range was between 5 and 50 ng in both transmission and reflectance on wetted plates. Linearity was very good over this limited range, with r^2 -values of >0.995 obtained. Intra-plate RSD values were between 1 and 4%, whilst inter-plate RSD values were higher (8–14%) due to plate-to-plate variability. Results for a similar system evaluated in transmission, after the plate had been dried, gave a better limit of detection of <0.5 ng and a linear range between 1 and 10 ng. The increased noise due to the solvent is a factor adversely affecting the LOD on wet plates compared to that of dry plates. Intra-plate RSD values (1–3%) were comparable with those obtained for wet plates.

Future work will aim to investigate real-time imaging of TLC plates whilst still wet with solvent.

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