Research Article

Journal of Labelled Compounds and Radiopharmaceuticals

Received 16 February 2013, Revised 22 March 2013, Accepted 22 March 2013 Published online 23 July 2013 in Wiley Online Library

(wileyonlinelibrary.com) DOI: 10.1002/jlcr.3052

Digitally enhanced thin layer chromatography: further development and some applications in isotopic chemistry

Daniel P. Manthorpe and William J. S. Lockley*

Improvements to thin layer chromatography (TLC) analysis can be made easily and cheaply by the application of digital colour photography and image analysis. The combined technique, digitally enhanced TLC (DE-TLC), is applicable to the accurate quantification of analytes in mixtures, to reaction monitoring and to other typical uses of TLC. Examples are given of the application of digitally enhanced TLC to: the deuteromethylations of theophylline to [methyl$-^2H_3$]caffeine and of umbelliferone to [$^2H_3$]7-methoxycoumarin; the selection of tertiary amine bases in deuterodechlorination reactions; stoichiometry optimisation in the borodeuteride reduction of quinizarin (1,4-dihydroxyanthraquinone) and to the assessment of xanthophyll yields in *Lepidium sativum* seedlings grown in deuterated media.

Keywords: digitally enhanced TLC; DE-TLC; deuteromethylation; *Lepidium sativum* carotenoids; methyl$-^2H_3$]caffeine; [$^2H_3$]7-methoxycoumarin; [9,10$-^2H_2$]1,4-anthraquinone; umbelliferone; quinizarin

Introduction

Thin layer chromatography (TLC) is still of great utility to chemists. However, it is normally considered as a qualitative technique. In contrast, quantitative TLC is usually carried out using high performance TLC plates in conjunction with expensive automated spotting apparatus and with multi-wavelength plate scanners costing tens of thousands of dollars. This paper describes how digital photography and public domain image processing software can be employed to enhance both the qualitative and quantitative uses of ordinary TLC.

The current work constitutes an extension of, and a substantial improvement upon, a previous study of digitally enhanced TLC (DE-TLC).[1] This previous study illustrated the utility of digital enhancement of TLC but was dependent on a bespoke data processing program (TLC-Analyzer), which was suitable for some types of DE-TLC analysis but limited in its ability to analyse parallel data. Unfortunately, this program is now outdated and will not operate on many modern computer systems. Hence, a re-evaluation and updating of the technique is overdue. This is particularly the case in view of the easy accessibility of digital cameras and of public domain image processing programs. When combined together, these developments have enabled a new approach to DE-TLC quantification and data manipulation, which is described herein. This new approach has proved, practical, versatile and inexpensive. As such, DE-TLC continues to be an economical procedure that is well suited to laboratories with a limited budget or to those laboratories where the potential contamination of expensive commercial high performance TLC systems currently precludes the use of quantitative TLC techniques.

It should be noted that DE-TLC maintains and extends all the advantages of TLC as an analytical method. It allows parallel analyses, all components of the mixture under analysis are visible and a variety of separation mechanisms can be employed. Moreover, the compounds or mixtures under investigation can be subjected to a very wide range of detection modes including fluorescence, fluorescence-quenching, chemical derivatisation, dye absorbtion, colour reactions[2] and so on. Hence, the DE-TLC procedure is applicable to compounds that are inappropriate analytes for HPLC or HPLC-MS.

Discussion

The methodology used for DE-TLC is simple, employing ordinary TLC tanks and plates. The improvements described in this paper arise from four primary sources; (a) the use of quantitative spotting via a syringe, microcap or micropipettor; (b) data acquisition via digital colour photography; (c) quantification via IMAGEJ image processing software (NIH, USA)[3]; and (d) application of a simple mathematical transform to convert the raw data to linear form. Essentially, the TLC plate is run, photographed (usually under visible or 254/360 nm ultraviolet light), the image quantified and the data is linearised via the transform. These stages are discussed in more detail below.

Quantitative thin layer chromatography procedure

Throughout this work, manual spotting via fixed volume microcaps (0.5–5 μl capacity) has been used and the spotting volumes for each analysis have been kept constant. Microcaps

Department of Chemistry, Faculty of Engineering and Physical Sciences, University of Surrey, Guildford, GU2 7XH, UK

**Correspondence to: William J. S. Lockley, Department of Chemistry, Faculty of Engineering and Physical Sciences, University of Surrey, Guildford, GU2 7XH, UK. E-mail: w.lockley@surrey.ac.uk*

were chosen because they are cheap and disposable but have a very high degree of dispensing precision. In our hands, similar analytical precision could be obtained using a fixed volume glass gas-liquid chromatography syringe or a good quality low volume micropettor set to a fixed volume (1–10 μl). The best data were obtained when the analyte was spotted in a solvent of lower polarity than the TLC mobile phase and when approximately circular spots were obtained. That said, good quality data were still obtainable when both these criteria could not be fully met. For quantitative analysis, duplicate sets of standards were prepared by spotting fixed volumes of a series of standard concentrations of the analyte. The test samples or quality control samples were placed between the two sets of standard sample spots. All the spots were allowed to dry before development. Typically, a 20×10 cm plate was used in landscape orientation with a development distance of 10 cm. The TLC tank was lined with filter paper and was thoroughly equilibrated with the eluent prior to chromatography.

Visualisation of the analytes

For coloured compounds, no specific technique of visualisation was necessary because the digital camera could record the spots directly and selection of the appropriate colour domain was straightforward. For compounds with chromophores that crossed 254 nm, visualisation was by fluorescence quenching using F_{254} TLC plates. In this case, the pictures were taken under 254 nm illumination from a 4 W ultraviolet lamp. For fluorescent compounds, the fluorescence was recorded under 4 W 'black-light' illumination at 360 nm. In the case of compounds detected by iodine adsorbtion, the TLC plates were allowed to stand in an atmosphere of iodine vapour until the spots were clearly visible against an essentially white background and photographed promptly. No other visualisation techniques were evaluated quantitatively in the current work, but colour detection of Ni^{2+} by dimethylglyoxime spray reagent worked well. Hence, in principle, the method should work with any TLC visualisation technique or development reagent.[2]

Digital photography and thin layer chromatography plate illumination

No special camera is required for the DE-TLC technique. The data in this paper were obtained using a 10.1 megapixel camera. It should be noted, however, that for good quantification, the photography of the plates does require very even lighting. For coloured analytes or iodine-developed plates, this is best achieved in outside daylight, although other lighting can be used provided that the lamp is at a significant (effectively infinite) distance from the plate and that no nearby surfaces are present to act as reflectors to reduce the evenness of the illumination. In the case of ultraviolet adsorbing or fluorescent compounds, siting the plate at a significant distance (*ca*. 1.5 m) from the ultraviolet lamp and using a manual exposure of several seconds at apertures around F8 worked well. The International Organization of Standardization/ American Standards Association (ISO/ASA) setting (if this was selectable) was left on auto to allow the camera to assess the best sensitivity for the photograph. The best settings for a particular camera and experimental set-up were determined by bracketing the exposures around the initial values above. A typical experimental arrangement is shown in Figure 1. The zoom function of the camera is used to ensure that the TLC plate image, or the area of the plate that is of interest, fills about 80% of the frame. This allows for any non-linearity (vignetting) towards the edge of the photograph, which can be experienced with inexpensive cameras. The ultraviolet lamps used in this study had fluorescent tubes of *ca*. 10 cm length, and the lamp was arranged such that the tubes were parallel to the line of spots on the TLC plate to maximise the uniformity of the illumination.

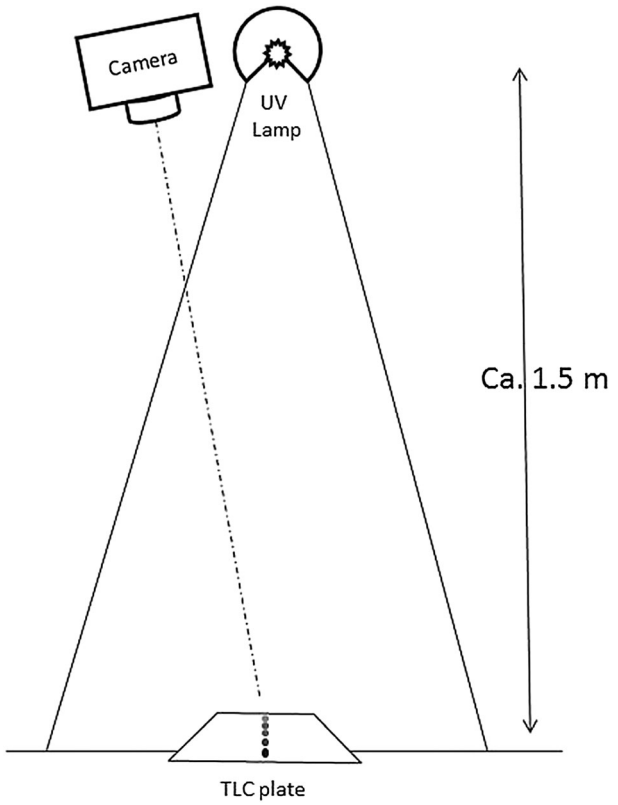


Figure 1. Camera and light source arrangement for fluorescence or fluorescence-quenching assay.

Image processing and spot quantification

Image processing was carried out using several public domain packages. In our hands, the IMAGEJ software[3] available from the US National Institute of Health proved the simplest, most straightforward and most versatile, although other packages could be utilised. For IMAGEJ, the photographic image may be input in joint photographic expert group (JPEG) or tagged image file format (TIFF) format. The spot image can be optimised (differentiated from the background or from interfering substances) by selection of the best colour domain (red, green or blue). Options are available within the program to invert the image, to subtract background and so on. The best option to select depends on the image. For example, an image from a TLC plate that involves fluorescence quenching by the analyte or detection by colour or by colour reactions will usually require selection of the appropriate colour domain and the image will need to be inverted such that the analyte pixels have large positive values against the background pixels, which will ideally have small values. Conversely, fluorescent analytes will require the appropriate selection of the colour domain but will not require inversion as the background will be dark and the fluorescent spots will be bright.

Quantification of the spots via IMAGEJ can utilise various approaches; two of which are simple and effective. These use different approaches to the selection and quantification of the spots. The spots may be selected, either individually by using an ellipse selection tool or as a batch by selection of a channel across or along the plate using a sub-routine designed for the quantification of gels. Either procedure can yield good results depending on the actual plate under analysis and they are often equivalent in terms of accuracy and precision. In the case of the gel sub-routine, the quantification is by integration of peaks derived by the subroutine from the strip of spots selected. In the case of individual spot selection, each spot is selected manually and a list of integrated densities compiled by the program. This integrated density, sometimes referred to as 'spot volume', is essentially a mean pixel value multiplied by the number of pixels in the spot area selected. This selection of individual spots generally functions well and is the best option for plate images that have a high background or where the spots are not circular. Although background subtraction can be carried out digitally by a subroutine in IMAGEJ, in our hands, we found that background subtraction was usually best achieved by quantification of equivalent blank areas of the plate near to the analyte spots, for example, a row of blank areas above, below or to the side of the spots. Keeping the area of the selection ellipse constant at that of the largest spot removes subjectivity in the analysis if this approach is used.

Data linearisation

Neither TLC nor photography are inherently linear techniques. In the case of TLC, the spot shape and analyte density within the spot can vary. Hence, it is essential to work within a reasonable concentration range. At the low end of the standard curve, the method is often limited by sensitivity and/or background; whereas at the top of the range, the limitations are usually spot shape and saturation. However, provided that a reasonable range is selected, the resulting standard curve can be linear. More generally, however, it follows a second order polynomial curve. Typically, a selection of a 10-fold to 20-fold concentration range proves most suitable for high quality analysis.

The raw data from the charge-coupled device detector of digital cameras is subjected to a significant amount of automated processing before the data is recorded as a joint photographic expert group or TIFF file. This manipulation enables the final file to have the correct intensities as judged by the human eye. Unfortunately, the eye does not respond to changes in light intensity in a linear fashion. Moreover, the absorbtion of light by the analyte on a TLC plate should follow an approximation to Beer's law, and hence the raw data from quantification of spot integrated intensity needs mathematical transformation in order to yield the linear standard curves desirable for an analytical method. Because several factors are involved, there is no straightforward method to linearise the data. The procedure utilised in this work to achieve the transformation is a modification of the method of Hess[1] in which an 'optical density' parameter is calculated from the determined spot integrated density such that:

$$'\text{Optical density}' = 2^{(\text{normalised spot integrated density/camera factor})}$$

where the spot integrated density is normalised such that the integrated density of the most intense spot (i.e. that of the highest concentration spot) has a value of 255 (for 8 bit images). The 'camera factor' is a measure of the change in the pixel response when the camera exposure is varied by a factor of 2. The factor may be determined experimentally as the change in integrated intensity of a fixed area of the camera image which is obtained upon doubling the camera exposure. Conversely, the factor can be varied, using the equation in the previous texts, so as to linearise the data as judged by the linear regression coefficient of the standard line obtained. In our hands, both methods work well and yield the same or very similar values for the camera factor. Once the transform is carried out, the resulting TLC data is linearised. Thus, the normalised data from an umbelliferone analysis (Figure 2a), which shows a typical standard curve with a negative second order coefficient, yields a linear standard curve after the transformation is carried out (Figure 2b). The transformation approach described in the previous texts enables the rapid and accurate analysis of many samples once the transformation has been incorporated into a spreadsheet such as Microsoft Excel. However, if only a few determinations are to be made, then simply reading the unknown concentrations from a plotted standard curve is straightforward and requires no mathematical or computational

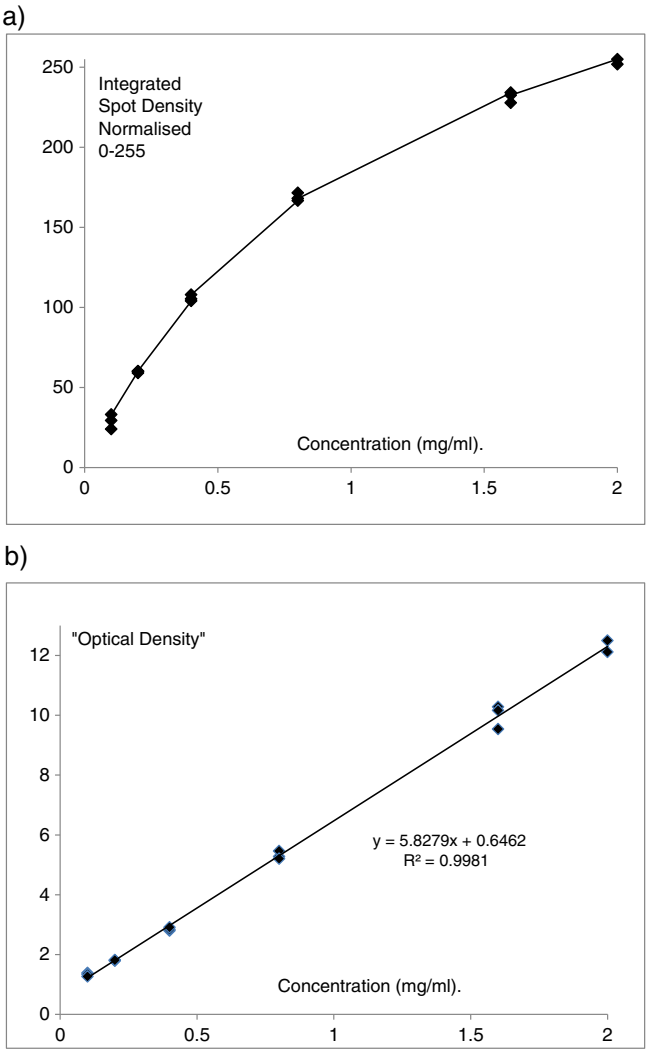


Figure 2. a. Normalised data from an umbelliferone DE-TLC analysis prior to transformation. b. 'Optical density' transformed (linearised) data from an umbelliferone DE-TLC analysis.

expertise. Indeed, if a simple quantification is required, such as that for the measurement of mass for specific radioactivity determination, then simply using a linear interpolation from standard samples set at multiples of 0.75-fold and 1.5-fold, the expected concentration will yield reasonable results due to the limited curvature over this narrow twofold range.

Results and discussion

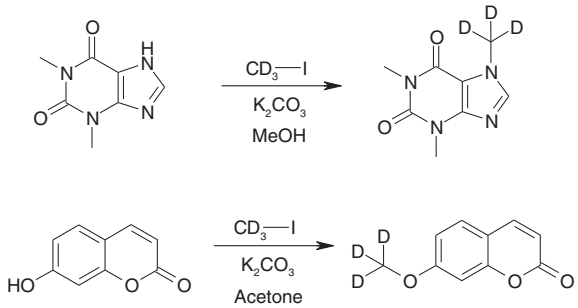
Determination of accuracy and precision

Table 1 shows the quality of analytical data for nine analytes across 11 *unoptimised* methods using various detection techniques. Accuracy and precision data were determined for all the analytes from the analysis of triplicate high and low concentration quality control samples against duplicate standard curves covering the standard range and were surprisingly good. Overall, a mean accuracy of 99 ± 6% and mean coefficient of variation (CV) of 4.1 ± 2.9% were obtained across all the methods.

To provide some practical examples of the use of the technique, several studies from the isotopic chemistry area are described in the succeeding texts. They illustrate the ways in which DE-TLC can enhance both the quantitative, semi-quantitative and qualitative uses of the TLC technique.

The first two examples are the uses of DE-TLC for monitoring reactions. In these cases, the technique is used to monitor the progress of the deuteromethylation reactions (Scheme 1) of theophylline and umbelliferone to yield trideuterated internal standards for the assay of caffeine and 7-methoxycoumarin by gas chromatography–mass spectrometry (GC-MS). Monitoring for reaction completion ensured that good yields of the internal standards were obtained by running the reactions at room temperature and monitoring by DE-TLC until the reaction was complete. The results of the monitoring are shown in Figures 3a and 3b.

Another example is the use of the technique to determine the reaction stoichiometry for the borodeuteride reduction of quinizarin to yield labelled 1,4-anthraquinone. In this case, a



Scheme 1. Deuteromethylations of theophylline and umbelliferone.

Table 1. Accuracy and precision data for nine analytes across 11 methods using digitally enhanced thin layer chromatography with various detection techniques

Analyte	Adsorbent eluent	Domain (detection)	Concentration range	Regression coefficient	QC CVs(%) high, low QC	Accuracies(%) high, low QC
Quinizarin	Silica gel	Blue	0.1-2.0 mg/ml	0.9954	2.55, 1.23	95, 106
	Toluene	(Orange colour)	0.1-2.0 mg/ml	0.9954	2.7, 2.24	92, 105
1,4-Diamino-	Silica gel	Green	0.5–4 µl	0.9993	4.2, 3.1	87, 101
anthraquinone	CH_2Cl_2	(Magenta colour)	0.5-4 µl	0.9994	4.0, 3.2	92, 105
Theophylline	Silica gel F254	Green	0.2–2.0 mg/ml	0.9962	5.1, 3.0	100, 102
	EtOAc/iPrOH/H_2O 10:7:6 v/v/v.	(254 nm)	0.2–2.0 mg/ml	0.9895	3.5, 4.6	100, 99
Caffeine	Silica gel F254	Green	0.2–2.0 mg/ml	0.9892	2.5, 3.0	101, 110
	EtOAc/iPrOH/H_2O 10:7:6 v/v/v.	(254 nm)	0.2–2.0 mg/ml	0.9897	2.0, 3.8	100, 102
Umbelliferone	Silica gel F254	Green				
	25% EtOAc/CH_2Cl_2	(254 nm)	0.2–2.0 mg/ml	0.9902	5.6, 12.8	98, 97
Umbelliferone	Silica gel F254	Green	0.1–2.0 mg/ml	0.9988	4.2, 0.91	100, 99
	25% EtOAc/CH_2Cl_2	(360 nm)				
Umbelliferone	Silica gel F254	Blue	0.1–2.0 mg/ml	0.9839	3.9.9.2	99,92
	25% EtOAc/CH_2Cl_2	(Iodine vapour.)				
Acetanilide	Silica gel F254	Green	0.125-1.0 mg/ml	0.9918	1.9, 1.4	95, 98
	25% EtOAc/CH_2Cl2	(254 nm)				
Riboflavin	Silica gel F254	Green	6.25-100 µg/ml	0.9969	6.9, 3.2	98, 101
	EtOAc/iPrOH/H_2O 10:7:6 v/v/v.	(360 nm)				
Nicotinamide	Silica gel F254	Green	0.1–2.0 mg/ml	0.9957	8.0, 3.4	89,94
	EtOAc/iPrOH/H_2O 10:7:6 v/v/v.	(254 nm)				
2′-Chloro-	Silica gel F254	Green	0.1–2.0 mg/ml	0.9808	11.9, 1.9	117,95
acetanilide	25% EtOAc/CH_2Cl_2	(254 nm)				

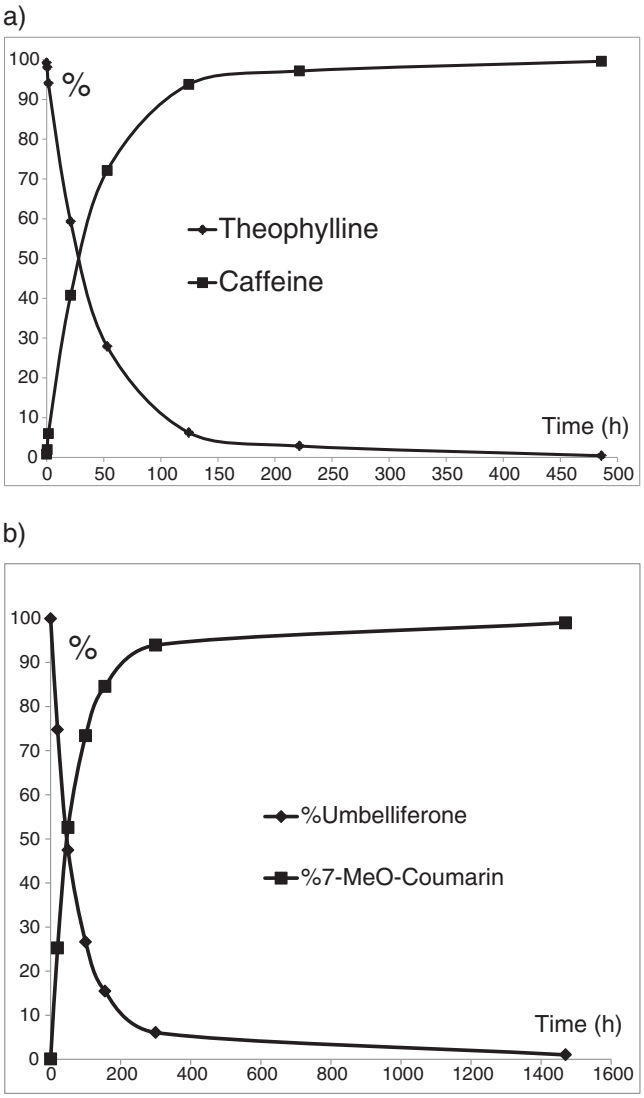


Figure 3. a. Monitoring of the deuteromethylation of theophylline to yield [methyl−$^{2}H_3$]caffeine. b. Monitoring of the deuteromethylation of umbelliferone to yield [methyl−$^{2}H_3$]7-methoxycoumarin.

semi-stable intermediate is formed, which is converted to the product by acid catalysed hydrolysis and dehydration (Scheme 2). Analysis of the reaction mixtures by DE-TLC allowed the optimum stoichiometry to be determined prior to a large scale reaction. Because all the components present were coloured, analysis required no specific detection method and was straightforward. Of course, in the absence of known extinction values for all the analytes, the method allowed only a semi-quantitative determination of concentrations but still yielded the information required to set a suitable stoichiometric ratio (Figure 4).

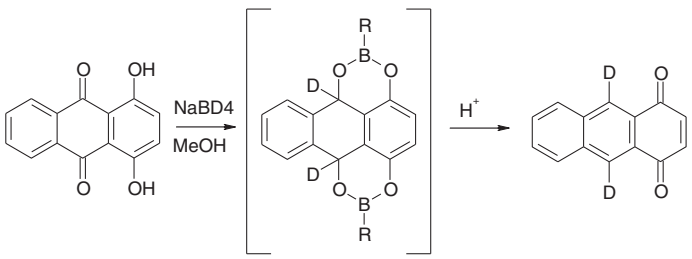
A similar use of the technique was in the optimisation of the deuterodechlorination of 2'-chloroacetanilide by deuterium gas, catalysed by 5% palladium on carbon, to yield [2'−^{2}H]acetanilide (Scheme 3). Here, extensive studies have been carried out[4] into the role played by the tertiary amine base, which is normally included in the reaction.[5] Both DE-TLC and quantitative nuclear magnetic resonance (NMR) methods were utilised to determine the yield of product in the presence of various tertiary amine bases (Figure 5) or in the presence of a single base at different times (Figure 6). There was a reasonable correlation between the DE-TLC determinations and the quantitative NMR method (Figure 7), which was based upon the integration of the methyl group resonances of the starting material and product in the proton NMR of the reaction mixture. However, the latter method was not validated and would clearly be susceptible to any interferences arising from minor undetected and/or unresolved by-products. Hence, the source of the observed differences cannot be definitively ascribed to one or other of the methods.

The aforementioned examples are included to show the ability of DE-TLC to carry out many determinations in parallel.

The selectivity conferred upon the DE-TLC technique by the ability to analyse TLC plates in the three different colour domains is shown clearly by some studies from the field of carotenoid chemistry. Figure 8 shows how the carotenoids, chlorophylls and pheophytin present in an aged acetone extract of cress seedlings are differentiated by the selection of the appropriate colour domain.

In a further carotenoid study designed to investigate the preparation of poly-deuterated carotenoids for HPLC-MS analysis, the reduction in carotene and xanthophyll yields occasioned by growing cress seedlings on a 1:1 mixture of water and deuterium oxide was easily quantified by DE-TLC analysis of a simple saponified extract (Figure 9). In this case, there were clear reductions in all the xanthophyls and carotenes; and in the case of two uncharacterised xanthophylls, the reductions were very marked (Figure 10).

Although the aforementioned studies were carried out as inexpensively as possible, some improvements to the technique might well be made for only small increases in cost. Thus the use of the JPEG file format was selected as it is the commonest file format used in inexpensive cameras. This format only has an 8 bit data depth (i.e. a maximum range of 0–255 per pixel per colour domain). Moreover, JPEG data are compressed, with the potential for some data loss, especially if files are saved several times. Nevertheless, JPEG files are very economical in the amount of memory used. The use of TIFF or raw files would avoid the possibility of data loss during the processing of the image and additionally could lead to a better dynamic range because the file format supports a greater data depth (typically 8,12,16



Scheme 2. Borodeuteride reduction of quinizarin.

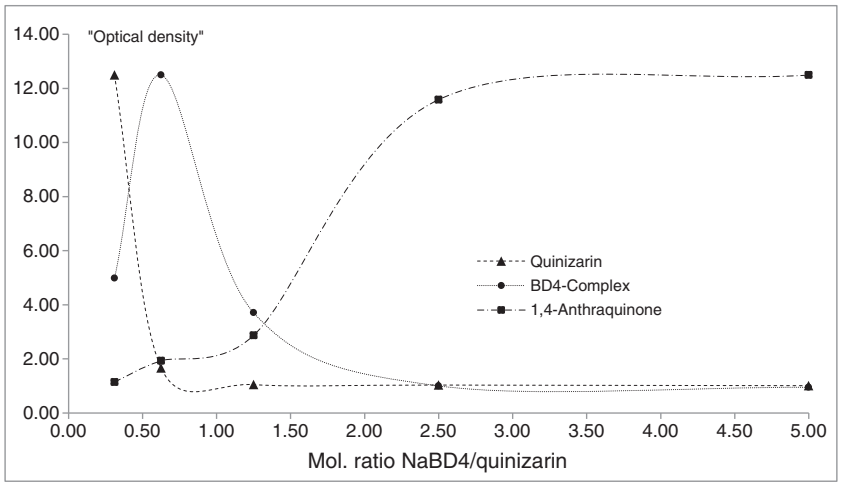
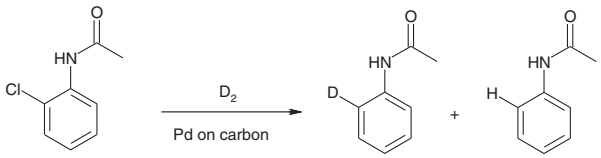


Figure 4. 'Optical density' of labelled 1,4-anthraquinone versus reaction stoichiometry.



Scheme 3. Deuterodechlorination of 2'-chloroacetanilide.

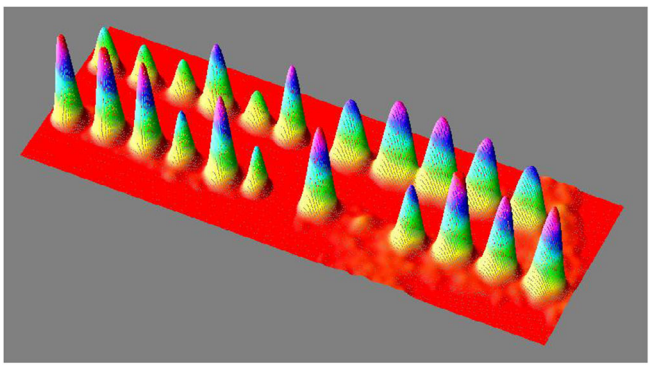


Figure 5. DE-TLC analysis of the efficiency of 11 tertiary amine bases in the deuterodechlorination of 2'-chloroacetanilide. The bottom row of spots is the labelled acetanilide product and the top row (higher Rf) is the 2'-chloroacetanilide starting material. The extreme right bottom spot is an acetanilide standard.

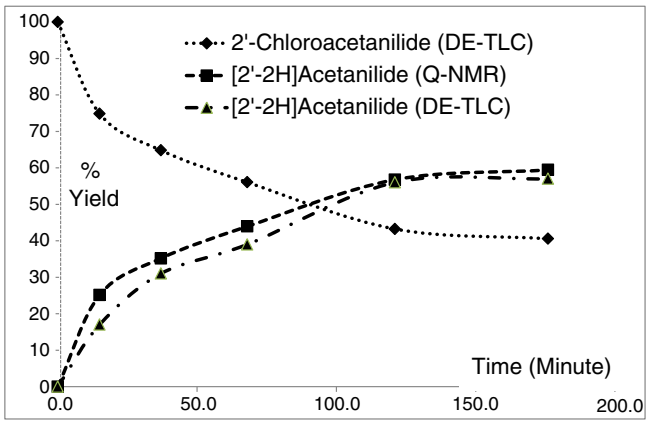


Figure 6. Percentage yield of labelled acetanilide against time (minutes) determined by DE-TLC or nuclear magnetic resonance.

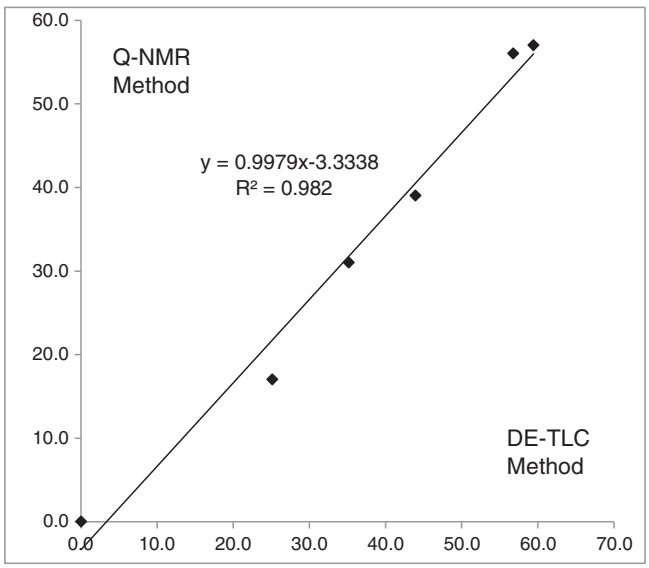


Figure 7. Correlation between DE-TLC and an unvalidated quantitative nuclear magnetic resonance method for the determination of labelled acetanilide.

or 24 bits). However, not all cameras can support these formats and the data files are considerably larger placing greater demands on the storage capacity of the camera.

Another possible improvement would be the addition of a cut-off filter to the ultraviolet light source to remove light other than the desired wavelength. Only one of the light sources used in this study (Mineral Light UVGL-25, (UVP Inc, San Gabriel, California, USA)) was equipped with such a filter, which should reduce the background light on the image and hence could improve contrast, sensitivity and selectivity.

Experimental

The TLC plates used throughout were Machery-Nagel aluminium-backed silica gel plates of thickness 0.1 mm containing a fluorescent indicator

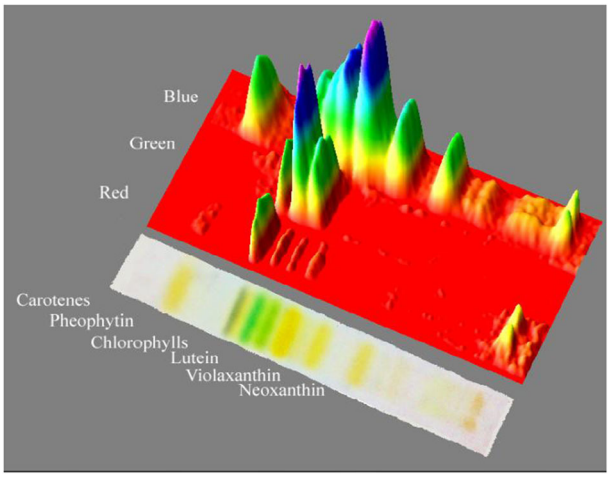


Figure 8. DE-TLC analysis of an aged acetone extract of cress (*Lepidium sativum*) seedlings in the various colour domains. Carotenes at highest Rf values.

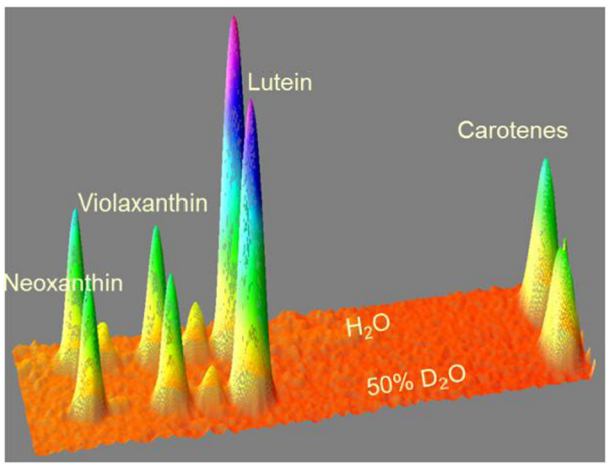


Figure 9. Variation of carotenoid recovery from cress (*Lepidium sativum*) seedlings grown using H_2O or 50%D_2O (= D_2O/H_2O 1:1 v/v). Carotenes are at the top of the plate (highest Rf value).

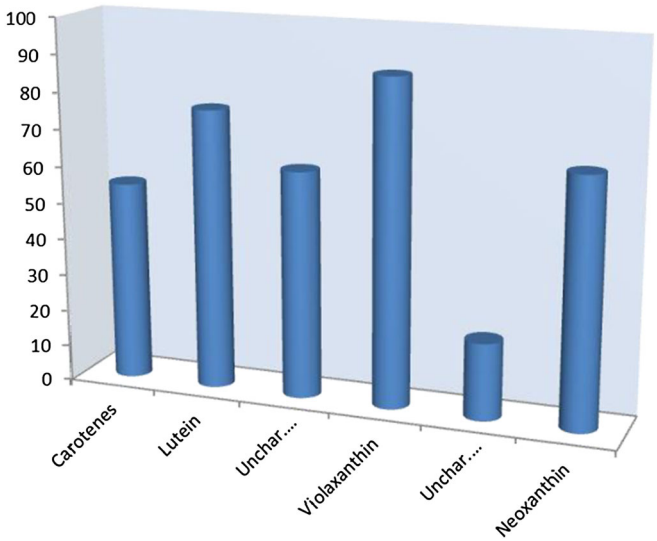


Figure 10. Relative yields of carotenoids from cress grown on a mixture of H_2O and D_2O (1:1 v/v) in comparison with cress grown on H_2O.

that could be excited at 254 nm and were obtained from Fisher Scientific, (Loughborough, UK).

The camera used was a Panasonic Lumix FZ28 model (Amazon.co.uk, Slough, Berkshire, SL1 1QP), athough similar results were obtained with other cameras.

The ultraviolet lamps used for 254 nm illumination were a Hygeniclean 4 W UV-C sterilising wand (UK Import & Export Ltd, Braithwell S66 7RL, UK or a 4 W Mineral Light UVGL-25, (UVP Inc, San Gabriel, California, USA). The latter was also used for fluorescence illumination at 360 nm. An alternative light source constructed from commercial 8 W 254 nm and 360 nm T5 300 mm screened fluorescent tubes was also evaluated, at 2 m distance from the TLC plate, and this arrangement also proved suitable for DE-TLC.

The IMAGEJ program was available free of charge from the National Institute of Health (NIH), USA and was downloaded from the website at http://rsbweb.nih.gov/ij.

The spotting of solutions was carried out using Drummond microcaps (Sigma-Aldrich Company Ltd, Dorset, England), although similar results were obtained using a 5 µl Hamilton syringe from the same supplier or an Eppendorf Reference variable volume pipette (1-10 µl), set at 5 µl (Fisher Scientific, Loughborough, UK).

Deuterium oxide, sodium borodeuteride, 2'-chloroacetanilide, acetanilide, caffeine, umbelliferone, theophylline and β-carotene were obtained from Sigma Aldrich Company Ltd, Dorset, England). Standard lutein was obtained by hydrolysis of Xangold® (Lifeplan Products Ltd, Lutterworth, Leics LE17 4ND) using sodium hydroxide in MeOH and was purified by TLC and stored at−20°. Deuteromethyl iodide was obtained from Cambridge Isotope Laboratories (UK agents CK Gas Products Ltd. Hook, Hampshire, RG27 7GR). All other reagents, chemicals and solvents were obtained from recognised chemical suppliers and used without purification.

Typical assay procedures

Determination of accuracy and precision values

A TLC plate (20 × 10 cm in landscape format) was spotted with aliquots (typically 5 µl) of the standard and quality control samples. Under these conditions, 18 spots can be accommodated per plate. Generally, the data in Table 1 were generated using six standard concentrations of analyte spotted in duplicate (12 spots) covering a 10-fold or 20-fold concentration ranges. Two quality control test samples with concentrations near the upper and lower ends of the assay standard curve were also spotted in triplicate (6 spots) making the 18 spots in total. The transformed data from all the standard spots were utilised to construct a standard line and the concentrations of the quality control samples were determined using the transformed data from the quality control samples and the equation of the standard line. Analysis of the resulting concentration data enabled the determination of accuracy and precision for the low and high quality control samples.

Reaction monitoring and optimisation

[methyl−$^2H_3$]caffeine from theophylline

To theophylline (184 mg,1.02 mmol) dissolved in MeOH (16 ml) and potassium carbonate (207 mg, 1.5 mmol) contained in a 20 ml screw-top glass vial complete with stirrer, was added [$^2H_3$]methyl iodide (128 µl, 2.0 mmol) and the reaction kept at room temperature and monitored by DE-TLC. For this procedure, 50 µl aliquots of the reaction mixture were evaporated and dissolved in water (1 ml). Aliquots (5 µl) of the resulting solution were analysed by DE-TLC using silica gel F_{254} eluted with ethyl acetate/propan-2-ol/water: 10:7:6 v/v/v). After the reaction was completed, the MeOH was evaporated and the resulting white

solid was triturated three times with dichloromethane (3 ml each time). The filtered extracts were combined and evaporated to yield [methyl−$^2H_3$]caffeine (186 mg, 92% based on theophylline, 46% based on CD_3I which was used in 100% excess). The compound was pure by TLC (silica gel F_{254}, EtOAc/iPrOH/H_20 10:7:6 v/v/v). The yield would have been 95% if the material utilised for DE-TLC monitoring was taken into account. The isolated material was characterised by GC-MS (*m/z* (%): 197 (100%), 168 (5%), 112 (40%), 85 (14%), 70 (11%), 58(16%) with no detectable peak at the unlabelled molecular ion of 194) and by ^{1}H-NMR, δ (500 MHz, $CDCl_3$) 3.41(3H), 3.59 (3H), 4.00 (absent, <0.06%), 7.51 (1H) ppm and ^{13}C-NMR (δ,$CDCl_3$, 27.9, 29.7, 33.6 (small multiplet only), 107.6, 141.4, 148.7, 151.7, 155.5 ppm. The deuterated batch assayed as 97.8% caffeine by GC-MS against authentic unlabelled caffeine (Sigma Aldrich) by comparison of the intensities of the 197 and 194 ions of the labelled and unlabelled caffeine.

[methyl−$^2H_3$]7-methoxycoumarin from umbelliferone

Potassium carbonate (236 mg, 1.71 mmol) was weighed into a 10 ml flask and umbelliferone (162 mg, 1.00 mmol) in acetone (5 ml) was added. Deuteromethyl iodide (200 µl, 3.15 mmol) was then added, and the reaction stirred slowly with monitoring by DE-TLC (5 µl aliquots analysed using silica gel F_{254}/dichloromethane) until the reaction was entirely complete. The solvent was removed under a stream of nitrogen, and the residue was triturated with three 2 ml portions of dichloromethane. The combined dichloromethane extracts were filtered and the filter cake also was washed with dichloromethane (2 ml). Removal of the dichloromethane under a stream of nitrogen yielded [$^2H_3$] 7-methoxycoumarin as a very pale yellow solid (180.2 mg, 100% based on umbelliferone, 31% based on CD_3I which was used in *ca*. threefold excess to speed the reaction). TLC, as aforementioned, showed only a single spot (Rf = 0.35) with no detectable umbelliferone (Rf = 0.1) and no other components. GC-MS similarly showed only a single peak. Crystallisation from dichloromethane (1.5 ml) by slow addition of hexane (6 × 1 ml) yielded diamond-shaped crystals (140.1 mg, 78% overall based on umbelliferone) along with material isolated from the mother liquors (38.8 mg), which still showed no other compounds present by TLC. The material was characterised by GC-MS (*m/z* (%): 179 (100%), 151 (80%), 133 (94%), 105 (10%), 77 (19), 51 (14%) and by NMR ^{1}H-NMR, δ (500 MHz, $CDCl_3$), 3.88 (not detectable), 6.25, (d,1H), 6.86–6.82 (complex multiplet, 2H),7.37(d,1H), 7.64 (d, 1H) ppm and ^{13}C-NMR (δ, CDCl3), 55.8 (not detectable),100.9, 112.6, 113.1, 128.8, 143.4, 156.0, 161.2,162.9 ppm.

Determination of the optimum stoichiometry for the sodium borodeuteride reduction of quinizarin to yield labelled [9,10−$^2H_2$]1,4-anthraquinone

Sodium borodeuteride (42 mg, 1.00 mmol) was dissolved in MeOH (2 ml) containing sodium hydroxide (1.2 µl of 10% w/v solution in MeOH) to ensure that the solution was alkaline, thus stabilising the borodeuteride. Aliquots of this solution (sufficient to achieve a final molar ratio of sodium borodeuteride to quinizarin of 0.3125, 0.625, 1.25 and 5.0) were added to five reactions vials each containing quinizarin (25 mg, 0.104 mmol) dissolved in sufficient MeOH to ensure that the final volume in each reaction was 2 ml. Aliquots (50 µl) from each reaction were treated with hydrochloric acid (2 N, 100 µl) and dissolved in acetone (0.5 ml). Aliquots of the resulting solution (5 µl) were analysed by DE-TLC (silica gel F_{254}, dichloromethane/toluene 1:1 v/v) at 0.5, 1.0 and 1.5 h. The analyses were identical at all the time-points showing that the reaction was rapid. Analysis of the samples from the five reactions at 1.0 h were used to construct Figure 4, which showed that a mol-ratio of *ca*. 2.5 or greater would be required to achieve a high yield of the desired product. The [9,10−$^2H_2$]1,4-anthraquinone product had *m/z* 210 (10%),182 (24%),154 (75%),128 (69%), 99 (4%) 77(16%) and δ (500 MHz, CDCl3) 7.05 (2H), 7.68 (2H), 8.10 (2H) and 8.6 (small residual traces) ppm. The ^{2}H-NMR ($CHCl_3$) showed only a single resonance at δ 8.59 ppm, the expected position of labelling at the nine and ten positions. The corresponding unlabelled material had the same retention time as the labelled compound, and the NMR and MS spectra were essentially identical with the literature mass spectrum[6], and the labelled compound showed only the differences associated with labelling in the nine and ten positions.

Efficiency of tertiary amine bases in facilitating the deuterodehalogenation of 2'-chloroacetanilide with deuterium gas over palladium

To 2'-chloroacetanilide (84.8 mg, 0.500 mmol) in tetrahydrofuran solution (2 ml) was added 5% 2.0 mmol, palladium supported on carbon (Aldrich item 27,670–7, 30 mg) and the chosen base (0.72 mmol) added. The reaction suspension was sealed in 10 ml capacity reaction tube, evacuated under vacuum then flushed twice with deuterium. Under these conditions, ca. 0.36 mmol of deuterium gas was present. The reaction was then stirred rapidly for 180 min. Under these conditions, the substrate was in excess over the deuterium gas and hence the maximum possible yield of acetanilide was 72%. After the reaction, the catalyst was removed by filtration and the solvent removed under a stream of nitrogen. The crude product was dissolved in dichloromethane (2 ml), washed, to remove base, with 2 N HCl, (2 ml) then with water (2 ml). The dichloromethane was dried and evaporated to yield the crude reaction product for evaluation by DE-TLC, NMR and GC-MS. Portions (2 mg) of the isolated material from reactions carried out with each base were separately dissolved in dichloromethane (2 ml) and aliquots of the solutions (5 µl) spotted for DE-TLC comparison of the extent of reaction. A standard sample of acetanilide at 1 mg/ml in dichloromethane was also spotted.

Growth of *Lepidium sativum* seedlings on deuterated medium and extraction of carotenes and xanthophylls

Into the bottom of each of four screw-top glass tubes (height 8 cm and diameter 1.5 cm) was placed a pad of sufficient surgical gauze to reach to 1.5 cm above the base. Aliquots of water (2 ml each) was added to two of the vials and a mixture of water and deuterium oxide (1:1 v/v, 2 ml each) was added to the other two vials. To each of the vials was added 15 seeds of *Lepidium sativum*, such that the seeds were spread over the surface of the gauze and the vials were capped. The seedlings were then allowed to germinate. Germination at 10 days was 26/30 for the 1:1 deuterium oxide/water vials and 29/30 for the water only vials. The seedlings were separated, blotted dry using filter paper, and the cotyledons were removed with scissors (yields, 301 mg for the deuterium oxide/water group and 270 mg for the water only group). To each group of cotyledons was added

10% w/v sodium hydroxide in MeOH (2 ml) and dichloromethane (2 ml) and the vials stirred in the dark for 18 h to destroy the chlorophyll and to hydrolyse the xanthophyll esters. Water (4 ml) was added to each of the hydrolysis reactions and the dichloromethane layer separated. The aqueous layer was washed with dichloromethane (1 ml) and the combined dichloromethane extracts were washed three times with water (3 × 5 ml) the dichloromethane layer was then dried by passing through a pad of cellulose tissue, washing through with a further 1 ml of dichloromethane, and evaporated to dryness at room temperature in the dark. The resulting extracts were each dissolved in dichloromethane (0.5 ml) and the carotene and xanthophyll contents compared by DE-TLC (silica gel F254, acetone/hexane 2:3 v/v or ethyl acetate/dichloromethane 1:1 v/v) by spotting equal fixed aliquots (15 μl).

Conclusions

The combination of digital photography with image processing and data transformation has enabled the easy and cost effective use of TLC as a quantitative technique for assay or as a semi-quantitative technique for reaction monitoring. The DE-TLC approach improves both the sensitivity and selectivity of TLC analysis and will enable the technique to play a greater role in chemical analysis. DE-TLC is particularly appropriate for laboratories without access to expensive quantitative TLC equipment or to those laboratories where contamination issues precludes the use of such equipment.

Conflict of Interest

The authors did not report any conflict of interest.

References

[1] A. V. I. Hess, *J. Chem. Educ.* **2007**, *84*, 842.
[2] R. Johnsson, G. Träff, M. Sundén, U. Ellervik, Evaluation Of quantitative thin layer chromatography using staining reagents, *J. Chromatogr. A* **2007**, *1164*, 298-305.
[3] The excellent ImageJ image processing software. http://rsbweb.nih.gov/ij
[4] a) W. J. S. Lockley, D. J. Wilkinson, J. R. Jones. *J. Label. Compd. Radiopharm.* **2006**, *49*, 401-402 and references therein;
b) W. J. S. Lockley, D. D. Jenkins, D. P. Manthorpe, *J. Label. Compd. Radiopharm.* **2013**, *56*(*2*), 50-63.
[5] Y. Monguchi, A. Kume, K. Hattori, T. Maegawa, H. Sajiki, *Tetrahedron* **2006**, *62*, 7926–7933.
[6] The Spectral Database for Organic Compounds: Advanced Industrial Science and Technology, Japan. http://sdbs.riodb.aist.go.jp [25 Jan 2013], spectrum 15066.