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Quantitative Ultraviolet Measurements on Wetted TLC Plates Using a Charge-Coupled Device Camera

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Abstract

This paper presents the first study of the UV imaging of spots on thin-layer chromatographic plates whilst still wet with solvent. Imaging of spots of benzophenone during and after development was carried out using a charge-coupled device camera. Limits of detection were found to be 5 ng on a wetted plate and 3 ng for a dry plate and the relationship between peak area and sample loading was found to be linear in the low nanogram range over an order of magnitude for both wet and dry modes with r^2 values > 0.99. It was found that UV measurements on wet glass-backed plates suffer from low sensitivity; however, the use of aluminium-backed plates gave increased sensitivity. The apparent absorption coefficient (ε_{app}) of 10 AU m^2 g^{-1} at 254 nm is consistent with reflection of the light from the aluminium surface with a double pass through the sorbent layer, and suggests that use of aluminium-backed plates should enable monitoring of separations by UV absorbance during TLC development.

Keywords

Thin-layer Chromatography, Videodensitometry, CCD Detection.

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1. Introduction

Making quantitative measurements in the ultra-violet region of the electromagnetic spectrum is useful because so many compounds absorb UV light. Making UV measurements on TLC plates using scanning densitometry is relatively straightforward, as the photomultiplier tubes used for detecting photons are UV sensitive [1]. Charge-coupled device (CCD) cameras are generally not sensitive to UV light, with response greatly reduced below 400 nm [2]. This problem has been surmounted by the use of UV-sensitive coatings which absorb then re-emit light at wavelengths amenable to CCD detection [3, 4, 5].

A CCD system fitted with UV and visible light sources to illuminate a planar surface has been reported [6]; however, the demand for UV sensitive CCD sensors has not yet justified the large-scale production of phosphor-coated devices, which are therefore still very expensive [7] and this contributes to the lack of ultraviolet CCD work on TLC plates [8].

Absorption of ultraviolet light by analytes in TLC can be determined on plates containing a fluorophore. However, this method (known as fluorescence quenching or quenching) is limited to compounds having a very strong absorption in the ultraviolet region [9].). Limits of detection (LODs) associated with fluorescence quenching are generally in the 50 ng region, with linear range of approximately one order of magnitude and precision < 3 % RSD [10].

Several authors have compared scanning densitometry with fluorescence-quenched videodensitometry in quantitative analysis [11, 12, 13]. In all cases the two detection methods were found to give similar results. Direct UV absorbance measurements by CCD camera would result in greater specificity than fluorescence-quenched measurements; however, there are no reports in the literature. In this work we describe direct UV absorbance measurements designed to test whether the method is appropriate for quantitative analysis in TLC.

2. Experimental

2.1 Chemicals

Benzophenone was supplied by Aldrich Chemicals (Poole, UK). A stock solution of benzophenone (200 mg L^{-1}) was prepared accurately in dichloromethane (DCM), and standards were made by dilution. The solvents acetonitrile, dichloromethane and hexane were all HPLC grade and supplied by Fisher Scientific (Loughborough, UK).

2.2 Chromatography

Thin layer chromatography was performed on 5 x 10 cm silica gel 60 and silica gel 60 F_{254} (containing fluorophore) TLC plates, (glass and aluminium backed varieties were used, layer thickness 200 μm. Plates were supplied by Merck (Darmstadt, Germany). 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). Focusing was carried out by developing with acetonitrile for a distance of approximately 1 cm and then allowing the plate to dry. Development was performed at room temperature in a 10 x 10 cm horizontal chamber (Camag, Muttenz, Switzerland). The mobile phases used were dichloromethane-hexane (50:50, v/v) and dichloromethane-hexane (75:25, v/v).

2.3 Imaging and Processing

The images were taken using an Antares Duo, LSR AstroCam CCD camera and the associated Imager 2 software (Cambridge, UK). The camera was fitted with an EEV CCD05-20 CCD chip; this has 770 by 1152 pixels each 22.5 μm square. The camera head cools the chip thermoelectrically to -40 °C. The UV-sensitive phosphor coating is 'Astrochrome 90'; this is a Lumogen coating, which is an organic phosphor that is excited below 400 nm and emits at 520-580 nm. The quantum efficiency of Lumogen coated devices below 400 nm have been measured to be up 25% [5]; the manufacturers claim 10-15% for the camera used here. The CCD was fitted with an A4869, 50 mm focal camera lens (Hamamatsu, Photonics UK Limited, Welwyn Garden City, UK). The camera was operated at maximum dynamic range and readout rate (165 kHz). All acquired images had a blank image (taken with the shutter closed) subtracted, to compensate for camera dark current and other fixed pattern noise. A Chance OX7 filter (Chance-Pilkington, St. Asaph, UK) with a pass band from 250 – 400 nm, was employed to block non-UV light.

The UV light was delivered from a 2 x 8 W 254/366 nm mercury lamp (Camag, Muttenz, Switzerland) positioned above the development tank for measurements in the reflectance mode in all experiments. A picture of the apparatus for the experiments is given in Fig. 1. The experiments were carried out with the lens aperture at f/ 3.5 and an exposure time of 200 ms. Image processing was achieved using Scion Image Beta 4.02 software (Scion Corporation, Frederick, MD, USA). Data smoothing and quantification was accomplished using PeakFit version 4 (Systat Software, London, UK) as in previous work [14].

Using the mercury lamp at 254 nm, off line experiments were undertaken on dried plates and also on wetted plates. In the dry plate experiments, the plates were imaged blank, spotted, focused with acetonitrile to a distance of approximately 1 cm from the bottom of the plate and allowed to dry. Subsequently the focused bands were developed for a further 4-5 cm with solvent, after which the plate was allowed to dry again before an image of the developed plate was then taken. A ratio of the two images was taken in order to compensate for fixed pattern effects and the logarithm calculated to generate a two-dimensional absorbance profile. In the wet plate experiments, an image was taken of the blank plate and then the plate was developed up to a predetermined distance (marked on the plate), and another 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, when the solvent front was at the same position as in the first image. Finally the plate was allowed to dry and imaged again. Absorbance images were calculated for the wet and dry plates using the two wet images and two dry images respectively. In this way two sets of results (wet and dry) were obtained for the same spots on the same plate.

2.4 Spectrophotometry

UV-visible spectrophotometry of benzophenone was carried out using a Shimadzu 1700 spectrophotometer and quartz cells with a path length of 1 cm. Measurements of absorbance were made between 200 and 400 nm. Benzophenone (λ_{max} = 252 nm) was dissolved in dichloromethane at a concentration of 12.5 mg L^{-1} with dichloromethane in the reference cell.

2.5 UV transparent plates

Thin-layer chromatography plates backed with UV transparent glass are not currently available commercially so it was necessary to make our own plates in-house. Two pieces (50 × 50 × 1 mm) of Spectrosil B quartz (Edmund Optics, York, UK), which has no significant absorbance above 200 nm, were coated with a silica gel slurry. The slurry was made by suspending 10 g of silica gel from Merck in 20 mL of water. Any air bubbles were removed by applying a partial vacuum in a Buchner flask. The slurry was then poured on the glass pieces. An aluminium jig was used, which allowed the glass plates to be given a sorbent layer of

known thickness by means of a scraper. The plates were then removed from the jig and left to dry at room temperature.

3. Results and Discussion

3.1 Dry imaged results

The first experiments undertaken were on dry glass-backed plates to investigate reproducibility and linearity. Fig. 2 is an example of the image taken; (A) is a raw image taken after development and (B) is the same image after correction for fixed pattern effects such as inhomogeneity in the light distribution across the plate surface and differences in the sorbent layer. Results obtained are given in Tables 1 and 2. Precision measurements (RSD) in Table 1 are for the full analytical procedure from spotting to evaluation ($n = 4$). The RSD values obtained were satisfactory (<5% in all cases).

In Table 2, the gradient (x^1 coefficient) is equivalent to the apparent absorption coefficient (ε_{app}), with units AU $m^2 g^{-1}$. The regression slopes from all the calibration experiments show good repeatability, with intercepts (x^0 coefficient) zero within experimental error. The average ε_{app} values from Table 2 may be compared with the value obtained spectrophotometrically. Absorbance of a 12.5 mg L^{-1} solution of benzophenone in dichloromethane at 254 nm was 1.1 AU, which corresponds to $\varepsilon = 8.6$ AU $m^2 g^{-1}$. The similarity indicates that for the light reflected from the dry TLC plate, the effective pathlength through the sample is approximately equivalent to a single straight pass through the sorbent layer.

3.2 UV imaging on wet glass backed plates

An attempt was made to undertake wet off-line experiments similar to those in previous work [14] but with the imaging being done in the UV region. Initial experiments were carried out on glass-backed plates with similar sample loadings but no spots were detectable when the plates were solvated. The spots became detectable if the plates were allowed to dry and the signals were of a similar size to those reported in section 3.1. Further attempts were made increasing the loadings until detection was possible, with 500 ng spots giving a S/N ratio of approximately 4:1 as illustrated in Fig. 3. The average result of 1.8×10^{-7} AU m^2 for the 500 ng peaks in Fig. 3 corresponds to a ε_{app} value of 0.36 AU $m^2 g^{-1}$. This value is a factor ~ 20 lower than those obtained from dry plates and spectrophotometrically at 254 nm (6-10 AU $m^2 g^{-1}$). These UV findings contrast with those obtained previously in visible light investigations where values of ε_{app} with a blue filter were found to exceed ε determined spectrophotometrically at 400 nm [14].

An investigation was made into the impact of light reflected back through the TLC plate on measured absorbance values by employing in-house (non-commercial) plates with a UV transparent support. Absorbance measurements were made with the development tank internally lined (base and sides) with polished aluminium in order to increase reflection and with the development tank similarly lined with black felt in order to decrease reflection. Measurements were made on the same spots wet and dry. A high sample loading (500 ng) was used in order to obtain a large signal and minimise effects caused by the layers produced not being as reproducible as commercially available layers. Absorption coefficients were measured from the dry plates to be 10.4 and 6.5 AU $m^2 g^{-1}$ and from the wet plates to be 5.5 and 3.6 AU $m^2 g^{-1}$ for the aluminium and felt linings, respectively.

All values of ε_{app} are a factor 10 or more greater than those obtained from images taken of a wet glass-backed plate. This suggests that when the plate is wet, UV light that enters the

sorbent layer traverses the whole of the layer and is absorbed by the glass. Using the fused silica plate, which does not absorb UV light, ε_{app} for benzophenone measured with the dry plate in the felt-lined tank is comparable to the values reported in Table 2. It can be seen that lining the tank with reflective or absorbent material has a significant effect on the ε_{app} values measured for both wet and dry plates.

3.3 Wet and dry imaged aluminium-backed plates

It was found that an increased signal could be obtained on wetted plates from absorbance experiments by using aluminium-backed plates instead of the glass-backed plates. Wet and dry results were obtained from the same spots, during and after chromatography. Linearity and precision were investigated using benzophenone and the results are given in Tables 3 and 4. Good intra-plate precision (<5% RSD) was obtained from wet and dry plates, with dry plates being the more repeatable; this is consistent with the results of experiments in the visible region where it was suggested that the presence of solvent made signal referencing less reproducible [14]. Linearity was over an order of magnitude; the slopes of the calibration plots were greater for wet than on dry plates and greater with the non fluorescing plates. The fluorophore present in the F254 plates will absorb a proportion of the incident UV light that passes through the sorbent layer and so reduce this as a proportion of the total reaching the detector, which includes light that has reflected off the top surface. This results in the lower ε_{app} values observed when using the F254 plates. All intercepts were positive, although the 95% confidence intervals of all intercepts from experiments on individual plates encompassed zero. However, the low errors at the 95% confidence level associated with the average intercepts from all plates shown in Table 4 suggested that the intercepts were in fact significantly different from zero. This is consistent with the presence of a systematic negative curvature in calibration curves, as observed in other studies [15]. Linearity in the loading range up to 50 ng was good, with r^2 values >0.993 in all cases.

Data in Tables 3 and 4 show that greater sensitivity is obtained on wetted plates than for dry plates when an aluminium support is employed, in contrast to the case with glass-backed plates where dry plates gave a greater signal than wet plates. For measurements made at visible wavelengths it was proposed that increased scatter (and hence longer effective path length) on dry layers was responsible for the greater signal [14]. For measurements in the UV, all results are consistent with light is being reflected from the backing of aluminium-backed plates, and that this reflection is more pronounced under wet than under dry conditions. The explanation for this would be that the sorbent layer becomes more transparent through decrease of the refractive index difference between silica and the surrounding medium (solvent as compared to air).

It is evident from Fig. 4 that a greater signal is obtained from a wet image than from a dry image. However, the noise is clearly worse, the result being that the signal to noise ratio (S/N) is higher (and the LOD lower) for the dry-imaged peak. The S/N ratio for the 'dry' peak is approximately 4:1 (peak-to-peak noise), whilst the S/N ratio for the 'wet' peak is approximately 2:1. The wet 5 ng peak is at the limit of detection, and the estimated LOD for the dry plate is 3 ng on a dry plate by this method for this compound under the test conditions. This compares well with sensitivity achieved with fluorescent quenching where LODs of ~50 ng have been reported [10].

Fig. 5 is a schematic showing UV light reflected from (A) glass, (B) aluminium and (C) fused-silica backed TLC plates considered as three groups. (i) Reflection from the sorbent layer top surface, (ii) reflection or scattering from the stationary phase within the sorbent layer and (iii) reflection from surfaces below the sorbent layer. The effective pathlength through the analyte containing mobile phase will depend on the relative proportions of these light paths with (i) contributing nothing and (iii) contributing the most. Table 5 lists the relative reflected

light levels measured by the camera for various plate types and experimental conditions. For a wet glass-backed plate ε_{app} is very low (very short effective pathlength) indicating that most of the light reaching the detector is from the top surface. Wet aluminium backed plates show the highest value of the relative reflected light level, suggesting most of the light reaching the camera has been reflected from the support after a double pass through the sorbent layer. Wet quartz plates are the intermediate case where the importance of the proportion of reflected light is indicated by the higher values obtained when a reflective lining is used. Glass and aluminium backed plates both have similar reflectance levels when dry, as the dominant effect is light reflected and scattered from the sorbent layer.

The level of the light component at the camera that has been reflected from the top surface of the sorbent layer from wet glass-backed plates can be estimated to be ~95% of the total reflected; this is based on the ε_{app} measured being ~5% of the spectrophotometer value. It is reasonable to assume that this light level component will be the same for all cases where the plate is wet with the same solvent, irrespective of the backing material. The relative reflected light levels listed in Table 5 would then indicate that for the wet aluminium-backed plate 95 % × 1/2.7 = 35 % of the total reflected light is from the top surface. If it is assumed that the remaining light has been reflected from the aluminium surface and has passed twice through the sorbent layer with little scattering, the predicted value for ε_{app} at small absorbance values would be 65% of double the spectrophometer value, which comes to 11 AU m^2 g^{-1}. This estimate is in good agreement with the observed value and lies between the values obtained from wet aluminium backed plated with and without fluorophore (Table 4).

Table 6 shows refractive index values of silica and solvents at visible (500 nm) and UV (254 nm) wavelengths. The closer the match between the two values, the less the scattering of light expected from within the sorbent layer. It can be seen that both sets of values for the mobile phase DCM-hexane (75:25, v/v) are close to those of silica at the corresponding wavelength. This suggests that there would be little scattering of light when the plate was solvated. At 254 nm the light would pass through the layer and be absorbed by the glass support. At 500 nm light would not be absorbed by the glass support and some would be reflected back up off the glass/air interface and from the bottom of the tank and result in a higher effective pathlength. This is the main reason why poor sensitivity was obtained using glass-backed plates with UV light compared with those using visible light. Additionally, the RI values are even better matched at 254 nm than they are at 500 nm, therefore even less scattering would be expected in the sorbent layer at 254 nm. All these figures are consistent with the suggestion of light being reflected from the backing surface with aluminium-backed plates and a double pass through the sorbent layer.

4. Conclusions

In this work, novel UV imaging of spots on a wetted TLC plate using a CCD camera is presented. Quantitative TLC has been carried out using UV light off-line and in the presence of solvent, with good linearity, precision and sensitivity. Glass-backed plates gave very poor sensitivity for measurements made in the presence of solvent. The value obtained for ε_{app} of the analyte benzophenone increased by an order of magnitude from < 1 to 10 AU m^2 g^{-1} by the use of an aluminium reflective backing. This is consistent with a significant proportion of incident light being transmitted through the layer and reflected back up through the chromatographic band, as opposed to being absorbed in the case of the glass support. This hypothesis was tested and confirmed using a non-absorbing fused silica plate to support the sorbent layer. The close refractive index match between the mobile and stationary phases at 254 nm and the resulting low scatter from within the sorbent layer suggests that most of the light reaches the detector by reflection from surfaces above and below the layer. This model accounts for the increase in the absorption coefficient measured from wet aluminium-backed plates when compared to the

value measured spectrophotometrically. Additionally, the low level of scatter means that the absorbance signal measured will be less sensitive to the position of the analyte within the sorbent layer than in the case of imaging of dry plates, where analyte quantification can be adversely affected by the phenomenon of secondary chromatography.

For aluminium backed plates the linear range was between 5 and 50 ng for both wet and dry plates; the limit of detection was 5 ng for wet plates, with better signal to noise ratios obtained for dry plates. Linearity was very good over this limited range, with r^2 values of > 0.995 obtained. Intra-plate RSD values were between 2.1 and 4.4 %, whilst inter-plate RSD values were higher (10-14%) due to plate-to-plate variability.

The ability to quantitatively measure the absorbance signal of analytes on TLC plates while wet with mobile phase should enable monitoring of separations during TLC development. Future work will aim to investigate real time imaging of TLC plate separations of UV absorbing analytes, analogous to studies carried out with visible light [15].

Table 1
Average signals of 4×25 ng benzophenone spots run with DCM-hexane (50:50, v/v), imaged offline on dry, glass backed silica gel 60 F254 TLC plates.

Average signal / 10^{-7} AU m^2	RSD / %
1.67 ± 0.05	3.1
2.06 ± 0.06	3.0
1.71 ± 0.02	1.2
1.99 ± 0.02	1.0
Average 1.86 ± 0.20	Interplate RSD = 11 %

Table 2
Coefficients from four point calibrations of benzophenone (5, 10, 25 and 50 ng) run with DCM-hexane (50:50, v/v) imaged on dry, glass-backed silica gel 60 F254 TLC plates.

Calibration	x^0 / 10^{-8} AU m^2	x^1 / AU m^2 g^{-1}	r^2
1	-0.7 ± 2.3	7.9 ± 1.7	0.9951
2	-0.4 ± 1.5	7.9 ± 1.0	0.9979
3	-0.4 ± 1.4	7.1 ± 1.0	0.9979
4	0.1 ± 2.4	7.4 ± 1.7	0.9942
average	*-0.4 ± 0.3*	*7.6 ± 0.4*	

Table 3
Signal level and reproducibility in quantification of peak area from 25 ng spots of benzophenone after chromatographic development with DCM-hexane (75:25, v/v). Aluminium backed Silica Gel 60 F254 and silica gel 60 TLC plates were used with four spots per plate and runs made with wet and dry imaging.

	Wet	Dry	Wet	Dry
	silica gel 60 F254		silica gel 60	
Average signal / 10^{-7} AU m^2	2.52	1.93	3.47	2.15
Average intra plate RSD / %	4.4	3.3	4.3	2.1
Inter plate RSD / % (n=4)	11	10	11	14

Table 4
Average coefficients from four replicate calibrations of benzophenone spotted at 5, 10, 25 and 50 ng at each of the four experimental conditions listed in Table 3.

	Wet	Dry	Wet	Dry
	silica gel 60 F254		silica gel 60	
x^0 / 10^{-8} AU m^2	3.0 ± 1.3	1.4 ± 0.3	3.2 ± 1.6	1.8 ± 0.7
x^1 / AU m^2 g^{-1}	10.0 ± 0.4	5.5 ± 0.7	12.6 ± 1.4	8.2 ± 1.5
r^2	0.9955	0.9971	0.9975	0.9957

Table 5
Relative reflected light levels measured at the camera and apparent absorbance coefficients of benzophenone measured at 254 nm from wet and dry TLC plates and for various support materials.

Support	Relative light level	ε_{app} / AU m^2 g^{-1}
Glass - wet	1	0.4
Glass - dry	2.4	7.6
Aluminium - wet	2.7	10.0
Aluminium - dry	2.5	5.5
Quartz with felt lined tank - wet	1.3	3.6
Quartz with aluminium lined tank - wet	2.4	5.5
Spectrophotometer measurement	-	8.6

Table 6
Refractive indices of silica gel and solvents at 500 and 254 nm.

Wavelength / nm	hexane[1]	DCM[2]	estimate for DCM-hexane (75:25, v/v)	silica[3]
500	1.380	1.435	1.421	1.462
254	1.426	1.499	1.480	1.508

1. Kozma *et al.,* 2005 [16]
2. Samoc, 2003 values for RI dispersion of chloromethane used, scaled by refractive index ratio of DCM to chloromethane at sodium D line [17].
3. Interpolated from Table VII.24 in James and Lord, 1992 [18].
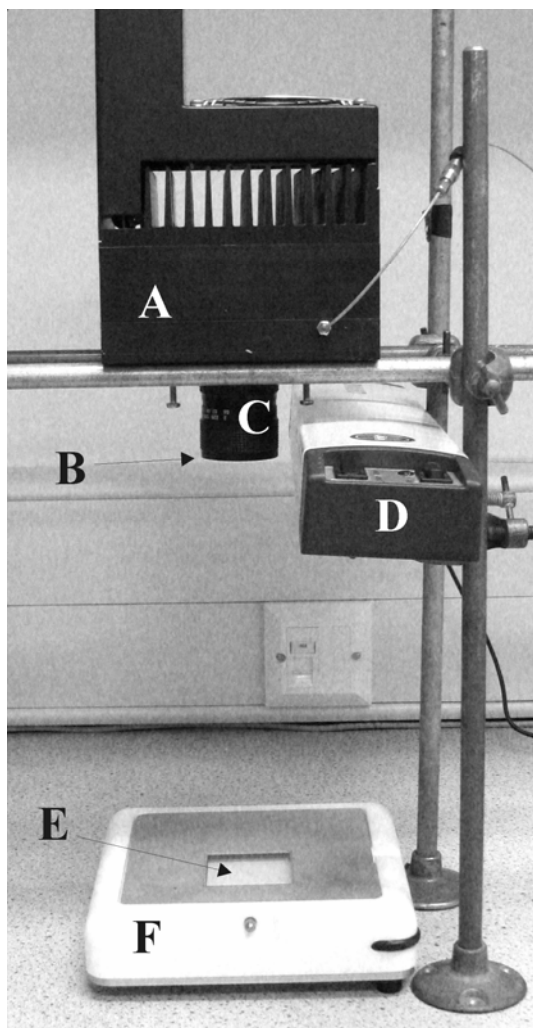
Fig. 1. Apparatus to image plates in UV in reflectance mode: A, CCD camera; B, UV pass filter; C, UV lens; D, mercury lamp; E, TLC plate; F, developing tank (fitted with UV transparent window). The whole system is enclosed to prevent stray light from entering.
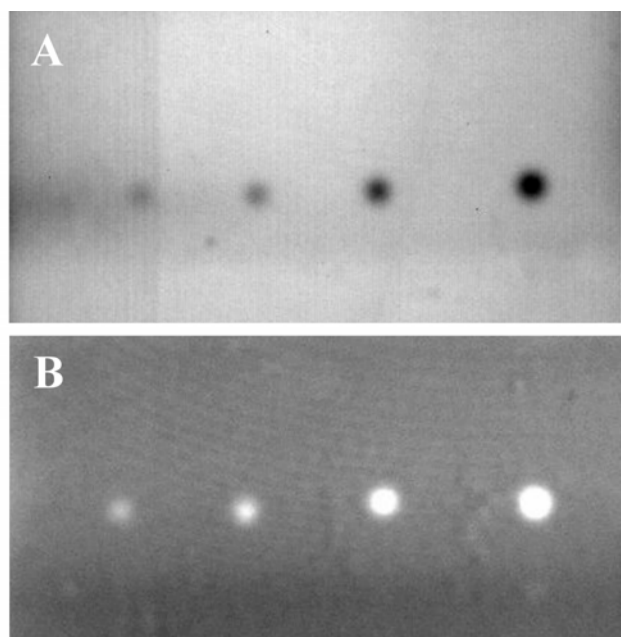
Fig. 2. (A) Raw, uncorrected image of a benzophenone calibration (5, 10. 25 and 50 ng) on a dry, glass-backed plate. (B) Image corrected for fixed pattern effects by ratioing (A) with an image of the blank plate.
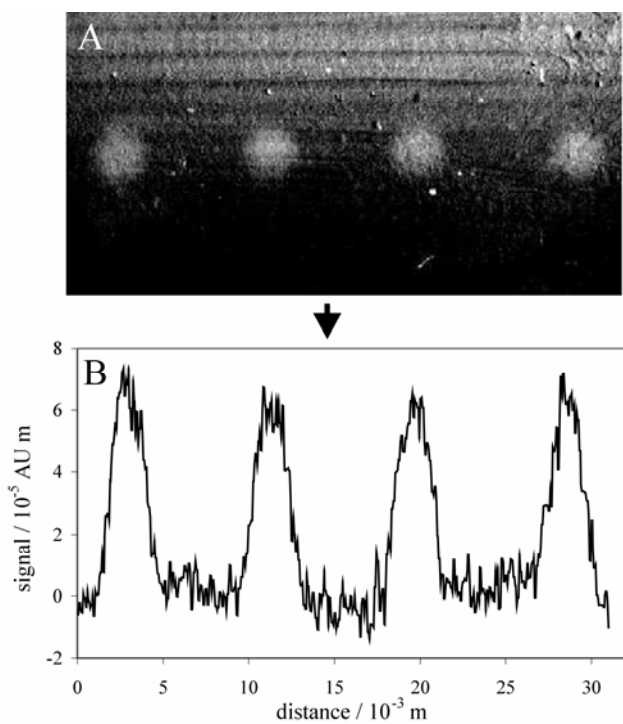
Fig. 3. (A) Image, corrected for fixed pattern effects, of four 500 ng spots of benzophenone on a wet, glass-backed Silica Gel 60 F_{254} TLC plate. (B) Peaks obtained by integration in the direction of chromatography (S/N ~4).
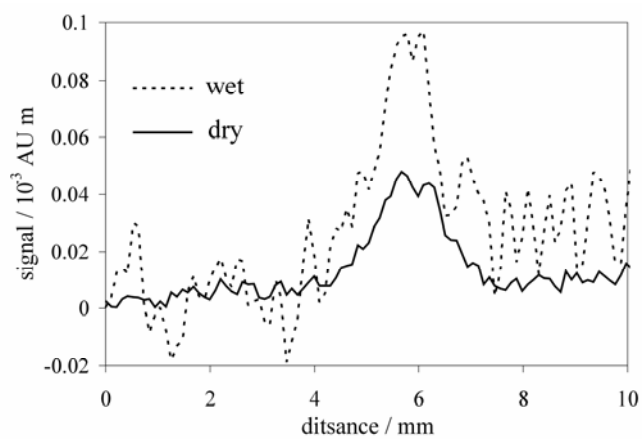
Fig. 4. Peaks obtained from the same 5 ng benzophenone spot imaged wet (during chromatography) and dry (after chromatography) on an aluminium-backed Silica Gel 60 F254 TLC plate.
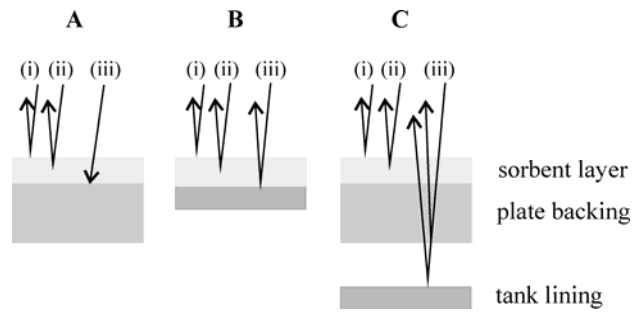
Fig. 5. Reflectance of incident UV light for (A) wet glass backed plate, (B) wet aluminium backed plate, (C) quartz backed plate. For details, see text.

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