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USE OF THE CHROMIMAGE FLATBED SCANNER FOR QUANTIFICATION OF HIGH-PERFORMANCE THIN LAYER CHROMATOGRAMS IN THE VISIBLE AND FLUORESCENCE-QUENCHING MODES

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SUMMARY

The ChromImage flatbed scanner densitometer with Galaxie-TLC software has been used for quantification of silica gel high-performance thin-layer chromatography (HPTLC). The visible mode was evaluated by determination of the recovery of a rhodamine B standard dye from a four-dye mixture and by determination of the precision of replicate analysis. Determinations of caffeine in multicomponent analgesic tablets and a cola beverage were performed in the fluorescence-quenching mode. Previously published methods for the tablet and beverage analyses were modified by using HPTLC plates with a brilliant ultraviolet indicator, and analysis of the beverage was further modified by automated application of standard and sample bands by use of a Linomat. Accuracy, precision, linearity, and sensitivity of these analyses with the ChromImage are reported.

INTRODUCTION

We have previously reported [1] use of an office flatbed scanner modified in-house for documentation and quantification of thin layer chromatograms detected by fluorescence quenching on silica gel layers containing an ultraviolet (UV) indicator. Since that work, the ChromImage has become available from AR2i (Le Plessis Robinson, France). This instrument, which, as far as we are aware, is the first commercial flatbed scanner densitometer, enables acquisition of TLC plate images and quantification of chromatograms by use of Galaxie-TLC software.

We tested the ChromImage for determination of a rhodamine B in a four-dye mixture at two concentrations on silica gel high-performance thin-layer chromatography (HPTLC) plates using the visible mode (white light source of the scanner). It was used with the 254 nm UV source, in

conjunction with modifications of previously-published procedures, for determination of caffeine in multicomponent extra-strength analgesic tablets [2] and in a cola beverage, without sample preparation [3]. Both determinations were modified by use of an HPTLC plate (Adamant) containing a brilliant UV indicator. Analysis of the beverage was further modernized by automated, spray-on band application of standard and sample solutions by use of a Linomat rather than manual application with a micropipet.

EXPERIMENTAL

Recovery of Rhodamine B Standard

The performance of the ChromImage in the visible mode was evaluated by determining the recovery of Rhodamine B from a four-dye mixture by use of an approach suggested by Aborashed [4]. HPTLC was performed on 20 cm × 10 cm silica gel $60F_{254}$ GLP plates (EMD Chemicals, Gibbstown, NJ, USA, an affiliate of Merck, Darmstadt, Germany; #5613/6), which were used as received. Test Dye Mixture I solution (Analtech, Newark, DE, USA), containing 4.00 mg mL^{-1} each of fast green FCF (aqua blue), rhodamine B (red), Bismark brown Y (yellow), and Sudan IV (violet-pink), was diluted to 0.175 mg mL^{-1} (standard S1) and 0.250 mg mL^{-1} (standard S2) with methanol. Five different volumes of S1 (2.00, 4.00, 6.00, 8.00, and 10.0 µL; 0.350–1.75 µg) were applied to produce the calibration plot, and duplicate 3.00-µL and 5.00-µL volumes were applied as 'unknown samples'. Sample zones were applied by use of a Linomat IV automated spray-on band applicator (Camag, Wilmington, NC, USA) fitted with a 100-µL syringe and operated with the settings: band length 6 mm, application rate 4 s μL^{-1}, table speed 10 mm s^{-1}, distance between bands 4 mm, distance from the bottom of the plate 1.0 cm, tracks 5–13 spotted. The plate was developed to 8 cm beyond the origin with ethyl acetate–methanol–water, 80:20:20, in the front trough of a Camag twin-trough chamber saturated with mobile phase vapor (saturation pad in the back trough). The plate was dried in a fume hood after development, scanned, and the rhodamine B was quantified by use of the ChromImage.

Determination of Caffeine in Analgesic Tablets

Caffeine standard was purchased from Aldrich (St Louis, MO, USA; #C0750) and a standard solution (0.100 mg mL^{-1}) was prepared in

methanol. To prepare a sample stock solution an extra-strength analgesic tablet with a label declaration of 65 mg caffeine, 250 mg acetaminophen, and 250 mg acetylsalicylic acid was ground to a fine powder with a mortar and pestle, the powder was transferred to a 100-mL volumetric flask by washing with approximately 70 mL methanol, and the mixture was stirred magnetically for 30 min. The stir bar was removed by use of a magnetic rod and the solution was diluted to volume with methanol and mixed by shaking. Undissolved excipients were removed by filtration through a Pall Gelman (Ann Arbor, MI, USA) Acrodisc LC 13 mm syringe filter, with a 0.45 μm PVDF membrane, into a capped vial. To prepare the TLC test solution from the sample the clear sample stock solution was diluted 1:10 by mixing 100 μL sample stock solution with 900 μL methanol. Digital Drummond (Broomall, PA, USA) microdispensers (100 and 1000 μL) were used to measure volumes.

A Nano-Adamant UV_{254} silica gel HPTLC plate (Macherey–Nagel, Easton, PA, USA; #821 120) was prewashed by development to the top with 1:1 dichloromethane–methanol, dried, and spotted on tracks 7–12 with 2.00, 4.00, 6.00, and 8.00 μL standard solution (0.200–0.800 μg caffeine) and duplicate 6.00-μL volumes of the sample solution (theoretically 0.390 μg, on the basis of the label declaration) by means of the Linomat with the settings listed above. The plate was developed in a saturated twin-trough chamber with ethyl acetate–glacial acetic acid, 95:5, as mobile phase, dried, and scanned and quantified with the ChromImage, using the 254-nm source.

The limits of detection (LOD) and quantification (LOQ) of caffeine were determined by spotting decreasing amounts of standard solution until the zone could no longer be detected by eye under 254-nm light in a Camag UV cabinet (LOD) or by use of the densitometer (LOQ).

Determination of Caffeine in a Beverage

The same volumes of the same caffeine standard solution were applied to a prewashed Adamant UV_{254} HPTLC plate by use of the Linomat, with four replicate 4.00-μL volumes of cola beverage that had previously been decarbonated by sonication (tracks 4–11). The initial standard and sample zones were placed further apart (6 mm instead of 4 mm) and the rate of application was reduced to 15 s μL^{-1} for the beverage. After application, the plate was dried for 5 min at 100°C on a Camag plate heater. The plate was predeveloped with methanol to a position 1 cm above the origin, dried for 2–3 min at 100°C on the plate heater, then developed to a

distance of 6 cm with ethyl acetate–methanol, 19:1, in a saturated twin-trough chamber. The plate was dried and then scanned and quantified with the ChromImage, using the 254 nm UV source.

Operation of the ChromImage Scanner and the Galaxie-TLC Software

The procedures described below are adapted and amplified from the *TLC Plug In User's Guide* received with the ChromImage and the *Help* section of the Galaxie software; these procedures were used throughout this research.

Scanning a Plate

The plate was placed face down on the scanner to fit the standard positioning cover (covers of three standard plate sizes were supplied with the instrument – 100 × 100, 100 × 200, and 200 × 200 mm^2). The knob at the back of the scanner was switched to either UVC (for ultraviolet C light, 254 nm) or VIS (for visible light) depending on the mode of scanning required for the plate. In the *Plug-ins* menu of the main Galaxie window, *TLC* was selected. In the pop-up window, the *Acquire* menu was selected from the *File* menu, and in the next screen acquisition conditions, for example wavelength, sample number, deposit front distance, etc., were entered. When all fields were filled, *OK* was selected to proceed to the *Twain Acquisition* window and *Acquire* was selected to initiate scanning. After the plate had been saved in the Galaxie database, as an .IEV file, *Export To Diamir* was selected from the *Picture Processing* menu as either *Automatic* or *Manual Selection*. *Automatic Selection* was selected for images of Linomat-spotted, unchanneled plates. When the lanes were defined the *Send* button was selected, then *OK* on the *Information* window that pops up listing all chromatograms created from the plate.

Chromatogram Processing

In the main Galaxie window, the *Open* tab was selected to open the chromatogram wanted. In the *Data* menu, the *Chromatogram Properties* menu was selected, then the *Variables* tab, and the solvent-front distance in centimeters was entered in the *DISTANCE_SOLVANT* [sic] field. The *Integrate* button at the top of the main window was selected to obtain the retardation factors for all peaks automatically defined by the software. These retardation factors would not be correct, however, because, by default, the migration distance of a zone from the bottom of the plate (instead of from the origin) is divided by the solvent front distance. To

obtain the correct R_F values the formula for the retardation factor must be changed manually every time a new chromatogram is being processed. This was done by selecting *Results* in the *Data* menu and in the table under the chromatogram right-clicking on the *Retardation_f* column. *Edit Variable RETARDATION FACTOR* was selected in the pop-up menu. In the *Edit Only RETARDATION FACTOR* window, the default formula for the retardation factor is RT/DISTANCE_SOLVANT, where RT is the retention time displayed on the x-axis.

[*Note*: Because Galaxie-TLC was adapted from software conceived for column liquid and gas chromatography, the name of the x-axis was apparently not changed from retention time, as in column chromatography, to migration distance, as is usual in TLC densitograms, even though the numbers on the x-axis correspond to centimeters on the plate. Thus, RT is the distance (cm) the zone traveled from the bottom of the plate (in the table of peak data below each chromatogram, these distances are also listed in centimeters, although the unit given in the table is mm).]

The formula was edited by subtracting the distance of the origin from the bottom of the plate (1.0 cm in this research) from the RT: (RT − 1.0)/DISTANCE_SOLVANT. *Apply* and *OK* were selected to close the window, then *Integrate* in the main Galaxie window to obtain the recalculated R_F values.

Peaks were then defined/deleted. Each unwanted peak was deleted by right-clicking on the peak and selecting *Peak*/*Delete*. If automatic integration had not defined the peaks of interest correctly, the peaks were redefined manually by selecting the *Move Marker Mode* icon and clicking and dragging the *Start*/*Stop Peak* and *Start*/*Stop Baseline* markers. When all peaks of interest had been defined, *Reprocess* was selected from the *Processing* menu to reprocess the chromatogram and save the changes. Before reprocessing, *Integration* in the *Options* tab was deselected, because otherwise all deleted peaks reappeared after reprocessing. This procedure was repeated for all the chromatograms on a plate.

Creating a Calibration Method

In the main Galaxie window, all the chromatograms were opened. For one of the chromatograms of the standards, *Calibration* in the *Method* menu was selected. The calibration method was configured by selecting *External Standard* as the calibration type and *Area* as the *Response*. *ug* was typed in as the *Standard Unit* and *Level number* was set to the number of standard aliquots (e.g. 4). *Initialize from ID tables* was selected to

import the defined peaks and enter the weights of the standards. After the calibration method had been configured, the method was saved under the name of the plate.

Calibration, and Interpolation of the Weights of the Unknowns

For each chromatogram, *Reprocess* was selected from the *Processing* menu. Under *Method File* the name of the calibration method was entered. For chromatograms of standards, the level of the standard (e.g. *Standard Level 1*) was selected under *Calibration*/*Level*. *Integration* in the *Options* tab was deselected and the chromatogram was reprocessed. For chromatograms of the unknowns, *Unknown* was left as the calibration level to obtain the weight of the sample after reprocessing. By default, the weights of the unknowns were interpolated from the first-order polynomial calibration curve that was forced through zero. To view the calibration curve and change the settings, the *Calibration* tab was selected in the lower left corner of the screen, *Open*/*Open Calibration Curve* was selected from the *File* menu, and the calibration curve was chosen by selecting the calibration method name. When the calibration curve file is opened the regression coefficient and the equation can be viewed under *Results*. Below the *Results* section, the *Polynomial Order* was set to 2 and the *Force Through (0,0)* option was deselected. In the *Calibration Points* table it was possible to uncheck the points in the *Used* column to exclude the points not wanted in the calibration curve. The regression coefficient and the equation were recalculated automatically. The calibration curve was saved and the reprocessing procedure was followed for the chromatograms of the unknowns to interpolate the weights from the saved calibration plot.

RESULTS

Recovery of Rhodamine B Standard

Figure 1 shows a densitogram obtained from the test dye mixture in the visible mode. The calibration plot (peak area against amount (μg) applied) for S1 was 0.999 using polynomial (second order) regression. The theoretical weights in the duplicate aliquots of S2 were 0.750 and 1.25 μg for 3.00 and 5.00 μL samples, respectively, and the mean recoveries after interpolation of the experimental weights were, respectively, 102 and 105% of the amount taken. These values were within the acceptance criteria for TLC procedures of the International Conference on Harmonization (ICH)

for accuracy of assay, content uniformity, and dissolution testing in pharmaceutical analysis [5]. They are also comparable with results for recovery of parthenolide standard by visible-mode slit-scanning densitometry reported elsewhere (97.3–106%) [4]. The differences between interpolated weights of duplicate aliquots of S2 were both 0%, because identical weights were obtained; this is indicative of good analytical precision.

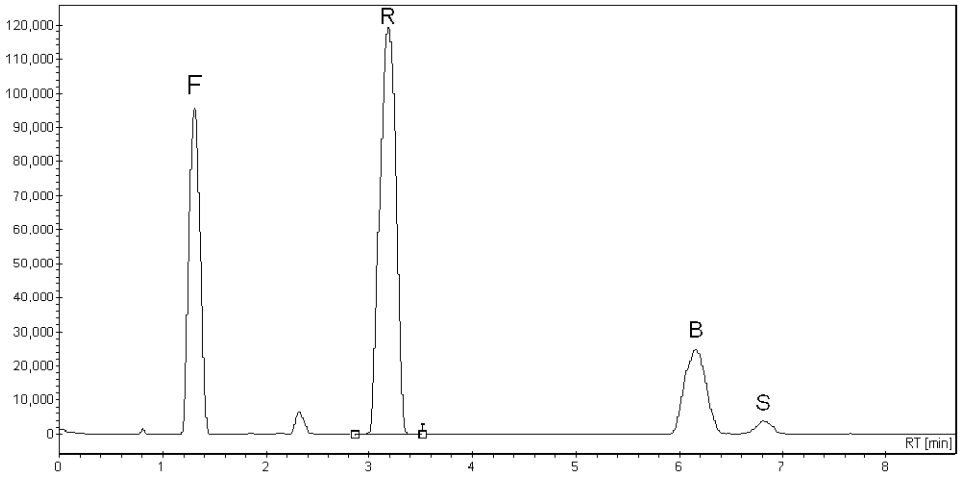

Fig. 1

Densitogram produced by the Galaxie-TLC software after separation of the dyes in Test Dye Mixture 1: F, fast green FCF (R_F 0.08); R, rhodamine B (0.33); B, Bismark brown Y (0.70); S, Sudan IV (0.78). A small impurity peak is seen between F and R. 4.00 μL of a 1.00 mg mL^{-1} solution was spotted for illustrative purposes; the solution spotted for quantification was more dilute, and the top two zones were too light to be photographed

To further assess precision, 6.00 μL S1 (1.05 μg) was spotted on each of tracks 5–13. The relative standard deviation (RSD) of the zone areas measured by the Galaxie-TLC software was 1.45%.

Determination of Caffeine in Analgesic Tablets

The calibration plot for caffeine had an *r*-value of 0.999 (second-order regression). Mean recovery of caffeine from the tablet was 100% of the theoretical (label) value, and the difference between interpolated weights in duplicate sample aliquots was 0.512%. When 4.00 μL volumes of standard solution (0.400 μg) were spotted on 15 of the 18 tracks available across the plate (tracks 3–17; Fig. 2), the RSD of the peak areas after de-

velopment was 1.66%. The LOD was found to be 0.100 μg and the LOQ 0.150 μg. These results are indicative of good linearity, accuracy, precision, and sensitivity for assay of caffeine in the tablet by use of the ChromImage.

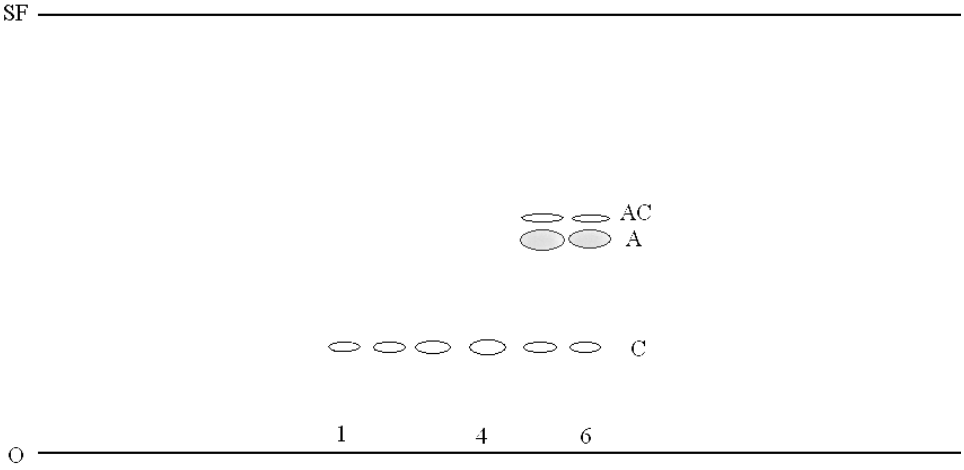

Fig. 2

Chromatograms produced by the ChromImage and used for analysis of caffeine in an analgesic tablet. SF, solvent front; O, origin; C, caffeine (R_F 0.27); A, acetaminophen (0.50); AC, acetylsalicylic acid (0.55). Lanes 1–4, 2.00–8.00 μL standard solution; lanes 5 and 6, duplicate 6.00-μL aliquots of sample solution. The zones are outlined because at the levels used for quantification they would not be readily visible after the plate image is reduced for publication

Determination of Caffeine in a Beverage

After predevelopment and analytical development as described in the experimental section, the R_F value of caffeine was 0.34; other zones were seen in the chromatograms at R_F 0.14 and 0.56, because other ingredients in the beverage absorb UV light and quench the fluorescence of the UV indicator. The *r*-value of the calibration plot was again 0.999 for second-order regression (Fig. 3). The mean weight of caffeine interpolated from the calibration plot for the four 4.00-μL volumes of cola was 0.399 μg, representing recovery of 98.8% relative to the value reported in the literature (36.0 mg per 12 ounces) [6]. The RSD of the four sample weights interpolated from the calibration plot was 2.50%. These data are

again indicative of good accuracy and precision for use of the ChromImage in the 254 nm UV mode.

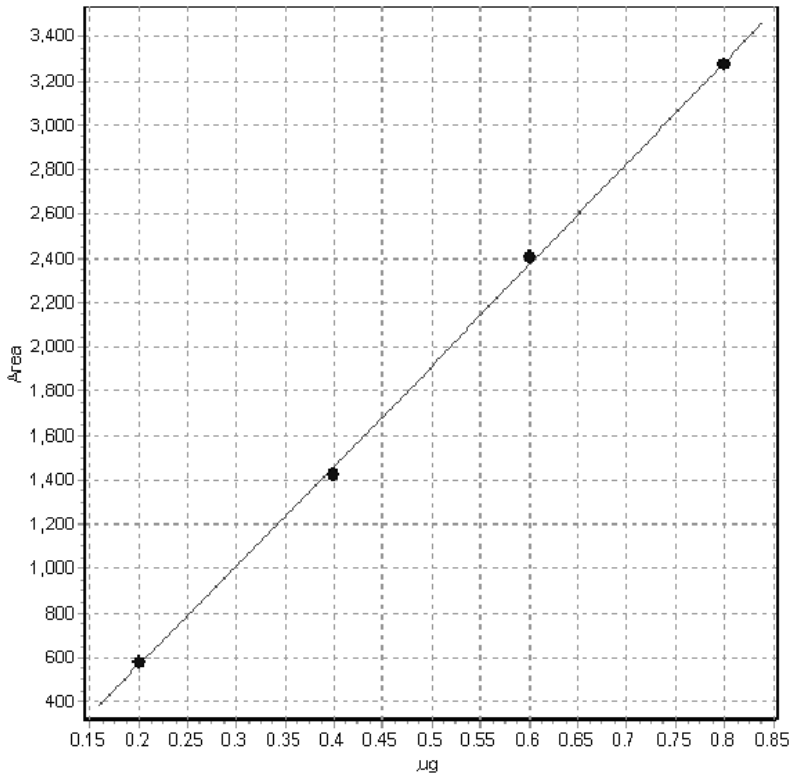

Fig. 3

Calibration plot for caffeine produced by the Galaxie-TLC software. The second-order regression equation was $y = 115.85x^2 + 4419.10x - 324.87$

DISCUSSION

In addition to the flatbed scanner used in this research, other types of densitometer are commercially available, including slit scanning densitometers, photodiode-array detectors, video cameras (CCD), and digital cameras [7]. Most quantitative TLC analysis reported in the literature has involved use of slit scanning densitometers which enable each lane to be scanned with monochromatic light of the optimum wavelength and spectra of separated zones to be acquired; these are regarded as providing the highest quality quantitative results. The analytical and validation results

obtained with the ChromImage in this study, however, compare favorably with slit scanner results for recovery of a standard in the visible mode [4] and with UV-mode determination of caffeine in the same analgesic tablets [2]. The results for caffeine in the beverage were superior to those obtained by use of an early double-beam fiber-optic densitometer (Kontes, Vineland, NJ, USA) [3]. All accuracy (comparison with known values), precision (repeatability), linearity, and sensitivity values obtained with the ChromImage for the visible and UV-mode analyses were consistent with recognized validation guidelines for pharmaceutical analysis by TLC.

The Galaxie-TLC software gives densitograms with relative peak height on the y-axis and R_T (retention time, min) on the x-axis (Fig. 1). Peak areas rather than heights were used in all the analysis reported in this paper. Densitograms of fluorescence-quenched zones were less smooth than that shown for colored zones in Fig. 1, but they were adequate for quantification using the manual integration approach described above.

Analysis was performed using the external standard method. First-order (linear) and second-order (polynomial) calibration were compared, and second-order without forcing through 0.0 resulted in superior accuracy and precision in all our analyses.

According to the manufacturer, the Adamant plate contains an enhanced UV indicator, a new and improved binding system, and a harder, abrasion-resistant layer. It was chosen for maximum brightness when illuminated by the 254 nm ChromImage source.

Caffeine was determined in the beverage without the need for sample extraction or cleanup. We found that direct application of the aqueous beverage with the Linomat was successful if an application rate of 15 s μL^{-1} was used, rather than the 4 s μL^{-1} rate normally used for standards and samples dissolved in organic solvents such as ethanol or methanol. It was found that up to 15 µL of the beverage can be applied as a compact band if this is necessary for the amount of caffeine to be bracketed within the calibration plot. There have been no previous descriptions in the literature of the conditions needed for direct application of beverage samples using the Linomat. Predevelopment for 1 cm with methanol served to further concentrate the initial zones obtained from 4.00 µL beverage before analytical development. It is likely that Linomat application to a plate with a concentration (preadsorbent) zone would obviate the need for the focusing predevelopment.

In summary, we have shown that the new ChromImage scanner densitometer gave good quantitative results in the visible and fluorescen-

ce-quenching modes. We have also modified previously-reported analyses for assay of an analgesic tablet and a beverage by use of a plate with enhanced UV indicator and use of automated spotting.

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