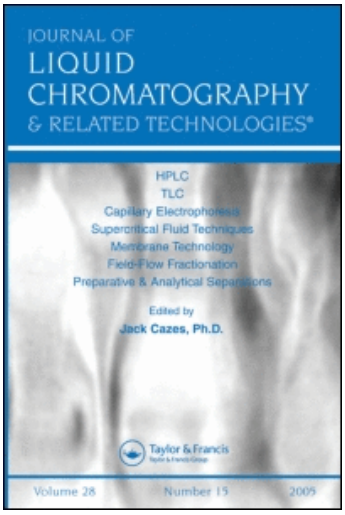

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Comparative Evaluation of TLC and HPTLC Plates Containing Standard and Enhanced UV Indicators for Efficiency, Resolution, Detection, and Densitometric Quantification Using Fluorescence Quenching

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ABSTRACT

Thin layer chromatography (TLC) and high performance TLC (HPTLC) commercially precoated silica gel plates, with enhanced brightness ultraviolet (UV)-indicator, were compared with comparable plates formulated with standard indicators. Caffeine, acetaminophen, and salicylamide were used as the model test compounds. Results showed that the increased UV-indicator plates had visually brighter backgrounds, however, the limits of detection (LOD) did not improve. Differences among the plates

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in terms of efficiency, resolution, and sensitivity and linearity of quantification with slit-scanning densitometry are also reported.

Key Words: Thin layer chromatography; Densitometry; Fluorescence quenching; Adamant plates; LuxPlates.

INTRODUCTION

Fluorescence-quenching is a widely used method of detection for compounds that absorb ultraviolet (UV) radiation, e.g., compounds containing conjugated π-electron systems including all aromatic compounds. Thin layer chromatography (TLC) and high performance TLC (HPTLC) plates containing a so-called UV-indicator (a phosphor such as manganese-activated zinc silicate) mixed with the sorbent emit a yellow-green fluorescence when irradiated with 254 nm light, and UV absorbing compounds become visible as dark zones on the bright emitting background. These are termed F_{254}-layers by some manufacturers. Two companies (Macherey-Nagel and Merck) now offer silica gel layers containing UV-indicators having increased brightness, with the claim that lower levels of detection are possible.

In previous publications, the development and validation of methods for quantitative TLC determination of caffeine as an ingredient or additive in beverages,[1] and for HPTLC determination in pharmaceutical alertness tablets[2] and extra strength analgesic tablets containing also acetaminophen and aspirin[3] by use of densitometry of fluorescence-quenched zones were reported. Because of our earlier experience with caffeine, this compound was chosen as the major model compound for comparing the bright plates with corresponding plates containing standard UV-indicator. Factors compared for the different plates were efficiency (number of theoretical plates, *N*), resolution (*R*), detection (limit of visual detection), and quantification (linearity and sensitivity of quantification with a slit-scanning densitometer).

EXPERIMENTAL

Layers

The following four precoated commercial plates were tested: two Merck (EMD Chemicals, Inc., Gibbstown, NJ, an affiliate of Merck KGaA, Darmstadt, Germany) 20 cm × 20 cm plates with 0.25 mm layers: silica gel 60

F_{254} TLC, catalog no. 5715-7; LuxPlate silica gel 60 F_{254} TLC, catalog no. 5805/1 (increased brightness); two Macherey-Nagel (Duren, Germany) 10 cm × 20 cm HPTLC plates with 0.2 mm layers: Nano-Sil-20 UV_{254}, catalog no. 811 023; Nano-ADAMANT UV_{254}, catalog no. 821 120 (increased brightness).

Preparation of Solutions

A caffeine (#C-0750, Sigma, St Louis, MO) stock standard solution (10.0 mg mL^{-1}) was prepared in methanol. Magnetic stirring for 30 min was required to complete dissolution. Three caffeine TLC standards were prepared by 1 : 10 dilution of the stock solution with methanol to a concentration of 1.00 mg mL^{-1}, 1 : 100 dilution to a concentration of 0.100 mg mL^{-1}, and 1 : 1000 dilution to a concentration of 0.0100 mg mL^{-1}.

An acetaminophen (#A-7085, Sigma) stock standard solution (1.00 mg mL^{-1}) was prepared in methanol. Two acetaminophen TLC standards were prepared by 1 : 50 dilution of the stock solution with methanol to a concentration of 0.0200 mg mL^{-1} and 1 : 10 dilution of that solution to a concentration of 0.00200 mg mL^{-1}.

A salicylamide (#S-0750, Sigma) TLC standard solution (1.00 mg mL^{-1}) was prepared in absolute ethanol. Magnetic stirring for 1 hr was required to complete dissolution. A second TLC standard was prepared by 1 : 10 dilution with absolute ethanol to a concentration of 0.100 mg mL^{-1}.

A stock standard of an extra strength analgesic tablet with label specifications of 65 mg caffeine, 250 mg acetaminophen, and 250 mg acetylsalicylic acid was prepared by grinding to a fine powder using a mortar and pestle, and completely transferring into a 100-mL volumetric flask by washing with about 70 mL of methanol. The solution was stirred magnetically for 30 min. The solution was diluted to the line with methanol and mixed by shaking, and then it was allowed to stand for 1 hr so the undissolved excipients settled to the bottom of the flask. The caffeine sample TLC test solution was prepared by mixing 100 μL of caffeine sample stock solution with 900 μL of methanol, measured with 100 μL and 1000 μL digital microdispensers (Drummond, Broomall, PA). The theoretical concentration of the caffeine test solution was 0.0650 mg mL^{-1} based on the label value.

Thin Layer Chromatography

Initial zones of 2.00, 4.00, 6.00, 8.00, and 10.0 μL of the three caffeine, two acetaminophen, and two salicylamide TLC standard solutions were

applied with a Camag (Wilmington, NC) Linomat IV automated spray-on band applicator having a 100 μL syringe and operated with the following settings: band length 6 mm, application rate 4 sec μL^{-1}, table speed 10 mm sec^{-1}, distance between bands 4 mm, distance from the plate edge 7 mm, and distance from the bottom of the plate 1.0 cm. The weights of these standard zones ranged from 0.0200 to 10.0 μg for caffeine and salicylamide and 0.00400 to 0.200 μg for acetaminophen.

The TLC plates were developed 12 cm beyond the origin and the HPTLC plates 6 cm beyond the origin with the mobile phase ethyl acetate–glacial acetic acid (95 : 5) for caffeine and acetaminophen, or methylene chloride–acetone (4 : 1) for salicylamide, in Camag twin-trough chambers containing a saturation pad (Analtech, Newark, DE). The development time was 15 min for the HPTLC plates and 25 min for the TLC plates. The migration sequence of the four compounds studied was the same on all plates, with R_F values ranging from 0.13–0.21 for caffeine, 0.40–0.55 for acetaminophen, 0.41–0.53 for salicylamide, and 0.57–0.78 for acetylsalicylic acid.

After development, the plates were dried in a fumehood on a TLC plate heater (Camag) for 5 min. The sample and standard zones were then quantified by use of a Camag TLC Scanner II with the deuterium source set at 254 nm, slit length 4, slit width 4, and scanning rate 4.0 mm sec^{-1}. The CATS-3 software controlling the densitometer produced a linear regression calibration curve relating the standard zone weights to their scan areas for each plate.

Evaluation Parameters and Calculations

The range of weights for caffeine and acetaminophen on each of the four plates required to generate a linear calibration curve with a correlation coefficient (*r*-value) equal to, or greater than, 0.99 was determined. This is the linearity requirement according to the guidelines of the International Conference on Harmonization (ICH).[4] To evaluate the limit of detection (LOD) on each plate, caffeine and acetaminophen zones were applied and developed in decreasing quantities, and the smallest visible weight was determined. The sensitivity of quantification (SOQ) was determined as the scan area obtained for the lowest weight on the linear calibration curve.

N and *R* values for caffeine were determined from direct measurements of the tablet sample solution zones on the layer, with a millimeter ruler. The tablet solution was applied in 2.00 μL aliquots to the HPTLC plates and 6.00 μL to the TLC plates, which yielded acetaminophen zones containing the lowest detectable weight. The upper acetylsalicylic acid and acetaminophen zones were used to obtain *R* data, and *N* was calculated from the caffeine

zone. The following equations, specified by Kowalska et al.,[5] were used to calculate N and R, respectively, from direct measurements of the zones on the layers:

$$N = \frac{16 \times l \times z}{w^2}$$

$$R = \frac{z_{ac} - z_a}{0.5(w_{ac} + w_a)}$$

where l is the migration distance from the origin to the mobile phase front, z is the migration distance from the origin to the center of the solute zone; w is the chromatographic zone width in the direction of mobile phase migration, and ac and a denote the acetylsalicylic acid and acetaminophen zones, respectively.

RESULTS

Results for the caffeine standard solution and the tablet solution on the four plates studied are summarized in Table 1. All data are average values for duplicate experiments that agreed closely.

Comparison of the Macherey-Nagel HPTLC Plates

Although, the Adamant plate background was visually brighter than the standard UV-indicator plate when viewed under 254 nm light in a UV cabinet, the LOD values and linear range for caffeine were the same on both plates. However, the SOQ was more favorable on the standard UV-indicator

Table 1. Results for caffeine on the Macherey-Nagel (MN) and Merck plates.

Plate	LOD (μg)	SOQ [area $(0.20\,\mu g)^{-1}$]	Range of linearity (μg)	N	R
MN Adamant HPTLC	0.20	353	0.20–1.0	4,331	2.6
MN standard HPTLC	0.20	484	0.20–1.0	8,880	6.3
Merck Lux TLC	0.20	466	0.20–1.0	4,050	6.9
Merck standard TLC	0.20	428	0.20–1.0	5,902	7.9

Macherey-Nagel plate. Also, the *N* value for the caffeine zone in the sample chromatograms and the *R* values for the acetylsalicylic acid and acetaminophen zones were better on the standard UV-indicator Macherey-Nagel plate.

Caffeine zones below the LOD could be measured on both the Adamant and standard plates. The lowest measurable zone weight was 0.060 μg on the Adamant plate and 0.040 μg on the standard plate, and these zones gave respective scan areas of 31 and 18 counts. The zones below the LOD did not fit on the linear calibration curve, but gave a 0.99 polynomial fit in the range of 0.060–1.0 μg for the Adamant plate and 0.040–0.80 μg for the standard plate.

One definition of a method's sensitivity is the slope of the calibration curve for the analyte. Calculation of the slopes (Δ area/Δ weight) of the linear plots for caffeine gave respective values of 1709 for the Adamant plate and 410 for the standard plate.

Comparison of the Merck TLC Plates

The Lux plate background was significantly brighter compared to the Merck standard plate when viewed under UV light, but the LOD and linearity values were identical. The SOQ was superior on the Lux plate, but the *N* and *R* values were higher on the standard plate (see Table 1).

Comparison of the Macherey-Nagel HPTLC and Merck TLC Plates

The Merck plates showed overall better *R* values and the Macherey-Nagel plates better *N* values, when tablet sample aliquots that resulted in acetaminophen zones with weights representing the LOD on each plate were applied. These volumes were 2.00 μL for the Macherey-Nagel plates and 6.00 μL for the Merck plates. The SOQ values were higher on the Merck plates.

DISCUSSION

Advertisements by Merck for their Lux plates layers claim they are "twice as bright when compared to conventional TLC plates" and that the benefit is "easy identification of the separated zones thanks to the higher content of fluorescent indicator." Macherey-Nagel writes that their Adamant plates feature "an increased brilliance, which in combination with the low-noise background, results in increased detection sensitivity." The purpose of this

study was to compare these new bright plates with the standard UV-indicator equivalent plates manufactured by each company, and with each other.

Although, the Adamant and Lux plates had an obviously brighter green fluorescence when viewed under 254 nm UV light, the visual LOD was not improved compared with plates with standard indicator (Table 1). This result for caffeine was confirmed by performing the same LOD study with acetaminophen, which has a structure that results in stronger UV absorption than caffeine, and salicylamide, which absorbs less strongly than caffeine. The LOD values were 0.080 μg for acetaminophen on the HPTLC plates and 0.12 μg on the TLC plates. As expected, detection limits for salicylamide were higher than for caffeine and acetaminophen, with LOD values of 0.60 μg on the Adamant plates and 2.0 μg on the Lux plates. Again, detection was not superior compared with the equivalent standard plates. Slopes of the linear calibration curves for caffeine, as well as for acetaminophen and salicylamide, were greater on the Adamant vs. the Macherey-Nagel standard plate.

The SOQ, which we define as the scan area of the lowest zones on the calibration curve, was increased for the Merck bright plate, but not for the Macherey-Nagel bright plate, vs. their standard counterparts. Pure solvent zones were applied with each series of standard zones used to generate calibration curves, but none of these blanks gave any scan areas. Therefore, noise was zero and the usual 10 : 1 signal (analyte area) : noise (blank area) ratio definition[4] could not be used to evaluate the quantification limit.

Linearity was defined as the range of weights, starting with the LOD, which gave a linear regression correlation coefficient of 0.99 or more. The linear range for caffeine was 0.20–1.0 μg on all plates. On some of the plates, (invisible) zones with weights below the LOD could be scanned, but including the scan areas of these zones did not yield linear calibration curves with the required 0.99 *r*-value for caffeine. As described above, caffeine zones below the LOD yielded an *r*-value of 0.99 with polynomial regression. Studies with acetaminophen showed that zones (down to 0.020 μg on the Macherey-Nagel HPTLC plates and 0.080 μg on the Merck TLC plates, both below the LOD values on these plates) gave calibration curves with linear correlation coefficients of 0.99.

It may be intuitively expected that the HPTLC plates studied would give higher *N* and *R* values than the TLC plates, because smaller-particle silica gel should be more efficient. However, Table 1 shows that the standard Merck plate had the second highest *N* value for caffeine and the highest *R* value. *N* values calculated for acetaminophen zones were higher on the Lux TLC and Merck standard plates (4956 and 4551, respectively) than on the Adamant HPTLC and standard Macherey-Nagel HPTLC plates (2884 and 3660, respectively). Higher *N* and *R* values on the TLC vs. HPTLC plates are apparently due to the way the experiments were run and the calculations

made, i.e., the z-values (zone migration distance, used for calculation of N) and Δz-values (zone separation distance, used for calculation of R) increased at a greater rate than the w-values for the longer mobile phase development distances (l-value) used for the TLC compared to HPTLC (see Fig. 1). The Adamant plate had a lower R value and lower N value for both caffeine and acetaminophen than its standard equivalent, and the Lux plate had lower R and N for caffeine, but higher N for acetaminophen.

N and R values can be calculated from measurements of the peaks from densitometric scanning, instead of the zones directly. The maximum of the scan peak was used as the center of the zone, and the zone width was measured as the width of the scan peak at the baseline. Measurements of the densitograms exaggerated the extents of the leading and trailing edges of the zones

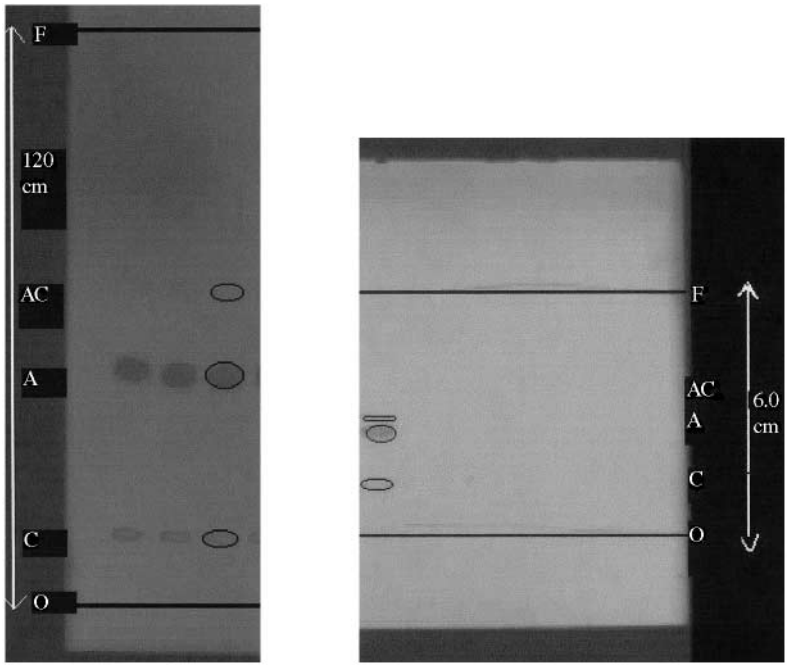

Figure 1. Chromatograms demonstrating the higher N and R values obtained on Merck standard TLC (left) compared to Adamant HPTLC (right) plates. A solution of the three-component analgesic tablets was spotted in 6 and 2 μL aliquots, respectively. AC, acetylsalicylic acid; A, acetaminophen; C, caffeine; F, mobile phase front; O, origin. The dark zones on the bright green fluorescent layer background were photographed under 254 nm UV light with a Camag VideoStore image documentation system, and the zones were outlined to be easily visible.

at the baseline compared with measurements of zone widths directly on the layer, but comparable zone-center measurements were obtained. This led to lower calculated *N* and *R* values, but the trends among the plates were the same. For example, the Adamant plate had respective *N* and *R* values of 472 and 1.6 and the MN standard plate 1210 and 4.7, when sample chromatograms scanned using a Shimadzu CS-930 densitometer in the reflectance mode with the deuterium source lamp set at 254 nm were measured. These values are lower but in the same order as the values in Table 1.

In TLC and HPTLC, the layer, mobile phase, and detection method are usually selected by controlled trial-and-error, based on previous experience or literature searching for the results of previous, similar separations. Although, we studied only a limited number of compounds, it is hoped that the data will be useful to help decide whether bright or standard TLC or HPTLC plates might be suitable for evaluation for a particular analytical application involving fluorescence quench detection. For example, for the densitometric assay of pharmaceutical preparations containing caffeine, acetaminophen, acetylsalicylic acid, and/or salicylamide, the Macherey-Nagel standard HPTLC plate would probably be the best among the four plates studied, because it has the highest *N* value and best SOQ compared with the other three plates, as well as a high *R* value and fast development time.

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