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Journal of Chromatography A, 771 (1997) 251–257

JOURNAL OF
CHROMATOGRAPHY A

Application of a colour analyzer in quantitative thin-layer chromatography

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Received 11 November 1996; accepted 7 January 1997

Abstract

Validation of quantitative TLC analysis of test dye samples using a Datacolor 3890 colour analyzer was performed. Chromatograms were recorded in the visible region and the results given were on-plate diffuse reflectance spectra of the chromatographic spot, its lightness, chromaticity and hue. The results showed low total R.S.D. for the determination of reflectance and lightness of the chromatographic spots (0.1–2.8% for low concentration level) with negligible instrumental error (0.01–0.04%). The applicability of the proposed method was tested with the quantitative TLC analysis of diffuse and distorted spots of amino acids obtained on non-homogeneous laboratory-prepared plates. Validation of the results has been made by comparison with image analysis and slit-scanning densitometry. The best results (the lowest R.S.D., detection limit and limit of quantification) were obtained by measuring reflectance with the Datacolor system. Densitometry and image analysis resulted in a lower precision (R.S.D.>10%) and significant instrumental error.

Keywords: Detection, TLC; Dyes; Amino acids

1. Introduction

Modern TLC has introduced sophisticated mechanical scanning densitometers in daily laboratory practice. Chromatograms are recorded in transmission or reflectance mode in the UV or Vis region or by fluorescence [1,2]. One of the disadvantages of conventional slit-scanning densitometry is difficult analysis of two-dimensional chromatograms and irregular or diffuse spots. Such irregular spots can appear near the second solvent front or when the analyte concentration is very low and large sample volumes are applied to the chromatographic plate. New techniques such as video densitometry or electronic scanning cannot compete with conventional transmission scanners in terms of cost, sensitivity and response range. Colour analyzers, originally designed for textile industry, offer new possibilities for quantitative TLC. Modern colorimetric systems used in the textile industry enable an intervention in production process such as making a recipe with brightening agents and shading with dyestuffs on a cotton fabric based on spectral analysis of colour [3,4]. One of the colour analyzers developed for this purpose is the Datacolor DC 3890 (Datacolor, Switzerland).

The aim of this work was to examine the possible application of the colour analyzer Datacolor DC

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PII S0021-9673(97)00036-8

3890 in laboratory practice for the determination of chromatographic spots in the visible range. The objectives were: (i) to examine the precision of the colour analyzer in quantitative chromatographic determination of coloured spots within the monitoring range (390–700 nm) and (ii) to test the method with the quantitative TLC analysis of diffuse and distorted spots.

For this purpose two chromatographic systems were used. The precision and accuracy of the proposed instrumental method were checked on test dye samples chromatographed on pre-coated silica gel 60 F_{254} TLC plates. As a model system for the determination of limiting factors in the quantitative analysis of diffuse spots, amino acids' samples were chromatographed on laboratory-prepared plates that gave well separated, but diffuse and irregular spots. The validation of the results obtained has been made by comparison with slit-scanning densitometry and image analysis.

2. Experimental

2.1. Chromatographic plates

(a) Pre-coated silica gel 60 F_{254} TLC plates (20×20 cm, 0.25 mm thick) were supplied by Merck (Darmstadt, Germany).

(b) Laboratory-prepared plates; A mixture (1:1, w/w) of microcrystalline cellulose (Merck) and natural tuff, mainly consisting of zeolite–clinoptilolite, was suspended in water, homogenized with an electric stirrer and spread on glass plates (20×20 cm) with a Camag applicator. The tuff was previously sieved and the fraction with particle size <40 μm was used for the layer preparation. The thickness of the wet layer was 300 μm.

2.2. Standard solutions

The dye samples Echtsaure gelb 3G, Ceres rot G and Lanasynrein rot were obtained from Bayer (Germany) and bromothymol blue and Sudan blue II from Macherey–Nagel (Düren, Germany), all the best available quality. Standard solutions of Ceres rot G, Lansynrein rot, Bromothymol blue and Sudan blue II were prepared by dissolving accurate amounts of powdered samples in ethanol. A standard solution of Echtsaure gelb 3G was prepared by dissolving the sample in water. These solutions were suitably diluted to give a mass concentration in the range of 10–500 μg/ml.

A mixed standard solution of α-alanine, glycine, leucine and valine was prepared by dissolving powdered amino acids (Merck) in ethanol–water (1:1, v/v). The solution was diluted to give the mass concentration of each compound in the range 5–500 μg/ml.

2.3. Chromatographic conditions

2.3.1. Test dyes

A 10-μl syringe (SGE, Australia) was used for sample spotting. Samples were applied as spots with an approximate diameter of 5 mm. The chromatograms were developed by ascending technique with previous chamber saturation to a distance of 10 cm in the solvent systems listed in Table 1. After development the plates were air dried.

2.3.2. Amino acids

The chromatograms were developed in phenol

Table 1
Solvent systems used

Dye sample	Solvent system	Volume ratio of components (v/v)
Echtsaure gelb 3G	2-Propanol–ethanol–water	30:20:10
Bromothymol blue	Benzene–ethanol	20:10
Ceres rot G	Benzen–acetone	100:1.5
Lanasynrein rot	2-Propanol–methanol–water	100:50:4
Sudan blue II	Benzene–acetone	100:1.5

(saturated with water)–ethanol–acetic acid–water (12:4:1:4, v/v). Spots of amino acids were detected by spraying with ninhydrin solution (1% in 1-butanol) and heating at 80°C for 20 min.

2.4. Apparatus

The Datacolor DC 3890 analyzer is a reflectance spectrometer equipped with an IBM-PC XT/AT computer; monitoring range 390–700 nm with 10-nm intervals; screen diameter 12–36 mm, The on-plate analyses of chromatographic spots are made on the basis of diffuse reflectance spectrometry [5,6]. The basic principle is as follows: the mat-surface of the chromatographic plate (chromatographic spot) is illuminated with polychromatic light (xenon lamp). The light reflected under the angle of 8° goes to the double-beam monochromator where it is dispersed into the spectrum and the intensity of each spectral component is measured separately. The results given were on plate reflectance spectra of the chromatographic spots and parameters of spot colour: lightness, chromaticity and hue. Data acquisition and processing were performed using the software FORMULA 2736 (Datacolor-Velebit). The instrument was calibrated with standard materials (100% reflectance was calibrated with $BaSO_4$ plate and 0% reflectance with black velvet). A Camag Turner fluorimeter 111 slit-scanning densitometer (Camag, Muttenz, Switzerland) (slit width, 1 mm; scanning speed, 20 mm/min; filter number, 826; λ=510 nm) and a Leco 2001 image analyzer (Leco, Germany) [equipped with a 486XT computer and high-resolution CCD camera (300 dpi) with zoom; determination of spot area in manual mode] were used.

3. Results

3.1. Quantitative analysis of test dye samples

3.1.1. Choice and optimization of chromatographic system

The quantitative TLC assay by means of the Datacolor DC 3890 is performed on the basis of spectral analysis of spot colour. The dye samples were chosen so that their reflectance minimum can cover the whole range in the Vis region of the spectrum that is also the monitoring range of colour analyzer used.

The main prerequisite of the validation procedure was to obtain approximately equal and round spots without "tail" with an R_F value between 0.4 and 0.6. For this reason each sample was chromatographed on a separate plate with specific developing system that gives such an R_F value. The optimization was carried out by varying the volume ratio of components in the basic solvent systems chosen on the basis of literature data until set demands were not satisfied.

3.1.2. Calibration curves

Each measurement of a chromatographic spot gave an on-plate reflectance spectrum (R, %) and three values that characterize spot colour: lightness (L), chromaticity (C) and hue (H). The lightness (intensity of whiteness) is defined as the intensity of white colour in the spot within the range from 0 (black) to 100 (white). Chromaticity or saturation of colour is the fraction of chromatic component in the colour. These two values are dependent on sample concentration while hue of the colour is the qualitative characteristic and is not dependent on concentration. Therefore these data were not significant from the aspect of quantitative chromatographic study.

The on-plate reflectance spectrum was recorded for all samples. The reflectance minimums of dye samples were as follows: Echtsaure gelb 3G at 420 nm, bromothymol blue at 440 nm, Ceres rot G at 500 nm, Lanasyrein rot at 530 nm and Sudan blue II at 620 nm. The calibration graphs of R, L and C measured at the wavelength of the reflectance minimum against sample concentration were plotted and regression equations and correlation coefficients were calculated. The on-plate reflectance spectrum and calibration graphs of R, L and C of Ceres rot G are given in Fig. 1 and Fig. 2. These results gave non-linear response functions with significant curvature at higher sample concentration. It is important to emphasize that sensitivity (signal/concentration) and operating range of the colour analyzer is dependent on sample type (intensity of its natural colour if it is coloured) or sensitivity of post-chromatographic detection reactions (for nonchromophores).

3.1.3. Validation procedure

The precision of the quantitative analysis was

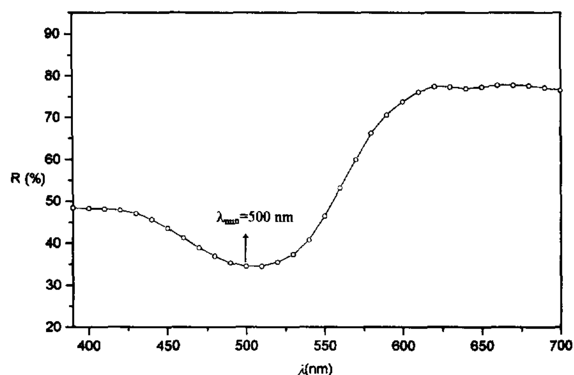

Fig. 1. On-plate reflectance spectrum of Ceres rot G.

checked by applying on the same plate seven loadings of the dye sample at two concentration levels. The low concentration level of each dye was defined as a concentration that gives 75% reflectance at wavelength of minimum reflectance, while the high concentration level was the concentration that gives 35% reflectance. Because of different colour intensity and sensitivity, the absolute concentration of each dye was different. After development and drying, each spot was recorded using the Datacolor 3890 seven times without resetting the screening position and seven times with resetting of the screening position. Relative standard deviation (R.S.D., %) of measurements was calculated. The total error (T) involved in the chromatographic and detection stages was calculated from the results of seven loadings. The error of quantitative measuring (M) was calculated on the basis of seven measurements of the same spot when resetting the screening position after each measurement. This value includes the error of setting the chromatographic spot in the center of the screening area which is largely dependent on operator skill. The instrumental error (I) was obtained by multiple measurements of the same spot without resetting the screening position and changing any experimental variables between measurements. This value represents the minimum error involved in the quantitative procedure and is attributable to the instrumentation system alone. Precision data for the determination of R, L and C for all samples are summarized in Tables 2–4.

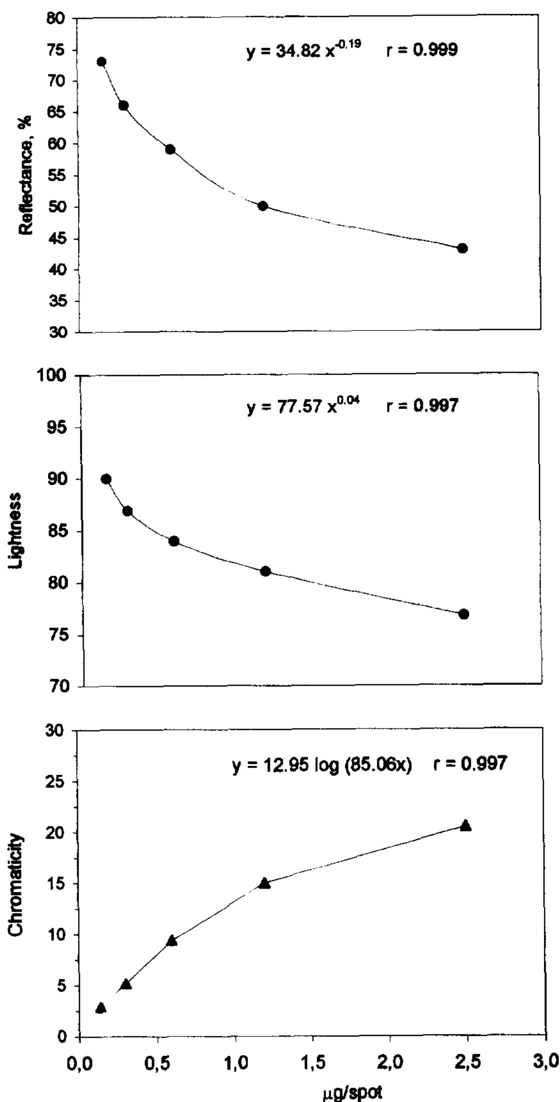

Fig. 2. Calibration curves of reflectance, lightness and chromaticity for Ceres rot G.

3.2. Application to the quantitative analysis of diffuse spots

Using laboratory-prepared layers the satisfactory separation of amino acids α-alanine, glycine, valine and leucine was achieved. The R_F values were as follows: glycine 0.42, α-alanine 0.58, valine 0.71 and leucine 0.85. However, the spots obtained, due

Table 2
Precision data (R.S.D., $n=7$) for the determination of reflectance of test dyes

Dye sample	R.S.D. (%) (low concentration)			R.S.D. (%) (high concentration)		
	T[a]	M[b]	I[c]	T	M	I
Echtsaure gelb 3G	2.79	0.60	0.01	3.70	1.50	0.02
Bromothymol blue	1.25	0.42	0.04	4.40	0.77	0.04
Ceres rot G	1.08	0.66	0.04	5.21	0.66	0.05
Lanasynrein rot	0.62	0.60	0.01	4.35	1.72	0.04
Sudan blue II	1.70	0.41	0.02	4.96	1.27	0.05

[a] T – total error.
[b] M – measuring error.
[c] I – instrumental error.

Table 3
Precision data (R.S.D., $n=7$) for the determination of lightness of test dyes

Dye sample	R.S.D. (%) (low concentration)			R.S.D. (%) (high concentration)		
	T	M	I	T	M	I
Echtsaure gelb 3G	0.14	0.02	0.01	0.43	0.02	0.01
Bromothymol blue	0.24	0.11	0.01	0.69	0.17	0.01
Ceres rot G	0.17	0.11	0.01	1.15	0.15	0.02
Lanasynrein rot	0.23	0.13	0.01	0.89	0.36	<0.01
Sudan blue II	0.51	0.10	<0.01	1.22	0.28	<0.01

to the high porosity and heterogeneity of the prepared layers, were diffuse and distorted. The reddish violet spots of amino acids were recorded in the visible region using a Datacolor reflectance spectrometer, slit-scanning densitometer and image analyzer. The precision, given as total and instrumental error, detection limit (mass of amino acids that gives a response equal to twice of background) and limit of quantification (mass of amino acids that allows the determination with 5% reliability) for each method were determined. The precision of the quantitative analysis was checked by applying on the same plate seven loadings of the mixture at two concentration levels (2.5 μg/spot of each compound and 0.25 μg/spot, respectively). The validation procedure for the colour analyzer was performed as described

Table 4
Precision data (R.S.D., $n=7$) for the determination of chromaticity of test dyes

Dye sample	R.S.D. (%) (low concentration)			R.S.D. (%) (high concentration)		
	T	M	I	T	M	I
Echtsaure gelb 3G	16.16	3.58	0.13	6.92	2.39	0.07
Bromothymol blue	21.37	8.50	0.89	7.35	0.79	0.04
Ceres rot G	13.27	7.83	0.11	7.87	1.09	0.04
Lanasynrein rot	6.51	5.69	0.14	5.47	2.04	0.09
Sudan blue II	10.76	1.45	0.06	4.44	1.17	0.04

Table 5
Quantitative analysis of diffuse spots of α-alanine (comparison of colour analysis, image analysis and densitometry)

		Colour analysis	Image analysis (spot area)	Image analysis (spot intensity)	Densitometry
Detection limit (μg/spot)		0.08	0.20	–	0.20
Limit of quantification (μg/spot)		0.16	1.00	–	0.50
R.S.D. (2.5 μg/spot)	Total	3.07	3.85	3.80	3.53
	Instrumental	<0.01	2.00	0.90	1.53
R.S.D. (0.25 μg/spot)	Total	2.71	10.31	9.45	12.08
	Instrumental	<0.01	7.40	5.90	8.99

above. The on-plate reflectance spectrum of each compound was recorded, the same as the lightness and chromaticity at the wavelength of the reflectance minimum (λ=500 nm for glycine, leucine and valine and λ=510 nm for α-alanine).

Using the image analyzer Leco 2001, the chromatograms were imaged by a charged-coupled video camera. The information obtained was processed in the form of a black and white picture, afterwards, the spot area and spot intensity, defined as the intensity of white colour in the spot within the range from 0 (100% black) to 255 (100% white) were determined. Two sets of experiments were conducted. In one experiment the samples were applied as a narrow band and in the other as a spot.

The same plates were repeatedly scanned (7 times) using a Camag Turner densitometer. The instrumental error was determined by multiple scanning (7×) of a single lane without changing any experimental variables between scans.

The comparison of precision, detection limit and limit of quantification obtained by three instrumental techniques is given in Table 5. Since similar results were obtained for all amino acids tested, the results are summarized for α-alanine.

4. Discussion

The colour analyzer is a powerful tool in analyzing and quantifying analytes that give coloured chromatographic spots. Although many analytes lack convenient chromophores, the use of numerous post chromatographic chemical reactions results in coloured spots that can be easily analyzed. The main characteristics of the Datacolor 3890 colour analyzer and, at the same time, its advantages in quantitative TLC assay are simple instrument design and fast data acquisition. An average chromatogram with 10–15 samples can be quantified in 10 min and the results given are reflectance spectra in the range 390–700 nm with 10-nm steps, lightness, chromaticity and hue of colour at every point of the reflectance spectra. The first step of measuring is the setting of the chromatographic spot in the center of the screening position whose diameter can be changed (max. 36 mm). The screen diameter must be bigger than the largest chromatographic spot on the plate and, in the case of the standard TLC precoated plates that we used, the optimal value of the screen diameter was 12 mm. From the methodological point of view it is important to emphasize that all data (R, L, C and H) refer to the whole field of sight and not only to the chromatographic spot.

Results presented in Tables 2–4 showed that M ranges from 0.4–1.7% for measuring reflectance, 0.02–0.5% for measuring lightness and 0.8–8.5% for measuring chromaticity of chromatographic spots of test dyes. The I value was low in all cases and it ranged from 0.01 to 0.04% for reflectance and lightness and from 0.03 to 1.0% for measuring chromaticity. Total R.S.D. (T) for the determination of reflectance ranged from 0.6–2.7% for low concentration to 3.7–5.2% for high concentration. Such unexpected better precision at low concentration can be explained in terms of smaller spots at low concentration that fit better in the screening position. The lowest total R.S.D. was obtained by measuring lightness of the chromatographic spot: 0.1–0.6% for low concentration and 0.2–1.4% for high concentration. Measuring chromaticity resulted in lower precision, especially at low concentration (R.S.D.

4.0–20.0%), with significant measuring and instrumental error. The difference between the total R.S.D. and measured R.S.D. corresponds to the error involved in the chromatographic stage. This error can be mainly attributed to the reproducibility of sample application while the reproducibility of chromatographic conditions such as heterogeneity of sorbent, deviations in layer thickness and non-linear solvent front was maintained by multiple determination on a single plate. The problem of a coloured and non-uniform layer that was often present after post chromatographic derivatizations, can also be minimized by parallel background colour analysis.

The comparison of quantitative determination of diffuse spots by the proposed technique and widely used slit-scanning densitometry and modern video systems confirms that diffuse and irregular spots are difficult to analyze by these conventional methods. The results obtained by image analysis and densitometry were not satisfactory, especially at low concentrations (total R.S.D.>10%, with a significant instrumental error) while colour analysis resulted in acceptable total (2.7–3.1%) and negligible instrumental error. The lowest limit of quantification and detection limit were also achieved using a colour analyzer (Table 5).

All these features make quantitative TLC analysis, by means of a colour analyzer, accurate and precise, especially in the case of distorted spots and two-dimensional chromatograms which are difficult to scan using slit-scanning densitometers. The main disadvantage is that the method can be applied in laboratory practice only for the determination of coloured chromatographic spots.

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