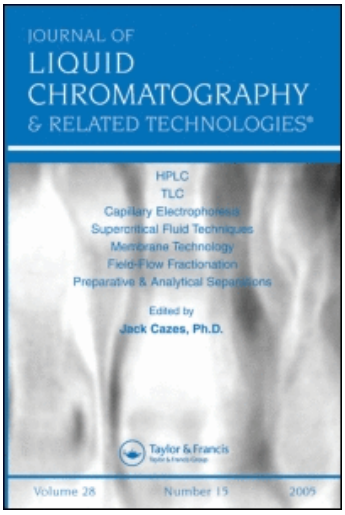

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Parameters Characterizing Chromatographic Spots and Separation Effects Determined by Visual and Densitometric Methods

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Parameters Characterizing Chromatographic Spots and Separation Effects Determined by Visual and Densitometric Methods

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Abstract: Phloroglucinol (P), niclosamide (N), salicylanilide (S) and thymol (T) were chromatographed on silica gel TLC plates with *n*-hexane-acetone in different volume proportions as mobile phases. Comparison and characterization of chromatographic spots of examined compounds on the basis of resolution (R_S), separation factor (α), constant of the pair separation (R_F^{α}), and ΔR_F values were discussed. The R_S parameter, serving to evaluate the substance separation, was determined by visual and densitometric methods. It was proven that the R_S parameter determined by the visual method for two adjacent substances is always larger than that determined by the densitometric method. It was stated that the densitometric method is correct, objective, and assures standard conditions for the parameter R_S determination.

Keywords: Normal phase thin layer chromatography, Densitometry, Spectrodensitometry, Separation parameters, Selected phenol drugs

INTRODUCTION

Separation of a mixture in liquid chromatography depends on the selective retention of the migrating components in the column by the stationary phase. Retention, arising from selective interactions of each component with stationary phase, is effected by simultaneous migration with the

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flowing mobile phase.[1–5] The quality of the obtained chromatographic separation is described by the values of retention parameters (R_F and R_M) and separation parameters (ΔR_F, R_S, α, R_F^{α}) in TLC.[6–12] The separation factor (α), constant of the pair separation (R_F^{α}) and ΔR_F, are calculated on the basis of the R_F values of the separated compounds. Only the resolution (R_S) takes into account the chromatographic spot broadening. Now and again, the R_S values are calculated by use of the visual method of a measurement.[7–9]

The aim of this work was application of the visual and densitomteric method to evaluate:

— the chromatographic band on the basis of peak height [AU], peak area [AU], and the angle (β) between the tangents at the inflection points to the curves of the densitometric peaks;
— the separation of particular substances on the basis of parameters: R_F, R_S, ΔR_F, R_F^{α}, and α.

EXPERIMENTAL

Chemicals and Sample Preparation

The components of the mobile phases: acetone (Chempur, Poland; analytical grade) and *n*-hexane (AnalaR, UK; analytical grade) were used for TLC analysis. The commercial samples of phloroglucinol (LOBA Feinchemie AG, Austria), niclosamide (Sigma-Aldrich, USA), salicylanilide (Sigma-Aldrich, USA), and thymol (Cefarm, Poland) were used as test solutes. The purity of the studied standard samples was at least 99%. The above mentioned compounds (about a concentration of 1 mg mL^{-1} of each standard) were dissolved in ethanol (POCh, Poland; 96%; analytical grade).

Thin Layer Chromatography

Adsorption Thin Layer Chromatography

Adsorption thin-layer chromatography (NP-TLC) was performed on 20 × 20 cm aluminium plates precoated with 0.2 mm layer of a silica gel $60F_{254}$ (E.Merck, #1.05554). The plates were prewashed with methanol and dried for 24 h at room temperature. The plates were then activated at 120°C for 30 min. The mixture solution of the studied drugs (2 μL) was spotted manually using a microcapillary (Camag, Switzerland) on the chromatographic plate. The mixture of investigated compounds was separated using *n*-hexane-acetone in volume compositions 45:5, 40:10, 35:15, 30:20, and 25:25 as mobile phases. The mobile phase (50 mL) was placed in a

classical chamber (Camag, Switzerland) and the chamber was saturated with the mobile phase for 30 min. The plates were developed to a distance of 14 cm at room temperature (18 $\pm$ 1°C). The plates were dried for 24 h at room temperature (18 $\pm$ 1°C) in a fume cupboard.

Visualization of Spots by Use of UV Lamp

The spots on a plate were visualized using a UV lamp (Cobrabid, Poland) at $\lambda = 254$ nm.

Visualization of Spots by Use of a Camag Densitometer

Densitometric scanning was then performed at 254 nm with a Camag Scanner TLC 3 operated in absorbance mode and controlled by winCATS 1.4.1 software. Next, densitometric scanning was then performed at multi wavelength in the range of 200 to 340 nm, at change of wavelength of 35 nm at every step. The radiation source was a deuterium lamp emitting a continuous UV spectrum between 190 and 450 nm. The slit dimensions were 8.00 $\times$ 0.30 mm, Macro; the optimize optical system was light; the scanning speed was 20 mm s^{-1}; the data resolution was 100 μm $step^{-1}$; the measurement type was remission; and the measurement mode was absorption; the optical filter was second order. Each track was scanned three times and a baseline correction (lowest slope) was used.

Spectrodensitometric Analysis

The spectrum was also performed using a Camag Scanner TLC 3. The radiation sources were a deuterium lamp emitting a continuous UV spectrum between 190 and 450 nm. Start wavelength was 200 nm and end wavelength was 450 nm. The slit dimensions were 8.00 $\times$ 0.40 nm, Micro; the optimized optical system was resolution; the scanning speed was 20 nm s^{-1}; the data resolution was 1 nm $step^{-1}$; the measurement type was remission; and the measurement mode was absorption; the optical filter was second order.

Separation Factors

The chromatograms were done in triplicate and each track was scanned three times, and the mean of the R_F values were calculated.

The separation factors, namely: ΔR_F values, selectivity (α),[6] and constant of the pair separation (R_F^{α})[10] were calculated for all the densitograms.

ΔR_F was calculated according to the formula:

$$\Delta R_{F(1,2)} = R_{F1} - R_{F2} \tag{1}$$

where R_{F1} and R_{F2} are the R_F values of two adjacent peaks on the densitogram; and $R_{F1} > R_{F2}$.

The selectivity (α) was calculated using the equation:

$$\alpha = \frac{1/R_{F1} - 1}{1/R_{F2} - 1} \tag{2}$$

where R_{F1} and R_{F2} are the R_F values of two adjacent peaks on the densitogram, and $R_{F1} < R_{F2}$.

The constant of the pair separation (R_F^{α}) was calculated for the investigated compounds as the ratio of the R_F values of the two adjacent peaks on the densitogram:

$$R^{\alpha}_{F(1,2)} = \frac{R_{F1}}{R_{F2}} \tag{3}$$

where R_{F1} and R_{F2} are the R_F values of two adjacent peaks on the densitogram; and $R_{F1} > R_{F2}$.

Resolution Factors

Visual Method of R_S Calculation

The visual method of the R_S calculation was based on the chromatographic parameters obtained directly from chromatogram. The resolution of two spots ($R_{S(c)}$) was calculated using the formula:[6]

$$R_{S(c)} = 2 \times \frac{d}{s} \tag{4}$$

where d is the distance between the centers of two adjacent spots on the chromatogram, and s is the sum of the widths of the two spots in the direction of flow of mobile phase.

Densitometric Method of R_S Calculation

The peak resolution ($R_{S(b)}$) was calculated using the equation:[11,12]

$$R_{S(b)} = \frac{2d}{w_{b1} + w_{b2}} \tag{5}$$

where d is the distance between the centers of two adjacent peaks on the densitogram, whereas w_{b1} and w_{b2} are the peaks width at the base.

The peak resolution ($R_{S(h)}$) was also calculated using the equation:[12]

$$R_{S(h)} = \frac{d}{w_{h1} + w_{h2}} \sqrt{\ln 4} \tag{6}$$

where d is the distance between the centers of two adjacent peaks on the densitogram, whereas w_{h1} and w_{h2} are the peaks width at half height.

The average values of peak resolution ($R_{S(a)}$) were also calculated according to the formula:

$$R_{S(a)} = \frac{R_{S(b)} + R_{S(h)}}{2} \tag{7}$$

RESULTS AND DISCUSSION

The separation of phloroglucinol (P), niclosamide (N), salicylanilide (S), and thymol (T) on silica gel using a *n*-hexane-acetone as mobile phase was investigated. It was affirmed that the R_F values of the studied compounds increase with an increase of acetone content in the *n*-hexane-acetone mobile phase. It influences the values of ΔR_F, R_F^{α}, and α parameters. The separation of all substances investigated from each other is possible using the above mentioned mobile phase in volume compositions: 45:5, 40:10, 35:15, and 30:20. Phloroglucinol remains at start using the above mentioned mobile phase in volume compositions: 45:5 and 40:10. When *n*-hexane-acetone mobile phase in volume composition of 30:20 was used the R_F of thymol was equal to 0.92. Using the above mentioned mobile phase in volume composition 25:25 the R_F values of niclosamide and salicylanilide are larger than 0.90, whilst thymol migrates with the front of mobile phase. It was affirmed that *n*-hexane-acetone mobile phase in the volume composition of 35:15 is optimum for the separation of the investigated compounds. Separation factors R_S, ΔR_F, R_F^{α}, and selectivity α of investigated substance were the basis of selection of a specified mobile phase.

After the chromatograms were dried and developed, the absorption bands were detected by using UV light at $\lambda = 254$ nm. It was stated that thymol is invisible on a chromatogram using a UV lamp at 254 nm. Next, the resolution of chromatographic spots, $R_{S(c)}$, from the chromatograms was calculated using the Eq. (4). The $R_{S(c)}$ values greater than 1 for pairs of the substance phloroglucinol–niclosamide and niclosamide-salicylanilide on the chromatogram were obtained using *n*-hexane-acetone as the mobile phase in all applied volume compositions. The R_S values obtained by use of this method were verified using densitometric analysis. The plates developed by the use of *n*-hexane-acetone mobile phase in the above mentioned volume compositions were also densitometric analyzed at $\lambda = 254$ nm. It was affirmed that densitometric analysis at $\lambda = 254$ nm enables the thymol detection. The R_F values of investigated substances were calculated from densitometric data. The separation

factors ΔR_F, R_F^{α}, and selectivity α were calculated from the R_F values. Moreover, the peak resolutions $R_{S(b)}$ and $R_{S(h)}$ were calculated from the Eqs (5) and (6) by the use of the obtained densitometric bands for the studied compounds. The obtained data using optimum mobile phase are presented in Table 1. The average $R_{S(a)}$ values calculated from Eq. (7), characteristic of densitometric peaks and visual evaluation of chromatographic spots detected in iodine vapor are also presented in Table 1. The characteristics of the densitometric peaks were done by determination of their height, area, and the angle between the tangents at the inflection points to the curves of the densitometric peaks (β). It was affirmed that $R_{S(b)}$, $R_{S(h)}$, and $R_{S(a)}$ values calculated on the basis of the densitograms are considerably lower than the $R_{S(c)}$ values calculated on the basis of the chromatograms. This shows that R_S values can be correctly marked exclusively on the basis of the densitograms. The scientific literature data indicate that at R_S values smaller than 0.8 we can not expect any good separations.

However, the R_S value is required to be larger than 1.5 to obtain the complete separation of the neighboring compounds on the densitograms. R_S values larger than 1.5, calculated on the basis of the densitograms, were obtained for the pairs of compounds P/N, N/S, and S/T by use of *n*-hexane-acetone in volume compositions 45:5, 40:10, and 35:15. Taking into consideration R_F and R_S values obtained by use of the densitometric method, it must be stated that the best separation of the studied substances was obtained by use of a *n*-hexane-acetone mobile phase in volume composition 35:15. The densitogram of substances investigated on plates precoated with silica gel by use of *n*-hexane-acetone mobile phase in volume composition 35:15 is presented in Figure 1. The angle value (β) is large for thymol (53°) in relation to the remaining substances. The area and height values of the chromatographic band for particular investigated compounds at $\lambda = 254$ nm is differentiated.

In a further part of this work, spectrodensitometric analysis of the examined substances was done. The spectrodensitograms of phloroglucinol ($\lambda_{max} = 204$ nm) and niclosamide ($\lambda_{max} = 338$ nm), as well as salicylanilide ($\lambda_{max} = 307$ nm) and thymol ($\lambda_{max} = 200$ nm) are presented in Figures 2 and 3, respectively. Therefore, densitometric scanning was then performed at multi wavelength in the range of 200 to 340 nm, with wavelength change at every step 35 nm. A three dimensional densitogram of investigated substances at different wavelengths (200, 235, 270, 305, and 340 nm) is presented in Figure 4. The resolutions of peaks $R_{S(b)}$, $R_{S(h)}$, and $R_{S(a)}$ were calculated by the use of the obtained densitometric bands for the studied pairs of compounds at particular wavelengths. Those values are presented in Table 2. The characteristics of the obtained densitometric bands are also presented in Table 2. It was affirmed that thymol can not be identified at wavelengths 305 and 340 nm. It was shown that resolutions $R_{S(b}$, $R_{S(h)}$, and $R_{S(a)}$ values larger than 1.5 were obtained at wavelengths 200, 235, and 270 nm for the studied pairs of substances P/N, N/S, and S/T. The values of resolutions $R_{S(b)}$, $R_{S(h)}$, and $R_{S(a)}$ obtained at these wavelengths are only slightly

Table 1. Characteristic of chromatographic spots and separation effect determined by densitometric method ($\lambda = 254$ nm) and visual method of compounds investigated using *n*-hexane-acetone, 35:15 (v/v) as mobile phase

		Separation factors			R_S values calculated from Eqs.				Characteristic of densitometric band			Visual description of chromato-graphic spot[b]
Compound	R_F	ΔR_F	α	R_F^{α}	(4)[a]	(5)	(6)	(7)	Height [AU]	Area [AU]	β [°]	
P	0.09								297	11318	4	Compact
N	0.37	0.28	5.94	4.11	11.14	5.62	5.58	5.60	196	9807	11	Light broadened
S	0.62	0.25	2.78	1.68	7.78	3.91	3.92	3.91	216	15371	14	Light broadened
T	0.82	0.20	2.79	1.32		2.25	2.15	2.20	52	4298	53	Broadened

[a]R_S−Calculated on the basis of parameters of spot position visualized in UV light.
[b]Visual evaluation of spot visualized in iodine vapor.

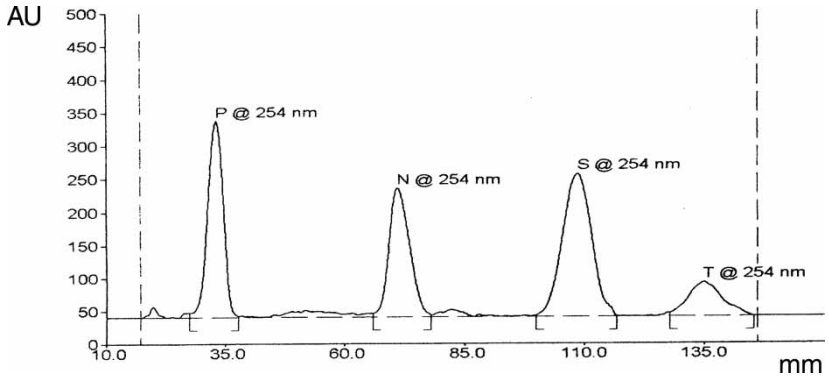

Figure 1. The densitogram of substance investigated at $\lambda = 254$ nm after their separation using a *n*-hexane–acetone mobile phase in volume composition 35:15.

different from resolution values obtained at $\lambda = 254$ nm using a densitometer. The heights and areas of chromatographic bands obtained at different wavelengths have differentiated values. The densitometric bands with the largest area were obtained at wavelengths in the neighborhood of absorption maximum (λ_{max}) for particular substances investigated.

Characteristics of the chromatographic band was realized using the densitometric method by determination of peak height, peak area, and the angle (β) between the tangents at the inflection points to the curves of the densitometric peak. From the obtained data, it is apparent that the band of

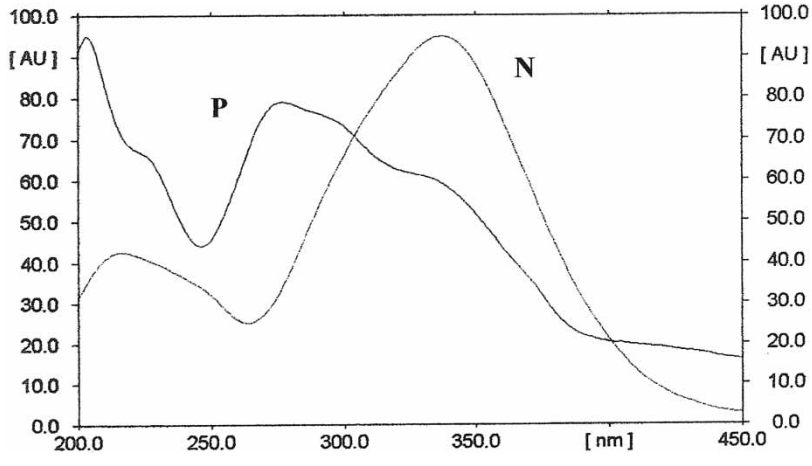

Figure 2. Spectrodensitograms of phloroglucinol (P) ($\lambda_{max} = 204$ nm and $\lambda = 278$ nm) and niclosamide (N) ($\lambda_{max} = 338$ nm and $\lambda = 217$ nm).

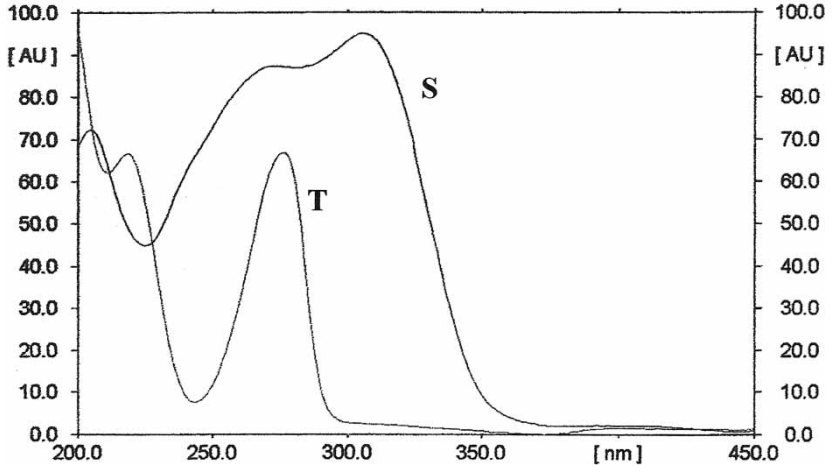

Figure 3. Spectrodensitograms of salicylanilide (S) ($\lambda_{max} = 307$ nm, $\lambda = 205$ nm, and $\lambda = 271$ nm) and thymol (T) ($\lambda_{max} = 200$ nm, $\lambda = 219$ nm, and $\lambda = 277$ nm).

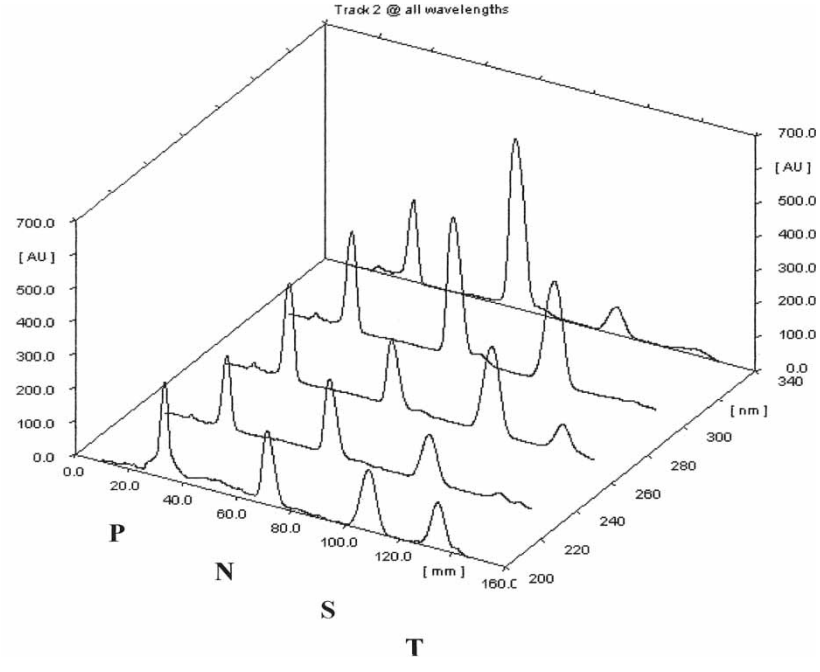

Figure 4. The densitograms of substances investigated (P–phloroglucinol, N–niclosamide, S–salicylanilide, T–thymol) at wavelengths 200, 235, 270, 305, and 340 nm after their separation using a *n*-hexane-acetone mobile phase in volume composition 35:15.

Table 2. Values of resolutions (R_S) and characteristic of denstitometric bands at various wavelength of investigated compounds by NP-TLC technique using *n*-hexane-acetone, 35 : 15 (v/v) as mobile phase

	R_S values calculated by use of Eqs.			Characteristic of densitometric band		
Symbol of compound	(5)	(6)	(7)	Height [AU]	Area [AU]	β [°]
$\lambda = 200$ nm						
P				288	13502	5
N	5.57	5.62	5.60	222	11771	10
S	3.78	3.74	3.76	186	12686	18
T	2.52	2.47	2.50	142	8869	19
$\lambda = 235$ nm						
P				216	8826	6
N	5.29	5.30	5.30	227	11716	9
S	3.83	3.78	3.80	140	9511	12
T	2.58	2.61	2.60	22	1184	70
$\lambda = 270$ nm						
P				287	12124	6
N	4.92	4.87	4.90	194	10004	13
S	3.71	3.74	3.72	252	18211	12
T	2.37	2.35	2.36	80	5472	36
$\lambda = 305$ nm						
P				299	13076	6
N	4.39	4.42	4.40	423	25634	8
S	3.40	3.31	3.36	309	23616	12
T	—	—	—	—	—	—
$\lambda = 340$ nm						
P				243	11297	11
N	4.39	4.42	4.40	504	32320	7
S	3.61	3.61	3.61	80	5511	49
T	—	—	—	—	—	—

phloroglucinol (P) has the lowest numerical value of angle β, whilst thymol (T) has the largest numerical value of angle β. It shows that the band of phloroglucinol is compact in spite of its large area. However, the band of thymol is broadened whereas area and height of its band are the smallest.

Analysis of chromatographic bands not by visual but by their densitometric characteristic which is a supplementary element of the separation effect evaluation. Each visual evaluation is subjective and a little precise in relation to the densitometric method. Only the densitometric method can be used for the objective evaluation of the separation effect and characteristic of particular chromatographic bands.

CONCLUSION

The comparison and characteristic of chromatographic bands of selected substances were presented on the basis of calculated separation factors: $R_{S(c)}$, $R_{S(b)}$, $R_{S(h)}$, and $R_{S(a)}$. The above mentioned parameters serving to evaluate the separation of substances were determined by visual method ($R_{S(c)}$) and densitometric method ($R_{S(b)}$, $R_{S(h)}$, and $R_{S(a)}$). It was affirmed that the densitometric method is correct and is the standard method to determine the above mentioned parameters. Furthermore, the R_S parameter determined by the visual method for two adjacent substances is always larger than determined by the densitometric method. Both height and area of the densitometric band and angle between the tangents at the inflection points to the curves of the densitometric peak (β) depend not only on amount of spotted substance but also on their physicochemical properties.

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