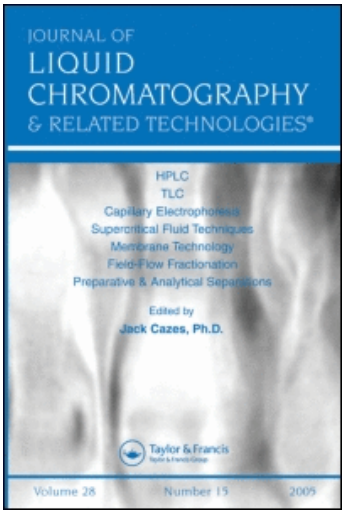

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The Application of Densitometry to Evaluate the Visualizing Effects of Salicylanilide using Brilliant Green

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Abstract: Salicylanilide was detected on glass plates precoated with a 0.50 mm layer of silica gel 60 F_{254} (E.Merck, #1.05744) and with a 0.25 mm layer of silica gel 60 F_{254} (E.Merck, #1.05715), as well as on aluminum plates precoated with a 0.20 mm layer of silica gel 60 F_{254} (E.Merck, #1.05554), silica gel 60 (E.Merck, #1.05553), and a mixture of silica gel 60 and kieselguhr F_{254} (E.Merck, #1.05567), with and without the use of brilliant green as a visualizing reagent. Spectrodensitograms of salicylanilide, with and without the use of brilliant green, on the particular chromatographic sorbents were presented. The limit of detection (detectability), detection index, broadening index, modified contrast index, densitometric visualizing index, and linearity range were used to evaluate the visualizing effects of salicylanilide. It was stated that the proposed new parameter within work, namely the densitometric visualizing index, is the objective parameter describing the visualizing effect of detected salicylanilide.

Keywords: Brilliant green, Broadening index, Densitometry, Densitometric visualizing index, Detectability, Detection index, Modified contrast index, NP-TLC, Salicylanilide

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INTRODUCTION

Many phenol derivatives have definite pharmacologic and biological properties.[1] For example, the salicylanilide and its derivatives have fungicidal,[1–3] antituberculotic,[3] and antimicrobial[4] activities. Salicylanilide is a crystalline compound used generally as a fungicide. It is applied either as an aqueous (weak alkaline) solution, or as a non-aqueous solution, at a concentration of 0.1% by weight of the fabric or paper.[2]

Currently, the most important field of application of thin-layer chromatography is pharmacy. The number of publications in the field of pharmacy has increased steadily.[5,6] The separated substances on thin-layers can be detected by the following methods: physical (individual color of substance or fluorescence of substance in UV light); chemical (colored reactions of separated substances with visualizing reagents); physicochemical (e.g., the application of isotopes as visualizing reagent); and biological (the application of biodetectors). The visualizing reagents have the special significance to detect separated compounds on thin-layers. In view of the detection mechanism of the compounds, the visualizing reagents can be sorted as follows: non-destructive reagents, which do not destroy the separated substances; and destructive reagents, which destroy or change the structure of the separated substances.[5–7]

There are good analytical and physicochemical reasons for describing the new visualizing reagents; these reasons, and the most important reagents and techniques for different types of organic compounds, including phenols, have been described elsewhere.[5,6,8–17] For example, Barton's reagent was used to detect dopamine; chloromine T-sodium hydroxide, sucrose-hydrochloric acid, and Pauly's reagents for the detection of phloroglucinol; Emerson reagent to detect thymol and eugenol; fast black salt K-sodium hydroxide reagent to detect terbutalina; potassium hexaiodoplatinate reagent to detect pentazocine; potassium hexacyanoferrate (III)-ethylendiamine reagent to detect dopamine, noradrenaline, and adrenaline;[5,6,9] aniline-diphenylamine-phosphoric acid, lead(IV) acetate-dichlorofluorescein, Gibb's, Millons reagents, Berlin blue reaction to detect arbutin; natural products-polyethylene glycol reagent to detect chlorogenic acid; vanillin-sulphuric acid reagent to detect eugenol, thymol, carvacrol; and anisaldehyde-sulphuric acid reagent to detect thymol.[18] Many indicators were also used as visualizing reagents for the detection of selected phenolic drugs in TLC.[10–17] It was stated, that brilliant green is the best visualizing reagent to detect salicylanilide.[10,11]

The limit of detection (detectability), detectability index, broadening index, contrast index, and modified contrast index were used earlier to

evaluate the visualizing effects of different compounds detected with the use of the visualizing reagents.[10–17,19–21]

The aim of this study was to propose the new index to evaluate objectively the visualizing effects of detected substances on thin layers using a densitometric method. This index was named the densitometric visualizing index. The densitometric visualizing index was used for the comparison of the visualizing effects of salicylanilide with and without the use of brilliant green as a visualizing reagent. Spectra of salicylanilide with and without the use of brilliant green on different sorts of chromatographic sorbents were compared.

EXPERIMENTAL

Thin Layer Chromatography

TLC was performed on 20 cm × 20 cm glass plates precoated with 0.50 mm layer of silica gel 60 F_{254} (E.Merck, #1.05744) and with 0.25 mm layer of silica gel 60 F_{254} (E.Merck, #1.05715), as well as on 20 cm × 20 cm aluminum plates precoated with 0.20 mm layer of: silica gel 60 F_{254} (E.Merck, #1.05554), silica gel 60 (E.Merck, #1.05553), and a mixture of silica gel 60 and kieselguhr F_{254} (E.Merck, #1.05567). The plates were prewashed with methanol and dried for 24 h at room temperature (18 ± 1°C). The plates were then activated at 120°C for 30 min. Standard solutions of salicylanilide (Sigma) containing 50.00, 45.00, 40.00, 35.00, 30.00, 25.00, 20.00, 15.00, 10.00, 7.00, 5.00, 3.00, 1.00, 0.70, 0.50, 0.30, 0.20, 0.10, and 0.07 mg were prepared in 5 mL methanol. Solutions of salicylanilide (5 μL) were spotted manually using a microcapillary (Camag, Switzerland) on the plates. Chloroform was used as mobile phase. Mobile phase (50 mL) was placed in a classical chromatographic chamber (Camag, Switzerland) and after saturation of the chamber with mobile-phase vapor for 30 min the plates were developed vertically, at room temperature (18 ± 1°C), to a distance of 14 cm. The plates were then dried for 24 h at room temperature (18 ± 1°C) in a fume cupboard.

Detection of Salicylanilide

Salicylanilide was detected without using a visualizing reagent and with the use of brilliant green as a visualizing reagent. Brilliant green was used as 50 mg/100mL solution in water.

The dried plates were dipped in the solution of brilliant green for 30 sec. The plates were then dried for 24 h at room temperature (18 ± 1°C).

Spectrodensitometric and Densitometric Analysis

A spectrum scan was performed using a Camag Scanner TLC 3 operated in absorbance mode and controlled by WinCATS 1.4.2 software. The radiation sources were a deuterium lamp emitting a continuous UV spectrum between 190 and 450 nm and a tungsten lamp-emitting a spectrum between 370 and 800 nm. Starting wavelength was 200 nm and ending wavelength were 350 nm and 700 nm for salicylanilide detected without and with the use of brilliant green, respectively. The slit dimensions were 10.00×0.40 mm, Macro; 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.

Densitometric scanning was then performed at the respective absorption maxima (Table 1). The radiation sources were a deuterium or wolfram lamps. The slit dimensions were 10.00×0.40 mm, Macro; the optimized 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 baseline correction (lowest slope) was used.

Table 1. The absorption maximum, broadening index and detection index for salicylanilide detected without and with the use of brilliant green on the particular chromatographic plates

	Without the use of a visualizing reagent			With the use of brilliant green as a visualizing reagent		
Chromatographic plates*	λ_{max}	Broadening index μg/[AU]	Detection index μg/[AU]	λ_{max}	Broadening index μg/[AU]	Detection index μg/[AU]
#1.05744	**307 nm**	0.686	0.07/1590	**597 nm**	0.544	0.70/11112
#1.05715	**307 nm**	0.557	0.07/1155	**268 nm**	0.544	1.00/20067
#1.05554	**307 nm**	0.608	0.07/1199	**268 nm**	0.977	0.70/4826
#1.05553	**308 nm**	0.434	0.07/1813	**268 nm**	0.595	1.00/14321
#1.05567	**312 nm**	1.021	0.70/3190	**305 nm**	0.800	1.00/26102

*1.05744–glass plates precoated with 0.50 mm layer of silica gel 60 F_{254}.
1.05715–glass plates precoated with 0.25 mm layer of silica gel 60 F_{254}.
1.05554–aluminum plates precoated with 0.20 mm layers of silica gel 60 F_{254}.
1.05553–aluminum plates precoated with 0.20 mm layer of silica gel 60.
1.05567–aluminum plates precoated with 0.20 mm layer of a mixture of silica gel 60 and kieselguhr F_{254}.

Broadening Index, Detection Index, Modified Contrast Index, and Densitometric Visualizing Index

Broadening Index[19,20]

The broadening index is defined as

$$I_{\text{broad}} = \frac{50}{p_2} \times 1000 \quad \left[\frac{\mu g}{\text{AU}}\right] \tag{1}$$

where 50 μg of the analyzed salicylanilide in 5 μL of solution was applied to the chromatographic plate, and p_2 is the spot area [AU] of 50 μg of analyzed salicylanilide.

Detection Index[19,20]

The detection index is defined as:

$$I_{\text{det}} = \frac{m_1}{p_1} \quad \left[\frac{\mu g}{\text{AU}}\right] \tag{2}$$

where m_1 is the smallest quantity of salicylanilide detected [μg] (limit of detection), and p_1 is the spot area of salicylanilide [AU] at the limit of detection of salicylanilide.

Modified Contrast Index[21]

The modified contrast index is defined as

$$I_{\text{Contr (mod if)}} = \frac{h}{\alpha} \quad \left[\frac{\text{AU}}{^\circ}\right] \tag{3}$$

where h is the height of the denstitometric band [AU] of 50 μg of analyzed salicylanilide, and α is the angle [°] between the tangents at the inflection points to the curves of the densitometric band of salicylanilide.

Densitometric Visualizing Index

The densitometric visualizing index (DVI) is proposed and defined in this work as

$$\mathbf{DVI} = \frac{p_2}{m_1 \times \alpha} \times 10^{-4} \quad \left[\frac{AU}{\mu g \cdot ^\circ}\right] \tag{4}$$

where m_1 is limit of detection of the analyzed salicylanilide [μg], p_2 is the spot area [AU] of 50 μg of analyzed salicylanilide, and α is the angle

[°] between the tangents at the inflection points to the curves of the densitometric band of 50 μg of analyzed salicylanilide.

The broadening indices, detection indices, modified contrast indices, and densitometric visualizing indices were calculated by use of Eqs. (1), (2), (3), and (4), respectively.

RESULTS AND DISCUSSION

Salicylanilide was densitometricly detected in five different chromatographic sorbents, namely on glass plates precoated with 0.50 mm layer of silica gel 60 F_{254} (E.Merck, #1.05744) and with 0.25 mm layer of silica gel 60 F_{254} (E.Merck, #1.05715), as well as on 20 cm × 20 cm aluminum plates precoated with 0.20 mm layers of: silica gel 60 F_{254} (E.Merck, #1.05554), silica gel 60 (E.Merck, #1.05553), and a mixture of silica gel 60 and kieselguhr F_{254} (E.Merck, #1.05567), without the use of a visualizing reagent and after the use of brilliant green as a visualizing reagent. It was stated, that salicylanilide bands without the use of a visualizing reagent are invisible on the chromatogram in a visible light. Dark green spots of salicylanilide on green background are visible on the chromatograms after an application of brilliant green as a visualizing reagent. The R_F values of salicylanilide are in the range from 0.75 to 0.88 in dependence on the applied chromatographic plates. Spectrodensitometric evaluation of salicylanilide with and without the use of a visualizing reagent was performed. Spectrodensitograms of salicylanide with and without the use of brilliant green as a visualizing reagent on the particular chromatographic plates are presented in Figures 1–5, respectively. However, the absorption maxima of salicylanilide without the use of a visualizing reagent and after the use of brilliant green on the particular chromatographic plates are presented in Table 1. It was stated, that salicylanilide without the use of a visualizing reagent has fundamental absorption band at the similar values of the wavelength equal to 307 nm or 308 nm on #1.05744, #1.05715, #1.05554, and #1.05553 plates. However, the absorption maximum of salicylanilide on a mixture of silica gel 60 and kieseguhr F_{254} without the use of a visualizing reagent is somewhat shifted and it is equal to 312 nm. Whereas, the obtained spectrodensitograms of salicylanilide without the use of a visualizing reagent differ in intensity of the additional absorption bands in a dependence on the chromatographic sorbents. The resultant spectrodensitograms of salicylanilide after the detection with brilliant green differ in the wavelength of absorption maximum. The absorption maximum of salicylanilide occurs at 268 nm on #1.05715, #1.05554 and 1.05553 plates; at 305 nm on #1.05567 plate, and at 597 nm on #1.05744 plate. Simultaneously, the resultant spectrodensitograms of salicylanilide

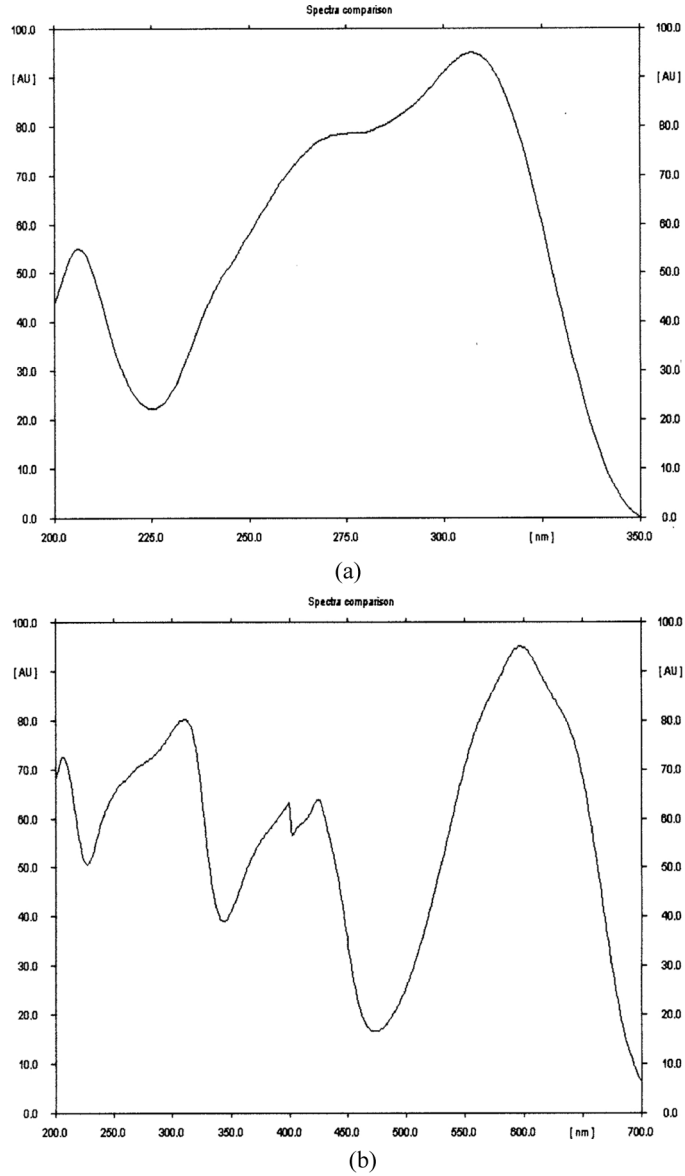


Figure 1. Spectrodensitograms of salicylanilide on glass plates precoated with 0.50 mm layer of silica gel 60 F_{254} (E.Merck, #1.05744): (a) without the use of a visualizing reagent; (b) with the use of brilliant green as a visualizing reagent.

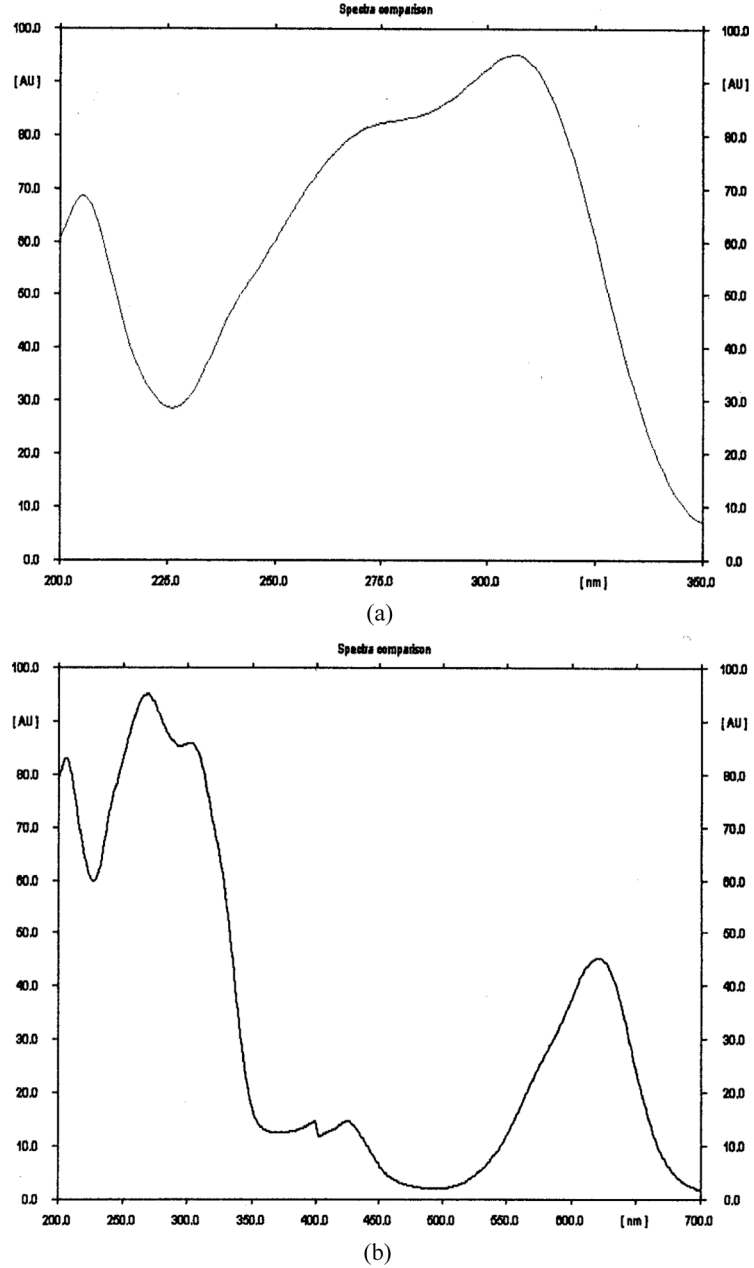
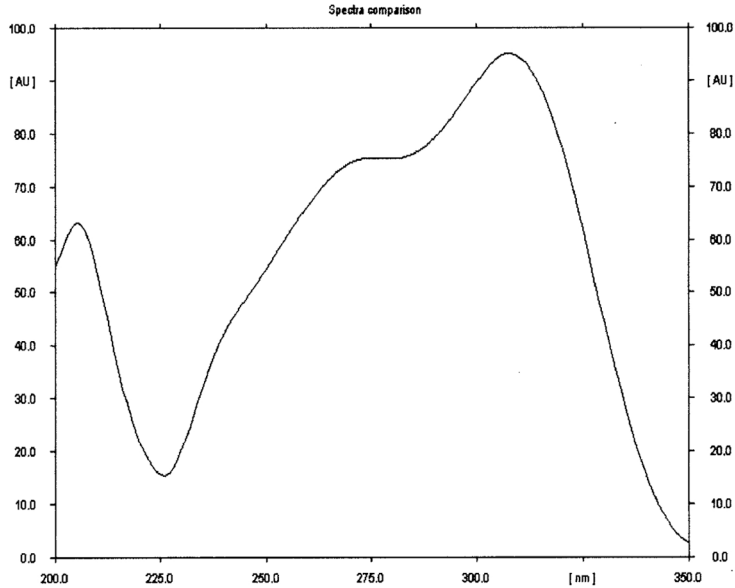
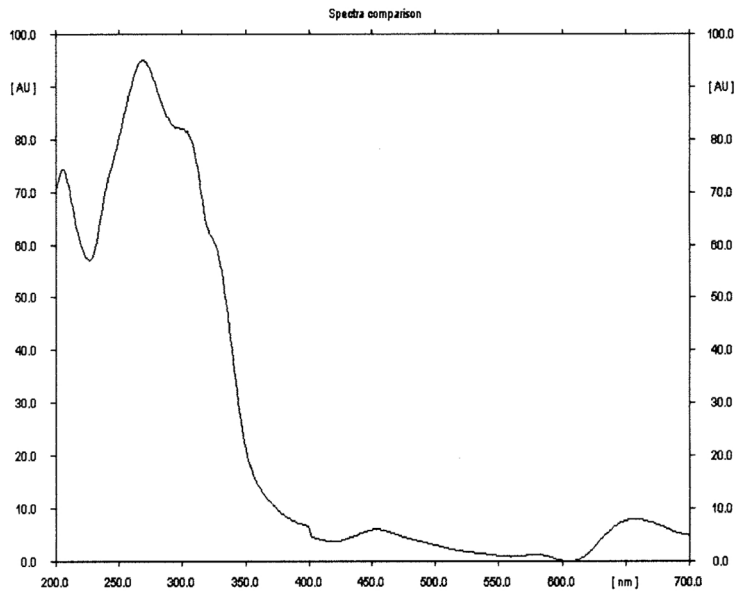


Figure 2. Spectrodensitograms of salicylanilide on glass plates precoated with 0.25 mm layer of silica gel 60 F_{254} (E.Merck, #1.05715): (a) without the use of a visualizing reagent; (b) with the use of brilliant green as a visualizing reagent.



(a)



(b)

Figure 3. Spectrodensitograms of salicylanilide on aluminum plates precoated with 0.20 mm layer of silica gel 60 F_{254} (E.Merck, #1.05554): (a) without the use of a visualizing reagent; (b) with the use of brilliant green as a visualizing reagent.

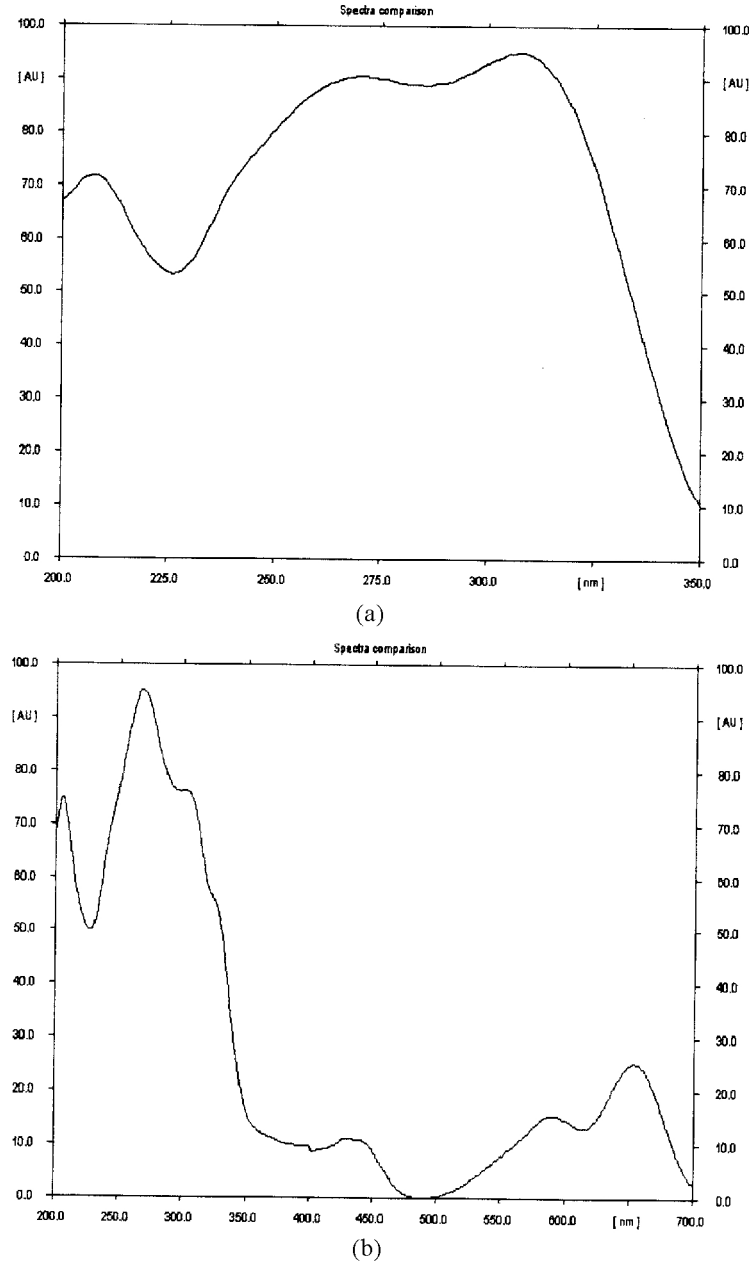
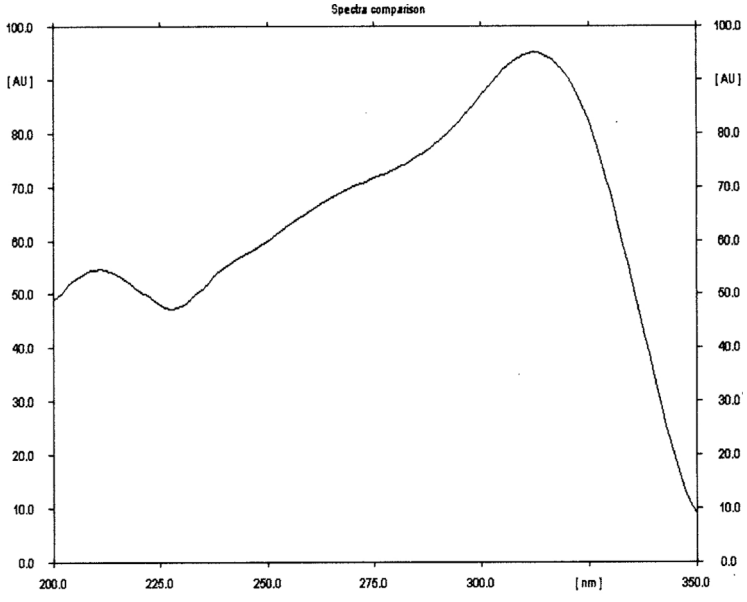
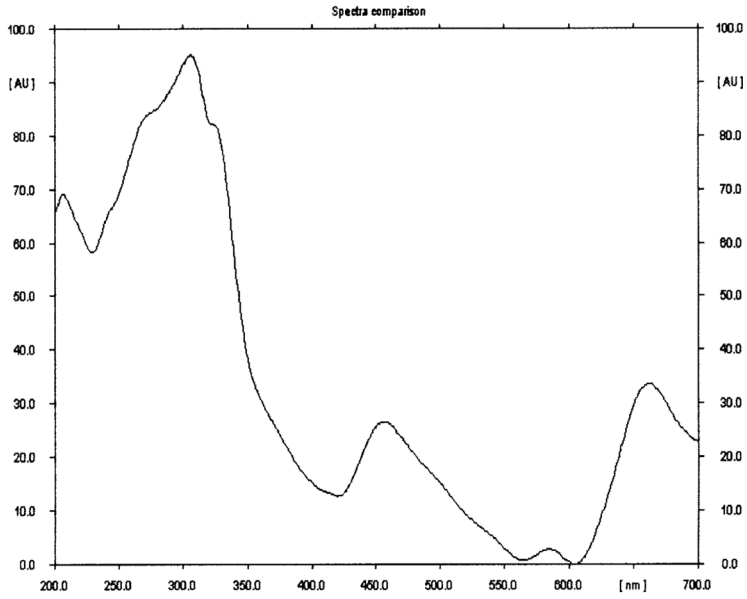


Figure 4. Spectrodensitograms of salicylanilide on aluminum plates precoated with 0.20 mm layer of silica gel 60 (E.Merck, #1.05553): (a) without the use of a visualizing reagent; (b) with the use of brilliant green as a visualizing reagent.



(a)



(b)

Figure 5. Spectrodensitograms of salicylanilide on aluminum plates precoated with 0.20 mm layer of a mixture of silica gel 60 and kieselguhr F_{254} (E.Merck, #1.05567): (a) without the use of a visualizing reagent; (b) with the use of brilliant green as a visualizing reagent.

after the use of brilliant differ also in the number and intensity of the additional absorption bands on the particular chromatographic plates. The obtained spectrodensitograms of the studied salicylanilide with and without the use of brilliant green as a visualizing reagent indicate, that applied sorbents influence on the wavelength of the obtained fundamental absorption band (λ_{max}) and the additional absorption bands, as well as on their intensity values [AU]. This fact indicates the necessary standaridization of the spectrodensitometric investigations regarding the applied chromatographic conditions. Therefore the spectrodensitograms of salicylanilide can be correctly compared only on the same chromatographic plate. This fact has fundamental significance in the identification analysis.

The densitometric analyses were performed at respective absorption maxima, which are given in Table 1. The obtained chromatographic spots of the investigated salicylanilide on the applied chromatographic sorbents after the use of brilliant green were durable and visible for over 6 weeks.

The broadening index was also calculated in this work. A good visualizing reagent has a relatively large numerical value of the broadening index for a particular substance detected (small spot area, which refers to 50 μg of substance detected). The broadening indices and the detection indices for the salicylanilide with and without the use of brilliant green as a visualizing reagent on the different chromatographic plates are presented in Table 1. The detection index indicates the ratio of the minimal number of micrograms of salicylanilide to the area of the chromatographic spot in AU. The area of the spot was assessed by the densitometric method.

The limits of detection of the salicylanilide investigated with and without the use of brilliant green as a visualizing reagent, linearity range, densitometric visualizing index, and densitometric band characteristic of 50 μg investigated salicylanilide on particular chromatographic sorbents are presented in Tables 2 and 3, respectively. It was stated, that the linearity range of salicylanilide is different in the dependence on the applied chromatographic sorbent and the detection of salicylanilide with and without the use of brilliant green as a visualizing reagent. Densitometric evaluation of the obtained densitometric bands of 50 μg salicylanilide was described by the area of densitometric band [AU], the densitometric band height [AU], and the angle (α) between the tangents at the inflection points to the curves of the densitometric band, formulated in degrees [°]. The contrast index represents two independent values, namely the angles (α) between the tangents at the inflection points to the curves of the densitometric band, formulated in degrees, and the densitometric band high [AU].[19] Earlier, we proposed the modified contrast index to the evaluation of the visualizing reagents to

Table 2. Characteristic of densitometric band, modified contrast index, limit of detection, densitometric visualizing index, and linearity range of salicylanilide on the particular chromatographic plates without the use of a visualizing reagent

Chromatographic plates	Densitometric band characteristic of 50 μg salicylanilide: Area [AU]	Height [AU]	α	Modified contrast index $\left[\frac{AU}{°}\right]$	Limit of detection [μg]	Densitometric visualizing index $\left[\frac{AU}{\mu g \cdot °}\right]$	Linearity range [μg spot^{-1}] [r, correlation coefficient]
#1.05744	72889	707	9	78.5	0.07	11.570	5.00÷30.00 ($r = 0.9802$)
#1.05715	89761	717	11	65.2	0.07	11.657	5.00 ÷ 50.00 ($r = 0.9904$)
#1.05554	82263	776	8	97.0	0.07	14.690	3.00 ÷ 20.00 ($r = 0.9832$)
#1.05553	115248	785	11	71.4	0.07	14.967	5.00 ÷ 25.00 ($r = 0.9917$)
#1.05567	48953	581	8	72.6	0.70	0.874	1.00 ÷ 30.00 ($r = 0.9902$)

Table 3. Characteristic of densitometric band, modified contrast index, limit of detection, densitometric visualizing index, and linearity range of salicylanilide on particular chromatographic plates with the use of brilliant green as a visualizing reagents

Chromatographic plates	Densitometric band characteristic of 50 μg salicylanilide: Area [AU]	Height [AU]	α	Modified contrast index $\left[\frac{AU}{°}\right]$	Limit of detection [μg]	Densitometric visualizing index $\left[\frac{AU}{\mu g \cdot °}\right]$	Linearity range [μg spot^{-1}] [r, correlation coefficient]
#1.05744	91842	704	8	88.0	0.70	1.640	3.00 ÷ 30.00 ($r = 0.9929$)
#1.05715	91765	592	16	37.0	1.00	0.574	3.00 ÷ 40.00 ($r = 0.9896$)
#1.05554	51183	463	9	51.4	0.70	0.812	1.00 ÷ 15.00 ($r = 0.9982$)
#1.05553	84087	583	13	44.8	1.00	0.647	3.00 ÷ 40.00 ($r = 0.9897$)
#1.05567	62492	566	9	62.9	1.00	0.694	3.00 ÷ 50.00 ($r = 0.9967$)

detect estradiol.[20] The modified contrast index indicates the ratio of the height of the densitometric band [AU] of detected subsatnce to the angle (α) between the tangents at the inflection points to the curves of the densitometric band formulated in degrees [°]. The best visualizing reagent has a highest value of the modified contrast index for particular substance detected.

In this work, we proposed a new index, namely the densitometric visualizing index, which is the objective parameter describing the visualizing effect of a detected substance. The densitometric visualizing index contains two most important characteristics of a densitometric band of a 50 μg studied substance, namely the area of the densitometric band [AU], and the angle (α) between the tangents at the inflection points to the curves of the densitometric band, formulated in degrees [°]. The densitometric visualizing index contains also a third most important element, namely a limit of detection of the studied substance. The best way of substance detection has higher values of the densitometric visualizing index. On the basis of values of densitometric visualizing index it was stated that the best detection effects of salicalanilide were obtained without the use of a visualizing reagent. The worst detection effect of salicylanilide without the use of a visualizing reagent was obtained on a mixture of silica gel 60 and kieselguhr F_{254}. However, on the remaining sorbents the similar visualizing effects of salicylanilide were obtained. Whereas, the best visualizing effect of salicylanilide with the use of brilliant green as a visualizing reagent was obtained on glass plates precoated with 0.50 mm layer of silica gel 60 F_{254} (E.Merck, #1.05744).

It was stated, that the densitometric visualizing index (DVI) is the objective parameter describing the visualizing effect of detected salicylanilide.

Further investigations are in progress and concern the confirmation of the significance of the densitometric visualizing index to the evaluation of the visualizing effects of different aliphatic compounds detected with the use of selected deys, namely gentian violet, methylene violet, methylene blue, methyl green, malachite green, and Janus blue.

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