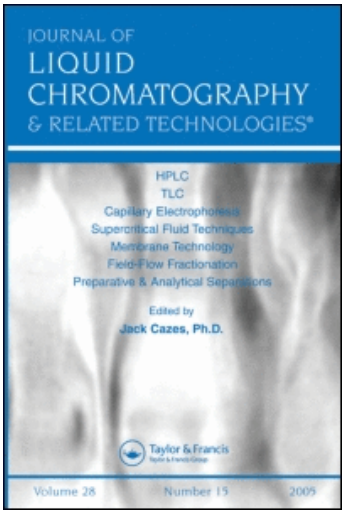

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Application of Densitometry for the Evaluation of the Separation Effect of Nicotinic Acid Derivatives. Part II. Nicotinic Acid and its Esters

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Application of Densitometry for the Evaluation of the Separation Effect of Nicotinic Acid Derivatives. Part II. Nicotinic Acid and its Esters

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Abstract: The nicotinic acid (1) and its esters, namely methyl nicotinate (2), ethyl nicotinate (3), isopropyl nicotinate (4), butyl nicotinate (5), hexyl nicotinate (6), and benzyl nicotinate (7) were investigated by NP-TLC, and RP-HPTLC. The R_F values were obtained from the densitometric analysis. The separation factors ΔR_F, R_F^{α} and selectivity α were calculated from the R_F values. The comparison and characteristic of chromatographic bands of the examined compounds were presented on the basis of calculated resolutions: $R_{S(c)}$, $R_{S(b)}$, $R_{S(h)}$, and $R_{S(a)}$. The resolutions 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. It was affirmed, that the best separation of the studied compounds was obtained by the RP-HPTLC technique on $RP18WF_{254}$ plates, and by use of dioxane-water in a volume composition of 50:50. However, butyl nicotinate from benzyl nicotinate cannot be separated by RP-HPTLC. Good separations of isopropyl nicotinate from ethyl nicotinate, as well as ethyl nicotinate from methyl nicotinate, were also not obtained by RP-HPTLC. It was affirmed, that adsorption thin-layer chromatography (NP-TLC) in the system of a neutral aluminum oxide $60F_{254}$ and the acetone-*n*-hexane mobile phase in a volume composition of 20:80 provided the optimum conditions for the complete separation of butyl nicotinate from benzyl nicotinate. These chromatographic conditions

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also allow better separations of methyl nicotinate from ethyl nicotinate, as well as ethyl nicotinate from isopropyl nicotinate, in relation to the separations of these compounds by RP-HPTLC.

Keywords: TLC, Densitometry, Separation parameters, Nicotinic acid, Nicotinic acid esters

INTRODUCTION

Nicotinic acid (1) has been used as a vasodilator for a long time.[1,2] The flush reaction to the nicotinic acid which occurs very rapidly is highly characteristic. The biochemical mechanism of the vascular response to the nicotinic acid is not well understood. However, some reports suggest that the nicotinic acid somehow enhances the biosynthesis or release of prostaglandin E_1, which stimulates adenylate cyclase and raises the level of cyclic adenosine monophosphate (cAMP).[3] Esters of the nicotinic acid, such as: methyl nicotinate (2), ethyl nicotinate (3), isopropyl nicotinate (4), butyl nicotinate (5), hexyl nicotinate (6) and benzyl nicotinate (7) are used in the pharmaceutical industry as ingredients of creams and ointments. These esters cross the skin rapidly and, on enzymatic hydrolysis, release nicotinic acid, which induces skin erythema. Nicotinate esters, which act as prodrugs, enhance the topical penetration of the active substances.[4–8] Simultaneous transport and metabolism of esters of the nicotinic acid were studied in the skin.[9,10] It was confirmed that a difference in the alkyl chain length of the ester prodrugs affected not only permeability but also metabolism in the skin permeation process. Various studies have shown significant enhancing effects of the lipophilic vehicles (isopropyl myristate, mineral oil) on the skin penetration of methyl nicotinate.[11–13] The explanation for the observed enhancing effects may be an interaction of the lipophilic liquids with the lipid bilayers of the stratum corneum that leads to a decrease of the barrier resistance. Enhancing effect of the vehicle (L-menthol) on the skin penetration of ethyl nicotinate has also been observed.[14] Isopropyl nicotinate is used as an analgetic and topical anesthetic agent.[15] Benzyl nicotinate in the presence of the liposomes improves skin oxygenation.[16–18] The composition of the liposomes significantly affects the time at which benzyl nicotinate starts to act and, to a lesser extent, the maximum increase of pO_2 in the skin and the effectiveness of the benzyl nicotinate action. However, the size of the liposomes influences both the effectiveness of benzyl nicotinate action and the time at which benzyl nicotinate starts to act.

We have previously investigated the optimum conditions for the separation of nicotinic acid, nicotinamide, N-methylnicotinamide, and N,N-diethylnicotinamide, which were studied by RP-HPTLC and NP-TLC. The evaluation of the separation effect of these compounds was based on the separation factors ΔR_F, R_F^{α}, α, and the resolution R_S values.[19]

The aim of this work was to use visual and densitomteric methods for the evaluation of:

—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 effects of particular substances on the basis of the parameters: R_F, R_S, ΔR_F, R_F^{α} and α.

The subjects of our study were the nicotinic acid and its esters.

EXPERIMENTAL

Chemicals and Sample Preparation

The components of the mobile phases: acetone, dioxane (POCh, Poland; analytical grade), methanol (Merck, Germany; for liquid chromatography), *n*-hexane (AnalaR, UK; analytical grade), and redistilled water were used for TLC analysis. The commercial samples of nicotinic acid (1), methyl nicotinate (2), ethyl nicotinate (3), butyl nicotinate (5) (Sigma-Aldrich, Germany), isopropyl nicotinate (4), hexyl nicotinate (6) (Aldrich, Germany) and benzyl nicotinate (7) (Fluka, Switzerland) were used as test solutes. The purity of the studied standard samples was at least 97%. The above mentioned compounds (about a concentration of 0.57 mg mL^{-1} of each standard) were dissolved in ethanol (POCh, Poland; 96%; analytical grade). The mixture solution of methyl nicotinate, ethyl nicotinate, and isopropyl nicotinate (about a concentration of 1.33 mg mL^{-1} of each standard) and a mixture solution of butyl nicotinate and benzyl nicotinate (about a concentration of 2 mg mL^{-1} of each standard) were also dissolved in ethanol (POCh, Poland; 96%; analytical grade).

Thin Layer Chromatography

Reversed-Phase High Performance Thin-Layer Chromatography

Reversed-phase high performance thin-layer chromatography (RP-HPTLC) was performed on 10×10 cm glass HPTLC plates, coated with RP-$18WF_{254}$ (Merck, #1.13124). The plates were prewashed with methanol and dried for 24 h at room temperature ($18 \pm 1^{\circ}C$). The mixture solution of the nicotinic acid and its esters (1 μL) and the mixture solution of methyl nicotinate, ethyl nicotinate, and isopropyl nicotinate (2 μL) was spotted manually using a microcapillary (Camag, Switzerland) on the chromatographic plate.

The methanol-water and dioxane-water in volume compositions of 100:0, 90:10, 80:20, 70:30, 60:40, 50:50, 40:60, 30:70, 20:80, 10:90, and 0:100 were used as mobile phases. Plates were developed to a distance of 7.5 cm at room temperature (18 ± 1°C) in a classical bottom chamber (Camag, Switzerland) previously saturated with the mobile phase for 30 min. After development the plates were dried for 24 h at room temperature (18 ± 1°C).

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 neutral aluminium oxide $60F_{254}$ (Type E) (E. Merck, #1.05550). 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 methyl nicotinate, ethyl nicotinate, and isopropyl nicotinate (2 μL) and the mixture solution of butyl nicotinate and benzyl nicotinate (2 μL) were spotted manually using a microcapillary (Camag, Switzerland) on the chromatographic plate. The mixtures of methyl nicotinate, ethyl nicotinate, and isopropyl nicotinate, as well as the mixture of butyl nicotinate and benzyl nicotinate, were separated using acetone + *n*-hexane in volume compositions of 100:0, 90:10, 80:20, 70:30, 60:40, 50:50, 40:60, 30:70, 20:80, 10:90, and 0:100 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 14 cm at room temperature (18 ± 1°C). The plates were dried for 24 h at room temperature (18 ± 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 $\lambda = 254$ nm with a Camag Scanner TLC 3 operated in the absorbance mode and controlled by winCATS 1.4.1 software. The radiation source was a deuterium lamp emitting a continuous UV spectrum between 190 and 450 nm. The slit dimensions were 6.00 × 0.30 mm, Micro for HPTLC plates, and 8.00 × 0.30 mm, Macro for TLC plates; 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.

Separation Factors

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

The separation factors, namely: ΔR_F values, selectivity (α),[20] and the constant of the pair separation (R_F^{α})[21] 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

R_S Calculation Using Visual Method on the Basis of Chromatogram

The resolution of two spots ($R_{S(c)}$) was calculated using the formula:[20]

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

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.

R_S Calculation on the Basis of Densitometric Analysis

The peak resolution ($R_{S(b)}$) was calculated using the equation:[22]

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

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:[23]

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

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

RP-HPTLC and NP-TLC techniques were used for the separation of nicotinic acid and its esters (0.57 μg of each standard). The studied compounds were separated on RP18WF$_{254}$ plates using a methanol-water and dioxane-water mobile phase in different volume compositions. It was affirmed, that the R_F values of the studied compounds decrease with an increase in water content in the mobile phase. Nicotinic acid (1) is an exception because its R_F values were changing in the narrow range. The compound bands were detected by use of UV light at $\lambda = 254$ nm, after chromatograms were developed and dried. Next, the resolutions of chromatographic spots $R_{S(c)}$ were calculated using the formula (4). It was affirmed, that butyl nicotinate (5) from benzyl nicotinate (7) cannot be separated by use of above mentioned chromatographic conditions. The $R_{S(c)}$ values greater than 1 for neighboring pair compounds ($6/5 + 7$, $5 + 7/4$, $4/3$, $3/2$, $2/1$) on the chromatogram were obtained by use of methanol-water in volume composition 50:50, as well as dioxane-water in volume compositions 50:50 and 40:60 as mobile phases. The densitograms of the studied compounds on RP18WF$_{254}$ plates using a methanol-water and dioxane-water mobile phases in a volume composition 50:50 are presented in Figure 1. The $R_{S(c)}$ values obtained by use of the visual method were verified using the densitometric method. The plates developed by the use of methanol-water and dioxane-water mobile phases in the above-mentioned volume compositions were densitometric analyzed

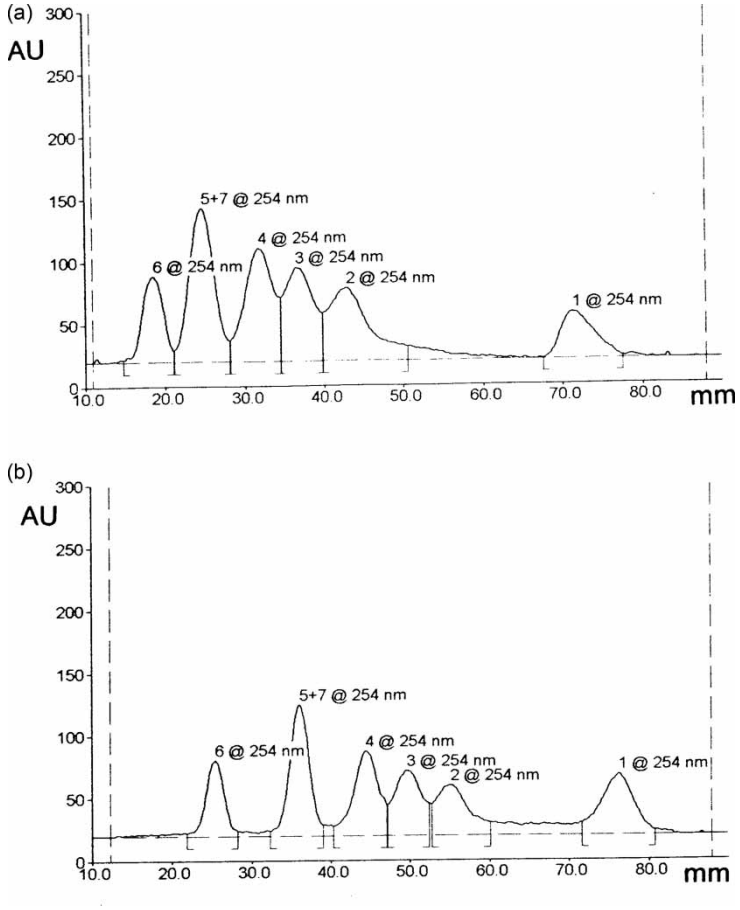

Figure 1. The densitograms of nicotinic acid and its esters (0.57 μg of each standard) on RP18WF$_{254}$ plates using a) methanol-water mobile phase with a volume composition of 50:50; b) dioxane-water mobile phase with a volume composition of 50:50; nicotinic acid (1), methyl nicotinate (2), ethyl nicotinate (3), isopropyl nicotinate (4), butyl nicotinate (5), hexyl nicotinate (6), and benzyl nicotinate (7).

at $\lambda = 254$ nm. The R_F values were obtained from the densitometric analysis. The separation factors ΔR_F, R_F^{α}, and selectivity α were calculated from the R_F values. Moreover, the resolutions of peaks $R_{S(b)}$ and $R_{S(h)}$ were calculated from the equations (5) and (6) by the use of the obtained densitometric bands for the studied compounds. The obtained data using methanol-water and dioxane-water mobile phases in the volume composition 50:50 are presented in Table 1. The average $R_{S(a)}$ values calculated by use of formula (7) and the characteristic densitometric peaks are also presented in Table 1. The characteristics of the densitometric peaks concern their height, area,

Table 1. R_F values, separation factors, resolutions and characteristic of densitometric bands of mixture of investigated compound by RP-HPTLC on $RP18WF_{254}$ plates

		Separation factors			R_S values calculated from eqs.				Characteristic densitometric band		
Comp. no[a]	R_F	ΔR_F	α	R_F^{α}	(4)	(5)	(6)	(7)	Height (AU)	Area (AU)	β (°)
Methanol - water, 50:50 (v/v)											
6	0.05								68	2125	17.5
5 + 7	0.13	0.08	2.84	2.60	1.71	1.13	1.14	1.14	122	4488	14
4	0.22	0.09	1.89	1.69	2.57	1.02	1.08	1.05	90	3823	26.5
3	0.29	0.07	1.45	1.32	1.33	0.51	0.54	0.52	75	3150	42
2	0.37	0.08	1.44	1.28	2.00	0.58	0.62	0.60	58	3526	57
1	0.75	0.38	5.11	2.03	8.31	3.02	3.05	3.04	37	1879	52
Dioxane - water, 50:50 (v/v)											
6	0.14								62	1717	19
5 + 7	0.28	0.14	2.39	2.00	3.08	2.47	2.41	2.44	106	3157	12.5
4	0.39	0.11	1.64	1.39	2.46	1.62	1.66	1.64	69	2580	27
3	0.46	0.07	1.33	1.18	2.00	0.77	0.77	0.77	53	2095	39
2	0.54	0.08	1.38	1.17	2.33	0.68	0.68	0.68	41	1971	61
1	0.82	0.28	3.88	1.52	6.67	2.58	2.64	2.61	49	2364	41.5

[a]The symbols of examined compounds are explained in Experimental part. Chemicals and sample preparation.

and the angle (β) between the tangents at the inflection points to the curves of the densitometric peaks. It was affirmed, that peak heights and peak areas have smaller values when the studied compounds were separated using dioxane-water mobile phase (50:50, v/v) in relation to the separation of these compounds by use of methanol-water mobile phase (50:50, v/v). Nicotinic acid is an exception to this rule. 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 using the visual method on the basis of the chromatograms. This shows that R_S values can be correctly marked first of all on the basis of the densitograms. The scientific literature data indicate that at R_S values smaller than 0.8 we cannot 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 densitogram. R_S values larger than 1.5 calculated on the basis of the densitograms were obtained for the pair of compounds $6/5+7$, $5+7/4$, and $2/1$ by use of dioxane-water in a volume composition 50:50. However, the separations of isopropyl nicotinate (4) from ethyl nicotinate (3), as well as ethyl nicotinate (3) from methyl nicotinate (2) are not good by using these chromatographic conditions. Therefore, the mixture of isopropyl nicotinate, ethyl nicotinate, and methyl nicotinate (2.66 μg of each standard) was separated on neutral aluminum oxide $60F_{254}$ and by use of an acetone-*n*-hexane mobile phase in various volume compositions. It was affirmed on the basis of $R_{S(c)}$ values calculated from the chromatograms, that the incomplete separation ($R_{S(c)} > 0.8$) of the studied compounds should be obtained on a neutral aluminum oxide $60F_{254}$ and by use of an acetone-*n*-hexane mobile phase with a volume composition of 10:90, 20:80, 30:70, and 40:60. However, the highest $R_{S(c)}$ values for the pair of studied compounds, isopropyl nicotinate (4)-ethyl nicotinate (3) and ethyl nicotinate (3)–methyl nicotinate (2) were obtained using an acetone-*n*-hexane mobile phase with a volume composition of 20:80. For comparison, the mixture of isopropyl nicotinate, ethyl nicotinate, and methyl nicotinate (2.66 μg of each standard) was also separated on $RP18WF_{254}$ plates and by use of a dioxane-water mobile phase with a volume composition of 50:50. The separation of these compounds was evaluated by the use of the visual and densitometric methods. The obtained results are presented in Table 2. The R_F values, the separation factors, and the characteristic chromatographic bands are also presented in Table 2. It was affirmed, that ΔR_F values are identical for examined compounds both by RP-HPTLC and NP-TLC techniques. In spite of this, the obtained R_S values for isopropyl nicotinate, ethyl nicotinate, and methyl nicotinate investigated by NP-TLC technique have higher values in relation to the R_S values for these compounds investigated by RP-HPTLC. The obtained R_S values using the densitometric method are considerably smaller than the R_S values obtained using the visual method. It was affirmed, that better separations of methyl nicotinate from ethyl nicotinate ($R_{S(a)(2/3)} = 1.06$), as well as ethyl nicotinate from ispropyl nicotinate ($R_{S(a)(3/4)} = 0.97$) were obtained using

Table 2. R_F values, separation factors, resolutions and characteristic of densitometric bands of investigated nicotinic acid esters by RP-HPTLC and NP-TLC techniques

		Separation factors			R_S values calculated from eqs.				Characteristic densitometric band		
Comp. no[a]	R_F	ΔR_F	α	R_F^{α}	(4)	(5)	(6)	(7)	Height (AU)	Area (AU)	β (°)
RP18WF_{254}, Dioxane-water, 50:50 (v/v)											
4	0.41								218	10206	13.5
3	0.48	0.07	1.33	1.17	1.54	0.61	0.66	0.64	179	7130	21
2	0.55	0.07	1.32	1.15	1.85	0.54	0.59	0.56	146	6447	24
Neutral aluminum oxide 60F_{254}, Acetone-*n*-hexane, 20:80 (v/v)											
2	0.54								77	4807	20
3	0.61	0.07	1.33	1.13	2.30	1.04	1.09	1.06	92	5413	16.5
4	0.68	0.07	1.36	1.11	1.80	0.94	1.00	0.97	115	6483	13
Neutral aluminum oxide 60F_{254}, Acetone-*n*-hexane, 20:80 (v/v)											
7	0.54								155	7566	8
5	0.71	0.17	2.09	1.31	5.33	2.92	2.92	2.92	134	7620	10.5

[a]The symbols of examined compounds are explained in experimental part. Chemicals and sample preparation.

an adsorption thin-layer chromatography (NP-TLC) on neutral aluminum oxide $60F_{254}$, and by use of an acetone-*n*-hexane mobile phase in volume composition of 20:80 in relation to the separations of these compounds by RP-HPTLC. The densitograms of methyl nicotinate (2), ethyl nicotinate (3), and isopropyl nicotinate (4) (2.66 μg of each standard) separated on $RP18WF_{254}$ plates using a dioxane-water mobile phase in a volume composition of 50:50, as well as on neutral aluminum oxide $60F_{254}$ using an acetone-*n*-hexane mobile phase in a volume composition of 20:80 are presented in Figure 2.

Butyl nicotinate (5) from benzyl nicotinate (7) cannot be separated by the RP-HPTLC technique. One chromatographic band is present on the

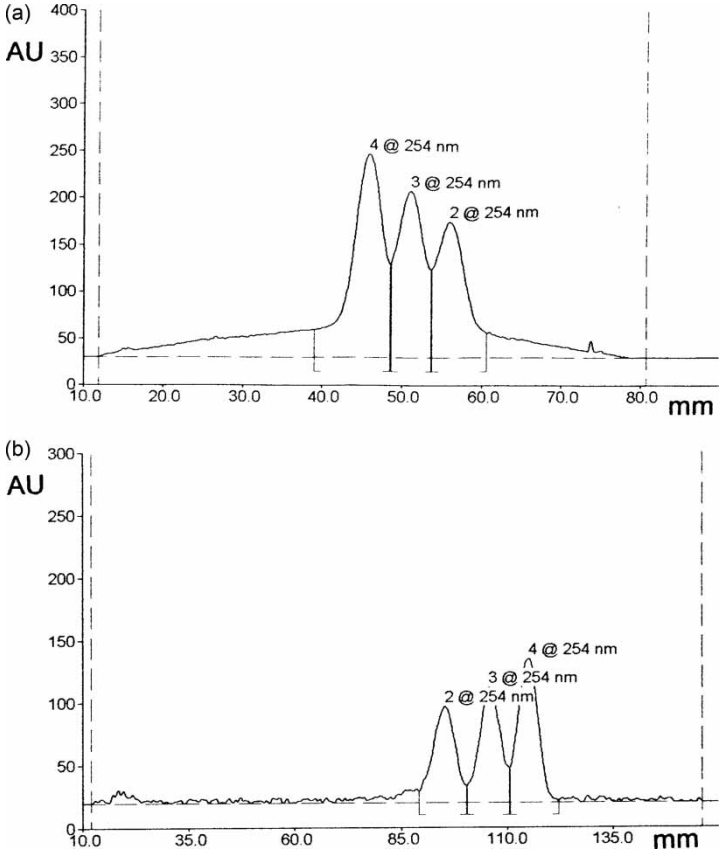

Figure 2. The densitograms of methyl nicotinate (2), ethyl nicotinate (3), and isopropyl nicotinate (4) (2.66 μg of each standard) on a) $RP18WF_{254}$ plates using a dioxane-water mobile phase with a volume composition of 50:50; b) on a neutral aluminum oxide $60F_{254}$ using an acetone-*n*-hexane mobile phase with a volume composition of 20:80.

densitograms of these compounds (Figure 1). Therefore, the mixture of butyl nicotinate and benzyl nicotinate (4 μg of each standard) was separated by the NP-TLC technique on neutral aluminum oxide $60F_{254}$ plates and by use of an acetone-*n*-hexane mobile phase in various volume compositions. It was affirmed, on the basis of $R_{S(c)}$ values calculated from the chromatograms using the visual method, that the complete separation of butyl nicotinate (5) and benzyl nicotinate (7) should be obtained on a neutral aluminum oxide $60F_{254}$ and by use of an acetone-*n*-hexane mobile phase with a volume composition of 10:90, 20:80, 30:70, and 40:60. The calculated $R_{S(b)}$, $R_{S(h)}$, i $R_{S(a)}$ values using the densitometric method also have values greater than 1.5 for the pair compounds 5/7. However, the highest R_S values calculated by use of the densitometric method were obtained for butyl nicotinate and benzyl nicotinate separated by use of an acetone-*n*-hexane mobile phase in a volume composition of 20:80. The R_F values, the separation factors, the resolutions calculated by the use of the visual and densitometric methods, and the characteristic densitometric bands for butyl nicotinate and benzyl nicotinate separated on neutral aluminum oxide $60F_{254}$, and by use of an acetone-*n*-hexane mobile phase in a volume composition of 20:80 are presented in Table 2. The densitogram of butyl nicotinate (5) and benzyl nicotinate (7) separated by use of the above mentioned chromatographic conditions is presented in Figure 3.

The obtained results unambiguously indicates that butyl nicotinate from benzyl nicotinate cannot be separated by RP-HPTLC. Good separations of isopropyl nicotinate from ethyl nicotinate, as well as ethyl nicotinate from methyl nicotinate were not also obtained by the RP-HPTLC technique. It was affirmed, that adsorption thin-layer chromatography (NP-TLC) in the system of a neutral aluminum oxide $60F_{254}$ and the acetone-*n*-hexane

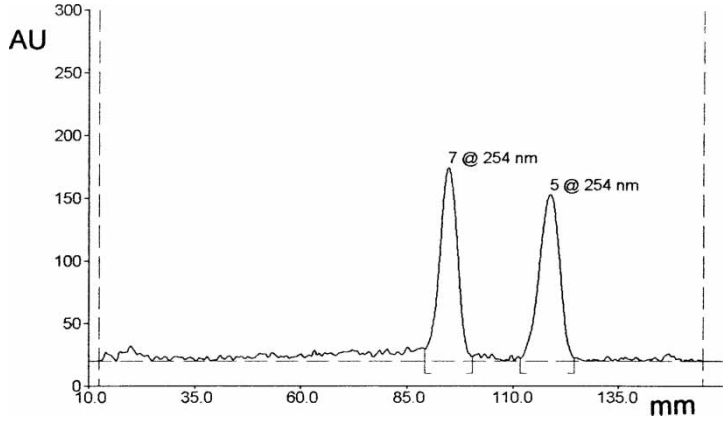

Figure 3. The densitogram of butyl nicotinate (5) and benzyl nicotinate (7) (4 μg of each standard) on a neutral aluminum oxide $60F_{254}$ using an acetone-*n*-hexane mobile phase with a volume composition of 20:80.

mobile phase in a volume composition of 20:80 provided the optimum conditions for the complete separation of butyl nicotinate (5) from benzyl nicotinate (7). These chromatographic conditions also allow better separations of methyl nicotinate from ethyl nicotinate, as well as ethyl nicotinate from isopropyl nicotinate in relation to the separations of these compounds by RP-HPTLC.

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

CONCLUSIONS

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 $R_{S(a)}$. Above mentioned parameters serving to evaluate the separation of substances were determined by the 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 a standard method to determine 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.

Further investigations are in progress and concern the separations of nicotinic acid and its other derivatives.

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