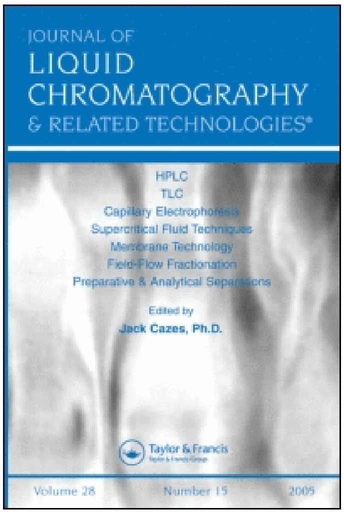

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Application of Densitometry for the Evaluation of the Separation Effect of Nicotinic Acid Derivatives. Part III. Nicotinic Acid and its Derivatives

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Abstract: Nicotinic acid (1) and its derivatives, namely nicotinamide (8), 3-pyridinecarbaldehyde (11), 3-pyridinecarbonitrile (12), 3-pyridylmethanol (13), and methyl 3-pirydyl ketone (14) 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 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 20:80. However, 3-pyridinecarbaldehyde (11) from 3-pryridinecarbonitrile (12) cannot be separated by the RP-HPTLC technique. It was also affirmed, that adsorption thin-layer chromatography (NP-TLC) in the system of a silica gel $60F_{254}$ and the acetone-*n*-hexane mobile phase in a volume composition of 80:20 provided the optimum conditions for the complete separation of 3-pyridinecarbaldehyde (11) from 3-pryridinecarbonitrile (12).

Keywords: NP-TLC, RP-HPTLC, Densitometry, Separation parameters, Nicotinic acid, Nicotinic acid derivatives

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INTRODUCTION

Nicotinic acid and nicotinamide (vitamin B_3, vitamin PP or niacin) are required for cell respiration, play an important role in the release of energy and the metabolism of carbohydrates, fat, and proteins, and are needed for proper circulation and healthy skin, for the functioning of the nervous system, and for the normal secretion of bile and stomach fluids. Nicotinic acid (1) is the oldest lipid modifying drug and induces a unique spectrum of changes in lipid and lipoprotein levels.[1] Besides its ability to decrease triglyceride and low density lipoprotein cholesterol levels, it has the strongest HDL cholesterol elevating activity among the currently available lipid modifying drugs. Nicotinamide (8) is used as a beneficial agent in the treatment of a variety of inflammatory skin disorders, such as acne vulgaris and bullous pemphigoid.[2–4] 3-Pyridinecarbaldehyde (11) is a niacin related compound, which induces apoptosis in HL-60 cells.[5] 3-Pyridinecarbonitrile (12) is the most commonly used as a precursor for nicotinamide and nicotinic acid manufacture. It is also used to produce agricultural chemicals and intermediates for the pharmaceutical industry.[6]

3-Pyridinecarbonitrile significantly irritates damaged skin and, in a solid state, produces an irreversible cornea opacity.[7] 3-Pyridylmethanol (13), also known as nicotinyl alcohol, acts directly on the walls of small blood vessels, causing them to widen. This results in increased blood supply to the extremities. 3-Pyridylmethanol is, therefore, useful in disorders of circulation, including peripheral vascular diseases, where the blood vessels in the extremities are narrowed.[8] Nicotinyl alcohol is used as a salt of tartaric or maleic acid in pharmaceutical preparations such as *Nicotol* and *Ronicol*.[9–11] Methyl 3-pyridyl ketone (3-acetylpyridine) (14), a niacinamide analog, is a potent neurotoxin which antagonizes nicotinamide for the synthesis of NAD (nicotinamide-adenine dinucleotide) and NADP (nicotinamide-adenine dinucleotide phosphate),[12] and the symptoms elicited by its administration resemble those of niacin deficiency.[13] Methyl 3-pyridyl ketone has been also shown to decrease activities of various enzymes such as tyrosine hydroxylase[14] and cysteine dioxygenase,[15] but had no effect on activities of fatty acid synthetase.[16] Furthermore, it tends to lower the concentrations of soluble proteins with high molecular mass in quail tissues, but to enhance the concentrations of soluble proteins with lower molecular mass.[17]

We have previously used normal phase thin layer chromatography and reversed-phase high performance thin layer chromatography on $RP18WF_{254}$ plates for the evaluation of separation of two groups of nicotinic acid derivatives, namely first group: nicotinic acid (1), methyl nicotinate (2), ethyl nicotinate (3), isopropyl nicotinate (4), butyl nicotinate (5), hexyl nicotinate (6), benzyl nicotinate (7),[18] and second group: nicotinic acid (1), nicotinamide (8), N-methylnicotinamide (9), N,N-diethylnicotinamide (10).[19]

The aim of this study was to use densitometry for the evaluation of the separation effects of third group: nicotinic acid (1) and its derivatives, namely nicotinamide (8), 3-pyridinecarbaldehyde (11), 3-pyridinecarbonitrile (12), 3-pyridylmethanol (13), and methyl 3-pirydyl ketone (14) investigated by adsorption and reversed-phase thin layer chromatography.

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, nicotinamide (Sigma-Aldrich, Germany), 3-pyridinecarbaldehyde, 3-pyridinecarbonitrile, 3-pyridylmethanol and methyl 3-pyridyl ketone (E. Merck, Germany) were used as test solutes. The purities of the studied standard samples were at least 98%. The mixture solution of nicotinic acid, nicotinamide, 3-pyridinecarbaldehyde, 3-pyridinecarbonitrile, methyl 3-pyridyl ketone (about a concentration of 0.67 mg mL^{-1} of each standard), and 3-pyridylmethanol (about a concentration of 0.22 mg mL^{-1}) dissolved in ethanol (POCh, Poland; 96%; analytical grade) was used in RP-HPTLC. The mixture solution of the above mentioned compounds (about a concentration of 2 mg mL^{-1} of each standard) dissolved in ethanol (POCh, Poland; 96%; analytical grade) was used in NP-TLC.

The mixture solution of the nicotinic acid and its derivatives was spotted manually using a microcapillary (Camag, Switzerland) on the chromatographic plate.

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 ± 1°C). The dioxane-water and methanol-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 cm × 20 cm glass backed, precoated with a 0.25 mm layer of a silica gel $60F_{254}$, TLC plates (E. Merck, 1.05715), on 20 cm × 20 cm aluminium backed plates precoated with 0.2 mm layer of a mixture of silica gel 60 and Kieselguhr F_{254} (E. Merck, 1.05567), and on 20 cm × 20 cm aluminium backed plates precoated with 0.2 mm layer of a neutral aluminum oxide $60F_{254}$ (Typ E) (E. Merck, 1.05550) plates. 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 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 was used as mobile phase. 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 ± 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 10.00 × 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_SCalculation 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:

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

RESULTS AND DISCUSSION

RP-HPTLC and NP-TLC techniques were used to separate mixtures of the nicotinic acid (1), nicotinamide (8), 3-pyridinecarbaldehyde (11), 3-pyridinecarbonitrile (12), methyl 3-pyridyl ketone (14). RP18WF$_{254}$ plates with spotted compounds were separated using a methanol + water and dioxane + water mobile phases in different volume compositions. Investigated compounds were separated also by use of the NP-TLC technique on silica gel 60F$_{254}$ (1.05715, Merck), a mixture of silica gel 60-Kieselguhr F$_{254}$ (1.05567, Merck), and neutral aluminum oxide 60F$_{254}$ (1.05550, Merck) plates using an acetone–*n*-hexane mobile phase in different volume compositions. The obtained results were presented as the dependence of R_F values vs. the volume composition of mobile phase. The results obtained by use of RP-HPTLC and NP-TLC techniques were presented in Figs. 1 and 2, respectively. It was affirmed, that the R_F values of the nicotinic acid are lower than 0.1 under applied chromatographic conditions using adsorption TLC. Taking into consideration R_F values, investigated compounds in adsorption TLC may be divided in three subgroups: a) first subgroup: nicotinic acid (1); b) second subgroup: nicotinamide (8) and 3-pyridylmethanol (13); c) third subgroup: 3-pyridinecarbaldehyde (11), 3-pyridinecarbonitrile (12), methyl 3-pyridyl ketone (14).

Nicotinic acid (1), nicotinamide (8) and 3-pyridylmethanol (13), on a neutral aluminum oxide 60F$_{254}$ using an acetone + *n*-hexane mobile phase with volume composition of 70:30 separate very well. The above mentioned compounds were separated from 3-pyridinecarbaldehyde (11), 3-pyridinecarbonitrile (12), and methyl 3-pyridyl ketone (14) under these conditions. But 3-pyridinecarbaldehyde (11), 3-pyridinecarbonitrile (12), and methyl 3-pyridyl ketone (14) separated on neutral aluminum oxide 60F$_{254}$, as well as a mixture of silica gel 60 and Kieselguhr F$_{254}$, using an acetone + *n*-hexane mobile phase gave one chromatographic band. It was observed that 3-pyridinecarbonitrile (12) separates from 3-pyridinecarbaldehyde (11) and methyl 3-pyridyl ketone (14) on silica gel 60F$_{254}$ using an acetone + *n*-hexane mobile phase with volume compositions of 40:60, 50:50, 60:40, 70:30, 80:20, 90:10, and 100:0. The R_F values of investigated compounds are in the range from 0.17 to 0.82. The $R_{S(c)}$ values obtained by use of the visual method were calculated using the Equation (4) for all pair compounds investigated under applied chromatographic conditions. It

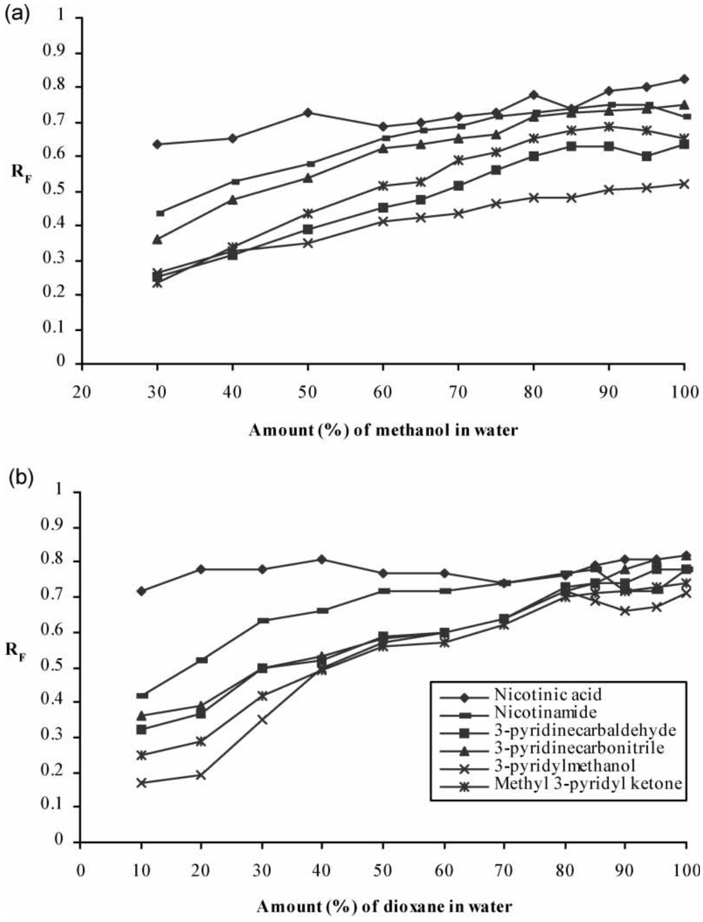

Figure 1. The dependence between R$_F$ values and the volume composition of mobile phases: a) methanol + water, b) dioxane + water for the third group of compounds (nicotinic acid and its derivatives) investigated on RP18WF$_{254}$ plates.

was affirmed, that the biggest amount of compound pairs separate completely or partly on RP18WF$_{254}$ plates using dioxane + water in volume composition 20:80 as mobile phase. However 3-pyridinecarbaldehyde (11) and 3-pyridinecarbonitrile (12) gave one chromatographic band under these chromatographic conditions. But, methyl 3-pyridyl ketone (14) separates incompletely from 3-pyridinecarbaldehyde (11) and 3-pyridinecarbonitrile (12). The R$_{S(c)}$ values obtained by use of this method were verified using densitometric analysis. The plates developed by the use of dioxane + water mobile phase in a volume composition 20:80 were densitometrically analyzed at $\lambda = 254$ nm. The R$_F$ values were obtained from the densitometric

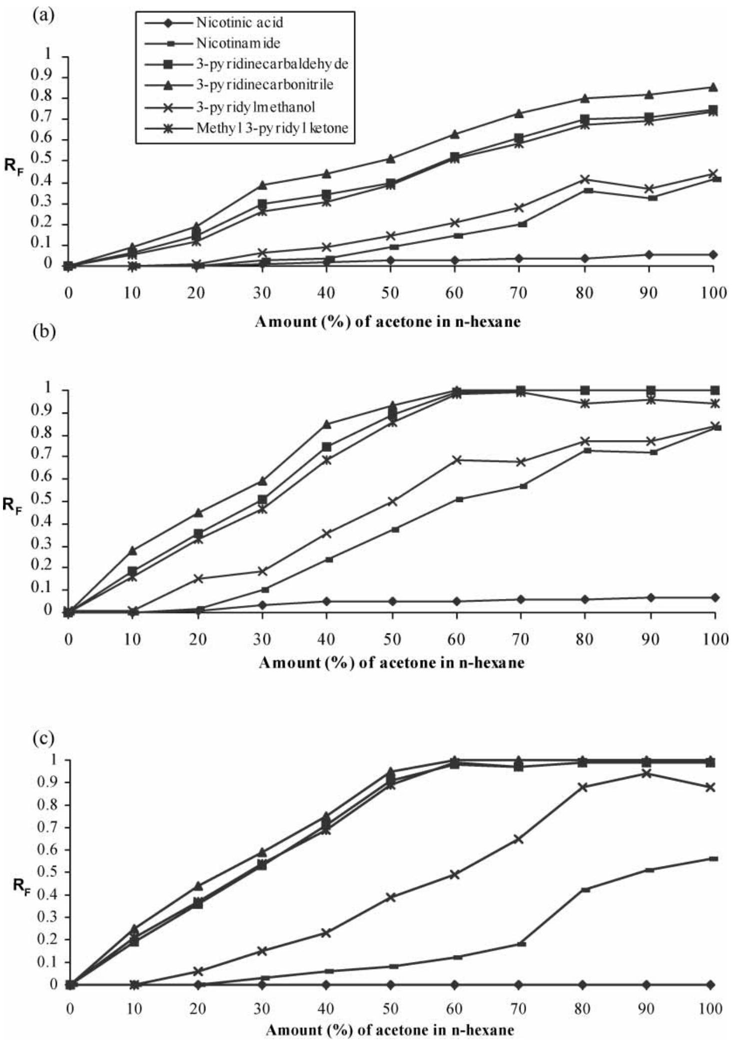

Figure 2. The dependence between R_F values and the volume composition of acetone-n-hexane mobile phases for the third group of compounds (nicotinic acid and its derivatives) investigated on: a) silica gel $60F_{254}$ (#1.05715, Merck); b) a mixture of silica gel 60-Kieselguhr F_{254} (#1.05567, Merck); c) neutral aluminium oxide $60F_{254}$ (#1.05550, Merck) plates.

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 are presented in Table 1.

Table 1. R_F values, separation factors, resolutions and characteristic of denstitometric bands of the third group of compounds (nicotinic acid and its derivatives) investigated by RP-HPTLC and NP-TLC techniques

		Separation factors			R_S values calculated from eqs.				Characteristic of densitometric band		
Comp. no.	R_F	ΔR_F	α	$R_F\alpha$	(4)	(5)	(6)	(7)	Height (AU)	Area (AU)	β (°)
$RP18WF_{254}$, Dioxane+water, 20:80 (v/v) (Compounds No.:1, 8, 11, 12, 14 in amount of 0.67 μg, and compound No 13 in amount of 0.22 μg)											
13	0.17								71	4324	33
14	0.31	0.14	2.19	1.82	1.41	1.33	1.31	1.32	112	4473	15
11 + 12	0.37	0.06	1.31	1.19	0.87	0.68	0.64	0.66	109	4890	18
8	0.50	0.13	1.70	1.35	1.81	1.45	1.41	1.44	94	3405	16
1	0.79	0.29	3.76	1.58	3.91	3.09	3.07	3.08	58	3138	40
Silica gel $60F_{254}$, Acetone+n-hexane, 80:20 (v/v) (Compounds No.: 11 and 12 in amount of 2 μg)											
11	0.70								93	7583	22
12	0.82	0.12	1.95	1.17	2.91	1.45	1.43	1.44	48	3107	29

where: nicotinic acid (1), nicotinamide (8), 3-pyridinecarbaldehyde (11), 3-pyridinecarbonitrile (12), 3-pyridylmethanol (13), methyl 3-pyridyl ketone (14).

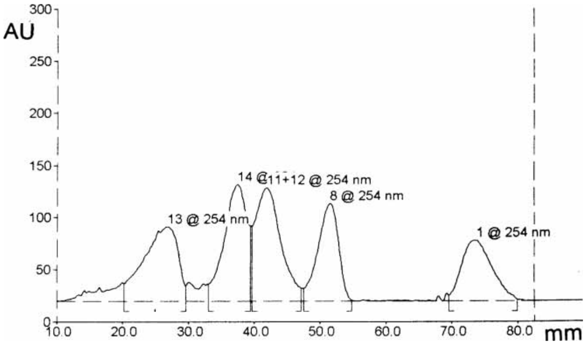

Figure 3. The densitogram of the studied compounds on a RP18WF$_{254}$ plates using a dioxane + water mobile phase with a volume composition of 20:80.

The average $R_{S(a)}$ values calculated by use of Equation (7) and the characteristic densitometric peaks are also presented in Table 1. The characteristics of the densitometric peaks concern their height, area, and the angle (β) between the tangents at the inflection points to the curves of the densitometric peaks. 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 densitogram of the studied compounds on RP18WF$_{254}$ plates and the use of dioxane + water mobile phase in a volume composition 20:80 are presented in Figure 3. However, these conditions do not provide for the separation of 3-pyridinecarbaldehyde (11) and 3-pyridinecarbonitrile (12). These studied compounds (each in

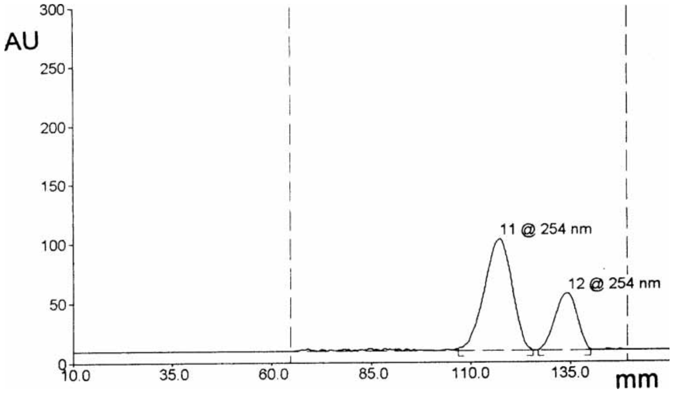

Figure 4. The densitogram of the studied compounds on a silica gel 60F$_{254}$ plates (#1.05715, Merck) using a dioxane + water mobile phase with a volume composition of 80:20.

amount of 2 μg) were then separated on silica gel $60F_{254}$ glass plates and by use of an acetone + *n*-hexane mobile phase with a volume composition of 80:20. The resolution values were calculated by use of visual and densitometric methods. The obtained results are presented in Table 1. It was affirmed, that 3-pyridinecarbaldehyde (11) separates from 3-pyridinecarbonitrile (12) under these conditions. The $R_{S(a)}$ value of these substances is equal to 1.44. The densitogram of the studied compounds on silica gel $60F_{254}$ glass plates and using an acetone + *n*-hexane mobile phase with a volume composition of 80:20 is presented in Figure 4.

It was generally affirmed, that NP-TLC and RP-HPTLC can be used for the separation of the third group of investigated compounds.

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