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ANALYSIS

A COMPARISON OF CHROMATOGRAPHIC SEPARATION OF SELECTED NICOTINIC ACID DERIVATIVES BY TLC AND HPLC TECHNIQUES

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Abstract: The separations of nicotinic acid and its derivatives analyzed by adsorption and reversed phase TLC and HPLC were compared. Results were presented as the dependencies of chromatographic retention parameters of studied compounds on the volume composition [%] of mobile phases. It was shown that there exists the possibility of the separation of compounds into specified groups (adsorption TLC and HPLC) and particular substances within individual groups (reversed phase TLC).

Keywords: TLC, HPLC, RPTLC, RPHPLC, nicotinic acid, nicotinic acid derivatives

Properties, application and separation of nicotinic acid and its derivatives have been the subject of chromatographic investigations (1–17). The high performance liquid chromatography technique deserves special attention. The HPLC method was applied for the determination of a small amount of niacin in foodstuffs (exactly in several kinds of vinegar and jam). The results of this analysis were compared with microbiological methods (6) By application of HPLC, niacin was also separated from Italian almond cultivators (5). The simultaneous determination of free nicotinic acid and niacinamide in meats was studied too (16). Nicotinamide was also successfully determinated in elemental diet by column – switching high performance liquid chromatography with a UV detector (8). And finally, an experimental system of nicotinamide was used to examine the effects of varying chromatographic resolutions on the quantitative accuracy of the curve method (13). Nicotinic acid (niacin, B_3 vitamin, pyridine–3–carboxylic acid) and nicotinamide (PP vitamin) are, e.g., widely distributed in living cells where they exist mainly in bound forms as part of nicotinamide adenine dinucleotides (NAD) that are coenzymes of dehydrogenases taking part in the transport of hydrogen (18–19). Nicotinamide can be produced from nicotinic acid or synthesized by microorganisms (among other things by bacterial flora). PP vitamin is of great importance for human organism. Its shortage causes weakness, headaches, apathy and pellagra (18). One should also take esters of nicotinic acid into consideration; some of them, for instance, methyl nicotinate, ethyl nicotinate, isopropyl nicotinate, hexyl nicotinate and benzyl nicotinate, are used as ingredients of pharmaceutical creams. They enhance the topical penetration of the active substances (20–21).

In our earlier work (22), we applied the separation factor (α) as well as the cluster analysis to evaluate the separation of chosen derivatives of nicotinic acid separated by means of TLC.

The aim of this work was a comparison of the separation of nicotinic acid and its derivatives using adsorption and reversed phase TLC and HPLC.

EXPERIMENTAL

Chemicals

Unless otherwise specified all chemicals used were of analytical grade. All solvents were obtained from E. Merck (Darmstadt, Germany). Solutions of commercial samples of nicotinic acid (NAC), methyl nicotinate (MN), ethyl nicotinate (EN), isopropyl nicotinate (IPN), butyl nicotinate (BN), hexyl nicotinate (HN), benzyl nicotinate (BNN), nicotinamide (NAM), N–methylnicotinamide (MNAM), and N,N–diethylnicotinamide (DENAM) (E.Merck, Darmstadt, Germany) were prepared in ethanol (99.8%).

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Thin Layer Chromatography

Adsorption Thin–Layer Chromatography

Adsorption TLC was performed on 20 cm × 20 cm glass–backed precoated silica gel $60F_{254}$ TLC plates (#5715, E. Merck, Darmstadt, Germany) and on aluminium–backed plates coated with a mixture of silica gel 60 and Kieselguhr F_{254} (#5567) and with polyamide 11 (#5555). Prior to use, the plates were activated at 120°C for 20 min. Normal–phase chromatography on silica gel and on the silica gel–Kieselguhr mixture was performed with a mixtures of acetone and n–hexane in different volume proportions as mobile phase. Mixtures of benzene and methanol in different volume proportions were used as mobile phase on polyamide 11 plates.

Reversed Phase – Thin Layer Chromatography

Reversed–phase TLC was performed on 10 cm × 10 cm C_{18s} reversed phase HPTLC plates (#1.15389, E. Merck, Darmstadt, Germany). Methanol and mixtures of methanol and water in the volume proportions 9+1, 8+2, and 7+3 were used as mobile phases.

Each nicotinic acid derivative (40 mg) was dissolved in 10 ml of ethanol. Solutions (5 µl) were spotted on chromatographic plates.

The plates were developed at room temperature in a classical flat bottom chamber (Camag, Switzerland) previously saturated for 30 min. The development distances were 15 cm and 8 cm for adsorption and partition TLC, respectively. The spots were detected under UV illumination at λ=254 nm.

High – Performance Liquid Chromatography

Investigations were conducted on a chromatograph (Knauer) with a UV – visis detector and an injector (Laboratorni Pristoje Praha). 0.01% solutions (20 µl) of the studied compounds were injected onto the chromatographic column. The detection was made at a wavelength of 254 nm.

Adsorption HPLC

The LiChrospher Si 60 column (5 µm) 250 mm in length (E. Merck), was applied. The mixtures of benzene and methanol in the volume proportions 0+10, 1+9, 2+8, 3+7, 4+6, and 5+5 were used as the mobile phases. The flow – rate was 0.8 ml/min.

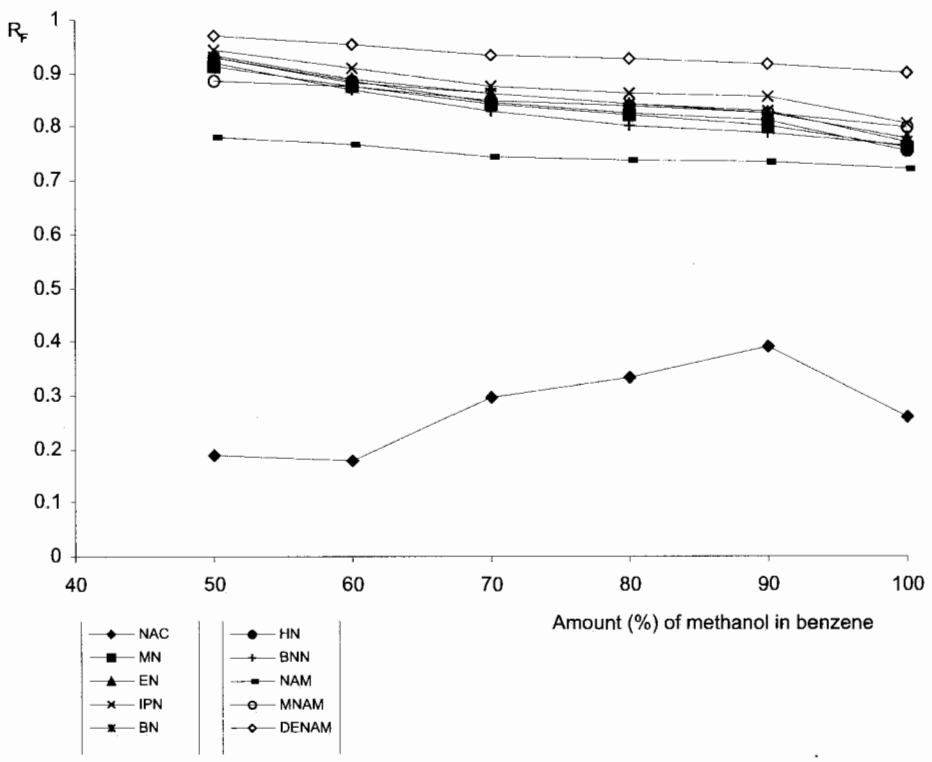

Figure 1. R_F values of the nicotinic acid derivatives on polyamide 11 plates with methanol – benzene mobile phases. The abbreviations are as given in „Experimental" section.

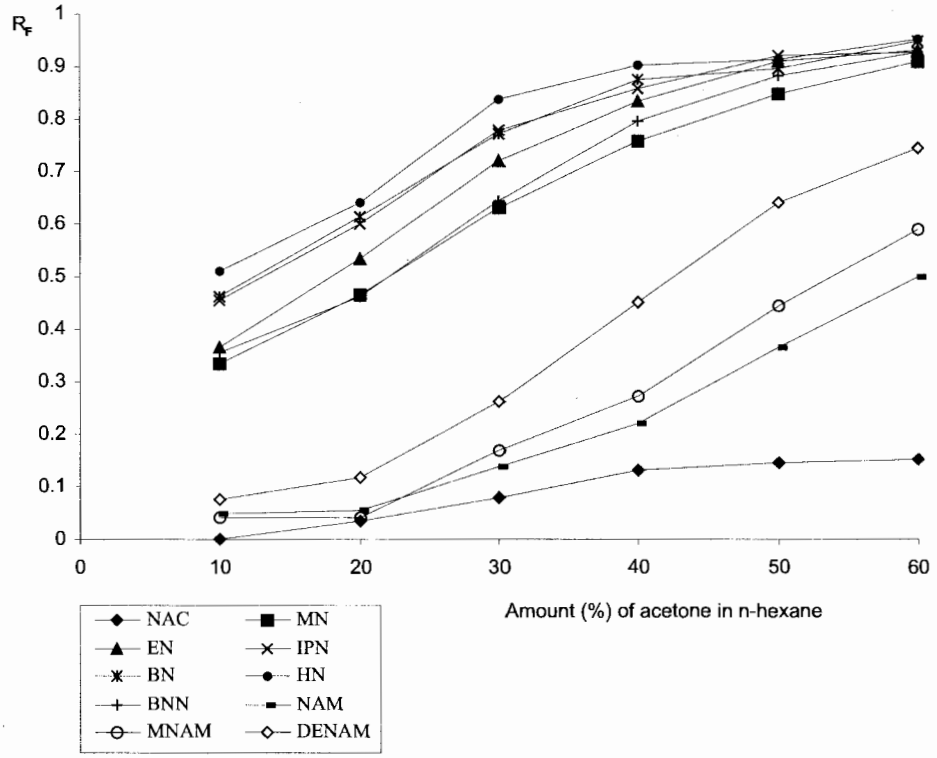

Figure 2. R_F values of the nicotinic acid derivatives on a mixture of silica gel–Kieselguhr plates with acetone – n–hexane mobile phases. The symbols are as given in Figure 1.

Reversed – phase HPLC

The C18 column 250 mm (E. Merck) in length, was used. The mixtures of methanol and water in the volume proportions 10+0, 9+1, 8+2, and 7+3 were used as the mobile phase. The pressure was changed from 4.3 MPa to 12.6 MPa. The flow – rate was 0.8 ml/min.

RESULTS AND DISCUSSION

The dependence of R_F values of the studied derivatives of nicotinic acid on the volume composition [%] of methanol in the benzene–methanol mobile phase with the application of polyamide stationary phase is shown in Figure 1. The separation of the investigated substances into the nicotinic acid, nicotinamide and N, N–diethylnicotinamide was observed. The remaining compounds create one chromatographic band. The best separation was obtained using the methanol – benzene mobile phase in the volume proportions 60+40, 70+30, 80+20, 90+10, and 100+0. The poorest separation was obtained using the methanol – benzene mobile phase (50+50, v/v). In this case only the nicotinic acid and nicotinamide were separated from one another.

Figure 2 shows the dependence of the R_F values of the studied derivatives of nicotinic acid on the volume composition [%] of acetone in the acetone – n–hexane mobile phase. As a stationary phase the silica gel – Kieselguhr mixture was applied. The stationary phase mentioned above and the acetone – n–hexane mobile phase in the volume proportion 40+60 separated studied compounds into three groups, i.e. nicotinic acid, amides (nicotinamide, N–methylnicotinamide, N,N–diethylnicotinamide) and esters that make a more compact group. However, in the group of these esters one can separate: methyl nicotinate, benzyl nicotinate, ethyl nicotinate, butyl nicotinate + isopropyl nicotinate and hexyl nicotinate. The separation mentioned above is the optimal one. The separation of the studied compound into nicotinic acid, nicotinamide, N,N–diethylnicotinamide, N–methylnicotinamide was also obtained using acetone – n–hexane in the volume proportions 30+70, 50+50, and 60+40 as mobile phases. Moreover, at the composition of the mobile phase 30+70, the separation of methyl nicotinate and benzyl nicotinate from ethyl nicotinate, from butyl nicotinate and isopropyl nicotinate as well as hexyl nicotinate is

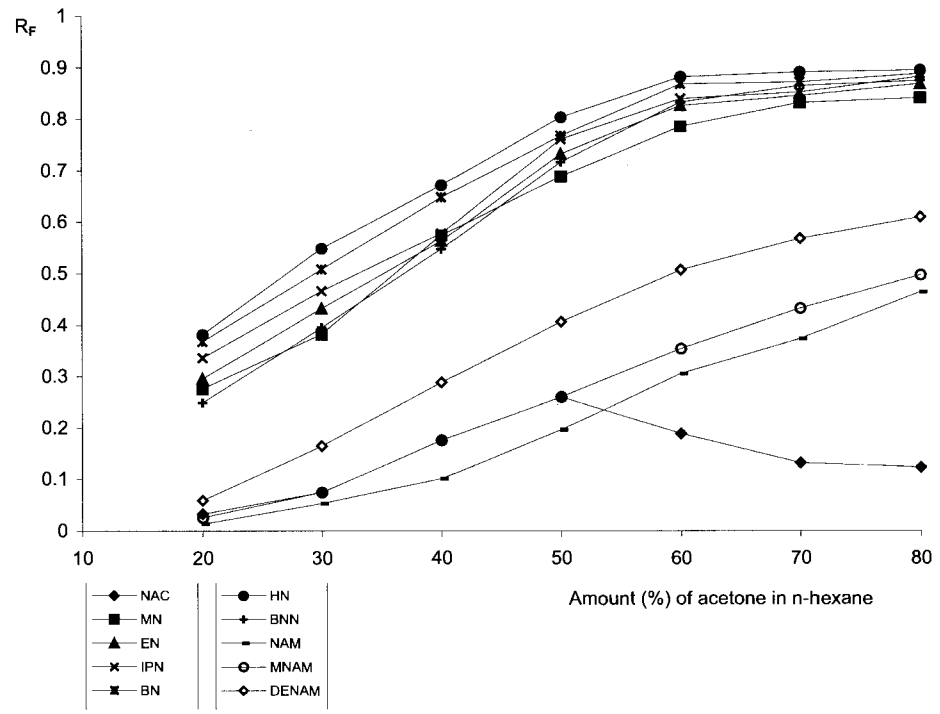

Figure 3. R_F values of the nicotinic acid derivatives on silica gel plates with acetone – n–hexane mobile phases. The symbols are as given in Figure 1.

possible. At the composition of the mobile phase 50+50 one can separate methyl nicotinate from the remaining esters, which make one chromatographic band. The separation of particular esters is not possible using the acetone – n–hexane mobile phase in the volume proportion 60+40. Finally, the poorest separation was obtained using the acetone – n–hexane mobile phase in the volume proportions 10+ 90 and 20+80. In no case did one manage to separate isopropyl nicotinate and butyl nicotinate from one another.

The dependence of the R_F values of the studies derivatives of nicotinic acid on the volume composition [%] of acetone in the acetone – n–hexane mobile phase with the application of silica gel as a stationary phase is shown in Figure 3. This figure presents that one can separate the studied compounds into three groups: nicotinic acid, amides and esters (the separations of the substances within the groups are also possible). On the basis of the studied dependences it appears that the best separation was obtained using the acetone – n–hexane mobile phase in the volume proportion 60+40. It is the separation into nicotinic acid, nicotinamide, N–methylnicotinamide, N,N–diethylnicotinamide and methyl nicotinate. The remaining esters have similar R_F values. The separation into nicotinic acid, and particular amides is also possible using the acetone – n–hexane mobile phase in the volume proportions 70+30, and 80+20. However, at such a composition of the mobile phase one cannot separate particular esters, which make one compact group. It was observed that it is possible to separate nicotinic acid, nicotinamide + N–methylnicotinamide, N,N–diethylnicotinamide, butyl nicotinate + hexyl nicotinate from one another using the acetone – n–hexane mobile phase in the volume proportion 20+80. Instead, the acetone – n–hexane mobile phase (30 + 70, v/v) makes it possible to separate nicotinic acid, nicotinamide, and N–methylnicotinamide from N,N–diethylnicotinamide. This composition of the mobile phase makes also the separation of the largest number of esters possible. These esters are ethyl nicotinate, isopropyl nicotinate, butyl nicotinate and hexyl nicotinate. Only benzyl nicotinate and methyl nicotinate cannot be separated from one another. Finally, the poorest separation was obtained using acetone – n–hexane mobile phase in the volume proportion 40+ 60.

The dependence of the R_F values of the studied derivatives of nicotinic acid on the volume composition [%] of methanol in the methanol – water mobile phase obtained on the RP–18 stationary

phase is shown in Figure 4. The best separation was obtained using methanol – water mobile phase (80+20, v/v). Such a composition of the mobile phase made the separation of all studied compounds possible excluding benzyl nicotinate and butyl nicotinate which, in these conditions, could not be separated. A good separation was also acquired at the composition of the methanol – water mobile phase (70+30, v/v). In these conditions, only butyl nicotinate and benzyl nicotinate, together with nicotinic acid and nicotinamide could not be separated as these compounds had similar R_F values. Undoubtedly, the poorest separation was obtained using the methanol – water mobile phase (90+10, v/v). It is the separation into hexyl nicotinate, butyl nicotinate + benzyl nicotinate, ethyl nicotinate + methyl nicotinate + isopropyl nicotinate, N,N–diethylnicotinamide, N–methylnicotinamide as well as nicotinic acid and nicotinamide. Using methanol (100%) as the mobile phase only the separation of the studied compounds into three groups was obtained. The first group is hexyl nicotinate; the remaining esters and N,N–diethylnicotinamide make the second group; and the third group is nicotinic acid together with the remaining amides. It is the porest separation since particular groups make one chromatographic band in which one cannot separate its compounds.

The dependence of the retention time (T_r) values of the studied derivatives of nicotinic acid by the HPLC technique on the volume composition [%] of benzene in the benzene – methanol mobile phase is shown in Figure 5. As a stationary phase Si 60 was applied. Figure 5 shows that the separation of the studied compounds into three groups is possible. The first group is nicotinic acid; amides make the second group; and esters create the third group (in suitable conditions there is also the possibility of separation between particular substances in the group). The best separation was acquired using the benzene + methanol mobile phase in the volume proportions 50+50, and 60+40. In these conditions, one can separate nicotinic acid, nicotinamide, N–methylnicotinamide, N,N–diethylnicotinamide and methyl nicotinate from one another; the remaining esters have similar retention times. Moreover, a good separation was also obtained using this mobile phase in the volume proportion 70+30. In these conditions, one can manage to separate nicotinic acid, particular amides, methyl nicotinate and hexyl nicotinate. Using the benzene + methanol mobile phase in the volu-

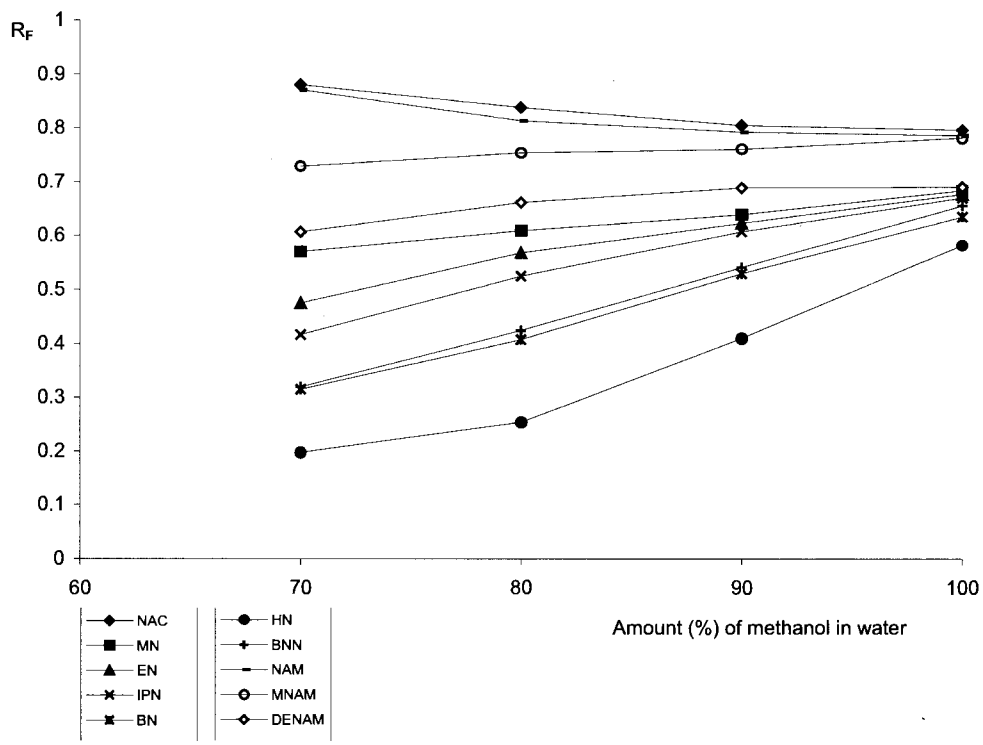

Figure 4. R_F values of the nicotinic acid derivatives on RP–18 plates with methanol – water mobile phases. The symbols are as given in Figure 1.

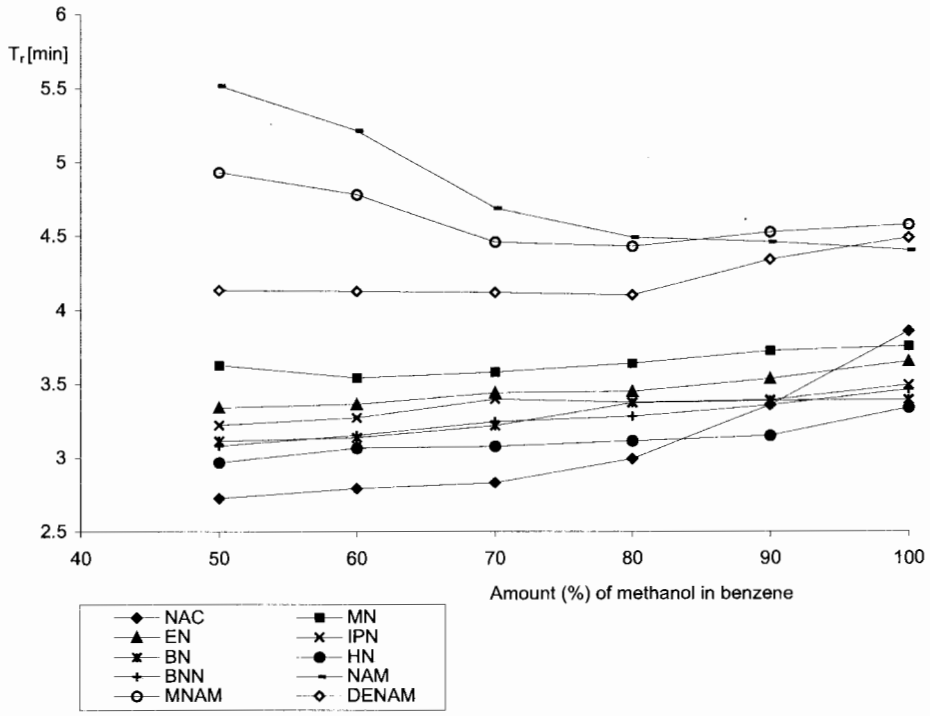

Figure 5. T_r values of the nicotinic acid derivatives on LiChrospher Si60 column with methanol – benzene mobile phases. The symbols are as given in Figure 1.

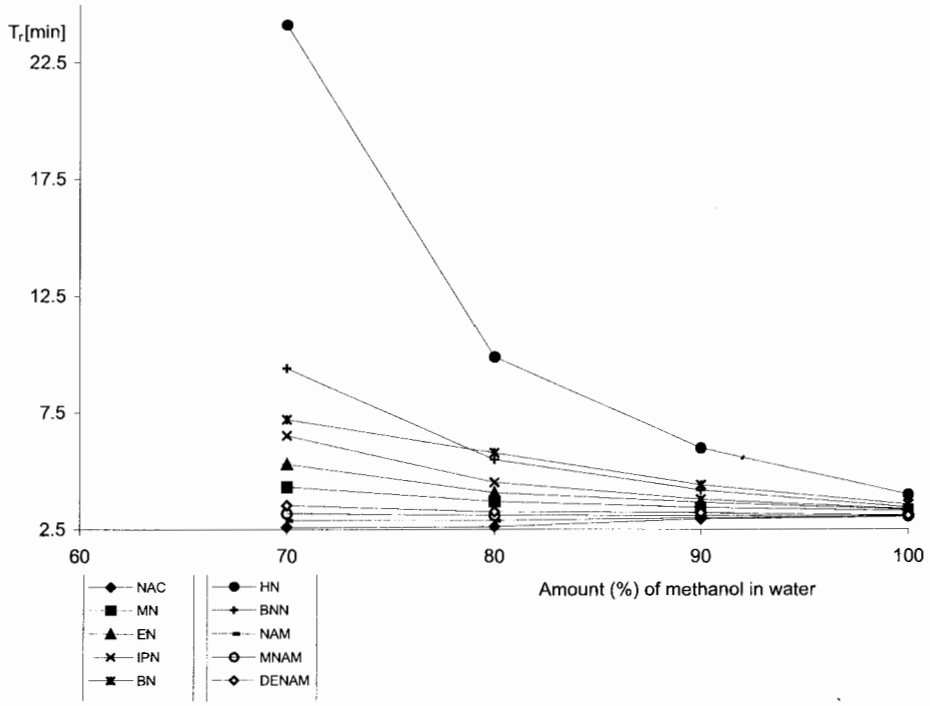

Figure 6. T_r values of the nicotinic acid derivatives on C18 column with methanol – water mobile phases. The symbols are as given in Figure 1.

me proportions 80+20, and 90+10 poor separations were obtained. In the first case, N,N–diethylnicotinamide, methyl nicotinate, nicotinic acid, hexyl nicotinate and N–methylnicotinamide as well as nicotinamide were separated from the remaining esters, which make one chromatographic band. In the second case, methyl nicotinate, ethyl nicotinate and hexyl nicotinate were separated; the remaining esters, similarly to amides, make a dense group together with nicotinic acid. The poorest separation was obtained using the mobile phase containing 100% of benzene. In these conditions, the studied compounds were separated into two groups. Amides belong to the first group, whereas esters and nicotinic acid make the second group. However, one did not manage to separate particular substances in any of these groups.

Figure 6 presents the dependence of the retention time (T_r) of the investigated compounds by RPHPLC on the volume composition [%] of methanol in the methanol – water mobile phase. The stationary phase was C18. The best separation was obtained using the methanol – water mobile phase in the volume proportion 70+30. In these conditions, the investigated compounds were separated into the hexyl nicotinate and benzyl nicotinate. The remaining compounds have similar T_r values and cannot be separated. Using the methanol – water mobile phases in the volume proportions 80+20, and 90+10, only hexyl nicotinate can be separated from the remaining substances. No separation was obtained using 100% methanol as the mobile phase. In these conditions, all compounds make one compact group having similar T_r values.

Further investigations are continued and concern the elaboration of such chromatographic conditions that will make the separation of all the investigated esters of nicotinic acid possible.

CONCLUSIONS

1. It was shown that there exist the possibility of the separation into specified groups of compounds (adsorption TLC and HPLC) and particular substances within individual groups (RP–18 TLC). It was shown simultaneously that both TLC and HPLC assure a similar separation of the studied nicotinic acid derivatives in particular chromatographic conditions.

2. The worked out chromatographic conditions of the separations of the investigated nicotinic acid derivatives can be used to their detection and determination in various biological samples.

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