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# HIGH-PERFORMANCE THIN-LAYER CHROMATOGRAPHY OF 2,4-DINITROPHENYL-AMINO ACIDS ON LAYERS OF RP-8, RP-18 AND AMMONIUM TUNGSTOPHOSPHATE

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## SUMMARY

The chromatographic characteristics of eighteen DNP-amino acids, of dinitrophenol and dinitroaniline were studied on RP-8 and RP-18 plates eluted with aqueous–organic solutions and with mixtures of organic solvents. The optimum conditions for the separation of the highest number of compounds were studied and a two-dimensional chromatogram is reported. On home-made layers of ammonium tungstophosphate, water, aqueous solutions of ammonium nitrate and nitric acid and water–methanol mixtures were used as eluents to separate the pairs of DNP-amino acids which exhibit the same chromatographic behaviour on RP-8 and RP-18 plates.

## INTRODUCTION

In the separation of 2,4-dinitrophenyl (DNP)-amino acids, chromatographic techniques have extensively been employed[1] for the identification of N-terminal amino acids and, therefore, for the determination of protein and peptide structure. Thin-layer chromatography of DNP-amino acids has been carried out on layers of cellulose[2,3], polyamide[4–6], silica gel[7,8] and wool cortical cells[9]. Recently, plates of silanized silica gel alone or impregnated with anionic and cationic detergents, and also layers of ammonium tungstophosphate, were successfully used in the separation of amino acids[10,11], peptides[12,13] and aromatic amines[14]. It was therefore of interest to investigate the behaviour of DNP-amino acids and to complete the studies carried out by the other researchers.

On layers of silanized silica gel, the two-dimensional technique can be used, eluting in the two directions with aqueous and non-aqueous mobile phases, so that the adsorption properties of the layer are changed and better separations can be achieved[15]. Besides water-soluble and ether-soluble DNP-amino acids, we examined also 2,4-dinitrophenol and 2,4-dinitroaniline, which are formed by photolysis or in the dinitrophenylation of the N-terminal amino acids.

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## EXPERIMENTAL

Standard solutions were prepared by dissolving the DNP-amino acids (Serva, Heidelberg, G.F.R.) in water–methanol (1:2). The sample volume used was 0.2–0.3 $\mu$l in the case of RP-8 and RP-18 plates (E. Merck, Darmstadt, G.F.R.) and 0.5 $\mu$l for the ammonium tungstophosphate layers. The compounds were visualized by exposure of the silanized silica gel layers to UV light (360 nm with dried plates or 254 nm with wett ones). On ammonium tungstophosphate the spots were identified by their yellow colour which deepens on exposure to ammonia vapours. The preparation of the last layers was carried out as described previously[14].

The migration distance was 6 cm in the case of RP-8 and RP-18 plates and 10 cm in the case of ammonium tungstophosphate, unless otherwise stated. All the measurements were carried out at 25°C using a Desaga thermostatic chamber.

The following abbreviations are used: DNP = dinitrophenyl; Gly = glycine; Ala = alanine; Ser = serine; Thr = threonine; Val = valine; Leu = leucine; Ile = isoleucine; Pro = proline; Met-$O_2$ = methionine sulphone; Trp = tryptophan; Phe = phenylalanine; Tyr = tyrosine; Asp = aspartic acid; Glu = glutamic acid; $CuSO_3Na$ = sodium cysteate; Lys = lysine; Arg = arginine; His = histidine; DNP-OH = dinitrophenol; DNP-$NH_2$ = dinitroaniline.

## RESULTS AND DISCUSSION

### *Silanized silica gel layers (RP-8 and RP-18)*

Table I lists the chromatographic characteristics of eighteen DNP-amino acids, dinitrophenol and dinitroaniline on RP-8 and RP-18 plates eluted with 1 *M* acetic acid in 60% methanol. The elution time in both cases is 90 min. Similar results are obtained on the two layers; in the case of RP-18 plates, however, more compact spots and a better resolution of the compounds are achieved. Subsequent experiments were therefore performed on RP-18 layers.

Since the DNP-amino acids contain a very weak[19] basic group, the use of more acidic eluents than those of column 2 (such as 1 *M* acetic acid + 1 *M* hydrochloric acid in 60% methanol) does not result in large differences in the chromatographic behaviour of most compounds, since the species in solution remain practically the same. In contrast, an increase of the apparent pH of the eluent, involving the deprotonation of the carboxyl group, results in different retentions of the compounds, with the exception of dinitroaniline.

The most remarkable differences in the chromatographic behaviour of the DNP-amino acids with respect to the acid medium are observed for elution with 1 *M* ammonia in 60% methanol. Under such conditions most compounds run with the solvent front or are less strongly retained than with acidic solutions owing to the presence in their molecules of one or more negative charges. The only exception is $\alpha$-N-DNP-Arg, which is more strongly retained than in acidic solution and, as the behaviour of DNP-$NH_2$ shows, also with decreasing percentage of the organic solvent in the eluent, owing to the replacement of acetic acid with ammonia. With the alkaline eluent, the elution time increases from 90 to 100 min.

The chromatographic behaviour of the DNP-amino acids on RP-18 plates eluted with aqueous–organic solutions seems to be controlled by a reversed-phase

partition mechanism since their affinity towards the stationary phase decreases the higher is the polarity of the compound (see the $R_F$ sequence of the series Ser, Thr, Gly, Ala, Val, Leu). The lower retention of a given compound having positive or negative charges compared to the neutral form supports this assumption.

The influence of the ionic strength on the chromatographic characteristics of the DNP-amino acids is shown by the data of columns 4 and 5; these data were obtained by adding 3% potassium chloride to the eluents of columns 2 and 3. The presence of this salt in the eluent does not result in large differences in $R_F$ values in acidic media, where the compounds are predominantly in the non-ionic form. In alkaline solution, a remarkable increase of the affinity towards the stationary phase for most DNP-amino acids and, particularly, for those with more marked hydrophobic characteristics, is observed. This can probably be ascribed to increasing hydrophobic interactions between the compounds having a negative charge and the stationary phase, and is similar to the behaviour of phenols on layers of silanized silica gel ($C_2$) alone or impregnated with anionic detergents[16], and on layers of Dowex 40-X4 ($Na^+$)[17].

The dependence of the $R_F$ values of DNP-amino acids on the ionic strength of the eluent in alkaline media can be used to obtain or to improve separations among the different compounds. The importance of this ionic strength effect had already been pointed out by us[18] in the separations of polypeptides on RP-2 plates.

With non-aqueous eluents, such as hexane–ethyl acetate–acetic acid (see columns 6 and 7 of Table I), interesting results are achieved, since the elution time decreases to 15 min and the affinity sequence of the DNP-amino acids is completely different from that observed with aqueous–organic eluents. The content of acetic acid is the same (2%) in both eluents employed and accounts for the compact spots observed. As the percentage of the non-polar compound, hexane is increased the $R_F$ values is generally decreased without a change in the affinity sequence of the DNP-amino acids.

With both eluents, the compounds with marked polar characteristics, such as DNP-$CySO_3Na$ and α-N-DNP-Arg, remain at the application point, while those with marked hydrophobic characteristics, such as DNP-OH, DNP-Leu and DNP-Ile, run practically with the solvent front. Furthermore, the sequence of $R_F$ values for members of homologous series (*e.g.*, Ser, Thr, Gly, Ala, Val and Leu) is opposite to that found with aqueous–organic eluents.

In order to explain the behaviour of DNP-amino acids in non-aqueous solvents we can assume that the free –OH groups of the silanized silica gel participate in an adsorption mechanism involving a stationary phase with polar characteristics[15]. Even the different solubilities of the DNP-amino acids in the mobile phase may affect the retention of those compounds such as $CySO_3Na$ and α-N-DNP-Arg, which are soluble only in polar solvents and which remain at the starting point when eluting with non-aqueous solutions containing high percentages of non-polar hydrocarbons.

Since the separation of all or most of the DNP-amino acids cannot be achieved with only one elution, either with aqueous–organic or non-aqueous eluents, we used the two-dimensional technique. The data of Table I show that the optimum conditions for the separation of the highest number of compounds can be achieved by eluting in the first direction with hexane–ethyl acetate–acetic acid (80:18:2) (see column 7) and in the second direction with 1 *M* ammonia + 3% potassium chloride in 60% methanol (see column 4).

TABLE I

$R_F$ VALUES OF DNP-AMINO ACIDS ON RP-8 AND RP-18 PLATES WITH DIFERENT ELUENTS

| *Compound* | *1 M Acetic acid in 60% methanol* | | *1 M $NH_3$ in 60% methanol RP-18* | *3% KCl + 1 M acetic acid in 60% methanol RP-18* | *3% KCl + 1 M $NH_3$ in 60% methanol RP-18* | *Hexane–ethyl acetate - acetic acid (75:23:2) RP-18* | *Hexane–ethyl acetate– acetic acid (80:18:2) RP-18* |
|---|---|---|---|---|---|---|---|
| | *RP-8* | *RP-18* | | | | | |
| 1 DNP-Gly | 0.62 | 0.57 | 0.86 | 0.58 | 0.68 | 0.71 | 0.56 |
| 2 DNP-Ala | 0.49 | 0.43 | 0.85 | 0.47 | 0.60 | 0.86 | 0.73 |
| 3 DNP-Ser | 0.74 | 0.70 | 0.89 | 0.71 | 0.78 | 0.37 | 0.25 |
| 4 DNP-Thr | 0.64 | 0.62 | 0.87 | 0.63 | 0.69 | 0.48 | 0.35 |
| 5 DNP-Val | 0.32 | 0.27 | 0.78 | 0.27 | 0.40 | 0.96 | 0.87 |
| 6 DNP-Leu | 0.22 | 0.17 | 0.68 | 0.18 | 0.26 | 0.99 | 0.94 |
| 7 DNP-Ile | 0.22 | 0.17 | 0.69 | 0.19 | 0.27 | 0.99 | 0.94 |
| 8 DNP-Pro | 0.52 | 0.53 | 0.87 | 0.52 | 0.62 | 0.83 | 0.72 |
| 9 DNP-Met-$O_2$ | 0.74 | 0.69 | 0.91 | 0.72 | 0.79 | 0.15 | 0.08 |
| 10 DNP-Trp | 0.33 | 0.30 | 0.83 | 0.30 | 0.43 | 0.85 | 0.71 |
| 11 DNP-Phe | 0.27 | 0.22 | 0.72 | 0.22 | 0.32 | 0.92 | 0.86 |
| 12 Di-DNP-Tyr | 0.16 | 0.10 | 0.48 | 0.10 | 0.12 | 0.66 | 0.51 |
| 13 DNP-Asp | 0.70 | 0.68 | 0.91 | 0.71 | 0.88 | 0.41 | 0.29 |
| 14 DNP-Glu | 0.65 | 0.62 | 0.91 | 0.64 | 0.88 | 0.49 | 0.36 |
| 15 DNP-$CySO_3Na$ | 0.94 | 0.91 | 0.92 | 0.85 | 0.93 | 0.00 | 0.00 |
| 16 Di-DNP-Lys | 0.24 | 0.18 | 0.64 | 0.20 | 0.26 | 0.57 | 0.38 |
| 17 α-N-DNP-Arg | 0.70 | 0.68 | 0.59 | 0.80 | 0.71 | 0.00 | 0.00 |
| 18 Di-DNP-His | 0.48 | 0.43 | 0.85 | 0.35 | 0.54 | 0.00 | 0.00 |
| 19 DNP-OH | 0.53 | 0.51 | 0.91 | 0.52 | 0.75 | 0.99 | 0.96 |
| 20 DNP-$NH_2$ | 0.48 | 0.48 | 0.35 | 0.51 | 0.41 | 0.92 | 0.75 |

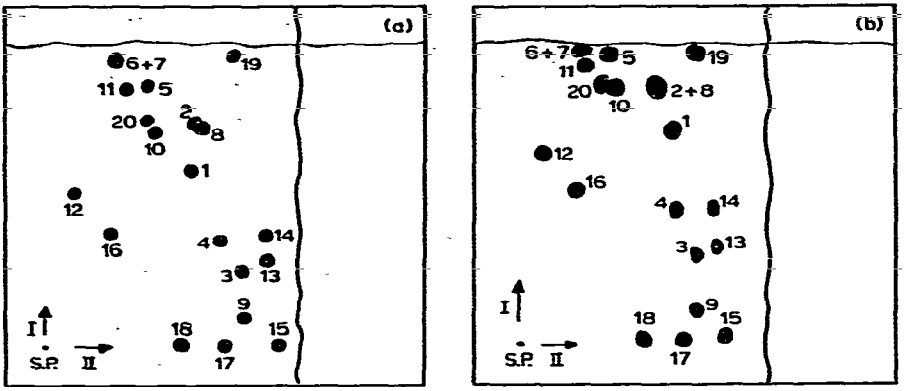


Fig. 1. Theoretical (a) and experimental (b) two-dimensional chromatograms on RP-18 plates. Eluents: in the first direction, hexane–ethyl acetate–acetic acid (80:18:2); in the second direction, 1 *M* ammonia + 3% potassium chloride in 60% methanol. DNP-amino acids as in Table I. S.P. = Starting point.

Fig. 1a indicates the theoretical separation and Fig. 1b shows the experimental one obtained with a mixture of all twenty compounds. This separation is on the whole better than those previously reported[2–9].

*Ammonium tungstophosphate (AWP) layers*

The data obtained on layers of AWP–$CaSO_4 \cdot \frac{1}{2}H_2O$ (0.5:2, 2:2, 4:2 and 8:2) eluted with aqueous and aqueous–organic solutions showed that, as in the case of amino acids[11] and primary aromatic amines[12,13], the DNP-amino acids are more strongly retained with increasing concentration of AWP on the layer. Compact spots, however, are observed only with AWP–$CaSO_4 \cdot \frac{1}{2}H_2O$ (4:2 and 8:2).

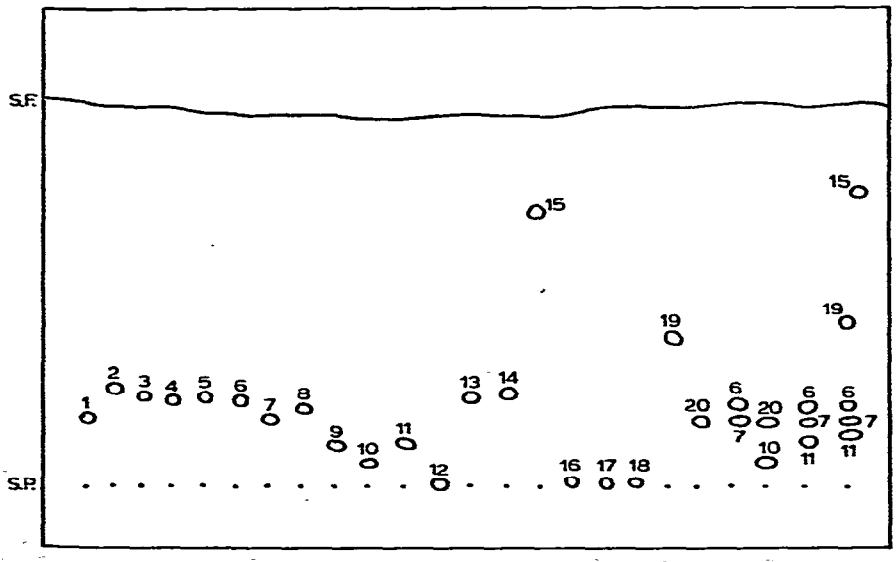


Fig. 2. Thin-layer chromatogram on AWP–$CaSO_4 \cdot \frac{1}{2}H_2O$ (4:2). Eluent: water–methanol (80:20). Elution time; 150 min. DNP-amino acids as in Table I. S.P. = Starting point; S.F. = solvent front.

Fig. 2 shows the chromatogram obtained on AWP–$CaSO_4 \cdot \frac{1}{2}H_2O$ (4:2) eluted with water–methanol (80:20 v/v). On replacing this eluent with water, solutions of ammonium nitrate and nitric acid, the sequence of the DNP-amino acids does not change. The compounds are strongly retained with the above eluents, with the exception of DNP-$CySO_3Na$ which is weakly retained when eluting with water or with water–methanol (80:20), whereas it exhibits a higher affinity towards the stationary phase in the presence of nitric acid or ammonium nitrate (*e.g.*, 1 *M*). The solubility of the compounds affects their chromatographic behavior; all three di-DNP-amino acids remain at the starting point when eluting with water and with water–methanol (80:20), owing to their low solubility.

Fig. 2 also shows some separations which are important from an analytical point of view, such as those concerning pairs of DNP-amino acids which are difficult to separate (Ala/Pro and Leu/Phe) or exhibit the same chromatographic behaviour on RP-18 plates under all experimental conditions (Leu/Ile).

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