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HIGH-PERFORMANCE THIN-LAYER CHROMATOGRAPHIC PRE-COATED PLATES WITH AMINO MODIFICATION AND SOME APPLICATIONS

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SUMMARY

High-performance thin-layer chromatographic pre-coated plates incorporating chemically bonded alkylamino groups have been developed. Their chromatographic properties are largely determined by the alkylamino groups chemically bonded to the surface of the silica gel. Because of the basic pK value of the amino group in aqueous media, the coating can be looked upon as a weak, basic anion exchanger. Polyvalent anions, *e.g.*, nucleotides or sulphonic acids, are retained according to their charge number: the more negative charges the molecule has the greater is the retention. The usage of these pre-coated plates is not restricted to aqueous solvent systems. The way in which organic solvents of various polarities can be used is demonstrated in a number of separations of steroids and phenols.

INTRODUCTION

In recent years non-polar reversed-phase (RP) sorbents have become increasingly important not only in high-performance liquid chromatography (HPLC) but also in thin-layer chromatography (TLC) and HPTLC. However, the available non-polar stationary phases are of a varying and, in most cases, fairly pronounced hydrophobic character depending on the alkyl or aryl groups bound to their surfaces. Such stationary phases are suitable for separating non-polar or only slightly polar substances by RP chromatography[1–3].

Beside the non-polar packing materials, in column chromatography use is made of polar-bonded stationary phases where the functional groups may of the diol, cyano or amino types. These stationary phases are used to separate hydrophilic or charged substances.

In analogy to the above hydrophilic modified stationary phases, an HPTLC pre-coated plate has now been developed in which amino groups are bonded to silica gel, *viz.*, the HPTLC NH_2 F254s pre-coated plate. Experimental separations are described which demonstrate the properties, chromatographic behaviour and potential applications of this plate.

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EXPERIMENTAL

Plates and solvents

The newly developed HPTLC NH_2 F254s pre-coated plate (Cat. No. 15647) was tested against HPTLC pre-coated plates, silica gel 60 F 254 (Cat. No. 5628), RP-2 F 254s (Cat. No. 13726), RP-8 F 254s (Cat. No. 13725) and RP-18 F 254s (Cat. No. 13724) from E. Merck (Darmstadt, G.F.R.). The solvents used (methanol, ethanol, acetone, chloroform, dichloromethane and acetonitrile, all from E. Merck) were of LiChrosolv® quality.

Samples and applied quantities

The following test substances from E. Merck were used: nucleoside polyphosphates (cAMP, AMP, ADP and ATP*), dinucleoside polyphosphates (NAD and $NADP^+$), naphthalenemono- and polysulphonic acids and phenols (*m*-cresol, 1-naphthol, hydroquinone, resorcinol and phloroglucinol). The steroids hydrocortisone, methyltestosterone and Reichstein's substance S were obtained from Serva (Heidelberg, G.F.R.). The individual substances were applied in quantities of between 100 and 300 nl with the aid of a Hamilton syringe. The concentrations of the solutions lay between 1 and 3 mg/ml. Evaluation was *in situ* using a TLC/HPTLC scanner with monochromator from Camag (Muttenz, Switzerland).

RESULTS AND DISCUSSION

Characteristics of the HPTLC NH_2 F254s pre-coated plate

The newly developed NH_2 F254s plate is based on the same silica gel 60 used in existing HPTLC silica gel 60 pre-coated plates and modified RP-2, RP-8 and RP-18 pre-coated plates from E. Merck. The layer incorporates an acid-stable fluorescent indicator which responds at 254 nm with a light blue fluorescence. The mean particle diameter of the sorbent as well as its narrow particle size distribution enable a high packing density and extremely good surface homogeneity on the plate, so that chromatograms can be evaluated *in situ* by optical means.

As in LiChrosorb NH_2 packing material for HPLC, the amino group takes the form of a γ-propylamino function bonded chemically to the surface of the silica gel. The chromatographic properties of the NH_2 F254s plate are largely governed by the polar, basic nature of the NH_2 group. Comparison of the pK values of short-chain aliphatic amines in aqueous solution[4] shows that the pK value of the amino group bonded to the silica gel should be in the order of pK = 9.5–11. The HPTLC NH_2 F254s plate is hence to be regarded as a weak, basic ion-exchanger plate[5].

Various additional surface properties result, however, from the nature of the modification. The alkylamino groups bonded to the silica gel surface may enter into weak hydrophobic interactions with sample substances of appropriate composition. Furthermore, selectivity may be affected by non-reacting silanol groups.

Two series of tests were conducted in order to categorize the polarity of the

* cAMP = cyclic adenosine 3′:5′-monophosphate; AMP = adenosine 5′-monophosphate; ADP = adenosine 5′-diphosphate; ATP = adenosine 5′-triphosphate; NAD = nicotinamide–adenine dinucleotide; NADP = nicotinamide–adenine dinucleotide phosphate.

amino-modified plate. The retention of three steroids (hydrocortisone, methyltestosterone and Reichstein's substance S) was investigated on HPTLC pre-coated silica gel 60, RP-2, RP-8, RP-18 and NH_2 plates using two different solvent systems, namely acetone–water (60:40) and chloroform–methanol (93:7). Fig. 1 shows the dependence of the R_F values of the three steroids on the type of plate used, comparing the two solvent systems. The test substances are seen to behave differently in each case. With the polar, aqueous eluent acetone–water (60:40) (Fig. 1a), the test substances are not separated on the silica gel plate nor on the amino-modified plate. In both cases they are eluted with the solvent front. Separation occurs, however, on the reversed-phase plates. The R_F values of the steroids decrease with increasing chain lengths of the reversed-phase layers. The elution sequence on RP plates arranged in order of increasing R_F values is: methyltestosterone, Reichstein's substance S and hydrocortisone. The opposite behaviour occurs when the non-polar eluent chloroform–methanol (93:7) is used (Fig. 1b). There is no retention on the RP plates and all three steroids are eluted with the solvent front. On the two polar plates, on the other hand, there are marked differences in retention. On the amino plate the R_F values of the three steroids are somewhat larger than on the silica gel 60 plate, although no distinct difference with regard to selectivity is discernible. On both polar plates, hydrocortisone has the smallest R_F value, followed by Reichstein's substance S and methyltestosterone with the highest value. The sequence is thus the exact opposite of that using reversed-phase plates with acetone–water as eluent. As the two diagrams in Fig. 1 show, the polarity of the amino plate is similar or possibly lower than that of silica gel 60. Its lipophilic character is less pronounced, however, than that of the HPTLC RP plates.

Owing to its hydrophilic modification, the amino plate can be wetted with pure water without added organic solvents or salts. Development is possible with pure water or with solvents of high water content. Furthermore, pure organic solvents or mixtures of any composition can be employed (see Fig. 2 and Table I).

Fig. 2 illustrates the velocity coefficient, κ, as a function of the water content of various solvent systems (methanol–water, ethanol–water, acetone–water and

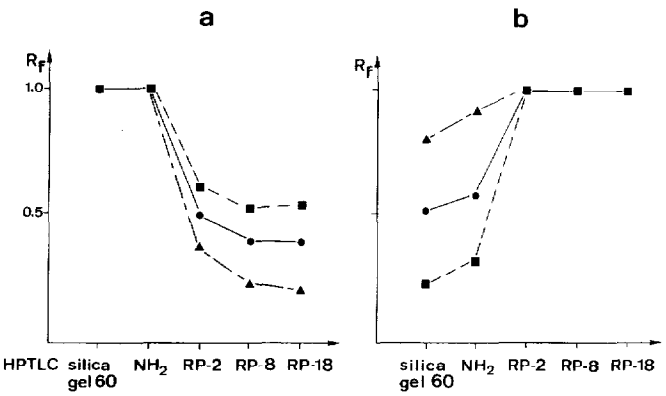

Fig. 1. R_F values of hydrocortisone (■), Reichstein's substance S (●) and methyltestosterone (▲) as a function of the type of HPTLC pre-coated plate used. Migration distance, z_f: 5 cm. Normal chamber without chamber saturation. Eluent: a, acetone–water (60:40); b, chloroform–methanol (93:7). Detection: UV, 254 nm.

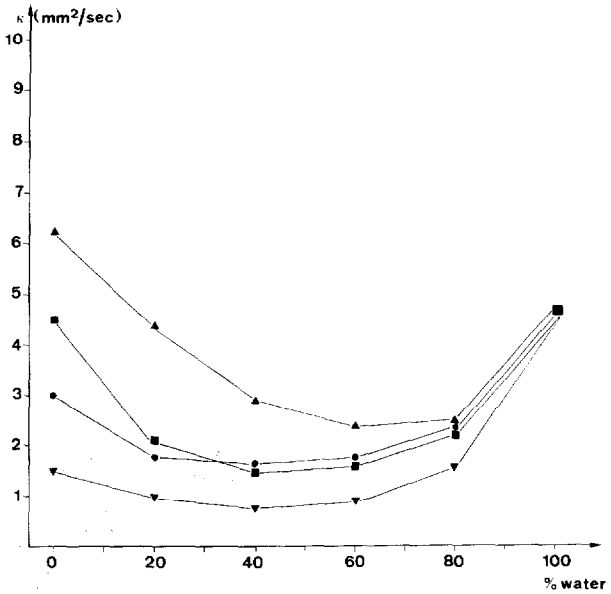

Fig. 2. Velocity coefficient, κ, as a function of the water content of the solvent system, for aqueous mixtures of methanol (●), ethanol (▼), acetone (■) and acetonitrile (▲). Plate: HPTLC NH_2 F254s, migration distance, z_f: 5.0 cm; normal chamber without chamber saturation.

acetonitrile–water mixtures). Curves with a minimum are obtained in all four cases. Of the solvents tested, acetonitrile–water has the highest κ values. The minimum for this curve is located at 70–80% of water. The minima for the other eluents are found at lower water contents, lying at between 40 and 60% of water. The κ values for the pure organic eluents methanol, ethanol and acetone are smaller than those for pure water.

In contrast, it is not possible to wet HPTLC RP plates with pure water. Indeed, for these plates only solvents with a relatively high proportion of organic components

TABLE I

κ VALUES (mm^2/sec) OF SOLVENTS ON VARIOUS HPTLC PRE-COATED LYAERS

The solvents are arranged in order of increasing polarity according to the eluotropic series of Halpaap and Ripphahn[6]. Migration distance, z_f = 5.0 cm; normal chamber without chamber saturation.

Solvent	*HPTLC pre-coated plate*				
	Silica gel 60	*NH_2*	*RP-2*	*RP-8*	*RP-18*
Heptane	3.2	3.6	3.5	3.7	3.8
Toluene	3.3	3.9	3.4	3.8	4.2
Chloroform	2.9	3.3	3.0	2.9	3.3
Acetonitrile	5.2	6.2	5.7	5.4	5.9
Acetone	3.9	4.5	4.7	4.9	5.9
Ethanol	1.5	1.5	1.4	1.4	1.6
Methanol	2.9	3.0	2.7	2.7	3.2
Water	—	4.7	—	—	—

or pure organic solvents are feasible. A comparison of the flow properties of the NH_2 F254s plate with those of the RP and silica gel 60 plates for HPTLC showed no essential difference in κ values for the five plates (see Table I). It is seen that amino modification of the silica gel results in little change in the flow properties as compared to those of the HPTLC silica gel 60 pre-coated plate.

Chromatography on the HPTLC NH_2 F254s pre-coated plate

As described, the chromatographic properties of the HPTLC NH_2 F254s pre-coated plates are mainly determined by the surface alkylamino groups. Hence it is to be expected that there will be special polyanion selectivity in aqueous solvents. Measurements were made using adenosine and its polyphosphates (cAMP, AMP, ADP and ATP) as well as naphthalenepolysulphonic acids in order to investigate this characteristic with regard to ion-exchanger properties. The solvents selected were mixtures of an alcohol (methanol or ethanol) and water as well as the components *per se*. Initial investigations did give the expected selectivity, but the spots formed were diffuse. However, addition of sodium chloride to the eluent suppresses secondary interactions and the consequent spreading effect.

The retention characteristics of adenosine, cAMP, AMP, ADP and ATP on an HPTLC NH_2 F254s pre-coated plate are shown in Fig. 3 as a function of the eluent water content in the methanol–water system. In each case 0.18 mol/l sodium chloride was added to the eluent. On account of the limited solubility of this salt in solvent systems of low water contents, saturated solutions were employed in the case of methanol as well as of methanol–water (90:10, 80:20 and 70:30). It is seen that an

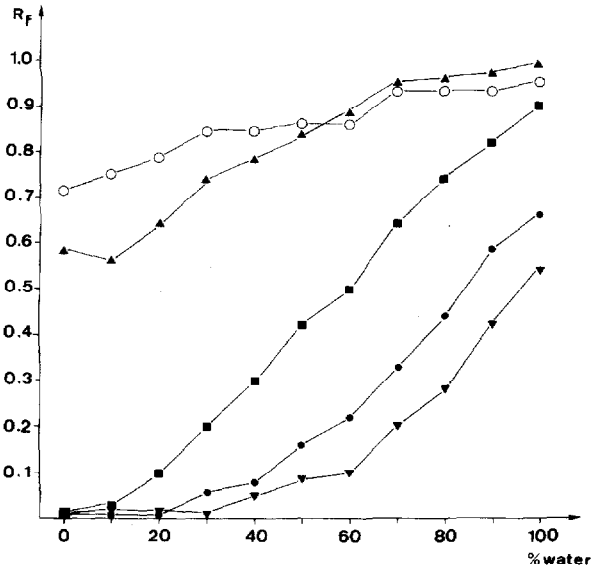

Fig. 3. R_F values of adenosine and adenosine mono- and polyphosphates as a function of the water content of the solvent system. Plate: HPTLC NH_2 F254s pre-coated plate. Eluent: methanol–water ranging from 0:100 to 100:0 with addition of 0.18 mol/l NaCl. Migration distance, z_f: 5 cm. Normal chamber without chamber saturation. Solutes: ○, adenosine, ▲, cAMP; ■, AMP; ●, ADP; ▼, ATP. Detection: UV, 254 nm.

increase in R_F value occurs with increasing water content of the solvent, both for negatively charged adenine nucleotides and for adenosine. The substances tested are highly polar and readily soluble in water. As the polarity of the eluent increases, so the nucleoside and nucleotide interactions with the stationary phase decrease, leading to a rise in R_F values. This rise in R_F values, and, in particular, the change in adenosine retention with increasing solvent polarity cannot be explained using a reversed-phase model of retention. The alkylamino chains can only undergo weak hydrophobic interactions with the nucleoside and the nucleotides, this being reflected in the high R_F of adenosine in this solvent system.

The elution sequence is determined chiefly by the number of the negative charges in the test substances. The charge number of the nucleotides varies from -1 to -4 in the order cAMP, AMP, ADP and ATP. The interaction of the weak basic ion-exchanger groups with the nucleotides of higher negative charge are stronger than with nucleoside phosphates of lower charge. Hence the elution sequence is fixed and, accordingly, the nucleotide cAMP shows least retention and ATP the most. Nucleotides undergo similar retention on PEI cellulose plates[7–9] or in column chromatography with anion exchangers[10–12].

The chromatographic separation of adenosine polyphosphates and of two dinucleoside polyphosphates (NAD and NADP) on an HPTLC NH_2 F254s plate is illustrated in Fig. 4. The nucleotides cAMP, AMP, ADP and ATP are retained to degrees determined by their charge numbers. NAD with a formal total charge number of -1 appears between cAMP and AMP. NADP has a formal triple negative charge

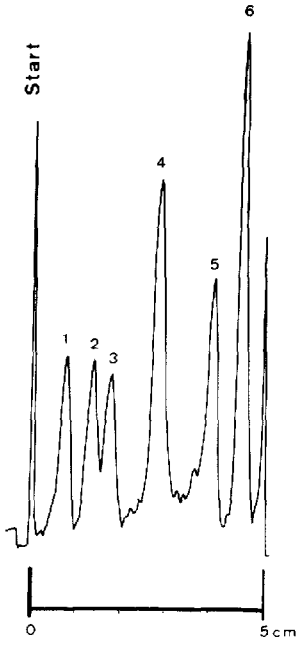

Fig. 4. Separation of various adenine nucleotides and polyphosphates on HPTLC NH_2 F254s pre-coated plate. Eluent: ethanol–water (30:70) with 0.2 mol/l NaCl. Migration distance, z_f: 5 cm. Normal chamber without chamber saturation. Detection: *in-situ* evaluation with a TLC/HPTLC scanner (Camag); UV, 254 nm. Peaks: 1 = ATP; 2 = ADP; 3 = NADP; 4 = AMP; 5 = NAD; 6 = cAMP.

but has a larger R_F value than ADP. At the neutral pH employed here, dinucleoside polyphosphates occur as zwitterions[13–15]. It is this zwitterionic character which explains the retention data of these substances relative to mononucleoside polyphosphates of the same total charge number.

Another way in which the retention of charged substances can be varied is by altering the pH of the eluent by addition of inorganic acids or bases. A splendid example of this is shown in Fig. 5, for the R_F values of anthracene and naphthalene-mono-, naphthalenedi- and naphthalenetrisulphonic acid. The eluent used was ethanol–water (40:60) plus 0.18 mol/l NaCl. The pH of the aqueous component was varied from 1 to 12. Adjustment in the acidic range was performed with hydrochloric acid and in the alkaline range with sodium hydroxide solution; in a second test series, ammonium hydroxide solution was used for adjustment in the alkaline range. Sulphonic acids are extremely strong acids and it can be assumed that in the pH range employed complete dissociation took place and that the charge number coincided with the number of sulphonic acid groups.

As in Fig. 3, the retention sequence of naphthalenesulphonic acids on the HPTLC NH_2 F254s pre-coated plate is determined by the number of their negative charges. Also the R_F values decrease with increasing number of sulphonic acid groups. Changing the pH with hydrochloric acid or sodium hydroxide solution has no effect on the R_F values of the five test substances. If, on the other hand, the pH in the alkaline range is adjusted with ammonium hydroxide solution, then the R_F

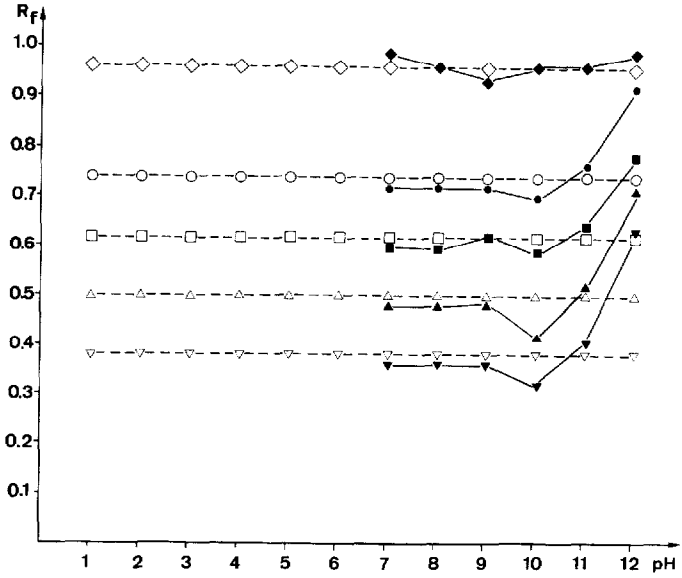

Fig. 5. The R_F values of anthracene and various naphthalenesulphonic acids as a function of the pH of the eluent. Plate: HPTLC NH_2 F254s pre-coated plate. Eluent: ethanol–water (40:60) with 0.18 mol/l NaCl; pH = 1–7 adjusted with hydrochloric acid (open symbols), pH = 8–12 adjusted with sodium hydroxide solution (open symbols), pH = 8–12 adjusted with ammonium hydroxide solution (filled symbols). Migration distance, z_f: 5 cm. Normal chamber without chamber saturation. Detection: UV, 254 nm. Solutes: ◇ ◆, anthracene; ○ ●, naphthalene-1-sulphonic acid; □ ■, naphthalene-1,5-disulphonic acid; △ ▲, naphthalene-1,3,6-trisulphonic acid; ▽ ▼, naphthalene-1,3,7-trisulphonic acid.

curves of the anions exhibit faintly discernible minima at pH 10 followed by a pronounced increase in R_F up to pH 12.

As is shown by the lack of effect achieved with sodium hydroxide solution, the rise in R_F values with increasing ammonium hydroxide concentration is not pH-dependent. Indeed, what is involved is a competition for the sulphonic acids between the free ammonium ions of the solvent and the alkylammonium ions bonded to the silica gel surface. A rise in the amount of ammonium hydroxide present in the eluent enables more and more free ammonium ions to associate with the sulphonic acids, whereby the naphthalenesulphonic acids are increasingly prevented from interacting with the stationary phase. The ultimate result is the observed rise in R_F values. Anthracene, a substance without charge, is not influenced either by a pH change or a variation of the ammonium hydroxide concentration in the eluent. The R_F value, which in any case is high, shows no change over the entire pH range investigated.

A similar example is demonstrated in Fig. 6 for the separation of some benzenecarboxylic acids. The mechanism of retention follows the same principle as discussed for the nucleotides and sulphonic acids respectively. The compound with the

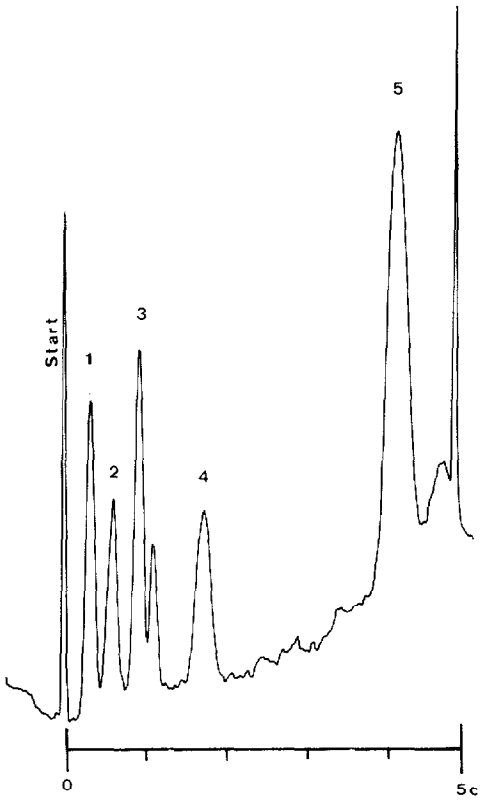

Fig. 6. Separation of some benzenecarboxylic acids on HPTLC NH_2 F254s pre-coated plate. Eluent: ethanol–ammonia pH 12 (60:40) with 0.18 mol/l NaCl. Migration distance, z_f: 5 cm. Normal chamber without chamber saturation. Detection: *in-situ* evaluation with TLC/HPTLC scanner (Camag); UV, 254 nm. Peaks: 1 = pyromellitic acid; 2 = benzene-1,2,3-tricarboxylic acid; 3 = benzene-1,2,4-tricarboxylic acid; 4 = phthalic acid; 5 = benzoic acid.

largest number of negative charges, pyromellitic acid, has the smallest R_F value and the singly charged benzoic acid is eluted near the solvent front. The other benzenecarboxylic acids with two or three carboxylic groups have intermediate retentions.

We have already described the ion-exchanger properties exhibited when aqueous solvents are used. It is also possible, however, to utilize the basic properties of the amino plate to separate polar substances by using pure organic solvents. Fig. 7 shows the separation of various phenols with acetone–chloroform (50:50). The chromatogram demonstrates a pronounced grouping effect. The monovalent phenols *m*-cresol and 1-naphthol have the highest R_F values, between 0.5 and 0.7; hydroquinone and resorcinol, both of which are bivalent species, have R_F value between 0.1 and 0.3; trivalent phloroglucinol remains at the point of application. This retention sequence is governed not only by the varying solubilities of the phenols in the solvent, but predominantly by the increasing interaction with the stationary phase as the number of hydroxyl groups in the molecule increases.

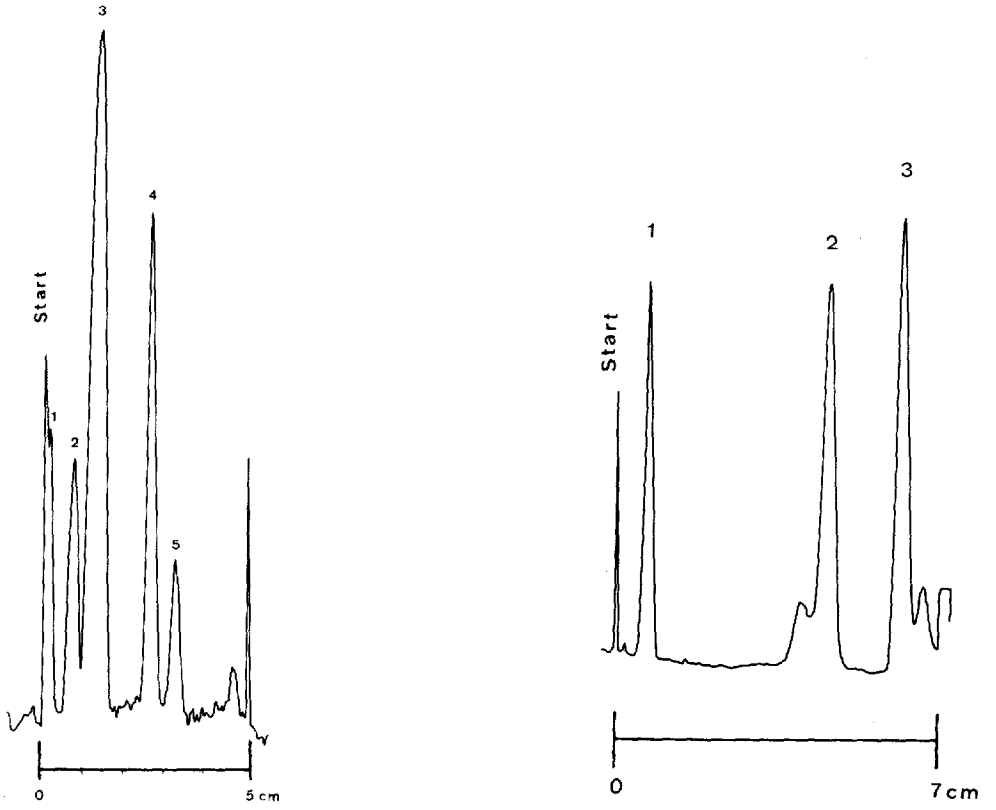

Fig. 7. Separation of various phenols on HPTLC NH_2 F254s pre-coated plate. Eluent: acetone–chloroform (50:50). Migration distance, z_f: 5 cm. Normal chamber without chamber saturation. Detection: *in-situ* evaluation with a TLC/HPTLC scanner (Camag); UV, 254 nm. Peaks: 1 = phloroglucinol; 2 = resorcinol; 3 = hydroquinone; 4 = 1-naphthol; 5 = *m*-cresol.

Fig. 8. Separation of the N-methyl derivatives of xanthine on HPTLC NH_2 F254s pre-coated plate. Eluent: methanol–acetonitrile–chloroform (10:80:10). Migration distance, z_f: 7 cm. Normal chamber without chamber saturation. Detection: *in-situ* evaluation with TLC/HPTLC scanner (Camag); UV, 254 nm. Peaks: 1 = theophylline; 2 = theobromine; 3 = caffeine.

A final example of the use of HPTLC pre-coated plate NH_2 F254s with non-aqueous solvents as eluent is the separation of the N-methyl derivatives of xanthine. A mixture of methanol, acetonitrile and chloroform was employed as mobile phase (see Fig. 8). The chromatogram demonstrates that the separation occurs without problems and with high selectivity.

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