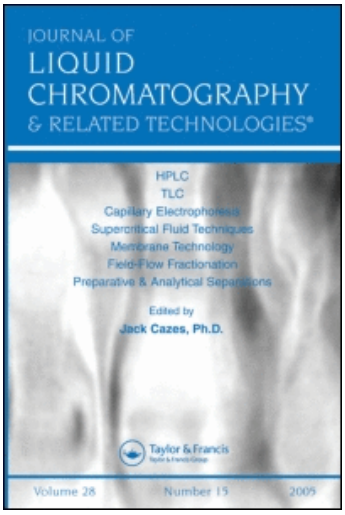

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THIN-LAYER CHROMATOGRAPHY OF PYRIDINIUM ALDOXIMES USING DISTINCT TECHNIQUES FOR DEVELOPMENT

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THIN-LAYER CHROMATOGRAPHY OF PYRIDINIUM ALDOXIMES USING DISTINCT TECHNIQUES FOR DEVELOPMENT

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□ *Twenty - two different pyridinium aldoximes were subjected to thin-layer chromatography using both classical chambers and also forced-flow technique for development. Running characteristics (such as* R_F *values, time versus front distance) were compared in saturated and unsaturated chambers, and an OPLC. A comparison deals with developments in classical (capillary-flow) and modern (forced-flow) thin-layer chromatography.*

Keywords classical chambers, forced-flow, OPLC, planar chromatography, pyridinium aldoxime, silica, UV detection

INTRODUCTION

Organophosphorous pesticides and warfare agents are known as strong inhibitors of cholinesterase enzymes. When a living organism gets in touch with an organophosphate, cholinesterase enzyme is inactivated, which results in a highly excessive amount of acetylcholine and/or butyrylcholine, causing serious symptoms and sometimes the death of human and animal subjects.[1–3] The treatment of organophosphate poisoning includes atropine (antagonist of acetylcholine), oxygen and a high extent of fluid supply.[4,5] Pyridinium aldoximes (such as either pralidoxime, or obidoxime or methoxime) can reactivate cholinesterase enzyme, so they have been widely used in the treatment of persons subjected to organophosphate

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poisoning.[6,7] Other pyridinium aldoximes (K-5, K-27, K-48, K-74, K-75, K-203, K-113, K-107, K-250, K-352, K-33, K-1000), that is, several compounds with the letter K- and a number in their names, have recently been synthesized by Kuca et al., and they show good performance in reactivating cholinesterase enzymes using in vitro and in vivo experiments.[8–10]

Separation of pyridinium aldoximes has recently been carried out from their formulation and also from various body fluids, tissue homogenates and excreted urine. The overwhelming majority of the analyses have been done using reversed-phase HPLC.[11–13] Other separation methods, such as classical column chromatography, thin-layer chromatography, and capillary electrophoresis have also been used.[14–16]

Overpressured-layer chromatography (OPLC, or another name of the same method is forced-flow thin-layer chromatography (FFTLC),[17–19] is an advanced method rooted both in thin-layer chromatography and HPLC.[19–22] OPLC is operated with specifically prepared thin-layer plates, where a covering membrane totally eliminates vapor phase, the whole procedure is done in a totally closed system. The mobile phase is supplied by a pump, so the progress of the mobile phase front is induced and regulated by forced-flow. Experimental "pressurized ultra-micro chamber" was constructed in the late 1970s,[19–22] and Chrompres 10, Chrompres 25, and the most recent Personal OPLC Basic System 50 have been the remarkable milestones in the progress of constructions.[20,21]

EXPERIMENTAL

Materials: Solvents and materials, such as acetonitrile, acetone, acetic acid, lithium chloride and sodium acetate were purchased from commercial sources in the best available quality.

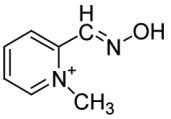
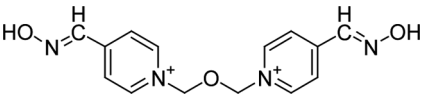
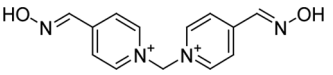
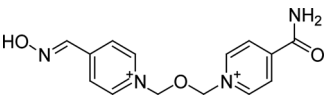
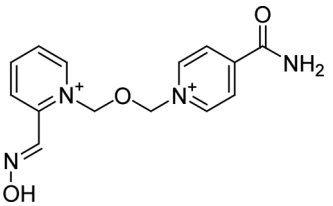
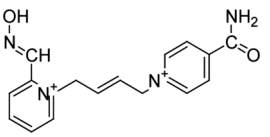
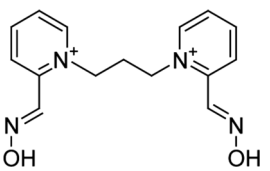
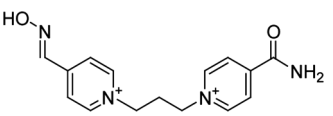
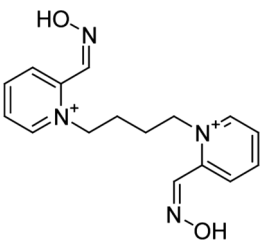
Standards of pyridinium aldoximes were supplied by Dr. Kuca. Their chemical structure, molecular size, total polar surface area (TPSA) and calculated logP value of each pyridinium aldoxime are given in Table 1.

Plain (underivatized) silica TLC plates were purchased from Merck (Darmstadt, Germany) TLC plates of 20×20 cm, silica gel 60 F_{254} and HPTLC plates of 20×20 cm, silica gel 60 F_{254} were used.

Methods

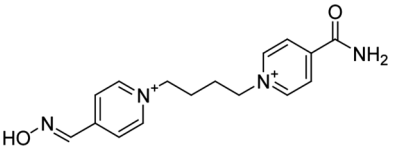
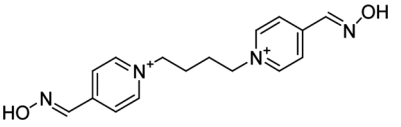
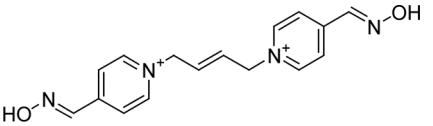
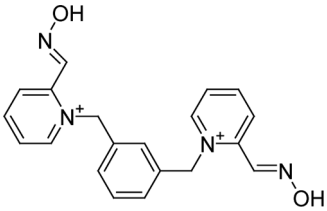
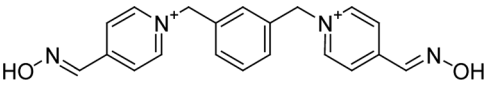
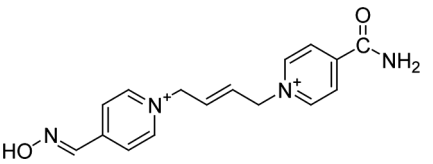
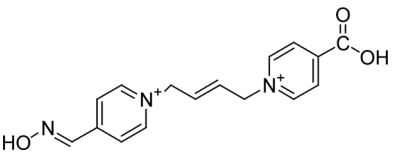
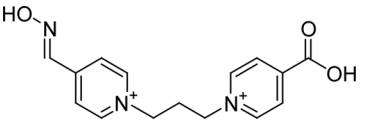
Thin-layer chromatography was performed in a two compartment all glass chambers of General Glassblowing Co. (Richmond, CA, USA) (30 cm × 10 cm × 27 cm). Chamber saturation was carried using a 20 cm × 20 cm filter paper strip that was developed 24 hours before the actual TLC using the same mobile phase. Filter paper strip was not used in the unsaturated chamber.

TABLE 1 Name, Chemical Structure, Molecular Mass, Total Polar Surface Area (TPSA, in Å^2) and the Calculated Index of Lipophilicity (logP)

Compound	Chemical Structure	MW (Da)	TPSA (Å^2)	LogP (calculated)
Pralidoxime		137.18	36.47	−2.56
Obidoxime		288.34	82.17	−2.87
Methoxime		258.31	72.94	−2.74
HS-6		288.34	92.67	−3.25
HI-6		288.34	92.67	−3.20
BI-6		298.38	83.44	−3.04
K-5		286.37	72.94	−2.26
K-27		286.37	83.44	−2.84
K-33		300.40	72.94	−2.22

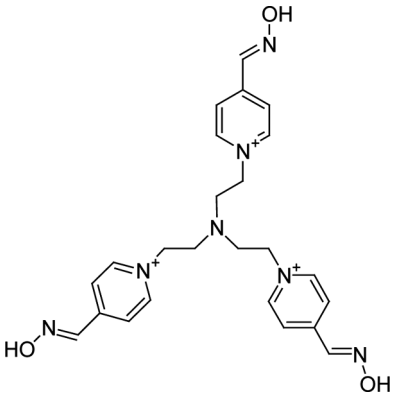
(*Continued*)

TABLE 1 Continued

Compound	Chemical Structure	MW (Da)	TPSA (Å^2)	LogP (calculated)
K-48		300.40	83.44	−2.79
K-74		300.40	72.94	−2.36
K-75		298.38	72.94	−2.46
K-107		348.44	72.94	−1.44
K-113		348.44	72.94	−1.66
K-203		298.38	83.44	−3.04
K-250		299.36	77.65	−3.09
K-352		287.35	83.44	−2.84

(*Continued*)

TABLE 1 Continued

Compound	Chemical Structure	MW (Da)	TPSA (Å^2)	LogP (calculated)
K-1000		464.61	112.65	−2.30

Forced-flow thin-layer chromatography run was done using a Personal OPLC Basic System 50 of OPLC-NIT Co., Ltd. For detailed information on the OPLC methods and about the chamber used here see our former publications.[19–22]

Three different mobile phase compositions were used:

1. Water – acetonitrile – acetic acid (8:1:1, v/v/v)
2. Water – acetone – acetic acid (8:1:1, v/v/v)
3. Acetone – 50 mM aqueous sodium acetate (2:8, v/v) + 0.1% LiCl.

Pyridinium aldoximes were spotted to TLC plate silica F254, 3 cm from the bottom, at a 20 mm distance of the side edges of the plates, also keeping a distance of 2 cm between the neighboring spots. Development was stopped when the mobile phase front reached the proper position, 2 cm from the top of the plate. Six types of TLC separations were performed:

1. TLC silica was developed with mobile phase No. 1 using saturated vapor phase.
2. TLC silica was developed with mobile phase No. 1 using unsaturated vapor phase.
3. TLC silica was developed with mobile phase No. 2 using saturated vapor phase.
4. TLC silica was developed with mobile phase No. 2 using unsaturated vapor phase.

5. HPTLC silica was developed with mobile phase No. 2 using an OPLC chamber.
6. HPTLC silica was developed with mobile phase No. 3 using an OPLC chamber.

All experiments were done in triplicate. Various types of detection can be used following TLC separations. Pyridinium aldoximes show light absorbance at 254 nm. Dark spots indicated the spots of pyridinium aldoximes when silica gel F_{254} plates were used. R_F values were calculated by the use of the regular methods: R_F = spot distance divided by front distance.

RESULTS

Solvent front distance versus time curves show definite decline either in a saturated or in an unsaturated chamber. Moreover, the progress of the mobile front is much slower in an unsaturated chamber than in a saturated one when the mobile phase is volatile. The slower mobile phase progress in an unsaturated chamber is remarkable when TLC silica plates are developed with hexane as mobile phase (not shown here). The difference is much less when the volatile solvent gives only a minor fraction of the mobile phase. Progress of the mobile phase front of water—acetic acid—acetone (8:1:1) on TLC silica plate is plotted in Fig. 1. Similar results were obtained when water—acetic acid—acetone (8:1:1) mobile phase was used, or the stationary phase was not TLC silica but either polyamide or cellulose (not shown here).

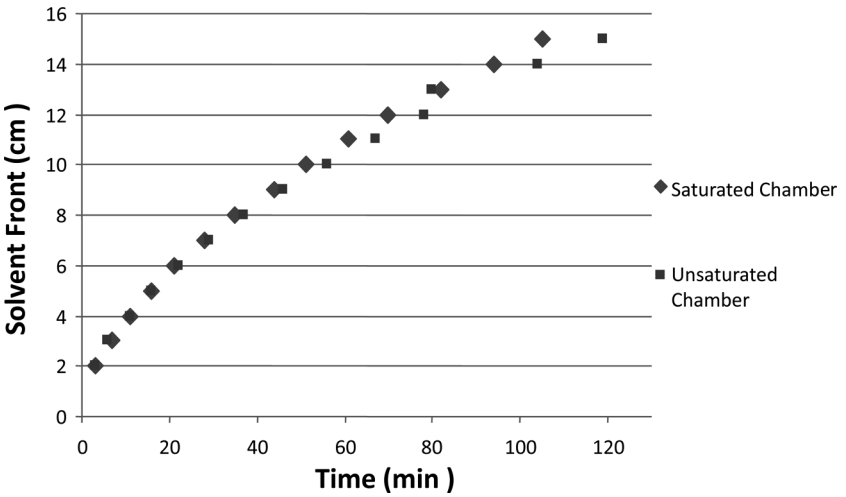


FIGURE 1 Time versus front distance in saturated and unsaturated TLC chamber when acetone—acetic acid—water (1:1:8) mobile phase is used for development.

The spots on TLC plates were visually located (Fig. 2.).

The R_F values of the pyridinium aldoximes were sometimes slightly influenced by the chamber saturation. Figure 3. shows the relations of R_F values when TLC was done in saturated versus unsaturated chambers using mobile phase No. 1. Figure 4 shows the outcome of similar experiments using mobile phase No. 2.

Figure 5 gives a comparison of R_F values obtained in saturated TLC chamber versus OPLC using mobile phase No. 1. A similar comparison demonstrates the relations between unsaturated chamber and OPLC (Fig. 6). To compare the possible separation differences between the two mobile phases (No. 1 and No. 2) the R_F values are compared in saturated chambers (Fig. 7), and in unsaturated chambers (Fig. 8). Using OPLC, R_F obtained in mobile phases Nos. 2. and 3. are compared in Fig 9.

Table 2 shows the statistical analysis for R_F values calculated following the compounds in different mobile phases, and/or different chambers (saturated, unsaturated, OPLC) were subjected to TLC. The absence of correspondence of R_F values (low statistical R and R^2) indicates that both TLC systems can be used consecutively as there are important differences between their ways of action.

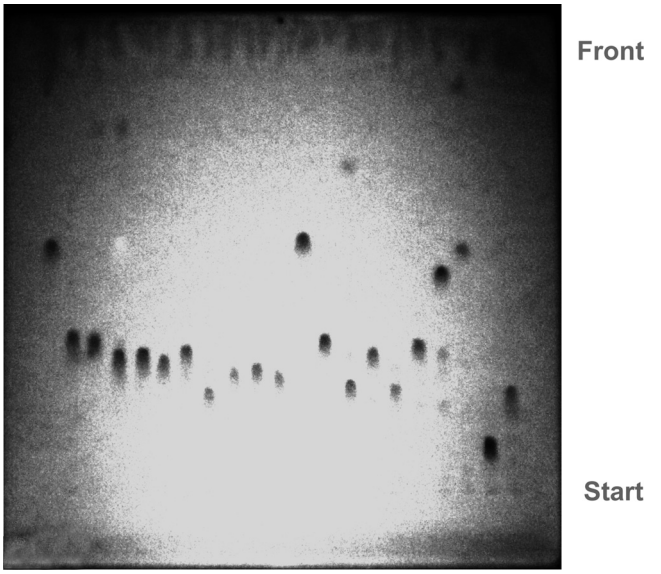


FIGURE 2 Thin-layer chromatogram of pyridinium aldoximes when Basic System 50 of OPLC-NIT was used for development. The stationary phase was TLC silica F_{254} and the mobile phase was acetone—acetic acid—water (1:1:8). The spots from left to right are: K-05, K-27, K-33, K-48, K-74, K-75, K-107, K-113, K-203, K-250, K-352, K-1000, Pralidoxime, Obidoxime, Methoxime, Trimethoxime, BI-6, HI-6, HS-6, Pralidoxime, HI-6. Visualization was done based on UV absorbance at 254 nm.

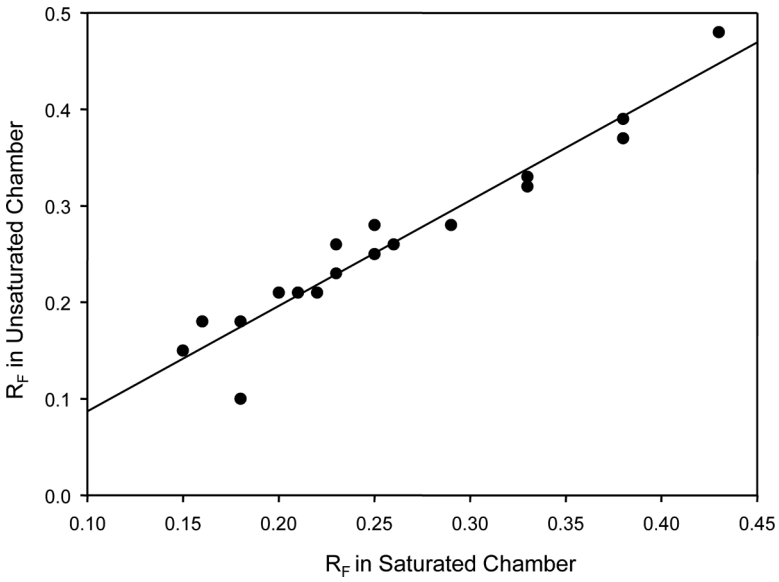


FIGURE 3 Comparison of R_F values: Water—Acetonitrile—Acetic Acid Mobile Phase (8:1:1) Saturated Vapor Phase vs. Water—Acetonitrile—Acetic Acid Mobile Phase (8:1:1) Unsaturated Vapor Phase, both TLC.

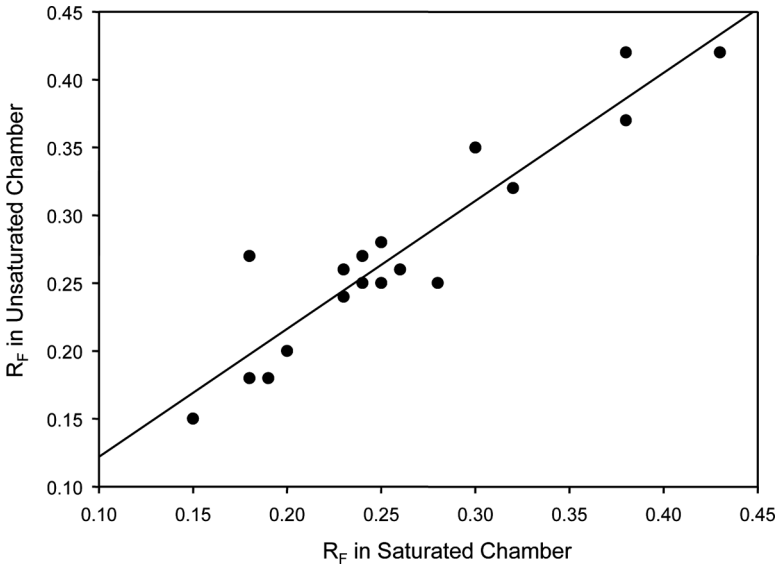


FIGURE 4 Comparison of R_F values: Water—Acetone—Acetic Acid Mobile Phase (8:1:1) Saturated Vapor Phase vs. Water—Acetone—Acetic Acid Mobile Phase (8:1:1) Unsaturated Vapor Phase, both TLC.

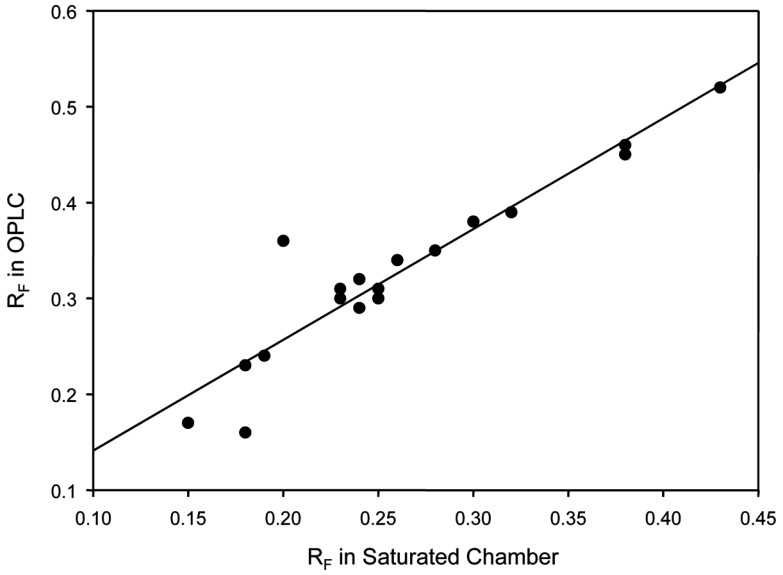


FIGURE 5 Comparison of R_F values: Water—Acetone—Acetic Acid Mobile Phase (8:1:1) Saturated Vapor Phase, TLC vs. Water—Acetone—Acetic Acid Mobile Phase (8:1:1) OPLC.

Figure 3 and its statistical evaluation indicate that the spread of spots in saturated/unsaturated chambers (using mobile phase No. 1) was similar, except the spot representing BI-6. This compound has specific, ortho- and

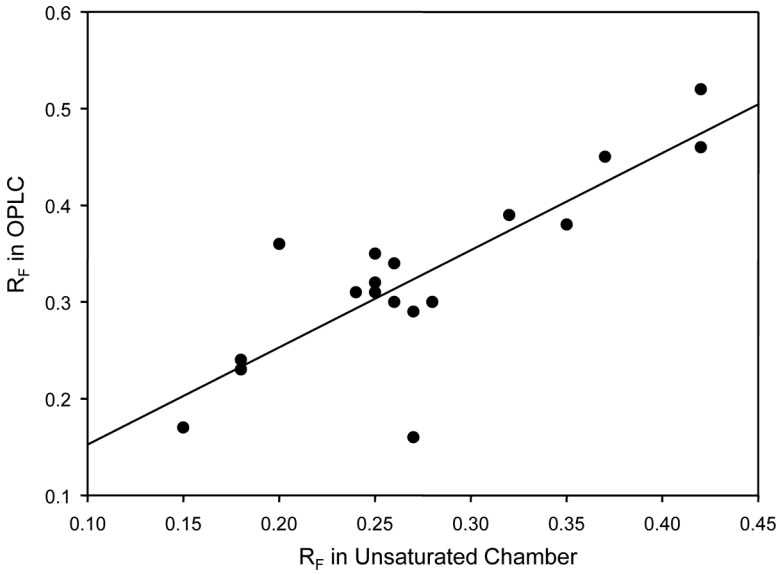


FIGURE 6 Comparison of R_F values: Water—Acetone—Acetic Acid Mobile Phase (8:1:1) Unsaturated Vapor Phase, TLC vs. Water—Acetone—Acetic Acid Mobile Phase (8:1:1) OPLC.

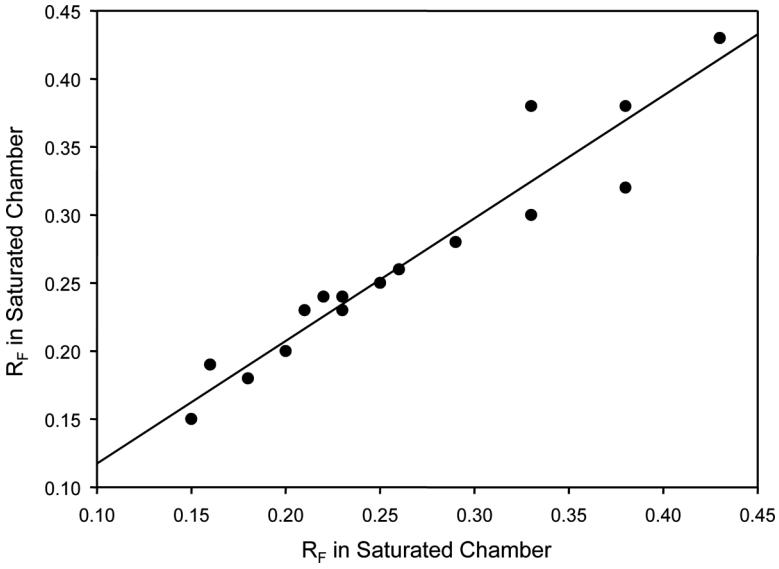


FIGURE 7 Comparison of R_F values: Water—Acetonitrile—Acetic Acid Mobile Phase (8:1:1) Saturated Vapor Phase vs. Water—Acetone—Acetic Acid Mobile Phase (8:1:1) Saturated Vapor Phase, both TLC.

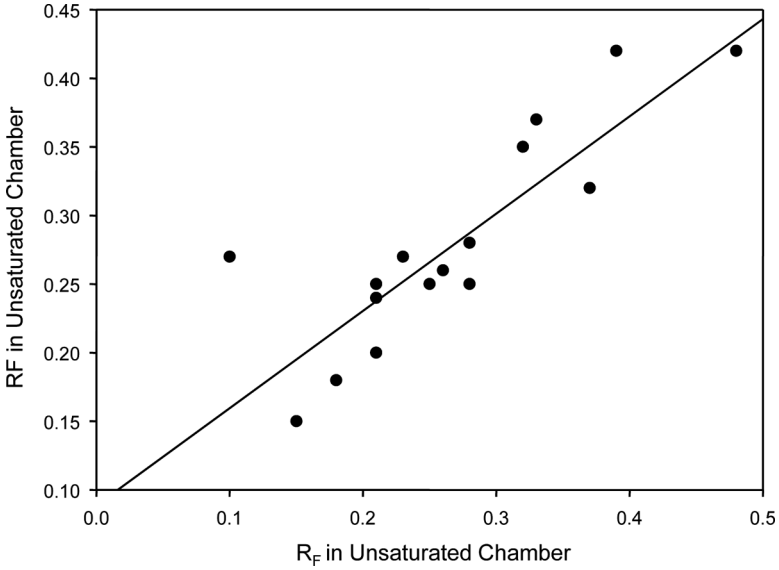


FIGURE 8 Comparison of R_F values: Water—Acetonitrile—Acetic Acid Mobile Phase (8:1:1) Unsaturated Vapor Phase vs. Water—Acetone—Acetic Acid Mobile Phase (8:1:1) Unsaturated Vapor Phase, both TLC.

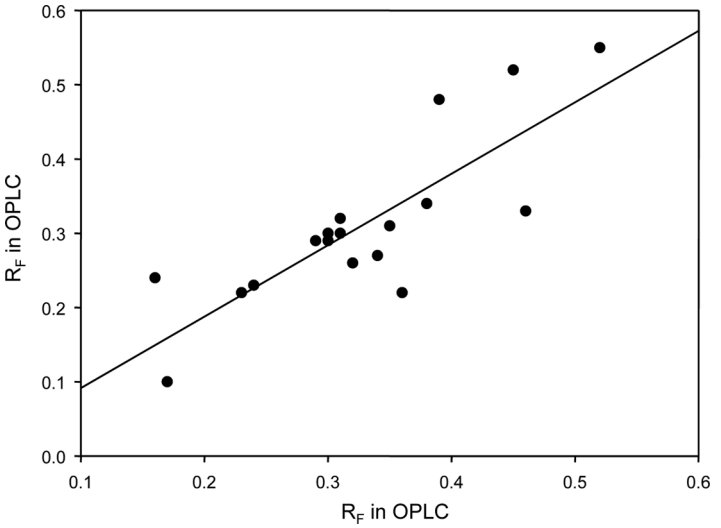


FIGURE 9 Comparison of R_F values: Water—Acetone—Acetic Acid Mobile Phase (8:1:1) vs. Acetone—Aqueous Sodium Acetate (2:8) plus 0.1% Lithium Chloride, both OPLC.

para- substitutions on the two pyridinium rings, thereby it diverges from all others (having either para and para- or ortho- and ortho-substitutions).

The same compound (BI-6) shows diverge behavior (Fig. 4) in comparison with saturated and unsaturated chambers using mobile phase No. 2 (water – acetone – acetic acid (8:1:1, v/v/v)). BI-6 shows an elongated spot,

TABLE 2 Statistical Evaluation of Differences in the R_F Values when TLC and OPLC Were Done

	y_0	a	R	R^2
Water—Acetonitrile—Acetic Acid Mobile Phase (8:1:1) Saturated Vapor Phase vs. Unsaturated Vapor Phase	−0.0569	1.1905	0.8889	0.7892
Water—Acetone—Acetic Acid Mobile Phase (8:1:1) Saturated Vapor Phase vs. Unsaturated Vapor Phase	0.0103	1.1905	0.9302	0.8653
Water—Acetone—Acetic Acid Mobile Phase (8:1:1) Saturated Vapor Phase vs OPLC	−0.0295	1.3427	0.9251	0.8558
Water—Acetone—Acetic Acid Mobile Phase (8:1:1) Unsaturated Vapor Phase vs OPLC	0.05	0.897	0.6357	0.4274
Water—Acetonitrile—Acetic Acid Mobile Phase (8:1:1) Saturated Vapor Phase vs. Water—Acetone—Acatic Acid Mobile Phase (8:1:1) Saturated Vapor Phase	0.027	0.8914	0.9646	0.9304
Water—Acetonitrile—Acetic Acid Mobile Phase (8:1:1) Unsaturated Vapor Phase vs. Water—Acetone—Acatic Acid Mobile Phase (8:1:1) Unsaturated Vapor Phase	0.062	0.7518	0.8302	0.6893
Water—Acetone—Acetic Acid Mobile Phase (8:1:1) vs. Acetone—Sodium Acetate (2:8) plus 0.1% Lithium Chloride, OPLC	−0.0302	0.9623	0.8484	0.7198

when R_F was calculated from the densest part (eye) of the spot. Anyway, the use of mobile phase No. 2 could not be suggested for TLC of BI-6. This anomaly of BI-6 is also present in other figures (Figs. 5, 6, 8 and 9). The most striking differences between the R_F values are found when the separations was done using the same mobile phase but different chamber systems (Fig. 6). Unsaturated mobile phase in the TLC chamber subjects the spots to behave another way than OPLC.

A poor relationship of R_F values is shown in Fig 8. These different mobile phases can also be used either for two-dimensional separation, or parallel experiments to separate either one of the pyridinium aldoximes from the background matrix, or from each other. The majority of the compounds shows different behavior using these different mobile phases (the organic modifiers are different) in the unsaturated chambers.

DISCUSSION

Thin-layer chromatography has several essential advantages over column (and capillary) technique of separation.

1. The progress of the solvent front can be visually followed.
2. Colored compounds can be visually located on the plate, the majority of others can be detected using specific color reagents. Certain compounds can also be detected under UV light either by their absorbance or fluorescence (Fig. 2).
3. Two dimensional separation can be easily done.
4. Spots of either no migration or very excessively fast movement can be detected at the place of load, or at the mobile phase front.
5. Certain techniques of thin-layer chromatography are simple and inexpensive, others are advanced methods that can offer high precision of analysis.

   A crucial factor of thin-layer chromatography is generally hidden in the presence of vapor phase that can essentially influence the separation. Geiss[23] published an in-depth review on the role of vapor phase in thin-layer chromatography. As our experiments definitely showed the saturation/non-saturation of the developing chamber has essential importance only when the mobile phase mainly contains volatile components. Fig. 3 indicates that using water – acetonitrile – acetic acid (8:1:1, v/v/v) either in saturated or in unsaturated chamber (but only one of them) should be used for development. The results of separation of pyridinium aldoximes are similar.

Both the far spread of the R_F from the resulting line and the low significance (R and R^2) indicate that these systems are worth using for both

two-dimensional developments, or for a consecutive use when the fate of pyridinium aldoximes are scouted in biological systems (study on either pharmacokinetics or metabolism).

In a classical TLC system the gross time of development may reach nearly two hours even when saturated chamber is used.

There is one more factor to be mentioned. Thin-layer chromatography, as a planar method, makes visual evaluation of the separated components possible. The light spots on Fig. 10 definitely indicate an anomaly. These spots are fluorescent, as it can easily be determined. For the time being it is not clear why these compounds (all of them have but-2-ene bridges) give extra spots, they can be either degradation products or something else. Structural evaluation of these fluorescent spots is in progress.

CONCLUSIONS

Separation systems can be found based on trial and error. It is especially valid when the influential role of vapor phase is questioned.

OPLC Basic System 50 of OPLC-NIT makes much faster speed of development possible. Time of development was 15 minutes using OPLC and 60 minutes in the classical TLC chamber. Moreover, the flow velocity of the mobile phase in the classical chambers showed a decline. In OPLC flow velocity of the mobile phase is constant and it can be regulated.

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