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Effects of saturation and temperature on the separation of some guanidino compounds by thin-layer chromatography on cellulose plates*

In general, standard procedures in thin-layer chromatography require a saturated chamber atmosphere and constant temperature (usually room temperature) to achieve optimum resolution and reproducible R_F values. There are, however, exceptions, and variable conditions produced by the gradient elution technique or vapour-programmed chromatography, for example, may improve the performance of a particular chromatographic separation[1–5]. During studies on the separation of some guanidino (amidino) compounds of biological interest by thin-layer chromatography on cellulose plates, it was noted that the migration of these compounds seemed to vary with both temperature and the degree of saturation of the chromatographic chamber with solvent vapour. We have therefore studied more systematically the effects of these factors on the resolution of creatine, creatinine, glycocyamine and arginine for practical as well as theoretical reasons.

Apart from variations in temperature and chamber saturation, other details of the procedures were standard and kept constant throughout. For all experiments, pre-coated cellulose plates with layers of 0.1 mm thickness (E. Merck, Darmstadt) were used. The test compounds were analytical-grade reagents and 10 μg amounts of each standard dissolved in 2 μl of distilled water were spotted individually on to the plates. The solvent system used was isopropanol–glacial acetic acid–water (60:45:15)[6]. Chromatographic runs were performed at three different temperatures: room temperature (20–22 °), 4° and 38°. For the separations at 4°, the tanks were placed in a thermostatically controlled cooling compartment (Colora), while at 38° the tanks were placed in an incubator; appropriate periods of time for equilibration and adjustment to the respective temperatures were allowed for in all instances. Rectangular chromatographic tanks measuring 8 × 20 × 20 cm (N-chambers[7]) were used. "Saturation" of the chambers included the usual measures to prevent solvent vapours from escaping from the chamber and to maintain a saturated atmosphere inside. With the "unsaturated" chambers, the lid was only loosely placed on top of the tank so as to allow solvent vapours to equilibrate rapidly with the external atmosphere. The separated substances were made visible by spraying the plates with an alkaline mixture of 10% (w/v) potassium ferricyanide and 10% sodium nitroferricyanide[8].

The experimental results are illustrated in Fig. 1. Developing times and spot diameters are listed in Table I. For the saturated system, the R_F values of the four test compounds were virtually unaffected by the different temperatures, the only differences being in the rates of migration of the solvent system. At 4°, almost twice as much time was necessary to complete the development than at room temperature, while the migration rate at 38° was only slightly faster than that at room temperature. The spot sizes also tended to increase with temperature.

With unsaturated tanks, all compounds migrated faster relative to the solvent front, which resulted in increased R_F values compared with the saturated system.

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These changes closely parallel the increases in temperature. However, depending on the temperature at which the separation was performed, increases in the R_F values were not uniform for all compounds. At 4°, for example, the R_F value of arginine was little affected by unsaturation while those of the other compounds showed increases that were more or less proportional to their relative migration

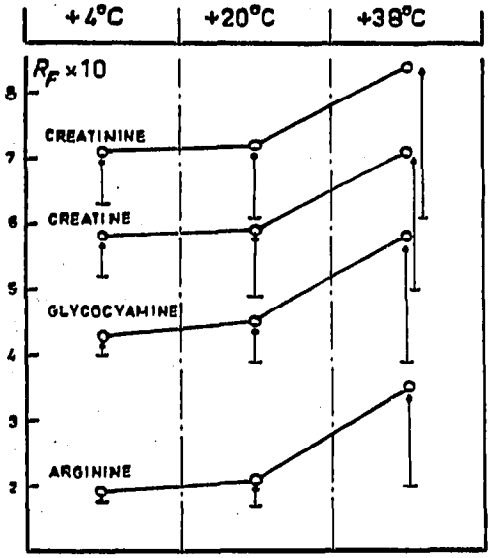

Fig. 1. Changes in R_F values of arginine, glycocyamine, creatine and creatinine brought about by chamber unsaturation and changes in temperature. R_F values are based on solvent fronts 10 cm from the starting line. The open circles indicate R_F values obtained with unsaturated chambers compared with R_F values of saturated chambers (bases of arrows), the length of the arrows illustrating changes due to unsaturation. R_F values of each compound are the means of three separate chromatographic runs.

TABLE I

EFFECT OF TEMPERATURE AND CHAMBER SATURATION ON DEVELOPING TIMES AND SPOT DIAMETERS

2 μl of standard solution were spotted 1 cm from the lower edge of the plate. Developing time refers to a distance of 10 cm from the starting line. Spot sizes are expressed in millimetres; as the solvent front was allowed to migrate exactly 10 cm, the numerical values are identical with the difference between the maximum and minimum R_F value (ΔR_F) of individual spots when applying a factor of 10^{-1}.

To statistically test the effects of temperature and saturation on the diameter of spots, all values were submitted to an analysis of variance. According to this test, neither temperature nor saturation affected significantly the spot diameters ($p > 0.05$ for temperature and saturation, respectively).

Property	*Temperature (°C)*			
	4	*20*	*38*	
Developing time				
Saturated	5 h	2 h 50 min	2 h 25 min	
Unsaturated	6 h	3 h 30 min	3 h 10 min	
Spot diameter (mm)				*Total means*
Saturated	6.0 ± 0.9	6.7 ± 1.1	6.5 ± 0.7	6.4 ± 1.0
Unsaturated	6.4 ± 1.1	7.1 ± 1.3	6.8 ± 1.0	6.8 ± 1.2
Total means	6.2 ± 1.0	6.9 ± 1.2	6.7 ± 0.9	—

rates. At room temperature and at 38°, there is less or no proportionality, the increase in the R_F value of arginine at 38° being almost as large as that of creatinine. Hence, resolution is best at 4°. Furthermore, the diameters of the spots were smallest on plates run at 4° (Table I), which adds to the advantages of a chromatographic separation at low temperature. Despite prolonged developing times, diffusion does not seem to interfere with sharp spots. There is, however, an overall tendency of spots to increase in size with unsaturation. A comparison of developing times observed under the various experimental conditions reveals that temperature rather than saturation or unsaturation exerts the most marked effects.

The advantages of unsaturated chambers in thin-layer chromatography have been pointed out by other workers[9,10]. Some controversy exists as to the theoretical basis of the observed effects of an unsaturated atmosphere on resolution[7,11]. A gradual change of adsorptivity due to adsorption of solvent vapours by the unwetted portions of the layer during chromatography, resulting in a gradient of the more polar vapour components of a multi-component system from bottom to top, has been suggested as one possibility[9,10]. With a single-component solvent, an increased solvent flow due to unsaturation is taken into consideration, although other factors are also involved[10]. On the other hand, an increased solvent flow might be plausibly adduced to explain increased R_F values, regardless of the complexity of the solvent system[7,12,13].

If the present results are evaluated from this point of view, their interpretation is made easier by the fact that cellulose layers were used instead of silica gel as in previous studies. Cellulose has little or no adsorptive properties, depending on the composition of the solvent system used. If the portion of the organic component of an organic–aqueous solvent mixture considerably exceeds that of the aqueous component, the chromatographic separation on cellulose layers is considered to follow the principle of partition chromatography[14], partition taking place between the stationary (aqueous) and mobile (organic) phases, depending on the partition coefficients of the solutes. The effects of unsaturation therefore appear to be the result of altered solvent flows rather than of altered adsorptivity of the layer, and it is obvious that unsaturation will affect the rate of solvent flow mainly by increasing the rate of evaporation, probably of the organic, *i.e.*, mobile phase. Changes of partition coefficients with temperature as a cause of altered R_F values can be disregarded in view of the constant R_F values obtained with saturated tanks irrespective of temperature.

At 38°, an apparent "pushing up"[10] of arginine has occurred. This phenomenon is thought to be brought about when evaporation is uniform throughout the surface of the layer and integral solvent flow decreases from the lower to the upper parts of the wetted layer. The "superproportional" increase of the R_F value of arginine therefore indicates that at 38° evaporation was uniform. Although it was observed that even in unsaturated tanks saturation is rapidly established near the bottom[12], this may not apply to a temperature of 38°. However, it may also be that due to increased vapour pressures at the elevated temperature, even an open tank may rapidly become completely saturated.

At room temperature, the pattern of chromatographic resolution approaches a more proportional one, which is most marked at 4°. This pattern implies that changes in solvent flow were uniform over the whole distance of the plate, affecting

the R_F values of all substances equally and proportionally to their individual migration rates. Therefore, solvent evaporation was likely to take place from a site that lies beyond the fastest migrating compound, *i.e.*, near the solvent front. Again, it is difficult to state what the effect of temperature was on the true state of saturation of the tank. Increased R_F values and their proportionality both indicate that the solvent flow was increased and that, whatever degree of saturation there was, the vapour pressure must have been uniform throughout the tank.

For practical purposes, unsaturation at low temperature might therefore improve the chromatographic resolution in that a mixture of compounds may be proportionally spread over a greater distance with spot sizes being smaller than on chromatograms run at normal temperature. As the discussion of the results has shown that the improved resolution is a result of increased solvent flow due to evaporation of solvent near the solvent front, similar results should be obtained by using the technique of continuous thin-layer chromatography[15,16]. Elevation of the temperature might shorten developing times considerably without interfering with the resolution.

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