INFLUENCE OF HUMIDITY VARIATIONS IN THE THIN-LAYER CHROMATOGRAPHY OF HYPNOTICS

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SUMMARY

The effect of humidity variations on the R_F values of some hypnotics was investigated under standardized conditions. It was found that considerable changes in R_F value can occur as a result of different amounts of water, present as vapour in the ambient atmosphere. With increasing humidity a distinct rise in R_F value is seen at first, but this is followed by a sharp fall at higher humidities. For reproducible work in thin-layer chromatography the availability of a constant-humidity-room is recommended.

In general in thin-layer chromatography (TLC), very little attention is paid to the influence of humidity on the reproducibility of the separations. After preparation of the plates they are heated to remove excess water and during this process, capillary bound water is also expelled from the adsorbent. Subsequently, in many instances, these activated plates are spotted in the ambient room atmosphere and, depending on the duration of this period and on the relative humidity, the plate will adsorb a certain amount of water vapour. The investigations of GEISS and co-workers[1] have very clearly demonstrated that the separation is very dependent on the amount of this absorbed water vapour and that considerable changes in R_F value can be observed due to the variations in the ambient relative humidity.

In our opinion the humidity effect is most noticeable in non-polar systems, for example the azo-dyes/benzene as used by GEISS *et al.*[1], whereas with more polar substances and solvents this effect will become smaller. However, it is always necessary to be aware of this problem, and to investigate the effect of changes in humidity upon the position of the spots, also in the case of more polar substances, as will be shown in this paper.

EXPERIMENTAL

Substances. 0.2% w/v solutions in chloroform of: (1) heptobarbital; (2) phenobarbital; (3) allobarbital; (4) hexobarbital; (5) methylphenobarbital; (6) bromisoval; (3–6) mixture of 3 + 4 + 5 + 6.

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The designation and quality of these substances is according to the Netherlands Pharmacopoeia, VIth Ed. (1958).

Adsorbent. Silica Gel GF_{254} (Merck, Darmstadt), 30 g in 60 ml distilled water for 5 plates; plate size 20 × 20 cm, layer thickness 0.25 mm when spread.

Activation. Air dried for 15 min, then heated for 30 min at 110° in an oven with a fan, cooled and stored in a desiccator.

Solvent. Chloroform Pro Analysi (Merck, Darmstadt) or free from ethanol.

Apparatus. Desaga, Heidelberg.

Documentation. Direct photography after activation with ammonia vapour, under 2 U.V. lamps (Camag, Muttenz), at 254 nm. Camera: Asahi Pentax, type SV, with a Super Takumar 1:1.8/55 lens with a 49 mm U.V. ghostless filter. Distance between camera and plate 70 cm, aperture 5.6, exposure time 15 sec. Film: Agfa Color CT-18 diapositive.

Standard conditions. Temperature 25°, relative humidity of the room 60%,

TABLE I

PROCEDURES OF PLATE DEVELOPMENT AT VARIOUS HUMIDITIES

Procedure	*Reheating*	*Drying agent during cooling*	*Contents of trough*
1	30′ at 110°	P_2O_5	P_2O_5
2	30′ at 110°	P_2O_5	H_2SO_4
3	30′ at 110°	P_2O_5	CaO
4	30′ at 110°	P_2O_5	no trough
5	30′ at 110°	none	no trough
6	30′ at 110°	none	H_2O

length of run 10 cm, spots 1.5 cm from bottom edge of the plate. After spotting the plate, the following three steps were carried out in six different ways (see Table I):

(1) reheating in the oven to expel the adsorbed water vapour;

(2) cooling for 24 h in a desiccator with or without drying agent;

(3) development in unsaturated chambers at various humidities.

These humidities were established by means of troughs containing drying agents or water, placed at the bottom of the chamber. These troughs were placed in the chambers 24 h prior to development and allowed to remain in the chamber during development.

TABLE II

AMOUNTS OF WATER INVOLVED IN THE DIFFERENT SEPARATION PROCEDURES

Procedure	*1*	*2*	*3*	*4*	*5*	*6*
Water in mg	<0.001	<0.05	<1	±48	±280	±350

RESULTS AND DISCUSSION

As a result of the differences in development procedure the effect of increasing humidity on the separation of the hypnotics can be observed. Knowledge of the capacities of the drying agents[2], and the volumes of the desiccator (26 l) and development chamber (3.6 l) enables the various amounts of water involved in the separations to be calculated. These are listed in Table II.

The separations obtained by the different procedures are shown in Figs. 1–6. The R_F values of the spots are listed in Table III. With increasing amounts of water,

TABLE III

R_F VALUES × 100 OBTAINED BY THE DIFFERENT SEPARATION PROCEDURES

Substance	*Procedure*					
	1	*2*	*3*	*4*	*5*	*6*
Heptobarbital	5	5	6	9	8	6
Phenobarbital	10	10	12	18	14	11
Allobarbital	12	13	15	26	17	13
Hexobarbital	18	23	29	70	27	24
Methylphenobarbital	23	31	37	84	35	31
Bromisoval	13	17	20	25	17	18

a rise in R_F value can be seen which is normal under these conditions and which is quite distinct in the separation with procedure 4. However, in procedures 5 and 6 with still increasing amounts of water, there is a very sharp fall in the R_F values; and in procedure 6, where development is in a relative humidity of nearly 100%, the R_F values have become so low that they are comparable to those obtained with the "dry" procedure 2. These observations are very surprising indeed, because one would not expect a decrease in R_F value with increasing humidity. We have no theoretical explanation at the moment for this phenomenon and do not know whether it is also found with other substances. The results underline, however, that a variable constant-humidity-room is almost indispensable in order to obtain reproducible work in TLC. Otherwise a very careful study into the effect of humidity variations has to be carried out.

At first in our investigations, after a rough comparison of the "dry" procedure 1 and the "wet" procedure 6, we thought that there was only a very small humidity effect, but a more systematic study showed us that this was not so. In our opinion, constant-humidity-rooms are to be preferred to the so called climate-chambers for development because of reasons of simplicity. With the former system the plates can be stored at the ambient room humidity, no precautions have to be taken during the spotting period, and there is no difference in the humidity inside and outside the development chamber and a whole range of desired humidities can easily be established by setting one simple knob.

It should also be noted that the special procedure[3] used by several authors, *i.e.*, of reheating the plates after spotting to obtain a more active plate, is very dangerous with respect to the reproducibility. Without suitable precautions, the active

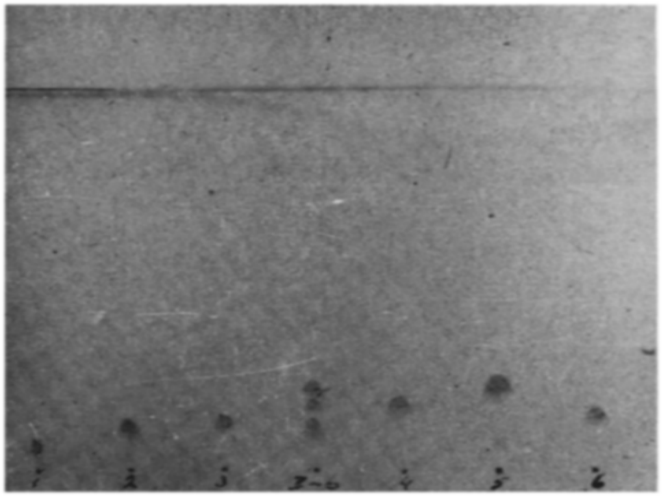

Fig. 1.

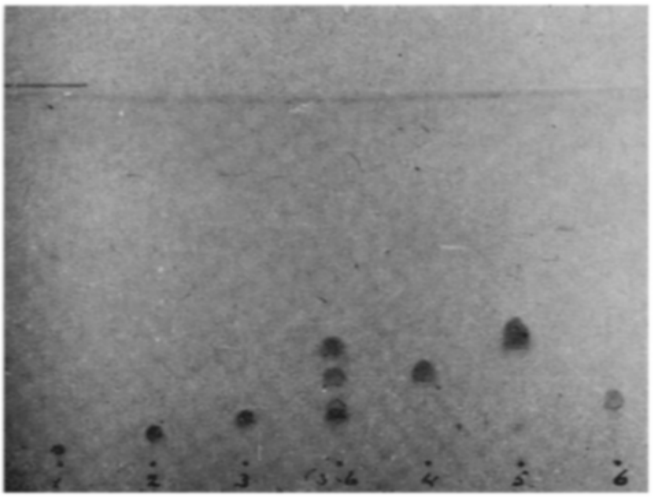

Fig. 2.

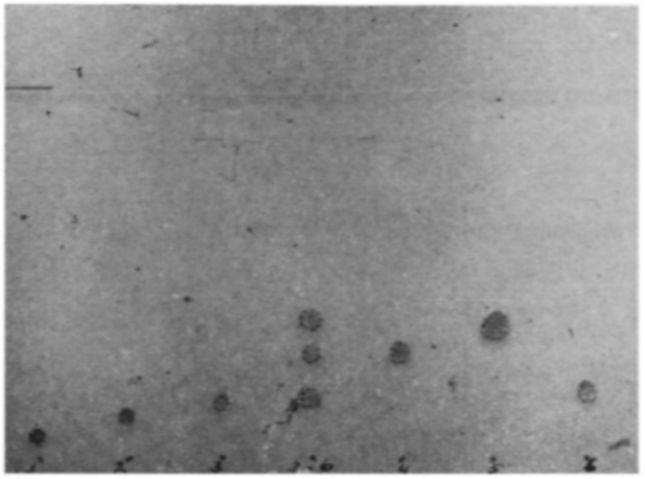

Fig. 3.

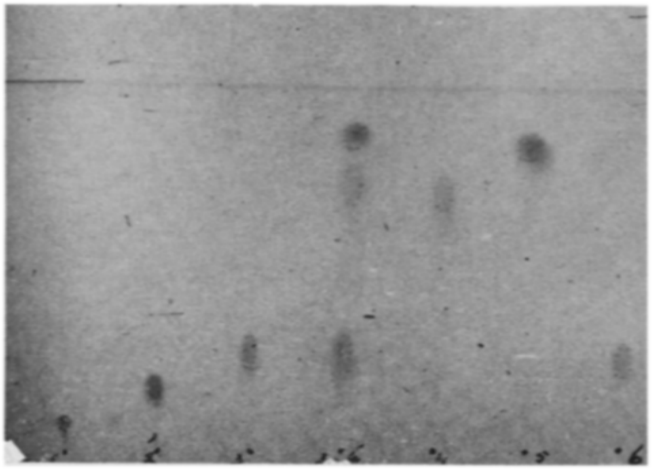

Fig. 4.

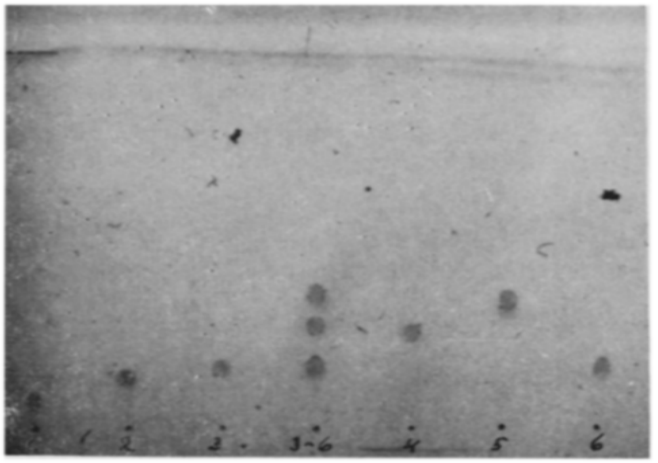

Fig. 5.

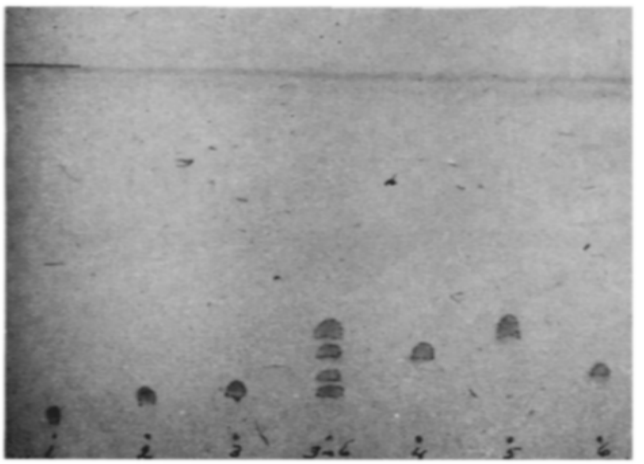

Fig. 6.

Fig. 1–6. Influence of increasing humidities on the separation of hypnotics. Different development procedures 1–6 with increasing humidities (*cf.* experimental part). Photography under U.V. light of 254 nm after activation with ammonia vapour. 1 = Heptobarbital; 2 = phenobarbital; 3 = allobarbital; 4 = hexobarbital; 5 = methylphenobarbital; 6 = bromisoval; 3–6 = mixture of 3 + 4 + 5 + 6.

plates will strongly adsorb water vapour and these small amounts of water can cause great changes in R_F values.

ACKNOWLEDGEMENTS

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DISCUSSION

BRENNER: Do you regard ammonia as a basic reagent or a polar solvent?

DE ZEEUW: I cannot give a definite answer. We have found, however, that with *e.g.* the solvent system isopropyl alcohol–chloroform–25% ammonia (45:45:10), the ammonia can be omitted from the solvent and can be replaced by a trough with ammonia on the bottom of the development chamber. Thus in this case the content of ammonia in water does not play an important role, as the effect is due to the ammonia from the gas phase.

GEISS: The decrease of some of the R_F values with increasing relative humidity might be explained as follows: Common ethanol-containing chloroform was used, which undergoes demixion on the layer. Some of the substances will migrate in the gradient of the transition zone. If the water content of the layer is high, alcohol becomes less soluble in chloroform and migrates at a lower rate; consequently, the second front retreats, the gradient becomes steeper and the substances which migrate in the gradient zone or below it show lower R_F values (*cf.* H. W. PRINZLER AND H. TAUCHMANN, *J. Chromatog.*, 29 (1967) 142).

DE ZEEUW: It did not matter whether we used chloroform containing 1% of ethanol or purified chloroform without ethanol. In both cases the same results were obtained. So the decrease of the R_F values cannot be explained by the presence of ethanol in the solvent.

GEISS: According to Dr. DE ZEEUW it is more expedient to work in climatized rooms than with development chambers which can be climatized, since the former are told to be more practical and cheaper. But the practically interesting and important humidity range is thus strongly limited, because, *i.a.*, the climatization system of the building cannot be brought to extreme values, simply for the reasons of human comfort. On the other hand, by the use of a broad humidity-controlled activity range, we can often manage with only one single solvent, and this is methodically of a fundamental advantage.

DE ZEEUW: In principal, I agree with your conclusion, but the climatized room I have in mind is a rather small one with a climatization system apart from the rest of the building. Of course there must be the possibility to change the humidity to extreme values in order to achieve the most convenient separation conditions. Incidentally, I did not mention the climatized rooms to be cheaper in my lecture.

JÄNCHEN: Although Dr. DE ZEEUW has demonstrated that unsaturated chambers can give better separations in certain cases, I should like to warn against their use. There certainly can also be found solvent systems that yield satisfactory separations in saturated chambers. Saturated development procedures have a definite advantage in quantitative TLC. No concentration effects towards the surface of the layer will occur and thus surface measurements *in situ* are improved, *i.e.* they show linear quantity dependence.

DE ZEEUW: I agree with you as far as quantitative TLC is concerned but it should also be observed that drying of the plate after development also gives surface concentration.

GEISS: I should even go further than Dr. JÄNCHEN in the criticism of the work with unsaturated N-chamber systems: The risk of non-reproducibility of the separation is inherent to the unsaturated N-chambers. (1) The evaporation from the plate takes place *during* the elution; thus it depends on the migration rate on the respective plate. (2) The evaporation from the wetted layer is more or less irregular. (3) With very slowly migrating layers the chamber can be gradually saturated from the trough; in such a case condensation from the vapour phase exceeds the evaporation.

One should not only avoid unsaturated chambers but, if possible, solvent mixtures as well. Pure elution solvents make the situation more simple and N-chambers—saturated or unsaturated—unnecessary. Thus a fundamental step towards improved reproducibility has been done.

DE ZEEUW and BRENNER: The practical analyst is less interested in reproducibility than in the separation!

GEISS: I should by no means advocate the determination of a reproducible absolute R_F. Il vaut ce qu'il vaut. Here goes reproducibility, there goes separation: What I mean is always *reproducibility of the separation*. Many of the separations shown at this symposium carry the germ of non-reproducibility inherent to the technique. Separations should be as reproducible in the higher latitudes as in the tropics. Earlier, we occasionally achieved surprisingly good separations with the so-called "hot elution" on alumina, such as could not be obtained with any other procedure. The incidence of these separations, however, was 50% at the most. Poorly reproducible humidity gradient elution was involved. We have thus given it up.

DE ZEEUW: I am glad of the reaction concerning my remarks on unsaturated chambers since this is, according to my opinion, a very important problem in TLC.

I should like to comment on Dr. GEISS' contribution. TLC has undergone tremendous development, yet obviously many separations, especially those of closely related substances, are only possible if multi-component solvents are used. It has also been shown that difficult separations of sizable classes of substances can only be obtained with unsaturated chambers. Thus practical TLC is primarily concerned with the feasibility of a separation. In order to achieve this it is not always possible to work under conditions which are optimum for the reproducibility.

What actually does the practical TLC analyst wish: Separation with less than optimum reproducibility or no separation with optimum reproducibility? According to my opinion the former is more important and hence we have studied reproducibility in these unsaturated chambers. I hope our lecture was able to show that rather good reproducibility can be achieved with this improved separation technique. This method can thus possibly represent a valuable extension of the development methods used so far.

PROCHÁZKA: Dr. PITRA has shown us, three years ago, that there exists an amount of water in silica gel at which the separation is optimum. At a very low and a very high water content the separation is poorer than with 10–20% of water.

PITRA: According to our ideas, which are partly based on published results, there really exists a separation optimum on silica gel layers, namely when 7–20% water have been sorbed. It should be noted, though, that the transition between adsorption and partition is far from sharp. In brief, either the LANGMUIR or the NERNST isotherm predominates. Undoubtedly, at more than 20% sorbed water, partition seems to be the prevailing mechanism of the separation process.

GÄNSHIRT: Is a partition chromatographic separation by means of PC possible? If so, this would support the hypothesis considered for the good separation of the respective series of substances at a very low and a very high humidity as a transition between the adsorption and the partition processes.

DE ZEEUW: We did not try it on paper. In my opinion, the possibility that partition plays a role, possibly an important one, cannot be excluded. It would be of interest to investigate this point further since partition does play a great role even in normal TLC which should not be unrestrictedly described as adsorption chromatography. We know that vapour adsorption plays an important role and forms a sort of stationary phase which is completed when the solvent reaches the respective areas; see *e.g.* R. A. DE ZEEUW, *J. Chromatog.*, 32 (1968) 43. We also know that the adsorbed amounts of vapour greatly determine the separation; these two groups of facts justify the conclusion that partition chromatography may play an important role.