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Note

Criteria for resolution by thin-layer and paper chromatography

I. Spot size, migration and R_F values: examples from analysis of nucleic acid hydrolyzates

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The compilation of R_F values in the literature is usually made without reference to the migration of the solvent. Most workers, in the author's experience, when looking for a solvent system to resolve a mixture or hydrolyzate apply a "rule of thumb" method for deciding their choice: if the R_F values of the substances concerned seem sufficiently different, then the solvent is tried. However, the difference between R_F values as a criterion of resolving power is dependent upon the distance the solvent is allowed to migrate, and upon the spot size, as shown below.

Let R_{F_1} and R_{F_2} be the R_F values ($R_{F_1} > R_{F_2}$) of substances 1 and 2 in chromatographic system S. Let the solvent migration be m cm on the developed chromatogram, and let r_1 and r_2 cm be the radii of the spots of substance 1 and substance 2, respectively. A condition of minimal separation or resolution is when the two spots are adjacent so that their centres are ($r_1 + r_2$) cm apart. If substance 1 migrates m_1 cm and 2 migrates m_2 cm, then

$$R_{F_1} = \frac{m_1}{m} \tag{1}$$

$$R_{F_2} = \frac{m_2}{m} \tag{2}$$

But, for minimal separation:

$$m_1 = m_2 + r_1 + r_2 \tag{3}$$

Therefore

$$(R_{F_1} - R_{F_2})\, m = r_1 + r_2 \tag{4}$$

and

$$m = \frac{r_1 + r_2}{R_{F_1} - R_{F_2}} \tag{5}$$

Hence, for separation

$$m \geqslant \frac{r_1 + r_2}{R_{F_1} - R_{F_2}} \tag{6}$$

Using cellulose layers and aqueous phosphate buffers, Harris[1] resolved purine and pyrimidine mixtures of nucleic acid hydrolyzates. The spots varied from 0.4 to 0.8 cm in diameter. Using 1.0 M NaH_2PO_4 solution as solvent, the published R_F values of adenine and guanine are 0.55 and 0.43, respectively. If a chromatogram is obtained with the smaller spots, then

$$r_1 + r_2 = 0.4 \tag{7}$$

$$R_{F_1} - R_{F_2} = 0.12 \tag{8}$$

Therefore

$$m \geqslant \frac{0.4}{0.12} = 3.33 \text{ cm} \tag{9}$$

Hence, provided that the solvent is allowed to migrate more than 3.33 cm, the separation of guanine and adenine would be achieved. In the same solvent system, the R_F values of uracil and thymine were 0.72 and 0.69, respectively.

$$m \geqslant \frac{0.4}{0.03} = 13.33 \text{ cm} \tag{10}$$

Therefore, in order to achieve the separation of the four bases, the solvent must migrate at least 13.33 cm.

Eqn. 6 shows, other things being equal, that thin-layer chromatography is usually superior to paper chromatography for resolution, because $r_1 + r_2$ is seldom more than 1.0 cm. Furthermore, it is common practice when using thin layers, especially commercially prepared thin-layer plates on a flexible backing, which are of a fixed size, to restrict the solvent migration to 10 cm. If small spots ($r_1 = r_2 = 0.2$ cm) are obtained, then from eqn. 6 we have

$$10 \geqslant \frac{0.4}{R_{F_1} - R_{F_2}} = \frac{0.4}{\Delta R_F} \tag{11}$$

Therefore

$$\Delta R_F \geqslant 0.04 \tag{12}$$

This value, ΔR_F, is the minimal difference in the R_F values of the substances to be resolved which will permit resolution under these conditions.

It may be allowed, therefore, when using thin layers, with careful control of spot size so that $r_1 + r_2 = 0.4$ cm, and restricting $m = 10$ cm, that the difference between the R_F values of the substances to be resolved must be not less than 0.04.

The general equation of resolution is

$$\Delta R_F \geqslant \frac{r_1 + r_2}{m} \tag{13}$$

and a decision can be made as to choice of solvent from published values by estimating r_1 and r_2 under the experimental conditions appertaining, and making a decision as to m, the length of migration to be used.

When it is desired to elute material quantitatively from the chromatogram, eqn. 13 can be modified to give a condition for resolution with no overlap of the resolved spots. If we take 1 cm as an arbitrary separation between the edges of adjacent spots, the modified equation becomes

$$\Delta R_F \geqslant \frac{r_1 + r_2 + 1}{m} \tag{14}$$

TABLE I

DIFFERENCE IN R_F VALUES (ΔR_F) FOR RESOLUTION OF TWO SUBSTANCES AS A FUNCTION OF MIGRATION OF SOLVENT, m cm, AND COMBINED SPOT SIZE, $(r_1 + r_2)$ cm

The R_F values in italic type refer to the theoretical separation when two spots touch edge to edge. The values in roman type refer to a separation of 1 cm between the circumference of spots or streaks.

m	$r_1 + r_2$							
	0.4	*0.50*	*0.60*	*0.80*	*1.00*	*2.50*	*3.00*	*5.00*
10	*0.04*	*0.05*	*0.06*	*0.08*	*0.10*	*0.25*	*0.30*	*0.50*
	0.14	0.15	0.16	0.18	0.20	0.35	0.40	0.60
20	*0.02*	*0.025*	*0.03*	*0.04*	*0.05*	*0.125*	*0.15*	*0.25*
	0.07	0.075	0.08	0.09	0.10	0.175	0.20	0.30
30	*0.014*	*0.017*	*0.023*	*0.027*	*0.034*	*0.084*	*0.100*	*0.167*
	0.047	0.050	0.053	0.06	0.067	0.117	0.133	0.20
40	*0.010*	*0.0125*	*0.015*	*0.020*	*0.025*	*0.062*	*0.075*	*0.125*
	0.035	0.0375	0.04	0.045	0.05	0.087	0.10	0.15
50	*0.008*	*0.005*	*0.012*	*0.016*	*0.02*	*0.05*	*0.06*	*0.10*
	0.028	0.030	0.032	0.036	0.04	0.07	0.08	0.12

It should be noted that eqns. 1–14 are also applicable to streaking material for resolution. Eqn. 14 can also be applied to paper chromatography, when m can be as high as 50 cm. Table I is a compilation for rapid reference for choosing likely solvent systems from published R_F values.

REFERENCE

1 A. B. Harris, *Biochim. Biophys. Acta*, 145 (1967) 520.