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# OVERLAPPING RESOLUTION MAPS AS AN AID IN PARALLEL DEVELOPMENT THIN-LAYER CHROMATOGRAPHY

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## SUMMARY

A method is described for constructing overlapping resolution maps for thin-layer chromatography. The axes of the maps are time, length and binary solvent composition. These maps can be used to identify sets of compounds within a mixture such that each set is fully separated under appropriate conditions. The separation of a mixture of fifteen steroids is described, using this technique.

## INTRODUCTION

Thin-layer chromatography (TLC) has gained acceptance in recent years as a rapid and reliable technique for the quantitative analysis of simple mixtures. Complex mixtures can be separated by TLC but due to the rather limited separating power of a TLC plate, multiple development TLC is usually required for the separation of such mixtures. This is usually a time-consuming procedure. An alternative approach for the separation of moderately complex mixtures is to divide the mixture into sets of compounds such that each compound in the mixture is represented in at least one set[1]. TLC conditions are then selected such that the components of each set are completely separated. Each set will require a separate TLC plate and will be run under different chromatographic conditions. Parallel development TLC has been suggested as a name for this method of analysis[1]. While it is also a multiple development technique, it has the advantage that the multiple developments can be performed simultaneously which results in reduction of the overall analysis time. The most significant problem associated with this method is that of assigning compounds to sets. A method, based on the inspection of plots of distance migrated *vs.* solvent composition, has already been described. An alternative method which utilizes the overlapping resolution map concept, which was introduced by Glajch *et al.*[2] for high-performance liquid chromatographic (HPLC) solvent optimization, is described below.

## EXPERIMENTAL

The solvents used were ethyl acetate and 1,1,2-trichlorotrifluoroethane, obtained from Aldrich (Milwaukee, WI, U.S.A.). Steroids were obtained from Sigma

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(St. Louis, MO, U.S.A.). Silica gel plates, catalogue No. 46011, were obtained from Analtech (Newark, DE, U.S.A.).

Chromatography was in a Camag Linear Chamber, modified for continuous development, as described elsewhere[3]. Plates were stored at a relative humidity of 60% until immediately before use.

Solute visualization was with 10% aqueous sulfuric acid, as described elsewhere[3].

## RESULTS AND DISCUSSION

The method of parallel development TLC relies on identifying sets of compounds such that each compound in a mixture is present in at least one set and that chromatographic conditions are available for separating the components of each set. Optimizing chromatographic conditions once a set of five to ten components is identified is generally straightforward provided a suitable binary solvent system is used. A more formidable problem is that of assigning compounds into sets. A method based on the inspection of a plot of distance migrated *vs.* solvent composition has already been described[1]. The method that we describe here is based on the overlapping resolution map (ORM) concept introduced by Glajch *et al.*[2].

In the ORM method six or seven solvent compositions are used to experimentally determine resolution for a given pair of compounds. These values of resolution are used to establish a statistical equation which expresses resolution as a function of ternary solvent composition. This equation is used to construct a resolution map (a ternary solvent diagram) for each pair of compounds in the mixture, showing which range of solvent compositions provides a minimum of baseline resolution. The resolution maps for all pairs are then overlaid to determine the range of concentrations that will yield the specified resolution or better between all components of the mixture.

The method that is described below differs in several respects from the original ORM method apart from the obvious difference that it is used here to select conditions for TLC whereas Glajch developed it for HPLC. The triangular diagram in the original ORM method describes ternary solvent composition whereas in the method described below the triangular diagram describes binary solvent composition, TLC plate length and time of analysis. Thus in addition to solvent composition, both TLC plate length and analysis time are variables. Furthermore an analytical equation is used to describe the acceptable region in the TLC parameter triangle rather than a statistical equation used in the original ORM method.

### *The equations*

The relationship between retardation factor, $R_F$, and capacity factor, $k$, is:

$$R_F = \frac{1}{1 + k} \tag{1}$$

For many binary solvent mixtures consisting of a polar and non-polar solvent the following relationship between capacity factor and the mole fraction, $X_s$, of the polar solvent holds

$$\ln k = a \ln X_s + b \tag{2}$$

where $a$ and $b$ are constants characteristic of a given compound. This relationship was originally derived in terms of $R_M$ by Soczewinski *et al.*[4].

The relationship between $z$, the distance in mm migrated by the solvent from the origin, $t$ the time in seconds required for this migration and $\kappa$ the solvent velocity constant is well approximated by:

$$z^2 = \kappa t \tag{3}$$

From this it follows that the rate of solvent migration decreases as the solvent front traverses the TLC plate. The value of $\kappa$ varies with mole fraction. For several binary solvent systems that were examined, the value of $\kappa$ can be expressed by the following quadratic expression:

$$\kappa = a_1 + a_2 X_s + a_3 (X_s)^2 \tag{4}$$

In continuous development TLC solvent is allowed to evaporate off the end of a TLC plate which extends out of a development chamber. Under these conditions $u_c$ the rate of solvent migration is constant

$$u_c = \kappa / 2l \tag{5}$$

where $l$ is the length of the TLC plate.

In continuous development TLC the total analysis time, $t_l$, consists of two components

$$t_l = t_1 + t_2 \tag{6}$$

where $t_1$ is the time during which the solvent front traverses the TLC plate and $t_2$ is the time during which continuous development occurs.

$M_D$ the distance migrated by each solute during $t_l$ is

$$M_D = d_1 + d_2 \tag{7}$$

where $d_1$ and $d_2$ are the respective distances migrated during $t_1$ and $t_2$. The distance $d_1$ is

$$d_1 = R_F (l - x) \tag{8}$$

where $x$ is the distance between spot origin and solvent origin. The distance $d_2$ is:

$$\begin{aligned} d_2 &= R_F u_c t_2 \\ &= R_F (\kappa / 2l) t_2 \end{aligned} \tag{9}$$

If $t_2$ is rewritten

$$\begin{aligned} t_2 &= t_l - t_1 \\ &= t_l - \frac{l^2}{\kappa} \end{aligned}$$

substituting into eqn. 9 we obtain:

$$d_2 = R_F\left(\frac{\kappa}{2l}\right)\left(t_l - \frac{l^2}{\kappa}\right)$$
$$= R_F\left(\frac{\kappa t_l}{2l} - \frac{l}{2}\right) \tag{10}$$

From eqns. 7, 8 and 10 it follows that:

$$M_D = R_F(l - x) + R_F\left(\frac{\kappa t_l}{2l} - \frac{l}{2}\right)$$
$$= R_F\left(\frac{l^2 - 2lx + \kappa t_l}{2l}\right) \tag{11}$$

From eqns. 1, 2 and 11 it follows that:

$$M_D = \frac{1}{1 + \exp(a \ln X_s + b)}\left(\frac{l^2 - 2lx + \kappa t_l}{2l}\right) \tag{12}$$

The center-to-center separation between two spots p and q is:

$$S_D^{p,q} = |M_D^p - M_D^q|$$
$$= \left|\frac{1}{1 + \exp(a_p \ln X_s + b_p)} - \frac{1}{1 + \exp(a_q \ln X_s + b_q)}\right| \times$$
$$\times \left(\frac{l^2 - 2lx + \kappa t_l}{2l}\right) \tag{13}$$

Thus the spot separation between any pair of compounds can be expressed as a function of plate length, $l$, analysis time, $t_l$, and binary solvent composition, $X_s$, provided the constants $a_1$, $a_2$ and $a_3$ in eqn. 4 and the constants $a_p$, $a_q$, $b_p$, $b_q$ in eqn. 13 are first determined from experimental data.

Eqn. 13 can be used to draw a resolution map showing an area on a triangular diagram where spot separation is greater than or equal to a specified distance. We have followed the convention introduced by Glajch *et al.*[2] and have shaded that part of the map where the specified separation is not obtained.

The number of possible pairs of solute combinations is $n!/(n-2)!2$. We discuss here the separation of a mixture containing the fifteen steroids listed in Table I. This would result in 105 resolution maps. This number can be significantly reduced by considering neighboring pairs only. At any given solvent composition there will be $n - 1$ neighboring pairs. However there are often spot inversions over a range of mole fractions. This increases the number of pairs that must be considered. Eqns. 1 and 2 can be used with a suitable computer program to determine elution orders for all the compounds in the mixture over the required range of mole fractions. This was done for the fifteen steroids discussed here for which it was found that there are 24

TABLE I

THE FIFTEEN STEROIDS GROUPED AS HIGH $R_F$ SOLUTES AND LOW $R_F$ SOLUTES

The underlined compounds in the high $R_F$ solutes are those present in maps 1 through 14 in Fig. 1; the underlined compounds in the low $R_F$ solutes are those present in maps 13 through 24.

| *Elution order at solvent composition for high $R_F$ steroids* | *Elution order at solvent composition for low $R_F$ steroids* |
|---|---|
| Mestranol | Mestranol |
| Cholesterol | Estrone |
| Ergosterol | Cholesterol |
| Estrone | Ergosterol |
| Androstanedione | Androstanedione |
| Progesterone | Progesterone |
| Ethisterone | Ethisterone |
| Androstanolone | Androstanolone |
| Acetoxyprogesterone | Acetoxyprogesterone |
| Epiandrosterone | Epiandrosterone |
| Methandriol | Methandriol |
| Adrostenediol | Androstenediol |
| Testosterone | Testosterone |
| Cortisone | Cortisone |
| Digoxin | Digoxin |

possible pairs of compounds that must be considered. The resolution maps for these pairs are shown in Fig. 1. The axes of each map are the same as those in Figs. 2 and 3. The maximum value of length, $l$, for each map is 200 mm and the maximum time is 145 min. There is no solvent composition within these maps where all components can be separated, *i.e.*, the entire area is shaded when all 24 maps are overlaid.

This mixture can however be separated by the technique of parallel development TLC. This refers to the technique of dividing the mixture into sets of components and using individual TLC plates under appropriate conditions for separating each set of compounds. The entire sample is spotted on each plate. Solutes that are not separated on a given plate either migrate into the solvent front or are of lower $R_F$ value than the members of the set that is separated. This technique has been used to separate the fifteen-component mixture as discussed here. In the original description, compounds were assigned to one of two sets by inspection of an $M_D$ *vs.* $X_s$ plot. The two sets can be more easily identified by overlapping the individual resolution maps shown in Fig. 1. Maps 1 through 14 can be overlaid as is shown in Fig. 2 and maps 13 through 24 can be overlaid as shown in Fig. 3. In our first description of the separation of this fifteen-steroid mixture by parallel development TLC it was necessary to calculate optimum separation conditions after identification of the two sets of compounds. This is not necessary with overlapping resolution maps. The conditions for the minimum time of analysis can be read directly from Figs. 2 and 3. For the high $R_F$ set of compounds the conditions are: $X_s = 0.22$, $l = 80.7$ mm and $t_l = 55.8$ min. For the low $R_F$ set the conditions are: $X_s = 0.48$, $l = 82.6$ mm and $t_l = 45.4$ min. These are the same conditions as found with the method described earlier in this paragraph. There is a good agreement between experimental and predicted results as has already been described.

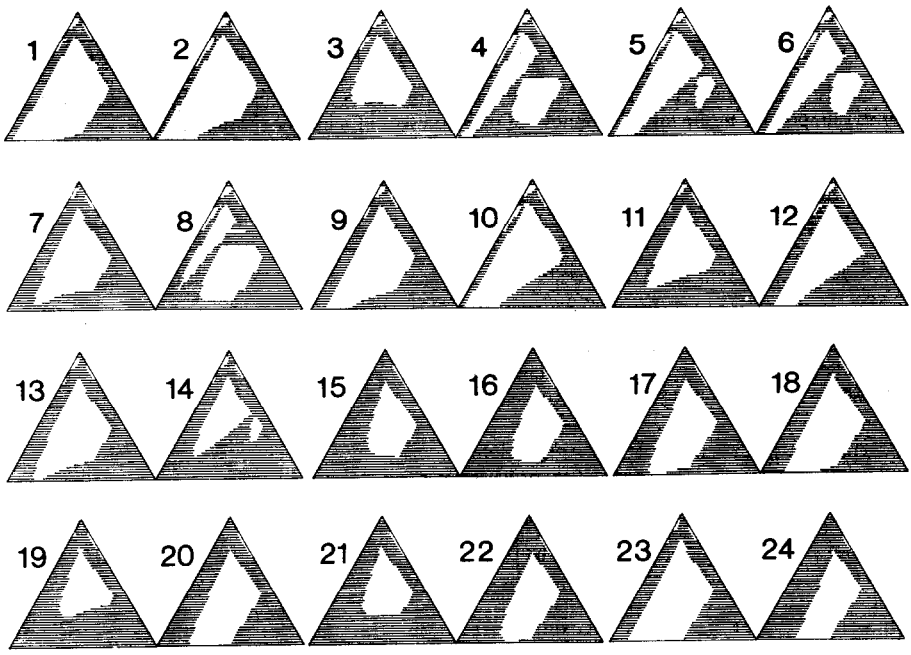


Fig. 1. Resolution maps for the 24 possible neighboring pairs of solutes for the separation of the fifteen steroids in Table I in ethyl acetate–1,1,2-trichlorotrifluoroethane. The three axes are as in Figs. 2 and 3. The identities of the pairs are: 1, mestranol/cholesterol; 2, mestranol/estrone; 3, cholesterol/ergosterol; 4, cholesterol/estrone; 5, cholesterol/androstanedione; 6, ergosterol/androstanedione; 7, esterone/androstanedione; 8, ergosterol/estrone; 9, androstanedione/progesterone; 10, ergosterol/progesterone; 11, progesterone/ethisterone; 12, progesterone/androstanolone; 13, ethisterone/acetoxyprogesterone; 14, ethisterone/androstanolone; 15, acetoxyprogesterone/epiandrosterone; 16, acetoxyprogesterone/androstanolone; 17, epiandrosterone/androstanolone; 18, androstanolone/methandriol; 19, epiandrosterone/methandriol; 20, methandriol/testosterone; 21, methandriol/androstendiol; 22, testosterone/androstendiol; 23, testosterone/cortisone; 24, cortisone/digoxin.

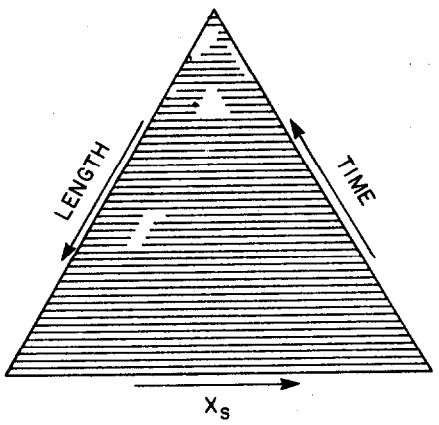


Fig. 2. The composite formed by overlapping maps 1 through 14 in Fig. 1.

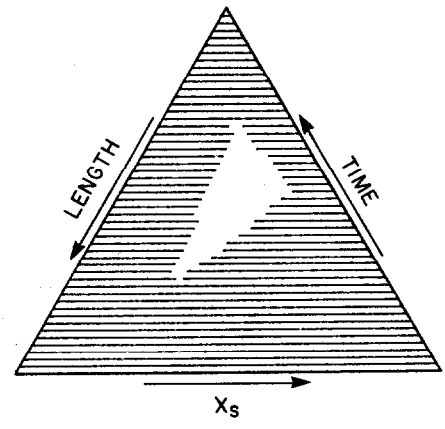


Fig. 3. The composite formed by overlapping maps 13 through 24 in Fig. 1.

Care must be exercised when using overlapping resolution maps to assign sets in parallel development TLC. The possibility exists that compounds that are not represented in a given overlapping resolution map nevertheless interfere with the separation of the given set of compounds. This can in principal occur due to spot inversions. This possibility can be checked by listing the elution order of all compounds in the mixture at the two solvent compositions at which the mixture is separated. This is easily done using eqns. 1 and 2. The listing of the compounds is shown in Table I. The compounds that are underlined in each column represent the low and high $R_F$ sets of compounds and are all represented in the individual maps comprising the corresponding overlaid map. Thus, it is seen that in this case there is no interference from compounds not represented in a given overlapping resolution map.

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