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Using the liquid nature of the stationary phase. VI. Theoretical study of multi-dual mode countercurrent chromatography

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ABSTRACT

Countercurrent chromatography (CCC) is a separation technique using a biphasic liquid system and centrifugal forces to maintain a support-free liquid stationary phase. Either one of the two phases can be the liquid stationary phase. It is even possible to switch the phase role during the separation. The dual-mode method is revisited recalling its theoretical background. The multi-dual mode (MDM) CCC method was introduced to enhance the resolution power of a CCC column. The theoretical study of the MDM method is validated by modeling the separation of two solutes. The basic hypothesis is that the forward step (partial classical elution) is followed by a backward step that returns the less retained solute to the column head. The equations show that the most important parameter to maximize resolution is not the number of MDM steps but the total volume of liquid phases used to elute the solutes. The model is validated calculating correctly the peak position of previously published MDM experiments.

1. Introduction

Countercurrent chromatography (CCC) is a separation technique using a biphasic liquid system to separate the components in a mixture [1–3]. Both the mobile phase and the stationary phase are liquid. It is difficult to maintain the selected liquid phase really stationary while the other liquid phase, the mobile phase, is pushed through it. Centrifugal fields are always used to generate a support-free liquid stationary phase [2]. The major use of the CCC technique is the purification of large amounts of compounds. CCC is a preparative technique [4]. Also, the liquid nature of the stationary phase allows for special processes that are not possible with any other chromatographic technique using a solid stationary phase. Five original uses of a liquid stationary phase were described in previous works of this series: (i) complexation reactions in the stationary phase [5], (ii) using a CCC column as a chemical reactor [6], (iii) elution-extrusion [7], a method that greatly extends the CCC hydrophobicity window [8], (iv) moving the two liquid phases in the same direction or co-current CCC [9], and (v) the back-extrusion CCC method [10].

The dual-mode method was the first real use of the liquid nature of the stationary phase. It consists of changing the elution mode during the separation process, switching the phase role and circulation direction. For example, the reversed phase mode can be initially used with an aqueous mobile phase and an organic apolar stationary liquid phase. The flowing direction must be from head to tail or descending since aqueous phases are always lower phases in non-chlorinated solvent systems [2,3]. Next, the normal phase mode can be used to complete the sample separation. After a measured elution time in reversed phase mode, the phase role and flowing direction are switched: the apolar liquid phase is used as the mobile phase flowing through the polar aqueous phase in a normal phase way and tail-to-head or ascending direction [2,3]. The dual mode method was used as soon as reliable CCC hydrostatic [11] or hydrodynamic columns were developed [2,4].

The theoretical modeling of the dual-mode method was first proposed independently by Menges et al. [12] and Gluck et al. [13] in 1990. It was further developed in 1997 by Agneli and Thiebaut [14]. The method was used to reduce the elution time of highly retained compounds [12,14] or to measure high solute liquid–liquid partition coefficients [13]. Recently, the dual-mode method was used to increase the resolution power of a CCC column in the case of two solutes with close partition coefficients. The change in phase role was performed several times calling the method "multiple dual-mode" (MDM) as first proposed by Delannay et al. [15]. The MDM method was later used to improve the separation of the naproxen enantiomers [16] and that of honokiol and magnolol [17]. In the later case, the method was called "intermittent" CCC instead of MDM because a continuous feed of the honokiol + magnolol mixture was introduced in the middle of two identical CCC hydrodynamic columns serially connected [17]. Continuous sample feed in between two hydrostatic CCC columns and associated with MDM elution was earlier called and even patented as "true moving bed" (TMB) [18]. The TMB naming is somewhat

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misleading since the patent does not describe the two liquid phases flowing opposite to one another at the same time; it describes a MDM process with alternating phases and flow directions. In all these uses, an increased resolution between compounds was obtained but the sample feed between two CCC columns add an essential advantage: it allows for continuous purification [17,18]. The MDM or "intermittent" CCC method equations were recently proposed and tested with model compounds [19]. However, the different peak widths are poorly considered [19] or even taken as constant [14] when band broadening is known to have a serious effect on chromatographic resolution. In a recent article, the change in peak width in CCC was fully studied [20]. In this work, the theory of the dual mode method is revisited and used to develop a theoretical approach for the MDM method taking in account the broadening of the solute bands inside the CCC column.

2. Theoretical models

2.1. The dual-mode method

2.1.1. Peak position

As previously modeled [12–14], the dual mode method must be described following its two steps (Fig. 1).

The first step is called "classical" meaning that the mobile phase is pumped into in the CCC column in the right direction: the upper mobile phase should flow in the tail-to-head or ascending direction through the lower stationary phase; the lower mobile phase should flow in the descending or head-to-tail direction through the upper stationary phase [1–4]. This classical mode step is performed using a mobile phase volume, V_{CM}. The solutes move inside the column by a distance x_i:

$$x_i = \frac{LV_{CM}}{V_{Ri}} \tag{1}$$

with the column length L and the solute retention volume, V_{Ri}, defined as:

$$V_{Ri} = V_M + K_{Di}V_S \tag{2}$$

The subscripts M and S stand for mobile and stationary phase volume inside the equilibrated CCC column of volume V_C with $V_M + V_S = V_C$. The dual mode method consists in switching the phase role: the stationary liquid phase becoming the mobile phase and *vice versa*. In the coming text, it will be avoided as far as possible to use the V_M and V_S notations that may be confusing, however, each time these notations are seen, they correspond to the phase volumes initially obtained when the CCC column was first created. The solute K_{Di} is the distribution ratio or partition coefficient expressed as *[concentration of i in all forms in the initial stationary phase]*$_S$ over *[concentration of i in all forms in the initial mobile phase]*$_M$ [1–3]. This value will be kept after phase switching using $1/K_{Di}$ as representing the Solute *i* distribution ratio.

Obviously, there are requirements: the solute must not leave the column before the phase switch. The condition is $x_i < L$ implying $V_{CM} < V_{Ri}$. If the later condition is passed, it simply means that the solute eluted from the column in the mobile phase forming a classical chromatogram. These eluted solutes will not be affected by the phase switch when the dual-mode method is performed. To exit the column, all solutes still in the column must travel back the distance x_i (Eq. (1)) but now eluted by the other phase with which they have the retention volume, V'_{Ri}:

$$V'_{Ri} = V_S + \left(\frac{1}{K_{Di}}\right)V_M = \frac{V_{Ri}}{K_{Di}} \tag{3}$$

Using Eqs. (1) and (3), the volume V_{DMi} to elute each solute is expressed by:

$$V_{DMi} = \frac{V'_{Ri}x_i}{L} = \frac{V_{CM}}{K_{Di}} \tag{4}$$

All solutes with retention volumes lower than V_{CM} have left the column during the classical mode step. The solutes eluting during the dual mode step fulfill the condition:

$$V_{Ri} > V_{CM} \tag{5}$$

Introducing *Sf*, the stationary phase retention ratio V_S/V_C, Eq. (5) can be rewritten as:

$$V_C[1 + (K_{Di} - 1)Sf] > V_{CM} \tag{6}$$

and developed for K_{Di} as:

$$K_{Di} > \frac{(V_{CM}/V_C) - 1}{Sf} + 1 \tag{7}$$

Eq. (7) is very interesting showing that, using a mobile phase volume exactly equal to the column volume during the classical step, i.e. $V_{CM} = V_C$, all solutes with a distribution ratio (or partition coefficient) lower than unity ($K_{Di} < 1$ or high affinity for the mobile phase) will be eluted in the classical mode (CM) step. All solutes having more affinity for the stationary phase ($K_{Di} > 1$) will remain inside the column and be eluted during the dual mode (DM) step with retention volumes given by Eq. (4). With $V_{CM} = V_C$, the maximum value for V_{DMi} is obtained for $K_{Di} = 1$ and is also exactly V_C (Eq. (4)). It means that with two V_C volumes of solvents, it is possible to elute all compounds contained in any sample. One column volume of mobile phase will elute in the classical way all solutes with K_{Di} lower than unity. A second column volume of the other phase used in the dual-mode way will elute on the other side of the column all remaining solutes [12–14].

This was observed experimentally. For example, a mixture containing five compounds with increasing partition coefficients K_{D1}–K_{D5} would elute in classical mode following the increasing order: 1, 2, 3, 4 and 5. If the dual mode method is used after the elution of Peak 1, the elution order will be 1 (eluted in classical mode with the mobile phase, Fig. 1) followed by 5, 4, 3 and 2 eluting in what was initially the stationary phase [1–4,13–15,20].

2.1.2. Peak width

It was shown that the solute bands broaden as they travel inside the CCC column. Using the peak variance, σ, and assuming that all peaks follow a Gaussian distribution, the band width at base is equal to 4σ expressed as [20]:

$$\sigma = \sqrt{xH} \tag{8}$$

with x the distance traveled and H the height equivalent to a theoretical plate ($H = L/N$), N being the number of theoretical plates of the CCC column. To be able to go on modeling, it will be also assumed that the column efficiency, i.e. the plate count, N, hence the height (length) equivalent to a theoretical plate, H, is the same for two successive compounds in the normal phase mode with an organic mobile phase, than that obtained in the reversed phase mode with the lower aqueous mobile phase. It is acknowledged that this assumption is highly questionable since it has been shown that the column efficiency was greatly depending on solute liquid–liquid distribution ratio as well as on stationary phase viscosity [2,3,21,22] and very often, the viscosities of the two phases of a biphasic liquid system are different. However, the constant efficiency assumption allows for a much simpler full theoretical development.

If the H term is unchanged by the switch of the role of the phases, then the band width, σ, increases with the distance traveled by the solute band regardless if it is going forward or backward. Eq. (8) can be used cumulating the solute displacements inside the column.

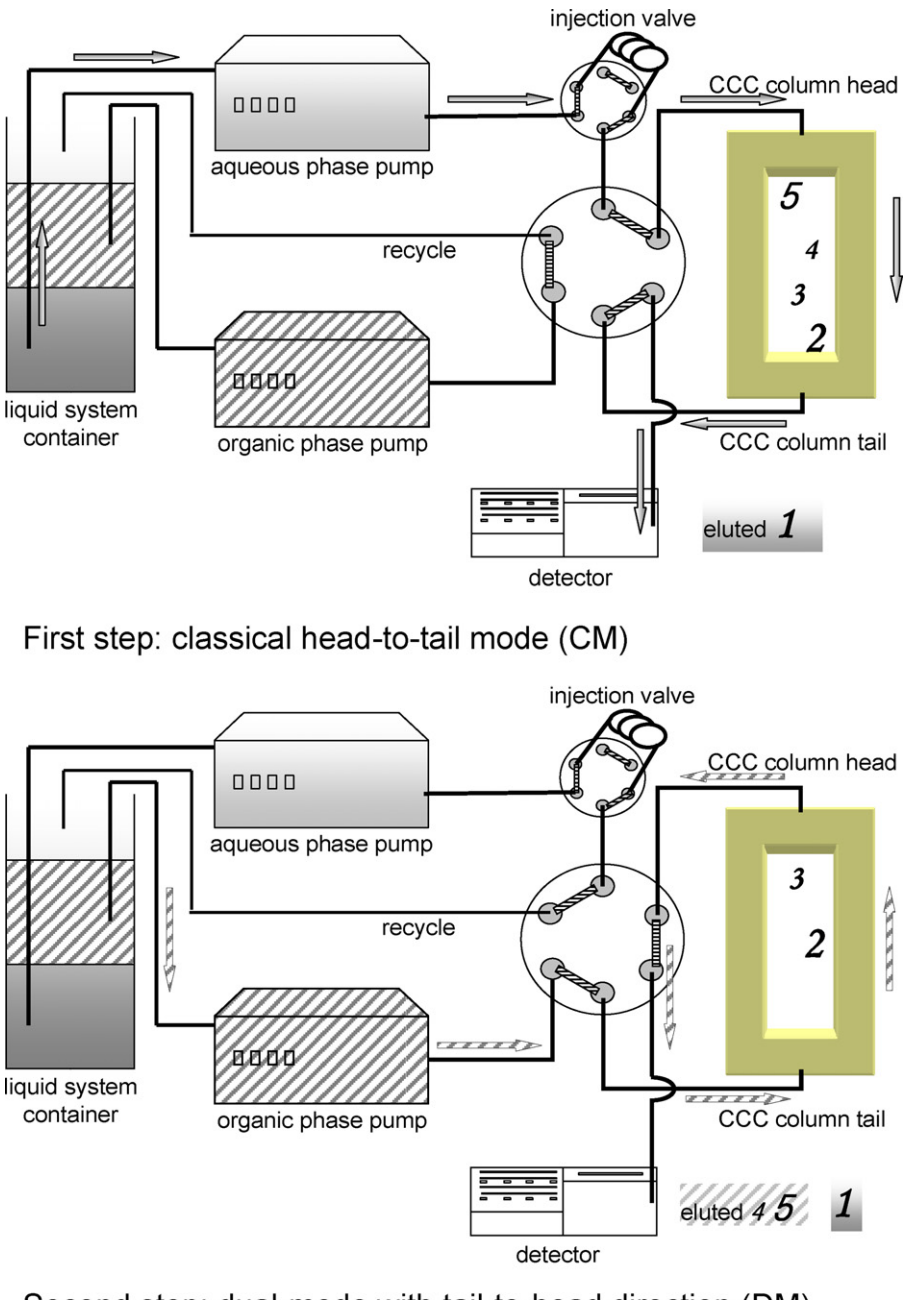

Fig. 1. Dual-mode scheme. (Top) Step 1, Solutes #1 to #5 were injected and the classical mode was started with the lower aqueous phase flown in the head-to-tail descending direction for a *Vcm* volume eluting only Solute #1. (Bottom) Step 2, the direction and phase valve is switched after *Vcm*. Both phase nature and flowing direction are reversed. Solute #5 elutes following Solute #1 and followed by Solute #4. Solutes #3 and #2 are still in the column but will elute in less than a column volume of organic phase (see text).

Expressing the total motion of the solute in the column by $\sum|x|$, the peak variance is simply:

$$\sigma = \sqrt{\sum |x|H} \tag{9}$$

2.1.3. Resolution factor

The resolution factor, *Rs*, is a measure of the quality of a chromatographic separation of two solutes eluted with the same mobile phase. The words "same mobile phase" are not relevant in any chromatographic techniques other than CCC. Indeed in CCC and using the dual-mode method, it was shown that it is possible to elute solutes in the classical way dissolved in the mobile phase and more solutes dissolved in the other phase exiting from the other side of the column. In that case, the resolution equation must be adapted as:

$$Rs = \frac{V_{R2} - V_{R1}}{(w_1 + w_2)/2} \tag{10}$$

The resolution factor between two peaks 1 and 2 is defined as the dimensionless ratio of the retention volume (or time) difference over the average peak width at base, *w*, expressed in volume (or time) unit. Eq. (1) shows that the distances traveled by the solutes inside the column and solvent volumes are linked. Eqs. (8) and (9) show that these distances are linked to the peak variance, σ, itself simply related to the peak width at base, *w*, by (Gaussian peaks):

$$w = 4\sigma \tag{11}$$

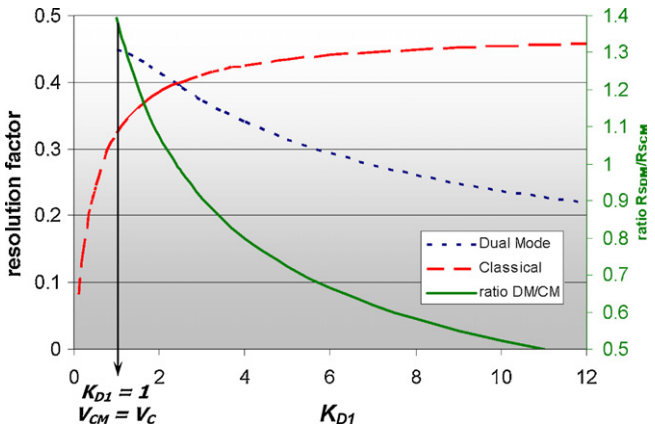

Fig. 2. Dual-mode resolution factor (Eq. (13); dotted line) compared to classical elution resolution factor (Eq. (15); dashed line) and the corresponding ratio value (solid line and right axis). CCC column volume V_C = 18 mL, Sf = 66.7% with V_M = 6 mL and V_S = 12 mL, N = 400 plates, selectivity K_{D2}/K_{D1} = 1.1, $V_{CM} = V_C$ = 18 mL. All solutes with $K_D > 1$ (arrow) are eluted in dual-mode.

Using Eqs. (1) and (8), it was demonstrated that the resolution factor could be expressed as [20]:

$$Rs = \frac{\left|\sqrt{x_2} - \sqrt{x_1}\right|}{2\sqrt{H}} \tag{12}$$

It is possible to use Eq. (12) to estimate the resolution factor obtained in dual-mode provided that Solute 1 did not elute before the phase role was switched (Eqs. (5) and (7)). Then, the distance x_i traveled by a given solute i in the classical step, V_{CM}, is the same that should be traveled backward during the dual-mode step. Using the plate height definition: $H = L/N$ we can form:

$$Rs = \sqrt{\frac{NV_{CM}}{2}}\left(\sqrt{\frac{1}{V_{R1}}} - \sqrt{\frac{1}{V_{R2}}}\right) \tag{13}$$

The dual-mode resolution factor was fully studied recently [20]. It was shown that it depended on the solute distribution ratio K_D as well as the liquid phase ratio inside the column ($\gamma = V_M/V_S$). With the single back and forth motion of the dual-mode method, it was found that the resolution factor increases compared to what would be obtained by the simple classical elution if the carrying by the mobile phase (low K_D solutes and classical mode) has more weight than the transport by the other phase (high K_D solutes and dual mode). The partition coefficient value, K_{De}, of a solute equally carried by the two phases in the dual mode procedure is as follows [20]:

$$K_{De} = \frac{1 - Sf}{Sf} = \frac{V_M}{V_S} = \gamma \tag{14}$$

2.1.4. Dual-mode versus classical elution mode

Agnely and Thiebaut extensively studied the advantages of the dual-mode method compared to those of classical elution CCC [14]. More than 20 complex equations were needed to show that there were drastic conditions required to obtain a better resolution factor with the dual mode method compared to a classical elution.

To make the choice simpler, the resolution factor obtained in classical CCC elution, Rs_{CM}, is recalled:

$$Rs_{CM} = Sf\frac{\sqrt{N}}{4}\frac{K_{D2} - K_{D1}}{1 - Sf[1 - (K_{D2} + K_{D1})/2]} \tag{15}$$

Fig. 2 shows the resolution factor, Rs_{CM}, obtained in classical CCC (dashed line) with two solutes and a selectivity factor of 1.1 ($\alpha = K_{D2}/K_{D1}$ = 1.1). This factor increases continuously as the solute partition coefficient increases. Fig. 2 also shows the resolution factor, Rs_{DM}, obtained in dual-mode (Eq. (13)) after one column volume of mobile phase in classical mode ($V_{CM} = V_C$) meaning that all solutes with K_{Di} lower than 1 eluted. Another column volume of what was initially the stationary phase is needed to elute all solutes remaining inside the column with the resolution factor shown by a dotted line in Fig. 1. Rs_{DM} decreases continuously starting at higher values than Rs_{CM} for low K_D solutes, but becoming clearly lower for high K_D solutes (Fig. 2 dotted line). The solid line is the ratio of the two resolution factors, Rs_{DM}/Rs_{CM}. If the ratio is higher than unity, using the dual-mode method will be beneficial producing better resolutions than the classical elution mode. This occurs for low K_D solutes. The resolution factors obtained for high K_D solutes are always better in classical mode elution than in dual-mode elution. However, it is important to consider all other experimental factors. The classical elution of high K_D solutes needs a large volume of mobile phase and likely long experiment duration. Many different experimental configurations were tested producing different resolution factors but a similar trend for all three presented curves.

2.2. The multi-dual-mode method

The denomination "multi-dual-mode" with the MDM acronym [15–16] will be used in this work preferentially to the naming "intermittent" CCC [17,19]. The MDM method consists in successive switching of both mobile phase nature and flowing direction. It was always used to increase the separation power of a CCC column [15–18]. To separate polyaromatic hydrocarbons or indole compounds, Delannay et al. used a succession of ascending and descending elution modes calling it MDM [15]. At each successive step, parts of purified compounds were collected ("shaving" method) at the respective column exits (head or tail), pushing back in the CCC column the remaining part of the mixture. Rubio et al. [16] and Yang et al. [19] separated enantiomers of leucine and naproxen or DNB-amino acids switching the phase role (back and forth liquid circulation) during the run without letting the enantiomers reach a column terminal.

The constant step MDM method consists in increasing the CCC column resolution capability switching the phase role several times. The method will be modeled saying that the initial step (and following odd numbered steps) are done in the reversed phase mode, i.e. the initial mobile phase is the lower aqueous phase of a biphasic liquid system flowing in the head-to-tail or descending direction with the upper organic stationary phase. The first dual mode step (and all even numbered steps) are done in normal phase with the upper organic phase being mobile flowing in the tail-to-head or ascending direction. The parameters selected are the liquid phase volumes V (Eq. (2)) and distances x traveled by the solute bands inside the column of length L (Eq. (1)). Since these distances are linked to the band widths (Eq. (9)), they allow for an easy resolution estimation (Eq. (12)). These distances will be noted $x_{i,j}$ with the first subscript i referring to Solute i and the second subscript j referring to Step j. It must be noted that odd numbered steps are done with the lower aqueous phase and positive values for x, the solutes moving toward column tail. Even numbered steps correspond to negative x values, the solutes regressing to the column head pushed by the upper organic phase (Fig. 3). However, for solute band broadening, the variances are additive and Eq. (9) is used cumulating all traveled distances [20].

2.2.1. Step 1

The less retained solute is the first to elute with the minimum lower phase V_{R1} retention volume and smallest K_{D1} distribution ratio. The aqueous mobile phase volume of the first MDM step, V_F with F for forward, should be a fraction of V_{R1} to be sure that Solute 1 does not elute immediately. Taking V_F as a nth fraction of V_{R1}, we

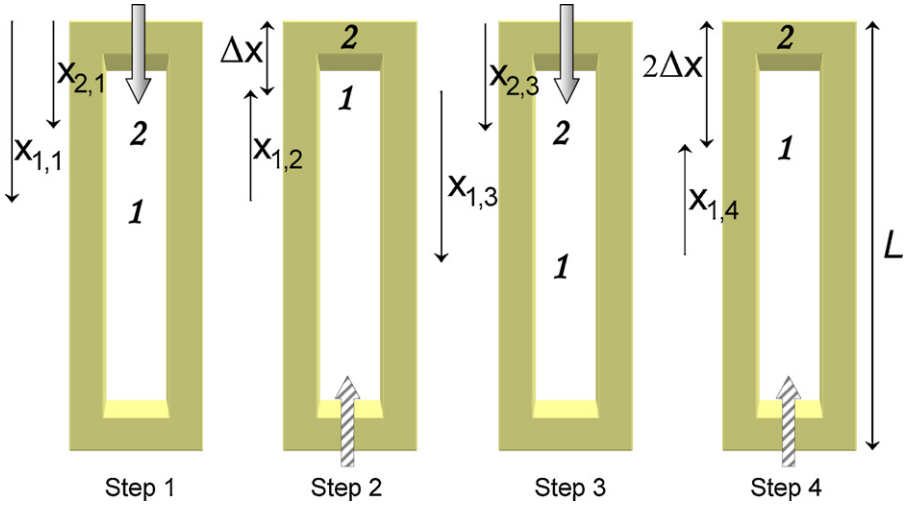

Fig. 3. The multi-dual-mode scheme in a CCC column of length L. Step 1, lower aqueous phase flown in the head-to-tail or descending direction (grey arrow). Solute 1 moved down by $x_{1,1}$; Solute 2 moved down by $x_{2,1}$. Step 2, first DM step with organic phase flown in the tail-to-head or ascending direction (hatched arrow). Solute 1 moved up by $x_{1,2}$; Solute 2 moved down by $x_{2,2}$ exactly equal to $x_{2,1}$ so putting it back to the column head when Solute 1 is down by Δx in the column. Steps 3 and 4 duplicate Steps 1 and 2 with $x_{1,1} = x_{1,3}$ and $x_{1,2} = x_{1,4}$ and Solute 1 is pushed down $2\Delta x$. All $x_{2,j}$ are the same in one direction or the other (see Eqs. (17), (18), (20) and (21)).

can trivially form:

$$V_F = \frac{V_{R1}}{n} \tag{16}$$

All solutes move a distance $x_{i,1}$ (Eq. (1)). The fastest Solute 1 has the smallest retention volume V_{R1}. It moves the longest distance $x_{1,1}$:

$$x_{1,1} = \frac{LV_F}{V_{R1}} = \frac{L}{n} \tag{17}$$

2.2.2. Step 2

Switching the phase role, Solute 1 becomes the slowest solute. To make things simple, let say that only two solutes are present in the mixture. Then Solute 2 was the slowest solute in Step 1; it moved a distance $x_{2,1} < x_{1,1}$:

$$x_{2,1} = \frac{LV_F}{V_{R2}} = \frac{LV_{R1}}{nV_{R2}} = \frac{L}{n} \cdot \frac{V_{R1}}{V_{R2}} = x_{1,1}\frac{V_{R1}}{V_{R2}} \tag{18}$$

It is the fasted solute in Step 2 that must not be expelled out of the column. The maximum possible volume of organic phase, V_B with B for backward, is the one that will put back Solute 2 at the column head ($x_{2,2} = -x_{2,1}$ so that it position is 0). V_B is expressed as:

$$V_B = \frac{V'_{R2}x_{2,1}}{L} = \frac{V_{R1}}{nK_{D2}} = \frac{V_F}{K_{D2}} \tag{19}$$

Solute 1 moved a distance $x_{1,2}$ eluted in normal phase mode with the volume V_B of organic phase:

$$x_{1,2} = \frac{LVB}{V'_{R1}} = \frac{x_{1,1}K_{D1}}{K_{D2}} \tag{20}$$

At the end of Step 2, Solute 1 is located at the distance Δx of the column head:

$$\Delta x = |x_{1,1}| - |x_{1,2}| = \frac{L}{n}\left(1 - \frac{K_{D1}}{K_{D2}}\right) \tag{21}$$

The process repeats itself with all Solute 2 motion being identical in absolute value (Eq. (18)). All odd steps for Solute 1 have Eq. (17) value. All even Solute 1 steps have Eq. (20) value as illustrated by Fig. 3.

2.2.3. Total number of steps needed to elute Solute 1

Δx allows calculating the number of steps needed to have Solute 1 located at the column exit ($x_{1,N} = L$). Two steps (one forward and one back) moves Solute 1 by Δx; however the total number of steps required N_{MDM} is not simply $2L/\Delta x$ since each forward phase step moves Solute 1 by the distance L/n toward the column end (tail) so Solute 1 will exit the column one more step after reaching the column distance $L - L/n$. So N_{MDM} is expressed by:

$$N_{MDM} = 2\frac{L - L/n}{\Delta x} + 1 = 2(n-1)\frac{K_{D2}}{K_{D2} - K_{D1}} + 1 = \frac{2(n-1)\alpha}{\alpha - 1} + 1 \tag{22}$$

N_{MDM} is related to the distance traveled by the solutes inside the column, hence to the band variance and peak width (Eq. (9)). It can be divided in N^F_{MDM} steps $x_{1,1}$ in the forward direction (aqueous mobile phase) and N^B_{MDM} steps in the backward direction with the organic normal phase with the relationships:

$$N^F_{MDM} - 1 = N^B_{MDM} = \frac{N_{MDM} - 1}{2} = \frac{(n-1)K_{D2}}{K_{D2} - K_{D1}} \tag{23}$$

2.2.4. Total elution volume, peak width and resolution factor

The distance, X_1, traveled by Solute 1 is:

$$X_1 = N^F_{MDM}|x_{1,1}| + N^B_{MDM}|x_{1,2}| = \frac{L}{n}\left(N^F_{MDM} + N^B_{MDM}\frac{V_{R1}}{V_{R2}}\right) \tag{24}$$

Eq. (9) gives immediately the Solute 1 variance σ_1 or peak width at base, $4\sigma_1$, as:

$$\sigma_1 = \sqrt{X_1 H} \tag{25}$$

The Solute 1 elution volume, V^{MDM}_{R1}, is simply the sum of the N^F_{MDM} aqueous lower phase volumes, V_F, used to push Solute 1 in the forward direction plus the sum of the N^B_{MDM} organic upper phase volumes, V_B, used to push it backward:

$$V^{MDM}_{R1} = N^F_{MDM}V_F + N^B_{MDM}V_B \tag{26}$$

The aqueous part of the Solute 1 elution volume is the first term of Eq. (26) and can be expressed using Eqs. (16), (19), (22) and (23) as:

$$N^F_{MDM}V_F = \frac{K_{D2} - (K_{D1}/n)}{K_{D2} - K_{D1}}V_{R1} \tag{27}$$

The organic part of the Solute 1 elution volume is the second term expressed as:

$$N^B_{MDM}V_B = \frac{1 - (1/n)}{K_{D2} - K_{D1}}V_{R1} \tag{28}$$

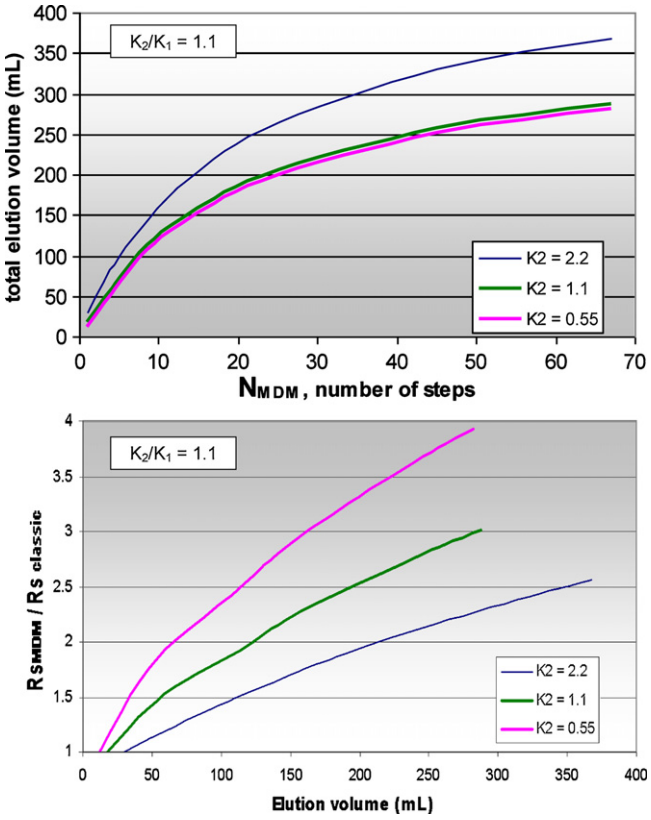

Fig. 4. Constant selectivity factor: $K_{D2}/K_{D1} = 1.1$. (Top) Change in Solute 1 total elution volume (lower aqueous phase plus upper organic phase) versus the MDM number of steps. (Bottom) Resolution gain expressed as the ratio of the MDM resolution factor (Eq. (30)) versus the resolution factor obtained after a classical elution. Column volume: 18 mL; Sf = 66.7%; Column efficiency: 200 plates. Numerical data is listed in Table 1.

The resolution factor between compounds 1 and 2 is calculated using Eq. (10) which implies the Solute 2 location in the column is known when Solute 1 leaves it (distance L). This is an easy task since, by the model design, we put back Solute 2 at the column head (distance 0) after each switch of phase mode (even step numbers). It means that one step before the last one, at Step $(N_{MDM} - 1)$, Solute 2 was still at the column head, distance 0. Hence, when Solute 1 leaves the column (Step N_{MDM}, distance L), Solute 2 is located inside the column at distance $x_{2,1}$ (Eq. (18)).

The cumulated distance, X_2, traveled by Solute 2 is simply:

$$X_2 = N_{MDM} x_{2,1} = N_{MDM} \frac{L}{n} \frac{V_{R1}}{V_{R2}} \tag{29}$$

Eq. (9) or (25) and X_2 give the Solute 2 band variance σ_2 that allows for the resolution factor calculation:

$$Rs_{MDM} = \frac{L - x_{2,1}}{2(\sigma_1 + \sigma_2)} \tag{30}$$

The full development of Eq. (30) produces a complicated combination of all parameters. Eq. (30) will be studied using practical cases.

2.2.5. Case studies

2.2.5.1. Constant selectivity. Three pairs of solutes were considered with distribution ratio being respectively 0.5 and 0.55 for polar compounds rapidly eluted, 1 and 1.1 for two compounds distributing equally between the two liquid phases and 2 and 2.2 for somewhat less polar compounds. The three pairs of compounds have exactly the same selectivity ratio: $K_{D2}/K_{D1} = 1.1$. Table 1 lists the Solute 1 elution volumes, indicating the aqueous and organic respective volumes obtained after different MDM steps. The V_F aqueous phase step volumes and the V_B organic phase step volumes are listed as well the MDM resolution factors and the resolution gains obtained.

Fig. 4, top, shows two important points: (i) as seen in Eq. (22), the number of MDM steps, N_{MDM}, is related to α, the selectivity factor. With a constant selectivity factor, the number of MDM steps is the same for all pairs of compounds, e.g. 45 steps with $n = 3$ (Table 1). (ii) The second point is that the increase in global retention volume is not directly proportional to the number of MDM steps. As shown by Eq. (26) and considering that the number of forward steps (N^F_{MDM}, aqueous phase) is just one step more that the number of backward steps (N^B_{MDM}, organic phase), the change in retention volume depends greatly on how the solute is carried. Low K_D solutes move preferentially with the aqueous phase which means that more organic phase volume will be needed to push them backward as seen in the "organic phase" column of Table 1 for the $K_D = 0.5$ solute. This is the opposite for hydrophobic solute (high K_D), i.e. the aqueous phase will not move them much, while a small volume of organic phase will push them backwards a lot. The volume weight of the two liquid phases is similar for the compounds with K_D equal or close to 1, where they move at a similar rate in both phases.

Fig. 4, bottom, relates to the gain in resolution, expressed as the ratio of Rs_{MDM} (Eq. (30)) over the resolution obtained in classical mode, to the elution volume. This figure clearly shows the advantage of the MDM method since there is always a gain in resolution. It also shows that the advantage is maximized for solutes with a low K_D value. It is possible to obtain with

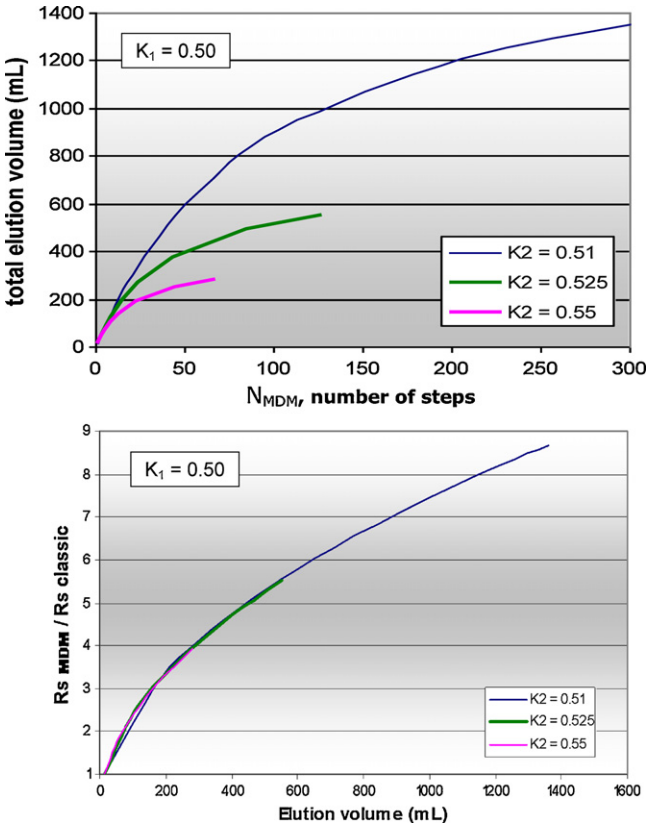

Fig. 5. Variable selectivity factor with Solute 1 $K_{D1} = 0.50$. (Top) Change in Solute 1 total elution volume (lower aqueous phase plus upper organic phase) versus the MDM number of steps. (Bottom) Resolution gain expressed as the ratio of the MDM resolution factor (Eq. (30)) versus the resolution factor obtained after a classical elution. Column volume: 18 mL; Sf = 66.7%; Column efficiency: 200 plates. Numerical data is listed in Table 2.

Table 1
Parameters of the multi-dual-mode (MDM) model for two solutes of selectivity 1.1.

n	N_{MDM}	V_F (mL)	V_B (mL)	V_{R1}^{MDM} (mL)	Aqueous* (mL)	Organic* (mL)	*Rs*	*Rs* gain*
K_{D1} = 0.5; K_{D2} = 0.55; V_{R1} = 12 mL; V_{R2} = 12.6 mL								
1	1	12.0	–	12.0	12.0	0.0	0.17	1.0
1.09	3	11.0	20.0	41.7	21.9	19.8	0.28	1.7
1.18	5	10.1	18.5	69.5	31.2	38.3	0.34	2.0
1.36	9	8.8	16.0	107	43.8	63.5	0.41	2.4
1.55	13	7.8	14.1	140	54.6	85.2	0.48	2.8
2	23	6.0	10.9	192	72.0	120	0.56	3.3
3	45	4.0	7.3	252	92.0	160	0.63	3.7
4	67	3.0	5.5	282	102	180	0.67	3.9
K_{D1} = 1.0; K_{D2} = 1.1; V_{R1} = 18 mL; V_{R2} = 19.2 mL								
1	1	18.0	–	18.0	18.0	0.0	0.23	1.0
1.09	3	16.5	15.0	47.7	32.9	14.8	0.31	1.4
1.18	5	15.3	13.9	72.9	45.4	27.5	0.36	1.6
1.36	9	13.2	12.0	113	65.6	47.5	0.44	1.9
1.55	13	11.6	10.6	146	81.9	63.9	0.49	2.2
2	23	9.0	8.2	198	108	90	0.57	2.5
3	45	6.0	5.4	258	138	120	0.64	2.9
4	67	4.5	4.1	288	153	135	0.68	3.0
K_{D1} = 2.0; K_{D2} = 2.2; V_{R1} = 30 mL; V_{R2} = 32.4 mL								
1	1	30.0	–	30.0	30.0	0.0	0.27	1.0
1.09	3	27.5	12.5	67.2	54.8	12.4	0.33	1.2
1.18	5	25.4	11.6	98.6	75.8	22.8	0.38	1.4
1.36	9	22.1	10.0	149	109	39.7	0.45	1.7
1.55	13	19.3	8.8	190	136	53.2	0.5	1.9
2	23	15.0	6.8	255	180	75.0	0.58	2.2
3	45	10.0	4.5	330	230	100	0.65	2.4
4	67	7.5	3.4	368	255	113	0.68	2.6

CCC column volume = 18 mL; V_M = 6 mL; V_S = 12 mL; *Sf* = 66.7% (organic upper phase); column efficiency N = 200 plates.

* "aqueous" is the aqueous part of the Solute 1 elution volume, V_{R1}^{MDM}; "organic" is the organic part of the Solute 1 elution volume; "*Rs* gain" is the ratio of the MDM resolution factor over the classical mode resolution factor listed for n = 1.

K_D = 0.5 and 67 steps MDM (corresponding to 282 mL, Table 1) a resolution factor four times higher that the one obtained by simple direct elution. For the K_D = 2 solute, the gain after the same number of 67 steps corresponding to the high volume of 368 mL is only 2.6 (Fig. 4 bottom and Table 1). It can be observed that the 282 mL volume corresponds to 24 retention volumes of the K_D = 0.5 solute (12 mL, Table 1) when 368 mL corresponds to only 12 retention volumes of the K_D = 2 solute (30 mL, Table 1). It was verified that this was true with different selectivity ratios.

Table 2
Parameters of the multi-dual-mode (MDM) model for two solutes of variable selectivities: 1.1, 1.05 and 1.02 with the same Solute 1 compound with K_{D1} = 0.50.

n	N_{MDM}	V_F (mL)	V_B (mL)	V_{R1}^{MDM} (mL)	Aqueous* (mL)	Organic* (mL)	*Rs*	*Rs* gain*
K_{D1} = 0.5; K_{D2} = 0.55; V_{R1} = 12 mL; V_{R2} = 12.6 mL; α = 1.1								
1	1	12.0	–	12.0	12.0	0.0	0.17	1.0
1.09	3	11.0	20.0	41.7	21.9	19.8	0.28	1.7
1.18	5	10.1	18.5	69.5	31.2	38.3	0.34	2.0
1.36	9	8.8	16.0	107	43.8	63.5	0.41	2.4
1.55	13	7.8	14.1	140	54.6	85.2	0.48	2.8
2	23	6.0	10.9	192	72.0	120	0.56	3.3
3	45	4.0	7.3	252	92.0	160	0.63	3.7
4	67	3.0	5.5	282	102	180	0.67	3.9
K_{D1} = 0.5; K_{D2} = 0.525; V_{R1} = 12 mL; V_{R2} = 12.3 mL; α = 1.05								
1	1	12.0	–	12.0	12.0	0.0	0.09	1.0
1.09	5	11.0	20.9	77.4	33.8	43.6	0.18	2.0
1.19	9	10.0	19.2	127	50.3	76.6	0.23	2.7
1.34	15	9.0	17.1	195	72.9	122	0.29	3.3
1.55	24	7.7	14.7	267	97.2	170	0.33	3.9
2	43	6.0	11.4	372	132	240	0.39	4.5
3	85	4.0	7.6	492	172	320	0.45	5.2
4	127	3.0	5.7	552	192	360	0.48	5.5
K_{D1} = 0.5; K_{D2} = 0.51; V_{R1} = 12 mL; V_{R2} = 12.1 mL; α = 1.02								
1	1	12.0	–	12.0	12.0	0.0	0.03	1.0
1.10	11	10.9	21.4	174	66.0	108	0.11	3.1
1.18	19	10.2	20.0	283	102	181	0.14	4.0
1.35	37	8.9	17.4	482	169	313	0.18	5.2
1.55	57	7.7	15.2	651	225	426	0.21	6.0
2	103	6.0	11.8	912	312	600	0.25	7.1
3	205	4.0	7.8	1212	412	800	0.29	8.2
4	307	3.0	5.9	1362	462	900	0.30	8.7

CCC column volume = 18 mL; V_M = 6 mL; V_S = 12 mL; *Sf* = 66.7% (organic upper phase); column efficiency N = 200 plates.

* "aqueous" is the aqueous part of the Solute 1 elution volume, V_{R1}^{MDM}; "organic" is the organic part of the Solute 1 elution volume; "*Rs* gain" is the ratio of the MDM resolution factor over the classical mode resolution factor listed for n = 1.

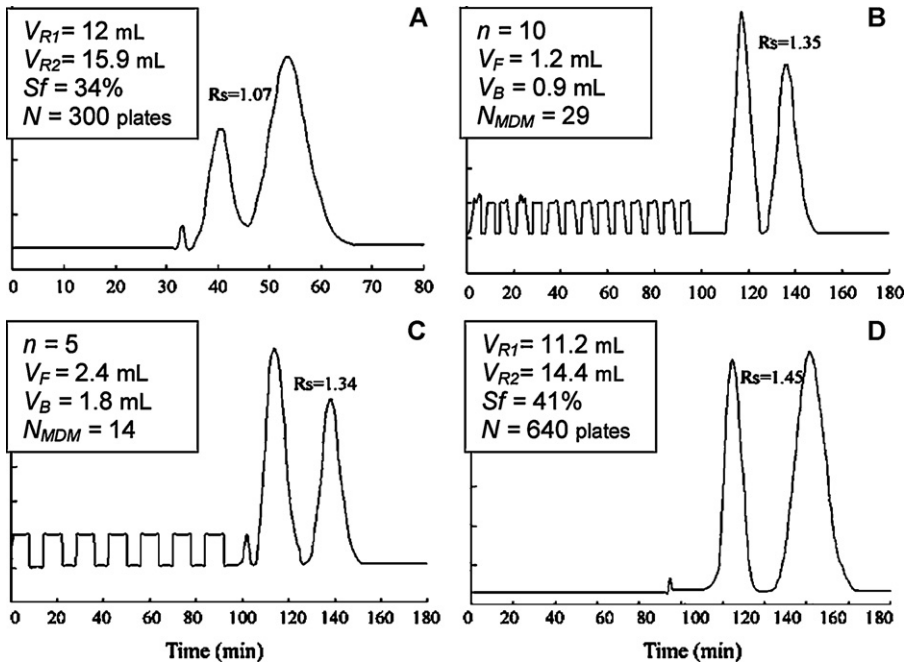

Fig. 6. Experimental separation of dinitrophenyl derivatives of alanine (first peak) and glutamine (second peak). (A) Classical separation with a 15 mL hydrostatic CCC column and the Arizona (AZ) N liquid system (hexane–ethyl acetate–methanol–aqueous HCl 0.1 M; 1:1:1:1, v/v), lower aqueous mobile phase in the descending or head-to-tail direction at 0.3 mL/min, V_S = 5.1 mL, V_M = 9.9 mL, 1000 rpm, detection UV 280 nm, K_{D1} = 0.42, K_{D2} = 1.18. (B) MDM mode with 29 steps as indicated performed with a constant flow rate of 0.3 mL/min for both liquid phases. (C) MDM mode with 14 steps of double volume and also constant flow of 0.3 mL/min. (D) Classical elution at 0.1 mL/min. Data and figure adapted from Ref. [19].

2.2.5.2. Changing selectivity with a constant K_{D1} for Solute 1. Fig. 5 should be compared with Fig. 4 since both figures use the same model. Fig. 5 shows the case of polar solutes with low K_D value and changing selectivity factors: 1.02. 1.05 and 1.1. Eq. (22) shows that the number of MDM steps depends on the selectivity factor α. As the two distribution ratios K_D become closer, their ratio, the selectivity factor α becomes close to unity and the number steps, N_{MDM}, needed to elute Solute 1 increases dramatically. If N_{MDM}, increases, the total solute retention volume increases as well as illustrated by Fig. 5, top. Fig. 5 bottom shows that the gain in resolution depends

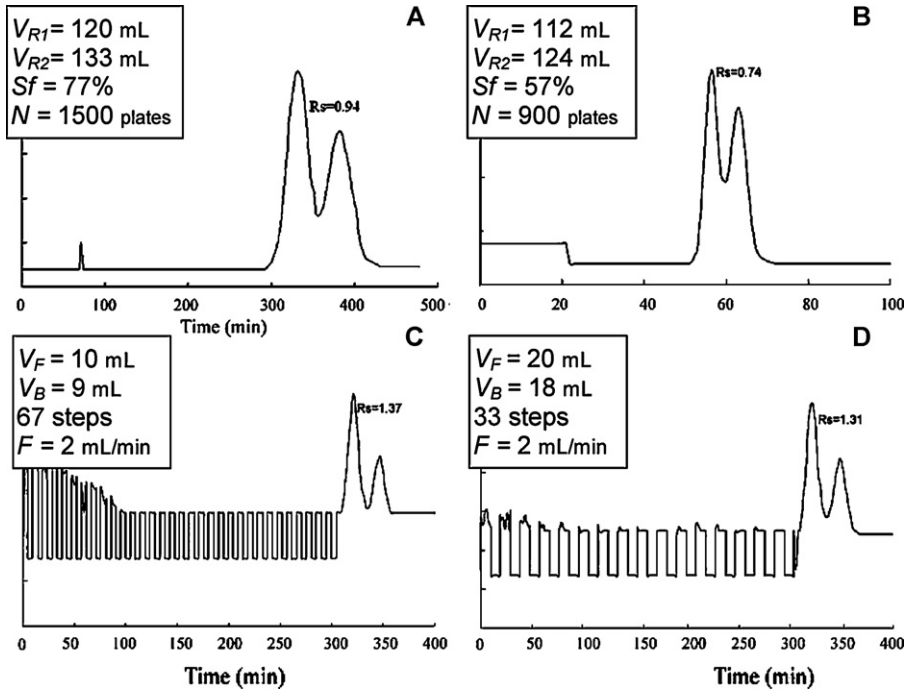

Fig. 7. Experimental separation of dinitrophenyl derivatives of serine (first peak) and aspartic acid (second peak). (A) Classical separation with a 130 mL hydrodynamic CCC column and the Arizona (AZ) L liquid system (hexane–ethyl acetate–methanol–aqueous HCl 0.1 M; 4:5:4:5, v/v), lower aqueous mobile phase in the descending or head-to-tail direction at 0.35 mL/min, V_S = 100 mL, V_M = 30 mL, 800 rpm, detection UV 280 nm, K_{D1} = 0.77, K_{D2} = 0.90. (B) Classical separation but with mobile phase flow rate 2 mL/min, V_S = 74 mL, V_M = 56 mL; (C) MDM mode with 67 steps as indicated performed with a constant flow rate of 2 mL/min for both liquid phases. (D) MDM mode with 33 steps of double volume and also constant flow of 2 mL/min. Data and figure adapted from Ref. [19].

Table 3
Full calculation of DNP-Serine and DNP-Aspartic acid position and band variance in a 130 mL hydrodynamic column during the MDM experiment of Fig. 7D.

Step #	DNP serine		DNP aspartic acid		Volume (mL)	Time (min)	Resolution factor
	Position	σ	Position	σ			
1	17.7%	1.4%	16.3%	1.3%	20.0	10.0	0.25
2	5.4%	1.8%	3.1%	1.8%	38.0	19.0	0.32
3	23.1%	2.3%	19.4%	2.3%	58.0	29.0	0.41
4	10.6%	2.6%	6.2%	2.6%	76.0	38.0	0.45
5	28.6%	2.9%	22.5%	2.9%	96.0	48.0	0.52
6	16.3%	3.2%	9.3%	3.1%	114.0	57.0	0.56
7	34.0%	3.5%	25.6%	3.4%	134.0	67.0	0.61
8	21.7%	3.6%	12.4%	3.6%	152.0	76.0	0.64
9	39.4%	3.9%	28.7%	3.9%	172.0	86.0	0.69
10	27.2%	4.1%	15.5%	4.1%	190.0	95.0	0.72
11	44.9%	4.3%	31.8%	4.3%	210.0	105.0	0.76
12	32.6%	4.5%	18.6%	4.4%	228.0	114.0	0.79
13	50.3%	4.7%	34.9%	4.6%	248.0	124.0	0.83
14	38.0%	4.8%	21.7%	4.8%	266.0	133.0	0.85
15	55.7%	5.0%	38.0%	5.0%	286.0	143.0	0.89
16	43.5%	5.2%	24.8%	5.1%	304.0	152.0	0.91
17	61.2%	5.3%	41.1%	5.3%	324.0	162.0	0.94
18	48.9%	5.5%	27.9%	5.4%	342.0	171.0	0.96
19	66.6%	5.7%	44.2%	5.6%	362.0	181.0	1.00
20	54.3%	5.8%	31.0%	5.7%	380.0	190.0	1.02
21	72.0%	5.9%	47.3%	5.9%	400.0	200.0	1.05
22	59.8%	6.1%	34.1%	6.0%	418.0	209.0	1.06
23	77.5%	6.2%	50.4%	6.2%	438.0	219.0	1.09
24	65.2%	6.3%	37.2%	6.3%	456.0	228.0	1.11
25	82.9%	6.5%	53.5%	6.4%	476.0	238.0	1.14
26	70.6%	6.6%	40.3%	6.5%	494.0	247.0	1.16
27	88.3%	6.7%	56.6%	6.7%	514.0	257.0	1.18
28	76.1%	6.8%	43.4%	6.8%	532.0	266.0	1.20
29	93.8%	7.0%	59.7%	6.9%	552.0	276.0	1.23
30	81.5%	7.1%	46.5%	7.0%	570.0	285.0	1.24
31	99.2%	7.2%	62.8%	7.1%	590.0	295.0	1.27
32	86.9%	7.3%	49.6%	7.2%	608.0	304.0	1.28
33	Elute	7.4%	65.9%	7.3%	628.0	314.0	1.31
	Elution			10 mL	652 Ser	326.0	1.31
	Elution			10 mL	700 Asp	350.0	1.31

Shaded lines and even steps correspond to backward elution with the organic upper phase in the backward tail-to-head ascending direction; open lines and odd steps correspond to forward elution with the aqueous phase in the forward head-to-tail descending direction. CCC hydrodynamic column of 130 mL. The indicated percentages correspond to the solute position inside the column expressed as percentage of column length or volume; e.g. 50% means that the solute is exactly at the middle point of the column or 65 mL. Parameters used: V_F = 20 mL; V_B = 18 mL; V_M = 56 mL; V_S = 74 mL; Sf = 57% (organic upper phase); N = 900 plates; K_{DSer} = 0.77; K_{DAsp} = 0.90.

only on the MDM step number: the lines corresponding to different selectivity ratios overlap exactly when the K_{D1} distribution ratio is constant (i.e. V_{R1} does not change).

Fig. 5 bottom and Table 2 both show a resolution gain of almost one order of magnitude for the two compounds with α = 1.02, K_{D1} = 0.5 and K_{D2} = 0.51. It is pointed out that these values were obtained by theoretical computation. Practically, it says that the initial resolution factor of 0.035 (a single perfect peak is seen) becomes 0.30 (a shoulder is seen) after 307 back and forth phase changes associated to 1.36 liter of phases (462 mL of aqueous phase and 900 mL of organic phase, Table 2). This has little practical interest. It is not worth using that much solvent with such a difficult task of changing the phases so many times just to be able to see a shoulder on the chromatogram.

From a theoretical point of view, this resolution improvement is very interesting and important: it says that it is possible to increase greatly the resolution factor obtained with difficult to separate compounds. All resolution factors depend on the square root of the column efficiency, N. This factor was kept constant in this study. In classical liquid chromatography, it is possible to increase the resolution factor of 0.035 obtained with a 200 plate column to Rs = 0.3 by using a 15,000 plate column. In CCC, the same gain in resolution can be obtained working with a 400-plate column and using the MDM method with 50 steps. Using modern columns that are able to have 1000 plate efficiency, the separation of enantiomers could be considered [16].

3. Confronting the model to literature experimental MDM separations

3.1. Alanine and glutamine separated on a hydrostatic small CCC column

Yang et al. fully described a complete MDM study including excellent experiments that can be revisited using the presented theoretical treatment [19]. Fig. 6 shows their experiment separating two alanine and glutamine derivatives using a 15 mL hydrostatic CCC column having roughly 300 plates. Fig. 6A shows their published classical elution chromatogram that allowed us to calculate some needed information, V_{R1}, V_{R2}, Sf and N were measured and are given in the figure caption and insert. The measured efficiency is not constant being 380 plates for the alanine first peak and only 220 plates for the glutamine second peak. An average value of 300 plates was taken for calculation. Fig. 6B shows the MDM chromatogram obtained after 29 steps (n = 10). The authors' noted Rs factor is 1.35 when it seems to be closer to 1.6 since the baseline is clearly touched between the two peaks. The model predicted an Rs_{MDM} value of 2.6. The total elution volume is about 45 mL including 14 backward steps of 0.9 mL organic upper phase (12.6 mL) and 15 forward steps of 1.2 mL of aqueous lower phase (18 mL) plus about 15 mL to elute the two compounds. Fig. 6C shows the experimental MDM done with 14 steps (n = 5) doubling the V_F and V_B volumes. The total elution volume is also about 45 mL. The authors noted an unchanged Rs factor (1.34). Here also, it seems closer to

1.6 with baseline return between peaks and the model predicted the same Rs_{MDM} value of 2.6. Fig. 6D shows that an even better resolution factor can be obtained using much less solvent volumes by simply reducing the flow rate to 0.1 mL/min. The authors list a resolution factor of 1.45 (Fig. 6D). Our own measurement on the figure returned a resolution factor of 1.8–2.0 using the tangent method to estimate graphically the peak widths at base. The experiment duration is the same. The elution volume is more than halved since only 17 mL of lower aqueous mobile phase are needed. The resolution increase is due first to the continuous slow classical elution (no extra broadening due to valve switching). The second reason is that CCC hydrostatic columns produce a higher efficiency at low flow rates having an inverted Van Deemter behavior [23]. It is verified that the doubling in column efficiency (300 plates at 0.3 mL/min and 640 plates at 0.1 mL/min) produces a $\sqrt{2}$ increase of resolution.

The first positive point shown by Fig. 6 is that the elution prediction of the model fits perfectly the experiment. As predicted and illustrated by Fig. 5, the gain in resolution depends only on the total elution volume. The MDM experiments presented by Fig. 6B and C were done with different numbers of steps but with the same total elution volume, hence showing the same resolution enhancement. The second positive point is the correct prediction of improved resolution. The resolution prediction is significantly higher than the experimentally observed one. This is easily explained considering possible extra-column band broadening induced by the switching valves and connecting tubing that could reduce the experimental resolution [19]. Another reason is the overestimation of resolution factors in the theoretical model: since the solute bands broaden, putting back Solute 2 exactly at the column head can just be done in theory. Practically, this would expel from the column head an increasing part of Solute 2 as its band become wider. We did model the MDM process using a smaller V_B volume so that no part of Solute 2 is lost. However, the equations obtained were much more complicated and difficult to handle. Since they produced very similar results, we decided to keep the MDM model as simple as possible and did not expose this work here.

3.2. Serine and aspartic acid separated on a hydrodynamic CCC column

Fig. 7 is another experimental MDM experiment done by the same authors but with an efficient hydrodynamic CCC column of 130 mL [19]. They separated the DNP derivatives of serine and aspartic acid. Fig. 7A and B show two classical CCC chromatograms done with two different flow rates. Obviously, the low flow rate of 0.35 mL/min (Fig. 7A) will need 7 h to complete when the faster flow rate of 2 mL/min is completed in a little more than 1 h. The small resolution gain is offset by the dramatic increase of experiment duration.

The MDM method is another way to improve resolution. Fig. 7C and D show two MDM experiments done with different conditions keeping the total elution volume constant. To illustrate the compound band progression inside the CCC column, we fully calculated the two solute band position and variance for Fig. 7D experiment as listed in Table 3. In this case, with 33 steps, the V_B volume to put back DNP-Asp at the column head would be 22.2 mL (Eq. (19)). Since the authors used a lower V_B volume of only 18 mL, they did not put back DNP-Asp at the column head but got it slowly moving ~3% of the column length toward the column tail at each DM step as seen reading the positions in Table 3 for odd numbered steps. The calculated variance σ allows computing the increasing resolution developing inside the CCC column (Eqs. (10) and (12)) as well as the peak width of the eluting solute using the classical Gaussian relationship (2σ at 60% of peak height and/or 4σ at peak base). The resolution factor is doubled by the MDM process at the cost of six times higher retention volumes and times (Table 3). If the added value of the separated compounds is worth the volume and time cost, the MDM process allows a higher resolution separation to be performed with a smaller column.

4. Conclusion

The MDM method can only be used with CCC column since the two phases must be liquid. The method allows for an artificial increase of the column length giving a significantly higher resolution power compared to the one obtained with the classical elution mode. The theoretical study showed that the best conditions to have a maximum gain in resolution with the MDM method were with low K_D solutes difficult to separate (low selectivity factor). The study showed that the resolution gain is offset by an increased elution volume. It is not the number of MDM steps that is the most important parameter; it is the total elution volume cumulating the volume of lower phase pushed in the head-to-tail or ascending direction plus the volume of upper phase pushed in the other direction. The theoretical study was done with polar solute and a reversed phase chromatographic liquid system (polar aqueous lower initial mobile phase and organic upper initial stationary phase). It would be necessary to reverse all forward, backward, head-to-tail and descending comments or mentions of the modeling of the normal phase chromatographic situation with very similar equations.

This work did not study the MDM case where the sample mixture is introduced in the middle of the CCC column or in between two identical CCC columns serially connected. This case was described as “intermittent” CCC with two hydrodynamic columns [17] and in a patent calling it “True Moving Bed” (TMB) with two hydrostatic columns [18]. The full theoretical study of these cases would be close to the present MDM theoretical description and could be the subject of a future study.

5. Nomenclature

AZ	Arizona biphasic liquid system. A range of 26 compositions, referred by the A–Z letters, of the quaternary hexane/ethyl acetate/methanol/water system
CCC	countercurrent chromatography
CM	classical mode
DM	dual mode
F	mobile phase flow rate (mL min^{-1})
H	height equivalent to a theoretical plate (m)
K_D	solute distribution ratio (or partition coefficient); ratio of the solute (all forms) concentration in the stationary phase over the solute concentration in the mobile phase
L	column length (m)
n	fraction of the Solute 1 retention volume used as the V_F forward mobile phase volume
N	plate number
N_{MDM}	total number of steps (forward plus backward) needed to start to elute Solute 1 at the column exit
N^B_{MDM}	number of backward steps (organic upper phase) in N_{MDM}
N^F_{MDM}	number of forward steps (aqueous lower phase) in N_{MDM}
MDM	multi-dual mode
Rs	resolution factor
Rs_{DM}	resolution factor obtained in dual-mode
Rs_{MDM}	resolution factor obtained with the MDM method

Sf stationary phase retention ratio of the classical mode (aqueous lower mobile phase and organic upper stationary phase)
t_R solute retention time (min)
TMB true moving bed method with two CCC columns
V_B backward organic phase volume used in one backward step (mL)
V_C CCC column (instrument) volume = $V_M + V_S$ (mL)
V_{CM} the mobile phase volume used in the first step of the dual-mode method (CM for classical mode) (mL)
V_{DMi} "stationary" phase volume needed to elute Solute i after the V_{CM} volume of mobile phase was used in classical mode (mL)
V_F forward mobile phase volume used in one forward step and taken as V_R/n (mL)
V_M mobile phase volume (mL)
V_S stationary or slower phase volume (mL)
V_R solute retention volume (mL)
V'_R solute retention volume in the other mode (switching the phases used for V_R) = V_R/K_D (mL)
V_{Ri}^{MDM} total retention volume for Solute i obtained using the MDM method (mL)
w_i peak width of Solute i at base (mL)
x position of the band inside the column (m)
$x_{i,j}$ position of the Solute i band inside the column after the MDM Step j (m)
X_i cumulative distance traveled by Solute i during the whole MDM process (m)

Greeks letters
α selectivity ratio = K_{D2}/K_{D1}
γ mobile phase over stationary phase ratio
σ solute standard deviation (m or mL)
Δx distance traveled by the Solute 1 band inside the column after two MDM steps: one forward and one backward (m)

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