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Optimum solvent selectivity and gradient mode for deoxyribonucleosides in reversed-phase high-performance liquid chromatography

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Abstract

The separation condition for five deoxyribonucleosides (dCyd, dUrd, dGuo, dThd, and dAdo) was determined by the optimization of mobile phase conditions. In this work, the binary system of water and methanol was applied in RP-HPLC. The elution profiles were calculated using plate theory based on the quadratic equation of retention factor, $\ln k = A + BF + CF^2$, and F was the volume percent of methanol. We modified the plate theory to calculate elution profile in both step and linear gradient mode. The optimal mobile phase composition was obtained by comparing the resolutions of the five deoxyribonucleosides and the separation times of last-eluting solute (dAdo) in step gradient elution. Also, in linear gradient elution, resolutions and separation time could be predicted by a slight modification of the relationship used in step gradient mode. The final calculated result for the resolution of five deoxyribonucleosides suggested that the appropriate first mobile phase composition was water/methanol 88/12 vol.%, then after 7 min the second composition of water/methanol, 65/35 vol.% was step-changed. Under experimental conditions, the agreement between the experimental data and the calculated values was relatively good.

Keywords: Solvent selectivity; Optimization; Gradient elution; Deoxyribonucleosides

1. Introduction

In liquid chromatography (LC) the mobile phase should be selective for the components and its composition is one of the most necessary variables influencing a separation. Many investigations on the relationship between HPLC retention and mobile phase composition were reported [1–3]. In isocratic elution, the mobile phase composition is unchanged during the separation. The various components of the sample have a wide range of k values. However, the disadvantages of isocratic mode are poor resolution of early-eluting bands, broadening of late-eluting bands to the point of difficult detection, tailing peaks, and unnecessarily long separation times. This is often overcome by changing the strength of the solvent during the operation.

Gradient elution is performed by changing the composition of the mobile phase. The changes in the solvent strength can be made stepwise or continuously. Gradient elution offers several advantages: total analysis time can be significantly reduced, overall resolution of a mixture is increased, peak shape is improved (less tailing) and effective sensitivity is increased since there is little variation in peak shape. More importantly, it provides the maximum resolution per unit time. The simplest way of varying the mobile phase composition is through stepwise

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rather than continuous elution. It is advantageous to make step changes in composition. All LC detectors can be used when mobile phase composition is not changing continuously. Moreover, step changes can be made without the need for complex equipment, so it can be used in process-scale LC.

In recent years a considerable amount of chromatographic work has been carried out in which gradient elution was used. Mathematical modeling and theoretical analysis play an important role in the application of analytical and preparative gradient chromatography. The plate theory [4] assumes that a solute is in equilibrium with both the mobile and stationary phase. Due to continuous exchange of solute between the two phases as it progresses down the column, equilibrium is hard to achieve. As a consequence, to develop plate theory, the column is considered to be divided into a number of plates. In plate theory, the concentration of a solute leaving a column can be calculated in terms of the volume of mobile phase that has passed through it.

The samples used in this work were deoxyribonucleosides, the building blocks of DNA, which differ from RNA nucleosides by the absence of one hydroxyl group in the sugar. The purine nucleosides are adenosine, deoxyadenosine, guanosine, and deoxyguanosine. The pyrimidine nucleosides are cytidine, deoxycytidine, thymidine, and uridine. Naturally occurring nucleosides show a wide variety of biological effects.

In this paper, a modified equation was suggested for calculating the distances migrated by the solutes in step and linear gradient mode. A quadratic dependency between ln k and mobile phase composition described in the preceding paper has been used to determine the retention factors of the deoxyribonucleosides [5]. The optimum composition of mobile phase for the separation of the five deoxyribonucleosides was obtained on the basis of resolution and separation times. The elution profiles in the optimal mobile phase condition and operating mode were calculated using plate theory to compare with experimental data.

2. Theoretical

In this work, the logarithmic retention factor, k, is correlated by a quadratic relationship involving the volume percent (F) of organic modifier [6,7].

$$\ln k = A + B \cdot F + C \cdot F^2 \tag{1}$$

where A, B, and C are empirical constants which should be experimentally determined. Eq. (1) was applied to ternary mobile phase in normal-phase HPLC [8].

Retention volume in isocratic mode is expressed by the retention factor as follows,

$$V_{r,n} = V_0(1 + k_n) \tag{2}$$

where $V_{r,n}$ and k_n are the retention volume and retention factor in the nth mobile phase composition respectively, and V_0 is the dead volume of unretained compound. The prediction of retention time under gradient conditions has been described by assuming that a gradient step is similar to a sequence of short isocratic steps [5,9,10]. The modified equation proposed for predicting the retention volume of step-gradient elution is:

$$V_{Rg} = V_0(1 + k_2) + \frac{V_{g,1}}{k_1}(k_1 - k_2) \tag{3}$$

where k_1 and k_2 are the retention factors in the first and second mobile phase compositions, and obtained by Eq. (1). $V_{g,1}$ is the volume of the first mobile phase in the step-gradient elution passing through an inlet of the chromatographic column until the second mobile phase is introduced to the column inlet [5,10]. It can be calculated by summation of a mixer volume installed in the HPLC and by gradient volume. In the case of linear-gradient mode, mobile phase composition is changing gradually and continuously. Linear-gradient mode may be envisaged as infinitely small segments of step gradient, Eq. (3) is modified and extended to linear-gradient mode. The retention volume in the linear-gradient mode can be calculated,

$$V_{Rg} = V_{r,\infty} + (V_{r,\infty} - V_0)\sum_{i=1}^{\infty}\frac{V_{g,i}(V_{r,i} - V_{r,i+1})}{(V_{r,i} - V_0)^2} \tag{4}$$

Eq. (3) and Eq. (4) are derived in the appendix.

The number of theoretical plates, N, is calculated in isocratic mode,

$$N = 16\left(\frac{t_R}{w}\right)^2 \tag{5}$$

The number of theoretical plates is assumed to be independent of the mobile phase composition throughout this work. It was obtained from the average value from several runs. In gradient mode, the number of theoretical plate was calculated by substituting t_R into t_{Rg} in Eq. (5). w was calculated by Eq. (5) substituting t_R into t_{Rg} or $t_{R,n}$ according to mobile phase shape and inserting average value of N.

The resolution between component 1 and 2 is given by

$$R_{12} = \frac{2(t_{R1} - t_{R2})}{(w_1 + w_2)} \tag{6}$$

The optimum resolution was obtained by calculating the retention time and peak width from Eq. (3), Eq. (4) and Eq. (5).

According to the plate theory, the chromatographic column is mathematically equivalent to a plate column where the total length is divided into N plates. It is assumed that instantaneous equilibrium is established for the solute between mobile and stationary phase. A material balance on solute around the plate N leads to the following equation [5,10]:

$$c_N = c_0 \sum_{i=N-r}^{N-1} \frac{(aV)^i}{i!} e^{-aV} \tag{7}$$

where c_N is the outlet concentration of solute, c_0 initial concentration, and a is constant,

$$a = \frac{1}{v_m + Kv_s} \tag{8}$$

where v_m and v_s are the volume of mobile phase and stationary phase in a theoretical plate, respectively. Eq. (7) enables to predict concentration elution profile of each component. The equilibrium constant (K) is correlated in terms of partition coefficient as

$$K = \left(\frac{\varepsilon}{1-\varepsilon}\right)k \tag{9}$$

where ε is the total porosity of the chromatographic column, and is assumed to be 0.75.

3. Experimental

A HPLC (Waters Assoc., Milford, MA, USA) equipped with a Waters 600E multisolvent delivery system, an U6K injector with 2 ml sample loop, and a 486 UV absorbance detector was used. Experimental data were recorded on a PC by CHROMATE (Ver.2.1, Interface Eng., Korea). μ-Bondapak C_{18} column (3.9 mm×300 mm) with 10 μm particle size (Waters Assoc.) was used.

Five deoxyribonucleosides of 2-deoxycytidine (dCyd), 2-deoxyuridine (dUrd), 2-deoxyguanosine (dGuo), thymidine (dThd), and 2-deoxyadenosine (dAdo) were purchased from Sigma (St. Louis, MO, USA). All the solutes were dissolved into water and the concentration was 100 μg ml^{-1}. Methanol, used as an organic modifier, was obtained from Baker (Phillipsburg NJ, USA). Water was distilled and deionized in a Milli-Q system (Millipore, Bedford, MA). All the solvents were filtered under vacuum prior to use. The eluents were degassed by sparging with helium in solvent reservoirs during separation. Standard volumes of 2.5 μl were injected directly for HPLC analysis. The elutions were performed by both isocratic and gradient modes at the flow-rates of 1 ml min^{-1}. UV absorption was measured at 254 nm with sensitivity of 0.01 absorbance units full scale (a.u.f.s.). All separations were done at ambient temperature. The dead volume (V_0) was determined as the retention volume of 2 ml of methanol.

4. Results and discussion

Five deoxyribonucleosides were separated in isocratic mode. The elution order of the five deoxyribonucleosides is dCyd, dUrd, dGuo, dThd, and dAdo. The elution order is independent of mobile phase composition. The organic modifier was methanol, and the amount was adjusted to separate the five components. In Fig. 1, the peaks were observed at the mobile phase composition 83/17, water–methanol (vol. %). Under this experimental condition, five deoxyribonucleosides were separated with minimum resolution of 1.39 between dCyd and dUrd within 12 min. The experimental and calculated profiles were in good agreement with not more than 5% difference of retention time as shown in Fig. 1.

Regressions were performed with Eq. (1) to obtain empirical constants for each solute from the experimental data of k and F listed in Table 1. The

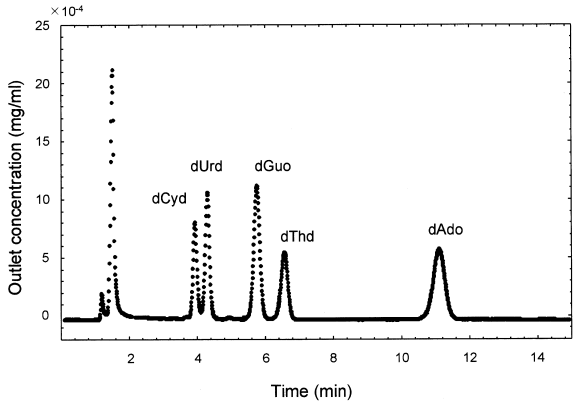

Fig. 1. Separation of deoxyribonucleosides in isocratic condition (F: 17%).

results were presented in Table 2. Regression coefficients of five deoxyribonucleosides are higher than 0.99.

To verify the validity of the theoretical equations developed and to find the optimum mobile phase composition and gradient conditions, the retention times of five deoxyribonucleosides were measured with different contents of methanol. The choice of an optimum mobile phase depends on the resolutions of all solutes and separation time of last eluting solute. The separation time was determined by summation of the retention time of last eluting solute and half of the band width. In this work, the optimum condition was obtained on the basis of two criteria. First, we considered the resolution of five deoxyribonucleosides. Thereafter, the separation time of last eluting solute was considered. The content of organic modifier and gradient time during the step gradient run increased by as much as 1% and 1 min, respectively. For linear gradient, actual gradient time

Table 2
Empirical constants and regression coefficients of deoxyribonucleosides

Material	$\ln k = A + BF + CF^2$			Regression coefficient
	A	B	C	
dCyd	1.47	−0.15	0.0021	0.9951
dUrd	1.73	−0.16	0.0018	0.9927
dGuo	2.77	−0.18	0.0019	0.9942
dThd	2.73	−0.17	0.0019	0.9924
dAdo	3.85	−0.19	0.014	0.9937

(t_g) is increased by as much as 0.2 min from 7 to 9 min. Single step gradient and linear gradient mode were limited in this work. Normally, a mixer is located between solvent reservoirs and column. As the mixer volume in our work was experimentally determined as 6.8 ml in the previous work [5], the value of gradient volume ($V_{g,1}$) was started at 7 ml. With a quadratic dependency between ln k and mobile phase composition, the retention factors in high content of organic modifier could be calculated. From this correlation, the retention factor increases with the higher content of organic modifier. To resolve this conflict, the maximum content of organic modifier was limited to 35 vol.%. The calculations were carried out using Mathematica (Ver. 2.2) on a Pentium® PC.

In step gradient mode, the optimal mobile phase condition was obtained by step-increase in methanol concentration from 12% to 35% with 7 min gradient time, and the chromatogram is shown in Fig. 2. Considering the mixer volume of 6.8 ml and dead volume of 2.7 ml, the first mobile phase exited the column after 9.5 min from injection at a flow of 1 ml min^{-1}. The second mobile phase arrives at the inlet of column 7 min after the injection of a sample.

Table 1
Retention factors of deoxyribonucleosides in binary mobile phases

Mobile phase (vol. %)		Retention factors (k)				
Water	Methanol	dCyd	dUrd	dGuo	dThd	dAdo
75	25	0.33	0.33	0.51	0.67	1.26
80	20	0.48	0.53	0.92	1.10	2.36
85	15	0.69	0.78	1.54	1.77	4.05
90	10	1.17	1.43	3.22	3.43	8.78
95	5	1.94	2.35	5.83	5.83	16.27
97	3	3.02	3.93	10.46	10.46	30.41

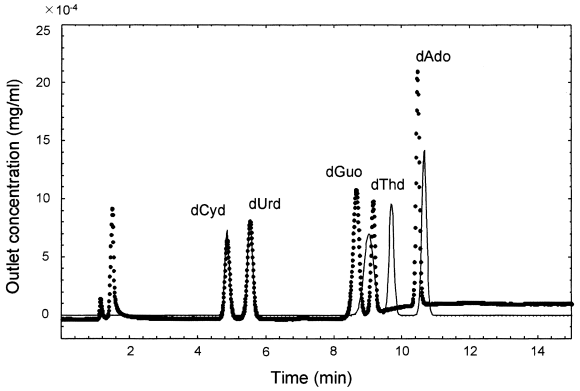

Fig. 2. Comparison of the experimental and calculated profiles in optimum step gradient condition (F_1: 12%, F_2: 35%, $V_{g,i}$: 7 min, solid line: calculated, dotted line: experimental).

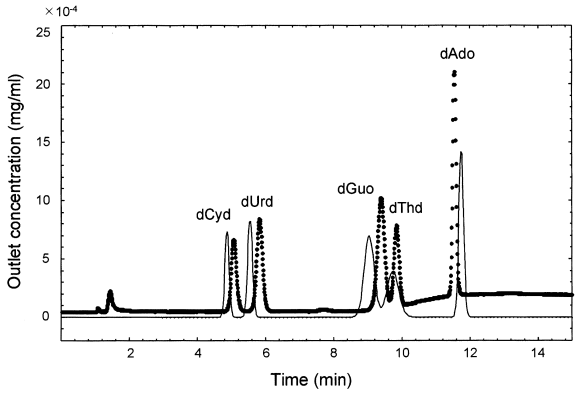

Fig. 3. Comparison of the experimental and calculated profiles in linear gradient condition (F_1: 12%, F_2: 35%, $V_{g,i}$: 7 min, $V_{g,\infty}$: 9 min, solid line: calculated, dotted line: experimental).

Before the step change to the second mobile phase, dCyd and dUrd were eluted in isocratic mode with 88/12 vol.% of water/methanol. The agreement between the experimental data and the calculated values of the two components was good. Around 9.5 min, a deviation of the calculated profiles of dGuo and dThd from the experimental data was obtained. In step gradient mode, it was assumed that the first mobile phase is changed instantly to the second at the gradient time. Actually the mobile phase composition changes with a slope, because the two mobile phases were miscible. Fig. 2 also shows that dAdo was eluted after the system had completely changed to second mobile phase composition and a relatively good agreement between the two profiles of dAdo was made. In linear gradient mode, the experimental and calculated profiles were compared in Fig. 3. The experimental conditions were that the initial mobile phase composition was 12% methanol and from 7 min to 9 min, the composition of methanol was linearly increased to 35%. The difference between the calculated and experimental values was 3%, and it was smaller than that of step mode, (10%). Comparisons of the calculated resolutions and separation times in step and linear gradients with the experimental variables are summarized in Table 3.

5. Conclusions

The five deoxyribonucleosides were separated by changes in mobile phase compositions. Based on the plate theory, elution profiles might be predicted by introducing the concept of solute migration in the mobile phase and the quadratic dependency of ln k in terms of the content of organic modifier. Compared to linear gradient mode, the step change in mobile phases resulted in some errors for prediction, especially around the time when the second mobile phase

Table 3
Comparison of resolutions and separation times between experimental data and calculated values

Resolution	Isocratic	Step gradient		Linear gradient	
	exp.	exp.	cal.	exp.	cal.
R_1	1.38	2.30	2.62	2.23	2.62
R_2	7.49	8.58	8.26	8.60	8.26
R_3	1.74	1.60	1.59	1.17	1.12
R_4	7.76	3.80	3.45	5.31	4.20
Separation time (min)	11.42	10.66	10.93	11.70	11.88

exits the column. The gradient volume in linear gradient mode could be easily estimated by modification of the relationship used in step gradient mode. Unfortunately, the superiority of the linear gradient mode was not confirmed in this work. This is mainly attributed to the fact that deoxyribonucleosides were eluted early enough so that the linear gradient mode could not be adequately applied.

6. Nomenclature

A, B, C:	Empirical constants used in Eq. (1)
a:	Constant used in Eq. (8)
c_0:	Concentration of injected solute [mg ml^{-1}]
c_N:	Concentration of solute in the Nth plate [mg ml^{-1}]
F, F_1, F_2:	Volume percent of organic modifier in mobile phase, that in the first and second mobile phase, respectively
K:	Equilibrium constant
k:	Retention factor
L:	Column length [cm]
N:	Number of theoretical plates
R_i,R_1,R_2,R_3,R_4:	Resolution, between dCyd and dUrd, dUrd and dGuo, dGuo and dThd, dThd and dAdo, respectively.
r:	Number of theoretical plate filled with solute at injection
t_R:	Retention time [min]
V:	Volume of mobile phase passing through the column [ml]
$V_{c,n}$:	Volume of mobile phase at solute migration meet with mobile phase migration in the nth mobile phase [ml]
$V_{g,i}$:	volume of the ith mobile phase passing through a column inlet before the introduction of the $(i+1)$th mobile phase into the column
V_0:	Dead volume [ml]
V_{Rg}:	Retention volume [ml]
$V_{r,1}$, $V_{r,\infty}$:	Retention volume in the initial and final mobile phase, respectively [ml]
v_m:	Volume of mobile phase in a theoretical plate [ml]
v_s:	Volume of the stationary phase in a theoretical plate [ml]
w:	Band width in the baseline [min]
Y_n:	Solute migration in nth mobile phase zone as a function of retention volume
$Y_{m,n}$:	Mobile phase migration in the nth mobile phase zone as a function of retention volume

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Appendix 1

The slope (a_1) of column length *versus* mobile phase volume in the first step of gradient elution is:

$$a_1 = \frac{L}{V_0(1+k_1)} \tag{A.1}$$

where L is column length and k_1 is retention factor in the first step of gradient elution. Solute migration (Y) in the first mobile phase zone as a function of retention volume is:

$$Y_1 = \frac{L}{V_0(1+k_1)}V \tag{A.2}$$

The relationship between the column length and retention volume in the second mobile phase zone is:

$$Y_{m,1} = \frac{L}{V_0}(V - V_{g,1}) \tag{A.3}$$

As shown in Fig. 4, Y_1 intersects $Y_{m,1}$ at ($V_{c,1}$, $Y_{c,1}$), so Y_1 is equal to $Y_{m,1}$, therefore

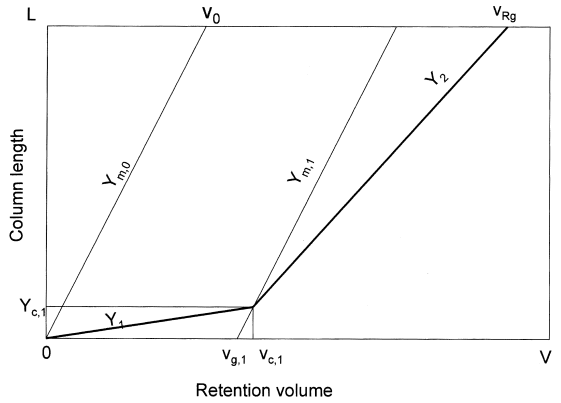

Fig. 4. Solute migration along column length and retention volume in step gradient mode.

$$V_{c,1} = \frac{V_{g,1}(1+k_1)}{k_1} \tag{A.4}$$

$$Y_{c,1} = \frac{V_{g,1}L}{V_0 k_1} \tag{A.5}$$

Solute migration (Y_2) in the second mobile phase zone through the point ($V_{c,1}$, $Y_{c,1}$) is:

$$Y_2 = \frac{L}{V_0(1+k_2)}V + \frac{V_{g,1}L}{V_0 k_1}\left(\frac{k_2-k_1}{1+k_2}\right) \tag{A.6}$$

Substituting V and Y_2 into V_{Rg} and L yields Eq. (A.7).

$$V_{Rg} = V_0(1+k_2) + \frac{V_{g,1}}{k_1}(k_1-k_2) \tag{A.7}$$

We can replace V_0 and k_1 of Eq. (A.7) with V_r:

$$V_{r,1} = V_0(1+k_1), V_{r,2} = V_0(1+k_2)$$

The resulting V_{Rg} is

$$V_{Rg} = V_{r,2} + V_{g,1}\frac{(V_{r,1}-V_{r,2})}{(V_{r,1}-V_0)} \tag{A.8}$$

Similarly, the method of Eq. (A.3)– Eq. (A.7) is extended to

$$Y_{m,2} = \frac{L}{V_0}(V - V_{g,2})$$

$$V_{c,2} = \frac{V_0 V_{g,1}(V_{r,2}-V_{r,1})}{(V_{r,2}-V_0)-(V_{r,1}-V_0)} = \frac{V_{r,2}V_{g,2}}{V_{r,2}-V_0} \tag{A.9}$$

$$Y_{c,2} = \frac{LV_{g,1}(V_{r,2}-V_{r,1})}{(V_{r,2}-V_0)(V_{r,1}-V_0)} + \frac{LV_{g,2}}{(V_{r,2}-V_0)} \tag{A.10}$$

$$Y_3 = \frac{L}{V_{r,3}}V + \frac{LV_{g,1}(V_{r,2}-V_{r,1})(V_{r,3}-V_0)}{(V_{r,2}-V_0)(V_{r,1}-V_0)V_{r,3}} + \frac{LV_{g,2}(V_{r,3}-V_{r,2})}{(V_{r,2}-V_0)V_{r,3}} \tag{A.11}$$

$$Y_{m,3} = \frac{L}{V_0}(V - V_{g,3})$$

$$V_{c,3} = \frac{V_0 V_{g,1}(V_{r,2}-V_{r,1})}{(V_{r,2}-V_0)-(V_{r,1}-V_0)} + \frac{V_0 V_{g,2}(V_{r,3}-V_{r,2})}{(V_{r,3}-V_0)-(V_{r,2}-V_0)} + \frac{V_{r,3}V_{g,3}}{V_{r,3}-V_0} \tag{A.12}$$

$$Y_{c,3} = \frac{LV_{g,1}(V_{r,2}-V_{r,1})}{(V_{r,2}-V_0)(V_{r,1}-V_0)} + \frac{LV_{g,2}(V_{r,3}-V_{r,2})}{(V_{r,3}-V_0)(V_{r,2}-V_0)} + \frac{LV_{g,3}}{(V_{r,3}-V_0)} \tag{A.13}$$

$$Y_{m,n-1} = \frac{L}{V_0}(V - V_{g,n-1})$$

$$V_{c,n-1} = \sum_{i=2}^{n-1}\frac{V_0 V_{g,i-1}(V_{r,i}-V_{r,i-1})}{(V_{r,i}-V_0)-(V_{r,i-1}-V_0)} + \frac{V_{r,n-1}V_{g,n-1}}{V_{r,n-1}-V_0} \tag{A.14}$$

$$Y_{c,n-1} = \sum_{i=2}^{n-1}\frac{LV_{g,i-1}(V_{r,i}-V_{r,i-1})}{(V_{r,i}-V_0)(V_{r,i-1}-V_0)} + \frac{LV_{g,n-1}}{V_{r,n-1}-V_0} \tag{A.15}$$

$$Y_{n-1} = \frac{L}{V_{r,n-1}}V + \frac{V_{r,n-1}-V_0}{V_{r,n-1}}\sum_{i=1}^{n-1}\frac{LV_{g,i}(V_{r,i+1}-V_{r,i})}{(V_{r,i+1}-V_0)(V_{r,i}-V_0)} + \frac{LV_{g,n-2}(V_{r,n-1}-V_{r,n-2})}{(V_{r,n-2}-V_0)V_{r,n-1}} \tag{A.16}$$

Refer to Fig. 5 for above procedures. So, V_{Rg} is expressed as follows,

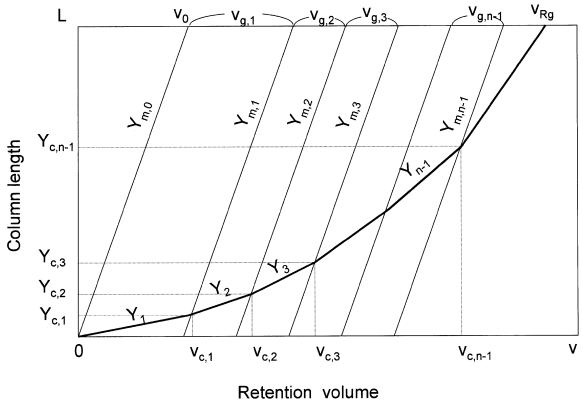

Fig. 5. Solute migration along column length and retention volume in linear gradient mode.

$$V_{Rg} = V_{r,n-1} + (V_{r,n-1} - V_0) \sum_{i=1}^{n-1} \frac{V_{g,i}(V_{r,i} - V_{r,i+1})}{(V_{r,i+1} - V_0)(V_{r,i} - V_0)} \quad (A.17)$$

In case of $n \to \infty$, $V_{r,i+1} \approx V_{r,i}$. Then, we finally obtain the following equation of the gradient volume,

$$V_{Rg} = V_{r,\infty} + (V_{r,\infty} - V_0) \sum_{i=1}^{\infty} \frac{V_{g,i}(V_{r,i} - V_{r,i+1})}{(V_{r,i} - V_0)^2} \quad (A.18)$$

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