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Multilinear gradient elution optimization in reversed-phase liquid chromatography based on logarithmic retention models: Application to separation of a set of purines, pyrimidines and nucleosides

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ABSTRACT

The analytical solutions of the fundamental equation of the multilinear gradient elution are derived in two cases, when the dependence of the logarithm of the solute retention ($\ln k$) upon the volume fraction of organic modifier (φ) is a three-parameter logarithmic expression, and when a simple linear relationship between $\ln k$ and $\ln \varphi$ is adopted. The derived theoretical expressions for retention times under multilinear gradient conditions are embodied to simple algorithms for fitting gradient data and especially for resolution optimization. Their performance was examined by using a mixture of 16 model compounds chosen among purines, pyrimidine and nucleosides in eluting systems modified by acetonitrile. It was found that the accuracy of the predicted gradient retention times is very satisfactory even if the simple logarithmic expression for the retention behavior of solutes, i.e. the linear dependence of $\ln k$ upon $\ln \varphi$, is used.

1. Introduction

In gradient elution chromatography a computer aided optimization procedure involves the solution of the fundamental equation of gradient elution with respect to the retention time, t_R, which in turn requires the dependence of the retention factor, k, upon the mobile phase composition. Up to now, in reversed-phase liquid chromatography (RP-LC), an analytical solution of the fundamental equation of gradient elution with respect to t_R, is available when $\ln k$ varies linearly with the volume fraction φ of an organic modifier in the hydro-organic eluents, and φ is programmed to vary linearly with the time. The combination of linear programmed gradient profile with a linear dependence of $\ln k$ upon φ is called linear solvent strength gradient and constitutes the base of DryLab, the most widespread package for gradient elution prediction and optimization to date [1,2]. However, due to the importance of using proper optimization algorithms for the determination of the optimum gradient profile at a certain separation, several other approaches have already been developed [3–9].

In a recent review article [10] we have shown that the logarithmic retention model

$$\ln\ k = \ln k_0 - r \ln(1 + b\varphi) \tag{1}$$

exhibits a good fitting performance which is comparable to that of the most popular retention models, the quadratic

$$\ln\ k = \ln k_0 + a\varphi + b\varphi^2 \tag{2}$$

and the rational one

$$\ln k = \ln k_0 - \frac{a\varphi}{1 + b} \tag{3}$$

In these equations k_0, r, a and b are adjustable parameters.

The aim of the present study is to develop analytical solutions of the fundamental equation of gradient elution when the retention model expressed by Eq. (1) as well as by limiting expressions of Eq. (1) and to use the derived expressions for t_R in algorithms for fitting gradient data and for multilinear gradient elution optimization. The effectiveness of the above optimization procedure is tested in the separation of 16 model compounds chosen among purine and pyrimidine bases as well as nucleosides due to their biomedical and pharmaceutical interest [11–15].

2. Theoretical section

The fundamental equation of gradient elution when the volume fraction φ of an organic modifier in the hydro-organic eluents varies with the time t is [6,9,16–19]

$$\int_0^{t_R - t_0} \frac{dt}{t_0 k} = 1 \tag{4}$$

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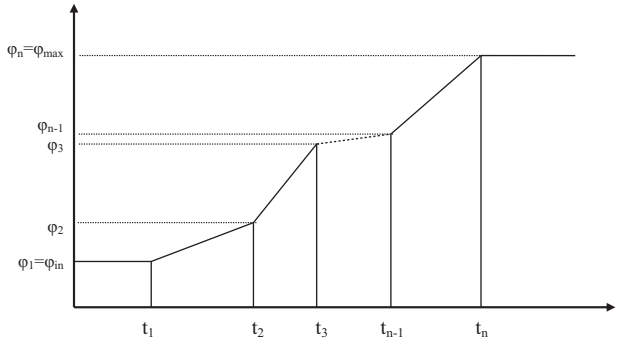

Fig. 1. Schematic multilinear gradient profile.

where t_R is the solute gradient elution time and t_0 is the column hold-up time. Eq. (4) has an analytical solution with respect to the analyte retention time t_R when a linear retention model, such as

$$\ln\ k = \ln k_0 - a\varphi \tag{5}$$

is adopted. The solution of Eq. (4) when the retention model is the linear one has been presented and discussed by Snyder and his colleague [1,2] and it constitutes the bases of the Drylab, which is the most widespread package for prediction and optimization.

It can be easily shown that the combination of the logarithmic model of Eq. (1) with the fundamental Eq. (4) results also in an analytical solution with respect to t_R. In addition, analytical solution is obtained from the model

$$\ln\ k = \ln k_0 - a\ln\varphi \tag{6}$$

which is a limiting expression of Eq. (1) when $b\varphi \gg 1$. Note that, the linear model, Eq. (5), is also a limiting case of Eq. (1) when $|b\varphi| \ll 1$. In this case $\ln(1+b\varphi)$ is expanded in a Taylor series and since $|b\varphi| \ll 1$, we obtain $\ln(1+b\varphi) \approx b\varphi$, which in combination with Eq. (1) yields Eq. (5).

Here we examine the solutions of the fundamental equation of gradient elution when the retention model is expressed by Eq. (1) or Eq. (6). It is evident that the solution of Eq. (4) when the retention model is Eq. (1) is expected to cover a much wider range of solutes than the solution of this equation with the very limiting linear model of Eq. (5). At this point we should clarify that analytical solutions of the fundamental equation (4) are needed only in optimization algorithms to suppress the computational time as much as possible.

For generality we examine the analytical solutions of Eq. (4) under the multilinear gradient profile depicted in Fig. 1. This profile is mathematically described by the following expression

$$\varphi = \begin{cases} \varphi_1 = \varphi_{in} & t < t_1 \\ \varphi_1 + \lambda_2(t-t_1) & t_1 < t < t_2 \\ \varphi_2 + \lambda_3(t-t_2) & t_2 < t < t_3 \\ \dots & \\ \varphi_{n-1} + \lambda_n(t-t_{n-1}) & t_{n-1} < t < t_n \\ \varphi_n = \varphi_{\max} & t > t_n \end{cases} \tag{7}$$

where λ_i is the slope of the φ vs. t at the i-th segment. In the treatment presented in this paper we assume that all slopes are different from zero, since a zero slope can be approximated in practice by an infinitesimally small slope. Note that in the times appeared in Eq. (7) we should add the dwell time t_D of the chromatographic system, i.e. the time needed for a certain change in the mixer to reach the inlet of the chromatographic column. Based on the multi-linear profile of Eq. (7) and provided that an analyte is eluted in the range $t_{p-1} < t < t_p$, where $p \le n$, Eq. (4) may be written as

$$\int_0^{t_R-t_0} \frac{dt}{t_0 k} = \int_0^{t_1+t_D} \frac{dt}{t_0 k} + \int_{t_1+t_D}^{t_2+t_D} \frac{dt}{t_0 k} + \dots + \int_{t_{p-2}+t_D}^{t_{p-1}+t_D} \frac{dt}{t_0 k} + \int_{t_{p-1}+t_D}^{t_R-t_0} \frac{dt}{t_0 k} = I_1 + I_2 + \dots + I_{p-1} + \int_{t_{p-1}+t_D}^{t_R-t_0} \frac{dt}{t_0 k} = 1 \tag{8}$$

where parameters $I_1, I_2, \dots$ represent the corresponding integrals in the above expression.

2.1. Logarithmic retention model expressed by Eq. (1)

When the retention model may be expressed by Eq. (1), all the integrals in Eq. (8) can be readily evaluated, since, according to Eq. (7), φ in each linear portion may be in general expressed as $\varphi = const + \lambda t$, and therefore

$$\int \frac{dt}{t_0 k} = \int \frac{(1+b\phi)^r dt}{t_0 k_0} = \frac{1}{t_0 k_0} \int [1 + b\cdot const + b\lambda t)]^r dt = \frac{(1+b\phi)^{r+1}}{t_0 k_0 (r+1) b\lambda} + const \tag{9}$$

From this integral we readily obtain that

$$I_i = \int_{t_{i-1}+t_D}^{t_i+t_D} \frac{dt}{t_0 k} = \frac{(1+b\phi_i)^{r+1} - (1+b\phi_{i-1})^{r+1}}{(r+1) t_0 k_0 b\lambda_i} \tag{10}$$

when $i = 2, 3, \dots, n$, whereas I_1 is given by

$$I_1 = \int_0^{t_D+t_1} \frac{dt}{t_0 k} = \frac{t_D + t_1}{t_0 k_{in}} \tag{11}$$

where k_{in} is the value of retention factor k when $\varphi = \varphi_1 = \varphi_{in}$.

Thus an analytical solution of Eq. (4) with respect to t_R is feasible but this solution depends on whether the analyte is eluted in the first isocratic portion, i.e. when $t_R < t_1 + t_D$, or in the last isocratic portion when $t_R > t_n + t_D$ or at an intermediate linear portion, say $t_{p-1} + t_D < t_R < t_p + t_D$. So we have to examine all these cases:

A) The analyte is eluted in the first isocratic portion. The condition that indicates that the analyte is eluted under isocratic conditions, i.e. before $t_1 + t_D$, is the validity of the following inequality

$$I_1 = \frac{t_D + t_1}{t_0 k_{in}} \ge 1 \tag{12}$$

Then from Eq. (4) we obtain

$$t_R = t_0(1 + k_{in}) \tag{13}$$

B) The analyte is eluted in the p-th linear portion, i.e. between t_{p-1} and t_p. When this happens, the following inequalities hold simultaneously

$$\int_0^{t_{p-1}+t_D} \frac{dt}{t_0 k} < 1 \quad \text{and} \quad \int_0^{t_p+t_D} \frac{dt}{t_0 k} \ge 1 \tag{14}$$

because

$$\int_0^{t_R-t_0} \frac{dt}{t_0 k} = \int_0^{t_{p-1}+t_D} \frac{dt}{t_0 k} + \int_{t_{p-1}+t_D}^{t_R-t_0} \frac{dt}{t_0 k} = 1 \Rightarrow \int_0^{t_{p-1}+t_D} \frac{dt}{t_0 k} < 1 \tag{15}$$

and

$$\int_0^{t_p+t_D}\frac{dt}{t_0k}=\int_0^{t_R-t_0}\frac{dt}{t_0k}+\int_{t_R-t_0}^{t_p+t_D}\frac{dt}{t_0k}=1+\int_{t_R-t_0}^{t_p+t_D}\frac{dt}{t_0k}>1 \tag{16}$$

Therefore, in order to determine the linear gradient in which an analyte is eluted, we simply determine p for which both inequalities (14) are valid. These inequalities may be expressed as

$$I_1+I_2+\cdots+I_{p-1}<1 \quad \text{and} \quad I_1+I_2+\cdots+I_{p-1}+I_p\geq 1 \tag{17}$$

and allow the direct determination of p. Now in order to calculate the retention time t_R of the analyte, we use again Eq. (8), which yields

$$\int_{t_{p-1}+t_D}^{t_R-t_0}\frac{dt}{t_0k}=1-I_1-I_2-\cdots-I_{p-1} \tag{18}$$

The integral of this expression is calculated again from Eq. (9). Thus we readily obtain

$$t_R=t_0+\frac{B^{1/(r+1)}-1-b\phi_{p-1}+b\lambda_p(t_{p-1}+t_D)}{b\lambda_p} \tag{19}$$

where

$$B=(r+1)t_0k_0b\lambda_p(1-I_1-I_2-\cdots-I_{p-1}+J_p) \tag{20}$$

and

$$J_p=\frac{(1+b\phi_{p-1})^{r+1}}{(r+1)t_0k_0b\lambda_p} \tag{21}$$

C) The analyte is eluted in the last isocratic portion, i.e. when $\varphi=\varphi_{\max}$. This happens when the following inequality is valid

$$\int_0^{t_n+t_D}\frac{dt}{t_0k}<1\Rightarrow I_1+I_2+\cdots+I_{n-1}+I_n<1 \tag{22}$$

For the retention time t_R of the analyte we have again

$$\int_0^{t_R-t_0}\frac{dt}{t_0k}=1\Rightarrow\int_0^{t_n+t_D}\frac{dt}{t_0k}+\int_{t_n+t_D}^{t_R-t_0}\frac{dt}{t_0k}=1 \Rightarrow\int_{t_n+t_D}^{t_R-t_0}\frac{dt}{t_0k}=1-I_1-I_2-\cdots-I_n \tag{23}$$

However now the last integral is calculated very easily because $k=k(\varphi_{\max})=k_{\max}$ is constant. Therefore,

$$t_R=t_0+t_n+t_0k_{\max}(1-I_1-I_2-\cdots-I_n) \tag{24}$$

2.2. Simple logarithmic retention model expressed by Eq. (6)

If we work as in the previous section, we obtain that Eqs. (13) and (24) are still valid when the analyte is eluted in the first and the last isocratic portions, respectively. When the analyte is eluted in the p-th linear portion, i.e. between $t_{p-1}+t_D$ and t_p+t_D, then the retention time is given by

$$t_R=t_0+\frac{\{(a+1)t_0k_0\lambda_p(1-I_1-\cdots-I_{p-1}+J_p)\}^{1/(a+1)}-\phi_{p-1}+\lambda_p(t_{p-1}+t_D)}{\lambda_p} \tag{25}$$

Here a and k_0 are the adjustable parameters of the retention model, Eq. (6), and

$$I_i=\frac{\varphi_i^{a+1}-\varphi_{i-1}^{a+1}}{(a+1)t_0k_0\lambda_i},\quad i=2,3,\ldots,n-1 \quad \text{and} \quad J_p=\frac{\varphi_{p-1}^{a+1}}{(a+1)t_0k_0\lambda_p} \tag{26}$$

arising from

$$\int\frac{dt}{t_0k}=\int\frac{\varphi^a dt}{t_0k_0}=\frac{1}{t_0k_0}\int(const+\lambda t)]^a dt=\frac{\varphi^{a+1}}{t_0k_0(a+1)\lambda}+const \tag{27}$$

2.3. Linear retention model expressed by Eq. (5)

Again Eqs. (13) and (24) are valid when the analyte is eluted in the first and the last isocratic portions, respectively, whereas the retention time in the p-th linear portion may be expressed as

$$t_R=t_0+\frac{\ln\{(1-I_1-\cdots-I_{p-1})/J_p+e^{a\lambda_p(t_{p-1}+t_D)}\}}{a\lambda_p} \tag{28}$$

where

$$I_i=\frac{e^{a\varphi_i}-e^{a\varphi_{i-1}}}{t_0k_0a\lambda_i},\quad i=2,3,\ldots,n-1 \quad \text{and} \quad J_p=\frac{e^{a\varphi_{p-1}-a\lambda_p(t_{p-1}+t_D)}}{t_0k_0a\lambda_p} \tag{29}$$

and a and k_0 are the adjustable parameters of the retention model, Eq. (5). Note that Eq. (28) is a generalization of the corresponding expression derived by Snyder et al. [20–23] for a single linear portion.

2.4. Algorithms for fitting and optimization

The fitting algorithm adopted to determine the adjustable parameters involved in the retention models, Eqs. (1), (5) or (6), was the R_LM algorithm proposed in [8]. According to this algorithm, an initial vector of the adjustable parameters is randomly selected from the search domain and the Levenberg–Marquardt method, using a rather small number of iterations, 500 in our applications, determines the local minimum of the cost function [8], which is stored. Then a new vector of adjustable parameters is randomly selected and the whole algorithm is repeated for a preset number of iterations, at least 100 iterations. The minimum of the stored local minima is determined and it presumably corresponds to the global minimum of the cost function.

For the optimization algorithm, we adopted the following very simple scheme. The algorithm using random numbers creates arbitrary multilinear gradient profiles (φ_{in}, φ_2, φ_3, …, φ_n, t_1, t_2, …, t_n) and at each gradient profile it calculates the retention times of all solutes through the corresponding analytical expressions derived in this paper. In addition, the algorithm calculates the minimum value of the absolute difference $\delta t=|t_{R,i}-t_{R,j}|$ between pairs of adjacent solutes, i and j, and the elution time of the most distant solute, $t_{R,\max}$. If δt is greater than a preset value and $t_{R,\max}$ is smaller than the maximum gradient elution time also preset by the researcher, then the vector (φ_{in}, φ_2, …, φ_n, t_1, t_2, …, t_n) along with the values of δt and $t_{R,\max}$ are stored. This procedure lasts for a preset time and then all stored data are shorted descending according to δt. The output is the first 100 (or any other preset number) of them together with the predicted retention times of the solutes. Note that the algorithm may use a certain retention model for all solutes or different retention models for each solute selected from Eqs. (1), (5) and (6). In the latter case the user defines through the input file which of the analytical expressions for t_R developed in the present paper will be used for the estimation of the elution time of each analyte. This algorithm scans about 1,000,000 points per min and for this reason it determines easily the optimum conditions at a certain separation. In contrast, if we use the approximate stepwise method proposed in [6] for the determination of t_R, the same algorithm scans about 16,000 points per min and for this reason it may fail to determine optimum conditions when we use a reasonable computational time (say 1 h).

Table 1
Experimental retention data (in min) of solutes studied under different linear and multilinear gradient profiles.

	No. of gradients															
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
$\varphi_1 = \varphi_{in}$	0.050	0.025	0.010	0.010	0.010	0.050	0.045	0.043	0.026	0.025	0.025	0.010	0.025	0.023	0.020	0.020
φ_2	0.150	0.150	0.150	0.150	0.150	0.150	0.150	0.150	0.150	0.150	0.150	0.150	0.093	0.085	0.093	0.079
φ_3	–	–	–	–	–	–	–	–	–	–	–	–	0.150	0.150	0.150	0.150
t_1 (φ_{in})	0	0	0	0	0	3.0	2.9	1.7	2.0	2.0	3.5	1.0	3.6	3.7	3.6	3.6
t_2 (φ_2)	20.0	10.0	5.0	10.0	20.0	13.0	3.7	4.0	8.4	7.0	7.8	3.5	3.7	4.9	3.7	4.9
t_3 (φ_3)	–	–	–	–	–	–	–	–	–	–	–	–	8.6	13.0	8.6	11.0
Solutes	t_R (min)															
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Cyt	3.40	3.85	4.23	4.23	4.23	3.41	3.44	3.51	3.80	3.84	3.83	4.23	3.84	3.88	3.96	3.96
Ura	3.93	4.52	5.34	5.27	5.28	3.93	3.96	3.98	4.43	4.52	4.45	5.34	4.51	4.57	4.72	4.71
UA	3.75	4.52	5.73	5.73	5.73	3.77	3.85	3.98	4.43	4.52	4.35	5.97	4.51	4.64	4.90	4.89
Cyd	3.93	5.00	5.84	6.17	6.46	3.93	3.96	3.98	4.87	5.00	4.93	6.44	4.99	5.12	5.43	5.43
Hyp	4.38	5.76	6.18	6.73	7.29	4.39	4.55	4.69	5.81	6.00	5.87	6.62	5.99	6.16	6.57	6.56
Urd	4.46	6.00	6.30	6.97	7.74	4.46	4.67	4.83	6.25	6.49	6.34	6.70	6.47	6.67	7.22	7.22
Xan	4.61	6.12	6.37	7.03	7.74	4.62	4.83	5.00	6.44	6.68	6.51	6.70	6.66	6.87	7.39	7.38
Thy	5.73	7.02	6.95	7.82	8.81	5.81	6.08	6.28	7.74	7.81	8.11	7.10	8.26	8.49	8.55	8.92
Ino	5.73	7.39	6.95	8.24	9.98	5.81	6.37	6.69	8.38	8.29	9.34	7.10	8.53	9.34	8.55	9.16
Guo	5.99	7.61	7.07	8.43	10.24	6.21	6.78	6.91	8.59	8.44	9.53	7.10	8.53	9.45	8.55	9.37
Ade	6.47	7.81	7.35	8.61	10.36	6.75	7.19	7.12	8.65	8.56	9.53	7.30	8.67	9.49	8.75	9.62
Thd	8.13	9.08	7.97	9.81	12.47	9.03	8.09	7.71	9.78	9.44	10.47	7.63	9.20	10.27	9.26	10.48
Ado	9.45	9.89	8.27	10.53	14.01	10.44	8.18	7.95	10.36	9.86	10.88	7.74	9.51	10.82	9.56	11.03
TB	10.69	10.72	8.85	11.32	15.01	11.42	8.53	8.40	11.03	10.47	11.42	8.19	10.24	11.67	10.30	11.82
TP	13.65	12.40	9.77	12.86	17.66	13.62	9.29	9.17	12.27	11.49	12.35	9.01	11.52	13.40	11.58	13.35
CF	19.10	15.32	12.24	15.63	22.21	17.19	11.67	11.53	14.71	13.95	14.67	11.51	14.13	16.79	14.18	16.17

3. Experimental

The liquid chromatography system consisted of a Shimadzu LC-20AD pump, a Shimadzu DGU-20A$_3$ degasser, a model 7125 syringe loading sample injector fitted with a 20 μL loop, a 250 mm × 4.6 mm MZ-Analytical column (MZ-Aqua Perfect C18 5 μm) thermostatted at 30 °C by a CTO-10AS Shimadzu column oven and a Shimadzu UV–visible spectrophotometric detector (Model SPD-10A) working at 260 nm. The solutes were the following 7 purines: uric acid (UA), hypoxanthine (Hyp), xanthine (Xan), adenine (Ade), theobromine (TB), theophylline (TP) and caffeine (CF); 3 pyrimidines: cytosine (Cyt), uracil (Ura) and thymine (Thy); and 6 nucleosides: cytidine (Cyd), uridine (Urd), inosine (Ino), guanosine (Guo) thymidine (Thd) and adenosine (Ado). A standard mixture of solutes at concentrations ranging from 2.7 to 8 μg/mL was used in a phosphate buffer of pH 5.0 by making appropriate dilutions of stock standard solutions of individual analytes in the same diluent except for UA and Xan, which were initially dissolved into a buffer of pH 9.1 because of their insolubility in pH 5.0.

In order to investigate the validity of the analytical expressions derived in Section 2 for the determination of t_R obtained under multilinear gradient conditions, different chromatographic runs were performed with mobile phases consisting of 0.02 M aqueous phosphate buffers (pH 5.0) modified with acetonitrile (MeCN). The concentration of the organic modifier in mobile phases was changed by mixing automatically two 0.02 M aqueous phosphate buffers (pH 5.0) containing $\varphi_{MeCN} = 0.01$ and 0.15, respectively. The different gradient profiles used and the obtained experimental retention data are shown in Table 1. Note that in this table all gradients, except for 16, have been selected empirically to cover an extended range of slopes and initial values of φ_{MeCN}. Gradient 16 has been selected by the optimization algorithm as the optimum gradient profile for a total elution time equal to 17 min.

The flow rate was 1.0 mL/min. The hold-up time and the dwell time were estimated to be $t_0 = 2.56$ and $t_D = 1.1$ min, respectively.

4. Results and discussion

The retention models expressed by Eqs. (1), (5) and (6), through their corresponding expressions of t_R, were used for both fitting and prediction in order to access their validity in the description of the elution times of the analytes under study. The adjustable parameters k_0, r, b and a were determined by fitting the theoretical expressions of t_R to the corresponding gradient data of the first five simple linear gradient runs of Table 1. Indicative results are presented in Tables 2 and 3. Table 2 shows the adjustable parameters of Eq. (6), whereas Table 3 depicts absolute percentage errors between experimental and calculated retention data. In particular, the latter table shows in detail the fitting and prediction errors according to Eq. (6) and the average absolute percentage errors due to the retention models of Eqs. (1), (5) and (6) at each gradient run.

In general, both Eqs. (1) and (6) exhibit a quite satisfactory fitting performance to gradient data since the overall absolute average percentage error between calculated and experimental retention

Table 2
Values of adjustable parameters of Eq. (6) and their standard deviations.

Solutes	$\ln k_0$	a
Cyt	−2.24 ± 0.10	0.40 ± 0.03
Ura	−2.38 ± 0.22	0.58 ± 0.06
UA	−3.38 ± 0.58	0.87 ± 0.16
Cyd	−3.63 ± 0.63	0.99 ± 0.18
Hyp	−3.02 ± 0.45	0.91 ± 0.13
Urd	−3.28 ± 0.45	1.02 ± 0.13
Xan	−3.02 ± 0.39	0.96 ± 0.11
Thy	−2.30 ± 0.30	0.87 ± 0.09
Ino	−3.94 ± 0.82	1.44 ± 0.27
Guo	−3.63 ± 0.70	1.37 ± 0.23
Ade	−2.66 ± 0.39	1.09 ± 0.13
Thd	−2.66 ± 0.21	1.23 ± 0.07
Ado	−3.49 ± 0.14	1.63 ± 0.05
TB	−3.13 ± 0.10	1.58 ± 0.04
TP	−3.53 ± 0.10	1.92 ± 0.04
CF	−3.79 ± 0.11	2.36 ± 0.05

Table 3
Absolute percentage errors between experimental and calculated retention data based on the simple logarithmic retention model, Eq. (6), and average absolute percentage errors obtained from Eqs. (6), (1) and (5) at each gradient run.

No. of gradients	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Cyt	2.0	2.1	1.1	0.0	0.8	1.7	1.9	0.5	1.4	1.8	1.7	1.8	1.7	2.0	1.9	1.9
Ura	0.7	1.1	7.3	1.5	2.6	0.7	0.6	1.2	1.7	0.9	2.2	0.5	1.4	1.4	2.4	2.6
UA	0.3	0.3	11.3	4.7	2.6	0.9	0.1	1.9	4.1	3.9	7.5	7.0	4.7	4.1	5.1	5.4
Cyd	0.9	2.9	8.8	5.3	0.0	0.8	2.0	3.5	4.5	4.2	5.1	10.4	5.1	5.4	8.2	8.2
Hyp	1.9	5.1	6.8	4.6	1.5	2.3	2.9	2.0	3.3	1.8	4.9	7.8	4.1	4.4	6.9	7.1
Urd	2.5	5.3	6.5	4.5	1.9	3.5	3.6	2.8	1.5	0.9	5.4	7.4	4.9	5.3	2.8	5.6
Xan	2.5	5.3	5.9	3.9	0.8	3.5	3.7	2.7	0.4	2.2	5.1	6.3	4.2	4.4	0.9	4.3
Thy	1.8	2.7	3.0	1.2	1.4	3.9	4.5	1.0	2.5	3.9	1.8	3.4	5.2	1.3	5.6	3.7
Ino	3.4	2.4	2.8	1.0	1.2	8.6	6.3	4.7	3.1	4.1	3.7	5.0	5.0	4.6	3.3	0.6
Guo	4.2	2.1	1.9	0.6	1.2	8.1	2.4	5.2	3.1	3.6	3.8	3.0	3.2	3.9	1.4	0.9
Ade	3.5	1.1	1.4	0.2	0.7	6.3	0.8	4.3	1.7	2.7	2.6	1.7	2.2	2.7	0.9	1.7
Thd	2.6	0.2	0.9	0.9	0.2	0.1	3.6	1.7	0.6	0.8	1.0	1.4	2.3	1.5	3.3	1.3
Ado	1.3	0.2	0.3	0.8	0.1	1.4	1.5	1.3	0.2	0.1	0.0	1.0	3.5	2.9	4.0	2.1
TB	0.6	0.5	0.5	0.3	0.6	1.1	0.2	0.7	0.1	0.6	0.2	1.7	2.4	2.2	2.8	1.4
TP	0.4	1.1	0.7	0.5	0.7	0.7	1.4	0.7	0.6	0.9	0.3	1.0	1.0	1.3	0.9	0.4
CF	0.5	1.2	0.9	0.6	0.1	0.7	1.2	1.1	0.4	0.4	1.4	0.8	0.5	0.1	0.3	0.1
Eq. (6)[a]	1.8	2.1	3.8	1.9	1.0	2.8	2.3	2.2	1.8	2.0	2.9	3.8	3.2	3.0	3.2	3.0
Eq. (1)[a]	1.9	1.9	3.8	2.0	0.9	2.7	2.3	2.2	1.9	2.0	3.1	3.7	2.9	3.1	3.1	2.8
Eq. (5)[a]	3.9	1.9	5.6	3.2	2.7	6.4	3.0	2.7	3.0	3.1	4.6	6.7	4.4	4.1	3.9	3.4

[a] Average absolute percentage errors between experimental and calculated retention data obtained at each gradient run.

data is only 2.1%. The overall predictive % error between calculated and experimental retention data increases to 2.7 for all gradient runs tested (see columns 6–15 of Table 3 for the predictive % errors). However, we observed that Eq. (1) exhibits a rather peculiar behavior. Every time we rerun the fitting algorithm changing the ranges of the adjustable parameters (search domain), the calculated b parameter always takes the maximum value of its range input, at least for certain solutes. Additionally, if we examine the statistical significance of the adjustable parameters of Eq. (1) from their t-ratio values, i.e. the absolute value of the ratio of each parameter to its standard deviation, it was found that the fitting parameter b was statistically insignificant for all solutes, since its calculated t-ratio parameters were less than 2 [24]. The above observations indicate that Eq. (1) could be replaced by the simple logarithm retention model, Eq. (6). Indeed, the fitting of same set of solute retention gradient data to Eq. (6) gave statistical significant coefficients and moreover the fitting and prediction performance of Eq. (6) is practically identical to that of Eq. (1) at least for the solutes under study.

Finally, we compared the fitting and prediction performance of the logarithm retention models, Eqs. (1) and (6), to that of the linear model, Eq. (5), by using the same set of gradient data. Table 3 shows that the average percentage errors between experimental and calculated retention data by means of Eq. (5) at each gradient run are systematically greater than those obtained from the logarithmic models.

Consequently, the simple two-parameter logarithmic Eq. (6) seems to be the proper choice for the retention model of the solutes under study and for this reason this model was used in the optimization algorithm in order to determine the linear or multilinear gradient profile that is expected to lead to optimum separation of the sample of interest, a difficultly separated set of analytes.

Note that the optimum gradient profiles determined by the optimization algorithm described in Section 2.4 mainly depend on the preset run time, i.e. total elution time. Moreover, the variation of φ should be restricted between two predefined values φ_{min} and φ_{max}, which are 0.01 and 0.15 for our experimental system. Thus, for example, the optimum gradient profile determined for a total elution time equal to 17 min was that denoted by No. 16 in Table 3. This is a multilinear gradient consisting of an isocratic part at the beginning and at the end of the elution as well as of two linear φ variations with different slopes. Indeed, a perfect resolution of the sample of interest is achieved within only 16.2 min in the chromatogram recorded under the above optimal gradient conditions, see Fig. 2. Note that the same optimum is obtained when we use the retention model of Eq. (1). In the same figure the superiority of the optimal gradient profile is also illustrated, since it is clear that two critical couples of peaks, Ura–UA and Guo–Ade, were not able to be separated in 16.8 min by the No. 14 gradient, a gradient profile similar to the optimal one.

To sum up, the simple logarithmic retention model expressed by Eq. (6) was demonstrated to be the proper choice for gradient retention prediction and optimization through the analytical expressions derived in this case for the solution of the fundamental equation of the multilinear gradient elution. This logarithmic model is as simple as the linear retention model, Eq. (5), but the analytical solution of the fundamental equation of gradient elution based on Eq. (6) gives better results in what concerns the predicted gradient retention times of solutes. The logarithmic retention model expressed by Eq. (1) exhibits precisely the same prediction and optimization performance with that of Eq. (6) but it uses three adjustable parameters, which, at least for the analytes under study, are not all statistically significant. Finally, the optimization procedure developed in this study should be a valuable alternative

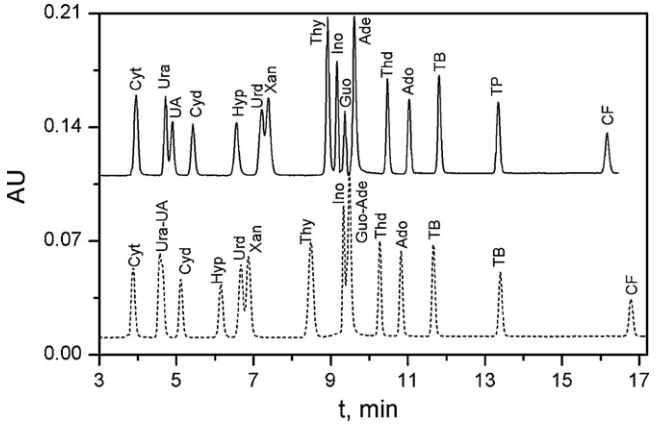

Fig. 2. UV detected chromatograms of a 16-component mixture of purines, pyrimidines and nucleosides obtained by using the optimal gradient profile, No. 16 (solid line) and the No. 14 gradient profile of Table 1 (dotted line). The elution order of solutes is shown in the figure.

to existing methods for the separation of the compounds concerned.

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