Global Optimization of Separation in High-Performance Liquid Chromatography

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Abstract

The statistical technique of optimizing mobile phases for liquid column chromatography using overlapping resolution maps is transformed into a nonstatistical multifactor optimizing method. The optimized parameters are the binary solvent composition, mobile phase flow rate, and analysis time. The main goal is to obtain the fastest analysis compatible with the desired separation. The principle is tested with two different sets of compounds in reversed-phase high-performance liquid chromatography: a mixture of seven herbicides and a mixture of nine benzodiazepines.

Introduction

The overlapping resolution map (ORM) optimization technique was introduced in the early 1980s by Glajch and co-workers (1,2) for high-performance liquid chromatography (HPLC) and was readily applied to high-performance thin-layer chromatography (TLC) (3,4). Originally developed as a mobile phase optimizing tool, it was transformed into a multifactor optimization ORM (MORM) by Tecklenburg and co-workers (5) and recently modified by us (6,7). The absolute requirement of a computer in ORM is probably the reason why, despite its efficiency, it is not more widely used.

Simultaneous optimization of the separation and analysis times has already been studied using the simplex method combined with chromatographic optimization functions (e.g., COF, CRF) (8). The problem with this optimization strategy is that it is a compromise between separation and analysis time, whereas MORM leads to the fastest separation of the compounds.

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A user-friendly software program was written to adapt MORM to HPLC. The method and the program were tested on mixtures of herbicides and benzodiazepines.

ORM

The basic principle of the triangular map of ORM is that the variables appearing on the three axes are related:

- In the original ORM developed by Glajch, the following equation applies:

$$X_1 + X_2 + X_3 = 1 \qquad \text{Eq 1}$$

where X_1, X_2, and X_3 are the volume fractions of the three solvents in the mobile phase.

- In the adaptation made by Tecklenburg for high-performance TLC, the migration distance, Z_f, the analysis time, t_A, and the volume fraction of the organic modifier in the binary mobile phase, X_S, are related:

$$Z_f^2 = (a_1 + a_2X_S + a_3X_S^2)t_A \qquad \text{Eq 2}$$

Table I. Results of the Preliminary Experiments and Calculated Parameters of Equations 3 and 7 for the Mixture of Herbicides*

	Experimental conditions									
X_S	0.5		0.6		0.7		0.7			
F(mL/min)	1		0.5		1.5		1			
	t_R	w_b	t_R	w_b	t_R	w_b	t_R	w_b	a	b
Simazine	8.39	0.223	8.14	0.24	1.63	0.068	2.45	0.125	–4.38	–0.65
Atrazine	10.34	0.314	10.48	0.309	2.07	–	3.10	–	–4.13	–0.24
2,4-D	13.12	0.316	12.36	0.310	2.21	–	3.32	–	–4.64	–0.33
MCPA	15.16	0.341	13.65	0.333	2.34	–	3.52	–	–4.87	–0.34
MCPP	26.39	–	20.65	0.438	3.05	–	4.60	–	–5.63	–0.28
2,4,5-T	27.26	–	22.09	0.498	3.29	–	4.96	–	–5.47	–0.14
MCPB	41.92	0.815	29.82	0.582	3.95	0.113	5.97	0.18	–6.15	–0.17

* The missing base width (w_b) values are due to limitations in the Varian software. Abbreviations: X_s, volume fraction of the organic modifier in the binary mobile phase; F, flow rate; t_R, retention time in minutes; w_b, width of the peak at the base in minutes. A, 0.0265; B', 0.00004; C', 0.34.

Table II. Results of the Preliminary Experiments and Calculated Parameters of Equations 3 and 7 for the Benzodiazepines*

Experimental conditions										
X_s	0.45		0.55		0.75		0.85			
F(mL/min)	1		0.5		1.5		1.8			
	t_R	w_b	t_R	w_b	t_R	w_b	t_R	w_b	a	b
Bromazepam	6.53	0.55	6.18	0.48	0.98	0.10	0.70	0.07	–4.846	–2.159
Nitrazepam	8.46	0.71	7.34	0.57	1.03	0.10	0.72	0.07	–5.117	–2.076
Flunitrazepam	9.66	0.82	7.67	0.60	1.00	0.10	0.70	0.07	–5.560	–2.281
Clobazam	11.81	1.02	9.09	0.72	1.09	0.11	0.73	0.07	–5.557	–2.057
Oxazepam	14.09	1.24	10.43	0.84	1.15	0.12	0.76	0.08	–5.646	–1.936
Tofisopam	20.66	1.98	12.91	1.07	1.17	0.12	0.74	0.08	–6.388	–2.122
Chlordiazepoxide	21.63	2.10	14.85	1.27	1.37	0.14	0.84	0.09	–5.814	–1.616
Dipotassic chlorazepate	25.62	2.60	16.38	1.43	1.38	0.14	0.83	0.09	–6.135	–1.695
Diazepam	32.73	3.60	20.22	1.86	1.55	0.16	0.89	0.10	–6.217	–1.507

* Abbreviations: X_s, volume fraction of the organic modifier in the binary mobile phase; F, flow rate; t_R, retention time in minutes; w_b, width of the peak at the base in minutes. A, 0.02; B', 0.002; C', 0.15.

Table III. Calculated and Experimentally Determined Retention Times (t_R) and Peak Width Values (w_b) for the Separation of Seven Herbicides

	Experimental		Calculated	
	t_R (min)	w_b (min)	t_R (min)	w_b (min)
Simazine	6.29	0.50	6.30	0.52
Atrazine	8.02	0.65	8.02	0.63
2,4-D	8.90	0.66	8.91	0.68
MCPA	9.60	0.77	9.61	0.72
MCPP	13.39	0.90	13.38	0.94
2,4,5-T	14.37	1.09	14.35	1.00
MCPB	18.18	1.17	18.16	1.21

* Conditions: flow rate, 0.49 mL/min; X_s, 0.652; analysis time, 18 min 53 s.

Table IV. Calculated and Experimentally Determined Retention Times (t_R) and Peak Width Values (w_b) for the Separation of Nine Benzodiazepines

	Experimental		Calculated	
	t_R (min)	w_b (min)	t_R	w_b (min)
Bromazepam	2.77	0.25	2.75	0.27
Nitrazepam	3.46	0.35	3.45	0.33
Flunitrazepam	3.81	0.33	3.79	0.36
Clobazam	4.61	0.46	4.59	0.42
Oxazepam	5.40	0.45	5.39	0.48
Tofisopam	7.41	0.67	7.38	0.65
Chlordiazepoxide	8.05	0.75	8.06	0.71
Dipotassic chlorazepate	9.32	0.78	9.27	0.82
Diazepam	11.75	1.10	11.71	1.05

* Conditions: flow rate, 1.71 mL/min; X_s, 0.488; analysis time, 12 min 20 s.

This paper adapts MORM to HPLC by replacing the development distance by the mobile phase velocity (or the flow rate, which could be more accessible to the operator). The triangular map was maintained because the three optimized parameters may be related in a single equation. Those parameters are the volume fraction of the organic modifier in the mobile phase, the flow rate (F), and the analysis time.

The method is based on the following principle: For each point of coordinates $\{X_S; F\}$ the resolution, R_S, of the least separated compounds is compared with a limit. A point is plotted at these coordinates if R_S is below this limit. The optimum is thus defined as the unplotted point of the minimal time coordinate.

The resolution is calculated from its formal definition:

$$R_S = \frac{2\,|\,t_{R2} - t_{R1}\,|}{w_{b2} + w_{b1}} \qquad \text{Eq 3}$$

where t_R is the retention time of the compound, w_b is the base width of the peak, and 1 and 2 are peak designations.

Retention times are calculated from the following equations:

$$t_R = t_0\,(k + 1) \qquad \text{Eq 4}$$

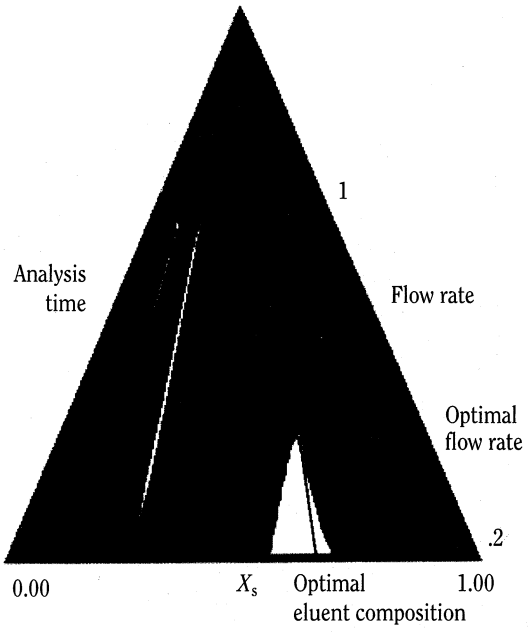

Figure 1. Screen print of the overlapping resolution map obtained for the optimization of the separation of seven herbicides. The white (or unplotted) zones correspond to coordinates (i.e., experimental conditions) leading to a satisfactory separation of all the compounds. In the shadowed (or plotted) zone, there is interference from at least two compounds. The optimal conditions are marked on the axis. They correspond to the coordinates of the point of the white zone, which have the lowest analysis time coordinates.

$$\ln k = a \times \ln X_S + b \qquad \text{Eq 5}$$

where k is the retention factor, t_0 is the dead time, and a and b are experimentally determined coefficients. The latter equation is derived from the work of Soczewinski and Golkiewicz (9) and was found suitable for the description of the behavior of most compounds in HPLC.

From Equations 2 and 3, the following is obtained:

$$t_R = t_0 (1 + e^{a (\ln X_S) + b}) \qquad \text{Eq 6}$$

Peak widths are calculated from the plate height, H:

$$H = \frac{L}{16}\left(\frac{w_b}{t_R}\right)^2 \qquad \text{Eq 7}$$

$$w_b = t_R\sqrt{\frac{16H}{L}} \qquad \text{Eq 8}$$

where L is the column length.

The Knox (10) equation is rearranged for the estimation of H as a function of the mobile phase velocity, u, the mobile phase composition, and compound behavior (through k)

$$H = Au^{1/3} + B'\left(\frac{1+k}{u}\right) + C'\left(\frac{k}{(1+k)^2}\right)u \qquad \text{Eq 9}$$

The variations in the diffusion coefficient were omitted on purpose, as their dependence on the mobile phase composition is still ill defined.

The analysis time, t_A, is the time required for the elution of the most retained peak (last); in other words,

$$t_A = t_{R(last)} + 1/2\, w_{b(last)} \qquad \text{Eq 10}$$

This ORM approach is superior to the original ...ion by Glajch and co-workers because optimization of the selectivity of the system (by varying the eluent composition) and the efficiency (by changing the eluent flow rate) is accomplished simultaneously. The drawback is that the method is limited to a binary eluent due to the bidimensional graphical representation and knowledge lattice of ternary eluent behavior.

Experimental

Apparatus

The experiments were carried out on a Varian chromatographic system (San Fernando, CA) equipped with an autosampler (Model 9095), a solvent delivery system (Model 9010), an ultraviolet–visible detector (Model 9050), and Varian LC Star Workstation software running on a compatible computer. Lichrospher 100 RP18 columns (Ref. 50943) were obtained from Merck (Darmstadt, Germany).

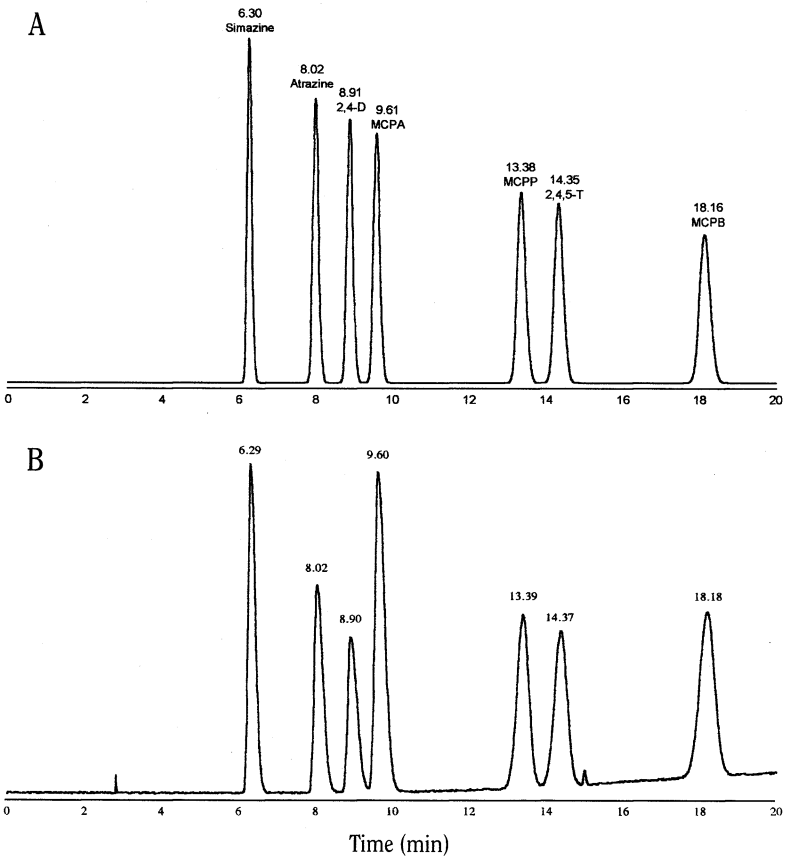

Figure 2. Chromatograms of the seven herbicides obtained under the following conditions: flow rate, 0.49 mL/min; X_S, 0.652. The top chromatogram (A) was calculated by the optimization software, and the bottom chromatogram (B) is a screen print of the experimental chromatogram.

Reagents and solutes

HPLC-grade methanol was obtained from Carlo Erba (Milan, Italy).

Sixteen test solutes were used to determine the capabilities of the program. Seven of the solutes were herbicides: atrazine (Cas No 1912-24-9); simazine (Cas No 122-34-9); MCPA or (4-chloro-*o*-tolyloxy) acetic acid (Cas No 94-74-6); MCPP or (±)-2-(4-chloro-2-methylphenoxy) propanoic acid (Cas No 7085-19-0); 2,4,5-T or (2,4,5-trichlorophenoxy) acetic acid (Cas No 93-76-5); MCPB or 4-(4-chloro-2-methylphenoxy) butanoic acid (Cas No 94-81-5); 2,4-D or (2,4-dichlorophenoxy) acetic acid (Cas No 94-75-7). All these compounds were purchased from Dr. Ehrenstorfen GmbH (Augsburg, Germany). Nine benzodiazepines were also used: bromazepam, nitrazepam, flunitrazepam, clobazam, oxazepam, tofisopam, chlordiazepoxide, dipotassic chlorazepate, and diazepam.

Preliminary experiments

Parameters a and b of each compound (Equation 3), and A, B', and C' (Equation 7) were determined by linear regression from preliminary experiments.

In order to reduce the number of preliminary experiments, the compounds were not injected as pure standards but split into mixtures of noninterfering solutes: mixture A, simazine, 2,4-D, 2,4,5-T, MCPB; mixture B, atrazine, MCPA, MCPP; mixture C, bromazepam, flunitrazepam, oxazepam, chlordiazepoxide, diazepam; mixture D, nitrazepam, clobazam, tofisopam, dipotassic chlorazepate.

These mixtures were injected using mobile phases of various methanol–water compositions at different flow rates. This reduced the number of preliminary experiments to four for both sets of compounds, as shown in Tables I and II.

Software

The optimization software was developed by one of the authors with Microsoft Visual Basic (Version 3.0 for Windows). It was designed to be as user friendly as possible; most of the operations were carried out by the computer (i.e., parameter calculations, chromatograms, and report editions), but all the chromatographic and plotting parameters were accessible to the user.

Experimental data were directly entered in a spreadsheet-like form as retention times and peak widths at various flow rates and eluent compositions. The program automatically extracted the parameters of Equation 6 (a and b for each compound) and Equation 9. The program calculated the resolution (Equation 3) using these parameters as a function of flow rate/composition couples and plotted the map. The results are presented as a theoretical chromatogram (see examples in Figures 2A and 2B) with the corresponding values for optimal conditions, expected retention times, and peak widths.

Results and Discussion

The results and the calculated parameters of the preliminary experiments are shown in Table I for the herbicides and in Table II for the benzodiazepines.

These results were processed by the opimization software (the ORM of the herbicide mixture is shown in Figure 1). The optimal conditions were tested on the mixtures. The calculated and experimental chromatograms of each mixture are compared in Figures 2 and 3. The results are shown in Tables III and IV.

The major differences between the calculated and experimental chromatograms are due to the fact that the software plotted Gaussian peaks of equal surfaces. The agreement in terms of retention times, peak widths, and separations is good, and the peaks are well separated on these optimal chromatograms.

Note that, in the case of the herbicides, the compounds were already adequately separated according to common criteria ($1 < k < 20$) in the second preliminary experiment (60% methanol at a flow rate of 0.5 mL/min) shown in Figure 4, but not in optimal conditions because the MORM optimum is one-third faster (18 min versus 30 min).

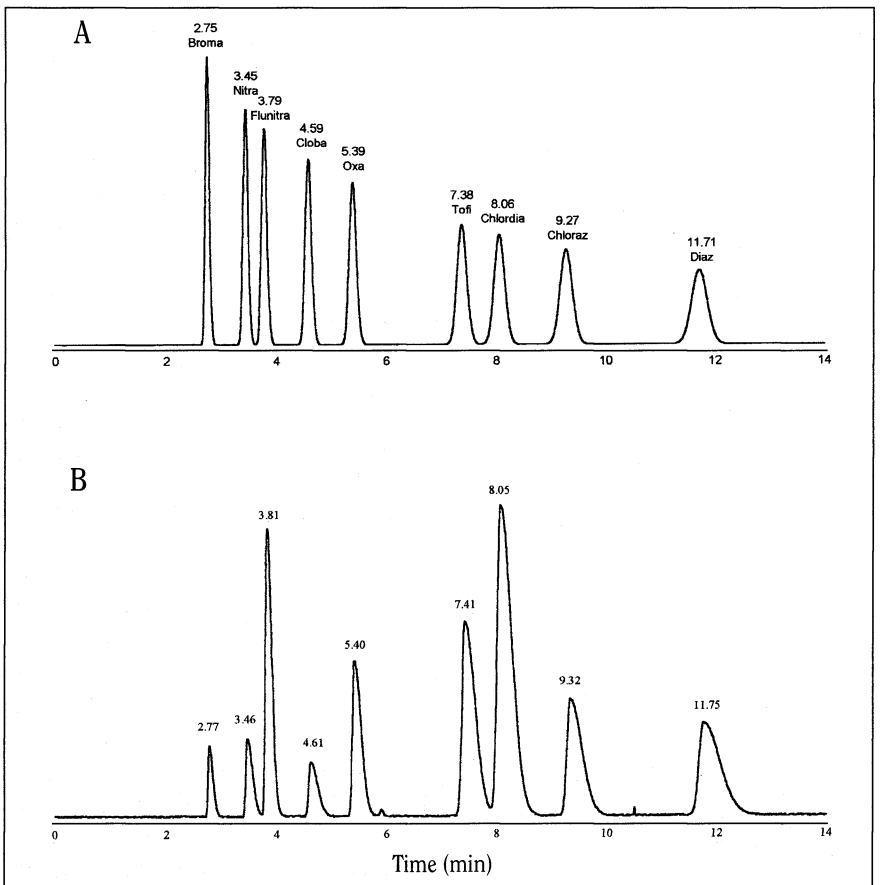

Figure 3. Chromatograms of the nine benzodiazepines obtained under the following conditions: flow rate, 1.71 mL/min; X_S, 0.488; analysis time, 12 min. The top chromatogram (A) was calculated by the optimization software, and the bottom chromatogram (B) is a screen print of the experimental chromatogram.

Conclusion

The optimization software proved to be an efficient tool for the optimization of separation and analysis time in HPLC. Further improvement could correct the nonlinear behavior of the compounds.

Appendix: the importance of accuracy

The ORM technique is based on the validity of the mathematical relationship between the experimental conditions and the compound behavior (t_R and w_b) and the precision of the parameters of this relationship.

In the case of the retention time, for ex-

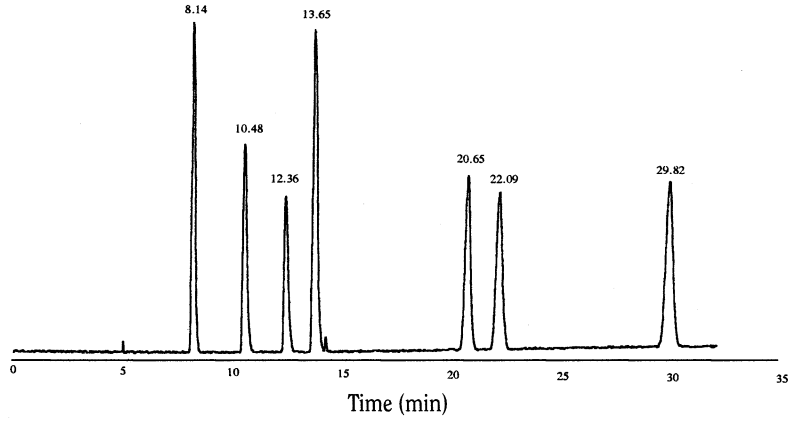

Figure 4. Screen print of a preliminary experimental chromatogram of the seven herbicides. It was obtained using the Varian LC Star Workstation software. Conditions: flow rate, 0.5 mL/min; X_S, 0.6.

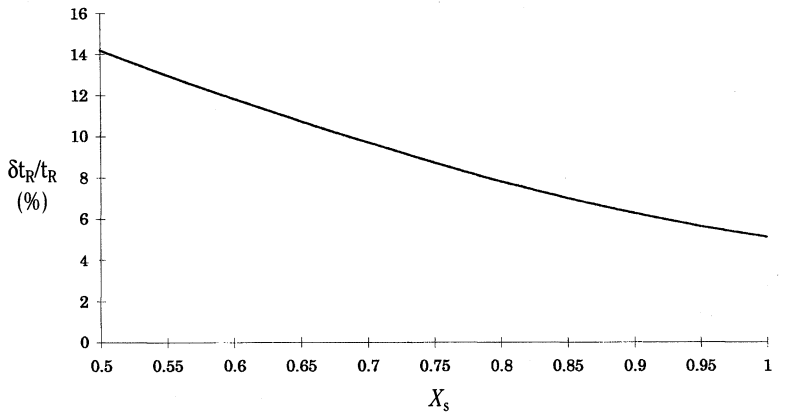

Figure 5. Plot of the percentage of error in the retention time of MCPA against X_S according to Equation 10. The following values were used: δX_S, 0.005; *a*, –4.87 ± 0.006; *b*, –0.34 ± 0.03; t_0, 0.70 ± 0.02.

ample, the determination coefficients of Equation 4 are generally above 0.999, which justifies the relationship. But errors in the determination of the parameters (*a*, *b*, or t_0) or in the conditions (X_S) lead to unrealistic forecasts:

Eq 11

$$\delta t_R = \left|\frac{\partial t_R}{\partial a} \times \delta a\right| + \left|\frac{\partial t_R}{\partial b} \times \delta b\right| + \left|\frac{\partial t_R}{\partial t_0} \times \delta t_0\right| + \left|\frac{\partial t_R}{\partial X_S} \times \delta X_S\right|$$

Eq 12

$$\delta t_R = e^{a \times \ln X_S + b} \times \left(\left|t_0 \times \ln X_S \times \delta a\right| + \left|t_0 \times \delta b\right| + \left|\frac{t_0 \times \partial}{X_S} \times \delta X_S\right| + \left|\delta t_0\right|\right) + \left|\delta t_0\right|$$

where δt_R is the error in the retention time.

Figure 5 shows the percentage of error that is obtained in the retention time if an error of ±0.5% of methanol is made in the mobile phase composition (the values of δa and δb correspond to a correlation coefficient of 0.9997 in Equation 4).

Therefore, experimental rigor is needed to limit this type of inaccuracy.

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