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SIMULTANEOUS OPTIMIZATION OF REAGENT CONCENTRATION AND pH IN REVERSED-PHASE ION-PAIRING CHROMATOGRAPHY

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SUMMARY

The procedure developed earlier by the authors for simultaneous two-parameter optimization in reversed-phase liquid chromatography has been adapted to ion-pair chromatography. From the many parameters controling reversed-phase ion-pair chromatography, the mobile phase concentration of the ion-pair reagent and the pH exert the largest effect on selectivity.

Initial chromatograms are chosen to cover the parameter space such that a good initial estimate of the optimum can be obtained. The true retention behaviour is approximated iteratively, and the optimum is located in a few additional chromatograms. The procedure can be followed through appropriate visualization of the results obtained in each step in the iterative procedure.

Two samples were subjected to the procedure, one containing only anions, the other containing cations, anions, and neutral molecules. The ion-pair reagents were sodium octylsulfonate and tetrabutylammonium bromide. A citrate buffer was used to control the pH between 2.5 and 6.

INTRODUCTION

The mobile phase composition in reversed-phase ion-pair (RP-IP) chromatography is often complex. In addition to the ion-pair reagent, buffers, neutral salts, and organic solvents can be used. The theoretical dependence of the capacity factor on the pK_a values of the solutes and the pH of the buffer, the counterion concentration, the mobile phase concentration of the ion-pair reagent, and the concentration of ion-pair reagent in the stationary phase has been studied in numerous papers by various authors[1–6]. Systematic studies on the influence of various parameters on the retention behaviour have been performed by Bartha and co-workers[7–11]. Such studies provide the key to systematic ion-pair optimization. In a previous study[12], we have described the application of an iterative regression procedure, developed by Drouen and co-workers[13–15], to the optimization of the ion-pair reagent concentration only. The present study describes an extension of the same procedure to the simultaneous optimization of two parameters. Alternative computer-aided procedures have been described by Goldberg *et al.*[16] and Lindberg *et al.*[17]. To select the two most appro-

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priate parameters, the influence of various parameters in ion-pair chromatography will be briefly reviewed.

The type of ion-pair reagent of the desired charge (either cation or anion) is, of course, sample-dependent and best selected on the basis of practical considerations, such as availability, solubility, purity, and stability. Hydrophobicity (chain length) seems important, but Bartha *et al.*[10] have shown that chain length is not a useful parameter to optimize. With increasing chain length of the ion-pair reagent less concentrated solutions are needed to reach the same coverage of the stationary phase. However, too long a chain makes it difficult to remove the ion-pair reagent from the column.

Studies by Bartha *et al.* and others[1–11] have shown that with increasing concentration of the ion-pair reagent, solute retention initially increases, but then levels off at a certain concentration, after which there is no further gain in selectivity.

The pH of the mobile phase directly influences the ionization of the solutes and of the ion-pair reagent and thus constitutes the second important parameter in ion-pair optimization. The choice of the buffer is dictated by its solubility in the mobile phase. Inorganic phosphate buffers have often been used for their wide pH ranges. However, organic buffers, like citric acid–citrate, are more soluble in mobile phases rich in organic solvents. Citric acid–citrate buffers have an even larger pH range than phosphate buffers.

The cations introduced with the buffer contribute to the total ionic strength of the mobile phase. A neutral salt can be added to keep the counter-ion concentration constant. Control of the counter-ion concentration results in the above-mentioned chain length independence and in a more linear relationship between log k and log P_m (the ion-pair concentration in the mobile phase)[9,10]. The salt is chosen for its solubility and non-corrosive properties.

In ion-pair chromatography, the addition of an organic modifier influences the overall retention in two ways: increased elution power towards the solutes as in ordinary reversed-phase chromatography and decreased adsorption of ion-pair reagent on the stationary phase[7,10]. Consequently, the concentration of organic solvent is a convenient parameter for controlling the retention of the last-eluted peak, but too high a solvent content must be avoided. The type of the organic solvent selected offers specificity as in reversed-phase liquid chromatography (LC)[18]. As a stationary phase, C_{18}-modified silica is preferred, because it accepts more ion-pair reagent per surface area than silicas modified with shorter chains. On the basis of these considerations it was decided to direct the simultaneous optimization of two parameters to the ion-pair reagent concentration and the pH of the mobile phase.

EXPERIMENTAL

Instrumentation

The liquid chromatograph consisted of a Waters (Millipore, Waters Chromatography Division, Milford, MA, U.S.A.) M6000A pump, a Rheodyne (Rheodyne, Cotati, CA, U.S.A.) 7125 sample injector with a 20-μl loop, a Waters RCM-100 radial compression unit, containing a Nova-Pak C_{18} column (10 cm × 8 mm from Waters) and a Waters M440 UV detector with a 254-nm filter.

The optimization program was developed in FORTRAN 77 on a Waters 880

data management system, equipped with 512 kbyte memory, a dual diskette drive (2 × 400 kbyte), integral 10-mbyte Winchester disk drive, extended bit-map graphics with colour monitor, a letter printer LA 100 (all from Digital, Maynard, MA, U.S.A.) and a Hewlett-Packard (Hewlett-Packard, San Diego, CA, U.S.A.) HP7470A graphics plotter.

Chemicals

Methanol was obtained from Rathburn Chemicals (Walkerburn, U.K.). Sodium bromide and citric acid monohydrate were "Baker Analyzed" reagents from J. T. Baker (Deventer, The Netherlands). Tetrabutylammonium bromide and anhydrous sodium octanesulphonate, were from Janssen Chimica (Beerse, Belgium). The solutes used were of the highest quality available.

Mobile phases

The composition of the five mobile phases used in the initial phase of the optimization of two different samples is presented in Table I. Between consecutive experiments the column was washed with a mobile phase containing the citrate buffer having the pH of the next experiment, but without the ion-pair reagent and the sodium bromide. The methanol concentration was increased (and decreased in the same way) by steps of 25% up to a maximum of 75%. Each step took 15 min. Finally, the column was equilibrated to the next mobile phase for at least 15 min. The total time needed to wash and equilibrate the column for the new mobile phase was 2 h. The detector base line was recorded to decide when washing and equilibration was completed. In all cases, the pH was adjusted to within 0.05 pH units in the aqueous solution. After that, methanol was added to the desired concentration.

TABLE I

PREPARATION OF BUFFERS WITH CONSTANT IONIC STRENGTH AND STARTING MOBILE PHASES

pH	*Citric acid (g/l)*	*NaOH (g/l)*	*NaBr (g/l)*
2.50	5.25	0.244	9.29
4.25	5.25	1.360	6.22
6.00	5.25	2.560	–

To prepare the five initial mobile phases, the following amounts of sodium octylsulphonate (Na-Oct) and NaBr were added to the buffers (mobile phase contains 5% methanol).

Mobile phase	*log P_m* (mmol)*	*Na-Oct (g/l)*	*NaBr (g/l)*	*log (P_m + 1)** (mmol)*	*TBA (g/l)*	*NaBr (g/l)*
pH = 2.50/6.00	0	0.216	7.10	0	0	0.926
pH = 4.25	0.923	1.812	6.34	0.5	0.696	0.704
pH = 2.50/6.00	1.845	15.140	–	1	2.902	–

* P_m = mobile phase concentration of ion-pair reagent.

** P_m + 1, to be able to take the zero ion-pair concentration.

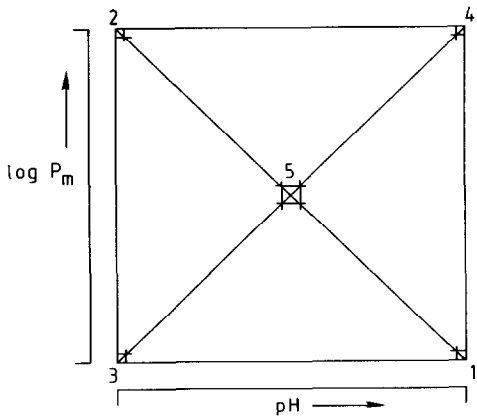

Fig. 1. Location of the five initial mobile phase compositions used to start the optimization procedure.

Software

Existing software for quaternary optimization in reversed-phase LC has been adapted in such a way that the parameter space can be calculated and presented over a square instead of a triangle[15]. Instead of calculating with 100 steps (1% resolution) over the range of each parameter, 30 steps (3.3%) were taken to reduce the computing time from 20 to 2 min. A further increase of the step size reduces the accuracy of the results while the gain in computing speed is marginal. The graphic presentation includes iso-response contour plots, pseudo-isometric 3D-plots of the response surfaces, and a simple indication of the data points and their confidence ranges in the parameter space.

RESULTS AND DISCUSSION

For an unambiguous description of the rectangular parameter space five initial mobile phase compositions were chosen as shown in Fig. 1 (ref. 19). In the present procedure the boundaries of the parameter space are set by the operator on the basis of previous experience. From the retention data of the corresponding chromatograms the complete retention behaviour and, hence, any desired optimization criterion can be calculated over the entire parameter space, and an optimum value can be predicted. In the next chromatogram the prediction is tested indirectly, because it has been shown previously that the use of a shifted mobile phase composition improves the efficiency of the procedure[14]. After each new data point, the calculation of the response surface of the optimization criterion is repeated, and the prediction of the optimum is updated. After a few such iterations the global optimum is found and the procedure stops[14].

It should be realized that the basis of the procedure is the calculation of retention surfaces. As in previous publications[15], the retention surface of a solute is calculated by linear interpolation between measured data points. For the present study, an approximately linear relationship can be safely assumed to exist between log k and log P_m[12], but the relationship between log k and pH will usually be S-shaped. As has been shown previously, however, such S-shapes can be readily de-

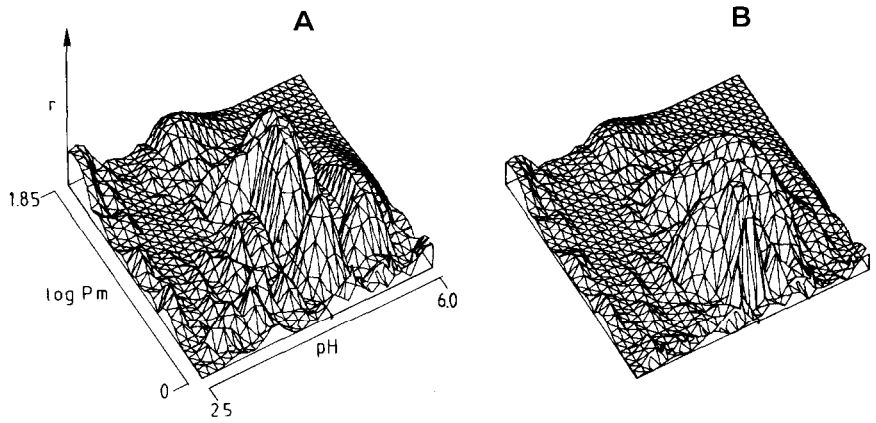

Fig. 2. (A) Representation in a 3D-plot of the criterion value (r) over the parameter space calculated from the five starting chromatograms of sample 1. (B) Representation of the final 3D-plot of the criterion value (r) for sample 1. A total of 10 chromatograms were used.

scribed by linear segments[19]. The procedure will now be illustrated by the optimization of two typical samples.

The first sample contained acidic, basic, and neutral solutes. The sodium octylsulphonate concentration ran from 1 to 70 mmol/l and the pH from 2.50 to 6.00. The optimization criterion used was the relative resolution product[13], which aims at an even spreading of the solutes over the chromatogram. Fig. 2A presents the 3D-plot, which shows that the predicted optimum is situated in an area with high values over a relatively broad range. This is desirable, as it is difficult to prepare the pH of the mobile phases with a precision better than 0.05 units. The program calculates the next mobile phase composition, and the procedure is repeated. Table II presents the composition of the successive, predicted "optimum" mobile phases used to refine the response surface. Obviously, the composition moves around a relatively small region,

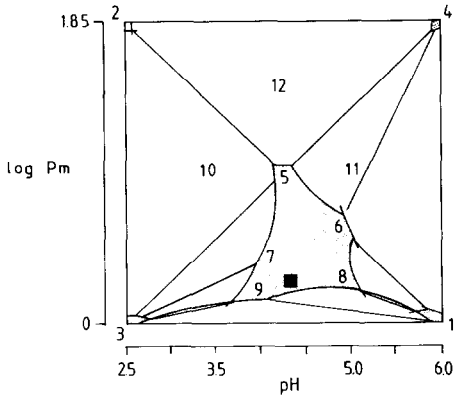

Fig. 3. Overview of the parameter space with indication where chromatograms were taken during the optimization procedure. ■ indicates the optimum. The shaded area represents the parameter space where a preset accuracy has been obtained.

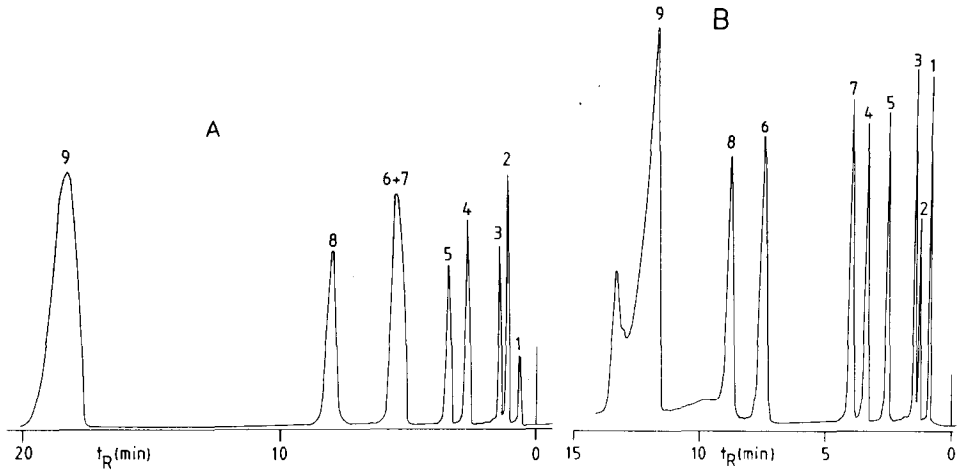

Fig. 4. (A) Chromatogram of sample 1, obtained with the first predicted optimum. Solutes: 1 = 2,4-dinitrobenzenesulphonic acid, 2 = L-DOPA, 3 = tyrosine, 4 = 3,4-dihydroxyphenylacetic acid, 5 = noradrenaline, 6 = 5-hydroxyindole-3-acetic acid, 7 = adrenaline, 8 = phenol, 9 = 3-hydroxytyramine. (B) Chromatogram obtained at the optimal mobile phase concentration (see Table II for conditions).

TABLE II

OPTIMIZATION OF SAMPLE 1

Ion-pair reagent is sodium octylsulphonate.

Chromatogram	*Composition*		*Criterion Rel. Res. Prod.**				
	pH	*log P_m*					
1	6.0	0	0.096				
2	2.5	1.85	0.132				
3	2.5	0	0.002				
4	6.0	1.85	0.000				
5	4.25	0.93	0.124				
	Predicted optimum			*Shifted composition*		*Criterion*	
	pH	*log P_m*	*Crit.*	*pH*	*log P_m*	*Predicted*	*Real*
6	4.46	0.73	0.457	4.87	0.60	0.004	0.382
7	4.69	0.49	0.543	4.13	0.41	0.381	0.444
8	4.23	0.37	0.663	4.81	0.26	0.367	0.198
9	4.23	0.31	0.720	3.91	0.16	0.127	0.098
Opt	4.35	0.24	0.687				
Real optimum	4.35	0.24	0.372				

* Relative resolution product (see ref. 14).

as illustrated in Fig. 3, and the procedure comes to a halt after four more chromatograms. As is clear from Fig. 3, a large area of the parameter space remains unsearched, but a few additional chromatograms taken in "open" areas (data points 10, 11 and 12 in Fig. 3) did not change the location of the global optimum. The final 3D-plot is presented in Fig. 2B. The gradual improvement of the description of the retention surfaces can be judged from a comparison between the criterion values predicted and measured for the shifted composition (the "optimum" composition is not measured until the procedure is completed). The initially poor agreement is already greatly improved in the next iteration and remains acceptable in the following steps.

Fig. 4 presents two chromatograms. The one in Fig. 4A resulted after the first predicted optimum and was taken against the advice of the program, which considered the information at this point insufficient. Indeed, solutes 6 and 7 were poorly resolved. The second chromatogram in Fig. 4B is the final result after four more iterations. The complexity of the separation can be judged from the many peak reversals. Clearly, the solute peaks are more evenly spread and better separated. Indeed, the criterion value has improved from 0.02 to 0.37. Also, the analysis time is reduced from 19 min to 14 min. However, this is unintentional, because the criterion does not aim for minimum analysis time. As can be seen from Table II, the criterion value observed in the final chromatogram of Fig. 4B ($r = 0.37$) is significantly lower than the value of 0.697 predicted by the procedure after run Opt (indicated in Table II). The reason is that the product of resolution of adjacent peak pairs is very sensitive to minor shifts in the position of ill-resolved peaks, *e.g.*, solutes 2 and 3 in Fig. 4B.

The second sample contained twelve acidic and neutral compounds. Tetrabutylammonium bromide was chosen as the ion-pair reagent. The concentration ran from 0 to 9 mmol/l, and the pH from 2.5 to 6.0. These boundaries were again chosen from chromatographic experience. To demonstrate the versatility of the procedure, the optimization criterion in this example was the minimal resolution of the critical peak pair (R_s min.).

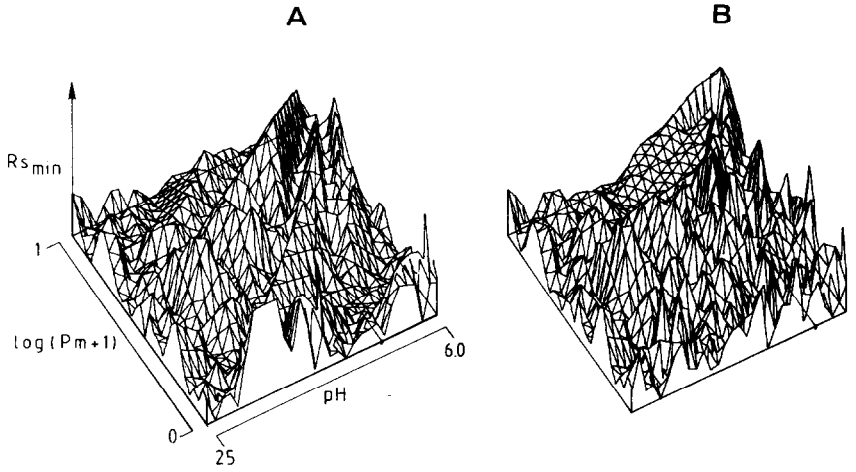

Fig. 5. Representation in a 3D-plot of the criterion value ($R_{s\,min}$) over the parameter space calculated (A) after five starting chromatograms and (B) at the optimum for sample 2.

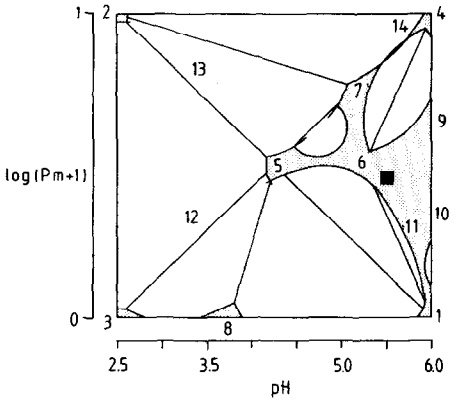

Fig. 6. Overview of measured chromatograms for sample 2 (see also Fig. 3). ■ indicates the optimum. The shaded area represents the confidence range.

The response surfaced calculated from the five initial chromatograms (Fig. 5A) predicted a highest value for $R_{s\ min}$ of 1.4, but due to many cross-overs of solute retention the surface was highly irregular and showed many secondary maxima of potentially similar importance. Probably, the surface was poorly defined at this early stage of the procedure, and the prediction should be judged with caution. Still, even for this complex sample only five additional chromatograms were needed for the

TABLE III

OPTIMIZATION OF SAMPLE 2

Ion-pair reagent is tetrabutylammonium bromide.

Chromatogram	*Composition*		*Criterion* $R_{s\ min}$				
	pH	*log* $(P_m + 1)$					
1	6.0	0	0.4				
2	2.5	1	0.6				
3	2.5	0	0.3				
4	6.0	1	0.1				
5	4.25	0.5	0.0				
	Predicted optimum			*Shifted composition*		*Criterion*	
	pH	*log* (P_m+1)	*Crit.*	*pH*	*log* $(P_m + 1)$	*Predicted*	*Real*
6	5.16	0.50	1.4	5.23	0.5	0.9	0.5
7	5.27	0.76	1.4	5.16	0.76	1.2	0.4
8	3.42	0.0	1.2	3.75	0.00	0.3	0.0
9	5.97	0.73	1.2	6.00	0.64	0.6	0.2
10	5.85	0.40	1.3	6.00	0.35	0.1	0.2
11	5.85	0.30	1.3	5.74	0.28	0.8	0.9
Opt	5.50	0.46	1.2				
Real optimum	5.50	0.46	0.9				

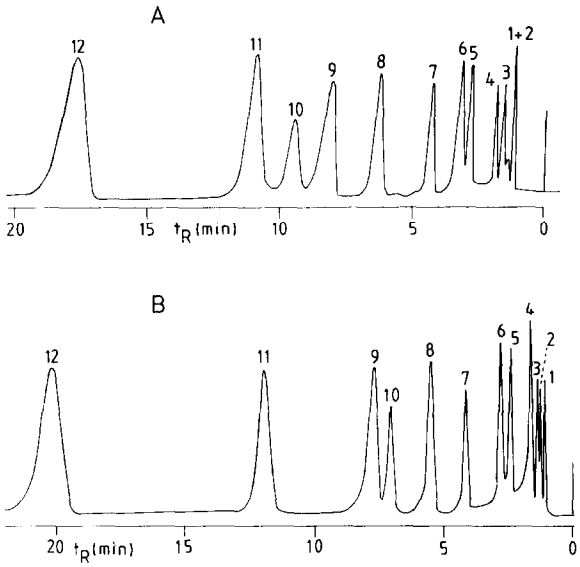

Fig. 7. Chromatograms of sample 2. (A) First optimum after five initial runs. Solutes: 1 = 3,4,5-trihydroxybenzoic acid, 2 = phenylalanine, 3 = *p*-aminobenzoic acid, 4 = 3,4-dihydroxybenzoic acid, 5 = *p*-hydroxybenzoic acid, 6 = 4-hydroxy-3-methoxybenzoic acid, 7 = *m*-hydroxybenzoic acid,8 = 3,4-dihydroxycinnamic acid, 9 = benzoic acid, 10 = phthalic acid, 11 = chlorogenic acid, 12 = DL-cathechine dihydrate. (B) Final chromatogram at the optimal mobile phase composition.

procedure to come to a halt. The successive mobile phase compositions are presented in Table III and their location in the parameter space in Fig. 6. In comparison to Fig. 3, a somewhat broader range of mobile phase compositions is covered, and the jump from composition 7 to composition 8 indicates the presence of an important secondary maximum. Nevertheless, the advice of the procedure to stop after data point 11 was correct, because additional chromatograms taken at compositions 12–14 did not change the final optimum. Fig. 5B presents the final 3D-plot, and Fig. 7 shows chromatograms taken for the first optimum (Fig. 7A) composition predicted on the basis of the five initial chromatograms ($R_{s\ min}$ equal to 0.5) and for the global optimum taken after six additional chromatograms ($R_{s\ min}$ equal to 0.90) (Fig. 7B). All twelve solutes in the sample are well separated.

CONCLUSIONS

The feasibility of simultaneous optimization of two parameters with the iterative regression design has been clearly demonstrated. The efficiency of the procedure, requiring only about ten chromatograms, is important in ion-pair optimization, because a fair amount of time is needed to change from one mobile phase composition to the next one. Because it is difficult to fine-tune the pH of the mobile phase, optimization criteria that produce response surfaces with very sharp maxima can be better avoided. In the present study, the ion-pair reagent concentration and the pH were selected as the most promising optimization parameters. However, organic solvent concentration could also have been chosen[13,18] and the type of solvent is ex-

pected to yield the same specificity as in ordinary RPLC. Computer calculation time has been brought back to acceptable proportions, certainly for a two-parameter optimization. In this study, the boundaries of the parameter space were selected from chromatographic experience. Efforts are now underway to derive the boundaries from well-planned preliminary experiments.

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