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REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION OF SACCHARIN, CAFFEINE AND BENZOIC ACID USING NON-LINEAR PROGRAMMING

J. W. WEYLAND*, H. ROLINK and D. A. DOORNBOS

Research Group Optimization, Laboratory for Pharmaceutical and Analytical Chemistry, State University, A. Deusinglaan 2, 9713 AW Groningen (The Netherlands)

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SUMMARY

A method is described which allows a chromatographic assay to be optimized with respect to the time needed to complete an analysis, while maintaining good resolution. The method introduces an operational research technique called non-linear programming. It was used to optimize the eluent composition for the assay of saccharin, caffeine and benzoic acid and resulted in a significant reduction in the analysis time.

INTRODUCTION

In routine chromatography, analysis time is an important optimization criterion, especially for automated equipment, requiring little or no operator action, as any savings in time will lower the cost per analysis by an equivalent amount. As most published high-performance liquid chromatographic (HPLC) determinations are probably not optimized with respect to either separation quality or time, it may be expected that many of these methods can be improved considerably. This study demonstrates this, using a non-linear programming technique to optimize the time needed to complete the separation of saccharin, caffeine and benzoic acid.

THEORETICAL

Once the column (with a particular stationary phase) has been chosen, the main variable influencing separation is the composition of the mobile phase. This variable also influences the time needed for an analysis, indicating an interaction between the quality of the separation and the analysis time. Retention of a solute depends on its capacity factor, which in turn depends on the "polarity" of the mobile phase.

Polarity has been defined in various ways[1], but can be understood as summarizing the ability of the solvent to interact with the solute. To quantify "polarity" several scales have been proposed, *e.g.*, Snyder's polarity parameter P' (ref. 2), based

on the Rohrschneider data set, and the Hildebrand solubility parameter[1,3,4]. Both scales hide as much as they reveal, as interactions between solute and solvent are combinations of several different types of interactions: dispersion, orientation, induction and acid–base. In solubility theory, the resultant of these interactions is called the solubility parameter, δ (Snyder[3] described a slightly different scheme, subdividing P' into different intramolecular contributions, x_i).

Schoenmakers[4,5] developed the solubility parameter model to account for the different types of interactions. In their model, each type of interaction is assigned its own solubility parameter. They derived the following equation for the separation factor of two solutes, j and i:

$$\frac{RT}{2v} \cdot \ln \alpha_{j,i} = (\bar{x}_i - \bar{x}_j)\bar{y}$$

where R is the gas constant, T is temperature, v is the molar volume of solutes ($v_i = v_j$ assumed) and $\bar{x}$ and $\bar{y}$ are vectors representing the partial solubility parameters for the solutes and a given mobile–stationary phase system:

$x_1 = \delta_d$, solubility parameter for dispersion $\qquad y_1 = (\delta_{d,m} - \delta_{ind,m}) - (\delta_{d,s} - \delta_{ind,s})$
$x_2 = \delta_o$, solubility parameter for orientation $\qquad y_2 = \delta_{o,m} - \delta_{o,s}$
$x_3 = \delta_{ind}$, solubility parameter for induction $\qquad y_3 = \delta_{d,m} - \delta_{d,s}$
$x_4 = \delta_a$, solubility parameter for acid–base interaction $\qquad y_4 = \delta_{b,m} - \delta_{b,s}$
$x_5 = \delta_b$, solubility parameter for base–acid interaction $\qquad y_5 = \delta_{a,m} - \delta_{a,s}$

where m = mobile phase and s = stationary phase.

Partial differentiation with respect to the different types of interactions yields

$$\frac{RT}{2v} \cdot \frac{1}{\alpha_{j,1}} \cdot \frac{\partial \alpha_{j,i}}{\partial y_n} = x_{n,i} - x_{n,j}$$

where n indicates the type of interaction[5]. This equation shows that the relative change in $\alpha_{j,i}$ resulting from a change in the nth type of interaction is directly proportional to the difference in the nth parameter of the two solutes. This offers the key to exploiting the differences between the solutes: find a mobile phase that maximizes α. In real chromatograms, where there are more peaks to separate, find a mobile phase in which the y-vector is such that it gives an optimum separation for all the peaks. This can be attained more or less by mixing solvents that differ in their "interaction patterns", or stated differently, that lie apart as much as possible in the five-dimensional vector space of the interactions.

Practically stable mixtures in reversed-phase (RP) HPLC can only be made of water, methanol, acetonitrile and tetrahydrofuran. In this study, a mixture of water, methanol and acetonitrile was chosen, largely from cost considerations. It proved adequate, however, for the purpose intended.

EXPERIMENTAL

As the purpose of this study was to optimize analysis time, while maintaining the overall quality of separation, *i.e.*, baseline resolution for all peaks or 99.8% resolution for all peaks, it is natural to constrain the variables to an area in which the desired separation is met, the feasible region, and then find within this area the minimum analysis time.

This indicates the use of an experimental design that allows the mapping of both the optimizing criterion and the resolutions between peaks at the same time, with only one set of experiments. For ternary mixtures a suitable design is the simplex lattice design, as described by Gorman and Hinman[6] or Snee[7]. In chromatography, this experimental design was used by Belinky[8] and Glajch *et al.*[9]. Belinky improved the resolutions between pairs of polynuclear aromatic hydrocarbons at near constant capacity factor, while Glajch *et al.* mapped the resolutions between all pairs of peaks in order to locate a region in the factor space comprised by a (pseudo-)quaternary mobile phase where every resolution meets a predetermined value. Glajch *et al.* did not try to minimize analysis time. Moreover, in their approach this would not be possible, as the water content of the mobile phase (the variable that influences the elution time the most) is not a free variable in their (pseudo-)quaternary system.

A simplex lattice design generally consists of a uniform distribution of points over all possible mixtures. At these points the responses are measured (Fig. 1). Response is then represented by polynomials fitted by least squares to the responses, which serve to map the response surfaces.

Because in mixtures the sum of fractions must add up to unity, it is possible to simplify the polynomials compared with those normally used. A full quadratic can be reduced using the constraint $x_1 + x_2 + x_3 = 1$ to the following relationship:

$$\eta = \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_{12} x_1 x_2 + \beta_{13} x_1 x_3 + \beta_{23} x_2 x_3$$

The minimum number of experiments necessary to obtain estimates of the regression parameters is seen to be six, as opposed to nine for a full quadratic polynomial. For higher order equations a similar reduction in the number of experiments is possible. Of course, this reduction is possible because the actual factor space is two-dimensional; the variables are interdependent.

As indicated before, a quadratic simplex lattice design consists of a minimum of six experimental points (Fig. 1). A few extra points should be measured in order to evaluate the goodness of fit of the model.

It is impossible to explore all the accessible factor space in the ternary mixtures used in RP-HPLC (mixtures of water, methanol, tetrahydrofuran and acetonitrile). Experiments with pure water exhibit extremely long elution times, while pure organic solvents will elute most peaks together with the solvent front. The region of interest is thus restricted to a small region of the available factor space.

Depending on the quality and analysis time of the initial chromatogram, a simplex lattice design is located around the original factor point, or a suitable region is located, whereafter the simplex lattice design is set up in this region.
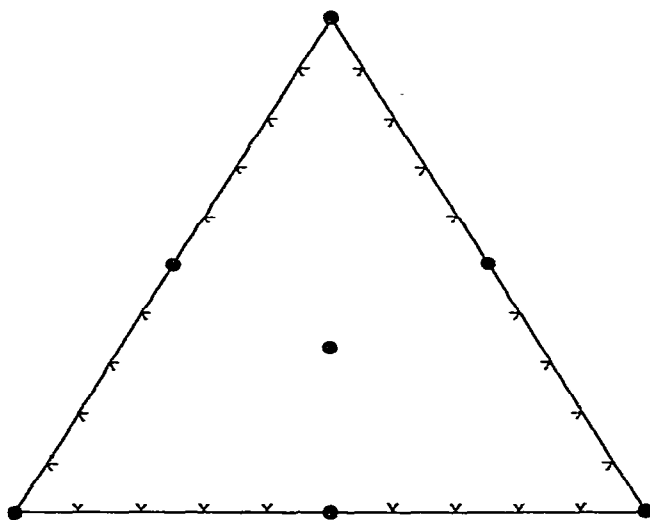
Fig. 1. Quadratic simplex lattice design, central point added.

Separation of sacharine, caffeine and benzoic acid in beverages

For every measured solvent composition the analysis time and the resolutions between all pairs of adjacent peaks were calculated. Analysis time was defined for the purpose of this study as the time it took to elute 99.8 % of the last peak, *i.e.*, time was measured at three times the width at 0.6 height after the top of the last peak. Resolutions were calculated from

$$R_{1,2} = \frac{2(t_2 - t_1)}{(w_1 + w_2)}$$

where w = peak width at the base.

Chemicals

For the mobile phase mixtures of deionized water and analytical-reagent grade methanol and acetonitrile (Merck, Darmstadt, G.F.R.) (degassed under vacuum, using an ultrasonic bath) were used, with 1 % of analytical-reagent grade acetic acid (Merck) added in order to convert the acids to their non-ionized forms. Standard solutions of saccharin (1.0 mg/ml), caffeine (1.0 mg/ml) and sodium benzoate (1.0 mg/ml) and a mixture containing 100 mg of these compounds in 100 ml were prepared using methanol as solvent. Standards were of European Pharmacopoeia quality and were used as received.

Instrumentation

The HPLC apparatus was constructed from a Milton-Roy pump and a Chromatronics 220 dual-channel UV absorbance detector (fixed wavelength, 254 nm). The column used was 25 cm × 4.6 mm I.D. stainless steel, packed with Li-Chrosorb RP-8, mean particle diameter 10 μm. Injections were made using a Valco 7000 p.s.i. injection valve, fitted with a 10-μl sample loop.

RESULTS AND DISCUSSION

The method for the determination of saccharin, caffeine and benzoic acid as described by Smiley et al.[10] gives impractically long retention times. The method uses a 6% acetic acid solution in water as the mobile phase. It was decided to use a simple simplex method[11], with analysis time as optimizing criterion, to locate a suitable region in which to situate the lattice design. Obviously the simplex tended to climb to higher organic solvent concentrations (Fig. 2), but the experiments involved at this stage gave valuable information over an extended region of the factor space. From these experiments it could be concluded that the resolution between both pairs of adjacent peaks approached 1.00 at approximately 60% of organic solvent. As the simplex favoured acetonitrile for shorter analysis times, a lattice design was located as indicated in Fig. 2. The results are given in Table I, and were used to calculate via polynomial regression the following polynomial equations:

$$T = -5.0013x_1 + 16.152x_2 + 8.802x_3 + 4.7564x_1x_2 - 2.7436x_1x_3 - 37.9756x_2x_3 \tag{1}$$

$$R_{12} = -11.1545x_1 + 2.7164x_2 + 0.9755x_3 + 18.5754x_1x_2 - 11.1676x_1x_3 - 2.5528x_2x_3 \tag{2}$$

$$R_{23} = -0.3792x_1 + 8.808x_2 + 2.4705x_3 - 2.9774x_1x_2 - 2.5573x_1x_3 - 17.1378x_2x_3 \tag{3}$$

where x_1 = volume fraction of methanol, x_2 = volume fraction of water, x_3 = volume fraction of acetonitrile and T = analysis time; for R_{12} and R_{23}, see Table I.

These expressions were used to calculate and plot the response surfaces in Fig. 3, where the constraint boundaries are also drawn in. It is possible to determine the
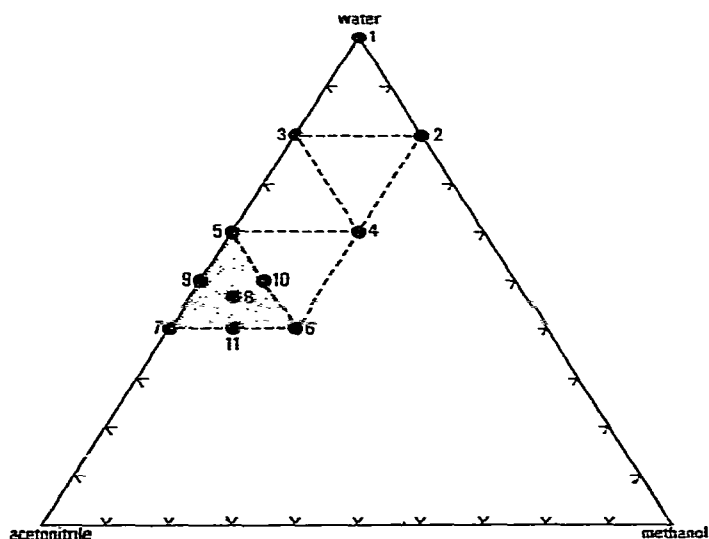

Fig. 2. Progress of simplex algorithms during optimization. Shaded area is the region where the simplex lattice design was located (points 5–11 from Table I).

TABLE I

ANALYSIS TIME AND RESOLUTIONS MEASURED BETWEEN SACCHARIN AND CAFFEINE
(R_{12}) AND CAFFEINE AND BENZOIC ACID (R_{23}) (SEE ALSO FIG. 2)

Experiment No.	Volume fraction			R_{12}	R_{23}	T (min)
	Water	Methanol	Acetonitrile			
1	1.00	0.00	0.00	>2	>2	>30
2	0.80	0.20	0.00	>2	>2	>20
3	0.80	0.00	0.20	1.96	4.80	10.13
4	0.60	0.20	0.20	2.00	3.19	6.50
5	0.60	0.00	0.40	1.36	1.82	4.07
6	0.40	0.20	0.40	1.19	1.24	3.06
7	0.40	0.00	0.60	0.94	0.88	2.63
8	0.466	0.066	0.466	1.47	1.27	3.08
9	0.50	0.00	0.50	1.33	1.40	3.00
10	0.50	0.10	0.40	1.29	1.70	3.66
11	0.40	0.10	0.50	1.23	1.02	2.85

optimum analysis time graphically from Fig. 3. Suppose one requires a minimum resolution of 1.25, corresponding to 99.8 % resolution of the peaks in the chromatogram. From the countour lines of the analysis time, one sees that increasing the amount of organic solvent in the mobile phase decreases the analysis time. However, compositions beneath the isocontour lines that indicate resolutions of 1.25 are not allowed (shaded area). It can easily be seen that the minimum analysis time, while maintaining a resolution of 1.25, is the intersection of the two constraints. From this the optimum composition is seen to be water–methanol–acetonitrile = 0.47:0.03:0.50, where the analysis time can be calculated from eqn. 1, its value being 2.94 min.

If a minimum resolution of 1.5 is required, the graphical approach is less suitable. The constraint will be met by the resolution between saccharin and caffeine becoming 1.5, the resolution between caffeine and benzoic acid still being far better than 1.5, and of no concern. The optimum can be located by finding the smallest distance between the constraint line and the next lower contour line of the analysis time. This is a tedious procedure, however, and it is possible to calculate the optimum from eqns. 1, 2 and 3 mathematically. Calculation of the optimum is also recommended when the number of peaks is so great that the contour plots become difficult to read. Once the models 1, 2 and 3 have been calculated, the problem of finding the minimum analysis time while maintaining a resolution of at least 1.5 can be stated as follows: Minimize

$$T = \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_{12} x_1 x_2 + \beta_{13} x_1 x_3 + \beta_{23} x_2 x_3$$

subject to the following constraints:

$$x_1 + x_2 + x_3 = 1$$
$$x_1 \geqslant 0$$
$$x_2 \geqslant 0$$
$$x_3 \geqslant 0$$
$$R_{ij} \geqslant \alpha_{1,ji} x_1 + \alpha_{2ji} x_2 + \alpha_{3,ji} x_3 + \alpha_{12,ji} x_1 x_2 + \alpha_{13,ji} x_1 x_3 + \alpha_{23,ji} x_2 x_3$$
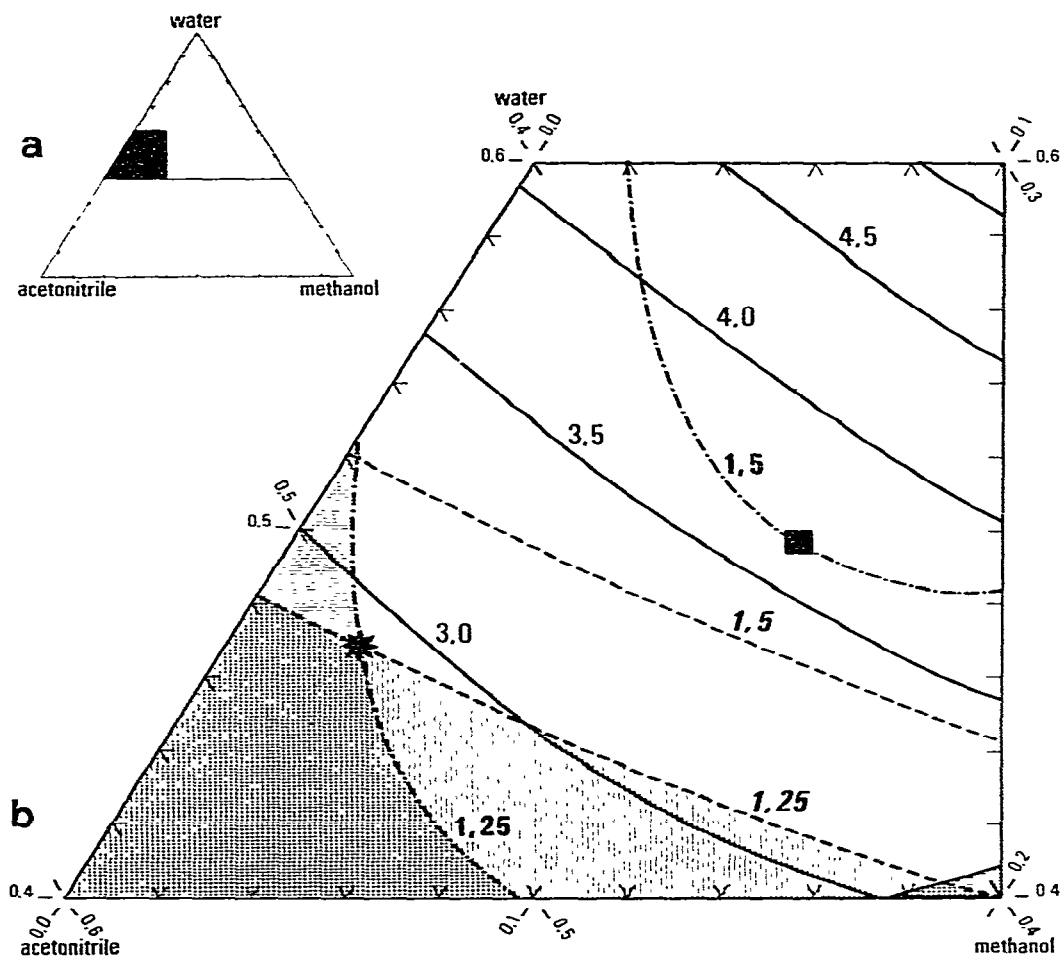
Fig. 3. (a) Accessible part of the factor space (shaded region) and part of the factor space shown in (b) (black region). (b) Contour plots over the region of the factor space indicated in (a): (————) analysis time: (-----------) resolution between saccharin and caffeine: (————) resolution between caffeine and benzoic acid). The shaded areas are outside the constraints for resolution 1.25. Optimum compositions for minimum resolution 1.25 denoted by ★. and for 1.5 by ■.

for $i = 1, n$ and $j = i + 1, n$ (R_{ji} = resolutions between each pair of peaks).

In our specific case there are only two resolutions to be considered, because in the region in which we are interested the elution sequence does not change. It is necessary to take into account only the separation between adjacent peaks, and not between peaks that have no overlap in the factor region.

The solution of this problem, minimize a non-linear objective function, subject to non-linear inequality constraints, is possible by an operations research technique called non-linear programming. An explanation of this technique is beyond the scope of this paper, but any good operational research textbook will provide a thorough discussion of the method[12]. The calculated solution to the stated non-linear programming problem is given in Table II for constraints of 1.25 and 1.5 on the resolution (R).

TABLE II

COMPARISON OF EXPERIMENTAL RESULTS WITH PREDICTIONS FROM THE MODEL AT CALCULATED OPTIMUM ELUENT COMPOSITIONS FOR $R_m > 1.25$ AND $R_m > 1.5$

Calculated optimum composition			Calculated time (min)	Measured time (min)	$R_{1,2}$ constraint	$R_{1,2}$ measured	$R_{2,3}$ constraint	$R_{2,3}$ measured
Water	Methanol	Acetonitrile						
0.470	0.027	0.503	2.93	2.94	1.25	1.18	1.25	1.29
0.496	0.108	0.396	3.63	3.23	1.50	1.42	1.50	1.80

The calculated optimum is found to be in good agreement with the optimum obtained graphically. Also summarized in Table II are the results of experiments carried out at the optimum composition. These results confirm the calculated predictions from the model with regard to analysis time and resolution well within experimental error; Fig. 4 shows chromatograms recorded during the progress of the simplex and at optimum composition. It appears that the method described is suitable for practical optimizations in chromatography. A final remark should be made. It is possible to substitute
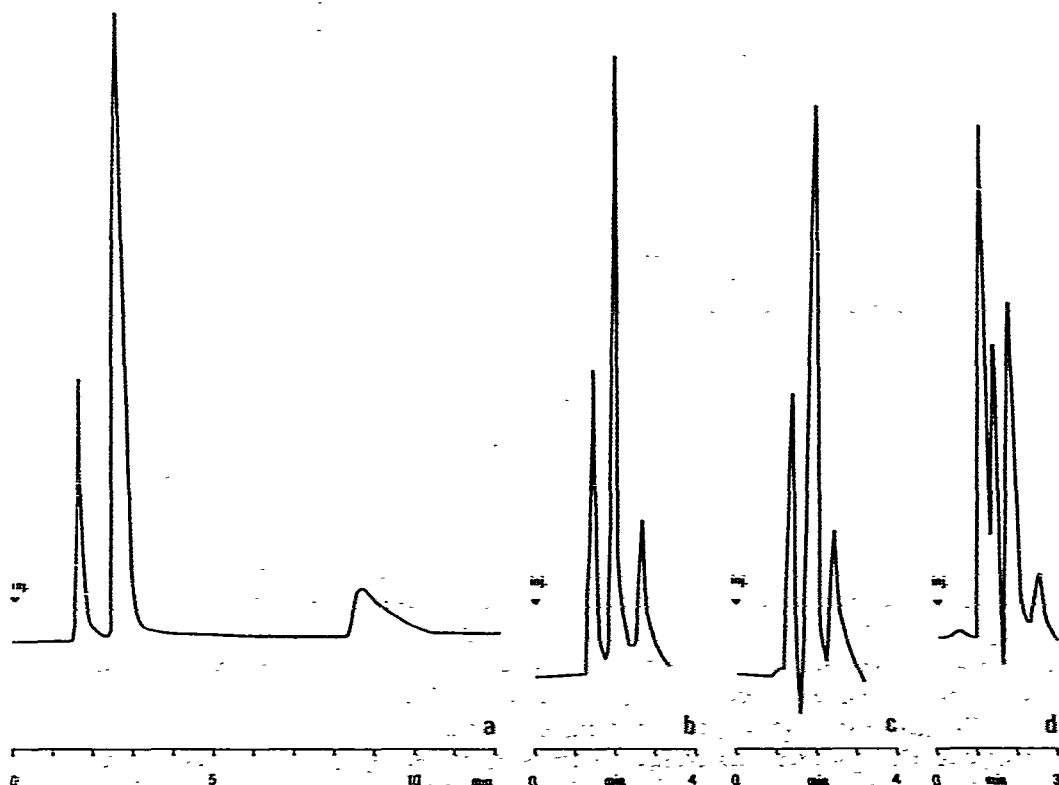

Fig. 4. Chromatograms of the standard mixture recorded during the optimization (a,b) and at optimum composition for $R = 1.25$ (c). Elution sequence: saccharin, caffeine, benzoic acid. (d) Chromatogram of dietary Cola at optimum composition. Eluent composition: water–methanol–acetonitrile = (a) 0.50:0.00:0.20, (b) 0.466:0.066:0.466, (c) and (d) 0.47:0.03:0.50 (optimum for $R = 1.25$).

cost per analysis for analysis time as the optimizing criterion, in which case probably a different optimum would have been found, trading off time against eluent cost.

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