AUTOMATION OF THE OPTIMIZATION OF THE SEPARATION OF PROTAMINES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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An automatic system for optimizing the conditions for the chromatographic separation of protamines has been developed using Nelder and Mead's modified simplex method. It is proposed to use penalty functions in order to take into account nonrigid constraints on the parameters to be optimized. The optimum conditions for the separation of the protamines from sturgeon gonads have been determined. It has been established that the greatest influence on the separation of the protamines is exerted by the concentration of the ion-pair reagent – trifluoroacetic acid. The system described permits the optimization of any chromatographic separations and the determinations to be performed thanks to the application of penalty functions to the optimization parameter.

High-performance liquid chromatography (HPLC) has come into wide use in the separation and analysis of proteins. We have previously reported the successful separation with the aid of reverse-phase HPLC on a Zorbax ODS column of the protamines from sturgeon gonads [1]. In this work we came up against a number of difficulties connected with the complexity of the chromatographic behavior of the protamines, and also the unreproducibility of the optimum conditions of separation becuase of certain differences in the characteristics of sorbents of one and the same type on passing from one column to another [2]. In view of this, it appeared to us to be desirable to use the optimization of the separation of these proteins with the aid of a computer.

The optimization of a process can be performed most effectively from its model. But the existing theory of the HPLC method on the whole is inadequate for the construction of reliable models based on physicochemical laws. Regression models proved to be polynomials of such a high order that they demand too great a volume of experimental material for their identification. Analysis of literature information has shown that to optimize the conditions for the chromatographic separation of components the successive simplex method (the method of a deformable polyhedron [4, 5]) is the most convenient and reliable.

We adopted the solution of using the sequential simplex procedure in the variant of Nelder and Mead [6] – one of the most effective methods in problems of nonlinear optimization. Characteristic for this method is fairly rapid convergence to the optimum, low sensitivity to the dimensions of the factor space, a relatively small number of returns to the target function, an acceleration of the search remote from the optimum (which leads to a considerable shortening of the number of experiments), and a high accuracy of the localization of the optimum. Not unimportant is the simplicity of the Nelder–Mead algorithm – it can readily be realized on a minicomputer in view of the inconsiderable demands on operative memory.

According to the Nelder–Mead method, after the reflection of the worst vertex of the polyhedron an attempt is made to expand or contract the simplex in the direction of improving the target function; in this process the simplex is deformed. Such deformation, on the one hand, ensures through expansion a marked acceleration of the search in a region remote from the optimum, and through contraction a more accurate localization of the optimum. We took constraints on the intervals of variation of the factors into account in the following way: if any point proved to be outside the region of permissible values even with respect to a single factor, it was returned to the disturbed face. In this way, we eliminated the main disadvantage of the methods of taking constraints into account [7, 8] – the difficulty in the search for an optimum located in the immediate vicinity to the RPV face or in the actual face of the region.

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The starting simplex was constructed in accordance with known recommendations [9]: the initial size of an edge was selected as 0.1-0.2 of the interval of change of the factor; so far as possible the ranges of change of the factors are scaled. The simplex was located in that region of the factor space relative to which it was known from preliminary experiments that a fairly good level of chromatography was ensured in it.

The list of factors affecting to any appreciable extent the process of chromatography [3] includes the parameters of the column (size of the sorbent particles, length of the column, activity of the sorbent, etc.) and also the rate of flow of the mobile phase, the temperature of the column, the composition of the mobile phase, changed by a given law, and the concentration of an ion-pair reagent.

On the basis of preliminary experiments, to separate the protamines we selected a sorbent based on silica gel modified with octadecylsilane (Zorbax ODS) and therefore the parameters of the column were excluded from the list of factors, and the temperature of the column, the amount of ion-pair reagent (in our case, trifluoroacetic acid (TFA) [1]), and the rate of change of the concentration of ethanol in the aqueous methanolic mobile phase were subjected to optimization. The region of permissible values and the initial level of the factors to be optimized are given below.

Factor	Range of permissible values	Initial level
Rate of increase in the concentration of ethanol, % per minute	0.2-2	0.3
Column temperature, °C	20-45	25
Concentration of trifluoroacetic acid, %	0-0.5	0.1

In view of the only slight influence of the rate of flow of the mobile phase on separation [10], its value was fixed at the level of 1 ml per minute.

The start of the simplex was made approximately in the middle of the region of permissible values of the factors, with some shift in the direction of their smaller values because of the specific nature of the construction of the initial polyhedron in space.

To estimate the quality of separation, in analogy with [4], we selected a chromatographic response function (CRF) in the following form:

$$CRF = \sum_{i=1}^{n-1} \min\left(2, \frac{2(t_{i+1} - t_i)}{w_{i+1} + w_i}\right) - n, \qquad (1)$$

where t_i and t_{i+1} are the retention times of the components; w_i and w_{i+1} are the widths of the peaks of the components at the level of the base line; and n is the number of peaks.

This form of the chromatographic response function permitted the separation of each pair of peaks to be taken into account and the splitting of one peak into peaks of components with close properties to be reacted to in an adequate manner, and, moreover, it is insensitive to the forced separation of a pair of reliably separated components.

The Nelder-Mead algorithm is oriented to the search for a minimum, while a maximum of the chromatographic response function corresponds to the best conditions of separation. It is therefore desirable to define the target function as the response function with reversed sign.

The chromatographic response function (1) does not take into account a number of additional constraints on the conditions of chromatography flowing from the specific nature of the object under study: in an acid medium, particularly with a rise in the temperature, the grafted-on phase of the sorbent breaks down [2], which limits the concentration of TFA and the temperature of the column. Furthermore, the time of analysis must not be too great.

In view of their lack of rigidity, such requirements cannot be taken into account by the superposition of additional constraints on a region of the factor space. Any constraint given nonrigidly may be broken, but only on the condition that this ensures an appreciable improvement in the quality of separation. It is therefore reasonable to impose a penalty on the response obtained under "undesirable" conditions of chromatography; the rigidity of this penalty must not rise with an approach to the optimum.

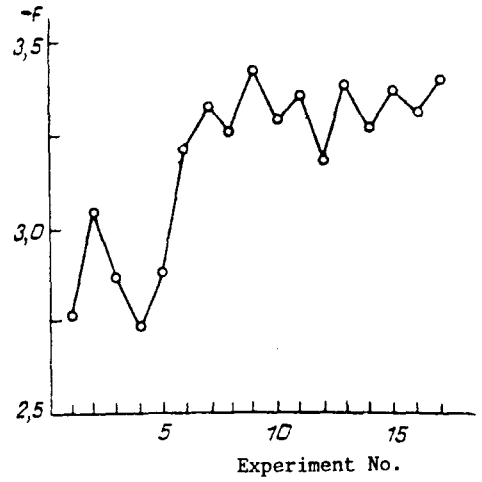

Fig. 1

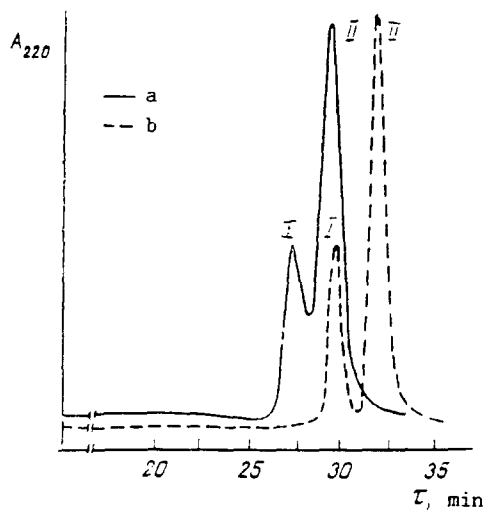

Fig. 2

Fig. 1. Changes in the target function in the process of finding the optimum.

Fig. 2. Chromatogram of the sturins in experiment 1 (a) and under the optimum conditions (b): I) sturin B; II) sturin A.

The penalty functions for each of the nonrigid constraints were given in the form

$$P_j = \begin{cases} 0, & \text{at} \quad x_j \leqslant x_j^* \\ a_j\left(x_j - x_j^*\right), & \text{at} \quad x_j > x_j^* \end{cases}. \tag{2}$$

where P_j is the magnitude of the penalty for the j-th index; x_j is the instantaneous value of the index; x^*_j is a threshold value established nonrigidly by the experiment; and a_j is a coefficient selected on the basis of expert evaluations.

The penalty functions finally acquired the following forms

$$\begin{aligned} &P_1(T^\circ\mathrm{C}): 0{,}04\,(T - 45), \\ &P_2(C_i): 4\,(C_i - 0{,}2), \\ &P_3(\tau_a): 0{.}03\,(\tau_a - 35). \end{aligned} \tag{3}$$

The optimization parameter (target function) can then be represented as

$$F = \sum_j P_j - \mathrm{CRF}, \tag{4}$$

where the CRF is determined from formula (1) and the penalties in accordance with (3).

The algorithms for the determination of the conditions by the modified simplex method of the parameters of chromatography and the treatment of the results of the chromatograms that have been described have been realized on a SP-4100 computer (USA) in the form of an automated system of optrimization of the conditions of chromatographic separation. All the calculations necessary for determining the target function were carried out automatically without manual treatment of the chromatograms. To calculate the width of a peak we used the empirical relation [8]:

$$w_i = \frac{2A_i}{h_i}$$

where A_i is the area of the i-th peak measured by an integrator; and h_i is the height of the peak at the moment corresponding to the retention time.

The procedure described has been used for the optimization of the chromatographic separation of the protamines from sturgeon gonads – sturins. A sturin consists of two components, A and B [11], the chromatographic separation of which is a complex problem because of

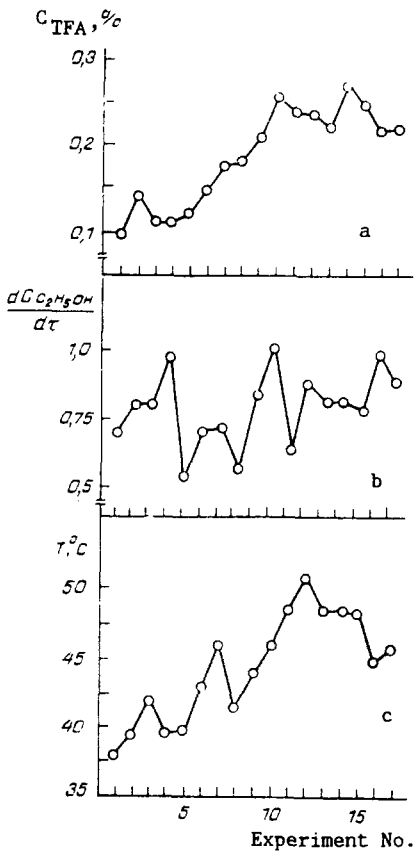

Fig. 3. Changes in the concentration of TFA (a), in the rate of change of the ethanol concentration (b), and in the column temperature (c) during the search for the optimum.

their high basicity. In the optimization of the separation of the sturins it was necessary to perform 17 chromatographic operations in order to determine the optimum combination of the factors that we were investigating. The rate of change of the concentration of ethanol in gradient elution was 0.86% per minute, the column temperature 44.3°C, and the concentration of TFA 0.21%.

It can be seen from Fig. 1 that the optimum combination of the factors was obtained as early as the ninth experiment, which shows the high efficacy of the proposed optimization procedure. In the subsequent experiments (10-17) a refinement of the optimum took place (the value of the target function at this point was 3.4). We may note that a penalty was imposed on the value of the target function at the optimum becaue the threshold of the concentration of TFA was exceeded. The value of the CRF amounted to 3.5, which shows a more than satisfactory separation (Fig. 2). According to the results of amino acid analysis, fraction I corresponded to sturin B and fraction II to sturin A; the yield of protein from the column under the optimum conditions averaged 96%.

As a comparative analysis of the change in the optimization factors during the process of finding the optimum showed (Fig. 3), the greatest influence on the separation of the protamines with the aid of reversed-phase HPLC is shown by the concentration of TFA, as is indicated by the uniform rise in the concentration of TFA in the first stage of the search (the subsequent fall to the limiting value of 0.2% was caused by the action of the penalty) and the sharp minimum in the target function with respect to the concentration of TFA. The proposed optimization procedure can be used to find the optimum conditions of the separation of various protein systems, but for systems of unknown composition the experiment must be repeated with the start of the simplex from a different point of the factor space.

EXPERIMENTAL

Sturins A and B were isolated by a procedure described previously [11]. The solvents for HPLC were purified by standard methods.

High-performance liquid chromatography was conducted on a Du Pont Model 8800 instrument (USA) using a Zorbax ODS column (0.46 × 25 cm). The proteins were detected by means of UV spectrophotometer with a flow-through cell at a wavelength of 220 nm. The protein fractions were selected with the aid of a ISCO Model 1220 fraction collector (USA) and were freeze-dried. The column was thermostated with an accuracy of ±0.2°C.

Amino acid analysis was carried out after the hydrolysis of the fractions with 6 N HCl (105°C, 24 h) on a Hitachi 835 amino acid analyzer (Japan). The working concentration of the sturins was 5 mg/ml, and the volume of sample introduced 10-20 μl.

The optimization program was drawn up in BASIC language and the volume of memory occupied was 3.5 kilobytes.

LITERATURE CITED

1. B. K. Rybin, N. N. Yaglitskaya, T. Yu. Matrosovich, and N. V. Makarov, Khim. Prir. Soedin., 744 (1986).
2. E. L. Styskin, L. B. Itsikson, and E. V Braude, Practical High-Performance Liquid Chromatography [in Russian], Khimiya, Moscow (1986), p. 33.
3. J. P. Bounine, G. Guiochon, and H. Colin, J. Chromatogr., 298, 1 (1984).
4. J. C. Berridge, Techniques for the Automated Optimization of HPLC Separations, Wiley, Chichester (1985), p. 19.
5. L. G. Sabate, A. M. Diaz, X. M. Tomas, and M. M. Gassiot, J. Chromatogr. Sci., 21, 439 (1983).
6. J. A. Nelder and R. Mead, Computer J., 7, 308 (1965).
7. J. C. Berridge, Trends Anal. Chem., 3, 5 (1984).
8. J. C. Berridge, J. Chromatogr., 244, 1 (1982).
9. D. Himmelblau, Applied Nonlinear Programming, McGraw-Hill, New York (1972).
10. M. Hermodson and W. C. Mahoney, Methods Enzymol., 91, 352 (1983).
11. E. P. Yulikova, L. K. Evseenko, L. A. Baratova, L. P. Belyanova, V. K. Rybin, and A. B. Silaev, Bioorg. Khim., 2, 1613 (1976).

AMINO ACID COMPOSITIONS OF PROTEINS SYNTHESIZED IN VITRO IN THE CELL NUCLEI OF EUKARYOTES

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The proteins synthesized in vitro by the nuclei of animal and plant cells differ in their amino acid compositions. The differences found show the species specificity of the products.

It has been shown previously (from the inclusion of [^{14}C]amino acids) that animal and plant cell nuclei synthesize proteins under different conditions [1, 5]. The products synthesized by the nuclei of neurons of animal brains and of plants (the cotton plant) coincide in molecular mass. It appeared of interest to compare their amino acid compositions.

Table 1 gives the results obtained in a determination of the amino acid compositions of the nuclear proteins.

A species specificity of the amino acid compositions of the proteins synthesized by the nuclei of rabbit and bovine brain neurons was found. We have established that in both proteins synthesized by brain neuron nuclei there are larger amounts of such amino acids as lysine, arginine, glutamic acid, proline, alanine, and valine in the bovine nuclear proteins with the amounts of histidine, aspartic acid, threonine, serine, glycine, and tyrosine 2-12 times higher than in the rabbit proteins.

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