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Chromatography + RECOVERY = superresolution chromatography

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Abstract

A method for improving the resolution of the chromatographic analysis based on deriving the point-spread function of a chromatographic column, i.e., a chromatogram of an individual compound, is described. The system of two data sets, namely, a chromatogram of a substance analyzed and a point-spread function of a chromatographic column in combination with the noise statistics, makes it possible to use the RECOVERY signal-reconstruction software package described in paper by Gelfgat et al. (Comp. Phys. Commun. 74 (1993) 335). The proposed method has been tested by chromatography of bovine serum albumin using gel filtration. The resultant resolution exceeds that reached using high-performance liquid chromatography (with the cost of the instruments being lower by a factor of 15–20).

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1. Introduction

Chromatography is a physicochemical method for investigating various substances. The method is based on the separation of the components moving through a chromatographic column [1], which is a calibrated tube of a 10 cm (high-performance liquid chromatography (HPLC)) to 30 m (gas chromatography) length. The column is filled with a special bed, which is silicagel, polymer, glass microparticles, etc.

Size exclusion chromatography demonstrates more clearly the process of substance separation. The separation is based on differences in the sizes and/or shapes of the analyte molecules, which governs the access of analytes to the pore volume inside the column-packing particles. The packing particles are formed by twisted polymer molecules (agarose, dextran, polyacrylamide, etc.) of a 100/μ diameter. According to their size, the smaller analytes have a nearly full access to the pore volume, the larger molecules with a lower access to the pore volume elute first, while the smallest molecules elute dernier.

The retention time t, which equals the time necessary for the molecules to pass through the column, is the main characteristic of the

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chromatography process. The following relation holds between that characteristics and the molecular weight M of a substance separated:

$$\log M = a - bt. \quad (1)$$

See Ref. [2]. The constants a and $b > 0$ are determined by calibration measurements of samples with known molecular weights.

A chromatogram is often represented by the sum of peaks, many of which are overlapped; this makes any accurate analysis difficult. The parametric estimation methods are widely used to raise the resolving power. They are based on the assumption that the peak shape of an individual substance can be described analytically, using, for example, the Gaussian function [3]. Unfortunately, many secondary processes (nonspecific sorbtion, column voiding, etc.) influence on the chromatography separation; then, the peak shape of an individual compound becomes not only asymmetric, but also time dependent. The shape of the peak observed in experiments does not often belong to a function from a large set of chromatography software packages. Therefore, the parametric techniques cannot give any reliable results.

We expect to accurately solve the problem of separating the overlapping peaks by a nonparametric method. In this case, the peak shape is determined by chromatography of an individual substance. Let, by analogy with optics, the peak shape be called the point-spread function of a chromatographic column. A chromatogram of a substance mixture is a superposition of individual peaks. If the shapes of the peaks are the same throughout the entire operation range of the device, the chromatogram obtained is a convolution of the sought molecular distribution with the point-spread function of the chromatographic column. To find the known molecular distribution, the input data must be deconvolved. The input data for this equation is a chromatogram, and the convolution operator kernel is a point-spread function of the chromatograph column obtained from measurements. The solutions for such problems can be found in Ref. [4].

2. Recovery software package

To solve this problem, we use the maximum likelihood principle-based RECOVERY software package, which restores the signals from the noisy data [5]. This package was especially developed to restore nonnegative functions. The sought spectra are always just such functions representing the molecular weight distribution for a substance under study. In the general case, the spectrum is an intermolecular-interaction distribution accounting for the separation of the compound studied. The RECOVERY package is documented in the paper [5] and full texts of all programs in the source Fortran code can be obtained from the CPC Program Library in http://cpc.cs.qub.ac.uk under the name ACLJ. Some recent examples of using this package are presented in paper [6]. Additional data on this software package can be obtained in http://kapitza.ras.ru/people/kosarev/recovery/recov.1.html.

3. Materials and methods

The experimental techniques were as follows: a protein mixture of a known composition (bovine serum albumin (BSA) monomer, dimer, and trimer) was separated by size exclusion chromatography. However, as known earlier the separating gel used cannot separate the mixture. So, a chromatogram with much overlapping peaks was obtained. The data were processed by the RECOVERY software package, and the result was compared with the finer separation data obtained using HPLC.

The bovine serum albumin (fraction No. 5, Sigma, USA) was dissolved in a phosphate buffer (NaCl-100 mM, NaH_2PO_4-40 mM, ethylenediaminetetraacetic acid-1 mM, pH = 6.8) and, after ultrafiltration (Millipore, 0.2 μm), was applied to a Fractogel TSK HW 55 gel (TOYO SODA, Japan). The separation was carried out at a solvent current rate of 1.5 ml/min, column (25×900 mm^2). The column eluent was monitored for absorbance at wavelength $\lambda = 280$ nm (Uvicord S 2138, LKB, Sweden). The output signal from the detector was connected with a computer via an interface [7].

The protein purity was checked by electrophoresis [8] in a polyacrylamide gel in the presence of sodium dodecylsulfate and mercaptoethanol (SDS-PAAG) in both the denaturing (boiling during 5 min) and natural conditions. The high-performance liquid chromatography was realized in a TSK G2000 SW Spherogel column, 10 mkm, 7.5 × 600 mm (LKB, Sweden) at a solvent current rate of 0.5 ml/min, the Beckman-Altex 334 chromatography system (USA). The columns were calibrated using the Pharmacia Gel Filtration Standard. The peak area was measured using the MicroCal Origin 4.1 software package.

Use was made of the **Dconv_** and **Dconv2_** programs from the RECOVERY software package designed for solving an integral convolution equation with a gaussian noise distribution.

4. BSA Chromatography and point-spread function determination

Fig. 1A illustrates a BSA chromatogram. It can be seen that the substance consists of at least two components (the second component can readily be seen on the right-hand side of the curve). The resolution of the gel bed used is insufficient for these components to be separated because the column used is designed for a wide molecular weight range of 10–700 kD [9]. With this resolution, therefore, a BSA monomer and a dimer with molecular weights of 67 and 134 kD, respectively, cannot be separated. Instead, the BSA monomer-containing fraction on the curve top was collected. The fraction obtained was repeatedly separated on the same column. The elution profile shown in Fig. 1B shows a relative purity (absence of the BSA dimer or trimer) of the resultant fraction. Electrophoresis in SDS-PAAG confirms the purity of the obtained fraction. The curve shown in Fig.1B was used as a point-spread function for the RECOVERY software package.

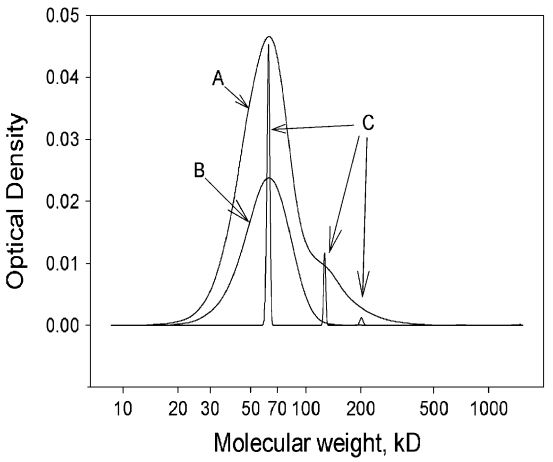

Fig. 1. The RECOVERY software package as applied to the chromatography separation data: (A) the BSA chromatogram obtained for a TSK-gel column; (B) the point-spread function (BSA monomer chromatogram); (C) the results of recovering the chromatographic separation data using the **Dconv2_** program from the RECOVERY software package.

5. Deconvolution with the recovery package

Asymmetry of the point-spread function does not prevent the nonparametric method from being used. It is possible to reach a superresolution with chromatographic separation. Fig. 1C demonstrates the narrow peaks, which result from recovering the chromatographic separation data using the **Dconv2_** program. Along the x-axis, the molecular weights calculated by formula (1) is plotted. The result is a set of three peaks, which correspond to the BSA monomer, dimer, and trimer. The HPLC separation of a mixture is much more effective than the TSK size exclusion chromatography and demonstrates the presence of three components, namely, monomer, dimer, and unresolved BSA trimer. The results given in the figure coincide with the data cited in literature [10].

Application of the RECOVERY software package improves significantly the chromatography results, namely, the molecular weights range (67–201 kD), which is not separated completely by the HPLC, is excellently separated by our method. The super-resolution obtained using the RECOVERY software package can be estimated by the data given in Fig. 1. The ratio of the width of the point-spread function to the BSA dimer–trimer distance has been obtained to be 1.1. This means that obtained resolution is better by a factor of 1.1

Table 1
Molecular weights of BSA components (kD) determined by different methods

A	1	2	3
B	67±1	134±1.4	201±1.7
C	67	—	—
D	68	135	190
E	66.4±0.53	127±1.62	200±5.78

A is the peak number, B is the tabulated value, C is the TSK gel, D is HPLC, E is RECOVERY.

Table 2
The percentage of the components of a separated mixture

A	1	2	3
B	100	—	—
C	84	13.7	2.3
D	83±6	14.7±6	2.3±1

A is the peak number, B is the TSK gel, C is HPLC, D is RECOVERY.

compared with the width of point-spread function (BSA monomer chromatogram shown in Fig. 1B). The signal-to-noise ratio for the data obtained is 47 dB. In principle, this makes it possible to have the super-resolution ratio equaling to about 5 [4]. The molecular weights of compounds (kD) obtained using the standard TSK size exclusion chromatography, HPLC, and the method described in this work are given (Table 1) as compared with the data from the Sigma-Aldrich catalog [11]. The row denoted RECOVERY contains the data averaged over five independent measurements with a probable error of 1%. The peak Nos. 1, 2, and 3 correspond to the BSA monomer, dimer, and trimer, respectively.

The proposed method demonstrates an excellent agreement of the monomer and trimer results with the published data. As regards the dimer mass, the accuracy is better than 5% and a possible reason of the small discrepancy arises from different shapes of molecules used for calibration and for chromatography.

Quantitative determination of the BSA components is shown in Table 2. The data demonstrate also a good agreement of the component percentage determined by the well known and new methods. Thus, we have shown that the proposed method essentially improves the quality of the chromatographic separation, making it possible to measure quantitatively the percentage of separate components of mixtures. Note that these new possibilities are achieved by reasonable processing the measured data with no complication in the instrumentation. The use of the RECOVERY software package to obtain gel filtration data has significantly increased the resolution of this method and improved the quality of the separation obtained with the HPLC technique. Note that the cost of the instrument complex used for gel filtration (US$ 1000) is roughly 15–20 times lower than that for the HPLC setup (20000 USD).

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