Journal of Chromatography, 538 (1991) 99–108
Elsevier Science Publishers B.V., Amsterdam

CHROM. 22 793

Systematic search for suitable two-phase solvent systems for high-speed counter-current chromatography

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ABSTRACT

We have introduced two series of two-phase solvent systems which facilitate the systematic search for the solvent systems suitable for high-speed counter-current chromatography. The *n*-hexane–ethyl acetate–*n*-butanol–methanol–water systems provide a broad range of hydrophobicity, while the chloroform–methanol–water systems are extremely useful for separations of various natural products with moderate hydrophobicity. The practical use of these solvent series was demonstrated with several test samples which include dinitrophenyl amino acids, S-triazine herbicides, indole auxins, and non-ionic organic solvents.

INTRODUCTION

High-speed counter-current chromatography (HSCCC) developed in the late 1970's produces highly efficient chromatographic separations of solutes without the use of solid supports [1–3]. Thus, the method eliminates all complications caused by the soliud support matrix such as adsorptive loss and deactivation of samples, tailing of solute peaks, contamination, etc. Recently, HSCCC has been widely used for separation and purification of various natural products with excellent results.

As in other CCC schemes, HSCCC utilizes a two immiscible solvent phases, one as a stationary phase and the other as a mobile phase, and the separation is highly dependent on the partition coefficient values of the solutes, *i.e.*, the ratio of the solute concentration between the mobile and stationary phases. Therefore, the successful separation necessitates a careful search for the suitable two-phase system which provides an ideal range of the partition coefficient values for the applied sample.

Generally speaking, the two-phase solvent system should satisfy the following three requirements.

(i) The solvent system should provide nearly equal volumes of the upper and the lower phases. This facilitates the choice of the mobile phase without excessive waste of

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0021-9673/91/$03.50

the solvent, and the two solvent phases are quickly separated into clear layers in a separatory funnel.

(ii) The two-phase solvent system should yield a reasonably short settling time (for the detailed description of the settling time, see Experimental). In HSCCC, the settling time should be shorter than 30 s for satisfactory retention of the stationary phase in the column [3,4].

(iii) The partition coefficient (K) of the desired compound should be close to one which gives the retention volume equal to the total column capacity. The smaller retention volume may result in a less efficient peak resolution while the greater retention volume tends to broaden the sample band by excessive mixing [5].

In the past, the search for the suitable two-phase solvent systems entirely relied on a laborious and time-consuming trial and error method which has often discouraged the users of CCC, while the method for the systematic solvent search has not been reported. This paper introduces two different series of two-phase solvent systems, one consisting of *n*-hexane–ethyl acetate–*n*-butanol–methanol–water and the other, chloroform–methanol–water, both of which lead to a systematic search for the suitable solvent systems. The search may be completed in several successive measurements of the partition coefficients by following the direction along the list of the two-phase solvent systems. Using these two-phase solvent systems, partition coefficient measurements were performed for the several groups of test samples with a broad range in hydrophobicity.

EXPERIMENTAL

Reagents

n-Hexane, ethyl acetate, *n*-butanol, methanol and chloroform were glass-distilled chromatographic grade and purchased from Burdick and Jackson (Muskegon, MI, U.S.A.). Acetone, 2-butanone and benzyl-alcohol were of reagent grade and obtained from Mallinckrodt (Paris, KY, U.S.A.), Eastman Kodak (Rochester, NY, U.S.A.) and Sigma (St. Louis, MO, U.S.A.), respectively. All dinitrophenyl (DNP) amino acids, dipeptides, and indole auxins were of reagent grade (Sigma), including N-2,4-DNP-δ-L-ornithine (DNP-orn), N-2,4-DNP-L-aspartic acid (DNP-asp), N-2,4-DNP-DL-glutamic acid (DNP-glu), N,N-di-(2,4-DNP)-L-cystine [diDNP-$(cys)_2$], N-2,4-DNP-β-alanine (DNP-β-ala), N-2,4-DNP-L-alanine (DNP-ala), N-2,4-DNP-L-proline (DNP-pro), N-2,4-DNP-L-valine (DNP-val), N-2,4-DNP-L-leucine (DNP-leu), L-tyrosyl–L-glycine (tyr–gly), L-tyrosyl–L-valine (tyr–val), L-tyrosyl–L-leucine (tyr–leu), indole-3-acetamide (IA), indole-3-acetic acid (IAA), indole-3-acetonitrile (IAN), indole-3-carboxylic acid (ICA), indole-3-acrylic acid (IAcA), indole-3-butyric acid (IBA), and indole-3-propionic acid (IPA). All S-triazine herbicides including atrazine, propazine, simazine, and trietazine were technical grade chemicals obtained from Ciba-Geigy (Greensboro, NC, U.S.A.).

Preparation of two-phase solvent systems

Twenty-seven volatile two-phase solvent systems shown in Tables I and II were prepared for the present studies. Each solvent system was thoroughly equilibrated in a separatory funnel at room temperature by repeated shaking and degassing by opening the stopcock. Then, the solvent mixture was transferred to a graduated

TABLE I

PHASE COMPOSITION, TWO-PHASE VOLUME RATIO, AND SETTLING TIME OF *n*-HEXANE–ETHYL ACETATE–*n*-BUTANOL–METHANOL–WATER SOLVENT SYSTEMS

No.	*n*-Hexane–ethyl acetate–*n*-buthanol–methanol–water					Volume ratio (U/L)[a]	Settling time (s)
1	10	0	0	5	5	1.05	5
2	9	1	0	5	5	0.96	8
3	8	2	0	5	5	0.88	14
4	7	3	0	5	5	0.82	20
5	6	4	0	5	5	0.77	22
6	5	5	0	5	5	0.74	26
7	4	5	0	4	5	0.80	28
8	3	5	0	3	5	0.86	30
9	2	5	0	2	5	0.93	30
10	1	5	0	1	5	0.92	30
11	0	5	0	0	5	0.88	32
12	0	4	1	0	5	0.91	20
13	0	3	2	0	5	0.99	15
14	0	2	3	0	5	1.09	12
15	0	1	4	0	5	1.16	14
16	0	0	5	0	5	1.22	17

[a] Volume of the upper phase divided by that of the lower phase.

cylinder to measure the volume ratio of the two phases. The solvent mixture was then returned into the separatory funnel where the two phases were stored until use.

Measurement of settling time [4]

Using the above equilibrated solvent phases, the settling time was measured as follows. A 2-ml volume of each phase, the total volume was 4 ml, was delivered into

TABLE II

PHASE COMPOSITION, TWO-PHASE VOLUME RATIO, AND SETTLING TIME OF CHLOROFORM–METHANOL–WATER SOLVENT SYSTEMS

Methanol–water (%)	Chloroform–methanol–water			Volume ratio (L/U)[a]	Settling time (s)
0	10	0	10	0.98	8
10	10	1	9	1.00	8
20	10	2	8	1.03	12
30	10	3	7	1.06	13
40	10	4	6	1.10	12
50	10	5	5	1.16	10
60	10	6	4	1.37	11
70	10	7	3	2.05	22
80[b]	10	8	2	–	–

[a] Volume of the lower phase divided by that of the upper phase.
[b] The solvent mixture formed a single phase.

a 5-ml capacity graduated glass cylinder which was then sealed with a glass stopper. The solvent in the cylinder was gently mixed by inverting the cylinder 5 times and the cylinder was immediately placed on a flat table in an upright position. Then, the time required for the solvent mixture to settle into two clear layers was measured. The experiment was repeated several times to obtain the mean value.

Measurement of partition coefficient

In the present study, the partition coefficient is expressed by $K_{(org/aq)}$: the solute concentration in the organic phase divided by that in the aqueous phase. The partition coefficient value for each component was determined as follows: For the non-volatile test samples, approximately 0.1 ml of methanol or water containing about 1 mg of the material was evaporated with air stream in a 13-mm diameter test tube. Then, exactly 2 ml each of the upper and the lower phases were pipetted into the test tube. For the volatile liquid samples such as acetone, 2-butanone, and benzylalcohol, 10 μl of each was separately delivered into a test tube and immediately added with 2 ml each of the upper and the lower phases. The test tube was stoppered with a teflon-lined cap, shaken vigorously for 1 min to thoroughly equilibrate the sample with the two phases, and briefly centrifuged to obtain clear layers of the two phases. Then, a 0.5-ml volume of each layer was transferred to the second test tube containing 2 ml of methanol, and the contents were thoroughly mixed. The absorbance of each solution was determined at a suitable wavelength with a Zeiss PM6 spectrophotometer.

RESULTS

n-Hexane–ethyl acetate–n-butanol–methanol–water solvent system

This solvent series provides 16 two-phase solvent systems which cover a wide range of hydrophobicity continuously from the non-polar *n*-hexane–methanol–water system to the polar *n*-butanol–water system. These solvent systems are numbered from 1 to 16 in the order of hydrophobicity and listed in Table I. All these solvent systems are volatile and yield a desirable two-phase volume ratio of near 1 so that either phase can be chosen as the mobile phase without excessive waste of the solvents. These solvent systems also give suitable settling times in which the longest value slightly exceeds the critical value of 30 s in the ethyl acetate–water system. This ensures a satisfactory retention of the stationary phase in HSCCC. The overall results indicate that all the solvent systems can be efficiently applied to any centrifugal CCC scheme.

Partition coefficient values of various test samples in these solvent systems are summarized in Fig. 1A, B, and C where the $K_{(org/aq)}$ of each component is plotted in a semilogarithmic scale against the applied volume ratio of the solvent system.

Among DNP amino acids tested (Fig. 1A), the most hydrophobic component of DNP-leu gives an ideal K value of 1.05 in solvent system 3 (*n*-hexane–ethyl acetate–*n*-butanol–methanol–water, 8:2:0:5:5) while the most polar component of diDNP-$(cys)_2$ gives the same K value in solvent system 14 (*n*-hexane–ethyl acetate–*n*-butanol–methanol–water, 0:2:3:0:5). Other DNP amino acids show similar K values in the solvent systems between the above two extremes. In the S-triazine herbicides (Fig. 1B), the hydrophobicity decreases in the order of trietazine, propazine, atrazine, and simazine. The first two components of trietazine and propazine give K values of over 1.5 even in the most hydrophobic solvent system 1 (*n*-hexane–ethyl acetate–*n*-butanol–

methanol–water, 10:0:0:5:5) whereas atrazine and simazine give the desired K values in solvent system 2 (*n*-hexane–ethyl acetate–*n*-butanol–methanol–water, 9:1:0:5:5) and solvent system 5 (*n*-hexane–ethyl acetate–*n*-butanol–methanol–water, 7:3:0:5:5), respectively. Three non-ionic solvents (acetone, 2-butanone, and benzylalcohol) (Fig.

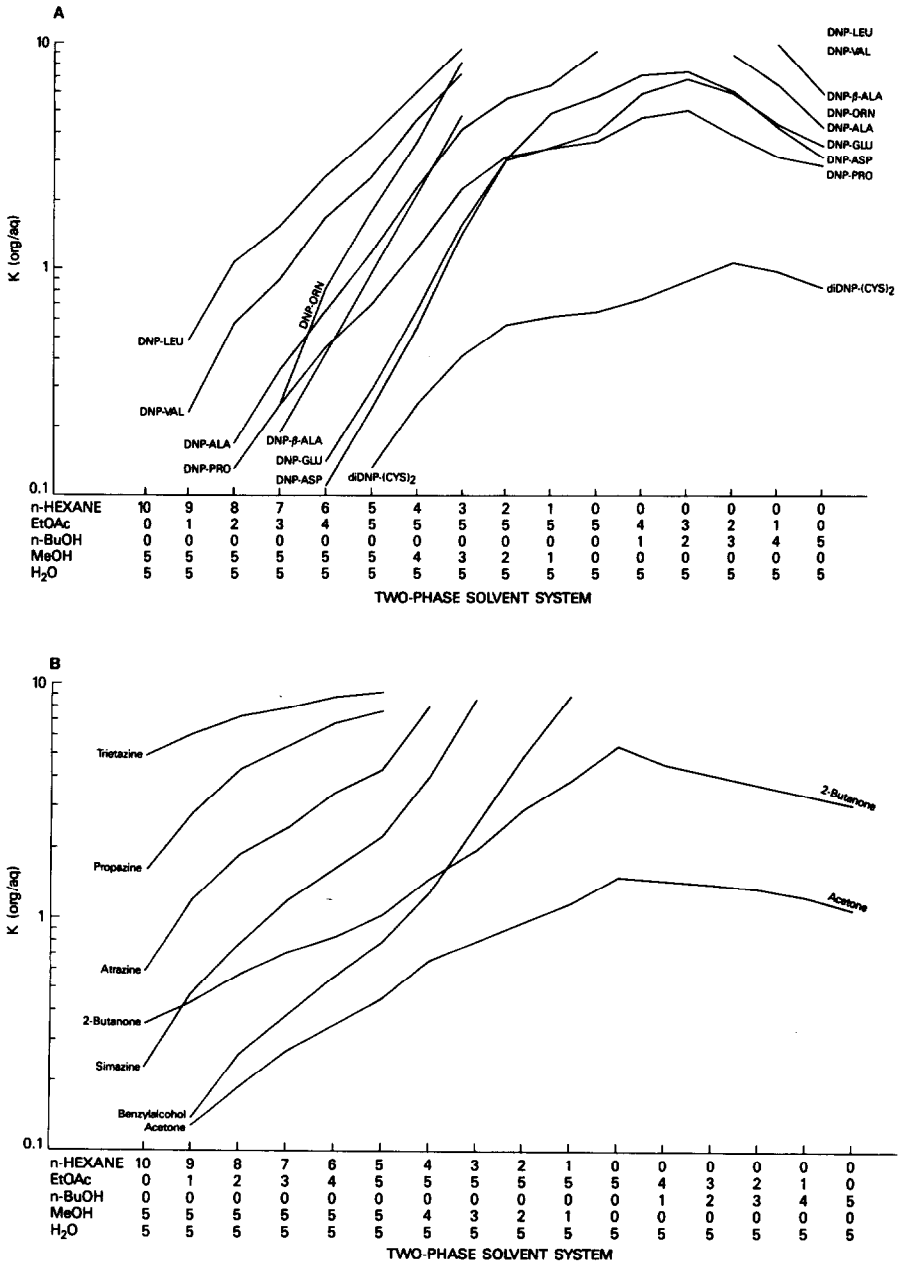

Fig. 1.

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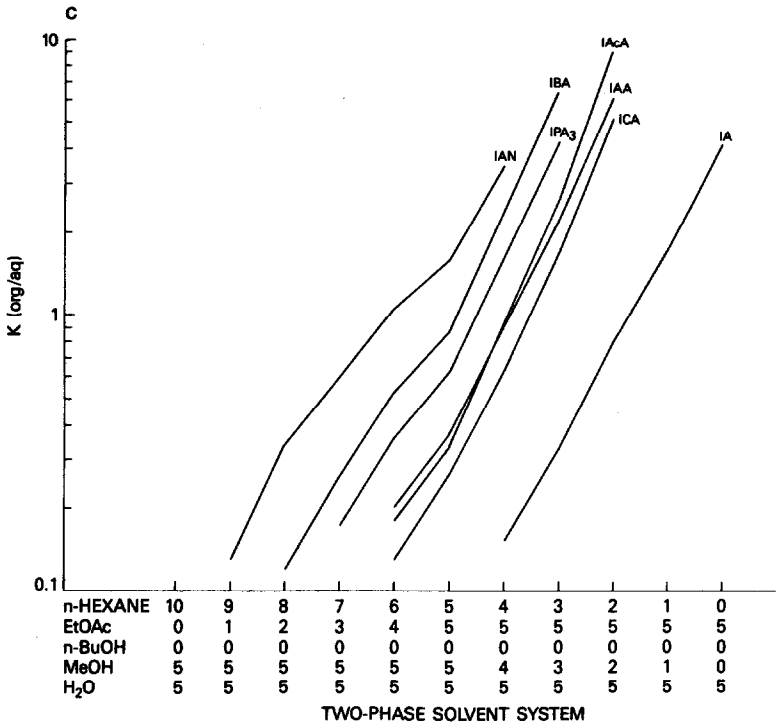

Fig. 1. (A) Partition coefficients, $K_{(org/aq)}$, of DNP amino acids in *n*-hexane–ethyl acetate–*n*-butanol–methanol–water system. (B) Partition coefficients, $K_{(org/aq)}$, of acetone, 2-butanone, benzylalcohol and S-triazine herbicides in *n*-hexane–ethyl acetate–*n*-butanol–methanol–water system. (C) Partition coefficients, $K_{(org/aq)}$, of indole auxins in *n*-hexane–ethyl acetate–*n*-butanol–methanol–water system. EtOAc = Ethyl acetate; *n*-BuOH = *n*-butanol; MeOH = methanol.

1B) and various indole auxins (Fig. 1C) give the desired K values each in the particular solvent system according to their polarity. All dipeptide samples tested showed low K values even in the most polar solvent system 16 (*n*-hexane–ethyl acetate–*n*-butanol–methanol–water, 0:0:5:0:5) and, therefore, more polar solvent systems are required for the separation of these compounds.

Chloroform–methanol–water solvent system

Listed in Table II are 11 chloroform–aqueous methanol (1:1) systems with various concentrations of methanol in water. The use of methanol concentration at 80% resulted in formation of a single phase, and 70% methanol concentration produced uneven volume distribution of the two phases. However, other solvent systems yielded near equal volumes of the two phases with excellent settling times of less than 15 s.

Partition coefficient values, $K_{(org/aq)}$, of various test samples in the chloroform solvent systems are illustrated in Fig. 2A, B, and C according to the format used in the *n*-hexane solvent systems. The partition coefficient values of the DNP amino acids (Fig. 2A) in these solvent systems varied greatly according to the hydrophobicity of the

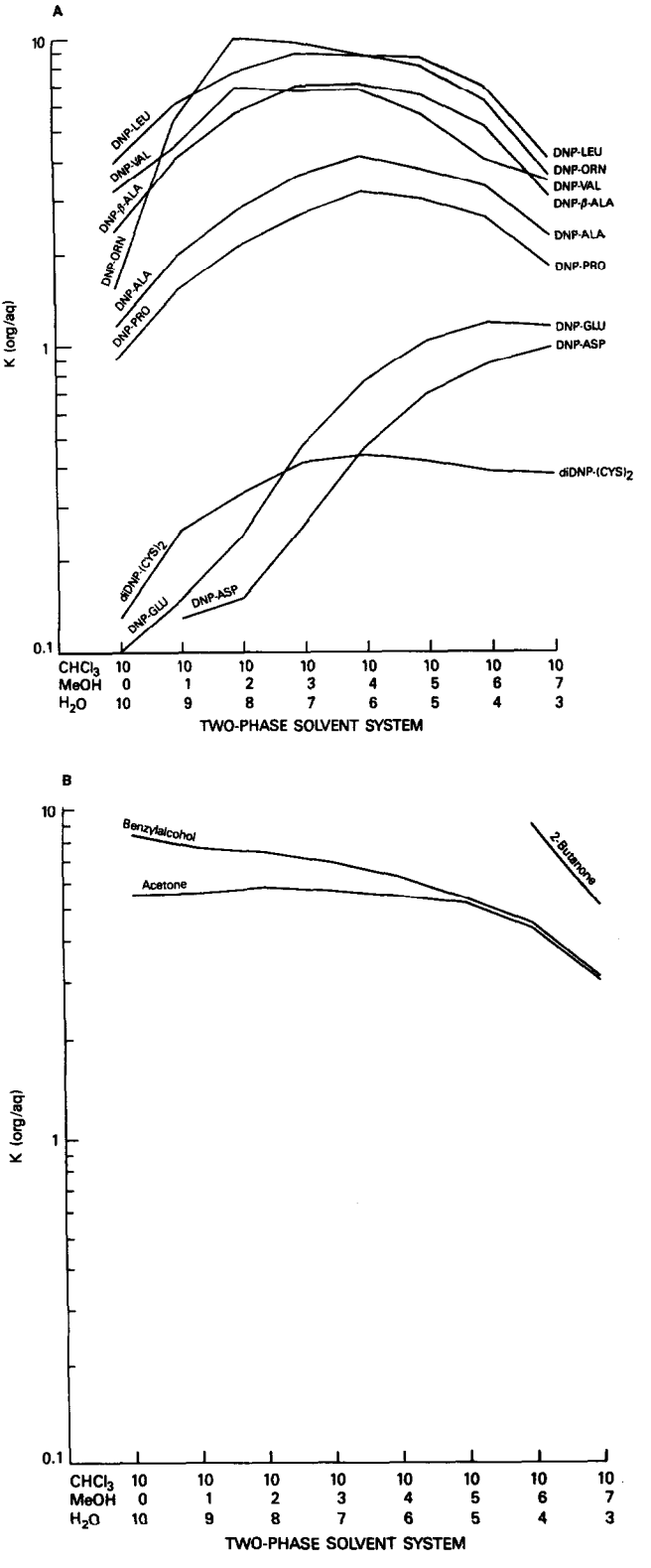

Fig. 2.

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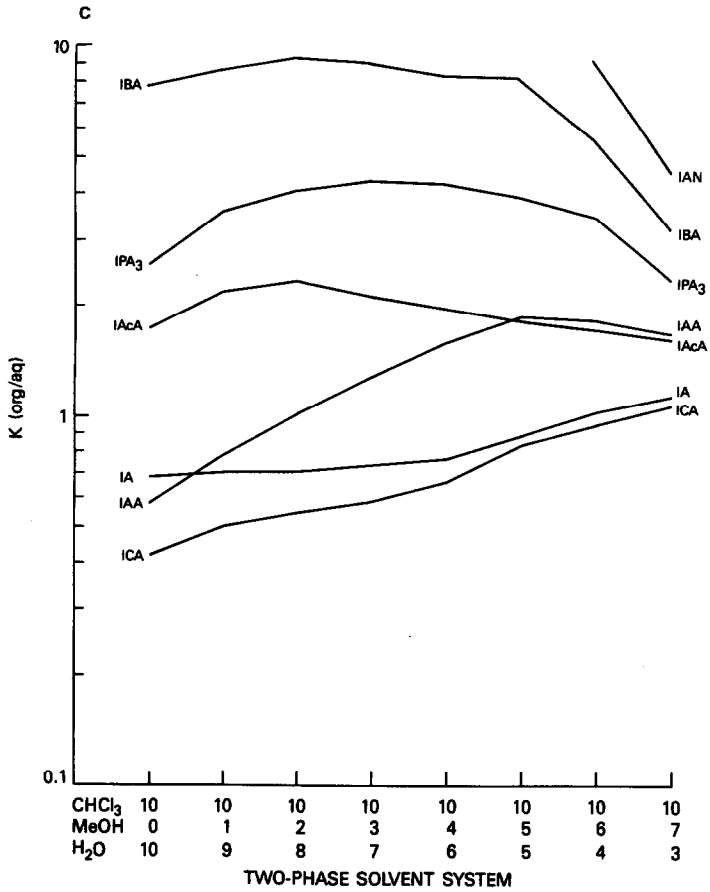

Fig. 2. (A) Partition coefficients, $K_{(org/aq)}$, of DNP amino acids in chloroform–methanol–water system. (B) Partition coefficients, $K_{(org/aq)}$, of acetone, 2-butanone, benzylalcohol in chloroform–methanol–water system. (C) Partition coefficients, $K_{(org/aq)}$, of indole auxins in chloroform–methanol–water system. $CHCl_3$ = Chloroform; MeOH = methanol.

sample. The hydrophobic components such as DNP-leu, DNP-val, DNP-orn and DNP-β-ala gave K values substantially greater than 1, while the hydrophilic components such as DNP-asp, DNP-glu, and diDNP-$(cys)_2$ gave K values smaller than 1. In most of the samples, K values became maximum at the 30–40% methanol concentration.

All chloroform solvent systems gave large K values for the non-ionic solvents (acetone, 2-butanone, and benzylalcohol) (Fig. 2B) and various S-triazine herbicides. Among the indole auxins (Fig. 2C), IAA, IA, and ICA gave K values of near 1 while other components showed much greater K values.

From the above results, it was observed that the hydrophobicity of the chloroform–methanol–water systems are corresponding to that of the *n*-hexane–ethyl acetate–*n*-butanol–methanol–water systems ranging in volume ratio between 1:1:0:1:1 and 3:5:0:3:5.

DISCUSSION

In order to facilitate the search for a suitable two-phase solvent system that provides an ideal range of the partition coefficient to the desired sample, we have selected two series of solvent systems: *n*-hexane–ethyl acetate–*n*-butanol–methanol–water and chloroform–methanol–water. In each solvent series, the partition coefficient of the sample can be finely adjusted by modifying the volume ratio of the components. The first series covers a broad range in both hydrophobicity and polarity continuously from *n*-hexane–methanol–water to *n*-butanol–water. The second series of chloroform–methanol–water provides moderate hydrophobicity and has proven extremely useful for separation of a variety of natural products simply by modifying the volume ratio between methanol and water. Most of these two-phase solvent systems provide near 1:1 volume ratios of the upper to the lower phases together with the reasonable range of settling times in 30 s or less so that they can be efficiently applied to HSCCC and other centrifugal CCC schemes.

To evaluate the usefulness of these two solvent series, we have examined the partition coefficients of the following 5 sets of test samples: 9 DNP amino acids with a wide range in hydrophobicity (which have been used as the standard samples in various CCC schemes); 7 indole auxins with a moderate range in hydrophobicity; 4 S-triazine herbicides with strong hydrophobicity; and 3 non-ionic solvents including acetone, 2-butanone, and benzylalcohol.

As described earlier, partition coefficient values, $K_{(org/aq)}$, of these test samples in the *n*-hexane and chloroform solvent systems are summarized in Figs. 1 and 2, respectively. These figures are extremely useful for selecting the proper solvent system for performing the HSCCC separations of the various sample mixtures. In these figures, the best separation will be obtained with the phase composition where the K values of the components are fairly evenly scattered around 1 at certain intervals. Some examples of the solvent selection from these figures are described below.

For the DNP amino acid separation, no single solvent system can achieve one-step separation of all compounds because of the wide range of the K values. However, the hydrophobic group, including DNP-leu, DNP-val, DNP-ala, and DNP-orn, can be separated with *n*-hexane–ethyl acetate–*n*-butanol–methanol–water (6:4:0:5:5) (Fig. 1A) and the hydrophilic group, including DNP-pro, DNP-β-ala, DNP-glu, DNP-asp, and diDNP-$(cys)_2$, can be separated with the above solvent mixture with a volume ratio of 4:5:0:4:5 (Fig. 1A). The chloroform solvent system can also be used for the separation of relatively polar DNP amino acids: DNP-ala, DNP-pro, DNP-glu, DNP-asp and diDNP-$(cys)_2$ may be separated with chloroform–methanol–water (10:7:3) (Fig. 2A).

Similarly, one can select *n*-hexane–ethyl acetate–*n*-butanol–methanol–water (3:5:0:3:5) for the separation of acetone, 2-butanone, and benzylalcohol and also the same solvent mixture (9:1:0:5:5) for the separation of S-triazine herbicides (Fig. 1B). The separation of the indole auxins may be successfully performed with *n*-hexane–ethyl acetate–*n*-butanol–methanol–water (4:5:0:4:5) (Fig. 1C) except for IAcA and IAA which can be well resolved with chloroform–methanol–water (10:2:8) (Fig. 2C).

As described above, the two solvent series we have introduced can provide the suitable K values for the separation of various samples with a broad spectrum in hydrophobicity. For the sample mixture with an unknown nature, the search for the

suitable solvent system may be initiated with the partition coefficient measurement with *n*-hexane–ethyl acetate–*n*-butanol–methanol–water (5:5:0:5:5) or chloroform–methanol–water (10:3:7). If the $K_{(org/aq)}$ value is too large, the search should be directed toward the more hydrophobic solvent systems in the hexane series and, if the K value is too small, the search should be directed toward the more hydrophilic solvent systems until the proper K values are obtained.

If the above solvent search reaches the solvent system 1 (*n*-hexane–methanol–water, 2:1:1, v/v/v) and a more hydrophobic solvent system is required, one may reduce the amount of water from the above solvent system and/or replace methanol with ethanol. Some useful solvent systems for the extremely hydrophobic compounds are *n*-hexane–ethanol–water (6:5:2, v/v/v) and *n*-hexane–methanol (2:1, v/v). On the other hand, if the solvent search ends at the solvent system 16 (*n*-butanol–water) and a still more hydrophilic solvent system is required, the above solvent system may be modified by the addition of an acid or salt: *n*-butanol–trifluoroacetic acid–water (1:0.01–0.001:1, v/v/v), *n*-butanol–acetic acid–water (4:1:5, v/v/v), and *n*-butanol–0.25 *M* ammonium acetate (1:1, v/v) have been successfully used for the separation of hydrophilic peptides [6]. Among those, the *n*-butanol–acetic acid–water (4:1:5, v/v/v) system exhibits a relatively long settling time and, therefore, the satisfactory retention of the stationary phase is obtained by applying a reversed elution mode, *i.e.*, eluting either the upper phase from the head toward the tail or the lower phase from the tail toward the head through a multilayer coil with β values less than 0.5 [4,7].

In the above experiments, the partition coefficient value of each component was obtained by measuring the absorbance of the pure sample in the upper and the lower phases. However, in practice, such a standard sample is not available, and the sample often contains various impurities or multiple components which interfere with the detection of individual components. In this case, the partition coefficient can be conveniently determined with high-performance liquid chromatography or thin-layer chromatography by chromatographing aliquots of the upper and the lower phases equilibrated with the sample. From the obtained pair of chromatograms, partition coefficient values for all resolved peaks can be determined by computing the ratio of the peak heights or areas between the corresponding peaks [8].

Although the present method has been devised for HSCCC, it may be effectively applied to other centrifugal CCC schemes.

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