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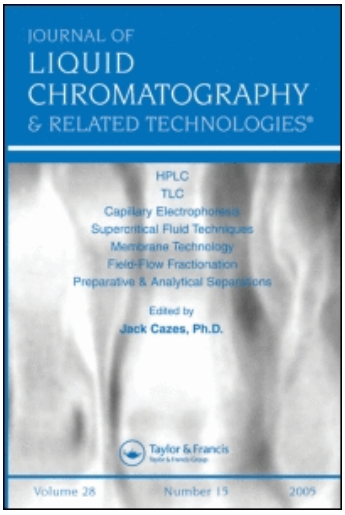

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OPTIMIZATION OF SOLVENT MIXTURE COMPOSITIONS FOR HIGH SPEED COUNTERCURRENT DISTRIBUTION[1]

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ABSTRACT

Seven solvents considered appropriate for use with relatively sensitive biosynthetic products undergoing separation by multilayer coil planet centrifuge high speed countercurrent distribution (HSCCD) have been evaluated in a new and systematic procedure for optimal solvent selection. The new method was illustrated by a complete HSCCD separation of five closely related dipeptides.

INTRODUCTION

Separation of animal and plant derived complex mixtures by various countercurrent distribution techniques[2] represents a generally powerful approach to such experimental problems. However, choice of a suitable solvent mixture and composition ratio(s) have remained the relatively time-consuming and

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uncertain steps of such procedures.[3-8] Application of high-speed countercurrent distribution (HSCCD) with a multilayer coil planet centrifuge (Ito coil) to semifinal isolation of naturally occurring antineoplastic substances has become a broadly utilized technique in our laboratory.[9-11] In turn, that has led us to devise a rapid and systematic method for selecting an optimal solvent mixture from a small series of solvents compatible with potentially sensitive compounds. The overall approach was based on the statistical technique we developed for optimizing a three component solvent for silica gel column chromatographic separation of isomeric mixtures.[12]

EXPERIMENTAL

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A mixture of five *N*-benzyloxycarbonyl-dipeptide methyl esters (supplied by SIGMA Chemical Co.) with similar polarity and structure was prepared. Solvent mixtures were prepared according to a variation matrix. Each solvent (1 ml) was placed in a graduated cylinder (5 ml), shaken 1 min., and the volume of each phase and total volume was recorded with the speed of resolution between phases (limit max. 30-50 sec.). The mixture of protected dipeptides (0.3-0.5 mg) was dissolved in a mixture of upper phase (0.5 ml) and lower phase (0.5 ml) and shaken well 1 min. A thin layer chromatographic (TLC) analysis was performed for the upper and lower phase with each solvent mixture. Small aliquots (20 µl) of upper and lower phase were chromatographed on "uniplate"-type 5 x 10 cm HPTLC plates supplied by Analtech, Inc., using the solvent system 5:2:3 *n*Hexane-acetonitrile-methylene chloride. Component positions were visualized by UV and by a 0.2% ninhydrin solution in ethanol (developed at 120°C). After observation of distribution between phases, solvent mixtures were chosen which gave the best distribution of compounds into the upper and lower phases (for best results a UV/VIS scanner was used).

A 300 µl aliquot of each phase (upper and lower) was transfered to a vial, solvent removed (sweeping with Argon),

and the residue dissolved in 100 µl of methylene chloride. A 10 µl sample was injected into the HPLC instrument and the analysis conducted. The HPLC analyses were performed employing a column of Phenomenex Partisil 5 SILICA (250 x 4.6 mm with 5:2:3 *n*heptane - acetonitrile - methylene chloride as solvent) controlled by an analytical Gilson HPLC (802B, 811, 2 x 302). The HPLC instrument was equipped with Rheodyne injection valve (7125 with a 10 µl loop), Apple IIe gradient manager (V 1.2 Gilson), UV detector and data station (Hewlett-Packard 1040A, 9000-300, 9153, ColorPro, ThinkJet; UV detection at 220 nm, range 210-400 nm).

After solvent optimization, the sample mixture (104.5 mg total) containing *N*-Z-L-Leu-L-Leu-OMe (20.9 mg), *N*-Z-L-Val-L-Leu-OMe (20.4 mg), *N*-Z-L-Val-L-Phe-OMe (20.9 mg), *N*-Z-L-Leu-L-Ala-OMe (21.3 mg), and *N*-Z-L-Ala-L-Val-OMe (21.0 mg), was resolved by HSCCD using the horizontal coil planet centrifuge P.C. Inc. Model #1 with the planet gear drive at 450 rpm, β=0.5-0.85. HSCCD column #10 (consisting of 60 m of 2.6 mm ID PTFE tubing) with a volume of 350 ml was used. Solvent was delivered with a FMI lab pump RP SY (Fluid Metering, Inc., Oyster Bay, N.Y.) with a flow of 6.2 ml/min. Column effluent

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Table 1

Five protected dipeptides separated by HSCCD with TLC and HPLC characteristics

	Rf[a]	Rf[b]	Rt(min)[c]	$K_{(i)}$
N-Z-L-Leu-L-Leu-OMe	0.60	0.41	3.76	K_1
N-Z-L-Val-L-Leu-OMe	0.57	0.36	3.89	K_2
N-Z-L-Val-L-Phe-OMe	0.53	0.30	4.05	K_3
N-Z-L-Leu-L-Ala-OMe	0.47	0.23	4.39	K_4
N-Z-L-Ala-L-Val-OMe	0.42	0.19	4.68	K_5

TLC solvents a, *n*Hexane-acetonitrile-methylene chloride 5:2:3; b, *n*Hexane-Toluene-Acetone-methylene chloride 4:4:1:1; and c, HPLC solvent (isocratic) *n*Heptane-acetonitrile-methylene chloride 5:2:3.

was monitored at 230 nm using a Gilson Holochrome UV-VIS detector (sensitivity 0.5 with a 10 mm cell corresponding to 70 µl) and a Linear recorder (Linear Instruments, Irvine, CA). The fractions were collected employing a Gilson 220 fraction collector, 800 drops/tube (6.8 ml/tube). The solvent system S-7 (see Scheme 2, lower phase water- 2-propanol, mobile phase H→T) was used with Rsf (retention volume of solvent front) = 120 ml.

METHODS AND DISCUSSION

Seven solvents for study were selected (*n*Hexane, toluene, methylene chloride, acetonitrile, *i*Propanol, methanol, and water) based on their well known utility in our anticancer constituents isolation investigations, wide range of dielectric constants, and miscibility behavior. An evaluation of the latter effects led to the potentially useful solvent combinations for HSCCD listed in Table 2.

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Five more combinations (*n*Hex-MeOH (2:1), *n*Hex-MeCN, *n*Hex-W, Tol-W, CH_2Cl_2-W) involving two immiscible solvents were not used. The protected dipeptides were sparingly soluble in water and required a bridging solvent.

The response criterion was derived from three considerations: in the field <F_{max}, 0> the partition coefficient K_{max} must be equal to F_{max} (A); the partition coefficient K_{min} must be equal to 0 (B); the partition coefficients $K_{(i)}$ must be of equal distance from each other and as far as possible (C) for maximum resolution and the F_{max} coefficient should be equal to 10 or 20 for reasonable separation time in the Ito coil. According to these conditions the response criterion "R" will be as summarized in Scheme 1.

Where F_{max} = maximal partition coefficient which determines the range for the calculation (suggested values are 10 and 20); K_{max}=maximal partition coefficient of a compound

Table 2
Potentially useful solvent mixtures for HSCCD derived from seven solvents (*n*Hex = *n*Hexane, Tol = Toluene, CH_2Cl_2 = Methylene chloride, MeCN = Acetonitrile, *i*PrOH = *i*Propanol, MeOH = Methanol, W = Water)

1	*n*Hex	MeCN	W		7	Tol	MeCN	W		
2	*n*Hex	*i*PrOH	W		8	Tol	*i*PrOH	W		
3	*n*Hex	MeOH	W		9	Tol	MeOH	W		
4	*n*Hex	MeCN	*i*PrOH	W	10	Tol	MeCN	*i*PrOH	W	
5	*n*Hex	*i*PrOH	MeOH	W	11	Tol	*i*PrOH	MeOH	W	
6	*n*Hex	MeCN	MeOH	W	12	Tol	MeCN	MeOH	W	

13	CH_2Cl_2	MeCN	W		
14	CH_2Cl_2	*i*PrOH	W		
15	CH_2Cl_2	MeOH	W		
16	CH_2Cl_2	MeCN	*i*PrOH	W	
17	CH_2Cl_2	*i*PrOH	MeOH	W	
18	CH_2Cl_2	MeCN	MeOH	W	

19	*n*Hex	Tol	MeCN	W	
20	*n*Hex	Tol	*i*PrOH	W	
21	*n*Hex	Tol	MeOH	W	
22	*n*Hex	Tol	MeCN	*i*PrOH	W
23	*n*Hex	Tol	*i*PrOH	MeOH	W
24	*n*Hex	Tol	MeCN	MeOH	W

25	Tol	CH_2Cl_2	MeCN	W	
26	Tol	CH_2Cl_2	*i*PrOH	W	
27	Tol	CH_2Cl_2	MeOH	W	
28	Tol	CH_2Cl_2	MeCN	*i*PrOH	W
29	Tol	CH_2Cl_2	*i*PrOH	MeOH	W
30	Tol	CH_2Cl_2	MeCN	MeOH	W

31	*n*Hex	CH_2Cl_2	MeCN	W	
32	*n*Hex	CH_2Cl_2	*i*PrOH	W	
33	*n*Hex	CH_2Cl_2	MeOH	W	
34	*n*Hex	CH_2Cl_2	MeCN	*i*PrOH	W
35	*n*Hex	CH_2Cl_2	*i*PrOH	MeOH	W
36	*n*Hex	CH_2Cl_2	MeCN	MeOH	W

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Scheme 1

With $R = A * B * C * 100$ [%]

$A = K_{max}/F_{max}$,

$B = (F_{max}-K_{min})/F_{max}$

Alignment (C) between $K_{(i)}$ can be expressed as:

$$C = \frac{\prod_{i=1}^{n-1} [K_{(i+1)}-K_{(i)}]}{[(K_{max}-K_{min})/(n-1)]^{(n-1)}}$$

The final equation becomes:

$$R = \frac{K_{max}\,(F_{max}-K_{min}) \prod_{i=1}^{n-1} [K_{(i+1)}-K_{(i)}]}{(F_{max})^2\ [(K_{max}-K_{min})/(n-1)]^{(n-1)}} \times 100\ [\%]$$

in a mixture (closest to F_{max}); K_{min} = minimal partition coefficient of a compound in mixture (closest to 0); and $K_{(i)}$ = partition coefficients of each compound in a mixture sorted from lowest to highest (K_{min} to K_{max}). Solvent mixtures (Table 2) 3, 11, 16, 17, 25, 26, 28, 35 were eliminated due to the prolonged (54-158 sec) time for separation of upper and lower phase. After TLC analysis in *n*hexane-acetonitrile-methylene chloride (5:2:3) only solvent mixtures (Table 2) 2, 4, 22, 23, 24 (15-27 sec. phase separation time) showed good distribution between upper and lower phases. These peptide mixtures were

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Table 3

Solvent mixtures and corresponding dipeptide partition coefficients

Solvent Mixt. No. (Table 2)	K1	K2	K3	K4	K5	R[%] <20,0>	R[%] <10,0>	C[%]
2	6.08	4.48	2.92	1.58	0.71	26.26	50.59	89.58
5	0.47	0.31	0.17	0.08	0.11	1.04	2.08	44.61
22	4.94	4.04	2.60	1.35	0.63	20.70	40.05	86.53
23	7.38	5.11	0.67	1.36	0.61	1.54	2.98	4.29
24	1.98	1.60	1.33	0.54	0.27	6.40	12.62	65.52

Table 4

Vol %					
*n*Hex	Tol	MeCN	*i*PrOH	W	R[%] <20,0>
20.0	20.0	20.0	20.0	20.0	20.70
50.0	0.	0.	25.0	25.0	21.12
33.3	0.	0.	33.3	33.3	26.26

analyzed by HPLC and partition coefficients were calculated by the equation: $K_{(i)}$ = Area upper(i) / Area lower(i). The results are summarized in Table 3.

Solvent mixtures 2 and 22 gave the best response. For detailed analysis solvent component variation by volume was conducted. The resultant partition coefficients ($K_{(i)}$) and responses (R,C) were calculated. By this means the three best solvent mixtures were uncovered and appear in Table 4.

The most appropriate four component solvent system *n*hexane- toluene- acetonitrile- 2-propanol- water 27.2:13.4:

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13.4:23.0:23.0 was calculated by the simplex optimization method[13] and the HPLC analysis was repeated. As this solvent mixture gave the poorer response (11.75%) attention was next directed to optimization of the *n*hexane- 2-propanol- water mixture. The following three steps (Scheme 2) illustrate the final procedure. Optimization was terminated at vertexes S-7 and S-8 when they gave the best responses. Because the P.C. Inc. Model #1 countercurrent unit was designed for resolution with $F_{max}=10$, solvent mixture S-7 was used for resolution of the protected peptides.

Separation (Table 5 and Fig. 1) of the five protected dipeptides by HSCCD employing solvent S-7 gave complete resolution between peaks without any overlapping. Each of the separated dipeptides was found to be identical (by TLC, infrared and 300 MHz ^{1}H-NMR comparisons) with the authentic specimens. As readily apparent in Fig. 1, separation of the five closely related dipeptides was achieved in an essentially ideal manner. Extension of these HSCCD solvent selection procedures to a number of current very difficult biosynthetic product, separation problems is in progress.

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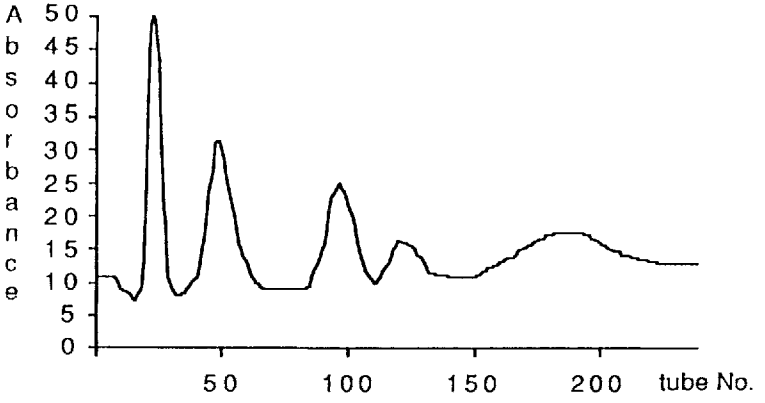

Figure 1

Scheme 2

Step 1. Variation by volume (S-2 to S-4) and simplex optimization (S-5 to S-8)

	Vol %			
Solvent	*n*Hex	*i*PrOH	W	R[%] <20,0>
S-2	25.0	50.0	25.0	5.23
S-3	50.0	25.0	25.0	21.12
S-4	33.3	33.3	33.3	26.26
S-5	41.6	29.2	29.2	32.20
S-6	30.5	43.1	26.4	14.30
S-7	37.4	31.3	31.3	28.79
S-8	45.8	27.1	27.1	44.65

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Step 2. Check the volume of each phase and separation time

	ml			
Solvent	Vu	Vl	Vt	t[s]
S-1	4.4	5.5	9.9	25
S-5	4.7	5.3	10.0	21
S-6	3.5	6.5	10.0	26
S-7	4.4	5.8	10.2	26
S-8	5.3	4.7	10.0	28

Vu=volume of upper phase; Vl=volume of lower phase; Vt=total volume; t[s]=separation time [seconds]

Step 3. Partition coefficients are determined from HPLC analyses and calculated responses

Solvent	K_1	K_2	K_3	K_4	K_5	R[%] <20,0>	R[%] <10,0>	C[%]
S-1	2.80	3.53	1.52	1.01	0.59	11.75	22.78	68.58
S-5	12.39	6.99	5.30	2.07	0.76	32.20	-	54.03
S-6	3.53	2.61	1.70	0.97	0.55	14.30	27.80	83.33
S-7	6.50	4.73	3.45	1.84	0.79	**28.79**	**55.21**	**92.23**
S-8	13.00	9.49	4.66	2.36	0.75	**44.65**	-	71.37

Table 5

Fraction tube No.	V(ml)	mg(isolated)[a]	compound
17-29	88.4	15.9	*N*-Z-L-Ala-L-Val-OMe
38-64	183.6	17.4	*N*-Z-L-Leu-L-Ala-OMe
89-109	142.8	16.5	*N*-Z-L-Val-L-Phe-OMe
112-136	170.0	17.6	*N*-Z-L-Val-L-Leu-OMe
150-245	652.8	18.1	*N*-Z-L-Leu-L-Leu-OMe

[a] the peaks were sharply cut

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