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GENERAL EVALUATION AND APPLICATION TO TRACE ANALYSIS OF A CHIRAL COLUMN FOR LIGAND-EXCHANGE CHROMATOGRAPHY

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SUMMARY

The direct optical resolution of more than twenty amino acids, including the separation of four isomers of *allo*-DL- and DL-isoleucine, can be achieved on a novel ligand-exchange column, MCI GEL CRS10W. The stationary phase, N,N-dioctyl-L-alanine-coated octadecylsilica, shows a stronger affinity for hydrophobic amino acids than for hydrophilic amino acids. General evaluation of the column clarified the effects of the column temperature and Cu^{II} concentration in the eluent on the chiral separation. The determination of trace enantiomers in some commercially available D- and L-amino acids was performed, revealing the presence of 0.03–0.5% of the trace enantiomer. The efficient resolution of strongly retained hydrophobic amino acids and hydroxycarboxylic acids was also achieved on a shortened column.

INTRODUCTION

The optical resolution of DL-amino acids by ligand-exchange chromatography has been intensively studied since the pioneering work of Davankov and co-workers in the late 1970s[1–5]. Following the modification of some of the initial ideas, several columns have become commercially available[6]. However, shortcomings of the commercially available ligand-exchange phases are that the number of amino acids separated on any given chiral column is limited and that the column temperature must be raised in order to increase the degree of resolution.

The column evaluated here, MCI GEL CRS10W, packed with N,N-dioctyl-L-alanine-coated octadecyl (ODS)-silica, demonstrated the efficient resolution of more than 20 amino acids on a single column at room temperature.

Considering reversed-phase packings coated with N-alkyl-L-amino acids, Davankov and Kurganov[5] first reported N-alkyl-L-hydroxyproline-coated ODS-silica as a novel stationary phase for ligand-exchange chromatography in 1980. Although the stationary phase demonstrated a very high selectivity for the optical resolution of various hydrophobic amino acids, the selectivity was not high for hydrophilic amino acids. This present paper describes a chiral stationary phase with a novel ligand that was developed to produce high selectivity for both hydrophilic and hydrophobic amino acids.

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EXPERIMENTAL

Reagents and reference materials

Amino acids and DL-mandelic acid were purchased from Sigma (St. Louis, MO, U.S.A.), DL-lactic acid from Kishida Kagaku (Osaka, Japan) and DL-leucic acid (DL-2-hydroxyisocaproic acid) from Tokyo Kasei (Tokyo, Japan). Copper(II) sulphate pentahydrate was purchased from Kishida Kagaku.

High-performance liquid chromatography

A Shimadzu (Kyoto, Japan) LC-6A system equipped with a Shimadzu SPD-6A UV spectrophotometric detector operating at 254 nm and a Waters Assoc. (Milford, MA, U.S.A.) column-temperature-controlling unit was used. The column, prepared by coating *ca.* 20 wt.-% of N,N-dioctyl-L-alanine on to ODS-silica, is commercially available as MCI GEL CRS10W (50 mm × 4.6 mm I.D.) from Mitsubishi Kasei (Tokyo, Japan). A column with a selectivity opposite to that of MCI GEL CRS10W, packed with N,N-dioctyl-D-alanine-coated ODS-silica, and a short column version (10 mm × 4.6 mm I.D.) of MCI GEL CRS10W were kindly supplied by Mitsubishi Kasei. The eluents evaluated included an aqueous solution of copper(II) sulphate

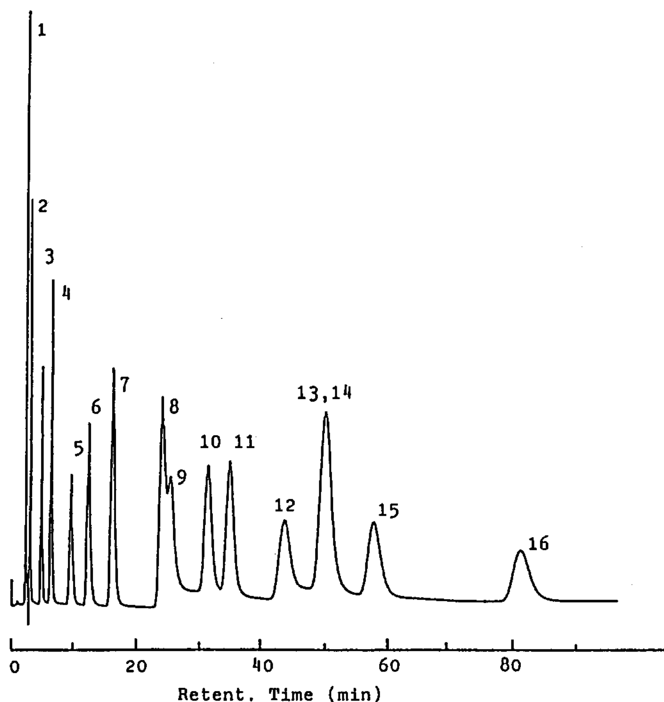

Fig. 1. Resolution of eight amino acid racemates. Column, MCI GEL CRS10W; eluent, 0.5 m*M* copper (II) sulphate solution; flow-rate, 1.0 ml/min; pressure, 110 kg/cm^2; column temperature, 32°C; UV detection, 254 nm. Peaks: 1 = D-Ala; 2 = L-Ala; 3 = D-Pro; 4 = D-Val; 5 = L-Pro; 6 = L-Val; 7 = D-Leu; 8 = D-Nle; 9 = D-Tyr; 10 = L-Leu; 11 = D-Eth; 12 = L-Tyr; 13 = L-Nle; 14 = D-Phe; 15 = L-Eth; 16 = L-Phe.

(between 0.1 and 2 m*M*) and a 2 m*M* solution of copper(II) sulphate in methanol–water (15:85, v/v). For trace analysis, a Shimadzu LC-5A system with a Shimadzu RF-530 fluorimetric detector was used.

RESULTS AND DISCUSSION

Resolution of amino acids

The MCI GEL CRS10W stationary phase is based on 3-μm ODS-silica of 100 Å mean pore diameter. It gave high separation factors, owing to a combination of ligand-exchange and hydrophobic interactions with N,N-dioctyl-L-alanine-coated ODS-silica. The column, although 50 mm long, gave sufficient retention and a high resolution even at ambient temperature (25–30°C). The durability of the column was also sufficient for practical use.

Fig. 1 shows the chiral separation of eight amino acids into their respective D- and L-enantiomers. The D-isomers were eluted ahead of the L-isomers in all instances, and hydrophilic amino acids were eluted ahead of hydrophobic amino acids. A remarkable baseline separation of four isomers of isoleucine was also achieved on this column, indicating that the amino acid contains 33% of *allo*-DL-isomers (Fig. 2).

The interaction between the amino acids to be resolved and the stationary phase can be understood in terms of a superposition of a complexing process and hydrophobic interaction. Hence the factors controlling retention and enantioselectivity, column temperature, Cu^{II} concentration in the eluent and the type of Cu^{II} salt, were varied and their effects were investigated.

Effect of column temperature

The column temperature was increased from 30 to 50°C at a constant eluent concentration [1 m*M* aqueous solution of copper(II) sulphate]. The chromatograms in Fig. 3 of a mixture of L-amino acids show a noticeable decrease in retention with increasing temperature. The separation factor also decreased with increasing temperature (Table I), suggesting that the temperature dependence of the complex stability must be greater for the later eluted isomer[1].

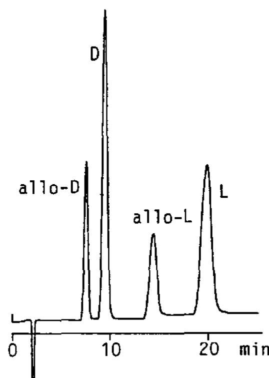

Fig. 2. Resolution of 20 μl of a 1 m*M* aqueous solution of DL-isoleucine. Conditions: eluent, 2 m*M* copper(II) sulphate solution; flow-rate, 1 ml/min; pressure, 100 kg/cm²; temperature, 30°C; UV detection, 254 nm (0.04 a.u.f.s.).

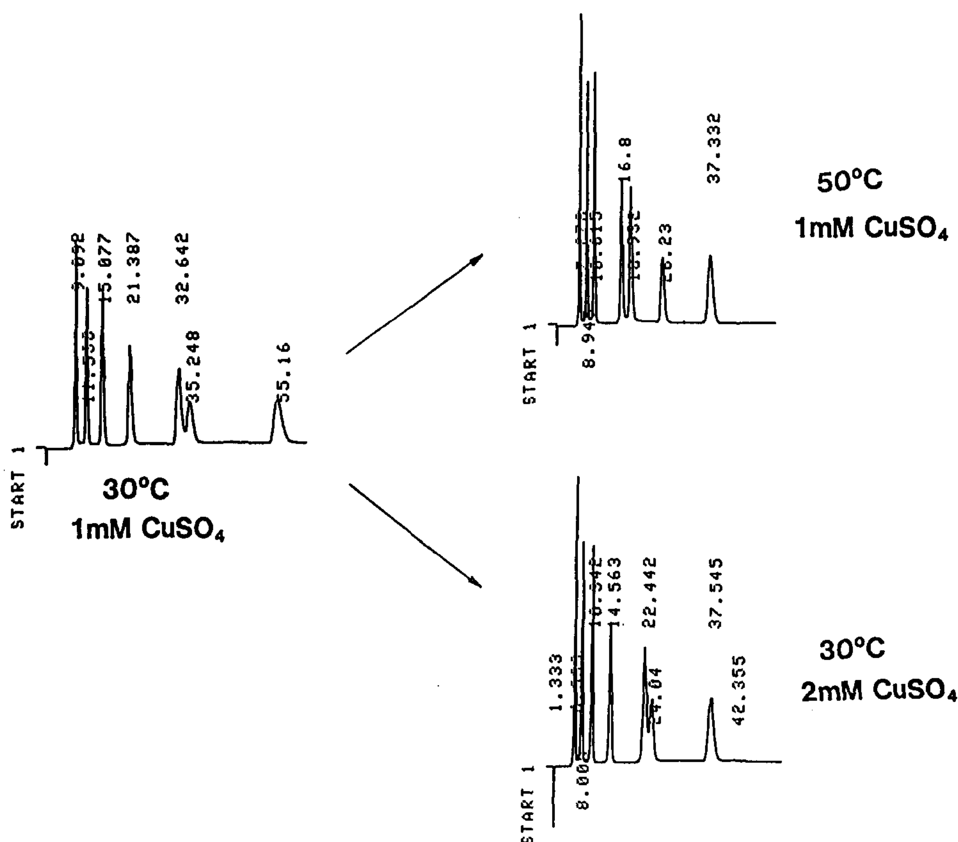

Fig. 3. Effect of temperature and copper(II) sulphate concentration on the resolution of an L-amino acid mixture. Order of elution: L-valine, L-norvaline, L-methionine, L-leucine, L-tyrosine, L-norleucine and L-phenylalanine.

Effect of Cu^{II} concentration and type of Cu^{II} salt

The copper(II) sulphate concentration in the eluent was increased from 1 to 2 m*M* at a constant temperature (30°C). It was observed that an increase in Cu^{II} con-

TABLE I

EFFECT OF TEMPERATURE AND COPPER(II) SULPHATE CONCENTRATION ON THE RESOLUTION OF DL-VALINE

Flow-rate, 1 ml/min; pressure, 110 kg/cm^2; detection, 254 nm.

$CuSO_4$ (m*M*)	*pH*	*Temperature* (°C)	k'_D	k'_L	α	R_s
1.0	5.30	30	7.32	14.93	2.04	5.01
1.0	5.30	50	6.05	11.68	1.93	4.26
2.0	5.15	30	5.04	10.30	2.04	4.59

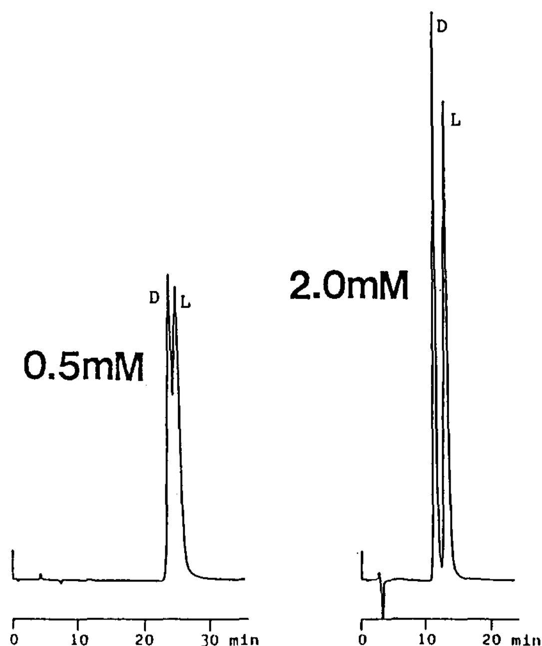

Fig. 4. Effect of copper(II) sulphate concentration in the eluent on the resolution of DL-aspartic acid (1 mM aqueous solution, 20 μl). Conditions: eluent, 0.5 mM (left) and 2 mM copper(II) sulphate solution (right); flow-rate, 1.0 ml/min; pressure, 110 kg/cm^2; temperature, 30°C; detection, 254 nm (0.16 a.u.f.s.).

centration decreases the retention time but has no effect on the resolution efficiency (Table I). The observed tendency can be explained by the fact that the lower the concentration of Cu^{II} in the eluent, the slower is the ligand exchange between the amino acid and the ligand on the stationary phase, which results in greater retention.

It is interesting to compare the changes in the pattern of the chromatograms in Fig. 3. It is clear that temperature affects the pattern of the chromatograms drastically, whereas the Cu^{II} concentration only affects the retention of the L-isomer. This tendency was also observed for D-isomers. Therefore, in order to weaken the interaction and consequently shorten the retention time, while maintaining the pattern of the chromatogram, an increase in the Cu^{II} concentration in the eluent seems favourable.

Subsequently, copper(II) sulphate was replaced with copper(II) acetate and the eluent concentration was altered. Comparison of the sulphate and acetate showed that the anions have no apparent effect on the enantioselectivity of the column, but in general copper(II) sulphate gave smaller retentions, probably owing to the lower ratio of copper(II) acetate [pH = 6.10 for 0.25 mM copper(II) acetate whereas pH = 5.50 for 0.25 mM copper(II) sulphate].

A striking effect of the copper(II) sulphate concentration on the resolution was observed for some acidic amino acids, especially DL-aspartic acid (Fig. 4). Copper(II) sulphate, rather than copper(II) acetate, and at a higher concentration gave a notably better resolution. Restriction of dissociation of the ω-carboxyl group of aspartic acid can be offered as an explanation.

TABLE II

OPTIMUM RESOLUTION CONDITIONS

Temperature, 32°C; detection, 254 nm.

Amino acid	*$CuSO_4$ (mM)*	*pH*	*Flow-rate (ml/min)*	*Retention time (min)*		k'_D	k'_L	α	R_s
				t_D	t_L				
Ornithine	0.1	5.60	0.2	6.00	6.83	1.15	1.45	1.26	–
Lysine	0.1	5.60	0.2	6.23	7.75	1.23	1.78	1.45	0.7
Alanine	0.1	5.60	0.5	8.22	10.99	6.34	8.81	1.39	1.4
Histidine	0.1	5.60	0.5	6.88	10.52	5.14	8.39	1.63	1.7
Serine	0.1	5.60	0.5	8.34	10.13	6.45	8.04	1.25	1.0
Threonine	0.1	5.60	0.5	9.04	11.34	7.07	9.13	1.29	1.3
Citrulline	0.5	5.35	0.5	6.42	10.40	4.73	8.29	1.75	2.3
Proline	1.0	5.25	1.0	3.72	7.31	5.64	12.04	2.13	4.5
Valine	1.0	5.25	1.0	4.66	8.92	7.32	14.93	2.04	5.0
Norvaline	1.0	5.25	1.0	5.85	11.51	9.45	19.55	2.07	4.7
Aspartic acid	2.0	5.15	0.5	11.52	13.22	5.20	6.11	1.18	0.9
Glutamic acid	2.0	5.15	1.0	10.76	16.22	18.21	27.96	1.54	2.3
Leucine	2.0	5.15	1.0	7.68	14.59	12.71	25.05	1.97	4.6
Tyrosine	2.0	5.15	1.0	12.42	22.45	21.18	39.09	1.85	5.3
Ethionine	2.0	5.15	1.0	15.84	26.37	27.29	46.09	1.69	5.0
Phenylalanine	2.0	5.15	1.0	20.75	37.79	36.05	66.48	1.84	6.3

Considering the effect of Cu^{II} concentration on retention and enantioselectivity, optimum resolution conditions for various racemic amino acids are summarized in Table II.

Effect of sample size on the number of theoretical plates

The maximum sample load capacity for a reasonable resolution was determined for L-methionine and L-alanine. The data in Fig. 5 indicate that, in order to attain a

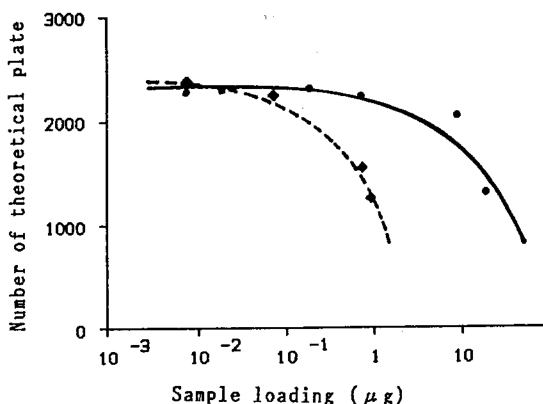

Fig. 5. Effect of sample load on the theoretical plate number for (◆) L-alanine and (●) L-methionine. Conditions for L-alanine: eluent, 0.1 m*M* copper(II) sulphate solution; flow-rate, 0.5 ml/min; pressure, 40 kg/cm^2; temperature, 30°C; detection, 254 nm. Conditions for L-methionine: eluent, 2 m*M* copper(II) sulphate solution; flow-rate, 1 ml/min; pressure, 97 kg/cm^2; temperature, 30°C; detection, 254 nm.

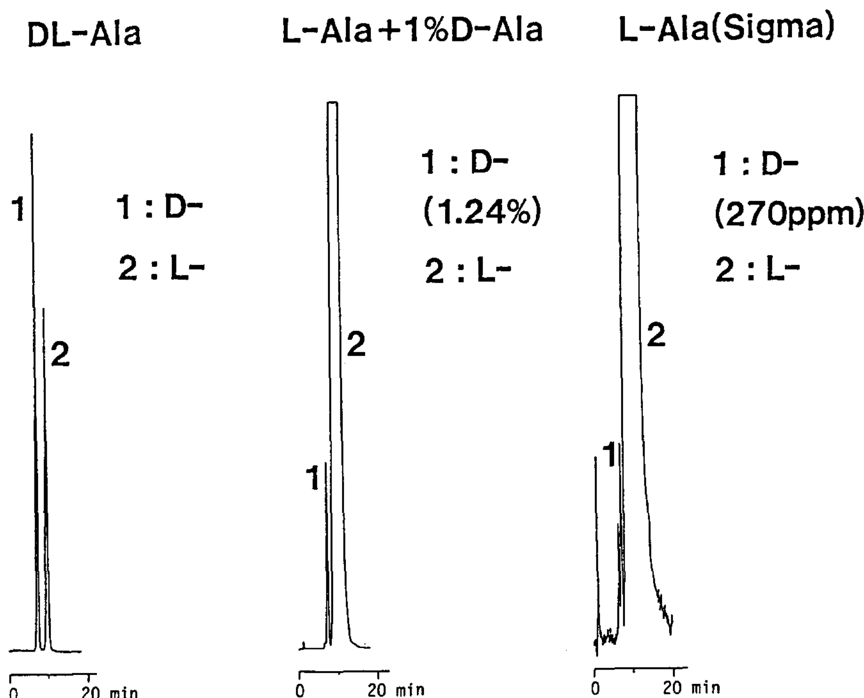

Fig. 6. Trace analysis of D-isomer content in L-alanine (100 ppm solution, 20 μl). Conditions: column, 50 mm × 4.6 mm I.D.; eluent, 0.1 m*M* copper(II) sulphate solution; flow-rate, 0.7 ml/min; temperature, ambient; fluorimetric detection (OPA post-column reaction method), exitation at 348 nm and emission at 450 nm, reaction temperature, 55°C.

theoretical plate number over 2000, 1 g of L-methionine and 0.1 g of L-alanine represent the maximum amount of sample.

After this investigation, the column was washed with distilled water. Subsequently, a decrease in the retention time for any given amino acid was observed. The retention was regained after passing 500 μl of 4% copper(II) acetate solution through the column. This indicates that Cu^{II} release from the Cu^{II}-complexed ligand on the stationary phase was the cause of the decrease in retention. It is not clear whether continuous overloading of the sample or the washing of the column causes the release of Cu^{II}. It should be noted that this was the only time that the column was washed with distilled water.

Effect of Cu^{II} salt in sample solutions

It was observed that sample solutions prepared with the eluent sometimes gave smaller peaks for the later eluted isomer, and not the expected 1:1 peak ratio for racemates. It was assumed that this phenomenon was due to the complex formation between the amino acids and Cu^{II} in the eluent solution and subsequent interaction with the stationary phase, causing elution of different ratios of Cu^{II}-complexed amino acids and non-complexed amino acids. This would create a difference in the mass of

the detectable Cu^{II}–amino acid complex. This assumption was verified by the 1:1 ratio of the peak areas of the resolved isomers when distilled water was used to dissolve the sample.

Resolution of hydroxycarboxylic acids

In addition to amino acids, organic acids that form complexes with Cu^{II} were also separated. A baseline separation of DL-lactic acid was achieved on this column by using 1 m*M* copper(II) sulphate solution as the eluent. In general, the elution of hydroxycarboxylic acids required a higher concentration of copper(II) sulphate in the eluent than did amino acids with similar side-chains. In most instances, faster elution was achieved by the addition of a small amount of methanol to the eluent. However, hydroxycarboxylic acids such as DL-leucic acid and DL-mandelic acid required more than 50 min for elution, even after addition of 15% (v/v) of methanol.

Table III summarizes the results for the resolution of some of the above-mentioned "long-retained" enantiomers on a short column (10 mm × 4.6 mm I.D.). Baseline separation of DL-leucic acid and DL-mandelic acid in less than 10 min was achieved on this column. The resolution of strongly retained hydrophobic amino acids, DL-phenylalanine and DL-tryptophane, was also possible.

Evaluation of the column durability

As the stationary phase is prepared by coating a hydrophobic ligand on to a reversed-phase packing, the stability of the immobilized ligand was of critical concern. The stability of the stationary phase was therefore evaluated. The changes in the capacity factors and separation coefficients after continuous analysis of some amino acids are shown in Table IV. When an aqueous solution was used as the eluent, no change was observed even after passing 21 l of the eluent. During that period, 800 analyses of the same sample were carried out, and no significant change of the chromatogram was obtained. On the other hand, when methanol was added to the eluent (15%, v/v), the capacity factors gradually decreased on increasing the elution volume. However, as the extent of the decrease was relatively small even after passing 21 l of

TABLE III

RESOLUTION OF AMINO ACIDS AND HYDROXYCARBOXYLIC ACIDS ON A SHORT COLUMN (10 mm × 4.6 mm I.D.)

Substance	*Conditions**	*Retention time (min)*		α	R_s
		t_D	t_L		
Phenylalanine	I	4.52	7.85	1.76	1.55
Tryptophan	II	3.49	5.95	1.71	1.14
Mandelic acid	II	4.35	6.68	1.54	0.94
Leucic acid	II	6.02	8.45	1.41	0.92

* (I) Sample, 1 m*M* aqueous solution, 20 μl; eluent, 2 m*M* copper(II) sulphate solution (pH 5.15); flow-rate, 1 ml/min; pressure, 7 kg/cm^2; temperature, 30°C; detection, 254 nm; (II) sample, 1 m*M* aqueous solution, 40 μl; eluent, 2 m*M* copper(II) sulphate solution in methanol–water (15:85, v/v) (pH 5.10); flow-rate, 2 ml/min; pressure, 37 kg/cm^2; temperature, 30°C; detection, 254 nm.

TABLE IV

CHANGE IN CAPACITY FACTORS AND ENANTIOSELECTIVITY WITH ELUTION VOLUME

Amino acid	*Conditions**	*Elution volume*							
		1 l				*21 l*			
		k'_D	k'_L	α	R_s	k'_D	k'_L	α	R_s
Proline	I	9.37	19.10	2.03	3.87	9.24	18.89	2.04	3.75
Valine	I	12.29	24.27	1.97	4.73	12.22	24.09	1.97	4.61
Phenylalanine	II	13.58	21.58	1.59	1.74	13.18	20.14	1.53	1.61

* (I) Sample, 0.5 mM aqueous solution, 20 μl; eluent, 0.5 mM copper(II) sulphate solution (pH 5.35); flow-rate, 1 ml/min; pressure, 110 kg/cm^2; temperature, 25°C; detection, 254 nm; (II) sample, 1.5 mM aqueous solution, 25 μl; eluent, 2 mM copper(II) sulphate solution in methanol–water (15:85, v/v) (pH 5.15); flow-rate, 1 ml/min; pressure, 126 kg/cm^2; temperature, 25°C; detection, 254 nm.

the eluent, and the separation coefficient remained almost constant, the decrease in the capacity factors would be a negligible problem in practical use.

Trace analysis

Sub-nanomole sensitivity is achieved by post-column derivatization with *o*-phthalaldehyde and subsequent fluorimetric detection[7], as shown in Fig. 6. For the trace analysis of L-isomers in D-amino acids, a column with opposite selectivity was used to enhance the detectability[8]. A straight line was obtained when the concentration of eluted L-isomer was plotted against incremental 100 ppm additions of L-isomer. The trace amount of initial L-isomer present in the corresponding D-isomer was determined by reading off the abscissa. The contents of L-isomers in D-aspartic acid and D-phenylalanine were 5000 and 800 ppm, respectively.

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