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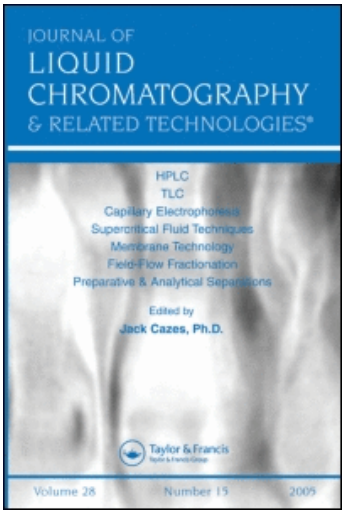

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A Method to Study Column Efficiency in Indirect HPLC Enantioseparation

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ABSTRACT

Four ways are proposed for the comparison of true column performance for various C_{18} columns: plotting the resolution vs. the retention factor, plotting the resolution rate *vs.* retention factor, calculating the retention time needed for a given resolution, or the resolution obtained in a given time. The utilization of these methods is shown for eleven C_{18} columns in the separation of the enantiomers of *erythro*-β-methyl amino acids. The indirect chromatographic method involved pre-column derivatization with new chiral derivatizing agents (CDAs), such as (1*S*,2*S*)-1,3-diacetoxy-1-(4-nitrophenyl)-2-propyl isothiocyanate [(*S,S*)-DANI], and (*S*)-*N*-(4-nitrophenoxycarbonyl)phenylalanine methoxyethyl ester [(*S*)-NIFE],

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and the separation of the diastereomers formed on different types of C_{18} columns. The different columns were compared in systematic chromatographic examinations. The influence of various parameters of the columns on the resolution and the time of analysis are discussed.

Key Words: High performance liquid chromatography; Column efficiency; *erythro*-β-Methyl amino acids; (1*S*,2*S*)-1,3-Diacetoxy-1-(4-nitrophenyl)-2-propyl isothiocyanate; (*S*,*S*)-DANI; (*S*)-*N*-(4-Nitrophenoxycarbonyl)phenylalanine methoxyethyl ester; (*S*,*S*)-NIFE.

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INTRODUCTION

Column efficiency is generally defined by the number of theoretical plates (N) or by the height equivalent to the theoretical plate (HETP). The number of theoretical plates gives only the efficiency of a column in terms of the band broadening of a compound during its passage through the column. The separating power of a column for two adjacent components can be described by the resolution R_S of the two peaks.

When one speaks about chromatography, separation and speed must always be considered together. In the early period of chromatography (gas chromatography), different terms were introduced. Golay proposed the performance index to express the relationship among the resolving power of a column, the time of analysis, and the pressure drop.[1] Purnell[2] introduced the separation factor, which is identical to the number of effective plates of Desty et al.,[3] described two years later in 1962. Desty et al. also proposed to define column performance as the rate of production of the effective plates, i.e., N/t_R, the number of effective plates per retention time; the higher this value, the better the column. Kaiser introduced the separation number (SN), Trennzahl (TZ), the number of peaks separated between two consecutive alkanes:[4]

$$\mathrm{SN} = \left[\frac{t_{R2} - t_{R1}}{w_{h1} + w_{h2}}\right] - 1 \tag{1}$$

where w_h is the peak width at half-height. The correlation with resolution reads:

$$\mathrm{SN} = 0.8495R_S - 1 \tag{2}$$

Hurrel and Perry used the effective peak number EPN:[5]

$$\mathrm{EPN} = R_S - 1 \tag{3}$$

The last two expressions have been demonstrated to be only modified forms of the resolution, R_S, and there is no reason for using them instead of R_S.

The problem with all of these terms is that one cannot demonstrate easily that the resolving power and the speed of analysis are characteristics, which can be traded against each other, nor can they be used, for example, to compare columns on an equal resolution or equal time basis.

Column performance may be characterized by the resolution rate (RR), introduced by Averill:[6]

$$\mathrm{RR} = \frac{R_S}{t_R} \tag{4}$$

which also includes the time needed for a given resolution, i.e., the rate of analysis. Ettre and March[7–9] and Szepesy and Lakszner,[10] in gas chromatography, proposed to express true column performance on the basis of resolution and time, and particularly by plotting resolution against retention time.

In liquid chromatography, the theory and practice of the various column types are well established, but few data are available in the literature, as regards comparisons of the performance of the various columns on resolution and time bases. Erni, in 1983, stated that the routine applications of HPLC are the main beneficiaries of increased efficiency in separation per unit time.[11] Similarly to Desty et al.,[3] Buszewski et al.[12] suggested expressing the efficiency of HPLC columns on the basis of the number of theoretical plates per time unit. Guillaume and Guinchard introduced a new chromatographic resolution function, which provided the most efficient separation.[13,14] Zhou described a new quantitative parameter S (column efficiency) for the column separation efficiency.[15] Kaiser and Kaiser discussed and compared the theoretical plate height, or theoretical plate number concept, in HPLC with the TZ and SN at the optimum speed.[16] Stringham developed a model that predicts a defined relation between chiral subcritical fluid chromatography resolution and analysis time.[17] The model predicts the value of the retention time required to obtain a desired resolution. In recent years, several papers have dealt with the efficiency and comparison of monolithic C_{18} columns, but few of them have discussed efficiency with relation to the time of analysis.[18–22]

In this paper, we report on a systematic investigation in which the performances of different C_{18} columns were compared with indirect chiral separation. For the comparisons, (1*S*,2*S*),(1*R*,2*R*)-*erythro*-β-methylphenylalanine (β-MePhe), (1*S*,2*S*),(1*R*,2*R*)-β-methyltyrosine (β-MeTyr), (1*S*,2*S*),(1*R*,2*R*)-β-methyltryptophane (β-MeTrp), and (1*S*,2*S*),(1*R*,2*R*)-β-methyl-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (β-MeTic) were selected and were

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derivatized with the new chiral derivatizing agents (CDAs) (1*S*,2*S*)-1,3-diacetoxy-1-(4-nitrophenyl)-2-propyl isothiocyanate [(*S,S*)-DANI], and (*S*)-*N*-(4-nitrophenoxycarbonyl)phenylalanine methoxyethyl ester [(*S*)-NIFE]. The diastereomers formed were separated under isocratic conditions with the application of a water/methanol (both contained 0.1% trifluoroacetic acid) mobile phase system. Our goal was to demonstrate the usefulness of comparing true column performances on four bases: resolution vs. retention factor plots, resolution rate vs. retention factor plots, the retention time needed to achieve a given resolution, and the resolution attained in a given time.

EXPERIMENTAL

Chemicals and Reagents

Racemic (1*S*,2*S*),(1*R*,2*R*)-*erythro*-β-MePhe, (1*S*,2*S*),(1*R*,2*R*)-β-MeTyr, (1*S*,2*S*),(1*R*,2*R*)-β-MeTrp, and (1*S*,2*S*),(1*R*,2*R*)-β-MeTic were from Acros Organics (Geel, Belgium). (*S*)-NIFE was from Solvay-Peptysyntha (Brussels, Belgium), and (*S,S*)-DANI was prepared in our laboratory.[23,24]

Acetonitrile (MeCN) and methanol (MeOH) of HPLC grade were purchased from Merck (Darmstadt, Germany). Triethylamine (TEA), trifluoroacetic acid (TFA), and other reagents of analytical reagent grade were also from Merck. The Milli-Q water was further purified by filtering it on a 0.45-μm filter, type HV, Millipore (Molsheim, France).

The eluents were degassed in an ultrasonic bath, and helium gas was purged through them during the analyses.

Apparatus

The HPLC measurements were carried out on a Waters HPLC system consisting of an M-600 low-pressure gradient pump, an M-996 photodiode-array detector and a Millenium^{32} Chromatography Manager data system. A second Waters Breeze system consisted of a 1525 binary pump, a 487 dual-channel absorbance detector, a 717 plus autosampler, and Breeze data manager software (both systems from Waters Chromatography, Milford, MA). Both chromatographic systems were equipped with Rheodyne Model 7125 injectors (Cotati, CA) with 20-μL loops.

The reversed-phase (RP) stationary phases used to perform the indirect analyses were octadecyl-modified, spherical, and end-capped silica-based phases: (**1**) Nucleosil, 5-μm particle size (150 × 4.6 mm) and (**2**) Nucleosil, 10-μm (250 × 4.6 mm) (Macherey-Nagel, Düren, Germany); (**3**) LiChrospher 100 RP18, 5-μm (125 × 4.0 mm) and (**4**) LiChrospher 300, 10-μm (250 × 4.0 mm)

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(Merck); (**5**) Vydac 218TP54, 5-μm (250 × 4.6 mm) and (**6**) Vydac 218TP104, 10-μm (250 × 4.6 mm) (The Separations Group, Hesperia, USA); (**7**) Nova Pak, 4-μm (150 × 3.9 mm) and (**8**) Symmetry, 5-μm (150 × 4.0 mm) (Waters, (Waters, Milford, USA); (**9**) Hypersil ODS, 5-μm (250 × 4.6 mm) (Shandon, Rucaron, Anglia); (**10**) Discovery, 5-μm (Supelco, Bellafonte, USA); (**11**) Hyperpep 300, 5-μm (250 × 4.6 mm), (Shandon). The most important physicochemical parameters of the C_{18} columns are listed in Table 1.

Derivatization Procedure

For derivatization the amino acid was dissolved in water (1 mg mL^{-1}). Derivatization of the investigated analytes with (*S,S*)-DANI[23,24] and with (S)-NIFE[25] was performed in accordance with the method reported in the literature. The derivatized amino acid was detected at 245 nm [(*S,S*)-DANI] and at 205 nm [(*S*)-NIFE].

RESULTS AND DISCUSSION

The retention of the (*S,S*)-DANI–*erythro*-β-MePhe diastereomers on the C_{18} columns exhibited typical RP behavior. With increasing organic modifier content in the mobile phase, the retention factors of both the first- and the second-eluted diastereomers decreased (data not shown). Increase of the MeOH content in the H_2O (0.1% TFA)/MeOH (0.1% TFA) mobile phase system from 45% to 70% (v/v), resulted in a 20- to 40-fold decreases in the retention factors. The largest decrease was observed for the Hypersil ODS column (**9**) (100-fold), and the smallest for the Nucleosil and LiChrospher columns (**1**), (**2**), and (**4**) (20-fold). It was also observed that the carbon content of the stationary phase had a great influence on the retention, which is typical in hydrophobic chromatography. At a given mobile phase composition, H_2O (0.1% TFA)/MeOH (0.1% TFA) = 40/60 (v/v), the stationary phases with the highest carbon content, i.e., (**1**), (**2**), (**3**), and (**8**), exhibited the largest retention factors. For the second-eluted diastereomer k_2, the values on these columns were $k_2 = 4.8$, 4.52, 6.42, and 11.54, respectively. Exceptions were column (**9**), which had the smallest one, $k_2 = 1.80$, and column (**11**), for which the carbon content was low (C% = 6.5), but $k_2 = 7.7$.

To compare the separating power of the columns investigated, the efficiency and resolution data were calculated. The data in Tables 2 and 3 originate from the curve fitting of different functions, e.g., N vs. k_2; R_S vs. t_2; R_S vs. k_2 and RR vs. k_2. The accuracy of the fits was calculated via the mean square errors (MSE = variance + bias^2).

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Table 1. Physicochemical parameters of C_{18} columns applied for the separation of (*S,S*)-DANI-derivatized *erythro*-β-MePhe diastereomers.

Column	Particle size (μm)	Pore size (Å)	Surface area (m^2 g^{-1})	Pore volume (cm^3 g^{-1})	Surface coverage (μmol/m^{-2})	Carbon content (%C)	End-capped	Column size (mm × mm I.D.)
(**1**) Nucleosil 5	5.0 ± 1.5	100	350	1.00	2.06	14.0	Y	150 × 4.6
(**2**) Nucleosil 10	10.0	100	350	1.00	2.06	14.0	Y	250 × 4.6
(**3**) LiChrospher 5	5.0	100	350	1.25	3.61	21.0	Y	125 × 4.0
(**4**) LiChrospher 10	10.0	300	80	1.00	3.89	5.0	Y	250 × 4.0
(**5**) Vydac218TP54	4.6–5.3	250–300	66–80	0.40–0.50	—	7.0–8.5	Y twice	250 × 4.6
(**6**) Vydac218TP104	10.0	250–300	90	0.75	—	7.0–8.5	Y	250 × 4.6
(**7**) Nova Pak	4.0	60	120	0.30	—	7.3	Y	150 × 3.9
(**8**) Symmetry	5.0	100	335	0.90	2.25	19.1	Y	150 × 4.6
(**9**) Hypersil ODS	5.0	120	170	0.65	2.84	10.0	Y	250 × 4.6
(**10**) Discovery	5.0	180	200	1.00	3.30	12.5	Y	250 × 4.6
(**11**) Hyperpep	5.0	300	80	0.90	2.12	6.5	Y	250 × 4.6

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Table 2. Theoretical plates (N_2), HETP, resolutions (R_S), resolution rates (RR) and separation factors (α) for the separation of (*S,S*)-DANI-derivatized *erythro*-β-MePhe diastereomers on different C_{18} columns ($k_2 = 20$).

	Column										
	(**1**) Nucleosil 5	(**2**) Nucleosil 10	(**3**) LiChrospher 5	(**4**) LiChrospher 10	(**5**) Vydac 218TP54	(**6**) Vydac 218TP104	(**7**) NovaPak	(**8**) Symmetry	(**9**) Hypersil ODS	(**10**) Discovery	(**11**) Hyperpep
N_2	190	160	80	900	900	2,400	300	970	5,000	540	1,420
HETP (mm)	0.79	1.56	1.56	0.28	0.28	0.10	0.50	0.15	0.05	0.46	0.18
N_2/L (m^{-1})	1,270	640	640	3,600	3,600	9,600	2,000	6,500	20,000	2,100	5,700
R_S	5.65	3.17	5.25	6.75	6.55	3.68	8.15	10.60	11.25	5.70	8.00
$R_S \times L^{-1/2}$ (cm^{-1})	1.46	0.63	1.49	1.35	1.31	0.74	2.10	2.12	2.25	1.14	1.60
RR (min^{-1})	0.131	0.051	0.159	0.114	0.089	0.049	0.290	0.252	0.193	0.062	0.113
RR $\times L^{-1/2}$ ($min^{-1}cm^{-1}$)	0.034	0.010	0.045	0.023	0.018	0.010	0.075	0.050	0.039	0.013	0.023
α	1.49	1.57	1.51	1.54	1.63	1.61	1.60	1.72	1.53	1.74	1.41

Note: Chromatographic conditions: flow rate, 0.8 mL min^{-1}; detection, 245 nm; mobile phase composition, H_2O (0.1% TFA)/MeOH (0.1% TFA) = 50/50 (v/v); $R_S \times L^{-1/2}$ and RR $\times L^{-1/2}$, resolution and resolution rate divided by the root square of the column length (*L*).

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Table 3. Comparison of column performances on equal resolution or equal time bases.

	Column										
	(**1**) Nucleosil 5	(**2**) Nucleosil 10	(**3**) LiChrospher 5	(**4**) LiChrospher 10	(**5**) Vydac 218TP54	(**6**) Vydac 218TP104	(**7**) Nova Pak	(**8**) Symmetry	(**9**) Hypersil ODS	(**10**) Discovery	(**11**) Hyperpep
Retention time needed to achieve equal resolution ($R_S = 1.5$)											
t_2 (min)	5.3	11.1	4.2	4.8	5.3	10.6	3.0	4.2	5.3	10.1	4.8
Resolution attained in equal time ($t_R = 10$ min)											
R_S	3.7	1.2	3.0	2.0	3.4	1.3	5.0	4.4	3.2	1.1	2.7

Note: Chromatographic conditions: flow rate, 0.8 mL min^{-1}; detection, 245 nm; mobile phase composition, H_2O (0.1%TFA)/MeOH (0.1% TFA) = 50/50 (v/v).

The efficiency data were calculated and are presented in Table 2. The number of theoretical plates and the HETP seems to differ considerably. The numbers of theoretical plates were compared at a constant value of $k_2 = 20$, where the resolution was almost independent of the retention factor of the second-eluted diastereomer (Figs. 1 and 2). As concerns the theoretical plate number and HETP values, the stationary phases of columns (**9**), (**6**), (**11**), (**8**), (**4**), and (**5**) seemed to be the most efficient. Most of these columns had a particle of size 5-μm (with the exceptions of (**4**) and (**6**), with a 10-μm particle size).

The theoretical plate number and HETP values expressed the column efficiency only in term of the band broadening. To express the separation powers of the different columns, the resolution was investigated as a function of the retention factor of the second-eluted diastereomer. Since the resolution is proportional to the square root of the column length (L), columns with different lengths should be compared by using the resolution divided by the square root of the column length: $R_S \times L^{-1/2}$. In Figs. 1 and 2, $R_S \times L^{-1/2}$ values have been plotted as a function of the retention factor of the second-eluted diastereomer, k_2. In Figs. 1 and 2, at low k_2 values, R_S increased steeply with increasing k_2. At about $k_2 \geq 20$, the curves flattened and R_S changed only slightly with increasing k_2. Different stationary phases can be compared only at the same retention factor k_2. Therefore, the separating power for the

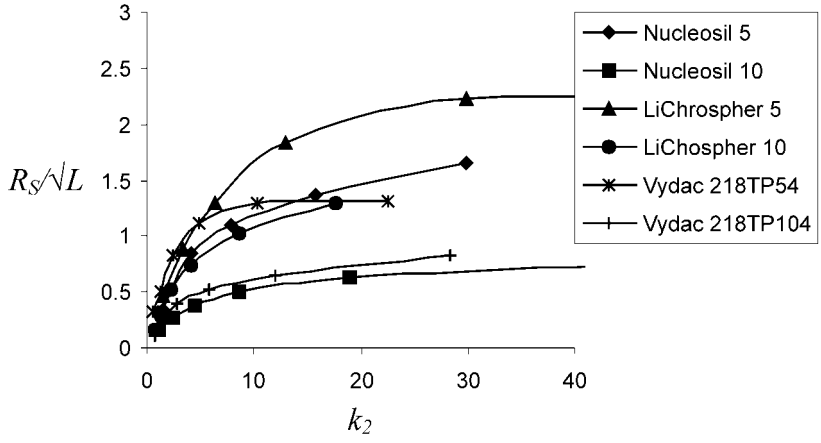

Figure 1. Resolution for different columns of (*S,S*)-DANI–*erythro*-β-MePhe diastereomers divided by the root square the of column length ($R_S/\sqrt{L}$) vs. the retention factor (k_2). Chromatographic conditions: columns—◆, (**1**); ■, (**2**); ▲, (**3**); ●, (**4**); ∗, (**5**); —|—, (**6**); detection, 245 nm; flow rate, 0.8 mL min^{-1}; mobile phase composition, H_2O (0.1% TFA)/MeOH (0.1% TFA).

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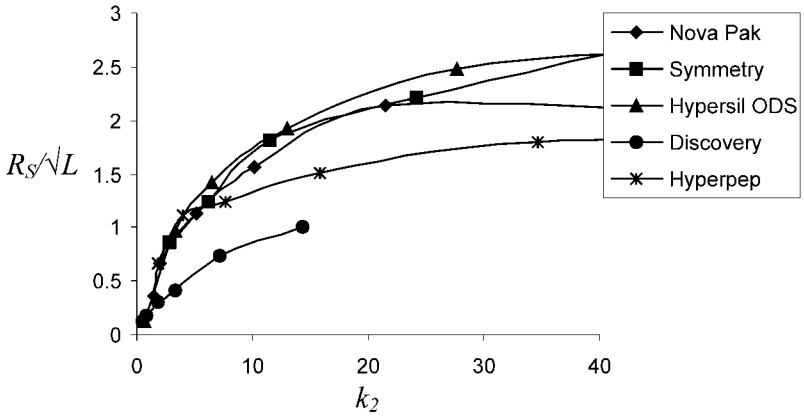

Figure 2. Resolution for different columns of (*S,S*)-DANI–*erythro*-β-MePhe diastereomers divided by the root square of the column length ($R_S/\sqrt{L}$) vs. the retention factor (k_2). Chromatographic conditions: columns—◆, (**7**); ■, (**8**); ▲, (**9**); ●, (**10**); ∗, (**11**); detection, 245 nm; flow rate, 0.8 mL min^{-1}; mobile phase composition, H_2O (0.1% TFA)/MeOH (0.1% TFA).

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separation of the (*S,S*)-DANI-*erythro*-β-MePhe diastereomers was calculated at $k_2 = 20$, where the resolution was almost independent of the retention factor. On this basis, the best columns were (**7**), (**8**), (**9**), and (**11**) (Table 2), all of which had a 5-μm particle size [column (**7**) had a 4-μm particle size]. Of the columns which had particle of sizes both 5 and 10 μm, i.e., columns (**1**) and (**2**); (**3**) and (**4**); and (**5**) and (**6**), the columns with 5-μm exhibited the higher separation power.

If the *rate of analysis* is of interest, for instance in the routine analysis of a high number of samples, column performance can be characterized by the resolution rate RR [Eq. (4)]. Columns with different lengths can be compared again by using RR divided by the square root of the column length: $RR \times L^{-1/2}$. Such plots are shown in Figs. 3 and 4 as a function of the retention factor k_2, and the numerical data are given in Table 2. From the curves in Figs. 3 and 4, two conclusions can be drawn. First, the maximum resolution rate can be achieved at around $k_2 \sim 1.0$ [with the exceptions of columns (**7**) and (**10**)]. Second, the order of the columns is changed with regard to the maximum resolution obtained. On the basis of the resolution rate, the best columns were (**7**), (**8**), (**3**), and (**9**); column (**7**), with a 4-μm particle size, exhibited the highest $RR \times L^{-1/2}$ value. For columns having particle of sizes both 5- and 10-μm, the columns with a 5-μm particle size again exhibited the better separation power [(**1**) vs. (**2**); (**3**) vs. (**4**); and (**5**) vs. (**6**)].

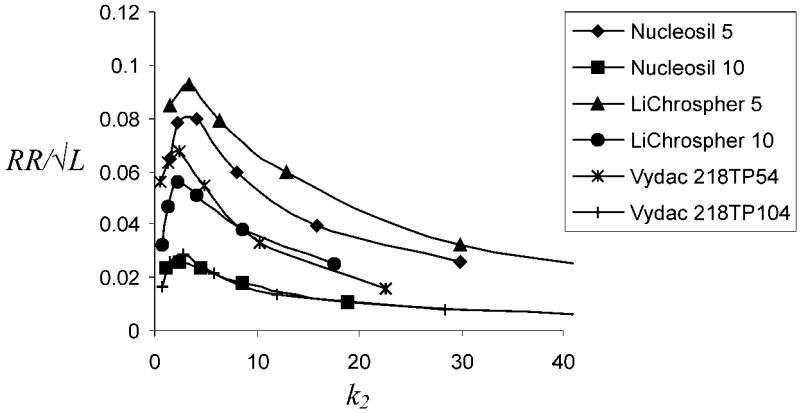

Figure 3. Resolution rates for different columns of (*S,S*)-DANI–*erythro*-β-MePhe diastereomers divided by the root square of the column length ($RR/\sqrt{L}$) vs. the retention factor (k_2). Chromatographic conditions: columns—◆, (**1**); ■, (**2**); ▲, (**3**); ●, (**4**); ∗, (**5**); ⊣⊢, (**6**); detection, 245 nm; flow rate, 0.8 mL min^{-1}; mobile phase composition, H_2O (0.1% TFA)/MeOH (0.1% TFA).

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Column performances can be compared on equal resolution or equal time bases. The results in Table 3 reveal the importance of such a comparison. The retention time needed to achieve baseline resolution ($R_S = 1.5$) was the smallest for columns (**7**), (**3**), and (**8**). The resolution attained in equal time ($t_2 = 10$ min) was again highest for columns (**7**) and (**8**). It seems that the particle size is one of the most important factors governing the separation efficiency based on the time scale.

Application (*S*)-NIFE as derivatizing agent, confirmed results mentioned earlier. At a mobile phase composition, H_2O (0.1% TFA)/MeOH (0.1% TFA) = 45/55 (v/v), comparison of the separation of (*S*)-NIFE–*erythro*-β-MePhe diastereomers on columns (**5**), (**7**), (**9**), and **(10)**, the RR $\times$ $L^{-1/2}$ values were 0.053, 0.104, 0.042, and 0.031, respectively, i.e., the best value was obtained for column **(7)**.

The column efficiency should be influenced not only by the particle size but also by the difference in chemical properties of stationary phases, caused by the difference in the modification method or the silica surface, too. Application of analytes *erythro*-β-MeTyr, *erythro*-β-MeTrp, and *erythro*-β-MeTic analogs confirmed these assumptions. For more hydrophobic β-MeTrp and β-MeTic analogs, column **(5)** was as efficient as column **(7)**, probably owing to the advantageous chemical properties (end-capping procedure). The hydrophobic interactions on the more hydrophobic surface of **(5)** probably

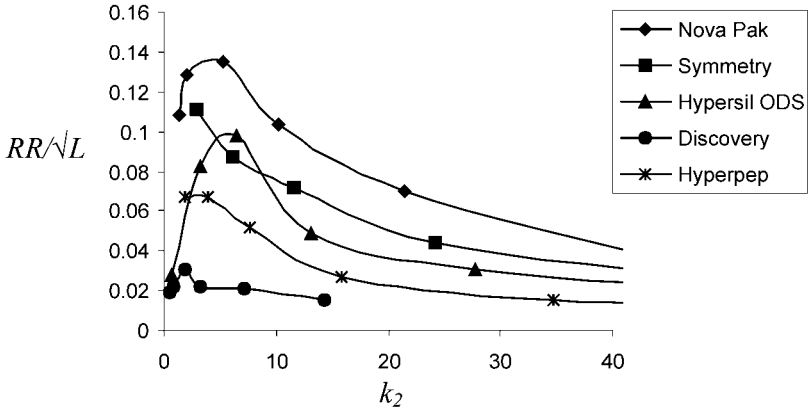

Figure 4. Resolution rates for different columns of (*S,S*)-DANI–*erythro*-β-MePhe diastereomers divided by the root square of the column length ($RR/\sqrt{L}$) vs. the retention factor (k_2). Chromatographic conditions: columns—◆, (**7**); ■, (**8**); ▲, (**9**); ●, (**10**); ∗, (**11**); detection, 245 nm; flow rate, 0.8 mL min^{-1}; mobile phase composition, H_2O (0.1% TFA)/MeOH (0.1% TFA).

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contributed to the chiral discrimination of more hydrophobic analogs. The $RR \times L^{-1/2}$ values for β-MeTrp and β-MeTic analogs were around 0.050 on both columns [(**5**) and (**7**)]. For more hydrophilic *erythro*-β-MeTyr, these values were 0.011 on column **(5)** and 0.063 on column **(7)**, indicating that the hydrophobic character of both the columns and analytes contributed to the chiral discrimination.

The sequence of elution of derivatized diastereomers was determined. The enantiomerically riched (2*R*,3*R*) analytes of β-methyl amino acids were prepared by enzymatic digestion with L-amino acid oxydase.[26] The elution sequence for diastereomers of (*S,S*)-DANI–*erythro*-β-methyl amino acids was (2*R*,3*R*) < (2*S*,3*S*), and for (*S*)-NIFE–*erythro*-β-methyl amino acids was (2*S*,3*S*) < (2*R*,3*R*).

CONCLUSION

A RP indirect high performance liquid chromatographic method was developed for the separation of the enantiomers of *erythro*-β-methyl amino acids, applying pre-column derivatization with new CDAs, (*S,S*)-DANI, and (*S*)-NIFE. The diastereomers formed were separated on different C_{18} columns, and the efficiencies of the columns in the separation of the diastereomers were determined.

The carbon content of the stationary phase determined the retention of diastereomers. Columns with higher carbon content resulted in a higher retention. The hydrophobic character of both the columns and analytes contributed to the chiral discrimination.

These experiments confirmed that the particle size of the columns influenced the resolution decisively. The retention time needed to achieve equal resolution and the resolution attained in equal time was also better on columns with smaller particle size.

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