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Journal of Chromatography A, 855 (1999) 157–170

JOURNAL OF CHROMATOGRAPHY A

www.elsevier.com/locate/chroma

Reproducibility of the separation of astaxanthin stereoisomers on Pirkle covalent L-leucine and D-phenylglycine columns

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Received 12 October 1998; received in revised form 31 May 1999; accepted 2 June 1999

Abstract

The reproducibility of the separation of astaxanthin stereoisomers on columns packed with Pirkle covalent L-leucine chiral stationary phase (CSP) was examined by comparing six columns purchased from the same manufacturer. Differences were found even for columns packed with CSP from the same lot. The reproducibility of columns packed with Pirkle covalent D-phenylglycine CSP was also examined by comparing columns purchased from the same manufacturer as well as from different manufacturers. Significant differences were found for columns packed by different manufacturers. Chiral column-to-column reproducibility for complex stereoisomeric separations should therefore not be taken for granted.

Keywords: Stereoisomer separation; Astaxanthin

1. Introduction

High-performance liquid chromatography (HPLC) is a widely used technique for the separation of enantiomers and diastereomers [1]. The development of chiral stationary phase (CSP) materials for enantioselective chromatography has been very rapid and today virtually any racemate can be at least partially resolved [2]. Although this is a complex and difficult problem, progress in understanding the phenomenon of chiral recognition at the molecular level is being made by the continuous improvement of nuclear magnetic resonance (NMR) techniques for studies of solution structures of model systems [3,4], as well as selector–selectand interactions in docking situations [5]. However, despite considerable progress in the understanding of chiral recognition, a detailed description of how retention and selectivity depends on the various mobile- and stationary-phase variables is still incomplete in many cases, and, therefore, cannot be used for predictive purposes with any degree of certainty [6–11]. Such an understanding is essential for the systematic control and manipulation of chiral separations. Consequently, chiral HPLC method development continues to be largely empirical [7,12,13], whereas in achiral chromatography, several software packages are now available to assist in

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0021-9673/99/$ – see front matter
PII: S0021-9673(99)00700-1

the optimization of resolution. These software packages, however, are still based on preliminary experimental retention and mobile phase data [8,14,15].

Once the appropriate CSP and HPLC conditions for a particular chromatographic separation have been determined, column-to-column reproducibility is paramount. Reproducible retention and resolution of the peaks in a chromatogram are crucial especially when the developed method is to be used routinely by multiple laboratories. When dealing with difficult separations such as the simultaneous chromatographic separation of over a dozen stereoisomers, serious problems arise if retention and resolution of the stereoisomeric peaks vary significantly from column-to-column. However, silica-based reversed-phase (RP) packings contain different amounts of residual silanols [16]. Columns of the same type (e.g., RP-ODS) from different manufacturers often show substantial differences in both retention and resolution due to variations in silica substrate and binding chemistry [14–17]. Columns from different manufacturers are therefore not always interchangeable. Although it has been reported that two columns prepared successively with the same material, using the same packing method, display different chromatography [18], columns packed with stationary phase from the same lot, and which have a similar number of theoretical plates as measured by the manufacturer, are generally expected to be identical or near identical with respect to retention and chromatographic performance. Because column reproducibility is beyond the chromatographer's control, development of methods where the separation factor of the stereoisomers is as large as possible, i.e., where the selectivity is high, is crucial. If the separation factor between the stereoisomers is large, the effects of minor differences in column selectivity are diminished.

Astaxanthin, 3,3′-dihydroxy-β,β-carotene-4,4′-dione, has two asymmetric carbons at the 3 and 3′ positions. All-*trans*-astaxanthin can exist as three stereoisomers: two enantiomers (3*R*,3′*R* and 3*S*,3′*S*) and a *meso* form (3*R*,3′*S* or 3*S*,3′*R*) [19]. Synthetic all-*trans*-astaxanthin consists of a racemic mixture of the two enantiomers (3*R*,3′*R* and 3*S*,3′*S*) and the meso form (3*R*,3′*S* and 3*S*,3′*R*) in the ratio 1:1:2, respectively. The structures of the three stereoisomers of all-*trans*-astaxanthin are shown in Fig. 1.

Astaxanthin gives wild salmon flesh its distinctive color, which is derived entirely from the fish's diet. Astaxanthin and canthaxanthin, β,β-carotene-4,4′-dione, are presently the only color additives that may be legally used in the United States as fish feed color additives to pigment the flesh of aquacultured salmonids [20]. Astaxanthin, which is essentially 3,3′-dihydroxy-canthaxanthin, can easily be distinguished from canthaxanthin by thin-layer chromatography (TLC) [21,22] or HPLC [22]. The US color additive astaxanthin is currently derived synthetically.

The principal *cis* astaxanthin isomers found in the flesh of wild salmon and in the color additive synthetic astaxanthin are the 9-*cis*-, 13-*cis*- and to a lesser extent, the 15-*cis*-astaxanthin (Fig. 2). Astaxanthin in wild salmon flesh has the same *cis:trans* ratio of approximately 1:4 as the color additive astaxanthin. However, the distribution of stereoisomers of all-*trans* astaxanthin in the color additive astaxanthin is different from the distribution of stereoisomers of all-*trans* astaxanthin found in the flesh of wild salmon [19,23–25]. Similarly, the distribution of stereoisomers of each of the *cis* forms of astaxanthin in synthetic astaxanthin is different from the stereoisomeric distribution of the corresponding *cis* forms of astaxanthin found in the flesh of wild salmon [19]. To enforce color additive regulations, the US Food and Drug Administration (FDA) needed a method to determine the presence of synthetic astaxanthin in fish and to distinguish between synthetic astaxanthin and astaxanthin present in wild salmon. A method was also needed to verify any labeling claims of ''wild salmon'' and to confirm the presence of synthetic astaxanthin.

To distinguish between wild salmon and aquacultured salmon fed synthetic astaxanthin, the developed HPLC method must separate the stereoisomers of all-*trans*-astaxanthin from one another and from *cis*-astaxanthin. Overlap of the leading *cis*-astaxanthin enantiomer pair with the trailing all-*trans* enantiomer interferes with the determination of the stereoisomeric ratios of the all-*trans*-astaxanthin. Moreover, for the method to qualify as a regulatory tool in multiple laboratories over an extended period of time, these separations must be maintained from column to column.

We had previously developed a method for the separation of the stereoisomers of all-*trans*-asta-

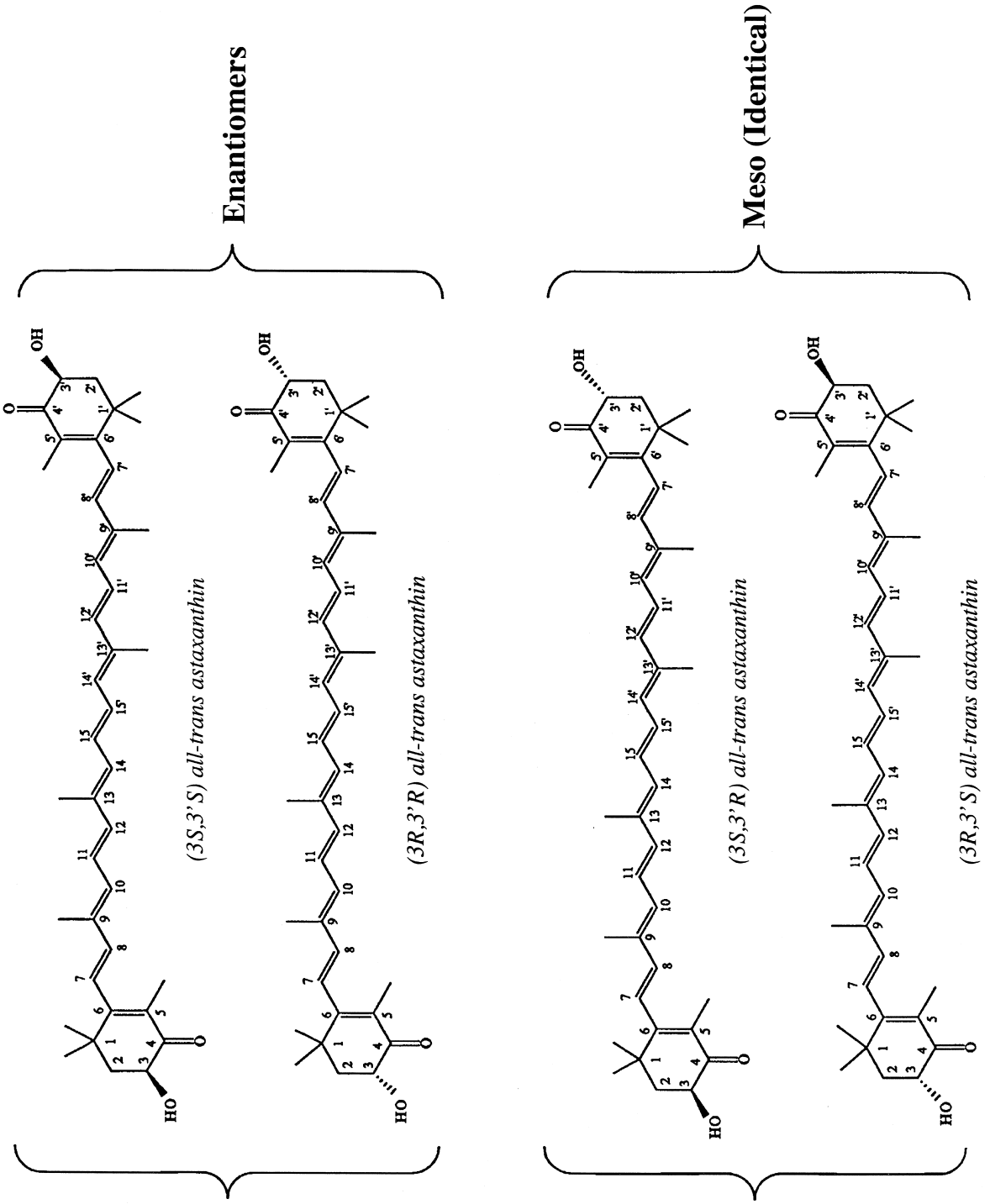
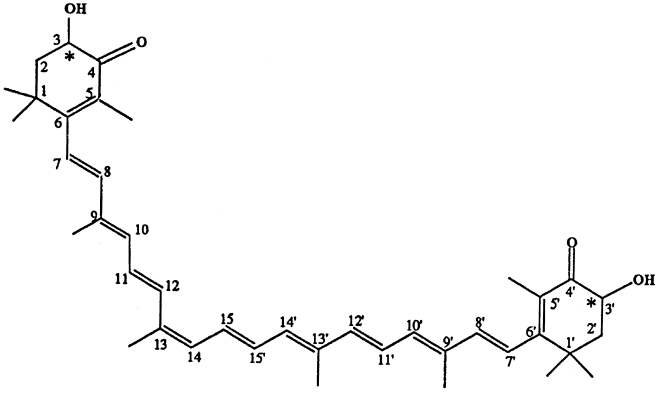
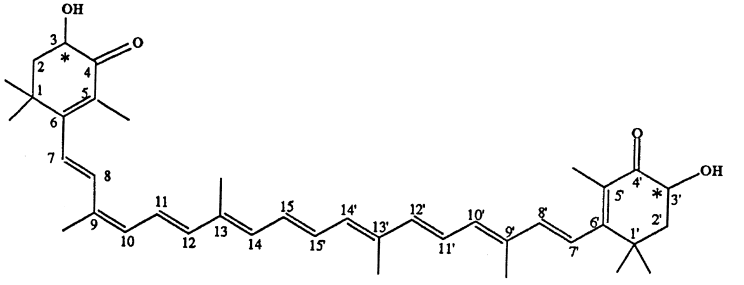


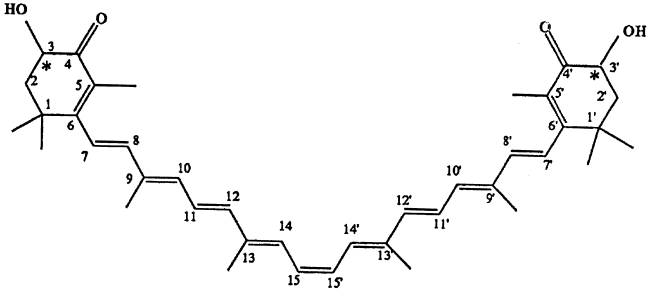
Fig. 1. The stereoisomers of all-*trans* astaxanthins.



13-cis astaxanthin



9-cis astaxanthin



15-cis astaxanthin

Fig. 2. The structures of 9-*cis*-, 13-*cis*- and 15-*cis*-astaxanthins. The chiral centers are designated by an asterisk (*).

xanthin and the partial separation of some of the *cis*-astaxanthin isomers on a Pirkle covalent L-leucine column [26]. The L-leucine method has been used to determine the distribution of the stereoisomers of all-*trans*-astaxanthin in marine-caught, authenticated wild salmon [24,25]. However, when the analysis was repeated with other L-leucine columns that were expected to be identical, a significant problem was encountered. Upon changing columns, overlap was observed between the tail of the last peak of all-*trans*-astaxanthin and the leading edge of the first peak of the *cis*-astaxanthin band profile. This was true for columns packed with CSP from the same lot and purchased from the same manufacturer (see Section 3.2). Each L-leucine column exhibited a different selectivity towards the astaxanthin stereoisomers.

The separation of the astaxanthin isomers on any of the six columns was adequate to distinguish between synthetic astaxanthin and astaxanthin extracted from the flesh of wild salmon. We were concerned, however, that with another batch of L-leucine columns, the leading *cis* isomer peak might totally overlap with the trailing all-*trans* isomer peak. Such a situation would lead to errors in the calculation of the ratios of enantiomers for all-*trans* astaxanthin, which is the basis of the method. Hence, in addition to optimizing the separation of the stereoisomers of all-*trans*-astaxanthin, we also focused on obtaining a large separation factor between the *cis*- and *trans*-astaxanthin isomers.

We subsequently reported a chiral HPLC method that separated the stereoisomers of all-*trans*-astaxanthin from each other and from the *cis*-astaxanthin isomers [27]. The predominant *cis* isomers were also partially separated from one another. We used a Pirkle covalent D-phenylglycine column (Sumichiral OA-2000) manufactured and packed in Japan. The separation obtained was much better than that of the published methods [26,28,29]. The selectivity factor (α) between the last all-*trans* (3*S*,3′*S*) peak and the first 9-*cis* (3*R*,3′*R*) peak is more than 1.5, which significantly reduced the risk of overlap between the *cis*/*trans* peak profiles upon changing to new columns. This method should therefore withstand small column-to-column variations. A similar separation was obtained with two other Sumichiral OA-2000 columns (chiral stationary phase made and packed by the same Japanese manufacturer). However, some of the columns packed with the same D-phenylglycine CSP but purchased from four different manufacturers failed to separate the astaxanthin stereoisomers under the same chromatographic conditions.

The reproducibility of complex stereoisomeric separations using chiral columns packed with Pirkle covalent CSP should therefore always be tested. Furthermore, comparisons between the selectivity of such columns packed with different CSPs should be made with caution.

2. Experimental

2.1. Chemicals and reagents

All the solvents were HPLC grade. *n*-Heptane, *n*-hexane, tetrahydrofuran (THF), methylene chloride and acetone were purchased from Baxter, IL, USA; 2-propanol (IPA) was purchased from Baker, NJ, USA. Synthetic astaxanthin and the commercial color additive mixture containing synthetic astaxanthin (stabilized synthetic astaxanthin, available commercially as Carophyll Pink beadlets) were obtained courtesy of Hoffmann-La Roche (Nutley, NJ, USA). A *cis*/*trans* mixture of astaxanthin isomers was obtained either by isomerization of synthetic all-*trans*-astaxanthin or by extraction of astaxanthin isomers from Carophyll Pink beadlets.

The *cis* isomers were generated by exposing all-*trans* synthetic astaxanthin dissolved in methylene chloride–IPA (1:1) to available light (daylight and fluorescent) for one week. The solvent was evaporated and the residue was dissolved in hexane and dried over silica gel. The astaxanthin was purified by loading the dehydrated hexane solution onto a Waters silica gel Sep-Pak cartridge (Millipore, Milford, MA, USA) and eluting the astaxanthin with chloroform. Alternatively, *cis*/*trans* isomers of astaxanthin were extracted from Carophyll Pink beadlets as previously described [27].

2.2. Instrumentation

The HPLC analysis was performed by using a Waters liquid chromatograph (Millipore), equipped with a photodiode array detector (Waters PDA 996),

three high-pressure pumps (two Model 510 and one 501), an IBM compatible Millennium 2010 chromatography manager for the analysis of data, a Waters 712 WISP autoinjector and autosampler.

2.3. Chiral columns

The three π-acceptor columns (designated as Sumichiral I, II and III, respectively, in the text, See Table 1) that resolved the stereoisomers of the predominant *cis*/*trans* isomers of astaxanthin contained Pirkle covalent D-phenylglycine CSP from different lots made by the same company in Japan: Sumichiral OA-2000 (5 μm), 250×4.0 mm I.D. (Sumika Chemical Analysis Service, YMC Inc., Japan). These three Sumichiral columns were also packed by the same company in Japan. Two other columns packed with Pirkle covalent D-phenylglycine CSP that was manufactured by the same company in Japan (YMC) were purchased from Phenomenex. The dimensions of these columns, designated as Chirex by the manufacturer, are slightly different from those of the Sumichiral columns; Chirex (*R*)-PGLY&DNB columns (5 μm): 250×3.2 mm I.D. (Chirex I), and 250×4.0 mm I.D. (Chirex II) (Phenomenex, Torrance, CA, USA). Another D-phenylglycine column, R(DNPG)-100-10SP (10 μm), 250×4.6 mm I.D. (Amicon Division, W.R. Grace, Danvers, MA, USA), was also tested. A Pirkle covalent D-phenylglycine column (Rexchrom, 5 μm, 100 Å), 250×4.6 mm I.D. was purchased from Regis, Morton Grove, IL, USA. The Pirkle covalent D-phenylglycine columns and their designation in the text are listed in Table 1.

The six columns used in the reproducibility study of the Pirkle covalent L-leucine columns were purchased from Regis. The dimensions of these columns are (5 μm, 100 Å), 250×4.6 mm I.D. Four columns were packed with CSP from one lot, whereas two were packed with CSP from another lot. The π-donor (*R*)-naphthylurea and (*R*)-phenylurea columns (5 μm), 250×4.0 mm I.D., were purchased from Supelco (Bellefonte, PA, USA). The π-acceptor π-donor Whelk-O-1 column (5 μm, 100 Å), 250×4.0 mm I.D., was purchased from Regis.

2.4. HPLC analysis procedure

All the analyses were run under isocratic conditions and at ambient temperature. The flow-rate

Table 1
Nomenclature and chromatographic parameters of Pirkle D-phenylglycine columns

Column trade name and type of CSP	Designation in text	Column dimensions	Manufacturer of CSP	Column packed by
OA-2000 Pirkle covalent D-phenylglycine (5 μm)	Sumichiral I	250×4.0 mm I.D.	YMC[a]	YMC[a]
OA-2000 Pirkle covalent D-phenylglycine (5 μm)	Sumichiral II	250×4.0 mm I.D.	YMC[a]	YMC[a]
OA-2000 Pirkle covalent D-phenylglycine (5 μm)	Sumichiral III	250×4.0 mm I.D.	YMC[a]	YMC[a]
Chirex Pirkle covalent D-phenylglycine (5 μm)	Chirex I	250×3.2 mm I.D.	YMC[a]	Phenomenex
Chirex Pirkle covalent D-phenylglycine (5 μm)	Chirex II	250×3.2 mm I.D.	YMC[a]	Phenomenex
Rexchrome Pirkle covalent D-phenylglycine (5 μm, 100 Å)	Rexchrom	250×4.6 mm I.D.	Regis[b]	Regis[b]
Pirkle covalent D-phenylglycine (10 μm)	Amicon	250×4.6 mm I.D.	W.R. Grace	

[a] Sumika Chemical Analysis Service, YMC, Japan.
[b] Regis Chemical Company (Morton Grove, IL, USA).

was 1 ml/min for the all D-phenylglycine columns and 1.5 ml/min for the L-leucine columns. The monitoring wavelength was 476 nm for the L-leucine columns and 480 nm for the D-phenylglycine columns. At the end of each analytical session the column was washed with IPA–*n*-heptane (1:1, v/v). After heavy use, the column was washed overnight with IPA at a flow-rate of 0.2 ml/min. When not in use, the column was stored in *n*-heptane–IPA (99:1, v/v). Before each injection, the column was equilibrated with the mobile phase until a steady state equilibrium was achieved. *n*-Hexane was initially used as the bulk solvent. It was replaced with *n*-heptane due to safety considerations. This change had no observable effect on retention or chromatographic separation.

3. Results and discussion

3.1. Effect of column aging on chromatographic separations

The resolution of the stereoisomers of all-*trans* astaxanthin and the separation of all-*trans*-astaxanthin from the *cis*-astaxanthins were adequate for the determination of the desired stereoisomeric ratio using the initial L-leucine column, column 1 [23–26]. The selectivity of the column, however, changed over time. Reoptimization of the mobile phase was needed to restore the separation of the astaxanthin stereoisomers. The most likely cause for the change in selectivity is the gradual loss of bonded CSP (i.e., bleeding) over the lifetime of a column, a commonly observed phenomenon for bonded phases [30]. However, when column 1 was in constant use for over five months during the analysis of salmon extracts [24,25], the selectivity of the column did not change (no change was observed in the retention time or the resolution of the astaxanthin stereoisomers). Other possible causes for the change in selectivity include the irreversible adsorption of analyte on the surface of the CSP, the presence of impurities (in the sample or in the mobile phase) that may interfere with the chromatographic separation, and a change in the packing density of the column with continued use.

It is unlikely, however, that the change in selectivity was due to the latter factors under the conditions of use, given the fact that hundreds of samples of astaxanthin extracted from the flesh of wild salmon had been analyzed over a period of over five months on the same column without making any change in the chromatographic parameters (mobile phase composition, flow-rate and temperature). For example, if astaxanthin adhered irreversibly on the CSP under those conditions, the selectivity would have changed markedly during the analysis of the extracts. The selectivity did not change during the analysis. Similarly, the presence of impurities in the analyte or in the mobile phase would have caused a change in selectivity during that period.

It has been reported that even well-packed columns may lose their efficiency with use due to a progressive consolidation of the column bed [18]. Such a change may have occurred for column 1 during the method development stage. However, no change in selectivity was observed for column 1 during subsequent multiple analyses of astaxanthin extracts from salmon flesh. Although the selectivity of column 1 decreased over time, it was always possible to obtain good resolution of the astaxanthin stereoisomers on column 1 after reoptimization of the mobile phase. Reoptimization was achieved by adjusting the ratios of the modifier (THF–IPA–triethylamine) in the mobile phase (hexane–THF–IPA–triethylamine). Even after prolonged use, the selectivity of column 1 compared favorably with the five new columns.

3.2. Effect of column packing on chromatographic separations

We performed further systematic optimization of the resolution of astaxanthin stereoisomers on column 1 by using a mobile phase consisting of *n*-hexane, THF and IPA. Maximum selectivity between *cis*/*trans* astaxanthin was obtained with a mobile phase composition consisting of *n*-hexane–THF–IPA (85:5:10, v/v/v) (column 1, Fig. 3). The separation was not duplicated, however, when five other L-leucine columns were used under identical chromatographic conditions. Columns 1–4 were packed with CSP from the same lot, while columns 5 and 6 were packed with CSP from a different lot (Table 2).

Column 1, the original column, was used in the

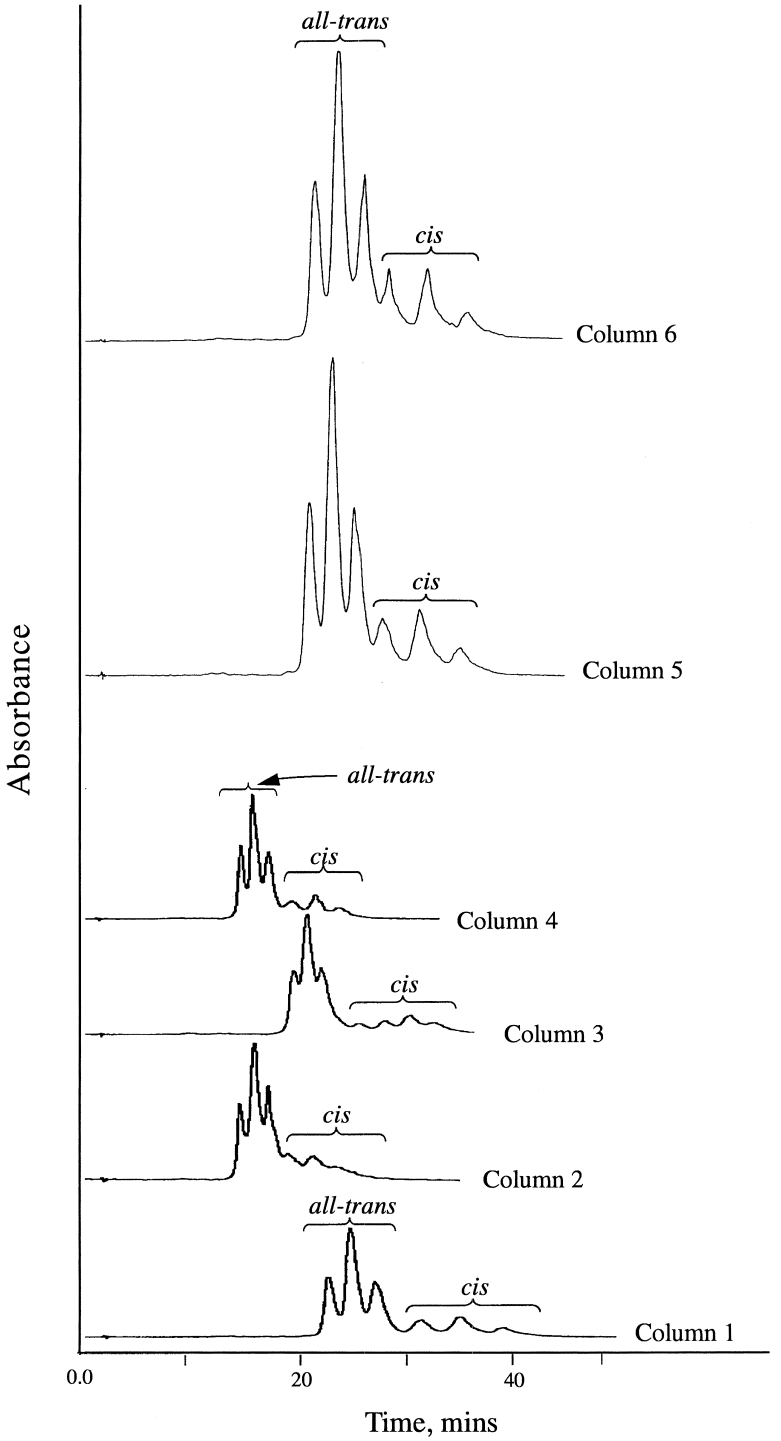


Fig. 3. Chromatograms of the astaxanthin stereoisomers on six L-leucine columns, run under the same LC conditions: columns 1, 2, 3 and 4, packed with CSP from the same lot and manufacturer (Regis); columns 5 and 6, packed with CSP from an alternative lot by the same manufacturer (Regis). Specimen: Synthetic astaxanthin exposed to daylight/fluorescent light for one week, mobile phase: *n*-hexane–THF–IPA (85:5:10, v/v/v), flow-rate=1.5 ml/min, monitoring wavelength=476 nm.

Table 2
The chromatographic parameters of all-*trans*- and *cis*-astaxanthin with *n*-hexane–THF–IPA, (85:5:10, v/v/v) as the mobile phase (flow-rate=1.5 ml/min; monitoring wavelength=476 nm; column: L-leucine, sample: synthetic all-*trans*-astaxanthin exposed to daylight/fluorescent light for one week)

Column	Lot No.[a]	N[b]	$k'_{\text{all-}trans_{meso}}$[c]	$k'_{\text{all-}trans_{RR}}$[c]	$k'_{cis_{SS}}$[c]	$\alpha_{trans_{RR}/trans_{meso}}$[d]	$\alpha_{cis_{SS}/trans_{RR}}$[d]
1	P 96-142-1	40 100	12.11	13.29	15.59	1.10	1.17
2	P 96-142-1	60 000	7.84	8.57	9.58	1.09	1.11
3	P 96-142-1	61 000	11.13	11.85	13.91	1.06	1.17
4	P 96-142-1	63 200	8.11	8.94	10.13	1.10	1.13
5	P 100-95	43 900	11.03	12.06	13.40	1.09	1.11
6	P 100-95	45 200	11.36	12.65	13.80	1.11	1.09

[a] Columns 1–4 are packed with CSP from the same lot, while columns 5 and 6 are packed with CSP from a different lot.
[b] N is the number of theoretical plates per meter as measured by the manufacturer, using 2,2,2-trifluoro-1-(9-anthryl) ethanol, as the standard.
[c] $k'=(t_R-t_0)/t_0$; t_R is the retention time, t_0 is the void time.
[d] $\alpha=k'_{(l)}/k'_{(e)}$; where the (l) is the later eluting isomer and (e) is the earlier eluting isomer for two consecutive peaks.

development of the L-leucine method [24,26] and for the analyses of hundreds of astaxanthin extracts from wild salmon flesh [23–26]. The surface chemistry of its CSP is therefore presumed to have been modified through use. Column 1 would therefore not be expected to be identical to new columns, including those packed with the same CSP. Nevertheless, it was expected that similar retention and resolution would be obtained with columns 2–4, which were packed with CSP from the same lot, and which had a similar number of theoretical plates as measured by the manufacturer (see Table 2). It has been reported, however, that column efficiency may not be a good measure of the reproducibility of column packing, especially for the comparison of columns packed with specialized stationary phases, or for columns used for difficult separations [31].

Peak shape, resolution and retention time varied for columns 1–4. Each of these columns had different chiral discrimination capabilities for the same set of astaxanthin stereoisomers. Column 1, which had the lowest number of theoretical plates as measured by the manufacturer, gave as good or better resolution of astaxanthin stereoisomers than columns packed with CSP from the same lot reported by the manufacturer to have 50% more theoretical plates.

The original separation of the *cis* isomers from the all-*trans* isomers, developed using column 1, was small. Therefore, minor column to column variations could result in overlap between the tail of the last stereoisomer peak of all-*trans*-astaxanthin and the leading edge of first stereoisomer peak of the *cis*-astaxanthin isomer band profile in column 2 (see Fig. 3). For example, there is significant overlap between the tail of the last stereoisomer peak of all-*trans*-astaxanthin and the leading edge of first stereoisomer peak of the *cis*-astaxanthin isomer band profile in column 2. The peak shape and separation of the stereoisomers of all-*trans*-astaxanthin obtained with column 3 are poorer than that of the other columns.

Columns 5 and 6 were packed with CSP from the same lot (Table 2), which was different from that of columns 1–4. Peak shape, resolution and retention time were similar, but not identical for column 5 and column 6 (Fig. 3). These two columns had similar chiral discrimination capabilities. However, their chiral discrimination capabilities were different from columns 1–4 for the same set of astaxanthin stereoisomers (see Fig. 3). Further attempts at improving the separation of the *cis* stereoisomers from the all-*trans* stereoisomers of astaxanthin by using different mobile phases were unsuccessful.

Differences in selectivity, such as those described above for the L-leucine columns packed with stationary phase from the same lot and from different lots, were reported for reversed-phase columns packed by the same manufacturer with stationary phase from the same lot [32]. Similarly, differences in column selectivity were reported for the same type of column purchased from different manufacturers [32]. These differences in selectivity of columns packed with CSP from the same lot may be explained by column heterogeneity. It has been reported that there is overwhelming evidence that the beds of the conven-

tional packed columns used in liquid chromatography are not homogeneous [33] and that the density of a packing material should not be regarded as a physical property in the same sense that density is a physical property of a solvent [18]. Although the possible origin of column heterogeneity has been discussed [33], no definitive conclusion has yet been reached [34]. It has also been reported that the phenomenon of column heterogeneity depends strongly on the packing procedure, and is most probably related to the interaction between the relatively high compressibility of pulverulent materials and the complex distribution of stress during the compression of the packing [34]. Our results are consistent with these reports of column heterogeneity.

Differences in chromatographic behavior for columns packed with CSP from different batches or by different manufacturers may be ascribed to the difference in the amount of residual silanols in different batches, and to the relative distribution of free, geminal and vicinal silanols [14,35]. The residual silanols could interact with astaxanthin through hydrogen bonding and dipole–dipole interactions. The resulting heterogeneous surface leads to mixed retention mechanisms, peak tailing and loss of resolution. The silica matrix may also contain metals, which would act as additional adsorption sites and enhance the activity of adjacent silanols [14,35].

3.3. Finding an alternative CSP

The search for an alternative CSP to separate the astaxanthin stereoisomers was limited to columns packed with Pirkle type covalently bonded CSP. Columns packed with π-electron acceptor CSP, such as D-phenylglycine; π-electron donor CSP, such as (*R*)-naphthylurea and (*R*)-phenylurea; and π-electron acceptor π-electron donor CSP, such as the Whelk-O-1 were studied. The investigation included the systematic modification of several mobile phase compositions. Only π-electron acceptor CSPs, namely D-phenylglycine and L-leucine, were capable of resolving the astaxanthin stereoisomers. The best separation of the astaxanthin stereoisomers was obtained on a Sumichiral OA-2000 column, which is based on (D)-*N*-(3,5-dinitrobenzoyl)-phenylglycine covalently bonded to silica gel [27].

3.4. Effect of CSP technology and column packing on chromatographic separations

Columns packed with Pirkle covalent D-phenylglycine CSP were obtained from four different companies and then compared. Two of the companies packed their columns with CSP manufactured by the same Japanese firm. The separation was optimized on the Sumichiral OA-2000, Sumichiral I, a column packed by the same company that manufactured the CSP. The best separation was obtained by using a mobile phase that consisted of a ternary solvent mixture of *n*-heptane, methylene chloride and IPA [27]. The separation obtained on the initial Sumichiral OA-2000 column (designated as Sumichiral I) by using 3% IPA is shown in Fig. 4A.

The Chirex columns, with D-phenylglycine CSP obtained from the same Japanese manufacturer but packed by a different vendor (Phenomenex), also separated the stereoisomers of the all-*trans* from the *cis* isomers of astaxanthin. However, the shape and resolution of the peaks were better on Sumichiral I than on the Chirex columns under the same chromatographic conditions. The separation obtained on the Chirex II column by using 3% IPA is shown in Fig. 5. The Rexchrom column, which contained D-phenylglycine CSP manufactured and packed by Regis, did not separate any of the astaxanthin stereoisomers.

The column packed with D-phenylglycine CSP bonded to 10 μm silica (W.R. Grace) separated the all-*trans* isomers from the *cis* isomers of astaxanthin. However, it did not separate the stereoisomers of the all-*trans*-astaxanthin or those of the *cis*-astaxanthin isomers.

These results indicate that columns from several manufacturers must be examined before making a general statement about the selectivity of a particular CSP (e.g., D-phenylglycine) in separating complex stereoisomeric mixtures. Moreover, columns containing the same CSP but packed by different manufacturers should also be investigated before any conclusive statement is made about the selectivity of the CSP toward the analytes in question. For similar reasons, the same precaution applies to making definitive comparisons between different CSPs, such as L-leucine or D-phenylglycine.

Conversely, if an attempt to separate a complex

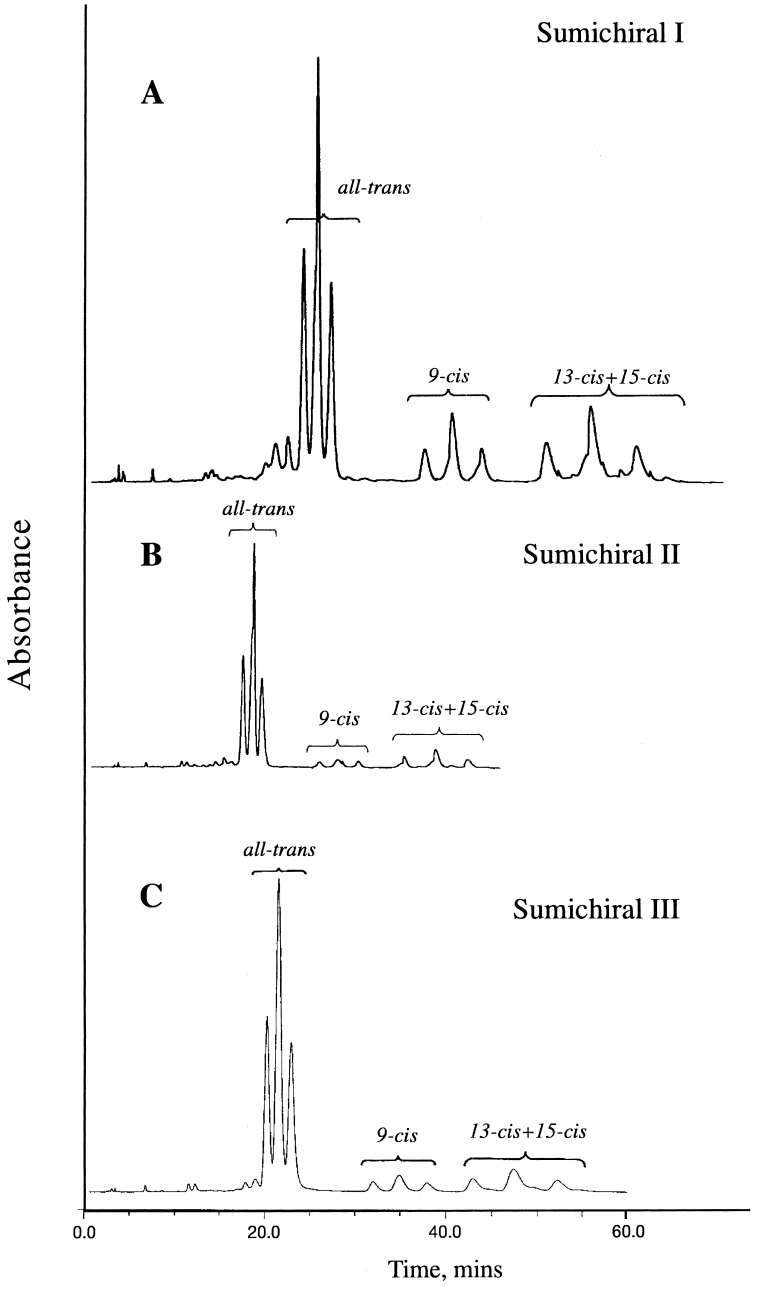


Fig. 4. Chromatograms of the stereoisomers of astaxanthin on three Sumichiral OA-2000 columns packed with Pirkle covalent D-phenylglycine CSP. Specimen: Astaxanthin extracted from Carophyll Pink. Flow-rate=1 ml/min, monitoring wavelength=480 nm, mobile phase: *n*-heptane–methylene chloride–IPA (73:25:3, v/v/v). (A) Sumichiral I; (B) Sumichiral II; (C) Sumichiral III.

mixture of stereoisomers with a specific chiral column were not successful under a variety of chromatographic conditions, it would be premature to conclude that the isomers could not be separated on other columns packed with the same CSP from different manufacturers. Such a conclusion could be

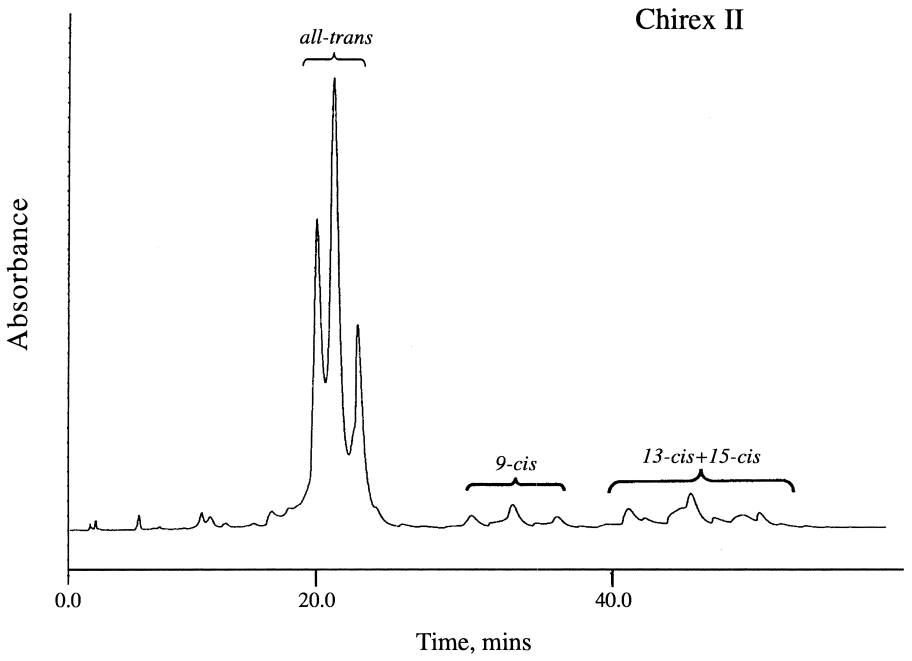


Fig. 5. Chromatogram of the astaxanthin stereoisomers on the Chirex II column (packed with Pirkle covalent D-phenylglycine CSP, column diameter: 4.0 mm). Specimen: Astaxanthin extracted from Carophyll Pink. Flow-rate=1 ml/min; monitoring wavelength=480 nm; mobile phase: *n*-heptane–methylene chloride–IPA (73:25:3, v/v/v).

Table 3
The chromatographic parameters of all-*trans*-, 9-*cis*- and 13-*cis*-astaxanthin with various solvent ratios (flow-rate=1 ml/min; monitoring wavelength=480 nm; column: Sumichiral OA-2000; sample: astaxanthin extracted from Carophyll Pink)

Column	Mobile phase[a]	k'				α		
		all-*trans*$_{meso}$	all-*trans*$_{SS}$	9-*cis*$_{RR}$	9-*cis*$_{SR,RS}$	all-*trans*$_{SS}$/all-*trans*$_{meso}$	9-*cis*$_{SR,RS}$/9-*cis*$_{RR}$	all-*trans*$_{RR}$/9-*cis*$_{SS}$
1	A	5.69	6.10	8.26	8.93	1.07	1.08	1.35
	B	9.01	9.62	13.74	14.93	1.07	1.09	1.53
	C	15.2	16.18	24.57	27.02	1.06	1.10	1.52
2	A	3.27	3.47	4.54	4.94	1.06	1.09	1.31
	B	5.26	5.60	7.78	8.49	1.06	1.09	1.39
3	A	4.88	5.23	7.20	7.88	1.07	1.09	1.38
	B	7.24	7.81	11.33	12.44	1.08	1.10	1.45
	C	11.25	12.17	18.64	20.50	1.08	1.08	1.53

[a] A=*n*-heptane–methylene chloride–IPA (70:25:5, v/v/v); B=*n*-heptane–methylene chloride–IPA (72:25:3, v/v/v); C=*n*-heptane–methylene chloride–IPA (74:24:2, v/v/v).

erroneous, as the case of the separation of astaxanthin stereoisomers on the D-phenylglycine column from one of the suppliers (Regis) has clearly shown.

3.5. Preliminary ruggedness test of separation on Sumichiral OA-2000 columns

Column-to-column reproducibility is a reflection of both bonding and packing technology. A preliminary test for the reproducibility of the separation of astaxanthin stereoisomers on the Sumichiral OA-2000 column was conducted by repeating the HPLC analysis with two new Sumichiral OA-2000 columns (Sumichiral II and III) packed with CSP from two different lots. The chromatographic separations were similar to those obtained under identical chromatographic conditions (Fig. 4B and C) with the first Sumichiral OA-2000 column (Sumichiral I, Fig. 4A). The chromatographic parameters for the three Sumichiral columns are listed in Table 3. The retention times are different, which indicates that the nonselective sites are different in the three columns. The peak shapes (Fig. 4A–C) and the separation of the stereoisomers (α, Table 3) are similar, however, which indicates that the chiral recognitions are fairly similar in the three columns.

4. Conclusion

Given the present state of both CSP manufacturing technology and of column-packing technology, it is not possible to directly compare the efficiency of chromatographic separations of complex stereoisomeric mixtures on columns packed with different types of CSP (such as L-leucine and D-phenylglycine) without an investigation of chiral columns obtained from different manufacturers. The column-to-column reproducibility of chromatographic separations of complex stereoisomeric mixtures on chiral columns packed with Pirkle covalent L-leucine and D-phenylglycine CSP should be verified experimentally.

A preliminary ruggedness test indicates that the chiral HPLC method for the separation of astaxanthin stereoisomers using a Sumichiral OA-2000 column is reproducible. This method could therefore be used as a convenient and facile regulatory tool.

Acknowledgements

We would like to thank Yehuda Shalon, MODcol Corp., St. Louis, MO, USA for the generous gift of the R-DNPG-100-10SP column.

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