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SELECTIVITY EFFECTS IN NON-AQUEOUS REVERSED-PHASE AND NORMAL-PHASE CHROMATOGRAPHY OF GEOMETRIC CANTHAXANTHIN ISOMERS

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SUMMARY

Non-aqueous reversed-phase and normal-phase systems are reported for the separation of geometric isomers of the carotenoid canthaxanthin. On Zorbax ODS, ternary mobile phases consisting of acetonitrile, methanol and a modifier, *e.g.*, dichloromethane, afforded baseline separation between the all-*trans* and a "total-*cis*" peak, the latter containing all unresolved *cis*-isomers. The methanol to acetonitrile ratio and, to a lesser extent the nature of the modifier, were found to be critical for optimal selectivity.

Individual *cis*-isomers were differentiated by normal-phase chromatography on silica. The influence of the surface properties of seven packings on the resolution between the four principal isomers was investigated. Selectivity factors for the pair all-*trans*/9-*cis*-canthaxanthin were widely divergent, whereas α-values for the 13/15-*cis* pair were virtually identical on all materials. Optimal resolution of all major isomers was achieved on 10-μm CP-Spher Si and 3-μm CP-Microspher Si columns, eluted with mixtures of dichloromethane and 2-propanol.

INTRODUCTION

Encapsulated embryos of certain lower Crustacea, *e.g.*, *Artemia*[1], *Branchipus*[2] and *Branchinecta*[2], have recently been shown to contain large amounts of *cis*-canthaxanthins. Current interest in the possible function of these unusual carotenoids[3] has led us to develop chromatographic methods for their separation, identification and quantitation. Although non-polar bonded phases do not generally lend themselves to the separation of geometric isomers, a previously reported non-aqueous reversed-phase system[4] was found to afford a partial resolution of all-*trans*-canthaxanthin and a composite "total-*cis*" peak, consisting of all unresolved *cis*-isomers[1]. Despite the incomplete resolution obtained, this system showed great promise as a basis for the quantitative analysis of canthaxanthins. Part of this paper is concerned with our efforts to improve the resolution of all-*trans*- and *cis*-canthaxanthins by optimizing the mobile phase selectivity.

For the differentiation of the individual *cis*-canthaxanthins, normal-phase

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chromatography was used. *cis*-Carotenoids have been chromatographed on silica[5,6], alumina[7] and calcium hydroxide[8]. Previous attempts to separate all major *cis*-canthaxanthins on silica were unsuccessful, one critical pair, tentatively identified as 13-*cis*-/15-*cis* canthaxanthin, being poorly resolved[1].

In this study, different silica materials and alumina were tested for their ability to resolve all-*trans*- and the major mono-*cis*-canthaxanthins, specifically the 13- and 15-*cis* forms.

EXPERIMENTAL

Chemicals

All-*trans*-canthaxanthin was a gift from Hoffmann-La Roche (Basle, Switzerland). *cis*-Canthaxanthins were prepared from all-*trans*-canthaxanthin by stereomutation[9] and purified as described previously[1]. Acetonitrile was "chemically pure" and came from Janssen Chimica (Beerse, Belgium). Dichloromethane and methanol (both "chemically pure") were obtained from Hoechst (Frankfurt, F.R.G.) and were redistilled in a spinning-band apparatus (B/R Instruments, Pasadena, MD, U.S.A.). All other solvents were of analytical-reagent grade and purchased from Merck (Darmstadt, F.R.G.).

Apparatus

The liquid chromatograph used for reversed-phase chromatography consisted of a Varian 5020 pump (Varian, Palo Alto, CA, U.S.A.), a Valco N60 valve injector (Valco, Houston, TX, U.S.A.), fitted with a 50-μl loop, an HP 1040A multi-channel photodiode array detector, set at 470 nm and connected to an HP 9121 dual-disc drive, an HP 7470A plotter (all from Hewlett-Packard, Palo Alto, CA, U.S.A.) and an SP 4100 integrator (Spectra-Physics, San Jose, CA, U.S.A.). The instrument used for normal-phase chromatography was equipped with an LKB 2150 double piston pump (LKB, Bromma, Sweden), an N60 Valco valve injector with a 50-μl loop (Valco), fitted with a capillary by-pass[10], a Varichrom variable-wavelength detector set at 470 nm and a Varian 9176 strip-chart recorder (both from Varian).

Columns

All columns were made of stainless steel and had the dimensions 25 × 0.46 cm I.D., except for the Zorbax ODS column, which was 15 × 0.46 cm I.D. They were either pre-packed (5-μm Zorbax ODS from Du Pont, Wilmington, DE, U.S.A.; 10-μm CP-Spher Si, 3-μm CP-Microspher Si and 5-μm Spherisorb alumina A5Y from Chrompack, Middelburg, The Netherlands; 5-μm ROSIL from Alltech Europe, Eke, Belgium) or laboratory-packed (10-μm LiChrospher Si-100 and 10-μm LiChrosorb Si-100, both from Merck, 10-μm Zorbax BP SIL from Du Pont and 10-μm Spherisorb S10W from Phase Separations, Queensferry, U.K.). The packing conditions were as follows: slurry medium, carbon tetrachloride; slurry concentration, 20%; packing pressure, 34.5 MPa (5000 p.s.i.); pressurizing liquid, hexane; and pump, Varian 8500, used at the maximum flow-rate (990 ml/h).

Chromatographic conditions

The Zorbax ODS column was eluted with ternary mixtures consisting of ace-

tonitrile as a base solvent, variable percentages of methanol and small concentrations of a modifier (diisopropyl ether, benzene, ethyl acetate, dioxane, tetrahydrofuran, chloroform, dichloromethane), either with or without a small amount of triethylamine (0.15%). The flow-rate was 0.7 ml/min and the temperature was ambient. For silica, the standard eluent consisted of dichloromethane–2-propanol (99.3:0.7). The dichloromethane had previously been dried over anhydrous magnesium sulphate. The CP-Microspher Si and the Spherisorb alumina column were eluted with solvent mixtures containing small amounts of water, *viz.*, 0.45% 2-propanol in dichloromethane, isohydric in water content[11], and 0.02% water in 2-propanol–dichloromethane (99.7:0.3), respectively.

Samples

Mixtures of all-*trans*- and *cis*-canthaxanthins were dissolved in the chromatographic solvent. Peaks were tentatively identified on the basis of their absorption spectra, recorded on-line and using the photodiode array detector. Characteristic features of the spectra include the wavelength of maximum absorption and the position and intensity of a *cis*-absorption band[1]. The structural formulae of canthaxanthins are depicted in Fig. 1.

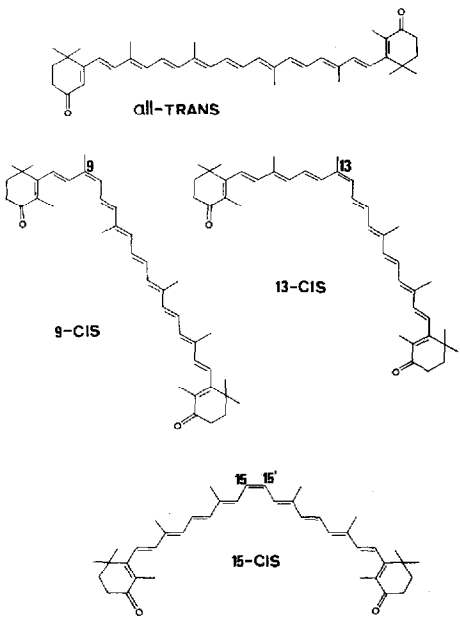


Fig. 1. Structural formulae of all-*trans*- and major mono-*cis*-isomers of canthaxanthin.

RESULTS AND DISCUSSION

Non-aqueous reversed-phase chromatography

The advantages of non-aqueous reversed-phase (NARP) chromatography on highly retentive packing materials, *e.g.*, Zorbax ODS, for the separation of low-polarity samples in general[12], and for vitamin A derivatives[13,14] and carotenoids[4] in

TABLE I

EFFECT OF MODIFIERS IN NARP CHROMATOGRAPHY OF ALL-*trans*- AND *cis*-CANTHAXANTHINS

Eluents contained the indicated percentages of organic modifier, 30% methanol and the remainder acetonitrile.

Organic modifier	*Content (%)*	*All-trans-canthaxanthin*			*'Total cis'-canthaxanthins*			α	*RS***
		k'	*N*	*AS**	*k'*	*N*	*AS**		
Tetrahydrofuran	7	2.28	9100	1.35	2.59	8900	0.97	1.13	1.93
Dioxane	13	2.08	8600	1.07	2.35	8500	0.80	1.13	1.88
Chloroform	9	2.03	8100	1.14	2.30	9300	1.10	1.13	1.83
Dichloromethane	9	2.38	9000	0.98	2.66	9000	1.11	1.12	1.82
Benzene	6	2.37	8300	0.95	2.62	9400	0.97	1.11	1.68
Ethyl acetate	11	2.33	9400	1.03	2.54	9000	0.75	1.09	1.30
Diisopropyl ether	4	2.87	8800	1.16	3.09	9300	0.88	1.08	1.19

* AS = asymmetry factor, calculated at 10% of peak height.
** RS = resolution between all-*trans*- and "total-*cis*"-canthaxanthins.

particular, have been thoroughly documented. In this study, ternary mixtures of acetonitrile as a base solvent, an organic modifier (to adjust the solvent strength) and methanol (to modulate the selectivity) were used. Surprisingly, even canthaxanthin *cis/trans* isomers could be partially resolved on Zorbax ODS, provided that a sufficiently high percentage of methanol was included in the eluent[1]. In order to improve this resolution, the effect of the organic modifier on selectivity, resolution, efficiency and peak symmetry was investigated (Table I). Cyclic ethers, *e.g.*, tetrahydrofuran and dioxane, unlike aliphatic ethers, were found to yield maximum selectivity. However, the superior resolution, as measured on the basis of peak widths at half-height, was overestimated as a result of either tailing of the all-*trans*-or leading of the *cis*-canthaxanthin peak. Addition of small amounts of triethylamine appeared to counteract this effect. Thus, near baseline separation between all-*trans*- and "total-*cis*"-canthaxanthins was obtained with acetonitrile–methanol–dioxane–triethylamine (50.85:30:18:0.15). Higher percentages of triethylamine reduced the capacity ratios, *k'*, but dit not improve the resolution any further. A more pronounced effect on selectivity (α) was observed on changing the methanol content of the eluent. A plot of α *versus* the percentage of methanol displayed a maximum at a concentration of 50–60% methanol (Fig. 2). Higher methanol contents resulted in a decrease not only in selectivity but also in plate count. The definitive eluent, composed of acetonitrile–methanol–dichloromethane (41:50:9), afforded baseline separation between all-*trans*- and "total-*cis*"-canthaxanthins (Fig. 3), even in the absence of triethylamine. Peaks 3 and 4 represent apo-carotenoids, *i.e.*, β-apo-8'-carotenal and β-apo-8'-carotenoic acid ethyl ester, to be considered as potential internal standards in the quantitative analysis of canthaxanthins.

Normal-phase chromatography

Chromatography of canthaxanthin stereomutation mixtures on ROSIL silica had previously shown that all but two major mono-*cis*-isomers, tentatively identified as 13- and 15-*cis*-canthaxanthin, could be readily resolved using mixtures of dichloromethane and 2-propanol[1]. Attempts to improve the selectivity by substituting

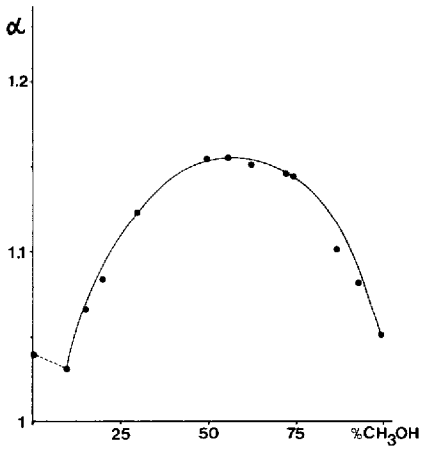


Fig. 2. Plot of selectivity (α) *versus* percentage of methanol in NARP separations of all-*trans*- and "total-*cis*"-canthaxanthins.

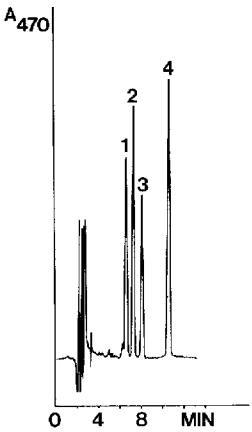


Fig. 3. NARP separation of all-*trans*- and "total-*cis*"-canthaxanthins and 2 apo-carotenoids (potential internal standards). Column, Zorbax ODS 5-μm (15 × 0.46 cm I.D.); eluent, acetonitrile–methanol–dichloromethane (41:50:9); flow-rate, 0.7 ml/min. Peaks: 1 = all-*trans*-canthaxanthin; 2 = "total-*cis*"-canthaxanthins; 3 = β-apo-8′-carotenal; 4 = β-apo-8′-carotenoic acid ethyl ester.

other polar modifiers, *e.g.*, acetonitrile for 2-propanol, or by the addition of water or triethylamine were unsuccessful. Retention, selectivity and efficiency data, and also peak-shape characteristics obtained on the various materials, are presented in Table II. The selectivity factors, α, for the critical pair 13-*cis*-/15-*cis*-canthaxanthin were virtually identical on all the materials tested, as opposed to the divergent α-values for the pair all-*trans*-/9-*cis*-canthaxanthin. The near baseline resolution between 13- and 15-*cis*-canthaxanthin on CP-Spher Si (Fig. 4) and CP-Microspher Si (Fig. 5) is attributable to the superior efficiency of these columns and the excellent peak symmetry. The performance of the 3-μm CP-Microspher Si column is particularly outstanding in both respects. Although its dimensions are unusual for a 3-μm

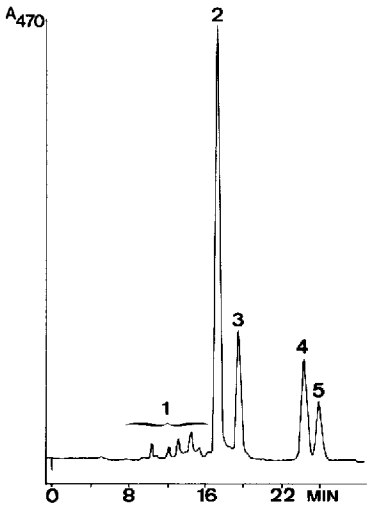
TABLE II

RETENTION, SELECTIVITY AND EFFICIENCY DATA FOR NORMAL-PHASE CHROMATOGRAPHY OF ALL-*trans*- AND *cis*-CANTHAXANTHINS ON DIFFERENT SILICAS

Eluent: dichloromethane–2-propanol (99.3:0.7).

Support	*Particle size (μm)*	*Capacity ratio (k')*				*α*		*RS**	*N***	*AS****
		All-trans	*9-cis*	*13-cis*	*15-cis*	*All-trans-/ 9-cis-*	*13-cis-/ 15-cis-*			
ROSIL	5	4.68	5.36	7.34	7.78	1.15	1.060	0.80	4600	0.66
CP-Spher Si	10	5.80	6.63	8.62	9.17	1.14	1.064	1.20	8300	1.11
Zorbax BP Sil	10	5.67	8.15	11.74	12.51	1.44	1.066	0.95	7400	3.04
Spherisorb S10W	10	2.34	4.39	6.04	6.37	1.88	1.055	0.60	3100	1.48
LiChrospher Si 100	10	2.61	5.14	7.39	7.84	1.97	1.061	0.65	1400	1.10
LiChrosorb Si 100	10	1.80	3.08	4.73	5.05	1.70	1.068	0.80	4200	1.11
CP-Microspher Si	3	2.54	5.52	8.08	8.58	2.17	1.062	1.77	21 100	1.19

* RS = resolution between 13- and 15-*cis*-canthaxanthin.
** N = calculated on the 13-*cis*-peak. Does not take peak asymmetry into account.
*** AS = asymmetry factor, calculated at 10% of the height of thw 13-*cis*-peak.



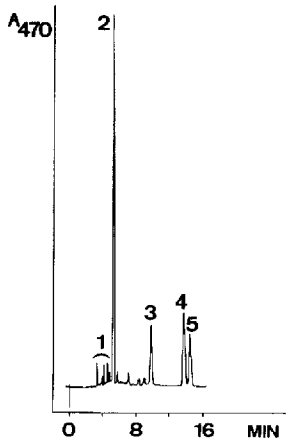


Fig. 4. Separation of geometric canthaxanthin isomers on 10-μm CP-Spher Si (25 × 0.46 cm I.D.). Eluent, dichloromethane–2-propanol (99.3:0.7); flow-rate, 1 ml/min. Peaks (tentative identification): 1 = di-*cis*-canthaxanthins; 2 = all-*trans*-canthaxanthin; 3 = 9-*cis*-canthaxanthin; 4 = 13-*cis*-canthaxanthin; 5 = 15-*cis*-canthaxanthin.

Fig. 5. Separation of geometric canthaxanthin isomers on 3-μm CP-Microspher Si (25 × 0.46 cm I.D.). Eluent: dichloromethane–2-propanol (99.55:0.45), isohydric[11]; flow-rate, 2 ml/min. Peaks (tentative identification): 1 = di-*cis*-canthaxanthins; 2 = all-*trans*-canthaxanthin; 3 = 9-*cis*-canthaxanthin; 4 = 13-*cis*-canthaxanthin; 5 = 15-*cis*-canthaxanthin.

column, the back-pressures were not unacceptably high (16.5 MPa or 2390 p.s.i. at 2 ml/min) and its mechanical stability was excellent. The latter may be partly associated with the use of the protective injector capillary by-pass[10].

Alumina was not capable of separating all four major isomers, the critical 13-/15-*cis* pair remaining unresolved (α = 1.00) in two different eluent systems

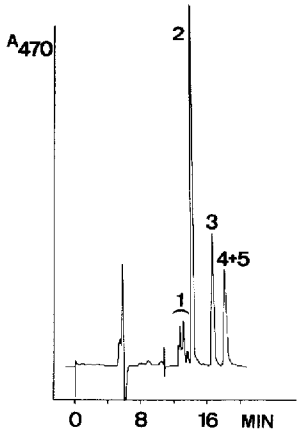


Fig. 6. Separation of geometric canthaxanthin isomers on 5-μm Spherisorb A5Y alumina. Eluent, dichloromethane–2-propanol–water (99.7:0.3:0.02); flow-rate, 1 ml/min. Peaks (tentative identification): 1 = di-*cis*-canthaxanthins; 2 = all-*trans*-canthaxanthin; 3 = 9-*cis*-canthaxanthin; 4 + 5 = 13- and 15-*cis*-canthaxanthin.

[dichloromethane–2-propanol (Fig. 6) and dichloromethane–acetonitrile, both containing 0.01% of water]. The capacity ratios on alumina were less reproducible than on silica, which is in keeping with the observations of Vecchi *et al.*, who took special precautions to control the water content of the eluent[7] by using a moisture control system[15].

Applications

Although NARP fails to resolve individual *cis*-isomers, this system is now being used routinely as a basis for the quantitation of all-*trans*- and *cis*-canthaxanthins in *Artemia*. A particular advantage of the technique is its compatibility with the direct injection of biological extracts in polar organic solvents, *e.g.*, methanol, without the need for additional elaborate sample pre-treatment. Owing to the high canthaxanthin levels in *Artemia*, *i.e.*, of the order of 100–400 μg/g, no concentration procedure is required. This guarantees the maximum possibility for the quantitative recovery of the highly labile *cis*-canthaxanthins, which after extraction readily isomerize to the more stable all-*trans* form. *Cis*→*trans* conversion is more likely to occur during the complex sample processing prior to normal-phase chromatography, which includes re-extraction with hexane, evaporation of the organic solvent and reconstitution of the residue. Isomerization is sometimes even observed on-column, especially with the acidic silicas. Thus, normal-phase chromatography is less suited to the quantitative determination of canthaxanthins, but remains valuable for qualitative purposes, *i.e.*, for the separation and characterization of individual *cis*-isomers.

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