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Chromatographic performance on a C_{30}-bonded stationary phase of monohydroxycarotenoids with variable chain length or degree of desaturation and of lycopene isomers synthesized by various carotene desaturases

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Abstract

Selectivity towards geometric isomers is a superior feature of a C_{30} polymeric stationary phase. Therefore, lycopene isomers synthesized in *Escherichia coli* transformants by catalysis of divers carotene desaturases were separated on this stationary phase. Due to their spectral characteristics and by co-chromatography with nuclear magnetic resonance-characterized carotene standards, some of them could be identified. Most of the lycopene isomers were cyclized by lycopene cyclase yielding mainly 9*Z*, 13*Z* and all-*E* β-carotene. In contrast, 7,9,7′,9′*Z* prolycopene is accumulating since it cannot be converted by this enzyme. Finally several acyclic hydroxycarotenoids with a chain of 30, 40 and 45 carbon atoms differing in the length of the polyene chain from 9 to 13 were separated on the C_{30} stationary phase. Longer retention times were observed when the length of the molecule increased and also when the conjugated double bond system was extended. Corresponding monocyclic carotenoids were less retained on the C_{30} stationary phase and derivatives with an ε-ionone end group eluted earlier than with a β-end group.

Keywords: Geometric isomers; Positional isomers; Hydroxycarotenoids; Carotenoids; Lycopenes; Prolycopene

1. Introduction

Natural carotenoids include C_{30}, C_{45} and C_{50} derivatives, but the majority of compounds consist of 40 carbon atoms. They are either acyclic or cyclic typically carrying β- or ε-end groups. Their color depends on the number of conjugated double bonds. Upon desaturation, some of these double bonds may be introduced both in a *Z* or *E* configuration leading to a variety of stereoisomers [1]. Depending on the absence or presence of hydroxy, epoxy and keto substituents, carotenoids range from non-polar carotenes to very polar xanthophylls. For the analysis of structurally different carotenoids, C_{18} stationary phases are most typically used. The different types of C_{18} materials as well as normal stationary phases and the variety of mobile phases have been extensively reviewed [2,3]. C_{18} reversed-phase high-performance liquid chromatography has been optimized to identify carotenoids from complex mixtures [4] for separation and purification prior to the identification of novel structures [5], or for quantitation of reaction products in cell-free carotenogenic reactions [6]. However, the resolution of geometric isomers is rather limited. Shape selectivity of carotenoids can

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be achieved on normal phases such as alumina and calcium hydroxide ([7] and references cited therein). However, these phases are difficult to handle, since control of water content may be a problem.

The introduction of a C_{30} stationary phase for carotenoid separation in 1994 [8] offered a new type of stationary phase which was helpful in an easy separation of polar and non-polar carotenoid geometric isomers and positional isomers like zeaxanthin and lutein [9]. The application potential of this phase has been demonstrated by the separation of carotenoids from complex biological samples (see Ref. [10] for a recent review) and by the simultaneous metabolic profiling of several plant isoprenoids [11]. For the development of the C_{30} phase, the size of bicyclic β-carotene was compared to the thickness of various monomeric and polymeric alkyl-modified silicas. Since carotenoids are larger than C_{18} monomeric and even C_{18} polymeric phases, a C_{30} phase exceeding the dimension of a C_{40} carotenoid was developed in order to improve selectivity for structurally related carotenoids [8,12]. The typical mobile phase contains methanol, 4% water and methyl *tert*.-butyl ether to increase the polarity. The organization of the C_{30}-alkyl chain was examined by solid state nuclear magnetic resonance (NMR) spectroscopy [13]. At room temperature the more ordered *trans* alkyl chain conformation dominates whereas higher temperatures increase the more disordered *gauche* conformations. By comparison of the NMR data with the temperature-dependent separation of geometrical isomers, an improved selectivity was obtained when the population of *trans* conformations exceeded the population of *gauche* conformations. A detailed study is given on the temperature dependence of the retention of various carotenoids and carotene isomers on several stationary phases included C_{30}-bonded material [14]. It was shown that on the latter the effect of increasing temperature which leads to shorter retention times is much more pronounced on linear than on bent carotenoids. Thus, temperature was suggested as a factor for the optimization of carotenoid separation. Factors were discussed which determine the shape recognition and separation of geometric isomers on alkyl stationary phases [12].

Combinations of selected genes from organisms which synthesize different carotenoid end products makes it possible to design and produce novel carotenoids in a heterologous host like *Escherichia coli*. In this approach, a carotenogenic pathway is assembled in a non-carotenogenic host in a modular way by transformation with the appropriate genes which encode the enzymes responsible for the individual catalytic steps. Depending on the combination of these genes, it is possible to synthesize desired carotenoid structures. The details of this strategy has been reviewed recently [15]. The potential of combinatorial biosynthesis has been exploited to obtain structurally diverse acyclic or monocyclic hydroxylated C_{40} carotenoids containing up to 13 conjugated double bonds [5]. Since the appropriate genes are available, C_{30} and C_{45} hydroxycarotenoids can also be designed. In most cases, the all-*E* isomer is the major product but *Z* isomers can also be found additionally. Structurally different carotene desaturases for the extension of the central polyene group exist [16]. Depending on the type, different geometric isomers are formed as products of the catalytic reaction [1,17].

In this study, a C_{30} stationary phase will be employed to separate and discriminate between the lycopene isomers formed by the individual desaturases. Characterized standards will allow the identification of some of these enzymatically formed isomers. Furthermore, the question of lycopene isomers selection by the lycopene cyclase CrtY for the formation of β-carotene will be addressed. Finally, examples are shown of how acyclic and monocyclic hydroxycarotenoids of different carbon chain length and with a variable polyene group separate on a C_{30} stationary phase.

2. Experimental

2.1. Source of carotenoids

All carotenoids were produced in *E. coli* JM101 as heterologous host. This strain was transformed with carotenogenic genes on compatible plasmids and grown as previously described [18]. The following plasmids mediated the formation of individual carotenoids: pACCRT-EBI$_{Eu}$+pRKCRT-C [18] – hydroxylycopene; pACCRT-EBI$_{Rc}$+pRKCRT-C [17] – hydroxyneurosporene; pACCRT-EBI$_{Eu}$+pRKCRT-C+pQEcrtD [5] – 1-hydroxy-3,4-didehydrolyco-

pene; pACCRT-EBal1+pRKCRT-C [5] – 1-hydroxy-3′,4′-didehydrolycopene; pACCAR25ΔcrtX+pRKCRT-C [5] – 1′-hydroxy-γ-carotene; pACCRT-EBI$_{Rc}$+pSO50 [18] – demethylspheroidene. 1′-HO-δ-carotene was formed by a transformant expressing pACCRT-EBI$_{Eu}$ together with the lycopene ε-cyclase from *Arabidopsis thaliana* [20] and pRKCRT-C. Nonaflavuxanthin was synthesized in a transformant expressing a lycopene elongase gene from *Brevibacterium flavum* together with pACCRT-EBI$_{Eu}$. Lycopene isomers as the products of the cyanobacterial ζ-carotene desaturases genes, *crtQa* from *Anabaena* and *crtQb* from *Synechocystis*, were formed in *E. coli* carrying plasmid pACCRT-EBP together with pZDS1B [21] or pTRC0940 [22], respectively. Lycopene in JM101/pACCRT-EBI$_{Eu}$ was synthesized by the gene product of *crtI* from *Erwinia uredovora*. Plasmid pRKCRT-Y carrying the lycopene cyclase gene *crtY* from *E. uredovora* [23] was used to establish β-carotene synthesis from lycopene in the transformants. The carotene fraction was isolated from *Synechocystis* PCC 6803 as previously described [24].

2.2. Extraction, purification and analysis

All carotenoids were extracted from freeze–dried *E. coli* cells with 6% KOH in methanol and heating to 60°C for 20 min. This procedure allowed complete extraction to a colorless residue but did not affect the isomer composition as demonstrated in control experiments at room temperature. Except for lycopene and β-carotene when formed by the involvement of individual carotene desaturases as single products, the carotenoids were purified from each other by column chromatography on alumina with increasing ether concentrations in petrol [25] or by thin-layer chromatography on silica with toluene–ethyl acetate–methanol (75:20:5, v/v) [26]. Carotenoids were separated on a non-endcapped polymeric 3 μm C$_{30}$ column (YMC Wilmington NC, USA) at 26°C, if not otherwise stated with a flow of 1 ml/min. All mobile phases used were based on mixtures of methanol and methyl *tert*.-butyl ether as the polar component with a constant water content of 4% [8]. In all our separations, the use of a step gradient allowed a very flexible optimization of isomer resolution keeping the retention times as short as possible. In the case of hydroxy carotenoids the mobile phase consisted of a first step of methanol–methyl *tert*.-butyl ether–water (56:40:4, v/v) for 65 min and then changed to a ratio of 11:85:4 (v/v). Lycopene isomers were separated using the same initial mobile phase but in the second step the solvent was changed to 26:70:4 (v/v) after 30 min. β-Carotene isomers were resolved at 11°C with the same initial concentration which was switched to methanol–methyl *tert*.-butyl ether–water (11:85:4, v/v) after 40 min. Absorbance spectra in the eluent were recorded on-line with a photodiode array detector 440 (Kontron, Straubenhard, Germany) and used for the calculation of the peak ratios. Besides the all-*E* isomers of most of the carotenoids, individual *Z* isomers as well as NMR characterized isomeric mixtures of β-carotene and lycopene were available for co-chromatography. The individual *Z* isomers were 7,9,7′,9′*Z* lycopene (=prolycopene), 9*Z* and 15*Z* β-carotene. The lycopene isomeric mixture contained all-*E*, 15*Z*, 5,13′*Z*, 9*Z*, 5,9′*Z* and 5*Z*. Their elution pattern on C$_{30}$ stationary phase and their spectral properties were annotated. The same was the case for two β-carotene isomeric mixtures which contained all-*E*, 9*Z*, 13*Z*, 13,15*Z*, 9,13*Z*, 9,9′13*Z* and 9,13,13′*Z*.

3. Results and discussion

Formation of lycopene can be catalyzed by three different desaturases. When the plant type ζ-carotene desaturase from *Synechocystis*, *crtQb*, converts the isomeric mixture of ζ-carotene produced by the phytoene desaturase *crtP*, two neurosporene and eight lycopene isomers can be separated as reaction products on the C$_{30}$ column (Fig. 1A). The lycopene isomers synthesized by the structurally unrelated ζ-carotene desaturase from *Anabaena* show a different qualitative and quantitative pattern (Fig. 1B). They resemble much more the isomeric composition obtained by the reaction of the phytoene desaturase from *E. uredovora* (Fig. 1C). The major difference to the products of the plant type ζ-carotene desaturase is the absence of prolycopene L1 and the complete conversion of all the neurosporene isomers. In addition, L2 and L3 are formed and L7 and L8 are absent. By comparison of Fig. 1 trace A and B, it is evident that lycopene and neurosporene isomers

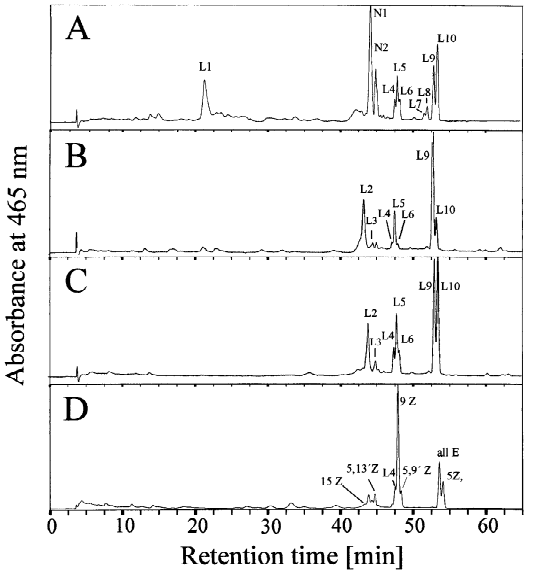

Fig. 1. Separation of the reaction products of different carotene desaturases which accumulate in *E. coli* transformants. (A) ζ-Carotene accumulating *E. coli* transformant co-transformed with the ζ-carotene desaturase gene from *Synechocystis*; (B). ζ-carotene accumulating *E. coli* transformant co-transformed with the ζ-carotene desaturase gene from *Anabaena*; (C) phytoene accumulating *E. coli* transformant co-transformed with the phytoene desaturase gene from *E. uredovora*; (D) lycopene isomer standard mixture. Separation was performed at 26°C.

overlap in the region between 40 and 45 min. This is especially the case for N1 and L2. These isomers were separated by decreasing the temperature to 5°C, yielding retention times of 86.0 and 86.4 min for N1 and L2, respectively, which made it possible to record their individual corrected spectra.

Co-chromatography with an authentic standard and the spectral characteristics identified N2 as all-*E* neurosporene. Neurosporene N1 could not be assigned by its spectral properties (Table 1) to any known neurosporene isomer listed in Ref. [27], nor to 5*Z* neurosporene which has been previously identified in ripe hips of *Rosa pomifera* [28].

Some of the lycopene isomers were identified by co-chromatography of an NMR characterized isomeric standard mixture (Fig. 1D) together with spectral properties (Table 1) and with authentic prolycopene (=7,9,7′,9′*Z*) [29]. Thus the following lycopene isomers could be identified: L1 as prolycopene, L3 as 5,13′*Z*, L5 as 9*Z*, L6 as 5,9′*Z*, L9 as all-*E* and L10 as 5*Z* isomer. Peaks L2 could only be tentatively assigned as 13*Z* [30] and L7 or L8 as 7*Z* [31] by comparison of spectra according to the isomerization shift of the main maximum, the *Z* peak ratio which is calculated from the height of the *Z* peak divided by the height of the main maximum [31] and by its retention on C_{30} phases relative to

Table 1
Spectral characteristics, distribution and assignment of the carotene isomers formed in *E. coli* transformants expressing different desaturase genes

Carotenoid assignment	Absorbance maxima (nm)	*Synechocystis crtQb*		*Anabaena crtQa*		*Erwinia crtI*	
		Ratio *Z*/main max.[a]	Distribution (%)[b]	Ratio *Z*/main max.	Distribution (%)	Ratio *Z*/main max.	Distribution (%)
N1	332, 423, **443**, 470	0.11	70.9		0		0
N2; all-*E*	332, 417, **442**, 470	0.07	29.1		0		0
L1; 7,9,7′,9′*Z*	420 (s), **439**, 460 (s)	0	29.4		0		0
L2; (13*Z*)[c]	345, 362, 441, **467**, 498		0	0.56	25.2	0.56	19.6
L3; 5,13′*Z*	345, 362, 445, **460**, 490.		0	0.25	4.5	0.27	6.0
L4	345, 362, 441, **467**, 497	0.13	6.5	0.13	3.0	0.12	6.3
L5; 9*Z*	345, 362, 442, **467**, 497	0.15	12.7	0.14	11.8	0.15	13.2
L6; 5,9′*Z*	345, 362, 442, **467**, 497	0.14	5.2	0.15	2.3	0.15	3.5
L7/L8; (7*Z*)	363, 445, **469**, 501	0.09	2.7/4.6	n.d.	0	n.d.	0
L9; all-*E*	363, 446, **472**, 504	0.07	16.0	0.07	43.8	0.07	25.9
L10; 5*Z*	363, 446, **473**, 504	0.07	22.9	0.07	9.4	0.07	25.5

[a] *Z*/main max. is the height of the *Z* peak (underlined) related to the height of the main maximum (in bold face).
[b] Of the isomers of individual carotenes.
[c] Tentative assignment in parentheses.

all-*E* [32]. A further indication for this assignment is the absence of both isomers after I_2-isomerization of all-*E* lycopene (data not shown) which produces only unhindered *Z* isomers like 5*Z*, 9*Z*, 13*Z* and 15*Z* [33]. As shown for β-carotene isomers [8], retention of mono *Z*-lycopenes on the C_{30} stationary phase decreased the more central the *Z*-double bonds are positioned. The all-*E* isomer eluted between 7*Z* and 5*Z*.

With the exception of trace A, all-*E* is among the dominant lycopene isomers formed in the experiments of Fig. 1. Characteristic for the structurally related desaturases from *E. uredovora* and *Anabaena* are the high amounts of 13*Z* lycopene and the absence of prolycopene (L1) whereas the *Synechocystis* desaturase forms predominantly the 7,9,7′,9′*Z* isomer and no detectable amounts of 13*Z* (Table 1).

The carotenogenic capacity of the lycopene-forming transformants carrying both different ζ-carotene desaturase genes was extended by the additional introduction of the lycopene cyclase gene *crtY* from *E. uredovora*. In Fig. 2 the resulting β-carotene isomers and the remaining lycopene isomers listed in Table 1 were separated. Four β-carotene isomers B1 to B4 were found in the transformant with the *Anabaena* ζ-carotene desaturase and only traces of lycopene isomers 9*Z* (L5) and all-*E* (L9) remained. With the individual standards, B3 was identified as 13*Z*, B7 as all-*E* and B9 as 9*Z* lycopene. With a thermally isomerized and NMR characterized β-carotene standard [34,35], B2 was identified as 9,13,13′*Z*, B4 as 9,13*Z* and B5 as 9,9′,13*Z*. The very low separation temperature of 11°C was chosen for all β-carotene containing samples in order to resolve B1 from B3, B5 from B6 and B7 from B8. At this temperature 9*Z* β-carotene (B9) and prolycopene (L1) eluted rather close together. They could be much better separated at 5°C with retention times of 53 and 58 min, respectively.

In the transformant with the ζ-carotene desaturase gene from *Synechocystis*, some of the lycopene isomers are converted to all-*E* β-carotene by the additional lycopene cyclase (Fig. 2B). In contrast to

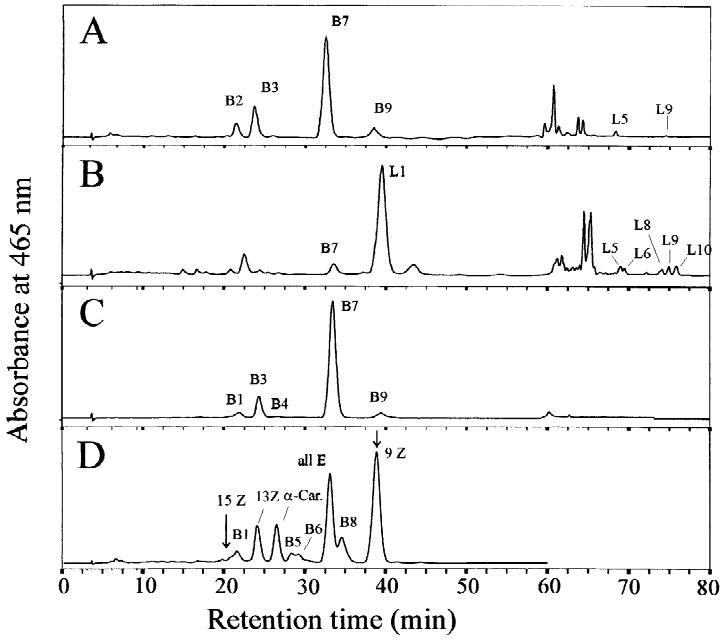

Fig. 2. Separation of β-carotene isomers. (A) Cyclization products from the isomers formed by the *Anabaena* ζ-carotene desaturase in *E. coli* transformants; (B) cyclization products from the isomers formed by the *Synechocystis* ζ-carotene desaturase in *E. coli* transformants; (C) β-carotene extract from *Synechocystis*; (D) β-carotene isomer standard and co-chromatography with 9*Z* and 15*Z* isomers (indicated by arrows). Separation was performed at 11°C. The lycopene isomers were labeled according to Fig. 1. Other peaks between 42 and 67 min are neurosporene isomers.

the transformant forming lycopene isomers as the end products (Fig. 1A), the residual amounts of prolycopene (L1) increased in relation to all the other lycopene isomers. This was observed in five independent transformants. Obviously, prolycopene cannot be directly or indirectly (via isomerization) converted to β-carotene in the *E. coli* transformant. In the carotene fraction from a *Synechocystis* culture (Fig. 2C), also all-*E* β-carotene is dominating with more than 75% of total β-carotene (Table 2). Of the four other mono β-carotene isomers, 9*Z* and 13*Z* could be identified by co-chromatography and spectral comparison to authentic standards (Fig. 2D).

With respect to the biosynthetic pathway of carotenogenesis [36], the initial formation of the 9 and 13 double bonds with the corresponding *Z* forms should already occur during the prenyl transferase reaction catalyzing the formation of geranylgeranyl pyrophosphate. Accordingly, some 11*Z* isomers would be formed during desaturation of phytoene and 7*Z* mono or poly isomers by the ζ-carotene desaturase reaction. 15*Z* β-carotene was absent in all cases. Since phytoene, the first carotene in the pathway to β-carotene, is predominantly formed as the 15*Z* isomer [37], isomerization may happen during the subsequent desaturation steps. Among the carotenes from *Synechocystis* cells, not any of the lycopene isomers including prolycopene (L1) which is the major product in the β-carotene producing *E. coli* transformant with the same ζ-carotene desaturase gene, is detectable. In the carotenogenic pathway of this cyanobacterium and related organisms with a similar ζ-carotene desaturase, a specific process may prevent the accumulation of some *Z* isomers of lycopene, especially of prolycopene or convert them in situ to all-*E* or other isomers which can be cyclized to β-carotene. Alternatives are either photoisomerization which is effective in vitro in the presence of chlorophyll [38] or enzymatic isomerization reactions. The latter possibility is supported by the existence of mutants with a poly*Z* carotenoid pathway which accumulates prolycopene with a 7,9,7′,9′*Z* configuration [29,39].

By combinatorial biosynthesis, several acyclic and monocyclic monohydroxy carotenoids were synthesized. They differ by their carbon chain length and the degree of desaturation (Fig. 3). Their retention behavior on a C_{30} stationary phase was assessed and compared. Separation of acyclic monohydroxycarotenoids with a chain length of 30, 40, and 45 carbons is shown in Fig. 4. The conjugated double bonds of the C_{40} carotenoids vary from 9 to 13 as indicated in Fig. 3. C_{45} nonaflavuxanthin (NFX) with the same polyene structure as C_{40} 1-hydroxylycopene (HOL) also carrying a terminal hydroxy group exhibited the longest retention time (Fig. 4A). Three minor isomers could be separated. Isomer NFX1 with the shortest retention time exhibited the highest isomerization shift, i.e., of the main absorbance maximum shown in bold face in Table 3.

Table 2
Spectral characteristics, distribution and assignment of the β-carotene isomers formed in *E. coli* transformant expressing different desaturase genes and an additionaly lycopene cyclase gene

Carotenoid assignment	Absorbance max. (nm)	*Anabaena crtQa*		*Synechocystis crtQb*		*Synechocystis* cells		β-Carotene standard	
		Z/main max.[a]	Distribution (%)	Z/main max.	Distribution (%)	Z/main max.[a]	Distribution (%)	Z/main max.	Distribution (%)
B1	338, 426 (s), **450**, 475					0.45	5.1		
B2, 9,13,13′*Z*	338, 420 (s), **444**, 470(s)	0.41	8.2					0.40	4.6
B3, 13*Z*	339, 422 (s), **445**, 470	0.43	17.8			0.45	12.0	0.39	9.8
B4, 9,13*Z*	338, 420 (s), **442**, 470					0.14	1.4	0.14	10.2
B5, 9,9′13*Z*	338 419, **442**, 470							0.10	2.5
B6	330, 420, **442**, 472							0.08	3.0
B7, all-*E*	338, 428 (s), **452**, 479	0.05	65.6	0.05	100	0.05	75.7	0.05	25.5
B8	340, 420, **442**, 468							0.08	8.2
B9, 9*Z*	340, 424 (s), **446,** 474	0.11	8.5			0.11	4.9	0.10	36.2
9*Z* standard	338, 424 (s), **449**, 475							0.10	
15*Z* standard	340, 425, **446**, 474							0.56	

[a] *Z*/main max. is the height of the *Z* peak (underlined) related to the height of the main maximum (in bold face). Tentative assignment in parentheses.

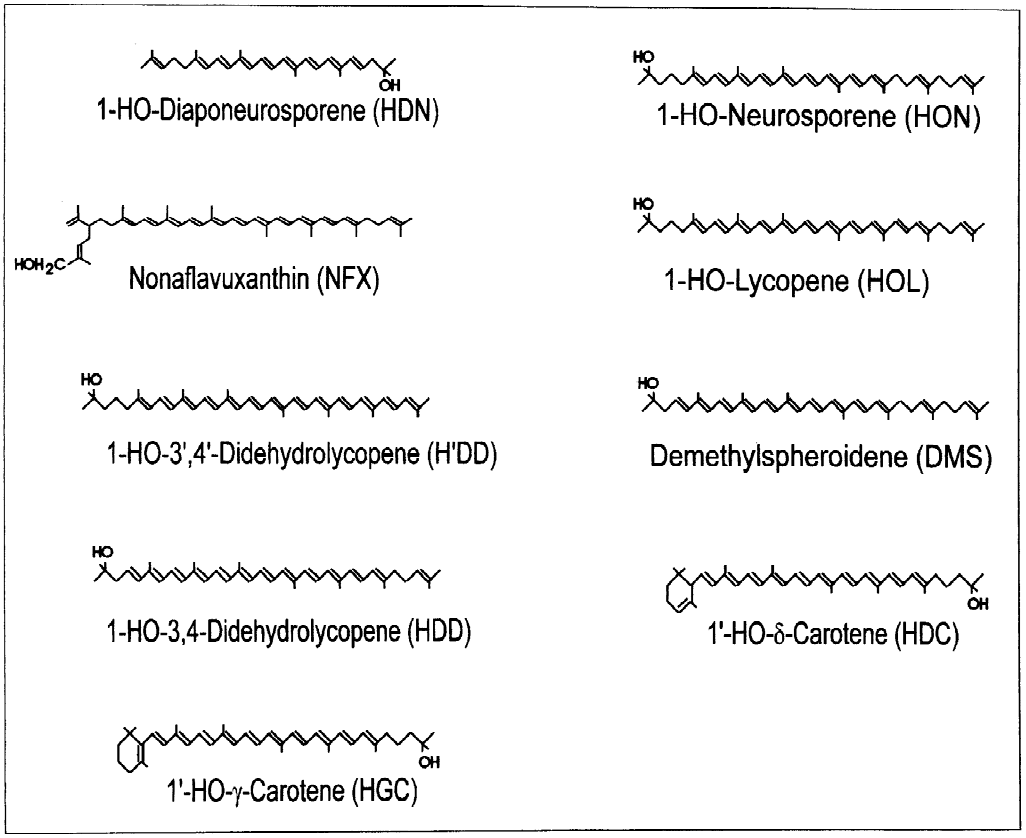

Fig. 3. Structures of carotenoids separated on C_{30} stationary phase: C_{30} hydroxydiaponeurosporene (HDN) with eight conjugated double bonds, C_{45} nonaflavuxanthin (NFX) with 11 conjugated double bonds, the C_{40} carotenoids 1-hydroxy-3′,4′-didehydrolycopene (H′DD) with 13 conjugated double bonds, 1-hydroxy-3,4-didehydrolycopene (HDD) with 12 conjugated double bonds, 1-hydroxyneurosporene (HON) with nine conjugated double bonds, 1-hydroxylycopene (HOL) with 11 conjugated double bonds, demethylspheroidene (DMS) with 10 conjugated double bonds, and the monocyclic 1′-hydroxy-δ-carotene (HDC) with 10 conjugated double bonds as well as 1′-hydroxy-γ-carotene (HGC) with 10 conjugated double bonds in the chain and one in the ionone ring.

Furthermore, the *Z* peak ratio is highest with 0.58. Compared to the *Z* peaks of lycopene and β-carotene isomers ([31,34]; Table 1), this value indicates a *Z* configuration of a central double bond, preferentially 13*Z* or 15*Z*. NFX2 and NFX3 with *Z* peak ratios around 0.15 could possess mono-*Z* or di-*Z* double bonds at the end of the conjugated chain. According to the spectral data, NFX4 or NFX5 with the longest retention times could be the all-*E* isomer. In comparison to the separation behavior of lycopene isomers listed in Table 1, it is likely that NFX4 is all-*E* and NFX5 a 5*Z* isomer. For the same reasons, the configuration of HOL6 (Fig. 4B) is assumed as all-*E* and HOL7 as 5*Z*. The peak of the all-*E* isomer of hydroxylycopene (HOL6) elutes more than 20 min earlier that NFX. The spectral characteristics including the *Z* peak ratio together with the relative retention behavior of the HOL isomers (Table 3A) resemble closely the lycopene isomers in Table 1. Based on this comparison it is proposed that HOL1 or HOL2 may be 13*Z*, HOL4 9*Z*, HOL5 5,9′*Z*, HOL6 all-*E* and HOL7 5*Z*.

1-Hydroxyneurosporene (HON) with nine conjugated double bonds is less retained than HOL with 11 conjugated double bonds (Fig. 4C). Among all HON isomers, HON1 and HON2 exhibited the highest *Z* peak ratios of 0.45 and 0.26, respectively (Table 3A). By comparison to spectral data of

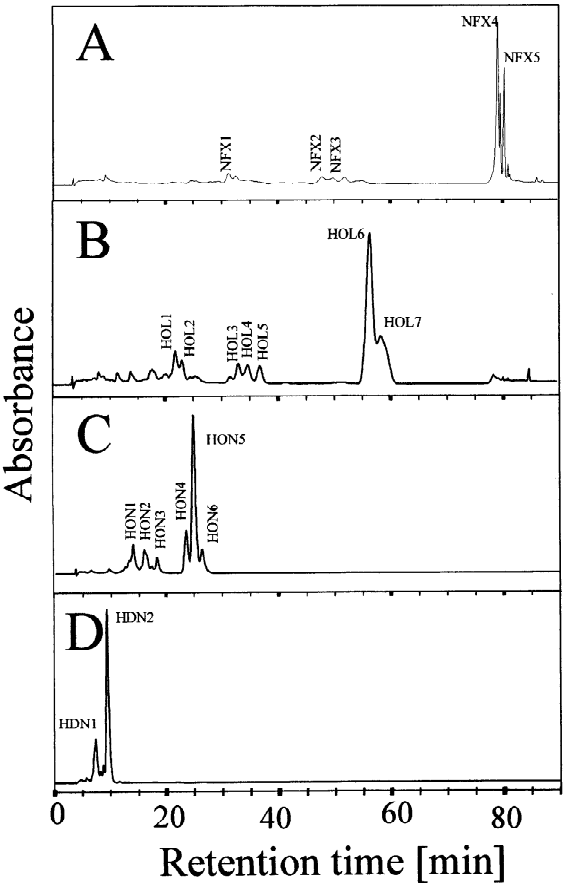

Fig. 4. Chromatogram of acyclic hydroxycarotenoids with different carbon chain length. The absorbance of (A) nonaflavuxanthin (NFX) and (B) hydroxylycopene (HOL) were recorded at 475 nm, (C) hydroxyneurosporene (HON) at 440 nm and (D) hydroxydiaponeurosporene (HDN) at 425 nm. Separation temperature was 26°C.

neurosporene isomers [27], these may be either 9*Z*, 13*Z* or 15*Z* isomers. HON4 to HON6 showed the lowest *Z* peak ratios and HON5 which is the most abundant isomer should be all-*E*. Hydroxydiaponeurosporene has the closest basic structure to hydroxyneurosporene apart from the chain length. The chemical characterization by Dr. S. Takaichi, Nippon Medical School, Kawasaki, Japan, of this C_{30} carotenoid of M_r 420 which is formed from diaponeurosporene revealed that it carries a secondary hydroxy group and a chain of eight conjugated double bonds (unpublished). Since this compound can be further desaturated to a product with 10 conjugated double bonds (data not shown), the most likely structure is the one given in Fig. 3. Two isomers could be separated (Fig. 4D). Both had a lower retention time than all the HON isomers. The main absorbance maximum of HDN1 was shifted by 5 nm to shorter wavelength compared to HDN2 and its *Z* peak ratio was high with a value of 0.3 (Table 3A). HDN1 is an isomer with a rather central *Z* double bond whereas the spectral data of HDN2 point to all-*E*.

With increasing carbon chain length, the hydroxycarotenoids are stronger retained. It could also be shown for all hydroxycarotenoids in Fig. 4 that the more central the *Z* double bond indicated by height of the *Z* peak ratio, the faster the isomers eluted from the C_{30} stationary phase. Although some of the *Z* isomers of NFX elute close to hydroxylycopene isomers and the HOL isomers close to the HON isomers, the major peaks were well-separated on the C_{30} stationary phase at a temperature of 26°C (Fig. 4).

Fig. 5 shows the hydroxycarotenoids separated according to the length of their polyene chain. Furthermore, hydroxy carotenes derived from lycopene by introduction of one β- or ϵ-ring, i.e., HGC and HDC, were included and compared. Demethylspheroidene differs from HON by an additional double bond at C-3 (Fig. 3). Three isomers could be separated in Fig. 5A. They all had a rather low *Z* peak and an assignment of the position of the *Z* double bond was not possible. Both major isomers had a retention time of 26 to 28 min compared to HON isomers which all eluted within 23 min.

Introduction of a C-3 double bond into HOL results in a 1-hydroxy-3,4-didehydrolycopene (HDD) molecule with 12 conjugated double bonds (Fig. 3). Three isomers of HDD could be separated (Fig. 5D) all with a rather low *Z* peak ratio. Beside of minor HDD1, two major peaks around 80 min were found. One of it may be all-*E*. All isomers are much stronger retained than HOL isomers. 1-Hydroxy-3′,4′-didehydrolycopene (H′DD) resembles HOL with an additional double bond at C-3′ extending the conjugated double bond system to 13 (Fig. 3). As for the previous carotenoids, three isomers of H′DD were detected according to the *Z* peak ratio and

Table 3
Spectral characteristics of the hydroxycarotene isomers separated in Fig. 4 (A) and Fig. 5 (B)

A			B		
Carotenoid isomer	Absorbance maxima (nm)	Ratio *Z*/main max.[a]	Carotenoid isomer	Absorbance maxima (nm)	Ratio *Z*/main max.[a]
NFX1	346, 360, 438, **463**, 496	0.58	DMS1	341, 423, **450**, 482	0.06
NFX2	347, 361, 440, **467**, 499	0.15	DMS2	332, 342, 428, **452**, 485	0.10
NFX3	347, 360, 439, **465**, 499	0.14	DMS3	333, 345, 430, 453, 486	0.10
NFX4	444, **472**, 502	0.07	HGC1	337, 349, 432, **453**, 478	0.57
NFX5	443, 472, 503	0.07	HGC2	338, 350, 434, 452, 481	0.59
HOL1	343, 361, 438, **463**, 494	0.59	HGC3	348, 431, **456**, 484	0.14
HOL2	342, 361, 439, **464**, 497	0.60	HGC4	434, **462**, 492	0.06
HOL3	343, 360, 439, **465**, 498	0.14	HGC5	438, **461**, 491	0.05
HOL4	345, 359, 439, **463**, 498	0.13	HDC1	331, 344, 426, **450**, 482	0.61
HOL5	342, 359, 440, **464**, 498	0.13	HDC2	332, 343, 430, **448**, 479	0.19
HOL6	442, 471, 503	0.07	HDC3	343, 430, **456**, 488	0.06
HOL7	443, **471**, 503	0.07	HDC4	343, 430, **453**, 487	0.06
HON1	318, 329, 410, **433**, 463	0.45	HDC5	329, 341, 427, **451**, 482	0.09
HON2	314, 329, 410, **434**, 462	0.26	HDD1	378, 453, **482**, 513	0.07
HON3	318, 330, 409, **432**, 462	0.10	HDD2	456, **483**, 515	0.09
HON4	314, 328, 412, **440**, 469	0.07	HDD3	459, **484**, 516	0.06
HON5	328, 413, **439**, 470,	0.06	H′DD1	392, 462, **491**, 524	0.12
HON6	328, 414, **440**, 469	0.06	H′DD2	368, 386, 432, 464, **490**, 525	0.20
HDN1	300, 313, 391, **415**, 442	0.30	H′DD3	370, 389, 438, 467, **496**, 529	0.12
HDN2	312, 398, **420**, 446	0.05			

[a] *Z*/main max. is the height of the *Z* peak (underlined) related to the height of the main maximum (in bold face).

especially the isomerization shift of the main absorbance maximum, of which H′DD3 seems to be the all-*E* isomer. The peaks around 60 min were HOL isomers and the peak at 82 min did not exhibit a carotenoid-like spectrum. All H′DD isomers including all-*E* eluted a few minutes later from the C_{30} column than the three HDD isomers.

The length-dependent separation behavior of the hydroxycarotenoids on the C_{30} stationary phase is similar to the C_{18} phase (Fig. 4). However, for each derivative several isomers could be resolved which previously could not be detected [18]. It seems to be a general feature that cyclic end groups decrease the retention time on all reversed-phases (Figs. 4 and 5). From the C_{30} column, hydroxy-δ-carotene with one ε-ionone end group elutes earlier than hydroxy-γ-carotene with a β-ionone end group. This corresponds to the faster elution of bicyclic lutein with one ε-end group and one β-end group versus zeaxanthin with two β-end groups [8].

The hydroxycarotenoids used in this study showed a continuous extension of their conjugated double bond system from 9 to 13 (Fig. 3). Much better than on C_{18} stationary phases [18,24], a direct relationship between the length of the polyene group of the hydroxycarotenoids and the retention time was observed with our C_{30} column (Figs. 4 and 5). The same behavior was found for non-substituted carotenes [11]. As indicated by the retention of 1-hydroxy-3,4-didehydrolycopene with 12 conjugated and an isolated double bond compared to 1-hydroxy-3′,4′-didehydrolycopene with 13 conjugated double bonds, the interaction of an isolated double bond with the C_{30} stationary phase is slightly less than a conjugated double bond.

The product of the *crtD* gene catalyzes the introduction of a single 3,4-double bond into 1-hydroxycarotenoids [19,40]. On C_{18} columns the separation of the product with an additional double bond is rather poor due to the polarity of the hydroxy group. We have demonstrated in the present study that with a C_{30} stationary phase the separation of the 3,4-desaturated products from the substrates is no longer a problem.

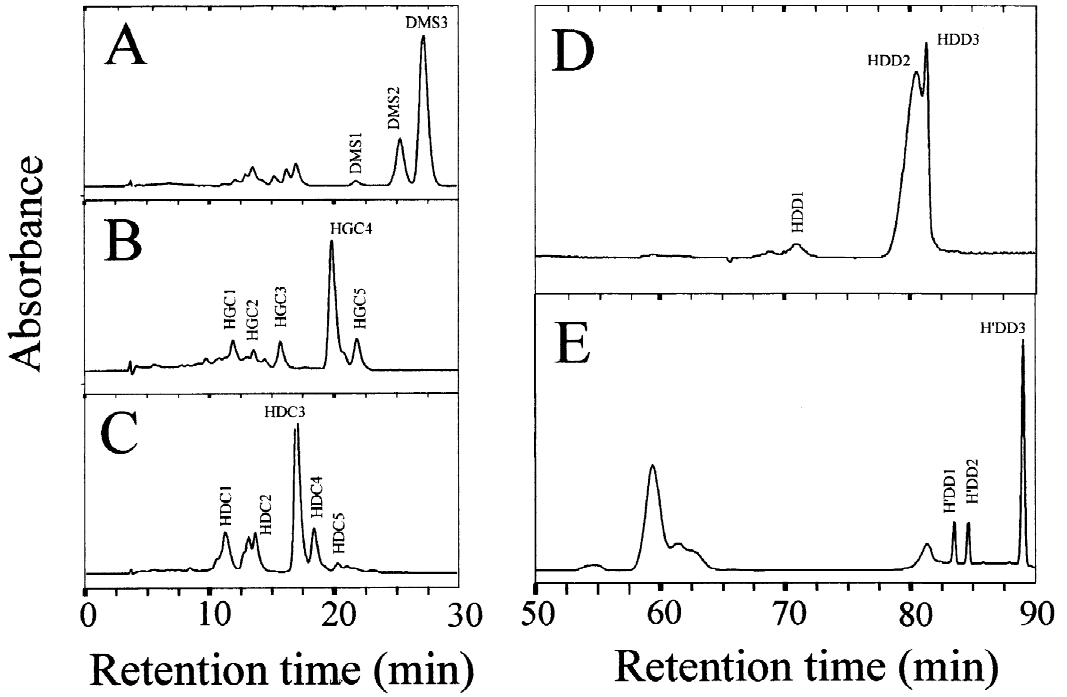

Fig. 5. Chromatogram of acyclic and monocyclic 1-hydroxy C_{40} carotenoids with different degree of desaturation. The absorbance of (A) demethylspheroidene (DMS) was recorded at 455 nm, (B) hydroxy-γ-carotene (HGC) at 460 nm and (C) hydroxy-δ-carotene (HDC) at 455 nm, (D) 1-hydroxy-3,4-didehydrolycopene (HDD) at 480 nm (E) 1-hydroxy-3′,4′-didehydrolycopene (H′DD) at 492 nm. Separation temperature was 26°C.

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