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Short communication

Reversed-phase high-performance liquid chromatographic identification of lutein and zeaxanthin stereoisomers in bovine retina using a C_{30} bonded phase

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Abstract

An efficient reversed-phase high-performance liquid chromatographic assay with ultraviolet detection at 450 nm for simultaneous determination of lutein and zeaxanthin stereoisomers in bovine retina is described. The procedure involves rapid and careful one-step hexane extraction of the carotenoids from the homogenized liquid and enrichment by on-line solid phase extraction on a polystyrene cartridge. The substances were eluted at a flow rate of 1 ml/min with acetone–water (85:15, v/v) on a C_{30} reversed-phase column. C_{30} phases exhibit superior shape selectivity for the separation of carotenoid stereoisomers compared with conventional C_{18} phases. For comparison a mixture of iodine-isomerized lutein and zeaxanthin standards was separated.

Keywords: Stereoisomer separation; Lutein; Zeaxanthin

1. Introduction

Lutein and zeaxanthin, two of the approximately 600 naturally occuring carotenoids and the major carotenoids of maize and melon, respectively, are constituents of the retina and are responsible for sharp and detailed vision [1,2]. New studies on age-related macular degeneration (AMD), one of the most important irreversible blinding diseases, show that the course of the disease is decisively influenced by the concentration of these two carotenoids in the retina [3]. In this context nutritional epidemiological reports have suggested that dietary intake of foods rich in certain carotenoids, in particular dark green, leafy vegetables (e.g. spinach, savoy, kale, parsley, broccoli and green peas) may protect against AMD [4]. It is supposed that lutein and zeaxanthin act as antioxidants protecting the retina from overexposure to short-wavelength visible light [5].

Taking into account the different biological effectiveness of carotenoid stereoisomers in treatment of AMD [6], knowledge of the presence of each available stereoisomer is of great importance. The distribution of lutein and zeaxanthin stereoisomers in the retina was examined by Bone et al. using conventional C_{18} reversed-phase high performance liquid chromatography (HPLC) [7]. The commonly used C_{18} phases for reversed-phase HPLC are well-suited for carotenoid separation, but the selectivity is inadequate for efficient separation of *cis* and *trans*

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isomers. Polymeric C_{30} phases have been specially designed by Sander et al. for separation of unpolar molecules and their geometric isomers [8,9]. The synthesis was optimized in our group by Pursch and Lacker [10,11] and the resulting C_{30} bonded phases have been used for the separation of stereoisomers of vitamin E, vitamin A derivatives and β-carotene [12–15].

With such a triacontyl phase all principal stereoisomers of lutein and zeaxanthin (Figs. 1 and 2) can be easily separated. In contrast to the centrosymmetric molecule zeaxanthin (derived from β-carotene), lutein (derived from α-carotene) has an unsymmetric structure, so that pairs of *cis* isomers exist as shown in Fig. 2.

This article reports on the identification of lutein and zeaxanthin *cis*/*trans* stereoisomers in bovine retina by reversed-phase HPLC. Separation of lutein and zeaxanthin stereoisomers on this specially designed triacontyl stationary phase is shown, demonstrating high selectivity and high resolution. A mixture of iodine-isomerized standards of lutein and zeaxanthin was also measured for comparison.

Because of the instability of carotenoid stereoisomers, a rapid and careful extraction method was developed, including one-step liquid–liquid extraction, followed by on-line solid phase extraction (SPE). The combined extracts were not evaporated, but injected directly into the injection valve of the HPLC-system in which the SPE cartridge was fixed as shown in Fig. 3. Along with faster consumption the advantage of this method is that the carotenoids are enriched directly in the HPLC-system, thus excluding light and oxygen. By switching the valve the sample elutes from the cartridge with the mobile phase. The *cis*/*trans* isomers of the carotenoids are then separated on the triacontyl stationary phase.

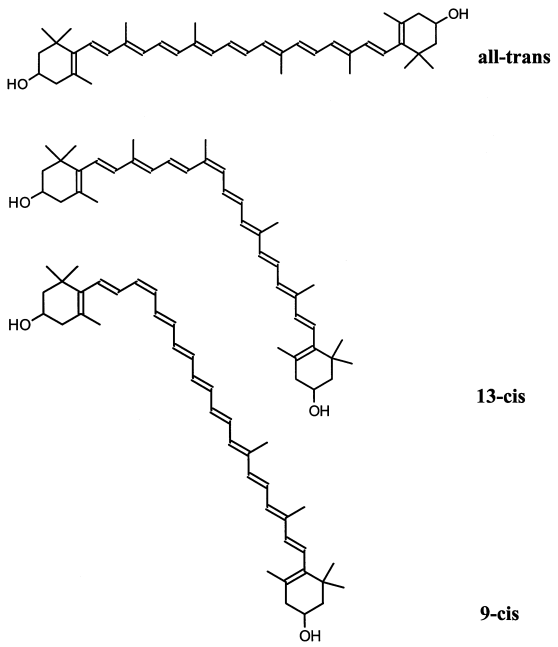

Fig. 1. Structures of the identified *cis*/*trans* isomers of zeaxanthin.

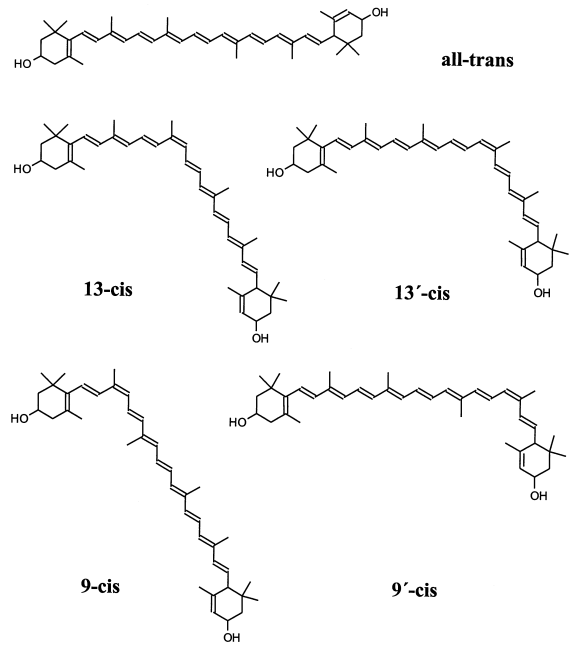

Fig. 2. Structures of the identified *cis*/*trans* isomers of lutein.

2. Experimental

2.1. Chemicals and standards

All solvents were HPLC grade, obtained from Merck (Darmstadt, Germany). The standards of lutein and zeaxanthin were a donation from Hoffmann LaRoche (Basel, Switzerland). 5 mg of the pure substances were dissolved each in 5 ml of chloroform and subsequently isomerized by adding

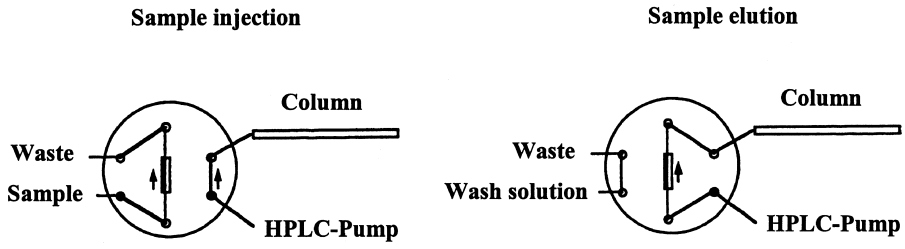

Fig. 3. Scheme of the enrichment of carotenoids by on-line SPE.

one drop of a solution of a iodine crystal in 2 ml hexane [16]. The solution was exposed to sunlight for about 5 h. The resulting isomerized solution was kept at −40°C.

2.2. Sample preparation and extraction

The eyes of bovines were immediately chilled in ice. At the University Eye Hospital Tübingen the retina was cut out rapidly. Two retinas were pooled in a culture tube and homogenized in a mixture of 4 ml of ethanol–water (50:50, v/v). The homogenized sample was centrifuged (6000 *g*) for 5 min. The supernatant liquid was carefully transferred into another culture tube. An aliquot of 2 ml hexane was added and the carotenoids were extracted. The mixture was centrifuged (6000 *g*) for 10 min for improved separation of the two phases. The extraction step was repeated. The pooled hexane phases were stored at −40°C.

2.3. On-line SPE

A 10 mm PLRP-S cartridge (Spark, The Netherlands) was placed into the injection loop as shown in Fig. 3. The sample was manually injected at a rate of about 1 ml/min. While the carotenoids were enriched on the cartridge, the solvent and other polar substances were carried to waste. The entire sample (4 ml) was injected. The enriched carotenoids were washed with 500 μl H_2O to reduce the hexane content. Switching the valves places the cartridge between the HPLC pump and the column. At this point the sample was eluted from the cartridge with the mobile phase acetone–water (85:15, v/v) at a flow rate of 1 ml/min, and separation on the C_{30} phase begins.

2.4. Chromatography

Analyses were performed on a Hewlett-Packard HP 1100 chromatographic system equipped with a binary pump G 1312A and UV detector G 1314A (Hewlett-Packard GmbH, Waldbronn, Germany). The *cis*-isomers were examined with a Waters 990 Photodiode Array Detector. Chromatography was performed on 250 mm×4.6 mm stainless steel columns (Bischoff, Leonberg, Germany) packed with ProntoSil 200 silica gel (Bischoff, Leonberg, Germany) and YMC silica gel (YMC Europe, Schermbeck, Germany). This silica gels were modified in our group with triacontyltrichlorosilane (ABCR, Karlsruhe, Germany) as stationary phase (200 Å, 3 μm). An isocratic mixture of acetone–water (85:15, v/v) was used as solvent. The flow rate was maintained at 1 ml/min, and elution was monitored by absorbance detection at 450 nm. 1 μl of a 0.01% solution (w/v) of isomerized standards of lutein and zeaxanthin were injected onto the column.

3. Results and discussion

Due to the small amount of carotenoids present in retina samples, careful treatment is essential. Additional problems are caused by sample oxidation and isomerization. Thus the retina should be excised quickly from the eyeball and protected from light to minimize oxidation and isomerization.

Liquid–liquid extraction is clearly the method of choice for isolation of the carotenoids lutein and zeaxanthin from the retina. Solid phase extraction (off-line and on-line) is unsuitable, because the homogenized sample tends to plug the cartridge and the column. But after isolation of the desired sub-

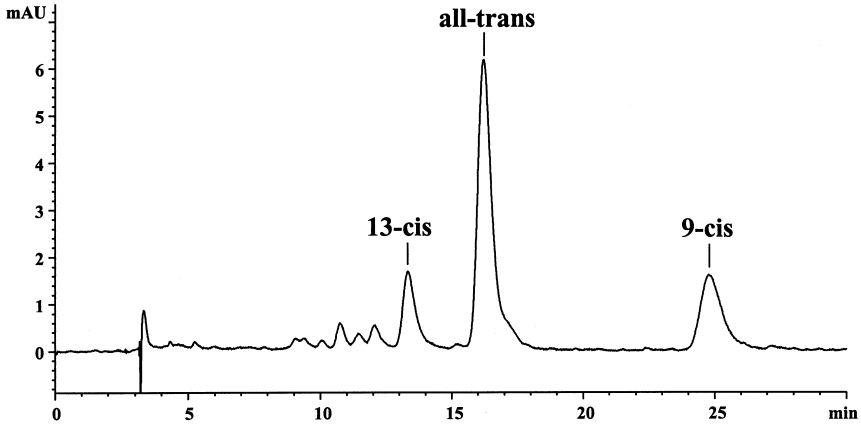

Fig. 4. HPLC chromatogram of the separation of zeaxanthin stereoisomers using a triacontyl stationary phase.

stances via liquid–liquid extraction on-line SPE offers the possibility to transfer the extract directly into the chromatographic system. Evaporation of the hexane extract would require too much time, and the carotenoids would be isomerized and oxidized. With on-line SPE the carotenoids are enriched directly on the cartridge in the chromatographic system, thus excluding light and oxygen. This direct transfer of the sample onto the column requires less sample preparation. As the cartridge can be cleaned analogous to a HPLC column the cartridge is used several times and simple automation is possible.

Figs. 4 and 5 show the separation of the *cis*/*trans* isomers of lutein and zeaxanthin on a triacontyl stationary phase (3 μm, 200 Å) exhibiting high selectivity and resolution. The structures of the principal stereoisomers in Figs. 1 and 2 are assigned to the chromatograms in Figs. 4 and 5, respectively. All major peaks in Figs. 4 and 5 have been identified by two-dimensional stopped-flow HPLC–NMR spectroscopy [17]. Besides the main stereoisomers, several minor compounds are observed, which are *cis* isomers, too. This was proofed by the small hypochromic shift of 1–3 nm and the significant hypochromic effect in the UV spectra [2]. Considering the influence of *cis*-bonded molecules on retention times, the following results have been found: Compared with all-*trans* zeaxanthin, elution of 13-*cis*

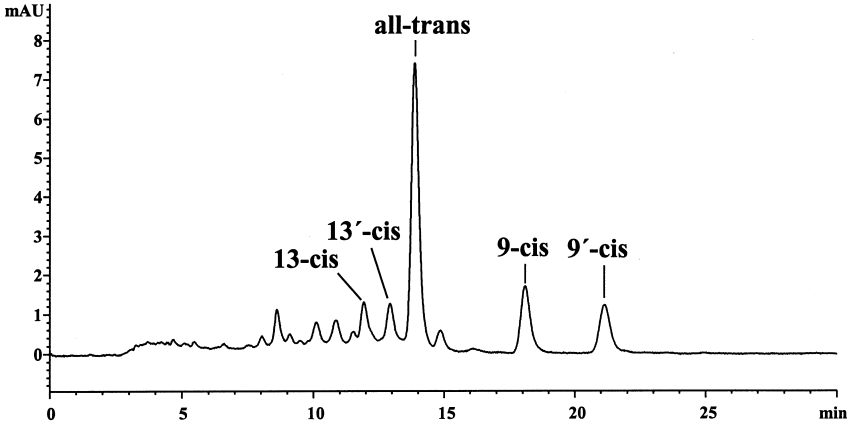

Fig. 5. HPLC chromatogram of the separation of lutein stereoisomers using a triacontyl stationary phase.

zeaxanthin was faster, while the 9-*cis* isomer interacted more efficiently with the stationary phase, resulting in a longer retention time. Lutein shows the same order of retention times. However, due to the unsymmetric structure of lutein, pairs of *cis* isomers were found.

The upper chromatogram in Fig. 6 depicts the separation of the standard mixture consisting of the iodine-isomerized retinal carotenoids lutein and zeaxanthin. Comparison with the lower chromatogram of the separation of the retina extract in Fig. 6 unequivocally shows that the retention times of the all-*trans* isomers do agree. The isomerization products can also be assigned. The peaks on the right side of the all-*trans* peaks are the 9-*cis* isomers of lutein and zeaxanthin in the following order: 9-*cis* lutein (18.4 min), 9′-*cis* lutein (21.7 min) and 9-*cis* zeaxanthin (23.8 min). The peaks on the left side near the all-*trans* peaks at retention times of about 12–13 min belong to the 13-*cis* isomers of lutein and zeaxanthin. Obviously, there exist several minor compounds besides the main stereoisomers, which are, considering the small hypsochromic shift of 1–3 nm, *cis*-isomers also [2]. The lower chromatogram in Fig. 6 exhibits additional peaks at retention times of about 6 min, which are oxidation products [12]. These oxidation and isomerization products, which are present in the retina, are not artifacts of extraction and chromatography. This was proven by studies with all-*trans* lutein and all-*trans* zeaxanthin,

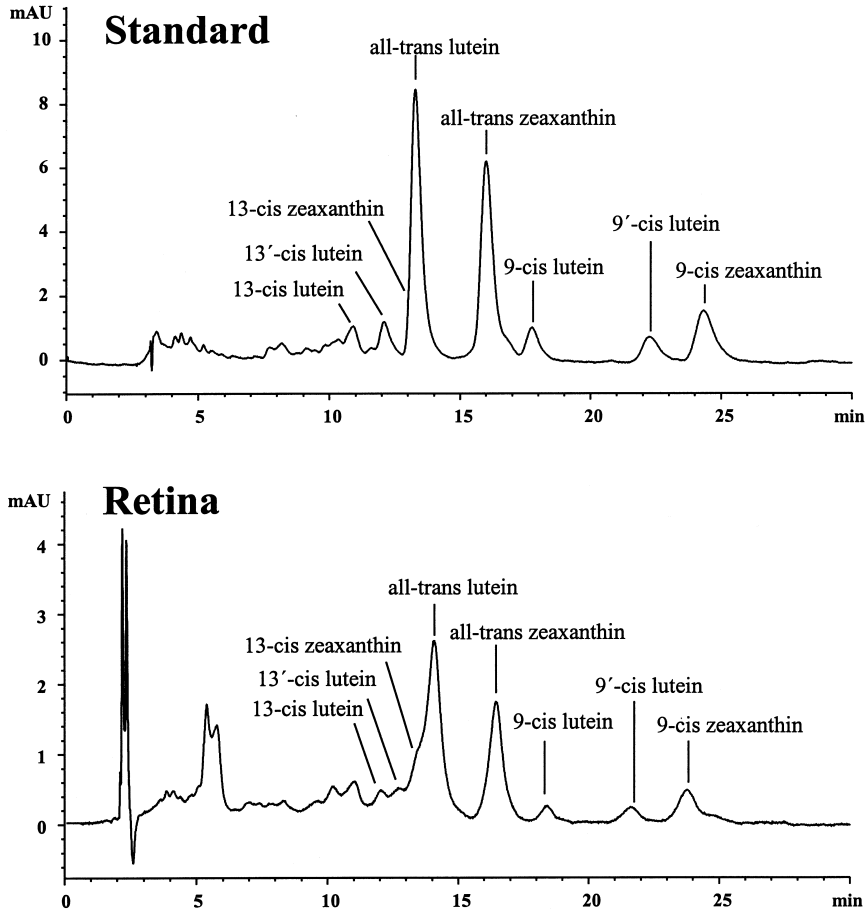

Fig. 6. HPLC chromatograms of the separation of isomerized standards of lutein/zeaxanthin stereoisomers (above) and of the separation of retina extracts (below) using a triacontyl stationary phase.

which show no isomerization or oxidation products after the described extraction and chromatographic procedure.

4. Conclusions

A simple, rapid and efficient extraction of carotenoids from the retina was developed, which minimizes oxidation and isomerization. A conventional off-line preparation of the sample with liquid–liquid extraction and evaporation of the solvents was not successful and only oxidation products were measured. With the technique of on-line SPE, the sample will not be destroyed and the stereoisomers are detectable. Using specially designed C_{30} stationary phases, high selectivity for the separation of lutein and zeaxanthin stereoisomers was achieved.

References

[1] J.J. Nussbaum, R.C. Pruett, F.C. Delori, Retina. 1 (1981) 296.
[2] G. Britton, S. Liaaen-Jensen, H. Pfander, Carotenoids, Vol. A: Isolation and Analysis, Birkhäuser Verlag, Basel, 1995.
[3] B.R. Hammond, E.J. Johnson, R.M. Russell, N.I. Krinsky, K. Yeum, R.B. Edwards, D.M. Snodderly, Invest. Ophthalmol. Vis. Sci. 38 (1997) 1795.
[4] J.M. Seddon, U.A. Ajani, R.D. Sperduto, R. Hiller, N. Blair, T.C. Burton, M.D. Farber, E.S. Gragoudas, J. Haller, D.T. Miller, L.A. Yannuzzi, W. Willett, J. Am. Med. Assoc. 18 (1994) 1413.
[5] F. Khachik, P.S. Bernstein, D.L. Garland, Invest. Ophthalmol. Vis. Sci. 38 (1997) 1802.
[6] H.K. Biesalski, C. Hemmes, W. Hopfenmüller, C. Schmidt, H. Gollnick, Free Radical Research. 24 (1996) 215.
[7] R.A. Bone, J.T. Landrum, L.M. Friedes, C.M. Gomez, M.D. Kilburn, E. Menendez, I. Vidal, W. Wang, Exp. Eye Res. 64 (1997) 211.
[8] L.C. Sander, K. Epler Sharpless, N.E. Craft, S.A. Wise, Anal. Chem. 66 (1994) 1667.
[9] C. Emenhiser, L.C. Sander, S.J. Schwartz, J. Chromatogr. A. 707 (1995) 205.
[10] M. Pursch, S. Strohschein, H. Händel, K. Albert, Anal. Chem. 68 (1996) 386.
[11] T. Lacker, M. Raitza, K. Albert, J. Chromatogr. A, in preparation.
[12] S. Strohschein, M. Pursch, H. Händel, K. Albert, Fres. J. Anal. Chem. 357 (1997) 498.
[13] S. Strohschein, G. Schlotterbeck, J. Richter, M. Pursch, L.-H. Tseng, H. Händel, K. Albert, J. Chromatogr. A. 765 (1997) 207.
[14] K. Albert, T. Lacker, M. Raitza, M. Pursch, H.-J. Egelhaaf, D. Oelkrug, Angew. Chem. Int. Ed. 37 (1998) 778.
[15] S. Strohschein, M. Pursch, D. Lubda, K. Albert, Anal. Chem. 70 (1998) 13.
[16] L. Zechmeister, Cis–trans Isomeric Carotenoids, Vitamin A and Arylpolyenes, Academic Press, New York, 1962.
[17] M. Dachtler, T. Glaser, K. Albert, Anal. Chem., in preparation.