Journal of Chromatography, 452 (1988) 535–542
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 20 756

REVERSED-PHASE LIQUID CHROMATOGRAPHY OF ASTACENE

H. J. C. F. NELIS and A. P. DE LEENHEER*
Laboratoria voor Medische Biochemie en voor Klinische Analyse, Rijksuniversiteit Gent, Harelbekestraat 72, B-9000 Ghent (Belgium)

SUMMARY

Carotenoids can be efficiently separated by non-aqueous reversed-phase liquid chromatography on Zorbax ODS. However, astacene, an acidic ketocarotenoid was found to display a peculiar behaviour in that it could not be eluted from this support using mixtures of acetonitrile–methanol–dichloromethane. The importance of astacene as a degradation product of astaxanthin warranted the development of a new liquid chromatographic system. Organic acids promoted the elution of the compound, but failed to suppress the accompanying excessive peak tailing. Efficient chromatography resulted from the incorporation of bis(2-ethylhexyl) phosphate (BEHP), an acid with a high degree of lipophilicity, in the mobile phase. Optimum separation of astacene and astaxanthin was achieved using methanol-free, semi-aqueous eluents, *i.e.*, acetonitrile–water, containing 0.01–0.05 *M* BEHP. Increasing amounts of BEHP significantly reduced retention while, unexpectedly, water had little effect over the concentration range studied (1–5%). To illustrate the practical usefulness of the new system, astacene was demonstrated in a saponified extract of flower petals of *Adonis annua*.

INTRODUCTION

We have previously reported non-aqueous reversed-phase liquid chromatography (NARP) on Zorbax ODS for the separation of carotenoids[1]. Typical eluents for these hydrophobic pigments contain acetonitrile as a base solvent, a non-polar modifier, usually dichloromethane, to adjust the eluotropic strength and methanol to modulate the selectivity[1,2]. Over the years this NARP system, with slight modifications, has formed the basis for the profiling and quantitation of carotenoids in a variety of biological materials, including *Artemia*[3], orange juice[4], serum[5], bacteria[6] and algae[7].

It was however soon recognized that under the standard chromatographic conditions the acidic ketocarotenoid astaxanthin failed to yield a symmetrical peak[1]. This particular carotenoid is of considerable academic and practical interest because of its abundance in nature[8] and its high economic value as a pigment in fish feeds[9]. Unlike astaxanthin, its close structural analogue astacene could not be eluted from Zorbax ODS using mixtures of acetonitrile–dichloromethane–methanol. Astacene is

0021-9673/88/$03.50

readily formed as an artefact from astaxanthin or astaxanthin esters in the course of alkaline saponification[10], an essential part of many isolation and analysis procedures for carotenoids. Therefore, a basic requirement of any chromatographic system for the determination of astaxanthin is its ability to include astacene and to separate both analogues.

Similar difficulties to obtain symmetrical peaks for ketocarotenoids possessing enolic hydroxyl groups are encountered in normal-phase chromatography. Derivatization, *e.g.*, acetylation of the acidic groups, is an obvious approach to suppress peak tailing[11]. Recently, coating of the silica support with phosphoric acid has been proposed as another useful remedy[12]. This can be carried out either *in situ* or by a slurry method prior to column packing. On such phosphoric acid-coated silica, astacene, semi-astacene, astaxanthin as well as three mono-*cis*-astaxanthins were readily resolved as perfectly symmetrical peaks.

We describe an alternative solution to the problem using reversed-phase chromatography with a mobile phase containing an organic, lipophilic phosphoric acid derivative. In general, reversed-phase is more attractive than normal-phase chromatography because it is more reproducible and less subject to variability.

EXPERIMENTAL

Chemicals

All-*trans*-astaxanthin and all-*trans*-astacene were gifts from Hoffmann-La Roche (Basle, Switzerland). Bis(2-ethylhexyl) phosphate (BEHP) was obtained from Fluka (Buchs, Switzerland). According to the manufacturer, the purity was approximately 50%, the remainder being mono(2-ethylhexyl) phosphate. Acetonitrile (Janssen Chimica, Beerse, Belgium), dichloromethane and methanol (both from Hoechst, Frankfurt, F.R.G.) were "chemically pure". The last two were redistilled in a spinning band apparatus. All other chemicals were reagent grade from Merck (Darmstadt, F.R.G.).

Chromatography

The HPLC system 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 an 100-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 correct elution order of astacene and astaxanthin was verified on the basis of their absorption spectra. The columns (15 cm $\times$ 0.46 cm I.D.) were packed with 5-μm Zorbax ODS (DuPont, Wilmington, DE, U.S.A.) or 5-μm Hypersil ODS (Shandon, Runcorn, U.K.). Elution was carried out with mixtures of acetonitrile–methanol–water–BEHP, the optimum mobile phase containing acetonitrile–water (97:3, v/v) and 0.05 *M* BEHP. The flow-rate was 1 ml/min and the temperature ambient.

Extraction of flower petals of Adonis annua

A few milligrams of flower petals of *Adonis annua* were homogenized with 3 ml of absolute ethanol in an all-glass Potter-Elvehjem tube. After addition of 0.8 ml of a

60% (w/v) aqueous potassium hydroxide solution, the mixture was saponified at 60°C for 45 min. A 3-ml volume of a 5% (w/v) sodium chloride solution was added and the carotenoids were extracted in 6 ml of diethyl ether. The organic layer was washed with water, dried over anhydrous sodium sulphate and evaporated to dryness under nitrogen. The residue was reconstituted with the chromatographic solvent and an 100-μl aliquot was injected on the liquid chromatographic column.

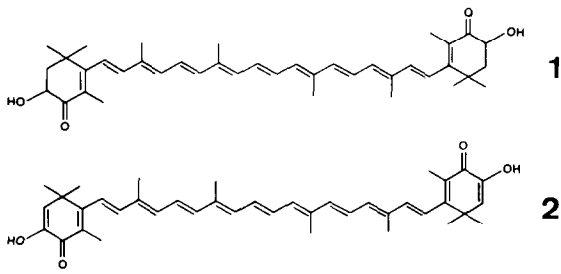
RESULTS AND DISCUSSION

Chromatographic behaviour of astaxanthin and astacene in NARP

Despite their structural similarity (Fig. 1), the chromatographic behaviour of astaxanthin and astacene in NARP was thoroughly different. Using the standard eluent acetonitrile–methanol–dichloromethane, astaxanthin was mostly eluted as a badly tailing peak from new Zorbax ODS columns[1], whereas astacene did not chromatograph at all. Even a gradient to 100% dichloromethane failed to bring about elution. At first, silanophilic interactions were thought to account for the erratic chromatographic behaviour of astacene in particular. However, when Zorbax ODS, which is rich in accessible silanol groups, was replaced by Hypersil ODS, allegedly a totally endcapped material, the same phenomenon was still observed.

Effect of acids on the chromatography of astaxanthin and astacene

The addition of a small amount of formic acid to the standard eluent resulted in an astaxanthin peak with acceptable symmetry[1], although on certain batches of (used) Zorbax ODS columns efficient chromatography could also be achieved with acid-free eluents. On the contrary, although the presence of acid indeed induced the elution of astacene, the peak shape invariably remained extremely poor, both on Zorbax ODS (not shown) and Hypersil ODS (Fig. 2A). Some mineral acids were excluded for practical reasons: phosphoric acid caused a progressive (reversible) de-



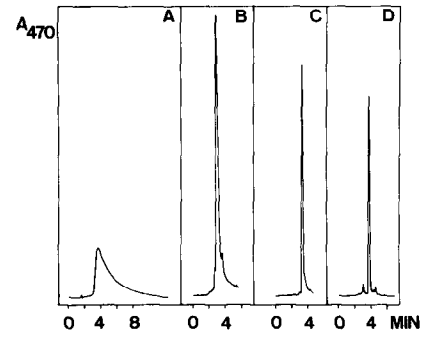


Fig. 1. Structural formulae of astaxanthin (1) and astacene (2).

Fig. 2. Chromatographic behaviour of astacene on a 5-μm Hypersil ODS column (15 cm × 0.46 cm I.D.) (A–C) and a 5-μm Zorbax ODS column (15 cm × 0.46 cm I.D.) (D) in various eluents: A, acetonitrile–methanol–dichloromethane (60:20:20, v/v/v) containing 0.2% formic acid; B, acetonitrile–methanol (80:20, v/v), containing 0.01 *M* BEHP; C, acetonitrile–methanol–water (79:20:1, v/v/v), containing 0.01 *M* BEHP; D, as C but Zorbax ODS instead of Hypersil ODS column. The flow-rate was 1 ml/min.

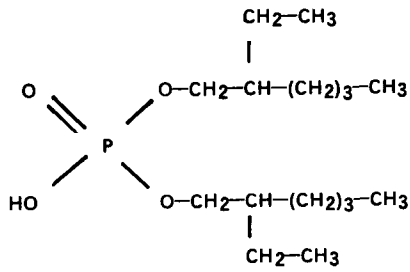


Fig. 3. Structural formula of bis(2-ethylhexyl) phosphate.

crease in column permeability, whereas sulphuric acid heavily promoted on-column carotenoid degradation. BEHP, structurally an organic phosphoric acid derivative (Fig. 3), proved to be a suitable agent to suppress peak tailing and to enhance the overall efficiency. The rationale for using this uncommon additive was the relatively strong acidity of the compound (pK_a 3.22) combined with an high degree of lipophilicity. These properties have previously underlied its use as a counter ion in ion-pair extraction of basic compounds[13]. A concentration of 0.01 *M* BEHP in an acetonitrile–methanol mixture led to a dramatic improvement of the astacene peak shape, both on Hypersil ODS (Fig. 2B) and Zorbax ODS. At the same time, however, BEHP significantly reduced the retention, which necessitated the addition of a small amount of water to neutralize this effect. Surprisingly, this further improved the peak symmetry (Fig. 2C, D). At this point, the term "NARP" is no longer applicable.

Separation of astacene from astaxanthin

For many carotenoids, the presence of methanol in a NARP eluent favourably affects resolution[1,2]. However, in the present system it was precisely methanol which counteracted the separation of astacene from astaxanthin. The effect of the incorporation of increasing amounts of methanol in the mobile phase is illustrated in Fig. 4. A methanol-free eluent yielded near-baseline separation (Fig. 4A). The addition of 20% methanol largely nullified this resolution and the elution order was reversed (Fig. 4B). Upon incorporation of an higher methanol content in the eluent this re-

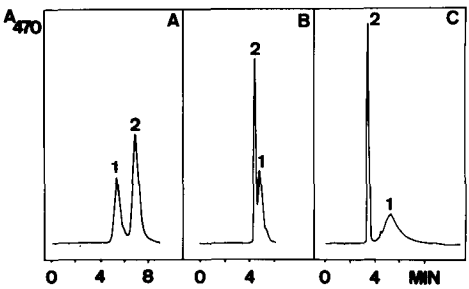


Fig. 4. Separation of astacene (1) and astaxanthin (2) on a 5-μm Zorbax ODS column (15 cm × 0.46 cm), eluted with different solvent mixtures: A, acetonitrile–water (98:2, v/v/v), containing 0.01 *M* BEHP; B, acetonitrile–methanol–water (78:20:2, v/v/v) containing 0.01 *M* BEHP; C, acetonitrile–methanol–water (48:50:2, v/v/v), containing 0.01 *M* BEHP. The flow-rate was 1 ml/min.

versed elution order was maintained, but astacene was no longer eluted as a symmetrical peak (Fig. 4C). A mobile phase consisting of methanol–water and 0.01 *M* BEHP still yielded a perfect astaxanthin peak, but astacene now appeared as a broad plateau. Therefore, the definitive eluent contained no methanol but only acetonitrile, water and BEHP.

Effect of eluent parameters on the retention of astaxanthin and astacene

The capacity factors, k', of both astacene and astaxanthin were inversely related to the concentration of BEHP in the mobile phase (Table I). Unlike this pronounced effect of BEHP, the water content of the eluent influenced the retention only marginally over the concentration range studied (1–5%) (Table II), which is highly surprising considering the lipophilicity of carotenoids. It contrasts indeed with earlier observations in NARP of other non-polar compounds, in which even a minimum amount of water led to a marked deterioration of the chromatographic performance[14].

Reproducibility

The results presented in Tables I and II were produced with a time interval of 2 months. It is seen that the k' values obtained with a given eluent (0.05 *M* BEHP in

TABLE I

EFFECT OF THE CONTENT OF BEHP (IN ACETONITRILE–WATER, 98:2, v/v) ON THE RETENTION OF ASTAXANTHIN AND ASTACENE

Concentration of BEHP (M)	*k' (astaxanthin)*	*k' (astacene)*
0.005	7.9	5.8
0.01	5.5	3.4
0.025	3.0	2.0
0.05	1.7*	1.2*
0.1	1.4	0.8

* Compare with the values in Table II.

TABLE II

EFFECT OF THE WATER CONTENT IN AN ELUENT CONSISTING OF ACETONITRILE AND 0.05 *M* BEHP (REFERRED TO THE TOTAL VOLUME) ON THE RETENTION OF ASTAXANTHIN AND ASTACENE

Concentration of water (%, v/v)	*k' (astaxanthin)*	*k' (astacene)*
1	2.3	1.6
2	2.4*	1.7*
3	2.7	2.1
4	3.1	2.5
5	3.3	2.7

* Compare with the values in Table I.

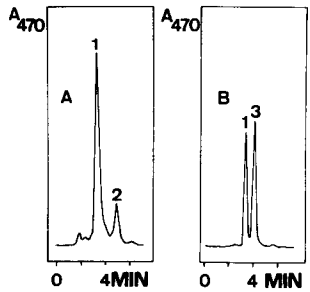


Fig. 5. (A) Chromatogram of a saponified extract of *Adonis annua* flower petals and (B) chromatogram of synthetic astacene and astaxanthin. Column: 5-μm Zorbax ODS (15 cm × 0.46 cm I.D.). Eluent: acetonitrile–water (97:3, v/v) containing 0.05 *M* BEHP; flow-rate, 1 ml/min. Peaks: 1 = astacene; 2 = unidentified; 3 = astaxanthin.

acetonitrile–water, 98:2, v/v) are different (*cf.*, the values marked with asterisks). It is significant in this respect that after this 2-month interruption the baseline separation from Fig. 4A could not at first be entirely reproduced. However, after flushing the column with dichloromethane to remove any adsorbed non-polar substances and prolonged equilibration with the mobile phase, the resolution progressively improved again.

Application

The present liquid chromatographic system could easily form the basis of analytical methods to determine astacene in a variety of organisms containing astaxanthin or astaxanthin esters. These include aquatic animals, *e.g.*, Crustacea[8,15], the fungus *Phaffia rhodozyma*[16], a bacterium[17], feathers of birds[18], algae[19,20] and the flower petals of *Adonis annua*[21]. During saponification of total lipid extracts under aerobic conditions, astaxanthin is readily converted into astacene[10]. To illustrate the practical usefulness of the system, astacene was demonstrated in saponified extracts of flower petals of *Adonis annua* (Fig. 5A). No trace of astaxanthin was detected. The major peak in the chromatogram was eluted in the same position as synthetic astacene (Fig. 5A and B). Confirmation of the peak identity was obtained from the corresponding absorption spectrum recorded with the photodiode array detector. As shown in Fig. 6 the spectrum did not however entirely coincide with that of synthetic astacene. The slight hypsochromic shift of the main maximum and the enhanced

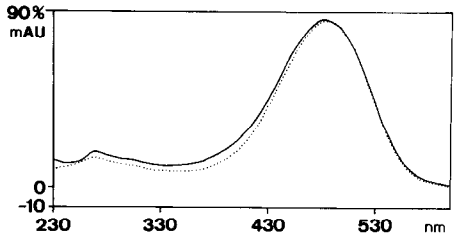


Fig. 6. Absorption spectra of peak 1 in Fig. 5A (——) and of synthetic astacene (----).

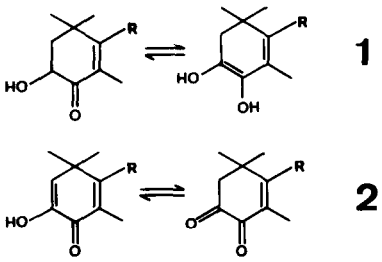


Fig. 7. Tautomeric forms of astaxanthin (1) and astacene (2).

absorption in the "*cis* peak" region suggest the presence of co-eluting *cis*-isomers of astacene[22], obviously formed at the elevated temperature of the saponification. The impurity of the chromatographic peak was also indicated by its composite appearance.

CONCLUSION

To the best of our knowledge, this report presents the first example of a reversed-phase separation of astacene and astaxanthin. The different chromatographic behaviour of these compounds under various conditions, *i.e.*, in the NARP standard eluent and in the presence of BEHP–methanol, is hard to rationalize, but may be linked to the existence of different tautomeric forms (Fig. 7) and, consequently, different degrees of acidity.

ACKNOWLEDGEMENTS

This work was supported by FGWO contract 3.0048.86. H. J. C. F. N. acknowledges financial support (permanent position of Research Associate) from the Belgian Foundation for Scientific Research (N.F.W.O.).

REFERENCES

1 H. J. C. F. Nelis and A. P. De Leenheer, *Anal. Chem.*, 55 (1983) 270.
2 H. J. C. F. Nelis, M. M. Z. Van Steenberge, M. F. Lefevere and A. P. De Leenheer, *J. Chromatogr.*, 353 (1986) 295.
3 H. J. C. F. Nelis, P. Lavens, L. Moens, P. Sorgeloos, J. A. Jonckheere, G. R. Criel and A. P. De Leenheer, *J. Biol. Chem.*, 259 (1984) 6063.
4 J. F. Fisher and R. L. Rouseff, *J. Agric. Food Chem.*, 34 (1986) 985.
5 J. G. Bieri, E. D. Brown and J. C. Smith, *J. Liq. Chromatogr.*, 8 (1985) 473.
6 H. J. C. F. Nelis and A. P. De Leenheer, *Abstracts 8th Int. Symp. on Carotenoids, Boston, MA, July 27—31, 1987*, p. 9.
7 H. J. Nelis and A. P. De Leenheer, *Abstracts 10th Int. Symp. Microchem. Techn., Antwerp, August 25–29, 1986*, p. 133.
8 T. W. Goodwin, *The Biochemistry of the Carotenoids*, Vol. II, *Animals*, Chapman and Hall, London, 2nd ed., 1984.
9 K. L. Simpson, T. Katayama and C. O. Chichester, in J. C. Bauernfeind (Editor), *Carotenoids as Colorants and Vitamin A Precursors*, Academic Press, New York, 1981, Ch. 4, p. 463.
10 S. Liaaen-Jensen, in O. Isler (Editor), *Carotenoids*, Birkhäuser, Basel, Switzerland, 1971, p. 88.
11 G. Englert and M. Vecchi, *Helv. Chim. Acta*, 63 (1980) 1711.

12 M. Vecchi, E. Glinz, V. Meduna and K. Schiedt, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 10 (1987) 348.
13 G. Hoogewijs and D. L. Massart, *Anal. Chim. Acta*, 106 (1979) 271.
14 M. F. Lefevere, *Ph.D. Thesis*, Gent, 1983.
15 R. Castillo, G. Nègre-Sadargues and R. Lenel, in G. Britton and T. W. Goodwin (Editors), *Carotenoid Chemistry and Biochemistry*, Pergamon, Oxford, 1982, p. 211.
16 A. G. Andrewes, H. J. Phaff and M. P. Starr, *Phytochemistry*, 15 (1976) 1003.
17 H. Iizuka and Y. Nishimura, *J. Gen. Appl. Microbiol.*, 15 (1969) 127.
18 A. H. Brush, in J. C. Bauernfeind (Editor), *Carotenoids as Food Colorants and Vitamin A Precursors*, Academic Press, New York, 1981, Ch. 5, p. 539.
19 F.-C. Czygan, *Arch. Mikrobiol.*, 61 (1968) 81.
20 F.-C. Czygen, *Arch. Mikrobiol.*, 62 (1968) 209.
21 A. Seybold and T. W. Goodwin, *Nature (London)*, 184 (1959) 1714.
22 L. Zechmeister, *Cis-Trans Isomeric Carotenoids, Vitamins A and Arylpolyenes*, Springer, Vienna, 1962, p. 25.