HPLC — a powerful tool in carotenoid research

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Abstract - This review demonstrates the basic principles of modern high
performance liquid chromatography and highlights the development and most
recent progress of this analytical and preparative separation technique in
carotenoid research.
Besides the high separation efficiency of this method, emphasis is drawn
on modern detection systems which give significant information about com-
plex mixtures with different chromophores, thus revealing the variety of
diastereomers (especially (E/Z)-isomers present in investigated natural
and synthetic sources.
Chromatographic systems covering a wide range of polarity and selectivity
are presented and illustrated with recent applications from plant and ani-
mal carotenoids.

Introduction

The invention and introduction of a new analytical method or the improvement
of an established one has always stimulated the expansion of scientific work,
and resulted in increased knowledge. In carotenoid chemistry this has been
well illustrated by Prof. Liaaen-Jensen's graph in Berne in 1975 (Ref. 1).
Such an increase was only possible when chromatographic methods for the iso-
lation of these labile natural products became available. This was emphasized
by Dr. Isler's remark in 'Carotenoids' (Ref. 2) and Prof. Weedon's statement
in his lecture in Madison in 1978 (Ref. 3). Today, about 500 different caro-
tenoids are known, and, owing to modern high performance liquid chromatography
(HPLC), their numbers are still growing.

Basic principles of HPLC

HPLC is not a new type of chromatography; it is rather a new technology for
the optimization of the kinetic effects, like zone-spreading due to diffusion,
mass transfer (sorption-desorption kinetics), etc. that work a priori against
a separation. These phenomena already have been known for a long time and were
theoretically treated in the fundamental work of Martin and Synge (Ref. 4), but
technology to overcome them was lacking until the late sixties when stable HPLC
systems were introduced. The first monograph concerning this topic appeared in
1971 (Ref. 5), and the 1st International Symposium on Column Liquid Chromato-
graphy in 1973 (Ref. 6) convincingly demonstrated the state-of-the-art and the
possibilities that this method offered.
As a consequence of the 'non-equilibrium' theory (Ref. 7), small $(3\mu, 5\mu)$
uniform (preferably spherical) particles with controlled porosity $(60\overset{\circ}{A}, 100\overset{\circ}{A})$
are indispensable, otherwise the resulting high pressure drop makes a closed
system necessary. Therefore, a high-pressure metering pump, injection device
and an appropriate detector to monitor the elution are required. The result of
this new technology is a high kinetic separation efficiency which can be num-
erically expressed by a HETP (height equivalent of a theoretical plate) of ca.
twice the particle diameter (i.e. a minimum of 6μ at the present time).
In addition to adsorption chromatography (liquid-solid chromatography, LSC),
chemically modified silica gels (bonded phase chromatography, BPC) offer the
possibility to choose between different normal and reverse phase systems. This
significantly enhances the thermodynamic selectivity of the method. Thus, also
racemates can be resolved by using either optically active stationary (Ref. 8)
or mobile (Ref. 9) phases (for a recent review see Ref. 10).
Moreover, each analytical separation can be transferred to a preparative one
(see e.g. Ref. 11). Working under exactly equal chromatographic conditions,

only columns with larger diameters need to be used and the flow-rate adjusted to maintain the same linear velocity (e.g. 250x4.6 mm, 1 ml/min.⟶250x25(40) mm, ca. 25(80) ml/min.), with the amount of sample to be injected being dependent on the initial resolution.

Development of HPLC in the carotenoid field

In carotenoid research the era of HPLC began in 1971 when Stewart and Wheaton (Ref. 12) reported their 'Continuous Flow Separation' of complex mixtures on precipitated zinc carbonate and magnesium oxide using steel columns and monitoring the chromatogram at 440 nm with a spectrophotometer. In 1974, Cadosch and Eugster (Ref. 13) published the separation of both natural and semisynthetic lutein-epoxide, flavoxanthin and chrysanthemaxanthin on a specially prepared MgO (2-6μ, Ref. 14) in order to establish the absolute configuration of the 5,6-epoxides and the correct structure of the latter four diastereomeric furanoxides (Ref. 15).

The early seventies was the age when totally porous irregular and spherical microparticles became available, superseding the relatively large superficially porous layer beads (37-50μ). Unfortunately their characteristics varied from batch to batch, and only very stable carotenoids could be separated to some extent. Compared with classical open column or thin-layer chromatography, the method offered no general advantages. Therefore, much work was done, not only in our laboratory (Ref. 14 & 16) to prepare such approved adsorbents like ZnCO$_3$, MgCO$_3$, MgO, etc. in a form suitable to HPLC. Most of the results were disillusioning because good separating columns lacked chemical or mechanical stability. Due to continuously decreasing permeability it was a time of real high-pressure chromatography, where a separation was only achieved by the thermodynamic selectivity of the adsorbent and the (sometimes rather peculiar) mobile phase.

Significant progress was only made feasible when reproducibly manufactured, stable microparticles for LSC and LLC (BPC) became available. Adsorption chromatography on different silica gels was shown by Liaaen-Jensen et al. (Ref. 17) to yield appreciable resolution in separating several groups of carotenoids (carotenes, diols, (E/Z)-isomers and other diastereomers) and by Moss (Ref. 18) who reported its benefits in his synthetic work on prolycopene.

For liquid-liquid partition chromatography, chemically modified silica displaced the 'classical' HPLC-LLC on pellicular supports or on microparticles with stationary phases like ODPN (3,3'-oxydipropionitrile) or ODS (C-18, reverse phase) adsorbed on the support.

Langer, in his 1976 thesis work (Ref. 19), thoroughly dealt with modern liquid chromatography of carotenoids and elaborated valuable isocratic and gradient elution procedures on reverse phase systems (ODS, 10μ). A wide range of polarity (crocetin and neoxanthin to β-carotene) could be covered; the method was used by several scientists (see e.g. Ref. 20) and became standard in routine analysis (Ref. 21). However, in the case of isomeric pairs (e.g. lutein/zeaxanthin, diastereomers) the limited selectivity was obvious.

Later, Pfander et al. (Ref. 22) reported the separation of lycopene, γ-, α-, and β-carotene by a non-aqueous reverse phase system (NARP, Ref. 23), a concept which was enlarged recently by Nelis and de Leenheer (Ref. 24).

In 1978, Vecchi (Ref. 25) suggested (in addition to adsorbents like silica or alumina) chemically bonded nitrile phases to be the most promising way for the separation of diastereomeric carotenoids. The result of a long effort of Vecchi and his coworkers in comparing and testing many different stationary and mobile phases must be considered the real break-through for HPLC in carotenoid research.

The main activities of this Roche group are now checking the optical purity of naturally occurring carotenoids (a commercially significant analytical method, too) and establishing the structures of diasteromers. Thus, accomplishing a separation via their diastereomeric (-)-camphanic acid diesters (Ref. 26), Vecchi et al. were able to prove the racemic and meso character of asthaxanthin from various natural sources (Ref. 27)(several (Z)-isomers being detected in the same analysis (Ref. 28)) and of the carotenoprotein mixture asteriarubin (Ref. 29). Recently, they reported the resolution of the optical isomers of zeaxanthin and lutein after derivatization by (S)-(+)-α-(1-naphthyl)ethylisocyanate (Ref. 30). LSC on silica (Si60, 5μ) with solvents of a hexane basis with several modifiers was shown to be the method of choice for these separations. Undoubtedly, extensions and improvements of this concept are under

current investigation.
The same authors (Ref. 31) demonstrated the advantages of LLC using nitrile
phases (e.g. Spherisorb S5 CN) by separating diastereomeric 3,3'-dioxo- (6,6'-
dihydrorhodoxanthins), 3,3'-dihydroxycarotenoids (tunaxanthins, luteins) and
some (Z)-isomers ((6Z)- and (6,6'-di-Z)-rhodoxanthins, see also Ref. 32 and
(13 or 13'Z)-luteins) from animal sources (Note a). The structures of these
compounds were established by ^{1}H- and ^{13}C-NMR techniques and by comparison of
their chiroptical data after preparative isolation. Similarly, based on a neat
preparative separation, the absolute configuration of antheraxanthin, (9Z)-
antheraxanthin, the (8R)- and (8S)-mutatoxanthins was confirmed (Ref. 34).

The Roche scientists also demonstrated the extreme sensitivity of alumina to
temperature and the water content of solvents in adsorption chromatography
(Ref. 35). Using the 'moisture control system' of Engelhardt and Boehme (Ref.
36), they were able to separate and isolate eleven different (Z)-β-carotenes
obtained after irradiation of all-(E)-β-carotene (see Ref. 37) and to assign
most of their structures by UV/VIS- and ^{1}H-NMR spectroscopy.
Recently, Tsukida et al. (Ref. 38) published a similar analysis where the ther-
mally and photochemically isomerized (Z)-β-carotenes were resolved on a lime
column (1-10μ). Owing to its relative insensitivity to moisture and tempera-
ture, high sample capacity and shorter retention times, this adsorbent seems
to represent a valuable compromise between the early approaches to fit classi-
cal basic adsorbents to HPLC (see Ref. 14 & 16) and microparticulate alumina.
From sixteen compounds, the Japanese authors characterized ten (Z)-β-carotenes,
including the hitherto unknown 'sterically hindered' (7Z)- and (7,13'-di-Z)-
isomers and two thermo-cyclized products by UV/VIS-, ^{1}H- and ^{13}C-NMR spectra.
Some of these structures were correlated with the provisional 'neo'-nomencla-
ture suggested by Zechmeister (Ref. 37).

These illustrations convincingly show HPLC as a powerful analytical and prepa-
rative separation technique in carotenoid research. As a consequence, many
routine analyses and quality controls, mainly in food chemistry, have been re-
ported in the last few years. To quote them all would be beyond the subject of
this brief summary. In his most recent review, Taylor (Ref. 21) lists many
applications on actual and older chromatographic systems in the carotenoid,
and, mainly in the retinoid field.

Detection and quantitation; recent applications
Due to the intense colour of most carotenoids, detection in the visible is the
best method. Although specific detection may be considered an advantage, it
has hampered the general development of HPLC concerning quantitation, sensiti-
vity and identification. Whereas quantitation requires both optimal resolution
and accurate calibration with suitable standards, the latter problem can gen-
erally be overcome by a combination of chromatography and spectroscopy.

Especially in carotenoid analysis, where the obvious singularities of the diff-
erent polyene chromophores involve particular difficulties, single wavelength
detection results in loss of important information. Therefore, the introduc-
tion of variable wavelength and, later, stopped-flow wavelength scanning UV/
VIS-detectors represented a great facilitation (see Ref. 17 & 39). The possi-
bility to run chromatograms consecutively at different monitoring wavelengths,
assign peaks on-line according to their spectra and to check the purity of a
peak, significantly enhanced detection efficiency.
A tremendous expansion of this concept has been due to the invention of diode-
array detection systems. Being comparable to ca. 250 different fixed-wavelength
detectors, this fully computerized system permits simultaneous detection at
several vavelengths, instantaneously and continuously memorizing spectra dur-
ing a the evolution of a peak and offering convenient raw data handling after
a chromatographic run (e.g. multi signal-, signal and spectra-, ratio of sig-
nals-, 3D-plots, derivative spectra, etc.). Thus, optimal qualitative and quan-
titative information revealing the bewildering complexity of a sample can be
gathered with one single injection.

Note a.   Some of these applications have been presented at the 6th Internati-
          onal Symposium on Carotenoids in Liverpool 1981 (Ref. 33).

Thorough HPLC analyses combined with the exhaustive application of the diode-array system enabled us to enlarge significantly our knowledge of carotenoid composition in many natural and synthetic samples. This may be best illustrated by resuming former investigations, and a few representative examples are presented in the following (further details and chromatograms being given in the lecture).

In 1977, Buchecker and Eugster (Ref. 40) reported the carotenoid composition of Rosa foetida. Isolation by classical methods yielded seven carotenoids: β-carotene, lutein, zeaxanthin, 'auroxanthin', 'luteoxanthin', violaxanthin and 'neochrome'. In continuation of the work on rose carotenoids, this analysis was recently repeated (Ref. 41). From the polarity range corresponding to the dihydroxyepoxy/-furanoxy carotenoids (see Table 1) at least fourteen different compounds could be identified(Fig. 1). In addition, relevant amounts of carotenes (besides the all-(E)-compounds, several (Z)-isomers of β-, ζ-, γ-carotene, β-zeacarotene, neurosporene and lycopene), monohydroxy carotenoids (β-cryptoxanthin and a trace of (Z)-isomers) and isomeric allenes ((8R)- and (8S)-neochromes, neoxanthin an its (9Z)-isomer) were detected. Moreover, novel tetrahydroxy-epoxides and -furanoxides were identified for the first time (for the elucidation of the structures, see Ref. 42).
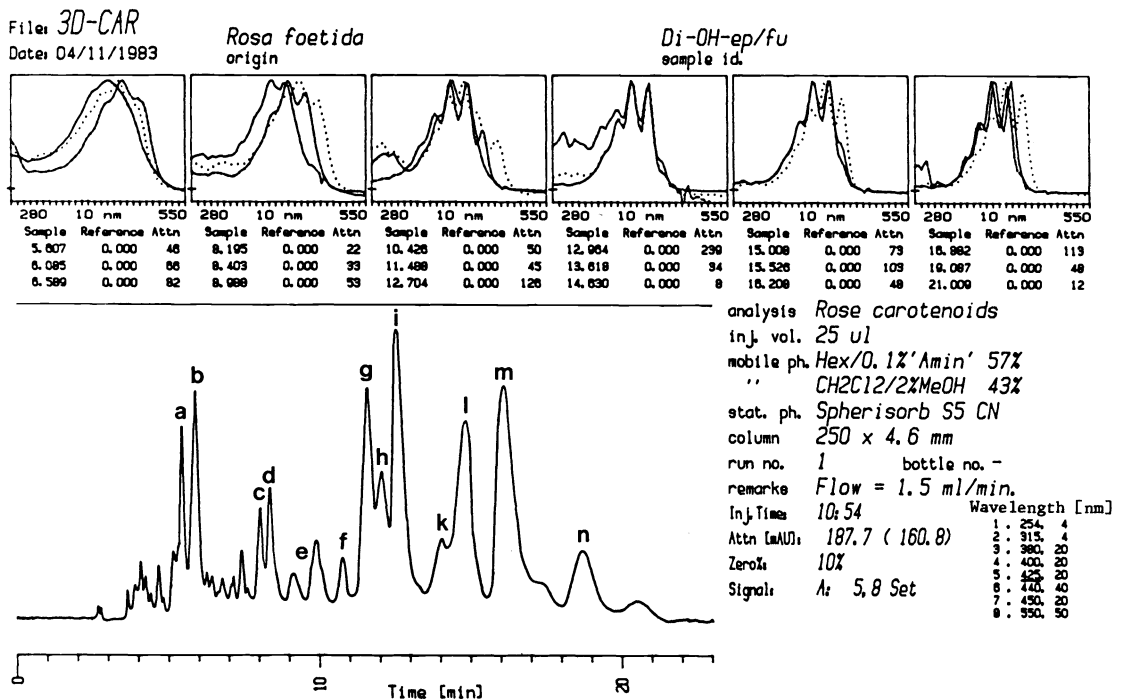

Fig. 1. Signal and spectra plot of a 'dihydroxy-fraction' of Rosa foetida. a lutein, b zeaxanthin, c all-(E)-antheraxanthin d (8R)- and (8S)-mutatoxanthins, e (Z)-auroxanthins, f (9Z)-antheraxanthin, g (8R)-luteoxanthin, h (8S)-luteoxanthin, i (8S, 8'R)-auroxanthin, j (8S,8'S)-auroxanthin, k (9'Z,8R)-luteoxanthin, l (8R,8'R)-auroxanthin, m (9Z)-violaxanthin, n (9'Z,8S)-luteoxanthin. The compounds g, h, i, j, k, l, and m were characterized for the first time (see Ref. 42).

Similarly, several other rose species were examined and shown to vary qualitatively only slightly, but the relative amounts of their carotenoids differed significantly (Ref. 39 & 41).

Recent growing interest in the stereochemical course of the biological carotene cyclization and the role of (Z)-isomers (Ref. 43) prompted us to search for adequate high efficiency separation procedures also for carotenoid hydrocarbons and their diastereomers (Ref. 44).
LSC and nitrile phases proved unsatisfactory, but appreciable resolution was

obtained using LLC systems 'A' and 'B' which are listed in Table 2. They mar-kedly differ in selectivity. The normal phase 'A' is quite sensitive to 'in-chain' (Z)-isomers (the all-(E)-compound always eluting last) but cannot dis-tinguish between β- and ε-endgroups. Whereas the reverse phase 'B' easily re-solves α-, β-, γ-, and δ-carotene, etc. and is more selective to isomers with the (Z)-double bond at the end of the chromophore (e.g. the stereospecifically synthesised (Ref. 45) (5Z)- and (5,5'-di-Z)-lycopenes, the (5Z)-, (5'Z)- and (9Z)-neurosporenes (Ref. 46), the all-(E)-isomer always eluting first). Although the neat separation of the individual all-(E)-carotenes is facile, their accompanying (Z)-isomers elute over a rather broad range. The resulting overlapping impedes an on-line full analysis and makes pre-separations on classical basic adsorbents necessary.

By joint application of the phase systems 'A' and 'B', numerous (Z)-isomers in the hydrocarbon fractions of rose species were detected (Ref. 44). Thus, it ccould be demonstrated that the 'lycopene' consisted mainly of (Z)-isomers (in-cluding prolycopene), the all-(E)-lycopene being only a minor diastereomer. Resolution of the equilibrium mixtures of photoisomerized all-(E)-, (5Z)- and (5,5'-di-Z)-lycopene on reverse phase 'B' yielded four compounds, whereas 'A' revealed at least ten different isomers (Ref. 44). Compared to Zechmeister's fundamental work (Ref. 37), four amongst them show a very intense cis-peak (ca. 50%) and can be assigned as 'neo-A-isomers'.
Purity control of crystalline, TLC pure synthetic diepoxylycopene (Ref. 47) surprisingly resulted in splitting into four isomers with each pair showing the same UV/VIS-spectra.

The possibility to distinguish easily between epimeric xanthophylls (e.g. 3'-epilutein) by HPLC induced us to look for such metabolic compounds in animals. Thus the two moth species Parnassius apollo (white) and P. phoebus (slightly yellowish) were examined and lutein was shown to be the main carotenoid. No trace of 3'-epilutein or unusual xanthophylls could be detected (Ref. 44). Similarly, resuming a former TLC analysis of the carotenoids present in the spider mite Tetranychus urticae (Ref. 48) by gradient elution HPLC (Fig. 2) impressively demonstrates the separation power of this technique. Due to the complexity of this fraction (the main carotenoid lutein already being removed) the identification of most of the compounds is still under investigation.
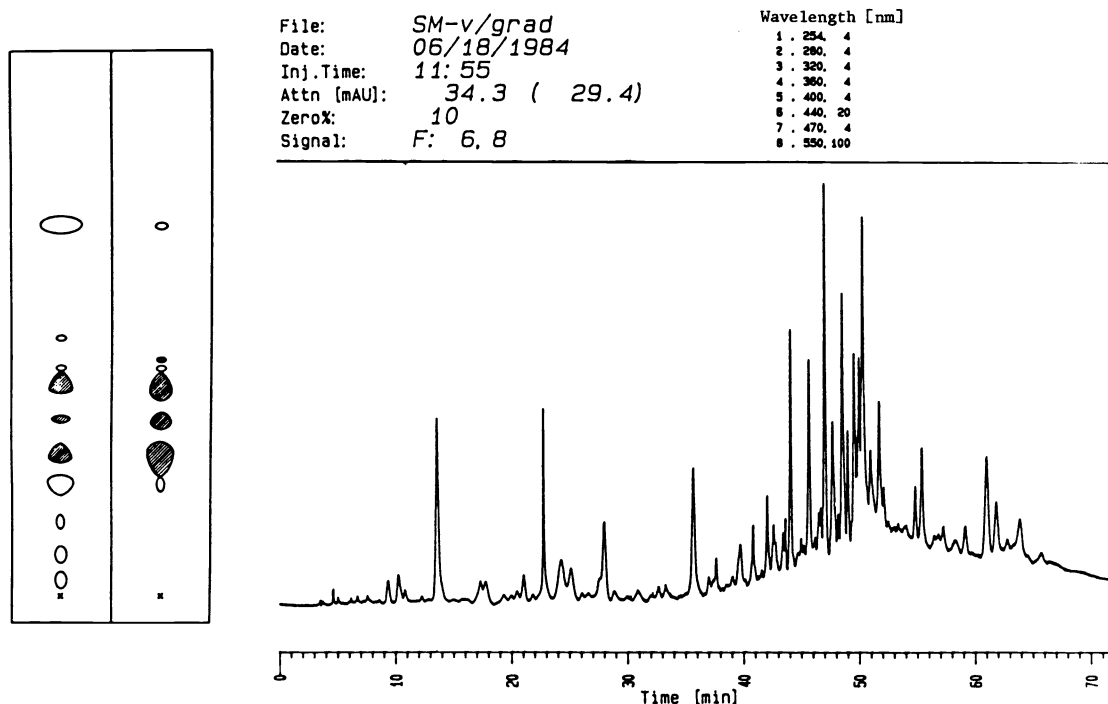

File:	SM-v/grad
Date:	06/18/1984
Inj.Time:	11:55
Attn [mAU]:	34.3 (29.4)
Zero%:	10
Signal:	F: 6, 8

Wavelength [nm]
1 . 254, 4
2 . 280, 4
3 . 320, 4
4 . 360, 4
5 . 400, 4
6 . 440, 20
7 . 470, 4
8 . 550, 100

Time [min]

Fig. 2. Comparison of TLC (silica gel, Ref. 48) and gradient elution HPLC (nitrile phase) of saponified extracts of Tetra-nychus urticae. Only the polarity range corresponding to Rf = 0.25-0.4 was analysed by HPLC.

Carotenoids which have been known for a long time without their constitutions being established are challenging objects. In order to characterize 'canary xanthophyll', extracts of feathers from two canary mutants (<u>Serinus canaria</u>, from the same breeding and taking the same food) and a greenfinch (<u>Carduelis chloris</u>), whose plumage is reported to consist chiefly of this not yet eluci- dated carotenoid (Ref. 49), were analysed by HPLC (Ref. 44). The main carote- noid of the orange mutant proved to be canthaxanthin, which after reduction yielded the (<u>meso</u>)- and (<u>rac</u>)-isozeaxanthins (separated in this elution order). The yellow mutant contained no canthaxanthin nor isozeaxanthins, but all the extracts revealed a main pigment whose all-(<u>E</u>)-isomer eluted immediately after α-cryptoxanthin. The same (<u>E</u>/<u>Z</u>)-mixture was detected in the green breast fea- thers of <u>Drepanoptila holosericea</u> (a New Caledonian fruit pigeon), whereas the more polar fraction was shown to consist of 3'-oxolutein, lutein, 3'-epilutein and zeaxanthin (Ref. 44).

TABLE 1. Polarity range of carotenoids on Spherisorb S5 CN
(250 x 4.6 mm)

Solv. A = hexane/0.1% N-ethyldiisopropylamine

Solv. B = dichloromethane (% MeOH)

Type of compound specific examples	%B (MeOH)	flow-rate (ml/min.)	approx. t_R (min.)
<u>Carotenes</u>	-		
β-carotene	-	0.5-0.7	5-6
lycopene	-	0.6-0.8	12-16
<u>Mono- and diepoxides/ -furanoxides</u>	2-6 (0.5)		
mutatochrom isomers	3 (0.5)	1.0	10-15
diepoxylycopene(s)	6 (0.5)	2.5	25-30
<u>Monohydroxy</u>	7-20 (0.5-1)		
α-,β-isocryptoxantins	15 (0.5)	1.5	12-14
α-,β-cryptoxanthins	15 (0.5)	1.5	19-21
<u>Dihydroxy</u>	35-40 (1-2)		
isozeaxanthin isomers	20 (1)	1.0	36-40
lutein, zeaxanthin	37 (1)	1.5	7-15
<u>Dihydroxyepoxides/ -furanoxides</u>	40-45 (2)		
luteoxanthin isomers	42-45 (2)	1.5	12-18
auroxanthin isomers	45 (2)	1.5	16-20
<u>Allenic trihydroxy- epoxides/-furanoxides</u>	50 (2-3)		
neoxanthin, neochrome isomers	50 (3)	1.5	12-17
<u>Tetrahydroxy</u>	50-55 (3-5)		
tetrolepoxides	55 (3)	1.5	22-25
tetrolfuranoxides	55 (4)	1.5	12-18
<u>Hexahydroxy</u>	55-60 (3-5)		
3,5-<u>trans</u>-hexol	60 (5)	1.5	ca.60

TABLE 2.   LLC systems for carotenes

'A': Spherisorb S5 NH₂   (250 x 4.6 mm)
      Hexane/0.1% N-ethyldiisopropylamine; 0.6 ml/min.

'B': Spherisorb S5 ODS·   (250 x 4.6 mm)
      Acetonitrile/THF 92:8 ; 1 ml/min.

Compound	System	approx. t_R (min.)
Phytoene	A B	3-5 (front) 40-45
β-carotene	A B	6-8 30-35
prolycopene	A B	11-13 20-22
lycopene	A B	35-38 18-20

Conclusions

During our investigations the phase systems which are listed in Tables 1 & 2 proved to be the most valuable ones, and they may serve as a guideline for starting carotenoid analyses. Owing to both complexity of natural samples and predominance of some pigments which can cover minor, significant compounds, on-line full analyses over the whole range of polarity are hardly feasible. In spite of thorough searching for useful gradient systems, this aim could not be reached satisfactorily. A possibility to overcome this problem may be real on-line multidimensional chromatography.
Nevertheless, the presented extrordinary advantages of modern HPLC (high efficiency, selectivity, speed and mildness) render it a powerful tool which should supersede classical chromatography also in carotenoid research.

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