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Review

Analysis of carotenoids by high-performance liquid chromatography and supercritical fluid chromatography

E. Lesellier* and A. Tchapla

Letiam, IUT Orsay, Plateau du Moulon, B.P. 127, 91403 Orsay (France)

C. Marty

Département SA, Ensia, 1 Avenue des Olympiades, 91305 Massy (France)

A. Lebert

Département GIA, Ensia 1, Avenue des Olympiades, 91305 Massy (France)

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ABSTRACT

The first part of this paper describes the chemical structures and the importance of carotenoids for health. Sample preparation for extracting the carotenoids from fruits and vegetable matrices is detailed in terms of pre-extraction treatment (enzyme inactivation, addition of antioxidants and acid neutralizers), extraction conditions with solvents or supercritical fluids and saponification. In the second part, HPLC and SFC separation methods are described. The efficiencies of different inorganic packings (silica, magnesium oxide, calcium hydroxide, alumina), bonded silica packings (cyano, octadecyl) and chiral phases (cellulose, cyclodextrins) are discussed. The choice of an appropriate method depending on the type of pigment to be separated (xanthophylls, catotenes, *cis–trans* isomers) is discussed. The effects of the mobile phase (specific interactions, hydrogen bonding) and of the stationary phase (nature and type of linkage: monofunctional or polyfunctional, end-capping of residual silanols) on the solute retention are reported and explained on the basis of the differences between the chemical structures of the pigments.

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* Corresponding author.

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1. INTRODUCTION

Because of their wide distribution in the plant and animal kingdoms, carotenoids are one of the main classes of natural pigments. They are found in a large number of fruits and vegetables (oranges, tomatoes, carrots, spinach, sweet potatoes, pumpkins), in spices and herbs (paprika, parsley, alfalfa) and in leaves, where their presence is masked by chlorophylls until autumn. They are also found in some animal products (eggs, butter, milk), and some seafoods.

Their basic structure is composed of eight isoprene units. The structural formula of all carotenoids is derived from that of lycopene, starting with different structural modifications [1]. The presence of oxygenated groups on this chain and ring closure at the extremes, and the partial dehydrogenation of the chain or its scission, define the structure of carotenoids, which makes it possible to divide them into two classes: (1) carotenes or hydrocarbon carotenoids, composed of only carbon and hydrogen, whose major components in foods are lycopene and α- and β-carotene (Fig. 1); and (2) xanthopylls or oxygenated carotenoids, which bear the following functions on their extremity or terminal ring: epoxy, carbonyl, hydroxyl ester or acids such as cryptoxanthin, the major pigment of citrus fruits, lutein or zeaxanthin present in green vegetables, or capsorubin and capsanthin the major pigments of paprika (Fig. 2).

The presence of numerous conjugated double bonds (eleven in β-carotene and zeaxanthin and ten in α-carotene and lutein) explains the intense colour of these molecules. Carotenoids can also be classified on the basis of the colour of the pigments: yellow-orange pigments such as β-carotene, lutein and zeaxanthin, whose absorbance maximum is about 450 nm; and red pigments such as cryptoxanthin, capsorubin and capsanthin, which have one or two oxygenated groups conjugated with the double bonds of the hydrocarbon chain and whose absorbance maximum is around 480 nm. This property is widely used in the food industry to increase the colour of a preparation. The pigments may be synthetic (β-carotene, β-apo-carotenal) or natural. Natural pigments are used as extracts or as dehydrated powders.

Carotenoids with a β-ionone terminal ring have provitamin A activity. Thus, β-carotene is the first vitamin A precursor, while α and γ-carotenes and cryptoxanthin have lower provitamin activity. Finally, it has been reported that β-carotene has antineoplastic activity, not only at the stage of onset of the disease [2] but also on existing tumours [3]. These properties depend to a great extent on the stereochemical structure of the pigments. In fresh plant tissues, carotenoids are generally present in the most stable structural form, all-*trans*, where all the double bonds are in the *trans* configuration [4–6]. The other forms are due to the *trans–cis* isomerization of the double bonds. This may involve a double bond (positions 7, 9, 13, 15, 13′, 9′) (Fig. 3) to form a mono-*cis* isomer or more rarely may occur on two double bonds and lead to the formation of a di-*cis* isomer. Chemically, this results in a disturbance of conjugation which has two effects of the visible spectrum: a hypochromic effect [7,8] and hypsochromic effect [7–9], causing degradation of the colour of the product and decreased provitamin activity.

In addition, *cis* isomers of carotenoid pigments are less stable to light [10] and can oxidize more rapidly than the *trans* compounds.

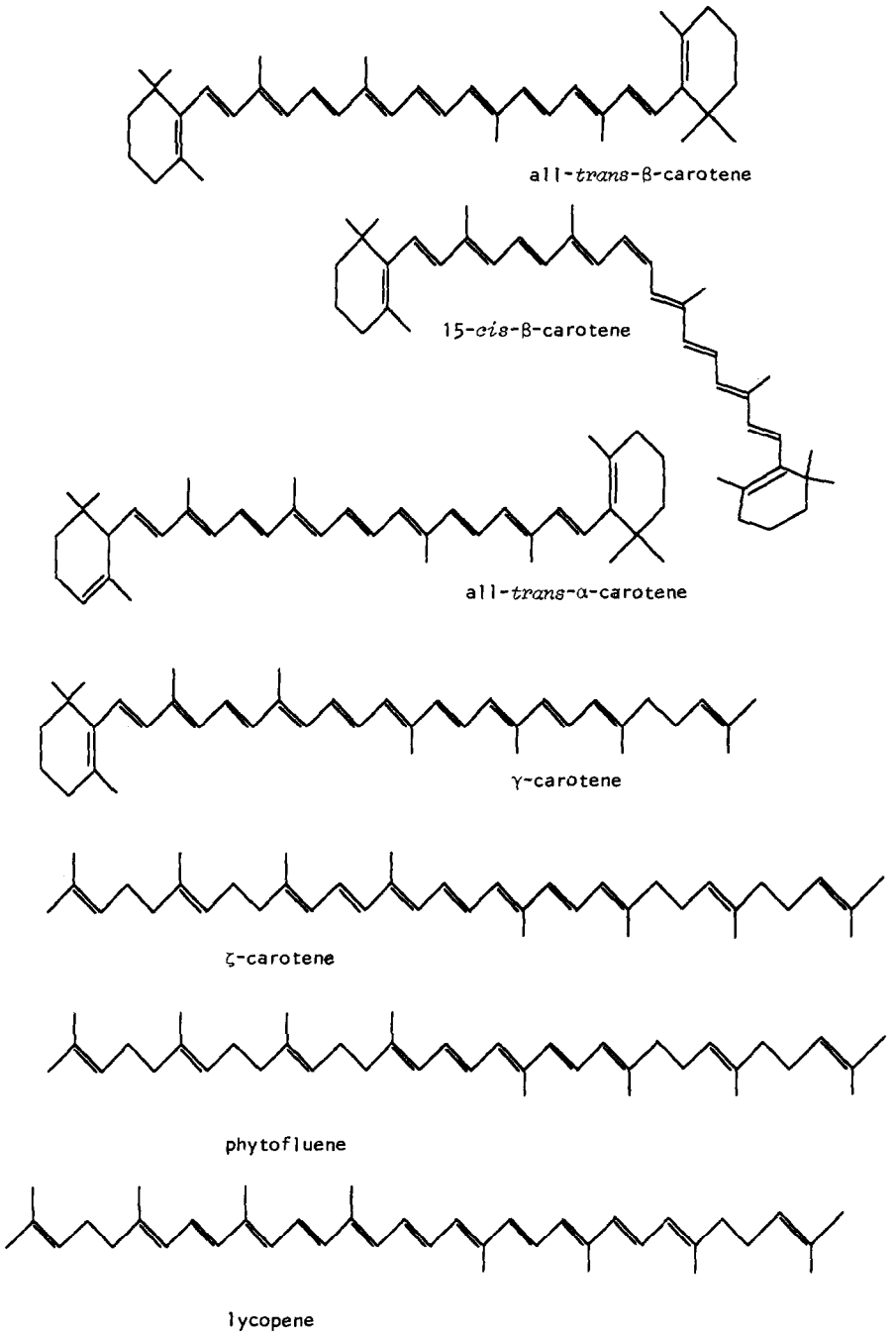

Fig. 1. Structures of some carotenes.

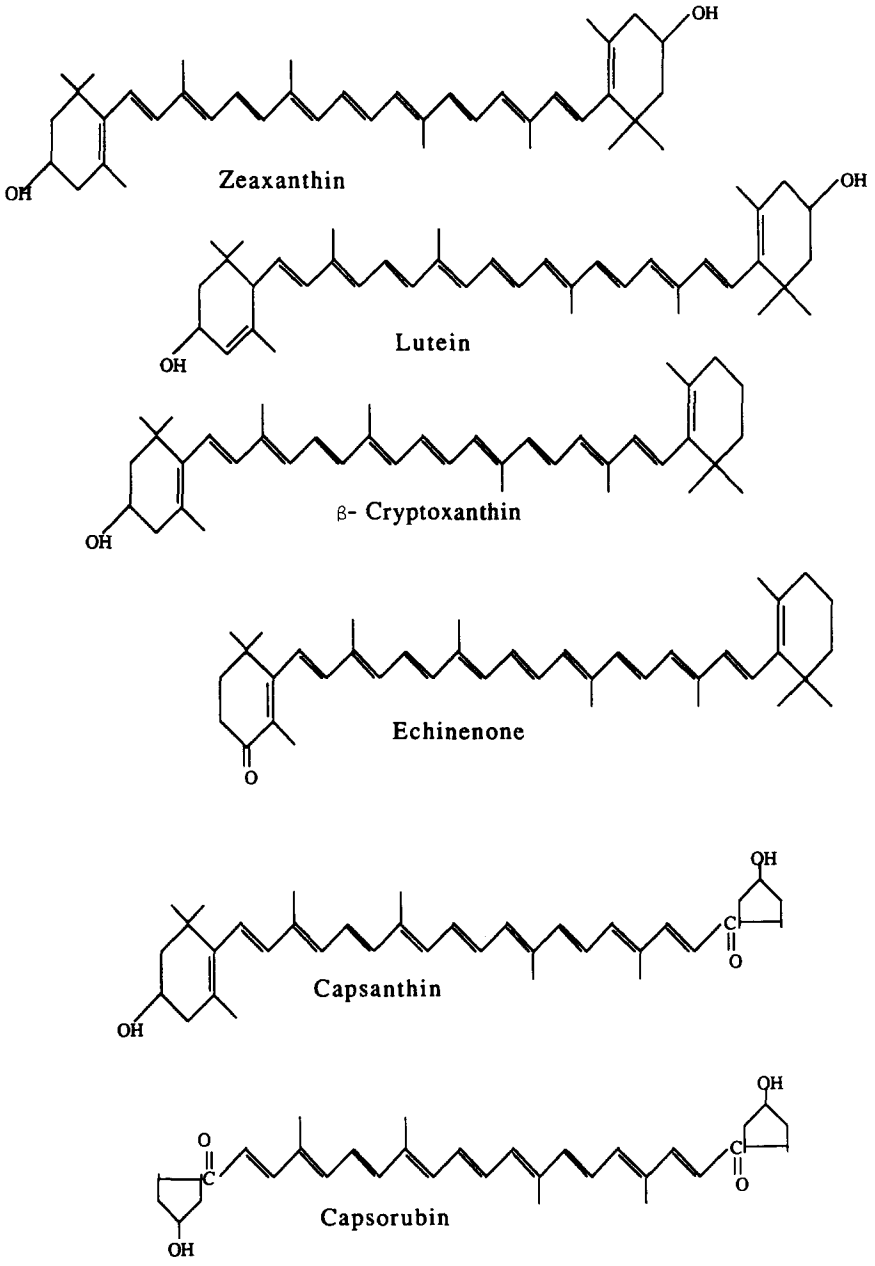

Fig. 2. Structures of some xanthophylls.

The importance of the constituents, in terms not only of colour but also nutrition, explains why many research groups are attempting to characterize and determine these pigments, especially stereoisomers that have different properties.

In this paper we describe and compare the techniques for the extraction and separation of carotenoid pigments in fruits and vegetables.

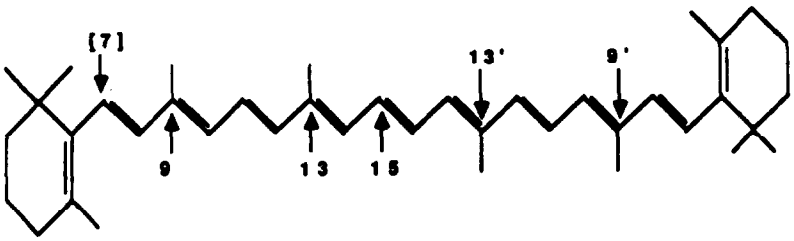

Fig. 3. Positions of isomerization for β-carotene.

2. SAMPLE PREPARATION

The lability of these unsaturated compounds is such that all operations must be conducted with special precautions (low temperature, attenuated light). Other factors can also favour structural modifications of the pigments in the sample, such as solubilization of carotenoids, presence of acids or enzymatic oxidation. This is why pretreatments are sometimes used to prevent or to minimize the degradation of carotenoid pigments during their extraction.

2.1. Pre-extraction treatments

2.1.1. Enzyme inactivation

In order to inactivate enzyme systems, such as lipoxygenase and peroxidase, vegetables are blanched before extraction. Daood and Biacs [11] reported the presence of lipoxygenase in paprika, particularly in the seeds, a portion of which is ground with the product. This enzyme has considerable denaturing activity towards the non-exterified pigments of paprika [12]. These enzymes have also been detected in green peas. Blanching in water for 90 s was required to destroy it [13].

2.1.2. Addition of antioxidants and acid neutralizers

Lipid oxidation activity in carrots has been reported [14,15]. The addition of antioxidants as butylhydrosylanisole (BHA) inhibits lipoxygenase [16]. Pinsky *et al.* [17], however, were unable to detect this activity, but did show the presence of antioxidant compounds in the root.

In order to limit pigment oxidation, antioxidants such as hydroquinone can be added to the sample [15]. Similarly, 2,6-di-*tert.*-butylhydroxytoluene [butylated hydroxytoluene (BHT)] is added to the diethyl ether used for extraction in order to inhibit the formation of peroxides. To the same end, Reeder and Park [18] proposed washing the diethyl ether with an aqueous solution of sodium hydrogensulphite ($NaHSO_3$).

The action of acids on the bleaching of carotenoids can ultimately cause the total decoloration of the pigment solution [7]. The isomerization of all-*trans*-β-carotene has also been observed in the presence of concentrated hydrochloric acid [7]. The solution is therefore made basic by adding 1% of magnesium carbonate ($MgCO_3$) [14] in order to neutralize acids released during extraction. This has also been recommended by the Association of Official Analytical Chemists (AOAC) [19].

2.2. Extraction conditions

The isomerization of carotenoid pigments begins as soon as they are solubilized and cannot be prevented by storing solutions in the cold. Zechmeister [7] reported that it depends on the solvent: the rate of β-carotene isomerization is greater in apolar than in polar solvents. This explains why extraction should be done as rapidly as possible.

In addition to precautions related to changing the pigment medium and to their contact with other substances during extraction, a large number of parameters have been taken into account in establishing extraction protocols: the nature of plant tissues, *i.e.*, fibrous (carrots) or relatively non-fibrous (citrus); the form in which the pigments are present, *i.e.*, free, esterified or complexed with matrix proteins; the chemical structure of different carotenoids together in the same extract; and the state of the product to be analysed, *e.g.*, fresh tissues with a high water content or dehydrated tisues.

We shall successively distinguish the extraction operations as a function of the type of sample.

2.2.1. Extraction with solvents

2.2.1.1. Fresh tissues. Regardless of the product being studied, the chronology of the steps in the extraction of carotenoid pigments is identical; the operational differences depend on the product.

Sample size depends on the occasionally non-homogeneous distribution of pigments in tissues and on their pigment content, variable from one to another. This size is virtually the same for carrots (2–20 g) [20–22], persimmon or papaya (10 g) [23] and fresh paprika (2–10 g) [12,24]. The volume of citrus juice extracted is 20–100 ml [18,19].

For sample preparation, tissues are generally mixed for several minutes alone, with water [4] or an organic solvent [5,21–23]. For juices, mixing is replaced with centrifugation [6], filtration [18,19] or even a prefractionation on an alumina column [26].

The principal extraction solvents, acetone, light petroleum, *n*-hexane, diethyl ether and tetrahydrofuran, have been used alone or in binary mixtures. The simultaneous presence of polar and non-polar solvents in an extraction mixture leads to the total recovery of pigments [5,20,23,24,27,28]. The AOAC recommends using acetone–*n*-hexane (60:40). Bauernfeind [29] indicated that the use of diethyl ether enabled te most highly polar compounds to be extracted into the organic phase, which otherwise could have been solubilized in the aqueous phase.

Grinding the residue is applied. Extraction is considered to be complete when the filtrates are colourless. The residue can be ground manually in methanol [21] and then extracted with acetone. It is important to grind samples to facilitate access of solvent to pigments located inside tissues and to break cell structures containing them (chloroplasts). This may be done at the beginning of the extraction if the tissues in question are fibrous, *e.g.*, carrots.

2.2.1.2. Dried tissues. The concentration of pigments resulting from the elimination of water is high (around tenfold for carrots); hence the sample size is smaller than with fresh tissues: 0.5–2 g for paprika powder [12,30,31] and about 1 g for dehydrated carrots [21].

As a result of their compact and solid nature, it is often necessary to saponify dry tissues before extraction [21,28] in order to increase the contact surface between the extraction solvent and pigments. The tissues are then extracted in the same way as fresh tissues.

2.2.2. Extraction with supercritical fluid

Supercritical CO_2 behaves as an non-polar solvent and solubilizes hydrocarbons. It has been used for the extraction and separation of carotenoids. One advantage of using it for extraction is that its residues in food extracts are not toxic, in contrast to organic extraction solvents.

The polarity of CO_2, related to its density, is determined by the pressure and temperature. Favati *et al.* [32] used this property to extract lutein or β-carotene selectively from a protein concentrate of alfalfa leaves (Fig. 4).

The solubility parameter of CO_2 (calculated ac-

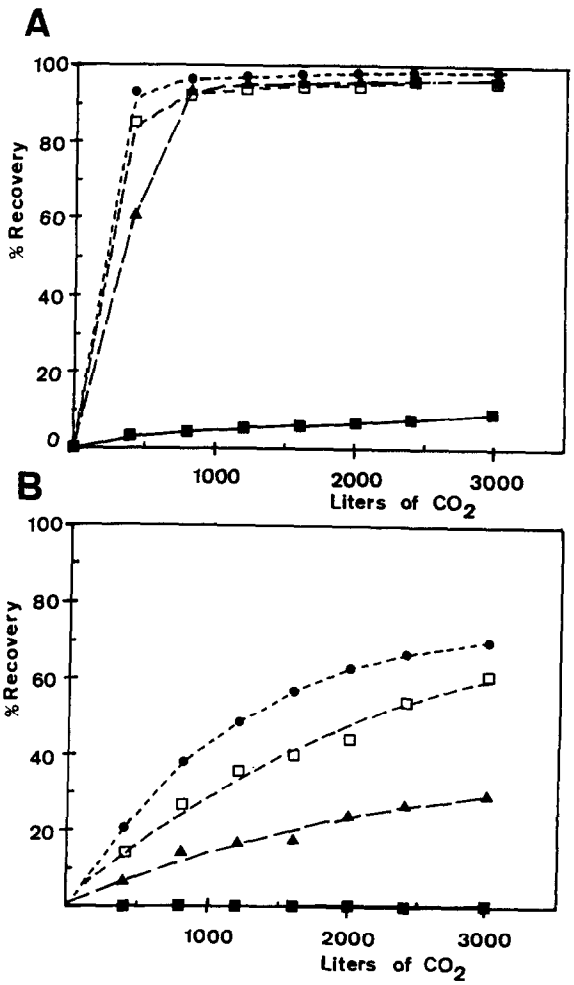

Fig. 4. Effect of pressure on the extraction of pigments as function of total CO_2 used. Temperature = 40°C. (a) β-Carotene; (b) lutein. Pressure: ■ = 10; ▲ = 30; □ = 50; ● = 70 MPa. From ref. 32.

cording to the equation of Giddings *et al.* [33]) was very close to that of β-carotene for pressures from 5 to 7 MPa and was close to that of lutein for 7 MPa. The stability of the pigments extracted under supercritical conditions was not studied. Yamagushi *et al.* [34], however, reported the degradation of astaxanthin esters extracted from shrimp at 80°C, which was apparently favoured by high extraction pressures (25 MPa).

Once the pigments have been extracted, chromatographic fractionation of the untreated extract is used for the subsequent identification and assay of the carotenoids.

2.3. Saponification

Saponification is carried out with the deal aim of eliminating chlorophylls that interfere in the spectrophotometric assay of carotenes and of releasing esterified xanthophylls [35]. This is done before or after extraction, depending on the nature of the plant material containing the pigments. For citrus fruits, saponification is done before extraction, whereas for more fibrous vegetables, in which pigments are not directly accessible, saponification is done after extraction. Methanolic sodium or potassium hydroxide (10–30%) is added to the pigment extract and the mixture is stirred either in an open container or under reflux for various time periods. Saponification itself can cause the aldo-condensation of β-8′-apo-carotenal with a ketone function [19]. A decrease in the carotenoid content has also been reported for extracts of squash [36], citrus [6], paprika [37] or broccoli [38].

This treatment is superfluous for a large number of plants containing carotenes as a major pigment, which by definition do not have esterifiable groups (carrots, tomatoes, pumpkin, sweet potatoes). In addition, these plants are relatively poor in lipids and thus saponification does not change the carotene content [22,25], nor does it cause the appearance of additional α- and β-carotene isomers [5].

In the light of this, most workers have chosen to avoid saponification, because even when justified, it can lead to degradation of the pigments being studied.

3. SEPARATION

The chemical diversity of carotenoids (polar and non-polar) or their similarity (positional isomers, *trans–cis* isomers and diastereoisomers) increase the problem of selecting a separation method, which is often a delicate step.

The instability of carotenoids towards heat and their low volatility make it difficult to use gas chromatography for analysis [39]. The less drastic operating conditions of liquid chromatography explain why this method is more widely used to analyse heat-sensitive components such as carotenoids. Numerous studies of the separation of carotenoids by HPLC have been performed. The large number of chromatographic parameters does not always lead to a clear understanding of the mechanisms governing separations. Therefore, it is the influence of these parameters related to the chemical strucure of pigments that we shall attempt to discern, particularly, with certain more complex separations.

3.1. High-performance liquid chromatography

3.1.1. Chromatography on inorganic supports

Historically, the first separations of carotenes were done by adsorption chromatography [40,41]. This method is generally adapted to the separation of certain isomers and compounds with different functional groups. The first nomenclature of *trans–cis* isomers was based on their retention in adsorption chromatography (neo A, neo B, all-*trans*, W, V, U) [42].

Four different adsorbents can been used [41]. Their capacity to separate different component is related to their polarity. In general, the main types of supports and the classes or compounds separated can be summarized as follows: silica is highly polar and very efficient for fractionating oxygen-containing pigments; magnesium oxide has intermediate polarity and can separate xanthophylls into several fractions (although with less resolution than silica) and carotenes (lycopene, α- and β-carotenes); calcium hydroxide is the phase with the lowest polarity and is better suited to the purification of the stereoisomers of carotenes, especially those of β-carotene; and the polarity of alumina depends on the degree of its activation state [43] and it can be used for separations of all carotenes: neutral alumina with activity 1 for fractionating *trans–cis* isomers, alumina with activity 2–3 inactivated by 4% water for oxygenated xanthophylls or alumina with activity 4 (inactivated by 10% of water).

Carotenes lacking oxygenated functions are eluted first. This shows their reduced affinity for these adsorbents. The retention of carotenes is apparently governed by interactions between the π-electrons of the pigments and the polar adsorbents. It depends both on the number of double bonds in the pigment and the accessibility to the stationary phase. Thus, increasing the number of double bonds increases the retention time while cyclization of the extremity of the hydrocarbon chain reduces it. Similarly, conjugation favours the retention of carotenes [44]. The decrease in conjugation sometimes competes with accessibility of the double bond, particularly in the separation of α- and β-carotenes; the retention time of α-carotene is lower than that of β-carotene. Xanthophylls are eluted later, in an order that depends on the functions they bear: monohydroxy, followed by dihydroxy.

The elution order of *trans–cis* isomers depends on the pigments, but is always related to the localization of the *cis* double bond, which increases or decreases interactions between the pigment and the stationary phase.

Results obtained with β-carotene show that the shift of the *cis* bond from the extremity (9-*cis*) towards the middle of the molecule (15-*cis*) decreases the retention of the compound [8,9,45,46]. As π interactions appear to be responsible for the retention

TABLE 1

ANALYTICAL CONDITIONS FOR THE SEPARATION OF CAROTENOID PIGMENTS BY NORMAL-PHASE CHROMATOGRAPHY

(i) = Isocratic elution; (g) = gradient elution.

Carotenoid	Origin	Stationary phase	Mobile phase	Flow-rate (ml/min)	Ref.
α- and β-carotenes, cryptoxanthin	Orange juice	Basic alumina, silica	(g) Benzene–hexane (37.5:62.5) (g) THF–*n*-hexane (16.6:83.3)	2 2	18
α- and β-carotenes, α- and β-cryptoxanthin	Orange juice	Magnesium oxide	(g) Acetone–hexane	3.5	19
Lycopene, α- and β-carotenes	Tomato	Magnesium oxide	(g) A = acetone–benzene (90:10), B = hexane, (A:B) = 2:98 → 100:0	–	55
All-*trans*-β-carotene, 8 *cis* isomers, α-carotene	Standard	Alumina	(i) *n*-Hexane	–	9
cis–trans-β-carotene, 9 *cis* isomers	Standard	$Ca(OH)_2$	(i) Acetone–*n*-hexane (0.5:99.5)	1	8
17 oxidized compounds of β-carotene	Extract of extruded starch	Silica	(i) *n*-hexane–diethyl ether (95:5)	2	54
cis–trans isomers of 1,2-epoxy-1,2-dihydrolycopene	Standard	Silica	(i) *n*-Hexane–TBME–NN-diisoPEamine[a] (100:4:0.1)	1.4	60
Dihydroxyxanthophylls, *cis–trans* isomers of lutein, diastereoisomers of lutein	Standard	Silica	(g) *n*-Hexane–methanol (99.9:0.1), acetone = 0 → 40%	1.25	61
cis–trans α- and β-carotene	Vegetables, sweet potatoes	$Ca(OH)_2$	(i) Acetone–hexane (3:97)	0.9	62
β-Carotene and xanthopylls	Sea sediments	Silica	(g) Acetone–hexane (1:99 → 75:25)	1	63
β-Carotene, ζ-carotene, phytoene, neurosporene, lycopene	Standard	Silica	(i) CH_3CN–hexane (0.12:99.8)	2	64
cis–trans isomers of neurosporene	Cellular	$Ca(OH)_2$	(i) Benzene–*n*-hexane (15:85)	2	45

[a] TBME = *tert.*-butylmethyl ether; PE = propylethyl.

of carotene, this shift of the *cis* double bond probably decreases the accessibility of π electrons to the adsorbent.

With xanthophylls such as astaxanthin [47] or canthaxanthin [48], on the other hand, the elution order is reversed. In this instance, retention is governed by the interactions between the hydroxyl groups and the adsorbents, which decrease when the *cis* double bond shifts towards the extremity.

Thin-layer chromatography [5,21,49,50–54] and open-column chromatography [20,28,29,55–59] have been widely employed. These methods are currently used in preliminary studies for the prefractionation of complex mixtures, or to prepare larger amounts of standards.

The main advantages of HPLC are easier quantification of products and speed of analysis, which with carotenoids reduces photochemical or oxidative degradation. These analyses are carried out on those supports classically used for pigment separation on open columns or on thin layers (Table 1). HPLC is generally carried out under isocratic conditions [4,8,9,44].

Fiksdhal *et al.* [61] reported the purification of xanthophylls and also certain *trans–cis* isomers and diastereoisomers of lutein 3'-ethyl ether. Nakazoe [64] reported the separation of two positional isomers, lutein (3,3'-hydroxy-β-carotene) and zeaxanthin (3,3'-hydroxy-α-carotene) on a column of Li-Chrosorb Si 100 with water–acetone as eluent. α- and β-carotenes have been separated on all the adsorbents cited except silica [19,44,61]. Rhodes *et al* [44] fractionated several carotenes on this adsorbent (phytoene, phytofluene, ζ-carotene, neurosporene and lycopene) as a result of the presence of 0.12% of acetonitrile in hexane mobile phase.

The properties of the supports used, combined with the efficiency of HPLC, explain the excellent results obtained in the separation of positional or *trans–cis* isomers. Thus, Vecchi *et al.* [9] isolated all-*trans*-β-carotene and eight of its isomers (Fig. 5). Tsukida *et al.* [8] separated nine *cis* isomers of all-*trans*-β-carotene on calcium hydroxide. They also demonstrated the formation of the 7-*cis* isomer, which was thought to be sterically impossible from a theoretical point of view. Using the same adsorbent, Chandler and Schwartz [4] fractionated the *trans–cis* isomers of α- and β-carotenes but noted a lack of resolution between the all-*trans* and *cis* isomers of α-carotene.

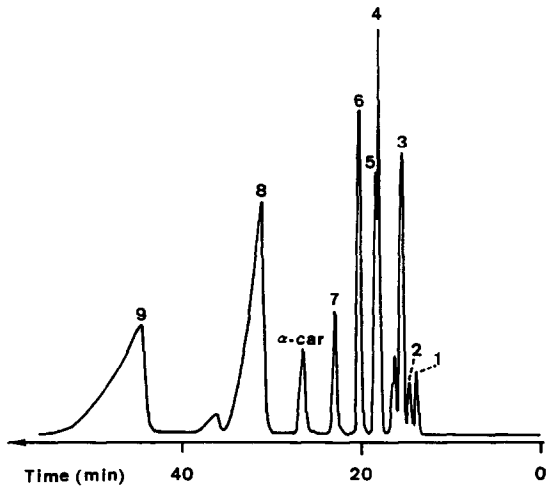

Fig. 5. Separation of nine stereoisomers of β-carotene by HPLC. Stationary phase, alumina; mobile phase, hexane; temperature, 19°C. 1 = 13,13'-di-*cis*-; 2 = 9,13,13'-tri-*cis*-; 3 = 9,13'-di-*cis*-; 4 = 15-*cis*-; 5 = 9,13-di-*cis*-; 6 = 13-*cis*-; 7 = 9,9'-di-*cis*-; 8 = all-*trans*-; 9 = 9-*cis*-. From ref. 9.

In general, the analytical conditions of adsorption chromatography, such as strict control of the water content in the mobile phase, which necessitates considerable control of the apparatus [9], the use of two different columns to separate xanthophylls and carotene [18] and the very long equilibration times between two injections make this technique difficult to use routinely with crude extracts.

3.1.2. Chromatography on bonded silica packings

An alternative is the use of silica bonded with functional groups, which presents a number of advantages: the interactions occurring in reversed-phase separations are different from those encountered in adsorption chromatography and will therefore have less impact on the structural modification of solutes; rapid equilibration of columns, permitting a change in composition in preliminary or optimization studies; the activity of the adsorbent is slightly modified by the water content of the mobile phase; and adsorption of impurities present in solvents or in samples on the stationary phase has little effect on the reproducibility of the results.

Satisfactory results have been obtained with cyano groups, particularly in the separation of enantiomers or of *trans–cis* isomers of xanthophylls [54,65–69].

Hydrocarbon chains are widely used for the separation of carotenoids. Octadecyl chains (C_{18}) are preferred to ethyl (C_2) or octyl (C_8) because of their greater hydrophobic interactions with carotenoid pigments. Elution solvents used are acetonitrile, methanol, water, methylene chloride, chloroform

TABLE 2

ANALYTICAL CONDITIONS FOR THE SEPARATION OF CAROTENOID PIGMENTS BY REVERSED-PHASE CHROMATOGRAPHY

(i) = Isocratic elution; (g) = gradient elution.

Carotenoid	Origin	Stationary phase	Mobile phase	Flow-rate (ml/min)	Ref.
Provitamin A compounds	Tomato	Partisil ODS 5	(i)CH_3CN–$CHCl_3$	2	70
Xanthophylls, carotenes	Spinach	Sil 60-RP 18	(g) CH_3OH–CH_3CN (25:75), H_2O 80 → 100%	1	59
α- and β-carotenes	Carrot, sweet potatoes	Partisil ODS 5	(i) CH_3CN–THF–H_2O (85:12.5:2.5)	2	22
α- and β-carotenes	Fruits, vegetables	μBondapak C_{18}	(i) CH_3CN–$CHCl_3$ (98:2)	1	27
Xanthophylls α- and β-carotenes	Human plasma	Zorbax ODS	(i) CH_3CN–CH_3OH–CH_2Cl_2 (70:10:20)	1	71
α- and β-carotenes, mixture of *cis* isomers	Plants	μBondapak C_{18}	(i) CH_3CN–CH_3OH–$CHCl_3$ (47:47:6)	2	5
5 carotenes	Standards	Ultrasphere ODS	(g) CH_3CN–H_2O (90:10), 2-propanol 30 → 55%	0.66	72
Canthaxanthin, β-cryptoxanthin, α- and β-carotenes, isomers of β-carotene	Standards	Vydax 201,	(i) Many solvent systems (i) CH_3OH–CH_3CN–THF: (40:52:8)	1	73
		Vydac 218,	(40:52:8)	1	
		Nova-Pak,	(35:50:15)	1	
		Zorbax ODS		1	
Neoxanthin, vialaxanthin, lutein, carotenes	Berries of grapes	Brownlee RP-18	(g) $(CH_3)_2CO$–H_2O (50:50) $(CH_3)_2CO$ 50 → 100%	1	74
Neoxanthin, violaxanthin, β-carotene	Alfalfa	Zorbac ODS, Hypersil ODS, Nova-Pak, Spherisorb ODS	(i) Ethyl acetate–CH_3CN (75:25 → 97:3)	1.6	75
β-Cryptoxanthin, α- and β-carotenes	Orange	Zorbax ODS	(i) CH_3CN–CH_2Cl_2–CH_3OH (65:25:10)	1	6
α- and β-carotenes, 9- and 15-*cis*-β-carotene	Fruits, vegetables	Vydac 218 TP 54	(i) CH_3OH–CH_3CN–THF (40:56:4)	1	76
β-Cryptoxanthin, α- and β-all-*trans* and-*cis* isomers β-carotene	Orange	Vydac 218 TP	(i) CH_3OH–$CHCl_3$ (94:6)	1	77
All-*trans*-neoxanthin, violaxanthin, lutein, β-carotene	Green vegetables	Microsorb	(g) CH_3OH–CH_3CN–CH_2Cl_2–hexane	0.5	38
α- and β-carotenes, non-apreno-β-carotene	Yellow plants	Brownlee RP 18	(i) CH_3OH–CH_3CN–CH_2Cl_2	1	78
α- and β-*trans*–*cis*-carotene	Standards	Vydac 201 TP	(g) CH_3OH 5 min, CH_3OH–$CHCl_3$ (94:6)	1	79
α- and β-*trans*–*cis*-carotene, lycopene	Carrot	Brownlee Spheri 5 ODS	(i) CH_3CN–CH_3OH–CH_2Cl_2 (63:27:10)	1	80
α- and β-carotenes, lutein, zeaxanthin	Standards	Brownlee RP 18	(i) CH_3CN–CH_3OH (85:15)	1.8 → 3.5	81
Carotene and xanthophylls	Barley	Hypersil	(g) THF–H_2O 50:50 → 100:0	1	82
Xanthopylls	Standards	Nova-Pak	(i) H_2O–CH_3CN–$CHCl_3$ (2:83:15)	1	83

and tetrahydrofuran, in binary or ternary mixtures, with isocratic or gradient elution (Table 2). All the results obtained with these stationary phases show that the order of elution depends on the hydrophobicity of pigments. Xanthophylls are more polar than carotenes, which are the least retained. The replacement of an alcohol function (neoxanthin) by an epoxy function (violaxanthin) increases the retention of the pigment, as does the esterification of xanthophylls. Monoesterified pigments are eluted first, followed by diesters [12,24,30,36]. If the same basic xanthophylls are considered, the elongating fatty acid chain length of esters increases the retention time of the compounds [36].

The retention times of carotenes are higher than those of xanthophylls, with lycopene being the first eluted. The elution order of α- and β-carotenes, on the other hand, is the same as that in adsorption chromatography [70,77].

Work done on the separation of *trans–cis* isomers of α- and β-carotenes has shown that the retention time of the *cis* isomer is greater than that of all-*trans* compound [73,79,80]. This has also been observed for canthaxanthin isomers [48,84].

3.1.2.1. Effect of the mobile phase. The presence of water in the mobile phase is the prime discriminating factor for the chromatographic system used. Braumann and Grimme [59] and Bushway and Wilson [22] reported separations done with low water contents, whereas Ruddat and Will [72] fractionated five standard carotenes (lycopene, α-, γ- and β-carotene and β-zeacarotene) and lutein from zeaxanthin in 25 min.

Other workers used only organic solvents in the mobile phase [70,71,73,76,81]. Thus, Nelis and De Leenheer [71] developed a system for separating carotenoids with a ternary mixture in a non-aqueous reversed phase (NARP) (Fig. 6). This avoids peak deformation and partial precipitation of the compounds on the column, and polar xanthophylls (lutein) and non-polar carotenoids (lycopene, α- and β-carotene) can be separated in the same analysis in less than 30 min [71]. Finally, this technique permitted the study of esterified xanthophylls, which could not be investigated by adsorption chromatography because of the strongly hydrophobic nature of the hydrocarbon chains of fatty acids.

The choice of organic solvents is dictated primarily by the eluent strength and the solubility of pigments. The specific properties of each solvent enable the results to be optimized. The presence of methanol (5–10%) masks residual silanol groups which interact with xanthophylls [75] or even with the double bonds of carotenes [80]. Thus, the presence of alcohol in the mobile phase leads to decreased selectivity between lutein and zeaxanthin which can cause an inversion of the elution order of the compounds, with zeaxanthin eluting first with an alcohol-free mobile phase [82,85], and lutein eluting first when alcohol is present [72,83,86,87].

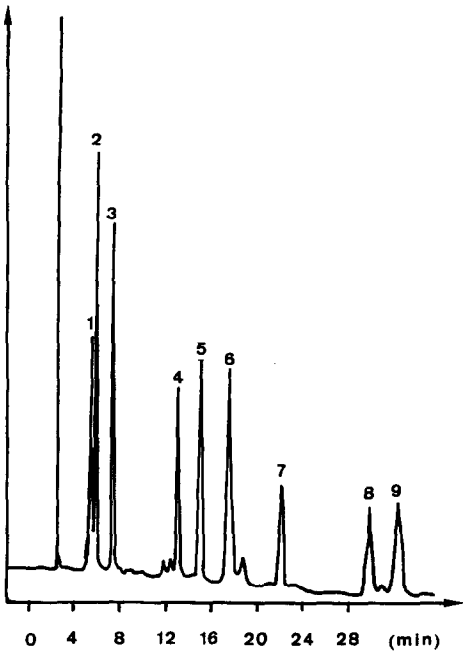

Fig. 6. Separation of a standard mixture of nine carotenoids by NARP-LC. Column, Zorbax ODS (250 × 4.6 mm I.D.); mobile phase, acetonitrile–methylene chloride–methanol (70:20:10, v/v/v); flow-rate, 1 ml min^{-1}; detection, 450 nm. 1 = Lutein; 2 = zeaxanthin; 3 = canthaxanthin; 4 = β-cryptoxanthin; 5 = echinenone; 6 = lycopene; 7 = torulene; 8 = α-carotene; 9 = β-carotene. From ref. 71.

In addition, the existence of π–π interactions between the double bonds of pigments and acetonitrile has been shown [80]. These interactions initially favour the solubility of carotenes in the mobile phase [80].

3.1.2.2. Characteristics of stationary phases. Lauren and McNaughton [75] investigated the effect of certain characteristics of C_{18}-bonded stationary phases on the separation of carotenes and xantho-

TABLE 3

EFFECT OF THE TYPE OF C_{18} COLUMN PROPERTIES ON THE SEPARATION OF α- AND *β-TRANS–CIS* ISOMERS

E = End-capped; NE = non-end-capped; M = monofunctional bonded silica; P = polyfunctional bonded silica; H = high (11%); L = low (7–8%).

Stationary phase	Carbon loading	E/NE	Type of bonded phase	Pore diameter of silica (Å)	CH_3OH in eluent (%)	Non-polar modifier in eluent (%)	Resolution (R_s) of α- and β-carotene	Resolution (R_s) of β-carotene *cis–trans* isomers	Ref.
Zorbax ODS	H	NE	M	70	<10	CH_2Cl_2 25	<1.25	–	6
Zorbax ODS	H	NE	M	70	<10	CH_2Cl_2 20	>1.25	–	71
Vydax 201 TP	L	NE	P	300	>10	THF 8	>1.25	>1.25	73
Zorbax ODS	H	NE	M	70	>10	THF 15	>1.25	<1.25	73
Vydax 218 TP	L	E	P	300	>10	THF 8	>1.25	>1.25	73
Vydac 201 TP	L	NE	P	300	>10	THF 8	>1.25	>1.25	79
Brownlee RP 18	–	E	M	80	10	CH_2Cl_2 10	>1.25	<1.25	80
Brownlee Spheri 5 ODS	–	E	P	80	10	CH_2Cl_2 10	>1.25	>1.25	80

phylls. They include the extent of bonding, varying from 7 to 11% (low and high density), and the degree of end-capping of residual silanol groups. The results showed that the optimum separation of a mixture of carotenes and xanthophylls was obtained with a non-end-capped bonded silica with a high bonding density (Zorbax ODS). Matus and Ohmacht [86] also observed an increased resolution of xanthophylls on non-end-capped columns. However, the retention of carotenes was decreased.

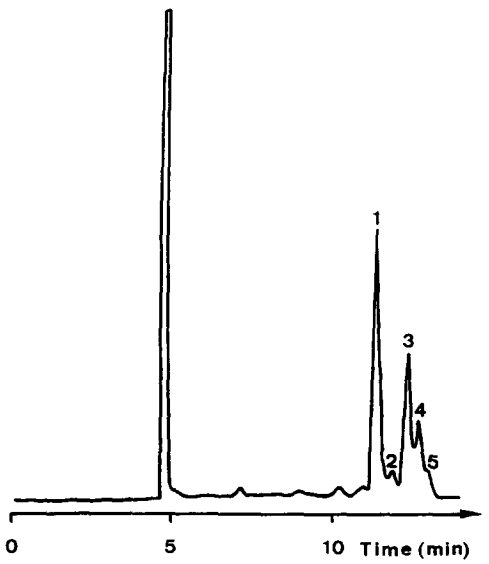

Fig. 7. HPLC separation of *cis–trans* isomers of β-carotene on a polyfunctional column. Column, Vydac 218 TP 54 (250 × 4.6 mm I.D.); mobile phase, acetonitrile–methanol–tetrahydrofuran (40:52:8, v/v/v); flow-rate, 1.0 ml min^{-1}; detection, 450 nm. 1 = All-*trans*-β-carotene; 2 = unknown; 3 = neo-U β-carotene; 4 = neo-B β-carotene; 5 = unknown. From ref. 73.

However, end-capping does not seem to affect the separation of *trans–cis* isomers of β-carotene (Table 3). Bushway [73,76] used different Vydac C_{18} columns to separate all-*trans*-β-carotene from its *cis* isomers (Fig. 7). All-*trans*-α- and -β-carotenes were not separated simultaneously, because of interference between the *cis* isomers of α-carotene and all-*trans*-β-carotene.

Using a Vydac C_{18} (201 TP) column, Quackenbush and Smallidge [77] were the first to report the separation of α-carotene from all-*trans*-β-carotene and from *cis*-β-carotenes. More recently, Quackenbush [79] reported the separation of all-*trans*-α- and -β-carotenes from their *cis* isomers by gradient elution, which improved the resolution between the compounds.

Lesellier *et al.* [80] reported the same separation under isocratic conditions using a column of Brownlee Spheri-5 ODS in NARP-LC. They showed that the type of bonding (mono- or polyfunctional) was very important for the separation of these compounds, both in HPLC [80] and in supercritical fluid chromatography (SFC) [88], where 22

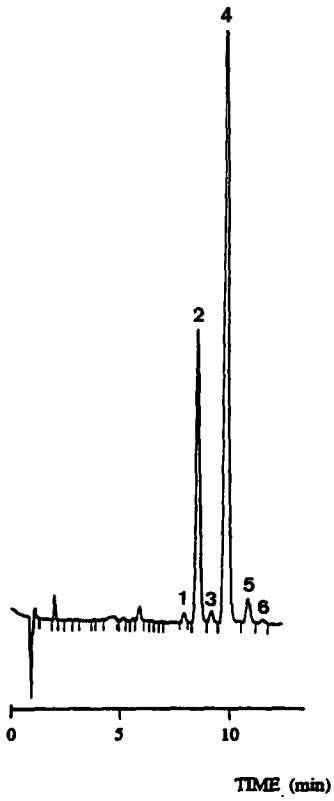

Fig. 8. Separation of a carrot extract by supercritical fluid chromatography. Column, Ultrabase UB 225 (250 × 4.6 mm I.D.); mobile phase, CO_2–methanol–acetonitrile (85:0.75:14.25, v/v/v); pressure, 15 MPa; temperature, 22°C; flow-rate, 3.0 ml/min; detection, 450 nm. 1 = γ- or ζ-all-*trans*-carotene; 2 = all-*trans*-α-carotene; 3 = *cis*-α-carotenes; 4 = all-*trans*-β-carotene; 5 = *cis*-β-carotene; 6 = *cis*-β-carotene. From ref. 96.

columns were tested. The use of a polyfunctional column is preferable for obtaining this separation. It is probable that the "network" structure of this phase was responsible for the separation, which can be explained in terms of flatness or non-flatness between compounds of identical chemical composition, as observed with polyaromatic hydrocarbons [89].

These compounds can, however, be fractionated with monofunctional columns at low temperature, although this is impossible at room temperature [80,90]. The temperature decrease should influence the conformation of hydrocarbon chains of the stationary phase and their mobility.

Monofunctional columns such as Ultrasphere ODS [84] and Zorbax ODS [48] enable *trans–cis* isomers of keto-carotenoids to be separated under the usual conditions.

Reversed-phase chromatography is well suited to the routine analysis of carotenoid pigments, regardless of the mixture being studied. Particular attention should be paid to the type of bonding, which can lead to considerable difference in the results.

3.1.3. Chromatography on chiral phases

This type of phase was first developed to separate optical isomers and is beginning to be used for the analysis of carotenoids. Moaka and co-workers [91,92] used cellulose supports (Chiracel OD) to separate the *trans–cis* isomers or diastereoisomers of carotenoids hydroxylated in position 2 or 4. A cyclodextrin support was used to fractionate lutein and zeaxanthin. The interactions between the hydroxy groups of carotenoids and the glucose molecules located at the periphery of the cyclodextrin cavity explain these results [93]. All-*trans*-β-carotene is also separated from the 15-*cis* isomer on cyclodextrin, but not from all-*trans*-α-carotene [93].

3.2. Supercritical fluid chromatography

Already used to extract pigments, CO_2 is also used in SFC for their separation. The relatively low critical temperature of CO_2 (31°C) enables heat-sensitive compounds such as carotenoids to be separated. It can be used equally well with capillary columns and packed columns. The first separation of α- and β-carotenes with packed columns in SFC was reported in 1968 by Giddings *et al.* [94]. More recently, Gere [95] separated lycopene and α- and β-carotenes in 5 min. He also fractionated a paprika oleoresin in 15 min and showed that the red pigments were eluted before carotene.

The simultaneous SFC separation of *trans–cis*-α- and -β-carotenes [88,96] has been achieved. Separations were more satisfactory than in NARP-LC [80] because two additional compounds could be separated, including one *cis* isomer of β-carotene (Fig. 8). The analogy between the separation mechanisms of these two techniques has been observed, in addition to the importance of π–π interactions [97]. Schmitz *et al.* [98] separated the *trans–cis* isomers of α- and β-carotenes on capillary columns. The results are very encouraging in terms of separation and gain in analysis time.

4. CONCLUSIONS

Progress in the analysis of carotenoid pigments during the last few years is due primarily to the introduction of new methods, such as NARP-LC, SFC and multi-wavelength on-line detection by coupling HPLC and photodiode-array spectrophotometry. Also, the more detailed understanding of pigment–stationary phase–mobile phase interactions and of the structures of stationary phases has enabled more complex separations to be carried out.

This understanding is of fundamental importance, as the diversity of stationary phases is constantly increasing (polymerization of linkages, mixed bonded phases) and will probably lead to highly efficient separations, provided that the choice of mobile phase and stationary phase parameters is optimized as a function of the pigments studied.

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