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Chromatographic separation of carotenoids

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SUMMARY. The carotenoids are extremely reactive and consequently unstable due to their long system of conjugated double bonds. Several precautions, such as protection against light and oxygen, use of low temperature and antioxidants, analysis in the shortest possible time, should be taken during isolation and chromatography. The food samples, preferably fresh, are homogenized and immediately extracted with a suitable organic solvent. Saponification has been employed in order to hydrolyze the carotenoid esters, remove fatty material and destroy chlorophyll. This optional step facilitates subsequent carotenoid separation, identification and quantification. The separation of carotenoids is usually carried out by column chromatography, thin layer chromatography and high performance liquid chromatography, in analytical or preparative scale, on many stationary phases such as silica-gel, alumina, MgO, $Ca(OH)_2$ and reversed-phase material (C_{18} and C_{30}). The choice of the most suitable chromatographic method depends on the amount of sample, carotenoid composition, resolution, speed and purity required. Examples of carotenoid separation in different stationary phases will be shown and discussed.
Key words: Carotenoids, isolation , chromatography.

RESUMO. Separação cromatográfica de carotenóides. Devido ao longo sistema de duplas ligações conjugadas, os carotenóides são altamente reativos e consequentemente instáveis. Várias precauções, tais como condução da análise no menor tempo possível, exclusão de oxigênio, proteção contra luz, uso de baixa temperatura e de antioxidantes, devem então serem tomadas durante o isolamento e cromatografia. Os alimentos, preferencialmente *in natura*, são homogenizados, e imediatamente os carotenóides são extraídos com solventes orgânicos. A saponificação é empregada com o objetivo de hidrolisar os ésteres de carotenóides, promover retirada de lipídeos e destruição de clorofila. Esta etapa opcional facilita a posterior separação, identificação e quantificação dos carotenóides. Os carotenóides são separados por cromatografia em coluna, cromatografia em camada delgada ou cromatografia líquida de alta eficiência (HPLC), tanto em escala analítica como preparativa. Várias fases estacionárias podem ser empregados tais como alumina, sílica, $Ca(OH)_2$, MgO e fase reversa (C_{18} e C_{30}). Os principais fatores que devem ser considerados na escolha do método cromatográfico são: quantidade de amostra, composição de carotenóides, pureza, resolução e velocidade necessárias. Serão apresentados e discutidos exemplos de separação de carotenóides em diversas fases estacionárias.
Palavras chave: Carotenóides, isolamento, cromatografia.

INTRODUCTION

The characteristic system of conjugated double bonds in the carotenoid molecule, in which the π electrons are delocalized over the whole polyene chain, is responsible for the long and straight shape of the molecule, for color due to absorption of visible light and for chemical reactivity, resulting in unstable and easily destroyed compounds.

Due to this fact, the following precautions must be taken during work-up:

- use of inert atmosphere, replacing air by vacuum or inert gas (N_2 or Ar),
- avoidance of high temperature, lower than 35°C for evaporation of large amount of solvent in the rotary evaporator; alternatively, evaporation of small volumes directly under N_2 or Ar,
- storage at very low temperature,
- all operations carried out in diffuse light; equipment and glassware covered by black cloth or aluminium foil,
- avoidance of acid and alkali, strongly acidic reagents not being used in the laboratory where the carotenoids are handled,
- all operations carried out in the shortest possible time.

Pre-chromatographic steps

The most common problem during work-up is *cis-trans* isomerization in solution catalyzed by heat, light, acids and active surfaces (1). Therefore, the pure carotenoid or even the crude extract should never be stored in solution, and preferably kept dry under inert atmosphere.

The steps involved in the preparation of the carotenoid extract are briefly discussed below.

Sample. It is important to use fresh and undamaged food samples, since unwanted reactions catalyzed by enzymes and acids may occur after harvesting.

Tissue homogenization should be preferably done immediately before analysis or during extraction because mechanical desintegration can introduce air and destruction of the cell wall liberates enzymes, such as lipoxygenase, and acids causing degradation of carotenoids and epoxide-furanoxide rearrangement, respectively.

Extraction. The choice of the best solvent for extraction depends on the sample, its pre-treatment and the carotenoid composition. Water-miscible organic solvents, usually acetone, are generally employed for extraction of fresh foods. Dried or lyophilized samples can be extracted with water immiscible solvents, such as ethyl acetate or diethyl ether (2), or preferably re-hydrated in the case of quantitative extraction, as for squashes and pumpkins (3) and pasta (4). The extraction of samples containing carotenoid glycosides demands a more polar solvent and usually ethanol (5) or methanol or its combination with acetone (6) is employed.

The addition of $NaHCO_3$, $MgCO_3$ or $CaCO_3$ (0.1 g/g sample) and antioxidants is recommended to neutralize tissues containing acids and to avoid oxidation, respectively.

The extraction can be carried out in a blender or simply with the aid of a mortar and pestle.

For qualitative extraction, large amounts of food are often used and there is no concern about loss of material. If the final objective is, for example, the isolation of a carotene, it is very useful to extract the sample first with a very polar solvent (e.g. methanol) that would remove water and the xanthophylls partially, both of which are discarded. The carotenes are then extracted with a suitable less polar solvent.

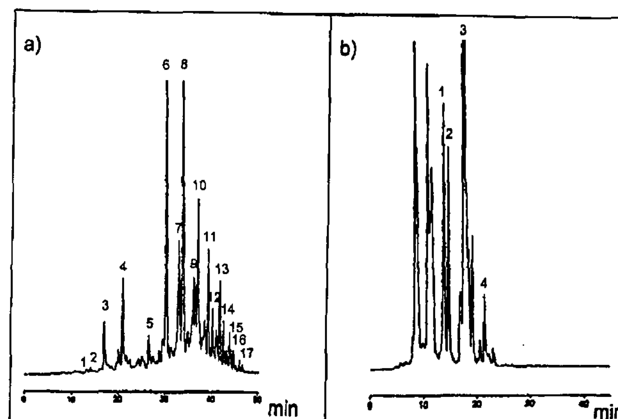
On the other hand, quantitative extraction requires complete and exhaustive extraction and no material can be lost. Usually three to four extractions are enough to remove the carotenoids completely from the sample.

Removal of water and solvents. The carotenoid extract obtained by extraction of the fresh sample with acetone contains large amounts of water which comes from the sample. Therefore, in order to remove water and acetone, the carotenoids are transferred to petroleum ether or diethyl ether by adding small portions of the acetone extract and large amount of water in a separatory funnel. The remaining traces of water can be removed either by addition of anhydrous Na_2SO_4 or drops of ethanol (by formation of an azeotropic mixture).

Saponification. Alkaline hydrolysis (saponification) has been used to remove contaminating lipids from fat-rich samples (e.g. palm oil), destroy chlorophyll (e.g. green vegetables) and hydrolyze carotenoid esters.

Xanthophylls esterified with a mixture of different fatty acids are typically found in fruits, and the use of saponification allows an easier chromatographic separation, identification and quantitation. This fact is exemplified with a carotenoid extract from tangerine, shown in Figure 1, where among the carotenoid esters, the unsaponificable extract contains free ß-cryptoxanthin and six esters of this major carotenoid. As expected, on saponification, the chromatogram turns to be much simpler and ß-cryptoxanthin becomes the major carotenoid (7).

FIGURE 1
HPLC chromatogram of a) unsaponified and b) saponified tangerine extract



Identification of numbered peaks: 1-lutein, 2-zeaxanthin, 3-ß-cryptoxanthin, 4-ß-carotene, 5 to 10- esters of ß-cryptoxanthin, 11 to 17-esters of di- and poly-hydroxy xanthophylls. Conditions: C_{18} Suplex pKb 100 (5µm, 4.6 x 250 mm) column and MeOH/acetonitrile/CH_2Cl_2/hexane (10:85:2.5:2.5) for 5 min, going to 10:45:22.5:22.5 from 5 to 40 min at 0.7 ml/min. Reference: Wingerath et al. (7).

Although saponification was found to be unnecessary for separation and quantification of carotenoids from leafy vegetables by column chromatography (CC) (8, 9), saponification is most of the time employed to clean the extract when subsequent identification is done by mass spectrometry and nuclear magnetic resonance (NMR) spectroscopy.

A general procedure that has been employed in our laboratory is the addition of an equal amount of methanolic 10% KOH to the hexanic carotenoid extract. This solution can be bubbled with N_2 or Ar and allowed to stand overnight at room temperature. Subsequently, the mixture is washed with water in a separatory funnel until free of alkali.

Carotenoids with 3-hydroxy-4-keto group, as astaxanthin, which is widespread in marine animals, microorganisms and algae, undergo oxidation in the presence of alkali and air. For such samples, saponification is not recommended or must be carried out under anaerobic conditions. For this purpose, a special apparatus and procedure were developed (10).

Aldol condensation is another undesirable reaction that can produce artifacts such as conjugated methyl ketones. Carotenals undergo aldol condensation during saponification in the presence of acetone that remains from the extraction

step. In fact, citranaxanthin and reticulaxanthin, reported as natural carotenoids from citrus, are probably aldol condensation products formed from ß-apo-8'-carotenal and ß-citraurin, respectively (11). In such samples the extraction can be performed with methanol and ethyl acetate.

It is recommended to verify if structural changes occur during saponification. Since the hydroxyl groups have no influence on the chromophore, the wavelength of the maximum absorption, shape and intensity of the UV-visible spectrum would be identical for the unsaponified and saponified samples.

The use of plastic material, filter paper and blender during the steps described above should be avoided, in order to prevent contamination, if the isolated carotenoid would be analyzed by direct insertion in the mass spectrometer.

Chromatography

The separation of carotenoids can be carried out by CC, thin layer chromatography (TLC), high performance liquid chromatography (HPLC) or a combination thereof. Since carotenoids are labile to high temperature, gas chromatography is not employed for separation.

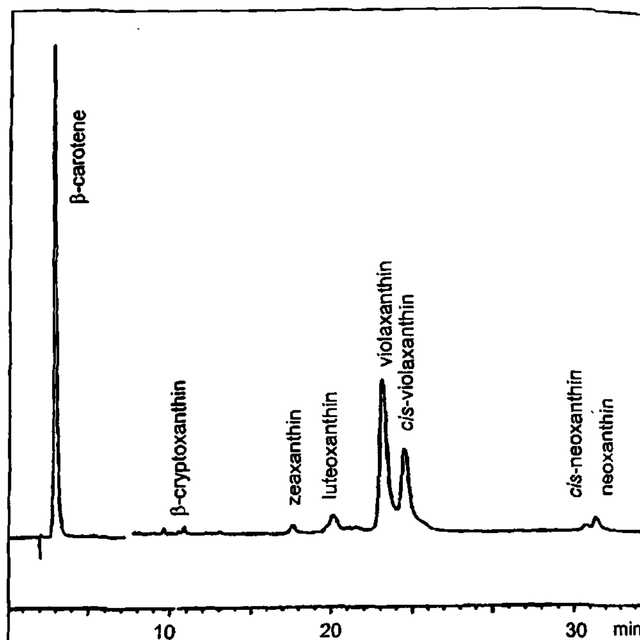
The choice of the most suitable chromatographic method depends on:

- complexity of the carotenoid composition of the sample;
- amount of sample, i.e. CC or large number of TLC plates for large amounts and TLC or HPLC for samples containing < 1 mg of total carotenoid;
- objective of analysis, e.g. for quantitative analysis CC or HPLC, since it is very difficult to totally recover the carotenoids from the TLC plates;
- resolution, purity and speed required;
- equipment available.

Chromatography is essentially a method of separation based on two phases, one stationary and one mobile. If the composition of the mobile phase is not changed during the separation, the term isocratic elution is used. For separation of complex mixtures with wide range of polarities, the composition of the mobile phase can be changed during separation, which is known as gradient elution. There is a large range of stationary phases available, and according to their polarity they can be divided into normal-phase and reversed-phase.

Normal-phase. Silica-gel and aluminum oxide are used to separate the carotenoids according to their polarity, compounds with more polar substituents being more strongly adsorbed. Both are commonly used as stationary phases for separation of carotenoids by CC and/or TLC. The bonded-phase nitrile (or cyano) also separates according to polarity and is employed for separation by HPLC. Figure 2 shows an example of the separation of mango carotenoids on a cyano column with gradient elution. As expected for normal phase, ß-carotene elutes first, followed by mono-, di- and poly-hydroxy carotenoids (12).

FIGURE 2
HPLC separation of carotenoids from mango extract



Conditions: Nitrile Spherisorb column (5µm, 4.6 x 150 mm) and as mobile phase acetone in n-hexane from 0 to 15% in 10 min, to 20% in 20 min, to 30% in 10 min and to 40% in 2 min at 1 ml/ min. Reference: Mercadante et al. (8).

Basic materials such as magnesium oxide (MgO) and calcium carbonate have affinity for conjugated double-bonds, polarity being less important. Thus, a greater number of conjugated double bonds implies stronger retention. The separation of α-carotene and ß-carotene can easily be achieved by CC or TLC using this kind of material (13,14). CC on MgO is also very useful for removing large amounts of colorless impurities from the sample.

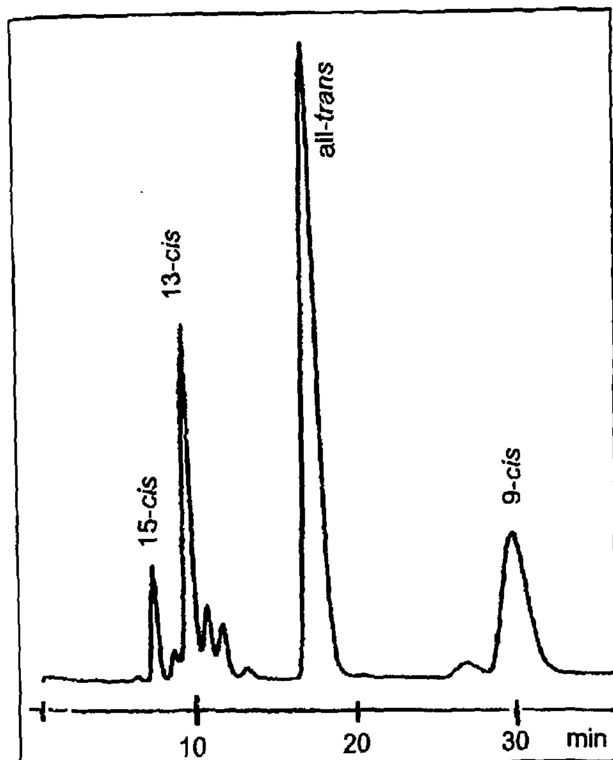
Calcium hydroxide ($Ca(OH)_2$) has been used to separate isomeric forms of ß-carotene (15,16) and ß-cryptoxanthin (16) by CC. Although not commercially available, this stationary phase was also employed in HPLC for separation of isomers of α-carotene, ß-carotene (Figure 3), ζ-carotene and γ-carotene (17).

The isolation of carotenoids from tomato (18-20), mango (12) and passion fruit (21) was successfully carried out using the above stationary phases for the purpose of identification by direct insertion in electron impact-mass spectrometry. Firstly, the saponified extract was separated by CC on alumina, giving three broad fractions (carotenes, mono- and polyhydroxy carotenoids), each of which was separated by TLC on silica, developed with petroleum ether, petroleum ether/diethyl ether (1:1 or 3:2), and diethyl ether according to increasing polarity of the fractions. Each fraction isolated from silica was further

purified by TLC on MgO/kieselguhr with combinations of acetone and petroleum ether as mobile phase.

The appropriate combination of such normal-phase adsorbents can also be employed to obtain standards for HPLC from natural sources.

FIGURE 3
HPLC separation on $Ca(OH)_2$ column of isomers from an isomerized solution of ß-carotene

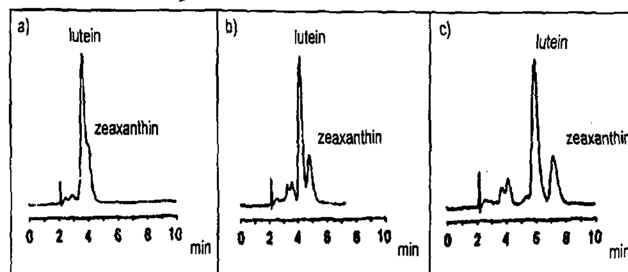


Conditions: $Ca(OH)_2$ (500mesh, 4.6 x 250 mm) column and as mobile phase 2% of *p*-methyl anisole in hexane at 0.7 ml/min. Reference: Schmitz et al. (17).

Reversed-phase. Nowadays, the reversed-phase material is the most popular for separation of carotenoids by HPLC, C_{18} bonded phase being the most employed. Many different C_{18} materials are available from different manufacturers. The difference lies in the:

- degree of carbon loading and end-capping. Silanol groups are expected to influence the retention behavior of polar compounds to a greater extent than nonpolar carotenes. In fact, the separation of α-carotene, ß-carotene and lycopene was little affected by endcapping since the seletivity is unrelated to polarity. On the other hand, the separation of the polar carotenoids lutein and zeaxanthin was influenced by silanol activity and better separation was achieved with the non-endcapped phase (22) (Figure 4).

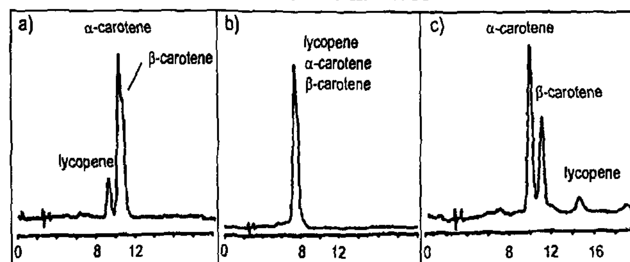
FIGURE 4
Effect of endcapping on polymeric C_{18} phases for separation of lutein and zeaxanthin



a) polymeric endcapped with hexamethyldisilazane, b) polymeric endcapped with trimethylchlorosilane c) polymeric not endcapped. Mobile phase: methanol at 1.5 ml/min. Reference: Sander et al. (22).

- nature of synthesis (monomeric - monofunctional or trifunctional or polymeric). Poorer resolution was observed for α-carotene, ß-carotene and lycopene using the trifunctional monomeric C_{18} phase and nearly baseline separation was achieved with the polymeric one (Figure 5). The polymeric synthesis improves the column seletivity towards form and groups with similar structure (22).

FIGURE 5
HPLC separation on different C_{18} phases of a carotene mixture



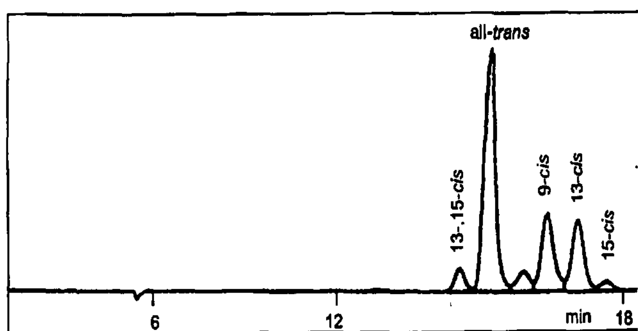
a) monomeric, monofunctional synthesis, b) monomeric, trifunctional synthesis and c) polymeric synthesis. Mobile phase: methanol/ethyl acetate (8:2) at 1.5 ml/min. Reference: Sander et al. (22).

The polymeric C_{18}, with the brand name Vydac, among more than 20 commercial reversed-phase columns, was also the only one that provided the separation of ß-carotene isomers (23). An example of the separation of all-*trans*-, 13,15-di*cis*, 9-*cis*-, 13-*cis* and 15-*cis*- isomers of ß-carotene can be seen in Figure 6. In this case the best separation was achieved at 30°C and resolution was strongly dependent on the column lot (24). The Vydac column has been widely employed for separation of carotenoids in processed foods such as carrot juice (25) and green vegetables (14, 26).

More recently, a C_{30} polymeric reversed-phase column was specially developed for carotenoids (22). This stationary phase shows adequate retention times for polar carotenoids and superior selectivity towards polar and nonpolar carotenoids, specially geometrical isomers. Among reversed-phase

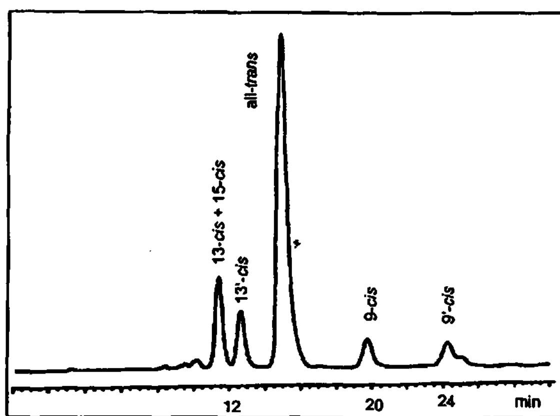
columns, the C_{30} is the only one that was capable of resolving geometrical isomers of asymmetrical carotenóids in which *cis* double bonds are present at the same position but at opposite ends of the molecule (27). This feature is shown in Figure 7, where 13-*cis*, 13'-*cis*, 9-*cis* and 9'-*cis* isomers of lutein were separated (28). This column has also been employed for separation of isomers in isomerized solutions of ß-carotene (28) (Figure 8), in processed vegetables and fruits (29) and of 39 carotenoids from orange juice (30).

FIGURE 6
HPLC separation on Vydac column of isomers from an isomerized solution of ß-carotene



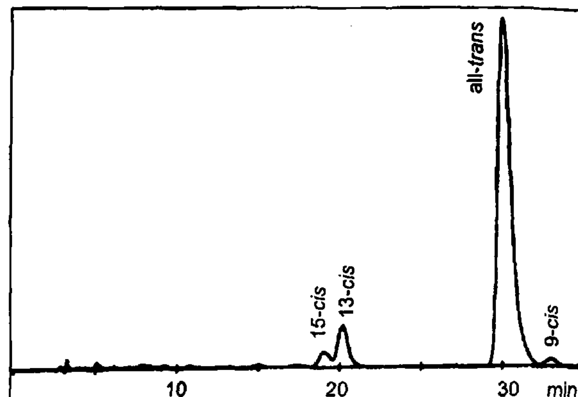
Conditions: C_{18} Vydac 218TP54 (5 µm, 4.6 x 250 mm) column and as mobile phase methanol/tetrahydrofuran (99:1) at 0.6 ml/min. Reference: Schierle et al. (20).

FIGURE 7
HPLC separation on a C_{30} column of isomers from an isomerized solution of lutein



Conditions: C_{30} YMC (3µm, 4.6 x 250 mm) column and as mobile phase methanol/*t*- butyl methyl ether (95:5) at 1 ml/ min. Reference: Brunner (28).

FIGURE 8
HPLC separation on a C_{30} column of isomers from an isomerized solution of ß-carotene



Conditions: C_{30} YMC (3µm, 4.6 x 250 mm) column and as mobile phase methanol/*t*- butyl methyl ether (8:2) at 1 ml/ min. Reference: Brunner (28).

Measurement of NMR spectrum requires very pure compound in higher amount than for UV-visible and mass spectra. For this purpose, the isolation employs CC and/or TLC, and the final purification step is usually carried out by crystallization or semipreparative HPLC. The optimization of HPLC mobile phase is preferably performed in an analytical column with the same characteristics as the semipreparative one. This procedure was used for the isolation and NMR identification of carotenoids from guava (2), annatto (31-33) and saffron (5).

CONCLUSION

Each stationary phase has a different mechanism of separation and therefore the analyst should apply the most suitable phase for a particular separation. A successful carotenoid purification should include chromatographic separation in at least two different kinds of adsorbent. Several examples of carotenoid separation can be found in the literature and it is very useful to look for previous experiences. Different solutions for the same problem may be found in the literature.

In order to enhance reproducibility, authors should provide clear and complete specification of mobile and stationary phases employed for carotenoid separation.

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