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Journal of Chromatography A, 697 (1995) 289–294

JOURNAL OF CHROMATOGRAPHY A

High-performance liquid chromatographic determination of the geometrical isomers of β-carotene in several foodstuffs

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Abstract

To better evaluate the provitamin A content of foodstuffs, an HPLC separation of the geometric isomers of β-carotene was developed. Of the several different stationary phases studied, the best separations of all-*trans*-, 9-*cis*- and 13-*cis*-β-carotene were obtained with calcium hydroxide columns using isocratic elution with *n*-hexane, isooctane or mixtures of these solvents. When xanthophyll pigments are also present, a gradient elution with acetone in isooctane is required. These techniques were used to determine the provitamin A content of squash, peaches, dendê oil (palm tree oil) and orange juice.

1. Introduction

Carotenoids, a class of naturally occurring hydrocarbons (commonly called carotenes) and their oxygenated derivatives (xanthophylls) are ubiquitous in nature. Those carotenoids with at least half of the β-carotene molecule, i.e., an unsubstituted β-ionone ring having an 11-carbon polyene side chain, are classified as metabolic precursors of vitamin A, which is considered important for vision and, possibly, as an anticarcinogenic agent. Thus, quantification of the provitamin A activity of foodstuffs is of primary importance, to access the contribution of the individual carotenoids present.

Traditional analyses of carotene mixtures have involved paper, thin-layer, normal phase gravity-flow column, and both normal- and reversed-phase HPLC [1]. One of the problems common to these chromatographic methods in determining the provitamin A content of foodstuffs is the separation of the different geometrical isomers, which have considerably different provitamin A activities [2]. For example, 13-mono-*cis*-β-carotene has 53% and 9-mono-*cis*-β-carotene has 38% of the activity of *trans*-β-carotene [3]. All *trans*-α, 13-mono-*cis*-α- and 9-mono-*cis*-α-carotene have 37%, 22% and 23%, respectively, of the activity of *trans*-β-carotene [4]. Other authors [5,6] have reported different values but the overall impression is the same: *trans*-β-carotene is an excellent source of provitamin A while the other carotenes contribute significantly lower quantities. Thus separation and quantification of the geometrical isomers is relevant in determining the provitamin A content of foodstuffs.

Currently, HPLC is considered the method of choice for provitamin A determination as it circumvents various analytical difficulties encountered with the classical methods. Most of the HPLC methods reported for provitamin A

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0021-9673/95/$09.50
SSDI 0021-9673(94)00752-7

determinations have used reversed-phase (C_{18}) columns [7], since these appear to reduce the risk of pigment degradation attributed to silica columns [8]. The use of alumina [9], magnesium oxide [10,11], calcium hydroxide [12,13], amino [14], and nitrile [15] columns has also been reported. However, most of these columns do not satisfactorily separate the geometrical isomers [16].

The objective of this work is to develop a separation of the geometrical isomers of β-carotene to better evaluate the provitamin A content of several foodstuffs.

2. Experimental

2.1. Chemicals

All reagents and solvents were of analytical or chromatographic grade. All solutions were stored in brown flasks or protected from light by aluminium foil. Pure samples of *trans*-β-carotene were obtained by extracting a carotenoid mixture from appropriate vegetable samples and separating this mixture by gravity-flow liquid chromatography with a mixture of calcium hydroxide and Hyflosupercel as stationary phase and elution with increasing quantities of ethyl ether in petroleum ether [2]. Isomerization, to obtain 9-*cis*- and 13-*cis*-β-carotene, was done by exposure to visible light in the presence of iodine dissolved in ethyl ether. The isomerized samples were also separated by gravity-flow chromatography and identified by UV–visible absorption spectra and appropriate chemical reactions [17]. The standards were used immediately after separation and identification.

2.2. Food samples

Oranges (*Citrus sinensis* Osbeck), yellow squash (*Curcubita maxima*), dendê oil (*Elaeis guineensis*, L.) and fresh peaches (*Prunus persica*) were purchased from supermarkets in Campinas, Brazil. These foods were chosen because of their varying carotenoid composition and their content of possible interfering substances. The squash and peach samples (10–30 g) were finely chopped and then homogenized for three min in a Waring blender containing 100 ml of acetone and 10 g of Hyflosupercel [18]. The resulting suspension was suction-filtered and the acetone extraction of the solids was repeated until complete removal of the pigments. The combined filtrates were partitioned with petroleum ether and this solution was washed with distilled water. The orange juice and dendê oil samples were similarly extracted with acetone, partitioned, and washed. The samples were saponified by treatment with 10% (v/v) methanolic potassium hydroxide overnight, followed by washing with distilled water. The final petroleum ether solution was dried by passing it through a short column containing anhydrous sodium sulfate. This solution was then concentrated on a rotary evaporator for injection into the chromatograph.

2.3. Chromatographic columns

Three commercial and two laboratory-packed columns were evaluated: a 125×4 mm I.D. LiChrocart column containing 5 μm LiChrosphere 100-RP-18 (Merck) with a C_{18} precolumn (Merck); a 125×4 mm I.D. LiChrocart column containing 5 μm LiChrosphere 100-NH_2 (Merck) with a NH_2 precolumn (Merck); a 250×2 mm I.D. column containing 10 μm Micropak-CN (Varian); a 150×4 mm I.D. laboratory-packed column containing 5 μm LiChrosorb Alox-T (Merck) and a 250×2.2 mm I.D. laboratory-packed column containing $Ca(OH)_2$ (Nakarri Chemicals).

2.4. Equipment

HPLC separations were carried out on a Varian Model 5000 liquid chromatograph equipped with a Rheodyne Model 7125 injection valve with a 10-μl loop and an oven with both heating and cooling capabilities. Detection was with a Varian Model 100 variable-wavelength spectrophotometric detector coupled to an Infralab Model 4290 integrator or with a Shimadzu Model SPDM6A diode array detector coupled to an Acer Model 915 V microcomputer. Absorption spectra were obtained with a VanDen Model PC2500 double beam UV–visible spectropho-

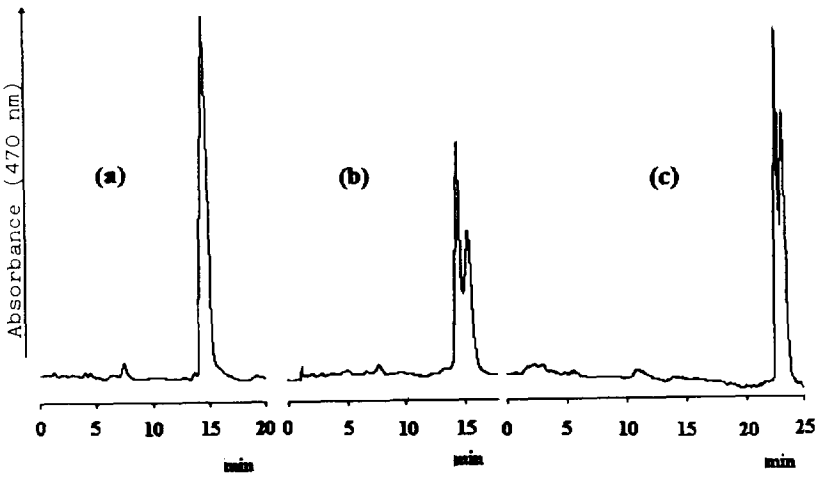

Fig. 1. Chromatograms of *trans*-β-carotene before (a) and after (b,c) isomerization. Column: 125 × 4 mm I.D. with LiChrospher 100 RP-18, 5 μm. Mobile phase: a,b = methanol–acetone (90:10, v/v) at 1.0 ml min^{-1}; c = pure methanol (15 min) then acetone 50% in 5 min, flow-rate 0.7 ml min^{-1}. Detection: absorbance at 470 nm.

tometer using 1 cm silica cells, coupled to an Intralab Model Omega recorder.

2.5. Calculation of the vitamin A value

One retinol equivalent (RE) corresponds to 6 μg of the provitamin A *trans*-β-carotene. The comparative provitamin A activity of the other carotenoids was taken from tabulated values [2,3].

3. Results and discussion

Separations on the C_{18} column were tested using different mixtures of methanol–water; methanol–acetone; methanol–chloroform, and

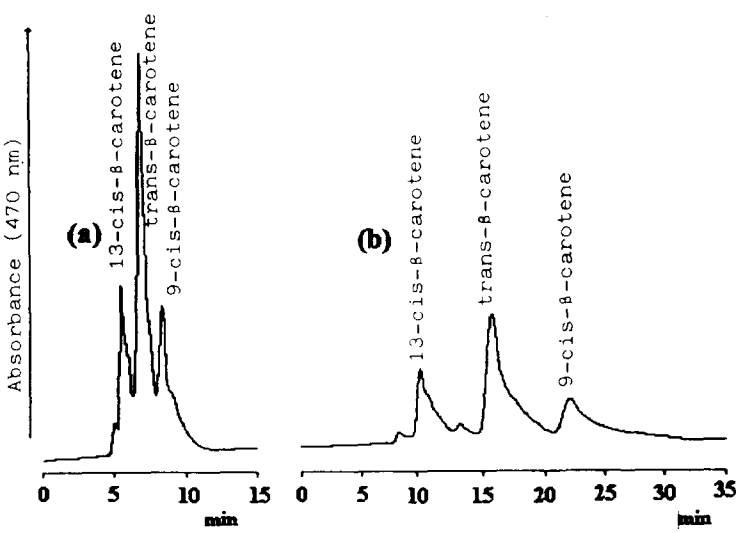

Fig. 2. Chromatograms of isomerized *trans*-β-carotene. Column: 250 × 2.2 mm I.D. with $Ca(OH)_2$. Mobile phase: (a) *n*-hexane; (b) isooctane, at 0.5 ml min^{-1}. Detection: absorbance at 470 nm.

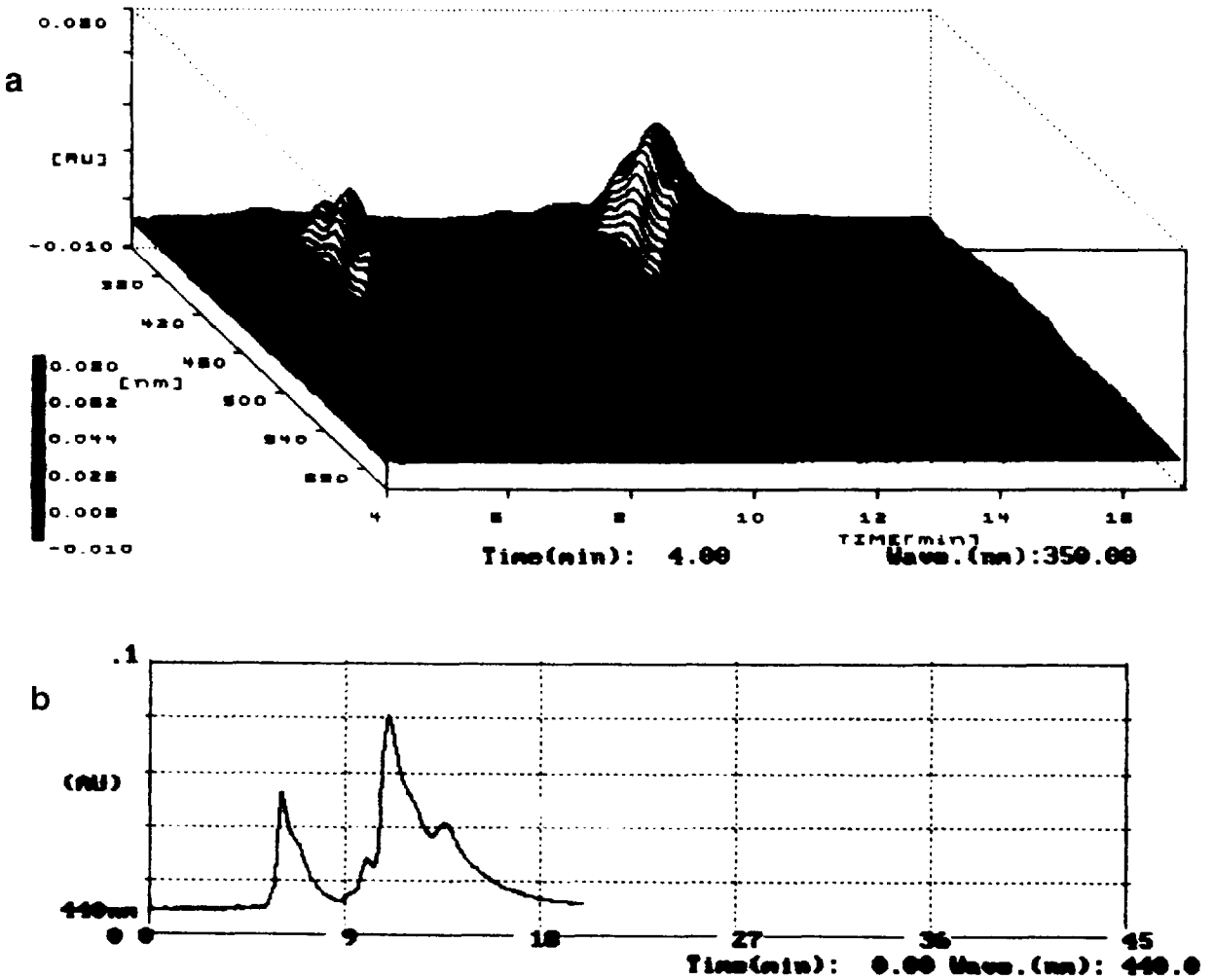

Fig. 3. Chromatograms of the carotenoids extracted from dendê oil. Column: 250 × 2.2 mm I.D. with $Ca(OH)_2$. Mobile phase: isooctane at 0.5 ml min^{-1}. (a) three-dimensional; (b) two-dimensional chromatogram at 440 nm.

ethanol–acetone. Partial separation of the *cis*- and *trans*-isomers was obtained using a 90:10 (v/v) methanol–acetone phase or with gradient elution with the same solvents. However, the two *cis* isomers were not separated under any conditions on this C_{18} phase (Fig. 1).

Separation of *cis* from *trans* isomers was also observed using the CN column with *n*-hexane. Mobile phase mixtures of hexane with dichloromethane, acetone or acetonitrile did not improve this separation. On the other hand, neither the NH_2 nor the Alox-T columns provided any separation of these geometrical isomers.

The best separations of all three geometrical isomers were observed with the calcium hydroxide column (Fig. 2), in agreement with previous literature [12,13]. Variation of the column temperature between −25°C and +40°C did not cause significant changes in the resolution although changes in retention times were observed. Thus, the column was used at room temperature with isooctane at a flow-rate of 0.5 ml min^{-1} for the quantification of the geometrical isomers in yellow squash and dendê oil (Fig. 3). A gradient from 100% isooctane to isooctane–acetone (80:20) in 60 min, also at a

flow-rate of 0.5 ml min $^{-1}$, was used for the orange and peach samples (Fig. 4) to separate β-cryptoxanthin from the β-carotenes.

Table 1 reports the provitamin A contents of the four foodstuffs studied in this work. The final column of this table lists the retinol equivalents (RE). For yellow squash, where only *trans*-β-carotene is observed, and orange juice, where only traces of the *cis*-β-carotene isomers are found, the RE values are similar to those previously determined [10,11]. The RE values reported here for dendê oil are less than those of the literature [19] since poor resolution of the geometrical isomers resulted in an overestimation of the *trans*-β-carotene content. Several recent studies using HPLC with extracts from peaches [20,21] have reported the separation and identification the β-carotene isomers. In the present work, β-cryptoxanthin was also quantified.

Despite the inconvenience of having to prepare the calcium hydroxide column in the laboratory, the resulting separations confirm this to be an excellent stationary phase for the separation

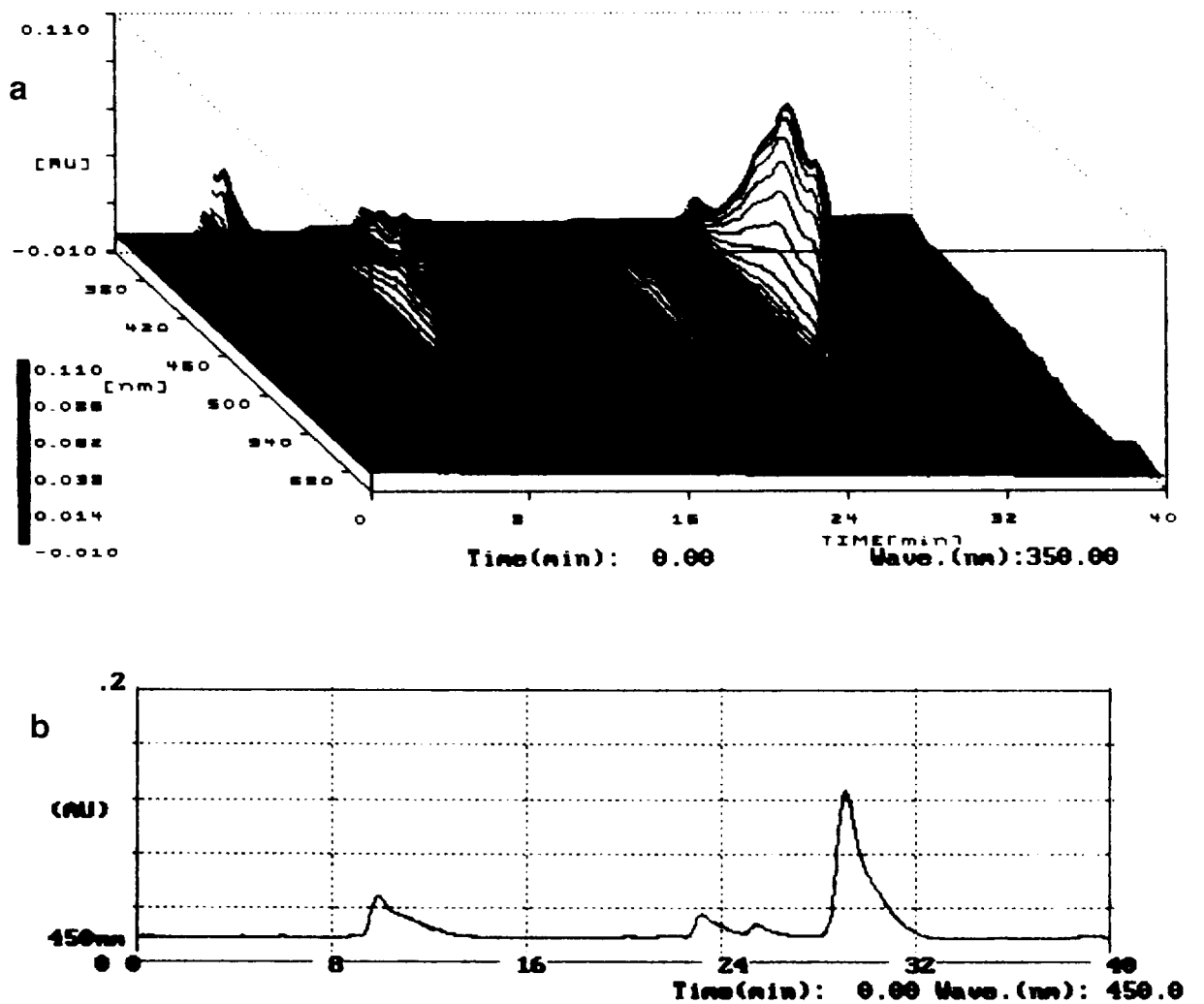

Fig. 4. Chromatogram of the carotenoids extracted from peaches. Mobile phase: gradient from isooctane to isooctane–acetone (80:20, v/v) in 60 min and detection at 450 nm. Other conditions as in Fig. 3.

Table 1
Provitamin A content of several foodstuffs

Foodstuff	Sample	Provitamin A found (mg/100 g)					Average retinol equivalent (RE/100 g)
		α-Carotene	13-*cis*-β-Carotene	*trans*-β-Carotene	9-*cis*-β-Carotene	β-Cryptoxanthin	
Yellow squash	1	–	–	0.93	–	–	
	2	–	–	0.82	–	–	150
	3	–	–	0.94	–	–	
Orange juice	1	0.007	tr[a]	0.011	–	0.016	
	2	0.009	tr	0.014	–	0.020	4.3
	3	0.009	tr	0.011	–	0.019	
Peaches	1	–	tr	0.17	–	0.58	
	2	–	tr	0.16	–	0.53	75
	3	–	tr	0.17	–	0.59	
Dendê oil	1	5.67	1.37	19.6	2.36	–	
	2	5.77	0.77	19.2	1.43	–	3960
	3	5.42	1.45	19.9	2.11	–	

[a] tr = Present at trace levels too low to quantify.

of the β-carotene isomers, permitting better estimation of the true provitamin A content of the foodstuffs tested. In the absence of xanthophylls, isocratic elution with isooctane is effective, however, elution of the more highly retained oxygenated compounds, after the β-carotene isomer separation, requires a gradient with a more polar component.

Acknowledgements

The authors express their appreciation to EMBRAPA for a fellowship to CRLC and to CNPq and FAEP (UNICAMP) for financial support.

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