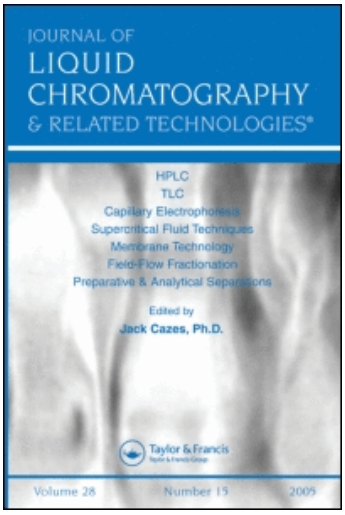

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SEPARATION OF CAROTENOIDS IN FRUITS AND VEGETABLES BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

Several isocratic high performance liquid chromatographic (HPLC) methods, including both normal and reversed-phase, have been developed to separate provitamin A compounds and other carotenoids - α-carotene, β-carotene, stereoisomers of β-carotene, γ-carotene, β-cryptoxanthin, canthaxanthin and lycopene- in fruits and vegetables. The normal phase systems used either an amino or alumina column with 99.5% isooctane and 0.5% tetrahydrofuran. Procedures employing reversed-phase used C18 columns- Vydac 201TP54, Vydac 218TP54, Zorbax ODS and NovaPak C18- with nonaqueous solvent systems comprised of various mixtures of acetonitrile, methanol, tetrahydrofuran and chloroform. All compounds were monitored at 470 nm or 450 nm. Each method has certain advantages and disadvantages for the analysis of carotenoids in fruits and vegetables.

INTRODUCTION

As researches learn more about the relationship of dietary intake and human health, an accurate and specific assessment of the nutrient content of foods is becoming more important. One such

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group of nutrients is the provitamin A carotenoids which are comprised of approximately 18 compounds (1,2) varying in vitamin A activity with the most prevalent and active ones consisting of β-carotene, α-carotene and their stereoisomers along with γ-carotene and β-cryptoxanthin (1,2). Besides their normal vitamin A function, recent research has shown that some provitamin A compounds have anticancer and antiulcer properties (3-9).

Antineoplastic evidence has been of two types. First, epidemiological studies have shown the existence of an inverse relationship between the risk of cancer and the consumption of foods containing β-carotene (3,4,6-8). Second, several laboratory experiments have demonstrated the inhibition of cancer cell lines and actual tumor regression in animals given β-carotene (3-5).

Antiulcer properties were observed by Mozsik et al. (9). They were able to show that β-carotene and β-cryptoxanthin were involved in the cytoprotective injury of the gastric mucosa.

Because of their important role in nutrition and in some cases disease and because of their different degrees of vitamin A activity, there is a need for a simple, rapid and specific method for individual provitamin A carotenoids in foods. Such a procedure would be beneficial to food scientists, horticulturists, nutritionists, cancer researchers, and epidemiologists. Of the 3 types of chromatographic methods used for carotenoid determinations- open column, thin-layer and high performance liquid chromatography- HPLC is the best. The other two procedures are very

time consuming; can transform carotenoids to their stereoisomers; and many methods cannot distinguish between stereoisomers.

High performance liquid chromatographic techniques are considered to be the quickest, simplest and most reproducible methods of analyzing complex mixtures of carotenoids in foods and other substances. The most extensive system has been developed by Sweeney and Marsh (2) for fruits and vegetables in which most of the prevalent provitamin A carotenoids can be separated. The disadvantages are that this method is lengthy due to an initial open column step and it employs 2 HPLC columns that are unavailable commercially. A similar method was developed for citrus fruit in that a column not commercially available was used (10).

Since 1977 HPLC methods for carotenoids have employed commercially available columns. Both normal and reversed-phase procedures have been developed (11-23) with and without gradients, but the most popular packing material has been C18. Of these numerous methods, only one, which was developed by Quackenbush and Smallidge (23), has begun to deal with the complexity of food carotenoids. They were able to rapidly separate several carotenoids including β-carotene and some of its stereoisomers using a Vydac column with a nonaqueous solvent system. The best results were obtained from a gradient procedure although in the isocratic mode one of the major β-carotene isomers was separated. Lycopene also interfered with β-carotene under certain conditions (23).

This paper describes several rapid isocratic HPLC methods (normal and reversed-phase) that were developed for provitamin A

carotenoids in fruits and vegetables including modifications of the Quackenbush and Smallidge procedure (23).

EXPERIMENTAL

Materials

Trans α- and β-carotene standards were obtained from Sigma Chemical Co. St. Louis, MO. γ-Carotene, β-cryptoxanthin and canthaxanthin were gifts from Hoffmann-LaRoche, Nutley, NJ and Basel, Switzerland. The stereoisomers of β-carotene were obtained by irradiating 25 mg/100 ml β-carotene solution using the procedure of Zechmeister (24). Magnesium carbonate anhydrous powder and sodium sulfate anhydrous granular form were purchased from Fisher Scientific Co., Fair Lawn, NJ. Stabilized tetrahydrofuran was bought from VWR Scientific, Bridgeport, NJ while the acetonitrile, methanol, isooctane and chloroform were all HPLC grade obtained from Fisher Scientific. All foods were purchased from local wholesalers and stores.

Preparation of Standards

Stock solutions of α-carotene, β-carotene, β-cryptoxanthin, γ-carotene and canthaxanthin were prepared by weighing 25 mg of each into 100 ml low actinic volumetric flasks and bringing each to volume with THF. Three working standrds were made by taking 1, 2 and 3 ml from each stock solution. Standards were stored at -20 C and were stable for 6 to 8 months. After which time, the standards should be recalibrated using Beer's Law. Irradiated β-carotene stock and working standards were prepared like the other carotenoids.

Liquid Chromatographic System

A liquid chromatograph equipped with a Waters Model 510 pump (Waters Associates, Milford, MA), a Waters U6K injector and a Schoeffel 450 variable-wavelength detector (Westwood, NJ) and an Omniscribe dual pen recorder (Houston Instruments, Austin, TX). The detector was set at 450 nm or 470 nm and 0.04 absorbance units full scale.

Liquid Chromatography Separations

Normal phase systems consisted of a 5 μ amino column (IBM Instruments, Wallingford, CT) 250 mm x 4.5 mm and a 5 μ Spherisorb alumina column (Phenomenex, Palos Verdes Dr., CA) 250 mm x 4.6 mm. The solvent system for both consisted of 99.5% isooctane and 0.5% stabilized THF with all flow rates at 1.0 ml/min.

Four different columns and 5 different solvent systems were used for the reversed-phase separations of carotenoids. These were as follows: (1) a 7-8 μ Zorbax ODS column (Phenomenex, Palos Verdes Dr., CA) 250 mm x 4.6 mm; solvent, acetonitrile-methanol-stabilized tetrahydrofuran 50/35/15; flow rate, 1 ml/min. (2) a 5 μ Vydac 201TP54 column (Anspec, Ann Arbor, MI) 250 mm x 4.6 mm; solvent, methanol-chloroform 90/10; flow rate, 1 ml/min. (3) a 5 μ Vydac 201TP54 column; solvent, methanol-stabilized tetrahydrofuran 90/10 ; flow rate 1 ml/min. (4) a 5 μ Vydac 210TP54 column; solvent, acetonitrile-methanol-stabilized tetrahydrofuran 40/52/8; flow rate, 1 ml/min. (5) a 5 μ Vydac 218TP54 column (Nest Group, Southboro, MA) 250 mm x 4.6 mm; solvent, acetonitrile-methanol-stabilized tetrahydrofuran 40/52/8; flow rate 1 ml/min. (6) a 4 μ

NovaPak C18 column (Waters Assoc., Milford, MA) 150 mm x 3.9 mm; solvent acetonitrile-methanol-stabilized tetrahydrofuran 58/35/7; flow rate, 1 ml/min.

Extraction and Analysis

All extractions were performed on 10 g of food. Each fruit or vegetable was weighed into a quart blender to which 20 g of anhydrous sodium sulfate, 1 g of magnesium carbonate and 100 ml of stabilized THF were added. Samples were blended at a moderate speed for 5 min followed by vacuum filtration through a Buchner funnel fitted with Whatman #42 filter paper. Most foods had to be extracted twice to remove all the carotenoids. The filtrate was brought to a final volume of 500 ml. A 100 ml aliquot was vacuumed evaporated to dryness at 40 C and taken to a final volume of 10 ml with stabilized tetrahydrofuran. If foods contained low levels of carotenoids like cabbage and celery, the entire filtrate was evaporated to dryness. Five µl of each sample and standards were injected into the HPLC using one of the separations described above. Peak height was employed for quantification since it was shown to be linear vs concentration.

This extraction procedure is very similar to the one developed by Bushway and Wilson (18) in 1982 with slight modifications. Therefore, recovery studies were not repeated.

Identification of the Carotenoids

Tentative identification was based on retention times of known standards. Further identification was made using UV-visible spectra and ratios at 470 nm vs 450 nm.

RESULTS AND DISCUSSION

Because of the carotenoid complexity of fruits and vegetables and the number of known carotenoids (approximately 500), it is doubtful that one HPLC method will be able to quantify all the major provitamin A compounds in every fruit and vegetable. For this reason, several carotenoid separations were developed. Of the numerous systems investigated, only 8 were observed to be beneficial in analyzing plant carotenoids. Two separations were normal phase while the others were reversed-phase.

The normal phase methods are shown in Figures 1 and 2. Figure 1 depicts results from an amino column with parts 1 and 2 representing a mix standard and irradiated β-carotene, respectively. The mix standard contained canthaxanthin, β-cryptoxanthin, α-carotene, β-carotene and γ-carotene, but as one might expect the polar carotenoids did not elute while α- and β-carotene co-eluted. As for the irradiated β-carotene, there were 6 peaks of which 3 (a, b, c) were unknowns while peak e was β-carotene and d and f have been tentatively identified from spectra as neo-β-carotene B and neo-β-carotene U, respectively. The other 2 chromatograms in this Figure are raw sweet potatoes (right) and baked sweet potatoes (left). A trace of neo-β-carotene B was observed in the raw tubers compared to a larger amount found in the cooked potatoes along with a trace of neo-β-carotene U. This was similar to what Sweeney and Marsh (2) reported in 1970 whereby yellow or red vegetables when cooked form more neo-β-carotene B.

Figure 2 shows the results of the other normal phase system (alumina). The mix standard (part 1) like Figure 1 only depicts 2

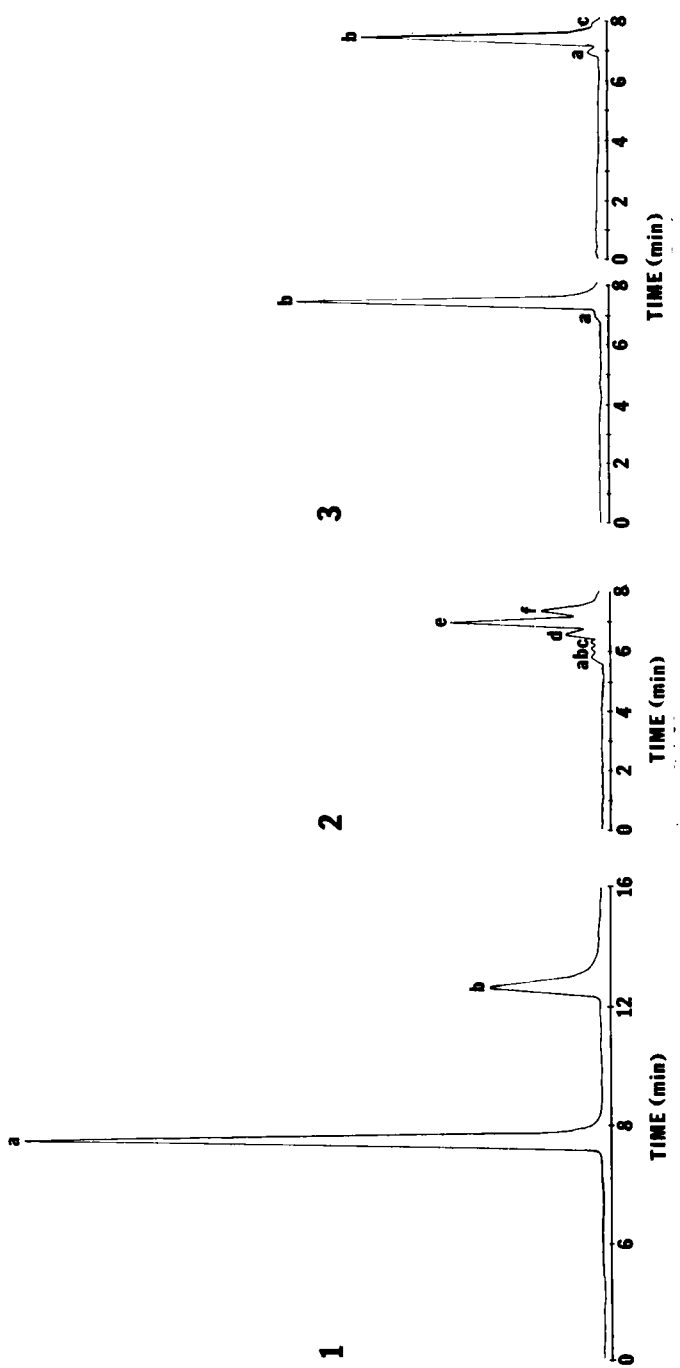

Figure 1. Separation of carotenoids on an IBM Amino column. Conditions are given in the text. Part 1: standard mixture; a, β-carotene and α-carotene; b, γ-carotene. Part 2: irradiated β-carotene; a, b, and c, unidentified β-carotene stereoisomers; d, neo-β-carotene B; e, β-carotene; f, neo-β-carotene U. Part 3: right, raw sweet potato; a, neo-β-carotene B; b, β-carotene and left, baked sweet potato; a, neo-β-carotene B; b, β-carotene; c, neo-β-carotene U.

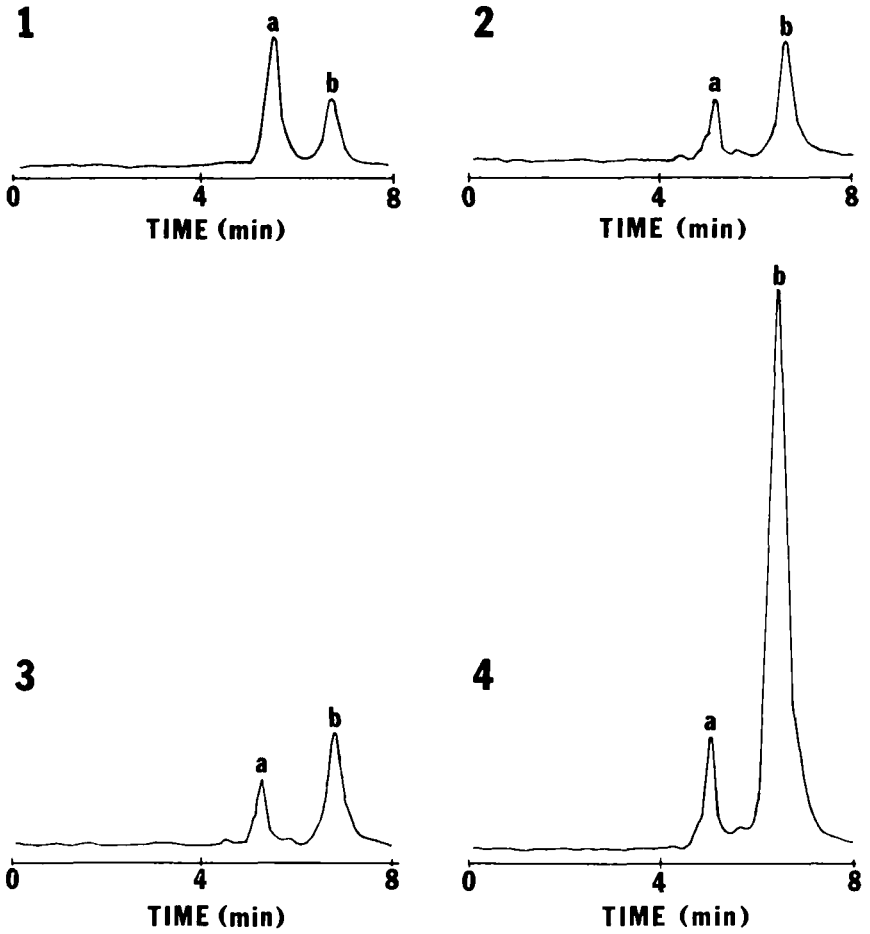

Figure 2. Separation of carotenoids on a Spherisorb Alumina column. Conditions are given in the text. Part 1: standard mixture; a, α-carotene; b, β-carotene. Part 2: irradiated β-carotene; a, mixture of β-carotene stereoisomers; b, β-carotene. Part 3: tomato paste; a, mixture of β-carotene stereoisomers; b, β-carotene. Part 4: raw kale; a, mixture of β-carotene stereoisomers; b, β-carotene.

chromatographic peaks (a and b) which represent α- and β-carotene. The other 3 carotenoids did not elute. Irradiated β-carotene is also different than above. All isomers elute before β-carotene with little resolution between stereoisomers. Parts 3 and 4 of Figure 2 are samples of tomato paste and raw kale. The β-carotene isomers are grouped into peak a and β-carotene is peak b.

Both normal phase systems offer a rapid means of analyzing β-carotene, the primary provitamin A compound in fruits and vegetables, without its stereoisomers interfering. However, the amino column separation is inadequate if α-carotene is present. Since high concentrations of α-carotene are not prevalent in most foods (exceptions some squashes, pumpkin, carrots and palm oil) this will not be much of a problem. Furthermore, if one wants to quantify or preparatively collect stereoisomers of β-carotene or γ-carotene, the amino method would be best. Even though α-carotene is separated from β-carotene with the alumina column, there will be some interference from the β-carotene isomers.

The reversed-phase carotenoid separations are shown in Figures 3-8. For these, there were 4 different columns and 5 different solvent systems employed. The best and quickest separations were observed with the Vydac columns (Figures 3-6). In this study 2 Vydac columns were employed (1) a Vydac 201TP54 column which was first used for carotenoid separations by Quackenbush and Smallidge (23) and (2) a Vydac 218TP54 column. Both columns have unique features.

As mentioned earlier the Vydac 201TP54 column was employed by Quackenbush and Smallidge (23). They developed 2 methods. The

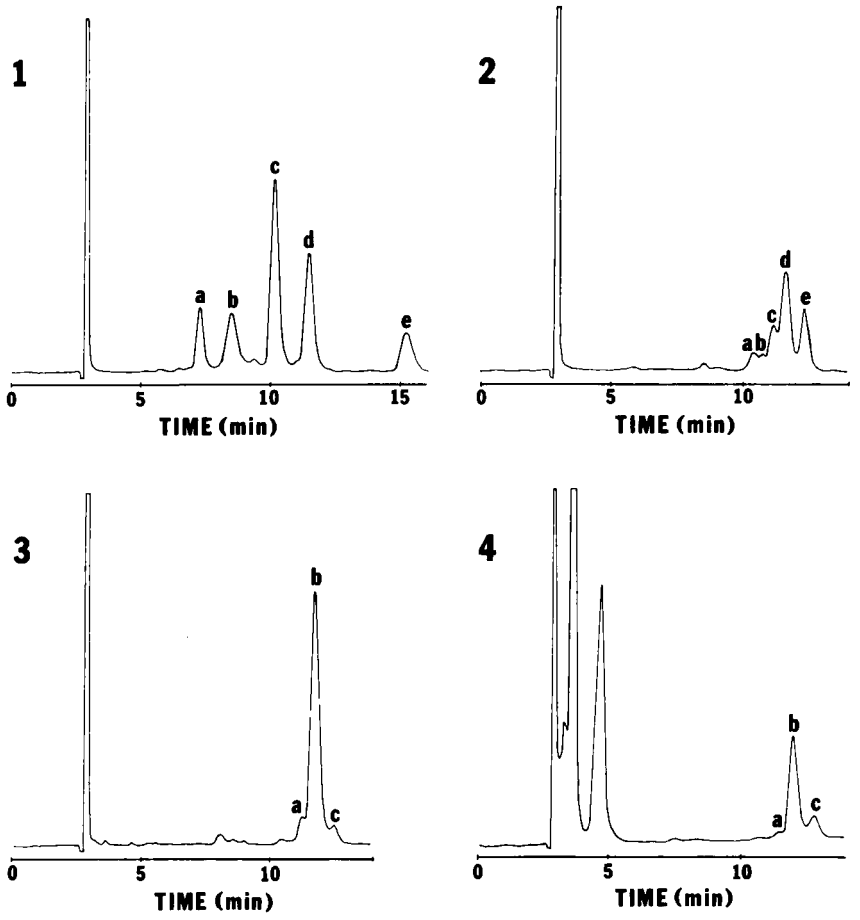

Figure 3. Separation of carotenoids on a Vydac 201TP54 column. Solvent methanol-chloroform 90/10. Other conditions are given in the text. Part 1: standard mixture; a, canthaxanthin; b, β-cryptoxanthin; c, α-carotene; d, β-carotene; e, γ-carotene. Part 2: irradiated β-carotene; a and b, unidentified β-carotene stereoisomers; c, neo-β-carotene B; d, β-carotene; e, neo-β-carotene U. Part 3: baby food sweet potatoes; a, neo-β-carotene B; b, β-carotene; c, neo-β-carotene U. Part 4: raw mustard greens; a, neo-β-carotene B; b, β-carotene; c, neo-β-carotene U.

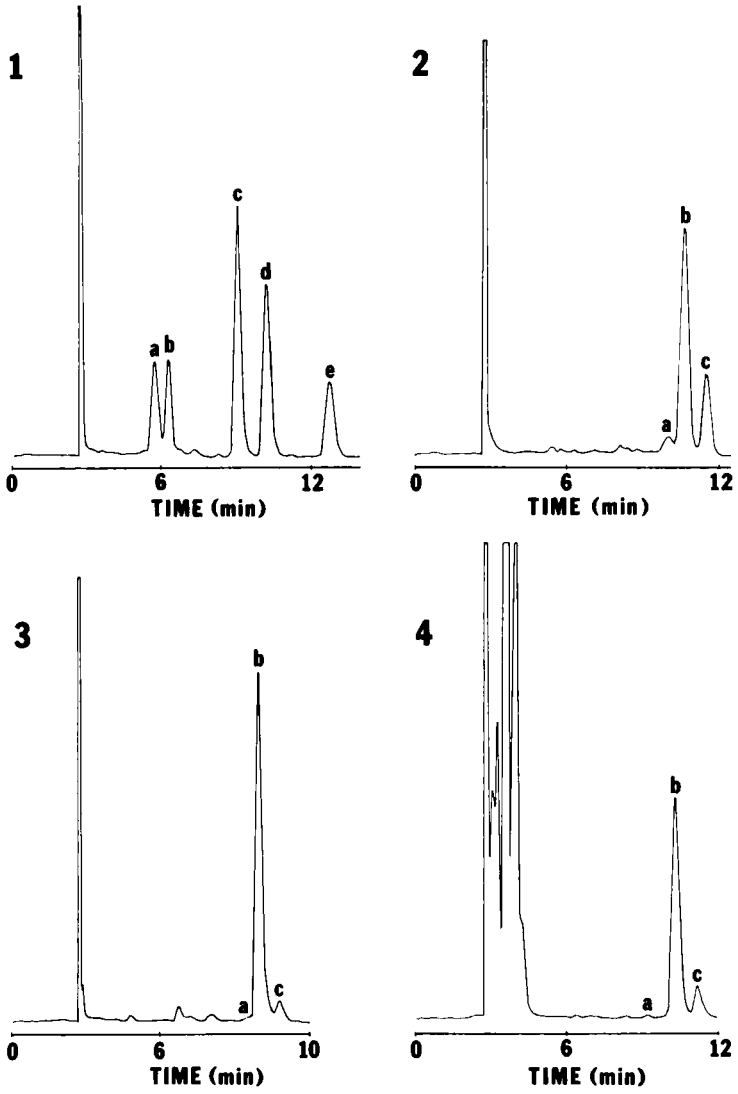

Figure 4. Separation of carotenoids on a Vydac 201TP54 column. Solvent methanol-tetrahydrofuran 90/10. Other conditions are given in the text. Part 1: standard mixture; a, canthaxanthin; b, β-cryptoxanthin; c, α-carotene; d, β-carotene; e, γ-carotene. Part 2: irradiated β-carotene; a, unidentified β-carotene stereoisomer ; b, β-carotene and neo-β-carotene B; c, neo-β-carotene U. Part 3 : baked sweet potato; a, unidentified β-carotene stereoisomer; b, β-carotene and neo-β-carotene B; c, neo-β-carotene U. Part 4: raw kale; a, α-carotene; b, β-carotene and neo-β-carotene B; c, neo-β-carotene U.

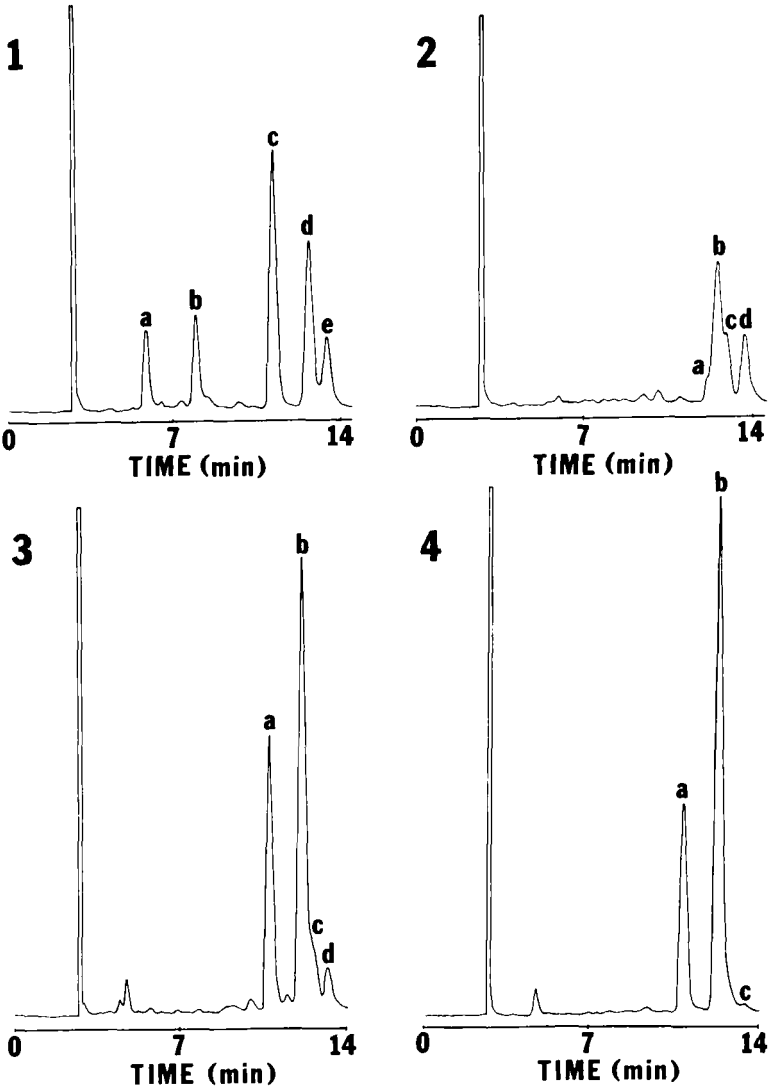

Figure 5. Separation of carotenoids on a Vydac 201TP54 column. Solvent acetonitrile-methanol-tetrahydrofuran 40/52/8. Other conditions are given in the text. Part 1: standard mixture; a, canthaxanthin; b, β-cryptoxanthin; c, α-carotene; d, β-carotene; e, γ-carotene. Part 2: irradiated β-carotene; a, unidentified β-carotene stereoisomer; b, β-carotene; c, neo-β-carotene B; d, neo-β-carotene U. Part 3: boiled carrots; a, α-carotene; b, β-carotene; c, neo-β-carotene B; d, neo-β-carotene U. Part 4: raw carrots; a, α-carotene; b, β-carotene; c, neo-β-carotene U.

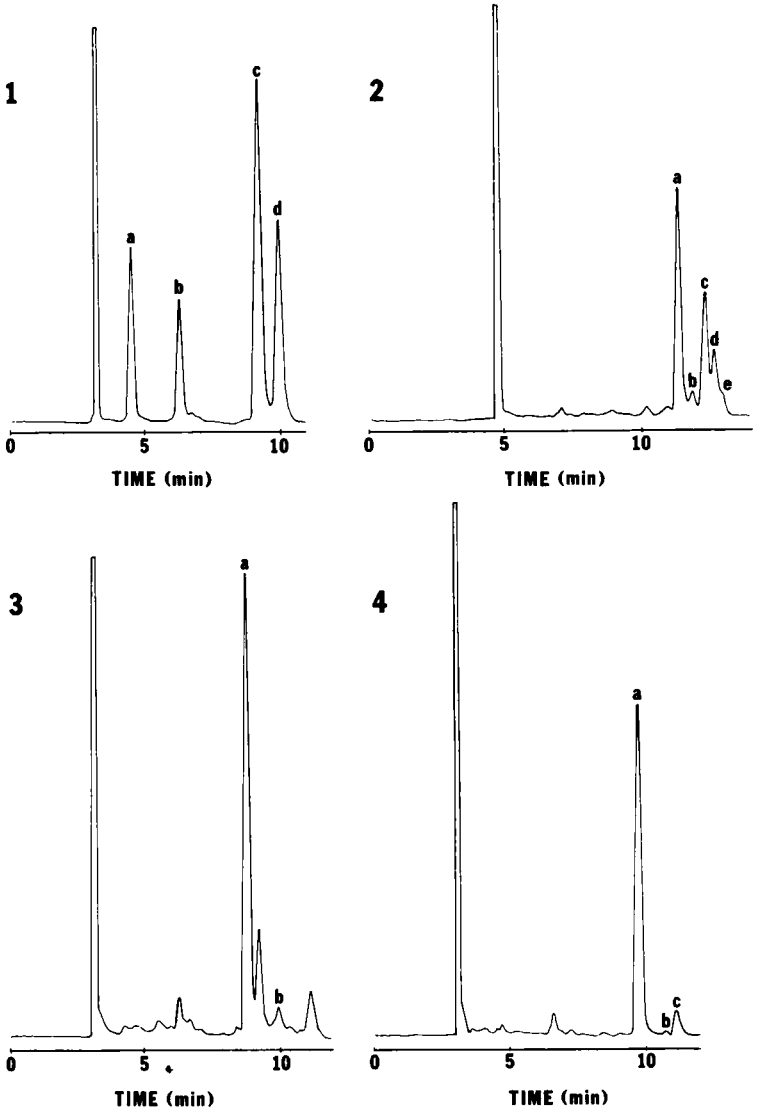

Figure 6. Separation of carotenoids on a Vydac 218TP54 column. Conditions are given in the text. Part 1: standard mixture; a, canthaxanthin; b, β-cryptoxanthin; c, α- and γ-carotene; d, β-carotene. Part 2: irradiated β-carotene; a, β-carotene; b, unidentified β-carotene stereoisomer; c, neo-β-carotene U; d, neo-β-carotene B; e, unidentified β-carotene stereoisomer. Part 3: tomato; a, lycopene; b, β-carotene. Part 4: baked sweet potato; a, β-carotene; b, neo-β-carotene U; c, neo-β-carotene B.

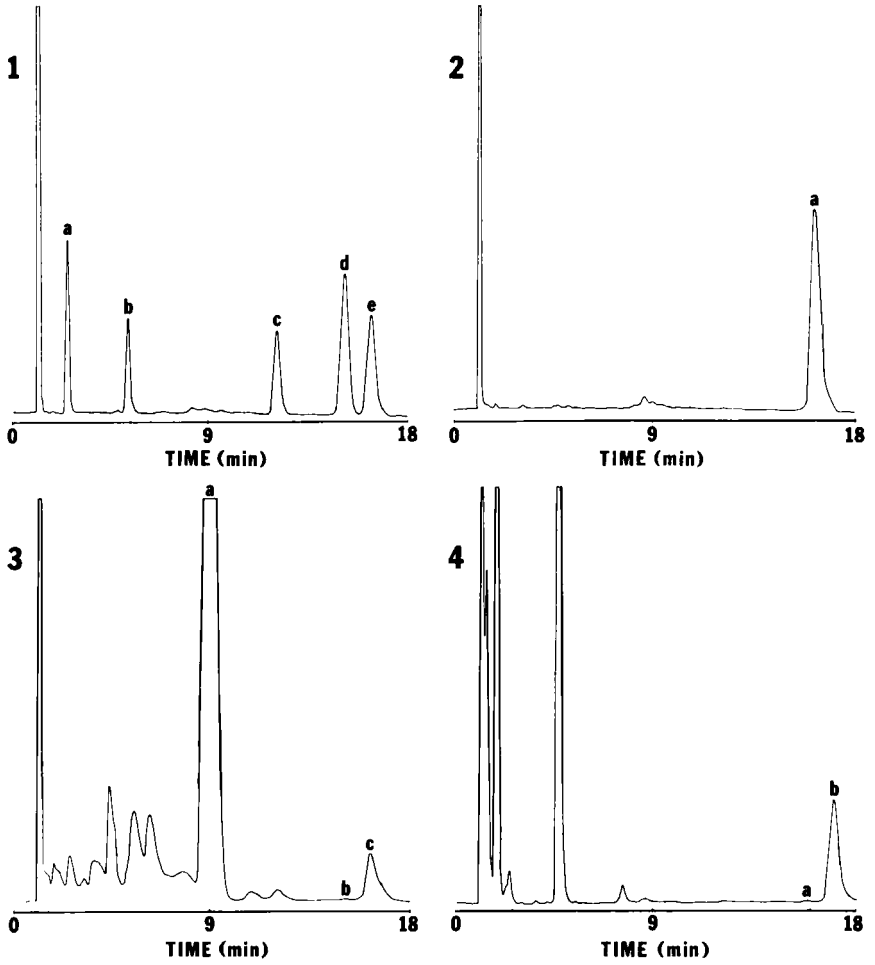

Figure 7. Separation of carotenoids on a NovaPak C18 column. Conditions are given in the text. Part 1: standard mixture; a, canthaxanthin; b, β-cryptoxanthin; c, γ-carotene; d, α-carotene; e, β-carotene. Part 2: irradiated β-carotene; a, mixture of β-carotene and its stereoisomers. Part 3: tomato paste; a, lycopene; b, α-carotene; c, β-carotene and its stereoisomers. Part 4: raw kale ; a, α-carotene; b, β-carotene and its stereoisomers.

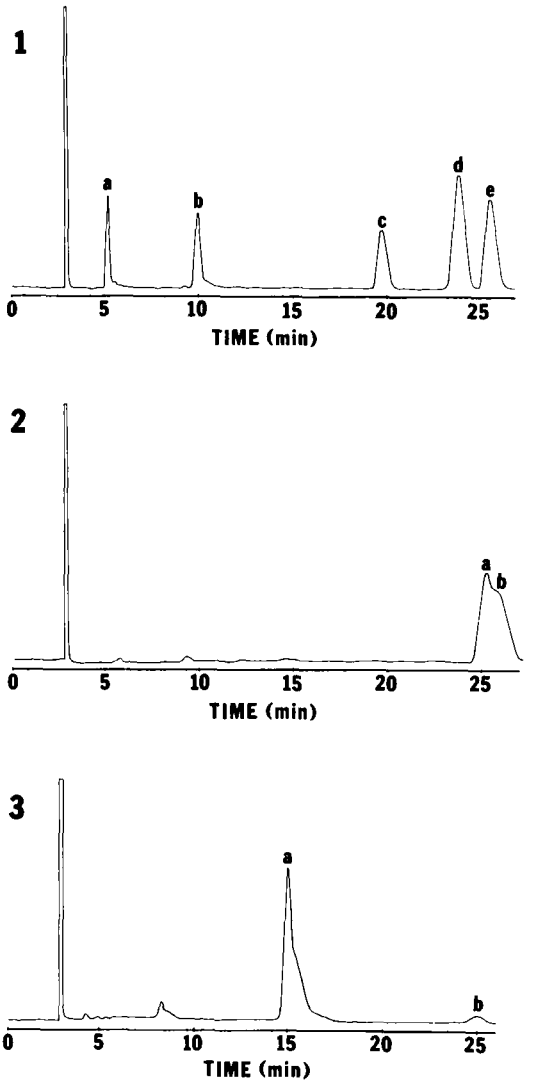

Figure 8. Separation of carotenoids on a Zorbax ODS column. Conditions are given in the text. Part 1: standard mixture; a, canthaxanthin; b, β-cryptoxanthin; c, γ-carotene; d, α-carotene; e, β-carotene. Part 2: irradiated β-carotene; a, β-carotene; b, stereoisomers of β-carotene. Part 3: tomato; a, lycopene; b, β-carotene.

first was an isocratic procedure using methanol-chloroform 90/10 and the second a gradient separation employing methanol-acetonitrile-chloroform. For the isocratic procedure, they reported the separation of 1 β-carotene isomer while 2 stereoisomers were resolved with the gradient system. A reexamination of the isocratic method of Quackenbush and Smallidge (23) was carried out here (Figure 3). The carotenoids in the mix standard were completely separated within 15 min (Figure 3 part 1) and it was observed that 4 β-carotene stereoisomers were partially resolved including the 2 major ones- neo-β-carotene B and neo-β-carotene U. Parts 3 and 4 of Figure 3 depicts separations of carotenoids in baby food sweet potatoes (part 3) and raw mustard greens (part 4). β-Carotene is partially resolved from neo-β-carotene B and neo-β-carotene U. Lycopene with this system will elute approximately 22 min.

Two other solvent systems were developed for the Vydac 201TP 54 column. The first was methanol-stabilized tetrahydrofuran 90/10 (Figure 4). This system shortened the analysis time considerably especially when lycopene was present. Also the lycopene peak is resolved into 3 components with this solvent mixture whereas just 2 components are separated using the above system. Figure 4 part 1 shows the mix carotenoid separation. All compounds were baseline resolved in 12.5 min. However, as can be seen in Figure 4 part 2 not all the major β-carotene stereoisomers are separated. Neo-β-carotene B co-elutes with β-carotene while neo-β-carotene U is separated along with one unidentified isomer. Examples of 2 foods chromatographed under these conditions are shown in part 3 (baked

sweet potatoes) and part 4 (raw kale). For samples containing neo-β-carotene B, there will be a high bias in the β-carotene value. This would especially be true of the red and yellow vegetables.

Figure 5 represents the last separation developed for the Vydac 201TP54 column. The carotenoid standards were all separated, but β- and γ-carotene were not completely resolved (Figure 5 part 1). The major β-carotene stereoisomers were partially resolved, but if γ-carotene were present then there would be interference. Carrots, raw and boiled, were choosen as food examples (Figure 5 parts 3 and 4). Separations were good except for the neo-β-carotene B present in cooked carrots. Unlike the acetonitrile system developed by Quackenbush and Smallidge (23), lycopene is separated from β-carotene (total time 18 min). Although lycopene has no provitamin A activity, it can cause interference especially with β-carotene.

The other Vydac column 218TP54 proved to be unique (Figure 6). Separation of the mix standard is shown in part 1 Figure 6. As can be seen, not all 5 compounds are separated. α-Carotene and γ-carotene co-elute. A problem would arise in foods containing high concentrations of both. This is not common. The main advantages of this procedure are its short analysis time (Figure 6 part 1) and the resolution of the major and minor β-carotene stereoisomers from the other carotenoids (Figure 6 part 2). Furthermore, each isomer is sufficiently resolved to be quantified using peak height. Parts 3 and 4 of Figure 6 show chromatograms of tomatoes and baked sweet potatoes, respectively. An interesting observation is that

lycopene will elute before β-carotene without interfering with it. If α-carotene and lycopene are present in detectable quantities then there will be an interference between them. However, not many foods would contain substantial quantities of both.

The last 2 reversed-phase separations are shown in Figures 7 and 8. Both procedures can resolve the 5 carotenoids in the mix standard (part 1 Figures 7 and 8), but neither can separate the stereoisomers of β-carotene (part 2 Figures 7 and 8) to the extend of the other methods. However the isomers are separated sufficiently (only a tailing peak with the NovaPak C18 system) so that when peak heights are used to quantify β-carotene only a slight high bias is obtained in most foods.

Recently another Zorbax system was developed for carotenoid separations by Nells and DeLeenheir (21) which is being used by many scientists for foods. A comparison was made between their system and the one developed here (Figure 8). It was demonstrated that the system of Nells and DeLeenheir (21) was longer and the β-carotene stereoisomers co-eluted.

In summary, several methods have been developed for the separation of carotenoids in fruits and vegetables. Before one can choose the best method for a particular application, one must select the food(s) that is to be analyzed and the carotenoids to be quantified. With this information, a system can be tried. However, before data is collected, spectra and ratios should be taken of each carotenoid peak of interest to confirm its purity.

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