Journal of Chromatography, 589 (1992) 171–176
Elsevier Science Publishers B.V., Amsterdam

CHROM. 23 735

Optimization of an isocratic high-performance liquid chromatographic separation of carotenoids

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(First received June 28th, 1991; revised manuscript received September 10th, 1991)

ABSTRACT

Using a polymeric C_{18} high-performance liquid chromatographic (HPLC) column, which demonstrated excellent separation selectivity toward carotenoid compounds in an earlier column evaluation, the effects of mobile phase modifier, modifier concentration, and column temperature were investigated. A seven-component carotenoid mixture was used to monitor changes in separation selectivity in response to variations in HPLC conditions. Both acetonitrile and tetrahydrofuran (THF) improved the resolution of echinenone and α-carotene; THF was selected for use as a modifier due to its solvating properties. At concentrations greater than 6% THF, the resolution of lutein and zeaxanthin deteriorated significantly. Temperature was varied from 15 to 35°C in 5°C increments. Resolution of lutein/zeaxanthin and β-cartone/lycopene were better at lower temperatures while echinenone/α-carotene separation improved as temperature increased. An acceptable separation of all seven carotenoids was achieved at 20°C using 5% THF as a mobile phase modifier. Method applicability is demonstrated for serum and food carotenoids.

INTRODUCTION

During the past decade carotenoid pigments have become highly publicized compounds due to their potential roles in cancer prevention [1–4], as antioxidants [5], and in the reduction of atherogenesis [6]. As a result, several publications have reported high-performance liquid chromatographic (HPLC) methods for the determination of carotenoids [7–12], but no publications have reported the influence of individual HPLC parameters on the separation of multiple carotenoids. Few laboratories have the time or the resources to evaluate a large sample of HPLC columns during analytical method development. Therefore, most "new" methods of separation are only slight adjustments in mobile phase composition. Recently, Epler *et al.* [13] evaluated 65 HPLC columns under standardized conditions with respect to carotenoid separation and recovery. In this study they observed, as others have reported [11,12] that polymeric C_{18} phases exhibited excellent selectivity for structurally similar carotenoids, whereas monomeric C_{18} phases, which represent the majority of the commercial C_{18} columns available, exhibited less selectivity for structurally similar carotenoids. Extensive investigations of the stationary phase characteristics responsible for differences in selectivity between monomeric and polymeric C_{18} phases for the separation of polycyclic aromatic hydrocarbon isomers have been reported by Sander and Wise [14]. Many of these same stationary phase characteristics influence the separation of carotenoids.

This report describes the optimization of an isocratic carotenoid HPLC separation using one HPLC column that exhibited very good separation

of the carotenoid mixture during the column evaluation study. The influence of multiple solvent modifiers and temperature on the separation of a carotenoid mixture was examined.

EXPERIMENTAL

Reagents

An ethanolic solution containing between 0.2 and 1.0 μg/ml of lutein [(3*R*,3′*R*,6′*R*)-β,ε-carotene-3,3′-diol, Kemin Industries, Des Moines, IA, USA], zeaxanthin[(3*R*,3′*R*)-β,β-carotene-3,3′-diol],β-cryptoxanthin [(3*R*)-β,β-caroten-3-ol, Atomergic Chemetals, Farmingdale, NY, USA], echinenone (β,β-caroten-4-one) (Hoffmann-La Roche, Nutley, NJ, USA), lycopene (ψ,ψ-carotene, extracted from tomato paste), α-carotene [(6′*R*)-β,ε-carotene], and β-carotene (β,β-carotene, Sigma, St. Louis, MO, USA) was prepared to monitor column separation selectivity. The following solvents were used in the mobile phase mixtures: acetone, acetonitrile, chloroform, diethyl ether, ethyl acetate, hexane, methanol, methylene chloride, tetrahydrofuran (THF) containing butylated hydroxytoluene (BHT) and toluene. All solvents were HPLC grade or equivalent and were used without further treatment.

HPLC

The HPLC system consisted of: a dual piston, quaternary low-pressure gradient solvent delivery system equipped with helium sparging and column temperature control; a high sensitivity, programmable, rapid scanning, UV–visible detector fitted with both deuterium and tungsten lamps and an 8-μl flow cell; and manual and automatic injection valves. Carotenoids were monitored at 445 nm and a computer-controlled data system was used to process data permitting review and manipulation of peak integration. The HPLC column contained 300 Å pore diameter, 5 μm particle size, silica polymerically modified with C_{18} (Vydac 201TP, 25 × 4.6 cm I.D. Separations Group, Hesperia, CA, USA). Other columns with similar chemistries could be substituted with minor modifications in mobile phase composition [13]. A 0.2 μm inline filter was placed between the pump and injection valve to trap particles resulting from piston seal wear. All stainless steel frits between the injection valve and the detector were replaced with biocompatible ceramic frits to minimize carotenoid precipitation and degradation [13,15]. A guard column containing 5-μm C_{18} material similar to the analytical column was directly attached to the analytical column inlet to remove particulate material and protect the column.

A methanol-based mobile phase was modified with 5 to 10% of the solvents listed under *Reagents* to determine the influence of each solvent on the separation of the carotenoid test mixture. After the modifier yielding the best selectivity was selected (THF), incremental portions were added to methanol to achieve the best separation of all seven carotenoids as determined by the shortest analysis time without compromising the resolution of all carotenoid peaks.

Column temperature was varied in 5°C increments between 15 and 35°C to investigate the influence of temperature on the separation of the carotenoid mixture. Temperature was regulated by the thermostatically controlled column oven on the HPLC system. The 15°C column temperature was achieved by placing a beaker of dry ice at the forced air inlet to reduce the chamber temperature to below ambient.

Carotenoid recovery

"Total recovery" was determined by flow injection analysis (FIA). To perform the FIA, the HPLC column was replaced by a 2000 mm × 0.8 mm I.D. PTFE tubing and the individual carotenoid solutions were introduced using a manual injector fitted with a 20-μl loop and recording the peak area at 445 nm. This process was repeated five times for each carotenoid. Then the HPLC column was replaced and each individual carotenoid solution was injected onto the column and "peak area" was recorded. Recovery of carotenoids from the HPLC column was determined by dividing the mean "peak area" by mean "total recovery" of the five injections of each carotenoid solution.

RESULTS AND DISCUSSION

The carotenoids included in the test mixture span a wide polarity range and represent β,β; β,ε; ψ,ψ; monoketo; monohydroxy; and dihydroxy carotenoids. These compounds account for approximately 90% of carotenoids in human blood and the

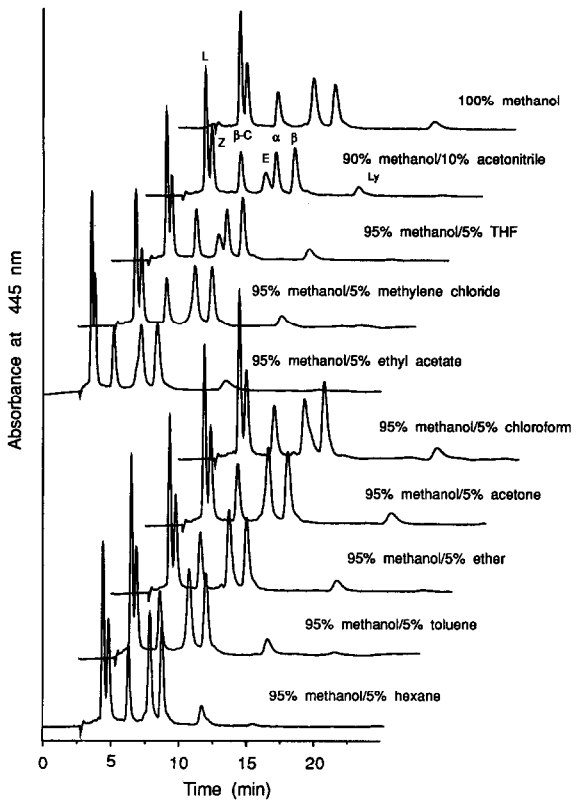

Fig. 1. The effect of nine solvent modifiers on the separation of seven carotenoids. Chromatographic conditions: Vydac 201TP C_{18}, 5 μm, 250 × 4.6 mm I.D. column; mobile phase as listed, 1.0 ml/min; UV–VIS at 445 nm; column temperature 25°C. Peaks: L = lutein; Z = zeaxanthin; β-C = β-cryptoxanthin; E = echinenone; α = α-carotene; β = β-carotene; Ly = lycopene.

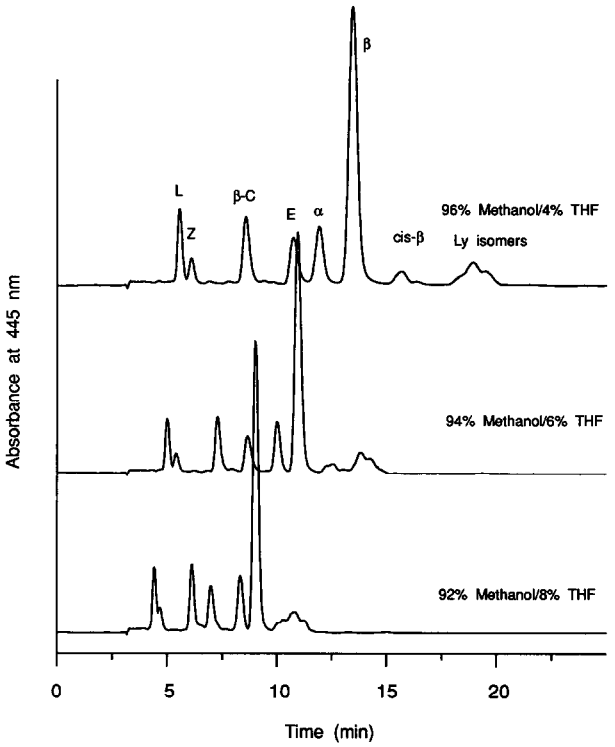

Fig. 2. The effect of three concentrations of THF on the separation of seven carotenoids. Chromatographic conditions as in Fig. 1. Peaks: *cis*-β = β-carotene *cis* isomers; Ly isomers = lycopene isomers; other peaks as in Fig. 1.

three major carotenoids in the US diet [7,16]. Therefore, an HPLC separation developed using this mix should find wide applicability to both food and serum carotenoid analyses.

A wide pore (300 Å), polymerically bonded C_{18} column with "biocompatible" frits was incorporated based on results of a previous column evaluation study in which polymerically modified C_{18} columns demonstrated greater resolution of the carotenoids in the ethanolic mixture [13]. Also for duplicate HPLC columns with different frit materials, recovery was improved in columns fitted with "biocompatible" materials (titanium and Hastelloy C) rather than stainless steel [13,15]. In the same study, total carotenoid recovery was found to be higher for nearly all columns when using methanol-based mobile phases rather than acetonitrile-based mobile phases. In the case of the polymeric C_{18} columns, the use of methanol actually resulted in improved carotenoid resolution. For this reason, our initial optimization began with a methanol-based mobile phase.

The influence of the mobile phase modifiers on the separation of the seven carotenoids in the test mix is illustrated in Fig. 1. With this particular column and 100% methanol as the mobile phase, echinenone coelutes with α-carotene, and lycopene is too far removed from β-carotene, substantially increasing analysis time. The only two modifiers that facilitated the separation of echinenone and α-carotene were acetonitrile and THF. Both solvents also selectively reduced the retention time of lycopene. The solubility of both the xantholphylls and carotenes is greater in THF than acetonitrile [17]. Therefore to improve carotenoid solubility, THF was selected as the modifier; although the resolution of the

lutein/zeaxanthin pair and echinenone/α-carotene pair was slightly decreased.

Mobile phases of methods previously developed using a similar C_{18} column have been modified with chloroform, THF, and a mixture of acetonitrile–THF [11,12,18]. Each of these reports illustrated the superior selectivity of the polymeric C_{18} column toward carotenoid compounds and especially geometric isomers, however, none of these reports addressed the separation of the carotenoid pairs discussed above.

The effect of varying the proportion of THF from 4 to 8% on the separation of the carotenoid mixture is illustrated in Fig. 2. As the THF concentration increases, resolution decreases between the pairs lutein/zeaxanthin and β-carotene/lycopene. At a column temperature of 25°C, 3 to 5% THF results in good separation of all the carotenoids in the mixture.

Previously, our laboratory has reported that column temperature alters column selectivity, specifically with respect to solute shape recognition [19,20]. Using 5% THF in methanol, column temperature was varied between 15 and 35°C to determine if the separation of structurally similar carotenoids could be improved (Fig. 3). At 15°C echinenone and lycopene are more strongly retained with respect to β-carotene ($\alpha_{E/\beta}$ 0.80 *vs* 0.67, and $\alpha_{ly/\beta}$ = 1.60 *vs.* 1.24, respectively, at 15 and 35°C). As a result, at 15°C, echinenone and α-carotene are not baseline resolved and lycopene elutes distant from the other carotenoids. Beginning at 25°C and

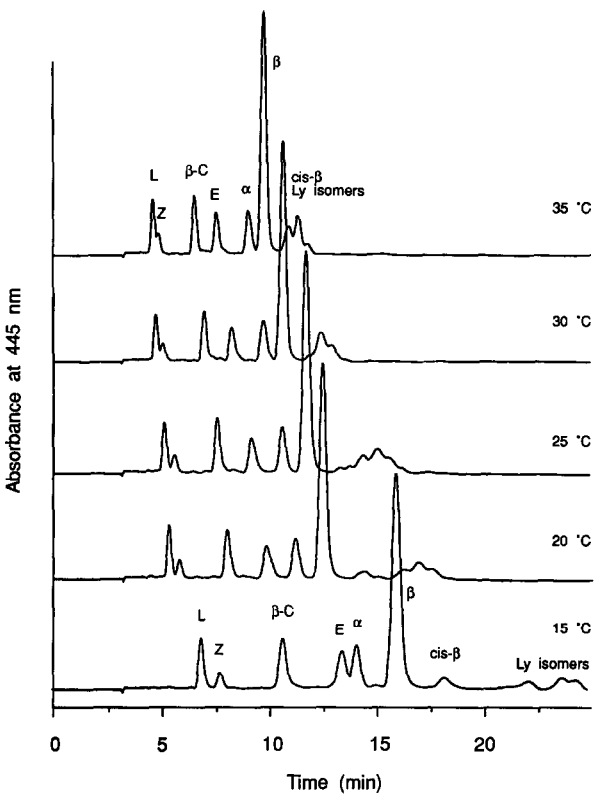

Fig. 3. The effect of five column temperatures on the separation of seven carotenoids. Chromatographic conditions: Vydac 201TP C_{18} column; 5% THF in methanol; 1.0 ml/min; UV/VIS at 445 nm; column temperature as listed. Peak identifications as in Fig. 2.

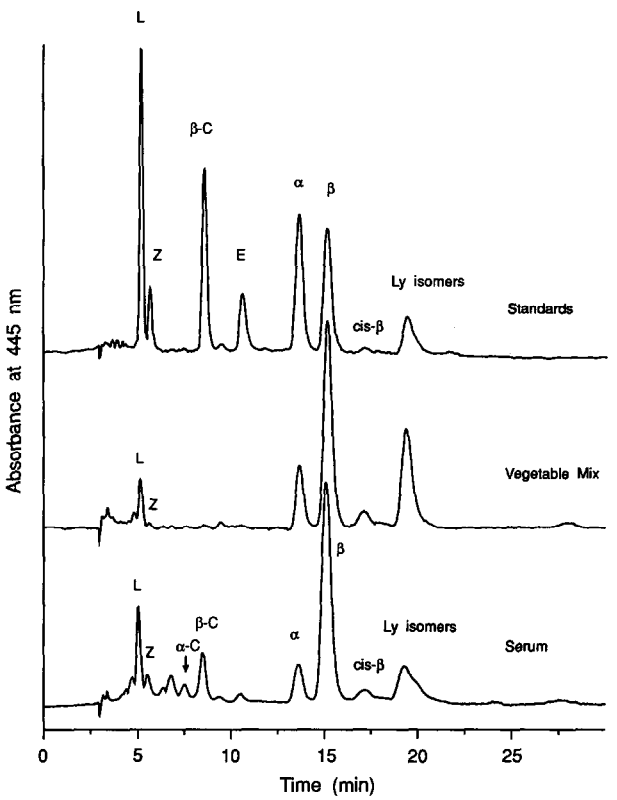

Fig. 4. The separation of serum and mixed diet carotenoids using the optimized HPLC method. Chromatographic conditions: Vydac 201TP C_{18} column; 5% THF in methanol; 1.0 ml/min; UV/VIS at 445 nm. Peaks: L = lutein; Z = zeaxanthin; α-C = α-cryptoxanthin; β-C = β-crytoxanthin; α = α-carotene; β = β-carotene; *cis-β* = β-carotene *cis* isomers; Ly isomers = lycopene isomers.

above, resolution decreases between the lutein/zeaxanthin pair and β-carotene/lycopene pair. A column temperature of 20°C was selected, because all carotenoids in the mixture are well resolved from each other, a baseline separation exists between the *trans* isomer of β-carotene and its geometric isomers, and there is partial resolution of the geometric isomers of lycopene. Lesellier *et al.* [21] recently described the separation of *trans/cis*-α- and β-carotenes and observed that temperature was a significant factor in optimizing the separation of these isomers. Using a narrow pore (80 Å) polymeric C_{18} column, they identified 21–22°C as the optimum temperature for the separation of *trans*-α- and β-carotene from their *cis* isomers. However, using the narrow pore column they were unable to resolve the *cis* isomers from each other. Previously, we have demonstrated the separation of individual geometric isomers of β-carotene using a wide pore polymeric C_{18} columns [22].

In our recent column evaluation study [13], we found that total carotenoid recovery from the HPLC column was influenced by the column packing, mobile phase, and frit material. The percent recovery and standard deviation of individual carotenoids measured by FIA using the optimized HPLC system described above are: lutein, 96 ± 6%; zeaxanthin, 107 ± 9%; β-cryptoxanthin, 94 ± 5%; echinenone, 92 ± 6%; α-carotene, 95 ± 3%; β-carotene, 96 ± 3%; and lycopene, 108 ± 6%. Recovery of each carotenoid was greater than 92% and total carotenoid recovery was 98 ± 6%. This demonstrates that the system not only resolves these major carotenoids but also elutes them quantitatively from the LC column.

The applicability of the method is demonstrated in Fig. 4 in which the separations of serum carotenoids and dietary carotenoids are illustrated. Most isocratic reversed-phase HPLC methods fail to resolve lutein and zeaxanthin when the capacity factor (k') for the hydrocarbon carotenoids is kept below 10 [7–9]. Previously, polymeric C_{18} phases have been reported to exhibit superior ability to recognize subtle differences in molecular structure [20,22,23]. In this application, the lutein/zeaxanthin pair, geometric isomers of β-carotene (*trans*/9-*cis*/13-*cis*), and geometric isomers of lycopene (*trans*/13-*cis*) are separated within 20 min. Although superior to monomeric C_{18} phases in many ways, polymeric C_{18} columns are not without problems. The total carbon load is lower in wide-pore polymeric phases (due to the lower surface areas of wide pore silicas) which results in weaker retention of the carotenoids and limits the amount and type of modifier used in the mobile phase. The large pore diameter and thick surface coating slows mass transfer resulting in slightly broader peaks. Lastly, column reproducibility from various production lots tends to be more variable than for monomeric C_{18} phases [13]. Because of these difficulties, duplication of the results illustrated in this manuscript with a different polymeric C_{18} column may require slight modifications in the mobile phase composition, but similar separations should be attainable.

In summary, the isocratic separation of carotenoids was optimized using a polymeric C_{18} column. Stainless steel frits were replaced with "biocompatible" frits to minimize oxidative degradation of the analytes and a methanol-based mobile phase was used to improve selectivity and recovery. Nine solvent modifiers were investigated to determine their effect on separation selectivity and THF was found to be the most beneficial modifier. Various combinations of THF composition and column temperature were assessed to achieve the best overall separation. The recovery of individual carotenoids and a mixture of seven carotenoids from the HPLC column was nearly 100%.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the helpful discussions with Lane C. Sander regarding stationary phase chemistry and the assistance of Katherine S. Epler with the initial column evaluations. The authors are grateful to Chris Nelson of Kemin Industries and Hemmige Bhagavan of Hoffmann-La Roche for their generous contributions of carotenoids.

This work is taken in part from a dissertation submitted by N.E.C. to the Graduate School of the University of Maryland in partial fulfillment of the requirements for the Ph.D. degree in Nutritional Sciences.

Certain commercial equipment, instruments, or materials are identified in this paper to specify adequately the experimental procedure. Such identification does not imply recommendation of endorse-

ment by the National Institute of Standards and Technology, nor does it imply that the materials or equipment identified are neccesarily the best available for the purpose.

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