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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND CAPILLARY SUPERCRITICAL-FLUID CHROMATOGRAPHY SEPARATION OF VEGETABLE CAROTENOIDS AND CAROTENOID ISOMERS

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SUMMARY

Carotenoids from carrots and tomatoes were separated with high-performance liquid chromatography (HPLC) and capillary supercritical fluid chromatography (SFC). All *trans* alpha- and beta-carotene were separated from their respective *cis*-isomers with capillary SFC. Carotenoids extracted from tomatoes included xanthophyll, lycopene and beta-carotene, while alpha- and beta-carotene were extracted from carrots. The HPLC separations were accomplished isocratically with a 25-cm column containing 5-μm ODS and methanol–acetonitrile–chloroform (47:47:6) or acetonitrile–dichloromethane (80:20). beta-Carotene *cis*-isomers were separated with SFC with a SB-cyanopropyl-25-polymethylsiloxane column, while alpha-carotene isomers were separated with two SB-cyanopropyl-50-polymethylsiloxane columns. Carotenoids from carrots and tomatoes were separated with a SB-phenyl-50-polymethylsiloxane column. Carbon dioxide with 1% ethanol was the SFC mobile phase. The eluent was monitored at 461 nm for HPLC and either 453 or 461 nm for SFC.

INTRODUCTION

Capillary column supercritical-fluid chromatography (SFC) has emerged as a very promising chromatographic technique that complements both high-performance liquid chromatography (HPLC) and gas–liquid chromatography (GLC). It is particularly effective for separations that are difficult due to limitations imposed by both HPLC and GLC[1]; *e.g.*, the thermal instability of the analyte, which would limit analysis on GLC and poor resolution due to limited theoretical plates, which can limit analysis on HPLC[2]. In addition, both selective (UV–VIS) and universal (flame

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ionization, FID) detectors can be used with capillary SFC. Supercritical carbon dioxide, with and without added modifiers, is an effective solvent for a wide variety of non-polar solutes. In addition to the normal column–solute interactions, the retention of an analyte on the column is dependent upon the density of the supercritical mobile phase[3], which can be easily altered to effect separations. The stationary phase composition and temperature, plus the solvent composition and density, provide a variety of parameters that can be programmed to separate a mixture of compounds. These conditions appear optimal for the separation of the thermally labile and difficult to separate carotenoids and their *cis-trans* isomers[4]. The high efficiency afforded by capillary columns is particularly important due to the large number of naturally occurring carotenoids (nearly 500, plus several isomers for many of them), most of which are closely related structurally[5].

Although SFC has been applied to a wide variety of compounds and mixtures in the past few years[6–11], there have been few reports on carotenoids and none on the separation of carotenoid *cis-trans* isomers[4,12,13]. Because of the relatively labile nature of the carotenoids, HPLC, rather than GLC, has been used extensively to separate and identify carotenoids[14–17] and their isomers[18,19] extracted from a variety of biological samples. The first application of SFC to carotenoids was in 1968 by Giddings *et al.*[12], who separated alpha- and beta-carotene. In 1987 Frew *et al.*[4] separated and identified several carotenoids with SFC in combination with mass spectrometry. Favati *et al.*[20] has utilized supercritical CO_2 for the extraction, but not the separation, of carotene and lutein from leaf protein concentrates. They illustrated the potential of supercritical CO_2 for selective carotenoid extraction under a variety of solvent densities.

The objectives of this work were three-fold: (1) to illustrate the carotenoid separation capabilities of SFC as compared to an isocratic HPLC system, (2) to illustrate the ability of SFC to separate closely related carotenoid geometric isomers and (3) to demonstrate the application of SFC to the analysis of carotenoids from biological systems.

EXPERIMENTAL

Standards and samples

Crystalline lycopene (L9875, from tomatoes), alpha-carotene (C0251, from carrots) and beta-carotene (C0126, from carrots) were obtained from Sigma (St. Louis, MO, U.S.A.). Carrots and tomatoes were purchased fresh locally. Carotenoid standards for SFC were dissolved in hexane (0.1%, w/v), while standards for HPLC were dissolved in chloroform at a concentration of 0.1% (w/v), and then diluted to a concentration range similar to that of the samples. Carotenoids were extracted from vegetables by blending 200 g (fresh weight) of vegetable sample in 200 ml of methanol for 5 min. The resulting puree was then filtered through a Büchner funnel with Whatman No. 1 filter-paper. The remaining vegetable tissue was extracted with 200 ml of hexane by stirring for 30 min under argon. The extraction mixture was coarsely filtered as above, and the hexane filtrate evaporated with a rotary evaporator to a volume of approximately 5 ml. The extract was stored under argon in a foil covered sample vial and stored in a freezer (−20°C) for analysis the following day.

cis-trans isomerization of the double bonds in the polyene chain for both alpha- and beta-carotene was catalyzed by iodine in hexane in daylight[21] with aliquots of the

0.1% (w/v) standard solutions. All standard and sample preparation was performed under yellow lighting (except when regular lighting was called for in the sample procedure). All solutions were filtered with a 0.2-μm filter prior to injection.

SFC apparatus

A Lee Scientific Model 501 capillary SFC (Salt Lake City, UT, U.S.A.) pump and oven were used with a Linear UV–VIS 204 detector (Reno, NV, U.S.A.). A Lee Scientific Model 501 extraction system was used, which utilizes cryofocusing to concentrate the analyte near the head of the column. The release of compressed gas through a sleeve around the cryofocusing unit results in rapid cooling to well below the supercritical point for CO_2. The analyte precipitates out of the extracting solvent (CO_2) at that point. A Model R40 refrigerated circulator (Precision Scientific Group, Chicago, IL, U.S.A.) was used to cool the pump, the Valco A90 injector (Houston, TX, U.S.A.), the extraction cell and the Linear UV/VIS detector. The Valco injection system contained a 200-nl internal loop operated in a time-split mode. The chromatographs were recorded on an Omniscribe Series B-5000 recorder (Houston Instruments, Austin, TX, U.S.A.) and a Hewlett-Packard (Avondale, PA, U.S.A.) Model 3390A integrator. The pump, oven, extraction cell and injection system was controlled by an ARC Turbo personal computer (American Research Corp., Monterey Park, CA, U.S.A.) with software written by Lee Scientific.

For the separation of the carotenoids extracted from vegetables, a SB-phenyl-50 column (10 m × 50 μm I.D., film thickness of 0.25 μm) was used with a cross-linked stationary phase containing 50% phenyl- and 50% polymethylsiloxane. For the separation of beta-carotene *cis-trans* isomers, a SB-cyanopropyl-25 column (7 m × 50 μm I.D., film thickness of 0.25 μm) was used with a cross-linked stationary phase consisting of 25% cyanopropyl and 75% polymethylsiloxane (Lee Scientific). For the separation of alpha-carotene *cis-trans* isomers, two SB-cyanopropyl-50 columns (10 m × 50 μm I.D., film thickness of 0.25 μm) were used with a cross-linked stationary phase consisting of 50% cyanopropyl- and 50% polymethylsiloxane (Lee Scientific). The mobile phase was SFC grade CO_2 containing 1% (v/v) ethanol (Scott Specialty Gases, Plumsteadville, PA, U.S.A.).

HPLC apparatus

The HPLC system consisted of a Tracor 950 chromatographic pump and 970A variable wavelength detector (Austin, TX, U.S.A.), a Rheodyne 7125 sample injector (Cotati, CA, U.S.A.), an Upchurch Uptight precolumn, packed with ODS C_{18} (Oak Harbor, WA, U.S.A.), a Supelco LC-18 (Bellefonte, PA, U.S.A.) stainless-steel column (25 cm × 4.6 mm I.D.) packed with 5-μm ODS and a Shimadzu (Kyoto, Japan) C-R3A Chromatopac integrator. The solvent system was comprised of either acetonitrile–dichloromethane (80:20)[22] or methanol–acetonitrile–chloroform (47:47:6)[23]. All solvents were filtered and degassed, and were of spectrograde quality.

SFC procedures

Alpha- and beta-carotene present in the carrot extract were separated at 50°C under isobaric conditions and a mobile phase density of 0.70 g/ml. The injection duration was 0.25 s. Carotenoids present in the tomato extract were separated at 45°C with an asymptotic density program with a 1/2 rise time constant of 30 min and

a termination time of 30 min. The initial density was 0.66 g/ml, while the convergence density was 0.72 g/ml. In effect, a mobile phase density of 0.69 g/ml was achieved after 30 min at which time the mobile phase density was held at 0.69 g/ml. Alpha- and beta-carotene *cis-trans* isomers were also separated at 50°C. The beta-carotene isomers were separated isobarically; the mobile phase density was 0.66 g/ml and the injection duration was 0.35 s. For the separation of alpha-carotene isomers the extraction cell was used as an injection system. A 1-μl volume of the isomerized alpha-carotene solution was loaded into the extraction cell. The extraction cell temperature was set at 50°C. After cryofocusing had been started and the mobile phase compressed to a density of 0.92 g/ml, a valve was adjusted to direct the supercritical carbon dioxide mobile phase through the extraction cell. During the extraction the oven was held at 45°C. After a 5-min extraction, the density was rapidly reduced to 0.52 g/ml. At that time a valve was adjusted that directed the mobile phase through the column, the cryofocusing was turned off and the temperature in the oven was increased rapidly to 50°C. A linear density ramp from 0.52 to 0.62 g/ml at 0.002 g/ml · min was run. Column eluents were monitored at 461 nm and the detector sensitivity was set at 0.02 a.u.f.s. for the vegetable and beta-carotene SFC analyses. However, for the alpha-carotene analysis the eluent was monitored at 453 nm at 0.06 a.u.f.s.

HPLC procedures

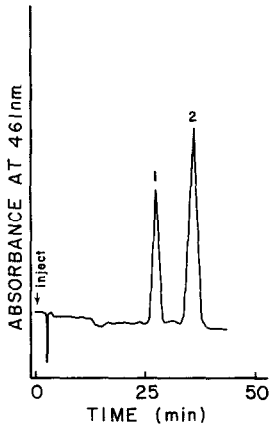
An isocratic system of methanol–acetonitrile–chloroform (47:47:6) at a flow-rate of 2.0 ml/min was used to separate alpha- and beta-carotene from carrots. An isocratic system of acetonitrile–dichloromethane (80:20) at a flow-rate of 2.0 ml/min was used to separate the carotenoids isolated from tomatoes. Column eluents were monitored at 461 nm. Lycopene, alpha-carotene and beta-carotene peaks were tentatively identified for both SFC and HPLC based on a comparison with the retention times of the standards.

RESULTS AND DISCUSSION

A comparison between the HPLC and SFC methods used to separate the vegetable carotenoids indicated improved separation with SFC. Fig. 1 shows the separation of alpha- and beta-carotene from the carrot extract achieved with SFC using the SB-phenyl column, which was clearly superior to the isocratic HPLC separation shown in Fig. 2. Fig. 3 demonstrates separation of carotenoids in the tomato extract by SFC with an asymptotic density program for the mobile phase. As with the carrot extract, the SFC separation was superior to the tomato extract separations using isocratic HPLC (Fig. 4).

In 1985, Bushway[14] investigated several isocratic HPLC methods for the separation of carotenoids from fruits and vegetables, with limited success in some cases. Ruddat and Will[15] and Wills *et al.*[17] found that gradient HPLC conditions, relative to isocratic conditions, improved the separation of complex mixtures of carotenoids and were better suited for the analysis of carotenoids from fruits and vegetables. As with gradient HPLC, SFC possesses the potential for efficiently separating complex carotenoid mixtures, and as method development continues improved separations are expected.

Figs. 5 and 6 show the separation of isomeric mixtures of alpha- and



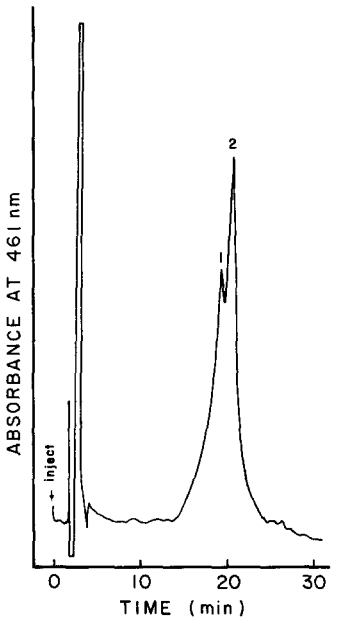
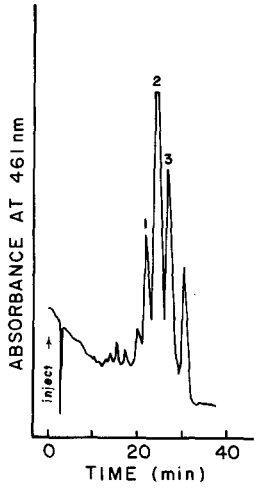


Fig. 1. SFC separation of alpha- (1) and beta-carotene (2) from carrot extract. Chromatographic conditions as described in text.

Fig. 2. HPLC separation of alpha- (1) and beta-carotene (2) from carrot extract. The mobile phase consisted of methanol–acetonitrile–chloroform (47:47:6).



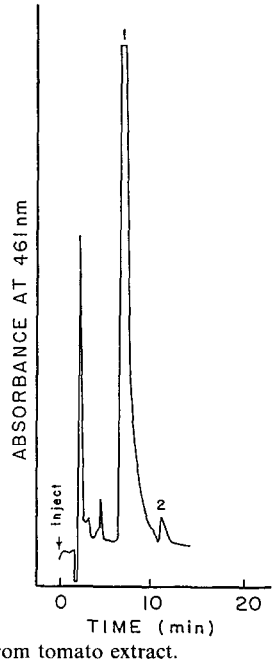


Fig. 3. SFC separation of lycopene (2), alpha- (1) and beta-carotene (3) from tomato extract.

Fig. 4. HPLC separation of lycopene (1) and beta-carotene (2) from tomato extract. The mobile phase consisted of acetonitrile–dichloroethane (80:20).

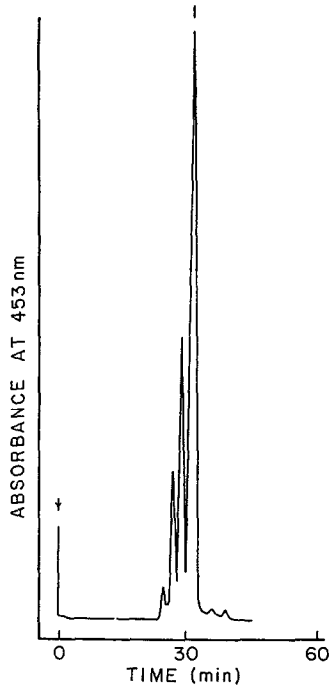


Fig. 5. SFC separation of all-*trans* alpha-carotene (1) and various *cis* isomers.

beta-carotene with SFC on cyanopropyl polymethylsiloxane column(s). The separations achieved are comparable to those obtained on HPLC for both alpha-carotene[18,19] and beta- carotene[14,18,19] isomers. Figs. 5 and 6 are the first examples of the separation of *cis-trans* carotenoid isomers with capillary SFC.

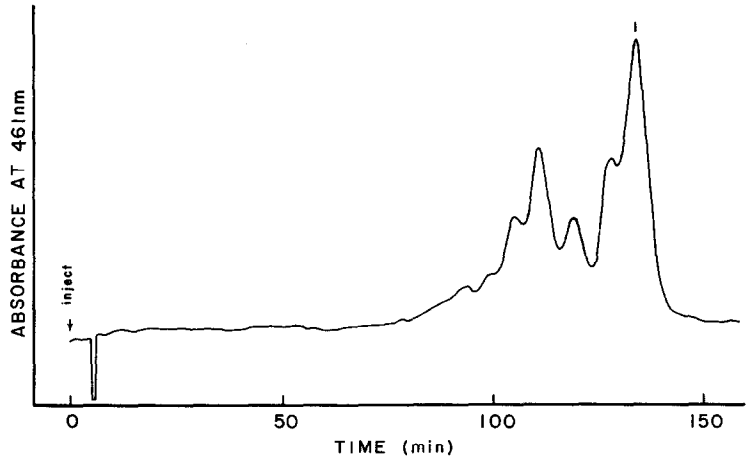


Fig. 6. SFC separation of all-*trans* beta-carotene (1) and various *cis* isomers.

Separation of *cis-trans* isomers of linoleic acid (*cis*-9,12 and *trans*-9,12) has been done with capillary SFC (12 m × 50 μm I.D., 50% cyanopropyl column)[11]. In addition, positional as well as stereoisomers, have been separated with capillary SFC[8–10]. Chang *et al.*[8] separated several series of both positional and stereoisomers with capillary SFC on a new liquid crystalline polysiloxane stationary phase. However, separation of *cis-trans* geometric isomers was not reported.

Frew *et al.*[4] suggested that the cyanopropyl stationary phase may be the most promising for carotenoid separations with SFC. The cyanopropyl column appeared to be the best of the SFC stationary phases available from Lee Scientific for the separation of carotenoids. However, due to the severe tailing of lycopene on the cyanopropyl columns, other stationary phases were examined. Consequently, a SB-phenyl-50 column was employed for the separation of samples containing lycopene, which provided separation without tailing of the lycopene in the mixtures examined.

Both pure supercritical CO_2 and supercritical CO_2 with 1% (v/v) ethanol were evaluated for their effect on the separations. Since no appreciable difference in separation efficiency was observed in the absence of the ethanol modifier with the capillary SFC columns examined, the results were not reported. During the last few years, many investigators have examined the effects of modifiers on SFC separations[24–26]. The addition of polar organic solvents as modifiers can cause significant changes in retention characteristics and peak shapes of analytes on packed column systems. Schmidt *et al.*[27] found that CO_2 with isopropanol (8%, v/v) enhanced the separation and peak shape of a series of relatively polar solutes on packed column SFC. While the addition of ethanol to the CO_2 for the separation of carotenoids in a capillary column devoid of exposed silanol or siloxane sites was not needed, a modifier may be required with packed columns or to enhance extraction from biological samples.

At the present time, the level of detection for compounds of interest in a biological matrix when using capillary SFC can often be restricted by the small amount of sample that can be injected on to the column (200–1000 nl). This problem can be largely overcome with the incorporation of an on-line extraction system, although the use of an extraction system just for injection complicates the process of injection. On-line supercritical-fluid extraction and chromatography is an interesting technique which has substantially expanded current chromatographic separation and quantitation capabilities. These techniques are currently under investigation.

CONCLUSIONS

This research illustrated some of the capabilities of SFC for carotenoid analysis. Capillary SFC has demonstrated significant potential for improvement of the separation of carotenoids and their *cis-trans* isomers relative to HPLC. A review of the literature indicates that capillary SFC is able to effect carotenoid separations that are comparable to the best current HPLC separations in other laboratories. SFC will never completely replace GLC or HPLC, but it will be an invaluable addition to the set of chromatographic tools used for separating and quantitating mixtures of compounds, particularly those compounds that are thermally sensitive, difficult to separate or exhibit problems with current extraction techniques.

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REFERENCES

1 D. W. Later, B. E. Richter and M. R. Andersen, *LC · GC*, 4 (1986) 992.
2 C. M. White and R. K. Houck, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 9(1986) 3.
3 J. C. Fjeldsted and M. L. Lee, *Anal. Chem.*, 56 (1984) 619A.
4 N. M. Frew, C. G. Johnson and R. H. Bromund, *ACS Symp. Ser.*, 366 (1987) 208.
5 J. A. Olson, in L. J. Machlin (Editor), *Handbook of Vitamins*, Marcel Dekker, New York, 1984, Ch. 1, p. 4.
6 D. W. Later, B. E. Richter, D. E. Knowles and M. R. Andersen, *J. Chromatogr. Sci.*, 24 (1986) 249.
7 D. W. Later, B. E. Richter, W. D. Felix, M. R. Andersen and D. E. Knowles, *Am. Lab. (Fairfield, Conn.)*, 18 (Aug. 1986) 108.
8 H.-C. K. Chang, K. E. Markides, J. S. Bradshaw and M. L. Lee, *J. Chromatogr. Sci.*, 26 (1988) 280.
9 H.-C. K. Chang and M. L. Lee, *J. Chromatogr. Sci.*, 26 (1988) 298.
10 L. T. Taylor, *J. Chromatogr. Sci.*, 26 (1988) 45.
11 Lee Scientific, Inc., *J. Chromatogr. Sci.*, 25 (1987) 179.
12 J. C. Giddings, L. McLaren and M. N. Meyers, *Science (Washington, D.C.)*, 159 (1968) 197.
13 D. R. Gere, *Hewlett-Packard Application Note*, 800-5, Hewlett-Packard, Avondale, PA, 1983.
14 R. J. Bushway, *J. Liq. Chromatogr.*, 8 (1985) 1527.
15 M. Ruddat and O. H. Will (III), *Methods Enzymol.*, 111 (1985) 189.
16 J. F. Fisher and R. L. Rouseff, *J. Agric. Food Chem.*, 34 (1986) 985.
17 R. B. H. Wills, H. Nurdin and M. Wootton, *J. Micronutr. Anal.*, 4 (1988) 87.
18 L. A. Chandler and S. J. Schwartz, *J. Food Sci.*, 52 (1987) 669.
19 F. W. Quackenbush, *J. Liq. Chromatogr.*, 10 (1987) 643.
20 F. Favati, J. W. King, J. P. Friedrich and K. Eskins, *J. Food Sci.*, 53 (1988) 1532.
21 L. Zechmeister, in *Cis-trans Isomeric Carotenoids, Vitamins A and Arylpolyenes*, Academic Press, New York, 1962, p. 51.
22 H. C. Furr, D. A. Copper and J. A. Olson, *J. Chromatogr.*, 378 (1986) 45.
23 C. R. Broich, L. E. Gerber and J. W. Erdman, Jr., *Lipids*, 18 (1983) 253.
24 J. R. Strubinger and M. I. Selim, *J. Chromatogr. Sci.*, 26 (1988) 579.
25 A. L. Billie and T. Griebrokk, *Anal. Chem.*, 57 (1985) 2239.
26 J. M. Levy and W. M. Ritchey, *J. Chromatogr. Sci.*, 24 (1986) 242.
27 S. Schmidt, L. G. Blomberg and E. R. Campbell, *Chromatographia*, 25 (1988) 775.