CHROM. 5161

CONTINUOUS FLOW SEPARATION OF CAROTENOIDS BY LIQUID CHROMATOGRAPHY*

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(Received November 12th, 1970)

SUMMARY

A liquid chromatographic system has been developed that gives good separation of complex mixtures of carotenoids. Carotenes are separated on magnesium oxide and xanthophylls on zinc carbonate. The columns are regenerated after each sample which virtually eliminates repacking. Submicrogram quantities of carotenoids are readily detected and many *cis–trans* isomers are separated. An antioxidant is included to reduce on-column losses and isomerization of carotenoids. The method is quantitative, reproducible, sensitive, moderately rapid, and suitable for routine analysis. Use of the system for the study of isomer formation of carotenoids and for the identification of citrus peel pigments is included.

INTRODUCTION

The technique of chromatography was developed in 1906 for the purpose of separating plant pigments. TSWETT, a Russian botanist, used columns of calcium carbonate and other materials for this purpose[1]. Later, KUHN AND LEDERER[2], STRAIN[3], CURL[4], and others developed improved procedures for separating carotenoids. More recently, thin-layer[5] and paper[6] chromatography have been used for rapid determinations.

DAVIES[7] and MONEGER[8] described continuous flow techniques for separating carotenoids. In our laboratory, however, we were unable to satisfactorily separate complex mixtures of citrus carotenoids with their techniques.

The purpose of this paper is to describe a liquid chromatographic system that is suitable for separation of carotenoid mixtures such as found in citrus peel. Standard adsorbents are used and the liquid chromatograph is assembled of materials and equipment readily available in many chromatographic laboratories. Careful attention to selection of adsorbents, methods of packing the column, selection of solvent systems, and inclusion of an antioxidant resulted in excellent separations of carotenoids and their isomers.

* Florida Agricultural Experiment Stations Journal Series No. 3796.

MATERIALS AND METHODS

Gradient systems

Several gradient devices were tried and found to be satisfactory. A simple linear gradient obtained by connecting two 125-ml erlenmeyer flasks together at their bottoms, using one as a reservoir and the other as a mixing chamber, gave good separations. A similar device using chambers of unequal size was also satisfactory (Kontes Glass Co., Vineland, N. J.). The reservoir and mixing chamber had diameters of 5.5 and 8.0 cm, respectively.

The most versatile gradient device was a nine-chamber assembly similar to the Technicon Chromatography Co. Autograd or the Phoenix Co. Varigrad, but constructed of material resistant to organic solvents. Ours was patterned after the Contigrad (Metaloglass, Inc., Boston, Mass.) but was machined from a block of aluminum 15 × 20 × 10 cm high. It was made with nine interconnected chambers each holding 90 ml of solvent. The gradient was mixed in each chamber by a metal paddle. In most separations, only the first three chambers were used. The added flexibility of this system allowed the production of nonlinear gradients tailored to the particular separation desired.

Pumps

A Milton Roy Chromatographic Mini pump, Model 196-31 (Milton Roy Co., St. Petersburg, Fla.), was used for most of these studies. This is a reciprocating plunger pump which uses either sapphire or Kennemetal plungers with various ring seals. Even with the recommended Rulon Tec ring seal, leakage around the plunger due to early failure of the seal was a problem under our operating conditions. The chromatograms shown in this paper were obtained with the sapphire plunger and solid teflon ring seal made in our machine shop. More recently we have been using a Milroyal D pump, Model HDB-1-30 R. This pump has adjustable graphite-impregnated teflon packing and sapphire ball checks and has worked well at 1800 p.s.i.

A Nester/Faust hi-pressure pump (Nester/Faust Mfg. Corp., Newark, Del.) was also tested. This pump uses digitally driven motors to drive pistons into stainless steel cylinders containing the solvents. By connecting two pumps in series and using one as the mixing chamber, a variety of gradients can be generated by regulating initial volumes and the relative speed of the two pumps. Although these pumps are rated at 2000 p.s.i., we have not yet achieved satisfactory operation without leaks. However, limited tests indicate the versatility of these pumps for generating various gradients and providing for more rapid separations of the carotenoids.

With both kinds of pumps, connections were made to a pressure gauge, and the column, using stainless steel tubing and Swagelok fittings. The pressure gauge (Frank W. Murphy Mfg., Inc., Tulsa, Okla., Model OPL-F-2000) was mounted on a diaphragm-seal transmitter and had both minimum and maximum pressure safety cut-off switches. A simple low-voltage latching circuit stopped the pump if either a high or low pressure setting was exceeded.

Columns

It has been traditional to use glass columns in the separation of plant pigments so that the various color bands could be observed as they were eluted. We found glass

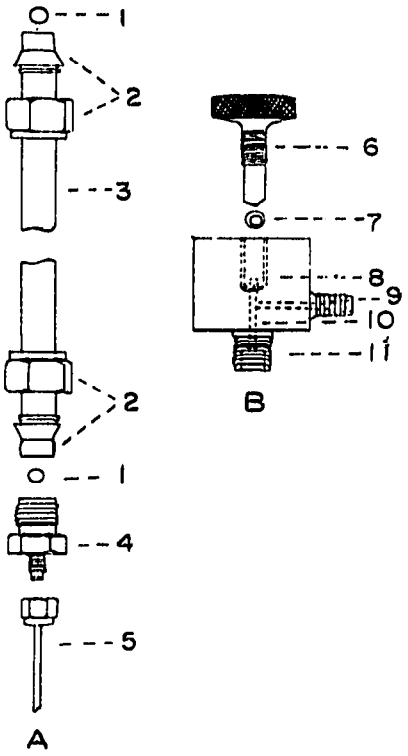

Fig. 1. (A) Chromatographic column (not drawn to scale). 1 = porous polyethylene disc; 2 = pressure fittings $^1/_4$ in.; 3 = stainless steel tubing $^1/_4$ in. O.D.; 4 = reducer $^1/_4$ in. to $^1/_{16}$ in.; 5 = polyethylene tubing 0.030 in. I.D. (B) Sample injection valve. 6 = thumb tightening screw $^1/_4$ in.; 7 = "O" ring; 8 = overflow spout $^1/_{16}$ in., open above "O" ring seat; 9 = inlet, threaded for pressure fitting; 10 = opening to column $^1/_{16}$ in.; 11 = column connection.

columns desirable for preliminary work in evaluating adsorbents and packing methods, and in fact used a 4 × 280 mm glass column for routine separation of the carotenes. However, stainless steel columns were preferable for routine work with the xanthophylls when higher operating pressures were encountered. The use of 1/4 in. tubing and standard Swagelok fittings provided columns that could be assembled or modified easily and provided leak-free operation up to 2000 p.s.i. (Fig. 1). The sample

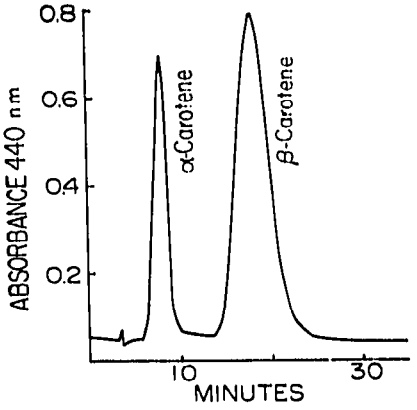

Fig. 2. Chromatogram from a carrot extract. Column: Sea Sorb 43, 4 mm I.D. × 280 mm long, glass; solvent: hexane containing 5% TPA; flow rate: 1.0 ml/min; temperature: room temperature.

injection valve was machined from brass, and although convenient, was not essential for satisfactory operation. Not shown is a water jacket for holding the column at a constant temperature of 15°. The use of the 1/4 × 1/16 in. reducer on the column and small diameter polyethylene tubing from the column to the detector gave minimum hold-up volume. Column lengths of 10–20 cm were satisfactory. Longer columns required excessive pressures for reasonable flow rates.

Detection and recording

Carotenoids in the column effluent were monitored with either a Beckman DB spectrophotometer and a Sargeant recorder, or a Technicon colorimeter and recorder. Both of these were satisfactory. The Beckman DB was used at 440 nm using a microflow cell with a 10 mm light path. A Sargeant SRL recorder with log gears was used to record absorbance with chart speeds of 0.1 or 0.2 in./min (Fig. 2).

The Technicon Model 1 colorimeter used an interference filter transmitting at 440 nm and a tubular flow cell with a 15 mm light path. The Bristol recorder supplied with the colorimeter was used to record absorbance at chart speeds of 0.1 or 0.2 in./min (Fig. 3).

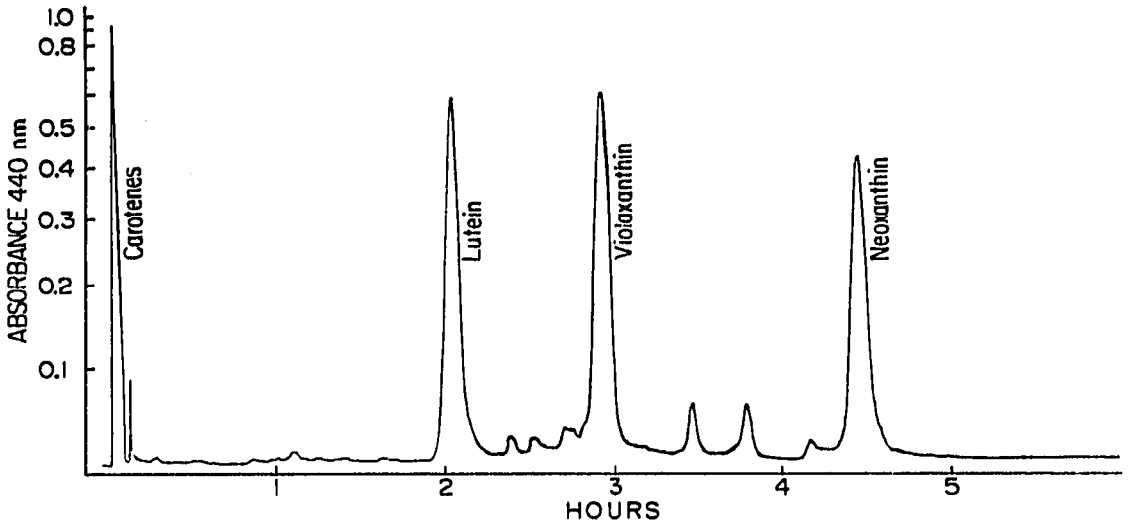

Fig. 3. Chromatogram of a spinach extract. Zinc carbonate column using a hexane TPA gradient. Column: $ZnCO_3$, 1/4 in. O.D. × 13.5 cm stainless steel. Solvent: chamber 1, 90 ml hexane, 1 g BHT; chamber 2, 89 ml hexane, 1 ml TPA; chamber 3, 65 ml hexane, 20 ml TPA. Flow rate: 1/8 ml/min; temperature: 15°.

With both types of equipment, the connection from the column to the flow cell was made with 0.03 in. I.D. polyethylene tubing. The length of this tubing was kept as short as possible to minimize mixing of the column effluent.

Adsorbents

A variety of adsorbents were tested for separation of carotenes and xanthophylls. These included silicic acid, infusorial earth, sugar, starches, cellulose, and several oxides, hydroxides, and carbonates of divalent cations.

Magnesium oxide (Sea Sorb 43, Fisher Scientific Co., S-120) was selected for the

separation of carotenes and was used as supplied. Precipitated zinc carbonate (Fisher Scientific Co., Z-29) was chosen for the separation of xanthophylls. Considerable variation existed in various batches of zinc carbonate giving materials of quite different physical and adsorptive characteristics. Lots were selected with fine, uniform particles. In using precipitated zinc carbonate for thin-layer or column chromatography, material from several sources should be obtained in order to compare their properties. Celite was tried as a diluent to increase flow rate. However, in each case resolution was greatly reduced.

Solvents

Separation of complex mixtures of carotenoids was achieved by gradually increasing the polarity of the solvent using a nonpolar and a polar solvent in one of the gradient devices described above. A variety of solvent combinations was tried. For the nonpolar solvent, petroleum ether, hexane, heptane, and higher boiling hydrocarbon fractions were evaluated. Freshly distilled hexane (b.p. 60–68°) was selected since the high volatility of petroleum ether created problems due to evaporation and formation of vapor bubbles in the system.

A number of alcohols were tested as polar solvents. Acetone, ethyl acetate, and some other commonly used solvents were not tested since portions of our liquid chromatograph did not have chemical resistance to these. Primary and secondary alcohols from methyl through hexyl were not entirely satisfactory and there was little difference in resolving properties among these. The tertiary alcohols, however, had unique properties for giving high resolution of carotenoids on both columns and thin layer plates. We used both *tert.*-butyl and *tert.*-pentyl alcohol (TPA). The TPA (Baker Chemical Co., 9046, *tert.*-amyl alcohol, reagent) was most satisfactory but had to be distilled over KOH before use to eliminate impurities (probably aldehydes) that absorbed light in the 300–350 nm range. TPA has a high boiling point (100°) but can be readily azeotroped at 30° in vacuum with benzene when it is necessary to concentrate carotenoid solutions.

Butylated hydroxytoluene (BHT) (Eastman Chemical Products, Tenox BHT) was routinely included in the solvent (1% w/v) to avoid losses and isomerization of carotenoids. Ethoxyquin (Monsanto Chemical Co.) was also effective as an antioxidant but was difficult to purify and caused some colorimetric interference because of its natural yellow color.

Packing the column

All of the standard methods of packing columns with dry and wet materials were tried. For the magnesium oxide and precipitated zinc carbonate finally selected, thick slurries in organic solvents packed rapidly and under pressure gave consistently good results. Since the initial loading of a column with a slurry under pressure presents certain technical difficulties, we used a simpler procedure.

This procedure started with a column that was three or four times longer than the final packed column. For glass columns, a standard 4 ft. length of glass tubing was used. For the stainless steel column, we attached a 4 ft. length of stainless steel tubing (same diameter as the column) to the column with a Swagelok coupling. The slurry was rapidly added to this extended column as a single charge and air pockets broken up with a piece of stiff plastic capillary tubing. The magnesium oxide slurry

was prepared in hexane and the zinc carbonate in hexane containing 5% TPA. The pump was then connected and solvent pumped as rapidly as possible to compact the column until the desired operating pressure was reached. The solvent was then pumped at a lower rate for about 1 h to complete packing of the adsorbent in the lower portion of the extended column. To provide the finished glass column, the tubing was broken in the lower packed region to provide a column of the desired length. For the stainless steel column, the upper tubing was removed leaving the lower column fully packed. A few mm of adsorbent was then removed from the top of the column and a thin porous polyethylene disc fitted on top of the packing.

Prior to running a sample of carotenoids, hexane containing BHT (1% w/v) was pumped through the column for 1 h. The same mixture was also used to regenerate the column after each sample had been run. It was found that with the use of this regeneration process, the column could be used repeatedly with little loss of resolution.

Loading the column

In order to make room for the sample, the excess solvent was removed from the top of the column. For loading, we used a microsyringe (No. 0010, Hamilton Co., Whittier, Calif.) with a No. 20 hypodermic needle. A piece of polyethylene tubing 0.030 in. I.D. and 36 cm long was attached to the end of the needle. The tubing was calibrated with water. Approximately 0.05 ml aliquots were used. The solvent used to dissolve the sample was very important in that good resolution could not be obtained with many common hydrocarbons and alcohols. The best results were obtained with ethyl ether. Following the application of the sample, the polyethylene tubing was washed with a small amount of hexane and the solvent was applied to the column.

Identification of carotenoids

The carotenoids, for the most part, were characterized from crystalline material and their properties compared with those from known sources[9–12]. Several kg of material was extracted with acetone and hexane. The esters were saponified and preliminary separations made with a 100 tube counter-current apparatus[4]. Further separations were made on large columns of Sea Sorb 43 or zinc carbonate using Celite as a diluent. The colored bands were collected in fractions and absorption spectra were determined on each. Fractions with similar spectra were combined, concentrated, and the carotenoids crystallized. Purity was also determined by monitoring on the column described in this paper. In this manner, good separation was achieved between the *trans* and *cis* isomers. All separations were carried out under low light intensity and whenever possible, systems were flushed with nitrogen. All materials were stored at −18°. However, in spite of these precautions it was obvious that artifacts formed profusely both in the counter-current apparatus and on the columns.

For identification purposes, some of the pigments were obtained from more than one source. The crystalline material was characterized with visible, IR and mass spectra[13]. The latter were made with a Bendix Time of Flight instrument with modifications to increase the resolution in the 400–700 mass range. In addition, identifications were based on TLC properties using the Hager method[5] and one developed in our laboratories using zinc carbonate. The zinc carbonate procedure consisted of

making plates from a slurry of precipitated $ZnCO_3$. They were heated in an oven at 110° for about 1 h or until thoroughly dry. The plates were then cooled slowly in order to prevent cracking, and were used immediately. The solvent consisted of hexane–triethylamine–TPA (90:5:5). The chromatograms were run at temperatures not exceeding 25°. This procedure gave very good resolution. The number of 5,6-epoxy groups were determined by the spectral shift following additions of HCl (ref. 14). Shifts in spectral curves due to isomerization were compared following treatment with iodine and light[14].

The chromatographic peaks of the "Dancy" tangerine extract (Fig. 4) were identified as follows:

Peak 1, carotene mixture and other unidentified compounds. Fraction chromatographed on Sea Sorb 43 column and on thin layer.

Peak 2, unknown.

Peaks 3, 4, unknown, probably cryptoxanthin *cis* isomers.

Peak 5, cryptoxanthin: crystallized from ethyl ether and methanol. Absorption maxima at 478, 452, and 426 nm in hexane. Mass 553. Cochromatographed on thin

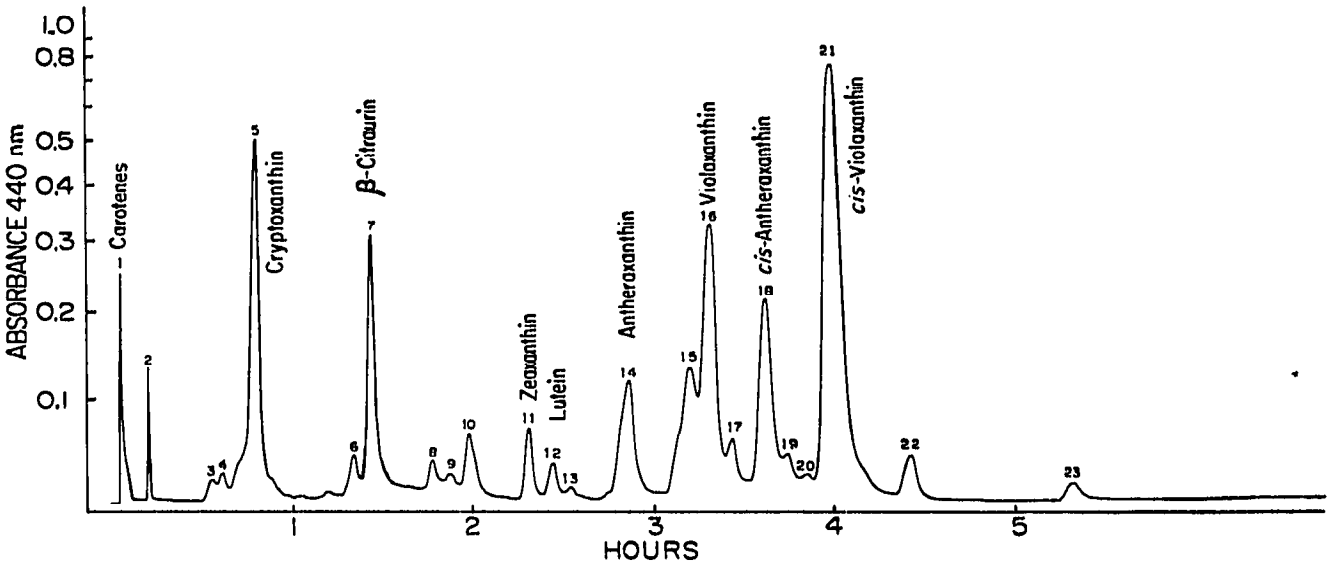

Fig. 4. Chromatogram of a Dancy tangerine peel saponified extract. Zinc carbonate column using a hexane–TPA gradient similar to that described in Fig. 3 except the flow rate was slightly lower.

layer with crystalline samples obtained from yellow papaya, egg yolks, and fruit of balsam apple (*Momordica charantia*).

Peak 6, unknown.

Peak 7, β-citraurin: crystallized from ethyl ether and hexane. Absorption maxima at 481, 454, and 429 (sh)nm. In ethanol, there was a smooth curve with no well-defined peaks. The IR spectrum showed peaks at 1665 and 1725 nm. The mass was 433.

Peaks 8, 9, 10, *cis* isomers of β-citraurin. We were unable to crystallize the compounds forming these peaks. Gels were formed in a mixture of ethyl ether and petroleum ether. Absorption maxima at 473, 447, and 428 nm. Impurities in all peel samples have interfered with the spectra in the *cis* region. When fractions collected from each peak were rechromatographed, all of the isomers including the trans were

formed. Similar absorption curves were obtained when the *trans* and the *cis* forms were treated with I_2 and light.

Peak 11, zeaxanthin: confirmation was based on absorption spectra, retention time of the *cis* isomers, R_F values, and color on thin layer. Comparisons were made with crystalline material obtained from yellow corn, balsam apple, and bacteria.

Peak 12, lutein: the column effluent gave absorption maxima at 473, 445, and 422 nm. The thin layer R_F values were similar to those obtained from crystalline samples obtained from spinach and orange marigold.

Peak 13, unknown.

Peak 14, antheraxanthin: fractions from the column had absorption maxima at 472, 443, and 423 (sh)nm. On treatment with HCl, the absorption maxima shifted to a shorter wavelength by 19 nm. This peak was formed when the effluent from peak 18 (*cis*-antheraxanthin) was treated with I_2 and light.

Peak 15, *cis*-violaxanthin: fractions from the column had absorption maxima at 457, 430, and 408 nm with a *cis* peak at 309 in ethanol. When treated with I_2 and light, the maximum shifted to a longer wavelength by 9 nm and had a similar absorption spectrum as the *trans* when treated in the same manner. When treated with HCl, the maximum shifted to a shorter wavelength by 32 nm.

Peak 16, Violaxanthin: absorption maxima of the column effluent in hexane was 470, 440, and 417 nm. When treated with HCl, the maximum shifted to a shorter wavelength by 44 nm. Chromatography on thin layer showed this compound to have similar R_F values to crystalline material obtained from spinach. This peak was formed when effluents from peaks 15, 21, or 22 were treated with iodine and light and rechromatographed.

Peak 17, unknown.

Peak 18, *cis*-antheraxanthin: the visible spectrum of crystalline material in ethanol was 469, 443, and 419 nm. There was no significant *cis* peak. However, it was not possible to obtain a pure *cis* form in that partial conversion to the *trans* (peak 14) took place in all samples. Following treatment with HCl, the maximum wavelength was 17 nm lower. When isomerized with I_2 and light, the absorption spectrum did not show a significant shift and the curve was similar to the *trans* when treated similarly. When the I_2-treated *cis* form was chromatographed on either thin layer or a $ZnCO_3$ column, the reversion to the *trans* form was observed. Because of the similarity between antheraxanthin and diadinoxanthin[15] additional steps were taken for identification. The IR spectra was not satisfactory for distinguishing these compounds. However, a high resolution mass spectra showed peaks at M 584.4229 and M-2 (loss of H_2).

Peaks 19, 20, unknown.

Peak 21, *cis*-violaxanthin: the crystalline material in hexane had absorption maxima at 468, 438, 415, and a small *cis* peak at 327 nm. When HCl in ethanol was added, the maximum shifted to a shorter wavelength by 39 nm. The absorption spectra of both *trans* and *cis* were similar when treated with I_2 and light. When the *cis* form was isomerized and rechromatographed, there was partial reversion to the *trans* as determined by retention time and absorption spectra. The mass was found to be 600.

Peak 22, *cis*-violaxanthin: the column eluate had absorption maxima at 463, 434, 410, and a high *cis* peak at 330 nm. When treated with I_2 and light, the amplitude

of the *cis* peak dropped and there was a shift of the maxima to a longer wavelength of 2 nm. The I_2-treated pigment had a similar spectrum to the *trans* which had been isomerized in the same way. When the isomerized *cis* form was again passed through the column, both the *trans* and *cis* forms separated.

Peak 23, unknown.

DISCUSSION

Basic requirements for the liquid chromatograph were: (a) a suitable gradient device; (b) a controlled volume pump capable of reproducible and leak-free operation at pressures up to 1000 or 2000 p.s.i.; (c) a jacketed column suitable for high pressures with minimal dead volume between column and detector; and (d) a low volume flow cell in a detector to monitor visible light absorption by the carotenoids. We used various types of standard and modified equipment with considerable success; the exact configurations described are mostly a matter of convenience and are probably not critical.

The adsorbents used, choice of solvents, and method of packing the columns were quite important, however. Magnesium oxide was the adsorbent of choice for the carotenes and zinc carbonate for the xanthophylls. Columns were packed rapidly with slurries of adsorbent in solvent under pressure. The most desirable solvent system was hexane containing TPA with BHT included as an antioxidant.

The method described gave excellent separation of the carotenes (Fig. 2) and xanthophylls (Figs. 3 and 4) in moderately short times. Separations like these were achieved routinely with leaf and citrus peel samples and also while monitoring the purity of preparative-scale samples for isolation and crystallization of individual pigments.

Most of our separations of complex mixtures of carotenoids such as that shown in Fig. 4 were done with a Milton Roy pump operating at approximately 800 p.s.i. Limited tests indicated that this time could be shortened considerably by using the Milton Roy pump at pressures up to 1600 p.s.i. or the Nester/Faust pump with pressures up to 2000 p.s.i. However, leakage became a serious problem at these higher operating pressures with the solvents used. These small leaks were difficult to detect but resulted in substantial changes in retention times.

The amounts of individual carotenoids were determined by measurement of peak areas on the chromatogram. Reproducibility of the system was demonstrated by running samples of a tangerine peel extract on the zinc carbonate column four times. Peak area measurements were made for the predominant carotenoids and absolute amounts calculated relative to standard areas obtained with crystalline pigments. The results shown in Table I demonstrate the ability of the method to determine quantitatively μg amounts with reproducible results. Retention times were also very reproducible. However, in order to make reproducible determinations on xanthophylls, it was necessary to maintain the column at a constant temperature. For this purpose, we used a water jacket through which water was circulated at 15°. Losses of carotenoids applied to the column were not studied extensively. Approximately 90% of two samples of lutein was recovered following standard chromatographic separation, but losses will undoubtedly vary among carotenoids.

TABLE I

RESULTS FROM 4 CHROMATOGRAMS OF DANCY TANGERINE PEEL EXTRACT SHOWING THE REPRODUCIBILITY OF THE METHOD

Zinc carbonate column with a hexane–TPA gradient.

Chromatogram	*Cryptoxanthin (μg)*	*β-Citraurin (μg)*	*cis-Antheraxanthin (μg)*	*cis-Violaxanthin (μg)*
1	2.49	1.72	1.34	9.17
2	2.52	1.72	1.36	9.78
3	2.44	1.59	1.24	8.76
4	2.55	1.66	1.20	9.13
Average	2.50	1.67	1.29	9.21
Std. error	± 0.02	± 0.03	± 0.04	± 0.21

Isomerization

The ease with which isomers are formed has long been a problem in carotenoid research. Although light, heat, iodine, and other agents are known to induce isomerization, the isomerization of pigments on the column has been disputed. ZECHMEISTER[16] maintained that isomerization of carotenoids was independent of the adsorption on an analytical column. "Indeed, it takes place spontaneously when a carotenoid solution is left at room temperature for several hours or days." Our research, however, indicates that carotenoids isomerized much more readily on columns than sitting on a laboratory bench. We also found that inclusion of an antioxidant in the solvent can greatly reduce this isomerization. The usefulness of our chromatographic

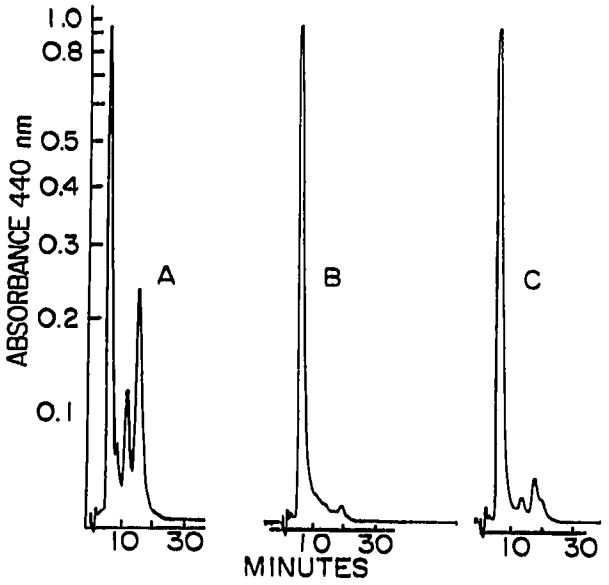

Fig. 5. Chromatogram of lutein using zinc carbonate column and 5% TPA in hexane. (A) *trans*-Lutein was put on the column and left for 2 h prior to developing the chromatogram. Note isomer formation. (B) *trans*-Lutein which remained on the laboratory bench while A was being run. (C) *trans*-Lutein which remained on the laboratory bench for about 4 h and was then adsorbed on the column, pretreated with BHT, for an additional 2 h. It was then run through the column with the above mentioned solvent containing 1 g BHT in 100 ml.

system in studying the formation of isomers and the benefits of adding an antioxidant were demonstrated with several pigments. Two examples follow: (a) a study of the on-column formation of lutein isomers and inhibition by antioxidants, and (b) the use of the chromatographic method for separation and identification of antheraxanthin isomers.

Demonstration of the formation of lutein isomers on the column was made using a nongradient chromatographic system that separated all the isomers observed in 25 min (Fig. 5). A sample of crystalline *trans*-lutein from marigold dissolved in ethyl ether and immediately run through the column gave a single peak. However, if the sample was adsorbed on the column, the pump turned off for 2 h, and the chromatogram then completed, at least three isomers were formed in substantial quantities (Fig. 5A). During the time the aliquot was on the column, the original sample was maintained in the dark at room temperature. Very little isomerization of this aged sample (about 3 h) occurred (Fig. 5B). We found that isomerization on the column could be greatly reduced by including 1% BHT (w/v) in the solvent. An aliquot of the lutein sample which had been in the dark at room temperature for about 4 h was adsorbed onto the column and allowed to remain for 2 h before completing the chromatogram (Fig. 5C). Much less isomerization occurred when an antioxidant was used.

Antheraxanthin isomers were also readily separated on the column. Treatment of *trans*-antheraxanthin with I_2 and light resulted in four peaks on the chromatogram (Fig. 6). The first was the *trans* and the last three were *cis*. The first two *cis* forms had similar absorption spectra and the last one had a very high *cis* peak at 332 nm. Proof that these last three peaks were actually antheraxanthin isomers was obtained by collecting them individually and treating them with iodine. Rechromatography showed that in each case a partial conversion back to the *trans* form had occurred. In addition, treatment with HCl shifted the absorption spectrum of each isomer downward about 20 nm indicating that these were isomers of antheraxanthin and not decomposition products.

Formation of isomers during extraction and chromatography is a major concern in attempting to establish the naturally occurring complement of pigments from

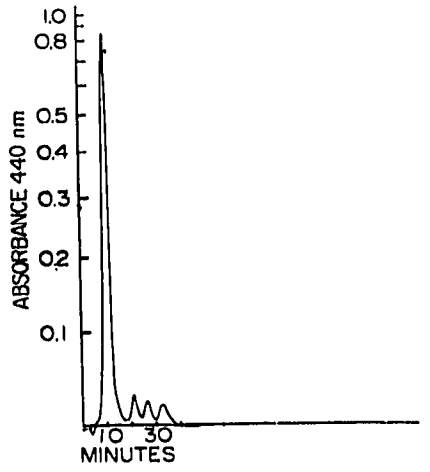

Fig. 6. Chromatogram of antheraxanthin following treatment with I_2 and light. Zinc carbonate column using 5% TPA in hexane. Left to right, *trans* and 3 *cis* isomers.

complex tissues such as citrus peel. Not only is it difficult to decide which are artifacts, but also large numbers of isomers result in incomplete separations during chromatography. We observed that highly purified extracts of citrus peel and crystalline carotenoids isomerized much more rapidly during chromatography than crude extracts. This probably is due to naturally occurring antioxidants that occur as contaminants in the less pure material. Incorporation of BHT as an antioxidant has greatly reduced problems related to isomer formation and has resulted in chromatographs with fewer artifacts.

Although the technique has been demonstrated to reduce isomerization, preliminary evidence would indicate that not all *cis*-isomers in citrus are artifacts (Fig. 4). Probably the predominant carotenoid in ripe citrus peel is *cis*-violaxanthin (peak 21). We have observed this from many cultivars with a variety of extraction procedures. This isomer has not been found in green peel, neither is it readily formed by treating the *trans* with iodine and light or by passing through a column.

Since carotenoids vary during a season, not all pigments found in tangerine peel are shown in Fig. 4. For example, we have isolated, crystallized, and characterized reticulataxanthin and found it to be the primary red pigment early in the season. Reticulataxanthin was readily separated from β-citraurin on the zinc carbonate column reported in this paper; the retention time of the latter pigment was slightly less than reticulataxanthin.

The method described here for the quantitative determination of carotenoids is simple and relatively rapid. It is believed that with slight modifications of gradients to give maximum resolution in minimum time for a particular mixture, the procedure can be used for routine determination of carotenoids from a variety of sources.

ACKNOWLEDGEMENTS

We thank the following for carotenoid samples: Dr. H. YOKOYAMA, Pasadena; Dr. G. BRITTON, Liverpool; and Dr. H. THOMMEN, Basle.

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