$$F_S = (A_{206})_{SDS=X}/(A_{206})_{SDS=0} \quad (2)$$

where $(A_{206})_{SDS=X}$ is the area at finite detergent concentration; $(A_{206})_{SDS=0}$ is the area at 0 detergent concentration.

From Table III, F_S decreases as SDS increases to 0.5 and then levels off for cut 1. Moreover, the arithmetic products of F_S and total nitrogen (i.e., total nitrogen resolubilized) in cut 1 are 11.8, 9.6, 6.4, and 11.7 mg, respectively at 0, 0.2, 0.5, and 1% SDS. Therefore, SDS increases extractability and initial solubility, but decreases resolubility for cut 1 (see F_S, Table III). Cuts 2 and 3 are completely soluble at all detergent concentrations within experimental error. The solubility results for all cuts agree with qualitative visual observations.

CONCLUSIONS

The nitrogenous constituents of freeze-dried Coastal Bermuda grass can be classified as insoluble (R_I), extractable-insoluble (R_{II}), extractable-temporarily soluble (SDS extractable cut 1), extractable-soluble (cut 1 at 0% SDS, cut 2, and cut 3). These classifications serve as a functional method of determining the end use of forage protein for maximum utilization as food or feedstuff. Cut 1 soluble ($N_T \simeq 11\%$); and possibly cuts 2 and 3 ($N_T \simeq 18\%$) might be made suitable for human use. Cut 1, SDS extractable ($N_T \simeq 28\%$ at 1% SDS), cut 2 and 3, and possibly R_{II} ($N_T \simeq 5\%$ at 1% SDS) might serve as a protein source for monogastric animals and R_I and R_{II} ($N_T \simeq 40\%$ at 1% SDS) for ruminants.

The degradation or precipitation of cut 1 could be minimized by separating it, before the chloroplastic proteins are solubilized and with the utmost rapidity, from cuts 2 and 3. Here, cut 1 was separated from cuts 2 and 3 in less than 4 h from the start of the extraction. Since N_T, percent CP, molecular weight distribution parameters (Table II), solubility parameters (Table III), and the content of aromatics (Table III) are independent of SDS concentration for cuts 2 and 3, a two-stage procedure apparently would be appropriate: stage 1, extract with buffers, antioxidant and mild conditions to separate R_I and R_{II} from cuts 1–3; stage 2, extract R_I and R_{II} with harsher conditions (e.g., detergent, pressure, heat, organic solvents, etc.) which would separate maximum chloroplastic protein.

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Determination of *all-trans* and *13-cis* Vitamin A in Food Products by High-Pressure Liquid Chromatography

David C. Egberg,* John C. Heroff, and Richard H. Potter

A high-pressure liquid chromatography (HPLC) procedure has been developed for the determination of retinol and its esters in food products. The method quantitates both the all-trans and 13-cis isomers. The presence of the 13-cis isomer in a number of food products was demonstrated. Recovery studies on different food products showed an average recovery of 94.6 ± 6.6%. Reproducibility data were generated showing a pooled relative standard deviation of 3.9%. The HPLC procedure was compared with an AOAC colorimetric procedure for six products; there was no statistical difference between the means.

A major challenge facing the food chemist is the development of more accurate and cost effective methods for nutrient analysis. High-pressure liquid chromatography (HPLC) is a technique which is finding many applications in this area. The technique is potentially fast, specific, and sensitive; it offers many advantages over more commonly used chemical methods (Clifford, 1976).

HPLC has been used extensively for the analysis of fat-soluble vitamins in concentrates, multivitamin preparations, and to a certain degree in food products. These

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applications have been reviewed (Conrad, 1975; Williams et al., 1972).

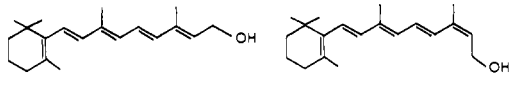
HPLC methods for vitamin A determination in food products have been reported (Van De Weerdhof et al., 1973; Dennison and Kirk, 1976). These methods require a partitioning step; thus, negating much of the potential time savings. When direct extraction and injection methods are reported, a scarcity of data exists concerning sample applicability, reproducibility, recovery, and comparison with standard methodology.

A primary objective of our study was to devise a relatively rapid, general HPLC procedure omitting the partitioning step and to obtain reproducibility, recovery, and comparative data on a number of food products.

It is well known that vitamin A derivatives are subject to facile isomerization to isomers with less biological activity. This isomerization can be induced photochemically or by acid catalysis (Harris, 1967). HPLC separation of vitamin A isomers in reaction mixtures has been reported (Vecchi et al., 1973). Because of this difference in biological activity, it would be desirable for a method to have the capability to quantitate all isomers found in food products.

EXPERIMENTAL SECTION

Reagents. Vitamin A acetate in cottonseed oil (equivalent to 29.1 mg of retinol/g of oil) (USP, Bethesda, Md.) was used as the primary standard. The *13-cis-* and *all-trans*-retinal were purchased from Eastman (Rochester, N.Y., No.'s 8082 and 8080, respectively). The acetonitrile used was nanograde. Spectrograde hexane, methylene chloride, and isopropyl alcohol were used.



ALL-TRANS RETINOL 13-CIS RETINOL

Vitamin A Standard Solutions. Solution A (equivalent to 26.2 μg of retinol/mL). The standard oil (90 mg) was dissolved in 10 mL of acetone and diluted to 100 mL in an amber volumetric flask with 95% ethanol. Solution B (2.62 μg/mL) was prepared by diluting 10 mL of solution A to 100 mL with 95% ethanol. The standards were prepared fresh daily.

Apparatus. *Reverse Phase Procedure.* A Dupont 830 liquid chromatograph (Wilmington, Del.) equipped with a Vari-Chrom variable wavelength detector (Varian, Palo Alto, Calif.) was used. A Valco valve (No. 830520-901, Dupont) fitted with a 40-μL sample loop was used. The column (25 cm × 3.2 mm i.d., 316SS) was slurry packed with Vydac 10-μm ODS packing (No. 201 TPX, Separations Group, Hesperia, Calif.) using a stirred slurry packer (No. 705, Micromeritics Corporation, Norcross, Ga.). A Metricel (No. 60170, Gelman Company, Ann Arbor, Mich.) filter with 0.45-μm pore size was used for sample filtration. The ultraviolet spectra were obtained with a Beckman DB-G.

Adsorption Chromatography Procedure. A Dupont 830 liquid chromatograph equipped with an Aminco Fluoro-Monitor detector (Silver Springs, Md.) was used. The Fluoro-Monitor employed a 20-μL flowcell, 4-W source (G.E. No. 4T4/BL), primary filter (Corning 7–51, 80% T at 365 nm), and a secondary filter (Wratten No. 8, sharp cut, 78% T above 510 nm). A Valco valve fitted with a 100-μL sample loop was used. A Zorbax (Dupont) 5-μm silica gel column (25 cm × 2.1 mm i.d., 316 SS) was used.

Procedure. *Reverse Phase.* The low-moisture samples were ground to pass a 40-mesh sieve. All samples were mixed to insure homogeneity. A portion of product containing about 75 μg of vitamin A (calculated as retinol, maximum sample weight 3.0 g) was added to a 125-mL amber Erlenmeyer flask equipped with reflux condenser and a nitrogen inlet. If samples contained sugar, 3 mL of water was added to form a slurry. About 50 mg of ascorbic acid was added as an antioxidant. Ethanol (95%, 30 mL for low-fat samples and 26 mL for high-fat samples) was added, and the Erlenmeyer was swept with nitrogen and heated to the reflux temperature. Aqueous potassium hydroxide (2 mL, 0.5 g/mL) was added to low-fat samples such as ready-to-eat (RTE) breakfast cereals and ethanolic potassium hydroxide (4 mL, 0.25 g/mL) was added to high-fat samples such as margarine. Two standards were carried through the procedure each day with the samples. The standards were prepared by pipetting 10 mL (262 μg retinol equivalent) of standard solution A and 20 mL of standard solution B (52.4 μg retinol equivalent) into two separate Erlenmeyer flasks and adding 20 mL and 10 mL of 95% ethanol, respectively. The standards were treated the same as the samples.

The samples and standards were heated at reflux temperature on a hot plate with occasional swirling for 45 min. The samples were cooled to room temperature and the potassium hydroxide neutralized by the addition of acetic acid in acetonitrile (10 mL, 0.1 g/mL) with swirling. The material was quantitatively transferred to a 100-mL amber volumetric flask with acetonitrile and diluted to volume with acetonitrile; fatty acid salts precipitated. About 5 mL of this material was filtered (0.45-μm filter) and a 4-mL aliquot was diluted to 5 mL with water. These samples and standards were injected (40-μL loop; mobile phase: acetonitrile–water, 65 + 35; flow rate approximately 1.5 mL/min) and the absorbance measured at 328 nm. The area of the standards (height × width at half-height) was used for quantitation. The absorptivity ratio of the all-trans to 13-cis at 328 nm (1.08, Harris, 1967) was used to calculate the 13-cis content.

Adsorption Chromatography Procedure. The samples and standards were prepared and saponified as described in the reverse phase procedure. After cooling to room temperature, the samples and standards were transferred to 250-mL amber separatory funnels with 30 mL of water and 160 mL of hexane–methylene chloride (3:1). After extraction and phase separation, the aqueous layer was discarded. The organic layer was washed with three 100-mL portions of water to remove any residual base.

Vitamin A oxime (200 μg) was added to the extract and a 100-μL portion of the extract, without concentration, was injected directly on the Zorbax column (mobile phase: hexane–methylene chloride–isopropanol; 300:200:1.5; flow rate: approximately 0.9 mL/min). The ratio of the retinol area (height × width at half-height) to vitamin A oxime area was used to quantitate the samples (see Figure 1). Under the conditions of this procedure, the fluorescence response of the 13-cis isomer was 75% of the *all-trans*-retinol.

Identification of 13-cis-Retinol. Peak A (Figure 1) was collected from the column. A portion was reinjected to insure that no *all-trans*-retinol was present. This material was reacted with antimony trichloride to give the characteristic blue color of a vitamin A isomer.

Peak A was collected and a portion reinjected as described above. Its ultraviolet spectrum (in mobile phase) showed a 328 nm maximum and a shoulder at 318 nm identical with that reported by Robeson et al. (1955) for the *13-cis*-retinol in ethanol.

Preparation and Injection of 13-cis-Retinol. A solution of *13-cis*-retinal in ethanol (4 mL, 100 μg, 0.35×10^{-6} mol)

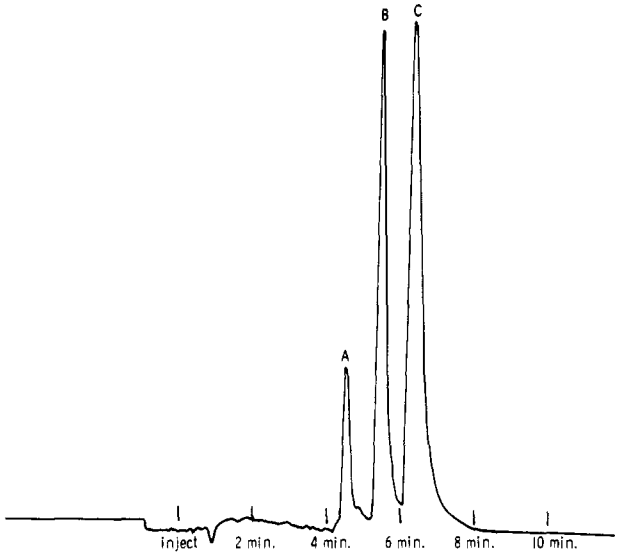


Figure 1. Chromatogram of cod liver oil extract using Zorbax silica gel, fluorescence detector, flow rate 0.90 mL/min. Peak A is 0.010 μg *13-cis*-retinol; peak B is 0.021 μg of *all-trans*-retinol; peak C is 0.032 μg of retinal oxime internal standard injected.

was added to an amber Erlenmeyer flask containing 21 mL of absolute ethanol. Sodium borohydride (0.5 mg, 13.2×10^{-6} mol) was added, and the solution was swept with nitrogen. The solution was allowed to sit at room temperature for 1 h. Acetic acid in acetonitrile (10 mL, 0.1 g/mL) was added to the solution with swirling, and the solution was transferred to a 100-mL volumetric and diluted to volume with acetonitrile. This material was used to spike a sample extract to identify the 13-cis peak using the reverse-phase procedure.

A solution of *13-cis*-retinal in ethanol was reduced with sodium borohydride as described above. However, after reduction with sodium borohydride, the mixture was transferred to a 250-mL amber separatory funnel and extracted as described in the adsorption chromatography procedure. This extract was used to identify the 13-cis peak observed in the adsorption procedure.

Preparation of Retinal Oxime Internal Standard. Hydroxylamine hydrochloride (2.0 g, 29 mmol) was added to a three-neck 100-mL flask equipped with condenser, magnetic stirrer, nitrogen inlet, and heating mantle. Sodium hydroxide (8 mL, 15% aqueous solution) was added to the solution. Retinal (1.0 g, 3.5 mmol) dissolved in 50 mL of 95% ethanol was added to the reaction solution with 10 mL of 95% ethanol. The system was swept with nitrogen and heated at the reflux temperature for 10 min. Water (35 mL) was added to the reaction solution, and the resulting mixture was transferred to a separatory funnel and extracted with two 60-mL portions of diethyl ether. The combined ether extracts were washed with 60 mL of 0.1 N HCl, followed by three 60-mL portions of water. The ether was dried ($MgSO_4$) and concentrated (rotary evaporator) to a crude yellow solid (1.07 g). This material was purified by column chromatography (100 g neutral alumina, activity I; particle size 0.05–0.2 mm, glass column 35 cm × 19 mm i.d.; eluent: stepwise gradient chloroform 10% ethyl acetate to ethyl acetate–3% methanol) to give a yellow solid (0.69 g, 66% yield, mp 138–140 °C). The crystalline material was stable when stored at 0 °C under nitrogen. Standard solutions were prepared fresh daily.

Carr-Price Method. The AOAC procedure (Association of Official Analytical Chemists, 1970) was used. Two retinol acetate standards were carried through the total procedure, including chromatographic cleanup, and used to quantitate the samples.

RESULTS AND DISCUSSION

Since retinol exists as different esters in food products, it is convenient to saponify the sample in the extraction step converting all esters to retinol. Complete conversion to the alcohol is evidenced by the lack of any ester peaks in the chromatograms.

Although the saponification and extraction procedure, as outlined in the Experimental Section is effective for vitamin A analysis, the number of steps involved detracts from its usefulness as a routine analytical method. The reverse-phase method, however, is comparatively simple and has many advantages over the methods currently used for vitamin A analysis.

The sample is extracted and saponified in a nitrogen atmosphere protected from light. Ascorbic acid was used as an antioxidant as suggested for tocopherol analysis (Christie et al., 1973). After addition of acetic acid equivalent to the potassium hydroxide, the mixture was brought to volume with acetonitrile precipitating fatty acid salts (characterized by GLC analysis of methyl ester derivatives). After filtration of the extract, an aliquot of filtrate was diluted with water; this was done to decrease the organic nature of the inject media to minimize band spreading at the head of the column. Two external standards were used with each group of samples.

Linear standard curves were observed in the region of interest (0 to 320 ng) with both detectors. Although the fluorescence detector was used with the adsorption procedure and the variable-wavelength detector with the reverse-phase procedure, these detectors could be used interchangeably.

Using a 3.0-g maximum sample weight, the detection limit was about 1 μg/g (1.6% RDA with a 1-oz serving size). For example, peak A (*13-cis*-retinol) in Figure 4 corresponds to 1.2 μg/g.

It was observed that a vitamin A isomer was present to an appreciable degree in a number of food products. It was of interest to identify this material since vitamin A isomers possess different biological activities (Harris, 1967). The 13-cis isomer (peak A in Figure 1) was isolated from the silica gel column and shown not to contain the all-trans isomer (peak B in Figure 1) by reinjecting a portion of the isolated material. The ultraviolet spectrum of the isolated material corresponded to the 13-cis isomer and the solution reacted with antimony trichloride to give a blue color characteristic of vitamin A vitamers and carotenoids. Commercially available *13-cis*-retinal was reduced to the retinol with sodium borohydride and the retention time of this material was shown to be identical with the unknown on both the reverse phase and silica gel columns. It is known that sodium borohydride reduces vitamin A aldehyde to the alcohol without changing the cis–trans stereochemistry (Brown and Wald, 1956). On the basis of these results, the unknown peak was assigned to the 13-cis isomer which is reported to have 75% of the biological activity of *all-trans*-retinol (Harris, 1967).

Although the resolution of the 13-cis and all-trans isomers was better on a silica gel column, the separation on the ODS column was sufficient for quantitation. Typical chromatograms are shown in Figures 2 through 5. The ease of operation of the reverse phase procedure made this technique the method of choice.

It is unlikely that the 13-cis isomer is an artifact of the method since it was not observed in any of the all-trans standards carried through the procedure. Also, in the recovery experiments, the 13-cis levels of samples spiked

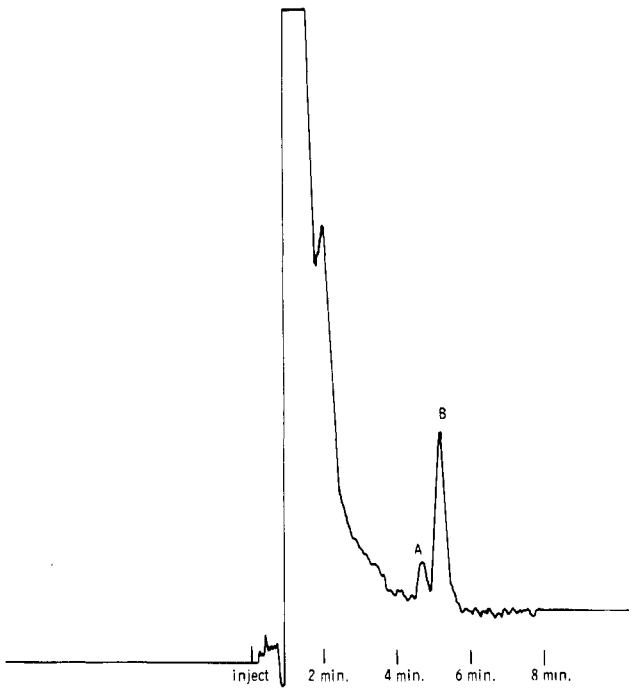


Figure 2. Chromatogram of butter using ODS column, 0.01 AUFS, flow rate 1.50 mL/min. Peak A is 0.002 μg 13-*cis*-retinol and peak B is 0.011 μg of *all-trans*-retinol injected.

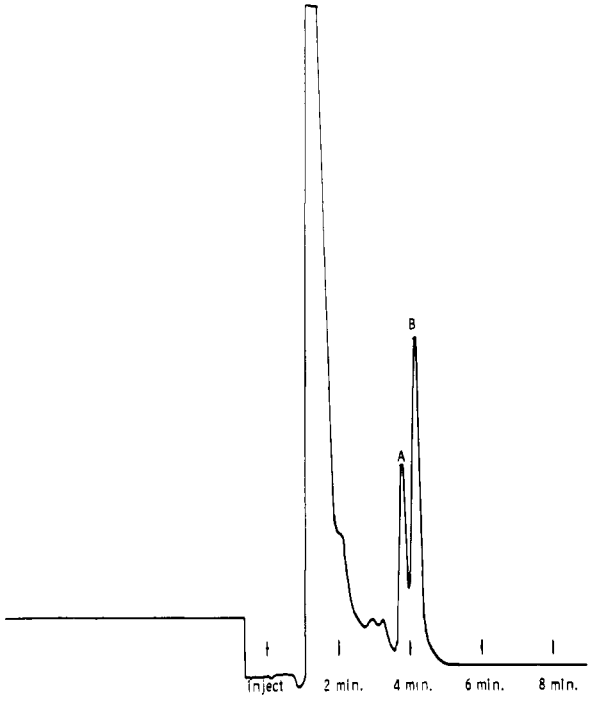


Figure 3. Chromatogram of cod liver oil using ODS, 0.05 AUFS, flow rate 1.75 mL/min. Peak A is 0.047 μg of 13-*cis*-retinol and peak B is 0.089 μg of *all-trans*-retinol injected.

with *all*-trans vitamin A acetate remained unchanged.

The 13-cis isomer was found to an appreciable degree in the food products studied as shown in Table I. It is not known if the 13-cis isomer was added to food products in the vitamin concentrate or to what extent it was formed in the food product. However, the cod liver oil and braunsweiger are unfortified products and showed a relatively large amount of the 13-cis isomer. A sample of ground RTE cereal (fortified with vitamin A palmitate) in a clear glass container placed in sunlight for 5 days showed a decrease in both all-trans (26.1 to 9.1 μg/g) and 13-cis (3.1 to 2.6 μg/g).

Figure 4. Chromatogram of dry cat food using ODS, 0.005 AUFS, flow rate 1.40 mL/min. Peak A is 0.002 μg of 13-*cis*-retinol and peak B is 0.007 μg of *all-trans*-retinol injected.

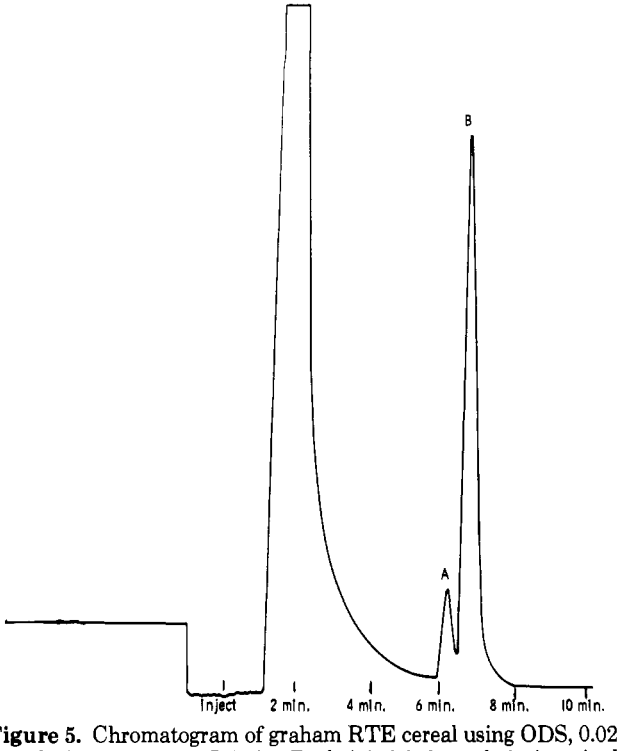


Figure 5. Chromatogram of graham RTE cereal using ODS, 0.02 AUFS, flow rate 1.25 mL/min. Peak A is 0.010 μg of 13-*cis*-retinol and peak B is 0.048 μg of *all-trans*-retinol injected.

When a standard solution of *all-trans*-retinol in clear glassware (2.76 μg/mL) was exposed to laboratory light for 12 h there was 14% degradation and 22% of the remaining material was the 13-cis isomer. Protected from light at room temperature, however, the extract from two RTE cereals and one margarine sample showed an average degradation of less than 0.3% in 4 h. Thus, the extract should be stable in an autosampling device provided it is protected from light. About seven samples/h can be injected.

The reproducibility of the reverse-phase method was ascertained by analyzing samples on different days; thus,

Table I. Reproducibility of HPLC Reverse-Phase Method for Vitamin A

Sample	13-cis[a] μg retinol/g	all-trans[a] μg retinol/g	Total[a] ± SD μg retinol/g	% 13-cis of total
Graham RTE cereal	4.79	13.3	18.1 ± 1.3	26.4
Wheat RTE cereal	2.48	13.5	16.0 ± 0.7	15.6
High sugar RTE wheat cereal	8.06	28.0	36.1 ± 1.5	22.4
Corn RTE cereal	6.05	18.4	24.4 ± 1.9	24.7
Cod liver oil	118	222	340 ± 3.9	34.8
High sugar energy bar		23.9	23.9 ± 1.9	
Margarine I		10.9	10.9 ± 1.4	
High sugar RTE corn cereal	2.89	14.7	17.6 ± 0.3	16.5
Braunsweiger	10.9	36.3	47.2 ± 1.7	23.1
Margarine II		6.71	6.71 ± 0.3	
Butter	1.17	5.23	6.40 ± 0.3	18.3
Oat RTE cereal	3.33	25.9	29.2 ± 0.1	11.4
Dry cat food	1.23	3.59	4.82 ± 0.6	25.6
Pooled within standard deviation	3.5% relative 22 degrees of freedom	2.7% relative 17 degrees of freedom	3.9% relative 18 degrees of freedom	

[a] Average of duplicate or triplicate analyses conducted on different days.

Table II. Recovery Data for HPLC Reverse-Phase Method

Sample	13-cis[a] μg retinol/g	all-trans[a] μg retinol/g ± SD	all-trans[a] added μg retinol/g	% recovery
High sugar corn RTE cereal	2.89	14.7 ± 0.1	14.4	95.7
Wheat RTE cereal	2.48	13.5 ± 0.4	18.4	92.0
Braunsweiger	10.9	36.3 ± 2.0	44.4	96.3
Graham RTE cereal	4.82	13.3 ± 0.3	14.5	95.4
Dry cat food	1.23	3.59 ± 0.04	2.60	75.9
High sugar wheat RTE cereal	8.06	28.0 ± 1.2	46.0	91.0
Corn RTE cereal	6.05	18.4 ± 2.3	18.4	94.1
Cod liver oil	119	222 ± 1.3	146	97.0
Oat RTE cereal	3.33	25.9 ± 0.2	26.4	101
High sugar energy bar		23.9 ± 1.9	21.0	99.4
Margarine I		10.9 ± 1.4	6.28	98.6
Butter	1.17	6.40 ± 0.3	6.39	98.3
			Av	94.6 ± 6.6%
Pooled within standard deviation	3.5% relative 22 degrees of freedom	3.6% relative 17 degrees of freedom		

[a] Average of duplicate or triplicate analyses conducted on different days.

Table III. Comparison of HPLC Reverse-Phase Method with Carr-Price Procedure

Sample	13-cis μg/g	all-trans μg retinol/g	Total[a] ± SD	AOAC[b] μg retinol/g
Dry cat food	0.89	2.32	3.21 ± 0.8	2.94
Cod liver oil	116	223	339 ± 0.9	339
High sugar corn RTE cereal	2.89	14.7	17.6 ± 0.3	17.6
High sugar oat RTE cereal	5.97	21.1	27.1 ± 1.7	26.6
Wheat RTE cereal	1.65	14.0	15.7 ± 0.4	16.9
Braunsweiger	10.9	36.3	47.2 ± 1.7	43.0
Av			75.0 μg/g	74.3 μg/g

[a] Average of duplicate and triplicate analyses conducted on different days. [b] Single analysis.

all sources of variability were taken into account. Duplicate and triplicate analyses were conducted and pooled relative standard deviations of 3.9% (18 degrees of freedom) and 3.6% (17 degrees of freedom) were observed for the combined 13-cis and all-trans value.

Samples were spiked prior to saponification with vitamin A acetate levels comparable to the level of vitamin A found to be present by analysis. An average recovery of 94.6 ± 6.6% was observed for the products shown in Table II.

The reverse-phase method was compared with the commonly used AOAC (Association of Official Analytical Chemists, 1970) Carr-Price procedure on six products. When the 13-cis isomer was taken into account, the HPLC method compared favorably with the colorimetric method for the food products studied. There was no statistical difference between the means (Student's t test, 22% α risk) and a standard error between methods of 2.6% relative was observed. The results are shown in Table III.

In conclusion, we have found this HPLC reverse-phase method to be an efficient technique for the determination of vitamin A and its 13-cis isomer in food products. The method is comparatively simple, sensitive, reproducible, and compares favorably with standard methodology.

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Provitamin A and Carotenoid Content of Citrus Juices

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The carotenoid content in juice of seven citrus cultivars was followed during the fruit maturation period. The analyses were performed with high-performance liquid chromatography (HPLC) procedures that are rapid and reproducible. Using these methods, isomers of cryptoxanthin as well as the carotenes were separated and determined for calculating provitamin A values. β-Cryptoxanthin readily separated from α-cryptoxanthin. The β isomer was found to be the main source of provitamin A in citrus juice while the structure of the α form would indicate that it is nonactive. The provitamin A content of citrus juice varies with fruit cultivar. Juice of the sweet orange cultivars had the lowest provitamin A carotenoid content while that of Dancy tangerine and Robinson were intermediate. The provitamin A content of Murcott was found to be the highest among all the cultivars studied.

The provitamin A value and color of citrus juice is due to a complex mixture of carotenoids (Curl, 1953; Curl and Bailey, 1956; Yokoyama and White, 1967; Gross et al., 1971; Subbarayan and Cama, 1965). Numerous papers published over the past 25 years on carotenoids in citrus have been concerned mainly with extraction and identification. There have been only fragmentary reports on the amounts of the various pigments that occur in the juice. The lack of quantitative information of these compounds has been due to their unstable nature and unsatisfactory analytical methods. Previously, separation has been by open column and thin-layer chromatography; and with these procedures, it has been difficult to obtain reproducible results. During the past 8 years, work has been going on in this laboratory using high-performance liquid chromatography (HPLC) for the quantitative determination of carotenoids (Stewart and Wheaton, 1971, 1972, 1973; Leuenberger and Stewart, 1976; Leuenberger et al., 1976). The purpose of this paper is to describe some of the procedures which have been developed and to report on the amounts of the major carotenoids found in the juice of several citrus cultivars.

EXPERIMENTAL SECTION

Samples. Fruit representing seven citrus cultivars, Hamlin, Pineapple, and Valencia orange, Dancy tangerines, and three hybrids, Orlando, Robinson, and Murcott were used. Sampling commenced in October when all varieties except Valencia were starting to mature and continued at approximately 2-week intervals through March. A sample consisting of 24 fruit was selected at random around the outside of the trees. For juice color determinations, 12 fruit were extracted with an electric hand reamer and the juice filtered through a 17 mesh stainless steel screen. A 75-mL aliquot was deaerated for 30 min in a desiccator jar using a water aspirator to draw a vacuum. Duplicate color readings were made with a Hunterlab Model D-45 Citrus Colorimeter (Huggart et al., 1969; Hunter, 1967). For carotenoid analyses, the remaining 12 fruit from the sample were extracted. The juice from the extractor flowed directly into 50 mL of a solution containing 10 g of KOH. The juice was made alkaline to reduce isomerization of the epoxide containing carotenoids. The remainder of the extraction procedure has previously been reported (Stewart, 1977).

Apparatus. The carotenes and cryptoxanthin with the exception of phytofluene were separated on a MgO column (Stewart, 1977) (Figure 1). Phytofluene was chromatographed on the same MgO column but eluted isocratically with 1% acetone in hexane at the rate of 2.5 mL/min. The detector was an Aminco Fluoro-Monitor using a primary filter, Corning 7-51, and a secondary filter Wratten No. 8.

The xanthophylls were chromatographed on a high-capacity silica pellicle, Pellosil (Reeve Angel), a method reported for the first time in this paper (Figure 2). The HPLC was carried out using a Milroyal D high-pressure pump (Milton Roy Co.) and a Technicon No. 1 detector with a 440-nm filter. The hookup was similar to that reported by Stewart and Wheaton (1971). The column was made of stainless steel tubing $^3/_{16}$ in. o.d. × ca. 3 mm i.d. × 13 cm with reducers (Swagelok) $^3/_{16}$ in. to $^1/_{16}$ in. on each end. Reducers were plugged with porous Teflon discs. The column was packed by tap-filling. A water jacket was constructed from $^1/_2$ in. copper tubing and the column maintained at 16 °C with a circulating water bath.

A drip-type gradient was constructed from two beakers (Konte K-42001, Konte Glass Co.) similar to that previously reported (Stewart, 1977). Some changes were necessary to give a satisfactory chromatogram. The polar solvent was dripped into hexane at the rate of 0.5 mL/min. The flow rate was achieved by raising the reservoir 20 cm above the end of the dripping tube. The diameter of the dripping tube also controlled the flow rate of the polar solvent. Teflon tubing, size 18, 0.042 in. o.d. × 9.5 cm (Allied Electronics) was attached to the reservoir. A

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