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GRAPEFRUIT PIGMENT DETERMINATION

Spectrophotometric Methods for Determining Pigmentation—Beta-Carotene and Lycopene—in Ruby Red Grapefruit

B. J. LIME and F. P. GRIFFITHS

U. S. Fruit and Vegetable Products Laboratory, Southern Utilization Research and Development Division, U. S. Department of Agriculture, Weslaco, Tex.

R. T. O'CONNOR, D. C. HEINZELMAN, and E. R. McCALL

Southern Regional Research Laboratory, Southern Utilization Research and Development Division, U. S. Department of Agriculture, New Orleans 19, La.

Two methods for the measurement of pigments occurring in colored grapefruit are outlined and the results compared. Method A involves extraction of the sample, separation of the major pigments—lycopene and carotene—on a magnesia-Super Cel column, elution, and spectrophotometric measurements of the separated pigments. By method B, a more rapid but less precise procedure, the pigment is extracted and the absorptivity of the extract determined at 451 mμ for carotene and 503 mμ for lycopene. Results of total concentration of the pigments, as determined by simultaneous equations, show method B averages 10.3% higher for lycopene and 16.2% higher for carotene than method A. During a 69-day period, January 26 to April 4, lycopene, in the edible fruit of Ruby Red grapefruit, decreased from 0.29 mg. % to 0.10 mg. %, and carotene from 0.34 mg. % to 0.21 mg. %.

PROBLEMS ASSOCIATED WITH THE COMMERICAL UTILIZATION of grapefruit are being studied by the U. S. Fruit and Vegetable Products Laboratory, Weslaco, Tex. The majority of the grapefruit trees which are now growing in the Lower Rio Grande Valley are either Ruby Red or Marsh Pink varieties, producing fruit which is colored light red or pink, and containing carotene and lycopene as the principal pigments.

The canning or freezing of juice from colored fruit presents problems in obtaining and standardizing the color of the product. Prior publications (*5, 6*) have discussed these difficulties and outlined chromatographic and reflectance methods for estimating the pigment content of the pulp and juice from colored grapefruit. Quantitative differences between the two methods of analysis and need for a more rapid chromatographic method for carotene and lycopene determinations stimulated the present work.

Comparisons are made between two methods: method A, a modification of the chromatographic-spectrophotometric method (*5*); and method B, the spectrophotometric method (*6*).

Method A is a relatively rapid chromatographic method for quantitatively measuring the carotene and lycopene extracted from the juice, suspended solids, and pulp of the fruit. The hexane-extracted pigments are separated by chromatographing on a magnesia–Super Cel column, eluted, and spectrophotometric measurements made of the pigment intensity of the separate eluates.

Method B is more rapid. The total carotenoid pigments are extracted in the same manner. Spectrophotometric measurements are made directly on the hexane extract at 451 and 503 mμ and the total pigment is calculated as carotene and lycopene by means of simultaneous equations (*6*).

Apparatus and Reagents

Waring Blendor. The Waring Blendor used was equipped with regular and microblender cups.

Spectrophotometer. A Beckman Model DU spectrophotometer, with 2- and 5-cm. cells for absorbance, was used in the New Orleans, La., laboratory. A Cenco-Sheard spectrophotometer was used for tests conducted in the Weslaco, Tex., laboratory. Each instrument was standardized with purified lycopene and carotene.

Chromatographic Column. A column 24 mm. in inside diameter and 245 mm. long, with a stem 4 mm. in inside diameter and 140 mm. long was used.

Chemicals and Reagents. Westvaco adsorptive powered magnesia, No. 2641 and 2642; Hyflo Super-Cel; commercial hexane, boiling point 66–69° C.; methanol, ACS grade; acetone; sodium sulfate, anhydrous; β-carotene, purified

Table I. Effect of Saponification of Hexane Extract

Extract No.	Nonsaponified Extract, Mg. %		Saponified Extract, Mg. %	
1 Carotene	0.30	0.30	0.29	0.29
1 Lycopene	0.45	0.45	0.43	0.43
2 Carotene	0.16	0.17	0.18	0.18
2 Lycopene	0.21	0.21	0.20	0.19

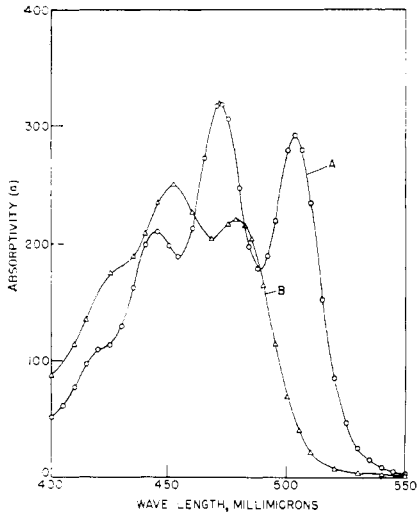

Figure 1. Spectral curves of purified lycopene (A) and purified carotene (B) in hexane

(AOAC) (*1*); lycopene prepared from tomato paste by the method of Davis (*3*); 10 and 50% by volume solutions of acetone in hexane; and 0.5 and 5% by volume solutions of methanol in hexane.

Methods

Preparation of Hexane Extract of Grapefruit Pigments

The pigmented material for extraction is prepared by halving 3 or 4 grapefruit and removing the edible meat and juice, while carefully excluding rag and seeds. The meat and juice are then blended in a Waring Blendor for 3 minutes. One hundred-gram portions are removed by pipet while the Blendor is operated at moderate speed. One hundred milliliters of methanol are added to each sample and the mixture allowed to stand for approximately 30 minutes. Two grams of Hyflo Super-Cel are then added to each portion and the samples filtered through a pad of this same filter aid on a Büchner funnel. The filtrate containing the methanol–water soluble substances is discarded.

The pigments are removed from the pulp by blending the filter cake in a microblender cup with 50 ml. of 50% acetone-hexane solution for 1 minute, filtering, and washing with 20 ml. of the extracting solution. The pulp and pad are blended, filtered, and washed twice more in the same manner; and the combined extracts are placed in a 500-ml. separatory funnel; 100 ml. of water are added and the aqueous acetone layer is removed and re-extracted with hexane until the hexane extract is colorless.

The combined hexane extracts are washed three times with 100-ml. portions of water, care being taken to prevent emulsions. After washing, the hexane extract is filtered through a pad of sodium sulfate on a medium-porosity fritted-glass funnel and made to a volume of 250 ml. with hexane. Portions of the extract are used in making the comparative analyses for carotene and lycopene with both methods A and B.

Method A. In preparation for chromatographing, a tube is packed to a depth of 10 cm. with a 1 to 1 weight mixture of Hyflo Super-Cel and magnesia and a 1-cm. layer of anhydrous sodium sulfate is placed above the absorbent. With the vacuum continuously applied, a mixture of 90 ml. of the above hexane extract plus 10 ml. of acetone is poured through the column. The carotene is eluted with 100 ml. of a solution of 10% acetone in hexane. The column is then washed with 100 ml. of a solution of 0.5% methanol in hexane, causing a small middle band to separate and move down the column ahead of the lycopene. The eluate containing this band is discarded. The lycopene is washed from the column with 200 ml. of 5% methanol in hexane. The carotene and lycopene eluates are transferred to separatory funnels and washed with 100-ml. portions of water (carotene twice and lycopene once) to remove acetone and methanol. Each fraction is then filtered through sodium sulfate on a fritted funnel to remove water and made to a volume of 200 ml. with hexane. Concentrations of the pigments are determined by measuring the absorbance of portions of the respective carotene and lycopene fractions in a Beckman Model DU spectrophotometer at a wave length of 451 mμ for carotene and 471 mμ for lycopene. The spectral curves of purified carotene and lycopene from which these maxima are taken are shown in Figure 1.

Method B. In method B the absorbance of a portion of the unchromatographed hexane extract is determined at 451 mμ (a maximum for carotene and a near minimum for lycopene), and at 503 mμ (a maximum for lycopene and a low absorbance value for carotene) (Figure 1). By means of simultaneous equations a measure of the total pigments present is obtained in terms of lycopene and carotene content. The pigment concentration of the unchromatographed extract used for method B is calculated as follows:

$$a = A/bc$$

where A = measured absorbance
b = cell length in centimeters
c = concentration of sample in grams per liter

and

$$\text{milligrams per cent of carotene} = 462\, a_{451} - 309\, a_{503}$$

$$\text{milligrams per cent of lycopene} = 395\, a_{503} - 80.5\, a_{451}$$

The constants in the above formulas were calculated using the specific absorbance of carotene at 451 and 503 mμ as 250.3 and 51.0, and lycopene at 451 and 503 mμ as 195.5 and 292.7. These values were obtained with a Beckman Model DU spectrophotometer.

Table II. Seasonal Carotene and Lycopene Content by Methods A and B

Date	Carotene, Mg. % Method A	Carotene, Mg. % Method B	Difference, % [(B − A)/B] × 100	Lycopene, Mg. % Method A	Lycopene, Mg. % Method B	Difference, % [(B − A)/B] × 100
1/26	0.27	0.33	18	0.23	0.24	4.2
2/2	0.34	0.41	17	0.28	0.29	3.4
2/9	0.31	0.37	16	0.19	0.20	5.0
2/23	0.24	0.31	23	0.09	0.11	18
2/29	0.22	0.28	21	0.17	0.19	11
3/8	0.24	0.29	17	0.14	0.19	26
3/25	0.27	0.33	18	0.16	0.17	5.9
3/22	0.28	0.34	18	0.15	0.16	6.2
3/28	0.22	0.28	21	0.09	0.12	25
4/4	0.21	0.27	22	0.10	0.10	0.0
Av.			19.1			10.5

Table III. Chromatography Recovery of Carotene and Lycopene by Method A

	Amount Added, Mg. %	Amount Originally Present, Mg. %	Total, Mg. %	Found, Mg. %	Recovery, %
Carotene	0.09	0.27	0.36	0.35	97.2
Carotene	0.09	0.27	0.36	0.35	97.2
Lycopene	0.06	0.16	0.22	0.22	100.0
Lycopene	0.06	0.17	0.23	0.22	95.7
Carotene	0.15	0.28	0.43	0.42	97.7
Carotene	0.15	0.28	0.43	0.42	97.7
Lycopene	0.13	0.15	0.28	0.27	96.4
Lycopene	0.13	0.15	0.28	0.25	89.3

Carotene av. = 97.5% recovery.
Lycopene av. = 95.4% recovery

Table IV. Reproducibility of Methods A and B[a]

Sample No.	Analyst	Carotene, Mg. % Method A	Carotene, Mg. % Method B	Difference, %, [(B − A)/A] × 100	Lycopene, Mg. % Method A	Lycopene, Mg. % Method B	Difference, %, [(B − A)/A] × 100
1	1	0.28, 0.28	0.28, 0.33	15.2	0.26, 0.26	0.28, 0.28	7.1
	2	0.26, 0.26	0.34, 0.34	17.6	0.26, 0.24	0.30, 0.29	15.3
	3	0.30, 0.31	0.33, 0.33	7.6	0.26, 0.26	0.28, 0.29	8.8
2	1	0.32, 0.31	0.35, 0.34	8.7	0.24, 0.23	0.26, 0.26	9.6
	2	0.30, 0.31	0.35, 0.33	10.3	0.23, 0.24	0.24, 0.26	6.0
	3	0.32, 0.32	0.35, 0.35	8.6	0.23, 0.23	0.26, 0.26	11.5
3	1	0.29, 0.29	0.36, 0.36	19.4	0.19, 0.20	0.22, 0.23	13.3
	2	0.27, 0.27	0.35, 0.34	21.7	0.20, 0.19	0.22, 0.23	11.4
	3	0.32, 0.32	0.36, 0.36	11.1	0.21, 0.21	0.23, 0.23	8.7
4	1	0.30, 0.29	0.34, 0.33	11.9	0.20, 0.21	0.22, 0.23	8.9
	2	0.30, 0.28	0.34, 0.34	14.7	0.21, 0.19	0.20, 0.22	5.0
	3	0.31, 0.31	0.36, 0.35	12.7	0.20, 0.17	0.23, 0.21	15.9
Av.				13.3			10.1
Av. of previous series Table II				19.1			10.5
Av. of two series				16.2			10.3

[a] A Cenco-Sheard spectrophotometer standardized with solutions of pure β-carotene and lycopene, was used in obtaining data reported in this table.

Table V. Comparison of Different Absorbents by Method A

Sample No.	Absorbent	Solvent	Mg. % Carotene	Mg. % Lycopene
1	MgO No. 2641	Hexane	0.26, 0.26	0.14, 0.15
	MgO No. 2642	Hexane	0.27, 0.27	0.15, 0.16
	Sea Sorb	Hexane	0.27, 0.27	0.15, 0.15
2	MgO No. 2642	Hexane	0.22, 0.23	0.18, 0.17
	Sea Sorb	Hexane	0.22, 0.23	0.15, 0.15
3	MgO No. 2641	Benzene	0.18	0.12
	MgO No. 2642	Benzene	0.18	0.14
4	MgO No. 2641	Hexane	0.24	0.08
	MgO No. 2642	Hexane	0.24	0.10
5	MgO No. 2641	Benzene	0.20	0.10
	MgO No. 2642	Benzene	0.17	0.10
6	MgO No. 2641	Hexane	0.31	0.18
	MgO No. 2642	Hexane	0.31	0.20

Table VI. Effect of Time in Solvent on Absorbance of Lycopene

Pigment	Solvent	Time in Solvent, Min.	Absorbance (A) at Maxima	Wave Length at Maxima, Mμ
Lycopene	Hexane	0	0.732	472
Lycopene	Hexane	75	0.719	472
Lycopene	Hexane	110	0.719	471
Lycopene	Hexane	170	0.678	471
Lycopene	Benzene	0	0.782	486
Lycopene	Benzene	60	0.769	485
Lycopene	Benzene	95	0.769	486

A more complete description of the method and its correlation with visual and reflectance measurements of fruit color has been reported (*6*).

Investigation of Methodology and Comparison of Methods

Saponification. Extracts of some plant materials require saponification before good analytical results can be obtained, but this was not observed to be the case with grapefruit. Early in the study, a comparison was made of the quantitative effect of saponification on the pigments. Saponified and nonsaponified samples were measured on the same day. Analytical data in Table I indicated that the tendency was for the nonsaponified samples to give insignificantly but consistently higher values; hence, the saponification step was omitted.

Methods A and B Applied to Analysis of Carotene and Lycopene in Seasonal Samples of Grapefruit. Data are given in Table II for Ruby Red grapefruit harvested at 1-week intervals between and including January 26 and April 4, 1956. Method A, the chromatographic-spectrophotometric method, gives analytical results for both carotene and lycopene which are below the values obtained on the same hexane extract samples with the spectrophotometric (binary) method B. In the series of analyses (Table II) the carotene values obtained by method A averaged 19.1%, and the lycopene values were 10.5%. In another series of analyses, to compare the reproducibility of the two methods, the carotene values averaged 13.3% and the lycopene were 10.1% (Table IV).

The sharp decreases in lycopene content in the fruit harvested over the 69-day period (0.29 mg. % to 0.10 mg. %) parallels the decrease in visual and reflectance measurements of color intensity observed in the seasonal study of the year before (*6*). Carotene is more stable than lycopene or at least does not decrease in grapefruit during the harvesting season to the same extent as lycopene. As shown in Table II, the decrease in 69 days was from 0.34 mg. % to 0.21 mg. %.

Pigment Recovery Test with Method A. Measured volumes of standard β-carotene and lycopene solutions in hexane were added to aliquots of a given hexane extract of grapefruit pigments to be chromatographed. Sufficient acetone was added in each case to make the mixture 10% in acetone with respect to hexane. Recovery of pigments after chromatographing averaged 97.5% for carotene and 95.4% for lycopene (Table III).

Reproducibility with Method A. To determine the reproducibility of method A, tests were made on independent samples of four lots of grapefruit pulp by three analysts. The results (Table IV) show close agreement between duplicate analyses of the same sample. Agreement between three analysts was also good. Statistical evaluation of the data indicate that the analyses are correct in milligrams per 100 grams of sample within ±0.012 mg. for lycopene and ±0.013 mg. for carotene at the 0.05 probability level.

Similar data for method B which measures total pigment content as carotene and lycopene, are included in Table IV for comparison.

Miscellaneous Observations Relative to Method A. A limited number of tests were made comparing the absorptives Westvaco Micron Brand No. 2641 and No. 2642 and Sea Sorb No. 43. Slightly lower lycopene values were obtained when magnesia No. 2641 was used in the chromatographic column (Table V).

The absorbance of lycopene at the maxima decreased slightly in both hexane and benzene solvents after standing an hour (Table VI).

Results and Discussion

Addition of methanol to the blended grapefruit sample during the preparation of the pigment extract serves to

precipitate the pectinaceous material and aggregate the cellular material containing the carotenoid pigments. Filter aid is added to facilitate filtration and removal of the water-soluble constituents. Extraction of the aqueous-methanol filtrate with hexane has shown that no lycopene or carotene are discarded with this filtrate. With adequate blending, three extractions of the pulp-filter aid mixture with a 50 to 50 acetone-hexane solution is sufficient to remove all of the carotene and lycopene. Removal of the acetone and drying the hexane is necessary before good adsorption of the pigments can be obtained upon chromatographing.

Saponification of the extract does not appear to have an effect on pigment values sufficiently great to justify the extra step. Carotene and lycopene are both relatively unstable pigments, and different absorbents and even different lots of the same absorbent may influence their measurement. Similarly, delay in analyzing the extracts for an hour or more may influence results. The experimental results reported in this paper emphasize the necessity of working through the analytical steps without undue delay.

Comparison of the values obtained for carotene and lycopene by the chromatographic-spectrophotometric method A, with those obtained by the spectrophotometric binary method B, show that the latter procedure gives values which are 10 to 15% higher. The reason is that the latter procedure, B, measures all the water-insoluble pigments present in terms of carotene and lycopene. Unpublished data obtained in the U. S. Fruit and Vegetable Products Laboratory confirm results reported by Khan (*4*) and Curl (*2*), that carotene and lycopene comprise approximately 80 to 90% of the total carotenoid pigments of Ruby Red grapefruit.

Method B offers a simple rapid procedure for estimating total pigment in terms of lycopene and carotene, for which most laboratories might be expected to have the skill and equipment required. A fairly close approximation of the analytical values for lycopene and carotene obtained with method A can be obtained with method B by subtracting correction factors from the results of the binary calculations, 10.3% for lycopene and 16.2% for carotene.

Method A is an analytical method. It requires the chromatographic separation of the pigments, the separate elution of the lycopene and carotene bands, and their quantitative spectrophotometric measurement. The absorption curves of these two pigments, separated in accordance with the recommended procedure, have been compared with absorption curves of pure β-carotene and lycopene. This comparison shows that the chromatographic procedure is successfully separating the two pigments. Precision and accuracy (recovery) tests have been made which show that method A is satisfactory.

Test data, as well as establishing the value and limitations of two methods for determining the pigment content of colored grapefruit, provide additional information confirming quantitative changes in the pigment content during the January–April period of the harvesting season (*5*, *6*). In this period the total pigment decreases as the season progresses; lycopene decreases regularly and carotene remains relatively constant until the latter part of the season. The trends are the same from year to year but the dates on which the pigment content changes significantly will vary. This fact undoubedly reflects changes in environmental conditions, sampling variables, and the like.

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MILK IRRADIATION

Irradiation Preservation of Milk and Milk Products

JOHN H. WERTHEIM, RATHINDRA N. ROYCHOUDHURY, JOHAN HOFF, SAMUEL A. GOLDBLITH, and BERNARD E. PROCTOR

Department of Food Technology, Massachusetts Institute of Technology, Cambridge, Mass.

A method was developed which increased the volatility of the off-flavor complex, or its precursors, of irradiated milk so that milk irradiated with 2 × 10^6 rep of gamma rays was obtained free from detectable off-flavors. The increase in the sensitivity of irradiated milk to browning, caused by the formation of reductones, was further studied. The production of chalky off-flavors from milk fat was caused partly by the formation of peroxides of the more saturated components, whereas oxidized off-flavors were caused by highly unsaturated fractions of the butterfat. A method was found by which milk or milk concentrates might be sterilized by cold sterilization—i.e., by the application of ionizing radiations.

Problems connected with the sterilization of milk by ionizing radiations have been delineated and described (*4*, *6*, *9–11*, *18*, *19*).

The study of the nature and origin of some of the chemical changes in milk and the possible development of means for preventing some of these changes is described herein.

Two reasons make it desirable to pay particular attention to the problems encountered in the radiation preservation of milk: The application of ionizing radiations results in stronger and more undesirable off-flavors than in any food as yet studied, and experience gained from research on these flavor changes in milk may prove applicable to other less difficult foods. Furthermore, milk is one of the few foods that can be separated into groups of components whose behavior can be studied independently, checked objectively, and then evaluated by recombining them into milk of the original composition.

Approximately 5 to 7.5 × 10^5 rep have been found to be sufficient for the