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Effect of freezing, canning and drying on the vitamin A (Carotene) content of spinach

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INTRODUCTION AND PURPOSE

The vitamin A activity of spinach has been determined by various workers, Eddy et al. (1925), (1929), Hanning (1935), Hume and Smith (1928), and Kifer and Munsell (1932). Carotene has been shown to be a precursor of vitamin A by Goldblatt and Barnet (1932), Kuhn and Brockmann (1933) and others, but little research has been carried out on a correlation between the carotene content and vitamin A potency.

This investigation was conducted to determine the relationship between carotene content and vitamin A activity of green leaf materials with special reference to spinach. In addition, the effect of canning, freezing and drying on the vitamin A potency was determined. The relationship between the carotene content and the vitamin A activity might provide a constant which would permit the utilization of a comparatively rapid chemical method to replace the long bioassay period. Commercially, the shorter method might provide a means for estimating the vitamin A constant of feeds and foodstuffs.

REVIEW OF LITERATURE

Three general methods for vitamin A or carotene determination have been proposed: 1. the animal or bioassay method which is the standard procedure; 2. spectrophotometric method which is too expensive for most laboratories; 3. the colorimetric which formerly has not been considered reliable.

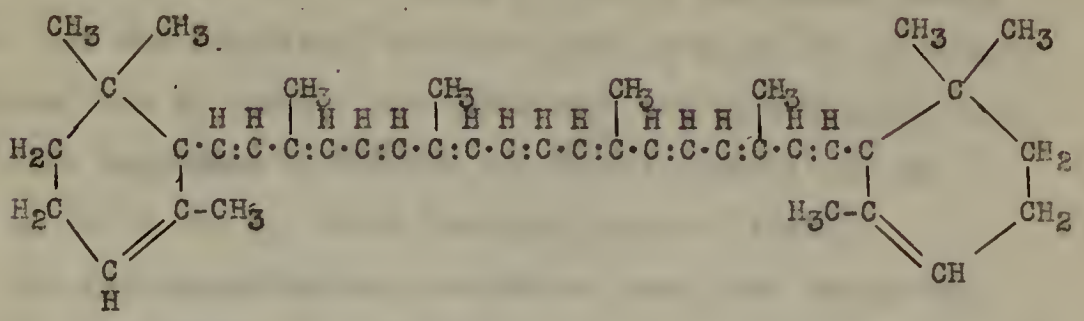
Green leaf plants were shown by Palmer (1922) to contain three predominant pigments; namely, chlorophyll, xanthophyll and carotene. Carotene is the only one of the three which has been shown to contain appreciable vitamin A activity. The problem carotene determination is the removal of these interfering pigments without loss or destruction of the carotene. At least three forms of carotene have been isolated from plants: alpha-carotene by Karrer et al. (1933), beta-carotene by Karrer and Morf (1931), and gamma-carotene by Kuhn and Lederer (1931). The latter state that spinach contains only beta-carotene, while Karrer and Schlientz (1934) claim traces of alpha-carotene; the presence of gamma-carotene in spinach has not been demonstrated.

Vitamin A as such is not found in plants. According to theory, the beta-carotene is broken down in the body, presumably in the liver. The split occurs at the position indicated in the beta-carotene formula. Formulas are given on the next page.

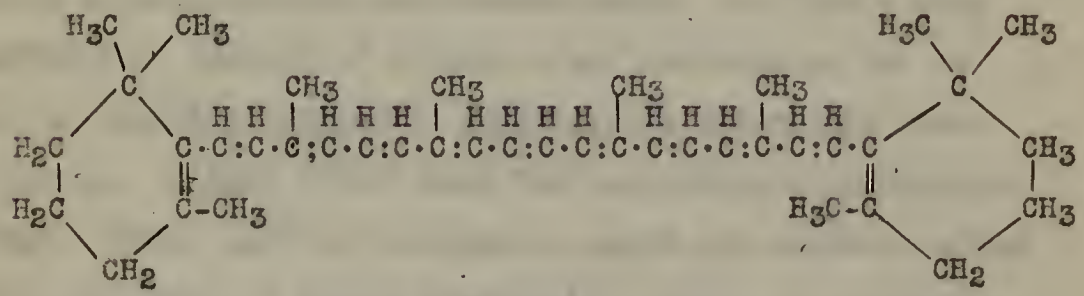
Three general methods for vitamin A or carotene determination have been proposed: (1) the animal or bioassay method; (2) the spectro-photometric method and (3) the colorimetric method.

The first of these, the rat or bioassay method is the standard procedure. The method was developed by Sherman and Munsell (1925) and substanially adopted as an official method by the United States Pharmacopeia, Tenth Revision (1934).

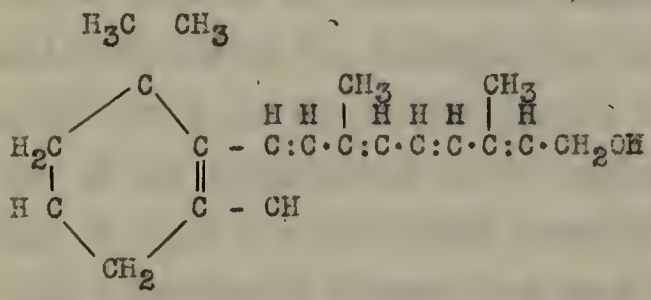
The formulae for alpha-carotene, beta-carotene and vitamin A are:



alpha-carotene from Karrer, Morf and Walker (1933)



beta-carotene from Karrer and Morf (1931)



According to this method, when 1.4 international units are fed daily, the depleted animals will show a weight gain of three grams per week. The assay period is four weeks during which time the individual rat must gain at least 12 grams and not more than 60 grams. The basal diet and salt mixture both are those suggested by Osborne and Mendel (1917), and now used in the U. S. P., Tenth Revision, Method (1934).

The spectrophotometric method has been used by various workers. De (1936) suggested the use of a spectrograph with sufficient dispersion to determine carotene in the presence of pigments closely related spectroscopically. De (1935) also suggested that amounts of vitamin A and carotene may be obtained by studying extinction coefficients before and after irradiation. Miller (1935) used the separation of Willstatter and Stoll (1913) and has developed a rapid and accurate method for determination of common carotenoids using a spectrograph. In another paper Miller (1934) gave quantitative absorption spectra for some of the common carotenoids. Schertz, (1932) an earlier worker in the determination of carotene, compared the colorimetric and spectrophotometric methods and decided in favor of the spectrograph partly because the colorimeter involves at least a five percent personal error.

Many colorimetric methods have been proposed and used, none of which, until recently, were considered accurate as measured by the corroboration of the vitamin A biossay. Arnaud (1889) is credited as having made the first colorimetric determination of carotene. Krogis (1934) proposed a

modified method of $K_2Cr_2O_7$ oxidation of carotene to give a color test. Ferguson and Bishop (1938), used a 20 percent solution of potassium hydroxide in methyl alcohol to extract carotene and xanthophyll directly. Guilbert's method (1934) is quite similar in principle to the method of Willstatter and Stoll (1918), except for the primary step in which ethyl ether used for the extraction must be of the highest purity to prevent oxidation. Russell et al. (1935) described a modified Willstatter method and gave as a standard a 0.0036 percent $K_2Cr_2O_7$ solution equivalent to a carotene solution containing 0.0021 milligrams per cubic centimeter. Strain (1934) proposed a qualitative colorimetric method based on the varying absorption of carotene on a magnesium oxide column. This dual absorption on magnesium oxide is a qualitative means of separating the various carotenes present in a specimen. Deleano and Dick (1933) found that carotene is ozonostrable and may be determined by dissolving the carotene in acetic acid, oxidizing in excess of potassium dichromate in the presence of hydrochloric acid, and then titrating the excess potassium dichromate iodometrically. This method might be feasible if there were no other oxidizing agents present. The reaction with antemony tri-chloride or the Carr- rice reaction has been proposed as a means of determining carotene and vitamin A as such, and also carotene, but Woolf and Moore (1938) and others found that an increase in intensity of the reaction does not mean formation of vitamin A.

EXPERIMENTAL PROCEDURE

Source and Preparation of Samples

In order to have comparable results, it was necessay to use the fresh, frozen canned and dried spinach from the same source. The same samples were used for the carotene determinations as well as for the rat biological assay for vitamin A in order to obtain a direct comparison of the two methods. A bushel of spinach brought in a local store was washed, cleaned, thoroughly mixed and divided into four equal parts. One part was stored in the fresh condition in a closed paper bag at 0-2°C. for the duration of the rat assay. The remainder was blanched with steam for four minutes and then one portion was canned in one-half pint glass jars at 115°C for 50 minutes. Another portion was frozen at 23°C, also in one-half pint glass jars and kept at that temperature until ready to be used. The remainder of the spinach was dried overnight in a tunnel drier at 60°C. After drying, the spinach was ground and sifted through a 40-mesh sieve and sealed in a two-quart jar in order to prevent the absorption of moisture. The moisture content of these samples of spinach was as follows:

Fresh	90.1 percent moisture
Frozen	85.6 percent moisture
Canned	92.0 percent moisture
Dried	5.0 percent moisture

EXPERIMENTAL METHODS

The biological assay was conducted according to the methods of Sherman and Munsell (1925), using eight rats per level. In the basal diet used in our laboratory, Crisco was substituted for vegetable oil; Crisco is a hydrogenated vegetable oil and therefore does not contain vitamin A. Dried yeast was purchased from the Standard Brands, Inc., New York.

Since the results (shown in Table 1.) obtained with the first lot of samples indicated higher values than those previously reported, the experiment was repeated using a second batch of spinach treated exactly the same as the first lot. By using two lots of spinach we were also enabled to compare market samples at two different periods of the year and also test the accuracy of the assay technic. In each of these experiments a control set of eight rats was maintained; the controls were fed with a U.S.P. standard reference oil so as to receive 1.4 international units daily in 0.1 grains of olive oil as a neutral, inert carrier. Coward and Key (1934) found that it was not necessary to feed the vitamin A supplement daily. The procedure used in this experiment was to feed the spinach at intervals of one week. The method used may be an explanation of the high results obtained for vitamin A value of spinach. The bioassay results are discussed in connection with the carotene data.

Basal Diet	Percent	Salt Mixture	Grams
Casein	18	$CaCO_3$	134.8
Salt Mixture	4	$MgCO_3$	24.2
Yeast Dried	8	Na_2CO_3	34.2
Starch	65	K_2CO_3	141.3
Crisco	5	Ortho-phosphoric acid	103.2
		H CL	53.4
		H_2SO_4	9.2
		Citric acid	111.1
		Ferric citrate $1\frac{1}{2}H_2O$	6.34
		KI	.02
		$MnSO_4$	.079
		Na F	.248
		Potash alum	.024

After a rather intensive survey of the available methods of carotene determination by means of the colorimeter, it was decided that a combination of the Russell, Taylor and Chichester (1935), Holmes (1932) and the original Willstätter and Stoll (1913) methods should be used.

Preliminary work with the colorimetric determination of carotene described by Russell, Taylor and Chichester (1935) showed the main difficulty to be the insufficient and varying degrees of carotene extraction from the leaf. The first modification was to triturate the sample with vitamin-free

sand. Although this additional step resulted in an increase of petroleum ether-extracted carotene in the final solution, the results were still varying. This was due to the spinach being imbedded in the sand. The petroleum ether was of such a low specific gravity that the sand and spinach mixture was not agitated sufficiently for the solvent to have easy access to the sample. Holmes and Leicester (1932) demonstrated that a three normal sodium hydroxide solution facilitated the disintegration of the plant cellulose and helped to hydrolyze the chlorophyll to a water-soluble substance. Consequently, 25 cubic centimeters of 3 N sodium hydroxide were added to each sample immediately after trituration. Optimum extraction conditions were dependent on the amount of shaking and the volume of the sample. Russell, Taylor and Chichester (1935) advocated that a final petroleum ether volume of 400 cubic centimeters was adequate.

The method as finally adopted, in detail, is as follows: A sample is taken which contains about 0.2 milligram of carotene. In this investigation .500 grams of spinach proved to be a satisfactory amount. The spinach is ground with 25 grams of finely divided quartz sand of the type used for vitamin C titrations and placed in a 350 cubic centimeter dilution bottle. To the sand-spinach mixture is added 25 cubic centimeters of 3 N sodium hydroxide and 100 cubic centimeters of petroleum ether. This combination is shaken mechanically for 90 minutes. The mixture is then

introduced into a liter separatory funnel, where the sand, alkali and ground spinach are allowed to settle to the bottom. The alkali, sand and spinach are returned to the dilution bottle and 100 cubic centimeters of fresh petroleum ether are added. The shaking is renewed for a 15 minute period, and the procedure of separation repeated. A third and fourth extraction of 15 minutes each remove all the petroleum ether-soluble material from the sample.

The chlorophylls are next removed by adding 50 cubic centimeter portions of 89 percent methyl alcohol and shaking mechanically for two minutes. The best procedure was found to be the addition of the 50 cubic centimeter portions of methyl alcohol to the total 400 cubic centimeters of petroleum ether-extract in the separatory funnel. This process is repeated until no coloring matter is ~~visible~~ visible extracted by the methyl alcohol.

The xanthophylls are removed by the addition of a 25 percent solution of potassium hydroxide in absolute methyl alcohol. A 25 cubic centimeter portion of this alcoholic potash is added to the solution in the separatory funnel, mechanically shaken for two minutes, and then immediately separated. Repeated extractions are made until no color is given in the colorimeter by an alcoholic potash extraction when compared against a standard containing the equivalent of a 0.0004 milligrams of carotene per cubic centimeter.

It was found best by experience to make one more extraction after the colorless one.

The alkali and water are removed from the petroleum ether with three, 50 cubic centimeter portions of distilled water and shaking a few times by hand remove the dissolved alkali and also carry down any sand which is in suspension. Washing with a 90 percent methyl alcohol solution serves to remove an excess of water present. The petroleum ether is then transferred to suitable beakers and evaporated in vacuo at room temperature (24°C.) to about 75 cubic centimeters: This volume is most easily attained by marking the beakers before starting the evaporation process. This petroleum ether solution is then transferred quantitatively to a 100 cubic centimeter volumetric flask, the beaker rinsed with small amounts of fresh petroleum ether, which are added up to the 100 cubic centimeter mark.

The petroleum ether solution is now compared against a standard of known concentration and the amount of carotene in milligrams is computed. The formula for calculating the amount of carotene is:

$$\frac{\text{Depth of Standard}}{\text{Colorimeter reading}} \times \frac{100}{\text{Amount of sample in grams}} \times \frac{\text{Total volume of unknown}}{1000} \times \text{Value of standard in milligrams of carotene per liter} = \text{milligrams of carotene in 100 grams of sample.}$$

The colorimeter used in this investigation was a Klett biological colorimeter having a daylight attachment. Each step of the method should be performed in the absence of direct sunlight; for, Quinn, Hartley and Derow (1930) showed that a three to five hour irradiation of a petroleum ether solution completely destroyed carotene activity.

The standard used was that of Russell, Taylor and Chichester (1935), in which a 0.0038 percent $K_2Cr_2O_7$ solution was found to be equivalent to 0.0021 milligrams of carotene per cubic centimeter. In the course of the investigation it was found that sometimes the concentrate was greater or lesser in intensity than the range of the standard. Since no data could be found to remedy this situation, the assumption was made that a change in the concentration of the potassium dichromate standard would be equivalent to a similar change in carotene concentration for a limited range. This was considered as justified later in that investigation with a Bully absorption tube and a Bausch and Lomb laboratory wave-length spectrometer, the readings for both the potassium dichromate and carotene solutions were found to be similar for the concentrations used in the experiment. Pieri (1935) and Miller (1934) both found that Beer's law holds over a small range in solutions containing carotene; i.e., the absorption varies with the concentration.

DISCUSSION OF RESULTS

The results of the biological assay and colorimeter methods are to be found in Table 1 and 2, respectively. The bioassay method showed fresh spinach to contain an average of 680 international units of vitamin A per gram as compared with 428 international units of vitamin A per gram for the canned spinach. The colorimetric method gave values of 430 micrograms of carotene per gram for fresh spinach as compared with 245 micrograms per gram for the canned. In order to compare the results of both methods, all values were converted to international units of vitamin A expressed on a dry basis. This comparison is shown in Table 2. An average percentage difference between the chemical and biological methods for each of the four sets of results is 20 percent in one case and less than ten percent in others.

The vitamin A potency results of spinach for this investigation are somewhat higher than those previously reported. The work of Hume and Smith (1928) placed the vitamin A potency of fresh spinach at about 100 international units. Manning (1933) found that 20 milligrams of canned spinach per day was the minimum requirement of the rat, so that her results give spinach a vitamin A potency of 75 international units. Kifer and Munsell (1932) showed that 12 milligrams daily gave a three gram per week gain, thereby indicating that the spinach assayed about 120 international units per gram. Eddy, Mohman and Carlsson (1925) stated that 0.026

grams daily were sufficient for normal growth, which, from their tables is about 20 grams in 25 days; this gives spinach a value of about 110 international units per gram. McLaughlin (1929) found that with small leaves 70 milligrams per week gave a 25 gram gain in eight weeks; from these figures the New Zealand spinach assays about 140 international units. Mitchell (1933) stated that ten milligrams of spinach are equivalent to one Sherman unit, or 140 international units per gram. Fraps and Treichler (1933) gave canned spinach as 100-140 Sherman units or therefore 140-200 international units. They also gave spinach that was dried in a vacuum and canned a value of 337 Sherman units or 480 international units per gram. This result compares favorably with the estimated vitamin A potency for dried spinach found in this investigation. Holmes and Leicester (1932) found that the carotene values for dehydrated spinach had 1.5 milligrams per 100 grams or 25 international units per gram. Fresh spinach had 4.8 milligrams per 100 grams or 80 international units per gram. Canned spinach had 15.4 milligrams per 100 grams or 260 international units per gram. Stanley and Stillman (1924), in a study of the effects of preparation on the vitamin A content of spinach found it necessary to feed four grams per day of raw, fresh spinach and five grams daily of boiled fresh spinach. These results would give spinach a value of 2 international units per gram. Steam cooked, commercially canned and commercially dried spinach could not be fed in

sufficient amounts to give normal growth for 60-100 gram rats. Such a variation in reported results leaves open to question of just how much spinach should be fed, and also how much carotene must be fed in order to obtain optimal growth.

Goldblatt and Barnett (1932) showed that five-tenths of a microgram of carotene equalled one Sherman unit or 1.4 international units. This result is 0.1 microgram less than the international standard given by Hume and Chick (1935) in the report of the British Medical Research Council. This bulletin defines the international standard of vitamin A as the potency of six-tenths of a microgram of pure beta-carotene. Green and Halliday (1930) fed 0.04 milligrams of carotene to obtain normal growth, while Hume and Smith (1928) fed only 0.003 milligrams per day. Kuhn and Brockmann (1933) showed that five micrograms of gamma-carotene per day could cure xerophthalmia. Olcovich and Mattill (1930) by feeding 0.005 milligrams per day of carotene obtained a daily growth of two grams in their rats. Polak and Stokvis (1931) found 0.5 micrograms per day to be sufficient. The international standard as defined above gives the relation of vitamin A potency of carotene to micrograms of carotene as 1.6 or a ratio of 1.7. The work of Fraps and Treichler (1936) was the only investigation reported that definitely attempted to correlate carotene content with vitamin A potency. They came to the conclusion that one microgram of carotene as determined in feedstuffs was equivalent to 1.3-2.2 units of vitamin A. Their average

results indicated a ratio of 1.6. Table 3 shows that the correlation for fresh and canned spinach in this investigation was found to be 1.7. Russell, Taylor and Chichester (1932) stated in one paper that carotene was much higher in potency when fed than when determined in the plant. In another paper Russell, Taylor and Chichester (1934) give a table showing the carotene determined, the gain in weight of rats, and the amounts of feed given daily. The liberty was taken with these figures to convert them into a ratio of vitamin A potency per gram of feed to micrograms of carotene per gram of feed. The ratio was found to vary from 1.8 - 4.0 and averaged 2.9. Although their results showed a rather wide variation in general, those samples which showed a higher carotene content gave the best growth.

At this point it seems pertinent to state that Ahmad (1931) demonstrated that in the conversion of small amounts of carotene into vitamin A as much as 90 percent of the carotene ingested was excreted. Moore (1933) called attention to the fact that minimum doses of supplement should be fed.

On the basis that vitamin A potency to carotene content in micrograms is about 1.7, it becomes possible to estimate vitamin A activity of feedstuffs from carotene content. In Table 2, this factor is used to calculate the results in international units of vitamin A for the frozen and dried

spinach as well as fresh and canned.

The storage results over a three month period may be found in Table 2. The storage was made in refrigerators unaffected by natural light, either direct or diffused; for, Quinn, Hartley and Perow (1930) found that dried spinach lost 70 percent of its vitamin A on storage for 18 months in diffused light. Schaunert, and Schlieblich showed that international standard carotene in oxygen-free sesame oil was unimpaired after 18 months. The canned and frozen spinach used in this investigation showed no loss of carotene on storage. These results on storage agree with the work of Eddy, Kohman and Halliday (1929) who found that the quantities of vitamins A and C in spinach stored for three years was practically the same as the content in freshly canned spinach.

Table I. Vitamin A Content of Fresh and Canned Spinach as Determined by Rat Bioassay

Weight of spinach fed daily		Average rat weights		Average gain in weight	Moisture content	Vitamin A potency in international units per gram	
		start	after 28 days			moist basis	dry basis
Date	milligrams	grams	grams	grams	per cent		
Lot 1 - fresh							
12/26/36	6	74	110	36	90.1	700	7070
12/26/36	5.7	78	110	32	90.1	654	6606
Lot 2 - fresh							
3/8/37	4.3	91	118	27	90.1	720	7272
3/8/37	4.3	98	121	23	90.1	650	6565
Lot 1 - canned							
12/26/36	7.8	72	112	40	92.0	590	7375
12/26/36	5.7	82	99	17	92.0	368	4600
Lot 2 - canned							
3/8/37	5.7	90	109	29	92.0	580	7250
Standard Reference Oil							
12/26/36	100	90	105	15		16	
3/8/37	100	104	118	14		15	

Table II. Carotene Content of Fresh, Frozen, Canned and Dried Spinach

Sample	Carotene content in micrograms per gram		Vitamin A potency using factor 1.7 international units		Bioassay results from Table I. international units	Percentage difference between chemical and biological methods
	12/26/36	3/8/37	moist basis	dry basis	dry basis	
Lot 1						
Fresh	415		784	7919	7070	
	426		682	6880	6606	8
Frozen	380	384	595	4131		
	402	420	656	4916		
Canned	239	240	384	4800	7375	
	252	259	410	5124	4600	20
Lot 2						
Fresh		400	640	6464	7272	
		480	768	7757	6565	3
Frozen		300	480	3333		
		282	451	3131		
Canned		345	552	6900	7250	5
		360	576	7200		
Dried		319	510	536	~~none~~	
		300	480	505		

Table III. Correlation of Vitamin A Potency to Carotene Content from Experimental Data

International Units of Vitamin A per gram = Ratio

Micrograms of carotene per gram

Fresh	$\frac{700}{415}$	= 1.7
	$\frac{654}{426}$	= 1.5
Canned	$\frac{590}{239}$	= 2.4
	$\frac{368}{252}$	= 1.4
Fresh	$\frac{720}{480}$	= 1.5
	$\frac{650}{400}$	= 1.6
Canned	$\frac{580}{352}$	= 1.6

7) 11.7

1.7 = Ratio

SUMMARY

1. A colorimetric method for carotene determination is given in detail. The modifications of the Willstätter and Stoll and other methods are: (1) addition of 25 cubic centimeters of three normal sodium hydroxide, (2) trituration with vitamin-free sand and, (3) mechanical agitation for optimum extraction. Optimum extraction occurs with a period of 90 minutes and three subsequent ones of 15 minutes each.

2. The vitamin A potency of fresh spinach is 600 international units per gram; canned spinach is 513 units per gram according to the biological assay.

3. The mean ratio of the vitamin A potency as determined by the U.S.P. method for rat assay, to carotene content as determined by the present method on two lots of spinach is 1.7. This figure closely approximates the figure 1.6 lately reported by Fraps, Treichler and Kemmerrer (1936).

4. The colorimetric standard used is a .0036 percent potassium dichromate solution equivalent to .0021 milligrams of carotene per cubic centimeter. Due to variation in carotene content of individual samples it was found necessary to compare the absorption range of the potassium dichromate solution with an equivalent carotene solution by means of a Bally absorption tube and a spectrograph. The results corroborated the work of Pieri (1935) and Miller (1935) who also found that carotene solutions follow Beer's Law for a limited range.

5. By use of the ratio figure 1.7, the vitamin A potency as determined by the carotene content of fresh, frozen canned and dried spinach is 718, 545, 475, and 495 international units per gram, respectively.

6. Storage for three months has no appreciable effect on the carotene content of frozen and canned spinach.

7. Weekly feeding rather than daily feeding is given as a possible reason for the high vitamin A potency of spinach assayed biologically.

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