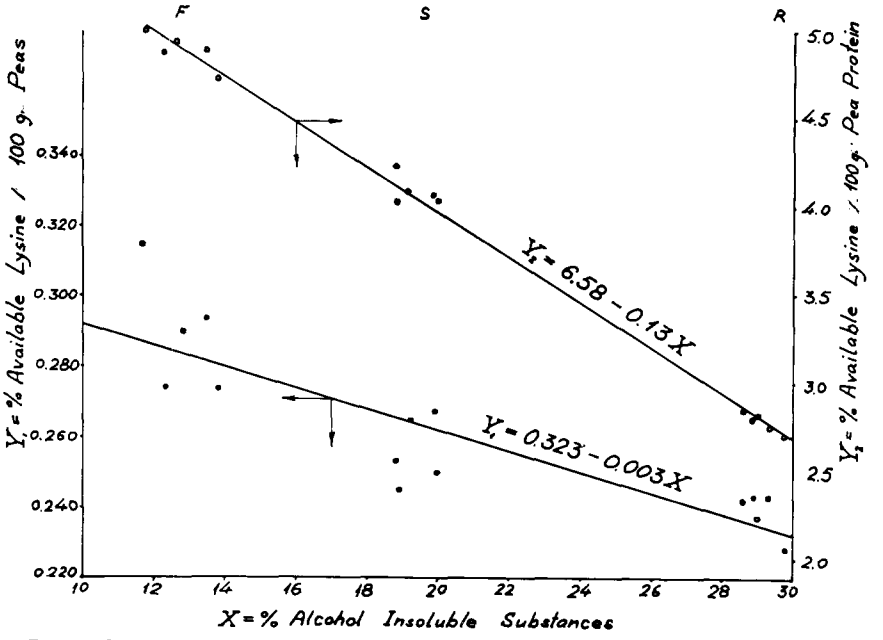

Figure 1. Relation of available lysine in peas and pea protein to alcohol-insoluble substances in peas

in canned peas and their protein, with decreasing grade (Equations 1 and 2).

$$Y_1 = 0.323 - 0.003\,X \quad (1)$$

$$Y_2 = 6.58 - 0.13\,X \quad (2)$$

where

Y_1 = concentration of available lysine in 100 grams of peas
Y_2 = concentration of available lysine in 100 grams of pea protein
X = AIS content in peas

It may be assumed, therefore, that the nutritive value of canned peas as a source of available lysine decreases with increasing maturity of the peas.

Figure 1 shows the regression lines and their equations between available lysine in the canned peas and their protein and the AIS content in the different grades. The correlation coefficient used as a measure of significance for the equations is 0.83 for the first equation (Y_1) and 0.99 for the second (Y_2), as against 0.641 at the 1% level of significance. The close negative correlation between both these parameters for the samples analyzed by the authors shows that the nutritive value of canned peas as a supplementary source of available lysine can be predicted from the AIS content. Reduced acceptability rating of canned peas corresponds to a reduced supplementary protein value.

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CAROTENOIDS DETERMINATION

Influence on the Estimation of β-Carotene by Other Carotenoids in Butternut Squashes at Harvest and during Storage

EDWARD P. LEWIS and SUSAN B. MERROW

Department of Home Economics, University of Vermont, Burlington, Vermont

The procedure of the Association of Official Agricultural Chemists (*1*), designed primarily for the rapid analyses of leaf and stem tissues, has been widely used to determine the β-carotene content of plant materials. The validity of this method depends upon the assumption that the major portion of the carotenoid hydrocarbons is in the form of β-carotene. However, if other carotenoids are present, they may be reflected in the values obtained.

Fujita and Ajisaka (*4*) reported only the α- and β-carotene content of *Cucurbita moschata* (Kintônasu) and *C. moschata* var. melonaeformis (Kabotya). To our knowledge the other carotenoids of *C. moschata* Duch., which include the hydroxycarotenoids eluted by the 10% acetone-hexane, have not been identified.

An early attempt at identification of the carotenoids of squashes using *C. moschata* Duch. (Giant squash) was made by Suginome and Ueno (*10*). They reported two carotenoids, "cucurbiten" and "cucurbitaxanthin," which they

This study was made to determine the extent to which other carotenoids influence the estimation of the β-carotene content of Butternut squashes by the AOAC method at harvest and during storage. The β-carotene was determined first by the AOAC method and then by partition chromatography. The β-carotene values after partitioning averaged from 17.6 to 26.7%, the α-carotene from 11.0 to 20.3%, and the hydroxycarotenoids from 43.3 to 46.4% of the AOAC β-carotene values.

thought were unknown. Zechmeister and Tuzcon (*11*) repeated the work of the Japanese authors. They found that "cucurbiten" was composed of 2% α-carotene and 98% β-carotene and "cucurbitaxanthin" was composed of lutein (xanthophyll) and violaxanthin.

In a previous study (*5*), the β-carotene content of Butternut and Buttercup squashes as determined by a modified AOAC method increased significantly after 5 and 10 weeks of storage, respectively. Among the factors considered in interpreting this increase was the possibility that the relative amounts of the carotenoid pigments might be changing during storage.

The method published by Purcell (*6*) offered a reasonably simple method of fractionation of carotenoids. In a preliminary investigation it was found that the β-carotene values obtained by the AOAC method were consistently higher than the values obtained by the Purcell method. The hydrocarbon fraction of Baby Blue, Silver Bell, and Sweet Meat squashes of the *C. maxima* species was found to contain chiefly β-carotene and no α-carotene. In contrast, the Butternut squashes of the *C. moschata* species contained both α-carotene and β-carotene.

This study was made to determine the extent to which the estimation of the β-carotene content of Butternut squashes by the AOAC method at harvest and during storage is influenced by other carotenoids.

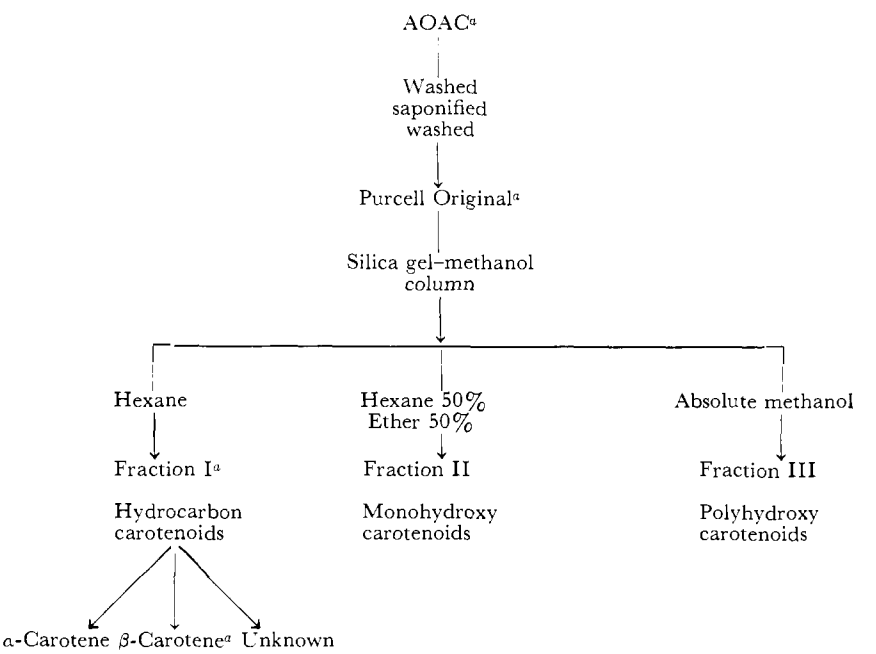

Figure 1. Diagram of fractionation of carotenoid pigments in Butternut squashes

[a] Absorbance determined in these solutions

Experimental

Butternut squashes were obtained from the University Horticultural Department. As part of another study in 1959, squashes were grown on the Horticultural farm in four replications of two fertilizer treatments. The seasonal rainfall was supplemented only when severe drought threatened the plants. The difference in fertilizer treatments had no effect on the carotenoid content and total solids, with the exception of the hydroxycarotenoid fraction. The two groups of four replications are treated as eight replications in this paper.

In September one representative fruit from each of the eight replications was analyzed on the day of harvest and the procedure was repeated after 1, 2, 3, 4, and 7 weeks of storage. The stored fruits were kept on slatted racks in a storage room. During the first half of the storage period the temperature averaged 22° C. (range 18° to 24° C.) and during the second half of the period, 14° C. (range 9° to 19° C.). Relative humidity averaged 58% during the 7 weeks of storage.

The samples analyzed were obtained from the neck portion of the fruits after the skin and the flesh around the seed cavity had been discarded. To obtain a representative primary sample the neck was quartered and cut into pieces about $^1/_4$ inch thick. These pieces were cubed in a French-fry cutter and thoroughly mixed in a crisper box. For the water blend, equal parts of distilled water and squash (500 grams) were slurried in a gallon-size Waring Blendor. On the fourth sampling date, the proportion of squash to water was changed to 2:3 to maintain a smooth slurry.

Total solids were determined by weighing about 20 grams of the water slurry into an aluminum weighing dish on a torsion balance and then obtaining an exact weight on an analytical balance. The weighed sample was placed in a forced draft oven for $4^1/_2$ hours at 70° C., cooled in a desiccator, and weighed. The sample was then heated for $^1/_2$ hour in the oven, cooled, and reweighed. This procedure was repeated until a constant weight was obtained (± 0.005 gram).

The Purcell method of extraction (*7*) was used, since it was considerably less time-consuming and similar results were obtained in preliminary work comparing the Purcell and AOAC methods of extraction. The eluate resulting from the AOAC procedure was fractionated by the Purcell method of partition chromatography in order to evaluate the extent to which carotenoids other than β-carotene were expressed as β-carotene in the results obtained by the AOAC procedure. The separation is diagrammed in Figure 1.

The predetermined amount (10 to 40 grams) of slurry was weighed exactly in a beaker on a torsion balance. Approximately 0.1 gram of magnesium

Table I. Carotenoids (Fresh Basis) and Total Solids in Butternut Squashes during Storage

(Mean of 8 replications expressed as mg. β-carotene in 100 grams)

Weeks of Storage	AOAC[a]	Loss due to Saponification[a]	Purcell Original[a]	Fraction I[a]	α-Carotene[a]	β-Carotene[a]	Fractions II and III[a]	Total Solids,[a] %
0	2.09 a	0.40 a	1.69 a	0.81 a	0.37 a	0.44 a	0.88 a	17.4 a
1	2.71 ab	0.64 ab	2.07 a	1.09 ab	0.45 ab	0.64 ab	0.98 ab	16.8 a
2	3.28 bc	0.76 abc	2.52 ab	1.23 abc	0.37 a	0.86 b	1.29 ab	16.3 a
3	3.90 cd	0.90 bc	3.00 bc	1.51 bc	0.68 bc	0.82 b	1.50 b	14.8 a
4	4.24 cd	1.16 c	3.08 bc	1.64 c	0.86 c	0.79 b	1.44 ab	15.3 a
7	4.59 d	1.15 c	3.44 c	1.31 bc	0.51 ab	0.80 b	2.14 c	15.5 a

[a] Means within column followed by same letter or letters are not significantly different ($P = 0.01$).

Table II. Carotenoids (Fresh Basis) of Butternut Squashes during Storage

(Expressed as per cent of AOAC values. Mean per cent of eight replications)

Weeks of Storage	Loss Due to Saponification[a]	Fractions II and III[a]	α-Carotene[a]	β-Carotene[a]
0	18.0 a	38.6 a	18.7 a	24.7 ab
1	23.0 ab	34.8 a	18.2 a	24.0 ab
2	23.1 ab	39.0 a	11.2 b	26.7 a
3	23.0 ab	38.0 a	17.8 a	21.2 ab
4	27.0 b	34.3 a	20.3 a	18.4 b
7	25.0 b	46.4 a	11.0 b	17.6 b

[a] Means within column followed by same letter or letters are not significantly different ($P = 0.01$).

carbonate (ca. 1/5 teaspoon) was added. The squash was mixed with an equal amount of absolute methanol (w./v.) and filter aid (2% of the sample weight). The mixture was filtered through a glass-fritted Büchner funnel covered with a pad of filter aid. The dried mat of squash residue and filter aid was scraped into the original beaker, covered with 100 ml. of acetone-hexane (Skellysolve B) (50:50), stirred, and allowed to stand 45 minutes in subdued light. The mixture was filtered through the same funnel and washed with just enough acetone-hexane to cover the mat. The mat was extracted a second time following the same procedure. The acetone-hexane filtrates and washings were combined.

This material was then prepared for chromatographing. The combined filtrates and washings were washed free of acetone in a separatory funnel with 0.06% sodium sulfate solution. Sodium sulfate solution was used instead of water to prevent emulsification. The hexane layer was dried in the separatory funnel with anhydrous sodium sulfate, decanted into a 500-ml. round-bottomed flask, and concentrated to about 50 ml. by means of a Rinco evaporator in a water bath at 30° C. The concentrated hexane solution was added to a 100-ml. volumetric flask containing 9 ml. of acetone and made to volume with hexane. The extract was passed through an AOAC column of Sea Sorb 43 and Hyflo Super-Cel (1:1, w./w.) and developed with 100 ml. of 1 + 9 acetone-hexane. The entire eluate was made to volume in a 200-ml. volumetric flask. The absorbance was determined in a Beckman Model B spectrophotometer at a wave length of 436 mμ.

The eluate was then prepared for use in the Purcell method by washing free of acetone. The solution of carotenoids was saponified with saturated methanolic potassium hydroxide, washed free of alkali, dried, and reduced to about 70 ml. by means of a Rinco evaporator as stated above. The concentrated hexane solution was made to 100 ml. in a volumetric flask with hexane. The solution was then ready for chromatographic fractionation by the Purcell method, which was followed from here on.

In Figure 1, the so-called Purcell Original reading includes hydrocarbons and hydroxy compounds. Fraction II contains the monohydroxy, and fraction III the polyhydroxy carotenoids. These two fractions were combined in this study. Fraction I, which contains the carotenoid hydrocarbons, was chromatographically broken down into its component carotenoids, as the validity of the β-carotene content as determined by the AOAC method was of primary interest. It contained α- and β-carotene and an unknown. The unknown appeared in approximately one half of the samples analyzed as a very faint yellow band at the top of the chromatographic column after removal of the α- and β-carotenes. The amount was negligible and impractical to measure because of the size of the sample used. When present it has been included in the values for α-carotene.

The values for AOAC β-carotene, Purcell Original, fraction I, and β-carotene were determined spectrophotometrically. The following values were obtained by difference: loss due to saponification (AOAC minus Purcell Original), fractions II and III (Purcell Original minus fraction I), and α-carotene (fraction I minus β-carotene). The sum of the values of the Purcell fractions I, II, and III and the loss due to saponification equals the AOAC value.

Results and Discussion

Table I presents the mean values expressed as milligram per cent and the per cent of total solids at harvest and during storage. In Table II the values of fractions II plus III, α-carotene, and β-carotene are expressed as per cent of the AOAC value. In both tables the effect of saponification has been presented.

The analysis of variance using Duncan's multiple range test (*3*) was used to test significance of differences between sampling dates. These are indicated at the 1% level of probability.

In Table I the carotenoid values are expressed as milligrams per 100 grams of fresh material.

It is obvious that the AOAC procedure overestimated the β-carotene value of Butternut squashes. At each sampling date the difference between AOAC β-carotene and Purcell β-carotene values was significant ($P = 0.01$). This overestimation was not constant for all sampling dates. The amount of Purcell β-carotene averaged from 17.6 to 36.7% of the AOAC β-carotene values. The amount of Purcell β-carotene after 2 weeks of storage and throughout the remainder of the storage period was significantly greater than at harvest but with a tendency to be a progressively smaller proportion of the AOAC β-carotene. Changes in the Purcell β-carotene and the AOAC β-carotene values during storage were dissimilar. The AOAC β-carotene values changed progressively, while the Purcell β-carotene increased during the first 2 weeks of storage and remained at that level thereafter.

The mean ratios between the two values at harvest and after 1, 2, 3, 4, and 7 weeks of storage were 4.82, 4.32, 3.81, 4.77, 5.54, and 5.72, respectively. The smallest ratio, 3.81, occurred after 2 weeks of storage and was significantly less than the ratios of 5.54 and 5.72, which occurred after 4 and 7 weeks of storage. However, since the Purcell β-carotene value after 2 weeks of storage was not significantly different from the values after 1, 3, 4, and 7 weeks of storage, this significance between ratios is probably of minor importance. The average ratio for all sampling dates was 4.83.

The mono- and polyhydroxycarot-

enoids made up the largest portion of the differences between the AOAC β-carotene and the Purcell β-carotene values and averaged from 34.3 to 46.4%. Because of the increased polarity of the 10% acetone-hexane solution as compared to hexane, the hydroxy carotenoids and the carotenoid esters in addition to the carotenoid hydrocarbons were eluted off and are reflected in the AOAC values. This might be remedied by using hexane instead of the 10% acetone-hexane, as suggested by Quackenbush (*8*). In this study the 10% acetone-hexane solution was used, in order that the extent of contamination of the subsequent eluent could be estimated by partition chromatography.

Cooley and Koehn (*2*) state that they believe that cryptoxanthin is eluted from a magnesia column (MgO and Hyflo Super-Cel) along with α- and β-carotenes when 10% acetone-hexane is used as a developing solution as in the AOAC method (*1*). This elution has been demonstrated by Quackenbush, who reported (*9*) the separation of cryptoxanthin from a corn grain extract with a 10% acetone-hexane solution on a magnesia column. Following fractionation, if cryptoxanthin was present in the Butternut squash it would probably occur in fraction II.

In the variety of squash studied the mono- and polyhydroxy carotenoids were significantly greater after 7 weeks of storage. However, there was no significant change in the percentage contribution by these fractions to the AOAC values among the sampling dates.

The differences between the mean AOAC β-carotene and the mean Purcell Original values, or a reduction of 18 to 25% during the storage period, could be due to either oxidation or saponification. If oxidation did occur, its effect on β-carotene appeared to be negligible, since an average of 97% of added β-carotene was recovered in a series of recovery tests. The reduction attributable to saponification, according to Purcell (*7*), was probably due to the partitioning of the hydroxy carotenoids between the methanolic KOH layer and the hexane layer. The differences between the mean AOAC β- carotene and the mean Purcell Original values, expressed as per cent of AOAC β-carotene, were significantly greater after 4 and 7 weeks of storage than at harvest. When expressed as milligram per cent, this loss or difference was significantly higher after 3 weeks of storage than at harvest.

Previous work with squash has demonstrated this loss, which occurs during saponification. Apparently this is in contrast to some other plant materials. Cooley and Koehn (*2*) found little or no effect of saponification with corn and alfalfa. Purcell (*6*) in fractionation of the carotenoid pigments of grapefruit compared the effect of saponified *vs.* unsaponified material and found no loss in total carotenoid content, but found a decrease in fraction I and an increase in fractions II and III. He states this is presumably due to the presence of carotenoid esters which are generally more soluble in hexane than are the free carotenoids released by saponification.

In contrast to the other carotenoids studied, which exhibited an increase during storage, α-carotene increased during 4 weeks of storage, then decreased to approximately the harvest value. The net effect was a significantly smaller percentage of the AOAC β-carotene value after 2 and 7 weeks of storage compared to the percentage on the other sampling dates.

The mean percentages of α-carotene and β-carotene in fraction I for all sampling dates were 43 ± 10 and 57 ± 11%, respectively. These percentages are approximately the same as the 39 and 61% found by Fujita and Ajisaka (*4*) in the edible portion of *C. moschata* var. melonaeformis (Kabotya). The same authors found a proportion of 10 to 90% in the *C. moschata* (Kintônasu). This difference in percentage composition could be due to varietal differences or possibly to length of storage.

Conclusions

The findings indicate that the procedure of the Association of Official Agricultural Chemists overestimated the β-carotene content of Butternut squashes at harvest and during storage. The largest portion of the differences between the AOAC β-carotene and Purcell β-carotene values consisted of mono- and polyhydroxy carotenoids. The α-carotene content and the unidentified carotenoid pigments which were lost during saponification also contributed significantly to this overestimation. The extent to which each factor affected the AOAC values varied, depending on the relative changes in these constituents during storage. The findings indicate that preliminary study of the characteristic carotenoid content of a sample of a plant material by partition chromatography is advisable in order to determine whether the more rapid AOAC method is appropriate for the estimation of its β-carotene content.

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