

INVESTIGATION OF THE METHODS FOR DETERMINING
CAROTENE IN DEHYDRATED ALFALFA MEAL

by

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TABLE OF CONTENTS

INTRODUCTION.....	1
LITERATURE REVIEW.....	1
EXPERIMENTAL.....	3
Comparison of Phasic and Chromatographic Methods.....	3
Comparison of the Extraction Processes of the Official and Silker Methods.....	7
Further Observations on the Official Method.....	9
A Suggested Method for Use with the Goldfish Extractor.....	10
Investigation of the Method of Silker, Schrenk, and King.....	12
A Modification of the Method of Silker, Schrenk, and King.....	13
Effect of Sample Size.....	14
Analysis of Pelleted Meals.....	15
Investigation of the A. O. A. C. Alternate Method.....	16
SUMMARY.....	17
ACKNOWLEDGMENT.....	19
LITERATURE CITED.....	20

INTRODUCTION

Since the carotene content of dehydrated alfalfa meal is a measure of its vitamin A potency, carotene determinations often are required by the feed distributor or consumer. Such determinations are of great interest to this laboratory, since they are involved in the alfalfa research being conducted at Kansas State College. Wide variations in analytical results have been obtained by different laboratories working with the same sample of meal. The factors responsible for such deviations are not understood as yet. Present data point to no single cause, but seem to indicate a variety of factors that might produce such variations. Thus, an investigation was made of the procedures now in use in an effort to determine some of the causative factors, and to make corrections and improvements if possible.

LITERATURE REVIEW

Early methods for the quantitative determination of carotene were based upon the suggestion of Borodin in 1833, as reported by Peterson (11), that the carotinoid pigments could be separated into alcohol-soluble and petroleum ether-soluble fractions. A method proposed by Willstatter and Stoll (18) in 1913 provided the basis for further work on phasic separation of the pigments. By their method the fresh plant material was ground in a mortar with quartz sand and 40 per cent acetone. The ground material was washed with 30 per cent acetone and the two extracts combined. Ethyl ether was added, the acetone was washed out with

water, and the resulting solution was saponified with methanolic potassium hydroxide. The alkali was removed by washing with water, the ether was evaporated, and the residue was taken up in petroleum ether. Xanthophylls were removed by washing with 85 to 92 per cent methanol. Carotene concentrations were determined colorimetrically, using a 0.2 per cent solution of potassium dichromate as a standard.

Various modifications of the Willstatter and Stoll method have been published. Coward (5) ground the plant material with solid potassium hydroxide to decompose the chlorophyll, after which the material was extracted for two or three days with light petroleum (B. P. below 40° C.) in a Soxhlet extractor. Russell et al. (14) described a method by which the plant tissue was extracted with petroleum ether alone, while Guilbert (9) presented a method which involved saponifying the chlorophylls and fats with 20 ml of saturated alcoholic potassium hydroxide. Ether was then added, and the alcohol and alkali were washed out with water. Xanthophylls were removed with 90 per cent methanol. Peterson, Hughes, and Freeman (12) modified the Guilbert method by eliminating the ether extraction and using petroleum ether (B. P. 40°-60° C.) instead.

As work in this field progressed, it was recognized that the phasic separation of carotene from other pigments was unsatisfactory. Chromatographic methods had been studied earlier by Tswett, as reported by Peterson (11), and were reported to be extremely satisfactory for the separation of carotene by Fraps and his as-

sociates (6, 7, 8), who made extensive studies of the methods of selective adsorption. Strain (16) reported a mixture of magnesium oxide and Hyflo Supercel to be effective in the separation of carotene from other pigments. Most of the methods now in use employ this adsorbent.

Wall and Kelly (17) extracted carotene from dehydrated alfalfa meal by refluxing the sample for 30 minutes with 30 per cent acetone in Skellysolve B. Kernohan (10) suggested extracting carotene from dehydrated meal by allowing the material to stand in petroleum ether. However, Silker, Schrenk, and King (15) found that this did not effect complete extraction of the carotene. They obtained complete extraction by allowing the sample to stand for 16 to 18 hours in a mixture of two parts Skellysolve B and one part acetone. This is the basis for their method of carotene determination.

Until 1949 a method of phasic separation was recognized as the official method of the Association of Official Agricultural Chemists (2). More recently, the official A. O. A. C. method (1) involves the refluxing of the material in a mixture of Skellysolve B and acetone, followed by adsorption on activated magnesia. A modification of the cold extraction method of Silker et al. (15) is recognized as an alternate method.

EXPERIMENTAL

Comparison of Phasic and Chromatographic Methods

The phasic method used in this study was essentially that

which was recognized as the official A. O. A. C. method (2) until 1949. The sample was refluxed for 20 minutes with 40 ml of 10 per cent methanolic potassium hydroxide. The solution was cooled, filtered through a Buchner funnel, and the residue was washed alternately with two 75 ml portions of methanol and Skellysolve B. The methanol-Skellysolve B extract was transferred to a 500 ml separatory funnel, and the funnel was shaken vigorously. After the two layers separated, the lower layer was drawn off into another separatory funnel containing 100 ml of Skellysolve B. The latter mixture was shaken and the lower layer discarded. The two Skellysolve B extracts were combined and washed with 90 per cent methanol saturated with Skellysolve B. These washings were continued until they were no longer colored. The extract was then washed with 100 ml of distilled water, dried over anhydrous sodium sulfate, and made to volume.

Two chromatographic methods were used in this comparison, one being the official A. O. A. C. method (1), which involves the refluxing of the sample with 30 ml of a 3:7 mixture of acetone and Skellysolve B. The Goldfish extractor was used for this purpose. The sample and solvent were placed directly into the flask of the extractor. After refluxing for one hour, the extract was cooled and filtered into a 100 ml volumetric flask. The residue was washed with Skellysolve B and the extract and washings were made to volume. This solution was chromatographed on an adsorption column composed of a mixture of activated magnesia (Micron brand No. 2642) and Hyflo Supercel in a ratio of 1:1 by weight. The carotene was eluted with a 9 per cent solution of

acetone in Skellysolve B.

The second chromatographic method used in the comparison was that of Silker, Schrenk, and King (15), in which the sample was allowed to stand in the dark in contact with 60 ml of a 1:2 mixture of acetone and Skellysolve B for a period of 16 to 18 hours. The extract was filtered through a Buchner funnel, and the residue was washed, by decantation, with several small portions of Skellysolve B. The filtrate was transferred to a beaker and heated on a steam plate to drive off the acetone and concentrate the solution to approximately 40 ml. This concentrate was chromatographed on an adsorption column consisting of 2 parts Hyflo Supercel and 1 part magnesia (Micron brand No. 2641). The carotene was eluted with 4 per cent acetone-Skellysolve B.

The carotene concentrations of the solutions obtained by the three methods were determined with the Beckman Spectrophotometer at a wavelength of 4360 Å (3).

Samples of dehydrated alfalfa meal were placed in paper bags and stored at a temperature of 25° C. The samples were analyzed for carotene by the three methods as they went into storage and at intervals of one month. From the results, presented in Fig. 1, it will be seen that the method of phasic separation gave higher results than the chromatographic methods. This was found to be true for all of the 16 samples that were stored and analyzed. These data seem to substantiate the report of Fraps and Kemmerer (6) that some non-carotenoid pigments remain after the process of phasic separation.

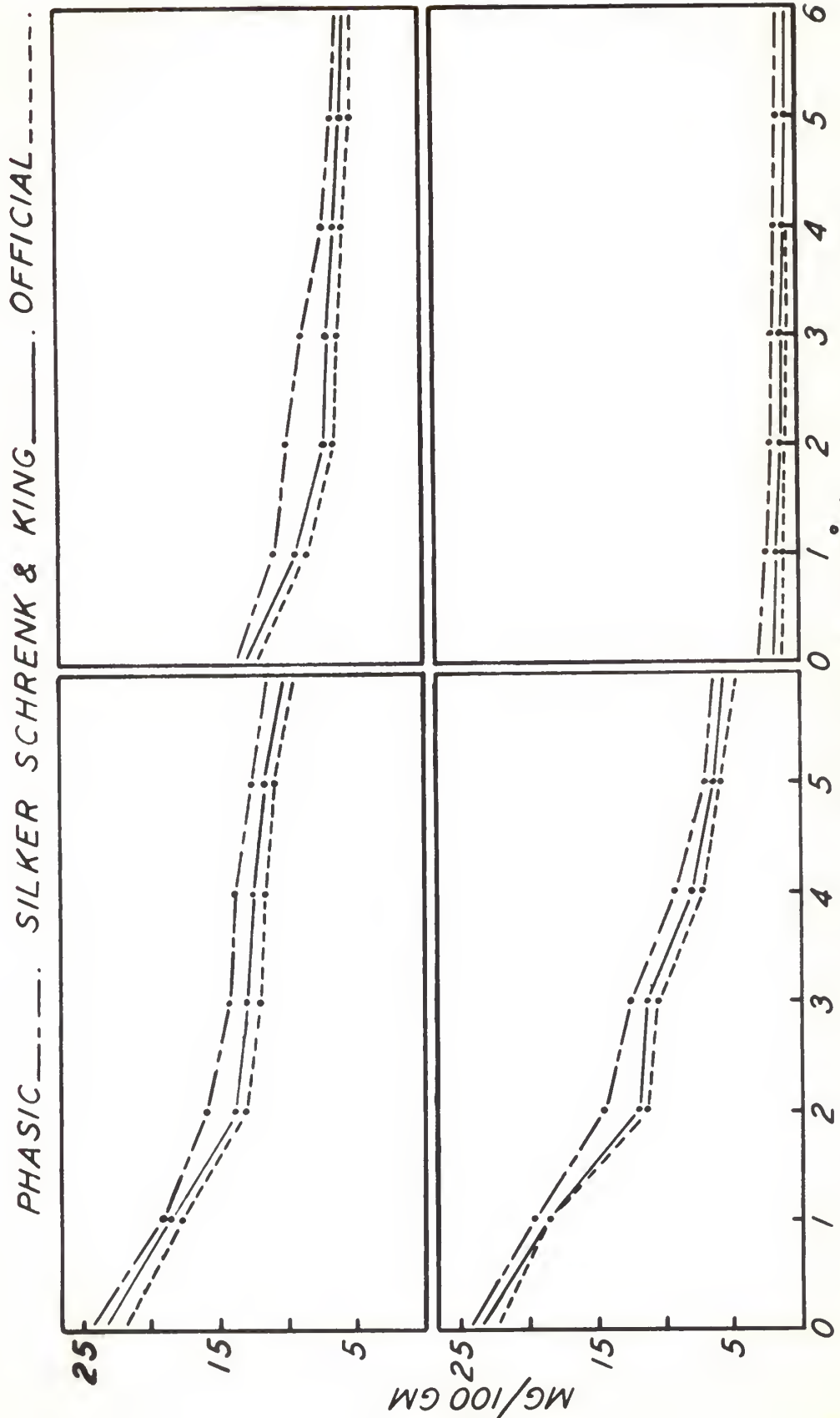


FIG. 1. COMPARISON OF THE PHASIC METHOD WITH THE SILKER SCHRENK & KING AND OFFICIAL METHODS

In an effort to determine if the higher results obtained with the phasic method were due to non-carotenoid pigments, determinations were made on the same sample by the three methods, and absorption curves were plotted for these three extracts. Figure 2 shows that the absorption maxima for the pigments separated by the phasic method are shifted slightly to the left from those obtained by chromatography. This indicated that the pigments extracted by the phasic method differ from those separated on the chromatographic column.

Comparison of the Extraction Processes of the Official and Silker Methods

From Fig. 1 it will be seen that slightly higher results were obtained by the method of Silker et al. (15) than were obtained by the official method. To determine if these differences were due to unequal extraction or to the chromatographic procedure employed, dehydrated alfalfa meal samples were analyzed by the two methods. In addition, they were analyzed by a modified method in which carotene was extracted by refluxing for one hour as in the official method, after which the samples were filtered and the analysis was completed according to the Silker, Schrenk, and King method. Thus, the modified method employed the extraction procedure of the official method and the chromatographic technique of the Silker, Schrenk, and King method.

The data presented in Table 1 show good agreement between the official and the modified method, while the Silker, Schrenk, and King method again gave slightly higher results. Thus, the

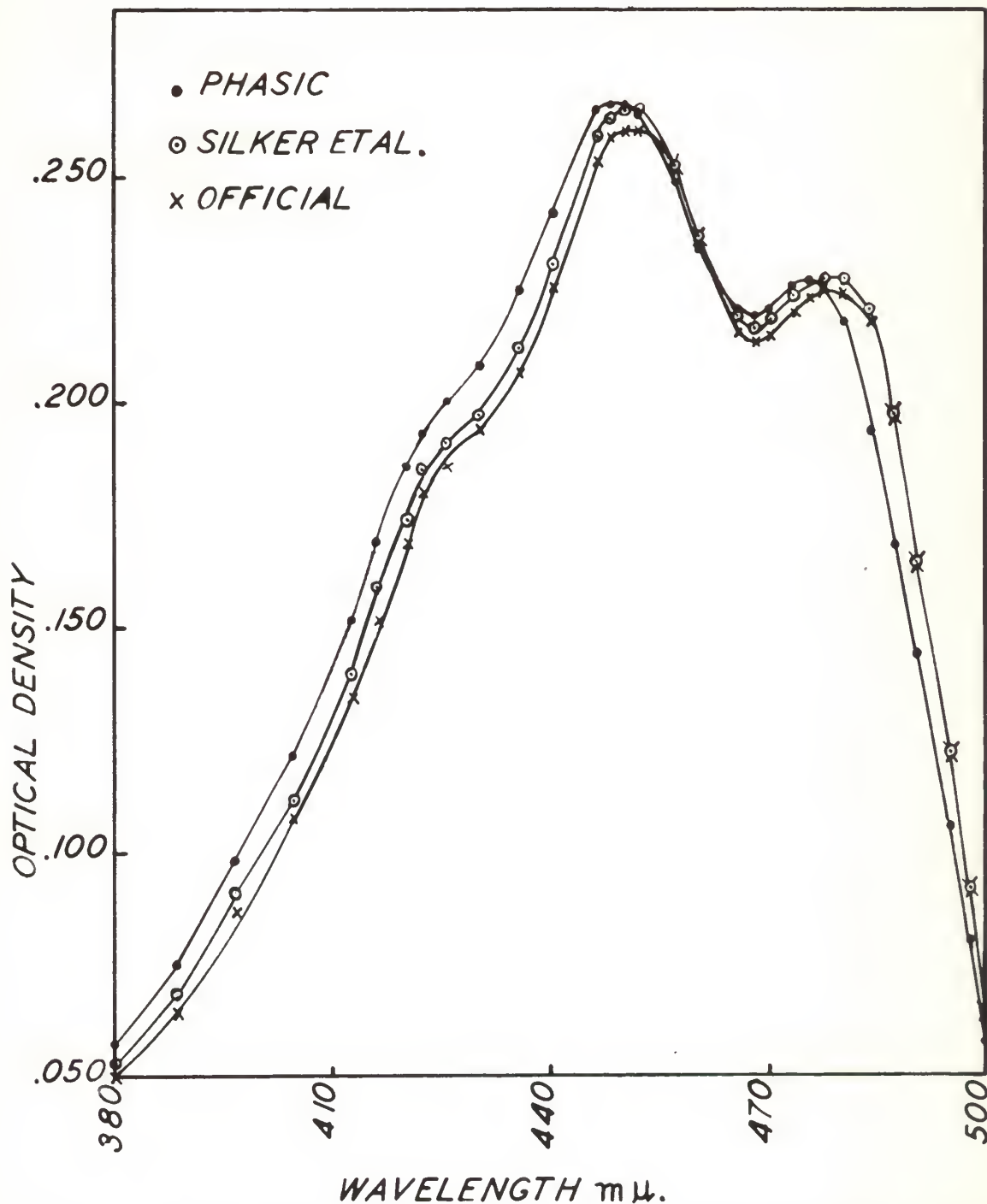


FIG 2 ABSORPTION SPECTRA OF PIGMENTS EXTRACTED BY THE PHASIC, SILKER ETAL., AND OFFICIAL METHODS

slightly lower values obtained by the official method appear to be due to the extraction phase of the method. Either extraction is less complete, or some carotene destruction occurred during the refluxing.

Table 1. Comparison of the results obtained by the method of Silker et al., the official method, and a combination of the two methods.

Sample no.	Silker, Schrenk, and King	Official	Combination
	mg/100 gm	mg/100 gm	mg/100 gm
1	6.9	6.5	6.2
2	10.8	10.0	9.7
3	22.1	21.4	21.8
4	22.6	21.8	22.2
5	25.1	24.6	24.7

Further Observations on the Official Method

In connection with the official method, it was observed that in some cases 50 ml of the 9 per cent acetone-Skellysolve B eluting solvent (as stated in the procedure) was not sufficient to elute completely the band of carotene from the column. Frequently the colored carotene pigments still could be seen on the column after the 50 ml of solvent had been used.

Samples were analyzed by the official method, and the results obtained by using 50 ml of the eluting agent were compared with those obtained by using 100 ml. Samples also were analyzed by the method of Silker et al. as a means of comparison.

From Table 2 it will be seen that the use of 100 ml of elut-

ing solvent yielded results that agreed closely with those obtained by the Silker method, while those obtained by using 50 ml were somewhat lower.

Table 2. Effect of volume of eluting agent on the determination of carotene by the official method.

Sample no.	Official 50 ml	Official 100 ml	Silker, Schrenk, and King
	mg/100 gm	mg/100 gm	mg/100 gm
1	5.9	6.3	6.9
2	10.8	10.9	10.8
3	19.8	21.2	21.9
4	20.0	21.8	22.1

All adsorption columns in this experiment were 10 cm long, as specified in the method. If the length of the column were shortened, 50 ml of the solvent might be adequate. However, due to the 9 per cent acetone concentration in the tissue extract and the use of 9 per cent acetone-Skellysolve B for elution, other pigments might be eluted if the column were shortened. In some cases a very light yellow band of pigment was observed near the lower part of the column after the carotene had been eluted. Any shortening of the column might allow this band to be eluted from the column. On the other hand, the use of too much solvent might sometimes elute this band even from the longer column.

A Suggested Method for Use with the Goldfish Extractor

The official method states that the Goldfish and Bailey-Walker extractors (1) may be used for the extraction of carotene

if an extraction thimble is not used. Thus, the sample is to be placed directly into the extraction flask along with the solvent. It was observed in this study that, during heating, a considerable amount of bumping occurred. As a result, alfalfa particles were thrown upon the sides of the flask and were not washed down by the condensed solvent. It was thought that the carotene from these particles might not have been extracted completely. Berkhout, Hekking, and Greeling (4) described an extraction method by which the sample was placed in an extraction tube between two plugs of wadding. This was inserted between a reflux condenser and an extraction flask so that condensing solvent would percolate through the sample. Such an extraction appeared to be desirable, and was tried with the Goldfish extractor. The sample was placed between two pieces of cotton in the extraction tube of the Goldfish extractor. It was then extracted with 30 ml of a 30 per cent acetone in Skellysolve B solution for one hour. This eliminated the annoying bumping and produced results that agreed very well with those of the official method (Table 3). It also eliminated a filtration which was necessary when the apparatus was used without an extraction tube.

Table 3. Comparison of carotene extractions by the Goldfish extractor when used with and without an extraction tube.

Sample no.	With extraction tube	Without extraction tube
	mg/100 gm	mg/100 gm
1	3.2	3.5
2	24.6	24.3
3	24.7	24.6
4	25.5	25.7
5	32.0	32.1

Investigation of the Method of
Silker, Schrenk, and King

Since it was found that the method of Silker et al. seemed to effect a more complete extraction of the carotene, a study of this procedure was made. According to this method the time necessary for complete extraction is 16 to 18 hours. The method states that the extraction is to be done at room temperature. Since the temperature of the laboratory varied with the season of the year, it was of interest to know what effect various temperatures would have upon the extraction process.

Table 4. Effect of temperature and time of extraction of carotene by the Silker, Schrenk, and King method.

Temperature Degrees C.	Hours of extraction							
	2	6	12	18	24	36	48	
4	10.7	11.7	12.1	12.3	12.0	12.8	13.2	
25	12.0	13.5	13.5	13.8	13.9	14.0	14.1	
Room	11.9	13.5	13.6	14.2	14.1	13.9	14.3	
37	12.9	13.4	14.2	14.2	14.3	14.4	14.6	
45	13.4	14.1	14.5	15.0	14.6	14.5	14.7	

Samples of dehydrated alfalfa meal were allowed to stand in 60 ml of a 30 per cent solution of acetone in Skellysolve B at temperatures of 4°, 25°, 37°, and 45° C., and at room temperature (30°-32° C.). Samples from each series were analyzed at the end of 2, 6, 12, 18, 24, 36, and 48 hours. The results, shown in Table 4, indicate that extraction at room temperature was complete at the end of 18 hours. At 37° and 45° C., complete extraction was accomplished in 12 hours. At temperatures of 25° C. or lower,

extraction was not completed after 48 hours of soaking. No destruction of carotene occurred upon prolonged extraction, even at the higher temperatures studied. Since the temperature of the laboratory normally is 30° to 35° C., temperature control usually is not a problem with the method. However, if temperatures should drop to 25° C. or below, lower values may result.

A Modification of the Method of Silker, Schrenk, and King

In the Silker, Schrenk, and King method the extracting solution is removed from the meal and is concentrated on a steam plate to about 40 ml to remove the acetone. Probably 300-400 ml of Skellysolve B are lost in this operation. A procedure was sought which would minimize these disadvantages.

A Claisen distilling apparatus with a detachable 500 ml round bottom flask was used. The sample was placed in the flask and was covered with 60 ml of 30 per cent acetone in Skellysolve B. After soaking overnight, the flask was attached to the Claisen head and vacuum was applied. Bumping was eliminated by introducing a limited amount of air through an ebullator tube. By immersing the flask in tap water to counteract cooling due to evaporation of the solvent, enough acetone could be removed in ten minutes to eliminate interference in the subsequent chromatographic operation. The remainder of the determination was identical with the Silker, Schrenk, and King procedure.

Results obtained by the above method were in good agreement with those obtained by the Silker, Schrenk, and King method

(Table 5). This technique should be adaptable to routine work. Since each extract can be concentrated in ten minutes, only a few Claisen heads would be needed to take care of a large number of samples, provided extra flasks were available. This technique would effect a considerable saving in the amount of Skellysolve B used, thus reducing the cost of the analysis.

Table 5. Comparison of the method involving no heat with the Silker, Schrenk, and King method.

Sample no.	No heat	Silker, Schrenk, and King
	<u>mg/100 gm</u>	<u>mg/100 gm</u>
1	22.9	22.6
2	23.0	22.6
3	23.5	23.5
4	25.2	25.2

Effect of Sample Size

For samples of low carotene content it may be necessary to use from 2 to 4 gm of sample for the determination. To find the effect of sample size on the methods of Silker et al. and the official method, a sample of low carotene content was chosen and determinations were made on 2, 3, and 4 gm samples.

Table 6. Effect of sample size on carotene determination by the official and by the Silker, Schrenk, and King methods.

Sample wt.	Silker et al.	Official
<u>gm</u>	<u>mg/100 gm</u>	<u>mg/100 gm</u>
2	3.0	2.8
3	3.0	2.8
4	3.0	2.7

From Table 6 it will be seen that sample size had no effect on the results obtained with a given method. No difficulties were encountered in the method of Silker et al., but serious bumping occurred when the 3 and 4 gm samples were run by the official method.

Analysis of Pelleted Meals

Frequently it is necessary to determine the carotene content of dehydrated alfalfa pellets. The question arose concerning how fine the pellets must be ground in order to effect complete extraction of the carotene. To determine this, a sample of pellets was ground to pass 10, 20, 40, and 60 mesh screens. After grinding, the samples were thoroughly mixed on a large sheet of paper. Carotene determinations were made by the Silker et al. and official methods. The data shown in Table 7 indicate that fineness of grinding had little effect upon the results obtained by the two methods. If the pellets are ground to pass a 20 mesh screen, the carotene will be easily extracted.

Table 7. Determination of carotene in ground alfalfa pellets.

Particle size	Silker et al.	Official
Mesh	mg/100 gm	mg/100 gm
10	22.6	20.6
20	22.6	20.4
40	22.6	20.8
60	22.4	21.3

Investigation of the A. O. A. C. Alternate Method

The Association of Official Agricultural Chemists (A. O. A. C.) also recognizes an alternate method for determining carotene, in which the sample is extracted by allowing it to stand in contact with 30 per cent acetone in Skellysolve B overnight. The extract is filtered and is made to a volume of 100 ml with Skellysolve B. The extract then is chromatographed as in the official method. This method was accepted by the A. O. A. C. as an alternate to the official method without first investigating its suitability. Hence, it was evaluated by comparing it with the Silker et al. and official methods.

As shown in Table 8, the alternate method gave higher values than either of the other methods. The extraction procedures of the alternate and of the Silker et al. methods are essentially the same. Hence, the differences in the results obtained by the two methods must be due to the chromatographic phase. Observations of the developing chromatograms supported this conclusion. When 2 gm samples were used with the alternate method, a wide

yellow band moved down the column above the carotene band as the column was washed with the 9 per cent acetone in Skellysolve B eluting agent. Although the band was approximately an inch above the bottom of the column by the time the carotene was eluted completely, some channeling could have occurred, allowing part of the pigments to pass into the eluate. This band was not observed when either the official or the Silker et al. method was employed. Thus, it would seem that the extract obtained with the alternate method contained enough acetone to cause excessive movement of some of the xanthophylls.

Table 8. Comparison of the alternate A. O. A. C. method with the Silker et al. and official methods.

Sample no.	Silker et al.	Official	Alternate
	mg/100 gm	mg/100 gm	mg/100 gm
1	6.0	5.5	6.9
2	6.5	6.4	7.3
3	14.5	14.0	16.3
4	22.6	20.4	22.8
5	27.3	25.4	28.4
6	27.3	25.7	28.6
7	27.4	25.0	28.4

SUMMARY

The method of phasic separation gave higher results than the chromatographic methods. Data obtained indicated that some non-carotenoid pigments remained after the process of phasic separation.

Lower values were obtained by the official method than were

obtained by the method of Silker et al. The differences appear to be due to the extraction phase of the methods.

In many cases 50 ml of eluting agent were not sufficient to elute completely the band of carotene from the column by the official method.

Bumping can be eliminated in the official method if the sample is placed in the extraction tube between two pieces of cotton in such a way that the solvent is allowed to percolate through the sample.

At ordinary laboratory temperatures, extraction of carotene was completed by the method of Silker et al. in 18 hours. At temperatures of 25° C. or lower, extraction was not completed after 48 hours of soaking. No destruction of carotene occurred during the 48 hours of extraction, even at temperatures of 45° C.

A method of extraction involving no heat was found to be useful in eliminating the loss of Skellysolve B that results when the method of Silker et al. is employed.

Sample size had no effect on the results obtained by the method of Silker et al. and by the official method.

The fineness to which pelleted samples were ground had little effect upon the results obtained by the Silker et al. and official methods.

The alternate A. O. A. C. method gave higher results than any of the other methods studied. These high values probably were due to the high acetone concentration in the extract.

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