THE USE OF THE ANTIMONY TRICHLORIDE METHOD FOR THE DETERMINATION OF VITAMIN A IN FEEDS, MILK AND BUTTER

Thesis for the Degree of M. S.
MICHIGAN STATE COLLEGE

Nelson W. Abernethy

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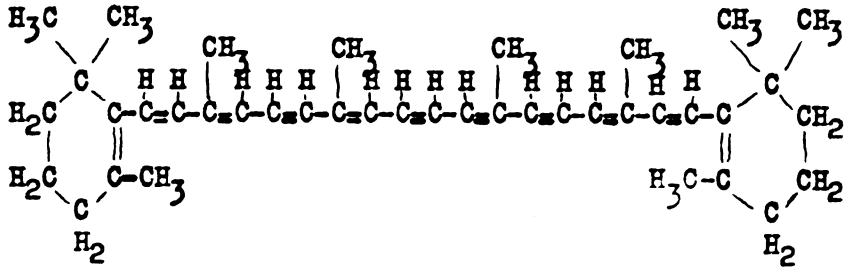
Acknowledgement

The author of this thesis wishes to express his indebtedness and gratitude to Dr. C. A. Hoppert, Professor of Chemistry, for his assistance in making this work possible.

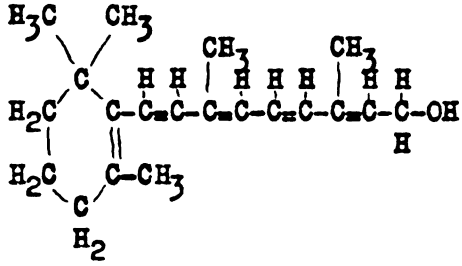
Introduction

Since the year 1912, when Hopkins (1) called attention to the fact that normal growth does not occur when purified food rations are used as feed, the study of vitamin A has been very important. Much has been learned since that time regarding the nature of the vitamin and the methods of assay.

Steenbock (2) has contributed a great deal to the chemistry of vitamin A, and the apparent relation between the vitamin A potency of plant and animal substances and the yellow pigment carotene. In the year 1926, Carr and Price (3) obtained a blue color when vitamin A was treated with antimony trichloride. The same color was obtained when carotene was used in place of the vitamin. This suggested a possible relation between the two. Euler has proven that carotene is very effective in supplementing diets deficient in vitamin A. Recently it has been quite definitely established that carotene is the precursor of vitamin A. It is believed that the conversion of the pigment into the vitamin takes place in the liver and that any excess carotene may be stored in this organ until needed. The following shows the possible structural relation between beta carotene and vitamin A. Only the beta form of carotene is given below, as it is now used as a basis for the international vitamin A unit.



Beta Carotene ($C_{40}H_{56}$)



Vitamin A ($C_{20}H_{30}O$)

It was pointed out above that a definite color is obtained when vitamin A is treated with antimony trichloride. While this reaction is not specific for the vitamin it does serve as a means of chemical assay. The intensity of the blue color is, in general, directly proportional to the concentration of the vitamin. Even though this reaction may be given by other substances, the latter are usually not present in materials which are practical sources of vitamin A so that the reaction is useful and convenient for the chemical determination of this vitamin. Wokes and Willimott (4) have suggested the use of a saturated solution of antimony trichloride in the test as this strength solution has been found to give the best results. Weaker solutions have been used but seem to give less accurate results. Norris and Church (6) have studied the effect of temperature upon the saturation point of antimony trichloride in chloroform and have shown that the concentration has a definite influence on the color reaction with vitamin A. They also studied the effect of light upon this reagent and found that strong light caused the reagent to deteriorate. Norris and Danielson (5) claim the color test checks within reasonable limits with the biolcgical method of assay. Besides the lack of specificity of the reaction there are several other shortcomings. The blue compound is very unstable so that the color fades within a very short period of time after it has been formed. Consequently the color intensity must be measured very soon after the reagent has been added to the solution to be tested. Moisture also interferes with the test causing cloudiness which makes accurate measurements impossible.

For a number of years cod liver oil has been used as a source

of vitamin A. When the oil is stored under favorable conditions the vitamin A potency of the oil may be retained for a long time. However, when the oil is mixed with feeds the destruction of vitamin A proceeds at a more or less rapid rate depending on the material with which the oil is associated, as well as on the storage temperature and accessibility of air. Consequently, it is necessary that a determination of the vitamin A content of feed mixtures be made. For the determination of vitamin A two methods of assay have been used, the chemical and biological. In as much as the biological method is costly and requires considerable time, it is being gradually replaced by the chemical method wherever the latter is applicable.

Accordingly, the present study concerns itself with the use of the Carr-Price reaction in the determination of the vitamin A content of various materials to which cod liver oil had been added, as well as in the determination of the vitamin A potency of milk and butter. The work also involved a study of the presence, in various foodstuffs, of substances which might interfere with the Carr-Price reaction.

For the measurement of the intensity of the blue color imparted by the treatment of vitamin A with antimony trichloride, use was made of the Lovibond tintometer, an instrument used internationally to measure the intensity of colors of various substances. The readings taken with this instrument, when determining the concentration of vitamin A, are expressed in terms of Lovibond blue units.

Procedure

A known quantity of cod liver oil, which had been previously standardized, was added to various feeds and mixed thoroughly. Various methods of extracting the oil from the feeds were tried.

The direct method of extraction gave nearly quantitative results in the case of cornmeal, but when oatmeal was used, considerable difficulty was encountered and another method had to be tried. Use was then made of the Soxhlet extraction apparatus, which proved to be more satisfactory than the former and was therefore resorted to for the extractions. With this apparatus it was found that approximately twenty extractions were made per hour, thereby enhancing the efficiency of the extraction and permitting the materials to be handled with less difficulty. Upon completion of extraction, 25 ml of water, 30 ml of ethyl alcohol, and 40 ml of 20% potassium hydroxide were added to the extract. The mixtures were then heated on a steam bath until saponification was completed, then transferred to a separatory funnel and 15 ml of ethyl alcohol added. Two extractions were made using 100-125 ml portions of ethyl ether. The ether extracts were then washed free from alkali with distilled water (phenolphthalein being used as an indicator), transferred to a flask containing 10 gm of anhydrous sodium sulfate and shaken vigorously to remove the water present. After decanting the solution and washing the sodium sulfate residue with anhydrous ether, the ether was removed by placing the flask in a bath at about 50° C and attaching it to a suction pump. The residue was taken up in chloroform, the volume of the latter depending on the amount of the vitamin A expected to be present. For the test, .2 ml of the chloroform solution was placed in the testing cell and 2 ml of the antimony trichloride reagent added. Readings were taken as soon as possible after the development of the color.

In the case of milk and butter no preliminary extraction with ether was made, these foods being directly saponified and the ether extractions then made as previously described.

Data

In order to determine the applicability of the Carr-Price reaction and the possible presence of interferring materials, mixtures of cod liver oil of known potency with cornmeal, oatmeal, linseed meal and alfalfa meal were prepared on the basis of 1 ml of oil to 99 gm of the meals and subjected to study.

To determine the potency of the cod liver oil used in these experiments, 1 ml of the oil was placed in a 50 ml volumetric flask and chloroform added to bring it up to volume. Several trials were made to determine the concentration of vitamin A in the solution, the average reading being 1.9 blue units. To show the effect of saponification upon the oil a similar sample was saponified according to the directions given in the procedure. In this case, several trials gave an average reading of 1.7 blue units, which is just slightly more than a 10% difference between the two readings. It might be, therefore, expected that some of the vitamin would likewise be lost when the cod liver oil-meal mixtures were saponified in a similar manner.

In the case of the cornmeal mixture, 30 gm samples were placed in extraction thimbles and extracted in a Soxhlet apparatus for periods of 2, 4, and 6 hours, to determine the time necessary for maximal recovery of the vitamin. The material extracted was saponified and further treated as previously described, the final unsaponifiable residue being taken up in 5 ml of chloroform.

Table 1

Sample #	Period of extraction	Blue units	Yellow units	B.U./gm of oil added	Expected potency in B.U./ gm of oil added
1	2 hrs	4.3	1.1	876	1038
2	2 hrs	4.4	1.2	896	1038
3	4 hrs	4.4	1.2	896	1038
4	4 hrs	4.5	1.2	901	1038
5	6 hrs	4.5	1.1	901	1038
6	6 hrs	4.4	1.1	896	1038

From the data given in Table 1 it may be seen that a 2 hr period of extraction was sufficient for maximal recovery of the vitamin A. The lovibond blue unit values obtained, represent approximately 90% of the expected recovery.

Reference should be made at this point to the inclusion of the yellow values given in all of the tables. It was necessary, in matching the color given by the samples with the standard colors of the tintometer, to introduce a certain amount of yellow. These yellow values may be of some future interest but no attempt is made to explain their significance.

An oatmeal mixture was prepared as in the case of the cornmeal. All samples were taken up in 5 ml of chloroform. The following data were obtained:

Table 11

Sample#	Period of extraction	Blue units	Yellow units	B. U./ gm of oil added	Expected potency in B.U./gm of oil added
1	2 hrs	3.6	1.3	733	1038
2	2 hrs	3.5	1.2	712	1038
3	4 hrs	3.7	1.2	753	1038
4	4 hrs	3.5	1.2	712	1038

The above data indicate a failure to account for an appreciable amount of the vitamin A added but that a two hour period of extraction was sufficient to remove whatever vitamin could be dissolved by the ether. It was observed that in the extraction of the oatmeal-cod liver oil mixture a considerable amount of finely divided material appeared in the ether. There seemed to be a direct relationship between the decrease in vitamin A recovery and the amount of the fine material appearing in the ether extract. However, no attempt was made to determine the manner of the interference.

Linseed meal was next treated in a similar way, maximal recovery being obtained with a two hour extraction period as shown in Table 111. As in the case of the cornmeal, approximately 90% of the expected potency was accounted for.

Table 111

Sample#	Period of extraction	Blue units	Yellow units	B. U./ gm of oil added	Expected potency in B.U./gm of oil added
1	2 hrs	4.6	2.6	937	1038
2	2 hrs	4.5	2.5	901	1038
3	2 hrs	4.5	2.6	901	1038

It is known that alfalfa contains a considerable quantity of the pigment carotene, which gives the Carr-Price reaction as in the case of vitamin A. Consequently, an initial test sample was prepared in order to determine the blue unit value that would be obtained without the addition of cod liver oil. A 20 gm sample was placed in each extraction thimble and treated as previously described, the final residue being taken up in 10 ml of chloroform. The results are given in Table IV.

Table IV

Sample#	Period of Extraction	Blue units	Yellow units	Blue units/gm of material
1	2 hrs	2.8	2.0	16
2	2 hrs	2.9	2.0	16.4

An alfalfa mixture was now prepared as in the case of oatmeal. The bulkiness of this material necessitated the use of 20 gm samples and the presence of carotene made the dilution of the final residue with chloroform differ from the previous cases. 20 ml of chloroform was added. Table V shows the results obtained.

Table V

Sample#	Period of extraction	Blue units	Yellow units	B. U./ gm of oil added	Expected potency in B.U./gm of oil added
1	2 hrs	2.1	1.3	788	1038
2	2 hrs	2.2	1.3	911	1038
3	2 hrs	2.2	1.3	911	1038

From the above data it may have been observed that the recovery of the vitamin from the alfalfa compares favorably with the results obtained with other feeds. This mixture, however, presented an additional step which involved a preliminary determination of blue units given by the alfalfa previous to the addition of the cod liver oil. The average of the blue units per gram of oil added is 870.

The Determination of Vitamin A in Milk and Butter

Milk and butter are valued, among other things, for their vitamin A content. The vitamin A potency is, however, quite variable depending on the supply of provitamin A in the ration of the dairy cows. Moreover, in recent years a practice has developed in which a cod liver concentrate has been added, chiefly with the intention of fortifying the milk with vitamin D. Attention is, however, now being called also to the increased vitamin A content resulting from the incorporation of the concentrate with milk. Consequently, it was considered of interest and importance to apply a chemical method to the determination of vitamin A in both milk and butter.

As a preliminary measure, several determinations were carried out on ordinary market milk, using 200 ml samples for saponification. A known amount of cod liver oil was added to a similar source of milk to determine the extent of recovery of vitamin A. Approximately the expected recovery was made and in addition, the natural occurring vitamin of the milk could be accounted for. In addition, several samples of market milk fortified with Vitex, a cod liver concentrate, were analyzed for vitamin A. In the case of the latter type of milk, 100 ml was found suitable for analysis. The vitamin A content of butter was determined by saponifying 5 and 10 gm samples, the remainder

of the procedure being similar to that used in all of the previous tests. The results of these analyses are summarized in the following table.

Table VI

	Sample #	Blue units	Yellow units	Blue units/ml
Market Milk	1	2.9	1.9	.80
	2	3.0	2.0	.83
	3	2.9	2.0	.80
Vitex Milk	1	3.3	1.5	1.81
	2	3.5	1.5	1.92
	3	3.1	1.2	1.70
	4	3.0	1.1	1.65
	5	3.3	1.1	1.81
	6	3.2	1.1	1.76
	7	3.3	1.2	1.81
Butter	1	2.5	.8	27.5 B.U./gm
	2	2.6	.7	28.6 "
	3	2.4	.7	26.4 "
	4	2.5	.7	27.5 "
	5	2.5	.7	27.5 "
	6	2.6	.7	28.6 "
	7	2.4	.7	26.4 "
	8	2.3	.5	25.3 "

The samples of market milk showed the presence of considerable natural occurring vitamin. The residues from the 200 ml samples were diluted with 5 ml of chloroform as were the samples of Vitex milk. From the data it is observed that the market milk shows the presence of .81 blue units per ml of milk, while the Vitex samples show an average reading of 1.78 blue units per ml of milk. The addition of Vitex to the milk therefore is responsible for .98 units per ml, which represents about 936 blue units per quart of milk. If this number is multiplied by $1\frac{1}{2}$, which is the conversion factor usually used when converting blue units into international units, it is found that 1404 international units of vitamin A are present in each quart of milk due to addition of Vitex.

The butter samples of 5 and 10 gm were treated according to the procedure, the final residues being taken up in 5 and 10 ml of chloroform respectively. The vitamin A potency found in these samples is an approximate agreement with values generally given for butter as determined by the biological method.

Discussion

Altho the method employing the Carr-Price reaction for the determination of vitamin A is not yet officially recognized, it is becoming more and more popular because of the obvious advantages in time and cost as compared with the biological method of assay. Various factors such as non-specificity of the reagent, loss of vitamin A incidental to the various steps employed in carrying out the determination and a fading color, account for a relatively low degree of accuracy. Nevertheless, the method in question does give results that compare favorably with those obtained by the biological method and it may therefore be expected that the former will replace the latter in many cases.

In general, it may be said that the results which were obtained in this study indicate that the method used is fairly reliable in determining the vitamin A that has been added to feeds through the use of cod liver oil. There is, however, a loss of the vitamin encountered, other than that explained by saponification. This additional loss was found in most cases. The fact that foodstuffs may contain some interfering materials may account at least in part for this additional loss. The expected recovery of the vitamin in each case was approximately 90% of the original amount added to the feeds. Of this 90%, there was another loss of 10% in the majority of the feeds tested. In the case of oatmeal, the recovery was considerably less than expected, although no explanation can be given.

In analyzing milk it was found that the percentage of added vitamin A, which could be accounted for was somewhat greater than in the case of the other foodstuffs. From this fact, it would appear that the use of the Carr-Price reaction in the determination of vitamin

A in milk, might become very useful in commercial fields as the demand arises for the testing of milk for this vitamin. When definite claims are made regarding the vitamin content of foods, it then becomes necessary to make a check on such materials. From the milk samples, to which Vitex had been added, a very high degree of recovery of the vitamin was obtained, the data showing this to be about 95%, using the initial saponified sample of cod liver oil as a basis.

As an indication of the reliability of the chemical method employed in this study the following information may be cited. The distributors of Vitex make the claim that when this product is used to fortify milk with vitamin D, it also adds 1600 international units of vitamin A per quart of milk, as determined by the biological method. In making a chemical assay of this milk it was found that by multiplying the number of blue units per quart of milk by the factor 1.5 that approximately 1400 international units could be accounted for. In so far as the relation between blue units and international units is not definitely established the factor 1.5 might cause some error in the conversion. Since the biological method is costly and time consuming, there is reason to believe that the chemical method will supplant the former in such work as indicated above.

Conclusions

The Carr-Price reaction may be used with a fair degree of accuracy to determine the vitamin A in foods that have been fortified by the addition of cod liver oil.

The degree of recovery of vitamin A by the method used is relatively high, the range generally lying between 80-95%.

This method is also applicable in the determination of vitamin A, in plain and fortified milk as well as in butter.

Bibliography

1. Physiol, J., 46, 173 (1913)

2. Steenbock, H., and Boutwell, P.W., J. Biol, Chem; 41, 81 (1920)

3. Carr, F.H., and Price E. A., Biochem. J. 20, 497 (1926)

4. Wokes, F., and Willimott, S.G., Analyst, 52, 515 (1927)

5. Norris, E. R., and Danielson, I. S., J. Biochem., 83, 469 (1929)

6. Norris, E. R., and Church, A. E., J. Biol. Chem., 89, 42 (1930)

T545 A146 118093

Abernethy

CHEMISTRY DEPT.

T545 A146 118093

Abernethy

The use of the antimony trichloride method for the determination of vitamin A in feeds, mild & butter.