

Determination of Vitamin A and Carotenoids in Butterfat

Spectroscopic Characteristics of Butterfat Fractions and Problems Involved in Biological Interpretations

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Results of tests made with modifications of the saponification and ether-extraction procedure (8) for determination of vitamin A in butterfat are reported, with a discussion and partial interpretation of the spectroscopic characteristics of such ether extracts.

NO SINGLE method for the direct spectroscopic determination of vitamin A in butterfat has been generally employed. A related application of spectroscopic methods (1) to the study of the vitamin A content of milk has been made by Dornbusch, Peterson, and Olson (3). However, little has been done in the study of errors involved with different butterfat samples or the correlation of spectroscopic data with biological assays.

In experiments in cooperation with the Technical Committee on Vitamin A Researches (8), a procedure of saponification and extraction with ether was developed. Details for spectrophotometric application have been reported (15). This paper reports the results of tests made with modifications of this procedure and presents a discussion and partial interpretation of the spectroscopic characteristics of such ether extracts. Attempts were made to correlate results calculated from direct spectroscopic data with those of the biological method in the case of samples (6, 7) which had been assayed by the rat-growth method.

EXPERIMENTAL

Since β -carotene and vitamin A per se are responsible for practically the entire vitamin A potency of normal butter, the analytical problem is primarily the determination of these two compounds in the presence of related carotenes and carotenols. The carotene content is no indication of the vitamin A alcohol content; hence, for direct determination of vitamin A, ultraviolet spectrophotometry of an extract is necessary.

Clarification of butter samples consisted of filtration of the melted fat through filter paper at 55° C. during 2 to 3 hours, to remove water and salt.

Carotenoids were estimated from absorption values at 4370 and 4360 Å., which are the coincident points for the absorption curves of β - and neo- β -carotenes in ether (15) and hexane (2), respectively. Wave lengths 4525 and 4675 Å. are of possible utility in the estimation of individual carotenoids (see Figures 1 and 2). They may be used with most spectrophotometers with a minimum possibility of error due to inexact wave-length calibration or to wide spectral regions isolated because the maximum of β -carotene occurs at 4525 Å. and the minimum of β -carotene at 4675 Å. [also near the flat region of the neo- β -carotene curve (2)].

The nature of the carotenoids found in butter is dependent largely upon the feed of the cows, as pointed out by Strain (13) and others. In Figure 1 are representative characteristic curves of the total carotenoids found in the butters studied. Sample 106 was specially churned at the Purdue creamery. The standard curves of β -carotene and its isomer neo- β -carotene (2) are included for reference. [This neo- β -carotene fraction probably consists largely of the isomer designated by Polgár and Zechmeister (10) as neo- β -carotene B.] All curves were placed to coincide at 4370 Å. It is noted that curve 8 is characterized by higher relative absorption than curve 7 in regions near 4000 and 4250 Å., which is in agreement with similar observations of Strain (13).

To obtain a characteristic absorption curve of the total carotenoids with a minimum amount of induced isomerization, a butter

sample from a cow fed a carrot-root carotene supplement was simply dissolved in ether, washed with water, dried with sodium sulfate, and filtered. The characteristic curve showed evidence of the presence of α -carotene and was very close to absorption curves of the ether extract after saponification, in spite of solvent differences due to the large amount of fat present. In this experiment a 7-cm. thickness was studied spectroscopically by comparing an 8-cm. cell with a 1-cm. cell, both filled with the butterfat solution to avoid differences in apparent absorption due to refractivity differences.

Another butterfat, 106, was dissolved in hexane, and washed free of carotenols with 94% diacetone alcohol (2). The characteristic curve (Figure 2) of the resultant carotene fraction corresponded well between 4360 and 4950 Å. with that of a mixture of β -carotene and neo- β -carotene. Upon analysis for these two components (2) the percentage of β -carotene was 77.1 at 4780 Å. and 79.1 at 4850 Å. Adsorption of this solution on columns of magnesia and alumina indicated the presence of two or more pigments. Figure 2 includes curves of the carotene fraction from each type of silage butter and from referee sample No. 3 (15), all of which were prepared by evaporating the ether extract almost to dryness, dissolving in hexane, and finally washing with 92% aqueous methanol. All curves were placed to coincide at 4360 Å.

Vitamin A was estimated from the absorption value at 3240 Å. which is the absorption maximum of vitamin A alcohol in ether solution (15). The characteristic curves of Figure 3 were arbitrarily placed to coincide with the standard curve of vitamin A alcohol at 3400 Å. The carotenoid content of butterfats is subject to wide variation and carotenoids other than β -carotene are

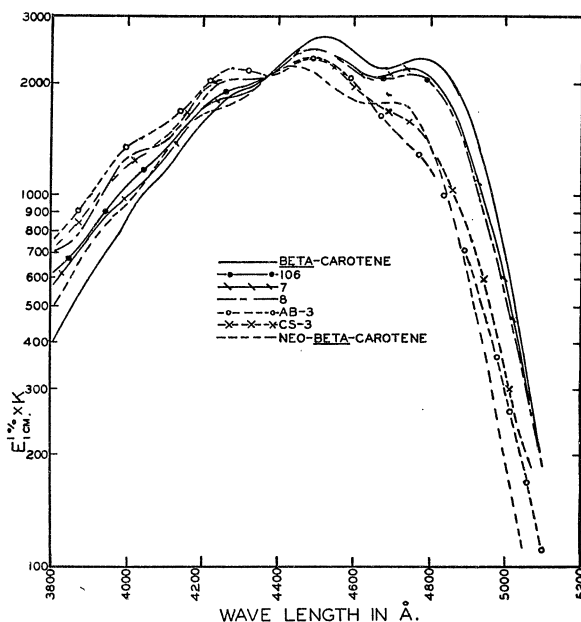


Figure 1. Absorption Spectra of Total Carotenoids in Ether Solution

- 106. Winter creamery butter, no artificial color added
- 7. Butter from cow fed alfalfa hay
- 8. Butter from cow fed carrot-root carotene supplement
- AB-3. Butter from cow fed alfalfa-brome silage
- CS-3. Butter from cow fed corn silage

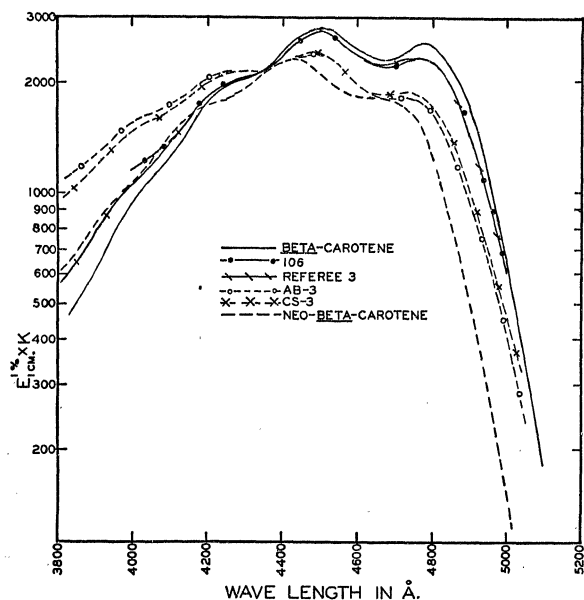


Figure 2. Absorption Spectra of Carotene Fractions in Hexane Solution

106. Winter creamery butter, no artificial color added
 AB-3. Butter from cow fed alfalfa-brome silage
 CS-3. Butter from cow fed corn silage

usually present. The ultraviolet absorption characteristics of such miscellaneous carotenoids have not been established. Colorless materials which absorb in the ultraviolet region probably also accompany vitamin A in the ether extract. Accurate corrections at 3240 Å. for absorption by carotenoids and other substances in such extracts are impossible at present. The necessity for some correction, even though it be somewhat arbitrary, is indicated by the curves of Figure 3, some of which agree fairly well with that of vitamin A below 3400 Å. They are invariably higher than the vitamin A curve at longer wave lengths, in part because of different carotenoid contents. Sample B3 was almost colorless and was obtained from a cow fed on a ration lacking carotenoids but supplemented with vitamin A in the form of a fish liver oil concentrate.

RELIABILITY OF EXPERIMENTAL PROCEDURE (8, 15). *Saponification Time.* A sample of commercial butter was clarified and saponified with aqueous methanolic potassium hydroxide in duplicate for 5, 10, 15, and 30 minutes. The eight absorption values of the ether extracts were constant to $\pm 2.5\%$ at 3240 Å. and to $\pm 1\%$ at 4500 Å. A 15-minute saponification with ethanolic instead of methanolic potassium hydroxide gave essentially the same result. Methanol is preferred because it is more stable with potassium hydroxide.

Number of Ether Extractions. Successive ether extracts were examined spectroscopically to determine their relative effectiveness in the removal of carotenoids and vitamin A. A rendered creamery butter with no artificial coloring matter was used. The first extract (100 ml.) removed 70% of the substance absorbing at 3240 Å. and about 91% of the carotenoids. The second extract (50 ml.) accounted for 20% additional absorption in the ultraviolet and removed all the remaining carotenoids. The third extract (50 ml.) accounted for 6.5% ultraviolet absorption and the fourth extract of 50 ml. left in the hypophase no appreciable quantity of material absorbing at 3240 Å.

Effect of Ordinary Laboratory Illumination. Commercial butter was clarified and analyzed in duplicate in two kinds of glassware. In one case, all manipulations were done in amber glassware (Kimble amber laboratory glassware), as recommended by Embree (4); in the other, ordinary Pyrex ware was used. Results on the two samples agreed within 1% in both the visible and ultraviolet regions, indicating that ordinary diffuse laboratory light does not affect the spectroscopic values obtained under these conditions. Absorption values of pure vitamin A in ethanol solution were not changed by fairly intense irradiation from an incandescent source (14).

Sampling Error. Many duplicate determinations made during this study indicate that butter is easily sampled after removal of water and salts. The surface butterfat exposed to air was avoided when possible. Spectroscopic results were easily duplicated with differences of 1% or less.

Recovery of Added Vitamin A. Two samples of pure vitamin A alcohol (0.325 mg.) were carried through the entire analytical procedure in the absence of butterfat. The resultant extract had a characteristic curve identical with that of vitamin A and a spectroscopic recovery of $93 \pm 1\%$ was obtained. In another experiment, 3 mg. of crystalline vitamin A alcohol were dissolved in 30 grams of melted butterfat that was very low in carotenoids. One gram of this butterfat-vitamin mixture was then diluted tenfold with more melted butterfat. After analysis the absorption values of the butterfat sample alone were subtracted from those of the enriched sample (in duplicate). Characteristic absorption curves that agree well with those of pure vitamin A alcohol were thus obtained. Recoveries of 97.0 to 95.5% were calculated. A similar experiment was performed on duplicate samples of a butterfat (sample 7) rich in carotenoids. Exact duplication of the characteristic vitamin A curve was obtained in this case. Recoveries of 99.4 and 94.2% were calculated.

Stability of Extracts. The ether solutions from sample 7 were examined spectroscopically after 2 weeks in cold storage at 4° C. While no change, qualitative or quantitative, was detected in the region above 4000 Å., a general decrease in absorption was found below this wave length. This decrease amounted to 9% at 3240 Å. A decrease of similar magnitude in 30 hours was noted in the extract from a relatively "colorless" sample. These results are typical and indicate the necessity for prompt spectroscopic readings in the ultraviolet, whereas observations in the visible region may be considerably delayed if extracts are stored under the proper conditions.

ATTEMPTS TO IMPROVE ULTRAVIOLET CHARACTERISTIC CURVES. Attempts were made to reduce the general absorption in the ultraviolet by purification of the vitamin A extract through removal of interfering substances.

Clarification. The authors' observations indicate that spectroscopic data on butters in the visible region may be converted to the butterfat basis by multiplying by the factor 1.20, as calculated from carotenoid absorption in the visible region. This is presumably due only to the removal of water and salt as colorless material by clarification. Clarification also removes certain materials which absorb in the ultraviolet, and the corresponding correction factor at 3240 Å. was ca. 1.35 for the three samples examined. It is advisable to clarify samples of butter before analysis because such materials may not be uniform in all butters and because the analytical data are thus placed on the fat basis, making variations in water or salt content unimportant.

Adsorption. When the ether extract was passed through a 10-cm. (4-inch) column of magnesia-Supercel (50-50) and the ether percolate made to volume, no change was noted in the absorption spectrum of the carotenoids in the visible region, or in the quantity of total carotenoids present. The ultraviolet absorption, however, increased about 10% at 3240 Å.

Treatment of a "colorless" butterfat with Lloyd's reagent, which has been used to decolorize butters and remove vitamin A (12), failed to provide a base curve which could be interpreted as representative of the general absorption—i.e., other than that represented by vitamin A. The difference between the butterfat curve and that of decolorized fat did not approach the curve of vitamin A.

Acid Extraction. When the final ether solution from sample 106 was extracted with 0.5 N hydrochloric acid, followed by 0.5 N sodium hydroxide and water, a small decrease in ultraviolet absorption occurred and small changes (perhaps isomeric) were found in the visible absorption.

Freezing. The ether extract was chilled in a dry ice-acetone bath and rapidly filtered on a cold filter in an effort to freeze out impurities. A 14% decrease occurred in the absorption value at 3240 Å. but the decrease was general in the ultraviolet and, therefore, no improvement in the characteristic curve resulted.

STABILITY OF CAROTENOIDS IN STORED SAMPLES. Ten months after the first spectroscopic observations, referee samples 6 (15) and 7 (both kept at -20° C.) were re-examined. For the former sample, the 4370 Å. absorption remained constant but the value at 4780 Å. decreased 5%. In the case of sample 7, however, the absorption at 4370 Å. decreased 5% but that at 4780 Å. decreased 28% and the resultant characteristic curves of the total carotenoids and the carotene fraction from this sample now resembled

those from CS-3, a silage butter. The cause of these changes was not investigated, but it is evident that the carotenoids in butterfat may undergo considerable change with time, even at low storage temperatures. Isomerization may be involved.

CORRELATION BETWEEN SPECTROSCOPIC AND BIOLOGICAL RESULTS. Twenty-four samples were assayed biologically by the rat-growth method. Fourteen relatively "colorless" samples were produced from two cows fed rations lacking in carotenoids but supplemented by various amounts of vitamin A in the form of a fish liver oil concentrate. Ten samples of yellow butter were from cows fed alfalfa hay or a diet supplemented by various amounts of carrot-root carotene (?). All samples were stored at -20°C . Unfortunately, the time intervals (1 to 21 months) were rather great between the dates of churning and the spectroscopic observations.

The vitamin A activity of the relatively "colorless" samples is attributed almost exclusively to vitamin A per se, derived from cod liver oil of the ration supplement. Corresponding values of $E_{1\text{cm}}^{1\%}$ (observed on unclarified butters) were corrected for water and salt content by multiplication of the observed value by 1.35 and for general absorption by 0.78, a factor discussed previously (15). The vitamin A contents corresponding to such absorption values were calculated on the assumption that the corrected absorption in the ultraviolet was due to vitamin A alcohol ($E_{1\text{cm}}^{1\%} = 1825$ at 3240 \AA).

Most of the samples high in carotenoids were clarified before analysis; for such samples the ultraviolet absorption values were simply multiplied by the factor 0.78 before calculation of vitamin A content, and total carotenoids were determined from absorption measurements at 4370 \AA . (15). The total carotenoid content was divided by 2 (9) and added to the vitamin A content.

With neither set of butters were spectroscopic calculations sufficiently well correlated with biological results to establish a clear cut relationship. When a line was drawn through the or-

gin to approximate the median line, individual samples of "colorless" butters deviated from this line a maximum of 20%, with a mean absolute deviation of 10%. With the yellow series, it was apparent that the two very different types of rations make a separate treatment desirable with regard to correction factors. Deviations from median lines similar to those from the "colorless" series could be obtained.

DISCUSSION

The varied nature of the curves for total carotenoids from different types of butter (Figure 1) and the changes which carotenoids may undergo during storage make difficult the optical estimation of the particular carotenoids that have vitamin A potency. The wave length 4370 \AA appears to be the best choice for total carotenoid estimation, at least until more detailed spectroscopic data are available on the various individual carotenoids present and on such of their isomers as may occur in butter.

It is reasonable that a measure of total carotenoids, such as obtained by the use of wave length 4370 \AA , should be inadequate in itself and should require a correction factor, the magnitude of which is dependent on the dietary source of the carotenoids.

Carrot-root carotene contains large amounts of α -carotene, and some γ -carotene in addition to β -carotene, as well as appreciable quantities of other carotenoids, while alfalfa hay contains β -carotene, various carotenols, and decomposition products of carotenoids. Recent developments in the application of isomerization methods to the study of carotenoids (2, 10) show the complexity of this problem.

The rather high content of carotenols found in the referee butters (15) causes the carotene fraction to be considerably more significant than the total carotenoids from the standpoint of measurement of vitamin A potency. It is possible that measurements on the carotene fraction would have required smaller correction factors than were applied to the total carotenoid absorption.

It is noted in Figure 2 that the curves for sample 106 and referee sample 3, creamery butters of January and July, respectively, are very close together and approximate the curve of a mixture of β -carotene and neo- β -carotene. Indeed, the characteristic curves for all six referee samples, taken at intervals throughout the year, were remarkably similar in both the ultraviolet and visible regions. Clarification and hot saponification may promote isomerization of β -carotene but it is not unlikely that neo- β -carotenes occur naturally in butterfat. Strain reported no spectroscopic change in the carotenoid spectrum after heating 3 hours at 50°C . during clarification (13). It is evident that the carotene fraction of butters from silage-fed cows is qualitatively different from those of butters from other sources, in agreement with previous work on this subject (11).

Ultraviolet characteristic curves of ether extracts from some butterfats show a decreasing (or at least not greatly increasing) absorption below 3240 \AA . Most of the interfering absorption in this region may be due to carotenoids. Removal of the carotenol fraction from referee sample 3 by 90% methanol decreased the ultraviolet absorption very much. However, the ultraviolet characteristic curve of the carotenol fraction (after transference to ether) did not resemble that of vitamin A any more closely than did the curve of the original ether extract.

Numerous other methods of handling the data were tried in an attempt to obtain a straight-line relationship which would result in smaller deviations, but no better results were obtained. In the case of the butters of the yellow series, the carotenoids were treated as a mixture of β -carotene and neo- β -carotene. Addition of the calculated β -carotene content (after division by 2) to the vitamin A content gave no better correlation. The maximum deviations for these samples are smaller than those reported by Fraps, Kemmerer, and Meinke (6), who applied corrections of a somewhat similar nature to butters.

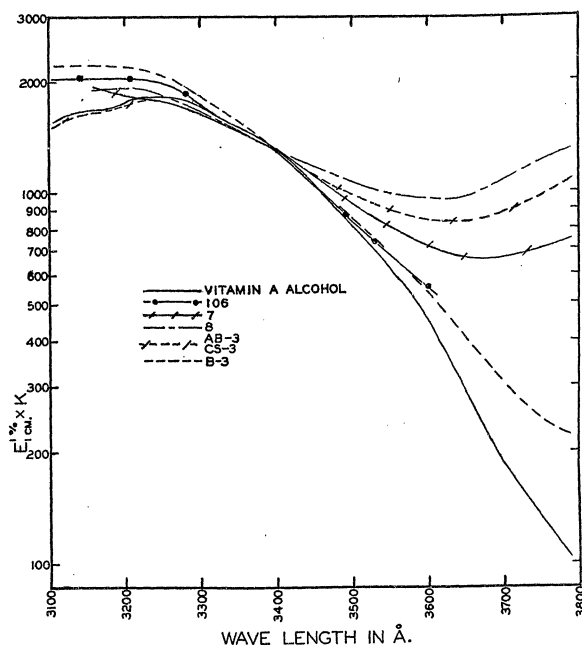


Figure 3. Ultraviolet Absorption Spectra of Ether Extracts

- 106. Winter creamery butter, no artificial color added
- 7. Butter from cow fed alfalfa hay
- 8. Butter from cow fed carrot-root carotene supplement
- AB-3. Butter from cow fed alfalfa-brome silage
- CS-3. Butter from cow fed corn silage
- B-3. "Colorless" butter from cow fed on ration lacking carotenoids

Efforts to obtain agreement between spectroscopic and biological results with six silage butters failed completely. The pigment system in such butters is too complex to permit a similar interpretation at the present time. This is recognized as a serious objection to the use of this method at present. Considerable improvement is needed before this method can be successfully employed on butterfats from cows on different diets, even for comparative purposes.

The calculation method presented above was based on numerous assumptions which are not necessarily better than others that might well be made. The necessity for such assumptions arises from the great complexity of the problem and the multitude of factors which interfere with a direct and simple spectrophotometric determination of vitamin A-potent substances in butterfat. Errors inherent in the biological method must also be considered in such correlations as were attempted here. An exact statistical treatment of the data was not considered profitable because of the many sources of error involved. It is hoped that these observations may be of assistance in the final development of a more direct treatment and more general application of absorption data to obtain contents of vitamin A-potent substances in butterfats. The extraction procedure employed is considered adequate and reliable. Further fractionation appears desirable, especially the separation of vitamin A from other carotenoids.

SUMMARY

Samples of butterfat produced under different dietary conditions were studied by the direct spectroscopic method. Total carotenoids were estimated and ultraviolet measurements were made on the unsaponifiable fraction. Characteristic curves of the total carotenoids and of the carotene fraction from very light "colorless" butters, yellow butters, and butters from cows fed alfalfa-brome grass and corn silages, were compared with that of β -carotene. Corresponding curves of the unsaponifiable fraction in the ultraviolet region were compared with that of vitamin A.

Effects of clarification, adsorption, acid extraction, and freezing upon the characteristic curves were studied, as well as various factors affecting the reliability of the experimental procedures.

Twenty-four samples were assayed biologically and attempts made to correlate spectroscopic with biological values. No clear-cut relationship could be established. The feed of the cows has a great influence on the nature of the carotenoids present in the butterfat. More extensive purification of the vitamin A fraction is desirable for the successful application of direct spectrophotometry to the determination of vitamin A in butterfats.

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