XIV. A MODIFIED SPECTROPHOTOMETRIC METHOD FOR THE ASSAY OF CAROTENE AND VITAMIN A IN BUTTER.

By ALBERT EDWARD GILLAM.

From the Department of Organic Chemistry, University of Manchester.

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In a previous investigation [Gillam *et al.*, 1933] a spectrophotometric method was developed for the evaluation of carotene, xanthophyll and vitamin A in butter. This involved the separation of xanthophyll from carotene by the well-known partition between methyl alcohol and light petroleum, but although necessary for an original research, it was felt that this method could now be considerably shortened for routine purposes. Experience shows that the carotene : xanthophyll ratio in butter averages 14 : 1 by weight, a fact which, as will be seen later, permits of the approximate estimation of the relatively unimportant xanthophyll without actual determination.

The spectrophotometric evaluation of carotene and xanthophyll depends on the fact that each of these substances exhibits a group of absorption bands in the visible region of the spectrum, the principal one occurring at $463m\mu$ for carotene and $455m\mu$ for xanthophyll (in chloroform solution). The amount of either carotenoid present in a solution containing only one of them is proportional to the intensity of its particular absorption band.

The following table shows the average light absorptive power (which is related to the yellow colour) of the carotene and xanthophyll fractions of some 70 samples of English butter, arranged in six sets but actually representing twenty small groups of cattle, of several pure breeds.

Group	No. of samples	* $E_{1\text{ cm.}}^{1\%}$ dry butter due to carotene ($463m\mu$)	$E_{1\text{ cm.}}^{1\%}$ dry butter due to xanthophyll ($455m\mu$)	% of total absorption due to carotene ($460m\mu$)
A	11	0.00522	0.000348	93.8
B	9	0.00395	0.000285	93.3
C	10	0.00878	0.000468	94.9
D	8	0.00526	0.000333	94.1
E	24	0.00502	0.000371	93.1
F	8	0.01159	0.000391	96.7
	70		Average value	94.0

* $E_{1\text{ cm.}}^{1\%} = \log I_0/I$ for a 1 cm. layer of a solution equivalent to 1 % of dry butter, I_0 = the intensity of the incident light, and I = the intensity of the transmitted light.

Allowing for the slightly different intensities of absorption of carotene and xanthophyll ($E_{1\text{ cm.}}^{1\%}$ $463m\mu = 1900$ for carotene, and $E_{1\text{ cm.}}^{1\%}$ $455m\mu = 1700$ for xanthophyll (lutein), when chloroform is the solvent) this absorption ratio of 94 : 6 becomes 14 : 1 for the ratio of carotene to xanthophyll by weight in butter.

These results, and the individual analyses from which they were summarised, show that:

(a) On the average, 94 % of the light absorption of butter-unsaponifiable matter in chloroform, at 455–460 $m\mu$, is due to carotene, the remainder being due to xanthophyll.

(b) The values for individual samples approximate to this mean value, and as xanthophyll is only a minor constituent of the butter pigment, small departures from constancy do not affect the resulting carotene percentage seriously, if the xanthophyll is estimated at approximately 6 % of the total yellow colour, instead of being actually determined.

The estimation of the carotene value at approximately 94 % of the total yellow colour of the butter, makes it possible to modify considerably the method of evaluation of carotene and vitamin A previously developed, with advantages which more than offset any error likely to occur through departures from constancy in the carotene : xanthophyll ratio.

(a) The elimination of the xanthophyll determination removes the need for the partition between light petroleum and methyl alcohol, with consequent saving of much time and material.

(b) The spectroscopic work is reduced to half because the unsaponifiable matter is examined as a whole instead of in two separate fractions.

(c) The accuracy of the method is increased by the much reduced handling of material well-known to be labile, as well as by the evaluation of the vitamin A in one fraction, instead of adding two results (and therefore two errors) together.

(d) The weight of butter required for the determination can be reduced, making a further economy in time and material.

Baumann and Steenbock [1933] have recently reported the results of an examination of fourteen American butters consisting of bulked samples representing the butter obtained in their particular district in each month of the year. They used a spectrophotometric method for both the carotene and vitamin A determinations but did not consider it necessary to correct their carotene values for xanthophyll. They pointed out, however, that in the case of the unsaponifiable matter of one particular August butter, approximately 95 % of the pigment was not extractable from light petroleum by methyl alcohol, and moreover, its absorption spectrum was characteristic of carotene. Thus the value they found for the carotene : xanthophyll ratio in this particular butter is very close to the value here shown to be the average of a large number of butters.

The modified procedure.

Preparation of the unsaponifiable matter. A weighed portion of the butter (100 g. of winter butter, or 25–50 g. of summer butter or half quantities if the antimony trichloride test is not being used) of known moisture content, is saponified for half an hour with a slight excess of colourless 20 % alcoholic potash, and the soaps are poured into about three volumes of water¹. The solution is extracted with ether until no more colour is removed, the extracts being bulked and washed carefully with several portions of water. The whole of the washed ether extract is then freed from solvent by evaporation in nitrogen. If the spectroscopic examination is not to be carried out at once this evaporation should be completed in a brown glass ampoule, the residue being finally sealed off in

¹ The colourless alcoholic potash recommended is obtained by using alcohol which has been previously boiled over, and distilled from, either metallic sodium or caustic soda.

nitrogen. Otherwise, the residue is dissolved in chloroform, made up to 10–20 ml. in a graduated flask and examined immediately.

Determination of the carotene. The solution now represents a known weight of butter per 100 ml. The intensity of absorption at 455–460 $m\mu$ is measured either photographically, using an apparatus such as the Hilger E 3 quartz spectrograph with a suitable photometer, or visually, using a spectrophotometer of the type of the Hilger-Nutting instrument¹.

We have found the visual method to be the better of these two, as there is less danger of destruction of the carotenoids by the ultra-violet rays of the light source (where an arc or spark is used with quartz apparatus) in the photographic method.

The value so obtained is expressed as $E_{1\text{ cm.}}^{1\% \text{ butter}}$ at 455–460 $m\mu$ and the absorption due to carotene is taken as 94 % of this value. To calculate the percentage of carotene in the butter, use is made of the fact that $E_{1\text{ cm.}}^{1\%} 463 m\mu (\text{CHCl}_3) = 1900$ for pure carotene [Gillam *et al.*, 1932]. The result is conveniently expressed as mg. carotene per 100 g. dry butter.

Determination of the vitamin A.

(a) *By means of the ultra-violet absorption band.* It is now generally accepted that the absorption band at 328 $m\mu$ shown by certain fish-liver oils [Morton and Heilbron, 1928] is a definite property of vitamin A. This is also exhibited by butter [Morton and Heilbron, 1930] and provides a means of evaluation of the vitamin A content. In the case of butter-unsaponifiable matter the absorption at 328 $m\mu$ is a summation of that due to vitamin A together with the smaller contributions due to carotene and xanthophyll. We have found that the absorption due to carotene *plus* xanthophyll, at 328 $m\mu$, is given by dividing the observed value of $E_{1\text{ cm.}}^{1\%}$ for these substances at 455–460 $m\mu$ by the factor 6.5. This correction is usually of the order 10–25 % of the gross absorption at 328 $m\mu$. From the value of $E_{1\text{ cm.}}^{1\% \text{ butter}}$ at 328 $m\mu$, due to vitamin A, it is possible to determine the vitamin A content in mg. per 100 g. dry butter by making use of the value $E_{1\text{ cm.}}^{1\%} = 1600$ given by Carr and Jewell [1933] for their richest concentrate. This value may need correction if vitamin A is concentrated further (*cf.* Karrer and Morf [1933], who have prepared a concentrate of $E_{1\text{ cm.}}^{1\%} (328 m\mu) = 1700$).

(b) *By the spectroscopic blue test with antimony trichloride.* If the solution of butter-unsaponifiable matter is treated with antimony chloride in chloroform the blue colour formed is largely due to vitamin A, but some small part is also due to carotene and, to a lesser extent, to xanthophyll. If, however, the intensity of absorption of the blue solution is determined at 585 $m\mu$ with a visual spectrophotometer, according to the technique already described for liver oils [Heilbron *et al.*, 1931] and applied to butters [Gillam *et al.*, 1933], the value so obtained can be regarded as being due to vitamin A and carotene only, the xanthophyll value here being negligible. As therefore the absolute carotene value is known from the direct absorption and as the blue value of pure carotene is also known ($E_{1\text{ cm.}}^{1\%} 585 m\mu = 420$), it is possible to calculate the contribution due to carotene. The difference gives the blue value due to vitamin A in terms of $E_{1\text{ cm.}}^{1\% \text{ butter}}$ at 585 $m\mu$, and as the purest distillates have a value $E_{1\text{ cm.}}^{1\%} 585 m\mu = 2600$ [Heilbron *et al.*, 1932] in chloroformic antimony trichloride, the vitamin A content can be

¹ Although pure carotene has its principal absorption band at 463 $m\mu$ for chloroform solution, in the presence of butter-unsaponifiable matter this lies nearer to 455 $m\mu$. The intensity of absorption however is not perceptibly altered by taking the measurement anywhere between 455 and 460 $m\mu$.

calculated in mg. per 100 g. butter. The results obtained in this way are always low, apparently owing to the presence in the original butter of substances which act as inhibitors of the blue colour. This is in agreement with the findings of Booth *et al.* [1933] who have shown that the blue values of butter (in Lovibond units) exhibit considerable inhibition and that the inhibition shows a seasonal variation.

In view of these facts the spectroscopic modification of the antimony tri-chloride blue test is not recommended for the general determination of the vitamin A content of butter, but it is included here as a secondary test if for any reason, such as the presence of unsaponifiable artificial colouring matter, the ultra-violet absorption spectrum method is interfered with.

An analysis of the carotene, xanthophyll and vitamin A values of some 70 English butters consisting largely of winter samples yields the following data (obtained by the full method):

	mg. per 100 g. dry butter			Estimated growth-promoting effect* in International units per g.
	Carotene (1900 basis)	Xanthophyll (1700 basis)	Vitamin A (1600 basis)	
Mean values (70 samples)	0.327	0.024	0.473	11.1
Maximum	0.88	0.075	0.922	24.2
Minimum	0.025	0.003	0.158	2.9
Mean value cows at grass (12 samples)	0.60	0.029	0.72	18.0
Mean value stall-fed cattle (58 samples)	0.272	0.0227	0.420	9.7

* Calculated from the numerical values on the assumption that vitamin A is 1.66 times as potent as carotene [Carr and Jewell, 1933]. This value may need revision when really representative values are available.

In connection with the application of this method the following points should be noted.

(a) All values recorded were obtained on unsalted butters and are expressed on dry butter-fat. To obtain comparable results on salted butter the water and salt should be estimated and the carotenoid values calculated on the butter-fat.

(b) The majority of these butters were made from the milk of pure-bred Shorthorn cows, with a sufficient number of samples from pure-bred Ayrshire and South Devon herds to show that the carotene : xanthophyll ratio applies approximately to these also. A few unclassified samples have also been tested with good results.

(c) When butters contain added colouring matter, any method for carotenoid determination depending on yellow colour will give faulty results, hence the treatment will need modification according to the nature of the colouring matter. If this should interfere with the absorption spectrum determination of vitamin A, the antimony chloride test can be called into service.

SUMMARY.

1. Values for the carotene, xanthophyll and vitamin A contents of seventy English butters have been obtained spectrophotometrically. Mean values are recorded.

2. It is shown that, on the average, the ratio of carotene to xanthophyll in butter is 14 : 1 by weight.

3. It is further pointed out that if 94 % of the light absorption of butter-unsaponifiable matter at 455–460 $m\mu$ is taken as being due to carotene, the results on individual samples are sufficiently close to those actually determined after removal of the xanthophyll to justify the approximation by the economy effected in time and material.

4. The spectrophotometric method previously developed for the determination of carotene and vitamin A in butter has been improved for routine purposes. Full details of the modified method are given.

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