The Determination of Carotene in Dried Grass

By W. A. G. NELSON

(*Read at the Meeting of the Society on April 2, 1947*)

A NEW method for the determination of carotene in dried grass is being put forward by a Carotene Committee,* which was originally formed through the Crop Driers Association, and in 1941 proposed a tentative method of analysis.[1] This was called Method B, two other methods, A and C, having been under consideration at that time.

In order to point out the defects of this old method and show the progress that has since been made, it is necessary to give an outline of the tentative method as published in 1941.

METHOD B OF 1941—

The sample of dried grass is ground to a fine powder with sand and extracted with a 3:1 mixture of light petroleum of b.p. 40° to 60° C. and acetone, in a Soxhlet or drip-type extractor. The extract is shaken up with a concentrated solution of potash in methyl alcohol to convert the chlorophyll present into potassium chlorophyllin, which is removed with water. The remaining extract now contains carotenoids and xanthophyll, and the xanthophyll is removed by means of a 9:1 mixture of methyl alcohol and water. The amount of carotene in the light petroleum solution thus obtained is then estimated colorimetrically.

From the work of Seaber,[2] Kon and Thompson,[3] Moore[4] and Mann,[5] it was demonstrated by chromatography that there is in grass a petrol-soluble carotenoid that does not exhibit the properties of β-carotene and has no biological activity. It was believed that this particular "carotene" forms on the average 30 per cent. of the total carotenoids in grasses and therefore analysis of a grass meal by Method B gives an untrue picture of its biological worth.

Furthermore, it was shown that it is not always possible to remove all the chlorophyll as potassium chlorophyllin, and a trace of chlorophyll in the final solution makes the colour estimation fallacious.

Any method which would give the true β-carotene content of a grass meal would, therefore, have to embody the following three important features:

(1) It must be capable of separating β-carotene completely from the non-active carotenoids of grass.

(2) It must yield for the final colorimetric estimation a solution entirely free from any forms of chlorophyll.

(3) It must be a method in which there is no likelihood of isomerisation taking place.

Points (1) and (2) have been mentioned above, and for (3) it is necessary to consider the work of Zechmeister.[6] He showed that β-carotene was capable of spontaneous isomerisation in solution and gave molecular extinction curves of the all-*trans* compound and mixtures of its stereoisomers (see Fig. 1).

Table I gives the $E^{1\%}_{1cm.}$ values of β-carotene over the wavelength range 451 to 447 mμ., calculated from the above molecular extinction curves.

TABLE I

Wavelength mμ.		$E^{1\%}_{1cm.}$
451·0	all *trans*	2575
450·5		2505
450·0		2435
449·5		2365
449·0		2295
448·5		2225
448·0		2155
447·5		2085
447·0	much isomerisation	2015

When a solution is being analysed colorimetrically for β-carotene the $E^{1\%}_{1cm.}$ value is usually taken to be 2500 at 450 mμ. From the figures in Table I it is obvious that, if isomerisation has taken place and the $E^{1\%}_{1cm.}$ at 450 mμ. is assumed to be 2500, inaccuracies will be incurred in determining the β-carotene present.

* Members of the Carotene Committee—Mr. R. O. Davies (*Chairman*), Dr. V. H. Booth, Dr. A. Green, Mr. J. Greenbaum, Mr. A. W. Hartley, Mr. T. Barton Mann, Dr. F. E. Moon, Dr. W. A. G. Nelson, Mr. W. M. Seaber, Mr. H. H. Ward, Dr. H. Wilkinson, Mr. R. F. Wright.

Zechmeister has postulated the possibility of the existence of twenty isomers of β-carotene within the range from all *trans* to all *cis* (see Fig. 2) and he has been able to identify several of them.

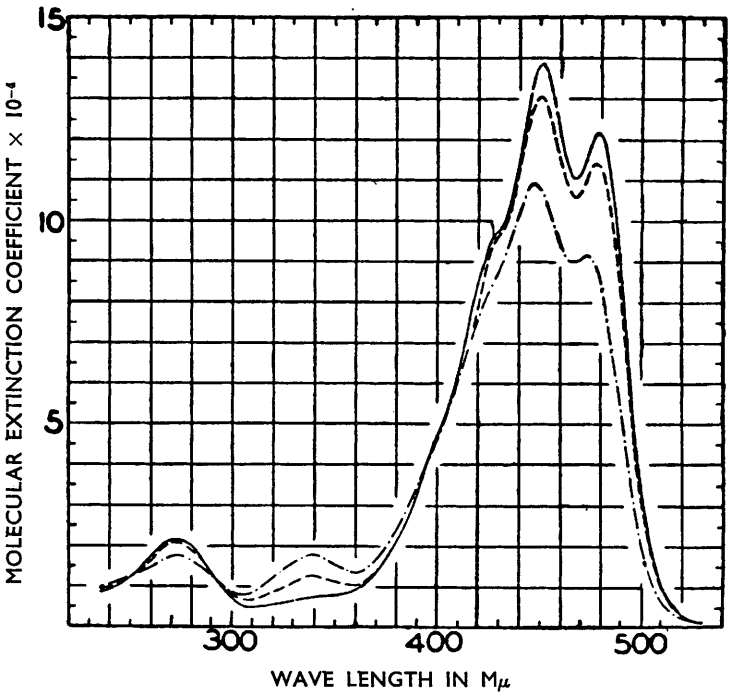

FIG. 1. Molecular extinction curves of β-carotene in hexane: ——, fresh solution of the all-*trans* compound; — — —, mixture of stereoisomers after heating under reflux in darkness for 45 min.; — · —, mixture of stereoisomers after iodine catalysis at room temperature in light. (From *J. Amer. Chem. Soc.*, 1943, **65**, 1523.

Bearing in mind the facts mentioned above, Mann, Seaber, Green and Hartley (who are members of the present Carotene Committee) collaborated with a view to finding an improved method for the determination of carotene in grasses. As a result of their investigations a chromatographic method called Method D was suggested.

METHOD D—

One g. of the sample is ground to a fine powder with sand and extracted with a 3:1 mixture of light petroleum of b.p. 40° to 60° C. and acetone, as in Method B. The mixed solvents are removed almost completely by evaporation on the water bath and the last traces are blown off with a stream of carbon dioxide to minimise oxidation. The dry residue is dissolved in light petroleum (b.p. 40° to 60° C.) and then passed through a column of bone meal (Mann's findings on the use of bone meal as an adsorbent have already been recorded[5]). The filtrate is then examined for β-carotene colorimetrically in the usual way.

The salient points emerging from the analysis of grass meal by Method D were:

(1) The filtrate from the bone meal column contained carotene which had the full biological activity of β-carotene, whilst the "carotene" fraction retained by the bone meal had no biological activity.
(2) The filtrate contained no chlorophyll.
(3) The absorption spectrum of the carotene in the filtrate coincided with that of all-*trans*-β-carotene.

It was now clear that a method had been found which gave a much more accurate determination of β-carotene in grass than the previous ones. By the combined efforts of the whole Committee, it was shown that Method D gave more reproducible results than those obtained by Method B.

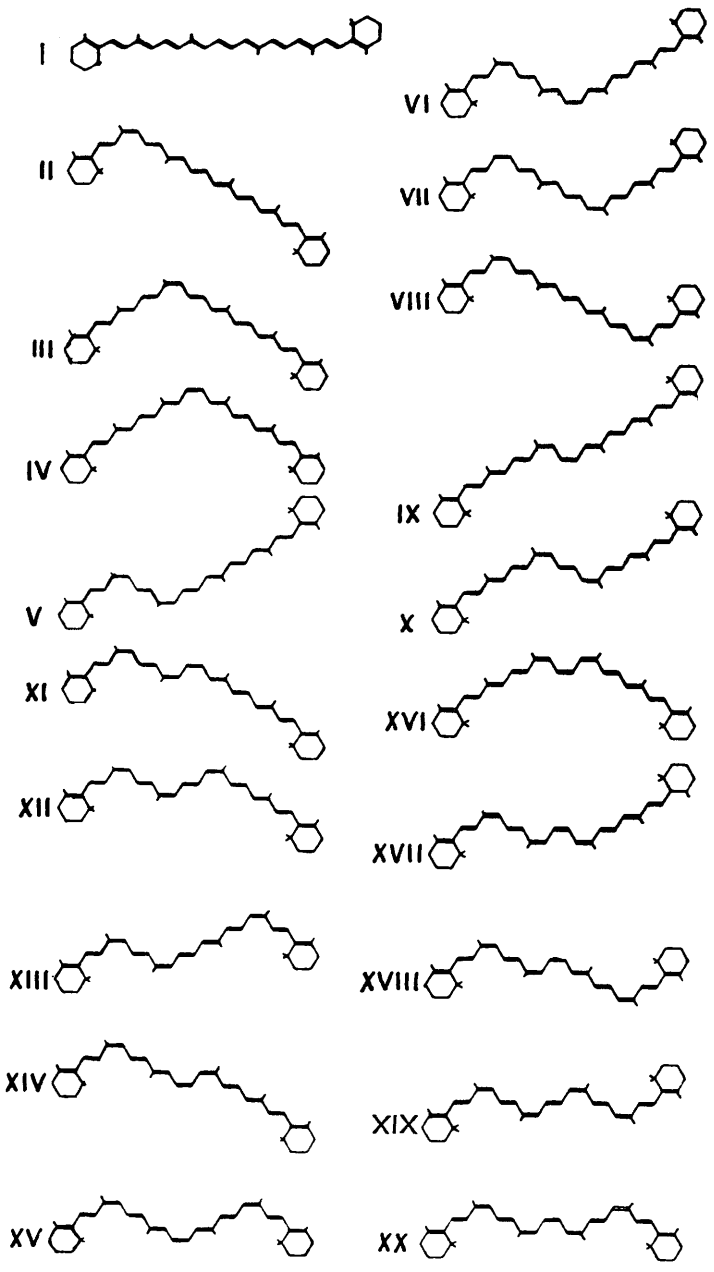

FIG. 2. Skeleton models of the twenty possible stereoisomers of β-carotene: all-*trans*-β-carotene, three mono-*cis*-β-carotenes, six di-*cis*-β-carotenes, six tri-*cis*-β carotenes, three tetra-*cis*-β-carotenes, and all-*cis*-β-carotene.

After this stage had been reached, an improvement in the method of extraction was brought about through the work of the Lever Bros. team consisting of Wilkinson, Edisbury and Gridgeman. The process of grinding with sand, followed by extraction with a 3:1 mixture of light petroleum of b.p. 40° to 60° C. and acetone, was replaced by extraction with light petroleum of b.p. 80° to 100° C. alone, without any initial grinding. This is now adopted in the method finally recommended by the Committee—Method E—which is described below.

METHOD E—NOW RECOMMENDED

Boil from 1 to 2 g. of grass meal with 50 to 60 ml. of light petroleum of b.p. 80° to 100° C. under reflux for 1 hour on a steam bath. Cool the flask and contents and filter the extract through sintered glass or any other suitable type of filter, or merely decant directly, on to a 2-inches by 1-inch column of bone meal. Rinse the flask and residue with small quantities of light petroleum (light petroleum of b.p. 40° to 60° C. may be used at this stage, for ease of subsequent removal if this happens to be desirable). Apply suction to the bone meal column, elute with light petroleum of b.p. 40° to 60° C. and concentrate if necessary. Estimate the carotene in the eluate, colorimetrically.

NOTES—

Boiling can be done in large Kjeldahl flasks, which have been found to provide sufficient condensing effect.

The bone meal should be extracted before use with a 3:1:1 mixture of petrol, acetone, and ether.

The bone meal should be of particle size passing a 120-line sieve but retained by a 200-line sieve. (Arrangements are being made for the preparation of a standard bone meal which can be supplied to anyone interested in this type of work.)

The bone meal column can be used for a large number of determinations. After repeated use it may be washed with acetone, which removes the bulk of the pigments. A further wash with light petroleum prepares the column for further tests.

The whole Committee carried out from 60 to 70 analyses on a sample of dried grass by Method D and an equal number by Method E. The average results by these two methods for all practical purposes can be considered to be identical. For this particular sample the average figures were:

By Method D: 335 mg. per kilo. By Method E: 330 mg. per kilo.

Further comparative average figures for a large number of tests were as follows:

By Method D: 129 mg. per kilo. By Method E: 135 mg. per kilo.
" " " 275 " " " " " " " 279 " " "

It has been shown that light petroleum of b.p. 80° to 100° C., without preliminary grinding of the sample with sand, is as efficient for the extraction of carotene as any other solvent or mixture of solvents. Its efficiency is only equalled by that of soaking the sample in the dark and in the cold for 48 hours with light petroleum of b.p. 40° to 60° C. This latter method, of course, is undesirable if an analysis is required quickly, as is usually the case. Previously, attempts to extract with boiling light petroleum of b.p. 40° to 60° C. had been made, but had been found to be inefficient. It seems that the light petroleum of b.p. 80° to 100° C. is much superior because of the higher temperature attained during extraction.

As mentioned previously, it was believed that about 30 per cent. of the so-called "carotene" obtained by Method B was not β-carotene. As a matter of academic interest as well as commercial importance, the Committee set out to get a more definite figure. For this purpose, 42 samples were analysed by Methods B and E, and it was found that the results by Method E were only 69 per cent. as great as those by Method B. Again this is an average figure, some meals giving, say, 60 per cent. and others 80 per cent., depending on the age of the meal and certain other factors.

From this work the following conclusions can be drawn:

(1) Light petroleum of b.p. 80° to 100° C. is an efficient solvent for the extraction of the carotenoids of grass.

(2) Bone meal is an efficient adsorbent for the separation of β-carotene from the other pigments in grass.

(3) Method E can be carried out more rapidly and easily than any other method.

REFERENCES

1. Carotene Committee of the Crop Driers Association, ANALYST, 1941, **66**, 334.
2. Seaber, W. M., *Ibid.*, 1940, **65**, 266.
3. Kon, S. K., and Thompson, S. Y., *J. Agric. Sci.*, 1940, **30**, Pt. IV, 636.
4. Moore, L. A., *Ind. Eng. Chem., Anal. Ed.*, 1940, **12**, 726.
5. Mann, T. Barton, ANALYST, 1944, **69**, 34.
6. Zechmeister, L., *Chem. Revs.*, 1944, **34**, No. 2, 267.

Discussion

Mr. W. M. Seaber said that in the past the estimation of carotene had been lengthy and tedious. He had been connected with this problem for a long time and had been interested to see the method develop in the direction of simplicity. He had tried the new method, not only on dried grass but also in general work, and had found it convenient and rapid. They owed a great deal to Mr. Barton Mann for bringing to light the advantages of bone meal as an adsorbent of non-carotene substances, and to the chemists at Messrs. Lever Brothers for introducing the use of light petroleum of higher boiling-point range than that previously used for the extraction. He was pleased to have taken part in the work of the Committee.

Mr. N. T. Gridgeman said that he had recently, on behalf of the Carotene Committee, statistically analysed the results of the final collaborative experiment in which methods D and E were compared. It had emerged that the reproducibility of the two methods was substantially the same (coefficient of variation = 4), and that method E yielded slightly, yet significantly, higher results (by about 3 per cent.) than method D. As in so many analytical techniques, reproducibility was found to be better within laboratories than between laboratories, and there may be good reason to believe that inter-laboratory differences (coefficient of variation = 8) were largely due to non-uniformity of instruments. Many laboratories perforce used "abridged" spectrophotometers, *e.g.*, photoelectric absorptiometers dependent upon optical filters and absorption standards. In β-carotene estimation potassium dichromate solution enjoyed an undeserved popularity as a suitable calibration medium—perhaps by false analogy with its undoubted virtues as a visual standard. The steepness of the slope of the absorption curve of potassium dichromate in the region of 450 mμ. rendered it highly unserviceable, and the position was worsened by the fact that most types of nominally identical optical filters exhibit marked variations in their transmission characteristics. He hoped that eventually a wholly satisfactory standard would be found; meantime perhaps the best expedient was to use petrol solutions of pure β-carotene.

Mr. K. A. Williams said he would like to support in the strongest possible terms Mr. Gridgeman's strictures on the use of potassium dichromate as a yellow colour standard. Whilst its use in visual colorimetry was defensible because of the limits of sensitivity of the human eye, it could not be defended for more accurate work. In spectroscopy or photo-electric measurements any standard must have an absorption curve very closely approximating to that of the test colour; it was but rarely that the curve of potassium dichromate did this.

Mr. T. Barton Mann said that from the point of view of spectroscopy one would naturally take readings at the peak of absorption. In photo-electric instruments employing filters he had found potassium dichromate relatively insensitive in so far as variations in concentration near that recommended, *i.e.*, 0·0158 per cent. ($\equiv$ 1 μg. of carotene per ml.) did not appreciably affect the graph. There was little difference between 0·0158 and 0·0164 per cent. solutions. He agreed that search should be made for an alternative standard, preferably a dye the colour of which would not be affected by change of pH.

Dr. C. H. Lea asked if differences in exposure to light in the course of analysis might cause a variable amount of change of the carotene.

Dr. Nelson pointed out that in method E there was no evaporation of solvent from the extract, as there was in method D.

Mr. Barton Mann said that oxidised carotene would be adsorbed on bone meal and not pass into the solution to be measured. The use of the higher-boiling light petroleum reduced risk of oxidation, because the higher temperature enabled the carotene to be extracted without previous grinding of the dried grass with sand. It would seem that the higher temperature removed the moisture of the grass meal and permitted the solvent to penetrate more readily.

Mr. Seaber remarked that light petroleum of b.p. 80° to 100° C. was apt to be rather variable in character and composition.

Mr. Williams suggested that perhaps *n*-heptane might prove more satisfactory. It had a boiling-point range of 98° to 100° C., and to ensure freedom from aromatic constituents it could be washed with sulphuric acid.

Mr. Barton Mann said that on the use of various petroleum fractions of lower or higher boiling-point ranges he would particularly like to draw the attention of analysts to a point that had arisen in the collaborative work of the Committee, *viz.*, that with change of solvent from one of lower to one of higher boiling-point there was a concomitant shift of peak absorption from a lower to a higher wavelength. For example, with light petroleum of b.p. 40° to 60° C. the E max. was 447 to 448 mμ., but with that of b.p. 80° to 100° C. it was 452 to 454 mμ. Dr. J. R. Edisbury had very kindly checked these findings and had furnished the speaker with the refractive indices of two such petroleum fractions, *viz.*, one of b.p. 40° to 60° C., 1·3656 and one of b.p. 80° to 100° C., 1·4002. These differences in absorption and refractive index were significant, perhaps not so much for users of spectrophotometers, by whom the extinction would normally be obtained at peak absorption, but certainly with colorimeters and instruments employing filters, where a change of wavelength would automatically introduce a change of colour. Pending further work along these lines it would seem advisable to adhere to the estimation "carotene in light petroleum of b.p. 80° to 100° C."

There could be no question that the method proposed did produce a solution containing the true carotene of grass meals and that intra-laboratory concordant results could be obtained. The question of inter-laboratory concordance appeared to be entirely a matter of instrument calibration, and it was to be

hoped that the Committee would tackle this problem, possibly along the lines of a master reference instrument of the photo-electric spectrophotometer type to which the owners of colorimeters could refer.

Dr. E. C. Wood asked if the accuracy of the new method had been checked by "recovery" experiments with known added amounts of pure β-carotene, and also if the method could be adapted to the analysis of substances containing vitamin A as well as carotenoids. He had read recently of an organic dyestuff, forming a stable solution, with an absorption spectrum sufficiently similar to that of carotene to make it a satisfactory standard instead of potassium dichromate. Perhaps one of the investigators could comment on this.

Mr. Barton Mann said he had no knowledge of any work on the recovery of carotene added to grass meals. The difficulty of such work would be very great. He had determined the efficacy of recovery of β-carotene from fat, using the bone meal treatment. A freshly prepared 0·03 per cent. standard, after saponification and extraction with ether, was subjected to the whole process of bone meal chromatography, and the figure returned was 0·0283 per cent. Having regard to the severity of the manipulation and the inherent errors of visual spectrophotometer estimation, one could conclude that the loss by such chromatography was nil or negligible.

With regard to vitamin A, Dr. T. W. Goodwin had found from his work with cod liver oil that vitamin A ester passes bone meal and vitamin A alcohol is adsorbed by it. If Dr. Wood had in mind the application of the method to biological material, for which saponification is usually necessary, then carotene would pass into the filtrate and vitamin A, as the alcohol after saponification, would remain adsorbed. He would refer Dr. Wood to "The separation of vitamin A from xanthophyll" (Mann, Analyst, 1943, **68**, 233); "A Chromatographic Method for Separating Free and Esterified Vitamin A" (Glover, Goodwin, and Morton, *Biochem. J.*, 1947, **41**, 94); "Relationship between Blood Vitamin A Level and Liver Stores in Rats" (*Ibid.*, 97).

Mr. Gridgeman said that the yellow substance referred to by Dr. Wood was methyl orange in aqueous solution, which had been suggested as a standard for β-carotene by R. J. Taylor in a recent paper (Analyst, 1946, **71**, 566). But it applied to β-carotene only in chloroform solution, in which the peak absorption occurred at about 460 mμ. No "control" experiments of the type Dr. Wood had in mind had been carried out, nor did he think they would be very valuable, as the separation problem was the extraction of carotene from grass cells.

In reply to a question by Mr. Bacharach, as to whether statistical analysis had isolated any inter-personal variations within laboratories, the speaker said that the inter-personal error had been studied in one laboratory and found to be negligible; but with colorimetric (in contrast with absorptiometric) methods some observational disagreements would almost certainly have to be reckoned with.

Later in the discussion Mr. Gridgeman deprecated the use of nitrogen for the removal of final traces of solvent, on the ground that it often contained some oxygen. Carbon dioxide was objectionable because of frosting of the cylinder valves. Hydrogen was always used in his laboratory and seemed to be the most suitable of the common gases for the purpose.