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STUDIES ON VITAMIN-A IN SOLUTION. PART IV. ESTIMATION OF ANTIOXIDANTS

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A modification of the Emmerie and Engel method for the determination of tocopherols is described for the assay of phenolic antioxidants like propyl gallate, ethyl gallate and monomethyl ether of hydroquinone when present in arachis oil or ethyl oleate. A correction is needed for the interference of colour formation due to the substrates.

During the studies on the stability of vitamin-A in solution (Basu and Bhattacharya, this *Journal*, 1949, **26**, 419, 459; 1950, **27**, 169) it has been found that vitamin-A alcohol, when dissolved in glycerides or esters, saturated or unsaturated, loses about 30% of its activity after about 200 hours of aeration even when incorporated with 0.05% of antioxidant. The antioxidants used were propyl gallate, ethyl gallate and monomethyl ether of hydroquinone. It was considered worthwhile to determine the quantity of antioxidant at intervals of aeration so as to ascertain if there were any relation between the loss of potency of vitamin-A and the quantity of antioxidant present. Lundberg and Halvorson (*Proc. Inst. Food Tech.*, 1945, p. 115) applied the ferrous tartrate reaction of Mattil and Filer (*Ind. Eng. Chem. Anal. Ed.*, 1944, **16**, 427) for the determination of propyl gallate, but a suitable and easy method applicable to the determination of all the above antioxidants is wanting. Accordingly attempts have been made for finding an improved procedure for the above purpose, with the result that the Emmerie and Engel method for the estimation of tocopherols (*Rec. trav. chim.*, 1938, **57**, 1351) has now been so modified that it can be successfully applied in the estimation of phenolic antioixidants of the type described above. The details of the process are presented below.

Experimental

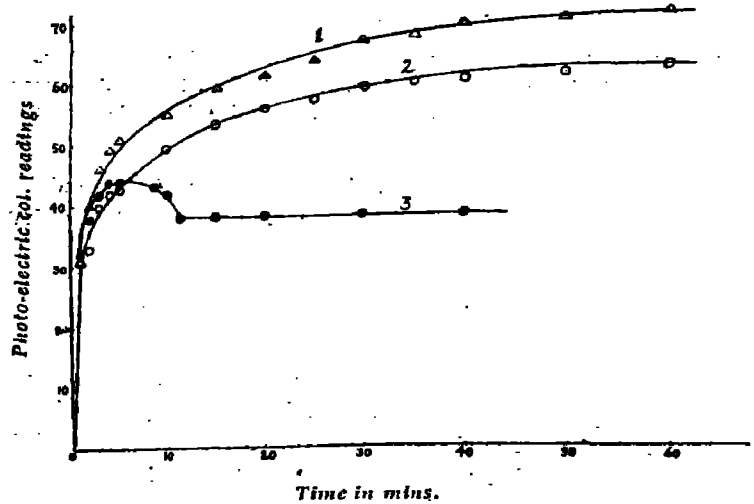
Time of Reaction.—The method of Emmerie and Engel consists in the reduction of ferric chloride by tocopherols and the measurement of the intensity of colour produced by the ferrous iron, thus formed, with 2:2′-bipyridine. This principle has been applied in the present investigation. But the time required in this case to form a colour, more or less of stable intensity, is different from the tocopherol reaction, as may be seen from Fig. 1.

Reagents used were (1) 80% pure alcohol; (2) ferric chloride of analytical reagent quality, dissolved in 95% alcohol so that each c.c. of the solution contained 0.832 mg. of $FeCl_3$, $6H_2O$ and (3) pure 2:2′-bipyridine, dissolved in 95% alcohol in such a way that each c.c. of the solution contained 1 mg. of bipyridine.

Procedure.—A solution of propyl gallate (1 c.c.) in 95% alcohol containing 40 microgram of the substance was diluted with 22 c.c. of 80% alcohol. 2:2′-Bipyridine solution (1 c.c.) was then added to it and finally 1 c.c. of ferric chloride solution. The container was shaken and the intensity of the colour produced was recorded immediately by a Klett-Summerson photo-electric colorimeter using filter No. 54, the zero of the

photo-electric colorimeter being adjusted with a blank solution containing 23 c.c. of 80% alcohol, 1 c.c. of 2:2'-bipyridine and 1 c.c. of ferric chloride solution. The variation of the intensity of the red colour was measured with time and the photo-electric colorimeter readings plotted against time are shown in Figure 1.

FIG. 1



Curves 1-3 refer respectively to ethyl gallate, propyl gallate and monomethyl ether of hydroquinone.

(1.) Similar experiments were carried out with ethyl gallate. But in the case of monomethyl ether of hydroquinone an aliquot equivalent to 120 micrograms was taken instead of 40 micrograms. The intensity readings for ethyl gallate as well as for monomethyl ether of hydroquinone, plotted against time, are also shown in Fig. 1.

From the figure it will be seen that the colour produced with monomethyl ether of hydroquinone is of much lower intensity than that with either ethyl gallate or propyl gallate under identical conditions. In the case of monomethyl ether of hydroquinone the intensity of the colour rises continuously up to the sixth minute and then gradually falls until 12.5 minutes is reached. From this point the intensity remains practically the same up to 40 minutes of reaction. So the time allowed for the reaction with monomethyl ether of hydroquinone during later experiments was 15 minutes.

In the case of propyl gallate or ethyl gallate, the intensity of colour rises sharply, initially up to 5 minutes and then slowly up to 30 minutes. But after 30 minutes the rise is very slow being only 3 or 4 % of the photo-electric colorimeter reading in the course of the next 30 minutes. So the time allowed for reaction in these two cases was 30 minutes.

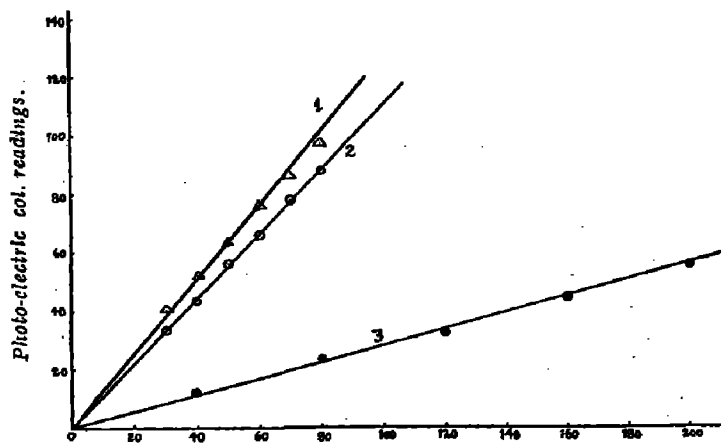
Reference Curve.—Pure propyl gallate (0.05 g.) having m.p. 146-48° was weighed accurately and dissolved in 95% alcohol, and the volume was made up to 100 c.c. with the same alcohol. Similarly 0.05% solutions of ethyl gallate and monomethyl ether of hydroquinone were made.

0.05% Solutions of ethyl gallate and propyl gallate (1 c.c. each) were diluted up to 25 c.c. and aliquots representing 30 to 100 micrograms were taken in 50 c.c. pyrex conical flasks. 80% Alcohol was added from a burette into the flasks to make the volume 23 c.c. and then 1 c.c. each of bipyridine and ferric chloride reagents was added. The operation was conducted away from direct light and after the addition of the reagents the flasks were kept in a dark place for half an hour and then the intensity of the colour was measured in a Klett-Summerson photo-electric colorimeter using filter No. 54, the zero of the colorimeter being adjusted with a blank solution containing the reagents and 80% alcohol only.

In the case of monomethyl ether of hydroquinone the dilution was similarly made but aliquots were taken representing 40 to 200 micrograms since the colour produced was of much lower intensity than that in the other two cases. After proceeding in the above way it was allowed to stand in the dark for 15 minutes and readings were similarly taken in the photo-electric colorimeter.

The photo-electric colorimeter readings have been plotted against the quantity of the antioxidant which are shown in Fig. 2. From the figure it may be seen, in all cases, that the intensity of the colour produced bears practically a linear relation with the quantity of antioxidant.

FIG. 2



Curves 1-3 refer to same as in Fig. 1.

Interference.—A major source of interference in the assay of antioxidants in synthetic esters and vegetable oils is the inhibition or enhancement of colour formation with the ferric chloride reagent. The inhibition effect in the estimation of tocopherols was studied by Kaunitz and Beaver (*J. Biol. Chem.*, 1944, **156**, 653, 661) who observed that when known amounts of synthetic α-tocopherol were added to a number of oils in low concentration, e.g. 0.1%, recoveries as small as 15% were obtained. These authors suggested that the inhibition might be due to a complex formed between ferrous ion and fat which retarded the formation of the coloured complex

between ferrous ion and bipyridine. To correct for inhibition Kaunitz and Beaver (*loc. cit.*) determined the extinction of a given weight of oil before and after the addition of a known amount of pure α-tocopherol. By proportion a corrected value for the tocopherol content of the oil was obtained.

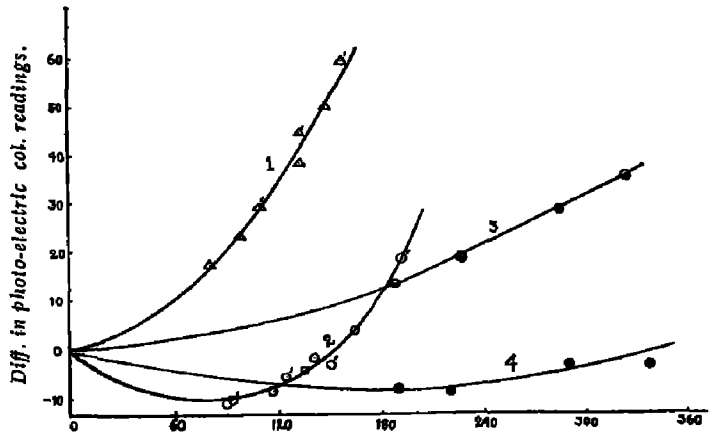
During this investigation it was found that ethyl oleate depressed the colour formation even when it was present in a small quantity, whereas contrary to the above observation, arachis oil at a very low concentration enhanced the colour formation but caused inhibition at higher concentrations. Moreover, the depression or enhancement of colour formation was not proportional to the quantity of the substrate. To ascertain whether the enhancement of colour at lower concentration of arachis oil was due to the presence of fine particles of oil in alcohol, the following experiment was carried out. After adding the reagents to small a portion of an antioxidant solution in arachis oil it was divided into 3 portions—one portion was kept in the dark as usual, the second was centrifuged with croks on the centrifuge tubes and the third portion was filtered through a filter paper which had been previously moistened with 80% alcohol so that this filter would not allow oil to pass through.

After the scheduled time, the portion kept as it was, the supernatant liquid of the centrifuged portion and the filtered solution gave the same readings in the photo-electric colorimeter. Thus it appears that the colour was not enhanced by the presence of any oily particles in suspension.

To find out the corrections for the inhibition or enhancement, known amounts of the various antioxidants were dissolved in weighed quantity of the substrates. Arachis oil and ethyl oleate used were of the quality as described in Part I of this series (Basu and Bhattacharya, *loc. cit.*). Small portions of each of (*i*) ethyl gallate in arachis oil, (*ii*) ethyl gallate in ethyl oleate, (*iii*) propyl gallate in arachis oil, (*iv*) propyl gallate in ethyl oleate, (*v*) monomethyl ether of hydroquinone in arachis oil and (*vi*) monomethyl ether of hydroquinone in ethyl oleate were accurately weighed into a 25 c.c. volumetric flask. Bipyridine and ferric chloride reagents (1 c.c. each) were added and the volume was made up to the mark with 80% alcohol. They were then kept in the dark for the required time and the intensity of colour was measured in the usual way. In this process ethyl oleate dissolved in 80% alcohol but arachis oil remained undissolved at the bottom. The difference between these readings and the corresponding photo-electric colorimeter readings for the same amounts of pure antioxidants dissolved in alcohol, which has been taken with the substrate solution, are plotted against the quantity of the substrates, This is shown in Fig. 3.

From Fig. 3 it will be seen that the inhibition or enhancement is not proportional to the amount of the substrate. But it may be seen that the extent of extinction (curve 1), caused by ethyl oleate both in case of propyl gallate and ethyl gallate is the same. This phenomenon is true for arachis oil also (curve 2), but in this case the colour formation is enhanced at the beginning which gradually decreases and ultimately inhibition is noticed when the weight of arachis oil exceeds 150 mg. But ethyl oleate and arachis oil behave in a different way in the case of monomethyl ether of hydroquinone, the interference being less than that in the other two cases (vide curves 3 and 4). This phenomenon may be due to the difference in the period of reaction in these cases.

FIG. 3



Curves 1-4 refer respectively to ethyl oleate with ethyl gallate + propyl gallate; arachis oil with ethyl gallate + propyl gallate; ethyl oleate with monomethyl ether of H.Q., and arachis oil with monomethyl ether of H.Q.

In this connection it may be mentioned that 80% alcohol was used with the idea that it would extract less amount of pigments from the arachis oil. Further, the arachis oil used was almost colorless and as such the correction for pigments was not considered necessary in the course of this investigation.

Precision.—To test the precision of the method, known amounts of antioxidants were added to the accurately weighed small portions of previously stated solutions of antioxidants which were used in finding out the interference of substrates. The recoveries after corrections obtained from Fig. 3 for the intereference caused by the substrate averaged 100.5% with a spread from 98.6 to 102.4%. The results of these experiments are shown in Table I.

TABLE I

Recoveries of added antioxidants.

Substrate.	Nature of antioxidant.	Amount (microgram). Orginally present.	Added.	Recovery (microgram) Calc.	Found.	%
Arachis oil	Propyl gallate	25	25	50	50.6	101.2
,,	Ethyl gallate	25	30	55	54.62	99.3
,,	Monomethyl ether of hydroquinone	50	50	100	98.60	98.6
Ethyl oleate	Propyl gallate	25	30	55	55.17	100.3
	Ethyl gallate	25	40	65	66.56	102.4
	Monomethyl ether of hydroquinone	50	75	125	126.50	101.2
					Mean.	100.5

From the table it may be seen that this method is fairly accurate and is suitable for the estimation of phenolic antioxidants of the above type.

Estimation.—The method of estimation of propyl gallate and monomethyl ether of hydroquinone, when present in arachis oil or ethyl oleate, is as follows.

Dissolve a known quantity of antioxidant (about 50 mg.) in 100 c.c. alcohol (95%) and draw the reference curve by taking aliquots representing 40 to 100 micrograms in the case of ethyl or propyl gallate and 60 to 250 micrograms in the case of monomethyl ether of hydroquinone and measuring by means of a photo-electric colorimeter the intefisity of the colour formed with ferric chloride and 2:2′-bipyridine reagents. The filter to be used in Klett-Summerson photo-electric colorimeter is No. 54 and the zero is to be adjusted with a blank solution containing the reagents and 80% alcohol only. Then draw the intenference curves for arachis oil and ethyl oleate by measuring the intensity of colour of known quantity of antioxidants which have been dissolved in the respective substrates. Take from an unkown solution an accurately weighed quantity of a substrate in such a way that the antioxidant present is between 40 and 150 micrograms in case of gallates or between 60 and 200 micrograms in case of monomethyl ether of hydroquinone and measure the apparent intensity of colour formed with the reagents. Adding or subtracting the correction value for the amount of substrate to the apparent intensity value, find out the corresponding value for antioxidant from the reference curve.

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