The Chemical Estimation of Vitamin E in Vegetable Oils

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The chemical methods for estimation of vitamin E, recommended in the literature, are all based on the oxidation of a hydroquinone nucleus which is present in all modifications of tocopherol. This nucleus can either be oxidized in such a way as to give a coloured product, or its reducing power may be measured and hence the quantity of tocopherol calculated. The latter principle is employed in the gold chloride potentiometric method of Karrer & Keller (1938, 1939) and in the colorimetric methods based on the $FeCl_3$-α:α'-dipyridyl reaction of Emmerie & Engel (1938).

The method of Furter & Meyer (1939) is not specific; the red colour which develops on treatment of tocopherol with concentrated HNO_3 is given also by its biologically inactive oxidation product (Lester Smith & Bailey, 1939). A method of assaying α-tocopherol acetate by hydrolyzing with sulphuric acid and titrating with ceric sulphate, recommended by the British Pharmaceutical Codex (1942), opens new possibilities, but apparently has not yet been applied to the estimations of tocopherol in its natural sources. The gold chloride potentiometric method requires elaborate apparatus, and is more complicated than the α:α'-dipyridyl method, which is simple, rapid and accurate if interfering substances are removed from the unsaponifiable matter before reagents are applied.

In this paper we describe means for preparing suitable extracts to which the α:α'-dipyridyl reaction can be successfully applied so as to secure results consistent with biological values for natural oils as obtained by other workers (Olcott, 1934; Drummond, Singer & MacWalter, 1935) and with such tests as we have so far performed. Preliminary accounts of the work have already been given (Moore & Tošić, 1943; Tošić & Moore, 1944).

EXPERIMENTAL

Experimental basis of chemical method for estimation of vitamin E

The ability to produce a red colour with ferric chloride and α:α'-dipyridyl is not a specific property of tocopherols, but is shown by many reducing agents. Emmerie & Engel (1938) found that both carotene and vitamin A produced colour, so that it was necessary either to remove them before the estimation of vitamin E, or to make an appropriate

correction. It is now clear that several other complications must be faced before it can be safely assumed that only vitamin E is being estimated. If the reaction is applied directly to oils without saponification our first consideration must be that esterified tocopherols do not produce colour. Since tocopherol is believed to be present in the free state in all its natural sources this criticism may not be serious except when we are dealing with oils artificially fortified with synthetic esters. Another objection is that oils may contain pigments which interfere with the assessment of the correction for carotenoids. These pigments, together with sterols, have been removed by Parker & McFarlane (1940) by means of extraction with 85 % H_2SO_4. We are, however, still faced with the finding of Kaunitz (1944) that the presence of oil seriously interferes with the development of colour by tocopherol. The objection to saponification, by ordinary methods, is that substantial losses of tocopherol may occur. Even in examining the unsaponifiable fraction, moreover, precautions have still to be taken. It may be necessary not only to correct for the presence of carotenoids, but in our experience to remove other reducing substances which may affect the accuracy of the method much more seriously.

For a successful application of the α:α'-dipyridyl reaction therefore it is necessary: (*a*) to saponify the oil under conditions which prevent the destruction of vitamin E, (*b*) to separate interfering substances from the unsaponifiable matter, (*c*) to make a correction for the reducing power of any carotenoids which cannot conveniently be removed, and (*d*) to protect the solution against sunlight during the estimation (Lester Smith & Bailey, 1939).

Choice of light filters

With the use of Zeiss Pulfrich Photometer, maximum transmission of the red ferrous-α:α'-dipyridyl complex formed by reaction with α-tocopherol was found to be at about $520 m\mu$. The most suitable filters for the colorimetric estimation should therefore be the green S 50 or S 53 filters. With an S 53 filter, a calibration curve for the reducing power of synthetic α-tocopherol proved to be a straight line for 0–168 μg. of α-tocopherol in the reagent mixture. The amount of tocopherol, as μg./ml. of solution, could be calculated by multiplying the extinction

coefficient as read on the instrument by a factor of 166·6.

In calculating the quantities of α-tocopherol, and other substances in unsaponifiable matter which reduce $FeCl_3$ in α:α'-dipyridyl reaction, this factor was always used. All the data regarding $FeCl_3$-reducing substances have been expressed in terms of 'apparent' synthetic α-tocopherol.

Stability of α-tocopherol during saponification in absence and presence of pyrogallol

Since Lester Smith & Bailey (1939) were unable to prevent the loss of tocopherol on saponification by working as far as possible in an atmosphere of inert gas, some other form of protection was necessary. A novel procedure involving the addition of pyrogallol was tried. It was expected that, during treatment of the oils with hot ethanolic potash, this substance would compete with tocopherol for the available oxygen, and so reduce the loss of vitamin. A series of recovery tests on synthetic α-tocopherol and its acetate after a saponification procedure was carried out in absence and presence of pyrogallol.

(a) *Recovery of α-tocopherol.* To 1 ml. of an ethanol solution of synthetic α-tocopherol, containing a quantity likely to be found in oil samples to be examined, 1 ml. freshly prepared 10% pyrogallol solution in ethanol was added. For each sample containing pyrogallol a control without pyrogallol (but with 1 ml. ethanol added) was prepared. The contents were heated on a water-bath, conc. KOH solution sufficient to saponify 1 g. of oil was added, and heating continued for a short time until saponification was complete. Distilled water was then added, and the vitamin extracted with diethyl ether. Excess of pyrogallol was readily removed by washing with water. The extracts were then evaporated under reduced pressure in a hot water-bath and the 'unsaponifiable' matter, in this case α-tocopherol only, was examined colorimetrically with $FeCl_3$-α:α'-dipyridyl reagent. The results of these tests indicate (Table 1 a) that pyrogallol eliminates the serious losses which occur during saponification.

(b) *Recovery of α-tocopherol from its acetate.* When α-tocopherol acetate is saponified as described under (a), the α-tocopherol formed can be estimated colorimetrically in the 'unsaponifiable' matter. Losses due to saponification in the absence of pyrogallol are considerable. On the other hand, the pyrogallol procedure secures a satisfactory recovery (Table 1 b).

The tests described under (a) and (b) show that the losses of α-tocopherol occurring during the alkaline treatment amounted on the average to about half of the vitamin added, while in the presence of pyrogallol recovery was almost complete.

Pyrogallol procedure applied to oils

The pyrogallol procedure was applied to natural oils to assess further the accuracy of the method.

Table 1. *Recovery of synthetic α-tocopherol in presence and absence of pyrogallol*

α-Toco-pherol (mg. added)	Without pyrogallol		With pyrogallol	
	mg. found	% recovery	mg. found	% recovery
(a) Recovery of α-tocopherol in absence of oil				
1·08	0·63	58·7	1·07	99·2
1·08	0·62	57·2	1·06	98·2
1·08	0·36*	33·3*	1·06	98·2
(b) Recovery of α-tocopherol from its acetate in absence of oil				
1·78	0·66	37·7	1·76	98·8
1·78	1·04	58·4	1·77	99·4
1·08	0·57	52·8	1·07	99·2
1·08	0·92	85·2	1·06	98·2
(c) Recovery of α-tocopherol from its acetate in presence of oil				
2·06†	1·84	89·3	2·02	98·2
2·06†	1·80	87·4	2·03	98·6
2·06†	1·72	83·2	2·01	97·5
2·06†	1·77	86·0	2·03	98·6

* In this case 1 ml. of distilled water was added to the ethanol solution of the vitamin.

† These figures represent total $FeCl_3$-reducing substances of the oil which are made up as follows: 1·82 mg. α-tocopherol, added in the form of its acetate, +0·24 mg. found in the arachis oil itself by our pyrogallol method.

When α-tocopherol acetate was dissolved in arachis oil, and the colorimetric estimation done on the unsaponifiable matter, the results (Table 1 c) indicate that during saponification pyrogallol quantitatively protects both tocopherol and the other substances which reduce $FeCl_3$. During the saponification in the absence of pyrogallol the losses amounted to 10·7–16·8% (average 13·5%). These losses were much smaller than those sustained when α-tocopherol or its acetate without oil were saponified in the absence of pyrogallol (Tables 1 a and 1 b). Probably the oil contains substances which partially protect the vitamin during the saponification.

Table 2. *Agreement of triplicates by the pyrogallol method*

Oil	$FeCl_3$-reducing substances* (μg./g. of oil)				Maximum spread as % of mean
	1	2	3	Mean	
Wheat-germ (freshly extracted)	3180	3080	3100	3120	3·20
Wheat-germ (old sample)†	558	549	541	549	3·10
Tung	2391	2361	2359	2370	1·36
Soya-bean	1355	1357	1361	1356	0·44
Cotton-seed (crude)	936	969	937	947	3·48
Cotton-seed	576	594	591	588	2·56

* Correction for carotenoid pigments has been deducted from the data in this table.

† A sample of wheat-germ oil, extracted over 10 years ago and kept in a store at room temperature.

The reproducibility of results in saponification with pyrogallol was tested on several vegetable oils (Table 2). A maximum difference between $FeCl_3$-reducing substances in triplicate estimations amounted to a maximum of 3·48 %, which is within the experimental error of estimation.

For comparing saponification procedures, with and without pyrogallol, we have examined a number of freshly extracted oils (Table 3). An average loss

Table 3. $FeCl_3$-reducing substances of freshly extracted oils

Oil*	$FeCl_3$-reducing substances† (μg./g. of oil)		% difference between the two methods
	With pyrogallol	Without pyrogallol	
Wheat-germ	3120	2470	20·8
Soya-bean	1356	1070	21·0
Firsts castor	909	814	10·4
Crude linseed	729	624	14·4
Cotton-seed	696	535	23·0
Crude rape	661	512	22·5
Crude sesame	527	430	18·3
Ground-nut	268	249	7·0
Crude cocoa-butter	220	148	32·7
Coconut	nil	nil	nil
Palm-kernel	nil	nil	nil

* Examined within 1 month of extraction.
† Corrections for carotenoid pigments have been deducted; the values of these corrections are given in Table 4.

of $FeCl_3$-reducing substances, amounting to 18·8 %, was incurred by the saponification procedure in which pyrogallol was omitted. It is of interest to notice the agreement with the data of Lester Smith & Bailey (1939), who obtained only an 87 % recovery of α-tocopherol after saponification of wheat-germ oil in which synthetic α-tocopherol was dissolved.

Correction for carotenoid pigments

Although the quantity of carotenoid pigments is in many cases negligible, a few oils, such as wheat-germ, soya-bean, crude linseed and rape oils contain sufficient quantities of the pigments to necessitate a correction in the total $FeCl_3$-reducing substances. For the determination of a factor which would transform the reducing activity of μg. of carotenoid pigments into μg. of α-tocopherol the following experiment was performed:

β-Carotene in ethanol in amounts ranging from 0–112 μg. was allowed to react with $FeCl_3$-α:α′-dipyridyl reagent under conditions identical with those employed in the estimation of vitamin E and the intensity of the red colour developed was determined colorimetrically. The readings were taken about 20 min. after mixing. The curve relating the concentration of carotene to the extinction coefficient was linear. 1 μg. of carotene was found to have reducing power equal to 2·5 μg. of α-tocopherol.

In our chromatographic procedure, to be described later, we found that carotene, which is always a small proportion of total carotenoids, passes readily through the column with vitamin E. Xanthophylls, however, remain adsorbed in the column, but can be eluted together with other $FeCl_3$-reducing substances. It is often desirable to estimate the reducing substances other than α-tocopherol and carotene. It is then necessary to make a correction for xanthophylls. For this purpose, having no specimen of pure xanthophyll available, we compromised by using the same correction factor of 2·5 as for carotene. The agreement of data in recovery tests before and after chromatographic separation (see later in Tables 8, 9 and 11) suggests that the error involved in this procedure is not large.

The correction for carotene is significant in the case of very few natural oils. Correction for xanthophylls found in the 'adsorbed' fraction is large, but it need be applied only in cases in which recovery of total $FeCl_3$-reducing substances is required. In several freshly extracted natural oils more than traces of carotenoid pigments, as estimated by yellow colour, were present in the unsaponifiable matter. The values of the corrections in the few samples which contained the pigments are given in Table 4. Higher values

Table 4. Correction for carotenoid pigments of some oils

Oil*	Carotenoid pigments correction (μg./g. of oil)	
	With pyrogallol	Without pyrogallol
Wheat-germ	289	271
Soya-bean	15	13
Crude linseed	30	27
Crude rape	37	35
Others	Traces or no carotenoids were detected	

* Freshly extracted oils given in Table 3.

for total carotenoid pigments were obtained if the oils were saponified in presence of pyrogallol than in its absence. This, therefore, suggests that a small proportion of carotenoid pigments may be destroyed during alkaline saponification and that this can be avoided by using pyrogallol.

Loss of $FeCl_3$-reducing substances on storage of oils

The values of total $FeCl_3$-reducing substances of some oil samples decreased with the time of storage of the oil. From Table 5, it will be seen that during storage the oils lost a portion of their $FeCl_3$-reducing substances, including carotenoid pigments. The loss was not uniform with all oils, and may depend on the type of oil and the storage conditions. A loss of 17·5 % was incurred in a sample of freshly extracted wheat-germ oil, even when this was stored at a low temperature (2–4°) for a period of only 2 months. It is evident, therefore, that in recording the data for vitamin E it is necessary to give the history of the samples examined.

Table 5. *Loss of FeCl₃-reducing substances on storage of oils*

Oil	Time of examination	FeCl$_3$-reducing substances (μg./g.)	Correction for carotenoid pigments (μg./g.)
Wheat-germ	1 day after extraction	3120	289
	After storage of 60 days at 2–4°	2576	181
Wheat-germ II	Over 10 years old	549	30
	17 months later*	491	29
Tung	Over 5 years old	2370	4
	15 months later*	2039	4
Soya-bean	Within 1 month of extraction	1356	15
	15 months later*	1222	11
Crude linseed	Within 1 month of extraction	729	30
	15 months later*	694	26
Cotton-seed	Within 1 month of extraction	696	0
	15 months later*	400	0
Crude rape	Within 1 month of extraction	661	37
	15 months later*	440	24
Crude sesame	Within 1 month of extraction	527	0
	15 months later*	420	0

* Stored at room temperature.

The biological inactivity of FeCl₃-reducing substances of tung oil

Tung oil was second only to wheat-germ oil in its content of substances reducing FeCl$_3$ (Tables 2 and 3). Since this oil contained only a small amount of carotenoid pigments (correction being 4 μg./1 g. of oil) and no vitamin A, it was necessary to decide whether the rest of 2370 μg. of the FeCl$_3$-reducing substances/g. of oil was either vitamin E or 'interfering substances', if it should prove inactive in biological experiments. The unsaponifiable matter of the oil, dissolved in arachis oil, was therefore tested biologically by the method of Evans & Burr (1927).

The data of these experiments (Table 6) show that total FeCl$_3$-reducing substances of tung oil have very little biological activity. Roughly 80–90 % of the FeCl$_3$-reducing substances are inactive and should be considered as interfering substances in the chemical estimation.

For example, at a dosage of 4·48 mg./animal, it was to be expected, if all the FeCl$_3$-reducing substances were α-tocopherol, that most of the rats used would have litters. Instead, out of six animals, four showed resorption, only

one gave a live litter, whilst one gave a dead litter. In another experiment, ten animals were used and they were dosed with 8·12 mg. each. It was thought that the dosage of 8·12 mg. of FeCl$_3$-reducing substances should give a 100 % response in biological experiments, even if FeCl$_3$-reducing substances are present in tung oil in the form of the less biologically active derivatives of tocopherol (β- and γ-tocopherol). But the experiments showed that out of ten animals dosed at this level only one gave a live litter, while in the rest (nine animals) resorption resulted.

Chromatographic separation of biologically inactive FeCl₃-reducing substances of tung oil

The existence of the interfering substances in the unsaponifiable matter of tung oil led to experiments with various chromatographic systems to find suitable conditions for the free passage of α-tocopherol with the removal by adsorption of 'interfering' substances.

'*Floridin XS earth*'-*benzene system*. With this system we confirmed the findings of Emmerie & Engel (1939a) that synthetic α-tocopherol freely passes through the column with benzene as solvent and eluent. Under such conditions 98·7 % of FeCl$_3$-reducing substances of unsaponifiable matter of tung oil unfortunately behaved in the same way. The 'Floridin XS earth'-benzene system cannot therefore

Table 6. *Biological test of unsaponifiable matter of tung oil*

Oil	Date of exp.	Dose of FeCl$_3$-reducing substances (mg./animal)	No. of animals	Resorption	Dead litters	Live litters
Tung in arachis	June 1943	4·48	6	4	1 (1)*	1 (1)*
Arachis (control)	,,	0·08	5	5	0	0
Tung in arachis	August 1943	8·12	10	9	0	1 (4)*
Arachis (control)	,,	0·08	3	3	0	0

* Figures in brackets denote the number of animals in each litter.

be used for the separation of α-tocopherol from the bio-
logically inactive 'interfering substances' in tung oil.

Aluminium oxide-light petroleum + ethanol system. In our
experience aluminium oxide, if left exposed to air, adsorbs
moisture, and its adsorption activity is reduced. For the
purpose of eliminating this trouble, we have used several
brands of standard activated aluminium oxide which
exhibited constant adsorption properties. Further, it was
found that with any standard Al_2O_3 the adsorption activity
was reduced with an increased concentration of ethanol
added to light petroleum, which served as solvent and
eluent. The degree of adsorption of synthetic α-tocopherol,
as well as of other $FeCl_3$-reducing substances on aluminium
oxide, will therefore depend *inter alia* on both these factors.

For more detailed study of this system the chromato-
graphic procedure was standardized. The alumina was acti-
vated by heating in a hot-air oven at 100° for 2-3 hr., just
before preparing the column, which was 50 mm. long by
10 mm. diameter. As solvent light petroleum A.R. (b.p.
40-60°) was used, with the addition of 0·0-1·5 % ethanol,
depending on the experiment. 10 ml. of solvent were used
for preparing the column, 5 ml. for dissolving α-tocopherol
or the $FeCl_3$-reducing substances, and 15 ml. for subsequent
washing of the column. The solvent was always added
before the top of the column became dry. The filtration rate
was 70-90 drops/min.

The latter procedure was successful when acti-
vated aluminium oxide (British Drug Houses Ltd.)
was used in combination with light petroleum and
1·0 % ethanol. After the preliminary experiments
the adsorption activity of this alumina was studied
in relation to the concentration of ethanol added to
light petroleum. Table 7a shows that α-tocopherol
was quantitatively adsorbed by the column if the
concentration of ethanol in the solvent ranges be-
tween 0-0·40 %. With ethanol concentrations rang-
ing between 0·60-0·90 % there was partial recovery,
and with 1·0 % or over a quantitative recovery.
With 1 % ethanol the standard column gave quanti-
tative recoveries with 173-1734 μg. of α-tocopherol.

When the $FeCl_3$-reducing substances of the un-
saponifiable matter of tung oil are passed through
this column, and the solvent used contained 1 % of
ethanol, 21·3 % of these are found in the unadsorbed
fraction. The adsorbed substances could be eluted
with 10 ml. of light petroleum containing 20 %
ethanol, and amounted to 75·7 % of the total $FeCl_3$-
reducing substances found in control samples before
chromatographic analysis. The total recovery after
chromatographic separation therefore amounted to
97·0 %.

The necessity of selecting the correct brand of aluminium
oxide is emphasized by our failure with two other prepara-
tions. A specimen made by Brockman's method adsorbed
too strongly, and failed to allow the quantitative passage of
α-tocopherol even at concentrations of ethanol which allow
the passage of the 'interfering substances' of tung oil. A
specimen from A. Wander Ltd. adsorbed weakly (Table 7b),
and allowed a passage through the column of about 70 %
of the 'interfering substances', at 0·8 % concentration of
ethanol.

Table 7. *Adsorption and recovery of α-tocopherol*

Aluminium oxide-light petroleum-ethanol system

Percentage of ethanol in solvent mixture	Added (μg.)	α-Tocopherol Found after passing through the column	
		(μg.)	Percentage of amount added
(a) 'B.D.H.' aluminium oxide for chromatographic analysis			
0·00	867	0	0
0·40	867	0	0
0·60	867	127	14·7
0·70	867	533	61·6
0·80	867	617	70·5
0·90	867	834	96·3
1·00	1734	1725	99·6
1·00	867	867	100·0
1·00	867	859	99·2
1·00	173	172	99·5
1·20	867	867	100·0
(b) Aluminium oxide (A. Wander Ltd.)			
0·00	867	0	0
0·30	867	64	7·4
0·45	867	513	59·2
0·60	867	681	78·5
0·70	867	784	90·4
0·80	867	867	100·0
0·90	867	874	100·8
1·00	867	862	99·3

*Influence of α-tocopherol on the adsorption of $FeCl_3$-
reducing substances of some oils, and the influence
of the latter on the passage of α-tocopherol*

Although the technique described in the preceding
section seemed promising it remained to be decided
(a) whether the column can separate the interfering
substances of oils other than tung oil in presence and
absence of synthetic α-tocopherol, and (b) whether
the relative concentration of α-tocopherol to that
of other reducing substances has any effect.

To test the influence of synthetic α-tocopherol on the
behaviour of the mixed reducing substances of plant oils,
wheat-germ oil, crude cotton-seed oil, and tung oil were
chosen as three widely different types which contain large
quantities of reducing substances. Mixtures were prepared
containing about the same quantities of α-tocopherol and
of the reducing substances present in the unsaponifiable
matter of each oil. Each mixture was then passed through
a column. The 'unadsorbed' fraction and an 'adsorbed'
fraction, which was eluted with 10 ml. of light petroleum
containing 20 % ethanol, were collected separately. For
each test, control experiments were done with separate
constituents of each system without mixing.

α-Tocopherol and ·the unsaponifiable matter of
oils were adsorbed to the same extent whether
passed through the column separately or as mix-
tures (Table 8). The 'adsorbed' and 'unadsorbed'
fractions, moreover, showed little change in their

Table 8. *Influence of synthetic α-tocopherol on the passage, through alumina columns, of $FeCl_3$-reducing substances of unsaponifiable matter of some oils*

	Before passing through the column (μg.)	In 'unadsorbed' fraction μg.	In 'unadsorbed' fraction Percentage of total	In 'adsorbed' fraction μg.	In 'adsorbed' fraction Percentage of total	Recovery μg.	Recovery Percentage of total
Sample							
1a Synthetic α-tocopherol alone	434	431	99·4	0	0	431	99·4
1b Unsaponifiable matter of tung oil alone	475	100	21·0	360	75·8	460	96·8
Total	909	531	58·5	360	39·6	891	98·1
I. Mixture of 1a and 1b	909	538	59·2	372	41·0	910	100·2
Difference		+7	+0·7	+12	+1·4	+19	+2·1
2a Synthetic α-tocopherol alone	476	470	98·8	0	0	470	98·8
2b Unsaponifiable matter of crude cotton-seed oil alone	518	242	46·7	274	52·9	516	99·6
Total	994	712	71·6	274	27·5	986	99·2
II. Mixture of 2a and 2b	994	727	73·1	273	27·4	1000	100·5
Difference		+15	+1·5	-1	-0·1	+14	+1·3
3a Synthetic α-tocopherol alone	434	432	99·6	0	0	432	99·6
3b Unsaponifiable matter of wheat-germ oil alone	514	330	64·3	194	37·7	524	102·0
Total	948	762	80·4	194	20·4	956	100·8
III. Mixture of 3a and 3b	948	736	77·7	196	20·7	932	98·4
Difference		-26	-2·7	+2	+0·3	-24	-2·4

Total $FeCl_3$-reducing substances* — After passing through the column

* Correction for carotenoid pigments deducted.

Table 9. *Recovery of synthetic α-tocopherol in presence of unsaponifiable substances of wheat-germ oil*

	Before passing through the column (μg.)	In 'unadsorbed' fraction μg.	In 'unadsorbed' fraction Percentage of total	In 'adsorbed' fraction μg.	In 'adsorbed' fraction Percentage of total	Recovery μg.	Recovery Percentage of total
Sample							
1a Synthetic α-tocopherol alone	434	432	99·6	0	0	432	99·6
1b Unsaponifiable fraction of wheat-germ oil alone	1028	628	61·1	401	39·0	1029	100·1
Total	1462	1060	72·6	401	27·4	1461	100·0
I. Mixture of 1a and 1b	1462	1034	70·8	395	27·0	1429	97·8
Difference		-26	-1·8	-6	-0·4	-32	-2·2
2a Synthetic α-tocopherol alone	434	432	99·6	0	0	432	99·6
2b Unsaponifiable fraction of wheat-germ oil alone	514	330	64·3	194	37·7	524	102·0
Total	948	762	80·4	194	20·4	956	100·8
II. Mixture of 2a and 2b	948	736	77·7	196	20·7	932	98·4
Difference		-26	-2·7	+2	+0·3	-24	-2·4
3a Synthetic α-tocopherol alone	434	432	99·6	0	0	432	99·6
3b Unsaponifiable fraction of wheat-germ oil alone	257	151	58·9	95	37·0	246	95·9
Total	691	583	84·3	95	13·7	678	98·1
III. Mixture of 3a and 3b	691	579	83·8	117	16·9	696	100·7
Difference		-4	-0·5	+22	+3·2	+18	+2·6

Total $FeCl_3$-reducing substances* — After passing through the column

* Correction for carotenoid pigments deducted.

adsorptive properties when passed through the column for the second time. The ratio 6f α-tocopherol to other reducing substances was also without effect on their chromatographic separation (Table 9). With our standardized column consistent results were obtained with amounts of total FeCl$_3$-reducing substances from 257 to 1462 μg.

Biological activity of the two fractions of wheat-germ oil

The unsaponifiable matter of wheat-germ oil (Table 8) contained some 40 % of reducing substances which were adsorbed on the column, and hence did not appear to be α-tocopherol. To confirm this conclusion biological tests were carried out upon the adsorbed and unadsorbed fractions.

Freshly extracted wheat-germ oil was saponified in presence of pyrogallol and the unsaponifiable matter was dissolved in sufficient light petroleum + 1·0 % ethanol, so that 5 ml. of this solution contained about 1 mg. of FeCl$_3$-reducing substances. 5 ml. portions of this solution were chromatographically separated, each portion with a freshly made column. The unadsorbed fractions were combined, the solvent evaporated under reduced pressure and the unsaponifiable matter obtained in this way was dissolved in a suitable volume of arachis oil. Each adsorbed fraction was eluted from the column with 10 ml. portions of light petroleum containing 20 % ethanol, and the eluates combined and evaporated under reduced pressure. The unadsorbed fraction obtained in this way was also dissolved in arachis oil. Before dosing, the reducing substances of the two preparations were estimated chemically.

The two fractions were tested biologically, as described earlier, against arachis oil as a control (Table 10). Treatment with the adsorbed fraction, at the dosage of 3·5 mg. of reducing substances after correction for carotenoid pigments,

tocopherols. Reducing substances which remain adsorbed in the column are either not tocopherol or are modifications of tocopherol which have only slight biological activity. This view is consistent with our previous finding that the reducing substances of tung oil which are mainly adsorbed on the column, show only slight activity in biological tests.

METHOD FOR THE CHEMICAL ESTIMATION OF VITAMIN E

Reagents

Pyrogallol solution. 5 % (w/v) freshly prepared, with ethanol as solvent.

KOH solution (for saponification). Aqueous solution containing 4 g. of KOH + 2·7 ml. H$_2$O.

α-Tocopherol standard (for calibration purposes). Ethanol solution containing about 1 mg. of synthetic α-tocopherol/1 ml.

FeCl$_3$ solution. Freshly prepared 0·2 % (w/v) ethanol solution.

α:α'-Dipyridyl solution. Freshly prepared 0·5 % (w/v) ethanol solution. This reagent, and the FeCl$_3$, may be stored in the dark for over 2 weeks.

Ethyl ether. A.R. for extraction purposes.

Ethanol. A.R.

Light petroleum. A.R. (b.p. 40–60°).

Aluminium oxide (British Drug Houses' Ltd., for chromatographic analysis). Activated by heating at 100° in an air oven for 2–3 hr. before use. It is essential to standardize each new batch of alumina against known materials, such as synthetic α-tocopherol and the unsaponifiable matter of tung oil.

Light petroleum + ethanol solvent. For chromatography, 1 % (v/v) of absolute ethanol in petroleum ether; for eluting the 'adsorbed fraction' 20 % (v/v) ethanol in petroleum ether.

Table 10. *Biological test of 'adsorbed' and 'unadsorbed' fractions of unsaponifiable matter of wheat-germ oil*

Sample	Date of exp.	Dose of FeCl$_3$-reducing substances (mg./animal)	No. of animals	Resorption	Dead litters	Live litters
Adsorbed fraction dissolved in arachis oil	May 1944	3·50	5	5	0	0
Unadsorbed fraction dissolved in arachis oil	„	4·00	7	2 or 3	1 or 2	3 (7) (7) (2)*
Arachis oil (control)	„	0·18	5	5	0	0

* Figures in brackets denote the number of animals in each litter.

gave no response in any of the five rats which were used. Resorption resulted in all cases. On the other hand, animals which were dosed with 4 mg. of FeCl$_3$-reducing substances from the unadsorbed fraction gave litters, with the exception of two animals in which resorption occurred. The controls, dosed with arachis oil only, gave no response, as resorption occurred in all of five animals.

Our experiments, therefore, support the conclusion that reducing substances which appear in the unadsorbed fraction of wheat-germ oil are mainly

Procedure

Saponification. 1 g. of oil is weighed in a conical flask, 2 ml. of 5 % pyrogallol are added, and the mixture is heated to 86° on a water-bath. After the addition of 0·44 ml. of the KOH solution, the flask is shaken in the bath for about 2 min., which usually causes complete saponification. To the mixture 8 ml. distilled water are added, the unsaponifiable matter is extracted in a separating funnel by shaking vigorously first with 10 ml. of ether and then with 5 ml. of ether. The two ether extracts are combined and washed

The Chemical Estimation of Vitamin E in Vegetable Oils.

By J. Tošić & T. Moore

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for   5 ml.   *read*   15 ml.

twice, each time with 5 ml. of 0·5 % aqueous KOH, vigorous shaking being avoided at this stage in view of the danger of emulsions. The ether extract is next washed several times with 10 ml. portions of distilled water, with only gentle shaking for the first and second times. Adequate washing of the extract is indicated by the quick separation of layers after vigorous shaking. The ether extract is evaporated on a water-bath, under reduced pressure, and the unsaponifiable matter dissolved in a volume ⸴ ᶜ ethanol, so that 1 or 2 ml. of such solution are equivalent to about 1 mg. of $FeCl_3$-reducing substances. The solution at this stage may be kept overnight in a refrigerator without harm.

If duplicate estimations are to be made, unsaponifiable matter equivalent to 2400–4000 μg. of reducing substances should be prepared. It is usually necessary to take more than 1 g. of oil, to increase proportionately the amounts of all the reagents used, and sometimes to extend slightly the time of saponification.

Chromatographic analysis. A portion of the solution of unsaponifiable matter, containing about 1 mg. of the reducing substances, is evaporated under reduced pressure to dryness on a hot water-bath, and care is taken to remove all ethanol. (a) *Solvent.* 30 ml. of light petroleum + 1 % ethanol solvent are prepared carefully for each chromatographic separation. (b) *Preparation of aluminium oxide column.* Activated aluminium oxide is packed in a tube of 10 mm. diameter and about 120 mm. long, so that length of the column is 50 mm. and diameter 10 mm. 10 ml. of light petroleum ether + 1 % ethanol solvent are passed through the column, which is now ready for use. For each estimation a new column should be made.

A portion of the unsaponifiable matter is dissolved in 5 ml. of the solvent mixture and passed through the column which is then washed with 15 ml. of the solvent. Throughout the analysis the top of the column should not be allowed to become dry, and the rate of filtration should be 70–90 drops/min. The 'unadsorbed fraction' of 20 ml. volume is collected in a clean flask, the solvent evaporated under reduced pressure on a hot water-bath, and the residue dissolved in ethanol and made up to a volume of not more than 25 ml. This solution is now ready for colorimetric estimations.

Colorimetric estimations. (a) *Estimation of carotenoid pigments.* In the unadsorbed fraction mainly carotene is found, and is estimated by its natural yellow colour by means of a Pulfrich photometer with the S 47 filter. The quantity of carotene is expressed in μg./1 g. of oil. (b) *Estimation of vitamin E.* A portion of the solution, containing between 100 and 160 μg. of $FeCl_3$-reducing substances, is pipetted out into a 25 ml. measuring flask. This is found out in a preliminary test, if the vitamin E content of the material examined is quite unknown. Sufficient ethanol is added to bring the volume to about 20 ml., then 1 ml. of $FeCl_3$ solution followed by 1 ml. of α:α'-dipyridyl solution. The volume is then made up to 25 ml. with ethanol, the contents being mixed by shaking the flasks after each addition. The control is prepared in the same way, but the unsaponifiable matter is omitted. The flasks are left in a dark place for 15 min. for full development of the colour. The unknown and the control solutions are transferred to 5 cm. long micro-cells, and their colours matched in a Pulfrich photometer with the use of the green S 53 filter. Since the solutions are sensitive to light the measurements must be made in diffused daylight or low artificial illumination.

Calculation of results. As an example, results with a sample of soya-bean oil may be quoted. 4·14 g. of oil were saponified, and the unsaponifiable matter dissolved in 4·14 ml. of ethanol. 1 ml. portions of this solution were chromatographed. The unadsorbed fraction containing vitamin E was eventually brought up to the volume of 20 ml. and carotene and vitamin E content of oil estimated.

Uncorrected vitamin E value. 6 ml. of this solution gave an extinction reading of 0·47, so that $FeCl_3$-reducing substances of this fraction amount to $0·47 \times 166·6 \times \frac{20}{6} = 261$ μg./ 1 g. oil. The factor 166·6 converts the step photometer reading into μg. of α-tocopherol (see p. 499).

Carotene correction. It was found that 1 g. of oil contained 1·17 μg. of carotene; thus the correction for this pigment amounts to 2·9 μg./1 g. of oil (see p. 500).

Vitamin E. After the carotene correction is deducted, the vitamin E content of the oil comes to 258 μg./1 g. of 'apparent' α-tocopherol.

Estimation of the reducing substances of the 'adsorbed' fraction and of total $FeCl_3$-reducing substances. Although these substances are discarded in the estimation of vitamin E, their evaluation is recommended for two reasons. First, if an estimation is also made of the total reducing substances of the unsaponifiable fraction before chromatographing, a useful check is available on the recovery of reducing substances, and on the behaviour of the adsorption column. Secondly, a high proportion of adsorbed reducing substances may suggest the desirability of biological tests for tocopherol isomers other than the α-form. These isomers, which may be more strongly adsorbed, have low biological activity.

The adsorbed substances are eluted from the adsorption column with 10 ml. of light petroleum containing 20 % ethanol, and estimations are made of the yellow colour and of $FeCl_3$-reducing substances in a similar way to that described for carotene and vitamin E.

Vitamin E content of oils

The contents of vitamin E and of reducing substances other than vitamin E of numerous vegetable oils are recorded in Table 11. Wheat-germ oil, in which vitamin E made up 60 % of the total reducing substances, was the richest source. Some other oils, e.g. tung oil, soya-bean oil, contained relatively large quantities of reducing substances, but only about 20 % of these were in the form of vitamin E. An old specimen of crude cotton-seed oil, a recognized source of vitamin E, contained about one-third of the total reducing substances of fresh wheat-germ oil, and 45 % appeared to be α-tocopherol. Coconut oil and palm-kernel oil contained no reducing substances.

Tocopherol isomers

The presence in natural fats of the β- and γ-isomers of tocopherol, and possibly also of other substances having slight vitamin E activity, raises a problem which we have been unable to investigate fully, mainly on account of difficulties in obtaining the isomers in this country. Through the kindness of Prof. A. R. Todd, however, we obtained a small

Table 11. *Vitamin E and other* $FeCl_3$*-reducing substances of plant oils*

		FeCl₃-reducing substances						
		Before passing through the column (μg./g. of oil)	After chromatographic separation				Recovery	
	Time of examination after extraction (months)		In 'unadsorbed' fraction*		In 'adsorbed' fraction			
Oil			μg./g. of oil	Percentage of total	μg./g. of oil	Percentage of total	μg./g. of oil	Percentage of total
Wheat-germ I	Within 2	2776†	1690†	60·9	1061†	38·2	2751†	99·1
Wheat-germ II	Over 144	491†	286†	58·3	160†	32·6	491†	92·0
Tung	Over 84	2039†	444†	21·8	1540	75·6	1984†	97·4
Soya-bean	15	1222†	250†	20·5	967†	79·3	1217†	99·8
Cotton-seed (crude)	Over 84	936†	425†	45·4	497†	53·1	922	98·5
Cotton-seed (refined)	15	400	144	36·0	254	63·5	398	99·5
First castor	15	693	184	26·5	493	71·2	677	97·7
Linseed (crude)	15	694†	110†	15·8	578†	83·3	688†	99·1
Maize	Over 84	458	274	59·8	168	36·7	442	96·5
Rape (crude)	15	440†	110†	25·0	331†	75·2	441†	100·2
Sesame	15	420	40	9·5	359	85·5	399	95·0
Arachis I	15	336	125	37·2	212	63·1	337	100·3
Arachis II	15	240	108	45·0	128	53·3	236	98·3
Cocoa-butter (crude)	15	212	47	22·2	156	73·6	203	95·8
Arachis III‡	15	123	39	31·7	81	65·8	120	97·5
Coconut	15	0	0	—	0	—	—	—
Palm-kernel	15	0	0	—	0	—	—	—

* Vitamin E fraction.

† Correction for carotenoid pigments has been deducted from the figures marked with asterisk; other oils contained either traces or no carotenoid pigments.

‡ A sample of refined undeodorized ground-nut oil for special white margarine.

specimen of the *p*-nitrophenyl-urethane of β-tocopherol. After saponification the free alcohol was run on to the adsorption column in the usual way. In contrast to the behaviour of the α-isomer only some 30 % passed through.

Further investigation into the separation of the tocopherol isomers is obviously desirable. It is fortunate, however, that the inefficient passage of β-tocopherol through the column, if confirmed, tends to compensate for its much lower biological activity, which according to Mason (1944) is only 40 % of that of α-tocopherol. As far as the α- and β-isomers are concerned, therefore, our routine method should give a good indication of the total biological activity, as usually expressed in terms of α-tocopherol.

SUMMARY

1. A method based on the α:α'-dipyridyl-FeCl₃ reaction has been evolved for the estimation of vitamin E in vegetable oils.

2. To avoid oxidation of the vitamin and other FeCl₃-reducing substances, the oils have been saponified in the presence of pyrogallol.

3. In addition to the known interference by carotenoid pigments, it has been found that the unsaponifiable fractions always contain varying amounts of other irrelevant substances which are

capable of reducing FeCl₃. These are presumably of low or negligible biological activity, and have been removed by adsorption on a column of activated alumina, under conditions which were carefully adjusted to allow the free passage of α-tocopherol.

4. Values for vitamin E, 'total reducing substances' and the interfering substances have been estimated for many oils. Tung oil gave a high value for total reducing substances, but only 20 % appeared to be in the 'α-tocopherol fraction', and the biological activity of the unsaponifiable fraction as a whole was correspondingly poor. In wheat-germ oil the 'α-tocopherol fraction' represented some 60 % of the total reducing substances. Biological tests were consistent with the concentration of activity in this fraction, and its absence from the remaining 40 % of reducing substances, which were separated by the adsorption column.

5. Further investigation into the separation of tocopherol isomers is obviously desirable. In our method β-tocopherol is partially adsorbed on the column. In practice, however, this inefficient passage through the column tends to compensate for its much lower biological activity.

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The Free Amino Groups of Insulin

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That the free amino groups in proteins may be the ε-amino group of lysine was first suggested by Skraup & Kaas (1906), who failed to isolate lysine from deaminized proteins. Van Slyke & Birchard (1913) later found an apparent equality between the free amino-N of proteins and one-half of their total lysine-N, which suggested that the free amino groups were due exclusively to such a source. Methods were then developed to confirm the presence of the free ε-amino group of lysine in proteins; these consisted in treating a protein with a reagent that would react with free amino groups to give derivatives that were stable to acid hydrolysis, so that on hydrolysis of the substituted protein the derivative of lysine could be isolated. The most satisfactory was that of Gurin & Clarke (1934), who treated gelatin with benzenesulphonyl chloride, hydrolyzed the resulting benzenesulphonyl gelatin and isolated ε-benzenesulphonyllysine from the hydrolysate.

In some proteins, and particularly in insulin, the number of free amino groups (Van Slyke) is far in excess of that which can now be ascribed to lysine, which suggests that the protein must contain residues of certain amino-acids which are condensed in such a way that their α-amino groups remain free. Jensen & Evans (1935) have, in fact, been able to isolate the phenylhydantoin of phenylalanine from a hydrolysate of insulin that had been treated with phenyl*iso*cyanate, thus demonstrating that some free amino groups of insulin are present on phenylalanyl residues. Chibnall (1942) has suggested that the free amino groups of proteins over and above the ε-amino groups of lysine are due to terminal

residues of polypeptide chains, and that the number of these groups must therefore give a measure of the number of polypeptide chains in the protein.

We found that methanesulphonyl amino-acids were stable to acid hydrolysis and that they could be fractionated by partition chromatography (Gordon, Martin & Synge, 1943) with an indicator in the aqueous phase of the column. The rates at which the bands moved on the columns were similar to those of the acetamino-acids. The method was satisfactory when applied to synthetic peptides, but with insulin the number of terminal groups estimated by titration of the methanesulphonyl amino-acids was far less than that suggested by the method of Van Slyke. The procedure was accordingly abandoned.

Abderhalden & Stix (1923) attempted to use 2:4-dinitrochlorobenzene (DNCB) for the identification of the terminal groups of a partial hydrolysate of silk fibroin. They did not meet with much success, chiefly owing to the presence of anhydrides in the hydrolysate and the difficulties of separating the products. It seemed, nevertheless, worth while to investigate this reagent, especially as all the 2:4-dinitrophenyl-amino-acids (referred to henceforth as DNP-amino-acids) produced are bright yellow, thereby facilitating chromatographic separation. DNCB will not react with amino-acids in $NaHCO_3$ solution unless heat is applied, and this brings about a certain amount of hydrolysis of the protein. Fortunately, however, the corresponding fluoro-compound, 2:4-dinitrofluorobenzene (DNFB) was found to react readily at room temperature, and the use of this has met with considerable success, for the DNP-amino-acids produced can be estimated