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Determination of α-Tocopherol by Chromatography on Secondary Magnesium Phosphate

(With Collaborative Tests in Four Laboratories)

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A description is given of a method for quantitative determination of α-tocopherol in natural and enriched products. The method, which is generally applicable and suitable for routine examinations, consists in isolation of α-tocopherol by chromatography on secondary magnesium phosphate of the unsaponifiable fraction of the sample with subsequent Emmerie and Engel reaction in equal parts of alcohol and petroleum ether. The chromatography results in a complete separation in one step of α-tocopherol from other tocopherols as well as from carotinoids, vitamin A, sterols and other reducing substances without any time-consuming preliminary treatment. The results obtained by the described method are given and shown to be in close agreement with previously published results.

The chemical methods originally used for the estimation of the vitamin E content of a product aimed in principle at a determination of the total content of vitamin E active tocopherols. When it was found, however, that the biological potency of the individual tocopherols varied considerably, it was realized that methods of this nature were highly unsatisfactory. The vitamin E activity of most of the natural vitamin E sources is chiefly due to their content of α-tocopherol, since the vitamin E activity of some of the other tocopherols is so low that considerable amounts are required to make the activity comparable to that of α-tocopherol (this applies, *e.g.*, to γ- and δ-tocopherol), while in the case of others they occur very sparsely in nature (*e.g.* β-tocopherol, which has so far been found in wheat germ and wheat germ oil only). In practice the determination of the value of a certain product as a source of vitamin E must therefore take the form of a determination of its content of α-tocopherol. When further bearing in mind the growing appreciation of the importance of vitamin E, *e.g.* in feeding stuffs, which has led to the marketing of a steadily increasing number of products enriched with

synthetic α-tocopherol, it will be understood that there is an urgent need of a method for the determination of α-tocopherol for routine purposes.

In the course of time a number of attempts have been made in order to determine the proportion of individual tocopherols present. Hove and Hove [1] thus developed a method for the determination of the total content of tocopherols and the content of α-tocopherol based on the varying rates of reaction displayed by the individual tocopherols with Emmerie and Engel's reagent. Quaife [2] determines the content of α-tocopherol as the difference between the total content of tocopherols and the content of β-, γ- and δ-tocopherol on the basis of the conversion of the latter into nitroso derivatives which are chromatographed on zinc carbonate-Celite. Tosic and Moore [3] determine the content of α-tocopherol by chromatography on alumina of the unsaponifiable matter. By this chromatographic process α-tocopherol is collected together with carotene, however, necessitating a correction for the contribution to the $FeCl_3$-dipyridyl reaction provided by the latter substance. It is further stated that β-tocopherol is not separated completely, involving an overestimation of the content of α-tocopherol.

The best methods for the estimation of the individual tocopherols hitherto developed seem to be those based on paper chromatography which have recently found comparatively extensive application [4-7]. Most of these methods, however, require the removal of certain substances prior to the chromatography to prevent interference with the running of the tocopherols on the chromatogram. It is, *e.g.*, necessary to remove sterols by crystallization from methanol, while vitamin A and carotene are removed by adsorption to Floridin Earth. It seems, furthermore, that one-dimensional, reversed-phase chromatography involves certain losses. Brown [4] has thus ascertained recoveries of only about 80 % of tocopherols. Eggitt and Ward [5] state that the recoveries achieved by them by chromatography were about 95 %. Frequently, however, recoveries of only 90 % are obtainable [7]. A number of the difficulties mentioned above seem, however, to have been overcome by applying two-dimensional chromatography as devised by Green *et al.*[7] By subjecting the extract to initial chromatography on paper impregnated with zinc carbonate it will in many cases be possible to eliminate a number of the time-consuming preliminary treatments, and the duration of the subsequent reversed-phase chromatography may be cut down and the losses consequently diminished. Any statement of the total loss involved in this cromatographic determination is not given, however.

In the following a description will be given of a column chromatographic method for the determination of α-tocopherol in natural and enriched products. In principle the method is simple, and it is readily applicable in routine examinations. The chromatography, in which quantitative recovery of α-tocopherol (98—100 %) is obtained, serves to remove in one step both carotene and vitamin A as well as other tocopherols without any preceding reduction of the quantity of sterols present being required.

For special purposes the method can readily be modified so as to be applicable to the determination of the individual tocopherols. This will be accounted for elsewhere [8].

EXPERIMENTAL

The principle of the method. The material is saponified by means of alcoholic potassium hydroxide to which pyrogallol has been added to protect the tocopherols against oxidation. The saponified solution is extracted repeatedly with ether, and the combined ether extracts washed with water and subsequently dried with anhydrous sodium sulphate. The ether extract is now evaporated to dryness in a carbon dioxide atmosphere and the residue is dissolved in petroleum ether. From this solution α-tocopherol is isolated from the remaining reducing components by chromatography on secondary magnesium phosphate. The amount of α-tocopherol present is finally determined colorimetrically by means of ferric chloride and α,α-dipyridyl (Emmerie and Engel's reaction).

Equipment

Saponification flask with reflux condenser } size to be applied depends on the content of
Separatory funnels } tocopherol in the sample
Evaporation equipment arranged for connection to carbon dioxide supply.
Various volumetric flasks and pipettes.
Chromatographic tube (see Fig. 1).
Spectrophotometer.

Reagents

1. *Pyrogallol*
2. *Potassium hydroxide solution, about 60 %.* Dissolve 60 g potassium hydroxide pellets in 40 ml water.
3. *Absolute alcohol.*
4. *96 % alcohol.*
5. *Ethyl ether.* Render peroxide free by allowing to stand over potassium hydroxide pellets. Distill before use.
6. *About 0.5 N potassium hydroxide solution in about 30 % alcohol.* To 30 ml of potassium hydroxide solution, about 60 % (2) add 300 ml 96 % alcohol and water to make 1 l.
7. *Sodium sulphate.* Anhydrous.
8. *Petroleum ether.* Boiling point below 70°C.
9. *Secondary magnesium phosphate* for chromatography (Merck: Magnesium phosphoricum purum — *cf.* subsequent comments). The commercial product is activated as follows: Dry a quantity of magnesium phosphate corresponding to 200 g anhydrous substance for 24 h at not less than 176°C and cool over concentrated sulphuric acid. Boil the powder for 15 min with a solution of 20 g secondary sodium phosphate (Na_2HPO_4, $2H_2O$) in 1.6 l distilled water. Cool somewhat and separate on Büchner funnel, washing with about 1 l water. Dry the magnesium phosphate for not less than 48 h at not less than 176°C and cool to room temperature over concentrated sulphuric acid. Grind gently in a mortar and sieve through a 120 mesh sieve. Store in a dry place in a well-closed container.
10. *0.1 % ferric chloride solution in absolute alcohol* } store in brown bottles at room
11. *0.25 % alcoholic solution of α,α-dipyridyl* } temperature. Will keep for at least two months.

Procedure

Saponification and extraction. Weigh into the saponification flask a quantity of the sample equivalent to 0.4—4 mg α-tocopherol, but not less than 2 g. Add one fourth of the sample's weight of pyrogallol and as many ml of 60 % potassium hydroxide solution as denoted by the weight of the sample in g and four times its volume of 96 % alcohol.

Saponify by bringing to the boil on water bath and boiling for 10 min. Cool somewhat and add a volume of water equal to three times the volume of alcohol used. Transfer into a separatory funnel, rinsing the flask with ether, using 100—200 ml according to the volume of the sample. When working with larger quantities of feeding stuffs, the alcoholic saponification solution may be decanted into a separatory funnel into which the required volume of water has already been transferred. In this case wash the precipitate successively with all the ether used for the extraction. Shake or swirl the separatory funnel for

one minute, allow the layers to separate and run the aqueous phase into another separatory funnel in which extraction is performed by means of 50—100 ml fresh ether. After a third extraction process, transfer the three ether extracts into one separatory funnel and wash with 100 ml water, 100 ml 0.5 N potassium hydroxide in 30 % alcohol and finally with three 100—200 ml portions of water.

Dry the ether extract by filtering through a 2—3 cm thick layer of anhydrous sodium sulphate placed on a glass filter, and reduce the volume by evaporation in a carbon dioxide atmosphere on water bath until only a few ml remain. Remove the last traces of ether by means of a current of carbon dioxide, but do not heat.

By means of petroleum ether transfer the residue into a volumetric flask of suitable size and fill to volume with petroleum ether.

Chromatography. A column is prepared from the activated magnesium phosphate by suspending a suitable quantity in petroleum ether and running the mixture into a chromatographic tube, the effective length of which is 20 cm and the internal diameter 12 mm (Fig. 1). This tube is connected by means of a rubber tube to a nitrogen cylinder, the nitrogen pressure being regulated so as to provide a suitable rate of flow (2—4 ml per min). When the column of magnesium phosphate has settled, it is covered with a thin layer of anhydrous sodium sulphate. The petroleum ether level must never be allowed to fall below the top of the magnesium phosphate column.

An aliquot of the petroleum ether extract corresponding to about 0.4 mg α-tocopherol (quantities down to 0.1 mg may, however, be determined) is now transferred to the column.

After rinsing with 50 ml petroleum ether, the receiver is exchanged and α-tocopherol is eluted with altogether 150 ml 2 % ethyl ether in petroleum ether. Carotene will not be adsorbed to the column, but may be collected with the petroleum ether. β-, γ-, and δ-tocopherol as well as vitamin A are adsorbed, but require to be eluted an ether concentration higher than 2 % (*cf.* Bro-Rasmussen and Hjarde [8]).

The α-tocopherol fraction is evaporated on a water bath (30°C) until a residue of 5—10 ml remains. This residue is transferred into a volumetric flask (if the quantity of α-tocopherol chromatographed is 0.4 mg, a 30 ml volumetric flask will be suitable), several portions of absolute alcohol and petroleum ether are used for rinsing, the rinsings are added and the flask filled up with absolute alcohol and petroleum ether resulting in an α-tocopherol solution in a solvent consisting of equal parts of alcohol and petroleum ether.

To check the effectivity of the chromatography the last 5 ml petroleum ether eluate plus the first 5 ml petroleum ether-ethyl ether eluate are collected in a 20 ml volumetric flask with 10 ml absolute alcohol. Now the main fraction is collected as described above and, finally, 10 ml eluate likewise in a 20 ml volumetric flask with 10 ml absolute alcohol. Eluate collected in this way before and after the main fraction must not give any reaction for α-tocopherol (*cf.* the subsequent comments).

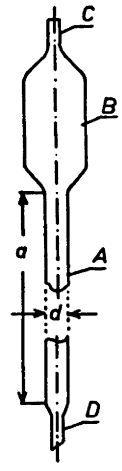

Fig. 1. Chromatographic column. A: column. B: reservoir for eluent. C: connection to pressure. D: outlet tube to retain plug of cottonwool at base of A.

The colorimetric measurement. To a 5 ml sample of the solution containing the isolated α-tocopherol are added 0.25 ml ferric chloride solution (reagent No. 10) and 0.25 ml α,α-dipyridyl solution (reagent No. 11).

The intensity of the colour is measured in a spectrophotometer (or suitable colorimeter) at 520 mμ exactly two min after the addition of the reagents, at which time the intensity must have reached a constant value.

A blank determination is run on 2.5 ml petroleum ether +2.5 ml absolute alcohol + 0.25 ml ferric chloride solution + 0.25 ml α,α-dipyridyl solution.

The value of $E^{1\%}_{1\,cm}$ 520 mμ for the sample can now be calculated and the content of α-tocopherol in the sample examined can be found. By measurements on spectrophotometer at 520 mμ the value of $E^{1\%}_{1\,cm}$ for pure α-tocopherol is taken to equal 400.

Comments

re secondary magnesium phosphate. It seems to be difficult to establish any certain criteria as regards the suitability of a commercial product for the purpose of chromatography. Examination of a number of products has shown that it is possible frequently, but not invariably from products of the above mentioned quality to obtain charges which can be activated. It appears to be necessary to test each charge directly by chromatographing pure α-tocopherol. In this process the α-tocopherol must not be eluted from a 20 cm column by means of 100 ml petroleum ether followed by 100 ml 0.5 % ethyl ether in petroleum ether, while elution should be quantitative when about 125 ml 2 % ethyl ether in petroleum ether is used. If α-tocopherol is eluted by means of petroleum ether or 0.5 % ethyl ether in petroleum ether, the charge of magnesium phosphate is unsuitable for the purpose, its activity being too low to allow for the adsorption of α-tocopherol from low-potency products with a large content of impurities.

re saponification. According to the method used in this case the sample to be tested is saponified directly without any preceding extraction of the fats. Pyrogallol is used to protect the tocopherol against oxidation in the alkaline solution. In agreement with previous investigations [3] pyrogallol was observed to have a marked effect, as will appear from Table 1.

However, even when using pyrogallol, it is not possible completely to eliminate losses in the α-tocopherol determination.

The losses involved in the analyses of individual products may be determined by parallel experiments in which known quantities of tocopherol corresponding to the contents in the products are added and recoveries determined. The following section on the results obtained by the present method gives examples of the losses involved in such determinations.

re chromatography. If the magnesium phosphate fulfills the above requirements, the efficiency of a chromatographic separation may be judged in the following way:

If the eluate collected before the main fraction gives reaction for reducing substances, this may be due either to a content in the extract of reducing substances which are eluted

Table 1. The protective effect of pyrogallol in the determination of α-tocopherol

	Quantity of α-tocopherol found (μg/g)		
	Butter	Wheat germ oil	Enriched fat emulsion
With pyrogallol	23.3	2 060	180
Without pyrogallol	21.5	1 570–1 720	139
Difference in %	8	17–24	20

immediately before α-tocopherol, or to an incomplete adsorption of α-tocopherol. If necessary, the identity of the reducing substances must be determined by chromatography of a parallel sample of the extract to which α-tocopherol has been added. According to the experiences gained hitherto it seems that in cases where this problem has been significant the reason has been an incomplete adsorption of α-tocopherol on account of extreme contents of impurities, chiefly sterols. In such cases a column of 5—10 cm greater length of the column is used.

If the fraction collected immediately after the main fraction displays a reducing action this indicates that tocopherol-like substances are eluted immediately after α-tocopherol.

The latter condition will generally manifest itself in the course of the colorimetric measurement in the form of an increase of the intensity of colour, since only α-tocopherol has been found to produce a colour of constant intensity in the course of 2 min in the mixture of alcohol and petroleum ether used here.

If it is not feasible in this way to eliminate the possibility that impurities have been collected together with α-tocopherol, the chromatography must be repeated, the eluate being collected in fractionation flasks half full of absolute alcohol. From each of the fractions which contain α-tocopherol an aliquot is drawn. These are combined and taken up with a suitable volume of equal parts of absolute alcohol and petroleum ether. If it is not immediately possible to ascertain which of the fractions contain α-tocopherol, parallel chromatography on part of the extract to which pure α-tocopherol has been added must, as referred to above, be resorted to.

RESULTS AND DISCUSSION

As mentioned above a number of determinations of the losses involved in the use of the method have been made in the course of the present work. Table 2 lists the total percentage recoveries for a number of samples with different α-tocopherol contents. It appears from this that the total recovery for the whole procedure decreases with decreasing content of α-tocopherol, the recovery in the case of wheat germ oil with a content of 2 100 μg/g being 98 %, while in the case of chicken feed containing 11 μg/g the loss is 15 %. It should be noted that the loss deriving from the chromatography only constitutes a small and constant part of the total loss (0—2 %), it having been endeavoured in all cases to use for the cromatography a quantity of the product containing about 0.4 mg α-tocopherol (in the case of chicken feed the quantity was only about 0.2 mg). The chief part of the losses in the case of the low-potency samples thus derive from the saponification and the extraction.

As already mentioned, the colorimetric measurement of the content of α-tocopherol is performed in solution in equal parts of alcohol and petroleum ether. For the purpose of the described method this solvent offers two advan-

Table 2. Total percentage recoveries in the α-tocopherol determination

Sample	α-Tocopherol found (μg/g)	Total recovery (%)	Corrected content (μg/g)
Wheat germ oil	2 060	98	2 100
Enriched feeding stuff	550	97	565
Enriched fat emulsion	180	95	189
Butter	23.5	90	26.1
Chicken feed	9.1	85	11

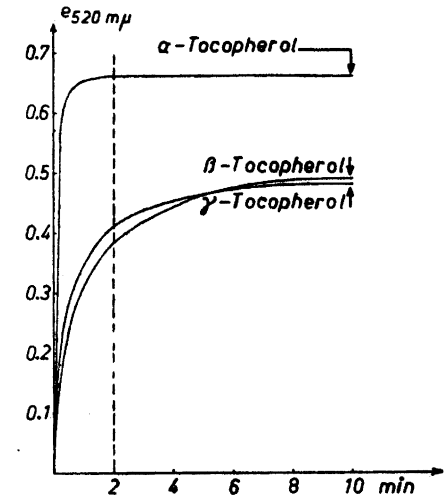

Fig. 2. The colour development at the Emmerie—Engel reaction for α-, β- and γ-tocopherol in petroleum ether/ethanol = 1/1.

tages above alcohol which is the solvent generally used. In the first place it is possible to use the eluate directly for the colorimetric measurement if it is collected in fractionation flasks half full of absolute alcohol, a procedure which is not feasible if petroleum ether only is used. This is of importance in cases when it proves to be necessary to perform a fractionate collection of α-tocopherol as mentioned above, and further in cases when a determination of the other tocopherols is required (*cf.* Bro-Rasmussen and Hjarde [8]). In the second place the use of this solvent gives a fairly certain criterion as to whether the reducing substance collected in the routine cromatography is identical with α-tocopherol, since, as already mentioned, it has been found that it is only the reduction by α-tocopherol of the ferric ions in Emmerie and Engel's reaction which is completed in less than 2 min. To illustrate this, Fig. 2 shows the relationship between colour intensity and time in the case of α-, β-, and γ-tocopherol. As it appears, most clearly in the case of β- and γ-tocopherol, the rate of reaction is reduced as compared to that prevailing in the case of corresponding measurements in alcohol. In the solvent used in this case full colour development is only obtained in the course of 7—10 min in the case of β- and γ-tocopherol, while in alcohol all the three tocopherols mentioned show full colour development in the course of less than 2 min [9].

The fact that the rate of reaction is thus less than when the reaction takes place in alcohol has no influence on the final colour intensity. In the case of synthetic α-, β-, and γ-tocopherol the $E^{1\%}_{1\,cm}$ 520 mμ values have thus in all cases been found to equal about 400 when measured after periods of 2, 10, and 10 min, *i.e.* the same value as that generally given for the reaction in alcohol. It should finally be mentioned that Lambert-Beer's law also applies unchanged when Emmerie and Engel's reaction is performed in a mixture of equal parts of alcohol and petroleum ether. δ-Tocopherol is the only tocopherol which does not show linear dependence on the concentration, for which reason a standard curve should be used in this case.

While the results of collaborative tests made in four laboratories to examine the reproducibility of the method are given in an addendum, a number of determinations of the α-tocopherol content of natural products will be given in the following (Table 3).

Table 3. Content of α-tocopherol in natural products (μg/g)

Material	Results obtained by present method	Results obtained by other workers
Wheat germ oil	2 100	1 680[4]
Wheat germ	220	—
Soybean oil	110	124[4]
Rapeseed oil	225	151[7]
Cottonseed oil	325	470[7]
Corn germ meal	73	—
Butter (summer)	26	24.8[10]
Lucerne	27	22—28[11]
Lucerne silage	36	30—31[11]
Chicken feed	11	—

Among the values given in the table only the determinations made on soybean oil and chicken feed have required additional chromatography with fractionate collection of α-tocopherol, since in these cases it has been found that α-tocopherol is followed immediately by a reducing substance which cannot be identified as a tocopherol [8]. In the case of all the other materials α-tocopherol has been collected directly in one fraction with 150 ml 2 % ether in petroleum ether.

With respect to some of the materials Table 3 lists α-tocopherol contents determined by other workers. A comparison shows that the values found in the present investigation agree well with those previously found.

The present method meets the demand for a simple and readily reproducible method for the determination of α-tocopherol independently of other tocopherols and other reducing constituents of natural and enriched products. The method has another advantage, *viz.* that it is generally applicable and at the same time provides a fairly good criterion as to the specificity of the method. Only in the case of products of very low potency will appreciable losses due to the saponification and extraction processes occur, necessitating parallel experiments with known α-tocopherol additions to correct for such losses. The chromatography, on the other hand, is practically quantitative.

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Addendum

COLLABORATIVE EXAMINATIONS OF A METHOD FOR THE DETERMINATION OF α-TOCOPHEROL BY CHROMATOGRAPHY ON SECONDARY MAGNESIUM PHOSPHATE

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The method described above for the determination of α-tocopherol has been checked through collaborative examinations in four laboratories.

The examinations comprised two experimental series as difficulties were experienced as regards the acquisition of suitable magnesium phosphate for the first series. The difficulties manifested themselves in particular with regard to the results obtained in one of the laboratories, for which reason these results have been omitted from Table 4.

Table 4. Results of collaborative tests

Experimental series	Sample of	Calculated addition of α-tocopherol	Results of tests in μg α-tocopherol per g			
			Laboratory			
			A	B	C	D
1	Wheat germ oil	0	2 040	1 800	1 960	2 020
	Soybean oil	0	—	180	240	165
	Soybean oil with an addition of 0.469 mg α-tocopherol acetate per g	410	—	530	580	540
	Feeding stuff x	0	—	70	65	60
	Feeding stuff x with an addition of 0.624 mg α-tocopherol acetate per g	546	—	480	560	570
	Feeding stuff x with an addition of 4.61 per cent wheat germ oil	90	—	160	130	145
2	Feeding stuff y (chiefly ground cereals)	0	6	6	6.6	9
	Feeding stuff y with an addition of 0.0486 mg α-tocopherol acetate per g	42.5	36	44	47	46—38
	Feeding stuff y with an addition of 0.481 mg α-tocopherol acetate per g	420	350	460	440	430

The materials selected for the tests as well as the results of the latter appear from Table 4. As it will be seen, some of the samples have been given an addition of known quantities of tocopherol acetate. For this purpose a commercial, synthetic preparation has been used, analysis of which showed it to contain 875 mg tocopherol per g, corresponding to 96 % pure α-tocopherol acetate. The figures given in the table as regards quantities of tocopherol added have been calculated on the basis of this analysis.

Results of the examinations

Consideration of the results of series 1 shows that maximum deviation occurs in connection with the examination of soybean oil. The reason is that this oil contains reducing substances which are eluted with 2 % ether immediately after α-tocopherol [8] and thus give rise to results which are too high when, as done here, the α-tocopherol fraction is delimited by the collection of a stipulated quantity of eluate. As already mentioned, the presence of this impurity in the α-tocopherol fraction will disclose itself, in that the intensity of the colour does not reach a constant value in the course of 2 min and, further, in that the fraction collected immediately after the main fraction will give a colour reaction with ferric chloride-dipyridyl. If collection in small fractions, as described above, is applied, enabling a delimitation of the α-fraction in each individual case, this source of error will be eliminated.

The presence of the impurities does not make itself felt so much in soybean oil to which α-tocopherol has been added, *i.a.* because a smaller amount of extract is required on the column due to the greater content of α-tocopherol with a correspondingly smaller content of interfering reducing substances.

As regards the results of series 2 the agreement is better although the values obtained by Laboratory A are lower than those of the other laboratories. However, Laboratory A's examinations were made at a somewhat later time than those of the other three laboratories, and it is possible that the lower results are caused by storage losses. The examinations made by Laboratory D of one of the samples suggest that this may be the case, the two values having been obtained by examinations performed at an interval of three months.

It will be seen that it is possible to determine a content of α-tocopherol of less than 10 μg per g, but the accuracy of this determination would no doubt have been better if collection in small fractions had been used for the delimitation of the volume of the α-fraction.

It should be added that the results of repeated examinations of the same material in one laboratory show considerably smaller variations than might have been expected from the discrepancies between the examinations in the four laboratories as given in the table.

From the examinations made the conclusion must be drawn that complete agreement cannot always be obtained from determinations made according to the present method in different laboratories. In cases of doubt, and always when dealing with feeding stuffs with a low content of α-tocopherol, the chromatography should comprise collection in small fractions so as to make it possible to plot the fractionation curve.