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Quantitative Determination of the Individual Tocopherols by Chromatography on Secondary Magnesium Phosphate

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A method is given for the determination of the distribution of tocopherols in natural products. The method depends on columnar chromatographic separation of the natural tocopherols in three groups in which the chief components are α-, $\beta+\gamma$-, and δ-tocopherol.

With regard to the recently described and more rarely occurring tocopherols, ζ-tocopherol (5,7-dimethyltocol) will be found together with α-tocopherol, while ε-tocopherol (5-methyltocol) is estimated together with β- and γ-tocopherol.

The possibility of obtaining further separation of the individual tocopherols and the importance in practice of such a separation are discussed, both with a view to the vitamin E activity of the tocopherols and to their action as antioxidants.

Examples are mentioned and the results discussed.

For the majority of practical purposes, in estimating the vitamin E activity of natural products, the greatest significance will be attached to the contents of α-tocopherol. The reasons for this are the higher biological potency of α-tocopherol and the fact that it is the most abundant of the tocopherols. Likewise, in the case of enriched products the tocopherol used will generally be α-tocopherol.

In spite of these facts, the problem of determining the content of other tocopherols in a certain product will at times be of considerable interest. It is thus well-known that the content of α-tocopherol in certain vegetable oils is fairly low compared to their content of other tocopherols, so that to obtain a reliable estimate of the value of such a product as a vitamin E source the contribution to the activity due to each individual tocopherol should be taken into consideration.

The same applies to some extent to tocopherol determinations, the purpose of which is to establish the antioxidant activity of natural products. In this connection it should be noted, however, that it is not possible concerning the antioxidant activity to ascribe a predominating effect to any one of the tocopherols as is possible with respect to the vitamin E potency. When

determining the antioxidant activity of the tocopherols of a certain product, it will thus frequently be insufficient to account for the content of α-tocopherol only.

In such cases a method for the determination of the individual tocopherols is essential. The development of methods for this purpose has commanded great interest during recent years. So far the best results seem to have been obtained by the application of paper chromatography [1-3]. These methods have made it possible to make quantitative determinations of the tocopherols hitherto found (α, β-, γ-, and δ-tocopherol) and also to demonstrate the presence of two new natural tocopherols, *viz.* ε-tocopherol (5-methyltocol) and ζ-tocopherol (5,7-dimethyltocol). On the basis of experience drawn from the paper chomatographic methods Eggitt and Norris [4] have quite recently developed a method for the separation of tocopherol mixtures by means of partition chromatography on paraffin-impregnated kieselguhr columns. This reversed-phase technique allows a separation of the tocopherols into three groups of isomers, *i.e.* mono-, di-, and tri-methyltocols. The authors of the present article have, however, only learned of this method after the conclusion of the present work.

In the following a description will be given of a method for the determination of the individual tocopherols depending on cromatography on secondary magnesium phosphate. This method is a further development of a method for the determination of α-tocopherol published previously (Bro-Rasmussen and Hjarde [5]). When chromatographing tocopherol mixtures on secondary magnesium phosphate, the tocopherols are adsorbed from a solution in petroleum ether. Elution with ethyl ether in petroleum ether in concentrations increasing gradually from 2 % to 6—7 % will result in successive elution of the individual tocopherols in the following order: α-, $\beta+\gamma$-, δ-tocopherol. Any carotene present will not be adsorbed, but will pass through the column with the petroleum ether, while any vitamin A present will require an eluent containing 10—15 % ether, *i.e.* it will be eluted after δ-tocopherol.

EXPERIMENTAL

The present investigations have all been performed according to the technique used for the determination of α-tocopherol as previously described [5] with the exception that the chromatography of the unsaponifiable fraction of the sample has been continued beyond elution with 2 % ether, using gradually increasing ether concentrations as mentioned in connection with each individual experiment.

In certain cases it proved to be convenient, in order to obtain maximum separation, to use a column of greater length than 20 cm. In such cases a 30 cm column has generally been used. This means that α-tocopherol is only eluted quantitatively when slightly more than 150 ml eluent with 2 % ether has been used, but it does not give rise to greater losses in connection with the chromatography than the 0—2 % losses usually met with.

It should finally be stated that fractional collection has been applied in all cases. Fractions have been collected by volume in volumetric flasks half-full of absolute alcohol. The Emmerie-Engel reaction has subsequently been applied to each individual fraction, the measurement being made in the case of α-tocopherol after a 2 min interval, in the case of β- and γ-tocopherol after 10 min, and in the case of δ-tocopherol after 20 min.

The adsorption of α- and β-tocopherol. The unsaponifiable fraction of about 2.5 g wheat germ was chromatographed on a 20 cm column. Elution with 2—4 % ether in petroleum ether. Fig. 1 shows the fractionation curve, the extinction at 520 mμ after the reaction with ferric chloride and α,α-dipyridyl being plotted for each individual fraction.

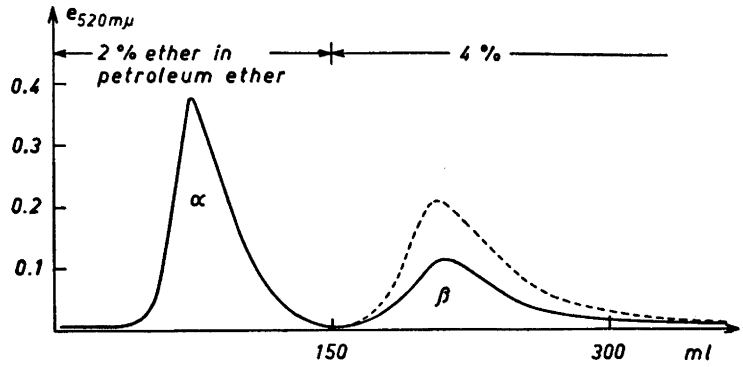

Fig. 1. Fractionation curve for wheat germ with a column 200 mm long. The dotted curve represents the result of addition of synthetic β-tocopherol.

It will be seen from the curve that the separation between the α- and β-tocopherol of wheat germ is complete, α-tocopherol being eluted with 2 % ether and β-tocopherol with about 4 % ether in petroleum ether. Summation shows the total contents in wheat germ to be:

α-tocopherol: 220 μg/g~66 %
β-tocopherol: 112 μg/g~34 %
Altogether: 332 μg/g~100 %

The identification of the two tocopherols of wheat germ was made by parallel runs with addition of the pure substances. To illustrate this the dotted curve in Fig. 1 represents the fractionation of wheat germ to which synthetic β-tocopherol (5,8-dimethyltocol) has been added.

The adsorption of γ-tocopherol. Experiments made with pure synthetic γ-tocopherol * show that this substance is adsorbed on secondary magnesium phosphate and that it is eluted with petroleum ether with about 4 % ether.

Chromatography on a 30 cm column of the unsaponifiable fraction of about 2.5 g wheat germ + about 0.5 mg γ-tocopherol was used to examine the adsorption of γ-toco-

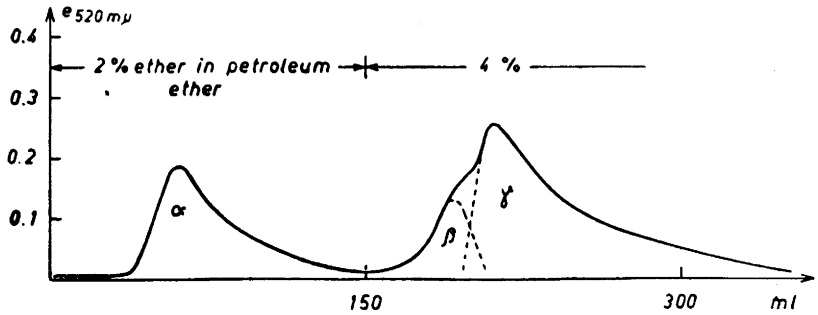

Fig. 2. Fractionation curve for wheat germ after addition of synthetic γ-tocopherol. Column 300 mm long.

* Bought from Distillation Products Industries.

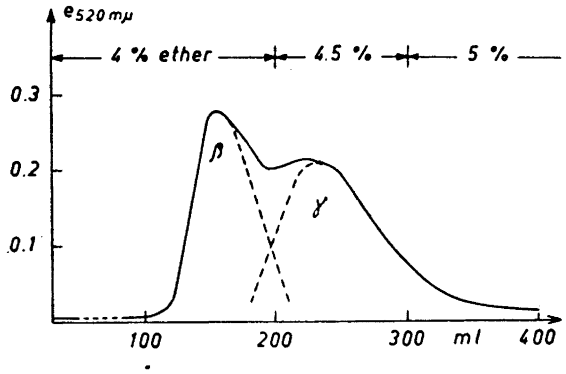

Fig. 3. Fractionation curve for mixture of synthetic β- and γ-tocopherol with a column 500 mm long.

pherol relative to that of β-tocopherol. Fig. 2 shows that β- and γ-tocopherol are on the whole eluted together by means of about 4 % ether. It appears from the figure, however, that there is a tendency towards separation, β-tocopherol being eluted together with the front of the γ-tocopherol zone.

That this tendency is an actual one appears more clearly from Fig. 3 which shows the fractionation curve obtained by chromatography of approximately equal quantities of pure β- and γ-tocopherol on a 50 cm column.

The adsorption of ζ-tocopherol. By means of two-dimensional paper chromatography Green *et al.*[3] have shown that 5,7-dimethyltocol is occasionally found in natural products, and they have given this substance the name of ζ-tocopherol. Since this tocopherol is known to possess a biological potency of the same order of magnitude as that of α-tocopherol (Jacob, Sutcliffe and Todd [6]), it is essential to examine the adsorption exhibited by this substance on chromatography with a view to vitamin E estimation.

By preliminary experiments it is found that synthetic 5,7-dimethyltocol is adsorbed on magnesium phosphate and eluted by means of 2 % ether in petroleum ether, *i.e.* as α-tocopherol. Chromatography on a 20 cm column together with α-tocopherol confirms

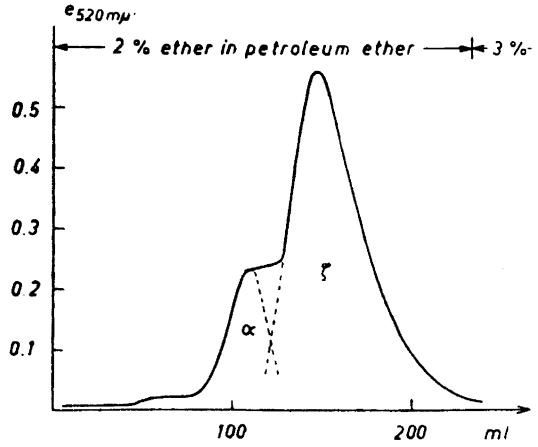

Fig. 4. Fractionation curve for mixture of synthetic α- and ζ-tocopherol with a column 300 mm long.

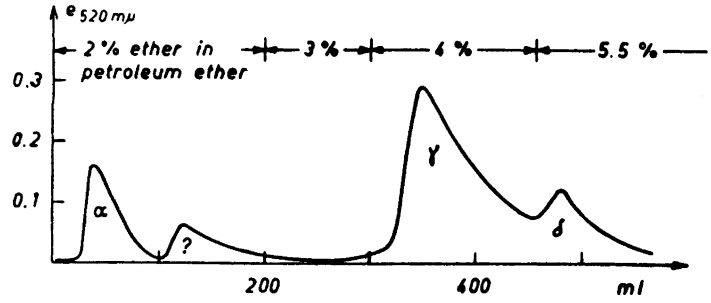

Fig. 5. Fractionation curve for unsaponifiable fraction of soybean oil with a column 300 mm long.

these results, α- as well as ζ-tocopherol being eluted quantitatively by means of 150 ml of 2 % ether in petroleum ether. In routine determinations of α-tocopherol any ζ-tocopherol present will thus be included in the estimate, a result which shows good agreement with the biological activities.

However, chromatography on a 30 cm column shows that there is a certain tendency towards separation between α- and ζ-tocopherol (Fig. 4), the adsorption of α-tocopherol being slightly weaker than that of ζ-tocopherol. Complete separation by this method does not seem to be possible, however.

δ-Tocopherol. Chromatography of soybean oil. To examine the asorption of δ-tocopherol the unsaponifiable fraction of about 2.5 g soybean oil was subjected to chromatography on a 30 cm column.

As appears from the fractionation curve (Fig. 5), a separation into four reducing substances is observed. Out of the four substances three may be identified as above in the case of β-tocopherol (Fig. 1) and are found to be α-, γ-, and δ-tocopherol, which may thus be separated completely, δ-tocopherol not being eluted until the ether concentration in the eluent has reached 6—7 %. The three tocopherols are found to be present in the following quantities:

α-tocopherol:	110 μg/g~13 %
γ-tocopherol:	430 μg/g~51.5 %
δ-tocopherol:	295 μg/g~35.5 %
Altogether:	835 μg/g~100 %

The possibility that the fourth reducing substance which is separated in the chromatography of soybean oil (marked with a ? in Fig. 5) is a tocopherol must apparently be ruled out. Judging from the course of the adsorption ζ-tocopherol is the only possibility, but chromatography of soybean oil with an addition of 5,7-dimethyltocol shows that the unknown substance is not identical with this substance. On the contrary, it appears from Fig. 6 that it is adsorbed between α-tocopherol and 5,7-dimethyltocol. By acting as "displacement carrier" the substance causes the above-mentioned, very incomplete separation between the two tocopherols to become complete. Thus there is no doubt that the unknown substance is not a tocopherol.

ε-Tocopherol. Chromatography of wheat bran. The last of the known natural tocopherols, 5-methyltocol, has hitherto been found in wheat bran and wheat germ only, in the former case in larger quantities, in the latter in small quantities [2,3]. It has been given the name ε-tocopherol.

Chromatography of the unsaponifiable fraction of about 10 g wheat bran showed that the total tocopherol content of 83 μg/g distributes itself between two elution peaks (Fig. 7), *viz*:

"α-tocopherol":	12.5 μg/g~15 %
"β-tocopherol":	70.5 μg/g~85 %
Altogether	83 μg/g~100 %

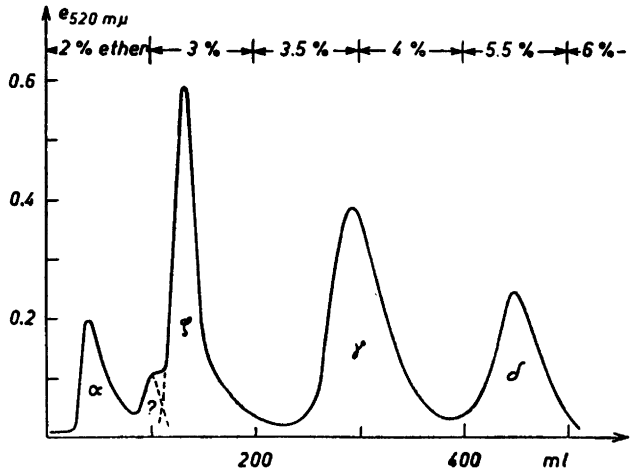

Fig. 6. Fractionation curve for soybean oil after addition of synthetic ζ-tocopherol. Column 300 mm long.

As previously shown, the "α-peak" may contain both α- and ζ-tocopherol. For the examination of the "β-peak" chromatography on paper impregnated with liquid paraffin was applied, the solvent used being 75 % alcohol. This showed that the "β-tocopherol" consisted of two components with $R_F = 0.55$ and $R_F = 0.75$, respectively. By means of parallel experiments in which pure β-tocopherol had been added the spot with $R_F = 0.55$ was identified as being due to β-tocopherol. The other spot ($R_F = 0.75$) thus seems to be identical with Brown's "fast spot" [1] as well as with the ε-tocopherol found by Eggitt and Ward [2] and by Green *et al.*[3]

The quantitative distribution of the components o f "β-tocopherol" was found to be as follows:

β-tocopherol: 11.5 μg/g~14 % of the total in wheat germ
ε-tocopherol: 59 μg/g~71 % » » » » » »

in good agreement with the results obtained by Green *et al.*[3]

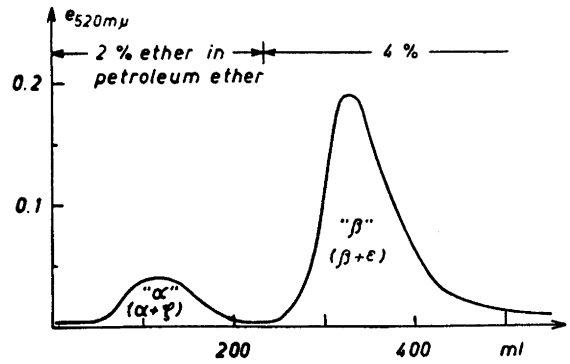

Fig. 7. Fractionation curve for wheat bran with a column 300 mm long.

DISCUSSION

The present experiments show that chromatography of mixtures of the known natural tocopherols on secondary magnesium phosphate results in a separation into three main groups, the number of methyl groups in *ortho* positions in relation to the 6-hydroxy group being the factor which chiefly determined the affinity to the adsorbent magnesium phosphate:

α-, and ζ-tocopherol are eluted with 2 % ether,
β-, γ-, and ε-tocopherol are eluted with 4 % ether,
δ-tocopherol is eluted with 6—7 % ether.

This separation is in perfect analogy with the conditions prevailing in chromatography on zinc carbonate-impregnated paper [3].

The possibility of obtaining further separation by means of the described chromatographic technique has been considered. The tendency, which is shown by Figs. 3 and 4, towards a separation of β- from γ-tocopherol and of α- from ζ-tocopherol suggests that the use of longer columns might enable further separation. In agreement with this attempts at a separation between β- and γ-tocopherol on a 50 cm column have shown improved separation as compared to that obtained on a 30 cm column. As, however, the separation on the 50 cm column is still very incomplete, the experiments have not so far been continued.

Another theoretical possibility of further separation has been demonstrated in connection with the chromatography of soybean oil. When, as shown in the case of α- and ζ-tocopherol there is a slight difference in the adsorption of the tocopherols, suitable substances may be used with success as "displacement carriers", causing a complete separation by being adsorbed between the two substances. The artefact found in soybean oil presents an example. Lacking knowledge of the chemical structure of this substance makes it impossible, however, at present to utilize this fact.

According to the technique used for the present investigations a more complete separation is apparently possible only when applying additional chromatography on paraffin-impregnated paper, as mentioned in connection with the adsorption of ε-tocopherol, a procedure which does not present any difficulties. In this connection it should be noted that by the chromatography on magnesium phosphate the sterols have been removed from the extract so that paper chromatography can be applied directly to the obtained tocopherol fractions. It should also be mentioned that a combination of the recently published reversed-phase partition chromatography on paraffin-impregnated kieselguhr [4] and chromatography on magnesium phosphate will give a separation corresponding to that obtained by two-dimensional paper chromatography. The determination might, *e.g.*, also be made by means of two parallel chromatographic separations of the unsaponifiable matter with subsequent calculation of the contents.

If the problem dealt with is the vitamin E activity of the tocopherols, it is a question, however, considering the biological activity of the individual tocopherols, whether there is any need of further differentiation beyond the three groups referred to above. As regards the biological activity of ζ-tocopherol it

is known that it is almost identical with that of α-tocopherol [6]. Even if the biological potency of ε-tocopherol has not as yet been determined, a consideration of its chemical structure will show that there is every reason to expect that it approximates that of γ- and β-tocopherol, which is generally stated to be 20—30 % of that of α-tocopherol [7]. Finally the biological potency of δ-tocopherol is taken to be 1 % (or less) of that of α-tocopherol. It will thus be seen that the separation obtained corresponds closely to the order of the biological potencies, so that when it is necessary to consider other tocopherols than α-tocopherol, a chromatographic separation on magnesium phosphate will be quite sufficient for most practical purposes.

Of course, it is not possible to apply these findings directly to the question of the antioxidant properties of the tocopherols. It cannot be excluded that for this purpose a complete elucidation of the composition of the tocopherol content may be of interest. In that case chromatography on magnesium phosphate may be combined with chromatography on paraffin-impregnated paper or reversed-phase partition chromatography on kieselguhr columns. It should be emphasized, however, in this connection that definite, quantitative figures for the relative antioxidant potencies of the tocopherols can hardly be given, since the values found depend to a great extent on methods of examination, temperature and criteria applied. [8,9] When taken together with our present lack of knowledge with regard to the antioxidant properties of the most recently described tocopherols, these circumstances tend to cause a determination of the content of each individual tocopherol to be rather illusory.

As mentioned previously in connection with the determination of α-tocopherol [5], column chromatography does not give rise to losses of any magnitude. Chromatography of a mixture of β- and γ-tocopherol on a 50 cm column and elution with 4—5 % ether in petroleum ether thus results in recoveries of 97—98 %.

Table 1. Distribution of tocopherols in natural products

Product	Total tocopherols (μg/g)	$\alpha+\zeta$ (%)	$\beta+\gamma+\varepsilon$ (%)	δ (%)
Wheat germ	332	66	34	
Wheat germ oil	2 300	91	9	
Wheat bran	83	15	85	
Corn germ meal	307	24	76	
Soybean oil	835	13	51.5	35.5
Rapeseed oil	691	32.5	68	
Cottonseed oil	888	36	64	
Mustard oil	525	16	84	

Table 1 lists the results of a number of examinations of natural products which are known to contain considerable quantities of the other tocopherols in addition to α-tocopherol.

Among the products mentioned in Table 1 both soybean oil and mustard oil contain artefacts which are eluted between α- and γ-tocopherol fractions. In soybean oil these substances occur in quantities corresponding to 8 % of

the total reducing power. Corresponding observations have been made by Green *et al.*[3] in twodimensional paper chromatography.

When considering the adsorption data, the artefacts observed in the course of the present investigation do not appear to be identical with those found by Green *et al.*, the latter authors stating that the reducing substances move beyond the α-tocopherol position on zinc carbonate paper, which should correspond to elution before α-tocopherol from the magnesium phosphate column.

The relative contents of α- and β-tocopherol in wheat germ correspond exactly to the values previously found [1,3]. It is remarkable, therefore, that the wheat germ oil examined displays such a low content of β-tocopherol, both as regards absolute and relative values. As the oil in question is a refined wheat germ oil, it cannot, of course, be excluded that the refining process has destroyed part of the β-tocopherol. Since, however, the content of α-tocopherol in the sample examined is slightly above the values usually found (values from 1 250 to 1 900 μg/g are given by Green *et al.*[3], Hove and Hove [10], Quaife [11], and Brown [1]), and since a *selective* destruction of β-tocopherol can hardly be expected to take place, it seems more probable that we are faced with individual variations in the tocopherol composition depending on the origin of the samples. This seems also to apply to cottonseed oil in the case of which Green *et al.*[3] find 58 % α-tocopherol (in the present investigation 36 %) and 42 % γ-tocopherol (in the present investigation 64 %).

Unlike previous researchers [3] the authors of the present work found no δ-tocopherol in mustard oil. It should be noted, however, that the present investigation has comprised one sample of mustard oil only.

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