Acta vet. scand. 1965, *6*, 101—108.

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A METHOD FOR DETERMINATION OF TOCOPHEROL IN BOVINE BLOOD

By

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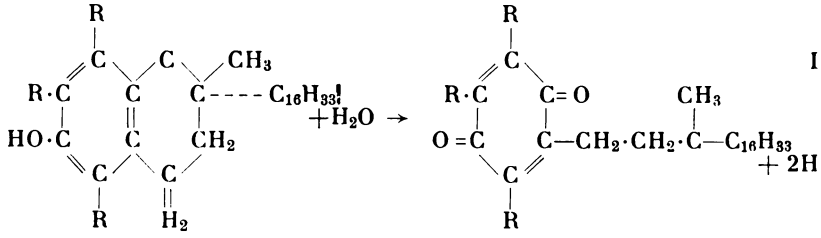
The determinations of the naturally occurring tocopherols, or Vitamin E, make as a rule use of their high reducing capacity. This reduction is also the most probable explanation of the biological effect of this vitamin. Being derivates of hydroquinone, the normal way of oxidation is:

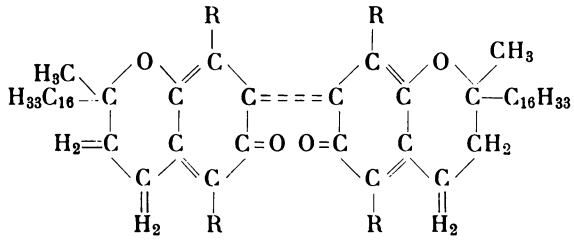


I

Tocopherol — Tocopherol quinone

(R = CH_3 or H)

This reaction is reversible. However active reducing agents, such as $LiAlH_4$, are necessary to bring the quinones back to the original chroman derivates. *Frampton, Skinner, Cambour & Bailey* (9) have isolated five products from methanolic solution of α-tocopherol treated with $FeCl_3$ at 50°C. On the alkaline oxidation with $K_3Fe(CN)_6$, *Csallany & Draper* (3) obtained the two isomers of di-α-tocopherone: The cis-di-α-tocopherone is:



II

On oxidation of two moles of α-tocopherol to this ortoquinone, two methyl groups are eliminated. The tocopherone cannot be reduced back to the original tocopherol. It follows from their findings on the analysis of these complicated oxidation reactions, which depend not only on the nature of the oxidant but also on the temperature and the solvent, that oxygen and oxidation catalysts must be eliminated as far as possible, not only during the extraction and purification but also during the transport of the blood samples.

The starting material for this investigation, venal blood, contains only 0,1 ml oxygen in 100 ml blood at 38°C. The water-insoluble tocopherols are associated with proteins and thus protected from rapid oxidation. The normal amount of the catalytic Fe^{3+}, 100 gamma/ml, may be increased at inconvenient sampling. Close contact of the sample with air is practically unavoidable at transporting the blood. Therefore rapid elimination of the iron catalyst is necessary. Hence a dilute solution of $K_4Fe(CN)_6$ must immediately be added to the sample.

After the sample has been taken, besides the destruction of tocopherol, other changes may occur. Thus the content of tocopherol may also increase, owing to splitting of tocopheryl-acetate. According to *Augustinsson* (1) the blood of all mammals contain arylesterase, that accelerates the splitting of aromatic acetates. Other kinds of such acetates than tocopheryl acetate occur seldom in normal blood. Activators for these esterases are ions of Ca, Ba and Sr. On the other hand the ions of heavy metals, especially ions of the rare earth-metals are inhibitors. If the calcium ions in the blood are precipitated by adding a solution of ammonium oxalate, that is saturated with La^{3+}-ions, the hydrolysis of the tocopheryl acetate will be sufficiently depressed. Then, after removing the blood corpuscles and treating the plasma with carbon dioxide, in order to eliminate the air from the vessels, stock solutions suitable for transport are obtained.

For the extraction of tocopherol from plasma, such solvents are often used that are known to dissolve considerable amounts of oxygen. According to *Fischer & Pfleiderer* (8), ethanol dissolves 14.3 ml, ether 41.5 ml, acetone 20.7 ml, and benzene 16.3 ml oxygen at room temperature and atmospheric pressure. Consequently, the risk for destruction of tocopherol will be great on the extraction with lipid solvents. The benzene and acetone used in this investigation were heated under boiling with reflux.

During the cooling argon was blown through the liquids. Shaking in separating funnels has been avoided. All the operations were performed under a layer of argon which is more convenient for this purpose than nitrogen, being 1.4 times denser than air. Extracts of tocopherol in oxygen-free benzene are stable for months, while dry extracts with free access to air and light are destroyed within a few days.

On this destruction the tocopherols vanish as rapidly as do the carotenes. The latter substances interfere also with solubility to the tocopherols. The carotenes, but not the tocopherols, give a characteristic extinction at 420 nm, a simple measurement of the colour density at this wave-length will not only give a good correction value for the carotene content but may also be used as a test for the efficiency of the extraction method. Hence it will serve as a control of the keeping of the samples on storage. Since the carotenes interfere in the living materials in the same way also, it is logical to raise the question whether determination of the tocopherol content without simultaneous annotation of the carotene content, including that of vitamin A, is useful for clinical purposes.

Benzene is the most suitable solvent for storing tocopherols. However extraction of blood or plasma with benzene will be incomplete due to the absorbtion of carotenes and tocopherols by the proteins of the plasma. The water layer on these proteins must first be replaced by an acetone layer. By gradual additions of an equal volume of acetone to the plasma, this exchange can be done without appreciable precipitation of proteins. In the subsequent addition of the same volume of benzene precipitation takes place. By centrifugation of the mixture, three layers are obtained. The top layer, that is coloured yellow by the carotenes, contains the tocopherols. The middle acetone-water-layer and the bottom layer, containing the precipitated proteins are again treated with benzene before being rejected. The collected benzene layers contain acetone and impurities that are soluble in this mixture. They are rinsed with water and dried with Na_2SO_4 sicc. No loss of carotenes and hence tocopherols occurs. The evaporation of benzene from these extracts should be done in an atmosphere of argon at a maximum-temperature of 40°C under reduced pressure. It has been found that up to one third of the carotenes are destroyed on evaporating at normal pressure and 80°C.

THE COLORIMETRIC METHOD

The principal part of the analysis of tocopherol is performed according to *Emmerie & Engel* (5, 6). Fe^{3+}-ions are reduced by tocopherol to Fe^{2+}-ions. These are incorporated into a complex with α-dipyridyl, $/Fe(dipyr.)_3/^{2+}$ and stabilized. This complex is red-coloured, with an extinction maximum at 520 nm. In the original Emmerie-Engel-method the sample and the reagents are dissolved in mixtures of ethanol and benzene. In dissolving $FeCl_3$ in ethanol its oxidation to aldehyde is initiated and further accelerated by illumination: unavoidable in colorimetric analysis. As the amount of tocopherol is determined by the Fe^{2+}-ions formed by the reduction, the presence of small amounts of other aldehydes of reducing potency will cause grave errors. There are many recommendations for eliminating these errors, such as redestilling the ethanol, measuring the colour at a fixed time after mixing, (discussed e.g. by *Feldheim*, 7) introducing a more effective complex-forming substance than the dipyridyl, proposed by *Canbäck & Wallenberg* (2), or buffering the reaction mixture to pH 4.1, introduced by *Domart* (4). We have tried to get away from this error by eliminating the aldehyde-forming substances. Tocopherols, $FeCl_3$ and α-α-dipyridyl may be dissolved in acetone-benzene mixtures. According to Formula I, water is necessary for oxidation of tocopherol to its quinone. A suitable mixture for developing the colour reaction has been found to be: 16 vol. of acetone, 4 of benzene, and 1 vol. of water. This solvent after mixing is also treated with argon in the described manner.

Already *Emmerie & Engel* found that carotenes interferred with tocopherols in reducing $FeCl_3$. Water is not necessary for this reaction. We have tried to differentiate between tocopherols and carotenes by using dry or wet solvents for colour reaction but without much success. The failure may be ascribed to the oxidation of tocopherols to tocopherones which reaction goes on in the absence of water (Formula II). We also found that careful exclusion of oxygen decreased the velocity of the development of colour at the Emmerie-Engel method to an extent that made the method unsuitable for analytical purposes. Thus the oxidation of tocopherols seem to be a rather complicated reaction. The extinction due to the reduction of the carotenes must be corrected for, by making dosage curves on pure β-carotene as well as on pure α-tocopherol. From the relation between the extinction of the original carotene solution in benzene at 420 nm and the

extinction at 520 nm of the same concentration of carotene, dissolved in acetone-benzene-water mixtures, which colour developed by $FeCl_3$ and α-α-dipyridyl, the correction factor is calculated. Three minutes after the mixing of the reagents the colour reaction is complete.

METHOD OF ANALYSIS

Immediately after pouring out the syringe, the stabilizer is added in an amount of 1 ml for 100 ml blood. The stabilizer is an aqueous solution that in 100 ml contains 4 mg $LaCl_3$, 4 mg $K_4/Fe(CN)_6/$ and 30 g $K_2C_2O_4$, dissolved in this order. After removing the blood corpuscles, the plasma is freed from air by blowing CO_2 into it. The samples are stored in refrigerator. They can be sent by mail without cooling.

For the analysis, 10.0 ml of this plasma is introduced in a centrifuge bottle. Only clear plasma is used. Protein precipitates due to careless handling are rejected. Magnetic stirring and argon blowing over the plasma surface are started before the addition drop by drop of 10 ml of acetone. Argon-saturated benzene is then added in the same way. The blowing and magnetic stirring are continued for further 10 minutes in order to keep the protein precipitate suspended. Centrifugation of this content of the bottle at 3000 r./min. for 10 to 15 minutes provides three sharply differentiated layers. The yellow top layer (about 20 ml) is pipetted into another centrifuge bottle. The remains of this layer are separated from the lower layers by washing them in the centrifuge with 10 ml benzene. The combined benzene — extract is washed thrice with 10 ml of aqua dest., dried with Na_2SO_4 sicc. and made up to 25 ml with benzene before measuring the extinction at 420 nm in a Klett-Summerson colorimeter. Presuming that the extinction is E_{42} colorimetric degrees, the correction for carotenes, including vitamin A, in a 10 ml sample is k.

$$k = 0.11 \cdot E_{42} \text{ degrees.} \qquad (1)$$

This benzene extract is taken to dryness at 40°C in a 100 ml test-tube, that has been filled with argon before exhaustion. The residue is dissolved in 9 ml of the solvent mixture (16 acetone + 4 benzene + 1 water). Then 0.5 ml of a 0.5 % α-α-dipyridyl-solution is added. On the following adding of 0.5 ml 0.1 % $FeCl_3$-solution (anhydrous $FeCl_3$, dissolved in a mixture of 4 vol. acetone + 1 vol. benzene), a stop-watch is started, and the solutions are thoroughly mixed by tipping the airated test-tube ten times. A sample of 4 ml is brought into the colorimeter cuvette and the extinction is measured at intervals of 4, 5, 6, 7 and 8 minutes after adding the $FeCl_3$-solution. If the mean value of these observations is E_{52} degrees, the amount of tocopherol in the 10 ml volume, or the concentration c, in the original plasma sample is

$$c = 0.85(E_{52} - 0.11 \cdot E_{42}) \text{gamma in 10 ml} = 8.5 \cdot 10^{-6}(E_{52} - 0.11 \cdot E_{42})\ \% \qquad (2)$$

EVALUATION AND CONTROL

Samples containing 0.2 ..... 40 gamma α-tocopherol pro ml were treated as described with $FeCl_3$ and α-α-dipyridyl. The extinctions were measured at 520 nm at intervals of 2, 3 ..... 7 minutes after addition of the iron chloride solution. In the concentration interval, c, of 4 to 40 gamma/ml, the extinction follows the formula:

$$I = c(11.7 \pm 0.2) \text{ col.units} \qquad (3)$$

By inversion of these figures, the factor 0.085 ± 0.002 gamma/ml col. unit is obtained. Within the limit of error, the colour of the preparations containing only α-tocopherol remains constant between 3 minutes and 2 hours. There is no sense in evaluating curves for the β-, γ-, ... tocopherol-solutions also since these substances are more reactive than α-tocopherol, their maximum extinction will be reached within 3 minutes.

Solutions containing carotenes will not attain a constant extinction within 10 minutes. The increment is about 1.8 % a minute and remarkably, the same for the colours of pure β-carotene-solutions and of plasma extracts. The degrees of extinction of the carotene-solutions are measured in the colorimeter using the filter for 420 nm, giving the value E_{42} col.units. If the Emmerie-Engel-method gives the extinction E_{52} col.units at 520 nm, the quotient E_{52}/E_{42} is found to be 0.42 ± 0.01 used in the correction. The magnitude of this correction is more than 20 %.

Table.

α-tocopherol added γ	Tocopherol found γ	Recovered γ	Tocopherol %	Correction for carotenes in γ tocopherol
0	38	0	—	27
50	85	47	95	26
99	133	96	96	36
149	180	143	96	30
0	36	0	—	30
40	73	31	94	37
79	122	87	109	32
119	170	135	113	30
0	30	0	—	22
50	83	53	106	20
99	128	98	98	18
149	182	152	102	21

For control, tocopherol in the form of stock solution in benzene was added in known amounts to the plasma immediately before the treating with acetone and benzene. The results are found in the table.

DISCUSSION

The method described here shall serve the veterinary routine. Hence it must be rapid, reproducible and simple. Separation of the carotenes and tocopherols by chromatography is not involved. Thus, the results from the analysis only show the actual amount of lipid-soluble Fe^{3+}-reducing substances in the blood plasma at the moment of sampling. In consideration of the interference of these substances not only at the analysis but also in vivo, a measure of the actual reducing capacity will give sufficient information for clinical purposes. The intentions of this investigation must be considered as attained: first to provide stabilization and extraction methods for tocopherol from plasma ensuring a practically complete recovery, and secondly to obtain a modification of the well known Emmerie-Engel analytic method giving extinction values stable for hours. If without such a stabilization destruction of these lipid-soluble reducing substances would occur more tocopherols would be destroyed than carotenes and more γ- than α-tocopherol. Thus, the proportions between the chromatographically separated substances would be disturbed and the analysis of less clinical value.

ACKNOWLEDGEMENTS

For making the survey of the immense litterature on this subject, and for the troublesome starting experiments I am much obliged to veterinary surgeon Evy Carlsson, Mariestad, I also wish to express my thanks to Miss Maarja Kippar and Mrs. Saga Elwe for technical assistance.

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SUMMARY

A salt solution for stabilization of the tocopherols in plasma is introduced. A lenient method for extraction of lipids of this kind is described. The method of colorimetric determination according to Emmerie and Engel is modified in using a mixture of acetone, benzene and water. These substances do not interfere with the reaction components, thus, giving extinction values constant for hours, and allowing exact determinations of tocopherols. Corrections for carotenes are to be done and annotated.

ZUSAMMENFASSUNG

Eine Methode zur Bestimmung von Tocopherol in Rinderblut.

Die E-Vitamine des Plasmas werden durch Zusatz einer Salzlösung geschützt. Eine schonende Extraktion dieser Stoffe nebst derartigen Lipiden ist beschrieben. Die Farbreaktion nach Emmerie und Engel ist insofern verändert dass sie in einer Mischung von Aceton, Benzen und Wasser ausgeführt wirt. Infolge der geringen Oxydier- und Reduzierbarkeit dieses Lösungsmittels werden die Extinktionswerte stundelang stabil. Für den Carotinoiden muss korrigiert werden, und die Grössen dieser Korrektionen angegeben.

SAMMANFATTNING

En bestämningsmetod för tokoferol i nötblod.

En saltlösning för stabilisering av tokoferoler i plasma har införts liksom en enkel och skonsam extraktionsmetod av dem och likartade lipider. Emmerie och Engels kolorimetriska bestämningsmetod har ändrats så, att den färgskapande reaktionen utförs i en blandning av aceton, bensen och vatten, vars komponenter varken oxideras eller reduceras av de däri lösta ämnena. Extinktionsvärdena blir därför stabila i timmar, vilket möjliggör noggranna bestämningar. För karotenerna måste korrektioner göras och anges.

(Received July 18, 1964).