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The Determination of α-Tocopherol in Animal Tissues by Column Chromatography

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A rapid method for the chromatographic separation and quantitative determination of small amounts of α-tocopherol in the unsaponifiable matter of animal tissue is described. The unsaponifiable material from 0.5—1.5 g of tissue, containing 1—20 μg of α-tocopherol, is put on a 0.5 × 5 cm column packed with a mixture of activated alumina, zinc carbonate and Celite. Twenty per cent benzene in *cyclo*hexane is passed through the column and eight fractions of 1 ml are collected. The Emmerie-Engel reaction is performed on each fraction. α-Tocopherol separates sharply in 3—4 fractions free of other reducing substances.

Although many procedures have been described for the determination of α-tocopherol, there appeared to be a need for a rapid method applicable to small quantities of animal tissues. Many methods which are suitable for materials with a relatively high content of α-tocopherol fail when applied to samples of animal origin. The molecular distillation [1,1a,2,14] of α-tocopherol, which is perhaps the most specific method currently used, requires preliminary removal of interfering materials; a specialized apparatus is also necessary. Paper chromatographic procedure [3–5], which separate some of the tocopherols in plant lipids, require a preliminary removal of sterols when extracts of animal unsaponifiable material with low tocopherol content are analyzed. The procedure described recently [6] which adapts previous paper chromatographic techniques to the analysis of animal tissues appears to require excessive amounts of mate-

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rial and also involves prolonged steps which greatly lengthen the time required for the analysis.

Column chromatographic methods which have been used for analyzing plant and feed materials [7,8] have not been adapted to small samples containing less than 20 μg of α-tocopherol. The use of floridin earth columns [2,9] for animal tissues in our experience is unsatisfactory because reducing substances other than α-tocopherol frequently appear in the eluate, especially when liver is analyzed [10]. Furthermore, it was felt that a method should be devised in which readily available adsorbents, not requiring special treatment, could be utilized.

Because of these various limitations in existing procedures, the present method was developed as an aid to studies of the metabolism and function of α-tocopherol. The procedure involves saponification of the sample, extraction of the unsaponifiable material and its chromatography on a column consisting of a mixture of alumina and zinc carbonate. The α-tocopherol is collected in the eluate free of other reducing substances and is quantitatively determined colorimetrically. Although the method has been used primarily for tissues from rats and chicks fed purified type diets, it should be applicable to other species under natural feeding conditions.

EXPERIMENTAL

Chemicals

1. Ethanol, 96 %, refluxed over KOH for 30 min and distilled.
2. Potassium hydroxide, 50 %. Dissolve 100 g of KOH in 100 ml water.
3. Hexane, purified. Although some commercial hexanes are pure enough after distillation, others have had to be washed with concentrated sulfuric acid, then with saturated potassium permanganate and finally distilled. *Cyclo*hexane may be used in place of hexane. This solvent from several sources has been found satisfactory without distillation.
4. Bipyridyl, 0.5 %, in ethanol.
5. Ferric chloride (hexahydrate), 0.2 %, in ethanol (kept in brown glass bottle).
6. Celite No. 545, John-Manville Corp., New York, N.Y. Celite Analytical Filter Aid also may be used.
7. Zinc carbonate, powdered, analytical reagent. Two brands which have been found to be satisfactory were: J. T. Baker Chemical Co., Phillipsburg, N. J., and May and Baker, Ltd., Dagenham, England (labelled approx. $ZnCO_3 - 2\ ZnO,3H_2O$). The product "Basic Zinc Carbonate" of Mallinkrodt Chemical Works, St. Louis, Mo., also was satisfactory but gave a very slow flow rate.
8. Activated alumina. Only alkaline aluminas have been found suitable. Merck & Co., Inc., Rahway, N. J. "Aluminum oxide, suitable for chromatography"; May and Baker, Ltd., "Aluminium Oxide, for chromatography". Aluminum Co. of America, Pittsburgh, Pa., "Activated Alumina F-20". All of these products gave satisfactory results. Several other activated aluminas gave recoveries of α-tocopherol below 90 %.
9. Pyrogallol, reagent grade.
10. Anhydrous sodium sulfate, reagent grade.
11. Benzene, reagent grade.

Apparatus

1. Chromatographic tubes. These are made from 19 cm lengths of glass tubing with an inside diameter of 4.5—5 mm. A constriction in the tube, to hold a cotton plug, is made about 5 cm from one end. A glass rod slightly smaller than the tube is used for tamping the column.
2. Glassware: 500 ml suction flasks, 50 and 100 ml Erlenmeyer flasks, separatory funnels.
3. Nitrogen gas.
4. A photoelectric colorimeter in which volumes of 3—4 ml of solution may be used. The Beckman model C colorimeter, the Coleman Jr. spectrophotometer, and the Klett-Summerson photoelectric colorimeter have been used. The Beckman DU spectrophotometer is also satisfactory.

PROCEDURE

Saponification and extraction of tissues. 0.5 to 3 g of tissue is homogenized in a tissue grinder with 5 to 10 ml of water and transferred to a 50 or 100 ml Erlenmeyer flask. To this are added an equal volume of ethanol, 50—100 mg pyrogallol and a few boiling stones. The flask is fitted with a reflux condensor (an air condensor is also satisfactory), placed on a hot plate or steam bath, and when the mixture is boiling, a volume of freshly boiled 50 % KOH which is equal to one-half the weight of the tissue is added through the condensor (very fatty tissues may require more). Gentle boiling is continued for 15—25 min, depending on the size and fat content of the sample.

An alternate procedure is to chop the tissue, preferably when frozen, into small pieces and saponify in 15—25 ml of ethanol without the addition of water.

When saponification is complete, the flask is cooled rapidly and the contents transferred to a separatory funnel, containing 10—15 ml hexane. If no water had been added previously, a volume equal to the saponified mixture is now added. This order of mixing reduces the tendency to form emulsions. The mixture is extracted by moderate shaking for one min; the extraction is repeated with 8 and 5 ml of hexane. The extract is washed five times with water, dried over anhydrous sodium sulfate, filtered or decanted and made to a convenient volume with hexane.

An aliquot of the extract equivalent to 0.5—1.5 g of tissue is evaporated to dryness in a conical centrifuge tube, using nitrogen and a warm water bath. Large volumes of solvent may be removed by a flash evaporator. The residue is dissolved in 0.05—0.1 ml of hexane and is ready for the column.

Chromatography. The three aluminas we have used required weakening by the addition of 4—12 % of water immediately before use. The actual amount needed is determined by observing the rate of migration of 10—50 μg of vitamin A alcohol on the mixed column. When 8—10 ml of the developing solvent (below) is passed through the column the vitamin A should move about 1 cm down the column. Usually, it will lie in a band 2—12 mm from the top. To check this, the column is sucked practically dry and the packing gently blown out by light air pressure. (A finger is held loosely over the end of the tube and compressed air applied at the bottom. When the column moves to the top of the tube, remove the air supply and blow on the end gently to push the packing out of the tube.) Antimony trichloride in chloroform (25 %) is then dropped onto the column from a pipet to locate the vitamin A.

To 0.3 g of alumina in a mortar is added the requisite volume of water (as indicated above) from a micro pipet and the two mixed by grinding gently with a pestle. 0.3 g of zinc carbonate and 0.15 g of Celite are added and mixed in. The mixed adsorbent must then be packed into the glass tube *within 10 minutes*, since on standing it becomes more strongly adsorptive. Once the column has been wet with hexane, it may stand for several hours.

The glass column, in a rubber stopper, is mounted on a suction flask connected to a water pump or to the house vacuum. A cotton or glass wool plug is inserted into the constriction in the tube and the adsorbent is added in portions of about 1.5 cm with the aid of a small funnel, using *very weak* suction. The top of the tube is tapped 2—3 times (not more) with a glass rod to facilitate settling of the adsorbent which is finally packed

firmly, but *lightly*, with the rod. The column is packed to a height of 5 cm; usually the adsorbent is put into the tube in 4—5 portions. A small amount of anhydrous sodium sulfate may be placed on top of the column.

Two or three ml of hexane are then passed through the column and the suction is increased until the flow rate is 1 ml per 2—2 1/2 min. With the vacuum off, the sample in 50—100 μl hexane is placed on the column using a micro pipet. Suction is reapplied and another 50—100 μl of hexane is used to rinse the sample tube. As soon as this has gone onto the column, 1 ml of the developing solvent, benzene in hexane (1:4 by volume), is added. The surface of the column is never permitted to become dry.

One ml fractions are collected in colorimeter tubes placed in the flask under the column. Seven to ten fractions are usually taken. As a check on the behavior of the column, after a liver sample has been run through, the column is extruded and treated with antimony trichloride. The vitamin A should be about one-half to two-thirds of the way down the column. If above or below this, then the column is too slow or too fast, and the water content of the alumina must be changed. This check is only necessary when a new batch of alumina is being used for the first time.

Colorimetric determination of α-tocopherol. The fractions are evaporated under nitrogen and the residue dissolved in 3 ml of ethanol. Then 0.2 ml of bipyridyl followed by 0.2 ml of ferric chloride are added and the density at 520 mμ determined 30 sec. after the addition of the ferric chloride. A room with artificial light should be used. The reagent blank is subtracted from the reading of the fractions containing α-tocopherol (see Table 1). Calculations are made from a standard curve employing pure α-tocopherol. These volumes may be varied depending on the instrument used. With the volumes indicated, the working range is 1—20 μg α-tocopherol.

To speed up the procedure, if the colorimeter tubes are marked at the 3 or 4 ml volume, the ethanol can be added to the mark without evaporating the solvent. This proportion of hexane-benzene solvent does not alter the Emmerie-Engel reaction.

COMMENTS AND RESULTS

Saponification. In agreement with other investigators [7,8,11], we have found α-tocopherol to be recovered quantitatively, as determined by the Emmerie-Engel reaction, after saponification if caution is used in the exclusion of air and pyrogallol is added as an antioxidant. When 5—60 μg of *dl*-α-tocopherol were saponified 20 min as described, recoveries were always above 93 %. However, we consistently found that with one source of potassium hydroxide the saponified tocopherol underwent a 10—15 % loss when put through the column. Since unsaponified tocopherol did not show this loss (below), it is apparent that the saponification procedure changed some of the tocopherol so that it was still reducing but would not pass through the column.

Packing the column. When properly packed, the α-tocopherol will come off sharply in two to four fractions. If the activity of the adsorbent is correct and not too much unsaponifiable matter is put on, the α-tocopherol should begin to appear in the 2nd, 3rd or 4th fractions. Two typical elution patterns are shown in Table 1. If the column is not packed uniformly the tocopherol will spread out over 5 or more fractions.

We have tried packing the column wet, *i.e.*, suspending the adsorbent in hexane and pouring the mixture into the tube. This procedure was found to give considerable variation in behavior of the column. Because of the different density and particle size of the three components in the adsorbent mixture, there was an uneven settling as the particles fell through the solvent.

Table 1. Typical elution patterns of unsaponified fractions from tissues.

Fraction *	Chick liver, 0.33 g			Rat muscle, 1 g		
	$A_{520m\mu}$	μg Tocopherol		$A_{520m\mu}$	μg Tocopherol	
1	0.059	—		0.062	—	
2	0.058	—		0.083	1.6	
3	0.063	—		0.143	6.0	10.5 μg/g
4	0.117	4.1	5.1 = 15.3 μg/g	0.101	2.9	
5	0.076	1.0		0.063	—	
6	0.062	—		0.064	—	
7	0.062	—		0.062	—	
8	0.058	—		0.058	—	
Reagent blank	0.062			0.062		

* Eluate fractions of 1 ml collected.

Recovery of α-tocopherol from the column. Samples of pure *dl*-α-tocopherol ranging from 8—30 μg were put through the column and the recovery in the eluate fractions determined. With the sources of alumina and zinc carbonate listed above, recoveries were from 94—99 % in fifteen tests. Other sources of activated aluminas gave recoveries from 76—85 %. When small amounts of α-tocopherol were added to the unsaponifiable material from rat or chick tissues which were either adequate or devoid of vitamin E, the recovery was quantitative (Table 2). If impure hexane is used, losses during chromatography can be appreciable.

When 15 to 60 μg of α-tocopherol were added to 0.5 g of vitamin E-free lard, or to 1.0 g of muscle from a vitamin E-depleted chick, and carried through the entire procedure, the recoveries were above 90 %.

Table 2. Recovery of α-tocopherol from the chromatographic column when added to tissue unsaponifiable fractions.

Expt.	Weight of tissue * (g)	Tocopherol present ** (μg)	Tocopherol added (μg)	Total tocopherol found (μg)	Recovery of added tocopherol (%)
1	0.5, rat liver	8.7	3.3	12.1	103
2	1.0, rat muscle	10.5	3.3	13.7	97
3	1.0, rat liver	0.0	14.6	13.1	90
4	1.0, rat liver	0.0	11.5	11.0	96
5	1.0, chick liver	0.0	14.6	13.9	95

* The hexane extract put onto the column was equivalent to this weight of tissue.
** Determined on a aliquot of the hexane extract.

Table 3. Comparison of column eluates with and without molecular distillation.

Tissue	Weight of tissue chromatographed (g)	α-Tocopherol content of tissue (μg/g)	
		Direct eluate	Distilled eluate
Rat A, liver	0.5	18.7	19.5
Rat B, testes	0.5	18.4	18.5
Rat B, muscle	1.0	10.5	9.4
Rat C, depot fat	0.5	8.8	7.8
Chick A, liver	0.5	18.7	19.4
Chick B, heart	0.5	26.6	23.3

Amount of unsaponifiable material put on the column. This has varied from less than 1 mg to 6 mg, representing the unsaponifiable matter from 0.4—1.5 g of tissue. No attempt was made to determine the upper limit of capacity. Before chromatographing the unsaponifiable matter from brain, it was necessary to desterolize the extract first by low-temperature freezing in methyl alcohol. The only interference encountered was from excessive vitamin A in livers from chicks dosed heavily with the vitamin. The column was found to be capable of holding 100 μg of vitamin A. If more than this amount is present in liver samples, a larger column can be used. It is desirable to use as small a sample as possible since better separation of the α-tocopherol is achieved. Thus, if the tocopherol content of a tissue exceeds 30 μg/g, a sample of 0.25—0.5 g is sufficient for the chromatography.

Separation of other unsaponifiable materials. β-Carotene passes through the column rapidly and appears in the first fraction. The only other substance which comes through in eight fractions is ubiquinone (coenzyme Q). This migrates at about the same rate as α-tocopherol but with the amounts present in tissues no significant color is given in the Emmerie-Engel Reaction. β-, δ- and γ-tocopherols stay on the column as do vitamin A and cholesterol. Reduced ubiquinone, prepared with $LiAlH_4$, is strongly adsorbed on the top of the column. Ubichromenol[13] did not come through the column even after 12 ml of developing solvent were used.

The reducing material in *Torula* yeast, which also occurs in the livers of animals fed the yeast, and which passes readily through Florex columns prepared with stannous chloride, was strongly adsorbed on the alumina-zinc carbonate column and did not appear after 16 ml of developing solvent were passed through.

Molecular distillation of the eluate. In order to determine if substances which might either enhance or inhibit the Emmerie-Engel reaction were coming through the column, eluates of a variety of tissues were molecularly distilled in the apparatus of Glavind, Heslet and Prange[14]. For this study, duplicate aliquots of tissue extracts were chromatographed and the Emmerie-Engel reaction run on the eluate fractions from one sample. The corresponding 3 or 4 fractions containing vitamin E, of the duplicate sample, were combined and

distilled for one hour at 100°C and 10^{-4} mm and the tocopherol content of the distillate determined. The results, shown in Table 3, indicate that no significant amount of interfering material comes through the column. As mentioned above, ubiquinone comes off the column with the α-tocopherol. We have found, as reported by Pudelkiewicz and Matterson [12], that ubiquinone does not distill under these conditions. (Reduced ubiquinone and ubichromenol similarly did not distill). The data in Table 3 provide evidence that ubiquinone in the amounts present normally in tissues does not interfere in the Emmerie-Engel reaction.

Analysis of tissues from vitamin E-deficient animals. Further evidence that the column effectively separates α-tocopherol from other Emmerie-Engel reducing substances was provided by analyzing tissues from young rats and chicks which had been fed purified diets, devoid of α-tocopherol, for 5—8 weeks. When the unsaponifiable matter from 1—2 g of tissue was analyzed there was no reducing material in the eluate fractions where α-tocopherol should appear. If the vitamin was added to such samples prior to chromatography quantitative recovery was achieved (Table 2). In several vitamin E-depleted rat and chick liver samples of one g, a small amount of reducing material, equivalent to 1—3 μg of tocopherol, came off the column in three fractions immediately after those containing the added α-tocopherol.

Application of the method. The following rat tissues have been analyzed satisfactorily: liver, kidney, spleen, heart, testes, lung, intestine, muscle and depot fat. When brain was analyzed, it was necessary to remove sterols prior to chromatography, as indicated above. Liver, heart and muscle from chicks also have been analyzed.

In the early development of the method, a column of adsorbent 1 × 6 cm was used with tissue samples from 1 to 3 g. Eluate fractions of 2—3 ml were collected. Results were similar to those found with the smaller column. Presumably, even larger columns could be adapted for isolation work or for problems involving interest in unsaponifiable constituents other than α-tocopherol. The eluate fractions may also be used for determining the ubiquinone (coenzyme Q) content of the sample. The solvent is evaporated, the residue dissolved in 3 ml ethanol, and the adsorbancy at 275 mμ before and after reduction with KBH_4 determined [15]. If the tocopherol content of the sample is sufficiently high, 6 ml of ethanol may be added to the colorimeter tubes; 3 ml are then removed for ubiquinone estimation while the Emmerie-Engel reaction is performed on the remaining 3 ml.

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