COLUMN CHROMATOGRAPHY IN THE DETERMINATION OF TOCOPHEROL: FLORISIL, SILICIC ACID, AND SECONDARY MAGNESIUM PHOSPHATE*

MARTHA W. DICKS-BUSHNELL**
Division of Biochemistry, University of Wyoming, Laramie, Wyo. 82070 (U.S.A.)
(Received September 9th, 1966)

Chemical determinations of tocopherols in biological tissues were initiated twenty-five years ago using columns of Florisil[1,2] prior to colorimetric analysis with $FeCl_3$–dipyridyl. However, eluates from these Florisil columns contained both vitamin A and cholesterol as well as tocopherols when benzene was the solvent. Recently Florisil columns eluted with hexane–benzene (9:1) have been used to qualitatively separate tocopherol from extracts of cigarette smoke[3]. Column chromatography with alumina[4,5] and secondary magnesium phosphate[6,7] has been undertaken using mixtures of ethyl ether with petroleum ether for separation of the tocopherols partially from each other and from carotenoids, vitamin A, sterols, and reducing substances for the purpose of analysis. Qualitative separation of the tocopherols from other lipid components using silicic acid columns eluted with Skellysolve B–ethyl ether (99:1)[8] or hexane–benzene (9:1)[9], and quantitative determination of tocopherol in milk using purification with columns of silicic acid eluted with benzene[10] have been reported.

The purpose of the work reported here was to compare methods of separation of the tocopherols from interfering substances and purification of biological materials for tocopherol analysis using column chromatography. Work was started using $MgHPO_4$. However, because of difficulty of purification of $MgHPO_4$ sufficient for column chromatography of tocopherol, studies were undertaken using Florisil or silicic acid. The present communication includes evidence for the need for hydrating Florisil to 20 % water for quantitative elution of the tocopherols and the separation of the tocopherols into 3 groups and from interfering substances using columns of Florisil eluted with mixtures of Skellysolve F with acetone, benzene, or ethyl ether.

EXPERIMENTAL

Procedures

A weighed sample of Florisil from the Floridin Company was hydrated by pipeting water onto it in a glass stoppered jar. After mixing and letting set for at least 1 h, the hydrated Florisil was poured with Skellysolve F into a 12 mm I.D. chromatography tube plugged with glass wool. The Florisil was then stirred with a glass rod and allowed to settle. The sample (see below) to be analyzed was dissolved in Skelly-

* Published with approval of the Director, Wyoming Agricultural Experiment Station, as Journal Paper No. 298.
** Present address 502 Ord Drive, Boulder, Colo. 80302.

solve F, pipeted or washed onto the column, and eluted with measured amounts of Skellysolve F or mixtures of Skellysolve F with ethyl ether, benzene, or acetone.

Columns containing 10 g of Florisil and mixed tocopherol concentrate were washed with 50 ml of Skellysolve F and then three groups of tocopherols were eluted with 100 ml of Skellysolve F–ethyl ether (199:1) to elute α-tocopherol, next with 100 ml of Skellysolve F–ethyl ether (99:1) to elute β- and γ-tocopherols, and last with 100 ml of Skellysolve F–ethyl ether (975:25) to elute δ-tocopherol. Total tocopherol was obtained by eluting other columns with 150 ml Skellysolve F–ethyl ether (975:25).

Silicic acid was washed and prepared to the dried form by three different methods independently as recommended for chromatography of lipids by HERNANDEZ *et al.*[11], HIRSCH AND AHRENS[12], and LIS *et al.*[13]. Then columns of silicic acid were prepared by two methods. One method was that recommended by HIRSCH AND AHRENS[12]. The second method was by hydrating the washed[11–13] and dried silicic acid to Brockmann activity grade III (15 % water)[11] by pipeting water onto weighed-out silicic acid in a glass stoppered bottle and then mixing. After 1 h, 10 g of the hydrated silicic acid was poured with Skellysolve F into a 12 mm I.D. chromatography tube plugged with glass wool. The silicic acid was stirred with a glass rod and allowed to settle in the column. A sample (see below) dissolved in Skellysolve F was pipeted or poured on to the column. The column was then washed with 100 ml Skellysolve F. Then total tocopherol was eluted with 150 ml of Skellysolve F–ethyl ether (98:2).

Secondary magnesium phosphate trihydrate was activated, dried, checked for activity with α-tocopherol, and used for chromatography of the tocopherols as described by others[6,7]. Low recoveries of tocopherol using columns of $MgHPO_4$ were initially obtained. With study it became evident that the Buchner-style, table-type funnel used for washing during activation of the $MgHPO_4$ did not allow for as complete washing and evacuation of water as the regular Buchner funnel used for previous studies[14]. Perhaps an increase in the number of washings would increase the purity of the activated $MgHPO_4$ with resultant increase in recovery of tocopherol from the salt. However, increasing the number of washes was not tried. Instead the salt was deactivated by the addition of water.

Tocopherols and the reducing coenzyme Q-type compounds that were eluted from columns with the tocopherols were determined in ethanol using ferric chloride–dipyridyl and reading in an Evelyn colorimeter with a 515 mμ filter[14]. The tocopherol equivalents of reducing coenzyme Q were estimated from a standard curve obtained for α-tocopherol in order to ascertain the degree of interference of coenzyme Q_6 and Q_{10}, with and without saponification, in the procedures studied.

Paper chromatography was used to check some samples eluted from certain columns or to separate the tocopherols in the mixed concentrate. The papers were either one-dimensional with paraffin infiltration[15] and development in 95 % methanol[16], or two-dimensional with the first phase zinc carbonate impregnation[15] and development in mixtures of either 3 % acetone[16] or 20 % benzene in Skellysolve B followed by paraffin infiltration and development in 95 % methanol.

Reagents and samples

Purification of Skellysolve F or petroleum ether, 30–60°, was executed by setting about 2 l of one of these crude solvents over about 1 l of concentrated sulfuric acid[17] in a bottle for at least two weeks and shaking a few seconds each day. Then the solvent

was extracted with about 500 ml of 10 % sodium carbonate solution, separated, distilled, and the first 5 % distillate and last 5 % residue discarded.

Purification of ethyl ether was accomplished by exposing Baker's analyzed reagent grade anhydrous product to about 100 g of Norit A with about 100 g of potassium hydroxide pellets in a 2 l distillation flask and distilling when needed.

Distilled Baker's analyzed reagent acetone was used. Benzene was the Baker's analyzed reagent grade.

Samples of *d*- or *dl*-α-, *d*-β-, *d*-γ-, and *d*-δ-tocopherols, B-carotene, vitamin A, vitamin K, rat liver extracts, and extracts of cereals and baby formulas were studied. The *dl*-β-tocopherylphenylazobenzoate and the 5,7-dimethyltocopherylphenylazobenzoate kindly supplied by Dr. J. GREEN, Walton Oaks Experimental Station, were also studied, as was a mixed tocopherol concentrate N. F. Type 4-34 kindly supplied by Dr. S. AMES, Distillation Products Industries. Also studied were coenzyme Q_6 from the California Corporation for Biochemical Research and coenzyme Q_{10} kindly supplied by Dr. K. FOLKERS, Merck Sharp and Dohme Research Laboratory.

Ethanol extracts of the cereal products and baby formulas were saponified in the presence of ascorbic acid, re-extracted into Skellysolve B, chromatographed through 20 g columns of Florisil and the tocopherols separated by two-dimensional paper chromatography. Rat liver tissues were extracted using chloroform–methanol (2:1), evaporated, saponified in the presence of pyrogallol[15], and chromatographed on 10 g columns of Florisil using 100 ml of Skellysolve F–ethyl ether (99:1) to elute the tocopherols, excepting δ-tocopherol.

RESULTS AND DISCUSSION

Florisil

The recovery of α-tocopherol was greater than 90 % when the 60/200 mesh Florisil was hydrated to 20 % water (see Table I). Similar results were obtained using 60/100 and 100/200 mesh Florisil. Recoveries of five tocopherols (α, β, γ, δ and ζ_2) from 20 % hydrated Florisil were on the average 95 %, except for β-tocopherol with only 90 % recoveries. When the amount of Florisil was increased to 20 g, the amount of eluting solvent had to be increased by about a third and the mixture of ethyl ether in Skellysolve F had to be raised from 2.5 to 3.5 % in order to elute similarly large amounts of all tocopherols. If these increases in amount of eluting solvent and concentration of ethyl ether were not carried out, δ-tocopherol would be left on the columns. Equal success was achieved using 125 ml of Skellysolve F–acetone (199:1 or 99:1).

The elution behavior of the tocopherols from columns of Florisil are presented in Table II. From a 10 g column of Florisil, α-tocopherol was eluted by 100 ml of mixtures of 6 % benzene or 0.5 % ethyl ether in Skellysolve F, γ- and β-tocopherols were eluted by 100 ml of mixtures of 10 % benzene or 1 % ethyl ether in Skellysolve F, and δ-tocopherol was eluted with 100 ml of mixtures of 15 % benzene or 2.5 % ethyl ether in Skellysolve F. Hence it was possible to separate the tocopherols in the mixed concentrate into the α, β and γ, and δ fractions (see Table III) using columns of Florisil and obtain results similar to those using two-dimensional paper chromatography or columns of $MgHPO_4$.

As coenzyme Q compounds are altered by saponification[18] and Florex-chroma-

TABLE I

RECOVERY OF TOCOPHEROLS FROM COLUMNS OF FLORISIL*

Eluant: Skellysolve F containing ethyl ether.

Water (%)	*Column wt.* (g)	*Eluant*		*Tocopherol*	*Recovery*** (%)
		Amount (ml)	*Per cent ethyl ether*		
0.2***	10	150	2	α	0
15	10	150	2	α	72
20	10	150	2	α	90–100 (5)
20	20	200	3.5	α	98
20	10	150	2	ζ_2	92–100 (4)
20	10	150	2.5	d-β	87–91 (5)
20	20	200	3.5	d-β	93
20	10	150	2.5	d-γ	92–101 (9)
20	20	200	3.5	d-γ	93–98 (3)
20	10	150	2.5	d-δ	90–100 (9)

* Data reported with 60/200 mesh Florisil, but similar results obtained with 60/100 and 100/200 mesh Florisil from Floridin Company, Tallahassee, Fla.

** Numbers in parentheses are numbers of columns run.

*** Moisture initially present in Florisil.

TABLE II

SEPARATION OF THE TOCOPHEROLS ON COLUMNS OF FLORISIL*

Eluant: Skellysolve F containing various solvents.

Mesh size	*Fraction number*	*Eluant*		*Tocopherol*	*Recovery* (%)
		Amount (ml)	*Per cent solvent*		
60/200	—	125	0.5% acetone	α	101
	—	125	1% ethyl ether		92
	—	125	6% benzene		94
60/200	First	50	Skellysolve F	α	0
	Second	100	0.5% ethyl ether		93
120/200	First	100	0.5% ethyl ether	dl-β	2
	Second		1%		89
	Third		2%		0
60/200	First	100	0.5% ethyl ether	d-γ	3
	Second		1%		88
	Third		2.5%		3
60/200	First	100	0.5% ethyl ether	d-δ	1
	Second		1%		3
	Third		2%		76

* All columns with 10 g Florisil hydrated with 20% water.

tography[19] and hence may interfere in analyses for the tocopherols, two were tested in these procedures. Coenzyme Q_6 and Q_{10} were saponified and chromatographed on Florisil columns. Large microgram quantities of saponified coenzyme Q_6 and Q_{10} were found to interfere (Table IV).

TABLE IV

INTERFERENCE OF COENZYME Q, WITH OR WITHOUT SAPONIFICATION, IN DETERMINATION OF TOCOPHEROL AFTER CHROMATOGRAPHY ON COLUMNS OF FLORISIL, SILICIC ACID, OR $MgHPO_4$

Eluant: Skellysolve F containing ethyl ether or Skellysolve F alone.

Adsorbent	*Coenzyme Q*		*Fraction number*	*Eluant*		*Interference in tocopherol equivalents***
	Treatment before chromatography	*Weight (mg)**		*Amount (ml)*	*Per cent ethyl ether*	
Florisil	Coenzyme Q_{10} saponified acidified	0.32	First	100	0	0.000
			Second	125	2.0	0.037
			Third	100	4.0	0.066
Florisil	Coenzyme Q_6	2.48	First	100	0.5	0.001
			Second	100	1.0	0.001
			Third	100	2.5	0.004
Florisil	Coenzyme Q_6 saponified ascorbic acid	1.10	First	100	0.5	0.000
			Second	100	1.0	0.032
			Third	100	2.5	0.004
Florisil	Coenzyme Q_6 saponified pyrogallol	3.44	First	100	0.5	0.000
			Second	100	1.0	0.013
			Third	100	2.5	0.007
Silicic acid	Coenzyme Q_{10} saponified acidified	1.30	First	100	0	0.000
			Second	150	2.0	0.128
			Third	100	4.0	0.052
$MgHPO_4$	α-tocopherol	0.102	—	250	2.0	0.091
$MgHPO_4$	Coenzyme Q_{10} saponified acidified	1.60	First	100	0	0.001
			Second	250	2.0	0.158
			Third	250	7.0	0.170
$MgHPO_4$	Coenzyme Q_{10}	1.80	First	100	0	0.002
			Second	250	2.0	0.000
			Third	250	7.0	0.020
$MgHPO_4$	α-tocopherol	0.093	—	250	2.0	0.088
$MgHPO_4$	Coenzyme Q_6 saponified pyrogallol	3.44	First	150	0	0.000
			Second	250	2.0	0.002
			Third	250	7.0	0.077
$MgHPO_4$	Coenzyme Q_6 saponified ascorbic acid	1.10	First	50	0	0.000
			Second	250	2.0	0.032
			Third	250	7.0	0.025
$MgHPO_4$	Coenzyme Q_6	2.48	First	50	0	0.000
			Second	250	2.0	0.001
			Third	250	7.0	0.044

* Weight of coenzyme Q in mg, as is or altered by saponification, on column of adsorbent.

** Amount of interference detected calculated using the calibration curve obtained for α-tocopherol to give tocopherol equivalents in mg.

TABLE V

ANALYSES OF RAT LIVER EXTRACTS FOR TOCOPHEROLS

Livers from rats fed:	*mg tocopherol/100 g liver*		
	*Florisil**	*$MgHPO_4$***	*Silicic acid**
Tocopherol-free diet	0.0	0.1	0.1
20 mg α-tocopherol/100 g diet	1.0	1.1	0.8

* 10 g columns eluted with 100 ml Skellysolve F–ethyl ether (99:1). Large amounts of non-tocopherol, reducing substances eluted from both livers with 100 ml of Skellysolve F–ethyl ether (98:2).

** 20 cm columns eluted with 250 ml Skellysolve F–ethyl ether (98:2). Non-tocopherol, reducing substances eluted from both livers subsequently using 250 ml of Skellysolve F–ethyl ether (96:4).

TABLE III

SEPARATION OF TOCOPHEROLS IN A MIXED CONCENTRATE SAMPLE

Tocopherol	*Per cent of total tocopherol*			*Eluant for Florisil column***
	Paper	*$MgHPO_4$**	*Florisil*	
α	49–53	49–56	49	100 ml 6% benzene
β and γ	39–42	39–43	42	100 ml 10% benzene
δ	6–10	4–10	9	100 ml 15% benzene

* α-, β- and γ-, and δ-tocopherol fractions eluted by 250 ml of mixtures of 2%, 4%, and 7% ethyl ether in Skellysolve F added consecutively. Moisture in $MgHPO_4$ was 1–2.9% in 7 columns run.

** Column of 10 g of 60/200 mesh Florisil.

DEVLIN AND MATTILL[1] reported that vitamin A, carotenoids, and sterols were not retained when Florisil was eluted with benzene. However, in the studies presented here when 10 g columns of hydrated Florisil were eluted using 150 ml of Skellysolve F–ethyl ether (975:25) no interference from vitamin A or vitamin K added to these columns was detected, while 9% of a sample of 100 μg of β-carotene was eluted. Hence liver samples only from animals fed no carotenoids were analyzed. Recovery of α-tocopherol from a column of Florisil also containing rat liver extract was 106%. Livers (Table V) from rats fed a tocopherol-deficient diet showed 0.0 mg tocopherol/100 g, while livers from rats fed supplements of 20 mg α-tocopherol/100 g diet showed 1.0 mg/100 g. When these Florisil columns were additionally washed using 100 ml of Skellysolve F–ethyl ether (975:25), large amounts of non-tocopherol, reducing substances were eluted. Similar results were obtained when the same liver samples were analyzed using columns of either $MgHPO_4$ or silicic acid. As sterols would also probably be adsorbed while tocopherols were eluted from Florisil columns[1,20,21] by the method proposed here, Florisil was studied as a purification adsorbent for saponified extracts of cereals and infant formulas prior to two-dimensional paper chromatography and found to allow satisfactory separation and purification of the tocopherols.

Silicic acid

When silicic acid prepared by the 3 methods of washing[11–13] and the respective columns of these dry adsorbents were eluted with Skellysolve F–ethyl ether (98:2), recoveries of tocopherol were zero. Columns of silicic acid prepared by the method of HIRSCH AND AHRENS[12] allowed only about 50% recovery of α-tocopherol using elution with Skellysolve F–ethyl ether (98:2). However, when these washed and dried silicic acid preparations were hydrated to 15% water and the 10 g columns eluted using 150 ml of Skellysolve F–ethyl ether (98:2), recoveries of α-tocopherol were 94–100%. No attempts were made to study the recovery of the other 4 tocopherols from this adsorbent nor were studies undertaken to see if the tocopherols could be separated on this adsorbent.

Other investigators[8,9,22] studying the separation of α-tocopherol on columns of silicic acid did not report the percentage recoveries obtained, while CSALLANY AND DRAPER[23] report only 57% recoveries of α-tocopherol from columns of silicic acid–Celite (2:1) eluted with Skellysolve F–ethyl ether (98:2).

However, ERICKSON AND DUNKLEY[10] obtained 95–100% recoveries of tocophe-

rol from columns of dry silicic acid using elution with benzene, but the resulting eluates would be expected from the work of others[8,12,13] to contain more interfering substances than in the method used here. However, when saponified extracts of cereals were purified on columns of silicic acid, the separation of the tocopherols from other contaminants by two-dimensional paper chromatography was not satisfactory.

Secondary magnesium phosphate

There was considerable variability in the recovery of α-tocopherol (see Table VI) from $MgHPO_4$ studied after activation by washing and drying. Secondary magnesium phosphate always gave lower recoveries of α-tocopherol when washed on the table-type, Buchner funnel than when washed on the regular Buchner funnel (Table VI). If the activated $MgHPO_4$ did not give a good recovery of α-tocopherol, this preparation was deactivated to 2 % water taking into consideration the moisture already absorbed. It was found that $MgHPO_4$ readily absorbed moisture even in a room with relative humidity less than 50 %. Too much moisture in the $MgHPO_4$ resulted in some elution of α-tocopherol when the columns were washed with 100 ml of Skellysolve F–ethyl ether (199:1). Hence these preparations of $MgHPO_4$ would not allow proper purification or separation of the tocopherols.

TABLE VI

RECOVERY OF α-TOCOPHEROL FROM DIFFERENT LOT NUMBERS OF $MgHPO_4$

Eluant: (A) Skellysolve F containing 0.5 % ethyl ether; (B) Skellysolve F containing 2 % ethyl ether.

Lot no.	*Per cent recovery of α-tocopherol*		
	Eluant A (100 ml)	*Eluant B (250 ml)*	*Moisture in ground salt (%)*
Buchner funnel–regular type			
1	0	71	0.9*
	0	97	1.8**
2	0, 0	97, 100	0.3, 0.4*
	17	84	2.0**
3	0	89	0.3*
	0	96	2.0–2.3**
*Buchner funnel–table type****			
1	0	52	0.8*
	0	90	2.0**
	0	93	2.8**
2	0	23	—
	0	1	0.5*
3	0	43	0.5*
	68	22	3.4**

* Moisture absorbed by $MgHPO_4$ during grinding, sieving, and setting in room in tightly closed jar.

** Sum of moisture added to $MgHPO_4$ plus that picked up during preparation*.

*** These three are lot numbers of $MgHPO_4$ from Amend Drug and Chemical Company.

Large microgram amounts of coenzyme Q_6 or Q_{10} (Table IV) were found to interfere in the analyses for α- and the other tocopherols, if these coenzymes were saponified before chromatography on columns of $MgHPO_4$, but only in determinations of total tocopherol by this method if not saponified.

SUMMARY

It has been shown that Florisil should be hydrated to 20% water to allow complete recovery of the tocopherols in column chromatography using eluting solvent of ethyl ether in Skellysolve F. Column chromatography with Florisil allows separation of the tocopherols into three groups (α, β and γ, and δ) using eluting solvents of ethyl ether or benzene in Skellysolve F. Saponified extracts of rat livers were analyzed directly for α-tocopherols after column chromatography with Florisil. However, total tocopherol could not be determined because large amounts of non-tocopherol, reducing substances were eluted from the columns along with δ-tocopherol.

It was found that silicic acid had to be hydrated to Brockmann activity grade III (15% water) to ensure good recoveries of α-tocopherol using elution of the columns with Skellysolve F–ethyl ether (98:2).

It has been shown that there is difficulty in washing the $MgHPO_4$ during activation and that moisture can be used to deactivate activated $MgHPO_4$ to allow complete recovery of α-tocopherol.

Large microgram amounts of coenzyme Q, changed by saponification into reducing compounds, were found to be partially eluted with tocopherol from columns of Florisil, silicic acid, or $MgHPO_4$. Also reducing coenzyme Q-type-compounds may be formed on columns of $MgHPO_4$ and eluted with the non-α-tocopherols.

REFERENCES

1 H. B. DEVLIN AND H. A. MATTILL, *J. Biol. Chem.*, 146 (1942) 123.
2 L. R. HINES AND H. A. MATTILL, *J. Biol. Chem.*, 149 (1943) 549.
3 A. RODGMAN AND L. C. COOK, *Tobacco Sci.*, 4 (1960) 7.
4 R. A. MORTON AND N. I. FAHMY, *Biochem. J.*, 72 (1959) 99.
5 A. EMMERIE, *Ann. N.Y. Acad. Sci.*, 52 (1949) 309.
6 F. BRO-RASMUSSEN AND W. HJARDE, *Acta Chem. Scand.*, 11 (1957) 34.
7 F. BRO-RASMUSSEN AND W. HJARDE, *Acta Chem. Scand.*, 11 (1957) 44.
8 M. F. SORRELS AND R. REISER, *J. Am. Oil Chemists' Soc.*, 34 (1957) 131.
9 R. L. ROWLAND, *J. Am. Chem. Soc.*, 80 (1958) 6130.
10 D. R. ERICKSON AND W. L. DUNKLEY, *Anal. Chem.*, 36 (1964) 1055.
11 R. HERNANDEZ, R. HERNANDEZ, JR. AND L. R. AXELROD, *Anal. Chem.*, 33 (1961) 370.
12 J. HIRSCH AND E. H. AHRENS, *J. Biol. Chem.*, 233 (1958) 311.
13 E. W. LIS, J. TINOCO AND R. OKEY, *Anal. Biochem.*, 2 (1961) 100.
14 M. W. DICKS AND L. D. MATTERSON, *J. Nutr.*, 75 (1961) 165.
15 ANALYTICAL METHODS COMMITTEE, *Analyst*, 84 (1959) 356.
16 V. H. BOOTH, *Chromatog. Rev.*, 7 (1965) 98.
17 W. R. BLOOR, *J. Biol. Chem.*, 77 (1928) 53.
18 H. H. DRAPER AND A. S. CSALLANY, *Biochem. Biophys. Res. Commun.*, 2 (1960) 307.
19 W. J. PUDELKIEWICZ AND L. D. MATTERSON, *J. Biol. Chem.*, 235 (1960) 496.
20 K. K. CARROLL, *J. Lipid Res.*, 2 (1961) 135.
21 J. EISNER AND D. FIRESTONE, *J. Assoc. Offic. Agr. Chemists*, 46 (1963) 542.
22 A. KUKIS AND J. M. R. BEVERIDGE, *J. Lipid Res.*, 1 (1960) 311.
23 A. S. CSALLANY AND H. H. DRAPER, *Arch. Biochem. Biophys.*, 92 (1961) 462.