THIN-LAYER CHROMATOGRAPHY WITH PRECOATED ALUMINA SHEETS

II. APPLICATION TO TOCOPHEROLS*

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INTRODUCTION

BAITSHOLTS AND ARDELL[1] have described general properties of precoated alumina sheets**. To illustrate the usefulness of these sheets, we describe below their application to the specific separation of α-tocopherol (vitamin E) from other tocopherols and non-saponifiable lipids in various biological materials.

EXPERIMENTAL

Samples were chosen to represent a variety of physical forms which might be encountered in both the plant and animal kingdoms. When possible, samples containing tri-, di- and monomethyl tocopherols were given preference to provide a better test of the procedure. In addition, we chose linseed oil because previous analyses[2] had shown an unidentified reducing material and alfalfa because this has consistently been a difficult material to analyze.

Oils were used as obtained. Individual and mixed plant foods were extracted with ethanol as described by QUAIFE AND HARRIS[3]. Colostrum, milk and the simulated milks (after dilution with water) were extracted by the Mojonnier method with the omission of NH_4OH. Liver fat was extracted from the homogenized tissue with Skellysolve F–ethanol[4]. Mesenteric fat was ground with granular anhydrous Na_2SO_4 and was extracted repeatedly with chloroform.

Saponification of the lipid extract and chromatography of a portion of the non-saponifiable fraction on $MgHPO_4$ were carried out as described previously[2]. In this study, however, we eluted the γ-, β- and δ-tocopherols simultaneously with 6 % diethyl ether in petroleum ether (b.p. 35–60°)*** and then monitored the eluates by chromatography on silica gel Chromagram sheets (K301R)[5] using a solvent system of petroleum ether (b.p. 60–71°)****–diethyl ether–acetic acid (90:10:1). Individual tocopherols were eluted from the sheets with ethanol.

Another portion of the non-saponifiable fraction was evaporated under N_2 to about 100 μl, streaked (2–3 cm) on unactivated alumina Chromagram sheets and

chromatographed in dim light in either a conventional chamber or a sandwich chamber using a solvent system of benzene–diethyl ether (50:50). It is reasonable to assume that under variable laboratory conditions, activation of the sheets for 30 min at 100° and slight adjustments in solvent polarity may be desirable for consistent and adequate performance.

For many of the samples, we also spotted portions of the non-saponifiable fraction on glass plates coated with Mannal TLC Alumina, Basic. The alumina Chromagram sheets were at least as good as, and usually superior to, the glass plates for resolution of the tocopherols and discreteness of the spots.

After chromatography, the sheets or plates were dried in the dark and sprayed lightly with a 0.004 % solution of 2′,7′-dichlorofluorescein (Eastman Organic Chemicals 373) in ethanol. When the tocopherol spots were *completely* dry (about 5 min), they were located with a 2537 Å (short wave) light. Under these conditions, R_F values for α-, γ- and/or β-, and δ-tocopherols were approximately 0.50–0.55, 0.35–0.40 and 0.20–0.25, respectively. The spots were circled with pencil, cut out with scissors and eluted with two successive 4 ml aliquots of ethanol. A blank was prepared in the same manner by eluting an unused area of the sheet. Colorimetric measurement of tocopherol with 2,2′-bipyridine–FeCl₃ reagents has been described[2]. TSEN's[6] substitution of 4,7-diphenyl-1,10-phenanthroline (bathophenanthroline) for 2,2′-bipyridine is particularly useful for small amounts of tocopherol.

Values obtained for the various tocopherols were corrected for losses observed during chromatography of known amounts of pure tocopherols either alone or added to tocopherol-free lipids previously obtained by preparative TLC on silica gel plates. Average recoveries for d-α-tocopherol (39 trials ranging from 3.2 to 32.4 μg), d-γ-tocopherol (40 trials ranging from 2.6 to 52.8 μg) and d-δ-tocopherol (15 trials ranging from 3.9 to 31.0 μg) were 90.0, 94.9 and 94.7 %, respectively. With ethanol as the eluting solvent, recoveries declined appreciably for amounts higher than the maximums cited.

RESULTS AND DISCUSSION

Analyses of 21 samples of diverse biological origins are given in Table I. With several scattered exceptions, results obtained by the two analytical methods showed good agreement. The absence of a method with verified accuracy for comparative purposes makes impossible any conclusion about which of these methods is "correct" or "more correct". For practical purposes, however, results obtained with alumina sheets and with MgHPO₄ are equally satisfactory; and the alumina sheets were both faster and more readily available than suitable MgHPO₄.

Details have not been given for 8 other samples of bovine milk and colostrum which were also analyzed by both methods. Equally good correlation was observed, however, on α-tocopherol concentrations ranging from 7 to 46 μg/g fat, with an average of 20 μg/g fat on MgHPO₄ and an average of 23 μg/g fat on alumina sheets.

The results with linseed oil and alfalfa illustrate a problem which may arise with the alumina sheets. Each of these materials contained an unidentified reducing substance(s) which chromatographed like α-tocopherol under the described conditions. The presence of these substances was observed during column chromatography when they were eluted with the non-α-tocopherol fraction (6 % diethyl ether) but sub-

TABLE I

ANALYSES OF BIOLOGICAL SAMPLES BY CHROMATOGRAPHY ON $MgHPO_4$ AND ON ALUMINA CHROMAGRAM SHEETS

Sample	Lipid content (%)	Concentration (mg/lb.)					
		α-Tocopherol		γ-Tocopherol		δ-Tocopherol	
		$MgHPO_4$	Alumina	$MgHPO_4$	Alumina	$MgHPO_4$	Alumina
Wheat	1.9	5.2	5.4	10.3[a]	9.1[a]	0	0
Corn	4.3	3.8	3.7	19.1	17.0	0	0
Corn	4.1	2.6	2.8	12.3	12.0	0	0
Alfalfa	5.0	7.8	8.5[b]	0[c]	0	0	0
Broiler feed	7.2	8.3	8.8	10.7	11.2	0	0
Layer feed	5.6	5.9	6.5	16.1[d]	16.0[d]	2.1	1.6
Cereal food	6.8	10.7	11.2	23.4[e]	17.3[d]	—	3.8
Cereal food	6.8	7.6	8.5	3.7[e]	4.6[d]	—	2.1
		Concentration (μg/g lipid)					
Soybean oil (refined)	(100)	83	106	706	717	118	110
Palm oil (crude)	(100)	405	406	271	274	0	0
Tung oil	(100)	174	192	647	600	34	54
Sesame oil (refined)	(100)	12	11	182	180	0	0
Linseed oil (raw)	(100)	0	0[f]	364[g]	375	31	31
Simulated milk	3.4[h]	208	217	372	358	0	0
Simulated milk	3.9[h]	55	58	310	287	0	0
Bovine colostrum	0.9	26	22	0	0	0	0
Bovine milk	3.1	14	17	1	0	0	0
Rat adipose tissue	(95)	42	39	11	11	0	0
Rat adipose tissue	(95)	54	50	18	13	0	0
		Concentration (mg/g lipid)					
Rat liver	3.8	19.5	21.0	0.06	0	0	0
Rat liver	4.0	49.5	47.1	0.06	0	0	0

[a] Assumed to be β-tocopherol but calculated as γ-tocopherol.

[b] Two-dimensional chromatography showed an unknown reducing material equivalent to 21.7 mg α-tocopherol/lb., which behaved like α-tocopherol in the first dimension but which separated from α-tocopherol in the second dimension.

[c] Also contained an unknown reducing material which behaved like α-tocopherol on alumina Chromagram sheets and was equivalent to 37.5 mg α-tocopherol/lb.

[d] May contain some β-tocopherol but entire fraction calculated as γ-tocopherol.

[e] γ- (and β- if present) and δ-tocopherols were not separated and were all calculated as γ-tocopherol.

[f] Two-dimensional chromatography showed an unknown reducing material equivalent to 184 μg α-tocopherol/g lipid, which behaved like α-tocopherol in the first dimension but which separated from α-tocopherol in the second dimension.

[g] Also contained an unknown reducing material which behaved like α-tocopherol on silica gel Chromagram sheets and was equivalent to 175 μg α-tocopherol/g lipid.

[h] Based on "normal" dilution.

sequently behaved like α-tocopherol during monitoring of the column eluates on silica gel precoated sheets. (The behavior of the unknown substance in this sample of linseed oil thus differed from that in a previous sample[2] when it was eluted with α-tocopherol in the 2 % diethyl ether fraction but was separated from it by two-dimensional paper chromatography. We do not know whether the substances are different or whether the $MgHPO_4$ behaved differently.)

These unknown substances were separated from α-tocopherol by two-dimensional chromatography on alumina sheets using benzene–diethyl ether (50:50) for the first dimension and benzene–chloroform (85:15) for the second dimension. Reverse-phase chromatography was unsuccessful and impractical for this purpose. Single-dimensional chromatography with benzene–chloroform (75:25) separated added α-tocopherol from the unknown substance in linseed oil but was not successful with alfalfa. Unfortunately, further increases in polarity of this system resulted in increasingly diffuse spots. If single-dimensional chromatography on alumina sheets with benzene–diethyl ether (50:50) were the sole method employed, both of these unknown substances would have been measured as α-tocopherol. Furthermore, even two-dimensional chromatography would have been unsatisfactory for measuring γ- and δ-tocopherols in alfalfa, had they been present, due to interference from other lipids.

Our customary method for analysis of plasma tocopherol entails chromatography of *whole* plasma lipids on silica gel Chromagram sheets[7]. Alumina Chromagram sheets were not as satisfactory for this purpose, primarily because the tocopherol bands were not as sharply defined. With *saponified* blood lipids, however, and with the samples in Table I, alumina sheets were superior to silica gel sheets for tocopherol analyses.

It should be emphasized that simple separation of tocopherols on thin layers of alumina is not novel. SEHER[8] reported this at least as early as 1961. Our work demonstrates, however, that tocopherols from biologic materials can be resolved satisfactorily on alumina Chromagram sheets and that the resolutions are usually superior to those obtained on glass plates coated with alumina.

SUMMARY

Tocopherols in biologic materials were isolated by extraction, saponification and chromatography of the non-saponifiable lipids on Eastman alumina Chromagram sheets. Resolutions were usually superior to those obtained on glass plates coated with alumina. Results obtained with 21 samples compared favorably with those obtained by column chromatography on $MgHPO_4$; and the alumina sheets were both faster and more readily available than suitable $MgHPO_4$.

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