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THE SEPARATION AND DETERMINATION OF α-TOCOPHEROL AND CAROTENOIDS IN SERUM OR PLASMA BY PAPER CHROMATOGRAPHY

R. F. BAYFIELD, R. H. FALK AND J. D. BARRETT
Veterinary Research Station, Department of Agriculture, Glenfield, New South Wales, 2167 (Australia)

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SUMMARY

A simple and relatively rapid method is described which avoids preliminary treatments in the determination of the lipid pattern, and in the quantitative analysis of constituents in human plasma and animal serum by two-dimensional paper chromatography.

Carotenoids were resolved into at least four components which were well separated from two other prominent lipid constituents, α-tocopherol and a vitamin A derivative.

Serum levels of β-carotene and α-tocopherol varied greatly between animals and were evidently related to the current diet.

The ratio of β-carotene to total carotenoids in human plasma was different to that in the serum of mature cattle but tocopherol levels were similar.

INTRODUCTION

In recent years a number of reports have appeared concerning the estimation of β-carotene, vitamin A and vitamin E in serum derived from humans[1–4] and animals[5].

Most of the analytical procedures have been based on a simple extraction of the lipids with hexane or similar solvent, after precipitation of proteins by ethanol, followed by further appropriate treatment.

LEITNER *et al.*[1] determined total carotenoids in light petroleum by measurement of their yellow colour. Vitamin E was then determined colorimetrically on the same extract after precipitation of sterols, a correction factor being applied for the contribution of carotenoids.

HANSEN's micro method[4] depended on a fluorescence measurement of a hexane extract of serum, while the method of BIERI[2] involved a thin-layer chromatographic separation of a hexane extract, after preliminary saponification of the whole serum.

Apart from saponification, other methods employed for the clarification of vitamin E extracts of biological materials have included hydrogenation for plasma[6], freezing techniques and column chromatography for tissues[7].

In an attempt to eliminate some of these "clean-up" complexities, HASHIM AND SCHUTTRINGER[3] recently proposed a rapid method for the determination of plasma tocopherols, in which the principal interfering substance, β-carotene, was estimated, and a more sensitive ferrous photophore used for measurement of tocopherol.

In the present report, advantage is taken of earlier experience with the separation and estimation of carotene, tocopherol and other lipids in animal faeces[8]. This method, based on that of BOOTH[9] for plant materials, has now been extended to the determination and examination of α-tocopherol, β-carotene and other carotenoids in animal serum and human plasma. In the proposed method the serum extract is chromatographed directly on paper. It is a simple, relatively rapid technique, which is free of any complex or time-consuming procedures for preliminary removal of interfering substances.

MATERIALS AND METHODS

Extraction of lipids

Following the practice of earlier workers, lipids were extracted with a hydrocarbon solvent, after precipitation of proteins. To duplicate samples of serum (5 ml)*, contained in a stoppered measuring cylinder (25 ml) was added an equal volume of ethanol (5 ml), and the mixture gently but thoroughly shaken 100 times.

Light petroleum (b.p. 60–80°), (10 ml), was added and the mixture further shaken 100 times. After separation of the layers, the cylinder was again shaken 100 times and allowed to settle.

When it was necessary to chromatograph more than 3 ml of the light petroleum extract, equivalent to 1.5 ml serum, the appropriate aliquot was pipetted into a small, round-bottomed flask and evaporated under reduced pressure on a warm water bath.

Chromatography of the extract

The light petroleum extract, 3 ml or less, in 1 ml portions for convenience, was carefully applied to the starting area, 7 cm × 3 cm of the square chromatography paper, prepared as previously described[8]. When larger volumes of the extract were evaporated, the residue was redissolved in three 1 ml portions of redistilled acetone and applied to the paper.

Development was carried out by standing the papers in simple rectangular specimen jars containing light petroleum (40–60°)–acetone (99:1)** for the first dimension. The papers were then quickly examined under U.V. light and positions of the various components outlined in pencil to ensure that they would be avoided during subsequent dipping of the unused portion of the paper in paraffin (3% in light petroleum (40–60°)). The paraffin-treated papers were then developed in the second dimension, using aqueous methanol (90%) as the mobile phase.

The air-dried papers were again examined under U.V. light for detection and marking of α-tocopherol and other fluorescing or absorbing spots.

α-Tocopherol was estimated with the Emmerie–Engel reagents after leaching the spot, and a corresponding blank area, with ethanol.

* The amount of serum is varied according to the expected carotene and tocopherol content.

** This concentration of acetone was found to be critical for serum.

β-Carotene was eluted from the paper with light petroleum (60–80°), preferably after the first dimension, and the concentration determined from its absorption at 450 mμ.

Where possible, other coloured or fluorescing spots were cut out, eluted with appropriate solvents and spectral data determined on a Beckman DU spectrophotometer.

Generally, identification procedures for the major constituents were similar to those previously described[8].

Total carotenoids and recoveries

Total carotenoids were determined on the excess light petroleum extract of some serum samples, both animal and human, by measuring the optical density at 450 mμ and calculating the result as β-carotene. This provided the opportunity to compare the ratio of β-carotene to total carotenoids in animal and human sera, as well as serving as a partial check on the recovery of β-carotene in animal serum.

Recoveries of pure β-carotene and α-tocopherol from paper chromatograms were determined under the same conditions as for serum.

Applications of the method

Animal serum. To test the procedure on serum from different classes of stock it was applied to samples from the following groups of experimental animals:

(i) Calves maintained on a mixture of hay and grain for six months, sampled twice at an interval of seven days.
(ii) Mature cows fed a similar mixture but with limited green pickings available.
(iii) Mature cows grazing good green pasture.
(iv) Merino lambs, aged six months, maintained on a mixture of hay and grain for four months.
(v) Mature sheep four and five weeks after changing from the hay and grain mixture to green pasture.

Samples of serum were stored at −20° while awaiting analysis for β-carotene and α-tocopherol.

Serum from six cows, two from each of three herds, grazing green pasture, was used to determine the ratio of β-carotene to total carotenoids in mature cattle.

Human plasma. Human plasma was prepared from heparinised blood, taken from six apparently-healthy, normal males. Precautions were taken to protect blood and plasma from light. The blood was treated within one hour of collection, and plasma stored at −20° for no longer than one week pending analysis.

RESULTS

The location of various serum constituents after chromatography of a light petroleum extract is illustrated in Fig. 1, the dotted areas representing positions occupied in the first dimension.

Table I indicates most of the serum components common to animal serum and human plasma, which were separated and detected by the chromatographic method. Positive identification[8] was restricted to the more prominent spots. Weaker spots,

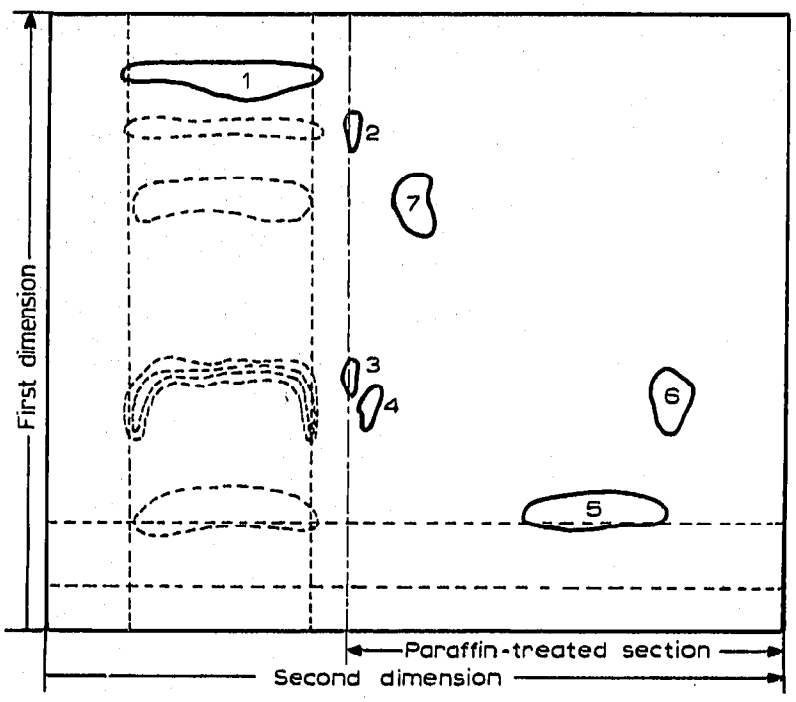

Fig. 1. Two-dimensional paper chromatogram of light petroleum extract of animal serum showing location of some components. (1) β-Carotene; (2,3,4) other carotenoids; (5) xanthophyll; (6) vitamin A derivative; (7) α-tocopherol.

TABLE I

COMPONENTS OF ANIMAL SERUM AND HUMAN PLASMA

Spot No. (see Fig. 1)	*Colour of spot*	*Effect of U.V. light*	*Solvent*	*Absorption maxima (mμ)*	*Identification*
1	Orange-yellow	Very weakly absorbing	Light petroleum (60–80°)	478, 450, 425	β-Carotene
			Ethanol	478, 452, 425, 340, 270	
2	Yellow	No effect	Chloroform	No definite peaks	A carotenoid (α-Carotene?)
3	Pale pink	Absorbing	Ethanol	No definite peaks	A carotenoid
4	Orange	Absorbing	Ethanol	475, 450, 285 270	A carotenoid (Cryptoxanthin?,
5	Yellow	Weakly absorbing	Ethanol	475, 445, 425, 265	Xanthophyll
6	Colourless	Yellow fluorescent	Ethanol	280–275	Vitamin A derivative (5, 8 epoxide?)
7	Colourless	Absorbing	Ethanol	292–291	α-Tocopherol
Spectral properties of direct extract of serum			Light petroleum (60–80°)	475, 450, 425	
			Ethanol	480, 452, 425, 340, 270	

such as 2 and 3, although distinctly visible, were present in such small amounts that even when spots from several papers were combined, absorption curves were too indefinite to indicate identity.

Tentative identification of spots 2, 3 and 4 as carotenoids, and spot 6 as a vitamin A derivative, was supported by the blue colour formed when these spots were treated with antimony trichloride.

Recoveries and total carotenoids

Recovery figures for pure β-carotene and α-tocopherol from paper chromatograms were generally of the order of 90%—for example, α-tocopherol 89.1%, 90.2%, and β-carotene 89.5%, 91.2%, 93.8%.

In two experiments, when both lipids were chromatographed together, the recovery of β-carotene improved in one instance from 86% to 91%, and in the other from 88% to 92%.

TABLE II

CAROTENOID AND TOCOPHEROL LEVELS IN HUMAN PLASMA

(Results are duplicate determinations on 2.5 ml plasma)

Subject	*Carotenoids*				*Tocopherol*	
	Spot size (μg)	*β-Carotene (μg/100 ml)*	*Total (μg/100 ml)*	*% β-Carotene of total*	*Spot size (μg)*	*α-Tocopherol (μg/100 ml)*
W.S.T.	0.55	22	66	33.3	13.5	540
	0.53	21	63	33.3	12.5	500
R.F.B.	0.82	33	97	34.0	13.0	520
	0.86	34	98	34.6	14.0	560
J.O.E.	1.06	43	99	43.4	21.5	860
	1.16	47	102	46.0	22.0	880
F.P.L.	1.06	43	132	32.5	16.5	660
	0.99	40	137	29.1	17.5	700
J.D.B.	1.12	45	163	27.6	26.0	1,040
	1.14	45	167	26.9	27.0	1,080
J.B.S.	1.23	49	181	27.0	20.5	820
	1.35	54	185	29.1	22.0	880

The good duplication obtainable by the method is clearly illustrated in the results given for human plasma (Table II).

The proportion of β-carotene in total carotenoids obtained for each of six cows was 84%, 87%, 86%, 90%, 85%, 92%, each figure being the mean of duplicate determinations.

Concentration in serum and plasma

The variation of serum β-carotene and α-tocopherol levels in animals on different diets is shown in Table III. The values range from zero in sheep, through very low in

calves, to a maximum of 3,200 μg/100 ml for β-carotene and 1,725 μg/100 ml for α-tocopherol in cows.

TABLE III

β-CAROTENE AND α-TOCOPHEROL LEVELS IN SERUM FROM CALVES, COWS AND SHEEP ON DIFFERENT DIETS

(All results are the means of duplicate determinations)

Animals	*No.*	*β-Carotene* (μg/100 ml)*		*α-Tocopherol* (μg/100 ml)*		*Diet*
		(a)	*(b)*	*(a)*	*(b)*	
Calves *(i)*	727	3.3	10.5	50	88	Chaff and grain
	728	8.4	6.6	100	70	
	733	2.5	4.5	80	65	
	734	1.2	11.6	30	64	
	730	5.1	9.4	60	64	
	732	0.8	11.4	40	92	
	729	4.8	0.8	33	30	
	731	4.8	2.8	50	—	
Cows *(ii)*	487	672		1,040		Dry feed with green pickings
	324	632		1,400		
	153	648		1,725		
(iii)	1	1,390		1,400		Good quality grazing grass
	5	1,280		1,330		
	8	3,200		1,530		
Sheep *(iv)*	225	N.D.**		N.D.		Dry feed (oaten chaff-grain)
	231	N.D.				
	224	N.D.		16		
	228	N.D.		33		
	227	N.D.		40		
	229	N.D.		65		
(v)	232	N.D.	N.D.	133	124	Fresh kikuyu
	226	N.D.	N.D.	152	158	Grass for 4–5 weeks

* Samples under (b) taken seven days after samples under (a).
** Not detected.

Results for the analysis of human plasma are shown in Table II. Mean total carotenoid levels ranged from 65 μg/100 ml–183 μg/100 ml, β-carotene from 22 μg/100 ml–52 μg/100 ml and the proportion of β-carotene in total carotenoids from 27.3%–44.7%. α-Tocopherol levels varied from 520 μg/100 ml–1,060 μg/100 ml.

DISCUSSION

The chromatographic method described reduced the risk of decomposition of lipids and other constituents by eliminating time-consuming preliminary steps such

as saponification. Using the method, several different components of serum or plasma were displayed on the one chromatogram. Because of this it was possible to observe more clearly the constituents contributing to the total carotenoid figure. Adequate separation was obtained by the direct application of the serum extract to the paper, and this allowed the convenient measurement of the more prominent spots such as β-carotene and α-tocopherol.

The measurement of individual carotenoids in plants, and their expression as a percentage of the total carotenoids, has recently been reported by BOND[10]. Earlier LEITNER *et al.*[11] determined the approximate proportion of β-carotene to xanthophyll, after separation by column chromatography, and regarded the sum of these components as total carotenoids.

In the method now described β-carotene was separated by paper chromatography and expressed as a percentage of total carotenoids, determined directly on the light petroleum extract. However, examination of the completed two-dimensional chromatogram (Fig. 1) revealed the presence of at least three other yellow spots and one pale pink area. Of these, probably the xanthophyll (spot 5) was most prominent, but from its intensity on the paper and its spectral properties (Table I) it is considered that another component (spot 4), probably cryptoxanthin, would also make an important contribution to the total carotenoid figure. The presence of cryptoxanthin in the serum of cows has also been reported by GILLAM AND EL RIDI[12].

Although satisfactory separation of β-carotene from other carotenoids can be achieved by the method, complete isolation of β-carotene from all other lipid constituents is not claimed. In fact it appeared that β-carotene was superimposed on another colourless, whitish-fluorescent constituent, possibly a steroid. This, however, did not appear to affect the estimation of β-carotene and, in order to ensure this, optical density readings were generally taken over the range 440 mμ–460 mμ to verify that the major peak occurred at 450 mμ, at which wavelength the concentration of carotene was determined. Full absorption curves on the β-carotene band were carried out on several occasions and these always coincided with published results or with our own previous findings for plants and animal faeces[8]. Absorption curves on the direct extract from cattle serum showed little or no variation from the curve for isolated β-carotene, but the true composition of the extract was revealed by chromatography as discussed above.

The intense yellow-fluorescent substance (spot 6) in the lower right hand corner of the chromatogram is apparently of vitamin A origin. The absorption curve in absolute ethanol (Table I) suggests an epoxide of vitamin A[13].

KAHAN[14] has recently described the thin-layer chromatographic separation of fluorescent vitamin A metabolites in human serum and discussed the possibility of the formation of decomposition products during analysis. Because of the extremely labile nature of vitamin A this possibility cannot be ignored. In the present case, however, it is considered unlikely that the yellow-fluorescent spot obtained from animal serum or human plasma is a decomposition product of vitamin A. When an extract of sheep liver was chromatographed under conditions identical with those used for serum or plasma, a yellow-fluorescent spot of similar location was obtained, the spectral characteristics of which were the same as those for authentic vitamin A alcohol (λ max. 324 mμ in ethanol).

Two-dimensional chromatographic systems are useful in eliminating uncertainty

about the presence of tocopherol isomers. The appearance of U.V.-absorbing areas above and below α-tocopherol in the first dimension on some of our chromatograms suggested the presence of such isomers. However, their failure to migrate past the paraffin line in the second dimension ruled out this possibility. In fact, the only tocopherol derivative we have observed with certainty in the biological materials so far examined is α-tocopherylquinone[8].

Recovery of pure α-tocopherol was lower than expected—recovery of 95% has been reported[15] for a similar technique used for plant analysis.

Although recoveries of β-carotene were satisfactory, it appeared that they were improved by about 4% in the presence of α-tocopherol. This suggests that α-tocopherol has a protective effect on β-carotene, as observed previously[8]. This effect is probably also operating during the analysis of normal serum.

Since the total carotenoids were determined on the untreated light petroleum extract of the serum, the carotene value obtained represented the maximum result, assuming complete extraction. Thus when the proportion of β-carotene was close to 90% the recovery of β-carotene from the paper chromatogram could be regarded as relatively high.

It is clear from Table II that the optimum spot size for α-tocopherol (10 μg) could be achieved by using a smaller amount of human plasma. However, a reduction in plasma volume would have an undesirable effect on the estimation of β-carotene by a corresponding reduction in its band size.

Spots of 10–12 μg of α-tocopherol are readily detectable, but spots containing 3–4 μg can also be found with a little more difficulty. The limit of detection would appear to be about 1 μg which agrees with other observations[16].

The measurement of β-carotene is probably most accurately and conveniently carried out when the carotene band contains about 10 μg and is eluted in 5 ml light petroleum. Although bands containing 1 μg of β-carotene are clearly detectable, it is preferable to aim for larger amounts to obtain the highest degree of accuracy.

In contrast to human plasma, the estimation of β-carotene and α-tocopherol in serum from mature cattle presents no serious problems, since 1 ml of serum gives spots of both lipids of about 10 μg, which are ideal for measurement.

The method seemed satisfactory when applied to serum and plasma of different origins. Thus, β-carotene values for calves on dry feed were low, values were higher in cows with limited access to green feed, and the highest values were obtained in cows grazing good quality pasture. These levels would be expected from diets which increased in β-carotene progressively. Similar trends were apparent for serum tocopherol.

Differences between human plasma and mature cattle serum in the ratio of β-carotene to total carotenoids were clearly revealed by the method.

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