DETERMINATION OF LIPIDS IN BIOLOGICAL MATERIALS BY PAPER CHROMATOGRAPHY

R. F. BAYFIELD, J. D. BARRETT AND R. H. FALK
Veterinary Research Station, Department of Agriculture, Glenfield, N.S.W. (Australia)
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INTRODUCTION

Although column[1,2] and thin-layer[3] chromatography have been used for the analysis of plant and animal products for lipid and provitamin components, the direct application of paper chromatography to untreated extracts does not appear to have been used extensively. In techniques reported, preliminary removal of interfering substances incurs the risk of losses, and involves time-consuming procedures unsuitable for routine examination of large numbers of samples.

Coloured pigments in marine algae have been separated by paper chromatography[4] and a two-dimensional paper chromatographic method has been employed for the analysis of tocopherols in foods and oils[5] and also in liver and faeces[6].

More recently, the separation of plant leaf pigments by paper chromatography has been described by BOOTH[7], and LICHTENTHALER[8] has developed systems for more rapid examination of plant materials.

Included in the current programme of research in this laboratory are studies on muscular dystrophy in grazing animals, and the definition of vitamin A requirements under drought conditions. For these purposes, more accurate and reliable techniques have been sought for the routine determination of vitamin A, β-carotene and the tocopherols in plant and animal products.

A technique employing two-dimensional paper chromatography, similar in principle to an earlier method for plants[7], but including minor modifications, has now been applied directly to extracts of sheep feeds and faeces without any preliminary treatment.

This technique has been extended to the quantitative measurement of α-tocopherol and β-carotene in these materials, and also in fresh pastures used by experimental animals.

Rates of decay of β-carotene and α-tocopherol in samples of pasture have also been determined by this method.

MATERIALS AND METHODS

Extraction of samples

Duplicate samples of freshly-collected feed or faeces, of about 0.3 g, were accurately weighed without previous drying and mixed with 1 g silica gel, 50–100 mesh chromatographic grade.

The prepared samples were then thoroughly stirred with several 5 ml portions of acetone–light petroleum (40–60°), 1:1, until no more colour was extracted. Usually a total of 50 ml of solvent was sufficient.

Each washing was decanted through a small pledget of cotton wool into a suitable flask. The solvent was evaporated under reduced pressure on a warm water bath, thereby eliminating the recommended drip-wash procedure for removing the acetone[7].

Paper chromatography

Chromatography papers, Whatman No. 4, were impregnated with zinc carbonate and sodium fluorescein, as recommended by the Analytical Methods Committee[9], and then cut to form a square, of side 28 cm.

The papers were drawn up to contain a starting area, 7 cm × 3 cm in the lower left-hand corner, 3 cm from the edge and 2 cm from the bottom.

The dried extract of feed or faeces was redissolved in the minimum of acetone, three 1 ml portions usually being sufficient, and applied to the starting area with the aid of a current of warm air.

Edges of the paper were stapled or pinned to form a cylinder, which was allowed to stand in a jar containing light petroleum (40–60°)–acetone (98.5:1.5) for development in the first dimension (75–90 min).

After impregnating the unused portion of the paper with medicinal paraffin (3 % in light petroleum) it was developed in a similar manner in the second dimension, using 90 % aqueous methanol (2 ½ h).

Chromatographic equipment consisted of rectangular specimen jars, 30 cm high and having a base 15 cm by 7.5 cm or 20 cm by 7.5 cm. These allowed rapid development and required only small quantities of developing solvents. Chromatography was complete in 4 h without the need to equilibrate papers or to line the developing tanks with papers saturated with solvent.

Papers impregnated with zinc carbonate were conveniently stored vertically in a sealed cylindrical jar containing silica gel desiccant.

Detection and estimation of lipids

Coloured pigments, such as the chlorophylls and carotenoids, were detected in the feed and faecal extracts by their characteristic appearance and their location on the chromatogram. Colourless substances, such as α-tocopherol, α-tocopherylquinone and other fluorescing materials, were detected by viewing the chromatogram under ultraviolet light. The more prominent constituents were identified by removing them from the chromatogram, eluting with appropriate solvents (Table I) and determining the absorption curve on a Beckman spectrophotometer, model DU.

Quantitative estimation of β-carotene was made from its absorbance at 452 mμ, in light petroleum, using appropriate conversion factors[10].

Quantitative estimation of α-tocopherol was carried out by the recommended colorimetric method[9]. Spots of α-tocopherol were cut out and eluted with 4 ml of ethanol to which was added 0.5 ml each of α,α′-dipyridyl (0.5 % w/v in ethanol) and ferric chloride (0.2 % w/v in ethanol). Optical density was read at 520 mμ after two minutes and concentrations were derived from a standard graph based on pure α-tocopherol.

TABLE I

ABSORPTION MAXIMA OF CONSTITUENTS SEPARATED FROM SHEEP FEED (KIKUYU GRASS) AND SHEEP FAECES BY PAPER CHROMATOGRAPHY

Constituent	*Solvent*	*Absorption maxima* ($m\mu$)	
		Green feed	*Sheep faeces*
Chlorophyll 1	Acetone	410, 420, 520, 570, 610, 660	410, 425, 530, 570, 610, 655
Chlorophyll 2	Acetone	420, 520, 570, 610, 660	410, 425, 530, 570, 610, 660
Chlorophyll 3	Acetone	410, 425, 500, 520, 570, 610, 660	410, 425, 480, 510, 530, 570, 610, 655
Chlorophyll 4	Acetone	430, 455, 600, 645	430, 455, 595, 645
Chlorophyll 5	Chloroform	420, 520, 540, 610, 660	420, 520, 560, 610, 655
Chlorophyll 6	Acetone	410, 425, 510, 530, 570, 610, 660	410, 425, 510, 530, 570, 610, 660
Xanthophyll	Ethanol	425, 445, 470	420, 440, 470
β-Carotene	Light petroleum	420, 452, 480	420, 452, 480
α-Tocopherol	Ethanol	290–292	290–292
α-Tocopheryl-quinone*	Ethanol	261, 268	262, 268

* Detected in green feed several hours after collection.

Materials examined

The basic analytical procedures described above were established using feed and faeces collected from sheep grazing Kikuyu grass (*Pennisetum clandestinum* Hochst.).

Subsequently, these methods were applied to feed and faecal samples collected from two merino lambs, maintained since birth on a dry ration based on lucerne hay (*Medicago sativa* L.) and also when the diet was later changed to fresh green Kikuyu grass.

Pastures grown specifically for the maintenance of experimental animal colonies were examined for their tocopherol and carotene contents.

The rates of decomposition of α-tocopherol and β-carotene were determined simultaneously in Kikuyu grass, stored in the laboratory at room temperature, sampled and analysed at weekly intervals.

RESULTS

Qualitative studies

Separation and detection of lipids. Chromatographic patterns obtained for extracts of Kikuyu grass and for extracts of sheep faeces from this diet were similar, with differences only in the concentrations of some constituents. A chromatogram, illustrating the separation of lipid constituents in extracts of sheep faeces from animals on green feed, is shown in Fig. 1. This chromatogram is also typical of that obtained for extracts of green feed and other samples of fresh pasture (see Table III).

Six green or yellow-green components were clearly separated on the chromatograms, and their absorption maxima corresponded with spectral data for various chlorophylls and closely related substances published elsewhere[4].

A pronounced orange-yellow band of β-carotene, isolated from feed and faeces, was located at the solvent front in the first dimension. The absorption spectrum in

light petroleum showed two principal maxima at 452 mμ and 480 mμ, in agreement with published data[4,10].

A yellow, xanthophyll-like spot and many other coloured and fluorescing spots were also noted in the feed and faecal extracts. Some of these appeared to correspond with carotenoid and quinone-like substances described in plants[7,8].

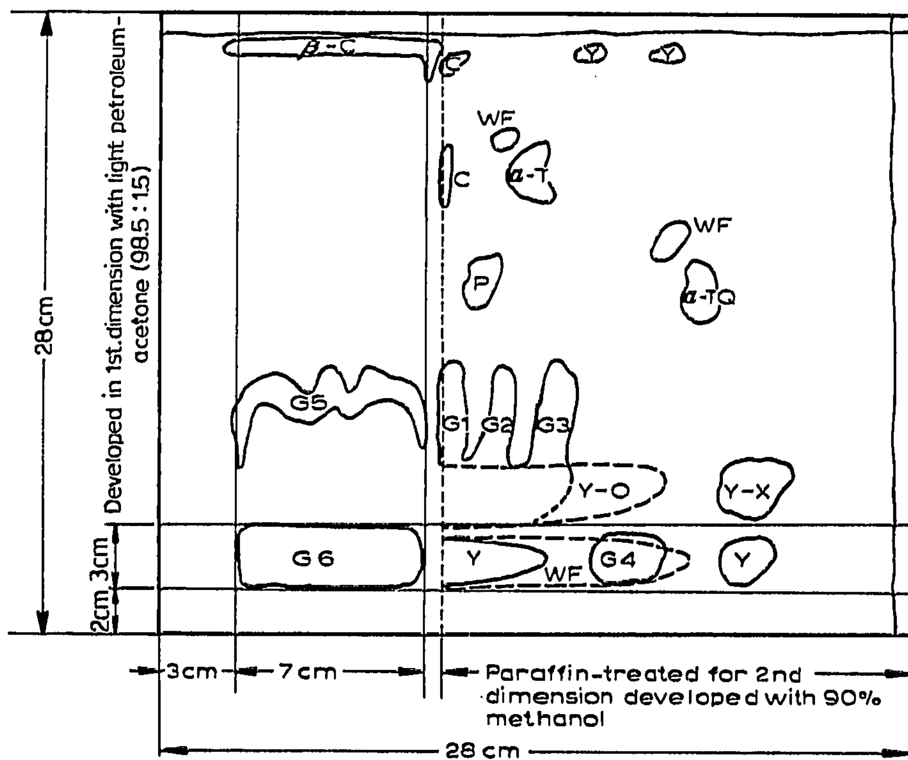

Fig. 1. Two-dimensional paper chromatogram showing separation of lipid components in extracts of sheep faeces (green feed diet). β-C = β-Carotene; α-T = α-tocopherol; α-TQ = α-tocopherylquinone; G1–G6 = green components (chlorophylls and closely related substances); Y-X = yellow xanthophyll; Y-O = yellow-orange pigments; Y = yellow pigments; P = pink pigments; W-F = white fluorescent substances; C = colourless, unknown, ultraviolet-absorbing substances.

α-Tocopherol was detected as a mauve spot under ultraviolet light, about midway between the chlorophyll front and β-carotene, and just inside the paraffin-treated area of the paper. The absorption spectrum in ethanol showed the expected maximum at 290–292 mμ[11].

A colourless spot with an intense absorption in the ultraviolet region (λ_{max} 261 mμ, 268 mμ) was detected between the chlorophylls and α-tocopherol. On the basis of its spectrum[12,13], and by comparison with the spectral and chromatographic properties of the quinone prepared from pure α-tocopherol, it was considered to be α-tocopherylquinone.

Absorption maxima for some of the constituents, isolated from green feed and faeces by paper chromatography, are shown in Table I.

Absorption curves for β-carotene, α-tocopherol and α-tocopherylquinone, isolated from sheep faeces, and typical of those obtained from green feed, fresh pastures and reference substances, are shown in Fig. 2.

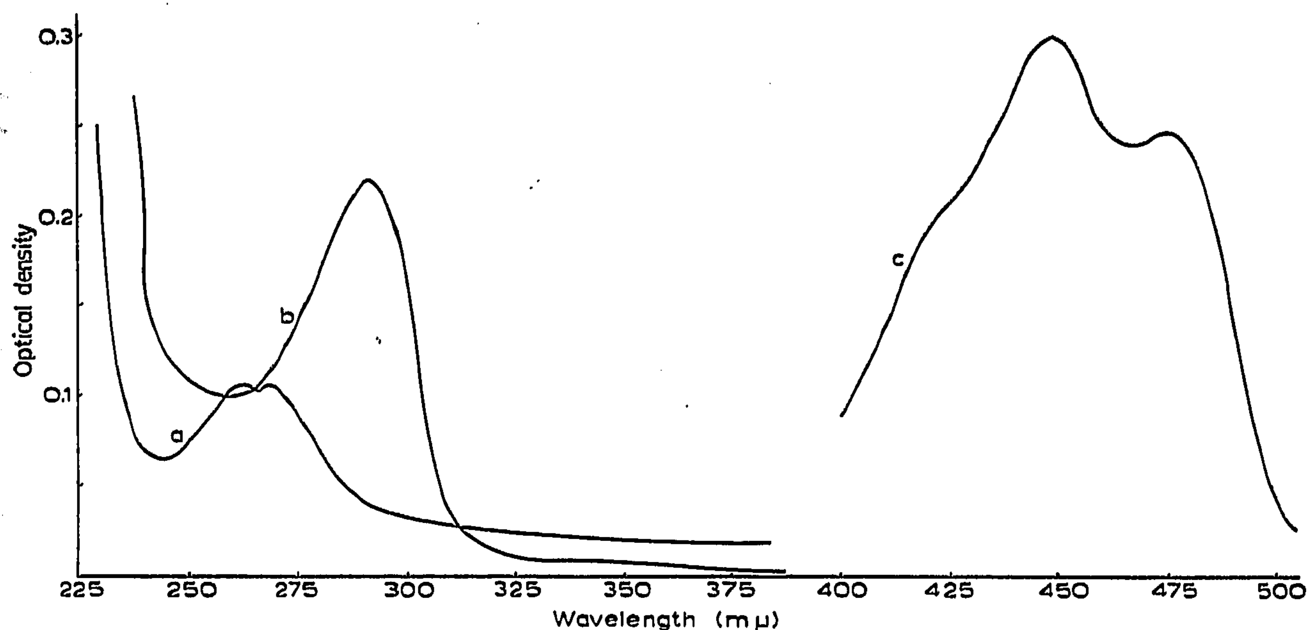

Fig. 2. Absorption curves of lipids, separated by paper chromatography, from extracts of sheep faeces. a = α-Tocopherylquinone; b = α-tocopherol; c = β-carotene.

Quantitative analysis of biological materials

Sheep feeds and faeces. Application of the method to feeds and faeces from lambs maintained under controlled experimental conditions showed differences in lipid concentrations.

On the dry ration, the concentration of β-carotene in the faeces was from 10 to 30 times that of the feed, on a dry weight basis. This feed contained no detectable α-tocopherol, but the faeces contained 1 to 2.3 mg per 100 g.

On the green feed, the concentration of β-carotene in the faeces was about 3 times that in the feed, and α-tocopherol levels were 4 to 5 times greater.

Concentrations obtained are shown in Table II and are expressed on a dry weight basis, as the means of duplicate determinations.

Fresh pastures. β-Carotene, and α-tocopherol contents of a range of pasture materials showed a wide variation and are listed in Table III.

TABLE II

CONCENTRATIONS OF β-CAROTENE AND α-TOCOPHEROL IN SHEEP FEEDS AND FAECES

All values are means of duplicate analyses and are expressed on a dry weight basis.

Component	*Dry feed*		*Green feed*		*Sheep No.*
	Feed (mg/100 g)	*Faeces (mg/100 g)*	*Feed (mg/100 g)*	*Faeces (mg/100 g)*	
β-Carotene	0.13	0.93	27.85	99.1	226
	0.13	3.73	27.85	89.2	232
α-Tocopherol	0.0	1.39	20.93	89.3	226
	0.0	2.31	20.93	89.8	232

TABLE III

β-CAROTENE AND α-TOCOPHEROL CONTENTS OF FRESH PASTURE
Values expressed on a wet weight basis.

Pasture	*β-Carotene*		*α-Tocopherol*	
	mg/g	*mg/lb*	*mg/g*	*mg/lb*
Kikuyu grass 1	0.064	29.1	0.085	38.5
Kikuyu grass 2	0.081	36.8	0.083	37.7
White clover leaf (*Trifolium repens* L.)	0.125	57.0	0.024	10.8
Berseem clover leaf (*Trifolium alexandrinum* L.)	0.170	77.2	0.052	23.7
Corn leaf	0.077	35.0	0.067	30.3
Oats leaf (mature) (*Avena sativa* L.)	0.081	36.8	0.010	4.5
Oats leaf (young)	0.046	20.9	0.015	6.8

Decomposition in drying pastures. Results for the determination of β-carotene and α-tocopherol in air-dried Kikuyu grass, reported in Table IV and illustrated in Fig. 3, showed that during the first two days deterioration was negligible, but beyond one week the decomposition was more rapid. α-Tocopherol deteriorated more readily than β-carotene.

TABLE IV

RATE OF DECOMPOSITION OF β-CAROTENE AND α-TOCOPHEROL IN KIKUYU GRASS
Values are means of duplicate determinations and are calculated on an oven-dry basis.

Time interval (days)	*% loss*	
	α-Tocopherol	*β-Carotene*
2	Negligible	Negligible
8	50%	30%
13	70%	50%
21	90%	70%
30	100%	80%

DISCUSSION

The use of column chromatography, employing a variety of adsorbents has been described for carotene[2] and the tocopherols[9,14], but frequently difficulties are encountered, such as destruction or retention of tocopherols on the column. This process can lead to spurious recoveries[15] and care has to be exercised in choice of adsorbent[16].

Direct application of the two-dimensional paper chromatographic method to feed and faecal extracts, though more time-consuming than unidimensional methods for plants[8], gives an added degree of separation of β-carotene and α-tocopherol from interfering substances. The accurate quantitative determination of α-tocopherol with

the iron–dipyridyl reagents[9] requires rigid freedom from reducing contaminants, as provided by the two-dimensional method.

The performance of labile constituents on the chromatograms was influenced by the amount of extract applied to the paper. In agreement with findings for plant extracts[7], it was observed for sheep faeces from green feed that, when the chlorophylls travelled about one-third the distance of the solvent front, α-tocopherol was well separated about midway between the chlorophylls and β-carotene.

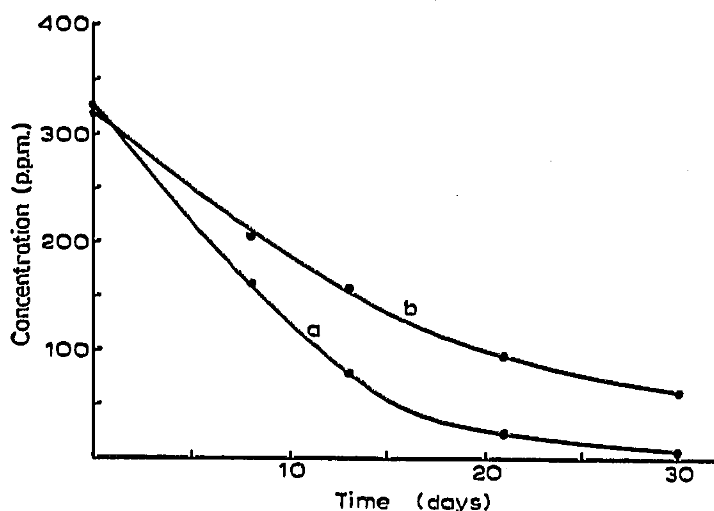

Fig. 3. Rate of decomposition of α-tocopherol (a) and β-carotene (b) in Kikuyu grass stored at room temperature.

Because of the high concentration of tocopherol in the faecal extracts, no difficulty was experienced in determining the absorption spectrum. However, for the green feed, several spots had to be combined before a satisfactory absorption curve could be obtained.

Location of the spots of α-tocopherol from both feed and faeces was always very similar and compared well with the position obtained for pure α-tocopherol.

Detection of α-tocopherylquinone in feeds and faeces, showing a typical bicuspid peak (261, 268 mμ), was of interest because of its position in metabolic pathways involving α-tocopherol.

Although α-tocopherylquinone was found as a prominent spot in chromatograms of fresh corn leaves (*Zea mays* L.) and has been reported in other plants[13], it was not detected in the fresh Kikuyu grass. However, as the drying process progressed and decomposition of lipids, including tocopherol, continued, the quinone appeared as a clearly detectable spot. α-Tocopherylquinone was also found in Kikuyu grass examined several hours after harvesting.

Studies using vitamin E as an antioxidant for β-carotene frequently measure the decay of the carotene only[17]. In contrast, the present method allows the convenient determination of both lipids simultaneously, after chromatographic separation. This technique is now being used in studies of digestibility in sheep.

SUMMARY

Two-dimensional paper chromatography was used, without preliminary removal of interfering substances, for examination of pastures, feeds and sheep faeces. α-Tocopherol, β-carotene and other lipids were adequately separated in a total chromatography time of four hours. α-Tocopherol and β-carotene were measured quantitatively in the diets and faeces. Rates of decay were also determined in samples of pasture.

In general, chromatographic patterns obtained from the faeces and the relevant diet were similar, but there were notable differences in the concentrations of both tocopherol and carotene.

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