APPLICATION OF REVERSED PHASE CIRCULAR PAPER CHROMATOGRAPHY TO THE ANALYSIS OF HIGHER FATTY ACIDS

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(Received August 7th, 1961)

INTRODUCTION

The necessity for the application of reversed phase systems for the separation of water-insoluble compounds was first recognised by BOLDINGH[1] who employed rubber coated papers for the separation of esters of higher fatty acids. We have recently considered the efficiency of this method and have investigated the possibility of using other nonpolar substances as paper impregnating materials and of various solvents as developers[2]. More recently we have carried out experiments with a few reversed phase systems and have studied their effect on the R_F values of higher fatty acids. The results of these studies, including the details of the quantitative method for the determination of higher fatty acids (saturated C_{12} to C_{18} acids and oleic, linoleic, and linolenic acids)[3], are presented in this paper.

MATERIALS AND METHODS

Apparatus

The apparatus in its simplest form consisted of a square wooden frame covered with a greased glass plate. A small Petri dish with developing solvent was placed at the centre of the tray. The paper was supported by glass rods which rested on the slots made in the wooden frame. A desiccator or bell-jar over a glass plate was also used in place of a wooden frame.

Materials for impregnating the paper

The materials used for impregnating the paper were: (1) liquid paraffin (190–220°) fraction; (2) vaseline (white petroleum jelly) obtained from Chesebrough Manufacturing Co. Consd., New York; (3) Dow Corning High Vacuum Grease; (4) Dow Corning Stopcock Grease (a Dow Corning silicone lubricant). The last two are products of Dow Corning Corporation, U.S.A., processed and distributed in India by Metropolitan Architects and Engineers Private Co. Ltd., Calcutta.

Method of impregnation

The material to be impregnated was dissolved in benzene 10% (w/v). Pieces of filter

paper of the desired size were impregnated by the capillary ascent technique. After air drying for 1/2 h the papers were ready for use.

Solvents

A number of solvents were investigated. Aqueous acetone, aqueous ethanol, aqueous methanol and aqueous acetic acid were successfully used for the separation of fatty acids. However, for the separation of "critical pairs" the solvents used were either glacial acetic acid–formic acid (88 %)–hydrogen peroxide (30 %) (6:1:1) or methanol–formic acid (88 %)–hydrogen peroxide (30 %) (6:1:1).

Choice of various grades of filter papers

Earlier investigators have used various grades of filter paper[4–6]. In the present work Whatman No. 1 and Whatman No. 3 sheets were compared, and Whatman No. 3 was found to be more suitable.

Micro-pipettes

The micro-pipettes required for measuring out the test liquid on to the filter paper were (1) Technico 861 BS 797 20°–0.2 ml, and (2) Technico 502 BS 797 20°–0.05 ml.

Analytical procedure

Circular paper of 24 cm diameter was used. The test solution (in ethanol) was applied as a spot on the circumference of a circle of 1 cm radius drawn around the centre of the circular filter paper. The amount applied at a spot varied from 50 to 100 μg. A filter paper wick was inserted into a hole at the centre of the filter paper. The length of the wick was adjusted in such a way that the distance between the surface of the filter paper and solvent was 1 cm. When the solvent had travelled a distance of 10 cm from the circumference of the inner circle, the chromatogram was removed and washed twice in tap water to remove the solvent. It was then kept immersed for 15 min in a 0.1 % mercuric acetate solution containing 0.5 ml of acetic acid per litre. Excess mercuric acetate was removed by washing the chromatogram in running tap water for 45 min. The chromatogram was air dried and then sprayed with 0.2 % solution of *s*-diphenyl carbazide in 95 % ethanol. The purple coloured bands obtained were cut into small pieces and eluted with 5 ml of 1:1 mixture of freshly distilled methanol and toluene. The absorption maximum was at 540 mμ when determined spectrophotometrically (Fig. 1); hence the estimations were carried out, using a Klett-Summerson photoelectric colorimeter with green filter (530 mμ).

EXPERIMENTAL RESULTS

Influence of the grade of filter papers

The easily available Whatman No. 1 and Whatman No. 3 filter papers were tried. They were impregnated with 10 % liquid paraffin in benzene. The four even-numbered saturated fatty acids (C_{12} to C_{18}) and oleic, linoleic and linolenic acids in 20 μg

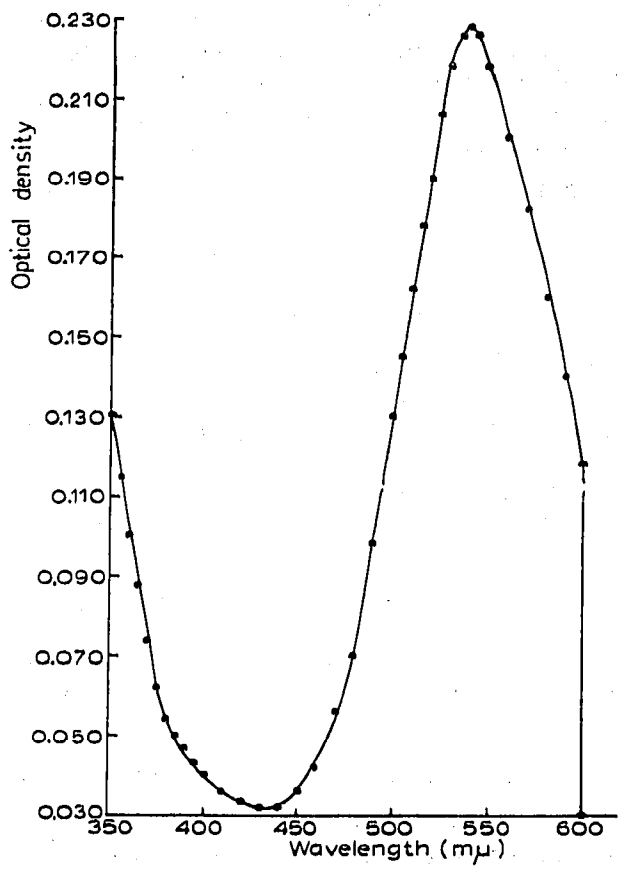

Fig. 1. Absorption spectrum of the mercury-*s*-diphenyl carbazide complex formed during the estimation of higher fatty acids. Solvent: methanol–toluene (1:1). Instrument: Beckman DU spectrophotometer.

quantities were spotted and separated by the circular paper chromatographic technique. The observations are given in Table I.

The results given in Table I indicate that though both grades of Whatman filter paper gave good separation, with Whatman No. 3 the separation was quicker. The R_F values were slightly higher for Whatman No. 3 filter paper with both the solvent systems. Acetic acid (90 %) gave higher R_F values than the glacial acetic acid–formic acid (88 %)–hydrogen peroxide (30 %) (6:1:1) system for the saturated higher fatty acids.

Solvent systems

The use of acetic acid, especially at high temperatures, gave rise to a very pungent odour. Hence the other solvent systems reported in the literature[2] were tried. Various concentrations of the aqueous solvents were also tried. It was observed that 80 % aqueous ethanol, 90 % aqueous acetone and 95 % aqueous methanol gave good separation of the fatty acids on 10 % liquid paraffin-impregnated Whatman No. 3 filter paper. The results are given in Table II.

Although all the three solvents tried gave a good separation, aqueous acetone and aqueous methanol were better as they effected the separation in a shorter time.

TABLE I

COMPARATIVE EFFICIENCY OF WHATMAN NO. 1 AND WHATMAN NO. 3 FILTER PAPERS IN THE SEPARATION OF HIGHER FATTY ACIDS BY REVERSED PHASE CIRCULAR PAPER CHROMATOGRAPHY

(a) With 90% acetic acid as developer

Grade of paper impregnated	Distance of development (cm)	Time of development (min)	R_F values of higher fatty acids						
			Saturated				Unsaturated		
			Lauric	Myristic	Palmitic	Stearic	Oleic	Linoleic	Linolenic
Whatman No. 1	10	315	0.81	0.67	0.50	0.39	0.52	0.66	0.79
Whatman No. 3	10	155	0.85	0.76	0.66	0.55	0.65	0.74	0.83

*(b) With glacial acetic acid–formic acid (88%)–hydrogen peroxide (30%) (6:1:1) as developer**

Grade of paper impregnated	Distance of development (cm)	Time of development (min)	R_F values of higher fatty acids (saturated)			
			Lauric	Myristic	Palmitic	Stearic
Whatman No. 1	10	330	0.74	0.60	0.43	0.32
Whatman No. 3	10	165	0.76	0.65	0.55	0.48

* This solvent oxidises the unsaturated higher fatty acids quantitatively. Hence their R_F values cannot be recorded.

TABLE II

COMPARATIVE EFFICIENCY OF VARIOUS SOLVENT SYSTEMS FOR THE SEPARATION OF HIGHER FATTY ACIDS BY CIRCULAR PAPER CHROMATOGRAPHY ON 10% LIQUID PARAFFIN IMPREGNATED WHATMAN NO. 3 FILTER PAPER

Solvent system	Distance of development (cm)	Time of development (min)	R_F values of higher fatty acids						
			Saturated				Unsaturated		
			Lauric	Myristic	Palmitic	Stearic	Oleic	Linoleic	Linolenic
Aqueous ethanol (80%)	10	195	0.79	0.72	0.64	0.51	0.63	0.70	0.79
Aqueous acetone (90%)	10	90	0.87	0.75	0.67	0.53	0.66	0.74	0.86
Aqueous methanol (95%)	10	75	0.81	0.73	0.62	0.50	0.63	0.72	0.80

Methanol is preferable to acetone for high temperature chromatography, since it has a high boiling point.

The use of methanol for the separation of fatty acids at high temperatures and for the separation of "critical pairs"

By using methanol–formic acid–hydrogen peroxide (6:1.5:1.5) the unsaturated fatty acids were oxidised and thus the saturated fatty acids could be effectively separated from their "critical partners".

When aqueous methanol (95%) was used for the separation of the fatty acids of the red seeds of *Adenanthera pavonina* at 55° (the higher temperature was necessary to separate the higher fatty acids beyond C_{18}), it was found to give five unsaturated and eight saturated higher fatty acids, as obtained earlier with 90% acetic acid solvent[3].

Use of other impregnating materials

Paper impregnated with 10 % Dow Corning Stopcock Grease (Silicone Lubricant). Three concentrations of aqueous methanol were tried for the separation of higher fatty acids on this paper. At a concentration of 95 % aqueous methanol the fatty acids (C_{12} to C_{18} and oleic, linoleic and linolenic) showed a tendency to separate but moved too close to the solvent front. At a concentration of 85 % methanol a good separation was obtained, but the movement of the fatty acids was slow. When 90 % aqueous methanol was used the movement was good and a separation of the fatty acids was effected (Table III).

TABLE III

R_F VALUES OF HIGHER FATTY ACIDS ON WHATMAN NO. 3 FILTER PAPER IMPREGNATED WITH 10 % DOW CORNING STOPCOCK GREASE (SILICONE) AND DEVELOPED WITH 90 % AQUEOUS METHANOL

Distance of development (cm)	*Time of development (min)*	*R_F values of higher fatty acids*						
		Saturated				*Unsaturated*		
		Lauric	*Myristic*	*Palmitic*	*Stearic*	*Oleic*	*Linoleic*	*Linolenic*
10	150	0.70	0.57	0.45	0.38	0.46	0.56	0.69

Paper impregnated with Dow Corning High Vacuum Grease. When Whatman No. 3 filter paper was impregnated with 10 % of this material, it was observed that the solvent (even 95 % aqueous methanol) moved very slowly and that the movement was also uniformly centripetal. When the paper was impregnated with 5 % of the material and used with 80 % aqueous methanol, the acids separated well but moved too close to the solvent front; when the concentration of methanol was reduced to 50 % there was very little movement and practically no separation of the fatty acids, but when the methanol concentration was raised to 65 %, a good movement and separation was obtained as indicated in Table IV.

TABLE IV

R_F VALUES OF HIGHER FATTY ACIDS ON WHATMAN NO. 3 FILTER PAPER IMPREGNATED WITH 5 % DOW CORNING HIGH VACUUM GREASE AND DEVELOPED WITH 65 % AQUEOUS METHANOL

Distance of development (cm)	*Time of development (min)*	*R_F values of higher fatty acids*						
		Saturated				*Unsaturated*		
		Lauric	*Myristic*	*Palmitic*	*Stearic*	*Oleic*	*Linoleic*	*Linolenic*
10	125	0.85	0.73	0.52	0.41	0.51	0.71	0.84

Paper impregnated with vaseline (white petroleum jelly). On the 10 % vaseline-impregnated paper the higher fatty acids moved very little when developed with 80 % aqueous methanol (Table Va); with 90 % aqueous methanol, there was good movement and separation of the fatty acids.

A good separation of the fatty acids could be effected by reducing the nonpolar phase content instead of increasing the polar phase concentration in the reversed phase system. This was achieved by reducing the vaseline concentration to 5 % and by using 80 % aqueous methanol as the developer (Table Vb).

TABLE V

EFFECT ON THE R_F VALUES OF HIGHER FATTY ACIDS OF THE CONCENTRATION OF VASELINE (NONPOLAR PHASE) AND OF AQUEOUS METHANOL (POLAR PHASE) IN THE REVERSED PHASE SYSTEMS

(*a*) *10 % Vaseline-coated paper*

Aqueous methanol (%)	Distance of development (cm)	Time of development (min)	R_F values of higher fatty acids						
			Saturated				Unsaturated		
			Lauric	Myristic	Palmitic	Stearic	Oleic	Linoleic	Linolenic
80	10	255	0.38	0.30	0.23	0.18	0.22	0.31	0.37
90	10	225	0.87	0.72	0.63	0.53	0.60	0.71	0.85

(*b*) *5 % Vaseline-coated paper*

Aqueous methanol (%)	Distance of development (cm)	Time of development (min)	R_F values of higher fatty acids						
			Saturated				Unsaturated		
			Lauric	Myristic	Palmitic	Stearic	Oleic	Linoleic	Linolenic
80	10	90	0.96	0.81	0.60	0.48	0.60	0.79	0.94
70	10	125	0.50	0.40	0.26	0.16	0.25	0.39	0.51

Quantitative estimation of higher fatty acids

The general procedure has been indicated previously[3]. The details as regards the spectral analysis curve (Fig. 1) and the Klett readings for the four saturated (lauric, myristic, palmitic and stearic) and the three unsaturated (oleic, linoleic and linolenic) acids (Table VIa and b) and for the three critical pairs (lauric–linolenic, myristic–linoleic and palmitic–oleic) of fatty acids (Table VIc) are given.

Recovery experiments

The results of the above analytical method were checked as follows. Known amounts of the fatty acids were added to those obtained from a strain of *Aspergillus niger*; after development of the respective chromatograms (with and without peracid modification[3]), the amounts of each fatty acid were determined before and after addition of the known amounts of fatty acids (Table VII).

DISCUSSION

As regards the use of various reversed phase systems for the qualitative analysis of fatty acids, it was observed that on Whatman No. 3 filter paper the separation of the fatty acids was more rapid than on Whatman No. 1 filter paper, although both

TABLE VI

VALUES OBTAINED USING A KLETT-SUMMERSON PHOTOELECTRIC COLORIMETER WITH GREEN FILTER (530 mμ), FOR THE HIGHER FATTY ACIDS SEPARATED BY VARIOUS SOLVENTS, AFTER EXTRACTION OF THE MERCURY-*s*-DIPHENYL CARBAZIDE COMPLEX WITH A 1:1 METHANOL–TOLUENE MIXTURE

(a) 90% Aqueous acetic acid as developer for individual fatty acids

Fatty acid concentration (μg/0.05 ml)	*Klett readings for*						
	Lauric	*Myristic*	*Palmitic*	*Stearic*	*Oleic*	*Linoleic*	*Linolenic*
10	8	13	16	6	8	13	22
20	17	25	31	11	14	25	45
30	25	38	48	16	20	38	65
40	34	53	63	21	26	50	90
50	42	64	78	26	32	65	114
60	50	79	93	32	37	80	135

(b) Glacial acetic acid–formic acid (88%)–hydrogen peroxide (30%) (6:1:1) as developer for the saturated fatty acids

Fatty acid concentration (μg/0.05 ml)	*Klett readings for*			
	Lauric	*Myristic*	*Palmitic*	*Stearic*
10	7	12	14	6
20	17	24	28	12
30	24	36	44	17
40	33	52	61	22
50	40	63	75	27
60	49	77	92	34

(c) 90% Aqueous acetic acid as developer for "critical pairs" of fatty acids

Concentration of each fatty acid in the mixture (μg/0.05 ml)	*Klett readings for*		
	Lauric–linolenic	*Myristic–linoleic*	*Palmitic–oleic*
10	29	26	23
20	60	52	44
30	88	79	66

grades of paper were equally sensitive. Among the impregnating materials examined and found to be equally useful for the separation of fatty acids, liquid paraffin may be preferred because of its easy solubility in the solvent used for impregnation and because of its cheapness.

All the developers studied gave good separation of the fatty acids; with aqueous methanol and aqueous acetone the separation was quicker; with the other developers, particularly aqueous ethanol, it was rather slow. At high temperatures it may be inconvenient to work with aqueous acetic acid as a developer, since it gives off a pungent odour. Under these conditions aqueous methanol may be preferred; this solvent has also the additional advantage of having a higher boiling point than acetone. At room temperature any of the quick developers may be used.

TABLE VII

RECOVERY OF FATTY ACIDS ADDED TO THOSE OBTAINED FROM *Aspergillus niger*

(a) 90% Aqueous acetic acid as developer

Fatty acids	*Fatty acids in Aspergillus niger (μg/0.05 ml)*	*Amount of fatty acids added (μg/0.05 ml)*	*Total amount of fatty acids expected (μg/0.05 ml)*	*Total amount of fatty acids recovered (μg/0.05 ml)*	*Percentage recovery of fatty acids*
Linolenic and lauric	Nil	20 Lauric	20	19.6	98.0
		20 Linolenic	20	19.4	97.0
Linoleic and myristic	32.4	20 Linoleic	52.4	50.9	97.2
		20 Myristic	52.4	51.3	97.9
Oleic and palmitic	45.8	20 Palmitic	65.8	64.6	98.2
		20 Oleic	65.8	63.9	97.1
Stearic	23.6	20 Stearic	43.6	42.8	98.2

(b) Glacial acetic acid–formic acid (88%)–hydrogen peroxide (30%) (6:1:1) as developer

Fatty acids	*Fatty acids in Aspergillus niger (μg/0.05 ml)*	*Amount of fatty acids added (μg/0.05 ml)*	*Total amount of fatty acids expected (μg/0.05 ml)*	*Total amount of fatty acids recovered (μg/0.05 ml)*	*Percentage recovery of fatty acids*
Lauric	Nil	20 Lauric	20.0	19.2	96.0
		20 Linolenic	Nil	Nil	—
Myristic	Nil	20 Myristic	20.0	19.3	96.5
		20 Linoleic	Nil	Nil	—
Palmitic	27.7	20 Palmitic	47.7	47.5	99.6
		20 Oleic	27.7	26.8	96.4
Stearic	22.8	20 Stearic	42.8	42.5	99.3

The R_F values for fatty acids could be altered suitably by adjusting the concentration of the impregnating material or the concentration of the developer. This is also a point of considerable interest in the chromatographic separation of fatty acids.

ACKNOWLEDGEMENTS

We are very grateful to Dr. S. C. PILLAI for his keen interest and advice. Our thanks are also due to Dr. P. L. NARASIMHA RAO and Mr. U. SITARAMA ACHARYA for their suggestions.

SUMMARY

A detailed study of various reversed phase systems for the qualitative analysis of fatty acids has been made and it has been shown, among other things, that the liquid paraffin–aqueous methanol system is a most convenient one.

The details of a fairly simple and rapid method for the quantitative estimation of higher fatty acids have also been discussed.

REFERENCES

1 J. BOLDINGH, *Experientia*, 4 (1948) 270.
2 C. V. VISWANATHAN, B. MEERA BAI AND U. SITARAMA ACHARYA, *Chromatographic Reviews*, Vol. 4, Elsevier, Amsterdam, 1962, p 160.
3 C. V. VISWANATHAN AND B. MEERA BAI, *J. Chromatog.*, 6 (1961) 264.
4 P. SAVARY, *Bull. soc. chim. biol.*, 36 (1954) 927.
5 R. G. BARKER, *Biochem. J.*, 54 (1953) Proc. xxxix.
6 V. P. SKIPSKI, S. M. ARFIN AND M. M. RAPPORT, *Arch. Biochem. Biophys.*, 87 (1960) 259.