IDENTIFICATION, ESTIMATION AND PREPARATION OF FATTY ACIDS BY CIRCULAR PAPER CHROMATOGRAPHY

C. V. VISWANATHAN AND MISS B. MEERA BAI
Department of Biochemistry, Indian Institute of Science, Bangalore (India)

(Received December 21st, 1960)

INTRODUCTION

During recent years a considerable amount of attention has been devoted to the development of rapid methods for the identification, isolation and quantitative estimation of fatty acids in biological materials. Reversed phase paper chromatography has been applied for the identification and estimation of fatty acids, using polarographic determination of copper[1], photometric estimation of copper as the dithiooxamide complex[2], microphotometric estimation of copper as a salt of rubeanic acid[3], or colorimetric estimation of copper as a salt of tetraethylthiuram disulphide[4]; in all these methods copper acetate was made to react with the fatty acids. The "Critical Pairs" of fatty acids have been analysed before and after hydrogenation of the mixture of fatty acids and before and after oxidation with alkaline permanganate[2], and small amounts of saturated acids occurring along with large amounts of unsaturated acids have been identified after oxidation of the unsaturated acids with peracid[5]. In the qualitative analysis of fatty acids by other methods various reagents have been used, *e.g.* bismuth sulphide[6], complex of mercury with *s*-diphenylcarbazide[5], and, specifically for unsaturated acids, osmium tetraoxide[7]. The use of high temperatures has been tried for the separation of higher saturated fatty acids, particularly those with chain lengths from twenty onwards[8].

All these methods either require special equipment or are not sufficiently rapid; moreover, the various methods differ only in regard to the reagents employed. Recently we have developed a comparatively simple method for the identification, isolation and quantitative estimation of saturated as well as unsaturated fatty acids, using the circular paper chromatographic technique and combining two of the conditions suggested in the literature[5,8]. The possibility of preparing the fatty acids was also examined. The results of these studies are briefly described in this paper.

EXPERIMENTAL

Quantitative estimation of fatty acids

The saturated straight chain fatty acids C_{18} and C_{16}, C_{14} and C_{12}, were separated from their corresponding critical components by the peracid procedure[5], using a 24 cm

circular Whatman No. 3 paper impregnated with 10% liquid paraffin. The C_{18} acid and the three critical pairs from C_{16} to C_{12} were identified after developing the chromatogram in 90% aqueous acetic acid and then treating it with mercuric acetate and *s*-diphenylcarbazide[5]. The fatty acids were estimated by extracting the mercury colour complex in a freshly distilled 1:1 mixture of methyl alcohol and toluene. As the colour complex had an absorption maximum at 540 mμ, it was estimated colorimetrically by using a Klett-Summerson photoelectric colorimeter with 530 mμ green filter. All the seven fatty acids gave a linear relationship between 5 μg and 60 μg. The colour was stable even at the end of 4 hours. The colour faded, however, in presence of the smallest amount of moisture.

With this circular paper chromatographic technique the fatty acids were separated very rapidly: in 2 1/2 hours the solvent travelled a distance of 10 cm and gave a very clear separation, the R_F values of the fatty acids differing by 0.1. In this method only linoleic acid gave a rose coloured mercury–*s*-diphenylcarbazide complex, whereas the rest of the fatty acids gave a purple colour. The linoleic acid was found between palmitic and myristic acid, more towards myristic acid.

This technique has been successfully used for the analysis of the fatty acids in biological materials such as *Aspergillus niger*, *Malbranchia pulchella*, activated sludge, sesame oil, mustard oil, and goat liver. The results are given in Table I.

TABLE I

FATTY ACID COMPOSITION OF VARIOUS BIOLOGICAL MATERIALS

(Results expressed as percentage)

Material	Saturated fatty acids				Unsaturated fatty acids		
	Lauric	Myristic	Palmitic	Stearic	Linolenic	Linoleic	Oleic
1. *Aspergillus niger*	—	—	28.3	22.0	—	31.3	15.1
2. *Malbranchia pulchella**	—	—	30.4	13.5	—	32.6	21.2
3. Activated sludge**	1.4	2.3	20.2	15.6	1.1	35.2	20.1
4. Sesame oil	—	—	8.5	5.3	—	35.2	48.1
5. Mustard oil***	—	—	1.0	—	4.1	8.7	33.0
6. Goat liver	—	—	25.4	30.3	—	12.3	30.2

* *Malbranchia pulchella* is a thermophilic strain from Indian compost; it was isolated by Miss M. PREMA BAI and Dr. P. L. NARASIMHA RAO of this Department.

** Activated sludge is a sewage sludge formed under aerobic conditions, which shows intense microbial activity. In this sludge the presence was observed of traces of three saturated fatty acids with R_F values lower than that of stearic acid, which still separate at room temperature.

*** The amount of erucic acid present in the mustard oil was 53.2% (determined by difference).

— Not present.

Isolation and preparation of the fatty acids

Reversed phase circular paper chromatography was used for the isolation and preparation of fatty acids. Forty mg of a commercial linoleic acid (containing 46.5% linolenic, 19.6% linoleic, 24.8% oleic and 8.2% palmitic acid) was spotted at the centre of the paraffin-impregnated paper. The mixture of the fatty acids was spread over a circle of 1.5 cm diameter. This was developed thrice with 90% aqueous acetic

acid and exposed to iodine vapours whereby a clear-cut separation of the unsaturated fatty acids occurred, showing three bands. A photograph of the chromatogram is shown in Fig. 1. The individual bands were cut out, eluted in ethyl alcohol and neutralised with $N/10$ sodium hydroxide using phenolphthalein as indicator. The alcoholic solution was made slightly alkaline and the unsaponifiable, *i.e.* liquid paraffin, was extracted with ethyl ether after evaporation of the alcohol and dilution

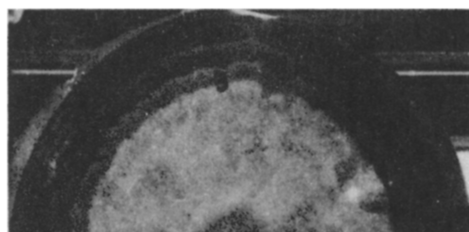

Fig. 1. Chromatogram of the unsaturated fatty acids from commercial linoleic acid, with three bands corresponding to: 1. Linolenic acid; 2. Linoleic acid; 3. Oleic acid.

of the soap solution with water. The soap solution was then acidified and the liberated fatty acids were extracted with ethyl ether. The ether solution was then washed free of mineral acid and after evaporation of the ether the weight of the fatty acids was determined. The recovery on the whole was 75 %, the amounts of the fatty acids recovered being: linolenic, 12 mg; linoleic, 6 mg; and oleic *plus* palmitic 12 mg (the amounts of the two last-mentioned acids, estimated by chromatography were 9.35 mg oleic and 2.70 mg palmitic acid). The linolenic acid had no impurities, whereas linoleic acid contained traces of linolenic acid, and oleic acid contained traces of linoleic acid. When twice the amounts of fatty acids were spotted the number of developments was increased twofold, *i.e.* to six. When 40 mg of a mixture containing only saturated fatty acids was spotted, the solvent did not move because of the solidification of the fatty acids and consequent blocking of the pores of the paper. When, however, saturated fatty acids were dissolved in an unsaturated acid in equal amounts (total 40 mg) and spotted, the solvent moved normally, and after four developments a clear-cut separation of the fatty acids was obtained. The yields were 15 mg of oleic acid and 16 mg of stearic acid from a mixture of 20 mg each of oleic acid and stearic acid. The recovery on the whole was 78 %.

The above procedure might be useful for the isolation of any new fatty acids present in biological materials.

Identification of higher saturated fatty acids

High temperatures up to 85° have been used for the chromatographic separation of higher saturated fatty acids from C_{20} onwards[8]. In the present study the red seeds of *Adenanthera pavonina*, which are known to be a good source, particularly of lignoceric acid[9], were used for analysis. When this material was analysed chromatographically at room temperature (25°), only two bands for unsaturated fatty acids corresponding

to oleic and linoleic acid were observed and three bands for the saturated acids corresponding to myristic, palmitic, and stearic acid. However, a purple streak from the starting point also appeared, indicating the presence of lignoceric acid. When the chromatogram was run at 55° and then exposed to iodine vapours, it was possible to observe five bands representing five unsaturated acids. Of these, three bands were prominent and the other two rather faint. A photograph of this chromatogram is given in Fig. 2, in which the two faint bands are not visible.

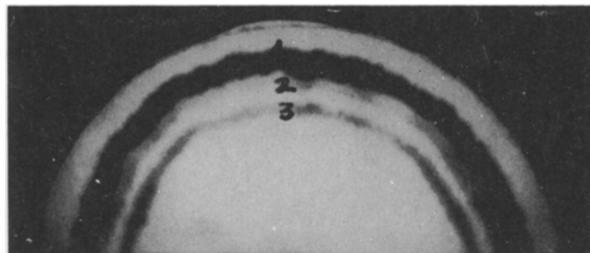

Fig. 2. Chromatogram (run at 55°) of the unsaturated fatty acids from the seeds of *Adenanthera pavonina*, with three bands (two other bands are not visible) corresponding to: 1. Linoleic acid; 2. Oleic acid; 3. Not identified.

The unsaturated acids in the seed material were destroyed by the peracid procedure[5], and another chromatogram was run for the analysis of saturated fatty acids at 55°. A photograph of this chromatogram is given in Fig. 3, in which bands for eight saturated fatty acids can be seen.

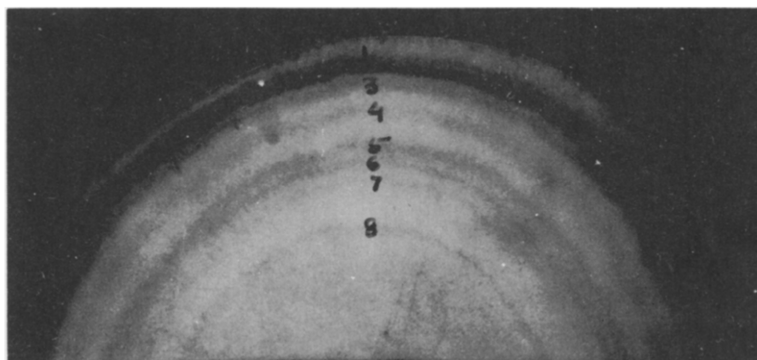

Fig. 3. Chromatogram (run at 55°, after peracid treatment) of the saturated fatty acids from the seeds of *Adenanthera pavonina*, with eight bands corresponding to: 1. Myristic acid; 2. Palmitic acid; 3. Stearic acid; 4 to 8. Not identified.

DISCUSSION

The circular paper chromatographic method described above for the quantitative estimation of fatty acids seems to be fairly simple and rapid. The time taken for the determination did not exceed 5 hours. The method is particularly useful for the analysis of the "Critical Pairs" of fatty acids; for such an analysis the method avail-

able up to the present is hydrogenation of the acids and oxidation with alkaline permanganate, which is somewhat tedious[2]. Another attractive feature of the circular paper chromatographic technique is its possible use as a preparative method also.

With regard to the use of high temperatures for the identification of higher saturated fatty acids, the circular paper chromatographic technique also offers advantages. For instance, by the earlier method[9], only two unsaturated and four saturated acids were identified in the seeds of *Adenanthera pavonina*, whereas with the technique described here, it was possible to identify five unsaturated and eight saturated fatty acids in the same material. Thus circular paper chromatography could be used for a more sensitive or effective analysis of the fatty acids in biological materials.

ACKNOWLEDGEMENTS

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

SUMMARY

A comparatively simple and rapid method for the identification, estimation and preparation of fatty acids has been developed, using reversed phase circular paper chromatography. The method is also suitable for the analysis of "Critical Pairs" of fatty acids and for the preparation of fatty acids. Further, when used at a higher temperature, the method is more sensitive in revealing the presence of even traces of higher fatty acids in the seeds of *Adenanthera pavonina*.

REFERENCES

1 H. P. KAUFMANN AND M. M. DESHPANDE, *Fette, Seifen, Anstrichmittel*, 60 (1958) 537.
2 P. E. BALLANCE AND W. MARY CROMBIE, *Biochem. J.*, 69 (1958) 632.
3 J. GRACIÁN, E. VIOQUE AND MA PILAR DE LA MAZA, *Nature*, 184 (1959) 1941.
4 M. NOVAK AND V. HROMADKOVA, *Die Nahrung*, 3 (1959) 1060; cited in *J. Inst. Brewing*, 66 (1960) 423.
5 M. A. BUCHANAN, *Anal. Chem.*, 31 (1959) 1616.
6 V. P. SKIPSKI, J. M. ARFIN AND M. M. RAPPORT, *Arch. Biochem. Biophys.*, 87 (1960) 259.
7 A. NOWOTNY, O. LÜDERITZ AND O. WESTPHAL, *Biochem. Z.*, 330 (1958) 47.
8 S. FIKER AND V. HÁJEK, *Chem. listy*, 52 (1958) 549.
9 S. M. MUDBIDRI, P. RAMASWAMI AYYAR AND H. E. WATSON, *J. Indian Inst. Sci.*, 11 (1928) 173.