*Procedure*

The chromatograms are drawn through the DNPH solution and held in the air for 10 min. The wet strips are then washed for 1 min in the neutralizing fluid, 10 min in the decolorizing fluid, 5 to 10 min in the reducing fluid and finally in tap water.

If the decolorization is not complete, decolorizing with subsequent washings repeated once or twice will be helpful. After the last washing in water the strips are air-dried and can be stored for further use.

The procedure described increases the sensitivity of the DNPH qualitative test about 5 times, as compared with the data of NEHER[4], when non-impregnated papers are used.

Owing to its simplicity and satisfactory results, the method described seems to be a convenient basis for quantitative analysis of ketosteroid hydrazones. These applications are now under detailed investigation and will be published later.

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## Two-dimensional paper chromatography of cholesteryl esters

### A new method for the separation of critical pairs

During recent years many authors have studied various chromatographic systems for the separation of cholesteryl esters[1-4]. Although in several cases good results were obtained no useful resolution of critical pairs of these substances by paper chromatography was described.

A two-dimensional technique in which adsorption and reversed-phase partition systems are combined enabled us to solve this problem.

*Experimental*

*Cholesteryl esters.* Esters of saturated fatty acids were synthetized by the method of KAUFMANN, MAKUS AND DEICKE[5]. Unsaturated esters were prepared enzymically according to MAHADEVAN AND GANGULY[6].

*Impregnation of paper with silica gel.* Whatman No. 3 paper (18 × 46 cm) was immersed in a mixture of about 2.5 % (v/v) solution of sodium silicate (prepared by dilution of a stock solution 36–38° Bé) and 5 % ammonium chloride in the ratio 100:30 (v/v). The excess of impregnating agent was removed by rubbing both sides of the paper with a glass rod. After drying in the air overnight the papers were washed three times in distilled water (*e.g.* using 1000 ml for six sheets of 18 × 46 cm) for

10 min. The chromatograms were then dried for 30 min at 80° and stored in a box. This procedure is a minor modification of HAMILTON's method[7].

*Impregnation of paper with paraffin oil.* The impregnation of silica gel papers with paraffin oil was performed according to MICHALEC AND STRAŠEK[1]. The scheme of this technique is given in Fig. 1.

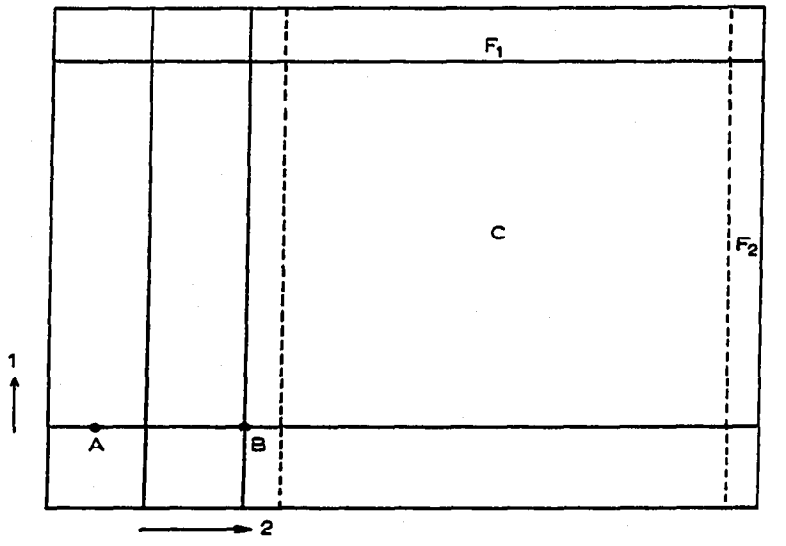


Fig. 1. Scheme of the two-dimensional technique. (A) Start for control chromatogram; (B) start; ($F_1$) front line for first run; ($F_2$) front line for second run; (C) part of chromatogram impregnated with paraffin oil (between the two dotted lines).

The mixture of esters (2–5 $\mu$g of each substance) was spotted on the start A and B, 2 cm from the lower end of the paper. The chromatogram was then developed with petroleum hydrocarbon (b.p. 60–90°) twice to the front line F 1, 1 cm from the upper end. After drying in air, part A was cut off and detection was carried out in order to check the separation in the first run. Then part C was impregnated up to the dotted line (about 0.5–1 cm from B) by immersing it in a 0.5 % solution of paraffin oil in ether. In the second run the mobile phase was acetic acid–chloroform–paraffin oil (80:15:5, v/v/v), development being carried out for 4–5 h to the second front line F 2.

All the chromatographic runs were performed at room temperature (20 ± 2°).

*Detection.* After drying at 80–90° the chromatogram was sprayed with phosphomolybdic acid (10 % in ethanol) and heated for about 5 min at the same temperature. Blue-green spots developed on a quickly darkening yellow-green background.

Besides this very sensitive detection, the reaction with 50 % antimony trichloride in acetic acid was also used. The mobilities of the cholesteryl esters are summarized in Table I.

*Results and discussion*

By applying this method we were able to separate some critical pairs of cholesteryl esters. In the first dimension on the paper impregnated with silica gel (adsorption chromatography) it is possible to separate the esters according to the degree of unsaturation. The mobilities ($R_F$ values) decrease in the order: saturated → monoethenoic → di-ethenoic → tri-ethenoic and tetra-ethenoic → more unsaturated esters.

In the second dimension on the paper impregnated with paraffin oil (reversed-phase partition chromatography) the esters were separated into the members of the homo-

TABLE I

$R_F$ VALUES OF CHOLESTERYL ESTERS

| Compound | $R_F$ | |
|---|---|---|
| | *silica gel* | *paraffin oil* |
| Cholesterol | 0.10 | 0.96 |
| Cholesteryl formate | 0.76 | 0.83 |
| Cholesteryl acetate | 0.52 | 0.84 |
| Cholesteryl butyrate | 0.63 | 0.77 |
| Cholesteryl caproate | 0.70 | 0.67 |
| Cholesteryl caprylate | 0.77 | 0.59 |
| Cholesteryl caprinate | 0.80 | 0.52 |
| Cholesteryl laurate | 0.82 | 0.45 |
| Cholesteryl myristate | 0.83 | 0.38 |
| Cholesteryl palmitate | 0.85 | 0.31 |
| Cholesteryl stearate | 0.87 | 0.24 |
| Cholesteryl arachidate | 0.87 | 0.19 |
| Cholesteryl behenate | 0.87 | 0.15 |
| Cholesteryl oleate | 0.72 | 0.31 |
| Cholesteryl palmitoleate | 0.71 | 0.37 |
| Cholesteryl linoleate | 0.58 | 0.38 |
| Cholesteryl linolenate | 0.40 | 0.44 |
| Cholesteryl arachidonate | 0.39 | 0.43 |
| Cholesteryl eicosenate | 0.72 | 0.23 |
| Cholesteryl erucate | 0.72 | 0.19 |
| Cholesteryl $C_{20:5}$ / Cholesteryl $C_{22:6}$ | 0.25 | 0.50 |

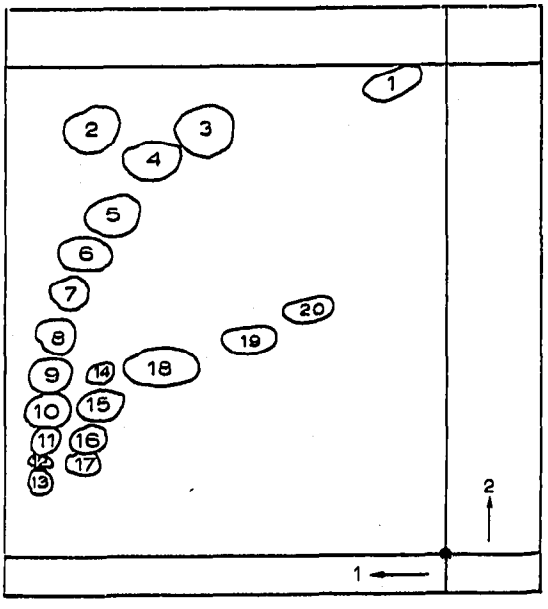


Fig. 2. Mixture of synthetic cholesteryl esters. (1) Cholesterol; (2) cholesteryl formate; (3) cholesteryl acetate; (4) cholesteryl butyrate; (5) cholesteryl caproate; (6) cholesteryl caprylate; (7) cholesteryl caprinate; (8) cholesteryl laurate; (9) cholesteryl myristate; (10) cholesteryl palmitate; (11) cholesteryl stearate; (12) cholesteryl arachidate; (13) cholesteryl behenate; (14) cholesteryl palmitoleate; (15) cholesteryl oleate; (16) cholesteryl eicosenate; (17) cholesteryl erucate; (18) cholesteryl linoleate; (19) cholesteryl linolenate and arachidonate; (20) cholesteryl $C_{20:5}$ and $C_{22:6}$.

logous series. It is well known that the problem of separating critical pairs of, for instance fatty acids, sterol esters, etc., by reversed-phase paper chromatography is difficult to solve. Chromatography at low temperatures imposes certain restrictions and with the other techniques, which include hydrogenation of unsaturated substances on the paper, oxidation with peracids and the addition of mercuri-methoxy

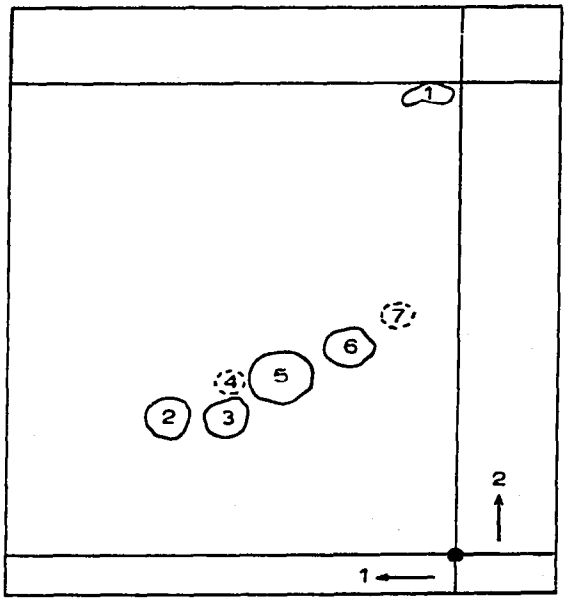


Fig. 3. Cholesteryl esters of human blood serum. (1) Cholesterol; (2) cholesteryl palmitate; (3) cholesteryl oleate; (4) cholesteryl palmitoleate; (5) cholesteryl linoleate; (6) cholesteryl arachidonate; (7) cholesteryl esters of more unsaturated fatty acids.

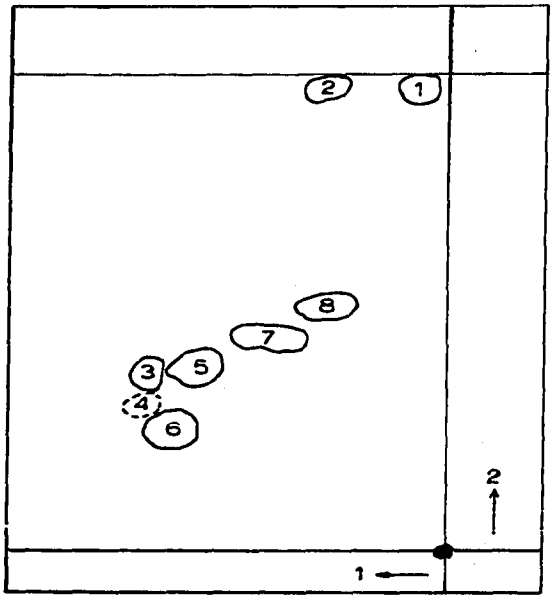


Fig. 4. Cholesteryl esters of fatty acids from rapeseed oil prepared enzymically. (1) Cholesterol; (2) unknown; (3) cholesteryl palmitate; (4) cholesteryl stearate; (5) cholesteryl oleate; (6) cholesteryl erucate; (7) cholesteryl linoleate; (8) cholesteryl linolenate.

groups to the double bounds, it is not possible to obtain on one chromatogram the whole spectrum of all the substances present in the material being analysed.

The two-dimensional system is very simple and, combined with a sensitive detection method, it constitutes a relatively quick and convenient method suitable for the separation of many critical pairs of cholesteryl esters.

This technique was used for the qualitative characterization of cholesteryl esters in various biological materials and for the identification of the spectrum of higher fatty acids in oils and fats after enzymic esterification with cholesterol.

These results will be discussed in another paper.

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# Notes

## The decomposition of nine amino acids during chromatography on paper

It has recently been shown[1] that some decomposition of glycine takes place during chromatography on Whatman No. 4 paper using phenol–water and *n*-butanol–propionic acid–water as developing solvents. Losses of up to 38% were obtained in this system; these losses could be minimized by distillation of the phenol before use, and by pre-treatment of the paper with oxalic acid. Filter paper pre-treated in this way had a pH of 4.9, compared with 6.7 for untreated paper. This study has now been extended to include eight other amino acids.

Before use, the following amino acids were purified chromatographically on oxalic acid-treated Whatman No. 4 paper, using distilled phenol[2]–water followed by *n*-butanol–propionic acid–water[3]: DL-[2-$^{14}$C]alanine (8.2 $\mu$C/mg); DL-[4-$^{14}$C]aspartic acid (10.7 $\mu$C/mg); DL-[1-$^{14}$C]glutamic acid (9.0 $\mu$C/mg); [2-$^{14}$C]glycine (8.9 $\mu$C/mg); L-[$^{14}$C]leucine (8.0 $\mu$C/mg); DL-[3-$^{14}$C]phenylalanine (21.1 $\mu$C/mg); DL-[3-$^{14}$C]serine (1.7 $\mu$C/mg); DL-[2-$^{14}$C]tyrosine (2.3 $\mu$C/mg); and D-[4,4'-$^{14}$C]valine (3.1 $\mu$C/mg).

Each amino acid was eluted with water, evaporated to dryness under reduced