# notes on methodology

## Automatic lipid extraction and thin-layer chromatography application with a programmed flow system

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SUMMARY An eight-channel programmed flow system for automatic lipid extraction and TLC application is described. Each channel has a container for lipid extraction connected by Acidflex tubing through an AutoAnalyzer pump to a TLC applicator needle. Extraction containers are prepared from disposable Oxford sampler pipet tips by inserting a small cotton filter into their lower, narrower end, which is connected to the pump tubing. The applicator needles are supported vertically in a manifold, and their tips rest on a TLC plate placed on a hot plate.

Serum is added to isopropanol in each extraction container, and proteins are completely precipitated in 2 min and retained in the extraction chambers by the cotton filters; lipid extracts are then transferred on to the heated TLC plate by intermittent pumping at a rate allowing for continuous evaporation of isopropanol under streams of warmed air or nitrogen. The lipids accumulate on the plate in eight small spots, one for each channel. Solvent is proportionally added into the extraction chambers from a common reservoir through Acidflex tubing in a second AutoAnalyzer pump. During the extraction procedure, both pump motors are automatically operated by a programmed timer with a solid-state switch.

Of several different solvents tested, isopropanol is the fastest for protein precipitation and lipid extraction and does not extract substances from the Acidflex tubing which interfere with chromatographic separation.

SUPPLEMENTARY KEY WORDS lipid extracting solvents · lipids in biological material

Improved ability to identify, separate, and measure the various classes of lipids facilitates the detection and investigation of abnormalities of lipid metabolism. TLC has become a standard method for separation of lipid extracts into major fractions for quantitative analyses. However, manual extraction of lipids and their application to TLC plates are tedious and time-consuming, and many intermediate steps are required. These facts challenged us to develop an automatic system which replaces the more cumbersome manual steps. The entire procedure from extraction to spotting of lipids from eight samples of serum is completed in 33 min (qualitative extraction) or 63 min (quantitative extraction), and no supervision is needed during that period. This system is a further development of an automatic eight-channel TLC spot applicator recently built in our laboratory (1).

*Description and Operation.* Eight extraction cups (Fig. 1, *A*) are connected individually through an AutoAnalyzer Pump I (*B*) (Technicon Co., Inc., Tarrytown, N. Y.) (2) by 0.02 in. i.d. Acidflex tubing (2) to 70 mm long, 23-gauge stainless steel needles. These needles are supported in a manifold (*C*) and are vertically spaced 16 mm apart over a TLC plate (*D*) which rests on a temperature-controlled hot plate (*E*). We prepared disposable extraction cups from Oxford sampler pipet tips (Oxford Lab, San Mateo, Calif.) (3) by inserting a small cotton filter into their lower, narrower end, which was then connected to the inlet side of the pump tubing. Separate 0.02 in. i.d. Acidflex tubing in a second pump I (*F*) carried solvent (isopropanol) from a common reservoir (*G*) to each of the extraction cups. A single pump II could accommodate all of the required tubing. A flow diagram of the extraction procedure is shown in Fig. 2.

Directing streams of warmed compressed air (or nitrogen) toward the area of the needle tips accelerates solvent evaporation on the TLC plate. The gas is warmed by passage through a heated coil of copper tubing immersed in a temperature-controlled water bath (Fig. 1, *H*), and distributed through the hollow applicator manifold.

Isopropanol extracts substances from both Standard (2) and Solvaflex (2) pump tubing, which react with phosphomolybdic acid and which fluoresce at 360 nm (Fig. 3, Plate *1*). No interfering substances are extracted from Acidflex tubing. The smallest Acidflex tubing available is 0.02 in. i.d. and delivers 0.16 ml/min with continuous pumping. This is too rapid a rate for continuous solvent evaporation on the TLC plate, but by using intermittent pumping, solvent can be applied at a rate suitable for continuous evaporation. A digital timer (Fig. 1, *K*) with solid-state switch turns the pump motors on and off according to a built-in, predetermined program.

Before processing new samples, we replace the used extraction cups with new ones and then wash the cups, tubing, and the needles with about 500 µl of isopropanol by continuous pumping until they are empty. We then fill each filter cup with 100 µl of isopropanol and 5–25 µl of serum and start the timer. The timing sequence is as follows: 2 min delay, 40 sec continuous pumping, and

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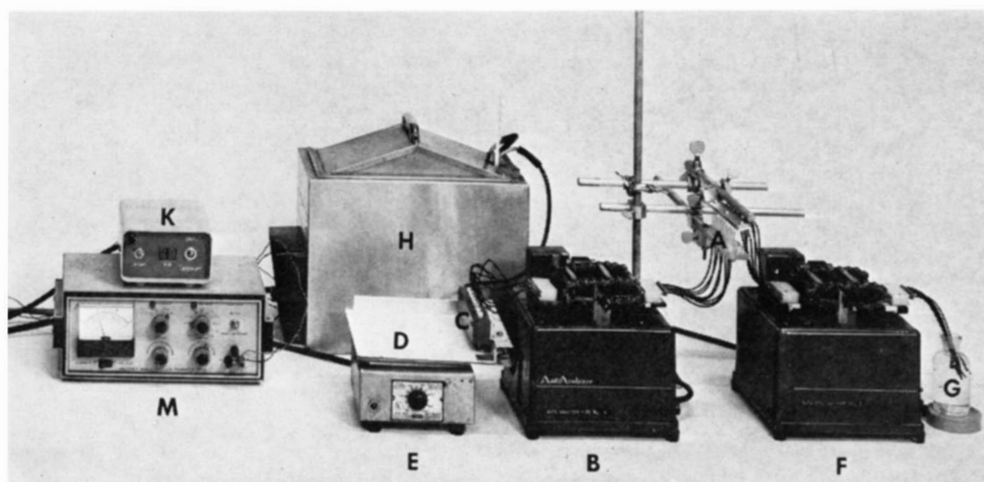


FIG. 1. Complete eight-channel system for automatic lipid extraction and TLC spot application. *A*, extraction containers; *B*, Pump I; *C*, applicator manifold; *D*, TLC plate; *E*, temperature-controlled plate; *F*, Pump I; *G*, solvent reservoir; *H*, temperature-controlled water bath with a heated coil for drying gas; *K*, programmed timer with solid-state switch; *M*, power supply for timer.

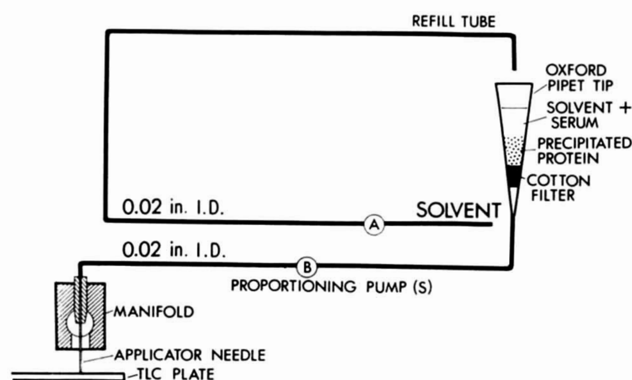


FIG. 2. Flow diagram for automatic lipid extraction and TLC application. Only one channel illustrated. See text for further explanation.

finally 30 min intermittent pumping for 0.3 sec every 6 sec.

The initial 2 min delay is required for complete protein precipitation in the filter cups. Otherwise some of the protein passes through the cotton filter, precipitates in the tubing (or needle), and plugs it. Continuous pumping for 40 sec transfers the first parts of extracts to the tips of the applicator needles. During the last 30 min, approximately 1.5 $\mu$l of lipid extract is spotted on to the TLC plate every 6 sec through all eight channels. By keeping the plate and drying gas at 60°C, the applied solvent continuously evaporates. The extracted lipids accumulate on the TLC plate in eight spots, in diameter 5 mm. During the extraction and application, the extraction containers are proportionally refilled with isopropanol through Acidflex tubing by the second AutoAnalyzer pump.

*Results and Discussion.* We selected isopropanol (4, 5) as the organic solvent in our automatic lipid extraction and TLC application system. Of the various solvents we tested, isopropanol shows the fastest protein precipitation and lipid extraction when used in our system. A 30 min lipid extraction with isopropanol (20 volumes per ml of serum) was found to be sufficient for qualitative and semiquantitative studies. In addition to isopropanol extraction (Fig. 3, Plates *2* and *3*), we tried alcohol–ether 3:1 (Bloor's mixture) and chloroform–methanol 2:1 (6, 7) (Fig. 3, Plates *4* and *5*).

From 5 to 40 $\mu$l of serum can be used (Fig. 3, Plate *6*). Whereas practically all free cholesterol, free fatty acids, and triglycerides are extracted from normal pooled serum using 20 volumes of isopropanol per volume of serum, (Fig. 3, Plate *3*), cholesteryl esters and phospholipids are not completely extracted. Phospholipids and cholesteryl esters can be completely extracted either by increasing the amount of isopropanol used for extraction, by reducing the amount of sample, or by combining both methods.

Since using more solvent increases the application time, use of less sample is the preferred way to extract cholesteryl esters and phospholipids completely. After a 30 min extraction of pooled serum with isopropanol (40 volumes per ml of serum) no phospholipids were reextracted on to a second plate.

Uniform extraction, application, and separation result when the same amount of the same serum is processed through each of the eight channels (Fig. 3, Plate *7*). We sprayed this plate with 10% phosphomolybdic acid in 96% ethanol, heated the plate at 90°C for 5 min, and scanned the plate by reflectance at 700 nm with a Zeiss chromatogram spectrophotometer (by

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TABLE 1 EXTRACTION OF $^3$H-LABELED CHOLESTEROL FROM MONKEY SERUM*

| | Automatic Procedure | | | | |
|---|---|---|---|---|---|
| | Proportional Filling | | | Static Filling | Manual Procedure† |
| | Chloroform–Methanol 2:1‡ | Ethanol–ether 3:1‡ | Isopropanol‡ | Isopropanol‡ | Isopropanol‡ |
| 30 min extraction | 2386 ± 372§ (73.8%)‖ | 2450 ± 333 (81.3%) | 2867 ± 211 (85.3%) | 2266 ± 174 (73.7%) | — |
| 60 min extraction | 3141 ± 354 (97.2%) | 2883 ± 461 (95.7%) | 3156 ± 228 (93.9%) | 2579 ± 193 (83.9%) | — — |
| Remaining in cup | 91 ± 51 | 129 ± 54 | 191 ± 108 | 493 ± 60 | — |
| Total | 3232 ± 367 | 3012 ± 473 | 3360 ± 250 | 3073 ± 182 | 3154 ± 162 |

* Eight samples of the serum were extracted in each experiment. The cholesterol concentration was 1024 mg/100 ml of serum.
† 0.1 ml of serum was shaken with 1.9 ml of isopropanol. The mixture was centrifuged, and 0.5 ml of the extract was taken for counting. See text for details.
‡ Extraction solvent.
§ Total cpm recovered ±SD. See text for details.
‖ % recovery.

courtesy of Carl Zeiss, Inc., New York) equipped with a recorder and integrator; a relative SD of 8.2% (at 70 mg/100 ml) was calculated for triglycerides.

Chromatographic isolation, separation of cholesteryl esters, and separation of phospholipids after automatic extraction and application are shown on Plates *8* and *9* (Fig. 3). For analysis of fecal fat, we prepare a suspension of 1 g of feces in 10 ml of water, and 25–50 µl of the suspension is used as a sample.

A study of the recovery of $^3$H-labeled cholesterol using different solvents during automatic extraction was performed. 250 µCi of tritiated cholesterol in orange juice was fed to a monkey, and blood was obtained by venipuncture 5 hr later. The serum cholesterol was extracted by the method of Abell, Levy, Brodie, and Kendall (8); the concentration was 1024 mg/100 ml as determined by the method of Zak, Moss, Boyle, and Zlatkis (9). 25 µl of the serum was used for each channel for the automatic extraction. The serum was pipetted into all filter cups with the same Oxford pipet by the same person. The extracts were pumped through the applicator needles directly into scintillation vials. Two successive extractions of 30 min each were performed, and the extracts were collected in separate vials. After evaporation of the solvent at 60°C, 10 ml of scintillation liquid (Bray's solution) (10) was added to each vial, and the radioactivity was measured (counting for 20 min in a Tri-Carb spectrometer) (Packard Instrument Co., Inc., Downers Grove, Ill.). The used filter cups were also dried and shaken in 10 ml of scintillation solution, and the activity of the cholesterol not extracted after 60 min of automatic extraction was measured. Clean, unused cups with filters were used as blanks and were treated in the same manner.

The results of the measurements after automatic extraction using proportional filling are shown for isopropanol, alcohol–ether 3:1, and for chloroform–methanol 2:1 (Table 1). Analyses of variance showed a significant difference between isopropanol and chloroform–methanol 2:1, and isopropanol and alcohol–ether 3:1 ($P < 0.01$) after 30 min of extraction; however, there were no significant differences after 60 min. Isopropanol, therefore, seemed to extract free cholesterol and cholesteryl esters the fastest and is preferable as solvent for qualitative chromatography using a 30 min extraction time.

There was no significant difference between manual extraction (using isopropanol) and automatic extraction with any of the solvents after a 60 min extraction time (Table 1). For quantitation of free cholesterol and cholesteryl esters, any of the above mentioned extraction solvents may, therefore, be used with 60 min extraction.

We tried to simplify our system using static filling instead of proportional refilling. In these experiments large sized pipet tips were used as extraction containers. After precipitation of the protein with 100 µl of extraction solvent, another 400 µl was added directly into the extraction containers. Results of isopropanol extraction using static filling are shown on Table 1. Static filling gives significantly less recovery after extraction for 30 and 60 min ($P < 0.01$).

Our automatic system replaces all manual steps from lipid extraction to TLC spot application of the lipid extracts. We have used the system for several months and believe it is an important step towards further automation in lipid analysis.

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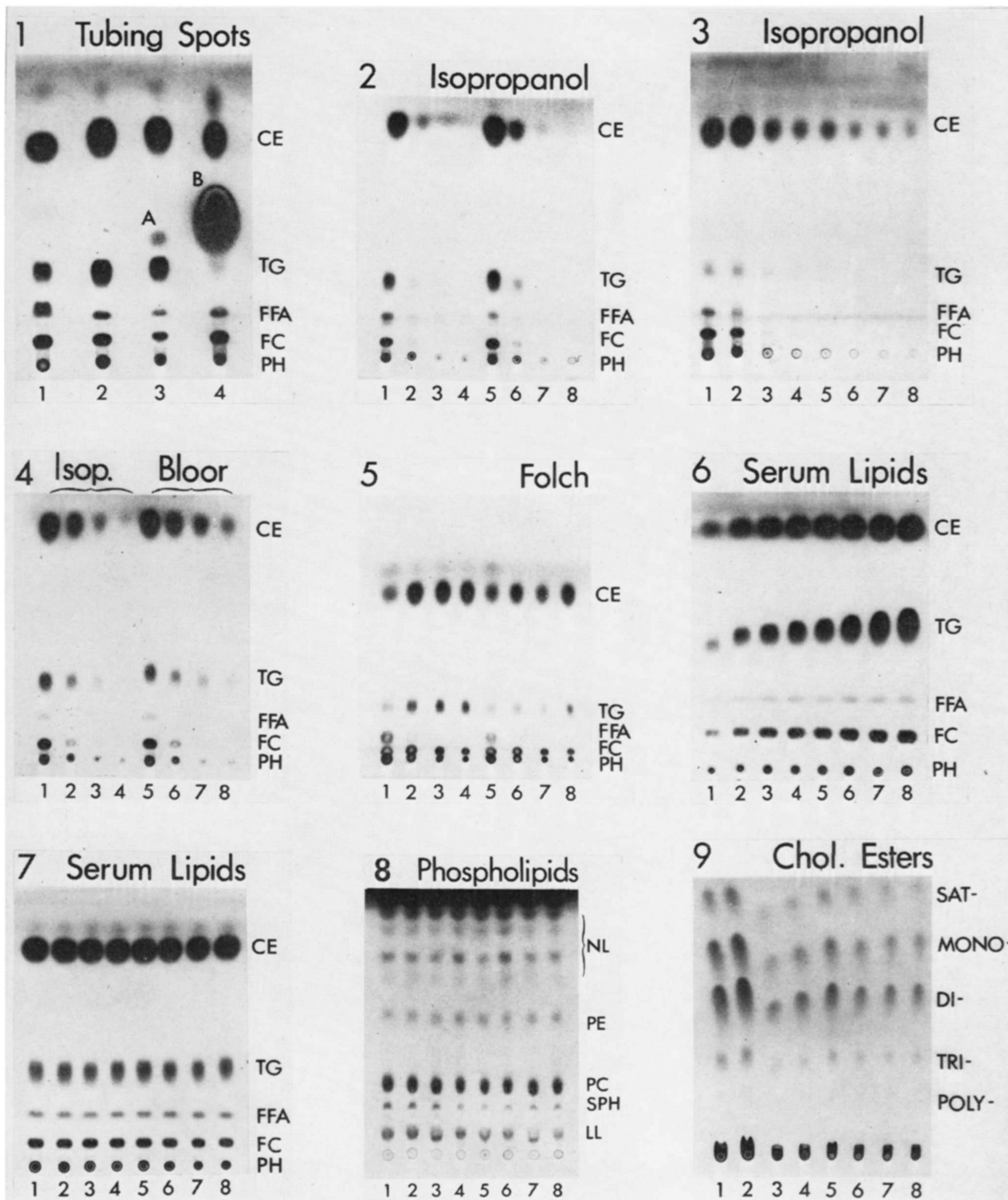


FIG. 3. Thin-layer chromatograms of lipids extracted and spotted automatically on precoated plates (Merck Silica Gel G, 20 × 20 cm). Unless otherwise specified, 25 μl of fresh pool serum was used for extraction in each channel. Extraction time: 30 min. For detection, the plates were sprayed with 10% phosphomolybdic acid in 96% ethanol and heated at 90°C for 5 min. The developing solvent system for Plates *1–7* was petroleum ether–diethyl ether–glacial acetic acid 90:10:1. *CE*, cholesteryl ester; *TG*, triglyceride; *FFA*, free fatty acid; *FC*, free cholesterol; *PH*, phospholipids; *NL*, neutral lipids; *PE*, phosphatidylethanolamine; *PC*, phosphatidylcholine; *SPH*, sphingomyelin; *LL*, lysolecithin.



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Plate *1:* "Tubing spot" *A* and *B* appear when Solvaflex (spot in lane 3) or Standard (lane 4) pump tubing is used, but not when Acidflex is used (lane 2). Standard solution of lipids through Acidflex in lane 1.

Plate *2:* Isopropanol extraction of serum lipids and application through the same channel in four successive spots by moving the applicator needle after 200 μl was spotted in lane 1, and after further 100-μl portions were spotted in each of lanes 2–4. The same procedure was followed with chylous serum in lanes 5–8.

Plate *3:* Isopropanol extraction of serum lipids through one channel, and successive application of 100 μl of extract in each of the lanes 1–8 by moving the applicator needle.

Plate *4:* Isopropanol (lanes 1–4) extracts more efficiently than alcohol–ether 3:1 (Bloor's mixture) (lanes 5–8). Procedure as for Plate 2, but with the same serum for both extraction solvents.

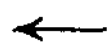
Plate *5:* Extraction with chloroform–methanol 2:1 (lanes 1–4) and 3:2 (lanes 5–8). Procedure as for Plate 4.

Plate *6:* Eight-channel operation. 5 μl (lane 1)–40 μl (lane 8) in increments of 5 μl of serum was extracted by 500 μl of isopropanol.

Plate *7:* Uniform extraction and application of 25 μl of serum using 500 μl of isopropanol for each channel.

Plate *8:* Phospholipids extracted with isopropanol. Developing solvent: chloroform–methanol–water 65:25:4.

Plate *9:* Separation of cholesteryl esters according to the degree of saturation of the fatty acids. The plate was developed twice in carbon tetrachloride.



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