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STRIPPING THIN LAYERS FROM CHROMATOGRAPHIC PLATES FOR RADIOTRACER MEASUREMENTS

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SUMMARY

We describe a method for stripping cellulose or cellulose–silica thin layers from glass chromatographic plates. A cellulose acetate mixture is used after chromatography to bind the particles into a porous, coherent film that detaches itself from the glass spontaneously and cleanly without the use of water. The intact layer can be stored, or the chromatographically separated spots can be cut out on the plate and recovered individually. The nature of the film and the stripping process make the method particularly suitable for radiotracer studies. Water-soluble and chloroform-soluble compounds can be studied, and radioactivity of ^{32}P, ^{14}C, ^{35}S or ^{3}H can each be measured in the bound layer by scintillation counting without significant quenching or loss of counting efficiency. Water-soluble compounds, and ninhydrin reaction products, can also be eluted from the intact piece of layer for other studies. We give details of the procedures used, and discuss the potential sources of error.

INTRODUCTION

For several years we have used a simple reliable technique for stripping cellulose layers or silica gel–cellulose mixed layers from glass thin-layer plates. Although the procedure has been briefly described, it has since become apparent that potential users have not found the method because it is buried in a paper on a different subject[1]. The method has proved to be particularly suitable for scintillation counting of water-soluble ^{14}C compounds, following their separation by thin-layer chromatography (TLC). It has also been used for measurement of radioactivity in water-soluble ^{3}H, ^{35}S and ^{32}P compounds, and in chloroform-soluble ^{32}P and ^{33}P compounds after TLC, by scintillation counting or Geiger-Müller counting. Other uses have included the stripping of complete layers for storage, and removal of spots, following ninhydrin spraying, for quantitative colorimetric estimation of amino acids[1]. Although a commercial stripping agent, Neatan (Merck, Darmstadt, B.R.D.), is available, it is very difficult to use on cellulose or silica gel–cellulose mixed layers. In addition, because water must be used to detach Neatan-bound layers from the supporting glass, loss of water-soluble materials occurs during stripping. We have not tested other commercial preparations. At least one is a lacquer. A similar lacquer which we tested gave variable counting efficiencies of ^{14}C because the lacquer coat caused some self-absorption losses. Collodion, used by Barrollier[2] for preserving layers, can, however, be

applied to tracer studies in much the same way as our mixture, although some count quenching occurs. The continued usefulness of our method prompts this report.

PROCEDURE

The stripping mixture (Stripmix) used to bind the thin-layer particles together is as follows: 7 g cellulose acetate (BDH, Poole, Dorset, Great Britain), 3 g diethylene glycol, 2 g camphor, 25 ml *n*-propanol and 75 ml acetone. As cellulose acetate varies in properties from one manufacturer to another, rather more or less may be needed: The final solution should have a viscosity like that of glycerol. The cellulose acetate binds the particles together, camphor and diethylene glycol keep the layer flexible so that it strips cleanly from the glass, while the chosen solvents dry rapidly and produce a porous film that is ideal for scintillation counting.

Stripmix is applied to the chromatogram as follows. The plate is laid flat, with paper underneath, on the bench. A "spacer" is placed on each side of the plate—we use either strips of adhesive insulation tape, Lasso (Smith and Nephew, Hull, Yorkshire, Great Britain), 0.2 mm thick, or 0.3-mm-diameter wires resting on the layer. A pool of Stripmix, about 20 ml, is poured on to the layer at one end of the plate, and then pushed evenly over the surface of the chromatogram by means of a glass rod resting horizontally on the spacer strips. About three passes of the rod, at about 10 cm/sec, usually give a layer of the desired quality (fewer passes and the layer may be rather brittle; more and the layer is unnecessarily thick for counting, though suitable for storage). Excess Stripmix is finally pushed over the edge of the plate and on to the paper beneath, and the plate is left to dry for 5–10 min. Subsequent handling is easier if the layer is not overdried. A cut is made around the margin of each spot by means of a scalpel or razor blade; at this stage the spot detaches itself cleanly from the glass and can be picked up with the tip of the blade and deposited directly into the vial (Fig. 1). Alternatively, the entire thin-layer surface can be taken off, by trimming around the edge of the plate. In this case, a rather thicker layer of Stripmix should be used.

Marking out of spots to be sampled can be done either before or after applying Stripmix. We normally prepare the autoradiograph, place it in the register on top of the plate so that the reference marks coincide, then draw around the boundary of each spot on the autoradiograph, using a ballpoint pen. Indentations are easily made on the chromatogram beneath and are still perfectly visible after the layer has been subsequently spread with Stripmix (Fig. 1). To avoid any chance of confusion, we label each spot with a soft pencil before cutting up the layer. Coloured spots (*e.g.* ninhydrin-sprayed amino acids) can be marked out after Stripmix has been applied.

The method used to measure radioactivity in the spots depends on the isotope used, and on the solubility of the compounds being studied. ^{32}P compounds can be assayed by Geiger-Müller counting, by using two-sided adhesive cellulose tape to stick each spot to its planchet. ^{14}C, ^{35}S and ^{33}P are better counted by scintillation procedures. The standard toluene–POPOP–PPO scintillant cocktail permeates the porous Stripmix layer, which becomes almost transparent, so that counting efficiency is high (about 70% for ^{14}C) and reproducible (Table I). This procedure is not suitable for ^{3}H. In general, we prefer to use a scintillation cocktail which accepts water, and which can be used for measuring radiactivity in experimental solutions as well as on thin-layer pieces. We have found the Triton scintillant[3] to be the simplest and most

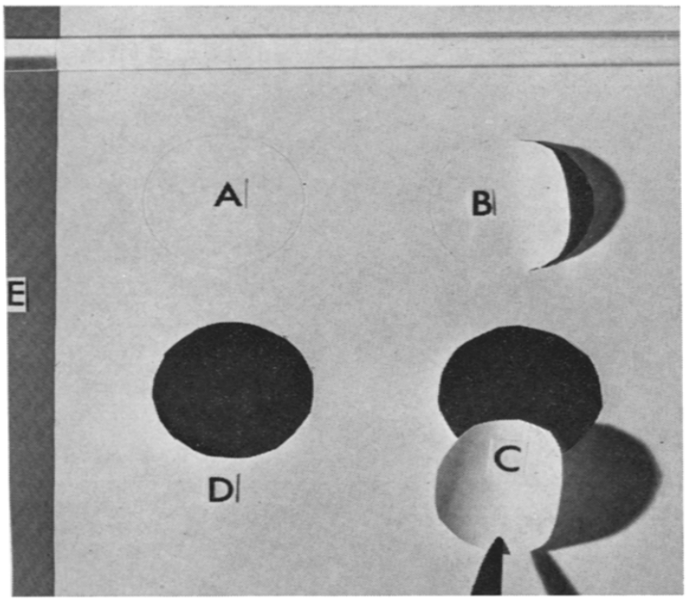

Fig. 1. Marking and removing spots from Stripmix-spread TLC plates. Spot A was outlined by pressure, by using a ballpoint pen to draw around the radioactive spot on the superposed autoradiograph. In spot B, part of this outline has been cut with a scalpel. Note the spontaneous peeling of the layer from the glass plate. With spot C, the cut has been completed and the spot is being transferred to the scintillation vial, using the scalpel tip. At D, observe that when a spot has been removed, the glass is completely free of any adhering thin-layer material. The adhesive tape spacer at E and the glass rod were used to spread the Stripmix.

TABLE I

COUNTING OF ^{14}C EXTRACT BY VARIOUS COUNTING PROCEDURES

Mixed layer is a cellulose–silica gel (5:2 w/w) mixed layer. Toluene scintillant is the conventional toluene–POPOP–PPO scintillant; Triton scintillant[3] and Bray's scintillant[5] are widely-used, water-accepting scintillants. Volume of aqueous sample supplied to vial: 10 μl.

Set	*Substrate and treatment*	*Water*	*Scintillant*	*Count rate*	*Std. dev. (%)*
I	Aqueous sample in vial	9%	Triton	1.000	± 1.0
	Aqueous sample in vial	Nil	Triton	1.037	± 1.4
	Cellulose layer+Stripmix	9%	Triton	1.028	± 0.5
	Cellulose layer+Stripmix	Nil	Triton	0.838	± 0.8
II	Mixed layer+Stripmix	9%	Triton	0.996	± 0.8
	Mixed layer+Stripmix	Nil	Triton	0.814	± 1.0
	Aqueous sample in vial	9%	Bray's	0.938	± 0.7
	Aqueous sample in vial	Nil	Bray's	1.027	± 2.2
	Mixed layer+Stripmix	9%	Bray's	0.906	± 0.8
	Mixed layer+Stripmix	Nil	Bray's	0.918	± 2.1
III	Mixed layer+Stripmix	Nil	Toluene	0.833	± 2.5
IV	Mixed layer, origin	9%	Triton	0.937	± 0.6
	Mixed layer, chromatographed	9%	Triton	0.926	± 1.4
V	Mixed layer+Neatan	9%	Triton	0.353	±29.3
	Mixed layer+Collodion	9%	Triton	0.968	± 4.7

reliable: it comprises two volumes of standard toluene–POPOP–PPO scintillant to one volume of Triton X-100 detergent, and has the great virtue that water can be added in amounts up to 14% of the total volume without affecting the ^{14}C counting efficiency[4]. The standard procedure which we use for measuring radioactivity in aqueous samples is as follows: The aqueous sample is pipetted into the vial, made up to 0.5 ml with water, then 5.0 ml of Triton scintillant is added and the vial is shaken gently, and then held in the counter at least 1 h before counting. The mixture thus contains 9% water. The standard procedure which we use for measuring radioactivity in pieces of paper chromatograms or segments of Stripmix-bound thin layer is as follows: The segment is placed in the vial, 0.5 ml of water is added and the vial is left for a few minutes so that the water-soluble compounds can dissolve, then 5.0 ml of Triton scintillant is added and the vial is shaken and counted as before.

RESULTS AND DISCUSSION

The results obtained by using different counting procedures were compared. In each treatment, replicates of ten samples were prepared and counted, and standard deviations were calculated. Total counts for each vial were $1–4 \times 10^5$ so that the standard error of counting was $\pm 0.2–0.3\%$. Much of the remaining variation can be attributed to errors in pipetting the radioactive sample. The ^{14}C solution used in these tests (Table I) contained water-soluble ^{14}C compounds (mainly sugars and amino acids) from an apricot leaf that had photosynthesized $^{14}CO_2$ for 30 min, while the ^{3}H test solution (Table II) contained ^{3}H-uridine plus carrier uridine. Each count rate has been expressed as a fraction of that obtained by the standard counting procedure for aqueous samples as outlined above. In Set I (Table I) the use of Triton scintillant is examined. When ^{14}C is in solution, it is counted at uniform efficiency whether or not 9% water is present in the scintillation cocktail. However, when the ^{14}C material is held in a dried extract on a thin layer or a paper substrate, counting efficiency is lower because there is considerable self-absorption of β-particles within the substrate and layer of extract (these losses are not corrected by quench-correction procedures). If water is added to the Triton scintillant, it can dissolve the extract from the substrate, and in this way full counting efficiency is achieved. It is preferable to add water to the sample before adding the scintillant, rather than as a water–scintillant mixture. The standard deviation of samples spotted by 10-μl microcapillary on to cellulose thin layer, $\pm 0.4\%$, was significantly less than that obtained when the samples were pipetted directly into the plastic scintillation vials, $\pm 1.0\%$. We attribute this to the greater ease and reproducibility of discharging the microcapillary on to the thin-layer material as compared with pipetting directly into a vial. Use of a conventional serological pipette to put 0.5-ml aliquots of a more dilute ^{14}C extract directly into the vials increased the standard deviation to about $\pm 2.2\%$.

Set II shows that a mixed layer, cellulose–silica gel (5:2) behaves in the same way as a cellulose layer towards inclusion of water in the Triton scintillant. Again the counting efficiency is higher and the reproducibility is better when water is included in the cocktail. Bray's scintillant[5] can be used in a similar way to the Triton scintillant. Results are also more consistent when water is included in the cocktail, although in this case the efficiency of counting is decreased by the addition of water. When a fixed proportion of water is used, the same counting efficiency is obtained whether the

sample is put directly into the vial, or is spotted onto a mixed layer and recovered by the Stripmix procedure.

Set III shows that ^{14}C can be counted on a Stripmix-spread layer using a standard toluene–POPOP–PPO scintillant which does not dissolve the compound, but which permeates the porous layer. In Set IV, a series of samples of plant extract were spotted on thin-layer plates. Half were not chromatographed, but were held in the vapour phase of the chromatography tank, while the remaining samples were chromatographed. All samples were autoradiographed and marked out in the usual way and individual chromatographically separated compounds were taken by the Stripmix procedure and counted. In this way the radioactivity in each of the chromatographed samples was measured as a total of eight individually collected and counted chromatogram areas. The total radioactivity recovered from the chromatographed samples was the same as that recovered from the unchromatographed origins, though the standard deviation was rather greater (note that about 7% of the ^{14}C in the extract, probably glycolate, was lost from the extract by volatilization into the chromatography tank with both unchromatographed and chromatographed samples). In Set V, the layer was stripped using either Collodion or Neatan instead of Stripmix. Collodion gave slight quenching and a rather greater standard deviation of counting, but could be used as a substitute for Stripmix. When Neatan was used, most of the radioactivity was lost into the water employed in stripping the layer: The resulting standard deviation was very high, and the method is clearly not suitable for water-soluble materials.

In another set of experiments, not detailed here, the various components of Stripmix were added to vials in amounts ten times those normally present in a Stripmix-spread chromatogram spot (20 mg cellulose acetate powder; 100 μl 10% diethylene glycol in acetone; 100 μl 5% camphor in acetone; or 50 μl *n*-propanol), and their effect on ^{14}C counting efficiency was determined. The camphor caused approximately 1.2% quenching, and the remaining components caused no detectable quenching.

Because of their similar radioactivity characteristics, ^{35}S, ^{33}P and ^{45}Ca would be expected to resemble ^{14}C in their counting behaviour. ^{3}H, as a very weak β-emitter, is rather a different matter. Other workers[3] have pointed out that water-accepting scintillation cocktails give greater count-rate variability with ^{3}H than with ^{14}C and that has been our experience. However, use of Stripmix does not cause any additional problems beyond those inherent in using ^{3}H as a tracer. Data in Set VI (Table II) reveal that ^{3}H compounds, like ^{14}C compounds, need to be dissolved from the thin-layer material in order that self-absorption losses can be minimized when Triton scintillant is used. When water is present in the cocktail, ^{3}H counting efficiency is essentially the same whether the extract is put directly into the vial, or is first spotted onto a thin-layer plate then removed for counting either by scraping it off as a powder, or peeling it off as a film by the Stripmix procedure. The standard deviation was consistently smaller when the Stripmix procedure was used. Results in Set VII show that Bray's scintillant can be used in a similar way to Triton scintillant for counting ^{3}H. Bray's scintillant may be able to partly dissolve water-soluble compounds even in the absence of added water, as the self-absorption effect is less pronounced than with Triton scintillant. Unlike the Triton scintillant, Bray's scintillant appears to be slightly sensitive to Stripmix components, as there is a 5% loss in counting efficiency when Stripmix is present. The results in Set VIII show that ^{3}H extracts can be recov-

TABLE II

COUNTING OF ^{3}H-URIDINE BY VARIOUS COUNTING PROCEDURES

Volume of aquous sample supplied to vial: 10 μl.

Set	*Substrate and treatment*	*Water*	*Scintillant*	*Count rate*	*Std. dev. (%)*
VI	Aqueous sample in vial	9%	Triton	1.000	±1.7
	Aqueous sample in vial	Nil	Triton	1.338	±2.4
	Mixed layer+Stripmix	9%	Triton	0.982	±1.5
	Mixed layer+Stripmix	Nil	Triton	0.433	±2.2
	Mixed layer, scraped off	9%	Triton	0.981	±1.9
	Mixed layer, scraped off	Nil	Triton	0.542	±9.0
VII	Aqueous sample in vial	9%	Bray's	0.533	±1.4
	Aqueous sample in vial	Nil	Bray's	0.833	±1.9
	Cellulose layer+Stripmix	9%	Bray's	0.505	±1.1
	Cellulose layer+Stripmix	Nil	Bray's	0.754	±1.6
VIII	Mixed layer, origin	9%	Triton	1.037	±4.5
	Mixed layer, chromatographed	9%	Triton	1.001	±3.6

ered quantitatively from thin-layer plates after chromatography by using the Stripmix procedure. Residues from the solvents used may increase counting variability.

An important requirement of a stripping procedure is that the radioactive spots should not be dissolved and become shifted or diffused when the stripping mixture is applied. Extracts containing water-soluble or chloroform-soluble ^{14}C compounds were prepared from an apricot leaf that had photosynthesized $^{14}CO_2$ for 30 min. Aliquots of each extract were applied as 1-cm spots to cellulose and mixed-layer plates. Each plate was autoradiographed, spread with Stripmix, and autoradiographed again. The two autoradiographs were compared to see how much movement of the extracts had occurred. Each spot was then marked out on the thin-layer plate from the first autoradiograph, the marking line being 1.5–2 mm outside the sharply defined edge of the spot. The spots were removed from the plates, each cut being made along the marking line; then a further ring, about 5 mm wide, was cut from around the periphery of each spot. In this way, material that was confined to the region of the original spot, during spreading of the Stripmix, was recovered in the spot, while material that had moved outside the spot boundary was recovered in the ring. Comparison of the two autoradiographs showed that water-soluble materials did not move during the spreading of Stripmix. Fine details in the original spot were still present after Stripmix spreading, and we estimate the maximum movement to be of the order of 0.2 mm. Over 99.7% of the tracer was recovered within the inner spot. On cellulose plates only 0.12±0.06% was recovered in the ring, and on mixed-layer plates, 0.28± 0.08%. The chloroform-soluble compounds are however soluble in Stripmix. Nonetheless, bad spot movement occurs only when an excessive amount of Stripmix is applied to the plate. With the normal spreading procedure described here, autoradiographs showed that the spot margins had moved a maximum of 0.5–1 mm; while in recovery experiments only 0.5% of the radioactivity was found in the surrounding ring as compared with the central spot (0.51±0.33% for cellulose and 0.52 ±0.25% for mixed layer).

Under normal conditions, errors through spot spreading should be less than those encountered here in a model system. In the first place, spot boundaries marked on the chromatogram are usually 3–4 mm from the edge of the spot. Secondly, on a chromatogram, the bulk of the radioactive material is in the very centre of the spot, well away from the margins; whereas in the artificial system studied here, much of the radioactive material became concentrated at the spot boundaries during the spotting procedure. In normal use, compounds that are soluble in Stripmix can still be recovered quantitatively by the Stripmix procedure.

An important feature of Stripmix is that when dry it forms a completely porous layer. Thus it is readily permeated by water, and it is possible to dissolve out all the water-soluble compound held in the layer. Individual spots were put in either 1 ml water or 1 ml 40% methanol and shaken gently for 30 sec, then the solvent was removed, leaving the intact film of layer behind. After three washes with water, only $1.28 \pm 0.58\%$ of the radioactivity remained within the layer, while three washes with 40% methanol removed all but $0.74 \pm 0.14\%$ of the water-soluble radioactivity. Material removed from the layer in this way can be used for re-chromatography. ^{14}C compounds in an apricot leaf extract were separated on cellulose and mixed layer plates by two-dimensional electrophoresis and chromatography[1]. Individual radioactive compounds, localized by autoradiography, were recovered from the thin-layer plates either by scraping in the conventional way, or by use of Stripmix. Each powder or piece of layer so obtained was washed with 0.2 ml water, then 0.1 ml of the resulting solution was spotted on a new plate for re-chromatography. Comparable results were obtained with each type of plate and each method of spot recovery. The components of Stripmix did not appear to hinder separation of the eluted compounds in any way. Both the recovery of the original spot from the thin-layer plate, and the subsequent elution were much simpler by the Stripmix procedure.

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