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A Low Temperature Cleanup Procedure for Pesticides and Their Metabolites in Biological Samples

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A low temperature cleanup procedure was developed to separate in one operation some polar and apolar pesticides from sample lipid, waxes, and water. Samples were extracted by refluxing with acetone–benzene (19 + 1) and extracts were cleaned up by low temperature precipitation at −78°C. A precipitation time of 30 min followed by filtration through Solka Floc (cellulose) was optimum. The water content of the extract did not adversely affect the recovery of polar residues but the presence of carbon in the filtration did. The cleaned-up extracts were suitable for glc determination with electron capture and flame photometric detectors. Recoveries of thirteen representative insecticides, fungicides, and herbicides (captan, diazinon, dinitrobutyl phenol anisole, 2,4-D acid, 2,4-D isopropyl ester, endosulfan, malathion, malaoxon, parathion, paraoxon, phosphamidon, fenitrothion, and fenitroxon) ranged from 80–116% when added separately or in combination to samples of carrots, peas, wheat, human fat, liver, and kidney.

Low temperature precipitation as a cleanup procedure for separating pesticide residues from sample extractives (lipids, waxes) has been widely reported but does not appear to have found wide acceptance.

Fairing and Warrington (1950) separated methoxychlor from fats and waxes by precipitation at −12°C. Dicofol, DDT, and methoxychlor were separated from plant waxes at −70°C (Anglin and McKinley, 1960). Later, McKinley and Savary (1962) and McCully and McKinley (1964a,b) extended the scope of the technique to include dieldrin, endrin, heptachlor, aldrin, isobenzan, and lindane in a variety of biological samples. Organophosphorus pesticide residues were determined in plant extracts following low temperature cleanup by Bates (1965). Recently, Grussendorf *et al.* (1970) described a semimicro apparatus and rapid cleanup method for hexane extracts of cereal grains, soils, and other types of samples. Most procedures use a liquid–liquid partition step interposed between extraction of the sample and low temperature cleanup. In general, apolar compounds are recovered but most polar residues remain in the aqueous fraction and are discarded.

This paper reports the development and evaluation of a low temperature cleanup procedure that separates polar and apolar residues from sample lipids, waxes, and water in a single step. Partition systems and drying agents are not required. Optimum analytical parameters, including recovery data, for pesticides and metabolites representative of insecticides, fungicides, and herbicides added to biological samples are given.

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MATERIALS

Apparatus. Micro-Tek gas chromatograph (MT220) fitted with a Melpar flame photometric detector for S394 and P526. The column was 5% DEGS on 60–80 mesh Chromosorb W, DMCS, packed in 3 ft × 1/8-in. i.d. borosilicate glass column. Operating parameters were as follows: Nitrogen flow, 80 ml/min. Oven temperature, 150 or 195°C. Hydrogen flow, 200 ml/min. Inlet temperature, 220°C. Air flow, 15 ml/min. Oxygen flow, 15 ml/min. Melpar temperature, 160°C. Electrometer settings: S394, Input 10^3, Output 8; P526, Input 10^3, Output 8; FID, Input 10^2, Output 16.

Varian Aerograph 600 gas chromatograph fitted with a Kovar cell (Tritium foil) electron capture detector and a 6% QF-1, plus 4% SE-30 on 60–80 mesh Chromosorb W, AW, packed in 4.5-ft × 1/4-in. i.d. pyrex glass column. Operating parameters were as follows: Nitrogen flow, 120 ml/min. Oven temperature, 195°C. Inlet temperature, 220°C. Range, 1 mV. Attenuation, 4.

Cold bath and accessories were custom-made from standard laboratory material and the construction details are described by McLeod (1972).

Vacuum pump with adjustable vacuum control.

Reagents. All organic solvents were pesticide analytical grade or analytical grade solvents redistilled in the laboratory to meet pesticide grade specifications. The extraction solvent was acetone–benzene (19 + 1). Carbon (DARCO

G60) and Solka Floc BW40 were prepared by the method of McCully and McKinley (1964a). The boron trifluoride methanol complex reagent was 14% boron trifluoride obtained from The British Drug Houses Ltd., Toronto, Ontario, Canada.

A stock solution of each pesticide was prepared by dissolving 25 mg in acetone and diluting to 25 ml. Pesticide mixtures were prepared by serial dilution of aliquots of the stock solutions. The concentration of pesticide solutions was not corrected for purity, as all standards were 95% or greater active component.

ANALYTICAL PROCEDURE

Sample Extraction. Five grams of animal tissue were refluxed for 1 hr with 20 ml of extraction solution and the supernatant was separated from the particulate matter by filtering through a medium porosity fritted glass funnel with the aid of negative pressure. The particulate matter was reextracted twice more for 10-min intervals with fresh 10-ml portions of the extraction solution. Filtrates were combined and concentrated to approximately 25 ml for further analysis.

Fifty grams of plant tissues were extracted by blending for 5 min, twice, with fresh 100-ml portions of the extraction solution. The supernatants were recovered by filtering through a medium porosity fritted glass funnel, combined, and made to 250 ml with acetone.

Low Temperature Bath Cleanup. The low temperature apparatus (Figure 1) is described elsewhere by McLeod (1972). CAUTION: it is preferable to locate the apparatus in a fume hood or, alternately, a well ventilated area because of methanol fumes. Methanol was added to the bath until the liquid surface was approximately 6 in. below the top of the box. Small portions of solid carbon dioxide (Dry Ice approximating the size of a 250-cm^3 beaker) were added cautiously, one at a time. The bath was cooled to −78°C in about 25 min and maintained at that temperature by addition of excess Dry Ice periodically during the time of use. The stainless steel top for holding six precipitation, six washing solutions and six filtering tubes was fitted into place. The bath temperature may be maintained overnight by partially filling with Dry Ice and covering with the insulated cover.

The filter tubes were prepared as follows and placed in the bath. Solka Floc sufficient to fill loosely a 10-ml beaker (*ca.* 1.5 g) was placed in each filter tube. Approximately 15 ml of acetone were added and the mixture was stirred with a glass rod and then allowed to settle. Particles of Solka Floc adhering to the sides of the filter tube were washed down with a minimum of acetone. The filters were allowed to temperature equilibrate in the bath, and the excess acetone was drawn off into a vacuum flask under 5 lb/in.^{2} negative pressure until the top of the filter pad was just exposed. This filtrate was discarded. A clean 125-ml vacuum flask and rubber tube with pinch cock was connected to each filter tube outlet, thence to a distribution manifold, and finally to the vacuum pump. Six 50-ml test tubes, each containing 20 ml of extraction solution, were placed in the bath to temperature equilibrate (wash solutions for each sample).

A 20-ml aliquot of each sample extract was transferred to its respective 50-ml precipitation test tube and then placed in a rack (six tubes in all). The rack of tubes was placed on the top of the prepared bath and a glass nitrogen bubbler tube inserted into each. The nitrogen gas flow was regulated to give a continuous and vigorous evolution of gas bubbles to agitate the sample extracts. Each tube was inserted through

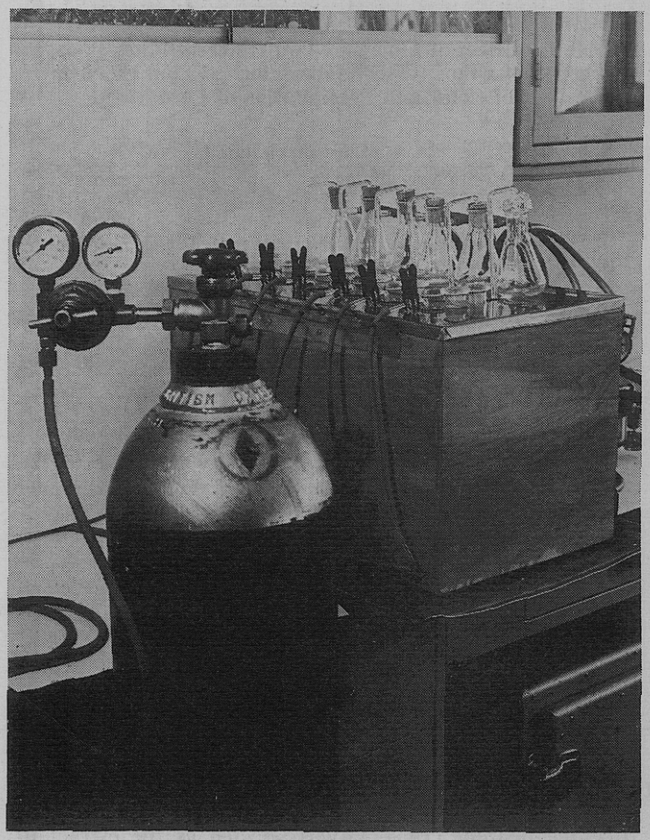

Figure 1. A photograph of the assembled low-temperature bath apparatus

the appropriate hole in the stainless steel top of the bath so that approximately two-thirds of the lower end was immersed in the cold methanol. Care was taken to insure that the gas bubbling was not interrupted and continued after placing the tubes in the bath.

After 30 min in the bath the nitrogen gas was disconnected and the contents of each precipitation test tube were transferred to their respective filter tubes. The supernatant was drawn off using a negative pressure of 5 lb/in.^{2} One-half of the cooled wash solution was transferred to the precipitation test tube, which was shaken vigorously to dislodge adhering precipitate; then the contents were transferred to the filter tube, but with the vacuum off. The nitrogen bubbler tube was used as a stirrer to mix the precipitate and wash solution in the filter tube; care was taken not to disturb the Solka Floc filter pad. Again the supernatant was drawn off and a second washing of the precipitate was carried out with the remaining wash solution.

Each vacuum flask containing approximately 40 ml of the combined filtrates for each sample was placed in a shallow water bath at 45°C, a gentle stream of nitrogen was inserted, and the contents were concentrated to approximately 0.5–1.0 ml. The concentrate of each flask was quantitatively transferred to 5-ml volumetric flasks with small portions of hexane and made to volume.

Glc Analysis. Five-microliter aliquots of the cleaned-up extracts were analyzed by glc. The pesticide recoveries were calculated by comparing the peak heights of compounds in the spiked sample extracts with those in standard solutions of the same concentration. When necessary, extracts were diluted to give a concentration of pesticides within the linear response range of the detector in use.

Table I. Effect of Time and Two Different Filter Pads, Carbon–Solka Floc (CSF), Solka Floc (SF), on Efficiency of Cold Bath Precipitation of Lard

Time in cold bath, min	Lard precipitated			
	Residue remaining, g		Percent removed	
	CSF	SF	CSF	SF
0	0.12080	0.12080	000.0	000.0
10	0.00010	0.00300	99.9	97.5
20	0.00085	0.00310	99.2	97.5
30	0.00005	0.00280	99.9	97.7
40	0.00025	0.00220	99.8	98.2
60	0.00010	0.00250	99.9	98.0

Table II. Percent Recovery of Some Pesticides With and Without Carbon (C) in the Solka Floc (SF) Filter Pad

Added water, g	Malathion, 11.25 μg		Parathion, 2.19 μg		2,4-D isopropyl ester, 5.25 μg	
	CSF	SF	CSF	SF	CSF	SF
0.00	97	95	63	98	62	93
0.25	89	92	69	92	57	89
0.75	98	92	60	101	61	99
2.00	98	96	59	98	57	90
3.00	87	86	60	94	24	73
4.00	92	94	42	92	46	93

DISCUSSION

Acetone–benzene (19 + 1) was selected as the extractant for polar and apolar pesticide residues in biological samples for several reasons. The combination contains a water-miscible solvent (acetone) and has been used for extracting organochlorine residues by McCully and McKinley (1964a). In addition, acetone has been used with other solvents such as hexane and ethyl ether for extracting many different pesticides and their metabolties: *e.g.*, aldrin, a cyclodiene by Goodwin *et al.* (1961); phygon, a dichloronaphthaquinone by Burchfield and McNew (1948); dichlobenil, a dichlorobenzonitrile by Beynon *et al.* (1966); 2,4-D, a dichlorophenoxy acid by Burchfield and McNew (1948); Zectran, an *N*-methyl carbamate by Niessen and Frehse (1963); and paraoxon, a diethyl *p*-nitrophenyl phosphate by Bates (1965). This extractant will not remove all conjugated residues such as those found with paraquat, organomercurials, 2,4-D, and 2,4,5-T. The release of conjugates such as these will, in many instances, be dependent on pH and this is presently under investigation.

Vigorous agitation or stirring during the low temperature precipitation is considered necessary in preventing occlusion of soluble compounds in the lipid and ice matrix. Hand stirring was used by McKinley and Savary (1962) and mechanical stirrers were part of the apparatus of McCully and McKinley (1964a,b). We have used nitrogen gas agitation of the mixture during precipitation to avoid the need for mechanical apparatus. Bates (1965) did not stir during precipitation nor during the wash procedure and this omission may have contributed to the low recoveries (mean 70%) of some pesticides added to various crops.

Optimum precipitation time and efficiency of the low temperature bath were determined by varying the times lard solutions were exposed to the low temperature and then determining the residue in the supernatant after evaporation and drying to constant weight. The data are given in Table I as grams of residue remaining in the supernatant and as the percent removed as precipitate. These values compare favorably with the 99.96% value reported by McCully and McKinley (1964a) who used a carbon–Solka Floc (CSF) filter pad in their procedure. Cleanup was essentially complete (99.91%) at 10 min for the CSF filter, but never reached this level for the Solka Floc (SF) filter (98.2% at 40 min). Thirty minutes was taken as the minimum time for precipitation.

Although carbon in the SF filter increased the efficiency of the low temperature cleanup, it had an adverse effect on the recovery of some pesticides. The data in Table II indicate malathion recovery was good (86–98%) for the CSF and SF filter pads. Parathion and 2,4-D isopropyl ester recoveries were low and variable (24–69%) for the CSF filter, but good (73–101%) for the SF filter. These data indicate that carbon should not be used in the SF filter pad, as it could retain pesticides other than parathion and 2,4-D isopropyl ester. Varying amounts of water were used in this experiment to simulate that present in biological samples. Its presence did not affect the recovery from CSF or SF filters.

The effect of various concentrations of water on the recovery of three pesticides and their metabolites was determined (Table III). The 2,4-D acid was converted to its methyl ester prior to glc analysis by a procedure described by Yip (1962), except a 14% boron trifluoride reagent was used and the methyl ester partitioned into hexane. The recoveries are based on a methyl ester standard, not put through the procedure. The data indicate a satisfactory recovery of all pesticides, at all water concentrations. As well, it was not necessary to dry, by the use of anhydrous sodium sulfate, the concentrated filtrates prior to glc analysis with E.C. This indicates that water was removed by low temperature precipitation to a satisfactory level. For compounds containing phosphorus and/or sulfur glc analysis with the Melpar detector, low levels of water can be tolerated.

Table IV gives the recovery data for pesticides added to three different plant extracts and analyzed by the procedure. The mean (two determinations) recovery values are good (81–116%), but precision (percent difference) varies from good (±5%) to poor (±10%). The values are considered satisfactory for screening purposes. Peas, carrots, and wheat are

Table III. Recovery (%)[a] of Some Pesticides Added to Different Concentrations of Water

Added water, g	2,4-D isopropyl acid, μg			Fenithrothion, μg		Fenitroxon, μg		Malathion, μg		Malaoxon, μg	
	20	40	25	20	40	20	40	20	40	20	40
0.00	97	98	97	96	98	102	96	88	95	93	88
0.25	95	92	89	94	95	101	94	86	92	87	86
0.75	100	90	89	90	95	86	97	92	91	81	92
2.00	99	98	97	88	97	93	81	86	96	89	86
3.00	98	84	88	92	98	99	94	90	86	90	90
4.00	92	79	96	99	95	98	87	84	87	80	84

[a] Each column of figures represents a lot of six determinations.

Table IV. Percent Recovery as Mean of Two Analyses With ± % Deviation of Some Pesticides Added to Plant Samples

Sample	Pesticide, ppm and recovery									
Peas	Parathion		Paraoxon		Phosphamidon		Endosulfan		Dinitrobutylphenol anisole	
	1.0		0.2		0.1		1.0		0.2	
	88 ± 8		91 ± 4		91 ± 4		93 ± 7		87 ± 1	
Carrots	Parathion				Malathion				Captan	
	0.5		1.0		4		8		20	40
	98 ± 2		88 ± 9		103 ± 7		81 ± 9		116 ± 7	85 ± 2
Wheat	Malathion				Malaoxon		2,4-D acid		2,4-D ester	
	4.0	9.0	4.0		8.0	4.0		8.0	4.0	8.0
	100 ± 0	95.2	108 ± 11		97 ± 2	108 ± 10		82 ± 2	102 ± 1	93 ± 3

Table V. Recovery (%) of Some Pesticides Alone and From Spiked 5-g Samples of Human Tissues

Pesticide	Nanogram per assay	% Recovery			
		No tissue	Kidney	Liver	Fat
Parathion	500	99	81	104	98
Malathion	400	102	86	81	83
Malaoxon	400	100	86	85	85
Diazinon	325	101	92	114	—[a]
Phosphamidon	500	93	97	—	—

[a] —Not analyzed.

more difficult to clean up by other procedures because of oils and waxes, and this may be a factor in causing wide variation in precision.

Recovery data for spiked human tissues are given in Table V, based on the response of phosphorus emssion at 526 mμ only. The values for each pesticide in the presence of various tissue types were considered satisfactory, as they ranged from 81% for parathion added to kidney and malathion added to liver to 114% for diazinon in liver. It was concluded that these pesticides are stable to the reflux extraction conditions used here, either alone or in the presence of tissue.

CONCLUSION

The low temperature precipitation of water, fats, and waxes in acetone–benzene extracts of samples gave a supernatant satisfactory for glc analysis. Polar and apolar pesticides were quantitatively recovered.

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