Analysis of Free and Bound Chlorophenoxy Acids in Cereals

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Several studies have confirmed the conjugation of chlorophenoxy acids with plant constituents, as reviewed by LOOS (1969) and by FEUNG et al. (1973). The identity of the major part of conjugates is suggested to be combinations of chlorophenoxy acids and amino acids. In spite of this the nessessary hydrolysis of these conjugates is not performed by most of the published methods for chlorophenoxy acids. CHOW et al. (1971) studied the original procedure of YIP and NEY (1966) which involves extraction without hydrolysis with 50% diethyl ether/hexane in presence of sulphuric acid. By heating wheat plant tissue under dilute alkaline conditions they found that less than 25% of the residue could be accounted for by analyzing only the organic phase of the YIP and NEY (1966) method.

As part of the investigations described here, initial experiments were made to adapt the CHOW et al. (1971) procedure to the analysis of cereal grains. Due to strong gelatinization of starches which excluded all further separation and clean up, these experiments, however, were unsuccessful. Attempts to eliminate this effect of starches through a complete hydrolysis by prolonged heating and increase of the alkali concentration were also unsuccessful due to a simultaneous destruction of the chlorophenoxy acids. To solve these problems a procedure has been developed by which the starch is hydrolyzed under acidic conditions followed by treatment with diastase and further hydrolysis of amino acid conjugates through the action of proteolytic enzymes. This extraction procedure will be referred to in the following as procedure A. By analysis of chlorophenoxy acid treated barley grains the procedure A was compared with the extraction procedure of YIP and NEY as described in FDA's Pesticide Analytical Manual (1968), which will be referred to as procedure B. Finally a later extraction procedure of YIP (1971) (procedure C), was compared with the present procedure A. In procedure C acetonitrile is used as extraction solvent.

Bulletin of Environmental Contamination & Toxicology,
Vol. 13, No. 6 © 1975 by Springer-Verlag New York Inc.

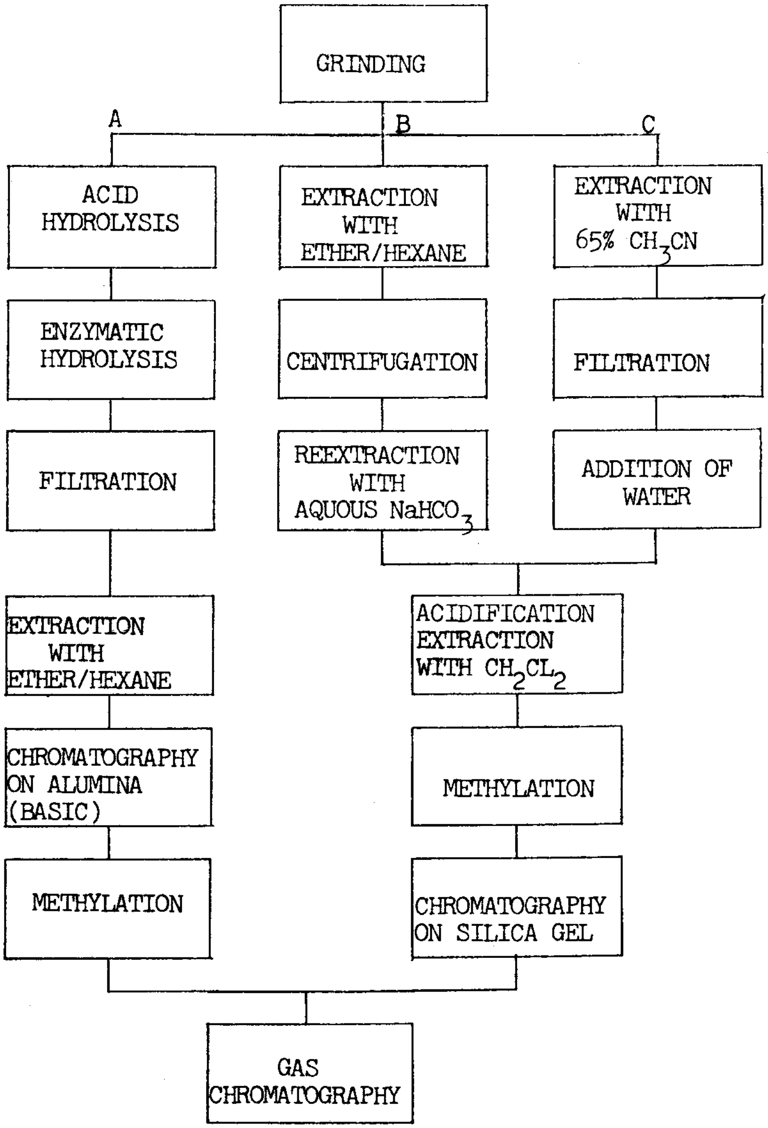

Fig. 1: Flow diagram showing the analytical operations in three different procedures.

Experimental

Methods of analysis. The diagram in fig. 1 shows the analytical operations in procedures A, B and C. After the aqueous hydrolytical step in method A and after the extraction with organic solvents in methods B and C, different procedures of clean-up are required. In procedure A alkaline alumina was used for purification of the extracts and in the procedures B and C silica gel was used for clean-up of the methylated chlorophenoxy acids. No significant differences between the recoveries from these two clean-up procedures were found when free chlorophenoxy acids were added to untreated barley as shown in table 1.

TABLE 1

Recoveries from study of three extraction procedures.

Procedure	mg/kg added to untreated barley	Ranges of recovery diclorprop	Ranges of recovery 2,4-D
A	o,1-1,o	75-99%	72-82%
B	o,1-1,o	76-8o%	72-95%
C	o,5	77-1oo%	68-1o2%

Reagents and solvents. Millipore Super Q purified water was used for all aqueous reagents. Sodium chloride, sodium hydroxide, sodium hydrogen carbonate, sodium sulphate, potassium chloride, orthophosphoric acid, phosphtotungstic acid, sulphuric acid, ethanol and iso-octane were all of reagent grade. The sodium sulphate was further purified by heating at 500°C for 5 hours followed by addition of 0.1% concentrated sulphuric acid to prevent the absorption of chlorophenoxy acids which takes place with batches of sodium sulphate with alkaline properties. Acetonitrile, petroleum ether and diethyl ether (commercial grade), hexane and dichloromethane (reagent grade) were purified by fractionated distillation. Alumina (basic, BDH, Brockmann grade 1), silica gel (Merck, 0,05-0.2 mm), diastase (BDH no. 39013) papain (BDH no. 39030) and ficin (Schwarz and Mann, no. 900182) were used without further purification. Hyflo Super-Cel was employed as a filter aid, having previously been purified by shaking in 1 N hydrochloric acid, filtering, rinsing in water and ethanol, and drying. Diazomethane in diethyl ether was prepared from Diazald (Aldrich Company) according to manufacturer's directions.

Extraction procedure A. 25 g of barley grains were milled and treated at 100°C for 60 minutes with 100 ml 5% phosphoric acid, followed by treatment with 0.5 g diastase at 50°C for 2 hours at pH 5,1 in the presence of chloride ions through addition of 10 ml 1 N potassium chloride. Mechanical stirring was employed. Additional treatment was performed with proteolytic enzymes (papain or ficin) overnight at room temperature and pH 5.1 in the presence of a few crystals of sodium thiosulphate. After addition of 10 ml 20% phosphotungstic acid and 3 g Hyflo Super-Cel the mixture was filtered through Hyflo Super-Cel in a Büchner funnel. The filter cake was rinsed carefully with water. The combined filtrates were extracted three times with 75 ml 50% diethyl ether/petroleum ether. The emulsion formed by shaking was removed by addition of ethanol or a saturated solution of sodium chloride in water. The combined organic phases were dried with anhydrous sodium sulphate.

Extraction procedure B. Extractions were performed as described in FDA's Pesticide Analytical Manual (1968) with diethyl ether/hexane/10% ethanolic sulfuric acid (5:5:1) followed by repeated centrifugation and re-extraction with a 1% aquous solution of sodium hydrogen carbonate. After acidification the chlorophenoxy acids were subsequently extracted into dichloromethane and the combined dichloromethane extracts were dried with anhydrous sodium sulphate.

Extraction procedure C was performed with 65% acetonitrile/water after the latest procedure of YIP (1971), followed by filtering of the extract on Hyflo Super-Cel in a Büchner funnel. To an aliquot of the combined filtrates were added a threefold amount of a 1% aquous solution of sodium hydrogen carbonate and 15 ml of a saturated aquous solution of sodium chloride. After washing three times with dichloromethane, the solution was acidified and extracted three times with dichloromethane. The combined dichloromethane extracts were dried with anhydrous sodium sulphate.

Alumina chromatography and methylation of extract A. The combined diethyl ether/petroleum ether extracts or a suitable aliquot from the extraction procedure A were purified by passage through a column containing 10 g alkaline alumina. The alumina was washed with 100 ml diethyl ether and dried with nitrogen. The chlorophenoxy acids were eluted with 100 ml 1% aqueuos sodium hydrogen carbonate. The aqueuos solution was acidified and extracted three times with dichloromethane. After addition of 1 ml iso-octane the combined dichloromethane extracts were evaporated to ½ ml. Further 1 ml iso-octane was added and partly evaporated to remove remaining dichloromethane. The chlorophenoxy acids were methylated by addition of 1 ml of diazomethane reagent and the reagent was allowed to evaporate at room temperature in a well-ventilated fume cupboard. The residue was dissolved in petroleum ether for gas chromatographic analysis.

Methylation and silica gel chromatography of extracts B and C. The combined dichloromethane extracts B or C were evaporated to ½ ml and methylated with diazomethane as described above. The methyl esters of chlorophenoxy acids were transferred to a column containing 10 g silica gel completely wetted with 10% diethyl ether/hexane. Elution was performed with 175 ml 10% diethyl ether in hexane. The first 25 ml eluate were discarded and the following 150 ml were evaporated to 10 ml for gas chromatographic analysis.

The final determination was performed by gas chromatography with electron capture detector. The peak heights of samples were compared with peak heights of methyl esters of chlorophenoxy acids produced by the same methylation procedure. Several blank peaks occasionally originating from reagents did not interfere on at least one of the following stationary phases: 2% OV 17, 2% OV 225, 1% Apiezon L or a mixture of equal amounts of 7.5% DC 200 and 5% QF 1, all coated on chromosorb G, 100/120 mesh. The limits of detection for dichlorprop and 2,4-D methyl esters were 0.02 mg/kg. The recoveries found by addition to untreated samples are shown in table 1.

Spraying experiments. Barley was treated in parallel with 4 kg 2,4-D per ha and with 8 kg dichlorprop per ha at the following three times:
(I) just before the spikes were formed
(II) when spikes were formed, but before grains were developed
(III) When green grains were fully developed.
In addition untreated barley was grown. In all experiments samples were taken at normal harvest time. It is noted that all applications were done at concentrations about 4 times higher than practised under normal use conditions and that the spraying times (II) and (III) are normally not to be expected.

Results.

The samples from the spraying experiments were analyzed by procedure A and B after a few months storage at -25°C. By repeating the analysis by procedure B supplemented with analysis by procedure C after 18 months storage at -25°C no decrease in residues was found by procedure B. The results of this study are shown in table 2. All figures are given as mg chlorophenoxy acid pr kg barley grain without correction for losses during analysis. It is apparent from the data in table 2 that the enzymatic hydrolytic procedure A is much more efficient than the procedure B. Approximately 10 times higher residues of dichlorprop and approximately 5 times higher residues of 2,4-D were found by analysis by procedure A in comparison with the procedure B. These results from the analysis of grains from barley are in accordance with the investigations of CHOW et al. (1971), who found that more than 75% of the residue in wheat plant material would be lost by analyzing only the organic phase in a method similar to procedure B. Further it is confirmed that the acetonitrile extraction procedure of YIP (1971) yields results of residues which are nearly as high as those found by the procedure A.

TABLE 2

Results in mg/kg from study of three extraction procedures.

Compound and treatment	Time of treatment	Extraction procedure A	B	C
Dichlorprop	I	0,89	0.16	0.51
8 kg a.i./ha	II	1.38	0.25	1.00
	III	9.0	1.76	6.8
2,4-D	I	0.06	<0.02	0.04
4 kg a.i./ha	II	0.45	0.04	0.35
	III	4.3	0.51	4.0

I	spikes not formed
II	spikes formed, but grains not developed
III	green grains fully developed
A	Extraction after acid hydrolysis and enzymatic degradation
B	Extraction with 50% diethyl ether/hexane after YIP and NEY (1966)
C	Extraction with acetonitrile after YIP (1971)

Discussion.

These investigations clearly demonstrate that the extraction procedure is of major importance in the determination of chlorophenoxy acid residues in grains. The conventional procedure of extraction with a mixture of diethyl ether and hexane (procedure B) is insufficient, while the procedures A and C yield nearly equal results at a much more efficient level. As a part of the present experiments it has been found that ommission of the treatment with either papain or ficin in procedure A gives rise to losses of 2,4-D and dichlorprop in the order of 50% of total residues at all levels. In the light of this efficient liberation of residues through hydrolysis by proteolytic enzymes, it is remarkable that the simple extraction with acetonitrile of procedure C is of a similar efficiency. Supplementary analysis of filter cakes and mild alkaline hydrolysis of extracts (not described here) from both of the procedures A and C have further indicated that still unhydrolyzed chlorophenoxy acid conjugates with plant material may be present even after combined acid hydrolysis and enzymatic treatment by procedure A. Neither the proposed method A, nor the comparable acetonitrile procedure C can for these reasons be characterized as exhaustive. They present, however, greatly improved attempts to determine free as well as bound chlorophenoxy acids in grains of cereals.

Summary.

Extraction of the chlorophenoxy acids 2,4-D and dichlorprop in cereals has been examined by analyzing barley from spraying experiments. A procedure has been set up by combination of acid hydrolysis and enzymatic degradation followed by extraction and clean up on either silica gel or basic aluminum oxide. The final determination is based on reaction with diazomethane and subsequently GLC with ECD. This procedure was compared with two different extraction procedures previously described in the literature. The one comparative procedure uses a mixture of 50% diethyl ether/hexane in presence of sulphuric acid and resulted in residues up to ten times lower than found after the combined acid hydrolysis/enzymatic degradation procedure. In the second comparison a direct extraction was made with a mixture of 65% (v/v) acetonitrile in water. No differences were found between this and the combined acid hydrolysis/enzymatic degradation procedure.

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