Determination of 2,3,6-Trichlorobenzoic Acid Residues in Grain and Straw with Electron-Capture Gas Chromatography

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The determination of 2,3,6-trichlorobenzoic acid in concentrations as low as 0.008 p.p.m. in 3-gram samples of grain and 0.003 p.p.m. in 5-gram samples of straw with a gas chromatographic procedure with electron-capture detector was made possible through development of an improved cleanup procedure. The sample is extracted with a solution of phosphoric acid in ether. Acid substances are then isolated by extraction with a phosphate buffer of pH 7. Substances with a high electron affinity, which would give interfering gas chromatographic peaks, are masked by catalytic hydrogenation with sodium borohydride in water solution. The water solution is then acidified and the trichlorobenzoic acid extracted into toluene. After blowing in gaseous diazomethane, the toluene solution is injected into the gas chromatograph. The use of small samples makes it possible to carry out reactions and extractions of a large series of samples in centrifuge tubes, which, with the use of transfers of aliquots instead of exhaustive extractions, makes the method fast and convenient. Recoveries from fortified samples are between 82 and 94%. Results from field-treated samples of grain, straw, and dried green herb are reported.

A method for the determination of 2,3,6-trichlorobenzoic acid (TBA) in oil was described by Yip (1964). Kirkland described methods for the determination of chlorinated benzoic acids in herbicidal formulations (1961), and Kirkland and Pease (1964) described a method for residues in grain, plant tissues, and soils.

Grain and especially straw contain compounds which behave like and interfere with TBA, and even elaborate extraction methods and chromatographic methods did not separate them from TBA. The interfering compounds do not, however, contain halogen. Yip (1964) and Kirkland and Pease (1964) therefore used a coulometric detector for residue detection and determination. The sensitivity of this detector is low. Therefore large samples (20 to 50 grams) must be used, and this makes the procedure tedious.

A simple and fast method for large numbers of samples was needed in our laboratory. The extraction method was therefore improved by use of more selective solvents, and, as this was not sufficient, the electron affinity of the interfering compounds was decreased by hydrogenation. These modifications made it possible to use the electron-capture detector for measurement.

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METHOD

Apparatus. The mill used was a Slago 200-A, Kamas Kvarnmaskiner A.B., Malmö, screen 0.8 mm. or similar mill, giving a particle size distribution such that at least 95% by weight are smaller than 500 microns and 50% of those are smaller than 177 microns.

An ultrasound generator was used for cleaning electron capture detectors, hydrogenating, etc.

GAS CHROMATOGRAPH. An Aerograph 600 D with a tritium source electron-capture detector and a back-flush valve was used. The column was 0.25 inch, 1 meter, 2% of polyethylene glycol adipate (PGA) on 100- to 120-mesh DMCS-treated, acid-washed Chromosorb W. Column temperature was 135° C., injector temperature 165° C., and nitrogen flow rate 70 ml. per minute. Columns used for confirmation of identity were: a 10% Versamid 900, Varian 82-1695 on 100- to 120-mesh DMCS-treated, acid-washed Chromosorb W, 1-meter, 0.125-inch steel column, and a 1-meter, 0.25-inch steel column with 20% silicone elastomer SE-30 on the same support.

Glass-fiber filter, Arthur H. Thomas, No. 5270-D grad 934-AH, 24 mm.

Reagents. Diethyl ether, distilled from sodium, AB Synthes, Nol, Sweden, or similar highly purified product.

TBA STANDARD I. Dissolve 5.00 mg. of TBA in ether and dilute to 100 ml.

TBA STANDARD II. Dilute 10.0 ml. of standard I to 100 ml. with ether.

TBA METHYL ESTER. Methylate 2.00 ml. of TBA standard according to the procedure described below and dilute to 50.0 ml. with toluene.

HEXACHLOROBENZENE STANDARD. Dissolve 0.15 mg. of hexachlorobenzene in 100 ml. of toluene.

PHOSPHORIC ACID–ETHER. Dissolve 50 ml. of phosphoric acid (specific gravity 1.71) in 800 ml. of redistilled anhydrous diethyl ether and dilute to 1000 ml. Prepare weekly.

PALLADIUM CHLORIDE. Dissolve 0.2 gram of palladium chloride, $PdCl_2$, in 5 ml. of 0.5*M* hydrochloric acid, if necessary with slight warming. Add 5 ml. of water after $PdCl_2$ is completely dissolved.

DIAZOMETHANE. Prepare diazomethane according to the procedure used by Kirkland (1961), but use *N*-nitroso-*N*-methylurethane instead of Diazald, and introduce a 30-cm. Vigreux column into the distillation apparatus between the distillation flask and the condenser. Keep the reagent in a freezer at −20° C. in a round-bottomed vacuum flask with a stoppered unlubricated Clearfit glass joint. It can be used as long as it is distinctly yellow, usually about 3 weeks. Be careful! Diazomethane is very poisonous!

Toluene and *n*-pentane are distilled slowly with a 50-cm. Vigreux column to remove higher boiling impurities.

BUFFER, pH 7.0. Titrate a solution of 0.5*M* potassium dihydrogen phosphate with 10*M* sodium hydroxide to pH 7.0. Extract 500 ml. of the solution with 10 ml. of toluene and then with 10 ml. of pentane.

1*M* PHOSPHORIC ACID AND 7.5*M* HYDROCHLORIC ACID. Extract 500 ml. of the solutions with 10 ml. of toluene and then with 10 ml. of pentane.

Procedure. GRAIN. Shake 3.0 grams of the ground and well mixed grain vigorously with 10.0 ml. of phosphoric acid–ether in a glass-stoppered test tube for 3 minutes. Add 4 ml. of 1*M* phosphoric acid, shake for 1 minute, stopper, and centrifuge at 2500 r.p.m. for 5 minutes. Pipet 5.0 ml. of the clear ether phase into another tube, add 6.0 ml. of the buffer, and shake vigorously for 2 minutes.

STRAW. Shake 5.0 grams of the ground and well mixed straw vigorously with 30.0 ml. of phosphoric acid–ether in a glass-stoppered test tube for 3 minutes. Add 30 ml. of 1*M* phosphoric acid and shake for 1 minute. Allow to settle for 2 minutes and pour the ether phase into a glass-stoppered centrifuge tube. Stopper and centrifuge for 2 minutes. Pipet 15.0 ml. of the clear ether phase into another tube, add 6.0 ml. of the buffer, and shake vigorously for 2 minutes.

CONTINUED TREATMENT OF EXTRACTS. Centrifuge for 3 minutes. Draw off the ether phase with a pipet connected to a water pump via a suction flask. Add 2 ml. of ether to the water phase of the grain sample and 10 ml. to that of the straw sample. Shake for 15 seconds. Centrifuge. Draw off the ether and shake the water phase with 2 ml. of pentane. Centrifuge, draw off the pentane in the same manner, and evaporate residual pentane with a gentle stream of air drawn over the water surface with the same pipet.

HYDROGENATION. Add 50 μl. of palladium chloride solution to the water phase and place the tubes in the ultrasonic bath. Add about 10 mg. of sodium borohydride and after 2 minutes about 5 mg. more. Acidify after 2 minutes with 2.0 ml. of 7.5*M* hydrochloric acid. Continue the ultrasonic treatment for another 2 minutes. Suck the solution through a dry glass fiber filter and pipet 6.0 ml. of the filtrate into a glass-stoppered test tube.

Add 0.50 ml. of toluene and shake for 2 minutes. Centrifuge for 2 minutes. Pipet 0.3 ml. of the toluene phase into a small test tube, avoiding the water phase.

METHYLATION. Use the distilling apparatus shown in Figure 1. Wear disposable plastic gloves and carry out the operation under a hood. Pipet about 0.5 ml. of diazomethane solution into test tube *A* and put on the inlet tube. Warm the solution (not the air space above it!) with the hand or by dipping it into a beaker of tepid water and introduce the tip of the tube into the sample solution. Continue until the toluene shows a weak but distinct yellowish tinge throughout the whole solution, which persists for at least 10 seconds.

Inject 5 μl. of the methylated solution into the gas chromatograph. Reverse the gas flow through the column with the back-flush valve after TBA-peak has been eluted, and allow the impurities to pass out. Reverse again and inject the next sample as soon as the base line is stable. Retention time of TBA is 8 minutes, necessary back-flush time about 10 minutes.

Check the sensitivity of the detector with 5 μl. of the TBA–methyl ester and include one TBA-free sample and two fortified samples with different TBA contents in every series of analyses. Compare the results of these runs with the calibration curve and calculate empiric correction factors for calculation of the TBA contents of the unknowns.

Calibration Curves. Add different volumes of TBA standard solution to samples of ground TBA-free grain or straw. Use the standard which corresponds to the level of TBA in the samples. Treat the samples according to the procedures described above.

IDENTIFICATION

In case of doubt, confirm the identity of the gas chromatographic peak by adding hexachlorobenzene to the

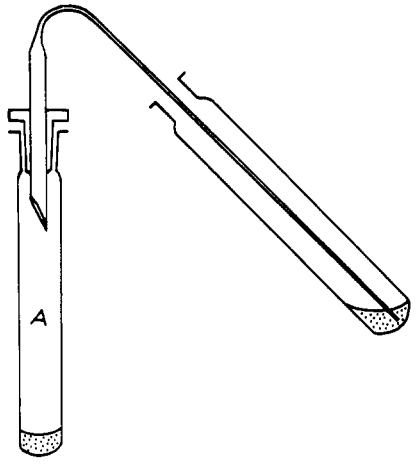

Figure 1. Apparatus for methylation with diazomethane

A. 5-ml. test tube with polyethylene stopper

sample and running the sample on different columns. Compare the relative retention times.

Procedure. Inject 5 μl. of the sample solution together with 1 μl. of the hexachlorobenzene solution. Reduce the sensitivity of the detector during the elution of hexachlorobenzene.

A large amount of hexachlorobenzene is used because the extracts of grain and straw contain a compound which has the same retention time as hexachlorobenzene, which interferes with the identification of a small amount. Data obtained with the confirmation procedure are given in Table I.

RESULTS

Samples of TBA-free grain and straw were fortified with TBA and analyzed according to the procedure described. Calibration curves are shown in Figures 2 and 3.

Figure 4 shows the chromatograms obtained in an experiment to evaluate the detection limits in grain and straw. Apparently 0.003 p.p.m. can safely be detected in straw, while the limit is about 0.008 p.p.m. in grain.

In a series of experiments TBA was added to samples or to extracts at different stages of the procedure. The yields and losses are reported in Table II. Analyses of field-treated grain, straw, and green herb are reported in Table III.

DISCUSSION

Extraction of Sample. In the procedure presented, phosphoric acid, which displaces TBA from the starch, is dissolved in anhydrous ether. Apparently, the phosphoric acid penetrates between and into the unswelled particles and liberates the TBA, which is taken up by the ether. Kirkland and Pease (1964) used a mixture of methyl ethyl ketone, phosphoric acid, and water for this extraction.

Table I. Isothermal Retention Data for TBA on Three Columns[a]

Column	Column Temp., ° C.	Retention Times, Min. TBA, t_1	Hexachlorobenzene, t_2	t_1/t_2
PGA	134	8.82	8.97	0.98
SE-30	136	18.75	40.70	0.46
Versamid	136	10.45	22.30	0.47

[a] Gas flow rate in all columns 70 ml./min.

Table II. Yields of TBA Added at Different Stages of Procedure[a]

TBA Added to	Ground Sample	Etheral Extract	Buffer: Before hydrogenation	Buffer: After filtration
Grain, p.p.m.				
0.67	94	83	98	105
0.20	90	81	81	101
0.10	83	78	81	90
Straw, p.p.m.				
0.67	94	87	95	95
0.20	88	73	97	91
0.10	82	86	80	89

[a] Calculations based upon yield of TBA added to toluene extract. This yield was set 100%.

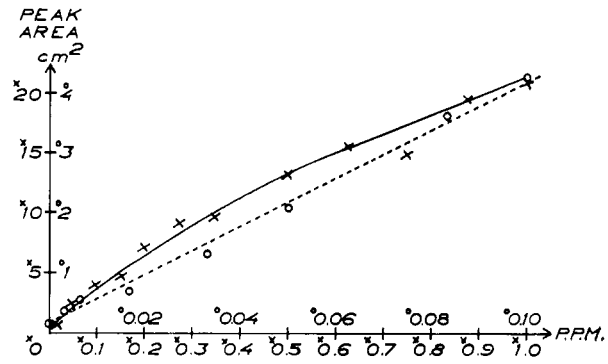

Figure 2. Calibration curves for TBA in grain

× High concentrations of TBA
○ Low concentrations of TBA

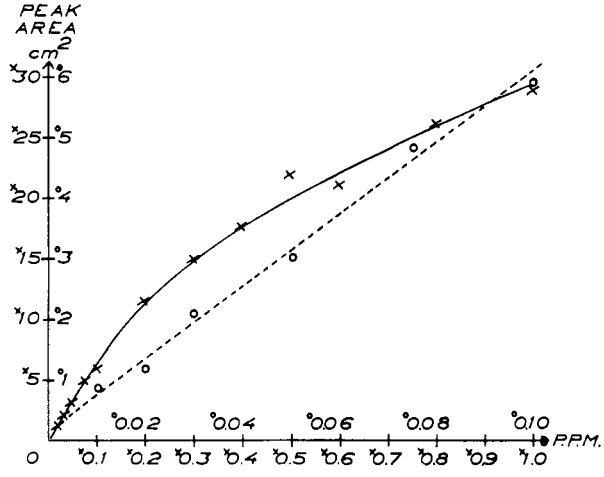

Figure 3. Calibration curves for TBA in straw

× High concentrations of TBA
○ Low concentrations of TBA

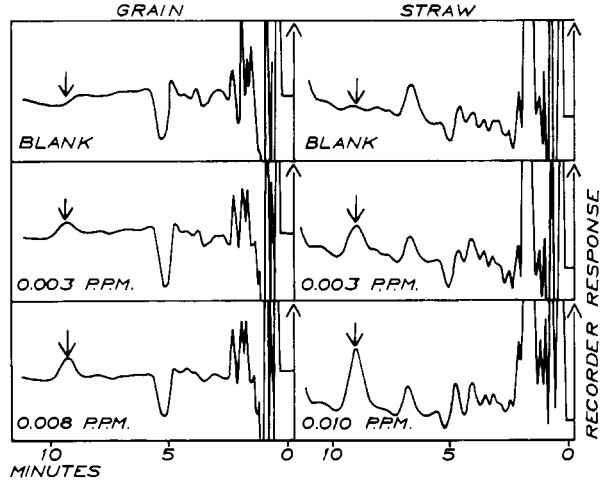

Figure 4. Tests for sensitivity of detection of TBA

Detector sensitivity attenuator 2

Table III. Analyses of Field-Treated Grain, Straw, and Dried Green Herb[a]

Fields sprayed with water solutions of TBA

Sample	Dosage, Grams per Acre	Results, P.P.M.				
Barley						
Grain A	0	<0.008	<0.008	<0.008		
Grain B	81	0.26	0.22	0.23		
Grain C	320	1.08	0.96			
Grain D	0 ?	0.26	0.26			
Grain E	0	<0.008	<0.008			
Grain F	81	0.13	0.13	0.14	0.14	0.14
Grain G	320	0.61	0.46	0.65	0.64	0.59
Straw H	0	0.008	0.008			
Straw I	65	0.010	0.008			
Green herb J	81	0.39	0.40	0.46		
Green herb K	320	3.8	3.3	3.6		
Wheat						
Grain L	121	0.02	0.03			
Grain M	121	0.03	0.03			
Straw N	121	0.16	0.22			
Straw O	121	0.18	0.16			

[a] Samples taken from small experimental fields. High TBA content in grain D might have been caused by wind drifting.

To evaluate the results of the extraction method, experiments were made to hydrolyze the finely ground grain by heating it with dilute acid and also with diastase and by exhaustive extraction of the TBA from the liquid hydrolyzates. Reproducible results were obtained in this manner with both fortified and field-treated samples. In no case were the results higher than those obtained with the method presented. The operations were more tedious, and the gas chromatograms obtained contained more and higher interfering peaks than those obtained with the method described.

The excess of phosphoric acid is removed from the ether phase by extracting it into a water solution of 1*M* phosphoric acid, which also displaces much of the ether from the interstices of the samples. The remaining concentration of phosphoric acid in the ether phase is less than 0.001*M*.

Cleanup. The distribution of TBA between ether and water solutions of different acidities was determined by extraction and spectrophotometric measurement (Figure 5). Complete extraction into the water phase is obtained at a pH value slightly below 7 and upward. Extraction from fortified sample extracts was then tried at a few pH values between 7 and 10. The results were measured with a gas chromatograph using the procedure described above. At pH 7, the interference from other extracted substances was least, and the extraction of TBA was still complete. To decrease the co-extraction of interfering substances, smaller volumes of water were tried. However, solid precipitates were obtained in the ether phase, and the analyses gave low recoveries. Analyses of the isolated precipitates showed that they contained much TBA.

In the extraction of TBA into toluene, low yields were obtained when the water phase was acidified with 0.5 ml. of

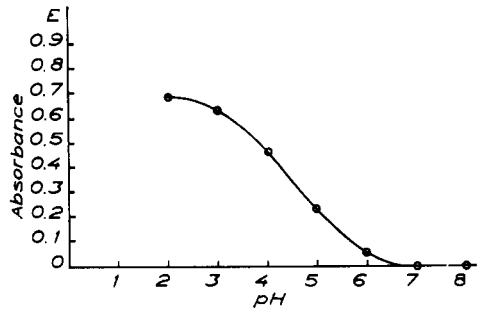

Figure 5. Partition of TBA at different acidities

5-ml. portions of ether solution containing 0.3 mg. of TBA per ml. extracted with 5-ml. portions of phosphate buffers of different acidities. Absorbances of ether solution measured in 1-cm. cuvette at 285 nm. after extraction

7.5*M* hydrochloric acid. One to 3 ml. gave complete extraction. Chloroform (Yip, 1964) and hexane (Kirkland and Pease, 1964) had been recommended for this extraction. Chloroform appeared inadvisable in our case because of its chlorine content. With hexane several extractions were necessary to obtain a quantitative yield. Ether gave a quantitative extraction, but extracted more extraneous material than toluene. Also the ether extract had to be dried before the methylation. Toluene extracts so little water that the extract can be methylated and injected directly into the gas chromatograph without drying.

With straw and a few samples of grain, the extractive cleanup was not sufficient; small interfering peaks were still obtained in the gas chromatograms. Experiments with thin-layer chromatography (Henkel, 1965) and column chromatography on aluminum oxide with dilute acetic acid as eluent did not give sufficient separation. Herrmann (1967) found *p*-cumaric acid, ferulic acid, sinapic acid, and caffeic acid in straw. These substances could be expected to pass through the extraction procedures and through the chromatographic procedures like TBA. They do not contain chlorine, as does TBA, but their high electron affinity is mainly due to their unsaturated character. Catalytic hydrogenation described by Brown and Brown (1966) was therefore modified for this method. Larger amounts of catalyst than described above decreased the yield of TBA and did not significantly improve the hydrogenation of extraneous materials. The procedure takes about 7 minutes. Prolonged hydrogenation does not improve the effect, nor does the addition of larger amounts of sodium borohydride. Ultrasonic agitation gives a better effect than conventional shaking and stirring, which is not surprising from kinetic considerations.

The methyl esters of the acids mentioned above were analyzed by gas chromatography. Considerable decomposition occurred in the column, and none of their peaks coincides with TBA. No further experiments were made to identify the interfering compounds. Figure 6 shows that the hydrogenation causes a considerable decrease of the total area below the recorder trace of straw extract. This cannot be explained by a mere displacement of the peaks. A masking effect due to a decrease of the electron affinity of the interfering compounds appears to be the most probable explanation.

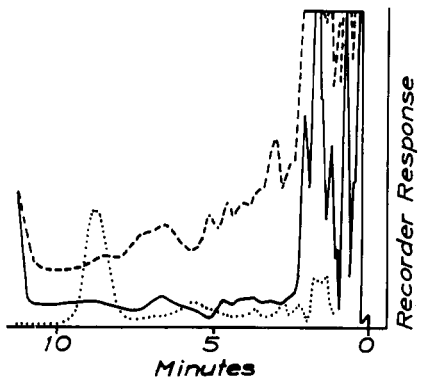

Figure 6. Chromatograms of extract from straw

--- Unhydrogenated extract
—— Hydrogenated extract
· · · TBA–methyl ester
Detector sensitivity attenuator 8

Several interfering peaks originated from impurities in the solvents. Therefore solvents must be distilled to remove high boiling electron-capturing materials. The buffer solution, the hydrochloric acid, and the phosphoric acid are extracted with toluene and pentane.

Apparatus for methylation with diazomethane shown in Figure 1 was convenient. There is no risk of contamination from high boiling compounds in the diazomethane solution, and the volume of the sample solution is not significantly affected. Experiments with repeated methylations and with methylation in other solvents showed that the methylation is complete as soon as the solution shows a slight yellowish tinge.

Gas Chromatography. The polyethylene glycol adipate column was the most efficient column tried.

The methylated extracts from straw and grain contain materials which give high peaks and have long retention times. It would, therefore, be necessary to purge the apparatus for a long time after elution of the TBA to get a stable base line. This is avoided by reversing the gas flow through the column with a back-flush valve after elution of TBA.

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