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Methods for the Determination of Diphenylamine Residues in Apples

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Two methods for the determination of diphenylamine residues in apple peel were devised and used during a fruit storage experiment. One was based on extraction in a Soxhlet apparatus and formation of the fluorobutyryl derivative for electron-capture gas chromatographic determination; the second method used steam distillation and direct determination of diphenylamine with a nitrogen-sensitive thermionic gas chromatographic detector. The latter method, after correction for recovery losses, gave residue levels ca. one-third higher during the 118 days of the storage experiment.

The possible withdrawal (in the United Kingdom) of the chemical now used for scald control has prompted further investigation of the levels of diphenylamine on apples, following laboratory and commercial scale storage trials. A number of methods based on spectrophotometric procedures have been described for the determination of diphenylamine residues. Yatsu (1956) and Harvey (1958) utilized the blue oxidation product produced with vanadium pentaoxide in sulfuric acid while Bruce et al. (1958) coupled diphenylamine with diazotized 2,4-dinitroaniline. As these methods appeared to lack the selectivity and sensitivity required, a gas chromatographic technique was sought. The method described by Gutenmann and Lisk (1963) in which diphenylamine is converted to an electron-capturing bromo derivative was unsatisfactory because it gave significant blank values with the fruit used and was insufficiently sensitive.

A method using another diphenylamine derivative which had greater specificity and a lower limit of detection was therefore developed. Subsequently, during the analysis of the prestorage samples, other apparatus and equipment became known and available to us which allowed a more simple method to be devised. Both methods are given as their utility depends on the equipment available.

METHODS

(i) General. Treatment of amines with heptafluorobutyric anhydride yields derivatives with excellent electron-capture sensitivity; this reaction has been utilized, for example, in the determination of ethoxyquin (Winell, 1976) and carbofuran (Lawrence et al., 1977). Diphenylamine also reacts in this way, but when used with spiked apple extracts no derivative was obtained, presumably due to interfering compounds extracted from the apple.

A cleanup step was therefore essential prior to reaction with the anhydride. A number of conventional procedures were tried, including partition with an acidic aqueous phase and column chromatography on alumina or magnesium oxide/Celite, but none of these were successful. A suitable procedure is given below.

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During the course of this work a GC with a heated bead nitrogen detector became available to us, making it possible to determine diphenylamine directly without cleanup and derivatization. In initial work with this detector, a Soxhlet extractor was used to extract the diphenylamine but the direct extracts caused some contamination of the GC column; however, a combined steam-distillation and solvent extraction apparatus was then used which gave clean extracts.

(ii) Extraction. (*a*) *Preparation of Fruit.* Diphenylamine was determined in apple peelings approximately 1 mm in thickness. For some poststorage samples the apple flesh (i.e., all the tissue remaining after removal of the peel) was also analyzed.

Each individual apple was weighed and its surface area obtained from tables (Turrell, 1946) after measurement of its major and minor axes. Peel from each apple was extracted either in a Soxhlet extractor or using a special steam-distillation and solvent extraction unit (Veith and Kiwus, 1977).

(*b*) *Soxhlet Extraction.* For Soxhlet extraction, 150 mL of petroleum ether (boiling range 60–80 °C) was placed in a 250-mL B24 round-bottom flask and a 100-mL capacity Soxhlet extractor containing the chopped sample was attached. The petroleum ether was allowed to boil under reflux for 3 h. The extract was dried over anhydrous sodium sulfate and then made up to 150 mL.

(*c*) *Steam Distillation/Solvent Extraction.* In this method the peelings or, in some investigations, the flesh was placed in a 500-mL B24 round-bottom flask, 200 mL of water was added, and the steam-distillation/solvent extraction apparatus was attached. The apparatus was filled with water to a depth of 10–20 mm and 20 mL of petroleum ether (boiling range 60–80 °C) added with a pipet to form an upper layer. The contents of the flask were allowed to boil vigorously for 60 min after which the petroleum ether layer was transferred. Three rinses of 5 mL of distilled water, 3 mL of petroleum ether, and 5 mL of distilled water were sufficient to transfer all the extract through a Whatman phase-separating paper into a 20-mL volumetric flask. The petroleum ether rinse was almost sufficient to replace solvent which was "lost" either as a

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surface layer in the apparatus or by evaporation.

(iii) Cleanup. A portion (usually 5 mL) of the petroleum ether apple extract was added to an equal volume of 5% aqueous sodium hydroxide solution in a stoppered tube. The mixture was heated in a water bath at 50 °C for 30 min and then cooled to room temperature. A 1-cm diameter chromatography column fitted with a sintered-glass disc and a tap was filled to a depth of 4 cm with sodium metabisulfite. A 2-mL portion of the base-treated petroleum ether extract was passed through the column and eluted with a further 10 mL of petroleum ether.

All the eluate was collected and concentrated to 2 mL in a stream of air. It has been reported elsewhere (Harvey, 1958; Gutenmann and Lisk, 1963) that diphenylamine is lost during evaporation in a stream of air; we found no significant loss provided that the sample was not allowed to evaporate to dryness.

(iv) Derivatization. The solution obtained after cleanup was treated with 20 μL of heptafluorobutyric anhydride and 0.1 mL of 0.05 M trimethylamine in benzene as catalyst. The mixture was heated at 50 °C for 30 min, cooled to room temperature, and shaken with 1 mL of water for ca. 1 min to hydrolyze any excess anhydride. One milliliter of 5% aqueous ammonia solution was then added and the mixture shaken for another ca. 1 min to extract all the heptafluorobutyric acid into the aqueous phase. An aliquot (2 mL) of the petroleum ether phase was used for GC.

Known amounts of pure diphenylamine, at similar concentrations to the samples, were derivatized in the same way for use as standards. The identity of the derivative was confirmed by preparation of a larger amount, multiple recrystallization from petroleum ether and analysis (mp 64 °C; IR absorption by the carbonyl group at 1690 cm^{-1}; GC-MS peaks: m/e 365 (M), 100%; m/e 168 ($C_{12}H_{10}N$), 88%; m/e 197 (C_4F_7O), 35%...).

(v) Gas Chromatography. (*a*) *Determination of Diphenylamine as Its Heptafluorobutyryl Derivative.* The chromatograph used was a Pye 104 with a GCV amplifier (which operates at constant current and a pulse frequency proportional to sample concentration) and a ^{63}Ni electron-capture detector. A 3 ft, 4-mm i.d., glass column of 2% phenyldiethanolamine succinate on 80–100 mesh Chromosorb G, AW-DMCS, was operated at 125 °C with an inlet temperature of ca. 180 °C, a detector temperature of 250 °C, and a carrier gas (nitrogen) flow rate of 125 mL min^{-1}. A typical chromatogram obtained after taking pure diphenylamine through the derivatization procedure is shown (Figure 1A), together with a chromatogram of a treated apple extract following cleanup and derivatization (Figure 1B). A derivatized untreated apple extract gave no interfering peak in the area corresponding to the diphenylamine derivative. The retention time of the diphenylamine derivative was 194 s. (For comparison, the retention time of Aldrin under the same conditions was 818 s.) The lower limit of detection (three times noise level) was 0.05 ng of diphenylamine, giving a limit of detection for quantitative measurement of ca. 0.001 ppm based on peel from a single apple. The detector response (peak height) was linear over at least the range of 0 to 10 ng.

(*b*) *Direct Determination of Diphenylamine.* A Perkin Elmer F33 gas chromatograph with a heated rubidium bead phosphorus–nitrogen detector was used. A 1 m, 3 mm i.d., glass column with a mixture of 3% OV-17 and 0.02% Epikote 1001 on 80–100 mesh Gas-Chrom Q was operated at 200 °C with a carrier gas (nitrogen) flow rate of 50 mL min^{-1} and an injector/detector temperature of 275 °C. The retention time of diphenylamine was ca. 210 s. Typical chromatograms of pure diphenylamine and of a treated apple extract (without cleanup) are shown (Figures 1C and 1D). An untreated apple extract gave no interfering peak in the area corresponding to diphenylamine. The lower limit of detection for quantitative measurement was very similar to the electron-capture method. Again the response (peak height) was linear over the range used (up to ca. 10 ng of diphenylamine).

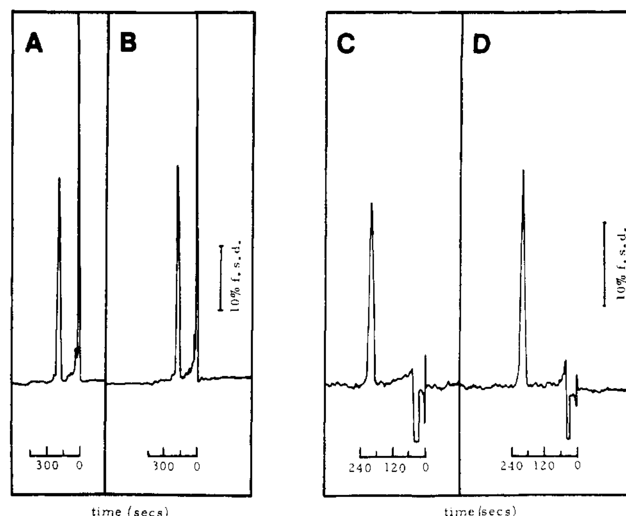

Figure 1. Typical chromatograms: (A) Pure diphenylamine heptafluorobutyryl derivative (2-μL injection, equivalent to 4 ng of diphenylamine). (B) Heptafluorobutyryl derivative of diphenylamine extracted from apple peel containing 2.5 ppm of diphenylamine (2-μL injection, equivalent to 5.3 ng of diphenylamine and 3 mg of extracted apple). (C) Pure diphenylamine (4-μL injection, 8 ng of diphenylamine). (D) Diphenylamine extracted from apple peel containing 3.6 ppm of diphenylamine (2-μL injection, equivalent to 9.6 ng of diphenylamine and 2.6 mg of extracted apple). A and B with an electron-capture detector; C and D with a nitrogen thermionic detector.

Table I. Recovery of Diphenylamine from Apple Peel

amount added,[a] ppm	recovery			
	method 1[b]		method 2[c]	
	mean, %	SD	mean, %	SD
0.1	82	5.7	90	5.1
1.0	80	13.1	92	10.7
5.0	85	10.1	93	10.3
10.0	92	3.3	66	7.4

[a] Based on weight of whole apple. [b] Method 1, Soxhlet extraction and derivative formation for electron-capture GC determination. [c] Method 2, steam distillation and direct GC determination.

When apple extracts obtained using a Soxhlet extractor were used in this method some contamination of the column occurred; this was seen as a gradual decrease in response during a run of samples. It may have been possible to overcome this effect by the introduction of a cleanup stage but the problem was obviated by using the Veith and Kiwus apparatus.

(vi) Recoveries. These were determined at levels between 0.1 and 10 ppm for both of the methods. The first method consisted of extraction of the peel in a Soxhlet apparatus, alkali/column cleanup, derivatization, and determination by GC with an electron-capture detector; the second consisted of extraction of the peel in a Veith and Kiwus apparatus and determination by GC with a nitrogen detector. In each case diphenylamine solution

Table II. Diphenylamine Residues (μg cm^{-2}) after Treatment and during Storage

treatment rate, ppm	method[a]	mean[b] diphenylamine residues (μg cm^{-2}) after storage for 0-118 days				
		0	35	63	90	118
500	1	1.33	0.40	0.28	0.21	0.08
	2	1.32	0.54	0.48	0.36	0.12
1000	1	2.46	1.28	0.16	0.16	0.13
	2	2.27	1.18	0.43	0.26	0.18
2000	1	2.59	1.35	0.60	0.37	0.22
	2	3.62	1.16	0.99	0.42	0.29
4000	1	4.11	5.14	1.06	0.53	0.45
	2	6.01	4.23	1.66	1.13	0.42

[a] Method 1, Soxhlet extraction and derivative formation for electron-capture GC determination; method 2, steam distillation and direct GC determination. [b] Mean of five values for 0 days of storage, two values for 35, 63, and 90 days of storage and four values for 118 days of storage.

in petroleum ether was added to the apple peel in the apparatus immediately before extraction.

The results are shown in Table I.

(vii) Statistical Methods. The results from the two methods were compared in two ways, using the pairs of values obtained from each sample. For the first comparison the fractional differences in the pairs of values were regressed against the means and in the second comparison the proportions of the pairs of values were regressed against the means [i.e., $2(b - a)/(a + b)$ or b/a were regressed against $(b + a)/2$, where a was the concentration obtained by Soxhlet extraction and the derivative method and b, the concentration obtained by extraction with the Veith and Kiwus apparatus and direct determination].

RESULTS

The two methods were used to determine diphenylamine on apples during a laboratory treatment and storage experiment designed to simulate commercial conditions. Full details of the treatments and biological effects, in which >200 apples were examined by both methods, are given elsewhere (Johnson et al., 1980). The results of the residue determinations are given in Table II. The residues were on fruit dipped in the specified dispersion and stored for various periods at 3.9 °C in an atmosphere of 8–10% CO_2 in air. Each result is the mean of separate determinations on two to five apples.

The variation between the two analytical methods, after correction for recovery losses was 25 and 32%, depending which of the above statistical methods was employed. The difference did not change significantly throughout the storage period.

A total of 60 pairs of determinations were used. The ratio b/a had a variance of 0.374 and a mean of 1.387, which differed significantly from 1 at the 0.1% level. The regression of $2(b - a)/a + b)$ against $(a + b)/2$ indicated that the variance of the former did not depend on the latter, hence the variation of the fractional difference between the results in a pair was similar whatever the absolute values, and the usual significance tests could be applied. These showed no significance for the slope of the regression, but a significance at the 0.1% level for the intercept, i.e., that b was, on the average, significantly greater than a. This was confirmed by an ordinary t test on the values of $2(b - a)/(a + b)$, which indicated a positive mean, significant at the 0.1% level.

DISCUSSION

Extraction with the Veith and Kiwus apparatus enabled the diphenylamine to be collected in a small volume of solvent, in a relatively short time (1 h) compared with Soxhlet extraction which required 3 h and subsequent concentration to low volume. Kennett (1961) described an apparatus based on the same principle in which a hexane and water mixture was distilled from the sample and the hexane collected in a modified reflux head to continuously extract the diphenylamine. Although this technique also presents the sample in a small volume of solvent, it requires a long reflux time for extraction. With the Veith and Kiwus apparatus most of the diphenylamine (at these levels) was transferred to the petroleum ether in 30 min and recovery was not increased by heating for longer than 1 h.

Investigation showed that before derivatization the alkali treatment in the cleanup stage was essential for acceptable recoveries. However, trimethylamine could on many occasions be omitted from the derivatization reaction without affecting the recovery, but it was included for all the results reported. The washing steps were required to prevent "tailing" fronts on the chromatographic peaks.

The two extraction methods (Veith and Kiwus or Soxhlet) may be combined with either of the analytical methods ("direct" or after derivative formation). All combinations were found to be satisfactory for concentrations ≤5 ppm but extraction with the Veith and Kiwus apparatus followed by direct determination using a GC with a nitrogen detector was preferable because of the savings in equipment, chemicals, and time—it was found that approximately 3 h was required for the cleanup and derivatization of a batch of eight samples (a convenient number to handle at one time).

The reason why one method consistently gave higher results than the other (ca. 29%) is not known. As this applies to results corrected for recovery losses, a possible explanation is that after a short interval DPA became "bound" in a form which was only released by one of the methods used. There was no evidence that "binding" changed during the storage period.

No other evidence for the existence or nature of this binding has been found, and the toxicological implications are unknown, but it would seem expedient to use the Veith and Kiwus apparatus and the direct method of determination because of the higher results given, as well as for the reasons given above.

CONCLUSION

Steam distillation/solvent extraction of the sample followed by GC using a heated bead nitrogen detector provides a relatively rapid and simple method for determination of diphenylamine residues in apples. Where this type of detector is not available, formation of the heptafluorobutyryl derivative and analysis by electron-capture GC is a useful alternative. Either method is more selective and sensitive than those previously available.

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A Method to Determine Dinoseb Residues in Crops and Soil by Gas Chromatography

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A method is described for the determination of residues of dinoseb (2-*sec*-butyl-4,6-dinitrophenol) in alfalfa, corn, cottonseed, field beans, almonds, peanuts, peas, potatoes, soybeans, grapes, oranges, peaches, pears, barley, wheat, and soil at levels ranging from 0.05 to 100 ppm. Dinoseb is first extracted by hot hydrolysis in methanol–sulfuric acid and subsequently partitioned into diethyl ether and adsorbed onto basic alumina. After elution with sodium bicarbonate, ether partition, and diazomethane methylation, the dinoseb methyl ether is adsorbed onto acidic alumina and eluted with ether. Electron-capture gas chromatography provides a sensitive means of quantifying residues of dinoseb down to 20 pg. Average recoveries ranged from 77 to 99%.

Dinoseb (2-*sec*-butyl-4,6-dinitrophenol) is the active ingredient in several herbicides which are formulated as the alkanolamine salts of the ethanol series, as the ammonium salt, or as the free phenol. [Typical formulations include PREMERGE 3 Dinitro Amine Herbicide, DOW Selective Week Killer, and DOW General Weed Killer, which are products of The Dow Chemical Company.] These herbicides are valuable and effective in the control of many broadleaf weeds in crops and have been used extensively for many years by farmers and state and federal experiment station investigators. The lack of translocation of dinoseb in plants (Bandal and Casida, 1972) together with its short residual life on plants and in soil allow its use in many crop situations without risk of residues.

The literature is deficient in extensive and well-validated methodology for dinoseb determination in crops. Yip and Howard (1968) reported work on several dinitrophenols in some fruits and legumes. McKellar (1971) reported a method for dinoseb determination in milk and cream. Guardigli et al. (1971) developed a TLC procedure for dinoseb residues. Dekker and Selling (1975) in the Netherlands presented a method for dinoterb (2-*tert*-butyl-4,6-dinitrophenol) in soil. Edgerton and Moseman (1978) applied the methodology of McKellar to determine dinoseb in feed and rat tissues and excreta.

The method described here has been practiced for 10 years by four analysts in some 32 projects on 16 different crops plus soil, involving 37 substrates which were succulent, oily, dry fibrous, cellulosic, highly carbohydrate, or ionic (soil). Large numbers of recovery determinations validating the method in these substrates have been condensed into tables of average values.

EXPERIMENTAL SECTION

Gas Chromatograph. A Tracor Model 222 equipped with a linearized nickel-63 electron-capture detector (ECD) was used and operated at 95:5 argon/methane flow of 70 mL/min through the column plus 20 mL/min as detector purge, with temperatures of 200–220 °C (column), 350 °C (detector), and 250 °C (injector). Earlier work utilized a Barber Colman Model 5000 equipped with a strontium-90 ECD, which was operated at 90 mL/min nitrogen flow, with temperatures of 200 °C (column), 250–350 °C (detector), and 225 °C (injector). In both instruments, a 1.8 m × 3 mm i.d. glass U-column packed with 5% DC-200 on 80–100 mesh Gas-Chrom Z was used. An alternate packing would be 3% OV-101. In these instruments, 20 pg of dinoseb methyl ether produced a 5–10% FSD, with a base line noise of 0.1–0.2%. Retention time was typically 3–4 min.

Reagents. Solvents used were either distilled in glass or pesticide residue quality.

Basic and acidic alumina, Woelm type, obtained from Waters Associates as activity grade 1, were stored continually in an oven at 130 °C. Prepared columns were cooled before use.

Standards of dinoseb and dinoseb methyl ether were obtained from the Agricultural Products Department of Dow Chemical U.S.A. in 99+% purity. Solutions of dinoseb were kept in the dark, and those of dinoseb methyl ether were refrigerated except just prior to use, when they were allowed to come to room temperature.

Diazomethane methylating solution was prepared in ether from Diazald according to the directions on the bottle from Aldrich Chemical Co., Milwaukee, WI. Caution should be exercised in the preparation and use of diazomethane because it is toxic and can cause skin sensitivity and is potentially explosive under certain conditions.

Sample Preparation and Extraction. Crops should receive a preliminary chopping (Hobart Food Cutter) or grinding (Wiley Laboratory Mill), as appropriate, and be thoroughly mixed to provide a homogeneous sample. Weigh 10 g of pulverized sample (5 g of low-density samples such as straw or fodder) into a 4-oz square bottle and add 40 mL of methanol containing 2 mL of 6 N sulfuric acid. (More methanol may be required to cover straw or fodder.) Prepare a recovery sample by spiking a duplicate control sample with 1 mL of the appropriate concentration of dinoseb in methanol and letting stand 15 min. After heating the bottles for 1 h at 70 °C in a water bath or oven and cooling to the touch, blend each sample using a Lourdes MM-1 multimixer or Brinkmann Polytron PT-20ST homogenizer for 3 or 1 min, respectively. Add 5 g

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