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FRUIT THINNER RESIDUES

Residue Determination of Naphthaleneacetic Acid in Olives

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A method for the determination of small residues of naphthaleneacetic acid in olives, which had been treated with 150 p.p.m. of this plant growth regulator shortly after bloom, has been developed. The chloroform extract of raw olives is purified by chromatography on alumina and silica gel, followed by esterification with diazomethane and separation of the methyl ester by gas-liquid chromatography. Final analysis is by spectrophotometry at 360 mμ with an over-all sensitivity of 0.1 p.p.m. Residues of naphthaleneacetic acid ranged during one season's treatment from an initial concentration of 0.5 p.p.m. to nondetectable at harvest time, 142 days later. Comparable results from the 1962 season ranged from 1.6 p.p.m. initially to nondetectable amounts at harvest time, 125 days later.

THE USE of naphthaleneacetic acid (NAA) as a fruit thinner for olives (*5, 6*) has prompted the development of a sensitive analytical method for residue determinations of this plant growth hormone. A recently developed method for NAA employs the nitration of the naphthalene ring and the subsequent development of a characteristic color (*1*). An adaptation of this method for the determination of NAA on olives proved unsuccessful due to high blanks, and a rigorous cleanup technique had to be devised, based on column and gas-liquid chromatography followed by nitration and spectrophotometry. The cleanup step involving gas liquid chromatography was an adaptation of the residue method for methyl ester naphthaleneacetic acid (MENA) in potatoes (*7*).

Experimental

Apparatus and Equipment. Gas liquid chromatograph Aerograph A-90-C, equipped with 6-foot, 1/4-in. o.d. copper column packed with 20% w./w. silicone high vacuum grease Dow 11 on acid-washed Chromosorb P. Heated exit port is modified with 7/15 standard taper stainless steel inner joint.

Fraction collectors are 1.0 × 14 cm. glass tubes, tapered at one end and fitted with a 7/15 standard taper outer joint at the other. A small piece of absorbent cotton is inserted into the collector and saturated with chloroform just prior to collecting (*8*).

Spectrophotometer, Beckman Model DU; cylindrical silica cells, 10 mm. o.d., 100 mm. light path (Pyrocell Mfg. Co., New York 28, N. Y.).

Reagents. DIAZALD-STABILIZED DIAZOMETHANE [*N*-methyl-*p*-tolylsulfonylnitrosamide (Aldrich Chemical Co.)]. Diazomethane is freshly generated once every 2 weeks by the following method. Seven grams of Diazald is dissolved in about 5 ml. of anhydrous diethyl ether and placed in a dropping funnel attached to a distillation flask. Ten grams of potassium hydroxide dissolved in 8 ml. of water and 20 ml. of 95% ethanol are transferred into the distillation flask kept at 0° C. The temperature is slowly raised to 70° C. in a water bath, and the Diazald solution is added dropwise. With continuous agitation provided by a magnetic stirrer, the ethereal solution of diazomethane is distilled into a 25-ml. volumetric receiving flask cooled in ice. Additional ethyl ether is slowly added through the dropping funnel until about 25 ml. of distillate has been collected. The diazomethane solution may be stored for several weeks in a desiccator at −10° C. Since diazomethane is toxic and explosive, only rubber connections are used on the apparatus, and all operations are carried out in a well-ventilated hood.

ALUMINA, basic, grade 1 (Woelm).

SILICIC ACID (Mallinckrodt 2844); activated at 160° C. for 4 hours and stored in a desiccator.

1-NAPHTHALENEACETIC ACID (Distillation Products).

NAA Calibration. One milliliter of a standard solution of NAA (1.00 mg. per ml. of chloroform) was esterified by the addition of 1.0 ml. of a freshly prepared diazomethane solution in ether. After the cessation of gas evolution, indicating the completion of the reaction, the solution was concentrated to 1.0 ml. in a graduated centrifuge tube with a stream of air.

Aliquot volumes of this solution ranging from 0 to 25 μl. were injected into a gas chromatography column by means of a 100-μl. Hamilton syringe. Operating conditions of gas chromatography were: 210° C. column temperature, 50 ml. per min. helium gas flow. The retention time for MENA as detected by gas conductivity katharometer was between 4 to 6 minutes. Fractions were collected manually at this predetermined retention time by attaching the glass collector to the heated exit port (*7*).

The condensed MENA was washed from the collector into a 50-ml. Erlenmeyer flask with 15 ml. of chloroform and evaporated to dryness with warm

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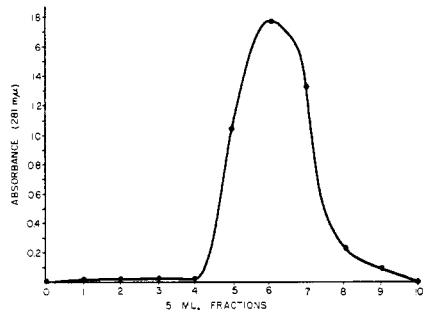

Figure 1. Elution curve, 1000 μg. of naphthaleneacetic acid; silica gel column; 5% v./v. *n*-butanol in chloroform elutrient

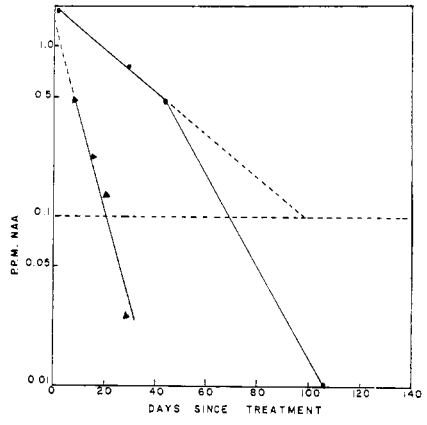

Figure 2. Degradation curve of naphthaleneacetic acid on olives

Details of treatment in Tables I and II (▲ 1960; ● 1962 series)

air. Six milliliters of 0.1*N* sodium nitrate in concentrated phosphoric acid were added and the solution was heated on a steam bath for 10 minutes. A glass marble placed on top of the Erlenmeyer flask served as a condenser. After the nitration was completed the absorbance of the cooled solution was measured at 360 mμ (*1*) and was plotted against micrograms NAA resulting in a straight line with a slope of 0.045 absorbance per microgram of NAA.

Residue Determination. Extraction. One hundred grams of olives were blended with 250 ml. of redistilled chloroform and 20 ml. of 3*N* hydrochloric acid and agitated on a rotary shaker for one-half hour. The slurry was centrifuged at 200 × G for 5 minutes, the top layer was decanted and the chloroform layer filtered through Whatman No. 1 paper. The resultant red filtrate was evaporated in vacuo to about 15 to 20 ml.

Cleanup. The concentrated extract was chromatographed on basic alumina using a 2.5 × 24 cm. glass column packed with 20 grams of adsorbent. The adsorbed sample was washed with 300 ml. of chloroform and the column dried under vacuum to remove traces of trapped chloroform. The acid was eluted with 50 ml. of 1% w./v. sodium bicarbonate followed by 20 ml. of distilled water.

The water phase was acidified by adding 30 ml. of 3*N* hydrochloric acid, and the NAA was extracted in a separatory funnel with three 50-ml. portions of chloroform. This solution was concentrated in vacuo to a final volume of about 5 ml.

A silica gel column was now prepared as follows. Twenty grams of dry silicic acid suspended in about 50 ml. of chloroform was slurried into a 2.5 × 24 cm. glass column. The column was washed with about 200 ml. of chloroform until a translucent gel was obtained.

After the concentrated chloroform extract containing possible NAA residues had been added to the top of the column, it was washed with 50 ml. of chloroform, and the washings were discarded. NAA was quantitatively eluted off the column with 100 ml. of 5% v./v. *n*-butanol in chloroform (*3*). The chloroform-butanol phase was taken to dryness under reduced pressure, and 1 ml. of ethereal diazomethane solution was added.

This cleanup was followed by gas chromatography and color development as described under "NAA calibration." Micrograms of NAA were directly read from the calibration curve and p.p.m. residues calculated on the basis of grams-aliquot analyzed.

Table I. Naphthaleneacetic Acid Residues in Olives

1960 Season (150 p.p.m. sprayed)

Sample No. (25-Gram Aliquot)	Days Since Application (June 1, 1960)	Absorbance[a]		P.P.M. NAA		% Recovered
		Gross	Net	Added	Found	
1084 (Check)		0.090	0	...	...	...
1084AR		0.155	0.065	0.1	0.06	59
1084B	8 (June 9)	0.637	0.547	...	0.50	...
841AR	15	0.542	0.465	0.5	0.42	84
841B	15 (June 15)	0.314	0.237	...	0.22	...
Check[b]		0.077	0	...	...	...
1268AR	21	0.492	0.415	0.5	0.38	76
1268B	21 (June 22)	0.222	0.145	...	0.13	...
Check[b]		0.077	...	...	...	...
842A (Check)	29	0.068	...	...	...	...
842 AR	29	0.148	0.080	0.1	0.07	...
842B	29 (June 30)	0.097	0.029	...	0.03	...
1269A (Check)	63	0.073				
1269AR	63	1.150	1.077	1.0	1.00	100
1269B	63 (Aug. 3)	0.076	0.003	...	0	
843A (Check)	142	0.078	0	...	...	...
843AR	142	0.221	1.143	1.0	1.04	104
843B	142 (Oct. 21)	0.102	0.024	...	0.02	

[a] Average of two replicated determinations, std. dev. = ±0.005.
[b] Check sample not available—for "net," average of 4 other check samples used.

Table II. Naphthaleneacetic Acid in Olives

1962 Series (150 p.p.m. active ingredient to run off; 50 grams per 8 oz. emulsifiable concentrate)

Sample No. (25-Gram Aliquot)	Days Since Application (June 6, 1962)	Absorbance[a]		P.P.M. NAA		% Recovered
		Gross	Net	Added	Found	
1101B	1 (6-7)	1.850	1.770	...	1.61	..
Check[b]		0.080	...	...	...	..
1116B	29 (7-15)	0.836	0.756		0.76	..
Check[b]		0.080	...	...	...	..
1142A (Check)	43 (7-19)	0.070	...	...	...	..
1142AR	43	0.525	0.455	0.50	0.41	82
1142B	43	0.594	0.524	...	0.47	..
1251A (Check)	106 (9-20)	0.103	...	...	...	..
1251AR	106	0.153	0.050	0.1	0.05	50
1251B	106	0.117	0.014	...	0.01	..
1267AR	125 (10-5)	0.505	0.425	0.50	0.38	76
Check[b]	125	0.080	...	...	...	..
1267B	125	0.079	0	...	0	..

[a] Average of two replicated determinations, std. dev. = ±0.005.
[b] Checks not available—for "net," average of 6 other checks used.

Results and Discussion

Cleanup Procedure. The determination of trace amounts of naphthaleneacetic acid in olives posed several serious problems. The high oil content and the presence of water-soluble pigments and organic acids required a three-step chromatographic cleanup to achieve a required sensitivity of at least 0.1 p.p.m. Attempts to apply two

Table III. Apparent NAA Residues on Untreated Olive Samples

(25-gram aliquots)

Sample No.[a]	Absorbance (360 mμ)	Apparent P.P.M.
1084A	0.090	0.08
842A	0.068	0.06
1269A	0.073	0.06
843A	0.078	0.07
1142A	0.070	0.06

[a] See Tables I and II for description.

published methods for the analysis of NAA (*1, 8*) to olives proved unsuccessful. Bache *et al.* (*1*) analyzed apples for NAA by extraction and a single-column cleanup using silica gel. Although Bache's colorimetric method was finally adopted, the cleanup would not remove olive oil. The gas chromatography–ultraviolet spectrophotometry technique (*7*) also proved inadequate for this purpose, again due to high oil content and interfering absorbances at 224 and 281 mμ.

The method finally adopted for NAA analysis in olives is a modified combination of the published methods.

After the initial extraction of olives with acidified chloroform, column chromatography on basic alumina permitted the complete removal of olive oil. This procedure was first suggested by Daoud and Luh (*4*) and was based on the quantitative adsorption of organic acids from nonpolar solvents. The alumina was washed with copious amounts of chloroform which removed all of the oil and some fat-soluble green pigments. Naphthaleneacetic acid was quantitatively eluted from alumina with 1% sodium bicarbonate solution.

However, the base also removed a water-soluble, purple pigment from the column which interfered with the colorimetric or spectrophotometric methods if applied after this step. This pigment could be removed by silica gel chromatography, and the acid was quantitatively eluted with 5% *n*-butanol in chloroform (*2, 3*). This step was checked by chromatographing 1000 μg. of NAA and reading the absorbance of 5-ml. fractions at 281 mμ (*7*). Based on the theoretical absorbance of 1000 μg. of NAA, 94.3% of the acid was recovered between 40 to 100 ml. elutriate (Figure 1).

Even after silica gel chromatography, direct colorimetry or spectrophotometry yielded relatively high blanks which did not yield an over-all sensitivity of 0.1 p.p.m. Consequently, the final solution was further purified by gas-liquid chromatography, analogous to one of the published methods (*7*). Since the colorimetric procedure based on nitration yielded considerably lower blanks than a direct reading at 281 mμ, the colorimetric technique was chosen for final analysis. The extinction coefficient of the color resulting from the nitrated naphthalene ring was low and optical cells with a 10-cm. light path were chosen to give ultimate sensitivity. If an absorbance of 0.11 per 2.5 μg. NAA was arbitrarily chosen as minimum readability, a sensitivity of 0.1 p.p.m. with 25-gram aliquot samples was achieved.

Residue Analysis. Olive trees were sprayed 2 weeks after bloom with an emulsifiable concentrate of NAA containing 150 p.p.m. active ingredient. These tests were conducted in May 1960 and 1962, but all analyses were performed in 1962. Samples from the 1960 series were stored at −10° C. Recoveries of NAA added to fruit prior to extraction were studied at three levels—0.1, 0.5, and 1.0 p.p.m. Recoveries of added NAA at the 1.0 and 0.5 p.p.m. levels ranged from 76 to 104% and at 0.1 p.p.m. from 50 to 76% (Tables I and II). Apparent NAA residues in untreated samples ranged from 0.06 to 0.08 p.p.m., which was below the stated sensitivity of the method (Table III).

Results for residues found in treated olives and harvested at various periods after treatment up to actual harvest time (mid-October) are shown in Tables I and II and plotted in Figure 2. Residues of NAA from the 1960 test were generally lower than those of 1962. However, in both cases no detectable residues (below 0.1 p.p.m.) were found at harvest time. The reason for lower 1960 residues may be the slow volatilization of NAA during almost 2 years' storage. If, however, the straight-line portion of the 1962 series (Figure 2) is extrapolated to 0.1 p.p.m., a calculated waiting period of 100 days after treatment is obtained.

Acknowledgment

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SUCKER CONTROL CHEMICALS

Detection of Compounds that Inhibit Vegetative Bud Growth of Tobacco

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Removing the flower parts of tobacco as the plants approach maturity is a recommended procedure to improve quality of the leaves (*2*). In commercial practice, a few days after the top has been removed the dormant buds at the base of the leaf petioles begin to grow vigorously and develop new shoots that must be removed, sometimes repeatedly at several weekly intervals to maintain high quality of the salable leaves. Mineral oil emulsions and maleic hydrazide preparations were developed to control sucker growth chemically and reduce labor costs (*9*). These methods have not proved entirely satisfactory, however. A new method of evaluating additional chemicals for sucker control is described here, and results obtained with the method are presented.

Methods

Preparation of Chemicals. Many of the chemicals were available for these tests in minute quantities. For this reason, lanolin paste was used as a carrier to conserve the amount of chemical required and thus make it possible to test a wide range of chemicals. Each chemical was prepared at approximately 1.0%