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Determination of Naphthaleneacetic Acid (NAA) in Oranges, Tangerines, and Processed Products: High-Performance Liquid Chromatography with Fluorometric Detection

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A rapid procedure involving high-performance liquid chromatography with fluorometric detection is described which is capable of analyzing tangerines and oranges, as well as their processed fractions, for residues of NAA as low as 0.008 ppm. Sulfuric acid is used to release conjugates for extraction with methylene chloride, followed by partitioning between ethyl ether and potassium hydrogen phosphate for cleanup. The insensitivity of the fluorometer to pulsatile flow allows the use of an economical single piston solvent pump for chromatography. Residues were measured in 'Dancy' tangerines and processed fractions in one disappearance experiment and in 'Pineapple' orange in another.

The inherently high fluorescent quantum yield for naphthaleneacetic acid (NAA) was utilized by Jolliffe and Coggins (1970) in the development of a sensitive and selective analytical procedure for the analysis of residues in citrus as low as 0.1 ppm. Their report summarizes the approaches of previous methods as well.

As has been reported for California citrus (Hield et al., 1966), NAA has been found to be effective in Florida for improving fruit size and reducing alternation of heavy and light crops of 'Dancy' tangerines (Wheaton and Stewart, 1973), as well as for preventing sprouting of 'Bearss' lemon trees when applied directly to tree trunks following pruning (Lundberg and Smith, 1974).

Metabolism studies of NAA, applied as a dip to fruiting 'Kinnow' mandarin branches, by Shindy et al. (1973) showed that major metabolites, appearing between 6 h and 4 days after application, were naphthaleneacetylaspartic acid and 1-β-D-glucose-α-naphthaleneacetate. Together, these two metabolites represented, at 4 days after application, 59% of total radioactivity in the whole fruit. Both of these metabolites were readily hydrolyzed by hydrochloric acid to give free NAA. Coggins et al. (1972) studied the disappearance of NAA in Kinnow mandarin fruit as well as determining residues in eight other types of citrus. Residues became immeasurable (<0.07 ppm) in fruit harvested 3 to 6 weeks after application.

This paper describes the residues of NAA in 'Dancy' tangerines and various types of fractions resulting from a typical commercial processor as well as in fresh 'Pineapple' oranges after a single application to trees containing immature fruit. Additionally, a high-performance liquid chromatographic procedure is described which provides for the simple, rapid, highly sensitive (0.008 ppm limit of detection) and highly selective analysis of fresh fruit as well as many types of processed products.

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EXPERIMENTAL SECTION

Apparatus. An American Instrument Co. (Silver Spring, MD) spectrophotofluorometer, Model 4-8202, equipped with a 150-W mercury–xenon lamp, Model B16-63019 flow cell (300-μL volume), and an A363-62140 adapter, was used as the fluorescence monitor for the eluant of a liquid chromatographic column. Wavelengths used were 288-nm excitation and 340-nm emission, with a slit program of 3, 3, 3, 3, 3, and 5 mm. Chromatograms were recorded on a Sargent Model MR recorder.

Separations were performed by reverse phase either on a 2 mm i.d. × 50 cm ETH (Dupont) column or on a 4 mm i.d. × 25 cm μ Bondapak CN (Waters Associates). Injection on the ETH column was by syringe (10 μL) via a Chromatronix Model 107B25 sample injection tee; a Chromatronix Model HPSV-20 sample injection valve with a 25-μL loop was used in conjunction with the μ Bondapak CN column. Mobile phase for the ETH column was 0.1 M pH 4.3 citrate at 1.3 mL/min (pH 4.7 was used for the molasses samples); a 0.1 M pH 7.0 phosphate buffer was used for the μ Bondapak CN column at 1.0 mL/min.

Mobile phase was pumped by a Waters Associates Model 6000 solvent delivery system or a Milton Roy Model 196-0042-028 single-piston pump.

Blending of the samples was accomplished with a Lourdes Model VM blender.

Concentration of extracts was performed on a steam bath by use of a 500-mL Kuderna-Danish flask fitted with a three-ball Snyder column and a 10-mL ampule.

Procedure for Fresh or Dried Fruit. Twenty-five grams of peel, pulp, whole, or dried fruit was placed in a 1-qt Mason jar along with 200 mL of methylene chloride and 5 mL of 18 N H_2SO_4; the sample was blended at medium speed for 4 min. It was then filtered under vacuum through a no. 2 porcelain Buchner funnel containing no. 1 filter paper. The Mason jar was rinsed with an additional 50 mL of methylene chloride which was poured through the filter cake. The filtrate was then

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quantitatively transferred to a 500-mL separatory funnel where it was washed with 100 mL of 0.01 N H_2SO_4 (pH 2), after which the methylene chloride phase was drained into a 500-mL Erlenmeyer flask where it was dried with anhydrous sodium sulfate. Concentration to approximately 1 mL was accomplished over steam in the Kuderna-Danish apparatus. After transfer to a 15-mL Teflon-lined cap culture tube by portions totaling 3 mL of methylene chloride, the solvent was gradually blown to dryness at ambient temperature with dry nitrogen. The residue was then dissolved in 5 mL of ethyl ether which was extracted with 2 × 4 mL of 0.2 M K_2HPO_4; centrifugation was required to separate the phases after shaking. The combined aqueous fractions (8 mL) were transferred to a clean culture tube and washed with 2 × 2 mL of chloroform; centrifugation allowed complete separation of the phases. Acidification of the aqueous phase (to pH 1) with 5 N H_2SO_4 permitted partitioning of the NAA into 2 × 3 mL portions of chloroform, after which the chloroform was removed under a stream of dry nitrogen and the residue redissolved in 10 mL of distilled water. Samples were allowed to stand at ambient temperature for 1 h prior to chromatography to insure complete solubilization of the residue.

Procedure for Water-Soluble By-products (Press Liquor, Juice, Emulsion Water, Water Rinses). Twenty-five grams of material was transferred from a small beaker to a 250-mL separatory funnel with a rinse of 20 mL of distilled water. This solution was acidified with 5 mL of concentrated H_2SO_4 and extracted with 2 × 200 mL of methylene chloride. The methylene chloride was drained into a 500-mL Erlenmeyer flask and dried with anhydrous sodium sulfate before concentration to 1 mL over steam in the Kuderna-Danish apparatus. After transfer to a 15-mL culture tube with a 5-mL methylene chloride wash, the solvent was evaporated to dryness under dry nitrogen before partitioning with 0.2 M K_2HPO_4 as for fresh fruit.

Procedure for Molasses. Exactly 2.5 g of molasses was weighed in a 25 mm × 200 mm culture tube with a Teflon-lined cap; 40 mL of distilled water and enough 5 N H_2SO_4 were added to bring the pH to 1. Ethyl ether (20 mL) was added to the tube and the tube was shaken gently; the formed emulsion broke upon centrifugation. The ether layer was transferred to a clean 15-mL culture tube by Pasteur pipet and the aqueous phase was reextracted with a similar portion of ether. Partitioning with K_2HPO_4 and further workup was done as for fresh fruit except only 1 mL of distilled water was used to dissolve the final residue before chromatography.

Procedure for Oil. Exactly 25.0 g of oil was weighed in a 25 mm × 200 mm culture tube to which was added 20 mL of ethyl ether. The ether–oil phase was extracted with 2 × 4 mL of 0.2 M K_2HPO_4, the combined fractions of which were subsequently handled as for fresh fruit.

Field Experiments. ' Dancy ' tangerines used for the disappearance studies were in a grove operated by Haines City (Florida) Citrus Growers Association located approximately 2 miles southwest of Haines City, FL. Exact tree age was unknown, but they were large mature trees in the 25–40-year age range. ' Pineapple ' orange trees were on University of Florida, Lake Alfred Agricultural Research and Education Center property, approximately 1.5 miles east of Lake Alfred, and were large, mature trees planted in January, 1925.

A randomized block experimental design was used consisting of single tree plots arranged in four blocks. Control trees and trees sprayed with NAA were separated from each other by one or more buffer trees. Fruit samples for residue analysis were randomly selected from the shoulder of the trees. Samples from two blocks were pooled to give duplicates.

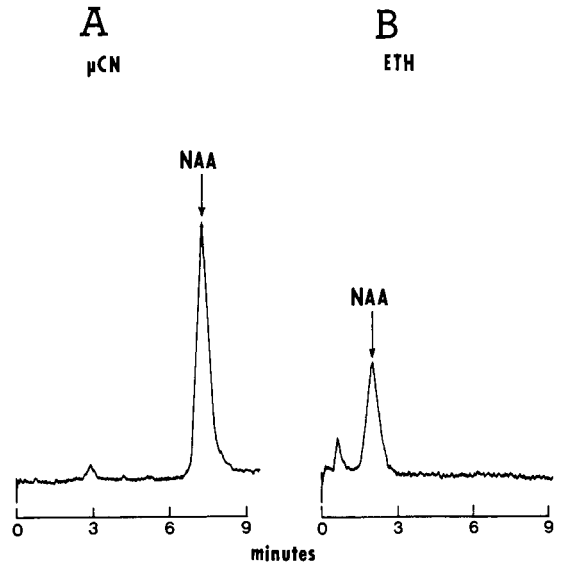

Figure 1. NAA standard injections, 2.5 ng; photometer, 0.01. (A) μ Bondapak CN, 4 mm i.d. × 25 cm, 1 mL/min of 0.1 M pH 7 phosphate buffer. (B) ETH, 2 mm i.d. × 50 cm 1.3 mL/min of 0.1 M pH 4.3 citrate buffer. Solvent delivery by Waters Model 6000.

NAA was applied to both tangerines and oranges using a Meyer Hi-Pressure Sprayer with Boyce Double Barrel Guns. The formulation used was Thompson Chemicals Fruit Fix Super Concentrate 800, USDA Reg. No. 642-150, 21.4% by weight solution of NAA-ammonium salt. This formulation was diluted, 448 mL/100 gal, with water along with 50 mL/100 gal of spreader, X-77 (Ortho), which consists of a mixture of alkylarylpolyoxyethylene glycols, free fatty acids, and isopropyl alcohol. Approximately 10 gal of mixture was applied per tree, providing the recommended rate of 1.5 lb of NAA/acre (250 ppm), or twice the NAA concentration was used to apply at a rate of 3.0 lb of NAA/acre (500 ppm).

Tangerine fruit were picked immediately after spraying (0 day) as well as at 1, 7, 14, 68, 128, and 156 days. ' Pineapple ' oranges were picked at 1, 9, 15, 68, 128, and 177 days after spraying.

Mature tangerine fruit were picked for processing 156 days after the single application of NAA and subjected to the previously described processing sequence, the final step of which is being carried through a pilot plant feed mill (Reitz, 1972).

Recovery Studies. Since ten different tangerine processed fractions were analyzed, recoveries were performed on three types of samples which would represent the extremes in sugar, oil, and water content. No processing was required of the ' Pineapple ' oranges, hence recoveries were performed only on whole immature fruit. Preliminary residue studies indicated that extremely low residues, less than 0.1 ppm, were to be expected; consequently, recoveries were done from 0 to 1 ppm.

RESULTS AND DISCUSSION

High-Performance Liquid Chromatography. Typical chromatograms of NAA standards on the two columns used are shown in Figure 1. Initial method development was done on a μ Bondapak CN, which produced almost 1700 theoretical plates for an NAA retention of 7.5 min. Inadvertent irreversible damage to this

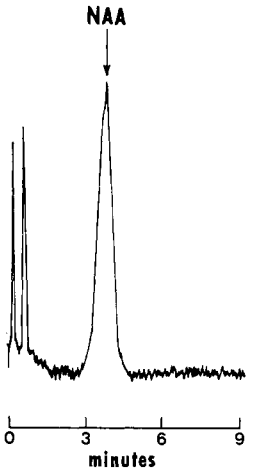

Figure 2. NAA standard injection, 2.5 ng; photometer, 0.01. ETH, 2 mm i.d. × 50 cm, 1.3 mL/min of 0.1 M pH 4.3 citrate buffer. Solvent delivery by Milton Roy Model 196-0042-028 pump.

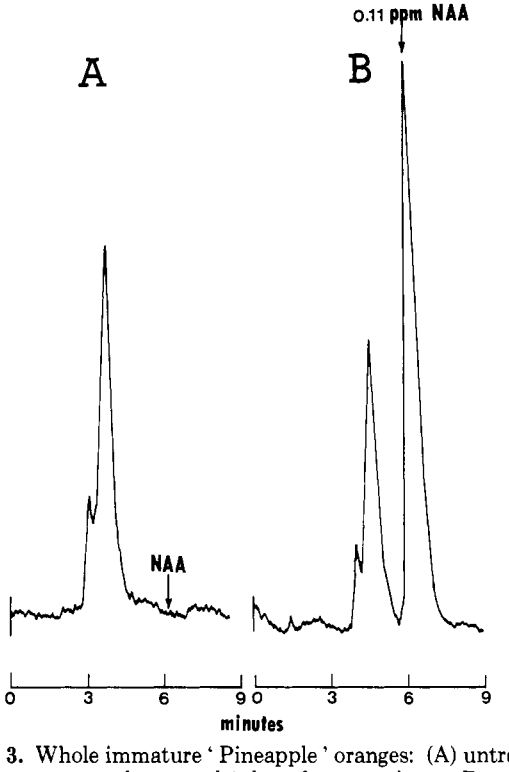

Figure 3. Whole immature ' Pineapple ' oranges: (A) untreated, (B) 250 ppm spray, harvested 1 day after spraying. μ Bondapak CN as in Figure 1.

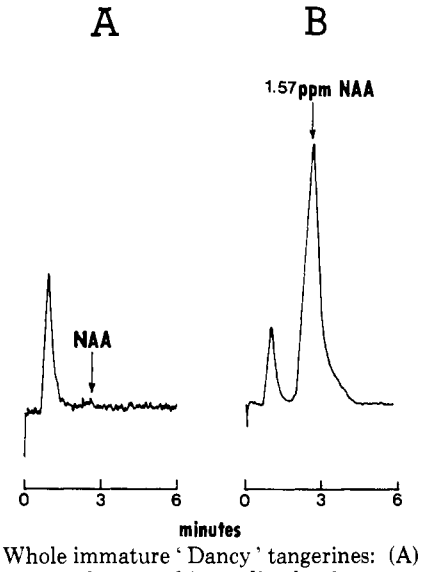

Figure 4. Whole immature ' Dancy ' tangerines: (A) untreated, (B) 500 ppm spray, harvested immediately after spraying. ETH, photometer 0.03, otherwise as in Figure 1.

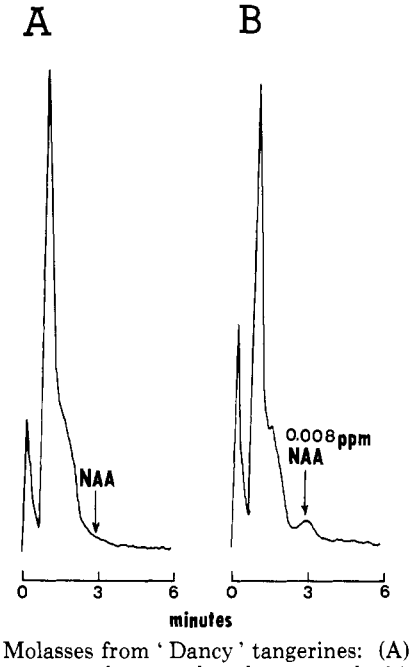

Figure 5. Molasses from ' Dancy ' tangerines: (A) untreated, (B) 500 ppm spray, harvested and processed 156 days after spraying. ETH as in Figure 1.

column forced utilization of the ETH column which gave only 60 theoretical plates, but allowed for shortened retention of about 2 min, providing a substantial percentage reduction in analysis time. Analytical curves were linear for both columns from 0.5 to 10 ng of NAA, as determined from peak heights. Use of the fluorometer as a detector provided for a signal to noise ratio (S/N) 200 times higher than that realized with a modern 254-nm absorbance detector designed for LC, without any measurable band broadening due to the larger cell volume. Limit of detection was approximately 0.1 ng (S/N = 4).

The insensitivity of the fluorometer to pulsatile flow with an aqueous based system is illustrated in the chromatogram of Figure 2 obtained with the Milton Roy single piston, pulsatile pump without the use of any type of pulse dampener. No loss in signal to noise, resulting from pulsatile flow through the cell of the fluorometer, can be seen when compared to the chromatograms in Figure 1 which were obtained with the Waters Model 6000 solvent delivery system. Nor was there any loss in efficiency observed although the contrary may be expected to occur with columns of significantly higher theoretical plates.

Recovery Studies. Fruit harvested up to 15 days after the single application was very immature, making it impossible to effectively separate the peel from the pulp. Consequently, recoveries and analysis were performed on the whole fruit of this type. Table I summarizes recoveries obtained for whole fruit as well as peel oil and molasses obtained from ' Dancy ' tangerines. Good recoveries were achieved even at the 0.008-ppm level.

Residues. Figures 3, 4, and 5 illustrate chromatograms from those types of samples in which residues were detected. Highest residues found were in the 0-day ' Dancy ' tangerines which received the 500-ppm spray (1.38 ppm average); the 250-ppm spray produced a lower residue (0.45 ppm average) at 0 day. Of the processed tangerine

Table I. Percent Recoveries of NAA from Citrus Fruit and Processed Fractions[a,b]

	recoveries, %						
	fortification levels, ppm						
sample type	0.008	0.01	0.05	0.1	0.2	0.5	1.0
fresh fruit[c]							
'Dancy' tangerines	75	78	70	e	73	63	76
'Pineapple' oranges	94		83	99		78	
peel oil[d]	66	85	83				
molasses[d]	64	71	83				

[a] Appropriate amount of free NAA acid added to control sample in 0.1 mL of methanol immediately before extraction. [b] Analyzed as duplicates with RSD of 11%. [c] Immature whole fruit. [d] Processed fractions prepared from mature untreated 'Dancy' tangerines. [e] Indicates recoveries were not performed at this level.

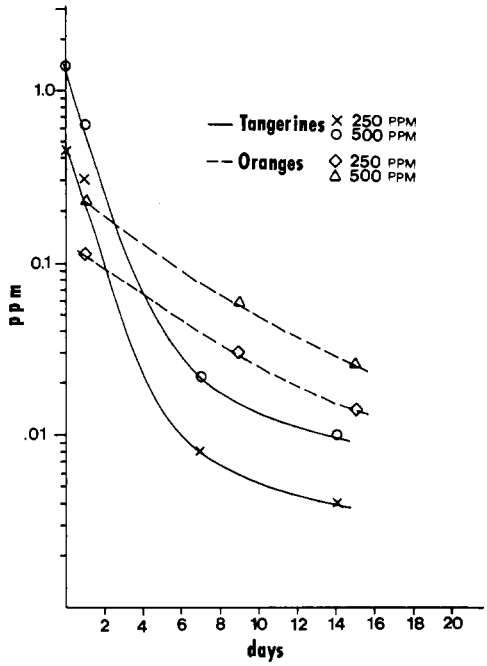

Figure 6. NAA residue disappearance curves for 'Dancy' tangerines and 'Pineapple' oranges as whole immature fruit. Points on the curves are averages of field duplicates; average relative standard deviation for tangerines was 11%; for oranges, 8%.

fractions analyzed only the tangerine molasses contained a measurable residue (0.008 ppm). No residues were found in the washed peel, washed pulp, unwashed peel, unwashed pulp, press liquor, emulsion water, peel frit, finisher pulp, fruit juice, after rinse water, prerinse water, peel oil, or chopped peel. This is understandable from the disappearance curve of the immature whole fruit (Figure 6) which shows low residues after 14 days, since the processed fractions were prepared from mature fruit harvested 156 days after the single application.

Residues in 'Pineapple' oranges were even lower after 1 day than in the tangerines (Figure 6), but were still detectable in the peel even after 68 days.

The fact that NAA residues were nearly undetectable in immature 'Dancy' tangerines only 14 days after spraying, were barely detectable in the molasses from mature fruit (0.008 ppm), and undetectable in all of the other processed fractions analyzed emphasizes the safeness of its use as administered in this experiment. Similarly, fresh 'Pineapple' oranges contained only a trace amount of NAA (0.008 ppm) in the peel of nearly mature fruit harvested 68 days after the single spraying; no detectable residue was found in the edible portion.

Although the quantum yield of fluorescence for NAA was not determined it is undoubtedly quite high, such that minimum cleanup of citrus extracts is required before chromatography, allowing for a simple, rapid, and highly sensitive analytical procedure. Utilization of the fluorometric detection obviates the need for the more expensive pulseless solvent delivery system at least in conjunction with the relatively inefficient columns which were used.

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