A Fluorometric Method for the Determination of Naphthaleneacetic Acid Residues in Citrus

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Fluorescence intensity as measured in a spectrophotofluorometer is used to determine 1-naphthaleneacetic acid in citrus tissues. The cleanup for this sensitive detection method is accomplished by selective extraction, liquid-liquid partitioning, and permanganate oxidation. Inclusion of a silica gel cleanup column is necessary for peel samples. The method has a sensitivity of 0.1 ppm based on a 25 g sample.

As is true for a number of other crops, 1-naphthaleneacetic acid (NAA) induces abscission of young citrus fruits. Many citrus varieties produce large numbers of fruit one year and low numbers the next. In some varieties, such as Wilking and Kinnow mandarin, alternate bearing is almost complete; few, if any, fruit are produced in alternate years. Appropriate degrees of fruit thinning have not only reduced certain problems associated with the heavy production portion of the cycle, such as excessively small fruit, but have caused trees that would otherwise be fruitless to produce a moderate crop of fruit during the next year (Hield *et al.*, 1966).

Prior to requesting registration for the use of NAA as a fruit thinning agent on citrus, residue data must be collected using an analytical method suitable for citrus. Bache *et al.* (1962) developed a residue method for NAA in apples using both ultraviolet absorption and colorimetry for quantitative measurements. Another method by Bache *et al.* (1964) determines NAA at 0.1 ppm using gas chromatography and detecting by electron affinity after nitration and esterification. An ultraviolet absorption microdetermination of NAA in pineapples with a sensitivity of 0.03 ppm was reported by Young *et al.* (1963). Methods for potatoes (Zweig *et al.*, 1962) and olives (Zweig *et al.*, 1964) make use of gas-liquid chromatography after esterification as a cleanup procedure, followed by a colorimetric determination. These methods were not directly applicable to citrus fruits. Regardless of the final method of detection, it was necessary to develop specific extraction and cleanup procedures for citrus. The fluorescence properties of NAA (Hornstein, 1958) provide a potentially sensitive and reasonably specific assay method. This paper describes the extraction, cleanup, and fluorescence assay method developed and tested on a number of citrus species and varieties.

EXPERIMENTAL

Extraction. Grind 25 g of thinly sliced peel with 75 ml of methylene chloride (CH_2Cl_2) in an Osterizer electric blender for 2 min. Add an additional 75 ml of CH_2Cl_2 and continue to blend for several minutes or as long as grinding is effective. Transfer the macerate, using 100 ml of CH_2Cl_2, into a jar. After the addition of 5 ml of 18 N sulfuric acid, place the sample on an oscillating shaker for 30 min. Vacuum-filter the slurry through Whatman 42 paper covered with 3 g of Celite 545. Place the filter paper and pad into the Osterizer with 100 ml of CH_2Cl_2 and grind for 2 min. Filter as before, rinse with 50 ml of CH_2Cl_2, and combine all filtrates.

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The extraction procedure for citrus pulp is similar to the procedure used for peel, but the sequence is different enough to require a description. The initial step is to place 25 g of pulp tissue, 125 ml of CH_2Cl_2 and 2 g of Solka Floc SW-40 (McKesson & Robbins, Inc.) in an Osterizer and macerate at high speed for 1 min. Vacuum-filter the slurry as for peel and save the filtrate. Place the filter paper and pad in the grinder with 100 ml of CH_2Cl_2 and blend for 2 min. Transfer the resulting mixture to a jar, add 100 ml of CH_2Cl_2 plus 5 ml of 18N H_2SO_4, and agitate on a shaker for 30 min. Vacuum-filter, rinse the filter pad with 50 ml of CH_2Cl_2, and combine all filtrates.

Cleanup. While the pulp extract is taken directly to a liquid-liquid partitioning step, it is necessary to take the peel extract through the following cleanup step. By use of a separatory funnel, water is removed from the combined peel filtrate, which is then concentrated to 5 to 10 ml (using a Rinco evaporator) under reduced pressure, as produced by a water aspirator, at 50° to 60° C and placed on a silica gel column. The column is prepared by adding 10 g of dry silicic acid (100 mesh, A.R.) to a 2.5 × 24 cm glass column containing 40 ml of CH_2Cl_2. A small glass wool plug is situated at either end of the silicic acid. The top plug is not added until after the silicic acid has settled into position and gas bubbles have been removed. The column is then washed with 50 ml of CH_2Cl_2, but care is taken to never permit the liquid level to drop below the upper glass wool plug.

The concentrated sample is transferred onto the column by using a total of 50 ml of CH_2Cl_2 in several increments. The solvent that goes through the column during this operation is discarded and then NAA is quantitatively eluted from the column with 100 ml of 5% (v/v) *n*-butanol in CH_2Cl_2.

The column eluate from the peel sample, or the pulp extract, is placed in a 250-ml separatory funnel and NAA is partitioned into aqueous bicarbonate by extracting the organic phase with three 50-ml aliquots of 2% (w/v) potassium bicarbonate. The combined bicarbonate extracts are returned to the separatory funnel and washed with two 25-ml portions of CH_2Cl_2 before being acidified with 10 ml of 25N phosphoric acid (H_3PO_4). During this step care is taken to gently shake the funnel and to frequently release pressure until all the CO_2 has evolved. Following the acidification step, 5 ml of 0.05N potassium permanganate is shaken into the liquid and the mixture is allowed to stand for 10 min. If the permanganate is excessively reduced, as shown by a change from purple to pink, more $KMnO_4$ may be added.

Extract the oxidized sample with three 10-ml portions of CH_2Cl_2 and place the combined extracts in a 125-ml separatory funnel. Shake gently with 10 ml of distilled water and dis-

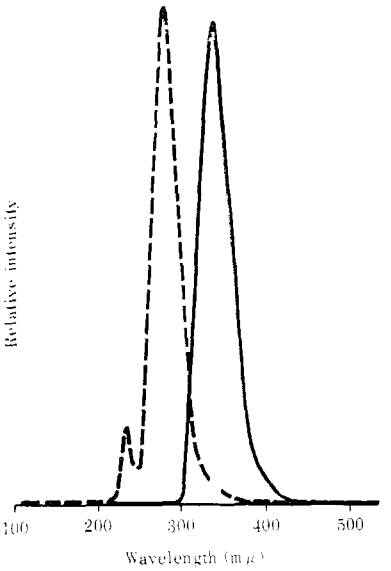

Figure 1. Excitation (- - -) and fluorescence (—) spectra for NAA dissolved in potassium bicarbonate (2%)

card the aqueous phase. The final step is to extract NAA from the organic solvent with 2% potassium bicarbonate. Two 10-ml and one 5-ml portion are used and the final volume of these combined aliquots is adjusted to 25 ml in a volumetric flask. This solution is subjected to fluorescence analysis.

Analysis. Determine fluorescence intensity of samples using an Aminco Bowman spectrophotofluorometer with a #3 slit arrangement at an excitation wavelength of 288 mμ and an emission wavelength of 340 mμ (Figure 1). Fluorescence readings are converted to quantity of NAA by use of a previously prepared calibration curve of fluorescence intensity *vs.* NAA concentrations throughout the range of 0.05 to 2.0 μg/ml in 2% potassium bicarbonate. Although a slight degree of fluorescence is obtained from 2% potassium bicarbonate, we prefer to subtract this value in the preparation of the curve. Such a procedure permits fluorescence readings obtained from untreated tissue to be subtracted from readings obtained from treated and fortified samples, followed by direct conversion to quantity of NAA from the calibration curve. In order to determine the reliability of fluorescence readings in a particular assay, samples of known concentrations of NAA are included in each series of readings.

Quenching, if present, was not a major factor in this study, but quenching should be evaluated in each assay. To do this, mix equal aliquots of solutions obtained from untreated tissue with NAA solutions at concentrations one to two times the concentration used for fortified samples. The percentage reduction in fluorescence beyond that of a 50% dilution indicates the degree of quenching (Pease and Gardiner, 1969). It is necessary to examine quenching at NAA concentrations similar to those used in fortified samples because the photomultiplier of the Aminco Bowman spectrophotofluorometer is not exactly linear when different instrument ranges are used. Therefore, small differences could incorrectly indicate a quenching effect.

RESULTS AND DISCUSSION

Extraction. Since NAA is quite soluble in ethanol and acetone and because these solvents penetrate plant tissue well, it is reasonable to conclude that they would be the preferred extracting solvents. However, we found that they were unsatisfactory for this analysis of citrus fruit tissue. These solvents extract substances that form emulsions and make the various solvent partition steps difficult, and they also extract phenolic-like compounds that interfere with the fluorescence analysis. These interference compounds possess partition coefficients similar to NAA and are difficult to remove once extracted. It was determined that CH_2Cl_2 extracts lesser amounts of the substances that form emulsions and those that cause interference. Since it provides such excellent selectivity in citrus tissue and is capable of dissolving 20% (w/v) of NAA readily at 25° C, CH_2Cl_2 was chosen as the extracting solvent. Thorough maceration of the tissue, to provide good solvent contact, was performed to assure quantitative extraction of NAA.

Table I. Recovery of NAA from Peel and Pulp of Mature Citrus Fruits[a]

Variety	Percent recovery[b] Peel	Pulp	
Valencia orange	70 ± 3	85 ± 1	88 ± 4[c]
Navel orange	71 ± 1	67 ± 3	88 ± 3
Marsh grapefruit	76 ± 5	71 ± 5	93 ± 2
Meyer lemon	77 ± 1	78 ± 2	95 ± 1
Kinnow mandarin	79 ± 3	71 ± 9	89 ± 3

[a] Tissue fortified to a level of 1.0 ppm NAA. [b] Values presented represent averages and observed ranges. [c] Solka Floc SW-40 used in extraction procedure for data presented in this column.

As mentioned earlier, it is necessary to extract citrus peel and pulp in slightly different ways. Solka Floc, a cellulosic material (Nelson *et al.*, 1968) is added, and then the pulp tissue ground for only a short period of time before filtering to prevent the formation of gel-like emulsions. Their formation in pulp extracts is probably related to the high water content as well as the presence of certain chemical constituents. The low and erratic recoveries obtained when gel-like emulsions were formed, and the effectiveness of Solka Floc in preventing them and improving recoveries are shown in Table I. Peel macerates were not filtered immediately because emulsions were not formed and maceration was more complete when the tissue was first shaken in the acidified extraction solvent before being ground a second time.

Although citrus tissues are inherently acidic, additional acid was added to macerated tissue to make certain that residues from the two commercial formulations used, as well as NAA salts or glucosides that may have formed in the tissue, were converted to the acid form. Glucosides are dissociated readily under highly acid conditions even at low temperatures (Giang and Beckman, 1968). It was also necessary to acidify to a pH lower than 3.5 to insure efficient transfer of NAA into CH_2Cl_2 from the aqueous phase.

Cleanup Procedure. Considerable selectivity during extraction occurred by using CH_2Cl_2, but further purification was obtained by partitioning between the organic solvent and aqueous solutions followed by oxidation with acidic permanganate. The oxidation step reduced fluorescence in untreated samples by at least a factor of two, and produced higher recoveries in fortified samples. Visual evidence of the action of permanganate was seen by the absence of yellow coloration in the final alkaline aqueous sample. Young *et al.* (1963) demonstrated that 0.05N $KMnO_4$ in phosphoric acid had no effect on NAA during a 10 min exposure period. We examined the stability of NAA to $KMnO_4$ in our system and found that it had no effect on NAA during the intervals used, but that measurable losses could be detected if exposures in excess of 20 min were used.

Table II. Recovery of NAA from Peel and Pulp of Immature Citrus Fruits[a]

Variety	Percent recovery	
	Peel	Pulp
Wilking mandarin	69 ± 2	89 ± 3
Orchard 1	60 ± 1	93 ± 1
	57 ± 2	87 ± 1
Orchard 2	62 ± 1	99 ± 1
	45 ± 1	98 ± 1
	58 ± 4	88 ± 2
Dancy tangerine	47 ± 1	94 ± 1
	48 ± 7	95 ± 1
	46 ± 1	90 ± 2
Kinnow mandarin	83 ± 1	95 ± 2
	85 ± 1	89 ± 1
	85 ± 2	92 ± 1

[a] Solka Floc was used in the extraction of pulp tissue. The silicic acid column was not used for cleanup of peel extracts. Tissue was fortified to a level of 1.0 ppm NAA.

Table III. The Effect of a Silicic Acid Column Cleanup Step on Recovery of NAA from the Peel of Immature Citrus Fruits[a]

Variety	Percent recovery	
	No column	Column
Satsuma mandarin	11	95
Wilking mandarin	59 ± 2	95 ± 1
Shamouti orange	25 ± 3	89 ± 2

[a] Tissue was fortified to a level of 1.0 ppm NAA.

Table IV. Sensitivity of the Final Method when Used on Immature Mandarin Fruits[a]

Residue fortification level (ppm)	Percent recovery	
	Peel	Pulp
1.0	93 ± 2	93 ± 1
0.2	91 ± 1	87 ± 1
0.1	86 ± 8	71 ± 2
0.05	55 ± 5	45 ± 5

[a] Solka Floc used for pulp and silicic acid column used in cleanup step for peel.

The above cleanup procedure was satisfactory for pulp extracts and initially we considered that it was satisfactory for peel extracts. The initial studies, however, were restricted to mature fruits (Table I). When analysis was performed on the peel of immature fruit, consistent results were obtained within a given orchard or variety, but apparent recoveries were frequently too low to be acceptable (Table II). Further investigation confirmed that this problem was related to maturity. The problem diminished and eventually disappeared as maturity advanced. Since it was desirable that the method be reliable for any stage of maturity, it was necessary to introduce an additional cleanup step for peel extracts to remove the variable interference factor.

Attempts to use Dowex 1-X8 ion exchange resin failed because of the difficulty of eluting NAA from the column; even strong concentrations of mineral acid and organic solvents were unsuccessful elutants. A silicic acid column proved satisfactory. Interference material was adsorbed strongly and NAA could be quantitatively eluted. Neither NAA nor the interference material came off in the preelution solvent, and the interference material did not come off during exhaustive extraction with the solution used to elute NAA. A 10 g silicic acid column finally was selected. The effectiveness of this column is shown in Table III. Attempts to shorten this cleanup step by using columns smaller than 10 g or batch style systems were unsuccessful.

Recovery and Sensitivity. The use of the spectrophotofluorometer affords the method some specificity because it allows the selection of the exact excitation and emission wavelengths. For example, the methyl esters of methyl anthranilic acid and anthranilic acid, the former occurring in mandarin oil and the latter in practically all citrus oils, are highly fluorescent compounds. If the cleanup procedure fails to eliminate these materials, they would not interfere with the analysis because their peak emission wavelengths are at 422 mμ and 400 mμ, respectively, and their fluorescence spectra do not overlap that of the NAA emission at 340 mμ.

While sensitivity of the fluorescence step can be used to indicate the lowest concentration of NAA that can be detected in the final solution, the overall sensitivity of the method depends on the quantity of tissue used and the degree of success obtained in extraction and cleanup. Concentrations at least as low as 0.02 ppm of pure NAA solutions could be detected. The overall method, however, was quantitatively reliable down to 0.1 ppm and appeared to be only qualitative at concentrations lower than this (Table IV). The percentage recovery from pulp samples decreased faster than that of the peel. We suggest that this is due to a low level of absolute adsorption by Solka Floc and/or the pulp tissue itself, which increases percentagewise as the fortification level is decreased. A typical set of relative fluorescence intensity values for untreated citrus tissues ranged between 0.019 and 0.023 for pulp and 0.059 and 0.064 for peel. These samples fortified at 0.2 ppm gave fluorescence intensities of 0.151 to 0.162 for pulp and 0.209 to 0.218 for peel.

The final sample solutions were quite stable when kept cool and in the dark. When samples were stored at room temperature and not protected from fluorescent light irradiation for periods as long as two weeks, occasional losses of 5 to 10% were found.

While we have demonstrated the adequacy of this method for a number of citrus fruits, we believe that it might also be applicable to other crops. A limited amount of work performed on apples has shown promise.

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