Identification of Scopoletin in Dried Bract of the Cotton Plant

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Scopoletin (7-hydroxy-6-methoxycoumarin), a blue-fluorescing compound, has been identified as a component of bract tissue from field-grown, frost-killed cotton. This identification is based on its chromatographic behavior on films of silica (three eluents) and on its infrared, mass, and fluorescence spectra. Also, the dansyl derivative of the unknown and authentic scopoletin was chromatographically identical. The total scopoletin content of dried bract was about 5 ppm. Investigations into the possible importance of scopoletin in byssinosis are suggested.

Bract, the leaf-like tissue under the boll of the cotton plant, and a prominent component of the "cotton dust" generated during textile processing, has been reported to contain the chemical that causes byssinosis (Antweiler, 1968; Bouhuys, 1966; Bouhuys and Nicholls, 1966; Hitchcock *et al.*, 1973; Taylor *et al.*, 1971). The causative agent is water soluble and partially deactivated by steam treatment of raw cotton (Hamilton *et al.*, 1973; Hitchcock *et al.*, 1973; Taylor *et al.*, 1971). Taylor and coworkers (1971) suggest that phenolic plant pigments are the active agents.

A study of the chemical composition of water-soluble pigments from dried bract of the mature cotton plant was undertaken. There are three major fluorescing pigments (365-nm uv light); two fluoresce yellow, and one fluoresces blue (Wakelyn, 1974). These three compounds are also present in the condensate obtained after raw cotton has been treated with steam. We have identified the blue-fluorescing compound as scopoletin.

EXPERIMENTAL SECTION

Isolation of the Blue-Fluorescing Pigment. Bracts from frost-killed, field-grown cotton (High Plains, Lubbock, Tex. area; varieties, Paymaster 111 and 909) were collected by hand in November of 1971 and February of 1973. The bract samples were ground in a Wiley mill (room temperature; 20 mesh) and stored in a dry state. Portions (10 g) were extracted with water (triple-distilled over $KMnO_4$ and H_3PO_4), either in a Soxhlet apparatus for 1 hr or at room temperature with constant stirring for 2 hr. The water extract was filtered through Whatman No. 41 and No. 42 paper and partitioned with ether (10×, 50 ml). The combined ether portions were passed through Na_2SO_4 in a sintered glass filter and evaporated to 100–200 μl. This crude preparation was purified by preparative chromatography on thin layers of silica gel, using ether (eluent I) as the eluent in lined tanks. The blue-fluorescent band was scraped off each chromatogram and eluted from the silica with methanol. The eluates were evaporated to dryness in a stream of nitrogen gas. This preparation (first) was then chromatographed as above by development in chloroform–acetone–formic acid (80:19:1, v/v/v) (eluent II). The blue-fluorescent portion was eluted from the chromatogram as described above and evaporated. This preparation (second) is called compound A.

Characterization of Compound A. Compound A and authentic scopoletin were chromatographed separately and mixed on silica gel. The solvent systems used as separate one-dimensional eluents were: ether, eluent II, and naphtha solvent–ether–formic acid (50:50:1, v/v/v) (eluent III). In addition, ether and eluent II and eluent II and eluent III were used as two-dimensional eluent pairs.

A portion of compound A and a sample of authentic scopoletin in methanol were evaporated in separate ampoules and dissolved in sodium bicarbonate solution (0.1 *N*); then a solution of 5-dimethylaminonaphthalene-1-sulfonyl (dansyl) chloride (3 mg/ml) in acetone was added to each. The ampoules were capped tightly, and the reactions were allowed to proceed at room temperature for 24 hr. A portion from each ampoule was spotted on silica thin layers and chromatographed, using ether and eluent II as separate one-dimensional eluents.

The infrared (ir) spectrum of compound A from the second preparation and authentic scopoletin were obtained in KBr discs, using a Beckman IR-18A infrared spectrophotometer. Mass spectral measurements were made with a Varian MAT CH-7 mass spectrometer with an ionizing voltage of 70 eV. Fluorescence spectra were recorded on a corrected microspectrofluorometer (Mayer and Novacek, 1974).

Quantitative Estimation of Scopoletin in Bracts. Samples (10 g) of ground bract were extracted twice in a Soxhlet apparatus using 85% isopropyl alcohol (475 ml) (Yang *et al.*, 1958) for 3 hr. The combined extracts were concentrated to about 100 ml, 5% aqueous NaCl solution (10 ml) was added, and the mixture was partitioned with chloroform (5 × 50 ml). The chloroform layers were combined, evaporated to dryness, and made up to a volume of 100 ml with chloroform. Crude bract extract samples and a standard scopoletin solution were spotted quantitatively on a silica thin layer that had been scored in 2-cm channels with a Brinkman No. 04-10-33 cutter. The quantities of standard that were spotted contained 2–50 ng of scopoletin. Chromatograms were developed for 30 min (about 12 cm), using eluent II. The fluorescence (excitation at 360 nm in the reflectance mode) on the chromatogram was measured with a spectrodensitometer, Model SD 3000, Schoeffel Instrument Corp., with a SDC 300 density computer (0.2 mg of scopoletin can be quantitatively measured). The relationship of peak area *vs.* concentration of scopoletin was linear in the range 0–50 ng, and the quantities of scopoletin in a sample were determined by comparing peak areas of the sample and standard. The use of scored channels allowed the centering marks of the detector head to be manually positioned in the center of each channel and obviated the need of critical adjustment before scans (Pons, 1971).

RESULTS AND DISCUSSION

Compound A cochromatographed with authentic scopoletin when developed with the three different eluents. Both showed the same changes in fluorescence (under 365-nm uv light) and visible color after treatment with ammonia. The dansyl derivatives of compound A and authentic scopoletin had the same fluorescence (purplish-red) and R_f values when chromatographed on silica gel with ether or eluent II. The ir, mass spectra, and fluores-

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cence spectra of authentic scopoletin and compound A were identical. The ir peaks were: 1570 and 1295 (vs), 1710, 1613, 1510, 1265, 1143, and 1020 (s), 1440, 1190, 925, and 865 (m), and 1380, 1224, 820, and 747 (w) cm^{-1}. Mass spectra were: *m/e* 50 (10%), 51 (18%), 65 (8%), 69 (36%), 79 (18%), 121 (17%), 149 (39%), 164 (21%), 177 (55%), 178 (6%), and 192 (100%), and corrected emission maximum, 380 nm (excitation, 365 nm). All of these observations show that compound A is scopoletin. The total scopoletin content of dried bract as estimated by tlc and fluorodensitometry was about 5 ppm.

Scopoletin occurs in cigarette smoke and tobacco (Yang *et al.*, 1958) and has been reported to increase greatly in fruits and vegetables infected by microorganisms (Hughes and Swain, 1960). Cotton plants affected with *Verticillium* wilt also apparently contain scopoletin (Caldwell *et al.*, 1966). Infection by the pathogen greatly enhances the production of scopoletin in the cotton plant, but physiological maturity of the plant determines the amount of scopoletin produced (Stith, 1969).

Steam treatment of raw cotton reduces byssinotic symptoms during carding (Merchant *et al.*, 1973). A similar treatment removes scopoletin from bract trash. Since phenols have been implicated in lung disease (Steinfeld, 1972), it should be determined whether scopoletin can contribute to byssinosis.

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A Fluorometric Method for the Determination of Residues of 1-Naphthaleneacetamide and 1-Naphthaleneacetic Acid on Apples

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1-Naphthaleneacetamide (NAAamide) and 1-naphthaleneacetic acid (NAA) were applied to apple trees to prevent fruit drop 4 weeks before harvest. Maximum residues detected by fluorescence spectrophotometry, on a whole fruit, fresh weight basis, did not exceed 0.049 ppm for NAAamide and 0.012 ppm for NAA. Compared to the toxicologically permitted amount of 1.5 ppm, the residues are small, but persistent. The sensitivities of the assay for NAAamide and NAA in apple extracts are 0.025 and 0.01 ppm, respectively. The recoveries for both growth regulators are 90-103%. No chromatographic steps are required using the assay procedure.

1-Naphthaleneacetamide (NAAamide) and 1-naphthaleneacetic acid (NAA) have been used for almost 30 years as growth regulators. Application shortly after blossom induces fruit thinning on apple trees and the same compounds applied 3-4 weeks before harvest prevent fruit drop. Replacement of these growth regulators with Sevin (*N*-methyl-1-naphthylcarbamate) as a fruit thinning agent failed, since the carbamate is extremely toxic to honeybees. Although NAAamide may cause less damage by the burning of leaves than NAA, it is less frequently used. Until now, sensitive methods of residue analysis for NAAamide have been unknown. For NAA, however, a previously reported uv absorption and colorimetric method could detect residues as low as 0.2-0.1 ppm in apples (Bache *et al.*, 1962). Similar procedures were developed for olives (Zweig *et al.*, 1964) and for pineapple (Young *et al.*, 1963) with a sensitivity of 0.03 ppm.

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To produce a sufficient biological effect, both growth regulators must be applied in a concentration of 20-100 ppm of the spraying solution. Consequently, residues below 0.1 ppm on apples may be expected. Therefore, it was necessary to develop a more sensitive detection procedure than those available. The fluorescence properties of NAAamide and NAA provide a specific method for the detection of NAA in microgram amounts (Hornstein, 1958). With this fact in mind, residue analyses of NAA have been conducted in citrus fruit (Jolliffe and Coggins, 1970) and, by a modified procedure, in Kinnow mandarin fruit (Coggins *et al.*, 1972) with a minimum detectable concentration of less than 0.1 ppm. In this paper a short and sensitive procedure is described for the determination of NAAamide and NAA on apples within 5-6 hr, omitting tedious chromatographic steps.

MATERIALS AND METHODS

Application of NAAamide and NAA to Apple Trees. To prevent fruit drop, a solution containing 10 g of