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Identification of Scopoletin in Cigarette Tobacco and Smoke

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Scopoletin (6-methoxy-7-hydroxycoumarin) has been identified after isolation in chromatographically pure form from the flowers, stems, and leaves of oven-dried, healthy, greenhouse-grown One-Sucker tobacco. Examination of the tobacco in 29 brands of cigarettes commonly used in the U. S. has shown that every one contained scopoletin. The mainstream smoke from every cigarette sample tested also was found to contain scopoletin.

A fluorescent substance accumulating in the roots of decapitated tobacco plants infected with virus of spotted tomato wilt, was isolated and identified by Best[1] as scopoletin (6-methoxy,7-hydroxycoumarin). Best[2] determined the histological distribution of blue-fluorescing material in the healthy tobacco plant and reported that this fluorescence was markedly brighter in the endodermis of the root-stock, stem, and leaf veins than in the other tissues of these organs, while the small roots were fluorescent throughout. Goodwin and Kavanagh[3] point out, however, that the blue fluorescence which Best reported in his later paper may not all be due to the presence of scopoletin.

Yang[4] found that oven-dried tobacco from healthy, One-Sucker tobacco plants, *Nicotiana tabacum*, grown either in the greenhouse or in an open field at the Argonne National Laboratory contained, in addition to scopoletin, more than eight different blue-fluorescing compounds. From this complex mixture of blue-fluorescing substances, Yang isolated in pure form and identified scopoletin from the dried leaves, stems, and flowers of this tobacco.

The scopoletin was identified, after purification, by comparison with an authentic sample prepared by synthesis, according to the procedure of Aghoramurthy and Seshadri,[5] and also with another authentic sample obtained by isolation of the scopoletin from oat roots, using cellulose powder and mass paper chromatographic techniques. Goodwin and Kavanagh[3] had previously identified scopoletin in oat roots.

Pollock, Goodwin, and Greene[6] in a study of external applications of scopoletin to *Avena* and *Phleum* roots have shown that scopoletin inhibits root growth.

It also appeared important to learn by experimentation whether scopoletin is present in tobacco after curing and incorporation into cigarettes, and, if so, whether any scopoletin, m.p. 204°, survives the smoking process to persist in the mainstream smoke from the cigarette. We have examined tobacco in 29 brands of cigarettes commonly used in the U. S. and have found that every one tested contained scopoletin. These included regular size, filter, "denicotinized," menthol, and king size cigarettes. We have discovered also that the mainstream smoke from every cigarette sample tested contained scopoletin. This was the case under every different smoking condition used. The amounts of scopoletin present in the smoke, however, are apparently different, and quantitative studies are now in progress.

EXPERIMENTAL

Scopoletin from cigarette tobacco. Each qualitative analysis on the tobacco was performed separately on approximately 2 g. of cigarette tobacco obtained from cigarettes in a freshly opened pack or box, purchased locally on the open, retail market. The paper from each cigarette was removed before extraction of the tobacco. In the case of filter cigarettes, the tobacco was separated from both the filter and paper. Each 2-g. extraction was carried out in a separate Soxhlet extractor, using 200 ml. of 85% isopropyl alcohol for approximately 3 hr. on a steam bath. A second extraction was made on each sample, using 200 ml. of 85% isopropyl alcohol for 3 hr. The two extracts of the 2-g. tobacco sample were combined, reduced to approximately 150 ml. *in vacuo*, and the volume was then adjusted with 85% isopropyl alcohol to the mark in a 200 ml. volumetric flask. Aliquots of this solution were then taken for one-dimensional and two-dimensional paper chromatographic analyses in comparison with authentic scopoletin, and also for additional paper chromatographic purification for further study on the scopoletin identification.

For the one-dimensional paper chromatograms, 0.5-ml. samples of each cigarette tobacco extract concentrate were spotted on Schleicher and Schuell No. 589 red ribbon chromatographic paper next to a similar amount of an authentic sample of scopoletin. Solvent systems used were 15% acetic acid–water; 60% acetic acid–water; *n*-butyl alcohol–acetic acid–water (6:1:2 v./v.); *n*-butyl alcohol–benzene-pyridine-water (5:1:3:3 v./v.); and nitromethane-benzene-water (2:3:5 v./v.). Typical R_f values for scopoletin in these solvent systems, respectively, using the S & S No. 589 red ribbon paper for chromatography and a temperature of 28° ± 3° were: 0.47; 0.74; 0.82; 0.82; and 0.69. After chromatography, the papers were examined under ultraviolet "black light" (3660 Å). A bright blue fluorescence is exhibited by scopoletin.

Although a one-dimensional chromatogram of the various cigarette tobacco extract concentrates prepared as just

(1) R. J. Best, *Australian J. Exptl. Biol. Med. Sci.*, **22**, 251 (1944).

(2) R. J. Best, *Australian J. Exptl. Biol. Med. Sci.*, **26**, 223 (1948).

(3) R. H. Goodwin and F. Kavanagh, *Bull. Torrey Botan. Club*, **76**, 255 (1949).

(4) C. H. Yang, M.S. Thesis, University of Oklahoma, August 1955.

(5) K. Aghoramurthy and T. R. Seshadri, *J. Sci. Ind. Research (India)*, **11B**, 411 (1952).

(6) B. M. Pollock, R. H. Goodwin, and Susan Greene, *Am. J. Botany*, **41**, 521 (1954).

described showed many spots when viewed by ultraviolet light, the scopoletin spot thereon could be readily detected and tentative identification made by cochromatography with authentic scopoletin.

Two-dimensional paper chromatograms of the cigarette tobacco extract concentrates also were made, using in one group the *n*-butyl alcohol–acetic acid–water system in the first direction, then 15% acetic acid–water in the second direction.

In the second group of experiments on two-dimensional chromatograms, we used the nitromethane-benzene-water system in the first direction, then 15% acetic acid water in the second direction. After chromatography, the scopoletin spot could be easily located in every case, even though other spots could be seen on the chromatogram under the ultraviolet light.

By the methods described, the tobacco from 29 brands of cigarettes was examined, and every one was found to contain scopoletin. Cigarettes studied were Camel, Cavalier, Chesterfield (both regular and king size), Dunhill, Encore, Herbert Tareyton (both king and filter), Hit Parade, Kent, Kool (both regular and filter), L & M, Lucky Strike, Marlboro, Oasis, Old Gold (both regular and filter), Pall Mall, Parliament, Philip Morris (both regular and king), Raleigh (king), Regent (filter), Salem, Sano (regular), Spud, Viceroy, and Winston.

Scopoletin in cigarette smoke. Each cigarette was smoked on a standard smoking apparatus (Phipps & Bird, Inc., Richmond, Va.) based on a design of the American Tobacco Co.

Our experiments on representative brands and types of cigarettes indicated that scopoletin was readily detectable in the smoke, when 10 individual cigarettes were smoked under all varying smoking machine conditions tested. (With practice scopoletin can be recognized on a chromatogram of the smoke from one individual cigarette.) Tried were a faster smoking rate (3.3-sec. duration; 54 ± 4 ml. volume; 60-sec. interval); a medium speed (2-sec. duration; 35 ± 4 ml. vol.; 60-sec. interval); and a slower speed (1-sec. duration; 16 ± 1 ml. vol.; 60-sec. interval). Also varied were the cigarette butt lengths; regular size cigarettes (2, 3.5, and 5 cm.) and king size and filters (3, 4, and 6.5 cm.) All combinations of the above showed the presence of scopoletin in the smoke. Judged by gross observation of the size and intensity of the scopoletin on the paper chromatograms of the smoke obtained under different smoking conditions, quantitative differences occurred. The quantitative studies are in progress in our laboratory. In that *qualitatively*, scopoletin was present under all the conditions tried for the selected representative cigarettes, the following conditions were arbitrarily selected for smoking all 29 brands: butt length of 2 cm. for regular size cigarettes and 3 cm. for king size and filters; volume, 54 ± 4 ml.; puff duration, 3.3 ± 0.2 sec. at 60-sec. intervals; and one pack or box of 20 cigarettes per sample for study.

The smoke from each 20 cigarettes of one brand was trapped, in part, in a 300-ml. Kjeldahl flask, immersed in a salt-ice mixture (av. temp., −18°). Some scopoletin was found to escape into a second and into a third trap, even when a "Dry-Ice"–acetone bath was used for cooling the first two traps. For the identification of scopoletin as reported in this paper, however, a sufficient amount was obtained in the first Kjeldahl flask, even with a salt-ice mixture, for clear-cut *qualitative* analysis.

The trapped smoke, in each case, was dissolved in dry acetone. To analyze for the presence of scopoletin, the acetone solution of the smoke was then subjected to both one-dimensional and two-dimensional chromatography according to the procedure already described for the extract from cigarette tobacco. In the case of every one of the 29 brands of cigarettes smoked, a bright blue fluorescent spot coinciding in color and R_f values with authentic scopoletin was observed. Additional spots, often 10 or more, usually could be found under the ultraviolet light. Two additional blue fluorescent compounds present on the chromatograms from the smoke are now being investigated.

In order to obtain a pure sample of scopoletin from the smoke for further identification studies, the acetone solution of the smoke was subjected to extended paper chromatographic separation. For this purpose, the acetone solution of the smoke was streaked across a sheet of S & S No. 589 red ribbon chromatography paper, size 19 × 58 cm., and first developed in a 15% acetic acid–water solution for 9 hr. by descending chromatography. The developed chromatograms were air-dried. The zone which contained scopoletin and fluoresced a bright blue color under ultraviolet light was cut from the chromatogram and sewed onto a new sheet of the S & S No. 589 paper, 19 × 58 cm. The *n*-butyl alcohol–acetic acid–water system was used for 30 hr. during this second chromatographic step. The resulting scopoletin zone (R_f of approximately 0.82) was cut from this chromatogram and sewed onto still another sheet of the S & S No. 589 paper. The third chromatographic run involved the use of 15% acetic acid–water as the developing solvent. The resulting scopoletin zone (R_f of about 0.47) was cut, sewed onto yet another new sheet of the chromatographic paper, and the *n*-butyl alcohol–acetic acid–water system was used for this fourth chromatographic step. After this extended chromatographic separation, the scopoletin zone appeared to be completely free of other compounds. It was, therefore, eluted off the paper with methyl alcohol in an elution chamber. This eluted scopoletin cochromatographed with authentic scopoletin in the solvent systems already described.

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