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COMPOSITION OF THE ODOROUS SECRETION OF TRIBOLIUM CASTANEUM

JOSEPH D. LOCONTI AND LOUIS M. ROTH¹

Pioneering Research Laboratories, U. S. Army Quartermaster Corps,
Philadelphia 45, Pennsylvania

The abdominal and thoracic odoriferous glands of adult flour beetles, *Tribolium castaneum* (Herbst), *T. confusum* J. du V., and *T. destructor* Uyttenb., contain a pungent, irritating liquid (Roth, 1943; Palm, 1946). Flour in which the beetles have built up a large population becomes pink in color due to contamination by this secretion (Chittenden, 1896; Payne, 1925; Chapman, 1926). This substance has been variously described as similar to cresols and phenols (Palm, 1946), aldehydes (Chapman, 1926), and quinones (Roth and Howland, 1941). Roth and Howland (1941) isolated the odorous material of *T. confusum* by passing dry air into a flask containing the beetles and then through a dry-ice trap in which the secretion collected as yellow-brown crystals. At room temperature the substance was a fairly volatile liquid with a pungent, quinone-like odor; it reduced KI starch paper readily.

Using this same method of isolation, Alexander and Barton (1943) obtained, from *T. castaneum*, yellow crystals melting at 32° C. The product was reduced with sulfurous acid to give a white crystalline product melting, after 4 recrystallizations, at 101-102° C. (2-ethyl-1,4-dihydroxybenzene m.p. 114° C.; 2-methyl-1,4-dihydroxybenzene m.p. 125° C.). Carbon and hydrogen analyses gave values intermediate between those of the methyl- and ethyl-derivatives. The ultraviolet absorption curve of the quinone showed a maximum at 245 m μ (2-methyl-1,4-benzoquinone, 245 m μ ; 2-ethyl-1,4-benzoquinone, 246 m μ). Electrometric titrations with N/25 ceric sulfate gave an oxidation equivalent of 67 (2-methyl-, 62; 2-ethyl-, 69). These data indicate a mixture of methyl- and ethylquinones. Essentially the same results were obtained from an analysis of the secretion of *T. confusum*.

In more recent work Hackman *et al.* (1948), using *T. confusum* and *T. castaneum*, isolated the secretion as the hydroquinones by immersing the beetles in sulfurous acid. They conclusively identified the presence of the main product as 2-ethyl-1,4-dihydroxybenzene from melting points, mixed melting points, and analyses of the dihydroxybenzene and its dibenzoate. In addition, they succeeded in tentatively identifying the presence of 2-methyl-1,4-dihydroxybenzene.

The odorous secretion of *T. castaneum* was found to act as a repellent toward these insects. The present work is concerned with the direct isolation of the chemically unchanged components of the secretion and the determination of those constituents responsible for repelling the beetles.

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ISOLATION OF THE SECRETION FROM *T. CASTANEUM*

About 74,000 adults of *T. castaneum* were placed in a 1-liter round bottom flask and the insects frozen by immersing the flask in a dry-ice-methylcellosolve bath. A nitrogen inlet tube reached to the bottom of the flask so that the gas, passing up through the mass of insects, carried with it the volatile secretion of the glands. Following the flask was a dry-ice trap through which the vapors passed. On evacuating the system to 1 mm. pressure and warming the beetles to 40° C., yellow crystals together with considerable quantities of ice began to collect in the trap. Sublimation was continued at 40° C. for 18 hours during which time most of the product was obtained. A small additional quantity was obtained by heating the beetles to 80° C. for 6 hours.

The crude product was dissolved in ether and after evaporation a 2-phase liquid system remained, a lower, brown layer and an upper, yellow layer. This crude material was sublimed, at room temperature and 0.1 mm. pressure, onto a dry-ice-cooled condenser. The end of the condenser next to the sublimation surface was insulated from the dry-ice by a one-inch plug of glass wool. In this way deep-yellow needles were collected on the cold portion of the condenser while a pale-yellow oil condensed on the warmer end of the condenser and dripped back into the sublimation tube. Thus a fairly clean separation of the crystalline product from the oil was obtained. The crystalline product, amounting to 1.8 g., melted at 33–49° C. It was subjected to batchwise countercurrent crystallization from diethyl ether and then from 35–60° C. petroleum ether. Two yellow crystalline products were isolated:

Compound A—Less soluble fraction; 113 mg.; m.p. 67–67.3° C.

Compound B—More soluble fraction; 1.4 g.; m.p. 37–38.7° C.

After removal of the oil layer (0.6 g.), a small amount of dark-brown, viscous material remained. Sublimation at 80° C. and 0.1 mm. pressure yielded a few greenish-yellow crystals (Compound C) melting at 143.5° C.

The isolation of these products from batches of 25,000 to 75,000 beetles was continued until from a total of approximately 1,850,000 insects (about 3238 g.) the following yields were obtained:

Mixture of Compounds A and B.....	30 g.
Compound C.....	44 mg.
Oil.....	15 g.

The crude material and the purified fractions were stored in a refrigerator.

CHEMICAL IDENTIFICATION OF THE COMPONENTS OF THE SECRETION

1. Compound A—Compound A, m.p. 67–67.3° C., gave no depression in melting point on admixture with authentic toluquinone (m.p. 68–69° C.).

Anal. Calcd. for $C_7H_8O_2$: C, 68.9, H, 4.95. Found: C, 68.9; H, 5.10.

Derivatives: (a) 2-Methyl-1,4-Dihydroxybenzene (Toluhydroquinone): One hundred mg. of Compound A were dissolved in 35 ml. water by warming slightly, and sulfur dioxide bubbled through for 5 minutes to produce a colorless solution. This was extracted 4 times

with 25 ml. volumes of ether; the ether extract was then dried and evaporated. A syrup remained which was dissolved in 5 ml. benzene and boiled to remove water. A white crystalline product was obtained on cooling. After recrystallization from benzene, the product melted at 125° C. Synthetic toluhydroquinone melted at 125–125.5° C., and no depression in melting point was observed on admixture with the derivative from the natural product.

(b) 4-5-Dibromo-1-methylcyclohexene-1-dione-3,6: To a solution of 100 mg. of Compound A in 0.5 ml. of dry chloroform, was added a solution of bromine in dry chloroform at 0° C. until no more bromine was taken up. The chloroform was allowed to evaporate leaving a syrup which, upon scratching and cooling, crystallized as pale-yellow prisms. Recrystallization from alcohol yielded a product with a m.p. of 55–59.5° C. The product obtained by the analogous bromination of synthetic toluquinone also melted at 59–59.5° C. with no depression in melting point upon admixture of the 2 products.

2. Compound B—This consisted of bright-yellow crystals, m.p. 37–38.7° C. which gave no melting point depression on admixture with a sample of synthetic ethylquinone (m.p. 39.5° C.).

Anal. Calcd. for $C_8H_{10}O_2$: C, 70.6; H, 5.92. Found: C, 70.0; H, 5.68.

Derivatives: (a) Ethylquinhydrone: To a solution of 100 mg. of Compound B and 100 mg. of synthetic ethylhydroquinone in 1 ml. of 95% alcohol was added 0.7 ml. of water. Upon cooling in a dry-ice bath, blue-black crystals of the quinhydrone formed readily. These were filtered on a cold funnel and washed with 0.5 ml. of 55% alcohol. The crystals melted at 79–80° C. while the analogous product from synthetic ethylquinone melted at 81–82° C. No depression in mixed melting point was observed.

(b) Benzyl Mercaptan Adduct: One hundred mg. of Compound B plus 100 mg. of benzyl mercaptan were dissolved in 5 ml. of ligroin (70–90° C.). On standing overnight and allowing part of the ligroin to evaporate, blue-black crystals, m.p. 76–78° C., were obtained. No melting point depression was observed on admixture with the analogous product from synthetic ethylquinone (m.p. 77–78° C.). On moistening with alcohol, both products turned to orange-colored crystals which regained their blue-black color on drying.

(c) Ethylhydroquinone: This was prepared by the same procedure as for toluhydroquinone. Crystallization from chloroform, benzene, and heptane as well as sublimation failed to give a pure product. Purification was finally achieved by rapidly crystallizing twice from water. The product melted at 115–115.5° C. and showed no depression on admixture with synthetic ethylhydroquinone.

3. Compound C—This compound was resublimed at 0.5 mm. pressure and 80–90° C. to give yellow crystals which melted at 143.5° C.

Anal. Calcd. for $C_7H_8O_2$: C, 60.9; H, 4.38. Found: C, 60.9; H, 4.41.

No derivatives were prepared because of the small amount of material available. No depression in melting point was observed on admixture with 2-methoxy-1,4-benzoquinone (m.p. 144.5–145° C.) obtained by oxidation of *o*-anisidine with chromic acid.

PHYSICAL IDENTIFICATION OF THE COMPONENTS OF THE SECRETION

In the determination of the ultraviolet absorption spectra of these quinones, using 95% alcohol as a solvent, it was noted that the quinones were unstable and the curves shifted with time (cf. Alexander and Barton, 1943). With ethylquinone, for example, the maximum at 247 m μ disappeared, while a new one appeared at 293 m μ . This change progressed more rapidly in the light and at room temperature than in a dark refrigerator. Figure 1 shows the effect of storage on the concentration of ethylquinone in alcohol. After storage for 24 hours at +5° C., there was a 67% loss of ethylquinone while after 6 days the total loss was 85%. As for the nature of this decomposition, it is possible that the ethylquinone was converted to ethylhydroquinone since a new maximum appeared at 293 m μ , at which point lies the maximum for ethylhydroquinone. In spite of this, however, alcohol was suitable as a solvent for quinones provided the curves were determined soon after preparation of the solution.

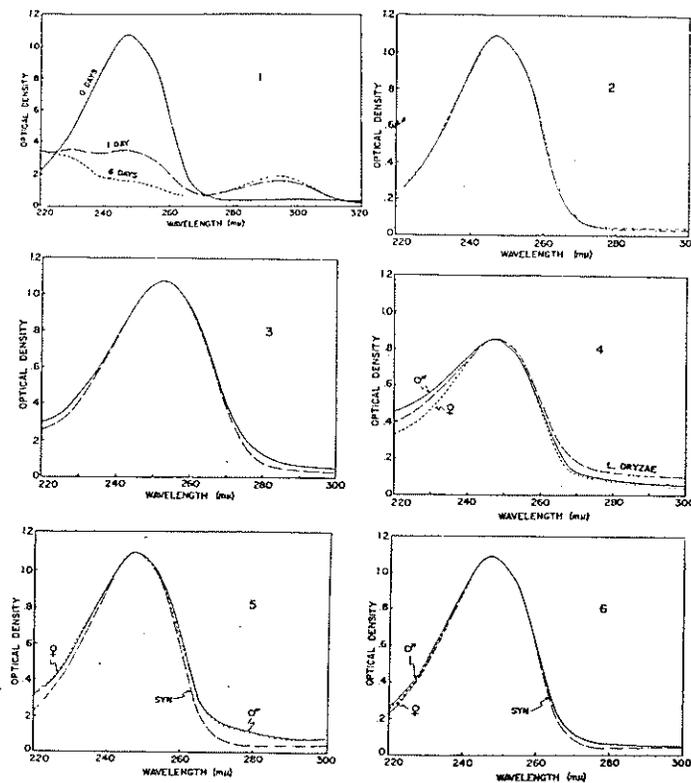
The ultraviolet absorption curves for synthetic ethylquinone and purified ethylquinone isolated from the beetles are shown in figure 2. Both curves are identical. Figures 5 and 6 show the results obtained with secretions from the abdominal and thoracic glands of males and females of *T. castaneum*. The curves for the secretions used in obtaining figures 5 and 6 were collected by cooling the insects in an ice-cooled Petri dish and picking off the crystalline material with a dissecting needle after the substance had flowed from the glands and solidified on the bodies of the beetles (Roth and Howland, 1941). Each curve was determined within a few minutes after the preparation of the solutions from material obtained from several insects. For comparative purposes each curve was adjusted to the same optical density at 247 m μ . Apparently the secretion is similar, as far as quinone content is concerned, in both the thoracic and abdominal reservoirs of both sexes.

The ultraviolet absorption curves of synthetic methoxyquinone and methoxyquinone isolated from *T. castaneum* are shown in figure 3. The curves are essentially identical, with maxima at 252.5 m μ .

The mild conditions under which the quinones were isolated is an indication that these compounds were present as quinones in the beetles and were not formed from other compounds during isolation. This indication is further supported by the ultraviolet absorption curves which were determined within a few minutes after the secretions were removed from the reservoirs of the glands.

Odoriferous glands are found in many tenebrionids (Roth, 1945). An examination of the adults of *Latheticus oryzae* Waterh. revealed that they too have thoracic and abdominal odoriferous glands situated in positions similar to those in *Tribolium*. Ultraviolet absorption curves (fig. 4) were made of the secretions of *T. destructor* by crushing the insects in the solvent and filtering off the solids. The curve for *Latheticus* (fig. 4) was obtained from crude material sublimed from a mixture of both males and females of this species. The maxima of the curves shown in figure 4 indicate that ethylquinone or ethyl- and toluquinones are present in both *T. destructor* and *Latheticus*. Thus at least 4 members of the Tenebrionidae (including *T. confusum*) secrete quinones. The myriapod *Julus terrestris* also secretes a quinone (Béhal and Phisalix, 1900).

The differentiation between toluquinone and ethylquinone by means of ultraviolet absorption is unsatisfactory. Curves for ethylquinone and toluquinone lie so close together with the maxima appearing at 246 m μ and 247 m μ respectively, that differentiation between them is not possible on the Beckman D. U. Quartz Spectrophotometer. However,



FIGURES 1-6. Ultraviolet absorption spectra. FIG. 1. Destruction, with time, of synthetic 2-ethyl-1,4-benzoquinone in alcohol. The solid line (0 days) represents the spectrum of the compound immediately after preparation of the solution. FIG. 2. Synthetic 2-ethyl-1,4-benzoquinone (broken line) and the natural product of *T. castaneum* (solid line). FIG. 3. Synthetic 2-methoxy-1,4-benzoquinone (broken line) and the natural product from *T. castaneum* (solid line). FIG. 4. Odorous secretions from males (σ) and females (φ) of *T. destructor*, and both sexes (combined) of *L. oryzae*. FIG. 5. Synthetic 2-ethyl-1,4-benzoquinone (SYN) and secretions from the abdominal odoriferous glands of males (σ) and females (φ) of *T. castaneum*. FIG. 6. Synthetic 2-ethyl-1,4-benzoquinone (SYN) and secretions from the thoracic odoriferous glands of males (σ) and females (φ) of *T. castaneum*.

the curves are useful for (a) substantiating the chemical evidence for the presence of one or both of the quinones cited, and (b) showing that these quinones are present in *T. destructor*, *L. oryzae*, and the abdominal and thoracic odoriferous glands of both males and females of *T. castaneum*.

POSSIBLE FUNCTION OF THE ODOROUS SECRETION

There has been some speculation concerning the function of the odorous secretion of *Tribolium*. Palm (1946) concluded that the substance serves as a defensive weapon since the secretion is highly toxic

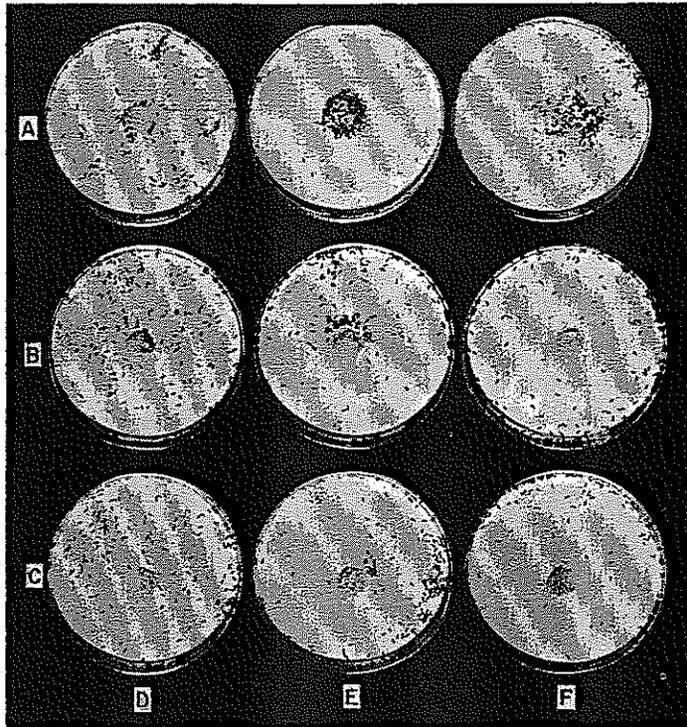


FIGURE 7. Repellent effect of synthetic and naturally occurring ethylquinone towards adults of *Tribolium castaneum*.

Horizontal rows: A = untreated flour; B = flour covering 5 mg. of ethylquinone obtained from adults of *T. castaneum*; C = flour covering 7 mg. of synthetic ethylquinone.

Vertical rows: Time after introduction of the beetles. D = 30 seconds; E = 10 minutes; F = 2 hours.

(Tests performed in darkness; 200 beetles per dish.)

to the beetles themselves (cf. Gough, 1939; Shepard, 1943), has a disagreeable odor, and is discharged when the beetles are disturbed. However, Roth (1943) has pointed out that there are relatively few insect enemies of *Tribolium* which infest flour and that the odorous secretion does not prevent the adult beetles from becoming infested with mites. Species of *Tribolium* have been found under the bark of trees and in old logs (Good, 1936; Butler, 1949), and it is possible that in these habitats the odorous secretion is employed as a means of defense. That the secretion from *T. castaneum* is a repellent was shown by the following experiments.

Adults of *T. castaneum* which had been starved for 5 days prior to performing the tests were used. Beetles in this state of nutrition are strongly attracted to flour (Willis and Roth, 1950). The test method was based on opposing this attraction toward flour by a repellent. A 15 cm. Petri dish having a disc of filter paper on the bottom contained the insects. Five mg. of powdered ethylquinone, obtained from the beetles, covered by about 0.2 g. whole wheat flour was placed in the center of the dish. Two hundred beetles were distributed at random around the edge of the dish and their behavior toward the flour was noted. Figure 7 shows the results of this test together with tests showing the behavior of beetles towards untreated flour and flour covering a powdered layer of 7 mg. of synthetic ethylquinone. Whereas the untreated flour was quickly invaded by the starved beetles, the mounds of flour placed over the synthetic and natural occurring ethylquinones were more or less intact after 2 hours. Tests with the naturally occurring toluquinone and methoxyquinone showed that the former was repellent while the latter failed to repel the beetles.

EFFECT OF THE ODOROUS SECRETION ON POPULATION GROWTH

It is well known that the activities of *Tribolium* in flour result in the medium becoming altered or "conditioned" (probably nutritive depletion and an increase in the concentration of waste products) and that the population declines largely through reduction of the reproductive rate (Allee *et al.*, 1949). It has been suggested that the odorous secretion may be one of the factors responsible for the decline of *Tribolium* populations in conditioned flour (Roth, 1943; Alexander and Barton, 1943). The following experiment was performed to test this hypothesis by observing the growth of populations in flour conditioned by the secretion. When recently-killed adults of *Tribolium* are placed in flour, the medium eventually becomes pink as a result of the volatile quinones being picked up by the flour. The pink flour fails to discolor KI starch paper, whereas discoloration of this paper is a test for the odorous secretion of *Tribolium* (Roth and Howland, 1941); also when mixed with hydroquinone the pink flour does not become blue-green (quinhydrone) which color is obtained when living beetles or crystals of synthetic ethylquinone are crushed with hydroquinone. It appears that the secretion no longer exists as quinones once it discolors the flour (cf. Alexander and Barton, 1943). Four different media were prepared by conditioning whole wheat flour with dead beetles. This method of conditioning the flour eliminated the nutritive depletion resulting from feeding insects. The beetles were

killed with chloroform vapor and placed in a refrigerator overnight. The dead insects were then mixed with finely ground flour and enclosed in weighing bottles for 3 days in an incubator at 30° C. and about 70% relative humidity. Each of the 4 media was prepared with one of the following concentrations of dead adults: (a) none (control); (b) 50 beetles/g. flour; (c) 150 beetles/g. flour; (d) 250 beetles/g. flour. At the end of 3 days the flour samples containing dead beetles were placed in open dishes in the incubator for 2 weeks. By this time the contaminated flour had become noticeably pink, those samples with the heaviest concentrations of dead beetles being the darkest in color. The flour was then sifted through an 80-mesh sieve and the dead beetles removed. A pair of living adults of *T. castaneum* was then introduced into 20 g. of each medium (4 replicates). Fecundity records of the females had been kept for 4 days prior to the start of the experiment; the oviposition rates of the females selected for the experi-

TABLE I

GROWTH OF POPULATIONS (ADULTS) OF *TRIBOLIUM CASTANEUM* IN FLOUR
CONDITIONED BY DEAD ADULTS

NOTE: The number is the mean of 4 populations; each replicate was seeded with 1 pair of adults; census taken after 163 days.

Extent of Conditioning of Flour	Mean Number ± Standard Error of Adults (Living + Dead)	Mean Number ± Standard Error of Dead Adults
Uncontaminated flour (control).....	142 ± 17.6	4.2 ± 1.9
50 dead beetles/g. flour.....	154 ± 3.1	5.2 ± 1.4
150 dead beetles/g. flour.....	104 ± 6.5	4.8 ± 1.0
250 dead beetles/g. flour.....	105 ± 13.7	6.0 ± 1.0

ment did not differ significantly from one another. The populations were allowed to build up for 163 days at the end of which time a census of adults was taken. The results are shown in Table I. Although the adult populations of the two most heavily conditioned media were somewhat lower than the controls, the differences between them and the control are not significant. Though this data is inconclusive it does indicate that, with the above experimental procedure, the conditioning of flour by quinones from flour beetles has relatively little influence on the increase of an adult population of *Tribolium*.

SUMMARY

The odorous secretion of *Tribolium castaneum* was sublimed directly from more than 1.5 million freeze-killed insects at 1 mm. pressure and 40° C. The yellow crystalline product, collected in a dry-ice trap, was purified by fractional crystallization and sublimation. Three different quinones were identified by melting points, suitable derivatives, and ultraviolet absorption spectra. Of the quinones present in the odorous secretion of *T. castaneum*, 80-90% was 2-ethyl-1,4-benzoquinone,

10-20% was 2-methyl-1,4-benzoquinone, and there was a trace of 2-methoxy-1,4-benzoquinone.

Ultraviolet absorption data showed that ethyl-and/or toluquinone were present in *Tribolium destructor*, *Latheticus oryzae*, and the abdominal and thoracic odoriferous glands of both males and females of *T. castaneum*.

Qualitative repellency tests, using *T. castaneum*, were made with the 3 quinones isolated from the adult beetles. The test method was based on the fact that starved beetles were strongly attracted to flour, and a repellent substance, when introduced into the flour, opposed or prevented this attraction. Of the 3 naturally occurring quinones tested, the ethyl- and toluquinones were repellent while the methoxyquinone failed to repel the beetles.

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