1337

The Composition of Honey. III. Detection of Acetylandromedol in Toxic Honeys

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Introduction

Toxicity of honey from certain sources is scarcely a new problem, having been recorded by Xenephon (Anabasis, Bk IV) and intermittently since. The occurrence of poisonous honey has been reviewed (1).

The reported toxicity of honey from such genera as *Rhododendron* and *Kalmia* has been related by inference to the toxic principle of the leaves of these and other Ericaceae (1, 2). The active principle in these plants (*Andromeda*, *Rhododendron*, *Kalmia*, *Leucothoe*, *Lyonia*, and *Pieris* spp.) has been shown to be acetylandromedol (andromedotoxin) (3, 4). Although flowers of *Rhododendron* (4) and *Azalea* (5) have been reported to contain acetylandromedol, and the physiological effects of the honey have been ascribed to this compound (2), no chemical procedure has been reported for the detection of acetylandromedol in honey.

As part of an extensive survey of the composition of American honeys, two samples of honey labeled "toxic" have been received;[1] the floral source of each was unknown to the producers. They were produced in the Great Smoky Mountain area of eastern Tennessee and at Banner Elk in western North Carolina. Each was "water-white" in color grade.

Experimental and Results

Chloroform extracts of the honeys gave a positive test with Godin's reagent. They were subjected to paper electrophoresis in borate buffer as described by Tallent, Riethof, and Horning (4) and gave positive tests for acetylandromedol. Crystalline acetylandromedol was obtained from one sample.

[1] Honey samples were supplied by Roy D. Brown, Newport, Tenn., and by Raymond Presnell, Banner Elk, N. C. via W. A. Stephen, Extension Apiarist, Raleigh, N. C. The acetylandromedol was kindly supplied by E. C. Horning, National Institutes of Health, Bethesda, Md. The infrared spectra were obtained by Carl Leander of this laboratory.

TABLE I

Paper Electrophoresis of Honey Extracts[a]

Material and source	0.01 *M* tetraborate		0.05 *M* tetraborate	
	Migration[b] *cm.*	Color[c]	Migration[b] *cm.*	Color[c]
N. Carolina	+4.8	Blue	+2.4	Blue
	+7.1	Blue		
Tennessee	+4.8	Blue	+2.4	Blue
	+7.2	Blue		
Acetylandromedol	+4.8	Blue	+2.4	Blue
	+7.2	Blue		

[a] See text for conditions.
[b] Corrected for electroosmosis.
[c] With Godin's reagent.

In a typical experiment, 45 g. honey was diluted with water to 130 ml., its pH was adjusted to 6.5, and it was extracted six times with chloroform. The extract was evaporated to dryness, taken up in 1 ml. of 95% ethanol, and 50 μl. was subjected to electrophoresis at 300 v. (5.6 v./cm.) for 6 hr. on Whatman No. 1 paper impregnated with 0.01 *M* sodium tetraborate. The apparatus was essentially that of Kunkel and Tiselius (6). After air-drying, the paper was treated with Godin's reagent. Electrophoresis was also carried out in 0.05 *M* tetraborate for 4 hr. Results are shown in Table I. The presence of two migrating species in 0.01 *M* and one in 0.05 *M* tetraborate is characteristic of acetylandromedol (4), as is the blue color with Godin's reagent.

Two other honey samples (a midwestern clover and a North Carolina mountain blend) gave negative results.

A sample of the North Carolina honey (116 g.) was diluted with 250 ml. water and neutralized to pH 7.0 with dilute sodium hydroxide. It was extracted with six portions (300 ml. each) of chloroform, using centrifugation to break emulsions. All extracts were combined and evaporated. The yellowish, waxy residue was extracted with water, which was filtered and evaporated *in vacuo*.

The water-soluble material was extracted several times with hot ethyl acetate, the solution filtered and allowed to evaporate. Of this 14.5 mg. residue, 11.8 mg. (0.010% of original honey sample) dissolved in ethyl acetate and was adsorbed on a column of 5 g. of acid-washed alumina. After washing with 4 ml. ethyl acetate the material was eluted with 30 ml. 10% methanol in ethyl acetate. It crystallized on evaporation and was recrystallized in fine needles from ethyl acetate-petroleum ether. The melting point of the air-dried material (capillary, Berl block, uncorr.) was 240–243° C.; that of an authentic sample (6) of acetylandromedol was 240–243°, as was the melting point of their mixture. The infrared spectrum of the material from honey coincided exactly with that of authentic material over the entire range examined (600–4000 cm^{-1}).

The yield of acetylandromedotoxin from the honey sample is comparable to that obtained by Wood *et al.* (3) from leaves of *Rhododendron maximum* (0.008%) and to the ++ concentration estimated by Tallent *et al.* (4) in leaves from *R. ponticum*, *R. luteum*, *R. catawbiense*, *Leucothoe editorum* and *Pernettya coriaceae*.

Pollen analyses of the honeys in Table I[2] indicated that both were principally derived from the mountain laurel (*Kalmia latifolia*), with considerable white clover pollen also present.

Summary

1. Paper electrophoresis of chloroform extracts of two honey samples suspected to be toxic has shown them to contain acetylandromedol, confirmed by the infrared spectrum of the isolated compound.

2. Both samples were shown by pollen analysis to originate largely from mountain laurel.

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[2] Pollen analysis was carried out by A. S. Michael, Field Crops Insects and Bee Culture Branch, Entomology Research Division, U. S. Dept. Agriculture, Beltsville, Md.

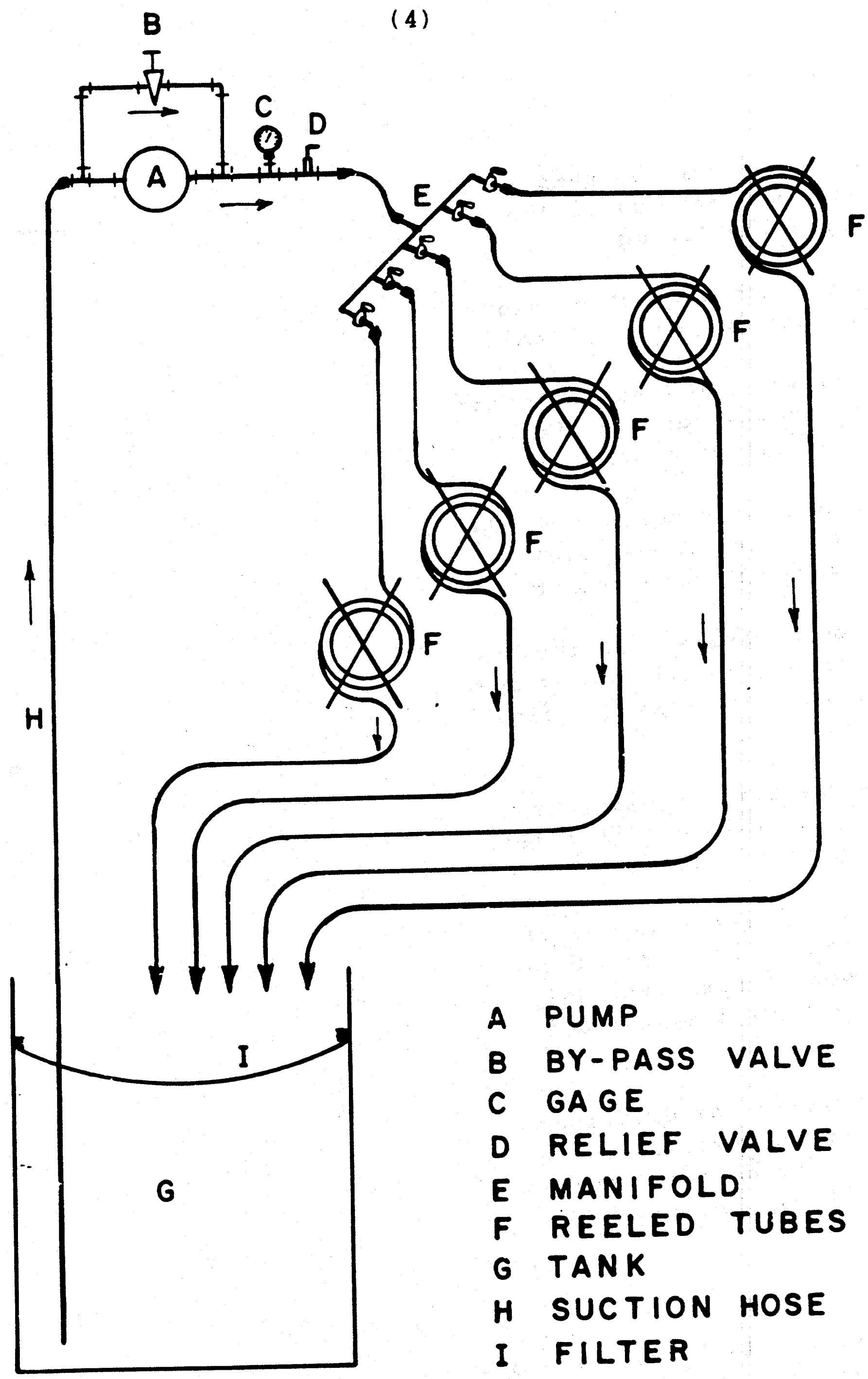

Figure 1.
Suggested assembly for washing plastic tubing.

(4) Start pump.

(5) Close bypass valve slowly until the pump is primed and then open it slowly until the liquid is flowing from the tubing.

(6) Measure the time required to collect an exact volume (1 quart, 1/2 gallon, or 1 gallon).

(7) If time required to collect 1 gallon is longer than 1 minute, close bypass valve a little and again measure the rate of discharge.

(8) Continue this process until the opening of the bypass allows 1 gallon of water per minute to be discharged from the 50-foot tubing.

(9) Note and record the gage pressure. If the by-pass-valve setting allows a discharge of more than 1 gallon per minute, decrease the flow rate by opening the valve.

(10) Measure and record the discharge pressure required to give 1-gallon-per-minute flow from each of the other lengths of tubing.

These pump gage pressures will deliver 1 gallon of liquid per minute regardless of whether 1 or more tubes are attached to the manifold and washed simultaneously providing all are approximately the same diameter and length.

Gage pressures must also be measured for different engths of lines containing different numbers of connect-rs. From the recorded data a calibration table can be onstructed. An example of typical calibration information is given in Table 1.

anifold

This is a metal 1-inch pipe, preferably brass, approx-nately 24 inches long with a number of cocks, for attach-nt of the plastic tubing, and an inlet connected to the scharge side of the pump. The 1/8-inch bore cocks should

TABLE I

Typical liquid pressures required
to yield flow rates of 1 gallon per minute
in various lengths of tubing with and without connectors.

LENGTH OF TUBING (FEET)	CONNECTORS IN LINE (NUMBER)	LIQUID GAGE PRESSURE (P.S.I.)
50	0	24
50	1-5	33
50	6-10	48
100	0	37
100	1-5	46
100	6-10	61
150	0	54
150	1-5	63
150	6-10	78
200	0	63
etc.	etc.	etc.

be of the long-handle, service type, with male inlet and tapered serrated outlets.

Tanks

Three tanks are required, one each for the disinfectant, the detergent, and the rinse water. These can be of the galvanized ashcan type. Suggested sizes required for the washing of different amounts of plastic tubing are 32-gallon size for 5,000 or more feet, 24-gallon for 2,000 to 5,000 feet, and 16-gallon for less than 2,000 feet.

Miscellaneous

Hose: The hose used for pump suction lines and for connecting pump to manifold must withstand 1,000 p.s.i.

Clamps: For hose and tubing connections.

Measures: For calibrating the rates of flow of wash liquid through the tubing. These can be of quart, 1/2-gallon, or 1-gallon size.

Reels: For coiling the plastic tubing during washing and storing. Reels should hold up to 500 feet of tubing.

WASHING TUBING AND FITTINGS

Plastic Tubing

(1) Wash the tubing by pumping successively the disinfectant, detergent, and the rinse water through the tubing at a rate of 1 gallon per minute. Cold wash solutions can be used, but better results are obtained if solutions are warm. Hot solutions, above 160° F. must not be used since they will soften and enlarge the tubing.

(2) All branch lines must be disassembled and all fittings, spouts, "T"s, "Y"s, etc., removed. Reassemble the tubing, using connectors, into continuous unbranched lines of 150 to 300 feet. Very long lines require excessive pump pressures to maintain the 1-gallon-per-minute flow rate (see method of pump calibration under Equipment) causing separation at the connections.

(3) Attach an assembled length of tubing to one or several of the cocks of the manifold, cocks open. Coil each of the lengths of tubing very loosely on a reel and place the discharge end in the tank containing Solution A so that the disinfectant can be recirculated through the system.

(4) Place the suction line of the pump in the disinfectant (Solution A), open the bypass valve of the pump and open all the cocks on the manifold to which tubing is attached. Start the pump.,

(5) Adjust to the desired pump pressure by adjustment of the bypass valve. Pump the disinfectant through the lines for 15 minutes.

(6) After pumping the disinfectant for 15 minutes, move the intake hose and the discharge lines into the detergent (Solution B).

(7) Pump the detergent through the tubes 15 minutes.

(8) After pumping the detergent through for 15 minutes, rinse the tubing with clear water by transferring the intake hose of the pump to a tank of clear water. Don't recirculate the rinse water; discharge it into a waste pipe. Pump the rinse water through the lines for at least 5 minutes.

(9) Disconnect the washed, rinsed tubing from the manifold. Drain and store the tubing. Draining is best accomplished either by suspending the tubing or by laying it on a slope. (The tubing will seldom drain completely dry). For storage, wind the tubing on reels and store out of the sun in a clean dry place.

Fittings (Spouts, "T"s, "Y"s, Connectors, Etc.)

Plastic:

(1) Soak in the disinfectant (Solution A) for 15 minutes. Remove from the disinfectant and allow to drain. Cold disinfecting solution can be used, but better results are obtained if solutions are warm. Hot solutions above 160° F., must not be used since they will soften and deform the plastic.

(2) Wash in the detergent (Solution B) for 15 minutes. The detergent must be agitated during this period so that it will be carried through all openings of the fittings. This can be done easily in a domestic clothes washing machine

(3) Following the 15-minute detergent wash, remove the fittings, drain, and rinse in clear flowing water or in three successive lots of clean water.

(4) Dry, preferably in the sun, on a tray lined with clean cloth or paper.

(5) Store in a covered, clean, dry container, such as a fiberboard box.

Metal:

(1) Soak in the disinfectant (Solution A) for 15 minutes.

(2) Remove and drain, place in the detergent (Solution B) in a metal container, and heat to a full rolling boil. Continue the boiling for 15 minutes. The boiling detergent will agitate the fittings sufficiently to insure thorough washing.

(3) After 15 minutes, remove the metal fittings, rinse well with clear water, drain, and dry on a tray in the sun. Place in a dry, covered container and store in a dry place.

DISCUSSION

The inner surfaces of plastic equipment which has been used to carry sap are not only dirty but may be covered wholly or in part with heavy deposits of microbes. Since many of these organisms are still living, the problem of cleaning the inner surfaces of the plastic involves killing these organisms as well as removal of firmly-held deposits. As in the case of washing clothing, best results are obtained when the wash solutions are agitated vigorously. The wash solutions in the tubing can be agitated only by pumping solutions through the tubes. Since higher flow rates provide greater agitation the minimum recommended rate for cleaning of plastic tubing is 1 gallon per minute.

The use of the unusually strong disinfectant solution is recommended to insure complete sterilization for the relatively short contact time (15 minutes) recommended. A longer contact time with a weaker disinfectant would also be satisfactory but, since this would be too time consuming, it is not practical. Most of the commercial disinfectants of the hypochlorite type also have some detergent action. A detergent following the disinfectant is recommended to insure the loosening and removal of dirt from the inner walls of the plastic.

The length of time, the order, and the manner of washing with the two solutions were established by experimental studies using highly contaminated plastic tubing that had been in use one season in an operating sugar bush.

To attempt to wash plastic tubing with all of the multiple branches and fittings intact is not recommended. Disassembling the tubing and fittings and then reconnecting the pieces of tubing into a single, continuous length of unbranched tubing assures that the volume of wash solution pumped per minute will be the same throughout the length of the tubing. Were the branches to be left intact, the flow of wash solutions would follow the path of least resistance, leaving some portions of the tubing poorly cleaned. For the same reason the fittings are best washed separately rather than while attached to the tubing.

The length of the tubing, either as one continuous piece or composed of short connected pieces, that can be washed at one time is dependent upon the pressure of the wash solution exerted in the lines and upon the strength of the connections between the pieces. Extremely long tubing and large number of connectors require higher pump pressure to maintain a flow rate of 1 gallon per minute. Excessively high pressures will cause separation of the lines at the connections, unless they are secured with clamps or wire. The use of clamps or wire is not recommended, because their installation and subsequent removal is tedious and time consuming.

Since a considerable amount of material is removed from the tubing, the wash solutions may become quite dirty. Consequently, the solutions should be filtered. This can be done by mounting several layers of cheesecloth or flannel between the discharge end of the tubing and the top of the tank, or by filtration of the solutions before each new set of tubes is attached to the manifold.

SIMPLIFIED WASHING METHOD

The strengths of the two wash solutions, disinfectant and detergent, and length of washing time were established by experimentation using excessively dirty tubing. If the tubing to be washed is relatively clean and free of dirt, the above directions can be modified by shortening the time of washing or by combining the disinfectant and the detergent into a single solution (Solution C) so that only one wash is necessary. Both the disinfectant and the detergent, when combined as directed to make Solution C, have the same concentration as the individual solutions. Use of this simplified method does not eliminate the final rinse with clean water.

The shortened time should never be less than 5 minutes for each of the two solutions and never less than 10 minutes if the combined solution is used.

For moderately dirty tubing, using Solution C with a washing time of 15 minutes and a flow rate of 1 gallon per minute is recommended.

When Solution C is used, considerable time can be saved if the pieces of tubing are attached to the outlets of the manifold in rotation. For a 5-outlet manifold, attach a new piece of tubing every 3 minutes. Thus, as each of the successively added tube has had its 15-minute washing, it is replaced without delay by a new one.

PRECAUTIONS

DO:

- Take down and wash tubing immediately at the end of the sap season. Tubing left in the woods or in storage becomes progressively dirtier and therefore harder to wash because of increased microbial activity with increasing season temperatures. After draining, inspect all tubing for evidence of unremoved material clinging to the inner surfaces. Rewash if necessary.

- Use new wash solutions for each 5,000 feet of tubing.

DON'T:

- Economize on strength of wash solutions by using less detergent or disinfectant than recommended.

- Economize on time of washing.

- Wash plastic tubing or fittings in water hotter than 160° F. Water above this temperature will soften and weaken the plastic. Tubing washed with very hot solutions will stretch, making attachments to the fittings difficult. Fittings may be deformed and thus become unsatisfactory.

- Wind tubing tightly on reels prior to washing because it will cause pinching of the tubes and thereby reduce rate of flow of wash solutions.

- Put metal fittings in a domestic clothes washing machine for they will damage the enamel surface of the machine.

- Overload the washing machine with plastic fittings.