Spectral Characterization of Cucurbitacins in a Bitter Mutant of Hawkesbury Watermelon (*Citrullus vulgaris* Schrad) that Elicit a Feeding Response to Diabroticite Beetles (Coleoptera: Chrysomelidae)[1,2]

A. B. DeMilo, C. J. Lee,[3] R. F. W. Schroder,[4] W. F. Schmidt[5] and D. J. Harrison[6]

Insect Chemical Ecology Laboratory, USDA, ARS, PSI, BARC-West, Beltsville, MD 20705 USA

J. Entomol. Sci. 33(4): 343-354 (October 1998)

Abstract Fractions obtained by open-column flash chromatography of a crude methanolic extract of the rind of a bitter mutant of Hawkesbury watermelon, *Citrullus vulgaris* Schrad, were further purified by preparative high-performance liquid chromatography (HPLC) to isolate chemical component(s) that elicit a visitation/feeding stimulancy response to the southern corn rootworm, *Diabrotica undecimpunctata howardi* Barber. Activity of chromatographic fractions were followed with a laboratory bioassay involving total insect-response counts. The chemical structure of the most active component in *C. vulgaris* was confirmed by chemical ionization mass spectrometry and proton nuclear magnetic resonance spectroscopy to be cucurbitacin-E glycoside. Two other cucurbitacin-like compounds were isolated and structures for them postulated. A procedure to prepare a crude, biologically active, extract of *C. vulgaris* is reported. Dose-response data for the crude extract in laboratory tests against two diabroticite beetles *D. undecimpunctata howardi* and *D. virgifera virgifera* LeConte are also reported.

Key Words Corn rootworm, southern corn rootworm, western corn rootworm, phagostimulant, cucurbitacin-E glycoside, *Citrullus vulgaris* Schrad, bait.

The most destructive pests of corn in North America are the northern corn rootworm, *Diabrotica barberi* Smith & Lawrence; the western corn rootworm, *D. virgifera virgifera* LeConte; the southern corn rootworm, *D. undecimpunctata howardi* Barber, and the Mexican corn rootworm, *D. virgifera zeae* Krysan and Smith. To control corn rootworms, farmers routinely spray 12 to 16 million ha or 50 to 60% of the corn acreage grown annually (Metcalf 1986). Usually, insecticide treatments are prophylactically applied, often unnecessarily, and consequently create health risks to humans, livestock, and wildlife (Sutter and Lance 1991).

By virtue of their ability to alter adult behavior, semiochemicals offer considerable promise for the management of corn rootworms (Levine and Oloumi-Sadeghi 1991,

[1]Received 22 October 1997; accepted for publication 02 February 1998.

[2]This article reports the results of research only. Mention of a proprietary product does not constitute endorsement or a recommendation for its use by the USDA nor does it imply registration under the FIFRA act as amended.

[3]Samsung Advanced Institute of Technology, CRICT, Taejon, Korea 305-380.

[4]Insect Biocontrol Laboratory, PSI, ARS, USDA, BARC-West.

[5]Environmental Chemistry Laboratory, NRI, ARS, USDA, BARC-West.

[6]Diet and Human Performance Laboratory, BHNC, ARS, USDA, BARC-East.

Sutter and Lance 1991, Metcalf and Metcalf 1992). Among the various types of semiochemicals investigated for this purpose are the curcurbitacins (oxygenated tetracyclic terpenes), non-volatile compounds that act as arrestants and phagostimulants to adult diabroticite beetles (Chambliss and Jones 1966a, Metcalf et al. 1980, Metcalf 1986, Metcalf and Metcalf 1992). Their extreme effectiveness and specific mode of action are important attributes that spurred a number of investigations over the last decade to evaluate them as key ingredients in baits laced with toxicants (Metcalf et al. 1987, Lance 1988, Weissling et al. 1989, Lance and Sutter 1990, 1991, 1992, Weissling and Meinke 1991, Brust and Foster 1995). These studies were the focus of an intensive research to find an alternative corn rootworm control agent to replace classical soil insecticide treatments that were generally ineffective and environmentally impacting. Optimized, target-specific baits, such as these, when applied in the field represent point sources of toxicant capable of killing 99% of corn rootworms that consume them (Tallamy and Halaweish 1993). Moreover, field cage and small-plot studies involving semiochemical-based baits with carbaryl or methomyl have demonstrated that the active ingredient can be reduced by more than 95% over recommended label rates for carbaryl for adult corn rootworm control (Metcalf et al. 1987, Lance and Sutter 1990).

Curcurbitacins represent a group of more than 20 class-related triterpenes that are biosynthesized in plants, principally Cucurbitaceae and certain suborders (Metcalf 1994), and they elicit powerful feeding stimulant and arrestant responses to a variety of corn rootworms (Metcalf and Metcalf 1992, Metcalf 1994). They are responsible for the bitter taste in most wild Cucurbitaceae (Rehm et al. 1957, Lavie and Glotter 1971, Guha and Sen 1975, Metcalf 1986). Moreover, Rehm et al. (1957) provided an excellent account of the distribution of the bitter principals in a number of Cucurbitaceae. Studies have been conducted to identify dependable, high yielding, cucurbitacins (Rhodes et al. 1980) and to demonstrate their utility in a ground-dry state for use in toxic baits (Metcalf et al. 1987).

As part of our research to develop a cucurbitacin-derived bait that would be compatible in formulations with water-soluble corn rootworm toxicants, we became interested in evaluating a bitter mutant of Hawkesbury watermelon, *Citrullus vulgaris* Schrad, for three reasons: it is known to contain cucurbitacin(s) (Chambliss and Jones 1966b, Rehm et al. 1957, Guha and Sen 1975); tissue extracts of it elicit strong visitation/feeding responses (Peterson and Schalk 1985); and its fruit can be readily cultivated to produce a high yield of aqueous extract (a potential formulation matrix) per kilogram of processed whole fruit (Schroder and DeMilo, unpubl. data).

Little has been published to date on the nature of the cucurbitacin in *C. vulgaris.* Rehm et al. (1957) and Guha and Sen (1975) report the presence of cucurbitacin-E glycoside in *C. vulgaris* and Peterson and Schalk (1985), more recently, characterized the cucurbitacin in *C. vulgaris* using thin layer chromatography (TLC) correlations of R_f values of their isolates to those reported in the literature of known cucurbitacins and standardized TLC solvent systems. By these methods, the glycoside of cucurbitacin E was reported as the major component in *C. vulgaris* along with its aglycone (cucurbitacin E) and a minor and extremely polar (R_f = 0 and 0.004 in two TLC solvent systems) component which they postulated as a conjugate of cucurbitacin-E glycoside.

We report here chromatographic and spectroscopic (GC-MS, NMR) methods used to isolate and identify the principal active component of *C. vulgaris* as well as bioassay data obtained for chromatographic fractions and isolates of *C. vulgaris* rind and

whole fruit extractives, in tests against adult diabroticite beetles, the southern corn rootworm and western corn rootworm. To our knowledge, this is the first report presenting GC-MS and NMR structural confirmation of the presence of cucurbitacin-E glycoside in *C. vulgaris.*

Materials and Methods

Insects. Mixed-sex teneral southern and western corn rootworm adults were purchased from French Agricultural Research, Inc. (Lamberton, MN) and maintained until needed in 8 × 8 × 8 in collapsible aluminum screen rearing cages (Bioquip Products, Inc., Gardina, CA). Western corn rootworms were also provided by the USDA's Northern Grain Insects Research Laboratory, Brookings, SD. They were fed a dry diet that consisted mainly of powdered-pollen substitute (Guss and Krysan 1973), and water was provided in 28-ml plastic cups to which was affixed a cotton wick. Cages were cleaned twice weekly and restocked with fresh food and water. The colonies were maintained at 24 to 27°C, 50 to 70% RH, and an hourly photoperiod of 16(L): 8(D).

Collection, identification and bioassay of *C. vulgaris* headspace volatiles (from rind). Cut pieces (100 g) of freshly thawed *C. vulgaris* rind were placed in a glass round-bottom flask equipped with gas inlet and outlet tubes. A stream of prepurified (gas passed through activated charcoal) nitrogen (300 ml/min) was passed over the rind for 20 h. The headspace volatiles entrained in the nitrogen stream were collected downstream in an activated-charcoal trap attached to the gas outlet tube. Details for preparing the trap are described by DeMilo et al. (1996). Following collection, volatiles adsorbed in the charcoal trap were removed by elution with 0.3 ml of methylene chloride. The volatiles were analyzed by GC-MS using a Hewlett-Packard Model 5980A GC-MS equipped with a Model 5971 MSD operating in the EI mode. GC-MS parameters: ionization voltage 70 eV, ion source temperature 180°C, GC column, HP5 (Hewlett-Packard, Avondale, PA) bonded fused-silica capilliary column (25 m × 0.2 mm i.d., 0.11 μm film thickness); structures were tentatively assigned based on spectral matches between unknowns in sample and known standards in the Wiley/NBS Spectral Database.

To assess feeding stimulation or attractiveness of volatiles, a 50 to 100 μl aliquot of the methylene chloride solution was applied to the center of a filter paper quadrant (solvent-only for control) and visitation/feeding responses were determined by the same methods used to assess activity in chromatographic fractions (see elsewhere in this section).

Small-scale extraction of *C. vulgaris* rind and liquid chromatography of extract. One gram of freeze-dried *C. vulgaris* rind was cut into small pieces (pea-size) and placed into an Erlenmeyer flask containing a solution of 40 ml of anhydrous diethyl ether and 10 ml of anhydrous methanol. The suspension was stirred magnetically at ambient temperature overnight (17 to 20 h) and then filtered to remove the rind. Evaporation of the filtrate by rotary evaporator (Buchi, Model RE 111) yielded 92 mg of a sticky semi-solid residue. The residue was redissolved in a small amount of solvent (ether: hexane: methanol 70: 30: 5) and subjected to flash chromatography on silica gel (230 to 400 mesh) using the same solvent mixture as the eluting solvent. Accordingly, five chromatography fractions (F1-5) were obtained with this solvent. Methanol was then used to elute the column, providing highly polar fraction F6. Removal of the elution solvent from each fraction by rotary evaporator provided a

residue that was bioassayed for feeding stimulation against corn rootworms. Amounts of recovered residue with corresponding R_f values: F1, 3.1 mg, 0.81; F2, 2.0 mg, 0.62; F3, 1.0 mg, 0.61; F4, 1.2 mg, 0.53; F5 1.3 mg, 0.24; F6, 77.8 mg, 0.00.

Preparative high performance liquid chomatography (HPLC) fraction of F6. Preparative HPLC of fraction F6 was conducted on a Waters Modular HPLC Unit consisting of two Model 6000A pumps, Model U6K injector, Model 441 UV detector, Model 680 Automated Gradient Controller, and a Shimadzu Model CR501 peak integrator. Chromatography was conducted on a 30 cm × 1 cm stainless-steel column packed with 5 μ C18-bonded silica gel (Spherisorb). HPLC parameters (isocratic): solvent, methanol:water (6:4), flow, 3 ml/min, monitoring wavelength, 254 nm. Fractions were collected manually with the start of each collection typically occurring at the beginning of a peak rise and ending on its downslope near the baseline. By these methods, four separate fractions were collected from a single chromatographic run each representing about 5 to 10 mg of residue from fraction F6. Pooled fractions were concentrated to dryness with a rotary evaporator and resulting residues bioassayed for feeding stimulation. Yields of residues recovered from 77 mg of F6 were: F6 · 1, 8.0 mg; F6 · 2, 23.8 mg; F6 · 3, 3.5 mg; F6 · 4, 4.8 mg.

Bioassay to determine activity of chromatographic fractions. Residues from chromatographic fractions F1-6 and subsequent HPLC fractions F6 · 1-4 were obtained by removing (rotary evaporator) the elution solvent from the fractions. Residues were dissolved in solvent (acetone or methanol) and aliquots containing the desired dose were applied to the center of a filter paper quadrant (¼ of a 4.25-cm diam Whatman No. 2 filter paper). A test involved placing one residue-treated and one untreated (solvent only) quadrant in a test arena (Petri dish, 100 mm × 15 mm) along with 1 (or 5) adult corn rootworms (16 to 20 days old). At pre-selected observation intervals (every 2 min for 10 min) visitation/feeding responses were recorded; (+) if insect was found feeding or resting on the filter paper or (–) if not. Positive responses were summed for all replicates of a given test and totals are presented in Tables 1 and 2. Tests were replicated 3, 6, or 9 times.

Large-scale extraction of *C. vulgaris* rind. The preparation of an extract for extended laboratory experiments was as follows: 1 kg of sliced (1-2 in squares) *C. vulgaris* rind (thawed from frozen stock) was charged into a 3-L capacity stainless-steel blender (Waring) along with 1 L methanol and some powdered dry ice. The rind was homogenized for 1 to 2 min and an additional 1 L methanol was added to help transfer the homogenate to a large Erylenmeyer flask. The flask and its contents were refrigerated (5 to 7°C) for 2 d. The homogenate was then filtered through a large medium-porosity sintered-glass funnel the bottom of which was lined to a 2 to 5 cm depth with glass wool to minimize plugging of the filter apparatus by finely suspended solids and tissue particles. The filtrate was stripped to dryness using a Buchi Model RE 111 rotary evaporator (waterbath temperature 45 ± 5°C) equipped with a dry-ice condenser. The residue (glass-like solid) on evaporation was further dried under high vacuum (0.1 Torr). Weight of residue was 26.1 g. This material was used for bioassay to determine its effectiveness as a feeding stimulant against corn rootworms.

Bioassay for dose-response determinations of crude extract derived from large-scale methanolic extraction of *C. vulgaris* rind. A 10% (w/v) aqueous solution of dry residue derived from the large-scale methanol extract of fresh *C. vulgaris* rind was prepared and a series of 10-fold dilutions were prepared from that solution.

Bioassay methods were as follows: six, 2.1-cm diam Whatman No. 1 filter papers,

equidistant and radially arranged, were glued, using white non-toxic paper glue, inside a 150 × 25 mm polystyrene Petri dish. With a Jencons Sealpette variable volume pipettor, 10 µl each of four dilutions (10%, 1.0%, 0.1%, and 0.01%), and a distilled water control were applied to the center of each circle. All dilutions were allowed to air dry. Ten, 4 to 6 days old southern or western corn rootworm adults (mixed sex), taken from a mixed population of 250 males and females, were then added to each dish. Each treatment was replicated five times.

Observations were made on the number of beetles in each circle, at 15-min intervals for 2 h; thereafter, observations were made every 30 min for 3 h. The beetles were allowed to remain in the dishes and feed for 24 h, at which time they were removed. A total of 14 observations was made during the test period. In addition to monitoring visitations, feeding activity at each circle was examined. Using a Biotron III Automatic Count/Area Totalizer, the particle (small spots of stain or regurgitate) count and surface area covered by brown feeding stain were determined at each concentration.

Mass spectrometric analysis. Mass spectra were obtained with a Finnigan model 4510 gas chromatograph-mass spectrometer (GC-MS) coupled with an INCOS data system. The samples were introduced via a direct exposure probe under the heating conditions of 0-1000 ma at a scan rate of 20 ma/sec. Chemical ionization spectra (CI-MS) were generated using ammonia (NH_3) and deutero-ammonia (ND_3) (both at a vacuum pressure of 0.6 Torr and a source temperature of 60°C) as the reagent gases of choice.

NMR spectroscopy. Proton NMR spectra were recorded on a Bruker QE 300 MHz spectrometer using Acetone-d_6 (Aldrich, Milwaukee, WI) solvent at 25°C. Presaturation of the HOD signal was required to fully observe proton frequencies close to the HOD frequency. All chemical shifts in the deuterated solvent are given in parts per million (ppm) relative to residual acetone-d_5 (at 2.05 ppm).

Results and Discussion

Cucurbitacins and their glycosides are highly oxygenated tetracyclic triterpenes that lack volatility by virtue of their high molecular weight and polarity. Despite this, we decided to investigate if the rind of *C. vulgaris* possessed any volatile components that might be attractive to corn rootworms. Thus, volatiles from the rind were collected by headspace techniques (DeMilo et al. 1996) and bioassayed against adult southern corn rootworms. No feeding stimulant or arrestant response was observed. From a GC-MS analysis, we were able to tentatively identify 17 volatile components, the six most abundant of which are (in increasing order of elution): hexanal, *E*-(or *Z*)-heptenal, (double bond assignment uncertain), *E*-2-octenal, nonanal, *E,Z*-2,6-nonadienal and 1,3-dimethoxybenzene. The high number of aldehydes in these volatiles is notable.

A freeze-dried sample of rind from *C. vulgaris* was used to isolate the feeding stimulant. An ether-methanol extraction of the rind followed by flash chromatography afforded 6 fractions (F1-6). Residues obtained from these fractions were bioassayed against the southern corn rootworm for feeding stimulation using two tests, varying only in the number of insects and replicates. Data show that fraction F6, the most polar fraction (R_f = 0.00) was the only active fraction in the series (Table 1). The feeding stimulant response for fraction F6 appeared fairly linear over the dose range,

Table 1. Visitation/feeding responses of adult southern corn rootworms to chromatographic fractions (F1-6) derived from small-scale methanolic extract of *C. vulgaris* Schrad

Fraction	Total number† visitation/feeding responses to 100 µg of fraction*	
	(3 replicates with 5 insects/rep.)	(6 replicates with 1 insect/rep.)
F1	3 (0)**	1 (0)**
F2	0 (1)	4 (0)
F3	4 (4)	3 (0)
F4	0 (6)	2 (0)
F5	2 (0)	2 (3)
F6 (100 µg)	58 (0)	16 (0)
F6 (10 µg)	19 (5)	
F6 (1 µg)	7 (0)	

* Tests were done on the residue obtained by removing elution from fraction solvent; each treatment involved dissolving 100 µg of test material in 100 µl of solvent. Resulting solution was applied to the filter paper.

** Numbers in parentheses are for solvent controls, 200 µl/disc (F1-5, acetone; F6, methanol).

† Totals were calculated by summing response observations (every 2 min for 10 min per replicate) for all replicates.

1 to 100 µg/disc. Feeding responses below 1 µg/disc were essentially nil with values paralleling those for controls.

Fraction F6 was subjected to further chromatographic separation using preparative-scale HPLC on C18-bonded silica gel column. Four fractions (F6 · 1-4) were collected from the column (Fig. 2) and residues obtained by solvent removal were bioassayed against adult southern corn rootworms for feeding stimulation. Data show that activity resided essentially in three fractions (F6 · 2-4) with the following order of relative activity: F6 · 4 > F6 · 3 > F6 · 2 (Table 2). Because only one insect responded to F6 · 1, this fraction was considered inactive. HPLC analysis of F6 · 4 on a C18-bonded silica gel analytical column showed it was composed of two components one major and one minor, the former being the center of focus for spectral characterization.

Data from chemical ionization mass spectrometry of (CI-MS) of the major component in F6 · 4 using ammonia (NH_3CI-MS) and deutero-ammonia gas (ND_3CI-MS) were consistent with the structure of cucurbitacin-E glycoside (Fig. 1). Ions generated by NH_3CI-MS at the lower mass range indicated the presence of a six-carbon sugar moiety. Multiple losses of water (M-18) indicated the presence of several hydroxy (OH) groups in the molecule. Combined data from NH_3CI-MS and ND_3CI-MS experiments confirmed the presence of six replaceable hydrogens in the molecule. The observed ions in NH_3CI-MS indicated an apparent molecular weight (MW) of m/z 718 (MW calculated for cucurbitacin-E glycoside = 718). Loss of the sugar moiety from the observed MW yielded a molecule with a molecular weight of m/z 556. Although in low amount, NH_3CI-MS yielded significant ammonia adduct ions at m/z 736 ($M + NH_4^+$),

Table 2. Visitation/feeding responses of adult southern corn rootworms to chromatographic fractions F6 · 1-4 resulting from HPLC fractionation of active fraction F6

Fraction	Total number* of visitation/feeding responses to 16 µg fraction** (9 replicates with 1 insect/rep.)
F6 · 1	1 (0)†
F6 · 2	4 (0)
F6 · 3	9 (0)
F6 · 4	18 (0)

* Totals were calculated by summing response observations (every 2 min for 10 min per replicate) for all replicates.

** Tests were done on the residue obtained by removing elution solvent from fraction; for each treatment 16 µg of test material was dissolved in 200 µl methanol and solution was applied to the filter paper.

† Numbers in parentheses are for solvent controls (methanol).

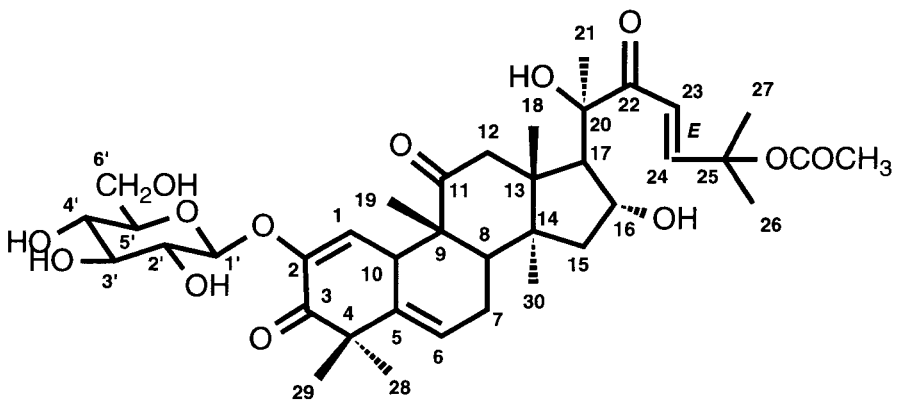

Fig. 1. Chemical structure and carbon numbering system for cucurbitacin-E glycoside.

718 (M + NH_4^+ – H_2O), 700 (M + NH_4^+ – $2H_2O$), 694 (M + NH_4^+ – C_2H_2O), 676 (M + NH_4^+ – HOAc), 658 (M + NH_4^+ – HOAc – H_2O), 574 (Cuc. E + NH_4^+) resulting from the loss of the sugar moiety, 514 (Cuc. E + NH_4^+ – HOAc), 496 (M + NH_4^+ – sugar – HOAc), 180 [(M – Cuc. E + NH_4^+) adduct ion of the hexose moiety], 197 (m/z 162 + [$(NH_3)_2$ + H]$^+$), 214 (m/z 162 + [$(NH_3)_3$ + H]$^+$). Interestingly, NH_3CI-MS and ND_3CI-MS for the minor component in F6 · 4 showed the presence of the same adduct ions as seen for the major component. Although weak at the higher mass end of the mass spectrum, the deutero-ammonia (ND_3) data of both the major and minor components, relative to the overall spectra of the ammonia (NH_3) data, indicate the presence of at least six exchangeable hydrogens (hydroxy groups) as well as multiple losses of D_2O.

Clearly, similar CI-MS fragmentation patterns and chromatographic properties

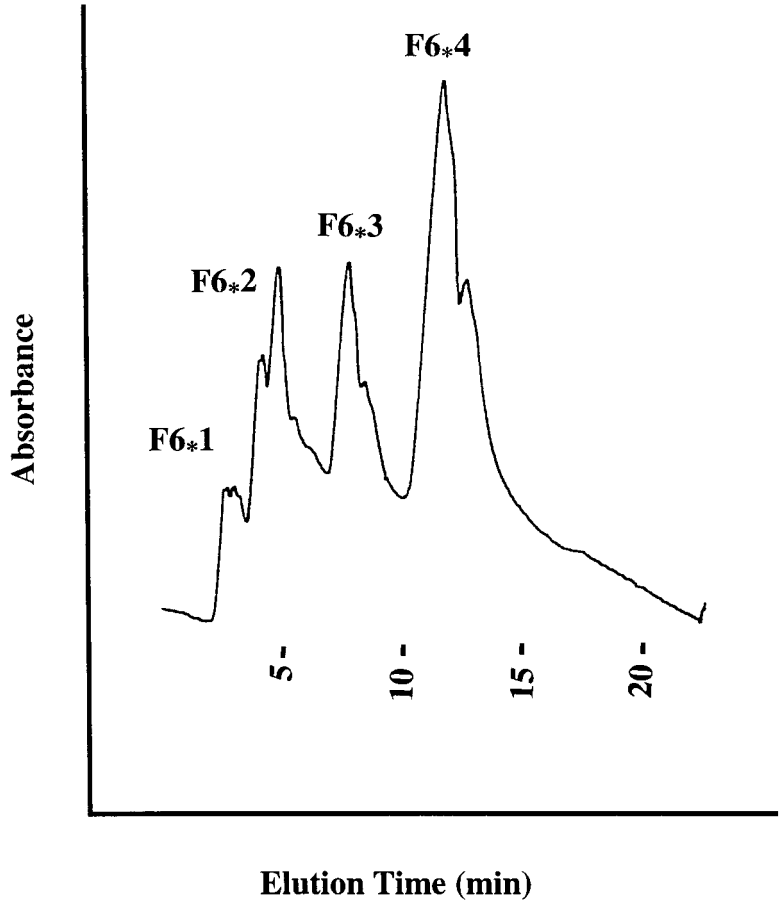

Fig. 2. Typical HPLC chromatogram of fraction F6 obtained with a preparative C18 bonded silica gel column under isocratic conditions: solvent, methanol:H_2O, 6:4; flow 3 ml/min, 254 nm.

(HPLC) suggest that the minor component in fraction F6 · 4 is also a cucurbitacin and one that is quite closely related to cucurbitacin-E glycoside (possibly a diastereomer or geometric isomer (i.e., shift of double bond or acetate moiety).

CI-MS analysis for fraction F6 · 3 also was conducted since F6 · 3 elicited about one-half of the feeding stimulancy observed for F6 · 4. Mass spectral data for F6 · 3 indicated an observed molecular weight of m/z 676. This would indicate a loss of an acetyl group from the major component believed to be cucurbitacin-E glycoside. Ammonia chemical ionization yielded significant ammonia adduct ions at m/z 694 (M + NH_4^+), 676 (M + NH_4^+ – H_2O), 658 (M + NH_4^+ – $2H_2O$), 514 [(M + NH_4^+ – sugar) readily loses the sugar moiety, then yields the adduct ions of m/z 532 & 549], 532 (m/z 514 + NH_4^+), 549 [(m/z 514 + $[(NH_3)_2 + H]^+$], 180 [(M – Cuc. I + NH_4^+) adduct ion of the hexose moiety], 197 (m/z 162 + $[(NH_3)_2 + H]^+$, 214 (m/z 162 + $[(NH_3)_3 + H]^+$. The

Table 3. Proton-NMR chemical shifts in parts per million for cucurbitacin-E glycoside*

CH_2 & CH	Assigned	Reported**	CH_3	Assigned	Reported**
H24	7.10 *d*	7.03 *d*	OAC	2.03 *s*	2.01
H23	6.75 *d*	6.54 *d*	$CH_3$26	1.64 *s*	1.55
H1	6.16 *m*	5.90 *d*	$CH_3$27	1.62 *s*	1.55
H6	5.80 *m*	5.70 *m*	$CH_3$28	1.47 *s*	1.38
H16	4.54 *m*	4.39 *t*	$CH_3$29	1.46 *s*	1.25
H12	3.44 *m*	3.20 *d*	$CH_3$19	1.38 *s*	1.42
H12	2.63 *m*	2.66 *d*	$CH_3$21	1.35 *s*	1.34
H7	2.34 *m*		$CH_3$30	1.04 *s*	1.02
H15	1.89 *m*		$CH_3$18	1.01 *s*	0.97
Sugar Protons	Assigned				
H1″	4.77 *d*				
H2′-6′	3.22–4.56				

* See Fig. 1 for structure and carbon numbering system. Peak pattern: *s* = singlet; *d* = doublet; *t* = triplet; *m* = multiplet.

** Values for the glycone of cucurbitacin E (Vande Velde and Lavie 1983); determined in $CDCl_3$ plus small quantity of CD_3OD to assist solubility.

deutero-ammonia (ND_3) data relative to the spectra of the ammonia (NH_3) data, indicate the presence of at least six (6Hx) exchangeable hydrogens (hydroxy). These data suggest that the component in F6 · 3 is cucurbitacin-I glycoside. However, further experiments will be required to further substantiate this compound and whether or not it co-exists with cucurbitacin-E glycoside in the tissue of *C. vulgaris* or was formed (i.e., deacetylated) during the chromatography.

Because the sample selected (F6 · 4 major) for NMR analysis was insoluble in deuterochloroform, the solvent of choice for many curcurbitacin aglycones (Vande Velde and Lavie 1983), we chose a more polar solvent such as acetone-d_6 (methanol-d_4 was also suitable for this purpose). Data from the NMR analysis of the major component in F6 · 4 are consistent with cucurbitacin-E glycoside (Fig. 1). For example, proton chemical shifts (Table 3) were consistent for cucurbitacin-E as the parent aglycone and compared favorably with values reported (Vande Velde and Lavie 1983) for cucurbitacin-E dissolved in deuterochloroform containing a small amount of methanol-d_4. Integration data (not published) were also consistent for cucurbitacin-E glycoside. The *J*-coupling pattern among adjacent protons is consistent with the assignments. The group of peaks observed at 3.22 to 4.56 ppm and 4.77 ppm along with respective integration data were consistent for glucose. The 0.3 ppm downfield chemical shift for H1 (6.18 ppm vs 5.90 ppm) is attributable to C2 as the site of glycosylic linkage.

NMR analysis of fraction F6 · 4 minor component provided a spectrum that included all proton signals associated with the major component, including an acetate

Table 4. Visitation/feeding responses of adult southern and western corn rootworms to aqueous solutions of *C. vulgaris* feeding stimulant from the large-scale methanolic extraction of rind of *C. vulgaris* Schrad*

% Concn. (w/v)	Total insects observed** on treated disc†	Avg. area (mm^2) covered by stain† on treated disc‡	Avg. particle count† on treated disc‡
		southern corn rootworm	
10.0	153	30.6 ± 10.2	107.4 ± 27.6
1.0	36	9.2 ± 4.1	42.2 ± 12.6
0.1	5	0.4 ± 0.5	5.0 ± 4.4
0.01	4	0	0.2 ± 0.4
control	8	0	0.8 ± 0.8
		western corn rootworm	
10.0	174	19.4 ± 9.5	85.2 ± 28.6
1.0	16	6.2 ± 5.3	29.2 ± 22.1
0.1	2	0	0.6 ± 0.9
control	4	0	0

* Ten beetles (mixed sex) were taken from a mixed population of 250 beetles and placed in test chamber with 6 treatments.

** Visitation/feeding responses at each disc made at 15 min intervals for 2 h and thereafter 30 min for 3 h (14 observations).

† Determined with a Biotron III Automatic Count/Area Totalyzer.

‡ 10 µl of test solution or water (control) applied per treatment.

methyl signal at 1.86 ppm. The only significant difference between spectra for the major and minor component was the relative size of the acetate methyl signal (it was much larger for the minor component). The observed chemical shift of 1.86 ppm rather than 2.03 ppm (Table 3) was due to spectra of F6 · 4 minor being recorded in a different solvent. Since mass spectral data showed that both components had identical molecular weights, then the difference may be rationalized by the minor component having an acetyl group at a different hydroxyl site.

Large-scale extraction of 1 kilogram of *C. vulgaris* rind with methanol afforded, after solvent evaporation, a 2.62% yield of tan-colored glass-like solid. The crude residue was dissolved in water and a series of 10-fold dilutions were prepared and bioassayed (Table 4) for visitation/feeding responses against the southern and western corn rootworm, using three different procedures (insect counts, stain area measurements, particle counts). All three methods proved useful in assessing the intensity of response. Data also indicated responses were directly proportional to concentration. While response of southern corn rootworm and western corn rootworm were roughly comparable at the 1% and 10% concentrations in two of the assays (insect counts, particle counts), response of western corn rootworm was approximately one-half of that observed using stain area for measurements. Regardless of method, the southern corn rootworm and western corn rootworm showed nil or minimal response to the stimulant at the 0.1% concentration.

In summary, GC-MS and NMR data confirm previous reports (Rehm et al. 1957, Guha and Sen 1975, Peterson and Schalk 1985) that cucurbitacin-E glycoside is the feeding stimulant in *C. vulgaris.* Chemical data in this study suggest that chromatographic fractions obtained during the isolation and purification of the principal feeding stimulant contain other cucurbitacins. Although Peterson and Schalk (1985) also reported the presence of cucurbitacin-E in *C. vulgaris*, we did not see any evidence of its presence in our studies. It is notable that Peterson and Schalk (1985) reported a highly polar ($R_f = 0$) unknown which they speculate was a conjugate of cucurbitacin-E glycoside. It is entirely possible that one of the partially characterized compounds in F6 · 3 or F6 · 4 may be related to their unknown.

Moreover, our laboratory feeding response data show that crude extracts of *C. vulgaris* and their isolates (i.e., chromotographic fractions) are highly effective as feeding stimulants/arrestants for two species of diabroticites (southern and western corn rootworm).

Because whole-melon extracts of *C. vulgaris* produce a high feeding stimulant response to the banded cucumber beetle (*D. balteata* LeConte) (Peterson and Schalk 1985), and because these melons are relatively easy to cultivate in temperate climates (Schroder, unpubl. data), we suggest that *C. vulgaris* may be a convenient, economical and high-yielding source of aqueous extracts that can be used to formulate toxicants into baits for management of corn rootworms.

Acknowledgments

We thank V. A. Levi and S. Dua, for helping with processing of *C. vulgaris* tissue extracts, and GC-MS and HPLC analyses. Thanks also go to M. Athanas and A. Sidor for conducting the bioassays and providing valuable field assistance to grow watermelons needed for this study. We also thank the USDA Vegetable Laboratory, Charleston, SC, for providing start-up seeds of *C. vulgaris* for cultivation of melons at Beltsville.

References Cited

Brust, G. E. and R. E. Foster. 1995. Semiochemical-based toxic baits for control of striped cucumber beetle (Coleoptera: Chrysomelidae) in canteloupe. J. Econ. Entomol. 88: 112-116.

Chambliss, O. L. and C. M. Jones. 1966a. Cucurbitacins: specific insect attractants in Cucurbitaceae. Science 153: 1392-1393.

1966b. Chemical and genetic basis for insect resistance in cucurbits. Am. Soc. Hort. Sci. 89: 394-405.

DeMilo, A. B., C. J. Lee, D. S. Moreno and A. J. Martinez. 1996. Identification of volatiles derived from *Citrobacter freundii* fermentation of a trypticase soy broth. J. Agric. Food Chem. 44: 607-612.

Guha, J. and S. P. Sen. 1975. The cucurbitacins–a review. The Plant Biochem. J. 2: 12-28.

Guss, P. L. and J. L. Krysan. 1973. Maintenance of the southern corn rootworm on a dry diet. J. Econ. Entomol. 66: 352-353.

Lance, D. R. 1988. Potential of 8-methyl-2-decyl propanoate and plant derived volatiles for attracting corn rootworm beetles (Coleoptera: Chrysomelidae) to toxic baits. J. Econ. Entomol. 81: 1359-1362.

Lance, D. R. and G. R. Sutter. 1990. Field cage and laboratory evaluations of a semiochemical-based bait for managing western corn rootworm beetles (Coleoptera: Chrysomelidae). J. Econ. Entomol. 83: 1085-1090.

1991. Semiochemical-based baits for *Diabrotica virgifera virgifera* (Coleoptera: Chrysomeli-

dae): effects of particle size, location, and attraction content. J. Econ. Entomol. 84: 1861-1868.

1992. Field tests of a semiochemical-based bait for suppression of corn rootworm beetles (Coleoptera: Chrysomelidae). J. Econ. Entomol. 85: 967-973.

Lavie, D. and E. Glotter. 1971. The cucurbitacins, a group of tetracyclic triterpenes. Forts. Chem. Organ. Naturestoffe. 29: 306-362.

Levine, E. and H. Oloumi-Sadeghi. 1991. Management of diabroticite rootworms in corn. Annu. Rev. Entomol. 36: 229-255.

Metcalf, R. L. 1986. Coevolutionary adaptations of rootworm beetles (Coleoptera: Chrysomelidae) to cucurbitacins. J. Chem. Ecol. 12: 1109-1124.

1994. Chemical ecology of diabroticites. Pp. 153-169. *In* P. H. Joliet, M. L. Cox and E. Petitpierre [eds.], Novel Aspects of the Biology of Chrysomelidae. Kluwer Academic Publishers, The Netherlands.

Metcalf, R. L., R. A. Metcalf and A. M. Rhodes. 1980. Cucurbitacins as Kairomones for diabroticite beetles. Proc. Natl. Acad. Sci. U. S. A. 77: 3679-3772.

Metcalf, R. L., J. E. Ferguson, R. L. Lampman and J. F. Andersen. 1987. Dry cucurbitacin-containing baits for controlling diabroticite beetles (Coleoptera: Chrysomelidae). J. Econ. Entomol. 80: 870-875.

Metcalf, R. L. and E. R. Metcalf. 1992. Diabroticite rootworm beetles, Pp. 64-108. *In* R. L. Metcalf and E. R. Metcalf [eds.], Plant Kairomones in Insect Ecology and Control. Chapman and Hall, NY.

Peterson, J. K. and J. M. Schalk. 1985. Semiquantitative bioassay for levels of cucurbitacins using the banded cucumber beetle (Coleoptera: Chrysomelidae). J. Econ. Entomol. 78: 738-741.

Rehm, S., P. R. Enslin, A. D. J. Meluse and J. H. Wessels. 1957. Bitter principals of the cucurbitaceae VII. The distribution of bitter principals in this plant family. J. Sci. Food Agric. 8: 679-686.

Rhodes, A. M., R. L. Metcalf and E. R. Metcalf. 1980. Diabroticite beetle responses to cucurbitacin kairomones in *Cucurbita* hybrids. J. Am. Hortic. Soc. 105: 838-842.

Sutter, G. R. and D. R. Lance. 1991. New strategies for reducing insecticide use in the corn belt, Pp. 231-249. *In* B. J. Rice [ed.], Sustainable Agriculture Research and Education in the Field. National Acad. Press, Washington, DC.

Tallamy, D. W. and F. T. Halaweish. 1993. Sensitivity to cucurbitacins in southern corn rootworm (Coleoptera: Chrysomelidae). Environ. Entomol. 22: 925-932.

Vande Velde, V. and D. Lavie. 1983. ^{13}C NMR spectroscopy of cucurbitacins. Tetrahedron 39: 317-321.

Weissling, T. J., L. J. Meinke, D. Trimnel and K. L. Golden. 1989. Behavioral responses of *Diabrotica* adults to plant-derived semiochemicals encapsulated in a starch borate matrix. Entomol. Exp. Appl. 53: 219-228.

Weissling, T. J. and L. J. Meinke. 1991. Semiochemical-insecticidal bait placement and vertical distribution of corn rootworm (Coleoptera: Chrysomelidae) adults: implications for management. Environ. Entomol. 20: 945-952.