Rapid High-Performance Liquid Chromatography Method To Quantitate Elaterinide in Juice and Reconstituted Residues from a Bitter Mutant of Hawkesbury Watermelon

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A C_{18} reversed-phase HPLC method has been developed to analyze the concentration of 2-*O*-*β*-D-glycopyranosyl-cucurbitacin E (synonym, elaterinide) in juice or reconstituted residues of juice derived from a bitter mutant of Hawkesbury watermelon, *Citrullus lanatus* (Thunb.) Matsum. & Nakai. The method requires only two steps (homogenation and filtration) for sample preparation prior to column injection and, thus, is amenable to processing large numbers of test samples. Percent elaterinide in juice samples from whole fruit, processed in various ways, ranged from 0.0230 to 0.0808%. Error differences in percent elaterinide for repetitive analyses within the same lot of juice ranged from 1.2 to 5.6%.

Keywords: *HPLC analysis; corn rootworm; elaterinide; cucurbitacin E glycoside; Hawkesbury watermelon; Citrullus vulgaris Schrad.; Citrullus lanatus (Thunb.) Matsum. & Nakai.*

INTRODUCTION

The most destructive pests of corn in North America are the members of the corn rootworm complex: the northern corn rootworm, *Diabrotica barberi* Smith and Lawrence; the western corn rootworm *D. virgifera virgifera* Le Conte; the southern corn rootworm *D. undecimpunctata howardi* Barber; and the Mexican corn rootworm *D. virgifera zea* Krysan and Smith. Semiochemicals offer considerable promise for the management of corn rootworms (Levine and Oloumi-Sadeghi, 1991; Sutter and Lance, 1991; Metcalf and Metcalf, 1992). Cucurbitacins (oxygenated tetracyclic terpenes) represent a discreet family of semiochemicals that have been investigated as arrestants and phagostimulants for adult diabroticite beetles (Metcalf et al., 1980; Metcalf, 1986; Metcalf and Metcalf, 1992). In the past decade cucurbitacin-derived semiochemicals have been evaluated as a key component for toxicant-laced baits that would specifically target diabroticites (Metcalf et al., 1987; Lance, 1988; Weissling et al., 1989; Lance and Sutter, 1990, 1992; Weissling and Meinke, 1991; Brust and Foster, 1995).

Recent studies (Schroder et al., 1997, 1998) have pointed to the utility of a bitter mutant of Hawkesbury watermelon, *Citrullus lanatus* (Thunb.) Matsum. & Nakai. (syn. *Citrullus vulgaris* Schrad.) as a feeding stimulant for incorporation into a water-soluble toxicant bait for controlling adult diabroticite beetles. The principal component in *C. lanatus* responsible for the feeding stimulant/arrestant response reportedly is cucurbitacin E glycoside, specifically 2-*O*-*β*-D-glucopyranosyl-cucurbitacin E or elaterinide (Rehm et al., 1957; Guha and Sen, 1975; Peterson and Schalk, 1985; DeMilo et al., 1998).

The emerging importance of *C. lanatus* as a cheap and readily harvestable source for a feeding stimulant to potentially manage populations of diabroticite beetles (Schroder et al., 1997, 1998) has stimulated a need for an analytical method to precisely determine concentrations of elaterinide in *C. lanatus* juice (processed or unprocessed) or in residues obtained by processing whole melons or their integral parts. It is anticipated that such a method will help provide quantitative chemical support to several research areas involving *C. lanatus*-derived baits, such as the development of efficacious formulations, quality control assessment in production and processing of watermelon, and matters relating to environmental safety.

High-performance liquid chromatography (HPLC) offers considerable promise as an analytical method for elaterinide due to the extremely polar nature of the highly functionalized molecule. Indeed, Halaweish and Tallamy (1993) have reported a C_{18} reversed-phase HPLC method for glucosides of cucurbitacin D and I from *Cucurbita texana*. Bauer and Wagner (1983) have also reported C_{18}-based HPLC methods to analyze for cucurbitacin glucoside B, E, I, and L in extracts from medicinal plants including an extract from fruit of the watermelon, *Citrullus colocynthis* (L.) Schrad. Spurred by the promise of these reports, we investigated reversed-phase HPLC as a method to determine elaterinide titers in juice from whole ripe fruit of *C. lanatus* as well as in solid residues obtained by processing whole or cut-up fruits.

MATERIALS AND METHODS

Plant Source. Bitter Hawkesbury watermelon seeds were germinated in flats in the greenhouse. Transplants were planted through black plastic mulch at the Beltsville Agricul-

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Table 1. HPLC Determination of Elaterinide in Processed Whole Fruit (*C. lanatus* Thunb.) Juice or Reconstituted Solid Residues

sample	source/description	% elaterinide
A	juice[a] (processed whole fruit)	0.0404
B	juice[a] (processed whole fruit)[b]	0.0342
C	juice[a] (processed whole fruit)[b]	0.0624
D	juice[c] (processed whole fruit, 7.5 kg single melon)	0.0808
E	juice[c] (processed whole fruit, 3.7 kg single melon)	0.0815
F	juice[c] (processed whole fruit, 2.8 kg single melon)	0.0676
G	reconstituted residue[d] (from drying of sample C)	0.0590
H	reconstituted residue[e] (from MeOH extract of rind)	0.0705
I	reconstituted residue[e] (from MeOH extract of pulp)	0.0340

[a] Obtained by homogenizing whole fruit with a meat grinder and then filtering the homogenate through cheesecloth. [b] Processed same as A but stored as a separate lot. [c] Obtained by homogenizing whole fruit with a Waring blender and then filtering through a steel-mesh screen. [d] Residue was redissolved in the same volume of water lost during drying. [e] Dissolved in a 1:1 (v/v) mixture of MeOH/H_2O.

tural Research Center during the summer of 1997. Spacing was 8 ft between rows with 3−4 ft between plants in the row. The plants were drip irrigated and fertilized to supply 25 lb of N, P_2O_5, and K_2O per acre. The crop was fertilized 2 weeks after planting and at fruit set. Watermelons were grown to full ripeness and harvested.

Solvents and Reagents. Solvents used for HPLC were of HPLC grade (EM Science, Gibbstown, NJ), and those for open column chromatography (EtOAc, 1-BuOH) were of reagent grade (EM Science). Acetophenone was purchased (J. T. Baker Chemical Co., Phillipsburg, NJ) and was >99% pure. The elaterinide standard used to identify peaks corresponding to elaterinide in test samples was isolated from *C. lanatus* and purified as follows: Freshly cut pieces of rind (5.6 kg total) were homogenized batchwise with MeOH (1 L/kg), and the homogenate was filtered through filter paper (Whatman No. 2) and the filtrate concentrated by rotary evaporator to dryness to yield 157 g of brown resin. The resin was redissolved in water (500 mL) and the resulting solution washed with hexane. The aqueous layer was extracted (3 × 100 mL) with a 1-BuOH/EtOAc mixture (1:2 v/v), and organic layers were combined. Evaporation of the solvent afforded 3.00 g of semisolid residue. The residue was dissolved in 100 mL of the 1-BuOH/EtOAc mixture and washed three times with 25 mL of H_2O. This gave 2.26 g of yellow powder after solvents were removed by rotary evaporation. Further purification by successive column chromatography treatments (first, elution from Florisil with EtOAc/MeOH solvent, and, second, elution from silica gel with EtOAc solvent) yielded 302 mg of elaterinide (pale yellow solid): mp 145−148 °C (micro hot stage); $[\alpha]_D$ −60.0° (EtOH, *c* 0.80); IR (KBr), λ (cm^{-1}) 3450, 1725, 1685, 1635, 1255. Ripperger and Seifert (1975) report mp 148−150 °C and $[\alpha]_D$ −63.5°. Chemical ionization mass spectrometry provided a molecular ion of 718; molecular weight for elaterinide ($C_{38}H_{54}O_{13}$) = 718.

Sample Preparation. *Juice Samples.* Test juice samples A−C (Table 1) and J−N (Table 2) were prepared from selected portions (lots) of a large quantity of juice that was stored in a frozen state for planned field studies. The processing method for juice involved grinding 230 whole watermelons with a commercial meat grinder (Falk Motoreducer, model 6E72-06A5, Milwaukee, WI) and then filtering the homogenate through cheesecloth filters with a commercial wine/cider press (Jaffrey model 8400, Jaffrey Manufacturing. Co., Shawnee Mission, KS) to get a clarified filtrate. Test juice samples D−F (Table 1) were prepared by homogenizing single whole fruit (harvested at different stages of maturity, i.e., 2.8−7.5 kg) with a 4 L stainless steel Waring blender (3 × 1 s pulses at 15500 rpm). The homogenate was filtered through a steel-mesh screen and analyzed without freezing. Pre-HPLC juice sample preparation briefly was follows: 1 mL of test juice from *C. lanatus* was mixed with 1 mL of 0.01% acetophenone in MeOH and then filtered through a 2.5 cm diameter (0.2 μm) syringe filter (Whatman Inc., Clifton, NJ). A 10 μL volume of final test solution was injected onto the HPLC column.

Table 2. Analysis Precision: HPLC Determination of Elaterinide in Selected Lots of Juice from *C. lanatus* Thunb.

sample[a]	n[b]	mean[c] % elaterinide in juice	std error (× 10^{-3})	% error
J	2	0.0230a	±1.3	5.6
K	4	0.0355b	±0.9	2.5
L	5	0.0605c	±0.8	1.2
M	3	0.0659c	±1.1	1.7
N	3	0.0781d	±1.1	1.4

[a] Obtained by processing 230 whole ripe *C. lanatus*. Frozen samples were thawed for analyses. Samples represent different storage lot numbers corresponding to the same process batch. [b] Number of HPLC determinations. [c] Means followed by the same letter are not statistically different at the 0.05% level using Bonnforoni mean comparison. Analysis was performed using the mixed procedure (SAS Institute, Inc., 1997) and the Bonnforoni option for multiple comparisons.

Reconstituted Residue Test Samples. The residue for reconstituted test sample G (Table 1) was obtained by evaporating 1 g of *C. lanatus* juice to dryness with a rotary evaporator (water bath temperature = 75 °C). The brown residue (58.5 mg) was reconstituted in 1 mL of water and then analyzed for percent elaterinide according to methods identical to those described for juice test samples A−F (see elsewhere in this section). Residues for test samples H and I (Table 1) were obtained as follows: 1 kg of freshly cut slices of rind or pulp was homogenized in a 3 L stainless steel Waring blender with 1 L of MeOH. The homogenate was filtered through a Celite 521 filterbed and the filtrate taken to dryness by rotary evaporator; yields (wt %) of residues were 3.69 and 4.05, respectively. Reconstitution of residue for analyses involved dissolving the calculated amount of residue to 1.0 mL of water and then adding 1.0 mL of 0.01% acetophenone standard solution. Determination of percent elaterinide in sample, from this point on, was exactly as described for juice in test samples A−C.

Reversed-Phase HPLC Analysis. The HPLC system consisted of two Waters (Waters Corp., Milford, MA) model 6000A pumps, a Waters model U6K injector, a Waters model 680 automated gradient controller, a Waters model 996 photodiode array detector, and a computerized data station equipped with Waters Millennium software to control the pumps, gradient controller, and detector and to process and print detector output. The HPLC column consisted of a 25 cm Supelcosil LC-18 (Supelco Inc., Bellefonte, PA) reversed-phase column packed with 5 μm spheres of ODS-bonded silica gel (100 Å pore size). The column was equipped with a 2 cm Pelliguard LC-18 guard column (Supelco).

HPLC parameters were as follows: pump mode, isocratic; solvent system, 2:1 (v/v) methanol in water; solvent flow rate, 1.5 mL/min; detection wavelength, 237 nm; sample injection volume, 10 μL; run time, 10.0 min.

Quantitation of Elaterinide. The following equations were used to determine the percent elaterinide in a test sample.

Equation 1, adapted from Beer's law relationship for an analyte and an internal standard, determines the weight of elaterinide (W_{ela}) in a 10 μL HPLC injection volume:

$$W_{ela} = W_{i.s.}(E_{i.s.}/E_{ela})(M_{ela}/M_{i.s.})(A_{ela}/A_{i.s.}) \quad (1)$$

In eq 1, ela is elaterinide, i.s. is internal standard (acetophenone), W is weight (in μg) per 10 μL injection volume, E is molar absorptivity (at λ_{max}), and A is peak area from the chromatogram [E for elaterinide = 17400 at 237 nm (Ripperger, 1976); E for acetophenone = 13000 at 240 nm (Silverstein et al., 1991)].

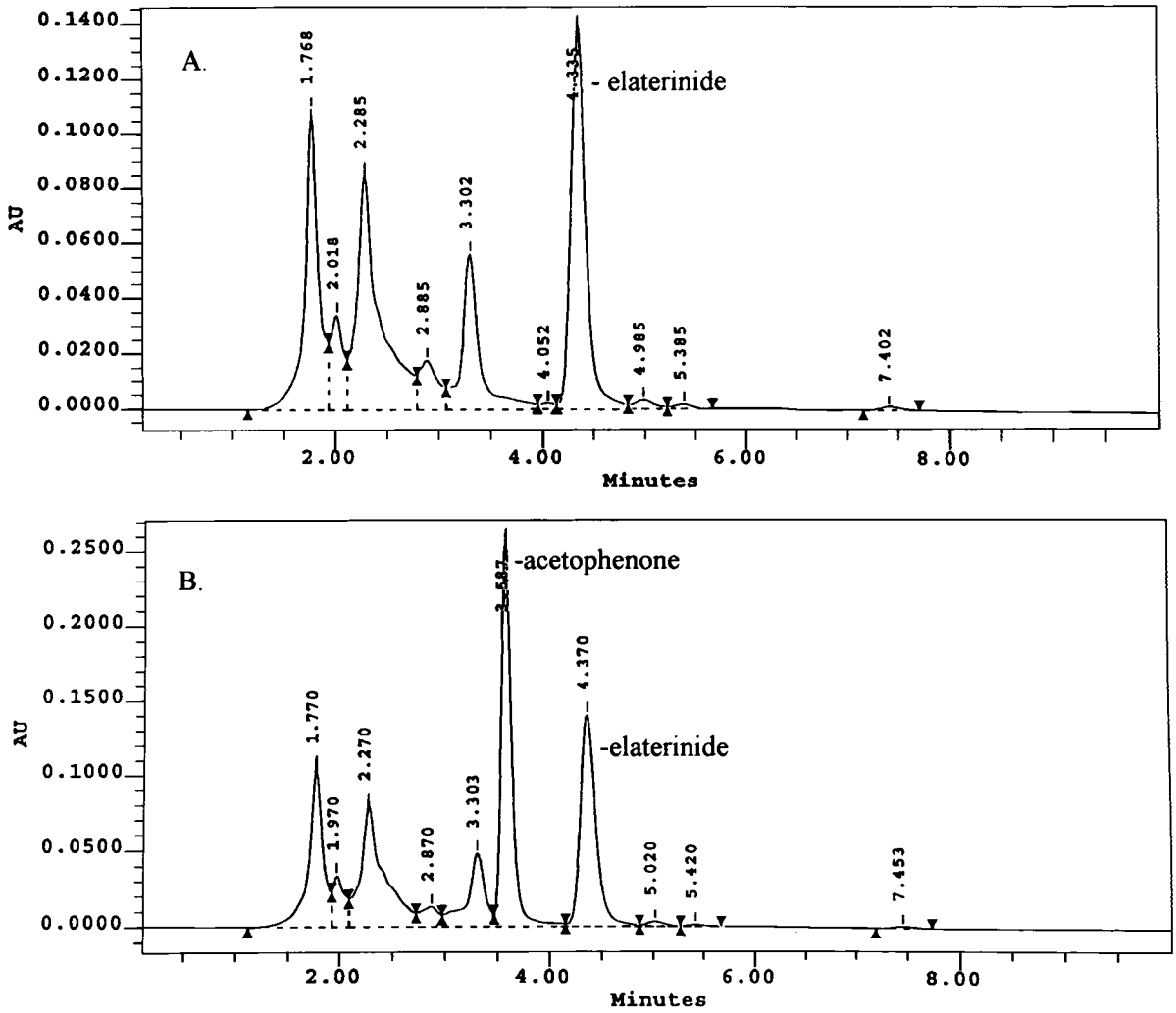

Figure 1. Typical HPLC chromatogram of fresh or freeze−thawed homogenates of whole fruit or reconstituted juice residues derived from bitter Hawkesbury watermelon, *C. lanatus* (Thunb.) Matsum. & Nakai.: (A) without acetophenone standard; (B) with acetophenone standard.

Because 10 μL of injection volume contains 5×10^{-7} g of i.s., eq 1 then can be simplified to eq 2, where the ratio $A_{ela}/A_{i.s.}$ is calculated from observed peak areas in the chromatogram.

$$W_{ela} = (2.235 \times 10^{-6}\ \text{g})(A_{ela}/A_{i.s.}) \quad (2)$$

Equation 3 is derived from eq 2 by applying appropriate volume correction factors. It allows for the determination of percent elaterinide in the juice or reconstituted residue test samples.

$$\%\ \text{elaterinide} = (2 \times 10^{4}\ \text{g}^{-1})W_{ela} \quad (3)$$

RESULTS AND DISCUSSION

Although HPLC methods to analyze elaterinide are reported (Bauer and Wagner, 1983; Halaweish and Tallamy, 1993), their complexity, especially in terms of the number of steps required for sample preparation, discouraged us from adopting those procedures to quantitate elaterinide in *C. lanatus*. Also influencing this decision was the fact that we realized at the outset of this project that we needed a method that could expedite fairly large numbers of test samples of *C. lanatus* juice or juice-derived residues that we knew we would be confronted with in ongoing research with toxicant baits. Thus, the method had to be simple with a minimum effort required for sample preparation.

As a result of some preliminary tests, we found that analysis of elaterinide based on C_{18} reversed-phase HPLC technology was also amenable for quantitation of elaterinide in whole-fruit juice homogenates or reconstituted juice residues derived from *C. lanatus*. However, unlike methods described by Bauer and Wagner (1983) and Halaweish and Tallamy (1993), precolumn sample preparation was greatly reduced. Specifically, the method required just two steps (homogenation followed by filtration) and, importantly, did not need an organic solvent to extract the host matrix, a step that was paramount in the earlier reported methods. Other advantages realized by our methods were utilization of isocratic elution of the analytes from the column and adoption of the inexpensive cosolvent methanol for column elution (earlier methods used acetonitrile).

Figure 1A illustrates a typical HPLC chromatogram of fresh or thawed homogenates of whole fruit or reconstituted solid residues derived from *C. lanatus*. The retention time observed for elaterinide was 4.34 min. Nine other peaks were observed in the chromatogram but remain unidentified. We speculate that they may

be sugars or other cucurbitacins (Ripperger and Seifert, 1975; Peterson and Schalk, 1985; DeMilo et al., 1998).

Figure 1B shows a typical HPLC chromatogram of a *C. lanatus* juice sample or reconstituted solid residue, but one containing the internal standard, acetophenone. The retention time for acetophenone was 3.59 min. Acetophenone was chosen for the internal standard because it eluted in an area of the chromatogram (Figure 1B, ~3.5−4 min) where there were no interfering peaks.

Table 1 shows the percent elaterinide found in three different sample types as determined by C_{18} reversed-phase HPLC. The elaterinide concentration in juice samples A−C, processed with production-scale equipment, ranged from 0.034 to 0.062% (av = 0.046%). The nearly 2-fold range in values was unexpected because all samples were processed from the same batch of watermelons (230 melons) and at the same time. Differences in elaterinide concentration can be rationalized by the possible presence of inadvertently introduced contaminants that could reduce the amount of elaterinide in certain lots by decomposition during storage.

Elaterinide concentration was also determined in juice samples derived from single whole melons; large (7.5 kg), medium (3.7 kg), and small (2.8 kg). Unlike samples A−C, for which juice was obtained by production-scale equipment and then frozen and subsequently thawed prior to analysis, test samples D−F represent juice obtained by laboratory-scale equipment (Waring blender) and that had been kept fresh prior to analysis. Accordingly, elaterinide concentrations determined for the large, medium, and small melons were 0.0808, 0.0815, and 0.0676%, respectively. Although the elaterinide concentration for the small melon was less than those for the medium and large melons, the difference may not be significant. Interestingly, the average concentration for test samples D−F (i.e., 0.0766%) was nearly 70% greater than the average calculated for samples A−C (i.e., 0.0457%). The strikingly lower concentration of elaterinide in samples A−C compared to that in samples D−F may be due to biological, chemical, agronomic, or meterological factors acting alone or in concert to reduce the concentration of elaterinide in samples A−C. Additional experiments would be needed to validate these results. For comparison purposes, concentrations reported for elaterinide or "cucurbitacin" in *Citrullus* spp. follow: Rehm et al. (1957) estimated a ≥0.1% concentration of elaterinide in *C. lanatus* fruit; Chambliss and Jones (1989) reported a 0.13−0.21% concentration of "cucurbitacin" in *C. lanatus* pulp (value varied from pink to red pulp) and a 0.25% concentration in the rind; Metcalf (1994) claimed that "cucurbitacin" concentration in *Citrullus* could reach 0.1%.

Elaterinide concentrations were also determined for reconstituted residue samples G−I (Table 1). The elaterinide concentration in sample G was 0.0590%, roughly 5% lower than the expected value of 0.0624% for juice sample C, from which G was derived. However, this small difference may fall within the limits of experimental error.

Elaterinide concentrations were also determined for reconstituted residue samples derived from methanolic extracts of the rind (sample H) and entire pulp (sample I). Of note, the rind contained exactly twice the amount of elaterinide found in the pulp (0.0705 vs 0.0340%). Similarly, Chambliss and Jones (1989) reported higher concentrations of elaterinide in the rind.

To assess the precision of the HPLC method to analyze elaterinide concentration in juice, repetitive analyses were conducted on five selected lots of juice (samples J−N) obtained by processing the melons with production-scale equipment. Data in Table 2 present mean values of percent elaterinide calculated for the repetitive runs along with the standard error and percent error. The means varied over a 3-fold range (i.e., 0.0230−0.0781%), and standard errors ranged from 1.2 to 5.6%. The highest variation was observed for sample J, which was analyzed only twice.

In summary, we have developed an HPLC method that is capable of rapid determination of elaterinide concentration for large numbers of samples of processed juice or reconstituted residues from *C. lanatus*. The method is based on C_{18} reversed-phase technology and requires minimal sample preparation. The precision determined for the method ranged from 1.2 to 5.6%. Percent elaterinide determined in whole fruit juice samples ranged from 0.0230 to 0.0808% and is generally slightly lower than earlier reported values. This difference may be rationalized by the fact that a small amount of elaterinide could have escaped detection because it was bound to tissues that were filtered away from the sample prior to analysis. Elaterinide concentration in fresh juice samples appeared to be higher than that in samples that were stored in the freezer and then thawed prior to analysis. The simplicity and speed with which this method analyzes elaterinide in test samples has greatly quickened the pace of ongoing research to develop toxic baits that focus on *C. lanatus* as the source of feeding stimulant for corn rootworms.

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