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Source: Journal of Insect Science, 2(19) : 1-6

Published By: Entomological Society of America

URL: https://doi.org/10.1673/031.002.1901

Martin A.W.P., Blackburn M., Schroder F.W.R., Matsuo K., and Li W.B. 2002. Stabilization of cucurbitacin E-glycoside, a feeding stimulant for diabroticite beetles, extracted from bitter Hawkesbury watermelon. 6pp. *Journal of Insect Science*, 2:19. Available online: insectscience.org/2.19

Journal of Insect Science
insectscience.org

Stabilization of cucurbitacin E-glycoside, a feeding stimulant for diabroticite beetles, extracted from bitter Hawkesbury watermelon

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Received 6 June 2002, Accepted 2 September 2002, Published 9 September 2002

Abstract

Cucurbitacins are feeding stimulants for diabroticite beetles, including corn rootworms and cucumber beetles, which can be added to a bait containing an insecticide thereby reducing the levels of other insecticide treatments needed to control these pests. One of them, cucurbitacin E- glycoside, is water soluble and easily processed from mutant bitter Hawkesbury watermelons (BHW) that express elevated levels of cucurbitacin. Storage of BHW extract at room temperature resulted in a 92% reduction of cucurbitacin E-glycoside over two months, while refrigeration or freezing resulted in a 60% loss of the active ingredient during this time. The loss of the active ingredient was correlated with an increase in BHW extract pH from 5 to greater than 9. The increase in pH of the BHW extracts at room temperature appeared to be due to the growth of certain bacteria, especially *Bacillus spp*. In refrigerated extracts, the pH remained relatively constant, and bacterial growth was dominated by bacteria such as *Lactobacilli*. An alternative to refrigeration is concentration of BHW extract. One means of concentration is spray drying, but the high sugar content of the BHW extract (20mg/ml glucose, 40mg/ml fructose) makes this technique impractical. Fermentation of the BHW extract by the yeast, *Saccharomyces boulardii*, eliminated the sugars and did not raise the pH nor alter the cucurbitacin E-glycoside content of the extract. Elimination of the sugars by fermentation produced an extract that could be successfully spray dried. BHW extract fermented by *S. boulardii* produced a higher level of feeding stimulation for spotted cucumber beetles in laboratory choice tests. When applied to cucumbers, there was no difference in control of spotted and striped cucumber beetles between baits of fresh or fermented juices combined with the same insecticide.

Keywords: *Diabrotica undecimpunctata howardi*, *Acalymma vittatum*, spotted cucumber beetle, striped cucumber beetle, feeding stimulant, fermentation

Abbreviation:

BHW	bitter Hawkesbury watermelon
cfu	colony forming unit
PDA	potato dextrose agar
RM	recovery medium

Introduction

Diabroticite beetles (Coleoptera: Chrysomelidae) are important pests of corn including, for example, corn rootworms. Certain species of these beetles also feed on soybeans, melons, cucumbers, and peanuts. Besides damaging the crop, the adult beetles transmit bacterial wilt and viral diseases (York, 1992). Much of the insecticide used to control the larvae of these insects is applied to soil prophylactically. Alternatives to soil control are needed to reduce pesticide usage.

Cucurbitacins, common secondary plant compounds of the Cucubitacea, are feeding stimulants for several species of beetles in the corn rootworm complex (Chamblis and Jones, 1966; Metcalf, 1994). These compounds can be added to a bait containing an insecticide thereby reducing the levels of other insecticide treatments needed to control these pests. But many of the curcubitacins occur as insoluble forms making concentration and formulation difficult.

Peterson and Schalk (1985) found that a water extract of a bitter mutant of Hawkesbury watermelon [*Citrullus lanatus* (Thumb.) Matsum. & Nakai (syn. *Citrullus vulgaris* Schad)] elicited a positive feeding response from banded cucumber beetles (*Diabrotica balteata* LeConte). This water-soluble cucurbitacin was

identified as cucurbitacin E-glycoside (DeMilo *et al.*, 1998). Extracts of homogenized whole bitter Hawkesbury watermelons (BHW) were used as a component of a bait for adult beetles (Schroder *et al.*, 1998). Cucurbitacin E-glycoside (0.05%) bait in combination with the insecticide phloxine B, successfully controlled spotted cucumber beetles on cucumbers (Schroder *et al.*, 2001).

When stored at room temperature, cucurbitacin E-glycoside is rapidly lost from crude watermelon extract that makes cold storage necessary. One source of degradation of cucurbitacin E-glycoside has been shown to be microbial (Maatooq *et al.*, 1995). Bitter Hawkesbury watermelon extract contains 0.03% to 0.1% cucurbitacin E-glycoside by fresh weight (Matsuo *et al*., 1999) and is composed of more than 90% water. The high water content of this watermelon extract in a bait would make shipping and storage costly.

Spray drying is one method to stabilize and concentrate the cucurbitacin content of the BHW extract, however, the high sugar content of the crude BHW extract complicates this process due to the decomposition of sugars during the heat step. In the present study, we examine the factors responsible for the degradation, develop methods to inhibit degradation, and describe a fermentation process that eliminates sugars from the extract that allows subsequent spray drying of the product.

Materials and Methods

Preparation of extracts

Bitter Hawkesbury watermelons were grown as recommended by Maryland Cooperative Extension Service, (1999), harvested when ripe, and stored at room temperature for less than a week before processing. Watermelons were washed in hot water (45 °C) to remove soil and loosely associated microbes. Individual watermelons were homogenized and the liquid collected by straining through cheese cloth (BHW extract).

For initial stability studies, the BHW extracts were divided into 10 ml aliquots, placed in sterile 20 ml scintillation vials, and incubated at 25 °C, 4 °C, or -20 °C to simulate storage at room, refrigerator and freezer temperatures. Four samples per storage condition were removed initially and at 4, 8, 13, and 17 weeks. These samples were analyzed for cucurbitacin E-glycoside concentration, numbers of microbes, and pH.

Cucurbitacin E-glycoside concentration

Cucurbitacin E-glycoside concentration was determined by HPLC (Matsuo *et al.*, 1999). Equal volumes of BHW extract and methanol, with acetophenone (0.01%) as an internal standard, were mixed, filtered, and applied to a C-18 HPLC column (Supelcosil LC 18 column; 250 mm x 4.6 mm; 5 micron packing; Supelco, www.sigmaaldrich.com/Brands/Supelco_Home.html). The column was run at room temperature, and eluted with a mobile phase of 66% methanol and 34% water on a HP1100 equipped with a diode array detector. Cucurbitacin E-glycoside was detected at 237 nm and quantified by comparison to the acetophenone standard.

Microbial analysis

Samples of BHW extract were serially diluted, spread in duplicate on half-strength L-agar (Miller 1972), or RM (5 grams tryptone, 2.5 grams yeast extract, 2.5 grams NaCl and 10 grams Oxoid agar/liter), and incubated aerobically at 30 °C for 48 hours. Recovery experiments for yeast were carried out on potato dextrose agar (PDA, Difco, www.voigtglobal.com/DIFCO.htm) under the same conditions. Microbial recovery is expressed as colony forming units (cfu) per ml.

Aerobic microbes isolated from extracts of BHW were identified using the Biolog system (version 3.5, Hayward, CA, USA) of substrate utilization for bacteria, which generates a similarity index relative to other strains in the database.

Effect of pH on cucurbitacin E-glycoside concentration

BHW extract was filter-sterilized (0.45 micron nylon filters) and the filtrate divided into five samples. One sample was used as control (pH 5.0), and the other samples were adjusted to pH 6.0, 7.0, 8.0 and 9.0 with 0.1 N KOH. Three 10 ml aliquots of each pH level of the BHW extract were placed in sterile scintillation vials at room temperature. Cucurbitacin E-glycoside concentrations were determined at 0, 1, 3 and 6 days, and corrected for the volume of KOH added. Additional extract was filter-sterilized and heated at 80 °C for 20 minutes to inactivate enzymes. These samples were adjusted to a pH of 5.13 or 9.03 with 0.1 N KOH (in triplicate). Cucurbitacin E-glycoside concentrations and pH were determined at 0, 1, 7, and 14 days.

Concentration and fermentation

Initial attempts to concentrate the BHW extract included spray drying 19 liters of the BHW extract in a spray dryer (1.2 m x 3 m) at the Dairy Laboratory at the University of Maryland. To 19 liters of crude BHW extract, 90 grams of Celite 521 filter aid was added and the mixture was filtered through a No. 2 Whatman filter paper under vacuum. Inlet temperature was set at 177 °C and the outlet temperature was 93.3 °C. Residence time was about 9 seconds. A successful attempt to spray dry BHW extract used 9.5 liters of BHW extract, fermented by the yeast *Saccharomyces boulardii* (Universal Food Corp., Milwaukee, WI, USA), and 900 grams of maltodextrin (STAR-DRI 1015, A.E. Staley Mfg.Co., www.aestaley.com/products/cereal/) as a carrier (162.8 °C inlet, 100 °C outlet).

BHW extract was fermented on a small scale in 20 ml vials. To begin the fermentation, *S. boulardii* was inoculated at 10^5 cfu/ml (grown on PDA) into 10 ml aliquots of BHW extract. Vials were incubated in triplicate at room temperature (ca. 25 °C) without shaking. Samples were taken at 1, 2 and 4 days for cell counts, sugar and cucurbitacin E-glycoside concentration.

For larger scale fermentation, *S. boulardii* was inoculated at the same concentration into a five gallon carboy containing 15 liters of BHW extract that had been heated at 80 °C for 30 minutes to kill indigenous microbes. Samples were taken initially, at 5, and at 7 days after inoculation when CO_2 production had ceased. Carbon dioxide production was monitored by bubble formation. Samples were analyzed for concentration of cucurbitacin E-glycoside and sugar.

Sugar concentration

The concentration of sugars in BHW extracts was determined by gas chromatography in triplicate (Li, 1996). Aliquots

(0.05 ml) of stored BHW extract were concentrated to dryness in a Speed Vac Concentrator (Savant Instruments, Inc. www.savec.com/apr01/savant/sav-home.htm). Pyridine reagent (0.5 ml) containing hydroxylamine hydrochloride and an internal standard, phenyl D glucopyranoside, was added to the dry sugar mixture, mixed, heated at 75 °C for 30 minutes, then cooled to room temperature. Hexamethyldisilazane (0.5 ml) and 4 drops of trifluoroacetic acid were added, samples mixed vigorously, and then centrifuged to obtain a clear supernatant containing the sugar derivatives and the excess hydroxylamine hydrochloride as a pellet. Samples were analyzed by gas chromatography on a cross-linked methyl silicone capillary column (Li, 1996).

Insect tests

We tested the properties of fresh versus fermented BHW extract in the laboratory using a preference test because fermentation altered the odor of the BHW extract. The feeding stimulant activity of cucurbitacin E-glycoside was tested by placing 100 microliters of fermented BHW extract, fresh BHW extract, or a water control on each of three 2 cm Whatman No. 1 filter paper circles in a 150 mm petri dish. Both fresh and fermented BHW extract contained 0.05 milligrams cucurbitacin E-glycoside. Fifteen spotted cucumber beetles, *Diabrotica undecimpunctata howardi* Barber, were placed into each of five dishes Counts of beetles on each paper circle were made every 15 minutes for 2 hours.

The efficacy of the fermented extract under field conditions was tested on cucumbers (*Cucumis sativus* (L.)) at Maryland University WYE Research and Education Center (Graysonville, MD, USA). A 4 x 4 Latin square design was used. Each treatment replicate consisted of three, 21 meter rows. Treatment rows were separated by 3 meter alleys and blocks were separated by 2.4 meter alleys.

Treatments consisted of a single application of: (1) bait prepared with fresh BHW extract (0.052%) and 213 grams/hectare phloxine B as toxicant (D&C Red No. 28,Warner Jenkins, St. Louis, MO, USA), (2) bait prepared with fermented BHW extract (0.053%) and 213 grams/hectare phloxine B, and (3) a water treated control. The microbes in the fermented bait were removed before application by centrifugation. Treatments were applied with a single TeeJet® Flood Jet wide angle, flat spray nozzle (EK4S5), to deliver bait in 100 micrometer droplets, delivering 85 liters/hectare. Boom height was 0.7 meter and coverage encompassed a 4 meter wide band. Cucumber plants had overlapping canopies and 20 cm long fruits. Adult spotted and striped cucumber beetles [*Acalymma vittatum* (Fabricus)] averaged 1/ 0.8 meter row prior to spray application.

In each treatment replicate, two randomly selected 3 meter sections of plants were inspected for beetles. Dead spotted and striped cucumber beetles were counted 1, 2 and 6 days after application. The number of dead beetles was recorded, the same sections were not sampled on subsequent days.

Statistical analysis

Mean comparisons of cucurbitacin E-glycoside concentrations over time and insect laboratory choice tests over time were performed using the MIXED procedure in SAS with repeated measures and Kenward-Rogers adjustment for degrees of freedom (SAS Institute, 1999). Microbial mean comparisons and mean comparisons for field experiments used the MIXED procedure in SAS with variance grouping, because of unequal variances, and paired t tests. Unless otherwise indicated, comparisons are made on a per volume basis. Means are expressed along with standard error of the mean.

Results

Decline of cucurbitacin E-glycoside concentration during storage

The initial concentration of cucurbitacin E-glycoside in BHW extract was 0.95 ± 0.11 mg/ml. When aliquots of BHW extract were stored at room temperature the cucurbitacin E-glycoside content declined to 8.4% of the initial concentration in 8 weeks and to 4.2% in 17 weeks (Figure 1). Cucurbitacin E-glycoside concentration in both refrigerated or frozen BHW extract declined to 79 % of the original concentration over an 8 week period. At 17 weeks, when the experiment was terminated, the concentration of cucurbitacin E-glycoside remaining in frozen BHW extract was 57.9% and in the refrigerated BHW extract was 47.4% of the original concentration. In single samples kept frozen or refrigerated for 1 year the remaining cucurbitacin E-glycoside was 34.8% and 14.5% of the original concentration respectively.

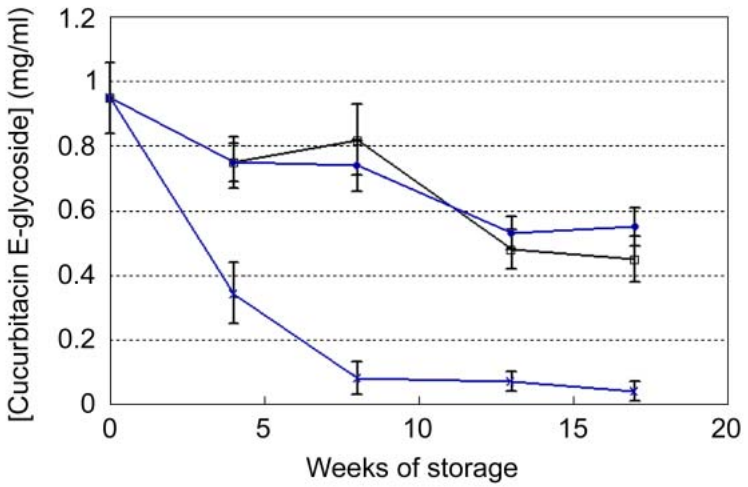

Figure 1. Concentration of cucurbitacin E-glycoside in BHW extract stored at various temperatures. (closed circles) –20 °C, (open squares) 4 °C, (crosses) 25 °C. Error bars represent standard error of the mean. N = 4.

Microbes in BHW extract

The initial microbial load in the BHW extract averaged 8.3 ± 1.4 x 10^4 cfu/ml. Microbial counts from the extract of individual watermelons ranged from 1.3 x 10^3 to 1.8 x 10^5 cfu/ml. In the samples stored at room temperature the number of microbes recovered increased to 9.86 ± 0.97 x 10^7 cfu/ml in four weeks and remained at this level throughout the experiment. During this time the average pH in these samples also rose from 5.98 to 8.24 (Figure 2). In 20 samples stored at room temperature in which the level of cucurbitacin was below detection limits, the pH averaged 9.22 ± 0.47and the microbial counts averaged 4.7 x 10^7 ± 0.72 cfu/ml. Conversely, in 6 samples where the cucurbitacin content was high (0.63 ± 0.34 mg/ml) the pH averaged 4.44 ± 1.3 and the microbial counts were lower at 1.7 ± 0.31 x 10^6 cfu/ml.

In the refrigerated samples, the microbial numbers increased to 1.48 ± 0.47 x 10^8 cfu/ml in 4 weeks. The average pH of these

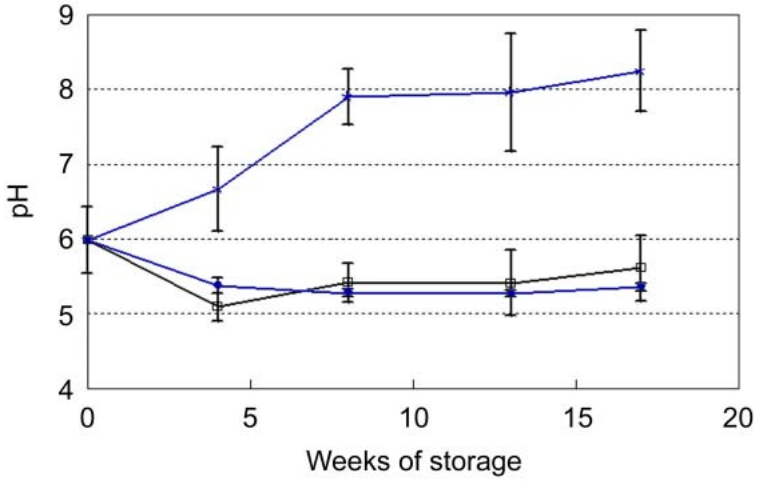

Figure 2. pH of BHW extract stored at various temperatures.(closed circles) –20 °C, (open squares) 4 °C, (crosses) 25 °C. Error bars represent standard error of the mean. N = 4

samples dropped to a low of 5 and then increased to 5.5 with only 6 samples above pH 7. In the frozen samples, the microbial numbers declined to 3.89 ± 2.23 x 10^3 cfu/ml. The pH remained relatively constant at 5.4 (Figure 2) with no samples above pH 6.

The types of microbes recovered from the 4 °C and 25 °C storage temperatures were different. The microbes recovered from 4 °C storage had only one or two colony types, which appeared to be *Lactobacilli* both by colony morphology, size, and distinctive odor. The pH of most of these samples was under 4, which indicated lactic acid formation. Microbes recovered from room temperature samples were more varied and included *Bacillus spp*. (Table 1).

Effect of pH on cucurbitacin E-glycoside concentration

Since very low or undetectable concentrations of cucurbitacin E-glycoside was observed in samples with high pH values, the effect of elevated pH on cucurbitacin E-glycoside concentration was tested in filter-sterilized samples. In initial experiments, cucurbitacin E-glycoside concentration in filter-sterilized extracts at the various pH levels from 5.0 to 9.0 declined at similar rates.

However, when extracts were also heated for 20 minutes at 80 °C (to deactivate enzymes that might be present in the extract) marked differences were observed in cucurbitacin E-glycoside degradation. In the pH 5.13 samples, the concentration of cucurbitacin E-glycoside remained constant (final concentration 0.541 ± 0.015 mg/ml). In the pH 9.03 samples, the cucurbitacin E-glycoside concentration declined from 0.561 ± 0.020 mg/ml to 0.307 ± 0.009 mg/ml in 14 days. Although the pH of the BHW sample without the addition of KOH remained constant over the 14 days of the experiment (pH 5.13 ± 0.017), the pH 9.03 test sample declined to pH 7.87.

Table 1. Correlation of pH and cucurbitacin content by dominant microbe from bitter Hawkesbury watermelon extracts stored at room temperature.

Microbe present	pH	[Cururbitacin E-glycoside] mg/ml	similarity index
Rhodotorula mucilaginosa	3.8	1.01	0.336
No id Gram -rod	6.9	0.5	
Bacillus sp.	7.9	0.013	
Bacillus sphaericus	9.8	0.03	0.662
Enterobacter cloacae A	9.7	0.018	0.586
Bacillus brevis	9.6	0	0.662
Stenotrophomonas maltophilia	9.8	0	0.801
No id Gram- rod	9.8	0	

Concentration of BHW extract

Initial attempts at spray drying led to a product with a taffy-like consistency and a burnt sugar odor. Much of the product stuck to the inner wall of the spray dryer. The addition of maltodextrin as a carrier did not eliminate stickiness and burnt sugar odor of product. The problem was suspected to be caused by the presence of free sugars in the BHW extract, which are hygroscopic and decompose at high temperatures.

Glucose and fructose were found in high concentrations in the stored BHW extract. The glucose content ranged from a high of 20.7 mg/ml in the juice of a ripe BHW to a low of 8.6 mg/ml in the juice in a small unripe melon. The fructose content likewise varied from 42.6 mg/ml in a ripe melon to 24.2 mg/ml in a small unripe melon. Stored ripe melons contained on average 20 mg/ml glucose and 40 mg/ml fructose.

In small scale replicated fermentations with *S. boulardii*, the cucurbitacin E-glycoside concentration remained constant or increased slightly, while the sugar concentration decreased to undetectable limits within two days (Table 2). In five gallon carboys, it took 5 days for the glucose and fructose to reach undetectable levels (less than 0.5 micrograms/ml) using *S. boulardii*. The level of cucurbitacin E-glycoside remained unchanged (0.52 mg/ml) in both small-scale fermentation and in the carboys.

Spray drying of 9.5 liters of the *S. boulardii* fermented BHW extract yielded 326 grams of a beige powder (36.2% recovery). This powder was intensely bitter and contained 1.95 grams cucurbitacin E-glycoside (as determined by HPLC) of the 5.2 grams cucurbitacin E-glycoside in the original BHW extract (37% recovery).

Effects on insects

Preference tests in which spotted cucumber beetles were

Table 2. The effect of fermentation of bitter Hawkesbury watermelon juice by *S. boulardii* on cucurbitacin E-glycoside, fructose and glucose concentration.

Time (days)	titer x 10^6*	[Curcurbitacin E-glycoside] (mg/ml)	Fructose (mg/ml)	Glucose (mg/ml)
0	0.2	0.594	46.9	24.7
0.5	1.5	0.824	34.6	19.4
1	13	0.546	29.2	9.7
2	210	0.570	0	0
3	300	0.488	0	0

* colony forming units of *S. boulardii* on PDA

exposed to paper disks treated with fresh extract, fermented extract, or water are shown in Figure 3. During each interval during the first 1.75 h observation period, the spotted cucumber beetles spent significantly more time on the disks treated with fermented BHW extract than either disks treated with fresh BHW extract or water. However by 2 h there were no differences in the number of visits of the beetles to the fresh or fermented extract. Spotted cucumber beetle visitations to the spray-dried product were greater than visitations to water control.

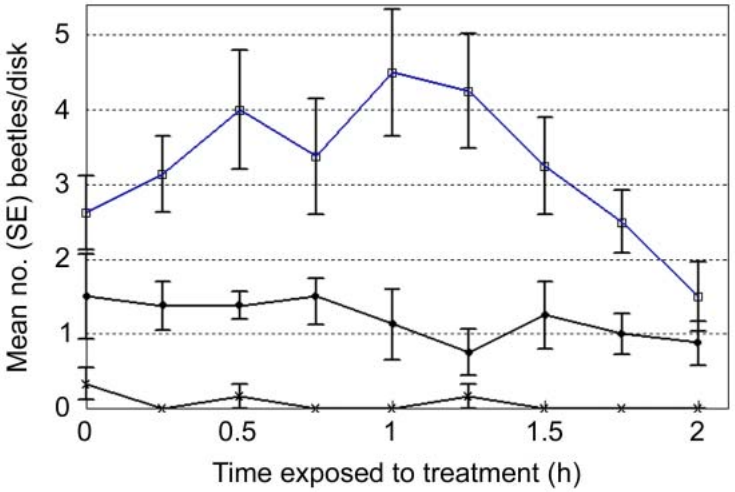

Figure 3. Response of spotted cucumber beetles adults to fresh and fermented BHW extract compared to controls. (crosses) control , (closed circles) fresh BHW extract, (open squares) fermented BHW extract. Error bars represent standard error of the mean. N=3.

In the field test there were no differences in numbers of dead spotted and striped cucumber beetles recovered from the blocks that were treated with the fresh or the fermented BHW extract + phloxine B over the course of the experiment. (Figure 4, $t = 0.37$, df = 11.9 $P = 0.7182$). Significantly more dead beetles were collected in the blocks treated with fresh BHW extract + phloxine B ($t = 5.69$, df = 13.1, $P < 0.0001$) and fermented BHW extract + phloxine B ($t = 3.31$, df = 6.73, $P = 0.0138$) than were collected from untreated control blocks. The dead beetles in the rows treated with BHW extract and phloxine B usually had red abdomens.

Discussion

Cucurbitacin E-glycoside is a highly effective feeding stimulant for diabroticite beetles within the corn rootworm complex, that can serve as the basis for insecticidal bait formulations (Schroder *et al.*, 2001). The bitter Hawksbury watermelon serves as a rich natural source of cucurbitacin E-glycoside, however, steps must be taken to stabilize the active ingredient during processing and storage of BHW extracts.

Refrigeration or freezing (4 °C and -20 °C, respectively) slowed the rate of cucurbitacin degradation in crude BHW extracts compared with extracts stored at 25?C, but did not eliminate degradation. Whether frozen or refrigerated, after 17 weeks of storage only ca. 50 % of the cucurbitacin E-glycoside remained. At 25 °C, less than 10% cucurbitacin E- glycoside remained after 8 weeks.

Microbial growth in extracts held at 25 °C had a major, but apparently indirect, impact on cucurbitacin E-glycoside degradation. The dominant microbes that grew in BHW extracts at 25 °C showed a strong tendency to raise the pH of the extracts to 9 or higher. It is known that aqueous alkali breaks down cucurbitacins by disrupting the ring structure (Lavie and Glotter, 1971), which would account for the lack of degradation products observed by HPLC.

In experiments designed to isolate the effect of pH on cucurbitacin E-glycoside stability, the pH of filter-sterilized BHW extract was raised by the addition of KOH. In these experiments cucurbitacin E-glycoside in sterile extracts declined at similar rates regardless of whether the pH was 5.5 (the initial pH) or 9.0. However, when similar experiments were conducted with heat treated (80 °C for 30 minutes) extracts, the cucurbitacin E-glycoside decreased by ca. 45% over a 2 week period at pH 9.0, while heat-treated extract left at the original pH of 5.13 retained over 96% of the original cucurbitacin E-glycoside content. A likely explanation for preservation of cucurbitacin E-glycoside after heating is that the heat treatment inactivates one or more enzymes present in extracts that can degrade cucurbitacin E-glycoside.

Enzymatic inactivation of cucurbitacin may explain the slow decline in concentration of cucurbitacin in BHW extracts stored at 4 °C and -20 °C, where the pH of the extracts remained low over time.Although the number of microbes recovered from extracts stored at 4 °C was similar to microbes recovered from extracts stored at 25 °C, but the types of microbes were different. Freezing the extracts at -20 °C did not protect cucurbitacin E-glycoside any better than refrigeration at 4 °C, suggesting an additional mechanism for degradation, perhaps enzymatic. Because BHW extracts were not filter-sterilized before storage, it is not clear whether the enzyme(s) responsible are of plant or microbial origin (Maatooq *et al.*, 1995).

While elevated pH due to microbial growth could be controlled by the addition of antimicrobial compounds (Deak and Beuchat, 1996), this would not slow the apparent enzymatic degradation of cucurbitacin E-glycoside. Heat treatment of BHW extract at 80 °C for 30 minutes effectively controled both degradation processes.

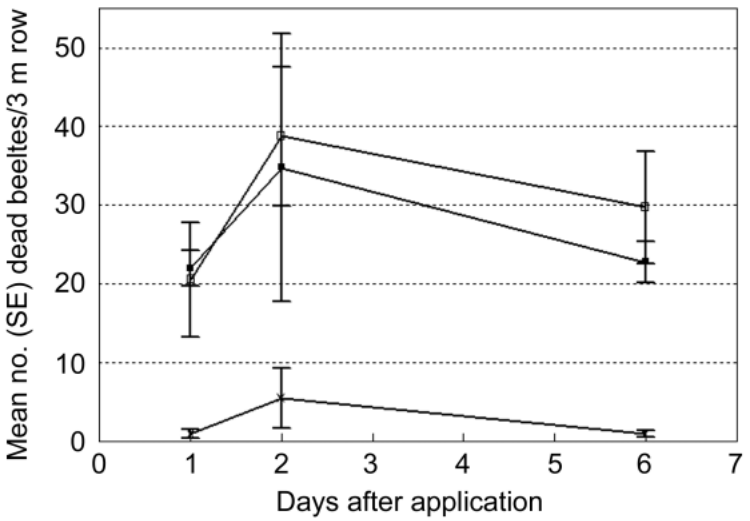

Figure 4. Number of dead diabroticite beetles collected from 3 meter rows on cucumbers sprayed with a combination of fresh (open squares) or fermented (closed circles) BHW extract with phloxine B compared to controls (crosses). N=4.

Another means of stabilizing the cucurbitacin E-glycoside content of BHW extracts is to dry the extract. Initial attempts to dry BHW extracts failed due to high levels of sugars in BHW extract. Spray drying resulted in caramelization of the product and fouling of the spray dryer. Fermentation of the BHW extract by *S. boulardii* successfully removed virtually all free sugars from the extract, while leaving the cucurbitacin E-glycoside content unchanged. Laboratory choice tests and field tests conducted with fermented BHW extracts indicated that the fermentation process did not adversely affect behavioral responses of spotted cucumber beetle adults to the extract. Spray drying of *S. boulardii* fermented extract resulted in a white powdery product. Although our overall yield of product was low (ca. 37%), this was due to the relatively small volume of the batch which was processed (the minimum volume that could be processed by the spray dryer). Larger volume batches would result in greater total recoveries.

The initial preference in the laboratory of fermented over fresh juice could be due to the presence of microbes that make metabolic by-products that have been identified as attractants for corn rootworm such as indole (Levine and Oloumi-Sadeghi, 1991). Microbes that were present in the laboratory assays were removed before the fermented bait was applied to cucumbers in the field. Thus the differences between the laboratory assays and field data may be due to slight differences in concentration of the cucurbitacin E-glycoside in the different preparations, absence of microbes in the field experiments, or some yet to be discovered factor or factors. The HPLC profiles do not suggest that other cucurbitacins were present that could also account for these preference differences.

In summary, cucurbitacin E-glycoside may be stabilized in BHW extracts by pasteurization at 80 ^{o}C for 30 min to kill the microbial flora associated with the melons and inactivate enzymes, fermentation by *S. boulardii* to remove sugars from the extract, and spray drying of the pasteurized, fermented extract. Our results indicate that extracts should be processed rapidly, as even freezing does not eliminate the degradation of cucurbitacin E-glycoside. In any processing scheme developed, high pH should be avoided. Spray drying results in a stable preparation, and since the weight of the extract is reduced by ca. 90%, transportation and storage expenses are greatly reduced.

Acknowledgments

Thanks to the team who made this research on a bait for corn rootworm possible: A. Shropshire and S. Woolard for microbiology, M. Athanas, and A. Sidor for the field and insect research, E. Howard, N. Tompkins and A. Manohar for summer help, S. Rankin for letting us use the spray drying facilities at U. Maryland, and the farm workers at the U. Maryland Vegetable Farm for hand harvesting an acre of melons. Mention of a commercial product does not imply endorsement of the U.S. Department of Agriculture.

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