

Toxicity of Fractionated and Degraded Mexican Marigold Floral Extract to Adult *Sitophilus zeamais* (Coleoptera: Curculionidae)

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ABSTRACT Floral extract of Mexican marigold, *Tagetes minuta* (L.), was toxic to adult male and female maize weevils, *Sitophilus zeamais* Motschulsky, under illuminated and dark conditions. The LD₅₀ values ranged from 35 to 47 µg per weevil across sex and photoperiod, whereas LD₉₉ values ranged from 319 to 816 µg per weevil. Fractionation tended to decrease toxicity. Generally, both sexes were similarly susceptible to a given treatment. Although the original extract and certain fractions probably contained photoactive thiophenes, which are toxic to certain larval Lepidoptera, there was no obvious effect of illumination on toxicity for this beetle species. Air exposure for 24 h, in the presence or absence of light, greatly reduced the toxicity of the unfractionated extract. Chromatographic profiles indicated that fractionation procedures had a significant effect on sample composition and on the amount of nonvolatile material. Air exposure for 24 h had a much more dramatic effect, with concomitant 365-nm UVA or incandescent light causing even greater degradation. Degradation under illumination is the end result of the process that causes photoactivation in susceptible species. Apparently, *S. zeamais* was not adversely affected by the amount of photoactivation that occurred as the extracts or fractions were degrading under our test conditions. These results are discussed in terms of maximizing the control potential of labile material in subsistence agriculture, particularly when purification is required to remove undesired compounds.

KEY WORDS *Sitophilus zeamais*, *Tagetes minuta*, floral extract, degradation, natural product

MARIGOLDS, *TAGETES* spp., are a source of insecticidal compounds (Maradufu et al. 1978; Arnason et al. 1981, 1986; Morallo-Rejesus and Decena 1982). Crude extracts from floral, foliar, and root tissues of Mexican marigolds, *Tagetes minuta* (L.), are insecticidal (Weaver et al. 1994). Photoactive thiophenes, present primarily in roots and flowers of several species of *Tagetes*, are biologically active against mosquito larvae (Arnason et al. 1981, Morallo-Rejesus and Decena 1982, Wells et al. 1993). Additionally, a study of the insecticidal activity of extracts of volatiles from *T. minuta* against the bruchid pest *Zabrotes subfasciatus* (Boheman) indicated that floral and foliar extracts, composed

predominantly of terpenoids, also are toxic (Weaver et al. 1994). In the context of subsistence agriculture, toxicity of newly collected, unfractionated plant extracts is of great interest because successful utilization of an insecticidal crop may depend on maximizing the amount of potential control available from all plant material grown rather than maximizing toxicity from purified material previously determined to be most efficacious. In addition, the practicality of using photoactive toxins as protectant insecticides in bulk stored products is limited because the storage environment does not allow penetration of light beneath the surface (Weaver et al. 1994).

These concerns suggest that simple chemical fractionation procedures may be useful in increasing the efficacy of particular preparations for control of stored-product pests. Purification and isolation of active compounds are beneficial because they could reduce the application rate and may be necessary to remove unwanted compounds that might alter the flavor and odor of the foodstuff. However, in terms of potential control by the total material available, the fractionation process results in the loss of at least some of the active material. In addition, marigold extracts contain components that are volatile and labile upon exposure to air and light. For thio-

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phenes, the photactivation that occurs is ultimately a degradative process. Here we evaluate these concerns by using a floral extract from *T. minuta* that had been fractionated or had been exposed to air or to air and light for 24 h. Topical toxicity under illuminated or dark conditions also was evaluated. These experiments were conducted with adult males and females of the maize weevil, *Sitophilus zeamais* Motschulsky, an important cosmopolitan pest of stored grain.

Materials and Methods

Fractionation and Degradation Procedures. The floral extract of Mexican marigold 'Muster John Henry' was prepared as described in Weaver et al. (1994) by using a simultaneous steam distillation and extraction method to obtain the essential oil (Wells 1992). Two 300-mg samples of the oil were placed into separate borosilicate vials and exposed to radiation in the UV or in the visible range that might exist in a storage facility where original extract was applied for insect control or in a crude pilot-scale facility where fractionation was conducted. The UV radiation was provided by a 4-W dual wavelength UV lamp (Fisher, Pittsburgh, PA) operated at a nominal wavelength of 365 nm (UVA). A 60-W incandescent bulb produced the visible radiation. The radiation sources were placed 10 cm above the open vials, and our test aliquot was collected after 24 h of exposure. One 300-mg sample was held in an open vial under darkness for 24 h. A 10-ml sample of the oil was placed into a 25-ml round bottom flask fitted with Liebig condensers and brought to reflux. Our test aliquot was collected after 3 h. A 7-g sample of the oil was placed into a 25-ml receiving flask and fractionated by vacuum distillation in a short-path unit (Kontes Glass, Vineland, NJ). Three fractions of lightly colored oil, weighing ≈ 1 g each, were collected in series, followed by a final 3-g fraction of dark brown, highly viscous material. All fractions and exposure aliquots were stored under a nitrogen headspace as described in Weaver et al. (1994).

Gas Chromatography. *T. minuta* extracts were analyzed on a Hewlett-Packard HP 5890 Series II gas chromatograph (GC) (Hewlett-Packard, Wilmington, DE) equipped with an autosampler, split/splitless injection port, and a flame ionization (FID) detector. An HP-5 (5:95%, diphenyl:dimethyl-polysiloxane) fused silica capillary column (25 m by 0.2 mm i.d. by 0.33 μ m film thickness) was used. Nitrogen carrier gas flow rate was 0.5 ml/min. Injector and detector temperatures were 250 and 300°C, respectively. The oven temperature was programmed from 50 to 300°C at 5°C/min, with a 10-min hold at 300°C. Data were recorded and plotted using HP 3365 ChemStation software. Default values were used for peak integration, except for the initial area reject parameter, which was set at 25,000 counts.

Extracts prepared for the bioassays (30 mg/150 μ L acetone) were diluted 20-fold in acetone and 2

μ L was injected into the GC. Split ratio was set at 50:1.

Insect Culture. *S. zeamais* were collected weekly from farm-stored corn in Bamberg and Barnwell counties, South Carolina, during winter and spring, 1993. Field-collected adults were reared on dried 'Pioneer 3320' corn from the 1992 crop at $25 \pm 1^\circ\text{C}$, $65 \pm 5\%$ RH, and a photoperiod of 12:12 (L:D) h. Cultures were maintained in 0.95-liter glass jars containing 0.7 liter of corn equilibrated to incubator conditions. Two days before bioassay, 2- to 3-wk old F_2 adults were placed on a small quantity of equilibrated corn held under culture conditions.

Bioassay. Adult maize weevils were briefly chilled and sexed according to Halstead (1963). Large numbers of weevils were required, so sexed individuals were incubated on a small quantity of corn at culture conditions until a sufficient number was available. A preliminary experiment was conducted to evaluate toxicity of each fraction and of the original extract. Extract (100 μ g in a 0.5- μ L aliquot of acetone) was applied to the dorsal thoracic surface of each individual by using an ISCO Model M Microapplicator (Instrument Specialties, Lincoln, NE). Insects were briefly anesthetized with carbon dioxide and handled with a vacuum needle for application. Control insects were treated with acetone only.

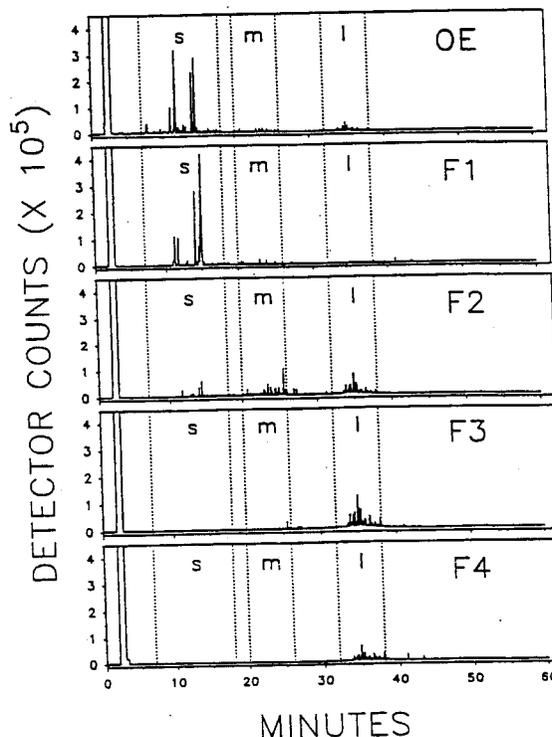


Fig. 1. Gas chromatograms of the original extract (OE) and fractions obtained by short-path distillation from the parent extract (F1-F4). Qualitative data on the various chromatograms are given in Table 1.

Table 1. Summary of GC analyses of various fractions and the original extract from *T. minus* flowers that were subjected to bioassay

Sample	Retention time			Counts ($\times 10^3$)
	Short	Medium	Long	
Extract ^a (OE)	89.6	3.1	4.9	8,821
Fraction 1 (F1)	94.7	3	0	8,737
Fraction 2 (F2)	12.6	35.8	40.6	5,452
Fraction 3 (F3)	0	1.96	90.9	4,844
Fraction 4 (F4)	0	0	79.8	2,212
3-h reflux ^b (OE-R)	88.3	3.8	0.6	4,631
Exposure 1 ^c (OE-I)	80.9	2.4	0	1,201
Exposure 2 ^d (OE-U)	78.4	0	6.6	397
Exposure 3 ^e (OE-D)	15.6	7.2	72.6	6,923

The GC analyses of fractions of the extract, a refluxed extract, and of the original extract taken before and after 24 h of exposure to air under various types of illumination are shown. Compositions of fractions of the extract are also given. Corresponding chromatograms are shown in Figs. 1 and 2. Eluting components were arbitrarily divided into 3 groups based on retention times in the profile for the original extract: short, 7–18 min; medium, 20–26 min; long, 32–38 min. The number shown for each retention time range is the percentage of total counts. Counts are the sum for the integrated peaks. Fractions were collected by short-path vacuum using an aspirator vacuum.

^a Extract was stored under dry N₂ in darkness at -20°C until chemical analysis or bioassay (Figs. 1 and 2, OE).

^b Extract was refluxed for 3 h (Fig. 2, OE-R).

^c Extract was exposed to air for 24 h at 20°C and irradiated by a 65-W incandescent bulb; distance from the radiation source was 10 cm (Fig. 2, OE-I).

^d Extract was exposed to air for 24 h at 20°C and irradiated by a 4-W 365-nm UVA light source; distance from the radiation source was 10 cm (Fig. 2, OE-U).

^e Extract was exposed to air for 24 h at 20°C and kept in darkness (Fig. 2, OE-D).

After treatment, groups of 10 insects were placed in 10-cm-diameter plastic petri dishes and incubated at 27 ± 1°C, 60 ± 5% RH, and a photoperiod of either 12:12 (L:D) h or 0:24 (L:D) h. Four replicate dishes (40 total individuals) of each treatment and a control were prepared for each sex/photoperiod combination. Mortality was assessed at 48 h by touching each insect with a blunt probe. Those that did not respond were considered dead. Extracts that did not cause at least 30% mortality under these conditions were considered nontoxic. Subsequently, a follow-up experiment tested the more toxic preparations. A 500-mg/mL stock solution in acetone was prepared for each material to provide dilutions to deliver doses ranging from 10 to 250 µg per weevil, depending on the initial toxicity of each extract or fraction. Dose-responses were determined with 4–6 doses. Incubation, mortality determination, and replication per dose were as above. Because anesthetizing and dosing individuals was time limiting, the experiment was conducted over a period of several days. Four replicate dishes (40 individuals) served as controls for each sex/photoperiod combination within a treatment.

Statistical Analyses. All mortality data were corrected by using Abbott's (1925) formula. Data were analyzed by regressing probit-transformed mortal-

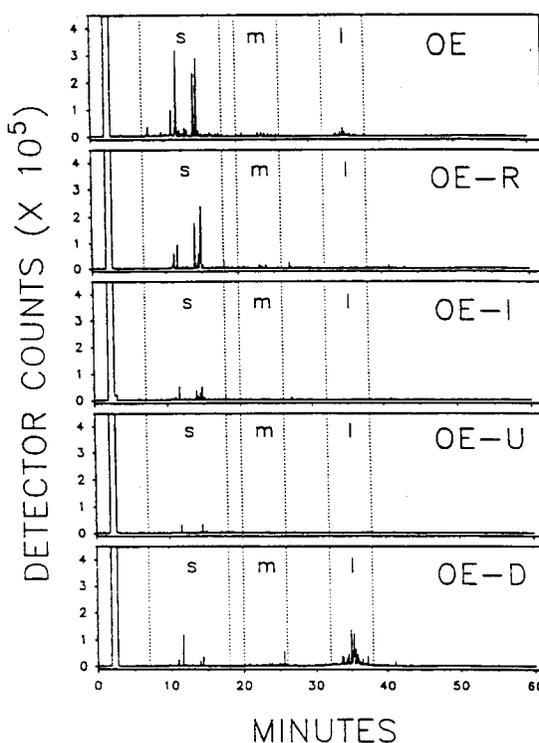


Fig. 2. Gas chromatograms of the original extract (OE) after various exposures: 3-h reflux, OE-R; incandescent light and air for 24 h, OE-I; UVA light and air for 24 h, OE-U; air in darkness for 24 h, OE-D. Qualitative data on the various chromatograms are given in Table 1.

ity against log-transformed dose according to the probit procedure in SAS (SAS Institute 1988). The parent floral extract was evaluated 3 times during the experimental period, yielding virtually identical dose responses, and tests of equality showed no significant differences between experimental replicates (SAS Institute 1988) so dose responses were pooled. Lethal-dose ratios (Robertson and Preisler 1992) were calculated at the LD₅₀ and LD₉₉ (LD₉₉ values were estimated by extrapolation when the highest dose did not cause 100% mortality) for each preparation relative to the original extract within a sex/photoperiod, for effect of photoperiod within each sex/extract, and for effect of sex within each extract/photoperiod. These are specific pairwise comparisons designed to detect any potential differences caused by these factors only.

Results

Gas chromatograms of the various fractions and the original extract are shown in Figs. 1 and 2. Each fraction differed from the original extract, not only in chromatographic profiles based on retention times and volatility but also in relative ion count of material (Fig. 1; Table 1). In addition, the fraction with the smallest quantity of material (Fig. 1; F4)

Table 2. Summary of probit analyses of mortality data for extracts and fractions of *T. minuta* that showed sufficient toxicity for dose-response assay

Material	Sex	Light	Slope ^a	Intercept ^a	LD ₅₀ ^b	LD ₉₉ ^b	χ ²
Original extract (OE)	M	Y	1.8 ± 0.5	-2.9 ± 0.5	40.8 (25.8-58.5)	816.2 (347.6-755)	12.5*
	M	N	2.6 ± 0.3	-4.3 ± 0.5	46.7 (36.2-58.6)	380.8 (236.4-865.3)	8.7*
	F	Y	2.4 ± 0.2	-3.7 ± 0.3	34.9 (30.7-39.3)	319.0 (245.5-448.4)	5.8
	F	N	2.1 ± 0.2	-3.3 ± 0.4	39.7 (28.8-51.8)	520.5 (285.1-1,573)	8.8*
Fractionation							
Fraction 1 (F1)	M	Y	3.7 ± 1.0	-6.7 ± 1.8	69.0 (34.1-109.5)	299.4 (156.3-15,149)	21.0*
	M	N	2.9 ± 0.7	-5.2 ± 1.3	68.4 (37.8-105.8)	447.0 (206.8-18,026)	13.8*
	F	Y	5.0 ± 0.6	-9.2 ± 2.1	70.4 (46.1-94.2)	206.8 (134.8-1,032)	16.3*
	F	N	4.1 ± 0.7	-7.3 ± 1.4	63.6 (44.5-81.8)	237.6 (153.2-802.9)	10.3*
Fraction 2 (F2)	M	Y	1.8 ± 0.3	-3.3 ± 0.6	68.8 (52.2-87.2)	1,336 (650.6-5,336)	2.9
	M	N	1.8 ± 0.3	-2.9 ± 0.6	41.7 (27.8-54.4)	869.4 (445.0-3,248)	6.0
	F	Y	3.2 ± 0.6	-6.0 ± 1.2	77.3 (43.0-125.1)	414.1 (206.6-6,187)	8.6*
	F	N	3.0 ± 0.6	-5.4 ± 1.2	60.6 (27.8-99.2)	354.9 (172.1-8,495)	9.0*
Fraction 3 (F3)	M	Y	4.2 ± 0.9	-8.6 ± 1.9	108.3 (75.6-145.3)	382.9 (233.2-2,397)	11.9*
	M	N	2.2 ± 0.4	-4.7 ± 0.7	126.1 (105.5-156.7)	1,378 (746.3-4,194)	5.0
	F	Y	4.1 ± 0.6	-7.9 ± 1.1	83.9 (72.0-94.5)	310.9 (242.6-468.3)	6.2
	F	N	4.1 ± 0.9	-7.8 ± 1.7	80.7 (50.3-110.3)	296.7 (183.3-1,466)	14.1*
Degradative process							
3-h reflux (OE-R)	M	Y	2.1 ± 0.6	-4.4 ± 1.3	112.1 (43.5-340.4)	1,364 (400.7-1.1 × 10 ⁶)	19.9*
	M	N	2.1 ± 0.3	-4.2 ± 0.6	115.4 (94.6-141.7)	1,563 (867.9-4,262)	1.8
	F	Y	1.4 ± 0.3	-2.8 ± 0.5	96.6 (71.6-128.4)	4,446 (1,614-35,904)	3.2
	F	N	1.9 ± 0.3	-4.0 ± 1.4	113.3 (91.9-140.7)	1,797 (952.1-5,404)	4.3

^a Number of individuals tested for each preparation: original extract (OE) on ♂♂, 600 illuminated and 601 in darkness; original extract on ♀♀, 602 illuminated and 601 in darkness; fraction 1 (F1) on ♂♂, 240 illuminated and 240 in darkness; fraction 1 on ♀♀, 240 illuminated and 240 in darkness; fraction 2 (F2) on ♂♂, 240 illuminated and 241 in darkness; fraction 2 on ♀♀, 240 illuminated and 240 in darkness; fraction 3 (F3) on ♂♂, 240 illuminated and 240 in darkness; fraction 3 on ♀♀, 240 illuminated and 240 in darkness; 3-h refluxed extract (OE-R) on ♂♂, 241 illuminated and 240 in darkness; 3-h refluxed extract on ♀♀, 240 illuminated and 240 in darkness. Chi-square values marked with an asterisk indicate that the variances and covariances have been multiplied by a heterogeneity factor, and that a value from the *t* distribution has been used to calculate the fiducial limits because the probability of a greater chi-square value is $P < 0.10$

^b Units are micrograms per weevil.

showed insufficient toxicity in our preliminary tests. Exposure to air in the presence (Fig. 2; OE-I, OE-U) or absence (Fig. 2; OE-D) of light resulted in significant changes in the chromatographic profiles (Table 1). These preparations lacked sufficient toxicity in the preliminary experiment so they were not studied further. Extract that was refluxed for 3 h (Fig. 2; OE-R) had reduced insecticidal activity and less low molecular weight material (Fig. 2; Table 1) but was sufficiently toxic for assay (Table 2). Results of probit analyses for all preparations with sufficient toxicity for the dose-response experiment are given in Table 2.

The LD₅₀ values for preparations that were sufficiently toxic to conduct dose-response experiments ranged from 30 to 130 μg per weevil (Table 2). The fractionation procedures resulted in little net gain or loss in toxicity of each fraction when compared with the parent extract (Table 3). Lethal dose ratios, within a particular sex/photoperiod combination, for the LD₅₀ values ranged from 0.9 to 2.9 times less toxic than the parent extract. Also, the confidence limits on the lethal dose ratios for many of the lower toxicity fractions or extracts did not include the number 1, indicating significantly lower toxicity. No fraction was significantly more toxic

Table 5. Lethal-dose ratios (95% CL) for effect of sex on toxicity of *T. minuta* floral extract and various fractions

Light	Extract (OE)	Fraction 1 (F1)	Fraction 2 (F2)	Fraction 3 (F3)	3-h Reflux (OE-R)
Y	1.1 (0.9, 1.6)	1.0 (0.8, 1.2)	0.9 (0.7, 1.2)	1.3 (1.1, 1.5)*	1.2 (0.8, 1.6)
	2.6 (1.0, 6.7)	1.5 (0.9, 2.3)	3.2 (1.0, 10.0)	1.2 (0.8, 2.0)	0.3 (0.1, 1.0)
N	1.2 (0.6, 2.5)	1.1 (0.9, 1.3)	0.7 (0.5, 0.9)*	1.6 (1.3, 2.0)*	1.0 (0.8, 1.4)
	0.7 (0.2, 2.7)	1.9 (1.0, 3.6)	2.4 (0.8, 7.1)	4.8 (1.7, 12.5)*	0.9 (0.3, 2.6)

All values reported are relative to females (1, no effect). Comparisons are made within the same photoperiod and extract or fraction only. For each photoperiod and extract or fraction the upper line is the LD₅₀ lethal-dose ratio, and the lower line is the LD₉₅ lethal-dose ratio. *Indicates that the confidence limits do not include 1; thus, the lethal-dose ratio is significant.

These results indicate that it may be most efficacious to use crude extracts of *Tagetes* spp. in control programs because the fractionation procedures probably reduce the amount of available biologically active material. For example, the potential for loss of activity during fractionation by short-path distillation is significant. This is evident from Fig. 1, which shows not only altered chromatographic profiles but also that the total amount of material in equivalent concentrations (wt:vol) is decreased (Table 1) when compared with the original extract. This is probably caused by increased thermal breakdown in each fraction. Close inspection of the chromatogram from the original oil (Figs. 1 and 2; OE) and fraction 1 (Fig. 1; F1) and the refluxed oil (Fig. 2, OE-R) shows the appearance of a peak at a retention time of 15 min. This peak resulted from the thermal exposure and is probably a rearrangement product. This qualitative comparison can be further supported by the large loss of material in the extract samples that were exposed to air or air and light for 24 h (Table 1; Fig. 1). These examples serve as worst-case scenarios for loss of activity caused by increased handling of material. Such exposure occurs after application of the material for insect control purposes as well, so it is apparent that toxicity of these preparations is short lived, and any residues remaining after several days of air exposure are likely to be nontoxic to insects. Therefore, subsistence farmers could use this material as initially collected (unfractionated) or when it had been carefully fractionated to provide only short-term control of an existing insect population.

Acknowledgments

We thank P. L. Lang, L. E. Fetzer, and S. M. Woo (Stored-Product Insects Research and Development Laboratory, Savannah, GA, [closed as of 1994]) for excellent technical assistance. Gratitude is expressed to T. W. Phillips (Oklahoma State University, Stillwater, OK), P. J. Landolt (USDA-ARS, Wapato, WA), and M. N. Parajulee (Texas Agricultural Experiment Station, Vernon, TX) for review of an earlier draft of the manuscript.

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Y	1.1 (0.9, 1.6) 2.6 (1.0, 6.7)	1.0 (0.8, 1.2) 1.5 (0.9, 2.3)	0.9 (0.7, 1.2) 3.2 (1.0, 10.0)	1.3 (1.1, 1.5)* 1.2 (0.8, 2.0)	1.2 (0.8, 1.6) 0.3 (0.1, 1.0)
N	1.2 (0.6, 2.5) 0.7 (0.2, 2.7)	1.1 (0.9, 1.3) 1.9 (1.0, 3.6)	0.7 (0.5, 0.9)* 2.4 (0.8, 7.1)	1.6 (1.3, 2.0)* 4.8 (1.7, 12.5)*	1.0 (0.8, 1.4) 0.9 (0.3, 2.6)

All values reported are relative to females (1, no effect). Comparisons are made within the same photoperiod and extract or fraction only. For each photoperiod and extract or fraction the upper line is the LD₅₀ lethal-dose ratio, and the lower line is the LD₉₅ lethal-dose ratio. *Indicates that the confidence limits do not include 1; thus, the lethal-dose ratio is significant.

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