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Detection of the phototoxic dye phloxine B in *Anastrepha ludens* (Loew) (Diptera: Tephritidae), *Apis mellifera* Linnaeus (Hymenoptera: Apidae) and honey

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DETECTION OF THE PHOTOTOXIC DYE PHLOXINE B IN *ANASTREPHA LUDENS* (LOEW) (DIPTERA: TEPHRITIDAE), *APIS MELLIFERA* LINNAEUS (HYMENOPTERA: APIDAE) AND HONEY

A Thesis

by

ALEENA M. TARSHIS MORENO

Submitted to the Graduate School of the
University of Texas-Pan American
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

May 2001

Major Subject: Biology

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DETECTION OF THE PHOTOTOXIC DYE PHLOXINE B IN *ANASTREPHA LUDENS* (LOEW) (DIPTERA: TEPHRITIDAE), *APIS MELLIFERA* LINNAEUS (HYMENOPTERA: APIDAE) AND HONEY

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ABSTRACT

Tarshis Moreno, Aleena M., Detection of the Phototoxic Dye Phloxine B in Anastrepha ludens (Loew) (Diptera: Tephritidae), Apis mellifera Linnaeus (Hymenoptera: Apidae) and Honey. Master of Science (MS), May, 2001, 50 pp., 3 tables, 13 illustrations, references, 51 titles.

A spectrophotometric method for detection of phloxine B, a phototoxic dye proposed as a replacement for malathion bait sprays, in extracted tissues of *Anastrepha ludens*, *Apis mellifera* and in honey was developed. Dye detection was increased with a pH change from 6 to 13.7 in tissues or from 3.7 to 8 in honey with 2% sodium hydroxide. An LC_{50} of 29.62 ppm phloxine B in 30% sucrose was obtained by feeding honey bees. A predictive model for dye in insect tissues and honey was developed and shown to be 89-92% effective. This study provides a forensic approach to determine if bees were killed or honey was contaminated by phloxine B sprays.

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INTRODUCTION

Flies of the genus *Anastrepha* (Diptera: Tephritidae) are among the world's most devastating agricultural pests. The genus comprises 185 described species. Seven of these cause serious economic damage due to fruit infestations and indirect loss of export markets because of quarantine barriers to agricultural crops. These species are *Anastrepha ludens* (Loew), *A. obliqua* (Macquart), *A. fraterculus* (Wiedemann), *A. serpentina* (Wiedemann), *A. striata* Schiner, *A. suspensa* (Loew) and *A. grandis* (Macquart) (Aluja 1994) and are found from the U.S. to the northern central part of Argentina and southern Peru. The genus *Anastrepha* appears to have originated in South America as 43% of the species are found there, 15% in Central America, only 4% in Mexico and the U.S., and 1% in the Lesser and Greater Antilles (Hernández-Ortiz and Aluja 1993). The Mexican fruit fly, *A. ludens*, is the most important economic fruit fly of the genus *Anastrepha* because of its wide range of hosts including citrus, mangos, and other subtropical fruits. Newer improved temperate varieties of apples and peaches grown in subtropical areas are also attacked by *A. ludens* (Norrbom and Kim 1988). *A. ludens* is tolerant to extreme environmental conditions and is found from the Lower Rio Grande Valley of Texas to Costa Rica (Plummer *et al.* 1941). The adult female lays eggs inside fleshy fruit tissues where the larvae develop.

Environmental Entomology is used as a model for syle and format.

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The damaged fruit drops from the tree about the time larvae complete their development. Larvae exit the fruit and burrow into the soil where they pupate and complete metamorphosis. Adult flies emerge from pupae in the soil and after emergence a maturity (premating) period ensues, this allows for development of eggs in the female and sperm in the male, before females mate and lay eggs. The length of development for each stage is temperature dependent.

Control of fruit flies depends largely on insecticides. A malathion-bait spray tactic was adopted about the time that the Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann), was eradicated from Brownsville, Texas in 1965 (Stephenson and McClung 1966). Malathion, a fumigant-contact-stomach poison, effectively kills fruit flies, other pests, and beneficial insects. Malathion sprays disrupt integrated pest management and have an adverse effect on the environment. There is a need to replace malathion with chemical compounds that are nontoxic to mammals, birds, and fish. Newer alternatives are plant and insect growth regulators, phototoxic dyes, toxins derived from microorganisms, and insecticides developed by using novel chemistries. The toxic-bait used from 1956 (Steiner *et al.* 1961) to the present for fruit fly control in the U.S. relies on the attraction of NuLure (a hydrolyzed corn protein) with malathion. If a noncontact, stomach toxicant is desired, this toxic bait has to be changed to prevent damage to beneficial insects such as bees and parasitoids but stimulate the target pest to eat it. The effectiveness of a selected chemical to a target fruit fly population will depend entirely on the bait's attractiveness, acceptability, and phagostimulatory properties. The bait has to be consumed to be toxic and have no contact toxicity.

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Moreno and Mangan (1995) addressed these concerns and produced a multicomponent bait that is an alternative for NuLure; they incorporated the phototoxic dye phloxine B into the new bait.

Phloxine B (D&C Red #28) (Fig. 1), a xanthene dye used in the drug and cosmetic industry, belongs to a class of photoactive dyes, some of which are phototoxic. Red #27, an acid form of phloxine B has no sodiums and is not as water soluble as Red #28. The phototoxicity of these dyes depends on photodynamic action. The photodynamic action of dyes can best be explained by using the xanthene dyes as a model. The xanthene dyes differ in the presence of the halogens, Cl, Br, or I.

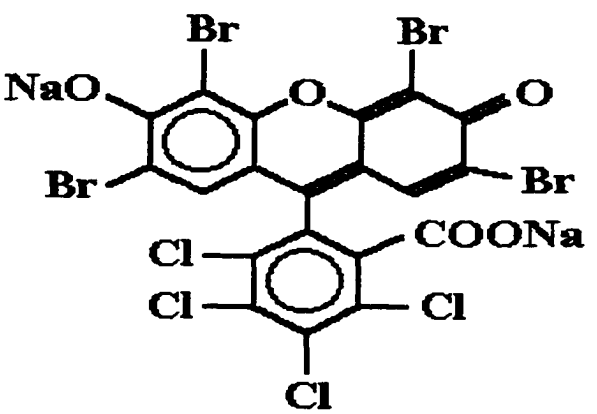

Fig. 1. Basic structure of phloxine B (D&C Red #28), disodium salt.

In light catalyzed toxicity, the primary toxic reaction in insects depends on absorption of visible light energy. The reactive dye collects light energy and converts it to a form that allows transfer to oxygen, thereby forming a reactive and excited, toxic singlet oxygen. The singlet state of oxygen rapidly decays to a triplet state, which has a longer life span than the singlet state. The highly energetic singlet oxygen molecule is able to oxidize many cellular components

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resulting in toxic action and death of an insect (Pimprikar *et al.* 1979). There are two mechanisms by which photodynamic systems function. In Type I mechanisms, dye absorbs a photon of light and rises first to the singlet, excited state and then drops to the excited triplet state. The energy of the photon is then added to the target substrate molecule making an activated form of the substrate. This activated molecule then adds to ground state oxygen or other oxygen radicals and becomes oxidized in the process. The Type II mechanism is similar except the photon rises to the excited triplet state giving energy to the ground state oxygen and thereby raising the oxygen to the excited singlet state. Lastly, the excited oxygen adds to the target substrate and oxidizes it. The Type II mechanism is considered to occur with the halogenated xanthene dyes (Heitz 1995). As the dye photosensitizer is a catalyst, it does not enter into any toxic reaction.

Previous Studies With Dyes

In 1928 Barbieri studied the action of several xanthene dyes (eosin, erythrosin, and rose bengal) against mosquito larvae and showed that several levels of visible light from various sources (electric and solar) could catalyze phototoxic reactions. Schildmacher (1950) reported on the light-catalyzed toxicity of fluorescein, erythrosin, eosin, rose bengal, and other dyes on *Anopheles maculipennis* Meigen, *A. superpictus* Grassi, and *Aedes aegypti* L. mosquito larvae. Solar light was used in both field tests with acridine red and in laboratory tests, with all the other dyes. Schildmacher found that rose bengal was more toxic than erythrosin, and, eosin and fluorescein were ineffective. The toxicity to

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codling moth, *Laspeyresia pomonella* L., larvae treated with methylene blue under short and long day photoperiods (16 h vs. 8 h) was quantified by Hayes and Schechter (1970). The diapausing larvae were adversely affected by exposure to long day photoperiods and higher dose levels. Yoho *et al.* (1971, 1973, 1976) studied the effects of photodynamic action on adult house flies, *Musca domestica* L., using fluorescent dyes of various concentrations in the food source. They observed mortality in dye-fed flies when the flies were exposed to either natural or artificial (fluorescent) light. Weaver *et al.* (1976) reported decreased hemolymph volumes and increased crop volumes in the American cockroach, *Periplaneta americana* L., and oriental cockroach, *Blatta orientalis* L., when fed or injected with rose bengal and erythrosin B and exposed to artificial light. The larvae of the yellow mealworm, *Tenebrio molitor* L., were killed by photodynamic action using methylene blue injected orally or ingested (Graham *et al.* 1972). The black imported fire ant, *Solenopsis richteri* (Forel), was shown to be susceptible to a lethal photooxidation reaction after ingesting lethal doses of rose bengal in the dark and then exposed to artificial light (Broome *et al.* 1975b). This reaction is dependent on dye concentration, incubation period prior to exposure, light intensity, and exposure time. Callaham *et al.* (1975) determined the susceptibility of the boll weevil, *Anthonomous grandis grandis* Boheman, to dye-sensitized photooxidation using various xanthenes (rose bengal, phloxine B, erythrosin B, and eosin yellow) as sensitizers. They observed decreased body weight with an increase in dye concentration as well as an increase in halogenation of the dye molecule. Broome *et al.* (1976) studied the biochemical

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effects in the boll weevil following rose bengal induced toxicity in the absence of light and found decreases in total lipid and protein concentration, wet and dry weights, and increases in total amino acid pool size.

Various xanthene dyes were evaluated for dye-induced toxicity in the face fly, *M. autumnalis* De Geer, by Fondren and Heitz (1978) and in the house fly (Fondren *et al.* 1978). They found that face flies are susceptible to a light independent toxic reaction that was also observed in house flies, imported fire ants, and boll weevils. Fairbrother *et al.* (1981) evaluated the toxic effects of rose bengal and erythrosin B to larval, pupal, and adult stages of the face fly when the larvae were exposed to dye-treated manure. They found that the extent of toxicity was dependent on the concentration of dye. Sakurai and Heitz (1982) studied the inhibition of larval growth and photooxidative toxicity in the adult house fly that was induced by rose bengal or erythrosin B mixed into an artificial agar medium and fed to the larval stages. Pupation was inhibited and pupal weight decreased as dye concentration increased. Adult flies that had consumed a nonlethal quantity of dye during the larval stage exhibited a high light-catalyzed toxicity. Pimprikar *et al.* (1979) tested for toxicity of rose bengal to the larvae of *Culex pipiens quinquefasciatus* Say and *Aedes triseriatus* (Say) as an alternative for mosquito control. *Culex* larvae are more susceptible to rose bengal than the larvae of *Aedes* species and mortality was found to be a function of dye concentration and light exposure time. They observed physiological and morphological abnormalities in the dye-treated larvae. Carpenter and Heitz (1980) correlated acute larval mortality with a combination of illumination time

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and rose bengal concentration. Pimprikar *et al.* (1984) studied the toxicity of rose bengal, phloxine B, erythrosin B, eosin yellow, rhodamine B, and fluorescein to larvae of *C. pipiens* L. and *A. triseriatus* and to predatory mosquitofish, *Gambusia affinis* Baird and Girard. Dyes were toxic to both species of mosquitoes and relatively nontoxic to predatory mosquitofish. Rose bengal-impregnated diets were used to kill the cabbage looper moth, *Trichoplusia ni* (Hübner), the pickleworm, *Diaphania nitidalis* (Stoll), and the corn earworm, *Helicoverpa zea* (Boddie) (Creighton *et al.* 1980). Clement *et al.* (1980) investigated the activity of rose bengal, erythrosin B, and phloxine B against 3rd instar of the black cutworm (*Agrotis ipsilon* [Hufnagel]). They found that dyes were most effective at light intensities of 4,400 and 9,200 lux. Carpenter *et al.* (1984) tested the joint toxicity of erythrosin B with rose bengal, eosin Y, diiodofluorescein, fluorescein, octabromofluorescein, and phloxine B against the adult house fly. The addition of these xanthene dyes increased the killing efficiency of erythrosin B against house fly. Koehler and Patterson (1986) evaluated erythrosin B for toxicity in adult and larval house flies. They found that by increasing dosage and light intensity, toxic action was increased. Burg *et al.* (1989) evaluated the effects of a liquid bait formulation of erythrosin B for the control of house flies and vinegar flies, *Drosophila robusta* Sturtevant, in an environmentally controlled poultry house. Fly populations were reduced and increasing light intensity made no difference. Krasnoff *et al.* (1994) studied the toxic effects of erythrosin B and light on adult apple maggot, *Rhagoletis*

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pomonella (Walsh), in the laboratory. They reported that mortality increased with higher doses of erythrosin B and also with increased exposure times.

Current Insect Studies

Moreno and Mangan (1995) and Mangan and Moreno (1995) developed a phototoxic dye-bait for the Mexican fruit fly that included a mixture of phloxine B and uranine. This mixture, known commercially as SureDye, was tested to determine its efficacy as a phototoxin against adult flies. Moreno and Mangan (1995) found that the phototoxic agent was phloxine B and uranine did not enhance phototoxicity but rather interfered with the phototoxicity of phloxine B. Liquido *et al.* (1995a) tested the phototoxicity of phloxine B on the Oriental fruit fly, *Bactrocera dorsalis* (Hendel) with and without fluorescein in methyl eugenol (a very powerful male oriental fruit fly attractant). Their data showed that phloxine B with or without fluorescein in methyl eugenol resulted in high male mortalities. In experiments with the Mediterranean fruit fly, Liquido *et al.* (1995b), also found that the addition of uranine did not enhance the toxicity of the bait. Moreno and Mangan (1995) found that efficacy of dyes changed with the addition of some adjuvants. They are currently conducting field tests on *A. ludens* and *C. capitata*, in Mexico, Guatemala, and Costa Rica; *A. suspensa*, in Florida; the Natal fruit fly, *C. rosa* Karsch, in South Africa, and *A. fraterculus*, in Brazil. Other investigators are also evaluating the effect of phloxine B on the southern corn rootworm, *Diabrotica undecimpunctata howardi* Barber (Schroder *et al.* 1998), Mexican bean beetle, *Epilachna varivestis* Mulsant (Contreras C. 1998), further evaluation

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with the boll weevil, and the diamondback moth, *Plutella xylostella* (L.) (Sánchez M. 2000).

Detection of Phloxine B. Broome *et al.* (1975a), Fondren *et al.* (1978), and Callaham *et al.* (1975, 1977) extracted xanthene dyes from black imported fire ants, house flies, and boll weevils, respectively using chloroform-methanol (2: 1; v/w) following the procedure of Radin (1969) and determined dye concentration on a Beckman Acta V Spectrophotometer. Broome *et al.* (1975a), Fondren *et al.* (1978), and Callaham *et al.* (1975, 1977) did not state which form of phloxine B, acid (Red #27) or salt (Red #28), was used.

This study is important because we wanted to know it we could determine, from a forensic approach, if Mexican fruit flies found dead in the field were killed because of ingested phloxine B sprays. The inquiries of this study were: (1) Can phloxine B (D&C Red #28, salt form) be extracted from *A. ludens* tissues in a relatively short time period (less than a day)? (2) Is there a correlation between the amount of dye fed to *A. ludens* and the amount recovered, if so, can a predictive regression model be developed based on the amount of phloxine B recovered from flies fed known amounts of phloxine B? (3) Can the dye be recovered from caged flies killed by phloxine B under greenhouse conditions to test the methodology and determine phloxine B residue? (4) Can the dye be detected in a non-target beneficial insect such as the honey bee, *Apis mellifera* to test extraction and recovery methodology? (5) Can an LC_{50} for phloxine B be determined for the honey bee? (6) Can the dye be detected in honey to determine possible contamination of phloxine B via honey bees?

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MATERIALS AND METHODS

Insects

Flies. Early experimental *A. ludens* were from a culture started in 1995 that originated from Chiapas, México. Later tests used a culture started in 1998 from Nuevo Leon, México. All rearing was done at the USDA, Agriculture Research Service, Kika de la Garza Subtropical Agricultural Research Center (ARS, KDLGSARC), Weslaco, TX. Laboratory conditions were maintained at 26.7 °C, 50% RH, and a photoperiod of 14:10 (L:D) h. Larvae were reared on an artificial diet of torula yeast, dried carrot powder, sugar, and fillers (Spishakoff and Hernández Davila 1968) and mature 3rd instar were induced to pupate in moist vermiculite. Upon emergence, adults were fed 4% (w/v) sugar-water only. The day prior to testing, 3 to 5 d-old adult female flies were aspirated and placed into an aluminum-frame screened rearing cage (22.5–cm^3) with 4% sugar-water.

Bees. All experimental honey bees were from local colonies of European stock maintained by the USDA-ARS-KDLGSARC-Honey Bee Research Unit (HBRU), Weslaco, TX. Frames with brood were placed into frame holders to obtain bees of known age (0-24 h) and held in the dark at 35 °C. Emerging bees were fed 30% (w/v) sugar-water. According to Herbert (1992), honey bees most readily accept sugars that are naturally found in nectar (sucrose, glucose, and fructose). In choice tests Waller (cited by Herbert 1992) found that sucrose was the most preferred and glucose least preferred.

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Instrumentation

A Perkin-Elmer Luminescence Spectrometer LS 50B (Perkin-Elmer Corp., UK) with an emission scan range of 500-600 nm and excitation at 490 nm was used to record the intensity of phloxine B. These values were used based on published properties of phloxine B (Green 1991). Intensity is the energy emitted by the light source and ranges from 0 to 1000. To determine if changes in pH change the peak intensity of the dye, a pH gradient from pH 2-13 using 0.75-ppm phloxine B was determined with two replications of three readings. Sodium hydroxide (NaOH) and hydrochloric acid (HCl) were used to adjust pH. The effective concentration (maximum and minimum intensities) limits and linearity for phloxine B from a selected optimum pH were determined.

Potential degradation of 4-ppm phloxine B in basic solutions was tested with concentrations of 0-8% NaOH. The intensity of phloxine B solutions was recorded at various time intervals from 0 to 96 h (2 replicates). Results from this test showed that NaOH degraded phloxine B. Therefore, a second test was conducted with 4-ppm phloxine B in 0 to 2% NaOH and intensity recorded from 0 to 48 h (2 replicates).

To test the chloroform-methanol (Chl:MeOH) extraction methodology of phloxine B used by Broome *et al.* (1975a), Fondren *et al.* (1978) and Callaham *et al.* (1975, 1977), a paired test with Chl:MeOH and 2% NaOH using 1 fly per replicate and 4 levels of phloxine B was set up. The intensity of fly extract in NaOH was read every 2 h (for 6 h total) until the intensity was less than 20. The

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intensity of fly extract in Chl:MeOH was read at 1, 3, 7, 9, 10, 15, 17, 20-24 and 27 d.

Procedures

Preparation of *A. ludens*. On the afternoon before testing, water replaced the sugar-water. On the morning of the test, individual female flies were aspirated into 1 oz. plastic containers (Jet Plastica Industries, Inc., PA) with cardboard lids. On the underside of each lid was a small preformed ring of paraffin. Inside this ring, a known volume of check or dye-bait (SolBait) (Drs. D.S. Moreno and R.L. Mangan, personal communication) formulation (Table 1) was placed. As used in these studies control refers to a group of insects given only essential food (i.e. 4% sugar water) and check refers to a group of insects receiving something in common to the rest of the treatments (i.e. bait). All chemicals were bought from Sigma Chemical Co. (St. Louis, MO) except for phloxine B (D&C Red #28, Warner Jenkinson Co., Inc., St. Louis, MO), and Solulys (spray-dried, hydrolyzed corn protein, Roquette Int., IA). The check contained no dye. After the flies ingested the treatment, they were placed into –20 °C for storage except for sun/shade flies.

Dye Extraction. Legs, wings, and fly heads were excised and discarded. Previous observations noted no dye in these areas as the dye enters the alimentary tract. The head was discarded due to pteridines that interfere with phloxine B absorbance (Dr. N. Tomic-Carruthers, personal communication).

Detection of Phloxine B in *A. ludens* with Tris Buffer pH 8. Tris buffer at pH 8 was used to extract the dye from *A. ludens* as it is a biological buffer, would

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keep the solution at a certain pH and would not harden the tissues. Flies were fed as above. The thorax and abdomen were ground up for 1 min with 100 µl of Tris buffer pH 8, in a 0.2-ml glass micro tissue homogenizer, then, transferred to a 1.5-ml microcentrifuge tube. The homogenizer was rinsed 2X with 100 µl of Tris and added to the microcentrifuge tube. An additional 700 µl of Tris was added for a total volume of 1 ml and centrifuged for 5 min at 2,000 X *g* and 4 °C. The intensity of the supernatant was recorded on the LS50B. There were 4 replicates with 1 fly per replicate.

Table 1. SolBait formulation for testing of *Anastrepha ludens*.

Ingredient	Concentration (w, v/v) %
Deionized water, qs ad	100.0
Phloxine B	0–0.5 (varies)
Invert sugar	20
Polysorbate 60	1.0
Soybean oil	0.25
Polyethylene glycol 200	1.0
Solulys, (2% Al amino acids)	4.4
Xanthan gum, prehydrated	0.4
Ammonium acetate	1.0

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Extraction of Phloxine B in Mexican Fruit Fly with Tris buffer pH 8 and NaOH. Flies were fed as a group with 0.5% phloxine B in SolBait for 24 h, killed and stored at –20 °C. A volume of 1 ml containing 0, 0.25, 0.5, 1, 2, or 4% NaOH in Tris buffer was used to resuspend and extract fly pellets (previously ground thorax and abdomen) that had first been extracted in Tris buffer pH 8 to establish a baseline intensity reading. Pellets were stored overnight at 4 °C and centrifuged (Eppendorf 5402, Brinkmann Inst., Inc, Westbury, NY) for 10 min at 16,000 X *g* and 4 °C. Supernatant intensities were recorded daily with fresh extraction buffer added to each pellet after intensities were recorded for 3 d. At this time, 2% NaOH was added to the controls to extract the dye. There were five replicates with one fly per NaOH concentration. Phloxine B fed flies ground up in water had a pH of 6 and flies ground in 2% NaOH had a pH of 13.6.

Extraction of Phloxine B in Mexican Fruit Fly with NaOH. The thorax and abdomen were placed into a 0.2-ml glass micro tissue homogenizer with 100 µl of 2% NaOH, ground for 1 min then transferred to a 1.5-ml microcentrifuge tube. The homogenizer was rinsed 2X with 100 µl of 2% NaOH, added to the microcentrifuge tube and an additional 700 µl of 2% NaOH was added for a total volume of 1 ml. The tubes sat for 105 min before centrifuging for 10 min at 16,000 X *g* and 4 °C. The supernatant was read and intensity recorded. One ml of 2% NaOH was added to the pellet, vortexed (Mistral Mixer, Lab-Line Inst. Inc., Melrose Park, IL), and allowed to sit for 45 min before centrifuging, as before. This process was repeated until the intensity of the phloxine B was less than 20, based on a scale of 1000. A minimum of 10 flies per phloxine B concentration

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were ground up. Concentrations used were 0, 0.3, 0.6, 1.2, 1.8, 2.4, 3.6, and 4.8 µg phloxine B.

Detection of Phloxine B in Mexican Fruit Fly Held Under Direct Sunlight or Under Tree-Shade in Greenhouse-Cage Studies. Flies were fed 1 µl of 0.48% SolBait and after eating, five flies were set aside as controls and stored at –20 °C and the rest were separated into two aluminum cages (22.5-cm^3) and placed into a greenhouse. The greenhouse was used to simulate field conditions to create a shaded canopy with six three-year-old potted grapefruit trees for one cage. The other cage was placed on a step ladder to about the same height as those flies in the shade and kept constantly exposed to direct sunlight. Temperature was not controlled except for the upper limit to ensure the health of the trees. Shading trees were arranged on a table and the legs of the table set in cups of an insecticide, chlorpyrifos, to ensure that ants did not reach experimental flies. Temperature, relative humidity and light intensity levels were recorded. Five flies from each cage were removed at 1, 2, 4 and 8 d and stored at –20 °C. Flies were processed as above with 2% NaOH.

Preparation of Honey Bees. Water replaced the sugar-water 2 h before feeding bees (24-48 h old). Five µl of 30% sucrose (w/v) (control) or 0.1875, 0.375, 0.75, 1.5 or 3 µg of phloxine B in 30% sucrose was hand-fed to individual bees. After ingestion, the bees were anesthetized with carbon dioxide (CO_2) and stored at –20 °C. A minimum of 15 bees was used for the control and each of the concentrations.

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Extraction and Recovery Methodology in *Apis mellifera.* The head and thorax of the bee were excised and discarded as observations showed there was no phloxine B visible in these areas. The honey sac, ventriculus (midgut), and rectum were dissected and the extraction procedure followed that of *A. ludens*.

Determination of the LC_{50} for Phloxine B in the Honey Bee. The LC_{50} is the estimated concentration of a toxicant needed to kill 50% of a healthy population of organisms. Fifteen to 20 honey bees, 0-24 h old were placed into standard honey bee pesticide testing cages (W. Wilson, USDA-ARS-KDGLSARC-HBRU), painted white (inside and out for maximum light reflection) and provided with 20 ml each of 30% sucrose (w/v) and water in vials. The vial caps had two 1-mm holes bored through them and the vials were inverted at one end of the cage. The cages were placed in the dark at 28 °C. The vials were replaced 30 h later with the treatment and the treatments were taken off the following morning before placing the cages outside directly in the sunlight (treatments available to bees for 16 h). Bees were offered 30% sugar-water and water during exposure to direct sunlight. Mortality counts were recorded prior to treatment and before placing the cages outside, and hourly or semi-hourly count of dead bees was made after mortality began. There were 3 to 5 replicates per treatment.

Determination of the LC_{50} for Dye-Bait in the Honey Bee. After the LC_{50} had been determined for phloxine B using 30% sucrose-water as the only carrier, a similar test was set up using varying concentrations of phloxine B in SolBait containing 30% sucrose. Treatments were check-bait (0% dye), 2.025, 4.05, 8.1,

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16.2 and 32.4-ppm dye in SolBait. There were four replicates per treatment with 20 bees per cage. Forty bees from the highest concentration were sampled at the end of the test for visual detection of the dye.

As the bees did not feed on the bait, a no choice test was set up to partition the SolBait ingredients to determine why the bees did not feed. Treatments were sucrose control – 30% sucrose; sucrose check – 5X LC_{50} phloxine B in 30% sucrose; bait control – Solulys (4.4%), sucrose (30%) and xanthan gum (0.4%); check – bait control + 5X LC_{50} phloxine B; PEG – check + polyethylene glycol 200 (1%); Soy oil – check + soybean oil (0.2%); Poly60 – check + polysorbate 60 (1%); or AmAce – check + ammonium acetate (1%). There were 3 to 4 replicates per treatment and 20 bees per cage. The methodology used was the same as for LC_{50} development for the honey bee. At the end of the test, 16 dead bees from each treatment were set aside and stored at –20 °C for dye detection.

A choice test was set up, offering bees a choice between 30% sucrose and a treatment. Treatments were as in the no-choice test with these changes: phloxine B at 6X LC_{50}; and SolBait – check + phloxine B + polyethylene glycol + soybean oil + polysorbate 60 + ammonium acetate was added as a treatment while the bait control was deleted. There were 3 to 4 replicates per treatment and 20 bees per cage. The methodology used was the same as for LC_{50} development in the honey bee. Bees that had died overnight were discarded in the morning. At the end of the test, 16 dead bees from each treatment except for the complete bait (SolBait), where live bees and dead bees were sampled, were stored at –20 °C for dye detection with the LS50B spectrophotometer.

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Detection of Phloxine B in Honey. A standard phloxine B was made in white clover honey (US Grade A, Fancy White) in the concentrations of 0.01, 0.1, 1 or 10-ppm to determine phloxine B recovery. The honey was heated for 10 sec in a 1,400W microwave oven and diluted to 10% with Milli-Q water (Millipore Corp., Bedford, MA) or known concentrations of phloxine B. The 10% honey solution (with and without phloxine B) was also adjusted to pH 8 with 2% NaOH. Intensity readings were taken using the LS 50B. Three replications were done at each concentration.

Statistics. Collected data were analyzed with Microsoft Excel 97 (Microsoft Corp.), DeltaGraph 4.5 (SPSS, Inc., Chicago, IL) and SuperAnova (Abacus Concepts, Berkeley, CA).

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RESULTS

Linearity of Phloxine B (pH 5.6, 8). There was a positive linear relationship between phloxine B intensity (y) and phloxine B concentration (ppm) (x) in water at pH 5.6 ($y = 648.6x + 11.3$; $R^2 = 0.998$) in the concentration range from 0 to 1.5 ppm and at pH 8 ($y = 728.35x + 8.08$; $R^2 = 0.999$) (Fig. 2) with the concentration range from 0 to 1 ppm phloxine B. The lowest detection of phloxine B occurred at 0.025 ppm for pH 5.6 and the highest detection occurred at 1.5 ppm for pH 5.6 and 1.3 ppm for pH 8. Dosages were selected to cover the intensity range of the spectrophotometer without having to dilute (extrapolate) samples.

Detection of Phloxine B in the Mexican Fruit Fly with Tris buffer pH 8 and NaOH. Addition of NaOH to the fly pellets resulted in an increase of phloxine B intensity compared to addition of Tris buffer. By comparing the change in phloxine B intensity between the baseline Tris wash and the first wash with NaOH, all treatments except for the control (0% NaOH) showed an average increase between 66 and 125% (Fig. 3). There was a 36% average decrease of phloxine B in the control group during this time period. Sodium hydroxide at 0.25% was strong enough to extract the dye from the tissue pellet.

Detection of Phloxine B in the Mexican Fruit Fly with 2% NaOH and Chl: MeOH. Both NaOH and Chl: MeOH extracted dye from flies, though the time in which this occurred was different. In 4 h, 98.7% of the dye was extracted from

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flies using NaOH and it took more than 27 d to extract 97.6% phloxine B from fly tissues using Chl: MeOH; after 27 d there was still residual dye left in the flies that had been extracted with Chl: MeOH. The total intensity of dye in both extraction methods was similar (Table 2).

Table 2. Comparison of two extraction methods to detect (intensity) phloxine B in Mexican fruit fly tissues by using an LS50B spectrophotometer.

Fly #	2% NaOH		Chloroform: Methanol (2:1)	
	μg[a]	Intensity[b] (6 h)	μg[a]	Intensity[b] (27 d)
1	1.35	716	1.25	326
2	4.10	607	4.08	725
3	4.40	983	4.45	1013
4	5.25	868	4.80	870

[a] Amount of phloxine B ingested.

[b] Total intensity.

Change in Intensity of Phloxine B with pH Change. According to Green (1991) there is a visual color change in 0.1% phloxine B in water at pH 3.3, where the dye is purple and turns colorless at pH 1.1. A color change was obtained by varying intensity with a change in pH. The intensity of 0.75-ppm phloxine B increased dramatically from pH 2 to pH 4 then stabilized to pH 9 and at higher pH levels decreased gradually (Fig. 4).

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NaOH Digestion of 4 ppm Phloxine B in Water. Preliminary tests using Chl: MeOH to degrade the phloxine B in *A. ludens* did not work as fast as using 2% NaOH with Tris. This led to the development of a method using NaOH for extraction of phloxine B. NaOH was a good solvent to extract phloxine B from *A. ludens*, but the length of time the fly could be left in NaOH before the dye was released was unknown. At time 0, all treatments except for 8% NaOH were pink in color and the 8% NaOH was purple. At 0.75 h, treatments from 0 to 1% had not lost any of the original intensity, but rather gained intensity (Fig. 5). Treatments of 2, 4 and 8% NaOH had lost intensity at 0.75 h. At 1.25 h, all treatments up to 2% NaOH either increased intensity or remained the same, while the 8% treatment had only one-half of its original phloxine B intensity and had turned light lavender. At 1.75 h, all treatments from 0 to 1% either increased intensity or remained the same. The 2% NaOH decreased intensity by 8%, 4% NaOH intensity decreased by 26% from the original reading, and the 8% treatment decreased by 64%. At 2.25 h, all treatments except for 0% had decreased intensity from the 1.75 h period, but treatments from 0 to 1% were greater than at T = 0. At 96 h, the intensities of 0 and 0.125% had changed little or increased from its original reading. Treatments from 1 to 4% NaOH showed little or no phloxine B. There was 22 and 63% less dye, based on intensity, in treatments 0.25 and 0.5% NaOH respectively at 96 h compared to T=0.

Data were analyzed comparing rates of extraction for different NaOH concentrations after different time intervals. Significance of resulting means were separated by using Tukey test of mean separation. The analysis showed no

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significant differences at T = 0 for treatments 0 to 4% NaOH. At T= 0.75 h there was no significant difference in treatments 0 to 2% and at T = 1.25 h treatments from 1 to 8% NaOH were significantly different from those of 0 to 0.5% NaOH. At T = 1.75 h and 2.25 h there was no significant difference between treatments containing 0 to 1% NaOH and treatments of 2 to 8% differed significantly from each other. At T = 96 h treatments of 0 to 0.25% NaOH were not significantly different from each other and treatments from 0.5 to 8% were significantly different from each other.

Previous results led to a second test of dye extraction to look at the NaOH range from 0 to 2% from 0 to 48 h. Data obtained in this test were submitted to the same analysis as the first test. The second test results show that at 4 h, 0 to 0.5% NaOH increased intensity; 1 and 2% NaOH decreased phloxine B intensity by 4 and 20%, respectively (Fig. 6). At 8 h, 0% has increased intensity, 0.25 and 0.5% intensities have remained the same, and 1 and 2% have decreased intensity by 12 and 40% respectively from T = 0. At 24 h all treatments except 0% have decreased intensity and at 48 h all treatments were lower than they were at the beginning. Analysis with Tukey's test for mean separation showed no significant differences among the treatments at T = 0 and 4 h. At T = 8, concentrations of 0 to 0.5% NaOH were significantly different from 1 and 2% NaOH which were significantly different from each other. At T = 24 and 48 h all treatments were significantly different from each other.

Detection of Phloxine B in *Anastrepha ludens* (Fig. 7). Based on the above information, the best extraction was obtained using 2% NaOH for 2 h in

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the 1st wash, and only 1 h in subsequent washes (2-5). The relationship between phloxine B intensity and μg phloxine B ingested was linear ($y = 193.47x - 29.612$, $R^2 = 0.9246$), where y = phloxine B intensity and x = μg phloxine B ingested.

Detection of Phloxine B in Mexican Fruit Fly Held Under Direct Sunlight or Under Tree-Shade in Greenhouse-Cage Studies (Fig. 8). Air temperatures ranged from 6.7 to 33.6 °C, relative humidity from 18.5 to 86.5%, and sunlight intensity from 315 to 980 μE m^{-2} sec^{-1} (1 μE m^{-2} sec^{-1} = 1μmol m^{-2} sec^{-1} = 6.02 x 10^{17} photons) in the sun and 50 to 65 μE m^{-2} sec^{-1} in the shade during the course of the 8 d experiment. All flies held in the sun cage died within 2 h, while 15% of the flies in the shade were alive by day 2. The longer the dead flies stayed in the sun cage before processing, the more brittle they were and more difficult to extract the dye from them. Shaded flies were not as brittle as flies in the sun cage.

In 43 out of 45 sun and shade flies, the dye was detected with the LS50B. Phloxine B intensities ranged from 0 to 627. Sun flies had decreasing amounts of dye from day 0 through day 8. There was no pattern to dye loss in the shade. The lowest dye intensity was 24 from a fly exposed to sun for 8 d and represents 0.27 μg of phloxine B and the highest dye intensity was 627 from a fly exposed to sunlight for 1 d and represents 3.39 μg of phloxine B, interpolated from the regression line from figure 2.

Detection of Phloxine B in the Honey Bee (Fig. 9). Extraction of phloxine B from honey bees was similar to that of *A. ludens*. A regression of y = 221.9x

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with an $R^2 = 0.8956$ was obtained, where y = phloxine B intensity and x = µg phloxine B ingested.

Determination of an LC_{50} for Phloxine B in the Honey Bee (Fig. 10). The LC_{50} for phloxine B in 30% sucrose in the honey bee was estimated to be 29.62 ppm. Air temperature ranged from 29 to 32.5 °C and solar energy ranged from 950 to 1,500 µE m^{-2} sec^{-1} during this period. Attempts to determine the LC_{50} for phloxine B in SolBait were difficult as very few bees died even at a concentration slightly higher (32.4 ppm) than the LC_{50} of phloxine B calculated from sugar-water fed bees. When these bees were dissected, 1/40 (2.5%) had phloxine B visible only in the honey sac, 12/40 (30%) had phloxine B visible in the honey sac, ventriculus and rectum, and 27/40 (67.5%) had no visible phloxine B. As one or more of the ingredients in the SolBait may have repelled the bees, the SolBait was partitioned and the phloxine B concentration was increased to 5X LC_{50} (148.1 ppm). Bees were not given a choice in this test among 30% sucrose and one of the other treatments. Bees consumed more of 30% sucrose with phloxine B (94.7% mortality) than any other treatment (Fig. 11). The sucrose control (30% sucrose) and the bait control (Solulys, xanthan gum and sucrose) had no mortality. There was no statistical difference in mortality rates (37.1–66.9%) among the other treatments.

Based on these tests, a choice test between 30% sucrose and all the other ingredients in SolBait were evaluated. Bees were given a choice between 30% sucrose and the sucrose check (6X LC_{50} = 177.72 ppm phloxine B in 30% sucrose) or the check (Solulys, xanthan gum, sucrose, phloxine B) containing

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polyethylene glycol, soybean oil, polysorbate 60, or ammonium acetate or all of the ingredients (SolBait). The bees preferred the sucrose check of 177.72 ppm phloxine B in 30% sucrose to any other treatment containing phloxine B (Fig. 12) (100% mortality). Least mortality occurred in the treatments with the Solbait (4.8%) and the sucrose control (30% sucrose, 2.8% mortality). The check with the ammonium acetate (mortality 27.4%) had the next lowest mortality and was not as preferred as the check itself or with polysorbate 60, soybean oil, and polyethylene glycol.

Honey bees from all treatments containing phloxine B in the no choice test had some level of dye detected with the LS50B (Table 3). All dead bees from the choice test had detectable levels of dye and live bees had no phloxine B detected. In the no choice test, dead bees were not discarded in the morning before the treatments were set outside, therefore those treatments that did not have 100% detection of the dye may have included some of these bees.

Detection of Phloxine B in Honey (Fig. 13). The 10% honey solutions had an initial pH of 3.7 and showed no peak for phloxine B. The addition of 2% NaOH brought the pH to 8 and there was no peak in the phloxine B area. Adding phloxine B to honey to obtain a phloxine B standard, concentration of 0.01 ppm, resulted in a very small peak and changing the pH to 8 increased the intensity by 1.7X. With a phloxine B level of 0.1 ppm in honey the peak intensity increased and the increase was even greater when the pH was changed to pH 8. Increasing phloxine B concentration increased the resulting intensities. With the addition of 10-ppm phloxine B to the honey, the intensity at pH 3.7 was on scale,

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but at pH 8 it went off scale (estimated at 5,840). At pH 3.7, y = 19.897x + 19.389, R^2 = 0.9486 and at pH 8, y = 583.25x + 7.3992, R^2 = 0.9996, where *y* = phloxine B intensity and *x* = ppm phloxine B added. The lower limit of detection of phloxine B in honey was 0.01 ppm at both pH 3.7 and 8.

Table 3. Percentage of honey bees from no choice and choice tests that had phloxine B detected with an LS50B spectrophotometer.

Treatment	% bees w/ dye	
	No choice[a]	Choice[b]
Sucrose check	100	100
Check	50	80
PEG	100	90
Soy Oil	90	90
Poly60	100	92
AmAce	60	40
SolBait (live)	--	0
SolBait (dead)	--	50

[a] Phloxine B = 148.1 ppm.

[b] Phloxine B = 177.72 ppm.

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DISCUSSION

Phloxine B is pH sensitive, as are other photoactive dyes (Green 1991), but not all pH sensitive dyes are phototoxic. Some dyes are very good diagnostic indicators. To maximize the detection intensity of phloxine B, the pH of the Mexican fruit fly and honey bee tissues and also test honey were increased. NaOH solutions were more efficient at extracting the dye at higher pH's due to the increased solubility of the dye. At lower pH's the dye would precipitate out. By standardizing the pH of insect tissues and test honey with sodium hydroxide, detection of phloxine B was optimized at lower levels of the dye, thereby, increasing dye sensitivity.

The methodology used to calculate the amount of dye fed to flies in the chloroform-methanol extraction was a modification from an earlier version of the current method. Although this method of extraction has been used in earlier studies, I found this method to be unsatisfactory. Flies were weighed before and after feeding and the weight gain and amount of dye present was calculated. Using this method sometimes resulted in loss of weight in flies even though flies had fed as evidenced by red abdomens. This may account for the discrepancy of my calculated phloxine B (Table 2). The methodology used by Broome *et al.* (1975a), Callaham *et al.* (1975) and Fondren *et al.* (1978) for extraction of xanthene dyes from the fire ant, boll weevil, and house fly, respectively was not

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explained clearly and, therefore, a different methodology was developed. The chloroform-methanol extraction methodology was very slow in dissolving fats and other tissues to allow rapid extraction of phloxine B. Phloxine B, D&C Red #28, is water soluble and a chloroform-methanol solvent extraction is not necessary. Chloroform and methanol are solvents useful in extracting animal and vegetable fats and oils but were very slow in dissolving insect fats and proteins where the phloxine B ostensibly had penetrated. Sodium hydroxide saponifies fats and hydrolyzes muscles and other tissues thereby releasing phloxine B.

Extraction of phloxine B in Mexican fruit fly tissues with Tris buffer was poor but was markedly improved with the addition of sodium hydroxide. Even though aqueous sodium hydroxide degradation studies with phloxine B showed that 1% sodium hydroxide degraded the dye less than 2% sodium hydroxide, in the periods of 1.75 and 2.25 h, a second test showed no significant difference between 1 and 2% in a 4-h period. Based on tissue studies, 2% was more effective than 1% sodium hydroxide in extracting phloxine B from fly tissues in a shorter period of time.

The regression line obtained for phloxine B in the Mexican fruit fly by feeding known amounts of phloxine B followed a linear path. This linearity is useful in determining if a questionable dead fly was killed by the ingestion of phloxine B and through interpolation, determine how much phloxine B the fly consumed. Thus, the method becomes a forensic approach that can provide information about phloxine B causing the death of a fly. By simulating a field exposure, the dye can be detected in flies after eight days exposure to the sun. I would have

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expected the shaded flies to hold the dye longer, but as these flies lived 2 d longer than sun flies they had ample time to regurgitate, excrete, and metabolize the dye, whereas the short lived (<2 h) sun flies did not have the time to do so. The variation in intensity of flies in the sun cage could be due to the fact that flies may have died in an area that received some shade during the daytime i.e. corners or next to walls. As each fly was fed the same amount of dye in the bait, it is possible that 0 intensity values of sun flies were exposed directly to the sun's energy and thus the dye was bleached out. The most reactive phototoxic dyes, such as esters, fade rather quickly under the sun's energy. The shaded flies lived longer and were given protection from the sun and it's likely that variation in intensities may be due to the fact that some flies may have a higher metabolic rate than others and also had more time to eliminate the dye through regurgitation and defecation than sun flies.

Bees were hand-fed individually because bees do not feed in the same manner as flies. The LC_{50} for phloxine B in 30% sucrose in honey bees was done outside using direct sunlight, as bees normally are outside foraging during the daytime and phloxine B requires light energy to activate it. This data cannot be compared to other xanthene compounds because this type of research has not been done with honey bees. Given the same amount of dye ingested, the intensity was higher for the bees since there was a shorter time span after feeding before they were killed as opposed to the flies that had time to excrete and regurgitate the dye before they were killed thereby eliminating a small portion of the dye. An LC_{50} for SolBait in the honey bee could not be determined

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because there was a strong negative affinity for the complete SolBait formulation. Moffett and Morton (1975) reported surfactant repellency by polysorbate 20 and polysorbate 81 to honey bees. According to data presented within, there was a significant difference in mortality rates between the check containing phloxine B with and without polysorbate 60. Polysorbate 20 is polyoxyethylene sorbitan monolaurate; polysorbate 81 is polyoxyethylene sorbitan monooleate and polysorbate 60 is polyoxyethylene sorbitan monostearate. They differ in their fatty acid composition but all contain some palmitic acid. According to reviews by Melksham *et al.* (1988), Atkins *et al.* (1975) and Woodrow *et al.* (1965) there was no information that polyethylene glycol 200 and soybean oil had been tested as repellents to bees. Woodrow *et al.* (1965) found that low concentrations of acetic acid had moderate to strong repellency toward bees but 10% acetic acid was not repellent. Some ammonium compounds were tested and found to have little or no repellency, but ammonium acetate was not tested. Aerial applications of SureDye-bait (phloxine B, uranine, fructose) over bee colonies in Guatemala while coffee plants were in bloom showed no difference in development of bee colonies inside or outside of the spray area (Wilson *et al.* 1997). Choice tests using Success 0.02 CB (Spinosad in SolBait) with field-caged bees in Guatemala had the same results with the bees staying away from the bait (Rendon *et al.* 2000). Honey bees were not deterred by phloxine B- sucrose-water and fed *ad libitum* but were deterred by SolBait. The data strongly suggests that honey bees would not feed on SolBait if it were available to them as long as they had a choice of nectar unless there were no flowers or other source of nectar. In

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addition, if bees were to ingest SolBait, it would have to be while in the liquid state. Once SolBait dries, the bees are not able to forage dry matter. Also, sucrose was substituted for invertose, which the bees do not prefer, in the original SolBait formulation to induce bees to feed on SolBait; thus, making the tests more conservative.

Based on bee behavior, there would not be many dead bees found near the hive as their foraging range is up to 1.5 km. If a bee were to ingest the dye, it is possible that they would not make it back to their hive as they fly in sunlight and the dye is activated by light energy. Thus, the affected bee would be killed or disoriented and would serve no purpose in the hive. Also, bees with erratic behavior are not allowed back into their hives by guard bees. If by chance surface contaminated phloxine B-affected bees make it back to their hives and contaminate honey, the methodology is in place to test for such contamination.

As with the Mexican fruit fly, the regression line for phloxine B in honey bees fed known amounts of the dye is linear; this information can be used as a forensic approach to determine if questionable dead bees were killed by the presence of phloxine B in their tissues. Therefore, the methodology that I developed applies to two separate orders of the class Insecta, Diptera and Hymenoptera, and potentially can be used for other arthropods as well.

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CONCLUSIONS

Sodium hydroxide was needed for detection of phloxine B in the tissues of Mexican fruit fly and honey bee. The impact of low pH (< 5) on phloxine B has a negative effect on its detection. Much better detection of phloxine B is achieved when the pH is between pH 6-13; this is possible because phloxine B is relatively pH stable. Even with the addition of 2% sodium hydroxide to tissues, phloxine B remained stable up to two hours. The grinding techniques developed to process insect tissues allowed for ease of dye detection in them. The techniques were verified by feeding flies known amounts of dye and placing them outside under direct and indirect sunlight. Simulated field conditions induced dehydration in flies before processing their tissues and successfully extracting phloxine B. Honey, because of its viscosity, needs to be diluted to 10% before use with this methodology. The extraction approach developed in this study appears to have a potentially broader use as insects in two different insect orders, Diptera and Hymenoptera, had phloxine B extracted from their tissues using the same methodology. This methodology was developed as a tool for a forensic approach to answer legal questions as to whether phloxine B used in field sprays against flies, also killed bees or contaminated honey.

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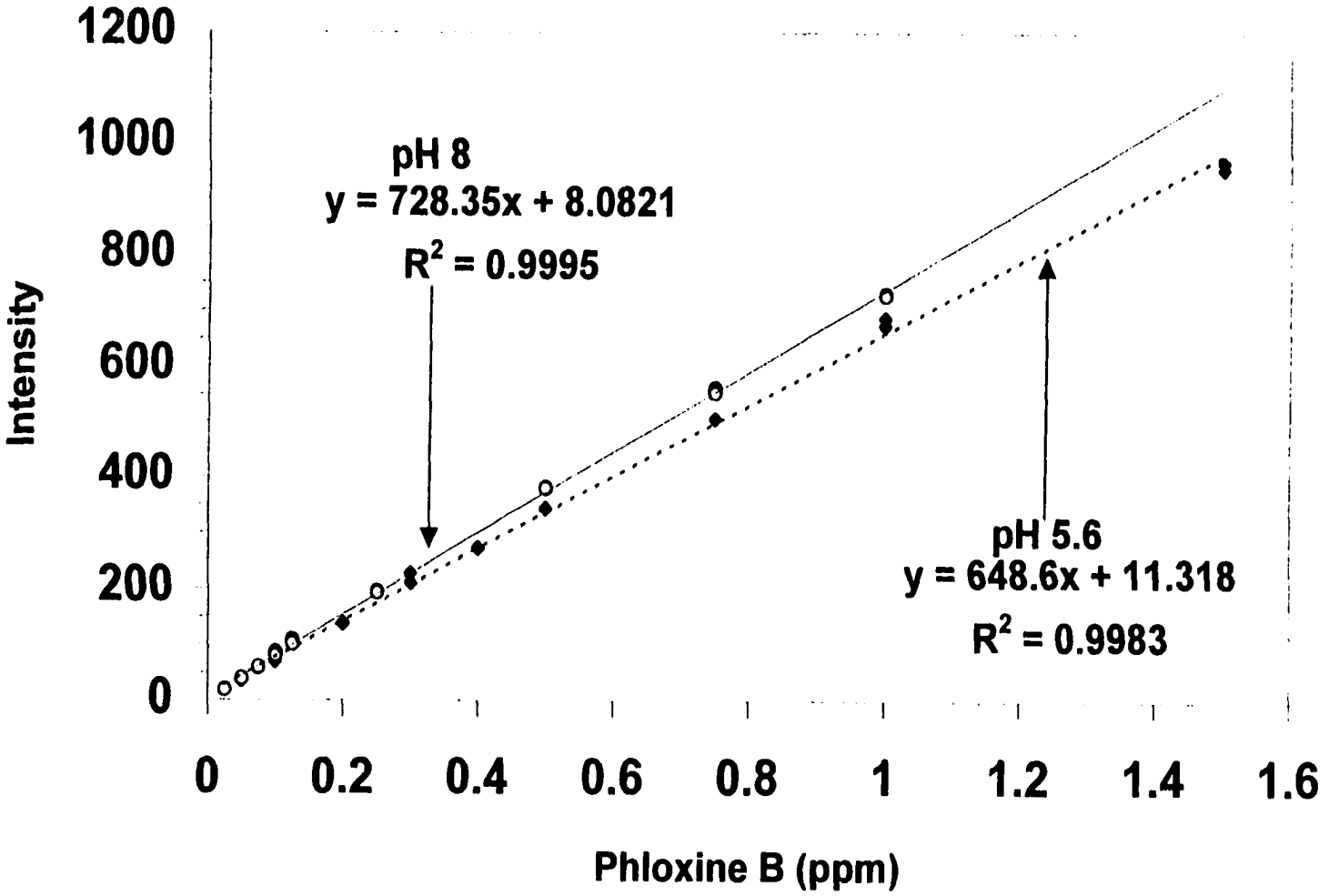

Fig. 2. Detection of phloxine B in an aqueous solution at two pH levels.

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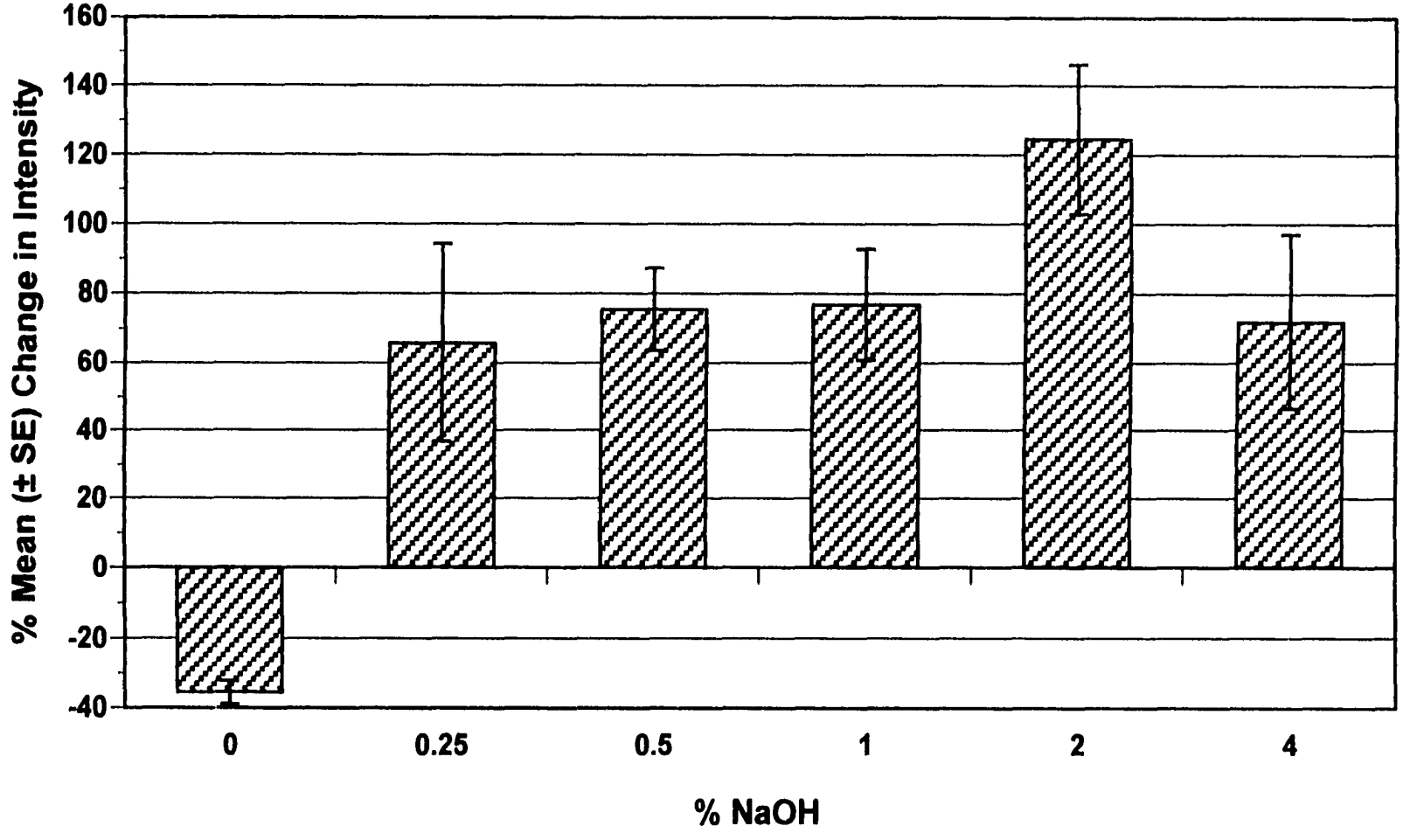

Fig. 3. Percent change in phloxine B intensity from 1st to 2nd extraction of Mexican fruit fly tissues. First extraction with Tris buffer only, 2nd extraction with Tris buffer and varying NaOH concentrations.

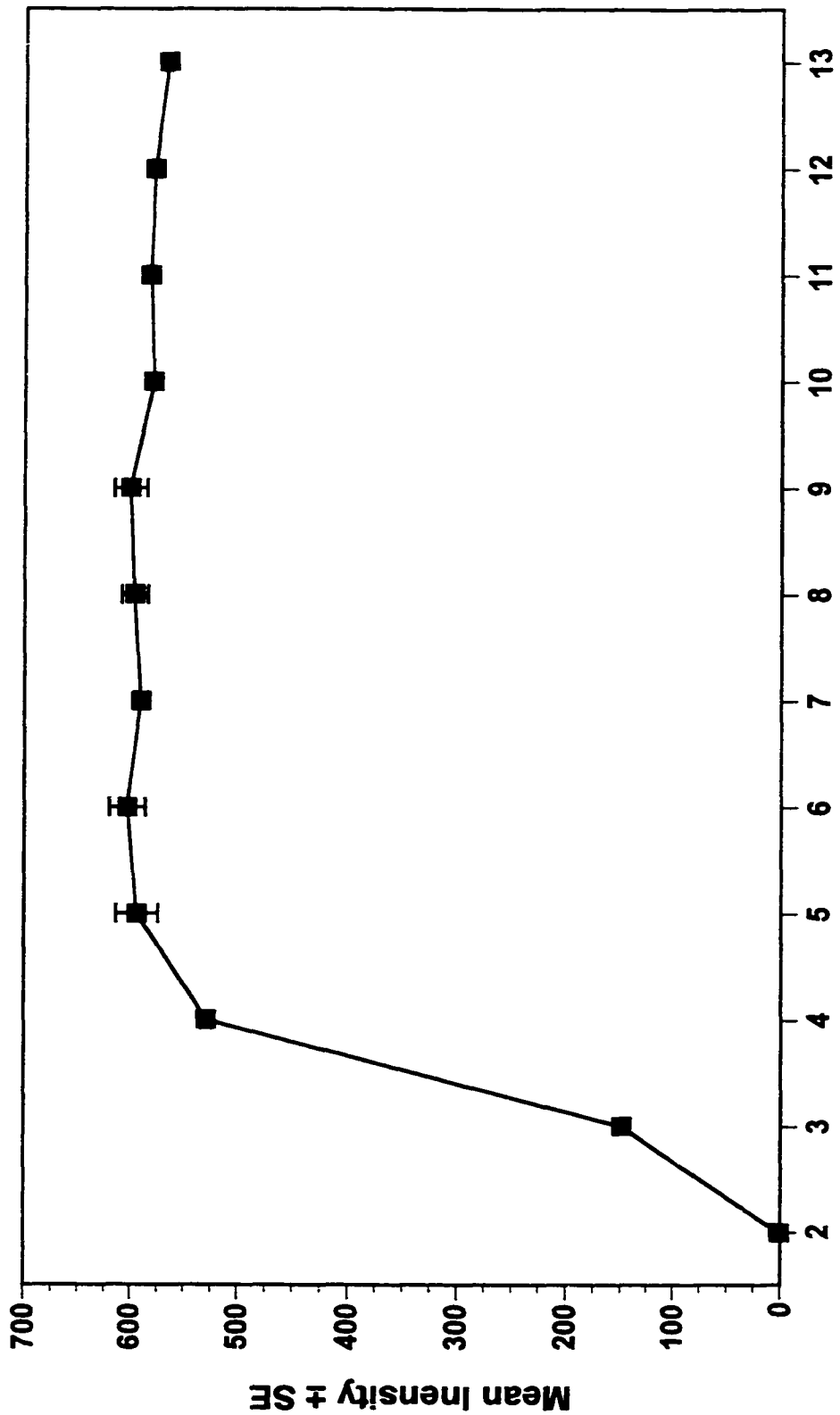

Fig. 4. Effect of pH on intensity of 0.75 ppm phloxine B in water.

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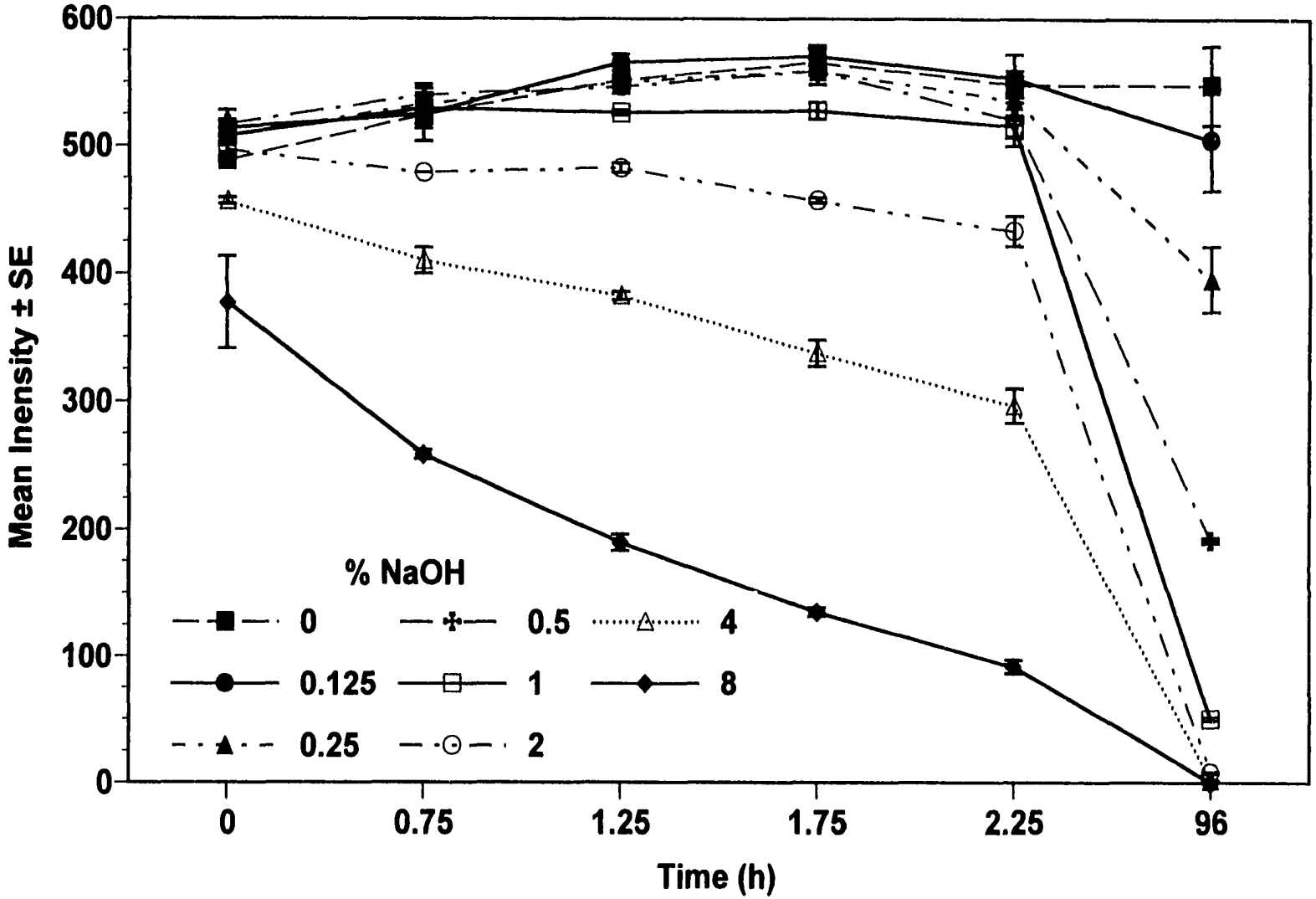

Fig. 5. Degradation of 4 ppm phloxine B over time with various concentrations of sodium hydroxide.

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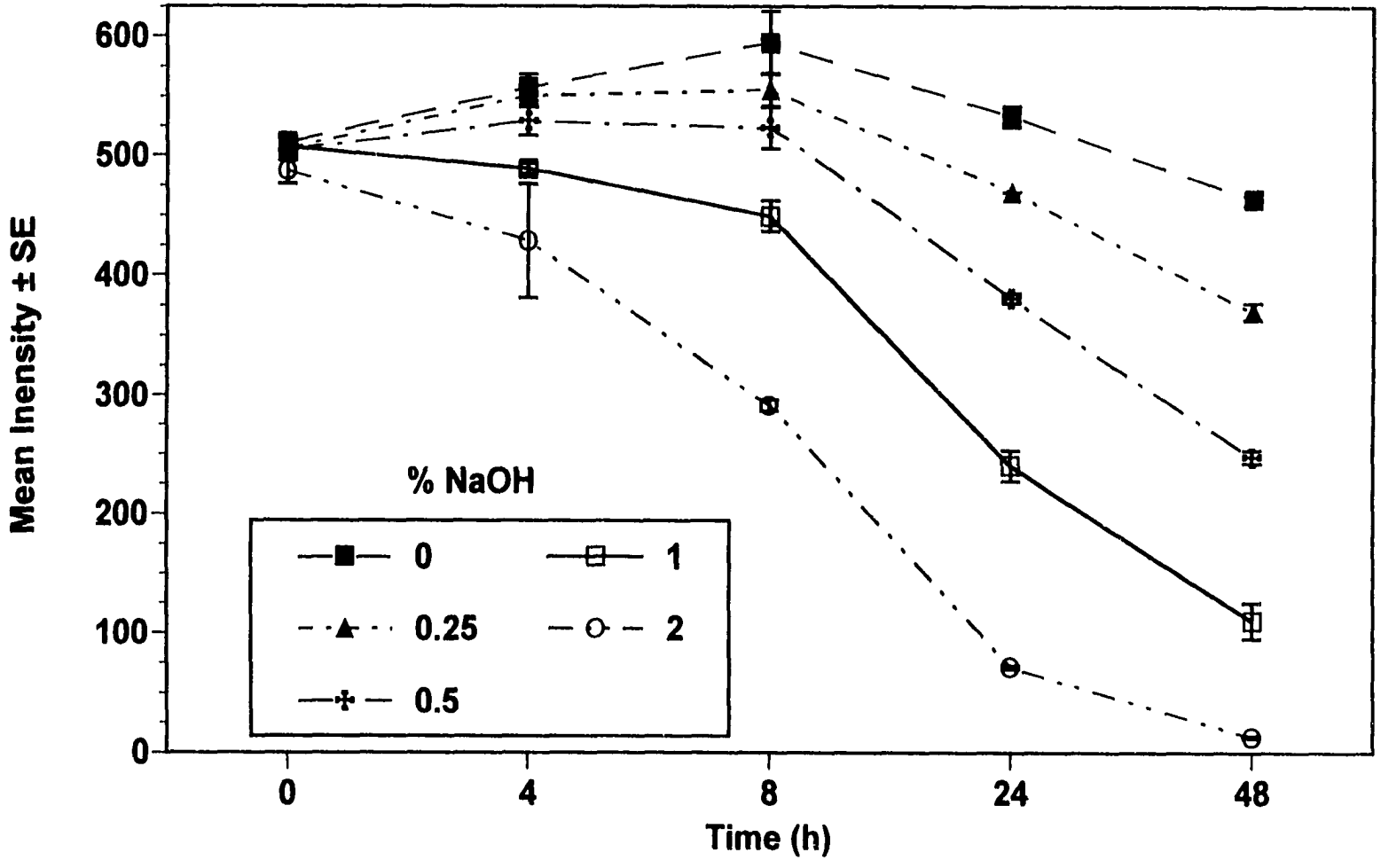

Fig. 6. Degradation of 4 ppm phloxine B over time with various concentrations of sodium hydroxide.

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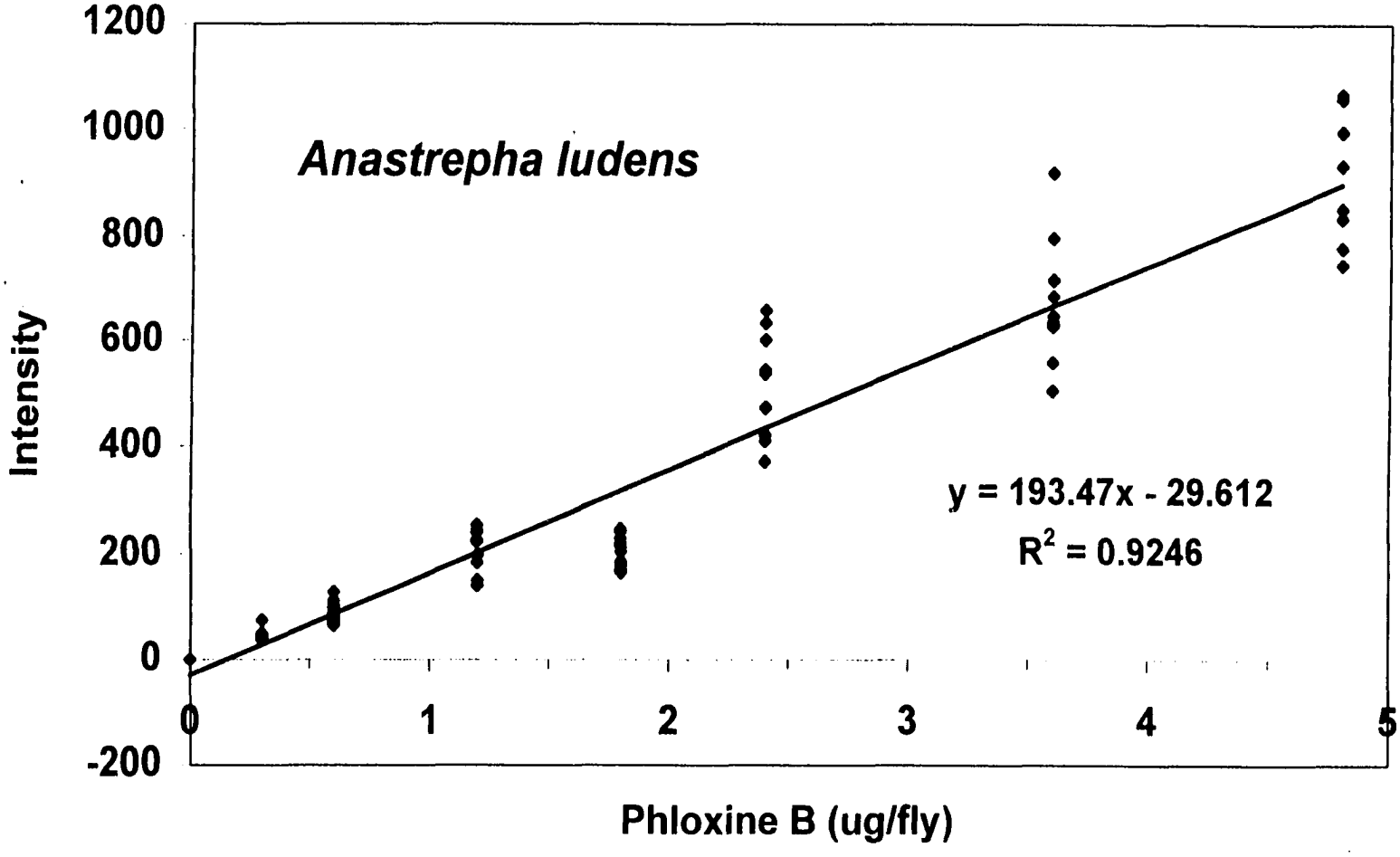

Fig. 7. Linear regression of intensity versus phloxine B concentration in Mexican fruit fly extracts.

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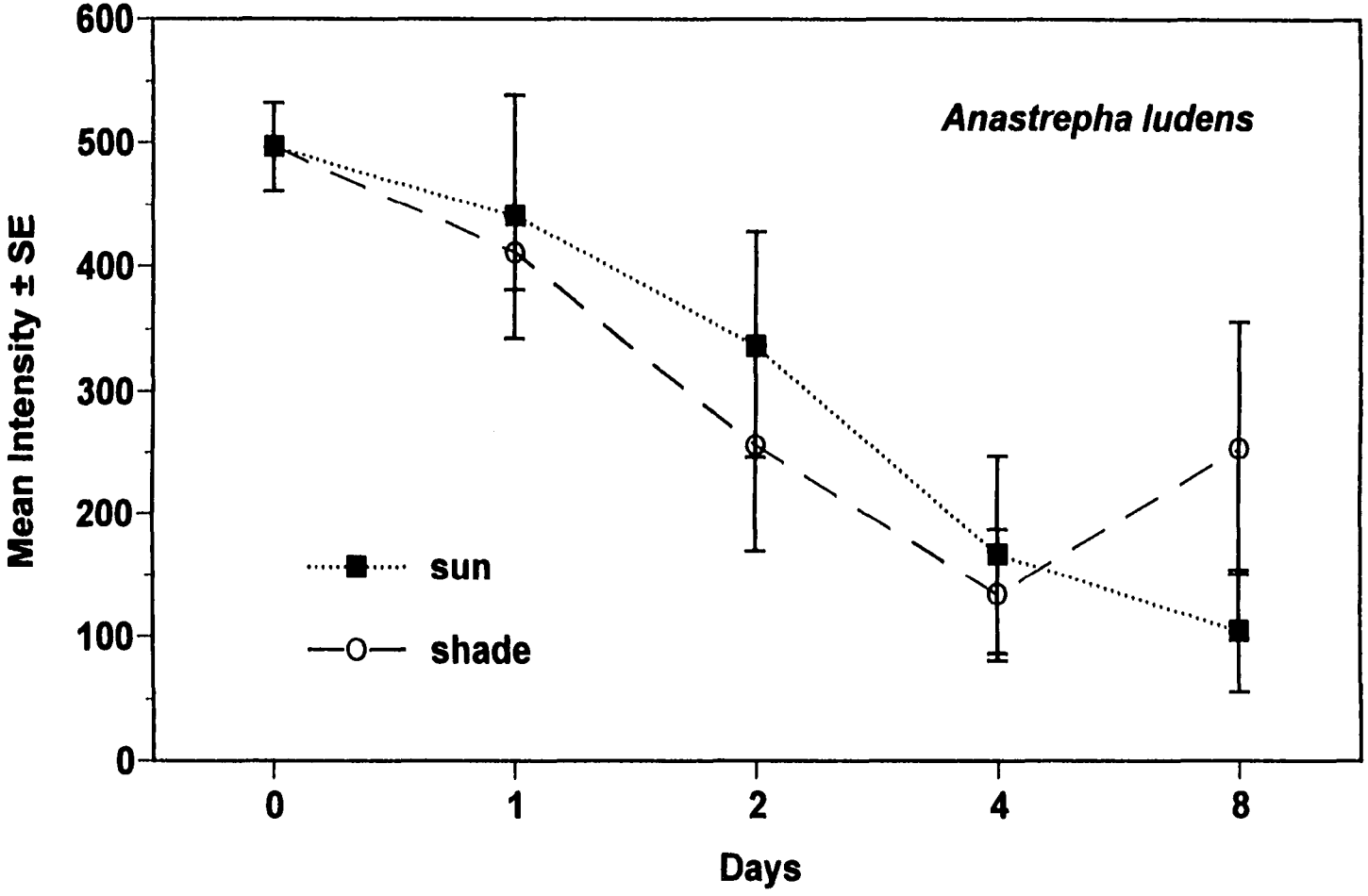

Fig. 8. Detection of phloxine B in the Mexican fruit fly under simulated field conditions.

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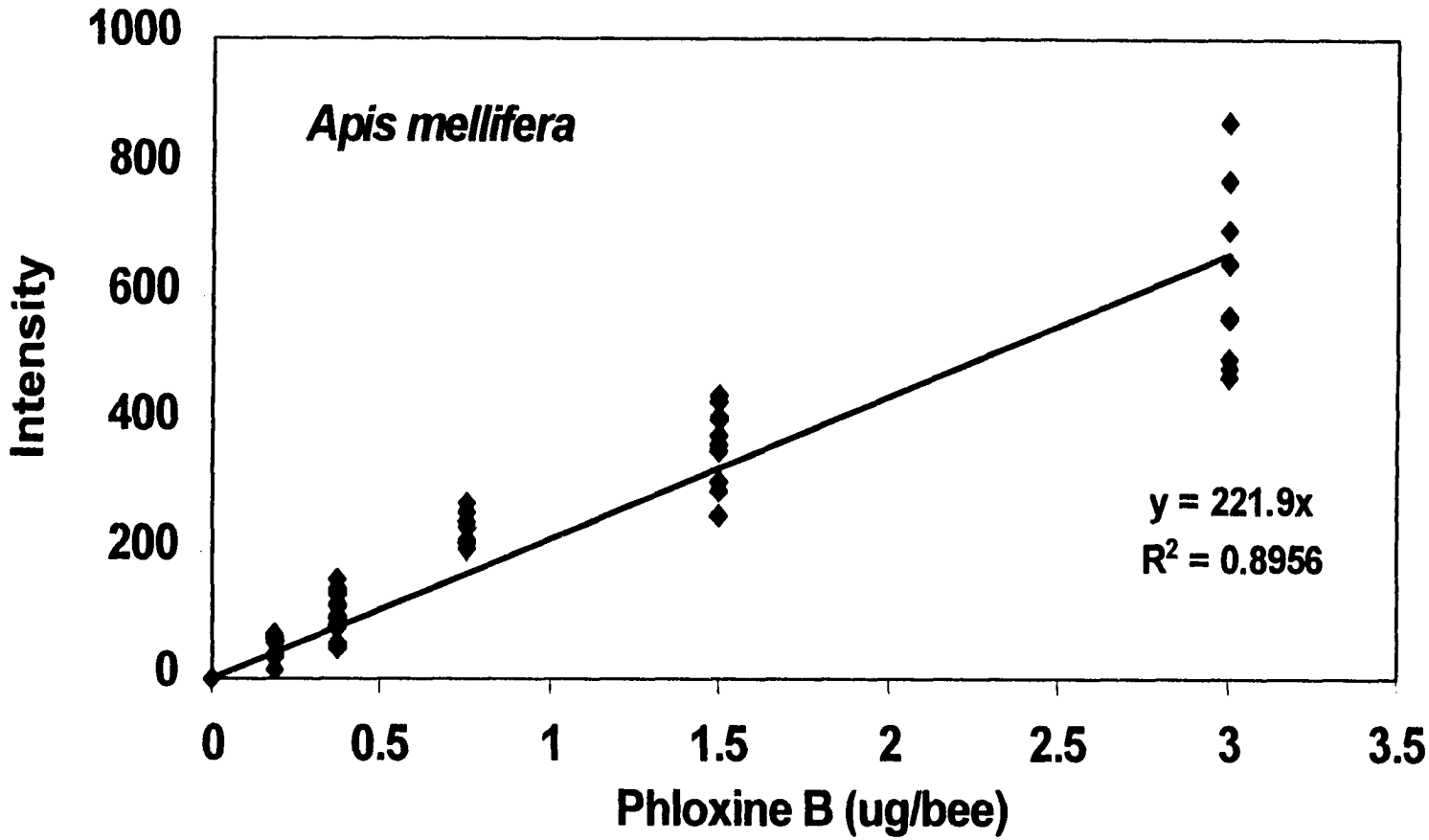

Fig. 9. Linear regression of intensity versus phloxine B concentration in honey bee extracts.

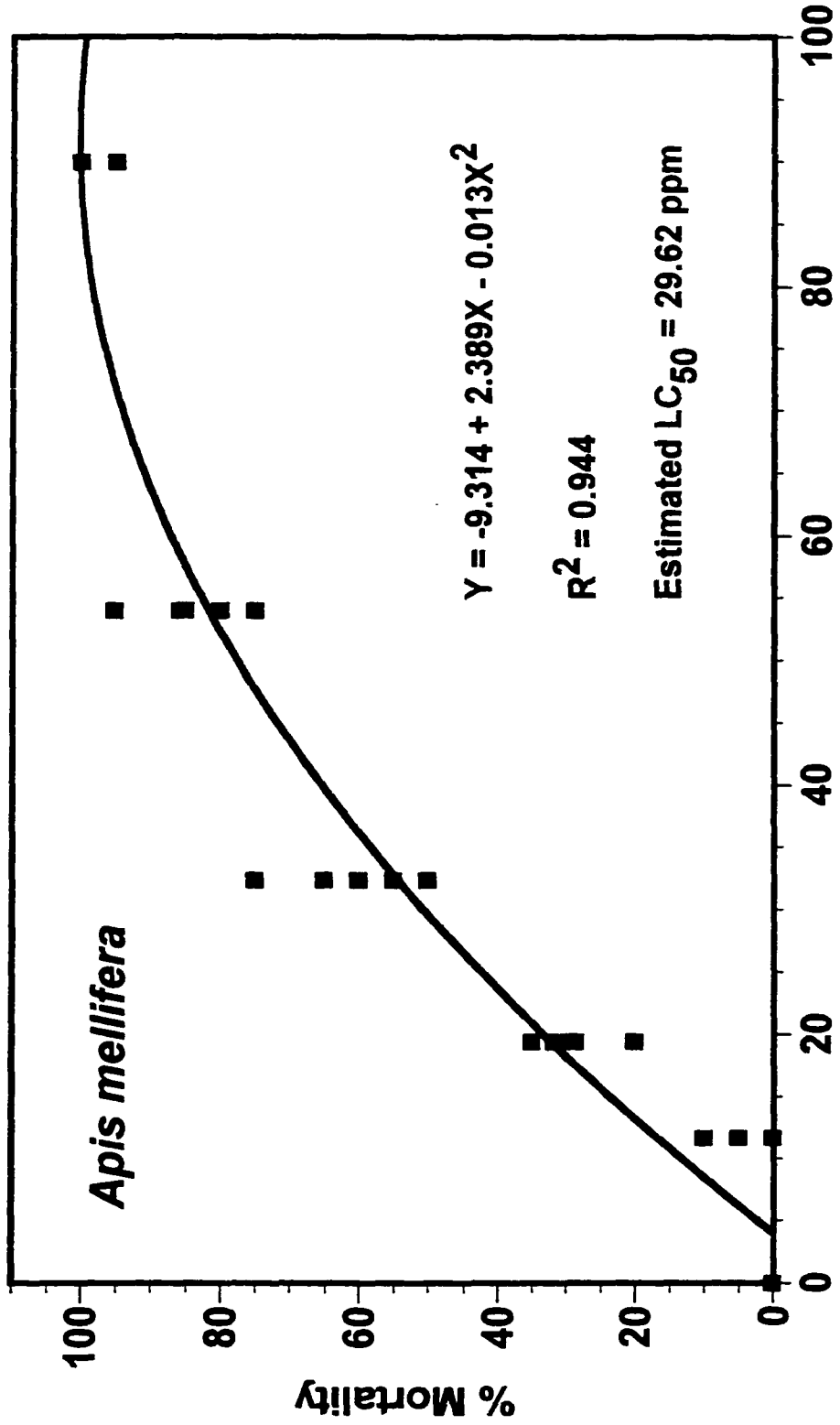

Fig. 10. Determination of LC_{50} for phloxine B in 30% sucrose in honey bees.

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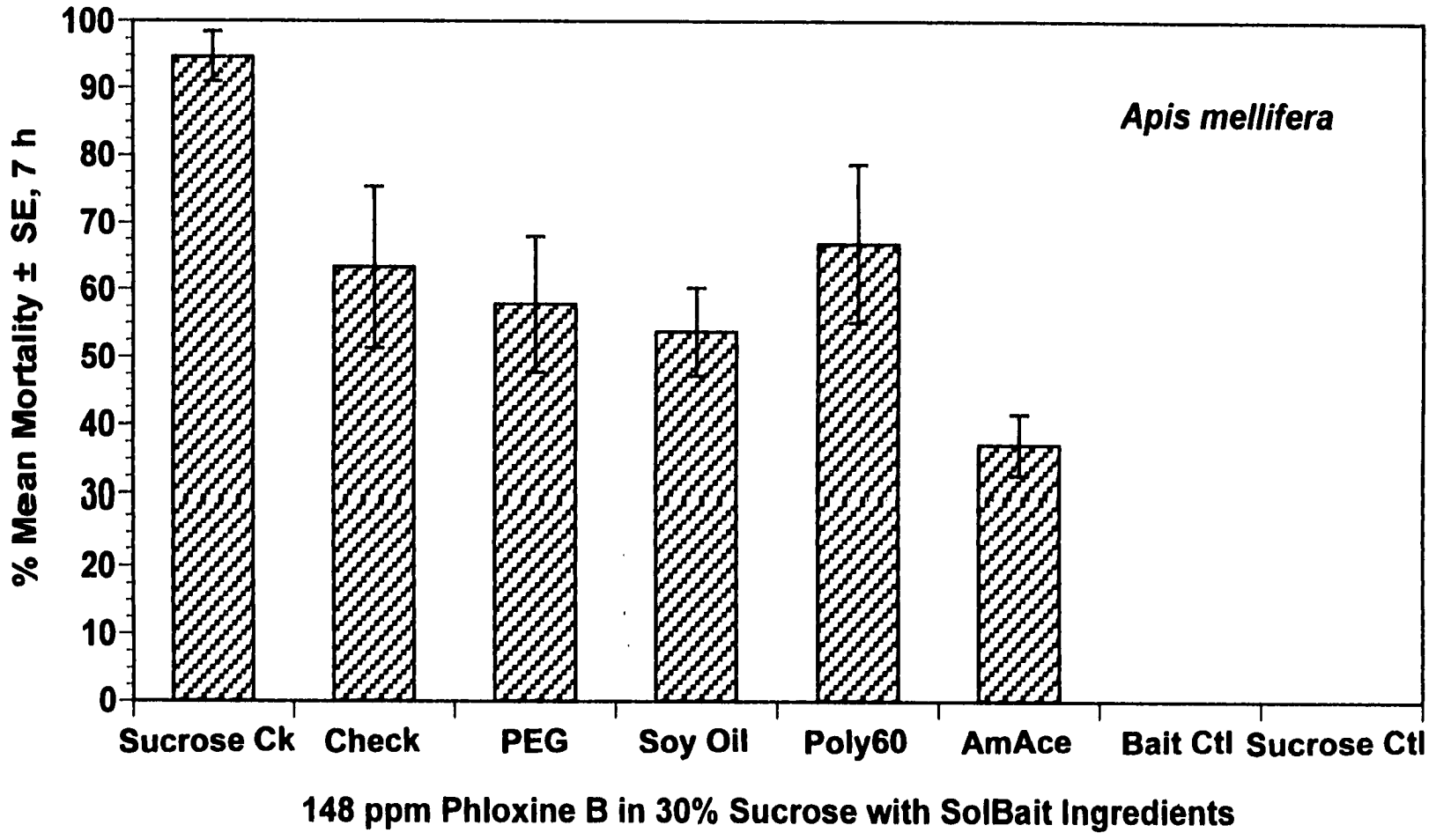

Fig. 11. Toxic effects of phloxine B in sucrose and with each of the ingredients that make up SolBait, in a no-choice test. Abbreviations: Ck = check; PEG = polyethylene glycol 200; Soy Oil = soybean oil; Poly60 = polysorbate 60; AmAce = ammonium acetate; Bait Ctl = SolBait w/out dye; Sucrose Ctl = 30% sucrose.

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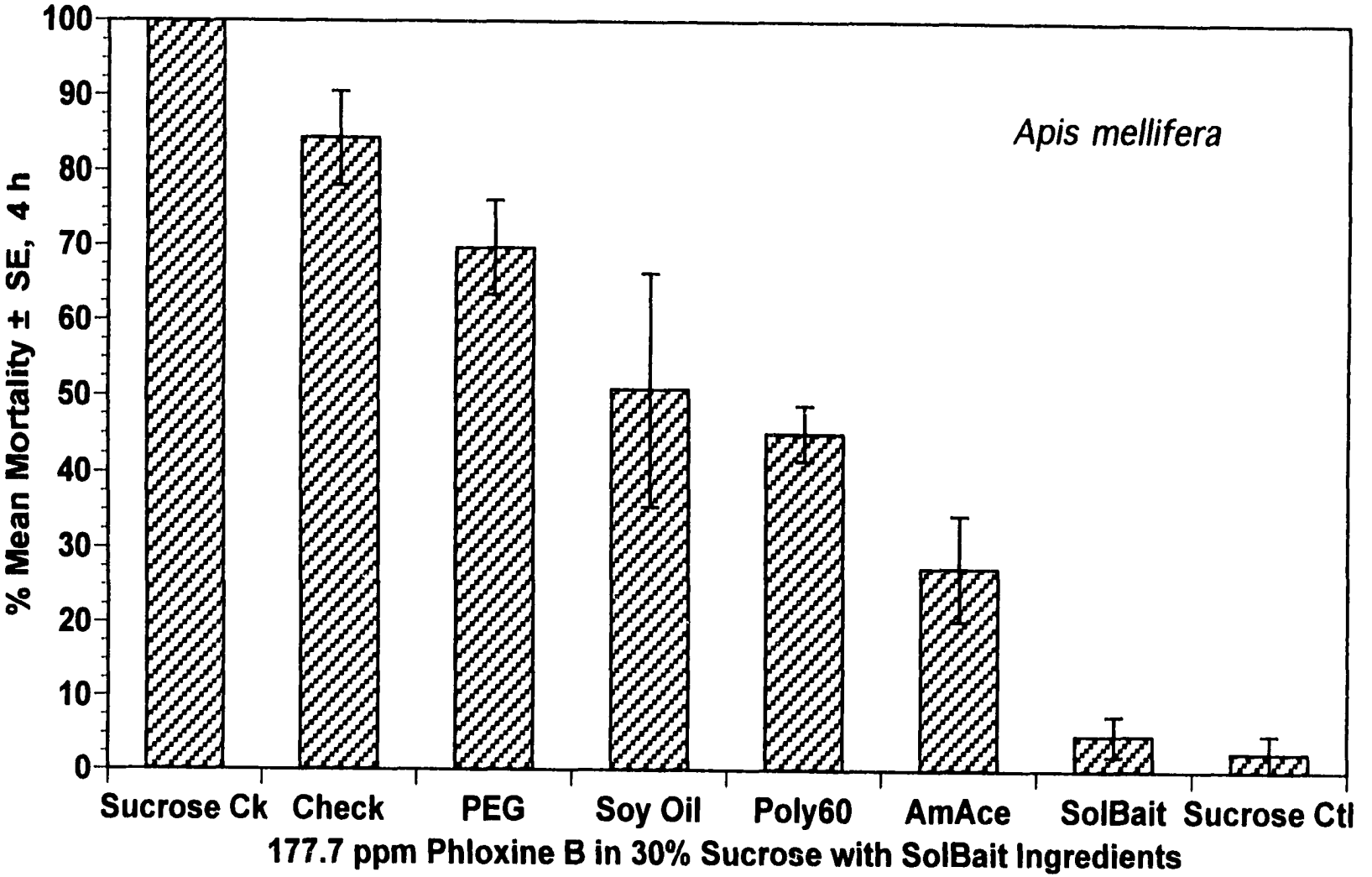

Fig. 12. Toxic effects of phloxine B in sucrose and with each of the ingredients that make up SoBait, in a choice test. Abbreviations: Ck = check; PEG = polyethylene glycol 200; Soy Oil = soybean oil; Poly60 = polysorbate 60; AmAce = ammonium acetate; Sucrose Ctl = 30% sucrose.

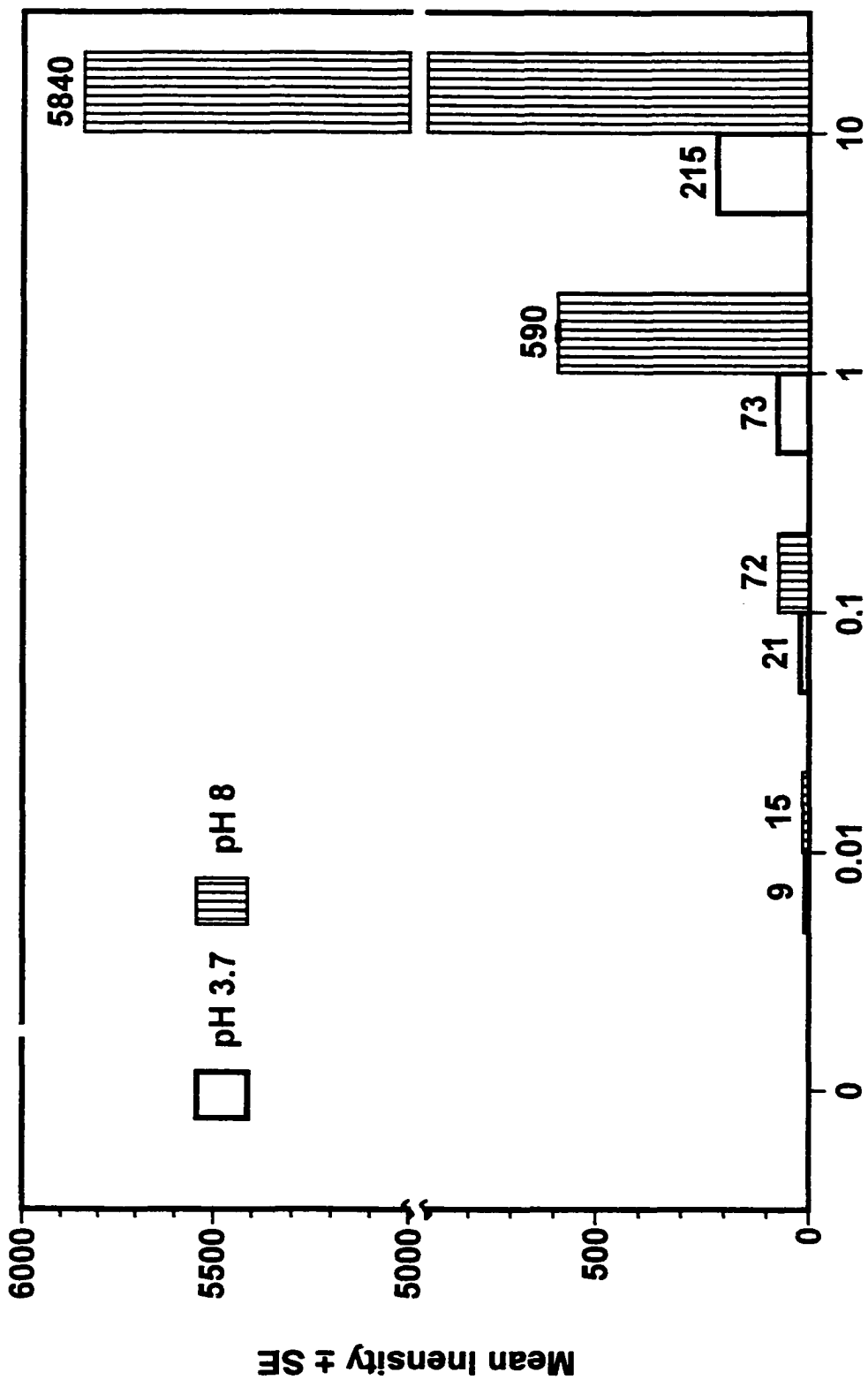

Fig. 13. Detection of phloxine B at two pH levels in a 10% aqueous honey solution.

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VITA

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