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Native Fluorescence from Juvenile Stages of Common Food Storage Insects

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Insect infestation of stored foods has significant economic and health consequences; the development of novel methods of detection thus presents considerable opportunities. The fluorescence from nine species of storage insects (beetles and moths) was studied; the juvenile stages of all nine species exhibited fluorescence under long-wave (365 nm) UV light; none of the adult insects emit fluorescence, so the fluorophore(s) might be a compound(s) associated with the unsclerotized cuticle. The spectra of larval stages of *Ephestia kuehniella*, *Oryzaephilus surinamensis*, *Corcyra cephalonica*, *Tribolium castaneum*, and *Tribolium confusum* exhibited excitation maxima in the range from 345 to 350 nm and emission maxima in the range from 421 to 427 nm, suggesting that fluorescence arises from a common chromophore; similarities in fluorescence properties implicate one of the many pteridine ring-containing compounds (pterins) commonly found in insects. Larvae and even eggs were readily imaged on foods using fluorescence under 365-nm excitation. Fluorescence thus appears to be ubiquitous in immature food storage insects, and fluorescence detection may be useful as a general method to detect insects in foods and agricultural commodities during storage or processing.

KEYWORDS: Fluorescence; storage insects; eggs; larvae; pupae; cuticle; stored products

INTRODUCTION

Grain, flour, dried fruits, tree nuts, peanuts, pulses, and spices are highly susceptible to infestation by insects. These insects are more than just offensive invaders of our food system. The annual postharvest losses due to insect pests amount to ∼20% in developing nations and ∼9% (∼36 million tons) in the United States (*1*, *2*). In 1990, mold and insect pests were responsible for postharvest grain losses in the United States in excess of $500 million (*3*), whereas in 2000, insect damage to stored wheat alone cost that sector of the food industry $500 million (*4*). Postharvest losses also stress water and energy resources. Insects contaminate food with excrement and other waste products, thus reducing the nutritional value as well as the market price (*5*).

The economic impact is not limited to the food destroyed by pests; it also includes lost productivity and the cost of pesticides, reconditioning, and replacement. Because grains constitute a major part of the diet, the impact of postharvest insect pests on human nutrition is enormous. Insect pests are vectors of microorganisms responsible for mycotoxins, spoilage, and foodborne illness. Some storage insects are vectors of *Aspergillus flavus*, and aflatoxin levels in grain have been shown to correlate with increases in insect population and insect damage (*6*−*8*). Exuviae from developing larvae and small insect fragments are inhalant allergens. Detached urticating hairs of dermestid beetle larvae cause mechanical injuries to skin, eyes, and nasal membranes. Quinones excreted by *Tribolium castaneum* and *Tribolium confusum* impart an unpleasant odor and contaminate stored food. The excreted quinones can cause conjunctivitis and dermatitis and are associated with tumors in mice (*9*, *10*). Consumers are typically outraged by insects or insect fragments in their food (*11*), and some religious dietary laws prohibit the consumption of insects.

Infested product unknowingly accepted into food-processing facilities presents a major sanitation problem. Storage insects, especially during incipient phases of infestation, are difficult to detect. These insects are very small and often the same color as the food they infest. The adult beetles are usually ∼3−4 mm in length and 1 mm in width, although a few species are about half that size. The beetle larvae are ∼1−6 mm depending on the instar and species. Both the adults and larvae of moths grow to about twice the size of the beetles. The eggs of these insects are generally 500−600 μm in length and ∼350 μm wide. These pests thus often go undetected until the population explodes and the insects begin to disburse.

We studied the eggs, larvae, and pupae of nine common species of storage insects (moths and beetles) and found that all exhibited fluorescence when exposed to 365 nm UV light, suggesting that fluorescence from juvenile stages of food storage insects is common. Fluorescence excitation and emission spectra of larvae of several species were nearly identical, suggesting a common chemical origin of the fluorescence. Because larvae, which are voracious feeders, cause most insect damage, these

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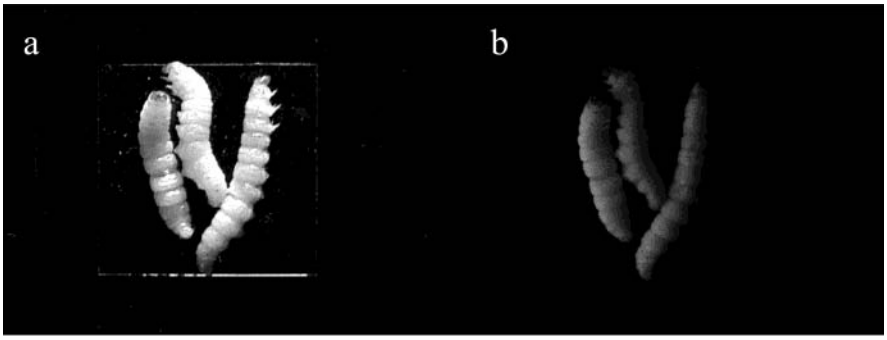

Figure 1. Last instar of *C. cephalonica* photographed under reflected room light (a) and long-wave (365 nm) excitation (b).

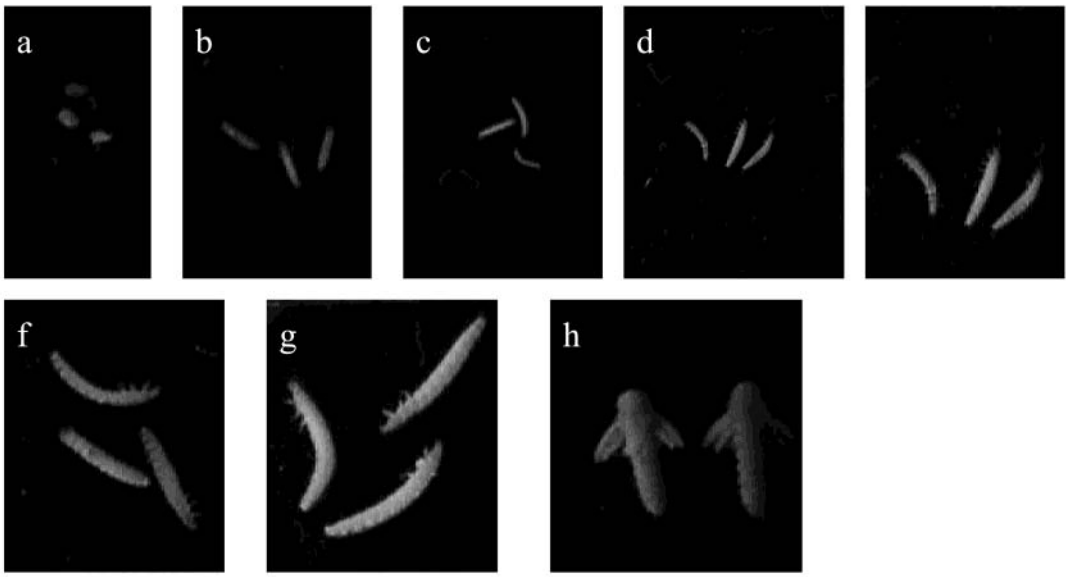

Figure 2. Fluorescence images of juvenile stages of *T. castaneum* under long-wave (365 nm) illumination: (a) eggs; (b) first instar; (c) second instar; (d) third instar; (e) fourth instar; (f) fifth instar; (g) sixth instar; (h) pupa.

results suggest that fluorescence might be used to sensitively monitor for insects in stored food or during processing.

MATERIALS AND METHODS

Insect colonies were supplied by the USDA-ARS at Kansas State University, Manhattan, KS. The colonies consisted of nine species: the red flour beetle, *Tribolium castaneum* (Herbst) and the confused four beetle, *Tribolium confusum* Duval (Coleoptera: Tenebrionidae); the saw-toothed grain beetle, *Oryzaephilus surinamensis* (L.) (Coleoptera: Sylvanidae); the cigarette beetle, *Lasioderma serricorne* (F.) (Coleoptera: Anobiidae); the lesser grain borer, *Rhyzopertha dominica* (F.) (Coleoptera: Bostrichidae); the Mediterranean flour moth, *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae); the rice moth, *Corcyra cephalonica* (Stainton) (Lepidoptera: Pyralidae); the almond moth, *Cadra cautella* (Walker) (Lepidoptera: Pyralidae); and the Indian meal moth, *Plodia interpunctella* (Hubner) (Lepidoptera; Pyralidae).

The insects were reared on diets recommended by the USDA-ARS. Each beetle colony was contained in a separate 1-pint Mason jar. Wire mesh was sandwiched between 7 cm diameter disks of filter paper and inserted in each jar closure in place of the lid. The insects were reared in a drybox that was modified for use as a growth chamber at 23−24 °C and 65% relative humidity. Eggs, larvae, and pupae were collected from the growth media by sieving; the eggs and first-instar larvae were collected using 50 and 80 mesh sieves and pans, whereas other instars and pupa stage specimens were collected with 18 and 25 mesh sieves. Larvae were separated by instar under a microscope. Specimens were preserved as follows: eggs, each larval instar, and the pupa stage insects of *T. castaneum*, *T. confusum*, *O. surinamensis*, and *L. serricorne*; eggs and last instar of *C. cautella*, *E. kuehniella*, *C. cephalonica*, and *P. interpunctella*; and eggs and first instar of *R. dominica*. Each set of specimens was placed in labeled and color-coded 2-dram vials with Pampel's solution: 95% ethanol, water, acetic acid, and formalin (15:30:4:6).

The preserved immature stages produce what appears as a white fluorescence by eye when examined under long-wave (365 nm) excitation with a hand-held UV lamp. However, when viewed through the digital camera this appears as a blue fluorescence; these images are depicted in **Figures 1** and **2**. To confirm that the camera was indeed recording blue fluorescence from the insect, and not scattered excitation light, a 380 nm long pass filter was positioned between the camera lens and specimen and above the UV light source; images collected through the long pass filter (**Figure 5**) were similar to those collected without the filter, albeit with slightly lower intensity. All color images were converted to gray scale images for publication using the Microsoft Bitmap imaging editor.

Three of each of the specimens of eggs, instars, and pupae were placed on slide covers for photographing. Larva and pupa stage insects were positioned so that when the three specimens were viewed from above, one was dorsal, another ventral, and the third lateral. Two digital photographs were taken of each slide against a black background. The specimens were first photographed under white light and then in a darkened room under 365-nm excitation.

The fluorescence spectra of last instar *T. castaneum*, *T. confusum*, *O. surinamensis*, *E. kuehniella*, and *C. cephalonica* were collected using a model F1T11i spectrofluorometer from Spex Industries (Metuchen, NJ). Larvae were packed into the well of a solid sample holder and held in place behind a quartz slide. The sample holder was oriented at an angle of 11° to the incident beam, and all slits were adjusted to 0.5 mm (1.9 nm bandwidth). Excitation spectra were collected using the measured maximum for that species (range from 420 to 430 nm); all

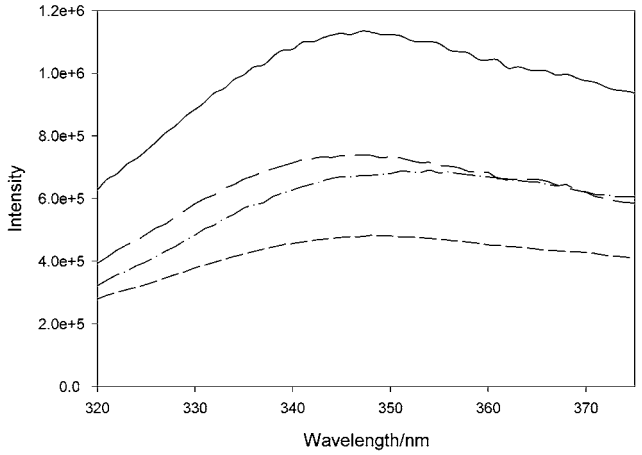

Figure 3. Excitation spectra for larvae of *E. kuehniella* (–•–), *O. surinamensis* (– – –), *C. cephalonica* (- - -), and *T. castaneun* (—).

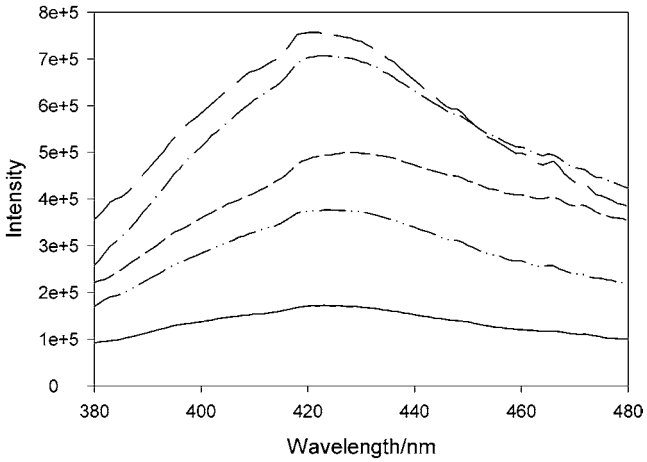

Figure 4. Emission spectra for larvae of *E. kuehniella* (–•–), *O. surinamensis* (– – –), *C. cephalonica* (- - -), *T. castaneum* (—), and *T. confusum* (–••–).

emission spectra were collected using excitation at 350 nm. All emission and excitation scans were collected from front face.

RESULTS

Heavily sclerotized adult insects of all species exhibited no fluorescence detectable by eye when examined under a hand-held long-wave UV lamp using 365-nm excitation, nor was fluorescence detectable with the spectrometer. The eggs, larval instars, and pupa stage specimens of all species examined, however, were visibly fluorescent under long-wave (365 nm) UV excitation. Comparison of images collected using reflected room light (**Figure 1a**) and emitted fluorescence (**Figure 1b**) of the last instar of *C. cephalonica* illustrates that the fluorescence was fairly uniform over the entire the surface of the insects; this was the case for every juvenile insect studied. Fluorescence images of all juvenile stages (egg, six instars, and pupa) of *T. castaneum* (**Figure 2**) clearly illustrate that surface fluorescence remains relatively intense during all juvenile stages; the fluorescence intensity may vary with developmental stage, however. Fluorescence images of the other seven insects examined were essentially identical to the illustrations in **Figures 1** and **2** (data not shown). Live insects illuminated by long-wave UV light also exhibited bright fluorescence that was indistinguishable by eye from that of insects treated with Pampel's solution; it was not possible to collect emission spectra of live insects due to their continuous movement during data collection.

Excitation and emission spectra were collected from larvae of three species of beetles (*E. kuehniella*, *O. surinamensis*, and *C. cephalonica*) and two species of moths (*T. castaneum* and *T. confusum*). Spectral overlays clearly indicate the similarities of both the excitation (**Figure 3**) and the emission (**Figure 4**) spectra in all species, suggesting a common chemical origin for the fluorescence. The excitation maxima for the larvae were in the range from 346 to 354 nm, whereas emission maxima ranged from 421 to 427 nm (**Table 1**).

The utility of using fluorescence to enhance the detection of insect larvae and eggs in foods was investigated by examining infested samples under full-spectrum room light and long-wave UV (365 nm) excitation. Larvae of the rice moth (*C. cephalonica*) were barely distinguishable on brown rice when imaged using reflected room light (**Figure 5a**) but easily detected when imaged through a 380-nm long pass filter using fluorescence under UV excitation (**Figure 5b**). UV illumination combined with fluorescence detection also enhanced the ability to distinguish larvae of the Indian meal moth (*P. interpunctella*) on cocoa beans (**Figure 6a**,**b**), the saw-toothed grain beetle (*O. surinamensis*) on raisins (**Figure 6c**,**d**), and the cigarette beetle (*L. serricorne*) on dried chili peppers (**Figure 6e**,**f**). Even the relatively small (~0.2 mm diameter) eggs of the almond moth (*C. cautella*) were easily imaged on cocoa beans under UV excitation (**Figure 6g**,**h**). In each of these cases the insect is a major pest of the product depicted. In addition to the examples shown here, larvae of saw-toothed grain beetles were readily detected by fluorescence on dates, brown rice, and apricots (data not shown). Autofluorescence may limit the utility of this technique to detect insect infestations in some foods. Blue autofluorescence, for example, severely interfered with the detection of larval *T. confusum*, a major pest, on wheat flour and larval *T. castaneum* on cashew kernels and basmati rice; similar interference occurred with the Mediterranean meal moth (*E. kuehniella*) and Indian meal moth larvae in wheat flour and almond moth eggs on cashew kernels (data not shown).

Table 1. Excitation and Emission Maxima for Several Species of Insect Larvae[a]

species	excitation/nm	emission/nm
Ephestia kuehniella	354	423
Oryzaephilus surinamensis	346	421
Corcyra cephalonica	349	427
Tribolium castaneum	347	423
Tribolium confusum	–	424

[a] Estimated error of each maximum is ±2 nm.

DISCUSSION

These results indicate that fluorescence is ubiquitous in the juvenile egg, larva, and pupa stages and undetectable in adult food storage insects; it thus appears to originate from unsclerotized cuticle. The insect cuticle is a structure of chitin filaments embedded in a protein matrix arranged in a helicoidal lamella. The process of sclerotization in the adult insect creates a hard, protective skin that is dark brown or amber in color; it is formed by a sequence of reactions within the protein matrix that generate both protein cross-links and light-absorbing melanin pigments derived from tyrosine (*12*, *13*). The inability to detect fluorescence from adult insects may reflect either a loss of the relevant chromophores during sclerotization or the masking of these chromophores by the high concentrations of strongly absorbing melanin pigments.

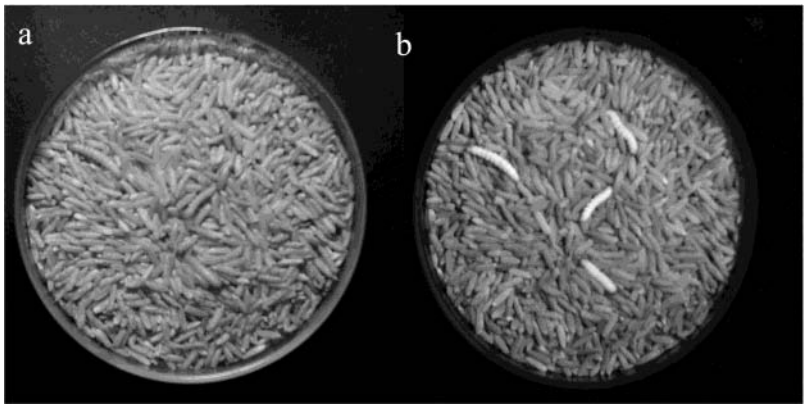

Figure 5. Larvae of the rice moth (*C. cephalonica*) on brown rice under reflected room light (a) and 365-nm UV excitation detected through a long pass filter (b).

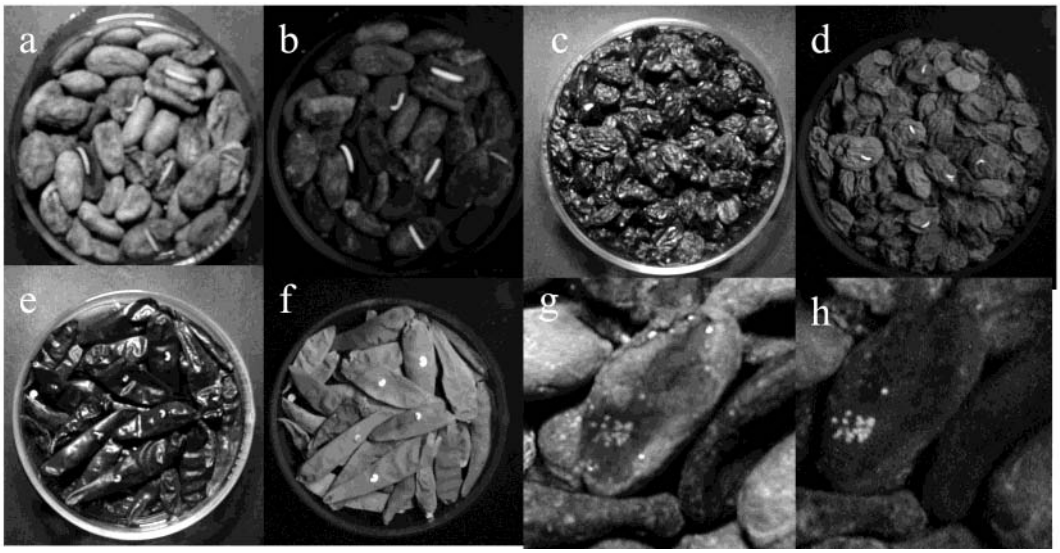

Figure 6. Larvae of the Indian meal moth (*P. interpunctella*) on cocoa beans under reflected room light (a) and 365-nm excitation detected through a long pass filter (b); of the saw-toothed grain beetle (*O. surinamensis*) on raisins under reflected room light (c) and 365-nm excitation detected through a long pass filter (d); of the cigarette beetle (*L. serricorne*) on dried chili peppers under reflected room light (e) and 365-nm excitation detected through a long pass filter (f); and of eggs (~0.2 mm diameter) of the almond moth (*C. cautella*) under reflected room light (g) and 365-nm excitation through a long pass filter (h).

Excitation and emission maxima fell within narrow ranges (346−354 and 421−427 nm, respectively), suggesting that fluorescence originated from a common chromophore in all insect species studied. There are several UV-absorbing chromophores or classes of chromophore that are potential sources of this fluorescence: the purine uric acid, indoles and their metabolites, tyrosine oligomers, and pterins, a diverse class of pteridine ring-containing compounds that are common in insects (*14*−*16*). The visible pigments (*16*, *17*) are unlikely candidates in these unpigmented insects.

Fluorescence from the purine uric acid (8-hydroxyxanthine) has been detected in both larvae and adults of species of *Dysdercus* butterflies (*18*) and in *Triatoma infestans* and *Panstrongylus megistus* (*19*). With excitation and emission maxima of approximately 290 and 360 nm (*20*), however, this chromophore cannot be the source of the measured fluorescence.

The fluorescence of scorpion has been associated with a compound derived from tryptophan involved in the cross-linking of sclerotized cuticle (*21*). Fluorescence from indole derivatives has also been identified in the fruitfly *D. melanogaster* (*22*) and in *Calliphora erythrocephala* (*23*). Fluorescence from kynurenine, a tryptophan metabolite, was identified in extracts of *Triatoma infestans* (*24*) and later located by microspectrofluorometery in the malpighian tubules of *T. infestans* and *Panstrongylus megistus* (*19*). Indole derivatives typically have excitation maxima of 280−300 nm and emission maxima near 350 nm, however (*25*), eliminating them as candidates. Kynurenine has a weakly absorbing ($\epsilon \approx 4500\ M^{-1}\ cm^{-1}$) band with a maximum at 360 nm and an emission maximum that varies from 435 to 460 nm depending upon solvent polarity (*26*); significant differences in both excitation and emission maxima from that observed here also make this chromophore an unlikely candidate.

Fluorescence from dityrosine and trityrosine has been observed in compounds found in the protein resilin from the desert locust *Schistocerca gregaria* (*27*, *28*). Fluorescence from the cuticle and hemolymph of the greater wax moth, *Galleria mellonella*, has been attributed to tyrosine oligomers (*29*), as has fluorescence from the cuticle of *Pennella elegans* (*30*). Dityrosine is also involved in cross-linking protein in the chorion of insect egg. Dityrosine has excitation at 320 nm and emission at 410 nm (*31*), both significantly different from the fluorescence maxima found here, making such chromophores unlikely candidates for the species giving rise to the insect fluorescence reported here.

Pterins, by far the most widely reported class of fluorescent chromophores found in insects (*14*, *15*), are a diverse group of pteridine ring-containing compounds that include leucopterin, xanthopterin, and erythropterin among others (*32*). They are found in a wide variety of insects including the moth *Gonepteryx rhamni* (*33*), many butterflies including the genera *Dysdercus* (*18*) and *Pieris* (*34*, *35*), the locusts *Carausius morosus* (*36*) and *Locust migratoria* (*37*), the Mediterranean fruit fly *Ceratitis capita* (*38*), the housefly *Musca domestica* (*39*), *Anopheles* (*40*), *Aedes*, and *Culex* (*41*) mosquito species, and the silkworm *Bombyx mori* (*42*), among others. Although the extent and nature of the substituents on the pteridine nucleus modulate both the absorption and emission of the pterin chromophores, the maxima in aqueous solution of pterin, 6-carboxypterin, 6-formylpterin, and other substituted pteridines are typically in the range from 340 to 360 nm for absorption and in the range from 430 to 445 nm for emission (*43*, *44*). Given the extremely wide diversity of insect species, tissues, and developmental stages in which pterins are found (*15*) and the near identity of fluorescence properties to those reported here, one or more of the pterins are the most likely candidate(s) for the fluorescent species in food storage insects.

Irrespective of the chemical origin of the fluorescence, even using excitation and emission conditions (365-nm long-wave UV excitation and >380 nm emission through a long pass filter) that were not optimized for distinguishing insect fluorescence from possible autofluorescence background, fluorescence imaging was found to significantly enhance detection of insect larvae and eggs on specific foods that are common targets of these insects. These results thus clearly demonstrate the principle of using fluorescence imaging to detect juvenile stage insects in specific food products during storage or processing. Additional work is obviously necessary, however, to define the optimal range of excitation and emission conditions necessary to effectively distinguish insect pests on specific foods.

There are existing models for such a strategy for detection of insect infestations. A device has been developed for the detection of aflatoxin in peanuts involving the passage of peanuts through a fluorescence sorter (*45*). Although this method was not effective for aflatoxin control when compared to color sorting by hand (*46*), it might be effective in detecting the larger fluorescent bodies of larvae in a processing stream. Near-infrared (NIR) spectroscopy has had a varying degree of accuracy in identifying specific stored-grain insects. The unique chemical composition of lipids in the cuticle of the different species was thought to be partially responsible for successful classifications (*47*). A probe combining fluorescence and NIR detection might be useful in both detecting and identifying both species and juvenile stage, as well as in estimating the insect population. Of particular interest is a second-generation prototype of an electronic grain probe insect counter being evaluated by the U.S. Department of Agriculture. It is an improved design of an IR detector that allows insect counts to be made electronically with the information relayed to a remote database (*48*).

There are several precedents for the use of fluorescence imaging to evaluate food quality. For example, fluorescence imaging under UV excitation has been used to evaluate the freshness of brown rice (*49*), postharvest imaging of chlorophyll fluorescence has been used to predict the quality of lemons (*50*), multispectral fluorescence imaging has been used to distinguish maize, pea, soybean, and wheat kernels (*51*), and Wold and colleagues have used fluorescence imaging to assess the extent of lipid oxidation in dairy products (*52*) and in chicken meat (*53*), to detect nematode parasites in cod fillets (*54*), and to detect intramuscular fat content in beef (*55*). In a recent review, Munck, a leader in the application of fluorescence analysis to foods (*56*), and Norgaard state with regard to the application of fluorescence imaging to food analysis that "there has never been a greater gap between the technical possibilities and their exploitation in practice" (*57*).

The economic impact of storage insects, combined with nutritional, sanitation, and quality concerns, motivates further investigation into the use of fluorescence imaging to detect these pests in food during storage and processing.

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