Development of immunoassays for quantitative detection of insects in stored products

G. B. Kitto*, F.A. Quinn* and W.E. Burkholder†

Abstract

Immunoassay procedures for the detection of insects and insect remains have been developed for several types of stored products. These tests, which are based on enzyme-linked immunosorbent assay (ELISA) procedures, couple excellent reproducibility with ease of use. One such assay is for use with whole grain and milled grain products. The test detects all major grain insect pests and gives a positive and linear response to larval and pupal life stages and adult insects. The assay is applicable to a wide variety of types of grain and milled grain products, including wheat, rice, barley, oats, and maize. A similar type of assay procedure has also been developed for the analysis of insect contamination of a broad variety of spices. Additional ELISA tests have been developed which are species specific. These provide information about the relative degrees of infestation by insect species that are either beneficial or harmful. Such species-specific assays are available for the granary weevil, *Sitophilus granarius*, and the kaphra beetle *Trogoderma granarium*, and for *Laelius pedatus*, *Bracon hebetor*, *Trichogramma pretiosum* and *Xylocoris flavipes*, which are used for biological control.

Introduction

The amount of insect contamination present in a stored-product sample provides an indication not just of the amount of contamination present at the time of testing, but also gives an indication of the quality of sanitation to which the material has been subjected in past storage and handling. Such testing additionally serves as a guide to the amount of insect infestation likely to be encountered in future storage and transport. For these reasons it is important to have test procedures which can reliably and quantitatively estimate the amount of insect material present in stored food product materials. It would be particularly convenient to have a test that could be applied across a very broad range of stored-food products and which was capable of detecting all the major stored-product insect species. It would also be desirable to have a single test which could be applied to both processed and unprocessed food products. Unfortunately, the array of tests presently available for detecting insect contamination are far from meeting these requirements. Most of these tests involve visual inspection elements, are highly variable and quite subjective. For example, the visual examination for live insects in whole grain for export provides no direct information about the degree of internal infestation. Yet, if present, these internally developing insects are likely to cause very serious problems during transportation and storage. Similarly, the assessment of insect damaged kernels only provides an indication that insects were present at some time in the past and again gives no reliable indication of the number of internally developing insects (Russell 1988).

With processed food products, such as flour, the primary test for insect contamination is the insect fragment count (U.S. Food and Drug Administration 1988). This assay too, is fraught with difficulties, since it evaluates only the number of insect fragments present without regard to their size. Thus, it is only an indirect measure of the mass of insect material present. Moreover, the test itself is highly variable (Kurtz 1965) and is highly dependent upon the type and life stage of the insects present in the food before processing. It has been demonstrated that the dead insects yield many more fragments than do live ones, and that dead adult insects can contribute as much as 50 times as many fragments as a dead larvae (Sachdeva 1978). While the insect fragment test has been a long-term standard for food analysis, it is worth noting that the test requires lengthy technician training, is time consuming and relatively costly.

It was against this background that we set out to develop modern biochemical assays, based on the immunological recognition of insect material in stored products. The aim was to develop assay procedures that combine a high degree of accuracy and repeatability with the requisite degree of sensitivity. Another goal was to devise tests that could be used with minimal training and at a variety of stored-food product handling and storage sites and which would be of low cost. One general type of procedure which has the potential of attaining these goals is the enzyme-linked immunosorbent assay (ELISA). This type of assay is used extensively in clinical diagnostics and is seeing increasing use in agriculture fields, such as the testing for pesticides (Mei et al. 1991) and aflatoxins (Vanderlaan et al. 1988).

One common feature required of all tests for insect contamination of stored-food products is that they be capable of detecting minute quantities of insect material in the presence of very large amounts of background material. The high selectivity of immunological assays makes them well-suited to this purpose. An overview of the immunoassay procedures that we have developed for insects in stored food products is shown in Figure 1. A measured sample of the foodstuff is briefly blended with an extraction fluid, in a common household blender, to solubilise any insect material present. An aliquot of this sample is then used in the ELISA test. Typically this test is carried out in plastic multi-well strips or plates. After the sequential addition of several reagents to the wells, colour develops in proportion to the amount of insect material present in the sample. The amount of colour in the wells is measured in a colorimetric ELISA reader and the data can either be printed out directly or stored for further analysis and manipulation in a computer database.

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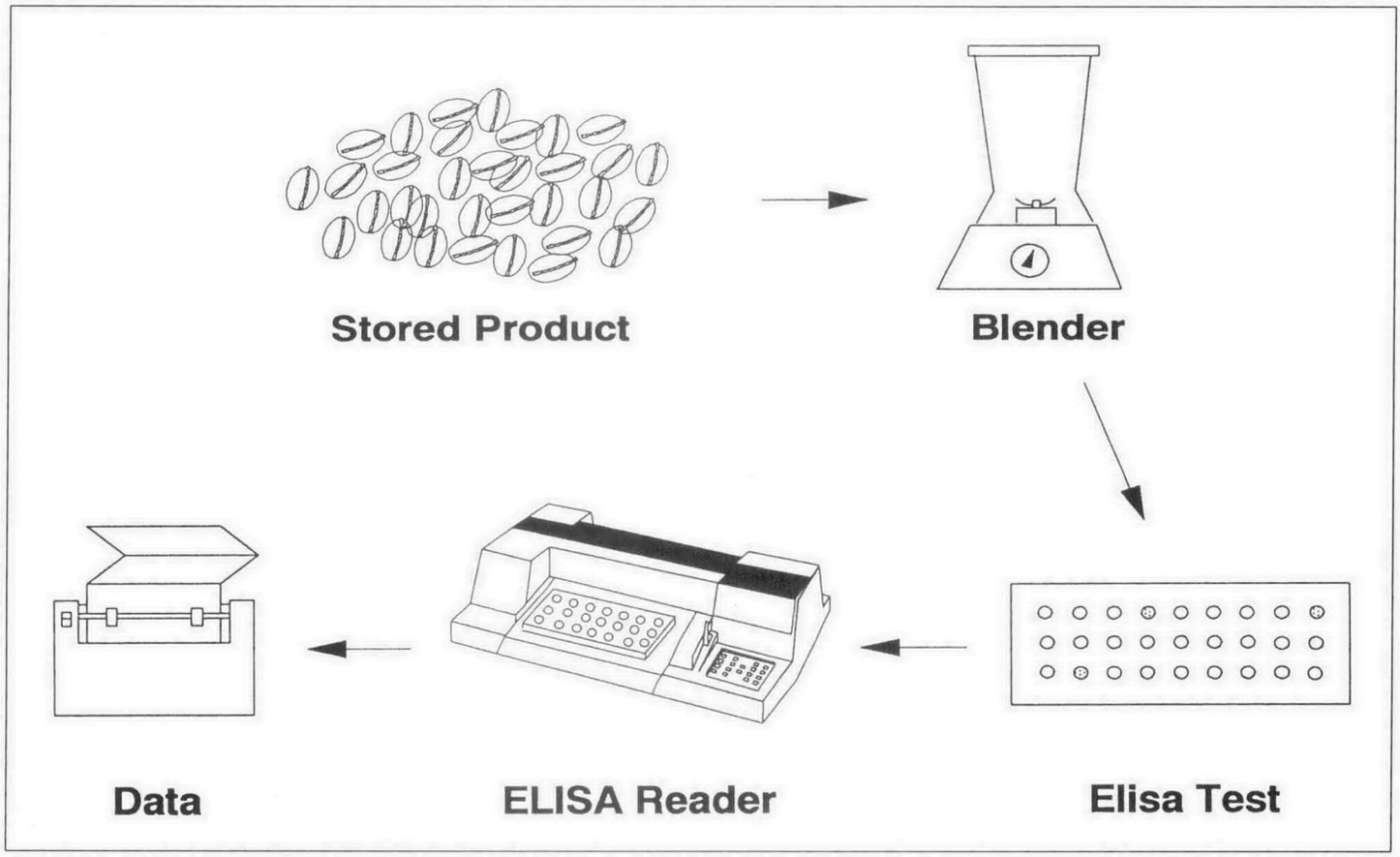

Fig. 1. An overview of the immunoassay procedure for the detection of insects, or insect remains, in stored products.

Development of Immunoassays for Insects in Whole and Milled Grain

For detecting grain insects by immunoassays, a suitable insect material to detect (antigen) had to be selected. Such an antigen would ideally be present in all of the common grain insects in substantial quantities and in all the major life stages, be easy to extract from whole grain and processed grain samples and differ little from insect to insect. The insect muscle protein myosin was chosen for immunoassay development since it meets the above criteria. This protein is present in all insect life stages except eggs, differs little in structure between all the common grain insect pests and is readily extracted using high salt solutions.

The general features of the ELISA assay that we developed using myosin as the test material are shown in Figure 2. Briefly, the walls of the wells of a plastic microtiter plate are precoated with rabbit polyclonal antibodies. When an extract of the grain or flour is added, any insect material present is selectively bound to the antibody coating the wells. After extraneous plant material is washed away, a second antibody, which is conjugated to an enzyme, is added to the wells. This second antibody also binds selectively to any insect myosin material present. After rinsing, substrate for the bound enzyme is added and colour subsequently develops in proportion to the amount of myosin present. The amount of myosin detected correlates well to the mass of insect material present. The mass of insect material present in turn correlates well to the number of insects present. A typical assay using this procedure is shown in Figure 3. In this case, samples of clean grain were spiked with the indicated number of granary weevils. The data indicate not only the linearity of the assay, in this case showing a coefficient of variation $r = 0.97$, but also the sensitivity of the assay which is readily capable of detecting as little as one granary weevil per 50 g sample of grain. The myosin ELISA assay has been shown to give positive linear results with all of the grain insect pests listed in Table 1, which represents a majority of the insects found in stored-grain products. The assay has been tested with all of the major wheat varieties as well as with barley, oats, rice, maize, soybeans, and sorghum and has been found to give a linear response to insect contamination in all cases (Kitto 1991; Quinn et al. 1992). Some slight variations in extraction procedures are required for specific types of grain. The assay has been shown to be highly reproducible, as indicated in Figure 4 which shows multiple replicate samples tested on a single day. In this case, the average reading was 0.975 with a standard derivation of 0.02.

The high degree of reproducibility of the myosin immunoassay was also established by assaying samples of grain of varying levels of insect contamination, obtained from grain mills across the United States, and assaying them repeatedly over several days. An example of this type of ruggedness testing of the assay is shown in Figure 5, using a series of hard red winter wheat samples which were assayed either two or three times with a high degree of reproducibility. The ability of the immunoassay to accurately assess the degree of internal infestation was also examined, in collaborative trials carried out with the U.S. Department of Agriculture. In these studies, which were carried out in a blind fashion, an excellent correlation was observed between the number of internally infesting granary weevils present, as determined by x-ray analysis, and the response in the immunoassay procedure (Schatzki et al. 1993).

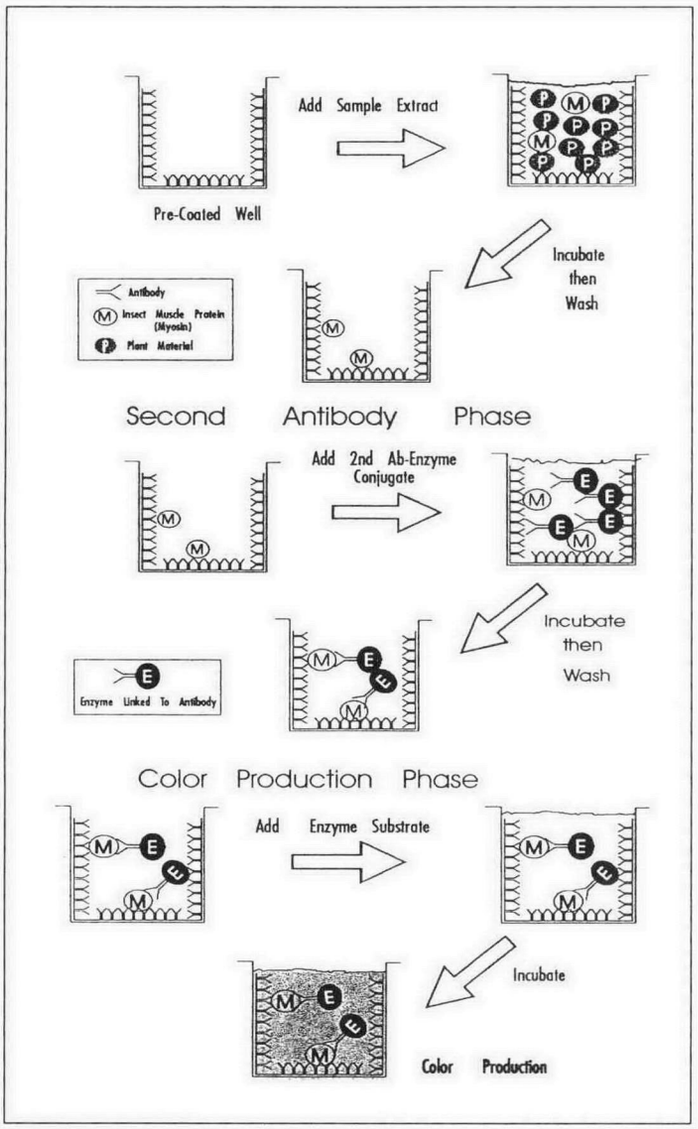

Fig. 2. The myosin sandwich ELISA procedure for quantitative detection of grain insects in stored products.

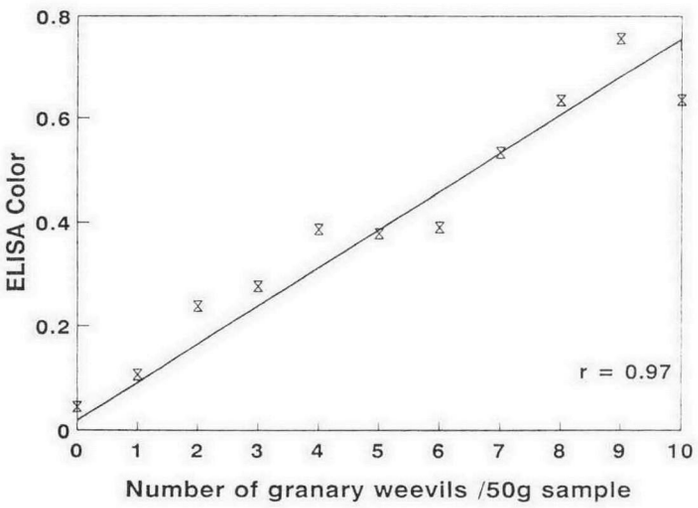

Fig. 3. Correlation between the amount of colour produced in the myosin sandwich ELISA (absorbance at 414 nm) and the number of granary weevils present in 50 g samples of hard red winter wheat. Points represent the average of three determinations.

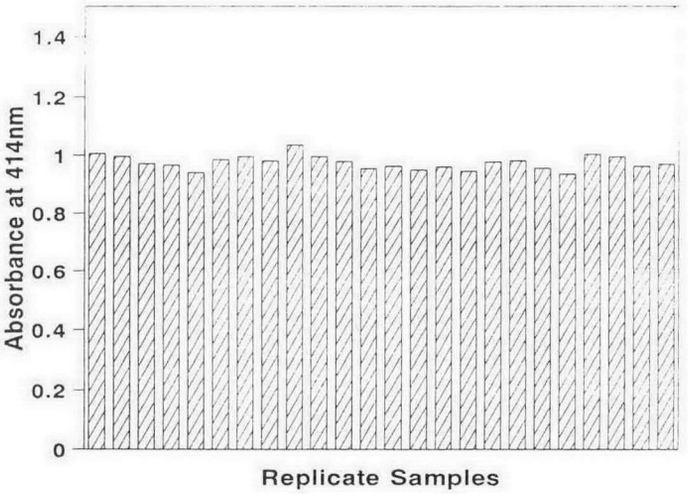

Fig. 4. Replicate samples assayed using the insect myosin sandwich ELISA procedure.

Table 1. Grain pests detectable using ELISA assay

Sitophilus zeamais	Maize weevil
Sitophilus granarius	Granary weevil
Sitophilus oryzae	Rice weevil
Trogoderma variabile	Warehouse beetle
Trogoderma glabrum	glabrous cabinet beetle
Tribolium castaneum	red flour beetle
Tribolium confusum	confused flour beetle
Oryzaephilus mercator	merchant grain beetle
Oryzaephilus surinamensis	saw toothed grain beetle
Rhyzopertha dominica	lesser grain borer
Prostephanus truncatus	large grain borer
Lasioderma serricorne	cigarette beetle
Stegobium paniceum	drugstore beetle
Callosobruchus maculatus	cowpea weevil
Attagenus megatoma	black carpet beetle
Alphitobius diaperinus	lesser mealworm
Plodia interpunctella	Indianmeal moth

Of particular significance is the fact that the immunoassay procedure can be applied equally well to either whole grain or milled grain materials. As shown in Figure 6, the total amount of insect material found in the flour, shorts, and bran fractions of a sample of milled wheat totalled the amount of insect material present in the original whole grain sample. This means that the immunoassay procedure can be used to accurately predict, by analysis of incoming whole grain, what amount of insect material will be present in the finished product. It should be noted that with the immunoassay procedure there is no difficulty in quantifying the amount of insect material present in the shorts and bran fractions which are extremely difficult to assay reliably using insect fragment counts.

Because of the accuracy and reliability of the immunoassay procedure and its ability to predict flour quality from whole grain analysis, this allows for the test to be used to quantitatively blend grain samples of differing levels of insect contamination to produce finished products of a desired

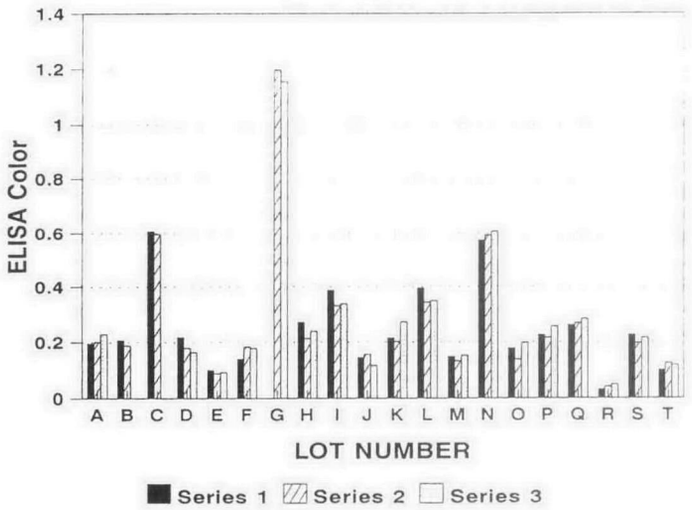

Fig. 5. Reproducibility of the myosin insect ELISA procedure using mill supplied grain samples with varying degrees of insect contamination. Samples of the grain were extracted and assayed separately either two or three times over a five-day period.

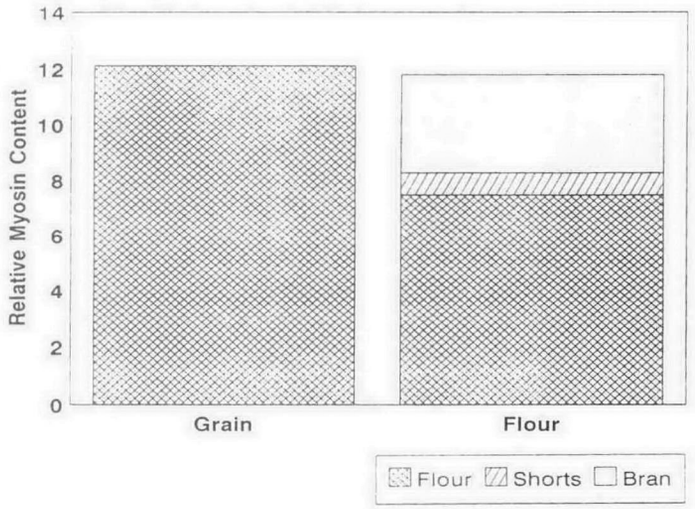

Fig. 6. The amount of insect myosin present in whole grain (hard red winter wheat) and in the flour, shorts and bran fractions milled from this grain, as assayed by the myosin ELISA procedure.

quality level. An example of the use of the myosin immunoassay for such blending is shown in Figure 7.

The microwell procedure described above is well suited for use in small laboratories, requires only about two days training, and is capable of evaluating approximately 20 samples in a two-hour period using our current methodologies. It would also be very useful if a simpler and quicker test was available for on-site testing of incoming grain and outgoing product at shipping docks and for checking the quality of export goods. For these reasons, we are engaged in developing a rapid, test-strip assay which can give quantitative results in a 15 minutes. An enhanced sensitivity immunoassay, using a biotin/streptavidin ELISA procedure, and which offers an approximately ten-fold enhancement over the regular ELISA is available for situations where detection of very low levels of insect contamination is necessary (I. Heller and G.B. Kitto, unpublished results).

Development of a Species-Specific Grain Insect Immunoassay

In addition to having available rapid tests which can accurately measure the total degree of insect contamination in stored-food products, it would be useful to have available species-specific tests which could provide information about the relative degrees of infestation by different insect pests. This type of information could be useful in selecting the most appropriate type of pesticide to be used on the stored product, or for providing feedback to the growers for more effective control regimes. As a model for this type of approach, a monoclonal antibody-based procedure has been developed for the quantitative detection of *Sitophilus granarius* (Chen and Kitto 1993). As illustrated by the example shown in Figure 8, this species-specific assay can accurately determine the degree of infestation caused by the granary weevil in a mixed insect population. In this particular case, a hard red winter wheat sample was spiked with the indicated number and variety of insects. The total degree of insect infestation was measured using the insect myosin ELISA, while the contribution of granary weevils to this total was estimated by the monoclonal antibody assay. The *S. granarius*-specific ELISA very accurately reflects the degree of granary weevil infestation. A similar species-specific assay has also been developed for the stored-grain pest *Trogoderma granarium*, the kaphra beetle (Stuart et al. 1994). This assay is capable of readily distinguishing the adults, pupae and larvae of this species from six other *Trogoderma* species and from other common grain pests.

Development of Immunoassays for Beneficial Insects

The protection of stored-food products against insect damage is clearly of major importance, yet the number of chemical agents for this purpose is very restrictive and insect resistance to these chemical insecticides is increasing. An alternative to the use of chemical pesticides is biological control using beneficial insects. Such beneficial insects can either kill deleterious insects by eating these pest insects directly, or more indirectly by infesting the egg or larvae of the pest species. A number of small-scale tests have shown the effectiveness of this biological control approach (Brower 1988; Keever et al. 1986). For such biological control methods to be used on a wide scale for stored-crop protection, much must be learned about their efficacy, the timing of insect release and about insect dispersal. Current assays for beneficial insects, like those for pest species, depend upon time consuming and expensive sieving and visual tests that require skilled personnel. A rapid and effective means for quantifying the number and spatial distribution of beneficial insects in stored products is highly desirable. In addition it would also be very helpful to have available rapid quantitative tests which could monitor the effectiveness of product cleaning in getting rid of any remaining beneficial insects (which are usually external feeders) when the product is processed.

Any test for beneficial insects necessarily requires a high degree of specificity. Advantage has been taken of the exquisite selectivity of monoclonal antibodies in our initial work on the development of species specific assays for beneficial insects. A monoclonal antibody based system has been developed for the parasitic wasps *Bracon hebetor* and *Laelius pedatus*, effective control agents for grain insect pests (Stuart and Burkholder 1991). These immunoassay systems have the

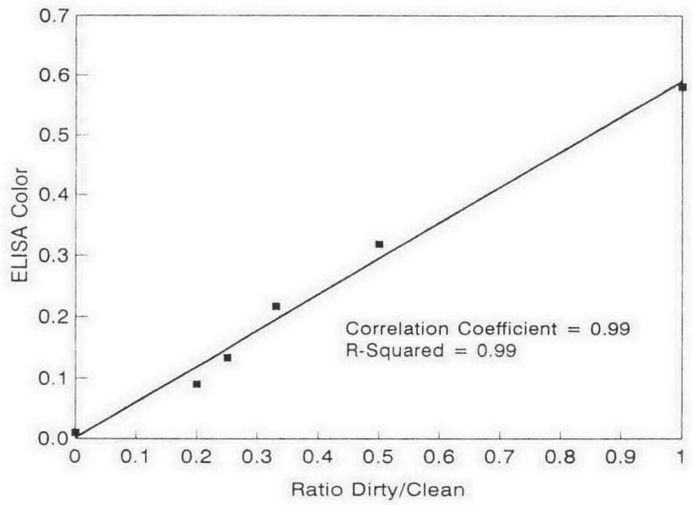

Fig. 7. Assessment of the application of the insect myosin ELISA for blending grain. Clean hard red winter wheat was spiked with granary weevils and ground. This spiked dirty wheat was mixed with ground clean grain in varying proportions to give the equivalent of 0–2.5 granary weevils per 50 g sample.

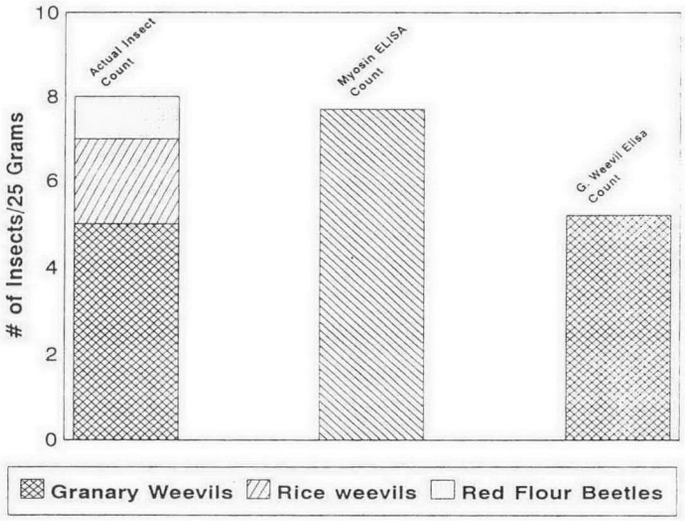

Fig. 8. Use of a species-specific ELISA to determine the proportion of granary weevils in a mixed insect population in hard red winter wheat. A sample of clean grain was spiked with the indicated number of insects. The total insect infestation was assessed by the myosin ELISA and the granary weevil content was assessed using a monoclonal antibody-based species-specific ELISA.

desired selectivity, cross-reacting only with the beneficial species and not with a range of grain insect pests. Similar monoclonal antibody-based immunoassay procedures have been developed in our laboratories for other beneficial insects, including the parasitic wasp *Trichogramma pretiosum* and the pirate bug *Xylocoris flavipes*.

Application of Insect Immunoassays to Other Stored Products

Because a variety of stored-food products harbor the same types of insect pests as grains, it was anticipated that the insect myosin immunoassay procedure might also be applicable to a wide range of food products. An extensive series of tests was therefore carried out to assess the applicability of this test to a broad spectrum of spices, nuts, and dried fruits. These studies included both an examination of whether the ELISA procedure might be affected by materials present in these food products and whether the assay procedure was of the requisite sensitivity and linearity. In the case of spices, the same type of extraction employed with grains was found to be satisfactory for analysis of these flavouring materials. A wide array of different spices of both the leafy and nut-like varieties proved amenable to insect analysis by the ELISA procedure, giving a linear and sensitive response. In a few types of spices, including star anise and lavender, however, inhibition of the immunoassay was observed. Preliminary investigations into this problem revealed that the diminution in signal is likely due to non-specific binding of the antibody by plant materials present in these spices and experimentation is underway to circumvent this difficulty.

A similar situation was found when a variety of nuts and dried fruits were tested for applicability of the insect ELISA assay. A large range of these food materials, including products as diverse as cashews and dried peaches, were perfectly suited to the myosin immunoassay, but a few have so far proven recalcitrant. Mites and aphids are also important destructive agents in several types of stored products. Preliminary investigations have shown that the myosin ELISA assay is also capable of measuring the degree of infestation by these organisms, including the detection of such commercially important species as tobacco mites.

Summary

The new immunologically based assays for insect contamination in stored products bring a much higher degree of objectivity and quantitation to the analytical process, since they eliminate most of the subjectivity present in the visually based tests that are currently being used. Not only do the ELISA assays provide for a highly reliable and repeatable inspection process, they also require little training time and are of moderate cost. At the present time, the ELISA tests which have been most thoroughly tested and documented require the use of a modestly equipped laboratory. If large numbers of samples need to be analysed, then the ELISA procedure can readily be automated, as has been done for clinical chemistry ELISA tests.

In many circumstances it would be highly desirable to have as an option extremely rapid test procedures through which the results could be obtained in a matter of minutes. This type of assay would be invaluable for evaluating incoming loads of stored product and for spot checking the samples for the export market. The test-strip assay that we have under development appears to be very well suited for this type of approach.

The use of these immunoassay techniques for measuring insect contamination in stored products does raise regulatory questions. The immunoassays detect the mass of a particular type of insect material in a sample. The detected material can be insect myosin, or other proteins in the case of the monoclonal antibody-based tests. The mass of this insect component has very definitively been shown to correlate with the total mass of insect material present in the samples, and this mass in turn, correlates very well with the number of insects present (see Figure 3 and Schatzki et al. 1993). Thus, the immunoassay procedures can be used with no difficulty as

a direct replacement for present assays which relate to numbers of insects in a sample. On the other hand, for processed stored products, such as wheat flour, where the primary current analytical procedure is the insect fragment count, a new type of regulatory unit will be needed for use with the immunoassays. This is because the insect fragment count simply measures the number of fragments present without regard to the actual mass of insect material present. Over the broad range, there is reasonable agreement between the ELISA procedure and insect fragment counts, but at low levels of contamination the correspondence between the two types of assays is weaker. This lack of correlation is due almost entirely to the variability of the insect fragment assay, as determined by direct comparisons of the two techniques using samples of known insect content (G.B. Kitto, P. Behrens and I. Heller, unpublished data). From studies based on the amount of insect test material present in different insect life stages and a knowledge of the length of time spent in each life stage, it is possible to define an 'average insect unit' for the immunoassay procedures (Schatzki et al. 1993). It is proposed that such a unit could be used for setting appropriate regulatory standards.

Acknowledgments

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