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DISTINGUISHING ADULT PECTINOPHORA SCUTIGERA (HOLDAWAY) ACCORDING TO LARVAL DIET BY X-RAY MICROANALYSIS

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Abstract

In an exploratory study using scanning electron microscope (SEM) based energy dispersive spectroscopy (EDS) of X-rays, differences were detected in the elemental compositions of adult Pectinophora scutigera raised on contrasting diets - cotton, Hibiscus tiliaceus and laboratory medium.

X-ray spectra were collected from the thorax and abdomen of 13 male and 13 female moths. X-ray yields are optimised by ashing the insects for 1 h at 400-600^{0}C, and by operating the SEM at 25 kV.

Spectrum matching, element matching, discriminant analysis and principal components analysis were used to classify the specimens on the basis of diet with 54-100% success. Spectra were considered as a whole or processed using either a digital filter to remove background or a ZAF program to compute semiquantitative elemental concentrations. Background corrected peaks is the favoured data format, having high discriminating power and being simple to obtain and interpret.

Better classification is apparent when subsets of the same sex, body part or both are employed; and when the data for each element is normalised to a mean of 0 and a standard deviation of 1. The chief discriminating elements appear to be F, Na, Mg, P, Cl, K and Ca with Si, S, Mn, Fe and Zn being of little assistance.

KEY WORDS: Insects, pink-spotted bollworm, X-ray microanalysis, bulk biological specimens, energy dispersive spectroscopy.

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Introduction

X-ray spectrometric methods have been applied to diverse biological species. For example, Calaprice (1971) studied sockeye salmon by X-ray fluorescence (XRF) energy dispersive spectroscopy (EDS). Using discriminant analysis of the spectral data he was successful in separating both adult and fry salmon on the basis of their geographical origins. Kelsall and Burton (1977), with some success, applied the same experimental and statistical techniques to feathers from lesser snow geese.

A number of X-ray spectrometric investigations have been directed towards insects.

McLean and Bennett (1978), using XRF/EDS and discriminant analysis, found that ambrosia beetles could be readily separated on the basis of geography, sex or the presence of adhesive from sticky traps. McLean (1980) reported on a practical extension, identifying the origins of beetles found in the Chemainus Sawmill. McLean et al. (1979) separated western spruce budworms on the basis of their source stands. Later, McLean et al. (1983) identified a partitioning of elements in the thoraces and abdomens of these insects, and discovered that elemental variations in adult insects were much smaller than those in the original food sources.

Another substantial contribution to the entomological exploitation of X-ray analysis is a series of papers by Bowden and co-workers. The first in the series (Bowden et al. 1984) concerned the moth Noctua pronuba, the subject of a previous study (Bowden et al. 1979). XRF/wavelength dispersive spectroscopy (WDS) was employed in both cases. Principal components analysis (PCA) gave no discrimination until subsets were considered. When this was done, not only could insects from different host plants be distinguished, but also insects from the same host plant growing on different soils. The second article (Bowden et al. 1985a) examined two species of aphid, using SEM/EDS. No discrimination was discernible for any aphid species, element, plant or soil subset. The third paper (Bowden et al. 1985b) again examined the same two aphid species, but collected from different host plants and with a refined PCA, and reported successful discrimination. Another moth, Agrotis segetum, was the subject of a fourth paper (Sherlock et al. 1985), in which robust means PCA was applied to XRF/WDS data. Provided host plant subsets were considered separately, the moths could be differentiated on the basis of soil type. A fifth paper (Sherlock et al. 1986) examined two aphid morphs collected at various localities mainly from the same host plant, using SEM/EDS. While the morphs were well differentiated, there was no clear separation, in either morph, on the basis of host plant locality.

Preliminary reports on another X-ray study of moths, Heliothis species, have been given by Fitt (1985, 1986). Dempster et al. (1986) have conducted XRF/WDS studies of the Brimstone butterfly. Compositional differences were observed between insects of different sexes, sites and seasons, but the host plant differences diminished as the adults fed and aged.

The broad conclusion that may be drawn from this literature survey is that X-ray methods for geographically discriminating insects have met with mixed success. In some instances, at least, for identifiable reasons or unknown, there is a lack of any measurable compositional variation in insects from different sources.

The subject of the present study is the pink-spotted bollworm, Pectinophora scutigera (Holdaway) (Lepidoptera:Gelechiidae), a serious pest of cotton in Central Queensland. Larvae tunnel into developing cotton bolls damaging lint and seed. Of particular interest is the question as to how populations carry over from one season to the next. Sabine (1969) stressed the importance of post-harvest cotton trash, containing infested bolls, and considered alternative host plants to be of little significance. However, the recent discovery of additional host plants (Walker and Harris 1985) suggests that alternative host plants may play a significant role in the carry over of moth populations. It is toward investigating this possibility that the method described here will be directed.

The purpose of this paper is to demonstrate that the larval diet of adult bollworms can be deduced with a good chance of success, and to suggest some ways to improve the success rate. The method set forth here is not claimed to be the best possible means of discriminating P. scutigera. A method meriting such a claim might be expected to include a complete quantitative elemental analysis of each moth as well as to take into account other variables - colouration, body weight, moisture content, age and so on. The proposed method does not require an accurate analysis of the detected elements. It is generally recognised that quantitative analysis of bulk biological specimens by EDS is difficult and prone to substantial error (Roomans 1980, Heinrich 1982, Boekestein et al. 1980). Detection of all the elements present in the specimens has not been attempted. Most EDS systems have a lower atomic number limit of Z=9, although windowless detectors may be pressed to Z=4 (Marshall 1984). Hydrocarbons are therefore difficult and hydrogen impossible to detect. An alternative spectroscopy, such as secondary ion mass spectrometry (SIMS), is required to probe for light elements.

Materials and Methods

Specimens

Adult P. scutigera were reared from larvae fed on 3 contrasting diets: artificial medium, cotton bolls and Hibiscus tiliaceus flowers.

A laboratory culture of P. scutigera, originally established from larvae already infesting cotton bolls, was maintained on artificial medium as described by Vickers (1982). The medium consisted mainly of soya beans (66.2% by weight of dry ingredients) with a small amount (2.3%) of ground cotton seed added to act as a larval feeding stimulant. The culture had been maintained on this medium for approximately 3 generations.

P. scutigera infested cotton bolls were collected from cotton (Deltapine 61) near Biloela, Central Queensland, in May 1985. Fallen H. tiliaceus flowers were collected from the base of a single tree in Biloela during March and April 1985. Cotton bolls and H. tiliaceus flowers were held in ventilated plastic boxes at 25^{0}C until the larvae had pupated. All pupae were sexed and incubated at 25^{0}C. On emergence adult moths were not fed, but immediately killed by freezing.

Specimens were deep frozen until required. To reduce the contribution of the hydrocarbon matrix to the X-ray spectrum, the specimens were ashed using a Townson and Mercer furnace. To establish the optimum ashing temperature, 5 entire air-dried moths (all males collected from cotton) were ashed for 1 h at each of the following temperatures: 200, 300, 400, 500, 600 and 700^{0}C.

Ashed moths were mounted on Al stubs using double-sided adhesive tape. From 1 to 3 moths were mounted on each stub. The moths were systematically aligned on the stubs to facilitate identification of body parts.

The main data base comprised X-ray spectra from 26 moths. Of the 26 moths, 5 of each sex (M, F) came from cotton (C), 5 of each sex from laboratory medium (L) and 3 of each sex from H. tiliaceus (H). The thorax (T) and abdomen (A) of each moth were examined separately, yielding a total of 52 spectra. The abbreviations we employ to refer to data subsets having the same sex and body part are MT, MA, FT and FA.

SEM/EDS system

The prepared specimens were placed in an ISI-60A SEM. For all the measurements, the working distance was fixed at 53 mm and the working magnification was 1000x. The specimens were irradiated with 30 keV electrons, the highest energy available, to ensure any elements with high atomic number were excited. During the acquisition of the data base, no elements with Z>29 were detected.

Later, to ascertain the optimum beam voltage, a series of X-ray spectra were obtained at electron energies of 15, 20, 25 and 30 keV. A single set of 5 specimens (male moths from cotton) was used in this investigation.

X-ray collection and sorting was via a PGT4 system. The X-ray detector was located 27 mm above the specimen and 21 mm from the beam axis; the take-off angle was 52^0. X-ray spectra were collected for 200 s. The beam current was measured using a Faraday cup before and after each spectrum. Each X-ray spectrum was transferred through an RS232 interface to an IBM Personal Computer (IBM PC) for further processing.

Data format

The X-ray spectral data was presented for statistical analysis in three forms. These will be described in order of increasing spectrum processing.

First, the raw channel-by-channel data was used. The energy calibration was 20 eV per channel. The lower threshold on the main amplifier rejected signals corresponding to less than 500 eV. No peaks were observed above 10 keV. So the data in channels 25 to 500 was used.

Second, background-corrected peak intensities were computed. The background was removed using a "top-hat" digital filter as described by Statham (1977). Given the detector resolution (152 eV at 5894 eV), the energy range over which peaks were observed (676 to 8630 eV) and the variation of resolution with energy (Goldstein et al. 1981), an optimal filter was chosen according to the prescription of Statham (1977). The filter had a central lobe 7 channels wide and side lobes 3.5 channels wide. Regions of interest were set up to record counts in peaks which consistently occurred, corresponding to the elements F, Na, Mg, Si, P, S, Cl, K, Ca, Mn, Fe, Cu and Zn. (To avoid interference from the K K-beta peak in measuring the Ca K-alpha peak, the Ca K-beta peak was used.)

Finally, semiquantitative elemental concentrations were calculated. An algorithm based on the FRAME C program (Myklebust et al. 1979) was used to determine the concentration of the elements Na, Mg, Al, P, S, Cl, K, Ca and Cu. The code uses the ZAF technique and background modelling. As all the assumptions of the program were not met (flat surface, at most 1 element unknown), the results are appropriately described as semiquantitative.

The data was subjected to various statistical treatments, which will be detailed in the Results and Discussion section. The simpler analyses were performed on the IBM PC. The filtered peak and semiquantitative concentration information was further analysed using the SPSS package (Nie et al. 1975) running on an HP3000 computer.

Results and Discussion

Ashing temperature

The effect of ashing temperature on X-ray yield is shown in Figure 1. The net-peak to background ratio of the K K-alpha line is

shown. A monotonic improvement in yield with temperature is observed, with a large step between 300 and 400°C.

As temperature increases, progressively more of the specimen is volatilised and oxidised, with it disappearing completely at 700°C. That ashing may cause irreproducible loss of elements from biological specimens has previously been noted (Goldstein et al. 1981, Turner and Bowden 1983). Our data lends some support to this suggestion, as larger absolute errors are associated with higher temperatures. However, the relative errors from 400-600°C ashing are smaller than those at lower temperatures.

A suitable ashing temperature will balance the gain in sensitivity due to matrix removal against the loss in precision due to the removal of analytic elements. The range 400-600°C fits these criteria: ashing at 400°C was employed in obtaining all the other data reported here.

Electron energy

Figure 2 shows the dependence of X-ray yield on electron energy over the range 15 to 30 keV. Of the four energies tried, 25 keV gave the greatest yield, in terms of the net-peak to background ratio for the K K-alpha line. This result is not explicable simply in terms of the over-voltage ratio increase, which would lead to an increase in yield with accelerating voltage. The decrease in detectability may be attributed to high energy backscattered electrons depositing energy in the Si(Li) X-ray detector. The further data reported in this paper had previously been obtained using 30 keV electrons.

Spectrum matching

Initial examination of the data base was by spectrum matching. The general principles of this technique are described by Russ (1984). In essence, the differences between a "test" spectrum and several "standard" spectra are computed - the smaller the difference, the better the match.

The test and standard spectra were generated as follows. Counting error spectra were generated for each of the original spectra by taking the square root of the counts in each channel. Each original spectrum and its associated error spectrum was then normalised with respect to the electron beam current flowing during collection. This produced the 52 test spectra and the 52 related error spectra. Standard spectra for cotton, hibiscus and laboratory medium were obtained by averaging the relevant test spectra. S(i) and T(i) denote the beam current corrected counts in channel i of the standard and test spectra respectively; s(i) and t(i) are the respective errors.

A number of statistics were used to sum, channel-by-channel, the differences between each of the 52 test spectra and each of the 3 standard diet spectra. The spectrum being tested was assigned to the diet from whose standard spectrum it differed least. The number of successful classifications was then tallied.

As expected, a squared difference statistic, $(S(i)-T(i))^2$, is more efficacious than either a difference, $(S(i)-T(i))$, or an absolute difference statistic, $|S(i)-T(i)|$, the classification success rates for these being respectively 67, 38 and 58%. These success rates may be compared with that expected on the basis of random guessing, 33%. The statistic favoured formally, $(S(i)-T(i))^2/(s(i)^2+t(i)^2)$, equivalent to the Mahalanobis D^2, gave a success rate of 71%.

The percentages of the previous paragraph are biased estimates of the success of matching. Each test spectrum contributes a small proportion to one of the standard diet spectra against which it is being matched. To measure this bias a "jackknife" (or "leave-one-out") procedure was followed. The last statistic was computed again with the contribution of the test spectrum under consideration being removed from the relevant standard spectrum. Proceeding this way gave the same success rate as before, 71%. This suggests that no great bias was introduced in using the same spectra to generate and evaluate the standard spectra.

Next, subsets of the data were considered separately. For instance, standard spectra for each diet were generated from the male spectra alone and the male spectra alone were matched with these standards. Classification success for each of the subsets is

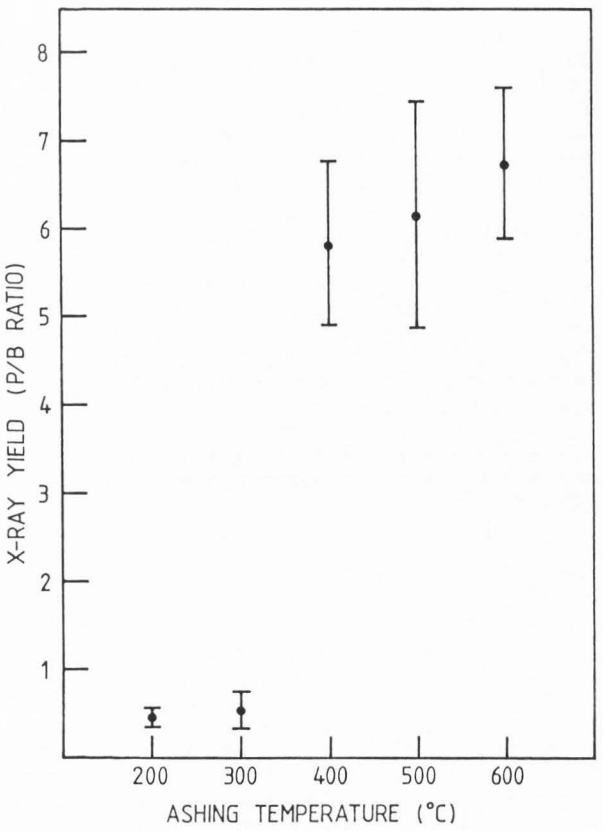

Figure 1. X-ray yield as a function of ashing temperature. The net-peak to background ratio for the K K-alpha line is shown. A bar representing the standard error of the mean is shown on either side of each data point.

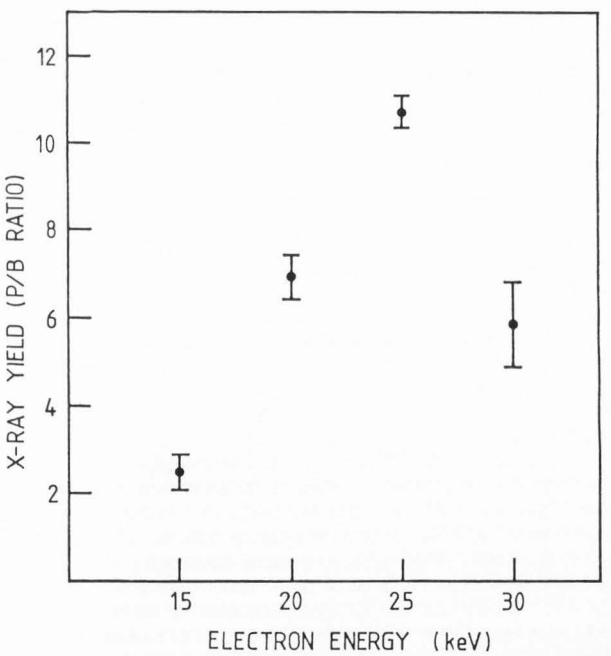

Figure 2. X-ray yield as a function of electron energy. The net-peak to background ratio for the K K-alpha line is shown. A bar representing the standard error of the mean is shown on either side of each data point.

given in Table 1. Several observations may be made concerning this table. Generally, the more homogeneous the subset, the higher the classification success rate. The spectra taken together were classified with 71% success: grouped according to sex and body part the success rate was 65-77%: grouped according to both sex and body part the success rate was 69-100%. The advantage in using subsets has previously been recognised with other moth species (Bowden et al. 1984, Sherlock et al. 1985). The same calculations were performed using a jackknife procedure and exactly the same success rates were found.

It is suggested that specimens of the same sex, or body part, or, preferably, of the same sex and same body part, be used whenever practical to improve the classification of P. scutigera. Although the data is not conclusive on this point (given the small sample sizes of the various sex-body part subsets), it appears that the male-thorax subset is the most homogeneous.

While not the main purpose of this study, it is of some interest to see how well the sex of adult P. scutigera may be distinguished and also how well the body parts (abdomen or thorax) may be distinguished.

This has been attempted using spectrum matching. No subsets were considered: the standard abdomen spectrum, for example, was the average of all abdominal spectra, regardless of sex or diet. A success rate of 50% would follow on the basis of random classification; spectrum matching did not yield much better. Using the statistic $(S(i)-T(i))^2/s(i)^2$, the correct sex of the moths was given in 52% of the cases, and the correct body part in 62%. These figures concern the data set as a whole and using more homogeneous groups (for example, of the same diet) may well increase the chance of correct classification, as it did previously. Moreover, other methods described below are shown to be superior to spectrum matching in classifying diets, and may similarly enjoy a greater success than spectrum matching in classifying moth sex and body part. However that may be, distinguishing sex and body part is not of practical entomological importance as these may be determined directly by inspection.

Element matching

Rather than match spectra channel-by-channel, the faster and more meaningful strategy of matching a small number of regions of interest, or elemental concentrations, may be employed (Russ 1984). Table 2 shows the results of such an approach using both regions of interest from digitally filtered spectra and semiquantitative elemental concentrations. The elements matched were Na, Mg, P, S, Cl, K, Ca and Cu. The statistic $(S(i)-T(i))^2/s(i)^2$ was used.

In one respect the results of Table 2 differ from those discussed under spectrum matching: in Table 2, the jackknife comparisons are not as successful as those that include the test specimen in a standard. This is expected; the earlier results are unusual in showing no difference with jackknifing.

In several respects the results shown in Tables 1 and 2 concur. Breaking the data base down into subsets increases the classification success rate. The success rates using all data formats - original spectra, digitally filtered spectra and semiquantitative concentrations - are similar, with not one of these clearly superior.

Other researchers have favoured our first and third data formats. Calaprice (1971) used a selection of channel-by-channel data as did Kelsall and Burton (1977). These workers used 40 and 80 channels respectively as variables in multivariate analyses. On the other hand, Turner and Bowden (1983), for example, examined a smaller number (10) of elemental concentrations. The results given in Tables 1 and 2 do not especially commend one or other of the data formats on the basis of classification success. The second data format - peaks from digitally filtered spectra - combines simplicity with interpretability. A small number of variables, identifiable with particular chemical elements, is readily derived from the original spectrum. This data format will be the main one used in the rest of the analyses reported here.

Character of the data base

A closer examination of the data base will precede the discussion of further classification strategies. The data base is here taken to comprise 52 cases containing backgound subtracted peaks for 13 elements.

A basic question is whether the data comes from a multivariate normal distribution. If it does, it is unlikely that moths from the 3 diets will be separable. Calculation of the skew and kurtosis for each of the elements across the 52 spectra suggested the data was not normally distributed. A more formal examination was afforded by performing on each element the Kolmogorov-Smirnov test (Hull and Nie 1979); only Fe and Cu appeared to be normally distributed. This leaves open the possibility of three distinct dietary subpopulations, with the elements Fe and Cu expected to be of little help in distinguishing them.

The same tests were run on each of the dietary subpopulations. For the purpose of distinguishing moths on the basis of their diets, it would be most convenient to find each diet subpopulation was multivariate normal, with the means widely separated in multivariate space. This was not the case. While some of the elements appeared to be normally distributed within each diet subpopulation (F, Na, K, Fe and Cu in cotton; Cl, K and Cu in hibiscus; Fe and Cu in laboratory) most of the elements show non-normality. To reduce non-normality due to skew, a logarithmic transformation may be applied (Srivastava and Carter 1983). A logarithmic transform also gives equal weighting to relative, rather than absolute, changes in the elemental data. The data within each diet was transformed according to $\ln(x+\bar{x}/10^4)$, where $\bar{x}$ is the mean of x, the concentration of the element under consideration. The Kolmogorov-Smirnov test was run on the transformed data for each element across each diet. As with the original data, only a few elements appeared to be normal in each diet (F, Na, Si and P in cotton; F, Mg, P and K in hibiscus; K in laboratory). The chief conclusion that can be drawn from these tests is that none of the three diets show marginal normality among most of the variables in it, let alone multivariate normality. A second conclusion is that transforming, by taking logarithms, the data within any diet changes which elements may be normal but does not improve the overall distribution.

Smaller groupings still (defined by sex or body part or both, as well as diet) were not tested for multivariate normality because of their small populations. Whether the elements in these prove to be normally distributed, or whether further factors still are needed to define normal populations, cannot be answered from our data.

The data for each element within each diet was also transformed to Z-scores (Nie et al. 1975). This is accomplished for each datum by subtracting the mean and dividing by the standard deviation. Thus the Z-score data for any element within any diet has a mean of 0 and a standard deviation of 1.

Discriminant analysis

Multivariate discriminant analysis aims to classify a multivariate observation into one of a number of distinct groups. As such, it seems well-suited to the present study and was used in similar circumstances by Calaprice (1971), Kelsall and Burton (1977) and McLean and Bennett (1978). However, certain problems accompany discriminant analysis in the current context. For a start, assumptions of multivariate normality underlie most discriminant programs (although Lachenbruch (1975) discusses methods not constrained by these assumptions). While in practice standard discriminant programs are generally robust to departures from normality (Jackson 1983), their suitability to the present data base must be regarded as tentative. More seriously, the discriminant method assumes that the distinct groups into which it classifies unknowns are comprehensive. However, it is gratuitous to assume that field-collected P. scutigera must have had a cotton or hibiscus larval diet. Field collected specimens may have come from an altogether different, perhaps as yet unknown, host plant. In spite of these reservations concerning its formal basis, discriminant

Table 1. Correct classification of moths according to larval diet using channel-by-channel data grouped in subsets.

Subset	Correct classifications (%)
ALL	71
M	77
F	65
T	77
A	77
MT	100
MA	69
FT	77
FA	92

Table 2. Correct classification (%) of moths according to larval diet using element-by-element matching.

Subset	Background corrected peaks		Semiquantitative concentrations	
		jackknife		jackknife
ALL	83	77	79	77
M	81	73	84	84
F	69	65	81	54
T	81	77	81	65
A	92	84	88	84
MT	84	69	92	77
MA	77	69	84	62
FT	77	62	77	54
FA	92	84	100	69

Table 3. Correct classification (%) of moths according to larval diet using discriminant analysis.

Subset	Background corrected peaks	Semiquantitative concentrations
ALL	67	67
M	81	81
F	88	85
T	92	89
A	92	85
MT	100	100
MA	100	100
FT	100	100
FA	100	100

analysis may be cautiously used as a descriptive tool with which to explore the data base.

Discriminant analysis was undertaken using both the background subtracted peaks and the semiquantitative elemental concentrations, with the aim of distinguishing moths on the basis of larval diet. The successful classifications ensuing are recorded in Table 3. It should be noted that the same data was used both to generate the discriminating functions and to evaluate them, so to this extent the success rates in Table 3 are biased. Table 3 is thus most directly comparable with Table 1 and columns 1 and 3 of Table 2. In comparing results across these tables, the discriminant analysis can be seen to be slightly more effective than the other methods. While discriminant analysis is not so powerful when all the data is considered together (67% success compared with 71-83%), it excels when subsets are considered separately (81-100% success compared with 65-100%). It is of interest to note that the background corrected peaks provide a slightly better discrimination than the semiquantitative concentrations; the extra spectral processing needed to obtain the concentrations again appears unwarranted.

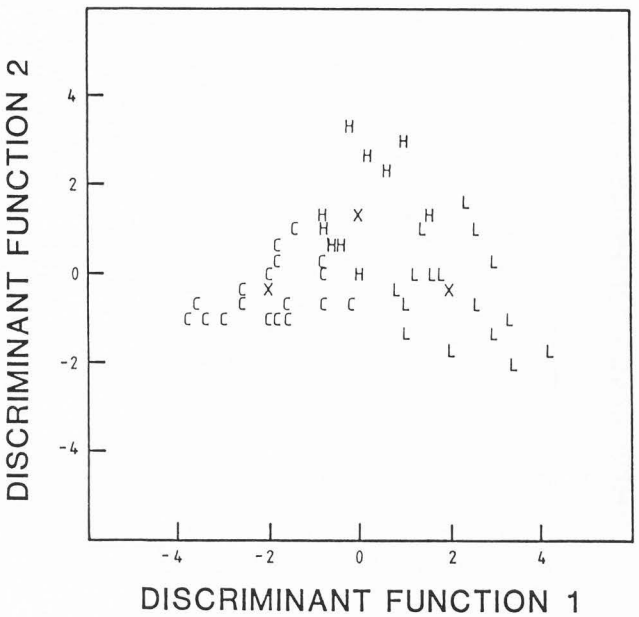

Figure 3. Discriminant analysis scatterplot for Z-score data. "C" stands for cotton, "H" for hibiscus, "L" for laboratory diet; "X" indicates group centroid.

Next, discriminant analysis was performed on the Z-score transformed data bases. Without grouping the data into subsets, greater differentiation was obtained than before: 94%. A plot of the Z transformed data in its chief discriminating plane is shown in Figure 3. Use of Z-scores, in which each element can contribute equally to the analysis, is thus seen to be preferable to using arbitrarily scaled or weight percent data.

Finally, stepwise discriminant analysis was carried out using the Z transformed data. In this approach, variables are added to the discriminating function one at a time, in order of discriminating power. Again, a final success rate of 94% was obtained. The variables used were, in order: P, Cu, K, Mg, Cl, F, Na and Ca. Thus as good results were obtained using 8 elements as 13. The nondiscriminating elements are Si, S, Mn, Zn and Fe. In the previous normality tests these appeared erratically across the specimens. They would seem to aid little in bollworm classification. As far as EDS analysis is concerned, the question of which are the best discriminating elements is of little importance as all elements are detected simultaneously.

Principal components analysis

The aim of PCA is to restate multivariate data in terms of new variables. In the common case in which much of the variance is accounted for by a few principal components, PCA allows the reduction of a many variable problem to a few-variable one. A difference between PCA and discriminant analysis is that the former does not presume the samples to be classified into distinct groups, whereas the latter does. Hence PCA gives a more natural representation of the data. However, PCA operates by minimising the difference between the data and the principal components and, as Jackson (1983) emphasises, this procedure may work against extracting the most important information from the data base.

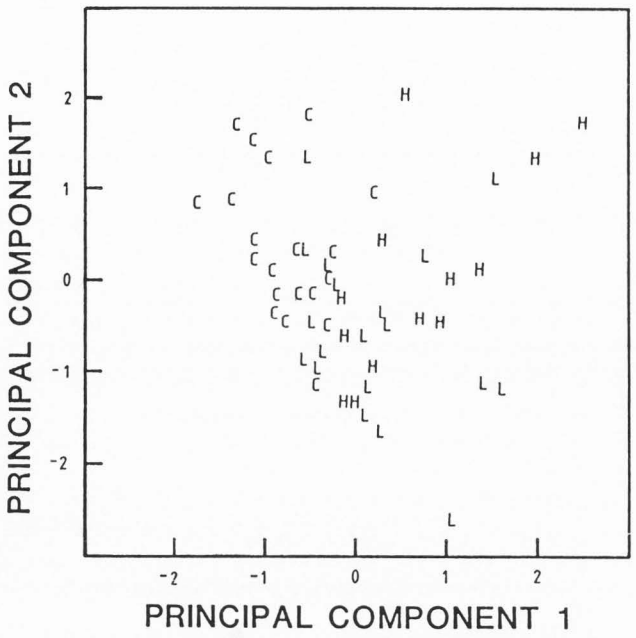

Figure 4. Principal components analysis scatterplot for Z-score data. "C" stands for cotton, "H" for hibiscus, "L" for laboratory diet.

The Z transformed data was subjected to PCA. The data is shown plotted against the first two principal components in Figure 4.

Extension

The results and discussion so far have been concerned with producing a data base and drawing descriptive information from it. The question now addressed is how and with what confidence this data base may be extended to classify field-collected specimens.

At the outset it is recognised that many factors, apart from the larval host plant, may affect elemental concentrations in P. scutigera. Just as the composition of a larva depends on the plant which nourished it so in turn the plant's composition depends on the soil on which it grew. In some cases, at least (Sherlock et al. 1985), the characteristics of the plant dominate differences in soil type, but the reverse may sometimes hold. We are presently investigating the elemental composition of insects reared on the same host plant grown on different soils. It is possible that the differences identified in this paper are merely differences between the soils on which the plants that made up the three diets grew.

Many other factors could conceivably affect elemental concentrations in moths. Bowden et al. (1984) list candidate contributors to the elemental composition of an insect, such as: soil - type, uniformity; host plant - species, variety, genetic makeup, stage of development, part consumed; insect - sex, physiological state, feeding in the adult stage, genetic makeup, disease, parasitism. Certainly the comprehensive data needed to determine the role played by all these factors is not yet at hand.

Conclusions

The major conclusion of this study is that adult P. scutigera may be distinguished on the basis of larval diet. This outcome is not trivial since similar studies have reported a range of results, from sharp distinction in some insects to no distinction in others.

The subsidiary conclusions drawn from this work concern the enhancement of the discrimination procedure. The first step in the procedure is data acquisition. Pretreating the specimen by ashing to remove the organic matrix improves X-ray yields. Too high a temperature leads to loss of analytic elements - ashing at 400-600^{o}C for 1 h is proposed as a suitable compromise. An electron energy of around 25 keV results in greatest X-ray yields. The preferred format for the data is as background corrected peaks, obtained by digitally filtering each of the original spectra. Little spectrum processing is needed to derive this data set, which then comprises a small number of variables identifiable with particular chemical elements.

The second step in the procedure is data analysis. For the larval diets studied, the data is unlikely to be multivariate normal. The use of Z-scores, rather than the original data, and the use of specimens of the same sex and body part, assist in the discriminant analysis.

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Discussion with Reviewers

A. Kiss: Criticism can be directed at the study in that the data base was small and limited.
K. Kiss: Do the authors plan to expand this work to obtain a wider data base which uses simpler and more reliable statistics?
J. A. McLean: My major concern is the very low 'n' in each category.
G. M. Roomans: The main problem with the paper is that the database is too small.
Authors: The work reported here is of a preliminary nature, undertaken to discover if x-ray microanalysis can be used to classify field-collected adult P. scutigera. Even with this relatively small sample, the results are quite encouraging, showing a high classification success for laboratory reared moths. Enough data has been gathered to refine specimen preparation and analysis conditions. We are presently using the methods developed in this paper to broaden the data base.

Our sample size of 52 spectra falls within the range reported in other studies - from 22 spectra (Bowden et al. 1979) to 728 (Bowden et al. 1985a).

K. Kiss: Did the authors attempt to use the "two-voltage" technique for quantitative EDX analysis? This yields a more precise and more accurate analysis of specimens with rough surfaces than the commonly used ZAF method. (See Kiss, K: "Quantitative" Electron Probe Analysis of Low-Atomic Number Samples with Irregular Surfaces. Applied Spectroscopy 37, 1, 1983.)
G. M. Roomans: In the case of a sample with a rough surface, a FRAME P based program would have been better than a FRAME C program.
A. J. Morgan: Have you considered ashing your specimens in a low-temperature oxygen plasma, since this may provide for both element retention and high analytical sensitivity?
Authors: We have not attempted any of these suggestions, all of which may well improve classification success.

A. J. Morgan: How large were the moths? Apart from PIXE and SIMS, would it be possible to analyse such specimens by other multielement chemical techniques, or does size preclude this possibility?
Authors: For an adult, the average body length is 7 mm and the width of the thorax about 2.5 mm. Other chemical techniques could be utilised: while the microscopic capabilities of SEM/EDS were one consideration, the speed and the simultaneous acquisition of multielement data were also important.

J. A. McLean: Did females oviposit any eggs? Had all moths voided their meconium? (This is often rich in elements not assimilated into the adult tissue.)
Authors: The females were unmated. As the moths void their meconium immediately after emergence we assume that all had done so before they were frozen.

G. M. Roomans: Since F can be measured in Teflon and related substances, couldn't the tape be the source of the signal?
J. A. McLean: Was an analysis of the adhesive tape carried out? Depending on the brand, this can contain Ti and/or Ca and Fe.
Authors: Prior to this work, spectra had been collected from the tape and no spurious signals were detected.

G. M. Roomans: Are the authors certain that they measure a true bulk specimen, i.e. that they do not excite the substrate? The lower P/B value at 30 kV may be due to the fact that the excitation volume exceeds the sample depth.
Authors: The excitation volume may affect the P/B ratio as suggested. The areas of the specimens analysed were chosen to minimise the possibility of probing the tape.

J. A. McLean: What effort was made to correct for different counting rates in a spectrum?
Authors: In each specimen, a suitably oriented residue was found to ensure a counting rate of around 20%.