THE QUANTITATIVE DETERMINATION OF INSECT INFESTATION IN POWDERED VEGETABLE DRUGS

By C. Melville

From the Department of Pharmacy, University of Manchester

Received May 24, 1951

Introduction

Vegetable drugs of commerce are liable to be infested by a variety of insect pests the detection of which in powdered materials is a matter of some difficulty. The methods at present in use for this purpose[1] depend upon separating the insect fragments from an aqueous suspension of the powder by flotation with an immiscible liquid such as petroleum, which is then filtered through paper. The number of fragments on the paper is counted under a low-power microscope and reported as the number per pound of the original powder, typical figures for spices[2] ranging from 0 to 5,000 per pound. Complete separation of the fragments by such methods is uncertain since they may remain entangled with the vegetable material. Furthermore, the reported figure for the extent of the infestation may be expected to vary with the degree of comminution of the powder since the size of the insect fragments is not taken into account. In a previous paper[3], the author has described a method based on acetolysis of the vegetable material, by which a complete separation of the insect fragments in a powdered drug can be attained. The present work was undertaken with the object of devising a quantitative method for relating the separated insect fragments to the number of insects originally infesting the unground drug, and is confined mainly to the two more common beetle pests *Stegobium paniceum* L. and *Ptinus tectus* Boie.

Experimental

(a) *Preliminary.* It was first necessary to decide which segment of the insect would be the most suitable one as a basis for a quantitative method. From those which might be used, the elytron was selected because its microscopical characters are such that it can be readily recognised and distinguished from the other segments when in fragments. It is also one of the larger segments and will therefore contribute a high proportion of the total number of fragments.

(b) *The numerical characteristics of the elytron.* Two numerical characteristics, the area and the number of strial punctures appeared worthy of investigation. For this purpose elytra removed from intact beetles, representative of the range in size of the specimens available, were cleared by heating in 5 per cent. w/v potassium hydroxide solution and mounted in gum-chloral. With the aid of a projection microscope, the outlines of the elytra were traced at a magnification of 80 × and the strial punctures marked on the tracings. The totals of the punctures were counted and the areas of the tracings measured with a planimeter.

The results are given in Table I and those for *S. paniceum* are illustrated graphically in Figure 1. From the latter graph it appears that the relationship between the two characteristics is nearly linear, and the

TABLE I

BEETLE ELYTRA—NUMBERS OF STRIAL PUNCTURES AND AREAS

Species	Puncture number range	Mean	Standard deviation	Area range sq. mm.	Mean	Standard deviation	Number of beetles
S. paniceum	285– 307– 408– 475	357·9	50·5	0·94–1·10–2·16–2·95	1·63	0·53	50
P. tectus ...	275– 290– 332– 359	311·4	21·0	1·91–2·10–2·72–3·17	2·41	0·31	25
N. hololeucus	271– 285– 332– 340	308·7	23·8	3·42–3·70–5·40–6·78	4·55	0·85	10
L. brunneus	900–1026–1382–1594	1204·5	178·0	1·44–1·72–2·48–2·68	2·10	0·38	15

equation of the line best fitting the points was calculated as $y = 204 + 94{\cdot}5\,x$ where y is the number of punctures, and x the area of the elytron in sq. mm. Theoretically therefore the two characteristics are equally suitable as a basis for a quantitative method. Reference to Table I, however, shows that in all cases the puncture numbers vary

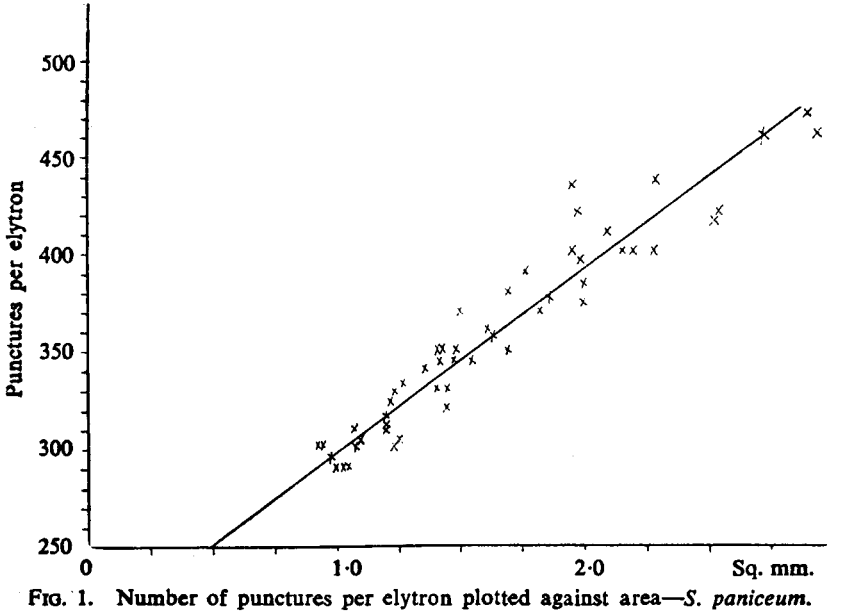

FIG. 1. Number of punctures per elytron plotted against area—*S. paniceum*.

over narrower ranges than the corresponding elytral areas and are therefore to be preferred. Futhermore the process of counting punctures is less complicated and time consuming than the measurement of areas. Accordingly it was decided to adopt the puncture number as the basis for subsequent investigations.

(c) *Variation of the strial puncture number with fragment size.* The

puncture number as determined on powdered materials may be expected to differ from the value obtained for intact elytra and to vary with the degree of comminution, due to the mechanical destruction which occurs and to error introduced by the presence of broken punctures. The numbers of the latter were found to be relatively few and it was decided to count all broken punctures as if they were intact. The extent of the variation of the puncture number with fragment size was determined using 200 specimens of *S. paniceum*. These were accurately weighed and then crushed in a mortar and triturated to a coarse powder with about 5 g., accurately weighed, of sucrose to facilitate powdering. Two separate approximately 0·5 g. quantities of the mixture were accurately weighed and the residue powdered more finely. From this two further approximately 0·5 g. quantities were taken and the residue powdered still further. Two samples were again weighed out and the procedure repeated until 10 such samples had been obtained, each pair of which represented an increasing degree of comminution of the original material. A few drops of water containing a trace of wetting agent were then added to each sample to dissolve the sucrose followed by 0·5 ml. of a suspension of about 0·25 g. of lycopodium accurately weighed in 25 ml. of suspending fluid. After thorough mixing, 3 slides were prepared from each of the 10 suspensions and examined following the method detailed later. The figure for the number of punctures per elytron was calculated for each slide and the mean of each 3 slides was taken as the value for the sample. The results are given in Table II, the degrees of comminution being indicated by the mean number of punctures per fragment of elytron, and are discussed later.

TABLE II

Degree of Fineness	Punctures per fragment	Strial punctures per elytron			Sample variation per cent.
		Sample series(*a*)	Sample series(*b*)	Mean	
1	10·3	289	327	308	± 5·8
2	6·4	300	330	315	± 4·5
3	4·2	341	289	315	± 7·6
4	3·5	308	322	315	± 4·8
5	2·7	343	310	326	± 2·2
	Mean	316·2	315·6	315·8	

It will be noticed from these results that the variation between samples is of the order of 5 per cent. of the mean with the exception of line 3 of Table II for which it is about ± 7·6 per cent. This was considered unduly high and as a check, and to provide a second estimation of the grand mean, a second quantity of 200 beetles was powdered to approximately the same degree of fineness with sucrose. From this mixture, 3 samples were weighed and the number of punctures per elytron determined as before.

The results appear in Table III and show a variation between samples

of the order of ± 2 per cent. of the mean which itself differs from the mean of Table II by less than 5 per cent. The two figures were, therefore, averaged to give a working mean of 322 punctures per elytron equivalent to 644 punctures per beetle. To obtain a working mean for *P. tectus*,

TABLE III

NUMBER OF PUNCTURES PER ELYTRON—SAMPLING VARIATIONS AND WORKING MEANS

Species	Number of beetles taken	Punctures per elytron			Mean	Working mean
		Sample 1	Sample 2	Sample 3		
S. paniceum	200	326	324	335	328·5	322·2
	200	see Table II			315·8	
P. tectus	100	308	305	322		311·7

100 beetles of this species were powdered, from which 3 samples were weighed and the number of punctures per elytron estimated as above. The results included in Table III are in good agreement with one another and give a working mean of 312 punctures per elytron equivalent to 624 punctures per beetle, which in this case is the same as the mean in Table I.

(d) *Isolation of the insect fragments from infested material.* The method used is, with minor modifications, the same as that described in an earlier paper. It consists of 4 stages:—(I) preliminary treatment with an organic solvent to remove soluble constituents such as fixed and volatile oils, chlorophyll, etc., which might otherwise interfere with subsequent treatment or be carried through to the final residue; (II) acid treatment to hydrolyse starch and oxidise lignin, hemicellulose and similar plant constituents; (III) alkali treatment to remove degradation products formed in the previous stage leaving a cellulosic residue; (IV) acetylation and solution of the cellulosic residue leaving the insect fragments. On applying the original method to the quantitative determination of *S. paniceum* it was found that results were sometimes lower than expected. This was due to the disappearance of the oval or rounded areas beneath the strial punctures on some of the recovered fragments making their counting difficult and to a tendency for the punctures, which in this insect are slit-like, to run one into the other. The number of punctures on these fragments could still be estimated from the number of associated tubercles, but with somewhat less accuracy. Hence it was decided to examine each stage of the method separately with a view to overcoming this difficulty. The areas beneath the punctures are relatively less impregnated with protective substances than the external layers, and are correspondingly less resistant to chemical attack. It was found from experiments on powdered insects that the areas beneath the punctures were not visibly affected by any single stage of the method, but that when this did occur it was due to the action of acid following that of alkali. Experiments were therefore made with the method using milder alkalis such as sodium carbonate and ammonia solution in place of

potassium hydroxide. Ammonia was found to be almost as effective as potassium hydroxide for removing the degradation products of the acid treatment and produced a considerable improvement in the microscopic appearance of the isolated insect fragments. On the other hand, replacement of sulphuric acid by perchloric acid in the final acetylation stage produced little improvement in the appearance of the fragments and was less effective in dissolving the cellulosic residue. It was decided therefore to use ammonia solution in the process when S. *paniceum* was known to be present. This modification was not necessary in the case of *P. tectus*, for which insect no difficulty was experienced in the recognition and counting of the punctures.

(e) *Method.* Weigh accurately about 5 to 10 g. of the infested powdered drug and macerate when necessary with light petroleum, ether, alcohol or other suitable solvent in a flask for a few hours or overnight. Filter through a No. 3 sintered glass crucible under reduced pressure, rinse the flask with a small volume of the solvent and add the rinsings to the filter. Partly dry the powder on the filter by suction for a few minutes and return it to the flask as a cake. Rinse any residue on the filter into the flask with a few ml. of water and shake well to break up the cake, add water sufficient to make the volume up to 90 ml. followed by 10 ml. of nitric acid. Heat gently, guarding against excessive frothing, and finally boil for 1 to 2 minutes. Alternatively where the drug is practically starch-free, frothing may be minimised by first boiling for about a minute before slowly adding the nitric acid. Filter through the original crucible, wash the residue with hot water, partly dry by suction and return to the flask as before. Make up the volume to 75 ml. with water, add 25 ml. of 10 per cent. w/v potassium hydroxide solution, bring to the boil and boil for about half a minute. Alternatively where *S. paniceum* is known to be present, adjust the volume to 90 ml. with water and add 10 ml. of strong solution of ammonia. Filter through the original crucible, wash with hot water, partly dry by suction and displace the last traces of water by allowing a few ml. of glacial acetic acid to pass through the residue. Transfer it to a dry flask of about 50 ml. capacity and remove any remaining fragments from the filter to the flask with the aid of 10 ml. of acetic anhydride and a small stiff-bristled brush. Shake well to disintegrate the residue, add a mixture of 8 ml. acetic anhydride with 2 ml. of concentrated sulphuric acid and warm gently until solution of the cellulosic material is complete. Transfer the liquid to a centrifuge tube, decant the supernatant liquid after centrifuging, and wash the residue once with glacial acetic acid and once with water, centrifuging each time. To the residue in the tube add from 0·1 ml. to 0·5 ml., accurately measured, of a 1 per cent. w/v suspension of lycopodium in suspending fluid, mix well and prepare slides following the method described in the British Pharmacopœia 1948 Appendix XIV, for the determination of foreign organic matter. Count the spores in 25 fields and examine the whole area of the mount systematically, counting the strial punctures on all the fragments of elytron observed, broken

punctures being counted as one. Calculate the number of beetles present from the elytral puncture number for the particular insect present.

RESULTS

Material was prepared for examination by triturating a known number of beetles to a moderately fine powder with sodium chloride to facilitate powdering, and adding the mixture to about 5 g. to 10 g. of a commercial drug powder known to be free from infestation. A number of such mixtures were prepared using *S. paniceum* and *P. tectus*, both separately and together; results appear in Table IV.

TABLE IV

RESULTS OF DETERMINATIONS ON INFESTED POWDERS

Insect	Drug	Numbers of insects		Recovery per cent.
		added	found	
S. paniceum	Liquorice root ...	15	14·4	95·9
	Hyoscyamus	10	9·2	91·9
	Belladonna herb ...	10	9·0	90·1
	Ginger...	5	4·7	93·8
P. tectus	Rhubarb	10	10·3	103·4
		5	5·2	103·4
S. paniceum	Rhubarb	5	4·9	97·6
P. tectus in admixture ...		5	5·0	100·3

DISCUSSION

The results in Table IV indicate that the method as described is successful in separating all the required insect fragments from the infested drug powder. The lower recovery figures for *S. paniceum* may be attributed to the use of a working mean calculated from counts on powdered beetles not subjected to the extraction process. It is during this process that partial destruction of the structures by which the strial punctures are recognised may occur, even though this is minimised by using ammonia in place of potassium hydroxide. No such difficulty is encountered in the case of *P. tectus*, for which the recovery figures are slightly over 100 per cent. This over-estimation is attributable mainly to error introduced by counting broken punctures, which in this beetle are easily recognisable as such even when considerably damaged and partly to confusion of punctures from the pronotum with those of the elytra. For either beetle, these errors may be allowed for by adjustment of the working mean after due experience of the method. Alternatively in the case of *S. paniceum* the determination could be based on the total area of the elytral fragments. The working value may be calculated by putting $y = 322$ punctures per elytron in the equation for the graph in Figure 1 giving a value of 1·25 sq. mm. per elytron or 2·5 sq. mm. per beetle. This figure, and not that of 1·63 sq. mm. from Table I, should be used, since the latter was determined on a relatively small number of beetles selected to cover the observed size range without

regard to their frequency of occurrence, and is not, therefore, a good estimate of the mean of a random sample. This alternative procedure, although more tedious, should improve the recovery percentage since elytral fragments can be recognised as such, even though the punctures may be so far damaged as to be uncountable.

The results in Table II for the variation of the number of punctures per elytron with the size of the fragment indicate a tendency towards over-estimation of the puncture number with decreasing fragment size. This increase, which is probably introduced by counting all broken punctures as whole ones, is small and negligible in comparison with that due to random effects between samples. In practice, therefore, the puncture number can be considered as independent of the fragment size and thus of the degree of comminution of the drug powder.

The use of the lycopodium method for obtaining an aliquot of the final suspension of the residue from the extraction process is open to objection since the insect fragments are relatively much larger and less numerous than the lycopodium spores. Thus for low infestations difficulties may arise in obtaining a uniform suspension for preparation of the slides. In such a case it would be preferable to transfer the residue completely to the slide and count every elytral fragment. The extension of this work to other beetle pests has not been possible to date due to lack of sufficient material, but the figures in Table I may be taken as a starting point for the insects concerned.

SUMMARY

1. The object of this work was to establish a method for the quantitative determination of beetle infestation in powdered drugs.

2. The elytron of the beetle was selected as the most suitable segment for this purpose and its numerical characteristics investigated.

3. The number of strial punctures per elytron varies over a narrower range than does the area, and is independent of the degree of comminution. The mean values are for *S. paniceum* 322 and for *P. tectus* 312.

4. A method for separating beetle fragments from powdered drugs and for calculating the number of beetles originally present is described.

5. Results of determinations on prepared mixtures containing *S. paniceum* and *P. tectus* indicate that the method is accurate and applicable to mixed infestations.

REFERENCES

1. *Official and Tentative Methods of Analysis, Association of Official Agricultural Chemists,* 1945.
2. Walker and Dalby, *Cereal Chemistry,* 24, 283.
3. Melville, *J. Pharm. Pharmacol.,* 1949, **1**, 649.

DISCUSSION

The paper was presented by DR. C. MELVILLE.

The CHAIRMAN described the paper as providing an ingenious quantitative estimation of beetles.

MR. T. C. DENSTON (London) pointed out that the damage to crude

drugs was caused very largely by larvæ, and in certain stages and at certain times of the year the insects were in a larval condition with relatively few adults. That being so, he wondered whether it was appropriate to refer to the method as giving a quantitative determination of insect infestation.

DR. J. W. FAIRBAIRN (London) said that scientifically it would have been more accurate to count the number of lumen punctures and divide the result by two, although the final answer would have been lower if that method were employed. Referring to Table IV, he said that the results were good if the figure of 14.4 was determined by examination of one side only. He suggested that the heading under Table I should read " Numbers of strial punctures and areas per elytron."

DR. G. FOSTER (Dartford) criticised the counting of fractions of insects as would appear to be the case in Table IV.

DR. J. M. ROWSON (London), in a written contribution to the discussion, said that the paper was a further step towards solving the problem of infestation control in powdered drugs, and he hoped that the author would continue that work with a view to the general application of the method to B.P. and B.P.C. drugs. With that in view and considering the range of values found in Table I, it would be interesting to know whether some average mean value for number of punctures for different insects would have to be taken so that the insect infestation could be expressed as *x* per cent. " calculated as dried *S. paniceum*." The author would doubtless bear in mind the need for some method of detecting other animal infestation where elytra were absent such as larvæ and moths.

DR. T. E. WALLIS (London), in a written contribution to the discussion, said that he would like to thank Dr. Melville for undertaking an investigation which was in urgent need of attention. So far as could be judged, the research had been developed on sound lines, establishing first fundamental criteria based upon the insects alone and then testing their application to known degrees of infestation. These results would enable some limit to be set to the amount of insects allowable and a suggestion by the author as to a suitable limit would have been welcome. A very heavy infestation would appear as a quite small percentage by weight, but Dr. Melville's work should enable some expression to be made in terms of the number of insects present in a given weight of drug.

DR. MELVILLE, in reply, said that in his opinion the number of beetles would correspond with the number of larvæ present, but the same principles could be applied relatively easily to the development of a quantitative estimation of larvæ. In reply to the suggestion that the broken punctures be counted and the number divided by 2 the difficulty was that they did not break evenly. The counts in Table IV were obtained by taking the average of 3 slides. As to the criticism of recording counts such as 9·4 beetles, it was maintained that fragments of beetles were being dealt with and not whole insects. The suggestion that it might be possible to calculate insect infestation on the basis of one specified kind of insect was feasible.