# MICROSCOPIC ANALYSIS OF FAECES, A TECHNIQUE FOR ASCERTAINING THE DIET OF HERBIVOROUS MAMMALS

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[*Manuscript received August* 11, 1960]

*Summary*

A technique is described that is being used in diet studies in kangaroos and wallabies. It is based on the identification of the epidermis of leaves and stems of plants recovered in faeces. Data obtained with it are qualitative and quantitative because (1) there is little or no digestion of epidermis that is encased in cutin, (2) epidermis is usually identifiable to species under low-power miscroscope, and (3) there is a determinable relation in each species of plant between the surface area and dry weight of its foliage.

## I. Introduction

Two events in 1954 stimulated research into the nutrition of the quokkas (*Setonix brachyurus*) of Rottnest I., W.A. First was the observation of a late-summer population "crash" (Waring 1956); then came the discovery by Moir, Somers, and Waring (1956) of the quokka's ruminant-like digestion. Among other projects, work was begun on the trace-element requirements of the quokkas and on the seasonal fluctuation in their health and condition (for preliminary accounts of this research see, respectively, Barker and Barker (1959) and Shield (1959). Development of both studies required a detailed knowledge of the animals' diet. Considerable information had been obtained by observing the incidence of grazing and browsing injury to plants; but such data were only crudely quantifiable and, moreover, were often unassignable to a precise time—a serious defect in view of the seasonal variation in the studied physiological characters.

The methods currently used in studies of livestock nutrition, including those that depend on indicator materials and faecal nitrogen (reviewed by Brown 1954), seemed unsuitable for our purpose. They measure total herbage intake, rather than the fractions of individual food items, which was needed in the Rottnest studies, where in a small area a number of plants of quite different nutritive value may be eaten. A technique was therefore sought that would yield both quantitative and qualitative data for particular times of the year.

Dr. A. R. Main then made the suggestion that quokka faeces, examined microscopically, might furnish the desired information. To test this, penned quokkas were fed for 5 days on a single (and different) item of natural herbage. Their faeces were collected on the sixth day, pounded up, washed, and temporarily mounted. All seven samples contained considerable amounts of epidermal tissue, which differed quite clearly from species to species. It was later found that epidermis from all perennial and a few annual plants passes undigested through the quokka's tract, and that there is no great difficulty in identifying to species the epidermis from plants growing in a restricted area.

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## II. Preparation of Permanent Slides of Plant Epidermis

The first requirement in a diet study using microscopic analysis is a reference collection of epidermis from food plants in the study area.

From large leaves an oblong of about 2 by 1 cm is cut; or if small, the leaves are merely trimmed round their edges. Unless this is done, the two surfaces do not separate in the subsequent treatment, which results in an unnecessarily indistinct picture of epidermal characters. Moreover, in plants with dorsiventral leaves it is necessary to have representative tissue from each surface, for they may be very different. As the epidermis from stems is usually modified (see Plate 1, Figs. 1 and 2), separate samples of these are required too, the stems being chopped into lengths of about 2 cm and split longitudinally.

The material is placed in a 150-ml flask containing 5 ml each of 10% nitric and chromic acid, fitted to a reflux condenser, and boiled until the mesophyll disintegrates and the two integuments of each leaf drift apart. This usually requires about 3 min, but in species with very fibrous leaves the operation may take more than 10 min. If a fume-cupboard is available, condensers may be dispensed with; but as there will be no recovery, more acid must be used initially.

After maceration the contents of the flask are poured into a 250-ml beaker, which is topped up with tap water. When the plant fragments have settled, as much fluid as possible is drained off and the beaker is refilled with water to which is added a few drops of ammonia. The supernatant fluid is again drained off and replaced with fresh water. The plant fragments are then transferred to a centrifuge tube, drained, and dyed with an alcoholic solution of gentian violet.

After standing for at least 30 min the tubes are topped up with 70% alcohol which is changed two or three times with upgraded alcohol until excess dye is removed. Changes are facilitated by centrifuging the tubes for 3 min at 3000 rev/min, by which time solids are sufficiently packed at the bottom for the tubes to be safely inverted for draining.

After a final change in absolute alcohol the contents of the tube are poured into a petri dish, whence epidermis is transferred to a slide with a small camel-hair brush. The epidermis is smoothed by gently stroking with a brush and, after removing excess alcohol with blotting-paper, it is mounted in euparal.

## III. Identification of Epidermis

The characters used in diagnosis may be grouped under one or another of five headings.

### (*a*) *Stain Reaction*

As all preparations of foliage and faeces are stained with gentian violet, differences in stain intensity and colour are often helpful. In many species only the vertical walls of cells are stained and the preparation has the appearance of lace (see Plate 1, Figs. 3 and 4). However, in most sclerophylls all parts of the epidermis are evenly and heavily stained. Epidermal tissues usually stain some shade or other of violet, but in a few species the colour is so altered as to be of diagnostic value, and in still fewer species the epidermis is completely unstained.

### (*b*) *Orientation*

Monocotyledons, especially, are characterized by a definite orientation of cells; in all species examined the ordinary epidermal cells are arranged in rows parallel to the venation and axis of the leaf. This has been observed only rarely in dicotyledons.

### (*c*) *Shape, Size, and Inclusions of Ordinary Cells*

The unspecialized epidermal cells may be described with respect to their shape, size, walls, and inclusions. Those of dicotyledons are typically hexagonal with a lesser or greater degree of rounding and elongation; in monocotyledons they are elongate rectangular. Within a leaf there is considerable range in cell size, the diameter of the largest commonly exceeding twice that of the smallest. However, the difference in mean cell size between two related species may be so marked as to be diagnostic. Width of cell walls varies from more than 10 to less than 1$\mu$. Walls may be straight, slightly or considerably curved, undulate, or, as in grasses and sedges, obtusely or acutely crenulate.

### (*d*) *Stomata*

These may be characterized by the presence or absence of subsidiary cells, orientation of the pore, location, and density. Metcalfe and Chalk (1950) classify the stomata of dicotyledons according to the number and arrangement of subsidiary cells. Three of their five categories are commonly encountered in Australian plants, viz. (1) *anomocytic*, where there are no subsidiary cells, i.e. guard cells are immediately surrounded by undifferentiated epidermal cells; (2) *paracytic*, where there are two subsidiary cells similar in size and shape and whose common wall is co-linear with the aperture; (3) *anisocytic*, where the subsidiary cells (usually three) are of indeterminate size and shape and whose common walls have no fixed relation to the stoma.

Within a species, stomatal cells are generally much more constant in size and shape than are ordinary epidermal cells. Since subsidiary cells may be obscure or absent, it is best for interspecific comparison to consider the size and shape of the area occupied by the two guard cells.

The location of stomata is indefinite in dicotyledons; they occur, if at all, haphazardly throughout the epidermis. The stomata of monocotyledons are usually restricted to definite sites—in most species to between the ends of ordinary cells, but occasionally to rows consisting exclusively of stomata.

In many species of shrubs stomata are completely absent from the dorsal surface of leaves. Elsewhere their density may be as low as 3 and as high as 450 per sq. mm.

### (*e*) *Trichomes*

The term trichome is a general one for all outgrowths of the epidermis. They are, however, frequently deciduous and so are generally less useful than stomata in diagnosis.

Clothing trichomes usually take the form of hairs, but in succulents they are commonly vesicular, and in a few species they are merely papillose projections from the epidermis. Hairs usually occur singly, but in certain genera and families they are clustered radially about a multicellular base or column. Single hairs may be 1-, 2-,

or 3-cellular. In shape they are typically long, narrow, distally pointed, and proximally wide and flat; exceptional are the short thorn-like hairs of *Spinifex*, the distally rounded hairs of *Frankenia*, the proximally pointed hairs of *Guichenotia*, and the branching hairs of *Solanum*. Vesicles may be sessile or attached to a short, unicellular stalk or a multicellular base. Density of trichomes ranges from 2 per sq. mm in species that are almost glabrous to 700 in species whose ventral leaf surface is completely obscured by hairs.

## IV. Preparation and Analysis of Slides of Faecal Residues

With a few modifications the preparation of faecal samples follows the procedure, described earlier, for plant material. Faeces should be first dried and thoroughly ground. Owing to its previous digestion the sample need only be boiled in the acid mixture for a minute. Since faecal residues adsorb more dye, one or two additional changes of alcohol are required for the removal of the excess. Furthermore, before changing alcohol it is advisable to centrifuge for at least 5 min lest light material be lost when tubes are inverted for draining.

Slides are examined under low power (45 ×) by systematically traversing zones 2·8 mm wide (i.e. the diameter of the field of view), whose centres are 5 mm apart. Fragments of epidermis are identified and their area is estimated in hundredths of a square millimetre by using a graduated eyepiece (an apparent millimetre divided into tenths and hundredths). The percentage by area of each species present is then obtained for each slide.

Relative area of epidermis is not in itself meaningful; what is wanted for further analysis is the relative weight of foliage of each species ingested. The relation between dry weight and surface area of leaf varies from species to species; nor is it constant throughout the life of a leaf, as will be seen in the equation

$$W/A = vd/A = td,$$

where $W$ is the dry weight, $A$ the area, $v$ the volume, $d$ the density, and $t$ the thickness of a leaf. However, intraspecific variation in the coefficient $W/A$ is small as compared with that possible between different species; consequently if it is ignored, certain categories of plants will be grossly overestimated in the analysis. A mean value of $W/A$ for each species may be obtained by dividing the total dry weight (in mg) of a representative shoot by its total surface area (in sq. mm). By applying the appropriate coefficients, areas of epidermis are converted into weight equivalent of foliage, and the proportion of each item in a sample is recalculated on a dry weight basis.

Whether data derived in this manner accurately reflect the proportion by weight of the various species ingested is not yet known. At present two assumptions are made: (1) that digestibility of epidermis is approximately the same in all perennial plants; and (2) that sampling errors can be nullified by replication and pooling of data.

## V. Digestibility of Epidermis

The assumption that there is no differential digestibility of epidermis among perennial species is based on the more general belief that digestibility of epidermis is all or nothing, the evidence for which is now discussed.

In the preparation of epidermis from annual plants, the foliage of most species disintegrates as soon as the acid mixture reaches boiling point; usually the only part of the epidermis that is recovered is the cuticle. Similar fragments of cuticle are recovered in faeces (where they are measured and recorded as "annual herbs"). On the other hand, the entire epidermis of all perennial species survives maceration and, when eaten, likewise survives digestion. Moreover, epidermis in faeces, apart from being fragmented into smaller clusters of cells, has the same appearance as that macerated *in vitro*; i.e. there is no indication of digestion, such as the erosion of cell walls.

The observed difference in digestibility between annuals and perennials is associated with the distribution rather than the thickness of cutin. Examination under polarized light of transverse sections of leaves of *Medicago* and other annuals

TABLE 1

MEAN PROPORTION BY AREA OF EPIDERMIS OF FOUR PLANTS IN FOOD AND FAECES

Standard deviations shown in parenthesis

| Species | Food | Faeces |
|---|---|---|
| *Acacia* | 0·246 (0·086) | 0·237 (0·090) |
| *Olearia* | 0·270 (0·100) | 0·268 (0·057) |
| *Rhagodia* | 0·288 (0·084) | 0·306 (0·099) |
| *Scaevola* | 0·196 (0·113) | 0·189 (0·072) |

reveals that only the outer wall of epidermal cells is covered by a layer of cutin. In the perennials examined, cutin is deposited on all walls of the cells; the cuticle, in effect, extends down between cells and completely surrounds them (see Plate 1, Figs. 5 and 6). Thus the epidermis of perennial plants seems to owe its resistance to both acid maceration and quokka digestion to its being completely encased in cutin.

To ensure further that there is no difference between perennials in digestibility of epidermis, the following experiment was made. A food mixture was prepared consisting of dried, finely chopped *Acacia rostellifera*, *Olearia axillaris*, *Rhagodia baccata*, and *Scaevola crassifolia* (250 g each), made cohesive and palatable with wheaten starch (300 g), sugar (200 g), molasses (130 g), sodium chloride (40 g), and casein (30 g). Three separately penned quokkas (adult females) were deprived of food for 2 days and then fed on the mixture for 5 days. On the morning of the sixth day freshly defecated pellets were collected. A sample of the offered food and a sample of the faeces from one quokka were macerated and stained. From each sample 10 temporary mounts were analysed, the material on a slide being returned to the appropriate sample after examination. The results are set out in Table 1.

In view of the large variation from slide to slide, differences between columns are clearly not significant. Indeed, inter-slide variability is so great that the method could only reveal relatively large interspecific differences in digestibility, which is certainly not the case here. Since the four species tested are a small but representative cross section of the local flora (they are respectively a tall shrub with large hard leaves; a medium shrub with small, soft, hirsute leaves; a scrambling shrub with small succulent leaves; and a low shrub with large subsucculent leaves) the results are consistent with the belief that there is little or no difference in digestibility of epidermis among local perennial plants.

## VI. Sampling Errors

For various reasons the author has always left intact that portion of a faecal, sample not immediately prepared into slides. That is, instead of grinding and mixing the whole of a sample and taking from it a convenient-sized subsample, the basic unit for preparation has been the faecal pellet (*c.* 2 for bettongs, 1 for quokkas and other wallabies, $\frac{1}{2}$ for kangaroos). Since the quokka defecates in the order of 20 pellets per day, our subsample would at first sight seem adequate for determining the diet during a night's feeding. It would be so, if the contents of the stomach were well mixed during digestion; but examination *in situ* of dyed foods shows that the passage of particles through the pylorus is roughly in the sequence of ingestion. Therefore at best, examination of a single pellet can only give information about the diet of an hour or two.

Information for a longer period may be obtained by analysing a subsample drawn from a homogeneous mixture of the whole sample; or, as is the author's practise, by pooling the data derived from analysis of at least 20 pellets, each from a different animal. The latter course is preferable in a population study, since it takes some account of individual variation in food preferences, and gives better coverage of a habitat. Even when the data are finally pooled, it is advisable to analyse separately the faeces of each animal. In this way we have, if required, some information on the diet of individuals; but more important, such data can be reorganized to answer various questions that may arise during the study, e.g. are there differences in diet due to age or sex?

From each quokka pellet two slides are prepared and examined. While usually the same plants are present in each, their proportions are sometimes very different. Discrepancies arise, not from failure to mix adequately the preparation before mounting, but from the great variation in size of epidermal fragments. The area of more than 90% of the fragments is less than 0·3 sq. mm, but occasionally much larger pieces are encountered. The frequency of the latter is so low that their number on any one slide is largely a matter of chance; yet by virtue of their size they may contribute more to the estimate than all other fragments together. The question, then, is how many slides must be pooled before the larger fragments of epidermis no longer greatly disturb mean proportions.

From the results of the feeding trial we have the standard deviations of the proportions of four species of plants in single slides of prepared faeces. These values may be pooled after expressing them as percentages of the appropriate means. Thus

the average standard deviation of data obtained from single slides is estimated as 32%. With this we may calculate the expected standard deviation of means of various numbers of slides from the formula $S_n = S_1/\sqrt{n}$. Expected percentage standard deviations of means of 2, 3, 4, 5, and 10 slides are 23, 19, 16, 14, and 10 respectively. In other words, the probability of an estimate based on the mean of 10 slides being within 10% of the true (or sample) proportion is approximately 67%; and within 20% of the true value, approximately 95%.

Analysis of a large number of slides prepared from a single sample of faeces will only be necessary when precise information is required about the diet of individual animals. More often information will be required about a population. As stated earlier, in population studies the author prepares only one faecal pellet from each quokka, the composition of the preparation being based on the mean of the proportions observed in two slides. Data on the diet of single animals will therefore have an expected standard deviation of 23%. Using again the formula $S_n = S_1/\sqrt{n}$ we calculate the expected standard deviation of means of 10 animals as 7% and of 20 animals as 5%. These estimates necessarily ignore differences between animals to food preferences and differences throughout the study area as to availability of food plants.

## VII. Discussion

Although this technique was devised to meet a particular situation, it could well be applied to a wide range of studies. Its special advantage is that the animal may be returned to the study area after a sample of its faeces has been collected. A technique not necessitating the death of the animal is desirable where there are concurrent population studies and is obligatory in research on livestock and on rare and protected animals, as in sanctuaries. The technique could also be valuable in subjects that are hard to trap or shoot but whose faeces are identifiable and easily procured. Even if the animal is shot it makes no difference, at least in macropods, whether material is collected from the stomach or rectum; the contents of all stomachs examined by the author were too thoroughly masticated for macroscopic determination of items.

The chief disadvantage of the technique is its inability to cope satisfactorily with the more tender annual plants, mostly introduced. This seldom matters in macropod studies; for over a large part of Australia this category of plant is absent or ephemeral. In other areas, such as the Wheat Belt or on Rottnest I., these plants may bulk considerably in the diet during winter and spring; but in late summer when the diet is most critical from a nutritional viewpoint, the category is ordinarily absent. It is only in the cooler and more humid areas of southern Australia, and especially where exotic pastures have been established, that the incidence of tender-leaved herbs will be so high that they cannot be ignored.

## VIII. Acknowledgments

The author is grateful to Dr. A. R. Main of this Department for first suggesting this work and for his subsequent supervision of it; to Mr. D. M. Churchill, Botany

Department, University of Western Australia, for advice in matters of plant histology; and to Mr. R. J. Moir, Institute of Agriculture, University of Western Australia, for criticism of the manuscript.

The work was financed by a research grant from the University of Western Australia and from a grant by C.S.I.R.O. to Professor H. Waring for marsupial studies.

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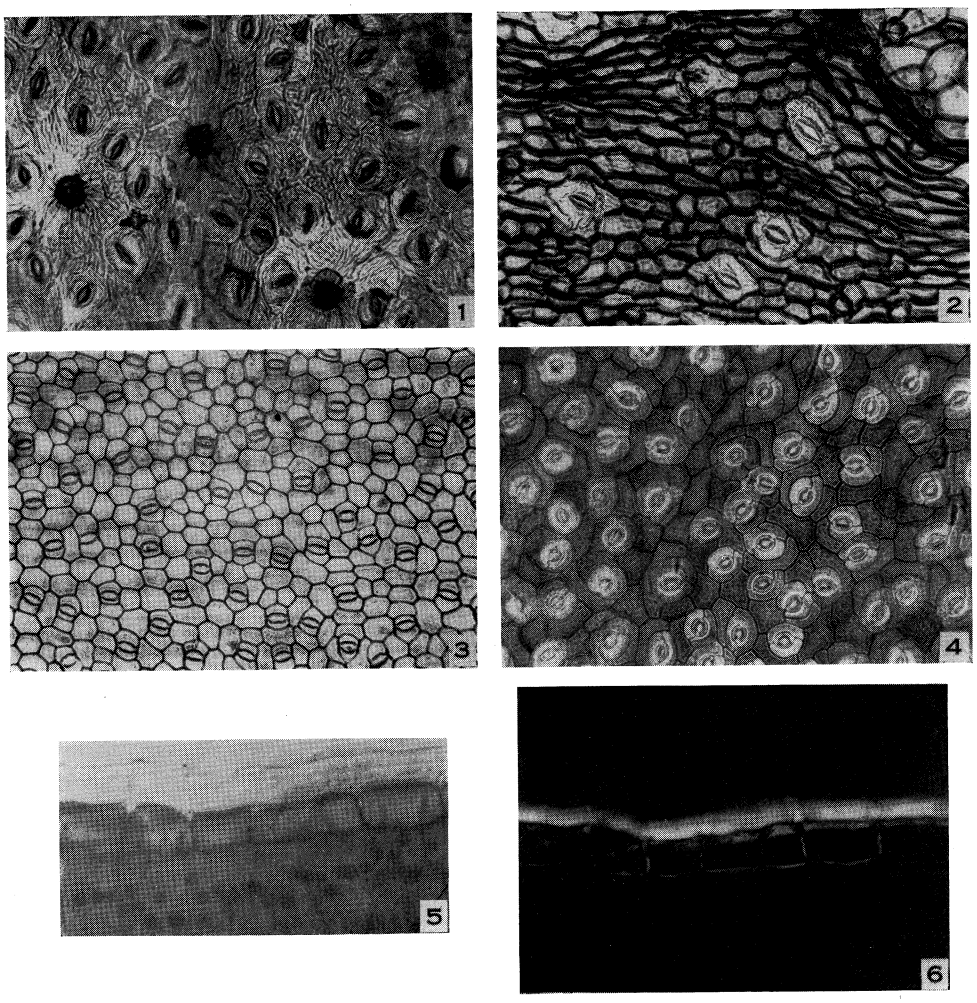
MICROSCOPIC ANALYSIS OF FAECES




Figs. 1 and 2.—*Myoporum insulare*, showing difference between epidermis from leaf (Fig. 1) and from stem (Fig. 2). In the latter, observe the absence of glands, the elongation of cells, and the thickening of cell walls.

Figs. 3 and 4.—Showing contrast in stain reaction between *Carpobrotus aequilaterus* (Fig. 3) and *Acacia cuneata* (Fig. 4). In the former only the vertical walls of epidermal cells are stained.

Figs. 5 and 6.—Transverse section through epidermis in leaf of *Alyxia buxifolia*, photographed with ordinary light (Fig. 5) and polarized light (Fig. 6).