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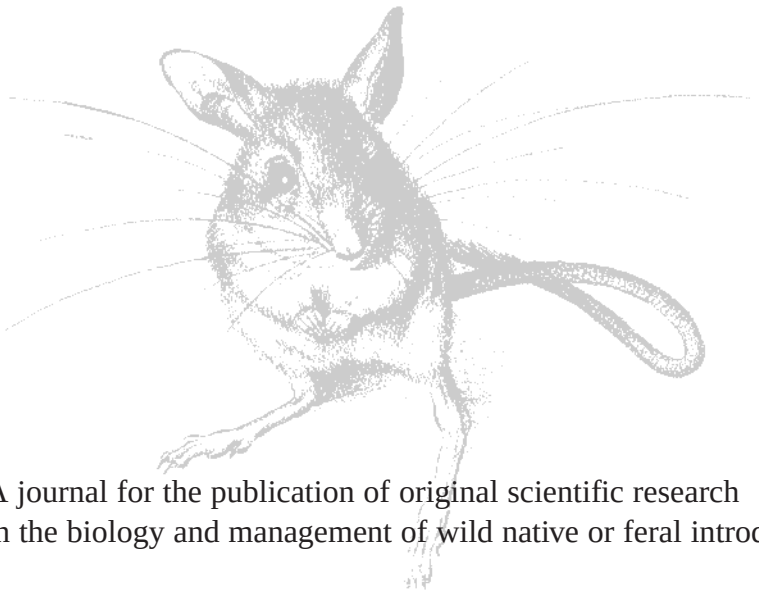

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# A new technique for measuring size distributions of fine ingesta/digesta particles

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*Abstract*. Techniques were developed to enable convenient, high-power image analysis of (ingested) food material. A constant volume of diluted gut sample was delivered to a large microscope slide before being slowly evaporated in still air to leave all particles statically on the same focal plane. Evaporation also allowed a meniscus to develop around each particle, forcing them to separate and thereby preventing overlap and aggregation of particles. Sub-samples were measured under four high-power magnifications (×2050, ×1290, ×510 and ×190) to permit precise estimates of size distributions of the very small particles. The techniques developed avoid the need for large ingesta/digesta samples, sieving, and filtering, all of which have limited previous studies.

## Introduction

The rate and extent of both *in vitro* and *in vivo* fermentation is known to be affected by the size of the food particles presented to the gut microflora (Pearce and Moir 1964; McLeod and Minson 1969). Hence, it is often desirable to determine the size distribution of ingesta particles that result from differential dental or feeding conditions.

Size distribution analysis of ingesta particles usually involves the sieving of gut samples through three or four different mesh sizes to separate the particles into broad size classes (e.g. Fujikura *et al.* 1989; Freudenberger 1992). Wet weights of each size class are then obtained and a histogram produced. However, there are many problems associated with such an approach. Since ingested particles are not perfectly spherical, size is not the only factor that determines whether or not a particle passes through, or is retained on, the mesh – the orientation of the particle as it approaches the mesh is also important. Furthermore, larger particles tend to block the mesh, preventing the smaller particles passing through, and the size of the smallest particles sampled depends on the size of the smallest mesh that is used. Thus, it is difficult to determine, with any accuracy, the fraction of very small particles in a gut sample. Particle size distributions that are based on the wet weights of broad dimensional classes do not allow the detection of any subtle differences in size distributions, and may be of limited value in feeding studies. Importantly, using sieving techniques to determine particle size distributions also requires large volumes of the material, often necessitating the death of subject animals.

In order to examine the dimensions of forage and digesta particles more closely, recent attention has focussed on the use of image analysis systems (e.g. Oura and Sekine 1989; Luginbuhl *et al.* 1991; van der Bilt *et al.* 1993). In preparing samples for image analysis, all particles must be separated from one another (i.e. they must not touch or overlap) in order to be measured individually. Manually separating particles from each other is an extremely laborious task, becoming increasingly more difficult for smaller particles. Consequently, samples are usually pre-sieved (1.0-mm or larger sieve) to exclude the small particles (Luginbuhl *et al.* 1991; van der Bilt *et al.* 1993). Once adequately spread, the samples are then analysed under a single, low magnification. Unfortunately, these methods also fail to deal with the very small particles. Firstly, the sieving actually removes the small particles from the sample. Secondly, even if these particles were present, the magnification used is not great enough to detect them. It is likely, however, that it is these very small particles that are of greatest importance (Pearce and Moir 1964; McLeod and Minson 1969) to the herbivore, since these particles provide the greatest relative surface area of assimilable tissue.

Various other constraints apply in the measurement of particle sizes produced by comminution. Accurate measurement of the dimensions of the wide range of particle sizes produced requires that samples must be examined at a number of different magnifications because at low magnifications the very small particles are not visible, whilst at higher magnifications the larger particles have a greater probability of extending beyond the edge of the viewing area so that they cannot be measured. Sub-sampling by examining a number of fields of view has the additional requirement that all particles be evenly distributed. Furthermore, depth-

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of-field constraints imposed under high-power light microscopy require that all particles be on the same plane. It is also necessary to prevent the Brownian motion of particles in suspension. Each sub-sample must be of the same dilution so that biases in the probabilities of encountering each particle size are consistent between all subsamples.

Hence, the aim of this study was to further develop the current methodologies used to measure size distributions of ingesta particles. A technique was developed that uses an autopipette to deliver a constant volume of gut solution to a microscope slide, dilute it and allow the fluid to evaporate.

## Methods

Ingesta was collected by removing the contents of the oesophagus just above the cardia from six adult male and six sub-adult male red kangaroos (*Macropus rufus*). Pre-cardial collection ensured that the size and shape of the particles resulted only from the current masticatory apparatus and not from extensive enzymatic digestion or mixing with older particles in the forestomach. Each sample was placed in 20 mL of FAA (formalin acetic acid) to preserve the integrity of the ingesta particles indefinitely (O'Brien and McCully 1981). In preparation for sub-sampling, oesophageal contents were transferred to larger bottles (1000 mL) and diluted to a density of 165 g $L^{-1}$ by the addition of more FAA solution.

### *Subsampling to slides*

A High Tech Lab V3-Series autopipette (200 μL with a 0.565-mm tip aperture diameter), was attached to a bottle lid to allow the tip of the autopipette to remain stationary with respect to the bottle, whilst the bottle was being agitated. Mechanical agitation devices were explored; however, they all produced some periodicity, thus inducing vortices and resulting in unequal densities within the suspension. The bottle containing the oesophageal sample was agitated by hand in all planes for at least 30 s in order to thoroughly mix the sample and yet prevent the initiation of vortices. The button on the partly submerged autopipette was then depressed and released, while still agitating, thereby drawing up exactly 0.1 mL of sample. The lid was then carefully removed and the subsample was delivered to a 76 × 50 × 1.2 mm microscope slide. Water (1 mL) was applied to the slide and thoroughly mixed with the sample, which was then spread across the whole slide. Other substances were tested for this dilution, including the FAA preservative, but all were found to either evaporate too fast or else leave a residue on the slides. Likewise, preliminary trials involving alternative sample volume to water volume ratios failed to separate particles adequately for image analysis.

Slides were placed on a perfectly flat surface at a room temperature of 17°C (still air) to allow the liquid component of the sample to slowly evaporate. Evaporating the liquid from the sample removed the problems of Brownian motion, particles being at different focal planes, and differences in settling rates that make it hard to view particles in solution. Moreover, as the liquid slowly evaporated, a meniscus formed around each particle, which prevented the particles from settling together. The rate of evaporation was found to be crucial to the settling locations of the very fine particles. Unless evaporation was very slow (at least 10 h), these particles tended to aggregate together and away from the other particles. In order to achieve these very slow evaporation rates, it was found that slides had to be left to evaporate at temperatures of 17°C or less and in still air.

Three replicate slides per oesophageal sample (resulting in 36 subsample slides) were produced to determine the variation within an oesophagus and to test the techniques of subsampling. All slides were protected from dust contamination by a plastic 'tent' during evaporation. Coverslips were not required, which avoided the problems of disturbing the samples with the coverslip-mounting medium.

### *Image analysis*

Computer-aided image analysis provides a powerful, non-subjective means of rapidly measuring the dimensions of numerous particles that are nongeometrical in shape. However, it is important to understand the limitations and requirements of image analysis systems. Image analysis is very sensitive to fluctuations in light levels, as these affect the grey level values of the pixels and therefore the size and shape of the objects to be measured. This becomes increasingly more critical with increased magnification and for particles with very thin, tapering edges. Therefore, great care should be taken to reduce fluctuations in light level.

A CCD (Panasonic WV-CD50) camera, mounted on a Leitz Orthoplan microscope, and connected to a Data Translation DT2867LC frame grabber board in association with Bioscan™ (Autoscan Pty Ltd) image analysis software was used to capture images for processing. Each captured image consisted of a 512 × 768 pixel array ranging in grey level from 0 (black) to 256 (white). Hence, each particle was represented on the display as an object with pixel values lower than that of the background. Once an image was captured, its grey-level pixel array was divided by the grey-level pixel array of reference image (an image of a blank slide under the same light conditions), before the result was multiplied by the average grey level of the reference image. This process standardises any slight fluctuations in the lighting conditions across an image. A circular, digital filter was then passed over the image so as to enhance object edges and to remove any granularity in the image. Grey-level thresholds were then set to arithmetically distinguish the objects (particles) from the background. Finally, the system was used to measure the two-dimensional area of every object on the display.

Image analysis software packages measure the area of a defined object by multiplying the number of pixels the screen object contains by the scaled area of a single pixel. Thus, due to the shape of the pixels and digital rounding errors, objects that are very small on the display, and therefore made up of only a few pixels, are not measured very accurately. Furthermore, very large objects have a higher probability of touching the edge of the display and therefore being unmeasurable. Consequently, in order to obtain an accurate estimate of the dimensions of particles that range greatly in size, it is necessary to analyse views from a range of different magnifications and for each view it is necessary to set upper and lower limits on the size of the objects to be measured.

Sub-sample slides were viewed with a bright, lightfield set-up under four magnifications (×2050, ×1290, ×510 and ×190) for which 10, 10, 5 and 5 fields of view were captured respectively. These magnifications resulted from the use of ×40, ×25, ×10 and ×3.5 objective lenses respectively, coupled with a large focal distance between the lens and the mounted CCD camera. Blue filters were used to reduce diffraction. Each field of view was approached by randomly stepping along the X–Y scale of the stage according to a random-coordinates generator. Hence the areas of all the objects from a total of 30 views per slide were measured.

For each sub-sample slide and each magnification, a particle size frequency distribution graph was produced (examples of which are shown in Fig. 1), and the median particle area was calculated. Particle size distributions resulting from material that has been comminuted tend to be highly positively skewed (Wilson *et al.* 1989). Hence, previous workers (Fujikura *et al.* 1989; Luginbuhl *et al.* 1991) have suggested that summary statistics based on the parameters of distributions (such as normal, lognormal and gamma) are inappropriate for analysing size distributions of ingesta particles. Whilst the median itself does not portray much information about the overall shape of a distribution, for the purpose of this study it adequately provides a means by which distributions can be statistically compared (Voon *et al.* 1986).

*Statistical evaluation*

For each magnification, variance components analysis was performed to compare the variance in median particle size between the subsamples within groups (individual kangaroos), with the between-groups variance. This provided a mechanism by which the precision of the developed methodologies could be statistically tested. All significance levels (*P* value) were set at 0.05, and all statistics were performed using SYSTAT (ver. 5.03) Statistical Package.

## Results and Discussion

*Image analysis*

Careful image analysis provides a number of advantages over wet sieving when estimating size distributions of ingesta particles. Firstly, there are no restrictions on size classes, and therefore size distributions can be analysed more finely and with greater resolution. Secondly, all the small particles are retained and measured. Finally, there are likely to be fewer errors associated with careful image analysis than there are involved in the fractionation and weighing procedures of sieving.

*Subsampling to slides*

Evaporating the gut solution onto a slide has the following advantages. Firstly, and perhaps most importantly, by utilising the physical drying properties of a liquid, it separates all of the particles so that they do not overlap or aggregate. This is a major advantage for preparing gut samples for image analysis, since in order for each of the particles to be accurately measured, no particle can touch another particle. Until now this has been a major limitation, with workers needing to separate the particles by hand (e.g. Luginbuhl *et al.* 1991**;** van der Bilt *et al.* 1993), which is not only tedious but impossible for the very small particles examined in this study. To permit accurate high-power analysis, all particles must be on the same focal plane, and static. This can be achieved only by evaporating the gut solution onto a microscope slide.

Auto-pipetting (to select a constant volume of a mixed gut sample) before any settling can occur ensures that each subsample is of the same concentration. This is important because of the different probabilities of encountering particles of different sizes and because of the desirability for consistency between subsamples.

Variance components analysis indicated that there was much higher variance in the between-groups components (individual kangaroos) than in the within-groups components for all magnifications (Table 1). The consistency between sub-samples demonstrates the precision of the technique.

Fig. 1 shows two typical frequency distribution curves that were produced using the described techniques. Note that the major differences occur in the percentage frequencies of particles between 4 $\mu m^2$ and 20 $\mu m^2$ in area. These particles are smaller than those previously quantified in food particle size analysis (e.g. Freudenberger 1992). Moreover, since digestion is a process that occurs at a very small scale, differences in such fine particles are likely to have large influences on the rate and extent of digestion (Pearce and Moir 1964; McLeod and Minson 1969).

**Table 1. ANOVA and subsequent variance components (VC) analysis table for each of the four magnifications**
The relative contributions of the between-groups (between individual kangaroos) and within-groups (between subsamples) variations can be seen

| Magnification | Source | d.f. | m.s. | *F* ratio | *P* | %VC |
|---|---|---|---|---|---|---|
| ×2050 | Between groups | 11 | 1.1930 | 22.1644 | 0.0000 | 87.6 |
| | Within groups | 24 | 0.0538 | | | 12.4 |
| ×1290 | Between groups | 11 | 6.6456 | 39.6322 | 0.0000 | 92.8 |
| | Within groups | 24 | 0.1677 | | | 7.2 |
| ×510 | Between groups | 11 | 87.4983 | 34.2581 | 0.0000 | 91.5 |
| | Within groups | 24 | 2.5541 | | | 8.5 |
| ×190 | Between groups | 11 | 106.1818 | 15.9273 | 0.0000 | 83.3 |
| | Within groups | 24 | 6.6667 | | | 16.7 |

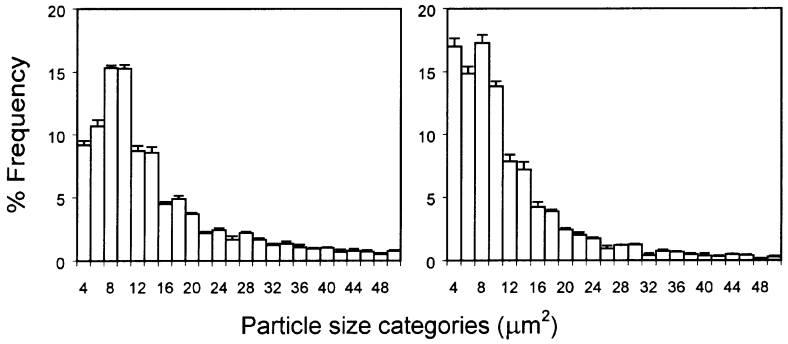


**Fig. 1.** Two contrasting frequency distributions of mean (+s.e.) percentage ingesta particle size ($mm^2$). Each displayed distribution was calculated from three subsamples. Individual subsamples were taken from material collected from the pre-cardial oesophageal region of two different red kangaroos (*Macropus rufus*) and analysed under ×510 magnification.

The technique developed is not intended to replace existing techniques of particle size analysis. Rather, it is designed to augment these methodologies by providing a means by which the fine particle fraction can be measured rather than discarded. Combining the new technique with existing techniques should therefore result in a more complete investigation of the particle size distributions. The new technique also provides a means by which the particle size distributions of smaller herbivorous animals (i.e. insects) could be measured. Such investigations have been very limited in the past due to requirements of large sample volumes and the scarcity of particle sizes available through sieving and filtering.

Whilst the technique described above was primarily designed to be used to investigate fine ingesta/digesta particles, the same principles could be applied on a larger scale to measure larger particles. The technique should be equally as effective at producing rapid, precise size distributions of virtually any small objects (such as seeds). Therefore this techniques has the potential to enhance all studies that investigate particle size distributions. Likewise, the slide preparation techniques developed should be appropriate for any application that requires small particles to be quickly and easily separated.

In conclusion, appropriate preparation of slides as well as careful image analysis allowed particle size distributions of ingesta to be investigated at a range of high-power magnifications as well as avoiding the loss of fine particles and the size class restrictions imposed by sieving and filtering.

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