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COMPARISON OF PHYSICAL SEPARATION AND ALKANE CONCENTRATIONS TO ESTIMATE THE SPECIES COMPOSITION OF HERBAGE SAMPLES FROM A PASTORAL ENVIRONMENT

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SUMMARY
The botanical composition of simple herbage mixes has been successfully estimated using the alkane profiles of pasture species. In this report, the ability of the n-alkane technique to identify the component species in more complex herbage mixes from a pastoral environment was examined. Herbage samples (quadrat cuts), collected from 3 heterogeneous pastures at Condobolin, were sorted by hand to identify the botanical components and their proportional representation. Within each pasture, 19-20 individual species were identified. Groups of species were subsequently formed on the basis of a principal components analysis of their alkane profiles. The botanical composition determined by hand sorting was then compared with the estimates obtained from a least-squares optimisation of the alkane profiles, and significant differences between the techniques were found. In general, the proportions of the *Medicago spp.* component, and of the component including *Avena spp.*, in the herbage mixes were underestimated.

Keywords: botanical composition, n-alkane, hand sorting

INTRODUCTION
Saturated hydrocarbons, n-alkanes, are a component of plant cuticular wax, and the composition of that component varies between plant species (Dove and Mayes 1991; Dove *et al*. 1996), and between plant organs and age of the plant (Dove *et al*. 1996). The botanical composition of herbage has been successfully determined in herbage mixes by using those differences in the pattern of alkane concentrations between species (Dove 1992). If applicable more generally, this would be much less labour intensive than physical separation and sorting of the components. It would also be more convenient as it need not be done immediately following sample collection. However, much of the early evaluation work has been conducted on artificial mixtures of herbage, with a limited number of components (e.g. Dove 1992; Vulich 1993).

The number of alkanes, and the differences in their concentration between the components, limits the number of botanical components that can be distinguished in a mix, whether determined by solving simultaneous equations (Dove 1992) or by least squares methods (Dove and Moore 1995; Newman *et al.* 1995). Extensively grazed pastures may have many more species present than the 8-12 alkanes usually available. To overcome this limitation, species with similar alkane compositions may be grouped, and each group considered a botanical component (Bugalho *et al.* 2002). The experiment described here compares alkane-based estimates of botanical composition with those obtained by physical separation of heterogeneous herbage samples collected in a pastoral environment.

MATERIALS AND METHODS
Location
This study was based on samples collected at the Agricultural Research and Advisory Station, Condobolin (33°04'S, 147°13'E) on the Central Western Plains of New South Wales. The Station has a mean annual rainfall of 427 mm that is non-seasonal and highly variable.

Samples
In June 1995, mixed herbage samples from sixteen 0.25 m^2 quadrats were cut to ground level in each of 3 paddocks and were refrigerated until hand-sorted to identify individual species. The separated components from each quadrat were then frozen, freeze-dried and weighed. The separated components were then recombined and ground. The concentration of alkanes in the recombined quadrat samples was determined by gas chromatography using the method described by Dove *et al.* (1996).

Pluck samples of the species present in each paddock were also collected at the same time as the quadrats were cut. These samples were collected from a number of sites across each paddock and pooled within each species. The pooled samples were immediately frozen and stored until freeze-dried and the alkane concentrations similarly determined.

The botanical composition of the herbage was estimated using a least-squares procedure (EatWhat; Dove and Moore 1995), to optimise the combination of species/components to the observed pattern of alkane concentrations in the quadrat herbage samples. As the number of species identified in the paddock exceeded the number of alkanes present in the herbage and samples, principal components analyses were conducted using S-PLUS (Statistical Sciences 1993) to identify, and then group, species that the principal components analysis indicated had similar alkane concentration patterns. As the scale of the concentrations of each of the alkanes varied, the principal components were calculated for the correlation matrix (Venables and Ripley 1994).

Differences between physical separation and the alkane technique in the estimates of the percentage of each botanical component present in the quadrat samples (within each paddock) were tested using least squares analysis of variance (REG; Gilmour 1988). The model fitted included method (physical separation *v.* alkane) and quadrat as fixed and random effects, respectively.

RESULTS

The major components, as determined by physical separation, on a DM basis, were the legume species (predominantly lucerne) and oat stubble in each paddock. All other components comprised less than 27% of the pastures.

Table 1. Botanical composition (% of DM) of quadrat samples from each of 3 paddocks estimated by physical separation and from the alkane concentrations of the herbage.

Paddock	Botanical components	% Composition		s.e.d.	
		Separated	Alkanes		
1	*Lolium spp.*	2.7	19.8	5.4	**
	Medicago truncatula and *M. sativa*	35.3	17.6	6.8	*
	Arctotheca calendula, Centipeda spp., Calotis lappulacea and *Trifolium spp.*	9.3	16.6	7.7	ns
	Raphanus raphanistrum	0.2	41.2	7.5	**
	Solanum esuriale	0.0	0.0	0.0	ns
	Avena spp., Carthamus lanatus, Erodium spp. and *Eclipta platyglossa*	52.6	4.8	9.1	**
2	*Trifolium spp.*	4.3	3.1	1.7	ns
	Avena spp.	19.4	3.4	3.4	**
	M. truncatula and *M. sativa*	60.5	29.9	6.3	**
	Lolium spp. and Hordeum spp.	8.2	1.7	2.8	*
	Polygonum spp. and *C. lappulacea*	0.2	33.3	3.9	**
	Sisymbrium spp.	0.3	2.6	1.4	ns
	C. lanatus, Centipeda spp., Convolvulus spp. and *Echium plantagineum*	5.6	0	3.0	ns
	Solanum esuriale	1.5	26.0	6.4	**
3	*Trifolium spp.*	3.0	0.0	0.7	**
	Lolium spp.	4.0	46.6	8.9	**
	M. truncatula and *M. sativa*	35.1	22.9	6.3	ns
	Eragrostis spp.	1.2	23.9	4.3	**
	Teucrium racemosum and *Vittadinia spp.*	0.2	3.8	2.8	ns
	Atriplex spp., Avena spp., C. lanatus and *Enteropogon acicularis*	56.5	2.9	4.6	**

* $P<0.05$; ** $P<0.01$; ns not significant

A total of 19, 20 and 19 species samples were collected in paddocks 1, 2 and 3, respectively, for which data on the alkane concentrations (C_{24-33} inclusive) were available. Following principal component analyses of the alkane concentrations of the individual species, the number of botanical associations was reduced to 9, 13 and 11, respectively. For each of the 3 paddocks, the associations of species were determined from the first 3 principal components, which accounted for 80% or more of the total variation. As the number of components identified equalled or exceeded the number of useful alkanes

available, these numbers were reduced to 7, 10 and 7 respectively, by omitting species only observed infrequently in the paddock.

For each of the paddocks, there were significant differences ($P<0.05$) between the proportions of the botanical components estimated using herbage alkanes and those determined by physical separation (Table 1). In all 3 sets of pasture quadrat samples, there was a trend for the proportions of the *Medicago spp.* component, and of the component including *Avena spp.,* to be underestimated. In 2 of the 3 paddocks (1 and 3), the proportion of the *Lolium spp.* component was overestimated, while in paddock 2, the proportion of the *Polygonum spp./C. lappulacea* component was overestimated.

The major herbage component present in a paddock was significantly underestimated in each case by the alkane technique relative to physical separation.

DISCUSSION

Clearly, the optimisation of botanical components on the alkane concentrations of the herbage samples inadequately described the botanical composition of the quadrat samples from these highly heterogeneous pastures. The magnitude of a component's presence in the pasture appeared unrelated to the accuracy of its predicted representation in the sample.

Previous estimates of the botanical composition of herbage mixtures from the concentration of alkanes have been based on a relatively small number of components in the mixtures (Dove 1992; Vulich 1993). In this experiment, at least 19 species were identified in the pasture of each paddock, which is much more than the number of alkanes for which data were available (up to 10). While principal component analyses and knowledge gained from field observations were used to reduce the number of botanical components to 7-10, this may be too many for the limitations of the technique. Dove and Mayes (1996) have suggested that the capacity to distinguish dietary components could be increased by using additional markers such as plant phenolic compounds or cuticular alkenes, fatty acids, alcohols or wax esters. Theoretically, the availability of additional markers would allow more species/botanical components to be distinguished. However, if there is insufficient difference in the concentrations of these markers between the components, there is no capacity to distinguish between the species.

In the comparisons shown in Table 1, species were grouped by principal components and the mean concentration of each alkane for that group of species was calculated for later use in a least-squares optimisation of botanical components. One problem in using such an approach is that equal weighting is given to each component of the group that may not be justified by its actual representation within the sample. The process effectively creates an artificial species, which may lead to errors in the composition estimates. The potential for error will depend on the magnitude of the variation in the alkane concentrations between the species within the association. The effect on the composition estimate will increase as the representation of the components of the association in the herbage mix increases. Simulations by Bugalho *et al*. (2002) indicate that errors in the estimated composition arising from an uneven distribution of species within components may be relatively small.

A number of factors may have been responsible for the errors in the estimates of botanical composition obtained using the alkane method. These include the extent of variation in alkane concentrations/profiles between species, the adequacy of the alkane characterisation of individual species/botanical components, and errors in estimating the alkane concentrations of the herbage mixtures (quadrat samples). The ability to distinguish between species/botanical components is dependent on the magnitude of differences in the concentration of individual alkanes between those species/components.

The procedure used to estimate the botanical composition optimises the combination of species to match the alkane concentration of the herbage samples (Dove and Moore 1995), and as such there was no unique solution. Only a small improvement in the residual values was reported by the EatWhat software for the best solution over that of the third best solution; the mean improvement across the quadrats in each of the 3 paddocks was only 4-10%. This indicates that the species/components were difficult to resolve algebraically, that patterns of alkane concentrations between species may not have been sufficiently different, and that the solution cannot be accepted confidently. Increasing the

number of botanical components is likely to exacerbate the difficulty of resolving the components when variation between the species is limiting.

Errors in estimating the alkane concentrations of the component species may lead to inappropriate grouping of species (following a principal component, or similar, analysis) and/or affect the solution of the least squares optimisation. Similarly, errors in estimating the alkane concentrations of the herbage mixtures (quadrats) will also influence the least-squares optimisation. Errors in characterising the alkane profiles of individual species would include those associated with both the sampling and with the analytical procedures. Smith *et al*. (2001) demonstrated large within-species variation in the alkane concentration pattern of grasses, which would include variation in plant parts/phenology (Dove *et al*. 1996), between sample variation, and possible spatial variation (Lee and Nolan 2003). Any inaccuracies in relation to the concentrations of alkanes in quadrat samples are likely to be attributable to analytical procedures.

The limitations of the alkane technique to accurately estimate the botanical composition of a highly heterogeneous herbage sample are likely to apply similarly to the estimation of the botanical composition of the diet of sheep grazing such pastures.

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