Report of Training Programme at NRI Laboratories at Wye and Chatham, United Kingdom

NRI Project A0729

Application of laboratory feed evaluation to identify methods of easing feed scarcity in North West India

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Background

In North West India tree leaves constitute major components of goat diets. Although 44 tree species have been identified, leaves of about 15 tree species are more commonly fed and these are potential feed resource for goats in the region. The feeding strategies adopted by the farmers vary considerably from village to village and farmer to farmer. The most common method is to feed tree loppings when goats are taken out for grazing or feed dry tree leaves when grazing is restricted due to some reason or other.

There is however a dearth of information on nutritive value of tree leaves and the data collected so far indicate that diets with large component of tree leaves could be protein deficient.

The project proposes to apply laboratory methods currently used at NRI to identify improved mixtures which will help select the farmers ideal feed combinations to ease out scarcity of feeds and deficiencies of nutrients.

In this context, training programme in laboratory techniques used for feed evaluation was planned at two laboratories of NRI at Wye and Chatham. This report gives an account of the activities and techniques used, together with an interim presentation of some of the data obtained.

Duration on training - 5 weeks (6 July to 10 August 1998)
Supervisor - Dr Chris Wood, Natural Resources Management Department

Training programme

Part I - Training at the NRI Nutrition Laboratory at Wye College.

At this laboratory I was exposed to two laboratory techniques used in feed evaluation. These were:

1. Use of in vitro gas production to evaluate rumen fermentation of goat feeds in nitrogen free and nitrogen rich media.

2. Detection of anti-nutritive factors by TLC/mould inhibition bioassay.

Detailed protocols of the methods are attached in the appendix.

In vitro gas production method.

The feed samples collected by Dr Chris Wood during his visits to the project area in February and May 1998 were subjected to evaluation using this technique. Seventeen feed samples collected in February and fifteen feed samples collected in May were studied for rumen fermentation pattern in nitrogen free (NF) and nitrogen rich (NR) media. The equation fitting (NR medium data) was done using the France model (France et al., 1993). The data is presented below.

Detection of anti-nutritional factors by TLC/mould inhibition bioassay

This method essentially involves use of thin layer chromatography on silica gel (Merck TLC 20x20 cm silica gel $60F_{254}$) for separation of alkaloids and other factors extracted first by 70% aqueous acetone and then by dichloromethane (DCM).

Part II - Training at NRI Laboratory at Chatham

At this laboratory I got an opportunity to learn two techniques:
1. Estimation of phenolic compounds in plants.

This method is based on reduction of iron (III) to iron (II) by tannins and other polyphenols by formation of a ferricyanide-iron (II) complex. The coloured product known as Prussian Blue absorbs maximally at 720 nm. The protocol is given in the Appendix.

2. Brine shrimp bioassay.

This technique is used for calculating lethal concentration 50 (LC50) values for toxic principles present in feedstuffs (protocol attached).

Familiarisation visits

Visits to Wye College Dairy Unit and the ADAS Feed Evaluation Unit were organised. These visits were useful in getting acquainted with feeding management practices applied at dairy farms in Britain.

Wye College Dairy Unit maintains a herd of 140 HF cows having an average lactation of 7300kg. The trials on comparative performance of animals on TMR (total mixed ration) system and simple diet (conventional system) were in progress at this farm.

Visit to ADAS Feed Evaluation Unit (FEU) is planned on 7 August which is a day after this interim report was prepared. The FEU is a leading centre for feed evaluation for the UK livestock industry and undertakes research for both commercial and government bodies.

Gas production data

Cumulative gas production data at selected incubation times is given in Table 1 for samples fermented in nitrogen rich (NR) medium. The various concentrate samples all yielded high gas productions at all selected times, indicating a rapid and extensive fermentation. Tree pods (IMy16 and 17) and cactus (IMy15) were also highly fermentable (over 300 ml gas g^{-1} for some samples), producing over 200 ml gas per g sample after 96h. This compares to a gas production of 238 ml g^{-1} for the good quality reference meadow hay. The gas productions from tree fodders were all lower, ranging from 177 ml g^{-1} for negad leaves (IMy4) down to 29 ml g^{-1} for khejri leaves (IMy18). The various dried leaves from the Udaipur hill slopes ranged from 106 to 126 ml g^{-1} (samples IF24 and 25, IMy5, 6, 8, 9, 10, 11). Bordi pala (samples IF6 and 7) had low gas productions (67 and 85 ml g^{-1}).

Table 2 gives the values for parameters obtained by fitting the France et al. (1993) model. The model did not fit the data for sample IF26 (Chenopodium alba), a sample which produced very little gas when fermented in the NR medium in spite of a proportional dry matter disappearance of 0.70 (Table 4). Otherwise the France model appeared to fit the data well. The gas pool A was linearly correlated to cumulative gas production at 96h fermentation ($R^2 = 0.99$). The France parameters indicate that the concentrates were fermented extensively (high gas pool A values) and quickly (high rate constant b values). The physiological significance of rate constant c is unclear. Lag times were relatively high compared with the other samples, but the physiological significance of this is unclear. Tree pod fermentation, although extensive, was relatively slow compared to the concentrates. *Prosopis juliflora* pods (IMy16) were fermented surprisingly slowly (b = 0.034), as tree pods are generally relatively rapidly fermentable. Other slowly fermented feeds (b <0.03) were bordi (dried or fresh; samples IF6, 7 and IMy19), and one of the Ziziphus mauritiana leaf samples (IF4), arunjia new leaves (IMy2) and one of the dried leaf samples (IMy8).

Table 3 presents dry matter disappearance (DMD) data during gas production and after acid pepsin treatment of the residue. DMD during gas production was linearly correlated with the 96h GP values ($R^2 = 0.89$). Interesting data to note is the very variable DMDs during acid pepsin treatment and GP per g DMD data. These data may indicate variable microbial biomass production and/or variable by-pass protein contents, but need further consideration.

Table 4 presents data on gas production and DMD of the feed samples fermented in nitrogen free (NF) medium; Table 5 presents the Nitrogen Deficiency Index (NDI) values calculated after 48 and 96 h fermentation, together with DMD and GP/g DMD data for

NR and NF data. The nitrogen deficiency index (at this stage experimental) was defined as:
100 x ($CG96_{N\text{-}rich}$ - $CG96_{N\text{-}free}$)/$CG96_{N\text{-}rich}$
where $CG96_{N\text{-}rich}$ = cumulative gas production after 96 h fermentation (CG96) in the feeds fermented in nitrogen-rich.
$CG96_{N\text{-}free}$ = cumulative gas production after 96 h fermentation (CG96) in the feeds fermented in nitrogen-free media
and similarly for NDI at 48h.
Nitrogen deficiency is indicated by a high positive NDI. Severely N deficient feeds were IF4 (Ziziphus mauritiana leaves), IMy 4 (negad leaves), IF23 (maize grain). There was evidence of some nitrogen deficiency in some concentrate samples, presumably because of their high content of fermentable carbohydrate; neem; bordi (but not sample IF6); some of the dried leaf samples; cactus. The negative NDI values appear to arise from samples where the GP/g DMD in NR medium is relatively low. This may be an indicator of a stimulation of microbial biomass production, but needs further consideration.

Total phenols

The samples collected in July were analysed for total phenols (presented as g gallic acid equivalent per kg sample). February and May samples will be analysed at the BAIF laboratories.

Bioassays

At the time of writing, the bioassay work has yet to be completed and data are not available. Samples IMy5 and IMy6 (dried leaves from beneath karanj) showed the presence of toxic principles. This however needs to be confirmed by repeating the analysis.

Future work

Goat monitoring data

Due to lack of time, it was only possible to have a very limited look at the goat monitoring data during the visit. The data could be read without problems by the software in use at NRI. One problem appears to be with the comments column, which has become far more complex than envisaged. Presenting named species as codes is a good idea, but many of the comments are a simple repetition of the activity codes. It was not intended that all observations would have comments; this column is for naming species where known and commenting on unusual events or for clarification. The result has been an impossibly complex multitude of codes which is unworkable. It is suggested that a named species code column is introduced for **existing** species codes and the comments column is used for additional comments in text. Such comments should not be particularly common nor a repetition of other coded information.

Gas production data

Nitrogen analysis of residues of low degradability have yet to be done. Samples collected in July to be analysed and repeats to be done of other selected samples. Data from earlier work to be added. Data to be organised by category of feed to facilitate analysis and interpretation of data.

Analytical data

Remaining chemical assays will be completed at the BAIF laboratories

Bioassays

A final technical report on the TLC/mould assays is expected shortly from Wye College. The brine shrimp assays will be continued at the NRI Chatham laboratories.

References

France J, Dhanoa M S, Theodorou M K, Lister S J, Davies D R and Isac D (1993) A model to interpret gas accumulation profiles associated with in vitro degradation of ruminant feeds. Journal of Theoretical Biology 163: 99 - 111.

Table 1 Cumulative gas production data (ml g^{-1} DM, corrected for no substrate control) at selected incubation times for samples fermented in nitrogen rich (NR) medium

		incubation time (h)			
		12	27	48	96
Sample code	Description of sample				
IF3	Azadirachta indica (Neem) leaves	55	98	134	159
IF4	Ziziphus mauritiana leaves	38	73	117	151
IF5	Cotton pala	25	67	97	110
IF6	Ziziphus nummularia, dried leaves (bordi pala)	16	36	49	67
IF7	Ziziphus nummularia, dried leaves (bordi pala)	18	43	57	85
IF8	Azadirachta indica (Neem) leaves	47	86	104	129
IF13	barley grain, ex BAIF buck breeding programme	162	237	264	281
IF14	wheat grain, ex Patiyo Ka Khera	168	267	295	310
IF15	barley grain, ex Patiyo Ka Khera	152	250	279	296
IF18	wheat grain + sun hemp seed "concentrate" ex Mr. Sava Roopaji Vadera, Khakad	111	198	215	224
IF19	barley grain ex Khakad	157	245	274	291
IF20	neem leaves	48	87	125	146
IF22	wheat grain, ex Kirat	117	221	252	269
IF23	dried maize, ex Gopir	95	251	303	330
IF24	dried leaves of "kadwa" from hill slopes	25	68	108	126
IF25	dried leaves of "kadwa" from hill slopes	22	66	102	121
IF26	whole weed from wheat field (Chenopodium alba)	38	69	77	89
IMy1	*Ziziphus mauritiana* (ber) leaves	17	45	61	81
IMy2	*Acacia leucophloea* (arunjia) new leaves	19	50	86	121
IMy4	*Derris indica* (negad) leaves	44	100	156	177
IMy5	Dried leaves collected underneath karanj tree, upper valley (location code 2)	26	66	92	106
IMy6	Dried leaves collected underneath karanj tree, lower slopes (location code 3)	30	70	100	117
IMy7	*Vitex negundo* (Khanni), tops of green shoots pooled from 5 trees (only part eaten by goats)	63	124	164	195
IMy8	Dried leaves from below tamat trees, top of hills (location code 4)	23	49	82	106
IMy9	Second sample as per IMy8	21	51	85	106
IMy10	Dried leaves from below karanj trees (mainly), but also *Vitex negundo* (khanni) and *Holorrena antidisentrica* (kadwa) trees, top of hills (location code 4)	31	73	106	123
IMy11	Dried leaves from below mainly *Holorrena antidisentrica* (kadwa) trees, lower slopes (location code 3)	27	68	103	119
IMy15	Cactus, *Opuntia spp.*? (thor), dried	71	163	206	229
IMy16	*Prosopis juliflora* pods	97	142	178	212
IMy17	*Prosopis cineraria* (khejri) pods	102	165	209	235
IMy18	*Prosopis cineraria* (khejri) leaves	6	16	25	29
IMy19	*Ziziphus nummularia* (bordi) fresh leaves	31	63	90	128
ref meadow hay		53	135	195	238
0.5 glucose		193	285	340	363

Table 2 Gas production data: France model parameters

Sample code	Gas Pool A (ml g^{-1}DM)	Rate Constant b (h^{-1})	Rate Constant c ($h^{-0.5}$)	Lag Time T-(h)
IF3	164	0.039	-0.018	0.5
IF4	172	0.024	-0.014	0.8[a]
IF5	112	0.053	-0.108	3.5
IF6	71	0.030	-0.027	1.2
IF7	94	0.024	-0.013	1.0
IF8	127	0.050	-0.041	1.3
IF13	269	0.130	-0.207	2.6
IF14	300	0.132	-0.240	3.2
IF15	287	0.114	-0.185	2.6
IF18	217	0.135	-0.259	3.5
IF19	280	0.124	-0.207	2.8
IF20	152	0.037	-0.022	0.5
IF22	261	0.101	-0.182	3.1
IF23	322	0.085	-0.183	4.2
IF24	130	0.046	-0.099	3.7
IF25	124	0.048	-0.104	4.1
IF26	n/a[b]	n/a[b]	n/a[b]	n/a[b]
IMy1	81	0.041	-0.065	2.7
IMy2	144	0.024	-0.043	2.6
IMy4	185	0.045	-0.079	2.5
IMy5	107	0.054	-0.105	3.4
IMy6	118	0.049	-0.087	2.7
IMy7	194	0.054	-0.080	2.5
IMy8	116	0.029	-0.042	1.7
IMy9	114	0.035	-0.065	2.8
IMy10	125	0.050	-0.089	2.9
IMy11	123	0.046	-0.089	2.8
IMy15	228	0.064	-0.103	2.3
IMy16	216	0.034	0.044	0.1
IMy17	236	0.051	-0.019	0.4
IMy18	30	0.042	-0.087	3.0
IMy19	150	0.022	-0.011	0.8
reference meadow hay	243	0.042	-0.070	2.2
glucose	351	0.112	-0.190	3.0

note a estimated using the “alternative” method of France et al.

note b values not obtained due to the poor fitting of the France et al. model.

Table 3 Dry matter disappearances (DMD) in nitrogen rich (NR) medium, before and after acid pepsin treatment

	DMD after GP	GP + acid pepsin	during acid pepsin	GP/g DMD (GP only)	GP/g DMD (GP plus acid pepsin)
IF3	0.53	0.66	0.116	302	240
IF4	0.52	0.63	0.103	289	240
IF5	0.42	0.52	0.097	265	212
IF6	0.38	0.51	0.068	177	131
IF7	0.40	0.51	0.080	211	166
IF8	0.55	0.67	0.116	235	194
IF13	0.89	0.90	0.009	318	314
IF14	0.95	0.96	0.006	327	324
IF15	0.93	0.94	0.009	317	314
IF18	0.84	0.85	0.009	267	262
IF19	0.91	0.92	0.007	321	318
IF20	0.48	0.62	0.113	307	234
IF22	0.93	0.98	0.015	288	275
IF23	0.97	0.98	0.010	342	338
IF24	0.52	0.59	0.060	240	213
IF25	0.51	0.61	0.095	239	198
IF26	0.70	0.78	0.082	127	113
IMy1	0.41	0.46	0.081	199	179
IMy2	0.37	0.47	0.087	322	259
IMy4	0.58	0.64	0.083	306	276
IMy5	0.49	0.63	0.127	217	170
IMy6	0.51	0.61	0.130	230	192
IMy7	0.76	0.82	0.080	259	239
IMy8	0.50	0.60	0.083	209	177
IMy9	0.51	0.59	0.070	206	178
IMy10	0.49	0.61	0.099	251	202
IMy11	0.52	0.58	0.057	228	207
IMy15	0.74	0.81	0.067	310	284
IMy16	0.77	0.81	0.021	275	262
IMy17	0.69	0.77	0.049	340	305
IMy18	0.23	0.30	0.047	125	98
IMy19	0.53	0.64	0.102	239	201
ref meadow hay	0.72	0.74	0.027	331	322
0.5 glucose	1			364	

Table 4 Cumulative gas production data (ml g^{-1} DM, corrected for no substrate control) at selected incubation times for samples fermented in nitrogen free (NF) medium

incubation time (h)	12	27	48	96	DMD after GP	GP/g DMD
IF3	41	68	98	141	0.51	279
IF4	32	49	62	77	0.34	225
IF5	30	63	96	111	0.43	257
IF6	24	40	52	65	0.31	213
IF7	20	34	45	56	0.29	197
IF8	41	66	84	102	0.49	206
IF13	86	182	276	314	0.87	359
IF14	127	251	309	332	0.95	351
IF15	82	152	245	324	0.93	349
IF18	161	244	260	266	0.85	314
IF19	96	245	313	340	0.91	376
IF20	42	71	100	136	0.45	303
IF22	57	115	195	286	0.92	312
IF23	40	81	156	282	0.94	299
IF24	27	49	80	125	0.51	244
IF25	28	56	94	127	0.51	250
IF26	42	76	114	141	0.76	184
IMy1	27	45	58	72	0.33	221
IMy2	21	47	68	109	0.38	285
IMy4	22	32	39	44	0.32	138
IMy5	31	66	99	119	0.50	238
IMy6	30	64	99	121	0.52	235
IMy7	78	148	202	216	0.72	300
IMy8	24	41	53	69	0.40	172
IMy9	25	44	62	100	0.52	192
IMy10	31	65	99	122	0.47	256
IMy11	28	53	83	117	0.51	229
IMy15	48	79	122	198	0.72	274
IMy16	95	143	180	216	0.71	305
IMy17	101	166	212	233	0.68	340
IMy18	9	18	26	30	0.26	114
IMy19	36	61	77	95	0.40	240
ref meadow hay	70	123	191	245	0.68	359

Table 5 Nitrogen Deficiency Indecis (NDI) after 48 and 96h incubation, Dry Matter Dissappearances (DMD) and gas production per g DMD in nitrogen rich (NR) and nitrogen free (NF) media

			NR medium		NF medium	
	NDI48	NDI96	DMD	GP/g DMD	DMD	GP/g DMD
IF3	24	10	0.53	302	0.51	279
IF4	41	43	0.52	289	0.34	225
IF5	1	-1	0.42	265	0.43	257
IF6	-4	2	0.38	177	0.31	213
IF7	17	27	0.40	211	0.29	197
IF8	17	18	0.55	235	0.49	206
IF13	-5	-11	0.89	318	0.87	359
IF14	-5	-7	0.95	327	0.95	351
IF15	12	-9	0.93	317	0.93	349
IF18	-20	-18	0.84	267	0.85	314
IF19	-14	-16	0.91	321	0.91	376
IF20	18	6	0.48	307	0.45	303
IF22	21	-6	0.93	288	0.92	312
IF23	46	14	0.97	342	0.94	299
IF24	22	1	0.52	240	0.51	244
IF25	6	-4	0.51	239	0.51	250
IF26	-39	-48	0.70	127	0.76	184
IMy1	4	10	0.41	199	0.33	221
IMy2	17	8	0.37	322	0.38	285
IMy4	68	67	0.58	306	0.32	138
IMy5	-6	-10	0.49	217	0.50	238
IMy6	1	-3	0.51	230	0.52	235
IMy7	-21	-9	0.76	259	0.72	300
IMy8	29	29	0.50	209	0.40	172
IMy9	22	5	0.51	206	0.52	192
IMy10	6	1	0.49	251	0.47	256
IMy11	17	2	0.52	228	0.51	229
IMy15	38	13	0.74	310	0.72	274
IMy16	-1	-2	0.77	275	0.71	305
IMy17	-1	1	0.69	340	0.68	340
IMy18	-3	-2	0.23	125	0.26	114
IMy19	11	22	0.53	239	0.40	240
ref meadow hay	2	-3	0.72	331	0.68	359
0.5 glucose	n/a		1	364		

Table 6 Analysis of tree leaves samples collected in July1998

Should have said %!

Sample code	Residual dry matter (g/kg DM)	Ash (g/kg DM)	Crude protein (g/kg DM)	Ether extract (g/kg DM)	NDF (g/kg DM)	ADF (g/kg DM)	CF (g/kg DM)	Lignin (g/kg DM)	Tannins (g eq. GA per kg)
IJy1	91.80			2.25	41.54	26.91	13.97	9.46	37
IJy2	94.20			3.82	42.52	37.06	19.63	10.39	63.7
IJy3	93.81			2.13	80.2	40.54	32.25	7.18	4.2
IJy4	94.31			4.5	41.01	28.45	19.59	15.52	16.4
IJy5	95.51			6.12	33.93	23.73	12.11	10.18	24
IJy6	91.07			1.97	26.84	17.98	10.84	3.67	8.9
IJy7	94.45			4.69	53.46	38.38	26.49	16.08	86.5
IJy8	94.70			2.71	54.75	44.07	24.61	13.69	44.2
IJy9	93.58			2.97	43.03	35.82	21.52	8.12	9.5
IJy10	95.50			1.97	26.11	12.86	5.58	2.88	16
IJy11	93.51			8.07	35.41	30.99	19.91	7.29	18.3
IJy12	90.03			2.63	35.65	21.75	12.17	3.84	
IJy13	92.43			2.23	44.27	38.18	17.74	12.99	88.7
IJy14	92.43			3.77	35.11	23.57	13.53	5.45	6.3

APPENDIX
Table 7 Samples obtained in India, February 1998

Code	Description	Analyses to do		
		Anti-nutritional factors	Gas production	Chemical analysis
	From Bhilwara			
IF1	A leucophloea pods, not toxic	x		
IF2	A leucophloea leaves from same branch as IF1	x		
1F3	Azadirachta indica (Neem) leaves	x	x	x
IF4	Ziziphus mauritiana leaves	x	x	x
IF5	Cotton pala	x	x	x
IF6	Ziziphus nummularia, dried leaves (bordi pala)	x	x	x
IF7	Ziziphus nummularia, dried leaves (bordi pala)	x	x	x
IF8	Azadirachta indica (Neem) leaves	x	x	x
IF9	A leucophloea pods, not toxic	x		
IF10	A leucophloea leaves from same branch as IF9	x		
IF11	as IF9 but from other branch of tree, pool with IF9 after weighing			
IF12	A leucophloea pods, said to be toxic (pods curled, reddy brown and look dry compared to non-toxic pods)	x		
IF13	barley grain, ex BAIF buck breeding programme		x	x
IF14	wheat grain, ex Patiyo Ka Khera		x	x
IF15	barley grain, ex Patiyo Ka Khera		x	x
	From Udaipur			
IF16	A leucophloea pods, not toxic[a]			
IF17	A leucophloea pods, said to be toxic (blotches on pod surface, said to due to disease, and indicate toxicity)[a]			
IF18	wheat grain + sun hemp seed "concentrate" ex Mr. Sava Roopaji Vadera, Khakad		x	x
IF19	barley grain ex Khakad		x	x
IF20	neem leaves	x	x	x
IF21	A leucophloea pods, said to be toxic (had ball-like growths, galls, on twigs, said to be caused by insects and to indicate toxicity)	x		
IF22	wheat grain, ex Kirat		x	x
IF23	dried maize, ex Gopir		x	x
IF24	dried leaves of "kadwa" from hill slopes	x	x	x
IF25	dried leaves of "kadwa" from hill slopes	x	x	x
IF26	whole weed from wheat field (Chenopodium alba)		x	x (CP)

Note a Samples attacked by mould whilst in transit to UK and will not be evaluated.

Table 8 Samples collected in India, May 1998

Code	Description	Analyses to do		
		Anti-nutritional factors	Gas production	Chemical analysis
	From Udaipur			
IMy1	*Ziziphus mauritiana* (ber) leaves	x	x	x
IMy2	*Acacia leucophloea* (arunjia) new leaves	x	x	x
IMy3	*Acacia nilotica* (babul) pods	x		
IMy4	*Derris indica* (negad) leaves	x	x	x
IMy5	Dried leaves collected underneath karanj tree, upper valley (location code 2)	x	x	x
IMy6	Dried leaves collected underneath karanj tree, lower slopes (location code 3)	x	x	x
IMy7	*Vitex negundo* (Khanni), tops of green shoots pooled from 5 trees (only part eaten by goats)	x	x	x
IMy8	Dried leaves from below tamat trees, top of hills (location code 4)	x	x	x
IMy9	Second sample as per IMy8	x	x	x
IMy10	Dried leaves from below karanj trees (mainly), but also *Vitex negundo* (khanni) and *Holorrena antidisentrica* (kadwa) trees, top of hills (location code 4)	x	x	x
IMy11	Dried leaves from below mainly *Holorrena antidisentrica* (kadwa) trees, lower slopes (location code 3)	x	x	x
IMy12	*Acacia nilotica* (babul) leaves	x		
IMy13	*Acacia nilotica* (babul) pods	x		
	From Bhilwara			
IMy14	*Acacia nilotica* (babul) pods	x		
IMy15	Cactus, *Opuntia spp.*? (thor), dried	x	x	x
IMy16	*Prosopis juliflora* pods	x	x	x
IMy17	*Prosopis cineraria* (khejri) pods	x	x	x
IMy18	*Prosopis cineraria* (khejri) leaves	x	x	x
IMy19	*Ziziphus nummularia* (bordi) fresh leaves	x	x	x

Table 9 Samples of tree leaf fodders collected by Mr Badve, Rajasthan, July 1998

Samples of fresh leaves, dried and ground to 1 mm before analysis

Code	Description	Analyses to do		
		Anti-nutritional factors	Gas production	Chemical analysis
IJy1	Ziziphus maritiana (ber)	x	x	x
IJy2	Mangitesa indica (mango)	x	x	x
IJy3	Bamboo	x	x	x
IJy4	Derris indica (negad)	x	x	x
IJy5	Holorrena antidisentrica (kadwa)	x	x	x
IJy6	Pongamia pinnata (karanj)	x	x	x
IJy7	Acacia leucophloea (runjiya)	x	x	x
IJy8	hitazi	x	x	x
IJy9	Ficus indica (pimpal)	x	x	x
IJy10	tamat	x	x	x
IJy11	khanni (all green leaves, not just shoots)	x	x	x
IJy12[a]	godla			x
IJy13	Acacia nilotica (babool)	x	x	x
IJy14	kalbi	x	x	x

Note a Samples attacked by mould whilst in transit to UK and will not be evaluated.

Table 10 Analysis of certain feed samples collected in February and May 1998

Sample code	Residual dry matter (g kg^{-1}DM)	Ash (g kg^{-1}DM)	Crude protein (g kg^{-1}DM)	Ether extract (g kg^{-1}DM)	NDF (g kg^{-1}DM)	ADF (g kg^{-1}DM)	CF (g kg^{-1}DM)	Lignin (g kg^{-1}DM)
IF3	950	91	116	30	265	184		85
IF4	961	78	139	18	333	217		50
IF5	942	127	132	32	457	342		143
IF6	947	135	125	13	339	220		83
IF7	943	126	117	34	325	213		79
IF8	948	73	156	56	287	179		89
IF20	939	116	124	35	231	178		90
IF24	945	107	66	47	317	315		118
IF25	938	139	93	22	361	250		88
Barley (IF13?)	927	30	99	13	239	100		27
Wheat (IF14?)	937	14	125	8	153	25		10
IMy1	940	151	145	17	361	270	118	60
IMy2	946	79	169	17	358	281	168	86
IMy3	953	52	133	21	332	278	169	81
IMy4	946	62	128	34	346	260	145	112
IMy5	963	164	114	14	293	270	91	102
IMy6	945	156	105	14	252	228	87	53
IMy7	946	71	216	57	205	150	122	40
IMy8	958	169	68	11	313	334	106	70
IMy9	966	164	74	11	310	380	106	87
IMy10	967	157	98	22	308	272	108	84
IMy11	963	99	91	47	348	328	128	136
IMy12	960	94	143	44	273	151	104	74
IMy13	959	40	136	20	272	250	156	74
IMy14	955	47	137	19	271	218	154	61
IMy15	950	101	51	58	313	281	199	70
IMy16	955	43	115	14	382	214	189	39
IMy17	941	40	164	15	325	213	136	44
IMy18	952	79	124	24	358	246	142	118
IMy19	948	57	185	21	292	196	129	64

Table 11 Literature values for composition of selected concentrate feeds in India[a]

Feed	Ash (g kg^{-1}DM)	Crude protein (g kg^{-1}DM)	Ether extract (g kg^{-1}DM)	Crude fibre (g kg^{-1}DM)	Nitrogen free extract (g kg^{-1}DM)	CP digestibility
Barley grain (*Hordeum vulgare*)	114	115	19	318	434	0.71
Wheet grain (*Triticum aestivum,* syn. *T. vulgare*)	87	73	13	347	480	n/a
Sun hemp seed (*Crotalaria juncea*)	80	142	25	333	386	n/a

Note a source: Sen, K.C., revised by Ray, S.N. and Ranjhan, S.K. (1978) Nutritive values of Indian cattle feeds and the feeding of animals. Published by the Indian Council of Agricultural Research, New Delhi, India, 6th edition 92pp.

GAS PRODUCTION PROTOCOL VERSION OF JUNE 1997

Wednesday

Grind substrate to pass through a 1 mm dry sieve (if not already ground). Weigh out substrate. Generally use 1g of substrate, weigh to a tolerance of $\pm$0.0020g (except if glucose is used as a standard in which case use 0.5 g glucose).

Thursday

Make up suitable amounts of medium as described overleaf. Bring medium to the boil, without allowing to boil for more than a few minutes, and allow to cool under CO_2. The medium should turn pink or go clear.

Friday

Dispense 90 mls of medium into 125 ml serum bottles using automatic pump and gassing with CO_2. Always fill a few spare bottles with medium. Seal with butyl rubber stoppers, but do not crimp. Store at 4^{o}C.

Monday

Transfer the substrate into the serum bottles containing the medium using a small wide bore funnel. Gas the bottles with CO_2. Reseal with butyl rubber stoppers and crimp with aluminium caps. Replace in the incubator at 4^{o}C and programme it to switch to 39^{o}C at about 2am to prewarm the bottles.

Tuesday

A minimum of 2 people are required to inoculate the bottles

Donor animals

species - sheep
diet - hay:concentrate 70:30 DM basis. Approx composition of concentrate: oil 5.5%, protein 18%, fibre 8%, ash 8%, vit A 9000iu/kg, vit D3 2000iu/kg, vit E 10iu/kg.

Prepare Inoculum

Restrain donor animals using collars. Remove fistulae bung and remove rumen fluid using a pump. Collect rumen fluid starting at 8.15 am before the sheep are fed and keep warm in a thermos flask. Pour about 500ml of rumen fluid and solids into a blender and blend for 20 seconds at a high rating. Filter fluid through 4 layers of muslin in a large

funnel and collect in a beaker under an atmosphere of CO_2. Inoculum is used undiluted, or if nitrogen-deficient fermentations are to be conducted, it is diluted as follows: one part rumen fluid to three parts N-free (Menke) medium (x4 dilution).

Keep the inoculum stirred and under CO_2.

Inoculation of the bottles

While the inoculum is being prepared, the serum bottles are adjusted to atmospheric pressure and the bottles are then returned to the incubator at 39^{o}C.

Using a 20 ml syringe and 21 gauge 1.5 in (0.8 x 40 mm) needles (colour code green), 10ml of inoculum is injected into each bottle. Shake bottles and return to the incubator.

Starting at 10 am, the bottles are readjusted to atmospheric pressure, shaken and returned to the incubator. This is taken as the starting point (Time = 0). Readings are then taken at the following times.

		Time	Hours After Inoculation
Day 1	Tuesday	1000	0
		1300	3
		1600	6
		1900	9
		2200	12
Day 2	Wednesday	0100	15
		0700	21
		1300	27
		1900	33
Day 3	Thursday	0100	39
		1000	48
		2200	60
Day 4	Friday	1000	72
Day 5	Saturday	1000	96

SAFETY NOTE Readings should be taken in a fume cupboard and gasses evolved disposed of inside the fume cupboard.

A pressure transducer (we use Bailey and Mackey Ltd, Birmingham B42 1DE, UK) is used to measure headspace pressure in the bottles. The transducer had a range of 0 - 25 psi, accuracy of 0.1 $\pm$ 2%, readings calibrated to read in units of psi (although such calibration is not essential). It was connected to a disposable luer lock 3-way tap

allowing a needle (23 gauge 1 in, 0.6 x 25 mm; colour coded blue) and syringe to be fitted to the other outlets.

Gas pressure is read by removing bottles tray by tray from the incubator, inserting the needle through the butyl rubber stopper into the headspace above the medium. Note pressure. Adjust the pressure to atmospheric by removing gas into the syringe and note volume of gas removed (read the syringe). Take readings from all the bottles in the tray, shake the bottles, and return them to the incubator.

Determination of Dry Matter Disappearance (DMD)

On Saturday at the end of the gas production run, vacuum filter through pre-weighed filter crucibles (Sintaglass, porosity 1 - regraded P160). Wash bottle with water to removed residues and wash residues on the filter. Oven dry overnight at 105^{o}C then allow to cool in desiccator and weigh. Express DMD as a proportion of the initial dry matter in the substrate.

MEDIA

SAFETY NOTE, calcium chloride, resazurin are classed as irritants. Sodium hydroxide, sodium sulphide, ferric chloride are corrosive. Avoid skin contact, wear gloves and laboratory coat.

Dihydrogen sulphide gas is very toxic and may be generated from sodium sulphide. Vent waste gas in fume cupboard when readings are taken.

Carbon dioxide can cause suffocation. Use only in a well ventilated laboratory, avoid breathing the gas.

Inoculum could contain harmful microbes. Animals used must be healthy. Wear gloves and laboratory coat when handling. Handle in fume cupboard as far as possible. Clean spillage, dispose of solid wastes by incineration.

NITROGEN (N) RICH (THEODOROU) MEDIUM

Component Solutions

1. Micromineral solution (g per 100 ml)
Make up stock solution and keep in fridge.

Calcium chloride ($CaCl_2.2H_20$)	13.2
Manganese chloride ($MnCl_2.4H_20$)	10.0
Cobalt chloride ($CoCl_2.6H_20$)	1.0
Iron chloride ($FeCl_3.6H_20$)	8.0

2. Buffer solution (g per 1itre)

This is made up in variable quantities for each fermentation run and can be stored in a fridge for a limited period. Calculate how much is required for each run.

Ammonium hydrogen carbonate (NH_4HCO_3)	4.0
Sodium hydrogen carbonate ($NaHCO_3$)	35.0

3. Macromineral solution (g per litre)
This is made up in variable quantities for each fermentation run and can be stored in a fridge for a limited period. Calculate how much is required for each run.

di Sodium hydrogen orthophosphate 12-hydrate ($Na_2HPO_4.12H_2O$)	9.45
Potassium di-hydrogen orthophosphate (KH_2PO_4)	6.20
Magnesium sulphate 7-hydrate ($Mg\ SO_4.7H_20$)	0.60

4. Reducing Solution (g per 100ml)
Make up freshly

Cysteine $HCL.1H_2O$	0.625

5. Anaerobic indicator (g per 100 ml)
Make up stock solution and keep in fridge

Resazurin	0.1

To make medium mix the component solution in the following amounts to make about 1 litre of medium.

1. Micromineral	0.1 ml
2. Buffer	200 ml
3. Macrominerals	200 ml
4. Reducing Solution	40 ml
5. Indicator	1 ml
6. Deionised water	559 ml

N-FREE (MENKE) MEDIUM

Component Solutions
1. Solution A (g per 100 ml)
(this is the same as the Micromineral solution for the N-rich medium)
Make up stock solution and keep in fridge.

Calcium chloride ($CaCl_2.2H_20$)	13.2
Manganese chloride ($MnCl_2.4H_20$)	10.0
Cobalt chloride ($CoCl_2.6H_20$)	1.0
Iron chloride ($FeCl_3.6H_20$)	8.0

2. Solution B (g per litre)
This is made up in variable quantities and can be stored in a fridge for a limited period. Calculate how much is required for each fermentation run.

Sodium hydrogen carbonate $NaHCO_3$	39.0

3. Macromineral solution (g per litre)
This is made up in variable quantities and can be stored in a fridge for a limited period. Calculate how much is required for each fermentation run.

Na_2HPO_4 (anhydrous)	5.70
Potassium di-hydrogen ortho-phosphate (KH_2PO_4)	6.20
Magnesium sulphate ($Mg\ SO_4.7H_20$)	0.60

4. Anaerobic indicator (g per 100 ml)
(this is the same as for the N-rich medium)
Make up stock solution and keep in fridge

Resazurin	0.1

To make medium mix the component solution in the following amounts to make about 1 litre of medium.

1. Solution A	0.1 ml
2. Solution B	200 ml
3. Solution C	200 ml
4. Indicator	1 ml
5. Deionised water	599 ml

NB There is no reducing solution in the Menke medium

If after boiling and cooling the medium does not turn pink the add some reducing agent - Two mls per litre of media

Reducing Agent
(Components for 100 mls)

Distilled Water	95ml
Sodium Hydroxide 1M Na OH	4ml
Sodium Sulphide	625mg

Equation fitting

France model

France, J., Dhanoa, M.S., Theodorou, M.K Lister, S.J Davies, D.R. and Isac, D. (1993) A model to interpret gas accumulation profiles associated with *in vitro* degradation of ruminant feeds. *Journal of Theoretical Biology* **163**: 99-111.

1. Using a spreadsheet, calculate gas production data per g DM and subtract values for the no substrate control. It is usual to fit the equation to data averaged over treatment replicates.

2. Equation for fitting:

$G = A - BQ^{t}Z^{\sqrt{t}}$ eqn 1

where G = cumulative gas production per g DM corrected for no substrate control
t = incubation time (h)

Initial estimates A = 200, B = 200, Q = 1, Z = 1.
A - B*(Q**t)*(Z**SQRT(t)) for SPSS
PARM[1]-PARM[2]*(PARM[3]^(t))*(PARM[4]^SQRT(t)) for Statgraphics, where PARM1,2,3,4 are A,B,Q,Z respectively.

This directly yields values for A = gas pool size (ml per g DM)

From the parameters estimated using equation 1, two rate constants and the lag time can be calculated as follows:

rate constant (h^{-1}) b = -lnQ eqn 2

rate constant ($h^{-0.5}$) c = -lnZ eqn 3

lag time (h) $\sqrt{T} = \dfrac{-\ln Z/2 \pm \sqrt{(\ln Z)^2/4 - \ln(B/A) \times \ln Q}}{\ln Q}$

For SPSS, the equation to fit (equation 1)has the form:

A - B*(Q**time)*Z**SQRT(time)), whence A, B, Q and Z

3. The other values can be calculated using a spreadsheet (e.g. excel). Set up as follows:

Parameter	**row**	**formula to type in**
A	2	(derived from equation 1)
B	3	(derived from equation 1)
Q	4	(derived from equation 1)
Z	5	(derived from equation 1)
b	6	-LN(Q)
c	7	-LN(Z)
lnZ	8	LN(Z)
lnB/A	9	LN(B/A)
lnQ	10	LN(Q)
square root function	11	(row8*row8/4)-(row9*row10)
square root T+	12	(-row8/2 + SQRT(row11))/row10
square root T-	13	(-row8/2 - SQRT(row11))/row10

T+	14	row12*row12
T-	15	row13*row13
function -c/2b	16	-row7/(2*row6)
alternative √T	17	-row8/(2*row10)
alternative T	18	row17*row17

EQUIPMENT

Essential major items

Incubator (to hold 39^{o}C, preferably programmable)
Pressure meter, preferably two, sensitive over range 0 - 25 psi
Inoculum donors (e.g. two rumen fistulated sheep)

Required general facilities, equipment

4 figure chemical balance
Fume cupboard
Carbon dioxide gas
weighing boats
hot plate
stirrer
thermos flasks
trays for bottles
large funnel
scinter glass crucibles, porosity 1 (P160)
drying oven
muffle furnace

Desirable major items

Vacuum pump (for inoculum collection)
Metered pump for medium dispensing

Specialist equipment

small wide bore funnel (can use plastic funnel with stem cut off)
gassing needles
125 ml serum bottles
butyl rubber stoppers
aluminium crimps
crimper
decapper
three way tap
needles
syringes

muslin

Chemicals required

Calcium chloride ($CaCl_2.2H_20$)
Manganese chloride ($MnCl_2.4H_20$)
Cobalt chloride ($CoCl_2.6H_20$)
Iron chloride ($FeCl_3.6H_20$)
Ammonium hydrogen carbonate (NH_4HCO_3)
Sodium hydrogen carbonate ($NaHCO_3$) **suggest 1 kg minimum**
di Sodium hydrogen orthophosphate 12-hydrate ($Na_2HPO_4.12H_2O$)
Potassium di-hydrogen orthophosphate (KH_2PO_4)
Magnesium sulphate 7-hydrate ($Mg\ SO_4.7H_20$)
Cysteine $HCL.1H_2O$
Resazurin (5 g)
Na_2HPO_4 (anhydrous)
Sodium Hydroxide
Sodium Sulphide

Prussian Blue Assay for Total Phenols

Principle

This method is based on the reduction by tannins and other polyphenols of iron (III) to iron (II), followed by the formation of a ferricyanide-iron (II) complex. The coloured product (commonly known as Prussian blue) absorbs maximally at 720nm.

The major difference between this method and the published method is the use of iron (III) ammonium sulphate instead of iron (III) chloride as the first reagent. Solubility problems are common with iron (III) chloride, but are eliminated by using iron (III) ammonium sulphate.

Equipment

1. 100ml Erlenmeyer flasks or test tubes

2. Spectrophotometer capable of taking absorbance readings at 720nm.

Reagents

1. 0.1M Hydrochloric acid

Add 8.3ml of conc. HCl to 500ml of distilled water and make up to 1L.

2. 0.1M Iron (III) ammonium sulphate in 0.1M HCl

Dissolve 48.2g of $FeNH_4(S0_4)_2.12H_2O$ in 1L of 0.1M HCl. This makes a pale yellow solution.

3. 0.008M Potassium ferricyanide

Dissolve 2.63g of $K_3Fe(CN)_6$ in 1L of distilled water. This makes a yellow solution.

4. Gallic acid standard, use 0.40g made up to 50mls with 70% acetone.

Procedure

1. Weigh accurately, approximately 500mg of sample and extract into 70% acetone using an ultraturrex (as per the radial diffusion method).

2. Dispense 50ml of deionised water into a 100ml Erlenmeyer flask.

3. Add 10µl of the acetone extract to the water. A solvent only blank and replicate standards should be included.

4. Add 3ml $FeNH_4(SO_4)_2$ and swirl. Additions should be timed; 30 sec intervals are convenient.

5. Exactly 20 mins after the addition of $FeNH_4(SO_4)_2$, start timed (30 sec intervals) additions of 3ml $K_3Fe(CN)_6$. Swirl.

6. Exactly 20 mins after the addition of $K_3Fe(CN)_6$, read the Absorbance at 720nm in a 1cm cuvette, making readings at 30 sec intervals.

7. The assay should be standardised against an appropriate phenolic, for example gallic acid.

Calculation

Subtract reading for blank from test and standard data.

$$\text{Total Phenols (mg gallic acid equivalent/g sample)} = \frac{\text{Abs sample}}{\text{Abs std}} \times 80 \times \frac{500}{\text{sample wt (mg)}}$$

Divide by dry matter to express per g DM sample.

Notes

A useful TLC spray or spot test can be made by mixing equal volumes of the two reagents, and spraying onto dry plates. Phenolics give bright blue spots. A blue

background eventually develops. The spray mixture must be made fresh, and should be brown in colour. It should be discarded if it turns blue.

Reference

Price, M.L. and Butler, L.G. (1977) J. Agric. Food Chem. Vol 25 No. 6

DETECTION OF ANTINUTRITIVE FACTORS BY TLC/MOULD INHIBITION BIOASSAY

Preparation Of Plant Material

Methods used for selection and preparation of fresh plant material resulted in dried, finely milled leaf for use in bioassay. Tree species and sample codes are identified in table 1.

Extraction of Crude Sample

The dried leaf material (1g) was extracted with 70% acetone (50ml). After 5 minutes the resultant solution was filtered by suction through watman filter paper (no.1). The remaining leaf material was re-extracted and filtered in the same way. The filtrate was mixed with ethanol and reduced to approximately 3ml in a rotary evaporator (35^oC) and de-ionised water (3ml) was added. The extract was washed twice with dichloromethane (2 x 6ml). The bottom dichloromethane extract (DCM ext) was removed with a pipette after each washing and collected.

The remaining aqueous extract (aq extr.) was mixed with an equal volume of ethanol to assist evaporation of water from the extract. The extract was evaporated to dryness in a rotary evaporator (35^oC). The remaining aq extr. was dissolved in 50% ethanol (2ml) and sealed.

The DCM extr. was mixed with anhydrous sodium sulphate to dry it. The extrr. was filtered through Watman filter paper (no.1) and collected. The remaining salt was rinsed with dichloromethane until colourless, filtered and collected. The extr. was reduced to dryness by rotary evaporation. The DCM extr. was re-dissolved in ethanol (1ml) and DCM (20 micro-l) and sealed.

Thin Layer Chromatography (TLC)

The plates used to run the samples were Merck TLC 20 x 20 cm silica gel 60 F_{254}. A pencil line was drawn on the plates 2 cm from the base. The samples were applied to the plate in a 2 cm streak along the line in 2 applications of 10 micro-l. The number and spacing of the extracts applied to the plates was variable and is detailed later with the number of repetitions.

The two types of extract were developed in two solvent systems in a chromatography tank. The DCM extracts were developed in hexane and ethyl acetate (50:50) The Aq extracts were developed in water, formic acid and ethyl acetate (1:1:18). A blank plate was developed in each solvent system as a control. The plates were developed in the solvents for approximately 1 hour. The plates were removed from the tank and left overnight in a fume hood to reduce their acidity.

Fungal Application

A potato dextrose slope with a culture of *Cladosporium herbarum* under sterile liquid paraffin was obtained from cold storage (4^oC). Sigma nutrient agar (100ml) was prepared according to the packet instructions sealed and autoclaved. The cool agar was reheated and poured into petri dishes at a depth of approximately 5mm. The fungus was cut from the slope and sub-cultured

onto the agar plates using a streak method under sterile conditions. The agar plates were sealed and incubated at a temperature of 20^{o}C for one week. The cultures were kept for use at a temperature of 4^{o}C for a period of 2 weeks to maintain a high number of viable spores. Fresh spores were sub cultured from the agar plates as needed using the same method.

Sigma potato dextrose broth was prepared according to the packet instructions. The broth (5ml) was poured on *Cladosporium* in agar. A drop of detergent (Tween no. 20) was added to bring the fungal spores into suspension. The spore suspension was filtered through a stainless steel mesh (London & England aperture 53) then centrifuged at 200g. The resultant pellet was re-suspended in broth (4ml) with 1ml being added to 20ml of broth per plate. The developed plates were sprayed until uniformly wet but not saturated with a mechanical pump spray (The Body Shop). The plates were left to dry and put into a sealed, darkened container separated from the dampened tissue with a plastic barrier. The container was incubated at 20^{o}C for two days. The plates were removed and left to air dry. The plates were examined visually for any zones on the plate where the fungi had been inhibited. Photographs of all the bioassay's were taken to provide a record of the results.

Record of Bioassay

A total number of 16 plant samples were extracted. Initially both phases of the extraction (DCM and aq) were streaked 8 per plate sequentially with a 3mm spacing. They were developed in the corresponding solvent and bioassayed, 4 repetitions of each extract were made. A blank plate was run in both solvents and bioassayed as a control.

Extracts which have resulted in inhibition zones were streaked on plates individually 6 per plate with 1cm intervals x 2 developed and given a second bioassay.

Chemical Detection of Active Spots

The extracts which contain active compounds detected with the bioassay have been redeveloped on additional plates for chemical detection of the active compound. Parallel plates were developed for each of the extracts (x 3) in the corresponding solvent. The extracts were streaked on the plates with a spacing >1cm. One representative of each developed extract was subjected to the three following detection techniques.

- A developed plate was placed in a chromatographic tank with Iodine crystals for 5 minutes and visually examined.

- The developed plates were sprayed with a Reagent which detects Tannins and visually examined.

Tannin Reagent A) 0.2% $K_3Fe(CN)_6$ Spray plate with A followed by
0.2% $FeCl_3.6H2O$ 2M HCl and De-I H_2O
A few grains of $KMnO_4$ Blue colour = Tannin

- The plates were visually examined under UV light (366 & 254nm) and the active spots were detected by fluorescence. A single plate was developed containing all the active spots detected in the Aq solvent system for comparison of the spots. Photographs have been taken as a hard record.

1.2 INTERPRETATION OF PLATES

Optical Density

The photographic results for the second bioassay of the extracts containing active compounds were scanned for optical density with a high resolution scanner to producing a measurable value for inhibition and an Rf value for the spot. One inhibition zone was scanned for each extract at a density of 0 - 1.5 (arbritry units) at a 100 microns resolution.

Scoring Of Inhibition Zones

A visual method has been used to score the inhibitory effect of the active spot. The size of the active spot has been compared with the size of the zone of inhibition on the second bioassay. The boundary of the active spots were marked on the parallel plates when visible under UV light and a size comparison has been made with the inhibitory zones. The inhibition was scored from 0-5 where 0 = no inhibition and 5 = complete inhibition.