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Effects of different forages on phenol and methylphenol formation in the rumen of sheep.

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ABSTRACT

Rumen concentrations of the undesirable phenol flavour compounds 4-methylphenol, 3-methylphenol, and phenol, were measured in pooled samples from six rumen-fistulated Romney wethers. Samples were collected at approximately 1 hr intervals for 8 hr after feeding white clover (WC), perennial ryegrass (PRG), or *Lotus corniculatus* (LC). For all three phenols, the time-course curves for the rumen concentration differed significantly between the three diets ($P<0.001$) with wide differences in maximum concentrations. For 4-methylphenol, concentrations normalised to crude protein intake (CPI) were very similar. Concentrations of phenol normalised to CPI were very similar for PRG and WC diets but much lower for the LC diet. No 3-methylphenol was detected in the rumen of WC-fed animals. For animals fed the PRG and LC diets, concentrations of 3-methylphenol normalised to acid detergent fibre intake were similar. Differences were also observed in the time to maximum concentration after the commencement of feeding, which was much shorter for 4-methylphenol and phenol (2-3.5 hr) than for 3-methylphenol (6 hr). This study established that feeding different forages resulted in differences in phenol metabolism in the rumen, and provides evidence that 4-methylphenol and 3-methylphenol derive from different dietary sources.

Keywords: diet; flavour; methylphenols; rumen; ADF.

INTRODUCTION

Meats and dairy products derived from animals raised on pasture have a characteristic flavour. At one extreme of preference, pastoral flavour is described as 'full-bodied', an attribute to be valued. At another, pastoral flavour is described as 'off', 'animal-like' or 'grassy'. These latter descriptions are from consumers accustomed to the flavour of meat from grain-finished animals, which has a relatively bland flavour (Keen, 1998; Young *et al*., 2003).

Phenol and the alkylphenols are a group of flavourful compounds that occur in meat and dairy products. The methylphenols in particular, have been implicated as contributing towards 'pastoral flavour' in both beef (Ha & Lindsay, 1991) and sheep meat (Young *et al*., 1997, 1999 and 2003), and in dairy fats (Urbach *et al*., 1972). As part of the process of metabolic detoxification in the animal, the phenols are conjugated and excreted in the urine as sulfates and glucuronides. These conjugates have been reported in skim milk (Brewington *et al*., 1973; Lopez & Lindsay, 1993), and are regarded as a source of 'flavour potential' in dairy products. In New Zealand, concentrations in skim milk of conjugates of 4-methylphenol, and 3-methylphenol have been found to be significantly higher for cows fed pasture than those fed a total-mixed-ration diet (Lane *et al*., 2002).

The formation of these compounds in the rumen is not well understood. Ha & Lindsay (1991) suggested that alkylphenols in beef fat volatiles may have links to specific components of a pasture diet, such as lignin and diterpenes, which might be converted to phenols in the rumen. They also noted that dietary protein was a potential source of phenol flavour compounds, as degradation in the rumen of the amino acid tyrosine could generate phenol, 4-methylphenol, and 4-ethylphenol. For 3-methylphenol there is no corresponding amino acid progenitor, but it has been suggested (Lane & Fraser, 1999) that 3-methylphenol is likely to derive from plant-associated fibre phenolic components. These could include lignin, lignans, and lignin precursors and other polyphenolics such as condensed tannins (Déprez *et al*., 2000).

The production by ruminants of 4-methylphenol and phenol is also of interest in relation to the environmental impact of intensive livestock agriculture. In studies in Europe, 4-methylphenol and phenol have been identified as markers of effluent pollution in waterways (Schussler & Nitschke, 1999).

In this study, the free phenol content of rumen fluid has been analysed in samples collected from sheep fed either white clover, perennial ryegrass or *Lotus corniculatus* (a condensed-tannin-containing legume (Foo *et al*., 1995)) prior to, during, and after feeding. Data has been compared with Near Infra-red Reflectance Spectrometry (NIRS) analyses of the diet (Feedtech, AgResearch Limited, Palmerston North, New Zealand) to investigate the possible relationships with dietary components.

MATERIALS AND METHODS

Animals, Forages and Sampling

The design and management of this trial were described by Schreurs *et al*., 2003. Briefly, six Romney wethers, fistulated in the rumen were fed white clover (WC) for three weeks, followed by perennial ryegrass (PRG) for three weeks, and finally *Lotus corniculatus* (LC) for three weeks. Feeding occurred daily at 0800 hr and 1600 hr and the animals were allowed 2 hours to feed at each time. After a period of adjustment to each of the forages, rumen contents were sampled at -0.25, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6.25, 7, and 8 hr after the start of the two-hour morning feeding. Rumen contents were squeezed through a double layer of cheesecloth and the fluid immediately frozen in liquid nitrogen. Prior to analysis, the samples were thawed and the samples from the six animals per treatment were

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pooled. The weight of feed offered and refused was recorded for each sheep, at each meal. Triplicate forage samples (200 g) of both feed offered and refused were taken to determine dry matter (DM) content by oven drying (90°C for 24 hr). Samples of the forage offered and refused were collected throughout the trial, frozen and freeze-dried to determine crude protein (CP), acid detergent fibre (ADF), and neutral detergent fibre (NDF) composition by NIRS (Feedtech, AgResearch Limited, Palmerston North, New Zealand).

Phenol analysis

Concentrations of phenol, 3-methylphenol and 4-methylphenol in rumen fluid were measured using an adaptation of the method of Lane & Fraser (1999). Rumen fluid (5 ml) in saturated NaCl (50 ml) was spiked with 2-ethylphenol (41.5 µg, internal standard) and extracted by simultaneous distillation extraction with *t*-butyl methyl ether (20 ml) for 90 min. The phenols were analysed by GC-MS (QP-2010, Shimadzu, Kyoto, Japan) utilizing the selected ion recording mode. Extracts were resolved on a ZB-WAX column (30 M x 0.25 mm I.D. x 0.25 µm film thickness, Phenomenex, Torrance, CA, USA) with Helium as the carrier gas (69 kPa). The injection (1 µl) was performed in splitless mode with a 0.5 min sampling time and an injection port temperature of 250°C. The column oven was initially held at 50°C for 1 min, then heated at 4°C min^{-1} to 220°C and held at that temperature for a further 5 min. The interface temperature was 250°C and the ion source temperature was 200°C. Selected ions monitored for each compound were (target ion/reference ion); 2-ethylphenol, *m/z* 107/122; *p*-cresol, *m/z* 107/108; *m*-cresol, *m/z* 108/107; phenol, *m/z* 94/66.

Statistical analysis

The data were analysed for the significance of differences between feed treatments by the statistical comparison of time-course curves for rumen concentration data and for concentrations normalised to intake of CP, NDF and ADF with Genstat version 6.0. Regression curves were fitted with a quadratic × quadratic equation (Ross, 1987). The model equation was:

$$y = \frac{a + (b + cx)}{(1 + dx + ex^2)}$$

From this equation, factors *a*, *b* and *c* are the linear terms and factors *d* and *e* are the non-linear terms. The significance of differences between the fitted time-course curves for the feed treatments was tested by non-linear regression and analysis of variance.

RESULTS

Feed parameters

The intake data presented in Table 1 refer to the AM feeding period only. Full daily intake data was tabulated by Schreurs *et al*., 2003. The concentrations and intakes of the feed parameters (DM, CP, ADF and NDF) for the diets were significantly different. The PRG diet contained the highest DM%, ADF and NDF concentrations and the lowest concentration of CP, while the concentrations for the WC and LC diets for these parameters were similar. The intake data showed that PRG contained the highest DM, ADF, and NDF intakes, and a similar amount of CP intake compared to the LC diet. Values of these parameters for WC were much lower than for the other diets.

TABLE 1: The means (SEM) of dry matter percentage (DM), concentrations (g/100g DM) of crude protein (CP), neutral (NDF) and acid (ADF) detergent fibre and intakes of DM, CP, NDF, and ADF (g/day) for white clover (WC), perennial ryegrass (PRG) and *Lotus corniculatus* (LC) fed to sheep.

	WC	PRG	LC	Significance
Concentration				
DM	12.2 (0.3)	23.3 (0.4)	14.8 (0.2)	P<0.001
CP	27.2 (0.7)	17.5 (0.3)	24.0 (0.8)	P<0.01
NDF	24.1 (0.1)	45.4 (0.2)	24.0 (1.3)	P<0.001
ADF	22.1 (1.0)	25.5 (0.1)	21.2 (0.7)	P<0.05
Intake				
DM	114.0 (26.6)	350.5 (50.6)	278.3 (45.2)	P<0.01
CP	30.9 (7.2)	61.5 (8.9)	66.9 (10.9)	P<0.05
NDF	27.4 (6.4)	159.1 (23.0)	66.7 (10.8)	P<0.001
ADF	25.2 (5.9)	89.5 (12.9)	59.0 (9.6)	P<0.001

Metabolism data

The metabolite concentrations in the rumen and concentrations corrected for either CP or ADF intake, together with the fitted quadratic × quadratic curves are presented in Figures 1a-3b. Pairwise comparisons of the data showed that in all but one case, the different curves were fitted with common *d* and *e* terms.

The time-course curves for 4-methylphenol concentration (Figure 1a) for the three feeds were significantly different (P<0.001). The time-course curves for 4-methylphenol concentration in the rumen showed a major difference in amplitude between the WC diet, and the LC and PRG diets, but the time to maximum (t_{max}) concentration was similar (approximately 2.5 hr). In pairwise comparisons, the curves for the LC and PRG diets were not significantly different. When the rumen concentration of 4-methylphenol was normalised to crude protein intake (Figure 1b), the amplitudes of the curves were very similar, although the equations for the curves remained significantly different (P<0.001) when the three feeds were considered together. Pairwise comparisons showed no significant treatment difference between the LC and WC curves. By contrast, the curves for 4-methylphenol concentrations normalised to either ADF or NDF intake exhibited widely different amplitudes (data not shown).

The time-course curves for phenol concentration (Figure 2a) for the three feeds were significantly different (P<0.001). The curves showed a similar t_{max} for the PRG and WC diets (approximately 2 hr), but the LC t_{max} was delayed (approximately 3.3 hr). The amplitudes of the curves were considerably different for all three diets (confirmed by pairwise comparisons, P<0.001). When the rumen concentration of phenol was normalised to crude protein intake (Figure 2b), the fitted curves remained significantly different (P<0.001). While the amplitudes for PRG and WC were similar, the amplitude for LC remained much lower. Pairwise comparisons of the data confirmed that these differences were significant (P<0.01).

FIGURE 1. a) Concentration of 4-methylphenol (mg/l rumen fluid) vs time (hr) from the beginning of feeding for animals fed white clover (■), perennial ryegrass (▲) or *Lotus corniculatus* (●). b) Ratio of 4-methylphenol/crude protein intake (mg/l/kg CPI/d) vs time (hr) from the beginning of feeding for animals fed white clover (■), perennial ryegrass (▲) or *Lotus corniculatus* (●). Curves fitted for both figures are quadratic × quadratic.

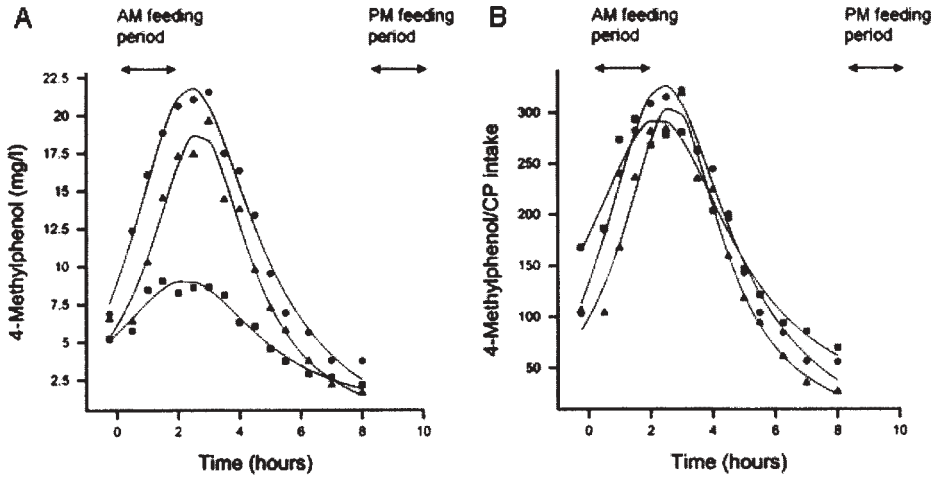


FIGURE 2. a) Concentration of phenol (mg/l rumen fluid) vs time (hr) from the beginning of feeding for animals fed white clover (■)perennial ryegrass (▲) or *Lotus corniculatus* (●). b) Ratio of phenol/crude protein intake (mg/l/kg CPI/d) vs time (hr) from the beginning of feeding for animals fed white clover (■)perennial ryegrass (▲) or *Lotus corniculatus* (●). Curves fitted for both figures are quadratic × quadratic.

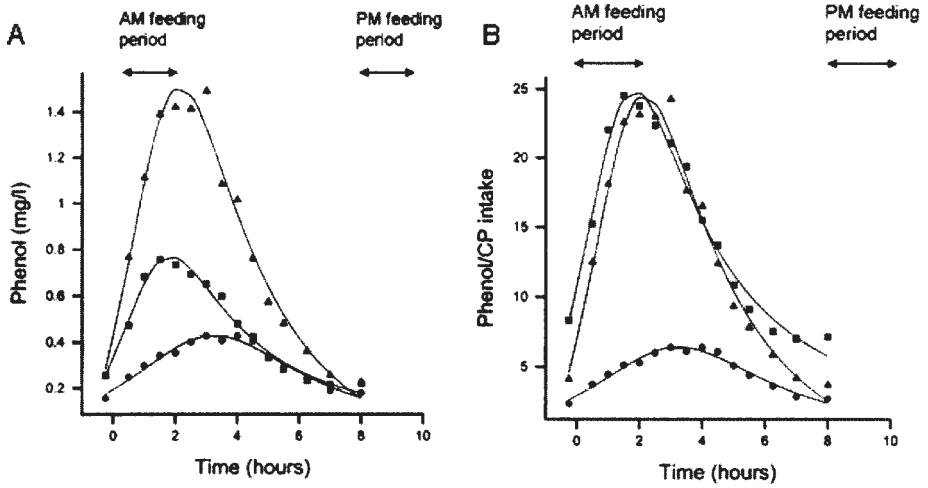
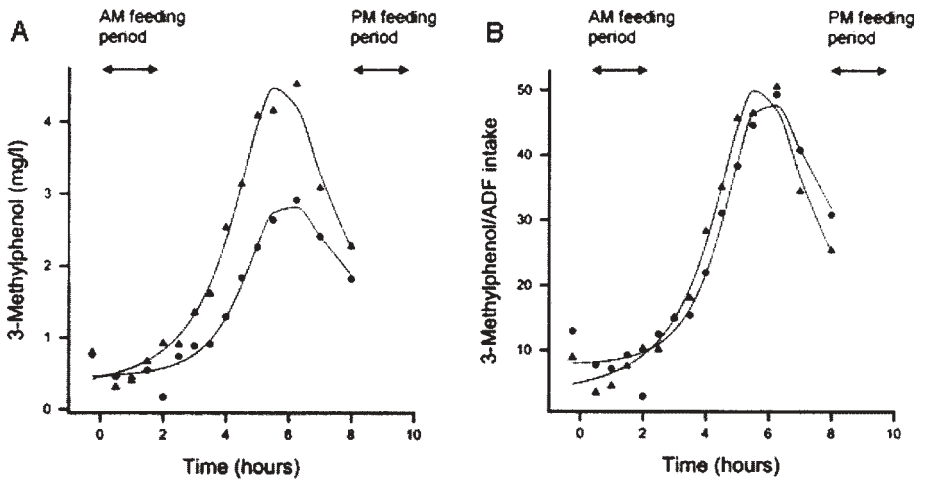


FIGURE 3. a) Concentration of 3-methylphenol (mg/l rumen fluid) vs time (hr) from the beginning of feeding for animals fed perennial ryegrass (▲) or *Lotus corniculatus* (●). b) Ratio of 3-methylphenol/acid detergent fibre intake (mg/l/kg CPI/d) vs time (hr) from the beginning of feeding for animals fed perennial ryegrass (▲) or *Lotus corniculatus* (●). Curves fitted for both figures are quadratic × quadratic.



Normalisation to either ADF or NDF intake did not remove differences between the time-course curves for phenol for the three diets (data not shown).

No detectable concentration of 3-methylphenol was observed in the rumen on the WC diet. The time-course curves for 3-methylphenol concentration (Figure 3a) in the rumen were significantly different ($P<0.001$), and differed in amplitude but not in t_{max} (approximately 6 hr). When the rumen concentration of 3-methylphenol for LC and PRG diets was normalised to ADF intake (Figure 3b), there was no significant treatment effect. Normalisation of the data to either CP or NDF intake did not eliminate the treatment effect (data not shown).

DISCUSSION

Two sources of 4-methylphenol in the rumen of forage-fed sheep have been identified (Martin, 1982), the deamination and decarboxylation of tyrosine, and the degradation of coumarate esters and other plant phenolics. The time-course curves for 4-methylphenol normalised to crude protein intake suggest that this factor largely accounts for the differences between the concentration of 4-methylphenol in the rumen for the three diets.

Phenol is also a product of tyrosine degradation (Ha & Lindsay, 1991; Martin, 1982). However, while the differences between rumen concentration of phenol on WC and PRG diets could be accounted for by crude protein intake, this was not the case for the LC diet, in which phenol concentration was relatively low. The LC diet appears to have a specific effect on the formation of phenol but not 4-methylphenol, despite their likely common origin. The possibility that the condensed tannins present in LC (Foo *et al*., 1995) have a differential effect on the rumen formation of the two phenols warrants further investigation. The pattern of feed effects on the rumen formation of these amino-acid-derived phenolic compounds differs notably from that observed for the amino-acid-derived indoles, skatole and indole (Schreurs *et al.*, 2003).

The dietary source of 3-methylphenol in ruminant products has not been identified although precursors have been observed as products of anaerobic fermentation of plant polyphenolics (Déprez *et al*., 2000). The process of 3-methylphenol formation in the rumen is relatively slower than that of 4-methylphenol, which is consistent with different pathway of synthesis. Previous studies had suggested an association with fibre content of the diet (Lane & Fraser, 1999; Young *et al*., 2003). The time-course curves for 3-methylphenol normalised to ADF intake for PRG and LC show that differences in 3-methylphenol concentration in the rumen are largely accounted for by the dietary fibre intake. This was not the case for NDF suggesting that the origin of 3-methylphenol is specifically related to the ADF portion of the total plant fibre. Despite the low ADF intake of the WC diet, the levels of 3-methylphenol in the rumen could be expected to be above the detection limit, by comparison with the data for the LC and PRG diets. The undetectable levels may be due to differences within the components of ADF, or indirect effects on digestion rate.

The results of this study indicate that the effects of different forage diets on the formation in the rumen of undesirable phenol flavour compounds are largely accounted for by broad measures of feed composition intake (CP, ADF intake). In particular, rumen concentrations of the major phenolic flavour compound, 4-methylphenol, appear to be determined by CPI. Concentrations of 4-methylphenol conjugates have been reported to be much higher in the milk of cows fed forage diets than that of TMR-fed cows (Lane *et al*., 2002). The current findings suggest that this can be accounted for by the higher protein (and hence tyrosine) content of the forage diets. Specific measures of tannin and lignin content may be required to account for the low levels of phenol formation on the LC diet, and the absence of 3-methylphenol on the WC diet.

While the use of *Lotus corniculatus* as a pasture legume has been suggested as a strategy to ameliorate pastoral flavour of meat and dairy products due to skatole and indole (Schreurs *et al*. 2003), this study suggests flavour effects due to 4-methylphenol are unlikely to be controlled by this strategy. Further investigation will be required to identify means of inhibiting 4-methylphenol formation in the rumen and control this component of pastoral flavour.

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REFERENCES

Brewington, C.R.; Parks, O.W.; Schwartz, D.P. 1973: Conjugated compounds in cows milk. *Journal of agriculture and food chemistry 21*: 38-39.

Déprez, S.; Brezillon, C.; Rabot, S.; Philippe, C.; Mila, I.; Lapierre, C.; Scalbert, A. 2000: Polymeric proanthocyanidins are catabolized by humen colonic microflora into low-molecular weight phenolic acids, *Journal of nutrition 130*: 2733-2738.

Foo, L.Y.; Newman, R.; Waghorn, G.; McNabb, W.C.; Ulyatt, M.J. 1995: Proanthocyanidins from *Lotus corniculatus*. *Phytochemistry 41*: 617-624.

Ha, J.K.; Lindsay, R.C. 1991: Volatile alkylphenols and thiophenol in species-related characterizing flavours of red meats. *Journal of food science 56*: 1197-1202.

Keen, A.R. 1998: Flavour compounds and their origin in dairy products. *Chemistry in New Zealand*, September/October issue: 5-13.

Lane, G.A.; Fraser, K. 1999: A comparison of phenol and indole flavour compounds in the fat, and of phenols in the urine of cattle fed pasture or grain. *New Zealand journal of agricultural research 42*: 289-296.

Lane, G.A.; Fraser, K.; Kolver, E.S.; Rowan, D.D.; Allen, J.M.; Mills, O.E.; Abraham, A.S.; Olney, S.D. 2002: Effect of a total mixed ration diet on the concentration of amino acid-derived volatiles in milk. *Proceedings of the New Zealand Society of Animal Production 62*: 242-245.

Lopez, V.; Lindsay, R.C. 1993: Metabolic conjugates as precursors for characterizing flavour compounds in ruminant milks. *Journal of agriculture and food chemistry 41*: 446-454.

Martin, A.K. 1982: The origin of urinary aromatic compounds excreted by ruminants 3. *British journal of nutrition 48*: 497-507.

Ross, G.J.S. 1987: Maximum likelihood program. Numeric Algorithms Group, Oxford. 5.13-5.14.

Schreurs, N.M.; Tavendale, M.H.; Lane, G.A.; Barry, T.N.; McNabb, W.C. 2003: Postprandial skatole and indole formation in the rumen. *Proceedings of the New Zealand Society of Animal Production 63*: 14-17.

Schussler, W.; Nitschke, L. 1999: Death of fish due to surface water pollution by liquid manure or untreated wastewater: analytical preservation of evidence by HPLC. *Water research 33*: 2884-2887.

Urbach, G.; Stark, W; Forss, D.A. 1972: Volatile compounds in butter oil. II. Flavour and flavour thresholds of lactones, fatty acids, phenols, indole and skatole in deodorized synthetic butter. *Journal of dairy research 39*: 35-47.

Young, O.A.; Berdagué, J.-L.; Viallon, C.; Rousset-Akrim, S.; Theriez, M. 1997: Fat-borne volatiles and sheepmeat odour. *Meat science 45*: 183-200.

Young, O.A.; Lane, G.A.; Priolo, A.; Fraser, K. 2003: Pastoral and species flavour in lambs raised on pasture, Lucerne or maize. *Journal of the science of food and agriculture 83*: 93-104.

Young, O.A.; Priolo, A.; Lane, G.A.; Knight, T.; Fraser, K. 1999: Causes of pastoral flavour in ruminant fat. *Proceedings of the 45th international congress of meat science and technology*, Yokohama, 420-421.