Formation and excretion of phenol flavour compounds in sheep

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ABSTRACT

Rumen, plasma and urinary concentration of the undesirable phenol flavour compound 4-methylphenol was measured in two rumen-fistulated Romney wethers fed perennial ryegrass. Following a one week adaptation period, samples were collected at ca. 1 hr intervals after feeding on two sampling days one week apart. Maximal concentration of 4-methylphenol in the rumen, blood and urine was reached at ca. 2, 3, and 4.5 hr respectively, declining to a minimal concentration by 9 hr (15% of maximum), 11 hr (16%), and 18 hr (<1%) respectively. Thus phenol compounds formed in the rumen are cleared rapidly and efficiently by excretion into the urine. In a separate trial the absorption and excretion of the exogenous phenol, thymol, was measured. Four groups of 4 lambs grazed on pasture were dosed daily with either a control or 3 different levels of thyme slurry for two weeks. After one week of dosing spot urine samples were collected on two consecutive days. Upon completion of the two week dosing period the animals were slaughtered and subcutaneous fat samples collected. The concentration of thymol (conjugates) in the urine was highly correlated with the thyme dosage ($r=0.848$, $P<0.001$), but there was no significant correlation between dose level and the concentration of (free) thymol in the fat. These results suggest that the concentration of phenol flavour compounds in ruminant products is affected by the efficiency of conjugation and excretion as much as by their formation in the rumen.

Keywords: diet; flavour; methylphenols.

INTRODUCTION

Meats and dairy products derived from animals raised on pasture have a characteristic flavour. This flavour has been described as 'full-bodied' and is an attribute valued by populations of consumers accustomed to pastoral flavour. However consumer groups more accustomed to the flavour of meat from grain-finished animals have described this pastoral flavour as 'off', 'animal-like' or 'grassy' (Keen, 1998; Young *et al*., 2003).

The alkylphenols are a group of flavourful compounds that occur in meat and dairy products and 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, and 2003), and in dairy fats (Urbach *et al*., 1972). Ha and Lindsay (1991) suggested that alkylphenols in beef fat volatiles may have links to specific components in a pasture diet such as lignin and diterpenes. The concentration of 4-methylphenol in the rumen has been postively correlated with crude protein intake (Fraser *et al*., 2003), while urinary concentration of 4-methylphenol has been linked to both the deamination and decarboxylation of tyrosine and the degradation of coumarate esters via phenolic acid intake (Martin, 1982). The pathway for metabolic detoxification and removal of phenolic compounds is via conjugation and excretion in the urine as sulfates and glucuronides (Scheline, 1991) with the main site of conjugation in the gastrointestinal epithelium (Powell *et al*., 1974; Lundh, 1990). These conjugates have also been reported in skim milk (Brewington *et al*., 1973; Lopez & Lindsay, 1993) and are regarded as a source of 'flavour potential' in dairy products. Significant dietary effects on methylphenol conjugate concentration in urine (Lane & Fraser, 1999) and skim milk (Lane *et al*., 2002) has been positively correlated to crude protein and fibre intake.

Concentrations of phenols in meat and dairy product will be determined by the level of exposure of the animal to phenols formed in the rumen (endogenous) or ingested as naturally occurring plant phenols (exogenous) and by the animal's ability to absorb, metabolize and excrete them.

The objective of this study was to monitor the production, transfer, excretion and adsorption of phenolic compounds. The first animal trial explores the concentration of 4-methylphenol in the rumen, blood and excretion in the urine with sheep fed perennial ryegrass. The second animal trial explores the uptake, absorption and clearance of exogenous phenols. Lambs were dosed increasing levels of the herb thyme (carried out in the course of studies of meat flavour modification; Keogh *et al*., unpublished) and the excretion and absorption of the phenols thymol and carvacrol were monitored.

MATERIALS AND METHOD

Animals, Forages and Sampling

Trial 1:

Animals and Feeding

Two Romney wethers with rumen fistula were placed in metabolism crates and housed indoors at Nutrition and Behaviour, AgResearch Limited, Grasslands Research Centre, Palmerston North. The animals were fed fresh perennial ryegrass for two 2 hr meal periods each day, at 09:00 and 16:00 hours. Following a 1 week adaptation period, for two days prior to sampling and on sampling day the animals were fed *ad libitum* in the morning (09:00) only.

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Samples were collected at approximately 1 hr intervals after feeding and then twice daily feeding resumed once sampling had concluded. A second sampling day occurred one week later. Animals had access to fresh, clean water at all times. Forage sample of both feed offered and refused were taken to determine dry matter content by oven drying (90°C for 24 hr). Crude protein (CP), neutral detergent fibre (NDF), and acid detergent fibre (ADF) composition was determined by NIRS (Feedtech, AgResearch Limited, Palmerston North).

Catheters and Blood Sampling

Temporary jugular catheters were inserted a week after the animals had been placed in metabolism crates (two days prior to the first sampling day). Two catheters were inserted into each sheep. One catheter was placed within the jugular vein and the other threaded down the jugular vein into the heart until the blood within the catheter was pulsating. Samples of blood were taken from each catheter of each sheep, one hour prior to feeding and then at hourly intervals from the start of feeding, for 15 hours. Blood was collected by flushing the catheter with approximately 2 ml of 50 iu heparin in saline then withdrawing 4-5 ml of blood which was discarded. The blood sample was then aspired from the catheter into a 7.5 ml LH S-Monovette tube (Lithium Heparin 15 iu/ml of blood, Sarstedt, Germany) using S-Monovette adapters. Blood samples were immediately placed on ice. After sampling, the catheter was flushed with 5-8 ml of 50 iu heparin in saline. Blood samples were then centrifuged (3270 g for 15 minutes) and the plasma removed and frozen at -20°C for the analysis of phenols at a later stage.

Urine and Rumen fluid Sampling

Both animals were fitted with faecal collection bags which allowed the collection of clean urine from the metabolism crate collection tray into a clean bucket. Hourly urine samples were collected beginning one hour prior to the two hour morning feeding period and continuing for 20 hours. The weight of the urine collected each hour was recorded and a 10 ml sample was centrifuged (11250 g for 15 minutes) and the supernatant frozen at -20°C for analysis of phenols.

Whole rumen contents were also sampled hourly for 15 hr via the rumen fistula and squeezed through a double layer of cheesecloth. A 4 ml sub-sample of the rumen fluid was then frozen in liquid nitrogen and stored in a -20°C freezer for analysis of phenols.

Trial 2:

Four groups of 4 Romney lambs grazed on pasture were dosed daily with 4 different levels (0, 100, 400, or 800 ml) of thyme slurry for two weeks. The thyme slurry was produced by blending freeze-dried thyme with water and the larger particulate material was removed by crude filtration. After one week of slurry dosing urine samples were collected 5 hr after dosing (shown to correspond to the maximum thymol excretion in a preliminary experiment) on two consecutive days and stored at -20ºC until analysed. At the end of the two week dosing period the animals were slaughtered and subcutaneous fat samples were collected and stored at -20ºC until analysed.

Analytical methods

The concentration of 4-methylphenol in rumen fluid was measured by steam distillation simultaneous extraction with a Likens-Nickerson apparatus and GC-MS (Fraser *et al*., 2003).

Plasma concentration of total 4-methylphenol was measured after enzyme hydrolysis and partitioning into ethyl acetate. Plasma (0.5 ml) spiked with 2-ethylphenol (3.27 μg, internal standard) was incubated with 0.5 ml of 0.2 M sodium acetate buffer (pH 5.0) containing 1000 units β-glucuronidase and 50 units of sulfatase activity (Sigma type H-5, *H. pomatia*) at 37°C for 24 hr. The protein precipitate was resuspended with 1 ml of 6 M guanidinium hydrochloride and the sample partitioned with 1 ml of ethyl acetate (containing 5.53 μg 2-isopropylphenol, secondary standard) in a vortex mixer (2 × 30 seconds). After centrifugation (2700 g, 5 min), disruption of the resulting clot in the ethyl acetate phase and re-centrifugation (2700 g , 5 min) an aliquot (1 μl) of the ethyl acetate phase was analysed by GC-FID (GC-17A, Shimadzu, Kyoto, Japan). The mixture was resolved on a ZB-WAX column (30 M × 0.32 mm I.D. × 0.25 μm film thickness, Phenomenex, Torrance, CA, USA) with helium as the carrier gas and a linear temperature programme from 50 to 250°C.

Urinary concentration of total 4-methylphenol, thymol and carvacrol was determined after hydrolysis of the conjugates. The hydrolysis procedure was performed as reported by Lane & Fraser (1999) and the extraction of the released phenols by solid phase extraction as documented by Lane *et al*. (2002). The phenols were analysed by GC-FID using the method described above for plasma and the concentration calculated relative to 2-ethylphenol (internal standard).

Adipose tissue concentration of 4-methylphenol, thymol and carvacrol was determined by simultaneous distillation extraction and GC-MS (Lane & Fraser, 1999).

Thyme slurry (25 ml) spiked with 2-ethylphenol (1550 μg) was shaken (3 × 30 sec) with 20 ml n-hexane. After centrifugation (400 g, 5 min) 5 ml of hexane was decanted into a flask containing anhydrous sodium sulphate and an aliquot (1 μl) was analysed for thymol and carvacrol by GC-FID using the method of McGimpsey *et al*. (1994) on a SPB-1 column (15 M × 0.32 mm I.D. × 0.25 μm film thickness, Supleco, USA).

Statistical analysis

The relationships between thymol dietary intake, urinary excretion, and adipose tissue accumulation were determined by correlation and by linear regression (weighted to compensate for unequal variances in the case of the excreted thymol data) with Minitab version 13.31 (Minitab Inc., PA, USA). Spline curves were fitted to the time-course data with Genstat version 6.2

(Lawes Educational Trust, Oxford, UK), and used to estimate both time to maximum concentration (t_{max}) and time to minimum concentration (t_{min}).

RESULTS

Trial 1:

The average dry matter intake for the two sheep on the two sampling days was 506 ± 39 g/two hr feeding period/animal. The CP, NDF and ADF intake was 114 ± 9, 124 ± 10, 231 ± 18 g/two hr feeding period/animal respectively.

The 4-methylphenol concentration in the rumen, plasma and urine together with fitted spline curves are presented in Figures 1-3.

FIGURE 1: Ruminal concentration of 4-methylphenol (μg/ml rumen fluid) vs time after morning feeding (hr) for animals fed perennial ryegrass. The fitted curve is a spline and accounted for 88.3% of the variance. ↓ indicates beginning of two hour feeding period.

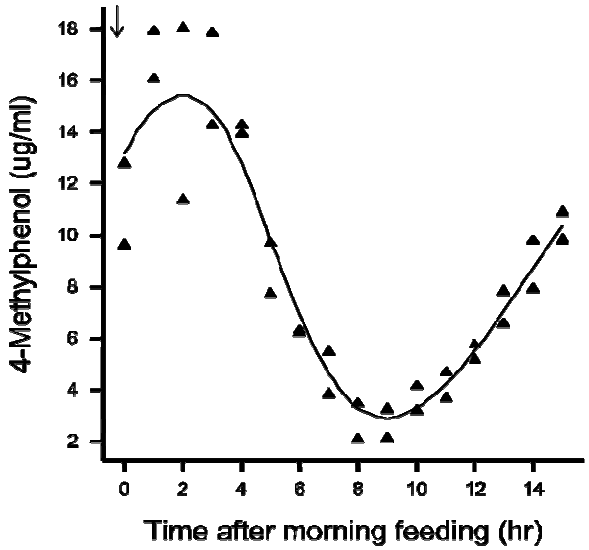
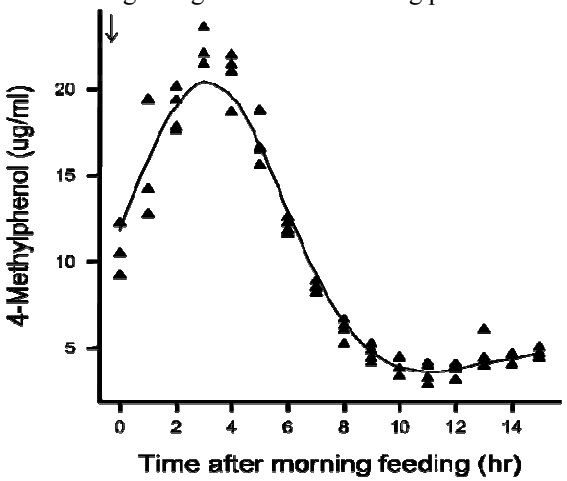


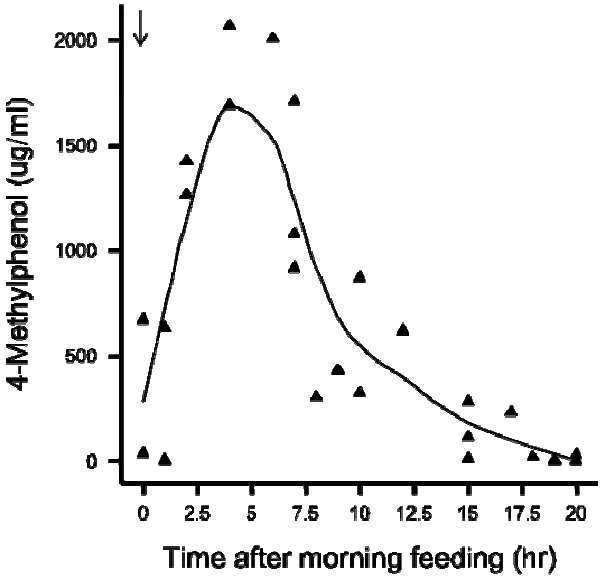
FIGURE 2: Blood concentration of 4-methylphenol (μg/ml plasma) vs time after morning feeding (hr) for animals fed perennial ryegrass. The fitted curve is a spline and accounted for 95.9% of the variance. ↓ indicates beginning of two hour feeding period.



The time-course curve for 4-methylphenol in the rumen was biphasic and had a t_{max} of approximately 2 hr, and a t_{min} of approximately 9 hr. The minimum concentration declined to 15% of the maximum and the concentration then increased to 60% of maximum at the 15 hr (final) time point.

The time-course curve for 4-methylphenol in the plasma had a t_{max} of approximately 3 hr, and a t_{min} of approximately 11 hr, with the minimum concentration decreasing to 16% of the maximum and then increasing to 21% of maximum after 15 hr.

FIGURE 3: Urinary concentration of 4-methylphenol (μg/ml urine) vs time after morning feeding (hr) for animals fed perennial ryegrass. The fitted curve is a spline and accounted for 74.3% of the variance. The missing time points occurred as animals did not urinate at each hourly interval. ↓ indicates beginning of two hour feeding period.

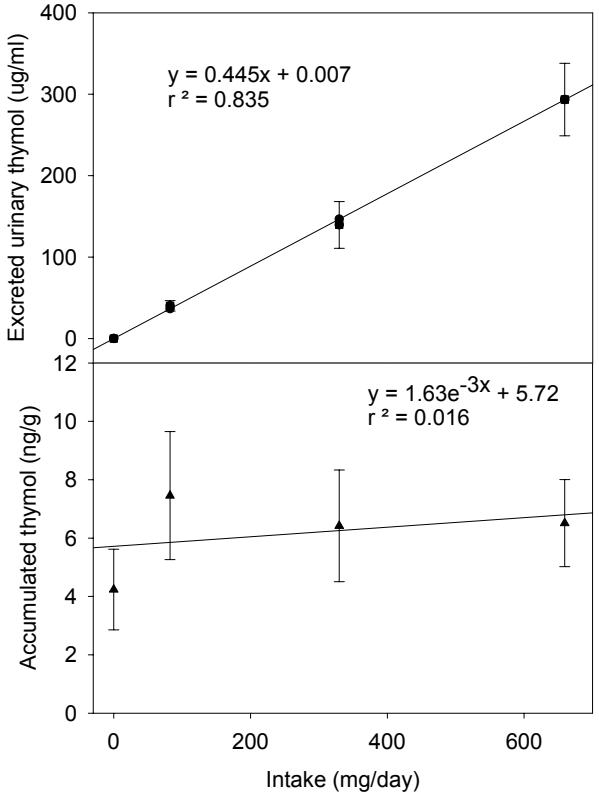


The time-course curve for 4-methylphenol in the urine had a t_{max} of approximately 4.5 hr, and a t_{min} of approximately 18 hr, with the minimum concentration decreasing to <1% of the maximum.

Trial 2:

Mean daily intakes of thymol for the 100 ml, 400 ml, and 800 ml/day thyme slurry dosings were 82 ± 4, 330 ± 15, 659 ± 30 mg/day respectively. No thymol was detected in the urine of control (0 ml dosed) animals, and there was no evidence of a daily difference in urinary thymol concentration between the two sampling days. The concentration of thymol in the urine was strongly dependent on the thymol intake (correlation coefficient (r) = 0.848, P<0.001) (Figure 4).

FIGURE 4: Concentration in sheep dosed thyme daily for two weeks of excreted thymol (μg/ml) in urine (■) and accumulated thymol (ng/g) in adipose tissue (▲) vs daily thymol intake (mg/day). Error bars are standard errors of the mean.



Mean concentration of thymol in the adipose tissue was higher in the dosed animals but not significantly different from the control animals and there was no significant dose-response trend (Figure 4). The concentration of carvacrol was lower but exhibited similar trends as for thymol (data not shown).

DISCUSSION

Trial 1:

The two potential sources of 4-methylphenol in the animal are tyrosine or phenolic acid degradation (from fibre and other plant phenolics). We have previously reported that the formation of 4-methylphenol is positively related to crude protein intake (Fraser *et al*., 2003), with similar concentrations and time-course curves to those reported here for the first phase (to 8 hr). The second phase (9-15 hr) in 4-methylphenol concentration in the rumen increases similarly to the 3-methylphenol concentrations in the rumen during this time period (data not shown). This delayed 3-methylphenol formation has previously been linked to acid-detergent fibre (Fraser *et al*., 2003) intake. Similarly the increase in 4-methylphenol concentration during the 9-15 hr phase may also be associated with the degradation of phenolic acids or glycosides associated with dietary fibre.

The conjugation and transfer of free 4-methylphenol from the rumen to the plasma occurs rapidly as there is approximately 1 hr between the t_{max} of each, and likewise the partitioning from the bloodstream to the urine is also highly efficient with a t_{max} difference of approximately 1.5 hr. The rapid, but not complete removal of 4-methylphenol to a concentration of approximately 3 μg/ml in both the rumen and plasma indicate a possible threshold concentration for the conjugation or transfer mechanism. There is only a small increase in 4-methylphenol concentration in the plasma after 11 hr, in marked contrast to the pattern in the rumen. Possible explanations for this anomaly are a lower pool size in the rumen 11 hr after feeding or dependence on rumen outflow and phenol uptake downstream of the rumen.

Trial 2:

Thymol and carvacrol are common constituents of herbal plants such as *Thymus vulgaris L*. (McGimpsey *et al*., 1994) but are not found in common forage species. The high correlation between dose amount and the concentration of thymol and carvacrol conjugates excreted shows that the animals were able to efficiently metabolise increased levels of exogenous phenolic compounds. The increase in the flux of compounds into the rumen and increased excretion did not result in significantly increased accumulation of free phenols in the adipose tissue. This indicates the rate of detoxification and excretion increases with increasing levels of exogenous phenols dosed. In addition the levels of thymol in the fat of control animals were higher than levels observed in New Zealand pasture-fed lambs in previous trials (mean > 1 ng/g, O. Young, G. Lane, unpublished data). A possible source could be recycling of excreted phenols through the pasture (R. Keogh, unpublished data).

Taking the results of the two trials together, they suggest phenols from endogenous sources behave similarly to exogenous phenols given at increasing doses. The concentration of phenol flavour compounds in ruminant products is probably affected by the efficiency of conjugation and excretion in the animal as much as by the formation of phenols in the rumen. Further research will be required to explore whether these processes can be exploited in a practical farming system to obtain dairy products with lower total phenol concentrations, and thus provide a technique to moderate 'pastoral flavour' in dairy products within a pasture-based production system.

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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.

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.

Fraser, K.; Lane, G.A.; Schreurs, N.M.; Tavendale, M.H.; McNabb, W.C.; Marotti, D.M. 2003: Effects of different forages on phenol and methylphenol formation in the rumen of sheep. *Proceedings of the New Zealand Society of Animal Production 63*: 40-44.

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.

Lundh, T.J.O. 1990: Conjugation of plant estrogens formononetin and daidzein and their metabolite equol by gastrointestinal epithelium from cattle and sheep. *Journal of agriculture and food chemistry 38*: 1012-1016.

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

McGimpsey, J.A.; Douglas, M.H.; van Klink, J.W.; Beauregard, D.A.; Perry, N.B. 1994: Seasonal variation in essential oil yield and composition from naturalized *Thymus vulgaris L*. in New Zealand. *Flavour and fragrance journal 9*: 347-352.

Powell, G.M.; Miller, J.J.; Olavesen, A.H.; Curtis, C.G. 1974: Liver as major organ of phenol detoxification? *Nature 252*: 234-235.

Scheline, R.R. 1991: CRC handbook of mammalian metabolism of plant compounds. Boca Raton, CRC Press.

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.