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Malate, Citrate, and Amino Acids in Tall Fescue Cultivars: Relationship to Animal Preference

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ABSTRACT

Grazing animals depend on little-understood chemical and physical cues when selecting forage diets. This study determined malate, citrate, and amino acid concentrations in endophyte-free tall fescue (*Festuca arundinacea* Schreb.) and related those concentrations to cultivar, harvest time, and grazing-animal preference. 'Barcel', 'Kenhy', 'Kentucky-31', 'Missouri-96', 'Mozark', 'Stargrazer', and the two accessions C1 and HiMag were established in three replicates within each of three pastures. Organic acids were determined on regrowth within each plot during four seasons and two years; amino acids were determined on regrowth of four cultivars across three replicates during both spring and fall seasons in one year. Malate and citrate were extracted with boiling water and quantified by high-performance liquid chromatography (HPLC) with an organic acid column. Amino acids were hydrolyzed, separated by ion-exchange HPLC, and quantified as their ninhydrin derivatives. Both malate and citrate concentrations differed between years. During one year only, malate concentrations were higher in Kenhy (68 g kg^{-1} dry matter [DM], most preferred) than in Mozark (54 g kg^{-1} DM, least preferred). Citrate concentrations (13 g kg^{-1} DM) were not different among cultivars. Eighteen amino acids (including tryptophan) accounted for 75% of total N. Thus, tissue N data were used as covariates to amino acid data in the ANOVA. Kenhy contained higher concentrations of eight amino acids than did other cultivars. These differences may reflect presence of *Lolium* genes in Kenhy. Cattle (*Bos taurus* L.) grazing preference (0 = not eaten; 10 = completely eaten) was not related to malate, citrate, or amino acid concentrations among cultivars.

ORGANIC ACIDS, such as malate and citrate, are fundamental to photosynthesis and aid cation absorption and sap-pH regulation in plants (Dijkshoorn, 1973). Concentrations of these acids likely differ among and within plant species and diurnally. Boland et al. (1976) found a large range in malate concentrations in leaf tissue of 22 genetically diverse tall fescue genotypes grown in a glasshouse. Differences in genotype, soil fertility, temperature, and maturity affect organic acid concentrations in grasses (Burns et al., 1968; Barta, 1973).

Genetic differences in malate or citrate concentration among forage cultivars might affect animal preference and overall forage palatability (Jones and Barnes, 1967). Malate appears to reduce acidosis in ruminants on high grain diets (Martin, 1998). Both malate and citrate increase salivary flow and intensify sweet flavors in diets of nonruminants (Gilbertson et al., 1997). Similar effects may occur in ruminants, increasing animal preference and altering dry matter intake and digestion.

Amino acid profiles differ among families and genera, and their total concentration is related to maturity and N nutrition of the plant (Yeoh and Watson, 1988). Boland et al. (1977) found large genotypic differences in amino acid concentration among tall fescue genotypes, but in neither of the above studies were amino acid profiles corrected for differences in N concentration. Relative proportions of each amino acid are often similar within a species or genus when corrected for plant N, unless there are large differences in total N or in nonprotein N in the tissue.

In his review of postingestive feedback, Provenza (1995) noted that deficits or imbalances of amino acids decrease intake and cause feed aversions in lambs (*Ovis aries*). Thus, forages containing higher concentrations of malate and citrate or perhaps certain amino acids might enhance flavor and nutrient utilization and provide positive postingestive feedback affecting ruminant preference.

We hypothesized that differences in malate, citrate, or amino acid concentrations among our tall fescue cultivars might serve as cues to grazing cattle. We report concentrations of malate and citrate in vegetative herbage of eight endophyte-free tall fescue cultivars and concentrations of 18 amino acids in four cultivars and relate these to cattle grazing preferences (Shewmaker et al., 1997) for those cultivars.

MATERIALS AND METHODS

Fescue Cultivars and Experiment Conditions

Eight cultivars of tall fescue (Shewmaker et al., 1997) were grown on irrigated Portneuf soil (coarse-silty, mixed, superactive, mesic Durinodic Xeric Haplocalcids) near Kimberly, ID (42°30′ N, 114°08′ W; elevation 1200 m). The cultivars, which were free of the fescue endophyte [*Neotyphodium coenophialum* (Morgan-Jones & W. Gams) Glenn, Bacon & Hanlin], were Barcel, Kenhy, Kentucky-31, Missouri-96, Mozark, Stargrazer, and first-generation (C1) and second-generation (HiMag) breeding populations selected for high Mg and Ca concentrations and low K/(Ca+Mg) ratios, as described previously by Mayland and Sleper (1993). The experiment area was divided into three pastures, each of which contained three replicates of eight plant cultivars in a randomized complete block design (Shewmaker et al., 1997). Each plot (cultivar) was composed of six rows; 56 cm apart and 6.7 m long. Six yearling Hereford and Hereford × Angus heifers (mean wt. = 286 kg) successively grazed each pasture for 48 h during May, June, August, and September of 1993 and 1994. Preference

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Abbreviations: EAA, essential amino acid; cyst(e)ine, cystine or its reduction product, cysteine; HPLC, high-performance liquid chromatography; NEAA, nonessential amino acid; TAA, total amino acid [measured in this study].

scores, described in detail by Shewmaker et al. (1997), were visual estimates of utilization (0 = no use; 10 = 100% use) at 48 h after initiating grazing. Grazing was followed by flail mowing to 8-cm stubble height, fertilizing with 56 kg N ha^{-1}, and irrigating as needed.

Fescue Harvesting and Sample Preparation

Forage subsamples were clipped at 8-cm stubble height in each plot within a pasture immediately before grazing. Tall fescue plants were in the 2.0 growth stage (leaf elongation and onset of stem elongation; Moore et al., 1991). Forage was harvested from a random 60-cm section in rows three and four in each plot and composited. Samples were kept at −5°C until freeze-dried, then ground to pass 1-mm screens first in a Wiley[1] shear-mill (A.H. Thomas, Philadelphia, PA) and then a Tecator–Cyclotec abrasion mill (Tecator, Höganäs, Sweden). Ground samples reserved for later analyses were stored in screw-capped high-density polyethylene bottles at about 10°C.

Organic Acid Methodology

Malate and citrate concentrations were determined in tissues of eight cultivars harvested from plots on three replicates, three pastures, four dates, and two years. Malate and citrate were extracted from 0.5-g plant samples with 25 mL of boiling water for 30 min, filtered through a Whatman no. 1 filter paper, centrifuged (10 000 × *g*, 10 min, 25°C), and filtered through a 0.45-μm membrane filter before HPLC analysis (Russell and Van Soest, 1984; Callaway et al., 1997). Acids were quantified by HPLC using a Shimadzu LC-10AS liquid chromatograph (Shimadzu Scientific Instruments, Columbia, MD), with the following Shimadzu accessories: RID-6A refractive index detector, SCL-10A system controller, SIL-10A autosampler, C-R5A integrator, 50-μL loop, 50°C, and a Bio-Rad HPX-87H organic acid column. Samples were eluted from the column with 0.0065 *M* H_2SO_4 at a flow rate of 0.5 mL min^{-1}.

Amino Acid Methodology

Amino acid concentrations were determined on plant tissue harvested in May and September 1993 from three replicates of Barcel, HiMag, Kenhy, and Mozark cultivars grown on Pasture 2. Protein in 30 to 50 mg of freeze-dried plant material was hydrolyzed according to AOAC methods 982.30, 988.15, and 985.28 (AOAC, 1990, p. 1096, 1101, 1105). Individual amino acids were determined by post-column detection of their ninhydrin derivatives (570 and 440 nm) after separation by ion-exchange HPLC. Norleucine and taurine were added before the hydrolysis to assess stability and recovery of acid stable and sulfur amino acids (cysteine and methionine), respectively. Recoveries were typically better than 95% across the range of amino acids. Instrumentation consisted of a Shimadzu LC 10A autosampler and HPLC system coupled with a Pickering Laboratories PCX3100 derivative system (Pickering Laboratories, Mountain View, CA) equipped with a Pickering 3 × 250 mm, 8-mm sodium cation exchange column. Operating conditions were optimized to allow separation and quantification of 18 amino acids in plant protein. Separate programs were used to analyze acid stable amino acids, sulfur amino acids, and tryptophan. Amino acid concentrations in the hydrolysate were determined relative to response factors of amino acid standards (Pierce amino acid calibration mixture H; Perstorp, Inc., Florence, MA). Using ninhydrin detection and measuring absorbency changes at 570 nm, sensitivity was 0.2 pmol (3 s detection limit). Repeated amino acid analyses of a standard casein sample showed that precision was better than 3% at 5 nmol. Coefficient of variation of repeated analyses of amino acids in standard casein ranged from 4.5 to 6.0%, with the exception of arginine, histidine, and valine (which ranged from 6 to 7.5%); isoleucine, methionine, and tyrosine (which ranged from 7.5 to 9.0%); and cyst(e)ine (19%).

Other Analyses

Potassium was determined by flame emission after digesting plant tissue in nitric–perchloric (3:1) acid (Greweling, 1976). Mineral, organic acid, and amino acid concentrations are expressed on a dry matter basis.

Statistical Analyses

Malate and citrate data were accepted as normally distributed only after transformation to log base 10 values and were analyzed by least squares to fit general linear models (SAS Inst., 1990). The model assumed year (Y), cultivar (C), and harvest (H) were fixed effects and pasture (P) and replicates (R) were random. Tests for differences among main effects used the following error terms: Y tested by P × Y, C tested by P × C, P × C tested by C × R(P), C × Y tested with C × P × Y, H tested by P × H which was tested with R × H(P) and P tested by R(P). The organic acid data were then back-transformed for presentation.

Amino acid data were normally distributed and analyzed by least squares, with N as a covariate, to fit a general linear model (SAS Inst., 1990). The model assumed C and H were fixed effects and R was random. Tests for differences among main effects used the following error terms: C and R tested with C × R and H tested with R × H. Essential amino acid (EAA) N, nonessential amino acid (NEAA) N, and total amino acid (TAA) N were sums of N represented by each of the respective amino acids; data were analyzed by harvest date and cultivar. Preference scores (Shewmaker et al., 1997) for each cultivar were regressed against arithmetic values of malate and citrate and for amino acid values corrected for N concentration. Organic acid data for plants harvested from the three pastures, four seasons, and two years were regressed on average minimum–maximum daily temperature and solar radiation data obtain near the field site for 1, 2, or 3 d prior to sampling.

RESULTS AND DISCUSSION

Malate and Citrate in Tall Fescue Forage

Malate concentrations differed ($P < 0.05$) among cultivars in 1993, but not in 1994, when concentrations were only 60% of the previous year (Table 1). Citrate concentrations were similar among cultivars in both 1993 and 1994, but values were about 26% higher in 1994 ($P < 0.05$; Table 1). In other studies (Boland et al., 1976), differences in organic acids have been associated with genetic differences. Additionally, differences in plant maturity, air temperatures, radiation, soil fertility, water stress, or time of day when harvested are thought to affect organic acid content (Burns et al., 1968). In this study, organic acid data were not correlated with

[1] Mention of a trademark or proprietary product in this paper does not constitute a guarantee or warranty of the product by the sponsoring organizations and does not imply its approval to the exclusion of other products that also may be suitable.

either minimum or maximum temperatures or solar radiation data for the 1, 2 or 3 d preceding harvest (data not shown), across years. Boland et al. (1977) found malic acid concentrations to be positively associated with total nonstructural carbohydrate and suggested that the two might serve as temporary C sinks. Burns et al. (1968) reported a diurnal effect such that concentrations of malate were greater and citrate less in alfalfa (*Medicago sativa* L.) harvested in the afternoon than in the morning. In our study, forage samples were harvested between 0830 and 0930 h (mountain daylight time) in both years, so sampling time did not contribute to variation in our citrate and malate values.

Barta (1973) reported that N fertilization increased citrate concentrations in bromegrass (*Bromus inermis* Leyss.) and orchardgrass (*Dactylis glomerata* L.), but increased malate concentrations only in bromegrass. Potassium fertilization decreased or did not affect either malate or citrate concentrations in either alfalfa or grasses (Burns et al., 1968; Barta, 1973). In our study, N was applied uniformly across all plots after each grazing period. However, the lower yields of Kenhy were accompanied by higher N concentrations. Even so, the organic acid data were not related ($r^2 < 0.2$; data not shown) to forage N. Other soil nutrients (including K) were uniform and adequate for forage production across the plots. Plots were uniformly irrigated, providing similar amounts of soil water in both years. Thus, the cause or causes for the significant year effects are not known.

Ting (1981) suggested that K concentrations drive the accumulation of CO_2 in malate and other organic acids. This relationship was not supported in this study, where the correlation of K (not shown) with malate concentration in tall fescue tissue was near zero. Such a relationship occurs when K limits growth. In this study, K concentrations exceeded the 20 to 30 g kg^{-1} critical minimum (Mayland and Wilkinson, 1996), suggesting high uptake in excess of plant requirements, so K did not limit maximum malate accumulation.

In our study, year effects for citrate concentrations were significant ($P < 0.05$), but the cultivar × year interaction was not. We detected differences in malate concentration only in 1993, when Kenhy, the most preferred cultivar, contained the most malate and Mozark, the least preferred cultivar, contained the least (Table 1). The range in malate concentration across both years was similar to that reported for alfalfa by Callaway et al. (1997). In their study, malate ranged between 29 and 75 g kg^{-1} and declined with increasing maturity. They also found differences in malate concentrations between some bermudagrass [*Cynodon dactylon* (L.) Pers.] varieties and noted a decline in malate concentrations as grass matured.

Jones and Barnes (1967), using hot water extraction, found between 4 and 17 g kg^{-1} malate and 2 and 8 g kg^{-1} citrate in six different grass species, including orchardgrass and perennial ryegrass (*Lolium perenne* L.). In that study, the decline in malate and citrate concentrations with plant maturity was not as dramatic as reported by Callaway et al. (1997). However, Martin (1970) reported that the concentration of organic acids in perennial ryegrass decreased with maturity in direct proportion to the decrease in leaf tissue.

In both years of our study, grazing and the accompanying harvests were conducted on regrowth forage at the stem elongation growth stage (Moore et al., 1991). Thus, plant maturity was expected to be similar across harvests.

Organic acids in our study and that of Callaway et al. (1997) were higher in concentration than previously reported for both malate and citrate in forages (Jones and Barnes, 1967; Burns et al., 1968; Barta, 1973; Boland et al., 1976). This difference is explained, in part, by differences in forage genotype and extraction procedure used. Jones and Barnes (1967) evaluated four methods of extracting organic acids from forage and reported that extraction with 80 to 85% ethanol gave lower concentrations of malate and citrate than extraction with hot water, or with acetone followed by 0.01 *M* HCl, or with 80 to 85% ethanol under reduced pressure.

Table 1. Arithmetic mean concentrations of malate and citrate in eight tall fescue cultivars by year.

	Malate		Citrate	
Entry	1993	1994	1993	1994
	g kg^{-1}			
Barcel	63.0	38.1	10.8	14.3
C1	64.8	36.2	11.7	13.5
HiMag	62.1	37.7	11.3	14.4
Kenhy	68.1	39.7	11.2	13.3
KY-31	64.2	39.8	11.6	14.2
MO-96	58.1	40.0	10.9	14.8
Mozark	54.0	37.2	10.8	13.9
Stargrazer	64.6	36.2	11.2	14.5
LSD (0.05)	5.4	NS	NS	NS
CV, %	18.5	22.4	19.2	19.4

Table 2. Least square means of essential (EAA) and nonessential (NEAA) amino acids adjusted for N covariate and the total N in four tall fescue cultivars across spring and fall harvests.

Amino acid	Barcel	HiMag	Kenhy	Mozark	CV
	g kg^{-1}				%
EAA					
Arginine	6.50b†	6.53b	7.10a	6.33b	4.3
Cyst(e)ine‡	2.28a	2.26a	2.33a	2.25a	4.4
Histidine	2.23a	2.47a	2.43a	2.15a	14.9
Isoleucine	4.33ab	4.40ab	4.62a	4.02b	7.0
Leucine	9.38b	9.38b	10.15a	8.92b	4.1
Lysine	6.30b	6.38b	6.85a	6.13b	4.4
Methionine	2.72a	2.58a	2.93a	2.63a	4.5
Phenylalanine	6.42b	6.33bc	6.83a	6.05c	4.2
Threonine	5.90b	5.85b	6.37a	5.60b	4.1
Tryptophan	1.95a	2.00a	2.18a	2.00a	5.0
Tyrosine‡	3.90b	3.80b	4.15a	3.60b	4.5
Valine	5.75ab	5.82ab	6.12a	5.40b	5.8
NEAA					
Alanine	8.50b	8.35b	9.18a	8.13b	4.2
Aspartic acid	12.25a	12.43a	13.40a	12.05a	2.6
Glutamic acid	14.62a	14.92a	16.30a	14.45a	2.8
Glycine	6.57b	6.65b	7.12a	6.30b	4.2
Proline	6.52a	6.68a	7.62a	6.37a	3.6
Serine	5.57a	5.60a	6.10a	5.43a	4.2
Nitrogen	20.6b	20.6b	22.7a	20.7b	6.1

† Within rows, means followed by the same letter are not statistically different ($P \leq 0.05$).

‡ The semiessential amino acids cyst(e)ine and tyrosine are conditionally essential if methionine or phenylalanine are limiting animal needs (Pisulewski et al., 1996).

Amino Acids in Tall Fescue Forage

Concentrations of amino acids in plants vary with total protein in the tissue and this is reflected by changes in the concentration of total N (except perhaps if nonprotein N is high; e.g., high nitrate). It follows that any statistical analyses of amino acid concentration data would be corrected for N concentration as a covariate. In this study, N concentration among cultivars was significantly ($P < 0.05$) different; therefore, amino acid concentration data were covariate adjusted. Differences between spring and fall harvest were significant ($P < 0.05$) only for the two sulfur amino acids, cyst(e)ine and methionine. Further analyses of these two amino acids revealed cultivar differences only for cyst(e)ine in spring-harvested samples. Kenhy contained less ($P < 0.05$) cyst(e)ine (2.15 g kg^{-1}) than other cultivars (2.36 to 2.40 g kg^{-1}; data not shown). Methionine concentrations were higher in spring-harvested (2.77 g kg^{-1}) than fall-harvested (2.66 g kg^{-1}) tissue. We cannot explain this difference.

Table 2 reports concentration of essential and nonessential amino acids (EAA and NEAA) in each of the four cultivars. There were no significant ($P < 0.05$) concentration differences among cultivars for the EAAs cyst(e)ine, histidine, methionine, and tryptophan, nor for the NEAAs aspartic acid, glutamic acid, proline, and serine. Of the remaining amino acids, only small differences occurred among the four cultivars. With one exception (phenylalanine), amino acid concentrations in Barcel, HiMag, and Mozark did not differ. Generally the concentration of amino acids in Kenhy were greater than those determined in the other cultivars. This likely reflects the presence of *Lolium* genes in Kenhy (Alderson and Sharp, 1994).

The similarity of amino acid concentrations among our cultivars is in contrast to previous reports of genotypic differences in tall fescue and may be attributed to differences in methodology. Using aqueous ethanol (50:50) extractant, Boland et al. (1977) found different amino acid profiles among tall fescue genotypes. However, their reported concentrations were as little as 1/20 of our concentrations. Miller et al. (1996) measured eight amino acids in grain and immature wheat (*Triticum aestivum* L. cv. Terral 817) and triticale (×*Triticosecale* Wittmack) forage. They did not report N concentration in their forage, but concentrations of respective amino acids in vegetative tissues were about twice those reported here for tall fescue. These examples illustrate the problem of comparing amino acid concentrations when N concentrations are different and N is not used as a covariate.

The sum of amino acid N measured in this study accounted for 15.5 of the 21.1 g N kg^{-1} in the four cultivars. Of the four cultivars, Mozark contained the lowest proportion of EAA N, NEAA N, and TAA N to total N in the plant (Table 3). In the fall, these values for Mozark were different ($P < 0.05$) from those for the other cultivars, but in spring the values for Mozark differed from those for Barcel and HiMag but not Kenhy. Recovery of amino acid N from the fescue tissue was considered to be better than 95% based on recovery of amino acids from casein, a standard quality control (QC) material that was analyzed in parallel. The remainder of N (5.6 g kg^{-1}) was assumed to be associated with inorganic forms of N including nitrate. The amino acid concentrations (uncorrected) were positively correlated with each other, having $r^2 > 0.95$ in all cases. This suggests that the amino acid profiles for these four tall fescue cultivars were quite similar.

Organic Acids and Amino Acids as Cues to Grazing Animals

Malate, and perhaps citrate, might serve as positive cues to grazing ruminants. Increasing the malate content of the diet stimulates lactate utilization and propionate production by the ruminal bacterium *Selenomonas ruminantium* (Martin and Streeter, 1995; Callaway and Martin, 1996). This effect could reduce the severity of acidosis arising from consumption of diets high in readily fermentable carbohydrates (Martin, 1998). Although acidosis is uncommon in grazing animals, malate-dependent stimulation of this bacterium might provide positive effects on ruminal fermentation.

Gilbertson et al. (1997) reported that both malate and citrate intensify sweet flavors in diets of nonruminants. If palatability is also stimulated in ruminants, then we might expect an increased preference for forages containing higher concentrations of these two organic acids. These acids stimulate production of saliva, and this could improve buffering capacity of the rumen and increase digesta turnover and nutrient utilization (Gilbertson et al., 1997).

Preference scores ranged from 1.5 to 8.8 (Shewmaker et al., 1997). These were poorly related to organic acid concentrations measured in this study. Preference scores

Table 3. Cultivar means of total N partitioned as essential amino acids (EAA N), nonessential amino acids (NEAA N), and total amino acids (TAA N)†.

Cultivar	Spring harvest			Fall harvest		
	EAA N: Total N	NEAA N: Total N	TAA N: Total N	EAA N: Total N	NEAA N: Total N	TAA N: Total N
	g kg^{-1}					
Barcel	426a†	320a	746a	450a	326a	776a
HiMag	434a	328a	761a	453a	327a	780a
Kenhy	401b	315a	716b	455a	330a	785a
Mozark	401b	312a	713b	435b	313b	749b
LSD (0.05)	24	17	40	13	10	16
CV, %	4.4	3.0	3.7	3.0	3.0	2.9

† Within columns, means followed by the same letter are not significantly different ($P \leq 0.05$).

regressed on malate, citrate, and malate + citrate concentrations yielded $r = 0.28$, 0.35, and 0.44 ($P = 0.11$), respectively. Thus, variability in the concentration of malate or citrate or both accounted for less than 20% of variation in animal grazing preference scores. These results suggest that grazing preferences among tall fescue cultivars are not related to malate or citrate concentrations in the forage.

Dietary amino acids consumed by ruminants are first metabolized by rumen microorganisms (degradation and synthesis) forming another set of amino acids whose profile does not resemble that of the diet (Schingoethe, 1996). Such outcomes are difficult to predict. It is possible that amino acids in the forages consumed by animals or some metabolic intermediate of digestion might have an immediate flavor effect.

CONCLUSION AND SUMMARY

Grazing preference scores (Shewmaker et al., 1997) averaged by cultivar for May and September 1993 were Kenhy > Barcel = HiMag > Mozark (7.7 > 5.5 = 5.5 > 3.4; $P < 0.05$). However, these scores were not related to concentrations of any essential or nonessential amino acid quantified in these four tall fescue cultivars. Thus, variation in malate, citrate, and amino acids measured in this study was not related to variation in grazing preference of tall fescue cultivars.

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