



***In vitro* ruminal degradation of carbohydrate fractions in tropical grasses fertilized with nitrogen**



Erika Andrea Hernández ^a

Francisco Indalecio Juárez Lagunes ^{a*}

Alice N. Pell ^b

Maribel Montero Lagunes ^c

Juan Manuel Pinos Rodríguez ^a

Robert W. Blake ^b

^a Universidad Veracruzana. Facultad de Medicina Veterinaria y Zootecnia. 91710 Veracruz, Ver. México.

^b Cornell University. Department of Animal Science. Ithaca, NY. USA.

^c INIFAP. Campo Experimental La Posta. 94277. Medellín, Ver. México.

*Corresponding author: juarezf@hotmail.com

Abstract:

The goal was to determine the digestion rates of carbohydrate fractions A (sugars, oligosaccharides and organic acids), B₁ (starch and soluble fiber), NSC (non-structural carbohydrates) and B₂ (available NDF) in four tropical grasses using the gas production technique. Samples of whole forage (WF), residue insoluble in 90% ethanol (EIR) and isolated NDF (iNDF) were fermented *in vitro* and gas production measured. Gas volumes were determined from the following fractions, A = WF minus EIR; B₁ = EIR - ND; NSC = WF - iNDF; and B₂ = iNDF. Grasses were *Andropogon gayanus*, *Urochloa brizantha*, *Cynodon plectostachyus*, and *Megathyrsus maximus* each grown in Veracruz, Mexico on four plots (5×5 m), fertilized (relationship equivalent to 0 and 100 kg N/ha) and clipped 35 d after the N

fertilization. A complete randomized block design with factorial arrangement 4×2 and two replicates per treatment was used. Factors were grass species and N fertilization. Data were fit using a single-pool exponential model with lag. The volume (mL gas/100 mg OM), rate (%/h) and lag (h) were: WF (22.8; 5.3; 2.1); A (3.2; 15.7; 0.5); B₁ (1.5; 15.7; 0.2); and B₂ (18.3; 6.6; 5.2). *Andropogon* and *Urochloa* had higher NSC content compared to *Megathyrsus* and *Cynodon* but lower gas yield per unit of NSC. Rates of digestion for the B₂ fraction ranged from 4 to 8 %/h; and NSC digestion rate averaged 15.7 %/h. Nitrogen fertilization reduced carbohydrate pool sizes but did not affect rates of digestion. It is concluded that the rates of digestion of the carbohydrate fractions differs by grass specie.

Key words: C₄ grasses, Carbohydrate fractions, Digestion rates, Gas production, CNCPS model.

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The energy content of forages that is available to the animal cannot be determined using standard analytical techniques. Therefore, other means are needed to estimate it. Past use of empirical prediction equations based on chemical composition, aided by detergent system analysis of fiber⁽¹⁾ has been in the foundation for a comprehensive system of forage evaluation⁽²⁾. However, the underlying relationship between energy content and chemical composition is inconsistent in tropical forages with high contents of lignin, silica, tannins and other secondary compounds, which may interfere with digestion.

An alternative approach uses the *In vitro* ruminal digestion method⁽³⁾. This technique is commonly used to predict the digestibility of a feedstuff. However, measuring the extent of digestion by substrate disappearance has limitations: The soluble components are assumed to be completely digested and with similar energy values regardless of their carbohydrate or organic acid profiles⁽¹⁾. The Cornell Net Carbohydrate and Protein System model (CNCPS) v.5 <http://blogs.cornell.edu/cncps/> fractionates carbohydrates into three major fractions: fraction A (sugars, oligosaccharides and organic acids), fraction B₁ (starch and soluble fiber), and fraction B₂ (digestible structural carbohydrates)^(4,5). The CNCPS further partitions carbohydrates into eight digestible fractions⁽⁶⁾: A₁ (volatile fatty acids); A₂ (lactic acid); A₃ (other organic acids); A₄ (sugars); B₁ (starch); B₂ (soluble fiber); B₃ (available NDF); C (unavailable NDF). However, the CNCPS v6.5.5⁽⁷⁾ <http://blogs.cornell.edu/cncps/> model considers information only about the digestion rates of four fractions, A₄, B₁, B₂ and B₃. In this model (version 6.5.5), the rate of digestion assigned to the A₄ fraction (40 to 60 %/h) was

obtained from data based on mixed ruminal microbes^(8,9) using the gas production technique⁽¹⁰⁾. This technique has been automated and used to estimate the digestion of the NDF⁽¹¹⁾ and non-structural carbohydrates (NSC)⁽¹²⁾. Accordingly, fractions B₁ and B₂ have rates of 20 to 40 %/h and the B₃ fraction rate varies between 1 and 18 %/h.

The feed library of the Nutrient Requirements of Beef Cattle⁽¹³⁾ (<https://www.nap.edu/download/19014>) does not include tropical grasses. However, the Large Ruminant Nutrition System (LRNS) v1.033⁽¹⁴⁾ (<http://nutritionmodels.com/lrns.html>) includes rates of digestion of carbohydrate fractions A, B₁ and B₂ for tropical grasses. In this library grasses from Mexico⁽¹⁵⁾ are differentiated from Brazil, Honduras and Florida. The updated tropical library of the CNCPS v.6.5.5⁽⁷⁾ validates the database from Mexico and correct the rates from Brazil, Honduras and Florida by assigning fixed values (%/h) of 40 for the A₄; 30 for B₁; 30 for B₂; and 3.0 for B₃ carbohydrate fractions. These last values are in agreement with previous reports⁽¹⁶⁻¹⁹⁾. However, more research is needed to update these rates.

Therefore, the objective of the present study was to chemically quantifying the carbohydrate fractions, A, B₁, B₂ and C, and to measure the digestion kinetics of each of these fractions by measuring gas production in four tropical grasses fertilized with nitrogen.

The study was conducted at La Posta Experimental Station of Mexico's Instituto Nacional de Investigaciones Forestales Agrícolas y Pecuarias (INIFAP). This research station is located on the southeastern coast of Mexico in the State of Veracruz at 19° 02' N and 96° 08' W, with an altitude of 12 m asl, a tropical subhumid Aw climate, with average annual rainfall of 1,728 mm, 25° C of average temperature and 81 % relative humidity. The soil is classified as Oxisol, a predominantly sandy loam with >15 % clay and 1.7 % organic matter, the pH was 5.35. The soil chemical analysis report showed the follow mineral content (ppm): P₂O₅, 12; K, 108; Mg, 115; Ca, 545; NO₃, 9.5; S, 16; Mn, 13; Fe, 53; Cu, 0.45; and B, 0.6.

The selected grasses *Andropogon gayanus*, *Urochloa brizantha*, *Cynodon plectostachyus*, and *Megathyrsus maximum* Var. Guinea, are commonly used species. At the onset of the rainy season, each grass was grown in four plots (5×5 m). Two plots were none fertilized, and the others were fertilized with N from urea (relationship equivalent to 100 kg N/ha). This dose is representative of that local livestock producers use. All plots were previously cut to a height of 5 cm. There were two sampling periods (June 20 and July 25). After 35 d of regrowth, one sample of 2 m² from the center of each plot was clipped at a height of 10 cm. Samples were taken between 0700 to 0900 h. A sub-sample of 500 g of green material was immediately frozen at -15 °C, and another was placed in a forced air oven at 100 °C for 24 h to determine DM content and discarded. At the end of the sampling period (July 25), four frozen samples

from each grass were lyophilized, placed in 30×25 cm heavy-duty freezer bags, and sent to Cornell University (USA) for chemical analysis.

All samples were ground through a 1-mm screen in a Wiley mill (Model 4, Arthur H. Thomas Co. Philadelphia, PA). Dry matter for correction was determined by direct oven-drying of samples at 100° C overnight. Crude protein (N×6.25) was determined by a Macrokjeldahl procedure⁽²⁰⁾, modified using Boric acid at 4% concentration during distillation. Neutral detergent fiber (NDF) (without sodium sulfite), acid detergent fiber (ADF), nonstructural carbohydrates (NSC), neutral detergent insoluble protein (NDIP), and acid detergent insoluble protein (ADIP) were determined⁽²¹⁾. Permanganate lignin, cellulose and acid insoluble ash were also determined⁽²²⁾. Hemicellulose was calculated as the difference of NDF minus ADF with appropriate correction for contents of ash and crude protein. Sugar content was determined by ethanol extraction (EIR)⁽²³⁾.

Total carbohydrates and its fractions (NSC, A, B₁, B₂, and C) were estimated as follows:

Total carbohydrates= 100 - CP - ash - fat.

C fraction= lignin/NDF * 2.4.

B₂ fraction= (NDF/OM) - NDIP - C fraction.

A fraction= (DM - CP - ash) - (ethanol insoluble residue – CP in ethanol insoluble residue – ash in ethanol insoluble residue).

NSC = 100 - CP - (NDF - NDIP) - ash - fat.

B₁ fraction = NDS - A.

The digestion kinetics of carbohydrate fractions were estimated from gas production measurements⁽¹¹⁾ using the curve subtraction procedure⁽¹²⁾. To achieve this, the whole forage, ethanol insoluble residue (EIR) and the isolated NDF were fermented separately. For EIR⁽²³⁾ five hundred milligrams of sample in 100 mL of 90% vol/vol ethanol were stirred for 4 h. The sample was filtered through a 37-μ nylon mesh (Tetko[®], Briarcliff Manor, NY) and thrice rinsed with 90% ethanol without vacuum and once with acetone under vacuum. The sample then was dried at 50 °C overnight to remove residual acetone.

For the isolated NDF⁽¹¹⁾ five hundred milligrams of sample and 100 mL of ND solution in 150-mL serum bottles were autoclaved for 1 h at 105 °C. This NDF isolate was rinsed with hot water and 100 mL of ethanol, and filtered through a 37-μ nylon mesh. Residual detergent was removed by soaking the isolate overnight at 39 °C in a solution of 1 M (NH₄)₂SO₄ (1 g NDF to

100 mL 1 M $(\text{NH}_4)_2\text{SO}_4$). The isolate was again rinsed with hot water followed by 100 mL each of ethanol and acetone and air dried.

For the *in vitro* digestion⁽²²⁾ the medium was boiled to remove dissolved gases and cooled, cysteine added, and pH adjusted to 6.8 as necessary. Sodium sulfide was replaced by an equal weight of cysteine hydrochloride to protect the pressure sensors used to monitor gas volume from traces of hydrogen sulfide. Ruminant fluid was collected approximately 4 h after feeding from two out of four mature, non-lactating, Holstein cows housed in the LARTU (Large Animal Research and Teaching Unit at Cornell University) and maintained on Timothy (*Phleum pratense*) hay Full Bloom (CP, 8%; FDN, 65%) similar quality than grasses of this study, in accordance with the Institutional Animal Care and Use Committee (IACUC) protocol.

At the outset of a fermentation, each 120 mL serum bottle contained 8 mL medium, 2 mL ruminant fluid, and 100 mg of either whole forage, EIR, or isolated NDF. Gas production was measured every 20 min during a 48 h fermentation using a computerized monitoring system^(11,12). The disappearance of NDF was determined at the end of each fermentation⁽¹¹⁾. All gas volumes were corrected to standard atmospheric pressure (760 mm Hg).

The estimation of digestion rates for the A, B₁, B₂, and NSC fractions by curve subtraction requires that the gas volume produced by the separate preparations (EIR and NDF) be adjusted to a common basis proportional to the content of each fraction within the whole forage⁽⁸⁾. Therefore, the gas volume produced was adjusted proportionally to the OM content of the whole forage.

Gas production during fermentation was recorded every 20 min for 48 h. On a point by point basis, data from the curve were subtracted from the gas produced by the larger fraction^(8,24). The gas from the A fraction was estimated by the difference between the gas yields from the whole forage sample and its EIR preparation. The B₁ fraction was estimated by the difference between the EIR preparation and the isolated NDF. The B₂ fraction is the gas produced from the fermentation of isolated NDF, and the NSC is the difference between the whole forage and its isolated NDF.

Kinetic analyses of cumulative gas production were obtained using a single pool exponential model with lag⁽²⁵⁾, $Y=a*(1-\exp(-b*(x-c)))$, where Y=volume of gas mL/100 mg OM at time x; a=maximum volume of gas, mL; b=rate constant of gas production, %/h; c=lag term, h. The gas curves obtained by subtraction for the A, B₁, and NSC fractions reached their asymptotes

between 12 and 24 h, indicating that these fractions had been depleted⁽¹²⁾. Afterwards changes in gas volume are related to microbial turnover, and potential non-additivity of the curve subtraction approach^(26,27). For this reason, the gas curves for the A, B₁ and NDS fractions were truncated for curve fitting after they plateaued⁽⁸⁾. All curves were fitted using the Table Curve (version 4.0, Jandel Scientific, San Rafael, CA).

A complete randomized block design with factorial arrangement and two replicates per treatment was used, where the factors were grass species and N fertilization. A laboratory standard of Guinea grass (*M. maximum*) was used to control for ruminal fluid variation among *in vitro* analyses. A 4×2 factorial arrangement of forage species (*A. gayanus*, *U. brizantha*, *C. plectostachyus*, or *M. maximum* Var. guinea) and N fertilization (0 and 100 Kg/ha) as factors was used. Planned comparisons among the forages were evaluated using Tukey's W procedure. Results were deemed significant at $P \leq 0.05$ for the effects of grass species and fertilization. The ANOVA analyses were performed using the MINITAB, Version 10 (Minitab Inc., State College, PA)⁽²⁸⁾. Because there were no interactions (grass*N fertilization) of the 4×2 factorial arrangement of treatments, only means of mean factors (grass or N fertilization) are shown in Tables 2 and 3.

Chemical composition by grass species and amount of N fertilization are presented in Table 1. Under the same conditions of management and environmental growing conditions, the chemical composition of the grasses differed by species. *Urochloa* contained less NDF, neutral detergent insoluble protein (NDIP) and lignin than the other grasses. *Andropogon* had high NDIP and NSC levels. *Megathyrsus*, however, was distinguished for its high content of ash and acid insoluble ashes (AIA), and its low content (7.2 %) of CP. These values mirrored those found in the same similar-age species with climate Aw₀ in Guerrero, Mexico⁽²⁹⁾. *Cynodon* had high NDF and low NSC contents. Grasses varied in their distributions of chemical constituents, which reflects differences in morphology and physiology. Previous reports have indicated variations in the chemical composition of tropical grasses due to species⁽³⁰⁾, season of year⁽³¹⁾ and plant age^(32,33). Across these studies high amounts of ash in *Megathyrsus*, low lignin in *Urochloa* and low amounts of crude protein in *Cynodon* were consistently observed. The chemical constituent findings are consistent with other reports for *Cynodon*⁽³⁴⁾, *Megathyrsus*⁽³⁵⁾, *Urochloa*⁽³⁶⁾ and *Andropogon*⁽²⁹⁾, suggesting potential inherent growth differences in their plant tissues⁽³³⁾.

Table 1: Chemical composition (g/100g DM) of four tropical grasses fertilized with Nitrogen

	<i>A.</i> <i>gayanus</i>	<i>U.</i> <i>brizantha</i>	<i>C.</i> <i>plectosta-</i> <i>chyus</i>	<i>M.</i> <i>maximus</i>	SEM	Non fertilized	Fertilized	SEM
Ash	8.2 ^c	9.6 ^b	9.5 ^b	11.3 ^a	0.12	8.3 ^b	10.9 ^a	0.06
EE	2.0 ^b	2.4 ^a	1.3 ^c	2.6 ^a	0.05	1.6 ^b	2.5 ^a	0.03
CP	9.1 ^a	9.0 ^a	8.3 ^{ab}	7.2 ^b	0.12	5.9 ^b	10.9 ^a	0.06
NDF	69.8 ^b	66.4 ^c	74.9 ^a	69.1 ^{bc}	0.36	72.6 ^a	67.5 ^b	0.18
NDIP	4.4 ^a	1.2 ^c	3.1 ^{ab}	2.9 ^b	0.14	2.2 ^b	3.6 ^a	0.07
ADF	41.0 ^a	36.5 ^b	41.2 ^a	42.3 ^a	0.18	40.3 ^a	40.2 ^a	0.09
ADIP	0.6 ^b	0.3 ^c	0.8 ^a	0.6 ^b	0.02	0.5 ^b	0.7 ^a	0.01
AIA	4.3 ^{ab}	3.1 ^c	3.3 ^{bc}	5.0 ^a	0.13	3.5 ^a	4.4 ^a	0.06
Cel	32.2 ^a	29.7 ^b	32.3 ^a	33.0 ^a	0.09	32.4 ^a	31.2 ^b	0.05
Hem	28.2 ^b	31.3 ^a	33.1 ^a	28.3 ^b	0.22	33.1 ^a	27.3 ^b	0.11
NSC	16.5 ^a	14.7 ^{ab}	10.7 ^c	14.0 ^b	0.22	14.6 ^a	13.3 ^b	0.11
Lig	4.4 ^b	3.7 ^c	5.6 ^a	4.3 ^{bc}	0.06	4.5 ^a	4.5 ^a	0.03
ETOH	87.2 ^{ab}	85.5 ^b	89.1 ^a	87.9 ^a	0.22	87.3 ^a	87.5 ^a	0.11

EE= ether extract; CP= crude protein; NDF= neutral detergent fiber; NDIP= neutral detergent insoluble protein; ADF= acid detergent fiber; ADIP= acid detergent insoluble protein; AIA= ash insoluble in acid; Cel= cellulose; Hem= hemicellulose; NSC= nonstructural carbohydrates; Lig= lignin; ETOH= residue insoluble in ethanol 90%.

^{a,b,c} Means with different superscript differ ($P \leq 0.05$) for the grass effect or for the fertilization effect.

Fertilization with N modified the amount and distribution pattern of the nutrients in these plants (Table 1). Protein contents were increased in both the cell wall and cell soluble fractions. Because amino acids and proteins in plants are synthesized from sugars⁽³⁷⁾, an increase in N supply depresses the sugar content (less NSC). Fertilization also reduces the NDF content with most of this decrease occurring in hemicellulose, most of which is deposited in the secondary wall as plants mature. An increase in CP and reduction of NDF has also been found in *Urochloa ruziziensis* fertilized with 120 kg/N/ha and harvested at 30 d of regrowth⁽³⁶⁾.

Chemical constituents of the plant cell have been used to mathematically predict the feedstuff energy available to the animal^(37,38). An alternative approach is to integrate digestion and passage rates using the relationship among different energy pools, $k_d = k_d / (k_d + k_p)$, where K_d is rate of digestion and K_p is rate of passage. The estimated carbohydrate pools of the grasses in our study are in Table 2. The total carbohydrate content ranged from 77.8 to 80.4 % OM. The digestible NDF content (B_2 fraction) ranged from 47.8 to 51.2 % on an OM basis with *Andropogon* containing the least and *Cynodon* the most. Conversely, the NSC content was greatest in *Andropogon* (17.4 % OM) and least in *Cynodon* (10.7 % OM). The C fraction (Lignin/NDF*2.4), which is assumed indigestible, ranged from 13.5 to 18.0 % with the largest

amount found in *Cynodon* and the least in *Urochloa*. As a proportion of NSC, the A fraction (sugars, organic acids and short chain polysaccharides) constituted 68% of the total with the B₁ fraction (starch and soluble fiber) made up the remainder. While the B₁ fraction in tropical forages contains the smallest pool of carbohydrates (mostly as starch), it nonetheless represents about one-third (30 %) of NSC. The carbohydrate pools in this study are in agreement with the values shown in the LRNS and CNCPS feed libraries. Grasses elsewhere of the same species have been found that the B₁ is the smallest CHO fraction and that is made of starch mainly⁽³⁹⁾. The NSC is a complex fraction where the starch is part of the non-fiber carbohydrates (NFC) and the pectin substances are part of the structural carbohydrates unaccounted in the B₂ fraction.

Table 2: Carbohydrate fractions (g/100g OM) of four tropical grasses fertilized with Nitrogen

	<i>A.</i> <i>gayanus</i>	<i>U.</i> <i>brizantha</i>	<i>C.</i> <i>Plectosta-</i> <i>chyus</i>	<i>M.</i> <i>maximus</i>	SEM	Non fertilized	Fertilized	SEM
CHO	80.4 ^a	79.7 ^a	79.9 ^a	77.8 ^b	0.12	83.7 ^a	75.2 ^b	0.06
A	10.6 ^a	10.8 ^a	7.9 ^b	9.6 ^{ab}	0.19	10.5 ^a	9.0 ^b	0.10
B ₁	6.8 ^a	4.7 ^{ab}	2.7 ^b	4.0 ^b	0.26	4.5 ^a	4.6 ^a	0.13
NSC	17.4 ^a	15.5 ^{ab}	10.7 ^c	13.6 ^b	0.24	15.0 ^a	13.6 ^b	0.12
B ₂	47.8 ^b	50.6 ^{ab}	51.2 ^a	49.4 ^{ab}	0.32	53.9 ^a	45.6 ^b	0.16
C	15.2 ^b	13.5 ^b	18.0 ^a	14.8 ^b	0.20	14.8 ^b	16.0 ^a	0.10

CHO= total carbohydrate content, % OM=100-CP-ash-fat; A= (dry matter corrected for CP and Ash) - (residue remaining after extraction with 90% ethanol corrected for CP and ash); B₁=NSC-A; NSC=Non-structural carbohydrates=100-Crude protein-(NDF-NDIP)-fat-ash; B₂=NDF on organic matter basis minus NDIP minus the C fraction; C =Lignin/NDF*2.4.

^{a,b,c} Class means with different superscript differ ($P<0.05$) for grass effect or for fertilizer effect.

Nitrogen fertilization had a dual negative impact on carbohydrate pools (Table 2). First, the total plant carbohydrate was reduced due to a smaller A pool. An increase in the N fractions requires a corresponding depression in non-nitrogen components, especially sugars⁽³⁷⁾. Second, the B₂ pool was reduced by 15.4 %. At different levels of N fertilization the same effect on NDF it has been demonstrated⁽³⁶⁾. The positive effect of N fertilization in reducing the NDF content is offset by a negative effect in increasing lignification. The net result is a reduction in the availability of the B₂ fraction and an increase in the indigestible (C) fraction. The overall effect on the plant is a reduction in the available total carbohydrates. This may be why there are no improvements in IVDMD with N fertilization⁽³⁶⁾. CNCPS predictions⁽¹⁵⁾ found that the lower NDF in nitrogen fertilized tropical grasses was offset by higher CP and ash, which lowered the content of NSC. As a result, nitrogen fertilization did not significantly change the ME allowable milk. However, it improved the MP allowable milk dramatically. Because N fertilization increased both the CP and soluble protein content of the grasses, both

the ruminal N balance and the peptide balance increased. Juarez-Lagunes *et al*⁽¹⁹⁾ concluded that N fertilization could be expected to improve MP allowable milk, primarily because of increased pool sizes of CP and soluble protein.

Another challenge is to establish a connection between carbohydrate pools, energy yield from rumen fermentation and gas production. Gas production is not only affected by the amount of carbohydrates in a given pool, but it is also by their availability. Ranges from 27 to 30 mL of gas per 100mg of OM were found in whole forages in this study. Similar gas production was observed in 24 tropical grass species in Ethiopia⁽⁴⁰⁾. *Cynodon* produced less gas than *Megathyrsus* (Table 3) because *Cynodon* contains a larger C fraction than *Megathyrsus* (Table 2). A large C fraction indicates low availability of the cell wall. However, the C fraction does not explain the low availability of NSC. It is generally assumed that the NSC fraction is highly digestible⁽³⁷⁾. Because *Andropogon* has more total carbohydrates with the same C fraction size as *Megathyrsus* (Table 2), *Andropogon* should be expected to yield more gas than *Megathyrsus*. However, gas yields were similar (Table 3). Something may interfere with gas production from *Andropogon*.

Volumes of gas produced by the NSC also are shown in Table 3. *Andropogon* and *Urochloa* contain more NSC than *Cynodon* and *Megathyrsus* (Table 2), but they produce the same volume of gas from the NSC fraction. Moreover, the amount of gas per 100 mg of NSC is reduced suggesting that fermentations of the NSC of *Andropogon* and *Urochloa* were inhibited. Based on the subtraction technique, the fermentability of the A fraction of *Urochloa* and the B₁ fraction of *Andropogon* seemingly were affected. We suspect that tannin-like substances (TLS)⁽⁴¹⁾ or other secondary compounds interfere in the fermentability of NSC. During the preparation of the isolated NDF; tannins, biogenic silica or other secondary compounds are washed out, so the fermentability of the isolated NDF would be affected only by lignin content.

Table 3: Gas production and digestion rates of four tropical grasses fertilized with Nitrogen

	<i>A.</i> <i>Gaya-</i> <i>nus</i>	<i>U.</i> <i>Brizan-</i> <i>tha</i>	<i>C.</i> <i>Plectosta-</i> <i>chyus</i>	<i>M.</i> <i>Maxi-</i> <i>mus</i>	SEM	Non fertilized	Fertili- zed	SEM
Total carbohydrates								
Total gas, mL	23.7 ^a	23.0 ^a	21.6 ^b	23.6 ^a	0.11	24.0 ^a	21.9 ^b	0.05
Gas, mL/100 mg OM	29.5 ^{ab}	28.9 ^b	27.1 ^c	30.3 ^a	0.15	28.7 ^a	29.2 ^a	0.07
Degradation rate, %/h	5.1 ^{ab}	5.2 ^{ab}	4.8 ^b	6.0 ^a	0.10	4.9 ^b	5.7 ^a	0.05
Lag phase, h	2.2 ^b	2.4 ^b	1.0 ^c	3.0 ^a	0.06	2.1 ^a	2.2 ^a	0.03
B ₂ fraction								
Total gas, mL	19.4 ^a	18.6 ^{ab}	17.5 ^c	18.4 ^{ab}	0.14	19.2 ^a	17.8 ^b	0.07
Gas, mL/100 mg OM	40.9 ^a	36.9 ^{ab}	34.0 ^b	37.3 ^{ab}	0.43	35.3 ^b	39.2 ^a	0.21
Degradation rate, %/h	7.3 ^{ab}	8.4 ^a	3.8 ^c	6.8 ^b	0.16	6.5 ^a	6.6 ^a	0.08
Lag phase, h	4.5 ^b	5.2 ^b	4.6 ^b	6.7 ^a	0.14	5.2 ^a	5.3 ^a	0.07
NSC fraction								
Total gas, mL	4.3 ^b	4.4 ^b	4.1 ^b	5.2 ^a	0.08	4.8 ^a	4.1 ^b	0.04
Gas, mL/100 mg OM	24.5 ^b	28.1 ^b	38.6 ^a	38.6 ^a	0.90	34.1 ^a	30.8 ^a	0.45
Degradation rate, %/h	13.8 ^b	27.4 ^a	13.2 ^b	8.6 ^b	0.77	17.5 ^a	14.0 ^a	0.38
Lag phase, h	1.2 ^a	0.5 ^a	0.1 ^b	0.6 ^a	0.11	0.3 ^a	0.8 ^a	0.05
A fraction ¹								
Total gas, mL	3.3 ^a	2.0 ^b	3.4 ^a	3.2 ^a	0.08	3.2 ^a	2.7 ^b	0.04
Gas, mL/100 mg OM	31.7 ^b	18.2 ^c	42.6 ^a	33.4 ^b	0.72	32.0 ^a	31.0 ^a	0.36
B ₁ fraction ¹								
Total gas, mL	0.9 ^b	2.4 ^a	0.7 ^b	2.0 ^a	0.09	1.6 ^a	1.4 ^a	0.05
Gas, mL/100 mg OM	13.8 ^b	54.7 ^a	24.4 ^{ab}	51.4 ^a	3.44	39.8 ^a	32.3 ^a	1.72

Total carbohydrates= 100 - CP - Ash - Fat.

B₂ fraction= digestible structural carbohydrates= NDF/OM - NDIP - C fraction.

NSC fraction= non-structural carbohydrates= 100 - CP - (NDF - NDIP) - Ash - Fat.

A fraction= sugars and short chain polysaccharides= (dry matter corrected for CP and Ash) - (residue remaining after extraction with 90% ethanol corrected for CP and Ash).

B₁ fraction= starch and soluble fiber= NSC - A.

¹Degradation rates (%/h) and Lag phases (h) for A fraction and B₁ fraction were similar to NSC fraction.

^{a,b,c} Means with different superscript differ ($P \leq 0.05$) for the grass effect and for the fertilization effect.

When it was applied curve subtraction to NSC (whole forage - isolated NDF) all potentially interfering substances (tannins, biogenic silica or secondary compounds) were accounted in the NSC fraction, thus reducing gas yield. In our case, digestibility of the isolated NDF was 6.6 % greater than for whole forage NDF. These differences were 6.9 % for *Andropogon* and *Urochloa*, and 6.2 % for *Cynodon* and *Megathyrsus*. As a result, it may have experienced some under-prediction of NSC gas production. Because the amounts of soluble silica were similar in *Cynodon* and *Megathyrsus* compared to *Andropogon* and *Urochloa* (see AIA in Table 1), it was assumed that the major source of variation in gas produced by the NSC fraction likely resulted from secondary compounds. In a botanical survey *Megathyrsus* did not contain TLS, which obtains maximum expression in *A. gayanus*⁽⁴¹⁾. In the study *Urochloa* did not appear to contain condensed tannins, however it is suspect that there indeed may be other interfering substances. These findings support the suggestion that lignin content should be added to the equation to estimate total carbohydrates by the CNCPS model. Therefore, this modified CNCPS equation would become:

$$\text{CHO (g/kg DM)} = 1000 - [\text{CP (g/kg DM)} + \text{EE (g/kg DM)} + \text{MM (g/kg DM)} + \text{Lignin (g/kg DM)}]$$

The interference by phenols in the digestion of legumes and grasses merits more study for better management of ruminant nutrition in the tropics.

Nitrogen fertilization reduced the total amount of carbohydrates available for rumen fermentation (Table 2). The volume of gas produced was proportionally diminished with the amount of carbohydrate (Table 3). For instance, there was no difference in the amount of gas per 100 mg of substrate from unfertilized and fertilized forages. In the B₂ fraction, fertilized forage (FE) produced less gas than unfertilized forage (NF) because FE contained less fermentable structural carbohydrates (SC). In this study of same-age forages, fertilized grasses contained less NDF and the same amount of lignin as a percentage of dry matter as unfertilized grasses (Table 1), as has been found by others^(42,43). Therefore, there was more lignin as a percentage of the NDF. On the other hand, the difference in SC content between NF and FE was due primarily to hemicellulose. It is known that hemicellulose has more complex linkages with lignin than cellulose⁽³⁷⁾. Therefore, hemicellulose should be less available, increasingly so as the plant cell wall matures from more linkages between hemicellulose and lignin⁽³³⁾. NF grasses contained more hemicellulose and more mature cell walls than FE⁽⁴⁴⁾. The linkages between lignin and hemicellulose was reflected by the reduction in the amount of gas per 100 mg of SC from the NF grasses (Table 3). In summary, fertilized grasses produced 7.3 % less total gas from a smaller SC pool. However, this was compensated by 10 % more gas per unit of SC because they are less mature than NF grasses at

the same age.

Rates of digestion are presented also in Table 3. The range of the digestion rates obtained by the exponential equation for the whole forage was from 4.8 to 6.0 %/h ($r^2=99.7 \pm 0.12$; t-value= 61.2 ± 12.04), which agrees with other reports⁽⁴⁵⁾. For isolated NDF, digestion rates ranged from 3.8 to 8.4 %/h ($r^2= 99.8 \pm 0.11$; t-value= 62.6 ± 14.07), values that were higher than in other reports^(4,18,19) of 2 to 4 %/h for the B₂ fraction, and aligned with NDF digestion rates between 5.16 and 9.34 for C4 grasses⁽⁴⁶⁾, and corn silages⁽⁴⁷⁾. Updated versions of nutrition models (CNCPS; LRNS; NRC) should incorporate these rates to more accurately estimate ruminally available energy from the SC in C4 grasses. In tropical grasses, the B₂ fraction is the largest pool of carbohydrates, so the impact on the ME available to the animal could be significant. The ME allowable milk predicted by the CNCPS⁽¹⁵⁾ was very sensitive to change in the rate of digestion of the B₂ carbohydrate fraction. The ME allowable milk increased 88 % when the rate increased from 3 to 6%/h, and it increased an additional 24% when the rate increased from 6 to 9%/h. The predicted MP allowable milk increased from a – 0.8 to 5.7 kg/d as the B₂ rate increased from 3 to 6%/h and increased to 9.9 kg/d with a B₂ rate of 9%/h. These increases are the result of greater rumen degradation of SC.

In this study, because the B₁ was less than 10 % of the total DM, it was combined the A and B₁ rates and used the combined NSC rate for both fractions (Table 3). The rates for the NSC were highly variable, ranging from 8.6 %/h in *Megathyrsus* to 27.4 %/h in *Urochloa* ($r^2= 99.2 \pm 0.52$; t-values= 13.7 ± 6.83), with an overall mean of 15.7 %/h. These values are near the average (13.7 %/h) for bromegrass, orchardgrass, and alfalfa, where rates of digestion were 13.9 %/h for the A fraction and 11.8 %/h for the B₁ fraction⁽⁸⁾, also from Brazilian tropical grasses with rates of digestion for the NSC fraction between 6 and 12 %/h⁽⁴⁸⁾. The CNCPS tabular values of digestion rates for the A fraction are fixed 40 %/h and for the B₁ fraction are 30 %/h in most tropical grasses. There is need for more research on the rates of digestion of carbohydrate pools in tropical grasses, and more frequent revision of tabular values for field use. Nitrogen fertilization did not have much apparent influence on rates of digestion (Table 3). These were apparently more affected by inherent plant physical structure. Chemical differentiation was more related to the extent of digestion and volume of gas produced⁽⁴⁸⁾. Tissue anatomy strongly affects degradation rates. Cell types with thickened secondary wall, such as vascular bundles, sclerenchyma strands, epidermis and parenchyma bundle sheath of C4 grass leaves form solid, multicellular blocks of cells that constitute a barrier to microbial access to wall surfaces⁽⁴⁹⁾. If all cells had only thin primary walls, (e.g., mesophyll, phloem and undifferentiated parenchyma tissues of leaves and young stems) then the cell wall would degrade rapidly.

In summary, chemically *Andropogon* and *Urochloa* had more NSC compared with *Megathyrus* and *Cynodon* but they produced less gas per unit of NSC. It is suspected interference from secondary compounds. The rates of digestion for the B₂ fraction ranged from 4 to 8 %/h and the rate of digestion for the NSC averaged 15.7 %/h. Nitrogen fertilization had a negative impact on carbohydrate pool sizes but did not affect rates of digestion.

Digestion rates found in this study suggest that the CNCPS, LRNS and NRC should update more frequently the ruminally available energy from SC and NSC in tropical forages. The impact on the prediction of the ME available to the animal could be significantly improved.

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