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AN *IN VITRO* TECHNIQUE FOR ESTIMATING DIGESTIBILITY OF TREATED AND UNTREATED WOOD [1,2]

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IN the United States, about 50 million tons of wood and bark residues, containing up to 75% carbohydrate are available annually as a potential energy source for ruminants. Except for modest digestibility of a few hardwood species, however, the carbohydrates of whole-wood residues are essentially unavailable to the ruminant animal. The close molecular association between the major constituents of wood — cellulose, hemicellulose, and lignin — appears to limit bacterial and enzymatic access to the carbohydrates of the cellwall.

Success in using wood residue carbohydrates as an energy source thus requires the development of an effective and practical means for enhancing the accessibility of the cell wall constituents to the action of rumen microorganisms. Of prime importance to this type of undertaking is a rapid, accurate, and inexpensive in vitro technique for assaying the large numbers of samples that will be generated.

The in vitro procedure of Tilley and Terry (1963) or one of its modifications has been used extensively and is one of the better laboratory methods for evaluating the nutritive quality of forages (Barnes, 1967; Oh, Baumgardt and Scholl, 1966; Van Soest, Wine and Moore, 1966). When used to estimate the digestibilities of modified woods and other high-cellulosic substrates, however, it yielded low and quite variable coefficients of digestibility.

The purpose here was to adapt the first stage of the Tilley and Terry technique to assay treated and untreated woods. Through critical investigation, each operational step was optimized. The resultant modified procedure lends itself with acceptable precision to routine processing of multiple samples. Examined during optimization were the following: Type of incubation vessel, ratio of inoculum to buffer, sample size, prewetting of substrate, tube orientation during incubation and length of incubation.

Methods and Materials

The donor cow for the inoculum was a 2-year-old ovariectomized, nonlactating Holstein heifer maintained on an all-alfalfa hay diet. Ruminal ingesta was collected via a ruminal fistula 4 to 5 hr. after the morning feeding. Feed and water were not withheld prior to collection. The sample of ruminal ingesta was transferred to the laboratory in a prewarmed thermos and strained through three layers of cheesecloth into a round-bottom flask supported in a 39 C water bath and equipped with a magnetic stirrer and a CO_2-inlet tube. The well-agitated inoculum was dispensed by means of a calibrated syringe to the incubation vessels that contained the wood substrate and McDougall's buffer (1948) adjusted to pH 6.8 with CO_2. After the addition of rumen fluid, each tube was gassed with CO_2, sealed tightly, and placed in a 39 C water bath for incubation. Total liquid volume was 38 ml per tube.

Substrates used during the development of a satisfactory in vitro procedure included aspen wood, cotton linters, alfalfa, ammoniated aspen wood and aspen bark. These substrates were all ground through a 40-mesh screen with a Wiley mill. The ammoniated aspen was prepared by steeping aspen sawdust in liquid ammonia for 1 hr. at 30 C in a pressure vessel (approx. 150 psi).

Fifty-ml polyethylene centrifuge tubes fitted with Bunsen valves were used initially as incubation vessels but were replaced with 50 ml screwcap glass culture tubes. A horizontal tube position during incubation was selected following comparison with a vertical or a continuously rotated position. Ratios of rumen fluid to McDougall's buffer solution of 1:10, 1:4, 1:1, 4:1 and 5:0 were compared.

A comparison of dry matter digestibilities was made between prewetted samples and

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samples that were not prewetted. Substrates were wetted by adding 3 ml of water to 0.25 g of substrate. The samples were placed under a 28-in Hg vacuum for 10 to 15 min. before the addition of rumen fluid and McDougall's buffer. Prewetting was adopted as a routine procedure following initial comparisons. The length or duration of the incubation period was examined. Within- and between-day variability and total digestibility were the criteria used to select an optimal incubation period.

To determine dry matter digestibility, the incubation mixture was centrifuged at 1800x gravity for 15 min. immediately following incubation. The supernatant was decanted by suction. The settled solids were resuspended in 35 ml H_2O and again centrifuged and decanted; then the residue was dried at 100 C for 48 hours. Tubes containing only rumen fluid and McDougall's buffer were used to correct for the dry matter contributed by the rumen fluid. The following formula was used to calculate dry matter digestibility:

$$\text{Digestible dry matter (\%)} = 100 \left[\frac{0.25\text{ g} - (\text{residual dry matter (g)} - \text{control dry matter (g)})}{0.25\text{ g}} \right]$$

Carbohydrate digestibility, in addition to dry matter disappearance, was determined. Carbohydrate analysis was performed on dried samples before and following a 96-hr. fermentation period. Substrates and fermentation residues were hydrolyzed, according to the quantitative saccharification procedures described in American Society for Testing and Materials Standard Method D 1915–63 (1969) using lime neutralization, and the resulting reducing sugars were measured colorimetrically, using Nelson's (1944) adaptation of the Somogyi method.

The forages used in the test analysis included bromegrass, alta fescue, orchardgrass, reed canarygrass, timothy, and alfalfa. All forages were ground through a 40-mesh screen.

Results and Discussion

Table 1 shows a comparison of the 48-hr. digestibilities with valved and sealed incubation tubes. Higher digestibility coefficients and decreased standard deviations were obtained for all substrates with the sealed tubes. This observation may be related to the 10 to 16 hr. digestion lag phase obtained with woods and purified celluloses. The valved tubes apparently lost sufficient CO_2 during this lag phase to allow media pH to rise above 7.0, with resultant impairment of cellulose digestibility (Baumgardt, 1959). The sealed tubes did not allow CO_2 loss, and the starting pH of 6.8 was held constant during the lag phase. This pH was within the range of maximum digestion of cellulose. Some gas accumulation was noted with the sealed tubes, but apparently this did not have a detrimental effect on substrate digestibility.

TABLE 1. EFFECT OF TYPE OF INCUBATION VESSEL ON *IN VITRO* DRY MATTER DIGESTIBILITY

Type of tube	Digestibility: Aspen wood	Cotton linters	Ammoniated aspen wood
	%		
Valved	11±6.5[a]	40±13.5	33±10.8
Sealed	20±2.9	63±5.4	37±2.1

[a] Standard deviation.

Initially, one part of rumen fluid was mixed with four parts of McDougall's solution. This ratio required additional nutrients such as urea, yeast, or glucose to achieve maximum and reproducible digestion coefficients from substrates such as wood. The added nutrients represented additional variables, the elimination of which increased digestibility. An examination of the different ratios of rumen fluid: McDougall's solution in table 2 revealed that substrates incubated with a 1:1 ratio without added nitrogen or glucose had the highest dry matter digestibilities. The 1:1 ratio apparently provided adequate nutrients, growth factors and numbers of microorganisms that were lacking in the 1:10 and

TABLE 2. EFFECT ON DIGESTIBILITY OF RATIO OF RUMEN FLUID TO McDOUGALL'S SOLUTION WITH AND WITHOUT ADDITIVES

Rumen fluid: McDougall's[a]	Dry matter digestibility: Aspen wood, Additives[b]	Aspen wood, No additives	Cotton linters, Additives[b]	Cotton linters, No additives
	%			
5:0	15.2	21.2	32.0	37.8
4:1	19.4	23.9	44.0	55.6
1:1	20.2	25.4	70.5	75.2
1:4	11.5	4.6	54.5	28.0
1:10	3.4	2.0	38.7	8.6

[a] Total volume was 35 milliliters.
[b] 70 mg yeast extract and 17.5 mg glucose were added to each tube.

TABLE 3. EFFECT OF SAMPLE SIZE ON *IN VITRO* DRY MATTER DIGESTIBILITY

Substrate	Digestibility		
	350 mg	250 mg	150 mg
	%		
Aspen wood	18.8±3.9[a]	18.5±3.5	19.6±5.3
Aspen bark	27.7±0	27.6±1.9	28.1±0
Cotton linters	77.1±1.7	81.6±0.4	83.9±1.5
Alfalfa	57.6±1.6	58.6±0.5	62.2±0.4

[a] Standard deviation.

1:4 ratios but still provided sufficient buffering capacity that was lacking in the 4:1 and 5:0 ratios. The pH values with the 4:1 and 5:0 ratios would drop below pH 6.0 at 18 to 21 hr. of incubation.

A 48-hr. incubation of the more digestible substrates, such as cotton linters, alfalfa, and treated woods, resulted in a final pH of 5.8 to 5.9. This pH was not conducive to maximum dry matter digestibility. For more adequate control of fermentation pH, sample size was decreased from 0.35 to 0.25 g, whereas the came volume of inoculum and buffer (35 ml) was retained. The 0.25 g sample size, when compared to the 0.35 g sample size, increased digestibility of cotton linters and tended to increase digestibility of alfalfa (table 3). The final fermentation pH for the 0.25 g samples remained around 6.2. The 0.15 g sample size, while yielding still higher digestibilities, was not considered satisfactory for routine use because of the larger weighing errors associated with this smaller quantity. The advantage of reduced sample size is indeed modest, but for more highly digestible treated woods it mav be advantageous.

Following the changes mentioned, collaborative in vitro studies with the Forest Products Laboratory revealed consistent discrepancies between digestibility values obtained from each of the laboratories. The Forest Products Laboratory prewetted samples prior to adding the inoculum and obtained higher digestibilities than the Dairy Science Laboratory, which did not prewet their samples. Prewetting fills the wood-fiber lumen with water; thus it allows an increased rate of enzyme and microbial penetration and digestion.

TABLE 4. EFFECT OF PREWETTING OS SUBSTRATE DRY MATTER DIGESTIBILITY

Treatment	Digestibility			
	Aspen wood	Aspen bark	Cotton linters	Alfalfa
	%			
Prewetted	20±1.8[a]	26±0.8	70±2.5	55±2.1
Not prewetted	15±2.3	23±0.8	72±7.5	49±3.2

[a] Standard deviation.

As shown by the data in table 4, prewetting resulted in higher digestibilities for aspen (wood), aspen bark and alfalfa, whereas digestibility of cotton linters remained about the same. The standard deviation of digestion coefficients was also decreased by prewetting. Because cotton linters are readily dispersed as single fibers, prewetting had no effect on the rate of digestion. Complete interlaboratory agreement on substrate digestibility was obtained following adoption of the prewetting technique.

An examination of tube position during fermentation was prompted when it was observed that tubes in a vertical position did not maintain good substrate-media contact. Tubes were not mixed during the night, and in those in a vertical position, a dry, crusty layer of material formed on top of the media solution. This led to varying values of digestibility. A comparison of three tube positions, vertical, horizontal and continuously rotated, suggested that the horizontal tube position provided the highest digestibility coefficients (table 5). The horizontal tubes required less agitation for maximum digestion because the media and substrate remained in continuous contact. However, variation in digestion coefficients was reduced with the horizontal tubes if they were gently shaken every 2 to 3 hr. during the day. The lowered digestion coefficients observed with the continuously rotated tubes probably were caused by the excessive agitation of samples, which severed microbial-substrate contact. Continuous microbial-substrate contact is essential for active cellulose digestion (Baker *et al.*, 1959; Quicke *et al.*, 1959).

A rate study (figure 1) revealed that the digestion of aspen, ammoniated aspen, alfalfa, and cotton linters was far from complete at 48 hours. The 48-hr. digestibilities were located on the rapidly ascending portions of the rate curves and were subject to considerable variability. As can be seen, attainment of a digestion plateau would require more than a 200-hr. incubation: however, a 96-hr. incubation was sufficient for digestion to reach 90% or more of the plateau value. A smaller error, therefore, would be associated with the selection of a 96-hr. rather than a 48-hr. fermentation period as the end point.

In table 6 in vitro dry matter digestibility is compared for 48- and 96-hr. periods. Dou-

TABLE 5. EFFECT OF TUBE POSITION ON *IN VITRO* DRY MATTER DIGESTIBILITY

	Digestibility					
	Aspen		Cotton linters		Alfalfa	
Treatment	Shaken	Not shaken	Shaken	Not shaken	Shaken	Not shaken
	%					
Horizontal	23±0.7[a]	24±1.4	75±2.5	70±7.4	52±0.6	52±2.9
Vertical	19±1.8	15±3.1	61±5.1	49±14.0	49±4.5	37±1.0
Rotated[b]	18±4.5		45±6.2			

[a] Standard deviation.
[b] Continuously rotated at six revolutions per minute.

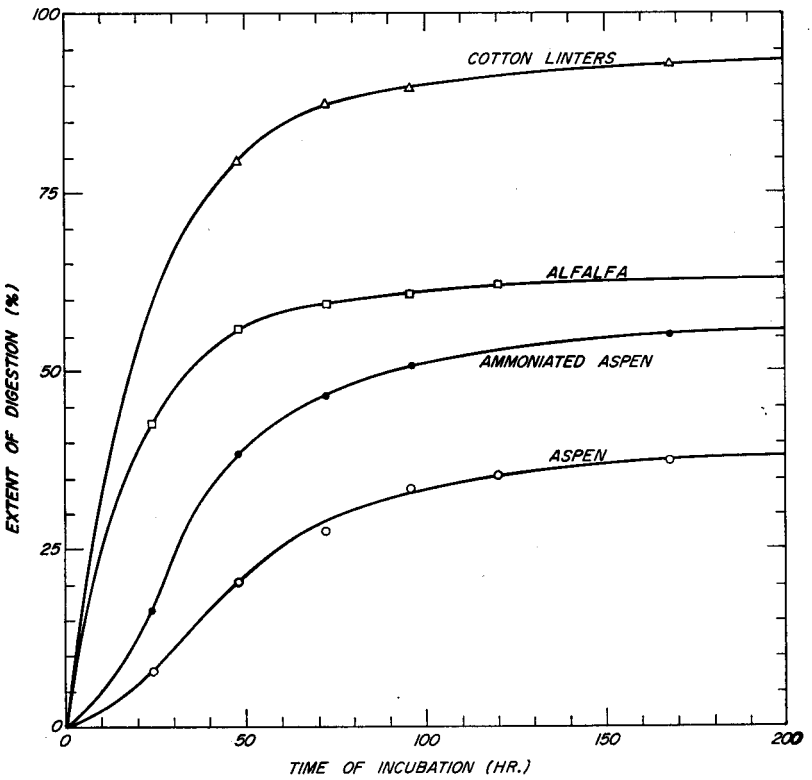

Figure 1. Relationship of percentage of in vitro digestion of various substrates to time of incubation.

TABLE 6. EFFECT OF INCUBATION TIME ON EXTENT AND VARIATION OF *IN VITRO* DIGESTIBILITY

	48-hr. incubation			96-hr. incubation		
		Coefficient of variation			Coefficient of variation	
Substrate	Digestibility	Within day	Between days	Digestibility	Within day	Between days
	%					
Aspen wood	18.3	12.9	17.9	30.3	5.0	4.7
NH_3 aspen (1 hr. at +30 C)	40.0	4.6	10.3	47.9	2.8	3.3
NH_3 aspen (1 hr. at +60 C)	39.7	9.7	14.1	52.3	1.2	6.5
Cotton linters	73.7	4.3	11.2	92.1	0.8	2.6
Alfalfa	54.5	1.1	...	61.4	0.8	...

bling the incubation time increased the digestibility of all substrates; the increases ranging from about 65% for untreated aspen to 12.5% for alfalfa. The digestibility of liquid ammonia-treated aspen was only about 10% lower than the digestibility of alfalfa at 96 hours. The digestibility of cotton linters was approaching the theoretical maximum. Complete digestion of cotton linters would probably not be possible because of the accumulated microbial cell mass and acidic by-products that result from substrate fermentation. The 96-hr. fermentation period also decreased both within and between day variation on all substrates as shown by the coefficients of variation in table 6. The 96-hr. *in vitro* digestibility for aspen more closely estimated its *in vivo* digestibility (*unpublished data*), whereas the apparent in vivo digestibility of alfalfa was better estimated by the 48-hr. fermentation period. An extended incubation period also reduced digestibility variation in fermentation studies on wood and cotton with isolated rumen bacteria (Dehority and Scott, 1967; Stranks, 1961).

A direct comparison of losses of carbohydrate and dry matter during a 48-hr. digestion proved that the disappearance of dry matter was more than simple solubilization of substrate. Actual carbohydrate digestion was taking place. Values for in vitro digestible dry matter were 16.9% for aspen wood, 70.4% for cotton linters and 55.6% for alfalfa, whereas comparable values for digestibility of carbohydrate were 17.9, 75.8 and 53.9%, respectively.

The final procedure consisted of the following four steps:

(1) Inoculum preparation: Ruminal ingesta obtained 4 to 5 hr. following feeding through a ruminal fistula from a cow fed an all-alfalfa hay ration was strained through three layers of cheesecloth. The inoculum was maintained at 39 C and was agitated both mechanically and with a stream of CO_2 gas during aliquoting to the sample tubes. A calibrated syringe pipette was used for the aliquoting.

(2) Sample preparation: 0.25 g of substrate previously ground through a 40-mesh screen with a Wiley mill and of known moisture content was weighed into a 50 ml screw-cap glass culture tube. The samples were prewetted by adding 3 ml of water and placing under 28-in Hg vacuum for 10 to 15 minutes.

(3) Incubation conditions: 17.5 ml of strained whole rumen fluid and 17.5 ml of McDougall's buffer, adjusted to pH 6.8 with CO_2 were added to each prewetted sample. The tubes were then flushed with CO_2, sealed tighly, and placed horizontally in a 39 C water bath. The tubes were gently shaken by hand every 2 to 3 hr. during the day throughout the 96-hr. fermentation.

(4) Determination of dry matter digestibility: At the end of fermentation the samples were centrifuged at 1,800 x gravity for 15 minutes. The supernatant was decanted by suction to within about 1/2 cm of the settled layer. The solids were redispersed in 35 ml H_2O, certrifuged, and the supernatant again decanted by suction. The residues were then dried at 100 C for 48 hours. Correction factors for the dry matter contributed by the rumen fluid were obtained by incubating control tubes containing only rumen fluid and McDougall's buffer for the same length of time as the other samples.

The in vitro procedure just described provided more reproducible determinations of dry matter digestibility of treated and untreated woods than did the Tilley and Terry method. As a further test it was desired to determine whether this method was suitable for estimating the *in vivo* dry matter digestibilities of selected forages. Fourteen forages, including grasses and legumes, and six complete rations with known *in vivo* digestibilities were evaluated with the modified in vitro procedure. Table 7 shows the comparison between the coefficients of in vitro and in vivo digestibility and the correlation between them.

The correlation between the in vivo and in vitro digestibility coefficients was highest for alfalfa (0.970) and complete rations (0.994). A correlation of 0.945 was obtained for the grass forages. The overall simple correlation between in vivo and in vitro digestibility coefficients was 0.955. The complete rations contained 0 to 40% aspen wood.

Oh *et al.* (1966), in an extensive comparison of forage evaluation methods, found the Tilley and Terry in vitro technique superior to the others examined, and noted simple correlation coefficients of 0.83 for grasses and 0.97 for legumes. From this it appears that the in vitro technique described here is superior to the Tilley and Terry technique for estimating the digestibility of woods and grasses, but is no better for estimating the digestibility of legumes.

Summary

A closed in vitro technique, utilizing whole

TABLE 7. COMPARISON OF *IN VIVO* AND *IN VITRO* 96-HR. DRY MATTER DIGESTIBILITIES OF SEVERAL FEEDSTUFFS

Feedstuff	Digestibility		Correlation between *in vivo* and *in vitro*	Overall Correlation between *in vivo* and *in vitro*
	In vivo	*In vitro*		
	%	%		
Grass forages				
Birdsfoot trefoil	60.0	56.6±1.3[a]		
Reed canarygrass	55.6	52.8±1.1		
Bromegrass (Mich.)	55.1	53.6±1.3		
Alta fescue	57.1	60.5±2.0	0.945	
Timothy	76.8	72.1±1.4		
Orchardgrass	73.1	73.6±1.3		
Bromegrass (Del.)	59.1	60.5±1.4		
Alfalfa forages				
Alfalfa (WD469)	71.3	67.4±2.0		
Alfalfa (WD460)	53.3	50.1±1.2		
Alfalfa (WD433)	69.9	61.7±3.2	0.970	0.955
Alfalfa (WD488)	57.4	54.2±0.1		
Alfalfa (WD388)	63.3	58.4±1.4		
High-roughage ration				
Ration A (0% wood)	58.0	60.0±0.8		
Ration B (20% wood)	54.5	56.1±0.5		
Ration C (40% wood)	51.1	54.0±0.4		
			.994	
High-concentrate ration				
Ration D (0% wood)	89.6	89.2±0.7		
Ration E (20% wood)	77.0	74.8±1.2		
Ration F (40% wood)	65.2	63.4±0.5		

[a] Standard deviation.

strained rumen fluid as the inoculum, was developed to estimate the dry matter digestibility of treated and untreated woods. The technique is a modification of the first stage of the Tilley and Terry procedure for assaying the nutritive qualities of forages.

Important steps of the modified procedure include the following: (1) Use of 50 ml glass screwcap culture tubes instead of Bunsen-valved tubes as the incubation vessels. (2) Reduction of sample size from 0.5 g to 0.25 g with final incubation volume at 38 milliliters. (3) Prewetting the substrate samples for 10 to 15 min. with 3 ml of water under 28 in Hg vacuum. (4) Changing the ratio of rumen fluid to McDougall's buffer from 1:4 to 1:1 to increase extent of digestion. (5) Placing the tubes horizontally rather than vertically in the water bath during incubation. (6) Extending the incubation period from 48 hr. to at least 96 hours.

The six changes in procedure maximized the dry matter digestibility of various woods as well as decreased the among trial variation in digestibility. The sealed tubes, the 1:1 rumen fluid to McDougall's buffer ratio, and the smaller sample size resulted in better pH control during fermentation, whereas the prewetting step and extended fermentation period resulted in digestibility values that were closer to those estimated for in vivo digestibility. The horizontal placement of the tubes permitted continuous substrate-media contact during fermentation.

The modified procedure, when used to estimate the digestibility of several feedstuffs, resulted in correlation coefficients of 0.970 for alfalfa forages, 0.945 for grass forages and 0.994 for complete rations containing aspen wood.

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