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Effects of incubation time and sodium sulfite upon in-vitro digestibility estimates and sample filtering time

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Abstract

We conducted 2 experiments to quantify the effects of incubation time, filtering method, forage type, and associated interactions on the precision and accuracy of in-vitro digestibility as estimates of in-vivo digestibility. Experiment I used 10 incubation times and alfalfa (*Medicago sativa* L.), kleingrass (*Panicum coloratum* L.), prairie grass, and wheat straw (*Triticum aestivum* L.) hays to determine whether a single incubation time should be employed to estimate digestibility of a variety of forages. Additionally, 2 second stage neutral detergent extraction methods were evaluated to determine sodium sulfite effect on fiber recovery and filter time. An interaction existed between incubation time and in-vitro estimates of digestibility. The use of sodium sulfite increased ($P<0.05$) digestibility estimates (1.3 units) across all hays and decreased filtering times by as much as 9.5 min/sample. Experiment II utilized 3 hays (alfalfa, kleingrass, and wheat straw), 4 incubation times and 4 neutral detergent extraction methods in an effort to isolate where the changes in neutral detergent fiber (NDF) estimates due to sodium sulfite occurred and if a method could be developed to maximize filtering speed without compromising the accuracy of digestibility estimates. Use of sodium sulfite in the rinse water did not affect apparent NDF recovery and decreased filtering time by approximately 10 min. when compared to no sulfite additions. Results of this study confirm previous observations that a single incubation period should not be used to estimate in-vivo digestibility. Addition of sodium sulfite to the rinse water provides a viable means to decrease sample analysis time without jeopardizing the accuracy of digestible NDF estimates.

Key Words: fiber recovery, organic matter digestibility, neutral detergent extraction

Laboratory methods of determining forage digestibility have received a great deal of attention as an alternative to in-vivo trials (Hadjipanayiotou et al. 1987). The in-vitro procedure developed by Tilley and Terry (1963), utilizing a 48-hour incubation in rumen inoculum and buffer followed by a 48-hour acid pepsin digestion, is a widely accepted in-vitro technique to estimate apparent in-vivo digestibility. Van Soest et al. (1966) modified this method by utilizing a neutral detergent solution (NDS) extraction in place of the second stage acid-pepsin digestion, which resulted in increased accuracy and decreased procedure time and estimated true digestibility.

The validity of using a set incubation period for all forages is questionable because rumen retention time varies among forage types having similar digestibilities (Ingalls et al. 1966). A 48-hour incubation may be too long for legumes and too short for grasses (Holechek et al. 1986). Maximum correlation of fermentation time with digestibility is approximately 36 hours for a wide range of forages (Van Soest, 1978).

Van Soest and Wine (1967) found NDS extraction could result in the formation of a gelatinous material that clogs filters and leads to increased neutral detergent fiber (NDF) yields. Addition of sodium sulfite to NDS reduces residual and keratinized proteins and increases filtering ease (Van Soest 1982). Unfortunately, sulfite inclusion during reflux also decreases apparent neutral detergent fiber (NDF) recovery, probably as a result of sulfite cleaving lignin bonds (Robertson and Van Soest 1981, Moir 1982, Van Soest 1982).

Based upon previous reports, this study was designed to address the following objectives.

1. Determine whether a single incubation period can accurately estimate in-vivo apparent digestibility of a range of hays.
2. Quantify the degree to which sulfite reduces recovery of undigested fiber and thereby over-estimates apparent digestibility.
3. Quantify the effect of sodium sulfite addition on filtering time.
4. Isolate where in the NDS procedure the effects of sulfite are manifested.

Methods

Experiment I

Four hays of known in-vivo digestibility by cattle (Hunt et al. 1990) were used to evaluate the combined effects of 10 in-vitro

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incubation times and 2 NDF extraction methods on the accuracy and precision of in- vitro estimates. The hays were, mid-bloom, alfalfa (*Medicago sativa* L.), kleingrass, mid-anthesis, (*Panicum coloratum* L.), prairie grass, mid-anthesis, (a mixture of *Panicum* spp. *Andropogon* spp.), and wheat straw (*Triticum aestivum* L.) with respective in-vivo organic matter digestibilities (OMD) of 76, 65, 59, and 54% (Table 1). Hays were fed at 95% of predetermined ad libitum levels, and total fecal collections were made from crossbred steers (252 kg) housed in individual metabolism crates. For in-vitro analyses, hays were ground to pass a 1-mm sieve in a Wiley mill. The experiment consisted of 2 in-vitro trials using a modified Tilley and Terry (1963) technique incorporating a NDF extraction as a second stage in lieu of an acid-pepsin digestion (Van Soest et al., 1966). Incubation times of 8, 16, 24, 36, 48, 60, 72, 96, 120, and 144 hours were used. The relationship of in-vitro digestibility estimates to the corresponding in-vivo value of apparent organic matter digestibility for each hay was determined. The 2 methods of NDF extraction used were according to Van Soest and Wine (1967) with the following modifications:

Method 1. Delete the use of decalin and sodium sulfite.

Method 2. Delete the use of decalin, add approximately 0.1 g of sodium sulfite to each sample and add approximately 1.0 g of sodium sulfite/liter of hot water used for rinsing.

Each hay incubation time/NDF method was triplicated within each in-vitro trial and randomized throughout 2 water baths to minimize bath effects. At each incubation time, 6 tubes/hay were removed from the water baths and dosed with 1 ml of a 5% mercuric chloride solution to halt fermentation. Tubes were then refrigerated at 3.4° C until extraction. Samples were filtered under vacuum in 50 ml, coarse, fritted glass gooch crucibles. Three tubes hay^{-1} incubation time^{-1} were subjected to each NDF method to determine differences in fiber recovery between the 2 NDF methods. Additionally, in Trial 2, filtering times were recorded as the time from the start of filtration to the removal of all visible soap from NDF residue. All results are presented on an organic matter basis.

Experiment II

Experiment II was conducted because the results of Experiment I indicated sulfite had significant effects on in-vitro digestibility estimates and filter time. Three of the previous hays (alfalfa, kleingrass, and wheat straw) and 4 incubation times (24, 48, 72, and 144 hours) were used to quantify the effects of sodium sulfite inclusion at different stages of the NDF extraction on in-vitro digestibility estimates and filter time. The in-vitro method employed was the same as Experiment 1 except that 4 NDF methods were used instead of 2. All methods deleted the use of decalin.

Method A. No sodium sulfite.

Method B. Add approximately 0.1 g of sodium sulfite to each sample before refluxing.

Method C. Add approximately 1.0 g of sodium sulfite/liter of hot water used for rinsing.

Method D. Combine Method B and C.

Five tubes hay^{-1} incubation time^{-1} NDF method^{-1} were randomized throughout 2 water baths. At each incubation time, 20 tubes hay^{-1} were removed from the water bath and fermentation terminated by the addition of 1 ml of 5% mercuric chloride. Tubes were refrigerated (3.4° C) until the NDF extraction was performed. Filtering times were recorded for each sample as in Trial 2 of Experiment I.

Statistical Analyses

A split-split plot analysis of variance was used to test the response of in-vitro digestibility and filter time. Tubes were used as replicates. Whole plot hay was tested with the hay × replicate interaction. In the split-plot, filter method and the filter method × hay interaction were tested with the filter method × hay × replicate interaction. Incubation time, incubation time × hay, incubation time × filter method and incubation time × hay × filter method interactions comprised the split-split plot and were tested by residual model error. A protected Student-Neuman-Keuls test was utilized for mean separation. A t-test was employed to determine at which incubation times within each hay in vitro digestibility differed from OMD. Unless otherwise noted, significance is at $P < 0.05$.

Results and Discussion

Experiment 1

There was an incubation time by hay interaction (Fig. 1) for in-vitro neutral detergent fiber digestibility. At 8 hours, in-vitro digestibility of alfalfa hay was greater than that of kleingrass, prairie hay, and wheat straw. Corresponding cumulative increases in in-vitro digestibility from 8 to 48 hours were greater for kleingrass, prairie hay, and wheat straw than alfalfa hay. The interaction resulted from the rapid disappearance of alfalfa hay through 8 hours and a slower rate through 144 hours in contrast to increased disappearance rates of the remaining hays.

Observed temporal differences in increasing in vitro digestibility among forages probably result from the in-vivo OMD of a forage determining potential in vitro digestibility at any given incubation period, with alfalfa hay being the most and wheat straw the least digestible (Table 1). Note that the relationship among forages does not change between incubation times for in-vitro digestibility, however, disappearance rates do. For example, the structural and anatomical characteristics of alfalfa hay, a dicot, harvested during early growth and with a high leaf to stem ratio, are conducive to rapid digestion. In contrast, wheat straw, a

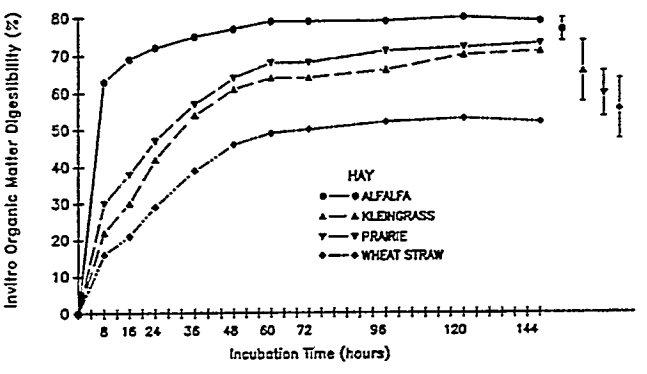

Fig. 1. Relationship between in-vitro organic matter disappearance (symbol-line) and incubation time for four hays compared to their in-vivo apparent digestibility (symbol + 1 SD) for Experiment I.

Table 1. Nutrient content and organic matter digestibility of alfalfa, kleingrass, prairie grass and wheat straw hays used in Experiments I and II.

Hay	Constituent				
	OMD[a]	CP[b]	NDF[c]	ADF[d]	OM[e]
	% of OM				
Alfalfa	76	25	40	30	88
Kleingrass	65	12	83	46	92
Prairiegrass	59	7	73	39	93
Wheat straw	54	3	86	53	92

[a]In-vivo apparent organic matter digestibility
[b]Crude protein/OM basis
[c]Neutral detergent fiber/OM basis
[d]Acid detergent fiber/OM basis
[e]Organic matter, % of dry matter

mature monocot, had less cell soluble and increased cell wall constituents and a lower leaf to stem ratio that resulted in a slower rate and extent of digestion. Similarly, Holechek et al. (1989) found that herbaceous plants had higher in-vitro digestibility estimates at any incubation time than did grasses.

Mean maximum in-vitro digestibility estimates of each hay, except wheat straw, exceeded the mean OMD estimates for that hay (Fig. 1). Except for prairie hay, maximum mean in-vitro digestibility estimates are within 1 standard deviation of the mean OMD of a hay. Overestimation of in-vitro digestibility at longer incubation times in prairie hay was attributed to the low crude protein (CP) content (<7%) of prairie grass hay, leading to depressed in-vivo OMD because of nitrogen restriction at the rumen microbial level (Van Soest 1982), even though prairie hay was comparatively low in NDF and acid detergent fiber (ADF) (Table 1). Elevated in-vitro digestibility estimates of prairie hay apparently resulted from nitrogen enrichment (urea addition) of the in-vitro incubation medium facilitating increased microbial degradation of structural carbohydrate (Schmid et al. 1969, Madsen and Hvelplund 1988). In contrast, wheat straw also low in CP, was high in NDF and ADF and nitrogen enrichment in-vitro did not increase estimates of digestibility.

Differences between in-vitro digestibility and OMD and in-vitro digestibility as a percent of OMD were calculated to determine the effect of incubation time on estimates of OMD (Table 2). For alfalfa hay, only the 36 hour incubation time did not differ from OMD. However, this estimate was not different ($P>0.05$) from the 24 hour or the 48 hour and greater incubation times. The in-vitro digestibility estimates from 60, 72, and 96 hour incubation times did not differ from OMD of kleingrass. These estimates, however, did not differ from in-vitro digestibility values obtained 48 hour incubation time. Only the 36 hour in-vitro digestibility estimate for prairie hay did not differ from OMD. Estimates of in-vitro digestibility from 36 hour onward did not differ from one another. The relatively short incubation time required to achieve OMD levels in prairie hay can be attributed to the nitrogen enriched incubation medium resulting in increased rates and extents of digestion in-vitro relative to in-vivo. In-vitro digestibility of wheat straw was estimated within 2 units of OMD after 144 hour incubation time. In-vitro digestibility estimates at 96, 120, and 144 hour incubation times did not differ from wheat straw OMD.

Across all hays and times, the use of sulfite significantly increased in-vitro digestibility estimates by 1.3 percentage units of disappearance. Significant increases in in-vitro digestibility of 0.87, 2.0, and 1.4 percentage units were found for alfalfa hay, prairie hay, and wheat straw, respectively. A 0.87 increase in in-vitro digestibility for kleingrass hay was not significant.

In Trial 2 sulfite decreased filtering time for all hays, ($P<0.05$). Filtering time per sample was reduced 8 min by using sulfite in alfalfa hay and kleingrass hay. Filtering times were reduced by 9.5 min and 3 min in kleingrass and wheat straw, respectively.

Experiment 2

Filtering method had a significant effect on in-vitro digestibility estimates across all hays and times (Table 3). The use of sulfite during refluxing (sulfite + NDS and sulfite + NDS + rinse water) significantly decreased residual neutral detergent fiber (increasing in-vitro digestibility estimates) when compared to the methods using no sulfite during refluxing (no sulfite and sulfite + rinse water). Quantitative loss of NDF when sulfite was used in the refluxing stage is in agreement with results from Experiment I, Robertson and Van Soest (1981), and Moir (1982). Interestingly, the use of sulfite in rinse water did not increase in-vitro digestibility estimates over that of no sulfite (Table 3). We pro-

Table 2. The difference of in-vitro organic matter digestibility (IVOMD) from in-vivo organic matter digestibility (OMD) (units of digestibility) and percentage of potential OMD at each in-vitro time step in Experiment I.

Item	Incubation Time (hours)									
	8	16	24	36	48	60	72	96	120	144
Alfalfa hay										
Units difference	-13^{d1}	-7^{c}	-4^{b}	-1^{ab*}	1^{a}	3^{a}	3^{a}	3^{a}	3^{a}	3^{a}
Relative IVOMD(%)[2]	83	91	95	99	101	104	104	104	104	104
Kleingrass hay										
Units difference	-43^{f}	-34^{e}	-22^{d}	-11^{c}	4^{b}	-1^{ab}	1^{ab*}	1^{ab}	5^{a}	6^{a}
Relative IVOMD(%)	34^{a}	46	66	83	94	99	99	102	108	109
Prairie grass hay										
Units difference	-29^{d}	-21^{cd}	-12^{c}	-2b*	5^{ab}	9^{ab}	9^{ab}	12^{ab}	13^{ab}	14^{a}
Relative IVOMD(%)	51	64	80	97	109	115	115	120	122	124
Wheat straw hay										
Units difference	-34^{c}	-34^{bc}	-25^{b}	-16^{a}	-9^{a}	-5^{a}	-4^{a}	-3^{a*}	-3^{a*}	-2^{a*}
Relative IVOMD (%)	37	37	54	70	83	91	03	94	96	94

[a-f] Means within a row not having a common superscript differ at ($P<0.05$).
*Means within a row denoted by an asterisk do not differ from the mean OMD for a hay ($P\geq0.05$).
[2] Relative IVOMD = $\frac{(\text{IVOMD} \times 100)}{(\text{OMD})}$

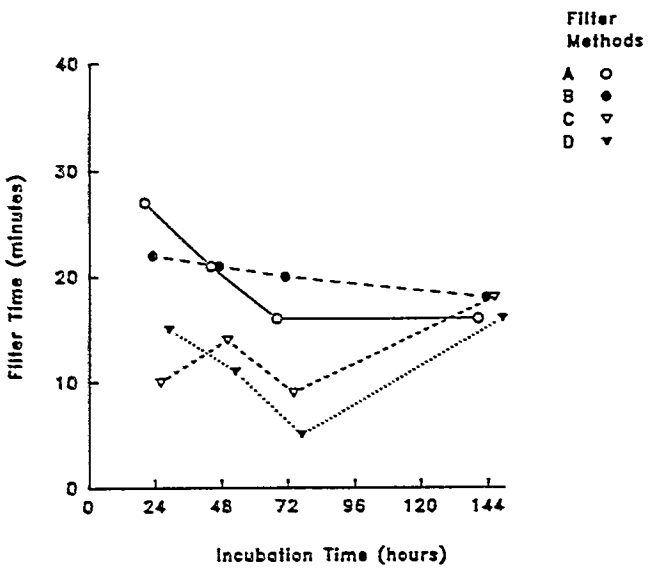

Fig. 2. Relationship between filter time (symbol) and incubation times for 4 filtering methods in Experiment II. A = No sulfite. B = sulfite + NDF. C = sulfite + rinse water. D = sulfite + NDF and rinse water.

pose that sulfite addition to rinse water did not change in-vitro digestibility values from no sulfite additions because of the reduced amount of time samples were exposed to sulfite. Apparently, sulfite in the rinse water was in contact with the sample a sufficient amount of time to break down the gelatinous starches and proteins in suspension, thus aiding filtration, yet short enough that sulfite did not cleave the lignin bonds of the undigested residue.

Filter method also had a significant effect on filter time (Table 3). No sulfite and sulfite + NDS had the slowest filter times but there were no difference between methods. Sulfite + rinse water and sulfite + NDS + rinse water were faster than sulfite and sulfite + NDS but not different from each other. This would suggest that the filtering clogging compounds that are broken down and removed by sulfite can be dissolved by adding sulfite to the rinse water only.

There was a significant filter method × incubation time interaction effect on filter time (Fig. 2). Mean filter times from 24 to 48 hour incubations decreased significantly for no sulfite and sulfite + NDS + rinse water, whereas in sulfite + rinse water filter time increased and sulfite + NDS exhibited no response. Except for the sulfite + NDS method, there was a significant decrease in filter time from 48 to 72 hours. No change in filter time relative to filter method at 144 hours. Absence of a filter method response beyond 72 hours may be attributed to the majority of cell solubles and more cell wall being digested or microbial accumulation limiting further in vitro fermentation.

Table 3.The effect of neutral detergent fiber extraction method on in-vitro organic matter disappearance (IVOMD) and filter time in Experiment II.

Treatment	IVOMD	Filter Time
	(%)	(minutes)
No Sulfite	58.3[c]	20.0[a]
Sulfite + NDS	59.9[a]	20.2[a]
Sulfite + rinse water	58.0[c]	10.3[b]
Sulfite + NDS and rinse water	59.2[b]	9.3[b]

[a-c] Means within a column not having a common superscript differ at ($P<0.05$).

Conclusions

We conclude that the use of a single incubation time does not give the best in-vitro estimation of the in-vivo digestibility. Consideration of the forage type being evaluated and preliminary forage quality analysis (CP, NDF, ADF) should allow one to select an incubation time that will closely estimate the proper OMD of that forage. However, the value of in-vitro estimates for more than relative comparisons of forages collected from a range of environmental and managerial conditions is suspect. If in-vitro techniques are to be utilized, we recommend the use of forages or hays with known in-vivo digestibilities to adjust for under or over-estimation.

Sulfite added to NDF rinse water offers an alternative to the problem of safely separating animal keratin and residual carbohydrates from lignin as stated by Robertson and Van Soest (1981). Use of this technique optimizes filter time relative to indigestible neutral detergent fiber recovery and analytical accuracy.

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