Acta Scientiarum

http://www.uem.br/acta
ISSN printed: 1806-2563
ISSN on-line: 1807-8664
Doi: 10.4025/actascianimsci.v34i3.10577

# Indigestible cellulose and lignin in determining feces production and apparent digestibility in horses

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**ABSTRACT.** Four crossbred geldings were used in a randomized blocks experimental design. The objective was to study the use of the internal markers indigestible cellulose (iCEL) and indigestible lignin (iLIG), obtained *in situ* (cattle) or *in vivo* (equine) to predict nutrient apparent digestibility in horses. Treatments consisted of different methodologies to determine digestibility: direct method with total feces collection (TC), and indirect method using internal markers iCEL and iLIG obtained either by *in situ* incubation in bovine rumen or i*n vivo* (IV) using the mobile nylon bag (MNB) technique in horses. Feces production was 2.80 kg in DM, and average recovery rate ($p > 0.05$) was 101%. Nutrient digestibility coefficient ($p > 0.05$) estimates were adequately predicted by iCEL and iLIG, obtained *in situ* or *in vivo*, with average values of 52.63, 54.17, 64.90, 43.73 and 98.28% for dry matter, organic matter, crude protein, neutral detergent fiber and starch, respectively. It can be concluded that iCEL and iLIG may be obtained *in vivo* by MNB in horses consuming a forage-concentrate diet, to predict nutrient digestibility coefficients.

**Keywords:** horse, indirect method, internal marker, mobile nylon bag.

## Celulose e lignina indigestíveis na estimativa da produção fecal e digestibilidade aparente em equinos

**RESUMO.** Foram utilizados quatro cavalos castrados sem raça definida, distribuídos em blocos casualizados. Objetivou-se estudar a viabilização dos indicadores internos, celulose (CELi) e lignina indigestíveis (LIGi), para predizer a digestibilidade em cavalos. Os tratamentos consistiram na determinação da digestibilidade por método direto com a coleta total de fezes (CT) e indireto pelo uso CELi e LIGi obtidos pelas técnicas "in situ" (IS) na cavidade ruminal de bovinos e "in vivo" (IV) nos equinos por meio do saco de náilon móvel (SNM). A produção fecal e taxa de recuperação ($p > 0,05$) médios encontrados foram de 2,80 kg na MS e 101%, respectivamente. As estimativas dos CD dos nutrientes ($p > 0,05$) foram adequadamente preditos pela CELi e LIGi, obtidos "in situ" e "in vivo", no qual os valores médios observados foram de 52,63, 54,17, 64,90, 43,73 e 98,28% para MS, MO, PB, FDN e Amido, respectivamente. Concluiu-se que a CELi e LIGi podem ser obtidas "in vivo" por meio do SNM em equinos, para predizer os coeficientes de digestibilidade de nutrientes, consumindo dieta mista.

**Palavras-chave:** cavalo, método indireto, indicador interno e saco de náilon móvel.

## Introduction

The nutritional value of a diet item is defined by the animal's ability to ingest and digest it. Intake is a difficult measurement to obtain in grazing animals, as it is assessed by estimates of feces production and digestibility. When evaluating digestion using the direct method, there is a need to accurately control intake and daily feces production, both of which are painstakingly difficult to monitor in grazing animals, even when using metabolic rooms (FREITAS et al., 2002).

*In vitro* digestibility of mixed diets, based on forages and grains, shows wide-ranging results depending on associative effects (MEHREZ et al., 1983), intake level and roughage presentation – fresh, dry or ensiled (LIPPKE, 2002). These persistent errors resulting from the *in vitro* technique justify efforts to develop new procedures featuring greater precision and accuracy. Thus, the development of new indirect methods of digestibility using internal markers emerges as an alternative for that need.

Internal markers, used extensively in experimentation with equines, involve acid-insoluble ash or acid detergent-insoluble ash (ARAÚJO et al., 2000; BERGERO et al., 2004; CUDDEFORD; HUGHES, 1990; MIRAGLIA et al., 1999; OLIVEIRA et al., 2003; STEIN et al., 2006) and indigestible cell wall components, such as neutral and acid detergent fibers (ALVARENGA et al., 1997; OLIVEIRA et al., 1998; OLIVEIRA et al., 2003, STEIN et al., 2006).

Nevertheless, the use of lignin as an internal marker to predict digestibility in horses has received criticism from the scientific community regarding its efficacy for that task, given the low feces recovery rate it provides, around 80% (ARAÚJO et al., 2000; MIRAGLIA et al., 1999; OLIVEIRA et al., 2003). The justification for that fact is that lignin is partially digestible (PENNING; JOHNSON, 1983), ranging from 20 to 40% in young grasses (VAN SOEST, 1994), making it inefficient and limiting its use as a marker. Assuming the possibility of partial digestibility of lignin, its precision as an internal marker can be improved by removing its digestible fraction. Therefore, it is worthwhile to investigate the capacity of the remaining fraction, named indigestible lignin (iLIG), to carry out the pertinent estimates for indigestible markers.

Indigestible cellulose (iCEL) is one of the most studied methods in equines, capable of consistently predicting the digestion of dry matter (DM) with *in vivo* methods (ALVARENGA et al., 1997; OLIVEIRA et al., 1998; OLIVEIRA et al., 2003, STEIN et al., 2006). In those trials, however, the methodology applied to obtain that marker is always *in vitro*, using rumen fluid as inoculant. Although usually precise, *in vitro* methods require more complex techniques and equipment, unlike indigestible residues obtained through *in situ* incubation of nylon bags in the ruminal cavity, which is a common procedure in cattle (BERCHIELLI et al., 2005; FREITAS et al., 2002; HUHTANEN et al., 1994; ÍTAVO et al., 2002a and b; LIPPKE et al., 1986).

Given the difficulty in performing surgical interventions in equines to reach the gastrointestinal tract, different methodologies have been developed or adapted to overcome these limitations. In that sense, we believe that, given adequate adaptations, the mobile nylon bag (MNB) used in that animal species especially to evaluate the digestibility of several diet items by the total digestive system (ARAÚJO et al., 1996; ARAÚJO et al., 2001; FOMBELLE et al., 2004; MACHEBOUEF et al., 1995) could make possible the collection of indigestible markers *in vivo*. As such, the feasibility of methods that can facilitate the acquisition of indigestible markers is essential to accelerate the evaluation process of the nutritional value of diet items in horse experiments.

In that context, the objective of the present work was to study the feasibility of indigestible markers cellulose and lignin, obtained through *in situ* techniques in cattle and *in vivo* techniques in equines by MNB, in predicting the nutrient apparent digestibility of horses fed coast cross hay plus corn grain, compared to total feces collection.

## Material and methods

The experiment was carried out at the Equine Metabolic Room belonging to the Animal Science Department of the Federal University of Lavras and at the Animal Nutrition Laboratory of the Animal Science Department, Esalq/USP. Four crossbred geldings (average age 6 years, body weight [BW] 340 kg) were used in a randomized blocks design containing four replications; each horse was used to originate the data on total feces collection and *in vivo* techniques using MNB. As such, each animal was regarded as a block, totaling four replications. The treatments consisted of different digestibility evaluation methodologies, consisting of a direct method with total feces collection (TC) and an indirect method using internal markers cellulose (iCEL) and indigestible lignin (iLIG), obtained through *in situ* (IS) techniques in cattle and *in vivo* (IV) techniques in equines using MNB, totaling five treatments.

The amount of feed given to the animals was determined according to the recommendations of the National Research Council (NRC, 1989), in order to meet nutritional requirements for the category. Daily DM was 2.0% BW, consisting of 70% coast cross hay and 30% corn grain, with mineral salt provided *ad libitum*. Meals were provided daily at 8 am, 12 pm and 5 pm, and leftovers were removed and weighed 15 minutes before each meal. The chemical compositions of the hay and corn can be found in Table 1.

**Table 1.** Chemical composition of diet items[1].

| Item | DM (%) | Nutrient (% DM) | | | | |
|---|---|---|---|---|---|---|
| | | OM | Starch | CP | NDF | ADF |
| Coast cross hay | 91.20 | 95.21 | 2.59 | 7.47 | 80.70 | 42.79 |
| Corn grain | 88.25 | 98.45 | 63.59 | 8.99 | 20.25 | 5.34 |

[1]Data obtained at the Animal Nutrition Laboratory of FMVZ/Unesp and Bromatology Laboratory of Esalq/USP.

The trial lasted for a total of 32 days, divided into two experimental phases, with 20 days for phase 1 and 12 days for phase 2. During phase 1 of the trial, used to determine digestibility by the direct method,

the first 15 days were used to acclimate the animals to the facilities, diets and handling conditions. The horses were kept in individual 2 x 3 m stalls, featuring bare cement flooring, with a feeding trough for feed and salt, and a drinker. After that period, the horses were placed in metabolism cages, featuring plastic buckets for water and mineral salt, a front feeding trough and feces collector, in which total collection was performed for five days. Feces collections was carried out four times a day (6 am, 12 pm, 6 pm and 12 am), weighed, homogenized and sampled at 10%, placed in labeled plastic bags and stored at -15°C, for later analyses. Before the start of the experiment, a broad spectrum vermifuge was given to the horses.

At the end of phase 1, the feces samples were thawed at room temperature, homogenized by treatment, to obtain a compound sample for each animal, of which 10% fractions were removed, weighed and pre-dried in a forced-air oven at 60ºC for 72h. After drying, samples were weighed again and then milled using a 1 mm screen. Bromatological analyses (DM, OM and CP) of the hay, corn and feces were carried out according to the methodology described by Silva (1989), and cell wall components (NDF and ADF) according to Van Soest et al. (1991), at the Animal Nutrition Laboratory of the School of Veterinary Medicine and Animal Science – Unesp – Botucatu campus, São Paulo State. Starch was determined according to Macrae and Armstrong (1968), at the Bromatology Laboratory belonging to the Animal Science Department of Esalq-USP. Phase II of the experiment was conducted to measure the indirect methodologies, with six days for each of the IS and IV techniques. To obtain the indigestible markers in cattle by the *in situ* method, nylon bags were used measuring 7 x 14 cm with 60 mm diameter, containing 7 g of sample milled at 1 mm to maintain the density of 10 to 20 mg DM of sample per $cm^2$ of bag surface (NOCEK, 1988). Samples of hay, corn and feces (for each horse of the previous phase) containing, respectively, six, 12 and four replications, were incubated in the rumen of a Holstein cow fistulated in the rumen for 144h, fed coast cross hay and mineral salt.

*In vivo* indigestible markers were determined in equines using the MNB technique. That phase of the experiment was carried out in conjunction with the digestibility trial by total feces collection, using the same horses stabled in masonry stalls and consuming coast cross hay, as previously described. White polyester nylon bags were used, 3.5 x 6.5 cm inner size, 60 microns porosity, prepared as described by Araújo et al. (1996). Inside each nylon bag were placed 1 g of 1-mm milled sample of hay, corn and feces from the direct method, maintaining the rate of 10 to 20 mg of dry matter of sample per $cm^2$ of bag surface, as recommended by Nocek (1988).

The insertion of the nylon bags was made in four horses by nasogastric tube, every other day during six days – one day with intubation and resting the next, until reaching 144h. At 1 pm of the first day of nasogastric tube insertion, 4, 5 and 16 bags were inserted per horse, containing, respectively, hay, corn and horse feces (four from each horse), totaling 25 bags/horse, all labeled. The number of replications for hay and feces was 16 bags each, and 20 bags for corn. The nylon bags were collected in feces four times a day, at 6 am, 12 pm, 6 pm and 12 am. They were then identified in a spreadsheet and immediately frozen at -15°C until the next intubation (reintubation). At the appropriate time, the bags were thawed at room temperature and pre-dried in an oven at 55ºC, to be then intubated again. Therefore, the nylon bags recovered in the feces were reintubated twice, by nasogastric tube, until totaling at least 144h of incubation, considering that the average passage time of nylon bags through the digestive tract of horses is 48h.

Following in situ or in vivo incubations, the bags were cleaned in a washing machine with running water until the water came out clear (40 minutes) and then taken to the forced-air oven during 72h at 60°C. Next, the nylon bags containing the digestion residues of hay, corn and feces, per animal and for each methodology, were opened forming a compound sample. The digestion residues were submitted to extraction by acid detergent, resulting in lignocellulose; the remaining fraction underwent cellulose solubilization by sulfuric acid at 72% (VAN SOEST et al., 1991), thereby obtaining markers iCEL and iLIG.

The feces production (FP) and digestibility coefficient (DC) of DM were estimated based on the equations proposed by Church (1993):

$$\text{FP in DM}\left(\text{g day}^{-1}\right)=\frac{\text{Marker Ingested}\left(\text{g d}^{-1}\right)}{\text{Marker Concentration in Feces}\left(\text{g g}^{-1}\text{ DM}\right)}$$

$$\text{DCDM (\%)}=100-\left(100\text{ X}\frac{\text{\% Marker in Diet}}{\text{\% Marker in Feces}}\right)$$

$$\text{DCN (\%)}=100-\left(100\text{ X}\frac{\text{\% Marker in Diet}}{\text{\% Marker in Feces}}\text{ x }\frac{\text{\% Nutrient in Feces}}{\text{\% Nutrient in Diet}}\right)$$

The recovery rate (RR) of the markers was assessed according to Krysl et al. (1988) through the following equation:

$$RR\ (\%) = \frac{\%\ Marker\ in\ Feces\ x\ FPobserved\ (g)}{Marker\ Ingested\ (g)}\ x\ 100$$

Feces production and the digestibility coefficients of the nutrients were subjected to analysis of variance using the Statistical Analysis System (SAS, 2000), according to the following model: $Yij = \mu + B_i + Tj + Eij$; in which Yij = feces production and apparent digestibility coefficient of the nutrients from horse *i* receiving treatments *j*; $\mu$ = overall constant; $B_i$ = effect of horse *i*, with *i* = 1,2,3,4; Tj = effect of treatment *j*, with *j* = 1,2,3,4,5; Eij = random error associated with each observation Yij.

Means were compared using Tukey's test, at 5% significance.

## Results and discussion

The concentration of indigestible markers present in the mixed diet, consisting of 70% hay plus 30% corn grain, showed significant variations according to collection methodology – *in situ* in cattle versus *in vivo* in horses – demonstrating the need for greater standardization of these techniques used in equine experiments (Table 2). iCEL levels of 6.77% *in situ* and 9.28% *in vivo* were intermediate to the values of 5.66% (OLIVEIRA et al., 2003) and 11.58% (STEIN et al., 2006) for horses consuming a 60:40 roughage:concentrate diet, determined by *in vitro* incubation using rumen fluid.

Despite the differences between the methodologies in assessing indigestible markers, the observed concentrations of iCEL and iLIG in the current study were adequate in enabling similar recovery rates to the TC group ($p > 0.05$). This response is interesting for identifying a good marker, as it demonstrates its ability to resist digestion during exposure in the gastrointestinal tract (FAICHNEY, 1975 apud COCHRAN et al., 1986). Thus, the relative indigestibility of markers iCEL and iLIG studied for horses ingesting a mixed diet gives these markers great ability to accurately predict digestibility.

Literature reports highlight the failure of lignin (LIG) as an indigestible marker in digestibility trials, given its apparent digestion and lack of a chemically defined structure (MUNTIFERING, 1982), resulting in low feces recovery rates ranging between 75 and 81% (ARAÚJO et al., 2000; KRYSL et al., 1988; OLIVEIRA et al., 2003). In an attempt to overcome the limitation regarding the disappearance of LIG during gastrointestinal transit, the present study proposed prior incubation in a microbial environment, and its chemical assessment, thereby obtaining iLIG. Thus, the levels of 3.77 and 4.79% for iLIG *in vivo* and *in situ*, respectively, were satisfactory, as confirmed by their high feces recovery rates.

**Table 2.** Concentration (% in DM), intake (g $d^{-1}$) and recovery rate (%) of cellulose (iCEL) and indigestible lignin (iLIG), under different methodologies, in horse diets consisting of coast cross hay plus corn grain.

| Variable | Methodology | | | | | CV (%) |
|---|---|---|---|---|---|---|
| | TC[1] | *In situ* | | *In vivo* | | |
| | | iCEL | iLIG | iCEL | iLIG | |
| Concentration | - | 6.77[b] | 3.77[d] | 9.28[a] | 4.79[c] | 1.61 |
| Intake | - | 39.93[b] | 22.23[c] | 54.63[a] | 28.63[d] | 3.22 |
| Recovery rate | 100 | 103.54[a] | 93.24[a] | 102.86[a] | 105.24[a] | 9.33 |

[1]TC = Total feces collection; Means with different letters in the same row differ ($p < 0.05$) by Tukey's test.

Estimates of feces production, presented in Table 3, were adequately predicted by iCEL and iLIG regardless of technique used, resulting in an average of 2.79 kg of feces in DM.

**Table 3.** Observed and estimated values for feces production expressed in kg (DM), % body weight (BW) and g $kg^{-1}$ of metabolic weight (MW) of horses fed coast cross hay plus corn grain under different methodologies.

| Feces production | Methodology | | | | | CV (%) |
|---|---|---|---|---|---|---|
| | TC[1] | *In situ* | | *In vivo* | | |
| | *TC* | iCEL[2] *iCEL* | iLIG[3] *iLIG* | iCEL *iCEL* | iLIG *iLIG* | |
| kg | 2.80 | 2.71 | 3.04 | 2.73 | 2.68 | 9.23 |
| % BW | 0.83 | 0.80 | 0.90 | 0.81 | 0.80 | 8.72 |
| g $kg^{-1}$ MW | 35.49 | 34.44 | 38.51 | 34.74 | 34.14 | 8.85 |

[1]TC = Total feces collection; [2]iCEL = Indigestible cellulose; [3]iLIG = Indigestible lignin; Means with different letters in the same row differ ($p < 0.05$) by Tukey's test.

The apparent digestibility coefficients of coast cross hay plus corn grain in horse diets, obtained with indigestible markers using *in situ* and *in vivo* methodologies, are shown in Table 4.

**Table 4.** Apparent digestibility coefficients (DC) of dry matter (DM), organic matter (OM), crude protein (CP), neutral detergent fiber (NDF) and starch, obtained using different methodologies for horses fed coast cross hay plus corn grain (% in DM).

| Variable | Methodology | | | | | CV (%) |
|---|---|---|---|---|---|---|
| | TC[1] | *In situ* | | *In vivo* | | |
| | | iCEL[2] | iLIG[3] | iCEL | iLIG | |
| DCDM | 52.63 | 54.04 | 48.62 | 53.58 | 55.14 | 7.90 |
| DCOM | 54.17 | 55.96 | 50.79 | 55.51 | 57.04 | 7.23 |
| DCCP | 64.90 | 65.94 | 61.99 | 65.58 | 66.81 | 4.69 |
| DCNDF | 43.73 | 45.50 | 38.97 | 46.86 | 44.90 | 11.45 |
| DCStarch | 98.28 | 98.35 | 98.18 | 98.33 | 98.40 | 0.13 |

[1]TC = Total feces collection; [2]iCEL = Indigestible cellulose; [3]iLIG = Indigestible lignin; Means with different letters in the same row differ ($p < 0.05$) by Tukey's test.

The DCs of DM, OM, CP, NDF and starch determined by TC were similar ($p > 0.05$) to those estimated by iCEL and iLIG in both techniques. Likewise, Oliveira et al. (2003) observed that iCEL was the best marker to estimate the DCs of DM, CP, NDF and GE for the same animal species fed a mixed diet containing up to 80% coast cross hay. Thus, they concluded that iCEL proved to be the most adequate internal marker in estimating nutrient apparent digestibility in horse diets.

In several studies with ruminants (COCHRAN et al., 1986; KRYSL et al., 1988; HUHTANEN et al., 1994) and equines (ARAÚJO et al., 2000; MIRAGLIA et al., 1999; OLIVEIRA et al., 1998; OLIVEIRA et al., 2003), LIG was rejected as an indigestible marker, for underestimating the DCs of nutrients. The efficiency of iLIG in estimating nutrient DCs in the present trial indicates that only the indigestible fraction of lignin should be used as a marker in digestibility trials. It is also worth mentioning that using MNB to obtain indigestible markers in equines shows promise in estimating feces production, as well as in the process of evaluating the nutritional value of diet items in horse experiments.

## Conclusion

The *in vivo* method in equines, using MNB, can be used to obtain indigestible markers, making it a promising technique in experiments with that animal species.

The iCEL and iLIG obtained *in vivo* and *in situ* were efficient in estimating feces production and digestibility coefficients of DM, OM, CP, NDF and GE for horses fed exclusively the mixed diets.

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*Received on July 2, 2010.*
*Accepted on March 22, 2011.*