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Fermentation kinetics of carbohydrate fractions of maize grains as determined by *in vitro* gas production curve subtraction technique*

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ABSTRACT

The objectives of this study were to study the fermentation kinetics of different carbohydrate fractions of maize grains of Chinese varieties based on *in vitro* gas production curve subtraction technique. Ten maize grain samples were extracted with either 80% ethanol or neutral detergent to obtain ethanol-insoluble residue (EIR) and isolated neutral detergent fibre (NDF). Then unfractionated maize grain (UCG), EIR and NDF were fermented *in vitro* and the gas production was recorded. Because fermentation characteristics of fraction A (sugars and organic acids) and B1 (starch and soluble fibre) were not directly measured, a curve subtraction technique was used to evaluate the gas production and fermentation of these soluble fractions. The results showed: 1. the proportion of different carbohydrate fractions averaged 10.3±1.1, 78.3±1.2, 11.8±0.6% and 0.6±0.2% on DM base for A, B1, B2 (digestible fibre) and C (indigestible residue) fractions, respectively; 2. there were high correlation coefficients among gas production of UCG, the A, B1 and B2 fractions; 3. there was no significant difference between observed kinetics parameters and those predicted by the Gompertz function for the A and/or B1 fractions; and 4. the A fraction had the significantly highest acetate to propionate ratio among fractions studied while the B2 fraction the lowest. In conclusion,

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the *in vitro* gas production curve subtraction technique was approved as a feasible approach to estimate fermentation kinetics of carbohydrate fractions of maize grains.

KEY WORDS: fermentation kinetics, carbohydrate fractions, curve subtraction technique, *in vitro*, maize grain

INTRODUCTION

With the widespread application of the Cornell Net Carbohydrate and Protein System (CNCPS) model, there is an increasing interest of CNCPS use in China. In the CNCPS model, carbohydrate is divided into four fractions: A - sugars and organic acids, B1 - starch and soluble fibre, B2 - digestible fibre and C - indigestible residue (Sniffen et al., 1992; Chen et al., 1999). In the case of experiment, the carbohydrate of maize grains could be fractionated into different pool size, including ethanol-insoluble residue (EIR) and NDF, and then the digestion kinetics of individual fractions can be determined by an *in vitro* gas production method (Menke and Steingass, 1988).

Maize is a second cereal crop source in China agricultural industry and provides a major energy feed ingredient for ruminant feeding. The digestion kinetics of feed or dietary DM and NDF may be determined by standard *in vitro* or *in situ* methods (Ørskov and McDonald, 1979). However, such methods generally cannot be directly utilized to evaluate the fermentation kinetics of the soluble fraction. Therefore, an indirect method-gas curve subtraction method has been developed (Schofield and Pell, 1995; Calabrò et al., 2001). This method is very important in determining fermentation kinetics for the application of CNCPS. However little has been studied on fermentation kinetics of different carbohydrate fractions of cereal concentrates, and limited information has been available on the VFA profiles after fermentation of various carbohydrate fractions of maize grains used as a ruminant feed. In China, although some researches have been conducted on the determination of fermentation characteristics of forage carbohydrate fractions (Zhao et al., 1994; Liu et al., 2002), there has been less research available on the determination of fermentation kinetics of cereal grains used for CNCPS application in ruminant feeding.

The objectives of the present paper were to partition carbohydrate fractions of maize grains of Chinese varieties, to determine the fermentation kinetics of their carbohydrate fractions by *in vitro* gas production curve subtraction technique.

MATERIAL AND METHODS

Sample preparation and carbohydrate fractionation schemes

Samples of ten different maize varieties (harvested at maturity stage) collected from different regions of China were used in this study. The sample set was selected to provide samples that varied in fibre content according to a laboratory scale wet-milling procedure (Eckhoff et al., 1996). The mean content of fibre obtained using this wet-milling method was 11.4%, with a range from 9.5 to 13.3%. All of these samples were ground to pass a 1-mm screen and saved for analysis. For each sample, the DM concentration of maize grains was determined by oven-drying at 105°C (AOAC, 1990). The content of the A fraction was determined by difference between unfractionated maize grain (UCG) and EIR, while the B1 fraction between EIR and NDF (NDF was the sum of the B2 and C fraction). Analogically, to obtain the gas production and fermentation kinetics of the A fraction, the gas production from the EIR fermentation was subtracted from that of UCG at scheduled time point. With regard to the B1 fraction, the similar approach was used by subtracting the isolated NDF gas production from the corresponding EIR gas production. The gas produced from the NDF residue represents that from the B2 fraction assuming that the C fraction cannot be fermented and be utilized by microorganisms.

Ethanol-insoluble residue analysis and preparation

About 0.5 g of maize grain samples of each variety were loaded into tared fibre bags and sealed, respectively. Then the bags were dipped in 80% ethanol (v/v) at room temperature with continuously stirring with about one rotation per sec for 4 h. After that, the bags were rinsed three times with 80% ethanol and one with acetone. The EIR was dried in a 100°C oven, and its percentage of the UCG was calculated. The UCG and their EIR were analysed for crude protein (CP), ether extract and ash according to Chen et al. (1999).

The ethanol extraction method described above was used to prepare EIR samples for fermentation except that the bags were dried at 60°C. The residues were saved for fermentation kinetics (Chen et al., 1999).

Neutral detergent fibre analysis and preparation

To avoid starch contamination of the fibre residue, a modified NDF procedure (Van Soest et al., 1991) was used in this study. Also for each of the ten varieties, about 0.5 g of maize grain samples were loaded separately into tared fibre bags and sealed. Then the bags were dipped in 100 ml neutral detergent (ND) plus

0.1 ml of heat-stable α-amylase and 0.5 g of sodium sulphite. After boiling the samples for 1 h, washed them with hot distilled water (several times until no foam was observed), ethanol (three times) and acetone (for the last time), dried in a 100°C oven and the NDF percentage of the maize grain was calculated (Pell and Schofield, 1993; Calabrò et al., 2005).

To obtain the NDF residue for fermentation, the same procedure was used except that the bags were dried in a 60°C oven overnight and the residues of NDF were saved for subsequent fermentation (Pell and Schofield, 1993; Chen et al., 1999).

In vitro gas production

All treatments involving animals were conducted under approval of the China Agricultural University Institutional Animal Care and Use Committee.

The *in vitro* incubation for gas production adopted the procedure described by Menke and Steingass (1988). Three cannulated mature Simmental×Luxi yellow cattle (27 months age, 600 ± 40 kg average body weight) were fed *ad libitum* a total mixed ration of concentrate (40% of DM), maize silage (35% of DM) and lucerne hay (25% of DM) twice daily for 8 d before rumen fluid collection. Rumen fluid, collected 2 h after morning feeding, was filtered through four layers of gauze into a bottle quickly. All laboratory handling of rumen fluid was carried out under a continuous flow of CO_2.

UCG, EIR and NDF of each maize grain sample were accurately weighed (150±10 mg) into 100 ml glass syringes fitted with plungers. *In vitro* incubations were conducted in two consecutive runs and each involving triplicates of samples. Syringes were filled with 30 ml medium consisting of 10 ml rumen fluid and 20 ml buffer solution as described by Menke and Steingass (1988). Three blanks containing 30 ml medium only were included in each assay as control. Then the syringes were incubated in thermostat incubator (39°C) and the gas production was recorded at 1, 2, 3, 4, 5, 6, 8, 10, 12, 16, 20, 24, 28, 32, 36, 40, 44 and 48 h of incubation. Gas production was not recorded after 48 h because of complete fermentation of the diet according to Wallace et al. (2001) and González-García et al. (2008). At the end of fermentation (48 h), the culture fluid (30 ml) of each syringe was carefully removed into centrifuge tubes (20 ml) and pH values were immediately measured. The remaining fluid (1 ml) was acidified with 25% metaphosphoric acid (200 μl) containing 2-ethyel butyric acid as an internal standard, and then frozen at -20°C. Upon thawing, the fluid sample was centrifuged at 10000 *g* for 15 min to remove any particulate matter, and the supernate was used for VFA analysis.

Volatile fatty acid analysis

The supernatant samples (0.6-μl portion) were analysed for acetic, propionic, isobutyric, butyric, isovaleric and valeric acids according to the method of Li and Meng (2006) by gas chromatography (Agilent 6890N) with a 30-m HP-INNOWax 19091N-213 (Agilent) capillary column (0.32 mm i.d. and 0.50 mm film thickness) in split mode (ratio, 1:100). Nitrogen was used as carrier gas with a flow rate of 2.0 ml/min and 2-ethylbutyric acid was used as an internal standard. The chromatograph oven was programmed as follows: 120°C for 3 min, 10°C min increment to 180°C, and then was held for 1 min. The injector port and FID detector were maintained at 220 and 250°C, respectively.

Curve subtraction and calculation of kinetics parameters

The curve subtraction technique was utilized to obtain the kinetics parameters of all these carbohydrate fractions fermented *in vitro*. The gas production of UCG, its respective EIR and NDF fractions were adjusted to represent the amount of each fraction present in the initial UCG sample (Calabrò et al., 2005).

To describe the dynamics of gas production over time, the Gompertz function of:

$$V = V_F \exp \{-\exp [1+\frac{\mu_m^e}{V_F} (\lambda - t)] \}$$

(Schofield et al., 1994) was chosen to calculate fermentation parameters. In the present study, some different letters were used according to Liu et al. (2002). So the equation is as following:

$$Y = B \exp \{-\exp [1+\frac{C^e}{B} (LAG-t)] \}$$

where: Y - cumulative gas volume of incubated substrate (adjusted to respective fraction of 100 mg UCG), B - the theoretical maximum of gas production (at $t=\infty$), C - the maximum rate of gas production ($ml \cdot h^{-1}$) that occurs at the point of inflection of the curse, and LAG - the lag time of fermentation (h).

Curve fitting and statistical analysis

The criteria used to judge the fitness of a given model were the fit statistic (F-values, mean square of regression/mean square error) (Schofied et al., 1994; Stefanon et al., 1996). A larger F-value indicates a better fit of the gas data to the mathematical model (Chen et al., 1999). The observed parameters of gas production were obtained by the subtraction approach as mentioned above. The

corresponding average parameters of the A fraction was obtained by the values on the curve of UCG gas production minus those on the curve of the EIR fraction gas production. The similar approach was used to obtain the average parameters of the B1 fraction calculated as the values on the curve of EIR gas production minus those on the curve of NDF gas production.

The observed and average kinetics parameters of the A or B1 fractions were compared using a paired t-test. The INSIGHT MODULE of SAS Version 8.0 was used to analyse the correlation coefficient of gas produced from different fractions. The Gompertz function was used to obtain dynamic fermentation parameters and the NON-LINEAR procedure of SAS V 8.0 was used to analyse these parameters. The one-ANOVA procedure was used to analyse the acetate to propionate (A: P) ratio and pH value of the incubated end products.

RESULTS AND DISCUSSION

Carbohydrate fractions and gas production. The amounts of the various fractions of maize grain are presented in Table 1. By the difference of UCG and EIR, the percentage of the A fraction (DM %) was calculated. The content of the A fraction averaged 10.3 ± 1.1%, which was different from the result of 7.1% as reported by Chen et al. (1999). That might be due to the different varieties of maize grain used in the experiment. However, both of the data overestimated the fraction A content, since ethanol extraction could also remove ether extract, some crude protein, a trace amount of ash as well as the A fraction. Owing to this chemical heterogeneity and solubility, it was impossible to get the gas production of the A fraction directly. So as proposed by some authors (Pell et al., 1997; Chen et al., 1999; Calabrò et al., 2005), fermentations of the 80% ethanol-soluble fraction corresponds to the A fraction and may be estimated approximately by subtracting the gas production of the EIR from that of UCG.

As mentioned above, the gas production subtraction technique was used to obtain the gas production data of the A and B1 fractions (A= UCG - EIR; B1= EIR - NDF). The gas production curves from the UCG, A, B1, and B2 fractions (NDF gas production), normalized to the amount of the fraction studied contained in 100 mg DM, are shown in Figure 1. The accumulated gas volume of UCG, the A, B1, and B2 fractions after 48 h fermentation were 38.95, 7.20, 27.86 and 3.56 ml/100 mg DM, respectively, which were in agreement with the previously researches (Opatpatanakit et al., 1994; Chen et al., 1999). The difference of gas production between UCG and EIR changed little after 6 h incubation (from 5.99 to 6.03 ml/100 mg DM) indicated the fermentation of the A fraction ended by 6 h.

Table 1. Chemical analysis and carbohydrate fractions of maize grain

Sample	Chemical analysis		Carbohydrate fraction, % DM				NDFD[1]
	DM, %	NDF	A[2]	B1[3]	B2[4]	C[5]	%
1	88.2	12.9	11.3	75.8	12.1	0.8	93.6
2	87.8	10.5	10.1	79.4	9.7	0.8	92.4
3	89.3	12.2	11.1	76.7	11.7	0.5	95.8
4	88.5	11.2	10.6	78.2	10.9	0.3	97.3
5	87.7	9.7	9.3	81.0	9.3	0.4	96.2
6	88.4	11.7	9.6	78.7	10.6	1.1	95.7
7	88.8	9.8	10.7	79.5	9.3	0.5	94.8
8	89.1	13.3	9.8	76.9	12.8	0.5	96.5
9	87.8	13.0	9.9	77.1	12.1	0.9	93.2
10	87.5	9.5	10.8	79.7	9.0	0.6	94.4
Mean	88.3	11.4	10.3	78.3	10.8	0.6	95.0
SD	0.6	0.6	1.1	1.2	0.6	0.2	1.3

[1] NDFD - neutral detergent fibre digestibility. It was calculated from the NDF disappearance during a 48 h fermentation of isolated NDF, and the residue was assayed for NDF after fermentation; [2] the A fraction - sugar and organic acid, equal to 100 - ethanol insoluble residue (EIR); [3] the B1 fraction - starch and soluble fibre, equal to EIR - NDF; [4] the B2 fraction - digestible fibre, equal to NDF×NDFD; [5] the fraction C - indigestible fibre, equal to NDF×(100-NDFD)/100

The content of the B1 fraction was 78.3 ± 1.2%. As expected, the B1 fraction, which mainly consisted of starch and a little soluble fibre, accounted for the largest proportion of carbohydrate fraction of maize grain. So it contributed the most to the total gas production (Figure 1).

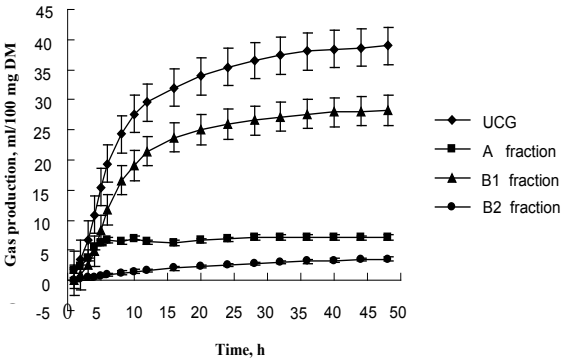

Figure 1. Gas production of unfractionated maize grain, A, B1 and B2 fractions. UCG - unfractionated maize grain, A fraction - sugars and organic acids, B1 fraction - starch and soluble fibre, B2 fraction - digestible fibre. Error bars represent ± 1 SD

The NDF fraction, including the B2 and C fractions, was 11.4±0.6%. Odle and Schaefer (1987) and Herrera-Saldana et al. (1990) declared that the NDF

content of maize grain was 10.8 and 9.3%, respectively. The NRC (1996) value was 10.8±3.6%. The slight discrepancy of NDF content in these studies might be resulted from different varieties, environmental factors or incomplete removal of soluble fractions during NDF analysis. We incubated the NDF to obtain the gas production of the B2 fraction as Chen et al. (1999) reported because of the C fraction was very little and assuming it cannot be fermented or utilized by microorganisms (Pozdíšek and Vaculová, 2008). The gas production curve of the B2 fraction came directly from NDF gas data and took the least contribution to total gas production (Figure 1).

Correlation analysis of gas produced from carbohydrate fractions. Little is known about the correlation of gas produced from different carbohydrate fractions of maize grain fermented *in viv*o or *in vitro*. Schofield and Pell (1995) reported that the gas produced by digestion of NDF is linearly related to the mass of fibre digested. Awati et al. (2006) reported that the cumulative gas produced at different time points showed a positive correlation with incubation time using the faecal inoculum of suckling piglets. In this study, the correlation coefficients among the gas production from different carbohydrate fractions were very high (Table 2). It should be mentioned that as the fermentation procedure of the A fraction finished rapidly (within 6 h), the correlation coefficients of the A fraction and the others parameters was presented at 6 h point while the correlation coefficients among other parameters were shown at 48 h point.

Table 2. Correlation coefficients among gas productions of UCG and its carbohydrate fractions

Item	A fraction [a]	B1 fraction	B2 fraction
UCG	0.989	0.988	0.955
A fraction		0.982	0.991
B1 fraction			0.977

UCG - unfractionated maize grain, A fraction - sugar and organic acid, equal to 100 - ethanol insoluble residue (EIR), B1 fraction- starch and soluble fibre, equal to EIR - neutral detergent fibre (NDF), B2 fraction - the digestible part of NDF, a - correlation coefficients among the A fraction and other parameters are the gas production values within the initial 6 h fermentation, while those among others are values at 48 h fermentation

Digestion kinetics parameters. The average and observed gas production were fitted to the Gompertz function to obtain kinetics parameters. The observed and average kinetics parameters of the A or B1 fractions were compared for model verification. There was no difference between the parameters obtained using the observed or average gas production curves of the A or B1 fraction (Table 3). This demonstrated that the exponential model (Gompertz function) was suitable for this study. Subsequently, the kinetics parameters of maize grain and its carbohydrate fractions were estimated (Table 4).

Approximately, the gas production of UCG was equal to the sum of the gas produced by the A, B1, and B2 fractions, which showed the division of maize grain into the three fractions was reasonable. The highest rate of gas production (0.33 h^{-1}) and the least lag time (0.17 h) of A fraction suggested that it could be fermented quickly, which is in agreement with that reported by Calabrò et al. (2005). However, Doane et al. (1998) argued that the fermentation rate of the A fraction was lower than the B1 fraction. The reason might be due to two aspects: one is different experimental condition; the other one might be related to the different mathematical model used for gas production analysis. The fermentation rate of the B2 fraction was lower than that of the A or B1 fractions. The order of fermentation rates of three fraction is in accordance with the previous report of CNCPS version 5.0 (0.05, 1.5 and 0.18/h for NDF, A, and B1 fractions, respectively; Fox et al., 2003), while the value had large differences. The discrepancy might be attributed to different variety, maturity stage of maize grain or different chemical extraction method employed in the studies.

Table 3. Comparison of fermentation parameters for the A and B1 fractions using observed or average data

Parameters	A fraction		SE[3]	P-value
	observed[1]	average[2]		
B, ml	6.99	6.99	0.056	0.950
C, h^{-1}	0.33	0.33	0.004	0.935
LAG, h	0.166	0.166	0.002	0.709
	B1 fraction		SE	P-value
	observed[4]	average		
B, ml	28.69	28.69	0.045	0.919
C, h^{-1}	0.11	0.11	0.002	0.567
LAG, h	1.09	1.09	0.002	0.342

[1] the observed gas production was computed by subtracting the actual gas production of the EIR fraction from that of the UCG; [2] the average gas production was estimated by subtracting the average gas production of the EIR fraction from that of the UCG; [3] SE, standard error; [4] the observed gas production was computed by subtracting the actual gas production of the NDF fraction from that of the EIR; [5] the average gas production was estimated by subtracting the average gas production of the NDF fraction from that of the EIR, B - the theoretical maximum of gas production, ml/100 mg maize grain DM, C - the rate of gas production, h^{-1}, LAG - the lag time (h)

Table 4. Kinetics parameters of *in vitro* gas production

Parameters	UCG	A fraction	B1 fraction	B2 fraction
B, ml	38.66 ± 3.15	6.99 ± 0.78	29.38 ± 5.12	3.913 ± 0.64
C, h^{-1}	0.12 ± 0.018	0.33 ± 0.016	0.08 ± 0.027	0.05 ± 0.007
LAG, h	0.61 ± 0.052	0.17 ± 0.012	1.35 ± 0.219	0.255 ± 0.046

UCG - unfractionated maize grain; B - the theoretical maximum of gas production, ml/100 mg maize grain DM; C- the rate of gas production; h^{-1}, LAG - the lag time (h)

Volatile fatty acid production and pH. The VFA profiles and pH of the fermented end-products were analysed at the end of fermentation (Table 5). We may use the fermentation balance equation of Wolin (1960) to compare the production of gas and VFA from glucose:

$$57.5 \text{ glucose} \rightarrow 100 \text{ VFA} + 60 \text{ CO}_2 + 35 \text{ CH}_4$$

where: VFA represents a mixture of acetic, propionic, and butyric acids (Schofield and Pell, 1995).

Table 5. VFA profiles (mmol l^{-1}100 mg^{-1} DM of maize grain) and pH values of different fractions after 48 h fermentation

Item	UCG [1]	A fraction[2]	B1 fraction[3]	B2 fraction[4]
TVFA	42.95 ± 5.09^{d}	5.81 ± 1.03	33.05 ± 4.34	4.07 ± 0.89
Acetate	25.11 ± 3.75	3.56 ± 0.64	19.12 ± 3.39	2.43 ± 0.53
Propionate	8.82 ± 1.4	1.21 ± 0.25	6.65 ± 0.77	0.96 ± 0.12
Isobutyrate	1.28 ± 0.34	0.04 ± 0.02	1.15 ± 0.26	0.09 ± 0.06
Butyrate	4.15 ± 0.78	0.94 ± 0.05	2.97 ± 0.81	0.35 ± 0.06
Isovalerate	1.76 ± 0.44	0.06 ± 0.02	1.54 ± 0.34	0.16 ± 0.05
Valerate	1.83 ± 0.15	0.13 ± 0.06	1.62 ± 0.09	0.08 ± 0.02
A:P	2.85^{b}	2.94^{c}	2.88^{bc}	2.53^{a}
pH^{e}	6.50 ± 0.08			6.51 ± 0.14

UCG - unfractionated maize grain; TVFA - total volatile fatty acid; EIR - ethanol-insoluble residue; A fraction: sugar and organic acid; B1 fraction - starch and soluble fibre; B2 fraction - digestion fibre; [1] the VFA profiles of UCG and EIR were the measured data at the end of 48 h fermentation; [2] the VFA profiles of the A and B1 fractions were the differences between UCG and EIR, and EIR and NDF, respectively; [3] the VFA profiles of the B2 fraction was the measured data of NDF; [4] mean ± SD, A:P=acetate to propionate ration, the different letters in the same row differ significantly at $P<0.05$; e - pH value of B2 fraction was the pH of NDF fermentation end production

The acetate, propionate and butyrate were the three main components of total VFA among all of these fractions. Because of phosphate-bicarbonate buffering *in vitro*, 1 mol of VFA produces approximately 0.8 mol of gas (Beuvink and Spoelstra, 1992). In our study, a similar result of 0.9 mole of gas was obtained (38.66/42.95; Tables 4 and 5).

The A fraction had the significantly highest acetate to propionate (A:P) ratio and the B2 fraction the lowest among fractions studied. Generally, the A:P ratio was used to evaluate substrate-related fermentation differences (Schofield and Pell, 1995). Higher propionate is associated with lower gas production because the extra carbon atom in propionate would otherwise have appeared as CO_2 while acetate contributes to gas production (1 mol of acetate = 1 mol of CO_2; Wolin, 1960; Calabrò et al., 2005). There were many literatures studying the differences of VFA profiles and A:P ratio in the ruminal fluid fed different feeds (Schofield et al., 1994). However, few studies had been taken on the effect of a single fraction.

The results of this study gave alternative information on the VFA characteristics of single carbohydrate fractions after 48 h fermentation for the first time. This could be useful for analysis of fermentation characteristic of carbohydrate fractions.

There was no significant difference among the pH values of products at the end of fermentation. Generally, the pH of the *in vitro* fermentation was checked to detect if its buffer capacity was surpassed (González-García et al., 2008). The pH in syringes in this study was maintained in the range of 6.4 to 6.7 for the different fractions. As a result, no effects were expected as a direct consequence of changes in buffer capacity.

As mentioned above, gas production mostly comes from fermentation of carbohydrate fractions because no ash fermentation occurs, no gas is available from fat fermentation, and little gas is produced from protein fermentation (Pozdíšek and Vaculová, 2008). Gas production techniques have a good potential to predict rumen OM degradation, in particular by the provision of kinetic information, and could be widely used to evaluate rumen fermentation kinetics of feeds (Umucalilar et al., 2002; Pozdíšek1and Vaculová, 2008). Combined with the CNCPS carbohydrate fractionation scheme (Sniffen et al, 1992; Chen et al., 1999), an *in vitro* gas production technology could provide some useful information on fermentation kinetic of different carbohydrate fractions of Chinese maize grains.

CONCLUSIONS

The *in vitro* gas production curve subtraction technique provides a suitable tool to evaluate the fermentation kinetics of soluble carbohydrate fractions of maize grains. By using this approach, the information about contents of carbohydrate fractions of Chinese maize grains, cumulative gas production and rate and contribution to volatile fatty acids of different fractions can be provided for the Cornell Net Carbohydrate and Protein System model application in direction of ruminant feeding in China.

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