Repeatability and Additivity of In Vitro Digestion Method for Determining Reducing Sugar Release from Swine Feed Ingredients: Postprint

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Abstract

This experiment aimed to establish a method for determining reducing sugar release from pig feed ingredients after digestion simulated by a monogastric animal biomimetic digestive system, to provide a reference for evaluating the biological efficacy of feed nutrients. The linear relationship between reducing sugar release and feed loading amount was studied using a corn-soybean meal type diet (75% corn + 25% soybean meal) as the test material, with five treatments of loading amounts at 0.2, 0.4, 0.6, 0.8, and 1.0 g, four replicates per treatment, and one digestion tube per replicate. The repeatability test of the method used corn-soybean meal diet, barley, peanut meal, and rice bran as test materials, with three batches per sample and four replicates per batch. The additivity test consisted of 19 treatments, where treatments 1–7 were diets of corn, barley, sorghum, soybean meal, peanut meal, cottonseed meal, and rice bran, respectively, and treatments 8–19 were 12 diets prepared by combining two or more feed ingredients at different ratios, with four replicates per treatment and one digestion tube per replicate; reducing sugar release of each treatment was measured after simulating pig digestion in the biomimetic digestive system. The results showed that when the feed loading amount was 0.2–0.8 g, the total reducing sugar release exhibited a significant linear relationship with the loading amount ($R^2 = 0.9992$), the relative reducing sugar release varied between 559.56–582.70 mg/g DM with a coefficient of variation of 1.66%, while at a loading amount of 1.0 g, the relative reducing sugar release decreased by 5.37% compared to the average value at loading amounts of 0.2–0.8 g. For barley, peanut meal, rice bran, and corn-soybean meal diets across three batches, the intra-batch, inter-batch, and total coefficients of variation for reducing sugar release were all no greater than 1.68%, with maximum inter-batch relative deviations of 0.68%, 1.50%, 1.39%, and 0.29%, respectively. The measured values of reducing sugar release for the 12 diets were significantly higher than the calculated values ($P < 0.05$), while the linear regression model between calculated

and measured values of reducing sugar release coincided with y = x (intercept P = 0.4805; slope P = 0.5141). It was concluded that when the feed loading amount was 0.2–0.8 g, there was a significant linear relationship between loading amount and reducing sugar release; the repeatability and additivity of the biomimetic digestion method for determining reducing sugar release in feed met the basic requirements for quantitative analysis.

Full Text

Study on the Repeatability and Additivity of Reducing Sugar Release from Pig Feed Ingredients Determined by Simulated Digestion Method

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Abstract: This study aimed to establish a method for determining the release amount of reducing sugar from feed ingredients after simulated digestion mimicking pig digestion, providing a reference for evaluating feed nutrient bioavailability. The linear relationship between sample volume and reducing sugar release was investigated using a corn-soybean meal diet (75% corn + 25% soybean meal) with five treatments of 0.2, 0.4, 0.6, 0.8, and 1.0 g, each with 4 replicates (one digestion tube per replicate). The repeatability of the method was tested using corn-soybean meal diet, barley, peanut meal, and rice bran, with each sample analyzed across 3 batches (4 replicates per batch). Additivity was assessed through 19 treatments: treatments 1–7 consisted of single feed ingredients (corn, barley, sorghum, soybean meal, peanut meal, cottonseed meal, and rice bran), while treatments 8–19 comprised 12 diets formulated by combining two or more feed ingredients at different ratios, each with 4 replicates (one digestion tube per replicate). Reducing sugar release was measured after simulated digestion in a system mimicking pig digestion. Results showed that when dietary sample volume ranged from 0.2 to 0.8 g, a significant linear relationship existed between total reducing sugar release and sample volume (R^2 = 0.9992), with relative release varying from 559.56 to 582.70 mg/g DM (coefficient of variation = 1.66%). At 1.0 g sample volume, relative reducing sugar release decreased by 5.37% compared to the average at 0.2–0.8 g. For barley, peanut meal, rice bran, and corn-soybean meal diet, intra-batch, inter-batch, and total coefficients of variation were all $ $1.68%, with maximum inter-batch relative deviations of 0.68%, 1.50%, 1.39%, and 0.29%, respectively. The determined values for 12 diets were significantly higher than calculated values (P < 0.05), yet the linear regression model between calculated and determined values coincided with the y = x line (intercept P = 0.4805; slope P = 0.5141). It is concluded that a significant linear relationship exists between sample volume (0.2–0.8 g) and reducing sugar release, and that the repeatability and additivity of this simulated digestion method meet the basic requirements for quantitative analysis.

Keywords: simulated digestion system; reducing sugar; repeatability; additivity

Carbohydrates constitute over 60% of pig diets, with starch content at approximately 40% [1] and non-starch polysaccharides ranging from 9.32% to 59.97% [2]. These carbohydrates can be hydrolyzed into aldehyde-containing reducing sugars under enzymatic catalysis, representing potentially available nutrients for animals. Therefore, establishing a simulated digestion method to determine reducing sugar release from feed ingredients is crucial for evaluating feed nutritional value and feed enzyme efficacy. Current methods for measuring reducing sugar release after enzymatic hydrolysis include approaches based on non-starch polysaccharide enzyme activity [3-4], with Shi et al. [5] establishing a method for determining reducing sugar release from feed ingredients catalyzed by four non-starch polysaccharide enzymes. Pedersen et al. [6] developed a method for measuring xylose release from piglet digesta after xylanase catalysis using digesta and dried chyme obtained from piglet stomachs and small intestines in 24-well plates. Xue et al. [7] outlined a method for determining reducing sugar release from feed after simulated poultry digestion in Erlenmeyer flasks using pepsin and pancreatin in a two-stage process. However, these studies did not investigate whether the repeatability and additivity of their methods meet the basic requirements for quantitative analysis. Furthermore, these methods often suffer from issues such as feed samples adhering to digestion tube walls, preventing adequate contact with digestive fluid, and microbial fermentation causing sharp pH drops in reaction solutions, all of which affect measurement precision. To address these problems, Zhao et al. [8] developed a module for determining reducing sugar release from feed ingredients using simulated digestion, building upon their previously developed computer-controlled simulated digestion system (SDS-2) for determining enzymatic hydrolysis energy values in monogastric animals. This study investigates sample volume linearity, repeatability, and additivity using this module to provide a reference for establishing methods to determine reducing sugar release after feed digestion by digestive enzymes.

1.1 Feed Ingredients

Representative feed ingredients including corn, barley, sorghum, rice bran, soybean meal, cottonseed meal, and peanut meal were selected. Samples were taken using the quartering method, ground to pass through a 40-mesh sieve, thoroughly mixed, and stored in sample bottles at -20°C until use. The nutritional composition of feed ingredients is presented in . Dry matter, crude protein, crude ash, crude fat, and crude fiber contents were determined according to GB/T 6435-2006 [9], GB/T 6432-1994 [10], GB/T 6438-2007 [11], GB/T 6433-2006 [12], and GB/T 6434-2006 [13], respectively. Nitrogen-free extract (NFE) was calculated as: NFE content = 100 - (crude protein content + crude fiber content + crude fat content + crude ash content).

1.2 Experimental Design

This study comprised three experiments. Experiment 1 examined the linear relationship between dietary sample volume and reducing sugar release using a corn-soybean meal diet (75% corn: 25% soybean meal) with five sample volumes (0.2, 0.4, 0.6, 0.8, and 1.0 g), each with 4 replicates (one digestion tube per replicate). Experiment 2 assessed repeatability across different batches for corn-soybean meal diet, barley, peanut meal, and rice bran, with each sample tested in 3 batches (4 replicates per batch, one digestion tube per replicate). Experiment 3 evaluated additivity among feed ingredients through 19 treatments , where treatments 1–7 were single feed ingredients and treatments 8–19 were 12 diets prepared by combining two or more ingredients at various ratios, each with 4 replicates (one digestion tube per replicate). Reducing sugar release was determined after simulated digestion using pig digestive simulation fluids and procedures.

1.3.1 Preparation of Simulated Pig Digestive Fluids and Reaction Solutions

Gastric buffer (pH 3.0): Dissolve 2.59 g sodium chloride and 0.25 g potassium chloride in 350 mL deionized water in a 500 mL beaker, adjust pH to 3.0 with 2 mol/L hydrochloric acid (HCl) at 39°C, cool, transfer to a 500 mL volumetric flask, and dilute to volume with deionized water.

Small intestinal buffer: Dissolve 0.52 g anhydrous disodium hydrogen phosphate, 2.57 g anhydrous sodium dihydrogen phosphate, and 120,000 U penicillin in 40 mL deionized water in a 100 mL beaker, adjust pH to 6.30 with 1 mol/L phosphoric acid or 1 mol/L sodium hydroxide at 39°C, cool, transfer to a 500 mL volumetric flask, and dilute to volume with deionized water.

Simulated gastric fluid (pepsin activity 737.5 U/mL): Dissolve 0.21 g pepsin (Sigma, P7000, 147.5 kU) in 200 mL gastric buffer (pH 3.0) with gentle stirring until dissolved. Prepare fresh before use.

Simulated small intestinal fluid: Dissolve 41.41 kU amylase (Sigma, A3306), 12.82 kU trypsin (Amresco, 0785), and 1.62 kU chymotrypsin (Amresco, 0164) in 17 mL deionized water with gentle stirring for at least 15 minutes until dissolved. Prepare fresh before use.

1.3.2 Determination Procedure for Reducing Sugar Release from Feed Using Simulated Digestion System

Weigh a feed sample ($ $1 g, accurate to 0.0002 g) into a glass simulated digestion tube, simultaneously determining the dry matter content of the sample. Add 10 mL simulated gastric fluid to the tube and install an electric stirrer at the other end of the digestion apparatus. Place the apparatus in a preheated monogastric animal simulated digestion system, connecting the tubing according to the principle of water inlet at the bottom and outlet at the top of each

digestion vessel. Connect five simulated digestion vessels in series within each group. Connect the digestive fluid and buffer addition tubes to the system via quick connectors, and plug the stirring motor into the power supply. In the control software, gastric phase parameters were: temperature 39°C, peristaltic pump speed 180 r/min, digestion time 4 h. At the end of gastric digestion, 6 mL small intestinal buffer was automatically injected into the digestion tube via pump #3, followed by 1.6 mL simulated small intestinal fluid via pump #4. Small intestinal phase parameters were: temperature 39°C, peristaltic pump speed 180 r/min, digestion time 16 h. After small intestinal digestion, transfer the digestion solution from the glass tube without loss to an appropriately sized clean volumetric flask, dilute to volume with deionized water, seal with parafilm, and mix well (enzyme blank groups were filtered directly without transfer). Filter 30 mL of the solution from the volumetric flask through a 0.22 m membrane filter using a disposable syringe, and dilute the filtrate appropriately. Add 2 mL diluted digestion solution to a test tube, add 2 mL deionized water, mix by vortexing, add 5 mL dinitrosalicylic acid (DNS) solution, heat in boiling water bath for 5 min, cool to room temperature, dilute to 25 mL, mix well, and measure absorbance (OD) at 540 nm. Prepare glucose standard curve and DNS solution according to GB/T 23881-2009.

1.4 Calculation of Reducing Sugar Release

Sample moisture content was determined and dry matter content calculated according to GB/T 6435-2006 [9]. The reducing sugar release was calculated as:

Reducing sugar release (mg/g DM) = [(a × OD_1 + b) × D × V - (a × OD_2 + b) × 17.6] / (w × DM)

where: a = standard curve regression coefficient; b = standard curve regression constant; OD_1 = OD value of each replicate tube; OD_2 = OD value of enzyme blank tube; D = sample dilution factor; V = final volume; w = feed sample mass per replicate tube; DM = dry matter content of feed sample.

1.5 Data Processing and Statistical Analysis

Basic statistics were analyzed using the MEANS module of SAS 9.0. Intra-batch, inter-batch, and total coefficients of variation were calculated according to Li et al. [14]. Maximum absolute deviation = Max{[(maximum value - mean) + (mean - minimum value)] / 2}, and maximum relative deviation = (maximum absolute deviation / mean) × 100. Data were analyzed by ANOVA using the GLM module. Linear regression between reducing sugar release and sample volume was performed using the REG module. Paired t-tests between determined and calculated values for dietary reducing sugar release were conducted using the TTEST module (Paired option). The TEST option in the REG module was used to analyze whether the regression slope and intercept differed significantly from 1 and 0, respectively, to determine if determined and calculated values were equal and thus test method additivity.

2.1 Linear Relationship Between Sample Volume and Reducing Sugar Release Determined by Simulated Digestion

The relationship between dietary reducing sugar release and sample volume is shown in [Figure 1: see original paper]. Total reducing sugar release increased linearly as sample volume increased from 0.2 to 1.0 g ($R^2 = 0.9979$, $P < 0.01$), with the linear relationship strengthening when sample volume ranged from 0.2 to 0.8 g ($R^2 = 0.9992$, $P < 0.01$). Regarding the effect of sample volume on relative reducing sugar release (per gram dietary DM), values ranged from 559.56 to 582.70 mg/g DM (CV = 1.66%) at 0.2–0.8 g sample volume. However, at 1.0 g sample volume, relative reducing sugar release was 540.20 mg/g DM, 5.37% lower than the average at 0.2–0.8 g.

Data columns with different letters indicate significant difference ($P < 0.05$).

Fig. 1 Effect of dietary sample volume on reducing sugar release amount

2.2 Repeatability of Reducing Sugar Release Determination by Simulated Digestion

As shown in , maximum relative deviations within the four replicates of the same batch were 1.40%, 2.19%, 0.88%, and 0.39% for barley, peanut meal, rice bran, and corn-soybean meal diet, respectively, with intra-batch CVs of 0.93%, 1.29%, 0.61%, and 0.34%. Maximum relative deviations between different batches were 0.68%, 1.50%, 1.39%, and 0.29%, with inter-batch CVs of 0.64%, 1.27%, 1.36%, and 0.25%. Total CVs across three batches were 1.02%, 1.68%, 1.46%, and 0.38%, respectively. Multiple comparison results of mean reducing sugar release across batches showed significant differences between batches for peanut meal and rice bran ($P < 0.05$).

2.3 Additivity of Reducing Sugar Release Among Feed Ingredients Determined by Simulated Digestion

As shown in , t-tests revealed that determined values for reducing sugar release from 12 diets formulated by mixing corn, barley, sorghum, soybean meal, peanut meal, cottonseed meal, and rice bran at different ratios were significantly higher than calculated values ($P < 0.05$). However, linear regression analysis between calculated and determined values yielded a coefficient of determination of 0.9996 ($P < 0.05$), with no significant difference in intercept from 0 (intercept = -1.99, $P = 0.4805$) or slope from 1 (slope = 0.99, $P = 0.5141$). This indicates that the simple linear regression model between determined and calculated values coincided with the y = x line.

3.1 Effect of Dietary Sample Volume on Reducing Sugar Release

Digestion in livestock involves physical, chemical, and microbial processes, with enzyme-mediated chemical digestion being dominant [15]. Except in young animals, healthy livestock secrete digestive enzymes in excess of amounts required

for complete hydrolysis of corresponding dietary substrates [16]. In simulated digestion based on dialysis separation of hydrolysis products, major digestive enzyme activities in simulated fluids are similar to those in vivo [8], and the ratio of digestive fluid volume (mL) to sample mass (g) exceeds 10:1, approaching or exceeding the ratio of intestinal chyme in vivo [17]. Thus, enzyme activity is excessive relative to sample volume. To accurately measure product formation during hydrolysis, the simulated digestion process in this study involved no material exchange with the environment. Therefore, with constant simulated digestive fluid volume, increasing sample volume may lead to product inhibition of enzymatic reactions. When enzyme activity is excessive and product inhibition is negligible, enzymatic reaction velocity follows first-order kinetics, i.e., product formation is linearly related to substrate concentration [18]. When substrate concentration increases further and significant product inhibition occurs, product formation deviates from this linear relationship. In this study, reducing sugar release increased linearly as dietary sample volume increased from 0.2 to 0.8 g. However, the 1.0 g treatment clearly affected the linear relationship between sample volume and reducing sugar release. When sample volume ranged from 0.2 to 0.8 g, calculated reducing sugar release per gram of sample remained relatively stable, whereas at 1.0 g, calculated relative reducing sugar release decreased significantly. These results indicate that in this simulated digestion method, product inhibition of enzymatic reactions becomes pronounced at sample volumes above 0.8 g, causing reducing sugar release to deviate from the stable linear relationship. Therefore, sample volume should be controlled between 0.2 and 0.8 g.

3.2 Repeatability and Additivity of Reducing Sugar Release Determination by Simulated Digestion

Repeatability and additivity are critical for validating quantitative analytical methods. Statistically, repeatability is defined as the consistency among independent results obtained under repeatability conditions [19], typically expressed through intra-batch, inter-batch, and total CVs [14]. This study determined reducing sugar release from corn-soybean meal diet, barley, peanut meal, and rice bran across three batches, with intra-batch, inter-batch, and total CVs all within 1.68%. This variation is similar to that for determining dry matter digestibility and enzymatic hydrolysis energy values by simulated digestion (within 1.40%) [14], slightly higher than inter-laboratory CVs for determining feed dry matter and gross energy (1.27% and 1.29%, respectively) [20], but lower than the CV for feed dry matter digestibility determined by flask-based simulated digestion (6.89%) [21]. This demonstrates that the variation in reducing sugar release determination by simulated digestion achieves repeatability comparable to proximate nutrient analysis. Although significant differences existed between batches for peanut meal and rice bran, maximum inter-batch absolute deviations were 1.15 and 4.75 mg/g DM, respectively, with relative deviations of 1.50% and 1.39%, which are lower than the allowable relative deviation for crude protein determination (within 3%) [10]. Therefore, the repeatability of this simulated

digestion method for determining reducing sugar release is satisfactory.

Method additivity refers to measured values equaling theoretical values when samples are combined at different ratios [8]. This is typically assessed by regressing determined values of newly formed samples against calculated values based on single component proportions and their determined values, then comparing the regression line to y = x [22]. Biological methods for determining metabolizable energy in poultry feed ingredients show satisfactory additivity [20], and simulated digestion methods for determining enzymatic hydrolysis energy values also demonstrate good additivity [23]. In this study, although paired t-tests indicated that determined values for reducing sugar release from 12 diets were slightly higher than calculated values, the regression model of determined versus calculated values did not differ significantly from y = x. This indicates that the simulated digestion method for determining reducing sugar release from feed ingredients has satisfactory additivity.

Conclusion

1. When determining dietary reducing sugar release by simulated digestion, a significant linear relationship exists between sample volume (0.2–0.8 g) and reducing sugar release.
2. The repeatability and additivity of reducing sugar release determination by simulated digestion meet the basic requirements for quantitative analysis.

References:

[1] HERRER-SALDANA R E, HUBER J T, POORE M H. Dry matter, crude protein, and starch degradability of five cereal grains[J]. Journal of Dairy Science, 1990, 73(9): 2386–2393.

[2] MALATHI V, DEVEGOWDA G. In vitro evaluation of nonstarch polysaccharide digestibility of feed ingredients by enzymes[J]. Poultry Science, 2001, 80(3): 302–305.

[3] General Administration of Quality Supervision, Inspection and Quarantine of the People' s Republic of China, Standardization Administration of China. GB/T 23874–2009 Determination of xylanase activity in feed additives (Spectrophotometric method)[S]. Beijing: Standards Press of China, 2009.

[4] General Administration of Quality Supervision, Inspection and Quarantine of the People' s Republic of China, Standardization Administration of China. GB/T 23881–2009 Determination of cellulase activity in feed (Filter paper method)[S]. Beijing: Standards Press of China, 2009.

[5] SHI Kunjing, ZHANG Zhaozuo, ZHANG Tieying, et al. Study on rapid evaluation of non-starch polysaccharidase effects on feed ingredients in vitro by DNS method[J]. Feed China, 2011(10): 40–43.

[6] PEDERSEN N R, AZEM E, BROZ J, et al. The degradation of arabinoxylan-rich cell walls in digesta obtained from piglets fed wheat-based diets varies depending on digesta collection site, type of cereal, and source of exogenous xylanase[J]. Journal of Animal Science, 2013, 90(Suppl. 4): 149–151.

[7] XUE Mei, SHI Xueping, ZHANG Tingrong, et al. Evaluation of four single enzyme combination effects in broiler wheat-based diets by in vitro method[J]. Chinese Journal of Animal Nutrition, 2014, 26(12): 3747–3756.

[8] ZHAO F, REN L Q, MI B M, et al. Developing a computer-controlled simulated digestion system to predict the concentration of metabolizable energy of feedstuffs for rooster[J]. Journal of Animal Science, 2014, 92(4): 1537–1547.

[9] General Administration of Quality Supervision, Inspection and Quarantine of the People' s Republic of China, Standardization Administration of China. GB/T 6435–2006 Determination of moisture and other volatile matter content in feeds[S]. Beijing: Standards Press of China, 2007.

[10] General Administration of Quality Supervision, Inspection and Quarantine of the People' s Republic of China, Standardization Administration of China. GB/T 6432–1994 Determination of crude protein in feeds[S]. Beijing: Standards Press of China, 1995.

[11] General Administration of Quality Supervision, Inspection and Quarantine of the People' s Republic of China, Standardization Administration of China. GB/T 6438–2007 Determination of crude ash in feeds[S]. Beijing: Standards Press of China, 2007.

[12] General Administration of Quality Supervision, Inspection and Quarantine of the People' s Republic of China, Standardization Administration of China. GB/T 6433–2006 Determination of crude fat in feeds[S]. Beijing: Standards Press of China, 2006.

[13] General Administration of Quality Supervision, Inspection and Quarantine of the People' s Republic of China, Standardization Administration of China. GB/T 6434–2006 Determination of crude fiber content in feeds[S]. Beijing: Standards Press of China, 2006.

[14] LI Hui, ZHAO Feng, JI Feng, et al. Repeatability and precision test of metabolizable energy determination of duck feed ingredients by simulated digestion system[J]. Chinese Journal of Animal Nutrition, 2010, 22(6): 1709–1716.

[15] YANG Feng. Animal Nutrition[M]. 2nd ed. Beijing: China Agriculture Press, 1999.

[16] LONGLAND A C. Digestive enzyme activities in pigs and poultry[R]. Wallingford: CABI, 1991: 3–18.

[17] ZHANG Jianzhi, ZHAO Feng, ZHANG Hongfu, et al. Study on components of small intestinal fluid and digestive characteristics of chyme in chickens based on T-cannulation[D]. Master' s thesis. Yangzhou: Yangzhou University, 2011.

[18] WANG Jingyan, ZHU Shenggeng, XU Changfa. Biochemistry[M]. 3rd ed. Beijing: Higher Education Press, 2006.

[19] General Administration of Quality Supervision, Inspection and Quarantine of the People' s Republic of China, Standardization Administration of China. GB/T 3358.2-2009 Statistics—Vocabulary and symbols—Part 2: Applied statistics[S]. Beijing: Standards Press of China, 2010.

[20] BOURDILLON A, CARRÉ B, CONAN L, et al. European reference method for the in vivo determination of metabolisable energy with adult cockerels: reproducibility, effect of food intake and comparison with individual laboratory methods[J]. British Poultry Science, 1990, 31(3): 557-565.

[21] CLUNIES M, LEESON S. In vitro estimation of dry matter and crude protein digestibility[J]. Poultry Science, 1984, 63(1): 89-96.

[22] ZHAO Feng, LI Hui, ZHANG Hongfu. Study on additivity of enzymatic hydrolysis energy values of duck feed determined by monogastric animal simulated digestion system[J]. Chinese Journal of Animal Nutrition, 2015, 27(2): 495-502.

[23] SIBBALD I R. A test of the additivity of true metabolizable energy values of feedingstuffs[J]. Poultry Science, 1977, 56(1): 363-366.

Note: Figure translations are in progress. See original paper for figures.