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Validation of a phenol-sulfuric acid method in a microplate format for the quantification of soluble sugars in ruminant feeds

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Abstract

Soluble sugars in feeds are important for ruminant production; however, performing numerous sugar analyses within a short period is a laborious task. Here, we developed a phenol-sulfuric acid (PSA) assay in a microplate format to quantify soluble sugars in ruminant feeds. This method is easy and quick and requires only a small quantity of harmful reagents. We found that assay measurements were not affected by the representative organic acids and sugar alcohol contained in feeds. The treatment of activated charcoal with ethanol extract prior to the PSA assay was effective in removing interfering compounds for a more accurate determination of soluble sugars in certain feeds. Furthermore, the inter-day and intra-day repeatability of the present method was acceptable. Hence, we conclude that the method developed in this study is suitable for routine analysis of soluble sugars content in ruminant feeds.

KEYWORDS

feed, phenol-sulfuric acid, ruminant, soluble sugars

1 | INTRODUCTION

Soluble sugars are important carbohydrates, similar to starch, cellulose, and hemicellulose, for forage science and ruminant nutrition. To preserve forage as silage, sufficient amounts of soluble sugars in forage are necessary to lower the pH of silage by lactic acid and acetic acid (McDonald et al., 1991). As nutrients for ruminants, soluble sugars are fermented rapidly in the rumen and used as an energy source for microbial growth and protein synthesis (Hall & Herejk, 2001). The soluble sugars content in feeds has been analyzed in various fields of research and extension, such as ruminant nutrition, breeding and cultivation of forage, and silage production. Several colorimetric methods such as the phenol-sulfuric acid (PSA) assay, anthrone-sulfuric acid assay, reducing sugar assay, and enzymatic assay are routinely performed (Hall, 2014; Pereira et al., 2019; Rivero et al., 2019; Zhao et al., 2020; Zhou et al., 2019).

The PSA assay is a relatively simple and easy colorimetric method for measuring soluble sugars in feeds (Hall, 2014). However, quantification of soluble sugars content from a large number of samples is necessary in forage science and ruminant nutrition research. It is desirable to improve the method when handling a large number of samples and to reduce the amounts of deleterious chemicals used in the assay for the safety of analysts and environmental aspects. The amounts of concentrated sulfuric acid and phenol using the PSA method with test tubes are not small. As an example of a common procedure, 1 ml of 50 g/L phenol solution, and 2.5 or 5 ml of concentrated sulfuric acid were used in a reaction (Chow & Landhäusser, 2004; Dubois et al., 1956). To resolve this issue, a PSA method in microplates was reported for pure chemical compounds and extracted juice from sorghum internodes (Li et al., 2018; Masuko et al., 2005; Wang et al., 2017). A scaled-down method using microplates requires approximately one-tenth of the chemicals compared to the method using test tubes. The PSA method in a microplate format has dual benefits: handling of a large number

of samples and reduction the amounts of chemicals used in the assay. However, the microplate format requires adjustment for ruminant feed analysis; the method should be validated because an assay performed on a small scale may result in lower repeatability or accuracy than that on a large scale.

To measure the content of soluble sugars in ruminant feeds, the effects of the matrix (e.g., non-sugar carbohydrates, organic acids, or other interfering substances) should be considered, as described in the validation guidance for sugar analysis (Szpylka et al., 2018). The matrix in the extract for sugar analysis of ruminant feeds is varied because various materials such as forage, silage, grains, byproducts from the food industry, and their mixtures are used. In ruminant feeds, fermented feeds such as silage contain lactic acid and acetic acid (Chen et al., 2020; Miyaji et al., 2020). Fujikawa et al. (1974) reported that some organic acids (e.g., lactic acid) can give color in the PSA assay. However, this has not been considered when determining the effects of organic acids on the PSA assay for ruminant feeds. As some ruminant feeds also contain mannitol, a sugar alcohol, which is classified as a non-sugar carbohydrate (Nishino, Harada, et al., 2003; Nishino, Yoshida, et al., 2003), the extent of the color reaction of sugar alcohol in the PSA assay should be evaluated. It has also been reported that alcohol-soluble substances present in plant tissues react with phenol and sulfuric acid, and the results potentially overestimate the sugar content (Chow & Landhäusser, 2004). Some studies have investigated the effect of activated charcoal in removing interfering substances (Haslemore & Roughan, 1976; Kaur et al., 2012), however, it is uncertain whether the procedure can be applied to the PSA assay for ruminant feeds.

The aim of this study was to develop and validate a PSA method in a microplate format for the quantification of soluble sugars in ruminant feeds. The effects of potential interference were also examined. Owing to the increasing research interest and extension to farmers in Japan, we examined the soluble sugars content in forage rice and ensiled total mixed ration (TMR) in particular (Kusa et al., 2018; Miyaji & Nonaka, 2018; Shibata et al., 2019; Tanno, 2020).

2 | MATERIALS AND METHODS

2.1 | Chemicals

All chemicals used were reagent-grade. Ethanol, phenol, sucrose, fructose, mannitol, lithium lactate, sodium acetate, and activated charcoal were obtained from FUJIFILM Wako Chemicals (Osaka, Japan), whereas concentrated sulfuric acid, maltose monohydrate were from Nacalai Tesque, and sodium butyrate was from Sigma-Aldrich.

2.2 | Feed samples

Eight types of ruminant feed were examined in total. Two cultivars of whole crop forage rice, "Hoshiaoba" as a large-panicle type and "Tachisuzuka" as a small-panicle and high-sugar type, were harvested 45 days after heading in Mie University. These forage rice cultivars were chopped and ensiled in sealed plastic bags at the laboratory and then subsequently opened 120 days after ensiling. The forage rice at harvest and their silages were oven-dried at 60℃ for 48 hr. Alfalfa hay and sudangrass hay, imported from USA, were collected from a local dairy farm and oven-dried at 60℃ for 24 hr. TMR for dairy cattle, composed of corn silage, Italian ryegrass silage, rolled corn grain, rolled barley grain, ensiled brewer's grain, soybean meal, beet pulp, and corn gluten feed, were collected after mixing the ingredients. The TMR was ensiled in a round bale silo with a stretched plastic film for 45 days from July to August in Mie prefecture and was collected. Both TMR (non-ensiled) and TMR silage were freeze-dried. All dried samples were ground through a Wiley mill to pass a 1-mm screen and subsequently finely ground using a milling machine (Wonder Blender WB-1; Osaka Chemical). The finely ground samples were passed through a 0.3-mm screen.

2.3 | Extraction of sugars

One hundred milligram of ground samples were weighed in a test tube (16 × 100 mm) having a screw cap. Samples were extracted in 10 ml of 80% (v/v) ethanol for 30 min at 80℃ in a water bath. During extraction, the contents of the tubes were mixed every 5 min. Following extraction, test tubes were cooled with tap water and subsequently centrifuged at 2,000 × *g* for 10 min. The supernatants were used for the PSA assay and high-performance liquid chromatography (HPLC) analysis.

2.4 | Pretreatment with activated charcoal

In 1.5 ml microtubes, 10 mg of activated charcoal was weighed, and 1 ml of the extract was added. If needed, dilution of the ethanol extract with pure water was performed in the tube. Following mixing, the tubes were placed at room temperature for 5 min and mixed again. The tubes were centrifuged at 10,000 × *g* for 5 min and the supernatants were obtained.

2.5 | PSA assay

The assay was performed according to the method of Masuko et al. (2005) with some modifications. Fifty microliters of pure compound solutions or extracts with or without activated charcoal pretreatment were added into a 96-well flat-bottomed microplate made of polypropylene (#655201; Greiner Bio-One). Thereafter, 150 μl of concentrated sulfuric acid was added into each well and the plate was shaken at approximately 500 rpm for 20 s using a plate mixer (FLX-M, FRONTLAB; AS ONE). Following shaking, 30 μl of 50 g/L phenol solution was added into each well, and the plate was shaken once again. Thereafter, the plate was sealed with a clear film made

of polyethylene terephthalate (2-3993-01; AS ONE), and heated at 105℃ for 30 min on an aluminum block heater (DTU-2C; TAITEC). After cooling of the plate to room temperature, it was read on a plate reader (SpectraMax ABS Plus; Molecular Devices) at an absorbance of 490 nm. To examine the absorbance of pure compounds, sucrose, glucose, fructose, maltose, mannitol, lactate, acetate, and butyrate were dissolved in water at 0.05 or 1 mg/ml and analyzed using the PSA method in five replicates. Maltose monohydrate, lithium lactate, sodium acetate, and sodium butyrate were weighed as maltose, lactic acid, acetic acid, and butyric acid basis respectively. As referred in the study of Hall (2014), sucrose was used as the standard at 0.02–0.2 mg/ml and measured in duplicate for feeds. To test the effect of activated charcoal treatment, three test tubes for sugar extraction were prepared and the extract from each test tube was dispensed into two wells of a microplate. The absorbance readings from the two wells were pooled as one data point from each test tube. The conventional PSA method using test tubes was also performed according to the method of Dubois et al. (1956), in which 500 μl of extracts and 500 μl of 50 g/L phenol solution were added to a test tube (18 × 180 mm). When necessary, the ethanol extract was diluted in the test tube with pure water. No activated charcoal treatment was carried out. Thereafter, 2.5 ml of concentrated sulfuric acid was added to the tube and mixed well. After cooling, the absorbance was detected using a spectrophotometer (CO7500 Colorimeter; Biochrom) at 490 nm.

2.6 | HPLC analysis

The contents of glucose, sucrose, fructose, maltose and mannitol in samples were quantified (Hall, 2014; Nishino et al., 2004). Ethanol extracts, as described above, were dried *in vacuo* and the solutes were dissolved in water and cleaned up using spin columns (MonoSpin C18; GL Science) previously conditioned with ethanol and water. The recovered solution was filtered through a nylon membrane (pore size, 0.45 μm; Shimadzu GLC) and subsequently used for HPLC analysis (LC-10; Shimadzu). HPLC conditions were isocratic with a flow rate of 0.4 ml/min using ultrapure water as the mobile phase. Twenty microliters of the sample was injected and the separation was performed using a CARBOSep CHO-682 column (7.8 mm I.D. × 300 mm; Concise Separations) at 80℃ in a column oven. Sugars and mannitol were detected using a refractive index detector (RID-10A; Shimadzu). The analysis was performed in triplicate. The recovery rates of sugars and mannitol from the spin column were >96.4%. Lactic acid, acetic acid, and butyric acid in dried samples were measured using HPLC with a conductivity detector (Kondo et al., 2016).

2.7 | Evaluation of assay precision

Four types of feed samples, forage rice (Tachisuzuka) at harvest and its silage, sudangrass hay and TMR (non-ensiled) were analyzed using the PSA method with or without activated charcoal treatment. For each sample, five test tubes were used for extraction and the assay from each test tube was performed in two wells of the microplate, as described above. These assays were repeated for three consecutive days in the same laboratory. Variation of the standard curve and limit of quantification were also calculated from these assays. Standards were analyzed on the 3 days, in two different microplates with two wells in each plate in each day. The limit of quantification for the present method was calculated from absorbance values as the mean reagent blank value + 10 × *SD* (AOAC International, 2013).

2.8 | Statistical analyses

The effect of activated charcoal treatment on the content of soluble sugars determined using the PSA method was confirmed with a *t*-test using the TTEST procedure of SAS 9.4. To calculate the relative standard deviation (RSD, %) of inter-day and intra-day assay, one-way analysis of variance was performed (Magnusson & Örnemark, 2014; Tabuchi et al., 2017). Inter-day standard deviation was calculated as the within-group variance, whereas the intra-day standard deviation was obtained as the square root of the sum of squares of the within-group and between-group variance. Both standard deviations were divided by the mean of all quantitative values, and inter-day and intra-day RSD were expressed as a percentage multiplying by 100 (%).

3 | RESULTS

The absorbance readings of sugars, sugar alcohol, and organic acids using the PSA method in a microplate are shown in Table 1. The absorbance of sucrose, glucose, fructose, and maltose varied within 10%. The absorbance of mannitol and lactic acid at 0.05 mg/ml was low when compared to sucrose. Lactic acid at 1 mg/ml showed evident absorbance; however, it was less than sucrose at 0.05 mg/ml. The absorbance of acetic acid and butyric acid, even at 1 mg/ml, was negligible.

TABLE 1 Absorbance response of different sugars, sugar alcohol, and organic acids ($n = 5$)

Chemicals	Concentration, mg/ml	Absorbance (at 490 nm)	Relative absorbance
Sucrose	0.05	0.333	100
Glucose	0.05	0.300	90
Fructose	0.05	0.321	96
Maltose	0.05	0.302	91
Mannitol	0.05	0.005	2
Lactic acid	0.05	0.014	4
	1	0.210	63
Acetic acid	1	0.003	1
Butyric acid	1	0.003	1

In the present assays, standard curves had an average (±*SD*) slope of 5.746 (±0.342), an intercept of 0.0648 (±0.0132), and an R^2 of 0.9932 (±0.0033) for the linear form of the curve. The mean absorbance (±*SD*) of blanks was 0.0667 ± 0.0033 for a quantification limit of 0.1000 absorbance. By calculation, the limit of quantification for the present method was 0.68 mg/kg dry matter (DM) based on a 100 mg sample of 900 g/kg DM.

A comparison of soluble sugars content in ruminant feeds measured using HPLC and PSA methods in both conventional and microplate formats are shown in Table 2. The soluble sugars content measured using PSA method in a microplate was close to the content obtained using the conventional method in a test tube. The sugars content measured using HPLC was lower in all samples compared to those measured using the present PSA method either with or without activated charcoal treatment. Sugars content measured using the PSA method without and with activated charcoal treatment showed 10–33 and 7–20 g/kg DM, respectively, higher than that determined using the HPLC method, indicating that differences between the PSA and HPLC methods were reduced by activated charcoal treatment. The activated charcoal treatment for both types of forage rice at harvest, Tachisuzuka silage and alfalfa hay lowered the measurements of sugar contents significantly ($p < .05$), and that for sudangrass tended to lower as well ($p < .1$).

Mannitol was present in forage rice silage of Tachisuzuka, non-ensiled TMR, and TMR silage at 7.7, 3.2, and 15.0 g/kg DM respectively. The lactic acid content in Tachisuzuka silage, Hoshiaoba silage, non-ensiled TMR, and TMR silage was 40.4, 27.0, 14.3, and 89.0 g/kg DM, respectively, and acetic acid content in these samples was 3.7, 3.3, 8.1, and 14.9 g/kg DM respectively. Butyric acid was not detected.

The results of intra-day and inter-day precision for four types of feeds analyzed using the PSA method with or without activated charcoal treatment are shown in Table 3. RSDs of intra-day and inter-day were 3.0%–4.9% and 3.0%–9.9%, respectively, among the feeds when analyzed without activated charcoal. These RSDs with activated charcoal treatment were 2.8%–3.6% and 2.9%–5.6% respectively.

4 | DISCUSSION

The present study examined the validity of measuring the soluble sugars content in ruminant feeds using the PSA method in a microplate format. Overall, the method was easy, reliable, and insensitive to coexisting materials, as described below.

As reported by Fujikawa et al. (1974), high concentrations of lactic acid can cause coloration in the present method. Among the ruminant feeds used in this study, TMR silage contains the highest lactic acid (89.0 g/kg DM) in the dried sample. In this case, lactic acid concentration of the extract prepared according to the present method was 0.81 mg/ml. Calculated from the relative absorbance of lactic acid to sucrose in Table 1, this amount of lactic acid results in an overestimation of sugars in the feed to only 2.8 g/kg DM. As this overestimate is extremely small, it is unlikely to be an issue in the actual analysis. In addition, acetic acid and butyric acid did not clearly give color in the PSA assay.

We also investigated the effect of sugar alcohol on the PSA method. It has been reported that mannitol is produced during silage fermentation (Nishino, Harada, et al., 2003; Nishino, Yoshida, et al., 2003) and is slowly metabolized in the rumen compared with sucrose and glucose (Ahmed et al., 2013). In the present study, Tachisuzuka silage, non-ensiled TMR and TMR silage contained mannitol, and the highest content was 15 g/kg DM in TMR silage. It was also reported that mannitol content in silage is 40–50 g/kg DM (Nishino, Harada, et al., 2003; Nishino, Yoshida, et al., 2003). However, the PSA assay did not evidently detect mannitol compared to sucrose, indicating that mannitol does not cause overestimation of soluble sugars content.

TABLE 2 Measurement of soluble sugars in feeds using HPLC or PSA method (g/kg DM, $n = 3$)

Samples	HPLC	PSA (conventional format)	PSA (microplate format)		
			Activated charcoal treatment		
			–	+	*p*-values[b]
Forage rice, Tachisuzuka (at harvest)	106.8	125.9	125.9 ± 4.3[a]	113.2 ± 1.7	<.01
Forage rice, Tachisuzuka (silage)	2.1	14.1	14.8 ± 0.1	10.9 ± 0.4	<.01
Forage rice, Hoshiaoba (at harvest)	44.1	60.7	58.1 ± 3.7	47.0 ± 2.4	.01
Forage rice, Hoshiaoba (silage)	14.1	25.3	27.5 ± 0.8	26.8 ± 0.7	.39
Sudangrass hay	64.6	85.1	76.8 ± 5.8	65.4 ± 5.4	.07
Alfalfa hay	56.2	73.6	79.6 ± 2.2	61.8 ± 7.5	.02
Total mixed ration (non-ensiled)	81.4	88.2	92.7 ± 4.6	87.4 ± 3.4	.18
Total mixed ration (silage)	0.0	11.3	10.2 ± 0.8	10.0 ± 0.5	.75

Abbreviations: DM, dry matter; HPLC, high-performance liquid chromatography; PSA, phenol-sulfuric acid.

[a]Mean ± *SD*.

[b]The effect of activated charcoal treatment on the content of soluble sugars determined using the PSA method in a microplate format was confirmed with a *t*-test.

TABLE 3 Mean concentrations and relative standard deviation of intra-day and inter-day for sugars in feeds determined using PSA method without or with activated charcoal treatment

	Mean (g/kg DM)			Relative standard deviation (%)	
Samples	Day 1	Day 2	Day 3	Intra-day	Inter-day
Without activated charcoal					
Forage rice, at harvest (Tachisuzuka)	127.2	128.5	127.1	3.0	3.0
Forage rice, silage (Tachisuzuka)	14.7	15.3	14.8	3.6	3.9
Sudangrass hay	75.1	75.0	68.0	4.6	6.9
Total mixed ration, non-ensiled	98.1	93.0	82.3	4.9	9.9
With activated charcoal					
Forage rice, at harvest (Tachisuzuka)	114.5	112.0	111.8	3.6	3.6
Forage rice, silage (Tachisuzuka)	11.7	11.4	11.5	2.8	2.9
Sudangrass hay	61.7	64.7	58.7	3.2	5.6
Total mixed ration, non-ensiled	81.1	81.7	75.9	3.6	5.1

Note: The number of replicates in each day was 5.

Abbreviations: DM, dry matter; PSA, phenol-sulfuric acid.

We compared the differences between the soluble sugars contents of ruminant feeds determined using the PSA method and HPLC. The HPLC values are the sum of the only four sugars (glucose, sucrose, fructose, maltose) and do not include any other carbohydrates. In contrast, the values using the PSA method represent the amount of various carbohydrates, including larger oligosaccharides, and might be affected by the presence of interferences (e.g. pigments) in some cases (Ebell, 1969; Hall, 2014; Haslemore & Roughan, 1976; Kaur et al., 2012). There were slight differences between the values of soluble sugars content measured using the two methods, which is likely due to the differences in the characteristics of the methods described above. Therefore, we believe that the PSA method used in this study is effective in examining the sugars content of ruminant feeds.

The treatment of activated charcoal with ethanol extract prior to the PSA assay slightly lowered the measurements of soluble sugars content in most feed samples. In a preliminary study, the recovery rate of glucose, sucrose, fructose, and maltose in 80% ethanol from activated charcoal treatment was higher than 98.6% in HPLC analysis (data not shown), indicating that the loss of sugars by activated charcoal treatment was negligible. Several studies has reported that removing interfering substances prior to colorimetric methods such as PSA assay and anthrone-sulfric acid assay would be suitable (Ebell, 1969; Haslemore & Roughan, 1976; Kaur et al., 2012). It seems that alcohol-soluble pigments (e.g. chlorophylls, carotenoids, phenolic compounds) in the non-charcoal treatment sample or their reactants with sulfuric acid might show absorbance at 490 nm, leading to an overestimation of the sugars content (Chen & Vaidyanathan, 2013; Ebell, 1969). In the present study, the activated charcoal treatment would be effective for removing some parts of alcohol-soluble interference compounds; the treatment can be adapted for the determination of soluble sugars in ruminant feeds using the PSA method in a microplate format.

We also evaluated the inter-day and intra-day repeatability of the developed method. According to the method performance requirement for sugars in animal feeds (Szpylka et al., 2018), it is recommended that inter-day RSD is lower than 7 and 5% when sugar content is 1–50 and 50–500 g/kg DM in samples respectively. For the PSA method with microplate format for sorghum juice, inter-day and intra-day RSDs were 3.7%–6.8% and 4.8%–9.5% respectively (Li et al., 2018). The RSD values in the present study were similar to those reported in the literature (Li et al., 2018; Szpylka et al., 2018; Zhang et al., 2014), indicating that the precision of the method was acceptable.

In this study, the PSA method was developed in a microplate format to quantify soluble sugars in ruminant feeds. This method allows the analysis of a large number of samples in one run with smaller amounts of chemicals as compared to assays in test tubes. This procedure is suitable for routine analysis of forage and TMR. Further research is needed to investigate more types of samples, particularly temperate forage containing fructan which cannot be extracted by ethanol. Precision by different operators on different instruments should also be evaluated in multiple laboratories.

CONFLICT OF INTEREST

All authors declare no conflict of interests.

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