BRIEF COMMUNICATION: Investigation of the water-soluble carbohydrates content of Plantain (*Plantago Lanceolata* L.)

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Abstract

Previous research has shown that the water-soluble carbohydrates (WSC) of Plantain (*Plantago lanceolata* L.) leaves differ from that of many other common forages. Analytical methods used to quantify WSC may underestimate the concentration of WSC in plantain herbage. To ascertain the suitability of current method, the conventional anthrone reaction method (AR) was compared against the phenol-sulphuric acid method (PSA). The WSC of eight plantain samples and one ryegrass sample were compared using AR and PSA against high-performance liquid chromatography (HPLC). The results showed that AR greatly underestimated the WSC content of plantain compared with PSA, which was similar to HPLC (7.18%, 13.5%, 14.7% DM, respectively, $P<0.001$). The discrepancy between the results from AR and PSA was due to sorbitol, which was not detected using AR.

Keywords: *Plantago lanceolata* L.; water-soluble carbohydrates; anthorone reaction method: phenol-sulphuric acid method; sorbitol

Introduction

Plantain is a herb species with a broad distribution in grasslands throughout the temperate world. Recently, selection of plantain genotypes, which are high yielding and tolerant of frequent grazing has led to adoption of plantain in livestock production systems in New Zealand (Powell 2007; Stewart 1996). Feed planning for livestock requires information on both quantity and nutrient quality of forages, including WSC, which is one of the important feed energy sources. Analytical procedures use to extract and determine WSC can vary between laboratories, but in New Zealand, it is common to use wet-chemistry based techniques on colorimetric methods using AR or PSA to detect WSC.

Previous research has shown that the WSC from Plantain leaves is composed of L-arabinose (20%) (Brautigam & Franz 1985). Kardošová (1992) reported that in the crude mucilage isolated from the leaves of *Plantago lanceolata* L., var. LIBOR, L-arabinose comprised 26% of the total WSC. According to Dubois (1956) and Ashwell (1957), the AR is of limited use for pentose detection, as the reaction is not sensitive to this sugar. Therefore, the AR may not detect pentose, hence, underestimate the WSC content of plantain. PSA method would be an option for detecting the WSC content of the samples with pentose (Dubois et al, 1956; Masuko et al. 2005). High-performance liquid chromatography is the most reliable technique as it has capacity to quantify a wide range of sugars, and sugar alcohol. However, routine analysis using HPLC requires specialist equipment and is more expensive than AR or PSA. Thus, the objective of this trial was to compare two commercial assays (AR and PSA) for WSC analysis of Plantain against HPLC method.

Materials and methods

Sample extraction

Eight finely ground and freeze-dried plantain leaves (P1-P8 Table 1) collected during spring and autumn sampling in 2014 and 2015 were used to compare methods. Approximately 25 mg of each sample in duplicate was mixed with 1 ml 80% ethanol in a 2ml screw-cap tube, shaken for 30 minutes at 65°C using shaking incubator (Labnet Vortemp56). The samples were then centrifuged for 15 minutes at 13000 rpm (Hermle Z216MK), and the supernatant was collected into Eppendorf tubes. The residue was further extracted using the same procedure with 80% ethanol and twice with deionized water (DI) water to obtain 2 ml ethanol and 2 ml water extract. The extraction and analysis included a laboratory herbage standard control sample, a perennial ryegrass (RG).

Colourmetric analysis

The AR and PSA methods previously described by Ashwell (1957) and DuBois et al. (1956) respectively, were used to analyse the extracts. Extracts were read on 96-well flat-bottom microplates using a microplate reader spectrophotometer (Theromo Scientific Multiskan go, Finland) at 620 nm for AR and 490 nm for PSA. Sucrose and inulin were used as a standard for low-molecular and high-molecular weight sugars, respectively. The extracts were diluted accordingly to fit the range of standards.

HPLC analysis

Each sample was diluted three times in water, transferred into an injection vial, and 10µl of the solution was injected in to Shimadzu HPLC system (Shimadzu Corporation, Kyoto. Japan). The HPLC had an Alltech 3300-evaporative light scattering (ELSD) detector and an Prevail™ Carbohydrate ES Columns (250 x 4.6mm). For

Table 1 Water-soluble carbohydrates (WSC) of plantain (P1-8) and ryegrass standard (RG), obtained from the anthrone reaction method (AR), phenol-sulphuric acid method (PSA) and high-performance liquid chromatography (HPLC) (SEM 0.69 for means of AR, PSA and HPLC).

Sample ID	WSC by AR % DM	WSC by PSA % DM	WSC by HPLC % DM	Sorbitol by HPLC % DM	WSC by HPLC without Sorbitol % DM	Sorbitol in WSC %
RG	16.56	18.10	14.09	0	14.09	0
P1	8.77	16.36	16.42	6.73	9.69	41.0
P2	9.91	21.46	21.10	7.60	13.50	36.0
P3	5.45	8.79	13.26	7.16	6.10	54.0
P4	10.09	15.14	18.75	7.55	11.19	40.3
P5	3.57	10.67	9.24	4.62	4.62	50.0
P6	6.39	15.95	11.86	4.05	7.80	34.2
P7	5.97	9.11	12.58	5.03	7.55	40.0
P8	7.32	10.76	14.09	5.78	8.31	41.0
Average (P1-P8)	7.18 [b]	13.53 [a]	14.66 [a]	-	-	42.1

[a-b] Means with different subscripts are significantly different (P<0.001)

the gradient mobile phases, tube A contained acetonitrile, tube B contained DI water, and the following gradient was used at a flow rate of 1.0 ml/ min: 0-5 min 20% B, 5-10 min 20%-50% B, 10-11 min 50%-20% B and 11-15 min 20% B. The column was kept at 20˚C during analysis. The ELSD detector flow rate of nitrogen was 1.4L/min at 38ºC and the gain was 4.

Carbohydrate standards: There were eight standards: α-L-rhamnose (Sigma, Aldrich, 99%), L(+)-arabinose (Sigma, Aldrich, 99%), D-fructose (Merck, 99%), sorbitol (Sigma-Aldrich, 99.5%), D-mannitol (Sigma, Aldrich, 99%), D-glucose (Merck, 99%), sucrose (Sigma, Aldrich, 99.5%), and D-raffinose (Sigma, Aldrich, 99%).

Data processing: each carbohydrate was identified by comparing retention time of standards, and and were quantified from the peak areas of chromatograms using external calibration standard curves. Data were processed using LC solution software version 1.22SP1.

Statistical analysis

To compare the differences among the methods (AR, PSA and HPLC assays), one-way analysis of variance (ANOVA) was performed using Genstat version 19.1 (VSN international, 2018), where the method of analysis was the fixed term and replicate was the random effect. Means were separated using Fishers protected LSD.

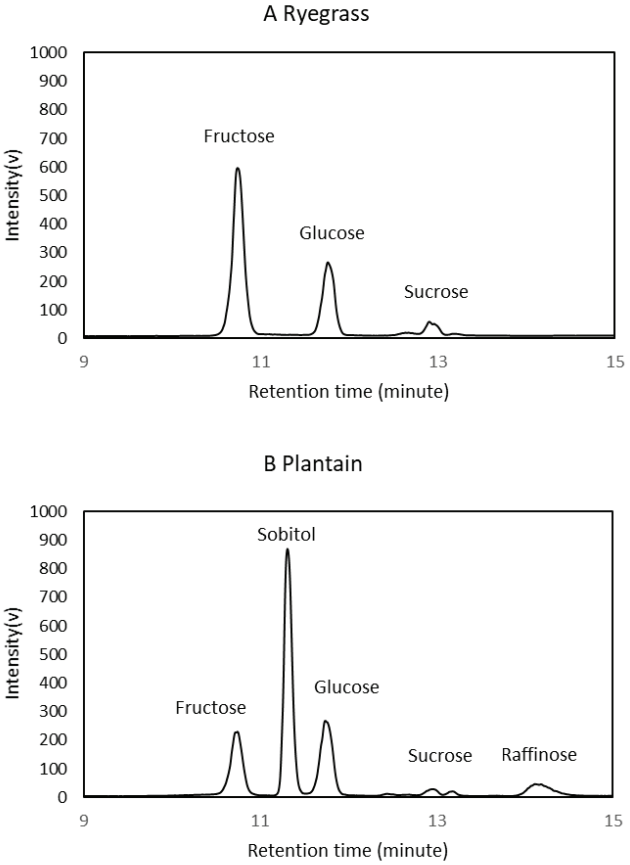
Results and discussion

The AR method greatly underestimated the WSC content compared with the HPLC method (Table 1). Compared with AR, the WSC value derived using PSA was closer to the HPLC value. The average WSC was 7.18±0.69 % DM from AR, 13.5±0.69 % DM from PSA and 14.66±0.69% DM from HPLC. However, there was no L-arabinose detected by HPLC (as expected). Instead, there was about 42.1 % sorbitol component of the total WSC (Figure 1 and Table 1).

Figure 1 shows the chromatography from the HPLC for ryegrass and plantain. Fructose, glucose, sucrose and minor quantities of raffinose in the extract from ryegrass were the main sugars present. For the plantain sample, apart from fructose, glucose, sucrose and raffinose, there was also on average of 6.07% DM sorbitol present, which accounted for a large proportion of total WSC.

Spectrophotometric assays used to measure total soluble sugar do not detect sugar alcohol. Sorbitol (sugar

Figure 1 High-performance liquid chromatography (HPLC) of (A) The Ryegrass standard-80:20 Ethanol:water extract; (B) The test material Plantain-80:20 ethanol: water extract



alcohol) produces no colour in the AR (Ashwell 1957). HPLC is able to quantify various types of sugars and also for sugar alcohol. The PSA method gives different responses with different carbohydrates (DuBois et al. 1956), so that the values obtained with either assay seems dependent upon the carbohydrate standard used. For greatest accuracy of measurement, it is preferable for the carbohydrate standard to be similar to (if not identical to) the carbohydrate being measured. Meeting this last criterion is not feasible when analysing feeds that contain unknown mixtures of soluble carbohydrates unless an extensive analysis is done beforehand, which would negate the need to use the empirical assays (Hall 2014).

Implications of sorbitol in livestock feeds

Whether the sorbitol is included in the WSC for livestock feed assessment remains a question. Sorbitol, also known as glucitol, is a sugar alcohol which is found mainly in the Rosaceae and Plantains (Lewis 1984). Some consider it play no role in animal metabolism (Koolman & Roehm 2005). While sorbitol is technically a carbohydrate, it has a lower energy density than sugar because of its incomplete absorption and it is argued that it should not be counted as part of the carbohydrate energy pool.

We conclude that the AR method is a suitable method for determining WSC in plantain if results are used for feed planning and estimating energy availability for livestock. However, if sorbitol needs to be included as a part of WSC, there are two ways to determine WSC according to our results: 1) Employ the PSA method for WSC analysis or 2) use WSC from AR plus sorbitol, which was 42.1% in average in this study.

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