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Contribution to the Method of Sugar Analysis in Legume Grains for Ensiling – A Pilot Study

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ABSTRACT

The quotient of sugar content and buffering capacity (S/BC quotient) serves as an important predictor for the ensilability of green feed and cereal or legume grains. For this, reference values were given which base on the anthrone method for sugar analysis. This method has largely been replaced today, but the consistency of results from different methods is questionable. In this study, the sugar content of legume grains was determined by the anthrone method and compared with results from nowadays more common methods. For this, grains from lupine (Lupinus spp.; var. 'Bora', 'Borlu'), pea (Pisum sativum; var. 'Lisa', 'Phönix') and field bean (Vicia faba; var. 'Limbo') were analysed (n = 4 each) for sugar via anthrone method, a gravimetric method (GRAVI) and HPLC. Following HPLC, either glucose, fructose and sucrose (HPLC-1) or these monomers plus galactose (HPLC-2) or HPLC-2 plus oligomeric carbohydrates (raffinose, stachyose, verbascose; HPLC-3) as sum were referred to as sugar. Results were compared by one-way analysis of variance. None of the alternative methods provided results that are at least similar to the sugar content detected by the anthrone method ($P > 0.05$). HPLC-3 caused a clear overestimation whereas the other methods (VDLUFA, HPLC-1, HPLC-2) resulted in a remarkable underestimation compared to the anthrone method. The results from legume grains suggest that different methods of sugar analysis provide remarkably different results even though all methods are accepted and applied in routine analysis. Thus, i) sugar contents should not be interpreted without knowledge of the applied method, and ii) as long as reference values base on anthrone method, the forecast of ensilability via the S/BC quotient should only be performed when anthrone method was used to determine the sugar content. The comparison of results from different methods of sugar analysis should be extended to grasses and further more conventional material for ensiling.

Keywords: Legume Grain, Ensiling, Sugar, Buffering Capacity, Anthrone Method, HPLC

I. INTRODUCTION

Legume grains such as lupine, field bean and pea are valuable feedstuffs particularly because of their remarkable content of essential amino acids. These grains, however, mature at different rates even on the same field and furthermore contain several anti-nutritional factors such as oligomeric carbohydrates, tannins and alkaloids ([13], [16], [15], [25], [29]). Ensiling of moistly harvested legume grains mitigates the problem of inconsistent maturity and furthermore microbial fermentation may decrease the amount of some anti-nutritional factors ([1], [2], [5], [6], [7], [8], [10], [17], [22], [24]). The problem however is the regularly low quotient of sugar content and buffering capacity (S/BC) in legume grains.

The S/BC serves as important predictor for the ensilability of plant material. The background is that S/BC represents the ratio between the content of carbohydrates in the feed, which are beneficial for the microbial production of lactic acid on the one hand and the buffering capacity that stands for the ability of the feed to counteract lactic acid-induced pH reduction on the other hand. The S/BC quotient further substantially supports the decision whether silage additives such as molasses or other carbohydrate sources easily available for lactate-producing bacteria (LAB) are necessary for

the preparation of high-quality silages or not. For this, dry matter (DM) dependent references about the S/BC quotient are given, which, in the individual case, is the minimum required for the preparation of high quality silages ([27], [28]). These references, or at least recommendations, have been established by Weißbach ([27], [28]) on the basis of sugars according to the anthrone method ([4], [30]). This method has been adapted to quantify the totality of carbohydrates available for microbial fermentation in the initial period of the ensiling process. According to the anthrone method ([4], [30]), simple sugars in a feed sample are determined colorimetrically. Anthrone assay is based on condensation of furaldehyde derivatives, generated by carbohydrates in presence of a strong oxidizing sulfuric acid, with a reagent anthrone (9,10-dihydro-9-ozoanthracene). This method determines both reducing and non-reducing sugars because of the presence of the strongly oxidizing sulfuric acid. In this case, an aliquot part of sugars reacts with the anthrone reagent to produce blue-green color compounds in linear relationship between the absorbance and the amount of sugar ([3]).

Because of several disadvantages, the anthrone method is at present only seldom used in both scientific and routine analysis. Alternatively, gravimetric or volumetric methods as described in the German key book for feed analysis (methods of the 'Verband Deutscher Landwirtschaftlicher Untersuchungs- und Forschungsanstalten' [VDLUFA] according to Naumann and Bassler, [18]) or high-pressure liquid chromatography (HPLC) are applied. The latter has the additional advantage that individual carbohydrate fractions can be both qualified and quantified.

Legume grains are particularly interesting in this concern because evidence exists that they contain large amounts of carbohydrates, which may be fermented by lactic acid producing bacteria, but not identified as sugar by common methods of sugar analysis besides anthrone method. Gefrom et al. ([6], [7], [8]) indeed produced high-quality silages from lupine grains, field beans and peas even without any silage additive although the sugar content (as sum of glucose, fructose and sucrose determined *via* HPLC) and thus the S/BC quotient of the lupine grains were critically low.

We hypothesized that different methods of sugar analysis and subsequent calculations may lead to dissimilar change with different contents of what is considered to as sugar and, thus, unequal and in individual cases inappropriate statements on the ensilability of the feed in question and the necessity of carbohydrate sources as silage additive. The aim of the recent pilot study was to compare sugar content and S/B of randomly selected legume grains of different sources and varieties determined by the anthrone method, as original method for S/BC calculation and references, with nowadays more common analytical methods.

II. METHODS AND MATERIAL

A. Feed samples and analytical fractions

Legume grains from lupine (*Lupinus spp.*, varieties 'Bora' and 'Borlu'), pea (*Pisum sativum*, varieties 'Lisa' and 'Phönix') and field bean (*Vicia faba*, variety 'Limbo') from the harvest year 2012 were sampled from four different areas each. The grain was analysed for dry matter (DM), crude ash, starch, the oligomeric carbohydrates raffinose, stachyose and verbascose, total sugar and individual sugar fractions (glucose, fructose, sucrose, galactose), and the buffering capacity (BC). Sugar as a total fraction was detected and defined in different ways: i) *via* anthrone method ([27]; ANTHR), ii) by a gravimetric VDLUFA method ([18]; GRAVI; except for field bean, variety 'Limbo'), and iii) as sum of HPLC fractions. Following HPLC, either the sum of glucose, fructose and sucrose (HPLC-1) or the sum of these sugars plus galactose (HPLC-2) or HPLC-2 plus the determined oligomeric carbohydrates (HPLC-3) were referred to as sugar. Each determination was performed in five replicates.

B. Analytical methods and calculations

Prior to analysis the air dry samples were either milled through a mash with a size of 1 mm or, for analysis of individual sugars and starch, with a swing mill (MM 200, Retsch GmbH & Co. KG, Haan, Germany) for 5 minutes with a frequency of 30/sec.

1) Dry matter and crude ash: The contents of DM and crude ash were analysed according to Naumann and Bassler ([18]).

2) Starch: For the determination of starch, an enzymatic procedure was chosen using a 0.2% solution of thermo-

stable amylase (Thermamyl 120, Novo Nordisk A/S, Bagsvaerd, Denmark). The milled sample was swung in a water bath (90 ºC) for 30 minutes and filtered (Rotilabo filter disc, Typ 13A, Carl Roth GmbH & Co. KG, 76185 Karlsruhe, Germany). 2 ml of 0.1% of amyloglucosidase solution was added to 2 mL of the filtrate and stored for 16 h in an incubator at 60 °C. Afterwards, the concentrations of mono- and dimeric carbohydrates were analysed by HPLC (Shimadzu-Deutschland GmbH, Duisburg, Germany; refraction index; column HPX-87P; Biorad, Hercules, CA, USA). From this, the likewise per HPLC determined content of mono- and dimeric sugars, which were already be present in the feed sample prior to amylase-treatment, was subtracted (starch = [mono- and dimeric sugars after amylase-treatment] – [mono- and dimeric sugars prior to amylase-treatment]). The procedure has previously been described by Schmidt et al. ([23]).

3) Glucose, fructose and sucrose: The milled material was transferred in a 100 mL volumetric flask. 100 mL of triple desalted water was added and mixed during the flask was swung in a water bath (23 °C) for 60 minutes, and afterwards filtered (Rotilabo filter disc, Type 13A, Carl Roth GmbH & Co. KG, Karlsruhe, Germany). The concentrations of fructose, glucose and sucrose were measured in the filtrate by HPLC as described above (see 'starch'; [7], [23]).

4) Galactose and oligomeric carbohydrates (stachyose, raffinose, verbascose): The chromatographic method for the detection of galactose and the oligomeric carbohydrates stachyose, raffinose and verbascose originates from Quemener ([21]) and was performed in a slightly modified way according to Kluge et al. ([14]). For this, 500 mg of the milled feed were transferred into 10 mL centrifugal glasses, 10 mL of distilled water were added and all together dispersed for 2 minutes (Ultra-Turrax; Polytron PT 1600E, Kinematika AG, 6014 Luzern, CH). After centrifugation (at 4,000 U/min for 5 min) 6 mL of the overlap were separated and centrifuged again (at 13,400 U/min for 5 min). To 5 mL of overlap 30 µl 1N HCl (pH 4.2) were added and the solution was centrifuged once more (at 13,400 U/min for 5 min). For precipitation of proteins, 30 µl Carrez I (21.9 g zinc acetate and 3 g vinegar (ice), dissolved in *aqua dest.*, filled up to 100 mL with water) and then 30 µl Carrez II (10.6 g potassium ferro-cyanide dissolved in water and filled up to 100 mL with water) were added and all together centrifuged (13,400 U/min, 5 min). The overlap was frozen (- 20 ºC) prior to analysis. The analysis was performed by HPLC (Shimadzu-Deutschland GmbH, Duisburg, Germany; refraction index; column HPX-87C; Biorad, Hercules, CA, USA; separation column: Merck KGaA 64271 Darmstadt; column chrospher 100 NH_2, 4 mm ID; 300 mm length; mobile phase: acetonitrile H_2O, 70 : 30; flow rate 1 ml/min; pressure 124 bar; temperature: 30 °C, RID detector).

5) Sugar via anthrone method: Anthrone assay was performed according to a modification described by Weißbach et al. ([27]). The method is non-stoichemetric and therefore it is necessary to prepare a calibration curve using a series of glucose standards of known carbohydrate concentration. For anthrone reagent 780 mL concentrated acid sulphur (w = 95 – 97%; ρ = 1.84 g/mL) were added under cool conditions to 330 mL *aqua dest.*, further 1 g thiourea and 1 g anthrone were added, and the solution kept cool in a brown bottle for 5 days. 1 g of dried material was added with 200 mL *aqua dest.*, mixed for 60 minutes at 180 vibrates per minute, filled up with *aqua dest*. to 500 mL and filtered in a 300 mL flask. 2 mL of Carrez-solution I and II were added to 50 mL (or 25 mL when a sugar content of > 18% of DM is expected) of filtered solution, 2 mL of Carrez-solution I and II were mixed and filled up with *aqua dest.* to 100 mL and filtered again. In every case 2 mL of the filtrate were pipetted in test tubes with screw-topped, cooled on ice and mixed with 10 mL of anthrone-reagent (by vortex for 30 seconds). The samples were boiled for 20 minutes and then cooled down for 10 minutes with help of a cold water bath. The value for absorbance was evaluated at 625 nm (1 cm cuvette, calibration by blank value). The content of water-soluble carbohydrates was calculated according to the calibration curve with glucose-standards.

6) Analysis of carbohydrates by gravimetric VDLUFA method: The analysis of sugars according to the official method of the VDLUFA was performed as described by Naumann and Bassler ([18]). For this, method no. 7.1.1 was taken ([18]). On principle, sugars are dissolved in diluted ethanol and clarified with Carrez-solutions I and II. After evaporation of the ethanol, sugars were determined before and after inversion with Luff-Schoorl reagent. As the result, the content of reducing sugars and total sugars following inversion was expressed as sucrose (after conversion of glucose with factor 0.95).

7) Buffering capacity: The BC was determined according to Weißbach (in-house method of the Institute of Crop and Soil Science, Julius Kühn-Institute, Federal Research Centre for Cultivated Plants). For this, in short, cocked distilled water was added to the sample of the dry and milled (1 g; sieve pore size 1 mm) feed in a 1 : 100 (feed : water) ratio and samples were mixed. 30 – 60 min thereafter, titration was performed with lactic acid (LA; 0.1 mol/L) until pH 4.0. The rate of total LA consumption ($LA_{feed+water}$) was documented 240 sec after reaching this pH endpoint. Differences between repeated analyses within the feed sample in question was accepted when less than 0.1 mL. In parallel the same titration procedure was performed with 100 mL of distilled water only and LA consumption until titration to pH 4.0 (LA_{water}) was noted. Factor of LA was daily determined. For this, 15 mL of lactic acid were diluted with 58 mL of distilled water and then titrated with sodium hydroxide solution (0.1 mol/L) until pH 9.0. The factor of LA was calculated as quotient of the set-point volume and the actual volume needed for titration. For calculation of BC the titration value ($LA_{feed+water}$) was corrected by blank value for water only (LA_{water}). The result was than based on feed DM (BC, in g LA/kg DM).

8) S/BC quotient: To calculate the S/BC quotient, the content of sugar according to the analytical method in question was divided by the BC.

C. Statistical Methods

Within each of the four individual legume grains, the sugar contents determined by different analytical methods were compared by one-way analysis of variance (SPSS 20.0; for Windows, Chicago, IL, USA). The level of significance was pre-set at $P < 0.05$. *Post hoc*-comparison of results was performed by the LSD test.

III. RESULT AND DISCUSSION

The legume grains tested here were characterized by contents of dry matter (DM), crude ash, and starch, with means ranging as follows: 890 – 905 g/kg, 28 – 36 g/kg DM, and 13 – 467 g/kg DM (table 1). Mean sugar contents analysed according to the different methods applied in this study are given in table 2. The sugar contents measured by the anthrone method varied between 56 – 95 g/kg DM with lowest and highest values with field bean and lupine grains, respectively. The gravimetric method and predominantly the HPLC methods, too, provided results that are not equivalent to that derived from the anthrone method. The sugar contents according to GRAVI, HPLC-1, HPLC-2 resulted in a remarkable underestimation ($P < 0.05$) compared to the anthrone method. HPLC-3 caused either a clear overestimation ($P < 0.05$) or a satisfactory agreement to anthrone sugar, but only in an individual case (for pea, var. 'Phönix'). The mean BC varied between 37.3 (field bean, var. 'Limbo') and 47.0 (pea, var. 'Phönix') g LA/kg DM (table 1).

Table 1: Contents of dry matter, crude ash and starch, and buffering capacity of legume grains from different species and varieties

Species (variety)	dry matter [g/kg]	crude ash [g/kg dry matter]	starch [g/kg dry matter]	buffering capacity [g LA/kg dry matter]
Lupine ('Bora')	904	36	13	37.4
Lupine ('Borlu')	905	35	13	44.1
Pea ('Lisa')	890	32	455	39.1
Pea ('Phönix')	892	28	467	47.0
Field bean ('Limbo')	899	36	436	37.3
± pooled s.d.	2.11	1.8	15.6	2.37

LA = latic acid

Table 2: Sugar content (in g/kg of dry matter) of legume grains analysed with different methods

Species (variety)	ANTHRO	GRAVI	HPLC-1	HPLC-2	HPLC-3	pooled s.d.
Lupine ('Bora')	86^{b}	53^{c}	35^{e}	44^{d}	110^{a}	± 0.53
Lupine ('Borlu')	95^{b}	58^{c}	34^{d}	39^{d}	104^{a}	± 0.36
Pea ('Lisa')	61^{b}	38^{c}	24^{d}	31^{c}	78^{a}	± 0.68
Pea ('Phönix')	77^{a}	54^{b}	23^{c}	23^{c}	81^{a}	± 0.40
Field bean ('Limbo')	56^{b}	n.a.	21^{d}	34^{c}	65^{a}	± 0.29

ANTHRO: anthrone method; HPLC-1: glucose, fructose, sucrose; HPLC-2: sugars according to HPLC-1 plus 'galactose'; HPLC-3: sugars according to HPLC-2 plus raffinose, stachyose and verbascose; n.a.: not analysed; GRAVI: gravimetric method according to Naumann and Bassler ([18]), calculated as sucrose; [abcd] Means within different superscripts within a line differ with $P < 0.05$.

From this, the S/BC quotient was calculated for each legume grain and sugar content according to the different analytical methods of sugar analysis (Fig. 1). Taking the anthrone method into account, S/BC quotients ranged between 1.46 (field bean, var. 'Limbo') and 2.30 (lupine, var. 'Bora'), with positive prognosis for ensilability (meaning S/BC quotient of > 2 g sugar/g lactic acid when ensiled with DM content of 65%; [27], [28]) with both lupine varieties only. The same prognosis was given for both lupine grains when the S/BC quotient was calculated by use of sugar determined via HPLC-3,

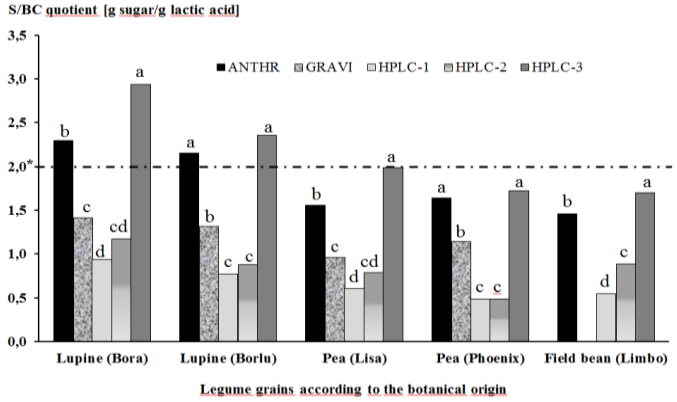

Figure 1: Quotient of sugar and buffering capacity (S/BC quotient; *the dashed line indicates positive prognosis for ensilability, meaning that the S/BC quotient amounted to at least 2 g sugar/g lactic acid [27], [28].)

but the S/BC quotient for lupine, *var.* 'Bora', was clearly higher than revealed following anthrone method. The S/BC quotients calculated by use of the other methods for sugar analysis applied here were in every case substantially lower ($P < 0.05$) compared to the anthrone-based S/BC quotient in question.

To produce high-quality silages with DM contents of around 65%, which might be achieved when harvested in an immature state under practical conditions, an S/BC quotient of 2 g sugar/g LA or more is required ([27]). Because of the low sugar but high protein content, however, the S/BC quotient of legume grains is frequently below this level, which in turn indicates poor ensilability. In the current study, the required S/BC quotient was only achieved by the grains of both lupine varieties, but the other legume sources were clearly below 2 g sugar/g LA. Calculations based on either reducing sugars or the sum of mono- and dimeric carbohydrates, with or without galactose, resulted in substantially lower S/BC quotients. The fraction appeared in the chromatogram as galactose, however, ranged from 0 (pea, *var.* 'Phönix') to 13 (field bean, *var.* 'Limbo') g/kg DM which was equivalent to up to 38% of the totally analyzed simple sugars. This is in a good agreement with Gefrom ([6], [7], [8]).

Despite of low S/BC quotients in the current study, all legume grains were nevertheless successfully ensiled which is in good agreement with the literature ([5], [6], [7], [8], [26], [28]). The authors demonstrated that not only simple sugars but also oligomeric carbohydrate fractions (raffinose, stachyose, verbascose) were decomposed to a remarkable degree by the fermentation process associated with ensiling. That might potentially also apply to carbohydrates with a degree of polymerization higher than verbascose. From literature it is known that about 3 and 15% of the DM of legume grains may consist of oligosaccharides ([6], [7], [8], [16]). Lowest (~ 3.0 – 3.5 g/kg DM) and highest contents (~ 5.5 – 7.0 g/kg DM) of stachyose plus raffinose and verbascose were found in field beans and lupine grains, respectively, with peas being in between. In the current study these oligomeric carbohydrates attributed after all with 48 – 72% to the totality of the low molecular sugars determined by HPLC, being a remarkably high percentage which should not be neglected.

The most prominent low molecular oligosaccharides in legume grains are indeed stachyose, raffinose and verbascose which are tri-, tetra- and pentasaccharides, respectively, with one, two and three galactose units within the molecule. Glucose and fructose units form the remaining of the individual molecule. In field bean, peas and lupine grains oligosaccharides seem to be dominated by varbascose, stachyolse and verbascose in an approximately balanced ratio, and stachyose, respectively ([6], [7], [8]).

Because previous studies already demonstrated that the oligosaccharides in question are largely be degraded by microbes involved in the process of ensiling ([5], [6], [7], [8], [26], [28]), it seems to be advisable to choose a method for sugar analysis that involves these oligosaccharides, particularly in legume grains. The S/BC quotient shall than be calculated on basis of that

kind of sugar analysis. The anthrone method is suitable to quantify simple sugars but can also be used for quantitative analysis of oligo- and polysaccharides provided they occur in the solution ([3]). Other polymer carbohydrates (> pentasaccharide), might not be determined although potentially useable for LA. Appropriate HPLC procedures may help to solve this problem. In terms of rapidity, specificity, sensitivity and precision, HPLC is currently one of the most powerful analytical techniques to characterize carbohydrates according to its type and quantity. However, it is a matter of choice and thus of our knowledge about fermentation biology which individual carbohydrate fractions are required to be analyzed and defined as sugars available for LAB in the early stage of ensiling. Beside of other factors the content of individual carbohydrates depends from the biological origin of the plant in question. In this way, when grasses from the local geographical latitude shell be characterized according to its S/BC quotient, fructans should additional recipients of HPLC analysis.

More sophisticated analytical methods may allow a highly specific sugar analysis, but the current S/BC recommendations were established on the basis of anthrone sugars. In the consequence this means that the progress in sugar analysis needs to be accompanied by experimentally based derivations on corresponding minimally required S/BC quotients and, thus, investigations on ensilability.

In this context one should be aware that added LAB may not only support the endophytic microbes by fermenting available sugars but they also may alter the kind of carbohydrate used for lactic acid production. As an example *Lactobacillus plantarum* (different strains) should be mentioned, which can ferment starch throughout the ensiling process ([7], [9], [11], [12], [19], [20]). Gefrom et al. ([7]) demonstrated that starch from field beans has been decomposed by ensiling without any additive to at most 14% whereby the use of *L. plantarum* (DSM 8862 and 8866) as silage additive increased the degree of starch degradation up to 47%. Despite all silages were of high quality, the lower pH in the silage prepared by use of *L. plantarum* reflected the use of extra carbohydrates. Contrary, the further addition of molasses had no continuing effect. Obviously interactions exist between the sugar source and the LAB in the plant material (whether added or not) according to their genotype, quantity and viability. It is questionable, however, whether that is a matter of interest when the initial time period of the ensiling process is particularly addressed.

IV. CONCLUSION

From the results of this study with selected legume grains it can be speculated that different methods of sugar analysis provide different results with consequences for the calculated S/BC quotient and, thus, the prognosis of ensilability. As long as recommendations for the minimal required S/BC to produce high-quality silages base on anthrone sugars, such a forecast of ensilability should only be performed when indeed the anthrone method was used to determine the sugar content. More powerful and sophisticated methods for sugar analysis are highly welcome, but further studies on the ensilability of the plant material are requested i) to identify the relevant sugar fractions which need to be taken into account in terms of ensilability and, ii) to adjust S/BC recommendations to these fractions.

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