The influence of sample preparation on the level of soluble and non-structural carbohydrates in forage crops and silages

P. Udén
Department of Animal Nutrition & Management, Kungsängen Research Centre, Swedish University of Agricultural Sciences, S-753 23 Uppsala, Sweden

Introduction

Oven drying at around 60°C is by far the most common method for preparing feed samples for analysis (e.g. Undersander et al., 1993) and is recommended by the European Union (71/393/EEC) for high-moisture solid feeds and for neutral detergent fibre analysis (ISO 16472:2006 IDT). Sugar losses during sample preparations are often ignored for reasons of not being volatile. Substantial losses were, however, seen after drying by Jones (1962), Lancaster et al. (1977), Deinum and Maassen (1994), Nielsen et al. (2007) and Pelletier et al. (2010). Deinum and Maassen (1994) investigated the effects of different oven drying temperatures on sugar recovery and attributed an increasing loss of sugars with decreasing oven temperature to respiratory losses. Similar results were reported by Pelletier et al. (2010) and the authors also reported that a 1-min microwave oven treatment before drying at 55°C gave similar values as for freeze drying by denaturing proteins that cause enzymatic conversions and respiratory losses. Metabolism of sugars during oven drying of silage is also likely to have been the reason for the 45% loss of glucose in maize silage (Nielsen et al., 2007).

Drying samples is not an option in the analysis of volatile silage components. Therefore, ammonia, organic acids, alcohols and other volatiles are extracted directly from the fresh forage or silage sample either after squeezing out the juice with a hydraulic press or after macerating the sample in a known amount of water. Little information is available on the possibility of using fresh crop or silage extracts for analysis of sugars, something which should reduce labor costs, particularly, in the case of subsequent analyses of fermentation products with the same type of method – chromatographic or spectroscopic. Normally, forage samples are extracted in water before the determination of soluble carbohydrates. However, many whole-crops contain starch requiring an acetate buffer for an enzymatic analysis of starch. In a sequential analysis of soluble sugars and starch it would be useful if acetate could replace water in the extraction of soluble carbohydrates.

In this study we compared: i) the effect of four extraction-preparation methods on the analyzed levels of water extractable soluble carbohydrates in grasses and legumes and three preparation methods for acetate extractable whole-crop cereals and ii) the ability of hot water and acetate to extract sugars in grasses and legumes.

Materials and methods

Samples
Samples were collected in Norway and Sweden and consisted of 24 direct-cut and wilted grasses (16), clovers (4), grass-clover mixtures (3), birdsfoot trefoil (1) and of 12 whole-crops of wheat (2), barley (2), oats (2), maize (4) and peas (2). Half of the samples were silages and half were crops. Approximately 2 kg of all samples were frozen at -25°C and ground in a meat grinder to pass a 13-mm sieve. The frozen and ground samples were thoroughly mixed and then immediately divided into three subsamples of equal size and prepared for analysis. One subsample was freeze dried (FDR), one was dried in a forced draught oven (ODR) and the remaining subsample was further divided into two sub-

subsamples and frozen fresh (FF) in two different ways. One sub-subsample was used to fill four 50-mL plastic test tubes with 10.000 g of the material, to be extracted later by hot water (grass-legume samples) or acetate buffer (whole-crops), and re-frozen. The second sub-subsample (grass-legume samples only) was put in duplicate in 'Ziplock' bags (100.00 g) and 100.0 mL water was added for the cold water extraction and re-frozen.

The samples for oven drying were placed on aluminum trays at a thickness of 1 cm and dried for 16 h at 60°C. Samples for freeze drying were first frozen to -80°C before starting the drying process. All dried samples were allowed to equilibrate at room temperature, ground in a knife mill (Brabender OHG, Duisburg, Germany) to pass a 1-mm sieve and bottled.

Extraction of soluble carbohydrates
Water (hot or cold) was used to extract all grass-legume preparations and acetate for all whole-crop samples and also, for comparative purposes, for the grass-legume ODR and FDR preparations. Specific sample amounts, extraction volumes, times and temperatures are specified in Table 1. Soluble carbohydrates [Su(s)] were assumed to consist of glucose, fructose, and sucrose and, as was discovered later, some "soluble" starch [St(s)]. Sub-samples taken were used for subsequent enzymatic analysis. Acetate extractions (0.0500 M, pH 5 with 280 mg CaCl2/L) were done on whole-crop samples to enable also the sequential analysis of starch and, for comparative purposes, on the grass-legume ODR and FDR preparations.

Table 1 Details on sample drying and extraction procedures for the samples which all had been stored frozen

Sample type	Treatment abbreviations[a]	Drying method	Solvent	Temp.[b]	Extraction time
Grasses and legumes	ODR, FDR	Oven or freeze drying	Water	100°C	3 min
	FF	None	Water	100°C	3 min
	FF	None	Water	-25°C/20°C	>24 h/0.5 h
	ODR, FDR	Oven or freeze drying	Acetate	60°C	40 min
Whole crops	ODR, FDR	Oven or freeze drying	Acetate	60°C	40 min
	FF	None	Acetate	60°C	40 min

[a]ODR = oven dried; FDR = freeze dried; FF = fresh-frozen; [b]-25°C/20°C means that the defrosted samples were re-frozen after adding water for >24 h, then defrosted again and kept in room temperature for 30 min before filtering.

Enzymatic analyses
The analysis of soluble sugars was based on an acid hydrolysis of sucrose and fructans, followed by enzymatic conversions of all fructose and glucose to glucose-6P. The amount of glucose equivalents was finally measured in a spectrophotometer from the absorbance change at 340 nm due to the conversion of NADP to NADPH. Whole-crop samples were analyzed for both soluble and insoluble starch [St(i)]. Soluble starch was defined as starch still in suspension after centrifugation for 5 min at 2000 x *g*. The starch analysis was similar to the soluble sugar analysis but included also two initial hydrolytic steps with amylase and amyloglucosidase.

Statistical analyses
All values were calculated per unit of fresh matter (FM) as these were considered unaffected

by losses of volatiles and would therefore be better for evaluating any possible loss of sugars by metabolism during sample preparation.

Analysis of variance for the effect of sample preparation and extraction method was done using the GLM procedure of Minitab (v. 15, Minitab Inc., State College, PA, USA).
Grass-legume samples:
1. Effects of sample preparation on Su(s): 96 observations (duplicate means) from the water extracted grass-legume samples were used which were made up of 24 samples and 4 preparations (FDR, ODR and FF extracted hot or cold). The fixed factors of the model were sample and preparation.
2. Effect of solvent on Su(s): 96 observations made up of 24 samples, 2 preparations (FDR and ODR) and 2 extractions (water and acetate) with sample and extraction method as fixed factors. Whole-crop samples:
Effects of preparation method on Su(s), St(s), St(i) and total non-structural carbohydrates: 36 observations from 12 samples and 3 preparations (FDR, ODR, FF). Fixed factors in the model were sample and extraction method.

Results and Discussion

Grass-legume samples
Table 2 shows results for the grass-legume Su(s) analysis. No clear differences were seen among the FDR and the two FF variants. The ODR preparation resulted, however, in lower levels than all the other preparations with a 19% lower level compared to the FF hot water treatment ($P<0.05$; total average) and for silage, this value was 29% lower ($P<0.05$). Extraction with either water or acetate buffer resulted in very similar Su(s) levels in the grass-legume samples with 25.3 and 24.4 g/kg FM for acetate and water, respectively. A possible explanation for the lack of effect on the grass-legume crops may have been a rapid dehydration, as a result of using only a 1-cm layer of sample material on the trays as opposed to 6 to 10-cm layers used by Pelletier et al. (2010). In silage, a more rapid metabolism due to the presence of microorganisms may have caused an increased loss of sugars.

Table 2 Comparisons of sample preparation of 24 water extracted grass-legume crop and silage samples on recovery of soluble sugars [Su(s)] expressed in g/kg of fresh matter

Form	Preparation: Extraction:	ODR Hot	FDR Hot	FF Hot	FF Cold	SEM	P=
Crop		25.9a	29.1	28.5	30.0b	0.85	0.012
Silage		19.2a	23.5ab	27.0b	23.9b	1.17	0.001
All		22.6a	26.3b	27.8b	27.0b	0.53	<0.001

ODR = oven drying; FDR = freeze drying; FF = fresh-frozen; Hot = hot water; Cold = cold water; a,b = treatment means followed by different letters differ significantly ($P<0.05$).

Whole-crop samples
Small differences were detected in Su(s) values with slightly (average 7%) lower values for the ODR preparation (Table 3), compared to FDR and FF ($P<0.05$). A surprisingly high proportion of the starch was soluble in the acetate buffer with mean of 0.34 for the FF preparation. It varied from very low (0.06) in pea silage to very high (0.85) in the early cut maize crop (not shown). More St(s) was solublized by the FF preparation than the FDR preparation ($P<0.05$) but there was no effect of sample preparation on total starch (Table 3).

This also resulted in a higher level of soluble carbohydrates (sugars and starch) for the FF preparation (P<0.05).

Table 3 Effect of sample preparation method on concentrations of soluble sugars and starch in 12 whole-crop crop and silage samples extracted in hot water (g/kg fresh matter)

	ODR	FDR	FF	SEM	P=
Soluble sugars	21.3a	22.7b	23.2b	0.41	0.008
Soluble starch	9.8a,b	6.4a	15.7b	1.84	0.006
Insoluble starch	51.2	54.7	46.9	2.34	0.086
Total	82.3a	83.8	85.9b	0.76	0.012

ODR = oven drying; FDR = freeze drying; FF = fresh-frozen; a,b = treatment means followed by different letters differ significantly (P<0.05).

Analytical considerations

For practical and economical reasons, it is advantageous to analyze soluble sugars and other soluble components, using a single preparation. This study showed that cold water gave similar results as for hot water extraction of the FF preparations. Cold water extraction of fresh-frozen samples can therefore be recommended for the analysis of soluble components. It is also likely that a procedure using a hydraulic press will give similar results. The best drying procedure seemed to be freeze drying, even though it did not always differ from ODR (Table 2). The risk of oven drying is a prolonged drying time when overloading the dryer with too thick sample layers and too many trays.

If both starch containing whole-crop samples and grass-legume samples are analyzed routinely, this study shows that the acetate buffer gives similar results as water extraction. The acetate buffer can therefore be recommended as a single extraction medium to avoid having separate protocols for the two sample categories. However, if silage fermentation products are also analyzed, water must be used as the acetate buffer would otherwise swamp the HPLC chromatogram. The most convenient method would then be to use the FF-cold water extraction procedure and add a higher strength acetate buffer after taking a sub-sample for fermentation end-products and before continuing with analysis of sugars and starch.

The presence of high levels of St(s) in the whole crop samples, particularly in the FF preparations, was surprising and has not been widely recognized in the feed science literature. Soluble starch, as a proportion of total starch, ranged from 0.06 in late cut maize silage to 0.85 in early cut maize crop with an average of 0.34 (data not shown). The forms of soluble starch (amylose, amylopectin or dextrins) or if it was part of any granular structure were not investigated. If this type of starch exists in the form of granules, these granules must be small enough to resist centrifugation at 2000 x g for 5 min. It is likely that St(s) has a more similar rate of degradation to Su(s) than to St(i) and that it should, therefore, be included in the water soluble carbohydrate fraction. If ignoring the presence of St(s), an underestimation of total starch will occur in a sequential analysis of Su(s) and St(tot), depending on the amount of extract that is removed for the Su(s) analysis.

Conclusions

Fresh, freeze dried or cold-water extracts are recommended for analysis of soluble sugars in forage crops and silages. Acetate buffer or water extract similar amounts of sugars and can be recommended for both grass-legume and whole-crop samples. A large proportion of the

starch may exist in a "soluble" form. This fraction should not be ignored in the analytical procedure and may also have other nutritional properties than the insoluble form.

References

Deinum, B., Maassen, A., 1994. Effects of drying temperature on chemical composition and in vitro digestibility of forages. Anim. Feed Sci. Technol. 46, 75-86.

Jones, D.I.H,1962. Note on the pre-treatment of herbage samples for determination of soluble carbohydrate constituents. J. Sci. Food Agr. 13, 83-86.

Lancaster, R.J., Wilson, R.K., Jury, K.E., Newth, R.P., 1977. Comparison of methods of preparing grass and silage samples for laboratory analysis. N. Z. J. Agr. Res. 20, 157-161.

Nielsen, T.S., Kristensen, N.B., Weisbjerg, M.R., 2007. Effect of harvest time on fermentation profiles of maize ensiled in laboratory silos and determination of losses at 60°C. Acta Agr. Scand. Sect. A 57, 30-37.

Pelletier, S., Tremblay, G.F., Bertrand, A., Bélanger, G., Castonguay, Y., Michaud, R., 2010. Drying procedures affect non-structural carbohydrates and other nutritive value attributes in forage samples. Anim. Feed Sci. Technol. (in press).

Undersander, D., Mertens, D., Theix, N., 1993. Forage Analyses Procedures. Natl. Forage Testing Assoc., Omaha, NE, USA.