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Water-Soluble Carbohydrates in Dried Plant

I. Kerepesi,* M. Tóth, and L. Boross

Department of Chemistry and Biochemistry, Janus Pannonius University, H-7601 Pécs, Hungary

Within the framework of studying the fructan metabolism of wheat, several comparative investigations of different preparation and extraction methods were accomplished to find the most suitable methods for quantitative determination of primary water-soluble carbohydrate contents of plant material. Changes in sugar content of oven-dried samples were detailed. Oven-dried plant materials have significantly lower total water-soluble carbohydrate content than fresh ones. The decreases were higher in monosaccharide level than in fructan and glucan. Sucrose concentration of dried samples was similar to fresh ones. Changes in sugar components were different, so their ratio in the fresh samples were not identical with the dried ones. The efficiency of extraction of soluble carbohydrate could be increased by using boiling water as the first extracting solution instead of ethanol. The TLC analysis show that oven-dried plant samples contain significantly lower amount of fructans with low degrees of polymerization (DP). The conclusion is, that neither glucose, fructose, or oligosaccharide content, nor the DP of fructans of dried plant are similar to the fresh one.

Keywords: *Carbohydrates; fructan; oligosaccharide; glucose; fructose; sucrose; TLC; wheat (Triticum aestivum L.); oven-dried*

INTRODUCTION

Vascular plants contain a wide range of water-soluble carbohydrates. Their chemical natures and the amounts present vary in both space and time, from species to species, organ to organ, and tissue to tissue, as well as diurnally, and with season. Studying the fructan metabolism requires exact determination the amount of glucose, fructose, sucrose, and oligosaccharides. Due to the development of many sophisticated methods of analysis the existing literature on fructans in plants is vast. At the same time there are a number of carbohydrate preparation and extraction methods in this field, based on mostly the modification of techniques developed by Wylam (1954) and Koch and Hehl (1975), which give incomparable or dissimilar results.

In all widespread methods the plant material is killed prior to extraction in order to stop enzymatic processes and thus right from the start to prevent, as far as possible, any changes in the concentration of carbohydrate components. Using hot water or ethanol (Sims *et al.*, 1991; Albrecht *et al.*, 1993; Simmen *et al.*, 1993) and liquid nitrogen (Solhaug, 1991; Bancal and Triboi, 1993), freeze-drying (Winzeler *et al.*, 1990; Praznik *et al.*, 1992), or oven-drying samples (Judel and Mengel, 1982; Virgona and Barlow, 1991) are the most popular methods to inactivate enzymes. This first step is very important and has a great effect on the detectable amount of carbohydrates in plant samples. It should however be mentioned that carbohydrate degrading enzymes are not inactivated by freeze-drying, so that they may recover their activity during extraction (Koch and Hehl, 1975). On the other hand there is also a risk of enzymatic changes in sugar concentration during drying (Koch and Hehl, 1975). In the daily practice the extraction method for quantitative determination of sugars is usually based on the extaction of samples in boiling water or/and ethanol (Livingstone, 1990; Farrar and Pollock, 1993).

This study is part of our experimental work dealing with the metabolism of fructans in plants. Our purpose was to determine the error of analyses based on the different, recently used carbohydrate preparation and extraction methods, especially to compare the water-soluble carbohydrate content of fresh and oven-dried plant material.

MATERIALS AND METHODS

Plant material was winter wheat (*Triticum aestivum* L. cv. Martonvásári 21).

The seeds were grown hydroponically over 21 days in a growth chamber containing complete nutrient solution, with a 16 h daylight period 23 °C, and an 8 h dark period 18 °C. The plants were removed on the 4th, 7th, 14th, and 21st day and separated into shoot and root or leaf, stem (crown plus whorl), and root. The different preparation and extraction methods were immediately started. In this study mostly data of leaves are detailed.

Extraction of Samples: (*a*) Samples of 2.5 g fresh weight were extracted twice in 40 mL of boiling water, and twice in 40 mL of aqueous (80% v/v) boiling ethanol with 15 min for each individual extraction.

(*b*) Samples of 2.5 g fresh weight were extracted twice in 40 mL aqueous (80% v/v) boiling ethanol and twice in 40 mL boiling water 15 min for each individual extraction.

(*c*) Samples of 2.5 g fresh weight were extracted four times in 40 mL boiling water with 15 min for each individual extraction.

(*d*) Samples of 2.5 g fresh weight were dried in preheated oven at 105 °C for one hour and overnight at 70 °C. The dried tissue was ground, and the water-soluble carbohydrates were extracted as in methods *a*, *b*, and *c*.

In all cases of extraction methods (*a*−*c*) boiling was performed under reflux, and the fractions were collected, cleared by filtering through Whatman no. 42 (pore size 2.5 μm) paper. Filtrates were dried under reduced pressure (40 °C), using vacuum evaporator (Buchy model SB, Sweden) and were dissolved in distilled water.

Carbohydrate Solution. Pure carbohydrate solution was a mixture that contained 5 mM glucose, fructose and sucrose (Sigma), 1 mg/mL fructan (inulo-oligosaccharides from tubers of *Helianthus tuberosus*), and glucan (from barley, Sigma). This solution was completed with 5 mg/mL BSA (bovine serum albumin, Sigma), or 50 mg/mL BSA, or 5 mg/mL amino acid (L-alanine, Sigma), or 50 mg/mL amino acid, respectively and were dried the same way than fresh plant samples.

Chemical Analysis. Oligosaccharides were hydrolyzed by boiling in 0.5% HCl for 60 min.

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Table 1. Glucose, Fructose, Oligoglucan, and Fructan Content (mg/g Fresh Weight) in the Samples of 7 Day Old Fresh and Oven-Dried Wheat Leaves Using Different Extraction Methods[a]

	glucose		oligoglucan		fructose		fructan	
	mean SD	$SD_{0.1\%}$	mean SD	$SD_{0.1\%}$	mean SD	$SD_{0.1\%}$	mean SD	$SD_{0.\%}$
a	11.10 ± 1.22		18.06 ± 2.13		1.88 ± 0.2		23.64 ± 3.11	
b	8.21 ± 0.93	1.95	12.61 ± 1.53	3.33	1.96 ± 0.2	−	15.73 ± 1.86	4.66
c	8.46 ± 0.89	1.90	17.31 ± 1.86	−	0.88 ± 0.1	0.3	16.81 ± 1.95	4.66
a^+	0.81 ± 0.09	1.55	7.51 ± 0.81	2.88	0.55 ± 0.07	0.27	12.52 ± 1.56	4.44
d b^+	0.93 ± 0.1	1.55	6.36 ± 0.72	2.88	0.65 ± 0.07	0.27	11.41 ± 1.46	4.26
c^+	0.88 ± 0.09	1.55	6.12 ± 0.69	2.88	0.65 ± 0.07	0.27	11.63 ± 1.48	4.39

[a] SD = standard deviation; $SD_{\%}$ = significant difference, $n = 12$.

The amount of free (analyzed before hydrolyses) and bound (oligosaccharides, analyzed after hydrolyses) glucose, fructose and sucrose was measured with the use of Boehringer Mannheim GmbH glucose/fructose/ sucrose, no. 716 260 kits (Wagner *et al.*, 1983).

Total water-soluble carbohydrate determination based on the phenol−sulfuric acid method (Dubois *et al*., 1956), involved adding 1 mL of 5% phenol solution and 5 mL of concentrated sulfuric acid to 200 μL of samples and reading the absorbance at 510 nm after 20 min. Sucrose was used as standard.

Before carbohydrate analyses the protein content, if was necessary, was precipitated (Chatterton *et al*., 1993) and centrifuged at 10000*g*.

Oligofructans were fractionated on silica gel TLC plates (Silica gel, HPTLC 60 F_{254}, layer thickness 0.2 mm, Merck, Germany) with 1-butanol−ethanol−water (5:3:2) four times (Suzuki 1989). Fructans were detected with thymol reagent using inulo-oligosaccharides from tuber of *Helianthus tuberosus* as standard (Praznik *et al.*, 1992). Lanes were analyzed with a densitometer (Sharp JX-325 Image Master System, Pharmacia Biotech) using fructose calibration curve.

Three replications of each experiment were performed. The data were analyzed by the STATGRAPHICS statistical package, using the *t*-test and ANOVA functions (STSC INT. 1991, Users Gide, Statgrafics Version 5.0) to assess significant differences between means.

RESULTS

In these experiments the influence of some preparation and extraction methods on the soluble carbohydrate content in plant material was studied. The effect of drying on water-soluble carbohydrate content of wheat seedling was especially detailed. Our previous results show (not shown) that the first four collected fractions of extracted plant material using the above detailed methods (*a*−*d*) contained almost all amounts of the soluble carbohydrates examined here.

Table 1 shows the concentration of free and bound glucose and fructose content in 7 day old seedling leaves using different preparation and extraction methods. According to these values not only boiled water but also ethanol had to be used to extract the carbohydrates. Furthermore, the efficacy increased, if the first extracting solution was boiling water instead of ethanol ($P > 0.001$), except for free fructose content in fresh samples which showed similar level using both methods (*a* and *b*). The greatest differences were measured between the soluble carbohydrate content of fresh and dry samples ($P > 0.001$).

To determine exactly the effect of drying, qualitative and quantitative analyses of primary water-soluble carbohydrates of 4−21 day old wheat seedlings were accomplished with the use of fresh and dried samples, in parallel. In this study the "*a*", and "a^+" methods were compared (Table 1), so drying—as treatment—was the only difference between samples.

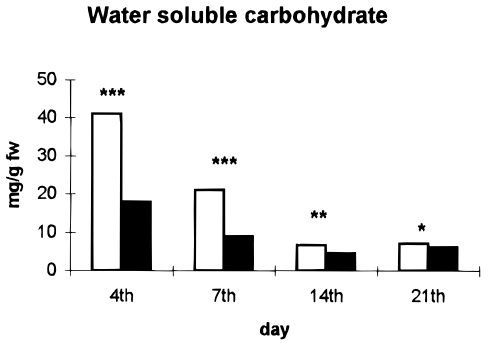

Figure 1. Total water-soluble carbohydrate content in the leaves of fresh and oven-dried 4−21 day old wheat seedlings. Symbols: fresh, □; dried, ■. The differences from the control were significant at the $P < 0.05$ (∗), $P < 0.01$ (∗∗), and $P < 0.001$ (∗∗∗) levels.

Table 2. Glucose, Fructose, and Oligosaccharide Content in Dried Wheat Leaves in the Percentage of Fresh Leaves

day	glucose (%)	fructose (%)	fructan (%)	glucan (%)
4th	3.08	15.91	65.81	27.47
7th	3.02	14.75	12.79	20.45
14th	2.31	3.61	5.10	11.41
21st	5.03	3.12	56.10	60.40

In 4−21 day old seedlings the total water-soluble carbohydrate content was significantly lower ($P > 0.001$−0.05) in dried samples than in fresh ones. These results were shown by the soluble carbohydrate content of leaves (Figure 1).

Considering the components of the total soluble carbohydrates, our results showed that the highest difference was measured in the free glucose (Figure 2a) and fructose level (Figure 2b). By describing these data in the percentage of fresh samples as control (Table 2), it can be seen, that effect of drying on free hexose content depended on maturity.

Figure 2c,d shows the time-dependent changes of oligoglucan and fructan level. The effect of drying on oligosaccharide content was not as dramatic as on monosaccharides: 5−65% were left (Table 2). In 4−14 day period, the degree of decrease in fructan and that in glucan content was different ($P < 0.001$): on the 4th day the glucan was greater after that fructan degradation showed a higher degree. As Table 3 shows changes in dry matter in the 4−21 day period are too small (72−75%, compare with 4 day value) to be responsible for differences in carbohydrate content.

Sucrose concentration of dried samples was 85−90% of the percentage in fresh ones (not shown); its changes were not significant.

These results were completed with TLC analysis. Considerable fructan content was detected only in the early developmental period (4−14 day). Wheat seedling

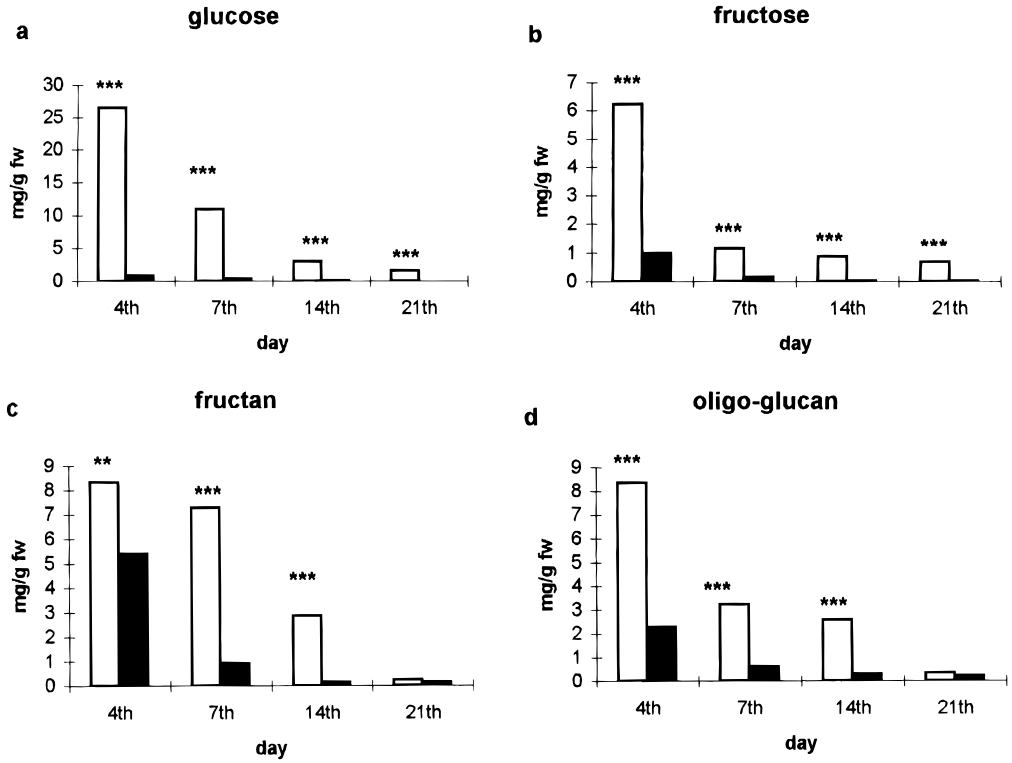

Figure 2. Glucose (a), fructose (b), fructan (c), and oligoglucan (d) content in the leaf of fresh and oven-dried 4−21 day old wheat seedlings. Symbols: fresh, □; dried, ■. The differences from the control (fresh) were significant at the $P < 0.05$ (∗), $P < 0.01$ (∗∗), and $P < 0.001$ (∗∗∗) levels.

Table 3. Dry Weight of 2 g of Fresh Wheat Leaf and Root in 4−21 Day Developmental Period[a]

	leaf (mg)		root (mg)	
day	mean SD	$SD_{0.1\%}$	mean SD	$SD_{0.1\%}$
4th	264 ± 32		196 ± 24	
7th	271 ± 31	–	200 ± 16	–
14th	202 ± 14	23.31	144 ± 20	20.86
21st	210 ± 21	25.54	169 ± 18	19.69

[a] SD = standard deviation; $SD_{\%}$ = significant difference, n = 30.

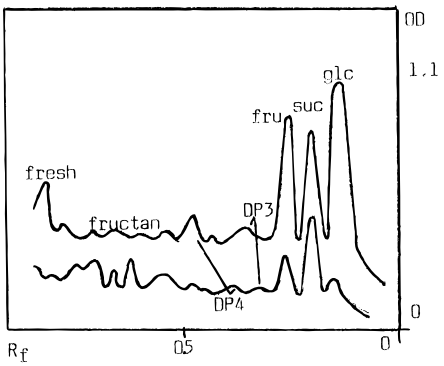

Figure 3. Separation of glucose (glc), sucrose (suc), fructose (fru), and oligofructans in 7 day old dried and fresh leaves of wheat seedlings.

contained a substantial low DP fructan pool in both plant material, but relative distribution of polymers in dried samples were different when compared with fresh ones. These data were represented with the densitogram of a 7 day old leaf (Figure 3). As the densitometrical investigation shows the concentration of DP 3 and DP 4 fructans were significantly lower ($P < 0.001$) in the dried plant samples.

To investigate the effect of drying on the sugar content of plant samples, the extracts (using "a" method) of 7 day old fresh plant material were dried in an oven instead of the plant itself. Carbohydrate contents of dried solutions were lower than control (not dried) ones: in the percentage of control glucose content of samples was 15%, fructose 43%, sucrose 92%, fructan 72%, and oligoglucan 86%. If pure carbohydrate mixture were dried in oven, a change in the sugar concentration, as was expected, was not detectable (not shown), but if protein or amino acid were added into these solutions before drying, the measured sugar content was lower than the control (sugar mixture without protein or amino acid). As Figure 4 shows, the detectable glucose and fructose content was significantly lower ($P < 0.001$) and depended on protein and amino acid concentration. The rate of decreasing was the highest

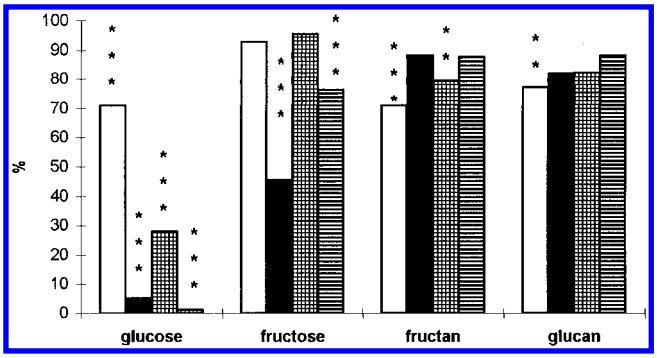

Figure 4. Glucose, fructose, fructan, and glucan content in oven-dried originally 5 mM glucose, fructose, and sucrose and 1 mg/mL fructan and glucan containing mixture completed with 5 mg/mL or 50 mg/mL protein or amino acid. Data are shown in the percentage of control (pure carbohydrate mixture without protein or amino acid) ones. The bars are from left to right 5 mg/mL BSA, 50 mg /mL BSA, 5 mg/mL amino acid, and 50 mg/mL amino acid. The differences from the control were significant at the $P < 0.05$ (∗); $P < 0.01$ (∗∗) and $P < 0.001$ (∗∗∗) levels.

in the glucose content, similar to those obtained in the dried plant samples.

DISCUSSION

In this study the soluble carbohydrate content and relative contributions of primary sugar components in oven-dried and fresh plant material was compared. Marked differences in the sugar concentration depending on the preparation and extraction methods.

In agreement with previous observation (Machado de Carvalho and Dietrich, 1993) we found that four extraction runs were needed both in dried and fresh plant samples to extract the carbohydrates examined here. In some works only three extraction steps were published (Bancal and Triboi 1993). In contrast Koch and Hehl (1975) obtain hardly any amounts of soluble carbohydrate in the third extraction of wheat seedlings. Praznik *et al.*(1992) used only boiling water for extraction of soluble carbohydrate, while hot ethanol followed by hot water is the most common method in literature (Wylam, 1954; Kühbauch and Schyder, 1989; Housley and Pollock, 1993; Farrar and Pollock, 1993). We measured higher sugar concentrations in the samples which were extracted first in boiling water instead of hot ethanol. Temperature is one of the most important factor for enzymes inactivation and boiled water has a higher temperature than hot ethanol.

Koch and Hehl (1975) published some preparation and extraction methods of plant carbohydrates (wheat seedlings, grass, and alfafa) and concluded that drying of plant material and subsequent 2-fold extraction in ethanol and water gives better results as compared to fresh samples. With reference to this work numerous authors used this method (Judel and Mengel, 1982; Nelson and Spollen, 1987) in spite of the fact that the results of Koch's experiment clearly show that only sucrose and oligosaccharide concentration of dried samples were higher or similar than these of fresh ones. The amount of monosaccharides and fructan was significantly lower in dried plant, showing a good coincidence with our results.

Degree of changes in various sugar components due to oven-drying method were different; consequently their relative contribution in fresh plant samples were not identical with those of the dried ones (Table 2).

The TLC analysis of our fresh samples were similar to previous results (Wagnet *et al.*, 1983; Jeong and Housley, 1990), but quantitative evaluation of different DP of oligofructans compared with dried ones are not referred to in the literature.

Explaining the molecular background of these results was not purpose of this work, but our results clearly show that the cause of drying-dependent changes in amount of soluble carbohydrate level should be researched not only in the different enzymatic activity of the certain parts of plant, and/or in the different heat stabilities of the enzymes but in the different protein and amino acid contents (Figure 4). Glucose participates in cross-linking reactions with protein by a complex glycosylation reaction between amino and carbonyl groups known as the Maillard reaction (Koster and Leopold, 1988; Lee, 1995; Van Soest, 1965; 1994). Furuseth *et al.* and Phillipou and Phillips referred to a nonenzymatic reaction between glucose and hemoglobin molecules, depending on the glucose concentration. Similar reactions could be proceeding because the concentration of sugars in plant tissues are increasing continuously during drying.

The conclusion is, that the extract of oven-dried plant material are not adequate to study the fructan metabolism. Neither glucose, fructose, and oligosaccharide content nor the DP of fructans of dried plant are similar to the fresh one. Experiments to study molecular background of these results can be the objects of further studies.

ABBREVIATIONS USED

Suc, sucrose; DP, degree of polymerization; Fru, fructose; Glc, glucose; TLC, thin-layer chromatography; wsc, water-soluble carbohydrate.

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