Determination of Water-Extractable Nonstructural Carbohydrates, including Inulin, in Grass Samples with High-Performance Anion Exchange Chromatography and Pulsed Amperometric Detection

Michael Raessler,* Bianka Wissuwa, Alexander Breul, Wolfgang Unger, and Torsten Grimm

Max-Planck-Institut für Biogeochemie, Hans-Knoell-Strasse 10, D-07745 Jena, Germany

The exact and reliable determination of carbohydrates in plant samples of different origin is of great importance with respect to plant physiology. Additionally, the identification and quantification of carbohydrates are necessary for the evaluation of the impact of these compounds on the biogeochemistry of carbon. To attain this goal, it is necessary to analyze a great number of samples with both high sensitivtiy and selectivity within a limited time frame. This paper presents a rugged and easy method that allows the isocratic chromatographic determination of 12 carbohydrates and sugar alcohols from one sample within 30 min. The method was successfully applied to a variety of plant materials with particular emphasis on perennial ryegrass samples of the species *Lolium perenne*. The method was easily extended to the analysis of the polysaccharide inulin after its acidic hydrolysis into the corresponding monomers without the need for substantial change of chromatographic conditions or even the use of enzymes. It therefore offers a fundamental advantage for the analysis of the complex mixture of nonstructural carbohydrates often found in plant samples.

KEYWORDS: Nonstructural carbohydrates (NSC); inulin; grass samples; HPLC-PAD

1. INTRODUCTION

The determination of carbohydrate composition and the exact quantitation of these carbohydrates are of growing importance in different kinds of environmental samples such as plants (*1*), soil (*2*), and water (*3*). Whereas carbohydrate analysis in nutritional products, such as fruit juices or ciders (*4*) or bakery products (*5*), serves both economic and health purposes, determination of carbohydrates in plant material is of mostly scientific interest, although economic aspects may also be important. It is known, for example, that the composition and amount of particular carbohydrates in grass may have a great impact on the efficiency of cattlery. Higher sucrose concentrations will have a positive effect on milk production in cows (*6*). On the other hand, the exact determination of carbohydrate distribution may add to the better understanding of the seasonal behavior of plant species (*7*). Their carbohydrate composition may vary with season, light availability, and vegetative stage. The composition of nonstructural carbohydrates (NSC) will reflect growth and variations in photosynthesis as well as abiotic stress phenomena. During photosynthesis, plants remove considerable amounts of carbon dioxide from the atmosphere to store the carbon either as monosaccharides (e.g., glucose), oligosaccharides (e.g., sucrose), or polysaccharides (e.g., inulin,

starch). On the other hand, plants also release a certain amount of carbon to the environment, for example, by degradation, humification, or the action of bush or forest fires. Consequently, carbohydrates in plants are part of the biogeochemical carbon cycle (*8*), and their exact and reliable determination is of great importance for the accurate assessment of biogeochemical carbon balances.

This implies that a great number of analyses have to be carried out because carbohydrate distribution and concentration not only vary with season, as mentioned above, but also with plant species and even the plant compartment, such as root, stem, trunk, or leaf. Moreover, the determination of NSC, being, in general, a mixture of sugar alcohols, monosaccharides, oligosaccharides, and polysaccharides in one sample, is a highly challenging task for both sample preparation and analysis.

Several techniques are applied for the analysis of carbohydrates. "Classical" UV−vis spectroscopy is performed after reaction with coloring reagents, such as anthrone (*9*) or potassium ferricyanide (*10*). Application of these methods, however, does not allow the identification and quantification of individual carbohydrates. Strictly speaking, only the sum of all the carbohydrates that are amenable to the specific method applied is obtained. There are several drawbacks of these methods: for exact quantification of carbohydrates, the different response factors of individual carbohydrates have to be taken

* Corresponding author (e-mail raessler@bgc-jena.mpg.de).

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into account during calibration. This is almost impossible in complex mixtures of which the composition is not known. Additionally, because results obtained depend on the applied method, comparability of data is limited. To overcome these drawbacks and to allow global comparability of results, unbiased identification and quantification of individual carbohydrates in plant extracts are prerequisites.

A very promising tool is liquid chromatography with either pulsed amperometric detection (HPLC-PAD) (*11*) or mass spectrometry (HPLC-MS) (*12*). Combination of these detectors with liquid chromatography allows selective determination of single carbohydrates down to the picomole level.

In the following, we will present a straightforward and rugged procedure for the determination of water-extractable NSC and inulin in plant samples of the species *Lolium perenne*. Apart from sugar alcohols, as well as glucose, fructose, and sucrose, polysaccharides of the fructan-type, such as inulin, are known to be very important reserve carbohydrates in several perennial ryegrass species, among them *L. perenne*, and have therefore to be taken into account (*13, 14*). Separation and quantitation of simple carbohydrates were based on isocratic high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) using 18 mM NaOH as eluent. After hot water extraction of plant samples at $T = 80$ °C and subsequent centrifugation, water-soluble mono- and oligosaccharides were directly determined by HPAEC-PAD. Determination of inulin was carried out in a second water extract after acidic hydrolysis of the polysaccharide into the monomers fructose and glucose. The data thus obtained were corrected for both glucose and fructose concentrations from the water extracts as well as glucose and fructose concentrations from hydrolysis of sucrose using an Excel program written in our laboratory.

2. MATERIALS AND METHODS

2.1. Reagents. NaOH 50%, HCl 30%, and HClO₄ 40%, suprapur, as well as fructose, galactose, glucose, and sucrose (all p.a.) were purchased from Merck, Darmstadt, Germany. Arabinose, fucose, raffinose, and rhamnose as well as inulin from chicory and dahlia tubers were purchased from Fluka, Buchs, Switzerland. Inositol, mannitol, salicin, and sorbitol were purchased from Sigma-Aldrich, Deisenhofen, Germany.

2.2. Preparation of Standard Solutions and Mixtures of Standard Solutions. Bulk standards of each carbohydrate and sugar alcohol (1 g/L = 1000 ppm) were prepared by weighing 1 g of carbohydrate or sugar alcohol in a weighing crucible, which was transferred to a 1000 mL flask and filled to the mark with doubly distilled water, 18 MΩ (Millipore, Eschborn, Germany). These solutions were stored in 1 L plastic bottles at 4 °C in the dark. Standard working solutions of both single standards and standard mixtures at the 0.5, 1, 2.5, 5, and 10 mg/L concentration levels were prepared from the bulk standard by appropriate dilution with doubly distilled water. Standard working solutions were prepared fresh daily prior to chromatographic analysis.

2.3. Chromatographic Separation of Carbohydrates. The chromatographic separation of the 12 carbohydrates and sugar alcohols arabinose, galactose, glucose, fructose, fucose, rhamnose, raffinose, salicin, and sucrose as well as inositol, mannitol, and sorbitol was carried out using a Dionex ICS 3000 ion chromatography system equipped with a gradient pump and a pulsed amperometric detector. Separation was carried out on a CarboPac PA 10 (250 × 4.6 mm) analytical column, which was preceded by a Carbo Pac PG 10 guard column. Both columns were kept at a constant temperature of $T = 40$ °C by a column oven. A 25 μL sample loop was used for sample injection. An isocratic elution profile with 18 mM NaOH at a flow rate of 1 mL/min was used for the separation of carbohydrates. One analytical run lasted 30 min, after which the column was rinsed with
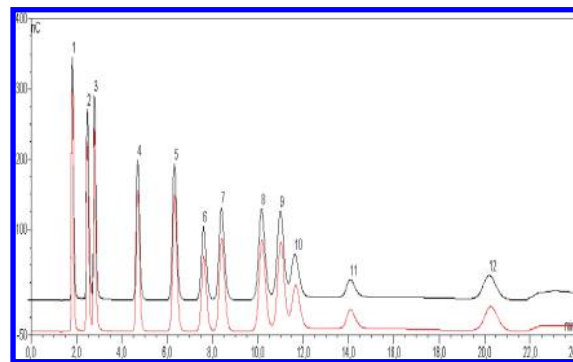

Figure 1. Analysis of the "standard mixture" including 12 carbohydrates and sugar alcohols; concentration = 10 mg/L for each carbohydrate. Peaks: 1, inositol; 2, sorbitol; 3, mannitol; 4, fucose; 5, salicin; 6, rhamnose; 7, arabinose; 8, galactose; 9, glucose; 10, sucrose; 11, fructose; 12, raffinose.

100 mM NaOH for cleaning. After the cleaning step, the column was re-equilibrated with 18 mmol NaOH before the next sample was injected onto the column. Regular column cleaning was mandatory for reproducibility of results, in particular, stability of retention times and separation of closely eluting peaks. In the case of sample extracts, additional column cleaning by rinsing with 250 mM NaOH at 1 mL/min for 1 h after 30 samples turned out to be necessary. All eluents were constantly purged with helium to avoid possible formation of carbonate from aerial carbon dioxide.

2.4. Preparation of Water Extracts for Determination of Water-Soluble Carbohydrates. Twenty milligrams of dried and ground plant material was weighed in a weighing crucible and transferred into a centrifugation tube. Five milliliters of doubly distilled water (Millipore) was added, and the tube was exposed to a shaking water bath (Julabo SW22, Seelbach, Germany) at $T = 80$ °C for 30 min. The centrifugation tube was cooled to room temperature and the water extract centrifuged at 4500 rpm (3260g) for 10 min (Sigma centrifuge 2-16, Osterode, Germany). After centrifugation, the liquid phase was separated from the undissolved sample pellet by pipetting into a 50 mL flask, which was filled to the mark with doubly distilled water. An aliquot of this solution was transferred to a sample vial and submitted to the chromatographic analysis of water-extractable carbohydrates.

2.5. Preparation of Water Extracts for Determination of Inulin. Sample preparation steps until centrifugation were the same as described under section 2.4. After centrifugation and separation of phases, the liquid phase was transferred in another centrifugation tube and brought to a volume of 10 mL by adding doubly distilled water. To this solution, 340 μL HCl 30% were added, resulting in an overall acid concentration of 1%. This solution was again placed in the shaking water bath at $T = 80$ °C for 60 min to allow for complete hydrolysis of inulin. After cooling the centrifugation tube to room temperature, the solution was transferred to a 50 mL flask and filled to the mark with doubly distilled water. An aliquot of this solution was transferred to a sample vial and submitted to chromatographic analysis of hydrolysis products of inulin, i.e. fructose and glucose.

3. RESULTS AND DISCUSSION

3.1. Chromatographic Separation of the Selected Carbohydrates and Sugar Alcohols. The chromatogram of the separation of the 12 carbohydrates and sugar alcohols arabinose, fructose, fucose, galactose, glucose, inositol, mannitol, raffinose, rhamnose, salicin, sorbitol, and sucrose is shown in **Figure 1**.

As can be seen from **Figure 1**, complete separation of all examined sugars was obtained under isocratic conditions; even the trisaccharide raffinose was eluted from the column using 18 mM NaOH at $T = 40$ °C. Completeness of elution was checked by recovery studies (cf. section 3.2.3 for details). Baseline separation was obtained for all components except glucose and sucrose, for which peaks eluted close to each other.

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For sufficient separation of these two compounds, the analytical column had to be carefully cleaned and reconditioned prior to analysis. If this was observed, distinct separation of these two carbohydrates was obtained that allowed their reliable and reproducible identification and quantification.

3.2. Method Validation. To verify the reliability of the method, both the chromatographic separation and the effectiveness of the extraction procedure, including the hydrolysis of inulin, were carefully examined. The appropriateness of the chromatographic method was checked using 12 carbohydrates and sugar alcohols often present in plant material. Once the isocratic separation of these carbohydrates was established, the stability of retention times and signal intensities were examined. Additionally, the linearity of the calibration range was checked. Linear calibration curves were used for calculating the limits of determination for each carbohydrate on the basis of the absolute amount of standard injected onto the column with the help of a 25 μL sample loop.

In a second step, the extraction procedure was verified for its effectiveness of quantitatively removing the water-soluble carbohydrates from the plant sample. Additionally, the effectiveness of the hydrolytic decomposition of the polysaccharide inulin, as well as the quantitative recovery of their monomeric congeners, fructose and glucose, was examined by the use of inulin standard solutions. After subsequent submission to hydrolytic decomposition and chromatographic separation, mass balances of both fructose and glucose were established, taking into account the hydrolytic equilibrium of the species in solution. This was done to prove the quantitative recovery of the monomers and thus the reliability of the method for the analysis of the nonstructural polysaccharides in the grass samples as well.

3.2.1. Stability of Retention Time and Signal Intensity. To verify the accuracy, reliability, and ruggedness of the chromatographic separation, both intraday ($N = 5$) and longer term stability ($N = 25$) of retention times, peak height, and peak area signals of each carbohydrate were determined. Whereas intraday stability values were determined as the means of five replicate analyses of each standard within 1 day, longer term stability was determined as the means of five intraday analyses recorded during a period of 5 weeks, resulting in a total of $5 \times 5 = 25$ analyses for each carbohydrate and sugar alcohol. Concentration of all standards was 10 mg/L. They were prepared fresh prior to each analytical series. Whereas intraday stability was high for all carbohydrates (RSD < 1%) and in accordance with observations of other authors (*15*), longer term examinations revealed shifts between 1 and 12% for retention times (raffinose, fucose), between 2 and 11% for peak areas (salicin, raffinose), and between 4 and 11% for peak heights (salicin, arabinose). All data for longer term stability are summarized in **Table 1**. Indicated are the mean values of the retention times, the peak heights, and peak areas for the examined carbohydrates, including their relative standard deviations.

The outcome of the longer term examinations clearly demonstrated the need for thorough cleaning and reconditioning of the column to keep the chromatographic process stable. This was even more pronounced when sample extracts were analyzed.

3.2.2. Linearity of the Calibration Range. For all 12 carbohydrates and sugar alcohols examined, the calibration range from 0.5 to 10 mg/L was checked for linearity. Calibration points were at 0.5, 1.0, 2.0, 4.0, 6.0, 8.0, and 10.0 mg/L. Regression coefficients r^2 for all carbohydrates were 0.997 (rhamnose, salicin) or better, except the sugar alcohols inositol, mannitol,

Table 1. Long-Range Examination of Stability of Retention and Signal Intensity ($N = 25$)

carbohydrate	t_R (min)	STD	RSD (%)	peak area	STD	RSD (%)	peak height	STD	RSD (%)
arabinose	8.4	0.4	4.5	26	2.5	9.6	89	9.9	11
fructose	14.3	0.9	5.8	14	1.1	8.4	27	2.6	9.5
fucose	4.7	0.6	12	25.3	1.5	6.1	139	14	10
galactose	10.2	0.6	5.6	38	2.2	6.3	103	7.1	7.6
glucose	11.1	0.6	5.1	41.1	1.9	4.8	100	6.2	6.1
inositol	1.8	0.01	0.5	29.2	1.9	6.6	273	22	8.3
mannitol	2.8	0.1	4.1	27.2	2.7	10.1	213	21	10
raffinose	20.6	0.2	1.0	18.9	2.1	11.5	43	3	7
rhamnose	7.6	0.1	2.2	17.4	0.9	5.5	58	3.9	6.7
sucrose	11.8	0.5	3.6	14.2	0.9	6.6	27	1.9	6.7
salicin	6.3	0.1	1.6	35.2	0.8	2.3	132	5.1	3.8
sorbitol	2.5	0.1	3.9	24.6	2.2	9.2	200	21	10

and sorbitol, for which regression coefficients r^2 were 0.96 and 0.98, respectively, indicating that linearity for these compounds was poor. Better linearity for the sugar alcohols ($r^2 > 0.99$) was obtained by restricting the calibration range to 0.25–2.5 mg/L.

3.2.3. Evaluation of Extraction Procedure for L. perenne. A particular sample of the grass species *L. perenne*, LP-STD, was used to evaluate the extraction procedure for the determination of water-extractable NSC. The chromatogram of the extract of LP-STD is shown in **Figure 2**, which is an overlay of six separate extractions of LP-STD carried out to check both the reproducibility and stability of the extraction procedure (see below). Identification of carbohydrates in the water extract of LP-STD was achieved by comparison of peak retention times with the known compounds in the standard mixture. This revealed the presence of the sugar alcohols inositol and sorbitol and of the carbohydrates glucose, sucrose, fructose, and raffinose in this sample. To check the effectiveness of the extraction procedure to recover all water-extractable carbohydrates, a second extraction step was carried out under identical conditions. After removal of the water phase of the first extraction step, 5 mL of doubly distilled water was added to the pellet, which was placed in the shaking water bath at $T = 80$ °C for another 30 min. After centrifugation, the water phase was separately analyzed by HPLC-PAD as described under section 2.3. The respective chromatogram did not reveal the presence of either glucose, fructose, or sucrose except for small signals referring to very small amounts of sugar alcohols that could be neglected. From these data, we concluded that the original extraction procedure was sufficiently effective to recover the water-extractable carbohydrates of interest. Additionally, no fructose peak was observed after the addition of acid to the second water extract, which confirmed the absence of inulin and therefore the effectiveness of the extraction procedure for these compounds, too (for details of inulin hydrolysis, cf. section 3.2.4). Moreover, the pH of the doubly distilled water used for the extractions was carefully checked to be >6.8, because hydrolysis of inulin caused by doubly distilled water at pH <6.8 has been reported (*16*).

Quantification of carbohydrates in the extract was carried out on the basis of the linear calibration curves for each sugar as described in section 3.2.2. To examine the stability and reproducibilty of the extraction procedure, six subsequent extractions of LP-STD were carried out and analyzed. As can be seen from the mean concentrations listed in **Table 2**, highly consistent results for the identified carbohydrates were obtained
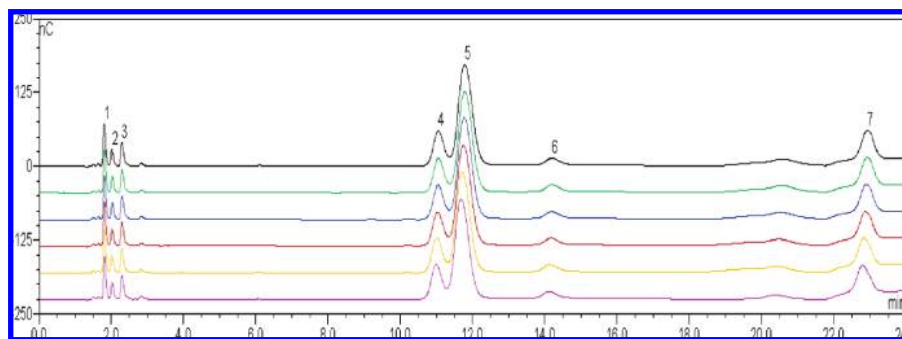
Figure 2. Overlay of chromatograms of six water extracts of LP-STD. Peaks: 1, inositol; 2, sorbitol; 3, mannitol; 4, glucose; 5, sucrose; 6, fructose; 7, raffinose.

Table 2. Replicate Analyses of LP-STD (Grams per Kilogram; $N = 6$)

|	inositol	sorbitol	glucose	sucrose	fructose	raffinose
mean	3.9	3.4	12	98.5	11.1	6.8
STD	0.05	0.01	0.12	0.9	0.3	0.5
RSD (%)	1.3	0.3	1.0	0.9	2.7	7.4

(RSD values < 3%, except raffinose, for which RSD = 7.4%), indicating a high degree of reproducibilty of the extraction procedure.

The composition of the water extract will be discussed in detail in section 3.3.

3.2.4. Hydrolysis of Inulin. Fructans, that is, oligosaccharides made of fructose units with varying degrees of polymerization (DP), as well as inulin, which bears an additional glucose unit, are known to be important reserve carbohydrates in several plant species, including ryegrass *L. perenne* (*17, 18*). Consequently, determination of water-extractable NSC in these plant species must include them. Although attempts at direct analysis of inulin in plant extracts by HPLC-PAD to assess their DP have been reported (*19*), these qualitative data are not applicable for quantification of inulin for a lack of adequate calibration standards. Consequently, inulin has to be hydrolyzed prior to analysis, which can be carried out either enzymatically or by the use of dilute acids. Enzymatic hydrolysis is time- and labor-consuming with incubation times of 24 h per sample, when exo/endo-inulinase is used (*19*). In addition, enzymatic hydrolysis does not necessarily lead to selective formation of fructose molecules. On the contrary, formation of fragments, such as F2 (made of two fructose molecules) or GF2 (made of two fructose and one glucose molecule, "kestose"), was observed (*19*). If not accounted for, fragmentation can therefore produce considerable errors in enzymatic inulin determination. Consequently, for a more accurate quantitation, acidic hydrolysis of the polysaccharides is more straightforward. However, acidic hydrolysis has to be carried out with care, by the use of dilute acids only. If acids are too strong, fructose may be degraded to form side products, such as oxymethylfurfural (*20*). The side-products are not detected by the analytical procedure, which will result in an underestimation of the inulin content.

To examine the most effective hydrolysis of inulin, standard solutions (volume = 10 mL) containing 10 g/L of chicory inulin were hydrolyzed using either HCl 0.5% or HCl 1.0%. These solutions were obtained by pipetting 170 or 340 μL, respectively, of HCl 30% to the inulin standard solutions. They were then exposed to a shaking water bath at $T = 80$ °C for 15, 30, 60, and 120 min, respectively. All solutions were analyzed in duplicate. At the end of hydrolysis time, the solutions were appropriately diluted (1:500) with doubly distilled water and analyzed by HPAEC-PAD. On the basis of the results for

glucose and fructose, mass balances were established and recovery rates calculated. The experiments revealed that the duration of the hydrolysis had only little effect on the recovery. More important was the concentration of the acid. HCl 0.5% led to recoveries of about 90% for 15, 30, and 60 min, with a slight increase to 93% for 120 min. When HCl 1% was used, recoveries were generally higher, with 93−95% for 15 and 30 min and 95% for 60 and 120 min. Consequently, we decided to carry out the hydrolysis of inulin in our samples by using HCl 1% and an incubation time of 60 min. The chromatogram of the hydrolysis products of chicory tuber inulin under these conditions is shown in **Figure 3**. Evaluation of concentrations revealed that glucose made up only 4% of hydrolysis products. The degree of polymerization (DP) of chicory tuber inulin is relatively small, ranging from 8 to 10, whereas the DP of inulin in *L. perenne* is, in general, >20 (*17*). Consequently, the inulin content of *L. perenne* in the extracts can be calculated on the basis of the measured fructose concentration only, without substantial loss of accuracy (*21*). However, for correct quantitation of inulin, both fructose concentrations in the water extract (water-soluble fructose) and from the hydrolysis of sucrose have to be accounted for. Whereas the amount of water-extractable fructose can be directly subtracted from the fructose concentration measured in the inulin hydrolysis extract, the amount of fructose from sucrose hydrolysis has to be calculated according to the equilibrium

$$\underset{\text{Mm 342.3}}{\text{sucrose}} + H_2O = \underset{\text{Mm 180}}{\text{fructose}} + \text{glucose}$$
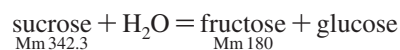
Using eq 1 gives the amount of fructose resulting from the hydrolysis of sucrose:

$$\text{sucrose (mg/L)} / 342.3 = \text{sucrose (mmol)} \times 180$$

$$= \text{fructose (mg/L)} \qquad (1)$$

The fructose content from inulin hydrolysis is then calculated according to eq 2 (mg/L):

$$\underset{\underset{F}{\text{(inulin)}}}{\text{fructose}} = \{\underset{\underset{H}{\text{(hydrolysis)}}}{\text{fructose}} - [\underset{\underset{W}{\text{(water-extractable)}}}{\text{fructose}} + \underset{\underset{S}{\text{(sucrose hydrolysis)}}}{\text{fructose}}]\}$$

$$(2)$$

H is determined in the second extract, and W and S are determined in the first extract.

The inulin content of the sample is finally determined by eq 3:

$$\text{inulin} = 0.91 \times \underset{\text{(inulin)}}{\text{fructose}} \qquad I = 0.91 \times F \qquad (3)$$

The factor 0.91 takes into account the hydrolytic equilibrium between inulin and the monomer; that is, it arises from the
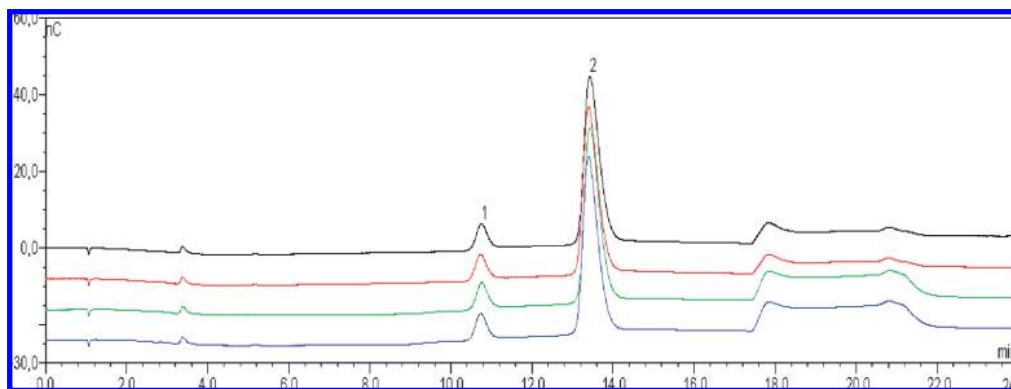
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Figure 3. Acidic hydrolysis of inulin from chicory tuber. Peaks: 1, glucose; 2, fructose.

Table 3. Quantification of Carbohydrates and Inulin in LP-STD and LP-1−LP-3 (Grams per Kilogram)

sample	inositol	sorbitol	glucose	sucrose	fructose	raffinose	inulin	NSC
LP-STD	3.9	3.4	12	98.5	11.1	6.8	166	302
LP-1	3.3	3.2	17.8	68.6	18.7	nd[a]	nd	111.6
LP-2	2.0	4.4	34	1.6	31.5	nd	175	248.5
LP-3	2.3	3.0	5.1	24	5.7	2.5	45.6	88.2

[a] nd, not detectable.

uptake of one molecule of water per fructose residue in the polysaccharide during hydrolysis. If water extracts reveal the presence of substantial amounts of oligosaccharides, such as raffinose, their fructose content has to be taken into consideration, if necessary (see below). This can be done easily, as all of the components taking part in the hydrolytic equilibrium are known. Additionally, for an even more precise inulin determination, the concentrations of glucose can also be taken into consideration. For this purpose, we wrote an Excel program to carry out the necessary calculations. Inulin data in **Table 3** and section 3.3 are reported and discussed on the basis of the calculations with the Excel program.

3.3. Analyses of Plant Extracts. Several samples of the ryegrass species *L. perenne* were submitted to the extraction procedures described in sections 2.4 and 2.5 and subsequently submitted to chromatographic analysis for the determination of water-extractable NSC and inulin after hydrolysis. In this section, information on both the qualitative and quantitative compositions of four selected samples, LP-STD, as well as LP-1−LP-3, will be given. LP-STD was used to evaluate the procedure (cf. section 3.2.4); the chromatogram of its water extract is shown in **Figure 2**. By far, the carbohydrate with the highest concentration in the water extract of LP-STD was sucrose (98.5 g/kg), making up for almost 75% of the sugars

found in the water extract. Concentrations of glucose and fructose were less pronounced, whereas those of raffinose and the sugar alcohols were almost negligible. This is in agreement with observations made by other authors (*10, 13*), who denoted sucrose as the key compound for carbohydrate transportation in perennial ryegrass. However, the concentration of sucrose was surpassed by that of inulin (166 g/kg), which made up >50% of all water-extractable NSC in LP-STD. The chromatogram of acidic hydrolysis of the water extract of LP-STD is shown in **Figure 4**. As expected, it is very similar to that of inulin hydrolysis of chicory tuber (cf. **Figure 3**). However, some differences can be observed. Contrary to the hydrolysis extract of chicory tuber inulin, the hydrolysis extract of LP-STD reveals the presence of the sugar alcohols inositol and sorbitol (as in the water extract). Moreover, the ratio of peak areas for glucose and fructose in **Figure 4** is different from that of pure chicory tuber inulin. This is due to the fact that, contrary to the hydroylsis of pure inulin in chicory tuber, the hydrolysis extract of LP-STD (and the other LP samples) contains glucose and fructose not only from inulin hydrolysis but also from hydrolysis of sucrose. Additionally, the amount of water-extractable glucose and fructose has to be taken into account. Consequently, the amount of glucose in the hydrolysis extract of LP-STD is much higher than in the hydrolysis extract of pure inulin, which can substantially change the ratio of fructose/glucose. Additionally, there is an important instrumental aspect that has to be taken into consideration. The amperometric response for glucose is higher by a factor of ca. 3 compared to an equal amount of fructose, resulting in a larger peak size of glucose for the same concentration (cf. **Figure 1**). On the other hand, the formation of additional glucose by acidic hydrolysis of starch is very unlikely under these conditions, as this would require the presence of concentrated acids (*22*).
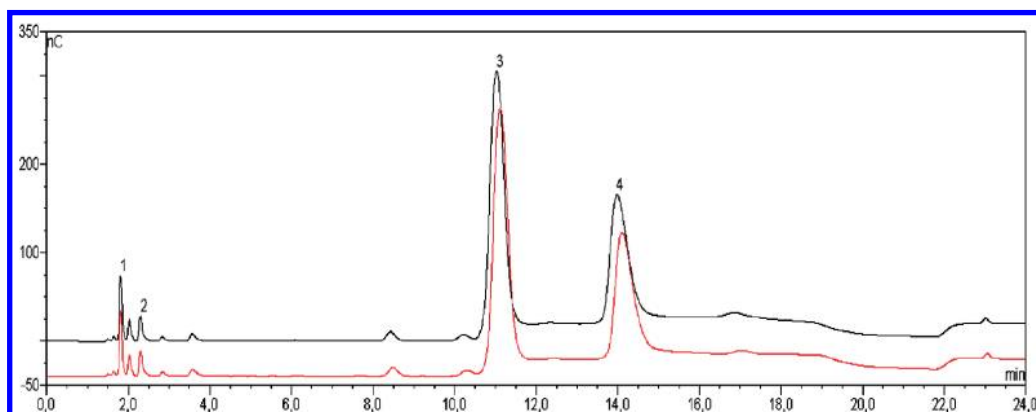

Figure 4. Inulin hydrolysis of LP-STD. Peaks: 1, inositol; 2, sorbitol; 3, glucose; 4, fructose.

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In the following, three other samples (LP-1−LP-3), from dozens that have been analyzed by our method, are discussed. They were characterized either by a high amount of sucrose and the absence of inulin (LP-1), only a little sucrose, and high amounts of both glucose and inulin (LP-2) or by moderate concentrations of the carbohydrates, including raffinose (LP-3). Quantitative evaluation of detected carbohydrates in these samples is summarized in **Table 3**, indicating that the amount of water-extractable in LP samples varied between 88 and 302 g/kg. It is worth mentioning in this context that the portion of sugar alcohols in the samples makes up between 2.5 and 6% of all NSC. If only water-extractable carbohydrates without inulin are observed, the portion of sugar alcohols rises to about 10% (LP-2, LP-3), an amount that cannot be neglected if reliable quantitation of NSC is desired. However, most enzymatic methods do not provide information on concentration of sugar alcohols. Consequently, this information is either lost, implying less accurate results, or has to be obtained by the application of additional instrumentation, such as gas chromatography after derivatization of samples (*23*), which is laborious and time-consuming.

The selection of data summarized in **Table 3** emphasizes the applicability and flexibility of the determination of water-extractable carbohydrates, including inulin, in samples of various compositions and concentrations. The ruggedness and reliability of the method have been carefully examined during method development. Information on water-extractable NSC of a sample can be obtained within 3−4 h. This is much faster than methods based on the enzymatic hydrolysis of carbohydrates. Moreover, it offers the advantage to easily determine all of the relevant carbohydrates individually. Application of the method is not restricted to grass samples but has been successfully applied to other types of plant samples, too (fruits, wood). It offers substantial progress in the determination of water-extractable carbohydrates.

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