Carbohydrate Research, 253 (1994) 287–291
Elsevier Science B.V., Amsterdam

Note

Separation of fructan isomers by high performance anion exchange chromatography †

L.H. Slaughter [a,*] and D.P. Livingston III [b]

[a] *Agronomy Department, University of Maryland, College Park, Maryland 20742 (USA)*
[b] *USDA-ARS, University Park, Pennsylvania (USA)*

(Received January 22nd, 1993; accepted June 14th, 1993)

Most cereal grasses accumulate substantial quantities of isomeric oligofructosyl-sucroses (fructan) during vegetative and reproductive growth[1–3]. While fructan involvement in sugar translocation, partitioning, and utilization has not been completely elucidated, previous studies have indicated that patterns of accumulation fluctuate diurnally and seasonally[1,4].

To examine fructan metabolism in cereal grasses an efficient method for isomer separation is necessary. Our investigation of seasonal growth patterns among several species of winter cereals led to the development of a method for separating fructan isomers, using high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD), that will be useful in future metabolic studies. Hitherto, combinations of reverse and normal phase HPLC methods have been utilized for fructan separation, but these often require extended separation times[5–8]. HPAEC-PAD has been used to quantify three fructan trisaccharides in *Helianthus tuberosus* L., *Aesculus hippocastanum* L., *Asparagus officinalis* L., and *Allium cepa* L.[9–11]; however, this methodology has not been used to characterize fructan in *Avena sativa* L. (oat). We now describe a chromatographic method for routine analysis of cereal fructan in leaf and crown tissues containing neutral sugars and fructan up to dp 7. The method requires minimal sample cleanup, and analyses take less than twenty minutes to complete.

Neutral sugars (glucose, fructose, and sucrose) and dp 3 and 4 fructans were chromatographed on a Carbo Pac-PA1 analytical column (4 × 250 mm). A sodium hydroxide–sodium acetate gradient was used to facilitate the separation of monosaccharides, disaccharides, and oligosaccharides. Because plant tissue extracts typically contain much higher concentrations of glucose, fructose, and

† Article A-6419, Contribution No. 8612 of the Maryland Agricultural Experiment Station.
* Corresponding author.

SSDI 0008-6215(93)E0289-D

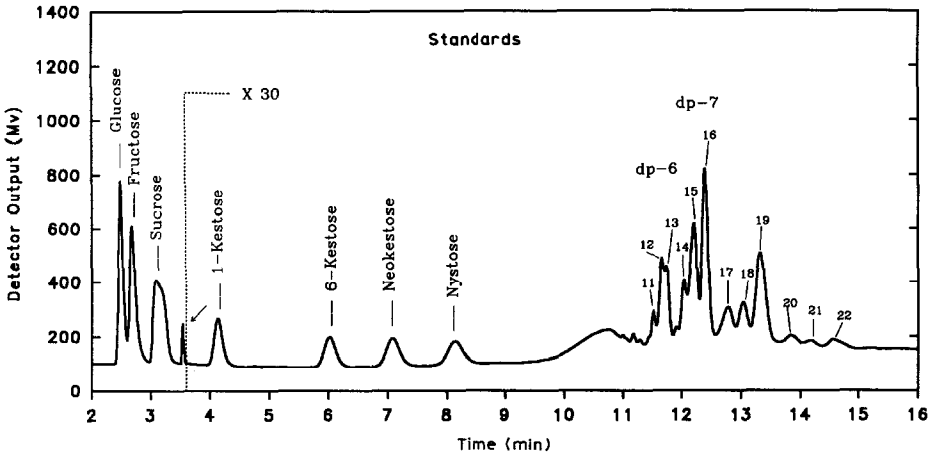

Fig. 1. A composite standard containing neutral sugars, fructan trisaccharides, nystose, and dp 6 and 7 fructans. Groups of signals corresponding to dp 6 (peaks 11–13) and dp 7 fructans (peaks 14–22) are from 0.05 mg mL^{-1} aggregate samples obtained from oat crowns. The peak at 3.5 min between sucrose and 1-kestose is due to the automatic shift in detector attenuation.

sucrose than of fructan, two detector attentuation settings were utilized during the chromatographic analysis.

A chromatogram of neutral sugars and fructan isomers up to dp 7 is shown in Fig. 1. Elution times (t_E), relative elution times (t_G; elution time of glucose = 1) and concentrations of neutral and fructan sugars are shown in Table I. Fructan oligomers larger than dp 4 were not quantified.

Our chromatographic method afforded excellent peak resolution and selectivity for glucose, fructose, sucrose, and fructan isomers of dp 3 and 4. Peak resolution decreased for dp 6 and 7 fructan oligomers, but peak selectivity remained acceptable for routine analysis (Fig. 1). Improved peak resolution in the dp 6 and 7 range was achieved by reducing the ionic strength of sodium acetate or by reducing the slope of the sodium acetate gradient, but this increased the analysis time (data not shown).

TABLE I

Elution times (t_E), relative elution times (t_G), and carbohydrate concentrations of the standard solution chromatographed to give Fig. 1

Carbohydrate	t_E (min)	t_G (min)	Concentration ($\mu g\ mL^{-1}$)
Glucose	2.48	1.00	69.7
Fructose	2.68	1.08	61.1
Sucrose	3.08	1.24	105.4
1-Kestose	4.12	1.66	3.9
6-Kestose	6.00	2.42	3.5
Neokestose	7.06	2.85	3.4
Nystose	8.12	3.27	6.7

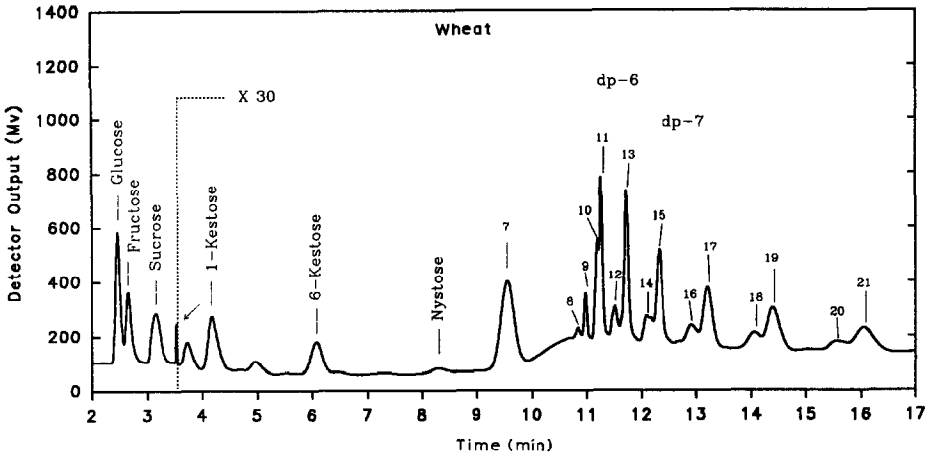

Fig. 2. Separation of neutral sugars and fructans from a 20-mg wheat-leaf sample.

Carbohydrate peaks corresponding to glucose, fructose, sucrose, 1-kestose, 6-kestose, neokestose, nystose, and other unidentified fructan oligomers were observed in chromatograms of both wheat and oat extracts (Figs. 2 and 3). However, the wheat and oat chromatograms are noticeably different in two major respects. Firstly, peak 7 (9.5 min) is present only in wheat extracts. Secondly, in the oat leaf extract, the elution profile of the dp 6 and 7 oligomers is very similar to that observed in the composite standard, which contained fructan extracted from oat crowns (Figs. 1 and 3). Additionally, the pattern of peak elution of the oat sample between 11 and 14 min seems to be characterized by groupings of three closely associated peaks (peaks 8–10, 11–13, 14–16, and 17–19). Peaks 8–10 may

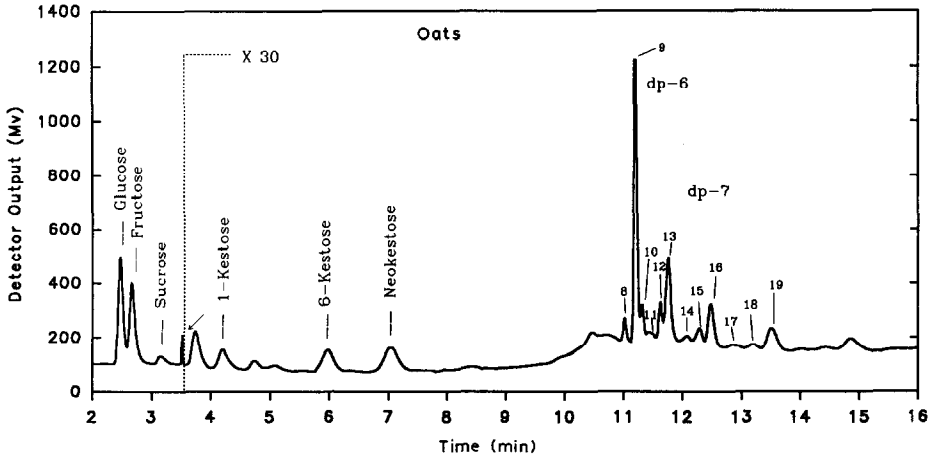

Fig. 3. Separation of neutral sugars and fructans from a 20-mg sample of oat-leaf tissue. Peaks 11–13 correspond to dp 6 fructans and peaks 14–19 to dp 7 fructans.

be dp 5 isomers. However, in the case of wheat, peaks in the dp 6 to 7 region of the chromatogram generally appear in groupings of two, a small peak followed by a larger peak. Whether the dp 6 and 7 fructan oligomers with similar retention times in the wheat and oat samples represent variation in isomeric form is uncertain and will require further study. With regard to peak 7 in wheat extracts, complete acid hydrolysis yielded a glucose : fructose ratio of 1 : 2.86, suggesting a dp 4 fructan. We speculate that this peak may represent the tetrasaccharide bifurcose, because of its retention time and proximity to nystose. Bancal and Gaudillère[1] identified bifurcose as the major dp 4 fructan in wheat.

The HPAEC-PAD methodology we describe is suitable for detecting neutral sugars and fructan in oat leaf and crown tissues at concentrations of 0.01 mg g^{-1} dry wt on a routine basis and will be extremely useful in future studies concerning fructan metabolism in winter cereals.

EXPERIMENTAL

Wheat (*Triticum aestivum* L. cv. Twain) and oat (*Avena sativa* L. cv. Fulghum) plants with 4–5 leaves were collected from field sites at the Western Maryland Research and Education Center (Keedysville Farm).

Neutral sugar standards (glucose, fructose, and sucrose) were purchased from Fisher Chemical Co.; pure 1-kestose (1^F-β-D-fructosylsucrose, dp 3), 6-kestose (6^F-β-D-fructosylsucrose, dp 3), neokestose (6^G-β-D-fructosylsucrose, dp 3), and nystose [1^F-β-D-fructosyl)$_2$sucrose, dp 4], which are not commercially available, were donated by Dr. N.J. Chatterton (Logan, UT).

Fructan isomers of dp 6 and 7 from cold-hardened oat crowns were separated at 21°C using a 20 × 300 mm Column of AG 50 W-X4 cation-exchange resin in the silver form (Bio-Rad, Richmond, CA)[2]. Individual peaks were collected and their dp confirmed by hydrolysis and determination of the glucose : fructose ratio of the hydrolyzate.

Carbohydrate extraction.—Freeze-dried wheat and oat tissue samples (20 mg) were combined with 10 mL of hot deionized water and homogenized for 30 s with a polytron blender. The homogenates were incubated for 30 min in a hot water bath (95°C) to halt enzyme activity. After incubation, the crude extracts were filtered through glass fiber filter discs and the volumes adjusted to 10 mL. Aliquots (1 mL) of the crude extracts were desalted with Amberlite MB-3 mixed-bed ion-exchange resin (Sigma Chemical Co., St. Louis, MO) (final volume 5 mL). At this point the extracts could be frozen at −25°C for up to 6 months with no detectable degradation.

Carbohydrate analysis using HPAEC-PAD.—Analysis of plant tissue samples was accomplished using the Dionex 4000 series Bio LC carbohydrate system (Dionex, Sunnyvale, CA). The analytical column packing used to separate carbohydrates is composed of microbeads coated with a porous sulfonated poly(styrene-divinylbenzene) latex carrying the ion-exchange functional groups (Carbo Pac-PA1, 10 μm)[12].

The pulsed amperometric detection (PAD) system, which depends on the electrochemical oxidation of sugars on a gold electrode surface, utilizes a repeating sequence of three potentials (E1, E2, and E3) that are applied for specific durations (t1, t2, and t3). The potentials and pulse-time settings of the detector were E1 = 0.05 V, t1 = 480 ms; E2 = 0.60 V, t2 = 120 ms; and E3 = −0.80 V, t3 = 300 ms.

The initial detector attentuation setting (30K μA) was maintained from the start of the chromatographic run for 3.5 min. Glucose, fructose, and sucrose elute from the analytical column in less than 3.5 min. The attentuation was automatically lowered to 1K μA at 3.6 min and maintained at this value for the remainder of the run. The detector output may be used to determine analyte concentration from either peak area or peak height; however, the concentration of an unknown analyte cannot be established, since different carbohydrates at the same concentration may produce different detector outputs.

A gradient elution is necessary to provide acceptable separation of neutral sugars and fructan isomers within a reasonable time for routine analysis. The gradient used consisted of two solutions: (*A*) 150 mM NaOH and (*B*) 150 mM NaOH–500 mM NaOAc. Initial conditions were 90% solution *A* and 10% solution *B*. These conditions were held constant from 0 to 3 min. From 3.1 to 6 min, the proportion of solution *A* was decreased to 85% and that of solution *B* increased to 15%. At 6.1 min, the proportion of solution *B* was increased to 45% and that of solution *A* reduced to 55%. These conditions were maintained until 15.1 min at which time the initial conditions were reestablished. The pump flow rate was maintained at 1.0 mL min^{-1} and operating pressure was ~ 7.3 MPa at ambient temperature.

REFERENCES

1 P. Bancal and J.P. Gaudillère, *Plant Physiol. Biochem.*, 27 (1989) 730–745.
2 D.P. Livingston III, *Crop Sci.*, 31 (1991) 751–755.
3 N. Shiomi, J. Yamada, and M. Izawa, *Agric. Biol. Chem.*, 40 (1976) 567–575.
4 L.H. Slaughter and D.J. Sammons, *Crop Sci.*, 33 (1993) 472–475.
5 P. Bancal and J.P. Gaudillere, *Plant Physiol. Biochem.*, 27 (1989) 751–760.
6 A. Heyraud, M. Rinaudo, and F. Taravel, *Carbohydr. Res.*, 128 (1984) 311–320.
7 P.C. Ivin and M.L. Clarke, *J. Chromatogr.*, 408 (1987) 393–398.
8 W. Praznik, T. Spies, and A. Hofinger, *Carbohydr. Res.*, 235 (1992) 231–238.
9 N.J. Chatterton, P.A. Harrison, W. Thronley, and J. Bennett, *Plant Physiol. Biochem.*, 27 (1989) 289–295.
10 N.J. Chatterton, W. Thornley, P.A. Harrison, and J.H. Bennett, *Plant Physiol. Biochem.*, 29 (1991) 367–372.
11 N. Shiomi, S. Onodera, N.J. Chatterton, and P.A. Harrison, *Agric. Biol. Chem.*, 55 (1991) 1427–1428.
12 R.D. Rocklin and C.A. Pohl, *J. Liq. Chromatogr.*, 6 (1983) 1577–1590.