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Research Article

Analysis of mono-, di- and oligosaccharides by CE using a two-stage derivatization method and LIF detection

A sensitive CE with LIF method has been developed for quantitative analysis of small carbohydrates. In this work, 17 carbohydrates including mono-, di- and oligosaccharides were simultaneously derivatized with 4-fluoro-7-nitrobenzofurazane (NBD-F) *via* a two-step reaction involving reductive amination with ammonia followed by condensation with NBD-F. Under the optimized derivatization conditions all carbohydrates were successfully derivatized within 2.5 h and separated within 15 min using borate buffer (90 mmol/L, pH 9.2). For sugar standards LODs were in the range of 49.7 to 243.6 nmol/L. Migration time and peak area reproducibility were better than RSD 0.1 and 3%, respectively. The method was applied to measure sugars in nanoliter volume samples of phloem sap obtained by stylectomy from wheat and to honeydew samples obtained from aphids feeding from wheat and willow.

Keywords:
Carbohydrates / CE / Honeydew / LIF / Phloem sap
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1 Introduction

Carbohydrates play important roles in metabolism, form key components of important molecules (such as DNA and glycoproteins) and, as oligosaccharides, make up critical components of natural structural polymers in plants such as cellulose; soluble carbohydrates are the initial product of photosynthesis. Furthermore, small carbohydrate molecules are increasingly being recognized as possessing signaling activities; some are being evaluated for treatment of human diseases [1]. Due to the lack of either chromophoric or fluorophoric functional groups and low extinction coefficients for both UV and fluorescent detection, the analysis of carbohydrates is often hampered and restricted to separation techniques such as HPLC, refractometry and the electrochemical methods using relatively insensitive mass responses [2, 3]. CE-LIF detection has attracted attention for the analysis of carbohydrates due to its high sensitivity, rapid analysis time, and low consumption of sample and reagent [4, 5]. However, the optimum choice of labeling reagent to react with carbohydrates has been a long-term challenge for CE-LIF method and a labeling reagent that is effective for one sugar might not be as suitable for another due to the wide structural diversities of carbohydrates [6].

As carbohydrates generally do not contain chromophoric or fluorophoric groups, pre-column derivatization is usually necessary in order to aid their detection in electrophoresis separations. At present, the most common derivatization scheme applied to carbohydrates is based on reductive amination in which aldehydes or ketones proceed in several consecutive steps to form stable secondary amines. 8-aminopyrene-1,3,6-trisulfonate (APTS), one of the most popular labeling reagents for the derivatization, was first introduced by Fraysse *et al.* [7]. Due to its extensive aromaticity, APTS–carbohydrate derivatives have a substantially higher molar absorptivity and quantum efficiency than most of the commonly used fluorophore carbohydrate derivatives [5]. However, the interference peaks resulting from the presence of cyanide ions or the degradation of labeling reagents [8, 9] and the requirement for substantial dilution to reduce the background interference coming from APTS make it difficult to identify component peaks in samples especially when the volumes and concentrations of interested samples are very limited; for example in plant phloem sap or aphid honeydew.

Concerns about safe handling of cyanide reagents coupled with the interference problems noted above have led to the development of alternative two-step derivatization schemes for reducing sugars. 3-(4-Carbobenzoyl)-2-quinoline-carboxaldehyde and 4-fluoro-7-nitrobenzofurazane (NBD-F) have been used as labeling reagents to react with reducing sugars [10, 11]. The advantage of these approaches is the relatively mild reaction conditions compared with the

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Abbreviations: APTS, 8-aminopyrene-1,3,6-trisulfonate; **NBD-F,** 4-fluoro-7-nitrobenzofurazane

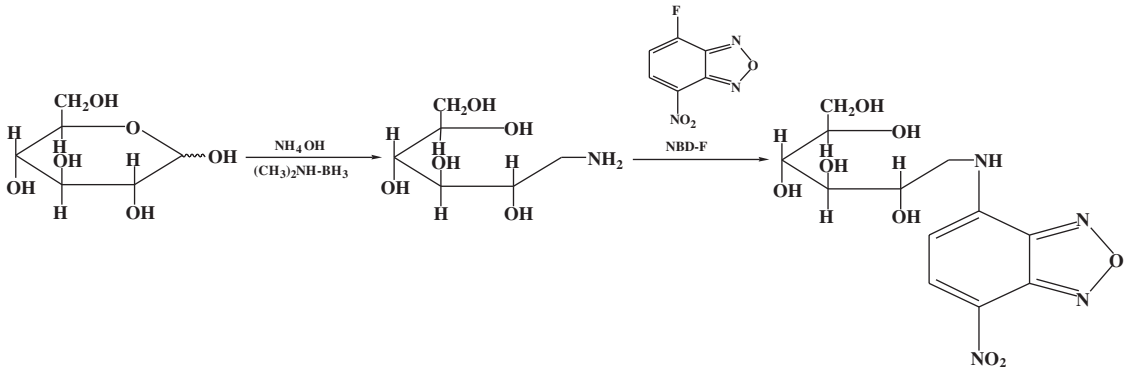

Figure 1. Reaction scheme for derivatization of reducing sugars with NBD-F.

more aggressive APTS derivatization system, which may cause release of sialic acid residues [12]. A disadvantage of the above two derivatization methods is that they lack the ability to react with ketose sugars. Derivatization of ketose sugars, such as fructose, presents a particular problem [12] and there is only a single previous reported method where ketose sugars have been derivatized by means of labeling reagents detectable by CE-LIF [13].

Carbohydrates produced by photosynthesis in leaves are predominantly distributed throughout the plants in the phloem sieve tubes that transport amino acids and sugars from sites of production (sources) to those of use (sinks). One current technique used to obtain pure phloem sap is termed aphid stylectomy, which involves severing the stylets of aphids feeding in the phloem. However, the volume of phloem sap obtained by means of this relatively noninvasive technique is very limited (generally 1–12 nL) [14, 15]. In this work, we report a novel two-step derivatization method (see Fig. 1) in which 17 sugars, including fructose, can be quantitatively derivatized with NBD-F and separated within 15 min. The method was applied to samples of phloem sap obtained from wheat and to honeydew samples collected from the aphids *Rhopalosiphum padi* L. feeding on wheat and *Tuberolachnus salignus* feeding on willow.

2 Materials and methods

2.1 Chemicals and materials

Unless otherwise stated, all chemicals and materials were of analytical-reagent grade. D-fructose, D-galactose, D-glucose, D-ribose, D-xylose, D-lyxose, maltotriose, maltoheptose, isomaltotriose, maltohexose, maltopentose, maltotetrose, dimethylamine–borane complex and glacial acetic acid were purchased from Sigma (St. Louis, MO, USA). D-melibiose, D-cellobiose and NBD-F were purchased from Fluka (Buchs, Switzerland). D-altrose and L-rhamnose were purchased from MP Biomedicals (Illkirch, France). Lactose, ammonia solution (35%), methanol and acetonitrile of chromatographic grade were purchased from Fisher Scientific (Loughborough, UK).

2.2 Instruments

CE experiments were performed with a Beckman P/ACE MDQ system (Beckman-Coulter, High Wycombe, UK) equipped with a 488 nm argon-ion laser module (Picometrics, France, 25 mW). The detection range was 0–2 relative fluorescence units. The data were collected and analyzed by Beckman P/ACE MDQ 1.5 or 1.2 software (Beckman-Coulter).

2.3 Electrophoretic conditions

Bare fused-silica capillaries (Composite Metal Services, Ilkley, UK) used were 50 μm (id) × 70 cm length (55 cm to window) and a short section of the external coating was burned to form the detection window. All new capillaries were conditioned sequentially with 1 mol/L NaOH for 20 min, 0.2 mol/L NaOH for 20 min, de-ionized water for 5 min and BGE for 10 min. Between each injection the capillary was reconditioned with 0.2 mol/L NaOH for 2 min, MeOH for 2 min and BGE for 2 min. The capillary was kept dry overnight. At the beginning of each day, the capillary was regenerated with 0.2 mol/L NaOH for 10 min, MeOH for 10 min and BGE for 10 min before the first injection. The BGE used in the experiment was 90 mmol/L borate buffer, pH 9.2, and the dissolving matrix, used to dissolve dried sugar standards or phloem sap samples, contained 20 mmol/L potassium hydrogen phosphate buffer, pH 8.0, with 50% v/v acetonitrile. All the experiments were performed at room temperature ($25 \pm 1^{\circ}C$). The sample was injected by pressure at 0.5 psi for 5 s and the separation voltage was 25 kV.

2.4 Derivatization procedure

An aliquot of 10 μL of a 30 μmol/L sugar solution in a 0.6 mL homo-polymer tube was mixed with 2 μL of

a 300 mmol/L ammonia and 50 mmol/L dimethylamine–borane complex solution adjusted to pH 4.5 with glacial acetic acid. Reaction mixtures were incubated at 70°C for 1.5 h. The solution at the bottom of the tube was evaporated to dryness by opening the cover of homopolymer tube and incubation at 70°C for 20 min. The residue was dissolved by 20 μL methanol and evaporated at 70°C for 10 min to dryness. The residue was then reacted with NBD-F, as reported in our previous work [16], but the incubation temperature was modified slightly to 70°C in order to reduce the derivatization time. Briefly, dissolving matrix (50 μL), used to dissolve dried sugar standards or phloem sap samples was mixed with 1.25 μL 50 mM NBD-F (dissolved in acetonitrile), incubated at 70°C for 15 min, then mixed with 50 μL H_2O and allowed to cool down at room temperature prior to analysis.

A sucrose pre-hydrolysis procedure was used based on a previous publication [17]. Briefly, a stock solution of 5 mmol/L sucrose was diluted with 0.5 mol/L HCl to a concentration of 30 μmol/L. The 10 μL diluted solution was incubated at 70°C for 15 min followed by opening the cover of homopolymer tube to evaporate to dryness. The residue was then derivatized by the two-step scheme as mentioned above.

2.5 Preparation of standard mixture and biological sample

Stock solutions (5 mmol/L) of 17 sugar standards were prepared with de-ionized water. A mixture of these solutions was diluted to 30 μmol/L with water prior to injection and analysis. Aphid honeydew was collected by placing an oil-filled Petri dish beneath ten caged aphids feeding on wheat and willow. An oil-filled capillary was used to collect the honeydew droplet. Wheat phloem sap was collected by means of the aphid stylectomy method, which has been described elsewhere [18]. The sample droplets (generally a few nanoliters) were allowed to dry at room temperature, sealed and stored at −20°C before analysis. At the time of analysis, the dried phloem or honeydew samples were dissolved and diluted with 10 μL of 50% methanol and incubated at 70°C to dryness before two-step derivatization as described above.

2.6 Validation procedures

In this work, a two-step derivatization method was used to validate linearity, LOD and precision using altrose as internal standard. Linearity was evaluated by analyzing five different concentrations in the range 300 nmol/L to 30 μmol/L. The normalized peak area (peak area divided by migration time) of each analyte, expressed as a ratio to the internal standard peak area, was then calculated and used to determine the linearity of the method. Intra-day precision, migration time and peak area ratio were determined by replicate analysis ($n = 6$) of 16 sugar standards with three different concentrations (300 nmol/L, 3 μmol/L and 30 μmol/L, respectively). An RSD% of 20% was regarded as acceptable for accuracy and precision at the lower LOQ and 15% RSD at higher analyte concentrations [19]. The LOD of each compound was defined as the concentration for which the S/N was greater than 3:1 after analysis from a stock solution.

3 Results and discussion

3.1 Optimization of derivatization procedure

The effect of the ammonia concentration on pre-column derivatization was examined by using three representative monosaccharides (glucose, fructose and galactose) and one disaccharide (melibiose). As shown in Fig. 2A, an increase in ammonia concentration resulted in improved derivatization efficiency for all the carbohydrates up to a concentration of 300 mmol/Land this ammonia concentration was chosen as optimum concentration. The influence of dimethylamine–borane complex concentration on derivatization efficiency was also carefully examined in this work. As shown in Fig. 2B, the optimum dimethylamine–borane complex concentration was 50 mmol/L.

The impact of reaction time in the first derivatization stage on the derivatization efficiency is shown in Fig. 3A. A maximum yield for all four tested carbohydrates was observed at 1.5 h and longer times resulted in a decline of system yield probably due to the degradation of the sugar–amine bond [20]. Hence 1.5 h reaction time for the first derivatization stage was used for further experiments. The impact of reaction time in the second derivatization stage (NBD-F) on the yield is shown in Fig. 3B. As expected, at 70°C NBD-F reacted with carbohydrates very quickly and saturation curves were obtained within 15 min. The result obtained in this work was very similar to the derivatization of amino acids using NBD-F as a labeling reagent [21]. The linkage between the sugars and NBD-F after the two-step derivatization was apparently stable and no obvious degradation was observed even though the derivatives were stored at −20°C for up to 1 month (data not shown).

3.2 CE separation of derivatized sugars

The CE separation of a mixture of 17 NBD-sugar standards is shown in Fig. 4. The identification of each labeled sugar was confirmed by spiking the mixture with the individual saccharide [22]. The large peaks observed at migration times before 6 min and after 10 min are reagent peaks due to the side products of NBD derivatization, NBD-NH_2 and NDB-OH [14, 23]. These reagent peaks do not interfere with the measurement of any of the analytes and hence were not considered to have any direct impact on the quantitative aspects of the

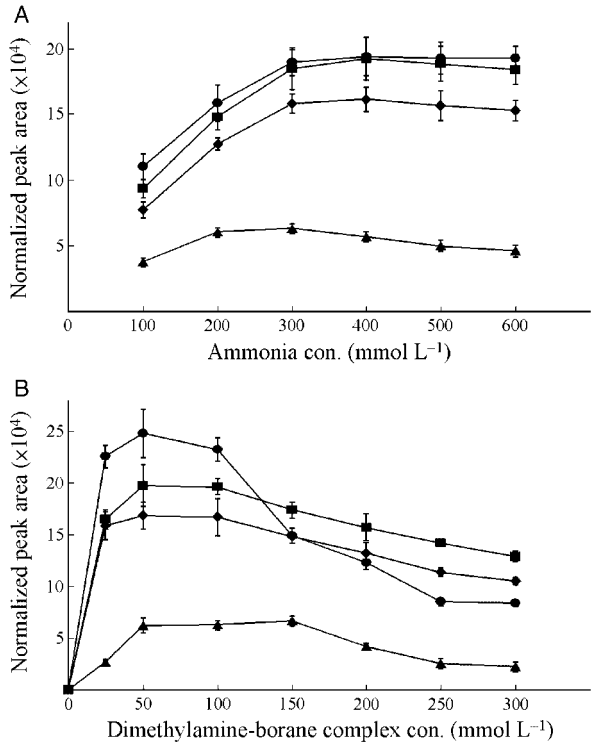

Figure 2. Effect of ammonia and dimethlyamine–borane complex concentration on pre-column derivatization of selected carbohydrates: (●) galactose; (■) glucose; (♦) melibiose; (▲) fructose. (A) in 50 mmol/L dimethylamine–borane complex; (B) in 300 mmol/L ammonia. Incubation times for the first-step and second-step were 90 and 15 min, respectively. The concentration of compounds was 30 μmol/L.

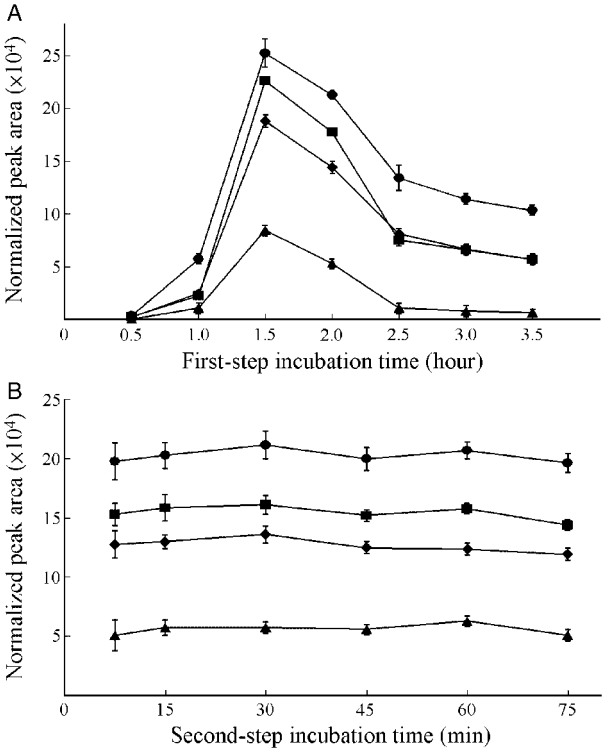

Figure 3. Effect of the first-stage and second-stage reaction time on pre-column derivatization of selected saccharides: (●) galactose; (■) glucose; (♦) melibiose; (▲) fructose. (A) At second-stage reaction of 15 min; (B) at first stage reaction of 1.5 h. The concentrations of ammonia and dimethylamine–borane complex were 300 and 50 mmol/L, respectively. The concentration of compounds was 30 μmol/L.

method. The sequence in order of increasing migration time was observed to be oligosaccharide first, followed by disaccharide and then monosaccharide. It was found that the NBD-derivatized sugar standards were readily separated using BGE containing 90 mmol/L borate buffer, and baseline separation was also achieved for the all saccharides. There were very few interfering peaks observed, often a problem with LIF methodology particularly with APTS derivatization of sugars. Compared with previously reported APTS derivatization methods, this new two-step protocol was successful in derivatization of not only aldose sugars, but also the ketose sugar fructose, which in the past was not thought to derivatize under reductive amination conditions. It is interesting to note that fructose resulted in two peaks after the two-step derivatization and the other saccharides resulted in single peaks. This phenomenon, which may be due to the instability of the ketose sugar skeleton, is in agreement with previous results [7, 24] using UV-detectable 4-aminobenzonitrile as a labeling reagent. Unlike a previous report of APTS labeling of fructose [13] we observed a substantially lower labeling efficiency for fructose compared with glucose. The reason for this is not known, but may relate to the different derivatization procedures used.

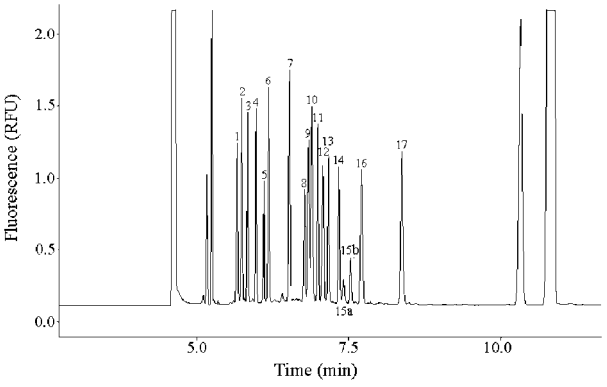

Figure 4. Typical electrophoretic separation of 17 NBD-derivatized sugar standard compounds in the optimized conditions. Peaks in the chromatogram: 1, maltoheptose; 2, maltohexose; 3, maltopentose; 4, maltotetrose; 5, isomaltototriose; 6, maltotriose; 7, cellobiose; 8, melibiose; 9, lactose; 10, rhamnose; 11, lyxose; 12, ribose; 13, xylose; 14, glucose; 15 a/b, fructose; 16, altrose; 17, galactose. Peaks migrating before 6 min and after 10 min are reagent peaks. The concentration of compounds was 30 μmol/L. Separation conditions: BGE, 90 mmol/L borate buffer, pH 9.2; separation voltage, 25 kV; sample injection, 0.5 psi for 5 s.

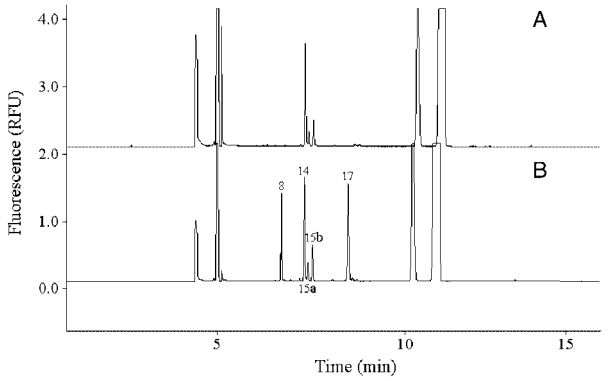

Figure 5. Electropherograms of NBD-derivatized sugar standards carried out in 90 mmol/L borate buffer, pH 9.2; (A) 30 μmol/L sucrose standard with 0.5 mol/L HCl hydrolysis pre-treatment; (B) 30 μmol/L four sugar standards without hydrolysis pre-treatment. Peak identifications and conditions were as for Fig. 4.

3.3 Hydrolysis of sucrose before derivatization

In theory, a peak for derivatized sucrose should be observed after the two-step derivatization as for the other disaccharides. However, for unknown reasons no sucrose peak was detected after derivatization even though the first-step incubation time was increased to more than 12 h. The same phenomenon was observed in the derivatization of raffinose, melezitose and stachyose, which all contain a fructose moiety. As a result, in order to derivatize sucrose a pre-hydrolysis procedure is necessary, as shown in Fig. 5. This results in three additional peaks appearing corresponding with glucose and fructose (two peaks). The result indicated that after hydrolysis pre-treatment sucrose was completely cleaved to glucose and fructose with 0.5 mol/L HCl.

3.4 Validation of the analytical method

Sixteen individual carbohydrates were used to validate the method. The LODs ($S/N = 3$) of the method ranged from 50 to 244 nmol/L (Table 1). The run-to-run repeatabilities ($n = 6$) of migration time with three different concentrations of sugars (300 nmol/L, 3 μmol/L and 30 μmol/L) using altrose as internal standard were all within 0.1% RSD and the run-to-run repeatabilities of peak-area ratio with three different concentrations were within 3.0, 5.9 and 15.7% RSD, respectively (see Table 2). Calibration plots were linear over the range investigated with r^2 typically greater than 0.99 for all analytes (Table 2). At injected concentrations of higher than 40 μM NBD-sugars had a stronger signal than the upper limit of the LIF detector, but this was well above the injected concentration observed in biofluid samples (where dilution resulted from sample processing and derivatization).

Table 1. Detection limits and migration times of individual sugar standards

Analytes	Migration time (min)	LOD (nmol/L)
Maltoheptose	5.68	70.3
Maltohexose	5.75	54.7
Maltopentose	5.85	53.4
Maltotetrose	5.99	54.0
Isomaltotriose	6.12	78.8
Maltotriose	6.20	49.9
Cellobiose	6.54	49.7
Melibiose	6.79	90.0
Lactose	6.86	64.4
Rhamnose	6.91	68.6
Lyxose	7.01	76.0
Ribose	7.09	91.4
Xylose	7.18	92.3
Glucose	7.35	85.8
Fructose	7.55	243.6
Altrose (internal standard)	7.73	95.1
Galactose	8.39	80.3

3.5 Determination of the sugar components in phloem sap and honeydew

In contrast with mammals, which use glucose for circulation in the vascular system, plants usually use sucrose as the dominant sugar for long-distance transport [25]. Sucrose is the predominant organic compound in phloem sap and is a critical carbon source for phloem-feeding insects such as aphids [26]. However, up to and often exceeding 1 mol/L sugar concentration, phloem sap poses an osmotic challenge for phloem-feeding insects that these animals must overcome in order to utilize it [27]. In this work, the two-step derivatization scheme without hydrolysis procedure was directly used to determine the sugar compositions from phloem sap obtained from the aphid stylectomy technique applied to wheat (Fig. 6). The glucose and fructose contents of these samples were very low and there were interfering peaks probably resulting from amino acids [28]. However, after hydrolysis pre-treatment and reducing the volume of phloem sap to 1 nL, two constituents coming from glucose and fructose moieties of sucrose were detected (Fig. 7). Quantitation was achieved by means of the internal standard method, using a single-point calibration for glucose linearity and minimum background interference. The content of sucrose in the analyzed sample was *ca.* 439 mmol/L. The measured sucrose concentration was in agreement with the typical range reported in the literature for wheat [28].

Phloem-feeding insects, including aphids, whitefly and psyllids, ingest phloem sugars at rates in excess of their requirement for carbon, and high concentrations of unassimilated sugars are voided in their honeydew, the egesta from the insect gut [29]. In this work, honeydew collected from aphid *R. padi* L. feeding on wheat and

Table 2. Assay validation data for individual analytes

Analytes	Slope ($\times 10^{-5}$)	Intercept	Correlation coefficient (r^2)	Migration time RSD% ($n = 6$)			Normalized peak area RSD% ($n = 6$)		
				0.3 μM	3 μM	30 μM	0.3 μM	3 μM	30 μM
Maltoheptose	2.3	−0.0101	0.999	0.09	0.05	0.07	13.6	4.6	2.2
Maltohexose	3.0	−0.0142	0.998	0.08	0.09	0.10	12.5	4.5	2.2
Maltopentose	3.0	−0.0131	0.998	0.09	0.09	0.10	14.6	3.6	2.1
Maltotetrose	2.8	−0.0094	0.999	0.09	0.07	0.10	13.5	3.3	2.4
Isomaltotriose	1.8	−0.0002	0.999	0.08	0.06	0.08	7.1	4.7	1.8
Maltotriose	3.3	−0.0029	0.999	0.06	0.06	0.07	10.3	2.8	1.8
Cellobiose	3.9	+0.0051	0.999	0.04	0.05	0.06	8.8	1.8	1.3
Melibiose	2.2	+0.0049	0.998	0.04	0.04	0.05	15.7	1.7	2.1
Lactose	3.3	−0.0008	0.999	0.04	0.05	0.06	12.6	2.3	2.8
Rhamnose	3.8	+0.0017	0.999	0.07	0.04	0.06	11.1	2.5	1.3
Lyxose	3.4	+0.0032	0.999	0.06	0.04	0.04	8.3	5.8	1.4
Ribose	2.9	−0.0051	0.999	0.06	0.02	0.03	12.3	2.8	1.6
Xylose	2.9	−0.0027	0.999	0.05	0.03	0.03	11.8	2.1	1.5
Glucose	3.0	−0.0044	0.999	0.03	0.03	0.02	15.5	1.9	1.3
Fructose	1.0	+0.0066	0.993	0.04	0.06	0.03	14.0	5.9	3.0
Galactose	4.9	−0.0028	0.999	0.03	0.05	0.04	7.9	2.8	1.5

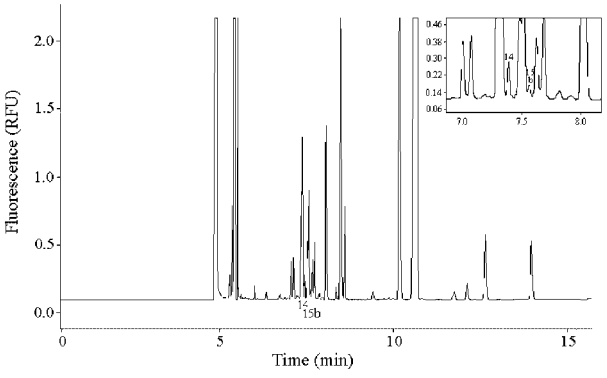

Figure 6. Typical electrophoretic separation of NBD-derivatized sugars in 10 nL wheat phloem sap. Peak identifications and conditions were as for Fig. 4.

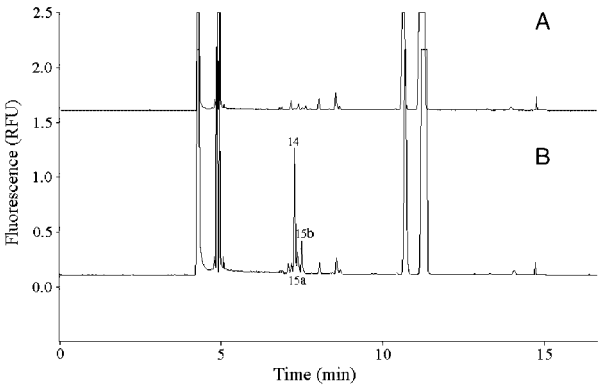

Figure 7. Typical electrophoretic separation of NBD-derivatized sugars in 1 nL wheat phloem sap. (A) Without 0.5 mol/L HCl hydrolysis pre-treatment; (B) with 0.5 mol/L HCl hydrolysis pre-treatment. Peak identifications and conditions were as for Fig. 4.

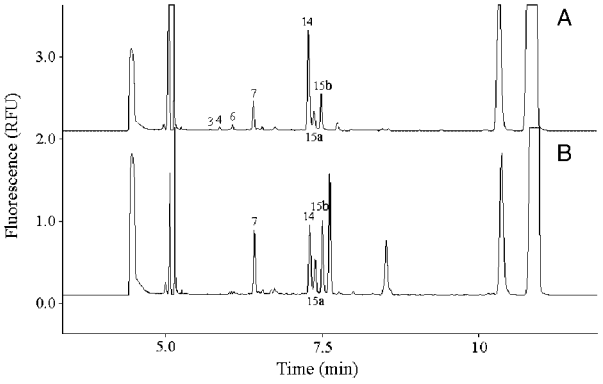

Figure 8. Typical electrophoretic separation of NBD-derivatized sugars in 10 nL aphid honeydew. (A) Aphids feeding on wheat; (B) aphids feeding on willow. Peak identifications and conditions were as for Fig. 4.

T. salignus feeding on willow was directly analyzed by this two-step derivatization scheme without the hydrolysis procedure (Fig. 8). Sugar peaks were identified by matching the migration time of sugar standards and confirmed by means of spiking with authentic sugar standards into the samples. The results (Table 3) indicate that six sugars including mono-, di- and oligosaccharides were detectable in the honeydew from wheat and three sugars belonging to mono- and disaccharides were present in the honeydew from willow. The observation suggests that the predominant carbohydrates in aphid honeydew are glucose, fructose, cellobiose and a series of oligosaccharides [30, 31]. Raffinose, stachyose, melezitose and trehalose have been previously reported as the major oligosaccharides and disaccharide in honeydew [32, 33] but could not be observed here due to the derivatization limitation mentioned above.

Table 3. Sugar concentrations measured in aphid honeydew samples; replicate samples from a single aphid

Analytes	Honeydew (wheat) con. (mmol/L ± SD; $n = 6$)	Honeydew (willow) con. (mmol/L ± SD; $n = 6$)
3 Maltopentose	1.48 ± 0.11	n.d.[a)]
4 Maltotetrose	2.54 ± 0.16	n.d.[a)]
6 Maltotriose	3.32 ± 0.18	n.d.[a)]
7 Cellobiose	12.8 ± 0.56	25.8 ± 1.8
14 Glucose	81.2 ± 2.3	49.0 ± 4.4
15 Fructose	75.2 ± 12.1	114.2 ± 8.0

a) n.d. = not detectable.

4 Concluding remarks

A novel two-step derivatization reaction for 17 reducing sugars including mono-, di- and oligosaccharides has been developed in this work to enable the quantitative determination of sugars in biological samples. The whole reaction scheme involves reductive amination with ammonia followed by condensation with NBD-F. With this method, 17 reducing sugars were successfully derivatized and separated within 15 min. Fructose, a ketohexose, which in the past was not thought to derivatize under reductive amination conditions, was also successfully derivatized with NBD-F. Sucrose, which contains a fructose moiety, cannot be derivatized directly by means of this reaction scheme but can still be identified and quantified indirectly by its hydrolysis products glucose and fructose. These data have confirmed that this new two-step derivatization scheme is a useful tool for determination of sugar composition in plant phloem sap, aphid honeydew and other volume-limited samples.

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The authors have declared no conflict of interest.

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