Liquid chromatography/mass spectrometry analysis of branched fructans produced in vitro with C-13-labeled substrates

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Published in:
Rapid Communications in Mass Spectrometry

Link to article, DOI:
10.1002/rcm.7013

Publication date:
2014

Document Version
Publisher's PDF, also known as Version of record

Link back to DTU Orbit

Citation (APA):

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Fructans are fructose based carbohydrates containing no or one glucose unit per molecule. They accumulate in about 15% of all flowering plants, and they are found in many economically important crops such as wheat, barley, and perennial ryegrass. Fructan synthesis starts by the addition of fructose to a primary hydroxyl group of sucrose. Hence, three trisaccharides can be formed, i.e. 1-kestotriose (1-kestose), 6-kestotriose (6-kestose) and 6G-kestotriose (neokestose), that form the template for further elongation. The resulting fructan structures can be divided into five classes based on their trisaccharide core and on the type of fructose-fructosyl linkage. Inulín-type fructans are linear structures with almost only \( \beta(2-1) \) linkages whereas neolevans are neofructans with \( \beta(2-6) \) linkages. Graminans are branched fructans with both \( \beta(2-1) \) and \( \beta(2-6) \) fructose-fructosyl linkages. It is believed that graminans are the major fructan type in the vegetative tissues of most cereals. Yet, both fructan concentration and structure may vary between tissue types and during the growing season. Several previous studies have correlated fructan structure to apparent health benefits and this is true of other types of dietary fiber as well. However, the traditional methods for the analysis of fructans are laborious and complex. The structural identification of graminan is complicated by the fact that for each degree of polymerization (DP), various graminan isomers can be formed. Thus, as the molecules increase in molecular weight the number of possible structures grows exponentially.

Traditionally, methylation analysis in combination with gas chromatography coupled to mass spectrometry (GC/MS) has been widely used for fructan structural characterization. Fructans are partly methylated, then hydrolyzed and the products are reacted to form alditol acetates. The relative proportions of the different fructan building blocks are revealed by GC/MS. In this way, an average composition of all fructans present in the sample can be obtained. If
the methylation steps are preceded by a separation step, information on individual fructan structures can be obtained but only the structure of low DP fructans (DP < 5) can be determined unambiguously.[12] Larger fructans are often hydrolyzed by specific enzymes and analyzed with GC/MS to obtain more structural information of the original compounds.[12] This approach is very time-consuming and inappropriate for large sample sets or for the study of many fructan structures. Nuclear magnetic resonance (NMR) can be used to study fructan structures and has the advantage that no derivatization step is required.[13] However, this method only gives an average composition of all fructan structures present in the sample unless it is preceded by a laborious purification to produce fractions of individual fructan structures. More recently, Suzuki et al. used a combination of direct infusion electrospray ionization mass spectrometry and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry to study the incorporation of glucose into fructans during fructan synthesis in asparagus tissue.[16] Harrison et al. reported the use of liquid chromatography coupled to mass spectrometry (LC/MS) to identify linear fructan structures.[17] They were able to separate inulin- and levan-type fructans and mapped their MS fragmentation patterns. The use of \(^{13}\text{C}\)-labeled substrates and specific fructan-synthesizing enzymes allowed the synthesis of fructan structures that are not commercially available, which were used to understand their MS\(^2\) fragmentation. Once this fragmentation pattern was established, it could be used to screen large sample sets for their presence without the need for fructan standards nor a preceding fructan purification step.[17] We hypothesize that LC/MS is suitable for the identification of branched fructans. The aim of the present study was to enzymatically synthesize branched, graminan-type fructans and to elucidate their MS\(^2\) fragmentation patterns. To this end, a heterologous sucrose:fructan 6-fructosyltransferase (6-SFT) from \textit{Pachysandra terminalis} was incubated with 1-kestotriose and either native sucrose or \(^{13}\text{C}\)-labeled sucrose. In the latter case, the fructose unit of sucrose contained a \(^{13}\text{C}\) isotope only on the first carbon. 1-Kestotriose, 1,1-kestotetraose and 1,1,1-kestopentaose were purchased from Megazyme (Bray, Ireland). 6-Kestotriose was a generous gift from Dr. Iizuka[18] and 6G-kestotriose was purified from a \textit{Xanthophyllomyces dendrorhous} culture broth.[19] 6-SFT from \textit{Pachysandra terminalis} was obtained by heterologous expression in \textit{Pichia pastoris},[20] and was donated by Prof. W. Van den Ende.

**EXPERIMENTAL**

**Materials**

All chemicals, solvents and reagents were purchased from Sigma-Aldrich (Bornem, Belgium) and were of analytical grade unless specified otherwise. Universally labeled sucrose or \([\text{UL-}^{13}\text{C}_6 \text{ fru}]\) sucrose (β-D-[UL-\(^{13}\text{C}_6\)] fructofuranosyl α-D-glucopyranoside) and \([\text{1-}^{13}\text{C}_1 \text{ fru}]\) sucrose (β-D-\([\text{1-}^{13}\text{C}_1\] fructofuranosyl α-D-glucopyranoside) were purchased from Campro Scientific (Veenendaal, The Netherlands). The fructose unit of \([\text{UL-}^{13}\text{C}_6 \text{ fru}]\) sucrose is composed of \(^{13}\text{C}\) isotopes only. The fructose unit of \([\text{1-}^{13}\text{C}_1 \text{ fru}]\) sucrose contains a \(^{13}\text{C}\) isotope only on the first carbon. 1-Kestotriose, 1,1-kestotetraose and 1,1,1-kestopentaose were purchased from Megazyme (Bray, Ireland). 6-Kestotriose was a generous gift from Dr. Iizuka[18] and 6G-kestotriose was purified from a \textit{Xanthophyllomyces dendrorhous} culture broth.[19] 6-SFT from \textit{Pachysandra terminalis} was obtained by heterologous expression in \textit{Pichia pastoris},[20] and was donated by Prof. W. Van den Ende.

**Methods**

**Enzymatic synthesis of branched fructans**

1-Kestotriose (300 mmol/L) was incubated with 6-SFT and either native sucrose or \(^{13}\text{C}\)-labeled sucrose (300 mmol/L) at 20°C in 500 or 1000 μL sodium acetate buffer (100 mmol/L, pH 5.5) containing 0.02% (w/v) NaN\(_3\). Reaction conditions 20°C in 500 or 1000 μL sodium acetate buffer (100 mmol/L, pH 5.5) containing 0.02% (w/v) NaN\(_3\). Reaction conditions were based on those described by Van den Ende et al.[20] Fructan formation was monitored with high-performance anion-exchange chromatography (HPAEC)[19] and a HPAEC profile of the fructan formation is shown in Supplementary Fig. S6 (see Supporting Information). Once at least 20% 1-kestotriose had been converted, 6-SFT was inactivated by pre-
incubating the reaction mixture at 100°C for 10 min after which it was filtered (0.2 μm) and 1000-fold diluted. The final reaction mixture contained at least 90 mmol/L glucose. Based on the detector response of 1,1-kestotetraose, the concentration of the formed DP 4 fructans was estimated to be at least 75 mmol/L.

Liquid chromatography/mass spectrometry

The fructan separation was performed using two trifunctional C18 alkyl columns (Acquity UPLC HSS T3, 100 × 2.1 mm, 1.8 μm particle size, Waters) placed in series. They were eluted isocratically with an 0.1% aqueous solution of formic acid as mobile phase for 15 min at a flow rate of 0.35 mL/min.[21] A 5 μL injection was used and the first 30 s of the analysis was diverted to waste. An orbitrap mass spectrometer (Orbitrap Fusion Tribrid; Thermo Scientific) was used as detector with data being acquired over a mass range of 500 to 2000 m/z. A heated electrospray ionization source was used at a temperature of 315°C in negative ion mode. For MS² analysis, the ions of interest were selected by the quadrupole using a window of 0.5 m/z. These ions were fragmented either by CID (30% collision energy) or by HCD (25% collision energy). Data were collected in centroid mode and analyzed with Xcalibur 3.0 software.

Mass spectral interpretation

The nomenclature of Domon and Costello[22] was used for the designation of the fructan fragments formed. For linear fructans, fragments containing the glucose unit are labeled as Ai, Bi, and Ci, depending on the cleavage site relative to the glycosidic bond. The subscript i represents the number of the glycosidic bond cleaved, counting from the glucose end. Fragments in which the

![Figure 1](image_url)

Figure 1. Molecular structures of 1&6-kestotetraose (1&6-KT) and 1,6-kestotetroase (1,6-KT) and their specific fragment ions formed in MS². The m/z values are indicated below the structural formula with between parentheses the m/z values of the corresponding [UL13C6 fru]-labeled species.
glucose unit was lost are designated as $X_j$, $Y_j$ and $Z_j$, depending on the cleavage site relative to the glycosidic bond, and the $j$ index counting starts from the intact fructose end. The letters A and X are used to designate fragments formed by cross ring cleavage, C and Y designate ions formed by breakage of the glycosidic bond with retention of water, whereas Z and B ions are formed by glycosidic bond cleavage involving a loss of water.\textsuperscript{[22]} For branched fructans, fragments with both a terminal glucose and a terminal fructose are labeled with $A_i, B_i$ and $C_i$, with an additional Greek letter in subscript when the fragmentation occurs in a branch and $\alpha, \beta$ and $\gamma$ represent the branch with the highest, the second highest and third highest molecular weight, respectively. The largest unbranched part containing glucose is called the core unit and its fragments receive no subscript Greek letter. In case this assignment of the core unit is ambiguous (when two potential core units with equal molecular weight are present), the portion with the most $\beta(2-1)$ linkages is designated as the core unit.

**RESULTS AND DISCUSSION**

**Enzymatic synthesis of branched fructans**

Branched fructans containing both $\beta(2-1)$ and $\beta(2-6)$ linkages were produced in vitro with a heterologously expressed 6-SFT from *Pachysandra terminalis*. The fructan synthesis was monitored using HPAEC (Supplementary Fig. S6, see Supporting Information). Once the reaction was complete the reaction mixture was analyzed by LC/MS and LC/MS\textsuperscript{2}. Authentic standards were used to confirm the identity of some of the low molecular weight fructans (Supplementary Fig. S7, see Supporting Information). Comparison of the fragmentation patterns in the MS\textsuperscript{2} spectra of the native and 13C-labeled fructans allowed the identification of the remaining DP 4, DP 5 and DP 6 structures (summarized in Table 1). By far the majority of the product of the reaction were DP 4 fructans, with the concentrations of higher DP fructans decreasing with size, i.e. the concentration of DP 4 > DP 5 > DP 6 (Supplementary Table S1, see Supporting Information) and the concentrations of higher molecular weight fructans (DP > 6) were too low to allow structural identification. 1-Kestotriose and sucrose were the reaction substrates but a small amount of 6G-kestotriose was seen to be present (Supplementary Fig. S1(a), see Supporting Information). HPAEC analysis showed that this 6G-kestotriose was already present at the start of the reaction and not accumulated thereafter (Supplementary Fig. S6, see Supporting Information). If 6G-kestotriose had been formed during the reaction, [UL-13C\textsubscript{6} fru] would have been incorporated in the newly formed 6G-kestotriose. However, although native 6G-kestotriose (m/z 503) was clearly present (Supplementary Fig. S1(a), see Supporting Information), no labeled 6G-kestotriose was observed in the extracted ion chromatogram (EIC) for 509 and 515, the m/z values of DP 3 fructans with one or two UL-13C\textsubscript{6} fructose units, respectively (Supplementary Figs. S1(b) and S1(c), see Supporting Information). Hence, *P. terminalis* 6-SFT appears to have no fructan:fructan 6G-fructosyltransferase (6G-FFT) side activity. Labeled 6-kestotriose was present in small amounts (as shown by the peak in the EIC trace for m/z 515), together with minor amounts of labeled 1-kestotriose. These results are in accordance with those of Van den Ende \textit{et al}.\textsuperscript{[20]} who observed that 6-SFT from *P. terminalis* has an inherent 6-SST (6-sucrose: sucrose fructosyltransferase) activity and a 1-SST (1-sucrose: sucrose fructosyltransferase) side activity. *P. terminalis* 6-SFT is known to prefer 1-kestotriose over sucrose as a fructose acceptor and, therefore, it preferentially produces graminans, rather than levan-type fructans.\textsuperscript{[20]}
Fragmentation of DP 4 fructans

6-SFT can potentially synthesize two DP 4 fructans or kestotetraoses (KT) when 1-kestotriose acts as fructose acceptor. The 1&6-kestotetraose (1&6-KT) is formed when fructose is attached to the 6 position of the fructose unit bonded to the glucose and 1,6-kestotetraose (1,6-KT) when fructose is added to the 6 position of the terminal fructose (Fig. 1). Two major peaks were observed in the EIC traces for m/z 665 and 671 (Supplementary Figs. S2(a) and S2(b), see Supporting Information), corresponding to the m/z values of DP 4 fructans with no or one UL-13C6 fructose unit, respectively. The MS² spectra of both 1&6-KT and 1,6-KT showed mainly C and Z fragments (Fig. 2), as previously seen for inulin- and levan-type fructans.\[17\] Comparison of the MS² spectra of native 1&6-KT (Fig. 2(a)) and [UL-13C₆ fru] 1&6-KT (Fig. 2(b)) indicated that only a small degree of B cleavage occurred. The major difference between the fragmentation patterns from 1&6-KT and 1,6-KT was the formation of a m/z 341 fragment. The m/z 341 fragment is released from 1,6-KT by a C₂ cleavage whereas it cannot be formed from 1&6-KT by a single bond cleavage (Fig. 1). Another point of differentiation between the two molecules is the production of the m/z 509 ion corresponding to a DP 3 fragment with one UL-labeled fructose. Labeled 6-kestotriose (m/z 509) is released from 1&6-KT (m/z 671) by a C₂ cleavage (Fig. 2(b)) but cannot be produced from 1,6-KT (m/z 671) (Fig. 2(c)) that can only form an unlabeled 1-kestotriose (m/z 503).

Figure 3. Molecular structures of the main DP 5 fructans formed by 6-SFT and the X₁ and C₂α fragment ions. The m/z values are indicated with between parentheses the m/z values of the corresponding [UL-13C₆ fru]-labeled species. X₁ (m/z 229) is released from two consecutively labeled fructose units whereas X₁ (m/z 227) is released from a labeled fructose bound to an unlabeled fructose. The C₂α fragment is only released from 1&6,6-KP.
In order to observe the low molecular mass fragments, MS² analysis was performed using HCD (Fig. 2(d)). The HCD spectrum of 1,6-KT (with one UL-labeled fructose unit) (m/z 671) contained an ion at m/z 227, corresponding to a labeled X₁ cross-ring cleavage product,[17] confirming that the labeled fructose is terminal and bonded β(2,6). Apart from 1&6-KT and 1,6-KT, three other DP 4 fructans were identified, although their concentrations in the reaction mixture were low (Supplementary Fig. S2(a), see Supporting Information). The identity of 6,6-KT and 1,1-KT (eluting at 1.9 and 8.7 min, respectively) was confirmed by comparing their MS² spectra with published data[17] and by use of authentic standards. As a result, the peak eluting at 5.5 min must have a 6G-kestotriose core, as all possible DP 4 fructans with a 1-kestotriose or 6-kestotriose core had already been identified. Only two structures could be formed by 6-SFT from 6G-kestotriose (i.e. 6G,6-KT and 6G&6-KT). The observation of the m/z 221 and 383 ions, corresponding to X₁ and X₂ cross-ring cleavage products, provides unequivocal evidence that 6G,6-KT is formed (Supplementary Fig. S3, see Supporting Information).

Fragmentation of DP 5 fructans

In theory, three kestopentaose (KP) fructan structures can be formed by 6-SFT starting from 1&6-KT or 1,6-KT, i.e. 1&6,6-KP, 1,6,6-KP and 1,6&6-KP (Fig. 3). Nevertheless, only two major peaks were observed in the EIC for DP 5 unlabeled fructans (m/z 827) or with two labeled fructose units (m/z 839) (Supplementary Fig. S4, see Supporting Information). The MS² spectrum of the first peak in the m/z 839 EIC contains a distinctive 677 fragment ion (Fig. 4(a)), indicating the presence of 1&6,6-KP. This fragment, containing two labeled fructose moieties, can be formed from 1&6,6-KP by a C₆α** cleavage, but not from 1,6,6-KP or 1,6&6-KP. Yet, the same MS² spectrum also contains a clear 509 peak which cannot be formed from 1&6,6-KP by a single bond cleavage, this can only be explained by a C₂α’’ fragmentation of 1,6,6-KP. Hence, this suggests that two of the three possible DP 5 fructans are coeluting. The MS² spectrum of the second peak in the m/z 839 EIC (Fig. 4(b)) comprises a X₂α’’ fragment ion (m/z 395) corresponding to a cross-ring cleavage product containing two labeled fructose units that were β(2,6)-linked to the initial fructan structure.[17] Only 1&6,6-KP (m/z 839) and 1,6,6-KP (m/z 839) can form this fragment, but the absence of the 677 fragment indicates that this is the MS² spectrum of 1,6,6-KP. Further evidence for the identity of these structures was found in the HCD spectra. The HCD MS² spectrum of the first peak in the m/z 839 EIC (which corresponds to the KP with 2 UL-labeled fructose units) contains both fragment ions at m/z 227 and 229 (Fig. 4(c)). Both of these fragments originate from cross-ring cleavage from a terminal β(2,6)-linked fructosyl-fructose moiety (Fig. 3). The ion (m/z 227) results from the fragmentation of a structure containing an unlabeled fructose that is bound to a terminal labeled fructosyl unit whereas the second ion (m/z 229) originates from a structure containing two consecutively labeled fructose units. This observation once more shows that this MS² spectrum contains fragments from two different DP 5 structures as the original compounds contained only two UL-labeled fructose units (Fig. 3). The X₁ ion (m/z 227) was released from doubly labeled 1,6&6-KP and the X₂ ion (m/z 229) from doubly labeled 1&6,6-KP. The HCD MS² spectrum of the second peak in the EIC (m/z 839) (Fig. 4(c)) contains only the 229 (X₁α”) and 395 (X₂α”) cross-ring cleavage products, originating from a β(2,6)-linked fructosyl-β(2,6)-fructose moiety. This is in agreement with the previously assigned identity of 1,6,6-KP.

Figure 4. Negative electrospray MS² spectra of the main DP 5 fructans formed by 6-SFT. (a) CID MS² (m/z 839) of [UL¹³C₆ fru]₂ DP 5 (tᵣ = 2.7 min). (b) CID MS² (m/z 839) of [UL¹³C₆ fru]₂ DP 5 fructans eluting at 3.5 min. (c) m/z 150–550 region of HCD MS² (m/z 839) of [UL¹³C₆ fru]₂ DP 5 fructans eluting at 2.7 min. (d) m/z 150–550 region of HCD MS² (m/z 839) of [UL⁺¹³C₆ fru]₂ DP 5 fructans eluting at 3.5 min.
Fragmentation of DP 6 fructans

Four DP 6 fructan (or kestohexaose (KH)) structures can be formed by 6-SFT starting from the three DP 5 fructans identified in the previous paragraph (Figs. 5 and 6). Four major peaks were observed in the EIC for DP 6 fructans (m/z 989, Supplementary Fig. S5, see Supporting Information). The MS² spectra of the DP 6 fructans containing three UL-labeled fructose units (m/z 1007) were not informative because of high levels of interference from (1-kestotriose-1-kestotriose)⁻ adducts. Therefore, MS² data were collected for 992, the m/z value of DP 6 fructans with three [1-¹³C₁] fructose moieties, generated from the reaction mixture containing [1-¹³C₁ fru] sucrose. The MS² spectra of the largest DP 6 peak (Fig. 7(a)) and the corresponding [1-¹³C₁]-labeled species (Fig. 7(b)) contain X₂ and X₃ cross-ring cleavage products, and an X₁ cross-ring cleavage product was seen in the HCD spectrum (result not shown). These ions are indicative for three consecutively β(2,6)-linked fructose units and can only originate from either 1,6,6,6-KH or 1&6,6,6-KH. The

![Diagram of molecular structures](image)

**Figure 5.** Molecular structures of 1,6,6,6-KH, 1&6,6,6-KH and the C₂α and X₃ fragment ions. The m/z values are indicated with between parentheses the m/z values of the corresponding [¹³C₁ fru]-labeled species.
Figure 6. Molecular structures of 1,6&6,6-KH, 6&1,6,6-KH and the C₂ fragment formed from 1,6&6,6-KH. The m/z values are indicated with between parentheses the m/z values of the corresponding [13C₁ fru]-labeled species.

Figure 7. Negative electrospray MS² spectra of the main DP 6 fructans formed by 6-SFT. (a) CID MS² (m/z 989) of unlabeled 1&6,6,6-KH. (b) CID MS² (m/z 992) of [13C₁ fru]₁, 1&6,6,6-KH. (c) CID MS² of (m/z 992) [13C₁ fru]₃, 1,6&6,6-KH. (d) CID MS² of (m/z 992) [13C₁ fru]₃, 1,6,6,6-KH.
formation of the C$_{2n}$*** (m/z 830) fragment in the MS$^2$ spectrum of the [1-13C$_1$]-labeled species excludes the first possibility (Fig. 5) and, consequently, it can be inferred that 1&6,6,6-KH was the major DP 6 fructan formed by 6-SFT. The MS$^2$ spectrum of the second peak (peak B in Supplementary Fig. S5(b), see Supporting Information) in the EIC for tri-labeled DP 6 fructans contained a distinctive m/z 667 ion (Fig. 7(c)). This fragment may result from a C$_2$*** cleavage of 1,6&6,6-KH (m/z 992) but cannot be formed from the other possible DP 6 fructan structures (Figs. 5 and 6) when only one bond is cleaved. In the MS$^2$ spectrum of the last DP 6 fructan (peak D in Supplementary Fig. S5(b), see Supporting Information), an X$_{3***}$ fragment (m/z 548) is observed, indicating that three labeled fructose units were β(2,6)-linked to the original compound. This observation together with the absence of a m/z 830 fragment demonstrates that the spectrum belongs to the unbranched 1,6,6,6-KH (Fig. 5). The MS$^2$ spectrum of the compound eluting first in the DP 6 EIC (peak A in Supplementary Fig. S5(b), see Supporting Information) contained a Z$^2$ fragment (m/z 812), together with a m/z 811 fragment (results not shown). The latter can be formed from 6&1,6,6-KH, the only remaining DP 6 fructan formed by 6-SFT (Fig. 6). Unfortunately, this conclusion could not be confirmed by the observation of distinctive fragments from the low m/z region because the ion current was too low to obtain reliable MS$^2$ spectra.

CONCLUSIONS

It can be concluded that incubation of 1-kestotriose with 13C-labeled sucrose and P. terminalis 6-SFT made it possible to elucidate the fragmentation pattern of all DP 4, 5 and 6 graminan-type fructans produced. Using the same approach, based on LC/MS analysis of 13C-labeled fructan oligomers, structural characterization of higher DP fructans should be feasible as long as the ion current and hence signal-to-noise ratio is sufficiently high. In this case, higher order MS$^n$ (n >2) analysis may be required for full structural elucidation since the number of theoretically possible isomers increases rapidly with increasing DP. In the present study, the major peaks in the DP 5 and DP 6 EICs contained 1&6,6,6-KP and 1&6,6,6-KH, respectively. These structures both have a 1&6-KT core to which one or two fructose units are attached via a β(2,6) linkage. This suggests that P. terminalis 6-SFT prefers to extend the existing branch above creating a new branch. Analysis of the DP 3 fructans in the reaction mixture with ULP-13C indicated that P. terminalis 6-SFT has no 6G,6-FFT activity but does have 6-ST and minor 1-ST side activities.

The method described above based on the MS$^2$ fragmentation routes enables not only the LC/MS-based characterization of fructan-metabolizing enzymes, but also a rapid screening of large sample sets for the presence of branched, graminan-type fructans. To this end, the presence of fructan oligosaccharides could be verified by MS$^1$ analysis. Next, their structures can be determined based on their LC elution order and by performing MS$^2$ analysis for the corresponding masses (summarized in Table 1). An interesting application is the study of the effects of food processing on this type of fructans. Alternatively, the effect of their fermentation by the colon microbiota can be investigated by LC/MS analysis of partially fermented fructans obtained from either in vitro or in vivo fermentation experiments. A third application is the study of fructan metabolism in plants by the structural characterization of fructans extracted from kernels or derived tissues from different phases of development as described for immature wheat grains.[225]

The EICs of the DP 3, 4, 5 and 6 of both native and 13C-labeled 6-SFT reaction products and DP 3 and DP 4 fructan standards are added as Supporting Information together with the negative electrospray MS$^2$ spectra of native and UL-labeled 6G,6-kestotetraose. In addition, HPAEC profiles showing the gradual formation of branched fructans are included. Finally, a summary of the 6-SFT reaction products and their ion intensities is provided.

Acknowledgements

This work was supported by the KU Leuven Methusalem program "Food for the future" (2007–2014). We gratefully acknowledge the "Fonds voor Wetenschappelijk Onderzoek – Vlaanderen“ (FWO, Brussels, Belgium) for the postdoctoral fellowship to E. Dornez. Instrumentation was purchased via funding from the Novo Nordisk Foundation.

REFERENCES


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