Food Chemistry 129 (2011) 305–311

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Structure and degree of polymerisation of fructooligosaccharides present in roots and leaves of *Stevia rebaudiana* (Bert.) Bertoni

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ARTICLE INFO

Article history:
Received 24 February 2011
Received in revised form 18 April 2011
Accepted 21 April 2011
Available online 1 May 2011

Keywords:
Stevia rebaudiana
Fructooligosaccharides
MALDI-TOF
ESI-MS
Inulin-like

ABSTRACT

Fructooligosaccharides have been isolated from roots and leaves of *Stevia rebaudiana*, by hot aqueous extraction, followed by precipitation with ethanol. Their structure has been determined using methylation and NMR analysis, MALDI-TOF, and ESI-MS. Fructooligosaccharides contained almost exclusively (2→1)-linked β-fructofuranosyl, with terminal α-glucopyranosyl and β-fructofuranosyl units. MALDI-TOF and ESI-MS analyses showed the wide range of degree of polymerisation (DP) present in various extracts. From roots and leaves, three different fractions gave profiles of homologous series, with DPs ranging up to 17 with MALDI-TOF and 19 using ESI-MS. These inulin-type fructooligosaccharides were the major component of extracts from *S. rebaudiana* roots and significant components from the leaves.

1. Introduction

Fructans, otherwise known as fructooligosaccharides, are important as storage components in many plant species, including several Asteraceae, Liliaceae and Poaceae, which are of great importance as forage grass cereals. The most common sources are underground organs of chicory, Jerusalem artichoke, asparagus, and members of the onion family (Wack & Blaschek, 2006). They contain (2→1)- and/or (2→6)-linked β-D-fructofuranosyl units with one internal or external glucosyl unit (Waterhouse & Chatterton, 1993). Some non-digestible fructans, namely those containing (2→1)-linkages, such as inulin and inulin-like oligosaccharides (Fig. 1), confer potentially interesting prebiotic properties in human and pet foods, and non-food applications (Fuchs, 1991).

Although there is a growing interest in fructans and fructan-producing species, there is little information on their biological proprieties. Some studies have shown the role of fructooligosaccharides (FOS) in defence functions (Buddington, Kelly-Quagliana, Buddington, & Kimura, 2002; Letellier, Messier, Lessard, & Quessy, 2000), lipid metabolism (Delzenne, Daubioul, Neyrinck, Lasa, & Taper, 2002), control of diabetes (Luo et al., 2000), and anti-cancer activity (Pool-Zobel, Loo, Rowland, & Roberfroid, 2002).

Stevia rebaudiana (Bert.) Bertoni belongs to the family Asteraceae and contains, in substantial quantities, several highly potent low-calorie sweeteners in its leaves (Brandle, 1998). The commercial exploitation of this plant has increased since the 1970's, when Japanese researchers developed a process for extraction and refinement of its components (Dacome et al., 2005).

However, only one study has been carried out on the molecular weight distribution of leaf extracts and a commercial *Stevia* supplement from *S. rebaudiana* (Jackson et al. 2009), where preliminary results were obtained using desorption electrospray ionisation mass spectrometry (DESI-MS).

We now describe an isolation procedure and structural analysis of fructooligosaccharides (FOS) from aqueous extracts of roots and leaves of *S. rebaudiana*, including determination of their degree of polymerisation (DP), using matrix-assisted laser desorption/ionisation mass spectrometry (MALDI-TOF) and electrospray ionisation mass spectrometry (ESI-MS).

2. Materials and methods

2.1. Plant material

Dried, powdered leaves of *Stevia rebaudiana* (Bert.) Bertoni were purchased from SteviaFarma, Maringá, Paraná State, Brazil. Roots were collected in the nearby Medicinal Plant Garden. Voucher

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0308-8146
doi:10.1016/j.foodchem.2011.04.057

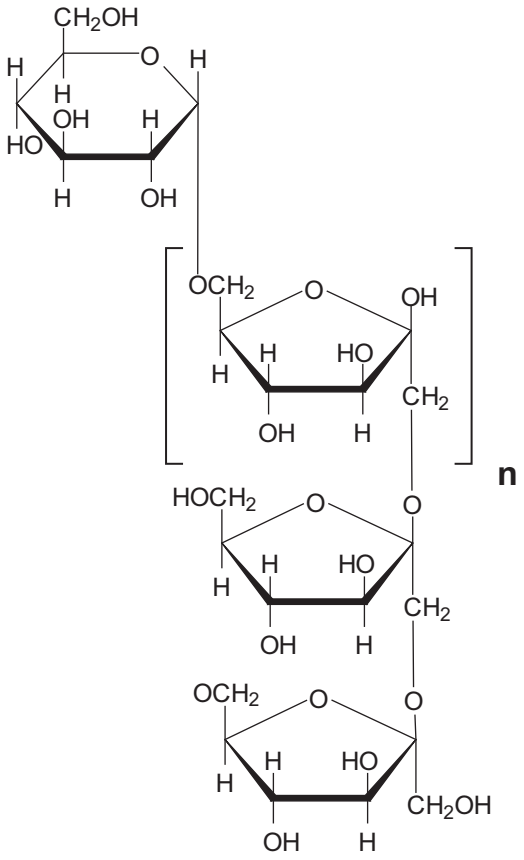

Fig. 1. General chemical structures for inulin and inulin-like oligosaccharides.

specimens of the plant material are deposited in the herbarium of the Department of Biology at the Maringá State University (identification number 14301-HUEM).

2.2. Reagents

All reagents were of analytical grade.

2.3. Isolation of fructooligosaccharides

Dried roots (100.0 g) were powdered and successively extracted with refluxing hexane (1000 mL) for 4 h and methanol (1000 mL) for 4 h. Insoluble material was separated and extracted with boiling water for 4 h (1000 mL). The extract was cooled, stirred for 3 h, and centrifuged (8000*g*, 30 min). The supernatant was evaporated to a small volume, added to EtOH (3×, v/v), left at 4 °C overnight; insoluble fructooligosaccharides were formed as a precipitate on addition to three volumes of EtOH (v/v). Centrifugation (8000*g*; 20 min) provided sediment, which was collected, washed twice with EtOH at the same 3:1 concentration and dried to give a product (15.7 g). This was dissolved in water (200 mL), and the solution then submitted to freezing followed by gentle thawing at 4 °C (Iacomini, Gorin, & Baron, 1988) until no more precipitate appeared. Centrifugation gave, following freeze-drying, the soluble component containing root fructooligosaccharides (RFOS, 4.6 g).

Dried leaves of *S. rebaudiana* (100 g) were extracted with refluxing acetone (1000 mL) for 2 h (3×). The residue was extracted with refluxing water (1000 mL) for 2 h (3×), which was evaporated to a small volume, and added to EtOH (3×, v/v). The resulting precipitate (4.75 g) was dissolved in water (100 mL), and the solution was treated with 10% aqueous TCA (100 mL) to precipitate protein. After centrifugation, the supernatant was neutralised with aq. NaOH, dialysed and freeze-dried. The residue was dissolved in H_2O (100 mL), and the solution was submitted to freeze–thawing until no more precipitate appeared. Centrifugation gave, following freeze-drying, the soluble component (2.68 g). Afterwards, 1.0 g was submitted to treatment using Sartorius ultrafiltration equipment (Model 16249). Commercial membranes with a molecular mass cut-off (MMCO) of 100 kDa and 30 kDa (Millipore) were used. Each one was cut to size and soaked overnight in deionised H_2O, prior to use.

Filtration experiments were carried out at a constant pressure of 4 bar. The dead-end ultrafiltration cell was filled with H_2O solution (100 mL) containing soluble components and ultrafiltration was allowed to proceed, at room temperature for 5–8 h, the time depending on the membrane. During the filtration process, fractions of average molecular mass (MMs) less than the MMCO of the used membrane passed through the membrane (Zpermeate), while those having larger MMs were collected as retained material. When approximately 100 mL of permeate had been collected, filtration was stopped. Both permeate and retained solutions were analysed by mass spectrometry and high-performance size-exclusion chromatography (HPSEC). The solution, permeated with a membrane of 30 kDa, was then dialysed against distilled H_2O in a closed system with a 15 kDa cut-off membrane. The water in the system (1 l) was renewed every 12 h (3×). The permeated fraction on dialysis contained leaf fructooligosaccharides (LFOS, 175 mg).

2.4. High-performance size-exclusion chromatography

High-performance size-exclusion chromatography (HPSEC) analysis of fructooligosaccharides was carried out with Wyatt Technology (Santa Barbara, CA) equipment coupled to a refractive index detector (Waters Model 2410; Waters Corporation, Milford, MA) and a multi-angle laser light scattering detector (MALLS; Model Dawn DSP) at 632.8 nm, using. Incorporated were four gel permeation ultrahydrogel columns in series, with exclusion sizes of 7×10^6, 4×10^5, 8×10^4, and 5×10^3 Da. Elution was carried out with 0.1 M aq. $NaNO_2$ containing 200 ppm aq. NaN_3 at 0.6 mL min^{-1}. The samples, previously filtered through a membrane (0.22 μm), were injected (250 μL loop) at a concentration of 1 mg mL^{-1}.

2.5. Determination of monosaccharide composition

Samples (0.1–1.0 mg) were hydrolysed in 500 μL 0.2 M TFA at 80 °C for 30 min. The TFA was evaporated under a stream of N_2 for 2 h at ambient temperature to give a residue. The hydrolysate was treated with $NaBH_4$ (2 mg), and after 18 h, AcOH was added, the solution evaporated to dryness, and remaining boric acid removed as trimethyl borate by co-evaporation with MeOH. Acetylation was carried out with Ac_2O:pyridine (1:1, v/v; 2 mL) at room temperature for 12 h, to give alditol acetates (Sassaki, Gorin, Souza, Czelusniak, & Iacomini, 2005). They were analysed by GC-MS using a Varian 3800 gas chromatograph coupled to a Varian Ion-Trap 2000R mass spectrometer (Varian, Palo Alto, CA). The column was DB-225MS (30 m × 0.25 mm i.d.; Agilent Santa Clara, CA) programmed from 50 to 220 °C at 40 °C/min, with helium as carrier gas, at a flow rate of 1 mL min^{-1}. The inlet temperature was 250 °C, and the MS transfer line was set at 250 °C. MS acquisition parameters included scanning from *m/z* 50 to 550 in electron ionisation mode (EI) at 70 eV. Components were identified by their retention times and EI spectra.

2.6. Glycoside linkage analysis

Fructooligosaccharides (1–3 mg) were solubilised in dry DMSO (460 μL) and per-*O*-methylated by the method of Ciucanu and Kerek (1984). The products were hydrolysed in 2 M TFA (500 μL) for 30 min at 60 °C and evaporated to dryness, after addition of 2-

methyl-2-propanol (500 μL). This minimised loss by decomposition of *O*-methyl fructofuranosyl derivatives, prior to reduction and acetylation. Resulting partially *O*-methylalditol acetates (PMAAs) were analysed by GC-MS, under conditions identical to those described for alditol acetates, except that the final temperature was 215 °C. The partially *O*-methylated alditol acetates were identified by comparison of their EI spectra with those of products obtained from fructooligosaccharides 1-kestose and nystose (Hayashi, Yoshiyama, Fuji, & Shinohara, 2000).

2.7. Electrospray ionisation mass spectrometric analysis

Samples of LFOS and RFOS were introduced into the mass spectrometer using a syringe pump, for offline ESI-MS analysis. Spectra were obtained in positive ionisation mode, using a triple quadrupole Quattro LC (Waters), setting the capillary voltage at 2300 V, cone voltage at 60 V and source at 100 °C. Each spectrum was produced by accumulation of data over 1 min.

2.8. Matrix-assisted laser desorption/ionisation-time of flight ((MALDI-TOF) analysis

Positive-ion MALDI–TOF mass spectra were acquired with MALDITOF/TOF Autoflex II (Bruker Daltonics, Billerica, MA) equipment. Analytes, co-crystallised with matrices on the probe, were ionised by using a nitrogen laser pulse (337 nm) and accelerated between 20 and 60 kV by using pulsed ion extraction before entering the time-of-flight mass spectrometer. The matrix was 2,5-dihy-

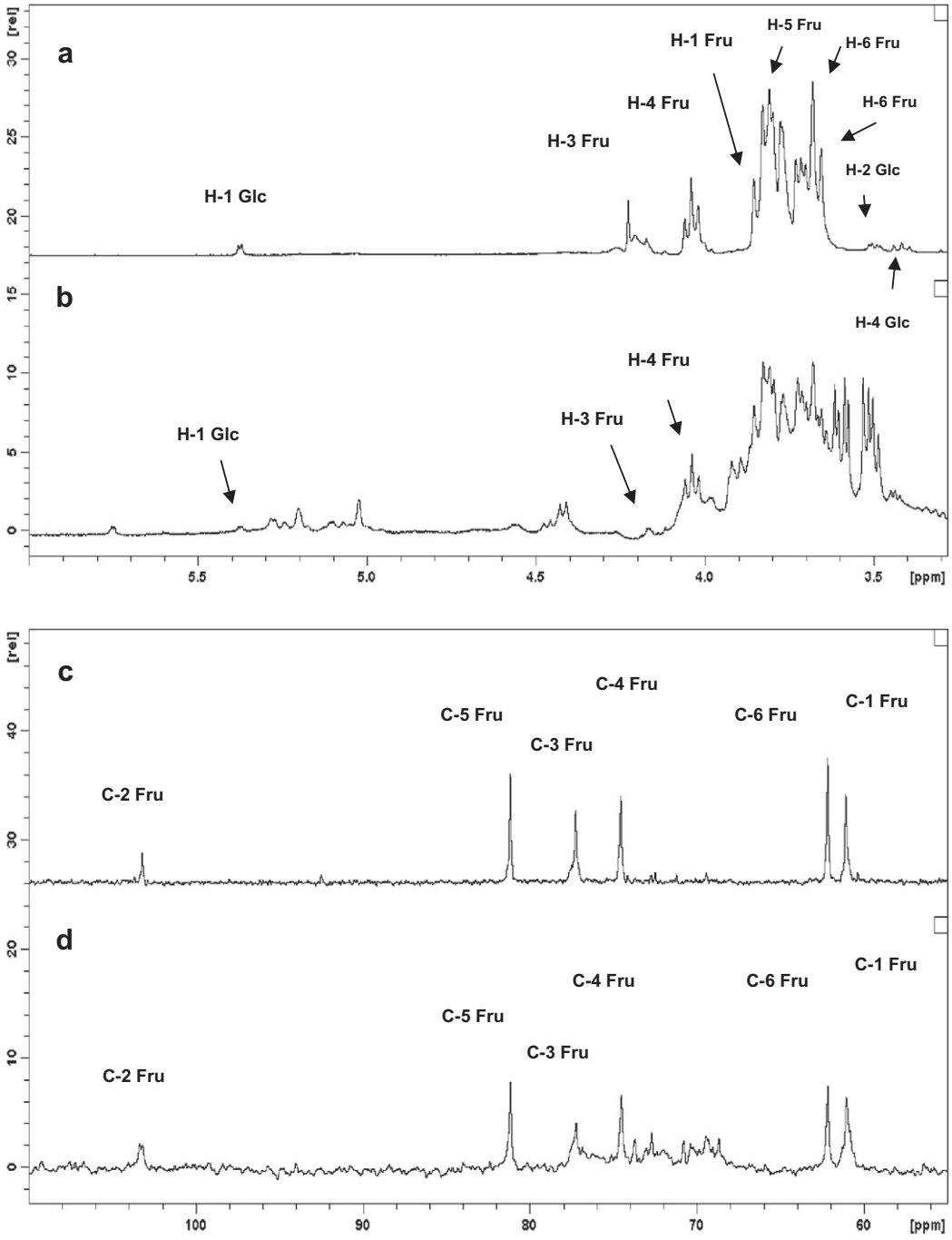

Fig. 2. ^{1}H NMR spectra of RFOS (a) and LFOS (b); ^{13}C NMR spectra of RFOS (c), and LFOS (d).

droxybenzoic acid (DHB). Each sample was dissolved at 4 mg mL^{-1} in deionised H_2O and those of matrix solutions were at 2.5 to 10 mg mL^{-1} DHB. The laser strength was selected to obtain the best signal-to-noise ratios. The number of laser pulses collected was chosen, as being necessary to obtain good responses for all oligosaccharides. The optimum experimental conditions (concentration of samples and matrices, convenient slide preparation, and laser power) were based on those determined for the positive-ion mode by Štikarovská and Chmelík (2004).

2.9. NMR spectroscopy

^1H and ^{13}C NMR spectra were obtained from samples in D_2O at 70 °C using a 400 MHz Bruker model DRX Avance III spectrometer, incorporating a 5-mm inverse probe. Chemical shifts (δ) are expressed in ppm relative to acetone, at δ 2.44 and 30.2 ($\underline{H_3C}CO\underline{C}H_3$) respectively. Two-dimensional spectra (HMQC and HMBC) were recorded using standard Bruker procedures (Cui, Eskin, Biliaderis, & Marat 1996).

3. Results and discussion

Fructooligosaccharides from roots (RFOS) and leaves (LFOS) of *S. rebaudiana* were extracted with hot water to inhibit enzyme activity. They were precipitated from aqueous solutions by addition to ethanol (3× vol.), and were purified by repeated dissolution and precipitation.

After purification, roots showed a yield of 4.6% and leave a yield of 0.46% of fructooligosaccharides (FOS). Different from RFOS, the LFOS purification protocol had additional steps, in order to eliminate the arabinogalactans, since the FOS from *S. rebaudiana* leaves appeared as a minor component, whereas the purification of RFOS was not necessary because the FOS was the only component isolated from aqueous extract of roots. FOS were analysed by GC-MS, following hydrolysis and derivatisation to alditol acetates of glucitol and mannitol, by methylation analysis, and ^{13}C-NMR spectroscopy.

Results of methylation analyses of RFOS provided further information on molecular structure. These indicated an overall linear structure with D-glucopyranosyl end-units, the major derivative of fructose being 3,4,6-tri-*O*-methylated (85.0%), indicating a β-(2→1)-linked backbone. The number of terminal non-reducing fructose residues was shown from the resulting 2,5-di-*O*-acetyl-1,3,4,6-tetra-*O*-methyl-mannitol and -glucitol (4.5%). Although some branching can exist, the amount of terminal fructose is smaller than that of terminal glucose (10%). This may be due to a greater instability of terminal fructose compared to terminal glucose fragments, in the hydrolysis step. Linear (2→6)-linkages between β-fructose residues can be excluded. Although partially *O*-methylated alditol acetates from (2→1)- and (2→6)-linked β-fructofuranosyl units have the same GC elution time, they can be distinguished by their mass spectra, based on the asymmetry introduced by reduction of the partially methylated fructoses at C-2 with sodium borodeuteride. Derivatives from (2→1)-linkages gave rise to ions of *m/z* 190 and *m/z* 161 as primary fragments. No significant *m/z* 189 and 162 ions, typical of products of (2→6)-linked fructofuranosyl units were detected, showing that such linear linkages were not present.

The ^1H-NMR spectrum of RFOS (Fig. 2a) showed the presence of one signal in the anomeric region at δ 5.37 (J = 3.8 Hz), others at δ 4.04 and 4.20 and between δ 3.60 and 3.90.

All resonances present in the ^{13}C-NMR spectrum of RFOS (Fig. 2c) could be assigned to fructooligosaccharides (Table 1). The C-2 resonance of fructofuranose from RFOS appears at δ 103.2 and the minor signal at δ 92.73 was assigned to an aldose-type residue, whereas those with shifts greater than δ 100 indicate ketose residues. These values agree with those obtained for C-2 signal intensities of chain fructose residues (δ 103.39) and the fructosyl moities (δ 103.91) of terminal sucrose in spectra (Wack & Blaschek, 2006).

In the 2D NMR spectra of RFOS, chemical shifts of the ^1H and ^{13}C of the main residues were fully assigned, based on literature data (Bock, Pedersen, & Pedersen, 1984; Bradbury & Jenkins, 1984; Cérantola et al., 2004), as arising from D-fructofuranosyl units with a β-configuration (Table 2). From their spectra, $^1H/^{13}C$ anomeric signals at δ 5.37/92.73 were assigned to α-D-glucopyranosyl units.

The ^1H-NMR spectrum of LFOS (Fig. 2b) contained one anomeric signal at δ 5.38 (J = 3.8 Hz), the other signals at δ 4.05 and 4.15 and between δ 3.60 and 3.90 (Fig. 1b).

The ^{13}C-NMR data for fractions RFOS and LFOS from *S. rebaudiana* (Table 1 and Fig. 2c, d) clearly contain the resonances of chicory inulin (Wack & Blaschek, 2006), with greater intensities for (2→1)-linked β-fructofuranosyl when compared with terminal fructosyl and glucosyl units.

HPSEC analysis of fractions RFOS and LFOS showed their heterogeneity, reflecting different degrees of polymerisation. In addition, integration of the H-1 signal of the glucose moiety at δ 5.44 and the H-3 and/or H-4 signals of the preponderant fructosyl units between δ 4.27 and 4.11 respectively, mean DPs of 8–9 (RFOS) and

Table 1
^{13}C NMR chemical shifts in parts per million of the β-D-Fru*f* units and α-D-Glc*p* units of *Stevia rebaudiana* fructooligosaccharides from leaves (LFOS), roots (RFOS) and the model chicory inulin.

Carbon		Chicory inulin[a]	LFOS	RFOS
β-Fructopyranose				
C-1	(2 → 1)-Linked	60.33	61.33	61.41
C-2	(2 → 1)-Linked	102.65	103.42	103.39
C-3	(2 → 1)-Linked	76.42	77.64	77.58
C-4	(2 → 1)-Linked	73.72	74.94	74.93
C-5	(2 → 1)-Linked	80.5	81.43	81.43
C-6	(2 → 1)-Linked	61.66	62.48	62.48
β-Glucopyranose				
C-1	Terminal	91.89	nd	92.73
C-2	Terminal	70.60	71.05	70.75
C-3	Terminal	72.03	nd	73.04
C-4	Terminal	68.66	68.96	69.78
C-5	Terminal	71.85	nd	71.46
C-6	Terminal	59.57	nd	60.75

[a] Chicory inulin chemical shifts (Wack & Blaschek, 2006); (nd) not detected.

Table 2
^1H and ^{13}C NMR chemical shifts of *Stevia rebaudian* fructooligosaccharides from roots (RFOS).

Residue[a]	H-1/C-1	H-2/C-2	H-3/C-3	H-4/C-4	H-5/C-5	H-6/C-6
RFOS						
→1)-β-D-Fru*f*-(2→	3.66–3.84		4.20	4.04	3.84	3.71
	61.41	103.39	77.58	74.93	81.43	62.48
α-D-Glc*p*-(1→	5.37	3.50	3.80	3.42	3.80	3.72
	92.73	70.75	73.04	69.78	71.46	60.75

[a] Due to overlapping signals, minor fructosyl signals, that is, those belonging to the glucose-linked and terminal residues, were omitted.

7–8 (LFOS) can be proposed, but in both ^{1}HNMR spectra there are signals of minor impurities that reduce the precision of this procedure for DP determination.

Other methodologies for DP determination are high-performance chromatography (HPLC), gas chromatography (GC), and principally high-performance anion-exchange chromatography with pulsed amperometric detection (HPAC-PAD), but the response for fructooligosaccharides (FOS) with HPAC-PAD can vary (Timmermans, van Leeuwen, Tournois, de Wit, & Vliegenthart, 1994) and analysis often requires considerable sample purification. Particularly significant was the analysis of FOS or inulin from some plants, using MALDI-MS (Wang, Sporn, & Low, 1999; Štikarovská & Chmelík, 2004; Lasytovicková & Chmelík, 2006; Arrizon, Morel, Gschaedler, & Monsan, 2010). We therefore applied this technique for determination of the DPs of fractions RFOS and LFOS.

The spectrum for each FOS is shown in Fig. 3. RFOS and LFOS ions had a mass difference of 162 Da, which corresponds to fructose/glucose residues. It gave rise to $[M + Na]^+$ and $[M + K]^+$ ions as the main distribution obtained in the +LIN mode. Almost all spectra exhibited monomodal molecular mass distributions. Often food samples, such as onions, shallots, and garlic, naturally contain a high concentration of potassium ions, and could be analysed without further addition of salts (Wang Sporn, & Low, 1999). The molecular ions seen in MALDI-MS for RFOS and LFOS samples were almost entirely the potassium adducts (Fig. 3).

Under these conditions, the DP distribution of FOS obtained for MALDI-MS ranged from 5 to 16 for RFOS and 4 to 9 for LFOS. These were consistent with their average-DPs obtained by integrating the ^{1}H signals from NMR spectra.

Carbohydrates ionise in a MALDI-MS source, only after cationisation with alkali ions (Börnsen, Mohr, & Widmer, 1995). For simplification of analysis, it is desirable that the carbohydrate sample contains predominantly only one metal ion, resulting in a single molecular ion peak. With no addition of another ion, the matrix and sample contained both sodium and potassium ions (Fig. 3), resulting in multiple ion signals. There have been some sample treatments, as with ion exchange membrane (Börnsen et al., 1995) and by dissolution of carbohydrates in 0.01 M solution of a particular metal salt (Wang Sporn, & Low, 1999), resulting in detection of a single adduct signal.

Since the DPs of RFOS and LFOS were between 4 and 16 units and ionic exchange can be readily carried out in the inlet system

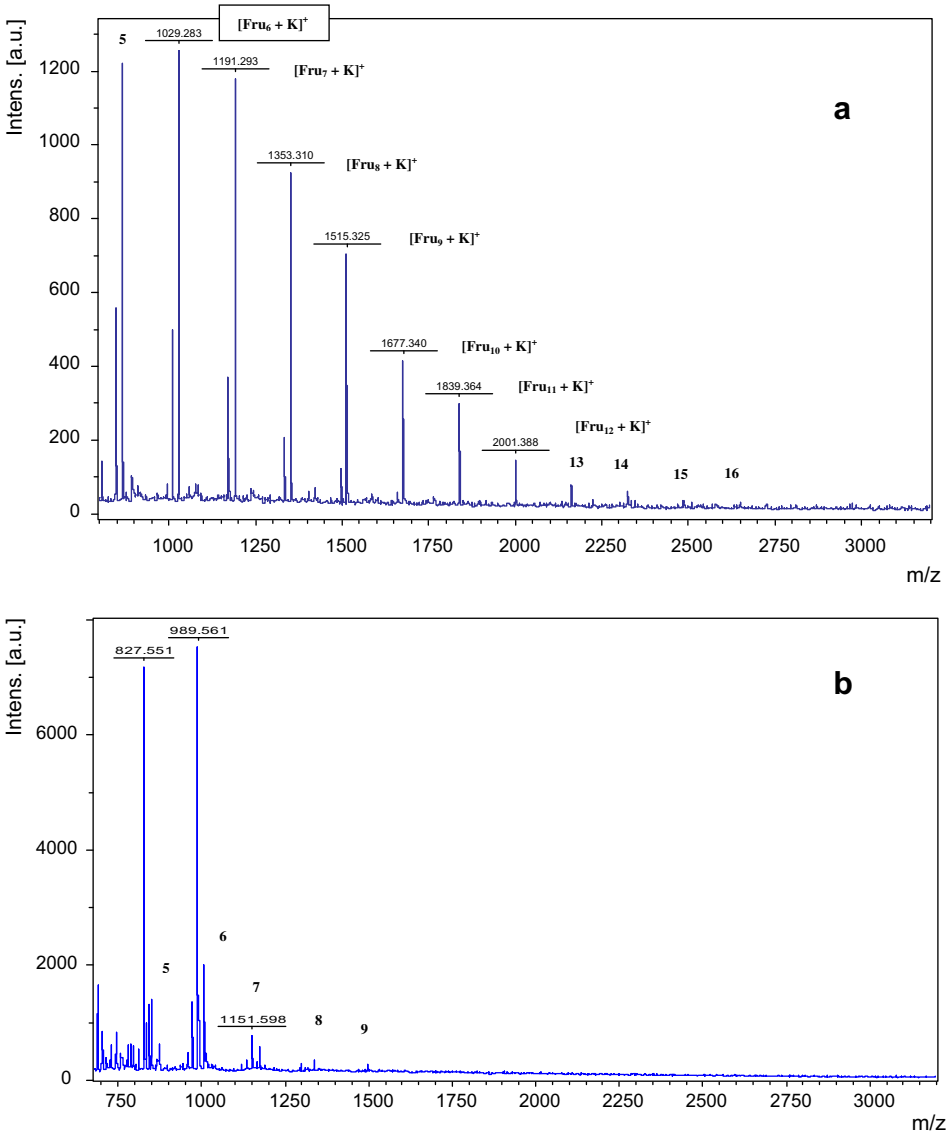

Fig. 3. MALDI-TOF-MS spectra of fructooligosaccharides from *S. rebaudiana*: RFOS (a) and LFOS (b).

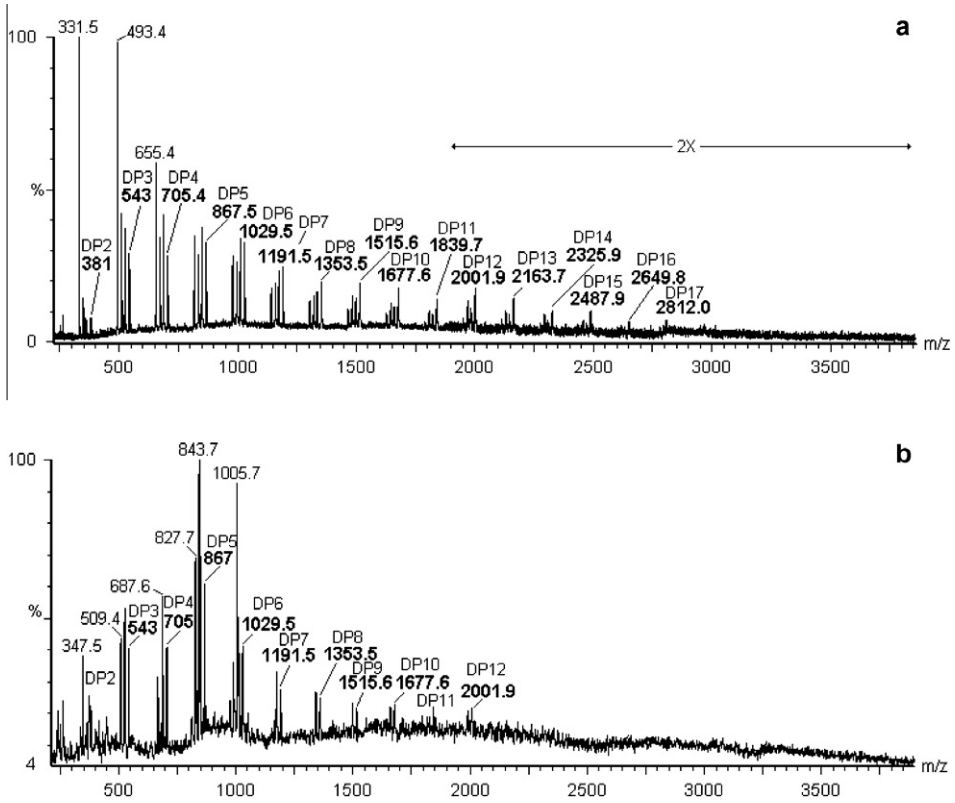

Fig. 4. ESI-MS spectra (+ve mode) of fructooligosaccharides from *S. rebaudiana*: RFOS (a) and LFOS (b).

of ESI-MS equipment, we analysed the FOS samples by this technique (Fig. 4). Under electrospray ionisation the FOS had DPs ranging from 2 to 17 for RFOS and 2 to 12 for LFOS that produced characteristic $[M + Na]^+$ and $[M + K]^+$ peaks, the sodium adducts being present even after ion exchange with potassium chloride, because of the significant concentration of this ion in leaves and roots extracts. However, in the FOS with *m/z* higher than 1500, K^+ ions were better produced.

The chain length distribution of FOS from root and leaves of *S. rebaudiana* was determined using ESI-MS (Fig. 4) and compared with those obtained on MALDI-TOF-MS analysis (Fig. 3). Similarities between the profiles of the two methods were found. However, ESI-MS seemed better for analysis of short- and long unit- chains, when DP < 20.

4. Conclusion

Based on GC-MS, NMR spectral, MALDI-TOF-MS and ESI-MS analysis of RFOS, SRFOS and LFOS, inulin-type fructooligosaccharides were the major component of *S. rebaudiana* roots and in its leaf extracts. This is of interest, since the inulin-type FOS is a naturally-occurring plant polysaccharide with important functional properties, related to prebiotics, dietary fibre, role lipid metabolism, and diabetes control.

Stevia rebaudiana roots can therefore be considered as a source of inulin-type FOS and its presence in the leaves indicates a possible application of extracts as a dietary supplement.

Acknowledgements

The authors thank the Brazilian agencies Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Fundação Araucária PRONEX-Carboidratos for their financial support.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.foodchem.2011.04.057.

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