Analysis of Food Oligosaccharides Using MALDI-MS: Quantification of Fructooligosaccharides

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Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) is a powerful new technique that will have a great impact on food analysis. This study demonstrates the applicability of MALDI-MS performed directly on an aqueous food extract for qualitative and quantitative analysis of food oligosaccharides. 2′,4′,6′-Trihydroxyacetophenone was found to be the best matrix for analysis of oligosaccharides in the foods examined. The relationship between laser strength, resolution, and the response factors of individual oligosaccharides using MALDI-MS was investigated. A MALDI-MS method for quantitative analysis of fructooligosaccharides with standard addition of a pure fructooligosaccharide was developed. High performance anion exchange chromatography with pulsed amperometric detection was compared to MALDI-MS for the analysis of fructooligosaccharides. The fructooligosaccharide analyses were performed on red onions, shallots, and elephant garlic.

Keywords: *Cyclodextrin; maltohexaose; fructan; inulin; kestose; nystose*

INTRODUCTION

Fructans are nonreducing water-soluble carbohydrates formed in higher plants composed of fructosyl units but usually containing one terminal glucose moiety per molecule. They occur as linear, branched, or, less frequently, cyclic molecules (Darbyshire and Henry, 1978, 1981; Bancal and Gaudillère, 1991; Stahl et al., 1997). Natural β-fructans have a degree of polymerization (DP) ranging from 2 to 55 or more. Lower mass (DP of 2−20) fructans are also called fructooligosaccharides (Figure 1), while higher mass polymers are known as inulin. It has been found that these nondigestible carbohydrates are effective in improving intestinal flora and increasing calcium and magnesium absorption (Ohta et al., 1995, 1998). Fructans have also been examined for their biological role in plant osmoregulation, adaptation to low-temperature photosynthesis, protection from freezing stress (Darbyshire and Henry, 1978; Pollock, 1984; Nelson and Smith, 1986; Chatterton et al., 1990; Livingston, 1990), and storage life of bulbs (Darbyshire and Henry, 1981; Suzuki and Cutcliffe, 1989).

Traditionally a variety of techniques have been used to analyze for fructooligosaccharides. Gel permeation chromatography (Darbyshire and Henry, 1978) has been used, but detection and identification of the separated fructooligosaccharides requires extensive additional methodology involving acid hydrolysis and various enzymatic and colorimetric procedures to identify the carbohydrates. The method by Manghi et al., 1995, detects fructooligosaccharides after various enzyme treatments and identification of the carbohydrates produced by high performance liquid chromatography (HPLC). However, the procedure is also lengthy and gives minimal information about which polymers are present. Loo et al., 1995, describe a number of analytical procedures for determination of fructooligosaccharides including HPLC for low DP fructooligosaccharides and a gas chromatography (GC) procedure. While the latter can be used for sensitive determination of fructooligosaccharides up to about DP of 12, extensive purification and derivatization with water sensitive reagents are required. In addition GC conditions of very high temperatures are required to volatilize the derivatized fructooligosaccharides. By far the most used procedure for analysis of fructooligosaccharides is high-performance anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD) (Chatterton et al., 1989, 1993; Shiomi et al., 1991; Timmermans et al., 1994; Loo et al., 1995). The fructooligosaccharide response with HPAE-PAD does vary (Timmermans et al., 1994) and the analyses often require significant sample purification.

Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) was originally developed for measuring the mass of large molecules such as proteins. MALDI-MS has also been applied to carbohydrates since about 1991 (Mock et al., 1991; Stahl et al., 1991). Fructooligosaccharides or inulin in some plants have been qualitatively analyzed by using MALDI-MS (Metzger et al., 1994; Stahl et al., 1997; Losso and Nakai, 1997) but there have been no reports using quantitative analysis. Our purpose in this study was to develop methodology for both qualitative and quantitative analysis of fructooligosaccharides in selected food samples.

MATERIALS AND METHODS

Materials and Reagents. Red onion bulbs (*Allium cepa* L.), shallot bulbs (*A. cepa* L. *var. ascalonicum*), and elephant garlic (*Allium ampeloprasum*) were purchased from local markets in Edmonton, Alberta, Canada. Inulin from Jerusalem

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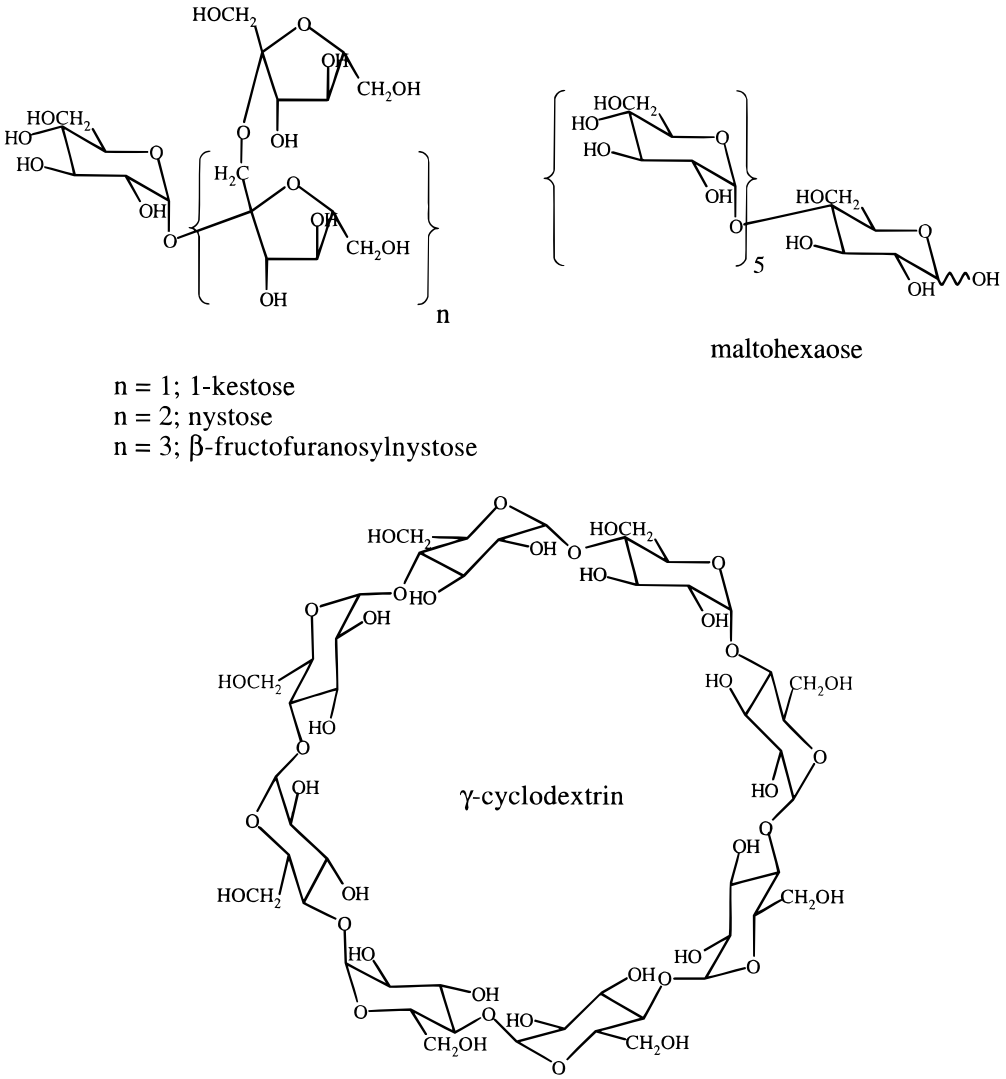

Figure 1. Chemical structures for fructooligosaccharides, maltohexaose, and γ-cyclodextrin.

artichokes, γ-cyclodextrin, maltohexaose, 4-hydroxy-α-cyanocinnamic acid (HCCA), and sinapinic acid were purchased from Sigma Chem. Co. (St. Louis, MO). A mixture of 1-kestose (GF_2, DP = 3), nystose (GF_3, DP = 4), and β-fructofuranosylnystose (GF_4, DP = 5) (34%, 53%, and 10%) and these individual fructooligosaccharides in pure form were a gift from Dr. A. Ohta, Nutritional Science Center, Meiji Seika Kaisha, Japan. 2′,4′,6′-Trihydroxyacetophenone monohydrate (THAP), [2-(4-hydroxyphenylazo)benzoic acid] (HABA), 3-aminoquinoline (3-AQ), 1-hydroxyisoquinoline, and 2,5-dihydroxybenzoic acid (DHB) were obtained from Aldrich Chem. Co. (Milwaukee, WI).

Extraction of Fructooligosaccharides from Onion, Shallot, and Garlic Samples. Fresh samples were peeled to remove the dry outer layers and then chopped using a food processor (Braun, UK 100, Type 4259, Germany) for 5 min. The mixed samples were freeze-dried. Each freeze-dried sample (1 g) was extracted with two portions of 40-mL double deionized water heated to reflux for 1 h. The cooled sample was centrifuged for 15 min at 10 000 rpm after each water extraction; the supernatants were combined and made up to 100 mL with water. The aqueous extract was then filtered with a Millipore HA 0.45-μm membrane (Chromatography Division/Millipore Corp., Milford, MA) and the extract kept frozen at −20 °C until needed.

Moisture Content. Sample moisture content was determined according to AOAC Official Method of Analysis (1990) by using a vacuum oven (National Appliance Co.) overnight in vacuo at 70 °C.

MALDI-MS. MALDI-MS was performed with the Proflex III, Bruker Analytical Systems Inc. (Billerica, MA). Analytes cocrystallized with matrixes on the probe were ionized by using a nitrogen laser pulse (337 nm) and accelerated under 20 kV by using pulsed ion extraction before entering the time-of-flight mass spectrometer. The preparation of matrixes and samples is shown in Table 1. Laser strength was selected to obtain the best signal-to-noise ratios. The number of laser pulses collected was determined as needed to obtain good responses of all oligosaccharides.

Quantification of fructooligosaccharides using MALDI-MS was achieved using standard addition. The frozen sample extracts were allowed to warm to room temperature. Samples were prepared by taking 50 μL of extract and mixing this with 50 μL of aqueous 0.01 M potassium chloride solution. The standard addition samples had GF_4 (7.9×10^{-4} M in 50 μL aqueous 0.01 M potassium chloride) added to 50-μL sample extracts. Samples and standard addition samples were each spotted in five separate positions on the probe. A single spectrum was then generated for each position on the probe (10 spectra in all, five for each sample and five for each standard addition sample) by random selection of three different spots for each probe position and collecting 60 laser pulses for each spot. That is, one spectrum represented the sum of 3 × 60 or 180 laser pulses. Peak heights (for potassium adducts) were determined for each fructooligosaccharide from each spectrum. These peak heights were then scaled relative to the GF_3 peak, which was arbitrarily set at a value of 1.0. Each of the five spectra for the sample was compared to a different spectrum from the five standard addition samples and the average increase in scaled relative peak height for GF_4 (standard fructooligosaccharide added) determined. This

Table 1. Performance of Matrixes for Desorption and Ionization of Maltohexaose and γ-Cyclodextrin

matrix	references	matrix concentration	preparation of matrix and sample	spectra quality	repeatability	matrix peaks	analyte molar ratio
2,5-dihydroxybenzoic acid (DHB)	Bruker, 1995; Mohr et al., 1995; Losso et al., 1997	1. 12.3 mg/mL in ethanol:water = 1:1	mix matrix and sample together in a ratio of 1:1	excellent	good	medium	normal
		2. 10.1 mg/mL in double deionized water	as above	good	good	medium	normal
2,4,6-trihydroxyacetophenone monohydrate (THAP)	Pieles et al., 1993	1. saturated in acetone	put matrix on the probe first and then sample on top of matrix	excellent	excellent	few	normal
		2. 12.5 mg/mL in acetonitrile:water = 1:1	mix matrix and sample together in a ratio of 1:1	excellent	excellent	few	normal
3-aminoquinoline	Metzger et al., 1994; Stahl et al., 1997	10.1 mg/mL in 10% ethanol	as above	good	poor	few	normal
4-hydroxy-α-cyanocinnamic acid (HCCA)	Bartsch et al., 1996	13.3 mg/mL in 50% ethanol or matrix saturated in ethanol	as above	good	poor	medium	normal
sinapinic acid	Bruker, 1995	14.2 mg/mL in acetonitrile:water = 1:1	as above	no	no	medium	
2-(4-hydroxyphenylazo)-benzoic acid (HABA)	Harvey et al., 1994	saturated in acetone	put matrix on the probe first and then sample on top of matrix	poor	good	lots	abnormal
2,5-dihydroxybenzoic acid (DHB)/1-hydroxyisoquinoline (HIC)	Mohr et al., 1995	0.2 M DHB/0.6 M HIC in acetonitrile:water = 1:1	mix matrix and sample together in a ratio of 1:1	good	poor	few	normal

gave a value for the average increased response due to the addition of GF_4. This response factor was then used to determine the average amount of each fructooligosaccharide in the five sample spectra. The acquisition of the MALDI-MS data took about 20 min and the calculations necessary were rapidly carried out by using Microsoft Excel 97. All samples were analyzed in duplicate. That is, an entirely new 10 spots (five samples and five standard addition samples) were analyzed.

HPAE-PAD. The frozen red onion, shallot, and garlic sample extracts were allowed to come to room temperature and diluted 1:5 (in 5-mL volumetric flask) with water. Fructooligosaccharide standards (10.0 mg) were prepared in a 50-mL volumetric flask with HPLC grade water. Each sample was passed through a 0.2-μm syringe filter (25 mm; Chromatographic Specialties, Brockville, ON). Filtered samples were analyzed on a Waters 625 metal free gradient HPLC (Waters Chromatography, Milford, MA). All samples and standards were injected (50 μL) with a Waters 712 Wisp autosampler. Carbohydrates were separated on a Carbo Pac PA1 column (250 × 4 mm; Dionex, Sunnyvale, CA) coupled with a Carbo Pac PA1 guard column (50 × 4 mm). The solvents used were 100 mM sodium hydroxide (solvent A), 100 mM sodium hydroxide/400 mM sodium acetate (solvent B), and 300 mM sodium hydroxide (solvent C). The mobile phase flow rate was maintained at 1 mL/min, with a linear gradient profile consisting of solvent A with the following proportions (v/v) of solvent B or C: 0−8 min, maintain 0% B and 0% C; 8−60 min, 100% B; 60−61 min, 100% C; 61−90 min, maintain 100% C; 90−91 min, 0% B and 0% C. Sodium hydroxide (300 mM) was added postcolumn (Waters Chromatography) at a flow rate of 0.70 mL/min to minimize baseline drift. Detection was achieved employing a Waters 464 pulsed amperometric detector (PAD) with a dual gold electrode and triple pulsed amperometry at a sensitivity of 50 μA. The electrode was maintained at the following potentials and durations: $E_1 = 0.05$ V ($T_1 = 0.2995$); $E_2 = 0.60$ V ($T_2 = 0.2995$); $E_3 = -0.80$ V ($T_3 = 0.4995$). Chromatograms were plotted employing Millenium 2010 chromatography manager software (Waters Chromatography). All samples were analyzed in duplicate. Quantification of fructooligosaccharides was determined from peak areas using sucrose as the external standard (the peak area of sucrose was used as the reference value to calculate all other response factors as in Timmermans et al., 1994).

RESULTS AND DISCUSSION

For quantification of carbohydrates using MALDI-MS, several factors must be examined individually, including the selection of matrixes, matrix and sample preparation, and the selection of an appropriate internal standard for quantification (Harvey, 1993; Jespersen et al., 1995; Gusev et al., 1995; Abell and Sporns, 1996; Bartsch et al., 1996; Wilkinson et al., 1997). The best matrix offers spot-to-spot and sample-to-sample repeatability and reproducibility, which makes quantitative analysis of the analytes of interest possible.

Selection of Matrixes. The main problems associated with matrix-assisted laser desorption/ionization (MALDI) quantitative analysis are poor shot-to-shot repeatability or crystal inhomogeneity (Gusev et al., 1995). Proper homogeneous crystallization over the entire probe area and a homogeneous embedding of analyte molecules in the matrix are the prime criteria for high repeatability and quantification. The selection of matrixes is usually based on a comparison of spot-to-spot or sample-to-sample repeatability and the ability to obtain a good quality spectrum with reasonable signal-to-noise ratios with the best possible resolution. 2,5-Dihydroxybenzoic acid (DHB) (Bruker, 1995; Mohr et al., 1995; Losso and Nakai, 1997), 3-aminoquinoline (3-AQ) (Metzger et al., 1994), 4-hydroxy-α-cyanocinnamic acid (HCCA) (Bartsch et al., 1996), and 2,5-dihydroxybenzoic acid (DHB)/1-hydroxyisoquinoline (HIC) (Mohr et al., 1995) have all been recommended as matrixes for carbohydrate analysis using MALDI-MS. These matrixes were tested for their suitability to ionize the oligosaccharides, maltohexaose and γ-cyclodextrin (Figure 1). The preparation of matrixes and their performance are listed in Table 1. DHB, 3-AQ, HCCA, and DHB/HIC matrixes all gave good quality spectra (Figure 2). The repeatability with DHB was acceptable, whereas the matrixes 3-AQ, HCCA, and DHB/HIC could not meet the need for high repeatability. DHB showed many matrix peaks in the low mass region, which could interfere with low molecular weight analytes of interest, such as kestose with a mass

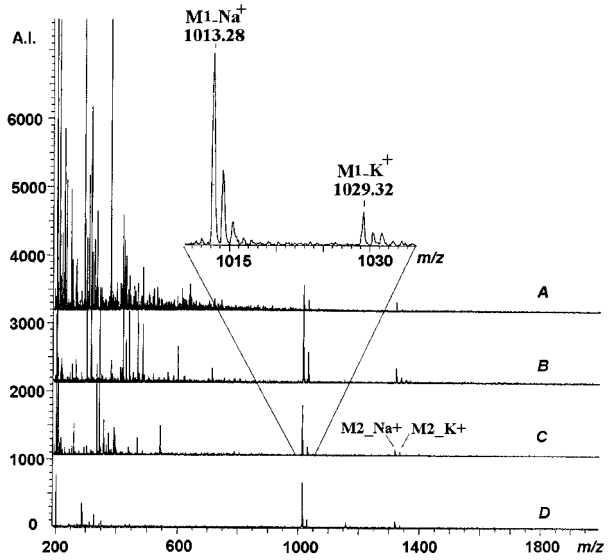

Figure 2. MALDI-MS positive ion spectra of γ-cyclodextrin and maltohexaose in various matrixes. A: 4-Hydroxy-α-cyanocinnamic acid, 13.3 mg/mL in ethanol:water (1:1). A 20 μL of sample mixture containing γ-cyclodextrin (2.5×10^{-5} M, marked M2) and maltohexaose (1.1×10^{-4} M, marked M1) in double deionized water was mixed with 20 μL of matrix in solution and vortexed for 30 s. Then 0.5-μL mixture of matrix and sample was applied to the probe. Laser strength was set at an attenuation of 44. Twenty shots were accumulated for the final spectrum. B: 2,5-Dihydroxybenzoic acid, 12.3 mg/mL in ethanol:water (1:1). Other parameters were the same as in A except the laser strength was set at an attenuation of 32. C: 2,4,6-Trihydroxyacetophenone monohydrate saturated in acetone. A 0.3-μL aliquot of matrix was applied to the probe first and air-dried. Then, 0.5 μL of sample was put on top of the matrix. Other parameters were the same as in B. D: 3-Aminoquinoline (10.1 mg/mL in 10% ethanol). Other parameters were the same as in B.

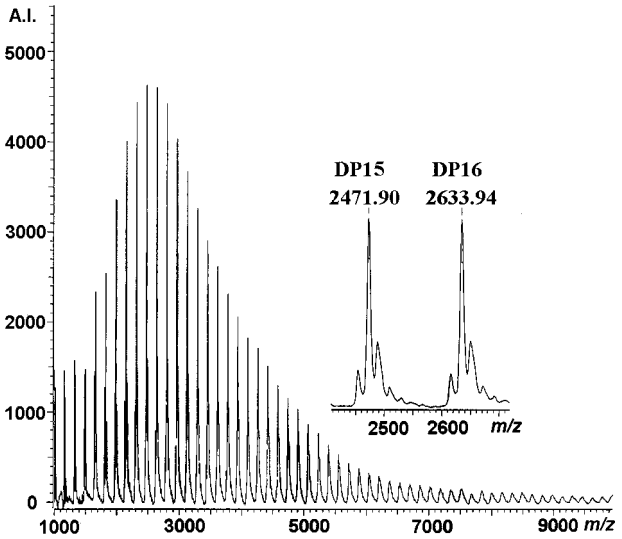

Figure 3. MALDI-MS positive ion spectrum of inulin from Jerusalem artichokes. Inulin was dissolved in double deionized water to give a final concentration of 4 mg/mL. A 0.3 μL of saturated THAP in acetone was first placed on the probe and a 0.5-μL inulin sample was put on top of the crystallized matrix to dry. Sixty laser pulses at an attenuation of 22 were accumulated for the final spectrum.

of 504. Metzger et al. (1994) first introduced 3-AQ as a matrix for inulin using MALDI-MS. Compared to DHB, 3-AQ showed sharper peaks (that is better resolution) and a lower background, but the high quality spectra could not be repeatedly obtained in our experiments. Mohr et al. (1995) noted that DHB crystals formed only near the rim of the probe, complicating the location of suitable laser ionization positions because in the center of the probe only a few crystals could be found. Stahl et al. (1997) found 3-AQ to be more sensitive to contaminants, such as salts. Naven et al. (1997) found the spectra acquired from DHB exhibited the most abundant fragmentation and that those using 3-AQ exhibited the least. Mohr et al. (1995) pointed out that DHB/HIC crystallized equally over the entire probe as a fine powder using vacuum-drying for a few seconds. This resulted in high quality spectra with few matrix peaks and intense analyte peaks. However, this procedure was not suitable for a multiple-position probe, because samples cannot be spotted and vacuum-dried simultaneously so irregular crystals are formed, leading to poor spot-to-spot repeatability.

The laser strength used for the matrixes 3-AQ, DHB, and DHB/HIC was almost the same, with an attenuation around 30−33, which was just above laser strength threshold values required to desorb and ionize analytes. (Note that for the MALDI-MS Proflex III, attenuation is opposite to laser strength; that is, the higher the attenuation the lower the laser strength.) At the same laser strength, DHB produced more matrix peaks than 3-AQ or DHB/HIC. Whereas a much lower laser strength (attenuation of 44 or 49) was used for HCCA or HABA to desorb and ionize maltohexaose and γ-cyclodextrin, both of these matrixes showed numerous matrix peaks making analysis of masses below 600 difficult. Furthermore, the peak ratio (0.30) of maltohexaose to γ-cyclodextrin obtained from HABA was quite far from the actual molar ratio (0.63).

The rate of the evaporation of the solvent affects the cocrystallization of matrix and sample. Fast evaporation leads to fine crystals and more homogeneous incorporation of sample. Improvement in sample homogeneity using the fast-evaporation method enhanced both shot-to-shot repeatability and sample-to-sample reproducibility (Nicola et al., 1995). Fast evaporation could be enhanced with THAP using acetone as the solvent. THAP is very soluble in acetone which then evaporates rapidly giving small homogeneous crystals. Previously, THAP was successfully used as a matrix for peptides and oligonucleotides (Kussmann et al., 1997, and Pieles et al., 1993). However, Mohr et al. (1995) indicated that THAP was not as good a matrix for carbohydrates such as DHB/HIC due to irregular crystallization and relative signal-to-noise ratios. However, they used water as solvent for this matrix. With water both the lower solubility of THAP and slower evaporation rate likely lead to the noted problems. To get an appropriate excess of matrix to analyte the THAP was first crystallized from acetone and then the aqueous sample applied on top of the formed crystals. The water redissolved some of the matrix and the remaining undissolved THAP acted as seed crystals for rapid recrystallization of analyte and matrix as the water evaporated. This technique resulted in high quality MALDI-MS spectra (Figure 2) with high spot-to-spot repeatability. The technique can be used to resolve the oligosaccharides in inulin up to a mass of 9000 (DP of about 55, Figure 3). Another advantage of this technique was its tolerance to small amounts of protein or other impurities in samples, with few interfering matrix peaks from THAP and reasonable signal-to-noise ratio at an attenuation of between 30 and 32. Therefore THAP with acetone

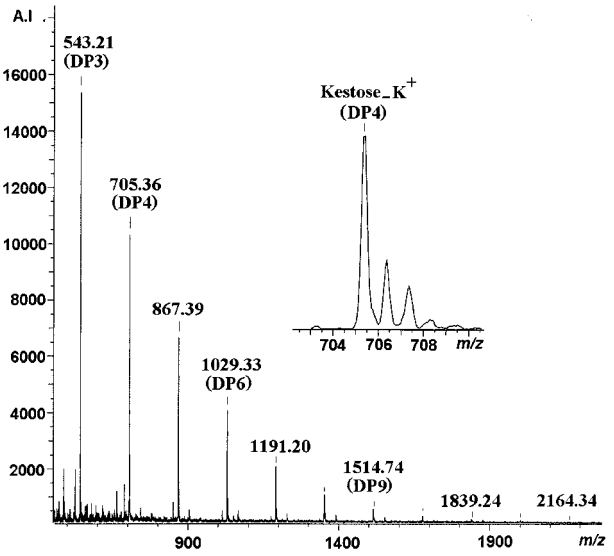

Figure 4. MALDI-MS positive ion spectra of fructooligosaccharides from shallots. The sample was prepared by first applying 0.3 μL of saturated THAP in acetone on the probe, air-drying, and then applying a 0.5 μL aqueous sample solution followed by further air-drying. Laser strength was set at attenuation of 31 and 180 laser pulses were accumulated in three random positions for the final spectrum.

prepared in a two step procedure was chosen as the matrix for later studies.

Alkali-Metal Adducts. In general, carbohydrates ionize in a MALDI-MS source only after cationization with alkali ions (Börnsen et al., 1995). For quantification it was desirable that the investigated carbohydrate sample contained predominantly one kind of alkali metal, resulting in a single molecular ion peak. With no modification the matrix and sample contain both sodium and potassium ions (Figure 2), resulting in multiple carbohydrate peaks. The peak intensity of carbohydrate alkali-metal ion adducts in an unmodified sample is dependent on the concentration of the alkali-metal ions in final solution applied to the probe and the affinity between the metal and the carbohydrate. It has been shown that the affinity of alkali metals to carbohydrates follows the order of H < Li < Na < K < Cs (Mohr et al., 1995; Börnsen et al., 1995). Ion exchange and purification of carbohydrates on a Nafion membrane has been successfully used as a sample pretreatment for MALDI-MS producing a single alkali ion adduct (Börnsen et al., 1995). However, there is another simpler method to obtain a single alkali ion adduct peak. By dissolving carbohydrates in a 0.01 M solution of the alkali ion salt (e.g., potassium chloride) we were able obtain a single alkali ion adduct peak. The concentration of alkali ions was crucial, since too high a concentration of salts would also suppress the molecular ions. Often food samples, such as onions, shallots, and garlic, naturally contain a high concentration of potassium ions, and could be analyzed without further addition of salts. The molecular ions seen in MALDI-MS for these food samples were almost entirely the potassium adducts (Figure 4).

Laser Strength. Laser strength determines the degree of the desorption and ionization of analytes in MALDI-MS. Usually, with increases of laser strength, more ions, including both matrix and molecular ions, are generated. Also higher laser strengths can lead to more fragmentation. For a high quality spectrum and quantitation, the ideal laser strength is very important.

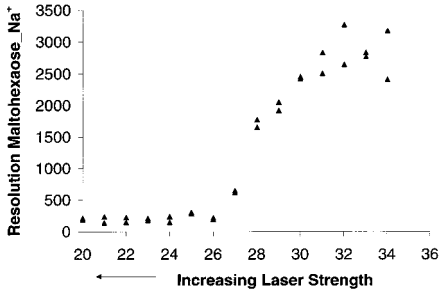

Figure 5. Relationship between laser strength and resolution. The sample was prepared by first applying 0.3 μL of saturated 2,4,6-trihydroxyacetophenenone monohydrate saturated in acetone, air-drying, and then applying 0.5 μL of maltohexaose (1.1×10^{-4} M) as described in Figure 4. The laser strength was changed from an attenuation of 34 down to 20 (x axis). At each attenuation, two spots were randomly chosen to collect two spectra (resolution shown for each spectrum) with 20 laser pulses totaled for each spot. The resolution of the molecular ion peaks was obtained from sodium adduct ion peak of maltohexaose (y axis).

Generally, laser strength has been chosen based on the signal-to-noise ratios (Bartsch et al., 1996; Naven et al., 1997). However, the relationships between laser strength, resolution, and the analyte response have not been thoroughly investigated for carbohydrates. We found that laser strength played a very important role in obtaining quality spectra. As the laser strength increased over a certain amount, the resolution deteriorated rapidly. Figure 5 indicates the trend of the resolution of maltohexaose sodium adduct peak with the variation in laser strength. At the attenuation between 30 and 33, high quality spectra were obtained with well resolved isotopic mass peaks having resolutions close to 3000 (full width at half-maximum, fwhm), and exact isotopic mass could be determined with an accuracy 100 ppm or less by using internal calibration. However, when the attenuation was decreased to 27, the resolution deteriorated rapidly to 500 (fwhm). This effect can be easily seen in spectra since isotopic resolution is lost. These isotopic peaks include the main peak plus one unit mass (M + 1) and M + 2 peaks due mainly to ^{13}C isotopes. At the same time, because of the loss of isotopic resolution, molecular ion peaks became broad and the measured masses of peaks were shifted to high masses with increasing laser strength (data not shown). More important, the molar peak ratio between maltohexaose and γ-cyclodextrin changed, making quantitative analysis impossible using one as the internal standard for the other, even though chemically these two molecules are very similar oligosaccharides. In Figure 6 the actual molar ratio of maltohexaose to γ-cyclodextrin was 4.4 and within the region of attenuation between 28 and 34, the peak ratio of maltohexaose and γ-cyclodextrin was very close to this molar ratio (4.1 $\pm$ 0.53) even though both sodium and potassium adduct peaks were used. However, with the increase in laser strength, the peak ratio decreased. This indicated that relatively more γ-cyclodextrin was desorbed and ionized during the ionization than maltohexaose. It can be concluded that the behavior of molecules, even when they are only slightly different in their molecular structure, can be quite different during the ionization process. While higher laser strength does result in a significant increase in ions formed, another advantage of lower laser strength is to limit any molecular fragmentation.

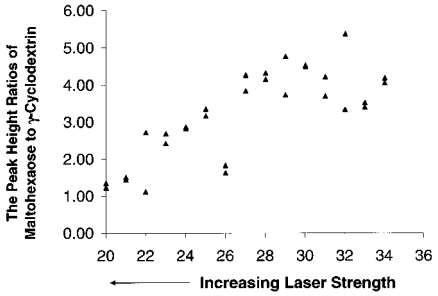

Figure 6. Relationship between laser strength and analyte peak ratios. The sample, preparation of matrix and sample, and other MALDI-MS parameters are the same as described in Figure 5. γ-Cyclodextrin was present at a concentration of 2.5×10^{-5} M. Total peak height of both sodium and potassium adduct peaks was used to plot this trend.

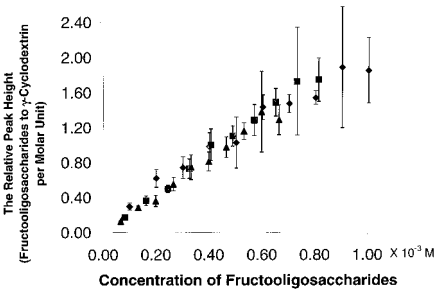

Figure 7. Responses of individual fructooligosaccharides in MALDI-MS. Diamond shapes: kestose (slope = 2.1, $R^2 = 0.93$). Squares: nystose (slope = 2.3, $R^2 = 0.99$). Triangles: GF_4 (slope = 2.1, $R^2 = 0.98$). Internal standard γ-cyclodextrin (4.0×10^{-5} M) was dissolved in 0.01 M potassium chloride solution. The concentration of individual fructooligosaccharides ranged from 1.0×10^{-4} to 1.0×10^{-3} M for kestose, 8.2×10^{-5} to 8.2×10^{-4} M for nystose and 6.7×10^{-5} to 6.7×10^{-4} M for GF_4 in 0.01 M aqueous potassium chloride. Other MALDI-MS parameters were the same as in Figure 4. Each data point was the mean of three random positions for a total of 180 laser pulses. Each spectrum from a single sample position was collected from three random spots for a total of three of the 180 laser pulses. Peak heights were used as for quantification. Each error bar stands for the standard deviation from the mean of three different spectra.

Oligosaccharide Response in MALDI-MS. The response of the analytes in MALDI-MS plays a very important role in quantifying analytes of interest. In theory, the intensity or response of an analyte should be linearly correlated to its molar ratios in the MALDI-MS sample. Figure 7 indicates the linearity between the concentration of individual fructooligosaccharides and their response in MALDI-MS using γ-cyclodextrin as an internal standard. The slopes or the relative response factors of the fructooligosaccharides using MALDI-MS were 2.1 for kestose, 2.1 for nystose, and 2.3 for GF_4. This indicated that the response of the individual fructooligosaccharides in MALDI-MS was very similar and was more than twice that of γ-cyclodextrin. Although the responses of γ-cyclodextrin and the fructooligosaccharides were different on a molar basis, the molar ratio of fructooligosaccharides to γ-cyclodextrin seemed to be consistent, making it possible to use γ-cyclodextrin as an internal standard. γ-Cyclodextrin was a useful internal standard for fructooligosaccharides since it is readily available in pure form, was likely similar in chemical stability to fructooligosaccharides since all compounds are nonreducing sugars, and had a unique mass that would not overlap with any other fructooligosaccharide (18 mass units less than the corresponding DP fructooligosaccharide because of its cyclic structure).

Analysis of Fructooligosaccharides in Food Samples. When γ-cyclodextrin was added as an internal standard to extracted food samples, the relative responses noted above (about a 2:1 molar ratio) for pure standards changed. It became obvious that in the different food extract environments γ-cyclodextrin responded differently than fructooligosaccharides. Food extracts from red onions, for example, completely suppressed the production of ions from added γ-cyclodextrin, even though the fructooligosaccharides could be seen. For this reason γ-cyclodextrin was abandoned as an internal standard.

While working with food extracts, however, one feature seemed very consistent and that was the relative ratios of the individual fructooligosaccharides. For this reason it was decided to attempt the use of a single purified fructoligosaccharide in a standard addition method to quantitate all of the fructooligosaccharides.

Table 2 shows the results for standard addition using nystose (GF_3) as reference peak (that is all other peak heights are compared to the peak height of this peak) and GF_4 as the standard added. Comparisons were carried out for two samples of known concentration, a supplied oligosaccharide mixture with known composition (standard A) and another mixture prepared from pure fructooligosaccharide standards (standard B). Even though differing amounts of internal standard (GF_4 added at both 0.72 and 0.33 mg/mL levels) were added, quantitation of all three oligosaccharide compounds compared nicely with known values.

Of course the ultimate test was to examine food extracts. Table 3 shows the MALDI-MS results for determining the fructooligosaccharide content in red onions, shallots, and garlic analyzed by using the standard addition method.

Comparison of MALDI-MS and HPAE-PAD Results. High performance anion exchange chromatography with pulsed amperometric detection (HPAE-PAD) has been used to quantitatively analyze fructooligosaccharides or inulin (Chatterton et al., 1989, 1993; Timmermans et al., 1994). Table 3 shows the result of analysis for fructooligosaccharides contained in onion and shallot with both HPAE-PAD and MALDI-MS and garlic with MALDI-MS. In general, the sensitivity of PAD detector decreases rapidly from DP = 2 to DP = 6, while for longer oligomers (DP = 7−17), the sensitivity of detector decreases only slightly (Timmermans et al., 1994). The calculation of fructooligosaccharides in food using HPAE-PAD was based on the response factors and linear relationship reported by Timmermans et al. (1994). Peak areas were integrated and compared to an external standard, sucrose (Figure 10) for quantification. Onion bulbs contain various isomeric fructans (Darbyshire et al., 1981; Bancal et al., 1989, 1991; Stahl et al., 1997). These isomer ions yielded a more complex chromatogram pattern than for MALDI-MS (Figure 8 is the HPAE-PAD chromatogram of shallots, while Figure 4 is the MALDI-MS spectrum for this sample). The retention times of individual peaks from both red onions and shallots were very comparable. Kestose (DP = 3), nystose (DP = 4), and GF_4 (DP = 5) in red onion and shallot samples were determined in comparison to the retention times of fructooligosaccharide standards. With higher DP fructooligosaccharides, because of the lack of individual standards, we assumed that adjacent

Table 2. Repeatability of MALDI-MS Analysis Data

concn of GF_4 (DP = 5) added standard, mg/mL	degree of polymerization	fructooligosaccharides standard A[a] actual value, mg/mL	fructooligosaccharides standard A[a] MALDI-MS data[c]	fructooligosaccharides standard B[b] actual value, mg/mL	fructooligosaccharides standard B[b] MALDI-MS data[c]
0.72	DP = 3 (kestose)	0.23	0.21 (0.01	0.37	0.41 (0.09)
	DP = 4[d] (nystose)	0.35	0.40	0.46	0.50
	DP = 5 (GF_4)	0.070	0.072 (0.01)	0.37	0.36 (0.03)
	total	0.67	0.68 (0.01)	1.20	1.26 (0.07)
0.33	DP = 3 (kestose)	0.23	0.20 (0.03)	0.37	0.36 (0.04)
	DP = 4[d] (nystose)	0.35	0.37	0.46	0.50
	DP = 5 (GF_4)	0.070	0.073 (0.01)	0.37	0.38 (0.04)
	total	0.67	0.65 (0.03)	1.20	1.23 (0.02)

[a] A mixture of 1-kestose (34%), nystose (53%), and GF_4 (10%) made in Nutritional Science Center, Meiji Seika Kaisha, Japan. [b] A mixture of 1-kestose, nystose, and GF_4 prepared in our experiment. [c] Mean of a duplicate with five replicates, each replicate from three of 60 laser pulses and standard deviations ($n = 2$). [d] The peak of fructooligosaccharide with DP = 4 (GF_3) was taken as the reference peak.

Table 3. Fructooligosaccharide Content Using HPAE-PAD and MALDI-MS

	fructooligosaccharide standard, %			red onions, mg/g fresh; water content = 88.3 (0.063) (fresh), 5.2 (0.58)[a] (freeze-dried)		shallots, mg/g fresh; water content = 84.2 (0.15) (fresh), 5.9 (0.22)[a] (freeze-dried)		garlic, mg/g fresh; water content = 63.1 (0.85) (fresh), 2.1 (0.25)[a] (fresh)
deg of polymn	actual value	HPAE-PAD mean[b]	MALDI-MS mean[c]	HPAE-PAD mean[b]	MALDI-MS mean[c]	HPAE-PAD mean[b]	MALDI-MS mean[c]	MALDI-MS mean[c]
3	34	37.06 (0.31)	30.83 (0.36)	3.92 (0.17)	6.81 (0.41)	8.43 (0.06)	14.88 (0.33)	14.69 (3.14)
4	53	49.98 (0.21)	58.01 (0.66)	3.99 (0.00)	3.88 (0.15)	8.27 (0.03)	11.37 (0.15)	10.44 (2.02)
5	10	12.96 (0.53)	11.24 (0.94)	2.67 (0.01)	2.19 (0.15)	8.15 (0.09)	7.69 (0.47)	9.34 (1.87)
6				3.42 (0.02)	1.29 (0.16)	10.86 (0.08)	4.84 (0.53)	8.67 (1.78)
7				1.96 (0.06)	0.82 (0.18)	7.86 (0.52)	3.24 (0.48)	7.92 (1.53)
8				1.25 (0.02)	0.52 (0.11)	5.73 (0.08)	2.07 (0.19)	6.81 (1.14)
9				0.96 (0.05)	0.43 (0.15)	4.37 (0.10)	1.43 (0.13)	5.55 (0.89)
10				0.40 (0.01)		2.73 (0.03)	1.01 (0.05)	4.64 (0.61)
11				0.30 (0.02)		2.02 (0.01)	0.87 (0.07)	4.07 (0.54)
12				0.18 (0.03)		1.33 (0.00)	0.90 (0.00)	3.54 (0.53)
13						0.78 (0.04)		3.11 (0.30)
14								2.80 (0.43)
15								2.37 (0.23)
16								2.14 (0.29)
17								1.87 (0.12)
18								1.64 (0.05)
19								1.53 (0.15)
total				19.06 (0.13)	15.93 (1.32)	60.55 (0.71)	48.3 (1.52)	91.74 (15.56)

[a] Numbers in parentheses indicate the standard deviation of triplicates ($n = 3$). [b] Mean of a duplicate and standard deviations ($n = 2$). [c] Mean of a duplicate (same extract) with five replicates, each replicate from three of 60 laser pulses and standard deviations ($n = 2$).

peaks (Figure 8) were isomers to calculate the amount of individual fructooligosaccharide content. The HPAE-PAD technique was more sensitive in terms of detection limit than MALDI-MS. The small amounts of higher DP fructooligosaccharides in onion or shallot samples were detected by HPAE-PAD (Table 3) and could only be seen with MALDI-MS by using higher laser strength where resolution and therefore quantitation suffered.

Both Loo et al. (1995) and Stahl et al. (1997) stated that for onion bulbs the major fructooligosaccharide had a DP = 5. However, using either analytical method, fructooligosaccharides other than DP = 5 were the major fructooligosaccharides in our onion sample. The distribution of fructooligosaccharides in red onions, shallots, and garlic seen by MALDI-MS followed a definite pattern and the amounts of individual fructooligosaccharides could be correlated to different exponential distributions (correlation coefficients ranged from 0.97 to 99, Figure 9). The natural symmetry of these exponential distributions for fructooligosaccharides in red onions, shallots, and garlic seems to support the relative amounts of fructooligosaccharides assigned by MALDI-MS. Also this distribution may be useful in predicating the amounts of fructooligosaccharides with higher DP in a sample or elucidating the changes of fructooligosaccharides in onions, shallots, and garlic during storage.

The fructooligosaccharides in the garlic sample, however, could not be analyzed by using HPAE-PAD because of problems with baseline drift, but they could be analyzed by using MALDI-MS with a slightly higher laser strength at an attenuation of 28 or 29. We are uncertain if the lack of sample purity affected the quantification of the other HPAE-PAD samples. It was clear that the best correlation between the two analysis methods was for the pure fructooligosaccharide standard mixture.

While there are obvious differences in the quantitation of fructooligosaccharides using HPAE-PAD and MALDI-MS, we feel that the MALDI-MS results more accurately reflect the true amounts of individual fructooligosaccharides in these food samples. As has already been noted the response of a pulsed amperometric detector (PAD) is different for different DP fructooligosaccharides (Timmermans et al., 1994). However, while we tried to account for this changing response, this is only the detector response for linear fructooligosaccharides. Onion bulbs contained various isomeric fructooligosaccharides, including branched fructooligosaccharides (Darbyshire et al., 1981; Bancal et al., 1989, 1991;

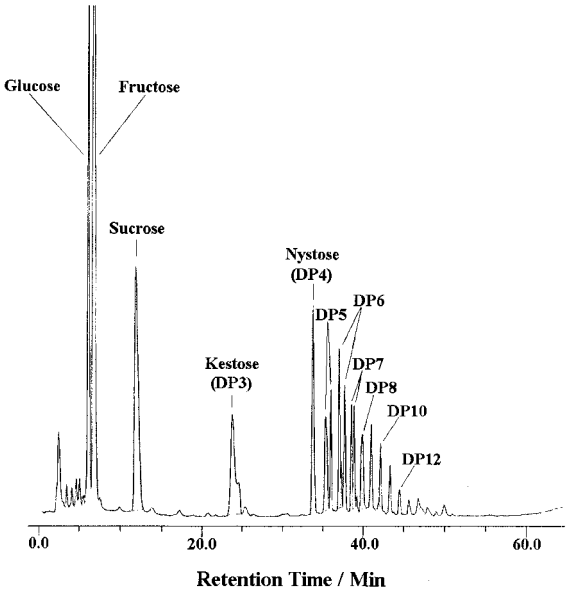

Figure 8. HPAE-PAD chromatogram of shallots. Peaks are identified by comparison of the retention time with standards. The higher DPs (DP = 6 or more) of fructooligosaccharides are labeled by retention time compared to DP = 5 and two adjacent peaks are treated as isomers.

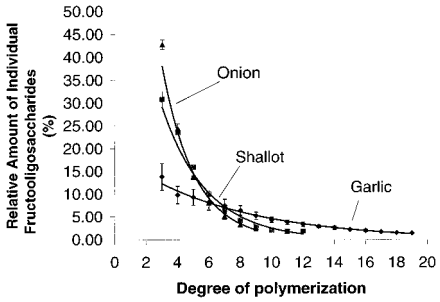

Figure 9. Distribution of individual fructooligosaccharides in red onions ($Y = 159.5e^{-0.48X}$, $R^2 = 0.99$), shallots ($Y = 82.8e^{-0.35X}$, $R^2 = 0.97$), and garlic ($Y = 18.73e^{-0.14X}$, $R^2 = 0.99$). Each data point and the error bars were determined from duplicate analyses.

Stahl et al., 1997), and nothing is known about the differing responses of the PAD detector to these differently linked (2→6 links) fructooligosaccharides. However, standards for similar mass branched and linear glucose oligosaccharides are available and their PAD responses vary a great deal (Figure 10). In fact the branched glucose oligosaccharides (that is 1→6 linked isomaltose and isomaltotriose; measured peak areas of 12.0 and 10.6 million, respectively) have significantly greater PAD responses than their linear mass equivalents (maltose and maltotriose; measured peak areas of 8.1 million and 6.8 million, respectively). It would therefore be expected that using linear oligosaccharide PAD response factors, one would overestimate fructooligosaccharides with branched forms present. As can be seen from Table 3, HPAE-PAD values are generally higher than corresponding MALDI-MS values.

MALDI-MS is a somewhat faster analysis method than HPAE-PAD, taking about 20 min (for determining peak heights for 10 probe positions) rather than an hour for each analysis and MALDI-MS is more tolerant to impurities (as noted for our garlic sample). Further reduction in MALDI-MS analysis time can be obtained by a less rigorous quantitation procedure (i.e., less laser pulses, fewer spots), but even with the numerous

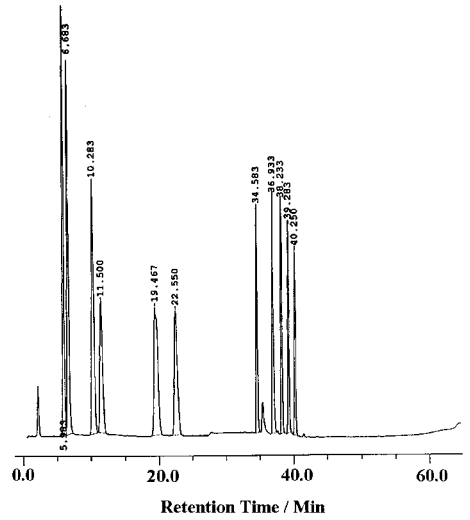

Figure 10. HPAE-PAD chromatogram of carbohydrate standards. Glucose, 98 ppm/5.98 min; fructose, 104 ppm/6.68 min; isomaltose, 98 ppm/10.28 min; sucrose, 98 ppm/11.50 min; isomaltotriose, 108 ppm/19.47 min; maltose, 102 ppm/22.55 min; maltotriose, 100 ppm/34.58 min; maltotetraose, 98 ppm/36.93 min; maltopentaose, 96 ppm/38.23 min; maltohexaose, 98 ppm/39.28 min; maltoheptaose, 102 ppm/40.25.

replicates that were performed in our experiments, standard deviations for MALDI-MS were still higher than for the HPAE-PAD method. Finally, MALDI-MS gives better assurance of correct molecular assignment since the isotopic mass of each peak is available, although because of similar masses branched and linear isomers cannot be distinguished. In fact with MALDI-MS the assignments can be further checked by substituting a different alkali metal in the sample preparation procedure to see expected mass shifts for each oligosaccharide adduct.

While we have concentrated on the analysis of fructooligosaccharides in this study, our feeling is that MALDI-MS can also be used to quantitate other oligosaccharides found in food by developing similar analytical methodology. To our knowledge this paper represents the first use of standard addition to quantitate using MALDI-MS.

LITERATURE CITED

Abell, D. C.; Sporns, P. Rapid quantitation of potato glycoalkaloids by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *J. Agric. Food Chem.* **1996**, *44*, 2292−2296.

AOAC Official Methods of Analysis **1990**.

Bancal, P.; Gaudillère, J. P. Fructan chemical structure and sensitivity to an exohydrolase. *Carbohydr. Res.* **1991**, *217*, 137−151.

Bancal, P.; Henson, C. A.; Gaudillère, J. P.; Carpita, N. P. Oiligofructan separation and quantification by high performance liquid chromatography. Application to *Asparagus officialis* and *Triticum aestivum*. *Plant Physiol. Biochem.* **1989**, 745−750.

Bartsch, H.; König, W. A.; Strassner, M.; Hintze, U. Quantitative determination of native and methylated cyclodextrins by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *Carbohydr. Res.* **1996**, *286*, 41−53.

Börnsen, K. O.; Mohr, M. D.; Widmer, H. M. Ion exchange and purification of carbohydrates on a Nafion membrane as a new sample pretreatment for matrix-assisted laser desorption/ionization mass spectrometry. *Rapid Commun. Mass Spectrom.* **1995**, *9*, 1031−1034.

Bruker. MALDI-TOF Mass Spectrometer. *User's Guide to the Proflex™* **1995**.

Chatterton, N. J.; Harrison, P. A.; Thornley, W. R.; Bennett, J. H. Sucrooligosaccharides and cool temperature growth in 14 forb species. *Plant Physiol. Biochem*. **1990**, *28*, 167−172.

Chatterton, N. J.; Harrison, P. A.; Thornley, W. R.; Bennett, J. H. Separation and quantification of Fructan (inulin) oligomers by anion exchange chromatography. *Inulin and Inulin-containing Crops* **1993**, 93−99.

Chatterton, N. J.; Harrison, P. A.; Thornley, W. R.; Bennett, J. H. Purification and quantification of kestoses (fructosylsucroses) by gel permeation and anion exchange chromatography. *Plant Physiol. Biochem*. **1989**, *27*, 289−295.

Darbyshire, B.; Henry, R. J. Differences in fructan content and synthesis in some *Allium* species. *New Phytol.* **1981**, *87*, 249−256.

Darbyshire, B.; Henry, R. J. The distribution of fructans in onions. *New Phytol.* **1978**, *81*, 29−34.

Gusev, A. I.; Wilkinson, W. R.; Proctor, A.; Hercules, D. M. Improvement of signal reproducibility and matrix/comatrix effects in MALDI analysis. *Anal. Chem*. **1995**, *67*, 1034−1041.

Harvey, D. J. Quantitative aspects of the matrix-assisted laser desorption mass spectrometry of complex oligosaccharides. *Rapid Commun. Mass Spectrom*. **1993**, *7*, 614−619.

Jespersen, S.; Niessen, W. M. A.; Tjaden, U. R.; vanderGreef, J. Quantitative bioanalysis using matrix-assisted laser desorption ionization mass spectrometry. *J. Mass Spectrom*. **1995**, *30*, 357−364.

Kussmann, M.; Nordhoff, E.; Rahbek-Nielsen, H.; Haebel, S.; Rossel-Larsen, M.; Jakobsen, L.; Gobom, J.; Mirgorodskaya, E.; Kroll-Kristensen, A.; Palm, L.; Roepstorff, P. Matrix-assisted laser desorption/ionization mass spectrometry sample preparation techniques designed for various peptide and protein analytes. *J. Mass Spectrom*. **1997**, *32*, 593−601.

Livingston, D. P., III. Fructan precipitation from water/ethanol extract of oats and barley. *Plant Physiol.* **1990**, *92*, 767−769.

Loo, J, V.; Coussement, P.; Leenheer, L. de; Hoebregs, H.; Smits, G. On the presence of inulin and oligofructose as natural ingredients in the western diet. *Crit. Rev. Food Sci. Nutr.* **1995**, *35*, 525−552.

Losso, J. N.; Nakai, S. Molecular size of garlic fructooligosaccharides and fructopolysaccharides by matrix-assisted laser desorption ionization mass spectrometry. *J. Agric. Food Chem*. **1997**, *45*, 4342−4346.

Manghi, P.; Vernazza, F.; Ferrarini, O.; Cagnasso, P.; Salvadori, C.; Fockedey, J. Analytical method for the determination of inulin in yogurt enriched with soluble fibre. *Eur. J. Clin. Nutr. Suppl.* **1995**, *3*, S296−S297.

Metzger, J. O.; Woisch, R.; Tuszynski, W.; Angermann, R. New type of matrix for matrix-assisted laser desorption mass spectrometry of polysaccharides and proteins. *Fresenius J. Anal. Chem*. **1994**, *349*, 473−474.

Mock, K. K.; Daevy, M.; Cottrell, J. S. The analysis of underivatized oligosaccharides by Matrix-Assisted Laser Desorption Mass Spectrometry. *Biochem. Biophys. Res. Commun*. **1991**, *177*, 644−651.

Mohr, M. D.; Börnsen, K. O.; Widmer, H. M. Matrix-assisted laser desorption/ionization mass spectrometry: improved matrix for oligosaccharides. *Rapid Commun. Mass Spectrom*. **1995**, *9*, 808−814.

Naven, T. J. P.; Harvey, D. J.; Brown, J.; Critchley, G. Fragmentation of complex carbohydrates following ionization by matrix-assisted laser desorption with an instrument fitted with time-lag focusing. *Rapid Commun. Mass Spectrom*. **1997**, *11*, 1681−1686.

Nelson, C. J.; Smith, D. Fructans: their nature and occurrence. *Curr. Top. Plant Biochem. Physiol.* **1986**, *5*, 1−16.

Nicola, A. J.; Gusev, A. I.; Proctor, A.; Jackson, E. K.; Hercules, D. M. Application of the fast-evaporation sample preparation method for improving quantification of angiotensin II by matrix-assisted laser desorption ionization. *Rapid Commun. Mass Spectrom*. **1995**, *9*, 1164−1171.

Ohta, A.; Ohtsuki, M.; Baba, S.; Hirayama, M.; Adachi, T. Comparison of the nutritional effects of fructooligosaccharides of different sugar chain length in rats. *Nutr. Res.* **1998**, *18*, 109−120.

Ohta, A.; Ohtsuki, M.; Baba, S.; Takizawa, T.; Adachi, T.; Kimura, S. Effects of fructooligosaccharides on the absorption of iron, calcium and magnesium in iron-deficient anemic rats. *J. Nutr. Sci. Vitaminol.* **1995**, *42*, 281−291.

Pieles, U.; Zuercher, W.; Schör, M.; Moser, H. E. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry: A powerful tool for the mass and sequences analysis of natural and modified oligonucleotides. *Nucleic Acids Res.* **1993**, *21*, 3191−3196.

Pollock, C. J. Sucrose accumulation and the initiation of fructan biosynthesis in *Lolium temulentum* L. *New Phytol.* **1984**, *96*, 527−534.

Shiomi, N.; Onodera, S.; Chatterton, N. J.; Harrison, P. A. Separation of fructooligosaccharide isomers by anion-exchange chromatography. *Agric. Biol. Chem*. **1991**, *5*, 1427−1428.

Stahl, B.; Lions, A.; Karas, M.; Hillenkamp, F.; Steup, M. Analysis of fructans from higher plants by matrix-assisted laser desorption/ionization mass spectrometry. *Anal. Chem*. **1997**, *246*, 1195−204.

Stahl, B.; Steup, M.; Karas, M.; Hillenkamp, F. Analysis of neutral oligosaccharides by matrix-assisted laser desorption/ionization mass spectrometry. *Anal. Chem*. **1991**, *63*, 1463−1466.

Suzuki, M.; Cutcliffe, J. A. Fructans in onion bulbs in relation to storage life. *Can. J. Plant Sci.* **1989**, *69*, 1327−1333.

Timmermans, J. W.; van Leeuwen, M. B.; Tournois, H.; de Wit, D.; Vliegenthart, J. F. G. Quantification analysis of the molecular weight distribution of inulin by means of anion exchange HPLC with pulsed amperometric detection. *J. Carbohydr. Chem*. **1994**, *13*, 881−888.

Wilkinson, W. R.; Gusev, A. I.; Proctor, A.; Houalla, M.; Hercules, D. M. Selection of internal standards for quantitative analysis by matrix-assisted laser desorption−ionization (MALDI) time-of-flight mass spectrometry. *Fresenius J. Anal. Chem*. **1997**, *357*, 241−248.

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