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Comparison of the water-soluble carbohydrate composition and fructan structures of *Agave tequilana* plants of different ages

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ABSTRACT

The composition of water-soluble carbohydrates from *Agave tequilana* plants of 2, 4 and 6½ years were compared by HPLC, HPAEC-PAD, MALDI-TOF-MS and GC–MS. The plants of 2 years exhibited the highest levels of free monosaccharide and low molecular weight fructans (DP 3–DP 6) with potential application as prebiotics. A maximum of fructan polymerisation was achieved at 4 years with mean DP from 3 to 30, then it was decreased at 6½ years with mean DP from 4 to 24. The linkage analysis showed an increase and decrease in the branching degree from 2 to 6½ years with a maximum at 4 years, correlated to changes in t-β-D-Fru*f* linkages with increased and decreased synthesis of (2→1) and (2→6)-β-D-Fru*f* as well as 1,6-di-β-D-Fru*f* linkages.

1. Introduction

Fructans are fructose-based oligo or polysaccharides present in many species of higher plants. In *Agave* species (CAM plants), fructans are reserve carbohydrates and they are synthesised and stored in the stems. The main function of these fructose polymers is storage before flowering and acts as osmoprotectans during drought (Hendry, 1987; Wang & Nobel, 1998). The principal difference of *Agave tequilana* with respect to other common fructan accumulating plants is that it takes from 8 to 12 years to perform the accumulation (Cedeño, 1995). Even though *A. tequilana* plants have the capacity for sexual reproduction, the cultivation starts with the plantation of *hijuelos* (plant clones derived from older agave plants), which are produced by asexual means with plant sprouting from rhizome (Rodríguez-Garay, Gutierrez-Mora, Flores-Berrios, & Loera-Quezada, 2004). Normally the production of *hijuelos* is carried out with a non controlled method, they could have 1–3 years since plant sprouting and normally a head diameter of the plant from 10 to 15 cm is considered by the *A. tequilana* producers as a parameter for separation from the mother plants and then the plantation is performed. *A. tequilana* fructans (ATF) are very important for the Mexican agroindustry, as they constitute the principal raw material for the elaboration of tequila after their hydrolysis for the subsequent fermentation and distillation processes (Sánchez-Marroquin & Hope, 1953). Normally the process of hydrolysis is carried out by cooking with incomplete break down of all fructans and with the production of Maillard compounds (Mancilla-Margalli & Lopez, 2002; Waleckx, Gschaedler, Colonna-Ceccaldi, & Monsan, 2008). Apart from the production of tequila, it has been found recently that ATF have potential for the elaboration of dietary products and drug delivery systems (Starbid, Zuñiga, Delgado, Saake, & Toriz, 2007; Urías-Silvas et al., 2008). Thus, to know the structure of ATF as well as the factors that affect their synthesis is important. The first characterisation of ATF by linkages analysis with GC–MS, as well as ^{13}C NMR, ^{1}H NMR and MALDI-TOF-MS methods applied to ATF of mature plants (8 years) showed that ATF is a complex mix of branched neo-fructans with β(2→1) and β(2→6) linkages, and these molecules have a degree of polymerisation (DP) ranging from 3 to 29 units (Lopez, Mancilla-Margalli, & Mendoza-Diaz, 2003; Mancilla-Margalli & Lopez, 2006). Similar structures have been found in *Agave veracruz* with a mixture of highly branched fructans with an internal glucose and containing both β(2→1) and β(2→6) linkages (Mancilla-Margalli & Lopez, 2006). In the case of *Agave deserti* it was reported the presence of a DP5 fructan in the vascular tissue with neokestose (DP3) as the principal fructooligosaccharide, a fructan with an internal glucose moiety (Wang & Nobel, 1998). Recently, it was found that fructan structures in agaves varied in function of plant species and region. The comparison of fructan linkage analysis between *A. tequilana*,

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Agave potatorum, *Agave cantala*, *Agave fourcroydes* and *Agave angustifolia* showed differences in the proportions of glucose polymerised, as well as in the proportions of β(2→6) and β(2→1) linkages of the principal chains (Mancilla-Margalli & Lopez, 2006). In addition, for other plants different to *Agavaceae* family, the synthesis of fructans is influenced by the production region, the soil nutrients, the plant variety, the seasonal changes, the water regime and the harvest time (Dias-Tagliacozzo, Itaya, Machado de Carvalho, Figueiredo-Ribeiro, & Dietrich, 2004; Faustini-Cuzzuol, Machado-Carvalho, Penteado-Zaidan, & Roberto-Furlani, 2005; Livingstone, Premakumar, & Tallury, 2006; Martínez-Noël, Tognetti, Nagaraj, Wiemken, & Pontis, 2006; Monti, Amaducci, Pritoni, & Venturi, 2005; Morcuende, Kostadinova, Pérez, & Martínez-Carrasco, 2005; Orthen & Wehrmeyer, 2004; Saengthongpinit & Sajjaanantakul, 2005; Shiomi, Benkeblia, Onodera, & Kawazoe, 2005; Wilson, Smith, & Yonts, 2004). To our knowledge, there is only one report about the influence on *A. tequilana* age over the tequila fermentation process (Pinal et al., 2009). Nevertheless, in the case of ATF no studies have been published on the effect of plant age over the structure of fructans and most of the information in other plants correspond to linear polymers with β(2→1) linkages principally. By the other hand, since an economically point of view and in particular for the *A. tequilana* producers, it is important to know how the composition of fructans changes in function of plant age, because by tradition the harvest for the tequila elaboration process is carried out from 8 to 12 years. Therefore, information over the accumulation of sugars is important in order to possibly decrease the time of harvest. Additionally, the utilisation of young agave plants for other purposes such as prebiotics production, could represent an economically important alternative. Thus, the objective of this work was to study the effect of *A. tequilana* plant age over the changes in the fructan structures for economical purposes.

2. Materials and methods

2.1. Standard material

D-Glucose, D-fructose and sucrose were purchased from Sigma–Aldrich. 1-kestose, nystose and inulin from Dahlia tubers were supplied by Fluka. All other reagents were of analytical grade.

2.2. Plant material

A. tequilana plants of different ages cultivated organically were collected at the same time from the same cultivation zone (10 km perimeter) of the Usmajac valley (Jalisco state, Mexico). Agave plants of 2, 4 and 6½ years of growth from this region were used. According to the information provided by the producers, the age of plants was considered since *hijuelos* plantation. The diameter and weight of the agave heads at different ages were measured. The sampling process was carried out as follows: the agave head was cut transversally in two halves, one half was then cut in slices from the centre to the exterior of the agave head, and 600 grams were taken from three slices. For each age group, this process of sampling was carried out from independent plants of *A. tequilana*. These samples were frozen for 2 days at −20 °C and the water-soluble carbohydrates (WSC) were extracted by mixing 66 ml of distilled water per 100 g of sample, the mix was blended in a mechanical device made of stainless steel and then stirred at 70 °C for 7 h. The WSC suspension was then centrifuged (5 kg for 30 min) and the supernatant was filtered under vacuum with Whatman paper (3 mm CHR). The filtered solution was then lyophilised and stored in a desiccator until they were analysed. Determination of total nitrogen was carried out in lyophilised samples in order to confirm the absence of proteins.

2.3. Total and free sugars quantification

Total sugar content was determined using anthrone: 100 μl of sample (WSC) were mixed with 200 μl of anthrone (200 mg of anthrone in 100 ml of H_2SO_4), and the mix was shaken and heated for 10 min at 100 °C. The reaction was stopped by immersion in ice for 5 min. The sugar content was obtained by comparing the absorbance of sample at 625 nm against a standard curve of sucrose (0 to 100 μg ml^{-1}). Free reducing sugars of all agave samples were determined using 3,5-dinitrosalicylic acid (DNS) reagent: 100 μl of sample (WSC) were mixed with 100 μl of DNS reagent (DNS 10 g l^{-1}, 300 g l^{-1} sodium and potassium tartrate and NaOH 16 g l^{-1}), the mix was shaken and then heated for 5 min at 100 °C. The reaction was stopped by immersion in ice for 5 min, then the free sugars was obtained comparing the absorbance of sample at 540 nm against a standard curve of fructose (0–2 g l^{-1}). The total fructan content was calculated by the difference between the total sugar content and the free reducing sugars.

2.4. High performance liquid chromatography

High performance liquid chromatography analysis device consisted in a Dionex ASI 100 (Sunnyvalle, CA, USA) with a Shodex 101 RI detector. One column was employed for the analysis of the carbohydrates; a Biorad HPLC Carbohydrate Analysis column: Aminex HPX-87 C column (300 × 7.8 mm; Biorad, Hercules, CA, USA) at 80 °C (elution with degassed ultrapure water at a flow rate of 0.5 ml min^{-1}). As standards, inulin from Dahlia tubers, glucose, fructose, sucrose and nystose were used. The samples of *A. tequilana* WSC from different ages were diluted in water distilled (100 g l^{-1}) and then filtered (membrane with 0.45 μm) before they were injected. After quantification of sugars, the percentage of free and polymerised sugars was calculated.

2.5. Liquid chromatography–mass spectrometry (LC–MS)

The molecular mass of fructooligosaccharides was determined by LC–MS using a Q-Trap mass spectrometer from Applied Biosystems. The separation was achieved with C18 RP-fusion column and fructans were ionised by Electron Spray Ionisation (ESI) at 450 °C, with a declustering potential of 60 V, separated by a quadripole and detected in positive ion mode.

2.6. High performance anion exchange chromatography (HPAEC-PAD)

Separation of the WSC of the lyophilised extracts obtained from *A. tequilana* stems of different ages was performed by HPAEC with pulsed amperometric detection (HPAEC-PAD, Bio-LC50 system, detector ED40, Dionex, Sunnyvalle, CA, USA) using an analytical CarboPac PA-100 column (4 × 250 mm; Dionex, Sunnyvalle, CA, USA). The column temperature was 35 °C, and a sodium acetate gradient in 150 mM NaOH was used at a flow rate of 1 ml min^{-1}. The elution programme consisted of 6 mM sodium acetate (0–10 min), 6–500 mM (10–190 min), and 6 mM (190–200 min). As standards, inulin from Dahlia tubers, glucose, fructose, sucrose, 1-kestose and nystose were used. The samples of *A. tequilana* WSC from different ages were diluted in water distilled (10 g l^{-1}) and then filtered (membrane with 0.45 μm) before they were injected. Inulin was injected at 5 g l^{-1}.