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Structural characterization of fructans from *Agave fourcroydes* (LEM.) with potential as prebiotic

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The use of prebiotics, like fructans, increases in animal production. They can be used as growth promoting additives because they benefit health and productive performance. *Agave fourcroydes* shows high concentrations of these compounds. Besides, structural characteristics of these carbohydrates may influence on animal response. The objective of this study was to determine structural characteristics of fructans present in *Agave fourcroydes* from Cuba, with potential as prebiotic in animal feeding. An amount of 10 stems of *Agave fourcroydes* were used, which belonged to the Empresa Henequenera "Eladio Hernández León", located in Matanzas province, Cuba. For structural characterization, thin layer chromatography, gas chromatography coupled to a mass spectrometry and anion-exchange chromatography of high resolution coupled to an amperometric detector. From the obtained results, a complex mixture of oligosaccharides, which present few ramifications and neo-kestose series, insulin (GFn) and inulo-n–ose (Fn), is proposed as structure for fructans of *Agave fourcroydes*. Structural characteristics of these carbohydrates are essential for animal nutrition. Fructans of *Agave fourcroydes* are considered as prebiotic candidate for being natural sources of oligofructans, estimated as the best substrates for acid-lactic bacteria.

Keywords: *chemical stuctures, Agave fourcroydes, fructan, prebiotic*

The use of prebiotic additives increases in animal nutrition because they benefit animal health and productive performance (Li and Kim 2013 and Zhao *et al.* 2013a).

Prebiotics are defined as nutritional ingredients, which are selectively fermented by the beneficial intestinal biota and modify their composition and metabolic activity, and, this way, improve health of the host (Roberfroid *et al.* 2010). Fructans, which are among the most studied compounds for these purposes, present a great structural diversity that influence on their prebiotic response (García-Curbelo 2010 and Ivarsson 2012).

Plants belonging to Agavaceae family are among those that synthesize and store fructans (Roberfroid 2007). *Agave fourcroydes* is grown in Cuba for obtaining fiber from its leaves. However, their stems constitute industry waste and remain without use in the field (Calixto and Mazorra 2008). Studies carried out by García-Curbelo *et al.* (2009) demonstrated the presence of high concentrations of fructans in stems of *Agave fourcroydes*. Under these conditions, obtaining these carbohydrates from this organ and knowing their structural characteristics are very important for biological evaluation of a new food additive for cattle rearing.

The objective of this study was to characterize chemical structures present in *Agave fourcroydes* from Cuba, with potential as prebiotic for animal nutrition.

Materials and methods

Biological material. Through the method of diagonals, 10 stems of *Agave fourcroydes* were collected, which were considered as industry wastes. *Agave fourcroydes* was grown in lands of Empresa Henequenera "Eladio Hernández León", located in Matanzas, Cuba. The soil was Lithosol type (data from Instituto de Geofísica, Matanzas). Collected agaves were 8 years old and were not emerged from the scape. Plant sampling was carried out in dry season and collection was performed during early hours in the morning. Stems used were those with 6.30 kg of mean weight. Fructans extraction was performed according to the methodology of López *et al.* (2003).

Thin layer chromatography (TLC). The thin layer chromatography was performed to the dry extract of *Agave fourcroydes*, according to the method of López *et al.* (2003), and it was compared to commercial fructans: Raftilose P95 (95 % of oligosaccharides with β bonds (2-1), linear), Raftiline HP (inulin polysaccharides of high purity) from ORAFTI brand and the mixture of standards: 1-kestose (Megazyme), nystose (Megazyme), kestopentaose (DP5, Megazyme), fructose (Fruc) (Sigma), glucose (Gluc) (Sigma) and sucrose (Sac) (Sigma).

Solutions of 20 mg. mL^{-1} were prepared with deionized water and a 1 µL of each solution was applied with small Hamilton syringes in analytic plaques of silica gel with aluminum stand (10 x 10 cm, Alldrich). The chromatological test was performed in four stages, with a system of solvents of butanol:propanol:water (3:12:4, v/v/v). Plaques were dried at room temperature and were sprinkled a reactive of aniline-diphenylamine-

phosphoric acid with an acetone basis (Anderson *et al.* 2000). Then, plaques were put into an oven at 100 oC for five minutes.

The compounds within the sample of *A. fourcroydes* were identified after comparing its retention factor (Rf) with the standard mixture and commercial products Raftilose P95 and Raftiline HP.

The Rf was calculated using the following formula:

Rf= Distance from application point to stain/distance before solvent

Gas chromatography coupled to a mass spectrometry. A total of 10 mg of dry extract of *Agave fourcroydes* and of commercial fructans Raftilose P95 and Raftiline HP were weighed. The extract was located in 4 mL reactivials. An amount of 500 µL of dimethyl sulfoxide (DMSO, Sigma) was added and was shaken for two hours, until achieving a total solution of the simple. The method of Mancilla (2006) was used for deriving carbohydrates into partially methylated alditol acetate.

In order to identify derivates using gas chromatography coupled to a mass spectrometry, derived samples were dissolved in 2 mL of CH2Cl2. For chromatographic separation, 1 µL was injected in a gas chromatographer (Hewlett Packard 5890 Series II) coupled to a mass spectrometer (Hewlett Packard 5972 Series). A capillary column of phenyl methyl silicon at 5 % (HP 1-DM, 25 m x 0.31 mm x 0.52 µm thick) and helium (He) as carrier gas were used, at a flow speed of 20 $mL.min^{-1}$ and a pressure of 7 PSI. Initial temperature of the oven was 80 ºC for trhee minutes and followed a slope of temperature of 5 $ºC.min^{-1}$ -- 160 $ºC.min^{-1}$; 0.5 $ºC.min^{-1}$ - 180 $ºC.min^{-1}$ and 14 $ºC.min^{-1}$ - 280 ºC.min-1. Total time was 60 min. Temperature of the injector was 270 ºC and that of the detector was 300 ºC. Identification of derivates was carried out according to their mass pattern.

Anion-exchange chromatography of high resolution coupled to an amperometric detector. Solutions at 1 % were prepared in water milli-Q from the dry extract of *Agave fourcroydes* and commercial fructans Raftilose P95 and Raftiline HP. These samples were filtered using a sieve of 0.22 micras of porosity. The methodology proposed by Shiomi (1991) was used. Identification of some compounds was determined with the standards: kestose, nystose and kestopentaose (DP5).

Results and Discussion

After analyzing the results of thin layer chromatography (figure 1), there was a presence of stains in *A. fourcroydes*, which corresponded to glucose/fructose (Gluc/Fruc), sucrose (Sac), inulobiose (F2), 1-kestosa (1-Kes), nystose and kestopentaose. In addition, it was demonstrated that fructans of dry extract have a low level of polymerization, as well as the commercial prebiotic Raftilose P95, although with a different chromatographic profile. The stain belonging to those of high level of polymerization, like in the case of the commercial prebiotic Raftiline HP, did not appear. This characteristic is important for evaluating *A. fourcroydes* as a prebiotic candidate because oligosaccarides are more used for obtaining these additives (Kanakupt *et al.* 2011).

In *A. fourcroydes*, there was a different stain, which was identified according to its Rf like neo-kestose (6 G-Kes), which was identified according to similar chromatographic studies (Cairns *et al.* 1999). These structures may contribute to a higher growth and metabolic activity of beneficial intestinal microorganisms (Gutiérrez 2013). Besides, they are compounds different from the inulin present in commercial prebiotics (Quiminet 2014).

The presence of 1-Kes, F2 and 6G-Kes indicates that fructans within dry extract of *A. fourcroydes* may be divided into different series: GFn, which is characteristic from *Cichorium intybus* (common chicory) and *Helianthus tuberosus* (Jerusalem artichoke) (van Laere and van den Ende 2002); Fn, structures without glucose molecule (Roberfroid 2005); and neo-fructan series, which was found in *Asparagus officinalis* (asparagus) and *Allium cepa* (onion) (Ritsema *et al.* 2004). This composition characterizes *A. fourcroydes* as a mixture of different structures. Studies of Bathia and Nandra (1979) showed different results. These authors identified fructans of inulin type in this genre, while García-Albornoz (2006) only reported presence of inulin and polyfructans in studies with *Agave fourcroydes* in Yucatán.

In the analysis of gas chromatography coupled to a mass spectrometry, which is essential for determining chemical bonds of derivates of fructans to partially methylated alditol acetate of *A. fourcroydes*, there were differences regarding commercial fructans Raftilose P95 and Raftiline HP (figure 2).

Peaks 1 and 2 corresponded to 2.5 Di-O-$acetyl^{-2}$-$deuterium^{-1}$, 3, 4, 6-tetra-O-methyl-D-mannitol and 2.5 Di-O-$acetyl^{-2}$-$deuterium^{-1}$, 3, 4, 6-tetra-O-methyl-D-glucitol, derived from reduction of terminal fructose within fructans (β-D-Fruc-t). Peak 3 belonged to terminal unit of α-D- glucopyranose (α-D-Gluc-t): 1, 5-Di-O-acetyl-$(1\text{-deuterium})^{-2}$, 3, 4, 6-tetra-O-methyil-glucitol. The base fragment of this molecule is m/z 101, unlike the base fragment m/z 129, which is a characteristic of Fru residues.

Peak 4 was identified as a derivate corresponding to mannitol configuration of derivate β (2-6)- D-fructofuranose: 2, 5, 6-Tri-O-acetyl-$(2\text{-deuterium})^{-1}$, 3, 4-tri-O-methyl-mannitol. This peak represents β (2-6) bonds. This bond has the lowest proportion among the samples.

Peak 5 indicates the presence of β (2-1) bonds, given by 1, 2, 5 –Tri-O-acetyl-$(2\text{-deuterium})^{-3}$, 4, 6-tri-O-methyl-mannitol. There was no evidence of separation of glucitol epimer, and, consequently, peak 6 contains glucitol configurations for both bond types: 2, 5, 6-Tri-O-acetyl-(2-deuterium)-1, 3, 4-tri-O-methyl-glucitol +

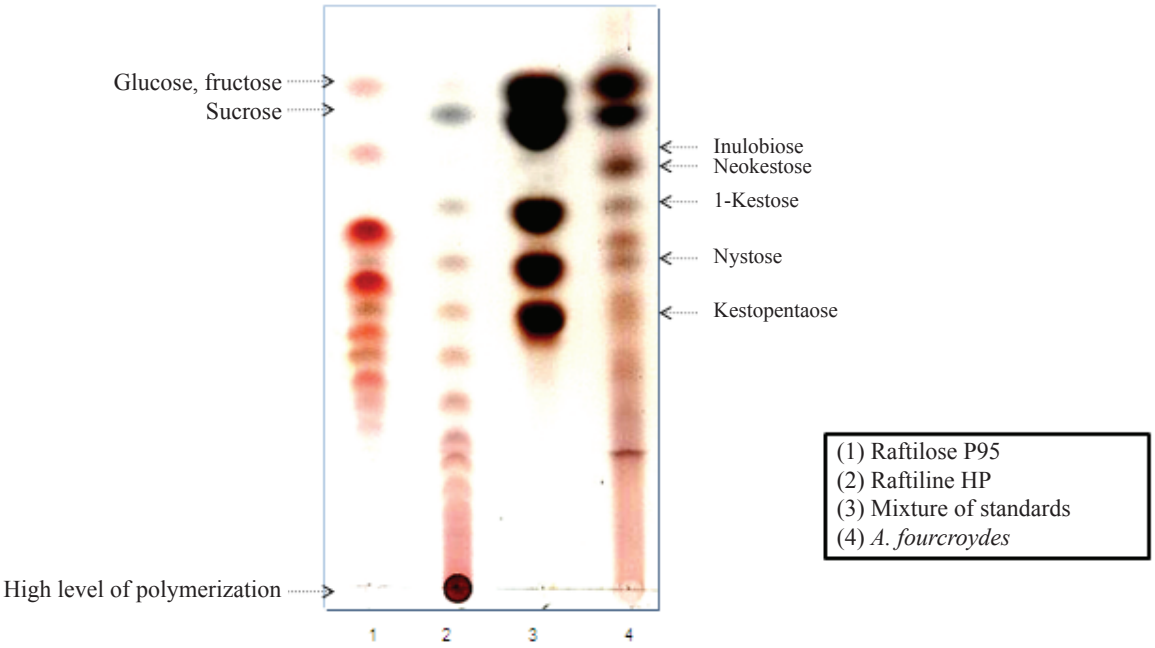


Figure 1. Identification of fructans from the dry extract of *Agave fourcroydes* by thin layer chromatography

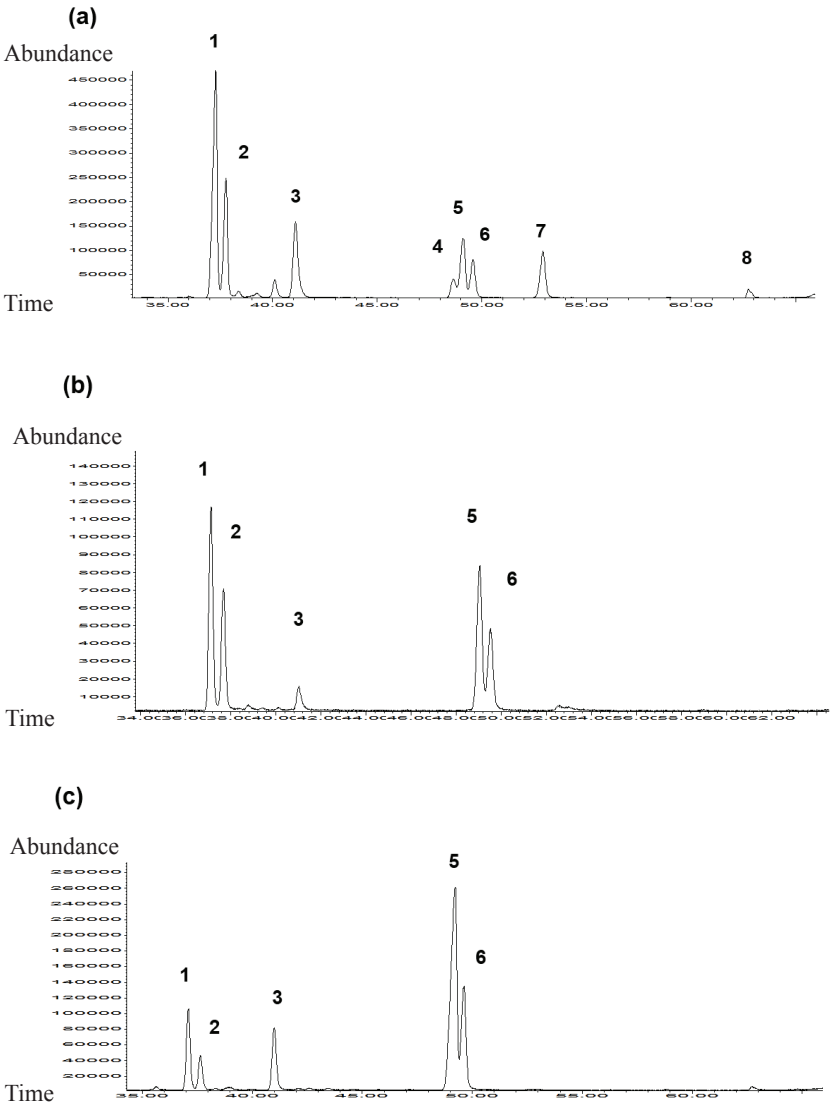


Figure 2. Profile of elusion of fructans derived to alditol, partially methylated acetates from dry extract of *Agave fourcroydes* (a), Raftilose P95 (b), Raftiline HP (c)

1, 2, 5-Tri-O-acetyl-(2-deuterium)$^{-3}$, 4, 6-tri-O-methyl-glucitol. Peak 7 confirms the presence of neo-fructans due to the presence of an internal glucose: 1, 5, 6-Tri-O-acetyl-(1-deuterium)$^{-2}$, 3, 4-tri-O-methyl-glucitol and peak 8 corresponds to ramifications 1, 2, 5, 6-Tetra-O-acetyl-(2-deuterium)$^{-3}$, 4-di-O-methyl-hexitol (-glucitol and –mannitol).

From chromatographic profiles of fructans of *A. fourcroydes*, it can be considered that there is a presence of internal glucose in these plants, which is a characteristic of neo-fructan series, as well as β (2-1) bonds and, in lower amount, β (2-6) bonds. In addition, the presence of few ramifications was confirmed.

Results demonstrate structural complexity of these compounds, which could be a characteristic of Agavaceae family, taking into account the study reports of López *et al.* (2003) in *Agave tequilana* Weber var. Azul. These authors identified fructans, β (2-1) bonds and β (2-6) bonds with abundant ramifications. The presence of complex structures was also confirmed in fructans of Urginea maritima (Spies *et al.* 1992), so they present similarities in their structure.

The presence of fructans with different β bonds within the dry extract of *Agave fourcroydes* makes possible to avoid their degradation by digestive enzymes of host and to be used as energy sources by lactobacilli and bifidobacteria, which are beneficial microorganisms of the gastrointestinal tract (Gibson and Roberfroid 2008).

Figure 3 shows the chromatographic profiles obtained by anion-exchange chromatography of high resolution of fructans of *A. fourcroydes* from Cuba (Raftilose P95 and Raftiline HP). According to Pavis *et al.* (2001), this method allows to know the distribution of fructans within the plant.

In chromatograms belonging to Raftilose P95 and Raftiline HP, there were fructans from inulin series, with well defined peaks (figure 3 b and c). However, when analyzing chromatographic profiles of dry extract of *Agave fourcroydes*, there was a great structural diversity. Only kestose, nystose y kestopentaose (DP5) were identified and when they were compared to the standards, a great difference between the peaks of the analyzed samples was confirmed. Fructans of A fourcroydes were more complex due to the presence of more peaks, so carbohydrates from inulin (GFn), neo-kestose and inulo-n–ose (Fn) series may be present. This analysis established the structural difference between fructans of *A. fourcroydes* and inulin-type prebiotics.

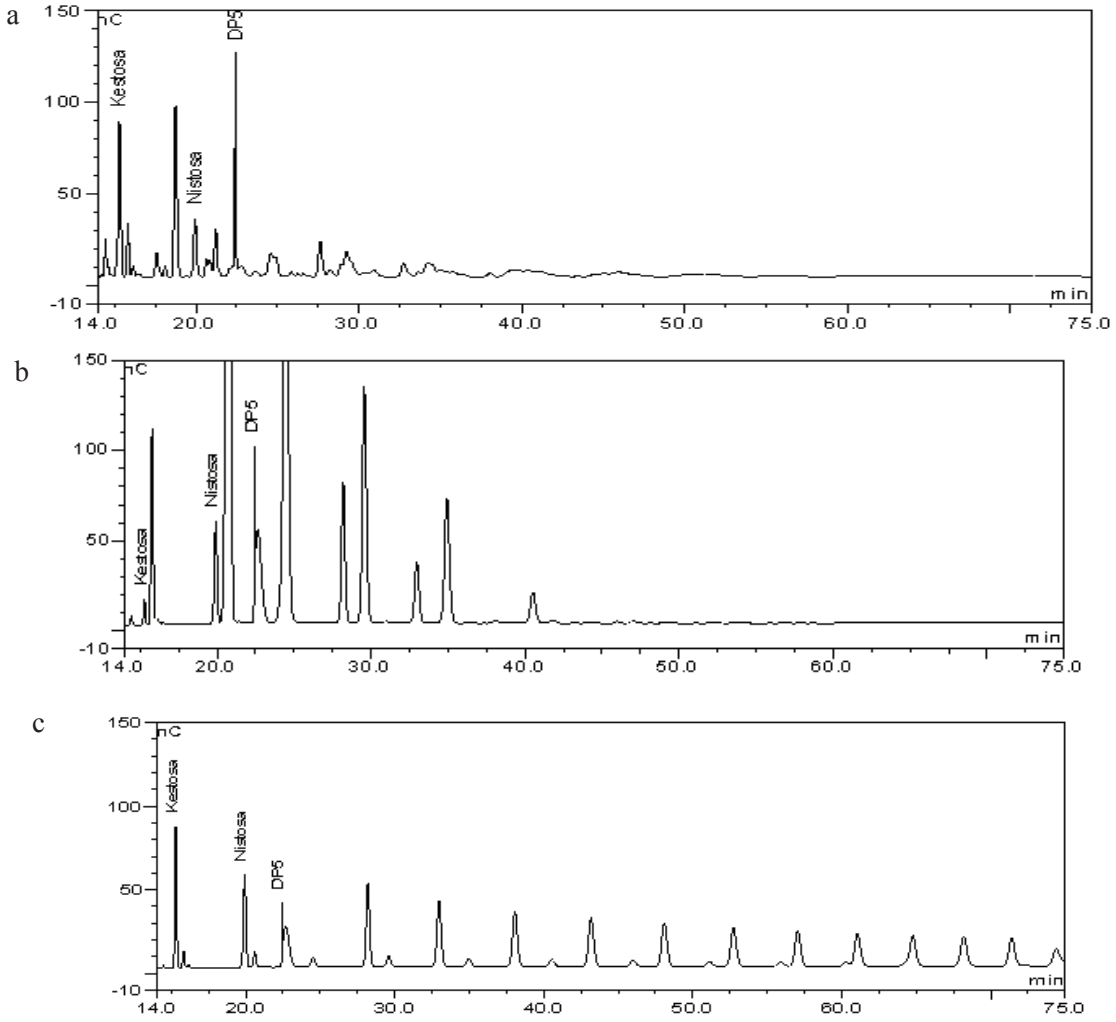
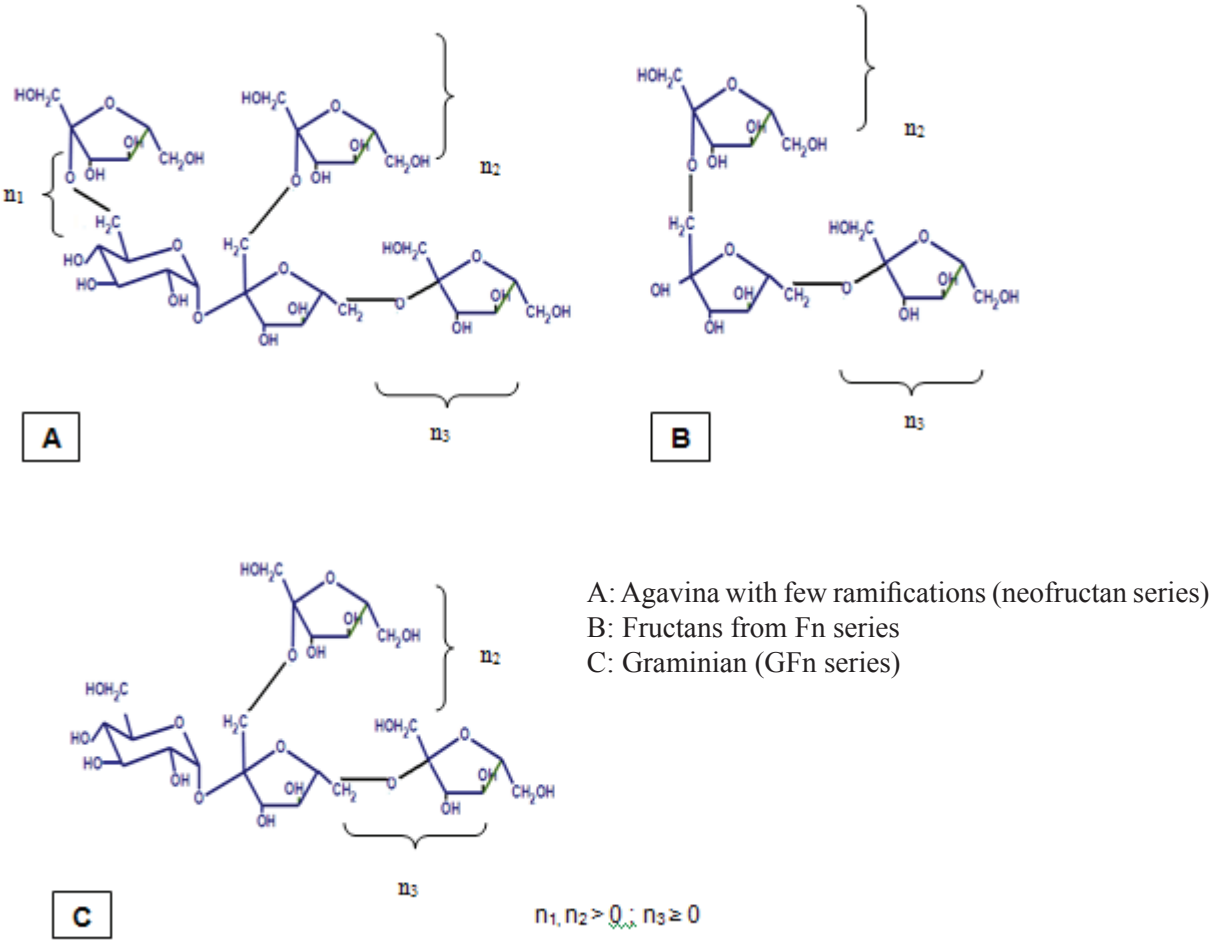


Figure 3. Chromatographic profile of fructans within the dry extract of *Agave fourcroydes* (a), Raftilose P95 (b) and Raftiline HP (c).



A: Agavina with few ramifications (neofructan series)
B: Fructans from Fn series
C: Graminian (GFn series)

Figure 4. Polydisperse mixture of oligofructans in the dry extract of *Agave fourcroydes* from Cuba.

Chromatological profile for fructans from dry extract of *A. fourcroydes* showed natural oligosaccharides, which confirms the results from thin layer chromatography. This chromatogram differed to that reported for *Agave tequilana*, *Agave potatorum* and *Agave angustifolia* by studies of Mancilla (2006), who stated fructans with high level of polymerization in the structure.

From the analysis of results, a complex mixture of oligosaccharides, which presents structures with few ramifications and Fn, GFn and neo-fructan series (figure 4), was proposed as structures for.

Structural characteristics of fructans of *Agave fourcroydes* confirmed in this research make them be very important for animal nutrition, and to be evaluated as prebiotic candidate because they constitute natural sources of oligofructans, which are considered as the best substrates for acid lactic bacteria regarding those with high level of polymerization (Gibson and Roberfroid 2008). Besides, fructans of *Agave fourcroydes* have different β-type bonds that make them indigestible by digestible enzymes from the superior part of the gastrointestinal tract of monogastric animals and, therefore, can be used as energy source for beneficial microorganisms from the caecum and colon of the host. This influenced on a better health state and on productive performance of the animal (Huyghebaert *et al.* 2011 and Zhao *et al.* 2013b).

These determinations allowed to perform the structural characterization of fructans from *Agave fourcroydes*, which are not from inulin type, and they constitute a polydisperse mixture of oligofructans. Due to their properties, fructans of *Agave fourcroydes* can be used as prebiotic in animal nutrition.

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References

Anderson, K., Li, S. & Li, Y. 2000. Diphenylamine-aniline-phosphoric acid reagent, a versatiloe spray reagent for revealing glycoconjugates on the layer chromatography plates. Anal. Biochem. 287: 337

Bathia, I. & Nandra, K. 1979. Studies on fructosyl transferase from Agave americana. Phytochemistry 18: 923

Cairns, A. J., Nash, R., Machado, M. A. & Sims, I. M. 1999. Characterization of enzymatic polymerization of 2,6-linked fructan by leaf extract of timothy grass (*Phleum prattense*). New Phytol 142:79

Calixto, B. & Mazorra, M. 2008. Planta para el desfibrado

del henequén. IV Simposio de Fibras Naturales, aprovechamiento integral y sus aplicaciones. FIBRATEC. Palacio de las Convenciones. Cuba. ISBN: 978-959-261-281-5

García-Albornoz, M. 2006. Determinación y caracterización de fructanos provenientes del henequén (*Agave fourcroydes*). Tesis de Maestría en Ciencia y Tecnología de las Plantas. Centro de Investigaciones Científicas de Yucatán. Mérida. Yucatán. México. 100 pp.

García-Curbelo, Y. 2010. Obtención de fructanos a partir del *Agave fourcroydes* (Lem.), caracterización estructural y evaluación biológica como prebiótico. PhD Thesis. Instituto de Ciencia Animal. La Habana, Cuba. 100 pp.

García-Curbelo, Y., López, G. M. & Bocourt, R. 2009. Fructans in *Agave fourcroydes*, potentialities for its utilization in animal feeding. Cuban Journal of Agricultural Science 43:169

Gibson, G. & Roberfroid, M. 2008. Handbook of prebiotic. Gibson, G and Roberfroid M. Eds. Bélgica, 85 pp.

Gutiérrez, P. 2013. Caracterización de dos glicosiltransferasas productoras de oligosacáridos prebióticos de las levaduras *Phaffia rhodozyma* y *Rhodotorula dairenensis*. PhD Thesis. Universidad Autónoma de Madrid. España, 120 pp.

Huyghebaert, G., Ducatelle, R. & Van Immerseel, F. 2011. An update on alternatives to antimicrobial growth promoters for broilers. The Veterinary J. 187:182

Ivarsson, E. 2012. Chicory (*Cichorium intybus* L) as fibre source in pig diets. Effects on Digestibility, Gut Microbiota and Performance. Doctoral Thesis. Swedish University of Agricultural Sciences, SLU Service/Repro, Uppsala. ISBN 978-91-576-7655-9.

Kanakupt, K., Vester Boler, B., Dunsford, B. & Fahey, G. 2011. Effects of short-chain fructooligosaccharides and galactooligosaccharides, individually and in combination, on nutrient digestibility, fecal fermentative metabolite concentrations, and large bowel microbial ecology of healthy adults cats. J. Anim. Sci. 89:1376

Li, J. & Kim, I.H. 2013. Effects of levan-type fructan supplementation on growth performance, digestibility, blood profile, fecal microbiota, and immune responses after lipopolysaccharide challenge in growing pigs. J. Anim. Sci. 91: 5336

López, M., Mancilla, N.A. & Mendoza-Díaz, G. 2003. Molecular structures of fructans from *Agave tequilana* Weber var azul. J. Agric. Food Chem. 51: 7835

Mancilla, N. A. 2006. Caracterización molecular de fructanos en Agave y Dasylirion spp identificación de fructosyl transferasas y su expresión en *Pichia pastoris*. PhD Thesis. CINVESTAVF-IPN, Unidad Irapuato. México, 120 pp.

Pavis, N., Chatterton, N. J., Harrison, P. A., Baumgartner, S., Praznik, W., Boucaud, J. & Prud, M. 2001. Structure of fructans in roots and leaf tissues of Lolium perenne. New Phytol. 150:83

Quiminet. 2014. Proveedores de: Inulina (Fibra Prebiótica). Available on: http://www.quiminet.com/productos. [Consulted: May 15th, 2014]

Ritsema, T., Verhaar, A., Vijn, I. & Smeekens, S. 2004. Fructosyltransferase mutants specify a function for the β-fructosidase motif of the sucrose-binding box in specifying the fructan type synthesized. Plant Mol. Biol. 54: 853

Roberfroid, M. 2005. Inulin: A fructan. In Inulin Type Fructans Functional Food Ingredients. Roberfroid, M. Ed. CRC Series in Modern Nutrition, CRC Press. Boca Raton. Belgica. p. 39

Roberfroid, M. 2007. Prebiotics: The concept revisited. J. Nutr. 137: 830

Roberfroid, M., Gibson, G., Hoyles, L., McCartney, A., Rastall, R., Rowland, I., Wolvers, D., Watzl, B., Szajewska, H., Stahl, B., Guarner, F., Respondek, F., Whelan, K., Coxam, V., Davicco, M., Léotoing, L., Wittrant, Y., Delzenne, N., Cani, P., Neyrinck, A. & Meheust, A. 2010. Prebiotic effects: metabolic and health benefits. Br. J. Nutr. 104 (2): S1

Shiomi, N., Onodera, S., Chatterton, J. & Harrison, P. 1991. Separation of fructooligosaccharide isomers by anion-exchange. Agric. Biol. Chem. 55:1427

Spies, T., Praznick, W., Hofinger, A., Altmann, F., Nitsch, E. & Wutka, R. 1992. The structure of the fructan sinistrin from Urginea maritima. Carbohydr. Res. 235: 221

Van Laere, A. & van den Ende, W. 2002. Inulin metabolism in dicots: chicory as a model system. Plant Cell and Environment 25: 803

Zhao, P., Wang, J. & Kim, I. 2013a. Effect of dietary levan fructan supplementation on growth performance, meat quality, relative organ weight, cecal microflora, and excreta noxious gas emission in broilers. J. Anim. Sci. 91:5287

Zhao, P., Wang, J. & Kim, I. 2013b. Evaluation of dietary fructan supplementation on growth performance, nutrient digestibility, meat quality, fecal microbial flora, and fecal noxious gas emission in finishing pigs. J. Anim. Sci. 91:5280

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