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PROFILING OF COMMMERCIAL AGAVE FRUCTOOLIGOSACCHARIDES USING ULTRAFILTRATION AND HIGH PERFORMANCE THIN LAYER CROMATOGRAPHY

PERFIL DE FRUCTOOLIGOSACÁRIDOS DE AGAVE COMERCIALES EMPLEANDO ULTRAFILTRACIÓN Y CROMATOGRAFÍA EN CAPA FINA DE ALTA RESOLUCIÓN

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Abstract

A set of ingredients from chicory and *Agave* were analyzed to obtain the carbohydrate distribution and band profiling through Ultrafiltration and High Performance Thin Layer Chromatography (HPTLC). For method standardization, reference materials and ingredients were used. Carbohydrate distribution was based in their separation by Ultrafiltration through 3 kDa membrane, allowing separation and quantification of long-chain fructans (degree of polymerization DP>10) from short-chain fructans (FOS DP<10). The relation of long-chain fructans versus short-chain fructans (long:short) resulted in 52-60:40-48 for *Agave* materials showing almost the same relation as the chicory ingredients. The retention factor (Rf) of each band by HPTLC from each sample was recorded and compared to generate a characteristic chicory and *Agave* profile. The bands of fructose, sucrose, 1-kestose and nystose were identified in all materials analyzed. Additionally, at least four distinctive bands were observed in chicory and three in the *Agave* materials. It was possible to differentiate between sources of fructans, since the band profiles were different. This approach to ingredients study showed to be useful to discriminate between *Agave* and chicory fructans and for following food industry quality control.

Keywords: fructans, fructooligosaccharides, *Agave* FOS, thin layer chromatography, nystose, kestose.

Resumen

Un grupo de ingredientes de achicoria y *Agave* se analizaron para obtener la distribución de carbohidratos y el perfil de bandeo mediante Ultrafiltración y Cromatografía en Capa Fina de Alta Resolución (HPTLC). La estandarización del método se realizó empleando materiales e ingredientes de referencia. La distribución de carbohidratos se basó en su separación por Ultrafiltración con membranas de 3 kDa, permitiendo la separación y cuantificación de fructanos de cadena larga (grado de polimerización (DP)>10) de los de cadena corta (FOS DP<10). La relación fructanos largos respecto de los cortos (largo:corto) resultó de 52-60:40-48 para materiales de *Agave*, mostrando una relación muy parecida a los de achicoria. Los factores de retención (Rf) de cada banda por HPTLC para cada muestra fueron registrados y comparados para generar un perfil característico para achicoria y *Agave*. Las bandas de fructosa, sacarosa, 1-kestosa y nistosa se identificaron en todos los materiales. Además se encontraron al menos cuatro bandas exclusivas en achicoria y tres en *Agave*. Fue posible diferenciar entre los orígenes de los fructanos ya que el perfil de bandeo fue diferente entre ingredientes. Este enfoque mostró ser útil para el estudio de ingredientes en la discriminación entre fructanos de *Agave* y achicoria así como para el control de calidad en la industria de alimentos.

Palabras clave: fructanos, fructooligosacáridos, FOS de *Agave*, cromatografía en capa fina, nistosa, kestosa.

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1 Introduction

Fructans are water-soluble polysaccharides based on fructose with β-fructofuranosyl residues that resist gastrointestinal human digestion. Although fructans have essentially three different types of linkages: fru-$\beta(2\rightarrow1)$, fru- $\beta(2\rightarrow6)$ and glu- $\beta(2\rightarrow6)$, there are a wide variety of lengths and structures depending on the source of the fructan. Linear fructan inulin with $\beta(2\rightarrow1)$ linkages are found in dicots, while linear fructan levan with $\beta(2\rightarrow6)$ linkages and branched fructan graminan with both $\beta(2\rightarrow1)$ and $\beta(2\rightarrow6)$ linkages found in bacteria and monocots (Saldaña *et al*., 2009; Yildiz, 2012). *Agave* genera is a monocot with high commercial value in Mexico, traditionally used for tequila and mezcal production (Molina-Guerrero *et al*., 2007; Tellez-Mora *et al*., 2012) and is currently being used for fructan extraction. The demand of fructan as a fiber has been growing giving the health concern of the population and the ease incorporation on functional food formulations (Beristain *et al*., 2006). Some *Agave* species studied for fructan content are *Agave tequilana, A. americana, A. angustifolia, A. potatorum, A. mapisaga* and *A. fourcroydes* (Mancilla-Margalli and López, 2006; Ortiz-Basurto *et al*., 2008; Ravenscroft, 2009).

Molecular structure from *Agave* fructans showed notable differences in comparison with chicory fructans. They contain not only inulin, but also branched fructans: levan $\beta(2\rightarrow6)$ linkages and neofructan structures consisting of a central sucrose to which $\beta(2\rightarrow1)$ and $\beta(2\rightarrow6)$ linkages to fructofuranosyl chains are attached (Bonett *et al*., 1994; Mancilla-Margalli and López., 2006; Arrizon *et al*., 2010; Ravenscroft *et al*., 2009). *Agave* fructans have an estimated degree of polymerization (DP) ranging from 3 to 29 units (Lopez *et al*., 2003; Ravenscroft *et al*., 2009).

Fructans in general can be divided in two fractions: long-chain fructans (>10 DP) and short-chain fructans (DP from 3 to 10) commonly named as fructo-oligosaccharides (FOS). Differential study of these fractions have demonstrated that short-chain fructans are responsible for improvement of mineral absorption linked to colon pH reduction (van den Heuvel *et al*., 1999; Zdunczyk *et al*., 2007), bifidogenic effect (Menne *et al*., 2000; Rao VA 2001; Kapiki *et al*., 2007) and production of short-chain fatty acids (Zdunczyk *et al*., 2007). Thus the FOS content and profiling of commercial ingredients is essential for potential claim detection and quality control assessment.

Highly polar fructo-oligosaccharides are notoriously difficult to separate by HPLC without prior derivatization or the use of highly alkaline eluent system (Cataldi *et al*., 2000; Robinson *et al*., 2007). Different combination of strategies have been reported for fructan characterization and include HPEAC-PAD, LC-MS, MALDI-TOF size exclusion, rpHPLC with combination of polymer permethylation, reductive cleavage, acetylation and GC/FID (Baumgartner *et al*., 2000; Praznik and Huber, 2005; Kocsis *et al*., 2007). All these methods are impractical at the industrial level for quality control purposes. There are two official methods for fructan quantitation in foods, the enzymatic/spectrophotometric AOAC 999.03 and ion exchange chromatography (AOAC 997.08), both are based on Prosky and Hoebregs (1999). The AOAC 997.08 has the principle to total hydrolysis of charbohydrates present in the sample, using amylase and inulinase, followed by quantification of free fructose and glucose produced, and a further series of subtraction steps. It has the inconvenience of showing a large standard deviation because of large corrections that have to be made. The AOAC 999.03 is based on a removal of monosaccharides by converting them into alditols, after amylases and sucrose treatment. The methodology of end point is colorimetric by measuring the total amount of reduced sugars present after inulinase treatment and has the disadvantage of not giving the fingerprint of the fructans. Both methods have the inconvenient that not fully hydrolyzed the *Agave* fructans, originating underestimation (Ortiz-Basurto *et al*., 2008 and own observation).

The objective of this work was to evaluate a simple and fast approach to analyze fructans combining Ultrafiltration, HPLC and HPTLC, in order to describe the carbohydrate distribution of commercial ingredients and to discriminate between two sources of fructans: chicory and *Agave*.

2 Materials and methods

2.1 Standards and materials from chicory and Agave

Fructose (F), glucose (G), sucrose (S), 1-kestose (1-K), nystose (N), isomaltotriose (I), maltopentaose (M5), maltoheptaose (M7) and chicory FOS (F8052) from SIGMA, were used as commercial standards. Two commercial ingredients of Beneo Orafti were used as references from inulin type fructans: Raftiline® GR as native inulin from chicory and

Beneo^{TM} P95 as short-chain fructooligosaccharides (FOS) from chicory. In order to obtain a reference material from *Agave*, FOS from *Agave tequilana* Weber var. azul were obtained and named as CIATEJ FOS. Water soluble carbohydrates (WSC) were obtained by the transversal cut of the *Agave* heads in halves, then smashed completely and mixed with water. The mixture was blended in a mechanical device of stainless steel at 70°C for 7 h, the WSC suspension was filtered through 80-100 mesh until a final concentration of 10-15 °Brix. Water soluble carbohydrates were purified using an ionic interchange column, resulting in a product with no color or minerals. Fructans were processed by tangential flow filtration (TFF) with a 3kDa molecular weight cut off membrane, which separates long-chain fructans (DP>10), from short-chain FOS (DP<10). The retentate containing FOS was then spray dried in order to obtain a white powder with a relative humidity of 4.1% (w/w). Three *Agave* ingredients verified by CIATEJ through audit, were obtained from industrial producers in order to use them as reference materials and were named as: Fag, Fan and Faa. Ten different materials claiming *Agave* origin were collected from commercial sources and compared with reference materials from *Agave* and chicory origin, coded as follow: Fac, Fap, Fas, Fam, Fai, Idm, Iol, Ipr, Ibn, Inv.

2.2 Sugar quantitation

S, F and G content of the different ingredients were quantified by HPLC using a BioRad Aminex 42-C column, and water as the mobile phase. Sugar concentration was obtained using the equation relating calibration curve from these sugars with the area under the curve using refraction index detector. N and 1-K were quantified using their calibration curves obtained by HPTLC linearized with logarithmic equations obtained by densitometry at 400 nm. Results were reported in dry weight.

2.3 Carbohydrate distribution

Fructans were characterized in order to obtain their distribution based on chain length. The samples were solubilized in water (40 mg/mL) and separated by ultrafiltration, using a 3 kDa membrane, in two different fractions: permeate (P) containing short-chain fructans with DP≤10~12 (FOS) and retentate (R) containing long-chain fructans with DP>10. Efficiency of the separation was confirmed by MALDI-TOF Bruker autoflex III TOF/TOF 200. FOS content was quantified subtracting the S, G and F concentration from the 3 kDa permeate dry weight (P) (ec. 1). Long-chain fructans (DP>10) were obtained through dry weight determination of the retentate. Total fructans ec. (2) were obtained by the addition of FOS plus R. Protein, lipid and ash were analyzed as a control.

$$FOS = P - S - G - F \quad (1)$$

$$Total\ \ fructans = FOS + R \quad (2)$$

2.4 High Performance Thin Layer Chromatography (HPTLC)

All the solvents (HPLC grade) and reagents were purchased from SIGMA. The HPTLC system used was from CAMAG, with a LINOMAT5 to load the sample into the cromatoplate, a TLC Scanner 3 to analyze the bands by densitometry at 400 nm and the winCATS software to evaluate the chromatograms obtained. Stationary phase for chromatography was HPTLC-Fertigplatten Nano-SIL NH2/UV254 (Macherey-Nagel, Germany) and mobile phase was a mixture of *n*-butanol:methanol:water:acetic acid, 50/25/20/1 (v/v/v/v). The cromatoplate was atomized with a solution of ethanol/H_2SO_4/anisaldehyde, 18/1/1 (v/v/v) and placed at 190 °C for 20 min for revelation. Selection of the most suitable measurement wavelength (λ_{max}) was conducted after scanning of standard solutions in a range from 360 nm to 540 nm. Retention factor (Rf) was calculated with the next formula: Rf = Distance of analyte (mm)/ Total distance of front solvent (mm). Calibration curves from F, S, 1-K, N, M5 and M7 were done although only were used for 1-K and N concentration. Precision measurements were conducted through Coefficient of Variation (%CV) in repeatability conditions with 5 replicates (same day, operator and apparatus) and reproducibility conditions for 4 days (different day, same operator and apparatus).

3 Results and discussion

3.1 Standardization of HPTLC method

The separation of standards was achieved successfully by HPTLC (Figure 1) with retention factors (Rf) of 0.09-0.11, 0.14-0.17, 0.25-0.27, 0.28-0.29, 0.36-039, 0.42-0.47 for M7 (DP 7), M5 (DP 5), N (DP 4), 1-K (DP 3), S (DP 2) and F (DP 1), respectively (Table 1).

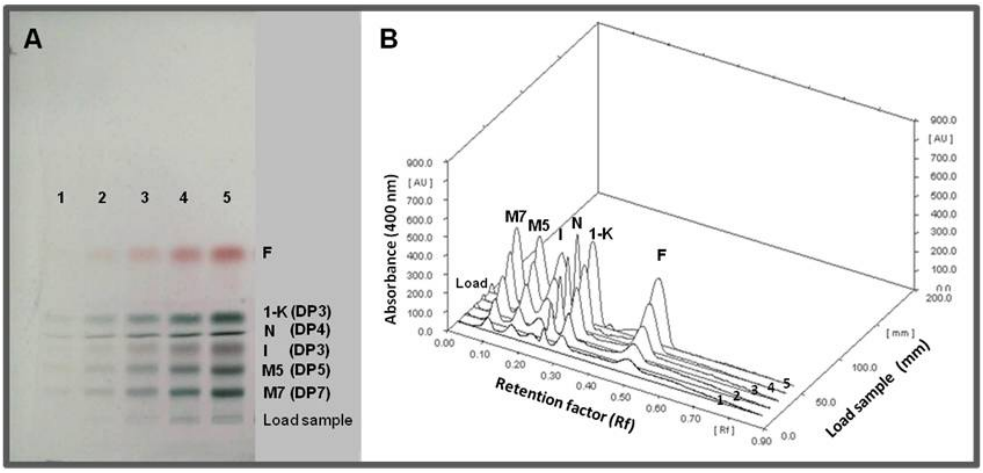

Fig. 1. Separation and quantification of oligosaccharides by HPTLC. A) Cromatoplate after revelation showing the oligosaccharides used as standards. Lanes 1-5: amount of each carbohydrate, 1=0.75 μg, 2=1.25 μg, 3=2.5 μg, 4=5 μg and 5=10 μg. DP: degree of polymerization. B) Chromatograms of standards at different concentrations obtained by densitometry at 400 nm.

Table 1. Retention factor (Rf) and percent of coefficient of variation (%CV) for repeatability and reproducibility evaluation from commercial standards by HPTLC.

COMMERCIAL STANDARD	DEGREE OF POLIMERIZATION (DP)	Rf (range)	% CV REPEATABILITY	% CV REPRODUCIBILITY
Fructose	1	0.38-0.47	0.00	8.34
Sucrose	2	0.36-0.39	1.74	9.15
1-Kestose	3	0.28 - 0.29	1.85	8.87
Nistose	4	0.25 - 0.27	2.66	12.25
Maltopentaose	5	0.14 - 0.17	3.24	11.27
Maltoheptaose	7	0.09 - 0.10	0.00	10.66

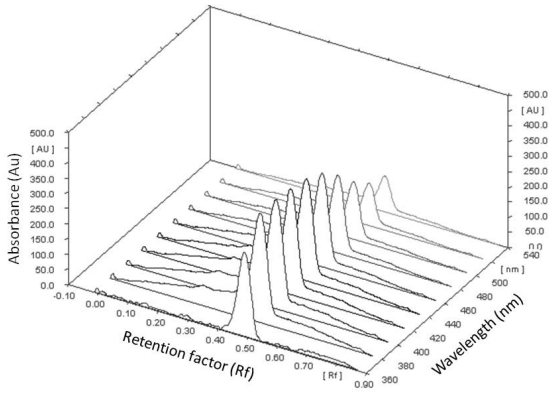

Fig. 2. Chromatogram of absorbance vs wavelength for fructose standard obtained by densitometry. Scanning was performed at wavelengths between 360 nm and 540 nm.

Scanning was performed at wavelengths between 360 nm and 540 nm and the maximal absorbance responses were observed at 400 and 420 nm (Figure 2). Standard curves were obtained from chromatograms generated by densitometry analysis after scanning of standard solution at 400 nm (Figure 1). Calibration curves were done in amount from 0.75 to 10 μg for F, S, 1-K, N, M5 and M7. The R^2 obtained for calibration curves ranged from 0.9885 (F) to 0.9455 (N).

Table 1 shows the Rf for every reference and %CV for repeatability and reproducibility conditions for commercial standards evaluated by HPTLC. The method shows good repeatability (%CV from 0 to 3%) and acceptable reproducibility (%CV from 8 to 12%), depending of the compound tested. Inclusion of appropriate references of pure substances F, S, 1K, N,

and chicory and *Agave* materials in every analysis is essential for %CV control and for reset of Rf.

3.2 Carbohydrate distribution

Tangential flow ultrafiltration method was implemented in order to quantify the carbohydrate distribution of reference and commercial fructans. It was possible to separate fructans, by ultrafiltration, in two different fractions: permeate containing short-chain fructans and retentate containing long-chain fructans. Efficiency of the separation was confirmed by MALDI-TOF, the maximum DP which is observed in the mass spectrum corresponds to a polysaccharide with a DP of 12 in the permeate fraction (Figure 3). Nanofiltration has been used to remove G, F and S from *Agave* juice fructans employing a 1 kDa membrane (Moreno-Vilet *et al*., 2013), however ultrafiltration through 3 kDa to separate *Agave* fructans in long and short chain has not been reported before.

Ultrafiltration by 3 kDa membrane for carbohydrate distribution analysis was successfully implemented. Table 2 shows the carbohydrate composition of *Agave* and chicory materials, classifying carbohydrates in sugars (G, F and S), long chain fructans (>12 DP) and FOS (≤ 10 DP). Chicory fructo-oligosaccharides standard from Sigma met their specification (≥ 90% FOS and inulin) resulting in 95.1% (addition of FOS plus large fructans). Raftiline® GR used as native chicory inulin reference, met their product specifications (>90% inulin, G+F ≤ 4% and S ≤ 8%) see Table 2 (Raftiline® GR). Beneo P95, an oligofructose type ingredient, was used as control for tangential flow ultrafiltration (TFU), resulting in 92.8% of FOS, close with specification sheet oligofructose of ≥ 93.2% (Beneo^{TM} P95). Minor differences are attributable to the method of analysis (Prosky and Hoebregs, 1999). Beneo P95 sugar content as G (1.8%), F (2.8%) and S (0.5%), were in agreement with specification sheet of the product: ($G + F + S$ <6.8%). General carbohydrate composition in chicory standard and reference materials showed consistency with enzymatic methods used for ingredient analysis (Beneo^{TM} P95; Coussement, 1999).

Commercial inulin always contains a small amount of mono and disaccharides, up to 10%. These sugars are naturally present in the chicory root, the raw material used for its extraction, and they are not result of processing. Low sugar and high performance inulin are obtained by removal of these mono- and disaccharides (Coussement, 1999; Madrigal and Sangronis, 2007).

Carbohydrate distribution of CIATEJ *Agave* FOS reference material was quantified founding 11.8% of sugars, 88.2% of short chain fructans and undetectable long chain fructans. MALDI-TOF study confirmed that *Agave* FOS obtained had the maximum DP between 10 to 12 (Figure 3). Three *Agave* reference materials showed a concentration of sugars range between 3.1 to 7.5%, with low concentrations of G and S (from < 0.5 to 1.6 %) and double amount of F (2.2 to 4.8%). This sugar pattern of G/F/S is summarized as 0-1.6/2.4-4.8/0.9-1.2, and is in agreement with Arrizon *et al*. (2010) and Mancilla-Margalli and Lopez (2006) that used materials from five different *Agave* species including *A. tequilana, A. angustifolia, A. potatorum, A. cantala* and *A. fourcroydes*. Native materials from chicory (Chicory FOS Sigma and Raftiline GR) showed a different sugar pattern than those from *Agave*, characterized by a low concentration of G and F, and a high concentration of S (0/0-1.9/2.7-4.7) (Table 2).

Except by Fas, all ingredients claiming *Agave* origin had the same general G/F/S pattern, than reference *Agave* materials with low G and S and high F, as is summarized in Table 2. A pattern of high F concentration and very low G concentration reflects a physiologic state of active hydrolysis of fructans in the *Agave* by fructan exohydrolase, which is a fructan-degradative enzyme that releases fructose moieties from non-reducing ends (Mancilla-Margalli and Lopez, 2006). Differences in sugar patterns with *A. tequilana* materials have been reported with age (Arrizon *et al*., 2010) and also when using the *Agave* leaves instead of the head (Wang and Nobel, 1998; Saldaña *et al*., 2009). According with our findings G/F/S pattern could be considered as one parameter to check the origin of the ingredients, although differences in fructan method extraction, as well as further addition of sugars may affect this distribution.

In general, the sugars in both, the *Agave* reference materials and the ingredients claiming *Agave* origin, met the Mexican Official Standard NMX-F-591-SCFI-2010. The official standard allows a maximum sugar content of G 4%, F 9% and S 2.5 % in commercial materials. Our results in *Agave* ingredients showed that G and F remained about 30 % below this standard.

Oligofructose was introduced as a synonym of FOS by Orafti and refers to a partial enzymatic hydrolysis of inulin from chicory.

Table 2. Carbohydrate composition of standards and ingredients from chicory and *Agave*. Results were classified in sugars (G, F and S), large fructans (>10 DP) and FOS (DP≤10~12).

INGREDIENTS	SUGARS			FRUCTANS			
	glucose	fructose	sucrose	fructans >10	FOS between 5 to ≈10 DP*	nystose	1-kestose
	g/100 g of ingredient (dry weight)						
CHYCORY STANDARD							
Chicory fructooligosachharides (Sigma)	<0.5	1.9	2.7	59.3	22.4	8.6	4.8
REFERENCE INGREDIENTS FROM CHICORY							
Beneo P95 (FOS)	1.8	2.8	0.5	2.1	38.5	35.9	18.4
Raftiline GR (native inulin)	<0.5	<0.5	4.7	53.3	31.9	6.2	3.9
CIATEJ AGAVE FOS REFERENCE MATERIAL							
CIATEJ FOS	3.6	8.2	<0.5	<0.5	75.7	7.4	5.1
REFERENCE INGREDIENTS FROM AGAVE							
Fag	<0.5	2.2	0.9	48.9	35.5	6.2	4.8
Fan	1.6	4.8	1.1	52.6	31.9	5.0	3.0
Faa	0.7	4.6	1.2	56.2	27.9	6.0	3.6
INGREDIENTS CLAIMING AGAVE ORIGIN							
9 differents**	0.6-3.1	1.4-7.5	<0.5 - 3.4	46-66	18.1-33.2	3.1-10.0	0.0-6.2

*DP: Degree of polymerization

**Fas ingredient was not included.

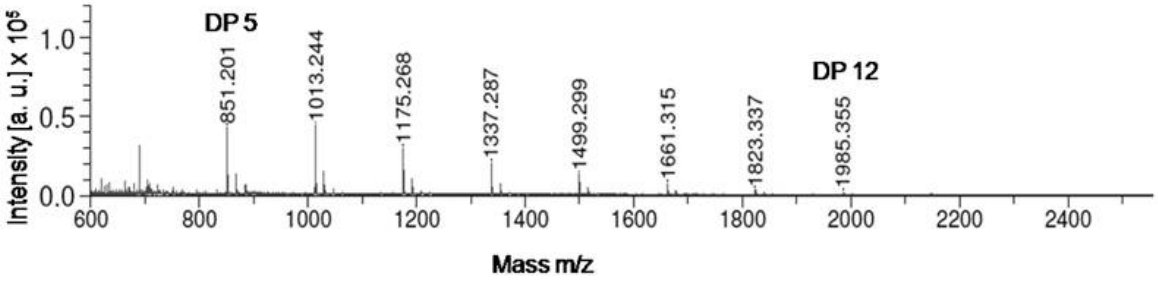

Fig. 3. Mass spectrum of *Agave* FOS obtained by ultrafiltration. DP: Degree of polymerization. The spectrum shows the peaks corresponding to *Agave* fructans permeate obtained with a 3kDa membrane. MALDI-TOF Brukerautoflex III TOF/TOF 200 was employed.

Chemically oligofructose has between 3-8 DP and some molecules contain fructose instead of glucose at the end of the molecule (Coussement, 1999; Madrigal and Sangronis, 2007). Typical composition of commercial oligrofructose products (dry matter) contain about 95% of oligosaccharides (Coussement, 1999; Beneo P95). Our results about Beneo^{TM} P95 meet this characteristic with 93% FOS (see Table 2).

Pattern of long-chain fructans content in relation with short-chain fructans (long:short) expressed as

percent of total fructans resulted in a relation 53-59:36-42 for native chicory inulin (Raftiline® GR), and 50-56:36-47 for *Agave* reference materials, showing almost the same relation in *Agave* and chicory. The characterization of FOS fraction in *Agave* ingredients is of great interest because it has been associated to bone health and bifidogenic effect (Menne *et al*., 2000; van den Heuvel *et al*., 1999). Chicory inulin enrichment with oligofructose has been prompted by industry, in order to achieve functional claims of ingredients.

3.3 HPTLC FOS profiling from chicory and Agave

Figure 4 shows the cromatoplate from *Agave* and chicory fructans obtained by HPTLC, and Figure 5 shows some chromatograms performed by densitometry. This method is able to separate molecules with DP ≤ 10~12. Table 3 summarizes the retention factor (Rf) of the observed spots in two set of results: chicory and *Agave*.

Lanes 2, 3 and 4 from Figure 4 show chicory materials profiling. At least six defined bands were found in chicory products and 1-K and N were confirmed. Table 3a shows F and S at Rf higher than 0.34 according with Sigma standards. Figure 4 shows at lanes 5 to 8 the reference *Agave* materials, and from 9 to 11 shows ingredients claiming *Agave* origin. Profiling of *Agave* materials showed Rf coincidences with chicory in the bands F, S, 1-K, N and a band with Rf 0.11-0.12. Some bands were observed in chicory materials at Rf 0.19-0.22, 0.16-0.17 and 0.12-0.15 with no coincidence with *Agave* bands (Table 3). Meanwhile, a couple of coarse spots at Rf 0.18-0.20 and 0.14-0.16 were found in *Agave* materials.

Differences between chicory and *Agave* profiles have been previously reported and fructans with DP 4 to at least 8 have been shown in *Agave* materials (Mancilla-Margalli and López, 2006; Ravenscroft *et al*., 2009; Saldaña *et al*., 2009; Arrizon *et al*. 2010). In the CIATEJ *Agave* FOS MALDI-TOF, were observed oligofructans from DP 5 to 12. These molecules may be included in *Agave* FOS but only five spots are possible to evaluate by HPTLC.

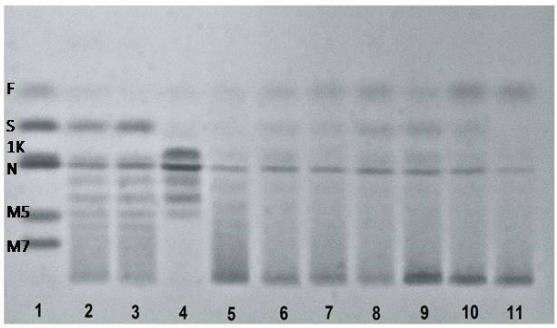

Fig. 4. Cromatoplate of standards, chicory and *Agave* FOS. Line 1: standards (F, fructose Rf 0.38-0.47; S, sucrose Rf 0.36-0.39; 1-K, 1-kestose Rf 0.28-0.29; N, nystose Rf 0.25-0.27; M5, maltopentaose Rf 0.14-0.17; M7, maltoheptaose Rf 0.09-0.10), 2: Chicory FOS Sigma, 3: Orafti GR, 4: P95 Orafti, 5: CIATEJ *Agave* FOS, 6 to 11 commercial *Agave* fructans, 6: Fag, 7: Fan, 8: Faa, 9: Fac, 10: Fap, 11: Fai.

Figure 6 shows the results from a couple of experiments of fructan hydrolysis using the enzyme cocktail Novozym ®960 (containing mainly an endo-inulinase activity) and revealed through HPTLC. A: showed the hydrolysis of inulin type fructans, the appearance over time of short fructans and then mono and disaccharides were observed. B: showed on the other hand, a very low rate of *Agave* fructans hydrolysis, evidenced by the null presence of spots in the area of short fructans as enzymatic product of hydrolysis, and a very low fructose production at four hours of reaction. The low activity of endo-inulinases over agave fructans is in accordance with the reported branched structure of *Agave* ingredients. This low hydrolysis rate may be a consequence of steric impediments that difficult the enzyme attack. At the same time, imply that the method of quantification based on hydrolysis with inulinase, like AOAC 997.08 may be underestimating the *Agave* fructans content and that HPTLC of FOS from *Agave* and the profiling method proposed in this study, represent good options for ingredient evaluation with industrial purposes.

All three *Agave* ingredients used as references showed 1-K and N, with higher proportion of N than 1-K (see Table 2). From nine commercial ingredients claiming *Agave* origin, two ingredients showed significant differences: Iol did not show 1-K (result not shown), and Idm showed only 50% of 1-K respect to found in the reference ingredients (Figure 5D).

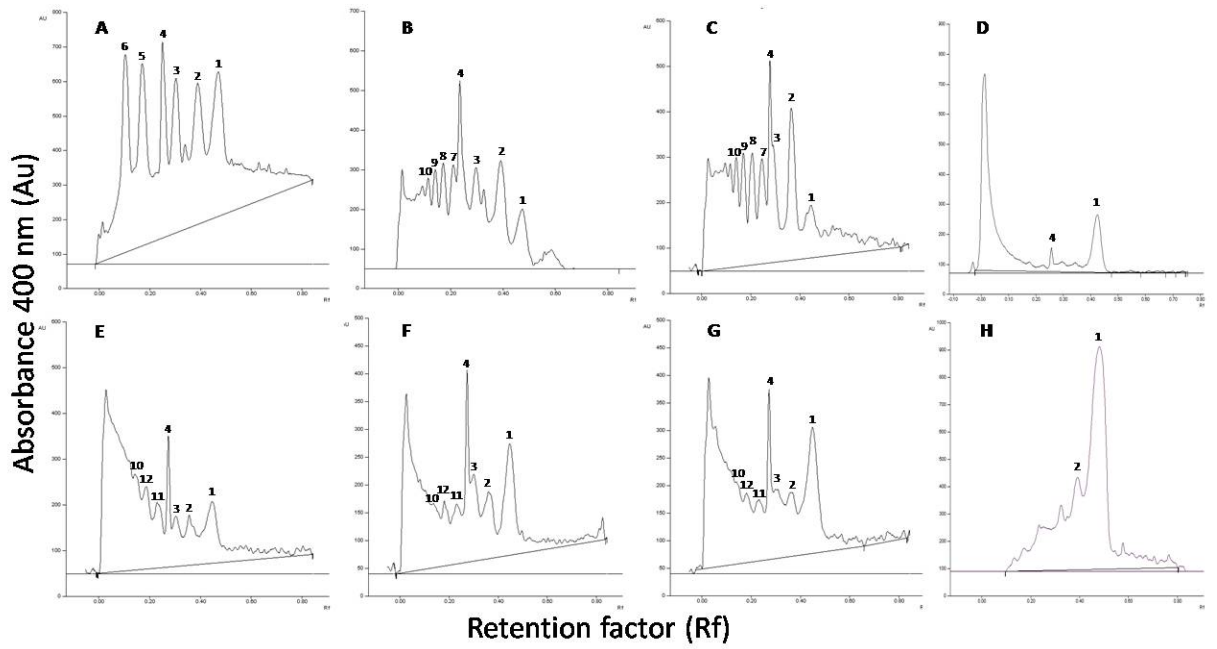

Fig. 5. Chromatograms of standards, *Agave* and chicory fructans obtained by densitometry at 400 nm. A: standards, B: Chicory FOS Sigma, C: GR Orafti, D: *Agave* ingredient Idm, E: CIATEJ *Agave* FOS, F: *Agave* ingredient Fag, G: *Agave* ingredient Fan, H: *Agave* ingredient Fas. 1: Fructose, 2: Sucrose, 3: 1-Kestose, 4: Nystose, 5: maltopentaose, 6: maltoheptaose, 7-12: unknown.

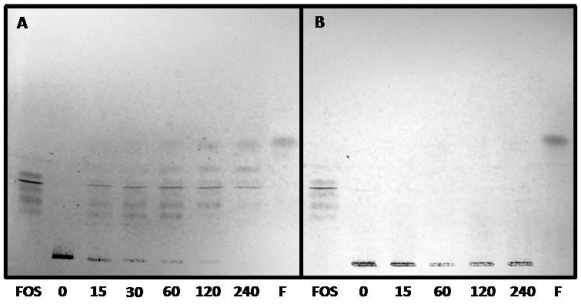

Fig. 6. HPTLC Cromatoplate of enzymatic hydrolysis of fructans with the enzyme Novozym ®960. A:Inulin-type fructans (SIGMA). B: Fructans from *Agave* tequilana Weber var. azul. FOS: Fructooligosaccharides (ORAFTI P95); 0, 15, 30, 60, 120 y 240: hydrolysis time (min); F: Fructose (SIGMA).

Iol and Idm ingredients showed a profile with reduced N and total FOS, and one might speculate that there may be an *Agave* fructan dilution with other ingredients or that the production process is affecting the FOS extraction. It is remarkable that the method was able to detect these types of differences. There is a widespread consensus in finding 1-K and N in *Agave* materials (Mancilla-Margalli and López, 2006; Ravenscroft *et al*., 2009; Saldaña *et al*., 2009; Arrizon *et al*., 2010). The 1-K is a lineal inulin fructan, however it has been proposed that neofructan 6G-kestose with an internal glucose are present in *Agave* materials (Wang and Nobel, 1998; Ravenscroft *et al*., 2009; Saldaña *et al*., 2009). In the chromatograms from chicory and *Agave* ingredients, only one spot with Rf about 0.28-0.29 corresponding to standard 1-K was found, so the present methodology is not able to discriminate between 1-K and 6G-kestose because the broadness of the bands. The scope of HPTLC does not include identification or differentiation between molecules with the same DP. The main objective was the general description of the FOS-sugars band pattern in commercial ingredients and the goal was achieved.

A couple of ingredients claiming *Agave* origin did not follow the general pattern observed by chicory or *Agave* fructans. Idm (Figure 5D) showed one band corresponding with F (Rf =0.43) and one very small spot on N (Rf = 0.26), see Table 1. Another sample labeled as Fas (Figure 5H) showed a big band corresponding with F (Rf = 0.45) and another with S (Rf = 0.37); nor FOS neither long-chain fructans were detected.

Table 3. Characteristic bands and Retention factor (Rf) from a) chicory and b) *Agave*.

a) Chicory ingredients			
Rf	% area	DP	Matches with Sigma standards
0.42-0.44	14-15	DP 1	fructose
0.36-0.39	6-10%	DP 2	sucrose
0.28-0.29	7-12%	DP 3	1-kestose
0.24-0.27	13-14%	DP 4	nystose
0.19-0.22	6%	DP ≥ 4	-
0.16-0.17	6%	DP ≥ 4	-
0.12-0.15	5%	DP ≥ 4	-
0.11-0.12	5%	DP ≥ 4	-
b) Agave ingredients			
Rf	% area	DP	Matches with Sigma standards
0.42-0.44	14-15	DP 1	fructose
0.36-0.39	6-10%	DP 2	sucrose
0.28-0.29	7-12%	DP 3	1-kestose
0.24-0.27	13-14%	DP 4	nystose
0.18-0.20	7-9%	DP ≥ 4	-
0.14-0.16	7	DP ≥ 4	-
0.11-0.12	7%	DP ≥ 4	-

Fas ingredient meets the characteristic of a product obtained from fructan hydrolysis. The HPTLC method was able to find unusual pattern profiles.

Conclusions

The methodology for studying fructan profiles based in ultrafiltration and HPTLC in food ingredients, was successfully implemented. In order to achieve a repeatability lesser than 3% CV, every run must include a mix of standards, *Agave* and chicory materials and the Rf of N and 1-K must be reset.

Although similar patterns of carbohydrate distribution in chicory and *Agave* ingredients were found by ultrafiltration, this is an effective method for screening and quality control evaluation.

The HPTLC method demonstrated effective selectivity of F, S, N and 1-K from the ingredients, so it is a useful method for quality control process and official standard evaluation. HPTLC profiling allowed the discrimination between *Agave* and chicory FOS, as well as finding uncommon profiles. A couple of coarse bands with Rf at 0.18-0.20 and at 0.14-0.16 were found in *Agave* ingredients, which did not coincide with bands from chicory materials. Studies about molecular weight, type of branches and identification of these compounds remain to be determined.

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References

Arrizon, J., Morel, S., Gschaedler A. and Monsan, P. (2010). Comparison of the water-soluble carbohydrate composition and fructan structures of *Agave tequilana* plants of different ages. *Food Chemistry 122*, 123-130.

AOAC Official Method 997.08. 2005. Fructans in food products. Ion Exchange Chromatographic Method. First action 1997. *AOAC International 45.4.06A*.

AOAC Official Method 999.03. 2007. Measurement of fructan in foods. Enzymatic/Spectrophotometric Method. First action 1999. Final action 2005. *AOAC International 45.4.06B*.

Baumgartner, S., Dax, T. G., Praznik, W. and Falk, H. (2000). Characterization of the high-molecular weight fructan isolated from garlic (*Allium satimum* L.). *Carbohydrate Research 328*, 177-183.

Beneo^{TM} P95. Hoja de especificaciones. Doc. CHA3-04-06-06.doc. Orafti. Tienen Belgium.

Beristain, C.I., Cruz-Sosa, F., Lobato-Calleros, C., Pedroza-Islas, R., Rodíguez-Hueso, M.E., Verde-Calvo, J.R. (2006). Applications of soluble dietary fibers in beverages. *Revista Mexicana de Ingeniería Qímica 5*, 81-95.

Bonett, G.D., Sims, I. M., Jhon, J.A. St. and Simpson, R. J. (1994). Purification and characterization of fructans with β-2,1 and β-2,6-glycosidic linkages suitable for enzyme studies. *New Phytologist 127*, 261-269.

Cataldi, T.R.I., Campa, C. and De Benedetto, G.E. (2000). Carbohydrate analysis by high-performance anion-exchange chromatography with pulsed amperometric detection: the

potential is still growing. *Fresenius Journal of Analytical Chemistry 368*, 739-758.

Coussement, P.A.A. (1999). Inulin and oligofrucose: safe intakes and legal status. *The Journal of Nutrition 129*, 1412S-1417S.

Kapiki, A., Costalos, C., Oikonomidou, C., Triantafyllidou, A., Loukatou, E andPertrohilou, V. (2007). The effect of a fructo-oligosaccharide supplemented formula on gut flora of preterm infants. *Early Human Development 83*, 335-339.

Kocsis, L., Liebhard, P. andPraznik, W. (2007).Effect of seasonal changes on content and profile of soluble carbohydrates in tubers of different varieties of Jerusalem Artichoke (Helianthus tuberosus L.). Journal of Agricultural and Food Chemistry 55, 9401-9408.

Lachenmeier, D.W., Sohnius, E.M., Attig, R. and Lopez, M.G. (2006). Quantification of selected volatile constituents and anions in Mexican *Agave* spirits (tequila, mezcal, sotol y bacanora). *Journal of Agricultural and Food Chemistry 54*, 3911-3915.

Lopez, M.G., Mancilla-Margalli, N.A. and Mendoza-Díaz, G. (2003).Molecular structures of fructans from *Agave tequilana* Weber var. azul. *Journal of Agricultural and Food Chemistry 51*, 7835-7840.

Lopez, M.G. and Urías-Silva, J.E. (2007).*Agave* fructans as prebiotics. *Recent Advances in Fructooligosaccharides Research 37*, 1-14.

Madrigal, L. and Sangronis, E. (2007).La inulina y derivados como ingredientes claves en alimentos funcionales. *Archivos Latinoamericanos de Nutrición 57*, 387-396.

Mancilla-Margalli, N. and Lopez, M. (2006).Water-soluble carbohydrates and fructan structure patterns from *Agave* and *Dasylirion* species. *Journal of Agricultural and Food Chemistry 54*, 7832-7839.

Menne, E.,Guggenbuhl, N. and Roberfroid, M. (2000). Fn-type chicory inulin hydrolysate has a prebiotic effect in humans. *The Journal of Nutrition 130*, 1197-1199.

Meyer, D. and Stasse-Wolthuis, M. (2009). The bifidogenic effect of inulin and oligofructose and its consequences for gut health. *European Journal of Clinical Nutrition 63*, 1277-1289. Molina-Guerrero, J.A., Botello-Álvarez, J.E., Estrada-Baltazar, A., Navarrete-Bolaños, J.L., Jiménez-Islas, H., Cárdenas-Manríquez, M. and Rico-Martínez, R. (2007). Compuestos volátiles en el mezcal. *Revista Mexicana de Ingeniería Química 6*, 41-50.

Moreno-Vilet, L., Moscosa-Santillan, M., Grajales-Lagunes, A., González-Chavez, M., Bonnin-Paris, J., Bostyn, S. and Ruiz-Cabrera, M. (2013). Sugars and fructans separation by nanofiltration from model sugar solution and comparative study with natural *Agave* juice. *Separation Science and Technology 48*, 1768-1776.

NMX-F-591-SCFI-2010. Alimentos - fructanos de *Agave* - especificaciones, etiquetado y métodos de ensayo (prueba). Diario Oficial de la Federación. Disponible en: http://200.77.231.100/work/normas/nmx/2010/nmx-f-591-scfi-2010.pdfAccessed in May, 2013.

Ortiz-Basurto, R. I., Porcelly, G., Doco, T., Williams, P., Dornier, M. and Belleville, A.(2008). Analysis of the main components of the Aguamiel produced by the Maguey-Pulquero (*Agave mapisaga*) throughout the harvest period. *Journal of Agricultural and Food Chemistry 56*, 3682-3687.

Praznik, W. and Huber, A. (2005). De facto molecular weight distributions of glucans by size-exclusion chromatography combined with mass/molar-detection of fluorescence labeled terminal hemiacetals. *Journal of Chromatography B 824*, 295-307.

Prosky, L. and Hoebregs, H. (1999). Methods to determine food and oligofructose. *The Journal of Nutrition 129*, 1418S-1423S.

Raftiline®GR. Product sheet. Doc. A4-03*01/02. Tienen, Belgium.

Rao, V. A. (2001). The prebiotic properties of oligofructose at low intake levels. *Nutrition Research 21*, 843-848.

Ravenscroft, N., Cescutti, P., Haershaw, M., Ramsout, R., Rizzo, R. and Timme, E. (2009).

Structural analysis of fructans from *Agave Americana* grown in South Africa for spirit production. *Journal of Agricultural and Food Chemistry 57*, 3995-4003.

Roberfroid, M.B. (2005). Introducing inulin-type fructans. *British Journal of Nutrition 93S*, S13-S25.

Robinson, S., Bergstrom, E., Seymour, M. and Thomas-Oates, J. (2007). Screening of underivatized oligosaccharides extracted from the stems of *Triricuma estivum* using porous graphitized carbon liquid chromatography-Mass spectrometry. *Analytical Chemistry 79*, 2437-2445.

Saldaña, O.I., Ritsema, T. and Pearce, S.R. (2009). Analysis and characterization of fructan oligosaccharides and enzymatic activities in the leaves of *Agave tequilana* (Weber) var. 'Azul'. *Dynamic Biochemistry, Process Biotechnology and Molecular Biology 3SI1*, 40-51.

Téllez-Mora, P., Peraza-Luna, F.A., Feria-Velasco, A. and Andrade-González, I. (2012). Optimización del proceso de fermentación para la producción de tequila, utilizando la metodología de superficie de respuesta (MSR). *Revista Mexicana de Ingeniería Química 11*, 163-176.

Van den Heuvel, E.G., Muys, T., van Dokkum, W. and Schaafsma, G. (1999). *The American Journal of Clinical Nutrition 69*, 544-548.

Wang, N. and Nobel, P.S. (1998). Phloem transport of fructans in the crassulacean acid metabolism species *Agave deserti. Plant Physiology 116*, 709-714.

Yildiz, S. (2011). The metabolism of fructooligosaccharides and fructooligosaccharide-related compounds in plants. *Food Reviews International 27*, 16-50.

Zdunczyk, Z., Juskiewicz, J., Stanczuk, J., Jankowski, J. and Król, B. (2007). *Poultry Science 86*, 1133-1139.