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Molecular and Physicochemical Characteristics of Fructan during Technological Processing of *Agave tequilana* Weber var. Azul

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ABSTRACT

Due to the high level of fructans, up to 72% of the dry matter, agave plants (Agavaceae) may be an excellent source to produce pure fructan powders as ingredients in functional food. Reliable analyses about content, quality profile and physicochemical properties are basic requirements for the development of appropriate sequences of processing steps. Samples of agave, harvested after 5-6 years (before flowering), from different plant sections (heart, basic and middle regions of leaves) and from consecutive steps of technological processing were investigated. Content of dry matter, protein and minerals were determined by means of AOAC methods. Content and composition of carbohydrates were analyzed using enzymatic and chromatographic methods. Solubility, viscosity behaviour and stability of solution were studied at different concentrations, pH and temperatures. The structure of fructan was investigated by methylation analysis. 28–32% DM was found in the heart of trunks and in the basic region of leaves. Total amount of carbohydrate was 65–83% of DM (up to 72% of fructan). Protein content was 4.3–5.0% and mineral content was 5.6–6.3% (high level of Ca^{2+} and Mg^{2+}). Protein and mineral content was lost during processing. Solubility was approx. 80% with high storage stability. Structure analysis proved agave fructan to be a β-(2,1)-linked main chain with a branching degree of 0.22 (22 mol% of alditol derivatives), forming predominantly β-(2,6)-linkages in the side chains.

Keywords: agave, analysis, fructan, processing
Abbreviations: **AAS**, atomic absorption spectroscopy; **DM**, dry matter; **DOT**, denomination origin of tequila; **DP**, degree of polymerisation; $\mathbf{DP_n}$, number average degree of polymerisation; $\mathbf{DP_w}$, weight average degree of polymerisation; **DRI**, differential refractive index; **DVS**, dynamic vapour sorption; **GLC/FID**, gas liquid chromatography/flame ionisation detection; **GLC/MS**, gas liquid chromatography/mass spectrometer; **kD**, kilo Dalton; **MW**, molecular weight; **Mw**, weight average molecular weight; **Mn**, number average molecular weight; **SEC**, size exclusion chromatography; **SEM**, scanning electron microscopy

INTRODUCTION

Among the great variety of agave plants in Mexico (more than 200 species) *Agave tequilana* Weber var. azul, is the raw material for the production of tequila, the Mexican alcoholic beverage originally denominated, with an annual production of about 200 millions of liters (CIATEJ 2004; Colunga-García *et al.* 2007). Tequila is only allowed to be produced in 5 counties of Mexico - Guanajuato, Jalisco, Michoacan, Nayarit and Tamaulipas, the tequila DOT zone, corresponding to the plantation zones of the blue agave (Ruiz-Corral *et al.* 1999). Similar alcoholic beverages from agave tequilana and different agave species produced in other counties of Mexico are named Mezcal (Colunga-García *et al.* 2007).

As to the properties of agave extract, mainly based on fructans, a series of scientific and technical studies have been done in recent years in order to determine the chemical composition and structure of the components (Spies *et al.* 1992; Praznik und Spies 1993; Baumgartner *et al.* 2000; Pavis *et al.* 2001; Lopez *et al.* 2003; Mancilla-Margalli *et al.* 2006) as well as to find alternative products such as agave syrup (having a low glycemic index) or pure "agave inulin" (agave fructan) being well known for its prebiotic properties (Praznik *et al.* 2003).

Today, quite a few entrepreneurs produce agave syrup, but only two produce agave fructan from *A. tequilana*. It will be advantageous to have an option of different agave products, because the tequila production has achieved an upper limit. On the other hand there is a great demand for health-promoting food and beverages; Mexicans are aware of this fact and increasingly produce fructan and derivatives from *A. tequilana*.

For convenient selection of agave plants and the development of appropriate sequencing of processing steps, reliable analyses of the content, quality profile (molecular composition, mineral and protein content) and physicochemical characteristics are required.

For the time being *A. tequilana* is taken as basis for the fructan production because of extended cultivations of this agave variety in the "Tequila Counties". However, more *Agave* species in Mexico might be found being qualified for the production of fructan and agave syrup using a comprehensive screening system – meaning evaluated analytical methods for the determination of plant components and different fructan profiles (Praznik *et al.* 2007) as well as studies of the physicochemical properties.

For application of the products from *A. tequilana* in food and non food aria, we developed analytical strategies for profiling the molecular weight distributions of sugar moieties, linkage type, and mineral content and physicochemical properties of fructans before, during and after processing.

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MATERIALS AND METHODS

Technological process of fructan production

Processing done in a pilot scale in a tequila company with the cooperation of the University of Guadalajara, was as follows:

1) Harvesting, peeling, rubbing of agave pines
2) Extraction with water (temp. about 75°C); counter flow principle
3) Clarification (charcoal, diatomean earth), filtration (filter panels)
4) Cleaning: ion–exchange chromatography
5) Thickening: evaporation under vacuum
6) Spray drying of the syrup (70% DM) to a white powder of fructan

Steps 1–5 were produced in a continuous process (rubbing, extraction, clarification/filtration, treatment with ion-exchange system and concentration/evaporation) from the raw material to the thickened syrup (70% DM). Step 6, the conversion of syrup to powder, was done discontinuously using a pilot spray dryer. For analysis of the carbohydrate profile, molecular weight distribution of fructan and status of mineral content, samples from different process steps were taken and freeze- dried.

Glucose, fructose, sucrose and fructan analysis by enzymatic assay

Lyophilized samples from different process steps were solved in bidest. H_2O (10 mg/ml) and the content of free glucose, fructose and sucrose was determined by means of enzyme kit (sucrose, D-fructose and D-glucose assay kit, Megazyme International Ireland Ltd., Co. Wicklow, Ireland). Fructan content was detected as well by measuring glucose- and fructose content after enzymatic hydrolysis (endo/exo-inulinase by Megazyme) at 37°C for 3 hrs (Kocsis *et al*. 2007; Praznik *et al*. 2007).

Analysis of molecular weight distribution of fructan by SEC

Calibrated size exclusion chromatography (SEC) with low system pressure (3–7 bar) (Praznik *et al.* 2007) was applied for detailed information of molecular weight distribution of fructan in agave samples and during processing. The established SEC-system is composed of columns with different separation range of molecular weight (MW): one column - Superose 12 (290 mm × 10 mm, Amersham) - crossed agarose gel with definite particle size of 10 nm - has a MW range between 1 to 200 kD and thus favours the separation of high polymer fructan; two columns - Toyopearl HW-40S (290 mm × 10 mm, Tosoh Bioscience, D) have a MW separation range < 5 kD inducing an excellent fractionation of low molecular fructan, di- and monosaccharides. After broad standard calibration with sucrose, fructose, low molecular fructan (nystose, 1-kestose from Megazyme, Ireland; self prepared fructooligosaccharides dp5 –10) and high polymer dextran (dextran 1 and dextran 25 - standard kit, Pharmacosmos, Denmark), this system ensures a total profile analysis of fructan samples from low to high molecular weight range.

Eluent: 0.05 M NaCl; flow rate: 0.6 mL/min; injected volume: 0.3 mL of 1% (w/v) sample solution (10 mg/mL eluent); mass detection: differential refractive index (DRI).

Data acquisition and data processing was done with CODAwin32 and CPCwin32 (a.h. group, Graz, Austria).

Sample preparation for SEC-analysis

Agave samples: Fresh samples of agave (different parts) were cut and freeze dried. Milled freeze dried powder (100 mg) were extracted with 5 mL deionised water (in 10 mL screwed tubes, 2 h at 80°C), residue was separated by centrifugation (3500 rpm for 10 min) and the supernatant was freeze dried again. For SEC analysis 10 mg of this powder/1 mL eluent was solved.

Process samples: 100 mL of process liquids (step 1–4), clarified by centrifugation (3500 rpm for 30 min) and concentrated syrup (step 5) were freeze dried and stored at 4°C. For SEC analysis 10 mg freeze dried powders/mL eluent were applied.

Methylation analysis with reductive cleavage

Determination of structure and elucidation of the sugar moieties and linkage types in the processed fructan was carried out by means of methylation analysis with reductive cleavage (Spies *et al.* 1992; Praznik *et al.* 2007). The lyophilized and well dried samples of fructans were dissolved in anhydrous dimethylsulfoxide. The solution was alkalized with thoroughly dried powdered sodium hydroxide, stirred till solutions are clear and finally methylated with iodomethane; after 4-5 h iodomethane was added anew (50% of the first time) and stirred over night. To quench the reaction, water was added to the mixture (2x repeated), the derivatives were partitioned into methylene chloride or chloroform and dried over dry sodium sulfate. After removal of the drying salt the organic phase was evaporated under nitrogen.

To permethylate the carbohydrates the reaction had to be carried out under *absolutely anhydrous conditions*, which means that samples, reagents and solvents need to be dried very well in advance.

Reductive cleavage: Dry samples were dissolved in a small amount of methylene chloride dried over molecular sieve, then trimethylsilane (reductive mean) and trimethylsilyltrifluor-methanesulphonate (catalyst) were added, stirred over night, followed by the addition of acetic acid anhydride and stirred for about 2 h. The reaction mixture was neutralized with saturated solution of sodium bicarbonate (with vigourous stirring to remove all CO_2); 1 ml chloroform was added and washed 2 times with water. After addition of 1 ml of methylene chlorid the aqueous phase was totally drawn away and the organic phase dried over dry sodium sulfate. After removal of the drying salt the organic phase had to be evaporated under nitrogen.

The dry methylated alditols were redissolved in methylene chloride and determined by GLC/MS and GLC/FID on GCMS - QP2010 Plus, Shimadzu, Japan.

Fused silica capillary column: DB-1701, 30 m × 0.25 mm I.D., 0.25 µm film thickness (122-0732 , J&W, Agilent, Austria); Temperature program: 80-140°C, 10°/min; 140–250°C, 4°/min; Carrier gas: Helium (pressure 1–1.2 bar); Temperature of injection: 230°C; Temperature of detection: 300°C.

Identification of the GC peaks and quantitative acquisition was done in comparison to fructan standards (fructooligomers of inulin series, high polymer inulin from chicory (*Cichorium intybus*) and Jerusalem artichoke (*Helianthus tuberosus*), fructan from garlic (*Allium sativum*) and sinistrin from Red squill (*Urginea maritima*) using MS detection with data analysis (Spies *et al.* 1992). Additional identification and quantification of the GC peaks were delivered from separate runs with FID-detection applying the carbon response method (Sweet *et al.* 1975). From these results the percentage of molar concentration of the obtained different alditol derivatives was calculated and used for the detection of structure.

Determination of mineral content

Mineral content was measured in "The Małapolska Centre of Food Monitoring and Certification", Krakow, Poland by means of atomic absorption spectroscopy (AAS) with standard methods according AOAC (AOAC 1997).

The content of Ca/Mg of processed samples were determined by titration with EDTA-solution (0.002 M/L), using Erio-T as indicator.

Measurement of physicochemical properties

Physicochemical characteristics of agave fructan powder were determined at the Faculty of Mathematics and Natural Sciences, Rheinische Friedrich-Wilhelms-Universität, Bonn, Germany (Nguyen 2008).

Table 1 Content of aqueous soluble carbohydrates and average DP_n of fructan in freeze dried process samples determined by means of enzymatic analysis.

Fructan of process steps	Fructose[a]	Glucose[a]	Sucrose[a]	Fructan[a]	DP_n
step 3	2.40 ± 0.08	0.40 ± 0.05	1.85 ± 0.07	90.90 ± 0.15	14.8 ± 0.3
step 4	2.31 ± 0.06	0.41 ± 0.04	1.58 ± 0.06	92.60 ± 0.25	15.0 ± 0.4
step 5	2.42 ± 0.08	0.40 ± 0.04	1.65 ± 0.08	92.75 ± 0.35	14.9 ± 0.4

[a] g/100 g

RESULTS AND DISCUSSION

Carbohydrate profile in the samples of continues process

Table 1 shows the content of fructose, glucose, sucrose and fructan in the freeze-dried processed samples. In step 3 – after extraction, clarification and filtration (light brown clear syrup with 18% DM) – low concentrations of glucose, fructose and sucrose were found by means of enzymatic analysis compared to the high content of fructan (> 90%). In steps 4 and 5 (removal of mineral salts by ion exchange chromatography and concentration of syrup by evaporation) no significant changes in the profile of carbohydrates could be detected confirming an acceptable stability of fructan during the applied process. Additionally, the number average of degree of polymerization (DP_n) of fructan was calculated by enzymatic analysis assuming that each molecule of fructan contains one glucose unit. The DP_n of fructans in the investigated process steps 3, 4 and 5 was found to be rather similar ranging 15 supporting the assumption that the processed fructan contains high molecular weight components in the fructan profile.

Molecular weight distribution of processed fructan

From the obtained distribution profiles mean molar mass values (number average molar mass Mn, weight average molar mass Mw) and an estimation of the polydispersity as the ratio of Mw/Mn may be calculated easily (Praznik *et al.* 2007). Similar results can be obtained by enzymatic analysis (see above). However, the advantage of the obtained fructan profile with the use of a DRI (mass detector) for the data acquisition is the negligible response correction between low and high polymer components and therefore easy handling of data for the molecular weight analysis. Concerning the mass profiles for the average values is to recognize that at equal mass fractions much more molecules (molar concentration) exist in the low molecular range than in the high molecular range and - consequently - the Mw (DP_w), Mn (DP_n) and their quotient Mw/Mn (DP_w/DP_n) (polydispersity index) differ according to the inhomogeneity of fructan samples.

Fig. 1 and **Fig. 2** show the elution profile and the molecular weight distribution of fructan at the beginning (step 1) and end (step 5) of continuous process and it is to see that no essential change had occurred in the profile. These figures generally show that the process hardly affected the fructan quality meaning that the distribution of degree of polymerisation was not changed. From this result the composition of the produced fructan is calculated to 20% of FOS (DP3 - DP12), 57% of high polymers in a range of DP20 - DP70 and 20% of polymers with intermediate DP; the remaining 3% can be explained by the content of mono- and disaccharides (glucose + fructose 2–3%, sucrose 2%). At step 1, there is a small peak observable in the range of FOS between DP 8 and DP12 that disappeared at the end of processing – a complex interaction of fructan with calcium ions as proofed by colouring with calconcarboxylic acid (red staining).

For further information about fructan from *A. tequilana* cultivated at different places in Jalisco additional profiles were established by SEC. Small differences in the polymer distribution of the plants could be detected indicating dependence on soil composition, frequency of irrigation and differences in climate (**Fig. 3, Table 2**).

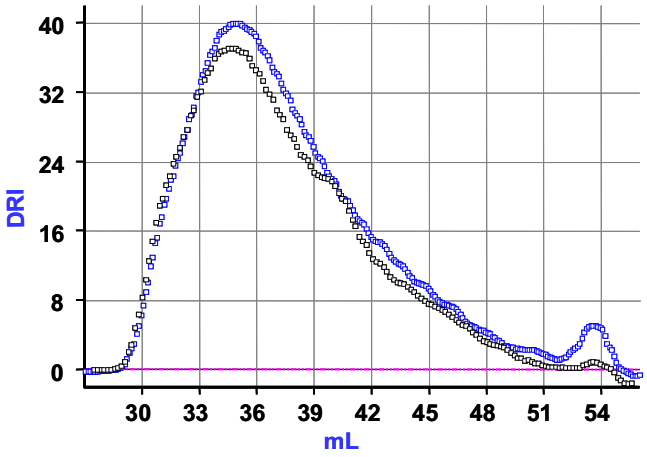

Fig. 1 Elution profile of processed fructan by means of SEC analysis (detail see in material and methods). Black – process step 1, after extraction from agave trunks and first clarification; blue – process step 5, high concentrated syrup after filtration, ionexchange cleaning and evaporation.

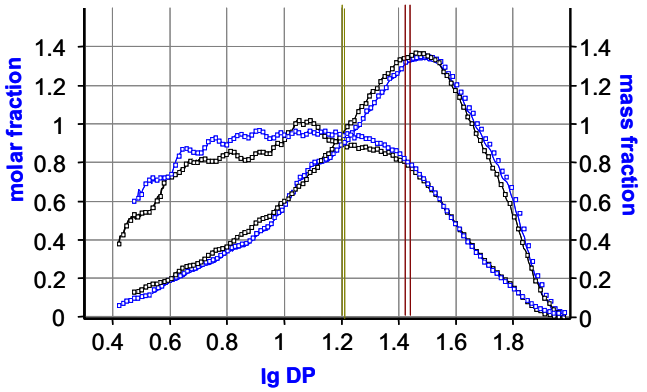

Fig. 2 Molar and mass fractions of log DP from calibrated elution profile (see Fig. 1), calculated with CPCwin32 (a.h. group, Graz/ Austria). Black – process step 1, blue – process step 5; average values for both profiles: $DP_w = 28$, $DP_n = 16$, $DP_w/DP_n = 1{,}8$.

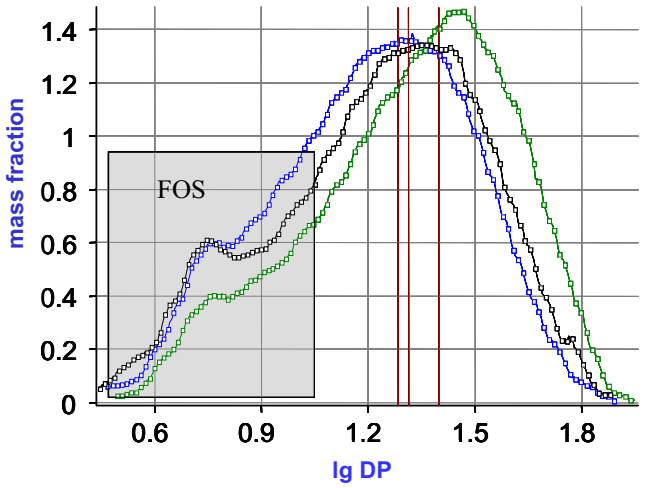

Fig. 3 Mass fraction profiles of agaves from different places of cultivation. A1 (blue), A2 (green), A3 (black); average values of molecular characteristics see **Table 2**.

Table 2 Molecular characteristics of agave plants from different places of cultivation.

Fructan of agave	DP_w	DP_n	DP_w/DP_n	FOS[a] (DP 3-12)
Agave 1	20	13	1.53	29%
Agave 2	25	16	1.56	19%
Agave 3	21	14	1.50	26%

[a] fructooligosaccharides

Table 3 Mineral content in different parts of agave plants.

	Ca[a]	Mg[a]	K[a]	Na[a]
heart	16241	1902	2346	57
	(1.62)	(0.19)	(0.235)	(0.057)
leaf-middle region	11145	2187	2481	48
	(1.115)	(0.219)	(0.248)	(0.048)
leaf - basic region	18124	4500	10220	65
	(1.812)	(0.45)	(1.02)	(0.065)

[a] mg/kg DM (%)

Table 4 Calcium/magnesium content in the different process steps

Process	g Ca/Mg per kg DM	% of DM
step 1	6.2123	0.62
step 2	6.5814	0.66
step 3	6.8217	0.68
step 4	0.00	_
step 5	0.00	_

Content of minerals in native agave plant and processed samples

Mineral content was always determined from the freeze dried sample. **Table 3** presents the results of AAS of the different parts of agave; compared to sodium the contents of calcium and magnesium, even potassium were rather high in the heart, the basic region of leaves and the middle region of leaves and it attracts attention that – in each case – the content of minerals in the basic region of leaves is the highest.

To control the mineral salts during the process calcium/magnesium was taken as indicator. The reason for calcium/magnesium as leading minerals is their high content in agave plants compared to other minerals and their easy determination by titration with EDTA. The result is shown in **Table 4**: steps 1, 2 and 3 of processing show an average content of 0.65% calcium whereas – in accordance with step 4 (removing of mineral salts by ion-exchange chromatography, syrup was light yellow and clear) – absolutely no calcium (as well as no other minerals) could be found in the investigated sample.

These results as well as carbohydrate analysis confirm that the processed fructan is free of mineral salts and contains only carbohydrate components. The spray dried powder is composed of 93% fructan, 2% monosaccharides (glucose + fructose), 2% sucrose and 3% water.

Structure of processed fructan

The results of the methylation analysis of a high polymer fructan fraction (isolated from processed fructan) are shown in **Table 5**. This agave fructan shows – besides terminal, linked and branched fructose units – glucose units predominantly linked, allowing the assumption of a mixed type fructan structure.

Seven terminal fructose residues and five to six branching points (branched fructose units) in the molecule confirm the branching structure as well (every branching point demands a further terminal fructose unit). Additionally seven β-(2,1)-linked and five β-(2,6)-linked fructose units were found. The amount of terminal, linked and branched fructose residues compared to the amount and position of glucose residues allows to calculate a mean DP of 23.9 and to design a favourite structure with β-(2,1)-linked fructose in the main chain, a branching degree of 0.22 (22 mol% of alditol derivatives) and β-(2,6)-linked fructose in the side chains of branching. It can be concluded generally that *A. tequilana* induces predominantly molecules of mixed type fructan with highly branched structure in their biosynthetic process.

Table 5 Calculated structure and DP of agave fructan after methylation analysis

Linkage type	% of alditol derivatives[a]	units/molecule
gluc terminal	0.62 ± 0.04	0.2
gluc 1-6 linked	3.53 ± 0.16	0.8
fruc 2-1, 2-6:		
fruc 2-1 linked	29.3 ± 0.90	7.0
fruc 2-6 linked	19.0 ± 1.5	4.6
fruc terminal	25.8 ± 1.3	6.8
fru branched	21.9 ± 1.6	5.3
DP	23.9	
degree of branching	0.22	

[a] three replications

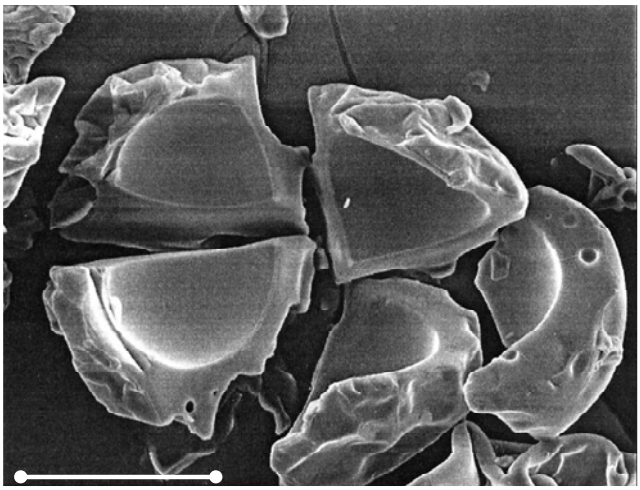

Fig. 4 SEM picture from spray dried agave fructan. Structure of particles with hollow spheres; bar scale: 60 µm.

Physicochemical properties of processed fructan

The analysis by X-ray diffraction reports that the product has an amorphous structure, whereas measurement of adsorption and desorption of water vapor by dynamic vapor sorption (DVS) attests the high hygroscopicity of the spray dried powder. Scanning electron microscopy (SEM) of the processed fructan proves the shape of spray dried particles revealing hollow spheres (**Fig. 4**); thus the surface of processed fructan is extended and may explain its high hygroscopicity.

CONCLUSION

Conclusively, it can be said that besides the production of tequila and because of regressive marketing of tequila the interest for fructan produced from the 'blue Agave' is increasing steadily. Accordingly, more and more companies may start fructan production in the near future in Mexico.

Results of molecular weight (degree of polymerization) distribution of fructan as well as the control of calcium content in processed samples allow to conclude that the produced fructan – purified and spray dried to the end product (step 6) – possesses a profile similar to fructan directly extracted from raw plant material except the content of minerals. Furthermore the unaltered molecular weight distribution of fructan in the different process steps is a striking demonstration that there occurs no breakdown of the fructan molecules during the applied process. Methylation analysis delivered a mean DP 24 for the fructan molecules in accordance with the DP, calculated from SEC–measurements (DP 25). The resulting fructan is a white amorphous powder with excellent aqueous solubility. The degree of

branching of 0.2 and the β-(2,1)/β-(2,6) linked structure of molecules (branched molecules) cause the excellent aqueous solubility of this fructan.

The obtained spray dried product consists of unregular spheres (SEM) with hollow space making it applicable as matrix for pharmaceutical drugs. Additionally high ability of water absorption due to the porous structure qualifies the fructan powder to be used in the compression of pills. However, the high hygroscopicity of the product requires specific packaging material for storage stability on one side and particular handling in application on the other side.

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