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Extraction optimization and inulin hydrolysis

MASTER DISSERTATION

Onofre Agapito da Silva Figueira
MASTER IN APPLIED BIOCHEMISTRY

UNIVERSIDADE da MADEIRA
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SUPERVISOR
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Esta dissertação foi desenvolvida no grupo de Produtos Naturais do Centro de Química da Madeira (CQM), sob a orientação da Professora Doutora Paula Cristina Machado Ferreira Castilho. Foi apresentada à Universidade da Madeira, para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Bioquímica Aplicada.

Onofre Figueira

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“Nothing in life is to be feared, it is only to be understood. Now is the time to understand more, so that we may fear less.”

Marie Curie

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Abstract

Fructo-oligosaccharides (FOS) and inulin are prebiotic fructose-based saccharides. They are structurally similar differing mainly in their molecular length: while FOS are short-chain linear molecules, inulin is a polysaccharide, with chains up to 60 or more fructose units. In the industry, FOS are synthetically produced through transfructosylation reactions. However, these reactions require difficult conditions to maintain the transfructosylation activity without hydrolytic activity. The use of natural matrices, such as yacon (*Smallanthus sonchifolius*) roots, is proposed for the extraction of FOS and inulin to overcome the disadvantages of synthetic production. This can be achieved through the extraction and purification of endogenous FOS, and by their production through the hydrolysis of inulin using endo-inulinase. In the present work, three different yacon extracts were prepared and analyzed by HPLC-RI, TLC and MALDI-TOF. The concentration of FOS obtained ranged from 57.1 mg/g to 150.9 mg/g. The DP of these FOS were determined to be over 7 by TLC. Inulin was precipitated using anti-solvents and analyzed by MALDI-TOF revealing inulin with maximum DP up to 20. Several purification methods were tested on an extract containing 74.5 mg/g of saccharides using *Saccharomyces cerevisiae*, activated charcoal and a combination of activated charcoal and Celite®. The best results were observed with activated charcoal with the recovery of 31.5% of inulin and 21.3% of FOS. Endo-inulinase was successfully immobilized in calcium alginate beads, with 97% immobilization yield, and used to hydrolyze the inulin in the yacon extract. The method proved effective, with total consumption of inulin and the consequent production of FOS, with no enzymatic leakage detected by the Bradford method. A TLC-densitometry method for the quantification of FOS in the hydrolysis was performed and proved to be a good, cheap and reliable method to follow the hydrolysis process, before advancing to more expensive techniques.

Keywords: FOS, inulin, yacon, endo-inulinase, TLC-densitometry.

Resumo

Os fructo-oligosacáridos (FOS) e a inulina são sacarídeos prebióticos à base de frutose, estruturalmente muito semelhantes diferindo, essencialmente, no seu comprimento molecular: os FOS são moléculas lineares e de cadeia curta, enquanto a inulina é um polissacárido, com cadeias longas contendo 60 ou mais unidades de frutose. Na indústria, os FOS são sinteticamente produzidos por enzimas através de reações de transfructosilação. No entanto, estas reações podem tornar-se dispendiosas uma vez que requerem condições especificas para a manutenção da atividade de transfructosilação sem que ocorra atividade hidrolítica. A utilização de matrizes naturais, como por exemplo o extrato de raízes de yacon (*Smallanthus sonchifolius*), é proposto como meio de obtenção de FOS e inulina, de modo a superar as desvantagens associadas à produção sintética. Tal poderá ser conseguido através da extração dos FOS, da sua purificação e produção através da hidrólise da inulina por intermédio da ação da enzima endo-inulinase. Neste trabalho, três extratos diferentes foram preparados e analisados por HPLC-RI, TLC e MALDI-TOF. Nesses extratos, as concentrações de FOS obtidas variaram de 57,1 mg/g a 150,9 mg/g. Analisando as TLC, observou-se graus de polimerização superiores a 7, enquanto as análises por MALDI-TOF, realizada para a inulina precipitada com anti-solventes, revelaram um grau de polimerização até 20. Foram ensaiados 3 métodos de purificação de um extrato contendo 74,5 mg/g de FOS e inulina usando *Saccharomyces cerevisiae*, carvão ativado e uma combinação de carvão ativado e Celite®. Os melhores resultados foram observados para o carvão ativado alcançando a recuperação de 31,5% de inulina e 21,3% de FOS. A enzima endo-inulinase foi imobilizada com sucesso em esferas de alginato de cálcio, com 97% de rendimento, sendo posteriormente utilizada para hidrolisar a inulina no extrato de yacon. O método revelou-se eficaz com o consumo de inulina a ser verificado, assim como a consequente produção de FOS, sem fugas de enzima detetáveis pelo método de Bradford. Foi ainda testado um método de quantificação de FOS na hidrólise por densitometria por TLC, que provou ser um método eficaz, barato e fiável para o acompanhamento da hidrólise, antes de avançar para a análise em técnicas mais caras.

Palavras-chave: FOS, inulina, yacon, endo-inulinase, densitometria por TLC.

List of Communications

List of Oral Communications

Costa M.C., Andrade J.M., Delsin S.D., Tavares B., Silva H., Rijo P., de Icaza Y., Rosado C., **Figueira O.**, Rodrigues L.M., (**2018**) New function discoveries for traditional probiotics in gut-skin axis; FISSIN - International Exhibition of Health, Dietary Supplements, Functional Ingredients and Nutraceuticals, LXFactory, Lisbon; 10th &11th November.

Figueira O., Martins G. N., **Castilho P. C.**, (**2019**) Extraction, purification and quantification of fructooligosaccharides in Yacon (*Smallanthus sonchifolius*) roots and syrup; 6th CQM Annual Meeting; Porto Moniz, Madeira; 26th & 27th April. ISBN: 978-989-54090-2-0.

Figueira O., **Castilho P. C.**, (**2020**) An analytical tool for inulin hydrolysis - can I use my smartphone in the lab?; 7th CQM Annual Meeting; Funchal, Madeira; 21st & 22nd September. ISBN: 978-989-54090-4-4.

List of Posters

Figueira O., Martins G. N., **Castilho P. C.**, (**2019**) Plant-based FOS and GOS: So easy, yet so difficult; XXVI Encontro Nacional da SPQ – XXVIENSPQ; 24th-26th July. ISBN: 978-989-8124-28-9, Porto, Portugal.

Figueira O., Martins G. N., A. Gomez-Zavaglia, **Castilho P. C.**, (**2019**) Fructo- and galacto-oligosaccharides purification: the hard step; 2nd Food Chemistry Conference; 17th-19th September, Seville, Spain.

List of Publications

Urreta M., Martins G. N., **Figueira O**., Pires P., **Castilho P. C.**, A. Gomez-Zavaglia, (**2020**) Recent advances in β-galactosidase and fructosyltransferase immobilization technology; Critical Reviews in Food Science and Nutrition. 1-32. DOI: https://doi.org/10.1080/10408398.2020.1783639.

Submitted (2020):

Martins G. N., **Figueira O.** & **Castilho P. C.**, Immobilization of β-galactosidase in calcium alginate beads. Basic Protocols in Encapsulation. Methods and Protocols in Food Sciences, Andrea Gómez-Zavaglia (editor), Springer Verlag. (*Accepted for publication*).

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Abbreviations

°Brix	Brix Degree
1-FFT	Fructan:fructan 1-fructosyltransferase
1-SST	Sucrose:sucrose 1-fructosyltransferase
6-SFT	Sucrose:fructan 6-fructosyltransferase
6G-FFT	Fructan:fructan 6G-fructosyltransferase
ACN	Acetonitrile
BSA	Bovine Serum Albumin
DHB	2,5-Dihydroxybenzoic acid
DP	Degree of Polymerization
DNS	3,5-Dinitrosalicylic Acid
Ethanol	EtOH
FEH	Fructan exohydrolase
FFase	Fructofuranosidase
FOS	Fructo-oligosaccharides
FTases	Fructosyltransferases
Fr	Fructose
Gal	Galactose
Glu	Glucose
GOS	Galacto-oligosaccharides
HMO	Human Milk Oligosaccharides
HPLC	High Performance Liquid Chromatography
HPLC-RI	High Performance Liquid Chromatography with Refractive Index
INU	Inulin
LOD	Limit of Detection
LOQ	Limit of Quantification
MALDI-TOF	Matrix-Assisted Laser Desorption/Ionization – Time of Flight
OS	Oligosaccharides
Rf	Retention Factor
RI	Refractive Index
RSD	Relative Standard Deviation
scFOS	Short Chain Fructo-oligosaccharides
T_g	Glass Transition Temperature
TLC	Thin Layer Chromatography
Xy	Xylose

I. Introduction

1. Functional Foods

The occurrence of several health disorders, such as diabetes, obesity and vascular diseases has increased the interest in health improvement through functional diets free of synthetic additives and/or modified substances. Therefore, an increase for the search and consumption of nutraceuticals or functional foods has been verified in the market. This reality has attracted the food industry to the development of new food products having health benefits as well as nutrition value [1–3]. According to Grand View Research, by 2019, the global functional foods market was expected to reach around 275.77 billion USD by 2025 (around 232.44 billion €) and expand at a CAGR (Compound Annual Growth Rate) of 7.9% during the 2014-2025 period [4]. The market of the functional foods started its development in a more contextualized manner in Japan in 1984, where new studies were establishing relations between nutrition, satisfaction and reinforcement of the physiological system. However, it was only in 1991 that Japan, the first country to highlight the importance of functional foods, established the term FOSHU - Foods for Special Health Use. By 2012, there were around 990 different products divided in 13 categories approved in Japan as functional foods. Following the Japanese interest, many European countries such as Germany, France and United Kingdom, and the United States started focusing some attention towards functional foods, consequently extending the global demand. Over the last three decades, foods with additives from natural sources have been formulated with specific goals towards beneficial health effects to the consumers. However, the regulation for these functional foods is not well-established varying from country to country. In Europe, for example, the legislation does not contemplate functional foods as a specific food category considering them only as a concept [2,5–7]. Functional food ingredients can be classified accordingly to their benefits or properties and divided into different groups, namely dietary fiber, prebiotics – mainly oligosaccharides, sugar alcohols, amino acids, peptides and proteins, vitamins, and probiotics, such as lactic acid bacteria [8].

1.1. Probiotics

Probiotics are nondigestible food ingredients that are essentially lactic acid producing bacteria considered to have a beneficial effect on the intestinal flora, for example the species from the genus *Lactobacillus* and *Bifidobacterium.* A probiotic is defined as a live microbial, a dietary supplement and/or ingredient that is beneficial to the health of the host through its effects in the intestinal tract. This way, a probiotic microorganism should survive the adverse conditions of the stomach and colonize the intestine, even temporarily, through adhesion to the intestinal epithelium [9,10].

1.2. Prebiotics

Prebiotics are also nondigestible food ingredients, substances that promote the good function and activity of a limited number of bacteria in the colon. They are generally oligosaccharides, which naturally resist digestion in the upper part of the gastrointestinal tract and exhibit a positive effect on the flora in the colon. Therefore, they impart a series of health benefits such as their calorie-free nature, the capability to act as sweeteners, their non-carcinogenic nature and the stimulation of the growth of *Lactobacillus* and *Bifidobacterium* in the colon. There are many studies about prebiotic foods and their ability to prevent colon cancer, and to reduce and regulate cholesterol, phospholipids and triglycerides levels in the serum [11,12]. Other nutraceutical effects resulting from the consumption of oligosaccharides with prebiotic activity have also been reported, such as a secondary role in obesity regulation through the increment of satiety and reduced hunger, increment in the expression or changes in the composition of short-chain fatty acids, increased expression of the binding proteins or active carriers related to mineral absorption (calcium and magnesium in particular), immune system regulation, prevention of gastroenteritis, reduction of Inflammatory Bowel Disease, reduction of cancer risk through the decrease of mutagenic or carcinogenic substances, thermal protection and cellular preservation [7,13].

1.2.1. Prebiotics in Cell Preservation

Bearing in mind the benefits of certain bacteria, such as lactic acid bacteria, and their role as probiotics, it is crucial to make efforts towards the extension of its shelf-life with adequate preservation processes. The implemented methods of preservation must prevent or minimize critical losses on cell viability and functionality. Freeze-drying and spray-drying are among the processes most widely used for preservation of these functional microorganisms [14,15].

Sugars have been used as protective agents during drying processes. Oligosaccharides are more effective than monosaccharides or disaccharides, which provide weak stabilization and consequent damages to the materials to preserve, and the presence of Maillard reaction products cause consequent repercussions of deterioration in the cell products when in storage [16].

Some hypothesis have been established trying to explain the protective role of sugars. One is the replacement of water molecules by sugars during the dehydration processes allowing the maintenance of the main biological structures in dehydrated conditions. Another hypothesis is based on the glass transition temperature (T_g) of sugars, their capability to form glass-like matrices with high viscosity and low molecular mobility, impairing some molecular interactions. Small polymers, with prebiotic activity, such as oligosaccharides, due to their higher T_g values, can increase the glass transition temperature of solutions. Blanch *et al.* (2012), reported some T_g values for some of the most common sugars found in strawberry, with fructose presenting 12.6 °C, glucose 35.5 °C, sucrose 68.3 °C. Fructo-oligosaccharides 1-kestose, nystose and

kestopentaose show T_g values of 91.2 °C, 102.2 °C and 120.8 °C, respectively [14,17]. Fructo-oligosaccharides with higher T_g values support the existence of a relationship between the structure of the sugar and the capability to modify the behavior of water at the required temperatures for cryo-, spray- and freeze-drying processes concerning cell preservation. Structure and properties of different types of sugars will be discussed in the next sections.

2. Carbohydrates

Carbohydrates are an important class of biomolecules. They are macronutrients which name derives from their composition (carbon, hydrogen and oxygen) with a general formula of $(CH_2O)_n$. They can be defined as polyhydroxyl-aldehydes or polyhydroxyl-ketones, which means they have an aldehyde or ketone in their composition, as well as several alcoholic functions. Other substances that, when hydrolyzed, originate these polyhydroxyl-aldehydes and polyhydroxyl-ketones are also considered carbohydrates. These molecules are synthesized through photosynthesis by plants, with CO_2 and H_2O as starting molecules and energy captured from a light source. In the human system, they are mainly used as cell fuel, being the main source of energy and representing between 50% and 60% of the total calorie intake needed for each individual. They can also be found widely distributed in the organism, in different molecular structures with diverse structural and functional importance, such as nucleic acids, proteoglycans, peptidoglycans and glycoproteins. According to their complexity, based on the number of units (degree of polymerization), carbohydrates can be classified into simple sugars, monosaccharides and disaccharides, and into complex sugars, oligosaccharides and polysaccharides [18,19].

2.1. Monosaccharides and Disaccharides

Monosaccharides are the simplest carbohydrates. Characterized by their high solubility in water and sweet taste, they are named with the suffix “ose”. Monosaccharides are classified according to three different characteristics: location of the carbonyl group, number of carbon atoms and chiral property. Chemically, they are aldehydes or ketones with two or more hydroxyl groups, hence monosaccharides can either be called an aldose or a ketose, respectively. In terms of classification by the number of carbon atoms, monosaccharides containing three, four, five and six carbons are referred to as trioses, tetraoses, pentoses and hexoses. Monosaccharides with more than 3 carbon chains have chirality, which implies the existence of nonsuperimposable mirror images known as enantiomers. Concerning their stereochemical classification, monosaccharides’ enantiomers are distinguished by the presence of symbols (D - dextrorotatory and L - levorotatory) in their names indicative of their rotational effect on the polarized light. D or L rotation is determined by the chiral carbon farthest from the functional group. When in their cyclic forms, monosaccharides are called furanoses (5 member rings) or pyranoses (6 member rings) and, chemically, these cyclic forms are hemiacetals [18–20].

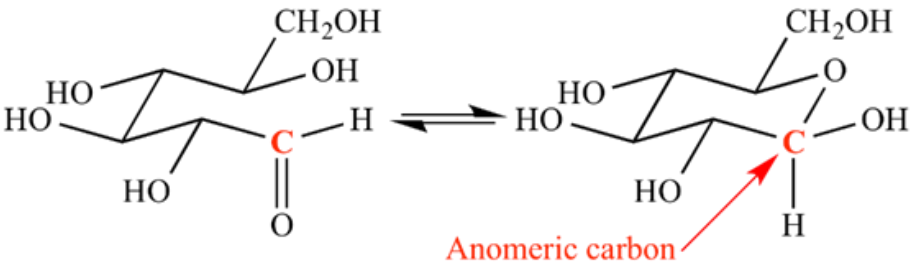

Figure 1 – Formation of the hemiacetal group [21].

Cyclization can lead to alpha (α) or beta (β) configurations, depending on the relative position of the OH groups in carbons 1 and 2: a trans position leads to β configuration, cis position leads to α configuration, as represented in the following **Figure 2**:

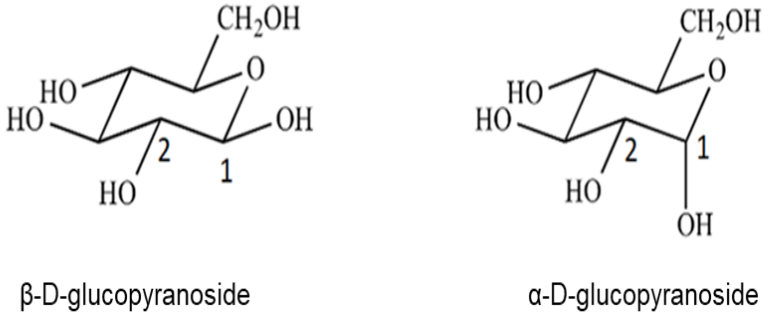

Figure 2 – Anomers of glucose formed in the cyclization process leading to α and β configurations of D-glucopyranoside, depending on the relative position of the OH group [22].

Disaccharides are a class of carbohydrates composed of two monosaccharide units linked by a glycosidic bond. Different glycosidic bonds can be found linking the two monosaccharides with the O-glycosidic bonds being the most common. In terms of its configuration relative to the anomeric carbon (**Figure 1**), the glycosidic bonds can use either an α- or β- anomeric OH. The most common disaccharides as shown in **Figure 3**, are sucrose/saccharose (β-D-fructofuranosyl-(2→1)-α-D-glucopyranoside), lactose (O-β-D-galactopyranosyl-(1→4)-α-D-glucopyranose) and maltose (O-α-D-glucopyranosyl-(1→4)-α-D-glucopyranose) [18,19].

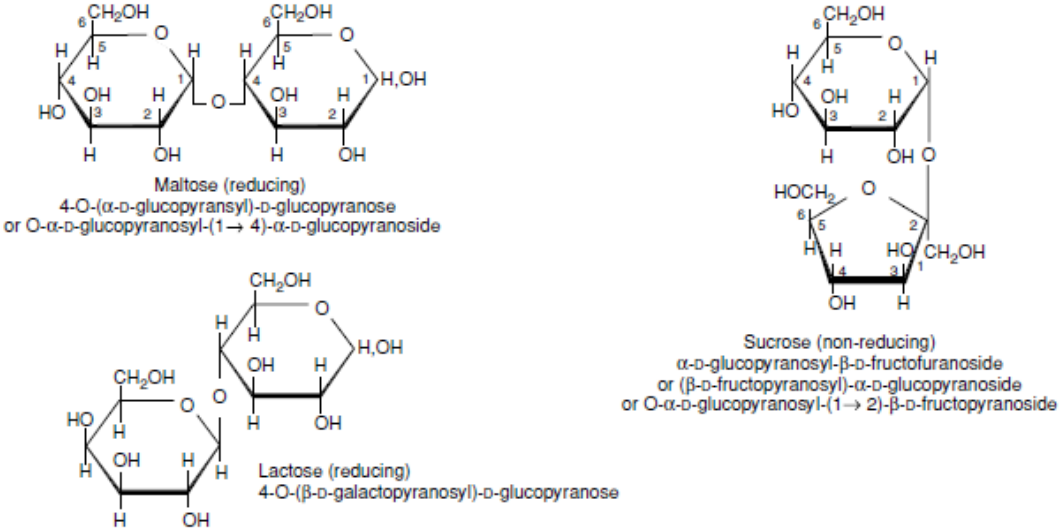

Figure 3 – Structure and glycosidic bonds of 3 of the most common disaccharides [23].

2.1.1. Reducing and Non-reducing Sugars

Monosaccharides (pentoses and hexoses) when in aqueous solutions, exist in a dynamic equilibrium between their open and cyclical forms. This interconversion between forms occurs constantly and at a high rate, affecting some properties like sweetness, crystallization, and the rate of some reactions. When monosaccharides are in their open form, and their carbonyl group – aldehyde in aldoses and keto in ketoses - becomes available for redox reactions, they are called reducing sugars. All monosaccharides such as glucose, fructose, galactose show these reducing properties as well as some disaccharides with lactose and maltose as examples. Non-reducing sugars are rarer: they have two acetal functions and no hemiacetal. The formation of disaccharides by condensation can use the -OH group of the hemiacetal of one monosaccharide unit and any -OH group of the second unit leading to an acetal and leaving one hemiacetal group that can still equilibrate with the open form of aldehyde or ketone being prone to oxidation, thus being a reducing sugar. If the condensation uses the -OH groups of the hemiacetals of both units, there will be a double acetal and the disaccharide will be non-reducing, such as happens with sucrose [19,24].

2.2. Oligosaccharides

The oligosaccharides are a class of carbohydrates that contain 3-10 moieties in their structure, and these can be linked through two types of glycoside bonds: O-glycosidic or N-glycosidic. As a result of their constitution, the oligosaccharides are low molecular weight sugars [25,26].

Oligosaccharides (OS) can be digestible or non-digestible depending on the anomeric carbon atom (C1 or C2) of their monosaccharides. Some OS in our diet have a configuration on the anomeric carbon (**Figure 1**) that leads to a glycosidic bond not susceptible to hydrolysis by

digestive enzymes in the upper digestive tract of humans and other mammals. These non-digestible saccharides are recognized as prebiotics, since they are fermented by beneficial bacteria in the colon of the host. **Table 1** shows some of the most common OS with prebiotic activity, from a list of 13 classes presented by Sako, Matsumoto and Tanaka (1999) [27] and they can be composed of one, two or three different types of monosaccharides such as fructose (Fr), glucose (Glu), galactose (Gal) and xylose (Xy). Besides the difference in the monomeric sugars, they can also differ in type of linkage, chain length and degree of branching. Among these, the most popular as prebiotic OS and as functional foods are the Fructo-oligosaccharides (FOS) and Galacto-oligosaccharides (GOS) [25,27–29].

Table 1 – Different classes of oligosaccharides according to their structure and composition [6].

Name	Building Blocks
Lactulose	Gal-Fr
Lactosucrose	Gal-Glu-Fr
Raffinose	Gal-Glu-Fr
Galacto-oligosaccharides	$(Gal)_n$-Glu
Fructo-oligosaccharides	$(Fr)_n$-Glu
Soybean oligosaccharides	$(Gal)_n$-Glu-Fr
Isomalto-oligosaccharides	$(Glu)_n$
Xylo-oligosaccharides	$(Xy)_n$
Malto-oligosaccharides	$(Glu)_n$
Cyclodextrins	$(Glu)_n$

2.2.1. Prebiotic Oligosaccharides

The majority of the low molecular weight OS are nondigestible, with resistance to the upper digestive tract enzymes and being fermented only in the colon, thus recognized as prebiotics. These functional saccharides include lactulose, lactosucrose, raffinose, FOS, GOS, soybean oligosaccharides, isomalto-oligosaccharides, gluco-oligosaccharides, xylo-oligosaccharides, gentio-oligosaccharides, arabinoxylan oligosaccharides, mannan oligosaccharides, pectic or pectin-derived oligosaccharides, chito-oligosaccharides, agaro-oligosaccharides, human milk oligosaccharides (HMO), xanthan-derived oligosaccharides, alginate-derived oligosaccharides and cyclodextrins [30].

2.2.1.1. Galacto-oligosaccharides

GOS and fructans (inulin and FOS) are the most common and most used prebiotic OS. The most commonly GOS are composed of 2-9 galactose units, and a terminal glucose unit, linked by β-glycosidic bonds, mostly β-(1→4) and β-(1→6), although β-(1→2) and β-(1→3) can also

be found. These are lactose derived and can be chemically produced by glycosylation or through biocatalysis and having the denomination of β-GOS or *trans*-GOS. The production of GOS can either occur with galactosyltransferases or β-galactosidases, with the first presenting higher selectivity and GOS yield but less advantages for commercial use. β-galactosidase allows the production of GOS either by synthesis or by transgalactosylation of pure lactose solutions. Another type of GOS, the α-GOS, can be naturally found in animal milk and some plants. These α-GOS have α-(1→6) linkages and are generally included in the groups of raffinose family OS and soybean OS [31–33].

2.2.1.2. Human Milk Oligosaccharides

More complex than the other OS, human milk oligosaccharides (HMOs) can contain in their structure up to five monosaccharides as building blocks, which can be glucose, galactose, N-acetylglucosamine, fucose and sialic acid. These blocks can form different OS with different structures in a number that surpasses the 150 HMO. All HMOs have lactose at the reducing end of the molecule and the chain can be elongated by some disaccharides, such as lacto-N-biose or N-acetyllactoseamine. Also, the linkages in the molecules can be modified either by sialylation with sialic acid in α-(2→3) and/or α-(2→6) linkages or by fucosylation with fucose in α-(1→2), α-(1→3) and/or α-(1→4) linkages, hence the variety of structures. Each HMO structure determines its function. In breast milk, around 35% to 50% of HMOs are fucosylated, 12% to 14% are sialylated and 42% to 55% are neutral nonfucosylated. Despite FOS and GOS being used in infant formulas to substitute HMOs, their structures are different [34,35].

2.2.1.3. Other Type of Oligosaccharides

Xylo-oligosaccharides are composed by xylose units linked by β-(1→4) glycosidic bonds. The number of xylose units can range between 2 and 10, but the majority of the xylo-oligosaccharides are the disaccharide xylobiose, the trisaccharide xylotriose and the tetrasaccharide xylo-tetraose. These saccharides are commonly found in some fruits and vegetables, honey and milk. They can also be obtained by enzymatic processes with the enzyme xylanase, with xylan as substrate. Their uses are diversified and they can be found in different food products, in pharmaceutical products and as dietary supplement products due to their prebiotic properties [36,37].

Malto-oligosaccharides are digestible OS composed by glucose units linked by α-(1→4) glycosidic bonds and can be composed by 2 up to 6 glucose units. These saccharides are produced by multiple enzymatic processes that start with the liquefaction of starch by α-amylase and pullanase. The first enzyme is used to break down the amylose, and the second to break the glycosidic bonds of amylopectin. Malto-oligosaccharides are generally used in food products, infants formulas and sports drinks [37].

Isomalto-oligosaccharides, are composed by glucose units, ranging from 3 to 6. These are nondigestible, due to their α-(1→6) glycosidic bonds, although some α-(1→4) glycosidic bonds can be found (e.g. panose). These saccharides can be found naturally in some products, such as honey, soy sauce, sake and some fermented foods. But, just like malto-oligosaccharides, they can also be obtained by enzymatic processes with starch as starting material. In this case, starch is liquified by amylases and a then transglucosidase converts the α-(1→4) bonds into α-(1→6) bonds. This process also generates impurities of free glucose, maltose and maltotriose. Contrary to malto-oligosaccharides, isomalto-oligosaccharides have prebiotic activity, promoting the growth of beneficial flora in the colon, helping reducing sugar cholesterol and assist in the absorption of minerals from foods [36,37].

Gentio-oligosaccharides are glucose-composed OS that can have 2 to 5 glucose units in their structure, linked by β-(1→6) glycosidic bonds. Since these bonds are not hydrolyzed in the stomach or small intestine, these are classified as prebiotic. The gentio-oligosaccharides generally result from enzymatic transglucosylation or from biocatalytic glycosylation with cultured cells, with glucose syrup as starting material [36].

Pectic-oligosaccharides are a novel group of OS obtained from the partial hydrolysis (depolymerization) of pectin. Pectins are ramified heteropolysaccharides constituted by a linear backbone of D-galacturonic acid units, both in acetylated and methylated forms, linked by α-(1→4) linkages. These pectic saccharides can be produced either by hydrothermal treatment, physical degradation or by acidic or enzymatic hydrolysis. Due to their complex structures, a different variety of enzymes need to be used, such as estereases, pectin lyases, and hydrolases in order to achieve pectin degradation. These fit in the prebiotic classification, since they promote the bifidogenic flora, apart from some other benefits for the consumers health, such as antioxidant and antibacterial activities, reduction of serum levels of total cholesterol and inhibition of the accumulation of body fat [32,36].

Particularly different from the other OS, cyclodextrins or cycloamyloses are cyclic OS composed by α-D-glucopyranose units linked by α-(1→4) linkages. These cyclic carbohydrates result from the degradation of starch and have a structure similar to a ring with an inner hydrophobic cavity and an external hydrophilic surface. These carbohydrates are widely used in food packaging and as carriers of food additives [37,38].

2.3. Polysaccharides

Structurally more complex than the other referred carbohydrates, polysaccharides are composed by many monosaccharides linked together by glycosidic bonds, with glucose being one of the most common monomers. When a polysaccharide is composed only by one type of monosaccharide is called homopolysaccharide, and when is composed by more than one class of monosaccharides it is a heteropolysaccharide. Heteropolysaccharides are often associated with

proteins and can form large molecular complexes. Similarly to some oligosaccharides, polysaccharides can be linear or highly branched [18,19,39].

The most abundant glycans are the storage polysaccharides starch (amylose and amylopectin) and glycogen, and the structural polysaccharide cellulose. Amylose, one of the glucans that compose starch, has its glucose units linked by α-(1→4) glycosidic bonds, from carbon 1 of one glucose to carbon 4 of the following (C1→C4), and is composed by 1000 to 5000 D-glucose units. Amylopectin, the other glucan that composes starch, can comprise over 600000 glucose units in its molecule. Its basic structure is similar to amylose, since it has the glucose units linked by α-(1→4) glycosidic bonds in the same manner (C1→C4). However, amylopectin presents ramifications that can comprise up to 26 glucose units and are linked together also by α-(1→4) glycosidic bonds. These ramifications are connected to the main chain by α-(1→6) linkages (carbon 1 of the glucose of the branch and carbon 6 of the glucose of the main chain). Glycogen is very similar to amylopectin, it can be composed up to 55000 glucose units and these are linked through α-(1→4) bonds. Glycogen also has branched chains (with glucoses linked by α-(1→4) bonds), linked to the main chain by α-(1→6) linkages. Regarding cellulose, this polysaccharide can be comprised by over 10000 glucose monomers linked by β-(1→4) glycosidic bonds. The presence of β-(1→4) bonds allows the molecule to form long linear straight chains, stabilized by hydrogen bonds. Besides the previously referred, other common polysaccharides can be named such as dextrins, inulin, chitin and hemicellulose [18–20,24].

3. Fructans

Fructans, also known as polyfructosylsucroses, are a family of fructose polymers that derives from sucrose and can either be linear or branched. They are composed by β-D-fructofuranosyl residues with or without a terminal glucose, and their DP can range from 3 up to 60 units, when found in plants, and can also be of very high molecular weight, with more than 100000 units, when originated by microorganisms. Depending on the linkage between each fructosyl moiety and the spatial arrangement of glucose residues, fructans can be divided into five categories: inulin-type, levan-type, graminan-type, neo-inulin-type, and neolevan-type fructans, as presented in **Figure 4.** There is a variety of enzymes, namely fructosyltransferases (FTases), involved in the synthesis of these polymers, responsible for the catalysis of the transference of fructose units from sucrose to a growing fructan chain. Fructans can be found in 15% of flowering plants as a reserve carbohydrate, and their production is directly correlated to the need to survive in harsh conditions, such as cold and dry environments. This protective role is achieved due to fructans insertion between the phospholipidic layers of the cell membrane, increasing the resistance to such conditions [40–44].

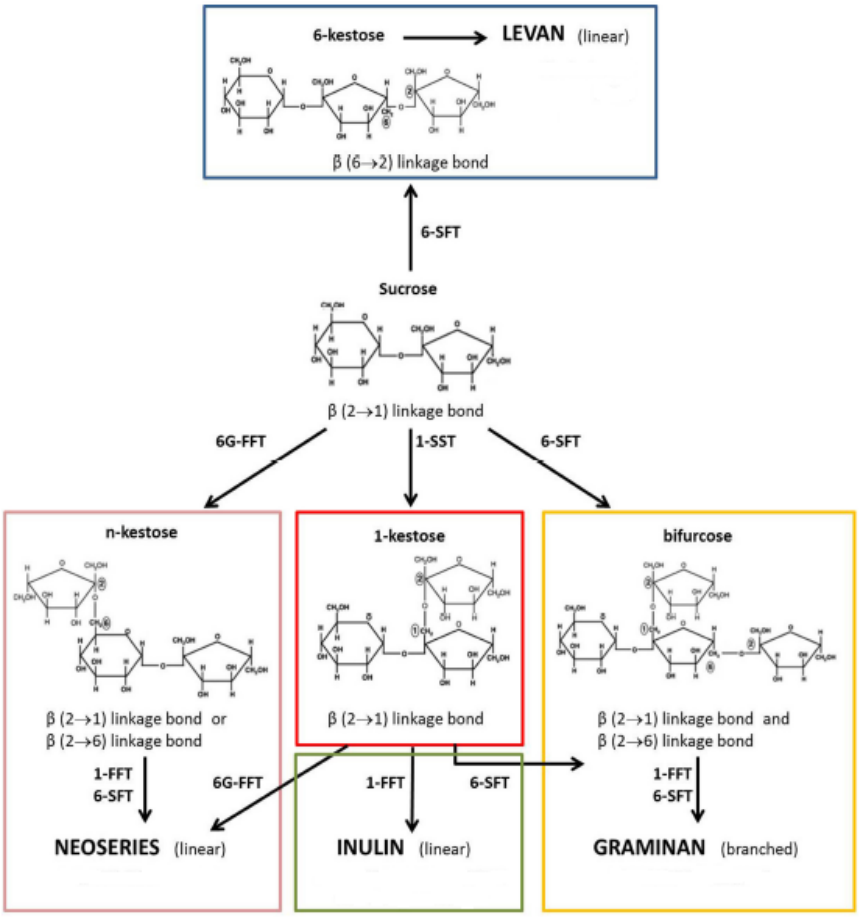

Figure 4 – Fructans types and structures from sucrose synthetization [45].

3.1. Types of Fructans

Fructans have different structures, either linear or branched, and have different chain lengths that can range from three up to nearly hundred or thousand fructose units, depending on the source. The different fructans' structures, and therefore different types, depend on the initial trisaccharide from which they originate, their glycosidic linkages and the presence or absence of branching and all is controlled by different fructosyltransferases [41,46,47]. The five types of fructans and structures are represented in **Figure 5** and those are the following:

- **Inulin-type** fructans will be further discussed in this work, in the **section 3.3**. This type of fructan is linear, with β-(2→1) glycosidic bonds linking the β-D-fructofuranosyl units and these fructofuranosyl units to sucrose. This group includes FOS with 1-kestose (DP3) as the smallest inulin type fructan [48].
- **Levan-type,** also known as phlein-type, is a linear fructan with β-(2→6) bonds linking its β-D-fructofuranosyl units and these linked to the 6 position of the sucrose. They can have up to 3000 fructose units linked to the glucose. 6-kestose (DP3) is the smallest in the levan-type [41,48].
- **Graminan-type** (also known as Gramminan) or mixed levan-type are branched fructans with both β-(2→1) and β-(2→6) glycosidic bonds in the same molecule between the β-D-fructofuranosyl units. The molecule bifurcose is the simplest example of a graminan fructan [46,48].
- **Neoseries-type** comprises neo-inulin-type and neolevan-type fructans. Different from the other types in which a terminal glucose is present, these linear fructans are characterized by having an internal glucose unit. In the neo-inulin-type the fructose units are bonded by β-(2→1) linkages and in the neo levan by β-(2→6) linkages but, in both cases, they are linked to the positions 1 and 6 of fructose and glucose respectively [48,49].

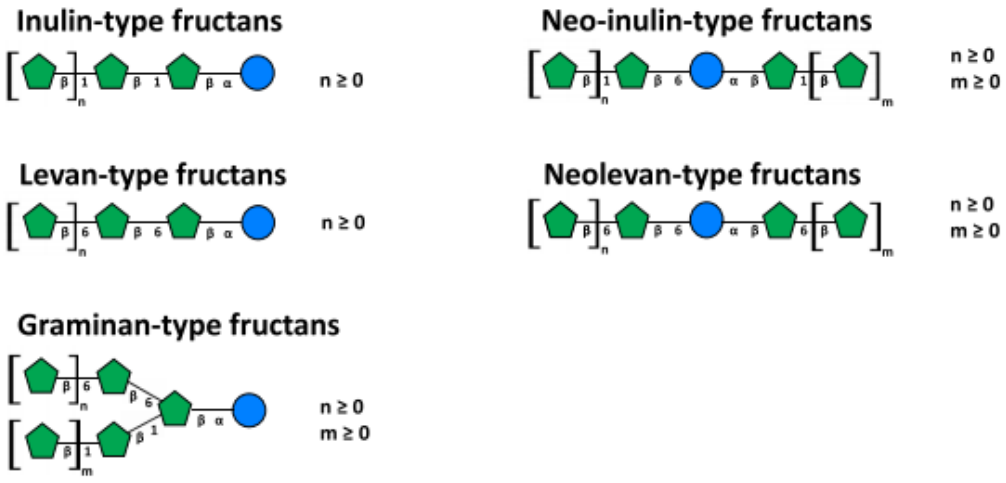

Figure 5 – Scheme of fructans types and respective structures [49].

3.2. Sources

After starch, fructans represent the second most common energy storage source and are extensively found in a variety of organisms. Among the different types of fructan-producing plants, the family with most producing species is the *Compositae* (or *Asteraceae*) family, followed by the *Liliaceae* family and the *Gramineae* family, with around 25000, 3500 and 1200 species respectively. For the *Compositae,* the richer in fructans are chicory, Jerusalem artichoke, yacon and dahlia; in the *Liliaceae* fructans are abundant in onion, garlic, leek and asparagus. In the *Gramineae* there are cereals such as barley, wheat and oat and some forage grasses such as *Lolium* and *Festuca*. In the *Compositae* family, inulin and inulin type fructans are the most abundant; in the *Liliaceae* family, the inulin neoseries type is frequently found, and in the *Gramineae* the most common are the levan and the graminan types [48,50,51].

Concerning microorganisms, a diversity of bacteria has been found to be fructan producers. These comprise plant pathogens and bacteria present in gastrointestinal flora of humans and other animals. Strains of *Bacillus*, *Streptococcus*, *Pseudomonas*, *Actinomyces* and *Lactobacillus* are relevant. Some fungi and yeasts are also able to produce fructans, in particular FOS. Fungi possess enzymes able to produce short-chain FOS. Some of the best well-known fungi FOS producers are *Aspergillus niger*, *Aspergillus japonicus*, *Aspergillus oryzae*, *Aerobasidium pullulans* and *Penicillium citrinum*. Some yeasts, such as *Rhodotorula spp.*, *Kluveromyces marxianus* and *Schwanniomyces occidentalis*, are capable to secrete extracellular enzymes, with FOS producing functions. Similarly to fungi, these yeasts produce short-chain FOS [48,50,51].

3.2.1. Yacon (*Smallanthus sonchifolius*)

Yacon (*Smallanthus sonchifolius*, also known as *Smallanthus sonchifolia* and formerly known as *Polymnia sonchifolia*) is an herbaceous perennial plant native to the Andean regions. This plant from the *Compositae*/*Asteraceae* family is related to sunflowers and Jerusalem artichoke (*Helianthus tuberosus*), the latter being a well-known source of FOS. Yacon produces large tuberous roots, with an appearance that resembles sweet potatoes. **Figure 6** shows all the plant components, including the whole plant, leaves, roots, and flowers. Yacon is extremely resilient and is able to develop under hot or cold conditions, can grow up to two meters, has large inversed arrowhead leaves with serrate margins and multiple yellowish-orange flowers. In spite of its resistance to both cold and hot environments, yacon generally grows in the warm conditions of the Andean valleys and in altitudes up to 3200 meters. The roots are the major component of the plant consumed, mainly due to its sweet- tasting and apple-like flavor and is mostly constituted of water and carbohydrates, with the water content surpassing 70% of the roots fresh weight. These roots can vary in color, from brown to purple, in size, and thickness. The main component

of the dry matter is FOS with contents ranging from 40% to 70%, differentiating these roots from most of the roots in which the main component is starch [52–54].

Yacon is produced in Portugal since 2013 mainly in the Coimbra district. The company *Yacon Portugal* sells two products, the fresh roots and yacon syrup obtained by shredding the roots at room temperature (juicing) and separation of the liquid, with partial evaporation of water.

Figure 6 – Yacon plant components [55].

3.3. Inulin Type Fructans

3.3.1. Inulin

Inulin is the most common name attributed to the polysaccharide α-D-glucopyranosyl-[β-D-fructofuranosyl] (n-1)-D-fructofuranoside. In food products, it is a very good substitute for simple sugars and fat, due to its sweet taste and thickening capacity with low caloric value, and is used as a prebiotic, acting in a similar way to dietary fibers improving the conditions of the gastrointestinal tract. Commercially, Jerusalem artichoke (*Helianthus tuberosus*), Chicory (*Cichorium intybus*) and Yacon (*Smallanthus sonchifolius*), are the main inulin sources [56–59].

The inulin and its inulin-type oligomers are fructose constituted with essentially β-(2→1) fructosyl-fructose linkages and the molecule usually ends with a (1,2) bonded α-D-glucosyl group, the same as in FOS. The length, composition and polydispersity of inulin is directly dependent on the plant species, phase in life cycle, harvesting time and extraction and post-extraction procedures. The DP and presence of branches in the inulin are important parameters that influence its physical and physiological properties [51,56,59–61].

3.3.2. FOS

Fructo-oligosaccharides (FOS) are very short-chained inulin that can also be referred to as oligofructose or inulin-type OS. They are OS of D-fructose with β-(2→1) fructosyl-fructose glycosidic bonds and generally carry a D-glucosyl moiety (glucose unit). Their degree of polymerization (DP) is comprehended between 2 and 10 and can be represented either as GFn or Fn, (G when a terminal glucose moiety is present and *n* being the number of fructose molecules present in the oligosaccharide chain). Among the most common FOS found in nature, there are 1-kestose (β-D-fructosyl-(2→1)-2-α-D-glucopyranoside, GF_2), nystose (β-D-fructosyl-(2→1)-3-α-D-glucopyranoside, GF_3) and 1-fructofuranosylnystose (β-D-fructosyl-(2→1)-4-α-D-glucopyranoside, GF_4) as represented in **Figure 7**. This small group, when present in a mixture can also be called short-chain FOS (scFOS). When FOS contain glucose, they can be represented by the chemical formula α-D-Glu-(1→2)-[β-D-Fru-(2→1)-]$_n$ (n=2-9) and are commonly known as FOS. When FOS only contain fructose in their structures (Fn) and therefore have a chemical formula of β-D-Fru-(2→1)-[β-D-Fru-(2→1)-]$_n$ (n=1-9), they are specifically known as inulo-oligosaccharides. The most commonly known inulo-oligosaccharides are inulobiose, inulotriose and inulotetraose. Besides the standard definition according to their DP, the term FOS are generally applied for GFn and Fn fructans with β-(2→1) linkages, despite their DP, excluding the other fructans with different linkages, such as levan, graminan and neo types, that were previously discussed [62–67].

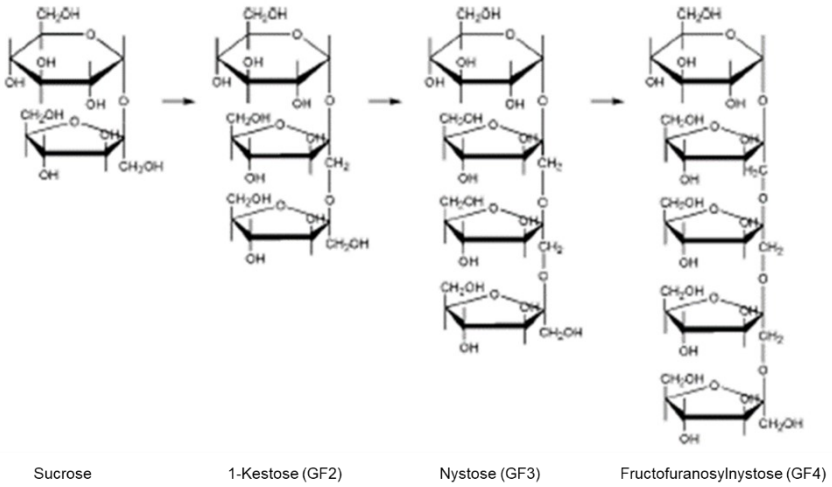

Figure 7 – Structures of the most common FOS [36].

3.4. Relevance of FOS

3.4.1. Functions in Plants and Biosynthesis

In plants, when photosynthesis outdoes their energetic demand, the amount of sucrose reaches critical levels in the storage organs. In this situation, the synthesis and storage of fructans (like FOS and inulin) occur in the vacuoles (**Figure 8**), reducing sucrose levels while maintaining the plant energy supplies. FOS are mainly used by plants as reserve carbohydrates, presenting, in this way, the same function as starch. In fact, some plant species store these two types of saccharides. The use and breakdown of FOS frequently occur when plants are growing (FOS maximum and initiation of breakdown during dormancy phase) or when in need to re-grow cut down organs, such as leaves. In plants with flowers, it is suggested that breakdown of FOS positively contributes to the osmotic driving force involved in the expansion of flowers. Besides that, different reports claimed the role of fructans as aids in the resistance of extreme conditions, such as temperature (extreme cold or extreme heat), excess salinity and dry environments [56,68,69].

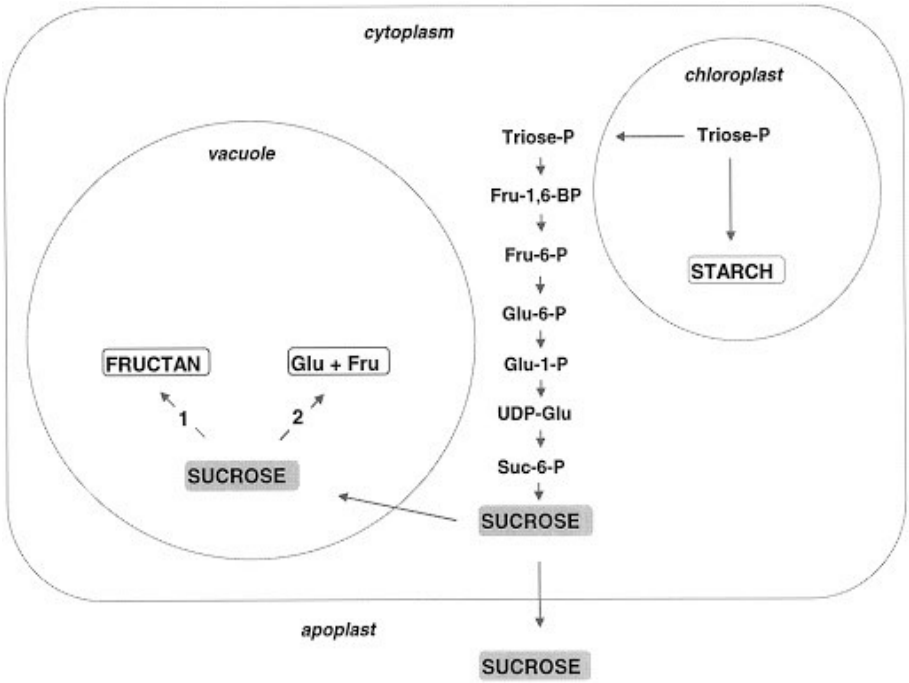

Figure 8 – Scheme of Fructan production in plant cells [50].

The biosynthesis of fructans/FOS is different from species to species regarding the establishing linkages, branching, chain size and type of fructosyltransferases (FTases) involved. For FOS and inulin, the synthesis is catalyzed essentially by three groups of enzymes, sucrose:sucrose 1-fructosyltransferase (1-SST), fructan:fructan 1-fructosyltransferase (1-FFT) and fructan exohydrolase (FEH). The production starts with a molecule of sucrose, which is responsible for the presence of a single glucose unit in FOS and inulin, and its relative inertness gives the resulting oligomers and polymers a certain degree of protection, preventing the fructan

from breaking down spontaneously. The 1-SST catalyzes the synthesis of 1-kestose, transferring a fructose unit from a sucrose donor to the C-1 of the fructose into another sucrose. As a second event, the 1-FFT is responsible for the elongation of the saccharide chain, transferring fructose to the 1-kestose or other fructans. These two enzymes are responsible for the diversity in chain length among FOS obtained by extraction from plants. FOS or inulin thus formed can even have their DP reduced by FEH enzymes, the ones responsible for the degradation of the produced saccharides by releasing terminal fructose units. These enzymes are regulated by the fructan content and there have been evidence that FOS and FEH can be found in the apoplast, being potentially involved in the production of apoplastic FOS, in a response to stress conditions, and in order to help in membrane stabilization. Other enzymes can also be found in plants namely sucrose:fructan 6-fructosyltransferase (6-SFT) and fructan:fructan 6G-fructosyltransferase (6F-FFT). The first is responsible for the production of levan and graminan types and also for the elongation of fructans with β-(2→6) linkages and the second for the production of neoseries type [50,56,69,70].

3.4.2. Fructans Processing in Human Body and Functional Properties

FOS, just like the majority of OS, are prebiotic agents. However, besides the improvement in the gastrointestinal conditions, FOS present a diversity of beneficial effects for the consumers, such as the improvement in mineral absorption, the modulation of the immune system, the positive effect on lipid metabolism, antioxidant effect and the preventive effects in bowel diseases and colon cancer, as presented in **Figure 9** [1,71].

Consumption of FOS has generally positive effects in the intestine. The short-chain fatty acids (SCFAs), end products of the metabolism of FOS in the intestine, are very easily absorbed by the epithelial cells in the colon. Consequently, their absorption stimulates the epithelial cells' growth as well as water and salt absorption. These factors allow the reduction of constipation, since they improve the intestinal mobility due to the increase in the humidity of the stool through osmotic pressure. Also, FOS act towards the inhibition of diarrhea, especially when induced by intestinal infections. The effect of fructans in the bifidobacteria has a potential indirect protective effect, as shown by studies that reported a secretion of a bacteriocin-type substance against harmful colon bacteria such as *Salmonella* and *Shigella* [48].

SCFAs produced in FOS' fermentation also change the colonic lumen by reducing its pH. As a result, an increment of certain minerals, such as calcium and magnesium, solubility occurs alongside an increment of the water content and absorptive capacity of the epithelium [48].

The functioning of the immune system strongly depends on the supply of nutrients. FOS' fermentation end products show excellent properties towards the modulation of the gut associated lymphoid tissue and the systemic immune system. For this modulation, FOS have showed an

increase in the activities of natural killer cells and phagocytes and the enhancement in lymphocyte functions [1,48].

There are claims that FOS affect the serum cholesterol and lipidic level, having hypocholesterolemic and hypotriglyceridemic effects. These effects are not confirmed to happen in humans. However, some studies revealed that the consumption of dietary fructans significantly decreases the serum triacylglycerols [48].

Bowel diseases and colon cancer can be related to the diet and some studies have revealed that the consumption of non-digestible OS, such as FOS can have positive effects on these individuals' health or even decrease the development of colon cancer. This anticarcinogenic effect seems to be related to improved cellular immunity and stimulation of the gut associated lymphoid tissue granted by the fermentation products of FOS, such as butyrate, that are able to stimulate the apoptosis in colonic cancer cells [48].

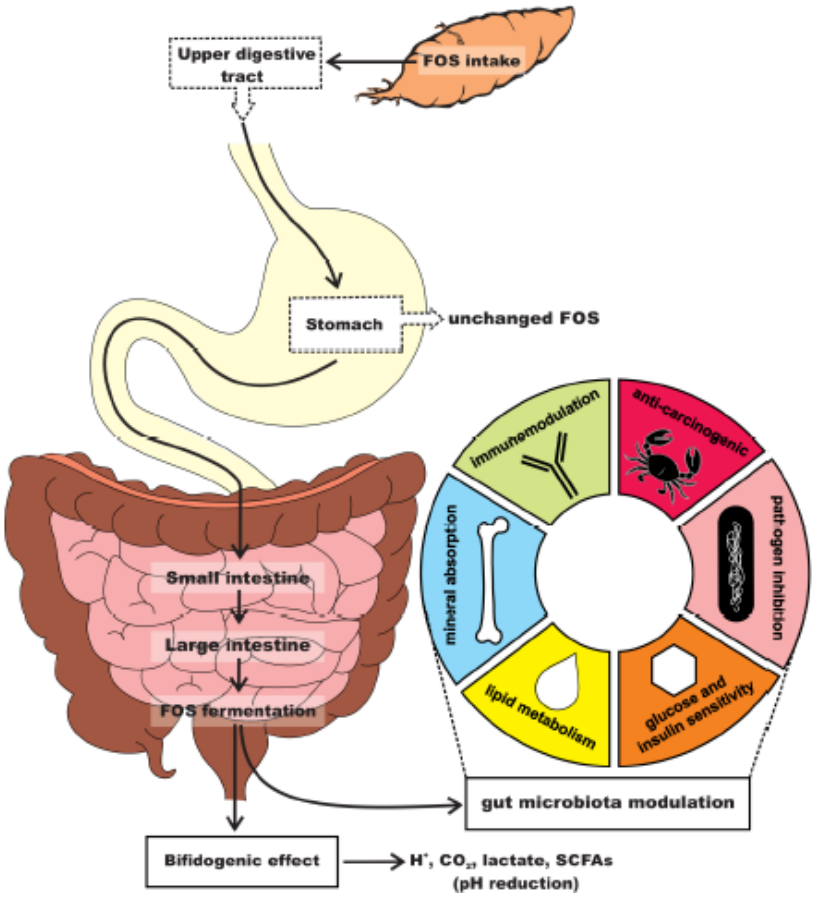

Figure 9 – Beneficial effects of FOS for the consumers health [72].

3.4.3. Fructans Industry Applications

With the functional food market rising and the increasing concerns about synthetic additives, the demand for FOS and other fructans in general has risen, as well as their applications in the food market. FOS produced by transfructosylation from sucrose are frequently used as prebiotic ingredients and longer-chain FOS produced by controlled hydrolysis with inulinase are commonly used as fat replacers. Commercially, FOS are frequently found as powder, syrup or as capsules, as supplements or incorporated into food products, such as beverages and health drinks, dairy products (milk powder in particular), infant formulas and weaning foods. They are also used as humectants to soften baked products, for decreasing the freezing point in frozen desserts, to provide crispness to low fat cookies and to replace sugar as binder in cereal bars, but with the benefits of having low caloric content. FOS have also been used as stability and preservative agents, prolonging the shelf life of the foods to which they are added [67,73,74].

4. FOS Production

4.1. Industrial Enzymatic Synthesis

As previously stated, FOS can be found and extracted from different natural sources. However, the overall FOS yields from these processes is low and is not enough for industrial demands. In order to surpass this and other limitations, such as seasonality of some of the FOS sources, they have been produced by biotechnological synthesis. FOS can be obtained by chemical glycosylation and *de novo* synthesis with glycosidase and glycosyltransferase, but this is a difficult multi-step method using hazardous and expensive chemicals and gives low FOS yields, not being economically feasible for implementation at industrial scale [75].

Therefore, in industry, FOS are synthetically produced by transfructosylation reactions with fructosyltransferases (fructosyltransferase and β-fructofuranosidase) as biocatalysts. In these reactions, the cleavage of β-(2→1) glycosidic bond is followed by a transfer of fructosyl units from a donor sucrose to an acceptor sucrose. This is a complex process, due to both hydrolytic and transfructosylation activities of β-fructofuranosidase, which means that both hydrolytic and synthetic reactions occur simultaneously, in a cycle of constant scFOS mixture production and hydrolysis. **Figure 10** presents a simplification of the process of FOS production through transfructosylation. This process is the basic principle of the formation of 1-kestose, followed by nystose and 1-fructofuranosylnystose. The first is produced by transfructosylation from sucrose and the following from transfructosylation from sucrose or from the lower DP FOS previously produced. Both sucrose and the recently formed scFOS act as substrate and as fructosyl acceptors. In this process, glucose is a concurrent product that can act as an inhibitor of the enzymatic reaction and must be removed either by an enzymatic reaction with glucose oxidase, or by mechanical purification processes. The final composition of the obtained FOS can be modulated

by the adjustment of different parameters, such as substrate concentration, source of the enzyme, time of the reaction, temperature and pH. In this method, the enzymes can be used in their free form, immobilized, or even as part of whole cells of microorganisms producers of fructosyltransferases [75,76].

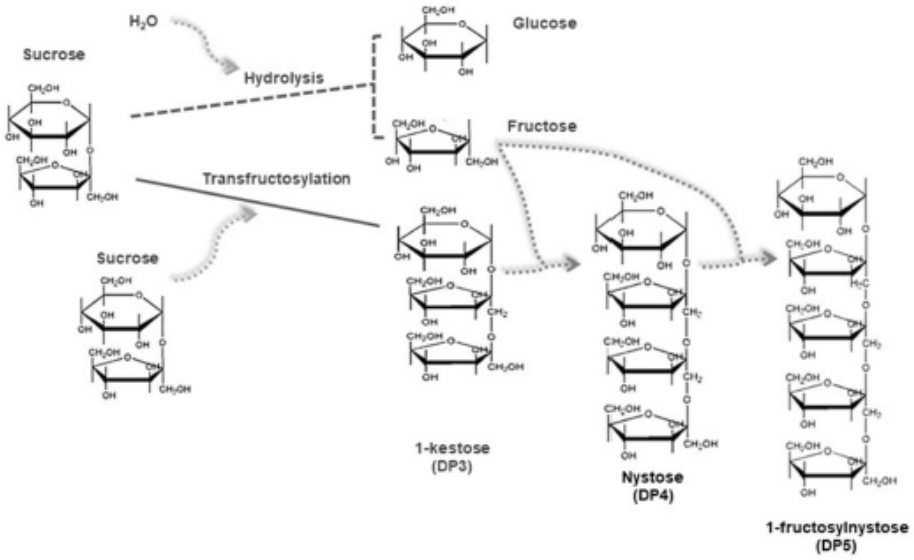

Figure 10 – Enzymatic production of FOS through transfructosylation [76].

4.1.1. Enzymes with Transfructosylation Activity

Fructosyltransferases (FTases), E.C. 2.4.1.9 and β-fructofuranosidase (FFase), E.C.3.2.1.26 are enzymes capable to catalyze the production of FOS. FTases only have transfructosylating activity and are responsible for the cleavage of the β-(2→1) linkage in sucrose (or in FOS) and the transference of the resulting fructosyl group to an acceptor, leading to the formation of FOS, with a release of glucose. Fructosyltransferase barely shows affinity towards water, which means that the hydrolytic activity of this enzyme is very low. On the other hand, FFase (also known as invertase) mainly catalyzes hydrolytic reactions but it also promotes transfructosylating reactions, with the latter activity being only evidenced at appropriate conditions, such as higher sucrose concentrations. The production of FOS by this enzyme can occur by two different mechanisms: reverse hydrolysis and transfructosylation. In the case of reverse hydrolysis, the equilibrium of the process is shifted towards the synthesis of FOS. First, a complex enzyme-donor is formed, and this will react with a hydroxyl group of the acceptor. The preferential acceptor is water (occurring normal hydrolysis), but in the case of having sucrose as an acceptor, the reverse hydrolysis occurs. The FOS production yield depends on the equilibrium constants of the two possible transformation reactions from the complex enzyme-fructosyl to either free enzyme and fructose or to the formation of 1-kestose and the following higher DP FOS. In the case of transfructosylation, a fructosyl group is transferred from an activated donor, usually

sucrose, to an acceptor, sucrose or FOS. The activation of the donor occurs by complexation with the enzyme, the fructosyl unit is transferred and a glucose unit is released. This process is influenced by the amount of FOS produced, meaning that a high concentration of FOS in the solution translates in a lower rate of transfructosylation [77,78].

These enzymes generally belong to the glycoside hydrolases family (GH) being either included into the GH-32 or GH-68 families. They can be commonly found in plants or they can be produced by microorganisms, essentially the fungi *Aspergillus pullulans* and *Aspergillus niger*, some yeasts like *Saccharomyces cerevisiae* and bacteria such as *Bacillus macerans.* The enzymes obtained from plants, yeasts and fungi are included in the family GH-32 and those of bacteria in the family GH-68 [76,77].

4.1.2. Inulin Hydrolysis for FOS Production

Extraction of inulin from natural matrices is relatively easy and always accompanied by extraction of FOS, sucrose, fructose and glucose. Since inulin is moderately soluble in water, and that solubility increases with temperature, the extraction of inulin is carried out by grinding and solubilization of the vegetable materials in hot water. A further enzymatic treatment can be employed in order to eliminate the undesired sugar impurities, such as sucrose, fructose and glucose. When the final goal of the procedure is the maximum obtention of FOS, in order to increase their yield, inulin can be degraded by acidic hydrolysis, autohydrolysis or enzymatic hydrolysis [56,76].

4.1.2.1. Acidic and Autohydrolysis

The hydrolysis of inulin can happen in a strong acidic environment, with pH ranging from 1 to 3, and relatively high temperatures (80 °C to 100 °C). The processes can occur either by homogeneous or heterogeneous catalysis. In this type of processes, due to the extremely acidic and high temperatures conditions, the resulting fructose is degraded and originates undesired by-products, such as di-fructose anhydride and hydroxymethylfurfural. Although some reports had stated the obtention of FOS through acidic hydrolysis, this is only viable with long chain inulin and branched fructans. Therefore, this type of hydrolysis is more suitable for fructose production, under controlled conditions to avoid undesired by-products formation, than for the production of FOS [76,79].

The autohydrolysis is a slow biological process that occurs in inulin, especially given the right conditions, and when inulin is stored for long periods of time. Older plants tend to have lower inulin and long-chain FOS contents, alongside mono-, di- and small OS. This happens due the enzymatic machinery developed by the plants capable to hydrolyze inulin [76].

4.1.2.2. Inulin Enzymatic Hydrolysis

Besides the enzymatic synthesis with sucrose as a starting material, another enzymatic process can be employed for FOS production. In this case, the hydrolysis of inulin occurs with

the enzymes inulinases, with specific endo-inulinases activity, whereas they will randomly act in the inulin and cleave internal β-(2→1) linkages. These random cleavages will originate a mixture of FOS and inulo-oligosaccharides, as represented in **Figure 11**. Some FOS obtained by inulin hydrolysis are similar to those obtained by transfructosylation while others will have only fructose in their structure. FOS produced from inulin have slightly longer chains when compared with FOS from synthesis, DP can range from 2 to 9 after hydrolysis and while from synthesis DP can range from 2 to 4. The hydrolysis of inulin generally happens at temperatures between 37 °C and 55 °C and at optimum pH comprehended between 6.0 and 7.0. The amount of FOS produced will depend not only on the hydrolysis conditions, but also on the source of the enzyme inulinase, which can also be immobilized allowing the hydrolysis to occur in batch or continuous processes and consequently granting higher FOS yields without significant enzyme activity loss [66,76,80].

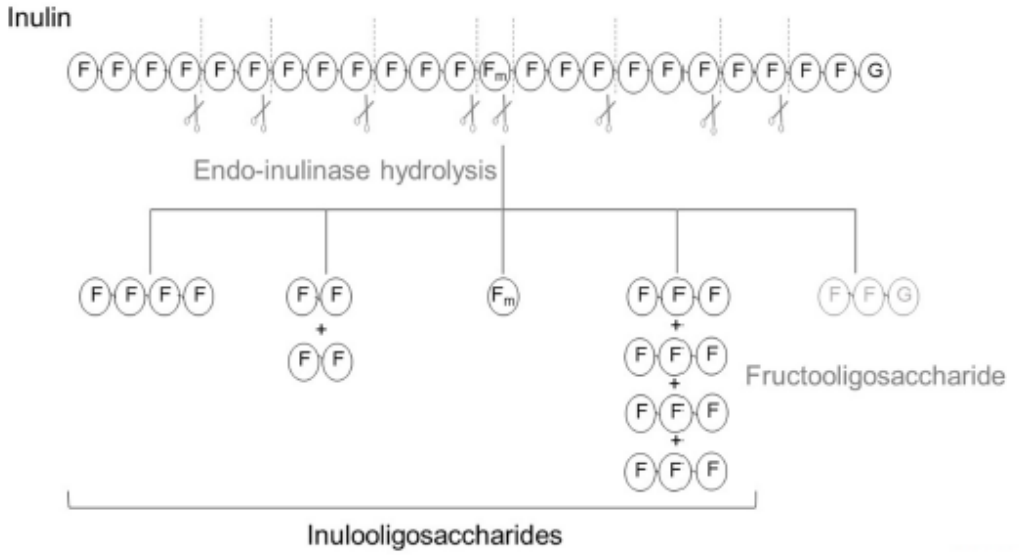

Figure 11 – FOS production from inulin with endo-inulinase [76].

4.2. Inulinases

Inulinases are a group of enzymes that belongs to the glycosidase hydrolase family 32 (GH-32) and can hydrolyze the O-glycosyl bonds. They are responsible for the hydrolysis of the β-(2→1) linkages in fructans, specially inulin. These enzymes can be produced either by plants or by a different variety of organisms such as bacteria, fungi and yeast, (such as *Xanthomonas* sp., *Aspergillus* sp. and *Kluyveromyces* sp. respectively) and, depending on the source, the outcome of the hydrolysis can be predicted. According to the cleavage pattern they can be divided into exo-inulinase (E.C. 3.2.1.80) and endo-inulinase (E.C. 3.2.1.7). Having an exo- and endo-action depends on the origin of the enzyme. Exo-inulinases' (β-D-fructan fructohydrolase) action begins at the non-reducing end of the inulin molecule and leads to the production of a high content fructose syrup (and some glucose). The resulting fructose syrup yields up to 95% fructose. Endo-inulinases (2,1-β-D-fructan fructanohydrolase) are specific for inulin and selectively degrade

inulin by endo-cleavage of internal 2,1-β-D-fructosidic linkages. This reaction originates FOS with DP ranging from 2 to 9 [81–83]. Both mechanisms of inulin degradation are represented in the **Figure 12**.

The majority of obtained inulinases are exo-inulinase and the expression of endo-inulinase is lower, which make them difficult to use in large-scale production of FOS. Besides that, the use of endo-inulinase requires low substrate concentration, long reaction time and a large quantity of enzyme consumption, making FOS production less lucrative [81]. In order to surpass this economical barrier, techniques such as enzyme immobilization can be used to improve catalytic properties and the ability to use the enzyme in a larger number of cycles with a high yield of the desired products [79,84,85].

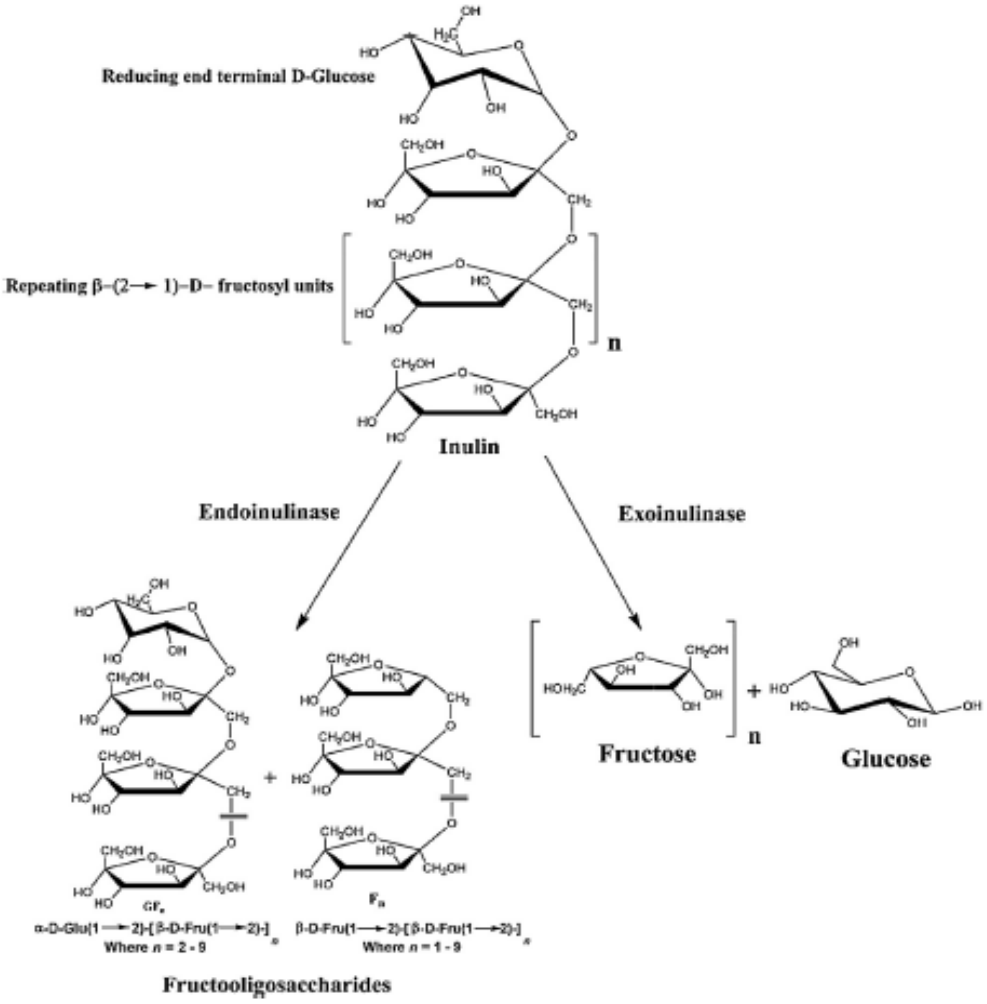

Figure 12 – Degradation mechanisms of inulin by inulinase [86].

4.2.1. Enzyme Immobilization

Enzymes are very important biocatalysts characterized by their high effectiveness and efficiency, high selectivity and activity and are widely used in the industry. From the industrial

point of view, some of the reasons for such importance are the reduction of reactional steps, reduction of the quantities of dangerous solvents which converts typically slow and expensive industrial processes into quicker, less expensive and more environmentally friendly ones. Despite these advantages, the majority of the enzymes have high production and separation costs, as they are relatively unstable and present a huge technical difficulty of recovery in their active state after being used in reaction procedures [87,88].

Enzyme immobilization is a strategy developed to overcome some of the major challenges of using enzymes in their free form. Immobilization is a simple and cheap process that allows the enhancement of the process reproducibility with the possibility to increment the enzyme activity, provides a better chance to reuse and recovery of the enzyme, allows for continuous processes, grants the enzyme structural stability to external changes (heat and organic solvents, for example), allows for the reduction of product inhibition and recovery of the desired product with higher purity. In the case of processes that involve the use of expensive, rare or difficult to purify enzymes, immobilization techniques that allow their reusability and recovery is crucial for industrial procedures. Immobilization involves the use of a matrix or carrier, where the enzymes will be attached or incorporated. The choice of the matrix should bear in mind the cost of the catalyst and of the total production. Immobilized enzymes are used in different areas, such as food, pharmaceuticals, medicine, biofuels production and waste water treatment [89–91].

4.2.1.1. Immobilization Methods

Different methods for immobilization can be performed (**Figure 13**), the most commonly applied are adsorption (mainly in water-insoluble carriers), covalent binding onto a surface, entrapment, or encapsulation in polymers and through carrier-free cross-linkages with the aid of multifunctional reagents such as glutaraldehyde. Cross-linking processes can be conjugated (or not) with some of the previous methods [89,92,93].

Adsorption is a simple, economic, generally reagent-free method, based on weak bonds, namely electrostatic interactions, hydrophobic interactions and van der Waal's forces. According to the interaction, this method can be subdivided into physical adsorption, electrostatic binding and hydrophobic adsorption. In this method, an enzymatic solution gets in contact with a solid support for a determined amount of time and under optimum conditions. The amount of enzyme adsorbed is limited and the non-adsorbed enzyme is then removed with a washing buffer. This method can have some disadvantages, such as poor storage stability, low reactional stability due to enzyme desorption or leaching caused by weak bonding that changes with drastic differences of temperature, pH and ionic strength [88].

Covalent binding is one of the most used immobilization methods, in which strong bindings are established between the functional groups of the enzymes and the supports. The enzymatic functional groups that establish those bonds should be non-essential for the enzymatic

activity. In order for the binding procedure to occur, the surface of the support is firstly activated with a cross-linker, a multifunctional reagent, such as glutaraldehyde. Following this step, the enzyme can covalently couple to the activated support. Due to the covalent bonds formed between the enzymes and the supports, very little enzyme leakage is verified. However, this method presents a high risk of denaturation due to the chemical alterations that enzymes go through to bind their functional groups. Generally, this procedure increases enzymatic stability but a reduction in enzymatic activity is verified [88].

Entrapment/encapsulation involves the retention of the enzymes by covalent or non-covalent binding within the polymeric network of the immobilization support. The enzyme is not directly attached to the support, i.e. there is no chemical interaction between the enzyme and the support. Instead, the structure of the support allows the diffusion of substrates and products without the dispersion of the enzyme to the medium. In this immobilization process, the enzyme is mixed with a solution of the monomer and then polymerization occurs through chemical reaction or through changes in the immobilization conditions. This method provides stability, minimizes enzyme leaching, if the pores have the correct size, and minimizes enzyme denaturation. However, some mass-transference resistance can occur, due to excessive matrix thickness, hindering the diffusion of the substrates and the products [88,89].

Cross-linking is an irreversible method in which covalent bonds are formed, with the aid of cross-linking agents, between the enzyme molecules. The cross-linking agents are generally multifunctional reagents that connect the enzymes into three dimensional aggregates. There are two types of aggregates, the cross-linking enzyme crystal (CLEC) and the cross-linking enzyme aggregate (CLEA). This method improves enzyme stability, allows for an easy removal from the reaction medium, avoids products contamination and provides better enzyme stability [88].

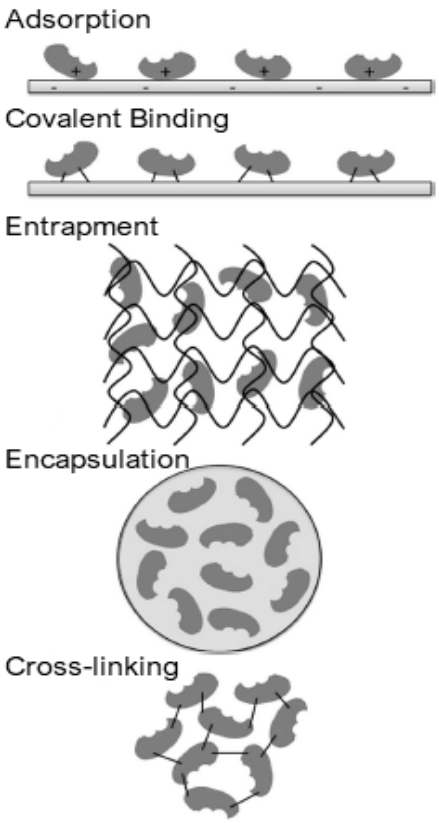

Figure 13 – Immobilization methods most commonly used for enzyme immobilization [94].

4.2.1.2. Support Materials for Immobilization

The success of the immobilization depends not only on the method of immobilization, but also on the support where that immobilization will occur. The choice of the immobilization support should take in consideration the material properties, but also some practical issues, such as cost, availability, stability, and the type of reactor where the reaction will happen. A good support should preserve the native structure and activity of the enzyme and resist the industrial conditions. The chosen material should provide a good affinity between the functional groups of the enzyme and the support, so a successful and effective binding can occur. There is no ideal and universal material, the choice of the support should always reflect the type of enzyme and method of immobilization, analyzing the advantages and drawbacks of its properties [95–98].

The spectrum of materials that can be used as immobilization supports is vast and can be divided in two major categories, based on their chemical composition: organic and inorganic materials. Organic materials can still be subdivided into natural and synthetic organic supports [98,99]. **Table 2** resumes some of the most used materials in enzyme immobilization techniques and some of the main properties, advantages, and disadvantages of each category.

Table 2 – Immobilization materials and main properties [87,89,95,96].

Organic Materials	**Properties**	
Natural	**Advantages**	**Disadvantages**
Alginate **Chitosan** **Starch** **Cellulose** **Carrageenan**	• Ability to be chemically modified • High biocompatibility • High affinity to biomolecules • Application in different immobilization methods	• Low chemical resistance • Low mechanical resistance (These impair immobilized systems to be used with aggressive thermal and pH conditions that may lead to the impossibility to regenerate the matrix)
Synthetic		
PVA (polyvinyl alcohol) **PVC (polyvinyl chloride)** **Polyurethane** **DEAE-cellulose** **Eupergit**		
Inorganic Materials	**Properties**	
Silica **Metal oxides (e.g. titanium)** **Hydroxyapatite** **Activated carbon** **Glass** **Ceramic** **Celite®**	**Advantages**	**Disadvantages**
	• Low reactivity • Thermal resistance • Mechanical resistance • High stability • High rigidity • High porosity	• Reduced biocompatibility • Low affinity to biomolecules • Inadaptability to be reshaped • Limited immobilization methods

The immobilization system used in this work is described in the following section.

4.2.1.3. Immobilization in Alginate Beads

Alginate is a natural polymer, generally extracted from brown seaweed, composed by two monomeric units: β-(1→4) D-mannuronic acid (M block) and α-(1→4) L-guluronic acid (G block). Alginate's structure consists of linear unbranched polymers composed by blocks M and G, either sequenced or alternated with regions that contain alternated M and G blocks. The basic structure will depend on the species of brown seaweed that the alginate originates from. This polysaccharide is widely used in the food industry, due to its capacity to change some food properties such as water binding capacity, emulsion stabilization and film formation, and is well known for its jellification properties. Due to these properties, this is one of the most used materials for encapsulation methods [100,101].

In the presence of calcium or sodium, alginate can form thermostable and biocompatible hydrogel beads that can encapsulate a variety of cells, enzymes, hormones, drugs and many others. The encapsulation success in the hydrogel alginate beads depends on the bead size, shape, swelling properties, solubility and mechanical and chemical stability. One of the most used and simpler method for production of alginate beads is through extrusion dripping (**Figure 14**). In this method, an alginate solution, with the material to be immobilized, is released from a syringe in the form of droplets onto a gelling solution, a calcium or sodium gelling bath; the spherical beads

are formed due to liquid surface tension. The size of the droplet can be modified according to the size of the tip of the syringe used for the extrusion, the flow rate of extrusion, viscosity of alginate and height of exit from the syringe to the gelling bath. The droplet surface gels from almost instantaneously when in touch with the gelling solution; however, in order to the cations completely diffuse to the inside of the bead to originate a strong and resistant material, beads need to be in contact with the gelling solution for long periods of time [100,101].

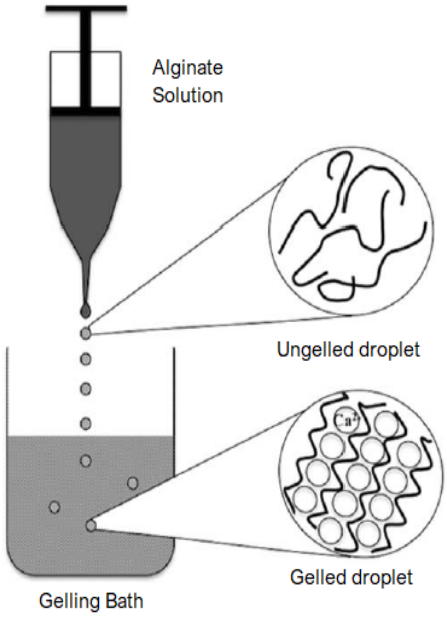

Figure 14 – Alginate beads formation by extrusion dripping [101].

5. Purification of FOS

As previously described, OS can be obtained either by extraction or by synthesis. The resulting OS present different impurities associated, such as mono- and disaccharides and, accordingly to the pretended use for OS, these can affect some of their beneficial properties. The purity of the obtained OS will also determine their shelf life. The removal of short chain saccharides became a necessity in order to allow for the application into a wide variety of products, such as pharmaceutical and food products. OS with none or low mono and disaccharides content can be incorporated into food products and contribute to different properties, such as sweetness, solubility and reactivity decrease of Maillard reactions [102].

There are numerous methods for OS purification. These can be based in affinity and molecule size, with the use of sorbents like activated charcoal, or based in ionic exchange chromatography, with ion-exchange resins. Membrane fraction techniques have also been employed in ultrafiltration or nanofiltration. Enzymatic oxidation making use of some microorganisms, such as yeasts, to selectively ferment some of the undesired saccharides. The choice of the method should take into consideration different aspects such as price, regeneration,

reusability of the material and capability for scale-up and use in industrial scale purifications [102,103].

In this work, several purification methods were assayed and these are described in the following sections.

5.1. Activated Charcoal

Activated charcoal is a well-known sorbent used in several catalytic reactions or in purification systems. This sorbent is one of the most widely used, due to its ready availability and reduced price besides being easily regenerated and having good desalting potential. Activated charcoal is a form of carbon that went through treatment with oxygen or steam, in order to create pores of high volume and in this way granting a large surface area for good sorption capacity. The sorption capacity is determined not only by the surface area, but also by the internal pore structure and the presence of functional groups in the pores [104–107].

Sugars, in general, can be physically adsorbed onto the activated charcoal surface, in a reversible process, due to van der Walls interactions. Since the major area of activated charcoal is non-polar or hydrophobic, and the hydrophobic character of sugars is related to the extent of the CH- groups, the adsorption of sugars will depend mainly on pore size. FOS and inulin are more adsorbed onto the activated charcoal than the smaller saccharides. For FOS separation and recovery, activated charcoal treatment can either be made in a slurry or in packed columns and it can be used as a single adsorbent or conjugated, for example, with Celite®. FOS purification with activated charcoal generally requires three main steps: a first, in which the activated charcoal and the OS are mixed, a middle step that comprises the washing of non-retained compounds with water and a final step, where the desired adsorbed compounds are selectively recovered through elution with ethanol: water gradients [75].

5.2. Microbial Purification (Free and Immobilized Yeast)

Some microorganisms, such as yeasts and bacteria, can ferment some mono- and disaccharides and convert them into ethanol and carbon dioxide. Since these organisms do not have carbohydrolases, enzymes capable to hydrolyze carbohydrates, such as OS, they can be used for their purification. *Saccharomyces cerevisiae*, for example, can completely remove fructose, glucose, galactose and sucrose from a mixture of sugars with OS, since OS with four or more units are not fermented. This type of purification methodology is a good way to increase the FOS content of a mixture, through the removal of monosaccharides and disaccharides. Nonetheless, despite the potential of this method, the use of these microorganisms involves an additional step for the removal of the biomass and products that resulted from these organism's metabolism [75,108].

In order to surpass the drawbacks of the technique, immobilization processes, like those used for enzymes, can be employed. Simple methods, such as immobilization in alginate beads,

can be utilized due to their good biocompatibility, availability, easy preparation and low cost. Immobilized microorganisms, were reported to grant an easier separation of the biomass from the reaction medium, higher substrate conversion yields, less inhibition by the sub-products and reduced reaction times [109].

5.3. Selective Precipitation of Inulin and High-DP FOS

Inulin and high-DP FOS can be separated and fractioned using anti-solvents, such as ethanol (EtOH) or other low alcohols, at different concentrations. A specific precipitation happens due to a gradual decrease of the solubility of these high-DP saccharides with the addition of the solvent. This fractioning occurs in two steps: the solubilization of the inulin and FOS in hot water followed by precipitation with the anti-solvent. To facilitate the precipitation, the solution can be kept under reduced temperatures. The precipitation occurs first for the longer saccharide's chains, enabling the separation of FOS and inulin from undesired mono- and disaccharides [110,111].

5.4. Enzymatic Purification

The use of enzymes has also been studied as a purification method [102]. This method has been used, mainly in mixed enzyme systems since some monosaccharides act as inhibitors for the OS producing enzymes. Glucose, for example, is an inhibitor of β-fructofuranosidase in FOS production. The major selective enzymatic oxidations reported are the ones with glucose oxidase, in which glucose is oxidized to gluconic acid, and cellobiose dehydrogenase, in which lactose is oxidized to lactobionic acid. These by-products must be further removed from the oligosaccharide mixture before use [102,112].

5.5. Chromatographic Purification

Chromatography, despite being mostly used for analytical purposes, can be a good technique for OS purification. Separations with chromatographic methods are based on the molecular differences of the saccharides instead of their macroscopic properties, as in other techniques. Ion exchange resins are typically used in simulated moving bed (SMB) chromatography and have stronger affinity towards monosaccharides than to OS. There are several reports of this technique for the fractioning of FOS and GOS [75]. Another commonly used chromatographic technique is the size-exclusion chromatography. This method is based on the permeation of the saccharides through a column filled with inert porous particles. The separation occurs due to molecular size differences which translates in different times of travel of the saccharides through the pores of the column. Besides, these chromatographic techniques can be combined with other techniques, such as microbial or enzymatic purification in order to remove not only the undesired monosaccharides, but also the undesired by-products of the purification methods [113,114].

6. Analytical Techniques for Carbohydrates

The analysis of sugars is performed mainly through spectroscopic and chromatographic techniques. Some of these techniques were used in this work: thin layer chromatography, high performance liquid chromatography with refractive index detector, Brix degree, DNS colorimetric method and MALDI-TOF mass spectrometry.

6.1. Thin Layer Chromatography – TLC

Thin Layer Chromatography (TLC) is widely used for the analysis of different products, either synthetic or natural, present in mixtures and for the assay of the purity of some compounds. The analytes ascend on the TLC plate through capillary action at different rates according to their affinity with the stationary and mobile phases. The behavior of the compounds and their movement in the plate can be expressed by the retention factor (Rf). This value is obtained dividing the distance traveled by each compound by the overall distance travelled by the eluent, also referred to as solvent front. The Rf is not always similar for the same samples, unless the same conditions are maintained, since it depends on the adsorbent and its thickness, the composition of the mobile phase, temperature, or the type of the chromatographic chamber used, for example [115–117].

After elution and separation of the compounds, each should appear as a spot on the plate, mainly if they are colored. Some plates already have a fluorescent compound, normally manganese-activated zinc silicate, that allows for the visualization of the spots under UV light. When none of this is possible, different methods for revelation of the spots can be applied, some more generic, like iodine vapors and other specific for the type of compounds being separated. For the latter the plate needs to be dipped or sprayed by the revealing solution [116].

TLC, in spite being a simple technique, has many advantages over the other most used chromatographic systems, such as HPLC, since it's a very low-cost and fast operation, requires a simple sample preparation, has good reproducibility, involves a lesser consumption of solvents and has a vast range of reagents for spot detection [118–120].

6.2. High Performance Liquid Chromatography – HPLC

High performance liquid chromatography is a chromatographic technique that requires high pressures to force the mobile phase to pass through the stationary phase conditioned in a column. Generally, the particles used as stationary phase, are small and compact allowing for an increase in resolution. This technique is not limited by volatility or stability of sample compounds [121,122].

Numerous columns can be found in the market, with different materials, including glass, fused silica and PEEK (polyethylethylketone), as some examples. Therefore, HPLC can have different separation mechanisms accordingly to the type of column and mobile phases used. There can be the normal phase chromatography, with an hydrophilic column packing and hydrophobic

mobile phase; reverse phase chromatography, characterized by a hydrophobic column packing; ion exchange chromatography, which uses an ion exchange material as stationary phase and size exclusion chromatography, that separates the analytes by molecular size, slowing the elution of smaller molecules by increasing their path through the porous sorbent, and the elution of larger molecules is increased due to their exclusion from the small porous in the support. The components of the samples are separated in the chromatographic run and are detected by a specific detector, that should be used according to the characteristics of the samples. There are different types of detectors, namely ultraviolet-visible (UV-Vis), diode array (DAD), refractive index (RI), evaporative light scattering (ELSD) fluorescence (FLD), mass spectrometry (MS), nuclear magnetic resonance (NMR) and pulse-amperometric detector (PAD) are some of the most frequently used [121–126].

HPLC's high precision and analytical selectivity have been used for the assessment of different components in food matrices, with it being an excellent instrument for their determination and quantification. For the separation of carbohydrates, almost every type of chromatography can be employed. For their detection, refractometry is the most conventional and reliable system used, since refractive index does not require additional treatment to the samples. However, it requires the availability of pure compounds to be used as standards. In their absence, mass spectrometry remains the more informative detection method [122,127].

6.2.1. RI Detector

Refractive index detectors measure the change in the refractive index of the mobile phase. When an analyte elutes from the column, a difference in the refraction of light is detected by the flow cell. This detector is a universal, non-specific detector, since it can detect any analyte with a refractive index different from the eluent. It is sensitive to changes in temperature, pressure and flow rate and it is not compatible with gradient elution. Since the measurements are purely differential, any change in the mobile phase requires recalibration. Despite its low sensitivity, the fact that carbohydrates are generally in high concentrations in the samples, this limitation is not a problem [124,125].

6.3. Brix Degree

The Brix degree (°Brix) is a calibration of the refractive index that measures the percentage by weight of sugar solids is a sucrose solution. This method is commonly used as a suitable value to express all soluble solids in syrups and sugar containing solutions, even if the sugars present are not all pure sucrose. For the measurement of °Brix, there are two instruments most regularly used. The Brix hydrometer and the refractometer. In these instruments, the Brix values are not only influenced by the amount of sugar in the samples but also by some acids and mineral content. When measured in a Brix hydrometer, or other gravimetric instruments such as the pycnometer, the results are frequently dubious, due to manual errors. Refractometers are more

reliable and accurate, since this method is based on the refraction of light or critical reflection of light. Although this technique is better, some attention is required, especially in the need to avoid damaging of the crystal, the presence of gas bubbles and the presence of suspended solids. Among the refractive techniques, critical angle based refractive index is the more appropriate since it is not affected by solid suspensions or by the color of the solutions. The basis of the Brix scale in the refractive index is the measure of grams of sucrose per 100 grams of sugar solution and is well known and established [128,129].

6.4. DNS Colorimetric Method

The colorimetric methods for carbohydrates determination are among the most versatile, easy and cheap methods. Its procedure is based on the reaction of hydrolyzed carbohydrates and a reagent that develops a characteristic color visible on the electromagnetic spectrum. The carbohydrates can be quantified in the UV-Vis spectrophotometer, although this quantification is not individual for each sugar, but a more generic one, total sugar or reducing sugars concentrations, for example. Amongst the most commonly used color development reagents, there are phenol, alkaline ferricyanide, anthrone and 3,5-dinitrosalicylic acid [130,131]. The Dubois method, also known as *phenol-sulphuric method*, is one of the most used colorimetric methods for total sugar quantification and the DNS (3,5-dinitrosalicylic acid) method one of the most used for quantification of reducing sugars.

The DNS method is a technique that allows the quantification of reducing sugars. The carbohydrates with reducing ends, either a free aldehyde or ketone group, are prone to oxidation, thus acting as reducing agents. When in alkaline medium, and in the presence of 3,5-dinitrosalicylic acid, the reducing sugars will reduce the acid to 3-amino-5-nitrosalicylic acid, and their aldehyde group is oxidized to aldonic acid. The initial yellow solution, due to the presence of the 3,5-dinitrosalicylic acid, switches to orange, the color of the product 3-amino-5-nitrosalicylic acid (**Figure 15**), with the intensity depending on the concentration of the reducing sugars. The concentration can be quantified by spectrophotometry with maximum absorbance at 540 nm. This technique is sensitive, ranging between 100 µg/mL and 500 µg/mL of reducing sugar, and the quantification is performed with a calibration curve with either glucose or fructose as standard solutions [132–134].

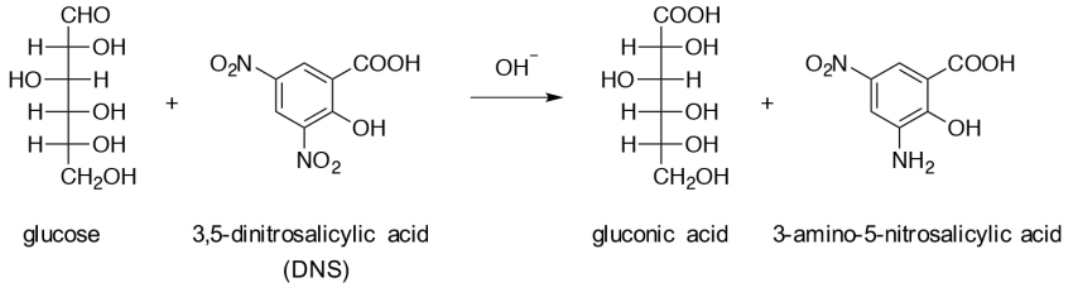

Figure 15 – DNS reaction scheme representing the reduction of the 3,5-dinitrosalicylic acid by glucose to 3-amino-5-nitrosalicylic acid [134].

6.5. MALDI-TOF Mass Spectrometry

MALDI-TOF (matrix-assisted laser desorption/ionization with time of flight) is a mass spectrometry technique thus requiring an ion source of ionization and transference of molecules into the gas phase, a mass analyzer capable to separate ions according to mass-to-charge ratio (m/z) and a detector for the separated ions [135].

In MALDI, the sample is mixed with or coated by a matrix. This matrix is composed by small acid molecules that have a strong optical adsorption in the range of the laser wavelength used. The matrix crystallizes when it dries and the sample, entrapped within, also co-crystalize. The matrix should be selected accordingly to the molecules to analyze and also to the type of laser. 2,5-dihydrobenzoic acid (DHB), sinapic acid (SA) and ferulic acid (FA) are the most common matrices. The sample in the matrix is ionized by a laser beam that generates singly protonated ions from the analytes. The protonated ions are accelerated and separated according to their mass-to-charge ratio (m/z), followed by detection and measurement in a mass analyzer like time of flight analyzer (TOF). TOF analyzers consists of flight tubes, in which the ions travel to reach the detector and the m/z ratio is measured by determination of the time of travel of the ions [135,136].

MALDI-TOF has been used for detection of different biopolymers and macromolecules. Saccharides are not an exception, despite the lower sensitivity and lower detectable mass range when compared with other molecules. MALDI-TOF has been used for determination of chain length of FOS and inulin. In terms of matrices for carbohydrates, DHB has been appointed as the best matrix for saccharides measurements [137,138].

II. Methods and Experimental Procedures

1. Materials and Equipment

1.1. Yacon Roots and Syrup

Yacon roots and syrup were purchased from *Yacon Portugal* and arrived at the laboratory in early December 2018. The roots were stored in the freezer at -20 °C until further use. The syrup was stored on a laboratory shelf at room temperature. Yacon syrup was used as a commercial extract and was diluted in water before use.

1.2. Reagents, Standards and Equipment

The regular laboratory reagents, standards and solvents used are described in **Supplementary Information** section **Table S2**. The equipment and materials used throughout the work are listed in **Table S1**.

2. Yacon Characterization and FOS Extraction

2.1. Raw Material Preparation – Yacon Roots' Treatment

Yacon roots were washed with running tap water to remove any surface dirt. Disinfection of the roots was performed by immersion in water containing sodium hypochlorite for 15 minutes. The roots were peeled and cut in slices, immersed in a cold water and acetic acid bath for 15 minutes to inhibit the activity of polyphenol oxidase (PPO). The roots slices were then cut into tiny pieces before the extraction.

2.2. Yacon Characterization Methodology

2.2.1. Yacon Juice

Four pre-treated yacon roots were weighed (230g) and the juice was made in an EXCEL juicer JE850 (Kenwood). Juice was collected and stored in the fridge at 8 °C until further use.

2.2.2. Moisture Content Determination

The determination of moisture content in yacon roots was performed after thawing and pre-treatment of the samples. Moisture content was measured using a moisture analyzer scale KERN DBS 60-3, with an automatic drying program, where the measuring chamber reaches the temperature of 120 °C, holds that temperature until the sample's weight is constant and stable for 30 seconds. The analysis was performed in triplicates with the result expressed as average ± standard deviation.

2.2.3. Ashes Content Determination

The ashes content of yacon was determined through calcination of the samples in a NABERTHERM L3/11/C6 furnace. Yacon pieces were added to weighed crucibles and all the weights were noted. After calcination, the crucibles were put in a desiccator until they reached room temperature and then the weight of the ashes was determined. The furnace was programmed

for a constant heating for 60 minutes until reaching 600 °C, maintaining that temperature for 8 hours. The process was performed in duplicate and the content determined according to the following equation:

$$(\%)\ Ashes = \frac{w(ashes)(g)}{w(yacon)(g)} \times 100 \quad (1)$$

2.2.4. Total Soluble Solids (°Brix)

Total soluble solids were determined for the raw yacon extracts, purified extracts and also hydrolyzed extracts, using a refractometer ATAGO RX-100 (Tokyo, Japan). Analysis were performed in triplicate and the results expressed in grams of fructose per 100 grams of solution. The results were compared with an already available calibration curve with fructose solutions.

2.3. Yacon FOS Extraction Methodology

For the present work, the extraction procedures aimed for the highest extraction possible of FOS with the lowest economic and ecological costs possible. With that in mind, all the extraction procedures tested used water as extraction solvent.

2.3.1. Extraction Method I

This extract was prepared through the adaptation of two extraction methods described by Contado *et al.* (2015) [139]. A sample of 50g of yacon roots, previously treated, was added to 250 mL of distilled water and heated to 75 °C, with continuous stirring for 1 hour. The mixture was then cooled down to room temperature, decanted and centrifuged for 10 minutes at 4000 rpm, followed by simple filtration. The recovered and filtered supernatant was alkalinized with sodium hydroxide (NaOH) to pH between 9 and 10. The solution was concentrated by heating in a water bath for 2 hours with continuous stirring. The mixture was cooled down to room temperature and 3 volumes of EtOH at 96% were added and cooled overnight in the refrigerator at 8 °C, for polysaccharides' precipitation. After precipitation, the mixture was centrifuged for 10 minutes at 4000 rpm, decanted and the excess of EtOH evaporated in the rotary evaporator. The final extract was frozen until further use.

2.3.2. Extraction Method II

The extraction was performed according to Cabello (2005) [140], 400g of pre-treated yacon roots were added to 200 mL of distilled water at 90 °C in a blender, blended for 2 minutes, followed by simple filtration. After that, the residues in the filter were washed with 300 mL of distilled water at 90 °C. The filtrate and the washing water were combined to form the extract. The final extract was frozen until further use.

2.3.3. Extraction Method III

This extraction method was based in the extraction I and some alterations were performed: 1 L of fresh distilled water was heated up to 75 °C in a water bath and 200g of yacon roots cut in pieces were added; the extraction took place for 1 hour with continuous stirring. After cooling down to room temperature, the mixture was placed in a water bath at 95 °C and a second extraction occurred for another 1 hour with continuous stirring. After cooling down to room temperature the yacon root pieces were separated by decantation and the extract centrifuged for 10 min at 4000 rpm. A light brown extract was obtained and frozen until further use.

3. Purification

3.1. Purification with Baker´s Yeast (*Saccharomyces cerevisiae*)

This purification method, represented in the **Figure 16,** was performed as described by Hernández *et al.* (2009) [141]. *Saccharomyces cerevisiae* was used for fermentation of undesired monosaccharides that are consumed with the formation of CO_2 and EtOH. Some alterations to the published method were introduced: 100 mL of extract was incubated at 37 °C for 24 hours with 1g of dried yeast in an Erlenmeyer flask plugged with cotton. The cotton allowed the maintenance of the aerobic environment inside the flask without building up of pressure. After the incubation time, the resulting EtOH from incubation was evaporated under nitrogen current. The solution was then centrifuged and filtered through 0.45 µm cellulose acetate syringe filter and stored at 8 °C until further analysis. The commercial baker´s yeast (Condi) was acquired at a local supermarket.

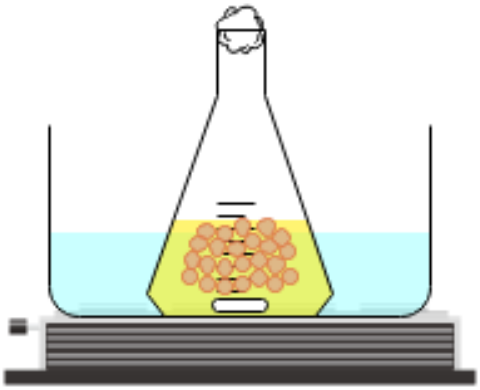

Figure 16 – Purification by yeast fermentation for 24h in a water bath at 37 °C.

3.2. Purification with Activated Charcoal

The process of purification using activated charcoal, based on the method of Hernández *et al.* (2009) [141] involved two steps. The first one was the adsorption of FOS that occurred by adding 3g of activated charcoal into a solution containing 500 mg of lyophilized yacon extract

and 100 mL of a 10% (v/v) ethanolic solution. The slurry was stirred at room temperature in an Erlenmeyer for 30 minutes. Past that time, the charcoal was recovered by vacuum filtration. For the second step, where the desorption of FOS happened, the previously recovered activated charcoal was stirred at room temperature in an Erlenmeyer with 100 mL of a 50% (v/v) ethanolic solution for 30 minutes. The mixture was filtrated at low pressure in porous plate funnel filter Nº2. The charcoal was washed 3 times with 10 mL of a 50% (v/v) ethanolic solution. The EtOH was evaporated in a rotary evaporator and the obtained solution re-filtered in cellulose acetate 0.45µm filters to eliminate the smaller charcoal particles still present. The solution was then lyophilized to obtain a dry purified extract.

3.2.1. FOS Adsorption into Activated Charcoal

Following the method used by Weston *et al.* (1999) [142] for oligosaccharide adsorption into activated charcoal, 4 g of commercial yacon syrup was dissolved in 80 mL of distilled water. 16 g of activated charcoal was added to the solution. Adsorption in a slurry method was tested for 24 hours with continuous stirring at room temperature. Aliquots were collected before the addition of the charcoal, every hour after its addition for 8 hours, and a final aliquot at the end of the study (24 hours). °Brix and reducing sugars by DNS method were measured, in order to evaluate rates of adsorption.

3.3. Purification with Activated Charcoal and Celite® 577

This purification process was performed using the principle of dry column vacuum chromatography, generally used for silica, and adapting the method of Whistler and Durso (1950) [143] and Hernández *et al.* (2009) [141]. Two filtering paper discs (Whatman® filtering paper number 1) were placed in a porous plate funnel filter Nº2, and 3 grams of Celite® 577 were distributed over the paper. Distilled water was added until Celite® was fully compacted.

The initial steps of this process were similar to those presented in the previous method. Firstly, 3 grams of activated charcoal were added into a solution containing 500 mg of lyophilized yacon extract and 100 mL of a 10% (v/v) ethanolic solution. After stirring for 30 minutes at room temperature, the solution was poured into the previously prepared column in the glass funnel filter Nº 2, as showed in **Figure 17.** The solution was filtered, and the liquid discarded. After this, for FOS desorption, 500 mL of a 50% (v/v) ethanolic solution was poured into the column and eluted by vacuum. The EtOH was evaporated in the rotary evaporator and the solution lyophilized and stored in the fridge until further analysis.

Figure 17 – Scheme of the purification with activated charcoal and Celite® 577.

3.4. Inulin Precipitation with Anti-solvents

This purification process consisted in the precipitation of inulin through the addition of anti-solvents with low toxicity. For this purpose, and following the method described by Ku *et al.* (2003) [144], the solvents chosen were EtOH, propanol and acetonitrile (ACN). Four ratios of solvent to extract were tested (1:1; 2:1; 3:1 and 4:1 v/v). Precipitations occurred overnight at 8 °C. Samples were centrifuged, dried and the amounts of inulin obtained were determined by gravimetry through the difference between masses before and after precipitation. The process leading to the highest amount of inulin were selected for analysis by MALDI-TOF to verify the maximum DP.

4. FOS Production from Inulin Hydrolysis

4.1. Immobilization in Alginate Beads

Endo-inulinase was immobilized in calcium alginate beads, adapting the method described by Zhou *et al*. (2010) [145]. 100 µL of endo-inulinase from *Aspergillus niger*, purchased from Megazyme, was dissolved in 2 mL of sodium acetate buffer (0.1 M pH 5.0). The choice of buffer solution followed the Megazyme instructions for the enzyme usage (**Supplementary Information**, **Table S3)**. This solution was then added to 20 mL of an alginate solution at 3% (w/v) prepared in sodium acetate buffer and stirred until complete dissolution. This solution was extruded from a syringe, droplet by droplet, to a 2% (w/v) calcium chloride solution, in an ice bath, in order to form the beads. The formed beads were washed 3 times with distilled

water and 1 time with sodium acetate buffer and stored in the buffer solution until further use. The beads were rinsed every time before using in the hydrolysis process.

The immobilization yield was determined by the following equation:

$$Immobilization\ Yield\ (\%) = \frac{Activity\ of\ the\ immobilized\ enzyme}{Activity\ of\ the\ free\ enzyme} \times 100 \qquad (2)$$

4.2. Enzymatic Assays using Free and Immobilized Enzyme

According to the data supplied by Megazyme for the endo-inulinase (**Supplementary Information**, **Table S3**) and applying a method adapted from Karimi *et al.* (2016) [146], endo-inulinase activity towards inulin was assayed. This activity was tested, in triplicate, adding 100 µL of enzyme solution to 400 µL of commercial inulin solution at 0.2% (w/v) prepared in sodium acetate buffer (0.1 M pH 5.0). Reaction occurred for 30 minutes at 45 °C. The reaction was stopped with the addition of 500 µL of DNS reagent, and heating in a boiling water bath for 5 minutes. Then, the mixture was cooled in an ice bath for 5 minutes to stop the DNS reaction. 200 µL of each solution were transferred to a well of a 96-well microplate and absorbance was read at 550 nm. The original method [146] described the readings to be performed at 540 nm, using a spectrophotometer and cuvettes. Since in the present work the option was to use small amounts and a microplate reader, the closest filter available was 550 nm. An experiment using the spectrophotometer showed no relevant differences in absorbance for the two wavelengths.

The same method was applied for the immobilized enzyme with a slight change: instead of 100 µL enzyme solution, a sphere of immobilized enzyme, with approximately 30 mg, was used.

One unit of inulinase was defined as the amount of fructose (1 µmol) produced per minute by the enzyme using this assay. Enzyme activity in µmol/min was obtained through the following equation:

$$Enzyme\ Activity\ (\mu mol/min) = \frac{\text{Concentration of Product (µmol/mL)}\times\text{Total Reaction Volume (mL)}}{\text{Reaction Time (min)}} \qquad (3)$$

4.3. FOS Production Intra- and Inter-day Analysis

Intra- and inter-day assessments of the immobilized system were performed to study the system capability to be reutilized. For the intra-day study, 4 cycles were tested and for the inter-day study the viability of the system to be stored at room temperature in the buffer solution for 4 consecutive days was also tested. All tests were performed with 1g of immobilized system and 10 mL of inulin solution at 1% (w/v) in 0.1 M sodium acetate buffer, for 30 minutes at 55 °C with continuous stirring.

4.4. Batch Production in Recirculating System

Batch production of FOS was carried as proposed by Nguyen *et al.* (2011) [147]. The hydrolysis occurred in a recirculating system for 24h at 55 °C with immobilized enzyme in calcium alginate beads. A column was prepared in a 10 mL syringe, packed with 7.5 g of loaded calcium alginate beads and covered with glass beads and cotton on both ends, to regulate the flow rate and to prevent the escape of calcium-alginate/inulinase beads. 200 mL of yacon extract, previously lyophilized, was dissolved in sodium acetate buffer 0.1 M pH 5.0 and was fed to the column with a peristaltic pump, in an up-flow rate of 2.5 $mL.min^{-1}$. Samples were collected hourly and were assayed for protein content and reducing sugars. The full system for the continuous FOS production is represented in **Figure 18**.

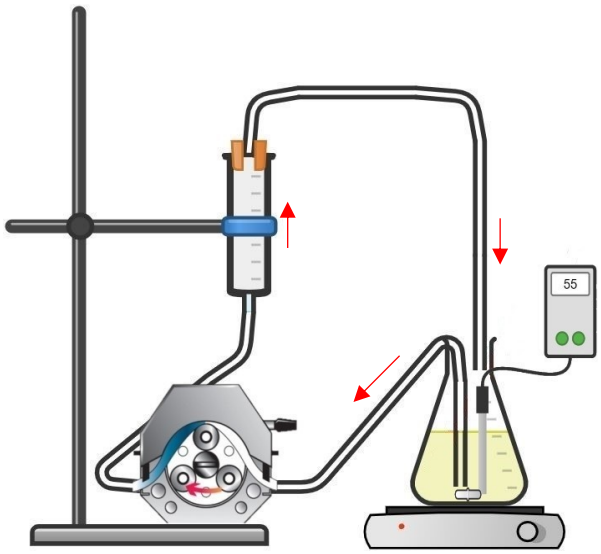

Figure 18 – Scheme of the batch production of FOS in a recirculatory system.

5. Analytical Methods

5.1. Reducing Sugars Quantification by DNS Method

The method for detection of reducing sugars was adapted from the one described by Arrizon *et al.* (2010) [148]. 250 µL of sample was mixed with 250 µL of DNS reagent (10 g/L 3,5-dinitrisalicylic acid –DNS, 300 g/L sodium and potassium tartrate and 16 g/L NaOH). The mixture was shaken and heated for 5 minutes at 100 °C. In order to stop the reaction, the mixture was immersed in an ice bath for 5 minutes. Then, 200 µL were added to 96-well microplate wells and absorbance was read at 550 nm in the microplate reader Perkin Elmer VICTOR^3 1420. The reducing sugars quantification was obtained comparing the absorbance of the samples against a standard curve determined for glucose from 0.125 g/L to 1.5 g/L (**Supplementary Information**, **Figure S1**).

5.2. HPLC-RI Compound Quantification

The resulting raw and purified extracts and the products of enzymatic hydrolysis were analyzed by HPLC (Dionex UltiMate 3000) using a ReproGel-Na column (250 x 8 mm, Maisch Gmbh) and a guard column CARBOSep CHO 411 (Concise Separations) at 80 °C and a Shodex RI-101 detector at 50 °C. The elution was isocratic with MiliQ Ultrapure Water (filtered with 0.22 µm cellulose acetate filters (Whatman®)) used as eluent at a flow rate of 500 µL/minute. Total FOS concentration was estimated as the sum of all FOS with DP ranging from 2 to 5. All detailed conditions can be found in **Table 3**.

Table 3 – Chromatographic conditions for HPLC-RI analysis used in the determination of the concentration of each saccharide present in the analyzed samples.

Column: **Stationary Phase:** **Guard column:**	ReproGel-Na, 9 µm 250 x 8 mm (Dr. Maisch Gmbh, Germany) PS/DVB (Polystyrene Divinylbenzene) CARBOSep CHO 411 Na 4.0 x 24 mm (Concise Separations)
Mobile Phase	MiliQ Ultrapure Water
Elution Gradient	Isocratic: 100% Ultrapure Water
Detector	Shodex RI-101 (50 °C)
Run Time	15 minutes
Analysis Temperature	80 °C
Injection Volume	5 µL
Flow Rate	500 µL/min
Retention Time	Fructose: ≈ 11.4 min Glucose: ≈ 10.4 min Sucrose: ≈ 7.8 min DP3: ≈ 6.7 min DP4: ≈ 6.1 min DP5: ≈ 5.9 min Inulin: ≈ 5.5 min

5.2.1. Development and Validation of the Analytical Method

For the development and validation of the HPLC-RI method, linearity, sensitivity (limits of detection, LOD, and limits of quantification, LOQ) and precision (intra-day and inter-day precisions) were assayed, for each standard. Standards solutions of fructose, glucose, sucrose, 1-kestose (DP3), 1,1-kestotetraose (DP4), 1,1,1-ketopentaose (DP5) and inulin prepared in the mobile phase ultrapure water were assayed.

5.2.1.1. Linearity

The linearity was tested for all standards available in the concentration range comprehended between 0.125 g/L – 1.5 g/L, with the exception of 1-kestose and 1,1-kestotriose that were tested in a concentration range between 0.125 g/L – 1 g/L.

5.2.1.2. Sensitivity

The sensitivity of the method was assessed through calculation of the LOD and LOQ for each of the previously stated standards.

The LOD were calculated using the following equation:

$$LOD = \frac{\sigma \times 3.3}{m} \quad (4)$$

where σ is the standard deviation of the intersection with the ordinate axis (Y) and *m* is the slope of the calibration curve.

The LOQ were calculated using the following equation:

$$LOQ = \frac{\sigma \times 10}{m} \quad (5)$$

where σ is the standard deviation of the intersection with the ordinate axis (Y) and *m* is the slope of the calibration curve.

5.2.1.3. Precision

The precision of the method was evaluated through the calculation of the percentual relative standard deviation (RSD%) of the standards when tested for their intra-day (repeatability) and inter-day (intermediary) precisions.

For the determination of the repeatability of the method, three different concentrations of each standard were prepared (0.25 g/L; 0.5 g/L and 1 g/L) and injected in sextuplicate (n=6) maintaining the experimental conditions, all in the same day.

The determination of the intermediary precision was also studied for three different concentrations of each standard (0.25 g/L; 0.5 g/L and 1 g/L), this time for two non-consecutive days, under the same experimental conditions. All analyses were performed in sextuplicate (n=6).

5.3. Thin Layer Chromatography

The extracts, the yacon juice and the resulting products from the hydrolysis and purification with activated charcoal were analyzed by TLC. Two applications (of 1.5 µL each) per sample and standards were placed in precoated silica gel plates (Silica gel 60 F_{254} from Merck). For the development of the TLC plates, three mobile phases were tested: ACN: water (85:15), ethyl acetate: acetic acid: 2-propnol: formic acid: water (20:10:5:1:15) and butanol: acetic acid: water (2:1:1) as presented by Reiffová (2014) [149]. TLC plates were developed using 0.3%

(w/v) N-(1-naphthyl)ethylenediamine and 5% (v/v) of concentrated sulfuric acid in methanol, and heating at 100 °C in an oven for 10 min [150].

5.3.1. TLC and Densitometry for Sugar Quantification

TLC is a simple and low-cost technique generally associated to qualitative analysis of compounds. Although some TLC techniques can be associated to some quantitative tools, such as flatbed scanners and densitometers, the incorporation of such methods into laboratory workstations in safe conditions can be challenging. The use of digital photography, through a smartphone, for example, emerges as an alternative route to obtain good and reliable images for quantitative analysis. Digital photography, associated to computer software, such as ImageJ, a known tool for electrophoretic gels, can be easily adapted and applied for TLC quantification [151].

After the elution and spot development on the TLC plate, the acquisition of the image was performed with a smartphone. The image was then uploaded to the ImageJ computer software. The software converted the image to black and white and the brightness and contrast were adjusted. The software required the bands containing the obtained TLC spots to be displayed vertically, in order to be selected for analysis. Each line of analysis (each application point and originated spots) was individually selected and the densitogram plotted with the areas of each spot in the TLC, calculated by the program according to their intensity and spot size in the plate. A calibration curve was drawn for fructose with four different concentrations ranging between 0.25 g/L and 1 g/L that were analyzed in tetraplicate (**Supplementary Information**, **Figure S3** and **Figure S4**).

5.4. MALDI-TOF

MALDI-TOF MS measurements were performed using a Bruker Autoflex maX Matrix-Assisted Laser Desorption and Ionization (Time-of-Flight) 2 Mass Spectrometer operating in the positive linear ion mode. Laser strength was varied from sample to sample to obtain the best signal. The matrix used for all samples was 2,5-dihydroxy benzoic acid (2,5-DHB) enriched with Na (sodium). External calibration was performed using the $[M + H]^+$ ions of a peptide mixture. Samples were at 10 mg/mL (in ultrapure water) and filtered through 0.45 µm cellulose acetate filters. Inulin precipitated with ACN, propanol and EtOH in the extract III were the samples analyzed by this technique.

5.5. Immobilization Efficiency Assayed by the Bradford Method

A totally efficient encapsulation of the inulinase by alginate would not permit the presence of protein in the solution. This efficiency must be tested to ensure that the observed activity is caused by the action of the immobilized enzyme and not by that of any enzyme in the free form eventually released from the calcium alginate beads. Colorimetric methods for protein

detection are generally used for enzyme detection. In this work, the detection of endo-inulinase was performed by the Bradford method (1976), with some adaptations [152]. 100 µL of samples or standard BSA solutions, with concentrations ranging from 0.05 g/L to 1 g/L, were added to 5 mL of Bradford reagent and were gently mixed. As a blank, a solution of 0.1 M pH 5.0 acetate buffer was used. After mixing the solutions, incubation occurred for 5 to 10 minutes at room temperature. Absorbance was measured at 595 nm in Perkin Elmer Lambda 2 for protein quantification, with a calibration curve of BSA as reference (**Supplementary Information**, **Figure S2**).

III. Results and Discussion

1. Yacon Characterization

Before any extractions and analysis were performed, yacon was characterized by assaying its moisture, ash, total soluble solids and reducing sugars contents. Moisture and ash contents were obtained from freshly cut yacon roots (wet basis) and total soluble solids and reducing sugars contents from freshly made yacon juice.

1.1. Moisture Content Determination

Small yacon samples were cut and analyzed in the moisture analyzer scale. Moisture content was determined, in triplicate, and the obtained mean value was 88.73 ± 0.64%. This value is in accordance to the value of 88.16 ± 0.20% reported by Alles *et al.* (2015) [153] and that reported by Silva *et al.* (2018) [154] that ranged between 88.86% and 91.21%.

1.2. Ash Content Determination

The determination of the ash content, by determining the mass of the ashes after calcination, was performed in triplicate, resulting in a mean value of 0.35 ± 0.11%. This complies with the literature since it is between the values presented by Lachman *et al.*(2003) [53], between 0.26% and 3.50%, and also those reported by Silva *et al.* (2018) [154] that ranged from 0.16% to 0.55%. The discrepancy in some of the values found in the literature are unsurprising, since the ash content is strictly related to the accumulation of minerals, such as sodium, magnesium, calcium and potassium, and that accumulation may depend on the type of soil, altitude of the cultivation, the cycle of cultivation and temperature [154].

1.3. Total Soluble Solids (°Brix)

In terms of the determination of the total soluble solids, the values were measured in triplicate consisting on a mean value of 10.1 ± 0.12%. 1 °Brix is defined as 1 gram of soluble solid in 100 grams of solution. Since yacon roots have high values of sugar content, and low values of other soluble components, it was assumed that the variations in the refractive index of the yacon juice was majorly due to the presence of those sugars. The °Brix obtained was consistent with the values found in the literature ranging from 8 to 12 and 9.9 to 12.6 as reported by Manrique *et al.*(2003) [155] and Herman *et al.* (1998) [156], respectively.

1.4. Saccharides Content

For saccharides content determination, yacon juice was diluted and injected in the HPLC and the concentrations of inulin, glucose and fructose in g/ 100g of yacon (dry mass) were determined. The concentrations of each saccharide in the concentrated yacon juice, were calculated: 9.06 ± 0.31 for inulin, 1.15 ± 0.03 for glucose and 1.39 ± 0.04 for fructose. The inulin percentage obtained was higher than the values reported by Lago *et al.* (2012) [54] and Brites and Noreña (2016) [157], with 1.07 ± 0.18 and 5.42 ± 0.01, respectively. Glucose and fructose

amounts were substantially lower than those described by the other studies. Lago *et al.* (2012) reported 3.30 ± 0.28 for glucose and 2.99 ± 0.18 for fructose, while Brites and Noreña (2016) reported 4.74 ± 0.01 for glucose and 11.00 ± 0.03 for fructose.

2. Yacon FOS Extraction and Quantification

2.1. TLC Analysis

A TLC method was optimized using three mobile phases, previously referred. **Figure 19** shows the difference in the spot definition for the three mobile phases, extracts and purified extracts (A: ACN: water (85:15); B: ethyl acetate: acetic acid: 2-propanol: formic acid: water (20:10:5:1:15) and C: butanol: acetic acid: water (2:1:1)).The ACN: water (85:15) mixture showed the lowest resolution, followed by the ethyl acetate: acetic acid: 2-propanol: formic acid: water (20:10:5:1:15) mixture. The mobile phase of choice with better definition in the resulting spots consisted in butanol: acetic acid: water (2:1:1).

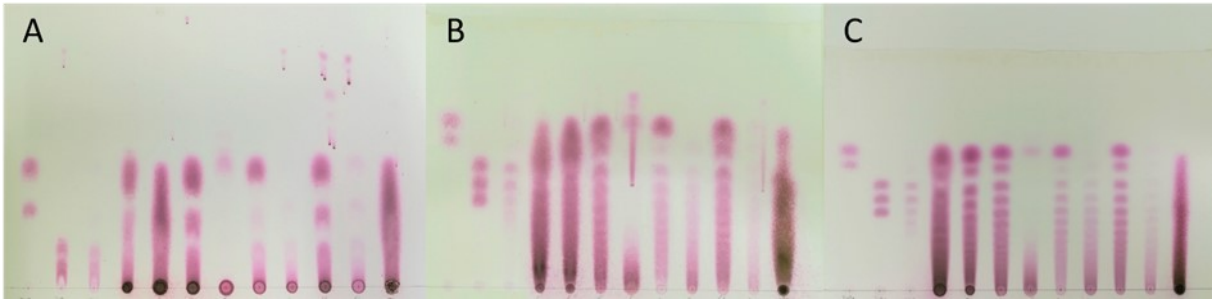

Figure 19 – Comparison between the three mobile phases tested. **A**: ACN: water (85:15); **B**: ethyl acetate: acetic acid: 2-propanol: formic acid: water (20:10:5:1:15) and **C**: butanol: acetic acid: water (2:1:1).

The best chromatographic method was applied to the available standards (lanes 1 to 3) and to the extracts (lanes 4 to 6) in the same plate (**Figure 20**). It is possible to verify the presence of inulin, several FOS, sucrose and monosaccharides in all three extracts. Inulin and monosaccharides appear with more intense spots in all of the three extracts, a strong indicative of higher concentrations of these carbohydrates.

As showed in **Figure 20**, and based on the spots' intensities, extract I (lane 4) appears to have the highest concentrations for all saccharides, mainly inulin, due to the high intensity bands and sample drag showed in the application point. Besides, the spots almost overlap, showing low definition and a strong indicative that the sample is too concentrate for the sensitivity of the method. The only spots correctly separated correspond to monosaccharides (DP1), disaccharides (DP2) and DP3. However, the extract could have been diluted for a better definition between the spots and sugars with higher DP's could be identified. Extract II (lane 5) comes in second in terms of spot intensity, although there is a higher definition than Extract I with the possibility to identify FOS up to DP5. This observed definition is due to lower concentration of the extract when compared to Extract I. Once more, the dilution of the extract could enable the separation of sugars with higher DP's in the extract. Extract III (lane 6) shows the lowest intensity in the bands, with

a higher definition and the possibility to identify FOS up to DP7. Furthermore, Extract III seems to have the most even distribution of the carbohydrates, with higher intensity for inulin and monosaccharides, possibly because it is significantly more diluted than the other extracts. These results are in accordance to those reported by Contado *et al.* (2015) [139], that have also identified the presence of FOS with DP up to 7 in this extracts by TLC, using 2-butane: 2-propanol: water (3:12:4 (v/v)) as mobile phase.

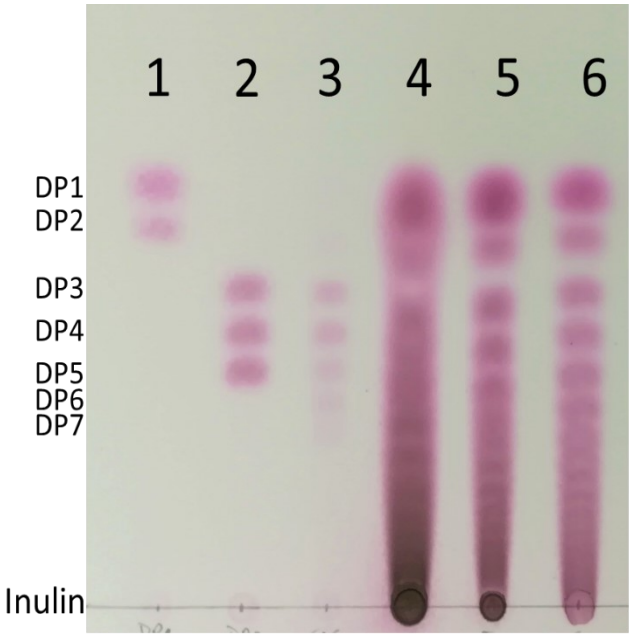

Figure 20 – TLC performed with butanol: acetic acid: water (2:1:1) for the standards (lanes 1 to 3) and yacon extracts (lanes 4 to 6). **Lane 1**: Mixture of fructose and sucrose; **Lane 2**: Mixture of 1-kestose, 1,1-kestotetraose and 1,1,1-kestopentaose; **Lane 3**: Commercial FOS mixture Beneo Orafti P95; **Lane 4**: Extract I; **Lane 5**: Extract II; **Lane 6**: Extract III.

For all the distinguishable spots, the retention factors (Rf's) were calculated for the extracts (lanes 4 to 6) and compared to the Rf's of the standards (lanes 1 to 3) as shown in **Table 4**. Despite it being possible to observe the presence of higher DP3 in all the three extracts, the Rf's were calculated only for those spots perfectly separated from each other. Since the three extracts were applied in the same amount to the plate (3 μL) it is safe to conclude that Extract I is the richest in all the sugars in solution. Furthermore, if Extracts I and II were diluted similar results to those obtain for Extract III could possibly be observed in terms of definition of the spots in the TLC plate.

Table 4 – Retention factors calculated for the saccharides standards and for the extracts.

Standards		
Lanes	**Correspondent Standard**	**Retention factor**
1	Fructose	0.58
	Sucrose	0.52
2	1-kestose (DP3)	0.43
	1,1-kestotetraose (DP4)	0.38
	1,1,1-kestopentaose (DP5)	0.32
3	Beneo Orafti P95 (DP3 to DP6)	0.43
		0.38
		0.32
		0.28
Extracts		
Lanes	**Correspondent Extract**	**Retention factor**
4	Extract I	0.58
		0.52
		0.43
5	Extract II	0.58
		0.52
		0.43
		0.38
		0.32
6	Extract III	0.58
		0.52
		0.43
		0.38
		0.32
		0.28
		0.23

2.2. HPLC Method Validation

All the HPLC conditions were established (**Table 3, II. Methods and Experimental Procedures**, page 46) based on the manufacturer's information for the chromatographic column available. The column used for the development and validation of the chromatographic method was a ReproGel-Na (Dr. Maisch Gmbh) filled with a PS/DVB (Polystyrene Divinylbenzene) copolymer, as mentioned in **Table 3**. The polymer can be functionalized into different ionic forms that can enhance the separation of the samples. In this case, the functionalization with sodium (Na) enhances the separation of sugars. This type of stationary phase is commonly used for this purpose, since it works as an ion-exchange resin and with a simple mobile phase such as water [158]. Batches of ultra-pure water freshly filtered through 0.22 μm membranes were used as mobile phase for each analysis. The column oven temperature was kept at 80 °C. The refractive index detector (Shodex RI-101) was kept at 50 °C. The analytical temperature's stability is crucial, since a major problem when using a RI detector lies in baseline instability, often caused by temperature fluctuations, since refraction depends on density which depends on temperature.

Baseline drifts can also be caused by the presence of dissolved gases in the mobile phase, differences in flow rates or back pressures that surpass the manufacturer's recommendation.

Using the previously stated conditions, with injections of 5 µL per sample and flow rate at 500 µL/min, the chromatograms obtained presented a stable baseline and a good resolution and separation of peaks, which were mostly symmetrical. Despite having an ion exchange filling, the column used, can also be considered of size exclusion, promoting the separation of sugars by size. Sugars with highest DP were the first eluted. Inulin was the first, with a retention time of 5.5 minutes, follow by DP5, DP4 and DP3 at 5.9, 6.1 and 6.7 minutes, respectively. Concerning smaller sugars, sucrose eluted first at 7.8 minutes followed by glucose and fructose at 10.4 and 11.4 minutes, respectively. Other peaks were either below the LOD and/or LOQ thresholds or impossible to identify due to lack of standards for higher DP FOS (DP>5), which are not commercially available.

The linearity of the method was assessed through external standardization. The calibration curves were drawn in concentrations ranging between 0.125 g/L and 1.5 g/L. The method proved to be linear with correlation factors higher than 0.98 (**Table 5**). Furthermore, for each reference substance, the standard deviation of the intersection with the ordinate axis was obtained and the sensitivity of the method determined through calculation of the LOD and LOQ. The proposed method showed very low sensitivity with the exception of DP4 that presented LOD and LOD of 0.0233 g/L and 0.0705 g/L respectively. Good values were verified for the detection of fructose and DP5 with 0.0588 g/L and 0.0732 g/L respectively. All other values surpassed the minimal concentration used of 0.125 g/L (**Table 5**). When compared with other results, these are higher than the values reported by Correia *et al.* (2014) [159] that ranged between 0.03 and 0.05 g/L for LOD and between 0.10 and 0.2 g/L for LOQ. Lima *et al.* (2019) [160] reported values for DP4 very similar to those of the present work 0.0704 g/L for LOD and 0.179 g/L for LOQ.

Table 5 – HPLC-RI validation method parameters.

Standards	Concentration Range (g/L)	Regression equation	Regression coefficient (R^2)	LOD (g/L)	LOQ (g/L)
Fructose	0.125 – 1.5	y = 1.3563x + 0.015	0.9990	0.059	0.178
Glucose	0.125 – 1.5	y = 1.1304x + 0.1301	0.9802	0.270	0.818
Sucrose	0.125 – 1.5	y = 0.9772x + 0.0504	0.9935	0.152	0.460
DP3	0.125 – 1	y = 1.3026x – 0.0166	0.9995	0.234	0.710
DP4	0.125 – 1	y = 1.2725x – 0.0255	0.9997	0.023	0.071
DP5	0.125 – 1.5	y = 1.1764x + 0.0007	0.9987	0.073	0.222
Inulin	0.125 – 1.5	y = 0.9703x + 0.0655	0.9821	0.254	0.770

For the validation of the method, it is necessary to consider random variations that can be estimated by the evaluation of the precision. The estimation of the precision is expressed by two parameters: the intra-day precision, also known as repeatability, and the inter-day precision (intermediate precision). According to Correia *et al.* (2014) [159] the relative standard deviation (RSD) should be lower than 5%, so the values can be accepted. As showed in **Table 6**, in both intra-day and inter-day injections, the variability is generally satisfactory since most of the values presented less than 5% in their RSD. The method showed good repeatability, since for the intra-day precision, the RSD ranged between 1.30% and 4.44%, and also revealed good intermediate precision, with exception of inulin that presented an RSD above 5% (5.04% and 7.61%). Considering that inulin is not a single molecule but a mixture of polymers with a range of sizes, somewhat pronounced RSD values are not unexpected. What is more, the detection is made by refractive index and, in a complex mixture, intermolecular rearrangement due to agitation, thermodynamic stabilization or even autohydrolysis are expected but not controllable factors that may affect the RI detection. Apart from inulin, the RSD for inter-day precision of the other standard's analyses ranged between 1.3% and 4.5%. These results are in accordance with those reported by Li *et al.* (2014) [161] that have obtained RSD values between from 1.2% and 4.6% for intra-day precision and between 2.9% and 7.7% for inter-day precision, with samples analyzed by UPLC-MS/MS.

Table 6 – Intra-day and inter-day precision determined for the applied HPLC-RI method.

Standards	Concentrations (g/L)	Intra-day precision (n=6)		Inter-day precision (n=6)	
		Mean	RSD (%)	Mean	RSD (%)
Fructose	0.25	0.24 ± 0.00	2.01	0.19 ± 0.01	3.02
	0.5	0.49 ± 0.01	1.30	0.36 ± 0.01	2.63
	1	0.99 ± 0.02	2.08	0.73 ± 0.02	3.23
Glucose	0.25	0.23 ± 0.01	3.36	0.14 ± 0.01	4.54
	0.5	0.54 ± 0.01	1.95	0.35 ± 0.01	3.57
	1	1.05 ± 0.03	2.44	0.72 ± 0.03	3.80
Sucrose	0.25	0.23 ± 0.01	3.69	0.17 ± 0.01	4.15
	0.5	0.49 ± 0.01	1.96	0.35 ± 0.01	3.27
	1	0.97 ± 0.02	2.06	0.70 ± 0.03	3.94
DP3	0.25	0.25 ± 0.01	2.26	0.21 ± 0.01	3.03
	0.5	0.48 ± 0.01	1.51	0.37 ± 0.01	3.80
	1	0.99 ± 0.03	3.45	0.78 ± 0.01	1.56
DP4	0.25	0.25 ± 0.00	1.38	0.21 ± 0.00	1.58
	0.5	0.47 ± 0.01	1.97	0.37 ± 0.01	3.36
	1	0.93 ± 0.03	2.71	0.74 ± 0.01	1.57
DP5	0.25	0.26 ± 0.01	2.00	0.21 ± 0.00	1.26
	0.5	0.50 ± 0.01	1.90	0.37 ± 0.02	4.34
	1	1.01 ± 0.04	3.56	0.77 ± 0.01	1.47
Inulin	0.25	0.23 ± 0.01	4.20	0.16 ± 0.01	7.61
	0.5	0.49 ± 0.02	3.31	0.39 ± 0.02	4.21
	1	1.08 ± 0.05	4.44	0.90 ± 0.05	5.04

2.3. FOS Content Quantification in Yacon Extracts

All three tested extracts were analyzed by the HPLC-RI method previously established and validated. Since all quantifications were performed by external standardization, the values for FOS accounted for DP3, DP4, DP5 and inulin, which were the only available standards of FOS.

TLC results suggest the presence of FOS with DP up to 7 in the yacon extracts analyzed, as did Li *et al.* (2014) [162] who reported the presence of FOS up to DP13 in yacon and other plants in the *Compositae* family through analysis by HPLC-CAD. Based on this information these compounds were expected to be detected in the HPLC-RI chromatograms, however this was not possible. The column used is appropriate for the separation of saccharides with DP1 to DP5. Those with DP≥5 are detected as a single peak, attributed to inulin in this case, insufficiently separated from that of single DP5. As a result, in this work, the samples' FOS content only accounted for DP3, DP4 and inulin as a whole, since the separation of the peak for DP5 was not ideal. **Table 7** shows the contents of all the quantifiable saccharides, including fructose, glucose and sucrose, in both g/L and mg/g of yacon.

Concerning the monosaccharides, Extract II presented the highest concentration values with 8.98 g/L, followed by Extract I with 7.15 g/L and lastly Extract III with 3.51 g/L. In terms of content of sucrose, the same extract sequence was observed with 1.93 g/L, 1.49 g/L and 0.96 g/L corresponding to Extract II, Extract I and Extract III, respectively. Regarding FOS content, the same order was not noticed. Extract I was the richest in FOS with 56.27 g/L, surpassing extract II and III with 35.15 g/L and 13.14 g/L, respectively. In terms of the expression of FOS content in mg/g, Extract III presented 74.51 mg/g, surpassing Extract II with 57.10 mg/g, and Extract I presented the higher content with 150.92 mg/g. Despite the increased complexity of the experimental procedure used to obtain Extract I, it was by far the most effective, yielding more FOS than Extracts II and III, with the latter being the worst method. Other studies showed a great variation in their FOS contents. Li *et al.* (2014) [162] reported total FOS contents of 145.13 mg/g and 157.17 mg/g, whilst Contado *et al.* (2015) [139] reported FOS content ranging from 91.03 mg/g to 170.48 mg/g. The obtained concentrations are in accordance with the previously reported studies, even despite the impossibility to distinguish DP higher than 4, through the used HPLC-RI method. The differences on FOS contents observed can be justified by physiological differences of the roots at the time of the harvest, the growth conditions, the weather conditions, the type of cultivar used and also the time and type of storage conditions of the roots. Both Graefe *et al.* (2004) [163] and Contado *et al.* (2015) [139] showed reduction in FOS content derived from storage for 12 and 20 days, respectively. The first reported a concentration decrease of about one third while the latter maintained more than 90% of the initial content.

Table 7 – Contents of all the quantifiable saccharides detected by HPLC. The columns for "g/L" refer to the concentration of the extract solution, while the columns for "mg/g" refer to the mg of said sugar per g of yacon.

Extract	Fructose		Glucose		Sucrose		DP3		DP4		Inulin[a]	
	g/L	mg/g	g/L	mg/g	g/L	mg/g	g/L	mg/g	g/L	mg/g	g/L	mg/g
Extract I	3.25	8.73	3.90	10.45	1.49	3.98	1.55	4.15	2.96	7.94	51.76	138.83
Extract II	4.81	7.81	4.17	6.78	1.93	3.13	1.61	2.62	1.83	2.96	31.71	51.52
Extract III	1.94	11.01	1.57	8.88	0.96	5.45	0.44[b]	2.51	0.68	3.85	12.02	68.14

[a] Include the values for DP5

[b] Value below the limit of quantification

3. Purification Methods

Extract III was chosen as a model to test the purification techniques, because there was a large amount of it available. All described purification techniques were tested with this extract, which was later also used for the enzymatic inulin hydrolysis, since the work to be performed was essentially a proof of concept.

3.1. Purification with Baker's Yeast

An attempt of FOS purification using commercial baker's yeast, for 24 hours, was tested. The commercial yeast contained dry *Saccharomyces cerevisiae* and the emulsifier sorbitan monostearate. The latter is generally present in these sort of baking yeasts to improve their activity when the rehydration occurs. This yeast was tested with the objective to remove monosaccharides and disaccharides from the extract, by fermentation, in order to purify the extracted FOS. This type of yeasts, when in aerobic conditions, ferment sugars, especially glucose and fructose, into EtOH and carbon dioxide [164]. This purification technique, despite consuming almost all the monosaccharides and disaccharides, as desired, also consumed FOS with DP4 and higher and drastically reduced the amounts of inulin, as observed in the TLC shown in **Figure 21A**. Concerning monosaccharides, fructose concentration decreased from 1.94 g/L to 0.26 g/L, glucose was almost fully consumed, showing only a vestigial peak at 10.4 min, as shown in **Figure 21B**, and the final concentration was way below LOD and LOQ. Sucrose also showed a very low prominence peak, representing very low amounts of this sugar, whose concentration was reduced from 0.96 g/L to a value below the LOD. In terms of FOS, only inulin could be truly quantified with its concentration reducing from 12.02 g/L to 7.87 g/L. In place of DP3 and DP4, a peak with tailing could be observed at around 6.7 minutes (**Figure 21**). This peak, along with the other two low prominence peaks that appear between 7.8 minutes and 10.4 minutes (sucrose and glucose) and the two peaks after 11.4 minutes (fructose) may be indicative of the presence of

secondary metabolites of the action of *Saccharomyces cerevisiae*, such as isopropanol, ethyl acetate, acetaldehyde, acetic acid, and glycerol [164].

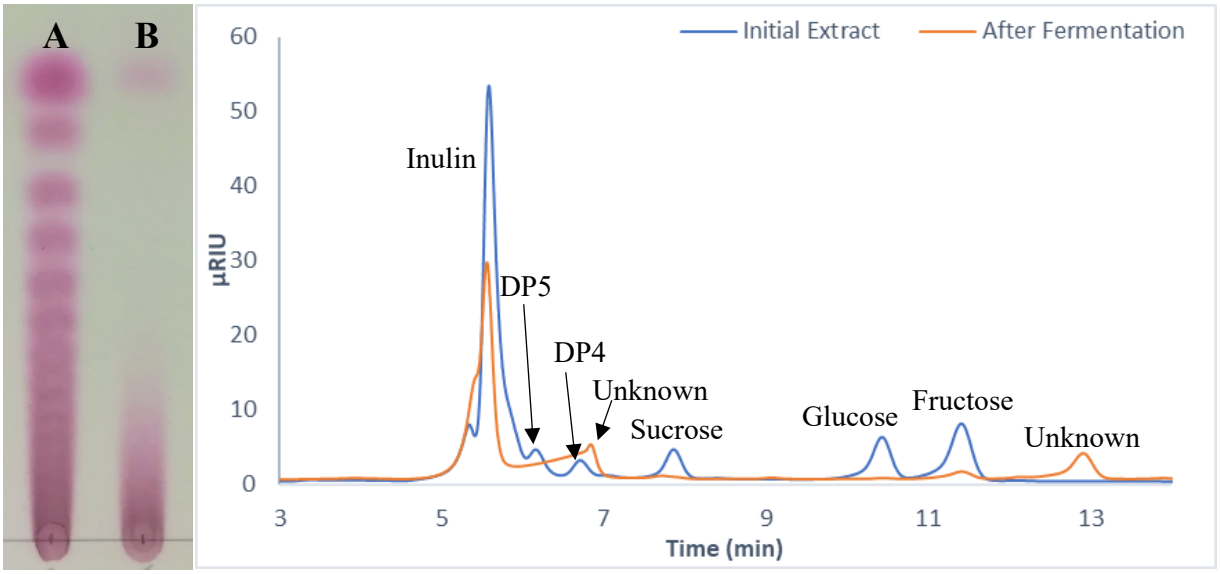

Figure 21 – Results of the purification of extract III with baker's yeast. **Left**: TLC before (**A**) and after (**B**) the fermentation; **Right**: Chromatogram for the analysis of the extract before and after the fermentation.

The presence of secondary metabolites resulting from the fermentation allied to the consumption and possibly adsorption of some FOS by the yeast deemed this method inappropriate for the purification of FOS. This was most-likely caused by the chosen experimental conditions applied, derived from published methods, that proved to be too harsh, rather than the method being ineffective: the ratio yeast/extract ratio was very high, the reaction period was too long, and the yeast cells were found to be too active. These discussed experimental parameters should be optimized.

3.2. Activated Charcoal

3.2.1. Study of Adsorption in Activated Charcoal

The study of adsorption in activated charcoal over time had the objective to assay the selectivity, retention capacity and time of adsorption required for sugars to be adsorbed.

The starting solution (4g of syrup dissolved in 80 mL of water) presented a °Brix of 4.2 which, assuming that the value of Brix is indicating the total sugar content, translates in a concentration of 42 g/L of total sugar in the solution. Concerning the reducing sugars, mainly monosaccharides, the solution presented a concentration of 7.2 g/L, measured by DNS method. Therefore, based on these values, it can be assumed that there is a concentration of 34.8 g/L of FOS (DP ≥2) in the diluted syrup solution.

The adsorption process was very fast, the equilibrium was already reached within the first hour for all the sugars present in the solution. Through the analysis of the adsorption curves (**Figure 22**), it is possible to verify that sugars are selectively adsorbed according to their molecular weights. After one hour has passed, some alterations were verified, although not

noteworthy, with a minimum increase in the concentrations of monosaccharides in the solution, from (51.39% to 55.56%) and a slight decrease in the concentration of OS, from (23.85% to 20.11%). A higher retention was achieved for OS than for monosaccharides. Monosaccharides were the least adsorbed with 50% (maximum for the first 4 hours). In the first 4 hours of the study, OS had an adsorption rate of 78.74% and a maximum adsorption rate achieved at the 8th hour with 79.89%.

For the 24 hours study, from total sugars in the solution, only 26.19% were not adsorbed and were initially discarded. This value was practically a constant throughout the whole study. From these sugars, 54.17% were monosaccharides, which is a good value since these should be eliminated in a FOS purification process. Regarding the FOS, about 20.40% were lost in this adsorption step.

Since the amounts of adsorbed monosaccharides and FOS do not change drastically over time, adsorption for more than 1 hour is not necessarily required. This information is critical, since, when envisioning a scale up procedure, a time-consuming process without significant changes is not a desired process. A further study of adsorption within the first hour should be performed, as well as a desorption study, in order to verify the total sugar loss using activated charcoal in the purification of FOS.

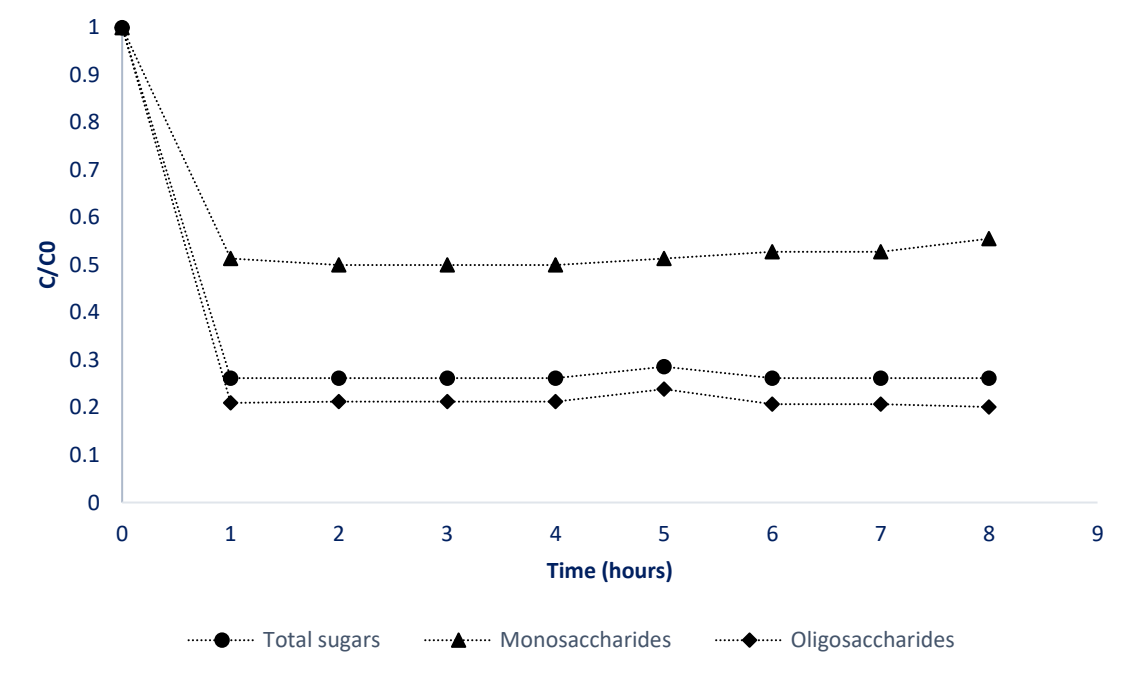

Figure 22 – Adsorption profile of monosaccharides, oligosaccharides and total sugars of the yacon syrup , adsorbed on activated charcoal, in a slurry method tested for 24 hours at room temperature.

3.2.2. Purification with Activated Charcoal

The purification of FOS from the yacon extract with activated charcoal was attempted using two different ethanolic solutions: 10% (v/v) followed by 50% (v/v). The ideal situation would be to have the OS adsorbed into the charcoal when the first solution was used with the mono- and disaccharides remaining dissolved. The 50% solution would remove the OS and inulin previously adsorbed onto the activated charcoal, after the removal of monosaccharides with the 10% solution, thus obtaining purified FOS. One of the main objectives with this method, was the possible application of a simple and economical method for FOS purification, using green processes. The solutions resulting from the purification were then analyzed by HPLC-RI for sugar detention and quantification.

Through the analysis of the chromatograms of the extract versus the purification after ethanolic elution at 10% and at 50% (**Figure 23**), it is possible to observe the initial extract concentration and the amounts desorbed from the activated charcoal for each ethanolic solution. The purification with EtOH at 10% desorbed, as expected, mainly monosaccharides with the desorption of 69.46% of fructose and 65.62% of glucose. It was also possible to obtain 41.24% of FOS with DP4 and 27.03% of the initial inulin, an unsatisfactory result since the desorption of FOS should only be obtained in the following step of the purification. No sucrose was recovered with this solution. The values for DP3 were not taken into consideration since the obtained concentration was below the LOQ of the method. All concentration data, before and after desorption, can be found in **Table 8**. Concerning the elution with the 50% ethanolic solution, after the desorption step with the 10% solution it was possible to recover even more 31.53% of inulin and 21.34% of FOS with DP4. The values obtained for DP3 and sucrose were not accounted for, since both values were below the LOD and LOQ. The obtained results are in line with those referred by Hernández *et al.* (2009) [141], in the sense that the author stated that ethanolic solution at 50% are preferably used for the recovery of FOS.

Overall, the method was not effective. The first step of the purification implied a significant loss of FOS and inulin, when it should remove only mono- and disaccharides. It is hypothesized that a way to solve this problem could be by making the initial slurry directly to the solution, and then apply an ethanolic gradient (5%, 10%, 15% (v/v)) in order to gradually remove the monosaccharides and disaccharides. Then, the recovery of FOS and inulin could be achieved by the application of an ethanolic solution with a higher concentration (e.g. EtOH at 50% (v/v)).

Table 8 – Comparison of the concentrations for the extract III before and after purification with activated charcoal in a slurry method.

Saccharide	Extract III (g/L)	Purified Extract EtOH 10% (v/v) (g/L)	Purified Extract EtOH 50% (v/v) (g/L)
Fructose	1.94 ± 0.05	1.35 ± 0.02	-
Glucose	1.57 ± 0.03	1.03 ± 0.01	-
Sucrose	0.96 ± 0.03	-	0.01[ab] ± 0.00
DP3	0.44[a] ± 0.12	0.16[ab] ± 0.01	0.07[ab] ± 0.00
DP4	0.68 ± 0.08	0.28 ± 0.01	0.14 ± 0.01
Inulin	12.02 ± 0.47	3.25 ± 0.06	3.79 ± 0.08

[a] Value below the LOQ; [b] Value below the LOD

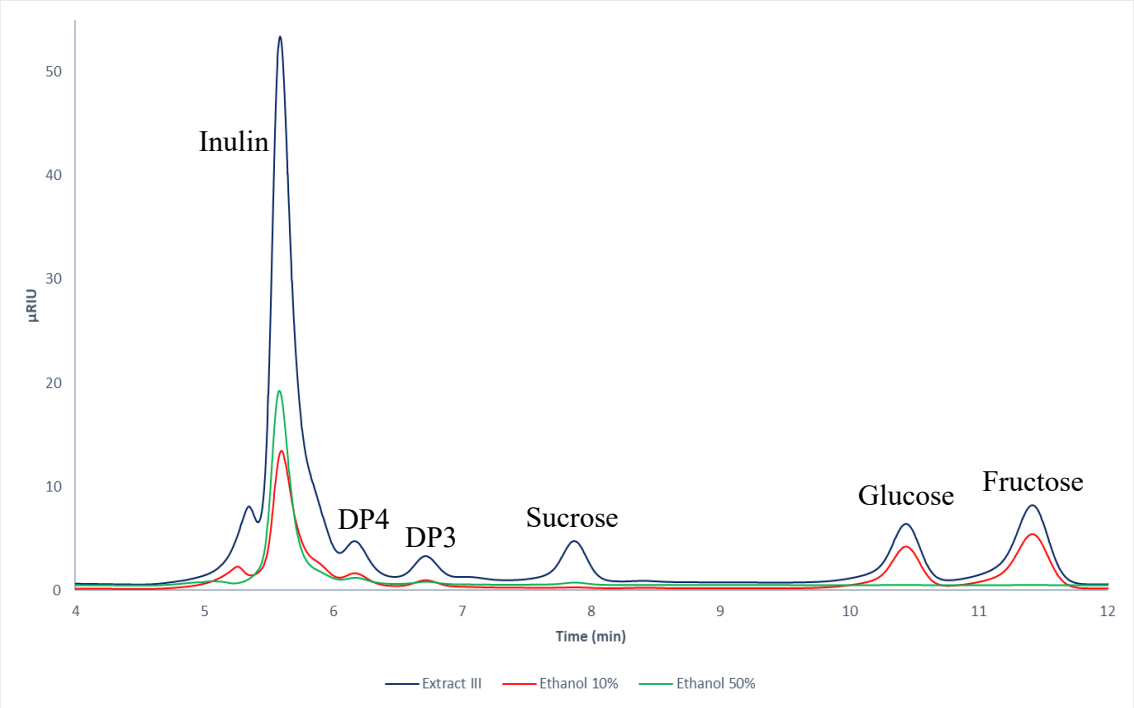

Figure 23 – Comparison of the chromatograms of the extract III and the ethanolic fractions (10% and 50%) of the purification with activated charcoal in a slurry method.

3.2.3. Purification with Activated Charcoal and Celite®

In an attempt to improve the purification of FOS with activated charcoal, Celite® 577 was used as a filtering aid. The use of Celite® aimed not only for a cleaner final product, without traces of activated charcoal, but purer. However, analyzing **Figure 24**, showing the chromatogram of the extract along with the chromatograms of the purification after ethanolic solution at 10% and at 50%, it is possible to verify an immense desorption of inulin and FOS alongside the expected good removal of monosaccharides and disaccharides with the ethanolic solution at 10%. This observation can be corroborated by the values of the recovered concentrations in comparison with the starting concentration, shown in **Table 9**. A more thorough analysis of the obtained concentrations revealed that, for the first ethanolic solution, there has been a recovery rate of 77.22% for inulin. The remaining sugars (fructose, glucose, sucrose and DP4) all showed recovery

rates “higher than 100%”. Values for FOS with DP3 were not accounted for since the obtained concentration was below the LOQ. These recovery rates higher than 100%, might be due to contamination with sugars entrapped in the porous plate of the used filtering funnel. Since the same funnel was used for the two types of purification tests with charcoal, some charcoal with adsorbed sugars might have been entrapped within the porous of the porous plate. Therefore, with the application of lower pressure and a new ethanolic solution, the entrapped sugars were released tampering the results for the purification with activated charcoal and Celite®. In order to surpass this, a more thorough cleaning of the porous plate funnel filter with vacuum and hot hydrochloric acid followed by rinsing with distilled water should be performed between purification with the same filtering funnel. Other cleaning solutions can be applied, depending on the filtrated material, as enlisted, for example by Aldrich® in their technical bulletin for the selection and utilization of fritted glassware [165].

Similarly to what happened in the previous method, with only charcoal, this purification method was not effective for the recovery of FOS and inulin. The solutions were contaminated with other sugars, so the obtained results were untrustworthy and the 50% (v/v) ethanolic solution only achieved recoveries of 14.86% for inulin and 17.76% for FOS with DP4. All the other concentrations were not taken into consideration, since those for fructose were below the LOQ, and those for FOS with DP3, sucrose and glucose, were below the LOD and LOQ.

Table 9 – Comparison of the concentrations for the extract III before and after the purification with activated charcoal and Celite® 577.

Saccharide	Extract III (g/L)	Purified Extract EtOH 10% (v/v) (g/L)	Purified Extract EtOH 50% (v/v) (g/L)
Fructose	1.94 ± 0.05	2.02 ± 0.04	0.05[a] ± 0.00
Glucose	1.57 ± 0.03	1.66 ± 0.04	-
Sucrose	0.96 ± 0.03	1.28 ± 0.20	0.10 [ab]± 0.01
DP3	0.44[a] ± 0.12	0.67[a] ± 0.10	0.11[ab] ± 0.02
DP4	0.68 ± 0.08	0.72 ± 0.05	0.12 ± 0.01
Inulin	12.02 ± 0.47	9.28 ± 0.13	1.78 ± 0.02

[a] Value below the LOQ; [b] Value below the LOD

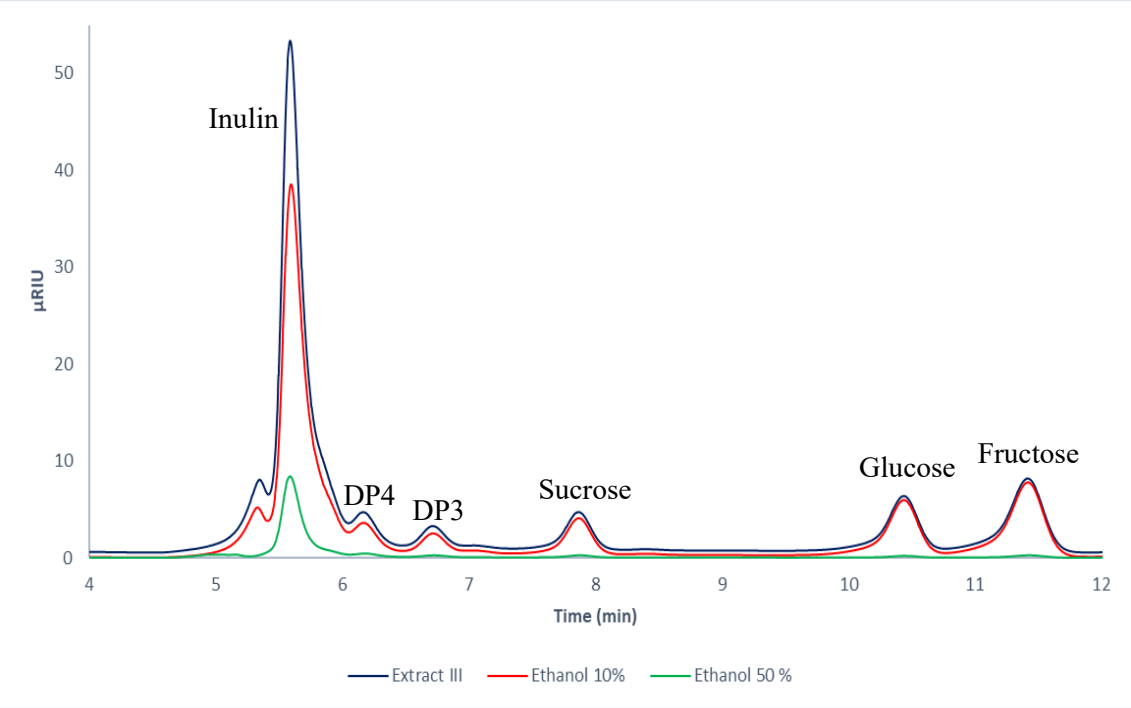

Figure 24 – Comparison of the chromatograms of the extract III and the ethanolic fractions (10% and 50%) of the purification with activated charcoal and Celite® 577.

The purification of FOS, i.e. the removal of monosaccharides, is crucial to increase their shelf life. Several methods were used, however this goal was not achieved, although it is clear that with additional studies there is a real possibility for all techniques. The application of other methods, such as the use of glucose oxidase to remove those particular monosaccharides was considered, but it was not possible to be performed.

3.3. Inulin Precipitation

Different ratios of solvent to extract were tested for the precipitation of inulin. The analysis performed by gravimetric method (page 43) revealed the ratio of 1:1 (solvent to extract) to be the one that produced the less desired results amongst the four ratios tested, with amounts of precipitated inulin lower than 0.005g for any of the solvents used. When the ratio was increased to 2:1, all samples showed an increase of precipitated inulin and from the ratio 2:1 to 3:1, the inulin amounts for propanol and EtOH kept increasing while a decrease was verified for ACN. The same was verified from ratio 3:1 to 4:1 where all the values increased. The ratio of 4:1 presented the highest precipitated amounts for all 3 solvents tested (**Figure 25**). Propanol showed the highest inulin precipitated with 0.0237 g from 1 mL of extract, followed by EtOH with 0.0164 g and finally ACN with 0.0142 g. Therefore, propanol at a 4:1 ratio of solvent to extract reveled to be the better inulin yielding method, although ethanol could be the ideal solvent since it is cheaper and more environmentally friendly

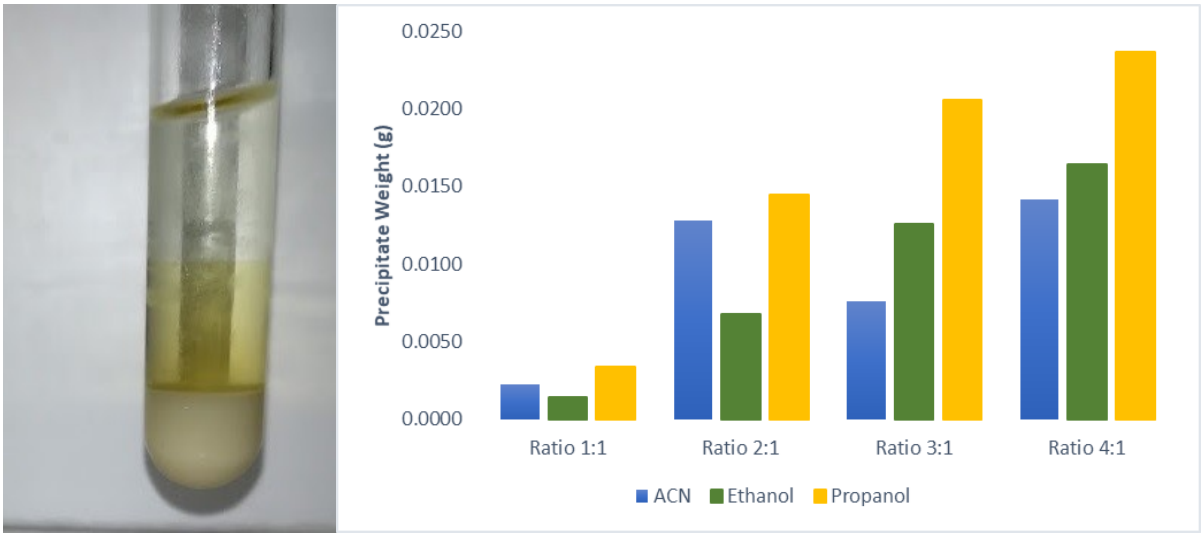

Figure 25 – Inulin precipitation results with ACN, ethanol and propanol as anti-solvents. Left: Resulting inulin precipitated with propanol 4:1; Right: Comparison between the gravimetric results for the three solvents tested.

The determination of the DP of each inulin obtained by precipitation was performed with the precipitates formed with the ratio of 4:1 solvent to extract and analyzed by MALDI-TOF MS (**Figure 26**). The resulting spectra showed the presence of sodium adduct that needed to be taken into consideration for the DP determination of the precipitated inulin and respective originated fragments. Analyzing the obtained mass spectra, it is possible to observe that each peak is separated by an approximate value of 160 Da, that can be ascribed to a hexose residue. The results for each analysis are registered in **Table 10**. These are in accordance to the values obtained by Borromei *et al.* (2009) [138], who stated a difference of 162 Da between each oligomer that corresponded to hexose residues. With ACN, the precipitate was not only formed in smaller amounts but also had the lowest DP obtained with a maximum of 16, verified in the last peak of the **Figure 26** (**A**) with m/z of 2629.281. Both precipitates with EtOH and propanol showed a maximum DP of 20, with m/z of 3270.986 Da and 3271.328 Da, respectively.

Similarly to what was found by Apolinário *et al.* (2017) [166], for *Agave sisalana*, the obtained inulin was a short-length polysaccharide with DP ranging from DP4 to DP20. For EtOH and propanol, both spectra showed a Gaussian distribution, with the most abundant distribution attributed to DP13. Also, both presented more peaks with higher masses but with lower intensity.

Table 10 – Degrees of polymerization identified for each resultant fragment from inulin with sodium adduct.

DP	ACN	EtOH	Propanol
	$[M + Na]^+$	$[M + Na]^+$	$[M + Na]^+$
4	702.657	-	702.683
5	863.385	-	863.394
6	1024.051	1024.073	1024.051
7	1184.572	1184.632	1184.659
8	1345.184	1345.244	1345.210
9	1505.902	1505.931	1505.778
10	1666.443	1666.320	1666.441
11	1826.967	1826.942	1827.036
12	1987.279	1987.328	1987.423
13	2147.952	2147.932	2147.881
14	2308.232	2308.284	2308.362
15	2468.690	2468.745	2468.753
16	2629.281	2629.233	2529.228
17	-	2789.734	2789.622
18	-	2950.076	2950.072
19	-	3110.541	3110.647
20	-	3270.986	3271.328

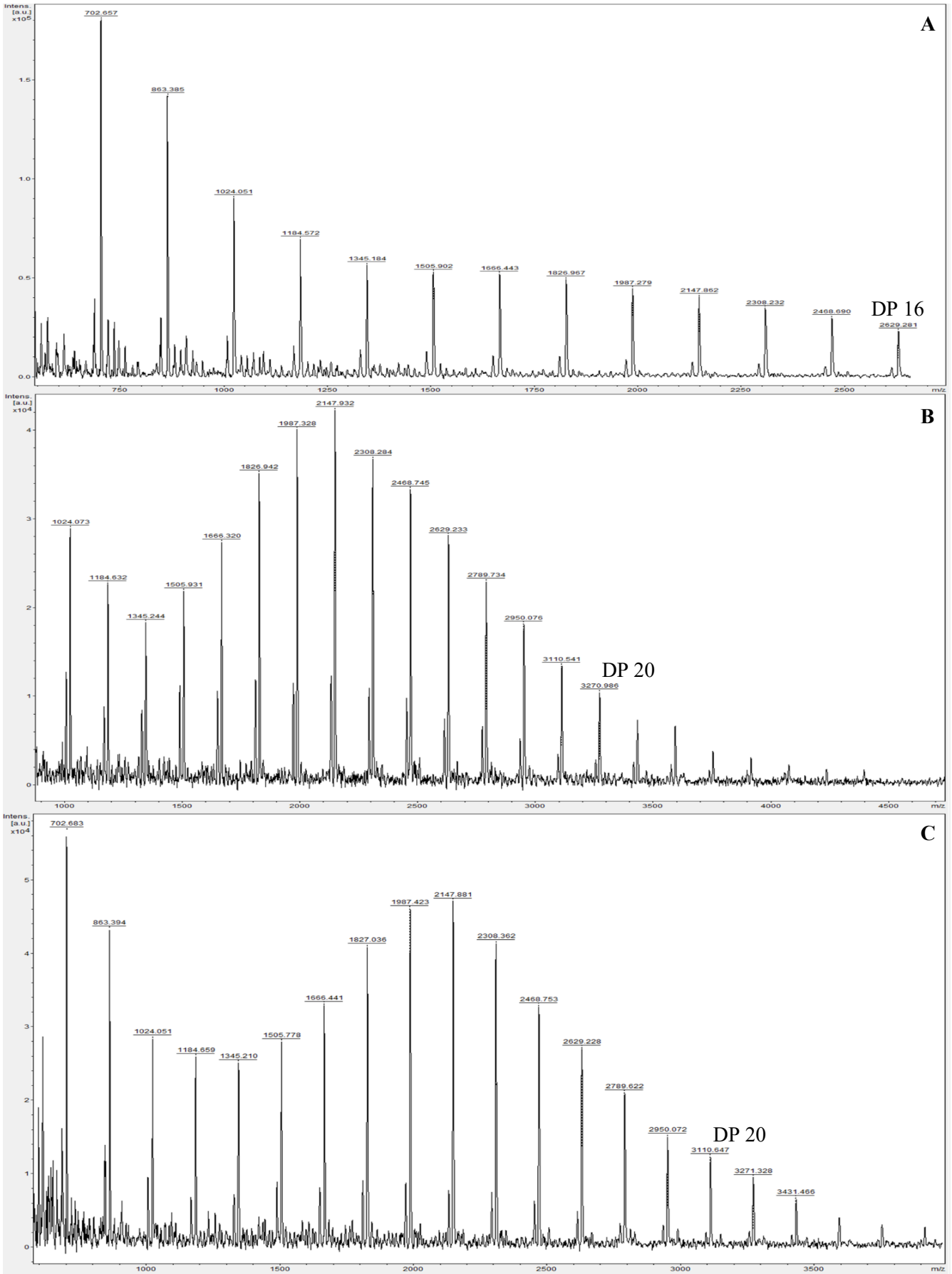

Figure 26 – MALDI-TOF MS results for the precipitated inulin in the ratios of 4:1 for ACN (**A**), EtOH (**B**) and propanol (**C**).

4. Enzymatic Assay for FOS Production

Since the obtention of FOS by direct extraction from yacon matrices was not satisfactory, yielding low amounts of FOS heavily contaminated with mono and disaccharides and large amounts of inulin, a different approach was assayed: the obtention of FOS by fraction of the long polymer inulin into small chains. An enzyme endo-inulinase was used and its activity in free and immobilized forms was compared.

4.1. Endo-inulinase Immobilization in Calcium Alginate

The method used for immobilization of endo-inulinase proved to be effective even if, contrary to what was performed in the original method [145], no chitosan or glutaraldehyde was added. Round beads were formed (**Figure 27**) with an average diameter of 4.24 ± 0.11 mm and average mass of 44.70 ± 2.01 mg (wet basis). Despite showing good mechanical resistance, the beads could not resist long exposure times under stirring conditions, even though the concentrations used for sodium alginate and calcium chloride were those referred by Missau *et al.* (2014) [167]. These authors also referred that the presence of chitosan and glutaraldehyde did not significantly change the results, at least in the range of 0.1% to 0.4% (v/v) and 0.1% to 0.9% (v/v), respectively.

Figure 27 – Obtained calcium-alginate beads with immobilized endo-inulinase.

4.2. Free and Immobilized Enzymatic Assay

Before advancing further in the work on the inulin hydrolysis, the endo-inulinase activity needed to be assayed. As previously stated, 1 unit of inulinase was defined as the amount of fructose, in 1 µmol per minute, produced in the assay. Free and immobilized endo-inulinase were both assayed, through the DNS test and the activity calculated[1]. The free and the immobilized endo-inulinase showed similar values of enzymatic activity when reacting with the commercial

[1] Activity was calculated using the equation (3) in page 44.

inulin solution at 0.2% (w/v) with 9.3 and 9.1 μmol/mL (U/ml), respectively. This revealed a highly effective immobilization, with 97.3% immobilization yield[2]. The remaining inulinase that was not immobilized may have been lost during the immobilization process, in the transference of the enzymatic solution to the alginate solution or even during the bead formation since the totality of the enzyme-alginate solution could not be completely extruded. Other immobilization studies reported different immobilization yields, although the yield is strongly dependent on the immobilization method, matrix and origin of the enzyme. Nguyen *et al.* (2011) [147] achieved 66% for the immobilization in chitosan for endo-inulinase from *Aspergillus niger*, de Paula *et al.* (2008) [168] achieved 82.60% for the immobilization in gelatin for endo-inulinase from *Kluyveromyces marxianus*, and Gill *et al.* (2006) [169] reported 100% yield for the immobilizations in DEAE-Sephacel, QAE-Sephadex and ConA-linked amino-activated silica beads for endo-inulinase from *Aspergillus fumigatus*.

4.3. Intra and Inter-day Study of Immobilized Enzyme for FOS Production

In this preliminary study, two different parameters were evaluated after hydrolysis with inulin solution at 1% (v/v) – cycles of reutilization intra-day and viability of storage at room temperature (inter-day). For the inter-day assays, the immobilized enzyme was stored at room temperature in sodium acetate buffer. All hydrolysates were analyzed for the presence of inulin by TLC.

In the intra-day study, the immobilized system was used for four consecutive hydrolysis cycles, lanes 1, 2, 3 and 6 in **Figure 28**. After the first use of the immobilized enzyme, hydrolysis fully occurred with no signs of inulin in the application point (lane 1), very similar to the spots of commercial FOS (FOS lane). The same was verified after the second use of the same beads (lane 2). For the third, the hydrolysis also occurred and a slight drag from the application point was noticeable (lane 3). Also, the gel beads were losing some of their shape and consistency. Finally, in the fourth consecutive use (lane 6), the gel had lost all its consistency and viscosity and no beads were visible, rendering impossible the recovery of the immobilized enzyme. As a consequence, visible in the TLC, the system no longer immobilized lost some of its activity, with a smear starting in the application point instead of the characteristic spots for FOS that appeared in the previous utilizations. With this immobilization method, it is possible to verify that the maximum reusability of the system is for three different consecutive cycles, with a slight gel consistency decrease but with a good hydrolytic activity.

As for the inter-day study, different beads with immobilized enzyme were stored at room temperature and tested its use for four consecutive days after the immobilization (lanes 1, 5, 7 and 8 in **Figure 28**). In the first day (lane 1), the same as in the previous study, the hydrolysis was complete with the spots in the TLC very similar to the commercial FOS (FOS lane). In the second

[2] Immobilization yield was calculated using the equation (2) in page 44.

day (lane 5), the same was verified, with no signs of sample drag along the elution. In the third day (lane 7), the immobilized system showed some activity loss with a lane dragging from the starting point that was not noticeable previously. Hydrolysis occurred but not completely. In the fourth and last day of the study (lane 8), the system showed no hydrolytic activity, with a drag from the application point very similar to the commercial inulin (INU lane). With this study, it was possible to verify that the immobilized enzyme maintains its activity for two days at room temperature without activity loss.

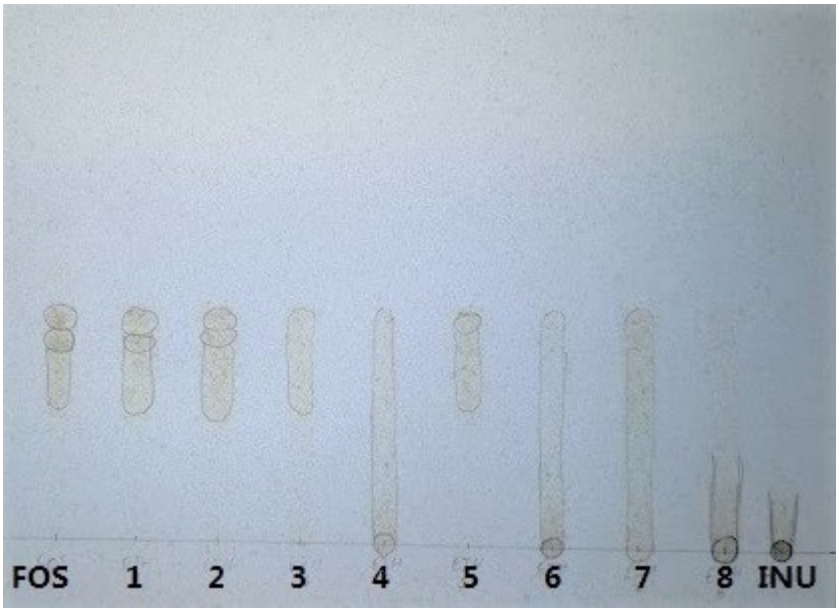

Figure 28 – Intra-day and inter-day analysis of the immobilized system. **Lane FOS**: commercial FOS (DP3-DP7) and fructose; **Lane 1**: first use of the immobilized enzyme**; Lane 2**: intra-day study, second use of the immobilized enzyme; **Lane 3**: intra-day study, third use of the immobilized enzyme; **Lane 4**: yacon extract; **Lane 5**: inter-day study, second use of the immobilized enzyme; **Lane 6**: intra-day study, fourth use of the immobilized enzyme; **Lane 7**: inter-day study, third use of the immobilized enzyme; **Lane 8**: inter-day study, fourth use of the immobilized enzyme; Lane INU: commercial inulin.

4.4. Batch Production in Recirculating System

After the 24h batch production, described in page 45, a TLC was performed using the aliquots collected at the 0, 1, 2, 3, 4, 20, 21, 22, 23 and 24 hours of the hydrolysis (**Figure 29**). Through the analysis of the obtained TLC, it is possible to verify that in the first four hours (lanes 2 to 5) of this FOS production, a reduction in the intensity of inulin spots was noticeable. It was also possible to observe an increment in the intensity of the spots for FOS, from DP3 to DP7 and possibly for FOS with higher DP that could not be identified. It was also possible to verify that, in this batch production system, by the 20th hour (lane 6) the hydrolysis was complete with all inulin transformed into FOS and monosaccharides as confirmed by lanes 6 - 10. From the 20th hour on the TLC presented two spots for monosaccharides (DP1) and disaccharides (DP2), representative of glucose, fructose and sucrose (or inulobiose) and FOS with DP between 3 and 7.

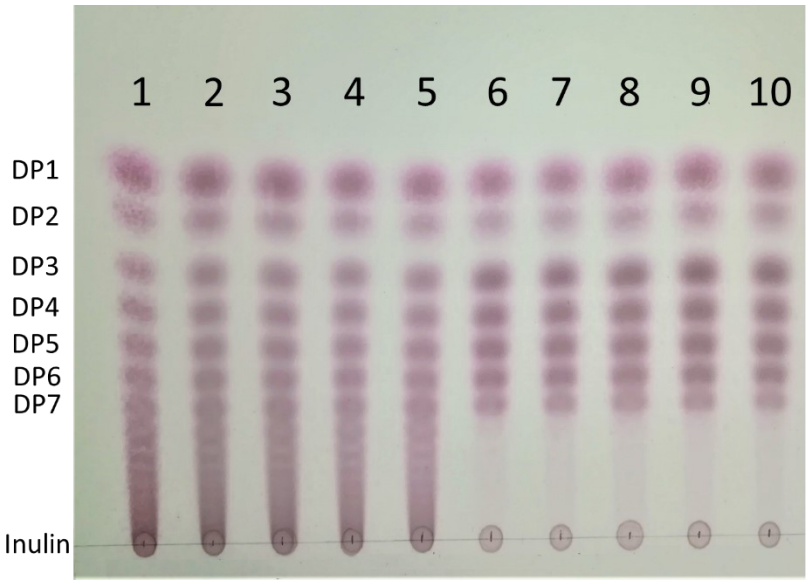

Figure 29 – TLC obtained for the inulin hydrolysis in the extract for 24h, with lanes 1 to 10 representing all the aliquots collected during the hydrolysis.
Lane 1: initial extract (hour 0 of the hydrolysis); **Lane 2**: 1st hour of the hydrolysis; **Lane 3**: 2nd hour of the hydrolysis; **Lane 4**: 3rd hour of the hydrolysis; **Lane 5**: 4th hour of the hydrolysis; **Lane 6**: 20th hour of the hydrolysis; **Lane 7**: 21st hour of the hydrolysis; **Lane 8**: 22nd hour of the hydrolysis; **Lane 9**: 23rd hour of the hydrolysis; **Lane 10**: 24th hour of the hydrolysis.

The aliquots were not only analyzed by TLC but also by refractometry to determine °Brix, and by HPLC-RI. The °Brix was used as an approximate quantitative method for the total sugar content in the samples. Since the samples are from a natural extract, they can also contain minerals, proteins and acids that may add to the resulting Brix value, but sugars generally account for >95%. °Brix were used to quickly check for either the consumption or the production of sugars by the used system. At the beginning of the reaction, the extract solution in sodium acetate buffer presented a °Brix of 1.6% that indicates 16 g/L of sugars in solution. Then, the system stabilized and from the 1st to the 22nd hours presented a °Brix of 1.7%. The last two hours of the hydrolysis showed values for °Brix of 1.8%. These slight alterations verified over time in the °Brix, can be due to the formation of FOS or to the presence of small particles of alginate that escaped the immobilized system. The °Brix of the sodium acetate buffer was verified before the sample analyses and considered as a blank.

Concerning the HPLC-RI, the initial extract, the first 4 hours and last 5 hours of the hydrolysis were analyzed for their monosaccharides, disaccharides, FOS, and inulin contents, as shown in **Table 11**. In the first four hours of the hydrolysis, small but not significant alterations in the concentrations of fructose and glucose were noticed. Despite those slight alterations over that period of time, the amounts of these saccharides were identical to the initial sample with 0.87 g/L for fructose and 0.65 g/L for glucose. A small increment was noticed for sucrose and DP4 from 0.54 g/L to 0.61 g/L and from 0.88 g/L to 1.26 g/L, respectively. On the other hand, a decrease was detected for DP3 and inulin that registered changes from 1.45 g/L to 1.15 g/L and from 5.83 g/L to 5.14 g/L. These small variations in concentration could be indicative of the start

of the hydrolysis with the consumption of inulin and DP3 alongside the formation of more sucrose and DP4. Regarding the last 5 hours (20th to 24th hour) of the hydrolysis, and in accordance to what was verified by TLC, no inulin was detected. Since in HPLC-RI the quantification is only possible for FOS up to DP5, after the consumption of long polymers of inulin, the shorter chains >5 can still be hydrolyzed. This can possibly justify the concentration increase for sugars with DP ≤5 observed until the 24th hour. Fructose, glucose and sucrose concentrations increased, respectively, from 0.82 g/L to 0.99 g/L, from 0.61 g/L to 0.75 g/L and from 0.56 g/L to 0.72 g/L. FOS with DP3 , DP4 and DP5 increased from 0.99 g/L to 1.01 g/L, from 1.58 g/L to 1.81 g/L and from 1.33 g/L to 1.60 g/L, respectively. All the resulting HPLC chromatograms (**Supplementary Information**, **Figure S5** to **Figure S9**) showed an unknown peak at 4.8 minutes. Besides this, the chromatograms for the collected samples from the 20th to the 24th hours showed an extra unknown peak at the 5.3 minutes. These unknown peaks were not attributed to any buffer component or alginate and their presence may be explained by the presence of other FOS that could not be identified due to the inexistence of related reference compounds. The same samples, from the 20th to the 24th hours, also presented two abnormal peak shapes, one peak splitting at the 5.8 minutes and a shoulder peak at the 6.8 minutes, as can be seen in the **Figure S7**, **Figure S8** and **Figure S9**. These might be due to the formation of new FOS during the hydrolysis; however, once again, this could not be quantitatively determined nor could have been without a good separation and good peak definition and a larger array of standards, which are not available commercially.

Table 11 – Concentrations (g/L) of each saccharide obtained by HPLC-RI for the inulin hydrolysis with immobilized endo-inulinase.

Time (h)	**0**	**1**	**2**	**3**	**4**
Fructose (g/L)	0.87 ± 0.04	0.79 ± 0.03	0.84 ± 0.04	0.82 ± 0.03	0.87 ± 0.00
Glucose (g/L)	0.65 ± 0.03	0.58 ± 0.03	0.62 ± 0.03	0.60 ± 0.03	0.65 ± 0.01
Sucrose (g/L)	0.54 ± 0.07	0.57 ± 0.02	0.61 ± 0.05	0.59 ± 0.02	0.61 ± 0.01
DP3 (g/L)	1.45 ± 0.05	1.26 ± 0.02	1.23 ± 0.03	1.18 ± 0.05	1.15 ± 0.01
DP4 (g/L)	0.88 ± 0.02	1.05 ± 0.03	1.12 ± 0.01	1.16 ± 0.01	1.26 ± 0.01
DP5 (g/L)	-	-	-	-	-
Inulin (g/L)	5.83 ± 0.25	5.12 ± 0.13	5.27 ± 0.15	5.06 ± 0.15	5.14 ± 0.04
Time (h)	**20**	**21**	**22**	**23**	**24**
Fructose (g/L)	0.82 ± 0.02	0.85 ± 0.02	0.94 ± 0.02	0.94 ± 0.01	0.99 ± 0.02
Glucose (g/L)	0.61 ± 0.02	0.64 ± 0.02	0.71 ± 0.02	0.71 ± 0.01	0.75 ± 0.01
Sucrose (g/L)	0.56 ± 0.12	0.64 ± 0.09	0.75 ± 0.02	0.71 ± 0.05	0.72 ± 0.03
DP3 (g/L)	0.99 ± 0.03	0.99 ± 0.04	0.98 ± 0.04	1.02 ± 0.04	1.01 ± 0.02
DP4 (g/L)	1.58 ± 0.07	1.66 ± 0.04	1.80 ± 0.04	1.77 ± 0.01	1.81 ± 0.03
DP5 (g/L)	1.33 ± 0.05	1.42 ± 0.06	1.54 ± 0.02	1.57 ± 0.02	1.60 ± 0.02
Inulin (g/L)	-	-	-	-	-

Since the alginate beads containing the enzyme showed a tendency to change shape and consistency, the possibility of enzyme leaching was evaluated by the Bradford test (page 48). The test was performed to the 24h of hydrolysis's sample. The same aliquots tested for the quantification of sugars were used for the Bradford methodology and the concentrations of protein in the samples were obtained through the calibration curve previously drawn (**Supplementary Information**, **Figure S2**). The results obtained were not significantly different form the values for the blank (performed with the buffer solution) with concentrations ranging from 0.0032 to 0.0181 g/L. The obtained values could be due to the presence of proteins from the initial extract and not from leached enzyme, since the highest value (0.0181 g/L) was found in the initial extract.

4.4.1. TLC Quantification by Densitometry

After the hydrolysis, as previously described, TLC and HPLC-RI analysis were performed for the resulting aliquots collected hourly. The formerly described TLC-densitometry method was applied to the resulting TLC and the concentrations of saccharides in each sample were obtained through the equation of the calibration curve obtained for fructose at four different concentrations (0.25 g/L to 1 g/L) (**Supplementary Information**, **Figure S4**). Densitograms were obtained for each analyzed sample, with a good differentiation between each DP, as showed in **Figure 30**, a densitogram plotted correspondent to the lane 6 in the TLC of the **Figure 29** (page 73).

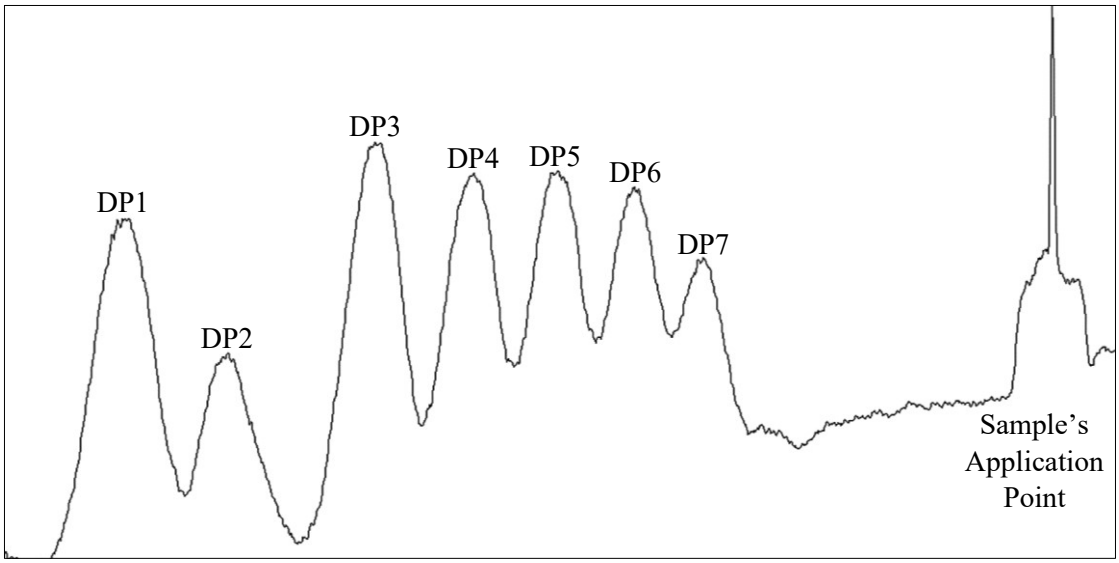

Figure 30 – Densitogram plotted by ImageJ for the 20th hour of the hydrolysis of inulin in the Extract III.

This method was performed as a proof of concept only. It was interesting to observe that this technique allowed for the quantification of FOS ≥5 but not for the differentiation of monosaccharides while in HPLC the opposite occurs. Also, the calculated concentrations by densitometry were not near those obtained by HPLC-RI. Despite this difference verified between the two techniques, the obtained concentration by the TLC-densitometry method were not totally

discarded and a comparative graphic with all the quantifiable saccharides was plotted (**Figure 31**). It was possible to verify that the inulin content gradually decreases over the first four hours of the hydrolysis and that decrease is followed by a slightly increase in the content of DP3, DP4 and DP5. As for the last five hours of the hydrolysis, it is observable the absence of inulin and an even higher increment in the contents of DP3, DP4 and DP5. Regardless of the differences in contents, the same was practically observed for the HPLC results. Similar results were verified for HPLC, as showed in **Figure 32**, with the major differences, besides the amounts, being the absence of initial DP5 (not detected with HPLC). Relatively to the last five hours of the reaction, no inulin was present as well as an increment in DP1, DP2, DP3 and DP4 was verified alongside the detection of DP5.

This TLC-densitometry method proved to be a good hydrolysis diagnostic method, showing good semi-quantitative results. It can be used as a fast, cheap and reliable preliminary methodology before advancing for more expensive techniques such as HPLC are applied.

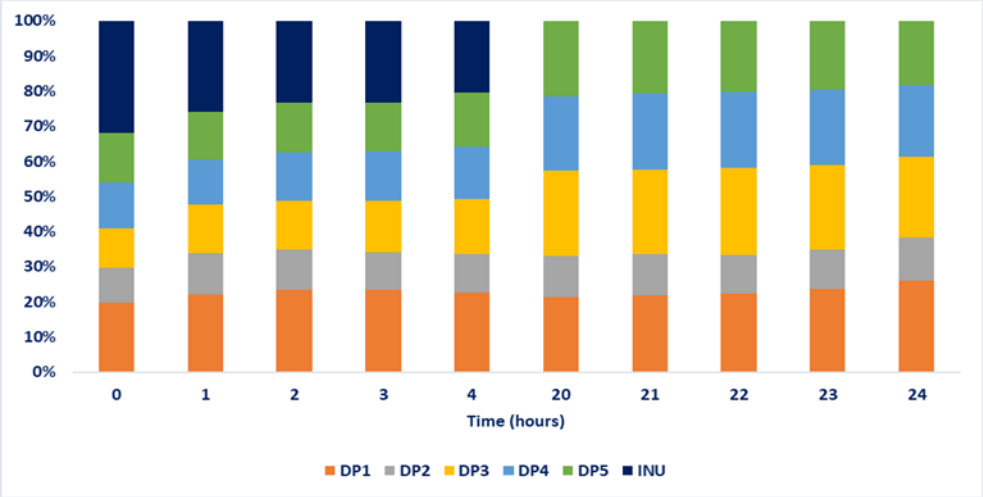

Figure 31 – Relative contents of the quantifiable saccharides determined by TLC-densitometry.

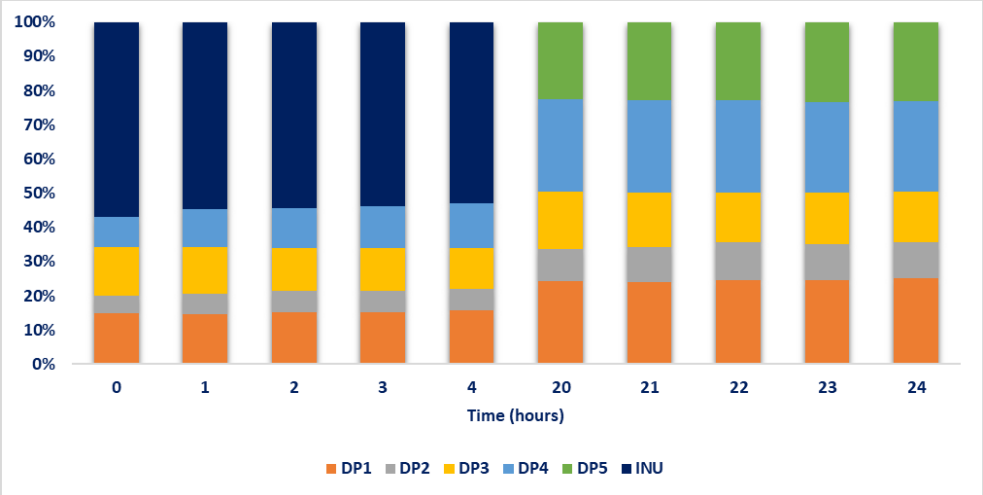

Figure 32 – Relative contents of the quantifiable saccharides determined by HPLC-RI.

IV. Conclusions and Future Work

FOS, known functional foods, are very sought after due to their prebiotic properties. Widely recognized for their benefits to the gastrointestinal flora, they also present other advantageous and nutritional properties for the consumers, such as low caloric content, increase of mineral absorption, antioxidant effect, modulation of the immune system and prevention of bowel diseases and cancer. Industrially, FOS are generally used as supplements, as fat and sugar replacers, humectants and as stabilizers and preservative agents. The valorization of some products, such as yacon can be achieved through the extraction and purification of FOS and inulin.

The most commonly found FOS in the market are from synthetic origin, produced by enzymes, namely fructosyltransferases, through transfructosylation reactions. However, this process is a cycle of constant production of scFOS and its hydrolysis and requires a strict control of the reaction. Therefore, the extraction of FOS and inulin from yacon, and the use of that inulin for the production of more FOS, with inulinase as a biocatalyst, emerged as a good alternative to the industrially synthetized FOS, for even more obtention of this OS from a natural source.

All the extractions tested aimed for the better FOS yield possible with the lower costs associated. Fast and simple extraction methods with water as solvent were developed, with the choice of the extract to be used in the other methodologies being based purely in the ratio of FOS/ monosaccharides and the final volume achieved.

Purification of the obtained extract with baker's yeast revealed ineffective with the consumption of the desired FOS and the production of sub products of the fermentation. Concerning the activated charcoal, the ethanolic solution at 50% (v/v) did not recover the majority of the FOS, as desired. Some FOS maintained adsorbed in the charcoal and the remaining desorbed with the ethanolic solution at 10% (v/v) alongside with the monosaccharides and disaccharides. Relatively to the purification with activated charcoal and Celite®, similar results were obtained with the difference that in this latter method all the monosaccharides and disaccharides were completely desorbed. For both methods, the inability to recover isolated fractions of FOS could possibly be overcome by the use of an ethanolic gradient.

The precipitation with antisolvents, proved to be an effective way to separate the inulin from the rest of the extract. The ratio 4:1 showed better yield results with propanol proving to be the better solvent among the three tested. The higher DP of inulin obtained was 20 as determined by the MALDI-TOF results.

Endo-inulinase from Megazyme was successfully and efficiently immobilized on calcium-alginate beads, without the need of cross-linkers. The verified immobilization efficiency of 97% was achieved with an alginate solution at 3% (w/v) and a calcium chloride solution at 2% (w/v). The immobilized calcium alginate-inulinase system showed good operational stability after two consecutive uses of the same beads and also good storage stability at room temperature when used for two consecutive days. In both cases, the third use showed a decrease of activity and for the fourth use very little to no activity was verified and a decrease in the bead's integrity.

The calcium alginate beads with immobilized endo-inulinase were successfully used in the hydrolysis of the inulin in the extract, in a recirculatory system, for 24h. The beads maintained their integrity and no protein was detected in any of the aliquots collected over time.

The TLC-densitometry method developed did not served the desired purpose, to be a reliable method for sugar quantification. However, the obtained results given by the method proved it to be a good, cheap and reliable preliminary methodology for some more expensive techniques.

As for future work and perspectives, it would be interesting to:

- Optimize the parameters of the adsorption and desorption of FOS into activated charcoal and study the behavior of FOS during the desorption when applying an ethanolic gradient;
- Use a different purification method, possibly flash chromatography, using silica, C-18 or amino cartridges (columns) in the Büchi Pure C-850 FlashPrep system, with ELSD detection;
- Improve the HPLC-RI method, trying to better separate FOS with a higher DP than 5 – test gradient mobile phases, increase the run time, increase volume of injection, increase/decrease flow rate;
- Perform sugar analysis by NMR and FTIR;
- Study the influence of some experimental parameters in the immobilization of the enzyme, such as: amount of enzyme to use, concentrations of alginate and $CaCl_2$, more thorough study of the resistance to temperature changes and reusability of the system, kinetic study of the immobilized system (determination of the Km and Vmax);
- Immobilization of the endo-inulinase in other matrices and with other techniques, such as PVDF through electrospinning.

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VI. Supplementary Information

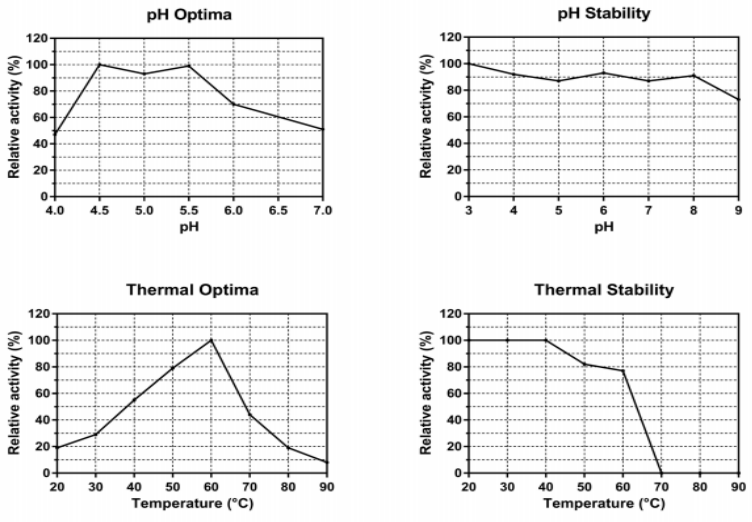
Table S1 – Equipment and materials used throughout the work.

Equipment/Materials	**Model, *Brand***
Heating and stirring plate	Isotemp, *Fisher Scientific*
Heating and stirring plate	MR Hei-Standard, *Heidolph*
Centrifuge	Rotofix 32 A, *Hettich Zentrifugen*
Freeze drier	Alpha 1-2 LD Plus, *Martin Christ*
Analytical scale	M14Ai, *Bel Engineering*
Moisture analyzer	DBS 60-3, *Kern*
Muffle Furnace	L3/11/C6, *Nabertherm*
Refractometer	RX-100, *Atago*
Peristaltic pump	REGLO Analog MS-4/8, *Ismatec*
HPLC	Dionex UltiMate 3000, *Thermo Fisher Scientific*
RI detector (for HPLC)	RI 101, *Shodex*
Column (HPLC)	ReproGel-Na, 9 µm 250 x 8 mm, *Dr. Maisch Gmbh*
Guard Column (HPLC)	CARBOSep CHO 411 Na 4.0 x 24 mm, *Concise Separations*
Microplate reader	Victor^3 1420 Multilabel Counter, *Perkin Elmer*
pH meter	744 pH Meter, *Metrohm*
0.45 µm cellulose Acetate sterile syringe filters	*Frilabo*
0.22 µm cellulose Acetate sterile filters	*Whatman®*
MALDI-TOF	Autoflex maX Matrix-Assisted Laser Desorption and Ionization (Time-of-Flight) 2 Mass Spectrometer, *Bruker*
TLC plates	TLC Silica gel 60 F_{254}, *Merck*
Circulating water vacuum pump	B-169 Vacuum-System, *Büchi*
Filtering paper number 1	*Whatman®*
Spectrophotometer	Lambda 2, *Perkin Elmer*
Juicer	EXCEL juicer JE850, *Kenwood*

Table S2 – Reagents and standards used in the experimental procedures.

Compounds	**Manufacturer**	**Purity**
Glucose	Riedel-de Haën	Analytical reagent grade
Fructose	Merck	Analytical reagent grade
Sucrose	Fisher Chemical	Analytical reagent grade
1-kestose	Sigma-Aldrich	Analytical reagent grade
1,1-kestotetraose	Sigma-Aldrich	Analytical reagent grade
1,1,1-kestopentaose	Sigma-Aldrich	Analytical reagent grade
Inulin from chicory	Sigma-Aldrich	Unknown
Acetonitrile	Carlo Erba	HPLC Plus Gradient grade 99.9%
Ethanol absolute	Panreac	Analytical reagent grade 99.8%
2-Propanol	Merck	Analytical reagent grade 99.9%
Acetic Acid Glacial	Fisher Scientific	Analytical reagent grade
Butan-1-ol	Lab-Scan	Analytical reagent grade 99.5%
Endo-Inulinase	Megazyme	High purity recombinant endo-Inulinase (Aspergillus niger) for use in research.
3,5-Dinitrosaliculic acid	Sigma-Aldrich	Analytical reagent grade 98.0%
BSA – Bovine serum albumin	Sigma-Aldrich	Biotechnology grade
Methanol	Fisher Scientific	Analytical reagent grade 99.9%
Sulphuric acid	PanReac AppliChem	Analytical reagent grade 95-98%
N-(1-naphthyl)ethylenediamine	Panreac	Analytical reagent grade 98.0%
Sodium hydroxide - NaOH	Panreac	Analytical reagent grade 98.0%
Potassium Sodium tartrate 4-hydrate	Panreac	Analytical reagent grade
Sodium acetate trihydrate	Riedel-de Haën	Analytical reagent grade
Alginate (Alginic acid from brown algae)	Sigma-Aldrich	Unknown
Calcium chloride	Panreac	p.a.>99.0%
Activated charcoal	Panreac	Unknown
Celite® 577	Fisher Scientific	Unknown

Table S3 – Endo-inulinase from Aspergillus niger (Megazyme) properties – data provided by the supplier.

Specific Activity	240 U/mg protein (on inulin) at pH 4.5 and 40 °C ~ 440 U/mg protein (on inulin) at pH 4.5 and 60 °C One Unit of endo-inulinase activity is defined as the amount of enzyme required to release one µg of β-D-fructose reducing-sugar equivalents per minute from inulin (20 mg/mL) in sodium acetate buffer.
Specificity	Endo-hydrolysis of β-2,1-D-fructosidic bonds of inulin
Relative Rates of Hydrolysis of Substrates	Inulin (Raftiline) (20 mg/mL) –> 100% Inulin (dahlia) (10 mg/mL) –> ~95% Action on all polysaccharides was determined in sodium acetate buffer (100mM), pH 4.5 at 60 °C
Physicochemical Properties	Recommended conditions of use are at pH 4.5 – 5.5 and 40 °C – 60 °C. pH Optima: 4.5 – 5.5 pH Stability: 3.0 – 8.0 (> 75% control activity after 24h at 4 °C) Temperature Optima: 60 °C (10 minutes reaction) Temperature Stability: up to 40 °C
Experimental Data	
Storage conditions	Enzyme is supplied as an ammonium sulphate suspension in 0.02% (w/v) sodium azide and should be stored at 4 °C. For assay, this enzyme should be diluted in sodium acetate buffer (100 mM), pH 4.5 containing 1mg/mL BSA. Swirl to mix the enzyme immediately prior to use.

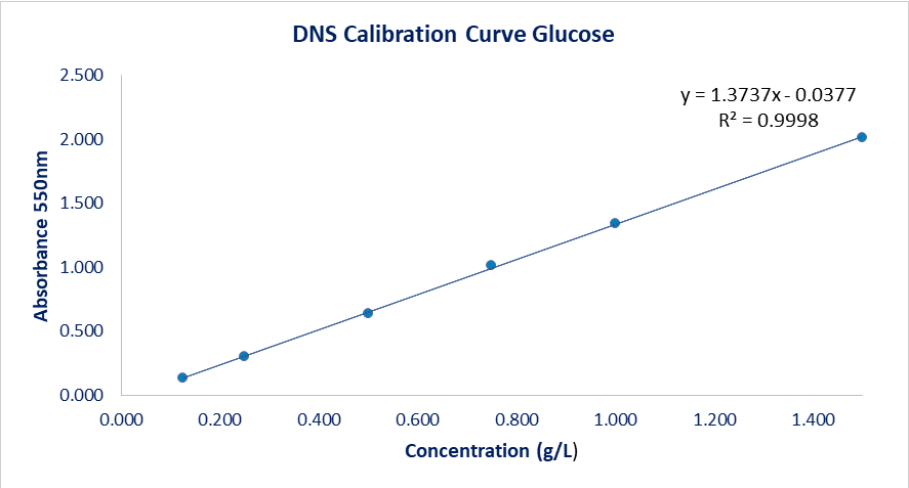


Figure S1 – DNS calibration curve for glucose concentrations ranging from 0.125 to 1.5 g/L.

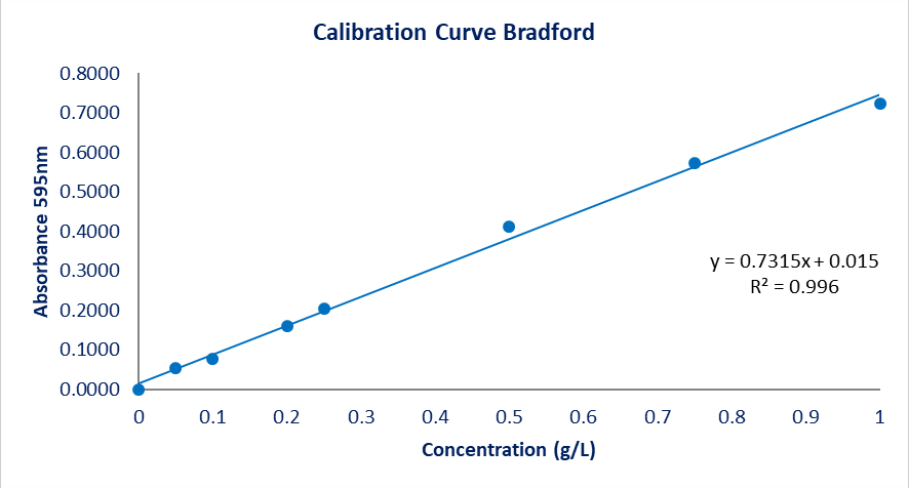


Figure S2 – Calibration curve used for total protein quantification, obtained with different BSA concentrations ranging from 0.05 to 1 g/L.

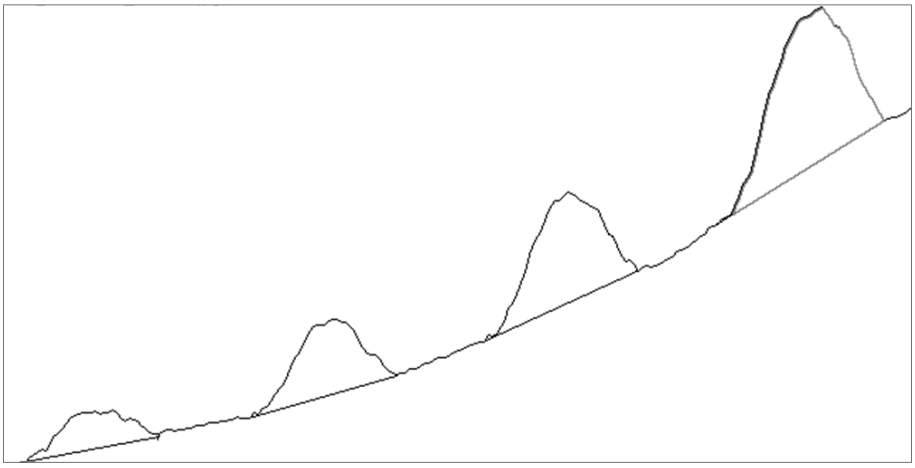


Figure S3 - Densitogram plotted by ImageJ for the calibration curved performed on the TLC with the fructose standards ranging from 0.25 to 1 g/L.

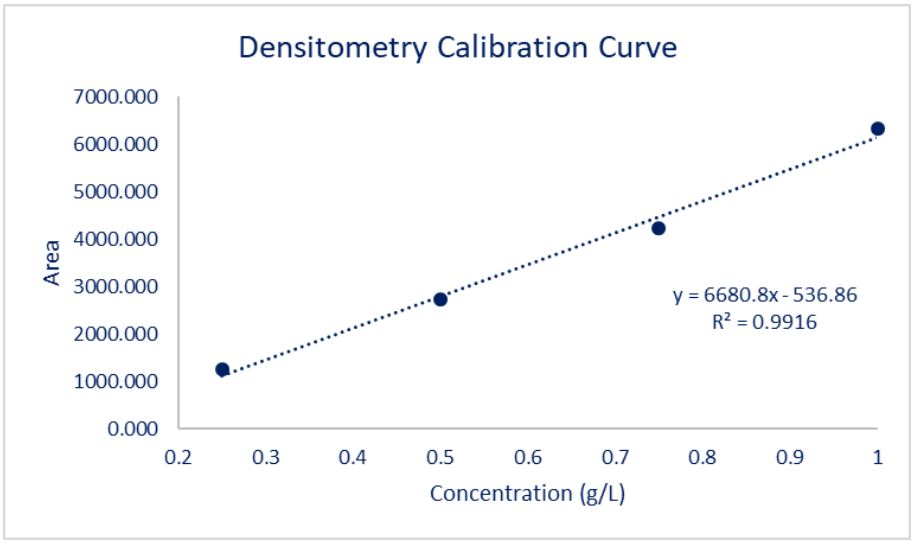


Figure S4 – TLC-densitometry calibration curve for fructose concentrations ranging from 0.25 to 1 g/L.

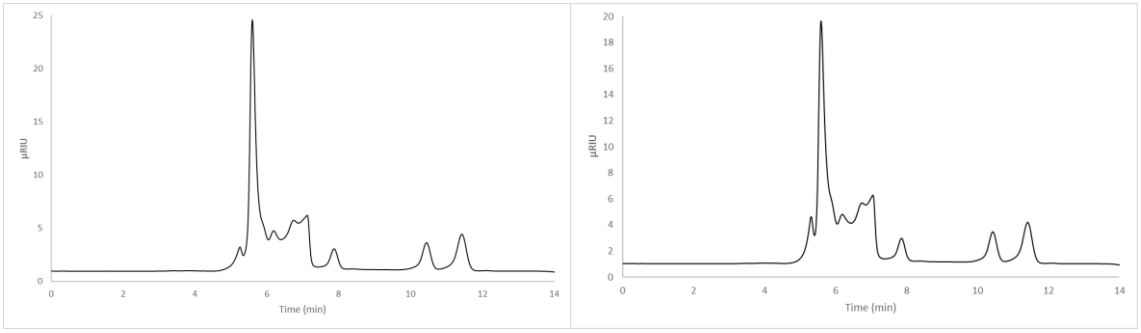


Figure S5 – Chromatogram of the inulin hydrolysis of the yacon extract in the beginning, at 0 hours (**Left**) and (**Right**) after 1 hour of the hydrolysis.

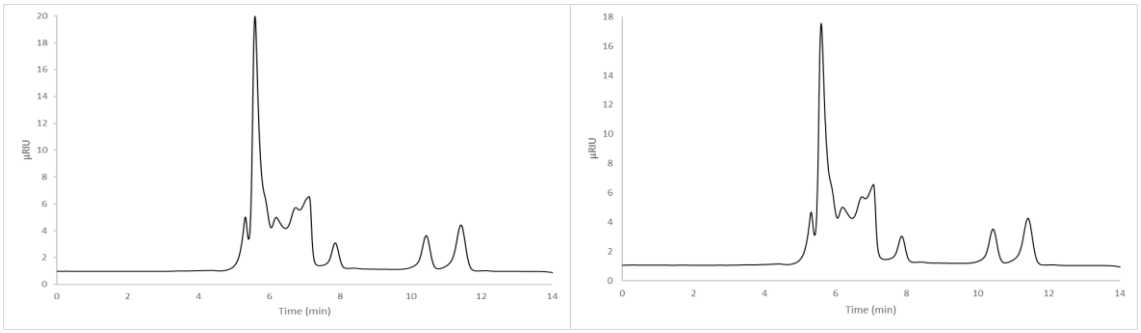


Figure S6 – Chromatogram of the inulin hydrolysis of the yacon extract after (**Left**) 2 hours and (**Right**) 3 hour of the hydrolysis.

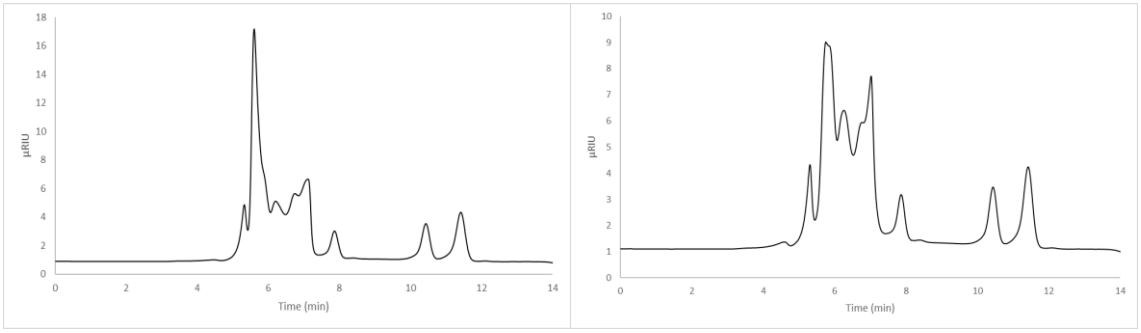


Figure S7 – Chromatogram of the inulin hydrolysis of the yacon extract after (**Left**) 4 hours and (**Right**) 20 hours of the hydrolysis.

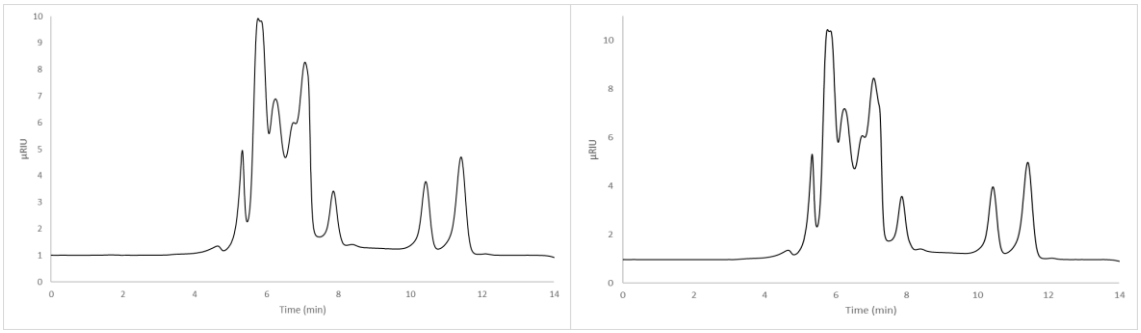


Figure S8 - Chromatogram of the inulin hydrolysis of the yacon extract after (**Left**) 21 hours and (**Right**) 22 hours of the hydrolysis.

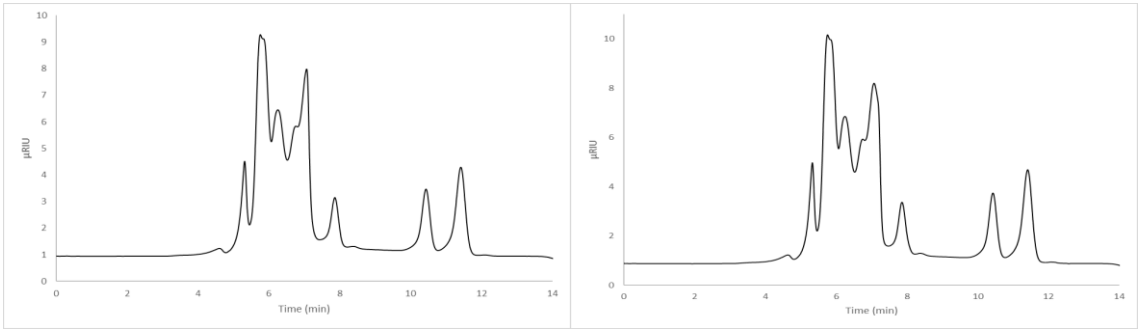


Figure S9 - Chromatogram of the inulin hydrolysis of the yacon extract after (**Left**) 23 hours and (**Right**) 24 hours of the hydrolysis.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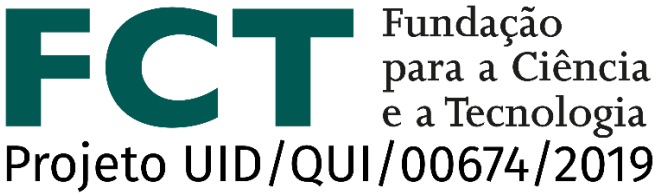
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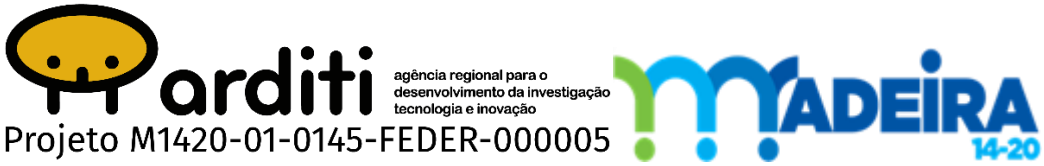
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