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„Structure and prebiotic potential of oligosaccharides isolated from *Opuntia ficus-indica*“

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Abstract

Opuntia ficus-indica is a plant belonging to the family of Cactaceae. The plant originates to Mexico but nowadays it is widespread as it can adapt very well to arid and semi-arid regions. Because people believed in its healing power *O. ficus-indica* was used in folk medicine for the treatment of ulcers, wounds or lots of chronic diseases. After scientific research confirmed the health benefits, there is more and more interest to use Opuntia in pharmaceutical industry.

In this thesis the dried and fresh *Opuntia ficus-indica* samples were examined with regard to its prebiotic potential. First the extracts were separated using SEC and the distribution between mono-, disaccharides and oligosaccharides was determined. Afterwards the mono- and oligosaccharide composition was clarified using thin-layer chromatography, reversed-phase high-performance liquid chromatography and high-performance anion-exchange chromatography. The composition of the dried and the fresh plant was very similar, with a few minor exceptions.

Prebiotics are used to have a positive effect on the host by stimulating the growth or activity of certain bacteria. To determine the prebiotic potential of *O. ficus-indica* the extracts and its fractions were added to bacteria. Subsequently, growth curves were determined by turbidity measurement. All samples tested showed prebiotic potential on the bacterial strains *Lactobacillus acidophilus*, *Lactobacillus rhamnosus GG*, *Bifidobacterium longum ssp. infantis* and *Bifidobacterium animalis ssp. lactis*.

Kurzfassung

Opuntia ficus-indica ist eine Pflanze, die zu der Familie der Cactaceen gehört. Ursprünglich stammt sie aus Mexiko, aber heutzutage ist sie weit verbreitet da sie sich sehr gut trockenem und halbtrockenem Klima anpassen kann. Da die Leute an die Heilkraft von *O. fiucs-indica* glaubten, wurde diese in der Volksmedizin für die Behandlung von Geschwüren, Wunden und vielen chronischen Erkrankungen eingesetzt. Nachdem auch wissenschaftliche Untersuchungen die positiven Auswirkungen auf die Gesundheit bestätigten, gibt es immer mehr Interesse daran Opuntia auch im pharmazeutischen Bereich einzusetzen.

In dieser Diplomarbeit wurde die getrocknete als auch frische Opuntia bezüglich ihres prebiotischen Potentials untersucht. Zu Beginn wurden die Extrakte mit Hilfe von Größenausschluss-Chromatographie aufgetrennt und die Verteilung zwischen Mono-, Disacchariden und Oligosacchariden ermittelt. Danach wurde die Mono- und Oligosaccharid Zusammensetzung mittels Dünnschichtchromatographie, Hochleitsungsflüssigkeitschromatographie und Hochleistungsanionenaustauscher-Chromatographie aufgeklärt. Die Zusammensetzung der getrockneten und der frischen Pflanze war bis auf wenige Ausnahmen sehr ähnlich.

Prebiotika sollen den Wirt positiv beeinflussen, da sie das Wachstum oder die Aktivität bestimmter Bakterien stimulieren. Um das prebiotische Potential von *O. ficus-indica* zu ermitteln wurden die Extrakte und deren Fraktionen mit Bakterien versetzt. Anschließend wurden Wachstumskurven mittels Turbiditätsmessung ermittelt. Die Extrakte zeigten alle prebiotisches Potential an den Bakterienstämmen *Lactobacillus acidophilus*, *Lactobacillus rhamnosus GG*, *Bifidobacterium longum ssp. infantis* und *Bifidobacterium animalis ssp. lactis*.

List of Abbreviations

FOS	fructooligosaccharides
GOS	galactooligosaccharides
dp	degree of polymerization
LAB	lactic acid bacteria
rpm	revolutions per minute
RT	room temperature
SEC	size exclusion chromatography
STD	standard
MW	molecular weight
DRI	differential refractive index
TLC	thin-layer chromatography
RP-HPLC	reversed-phase high-performance liquid chromatography
HPAEC	high-performance anion-exchange chromatography
TFA	trifluoroacetic acid
PMP	1-Phenyl-3-methyl-5-pyrazolone
PBS	phosphate-buffered saline
MRS+	Man-Rogosa-Sharpe medium with glucose
MRS-	Man-Rogosa-Sharpe medium without glucose
MeOH	methanol
F	fraction
OD_{600}	optical density at 600 nm
OFI	*Opuntia ficus-indica*

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1. Introduction

1.1 Mucilage

Mucilages are hydrophilic with water extractable carbohydrates, which consist of heteropolysaccharides and generally contain monosaccharides, glucuronic- and galacturonic acids. Those uronic acids are branched and can be methylated or acetylated. In plants they are one of the most important reserve substances binding water. Since mucilage is very viscous and the swelling capacity is very high the quality can be tested by a viscosimeter and determined by the swelling index. [1,2]

The reason why mucilage is important for pharmaceutical use is because it has lots of effects. For instance, it acts like a buffer because of the uronic acids, but it also has an irritation reducing effect, which can be useful for treatment of inflammatory bowel disease, ulcerative colitis or gastritis as well as haemorrhoids, anal fissure or other diseases of the rectum since it can facilitate the defecation. Because of the high swelling capacity, it can as well be used as a laxative or dietetic. Plants, which contain mucilage are often used as antitussives in mouth and throat area because they produce a protective film on top of the bronchial mucosa. Mucilage can also be helpful in pharmaceutical galenics as exploders, thickening agent or stabilizer. [1,2]

The mucilage of *Opuntia ficus-indica* is a high molecular weight polysaccharide, containing a molecular structure up to 30,000 different sugars. It contains polygalacturonic acid and residues of five natural sugars (such as D-galactose, D-xylose, L-arabinose, L-rhamnose and D-galacturonic acid). An experiment showed the mucilage sugar composition and revealed that the main sugar component of the mucilage of *Opuntia ficus-indica* is arabinose followed by xylose. [3,4]

1.2 Opuntia / Cactaceae

A cactus is a member of the plant family Cactaceae of the order Caryophyllales. The term “cactus” derives from the Ancient Greek and has been used for a spiny plant. Cacti are populated in the most diverse habitats. They can have lots of different shapes and sizes. Cactaceae are succulent perennials, which means they have thickened, fleshy parts to store water. The cactus can occur in many different forms for example as trees, shrubs or climbers. The leaves are often non-existent, but if there are leaves they are spirally arranged and usually transformed into spines. Those spines can help the cacti to defend themselves from herbivores but also can help to prevent water loss because it reduces the air flow close to the cactus. The flowers of a cactus are mostly solitary but sometimes clustered too. Cacti can be used as food for human but also as fodder for animals. Whenever consuming cacti, it is important to consider that some can contain psychoactive agents which may be used in herbal medicine. [5,6]

Opuntia belongs to the Cactaceae family, which has the special property to adapt to arid and semi-arid climates in tropical and subtropical regions. The family includes about 1500 species of cactus. Since it is clear that Opuntia extracts may have a positive effect on human health the interest in development for medical use increased a lot. [7]

1.3 *Opuntia ficus-indica*

Opuntia ficus-indica is generally called “prickly-pear cactus” in the Southern United States, “nopal” in Mexico and “Indian fig cactus” in Europe. (abbr. NP). [8–10] It is a dicotyledonous angiosperm, drought resistant succulent plant. [7,8,10] *O. ficus-indica* can be cultivated in arid and semiarid regions because of its adaptation mechanisms, for instance nocturnal stomata opening and an extraordinary CO_2 fixation pathway, which is called crassulacean acid metabolism. Other important characteristics are the shallow root system that permits rapid water uptake and a thick waxy cuticle that prevents extreme water loss. [11] Due to those characterizes it can tolerate a wide range of climatic conditions. [8] Therefore, the plant can be found worldwide, like in South America, Mediterranean area, Middle East and India or on Jeju Island, in the southern of Korea. [8,10,12–14] The amount of cladode production depends on the region, meteorological conditions and kind of the plant. [13]

Figure 1 Ripe fruits on a spineless *Opuntia ficus-indica*, Photograph by: Frank Schulenburg
Picture taken from: http://tropical.theferns.info/image.php?id=Opuntia+ficus-indica (24.05.18)

The nopal cladodes of Opuntia plants have already been used in Mexico by the Mesoamerican people over 7000 years not just as religious celebrations, food or forage but also as medical treatment. [12] In folk medicine the people believed in the healing power, so the plant was used for the treatment of lots of chronic diseases but also often used for the treatment of ulcers, wounds, burns, edema and even rheumatic pain and asthma. [10,13] Academic scientists, private companies and scientific investigations confirmed the health benefits and provided evidence for the potential of health benefit of this cactus. [7,13] Nowadays the young cladodes are eaten as vegetables but still are just rarely used in the modern nutrition. [12,13]

There is more and more interest in the cactus plant Opuntia because of the potential to use it in food, pharmaceutical and cosmetic industries. [12] *Opuntia ficus-indica* has a high potential to be helpful in human health and medicine. The cladodes as functional food could become a very important part of prevention of many chronic diseases, which are related to oxidative stress. The risk reducing effect is based on the highly potent antioxidant compounds and radical scavenging activities. [7,13]

Biologically relevant activities are: [7,14]

- hypolipidemic
- anti-inflammatory
- antioxidant
- hypoglycemic
- convey wound healing
- antimicrobial
- neuroprotective
- antiallergic
- anticancer
- reduce gastric damage

Due to those activities a therapeutic potential has been suggested for: dyslipidaemia, cardiovascular diseases, metabolic syndrome, non-alcoholic fatty liver disease, rheumatism, cerebral ischemia, cancer, virological and bacterial infections. [7,13]

A study evaluated the use of the cladodes from *Opuntia ficus-indica* as a potential health promoting functional ingredient in bakery products. [13] The nopal is low in calories and has low glycemic indexes. [15]

Chemical composition: The cladodes are very rich in pectin, mucilage and minerals and have a high amount of water and hydrophilic polysaccharide of large molecular weight, such as dietary fiber. [10–12] Dietary fiber can be classified as water-soluble fiber and non-water-soluble dietary fiber. It is widely known that the genus Opuntia produces lots of mucus, which is a part of dietary fiber that humans cannot digest. [9]

Opuntia ficus-indica contains calcium, potassium, magnesium, sodium and traces of iron and manganese, but also carbonates, chlorides, sulfates, oxalates and phosphates. [12] Further it contains vitamins (cactus pear has higher content than other common fruits like apple, banana or grape), polyunsaturared fatty acids and amino acids (major amino acid detected in the cladodes are glutamine, leucine, lysine, valine, arginine, phenylalanine and isoleucine). [7] The cladode of *Opuntia ficus-indica* is also rich in antioxidative compounds, such as phenolics, flavonoids, flavonols, carotenes, ascorbic acid, betaxanthin, betacyanin, vitamin E and alkaloids. [7,13,15] These compounds have a positive impact on health, especially the flavonoids have shown anti-inflammatory and chemo preventive activities. The most common flavonoid in *O. ficus-indica* is isorhamnetin. [16] Polyphenolics are also important for the positive effects, such as the prevention of cardiovascular dysregulation, neurodegenerative dysregulation and inflammation and they have proven anticancer activity as well. [7]
The composition of the cladodes can be different. It varies according to the season, climate, environment, cultivation site, soil type and age of the plant. [7,12]

1.4 Probiotics, Prebiotics, Synbiotics

1.4.1 Probiotics

The word *probiotic* comes from the Greek and means “for life”. In the 1960s it was used to describe a substance of a microorganism which stimulates the growth of another. Today we use the definition of Fuller, who defined the term *probiotic* as “a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance” [17,18]. Ideal probiotics should have the following properties: It is important that it is not pathogen. They are supposed to be resistant to gastric acid and bile and withstand all treatments and storage. Furthermore, it should persist in the intestinal tract, adhere to the epithelium or mucus and modulate immune response. [19]

Lots of microbial species have been used, for instance species of Bacillus, Bifidobacterium, Enterococcus, Escherichia, Lactobacillus, Lactococcus, Streptococcus and some yeast species. In humans Lactobacillus and Bifidobacterium species have been used the most. [19] Probiotics are more and more added into fermented dairy products like yogurt or freeze-dried cultures as well as in fermented vegetables and meats. The intake of probiotics has positive effects of human health and can be used therapeutically, for example reducing rotavirus-induced diarrhea and may even prevent colon cancer. [20]

1.4.2 Prebiotics

When it comes to the term prebiotic the “pro” was exchanged for “pre”, which means “before”. A prebiotic is defined as a “non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon.” [17] The bacterial strains, which benefit the most are bifidobacteria and lactobacilli. [21]

Those indigestible oligosaccharides are used as substrate by microorganisms, so they can produce energy and provide metabolites and micronutrients for the host. The aim is to improve the host health. An ideal prebiotic should not be hydrolyzed or absorbed in the upper part of the gastrointestinal tract. Examples of common prebiotics are fructooligosaccharides (FOS), galactooligosaccharides (GOS), inulin, lactilol and lactulose. [21–23]

1.4.3 Synbiotics

Synbiotics

A product is called synbiotic when it contains probiotics and prebiotics. [17] This combination might improve the survival of the probiotic organism when crossing the upper part of the gastrointestinal tract, because the specific substrate is easily available for fermentation and at the same time it can enhance the effects of the bacteria in the large bowel. [20,23] It should be noted that the term synbiotic is only used for products in which the prebiotic compound favors the probiotic compound. [17] Possible combinations are: Bifidobacteria + FOS, bifidobacteria + GOS, lactobacilli + lactilol. [23]

1.5 Bacterial strains

1.5.1 *Lactobacillus acidophilus*

The human gastrointestinal tract is a complex ecosystem, due to the trillions of organisms. One of them is *Lactobacillus acidophilus*. It belongs to the group of lactic acid bacteria (LAB), which are all gram-positive and non-sporing. They can appear as cocci, coccobacilli or rods. A lack of catalase is a characteristic property of *Lactobacillus acidophilus* and they need fermentable carbohydrates for growth. [24]

Lactobacillus acidophilus is one of the few bacteria, which can survive in the human gastrointestinal system. It produces lactic acid, is resistant to acid conditions in the stomach, to digestive enzymes and to the bactericidal activity of bile salts. These properties are responsible for the survival in the gut. Lactic acid bacteria are often used as probiotic agents in dairy products and functional food. The bacteria adhere to human epithelial cell lines and to human intestinal mucus. Therefore, they can prevent the adhesion of some pathogens and act as antimicrobial agens. This effect can be improved by the addition of Ca^{2+}. [25,26]

1.5.2 Lactobacillus rhamnosus GG

Lactobacillus rhamnosus GG is another well-known probiotic strain, which belongs to the lactic acid bacteria group. The name comes from the first letters of the surnames of the scientists Sherwood Gorbach and Barry Goldin who discovered the bacterium. This bacterial strain is also very resistant and survives in the gastrointestinal system of human and adheres on intestinal mucus. It lowers the activity of procarcinogenic enzymes, normalizes the microflora balance, the mucosa barrier and short-chain fatty acid levels. In fact, it has been successful in treating acute diarrhea caused by viruses and bacteria in adults and children. Additionally, there have already been positive results in treatment and primary prevention of atopic dermatitis. *Lactobacillus rhamnosus GG* is already used in lots of products based on diary, such as yogurt, fermented milk or semihard cheese. [26,27]

1.5.3 Bifidobacterium longum ssp. infantis

Bifidobacterium longum subsp. infantis is an anaerobic, gram-positive bacterium belonging to the genus Bifidobacterium. It is known that bifidobacteria are very important and helpful for the human gut and intestinal health. They have many positive effects such as diarrhea prevention in antibiotic treated patients, cholesterol reduction, cancer prevention or immune stimulation. Due to their various physiological functions, including producing organic acids such as lactic acid and acetic acid they can regulate the intestinal environment and even prevent the growth of harmful bacteria. The acetic acid is more potent than other acids for inhibiting the growth of harmful bacteria, for example *E. coli*. When *Bifidobacterium longum ssp. infantis* was first isolated it was quickly associated with a healthy gastrointestinal tract because it showed a high dominance in breast fed infants compared to bottle fed infants. It turned out that *Bifidobacterium longum ssp. infantis* is unique because it has the capacity to digest human milk oligosaccharide structures. [28,29]

1.5.4 *Bifidobacterium animalis ssp. lactis*

Bifidobacterium animalis subsp. lactis is a bacterium which also belongs to the genus Bifidobacterium, which are considered to be probiotic microorganisms. This bacterial strain has similar health benefits as *Bifidobacterium longum ssp. infantis*. These positive effects are attributed to their pH-reducing activity. It has been shown that *Bifidobacterium animalis ssp. lactis* is more stress resistant than other strains because it is resistant to acid and bile and is still viable after extended refrigerated storage. [30,31]

1.5.5 Comparison between lactobacilli and bifidobacteria

Bifidobacteria are the predominant beneficial bacteria in the human intestinal flora. The population of lactic acid bacteria is much lower than the population of bifidobacteria, which have adapted to live in the human intestinal tract. [32]

Starting with the appearance, the two bacterial strains differ, but also their habitat and sensitivity to oxygen is not the same. Another important difference are the main metabolites, because bifidobacteria produce acetic acid in addition to lactic acid. [32] The most significant differences between the two bacterial strains are shown in Figure 2.

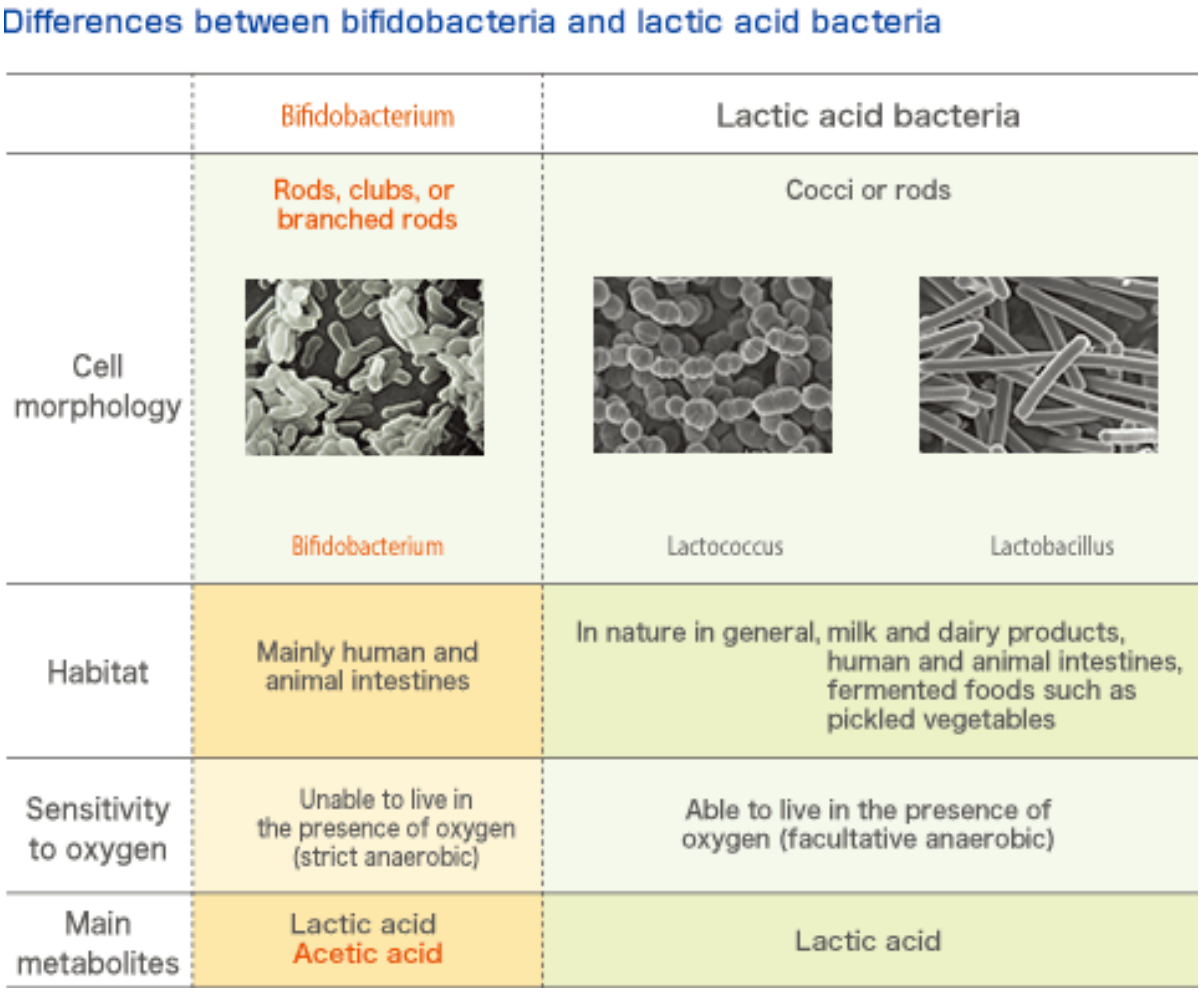

Figure 2 Differences between bifidobacteria and lactic acid bacteria, Provided by Morinaga Milk Industry

1.6 Fermentability of prebiotics

Some dietary fibers are declared as prebiotics, such as inulin-type fructans and galacto-oligosaccharides. The term “dietary fiber” is used to describe non-digestible components. Because of their resistance to digestion in the human small intestine they can reach the colon, where they are fermented by the gastrointestinal microflora. The fermentation of dietary fiber by microbacteria in the colon provides active products, which are locally in the gut or can be absorbed from the large bowel. In such a way they can exert a systemic influence on various parts of the body. They have a positive effect on human health by increasing the number or activity of the microbiota in the gastrointestinal tract. [33,34]

The fermentation of prebiotics by intestinal microbiota is of high impact. This so-called fermentability is determined by the monosaccharide units, the structure, the glycosidic linkage between the monosaccharide residues and the degree of polymerization (dp). Prebiotics with lower dp lead to an earlier growth than those with higher dp. [35,36]

1.7 Growth curves

As microorganism grow, they increase the turbidity of their growth medium. The more turbid the medium, the higher the concentration of the organism. The growth of the microorganism can be determined with a turbidity measurement. Growth curve can be generated where the optical density is plotted vs. time. [37,38]

2. Materials and Methods

2.1 Dried samples of *Opuntia ficus-indica*

The fractions were received from the colleague Jose Manuel Cruz Rubio. Summarily the first step was the extraction with acetone, then with 95% methanol and later with water. The mucilage was dissolved in water, and methanol was added until the methanol concentration reached 50%. The resulting precipitate was separated and was called fraction 50-0. More methanol was added to the solution until a concentration of 80% was reached. The precipitate was separated, and it became fraction 50-80. The methanol was removed of the solution by evaporation and then lyophilized, which led to fraction 100-80. An additional fractionation of the 100-80 fraction in sub-fractions F1-F8 was conducted using size exclusion chromatography (SEC) to obtain a separation by size. Further and more detailed information can be found in a colleague´s thesis: “Evaluation of the biological activity and the composition of extracts from two Opuntia species”. [39]

2.2 Sample preparation of fresh *Opuntia ficus-indica*

2.2.1 Extraction

The cladodes were obtained from Mexico and stored at -80°C for further use. For analysis the samples were thawed.

Figure 3 Cladode *of Opuntia ficus-indica* (Picture taken on May 22nd, 2018)

Figure 4 View of sliced *Opuntia ficus-indica* (Picture taken on May 22nd, 2018)

To remove chlorophyll and salts the fresh cladodes were sliced into thin stripes (approximately 3-4 mm) and then incubated with 100% methanol under continuous stirring overnight. Afterwards the methanol was decanted and the nopal was transferred into fresh methanol and stirred for 4 hours; this process was repeated twice. The nopal was milled and mixed with deionized water at 40°C and stirred for 2 hours. The mixture was filtrated, the remaining solid extracted with H_2O twice. The cellular plant compounds were separated by centrifugation at 13000 rpm and 20°C. The mucilage was frozen, lyophilized and stored until further use.

2.2.2 Mucilage fractionation

The lyophilized mucilaginous extract was mixed with deionized water and methanol (20:80 V/V) to obtain the 80-100 fraction of the fresh Opuntia. Separation of the supernatant and the precipitate was achieved by centrifugation at 9000 rpm and 20°C. The methanol was removed on a rotavapor, and then lyophilized and stored in a desiccator.

2.3 Poly- and oligosaccharide analysis

2.3.1 Molecular weight distribution

Size exclusion chromatography is a common method to determine the molecular weight of a sample. The principle is to separate based on molecular size. For the procedure it is necessary to dissolve the polymer in an appropriate solvent. Then it is injected into a column packed with porous particles of defined pore size. The polymer elutes through the column and molecules that are too large to penetrate the pores of the packing elute first and smaller molecules elute at a later time. To determine the molecular weight, the columns must be calibrated with polymer standards of known molecular weight. This is very important because SEC is a relative and not an absolute molecular weight technique. [40]

The system used for the analysis of the dry *Opuntia ficus-indica* samples consists of a pre-column (Fractogel HW40) and a column packed with Superose 6, followed by a column with Superose 12 and last with Toyopearl HW40S. Eluent consisted of 0.05 % NaN_3 and 50 mM NaCl were dissolved in 5 l deionized water.
Around 5 mg sample were dissolved in 0.350 ml of eluent and centrifuged for 10 minutes at 13000 rpm at room temperature. Then the supernatant was loaded to the analytical SEC system and the separation was performed under following conditions:

Flow rate: 0.6 ml/min
Duration: 130 minutes
Detection: by differential refractive index-detector (Knaur)

Calibration of the SEC system with:

Standard 1:	Glucose, Dextran 1 (1080 Da), Dextran 50 (43500 Da)
Standard 2:	Maltose, Dextran 5 (4440 Da), Dextran 80 (66700 Da)

Table 1 Standards for SEC calibration

2.3.2 Thin-layer Chromatography

Thin-layer chromatography is a simple chromatographic method. To perform a qualitative or semiquantitative analyses with TLC a closed vessel containing solvent and a coated plate are required. The plate is coated with the stationary phase, a thin layer sorbent. A small amount of sample is placed near one end of the plate and the sample is then dried. Now the plate is placed into the mobile phase, which is commonly a mixture of two to four solvents. Depending on how the layer and mobile phase were chosen the components of the sample move with the mobile phase through the stationary phase. The mobile phase must move an appropriate distance and then can be removed and dried. Afterwards the zones can be detected in with the application of a convenient visualization reagent. The result shows different migration because the components have different affinity to the mixture of the stationary and mobile phase. The identification of the compounds is based on comparison of R_f values to authentic reference standards. [41]

Samples and standards were dissolved in 60% methanol at a concentration of 4mg/ml and standards at a concentration of 1mg/ml (except glucuronic acid and galacturonic acid 3mg/ml) were injected on thin-layer chromatography plates (Merck, HPTLC plates silica gel 60, 20 x 10 cm) using a Linomat (CAMAG) system. The plates were developed three times.

Mobile phase for oligosaccharide analysis: 1-Propanol:deionized water 7:3.5

Carbohydrates were detected by immersion in thymol-sulfuric derivatization reagent (1.25mg thymol, 12.5ml sulfuric acid in 250ml methanol). Afterwards the plate was dried with a hot airstream and to visualize the bands the plates were incubated in the drying oven for 5 minutes at 100°C.

Two different standards were chosen to compare and identify the bands. The components and their concentration are listed in Table 2.

	Carbohydrate	**concentration**
STD 1	Rhamnose	1 mg/ml
	Xylose	1 mg/ml
	Arabinose	1 mg/ml
	Mannose	1 mg/ml
	Glucose	1 mg/ml
	Galactose	1 mg/ml
	Sucrose	1 mg/ml
	Glucuronic acid	3 mg/ml
	Galacturonic acid	3 mg/ml
STD 2	Fucose	1 mg/ml
	Fructose	1 mg/ml
	Maltose	1 mg/ml
	Lactose	1 mg/ml
	Raffinose	1 mg/ml

Table 2 Standards for thin-layer chromatography

2.4 Monosaccharide analysis

To determine the monosaccharide composition of the oligosaccharides a chemical hydrolysis step was performed. 4 mg of the samples were dissolved in 200 µl 2M trifluoroacetic acid (TFA) and incubated at 105°C for 2 hours. After the samples were cooled, they were centrifuged. TFA was removed by N_2 vaporization, by adding 1 ml of 100% methanol and evaporated by bubbling nitrogen gas. This step was repeated twelve times to ensure that all the TFA had been removed. The residue was dissolved in 1 ml of 60% methanol and then centrifugated at 13000 rpm for 10 minutes. The supernatant of each sample was used for further analysis and was stored in the freezer.

2.4.1 Monosaccharide composition analysis by thin-layer chromatography

The hydrolysate was analyzed the same way as the samples for poly- and oligosaccharide analysis by the described methods before. The only difference is that another mobile phase was used.

Mobile phase for monosaccharide analysis:

acetonitrile:0.3% ammonium hydroxide 17:3

2.4.2 Monosaccharide composition analysis by RP-HPLC

Reversed-phase high pressure liquid chromatography (HPLC) is a very efficient chromatographic method. The sample mixture is distributed between the mobile and the stationary phase. In the column the substances are separated from each other by interacting with the stationary phase. Then the substances are detected by a detector, which is connected to a computer and thus enables evaluation via software. [42]

1-Phenyl-3-methyl-5-pyrazolone (PMP) solution:

For the derivatization of monosaccharides, a PMP MeOH solution 0.3 M was prepared.

Standard and sample preparation:

Standards were weighted and dissolved in ammonia 32%. 200 µl aliquots of each standard and hydrolyzed sample were taken and mixed with 200 µl of 0.3 M methanolic PMP. Afterwards the mixture was incubated at 70°C for 30 minutes. To remove the ammonia 1 ml of deionized water was added and evaporated to dryness in the vacuum oven three times. To remove the PMP residues 1 ml chloroform ($CHCl_3$) was added and in the next step the $CHCl_3$ phase was discarded; this step was repeated three times. Before injection in the RP-HPLC the aqueous layer was centrifugated at 13500 rpm.

Mobile phase preparation:

A) Phosphate buffer 0.1 M pH 6.7
B) Acetonitrile

⇨ Mobile phase A : B 83:17

RP-HPLC conditions:

Flow rate:	1 ml/min
Injection volume:	10 µl
Detection:	245 nm
Column temperature:	30°C

Column: Zorbax Eclipse XDB-C18 4.6 x 250mm + precolumn 4.6 x 12.5mm

2.4.3 HPAEC-PAD

High-performance anion-exchange chromatography (HPAEC) can be used for many carbohydrates which are weak acids with pKa values in the range 12-14 and therefore their hydroxyl groups can partially or totally be transformed into oxyanions. These can be selectively eluted as anions by highly efficient anion-exchange columns. The HPAEC can be combined with the Pulsed Amperometic detection (PAD), which is performed at a solid anode, for instance gold, platinum and glassy carbon. [43]

Sample preparation:

The hydrolysate-samples were diluted and then injected in the IC-3000 ion chromatography system (Dionex, Thermo Scientific).

Standard preparation:

Standards (fucose, rhamnose, arabinose, galactose, glucose, xylose, fructose, galacturonic acid, glucuronic acid) were prepared and injected to generate a calibration curve.

Mobile phase preparation

Solvent A: 0.2 M sodium hydroxide
Solvent B: 0.2 M sodium hydroxide + 0.2 M sodium acetate
Solvent C: water

HPAEC conditions:

Column system: CarboPac PA1 guard columnn (4 x 50 mm) and analytical column (4 x 250 mm)
Injection volume: 10µl
Temperature: 30°C
Flow rate: 1 ml/min
Detection: standard carbohydrate waveform, by an amperometric detector (PAD-detector; Dionex)

Samples were separated using a multi-step gradient starting with regeneration with 200 mM NaOH (100% A) from -20.0 to -2.5 min, followed by 10 mM NaOH + 2 mM NaOAc (4% A + 1% B) from -2.5 to 22 min. At 22 min the eluent was changed to 10 mM NaOH + 10 mM NaOAc (5% B) and a gradient started to 200 mM NaOh + 200 mM NaOAc (100% B) until 40 min. Finally, the column was washed for 15 min with 200 mM NaOH (100% A)

2.4 Determination of bacterial growth curves

The growth curves of the bacteria were determined by using Bioscreen C (Labsystems Helsinki Finland), which is a technical device combining an incubator with a photometer. The sample were placed in the device and incubated at 37°C. Before each measurement, the samples were shaken to prevent bacteria from settling at the bottom of the well and turbidity was measured every hour at a wavelength of 600nm. The measurement was conducted for 120 hours (5 days).

To avoid contamination sterile work with bacteria is very important. All work steps that required a low-germ environment were carried out in a Laminar Air Flow.
The medium used, and all other solutions required for the work were autoclaved or sterile filtered and stored at 4°C in the refrigerator. Glassware and other materials were autoclaved or used as sterile disposable equipment. All work surfaces were cleaned with ethanol.

2.4.1 Bacterial strains

Four different bacterial strains were used for the determination of growth curves. The bacterial strains used were available as frozen glycerol stock, which was thawed at room temperature. The bacteria suspension was then centrifuged at 10000 rpm at RT for 3 minutes. To remove possible residues of glycerol, the pellet was washed twice with a phosphate buffered saline – cysteine hydrochloride solution. For this purpose, the supernatant was discarded after centrifugation and the residue absorbed in 1ml MRS+ medium and then carefully suspended again.

The used bacterial strains are listed in Table 3.

Bacterial strain	Abbreviation	Number
Lactobacillus acidophilus LA-5	La 601	DSM 13241
Lactobacillus rhamnosus GG	Lb 29	ATCC 53103
Bifidobacterium longum ssp. infantis	B inf	ATCC 15697
Bifidobacterium animalis ssp lactis	BB-12	DSM 15954

Table 3 Used bacterial strains

Cultivation of the bacterial strains:

Day 1: 1 ml bacteria suspension and 4 ml MRS+ media (containing glucose as carbohydrate source) were incubated overnight at 37°C under anaerobic conditions.

Day 2: 1 ml of each culture was diluted with 4 ml MRS- media (no carbohydrate source) and incubated overnight in an anaerobic incubator.

2.4.2 Preparation of media and solutions

MRS-Media (= Man-Rogosa-Sharpe Media):

casein peptone	10.0 g
meat extract	8.0 g
yeast extract	4.0 g
potassium phosphate dibasic	2.0 g
tween 80	1.0 g
ammonium citrate dibasic	2.0 g
sodium acetate	5.0 g
magnesium sulfate	0.2 g
L-cysteine hydrochloride	0.56 g
manganese sulfate	0.05 g

Table 4 Required material for MRS media

A medium without glucose (MRS-) and a medium with glucose (MRS+) were produced. The preparation of the media took place by weighing the substances (Sigma Aldrich) shown in Table 4.

MRS - The substances were weighted, then dissolved in 1000 ml distilled water

MRS + The substances were weighted, then dissolved in 900 ml distilled water, as 100 ml of a 20% glucose solution was added after the autoclaving process, so that the final concentration of glucose in the medium corresponded to 2%

2x MRS - The substances were weighted, then dissolved in 500 ml distilled water

After filling with distilled water, the measuring cylinder was left on a magnetic stirrer until all components were dissolved. The solution was transferred to a suitable autoclave vessel and then the media was autoclaved at 121°C for 20 minutes.
The glucose solution was prepared separately from the medium by dissolving 20 g glucose in 100 ml water. This solution was also autoclaved at 121°C for 20 minutes.

Preparation of PBS-cysteine hydrochloride solution

For washing the bacteria, a PBS-cysteine hydrochloride solution was prepared:
50 ml 10x PBS + 50 ml 10x cysteine-hydrochloride filled up to 500 ml in a measuring cylinder.
To prepare the 10x cysteine-hydrochloride solution 1 g cysteine-hydrochloride was dissolved in 200 ml distilled water and then autoclaved at 121°C for 20 minutes.

Preparation of the carbohydrate solutions

Carbohydrate stocks (2%) were prepared and used as standards. Eight standards were used: glucose, galactose, xylose, rhamnose, arabinose, fucose, glucuronic acid and galacturonic acid.
The carbohydrates were weighted, dissolved in water, sterile filtered and stored at 4°C.

Opuntia sample preparation

The solutions of dried and fresh *Opuntia ficus-indica* (2%) were prepared by dissolving 20 mg of the sample in 1 ml deionized water and were then sterile filtered and stored at 4°C.

Bacteria sample preparation

A method to determine the growth of bacteria is turbidity measurement. The optical density at 600 nm of the bacterial culture was determined by using a photometer (U-1100 Spectrophotometer, HITACHI).

Then required amount of each bacterial strain was calculated (to start the measurement with an OD_{600} of 0.1), transferred into a sterile micro test tube and centrifuged at 12500 rpm and room temperature for 3 minutes. The supernatant was discarded, and the bacterial cell pellet was washed with 1 ml PBS-cysteine hydrochloride solution and then centrifuged again at 12500 rpm and room temperature for 3 minutes, this step was repeated 2 more times. Afterwards the pellet was dissolved in the calculated amount of 2xMRS- media.

2.4.3 Determination of the effect of different *Opuntia ficus-indica* fractions on the growth of probiotics

To determine the growth of the bacteria each well of a sterile microtiter plate (honeycomb) was filled with a final volume of 200 µl (100 µl of carbohydrate stock or sample stock and 100 µl of bacteria culture). Negative controls of each bacterial strain were carried out to determine how much the bacterium grows without any carbohydrates. The difference here was that autoclaved deionized water was used instead of the carbohydrate source. For further control, 2 wells each were filled with MRS-and MRS+ without bacteria. If there was no increase in turbidity in these samples, a sterile process could be guaranteed.

The filled microtiter plate was incubated at 37 °C for 5 days in the “Bioscreen C”. The optical density (at 600 nm) was measured every hour after shaking the plate for 20 seconds. All samples were tested in duplicates, averages were calculated, and graphs, charts and tables were created.

3. Results and Discussion

3.1 Analysis of poly- and oligosaccharides of *Opuntia ficus-indica*

The extracts of dried Opuntia ficus-indica (OFI F100-80, F80-50, and F50-0) as well as the extracts of fresh Opuntia (OFI fresh F100-80) were examined.
For more detailed analysis of OFI F100-80 of the dried plant the sub-fractions OFI F1 - OFI F8 were used, which were collected and pooled from the preparative SEC. F8 was collected first and F1 last.

3.1.2 Molecular weight distribution

The distribution profile was examined by using analytical SEC. To evaluate the data the program CPCWin32 was used. To determine the molecular distribution, a first differentiation was made between mono- and disaccharides and oligosaccharides. If necessary, a distinction was made between several oligosaccharide peaks. Then, the mean molecular weight (MW), the polydispersity index (Mw/Mn), the weight and number average degree of polymerization (DPw, DPn) and the range of molecular weight and dp were determined from the oligosaccharide peaks.

Analysis of OFI F8 – OFI F1:
The results of OFI F8 – OFI F1 can be found in Table 5 and 6.

	OFI F8	**OFI F7a**	**OFI F6**	**OFI F5**
Mono- and disaccharide peak				
content	1.9%	5.1%	18.2%	21.6%
Oligosaccharide peak				
content	94.7%	94.9%	81.8%	78.4%
MW (Da)	6010	2640	1190	1190
Mw/Mn	2.116	1.714	1.417	1.368
DPw	38	16	7	7
DPn	17	10	5	5
DP range	1-490	1-170	1-40	1-32
MW range (Da)	194-79432	239-27542	208-6456	229-5128
Polysaccharide peak				
content	3.4%			

Table 5 SEC results of OFI F8, F7a, 6 and 5

	OFI F4	OFI F3	OFI F2	OFI F1
Mono- and disaccharide peak				
content	29.2%	32.5%	40.9%	49.4%
Oligosaccharide peak				
content	70.8%	67.5%	59.1%	50.6%
MW (Da)	1350	1010	830	840
Mw/Mn	1.588	1.554	1.317	1.2
DPw	8	6	5	5
DPn	5	4	4	4
DP range	1-49	1-43	1-39	2-32
MW range (Da)	251-7943	213-6918	281-6309	338-5128

Table 6 SEC results of OFI F4, F3, 2 and 1

From the values determined it can be clearly seen that F8 contains the least mono- and disaccharides and the most oligosaccharides, even polysaccharides. F1, on the other hand, has the highest mono- and disaccharide and the lowest oligosaccharide content.

The later the fraction is eluted from the SEC, the smaller molecules the fraction contains. The distribution between mono-/disaccharides and oligosaccharides in the fractions F8 – F1 are shown in Figure 5.

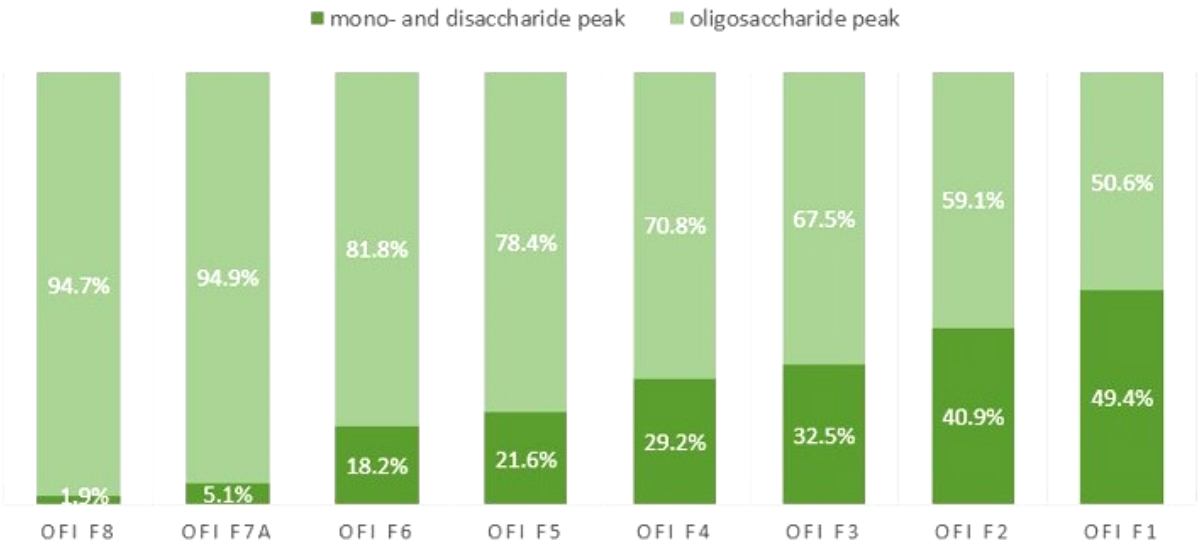

Figure 5 Distribution between mono-/disaccharides and oligosaccharides in the fractions F8 – F1

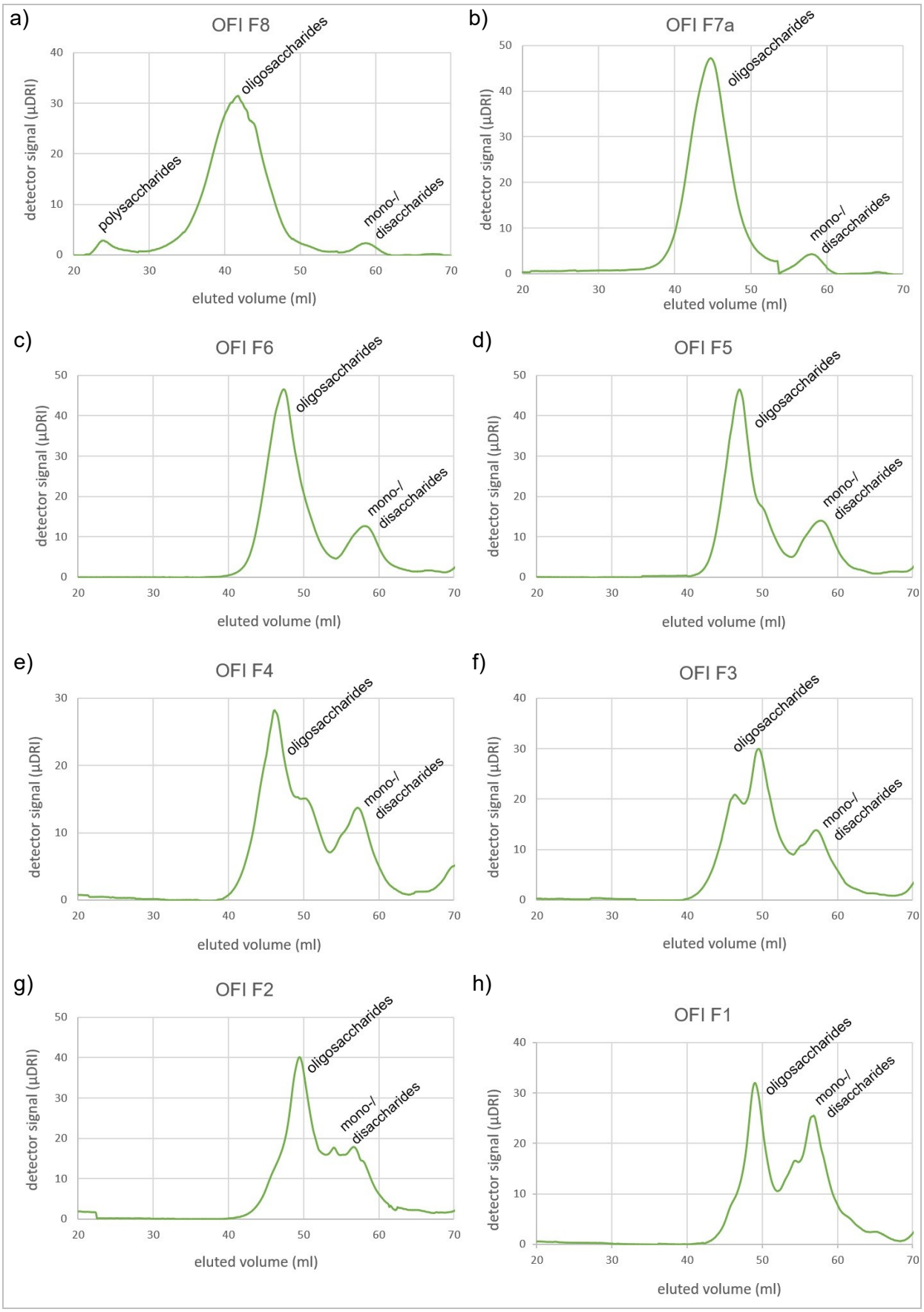

Figure 6 SEC analysis of *Opuntia ficus-indica* fractions a) OFI F8, b) OFI F7a, c) OFI F6, d) OFI F5, e) OFI F4, f) OFI F3, g) OFI F2, h) OFI F1; DRI = differential refractive index

The chromatograms obtained from the analytical size exclusion chromatography are shown in Figure 6.

The results also reveal that the individual oligosaccharide peaks have different degrees of polymerization. The higher the fraction number, the higher the average degree of polymerization. In detail fraction 2 the oligosaccharide peak has an average dp of 5 and dp range of 1-39. In contrast in fraction 8 oligosaccharides have an average dp of 38 and dp range of 1-490.

To compare the fractions and to make the difference even clearer, the graphs of several fractions were overlaid. Figure 7 shows the SEC analysis of OFI F8, F5 and F1.

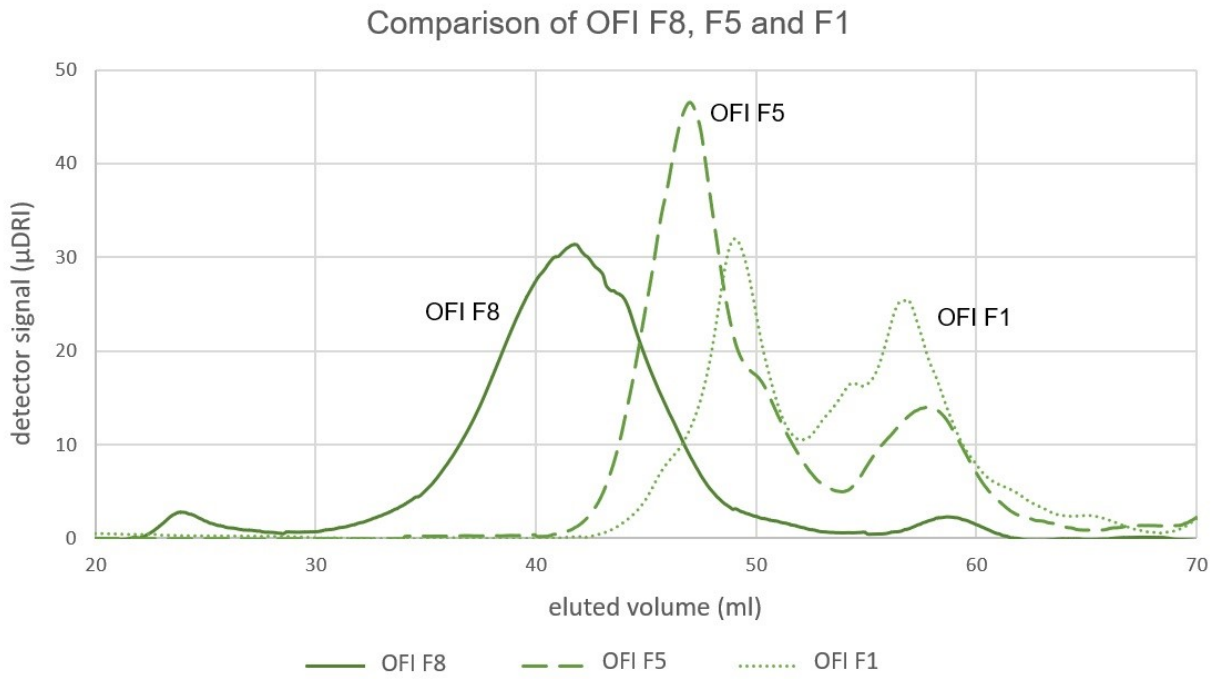

Figure 7 Comparison of SEC analysis of OFI F8, F5 and F1

Analysis of OFI F100-80, F80-50 and F50-0:

	100-80	80-50	50-0
Mono- and disaccharide peak			
content	28.4%	6.7%	10.6%
Oligosaccharide peak			
content	24.2%	25.9%	37.7%
MW (Da)	690	2020	1790
Mw/Mn	1.113	1.53	1.55
DPw	10	12	11
DPn	4	8	17
DP range	1-7	1-30	1-32
MW range (Da)	316-1071	301-4786	263-5128
Polysaccharide peak 1			
content	47.4%	19.1%	16.3%
MW (Da)	3100	13210	12660
Mw/Mn	1.442	1.276	1.219
DPw	19	82	78
DPn	13	63	64
DP range	6-152	30-178	33-166
MW range	1096-24547	4897-28840	5370-26915
Polysaccharide peak 2			
content		48.3%	35.4%
MW (Da)		213910	156120
Mw/Mn		1.708	1.584
DPw		1320	964
DPn		773	608
DP range		186-4078	170-3392
MW range (Da)		30199-660693	27542-549540

Table 7 SEC results of OFI F100-80, 80-50 and 50-0

The results of the extracts of dried Opuntia are listed in Table 7. It has been shown that F100-80 contains more mono- and disaccharides than the other two extracts. This result confirms the theory, since smaller sugars are more soluble in methanol. Therefore, most molecules with dp1 and dp2 should be present in fraction 100-80. The distribution also shows that fraction 80-50 contains larger molecules than fraction 100-80, which is clearly demonstrated in the SEC chromatogram because F80-50 is eluted later.

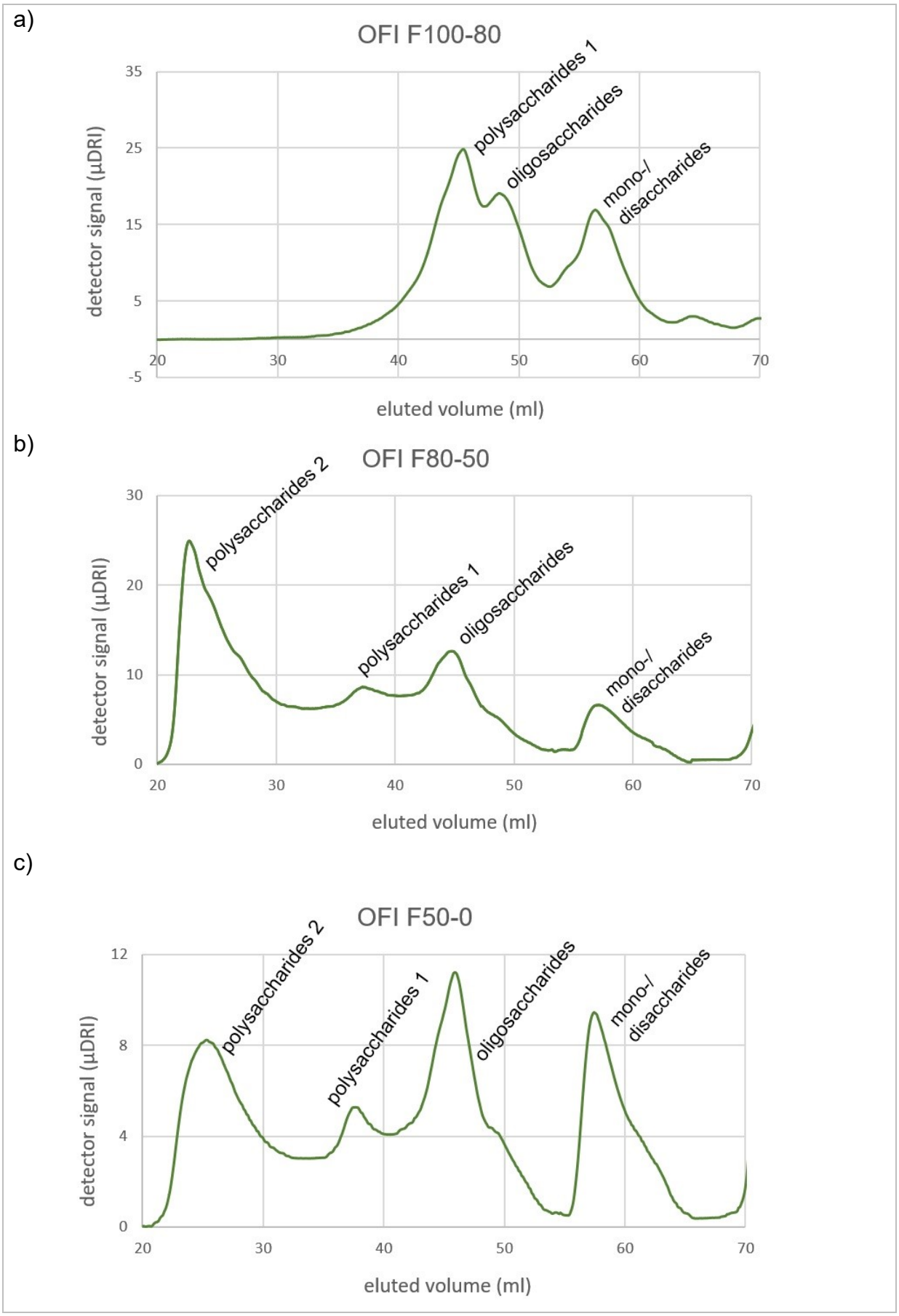

Figure 8 SEC analysis of *Opuntia ficus-indica* fractions a) F100-80, b) F80-50, c) F50-0

To compare the extracts and to illustrate the differences in Figure 9 the chromatograms of the extracts F100-80, F80-50 and F50-0 were superimposed.

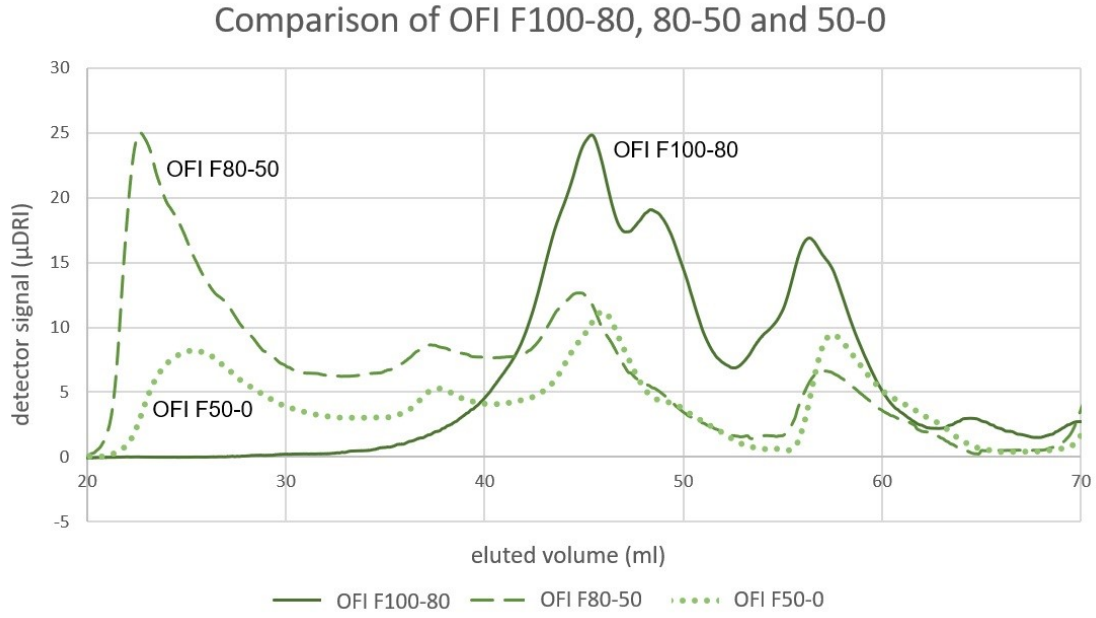

Figure 9 Comparison of SEC analysis of F100-80, F80-50 and F50-0

Analysis of fresh OFI F100-80

	OFI fresh 100-80
Mono- and disaccharide peak	
content	19.3%
Oligosaccharide peak	
content	66.4%
MW	1820
Mw/Mn	1.492
DPw	11
DPn	7
DP range	2-55
MW range	323-8912
Polysaccharide peak	
content	1.5%
MW	404590
Mw/Mn	1.079
DPw	2497
DPn	2314
DP range	1447-5134
MW range	234422-831763

Table 8 SEC results of fresh OFI F100-80

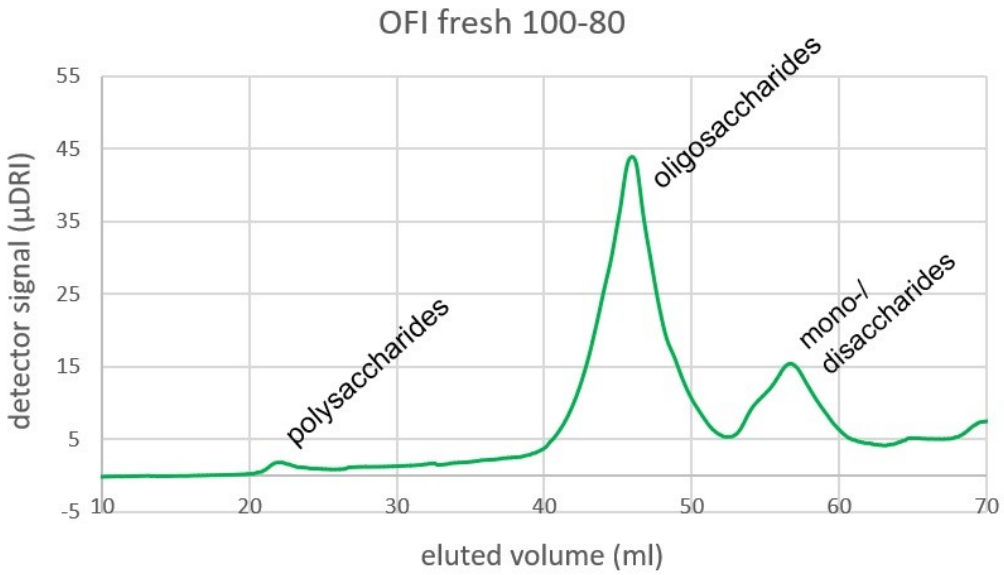

Figure 10 SEC analysis of fresh OFI F100-80

The results indicate that the extract of fresh *Opuntia ficus-indica* also contains more oligosaccharides than monosaccharides. It even contains a small amount of polysaccharides of relatively high molecular weight and DP.

In order to compare the extracts of the dried and the fresh cactus, the respective chromatograms were also superimposed, which is illustrated in Figure 11.

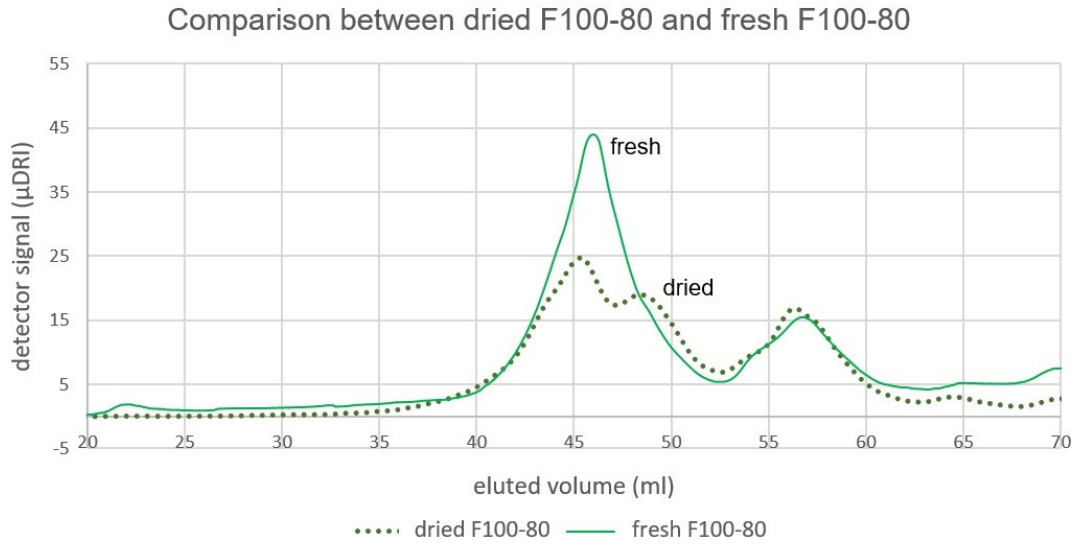

Figure 11 Comparison of SEC analysis of dried F100-80 and fresh F100-80

Comparing the composition of fresh and dry extracts, the oligosaccharide content of both is significantly higher than the monosaccharide content. The results show that the extract of the fresh plant contains less mono- and disaccharides. Yet this extract has a small amount of sugars of comparable high molecular weight and degree of polymerization, which were not detected in the dry extract. Since these were only found in the fresh Opuntia, it may be possible that these components are destroyed during treatment with heat.

3.1.3 Thin-layer chromatography

The samples were further analyzed using thin-layer chromatography. A suitable eluent was used to visualize the separation of the poly- and oligosaccharides.

All TLC plate images have been edited (contrast and brightness were changed) to make the bands clearly visible. But all images have been treated in the same way to make them comparable.

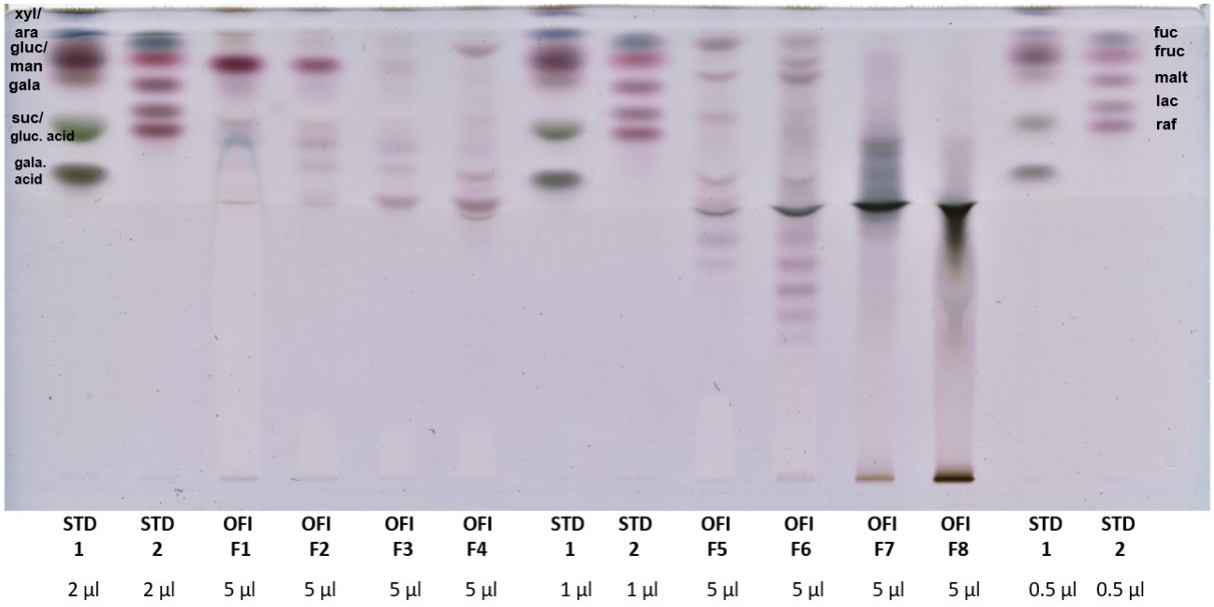

Figure 12 TLC plate of the fractions F1-F8 displaying the contained oligosaccharides

Figure 12 shows the mono, di- and oligosaccharides of fractions F1-F8. Because 1-propanol:distilled water 7:3.5 was used as an eluent the oligosaccharides are found in the lower half and the mono- and disaccharides are located in the upper half.

Fraction 1-4 do not show any oligosaccharides. On the other hand, F5 and especially F6 reveal the presence of oligosaccharides. Because of the exact separation of the carbohydrates it can be assumed that they consist of neutral oligosaccharides. It can also be speculated if it is an oligo-homologous series. To determine the monosaccharide components of the homooligosaccharides further tests are needed.

In contrast F8 shows blurred bands leading to the suggestion of the presence of heterooligosaccharides. It can be hypothesized that they consist of acidic oligosaccharides, which are not possible to separate with TLC. To determine the composition of the heteroligosaccharides further analysis would be necessary.

3.2 Monosaccharide analysis of *Opuntia ficus-indica*

All the samples were further analyzed on their monosaccharide composition using TLC, RP-HPLC and HPAEC-PAD.

3.2.1 Analysis of fraction F1 – F8

Thin-layer chromatography

The components of the unhydrolyzed fractions F1 – F8 were separated by TLC to determine which monosaccharides were originally present in *Opuntia ficus-indica*.

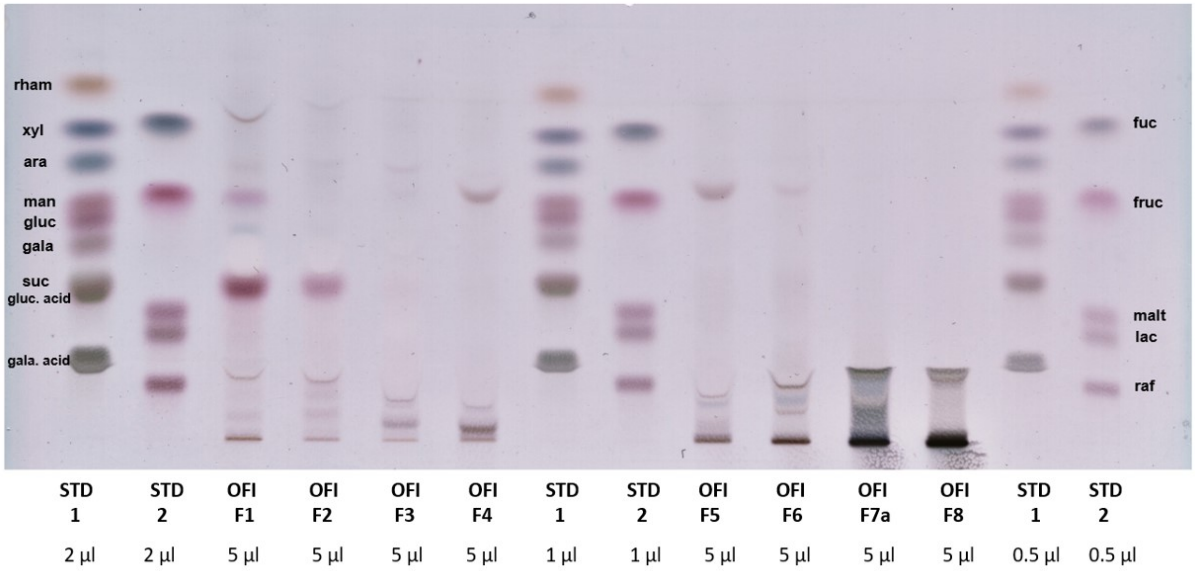

Figure 13 TLC plate of F1-F8 displaying the monosaccharides without hydrolysis

Figure 13 illustrates the mono-, disaccharides and trisaccharides without hydrolysis. As an eluent acetonitrile:0.3% ammonium hydroxide 17:3 was used. All the monosaccharides could be separated, but with this eluent the oligosaccharides cannot move, and therefore they stay at the bottom of the TLC plate.

The TLC displays that F1 contains fructose and an even higher concentration of sucrose. According to the results of the SEC, it can be assumed that sucrose and fructose make up almost 50% of F1. F2 shows only traces of sucrose.

The fractions with higher numbers show less monosaccharides and more oligosaccharides, which also correlates well with the results of the SEC.

For further analysis of the oligosaccharides and to determine the monosaccharide composition a chemical hydrolysis was performed.

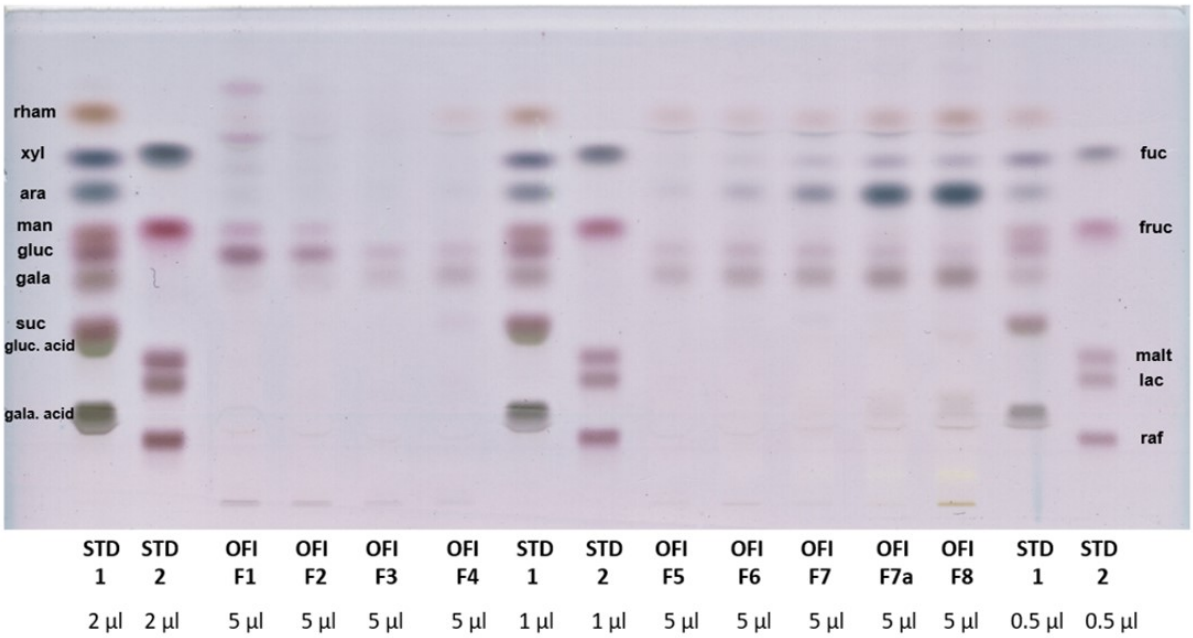

Figure 14 TLC plates of F1-F8 after hydrolysis

The results of TLC after hydrolysis are presented in tabular form in Table 9.

	F1	F2	F3	F4	F5	F6	F7	F7a	F8
Rhamnose				✓	✓	✓	✓	✓	✓
Xylose					✓	✓	✓	✓	✓
Arabinose					✓	✓	✓	✓	✓
Mannose	✓	✓							
Glucose	✓	✓	✓	✓	✓	✓	✓	✓	✓
Galactose	✓	✓	✓	✓	✓	✓	✓	✓	✓
Sucrose									
Glucuronic acid									
Galacturonic acid								✓	✓
Fucose									
Fructose									

Table 9 TLC results of F1-F8 after hydrolysis

Since no sucrose, maltose, lactose or raffinose can be seen, it can be assumed that the hydrolysis has worked, and all sugars have been split into monosaccharides.
The TLC has shown that the fractions consist of the following main components: glucose, galactose, rhamnose, xylose, arabinose, mannose and galacturonic acid.
A more complete representation of the monosaccharide composition and a comparison with the results of the RP-HPLC and HPAEC-PAD will be presented later in another table.

RP-HPLC

The monosaccharide composition was also determined using RP-HPLC. The peaks were assigned to the respective sugars and the concentration was calculated. The chromatograms of the fractions F1-F8 are presented in Figure 15-23.

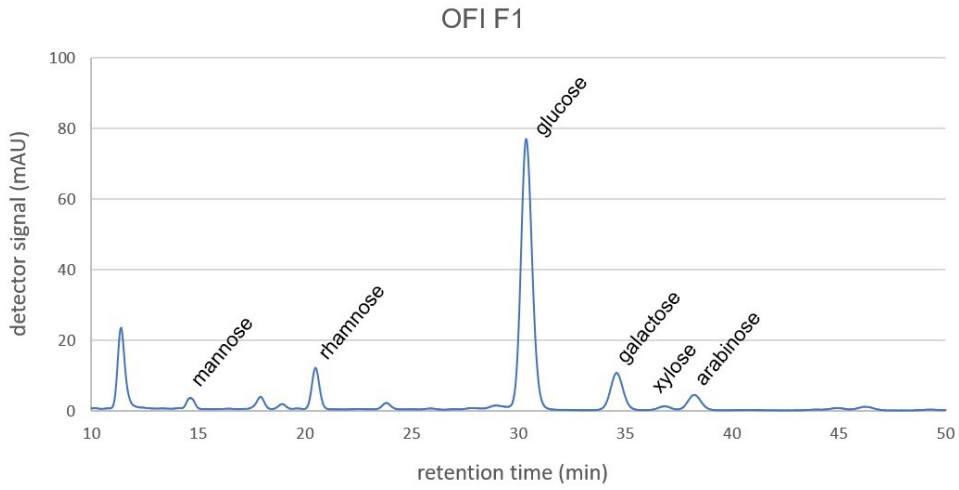

Figure 15 RP-HPLC chromatogram of F1

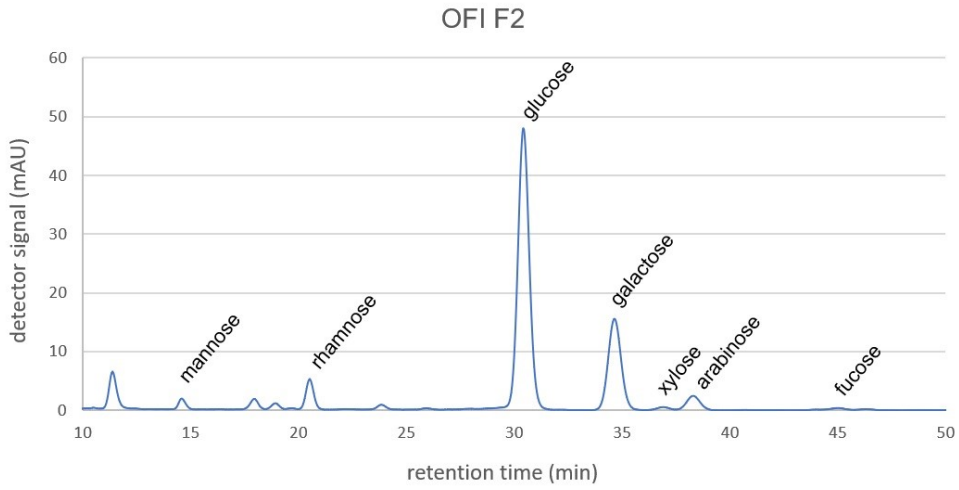

Figure 16 RP-HPLC chromatogram of F2

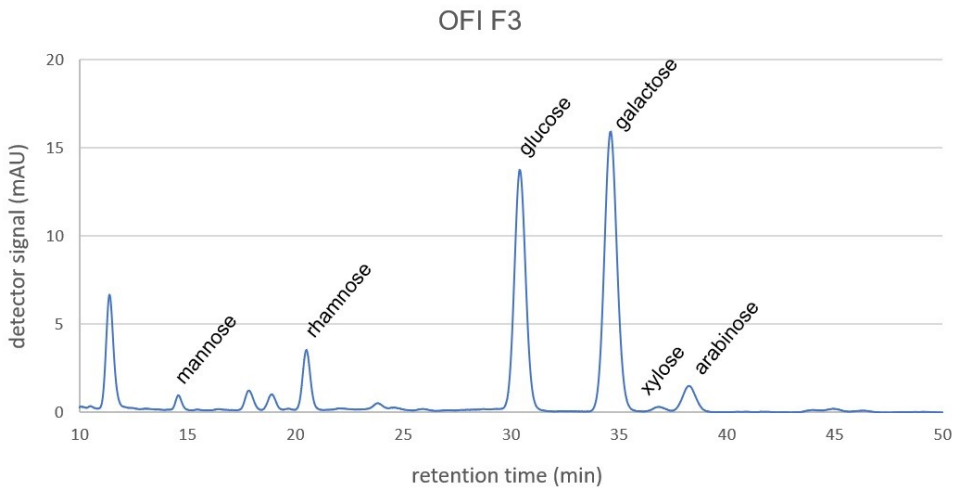

Figure 17 RP-HPLC chromatogram of F3

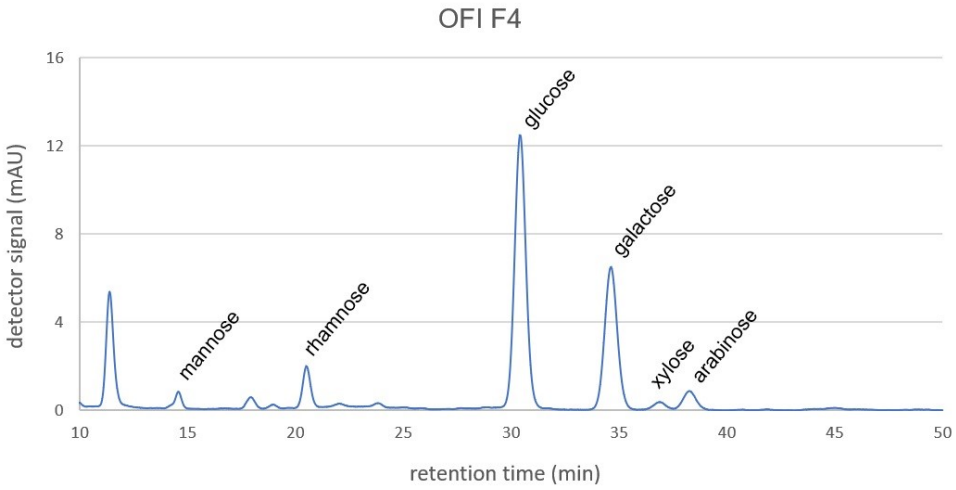

Figure 18 RP-HPLC chromatogram of F4

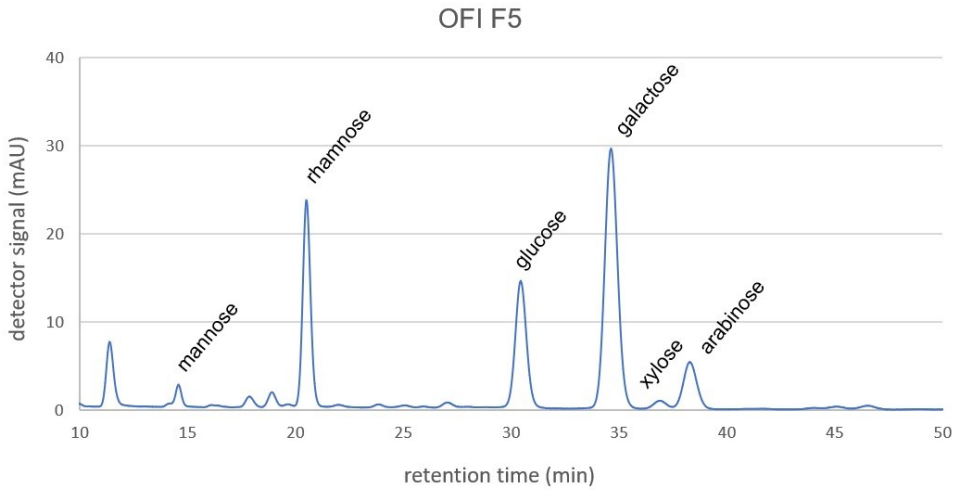

Figure 19 RP-HPLC chromatogram of F5

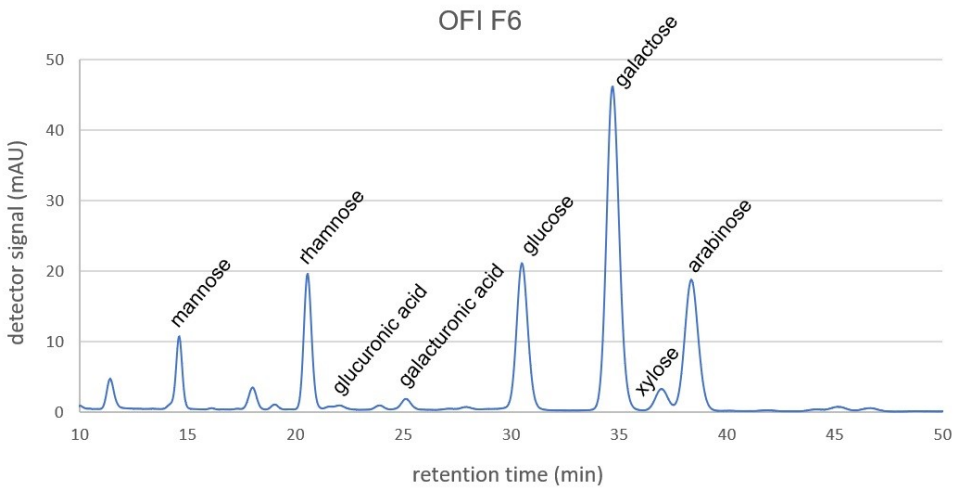

Figure 20 RP-HPLC chromatogram of F6

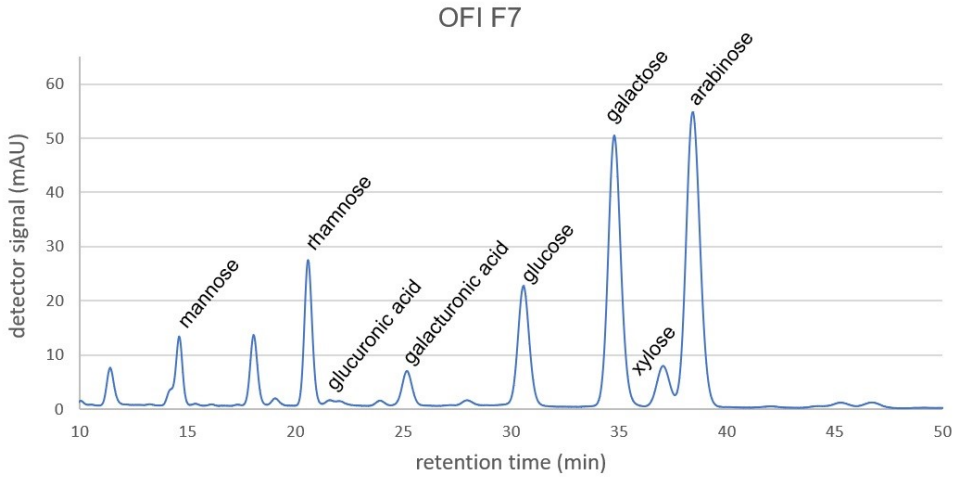

Figure 21 RP-HPLC chromatogram of F7

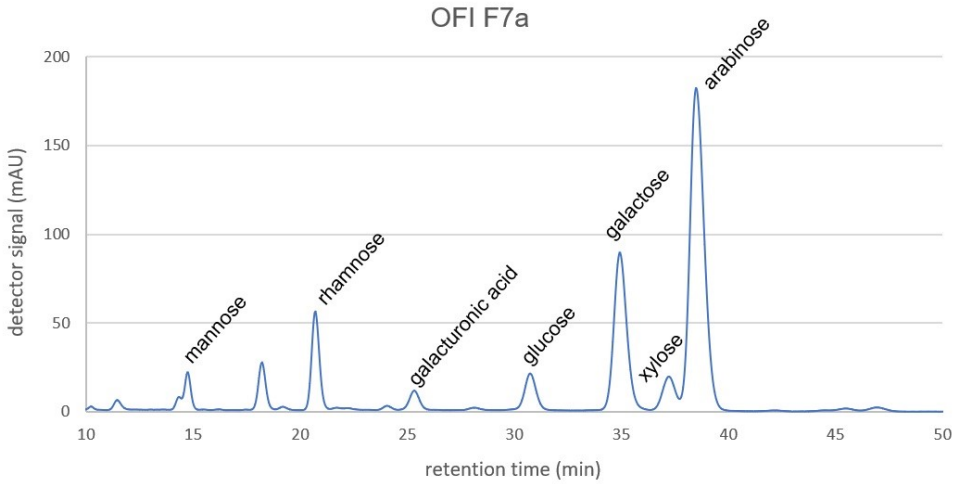

Figure 22 RP-HPLC chromatogram of F7a

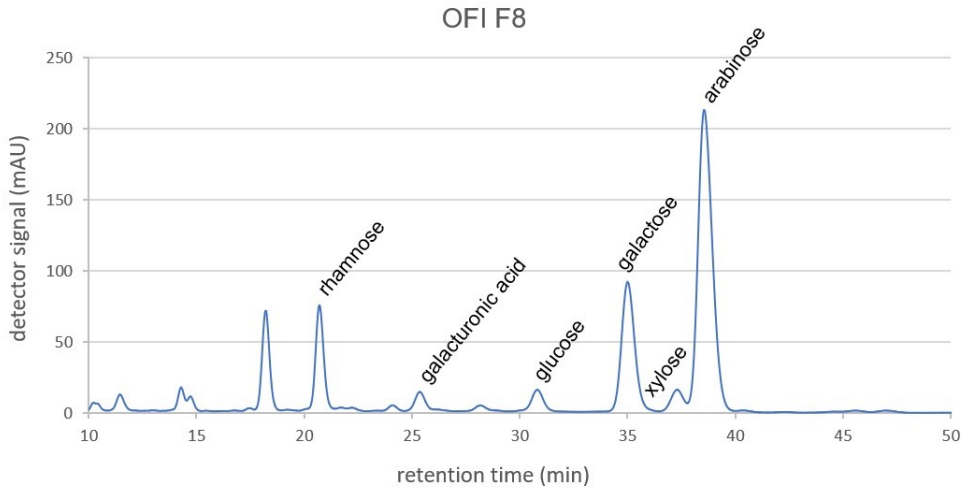

Figure 23 RP-HPLC chromatogram of F8

HPAEC-PAD

The monosaccharide analysis was also conducted using HPAEC-PAD. As with RP-HPLC, concentrations were calculated to compare the results. The chromatograms of the fractions F1-F8 are presented in Figure 24-32.

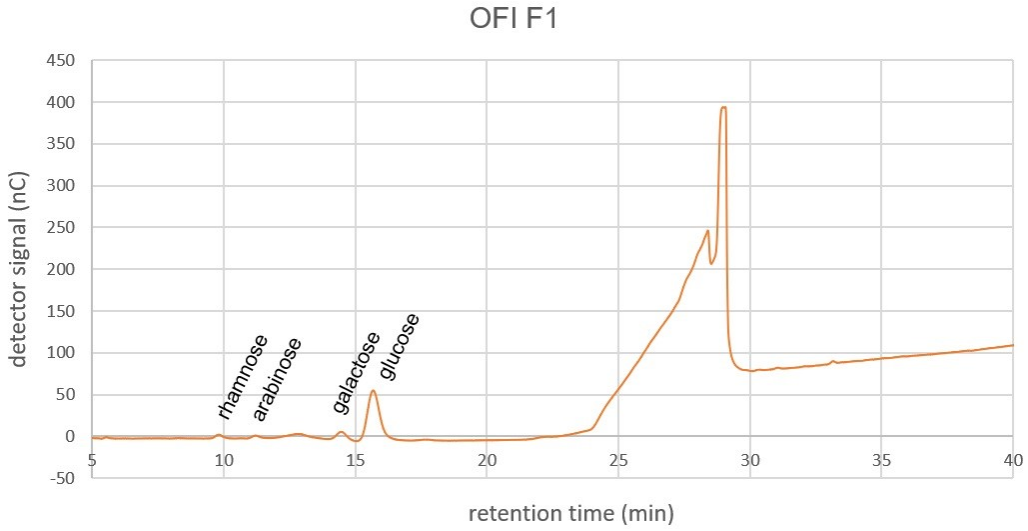

Figure 24 HPAEC-PAD chromatogram F1

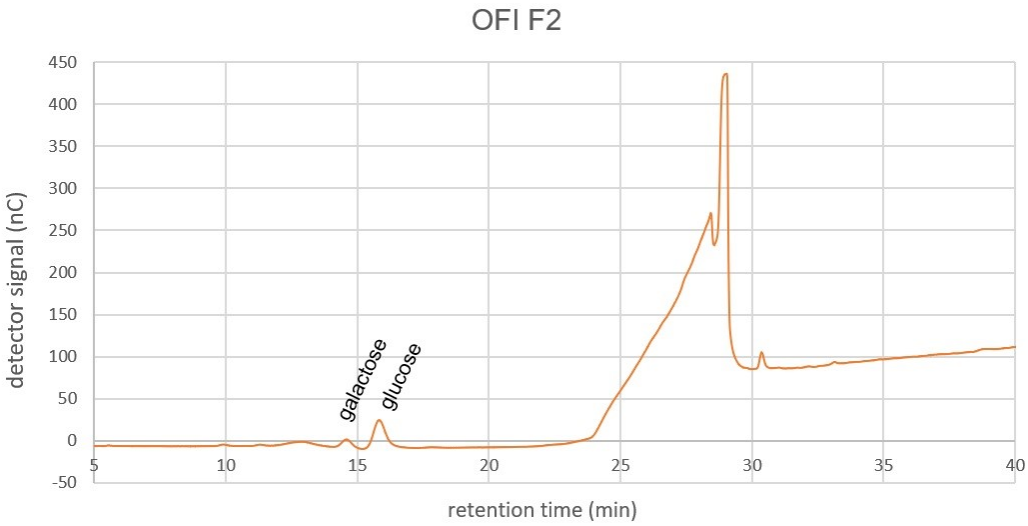

Figure 25 HPAEC-PAD chromatogram of F2

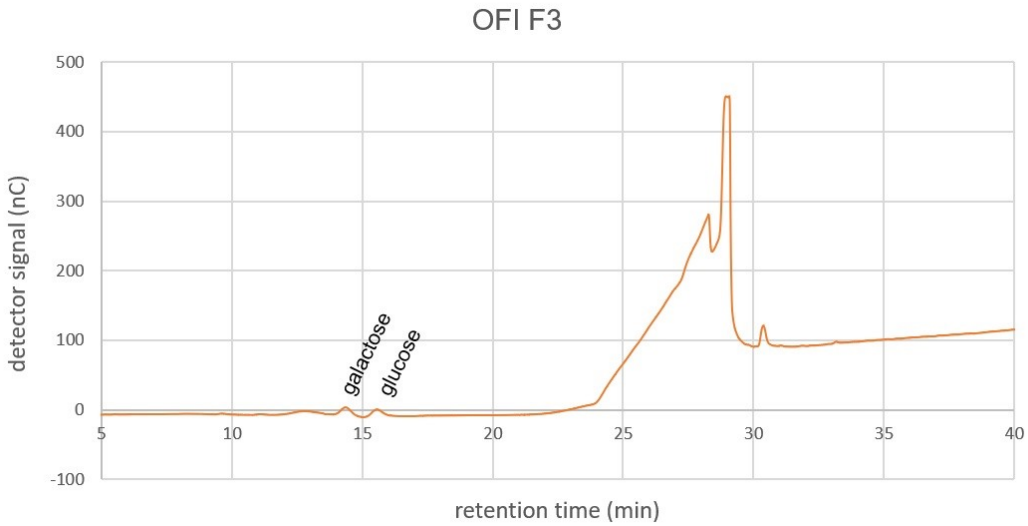

Figure 26 HPAEC-PAD chromatogram of F3

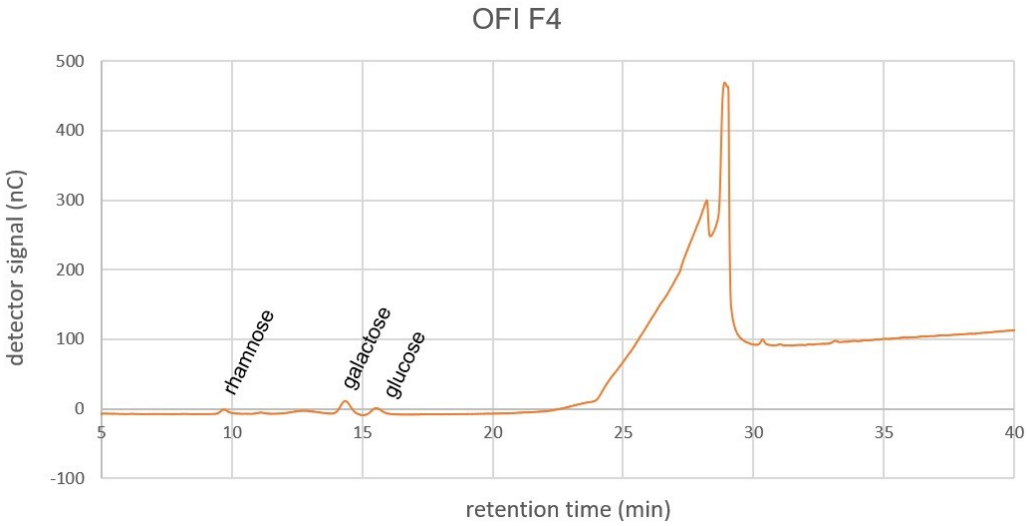

Figure 27 HPAEC-PAD chromatogram of F4

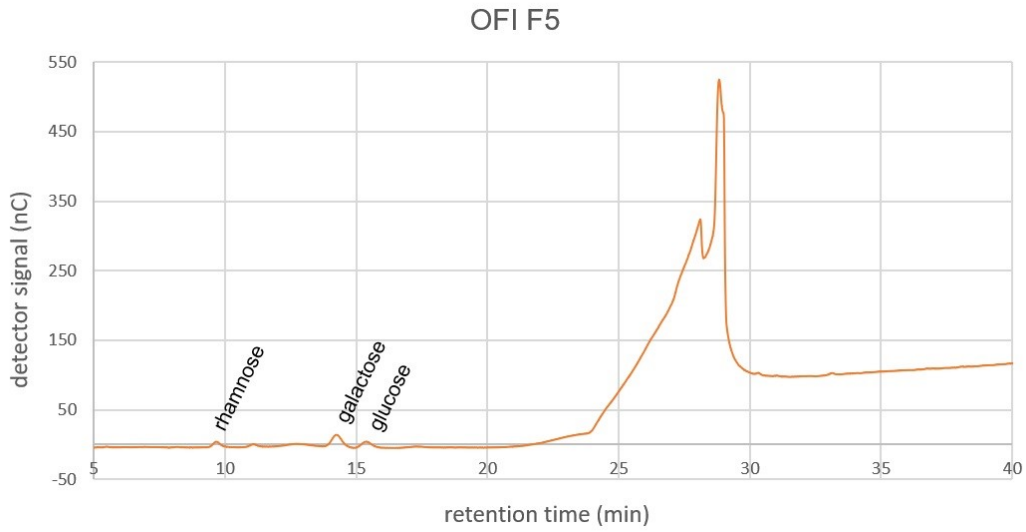

Figure 28 HPAEC-PAD chromatogram of F5

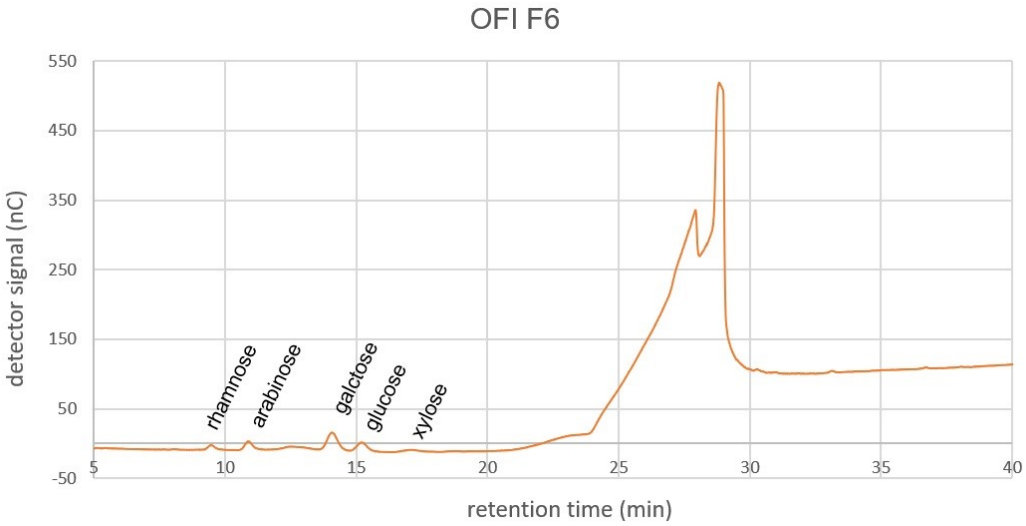

Figure 29 HPAEC-PAD chromatogram of F6

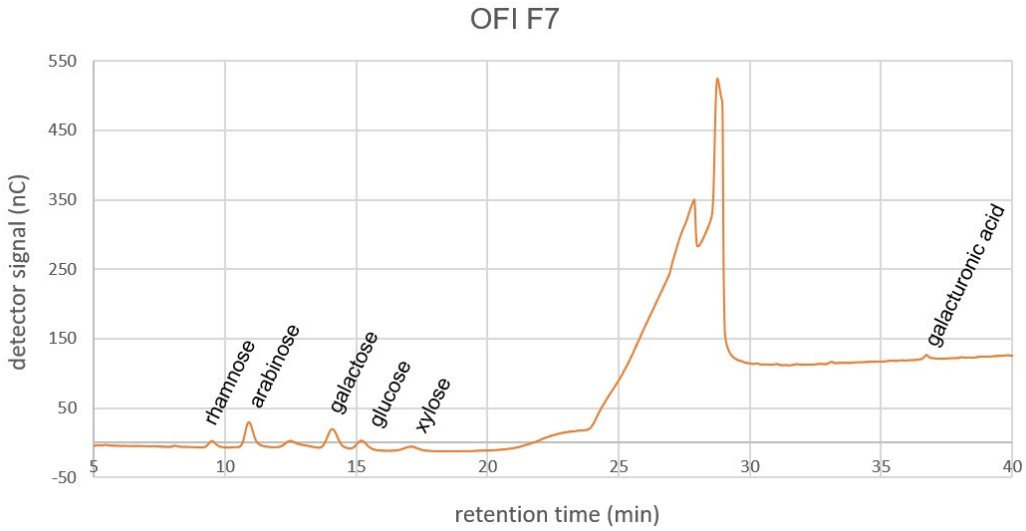

Figure 30 HPAEC-PAD chromatogram of F7

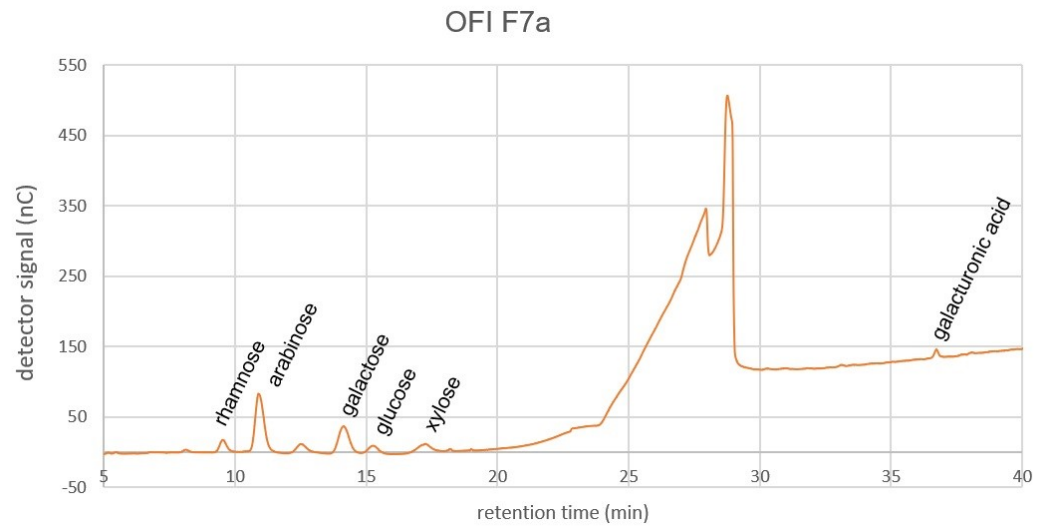

Figure 31 HPAEC-PAD chromatogram of F7a

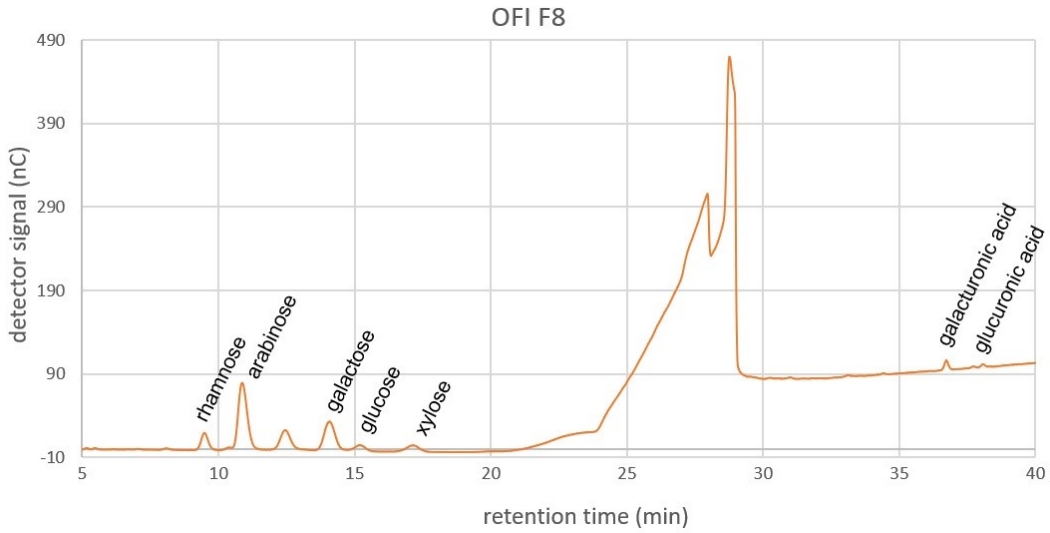

Figure 32 HPAEC-PAD chromatogram of F8

The samples were analyzed using three different methods (RP-HPLC, HPAEC-PAD and TLC). Since every chromatographic system has its advantages and disadvantages and has different sensitivities, different results may be obtained. In addition, the samples were prepared in diverse ways, which makes it possible to have deviating results.

To compare the results of the different analysis methods, the outcomes have been presented in tables.

Concentration of carbohydrates of OFI F1 - F8

	F8			F7a		
	RP-HPLC	HPAEC-PAD	TLC	RP-HPLC	HPAEC-PAD	TLC
Glucose	10.0	2.3	+	12.6	3.8	+
Galactose	54.3	13.5	++	52.0	15.0	++
Rhamnose	30.7	8.8	+	22.4	7.6	+
Xylose	6.4	7.2	+	8.1	8.4	+
Fucose						
Arabinose	111.5	23.5	+++	94.8	25.5	++
Glucuronic acid		2.9				
Galacturonic acid	12.3	3.8	+	10.0	3.5	+
Mannose				5.3		

Table 10 Concentration (µg/ml) of carbohydrates in F8 and 7a

	F7			F6		
	RP-HPLC	HPAEC-PAD	TLC	RP-HPLC	HPAEC-PAD	TLC
Glucose	13.3	3.5	+	12.5	3.8	+
Galactose	29.1	9.0	+	29.4	9.0	+
Rhamnose	11.3	2.5	+	8.5	1.6	+
Xylose	4.3	6.7	+	3.3	6.4	+
Fucose						
Arabinose	28.8	8.9	+	11.4	2.3	+
Glucuronic acid	2.5			2.4		
Galacturonic acid	7.7	2.4		5.1		
Mannose	5.8			4.4		

Table 11 Concentration (µg/ml) of carbohydrates in F7 and F6

	F5			F4		
	RP-HPLC	HPAEC-PAD	TLC	RP-HPLC	HPAEC-PAD	TLC
Glucose	8.8	3.0	+	7.8	3.4	+
Galactose	18.3	4.9	+	5.1	6.9	+
Rhamnose	10.0	2.9	+	1.6	1.7	+
Xylose	1.9		+	1.7		
Fucose						
Arabinose	4.2		+	1.9		
Glucuronic acid						
Galacturonic acid						
Mannose	2.1			1.5		

Table 12 Concentration (µg/ml) of carbohydrates in F5 and F4

	F3			F2		
	RP-HPLC	HPAEC-PAD	TLC	RP-HPLC	HPAEC-PAD	TLC
Glucose	8.5	3.3	+	27.1	11.5	+
Galactose	10.4	4.0	+	10.4	2.5	+
Rhamnose	2.2			2.8		
Xylose	1.7			1.7		
Fucose				3.1		
Arabinose	2.2			2.7		
Glucuronic acid						
Galacturonic acid						
Mannose	1.6			1.9		+

Table 13 Concentration (µg/ml) of carbohydrates in F3 and F2

	F1		
	RP-HPLC	HPAEC-PAD	TLC
Glucose	42.3	19.5	++
Galactose	7.5	3.0	+
Rhamnose	5.4	0.9	
Xylose	2.3		
Fucose			
Arabinose	3.7		
Glucuronic acid			
Galacturonic acid			
Mannose	2.4		+

Table 14 Concentration (µg/ml) of carbohydrates in F1

The analyses have shown that the composition of sugars varies from fraction to fraction. The results of the monosaccharide analysis are mostly consistent. Small deviations may occur due to the type of chromatographic methods. This can be illustrated by the analysis of fraction 8. While glucose, galactose, rhamnose, xylose, arabinose and galacturonic acid were detected in all analyses, glucuronic acid was only detected in HPAEC. The reason for this is that anion-exchange chromatography is higher sensitive to uronic acids.

It is also evident, that some carbohydrates were not detectable during the examination with HPAEC-PAD but were already detected by the TLC and RP-HPLC. This may be due to a dilution error.

Still, some of the substances are even non-visible in the TLC. This could be since the concentration of the sample was chosen too low and therefore the bands are not colored intensely enough to be recognized. It is also possible, that information has been lost due to the digitization of the plates.

3.2.2 Analysis of fraction F100-80, 80-50 and 50-0 and fresh F100-80

Thin-layer chromatography

To determine the monosaccharide composition of Opuntia extracts F100-80, 80-50 and 50-0 a chemical hydrolysis step was performed. Afterwards the samples were separated by TLC. As an eluent acetonitrile:0.3% ammonium hydroxide 17:3 was used.

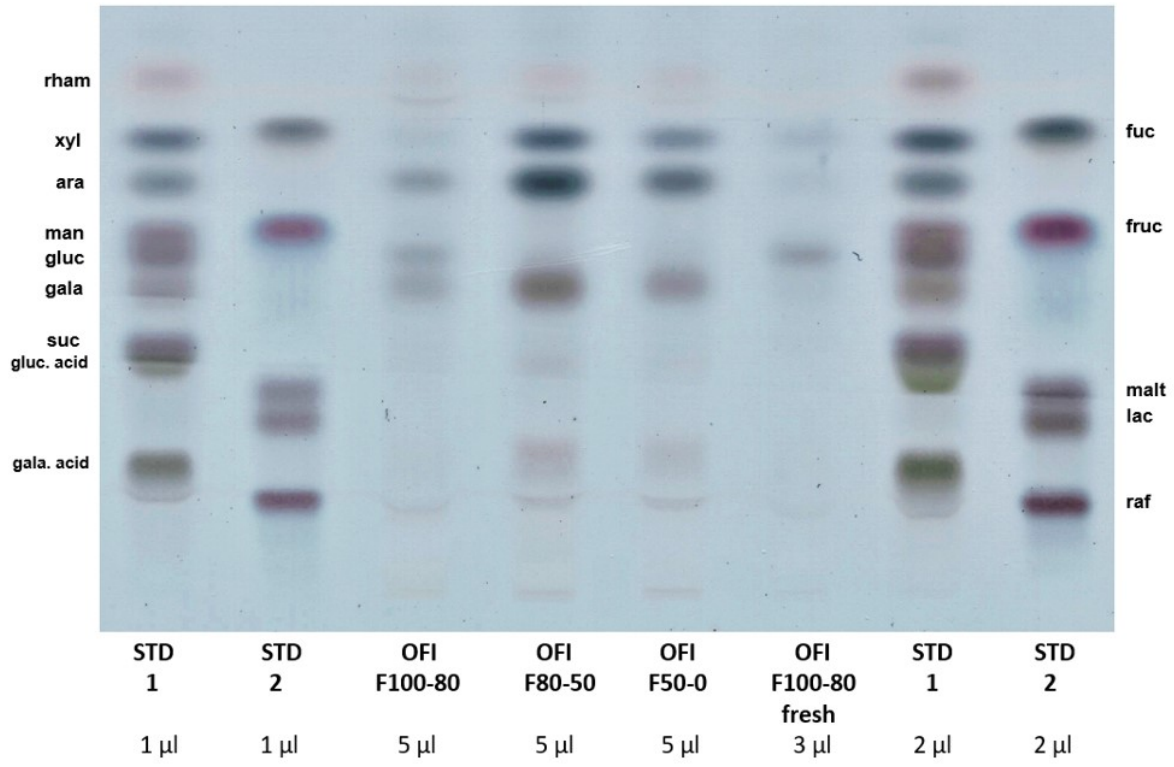

Figure 33 TLC plate of F100-80, 80-50, 50-0 and fresh 100-80 displaying the monosaccharides after hydrolysis

When comparing the extracts with each other, it is evident that F80-50 contains very high and F50-0 a slightly lower concentration of galactose, arabinose and xylose.

Since the carbohydrates of fresh F100-80 are very difficult to identify, it can be assumed that the concentration of OFI fresh was too low. Therefore, another TLC with higher concentrations was performed.

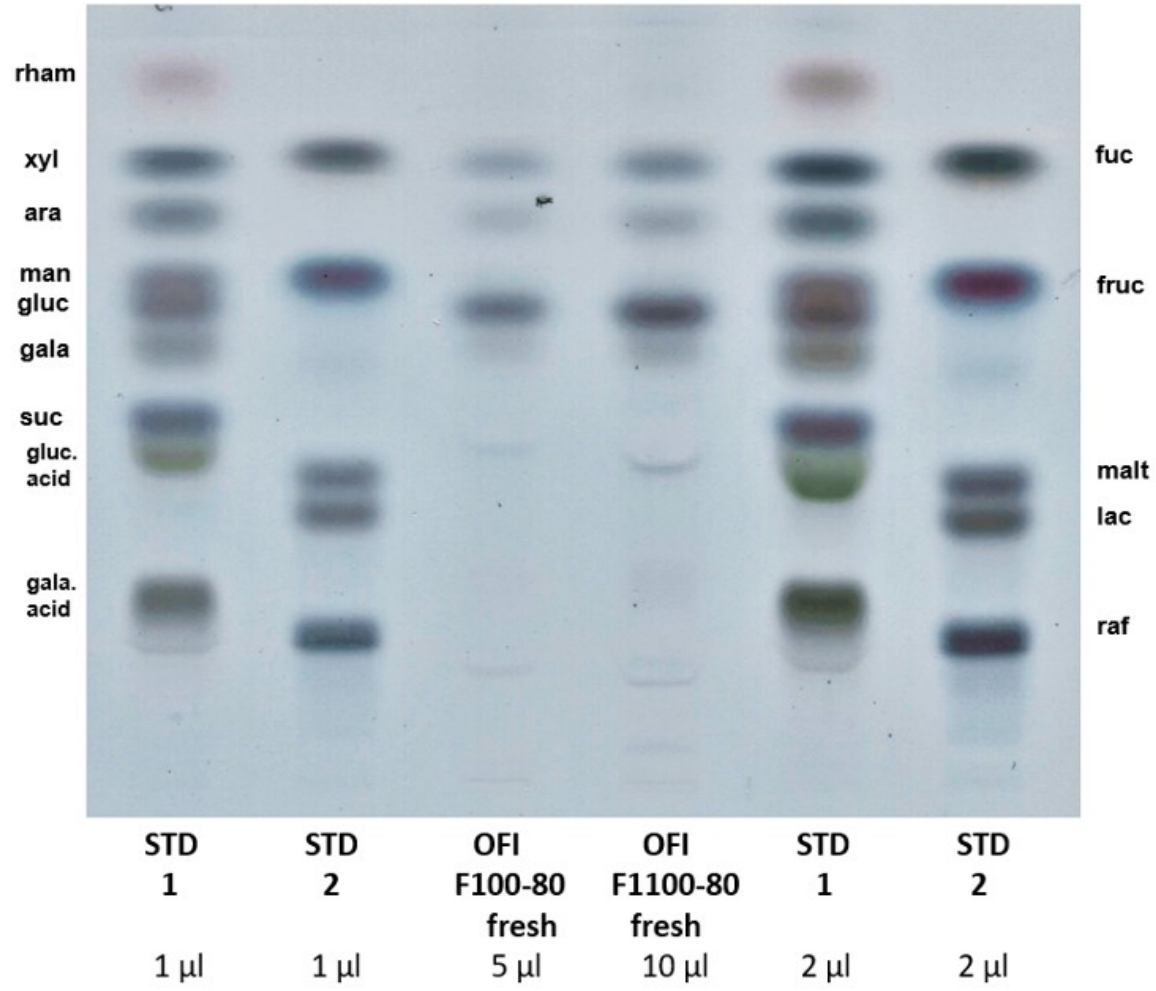

Figure 34 TLC plate of fresh F100-80 5 µl and 10µl

The fresh OFI F100-80 fraction has more glucose than galactose compared to the dried Opuntia extract. Besides, the fresh OFI F100-80 has no rhamnose. These differences in sugar composition may have been caused by heat and processing.
A more detailed analysis of the extracts and their carbohydrates was conducted with HPAEC-PAD.

HPAEC-PAD

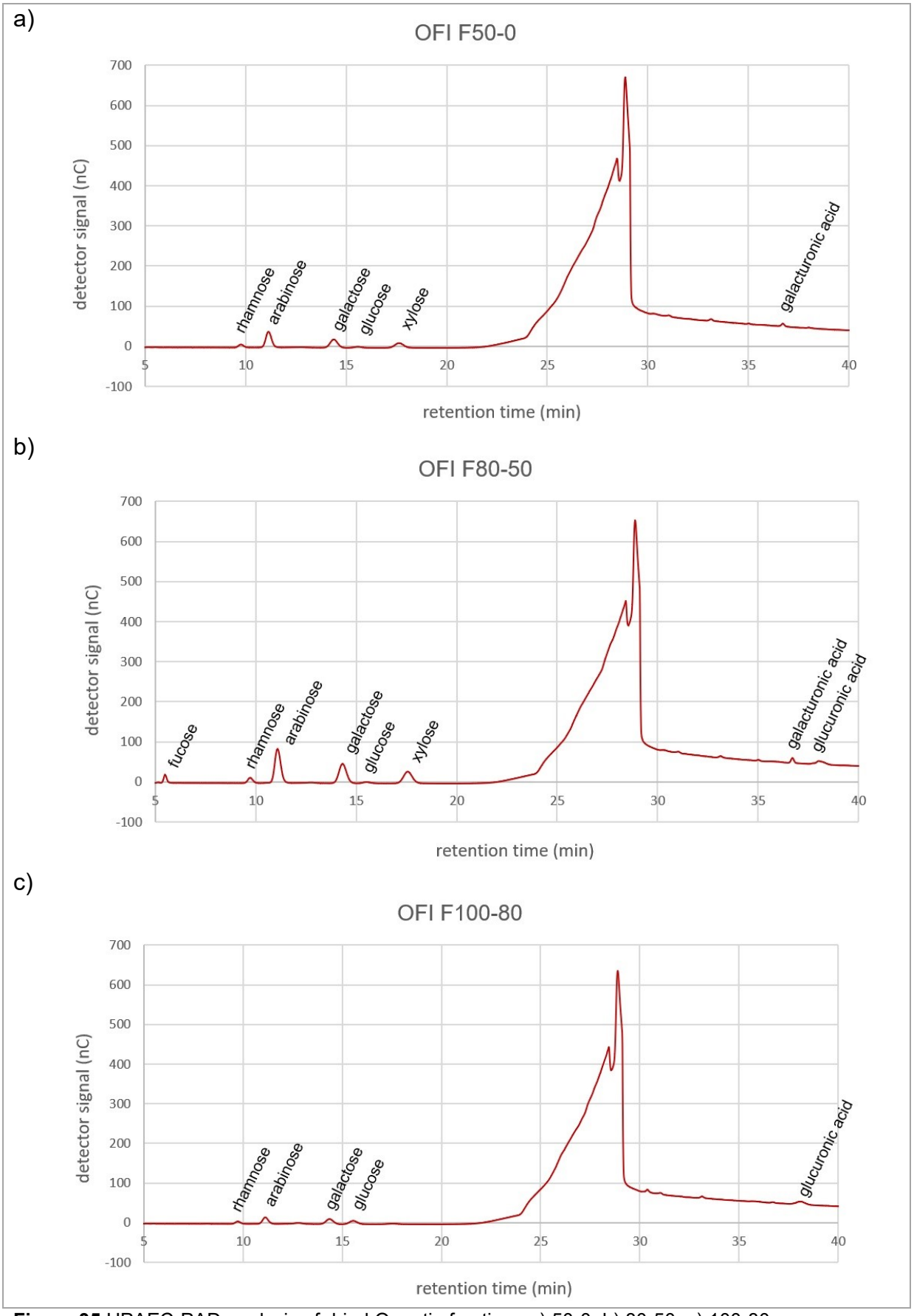

Figure 35 HPAEC-PAD analysis of dried Opuntia fractions a) 50-0, b) 80-50, c) 100-80

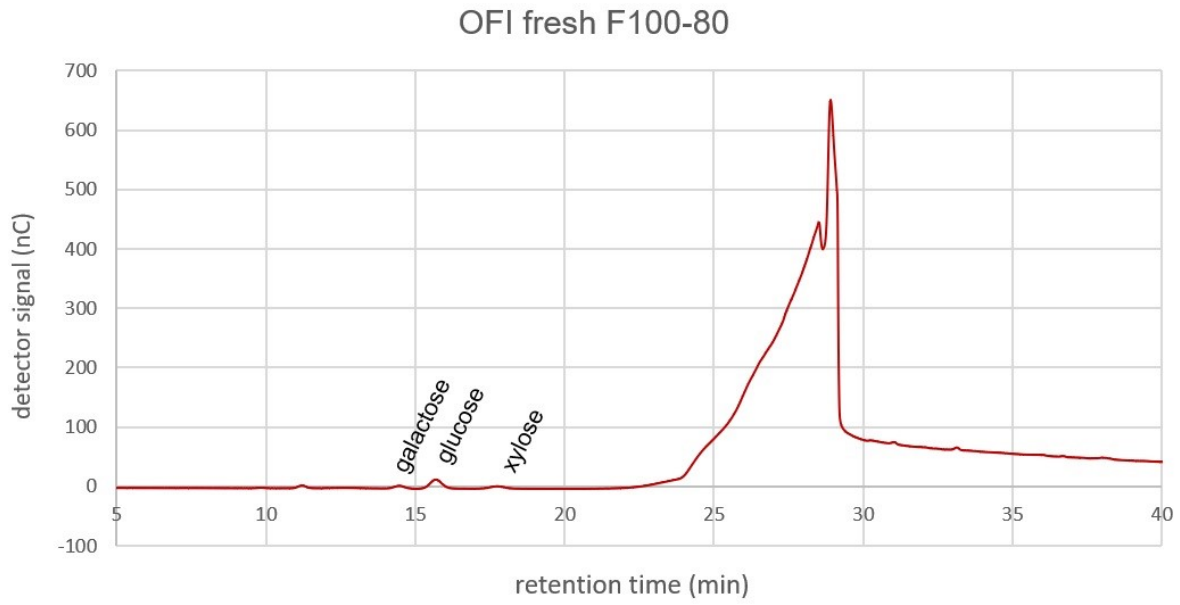

Figure 36 HPAEC-PAD analysis of fresh F100-80

The concentration of each carbohydrate was calculated. The comparison of the results obtained from the HPAEC and TLC is presented in Table 15.

	OFI F50-0		**OFI F80-50**		**OFI F100-80**		**OFI fresh F100-80**	
	HPAEC-PAD	TLC	HPAEC - PAD	TLC	HPAEC-PAD	TLC	HPAEC-PAD	TLC
Fucose			2.2 µg/ml		1.4 µg/ml			
Rhamnose	2.1 µg/ml	+	5.0 µg/ml	+	3.4 µg/ml	+		
Arabinose	10.0 µg/ml	++	24.1 µg/ml	+++	4.0 µg/ml	+	1.1 µg/ml	+
Galactose	6.9 µg/ml	+	17.3 µg/ml	++	3.0 µg/ml	+	4.9 µg/ml	+
Glucose	1.4 µg/ml	+	1.5 µg/ml	+			6.6 µg/ml	+
Xylose	7.9 µg/ml	+	11.0 µg/ml	++		+		+
Galacturonic acid	2.5 µg/ml	+	3.6 µg/ml	+				+
Glucuronic acid			4.1 µg/ml	+	4.9 µg/ml	+		

Table 15 Concentration of carbohydrates and TLC results of F50-0, 80-50, 100-80 and fresh F100-80

The results were obtained using different chromatographic methods, but they do correlate with each other.

Analyzing F50-0, the fraction shows all components in the TLC which were also recorded in the HPAEC. The quantity is also the same, as can be seen for example with arabinose, because here the concentration is the highest and with the TLC this carbohydrate zone is most intensively colored. The same applies to F80-50. Only fucose is not visible in the TLC. This could be since the concentration of the sample was chosen too low and therefore the bands are not colored intensely enough to be recognized. It is also possible, that information has been lost due to the digitization of the plates.

For fraction F100-80, xylose is clearly visible in the TLC, but not in the HPAEC. To make a clear statement about the composition, further analyses are necessary. The same applies to fresh F100-80.
The fact that fucose cannot be detected in the TLC may be because the concentration is low, and the band may overlap with xylose. To be sure, further tests should be carried out.

Comparing F100-80 of the fresh plant with F100-80 of the dried Opuntia, the composition of the carbohydrates is different. Fresh 100-80 contains neither fucose nor rhamnose nor glucuronic acid. But it does have glucose and traces of galacturonic acid, which could not be detected in the dried extract.
Surprisingly the extract F100-80 of dried *Opuntia ficus-indica* seems to contain no glucose. This can be accomplished by destroying or removing the glucose during the extraction procedure.

3.3 Bacterial growth

Growth of the bacteria was determined by using a turbidity measurement. For this a negative control of each bacterial strain was carried out using autoclaved deionized water instead of the carbohydrate source. Samples or standard, which were more turbid than the negative control have a possible prebiotic potential. The higher the optical density, the higher prebiotic potential is to be expected.

Additionally, negative controls consisting of Man-Rogosa-Sharpes media without glucose and without bacteria (MRS-) and Man-Rogosa-Sharpes media with glucose but without bacteria (MRS+) were conducted. Since these negative controls have been tested negative, those aren't shown in the following diagrams.

The growth of the carbohydrates was tested as standards. This was conducted for all four bacterial strains. Figure 37 is a representative illustration and shows the bacterial growth of *L.acidophilus* with standards (glucose, galactose, rhamnose, xylose, fucose, arabinose, glucuronic acid, galacturonic acid). The remaining results were summarized in tables to provide a convenient overview. Table 16 shows the percentage of growth of the standards.

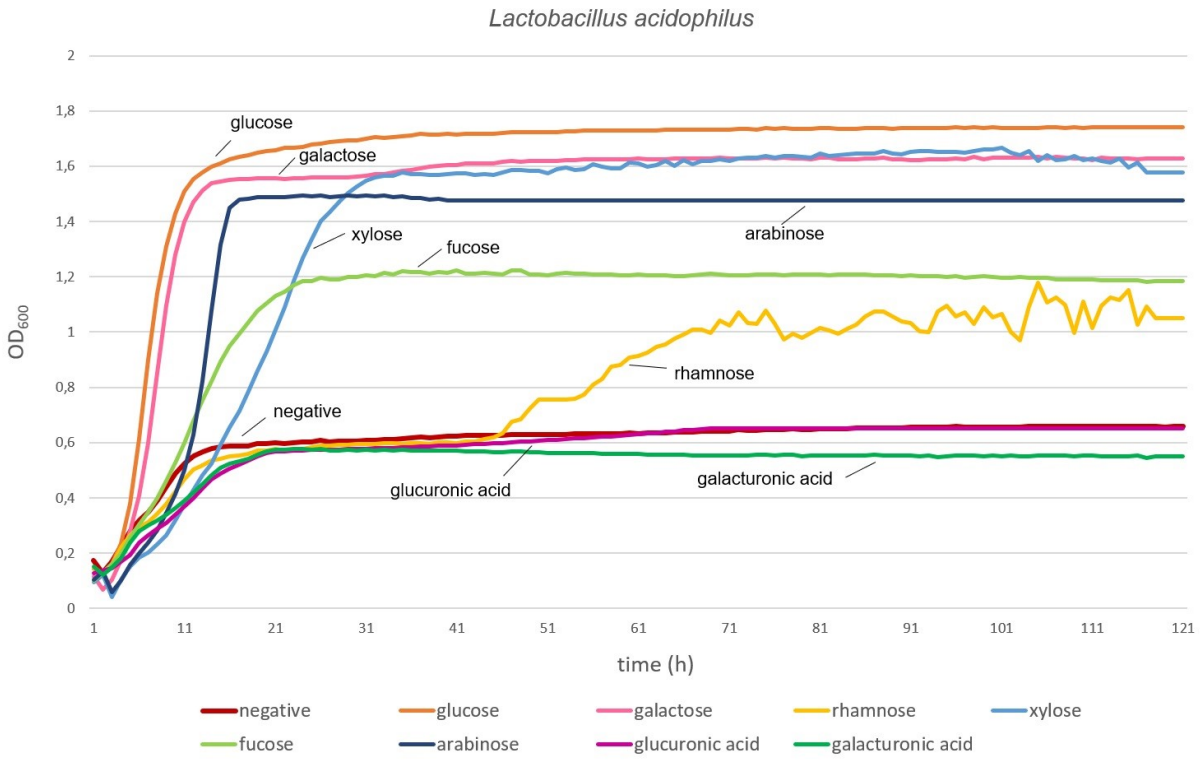

Figure 37 Bacterial growth curve of *L. acidophilus* with standards

The growth was calculated in percent by setting the positive control (glucose) to 100%. The other values were then set in relation to glucose.

	Lactobacillus acidophilus	***Lactobacillus rhamnosus GG***	***Bifidobacterium longum ssp. infantis***	***Bifidobacterium animalis ssp. lactis***
Negative control	0%	0%	0%	0%
Glucose	100%	100%	100%	100%
Galactose	90%	93%	91%	83%
Rhamnose	48%	15%	38%	3%
Xylose	93%	0 %	88%	97%
Fucose	53%	31%	45%	24%
Arabinose	77%	0%	69%	81%
Glucuronic acid	0%	0%	34%	1%
Galacturonic acid	0%	0%	0%	0%

Table 16 Percentage bacterial growth of the standard-solutions in relation to glucose (100%)

		L. acidophilus	***L. rhamnosus GG***	***B. longum ssp. infantis***	***B. animalis ssp. lactis***
Time after half maximum OD_{600} of glucose is reached		8 h	7 h	4 h	n. d.
Increase of turbidity in relation to glucose	Galactose	9 h	8 h	6 h	n. d.
	Rhamnose	-	-	-	n. d.
	Xylose	23 h	-	22 h	n. d.
	Fucose	30 h	-	-	n. d.
	Arabinose	14 h	-	15 h	n. d.
	Glucuronic acid	-	-	-	n. d.
	Galacturonic acid	-	-	-	n. d.

Table 17 Half maximum time of glucose and the standard solutions in relation to glucose; "-" means half maximum OD_{600} of glucose was not reached, "n.d" means not detected

The measurements show the ability of bacterial strains to ferment different carbohydrates. They also demonstrate to what extent the sugars can be metabolized. The results vary from strain to strain.

- *Lactobacillus acidophilus*

Glucose, galactose, xylose and arabinose are fermented well. The growth curves reached a maximum with an OD_{600} of 1.7 and 1.6 for glucose, galactose. Xylose also reached a peak value of 1.6 but rising with a delay. With the presence of arabinose, the bacterial growth curve achieved an OD_{600} of approximately 1.5. Fucose and rhamnose were metabolized by the bacteria as well but the bacterial strain didn't display such high OD_{600}. Glucuronic acid and galacturonic acid appear to be unfermentable for *Lactobacillus acidophilus*.

- *Lactobacillus rhamnosus GG*

Glucose and galactose were utilized the best followed by fucose and rhamnose. Glucuronic acid, galacturonic acid, arabinose and xylose couldn't be fermented by the bacterial strain.

- *Bifidobacterium longum ssp. infantis*

Glucose and galactose showed the most fermentation, xylose and arabinose were utilized slightly less. Rhamnose und fucose were metabolized by the bacteria as well but the bacterial strain didn't display such high OD_{600}. Glucuronic acid was fermented better than usual.

- *Bifidobacterium animalis ssp. lactis*

The presence of glucose and xylose led to the highest OD_{600} followed by arabinose. Fucose, rhamnose, glucuronic acid and galacturonic could be metabolized very little or even not at all.

Bacterial growth with fractions of *O. ficus-indica*

Figure 38 is a representative illustration and shows the bacterial growth of *L. acidophilus* with fractions of *Opuntia ficus-indica* (F1 – F8). Figure 39 shows the growth curves of *Lactobacillus acidophilus* with fractions of *O. ficus-indica* (F100-80, F80-50, F50-0 and fresh F100-80). The entire results were summarized in tables to give an overview. Table 17 and 18 show the percentage of growth of the bacterial strain in presence of the samples of *O. ficus-indica*.

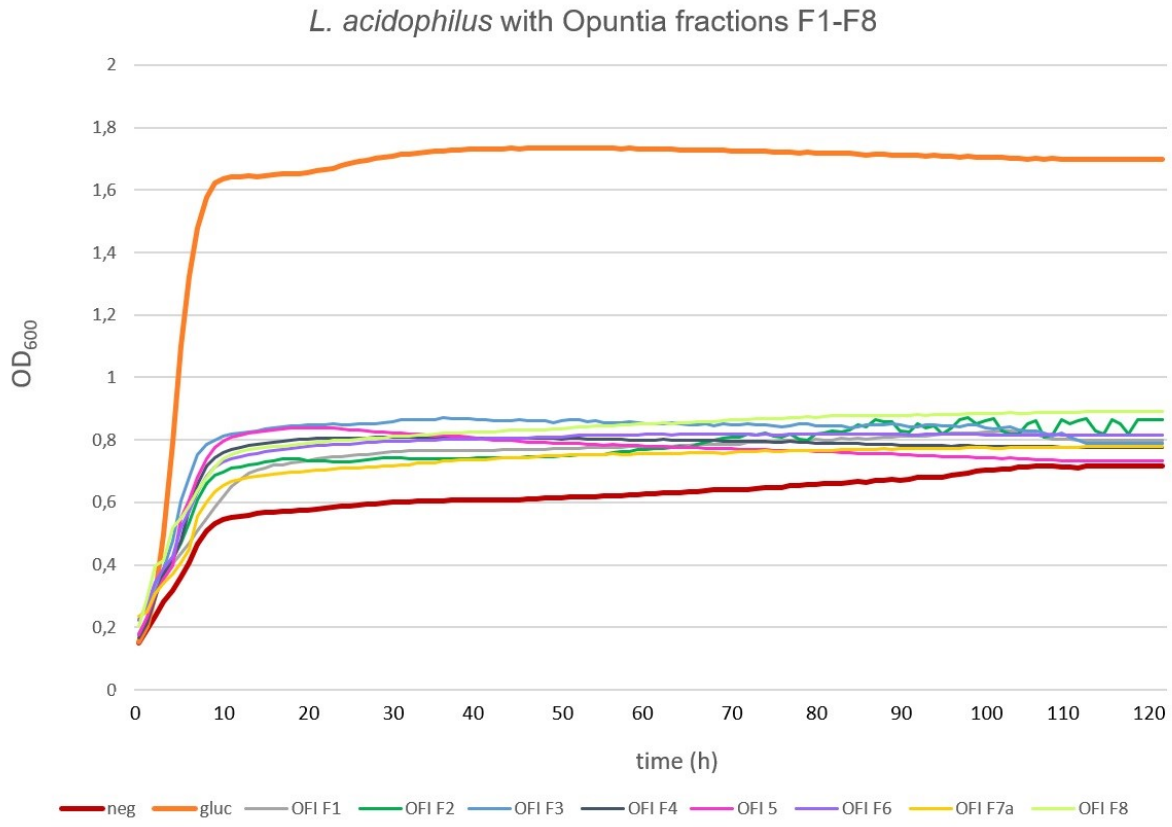

Figure 38 Bacterial growth curve of *L. acidophilus* with fractions F1-F8 of *O. ficus-indica*

	L. acidophilus		*L. rhamnosus GG*		*B. longum ssp. infantis*		*B. animalis ssp. lactis*	
Negative control	0%		0%		0%		0%	
	Percentage bacterial growth	Time after maximum was reached	Percentage bacterial growth	Time after maximum was reached	Percentage bacterial growth	Time after maximum was reached	Percentage bacterial growth	Time after maximum was reached
OFI F1	11%	77 h	7%	57 h	19%	17 h	19%	29 h
OFI F2	15%	68 h	16%	68 h	28%	87 h	19%	29 h
OFI F3	15%	10 h	8%	6 h	18%	87 h	11%	21 h
OFI F4	9%	19 h	13%	10 h	14%	11 h	10%	21 h
OFI F5	12%	11 h	20%	36 h	10%	15 h	4%	29 h
OFI F6	10%	36 h	14%	22 h	13%	18 h	10%	29 h
OFI F7a	6%	20 h	n. d.		9%	46 h	n. d.	
OFI F8	17%	25 h	n. d.		12%	99 h	n. d.	

Table 18 Percentage bacterial growth and time after maximum was reached of the fractions of *O. ficus-indica* in relation to glucose (100%)

All fractions have shown an increase in turbidity, which can be observed from the growth curves illustrated in figure 38 and the calculated percentages listed in table 18.

The bacterial strains have utilized the fractions differently but for none of the fractions a dp dependence of the best fermentability could be determined. The values of growth of *L.acidophilus* are in the range of 6-17%, of *L. rhamnosus* GG 7-20%, of *Bifidobacterium longum ssp. infantis* 9-28% and of *Bifidobacterium animalis ssp. lactis* 4-19%. No clear correlation between degree of polymerization and fermentability could be shown.

One point all bacterial strains have in common is that Fraction 2 led to the best or second-best fermentation. It even led to the maximum detected value of 28% growth of *Bifidobacterium longum ssp. infantis*.

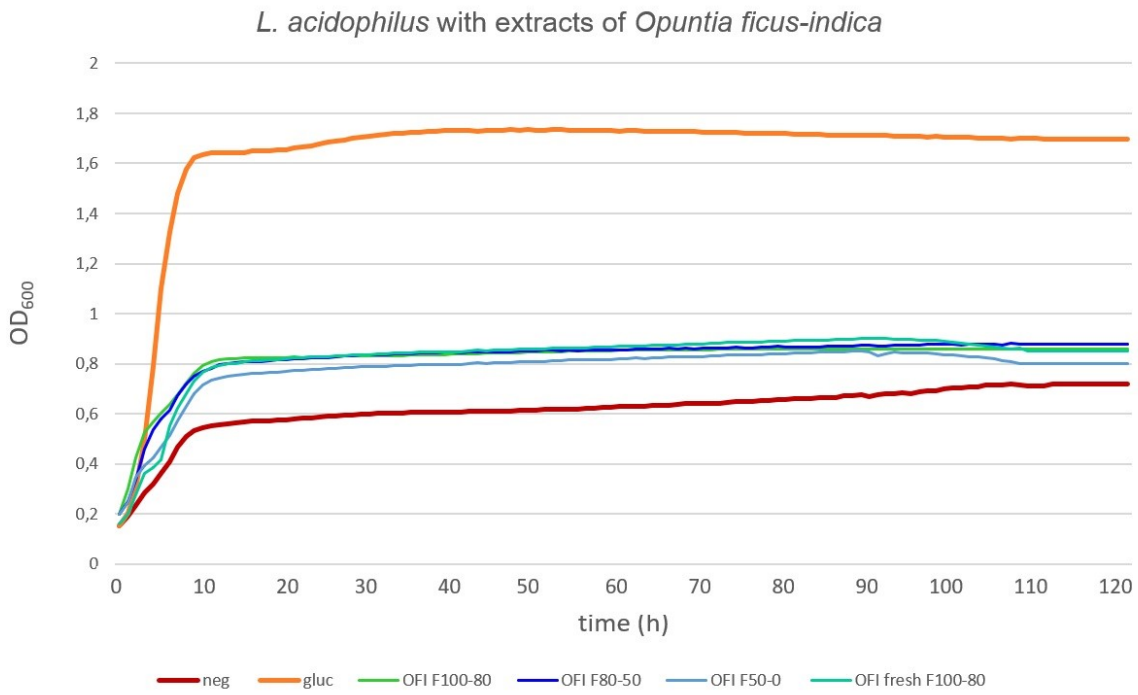

Figure 39 Bacterial growth curve of *L. acidophilus* with fractions F100-80, F80-50, F50-0 and fresh F100-80 of *O. ficus-indica*

	L. acidophilus		***L. rhamnosus GG***		***B. longum ssp. infantis***		***B. animalis ssp. lactis***	
	Percentage bacterial growth	Time after maximum was reached	Percentage bacterial growth	Time after maximum was reached	Percentage bacterial growth	Time after maximum was reached	Percentage bacterial growth	Time after maximum was reached
OFI fresh F100-80	18%	90 h	9%	23 h	16%	48 h	14%	21 h
OFI F0-50	13%	92 h	10%	14 h	9%	28 h	17%	29 h
OFI F50-80	16%	18 h	9%	32 h	13%	54 h	53%	29 h
OFI F100-80	14%	12 h	8%	77 h	18%	44 h	19%	21 h

Table 19 Percentage bacterial growth and time after maximum was reached of the extracts of *O. ficus-indica* in relation to glucose (100%)

Each fraction has caused an increase in turbidity, which can be seen from the growth curves illustrated in figure 39 and the calculated percentages listed in table 19.

Comparing bacterial growth in presence of the extracts of the dried *O. ficus-indica* it seems to be that F50-80 are the most fermentable samples of *L. acidophilus* and *Bifidobacterium animalis ssp. lactis*. *Bifidobacteriom longum ssp. infantis* utilized F100-80 the best. Fraction F50-0 was metabolized less in all the tested bacterial strains except for *L. rhamnosus GG*, where however the values of percentage growth of this strain are very similar.

OFI fresh F100-80 compared to F100-80 from dried Opuntia similar increase in turbidity was achieved.

The examination of the prebiotic potential of *Opuntia ficus-indica* revealed that all fractions do have prebiotic potential. Still, no exact correspondence between dp and fermentability could be found. It remains to be explored why some fractions lead to a better grow than others. Not only the dp is relevant for bacterial growth but also the monosaccharide composition may be of high importance. To find out the relevance of the composition more research needs to be done.
Since the structure and the glycosidic linkage between the monosaccharide residues do have an influence of bacterial growth these aspects also have to be identified with further analysis.

To determine whether *O. ficus-indica* can be incorporated in food or used as food supplement more research and analysis is needed.

4. Summary

The here examined fresh and dried *Opuntia ficus-indica* contain mono-/ disaccharides and oligosaccharides. The distribution of molecular weight and degree of polymerization was analyzed by using size exclusion chromatography. The composition of the mono- and oligosaccharides has been evaluated with three different chromatographic systems (TLC, RP-HPLC and HPAEC-PAD) to obtain the most information. The carbohydrate composition is fully analyzed. The exact oligosaccharide composition and linkage needs to be investigated in further analysis.

O. ficus-indica showed prebiotic potential on the used bacteria stains (*L. acidophilus, L. rhamnosus GG, B. longum ssp. infantis* and *B. animalis ssp. lactis*). The growth curves were measured with a turbidimetric method. For comparison standards were examined as well and caused a strain dependent fermentation as well as the fractions. In order to make more detailed conclusions about the prebiotic potential of the individual fractions also the structure and glycosidic linkage between the carbohydrates needs to be evaluated. To generalize the statement of prebiotic potential analysis with more bacterial strains are necessary.

To guarantee the effectiveness of the nopal stability to gastric- and bile needs to be tested.

Since the fresh nopal gets very viscous during separation procedure a greater outcome at the extraction should be in focus to be able to compare F80-50 and F50-0 as well.

5. List of Tables and Figures

6. References

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7. Granted figures

Concerning Figure 1: