Hamburg Unversity of Technology
Institute of Environmental Technology and Energy Economics

Bachelor's thesis

Extraction of fructans from Silphium Perfoliatum - Influence of temperature, extraction time and liquid-to-solid ratio

Name
María Renovell Sala

Matriculation number: 53460

Study programme: Chemical Engineering

First examiner: Martin Kaltschmitt

Second examiner: Andreas Zimmermann

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Statutory Declaration

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María Renovell Sala

Preamble

This bachelor`s thesis was executed at the Institute of Environmental Technology and Energy Economics at Hamburg University of Technology in the course of a term abroad.

Hamburg, 12/09/2019

María Renovell Sala

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List of abbreviations and symbols **Roman letters**

$\triangle$G	Glucose released from fructans	
$\triangle$F	Fructose released from fructans	
n	Average of the degree of polymerization	-
k	correction factor for water uptake during hydrolysis	-

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1 Introduction

1.1 Background

The emergence of the term of biorefinery appeared in response to the increase of world population and, therefore, of the energetic demand. Meanwhile, the availability of arable land and fossil resources was decreasing. Combining these facts with a significant increase in the awareness of using biomass more efficiently in an economical and environmental way and a growing interest in higher-value usage of especially non-food lignocellulosic biomass, there was a need to develop higher quality products and diversify to respond to global competition. In a biorefinery, biomass is converted into one or more valuable products like fuels, chemical materials, electricity, etc. Moreover, all types of biomass can be used from wood to waste and algae, but just some them can be used in an industrial scale as wood for producing electricity and solid waste in order to produce energy. [1, 2]

The use of biomass will become more important as it is the raw material in biorefineries and they are considered to be the future due to the limited availability of fossil resources and arable land, while the population growth is rising with its consequent demand for energy, materials and food. On the other hand, due to the increase of the awareness in using cleaner energies, the search for more efficient and environmentally friendly renewable energy material has been stimulated. This situation coupled with the need to reduce dependence on other countries and to promote environmental awareness has promoted the search for alternatives to fossil resources, based on renewable sources.

Therefore, the cascaded utilization is a good option, especially linked conversion pathways for the energetic and substantial utilization of biomass. Plant residues and waste materials, using innovative biotechnological treatments can produce energy and raw materials in integrated biorefineries. [1–4]

At the Technical University of Hamburg (TUHH) a biorefinery process has been developed during the last ten years using lignocellulosic materials such as straw as a raw material. In the scope of the current project phase ELBE-NH, further raw materials shall be investigated in order to obtain new products but also to show the flexibility of the developed process.

1.2 Aim of this work

In order to increase the economic effectiveness of biorefinery processes the product range should be extended; therefore, the goal of this project is to produce fructans as the target product. Fructans are sugars which are exclusively built from fructose monomers with one terminal glucose unit and they have several applications like food due to their prebiotic properties. These properties can induce the growth or activity of beneficial microorganisms.

For this work, fructans are going to be obtained from a plant called *Silphium perfoliatum*, also known as Silphie. This plant is basically used for biogas production, thus could be interesting as fructans are normally obtained from food-compatible plants and this might be a problem as these materials can be needed for another applications. One alternative concept could be the prior extraction of fructans and subsequent biogas production from the residues, which would increase the product range in course of processing Silphie.

Therefore, the aim of this work is to investigate the influence of extraction parameters on the yield of fructans. Based on this, it is the aim to optimise the yield of aqueous extraction of fructans from Silphie by variation of temperature, extraction time and solid-liquid-ratio.

2 Theory

2.1 Silphium perfoliatum

Silphium perfoliatum is the botanical name of the plant commonly known as Silphie, which belongs to the family *Asteracea*. It is originally from the eastern part of North America growing in moist riverbanks and on prairie land and can reach about 2.5 m height; leaves are green, long and tapering. Silphie is adaptable to changing weather conditions, once that it is set can tolerate short periods of time of drought, the shape of the leaves can store rain water and dew due to its cup shape; For that reason they are also called cup plants, with this conditions Silphie can survive dry and hot summers. [5, 6]

This type of plant, since it is perennial, the roots stay in the ground and it can be productive for up to ten years. Therefore, Silphie requires a long-term planning. Soil conditions should be moist and with a pH above 5.5. The cup plant has optimum conditions of growing at full sun exposure and at 20 ºC , but can also withstand frost at - 30 ºC. [6] It is recommended to harvest twice a year; the first harvest should be in the middle of June and the second harvest in September. Fertilizer application is not required during the first year if Silphie is planted on sites with high nutrient content. However, if the availability of nutrients is low an application of 60 to 80 kg of nitrogen per hectare is helpful for the establishment. For the following years it is recommended to match the applications of nitrogen with the biomass removal, with an average of 120 to 150 kg of nitrogen per hectare every year. There are some studies that also use phosphorous and potassium as fertilizer, with an average of 25 to 30 kg of phosphorous per hectare and 200 to 250 kg of potassium per hectare every year (Figure 56 in the Appendix).[6, 7]

Moist weather conditions can cause the growth of fungi and this can damage the stems and seeds but also Silphie is susceptible to pests and infection with pathogens. However, fungicides with specific applications to Silphie are not available. Silphie provides food sources for insects as it has flowers, therefore can help to diversify land use in areas with non-flowering crops such as areas with grass and maize production. During the flowering

season, July to September, pollen and nectar are produced; pollen of the Silphium has a high content in sugars and also a range of valuable amino acids which can ensure the health of the bees and ensure their food for winter. Moreover, the availability of water is another limitation for the provisions of flowers for the insects; the plant can be quickly adapted to changing weather conditions or drought periods by producing less flowers, this can affect insects like bumble bees due to the reduction of the sugars. It is demonstrated the variability in flower productions in the same season and the need of nutrient rich soils or the addition of fertilizer in order to guarantee a stable production during the year. [6] Figure 1 and Figure 2 show the quantity of Silphie biomass yield and the acreage in Germany during the last years.

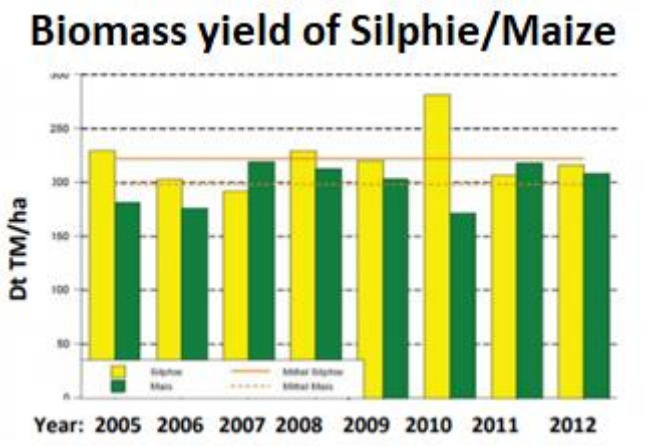

Figure 1: Biomass yield of Silphie and maize during 2005 to 2012 **[8]**

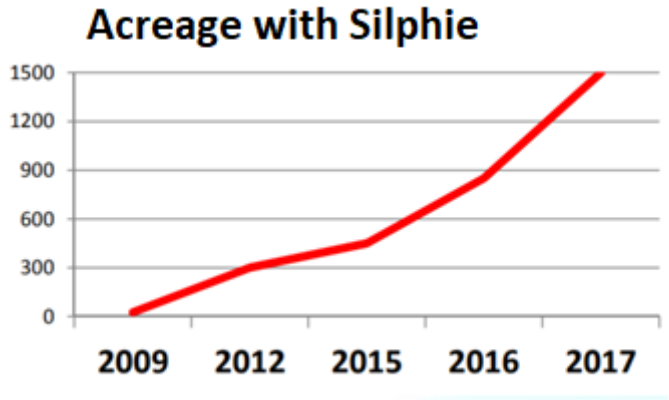

Figure 2: Acreage with Silphie **[8]**

Currently *Silphium perfoliatum* has several applications:

- Use for biogas production

Silphie can be used as a co-substrate for biogas production in biogas plants. A study of the product profitability shows that the Silphie present similar or even higher profit than silo maize. In the Figure 56 in the Appendix can be observed that for Silphie the biogas production is more economically profitable than for silo maize. [9]

- Use as shredded material for Silage

The high content of protein makes Silphie a good option for feeding cattle and dairy cows. For the first harvest the crude protein content is around 11.9% and for the second harvest the crude protein content is about 9.2%. The water content of the harvested Silphie can decrease by a short wilting phase after harvesting. [9]

- Direct feeding to farm animals

For direct feeding, the plant has to be harvested twice a year because if there is just one harvest, the stems and the lower leaves are too hard for feeding and it is rejected by the cattle. As a result of the long growing period and the permanently living roots, Silphie is really rich in minerals and trace elements. Thus, less quantity of food is needed for feeding the animals. [9]

- Bee pasture

Silphie flowering season lasts about eight weeks, from end of July to end of September and during this period the yields are visited by large number of insects such as bees. Therefore, it is a good alternative for honey production to regular sources due to the intensive agricultural use of the prairie today. [9]

- Cultivation in groundwater protection areas

The quantity of nitrate content of the soil is quite low after harvesting, around 5 to 9 kg per hectare, the roots of the Silphie can consume all the nitrogen fertilizer that have been applied in the previous months and therefore, can be cultivated in drinking water protection areas. It can be compared with Maize fields where the remaining of nitrates after harvest amounts typically to 30-70 kg per hectare. High nitrate concentration in drinking water is hazardous and can cause cyanosis to infants and seniors. [9]

2.2 Fructans

2.2.1 Structure and occurrence of fructans

There are many types of sugars, but the most familiar form is sucrose as it is the one used for baking at home. However, different types of sugars are classified according to their chemical structure. Oligosaccharides can differ in their nature of monomeric sugars and

in the sources of origin. Thus, depending on the type, they can differ in the benefits for the consumer. The most important types of oligosaccharides in the context of prebiotics are Fructooligosaccharides (FOS), Galactooligosaccharides (GOS), Lactulose derived Galactooligosaccharides (LDGOS), Xylooligosaccharides (XOS), Arabinooligosaccharides (AOS), algae derived marine oligosaccharides (ADMO). [10] The non-digestible oligosaccharides differ from the digestible ones as they have important physiological and physicochemical properties; hence, they can be used to improve the gut microecology. Fructooligosaccharides are polymers with different extension composed of fructose units and they are linked by β-2,1 position of sucrose. [10] Figure 3 presents an example of the structure of the fructooligosaccharides.

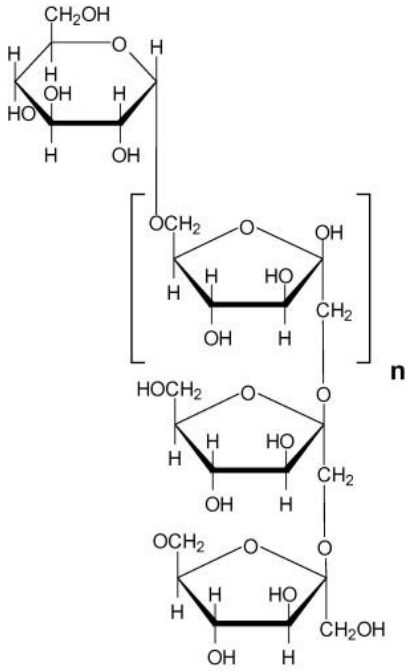

Figure 3: Structure of Fructooligosaccharides [11]

Fructans can be described as oligosaccharides (<10 monosaccharides units) or polysaccharides (>10 monosaccharides units). Fructans can be extracted by hot water without any chemical treatment. Fructans have different structures and lengths of chain, thus they are branched or linear; [12] However, the general structure of fructans consist of a glucose molecule linked to multiple units of fructose, which can reach 2 to 60 units of fructose in a molecule [13]. Fructose units are linked by β (2→1) linkages and with a terminal glucose molecule linked by α-D-l (1→2). The linkages between the fructose units are the most important, because they are responsible for the non-digestibility of fructans and therefore, for the low calorific value and the prebiotic character of fructans, which gives benefits for the health of the host. The length of the chain depends on different factors as the growth conditions of the plant, the storage time after harvest and the maturity stage. Short fractions have higher solubility and they are sweeter than long-chain fructans and long fractions are more viscous and thermostable. [14]

Fructans are natural carbohydrates which are used as reserves and that can be found in several plants, particularly in the family of Compositae. Moreover, they can also be found in different natural sources as its natural component like wheat, honey, onion, garlic and banana. However, they are commonly found as a component of cereals, fruits and vegetables next to starch specified. Figure 4 presents an example of the distribution of fructooligosaccharides in natural products.

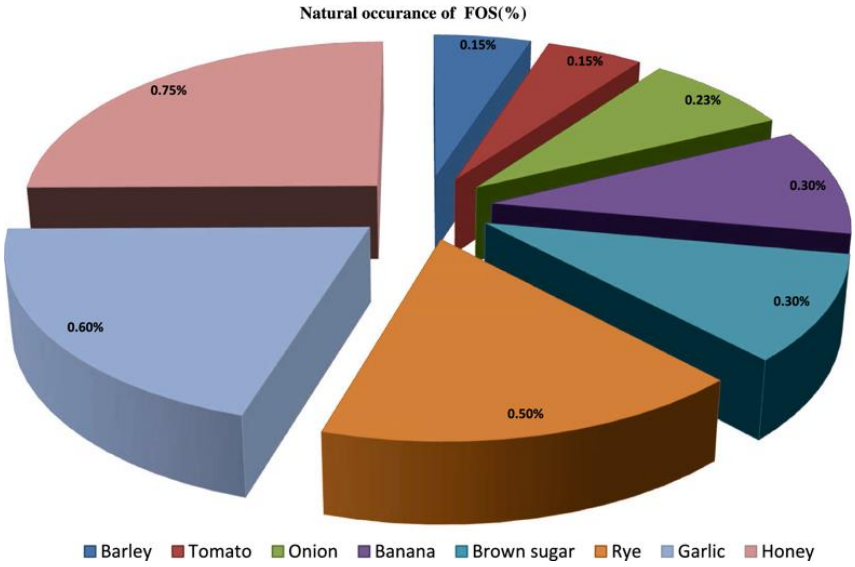

Figure 4: Natural occurrence of FOS [10]

2.2.2 Properties and benefits of fructans

❖ Non-digestible

Human enzymes can not hydrolyse the β (2→1) links of fructans. Thus, these saccharides reach the colon and therefore they are part of the soluble dietary fiber. This was tested in studies with humans using the ileostomy model with an intubation technique that was performed on volunteers. In this model, quantities of 17 g of inulin or pure oligofructose were administered with a recovery of 88 and 89%. In the intubation model, 20 g of oligofructose were ingested and was obtained a recovery of the 89%. The loss of 10% may be due to the hydrolysis of the links between glucose and fructose of the sucrose molecule, a very limited absorption of the small chains and a minimal fermentation of inulin and oligofructose by the microbial population. [14]

❖ Fermentability

Once that fructans reach the colon, where usually fermentation processes take place, they are metabolized by the microbiota that it is predominant found in the ascending part or near the colon. Once that fructans are completely converted into bacterial biomass, organic acids such as lactic acid and short-chain fatty acids (acetic, propionic and butyric acid) and gases (CO_2, H_2 and CH_4) and lactate contribute to the host energy metabolism. The short-chain fatty acids and lactate are partly used by the bacteria and

partly by the host. Anyway, they are less effective energy substrates than sugars and these factors together can explain the low caloric value of fructans.; the velocity of fermentation depends on the length of the chain, being faster in short chains. [14, 15]

❖ Prebiotic effect

Ingredients that produce a selective stimulation of growth and/or activities of microorganisms in the intestinal microbiota are called prebiotics. These prebiotics conferring benefits for the health of the host.

Based on the scientific consensus on prebiotics, a food with prebiotic properties requires the fulfilment of the following requirements:

- Prebiotics are not to hydrolysed or absorbed in the upper gastrointestinal tract and, therefore resistant to gastric acidity and not to be absorbed in the small intestine.
- Prebiotics are fermented by beneficial bacteria of the intestinal microbiota.
- Prebiotics induce beneficial physiological effects for health and the well-being of the host.

It is this prebiotic function that has aroused growing interest in fructans, since it has positive effects on health, as it produces a decrease of the lipid and glucose levels in blood and an improvement in intestinal transit. In addition, it also presents an increase in the absorption of minerals at the level of the large intestine, tested in research with rats and humans, where an increase in the absorption of calcium and other minerals was detected; the use of fructans in the diet shows a positive influence for the calcium content of the bones and their density. It is also demonstrated in animal models, that prebiotics decrease the development of colon cancer. Although the mechanism of action has not been elucidated yet, this preventive capacity could be due to two factors: the increase of the short chain fatty acids and the decreased of the proliferation of enzymes involved in cancer pathogenesis. Moreover, an inhibition of breast cancer has been observed in rats with a diet rich in fructans; also, an effect against the skin cancer has been demonstrated. All this makes fructans interesting as a factor in cancer treatment therapies. [14]

Finally, fructans can be resistant to ill effects of biles salts on Bifidus group of intestinal inhabitants. A study showed that the presence of fructooligosaccharides in the medium the Bifidobacterium improved the resistance and a better growth in company of bile salts. Their health benefits are such as anti-cancer properties, mineral absorption, lipid metabolism, anti-inflammatory and other immune effects (atopic disease). Moreover, fructans supplemented with calcium could have beneficial effects in bone mineral density in post-menopausal women; this is quite significant in osteoporosis. [10, 14]

2.2.3 Applications of fructans

❖ Food applications

Fructans are a natural compound of our diet as it is present in some foods such as onions, garlic, bananas, flour or even wheat. The daily intake of fructan per capita varies between 3 to 10 g. Besides, inulin was confirmed to be a food ingredient and not an additive in Europe, Australia, Japan, Sweden and Switzerland. An important property of inulin is that can be used as a fat-replacer. In order to obtain it has to be thoroughly mixed with water, or another aqueous liquid, a white, creamy structure results, which can be incorporated into foods to replace fat. Fructans also improves the stability of foams and emulsions, such as aerated dairy desserts, ice creams, table spreads, and sauces. Therefore it can replace other stabilizers in different food products. This could be a successful product due to the need of healthy products with a nutritional bonus. [15, 16]

Fructans can also be used as sweetener, it is sweet (35% compared to sucrose) and has a sweetening profile closely approaching that of sugar with a clean taste. Oligofructose has technological properties close to sucrose and glucose syrups, and, therefore, together with its sweetness profile, is frequently used as a sugar alternative. However, long-chain fructans have no sweetness. Currently, the demand of sweeteners is high due to the need for diabetics and the increase number of conscious consumers, therefore, this property has become more important. It behaves like a bulk ingredient, and contributes to body and mouthfeel, provides a better-sustained flavor with reduced aftertaste, and improves stability. Fructans are moderately soluble in water (maximum 10% solubility at room temperature), which allows its incorporation into watery systems. [10, 15]

❖ Pharmaceutical use

Fructans are considered to be a standard for Glomerular filtration rate (GFR), an indication of the ability of the kidneys to remove a substance from the blood and to deliver it into urine. This is possible because fructan is not metabolized, not toxic and freely filtered through the kidneys. Maybe in the future fructans also have application as a drug carrier. Some drugs loose efficiency in vivo due to poor chemical or biological stability but coupling a drug to an activated inulin could be a solution to this problem.[16]

❖ Further applications

All the non-food applications require transformation such as fermentation, enzymatic treatment or chemical modification. In a laboratory or in a pilot-scale can be produce products such as ethanol, acetone-butanol, 2-3-butanediol, lactate, succinate and the synthesis of hydroxymethylfurfural but due to the economic feasibility it is not possible to do it in an industrial scale yet. [16]

On the other hand, Inulin could be used to produce a fully oxidized ring-opened polycarboxylate to replace the builder or co-builder in detergent formulations as it has good calcium complexing properties. According to Kim et al. this product could be the most important of inulin application in the non-food sector. [16]

2.3 State-of-the-art regarding fructan production

2.3.1 Conventional fructan production

Fructans may be produced from either plant by extraction or systematically synthesised by means of microbial enzymes. For the presented work plant-derived fructans are of interest and therefore illuminated in the following section. In Figure 5 can be observed the diagram of the fructans production [17]:

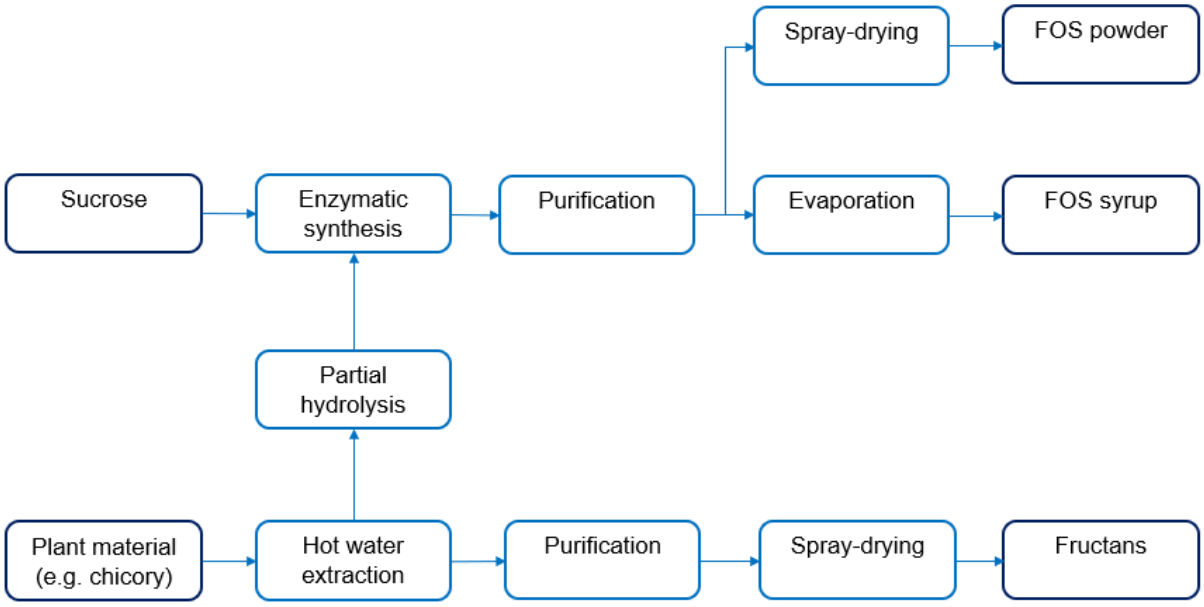

Figure 5: Porcess of the fructans production

Fructans production goes through different phases. The first step is to harvest and store the plant material in small piles on the field; then, after no more than seven days the plant is transported to the factory and then weighed and stored carefully. Subsequent, it is washed and sliced. The raw inulin is extracted with hot water in a diffuser from the "chips". The leached chips are dried and sold as feed and a first purification step is applied to the extract by carbonation of limewater. The formed CaCO3 precipitates and impurities like proteins are trapped in the flocks. This creates a foam-type product which it is rich in Calcium and organic matter and therefore, it is used by farmers to improve the quality of the soil. However, to process chicory for producing inulin is more difficult than processing sugar beets as chicory suffers more losses during the storage and processing than sugar beets. Consequently, it is a priority balancing the degradation of the inulin chain against infection, colour formation, Maillard reaction or the incorrect removal of the impurities formed in the first purification step. [18]

The second step is to refine the extract using cationic and anionic ion exchangers for demineralization and active carbon for the decolouration. The priority is to control the pH and the temperature values as they can be problematic for the process. After purification, the juice is passed over a 0.2 μm filter to be sterilized, subsequently the sterile juice is evaporated and spray dried. For converting the refined product into a stable end-product the spray-drying technology is being used. Ion exchange processes for demineralization involve high investment costs because the effluents must be treated in order to not be hazardous to the environment; therefore, they are collected and evaporated. At high concentrations crystallized salts like $(NH_4)_2SO_4$ and K_2SO_4 precipitate and they can be separated from the liquor by centrifuging and be sold as fertilizer; the mother liquor is evaporated into a storable end-product with high organic matter content and sold as feed. The condensates that have been generated in the evaporation can be re-used as process waters. [18]

Besides the production of native fructans, it can also be produced FOS syrup and FOS powder from sucrose or plant material as raw material. The only step that differs the production of inulin from the production of FOS syrups is a hydrolysis that can be done using enzymes. The goal of these enzymes is to produce as much inulin oligomers as possible with the least formation of monomers. Nevertheless, there is always going to be a small quantity of glucose, fructose and sucrose as they are present in the chicory as well. However, the market of inulin is not well-stablished as, for example, the market of fructose syrups. [18]

2.3.2 Research in the fields of fructan production

It has been observed an increase of scientific papers concerning to fructans over the last 10 years and it is expected to continue increasing. Moreover, the interest about how the consumption of fructans affect human health is becoming more important.

ORAFTI started a European Research project called ENDO (European Non-Digestible Oligosaccharides) in which fundamental biochemical mechanisms governing the altered lipid metabolism, the impact of inulin on the composition of the colonic flora and the influence of the altered bacterial interaction on the host health are going to be investigated. Moreover, the impact of non-digestible oligosaccharides on cholesterol metabolisms, mineral absorption and intestinal function is going to be tested on volunteers. [18]

The use of genetic engineering is becoming more important and some companies are working on projects that can transform the original plant into a transgenic one with further abilities. The aim is to obtain fructans with a high degree of polymerization (DP) expressed in chicory or sugar beets. In order to obtain high expression of the product and to have it in the right place. It is observed a difference between a DP of 10 than a fractioned inulin with mean DP of 23, only half of the quantity is needed to have the same fat replacement abilities or mouth-feel qualities. However, only environmentally friendly

processes will have future due to environmental requirements demand and that people day by day is more aware of the climate problems. [18]

As mentioned before, fructans are interesting due to their nutritional properties but mainly because of their prebiotic character, which contributes to many beneficial effects on human health and well-being. For all that, it is interesting to study the extraction of fructans from alternative plant materials. Therefore, Silphie, a plant generally used for biogas production, shall be investigated as potential fructan source in order to generate additional value since biogas can still be produced of the residues after the extraction. [18]

3 Materials and methods

3.1 Chemicals and equipment

3.1.1 Chemicals

- Ethanol 80%,

Preparation from 96% ethanol (Carl Roth).

- Sulfuric acid 72%

Preparation from a 96% sulfuric acid (Carl Roth, ROTIPURAN).

- Calcium carbonate

Manufacturer: Honeywell Fluka; Purity: ≥ 99%

- Glucose and fructose

The sugars were used for the calibration of the HPLC (High-performance liquid chromatography); Manufacturer: Sigma Aldrich/Merck, analytical grade

- Destilled water

3.1.2 Equipment

- Freeze-dryer

After harvesting on a field near Wilhelmshaven, Germany plant samples are freeze-dried in order to be dehydrated and prevent it from withering. Freeze drying is a water removal process normally used to extend shelf life, make the material better for the transport or to preserve perishable materials. The process works by freezing the material, then reducing the pressure and adding heat to make the frozen water to sublimate (Figure 6). Freeze-drying was performed at the Institute of technical microbiology at TU Hamburg with self-constructed apparatus.

The freeze-drying takes place in three phases:

- Freeze phase: Freezing is done in the freeze dryer directly or in any other freezer or chilled bath. The material must be cooled down below its triple point; therefore, sublimation can be ensured. Freeze drying is easiest to accomplish using large ice crystals; nevertheless, in case of freeze-drying biological materials, if crystals are too large, they can break the cell walls. For preventing this the freezing must be done rapidly.
- Primary drying phase: The pressure is lowered, and heat is added to the material in order to sublimate water; too much heat can alter the structure of the material, therefore the process can be slow.
- Secondary drying phase: The ionically bound of water molecules are removed by raising the temperature higher than in the primary drying phase. [19]

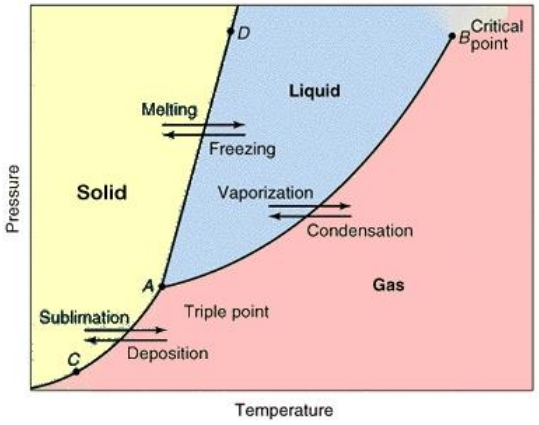

Figure 6: Phase diagram [20]

❖ Grinder and Mill

After the freeze-drying a customary grinder was used as pretreatment prior to milling. Milling was performed in a Fritsch planetary Ball Mill (Pulverisette 5) with four balls in each container (250 ml) at 360 rpm for 3 min.

❖ Sieve

Sieves were used for separating the particles in order to homogenize their size after the milling. A sieve of 1 mm was used to obtain a sample with particle sizes < 1 mm

❖ Water bath (Julabo-SW23)

For the extraction process the shaking was activated at 150 rpm and the temperature varied from 20 to 100 °C.

❖ Centrifuge (Hettich-Rotixa 50 RS and Eppendorf centrifuge 5417R)

Centrifugation was used for all the samples after the extraction step and after the neutralization (in case of analytical hydrolysis). First was used the Hettich-Rotixa 50

RS and then the Eppendorf centrifuge 5417R in order to minimized the suspended particles for the used of the HPLC. This equipment sorts out the solid part of the Silphie, which stays in the bottom of the tube, from the liquid phase. The parameters used were 4,500 rpm for 30 min at 20 ºC for the first centrifuge and 14,000 rpm for 10 min at 20 ºC for the second centrifuge.

- Drying furnance (WTC binder/C&S Kälte)

The dryer was used for drying the wet mass after the extraction in order to eliminate the water content from the samples. Ther samples were introduced at a temperature of 40 ºC until the mass was completely dried. Then, the mass was weighed for determining the dry mass of the samples.

- Furnace (Memmert)

The furnace was used in the analytical part for the hydrolysis, after adding the sulfuric acid the sample had to stay in the furnace for 30 min at 100ºC.

- HPLC (High-performance liquid chromatography)

The HPLC is used in order to quantify the sugars of the plant. The used system is a Agilent Technologies HPLC 1260 II infinity series HPLC with refractive index detection (RID) operated at 55 °C. A Bio-Rad Aminex HPX-87H (300 x 7.5 mm) column is used at 60 °C for separation with 5 mM sulphuric acid as eluent (flow rate 0.6 ml/min). The runtime per sample is 1 h.

In general, this technique is used for separating the components of a mixture and consist of a polar phase with H^+ ions, the column, and a mobile phase. The sample is injected in the mobile phase and the components of the sample pass and elute from the column depending on their interactions with the used resin. These chemical interactions determine the separation of the contents in the sample. The use of the different detectors will depend on the nature of the compounds to be determined. [21]

3.2 Experimental procedure

3.2.1 Extraction of fructans

First, the freeze-dried Silphie sample was milled in order to increase the specific surface and to obtain particles with a defined size (< 1 mm). Then, a defined amount of sample was transferred to a tube and distilled water was added corresponding to the desire liquid to solid ratio. The tube was shaken with a vortex mixer and immediately added to a heated and shaking water bath for the extraction. Afterwards the extract was filtered and then transferred to another tube and centrifuged. The solid residue was dried and weight for determining the total yield of extraction. Finally, the liquid extract sample was analysed by means of HPLC.

3.2.2 Sugar analysis

As there are no commercial standards of fructans in the market, direct determination by HPLC is not possible. Therefore, it is necessary to do an indirect procedure: sugar monomer determination before and after hydrolysis. The difference between the obtained results gives an estimation for the fructan content (Chapter 3.3.3).

- Hydrolysis

  The method for fructan hydrolysis is based on the NREL procedure for hemicelluloses in biomass. For the conducted hydrolysis it is necessary to adjust a final sulphuric acid concentration of 4% in the sample, therefore 174 µL of sulphuric acid are added to five millilitres of the extracted sample. Then, the sample is introduced to the furnace at 100 °C for 30 min in order to the hydrolysis to occur. Once that the 30 min are over the samples are introduced in a bath with cold water in order to cooling them down. [22]

- Neutralization

  After ten minutes the samples are cold enough to start the neutralization step; the calcium carbonate is added in small amounts until the pH reached a value of approx. 6. Then, the samples are treated first in the Hettich-Rotixa 50 RS (4,500 rpm) and then, in the Eppendorf centrifuge 5417R (14,000 rpm) in order to remove all the particles and protect the HPLC for the analysis afterwards.

3.3 Experiment evaluations

3.3.1 Design of experiment

Design of experiments was used for the maximisation of the yield. The corresponding experimental plan was created in the software Design-Expert which it is used for evaluation purposes. It is a piece of software designed to help the design and interpretation of multi-factor experiments; It offers a wide range of designs, including factorials, fractional factorials and response surface. Once the experiment has been carried out and the response values are entered, it can be analysed the response as there is access to various techniques for analysing and interpreting the fitted models. One of the most important techniques for analysing is the ANOVA test, it assesses the importance of one or more factors comparing the response variable means at the different factor levels. The null hypothesis states that all population means are equal while the alternative hypothesis states that at least one is different. Anovas require data from normally distributed populations with equal variances between factor levels. [23, 24]

Box-Behnken design was used for creating an experimental plan in order to obtain reliable results or statements on the reliability of the results, respectively. Box-Behnken design is a type of response surface design that does not contain an embedded factorial or fractional design. It has treatment combinations that are at the midpoints of the edges

of the experimental space and require at least three continuous factors. Figure 7 shows a three-factor box-behnken design. [25]

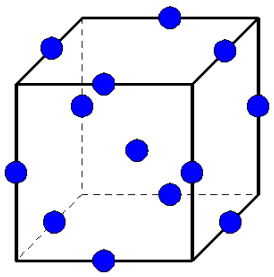

Figure 7: Box-Behnken design for three factors [26]

These designs allow efficient estimation of the first and second order coefficients. As they have fewer points than for example, central composite designs with the same number of factors it can be less expensive. Box Behnken design do not have axial points, thus, all design points are found in the safe operating zone. Also, it can ensure that all factors are not set at their high levels at the same time. [25]

Blocks are used in order to conduct the experiments separately and independently as when the number of runs is too large to be conducted under steady conditions it can be introduced an error into the experiment. The number of blocks depends on the number of factors. Factors are the independent variable, in this case, it is going to be used 3 factors: temperature, extraction time and liquid-solid-ratio. Factors as the solvent and the mixing were also raised, but according to the literature the was deduced that those three factors were the most important to have in account. Furthermore, the water as solvent was the best option as it is good for the environment and it is cheap to obtain. [27]

3.3.2 Calculation for the hydrolysis

In order to proceed with the hydrolysis, it is necessary to adjust a final sulphuric acid concentration of 4% in the sample. On the basis of sulphuric acid 72%, it is going to be calculated the volume of sulphuric acid needed for 5 mL of the sample using the following equation [22]:

$$V_{H2SO4} = ((C_{4\%\ H2SO4} * V_{sample}) - ((V_{sample} * C_{H^+} * 98.08\ (g)) / 2\ (mol\ H^+))) / C_{72\%H2SO4} \quad \textbf{(Eq. 1)}$$

Where:

$C_{4\%\ H2SO4}$=41 g/L

V_{sample}=5 mL

$C_{72\%H2SO4}$=1176.3 g/L

$C_{H^+}=10^{-pH}=10^{-6}$

Therefore, using a pH value of 6 in order to calculate the concentration of H^+, the quantity added of sulfuric acid 72% is 174 µL.

3.3.3 Extraction yields

The methods known for measuring the amount of fructan in plants are time-consuming. The method consists of an extraction step in a water bath and subsequent hydrolysis of fructans requiring two HPLC runs to determine the free monomers before and after the hydrolysis. The amount of fructans can be calculated with the following equations [22]:

$$M_f = k * (\triangle G + \triangle F) \quad \textbf{(Eq.2)}$$

$$K = (180 + 162*(n-1)) / (180 * n) \quad \textbf{(Eq.3)}$$

$$n = (\triangle F / \triangle G) + 1 \quad \textbf{(Eq.4)}$$

Where:

k=correction factor for water uptake during hydrolysis

n=Average of the degree of polymerization (DP)

△G=Glucose released from fructans

△F=Fructose released from fructans

With the determined amount of fructans, the **yield of fructans** can be calculated:

$$\text{Yield of fructans (\%)} = (M_f / \text{weight of the dried sample}) * 100 \quad \textbf{(Eq.5)}$$

It must be mentioned that for the extraction volume equals the amount of distilled water added in the extraction step. Thus, the quantity of added sulfuric acid in the hydrolysis is not considered. This simplification is acceptable due to the phenomenon known as volume contraction, which explains the volume of the reactants is greater than the volume of the products but as the amount of sulfuric acid is really small it can be scorned, this phenomenon can be observed adding a specific amount of ethanol and water, the final volume is less than sum of the volumes of ethanol and water separately.

For calculating the **yield of fructose and glucose** it is analogous to the yield of fructans but without the correction factor, it can be calculated with the equation 6:

$$\text{Yield of Fructose + Glucose (\%)} = (((\text{Fructose + Glucose}) * \text{extraction volume}) / \text{weight of the dried sample}) * 100 \quad \textbf{(Eq. 6)}$$

The yield of fructose and glucose provides information about how much molecules of fructose have been extracted before of the hydrolysis, the so-called free sugars.

Moreover, by measuring the weight of dried Silphie before and after the extraction it is possible to calculate the **total yield of extraction** following the next equation:

*Total yield of extraction (%) = 100 - (Dry mass / weight of the dried sample) * 100* ***(Eq. 7)***

This equation can give us the information about the percentage of the quantity of the sample that has been extracted in total and thus allows a statement on the share of impurities.

4 Results and discussion

4.1 Preliminary experiments

The first experiment was conducted with fresh Silphie for the parameters shown in the Table 1. The parameters were chosen according to literature on comparable fructan extractions with other raw materials. The optimum parameters in literature were in the range of 70 to 90 ºC and extraction times of < 60 min; the used parameter levels are based on these literature values. [28, 29]

Table 1: Parameters for the preliminary experiment

ratio	time	temperature
-	min	ºC
5	30	50
10	60	70
20	90	90

After the choice of the parameters the different extractions were conducted as explained in chapter 3.2, followed by the hydrolysis, neutralization and analysis. In this case the total yield of extraction was not calculated due to some problems in the filtration. Thus, the following results were achieved using the determined dry mass of xx% for calculating the yield.

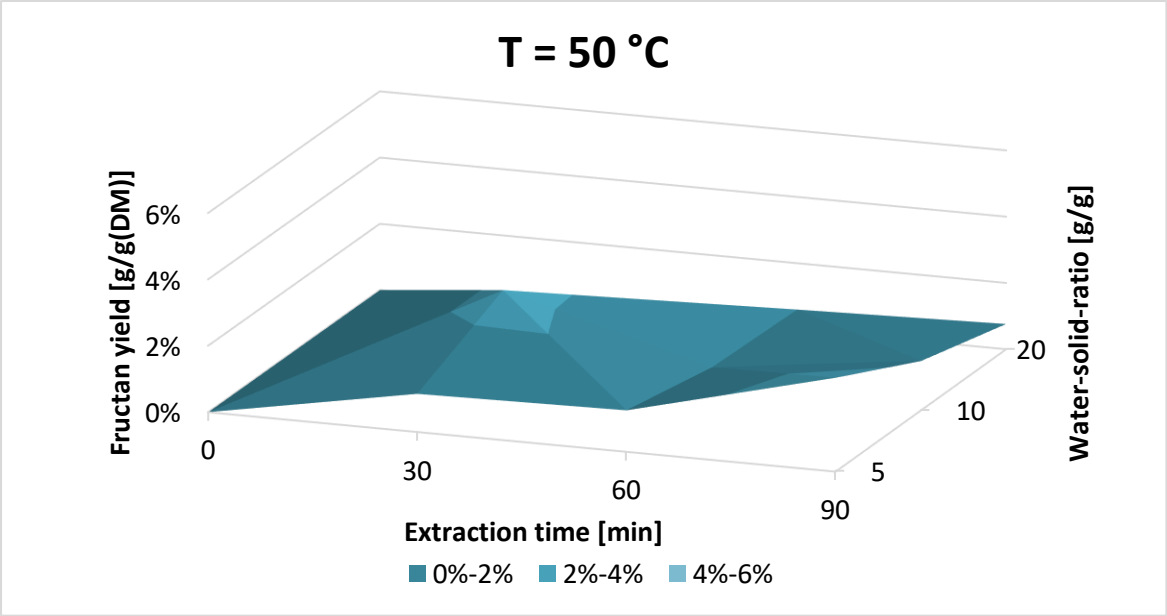

Figure 8: Yield of fructans from fresh Silphie for a temperature of 50ºC

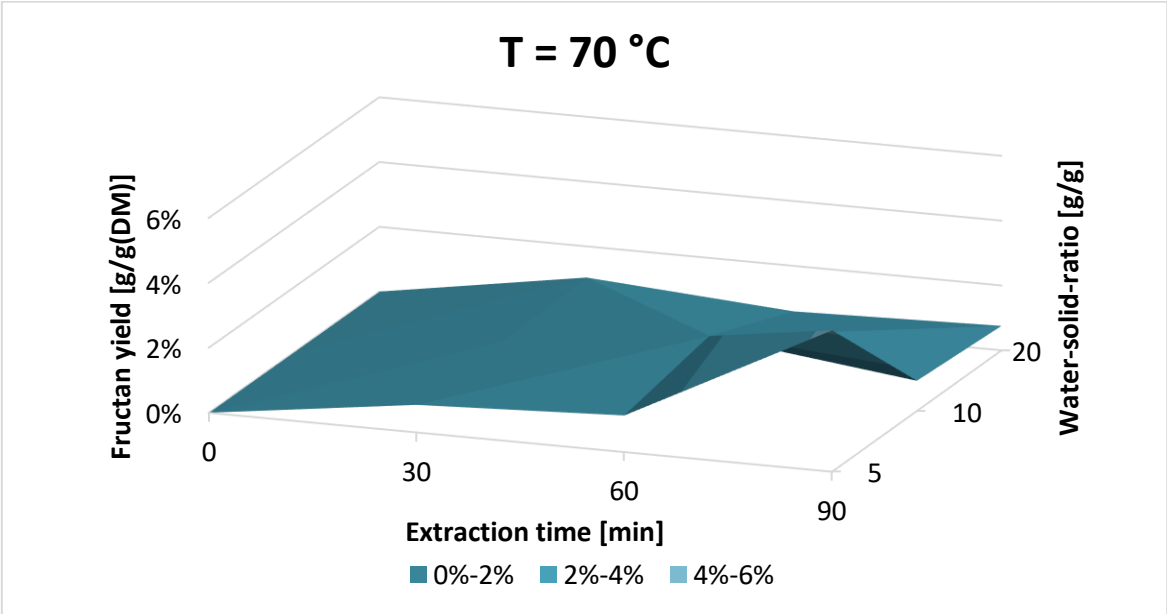

Figure 9: Yield of fructans from fresh Silphie for a temperature of 70ºC

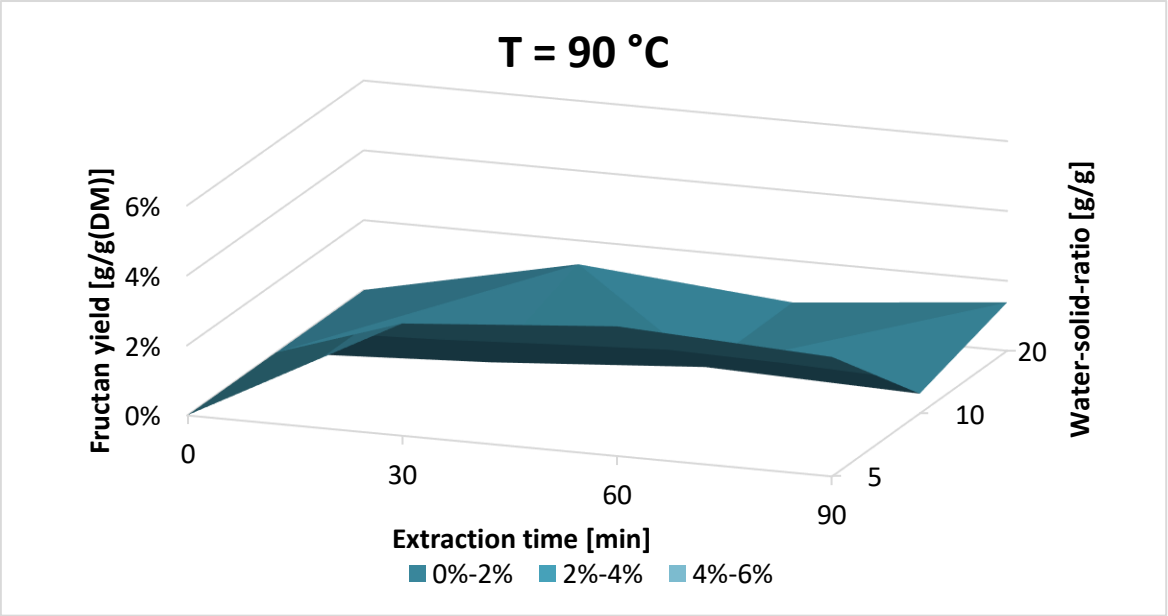

Figure 10: Yield of fructans from fresh Silphie for a temperature of 90ºC

As can be observed there is not a clear trend in the graphs as one would expect. Therefore, it is difficult to compare the results from the different parameters, but the data shows that for a liquid- solid-ratio (hereafter called ratio) of 5 the yields are higher than for 10 or 20. This is not reasonable, but would be also positive if it is considered in an industrial scale as would be used less amount of water resulting in a more environmental-friendly and cheaper process. On the other hand, the time does not show an optimum parameter, but increasing time leads to increasing yields. Depending on the temperature and the ratio the maximum value change. For the temperature, although there is no trend, the highest values are found for the maximum temperature of 90ºC. The maximum point can be observed for a temperature of 70ºC, a ratio of 5 and an extraction time of 90 minutes with a maximum yield of 4,35%. Nevertheless, the obtained yields are quite low, this might be due to the used of fresh Silphie. The fresh plant could have withered and, also the experiments were not carried out on the same day as harvesting. Another reason could be that the enzymes were degrading the fructans. Therefore, in the next chapter the enzyme influence will be studied.

4.2 Enzyme influence

In order to investigate a potential influence of the plant-based enzymes, a deactivation step is preformed prior to the extraction and the yield of fructans are compared to an extraction without enzyme deactivation. For the enzyme deactivation the procedure was the same than for the extraction, but some steps were added. The enzyme deactivation was tested with ethanol 80%, boiling water and for normal conditions in order to compare the obtained results. In general, an increase of temperature also means an increase in the reaction rate and extraction speed. However, as the enzymes are proteins, they are denatured by heat. [30]

Ethanol 80% was heated up to 70ºC, then added to the sample and immediately shake it and introduced to the water bath. On the other hand, distilled water was heated up to 100ºC, but as the boiling water was not possible to be transferred to the pipette due to the vapour it was necessary to wait until the water was not boiling anymore. The determined yields for different deactivation methods are shown below (Figure 11, Figure 12 and Figure 13)

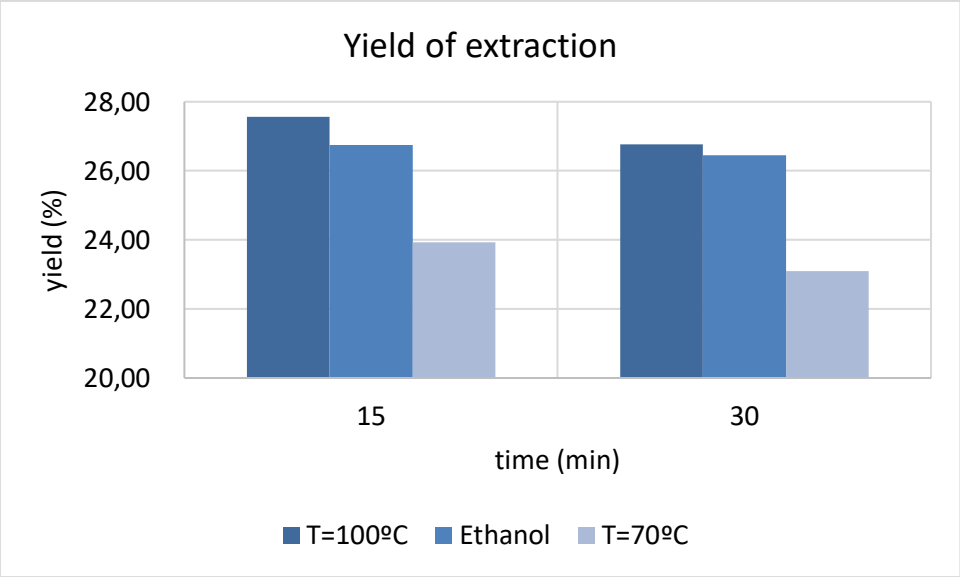

Figure 11: Yield of extraction in case of prior enzyme deactivation

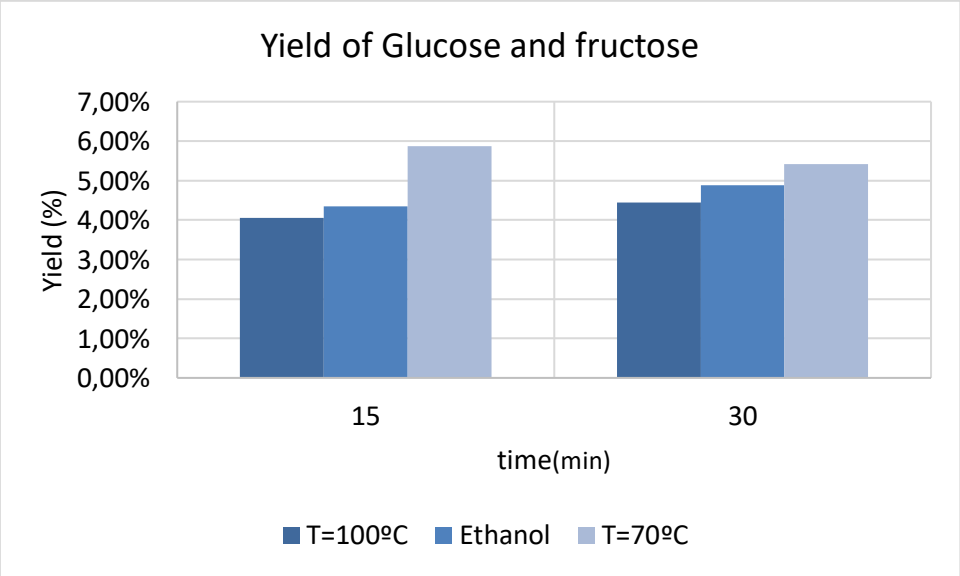

Figure 12: Yield of Glucose and fructose in case of prior enzyme deactivation

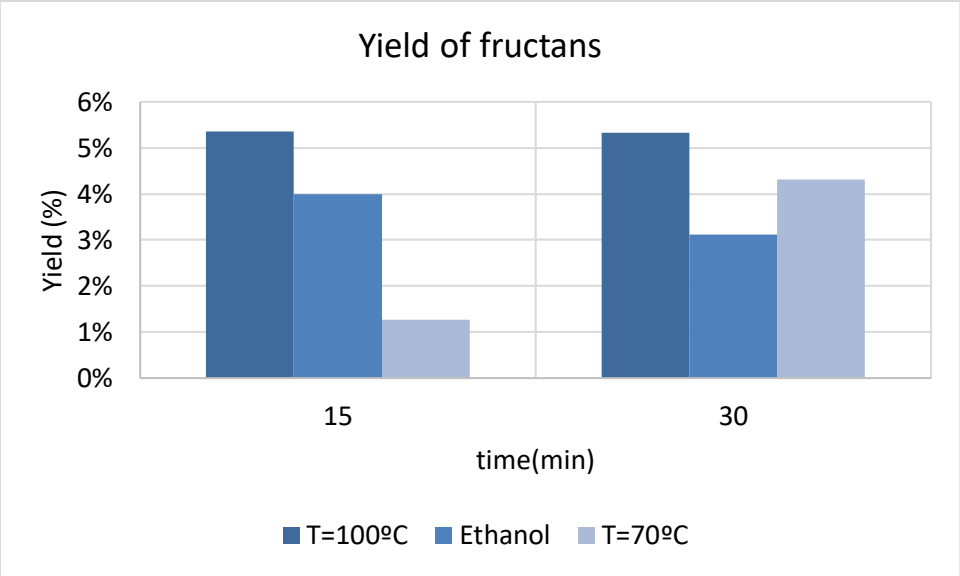

Figure 13: Yield of fructans for the enzyme deactivation

The yield of extraction is higher for boiling water and ethanol 80%, this makes sense as solubility in boiling water is better and ethanol can extract different components as we can see in the Figure 14 the colour of the extract is dark green comparing to the brown extract of water as solvent, this indicates that pigments like Chlorphyll have been extracted as well. On the other hand, the yield of glucose and fructose (Figure 12) is higher for a temperature of 70 ºC than for boiling water and ethanol 80%, as the free monomers are easy to be extracted, the differences could be due to the variety of Silphie sample composition. In contrast, the yield of fructans is higher for boiling water this might be due to the enzyme deactivation, the differences between the Silphie sample composition or the better solubility at higher temperatures.

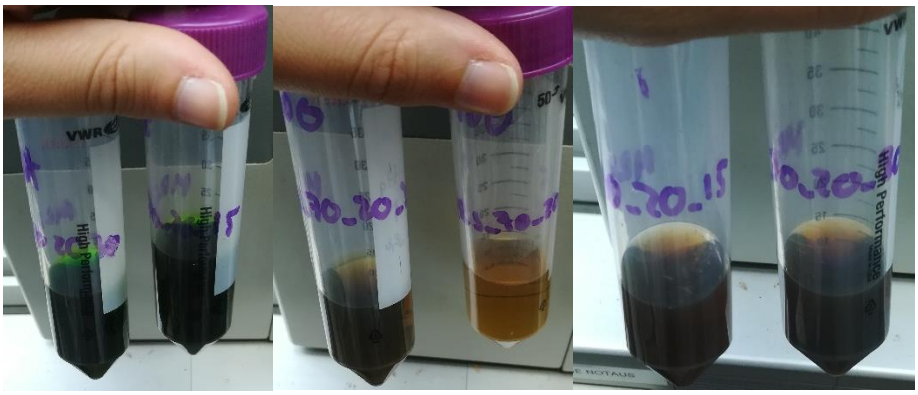

Figure 14: Comparison of the obtained extracts (Ethanol (left); boiling water (middle); 70 ºC (left))

In the case of the yield of fructose and glucosethere are no significant differences between ethanol and 70 °C water and those that can be found, can be due to small experimental

and analytical errors. For the yield of fructans in the Figure 13, for a time of extraction of 15 min there might a trend where without deactivation the yield is smaller than with deactivation, this can be also observed for the total yield of extraction. Comparing the deactivation methods, the boiling water is more effective regarding to fructan yield, but in case of 30 min of extraction time this trend is not visible anymore, it might be due to errors, so in order to make it reliable, the experiments should be repeated. Therefore, as there were some problems in the realization of the experiment with boiling water and had the higher yield of extraction it was decided to repeat that experiment but changing the method.

At first place, the water was boiled directly in the tube and after the 100 ºC were reached the sample was added to the tube carefully in order to not lose any mass of the sample, then was boiling during 0.5 to 15 min and after that the samples were shaken and finally put in the water bath to complete the 30 min of extraction time in total. The determined yields for different deactivation methods are shown below (Figure 15 and Figure 16)

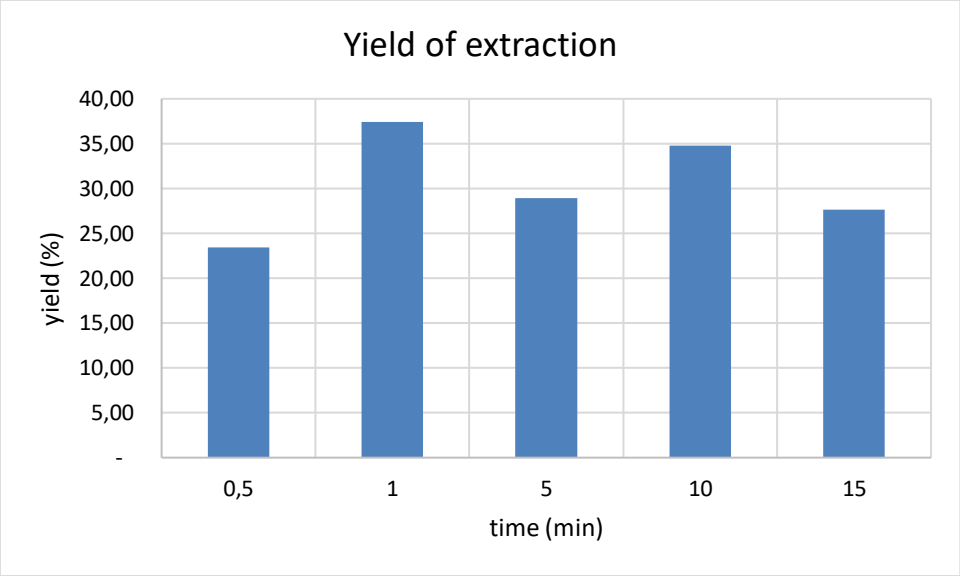

Figure 15: Total yield of extraction for the deactivation of enzymes with boiling water

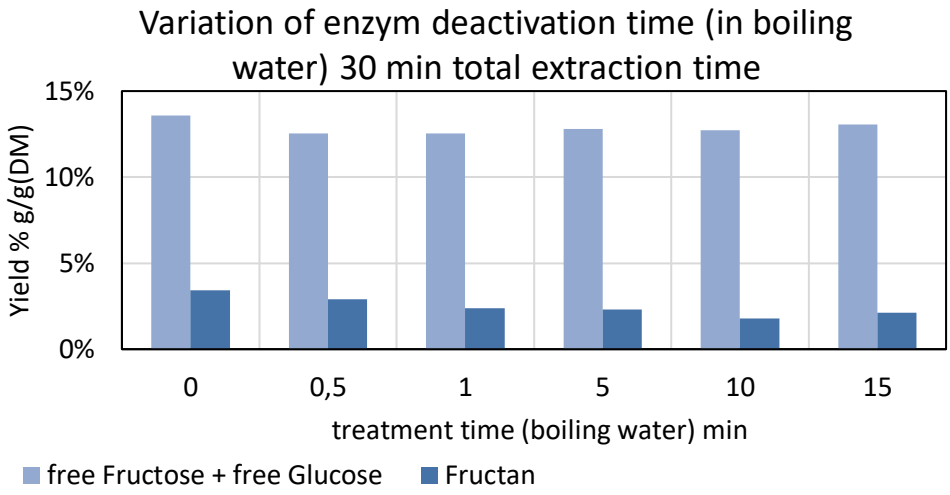

Figure 16: Yield of fructans and glucose and fructose after deactivation of enzymes with boiling water

This experiment is demonstrating that the enzymes have no effect on this process as it is observed that there are not significant differences between doing the deactivation and not doing it as all the obtained values are quite similar (Figure 16). Therefore, can be deduced that enzyme deactivation will be not necessary to perform these steps in the next experiments. The enzymes may have no effect because they are already deactivated because of the freeze-drying, or because in this case, they are not playing any role for the extraction (slow enzymatic reaction).

The findings of the preliminary experiments were used in the next step in order to find the maximum yield of extraction. This important not only for fructan production but also for determining the fructan content in Silphie reliable, what is not described in literature for Silphie, so far.

4.3 Optimisation of the fructan yield

4.3.1 Effect of the temperature

First, the effect of the temperature was studied separately in order to find out how relevant is this parameter for the extraction. According to literature temperature is one of the most important parameters. In this case, the total yield of extraction was calculated, as well as the yield of glucose and fructose and the yield of fructans for varying temperature of 20ºC, 40ºC, 60ºC and 80ºC and with an extraction time of 0.5 min. Therefore, the water bath was not used as the time was too short; the boiling water was transferred to the tube and then the samples were shaken for 0.5 min and filtered. The experiments were performed in duplicate in order to calculate the standard deviation to make more reliable the experiment. The following results were obtained [28]:

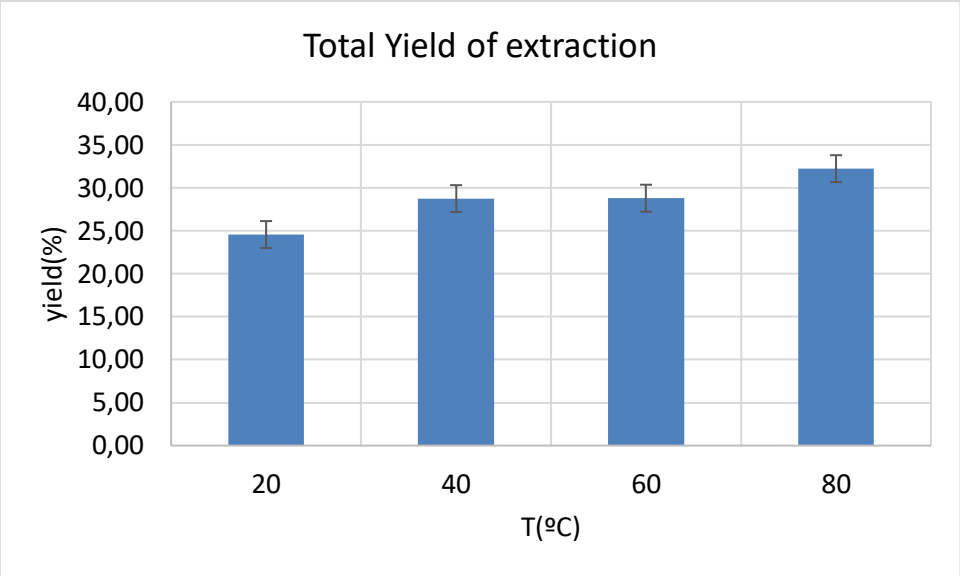

Figure 17: Yield of extraction for different temperatures (l/s=40; t=30 s)

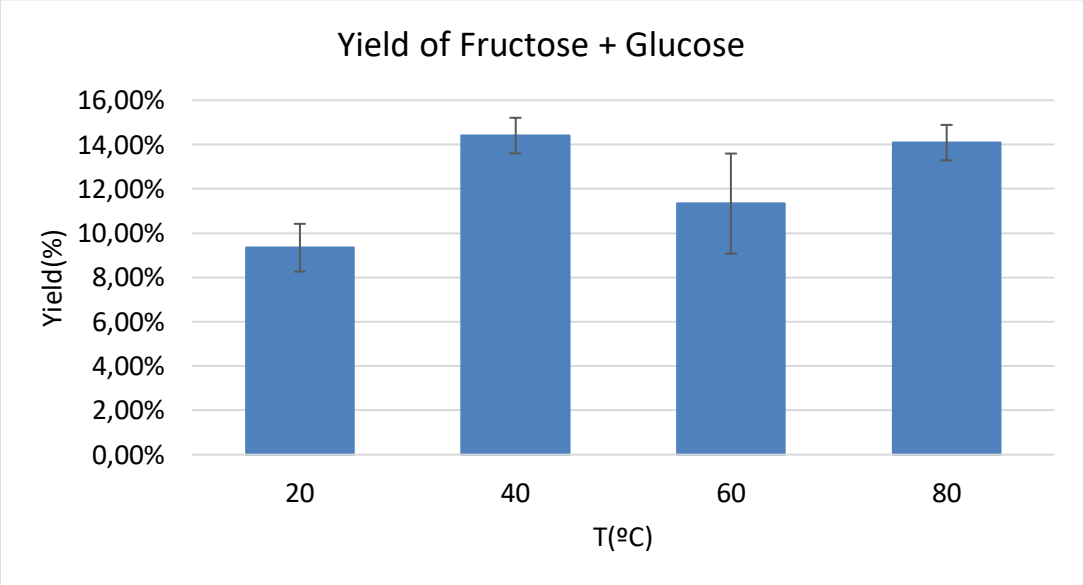

Figure 18: Yield of fructose and glucose for different temperatures (l/s=40; t=30 s)

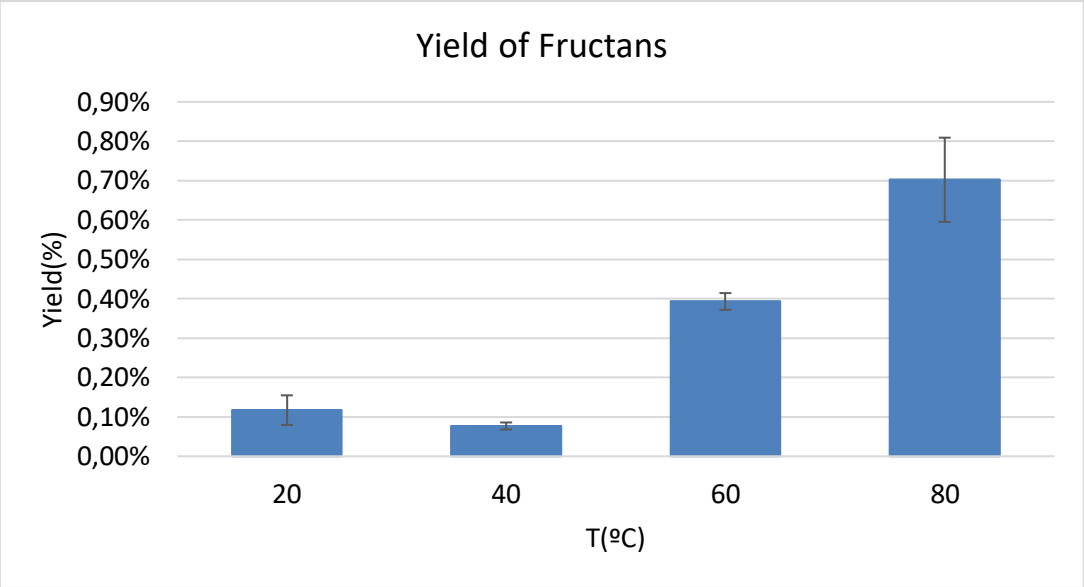

Figure 19: Yield of fructans for different temperatures (l/s=40; t=30 s)

In case of the total yield of extraction (Figure 17) the yield is similar for every temperature but there is a slight trend showing that the yield increases with the temperature, this make sense as the total yield of extraction shows everything that has been extracted, thus for higher temperatures the extraction is faster. On the other hand, the yield of fructose and glucose (Figure 18) seems to not follow a trend, but thanks to the standard deviation it can be observed that the yield of fructose and glucose for every temperature is similar. Sugar monomers are really easy to be extracted as after the milling the cell walls are damaged and the samples were obtained from the same harvesting and they were stored in the same way, therefore, the total amount of free monomers should be similar. However, for a temperature of 20 ºC the obtained yield is lower than for the other temperatures, this can be explained as was used the room temperature and maybe the extraction at that temperature is slower.

For the yield of fructans Figure 19, the results show that the temperature does have a relevant effect on the extraction. It is observed a clear trend, that for higher temperatures the extraction is better; that makes sense due to higher temperatures improving the extraction. As the extraction was conducted for a very short time the effect of the temperature is obvious.

In the following, design of experiments was used to find the optimum conditions for frutans extraction varying temperature, time and liquid-solid-ratio. For evaluation a quadratic model was expected and therefore Box-Behnken design was used. Three Silphie samples with different harvesting times were used for extraction and finally compared.

4.3.2 Extraction of Silphie harvest June 2019

For this experiment was used the dried Silphie harvested in June 2019. The parameters where chosen according to the literature. As was observed in the preliminary experiments

for high extraction time the extraction might be complete, therefore for this experiment the extraction time was reduced. The parameters that were used are shown in Table 2:

Table 2: Parameters for Silphie harvested in June

ratio	time	temperature
-	min	ºC
20	5	50
40	17,5	70
60	30	90

The parameters were inserted in Design-Expert and was decided to follow the box-Behnken design with a total of 17 experiments, having as a result the total extraction yield, the yield of fructose and glucose and the fructan yield. The following results were obtained:

Total yield of extraction

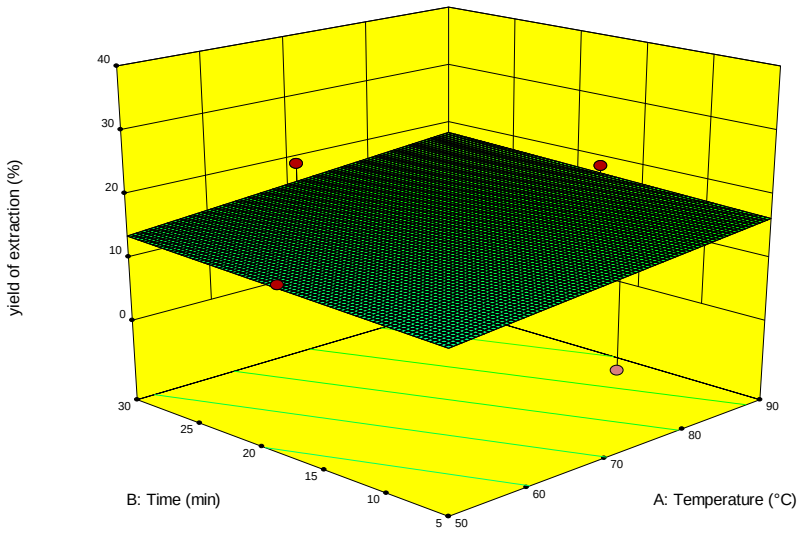

Figure 20: Yield of extraction for Silphie harvested in June for a ratio of 20 varying time and temperature for Silphie harvested in June

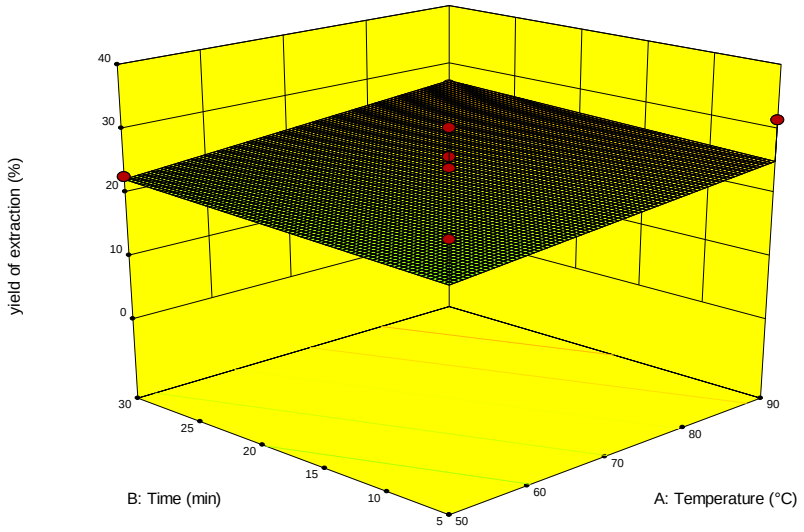

Figure 21: Yield of extraction for Silphie harvested in June for a ratio of 40 varying time and temperature for Silphie harvested in June

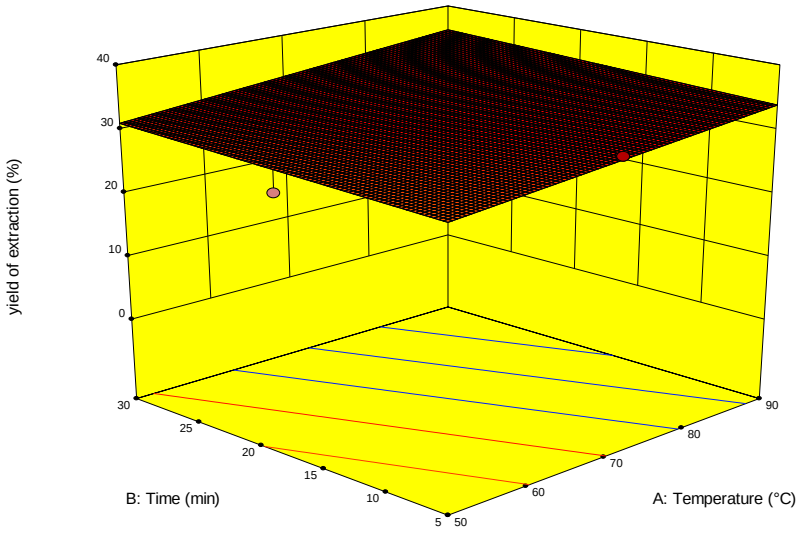

Figure 22: Yield of extraction for Silphie harvested in June for a ratio of 60 varying time and temperature for Silphie harvested in June

The values differ from each other depending on the conditions of the extraction (Figure 20, Figure 21 and Figure 22), however this strong difference might be due to experimental errors during the procedure (see error discussion). In the case of a ratio of 20, 5 mins and 70ºC the value is not making sense as it is negative. This can be due to an error while weighting the dried samples, as for a ratio of 20 the mass needed was higher (2 g) than for the other ratios (0.5 and 1 g). Therefore, the samples of a ratio of 20 needed more time to be completely dried than the other samples for ratio 40 and 60. This can be also observed as for a ratio of 20 there are significant differences in all the values comparing to the other ratios. These differences make sense as for a higher amount of water the

extraction is easier due to the concentration gradient, but such significant difference might be due to errors.

Moreover, the value obtained for a ratio of 40, time of 17.5 minutes and 70 ºC is an average of the five values measured in the experiment as was the central point; with a standard deviation of 25,1 ± 2.93.

Yield of free glucose and gructose

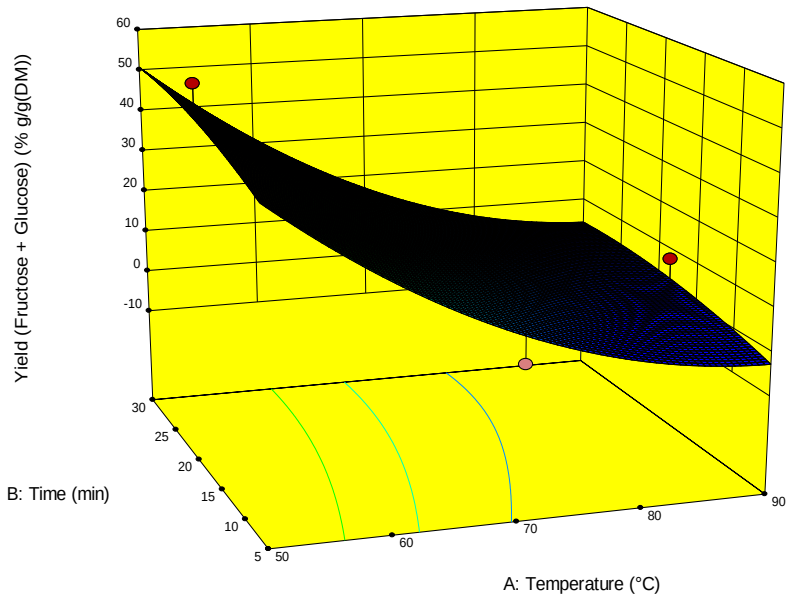

Figure 23: Yield of Glucose and Fructose for a ratio of 20 with varying time and temperature for Silphie harvested in June

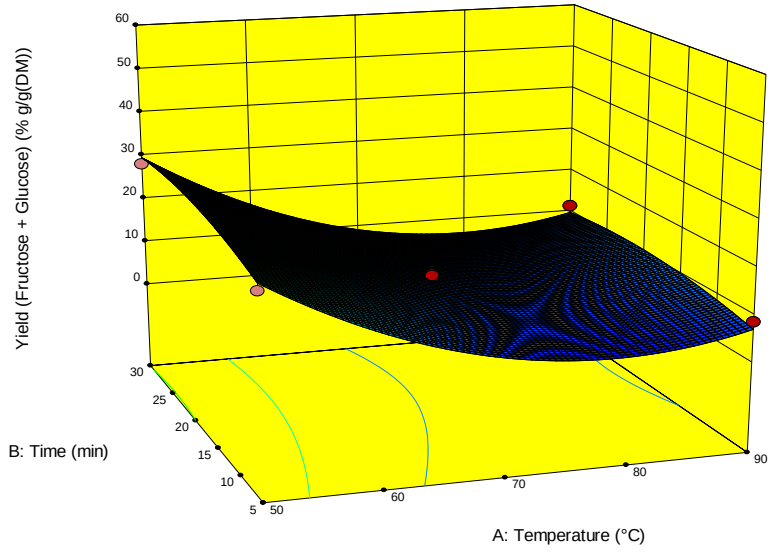

Figure 24: Yield of Glucose and Fructose for a ratio of 40 with varying time and temperature for Silphie harvested in June

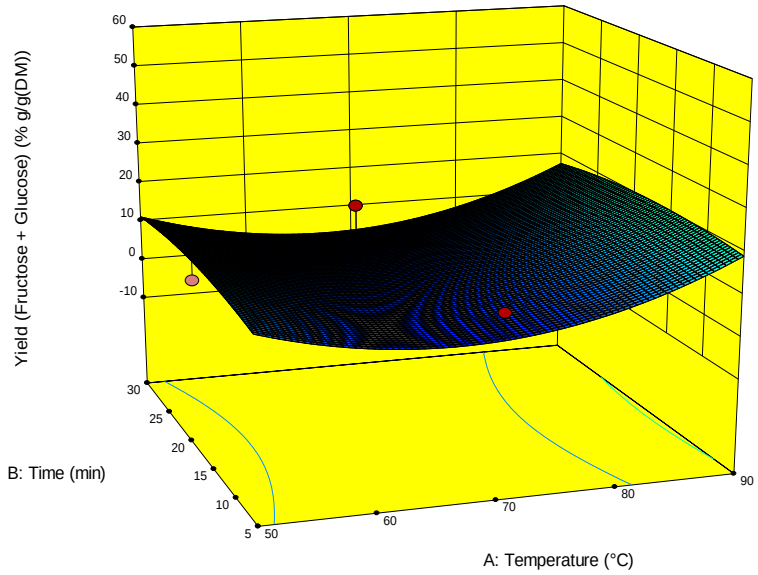

Figure 25: Yield of Glucose and Fructose for a ratio of 60 with varying time and temperature for Silphie harvested in June

From these graphs (Figure 23, Figure 24 and Figure 25) it can be appreciate that the higher the temperature is, the lower the yield is. This is not reasonable, however, for a ratio of 60 the yield is increasing a little. Moreover, it can be observed that the yield of fructose and glucose does not change over the time. This phenomenon can be explained because after the milling the material was observed with a microscope and the walls of the cell were damaged and due to this fact extraction of the sugars is easily and hardly limited as the solubility is very high.

Yield of fructans

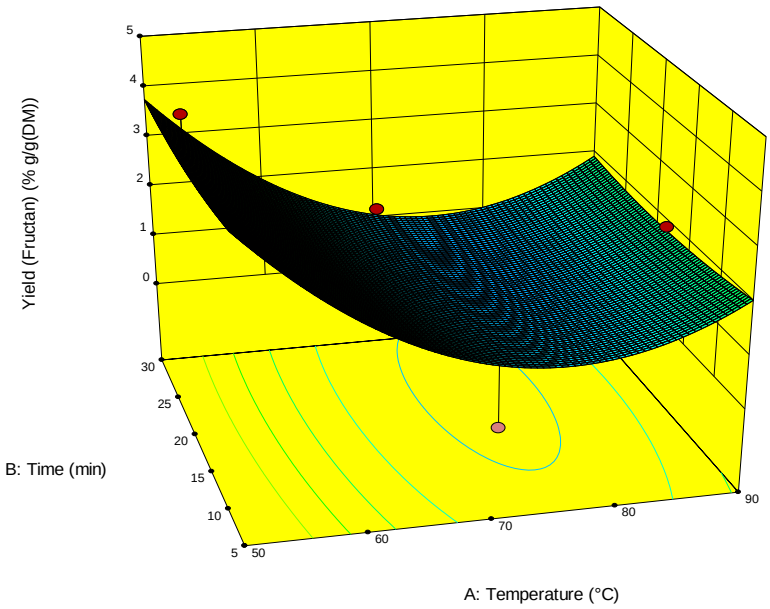

Figure 26: Yield of fructans for a ratio of 20 with varying time and temperature for Silphie harvested in June

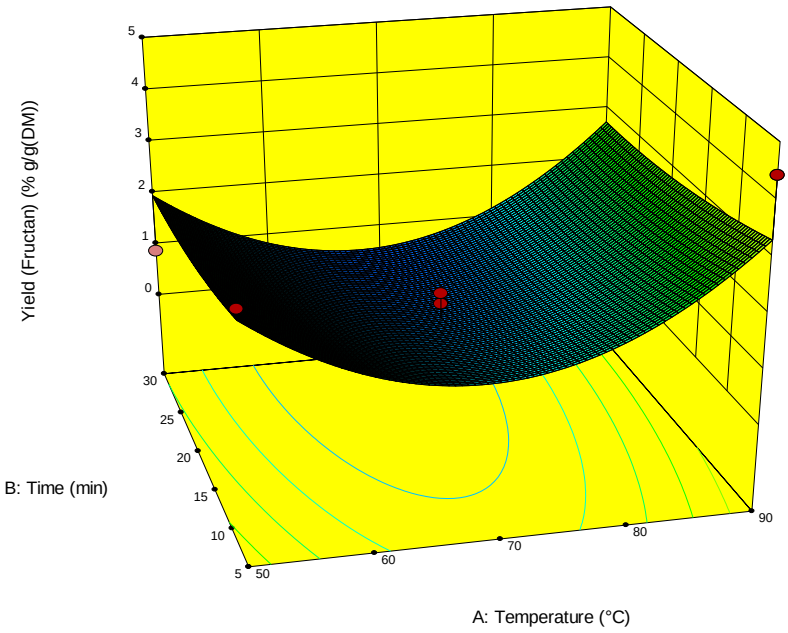

Figure 27: Yield of fructans for a ratio of 40 with varying time and temperature for Silphie harvested in June

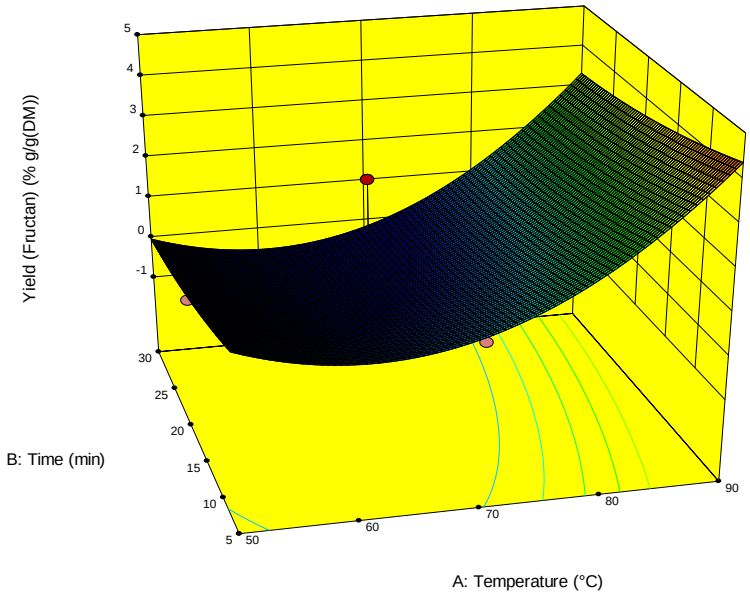

Figure 28: Yield of fructans for a ratio of 60 with varying time and temperature for Silphie harvested in June

From the graphs (Figure 26, Figure 27 and Figure 28) of the yield of fructans can be observed that the time is not having influence on the yield like in case of the free sugars. It is also shown that for a ratio of 20 the yield is decreasing with increasing temperature, this behaviour is not reasonable and was not expected to obtain such a trend in Figure 26. However, for a ratio of 60 the yield is increasing with the temperature, having the maximum for 90ºC. Furthermore, it would be expected to increase the yield when the ratio increases because of the concentration gradient, the more solvent you have the easier the transport will be. Therefore, we can not say that there is shown any trend in these graphs.

4.3.3 Extraction of Silphie harvest July 2019

The experiment with Silphie harvested in June (chapter 4.3.2) was repeated for the dried Silphie harvested in the month of July 2019 to see if there were any differences in order to the content of fructans. Thus, the same experimental plan as before was carried out and the following results were obtained:

Total yield of extraction

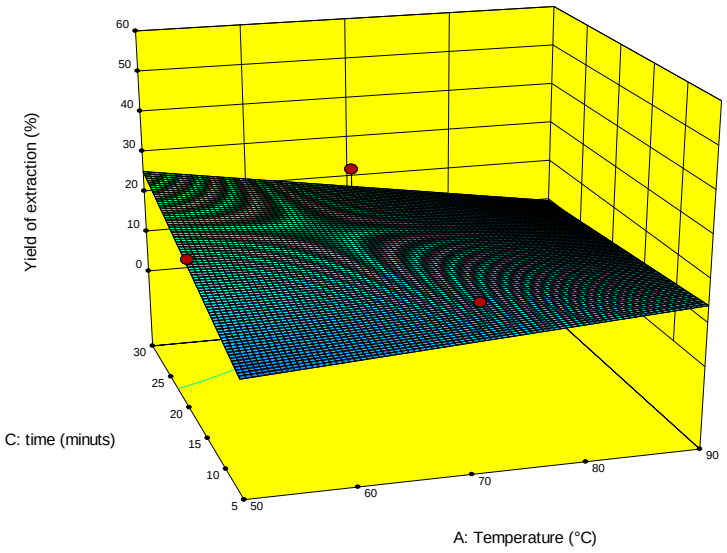

Figure 29: Yield of extraction for Silphie harvested in June for a ratio of 20 varying time and temperature for Silphie harvested in July

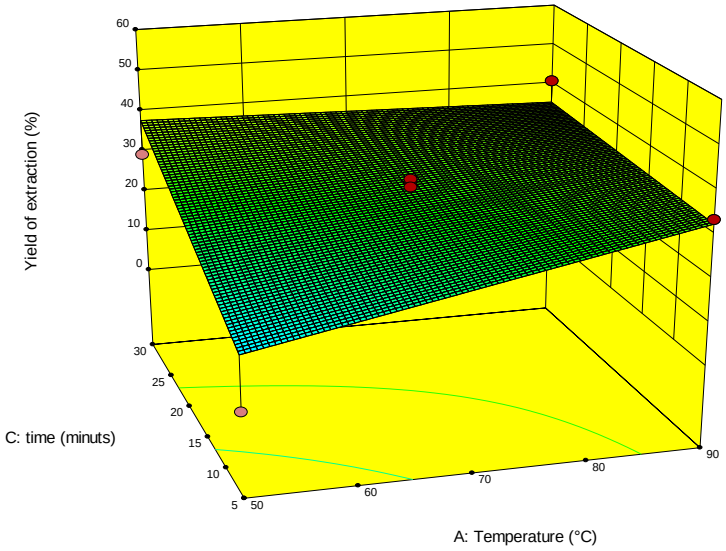

Figure 30: Yield of extraction for Silphie harvested in June for a ratio of 40 varying time and temperature for Silphie harvested in July

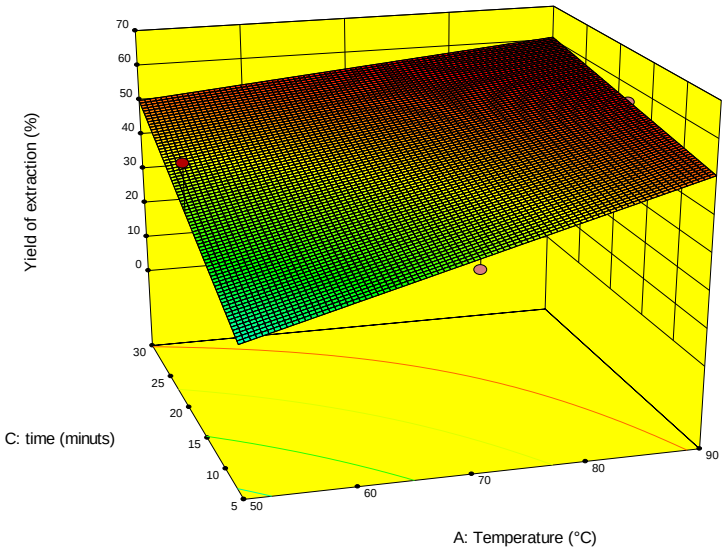

Figure 31: Yield of extraction for Silphie harvested in June for a ratio of 60 varying time and temperature for Silphie harvested in July

From the results (Figure 29, Figure 30 and Figure 31) it can be observed that there are some points that differ from the others such as the one of ratio 20, 17.5 minutes and 90ºC, the value is negative and the absolute value is really high (see value in the appendix Table 16) therefore, as in the previous experiment, was observed the same possible error for the samples with a ratio of 20. The mass of Silphie needed was higher for a ratio of 20 than for the other ratios. Consequently, the samples of a ratio of 20 needed more time to be completely dried than the other samples of ratio 40 and 60. In comparison with the experiment with Silphie harvested in June (Chapter 3.3.2), the yield of extraction seems to be more homogenous in the second experiment, this fact somehow makes sense due to the time of the harvesting; from the literature it is known that it is better to harvest the Silphie during the month of August, therefore July would be better harvesting than June.

Moreover, the value obtained for a ratio of 40, time of 17.5 minutes and 70ºC is an average of the five values measured in the experiment as was the central point; with a standard deviation of 31.6 ± 1.15.

Yield of free glucose and gructose

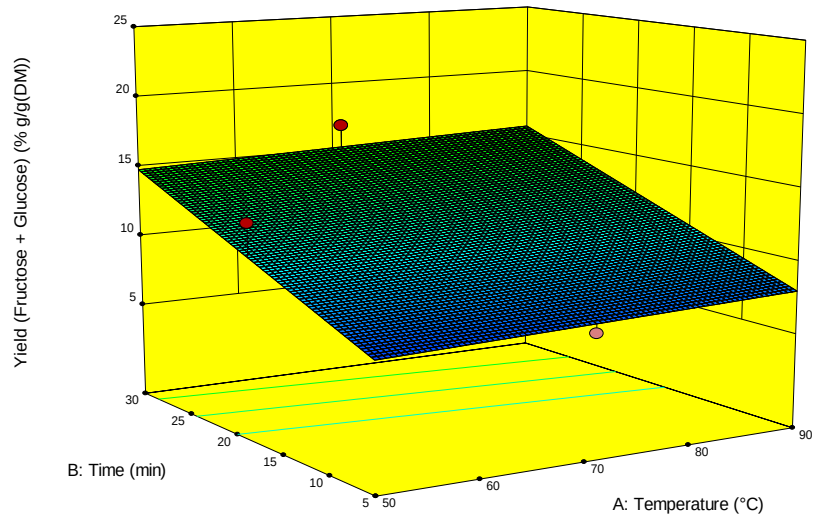

Figure 32: Yield of Glucose and Fructose for a ratio of 20 with varying time and temperature for Silphie harvested in July

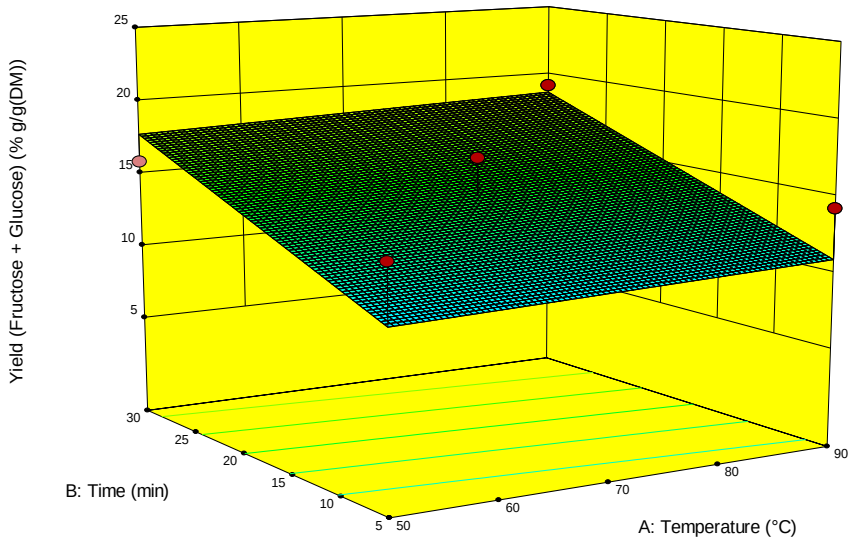

Figure 33: Yield of Glucose and Fructose for a ratio of 40 with varying time and temperature for Silphie harvested in July

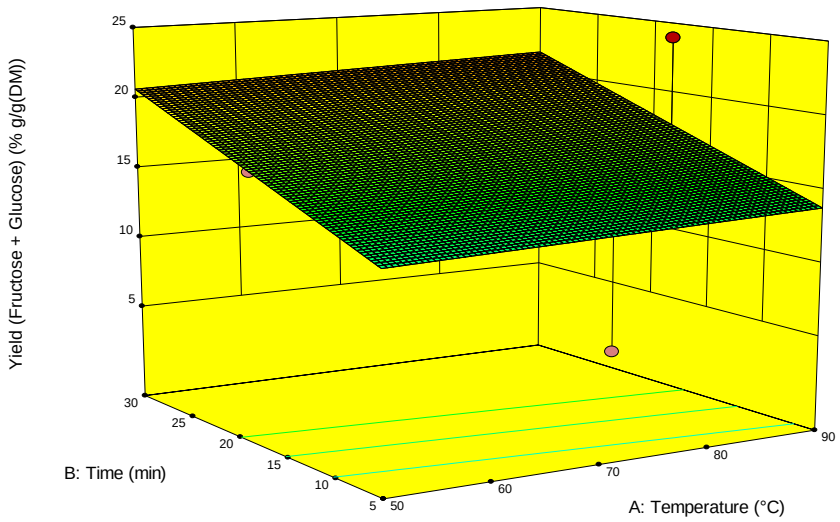

Figure 34: Yield of Glucose and Fructose for a ratio of 60 with varying time and temperature for Silphie harvested in July

In case of yield of fructose and glucose (Figure 32, Figure 33 and Figure 34), comparing with the experiment of Silphie harvested in June, it can be observed that when the yield changes so does the ratio, the higher the ratio the greater the yield. Also, in this case there is an influence between the time and the yield, it increases with the time.

Yield of fructans

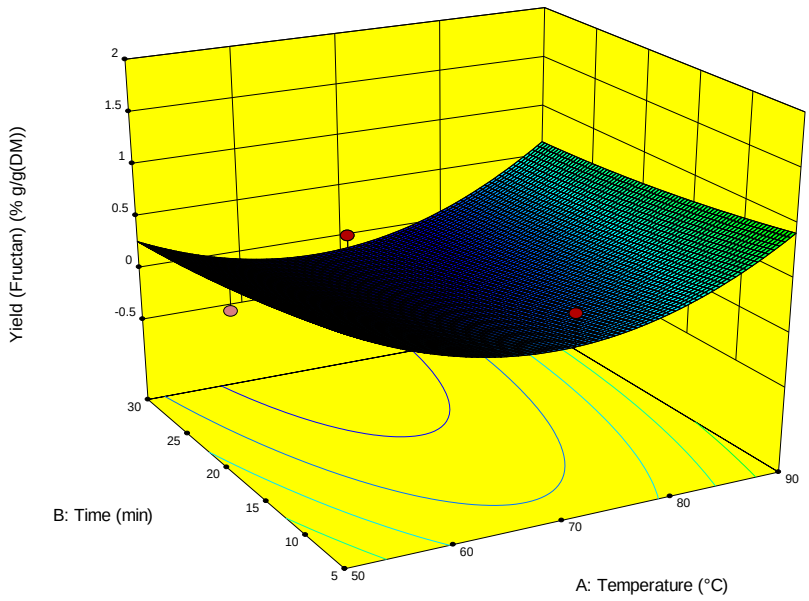

Figure 35: Yield of fructans for a ratio of 20 with varying time and temperature for Silphie harvested in July

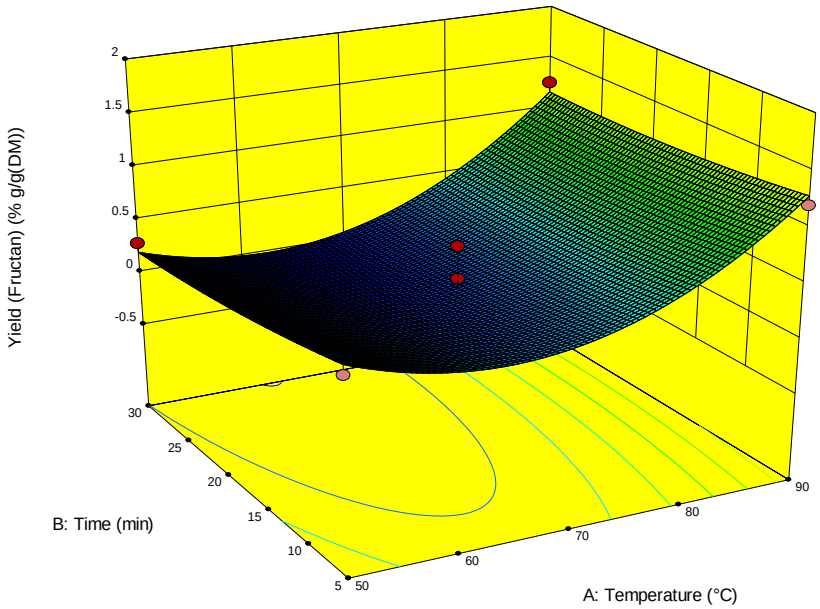

Figure 36: Yield of fructans for a ratio of 40 with varying time and temperature for Silphie harvested in July

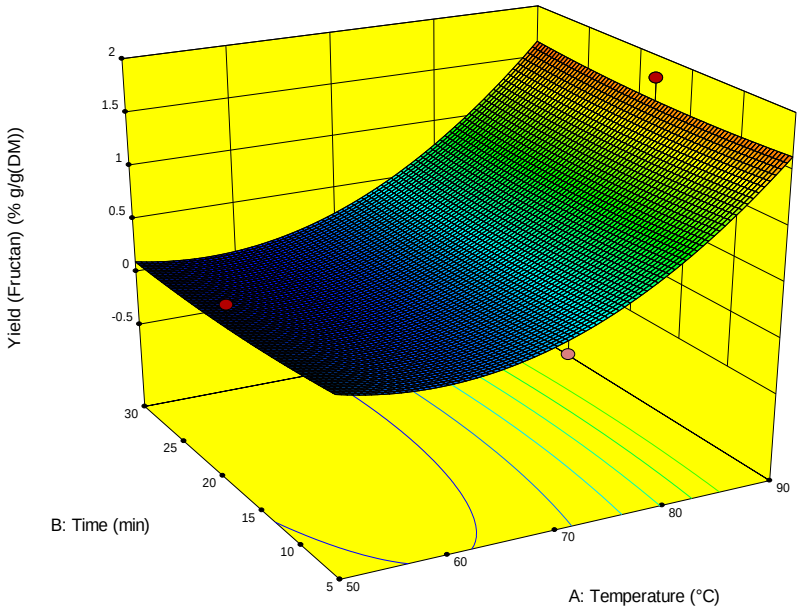

Figure 37: Yield of fructans for a ratio of 60 with varying time and temperature for Silphie harvested in July

The yield of fructans of this experiment (Figure 35, Figure 36 and Figure 37)differs from the experiment of Silphie harvested in June. It can be observed that the yield increases with the temperature and the ratio; the maximum point is found for a ratio of 60, and a temperature of 90ºC.

4.3.4 Extraction of Silphie harvest July 2019 with reduction of time

In this experiment was used the dried Silphie harvested in July 2019 as in the previous experiment (chapter 4.3.3) but with a reduction of the extraction time. The parameters were the ones show in the Table 3:

Table 3: Parameters for the with Silphie harvested in July and reduction of time

ratio	time	temperature
-	min	ºC
20	0.5	30
40	2.75	60
60	5	90

The time was reduced since it is not necessary that much time to perform the extraction of fructans; therefore, with a shorter time the other parameters can be better observed since not all the amount of sugars from the Silphie will be extracted due to the long extraction time. Moreover, the temperature range was extended in order to appreciate better its influence. The following results were obtained:

Total yield of extraction

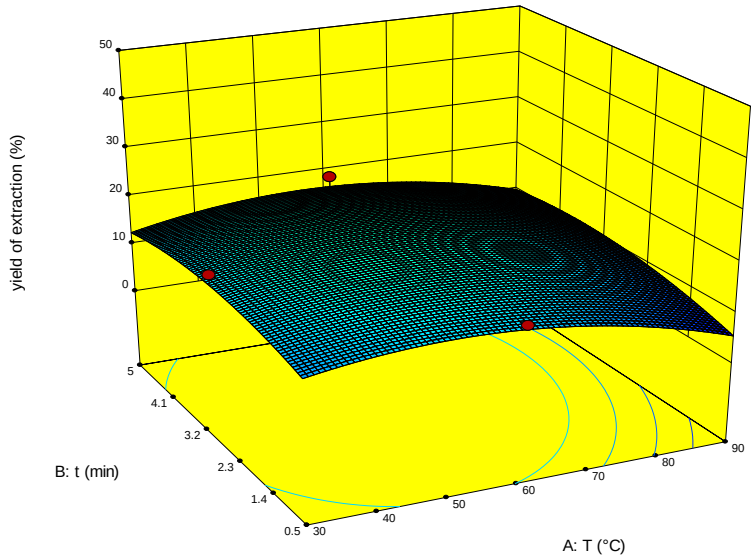

Figure 38: Yield of extraction for Silphie harvested in June for a ratio of 20 varying time and temperature for Silphie harvested in July and reduction of time

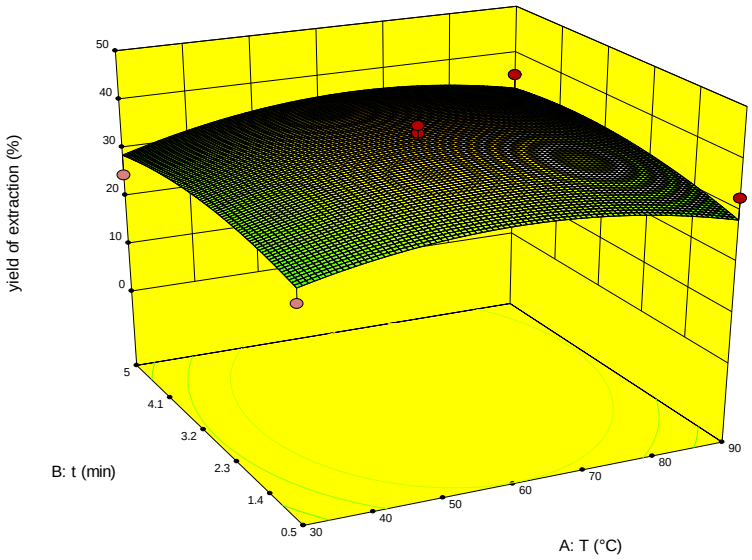

Figure 39: Yield of extraction for Silphie harvested in June for a ratio of 40 varying time and temperature for Silphie harvested in July and reduction of time

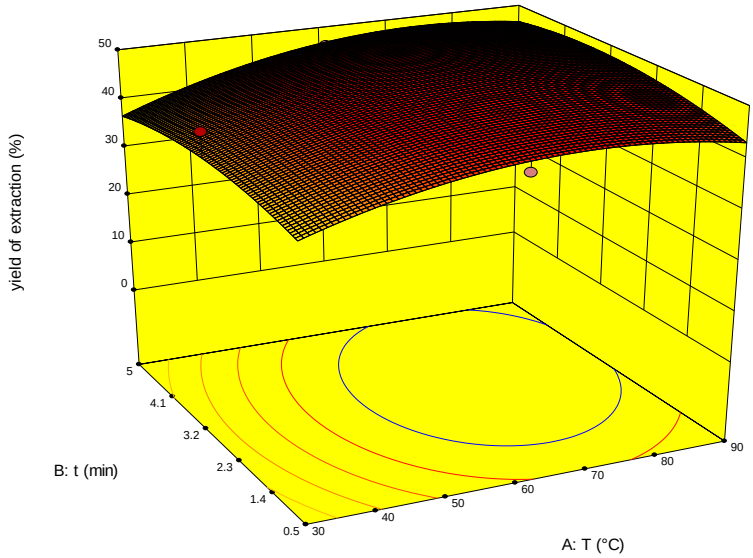

Figure 40: Yield of extraction for Silphie harvested in June for a ratio of 60 varying time and temperature for Silphie harvested in July and reduction of time

In case of total yield of extraction (Figure 38, Figure 39 and Figure 40), no negative values were obtained at any point. However, for the point of ratio 20, time 2,75 minutes and temperature of 90ºC the value was considerably lower than the others (see value in the appendix (Table 21). This might be due to the same error than for the previous experiments. The mass of Silphie needed was higher for a ratio of 20 than for the other ratios. Consequently, the samples of a ratio of 20 needed more time to be completely dried than the other samples of ratio 40 and 60.

Moreover, as in the other experiments the value obtained for a ratio of 40, time of 2,75 minutes and 60ºC is an average of the five values measured in the experiment because it was the central point; with a standard deviation of 37,8±2,35.

Yield of free glucose and gructose

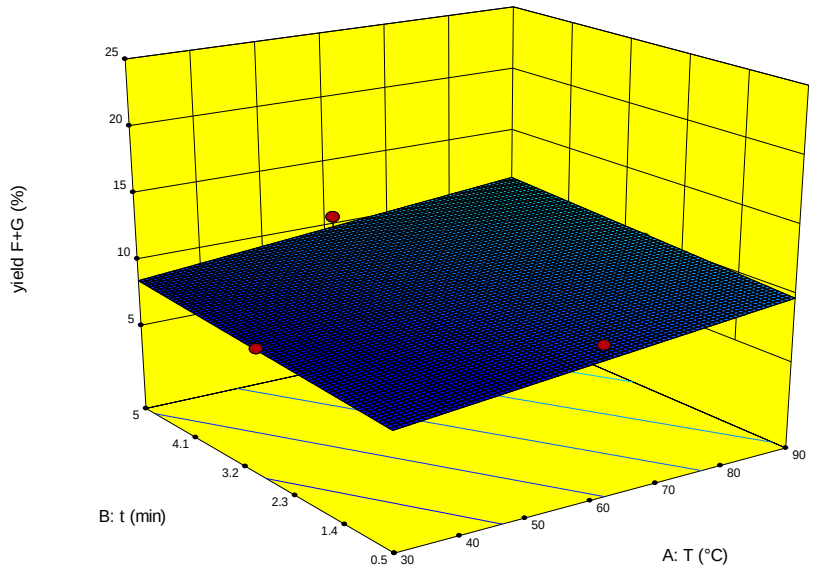

Figure 41: Yield of fructose and glucose for a ratio of 20 with varying time and temperature for Silphie harvested in July and reduction of time

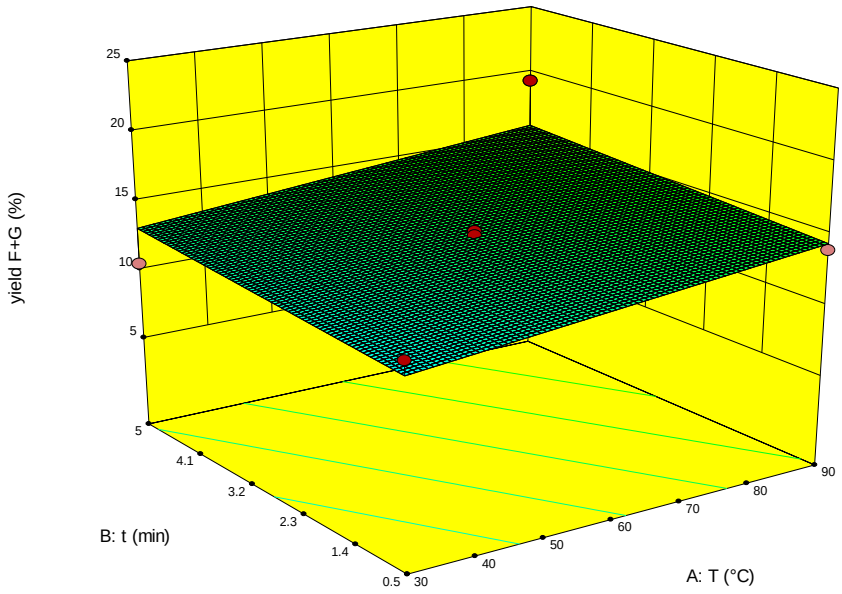

Figure 42: Yield of fructose and glucose for a ratio of 40 with varying time and temperature for Silphie harvested in July and reduction of time

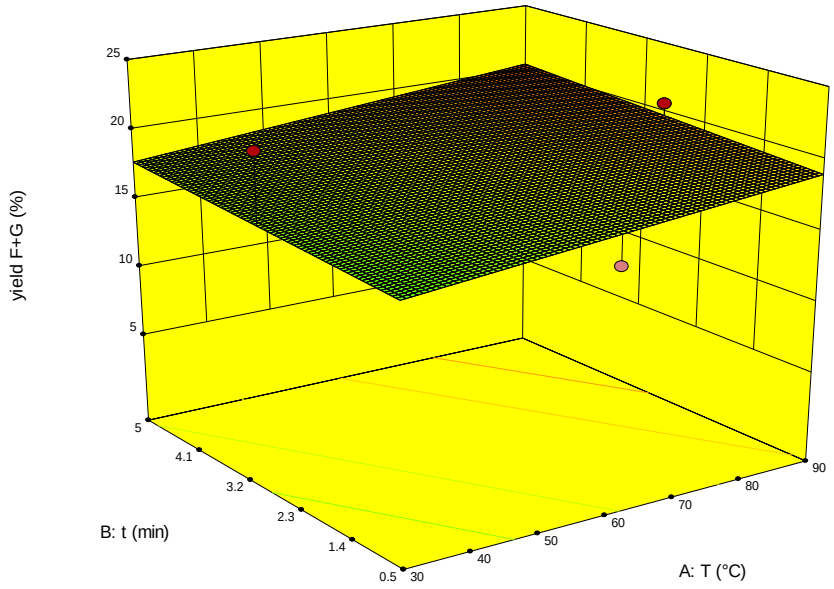

Figure 43: Yield of fructose and glucose for a ratio of 60 with varying time and temperature for Silphie harvested in July and reduction of time

The yield of fructose and glucose (Figure 41, Figure 42 and Figure 43), in comparison with the experiment of Silphie harvested in July without reduction of time, is not being influenced by the time but it is increasing when the ratio and the temperature do. Nevertheless, the differences between the different temperatures are not very significant and that makes sense, since, as explained above, the material is the same and the extraction for free monomers is easy, most of the glucose and fructose molecules are extracted.

Yield of fructans

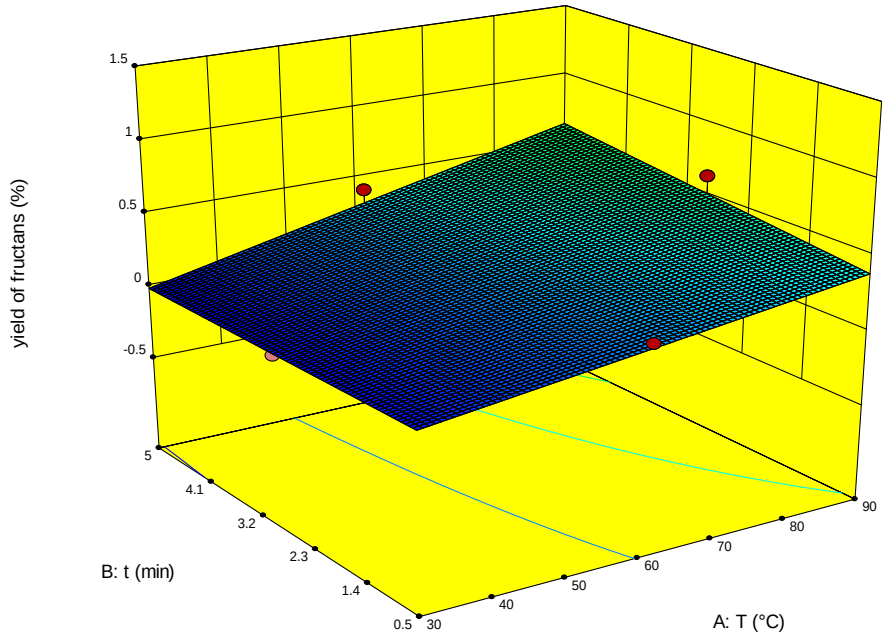

Figure 44: Yield of fructans for a ratio of 20 with varying time and temperature for Silphie harvested in July and reduction of time

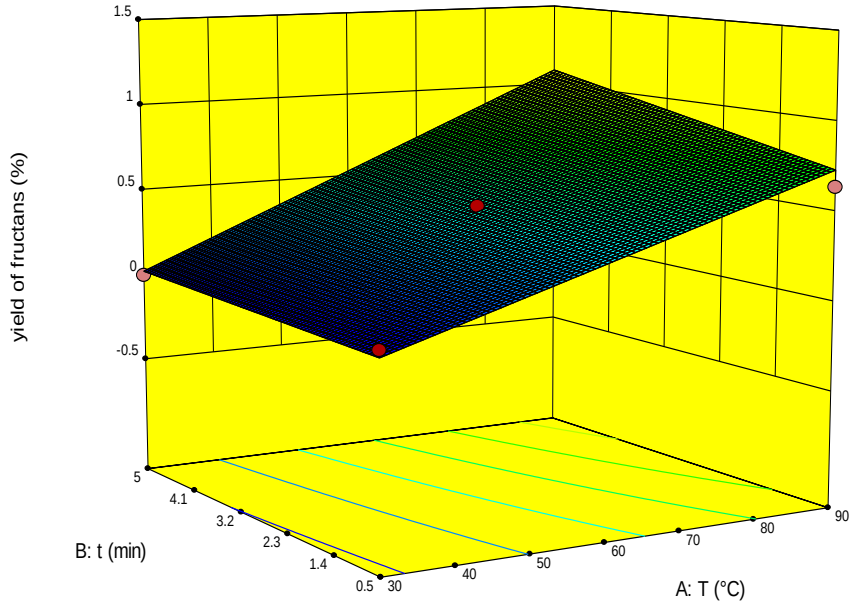

Figure 45: Yield of fructans for a ratio of 40 with varying time and temperature with varying time and temperature for Silphie harvested in July and reduction of time

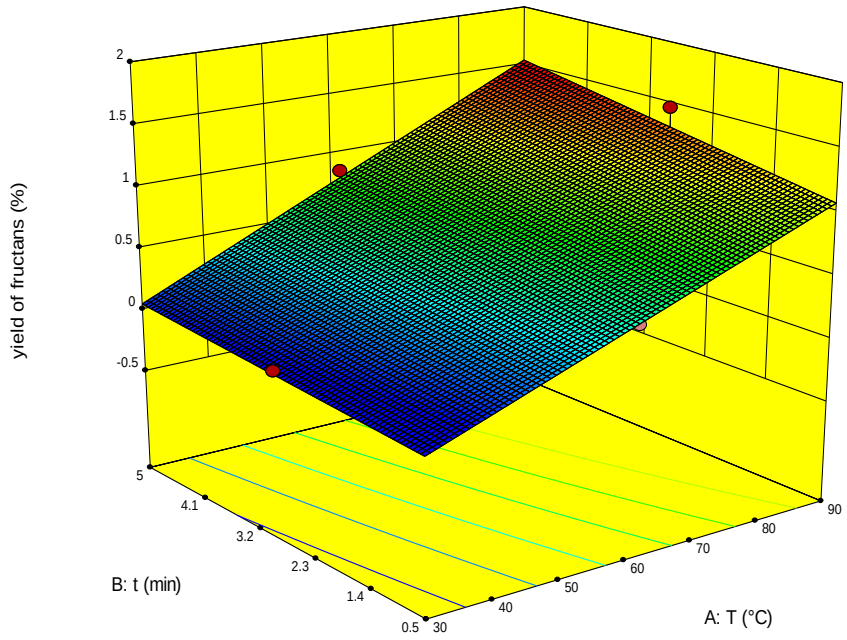

Figure 46: Yield of fructans for a ratio of 60 with varying time and temperature with varying time and temperature for Silphie harvested in July and reduction of time

In this case, the model is lineal not like in the other experiments but the yield of fructans also increases with the temperature and the ratio (Figure 44, Figure 45 and Figure 46), although as in the other experiments there is not a clear optimum point as was expected from the literature.

4.3.5 Extraction of Silphie harvest August 2019 with reduction of time

The experiment with Silphie harvested in July 2019 with reduction of time (chapter 4.3.4) was repeated for the dried Silphie harvested in the month of August 2019 in order to see if there were any differences regarding to the content of fructans. In this case, the total yield of extraction was not calculated due to some inconvenient during the experimental procedure. Thus, the same experimental plan as before was carried out and the following results were obtained:

Yield of free glucose and gructose

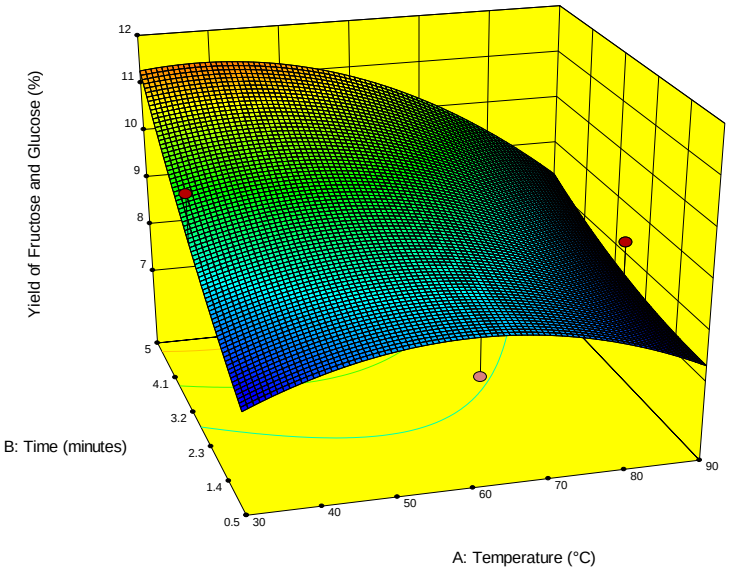

Figure 47: Yield of fructose and glucose for a ratio of 20 with varying time and temperature for Silphie harvested in August and reduction of time

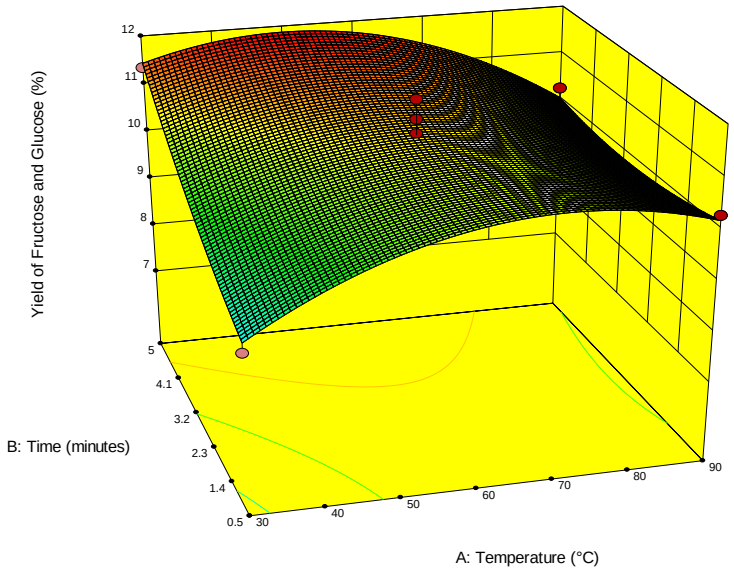

Figure 48: Yield of fructose and glucose for a ratio of 40 with varying time and temperature for Silphie harvested in August and reduction of time

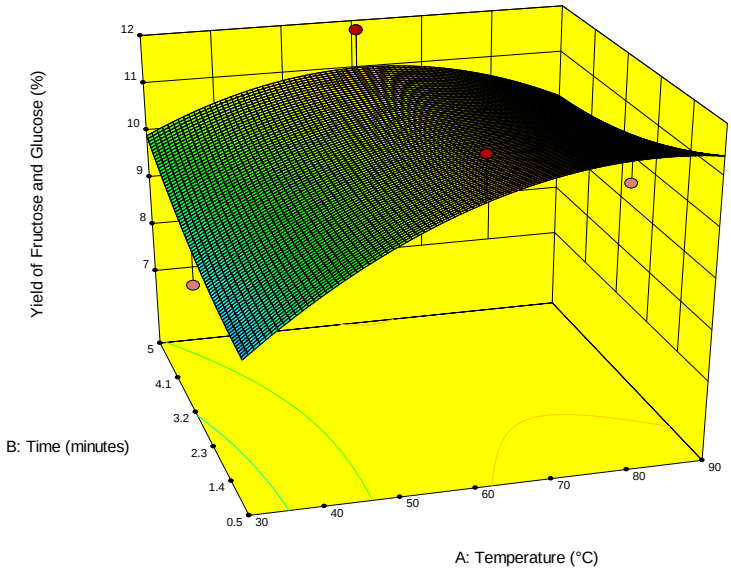

Figure 49: Yield of fructose and glucose for a ratio of 60 with varying time and temperature for Silphie harvested in August and reduction of time

The yield of fructose and glucose (Figure 47, Figure 48 and Figure 49), in comparison with the experiment of Silphie harvested in July with reduction of time, is not following any trend. This might be due to errors in the experimental procedure as the plant was not freeze-dried until 5 days since the Silphie was harvested and this can lead to errors. Also, due to the hot weather the yield from the Silphie that was used in the prior experiments suffered some degradation and therefore, for a temperature of 90 ºC was used Silphie from another yield.

Yield of fructans

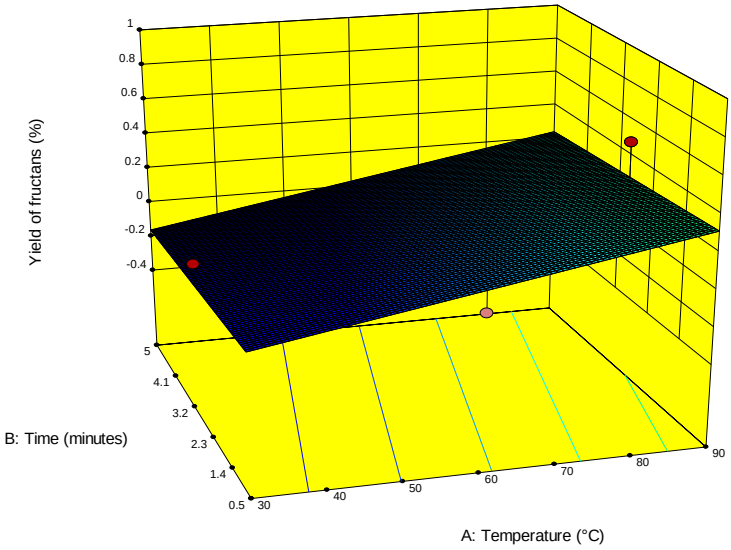

Figure 50: Yield of fructans for a ratio of 20 with varying time and temperature for Silphie harvested in August and reduction of time

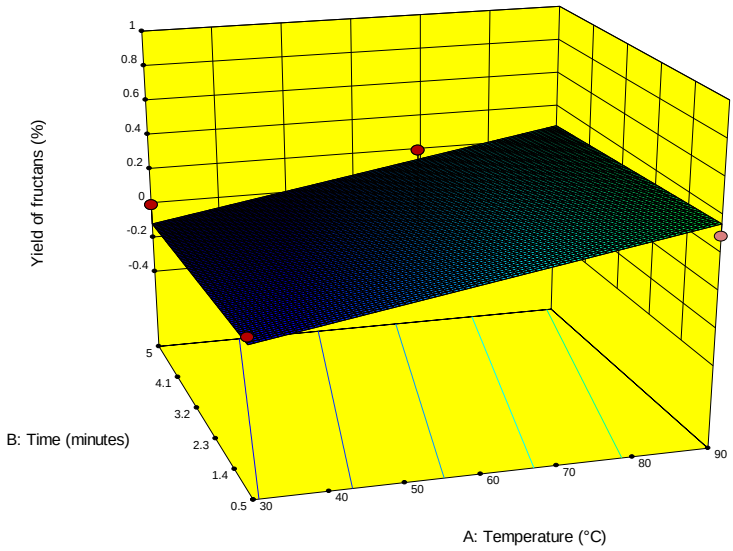

Figure 51: Yield of fructans for a ratio of 40 with varying time and temperature for Silphie harvested in August and reduction of time

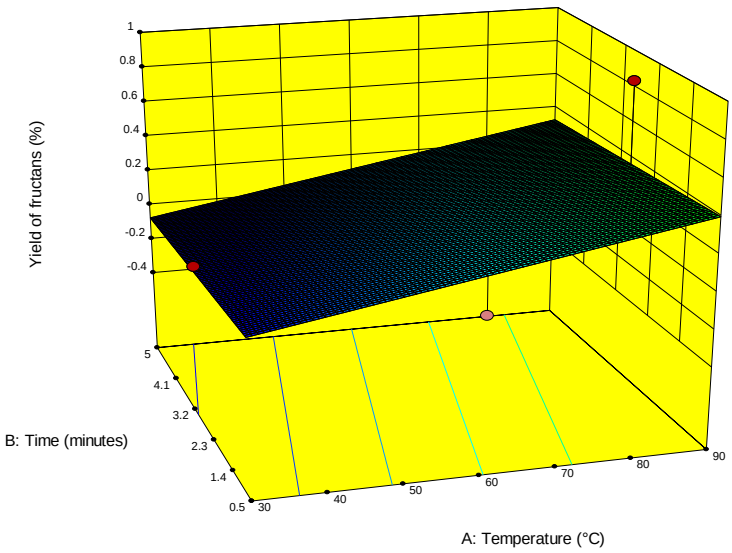

Figure 52: Yield of fructans for a ratio of 60 with varying time and temperature for Silphie harvested in August and reduction of time

In this case, the model is lineal but the yield of fructans has negative values (Figure 50, Figure 51 and Figure 52). Nevertheless, the yield increases with the temperature and with the ratio. In order to make more reliable this experiment should be repeated as the obtained values were not realistic and really different from the previous experiments. Although, it could also be influenced by the long time before freeze-drying the plant as explain above.

4.4 Discussion of errors

The obtained results were not as was expected to be from the theory, there was not an optimum point in the graphs. The graphs of the yield of fructans was expected to increase until a specific temperature (the optimum) and then decrease as in Figure 53 [31]

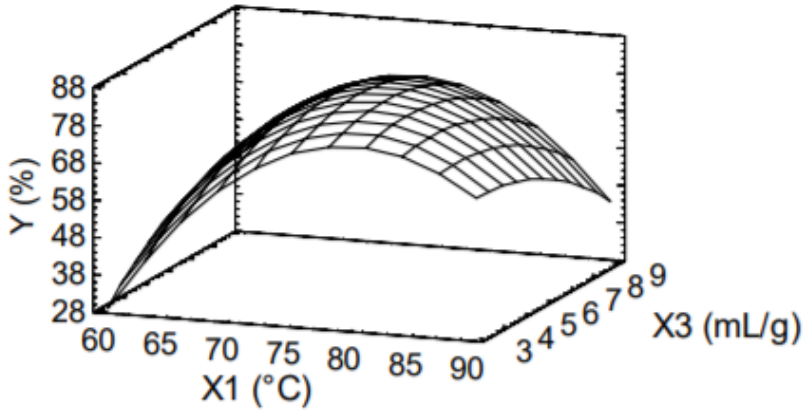

Figure 53: Representation of the expected yield of fructans varying the ratio and temperature [31]

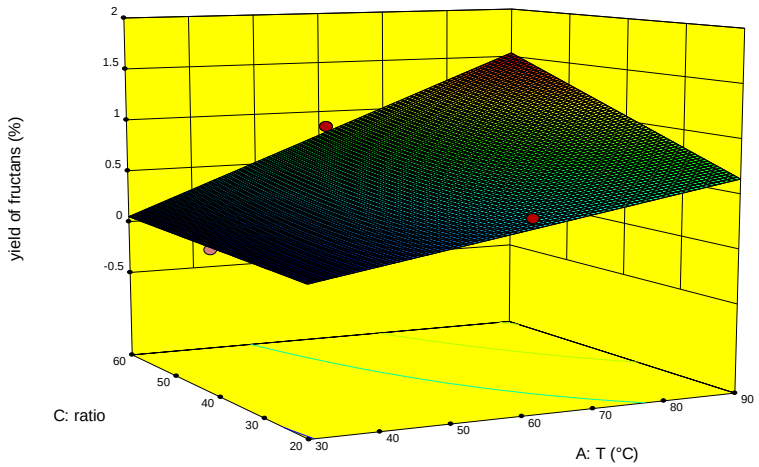

Figure 54: Representation of the obtained yield of fructans varying the ratio and temperature

This probably was not achieved due to different reasons as the material used for the extraction was biomass and it is not a homogeneous product so the results can differ a lot from each other and it was not separated the stems from the leaves and they probably have different fractions of components. Another potential reason might be the mixing, the sample was mixed during the extraction in the water bath and also before of introducing the sample to the water bath was shaken in a vortex mixer; however, maybe that was not enough and would have been better using another type of equipment as for example an ultrasonic equipment. Furthermore, Design of Experiments is also a main reason that can lead to an error as can be observed in the appendix in the Table 12, Table 17 and Table 22. ANOVA test, given by the program, in some points give a p-value not significant and a lack of fit significant, this can be because the experiments were measured just once, in exception of the central point, therefore, there might be an error preparing that sample and that point becomes a potential outliner and it could change all the model. To finish, another reason for error could be the experimental procedure, the extraction and the analysis in the HPLC; for example, to measure the weight of the samples without being completely dried for the calculation of the yield of extraction.

5 Conclusions

Fructans have been extracted from a plant called *Silphium perfoliatum*, which is typically used for biogas production, in order to make the process of the production of biogas more economical profitable. The extraction parameters temperature, extraction time and liquid-solid-ratio have been changed in order to study their influence on the extraction conditions.

Therefore, it can be concluded that for higher temperatures and higher liquid-solid-ratio the yield of fructans increases. However, the maximum point has not been obtained as was expected from the literature (Figure 53) The total yield of extraction obtained was around 45% (DM) while the yield of fructans was just 1.47% (DM). Therefore, just around the 3% of the total extraction are fructans.

In order to improve the results obtained and try to find an optimum point in a future, it would be necessary to repeat the experiments to make more reliable the experiments. However, the central point was repeated 5 times to be able to calculate the standard deviation. Also, it would be necessary to separate the stems from the leaves in the extraction as they might have different fractions of components and that can alter the results, however, the plant material used for the extraction was mixed and homogenized in order to have every sample with a similar composition. After this, it would be expected to decrease the standard deviation and then, be able to find the optimum parameters for the extraction of fructans from *Silphium perfoliatum*.

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7 Appendix

Table 4: Yield of extract for the enzyme deactivation

Yield of Extract in %			
time(min)	T=100ºC	Ethanol 80%	T=70ºC
15	27,56	26,74	23,93
30	26,75	26,44	23,09

Table 5: Yield of fructans for the enzyme deactivation

Yield_Fructan in %			
time(min)	T=100ºC	Ethanol 80%	T=70ºC
15	11%	8%	2,53%
30	10,66%	6,25%	8,62%

Table 6: Yield of extract for the deactivation of enzymes with boiling water

Yield of Extract in %	
time(min)	T=100ºC
0	-
0,5	23,45
1	37,44
5	28,96
10	34,79
15	27,61

Table 7: Yield of fructans for the deactivation of enzymes with boiling water

Yield_Fructan in %	
time(min)	T=100ºC
0	12,01
0,5	5,82
1	4,78
5	4,58
10	3,59
15	4,24

Table 8: Yield of extraction for different temperatures

T(ºC)	Yield of Extraction in %
20	27,33
20	21,80
40	26,93
40	30,55
60	27,24
60	30,34
80	31,11
80	33,34

Table 9: Yield of extraction for Silphie harvested in June for a temperature of 50ºC

Yield of extraction %			
T=50			
-	ratio 20	ratio 40	ratio 60
t=5 min	-	26,66	-
t=17,5 min	13,16	-	39,90
t=30 min	-	22,78	-

Table 10: Yield of extraction for for Silphie harvested in June for a temperature of 70ºC

Yield of extraction %			
T=70			
-	ratio 20	ratio 40	ratio 60
t=5 min	-172,45	-	31,96
t=17,5 min	-	25,10	-
t=30 min	19,05	-	30,17

Table 11: Yield of extraction for for Silphie harvested in June for a temperature of 90ºC

Yield of extraction %			
T=90			
-	ratio 20	ratio 40	ratio 60
t=5 min	-	31,57	-
t=17,5 min	18,69	-	31,96
t=30 min	-	26,48	-

Table 12: Anova test for fructans for the experiment 1

Anova test for fructans						
Source	Sum of squares	df	Mean square	F value	p-value (prob>F)	-
Model	23,66	9	2,63	2,37	0,1337	significant
A-T	0,89	1	0,89	0,8	0,4	
B-t	0,92	1	0,92	0,83	0,3933	
C-ratio	1,11	1	1,11	1	0,3508	
AB	0,01	1	0,01	0,009202	0,9263	
AC	6,72	1	6,72	6,06	0,0433	
BC	0,21	1	0,21	0,19	0,6792	
A^2	13,53	1	13,53	12,21	0,0101	
B^2	0,12	1	0,12	0,11	0,7517	
C^2	0,051	1	0,051	0,046	0,8355	
Residual	7,75	7	1,11			
Lack of fit	7,3	3	2,43	21,67	0,0062	not significant
Pure error	0,45	4	0,11			
Cor total	31,42	16				

Table 13: Anova test for fructose and glucose for the experiment 1

Anova test for fructose and glucose						
Source	Sum of squares	df	Mean square	F value	p-value (prob>F)	-
Model	2144,34	9	238,26	4,75	0,026	significant
A-T	731,76	1	731,76	14,59	0,0065	
B-t	26,92	1	26,92	0,54	0,4875	
C-ratio	152,09	1	152,09	3,03	0,1251	
AB	0,96	1	0,96	0,019	0,8937	
AC	699,44	1	699,44	13,95	0,0073	
BC	18,54	1	18,54	0,37	0,5624	
A^2	489,71	1	489,71	9,77	0,0167	
B^2	20,99	1	20,99	0,42	0,5383	
C^2	7,02	1	7,02	0,14	0,7193	
Residual	351	7	50,14			
Lack of fit	343,95	3	114,65	65,07	0,0008	significant
Pure error	7,05	4	1,76			
Cor total	2495,35	16				

Table 14: Yield of extraction for the second experiment for a temperature of 50ºC

Yield of extraction %			
T=50			
-	ratio 20	ratio 40	ratio 60
t=5 min	-	-6,50	-
t=17,5 min	18,46	-	47,68
t=30 min	-	29,41	-

Table 15: Yield of extraction for the second experiment for a temperature of 70ºC

Yield of extraction %			
T=70			
-	ratio 20	ratio 40	ratio 60
t=5 min	19,97	-	31,68
t=17,5 min	-	31,56	-
t=30 min	22,15	-	48,33

Table 16: Yield of extraction for the second experiment for a temperature of 90ºC

Yield of extraction %			
T=90			
-	ratio 20	ratio 40	ratio 60
t=5 min	-	33,60	-
t=17,5 min	-115,85	-	55,55
t=30 min	-	40,81	-

Table 17: Anova test for fructans for the experiment 2

Anova test for fructans						
Source	Sum of squares	df	Mean square	F value	p-value (prob>F)	-
Model	3,57	9	0,4	4,24	0,035	significant
A-T	1,45	1	1,45	15,54	0,0056	
B-t	0,11	1	0,11	1,16	0,3168	
C-ratio	0,14	1	0,14	1,49	0,2623	
AB	0,013	1	0,013	0,13	0,7249	
AC	0,34	1	0,34	3,64	0,0982	
BC	0,025	1	0,025	0,26	0,6233	
A^2	1,46	1	1,46	15,59	0,0055	
B^2	0,012	1	0,012	0,13	0,7294	
C^2	0,0003681	1	0,0003681	0,003939	0,9517	
Residual	0,65	7	0,093			
Lack of fit	0,42	3	0,14	2,33	0,2164	not significant
Pure error	0,24	4	0,06			
Cor total	4,22	16				

Table 18: Anova test for fructose and glucose for the experiment 2

Anova test for Glucose and fructose						
Source	Sum of squares	df	Mean square	F value	p-value (prob>F)	-
Model	191,07	3	63,69	3,98	0,0325	significant
A-T	1,72	1	1,72	0,11	0,7481	
B-t	121,13	1	121,13	7,58	0,0165	
C-ratio	68,21	1	68,21	4,27	0,0594	
Residual	207,86	13	15,99			
Lack of fit	169,79	9	18,87	1,98	0,266	not significant
Pure error	38,07	4	9,52			
Cor total	398,93	16				

Table 19: Yield of extraction for the third experiment for a temperature of 30ºC

Yield of extraction %			
T=30			
-	ratio 20	ratio 40	ratio 60
t=0,5 min	-	25,98	-
t=2,75 min	17,25	-	44,71
t=5min	-	24,77	-

Table 20: Yield of extraction for the third experiment for a temperature of 60ºC

Yield of extraction %			
T=60			
-	ratio 20	ratio 40	ratio 60
t=0,5 min	14,76	-	43,26
t=2,75 min	-	37,80	-
t=5min	18,54	-	46,10

Table 21: Yield of extraction for the third experiment for a temperature of 90ºC

Yield of extraction %			
T=90			
-	ratio 20	ratio 40	ratio 60
t=0,5 min	-	32,74	-
t=2,75 min	5,63	-	45,60
t=5min	-	35,39	-

Table 22: Anova test for fructans for the experiment 3

Anova test for fructans						
Source	Sum of squares	df	Mean square	F value	p-value (prob>F)	-
Model	2,28	6	0,38	34,12	<0,0001	significant
A-T	1,7	1	1,7	152,68	<0,0001	
B-t	0,089	1	0,089	8	0,0179	
C-ratio	0,29	1	0,29	25,64	0,0005	
AB	0,022	1	0,022	1,98	0,1896	
AC	0,17	1	0,17	15,48	0,0028	
BC	0,011	1	0,011	0,94	0,3542	
Residual	0,11	10	0,011			
Lack of fit	0,098	6	0,016	5,04	0,0697	Not significant
Pure error	0,013	4	0,003251			
Cor total	2,39	16				

Table 23: Anova test for fructose and glucose for the experiment 3

Anova test for Glucose and fructose						
Source	Sum of squares	df	Mean square	F value	p-value (prob>F)	-
Model	191,85	3	63,95	12,79	0,0004	significant
A-T	15,48	1	15,48	3,1	0,102	
B-t	4,41	1	4,41	0,88	0,3648	
C-ratio	171,96	1	171,96	34,38	<0,0001	
Residual	65,01	13	5			
Lack of fit	60,57	9	6,73	6,06	0,0491	significant
Pure error	4,44	4	1,11			
Cor total	256,87	16				

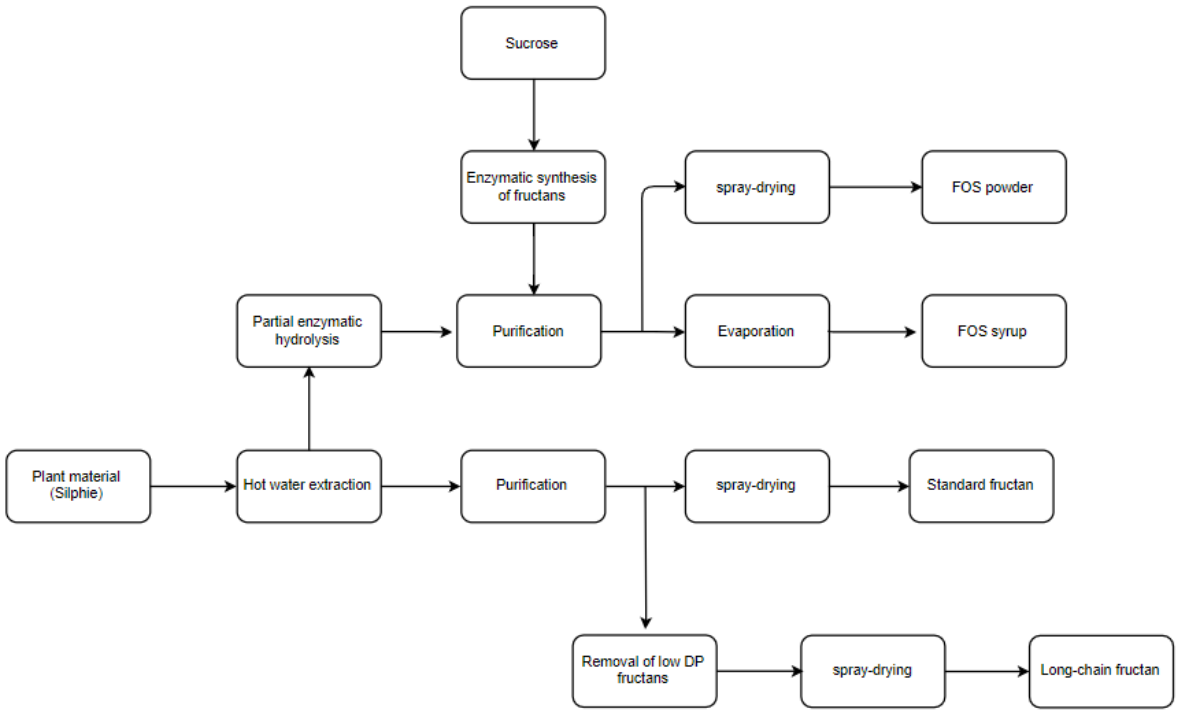

Figure 55: Diagram of fructan production

calculated by **Friedrich Asen** (AELF Bayreuth, section agriculture) and **Pedro Gerstberger** (University of Bayreuth, Department of plant ecology)

	Silomaize	Silomaize	Silomaize	Cup-plant	Cup-plant	Cup-plant
Yield	minor yield	medium yield	high yield	minor yield	medium yield	high yield
				2 x watering in first year		
years of production	annual			15 years of production		
fresh biomass tons/ha	43.5	50.0	58.0	46.5	53.5	62.0
dry matter (DM) %	31	31	31	29	29	29
yield DM tons /ha netto	13.5	15.5	18	13.5	15.5	18
variable cost of production:						
seeds / plantlets	281	281	281	213	213	213
fertilization						
Nitrogen	182	197	210	109	122	137
Phosphorus	73	84	98	24	29	33
Potassium	195	224	260	194	226	259
plant pest protection	79	79	79	4	4	4
variable engine costs incl. harvesting	599	662	739	511	577	658
costs of silage	42	48	56	45	52	60
insurance	22	23	24	20	21	22
sum of variable costs	1474	1599	1748	1120	1245	1387
+ subsidies	45	45	45	45	45	45
+ subsidies (Bavaria)						
+ subsidies (European Union)	300	300	300	300	300	300
variable costs with subsidies	1129	1254	1403	775	900	1042
hours of manpower/ha	9.3	9.5	9.7	5.2	5.4	5.6
variable cost recovery/hours of manpower	121	132	145	149	167	186
+ correction methane yield				320	320	320
- correction reduction of man power				60	60	60
+ correction loss of subsidies of first year				35	35	35
final costs €/ha	1129	1254	1403	1070	1195	1337

Figure 56: Profitability of biogas production for Maize and Silphie