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Optimization of Inulin Extraction from Chicory Roots and an Ultrafiltration Application to Obtain Purified Inulin and Hydrolyzed Fructooligosaccharides

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ABSTRACT
Inulin and fructooligosaccharides (FOS) are prominent functional components in the food industry due to prebiotic and other pharmaceutical properties. Inulin is a storage polysaccharide in various plants. FOS are naturally present in various plants and can be obtained by partial hydrolysis of inulin. In this study, ground and sieved chicory roots (*Cichorium intybus* L.) were used as starting material for inulin extraction under optimized conditions determined by Response Surface Methodology (RSM) with a Box-Behnken design. Optimum inulin extraction conditions from chicory roots were; temperature of 90 °C, extraction time of 30 min., and liquid-to-solid (LS) ratio of 10:1 mL/g. Inulin extract was further hydrolyzed to FOS by enzymatic or acid treatment, separately. Purification of inulin extract and FOS hydrolysate was performed by ultrafiltration with a 10 kDa membrane under the pressure of 2 bar with continuous stirring. As a result, inulin and FOS were obtained at 90% and 76% purity, respectively.

Keywords: *Cichorium intybus*; fructooligosaccharides, Response surface methodology, HPLC-RID analysis, Acid-enzyme hydrolysis, Membrane filtration

1. Introduction

The chicory plant *(Cichorium intybus* L.*)* is a natural source of inulin from the fresh roots and can grow in many different climates (Chandra et al. 2016; El-Kholy et al. 2020). Inulin is a biological macromolecule composed of a linear polymer of D-fructose with β-(2-1) glycosidic linkages, and a terminal glucose moiety which is linked to fructosyl unit via α-(1-4) linkage (Öztürk 2016; Fu et al. 2018; Singh et al. 2020a). The polymerization degree (DP) of inulin varies between 2-60 according to species, harvest time, and time of storage. Fructooligosaccharides (FOS) are fructans with DP<10 that naturally exist in various plants such as chicory, Jerusalem artichoke, yacon, and burdock (Li et al. 2013; Singh et al. 2020c; Redondo-Cuenca et al. 2021; Stökle et al. 2023). Additionally, FOS can be obtained by transfructosylation of sucrose by β-fructofuranosidases or by partial enzymatic hydrolysis of inulin using endoinulinases acting on internal β-(2, 1) glycosidic linkages (Beirão-Da-Costa et al. 2009; Li et al. 2013; Khuenpet et al. 2017). Transfructosylation by β-fructofuranosidases forms small chain length (2-4 fructosyl units) FOSs, whereas hydrolysis of inulin using endoinulinases forms long chain length (1-9 fructosyl units) FOSs (Singh et al. 2020a).

In the food industry, chicory stands out due to its content of inulin and derivative FOS and is a natural food ingredient. Both inulin and fructooligosaccharides have prebiotic activity that can promote probiotic growth, especially *Bifidobacteria*. Inulin also has pharmaceutical effects such as modulation of intestinal microbiota, anti-cancer and antioxidant activity, immunological enhancement, antidiabetic effect, hepatoprotective activity, and many others (Li et al. 2013; Dominguez et al. 2014; Fu et al. 2018; Perović et al. 2021). In addition to its serum cholesterol reducing and pathogenic bacteria inhibitory effects, FOS has cerebral protective activity and improves cognition (Li et al. 2013; Dominguez et al. 2014; Fu et al. 2018; Perović et al. 2021). Inulin and FOS are used as sugar and fat replacers in low-calorie foods to decrease obesity and diabetes risks (Dominguez et al. 2014; Khuenpet et al. 2017; Beirão-Da-Costa et al. 2009). Mostly they are preferred in desserts, infant formula, baked products, and fermented dairy products (Lopes et al. 2017; Kralj et al. 2018; Redondo-Cuenca et al. 2021). Inulin is used as dietary fiber,

gel-forming, and viscosity regulator agent in the food industry (El-Kholy et al. 2020). Due to these properties, food and pharmaceutical industries have utilized inulin and FOS for functional foods, nutritional composites, and drugs (Singh et al. 2020a; Perović et al. 2021; Singh & Singh 2022a).

Inulin can be obtained by extraction of plant materials such as chicory, Jerusalem artichoke, salsify, and dahlia, and by biosynthesis pathways (Beirão-Da-Costa et al. 2009; Apolinário et al. 2014; Kralj et al. 2018). According to Barclay et al. (2014), inulin is synthesized from sucrose by a polymerization reaction that transfers fructose from another sucrose molecule with catalytic enzymes in plants. Differing from this pathway, Apolinario et al. (2014) proposed distinctive biosynthetic pathway models in detail. FOS was obtained from various plants by partial enzymatic hydrolysis of inulin or sucrose transfructosylation by using fungal fructosyltransferases (Ávila-Fernández et al. 2011; Dominguez et al. 2014; Muñiz-Márquez et al. 2019). Also, FOS production from inulin by acidic hydrolysis was demonstrated in a previous study with an alternative methodology (Grzybowski et al. 2014).

Extraction is an important step for inulin production and hot water extraction is the method of choice due to the water-soluble property of inulin (Khuenpet et al. 2017). Extraction time and temperature are critical factors as they can cause either transfer of inulin from plant to water or degradation of inulin to reducing sugars. For this reason, extraction parameters should be selected properly. In the conventional method, to observe the effect of each experimental factor, one variable at a time is changed but this approach is time consuming and labor intensive (Singh et al. 2020c). To deal with these problems, the response surface methodology (RSM) technique is used for optimization processes. The Box Behnken experimental design (BBD) in RSM was used in this study for the optimization of process variables for inulin extraction from chicory roots.

In addition to extraction procedures, a further purification step is necessary to obtain high-purity ingredients for the food and pharmaceutical industries. To remove the impurities such as reducing sugars, high molecular weight carbohydrates, proteins, and fibers from crude inulin and FOS extracts, some physicochemical methods and chromatographic techniques were described previously (Li et al. 2004; 2013; Apolinário et al. 2014). However, recent studies focused on alternative membrane technology for the purification of these components. The membrane separation technique stands out with advantages of high productivity, low operation costs, and high product quality (Barclay et al. 2014; Muñiz-Márquez et al. 2019). In previous studies of inulin purification using membrane filtration, membranes with 5-50 kDa pore size were used with continuous stirring under 1-2.6 bar pressure to prevent membrane clogging (Zhu et al. 2018; Murtiningrum et al. 2020). In the studies of FOS purification, 1-10 kDa membrane filters were used (Qing et al. 2013). More studies are required to determine the effect of different separation applications during the extraction of inulin from its plant source. From this perspective, this study aimed to discover the most efficient and easiest way of obtaining inulin and FOS from chicory roots. This study explains how to obtain two different end products step by step in detail, starting from the chicory plant. At first, the inulin extraction conditions from chicory roots were optimized by the BBD in RSM. Inulin extract was obtained with these optimized conditions and then hydrolyzed to FOS by acid or enzyme treatment. A membrane filtration system was used to maximize the purity of inulin and FOS separately. Also, total carbohydrate and total protein content were investigated before and after filtration to calculate the purity and efficiency of the filtration system. At the end of the study, two different end products with relatively high purity were obtained from chicory. Considering that there is no extensive information about the purification of inulin and FOS using a membrane filtration system in the literature, and that this purification is generally performed by chromatographic techniques, this study is an example of the feasibility of a membrane filtration system for inulin and FOS purification and will be a pioneer for more detailed studies.

2. Material and Methods

2.1. Chicory root samples

The chicory roots were collected from Isparta province (located in the western Mediterranean region) in Türkiye during the flowering stage. The chicory roots were identified by Prof. Dr. Huseyin Fakir, Director of the Herbarium Section, Applied Sciences University of Isparta, Türkiye. The roots were conventionally dried (final moisture content was 7.5%), cleaned of soil, and ground, sieved (1 mm), and stored at 4 ºC until use.

2.2. Chemicals

The endo-inulinase enzyme from *Aspergillus niger* (300 U/mL, 60 ºC, 4.5 pH) was purchased from Megazyme, USA. Commercial inulin from chicory, that was kindly provided by Artisan Gıda (Türkiye), was a product of Orafti (Belgium). Glucose, fructose, sucrose, 1-kestose (GF_2), and nystose (GF_3) standards of analytical grade were purchased from Sigma-Aldrich (St. Louis, MO, USA). 1^F-fructofuranosylnystose (GF_4) analytical standard was purchased from Novagentek (England). All other reagents and solvents were of analytical reagent grade. All aqueous solutions were prepared using deionized water.

2.3. Experimental design

Design Expert-13 was used to optimize the extraction conditions of inulin from chicory roots. In RSM, a 3-factor BBD was used. Independent variables were temperature (ºC, A), time (min, B), and liquid-to-solid ratio (mL/g, C). The dependent variable was

inulin content (g/100 g dry weight). Independent variables were defined according to our preliminary trials and optimum extraction conditions in the literature (Dobre et al. 2008; Beirão-Da-Costa et al. 2009; Apolinário et al. 2014; Tewari et al. 2015; Balzarini et al. 2018; Başaran et al. 2018).

The complete design consisted of 18 experimental points (including six replicates of the center point), and the experiments were performed randomly. Experimental data were fitted to a quadratic polynomial model (1) to obtain regression coefficients.

$$Z = \beta_0 + \sum_{i=1}^{3} \beta_i x_i + \sum_{i=1}^{3} \beta_{ii} x_i^2 + \sum_{i=1}^{2} \sum_{j=1}^{3} \beta_{ij} x_i x_j \quad (1)$$

Where; Z was the dependent variable to be modeled, x_i and x_j were the independent variables, β_0 was the constant coefficient, β_i was the coefficient of linear effect, β_{ii} was the coefficient of quadratic effect, and β_{ij} was the coefficient of interaction effect.

2.4. Extraction of chicory roots

Inulin extractions were performed by using 2 g chicory root samples (described above). The calculated ratio of hot water was added to each sample. Water temperature of 60-90 °C, extraction time of 30-90 min, and liquid-to-solid ratio (water: root sample) of 10:1-30:1 v/w were used as extraction conditions. Extractions were carried out in a water bath running at the extraction temperature with 100 rpm shaking. After extraction, chicory root extract was filtered firstly through cotton cheesecloth and then centrifuged at 1400 rpm for 10 min. Supernatants of each extract were stored at -20 ºC until analysis.

2.5. HPLC-RID analysis of inulin and FOS

Inulin and FOS content determination was performed with an HPLC (High Pressure Liquid Chromatography) Shimadzu (Kyoto, Japan) system (LC-20A pump, SIL-20A HT autosampler) combined with a refractive index detector (RID-20A). Inulin separation was performed on an Inertsil-NH_2 column (5µm, 4.6 × 250 mm). The measurements were performed at a column temperature of 80 °C and detector temperature of 60 °C, using deionized water as mobile phase at flow rate of 1 mL/min and injection volume of 20 µL (Başaran et al. 2018).

FOS determination on HPLC was performed according to the methods of Nobre et al. (2018) and Muñiz-Márquez et al. (2019). 1-kestose (GF_2), 1-nystose (GF_3), and 1^F-fructofuranosylnystose (GF_4) were used as FOS standards, and glucose, fructose, and sucrose were also analyzed as residual sugars. Total FOS content was calculated as the sum of GF_2, GF_3, and GF_4. For the quantification of FOS and sugars, calibration curves were obtained with known concentrations of each compound. Samples were analyzed with a ReproSil Carbohydrate-Plus column (5µm, 4.6 × 250 mm) at 30 °C. The operating temperature of the refractive index detector (RID) was 35 ºC. A mixture of acetonitrile/water 80:20 (v/v) was used as the mobile phase at flow rate of 1 mL/min. Before analyses, all samples were filtered through a 0.45 µm polytetrafluoroethylene (PTFE) filter.

2.6. Hydrolysis of inulin to FOS

The enzymatic hydrolysis of inulin was carried out following the methods of Ricca et al. (2009), Yi et al. (2010) and Sarchami & Rehmann (2014) with slight modifications. Inulin extract, obtained in optimum extraction conditions according to our experimental design, was used as substrate for hydrolysis. Enzymatic hydrolysis was performed for FOS production in different experimental conditions according to the literature. These experimental conditions are given in Table 1. The appropriate enzyme concentration that gives the highest FOS yield was selected through our preliminary studies.

Table 1- Conditions for acid (A) and enzymatic (E) hydrolysis

Sample Name	*Temperature (°C)*	*Time (min)*	*Buffer volume (mL)**	*pH***
A-1	85	15	-	2.5
A-2	85	25	-	2.5
E-1	40	60	-	5.28
E-2	40	60	4	4.50
E-3	40	30	4	4.50
E-4	60	60	-	5.28
E-5	60	60	4	4.50
E-6	60	30	4	4.50

*: 0.05M sodium acetate was used as a buffer; **: 0.5 M citric acid was used in pH adjustment

For enzyme hydrolysis, 0.2 mL of the endo-inulinase enzyme (60 U) was added to 10 mL of inulin extract at room temperature and incubated in a water bath at the temperature and time in Table 1 with 100 rpm shaking. The hydrolysates were left at 100 °C for 15 min to inactivate the enzyme and cooled immediately for later study.

To compare the results, acid hydrolysis was also carried out following the method of Grzybowski et al. (2014). For acid hydrolysis, 10 mL of inulin extract, obtained in optimum conditions, was used. The pH value of each extract was adjusted to 2.5 with 0.5 M citric acid solution. Acid hydrolysis was performed in a 100 rpm shaking water bath at temperatures and times given in Table 1. At the end of the relevant period, the hydrolysates were cooled rapidly and stored at -20 °C.

2.7. Purification by membrane filtration

To separate high molecular weight sugars, proteins, and other impurities from inulin and FOS extracts and to obtain high-purity products, the ultrafiltration technique was used. Dead-end ultra-filtration runs were carried out in a filtration module that was manufactured for this aim (maximal volume 200 mL) (Fig. 1). The membrane filtration system was manually installed and operated under high-purity nitrogen gas, accompanied by a magnetic stirrer. Polyethersulfone ultrafiltration membranes (Sartorius Stedim Biotech GmbH, Germany) with 1, 5, and 10 kDa pore sizes were used individually.

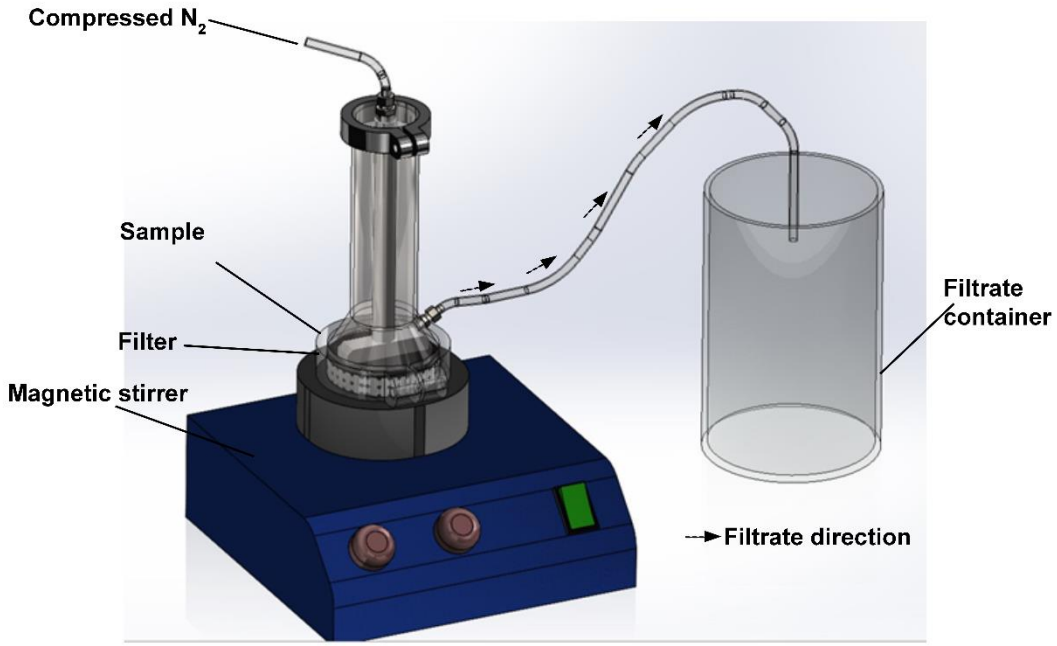

Figure 1- Filtration module

For the inulin filtration step, inulin extract was prepared under the previously determined optimum inulin extraction conditions (90 ºC-30 min-10 mL/g). The volume of the extract was determined as 40 mL for effective filtration and a 4g chicory root sample was studied for each trial. A membrane filtration system was installed using a membrane filter and loading the inulin extract into the system. 1, 5, and 10 kDa membranes were used in different trials. Nitrogen gas was operated at a pressure of 2 bar and the magnetic stirrer placed under the filtration system was operated at 400 rpm. The filtrate was collected in a test tube placed at the outlet of the filtration system. The inulin contents of the extract and final filtrate were determined by HPLC analysis. Filtration of FOS was carried out with hydrolyzed inulin extract (FOS hydrolysate). Hydrolysis conditions were 40 °C, 60 min and 60 U enzyme concentration. The membrane filtration system was set up as for the inulin purification. FOS contents of the hydrolysate and final filtrate were determined by HPLC analysis. Experiments were carried out as two replicates, using a new membrane for each experiment.

Purities of inulin and FOS were calculated according to Zhu et al. (2018).

$$\text{Inulin or FOS purity (\%)} = \frac{inulin\ or\ fos\ content}{total\ carbohydrate\ content + protein\ content} \times 100 \quad (2)$$

2.8. Total carbohydrate analysis

Total carbohydrate quantities of samples were measured by the phenol-sulfuric acid technique using D-glucose as standard (Albalasmeh et al. 2013). Briefly, 1 mL of phenol (5%) was added to 1 mL of the sample in a test tube. Afterwards, 5 mL of sulfuric acid (98%) was rapidly added and the tube was left standing for 10 minutes. After stirring, the tubes were placed in a water bath at room temperature for 20 minutes. Color development was measured at 490 nm on a spectrophotometer (Biotek-Synergy/HTX). The calibration curve was prepared with 0.01-0.75 mg/mL concentrations of standard glucose.

2.9. Total protein analysis

The protein concentration of samples was measured by the bicinchoninic acid (BCA) protein assay kit (Pierce, Thermo Fisher Scientific, USA). The calibration curve was prepared with between 20-2000 µg/mL of bovine serum albumin (BSA) standard that was supplied with the kit. Then, 0.1 mL of each standard and sample were transferred into test tubes. After this, 2 mL of working reagent (WR), that was prepared with supplied reagents, was added to each tube and mixed. Tubes were covered and incubated at 37 °C for 30 minutes. Color development was measured at 562 nm on a spectrophotometer.

2.10. Statistical analysis

The responses obtained from experimental designs underwent regression analysis by using the Design Expert Software (version 13). As a result, the determination coefficient (R^2), regression constants, and analysis of variance (ANOVA) were determined. The significance of the regression coefficient was verified by F-test and p-value. The statistical analyses were performed using JMP 9 (SAS, NC, USA). In the 0.95 confidence interval, the data were analyzed using one-way analysis of variance followed by Tukey's test.

3. Results and Discussion

3.1. Effects of temperature, time, and LS ratio on the inulin concentration

Effects of temperature (2A), time (2B), and LS ratio (2C) on the inulin content are presented in the Figure 2. As shown in Fig. 2A, increasing the extraction temperature from 60 to 90 °C resulted in the increase in inulin content when the other extraction parameters were fixed to 60 min (time) and 20:1 LS (ratio). This result is compatible with other studies about the extraction of inulin either from chicory roots or other plant materials (Lingyun et al. 2007; Tewari et al. 2015; Fu et al. 2018). Many of the previous studies extracted inulin at the temperature range of 80–90 °C from chicory roots as the water solubility of inulin increases with temperature (Apolinário et al. 2014). Although high temperatures positively affect inulin extraction, temperatures close to boiling point may increase undesired co-extract materials and cause release of simple sugars by hydrolysis (Redondo-Cuenca et al. 2021).

As can be seen in Fig. 2B, when extraction time increased from 30 to 90 min, the inulin content decreased significantly ($P<0.05$), while the other extraction parameters were fixed to 75 °C (temperature) and 20:1 LS (ratio). In the study by Fu et al., with *Codonopsis pilosula* roots (Fu et al. 2018), inulin yield increased until 120 min and then started to decrease. Tewari et al. (2014) indicated that inulin yield reduced as the time increased from 30 min to 50 min with microwave-assisted extraction. They also obtained maximum inulin yield at 30 min, similar to our finding. According to previous reports, mostly extraction periods of 30-60 min were used for inulin extractions from various plants (Apolinário et al. 2014). Since inulin is a polydisperse carbohydrate, prolonging the extraction time may lead to hydrolysis of inulin to monosaccharides.

The effect of LS ratio on inulin content is presented in Fig. 2C. Different LS ratios (10:1 to 30:1 mL/g) were examined, while other parameters were kept constant at temperature of 75 °C and time of 60 min. The inulin content was found to decrease as the LS ratio increased. At a higher LS ratio, the problem of insufficient solute (dilute solution) may occur and intermolecular forces between the inulin and the solvent become unable to provide sufficient solubility (Noori 2014; Esmaeili et al. 2021). The lower LS ratio (10:1) resulted in the highest inulin yield in this study. In contrast to our results, Tewari et al. (2015) found that a higher volume of solvent led to a higher response with microwave-assisted extraction. Another study, that focused on LS ratios of 5 to 12, found that inulin yield increased up to an LS ratio of 10, and then decreased (Lingyun et al. 2007). This result also showed that solvent ratios less than 10:1 v/w were insufficient to ensure immersion of the entire sample. From an economic point of view, the use of less solvent for the extraction process is preferable for large-scale production.

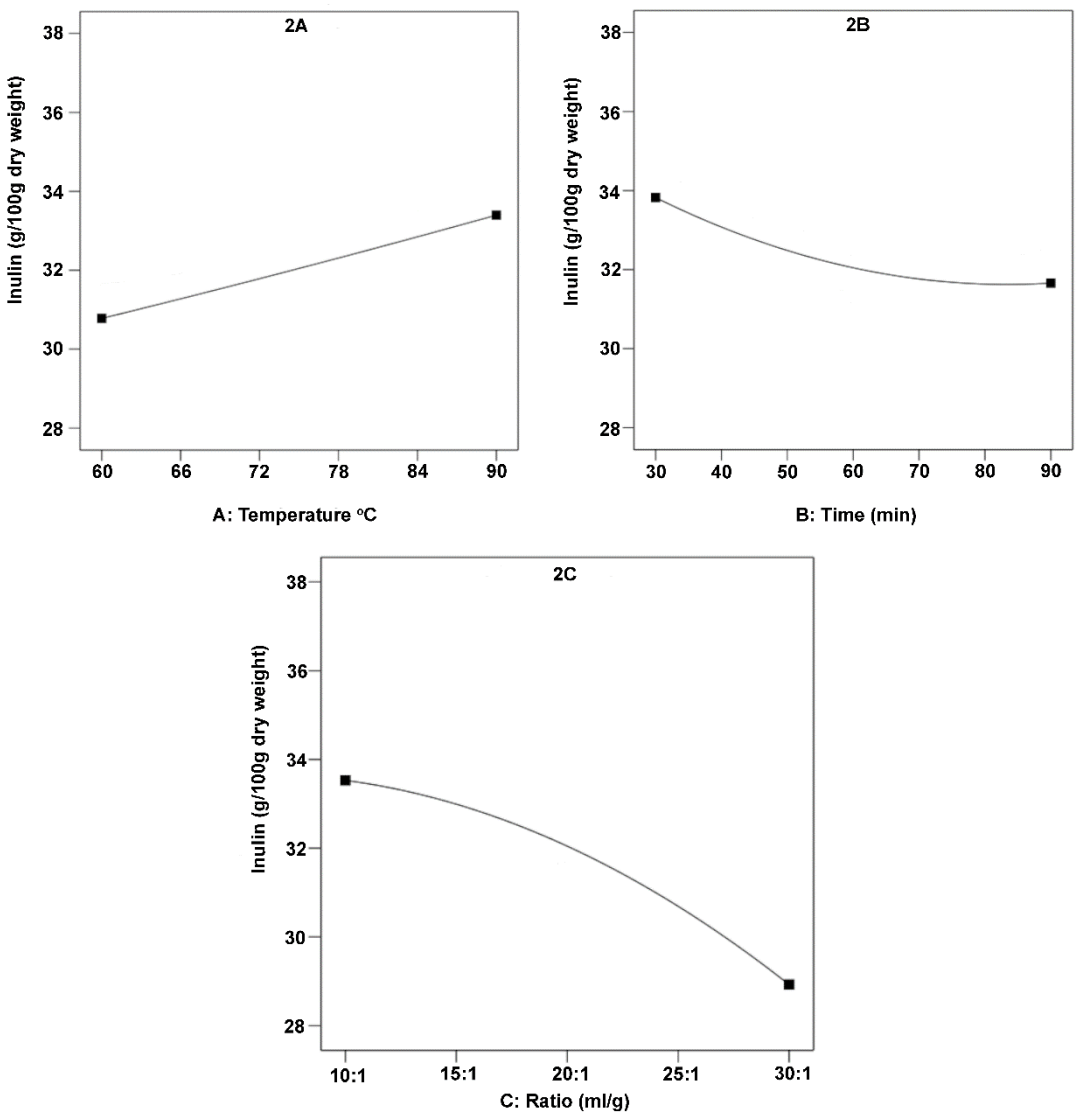

Figure 2- Effects of extraction parameters on inulin content

3.2. Optimization of inulin extraction conditions by BBD

Extraction results according to BBD are shown in Table 2. Inulin contents of extracts varied between 28.45-36.26 g/100 g dry weight (dw). In the literature, inulin contents of chicory extract were reported to be between 11.67% to 65.60% (Figueira et al. 2004; Dobre et al. 2008; Beirão-Da-Costa et al. 2009; Tewari et al. 2015; Redondo-Cuenca et al. 2021). Although our results are in this range, different inulin contents obtained in different studies may be due to the variety of plant, geographic location, harvest time, and length of storage. Different physiological processes that occur in each plant tissue might also affect inulin content (Apolinário et al. 2014; Redondo-Cuenca et al. 2021).

Table 2- Box-Behnken experimental design with coded and uncoded factors and results for inulin content*

Run	Factor 1	Factor 2	Factor 3	Experimental Values	Predicted Values
	A:Temperature (°C)	B:Time (min)	C:Ratio (mL/g)	Inulin (g/100g)**	Inulin (g/100g)
1	60(-1)	60(0)	10:1(-1)	31.83±1.02	31.16
2	60(-1)	60(0)	30:1(1)	28.45±0.95	28.76
3	90(1)	90(1)	20:1(0)	33.25±1.32	32.85
4	75(0)	60(0)	20:1(0)	32.50±0.84	32.05
5	90(1)	30(-1)	20:1(0)	35.29±1.05	35.34
6	75(0)	60(0)	20:1(0)	31.31±1.27	32.05
7	75(0)	90(1)	30:1(1)	28.61±0.92	28.36
8	75(0)	60(0)	20:1(0)	32.57±1.08	32.05
9	75(0)	30(-1)	30:1(1)	31.61±1.10	30.89
10	90(1)	60(0)	30:1(1)	28.51±1.52	29.18
11	75(0)	60(0)	20:1(0)	32.80±1.23	32.05
12	75(0)	30(-1)	10:1(-1)	34.86±0.89	35.11
13	60(-1)	90(1)	20:1(0)	30.61±1.05	30.23
14	60(-1)	30(-1)	20:1(0)	31.98±1.16	32.39
15	75 (0)	60(0)	20:1(0)	31.55±1.12	32.05
16	90(1)	60(0)	10:1(-1)	36.29±1.02	35.98
17	75(0)	90(1)	10:1(-1)	32.61±1.45	33.33
18	75(0)	60(0)	20:1(0)	31.55±1.53	32.05

*: Mean of triplicate determinations;**: Inulin content presented on dry weight basis

Table 3- Analysis of variance for the fitted quadratic polynomial model of extraction of inulin

Source	***p-value Prob>F***	***DF***	***Mean Square***	***Sum of Squares***
Model	0.000	9	8.35	75.15
A-Temperature	0.001	1	13.70	13.70
B-Time	0.004	1	9.37	9.37
C-Ratio	< 0.0001	1	42.37	42.37
AB	0.673	1	0.11	0.11
AC	0.021	1	4.84	4.84
BC	0.637	1	0.14	0.14
A^2	0.912	1	0.01	0.01
B^2	0.094	1	2.10	2.10
C^2	0.056	1	2.92	2.92
Residual	0.223	8	0.58	4.67
Lack of Fit		3	0.86	2.59
Pure Error		5	0.42	2.08
Cor Total		17		79.82
$\mathbf{R^2}$	0.942			
$\mathbf{R^2_{adj}}$	0.876			
Adeq. precision	13.39			

The *p*-value (Prob > F) serves as an important tool in ANOVA and it is used to check the significance of the model and variables (Karadag et al. 2019; Singh & Singh 2022a). A value of *p* less than 0.05 shows a significant coefficient of model terms (Singh et al. 2021). According to Table 3, the *p*-value for the model and independent variable of C-Ratio was found to be < 0.0001, which confirms the fitness of the designated model (Singh & Singh 2022a). Model terms A, B, and AC had *p* value of < 0.05, which justifies their significance (P<0.05). Lack of fit value is essential to define the authenticity of the model and the fitness of the model is represented by insignificant (P>0.05) 'lack of fit' (Singh et al. 2021). A low lack of fit (0.223) confirms that the model is adequate for predicting inulin concentration. Degree of freedom (DF), which is an important interpretation of ANOVA, is found by the number of times a specific run with similar values for all selected independent variables is repeated in the designed model (Singh & Singh 2022b). In the current study, DF 5 for pure error confirms the fitness of the selected model, which was further confirmed by the coefficient of determination (R^2) of 0.942, indicating that only 5.8% of total variation cannot be explained by our model. Adequate precision that measures the signal to noise (S/N) ratio of the designed model also authenticates the model fitness (Singh & Singh 2022b). An adequate precision value greater than 4 is desirable for a fitted model. In this model, an adequate precision value of 13.392 indicates a sufficient signal.

The final equation obtained in terms of coded factors (A: temperature (°C), B: time (min), C: liquid-to-solid (LS) ratio (mL/g)) is:

$$\text{Inulin (g/100g)} = 32.050 + 1.310A - 1.080B - 2.300C - 0.167AB - 1.100AC - 0.187BC + 0.042A^2 + 0.694B^2 - 0.818C^2 \quad (3)$$

3D response surface plots of the graphical representations of regression equations are presented in Fig. 3. They show the relationship between responses and experimental levels and the type of interactions between two test variables. Each figure represents the effect of two factors on the inulin content, while the other factor was kept at zero level (center value of the testing range). The interactive effects of extraction temperature (°C) and time (min), extraction temperature (°C) and liquid: solid ratio (mL/g) and time (min) and liquid: solid ratio (mL/g) are given in Figure 3A, 3B and 3C, respectively. In the 3D plots, the navy-blue color represents lower and the raspberry red color represents upper range variables, whereas light green color, yellow color, and sky blue color represent the intermediate range of the selected variables. In our model, only the interaction between the LS ratio and the temperature was significant (p=0.021). These figures show that an increase in the inulin content was observed when temperature increased and other factors (time and LS ratio) decreased.

The plots showed that inulin extraction reached its maximum at a combination of temperature of 90 °C, extraction time of 30 min and LS ratio of 10:1 mL/g. Maximum inulin content of 37.62 g/100 g dw was obtained under these parameters. Redondo-Cuenca et al. (2021) examined optimal conditions for the extraction of inulin plus FOS from chicory root and found optimal parameters were 77.4 °C, 59.4 min, and 27.8 mL/g LS ratio. Tewari et al. (2015) investigated optimum conditions for microwave-assisted extraction of inulin from chicory roots and found 90 °C, 30 min, and 40 mL/g LS ratio were the optimum parameters. Lingyun et al. (2007) also reported optimum conditions for inulin from Jerusalem artichoke tubers as 76.65 °C, 20 min, and 10:1 mL/g LS ratio.

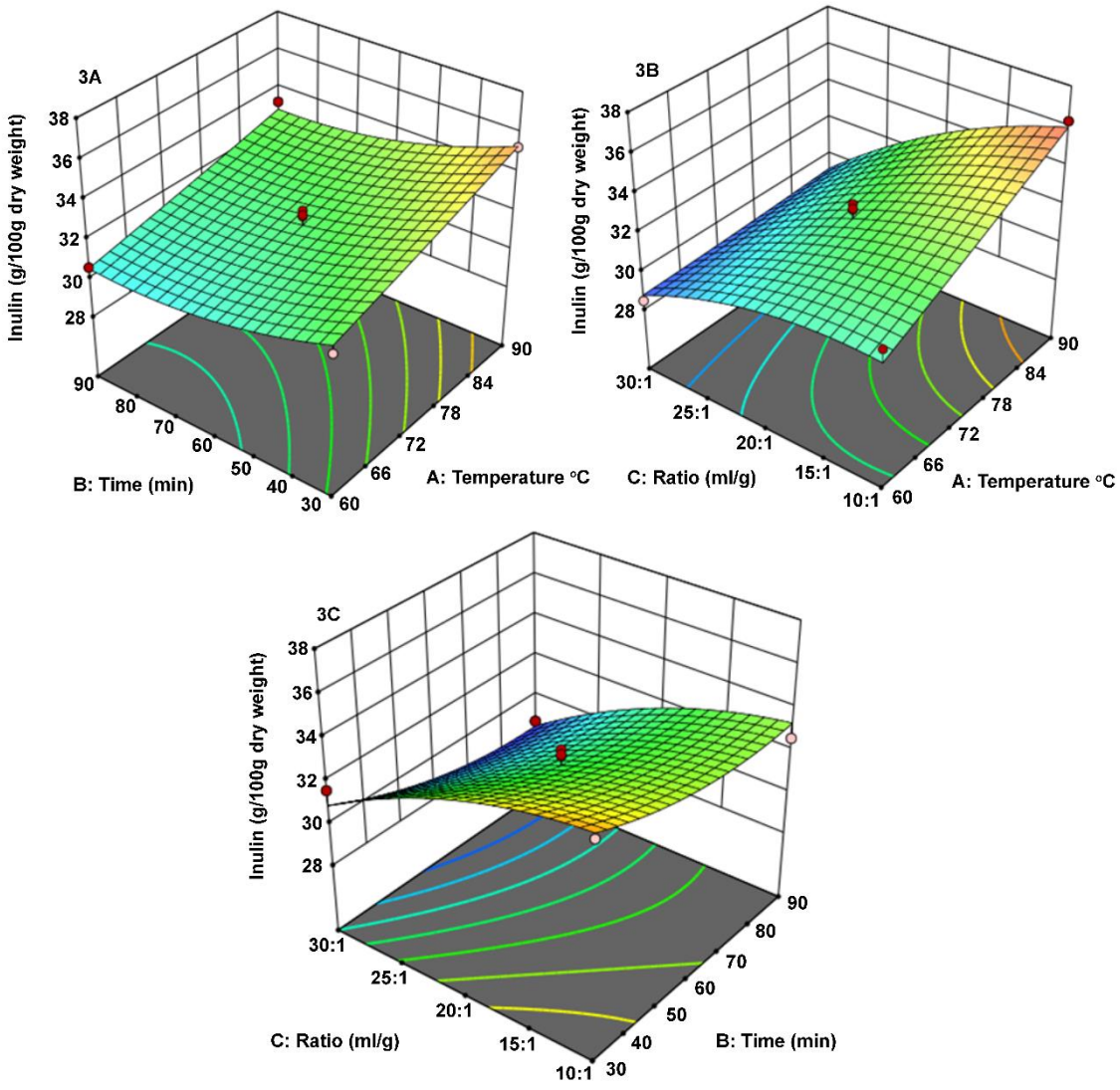

Figure 3- 3D response surface plots

3.3. FOS content of hydrolysates

FOS hydrolysates were analyzed for inulin, FOS, and residual sugar content. Also, the inulin extract used as the substrate was analyzed. Total FOS content was calculated as the sum of GF_2, GF_3, and GF_4. HPLC chromatograms of FOS and residual sugars are shown in Figure 4. FOS, inulin, residual sugar contents of hydrolysates, and inulin extracts are given in Table 4.

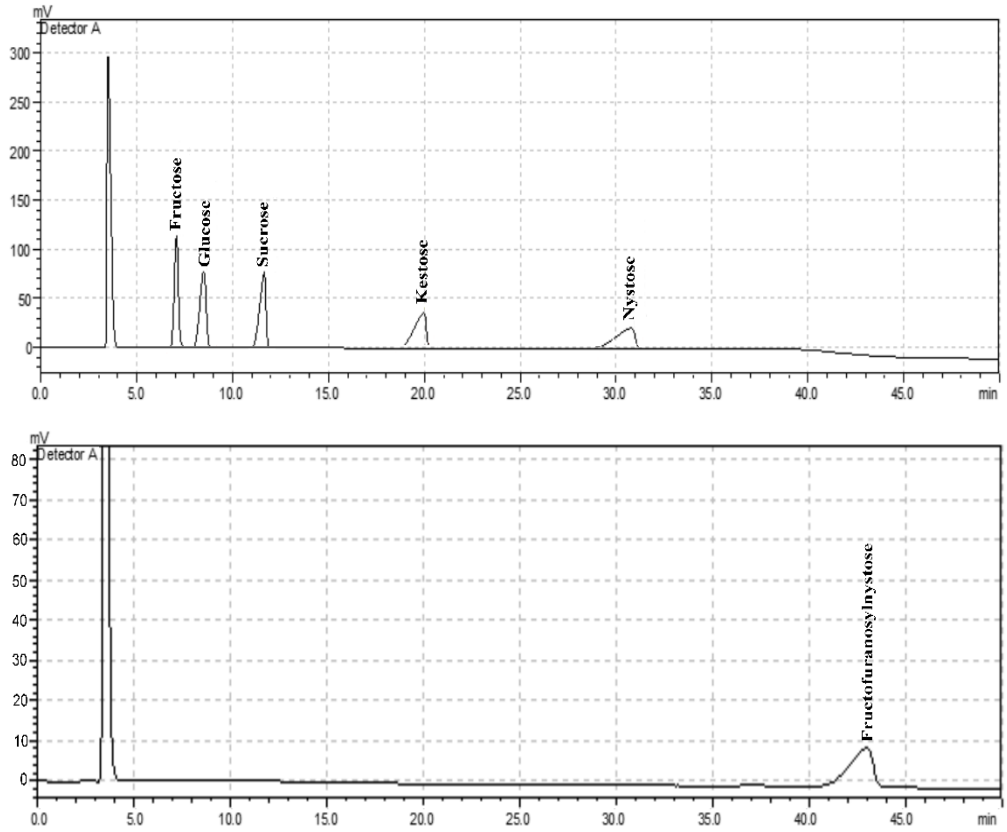

Figure 4- HPLC chromatograms of FOS and residual sugars

Table 4- FOS, inulin and residual sugar contents of hydrolysates and inulin extract

Contents (mg/mL)	*Samples**								
	Inulin extract	***E-1***	***E-2***	***E-3***	***E-4***	***E-5***	***E-6***	***A-1***	***A-2***
Inulin	16.97 ± 1.22^{A}	10.82 ± 0.75^{AB}	7.53 ± 0.63^{C}	8.32 ± 1.18^{BC}	10.94 ± 0.31^{ABC}	8.33 ± 0.51^{BC}	9.36 ± 0.58^{ABC}	11.14 ± 1.20^{ABC}	12.84 ± 0.95^{ABC}
Fructose	6.40 ± 0.39^{C}	6.47 ± 0.89^{C}	3.88 ± 0.15^{D}	3.77 ± 0.36^{D}	6.99 ± 0.28^{C}	3.72 ± 0.52^{D}	4.02 ± 0.51^{D}	8.83 ± 0.37^{B}	15.06 ± 0.19^{A}
Glucose	1.95 ± 0.32^{ABC}	2.22 ± 0.57^{A}	1.17 ± 0.17^{BC}	1.07 ± 0.13^{BC}	2.02 ± 0.22^{AB}	0.99 ± 0.40^{C}	1.07 ± 0.24^{BC}	1.59 ± 0.30^{ABC}	2.25 ± 0.45^{A}
Sucrose	1.95 ± 0.49^{A}	2.21 ± 0.29^{A}	1.31 ± 0.24^{A}	1.15 ± 0.31^{A}	1.96 ± 0.96^{A}	1.05 ± 0.24^{A}	1.15 ± 0.23^{A}	1.61 ± 0.30^{A}	1.73 ± 0.24^{A}
1-kestose(GF_2)	0.78 ± 0.14^{ABC}	1.16 ± 0.31^{A}	0.55 ± 0.13^{BC}	0.44 ± 0.16^{C}	1.12 ± 0.34^{AB}	0.48 ± 0.28^{C}	0.53 ± 0.15^{BC}	0.32 ± 0.09^{C}	0.38 ± 0.14^{C}
1-nystose(GF_3)	0.82 ± 0.10^{CDE}	2.35 ± 0.27^{A}	1.23 ± 0.39^{BCD}	1.41 ± 0.20^{BC}	1.69 ± 0.17^{AB}	0.93 ± 0.22^{CD}	1.02 ± 0.22^{BCD}	0.64 ± 0.27^{DE}	0.23 ± 0.20^{E}
1^{F}-fructofuranosylnystose (GF_4)	1.22 ± 0.19^{BCD}	2.60 ± 0.49^{A}	1.83 ± 0.43^{ABC}	1.98 ± 0.29^{AB}	0.00 ± 0.01^{E}	1.12 ± 0.26^{CD}	1.28 ± 0.24^{BCD}	1.03 ± 0.24^{CD}	0.99 ± 0.22^{D}
Total FOS	2.82 ± 0.35^{CD}	6.11 ± 0.38^{A}	3.61 ± 0.29^{BC}	3.83 ± 0.37^{B}	2.81 ± 0.18^{CD}	2.53 ± 0.27^{D}	2.83 ± 0.20^{CD}	1.99 ± 0.20^{DE}	1.60 ± 0.52^{E}

***:** Sample names were coded as defined in Table 1. Inulin extract refers to unhydrolyzed extract obtained in the optimum conditions. All results are mean of triplicate determinations. Different uppercase letters represent statistical difference between different samples for each component.

According to Table 4, the highest amount of FOS (statistically significant ($P<0.05$)) was obtained from sample E-1 (40 °C-60 min-enzyme hydrolysis-without buffer and acid). Although the inulin content was lower in hydrolysates using acid and buffer solution (E-2, 3, 5, and 6), no statistically significant increase in FOS content was observed in hydrolysates E-5 and E-6.

Acid hydrolysis results showed not only the lowest FOS formation but also the highest fructose content. This implies that acid hydrolysis might cause full hydrolysis of inulin to fructose units. The degradation of inulin to its monosaccharides was not targeted in this study since the functional properties are lost with degradation. Stökle et al. (2023) tested the acid hydrolysis of inulin to obtain fructose-enriched extracts but observed the formation of undesirable components, especially hydroxymethylfurfural (HMF), indicating that acid hydrolysis was not suitable to obtain FOS. Another methodology to obtain FOS is the transfructosylation of sucrose but previous studies reported that FOS produced from inulin hydrolysis might contain longer fructo-oligomer chains (DP 5-9) than the ones produced by the sucrose transfructosylation process (DP 2-4) (Dominguez et al. 2014; Singh et al. 2020b). Additionally, in the trans-fructosylation process, a considerable amount of by-products (unreacted sucrose, generated fructose and glucose) were produced, which decreases the amount of product and increases the purification cost (Singh et al. 2020b).

Han et al. (2017) found temperature of 35 °C and pH value of 4.5 were appropriate conditions for FOS production from inulin, verifying the hydrolyzation at low temperatures. In contrast to our study, a lower pH value was found to be optimum for hydrolyzation of inulin treated with recombinant endo-inulinase enzyme-engineered *Yarrowia lipolytica* strain in the aforementioned study.

3.4. Inulin purification

The extraction method for inulin usually results in solutions comprising a mixture of inulin, other polysaccharides, and non-carbohydrate compounds, mainly proteins, which should be further purified for industrial purposes (Apolinário et al. 2014; Perović et al. 2021). For this reason, inulin, total carbohydrate, and total protein contents of both inulin extract (before filtration) and inulin filtrate (after filtration) were analyzed to determine the yield of purification.
Ultrafiltration tests with 1 and 5 kDa membranes resulted in lower inulin recovery than with 10 kDa. For this reason, only 10 kDa membrane results are presented in this study (Table 5).

Table 5- Purification* of inulin and FOS****

Sample	***Inulin or FOS content (mg/mL)***	***Total carbohydrate (mg/mL)***	***Total protein (mg/mL)***	***Purity (%)***
Inulin extract (before filtration)	23.72 ± 1.21^{a}**	41.74 ± 3.05^{a}	1.36 ± 0.40^{a}	55.04
Inulin filtrate (after filtration)	17.15 ± 1.25^{b}**	18.17 ± 1.77^{b}	0.77 ± 0.21^{a}	90.54
FOS hydrolysate (before filtration)	13.70 ± 1.22^{A}***	45.38 ± 3.86^{A}	1.77 ± 0.49^{A}	29.06
FOS filtrate (after filtration)	13.41 ± 0.77^{A}***	17.35 ± 2.27^{B}	0.68 ± 0.18^{B}	76.02

*: All results are mean of triplicate determinations. Different lowercase letters indicate statistical difference between inulin samples in the same column, different uppercase letters show statistical difference between FOS samples in the same column

According to Table 5, purification by ultrafiltration resulted in a statistically significant ($P<0.05$) reduction in the total carbohydrate content of inulin extract. After filtration, 94% of the total carbohydrate content was composed of inulin, proving that other polysaccharides were mostly removed. In the filtrate, total carbohydrate content decreased by 56.47% and protein content decreased by 43.39%. The purity of inulin reached 90.54% in the filtrate.

There are several studies related to purification of chicory inulin by chromatographic techniques (Mavumengwana 2004), ion exchange resins (Zhang et al. 2022), and ultrafiltration for Jerusalem artichoke (Zhu et al. 2018), and red fruit (*Pandanus conoideus*) (Murtiningrum et al. 2020) inulin extracts. Mavumengwana et al. (2004) investigated the purification of inulin suspension by chromatographic techniques for color removal and tannin elimination. Zhu et al. (2018) studied ultrafiltration of Jerusalem artichoke extract, using a 50-kDa membrane with trans-membrane pressure of 0.3 MPa and a rotation speed of 800 rpm. The researchers were able to obtain a filtrate with 98% inulin purity, and showed that membrane type, pressure, and rotation speed were factors affecting the purity.

3.5. FOS purification

FOS hydrolysate was purified for the removal of residual enzymes, polysaccharides, and proteins. FOS purity (%) was calculated as for inulin purity. Purification with the 10 kDa membrane filter resulted in the highest recovery of FOS. As can be seen in Table 5, FOS purification with 10 kDa membrane resulted in 97.88% FOS recovery, a decrease of 61.77% of total carbohydrate content, and 61.59% of protein content in the filtrate. The reduction in the total carbohydrate and protein content was statistically significant ($P<0.05$). The final purity of FOS reached 76.02%.

A fast protein liquid chromatography method was used by Li et al. (2013) to purify FOS from burdock and 75-95% FOS purity was found at different conditions. Kuhn et al. (2014) purified FOS by column chromatography to 94% purity. In the literature, most of the purification studies about inulin and FOS were performed using chromatographic techniques such as size exclusion chromatography, ion exchange chromatography, and gel permeation chromatography (Muñiz-Márquez et al. 2019). Although higher purity levels were reported in studies with chromatographic techniques, the main disadvantage of chromatographic techniques for the purification of inulin and FOS is their relatively high installation cost, energy requirement, and resin requirement (Muñiz-Márquez et al. 2019). Purification by membrane techniques is simpler, and easily adaptable to large-scale processes, and although it involves installation costs, membrane filtration technique is more cost-effective than chromatographic techniques (Barclay et al. 2010; Muñiz-Márquez et al. 2019).

4. Conclusions

The process conditions for the extraction of inulin from chicory roots were optimized by using RSM design. The results verified that the RSM model was suitable and reliable for actualizing the expected optimization. Hydrolyzation of inulin to FOS was carried out by using acid and enzyme treatments in different trials. Acid hydrolysis resulted in the degradation of inulin to fructose. To obtain a high-quality product, purification is necessary. In this study, the membrane filtration technique was preferred because of its advantages such as easy scale-up and easily manipulated critical operational variables. However, membrane applications to purify inulin and FOS are limited in the literature. Mostly, chromatographic separation techniques that require high installation and operational costs and qualified employees were used for the purification of these components. In contrast to complex techniques, the membrane filtration method offers a simpler, more rapid, and economical way to obtain both inulin and FOS from chicory roots.

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