SALAZAR-LEYVA, Jesús Aarón; OSUNA-RUIZ, Idalia; RODRÍGUEZ-TIRADO, Víctor
Alfonso; ZAZUETA-PATRÓN, Iris Eréndira; BRITO-ROJAS, Héctor Daniel
Optimization study of fructans extraction from Agave tequilana Weber azul variety

Optimization study of fructans extraction from *Agave tequilana* Weber *azul* variety

Jesús Aarón SALAZAR-LEYVA[1]*, Idalia OSUNA-RUIZ[1], Víctor Alfonso RODRÍGUEZ-TIRADO[1],
Iris Eréndira ZAZUETA-PATRÓN[1], Héctor Daniel BRITO-ROJAS[1]

Abstract

Agave tequilana Weber *azul* variety is a valuable source of fructans. In this study, a response surface methodology was employed to optimize the extraction yield of fructans from agave pines. A Box Behnken factorial design was applied to evaluate the effects of several conditions such as extraction temperature, water raw material ratio and extraction time on extraction yield. Under the optimum extraction conditions (extraction temperature of 79.1 °C, water raw material ratio of 5.13 mL/g, and extraction time of 1.48 h) fructans extraction yield was 83.12%. The chromatographic profile of the agave crude extract presented four peaks, out of which fructans were the most preponderant (~87%). The obtained results suggest that the response surface methodology is adequate to optimize fructans extraction from *Agave tequilana* Weber *azul* variety.

Keywords: fructans; agave; extraction yield; optimization; response surface methodology.

Practical Application: Response surface methodology allows the optimization of fructans extraction from *Agave azul*.

1 Introduction

Fructans are carbohydrates that consist mostly of fructose, and in a minor proportion of glucose. These kinds of compounds have one or more fructosyl-fructose linkages which constitutes the majority of them. Fructans structures can be linear or branched, and can have different degrees of polymerization (DP). According to the way that fructofuranosyl units are linked, these biomolecules are classified as inulin, levan, phlein, graminan, and kestoses (Roberfroid, 2005). Due to the fact, that fructans cannot be digested by humans they are low-calorie food, stimulating the growth and activity of beneficial bacteria in the colon (Gomez et al., 2010; Robinson, 1995). Moreover, many authors have been reporting that fructans may act as fat substitutes because of their ability to add texture and to improve rheological characteristics of dairy products (Akın et al., 2007; Pimentel et al., 2013).

Most plants store starch as a reserve carbohydrates, however at least 10 families of higher plants store fructans in a soluble form in vacuoles, leaves, roots, stems, tubers, or kernels (Roberfroid, 2005; Vijn & Smeekens, 1999). So far, the plants that have been used industrially for the extraction of fructans are chicory (*Cichorium intybus*) and Jerusalem artichoke (*Helianthus tuberosus*) (Paseephol et al., 2007; Toneli et al., 2007). A natural source, rich in fructans is the *Agave* plant. These kinds of vegetables grow in Mediterranean and hot climates, being Mexico considered as the center of origin and biodiversity of *Agave* species (Ávila-Fernández et al., 2011; Furlán et al., 2014). Heads of plants belonging to the agave genus have high contents of fructan oligomers, composed mainly of fructose units linked to a sucrose molecule. These agave fructans consist of a complex

mixture of fructooligosaccharides, containing principally β (2-1) and β (2-6) linkages (Lopez et al., 2003; Mancilla-Margalli & López, 2006). Mexico has a considerable production of agave, and particularly the *Agave tequilana* Weber *azul* variety is grown commercially for the production of the worldwide famous alcoholic beverage tequila (Bautista-Justo et al., 2001). Recently, alternative industrial usages for agave plants have emerged, such as the production of syrups and agave fructans, being *Agave tequilana* Weber *azul* variety the raw material most utilized for these purposes (Ávila-Fernández et al., 2011; Narváez-Zapata & Sánchez-Teyer, 2009).

The industrial extraction process of fructans from vegetal sources, is generally based on diffusion of polysaccharides in hot water (Apolinário et al., 2014; Kim et al., 2001). In this sense, several investigations have been developed to set optimum extraction conditions of fructans extraction from plants, and temperature, extraction time, and water raw material ratio were identified as the most important factors influencing the extraction yield (Abozed et al., 2009; Lingyun et al., 2007; Paseephol et al., 2007). Despite the important role of *Agave tequilana* Weber *azul* variety as raw material to obtain fructans, little information is available about their extraction by hot water diffusion. Therefore, the objective of this study was to optimize the extraction process of *Agave tequilana* fructans by using hot water as a solvent. The response surface methodology was used to optimize extraction conditions (temperature, extraction time and water raw material ratio) in order to obtain the maximal fructans extraction yield. In addition *Agave tequilana* fructans obtained under the optimal

conditions were analyzed using High Performance Liquid Chromatography (HPLC).

2 Materials and methods

2.1 Plant material

Six-year-old *Agave tequilana* Weber *azul* variety plants were harvested in an agricultural field near Mazatlan, Sinaloa, Mexico. The stem of the plant (usually called "head" or "pine") was fragmented, washed with distilled water, and maintained at –22 °C for further use.

2.2 Reagents

Standards of chicory inulin, sacarose, fructose and glucose were purchased from Sigma-Aldrich (St. Louis, MO, USA). The water used for the chromatographic analyses was HPLC grade (Fisher-scientific, Pittsburgh, USA). A commercial kit for fructans determination was purchased from Megazyme (Wicklow, Ireland). All other chemicals and solvents used were of analytical grade.

2.3 Extraction of *Agave tequilana* **fructans**

In this study, a solid liquid extraction was done by using distilled water, considering the high solubility of fructans in this solvent. Agave pines were thawed at room temperature, and homogenized with water in a blender (International, LI-3, Mexico). The pH solution was adjusted to 5.5 to avoid acid hydrolysis of polysaccharides. Different extractions conditions were evaluated (temperature, extraction time and water raw material ratio), as it is detailed in section 2.7. The liquid extract obtained at the end of the extraction process was filtered to eliminate the solid fraction, and centrifuged (Thermo-Fisher Scientific, Sorvall, Waltham, USA) at $10,000 \times g$, for 10 min to remove suspended particles. The supernatant was considered as the fructans crude extract. All extraction experiments were performed in duplicate.

2.4 Determination of total carbohydrate

Total carbohydrate was determined by the phenol sulphuric acid method (Dubois et al., 1956) with slight modifications proposed by Fox & Robyt (1991). Samples of agave fructans (300 µL) were mixed with 300 µL of 5% phenol solution. The mixture was placed in an ice bath and 1500 µL of 96% sulphuric acid was added. After an incubation at 80 °C for 30 min, the solution appeared as orange color and its absorbance was measured at 492 nm using a UV–vis spectrophotometer (Jenway, 6305, Staffordshire, UK). Series of fructose solutions of known concentration were used to establish a standard curve.

2.5 Reducing sugar measurement

Reducing sugar content in agave fructans was measured using the dinitrosalicylic acid (DNS) method (Miller, 1959) with slight modifications. Briefly, 100 µL of agave fructans solution was mixed with 100 µL of DNS reagent. After mixing it with a vortex, samples were heated in a water bath at 100 °C for 5 min.,

followed by cooling to room temperature. Later 1600 µL of distilled water was added. The absorbance was recorded at 535 nm. A calibration curve was prepared using fructose as standard.

2.6 Determination of fructans extraction yield

Fructans content in the crude extract was calculated with the difference between total carbohydrate and reducing sugar (Lingyun et al., 2007), whereas the quantification of fructans in the plant material was assessed by the fructan assay procedure kit Megazyme (McCleary et al., 2000). Then, fructans extraction yield (FEY) was calculated as shown below (Equation 1):

$$FEY(\%) = \frac{Fructans\,crude\,extract\,(g)}{Fructans\,plant\,material\,(g)} \times 100 \qquad (1)$$

2.7 Experimental design and statistical analysis

Prior to the optimization study, a preliminary screening was conducted to determine the effect of extraction temperature, extraction time, and water raw material ratio over the variable response fructans extraction yield (FEY). To evaluate the effect of temperature, this factor was set at 60, 70, 80, and 90 °C. The other extraction condition was water raw material ratio fixed at 4 mL/g, and extraction time 1 hour. Also extraction time was evaluated, setting this factor at 1, 2, 3 and 4 h whereas water raw material ratio and extraction temperature were maintained at 4 mL/g and 80 °C respectively. In addition, water raw material ratio was evaluated at 1, 3, 6, 9, and 12 mL/g. Other extraction conditions were maintained as follows: extraction time 1 h and extraction temperature 80 °C. Based on the results of the preliminary screening, a three-level, and three variable Box-Behnken factorial design (BBD) was applied. The evaluated factors and their coded and uncoded levels are shown in Table 1. Response Surface Methodology (RSM) was used to investigate the optimum levels of the factors and their relationships. The complete design consisted of fifteen combinations including three replicates of the center point. The experimental runs were performed in a totally random order, and the experimental data was analyzed by means of a second order model (Equation 2):

$$Y = \sum A_0 + \sum_{i=1}^{3} A_i X_i + \sum_{i=1}^{3} A_{ii} X_i^2 + \sum_{i=1}^{2} \sum_{j=i+1}^{3} A_{ij} X_i X_j \qquad (2)$$

where Y is the response variable, A_0, A_i, A_{ii}, A_{ij} are the regression coefficients of variables for intercept, linear, quadratic and interaction coefficients respectively, and X_i and X_j are independent variables.

Table 1. Coded levels of the experimental factors for Box-Behnken design matrix.

Factor	Coded symbols	Levels		
		-1	0	1
Temperature (°C)	X1	60	75	90
Time (h)	X2	1	1.5	2
Ratio of water to raw material (mL/g)	X3	3	6	9

The verification of the model adequacy with the experimental data was done using the coefficient of determination (R^2), Absolute Average Deviation value (AAD) and the lack of fit test. AAD was calculated according to the Equation 3:

$$AAD = \left\{ \left[\sum_{i=1}^{p} \left(\left| y_{i,\exp} - y_{i,cal} \right| / y_{i,\exp} \right) \right] \Big/ p \right\} \times 100 \quad (3)$$

where $y_{i,\exp}$ and $y_{i,cal}$ are the experimental and calculated responses respectively, and p is the number of experimental run. The software package used was STATGRAPHICS Plus Version 5.1 (StatPoint Inc., Warrenton, USA).

2.8 High performance liquid chromatographic analysis of fructans crude extract

The crude extract obtained under the optimal conditions of extraction, were analyzed using the method of Marsilio et al. (2000) with slight modifications. The liquid chromatography apparatus consisted of a Varian Polaris HPLC system with an evaporative light scattering detector (ELS-2100) (Varian Inc., Palo Alto, USA). Parameters of the ELS-2100 detector were as follows: gas flow rate 0.9 slm (standard liter per minute), evaporation

and nebulization temperature at 90 and 80 °C respectively. The extracts were diluted until it was necessary and filtered prior to analysis, by using a nylon syringe filter (4 mm diameter, 0.2 µm size pore). The chromatographic separation was carried out using a MetaCarb 87 C column (300 × 7.8 mm) (Varian Inc., Palo Alto, USA). HPLC grade water was used as mobile phase at a flow rate of 0.6 mL/min keeping the column temperature at 85 °C. The injected sample volume was 20 µL. Chicory inulin, sacarose, glucose and fructose standards were used to compare it with the carbohydrates contained in the analyzed sample. In addition, a calibration curve was constructed using chicory inulin as a external standard in a concentration range of 0.05 to 0.45 mg/ mL.

3 Results and discussion

3.1 Preliminary screening

As it was previously stated in section 2.7., a single factor test was done, exploring the factors of extraction temperature, extraction time, and water raw material ratio. According to Figure 1a the highest fructans extraction yield (FEY) was obtained at a temperature of 70 °C, whereas at 90 °C FEY decreased drastically. Cai et al. (2008) found a similar behaviour in a crude
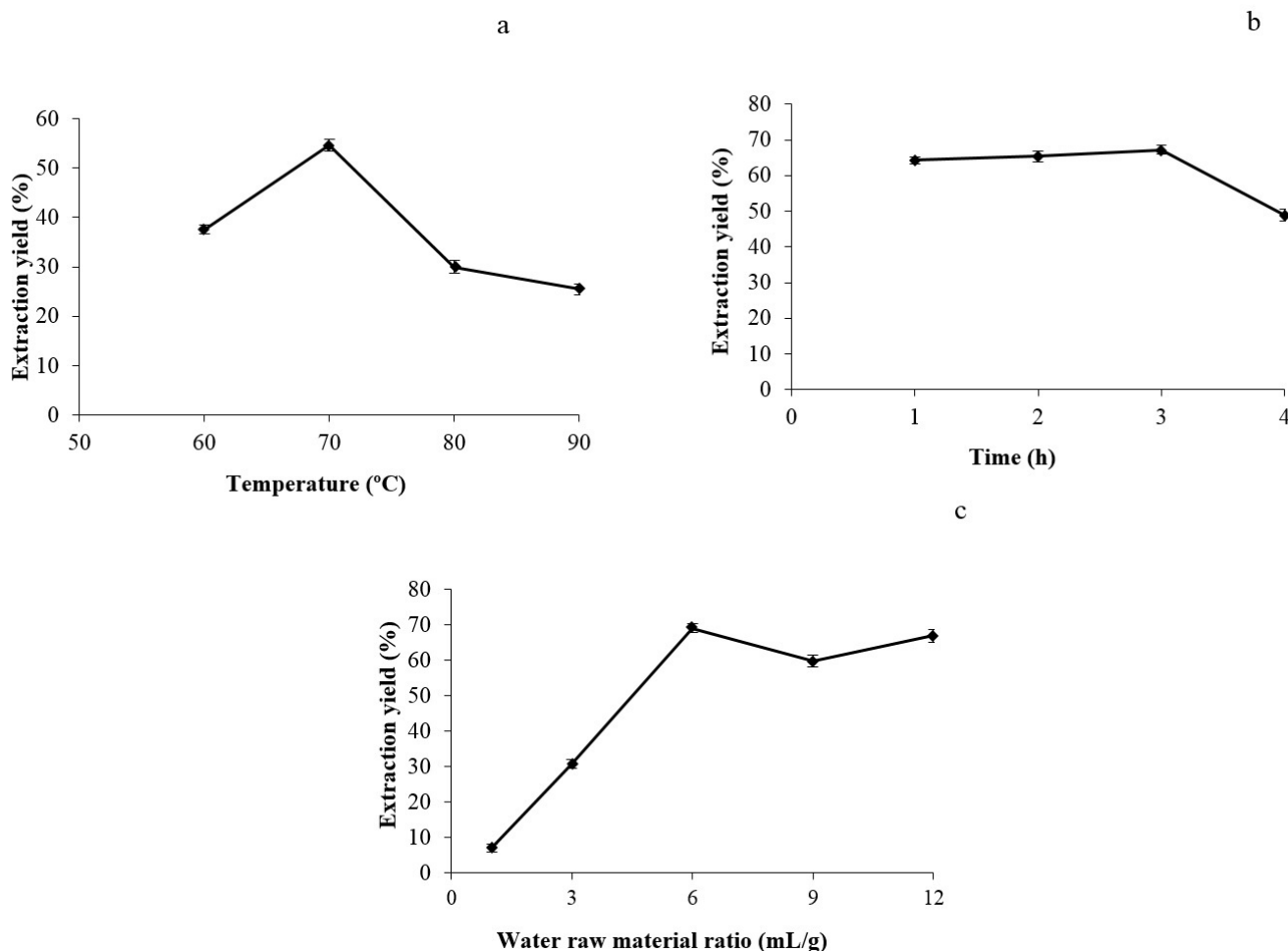

Figure 1. Effect of extraction temperature (a), extraction time (b) and water raw material ratio (c) on fructans extraction yield. Bars represent the standard deviation. Each value is a mean of three replicates (n = 3).

extract of polysaccharides from *Opuntia milpa alta* and reported that the decrease in extraction yield was due to the hydrolysis of polysaccharides at higher temperatures. The effect of time on fructans extraction yield is depicted in Figure 1b, and from this graph is possible to observe a slight increase on FEY during the first three hours. Later, extraction yield was decreasing. These results suggest that in the three first hours, fructans extraction yield maintain a dynamic equilibrium, nevertheless when extraction time was increased, fructans extraction yield decreased. Previous studies (Cai et al., 2008; Yin & Dang, 2008) reported that excessive extraction time induces polysaccharides extraction yield to decrease probably, due to hydrolysis of carbohydrate molecules. Aditionally, the effect of water raw material ratio on fructans extraction yield was evaluated. In this regard, in Figure 1c, it is observed that within the range of 1 to 6 mL/g of water raw material ratio, the extraction yield increased, and reached the highest value when the ratio was 6 mL/g. Similar results were observed by Yin & Dang (2008) and Yan et al. (2014) when extracted polysaccharides from BaChu mushroom and leaves of *Gynura bicolor* respectively. These authors mentioned that the increase of water raw material ratio may increase diffusivity of the solvent into vegetable cells, so desorption of the polysaccharides from the cells is enhanced. However, when the ratio is higher than 6 mL/g, fructans extraction yield slightly decreased and no longer changed.

3.2 Optimization study of crude fructans extraction using the Box-Behnken factorial design

According to the levels and factors showed in Table 1, the software package STATGRAPHICS Plus Version 5.1 (StatPoint Inc., Warrenton, VA, USA) proposed a matrix design with 15 experimental combinations. Table 2 shows the matrix design with the experimental and predicted results of the response fructans extraction yield (Y). In this research, the fructans extraction yield (FEY) ranged from 19.21% to 82.14%. A maximum value was found in experiment number 13, under the experimental conditions of temperature 75 °C, extraction time 1.5 h, and water raw material ratio 6 mL/g (Table 2). Furthermore, a regression analysis was applied to the experimental data, the response variable, and the studied factors, resulting in the following Equation 4:

$$Y = -272.939 + 6.27368 * X1 + 48.4315 * X2 + 7.98812 * X3 - 0.0377392 * X1 * X1 - 0.074725 * X1 * X2 + 0.00274709 * X1 * X3 - 17.6833 * X2 * X2 - 0.0108416 * X2 * X3 - 0.1986646 * X3 * X3 \quad (4)$$

where Y is the response variable (%), X1 is temperature extraction (°C), X2 is extraction time (h), and X3 is water raw material ratio (mL/g).

Table 3 shows a summary of the analysis of variance (ANOVA) applied to the second order model. The p value of the model was less than 0.05, indicating that it is a significant model. Moreover, the lack of fit was non-significant ($p \geq 0.05$) implying that the model accurately represents the data in the experimental region. Besides the lack of fit test, the coefficient of determination R^2 is a widely accepted parameter for verification of the model adequacy (Baş & Boyacı, 2007). In this regard, the R^2 was calculated as 0.9145 indicating that the statistical model explains 91.45% of the variability in the fructans extraction yield. When the statistical

Table 2. Matrix design and results from Box-Behnken factorial design.

Experiment	X1	X2	X3	Fructans extraction yield (%)[a] Experimental	Predicted
1	0	1	1	48.52 ± 1.23	54.21
2	1	0	1	57.23 ± 0.53	48.45
3	0	1	-1	66.45 ± 0.95	55.17
4	1	0	-1	55.34 ± 0.91	63.23
5	0	-1	1	61.05 ± 1.82	66.19
6	-1	0	-1	31.89 ± 0.55	29.23
7	0	0	0	80.15 ± 0.74	81.94
8	-1	1	0	19.21 ± 0.85	26.17
9	-1	-1	0	31.12 ± 0.25	35.11
10	0	0	0	73.34 ± 1.19	75.03
11	1	-1	0	62.21 ± 0.74	53.12
12	1	0	1	44.70 ± 1.25	47.87
13	0	0	0	82.14 ± 0.7	79.91
14	1	1	0	39.65 ± 0.34	37.15
15	0	-1	-1	69.02 ± 2.1	69.23

[a]Experimental fructans extraction yield are averages ± standard deviation from three independent experiments.

Table 3. Summary of the analysis of variance of the polynomial model for the response fructans extraction yield (Y).

Source	p value
Model	0.0123*
X1	0.0254*
X2	0.0319*
X1* X1	0.0018*
X1* X3	0.0274*
X2* X2	0.0128*
X3* X3	0.1745
Lack of fit test	0.2744

*Significant at $p < 0.05$.

model has several terms, it is recommendable to calculate the adjusted R^2 (R^2_{Adj}), because this adjusted value corrects the R^2 value for the sample size and the numbers of terms of the model (Box et al., 1978). In this context, the obtained R^2_{Adj} value from the regression analysis applied to our model was 0.8413 and was considered adequate to advocate the significance and prediction of our model. Other parameter that was used to check the accuracy of the model is the Absolute Average Deviation (AAD). The AAD between the predicted and experimental data must be as small as possible. In this regard, the AAD value was very small (0.000365) indicating that the model has a good fitness and it is adequate to describe the observed data of the response Y. In addition, from the ANOVA table it is possible to establish the significance level of each coefficient of the model and the interaction between the evaluated factors. In this study, the linear terms X1 (temperature extraction) and X2 (extraction time) affect significantly ($p < 0.05$) the response FEY. Other studies reporting the significant influence of temperature and time on extraction yield of polysaccharides from vegetable sources are those conducted by XuJie & Wei (2008) and Yan et al. (2014). Also, Bouaziz, et al. (2014) observed that water temperature had a significant influence on the extraction yield of polysaccharides from *Agave americana* leaves. Moreover, a significant interaction ($p < 0.05$) was observed for the quadratic terms X1*X1, X2*X2

and X1*X3. (Table 3). And in fact, the high significance of the X1 and X2 second order model indicates that even small variations in temperature and extraction time, can cause significantly alterations on fructans extraction yield.

The relationships between the response *Y* (fructans extraction yield) and the independent variables can be better understood by examining the response surface plots generated from the prediction model (Equation 4). Figure 2a shows the effect of extraction time (X2) and water raw material ratio (X3) on fructans extraction yield (Y). Temperature was fixed at level 0. Fructans extraction yield decreased dramatically when extraction time increased beyond 1.4 h. The water raw material ratio also affects the evaluated response; while this factor increased beyond 6 mL/g, extraction yields decreased. Figure 2b shows the effect of extraction temperature (X1) and extraction time (X2) at a fixed level of water raw material ratio, suggests that the maximum fructans extraction yield is near 80 °C, and beyond this temperature the extraction yield decreased. This behaviour could be related with the susceptibility of agave fructans to hydrolysis at elevated temperatures. Interestingly, by observing Figure 2b it is evident the significant influence ($p < 0.05$) of temperature and extraction time over the variable response. Figure 2c shows the influence of extraction temperature (X1) and water raw material ratio (X3) at a fixed extraction time (0 level). From this figure, it can be observed that small variations on extraction temperature cause significantly changes on fructans extraction. This result is related to the second order interaction (X1*X1) resulting from the ANOVA.

The optimum conditions of the different independent variables (extraction temperature of 79.1 °C, water raw material ratio of 5.13 mL/g, and extraction time of 1.48 h) were obtained by solving the model equation (Equation 4), where the maximum predicted *Y* value was 82.17%. The model was validated with additional experiments under the predicted conditions and the experimental value of the variable response was 83.12% ± 1.99 (average of three replicates), which was in agreement with the prediction of the model.

3.3 Analysis of agave crude extract by HPLC

The fructans crude extract obtained under the optimal extraction conditions was analyzed by HPLC. It is important to mention that the chromatographic analysis used in this research is not able to show detailed structural information of the polysaccharides present on the sample. However, by comparing the retention times of the utilized standards and the carbohydrates of the agave crude extract, it is possible to confirm that the studied sample contains fructans and other sugars. Then, the retention time (RT) of peak 1 is very close to the RT of chicory inulin. On the other hand, peaks 2, 3, and 4 could be related with the presence of sacarose, glucose, and fructose, respectively on the agave crude extract (Table 4). The quantification of agave fructans (peak 1) was done using a calibration curve of chicory inulin, and fructans where the principal carbohydrates contained on the sample, representing the 86.19% respect to total carbohydrates content. These results agree with the investigation of Mancilla-Margalli & López (2006), where they reported that fructans represents more than 60% of water soluble carbohydrates in five different agave species.
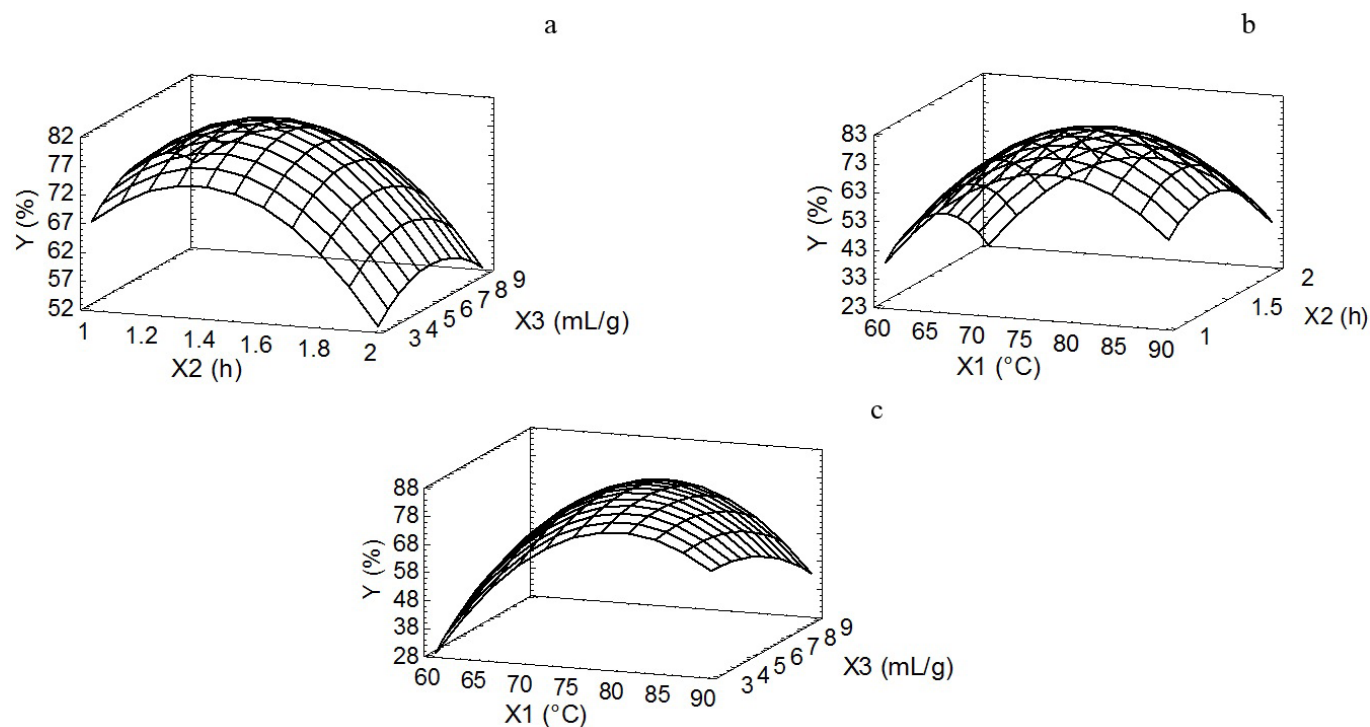
a

b

c

Figure 2. Response surface plots showing the effect of experimental factors on the response *Y*. (a) Extraction time, X2 vs. water raw material ratio, X3; (b) Extraction temperature, X1 vs. extraction time, X2; (c) Extraction temperature, X1 vs. water raw material ratio, X3.

Table 4. Retention time of standards and carbohydrates present in the agave crude extract.

Sample	Retention time (min)[a]
Peak 1	6.31 ± 0.001
Chicory inulin	6.33 ± 0.001
Peak 2	7.92 ± 0.003
Sacarose	7.80 ± 0.127
Peak 3	9.26 ± 0.040
Glucose	9.5 ± 0.213
Peak 4	12.03 ± 0.61
Fructose	12.01 ± 0.028

[a]Values of retention time are averages ± standard deviation from triplicates (n = 3) injected in the HPLC system.

4 Conclusions

Fructans extraction process by hot water diffusion from *Agave azul* pines was optimized using the response surface methodology. The independent factors that have a significant influence ($p < 0.05$) on fructans extraction yield were temperature (X1) and extraction time (X2). Besides, several second order interactions were detected (X1*X1, X2*X2 and X1*X3). A fructans extraction yield of 83.12% was achieved when optimum conditions of extraction temperature, water raw material ratio and extraction time were 79.1ºC, 5.13 mL/g and 1.48 h respectively. The analysis of agave fructans by HPLC allowed us to identify four peaks in the crude extract, matching with the retention time of chicory inulin, sacarose, glucose and fructose standards. Interestingly, fructans represented the majority of the carbohydrates in the agave crude extract. These results would contribute with the adequate exploitation of agave plants as raw material for the extraction and isolation of agave fructans which are valuable molecules that could be used as supplement or additive in the food industry.

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