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ABSTRACT

The online monitoring of chemical reactions by using benchtop Nuclear Magnetic Resonance (NMR) spectroscopy has become increasingly attractive for the past few years. The use of quantitative online NMR spectroscopy is a promising alternative to traditional analytical methods with its rapid, quantitative and non-invasive nature that makes it applicable to complex and diverse biochemical mixtures like food systems. In this study, sucrose hydrolysis by invertase was chosen as a model reaction for online monitoring. Rather than conventional NMR spectroscopy experiments that rely on the use of deuterated water, the benchtop setting allows working in protonated solvents, and tailored water suppression techniques were used to make quantification more accurate. For the hydrolysis reaction, a 10% sucrose solution was hydrolyzed. The kinetic constants determined by the fractional conversion model were comparable to the results obtained in other studies. Quantitative online NMR spectroscopy is seen as a promising tool for monitoring food processes in a continuous mode ~~and could be applied to different reactions like lactose free milk production, corn syrup production and hydrolysis of triglycerides.~~ .

Commenté [PG1]: you sid in the response that this sentence was removed!

Keywords: Benchtop NMR spectroscopy, sucrose, invertase, flow NMR, online monitoring, food applications

INTRODUCTION

Nuclear magnetic resonance (NMR) spectroscopy is one of the most common techniques used by both chemists and biochemists to identify molecular structures as well as to study the progress of chemical reactions (Marcone et al. 2013). NMR spectroscopy has several advantages over commonly used analytical techniques like high pressure liquid chromatography (HPLC), Raman spectroscopy, mass spectroscopy (MS), gas chromatography (GC). NMR spectroscopy allows non-destructive, quantitative analysis of liquid and solid samples requiring very small sample volumes. It is also characterized by a short analysis time and a high reproducibility (Kumar et al. 2015). Due to the fact that many foods contain organic compounds, with protons originating, e.g., from water, fat, carbohydrates, and proteins; proton NMR has become the most common type of NMR to determine these abundant food components. Its non-destructive and non-invasive nature has made NMR spectroscopy very popular in the fields of food chemistry, food microbiology and food packaging (Pentimalli et al. 2000; Nestor et al. 2010; Picone et al. 2011). The most studied areas are water/moisture contents, lipid content, adulteration and authentication of foods (Keeton et al. 2003; Chen et al. 2010; Hu et al. 2017; Gouilleux et al. 2018).

Despite the fact that NMR instruments date back to 1940s, because of its high capital cost, operating expenditures, size and infrastructure needs, NMR found its place in food research and industry after the first benchtop NMR relaxometry instrument was introduced by Bruker in 1973. Although benchtop relaxometry instruments have been in use in food research over 40 years, benchtop NMR spectrometers are very recent and their potential in food industry is largely unexplored (Blümich and Singh 2018). Benchtop NMR spectrometers can be used for process control purposes either by online-monitoring in a flow cell or in an NMR tube (Dalitz et al. 2012; Meyer et al.

46 2016).

47 Quantitative NMR (qNMR) was first introduced as an analytical tool for quantitative
48 analysis in 1963 (Jungnickel and Forbes 1963). ^{1}H qNMR is the most widely used
49 technique for the quantitative measurement of multi-components in complex mixtures
50 without separation of the individual components (Bharti and Roy 2012). One of the
51 most used techniques in qNMR is to use an internal standard (Giraudeau et al. 2014).
52 That also removes the need to construct a calibration curve for the experiment. The
53 most important parameters for choosing the internal reference are its solubility, the
54 absence of chemical interactions and the similar relaxation times with the analytes and
55 a distinct chemical shift.

56 Hydrolysis reactions are an important class of reactions in food industry since invert
57 sugar, corn syrup, lactose-free milk are all produced through enzyme-catalyzed
58 reactions. Sucrose is a disaccharide composed of two monosaccharides which are
59 glucose and fructose. The hydrolysis of sucrose produces equal molar amounts of
60 glucose and fructose, forming a mixture which is called invert sugar (Figure 1). This
61 invert sugar is sweeter and less prone to crystallization compared to pure sucrose syrup
62 (Cotton et al. 1955). The resulting invert sugar is used in many applications such as
63 beverage and baking industry but most commonly to produce a soft fondant center in
64 confectionery (Edwards 2009). The production of invert sugar can be achieved either
65 with acid or enzymatic hydrolysis of sucrose. Although acid hydrolysis is used
66 traditionally, it has a low conversion efficiency, needs high energy consumption, causes
67 browning of the product and could result in the formation of carcinogenic by-products
68 like hydroxymethylfurfural (HMF) (Bailey 1966). A better method for producing invert

sugar is to use invertase enzyme resulting in higher yields with none of the disadvantages of acid hydrolysis.

The competitive nature of process industry, especially food industry, requires to produce high quality products with competitive prices and to adapt the process quickly to changing customer needs. Many analytical methods are used in the food processing industry. However, most of the traditional analytical methods are time-consuming and sample-destructive. The use of quantitative online NMR spectroscopy is one such promising alternative with its rapid and non-invasive properties. It can be easily integrated to the process line in the factory with its high linearity feature between absolute signal area and sample concentration.

In this study a method for the online monitoring of one of the most widely used enzymatic reactions in food industry, sucrose hydrolysis, was tested by benchtop NMR spectroscopy, and the method quantitative performance was evaluated.

MATERIALS AND METHODS

Materials

D-(+)-Glucose, D-(−)-Fructose, Sucrose sugar and 3-(Trimethylsilyl) propionic-2,2,3,3-d_4 acid (TSP) were provided from Sigma-Aldrich Co., St. Louis, MO, USA. HPLC grade water from a water purification system (Nanopure Infinity, Barnstead International, IA) was used for the preparation of the solutions. Acetic acid glacial from VWR, Radnor, PA, USA was used for the pH adjustment. Invertase for the sucrose hydrolysis was supplied from Sigma-Aldrich Co., St. Louis, MO, USA.

Sample Preparations

10% (w/w) glucose, fructose and sucrose solutions and their mixtures (Glucose + Fructose+ Sucrose with a concentration of 10% (w/w)) were prepared separately by dissolving in non-deuterated HPLC grade water in a 50 mL tube. During solution preparation, 1% (w/w) TSP was added to all sugar solutions as the internal standard. All solutions were stirred for 2 minutes for complete dissolution of sugars and TSP. pH of the sugar solutions was adjusted to optimum working pH of the invertase enzyme (pH=4.5) with glacial acetic acid. TSP with ^{1}H chemical shift of ~~4.810~~ ppm was chosen as the chemical shift and concentration reference.

Commenté [PG2]: be careful!!

NMR Experiments

NMR experiments were performed on a low-field spectrometer operating at a 43 MHz frequency with a compact permanent magnet based on the Halbach design (Magritek Spinsolve, Wellington , NZ) (Danieli et al. 2010, 2013). This spectrometer is equipped with a gradient coil along the transverse plane of the NMR tube that can produce a maximum field gradient of 0.16 $T.m^{-1}$ and also has an external lock system which allows the use of non-deuterated solvents. For on-line monitoring, the system includes a glass flow cell with 5 mm outer diameter, a peristaltic pump (Reglo Digital, Ismatec, Wertheim, Germany) and PEEK tubing (Figure 2). The inner diameter of the flow cell is 4 mm in the measurement region (10 mm in length) and decreases to 1 mm in upper and lower regions of the measurement region to shorten the flow time during the experiment. A heating plate (RCT Basic, IKA-Werke GmbH & Co. KG, Staufen, Germany) was added to the system to control the temperature of the hydrolysis reaction. The flow rate was also optimized as explained afterwards.

Spin-Lattice Relaxation Time (T_1) Measurements

An inversion recovery sequence was used for measuring longitudinal relaxation times T_1 using an inversion time range, *t* of 0.1-10,000 ms with 15 points. The recovery curve was fitted using Equation 1.

$$I = I_0(1 - 2 \cdot exp\left(-\frac{t}{T1}\right)) \quad \text{(Eq. 1)}$$

NMR Spectroscopy Experiments

NMR experiments were carried out at 0.5 ml/min flow rate (see results and discussion for optimization of this value). The experiments were performed at 29 °C since this corresponds to the temperature at which the magnet stability is optimal. The determination of appropriate solvent suppression pulse sequence was crucial while working with non-deuterated solvents especially water. The gradient coil in the spectrometer used enabled us to use recently developed solvent suppression methods like the WET-180-NOESY pulse sequence (Gouilleux et al. 2017). This sequence gives an optimal solvent suppression for small molecules on a benchtop spectrometer, leading to a lower and narrower water signal with a clean phase with a minimal impact on nearby peaks.

The Signal-to-Noise Ratios (SNR) were calculated using the SNR calculation script of MestReNova software with 12.0.3 version (Mestrelab Research, Spain) by measuring the height of the peaks of interest and dividing these values by the noise level. The SNR has been calculated and measured as 260 at the beginning of the experiments. During the reaction, the~~.~~ ~~C~~concentration of sucrose decreased to 10 mg/g water starting from 60 mg/g ~~water~~. SNR at this concentration was calculated as 125. The limit of detection was found as 4 mg/g –corresponding to an SNR of 10– water by extrapolation. Here, 128 scans were found sufficient to yield an acceptable SNR for quantification. The 1D

^{1}H spectra were obtained with 128 scans for a total experiment time of 12 min. The 90^{o} pulse angle was achieved by a pulse length of 6.7 µs at 0 dB. The FIDs were recorded with 16 K points, a dwell time of 200 µs, and a repetition time of 6 s, corresponding to 5 times the longest T_1 (see below). The 1D data were processed with MestReNova software. All spectra were processed with a 0.2 Hz exponential apodization, an automatic phase correction and an automatic baseline correction via a Whittaker smoother algorithm. To align all the spectra correctly, TSP was used for ppm-scaling reference and set at 0 ppm. The resulting ^{1}H spectra from the different time points of hydrolysis experiment were stacked too see the progress of hydrolysis.

Quantitative Analysis

For the quantitative NMR measurements, the glycosidic proton peak of sucrose and the anomeric proton~~carbon~~ peak of glucose were chosen for hydrolysis follow up and for the quantification. Before peak integration, manual phase correction and manual baseline correction were performed in addition to the automatic correction. This procedure was found necessary to obtain a similar baseline for all the peaks that were used for integration. The resulting spectra from hydrolysis experiments were rearranged as a superimposed plot. The integral areas of the internal reference (TSP), sucrose and glucose were calculated by integration with the MestReNova software. Deconvolution tools were also evaluated but yielded a slightly lower performance than integration, probably due to the non-ideal line shapes. The integral areas of sucrose and glucose were normalized according to the integral area of TSP. Concentrations of sucrose and glucose for each time point were calculated using equation 2. The number of protons contributing to the signal for TSP was taken as 9, for glucose and sucrose, it was taken as 1.

Commenté [UW3]: This is also the anomeric 1H of sucrose ! replace gycosidic by anomeric

Commenté [UW4]: It is hard to detect 13C with a 1H/19F probehead ;)

$$C_x = \frac{A_x/N_x}{A_{TSP}/N_{TSP}} * \frac{MW_x}{MW_{TSP}} * C_{TSP} \quad \text{(Eq.2)}$$

C, *A*, *N*, and *MW* are the concentration in mg/g, the integral area in a fully relaxed ^{1}H NMR spectrum, the number of hydrogens contributing to the signals, and the molecular weight. The *x* index refers to the analyte (sucrose or glucose) while the *TSP* index refers to the internal reference.

Kinetic Modelling

The change of concentration of sucrose by time during hydrolysis was modelled using a fractional conversion model kinetics equation (Equation 3) to find the rate constant.

$$\frac{C - C_\infty}{C_o - C_\infty} = e^{-kt} \quad \text{(Eq. 3)}$$

C represents the concentration at time *t*, C_0 and C_∞ represents the initial and equilibrium concentration respectively, and k represents the rate constant.

RESULTS AND DISCUSSION

Exploring the 1D NMR Spectra

1D NMR experiments were first conducted to identify the peaks which could be used to monitor the sucrose hydrolysis reactions. Figure 3 shows the spectra of sucrose, glucose and fructose solutions and the mixture of all sugars. Because of the relatively low magnetic field, the resulting spectrum suffered from numerous overlaps between 3-4.5 ppm. The suppressed water peak can be seen at 4.9 ppm. Suppressing the water signal is critical especially at low-field because overlapping issues become more stringent due to the reduced spectral width at low frequency. Although the beta anomeric proton of glucose is overlapped with the water peak, the alpha anomeric

proton of glucose is clearly seen at 5.3 ppm. In addition, the glycosidic proton of sucrose at 5.48 ppm is very distinct. These two peaks can also be seen clearly in the NMR spectrum of the mixture solution. Therefore, they were chosen to monitor the hydrolysis of sucrose by the invertase enzyme.

Determination of Optimal Flow Rate

The choice of the optimal flow rate is one of the critical parameters in flow NMR experiments as the signal becomes broader and the resolution decreases with increasing flow rates. In flow NMR, 'inflow' and 'outflow' effects need to be considered carefully. During the experiment, the excited spins in the sensitive volume are refreshed by unexcited ones, leading to shorter apparent T_1. This 'inflow' effect allows shorter repetition times (TR) to reach quantitative conditions (Nordon et al. 2001). But when the flow rate is significantly high, excited spins leave the sensitive volume before the acquisition of the FID is finished. This is called 'outflow' effect. This causes severe line broadening and affects the resolution of the NMR spectra (Gouilleux et al. 2017). To determine the optimum flow rate to be used in hydrolysis experiments, NMR spectra of the model mixture were recorded in non-flow conditions and in flow with 0.5 ml/min, 1 ml/min and 2 ml/min flow rates. As observed in Figure 4, when the flow rate increases from 0 ml/min to 2 ml/min, the peaks became broader and the glucose peak overlapped with the water peak, as a consequence of the outflow effect. Therefore, it was decided to use 0.5 ml/min flow rate in the online monitoring of hydrolysis reactions, which resulted in a modest reduction of longitudinal relaxation times (see below) resulting from the inflow effect.

Spin-Lattice Relaxation Time (T_1) and Repetition Time (T_R) Measurements

As stated before, spin-lattice relaxation times (T_1) were measured by the Inversion

Recovery pulse sequence. For quantitative measurements, the peak of interest with the longest T_1 is the limiting factor, in our case TSP. For the corresponding peak, T_1 values were measured as 1.2, 1.1, 0.97 and 0.93 s in non-flow and in flow conditions with the flow rates of 0.5 ml/min, 1 ml/min and 2 ml/min, respectively. Here, the choice of a 0.5 ml/min flow rate resulted in a negligible reduction of the relaxation time, but was necessary to avoid significant broadening (see above). In order to ensure a 1% accuracy on the quantitative measurements, the repetition time TR was set to about 5 times the longest T_1, leading to TR = 6 s.

Online Monitoring and Kinetic Modelling of Sucrose Hydrolysis with qNMR Measurements

In Figure 5, the consumption of sucrose and the production of glucose in the course of the hydrolysis are clearly observable. At time around 240 min, the integral area of sucrose reaches to a constant value and does not change after this time.

Hydrolysis experiments were repeated 3 times and the change of the mean concentrations of sucrose and glucose as a function of time can be seen in Figure 6, showing the good repeatability of the experiments. In thise figure, it is seen that 1/6 of the sucrose ~~not being~~was not converted. This might have been caused by invertase optimum temperature and substrate inhibition. The optimum temperature for the activity of invertase is between 50 °C -60 °C. However, the experiments were performed at 29 °C because this corresponds to the temperature at which the magnet stability is optimal. This may affect the reaction rate and the amount sucrose converted. Also, the activity of invertase is affected by the substrate concentration. The maximum rate of hydrolysis is obtained with a sucrose concentration of 5 to 10%, and then with higher concentrations, the rate decreases (Bergmeyer, 1965). In the experiments, 10%

sucrose concentration has been used and this may affect the amount of sucrose being converted.

The mean concentrations of TSP were also plotted as a function of time to demonstrate baseline variation of the experiments (Figure 7). The mean curve was plotted for sucrose concentration and the data were fitted by equation 3. The average rate constant was found as $k = [12.88 \pm 1.96] \times 10^{-3}$ min^{-1} (Figure 8) at 29°C. -Vu and Le (2008) studied the kinetics of the immobilized and free invertase and found the kinetic constant for free invertase as 1.8×10^{-3} min^{-1}, 4.1×10^{-3} min^{-1}, 3.2×10^{-2} min^{-1} at 50°C, 55°C, 60°C respectively (Vu and Le 2008). In the aforementioned study, although a different approach (a new enzyme-catalyzed reaction was performed every time to see how the activity changed with respect to the initial enzyme-catalyzed reaction) was followed to calculate the activity of the enzyme, in terms of order of magnitude, values were found to be reasonably close to the ones obtained in this study (~10^{-3}). The differences might have been caused by the temperature, the initial concentration or the activity of the enzyme used in this study.

CONCLUSION

This study shows that benchtop quantitative ^{1}H NMR is a relatively easy and rapid method for the online monitoring and quantification of enzymatic reactions, which can easily be implemented for a variety of food chemistry applications and it could play a central role in the control of production processes in food industry.

Although not performed here, since it is now shown that such reactions can easily be monitored with this technique, as a further study the reaction parameters ~~can~~ could be changed and Michaelis Menten kinetic analysis could be conducted to find the kinetic parameters.

Compliance with Ethical Standards

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Conflict of Interest: Alper Soyler declares that he has no conflict of interest. Dylan Bouillaud declares that he has no conflict of interest. Jonathan Farjon declares that he has no conflict of interest. Patrick Giraudeau declares that he has no conflict of interest. Mecit H. Oztop declares that he has no conflict of interest

Commenté [PG5]: why not writing that all the authors declare that they have no conflict of interest?

Ethical Approval: This article does not contain any studies with human or animal subjects.

Informed Consent: Publication has been approved by all individual participants.

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FIGURE CAPTIONS

Figure 1. Hydrolysis of sucrose into glucose and fructose.

Figure 2. System setup for online reaction monitoring

Figure 3. 1D ^{1}H NMR spectra of sucrose solution, glucose solution, fructose solution and mixture of all sugars recorded at 43 MHz with water signal suppression.

Figure 4. 1D ^{1}H NMR spectra of model mixture solution recorded at 43 MHz in different flow rates of 0 ml/min, 0.5 ml/min, 1.0 ml/min, 2 ml/min, with water signal suppression.

Figure 5. 1D ^{1}H NMR spectra of sucrose hydrolysis recorded at 43 MHz.

Figure 6. Sucrose and glucose concentration changes in the course of sucrose hydrolysis by invertase, monitored by quantitative ^{1}H NMR benchtop spectroscopy. Error bars are standard deviations calculated on 3 successive experiments performed in the same conditions.

Figure 7. The mean concentration changes of TSP as a function of time. Error bars are standard deviations calculated on 3 successive experiments performed in the same conditions.

Figure 8. First order reaction kinetics obtained by adjusting the time evolution of sucrose concentration by a fractional conversion model.

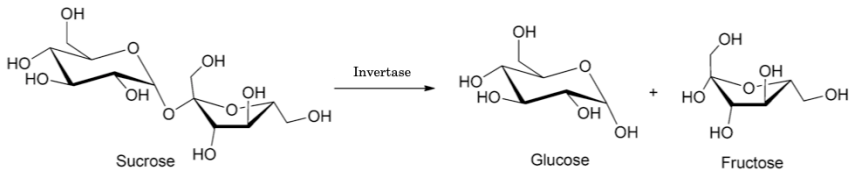


Figure 1

Figure 2

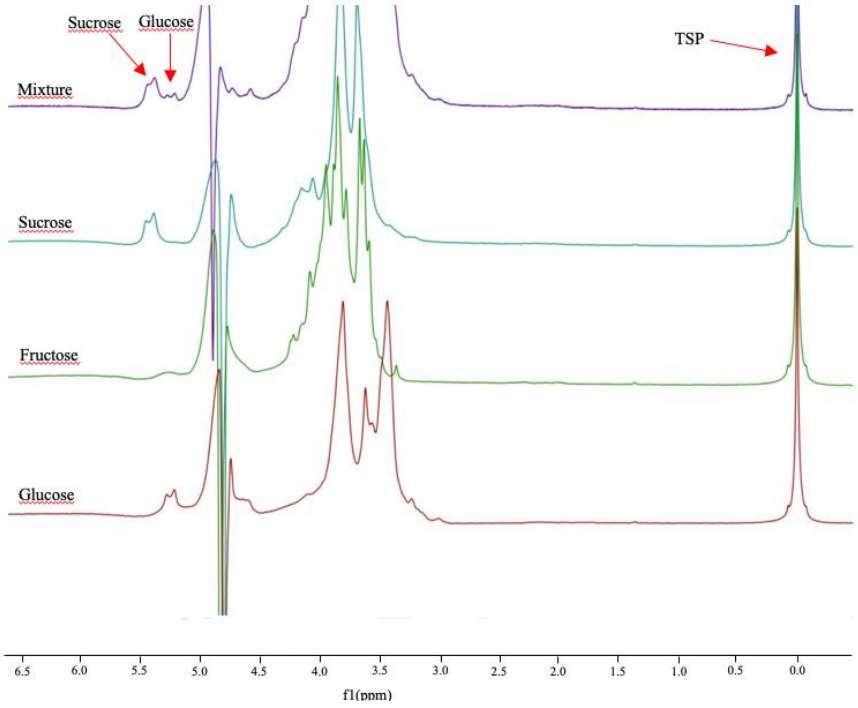

Sucrose
Glucose
TSP
Mixture
Sucrose
Fructose
Glucose
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
f1(ppm)

Figure 3

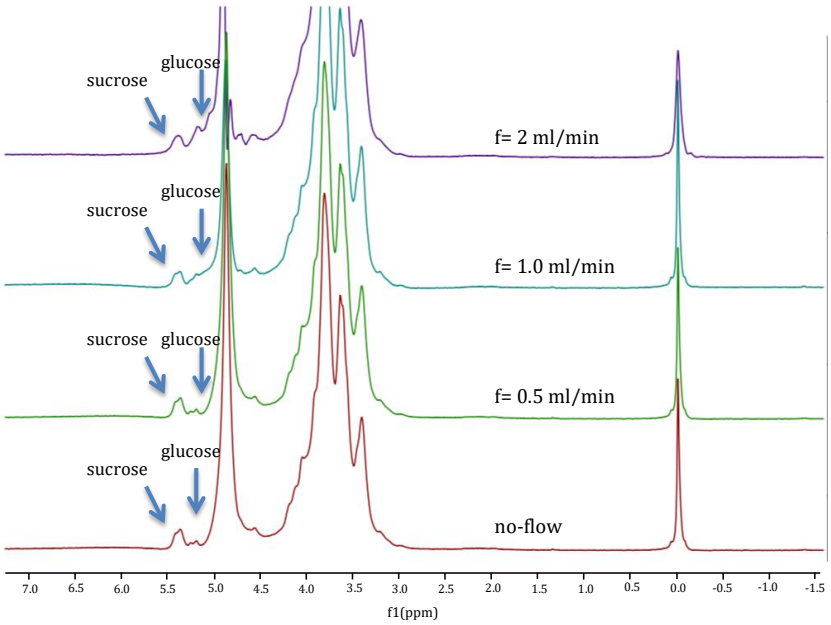

sucrose
glucose
f= 2 ml/min
sucrose
glucose
f= 1.0 ml/min
sucrose
glucose
f= 0.5 ml/min
sucrose
glucose
no-flow
7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0 -0.5 -1.0 -1.5
f1(ppm)

Figure 4

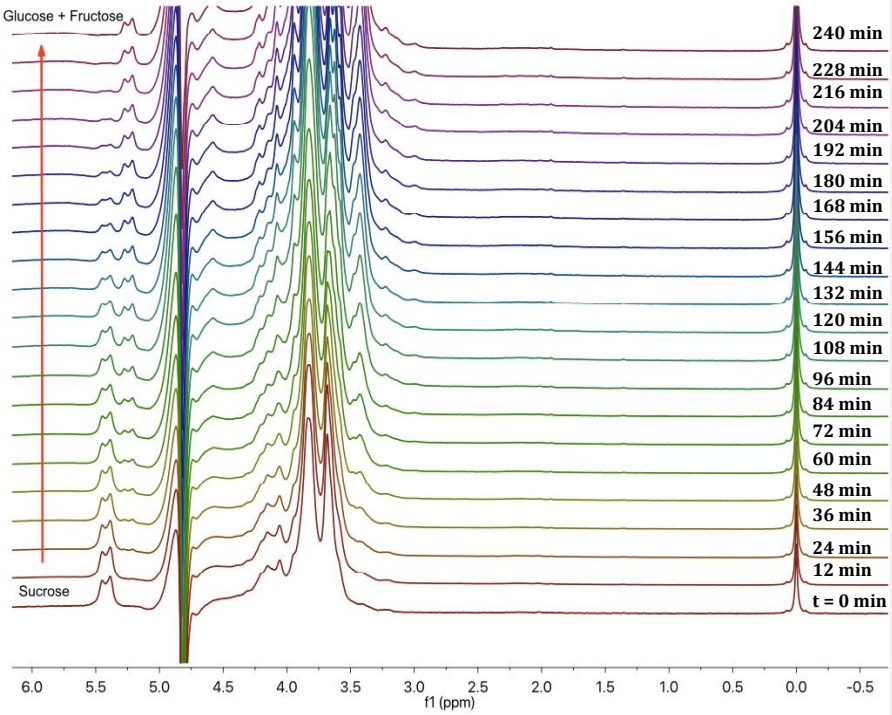

Glucose + Fructose
Sucrose
240 min
228 min
216 min
204 min
192 min
180 min
168 min
156 min
144 min
132 min
120 min
108 min
96 min
84 min
72 min
60 min
48 min
36 min
24 min
12 min
t = 0 min
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
f1 (ppm)

Figure 5

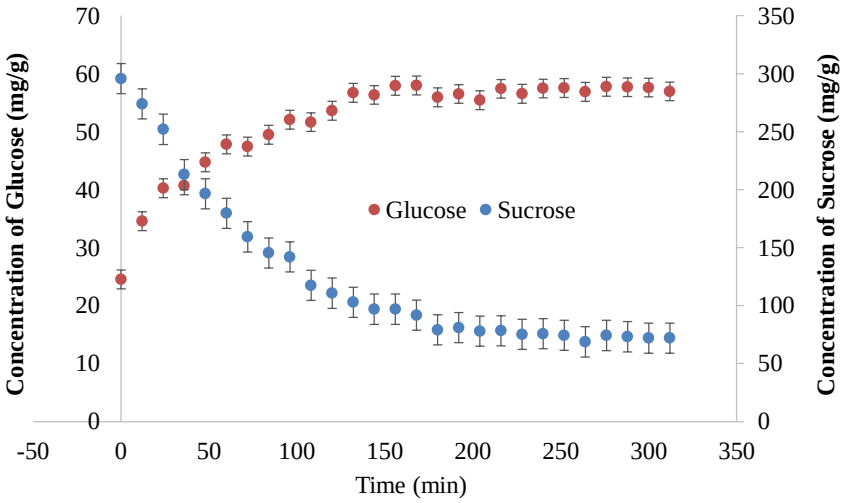

Concentration of Glucose (mg/g)
Concentration of Sucrose (mg/g)
Glucose
Sucrose
Time (min)
70
60
50
40
30
20
10
0
350
300
250
200
150
100
50
0
-50
0
50
100
150
200
250
300
350

Figure 6

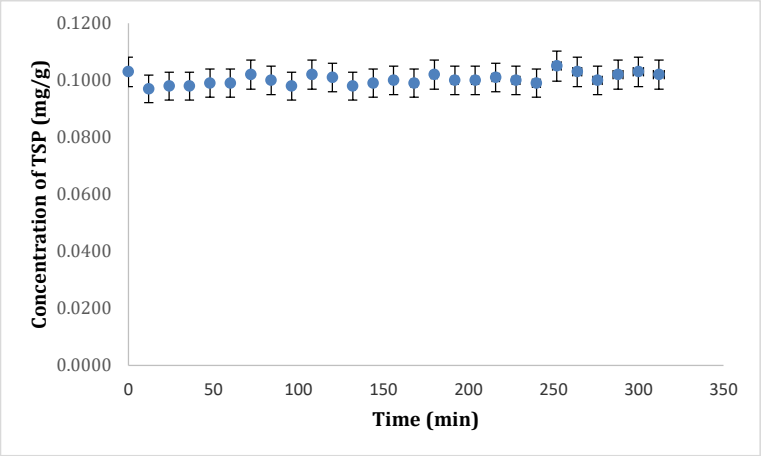
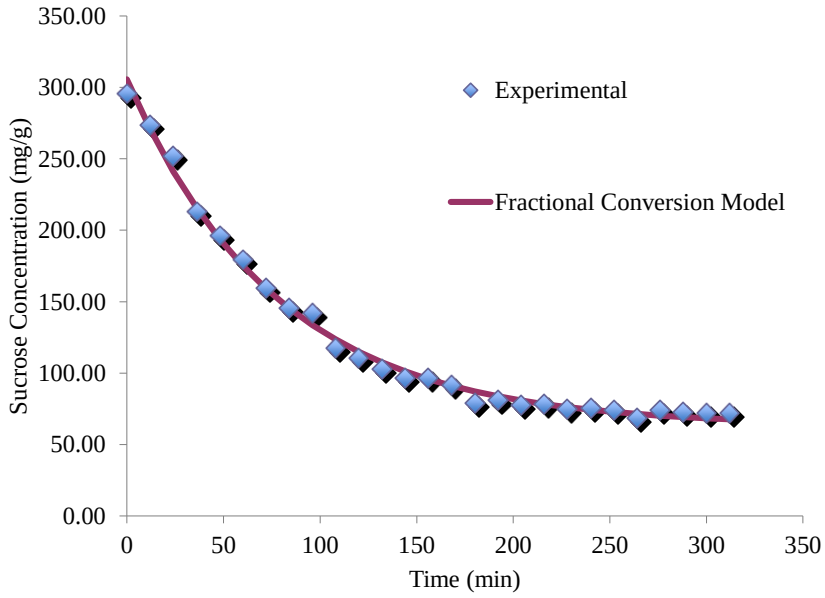

0.1200
0.1000
0.0800
0.0600
0.0400
0.0200
0.0000
Concentration of TSP (mg/g)
0
50
100
150
200
250
300
350
Time (min)

Figure 7

473

474



475

476 **Figure 8**

477