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Real-time benchtop NMR spectroscopy for the online monitoring of sucrose

hydrolysis

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1 ABSTRACT

2 The online monitoring of chemical reactions by using benchtop Nuclear Magnetic 3 Resonance (NMR) spectroscopy has become increasingly attractive for the past few 4 years. The use of quantitative online NMR spectroscopy is a promising alternative to 5 traditional analytical methods with its rapid, quantitative and non-invasive nature that 6 makes it applicable to complex and diverse biochemical mixtures like food systems. In 7 this study, sucrose hydrolysis by invertase was chosen as a model reaction for online 8 monitoring. Rather than conventional NMR spectroscopy experiments that rely on the 9 use of deuterated water, the benchtop setting allows working in protonated solvents, 10 and tailored water suppression techniques were used to make quantification more 11 accurate. For the hydrolysis reaction, a 10% sucrose solution was hydrolyzed. The 12 kinetic constants determined by the fractional conversion model were comparable to 13 the results obtained in other studies. Quantitative online NMR spectroscopy is seen as 14 a promising tool for monitoring food processes in a continuous mode and could be 15 applied to different reactions like lactose free milk production, corn syrup production 16 and hydrolysis of triglycerides.

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

17

18 Keywords: Benchtop NMR spectroscopy, sucrose, invertase, flow NMR, online

- 19 monitoring, food applications
- 20

21 INTRODUCTION

22 Nuclear magnetic resonance (NMR) spectroscopy is one of the most common 23 techniques used by both chemists and biochemists to identify molecular structures as 24 well as to study the progress of chemical reactions (Marcone et al. 2013). NMR 25 spectroscopy has several advantages over commonly used analytical techniques like 26 high pressure liquid chromatography (HPLC), Raman spectroscopy, mass spectroscopy 27 (MS), gas chromatography (GC). NMR spectroscopy allows non-destructive, 28 quantitative analysis of liquid and solid samples requiring very small sample volumes. 29 It is also characterized by a short analysis time and a high reproducibility (Kumar et al. 30 2015). Due to the fact that many foods contain organic compounds, with protons 31 originating, e.g., from water, fat, carbohydrates, and proteins; proton NMR has become 32 the most common type of NMR to determine these abundant food components. Its non-33 destructive and non-invasive nature has made NMR spectroscopy very popular in the 34 fields of food chemistry, food microbiology and food packaging (Pentimalli et al. 2000; 35 Nestor et al. 2010; Picone et al. 2011). The most studied areas are water/moisture 36 contents, lipid content, adulteration and authentication of foods (Keeton et al. 2003; 37 Chen et al. 2010; Hu et al. 2017; Gouilleux et al. 2018).

38 Despite the fact that NMR instruments date back to 1940s, because of its high capital 39 cost, operating expenditures, size and infrastructure needs, NMR found its place in food 40 research and industry after the first benchtop NMR relaxometry instrument was 41 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 42 43 their potential in food industry is largely unexplored (Blümich and Singh 2018). 44 Benchtop NMR spectrometers can be used for process control purposes either by 45 online-monitoring in a flow cell or in an NMR tube (Dalitz et al. 2012; Meyer et al. 3 46 2016).

47 Quantitative NMR (qNMR) was first introduced as an analytical tool for quantitative 48 analysis in 1963 (Jungnickel and Forbes 1963). ¹H 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 thedisadvantages of acid hydrolysis.

71 The competitive nature of process industry, especially food industry, requires to 72 produce high quality products with competitive prices and to adapt the process quickly 73 to changing customer needs. Many analytical methods are used in the food processing 74 industry. However, most of the traditional analytical methods are time-consuming and 75 sample-destructive. The use of quantitative online NMR spectroscopy is one such 76 promising alternative with its rapid and non-invasive properties. It can be easily 77 integrated to the process line in the factory with its high linearity feature between 78 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.

82 MATERIALS AND METHODS

83 Materials

D-(+)-Glucose, D-(-)-Fructose, Sucrose sugar and 3-(Trimethylsilyl) propionic2,2,3,3-d₄ 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.

90

92 Sample Preparations

93 10% (w/w) glucose, fructose and sucrose solutions and their mixtures (Glucose +
94 Fructose+ Sucrose with a concentration of 10% (w/w)) were prepared separately by
95 dissolving in non-deuterated HPLC grade water in a 50 mL tube. During solution
96 preparation, 1% (w/w) TSP was added to all sugar solutions as the internal standard.
97 All solutions were stirred for 2 minutes for complete dissolution of sugars and TSP. pH
98 of the sugar solutions was adjusted to optimum working pH of the invertase enzyme
99 (pH=4.5) with glacial acetic acid. TSP with ¹H chemical shift of 4-810 ppm was chosen

Commenté [PG2]: be careful!!

101 NMR Experiments

as the chemical shift and concentration reference.

100

102 NMR experiments were performed on a low-field spectrometer operating at a 43 MHz 103 frequency with a compact permanent magnet based on the Halbach design (Magritek 104 Spinsolve, Wellington, NZ) (Danieli et al. 2010, 2013). This spectrometer is equipped 105 with a gradient coil along the transverse plane of the NMR tube that can produce a 106 maximum field gradient of 0.16 T.m-1 and also has an external lock system which 107 allows the use of non-deuterated solvents. For on-line monitoring, the system includes 108 a glass flow cell with 5 mm outer diameter, a peristaltic pump (Reglo Digital, Ismatec, 109 Wertheim, Germany) and PEEK tubing (Figure 2). The inner diameter of the flow cell 110 is 4 mm in the measurement region (10 mm in length) and decreases to 1 mm in upper 111 and lower regions of the measurement region to shorten the flow time during the 112 experiment. A heating plate (RCT Basic, IKA-Werke GmbH & Co. KG, Staufen, 113 Germany) was added to the system to control the temperature of the hydrolysis reaction. 114 The flow rate was also optimized as explained afterwards.

116 Spin-Lattice Relaxation Time (T₁) Measurements

An inversion recovery sequence was used for measuring longitudinal relaxation times
T₁ using an inversion time range, *t* of 0.1-10,000 ms with 15 points. The recovery curve
was fitted using Equation 1.

120
$$I = I_0 (1 - 2 \cdot exp\left(-\frac{t}{T_1}\right))$$
 (Eq. 1)

121 NMR Spectroscopy Experiments

122 NMR experiments were carried out at 0.5 ml/min flow rate (see results and discussion 123 for optimization of this value). The experiments were performed at 29 °C since this 124 corresponds to the temperature at which the magnet stability is optimal. The 125 determination of appropriate solvent suppression pulse sequence was crucial while 126 working with non-deuterated solvents especially water. The gradient coil in the 127 spectrometer used enabled us to use recently developed solvent suppression methods 128 like the WET-180-NOESY pulse sequence (Gouilleux et al. 2017). This sequence gives 129 an optimal solvent suppression for small molecules on a benchtop spectrometer, leading 130 to a lower and narrower water signal with a clean phase with a minimal impact on 131 nearby peaks.

132	The Signal-to-Noise Ratios (SNR) were calculated using the SNR calculation script of
133	MestReNova software with 12.0.3 version (Mestrelab Research, Spain) by measuring
134	the height of the peaks of interest and dividing these values by the noise level. The SNR
135	has been calculated and measured as 260 at the beginning of the experiments. During
136	the reaction. the- Concentration of sucrose decreased to 10_mg/g water starting from
137	60 mg/g-water. SNR at this concentration was calculated as 125. The limit of detection
138	was found as 4 mg/g <u>–corresponding to an SNR of 10–</u> water by extrapolation. Here,
139	128 scans were found sufficient to yield an acceptable SNR for quantification. The 1D
	7

140 ¹H spectra were obtained with 128 scans for a total experiment time of 12 min. The 90° 141 pulse angle was achieved by a pulse length of 6.7 µs at 0 dB. The FIDs were recorded 142 with 16 K points, a dwell time of 200 µs, and a repetition time of 6 s, corresponding to 143 5 times the longest T1 (see below). The 1D data were processed with MestReNova 144 software. All spectra were processed with a 0.2 Hz exponential apodization, an 145 automatic phase correction and an automatic baseline correction via a Whittaker 146 smoother algorithm. To align all the spectra correctly, TSP was used for ppm-scaling 147 reference and set at 0 ppm. The resulting ¹H spectra from the different time points of

148 hydrolysis experiment were stacked too see the progress of hydrolysis.

149 Quantitative Analysis

150 For the quantitative NMR measurements, the glycosidic proton peak of sucrose and the 151 anomeric proton carbon peak of glucose were chosen for hydrolysis follow up and for 152 the quantification. Before peak integration, manual phase correction and manual 153 baseline correction were performed in addition to the automatic correction. This 154 procedure was found necessary to obtain a similar baseline for all the peaks that were 155 used for integration. The resulting spectra from hydrolysis experiments were rearranged 156 as a superimposed plot. The integral areas of the internal reference (TSP), sucrose and 157 glucose were calculated by integration with the MestReNova software. Deconvolution 158 tools were also evaluated but yielded a slightly lower performance than integration, 159 probably due to the non-ideal line shapes. The integral areas of sucrose and glucose 160 were normalized according to the integral area of TSP. Concentrations of sucrose and 161 glucose for each time point were calculated using equation 2. The number of protons 162 contributing to the signal for TSP was taken as 9, for glucose and sucrose, it was taken 163 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 ;)

164
$$C_{\chi} = \frac{A_{\chi}/N_{\chi}}{A_{TSP}/N_{TSP}} * \frac{MW_{\chi}}{MW_{TSP}} * C_{TSP} \quad (Eq.2)$$

165 *C*, *A*, *N*, and *MW* are the concentration in mg/g, the integral area in a fully relaxed ¹H 166 NMR spectrum, the number of hydrogens contributing to the signals, and the molecular 167 weight. The *x* index refers to the analyte (sucrose or glucose) while the *TSP* index refers 168 to the internal reference.

169 Kinetic Modelling

170 The change of concentration of sucrose by time during hydrolysis was modelled using

171 a fractional conversion model kinetics equation (Equation 3) to find the rate constant.

172
$$\frac{c-c_{\infty}}{c_o-c_{\infty}} = e^{-kt}$$
 (Eq. 3)

173 *C* represents the concentration at time *t*, C_0 and C_∞ represents the initial and equilibrium 174 concentration respectively, and k represents the rate constant.

175

176 RESULTS AND DISCUSSION

177 Exploring the 1D NMR Spectra

178 1D NMR experiments were first conducted to identify the peaks which could be used 179 to monitor the sucrose hydrolysis reactions. Figure 3 shows the spectra of sucrose, 180 glucose and fructose solutions and the mixture of all sugars. Because of the relatively 181 low magnetic field, the resulting spectrum suffered from numerous overlaps between 182 3-4.5 ppm. The suppressed water peak can be seen at 4.9 ppm. Suppressing the water 183 signal is critical especially at low-field because overlapping issues become more 184 stringent due to the reduced spectral width at low frequency. Although the beta 185 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.

190 Determination of Optimal Flow Rate

191 The choice of the optimal flow rate is one of the critical parameters in flow NMR 192 experiments as the signal becomes broader and the resolution decreases with increasing 193 flow rates. In flow NMR, 'inflow' and 'outflow' effects need to be considered carefully. 194 During the experiment, the excited spins in the sensitive volume are refreshed by 195 unexcited ones, leading to shorter apparent T1. This 'inflow' effect allows shorter 196 repetition times (TR) to reach quantitative conditions (Nordon et al. 2001). But when 197 the flow rate is significantly high, excited spins leave the sensitive volume before the 198 acquisition of the FID is finished. This is called 'outflow' effect. This causes severe 199 line broadening and affects the resolution of the NMR spectra (Gouilleux et al. 2017). 200 To determine the optimum flow rate to be used in hydrolysis experiments, NMR spectra 201 of the model mixture were recorded in non-flow conditions and in flow with 0.5 ml/min, 202 1 ml/min and 2 ml/min flow rates. As observed in Figure 4, when the flow rate increases 203 from 0 ml/min to 2 ml/min, the peaks became broader and the glucose peak overlapped 204 with the water peak, as a consequence of the outflow effect. Therefore, it was decided 205 to use 0.5 ml/min flow rate in the online monitoring of hydrolysis reactions, which 206 resulted in a modest reduction of longitudinal relaxation times (see below) resulting 207 from the inflow effect.

208 Spin-Lattice Relaxation Time (T_I) and Repetition Time (T_R) Measurements

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

210	Recovery pulse sequence. For quantitative measurements, the peak of interest with the				
211	longest T_1 is the limiting factor, in our case TSP. For the corresponding peak, T_1 values				
212	were measured as 1.2, 1.1, 0.97 and 0.93 s in non-flow and in flow conditions with the				
213	flow rates of 0.5 ml/min, 1 ml/min and 2 ml/min, respectively. Here, the choice of a 0.5				
214	ml/min flow rate resulted in a negligible reduction of the relaxation time, but was				
215	necessary to avoid significant broadening (see above). In order to ensure a 1% accuracy				
216	on the quantitative measurements, the repetition time TR was set to about 5 times the				
217	longest T_1 , leading to $TR = 6$ s.				
218					
210	Online Monitoring and Kinetic Modelling of Sucrose Hydrolysis with qNMR				
210	Measurements				
219 220	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				
213 219 220 221	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				
 210 219 220 221 222 	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.				

224 concentrations of sucrose and glucose as a function of time can be seen in Figure 6, 225 showing the good repeatability of the experiments. In thise figure, it is seen that 1/6 of 226 the sucrose not being was not converted. This might have been caused by invertase 227 optimum temperature and substrate inhibition. The optimum temperature for the 228 activity of invertase is between 50 °C -60 °C. However, the experiments were 229 performed at 29 °C because this corresponds to the temperature at which the magnet 230 stability is optimal. This may affect the reaction rate and the amount sucrose converted. 231 Also, the activity of invertase is affected by the substrate concentration. The maximum 232 rate of hydrolysis is obtained with a sucrose concentration of 5 to 10%, and then with 233 higher concentrations, the rate decreases (Bergmeyer, 1965). In the experiments, 10%

234	sucrose concentration has been used and this may affect the amount of sucrose being
235	converted.
236	The mean concentrations of TSP were also plotted as a function of time to demonstrate
237	baseline variation of the experiments (Figure 7). The mean curve was plotted for
238	sucrose concentration and the data were fitted by equation 3. The average rate constant
239	was found as k_=_[12.88_±_1.96]_x_10 ⁻³ min ⁻¹ (Figure 8) at 29°CVu and Le (2008)
240	studied the kinetics of the immobilized and free invertase and found the kinetic constant
241	for free invertase as 1.8 _x 10 ⁻³ min ⁻¹ , 4.1 _x 10 ⁻³ min ⁻¹ , 3.2 _x 10 ⁻² min ⁻¹ at 50°C, 55°C,
242	60°C respectively (Vu and Le 2008). In the aforementioned study, although a different
243	approach (a new enzyme-catalyzed reaction was performed every time to see
244	how the activity changed with respect to the initial enzyme-catalyzed reaction) was
245	followed to calculate the activity of the enzyme, in terms of order of magnitude, values
246	were found to be reasonably close to the ones obtained in this study ($\sim 10^{-3}$). The
247	differences might have been caused by the temperature, the initial concentration or the
248	activity of the enzyme used in this study.
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257 CONCLUSION

- 258 This study shows that benchtop quantitative ¹H NMR is a relatively easy and rapid
- 259 method for the online monitoring and quantification of enzymatic reactions, which can
- 260 easily be implemented for a variety of food chemistry applications and it could play a
- 261 central role in the control of production processes in food industry.
- 262 Although not performed here, since it is now shown that such reactions can easily be
- 263 monitored with this technique, as a further study the reaction parameters can-could be
- 264 changed and Michaelis Menten kinetic analysis could be conducted to find the kinetic
- 265 parameters.

266 Compliance with Ethical Standards

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- 272 **Conflict of Interest:** Alper Soyler declares that he has no conflict of interest. Dylan
- 273 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.
- 275 Mecit H. Oztop declares that he has no conflict of interest
- 276 Ethical Approval: This article does not contain any studies with human or animal
- 277 subjects.
- 278 Informed Consent: Publication has been approved by all individual participants.

279

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

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- 345
- 346

348 FIGURE CAPTIONS

- 349 Figure 1. Hydrolysis of sucrose into glucose and fructose.
- 350 Figure 2. System setup for online reaction monitoring
- **Figure 3.** 1D ¹H NMR spectra of sucrose solution, glucose solution, fructose solution
- and mixture of all sugars recorded at 43 MHz with water signal suppression.
- 353 Figure 4. 1D ¹H NMR spectra of model mixture solution recorded at 43 MHz in
- 354 different flow rates of 0 ml/min, 0.5 ml/min, 1.0 ml/min, 2 ml/min, with water signal
- 355 suppression.
- **Figure 5.** 1D ¹H NMR spectra of sucrose hydrolysis recorded at 43 MHz.
- 357 Figure 6. Sucrose and glucose concentration changes in the course of sucrose
- 358 hydrolysis by invertase, monitored by quantitative ¹H NMR benchtop spectroscopy.
- 359 Error bars are standard deviations calculated on 3 successive experiments performed in
- the same conditions.
- 361 Figure 7. The mean concentration changes of TSP as a function of time. Error bars are
- 362 standard deviations calculated on 3 successive experiments performed in the same363 conditions.
- 364 Figure 8. First order reaction kinetics obtained by adjusting the time evolution of365 sucrose concentration by a fractional conversion model.
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- Figure 2























