Online monitoring of Enzymatic Hydrolysis of Marine By-products using Benchtop Nuclear Magnetic Resonance Spectroscopy

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Abstract

Enzymatic hydrolysis is becoming a more commonly used method to create high value products from traditionally low value marine by-products. However, improvement to processing is hampered by a lack of ways to characterize the reaction in real time. Current methods of analysis rely on taking offline samples, deactivating the enzymes, and performing analysis on the products afterwards. Nuclear magnetic resonance benchtop spectroscopy was investigated as a method for online process monitoring of enzymatic hydrolysis. Online and offline NMR measurements were performed for enzymatic hydrolysis reactions on red cod, salmon and shrimp. Both the online and offline measurements were able to follow the reaction process and showed good agreement in their calculated reaction rate. Application of the methodology to several types of raw materials indicates the technique is robust with regards to sample type. Advantages and disadvantages of low-field versus high-field NMR spectroscopy are discussed as well as practical considerations needed in order to apply the method industrially.

Keywords: enzymatic hydrolysis; nuclear magnetic resonance spectroscopy; process monitoring; marine byproducts

1 INTRODUCTION

Focus on sustainability in marine resources has increased research into ways to obtain more value from them without increasing catch sizes. One effort is to find more profitable uses of by-products (Shahidi 2006). Portions of marine products leftover after food production, such as heads, entrails, or skin, have historically been used mainly as fish feed, a low value product (Rustad et al. 2011). In recent years, functional peptides and amino acids have seen increased use in high value products such as functional foods, health food supplements, pharmaceuticals, and cosmetics (Aspevik et al. 2017). These molecules can be produced efficiently from marine by-products by enzymatic hydrolysis (Kristinsson and Rasco 2000a; Sathivel et al. 2005; Slizyte et al. 2009). Peptidases are enzymes that cut the peptide bonds in proteins and therefore can be used to cleave the functional molecules from the large proteins in the by-products. Different enzymes have different behaviors. Exopeptidase cut from the ends of proteins while endopeptidase can cleave the proteins in the middle. Exopeptidase tend to be general in the peptide bonds they can cleave, while endopeptidase tend to be specific to the amino acids adjacent to the peptide bonds they cut. How an enzymatic reaction proceeds and the functional molecules it creates is dependent on the starting materials, the enzymes used, and the reaction conditions (Mahmoud et al. 1992, Hoyle and Merritt 1994; Maehashi et al. 1999; Ovissipour 2009). Therefore, considerable research is underway on enzymatic hydrolysis to better understand how these factors influence the final products in order to improve control over the process (Kristinsson and Rasco 2000b; Pagan et al. 2013; Dong et al. 2014; Piazza et al. 2014; Klomklao and Benjakul 2016; Galanakis 2019).

A challenge of research into enzymatic hydrolysis is that it is difficult to monitor the reaction in real time (Galanakis 2019). Degree of hydrolysis (DH), one of the most commonly used methods to describe

the progress of enzymatic hydrolysis, is the ratio of the cleaved peptide bonds in a hydrolysate to the total peptide bonds (Rutherfurd 2010). This measures how broken down the protein is relative to its initial state at several points in time, and then is used to calculate a rate constant. However, several methods for measuring DH (e.g. OPA and TNBS) cannot be performed in real time. While the pH-Stat method can be done in real time, the need to titrate sodium hydroxide limits the method to small test reactions and therefore cannot be used for monitoring on an industrial scale (Rutherford 2010). The pH-Stat method also does not give information about protein concentration. Common methods to determine protein concentration in hydrolysate are all performed offline. Samples must be taken, the enzymes thermally deactivated, and then frequently freeze-dried prior to some methods of determining protein recovery. Other methods, such as UV, require the samples to be diluted in order to measure protein concentration (Wider and Dreier, 2006). Both approaches are time consuming, difficult to automate, and limiting in terms of numbers of samples that can be analysed. It is controversial whether offline samples directly relate to the online conditions, as the enzymatic hydrolysis process will proceed for a period of time during the deactivation process and the elevated heating of the samples may also produce changes. All these issues make the goal of finding a method to perform on-line monitoring of reaction progress and protein concentration appealing. However, in order to monitor enzymatic hydrolysis in real time, a technique must meet several aspects of operational conditions. The method must be rapid, non-invasive, and able to be performed on opaque multiphase samples that often contain solid, liquid, and gas (i.e. air bubbles).

One technique that shows promise for online monitoring of enzymatic hydrolysis is nuclear magnetic resonance. Nuclear magnetic resonance (NMR) functions by placing a sample in a strong applied magnetic field (Levitt 2001). NMR experiments are then performed using what is referred to as a “pulse sequence”, which is used to measure a specific property about the sample. In chemistry, NMR can be used to give information about a sample’s molecular composition and structure. The electron density of bonds in molecules will cause small changes in the applied magnetic field, called “chemical shift”. Looking at the signal intensity at different chemical shifts can be used to identify constituents like

metabolites (Dona et al. 2016) and amino acids (Wüthrich 1986). NMR is frequently used to measure the quantity and structure of molecules containing hydrogen in samples, but may also be used to measure these properties for other biologically relevant elements such as carbon, nitrogen, or phosphorus. Quantitative NMR, often called qNMR, uses the signal intensity to determine concentration of species in solution (Wider and Dreier 2006; Bharti and Roy, 2012). This ability has made nuclear magnetic resonance a common technique in characterization of proteins.

Researchers have recently used high-field NMR spectroscopy to perform real-time monitoring of enzymatic hydrolysis (Sundekilde et al. 2018). They placed a small amount of minced chicken in a test tube along with enzymes and deuterated water and heated the mixture inside a 600MHz NMR magnet. Measurements at several points in time were made to observe the production of 40 different metabolites in the hydrolysate. The signal from hydrogen in undigested protein decays very rapidly and therefore disappears before the NMR signal can be acquired. In contrast, the signal from protein in solution decays more slowly and can be easily measured by NMR equipment. By monitoring the increase in signal of the amino acids of the peptides in the solution, the researchers were able to estimate the reaction rate for the two different types of enzymes used.

However, there have been practical limitations for using NMR spectroscopy as a process monitoring technique outside research applications (Colnago et al. 2014). Firstly, equipment has been prohibitively expensive. Typical high-field NMR spectroscopy equipment can cost millions of dollars, which has limited it to mainly universities and industrial research centers. The superconducting magnets also require dedicated facilities and staff, as well as regular filling with liquid helium and liquid nitrogen. This makes high-field spectroscopy equipment both expensive and impractical to maintain, particularly given processing plants often are located in remote areas.

In the past five years, a new type of NMR equipment has become available, the benchtop NMR spectrometer (Blumich 2016). While benchtop NMR equipment has been available for decades, it has been limited to relaxometry and diffusion measurements, as the magnetic field strengths possible

were too weak and inhomogeneous for spectroscopy. Benchtop NMR spectroscopy equipment has several advantages over the traditional high-field superconducting equipment (Blumich 2018). The price of equipment is an order of magnitude lower. The benchtop spectroscopy equipment also relies on permanent rare earth magnets instead of superconducting magnets to produce the necessary magnetic field. This removes the need for cryogen cooling, such that the required upkeep of the system is much simpler and the systems are much more amenable for use in remote locations. Another advantage of benchtop spectroscopy equipment is the external lock. Small variations in the magnetic field can lead to drift in the spectra between measurements, degrading signal quality of averaged measurements. Locking is a method used to correct for this problem. Typically, this is performed by using a deuterated solvent. The frequency of the deuterium signal is used as a reference to align each measured spectrum, correcting for drift. While this method works well for research, it is not practical in an industrial setting. In contrast, some benchtop spectroscopy equipment uses what is called an external lock and does not require anything to be added to the solution. These developments mean that NMR is beginning to be more commonly used in a process monitoring method (Meyer et al. 2016; Giraudeau and Felpin 2018). Benchtop NMR has been used as a process monitoring method for a variety of chemical reactions [Bernhard's fermentation, BAM reactions], but to date has not been used on enzymatic hydrolysis (Gouilleux et al. 2015; Giraudeau and Felpin 2018).

Another challenge to be overcome for NMR to be used industrially is solvent suppression (Gouilleux et al 2017). Both the water and solubilized protein will contribute to the measured NMR signal. For hydrolysate, the majority of the sample consists of water, with only a few percent protein. This means that the signal from the water will dominate over the weaker signal from the proteins, making interpretation and quantification challenging. The common way to avoid the issue of solvent suppression is to run measurements using deuterium oxide (Sundekilde et al. 2018). Again, this is not a practical solution for real-world monitoring of industrial processes. Therefore, NMR measurements need to be performed in a manner that the signal from water in the hydrolysate is sufficiently suppressed in order to adequately identify and quantify the weaker peptide signals.

126 And finally, perhaps ones of the most significant challenges to the adoption of NMR for industrial use
127 has been the perceived complexity of the method (Colnago et al. 2014). NMR is an extremely flexible
128 technique and able to characterize samples in many ways. High-field systems have been designed to
129 enable researchers to take advantage of this flexibility and the software gives them the ability to
130 control every minutia of a measurement. The downside of this is that it frequently requires detailed
131 understanding of NMR physics and specialized training to run even basic experiments. In contrast,
132 many of the benchtop systems are designed with students and the industrial user in mind and come
133 with push button measurements built in the software. This enables users to focus more on the data
134 produced and less on the details behind data acquisition with only minor losses in experiment
135 efficiency.

136 This study aimed to combine benchtop NMR spectroscopy with a solvent suppression measurement
137 method to evaluate how the technique might function for monitoring enzymatic hydrolysis under
138 industrial conditions. Therefore, experimental conditions were kept as close to actual conditions used
139 for production of commercial hydrolysate as possible. This meant that some methods commonly used
140 to improve interpretation of NMR data were not able to be used in the study. For example, the pH of
141 a sample may change under hydrolysis and can affect the chemical shift location of constituent peaks
142 in the NMR spectrum. This is usually corrected for in research studies by the addition of
143 trimethylsilylpropanoic acid (TSP) to the solvent to act as an internal chemical shift reference (Wishart
144 et al. 1995). However, such an addition would not be practical for commercial scale enzymatic
145 hydrolysate production, such that it was not used here.

2 MATERIALS AND METHODS

2.1 NMR EQUIPMENT

The system setup is shown in Figure 1. The NMR equipment used was a 43 MHz Spinsolve (Magritek, New Zealand) equipped with a custom 1 Tesla/meter gradient coil. The NMR system uses an external fluorine lock to maintain frequency stability between measurements. Silicone tubing was used for the transport of the hydrolysate between NMR instrument and the reaction vessel. However, silicon tubing has a strong, broad NMR signal. To avoid unwanted signal from the silicone tubing, glass tubing with a 5mm outer diameter and 3mm inner diameter was run through the NMR coil and the silicon tubing was connected to both ends. Material was pumped from the reaction vessel up and through the bore of the magnet using a minipulse 3 pump (Gilson, USA). Flow was stopped during NMR measurement.

2.2 NMR EXPERIMENTS

The NMR pulse sequence chosen for measurement was the WET-NOESY sequence. This pulse sequence combines WET solvent suppression and the NOE signal detection into a single measurement. It has been shown to provide effective suppression of the water peak while minimizing distortion to the spectra (Gouilleux et al. 2017). More detailed information on the pulse sequence can be found in the aforementioned reference. Shimming of the magnetic field was performed using a 90 % D_2O-10% H_2O reference sample before the start of measurements and prior to insertion of the flow tube, but not on the hydrolysate samples themselves. For the 90-degree pulse, the standard duration of 7 μs was used. Because the NOE is only used for water suppression, pulse length optimization is not critical. For the online measurements, 8 scans were used. A 5 second relaxation delay was used between scans. Measurement time was approximately half a minute. For the offline measurements, 16 scans were used and took approximately a minute to perform. The lower number of scans for online measurement trades a small penalty in the signal to noise of the data for more rapid acquisition speed. While this

sequence is non-standard, it could easily be imported into a routine measurement interface for later use by a non-expert or as an automated measurement. The NMR spectra were processed in MNova (MestraLab Research) using manual phase correction, 2 Hz exponential line broadening, and either a polynomial or Bernstein polynomial baseline correction. While performed manually here, an algorithm could easily be developed to perform this type of preprocessing automatically.

2.3 Hydrolysis Procedures

Five different enzymatic hydrolysis reactions were performed using three different raw materials: red cod (Pseudophycis bachus) fillet, salmon (Oncorhynchus tshawytscha) fillet and unpeeled shrimp (Litopenaeus vannamei). Raw materials were sourced from a local fishmonger (Wellington Trawling Company, Wellington NZ). Red cod fillets had been trimmed, but salmon fillets were not, such that skin, tendons and bones were all included in the hydrolysis process. Water was added in a 3:1 ratio to 250g of raw material and blended to produce a homogenized slurry approximately 1 liter in volume. Samples were heated to 60°C and a magnetic stir bar was used to mix the samples during the reaction. 0.15% by weight of Flavourzyme 1000L and Alcalase 2.4L (Novozymes, Denmark) were added to the mixture. In addition, measurements of the red cod hydrolysis were performed using the Alcalase or Flavourzyme enzymes individually. Replicates were not performed. Online measurements were performed at 0, 1, 3, 5, 7.5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, and 60 minutes. In addition to the online measurements, samples were collected for offline measurements at 0, 1, 3, 5, 7.5, 10, 15, 20, 30, 45, and 60 minutes. These samples were incubated at 90°C for ten minutes to deactivate the hydrolytic enzymes. Samples were then centrifuged to separate the solid and liquid phases and 600 μl of the supernatant was pipetted into a 5mm NMR tube. For the offline measurements on red cod, a 65 μL aliquot of 20 mM TSP-d_6 in D_2O was added to each sample as a chemical shift reference. Measurement on the offline samples was performed immediately after the online measurements were complete.

2.4 REACTION MONITORING

Enzymatic hydrolysis consists of multiple, simultaneous processes that affect the reaction rate such as substrate depletion, thermal denaturation of the enzyme, pH drift, and catalytic poisoning (Tipton 2002). For single enzyme, single substrate reactions, these reaction kinetics are well understood and can be described analytically (Jovanovic et al. 2016; Zhou et al. 2016). However, industrial processes to obtain peptides from by-products deviate from the idealized situation in many ways. Commercial enzymes often consist of multiple proteases, which may have a synergistic or inhibitory effect on one another. For example, Alcalase consists of four different proteases. Additionally, as the enzymes are produced naturally by bacteria, there will be some variation in between batches. The starting materials for enzymatic hydrolysis will consist of multiple substrates (e.g. muscle, skin, tendons, etc). The different ratios between these substrates can also vary from batch to batch. Temperature variations may also occur in the reactor. All these factors lead the process to deviate from the standard enzymatic kinetics equations.

Therefore, in order to quantify reaction rate, we take a more generalized process monitoring approach. Instead of attempting to provide a complete description of the enzymatic process, we focus on quantifying the reaction products. The signal from insoluble protein relaxes too quickly to be measured by the NMR equipment used in this study. As such, only the solubilized protein will produce a measurable NMR signal and therefore is easily distinguished from the undigested protein in the raw material. We use the quantification of this solubilized protein as a measure of the reaction progress. The production of solubilized protein via enzymatic hydrolysis is a first order process (Tipton 2002) and therefore, can be described by the equation:

$$A = A_f - A_{0'} e^{-kt}$$

where k is the hydrolysis rate constant, A_f is the final NMR signal intensity, and $A_{0'}$ is the difference between the initial and final signal intensities. Uncertainty in the reaction rate is calculated from the 95% confidence interval.

This is a different approach to characterize a hydrolysis reaction than is typically used. Degree of hydrolysis is the most common way to calculate a hydrolysis rate, by measuring the cleaving of peptide bonds as a function of time. Instead, in this study we aim to use NMR to measure the concentration of solubilized protein at several points in time and, from this, calculate a rate constant. This means the two methods are measuring on two different processes. DH is measuring how broken down the protein is relative to its initial state and the NMR technique is measuring solubilization of protein. While protein breakdown and protein solubilization are related during hydrolysis, they are different aspects of the reaction. Therefore, caution needs to be taken when comparing rate constants from this NMR method to reaction rates calculated from DH. A method to measure both degree of hydrolysis and protein concentration in real time by NMR would be valuable and is currently under development.

2.5 Calibration of NMR signal

One property of NMR is that the signal observed is linearly proportional to the concentration of component being measured. Quantitative NMR is commonly performed to measure concentrations and purities of pharmaceuticals with great accuracy (Bharti and Roy, 2012; Pauli et al. 2014) using internal standards such as maleic acid. The NMR signal and concentration of the standard can then be used to calculate the amount of another constituent in the sample whose concentration is unknown. Alternatively, external calibration samples can be used, which have known concentrations that are either physically separated from the sample of interest and measured at the same time, or measured sequentially in separate test tubes. The signal from these external calibration samples will be less accurate than for the internal standards, but are often more convenient and the accuracy is typically sufficient.

In the current study, the concentration of hydrolysate will be proportional to the measured NMR signal of the hydrolysate. We use reference samples created from freeze-dried snow crab hydrolysate samples from 0, 5, 15, 30, 45, and 60 minutes after adding hydrolytic enzymes. These samples were reconstituted to known concentrations that approximate the concentration at that time of hydrolysis

and, therefore, are useful standards for determining the concentrations of fresh hydrolysate samples over the course of hydrolysis. Ideally for industrial application, calibration samples should be created from hydrolyzed protein of the same type of raw material as the intended hydrolysis feed stock. Figure 2 shows the concentration of hydrolysate plotted against the integrals from either the aliphatic region or aromatic region of the NMR spectra. The aliphatic integral was 0.5 ppm wide centered around the peak furthest up field and this is associated with the aliphatic region of several amino acids. The aromatic region was 2.2 ppm wide and centered around the two furthest down field peaks, which are associated with aromatic amino acids and amide protons. The fits of these data were used to convert NMR signal to hydrolysate concentrations. The aliphatic region has less absolute error (RMSE 0.7 mg/mL, R^2 = 0.994) than the aromatic region (RMSE 2 mg/mL, R^2 = 0.94), due to the higher signal of the former, and was used as the predictor when the raw materials were lean.

3 RESULTS

The hydrolysis data was comprised of a series of time and integral pairs over the hydrolysis reaction. The integrals were transformed to hydrolysate concentrations using the models described in the previous section. Significant outliers arising from air bubbles or particulate transiting the sample volume during data acquisition were not included in rate calculations. The calibration data were acquired from samples in 5 mm NMR tubes, just as the offline samples were. The online samples were collected on a 3mm inner diameter flow tube and thus have a reduced sample volume and signal intensity. The final online and offline point was collected after deactivating the reaction at 90° C for more than 10 minutes. It was assumed that at this point the concentration of the online and offline samples were equivalent. To convert the flow samples to concentration using the snow crab model, it was necessary to correct the online concentration by the ratio of the integral of these points collected on the final hydrolysate. These data were collected after the final time points in the hydrolysis.

3.1 Red Cod Hydrolysis

Red cod is a lean white fish (Vlieg and Body 1988). As such, little oil production was expected during hydrolysis. This was reflected in both the measured NMR spectra and the resulting hydrolysis product. No oil layer was observed in the centrifuged offline samples. Figure 3 shows spectra from both online and offline measurements. The peak at 0 ppm in the offline sample is from the TSP added to correct for chemical shift drift. The aliphatic region of these spectra was used for analysis.

This part of the study sought to test the NMR method's ability to measure the effect of different types of enzymes on the rate and extent of hydrolysis. Alcalase is an endopeptidase while the Flavourzyme is an exopeptidase, so the two enzymes will have different rates of reactions and protein yields. An additional reaction combining Alcalase and Flavourzyme was performed to create a third test case. While one might expect that a combination of an endopeptidase with an exopeptidase would lead to more efficient breakdown of the material, comparison of the results (Figure 4, Table 1) indicates that in terms of reaction rate and amount of solubilized protein, the endopeptidase alone is more efficient. Flavourzyme alone solubilized the least amount of protein. These results are in line with findings of previous researchers and demonstrate the ability of benchtop NMR spectroscopy to monitor the efficiency of different enzymes.

3.2 Salmon Hydrolysis

Unlike the lean red cod, salmon is high in fat and, as expected, oil was produced during the hydrolysis process. This was observed in both the online NMR spectra and the offline samples. After centrifugation of the offline samples, a layer of oil was observed above the aqueous phase in the tube. For the offline measurement, only the aqueous phase was sampled. Figure 5 compares the online and offline spectra for salmon hydrolysate after 60 minutes and the fat layer in deuterated chloroform. The online sample contains an emulsion of fat and water. This leads to a large amount of signal in the upfield portion of the spectrum from the produced oil. In contrast, in the offline measurement on the aqueous phase, the signal from the underlying peptides can be seen. Because of the interference from

the oil signal, the reaction rate was calculated (Table 2) from the downfield signal associated with amide bonds between 7.8 and 9.2 ppm where the fat signal is absent (Figure 5 inset). Despite the weaker signal intensity from the peaks, this still allowed reasonable monitoring, as shown in Figure 6.

3.3 Shrimp Hydrolysis

The hydrolysis reaction on the shrimp was similar to the red cod. No confounding oil signal was noted in the NMR spectrum and no oil layer was observed in the centrifuged offline samples, as shown in Figure 7. Both the aliphatic and aromatic regions were used for calculation of the reaction rates (Figure 8, Table 3).

4 Discussion

4.1 Reaction monitoring and Rate Calculation

The initial intensity at time zero was treated as background and not used in the determination of reaction rates. Because the point measures the soluble material in the water phase before any enzyme is added, it is not actually a description of the hydrolysis process. Instead, the amount of solubilized protein before the start of hydrolysis is most likely dependent on the substrate used and the duration and intensity of the homogenization process. Figures 4, 6, and 8 show a noticeable jump from zero to the first time point for all reaction profiles, but this is much more significant for the offline samples. This is what one would expect, as the offline sample will have experienced additional degradation of the substrate during the deactivation of the enzyme as compared to the online measurements. As an intellectual exercise, if the zero-time point is used in the calculation of the rate constant, this leads to a faster calculated reaction rate for the offline samples due to the larger difference in the first two points. If the zero-point is included, this appears as if the reaction has progressed more quickly. For these reasons, a decision was made not to use the zero-time point in the fitting of the rate constant.

313 The calculated reaction rates approximate rates found in the literature for hydrolysis using Alcalase
314 and Flavourzyme enzymes under similar experimental conditions (Ahmadifard 2016; Sundekilde et al.
315 2018; Kristoffersen et al. 2019). It was observed that the combined Alcalase and Flavourzyme
316 measurement on the red cod produced a slower reaction and a less efficient hydrolyzation than for
317 the Alcalase alone. This effect has also been seen by other researchers (Nchienzia et al. 2010), who
318 noted that the combined enzymes were less effective than the Alcalase alone. Flavourzyme alone was
319 much less efficient at hydrolyzing the sample, which is also in line with the findings of other researchers
320 (Nchienzia et al. 2010; Sbroggio et al. 2016; Lidh and Thuy 2016). For all types of samples, benchtop
321 NMR spectroscopy appeared able to perform online monitoring of the enzymatic hydrolysis reaction
322 with good precision. This indicates the method is robust with regards to sample material and it should
323 be broadly applicable for monitoring of different types of enzymatic hydrolysis. Even though the online
324 and offline measurements showed similar rates, there was more information about the early
325 hydrolysis in the online measurements. The offline measurements show a considerable time lag while
326 keeping the same dominant rate constant. This means that online measurements will be much more
327 valuable in determining early hydrolysis events or higher order rates in the laboratory (Tipton 2002) or
328 intervening in the case of process monitoring or control.

329 Analysis using the aliphatic region has the advantage of higher signal intensity, but the amide and
330 aromatic region has the advantage of the lack of interference from fat (Figure 5). The reaction rates
331 determined from both the aliphatic and aromatic regions were found to be the same in the high signal
332 to noise offline measurements, with the exception of when Flavourzyme was used alone (Figures S1-
333 S4). Flavourzyme may have differing apparent rates for the aliphatic region and aromatic region
334 because its dominance by exopeptidases, which has been shown to preferentially produce solubilized
335 proteins containing hydrophilic amino acids (Tang et al. 2018). The online data for the lean samples
336 lacked the signal to noise to fit the aromatic peaks to sensible reaction rates. Due to the narrow flow
337 tube and fewer scans, the online data will have a roughly a factor of four less signal to noise. This could
338 be significantly improved by fitting the system with a thin-walled flow tube. Despite the agreement in

reaction rate, the concentrations measured from the aromatic peaks have a systematic difference from the aliphatic peaks. This appears to be a problem in the calibration process. There are several possible sources of this difference. We treat both the aromatic and aliphatic regions as equivalent proxies for the protein concentration. However, different amino acids will have different ratios of aromatics and aliphatics. Therefore, the ratio of aromatics to aliphatics may differ per milligram of protein between the calibration samples and the test samples. While not possible due to the constraints of this study, future work is planned to improve calibration of protein content to take this into effect. We also anticipate that creating calibration samples from hydrolyzed protein from the same type of raw materials as the intended hydrolysis feed stock will improve accuracy. Another possible cause for the discrepancy could be that the freeze-drying process changed the samples and so that this region of the spectra was not directly comparable to the fresh samples.

Despite the lack of an internal reference like TSP for the online measurements, the shift in peaks due to evolving pH during hydrolysis appeared minor, suggesting the method is amenable to automated analysis. While peaks associated with different types of amino acids (*e.g.* aromatic vs. aliphatic) can be identified, there does not appear to be enough chemical shift resolution to identify individual amino acids themselves, as is possible on high field NMR systems.

One drawback to the investigated NMR methodology is that it measures the soluble protein concentration in the aqueous phase. However, the enzymes will both solubilize protein and break the solubilized protein into smaller molecules and benchtop NMR will struggle to distinguish, for example, a dipeptide from a small protein within a hydrolysate mixture. Comparing results, it appears that the reactions monitored via NMR reach equilibrium more quickly than those in the literature monitored by other methods. In particular, while both the NMR measurements here and measurements in the literature (Pagan et al. 2015; Ahmadifard et al. 2016) showed a similar rapid increase at the beginning of hydrolysis, the results from the NMR plateaued sooner than results measured by other methods. This suggests that after the enzymes have managed to solubilize most of the available protein,

unsurprisingly the reaction continues to breakdown the larger peptides into smaller molecules with little impact on the total concentration of protein in solution. While monitoring protein concentration in solution is important in terms of efficient utilization of raw materials, the final molecular size of the proteins is important for many functional properties, such as solubility, taste or emulsion stability. Further research is underway to use NMR to estimate protein sizes in solution. Combining the two abilities would allow for unprecedented control over the enzymatic hydrolysis process, ensuring as much of the available raw material is digested as possible while enabling the reaction to be stopped when the desired molecular weight has been achieved.

4.2 Practical Considerations for Online Measurement

Several practical challenges exist with using NMR as an online measurement. Some concerns existed before testing whether the elevated temperature of the hydrolysate flowing through the magnet could adversely affect NMR equipment. The field strength of permanent magnets is temperature dependent and therefore benchtop systems are temperature stabilized in order to avoid magnetic field drift. While there was some temperature change observed during measurement, the impact was mitigated by frequently resetting the lock and center frequency to follow the magnetic field drift. Therefore, common reaction temperatures used for enzymatic hydrolysis do not appear to be problematic for the benchtop NMR equipment.

Another challenge that arose with online measurements was drifting of solid material through the coil during measurement. The signal from static solids in the coil will simply decay so quickly that it does not interfere with the measurement, but moving solids through the magnetic field during measurement will lead to a distortion in the magnetic field, leading to line broadening and inefficient solvent suppression. Setting a weak flow rate during measurement to counter gravity induced flow may help prevent solids from drifting through the coil during the measurement. Alternatively, some type of filter could be installed to prevent the movement of large solids (e.g. bits of bone, shell, etc) through the NMR coil during measurement. Another practical issue discovered during measurement

was intake of air into the flow loop. As the enzymatic reaction proceeds, the viscosity of the system decreases, and the surfactant properties of the soluble proteins cause more bubbles to appear in the flow line. As with solids, a moving bubble through the coil during measurement will lead to line broadening. It was found that reducing the stirring speed as the reaction progressed minimized the uptake of air into the flow loop. Also, while the equipment used here was limited to a vertical flow configuration, using a magnet with a horizontal bore would help minimize issues with both solids and bubbles during measurement.

4.3 High field and Low Field NMR Comparison

Despite its advantages, benchtop NMR has several drawbacks compared to high-field. One of the biggest disadvantages is that the chemical shift resolution is lower. Therefore, what would be distinct, narrow peaks in a high-field magnetic field may be broader, overlapping peaks at a lower field (example shown Figure 9).

This limits the ability to identify constituents in a system. Examination of the low-field spectra show much worse spectral resolution when compared to high-field NMR results obtained by other researchers (Sundekilde et al. 2018). While functional groups like alkanes and aromatics of the amino acids could be associated with regions of the spectra, the resolution was not sufficient to identify individual amino acids themselves. Higher magnetic field benchtop spectroscopy systems exist than the one used in this study, but they are still well below the super conducting field strengths commonly used in the related field of metabolomics, such that it is unlikely that the magnetic field gain will be enough to provide the necessary chemical shift dispersion. Therefore, identification and quantification of individual amino acids during enzymatic hydrolysis using currently available NMR benchtop spectroscopy equipment does not seem possible. However, it is possible that measurements using carbon to provide specificity could enable identification of individual amino acids. Because of the weak NMR signal of carbon, these measurements would take too long to be performed online, but could potentially be used after the hydrolysis measurement is complete to estimate final products. Lower

magnetic field also means the signal to noise is lower, such that more scans may be necessary in order to get a good quality signal, increasing the required measurement time. Lastly, several methods for solvent suppression are less effective at lower field due to a less efficient saturation of magnetization and decreased specificity of the suppressed signal.

5 CONCLUSIONS

Benchtop NMR spectroscopy is a promising method for monitoring of enzymatic hydrolysis in real time. The calculated reaction rates show good agreement between the online and offline measurements and are also in line with reaction rates estimated by other methods found in the literature for the Alcalase and Flavourzyme enzymes. The WET-NOE pulse sequence allowed sufficient solvent suppression such that the much weaker protein phase could be quantified under conditions similar to what would be expected for industrial measurement. The technique performs best on lean by-products, but monitoring is still possible for hydrolysis of fatty materials.

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Figures

Figure 1: Image of the online NMR setup for monitoring of enzymatic hydrolysis

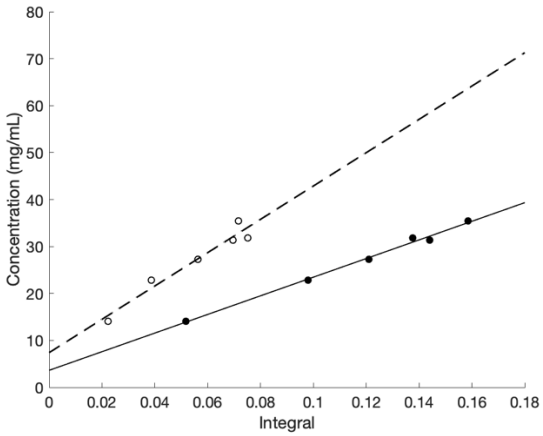

Figure 2: The concentration of hydrolysate plotted against the integrals from either the aliphatic region (filled circles) or aromatic region (open) of the NMR spectra.

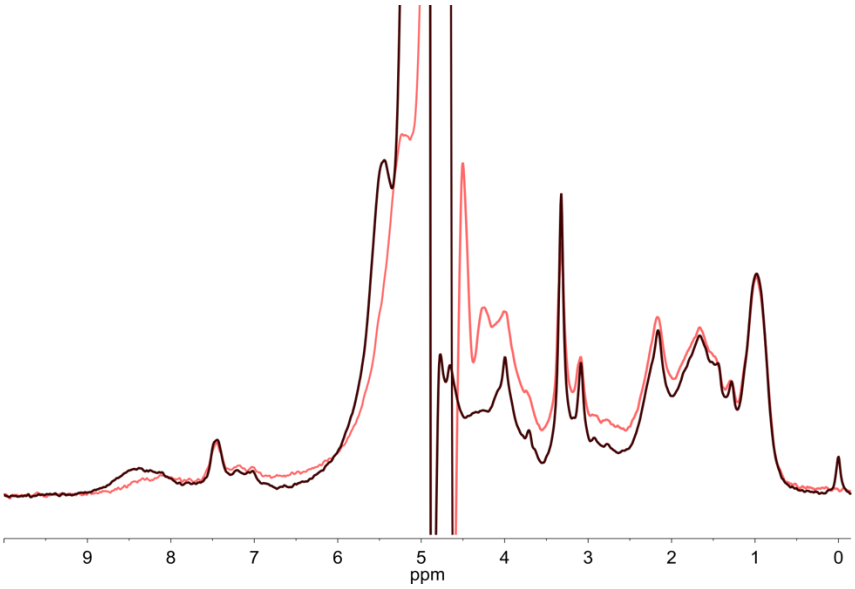

Figure 3: NMR spectra of final hydrolysate products from on-line (red) and off-line (black) measurements.

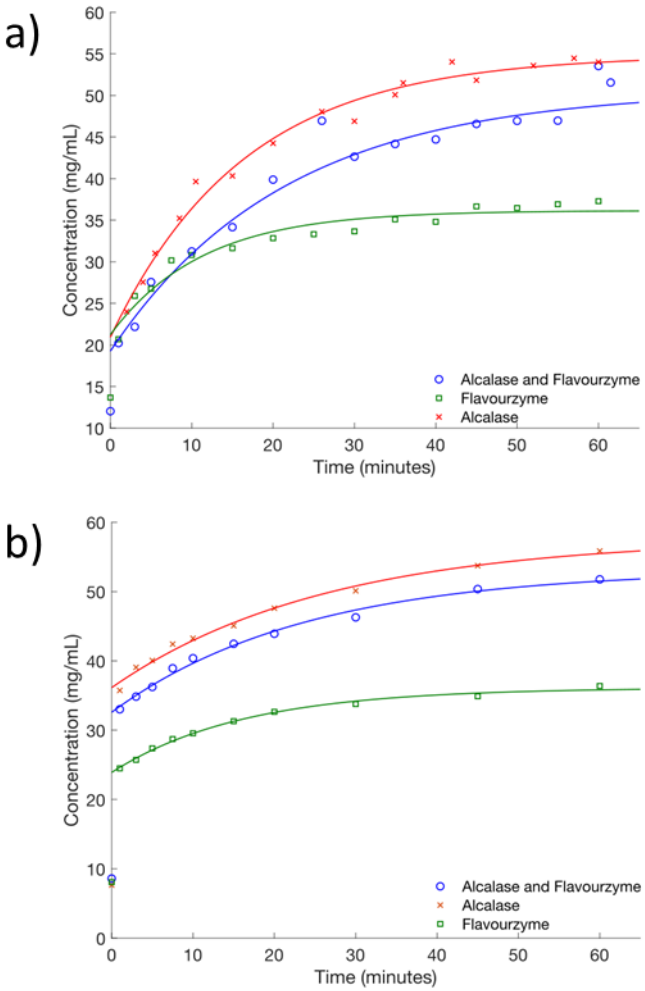

Figure 4: a) Reaction monitoring for hydrolysis of red cod samples online using alcalase (red crosses), Flavourzyme (green boxes), and an equal concentrations of both enzymes (blue circles) b) Reaction monitoring for hydrolysis of red cod samples offline.

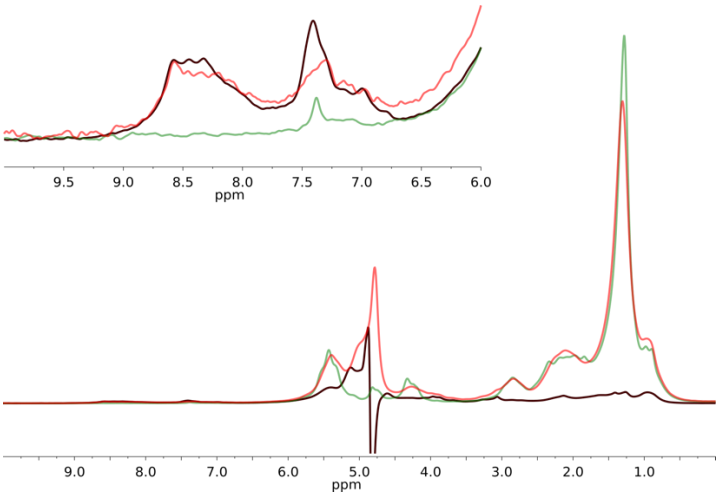

Figure 5: Comparison of salmon hydrolysate spectra from online (red) and offline (black) measurements and the produced oil (green). The inset shows the aromatic groups and amide bond resonances of the amino acids and peptides. The signal at 7.3 ppm in the fat spectrum is from residual chloroform.

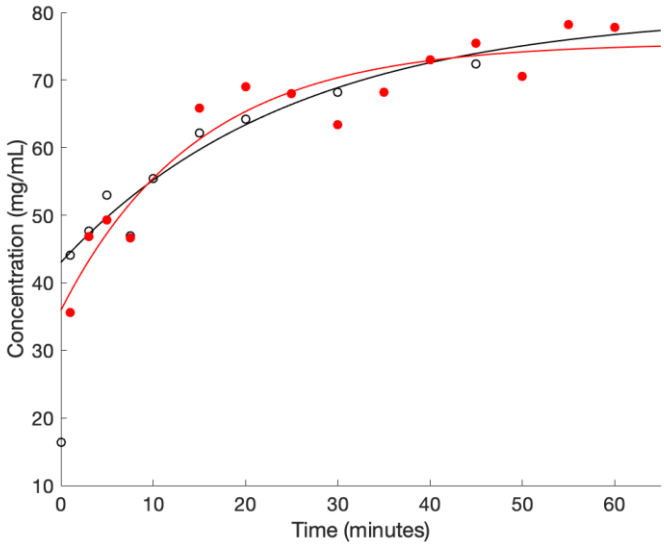

Figure 6: Reaction rate for online (red filled markers) and offline (open black markers) measurements of salmon hydrolysis.

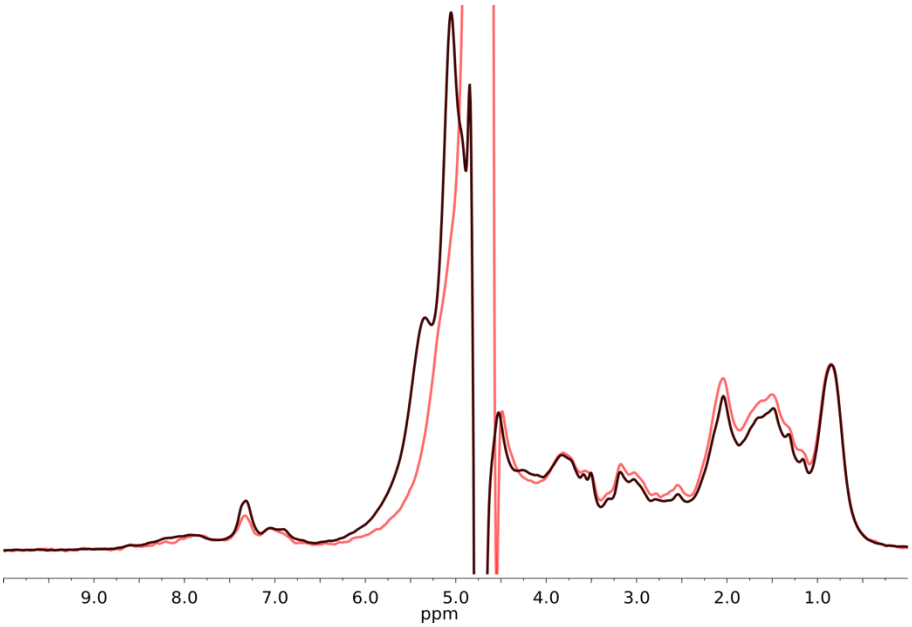

Figure 7: Comparison of shrimp hydrolysate spectra from online (red) and offline (black) measurements.

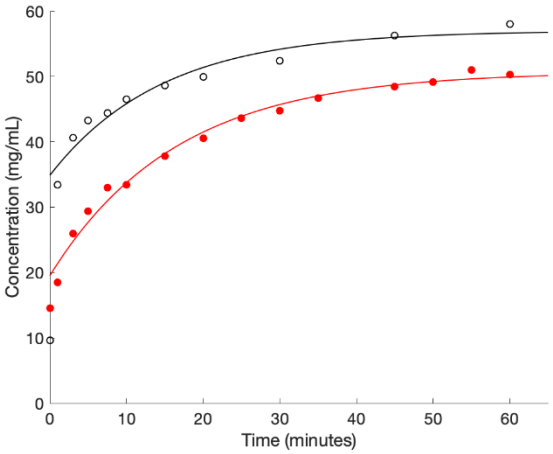

Figure 8: Reaction rate for online (red markers) and offline (black open markers) measurements of shrimp hydrolysis

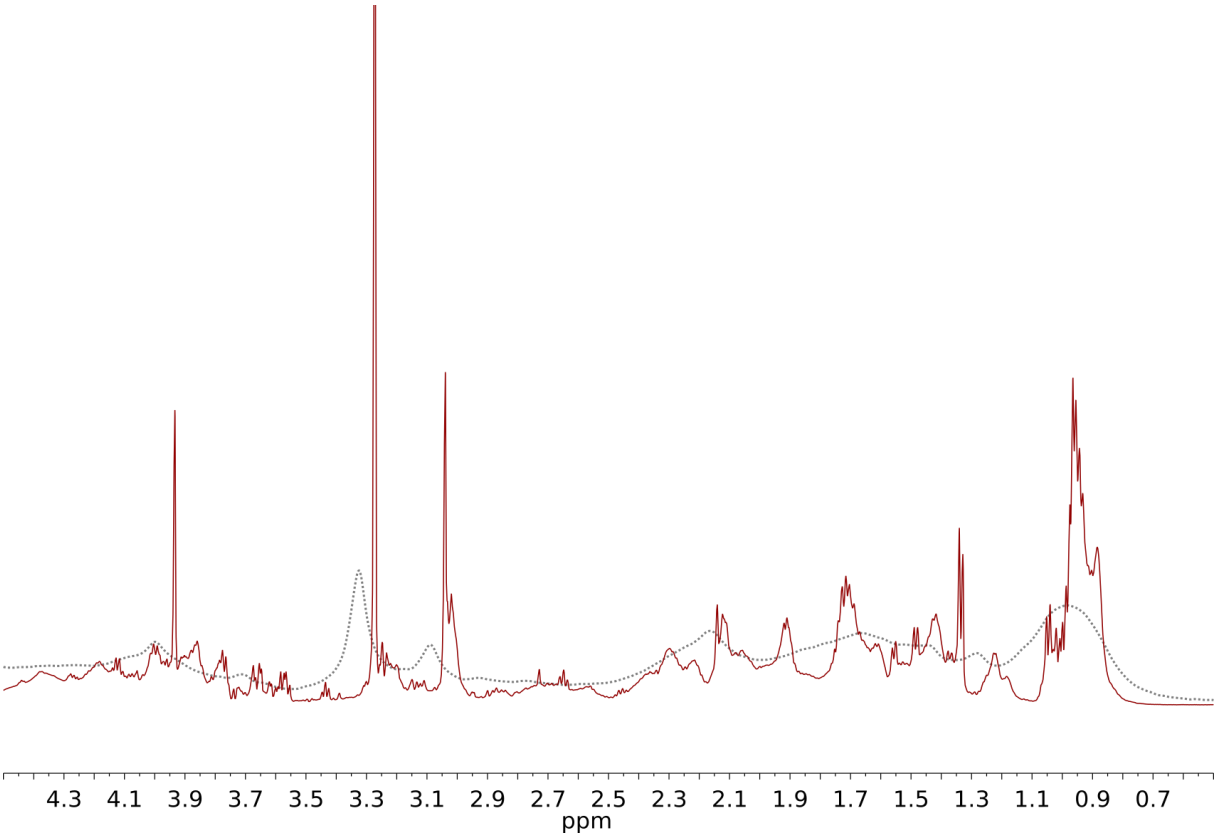

Figure 9: Comparison of 600 MHz high-field (red solid) and 43 MHz low-field (black dashed) NMR spectra of red cod hydrolysate.