**Development of Analytical Methodology for Microwave-assisted Hydrolysis of Glycans to Monosaccharides**

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**Abstract**

Determining monosaccharide compositions is a key step in identifying unknown biologically-derived oligosaccharides, which can be useful in determining their biological function. In order to test a new method for monosaccharides composition determination, the researcher hydrolyzed sucrose and maltooctaose using 1.0 M HCl and 2.0 M TFA in a microwave set at 95˚ C for 1 to 2 hours and then analyzed for monosaccharides via HPLC-CAD. Sucrose was completely hydrolyzed with good precision and maltooctaose was hydrolyzed to 67% glucose. This independent method has the potential to be simpler and more economical than other methods currently used for determining monosaccharide composition, and will be used to quantify oligosaccharides standards in a related project.

In the United States, 7.3 million women between the ages of 15 to 44 are infertile (Office of Information Services 2009). Although it may not affect the majority of women, those who are unable to conceive find infertility to be a significant problem. A more complete understanding of the molecular basis of fertilization is essential in order to assist couples having reproductive difficulties.

The process of fertilization is neither trivial nor completely understood. Fertilization is a highly organized process that occurs via recognition, binding, and fusion of an egg and a sperm (see Figure 1).

**Figure 1.** The fertilization process begins with sperm attachment and finishes with the block to polyspermy (Takasaki 1998).
The egg secretes an extracellular matrix, a protective layer that surrounds the egg and serves as a barrier to ensure successful fertilization. The egg’s extracellular matrix found closest to the egg is referred to as the vitelline envelope, which serves as a primary defense mechanism for proper recognition of sperm from the same species, and for preventing more than one sperm from entering the egg, in a process termed the block to polyspermy.

The block to polyspermy occurs by way of a series of reactions that alter the vitelline envelope and block additional sperm from recognizing and penetrating into the egg. The consequences are dire if more than one sperm enter the egg, since additional sperm nuclei would unbalance the total contribution of genetic information within the embryo and cause development to terminate. Following the fusion of a single sperm, the egg releases the contents of secretory vesicles known as the cortical granules, which contain an important block to polyspermy component termed the cortical granule lectin (CGL). CGL is a glycoprotein, a protein with carbohydrates added to its structure that will bind to a partner termed a ligand located on the outer side of the vitelline envelope. The interaction between the lectin and its ligand partner establishes the block to polyspermy.

Studying the process of fertilization in mammals is difficult, since mammals produce a limited amount of eggs and fertilization occurs internally. Therefore, a model organism is needed to study this process. The ideal organism would produce a plethora of eggs for research, which can be collected easily, its fertilization would occur externally, and it would have the same basic fertilization mechanisms as that of mammals. Amphibians were chosen because they produce vast amounts of eggs, about 3,000 eggs each cycle, and fertilization occurs externally, making fertilization easier to study. More specifically, the most commonly studied amphibian is the *Xenopus laevis* frog. In addition to producing a considerable amount of eggs, the block to polyspermy process is known in great detail in *X. laevis*. Furthermore, the same basic extracellular structures exist in mammals and amphibians, (i.e., the mammalian zona pellucida and frog vitelline envelope) and serve the same biological roles. Most importantly, CGL is also found in mammalian cortical granules and functions in the block to polyspermy. Since biochemical purification and biological assays generally require a significant amount of preparatory materials (i.e., eggs), the researcher has chosen to study the block to polyspermy process in *X. laevis*, which will more than likely be applicable to the mammalian fertilization process.

In *X. laevis*, much is known about the basic interaction of CGL and its ligand partner. Lectins are defined as proteins that bind to specific carbohydrates and are classified into various categories depending on their binding specificity.
Ligands are molecules that can bind to specific receptors. The ligand for CGL is also a glycoprotein and is largely comprised of carbohydrate chains termed oligosaccharides (Quill et al. 1996; Strecker et al. 1995; Tseng et al. 2001; Zhang et al. 2004). The ligand oligosaccharides that terminate in galactose residues appear to be the most important in the lectin-ligand binding interaction, which leads to the block to polyspermy. However, not much else is known about the structural composition of these essential ligand oligosaccharides and their relative quantities.

In general, glycoproteins make up half of all eukaryotic proteins (Rebecchi 2009) and the carbohydrate chains, also known as glycans, often serve biological roles (e.g., binding interactions, development, cancer). Consequently, it is important to be able to analyze the structure of glycans found on glycoproteins, but, unfortunately, current methods are inadequate. In order to analyze the glycans or oligosaccharides from glycoproteins, isolation, quantification, and compositional analysis is necessary to determine the monosaccharides that are present within the glycans since they can be comprised of many different sugars and be linked together in a multitude of ways. The goal of this research project is to develop a method to determine the monosaccharide composition of glycans derived from the ligand that binds to the X. laevis CGL, and is universally applicable to other biologically-relevant glycans that other researchers might want to study. Thus, the development of a methodology to study the monosaccharide composition of glycans found on the X. laevis ligand will serve as a model system for the study of other types of glycoproteins.

With regards to methodology, there are only a few existing techniques available to study carbohydrates (i.e., HPLC-PAD, derivatization, LC-MS, HPLC-UV); however, they are inadequate in one or more of the criteria necessary for these types of analyses. Specifically, compositional assays need to be sensitive, cost-efficient, fast, and quantifiable. The present research is designed to determine the monosaccharide composition of biologically-derived oligosaccharides using high performance liquid chromatography with a charged aerosol detector (HPLC-CAD). Ultimately, this method will quantitatively determine the type of monosaccharide subunits present in the carbohydrate structure derived from the glycoprotein. This new methodology is expected to fit all the criteria discussed previously and most importantly, to be quantifiable. Thus, it will be a significant contribution to the study of glycoproteins. As for the X. laevis CGL ligand, it is anticipated that this methodology will enable the researcher to determine the monosaccharide composition of the oligosaccharides that bind to CGL and will contribute to a greater understanding of the block to polyspermic fertilization.
LITERATURE REVIEW

One of the purposes of studying carbohydrates, as discussed previously, is to aid in determining the binding specificity of lectins to ligands. In order to do so, a method to analyze glycan components is needed that uses a method of complete acid hydrolysis. The method for monosaccharide and oligosaccharide composition used in this study is performed by high performance liquid chromatography (HPLC) with a charged aerosol detector (CAD) (Dixon and Peterson 2002; Gamanche et al. 2005). Existing methods for hydrolysis and compositional analysis are inadequate for this study’s purposes (fast, sensitive, cost-efficient, quantifiable, and readily available). Furthermore, the HPLC-CAD instrument is being used for oligosaccharide isolation and quantification.

Microwave-Assisted Hydrolysis

Hydrolysis is a classical method that is used for the synthesis or break down of molecules. Figure 2 illustrates how hydrolysis occurs under acidic conditions. For example, when sucrose, composed of one fructose and glucose, is exposed to heat and acid, a reaction occurs in which sucrose gets broken down into its subcomponents.

![Diagram of the hydrolysis of sucrose using hydrochloric acid (HCl).](image)

There are a few types of method for hydrolysis: enzymatic, basic, and acidic. Enzymatic hydrolysis uses enzymes to break down specific parts of a molecule and can be expensive and limited to specific linkages. Basic hydrolysis utilizes bases to break down molecules. Lastly, acidic hydrolysis uses acids to break down molecules.

For the purposes of this research, the researcher chose to apply acidic hydrolysis to break down carbohydrates. Acidic hydrolysis is inexpensive because acids are widely used for various applications, and are easier to remove from carbohydrates than bases.

Microwaves have been used for the synthesis of molecules since the mid-1960s. The use of microwaves emerged as an application widely used for
various reactions (including organic and biochemical reactions) because it is a quick and efficient method (Lee et al. 2005; Corsaro et al. 2004). Several advantages for using microwaves could be improved yields, decreased reaction time, reduction of the solvent volume, and decreasing or eliminating the use of catalysts (Roberts and Strauss 2004).

Heating blocks, conventional ovens, and conventional microwaves have been applied for hydrolyzing molecules. Heating blocks and conventional ovens can require extensive hours or days of heating or very acidic conditions to achieve nearly complete or complete hydrolysis. Moreover, conventional microwaves, although time-efficient, are not efficient in reproducing results. In previous studies, this researcher utilized a conventional oven and attained good hydrolysis results. However, in attempting to reproduce the results, this researcher attained poor hydrolysis results.

There are a couple advantages for performing hydrolysis in an industrial microwave. The temperature can be controlled in a solution to achieve a desired constant temperature. Controlling the temperature is a significant advantage for acquiring reproducible results. Another advantage is setting a method for which the microwave will control the power, time, and temperature accordingly. Setting a method also improves reproducibility due to the constant level of irradiation that can be absorbed.

Existing methods are aimed for partial hydrolysis (Lee et al. 2005; Patane et al. 2009; Zhao and Monteiro 2008). Partial hydrolysis methods are inadequate for a significant reason. In order to determine the composition and quantity of carbohydrates, complete hydrolysis is required. For this reason, the current study is aimed at developing a method for complete hydrolysis. The current method, although still being optimized, will be applied to glycans isolated from the *X. laevis* glycoprotein for compositional and quantification determination.

Carbohydrate Analysis

Glycans are carbohydrates, or sugars, that can be simple or highly branched—making them complex. Given that glycoproteins make up half of all eukaryotic proteins, it is important to study these carbohydrates. Carbohydrates are difficult to study because they lack chromophores. Chromophores are parts of molecules that absorb light. They are generally highly conjugated or aromatic; that is they contain many double bonds. There are relatively few existing methods that are used to study carbohydrates, including the following: high performance liquid chromatography (HPLC) with pulsed amperometric detection (PAD), HPLC-UV, derivatization, HPLC with charged aerosol detector (CAD), and liquid chromatography with mass spectrometry (LC-MS) (Dixon and Baltzell 2006; Nozal et al. 2005; Lv et
al. 2009; Nana et al. 2008). In order to apply a method to determine the composition and quantities of carbohydrates, a method that is fast, sensitive, cost-efficient, and quantifiable is needed.

**HPLC** is a separation technique that is used to separate compounds based on their affinity to the column matrix. Depending on the column matrix, compounds can bind to the matrix with greater or lower affinity, which is how compounds separate. HPLC can be used alone or in tandem with different detectors (PAD, UV, CAD).

**HPLC-CAD** is a separation technique that separates molecules and detects molecules of low volatility. Figure 3 shows how molecules are separated and detected by the HPLC-CAD. A sample is injected into the HPLC column and separated. Subsequently, molecules leaving the column are sprayed into a chamber with droplets dried in a heated drift zone producing aerosol particles. The aerosol particles are charged and then collected on a filter. The charged particles are then measured as a current.

![Figure 3. HPLC instrument and the detection method of the CAD.](Image)

One of the most sensitive separation techniques is HPLC-PAD because the instrument detects low concentrations, increasing the sensitivity of the detector (Nozal et al. 2005). However, the equipment required for this instrument is highly specialized and expensive. In addition, the separation method can be time-consuming, requiring about 100 minutes to achieve baseline separation of monosaccharides (Lv et al. 2009). HPLC-CAD utilizes the sample equipment for separation, detection, and quantification of oligosaccharides, eliminating the need for specialized equipment. Not only is HPLC-CAD cost-efficient, the instrument is also fast. For example, during previous studies, monosaccharides and oligosaccharides (containing at least 13 monosaccharide units) have been separated in less than 16 minutes.

HPLC-UV requires derivatization of carbohydrates because they are poor chromophores (Nozal et al. 2005; Lv et al. 2009). Like HPLC-UV, derivatization, as the name implies, requires derivatization of carbohydrates for their detection. HPLC-UV and derivatization methods require extra procedures to analyze carbohydrates to attain adequate sensitivity. Extra procedures are often tedious and time-consuming, which render the two methods disadvantageous in comparison to HPLC-CAD. Using HPLC-
CAD, samples can be directly injected into the column at low concentrations, eliminating the need for labor-intensive preparation.

The main disadvantages of mass spectrometry (MS) are: 1) cost; 2) not always as quantitative (poorer precision); and 3) difficulties in getting carbohydrates to charge using standard ionization methods. In addition, problems such as reproducibility and quantification have been problematic when characterizing carbohydrates with MS. This is extremely disadvantageous when a method is needed to determine the composition of carbohydrates and quantify the subcomponents (Nana et al. 2008). HPLC-CAD can separate and detect neutral monosaccharides so that glucose and galactose can be distinguished as determined by their retention time. Moreover, in previous studies, HPLC-CAD has been used for quantification of monosaccharides by the use of calibration curves and standards (Dixon and Baltzell 2006; Dixon and Peterson 2002).

Another advantage of using the CAD is its sensitivity in detecting compounds. The CAD detection limit, which is the lowest concentration that can be detected, was calculated to be less than 1 nanogram (ng). The sensitivity was also calculated to be ten times more sensitive than ELSD and five times more sensitive than UV detection (Inagaki et al. 2007). Furthermore, reported detection of non-volatile compounds resulted in a limit of detection of about 1 ng of the compound (Gorecki et al. 2006). Most, if not all, detectors have limitations that do not allow all types of molecules to be detected quickly and with great sensitivity. CAD is considered to be a universal detector with great potential for quantification (Gorecki et al. 2006; Hazotte et al. 2007; Dixon and Baltzell 2006). The potential universality of the detector is due to the process by which the analytes are converted to charged particles. Literature using the CAD has supported the universality of the detector (Gamanche et al. 2005; Gorecki et al. 2006; Hazotte et al. 2007; Dixon and Baltzell 2006).

One limitation of the detector is that the signal to noise ratio and response is dependent on the organic eluent content (Gorecki et al. 2006). This is not a significant limitation because the organic content can be increased for greater response, therefore, greater sensitivity.

Microwave-assisted hydrolysis is a quicker and more efficient method to achieve complete hydrolysis of test compounds, sucrose and DP 8. Additionally, HPLC-CAD has shown to be a fast, sensitive, cost-efficient and quantifiable approach for separating and detecting carbohydrates.
MATERIALS AND METHODS

The following details the materials and methods used in the present study.

Materials

The researcher purchased HPLC grade acetonitrile and certified ACS grade formic acid (88%) from Fisher Scientific (Pittsburg, PA) and ACS grade sucrose and HPLC grade acetonitrile from Acros Organics (Morris Plains, NJ). ACS grade hydrochloric acid (36.5-38.0%) was purchased from EM Science (Gibbstown, NJ). Maltoolctaose were purchased from Carbosynth Limited (Compton, UK). Maltose was purchased from Fluka (Milwaukee, WI), glucose from Spectum (Redondo Beach, CA) and DP 3 to DP 7 was purchased as a kit from Supelco (Bellefonte, PA). Fucose and N-acetylglucosamine were purchased from Sigma (St. Louis, MO). Galactose was purchased from Fisher Scientific (Fair Lawn, NJ). Fructose was purchased from Matheson Coleman and Bell (Norwood, OH). Water was purified by a Nanopure Infinity UV/UF system.

Equipment

Carbohydrates were hydrolyzed in a CEM MARS industrial microwave. An Alltech prevail carbohydrate ES column (250 x 4.6 mm) was used to separate carbohydrates and an Agilent 1100 HPLC with a custom-built charged aerosol detector similar to that described in Abhyankar (2007) was used for carbohydrate detection.

Microwave-assisted Hydrolysis

Sucrose (520 µg) was hydrolyzed with formic acid (0.95 M) and DP 8 (543 µg) with HCl (0.95 M). Hydrolysis was performed in a conical vial with a hole-punched Teflon septum. A PTFE membrane was inserted on top of the Teflon septum and few holes were poked to minimize chances of the membrane tearing. The sample containing the temperature probe did not have a PTFE membrane. The temperature probe was covered by a glass slip to prevent corrosion. Samples were irradiated at 400W, 45% power, and 95°C for over 1 hour or over 2 hours, for sucrose and DP 8 respectively. Hydrolyzed samples were dried via lyophilization.

Carbohydrate Analysis

Hydrolyzed samples were reconstituted in a mixture of 50% acetonitrile and 50% water, diluted to low concentrations and injected into the column. An isocratic method of 75% acetonitrile and 25% water, held constant, was used to separate monosaccharides. Oligosaccharides were separated using a gradient method with decreasing percent acetonitrile from 65% to 55% over a nineteen-minute period.
RESULTS AND DISCUSSION

Microwave-assisted Hydrolysis of Sucrose and DP 8—Complete hydrolysis of sucrose was achieved by microwave irradiation at 95˚ C for 1 hour and is demonstrated by the absence of sucrose in Figure 4. Formic acid has the ability to overheat, which was observed when hydrolyzing sucrose in a domestic microwave at 10% power. Obtaining the temperature in a conventional microwave is difficult and time-consuming. The overheating of formic acid was overcome by using a temperature probe in the industrial microwave that aids in controlling temperature. The temperature was controlled at 95˚ C ± 2˚ C, thus mitigating the chances of formic acid overheating.

![Image](https://example.com/image.png)

**Figure 4.** Overlaid chromatograms of diluted hydrolyzed sucrose (3 µg mL⁻¹, top trace) and fructose, glucose, and sucrose mixed standard (1 µg mL⁻¹, bottom trace).

DP 8, because of its seven bonds, was more difficult to completely hydrolyze to monosaccharides than sucrose. Figure 5 shows the products (glucose with small amounts of small oligomers) resulting from 2 hours of microwave irradiation with 0.95 M HCl. 77% of the moles of hydrolyzed DP 8 were recovered as glucose and smaller oligosaccharides. It is uncertain what was causing the loss of DP 8 products, but loss may have occurred during lyophilization or incomplete suspension of lyophilized sample in an ACN and H₂O mixture. Alternatively, the purity of the DP 8 standard may have been less than expected.

Hydrolysis of sucrose required less time and energy due to the single glycolytic linkage between glucose and fructose. Approximately one hour was needed to hydrolyze sucrose because formic acid, a weaker acid than HCl, was used. DP 8 required more energy, thus more time, to hydrolyze due to the increase of glycolytic bonds. Fructose and glucose are linked via an α,β-1,2 glycolytic linkage to form sucrose. DP 8 is linked via seven α-1,4
glycolytic linkages, which may be more difficult to hydrolyze, thus requiring a stronger acid and more water and time.

![Figure 5. Chromatogram of diluted hydrolyzed DP 8 (5.6 µg mL⁻¹).](image)

The reproducibility of the hydrolysis of sucrose was achieved using an industrial microwave. As shown in Table 1, hydrolysis was found to be very reproducible as the three microwave hydrolysis experiments gave very similar product concentrations.

<table>
<thead>
<tr>
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<th>Concentrations</th>
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<tbody>
<tr>
<td>DP 1</td>
<td>5.566</td>
<td>2072.4</td>
</tr>
<tr>
<td>DP 2</td>
<td>6.664</td>
<td>495.4</td>
</tr>
<tr>
<td>DP 3</td>
<td>8.079</td>
<td>62.5</td>
</tr>
</tbody>
</table>

*Table 1. Measured concentration of fructose and glucose released from hydrolysis of 3 µg mL⁻¹ of sucrose. The expected concentrations are 1.58 µg mL⁻¹.*

The researcher previously studied the hydrolysis of sucrose using a conventional microwave, which resulted in poor reproducibility. The poor reproducibility could be due to the lack of controlled variables such as temperature. Using an industrial microwave for hydrolysis resulted in reproducible results, and time efficiency, which were due to controlled time, power, and temperature. Sucrose was hydrolyzed three times, consecutively, to test the reproducibility. The reproducibility was calculated by the concentration (mg mL⁻¹) of sucrose components, fructose and glucose. The relative standard deviation of fructose and glucose was 1.65% and 1.26%, respectively.

Separation of Carbohydrates
The separation of carbohydrates is often tedious due to the pre-treatments needed to visualize carbohydrates. The researcher’s previous and current studies have shown that HPLC results in good sensitivity using the
Prevail column for separation of monosaccharides, sucrose and glucose oligomers shown in Figures 4, 5, 6 and 7. Good sensitivity in the analysis of monosaccharides is demonstrated by high signal to noise levels in low concentration level standards analyzed in Figures 4 and 6. The high signal to noise ratio indicates that HPLC-CAD has good sensitivity for detecting carbohydrates at low concentrations. A high signal to noise ratio and good sensitivity is important for the possible application of biologically derived carbohydrates. The linear response was observed for monosaccharide detection (see Figure 8). Quantification of carbohydrates is possible using the CAD as indicated by the linear response of previous studies and glucose. Analysis of carbohydrates is simple compared to methods that require additional steps.

![Figure 6. Chromatogram of fucose, N-acetylglucosamine, galactose, and glucose standards at 10 µg mL⁻¹.](image1)

![Figure 7. Separation and detection of 1 µg mL⁻¹ (20 ng injected) DP 1 to 8 glucose oligomers. A contaminant overlapped with the DP 4 peak.](image2)
Quality of Hydrolysis Yields
Complete hydrolysis of sucrose is demonstrated by the absence of sucrose as shown previously in Figure 4. Sucrose reaction product concentrations were close to that expected. As shown in Table 2, 77 % DP 8 was recovered after hydrolysis, with glucose being the predominant product.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Retention Time</th>
<th>Peak Area</th>
<th>% Glucose Oligomer</th>
</tr>
</thead>
<tbody>
<tr>
<td>DP 1</td>
<td>5.566</td>
<td>2072.4</td>
<td>67</td>
</tr>
<tr>
<td>DP 2</td>
<td>6.664</td>
<td>495.4</td>
<td>9</td>
</tr>
<tr>
<td>DP 3</td>
<td>8.079</td>
<td>62.5</td>
<td>1</td>
</tr>
</tbody>
</table>

Table 2. Results of DP 8 hydrolysis. The right column shows the mole percent yield of hydrolyzed products.

**Future Research**

Future studies will include separation of the monosaccharides fucose, N-acetylgalactosamine, N-acetylglucosamine (and their hydrolysis products), mannose, and galactose, found in glycans, as they are the most common monosaccharides present in glycans. To further optimize the method the researcher would decrease the sample size to be hydrolyzed and to apply the method to isolated glycans due to the limited material (µg amounts) available to analyze for hydrolysis and compositional analysis.

Once the methodology has been sufficiently worked out, the researcher plans to determine the composition of glycans that have been recently isolated from the *X. laevis* glycoprotein by Noah Kiedrowski. Binding assays will be used to determine which monosaccharides react with the CGL lectin. The researcher expects to get basic structural information on the important ligand constituents.
CONCLUSION

Microwave-assisted hydrolysis proves to be a useful method to hydrolyze sucrose and DP 8. Microwave-assisted hydrolysis, although still being optimized, will continue to be used as a method to analyze monosaccharide content. The researcher’s previous studies, including the data shown, demonstrate the simplicity of HPLC-CAD as a method to analyze carbohydrates. The results illustrate the effectiveness of hydrolysis and the sensitive detection of carbohydrates, which may be applied universally.
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