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# The Antimicrobial Properties of Honey and Their Effect on Pathogenic Bacteria

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A thesis submitted to the faculty of
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Master of Science

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ABSTRACT

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Effects on Pathogenic Bacteria

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Honey has been used to heal wounds since ancient times and there are many references in ancient literature that cite honey for its medicinal uses. It is used as an alternative agent to cure infections of wounds, burns, ulcers etc. Researchers have shown some of the antimicrobial properties of honey when used as an ointment. The purpose of this study was to examine the antimicrobial properties of honey from Utah and other locales, and to identify promising antimicrobial activities that could be useful in treating infections caused by resistant bacteria. Five different bacteria and eight different honey samples were used. To see the effects of honey on bacteria, various methods were employed. A disk diffusion assay was used to measure zones of inhibition. Osmolarity was measured to examine total solute differences. An Amplex® Red hydrogen peroxide/peroxidase assay kit was used to measure the amounts of hydrogen peroxide in the various honey samples. Protein assays were performed to examine total protein content and also to identify the presence of known antimicrobial proteins. The pH of each honey sample was also measured. Honeys used in this study showed relatively similar sugar contents and pH levels. One honey sample, NY, did not show any antimicrobial activity when it was tested against several bacterial pathogens. It also possessed a lower content of protein and hydrogen peroxide. Major Royal Jelly Protein 1 (MRJP1) was found in abundance in all honey samples. Sample 13 showed good antimicrobial activity even though it had lower concentrations of hydrogen peroxide than sample 14 and M+20. Sample 13 also had a slightly lower protein content than the other samples that displayed significant antimicrobial activity. The catalase inhibition studies showed that sample 13 displayed significant hydrogen peroxide activity. A more detailed study of the antimicrobial properties of these components may lead to the identification of useful therapeutics that can be used in our never-ending war against microbial infections.

Keywords: honey, anti-microbial, monofloral, polyfloral, Manuka honey, zone of inhibition, Bradford assay, HPLC-MS-MS, sugar content, osmolarity, hydrogen peroxide, proteins

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# INTRODUCTION

Infection rates are currently increasing at an alarming rate in both immune compromised and healthy people (Levy & Bonnie, 2004). Preventive measures such as antimicrobial agents which are antifungal, antiviral, and antibacterial are taken (Levy & Bonnie, 2004). Many microbes have become resistant to these drugs and this trend is expected to increase (Levy & Bonnie, 2004). Antimicrobial agents are currently being used to combat the rising burden of global infections, but due to the increase in resistant bacteria, alternative treatments are being sought (S. Mandal, Pal, Chowdhury, & Debmandal, 2009; Kwakman et al., 2008). Many researchers have focused on natural products like plant-based extracts and honey (S. Mandal, DebMandal, Pal, & Saha, 2010; Basualdo, Sgroy, Finola, & Marioli, 2007).

Some of the agents from nature have been used since ancient times to treat and cure infections that are caused by burns and other injuries (Levy & Bonnie, 2004). One of the medicines that have been used traditionally is honey (S. Mandal et al., 2009). It is a natural product formed from the nectar of flowers by honeybees (*Apis mellifera*) which has a wide range of therapeutic effects (S. Mandal et al., 2009). It has been cited as one of the most effective agents in ancient literature (A. Ahmed et al., 2003; Ndip et al., 2007). However, it has a limited use in modern medicine due to lack of scientific support.

## *Traditional uses of honey*

The use of honey has been traced down to some 7000 years ago. Here, some of the beneficial effects of honey used in ancient culture are summarized. Honey was used by ancient Egyptians, Greeks, Indians, and Chinese to treat wounds, infections, and various diseases (Eteraf-Oskouei & Najafi, 2013).

*Honey used in ancient Egypt*

Smith papyrus which is an Egyptian text dated between 2600 and 2200 BC shows a standard prescription for wounds. The mixture contains grease (mrht), honey (byt), and lint/fiber (ftt) (Zumla & Lulat, 1989). It was the most effective and popular drug among the Egyptians and was also mentioned in many remedies (Zumla & Lulat, 1989). Most of the Egyptian medicines included honey, milk, and wine. Honey was offered to their deities as a sacrifice. They also used honey to embalm the dead body (Eteraf-Oskouei & Najafi, 2013).

*Honey used in ancient Greece*

The ancient Greek beverage known as oenomel consisted of honey and unfermented grape juice and was used as a remedy for gout and certain nervous disorders (Eteraf-Oskouei & Najafi, 2013). In Greece and in many other areas of the world, honey has been used as a preservative of food (Voidarou et al., 2011).

The great Greek scientist and physician Hippocrates promoted these simple remedies (Eteraf-Oskouei & Najafi, 2013; Zumla & Lulat, 1989), that when honey and vinegar are taken together it reduced the pain. When honey and water are swallowed together it quenches thirst. Mixture of honey, water and other medicines are taken it helps in curing acute fevers. He also suggested honey to cure baldness, eye disease, cough, and sore throat. It was also used in topical antisepsis and in preventing and treating scars (Zumla & Lulat, 1989).

*Honey used in the ancient Indian system of Ayurveda*

In the Indian system of Ayurveda, which means “knowledge of life” (Telles, S., Puthige, R., & Kalkuni Visweswaraiah, N., 2007), honey is a blessing to those with weak digestion system (Eteraf-Oskouei & Najafi, 2013). The *Vedic* civilization considered honey to be one of

nature's most remarkable gifts to mankind (Eteraf-Oskouei & Najafi, 2013). The experts of Ayurveda recommend honey as valuable in keeping gums and teeth healthy, and as a treatment for skin disorders, cardiac pain, anemia, and imbalances of the lungs (Eteraf-Oskouei & Najafi, 2013).

*Honey used in ancient China*

Honey, according to Chinese traditional medicine, has a balanced character and acts as the principles of the Earth element. It enters the lung, spleen, and large intestine (Kuropatnicki, Kłósek, & Kucharzewski, 2018). Chinese tradition also has many scriptures and original prescriptions that suggest the use of honey. In these traditions, honey was used in combination with bee venom, pollen, royal jellies and other natural medicines to treat infections (Kuropatnicki et al., 2018).

In 1892 the Dutch scientist Van Ketel showed that honey exhibited antibacterial effects (Dustmann, 1979). Currently, researchers are paying more attention to medicines which have natural origins, and they believe that some natural products may be as effective as newer synthetic drugs (Abuharfeil, Al-Oran, & Abo-Shehada, 1999). Researchers and medical professionals have rediscovered some therapeutic uses of honey, especially where antimicrobial agents have failed to cure infections (Abuharfeil et al., 1999).

There are many reports that show the antimicrobial activity of honey against pathogenic microorganisms. A new medical branch called *apitherapy,* (M. D. Mandal & Mandal, 2011) in which all of the therapeutic products are bee-derived, is gaining popularity in modern day medicine (M. D. Mandal & Mandal, 2011). The reference to honey was first written in a Sumerian tablet writing, dating back to 2100-2000 BC, where they mentioned the use of honey as a drug and ointment (M. D. Mandal & Mandal, 2011). Presently, there are many types of

honey available which claim to have antibacterial properties. One of the most famous is Manuka honey, which is derived from *Leptospermum scoparium* trees found in New Zealand and eastern Australia. Figure 1 shows the Manuka tree with its flowers, from which honeybees obtain the nectar to make this honey.

Figure 1. Manuka tree and blossoms. (http://www.campermate.co.nz/wp-content/uploads/2012/11/IMG_9486.jpg)

*Sensitivity of bacteria to honey*

Many scientists and researchers have tested Manuka honey against various bacteria and it has been shown to be antimicrobial against 60 species of bacteria, including gram-positive, gram-negative, aerobes, and anaerobes (Dalgleish et al., 2007; Olaitan, Adeleke, & Ola, 2007). The reports have also shown that it is effective against pathogenic bacteria such as methicillin resistant *Staphylococcus aureus* (MRSA) and *Helicobacter pylori*, making Manuka honey a promising therapeutic for some types of wounds and infections (M. D. Mandal & Mandal, 2011). One specific case involved a boy who contracted a mixed infection with *Pseudomonas aeruginosa* and *Staphylococcus aureus* during his knee implantation surgery. Application of sterilized Manuka honey to the dressing pads led to a complete recovery within 11 weeks

(Dunford, C., Cooper, R., & Molan, P., 2000). Other studies have shown the healing effect of honey on infections that do not respond to antibiotics or synthetic medications and antiseptics (Subrahmanyam, M., 1991). Honey can act as a bacteriostatic agent (stops the reproduction of cells, without necessarily killing them) or a bactericidal agent (kills the bacteria), depending on the concentration of honey used to treat antibiotic-resistant pathogens (Patton, Barrett, Brennan, & Moran, 2006). More importantly honey was shown to be capable of killing bacteria in their highly resistant biofilm state (Wang, Starkey, Hazan, & Rahme, 2012). V. Bansal et al., showed that 3-7% pasteurized honey and 4-10% Manuka honey were bacteriostatic, whereas at concentrations of 5-9% and 7-14%, respectively, bactericidal activity was achieved (Bansal, V., Medhi, B., & Pandhi, P., 2005). In contrast, when a sugar solution which was similar in composition to that of honey (artificial honey) was used, it was bacteriostatic at 20-30% and was not bactericidal at all (Bansal, V., Medhi, B., & Pandhi, P., 2005). Previous research has shown that Manuka honey has specific antimicrobial activity which is due to a non-peroxide mechanism known as Unique Manuka Factor (UMF) (Patton et al., 2006; Orla Sherlock1, 4, Anthony Dolan1*, Rahma Athman1, Alice Power1, Georgina Gethin2, Seamus Cowman2, Hilary Humphreys1, 2010). The other recognized medicinal honey, besides Manuka honey, is Tualang honey, which is found in the Malaysian forests. This honey is also receiving attention from researchers and scientists because of its medicinal properties which are similar to those of Manuka honey (Patton et al., 2006).

*Properties of honey*

There are various types of honey available which come from different sources and can vary as much as 100-fold from each other relative to their thickness, color, and antibacterial potency (G Vallianou, 2014). G Vallianou also showed that much of the antibacterial activity in

honey is due to the presence of hydrogen peroxide ($H_2O_2$) (G Vallianou, 2014). Honey also has antioxidant properties, which may play an important role in food preservation (Aween, Hassan, Huda-Faujani, Emdakim, & Muhialdin, 2014). A variety of minerals and trace elements can be found in honey, depending on the floral source and geographic location (R, A., & EM, T., 2016). The exact composition of honey varies with the type of flower, soil, the seasons, and weather conditions at the time of collection. Color varies from very dark brown to colorless. The consistency of honey can vary between thin, viscous, very viscous, and partly to mostly crystallized. Flavors and aroma also depend on the floral source and the origin of the plant. Additionally, honey is hygroscopic, meaning that it draws moisture from cells and thereby dehydrates them (Eteraf-Oskouei & Najafi, 2013; Simon et al., 2009). The sugar concentration of honey is high. It also has a relatively low pH (between 3.3 and 4.5). Both of these properties can, prevent the growth of microorganisms. The healing properties of honey may be related to its antibacterial properties, its ability to -maintain a moist environment around a wound, which may help promote healing and the growth of tissues, and its high viscosity that provides a protective barrier to prevent infection (Lusby, Coombes, & Wilkinson, 2005; M. D. Mandal & Mandal, 2011). Honey may also assist the healing of tissue by inducing leukocytes to release cytokines at the site of infection (Patton et al., 2006).

*Enzymes found in honey*

Enzymes are additional important components of honey which play essential roles in wound healing, and also significantly contribute to the antimicrobial properties of honey. One of the antibacterial properties of honey arises from the enzyme glucose oxidase, which converts glucose to gluconolactone, which leads to the information of hydrogen peroxide and gluconic acid (Rossano et al., 2012). Therefore, various enzymes may be involved in the killing action of

honey on certain pathogens. There are many different types of enzymes in honey such as; diastase, amylase, invertase, catalase, and protease (Pontoh & Low, 2002; S.Babacan, L.F.Pivarnik, 2002). The presence and amounts of these enzymes is influenced by the plants on which the bees feed (Rossano et al., 2012).

Phytochemical factors in honey are described as non-peroxide antibacterial factors, which are believed to include many different complex phenols (also known as flavonoids) and organic acids. These substances do not break down easily under heat or light, or when honey is diluted. Volatile organic compounds including some organic acids, lysozyme, beeswax, nectar, and propolis are additional chemical factors that contribute antibacterial properties to honey (Bansal, V., Medhi, B., & Pandhi, P., 2005; Estevinho, Pereira, Moreira, Dias, & Pereira, 2008; Küçük et al., 2007).

There are two types of honey available based on the floral resources used by bees (R & EM, 2016); monofloral honey and polyfloral honey. Monofloral honeys come from single plant species providing the source of nectar, while polyfloral honeys are derive from nectars of multiple plant species (Rossano et al., 2012). Monofloral honeys have characteristic aromas, which usually indicate that they contain volatile compounds that originate from the sources of nectar (Soler, Gil, García-Viguera, & Tomás-Barberán, 1995). Some monofloral honeys also have stronger antibacterial properties (Soler et al., 1995). Some pathogens are more susceptible to monofloral honeys. Zafar showed that when a monofloral honey was applied to a wound, bacterial attachment to the tissue was blocked, thereby inhibiting the formation of biofilms at the wound site (Zafar, 2014). He also showed that when honey was used with antibiotics like oxacillin, it synergistically increased the antibiotic's effect (Zafar, 2014). A resistant pathogen such as MRSA became susceptible to the combination of honey and oxacillin (Zafar, 2014).

Research has also shown that honey is effective in veterinary medicine, and has cured diseases like mastitis, foot and mouth infections, gastrointestinal disorders, and otitis (Allen & Molan, 1997; Zafar, 2014). Honey has also shown antifungal activity and has been effective in treating dermatophytosis such as onychomycosis, and athlete's foot (Eteraf-Oskouei & Najafi, 2013). Additionally, honey has shown antiviral effects (Zafar, 2014). It has been used topically to successfully treat herpes simplex lesions, rubella rash, and varicella zoster lesions (Zafar, 2014). Honey has also shown some anti-mycobacterial properties (Zafar, 2014). In one study, Zafar showed that the addition of 10% or 20% honey to the medium, inhibited the growth of clinical TB isolates, but growth was not inhibited in media containing lower concentrations (Zafar, 2014). Some have suggested that including honey in one's diet would be beneficial in preventing mycobacterial infections (Mundo, Padilla-Zakour, & Worobo, 2004).

Induced resistance of pathogens to honey has never been shown, which makes honey a promising treatment consideration for infections of wounds, ulcers, burns, etc., which do not respond to antibiotics alone (Simon et al., 2009). Presently, infections of burns and wounds can be very challenging to treat, especially when infections are caused by antibiotic-resistant bacteria (Simon et al., 2009). These infections have been shown to respond to treatment with documented antimicrobial honeys, i.e. Manuka honey, but it is still unknown whether other honeys can perform similarly (Simon et al., 2009). Therefore, it is important to study different types of honey which have not been studied before, and also to examine locally produced honeys for their antimicrobial activities. Our hypotheses are that honeys from different locations, including ones from Utah have similar antimicrobial activity, and that the antimicrobial properties in honey are due to combination of $H_2O_2$, certain proteins, and high osmolarity, mostly from simple sugars.

Various types of honey have been purported to have antimicrobial activity. In order to document these effects, zone of inhibition tests was performed using various pathogenic bacteria. In these tests, 6 mm blank filter paper discs from BD were used. Various honey samples were added to the disks, which were placed on agar plates previously inoculated with various pathogenic bacteria. These plates were then incubated, and zones of inhibition were recorded. The size of the inhibition zones reflected the collective antimicrobial properties (inhibitory or bactericidal) of the honey samples against the specific bacterium.

*Proteins in honey*

Many studies have reported on the major constituents of honey such as sugars, flavonoids, enzymes, minerals and proteins (Cordella, Militão, Clément, & Cabrol-Bass, 2003; Kushnir, I., 1979; Tewari & Irudayaraj, 2004). However, there has been very little published to date on the proteins present in honey (Chua, Lee, & Chan, 2013). The relative amount of protein present in honey is very low, approximately 0.1-0.5%, with molecular weights of these proteins ranging from 20 to 80 kDa (Tewari & Irudayaraj, 2004). Many of these proteins are enzymes, such as alpha-glucosidase, beta-glucosidase, amylase, and glucose oxidase which are important in sugar metabolism (Baroni, Chiabrando, Costa, & Wunderlin, 2002); Won, Lee, Ko, Kim, & Rhee, 2008). These proteins in honey are naturally formed by bees and are important in the enzymatic breakdown of pollen and nectar (Chua et al., 2013; White, J. W., & Winters, K., 1989). Many research papers have shown that, honey proteins (Major Royal Jelly proteins (MRJPs)) contribute to the pharmacological properties of honey including anti-inflammatory, anti-microbial, and anti-cancer activities (Tonks et al., 2003; Molan, 2001). Major royal jelly proteins are family of proteins that are secreted by honey bee.

The family of MRJP consists of nine proteins, MRJP 1-9 (Guo, Kouzuma, and Yonekura, 2009). MRJP-1 is found in highest abundance in honey compared to the others (Šimúth, Bíliková, Kováčová, Kuzmová, & Schroder, 2004). All of the MRJPs are found in royal jelly (RJ) of *Apis mellifera* in various proteome analysis, except of MRJP8 (Buttstedt, Moritz, & Erler, 2014). MRJP-3 was shown to be capable of modulating an immune response in humans (Okamoto et al., 2003). Two of the proteins MRJP1 and MRJP2 have shown to be highly glycosylated, and the important difference between the two is the presence of antimicrobial peptide on C-terminal of MRJP1 (Brudzynski, Lannigan, & Sjaarda, 2015). It has been shown that MRJP1 has 3 precursor antimicrobial peptides: Jelleins 1, 2, and 4 (Brudzynski & Sjaarda, 2015). Most of the studies related to MRJPs have focused on royal jelly. There is a very limited amount of work done on protein identification in honey, using mainly High-Performance Liquid Chromatography with tandem mass spectrometry (HPLC-MS-MS) (Chua, Lee, & Chan, 2015). Chua et al. showed that this hybrid system is suitable for characterizing proteins in honey because of its high sensitivity (Chua et al., 2015). The methods used to extract proteins from honey and characterize them is very important. Similar methodology was used in these studies to characterize the proteins present in our samples.

## Materials and methods

### *The total antimicrobial effect of honey samples on various pathogenic bacteria*

Luria-Bertani (LB) agar (Fisher-scientific) and Muller-Hinton (MHA) agar (Thermo-Fischer) were used for these studies. In these tests, filter paper discs containing known amounts of various honey samples were placed on agar plates previously inoculated with various pathogenic bacteria. These plates were incubated, and zones of inhibition (ZOI) were recorded.

The size of the inhibition zone depended on the individual antimicrobial properties present in the honey. This provided a quick measurement of the antimicrobial properties (inhibitory or bactericidal) of the honey samples against pathogenic bacteria. A list of bacteria used is shown in Table 2. Bacteria were grown at 37°C for 24 hours. Bacteria were then suspended in PSS to a McFarland standard of 0.5. Plates were inoculated as previously described. Blank filter paper disks (6 mm BD) were placed onto the agar plate with sterile tweezers. A 10µl aliquot of different undiluted honey samples were dropped on to the filter paper disks. The plates were allowed to incubate at 37°C for 18 to 24 hours. Zones of inhibition were then recorded.

The honeys evaluated in this study are listed in Table 1. Local honey samples were collected from Lehi and Saratoga Springs, Utah. Other samples came from New york and New Zealand.

Table 1. Characteristics of honey samples evaluated in this study and their source locations.

| Honey samples | Viscosity | Color | Location |
|---|---|---|---|
| 12 | Viscous | Brown | Saratoga springs, Utah, USA |
| 12-w | Viscous and crystallized | Light brown | Saratoga springs, Utah, USA |
| 13 | Thin | Dark brown | Saratoga springs, Utah, USA |
| 14 | Viscous | Dark brown | Saratoga springs, Utah, USA |
| 15 | Crystallized | Light brown | Saratoga springs, Utah, USA |
| New York (NY) | Thin | Colorless | New York, USA |
| Manuka +5 (M+5) | Very viscous | Dark brown | New Zealand |
| Manuka +20 (M+20) | Very viscous | Dark brown | New Zealand |

*Concentration of $H_2O_2$ in honey*

The Amplex® Red Hydrogen Peroxide/Peroxidase Assay kit (Invitrogen catalog no. A22188) was used to measure the concentration of $H_2O_2$ in each of the honey samples. Stock solutions were prepared using the protocol provided by the manufacturer. The presence of $H_2O_2$ was also estimated using Quantofix peroxide strips (refer to Appendix C for more detail on how honey samples were prepared for this assay). Honey samples were diluted for the Amplex Red assay based on the estimation from the strips (refer to Appendix C for more detail).

After honey samples were diluted, 50µl of each dilution was pipetted into wells of a 96-well plate containing 50 µl of 100 µM Amplex Red reagent. The 96 well plate was allowed to incubate for 30 minutes at room temperature. After 30 minutes of incubation, fluorescence emission readings at ~590 nm were recorded using an excitation between 530 and 560 nm. A BioTek plate reader (Synergy HT) was used. All assays were done in triplicate.

*The effects of catalase*

Catalase from bovine liver (Sigma-Aldrich; C1345-1g; LOT# SLBW3156; 2000-5000 units/mg protein) was used in determining the antibacterial activity of $H_2O_2$ in honey. According to the certificate of analysis (COA) from Sigma-Aldrich, the activity of the catalase used was 4918 units/mg for the above identified lot number. Different concentrations of catalase were made to determine the neutralization effects they would have on the $H_2O_2$ in the honey samples. The different concentrations made were: 1,000 units/ml, 100 units/ml, and 10 units/ml, along with a zero-unit control and final concentration of catalase after adding it to 1 gram of honey was 100 units/ml, 10 units/ml, and 1 units/ml as shown in Figure 2. The final concentration were then placed onto the plates with disks that were previously inoculated with bacteria.

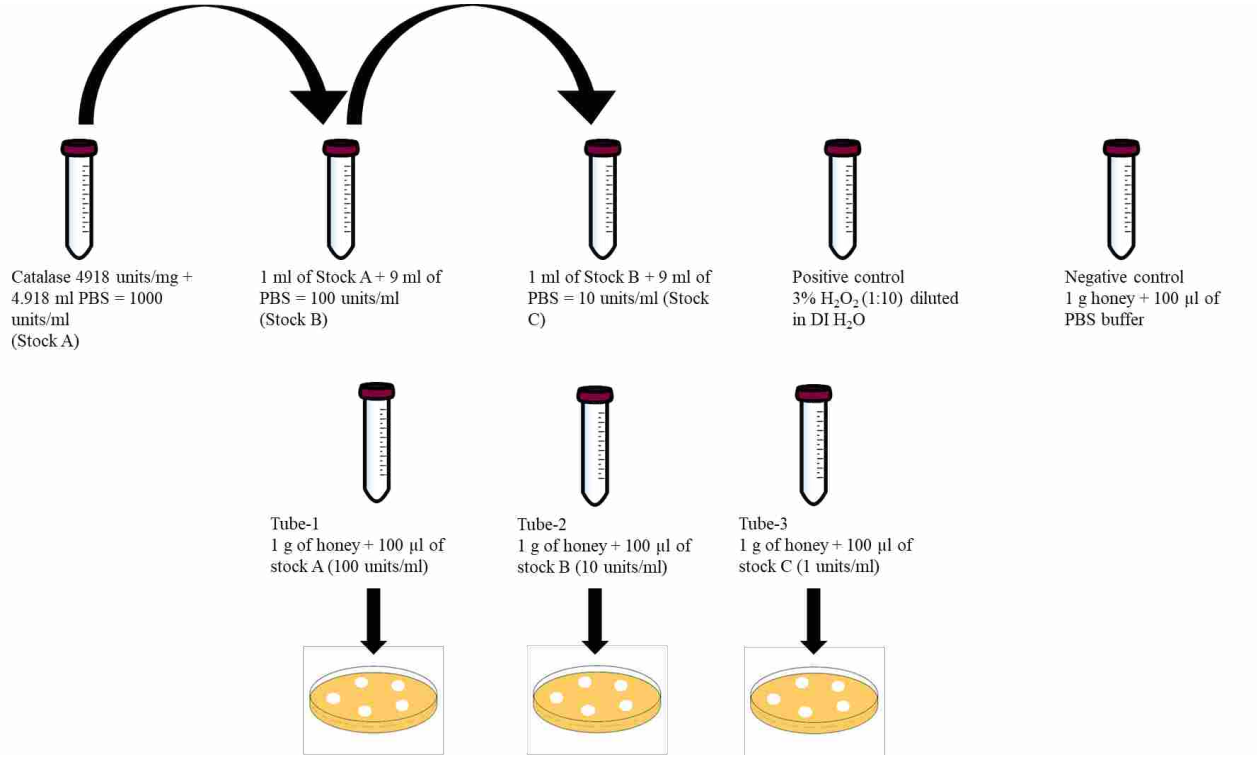


Figure 2. Schematic showing the preparation of the different concentrations of catalase used in these experiments. Tubes 1, 2 and 3 were used to inoculate blank disks on agar plates seeded with pathogenic bacteria.

All of the 8 samples of honey were evaluated in this manner to examine their $H_2O_2$ content. Each sample was done in duplicate and the experiments were repeated 3 times.

Table 2. Pathogenic bacteria used in the growth inhibition experiments.

| Bacteria | Strain# | Origin | Gram reaction |
|---|---|---|---|
| *Staphylococcus aureus* | 29213† | ATCC | Gram positive |
| Methicillin resistant *Staphylococcus aureus (MRSA)* | 1* | ATCC | Gram positive |
| *Escherichia coli* | 25922† | ATCC | Gram negative |
| *Yersinia enterocolitica* | 9610† | ATCC | Gram negative |
| *Pseudomonas aeruginosa* | 15422† | ATCC | Gram negative |

†ATCC number
*Clinical isolate

The bacteria used are listed in Table 2. The experimental design setup is shown in Figure 3.

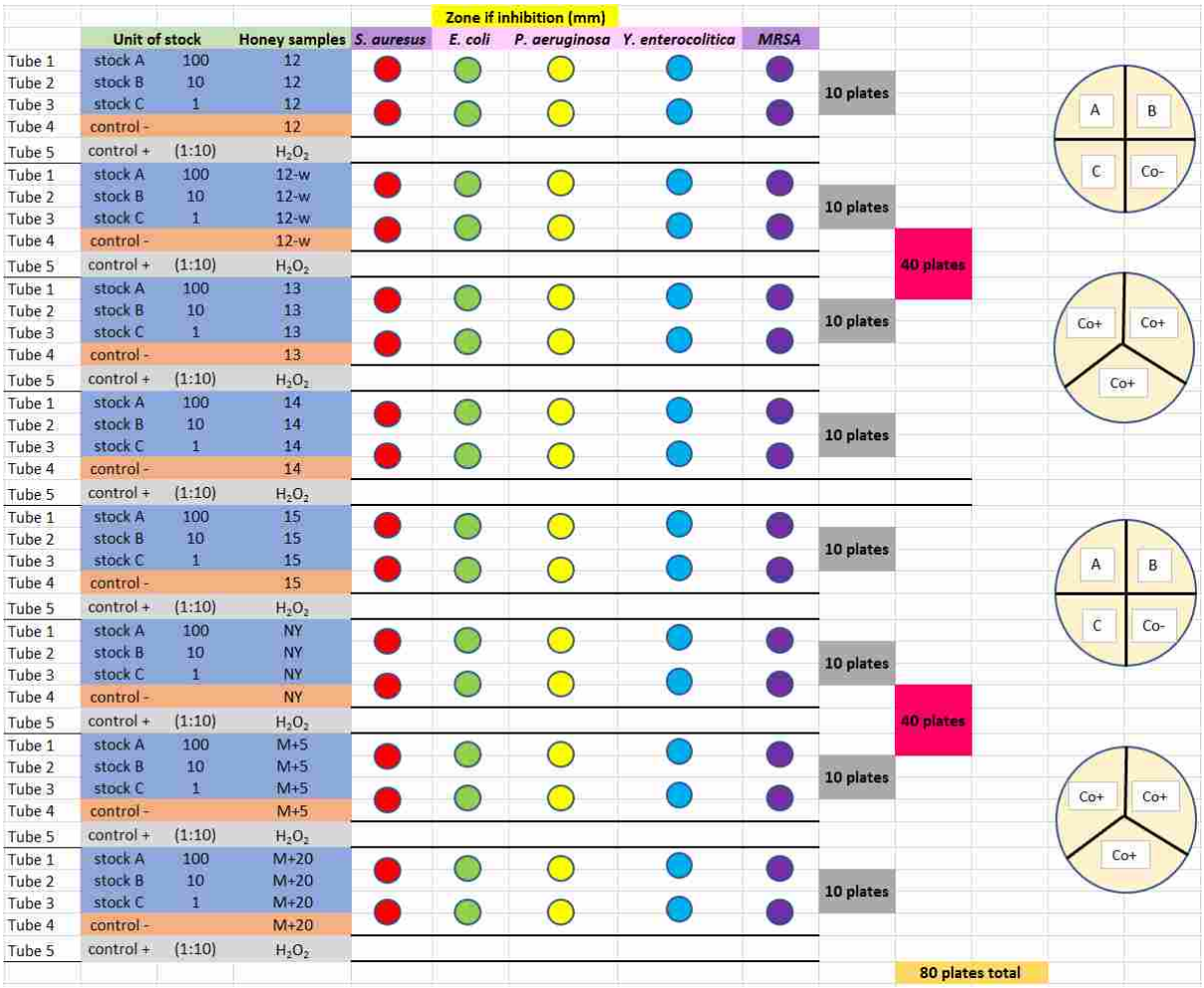


Figure 3. Design of the $H_2O_2$ /catalase experiments. Different concentrations of catalase stock solutions were combined with honey samples and bacteria. Both positive (Co+) and negative (Co-) controls were included.

Luria-Bertani (LB) agar was used for these experiments. Filter paper discs containing known concentrations of catalase mixed with honey were placed on blank disks on agar plates previously inoculated with various pathogenic bacteria. These plates were incubated, and zones of inhibition (ZOI) were recorded. The size of the inhibition zone was dependent on the honey sample and the concentrations of catalase added to the honey. These experiments were done to provide a quick measurement of the $H_2O_2$ activity in each honey sample and its contribution to the antimicrobial properties of that sample.

Bacteria were grown at 37°C for 24 hours. Bacteria were then suspended in physiological saline solution (PSS, 0.85% NaCl). A McFarland standard of 0.5 (1.175% wt/vol barium chloride dihydrate ($BaCl_2 \cdot 2H_2O$) with 1% sulfuric acid vol/vol ($H_2SO_4$)) was used to make bacterial suspension with an approximate cell density of $1.5 \times 10^8$ CFU/ml. The McFarland standard was made by adding 0.5 ml of $BaCl_2 \cdot 2H_2O$ to 85 ml of $H_2SO_4$ and bringing the final volume to 100 ml by adding DI water. A 50 µl aliquot of the bacterial suspension was spread with a cell spreader onto the LB agar plate. Blank filter paper disks (6 mm BD) were placed onto the inoculated agar plate with sterile tweezers.

*Osmolarity measurements of honey samples*

One gram of each honey sample was weighed into a separate 15 ml conical tube using a PL202-s analytical balance (Mettler Toledo). Tubes were done in duplicate in order to prepare two different dilutions. The first dilution was 1:10 dilution in DI $H_2O$. The honey and water were vortexed to obtain a homogeneous mixture. A 10 µl aliquot of this mixture was pipetted onto a 6 mm thin paper disk on the reading plate of a vapor pressure osmometer (Wescor Vapro model 5520). These assays were done in triplicate. The second dilution was a 1:100 dilution in DI $H_2O$, which was processed similarly. The osmometer was calibrated with 290 mmol/Kg and 1000 mmol/Kg standards according to the manufacturer's direction. This instrumentation was kindly provided by Dr. Dixon Woodberry, Department of Physiology and Developmental Biology, BYU.

*Identification of sugars present in honey using gas chromatography (GC)*

All 8 honey samples were diluted 1:100 with DI $H_2O$ in 1 ml conical microcentrifuge tubes. Honey samples were mixed by vortexing for 10 seconds. The samples were then transferred to small gas chromatography tubes and were allowed to dry completely under

nitrogen gas for 24 to 48 hours. After the samples were dried 50 µl of dimethylformamide was added (DMF No. 20672 dimethylformamide silylation grade, MW- 73.09, brand- PIERCE, Lot # 97041170) and 50 µl of (N, O-bis(trimethylsilyl)trifluoroacetamide) was added to derivatize the mixture (BSTFA with 1% TMCS, product # TS- 38833, Lot # MD153610, brand Thermo Fisher). The GC tubes were then sealed with metallic caps and placed an in oven at 75°C for 15 minutes. The tubes were then analyzed for carbohydrates using gas chromatography (GC) by the BYU Department of Food Science and Nutrition.

*Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)*

A 12.5% SDS-PAGE was prepared and used for honey protein separation (refer to Appendix C to see how these gels were made). A 10 µl aliquot of honey was mixed with 10 µl of PBS solution and 20 µl of bromophenol blue dye. The mixture was vortexed and was heated at 100 °C for 10 minutes to denature proteins. Ten µl of this mixture was loaded into the wells of a 4% stacking gel for electrophoresis at 100 V for 30 minutes or until the bromophenol blue dye had migrated 1 cm from the bottom of the gel. A similar treatment was applied to the protein ladder which was loaded into the first well of the gel. The protein ladder contained proteins from 10 kD to 250 kD. After electrophoresis was finished, the gel was carefully washed under running DI water for a few seconds. The gel was then carefully removed from the box and was allowed to stain in Coomassie brilliant blue dye for 10 minutes. After the staining, the gel was left to de-stain in de-staining solution (10% acetic acid, 30% methanol and DI $H_2O$), and was microwaved to speed up the process for at least 1 minute until the de-staining solution came to boil. The folded paper napkin was placed inside the container for the gel to de-stain. The container with gel and de-staining solution was placed onto the gel rocker and the gel was allowed to de-stain

for 30 minutes. The process was repeated 2 to 3 times until the desired level of de-staining was obtained. The gel was then viewed and imaged under visible light.

*In-gel digestion*

The abundant visible protein bands ranging from 50 to 75 kD were cut into small pieces and were submitted to the University of California San Diego (UCSD) Biomolecular & Proteomics Mass Spectrometry Facility for high performance liquid chromatography integrated with tandem mass spectrometry (HPLC-MS-MS) analysis.

*Protein assay (Bradford assay)*

Total honey protein concentrations were measured using the Bradford assay. A Pierce™ Coomassie (Bradford) Protein Assay Kit was used (Thermofisher Cat # 23200).

For the Bradford assay, 5 µl honey samples were diluted in 495 µl of DI $H_2O$ and the mixtures were vortexed thoroughly to get homogeneous solutions. To these tubes, 500 µl of Coomassie G-250 dye was added and mixed. Tubes were allowed to incubate for 10 minutes at room temperature and was covered with aluminum foil to keep it away from light, this was done to get consistent results. Following incubation, readings were taken in a spectrophotometer at 595 nm. The spectrophotometer was blanked with 500 µl of DI $H_2O$ and 500 µl of Coomassie G-250 dye before samples were measured. The bovine serum albumin (BSA) standard provided with the kit was used to calibrate the assay (refer to Appendix C for BSA standard set up). The samples were done in duplicate and the experiment was repeated three times.

*Measuring the pH of honey*

All of the 8 honey samples were diluted 1:2 in DI $H_2O$ and vortexed until homogeneous solution was obtained. The pH meter was calibrated using known pH 1.0 and 4.0 standards. The

readings were taken at room temperature, in triplicates using a SevenEasy 20 pH meter (Mettler Toledo).

## Results

### *Hydrogen peroxide activity*

$H_2O_2$ content of each honey sample was measured using Amplex Red Hydrogen Peroxide/Peroxidase assay in 96 well plates. The concentration of $H_2O_2$ in the honey samples was calculated from the standard curve (refer to Appendix C). The $H_2O_2$ content of the honey samples was measured at different dilutions in DI $H_2O$ (1:2, 1:4, 1:10 and 1:20) to get a more precise concentration determination, since glucose oxidase, the enzyme that produces $H_2O_2$ is practically inactive until honey is diluted. Figure 4 shows the concentration of $H_2O_2$ increased in most samples when they were diluted 1:2 and 1:4, then decreased at subsequent dilutions.

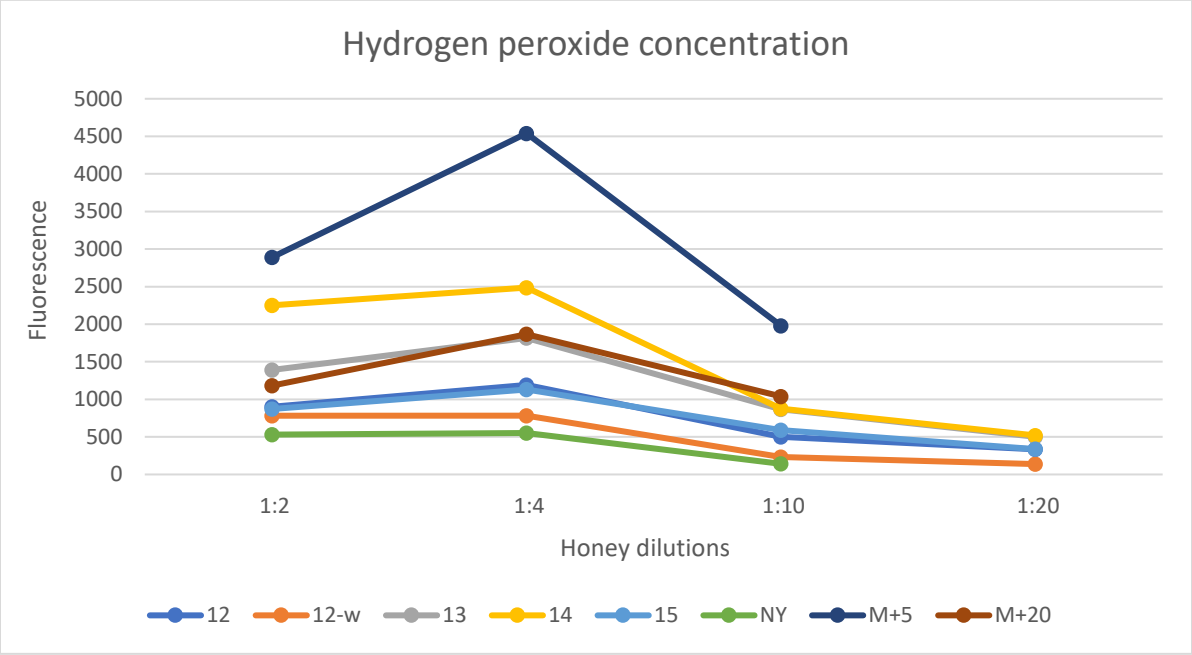


Figure 4. Hydrogen peroxide concentrations in different honey samples at different dilutions in DI $H_2O$. Assay were performed using the Amplex Red hydrogen peroxide/peroxidase assay.

Therefore, the fluorescence value close to each other for 1:2, 1:4, and 1:10 dilution was used to calculate the total concentration of $H_2O_2$ in all of the 8 honey samples. The concentration of $H_2O_2$ in different honeys ranged from 12.5 to 118.8 μM as shown in Table 3.

Table 3. Hydrogen peroxide content of the different honey samples.

| Honey samples | $H_2O_2$ concentration (μM) |
|---|---|
| 12 | 28.2 |
| 12-w | 18.5 |
| 13 | 47.7 |
| 14 | 62.2 |
| 15 | 34.4 |
| NY | 12.5 |
| M+5 | 118.8 |
| M+20 | 46.2 |

Honey samples 13 and M+5 consistently showed the highest concentrations of $H_2O_2$. Other samples showed lower levels of $H_2O_2$. The NY sample consistently showed the lowest levels of $H_2O_2$. The $H_2O_2$ levels of all 8 honey samples are shown in Table 3.

*Effects of catalase on the antibacterial activity of honey*

To further elucidate the antimicrobial contribution of $H_2O_2$ in honey, samples were treated with catalase. Addition of catalase to the honey samples rapidly neutralized the $H_2O_2$ and terminated its antibacterial activity as shown in Figure 5 (a-e). Neutralization of $H_2O_2$ with different concentrations of catalase reduced the antibacterial activity of the honey samples when

tested against different bacteria. This showed the importance of $H_2O_2$ in the antimicrobial activity of most honey samples.

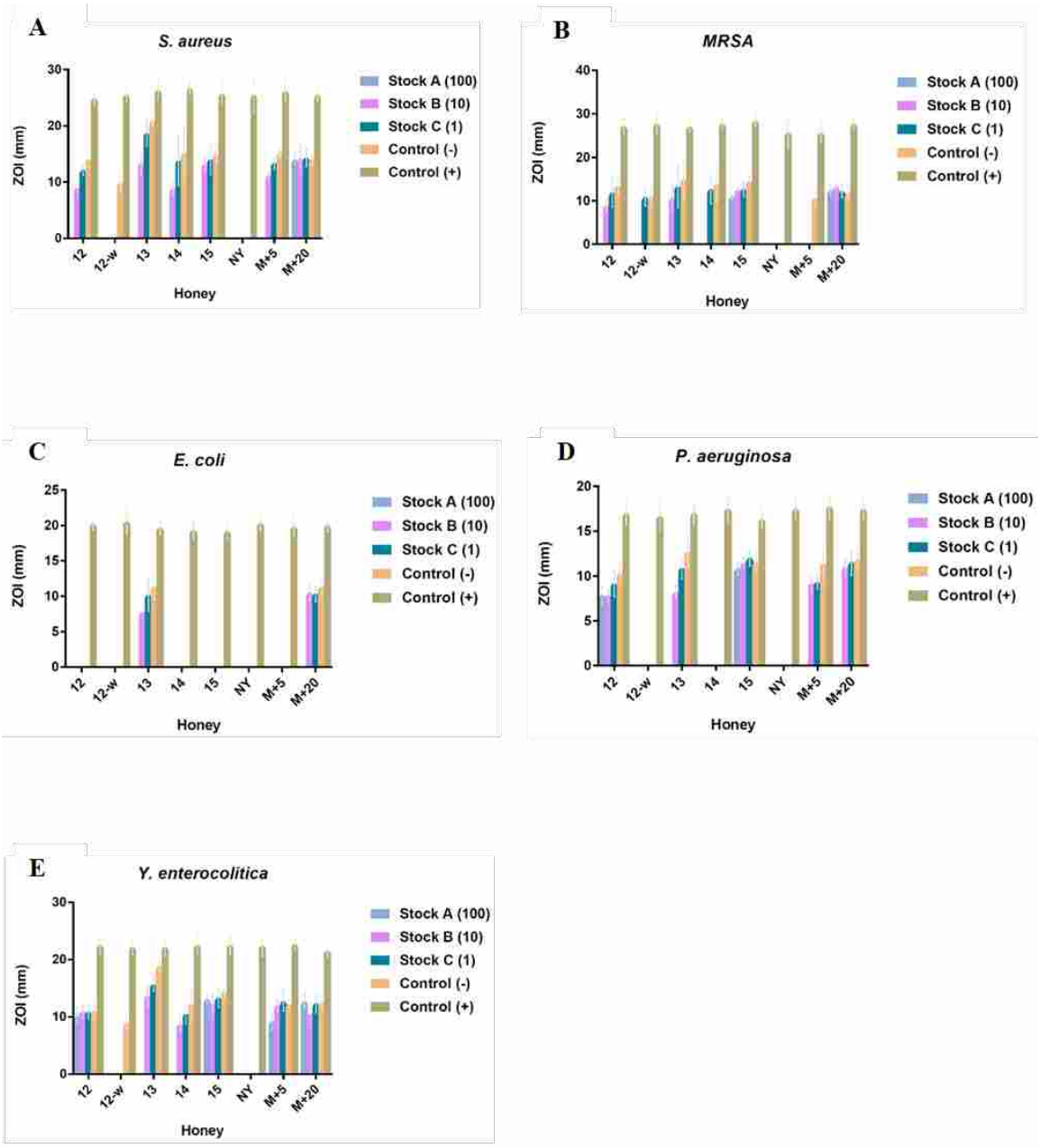


Figure 5. The effect of catalase on the antimicrobial activity of honey. Three different concentrations of catalase (100, 10 and 1 units) were added to various honey samples and their zones of inhibition (ZOI) against five different bacterial pathogens were measured (A). *S. aureus*. (B). *MRSA**. (C). *E. coli*. (D). *P. aeruginosa*. (E). *Y. enterocolitica*. The positive control was 0.3% $H_2O_2$ and the negative control was 1g honey + 100 µl of PBS buffer.

As seen in Figure 5 (a-e), 100 units of catalase neutralized the antimicrobial activity of most honey samples against most bacterial pathogens tested. *Y. enterocolitica* seemed to be most sensitive to the antibacterial action of honey samples neutralized with catalase. The zones of inhibition created by honey samples mixed with stock B (10 units) and C (1 unit) were more prominent in the various honey sample/pathogen combinations. For negative controls, no catalase was added, so antimicrobial activity due to $H_2O_2$ was seen in most of the honey sample/pathogen combinations. The positive control (0.3% $H_2O_2$) produced large zones of inhibition across all pathogens tested. Honey sample 13 seemed to possess the most $H_2O_2$-associated antimicrobial activity across all five pathogens tested.

In contrast, the honey sample which did not show any $H_2O_2$-associated antimicrobial activity across all five pathogens was NY.

*Osmolarity of various honey samples*

Honey has a very high solute concentration (mostly sugars), and it is well-known that its high osmolarity prevents almost all microbial growth. In order to establish that microbial inhibition differences between honey samples seen in Figure 5 were not due to osmolarity differences, we measured the osmolarity of each sample. These results are shown in Figure 6. The various honey samples did not differ significantly is osmolarity. But, the osmolarity of honey sample 13 was found to be highest, while honey sample NY showed the lowest osmolarity, in comparison to other samples.

When honey samples were diluted 1:10, the osmotic effect on bacteria was understandably reduced, and these diluted samples had reduced antibacterial effects. But, when samples were

diluted 1:100, the osmotic effect was reduced even further and antibacterial activity was not seen at all.

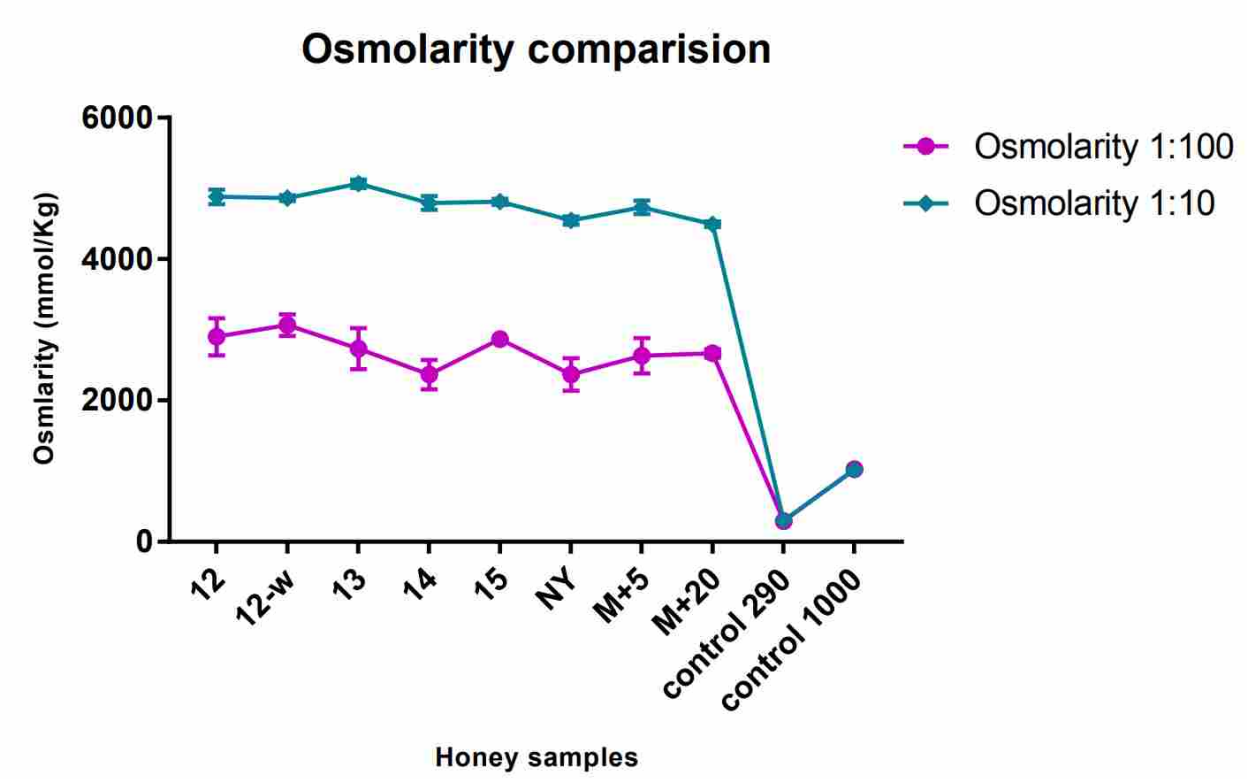


Figure 6. Osmolarity values for 1:10 and 1:100 dilutions of the various honey samples. The control standards of 290 mmol/Kg and 1000 mmol/Kg were used per manufacturer's instruction. Each sample was measured 3 times for two different dilutions. The repeated values were so close to each other that error bars are very small.

Figure 7 shows that when full strength honey was used bacterial growth is hindered and the zones of inhibition increased. As seen in Figures 7a and 7b, the zones of inhibition for *S. aureus* and *MRSA*-1 are largest when compared to the gram-negative bacteria *P. aeruginosa*, *Y. enterocolitica*, and *E. coli*, as seen in Figures 7c, 7d and 7e.

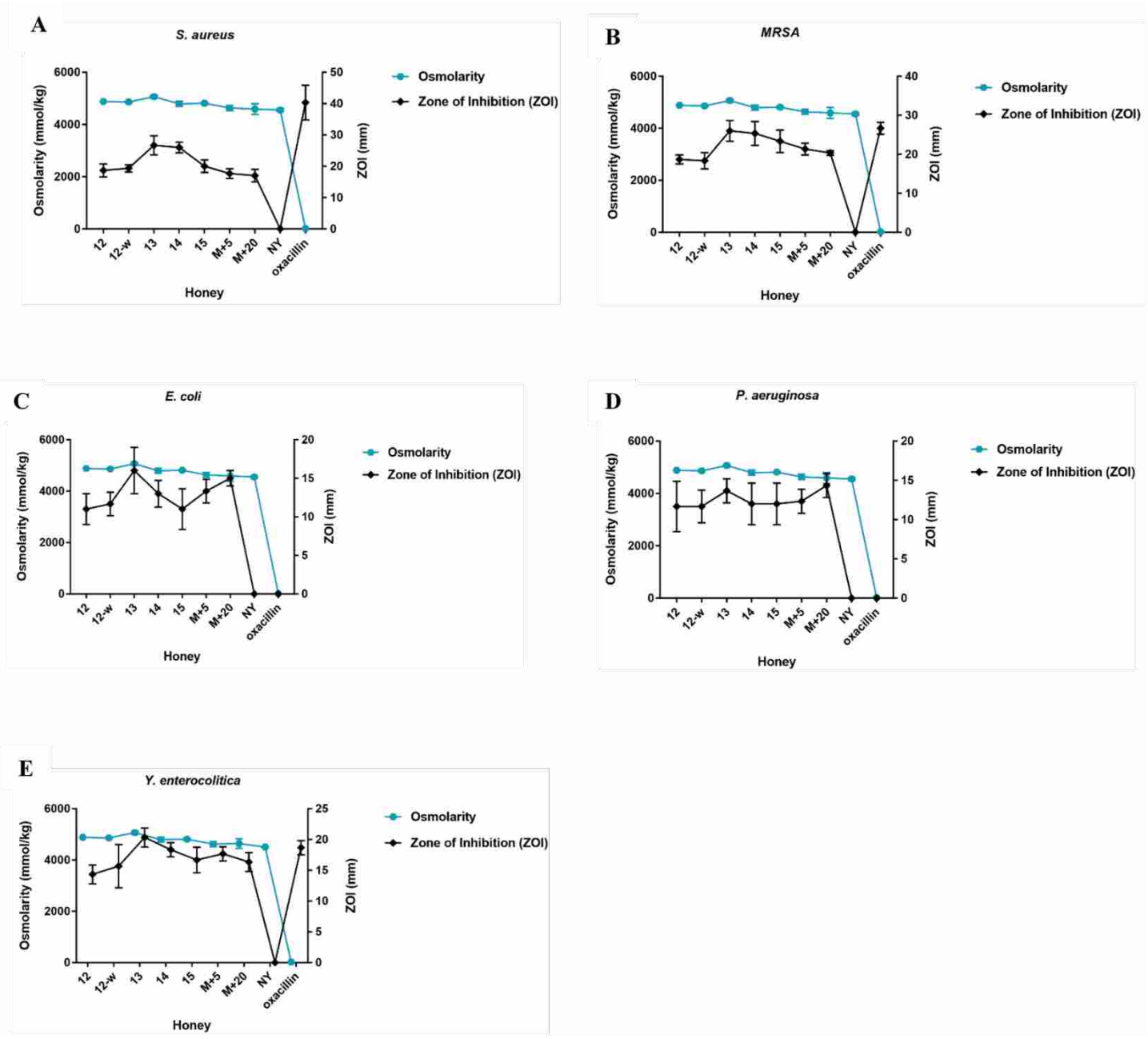


Figure 7. Osmolarity and zone of inhibition values for the different honey samples across 5 different bacterial pathogens. (A). *S. aureus*. (B). *MRSA**. (C). *E. coli*. (D). *P. aeruginosa*. (E). *Y. enterocolitica.*

(*clinical isolate).

*Carbohydrate composition*

Honey is composed mostly of carbohydrates with a small amount of water. The most abundant sugars found in honey are the monosaccharides glucose and fructose, with the disaccharide sucrose found at very low concentrations. Each of the honey samples used in this study was assayed for the amounts of these common sugars by GC. Table 4 shows these results (refer to Figures 9 through 16 in Appendix A for complete carbohydrate profiles of all samples) for the main carbohydrates present in the honey samples.

Table 4. Carbohydrate composition of each honey sample.

| Honey (1:100) | Amount of sugar present in (mg/ml) | | |
|---|---|---|---|
| | Glucose | Fructose | Sucrose |
| 12 | 3.21 | 5.06 | $2.62 \times 10^{-3}$ |
| 12-w | 3.12 | 3.28 | $2.28 \times 10^{-2}$ |
| 13 | 3.04 | 4.30 | $4.67 \times 10^{-3}$ |
| 14 | 3.00 | 2.85 | $1.78 \times 10^{-2}$ |
| 15 | 2.84 | 3.68 | $1.15 \times 10^{-2}$ |
| NY | 2.50 | 2.12 | $1.90 \times 10^{-2}$ |
| M+5 | 3.00 | 2.24 | $2.24 \times 10^{-2}$ |
| M+20 | 3.32 | 4.00 | $1.01 \times 10^{-2}$ |

As can be seen from Table 4, the sugar concentration for all of the honey sample is similar. Honey sample 13, which was the most antimicrobial sample in this study, had similar sugar concentrations to NY, which displayed the least antimicrobial activity.

*Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)*

A representative SDS-PAGE image for the honey samples is shown in Figure 8. The most abundant proteins are between 50 to 75 kD. The analyses of these proteins are shown in Figure 8 (refer to Figure 17 through 24 in Appendix B for the complete protein profiles). The mass spectrometer results for these abundant bands showed that MRJP1 was present in highest amount. MRJP1 was more prevalent than any other protein in these honey sample.

Besides MRJP1 protein, there were also other royal jelly protein family members identified as MRJP2 to MRJP7 and MRJP9, MRJP8 was absent in all honey samples.

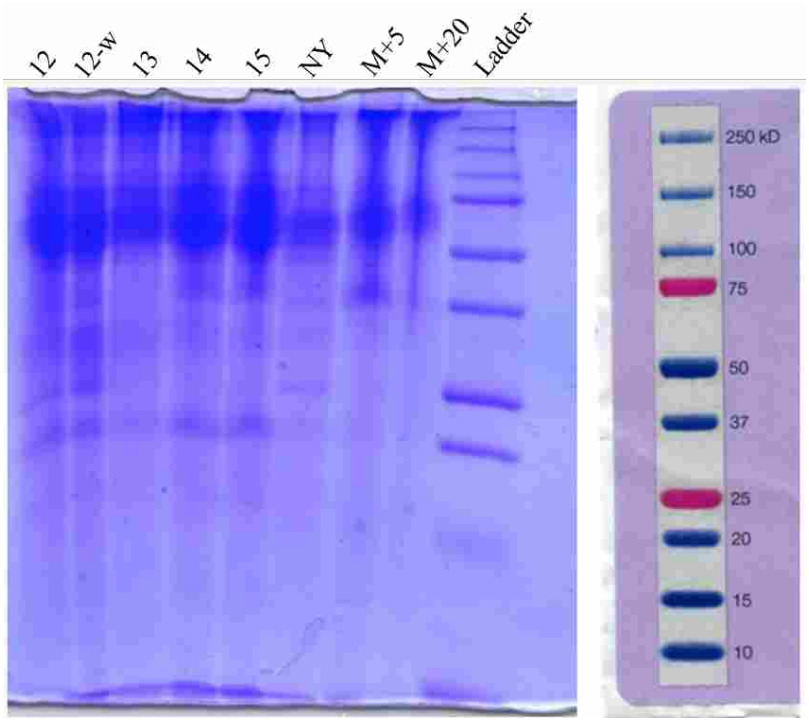


Figure 8. SDS-PAGE gel image of all 8 honey samples. The maximum ladder protein size is 250 kD. The gel was allowed to run at 100 V for 30 minutes and was stained in bromophenol blue dye for 10 minutes.

Coverage % is calculated by mapping the identified peptides to the open reading frame (ORF) sequences for that protein and determining the % of the sequence that is covered by the identified peptides.

Table 5. Major proteins and enzymes identified in the honey samples with coverage percent.

| | Proteins/enzymes | 12 | 12-w | 13 | 14 | 15 | NY | M+5 | M+20 |
|---|---|---|---|---|---|---|---|---|---|
| Coverage (%) | MRJP-1 | 84.49 | 84.49 | 69.68 | 84.49 | 81.94 | 72.45 | 85.88 | 82.18 |
| | MRJP-2 | 79.42 | 82.08 | 69.25 | 79.87 | 79.42 | 68.36 | 76.77 | 77.21 |
| | MRJP-3 | 58.46 | 64.15 | 55.88 | 61.03 | 69.49 | 49.82 | 64.34 | 58.09 |
| | MRJP-4 | 44.40 | 45.04 | 42.46 | 49.78 | 40.09 | 46.77 | 43.53 | 5.82/(*31.25) |
| | MRJP-5 | 33.28 | 45.82 | 36.12 | 43.31 | 40.30 | 29.26 | 27.93 | 30.77 |
| | MRJP-6 | 49.66 | 44.39 | 35.93 | 45.08 | 41.19 | 33.87 | 43.94 | 28.83 |
| | MRJP-7 | 51.69 | 57.11 | 48.53 | 55.98 | 56.43 | 49.66 | 58.92 | 37.92 |
| | MRJP-8 | - | - | - | - | - | - | - | - |
| | MRJP-9 | - | 12.06 | 18.68 | - | 13.71 | 19.62 | - | 11.58 |
| | Glucose oxidase | 52.85 | 52.68 | 30.89 | 62.11 | 43.41 | 31.38 | 27.8 | 23.41 |
| | Alpha amylase | 59.23 | 62.88 | 48.28 | 55.38 | 50.1 | 52.54 | 39.96 | 30.63 |
| | Glucose dehydrogenase | 30.88 | 35.68 | 16.8 | 35.2 | 30.4 | 15.52 | 14.4 | 5.6 |
| | Alpha glucosidase | 74.96 | 70.02 | 57.5 | 72.31 | 61.55 | 45.86 | 60.14 | 39.51 |

(*) MRJP-4 isoform

*Bradford assay*

To confirm the presence of proteins, Bradford assays were performed. The results of the Bradford assays in Table 6, indicated that the protein concentrations for most of the honey samples were similar. The protein content of the New York (NY) honey sample was the lowest when compared to the other honey samples. Moreover, the NY honey also produced the smallest number of protein bands in the SDS-PAGE gel. The other honey samples showed similar amounts of protein as detected by the Bradford assay.

Table 6. Bradford assay results of honey samples.

| Honey samples | Proteins in original honey sample (mg/ml) |
|---|---|
| 12 | 8.64 (± 0.01) |
| 12-w | 10.51 (± 0.05) |
| 13 | 8.39 (± 0.03) |
| 14 | 8.98 (± 0.03) |
| 15 | 9.48 (± 0.03) |
| NY | 4.34 (± 0.01) |
| M+5 | 7.26 (± 0.02) |
| M+20 | 10.49 (± 0.03) |

(±) standard error

*pH readings in honey*

The pH values for all of the honey samples in this study are shown in Table 7. These values ranged from 3.54 (NY) to 4.6 (13). This was fairly narrow range, indicating that most honey samples are slightly acidic in nature, which prevents the growth of microbes in them.

Table 7. pH values of honey samples diluted 1:2 with DI $H_2O$.

| Honey samples | pH values |
|---|---|
| 12 | 3.55 (± 0.018) |
| 12-w | 3.68 (± 0.008) |
| 13 | 4.16 (± 0.015) |
| 14 | 3.81 (± 0.014) |
| 15 | 3.75 (± 0.011) |
| NY | 3.54 (± 0.005) |
| M+5 | 3.96 (± 0.008) |
| M+20 | 3.91 (± 0.003) |

(±) standard error and range of values from multiple reading

The relative amounts of the known antimicrobial properties in honey were summed and compared with their observed antimicrobial properties. Figure 9 shows the relative amounts of three known antimicrobial properties in our honey samples, and how they relate to their antimicrobial properties as measured by the zones of inhibition. The composite antimicrobial content was ranked from highest to lowest (left to right) and compared with the mean zone of inhibition values of the five bacterial pathogens tested. There was a general correlation between these values except for samples 13 and 14 for the gram positives pathogens and sample 13 for the gram negatives pathogens. These samples produced larger zones of inhibition than the sum of their antimicrobial properties predicted.

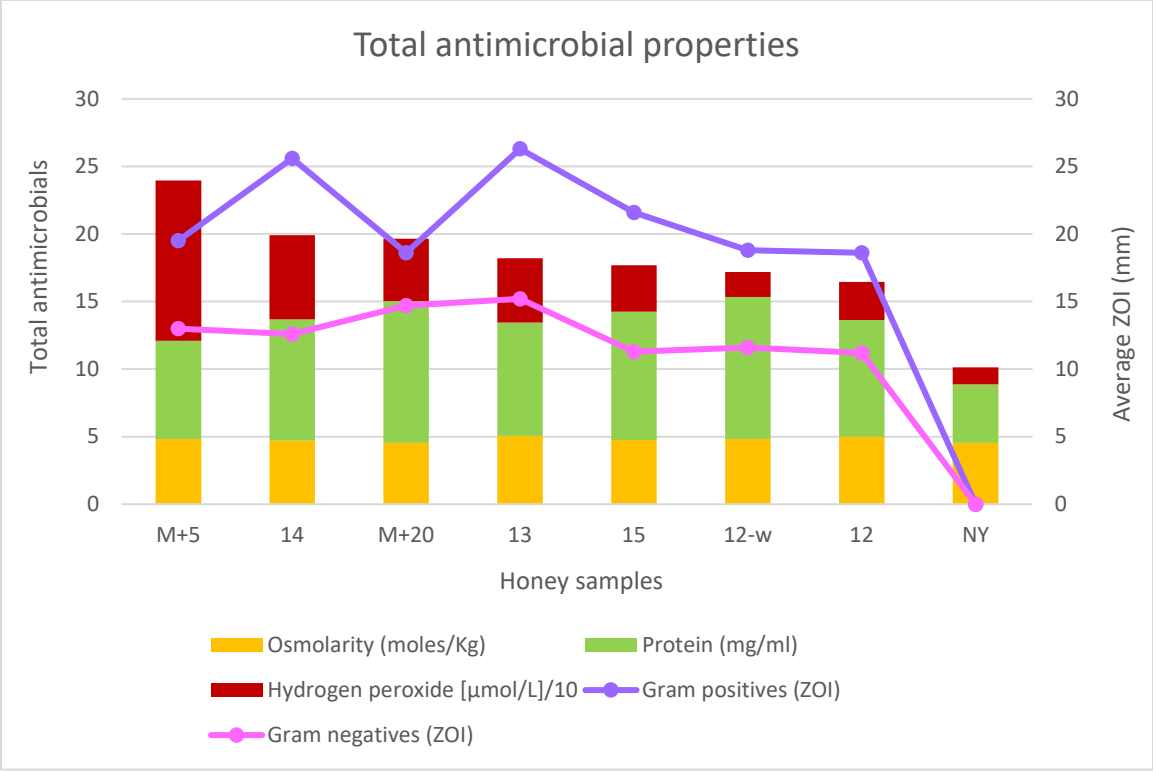


Figure 9. Total antimicrobial properties of each of the honey samples. Osmolarity was converted to moles/kg for representation purposes. The values of hydrogen peroxide were in (µmol/L)/10 for representation purposes, and protein values were mg/ml. Zones of inhibition (ZOI) was averaged across the five bacterial pathogens for all honey samples.

## Discussion

The results from these studies illuminate how certain components of honey may be related to its antimicrobial properties. Although honey's osmotic characteristics contribute to its antimicrobial properties, there are additional factors involved. The complex composition of honey makes it a promising substance to mine for possible therapeutic antimicrobial factors. Part of the antimicrobial activity of honey is related to the presence of $H_2O_2$.

As shown in Table 3, some of the honey samples showed low concentration of $H_2O_2$, indicating that, some types of honey do not accumulate high levels of $H_2O_2$. According to Bang

et al, the production of hydrogen peroxide by the enzyme glucose oxidase happens during the production or dilution and little or no $H_2O_2$ is produced in full strength honey (Bang, Buntting, & Molan, 2003). In these experiments, involving eight honey samples, we showed that the maximum production of $H_2O_2$ was seen at a 25% concentration and low levels of $H_2O_2$ was detected at a 50% concentration, despite the fact that concentration of the enzyme glucose oxidase and its substrate decreased when the honey was diluted from full strength ( Peter C Molan, 1992).

The reason for low glucose oxidase activity in undiluted honey is unknown, but previous studies have shown that it is not because of glucose oxidation inhibition by constituents of honey such as carbohydrates, enzymes or other minerals (Bang et al., 2003; Schepartz, A. I., & Subers, M. H., 1964). It has been suggested that the reason for low glucose oxidase activity in full strength honey is the low pH (White, Subers, & Schepartz, 1963; Bang et al., 2003). As shown in Table 7, pH values for the honey samples used in this study ranges between 3.5 to 4.2. The slightly acidic properties of honey may contribute to the growth inhibition effects seen on bacteria. The levels of $H_2O_2$ obtained in this study were similar to the results found in other studies, with different honeys. The levels of $H_2O_2$ ranged from 12.5 to 118.8 µM for the eight honey samples. Similar results were seen in a study involving 18 Canadian honey samples when diluted to 12.5%, where values ranged from 29.4 to 238.5 µM/L when calculated to 1 ml of undiluted honey (Brudzynski, 2006). Another study reported similar values ranging from 0 mmol - 0.95 mmol for 31 honey samples (Bogdanov S., 1984). The levels of $H_2O_2$ in a study which included 90 honey samples had similar levels of $H_2O_2$ when diluted to 14% ranging from 0 mmol to 2.12 mmol (Bang et al., 2003). Such wide variations in peroxide levels of honey may be due to the action of catalase in honey samples. Collectively, these results show that the levels of $H_2O_2$

do not continue to increase in honey over time, rather they increase before the final product is achieved or when its honey is diluted.

In addition, this study showed that honey, when diluted to 50%, did not result in the maximum production of $H_2O_2$ in any of the eight honey samples tested, but the maximum production of $H_2O_2$ was seen at 25% (a 1:4 dilution). A similar result was reported by Bang et al., where no $H_2O_2$ were detected in 50% honey (Bang et al., 2003). At a 10% concentration, honey showed detectable levels of $H_2O_2$ in most of the samples, after allowing them to incubate for 1 hour at room temperature. Incubating honey samples for more than 1 hour did not result in more $H_2O_2$ production (data not shown). Some factors which are known to affect the antibacterial activity of $H_2O_2$ in honey are exposure to excessive heat (>55°C) or light, or degradation of $H_2O_2$ within the honey itself (Majtan, Bohova, Prochazka, & Klaudiny, 2014). Moreover, the concentration of $H_2O_2$ in honey is approximately 900-fold lower than concentrations commonly used in hospital settings to disinfect medical equipment (Bizerra, Da Silva, & Hayashi, 2012).

The contribution of $H_2O_2$ to the antibacterial activity of honey can be determined by the effect of neutralization of this compound by adding the enzyme catalase (Majtan et al., 2014). Figure 5 showed that neutralizing $H_2O_2$ by adding catalase, abrogated the antibacterial activity of most honey samples when tested against pathogens. Similar results have been reported by others where they used 1000 units of catalase and further dilutions. Higher concentrations of $H_2O_2$ were completely removed from their standard curve samples, but not from honey samples treated similarly. (Allen, K. L., Molan, P. C., & Reid, G. M., 1991; Brudzynski, 2006). Our work showed similar results. When higher concentrations of catalase were added to most honey samples, it completely removed the $H_2O_2$ and also their antimicrobial activity. But, when lower

concentrations of catalase were used, complete removal of antimicrobial activity was not seen. The major variations seen in overall antibacterial activity of the honey samples were most likely due mainly to differences in the levels of $H_2O_2$. However, for some samples the antibacterial activity was due to non-peroxide factors. The concentration of $H_2O_2$ in the honey samples shown in Table 3 were 1,000-fold less than the 3% $H_2O_2$ commonly used as an antiseptic. This suggests that $H_2O_2$ is an important part of honey's antimicrobial activity. In the absence of glucose oxidase, honey may retain its antibacterial activity, even when catalase is present. This type of honey is known as "non-peroxide" honey (M. D. Mandal & Mandal, 2011).

When honey is diluted from full strength, both the viscosity and osmotic pressure drops (Figure 6) and this can result in growth of microorganisms which can cause spoilage (Molan, P. C., & Betts, J. A., 2004). Honey's composition consists of 90-95% sugar, mainly the monosaccharides glucose and fructose, and some disaccharides like sucrose and maltose. Other oligosaccharides are also present (Kwakman & Zaat, 2012). The amount of water in honey is only about 5-10%. As seen in Table 4 and Figure 6, sugar concentration does not directly correlate with antimicrobial activity. Honey sample NY showed the least antibacterial properties, despite the fact that its sugar concentrations were similar. A high sugar concentration with low available water results in a hypertonic solution, which prevents the growth of microorganism in honey (Kwakman & Zaat, 2012; Simon et al., 2009). When honey is applied to a wound site it draws the moisture out of the microbes which causes them to dehydrate and die, while allowing the wound to heal (Mandal & Mandal, 2011; Simon et al., 2009).

Since proteins in honey have been identified as a possible source of antimicrobial activity, we evaluated the protein content of each honey sample and identified the major proteins present by mass spectrometry. These results indicated that MRJP1 protein was found in greatest

abundance in all 8 honey samples that were evaluated (including six from the United Sates and two from New Zealand). These results were similar to those obtained in Won et al. (Won et al., 2008). Chua, Lee, and Chan also showed that MRJP-1 protein was present with other proteins in honey, with molecular weights ranging between 20 kDa to 76 kDa (Chua et al., 2015). Similar results were obtained in this study, as the molecular weight of MRJP-1 and other proteins ranged between 50 kDa and 75 kDa. Research has shown that antimicrobial properties in honey with abundant MRJP1 may be due to the co-presence of jelleins (Brudzynski & Sjaarda, 2015). Katrina Brudzynski showed the presence of 3 jelleins in MRJP1 and described how they contributed to the antimicrobial activity of honey. Jelleins are considered precursor antimicrobial peptides and were found with MRJP1. Brudzynski identified these peptides as: Jelleins 1, 2, and 4 (Brudzynski & Sjaarda, 2015). The presence of these jelleins might also explain the bacterial cell wall disruption properties of honey. The zones of inhibition seen in this study, through disk diffusion assays, might be explained by the damage that these substances effect on the bacterial cell. Besides MRJP1 protein, we detected other MRJP proteins from the same family, including MRJP2 to MRJP7. In some of our samples there was no MRJP9, and MRJP8 was absent in all (Table 5). In addition, some uncharacterized proteins were also present. It is possible that some of these proteins may also contribute to the antimicrobial properties of honey. This was similar to other studies (Chua et al., 2013).

To further confirm the presence of protein in honey samples Bradford assays were used to measure the total protein content of each honey sample. Results showed that the NY sample had the lowest concentration of protein, NY also displayed the lowest antioxidant properties, the lowest concentration of MRJPs and little-to-no accumulation of $H_2O_2$. Honey sample 13, which showed consistently high antimicrobial activity on all bacteria, also displayed the lowest MRJP1

coverage (Table 5), which suggests that more of its antimicrobial activity may be due to its peroxide activity (Table 3 and Figure 5).

Figure 9 shows the sum of antimicrobial properties, ranked from highest to lowest (left to right). This correlates generally with the mean zone of inhibition size on the five pathogens tested, except for samples 13 and 12. It is likely that these two honey samples have antimicrobial factors, other than the ones we have identified, or have antimicrobial synergies operating that have yet to be defined.

## Conclusion

In conclusion, the findings of this work suggest that the antimicrobial activities of honey differ substantially, depending on the type of honey, and are due to multiple factors present in the honey, which also vary considerably from one honey sample to another. As previously discussed, the activity of hydrogen peroxide was seen to be effective in some of the samples, while other samples did not show as much peroxide activity against bacteria.

From these studies, it can be concluded that honey samples from Utah have similar antimicrobial properties to those found in other honeys, even Manuka honey that is purported to have the highest antimicrobial effects. We have also shown that the antimicrobial properties of honey are likely due to peroxides, antimicrobials proteins, and osmotic effects.

In addition, there are likely many other antimicrobial factors in honey which have yet to be identified, as the contributions of peroxide and MRJPs do not account for the total antimicrobial effects demonstrated in this food. This is dramatically demonstrated in sample 13 which had a much higher antimicrobial activity than could be predicted from its defined

antimicrobial factors (Figure 9). It should also be mentioned that additional methods are needed that can accurately measure antimicrobial activities in honey, especially in in-use situations.

Besides its antimicrobial properties, honey has been shown by others to have additional beneficial effects such as boosting the immune system, anti-inflammatory properties, anti-oxidant activities, and aiding tissue regeneration and growth (Ballal, Bairy, Shenoy, & Shivananda, 2012).

This work has highlighted the complex nature and antimicrobial activity of honey. The main antimicrobial components in honey are its high sugar concentration, and corresponding high osmolarity, antimicrobial proteins, enzymes, and assorted chemicals.

A more detailed study of the antimicrobial properties of these components may lead to the identification of useful therapeutics that can be used in our never-ending war against microbial infections.

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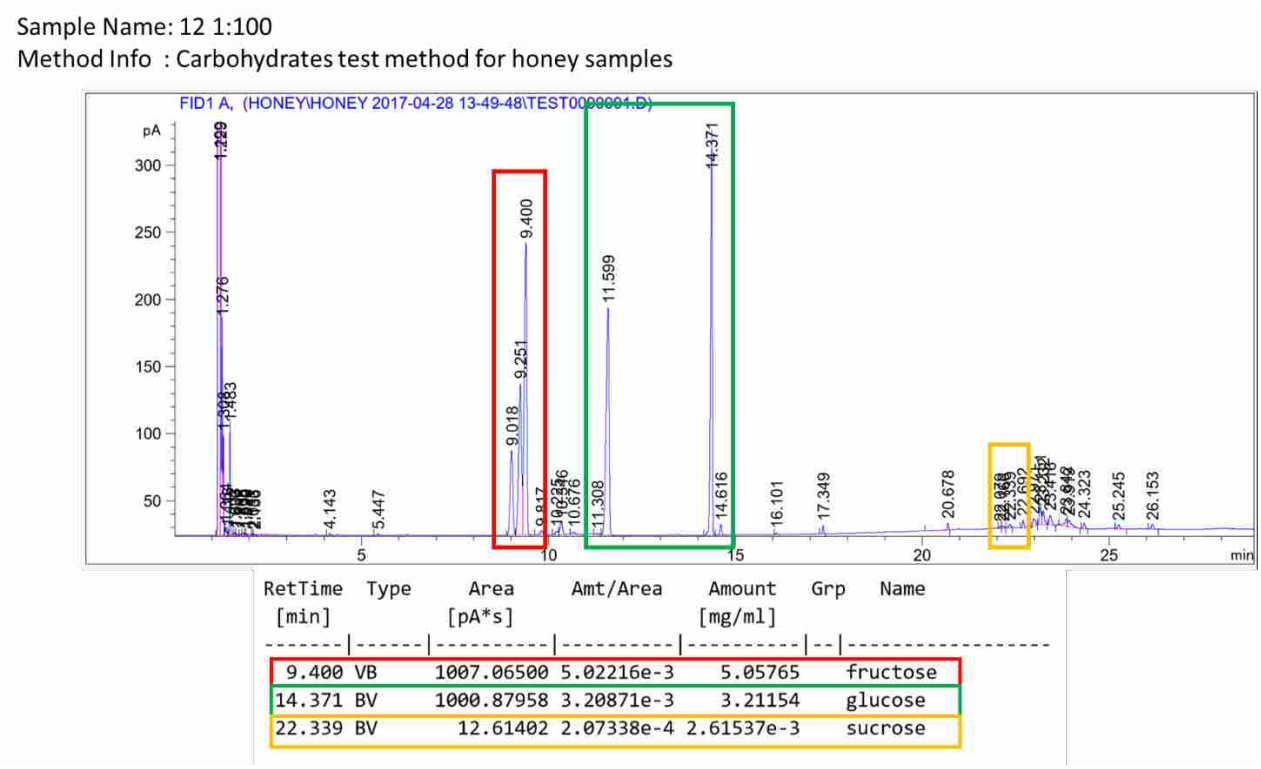
# Appendix A

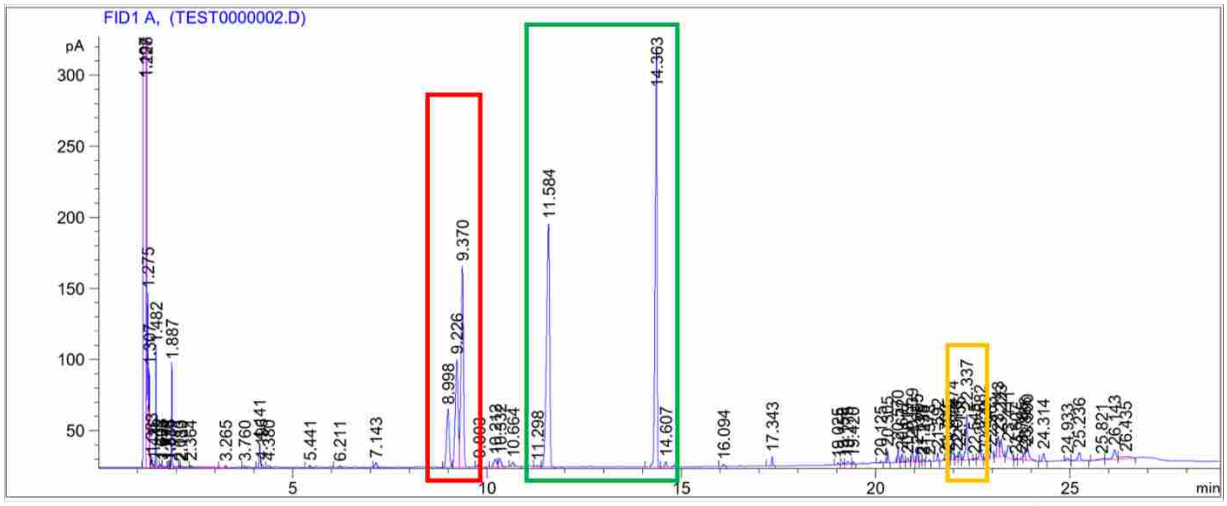
Identification of sugars present in honey samples using gas chromatography (GC).




| RetTime [min] | Type | Area [pA*s] | Amt/Area | Amount [mg/ml] | Grp | Name |
|---|---|---|---|---|---|---|
| 9.400 | VB | 1007.06500 | 5.02216e-3 | 5.05765 | | fructose |
| 14.371 | BV | 1000.87958 | 3.20871e-3 | 3.21154 | | glucose |
| 22.339 | BV | 12.61402 | 2.07338e-4 | 2.61537e-3 | | sucrose |

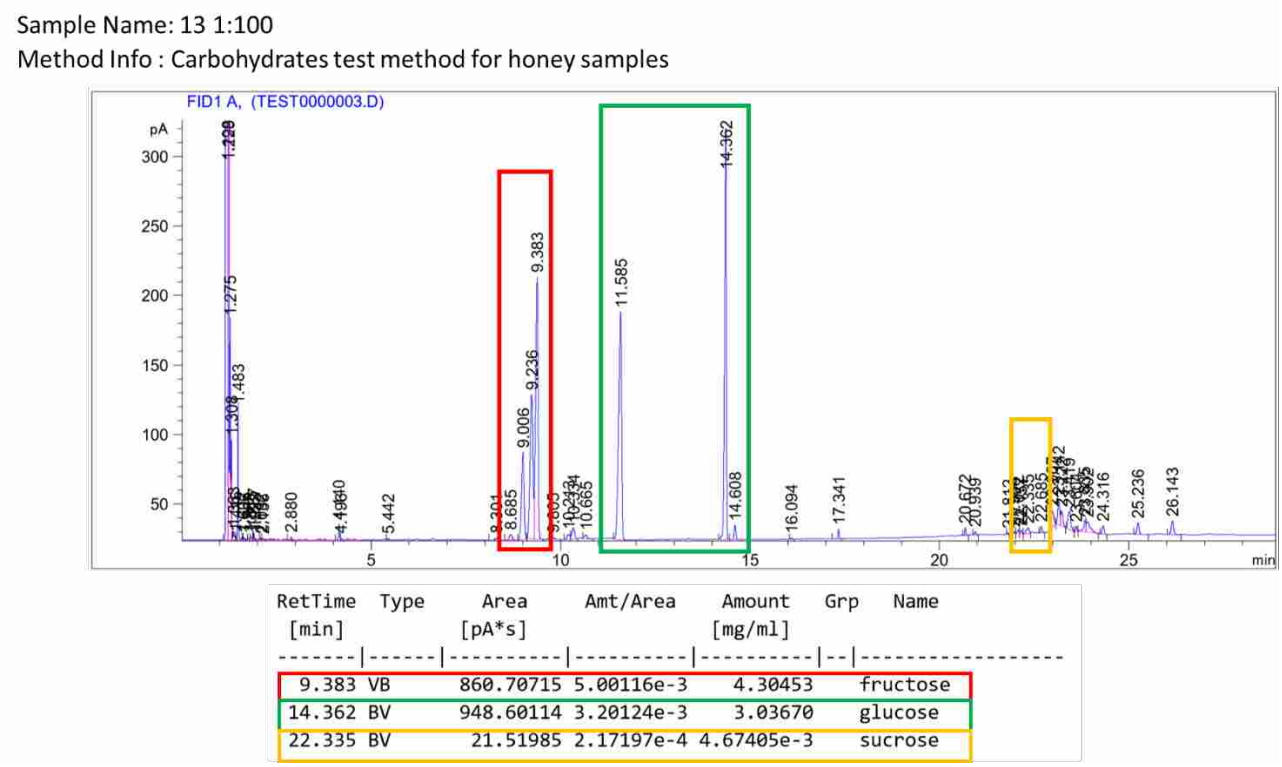
Figure 10. GC Carbohydrate analysis of honey sample 12. The red box shows the presence of fructose, the green box shows the presence of glucose, and the yellow box shows the presence of sucrose.

Sample Name: 12-w 1:100
Method Info : Carbohydrates test method for honey samples



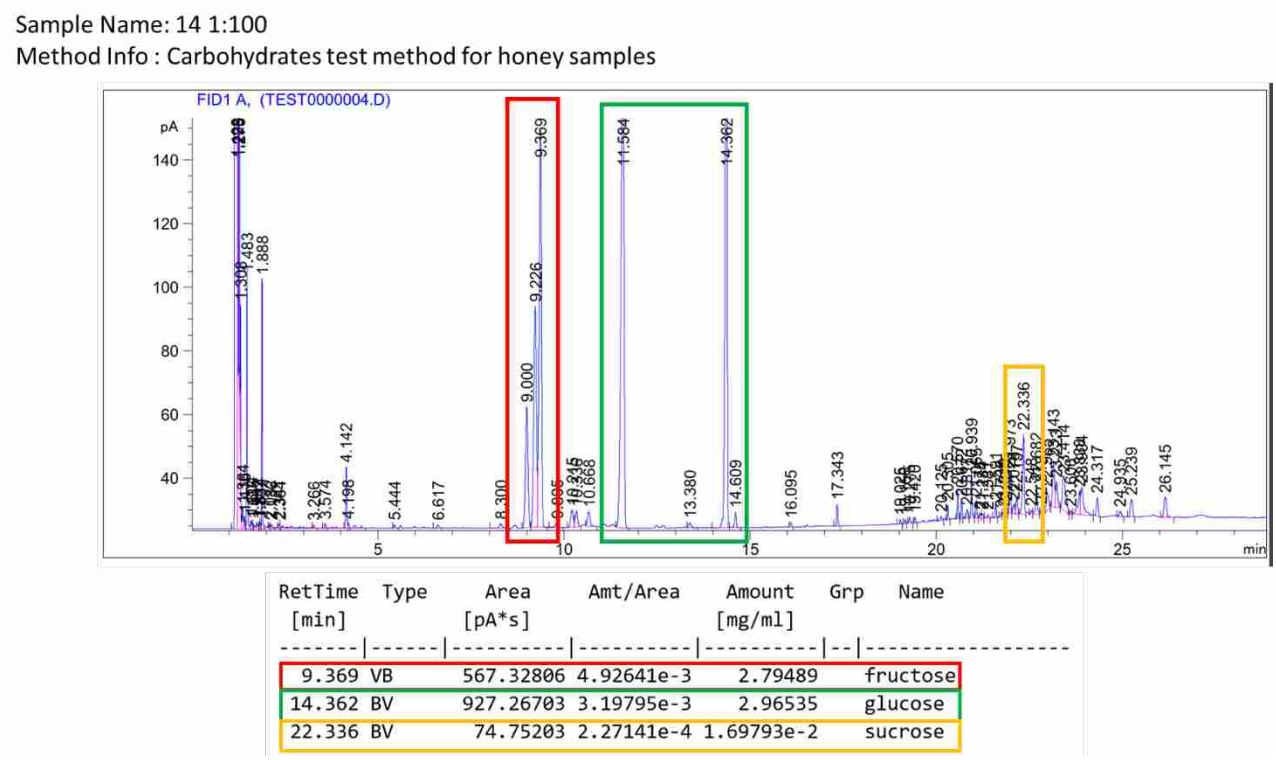

| RetTime [min] | Type | Area [pA*s] | Amt/Area | Amount [mg/ml] | Grp | Name |
|---|---|---|---|---|---|---|
| 9.370 | VB | 642.01868 | 4.95192e-3 | 3.17923 | | fructose |
| 14.363 | BV | 989.10828 | 3.20710e-3 | 3.17217 | | glucose |
| 22.337 | BV | 95.92818 | 2.28029e-4 | 2.18744e-2 | | sucrose |

Figure 11. GC Carbohydrate analysis of honey sample 12-w. The red box shows the presence of fructose, the green box shows the presence of glucose, and the yellow box shows the presence of sucrose.




| RetTime [min] | Type | Area [pA*s] | Amt/Area | Amount [mg/ml] | Grp | Name |
|---|---|---|---|---|---|---|
| 9.383 | VB | 860.70715 | 5.00116e-3 | 4.30453 | | fructose |
| 14.362 | BV | 948.60114 | 3.20124e-3 | 3.03670 | | glucose |
| 22.335 | BV | 21.51985 | 2.17197e-4 | 4.67405e-3 | | sucrose |

Figure 12. GC Carbohydrates analysis of honey sample 13. The red box shows the presence of fructose, the green box shows the presence of glucose, and the yellow box shows the presence of sucrose.




| RetTime [min] | Type | Area [pA*s] | Amt/Area | Amount [mg/ml] | Grp | Name |
|---|---|---|---|---|---|---|
| 9.369 | VB | 567.32806 | 4.92641e-3 | 2.79489 | | fructose |
| 14.362 | BV | 927.26703 | 3.19795e-3 | 2.96535 | | glucose |
| 22.336 | BV | 74.75203 | 2.27141e-4 | 1.69793e-2 | | sucrose |

Figure 13. GC Carbohydrate analysis of honey sample 14. The red box shows the presence of fructose, the green box shows the presence of glucose, and the yellow box shows the presence of sucrose.

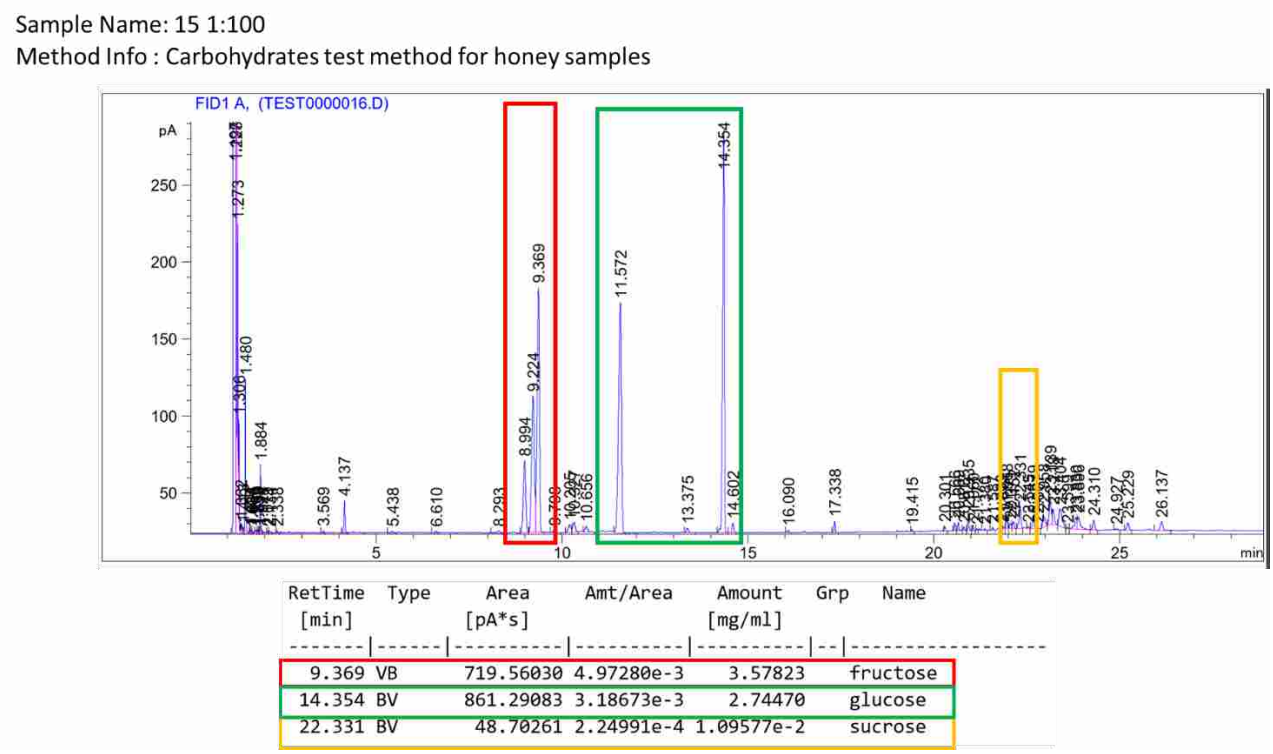



Figure 14. GC Carbohydrate analysis of honey sample 15. The red box shows the presence of fructose, the green box shows the presence of glucose, and the yellow box shows the presence of sucrose.

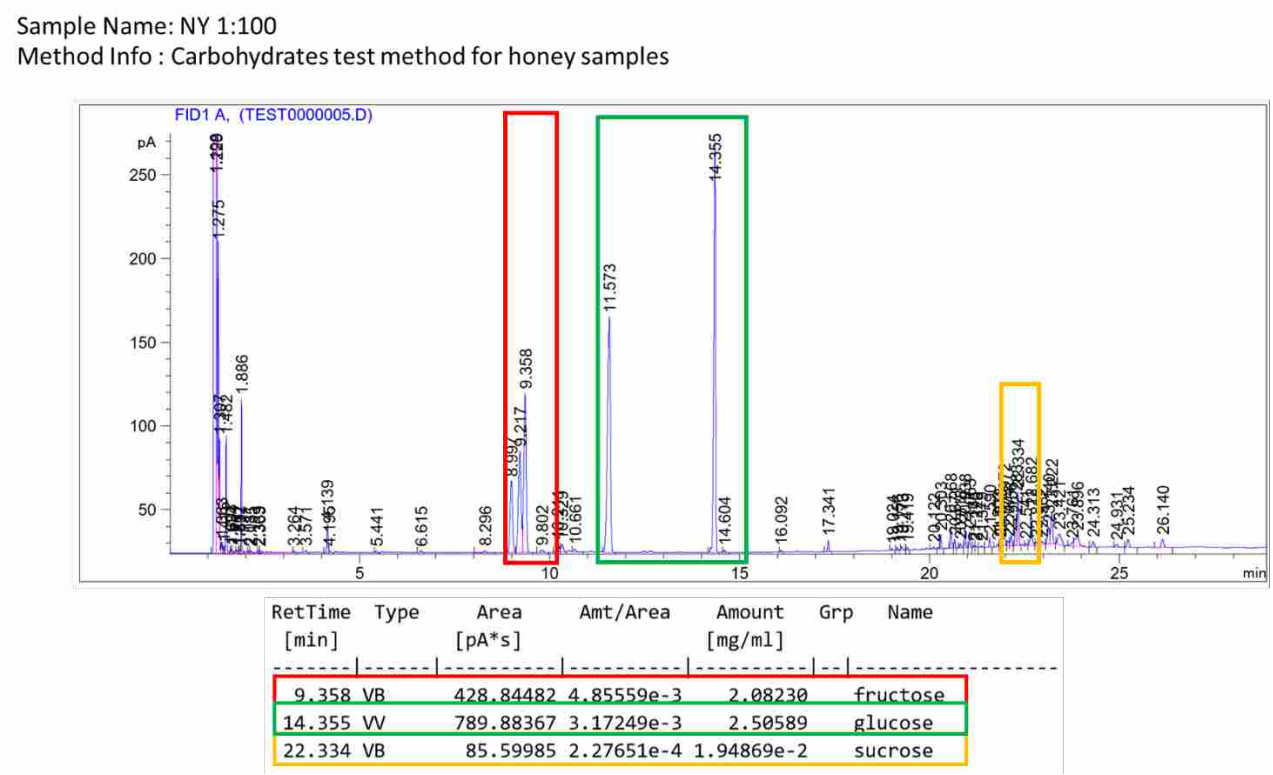



Figure 15. GC Carbohydrate analysis of honey sample NY. The red box shows the presence of fructose, the green box shows the presence of glucose, and the yellow box shows the presence of sucrose.

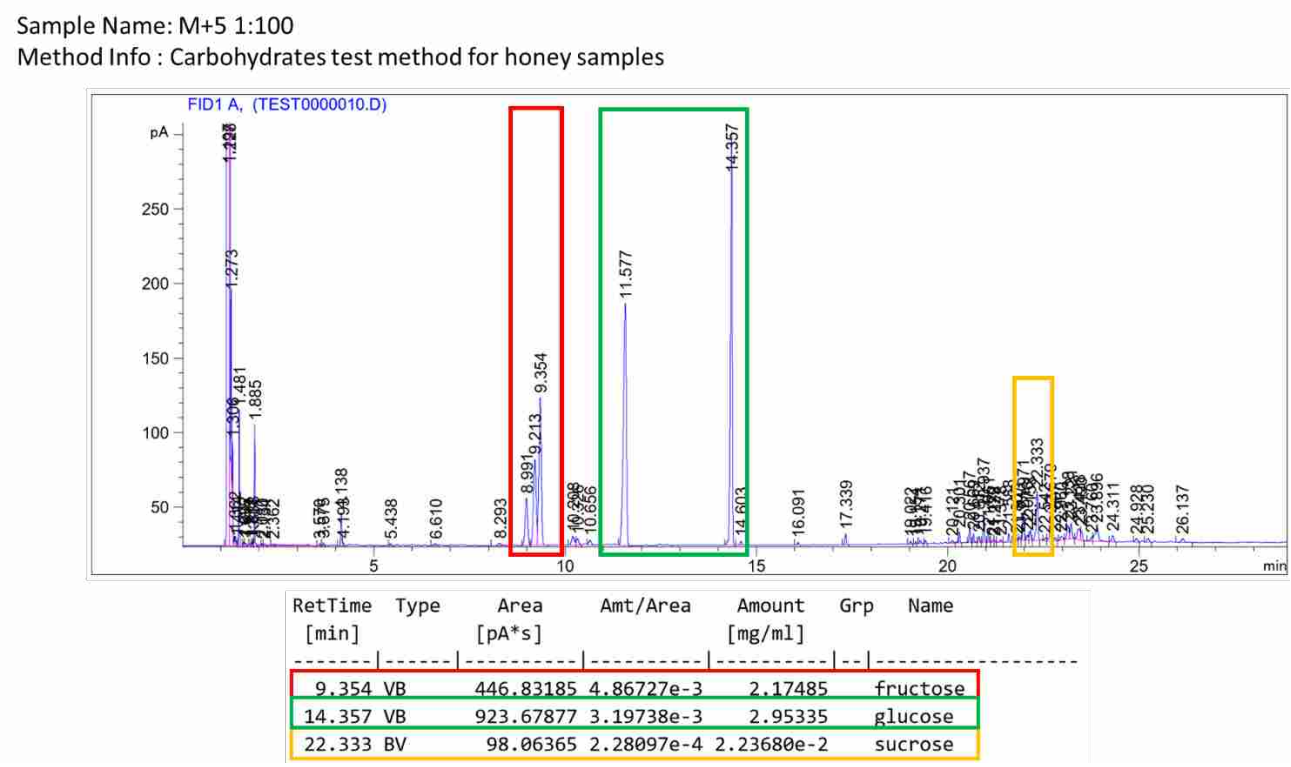



Figure 16. GC Carbohydrate analysis of honey sample M+5. The red box shows the presence of fructose, the green box shows the presence of glucose, and the yellow box shows the presence of sucrose.

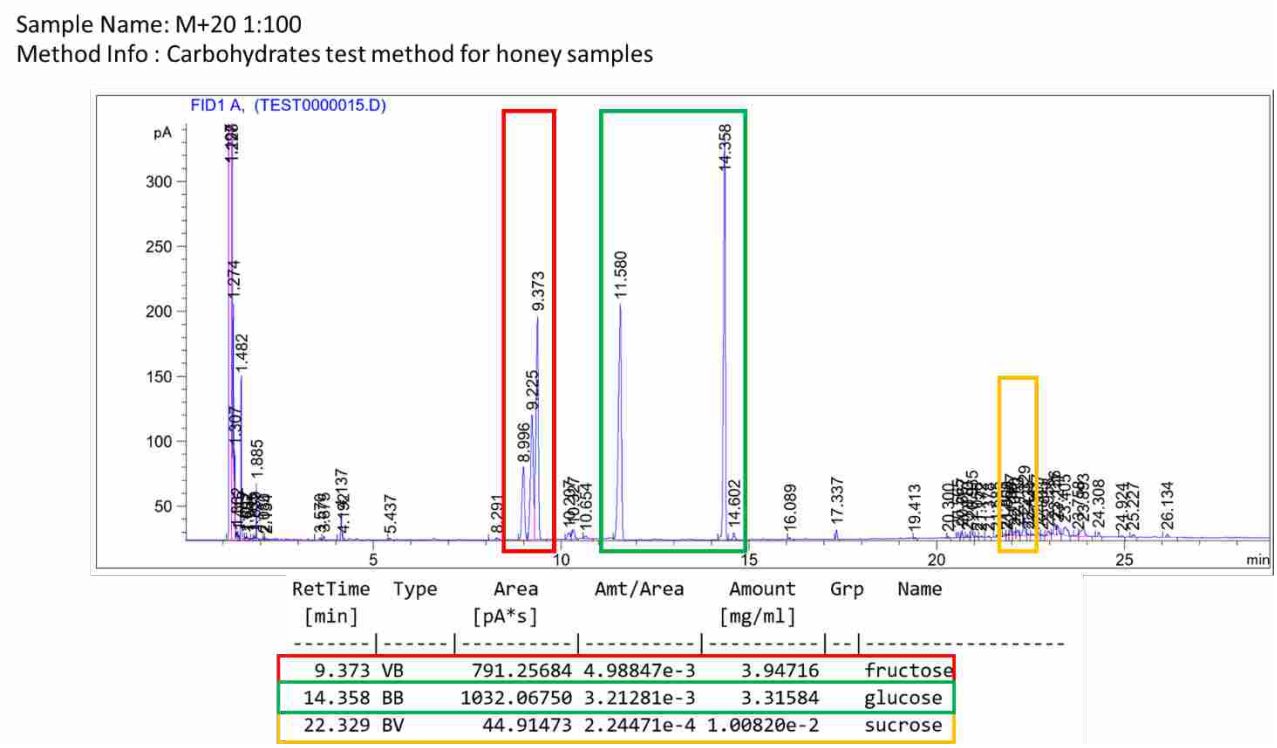
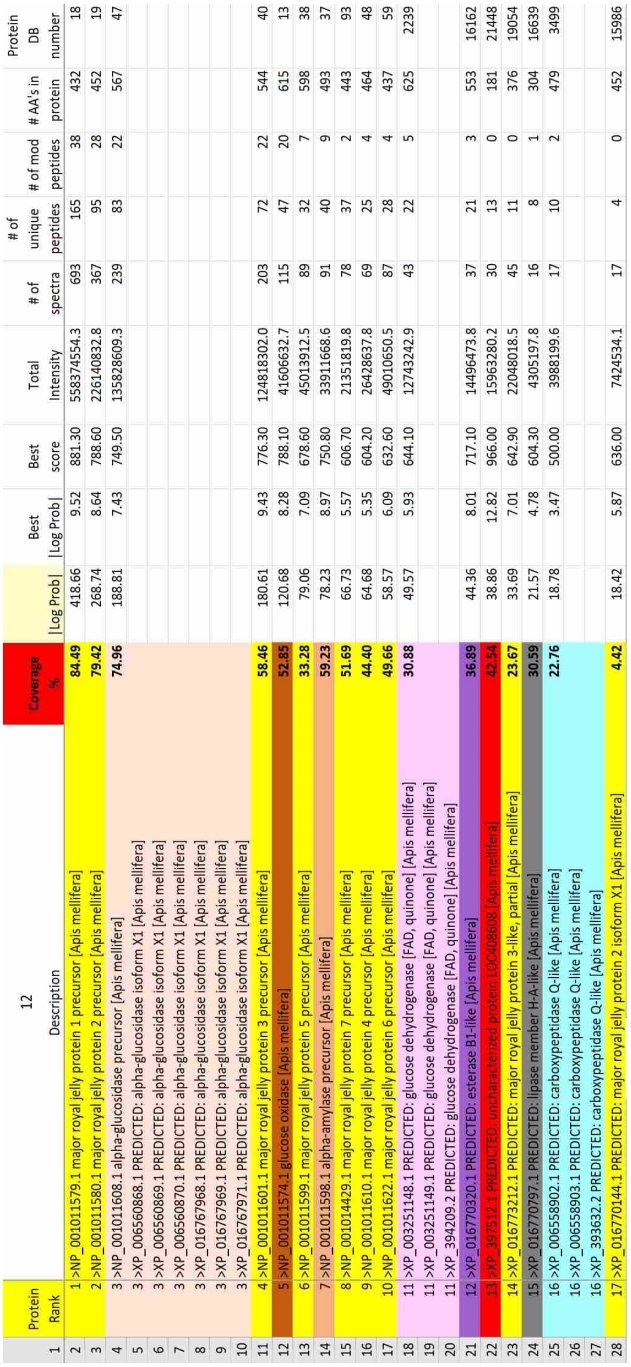



Figure 17. GC Carbohydrate analysis of honey sample M+20. The red box shows the presence of fructose, the green box shows the presence of glucose, and the yellow box shows the presence of sucrose.

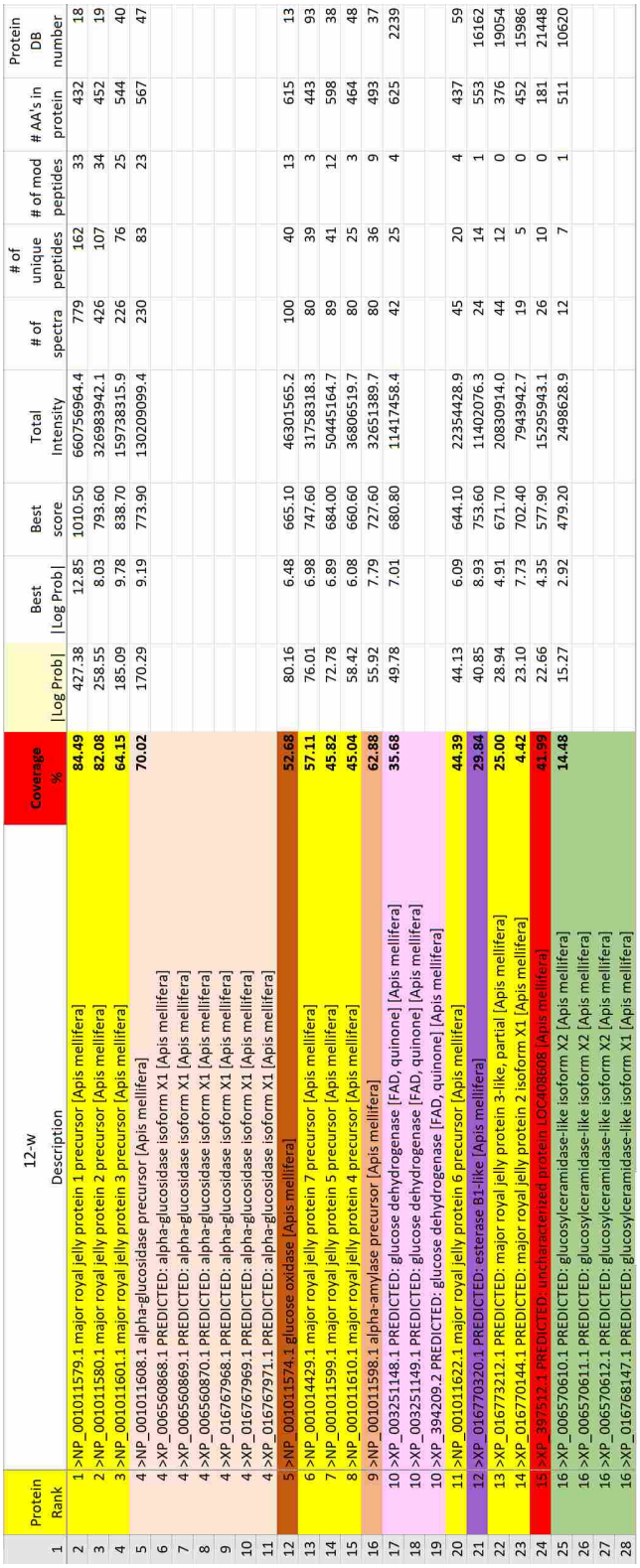
# Appendix B

Identification of proteins in honey samples using high-performance liquid chromatography coupled with mass spectrometry. The color code represents different proteins.



| | 1 | 12 | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Protein Rank | Description | Coverage % | \|Log Prob\| | Best \|Log Prob\| | Best score | Total Intensity | # of spectra | # of unique peptides | # of mod peptides | # AA's in protein | Protein DB number |
| 2 | 1 | >NP_001011579.1 major royal jelly protein 1 precursor [Apis mellifera] | **84.49** | 418.66 | 9.52 | 881.30 | 558374554.3 | 693 | 165 | 38 | 432 | 18 |
| 3 | 2 | >NP_001011580.1 major royal jelly protein 2 precursor [Apis mellifera] | **79.42** | 268.74 | 8.64 | 788.60 | 226140832.8 | 367 | 95 | 28 | 452 | 19 |
| 4 | 3 | >NP_001011608.1 alpha-glucosidase precursor [Apis mellifera] | **74.96** | 188.81 | 7.43 | 749.50 | 135828609.3 | 239 | 83 | 22 | 567 | 47 |
| 5 | 3 | >XP_006560868.1 PREDICTED: alpha-glucosidase isoform X1 [Apis mellifera] | | | | | | | | | | |
| 6 | 3 | >XP_006560869.1 PREDICTED: alpha-glucosidase isoform X1 [Apis mellifera] | | | | | | | | | | |
| 7 | 3 | >XP_006560870.1 PREDICTED: alpha-glucosidase isoform X1 [Apis mellifera] | | | | | | | | | | |
| 8 | 3 | >XP_016767968.1 PREDICTED: alpha-glucosidase isoform X1 [Apis mellifera] | | | | | | | | | | |
| 9 | 3 | >XP_016767969.1 PREDICTED: alpha-glucosidase isoform X1 [Apis mellifera] | | | | | | | | | | |
| 10 | 3 | >XP_016767971.1 PREDICTED: alpha-glucosidase isoform X1 [Apis mellifera] | | | | | | | | | | |
| 11 | 4 | >NP_001011601.1 major royal jelly protein 3 precursor [Apis mellifera] | **58.46** | 180.61 | 9.43 | 776.30 | 124818302.0 | 203 | 72 | 22 | 544 | 40 |
| 12 | 5 | >NP_001011574.1 glucose oxidase [Apis mellifera] | **52.85** | 120.68 | 8.28 | 788.10 | 41606632.7 | 115 | 47 | 20 | 615 | 13 |
| 13 | 6 | >NP_001011599.1 major royal jelly protein 5 precursor [Apis mellifera] | **33.28** | 79.06 | 7.09 | 678.60 | 45013912.5 | 89 | 32 | 7 | 598 | 38 |
| 14 | 7 | >NP_001011598.1 alpha-amylase precursor [Apis mellifera] | **59.23** | 78.23 | 8.97 | 750.80 | 33911668.6 | 91 | 40 | 9 | 493 | 37 |
| 15 | 8 | >NP_001014429.1 major royal jelly protein 7 precursor [Apis mellifera] | **51.69** | 66.73 | 5.57 | 606.70 | 21351819.8 | 78 | 37 | 2 | 443 | 93 |
| 16 | 9 | >NP_001011610.1 major royal jelly protein 4 precursor [Apis mellifera] | **44.40** | 64.68 | 5.35 | 604.20 | 26428637.8 | 69 | 25 | 4 | 464 | 48 |
| 17 | 10 | >NP_001011622.1 major royal jelly protein 6 precursor [Apis mellifera] | **49.66** | 58.57 | 6.09 | 632.60 | 49010650.5 | 87 | 28 | 4 | 437 | 59 |
| 18 | 11 | >XP_003251148.1 PREDICTED: glucose dehydrogenase [FAD, quinone] [Apis mellifera] | **30.88** | 49.57 | 5.93 | 644.10 | 12743242.9 | 43 | 22 | 5 | 625 | 2239 |
| 19 | 11 | >XP_003251149.1 PREDICTED: glucose dehydrogenase [FAD, quinone] [Apis mellifera] | | | | | | | | | | |
| 20 | 11 | >XP_394209.2 PREDICTED: glucose dehydrogenase [FAD, quinone] [Apis mellifera] | | | | | | | | | | |
| 21 | 12 | >XP_016770320.1 PREDICTED: esterase B1-like [Apis mellifera] | **36.89** | 44.36 | 8.01 | 717.10 | 14496473.8 | 37 | 21 | 3 | 553 | 16162 |
| 22 | 13 | >XP_397512.1 PREDICTED: uncharacterized protein LOC408618 [Apis mellifera] | **42.54** | 38.86 | 12.82 | 966.00 | 15963280.2 | 30 | 13 | 0 | 181 | 21448 |
| 23 | 14 | >XP_016773212.1 PREDICTED: major royal jelly protein 3-like, partial [Apis mellifera] | **23.67** | 33.69 | 7.01 | 642.90 | 22048018.5 | 45 | 11 | 0 | 376 | 19054 |
| 24 | 15 | >XP_016770797.1 PREDICTED: lipase member H-A-like [Apis mellifera] | **30.59** | 21.57 | 4.78 | 604.30 | 4305197.8 | 16 | 8 | 1 | 304 | 16639 |
| 25 | 16 | >XP_006558902.1 PREDICTED: carboxypeptidase Q-like [Apis mellifera] | **22.76** | 18.78 | 3.47 | 500.00 | 39881199.6 | 17 | 10 | 2 | 479 | 3499 |
| 26 | 16 | >XP_006558903.1 PREDICTED: carboxypeptidase Q-like [Apis mellifera] | | | | | | | | | | |
| 27 | 16 | >XP_393632.2 PREDICTED: carboxypeptidase Q-like [Apis mellifera] | | | | | | | | | | |
| 28 | 17 | >XP_016770144.1 PREDICTED: major royal jelly protein 2 isoform X1 [Apis mellifera] | **4.42** | 18.42 | 5.87 | 636.00 | 7424534.1 | 17 | 4 | 0 | 452 | 15986 |

Figure 18. HPLC-MS-MS analysis of the total proteins present in honey sample 12. Major royal jelly protein 1 was found in greatest abundance.



| | Protein Rank | 12-w Description | Coverage % | \| Log Prob \| | Best \| Log Prob \| | Best score | Total Intensity | # of spectra | # of unique peptides | # of mod peptides | # AA's in protein | Protein DB number |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 1 | >NP_001011579.1 major royal jelly protein 1 precursor [Apis mellifera] | **84.49** | 427.38 | 12.85 | 1010.50 | 660756964.4 | 779 | 162 | 33 | 432 | 18 |
| 2 | 2 | >NP_001011580.1 major royal jelly protein 2 precursor [Apis mellifera] | **82.08** | 258.55 | 8.03 | 793.60 | 326983942.1 | 426 | 107 | 34 | 452 | 19 |
| 3 | 3 | >NP_001011601.1 major royal jelly protein 3 precursor [Apis mellifera] | **64.15** | 185.09 | 9.78 | 838.70 | 159738315.9 | 226 | 76 | 25 | 544 | 40 |
| 4 | 4 | >NP_001011608.1 alpha-glucosidase precursor [Apis mellifera] | **70.02** | 170.29 | 9.19 | 773.90 | 130209099.4 | 230 | 83 | 23 | 567 | 47 |
| 5 | 4 | >XP_006560868.1 PREDICTED: alpha-glucosidase isoform X1 [Apis mellifera] | | | | | | | | | | |
| 6 | 4 | >XP_006560869.1 PREDICTED: alpha-glucosidase isoform X1 [Apis mellifera] | | | | | | | | | | |
| 7 | 4 | >XP_006560870.1 PREDICTED: alpha-glucosidase isoform X1 [Apis mellifera] | | | | | | | | | | |
| 8 | 4 | >XP_016767968.1 PREDICTED: alpha-glucosidase isoform X1 [Apis mellifera] | | | | | | | | | | |
| 9 | 4 | >XP_016767969.1 PREDICTED: alpha-glucosidase isoform X1 [Apis mellifera] | | | | | | | | | | |
| 10 | 4 | >XP_016767971.1 PREDICTED: alpha-glucosidase isoform X1 [Apis mellifera] | | | | | | | | | | |
| 11 | 5 | >NP_001011574.1 glucose oxidase [Apis mellifera] | **52.68** | 80.16 | 6.48 | 665.10 | 46301565.2 | 100 | 40 | 13 | 615 | 13 |
| 12 | 6 | >NP_001014429.1 major royal jelly protein 7 precursor [Apis mellifera] | **57.11** | 76.01 | 6.98 | 747.60 | 31758318.3 | 80 | 39 | 3 | 443 | 93 |
| 13 | 7 | >NP_001011599.1 major royal jelly protein 5 precursor [Apis mellifera] | **45.82** | 72.78 | 6.89 | 684.00 | 50445164.7 | 89 | 41 | 12 | 598 | 38 |
| 14 | 8 | >NP_001011610.1 major royal jelly protein 4 precursor [Apis mellifera] | **45.04** | 58.42 | 6.08 | 660.60 | 36806519.7 | 80 | 25 | 3 | 464 | 48 |
| 15 | 9 | >NP_001011598.1 alpha-amylase precursor [Apis mellifera] | **62.88** | 55.92 | 7.79 | 727.60 | 32651389.7 | 80 | 36 | 9 | 493 | 37 |
| 16 | 10 | >XP_003251148.1 PREDICTED: glucose dehydrogenase [FAD, quinone] [Apis mellifera] | **35.68** | 49.78 | 7.01 | 680.80 | 11417458.4 | 42 | 25 | 4 | 625 | 2239 |
| 17 | 10 | >XP_003251149.1 PREDICTED: glucose dehydrogenase [FAD, quinone] [Apis mellifera] | | | | | | | | | | |
| 18 | 10 | >XP_394209.2 PREDICTED: glucose dehydrogenase [FAD, quinone] [Apis mellifera] | | | | | | | | | | |
| 19 | 11 | >NP_001011622.1 major royal jelly protein 6 precursor [Apis mellifera] | **44.39** | 44.13 | 6.09 | 644.10 | 22354428.9 | 45 | 20 | 4 | 437 | 59 |
| 20 | 12 | >XP_016770320.1 PREDICTED: esterase B1-like [Apis mellifera] | **29.84** | 40.85 | 8.93 | 753.60 | 11402076.3 | 24 | 14 | 1 | 553 | 16162 |
| 21 | 13 | >XP_016773212.1 PREDICTED: major royal jelly protein 3-like, partial [Apis mellifera] | **25.00** | 28.94 | 4.91 | 671.70 | 20830914.0 | 44 | 12 | 0 | 376 | 19054 |
| 22 | 14 | >XP_016770144.1 PREDICTED: major royal jelly protein 2 isoform X1 [Apis mellifera] | **4.42** | 23.10 | 7.73 | 702.40 | 7943942.7 | 19 | 5 | 0 | 452 | 15986 |
| 23 | 15 | >XP_397512.1 PREDICTED: uncharacterized protein LOC409808 [Apis mellifera] | **41.99** | 22.66 | 4.35 | 577.90 | 15295943.1 | 26 | 10 | 0 | 181 | 21448 |
| 24 | 16 | >XP_006570610.1 PREDICTED: glucosylceramidase-like isoform X2 [Apis mellifera] | **14.48** | 15.27 | 2.92 | 479.20 | 2498628.9 | 12 | 7 | 1 | 511 | 10620 |
| 25 | 16 | >XP_006570611.1 PREDICTED: glucosylceramidase-like isoform X2 [Apis mellifera] | | | | | | | | | | |
| 26 | 16 | >XP_006570612.1 PREDICTED: glucosylceramidase-like isoform X2 [Apis mellifera] | | | | | | | | | | |
| 27 | 16 | >XP_016768147.1 PREDICTED: glucosylceramidase-like isoform X1 [Apis mellifera] | | | | | | | | | | |
| 28 | | | | | | | | | | | | |

Figure 19. HPLC-MS-MS analysis of the total proteins present in honey sample 12-w. Major royal jelly protein 1 was found in greatest abundance.

| | Protein Rank | 13 Description | Coverage % | \|Log Prob\| | Best \|Log Prob\| | Best score | Total Intensity | # of spectra | # of unique peptides | # of mod peptides | # AA's in protein | Protein DB number |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | | | | | | | | | | | | |
| 2 | 1 | >NP_001011579.1 major royal jelly protein 1 precursor [Apis mellifera] | **69.68** | 237.95 | 12.84 | 835.10 | 412742315.6 | 568 | 65 | 40 | 432 | 18 |
| 3 | 2 | >NP_001011580.1 major royal jelly protein 2 precursor [Apis mellifera] | **69.25** | 173.87 | 9.96 | 688.30 | 222310269.0 | 300 | 54 | 26 | 452 | 19 |
| 4 | 3 | >NP_001011601.1 major royal jelly protein 3 precursor [Apis mellifera] | **55.88** | 150.95 | 14.00 | 871.60 | 67888021.6 | 163 | 52 | 33 | 544 | 40 |
| 5 | 4 | >NP_001011610.1 major royal jelly protein 4 precursor [Apis mellifera] | **42.46** | 93.44 | 9.50 | 639.60 | 27583722.8 | 73 | 20 | 5 | 464 | 48 |
| 6 | 5 | >NP_001011608.1 alpha-glucosidase precursor [Apis mellifera] | **57.50** | 86.50 | 8.34 | 615.70 | 62763347.7 | 80 | 31 | 14 | 567 | 47 |
| 7 | 5 | >XP_006560868.1 PREDICTED: alpha-glucosidase isoform X1 [Apis mellifera] | | | | | | | | | | |
| 8 | 5 | >XP_006560869.1 PREDICTED: alpha-glucosidase isoform X1 [Apis mellifera] | | | | | | | | | | |
| 9 | 5 | >XP_006560870.1 PREDICTED: alpha-glucosidase isoform X1 [Apis mellifera] | | | | | | | | | | |
| 10 | 5 | >XP_016767968.1 PREDICTED: alpha-glucosidase isoform X1 [Apis mellifera] | | | | | | | | | | |
| 11 | 5 | >XP_016767969.1 PREDICTED: alpha-glucosidase isoform X1 [Apis mellifera] | | | | | | | | | | |
| 12 | 5 | >XP_016767971.1 PREDICTED: alpha-glucosidase isoform X1 [Apis mellifera] | | | | | | | | | | |
| 13 | 6 | >gi\|166900097\|sp\|P04259.4\|K2C6B_HUMAN Keratin, type II cytoskeletal 6B (Cytokeratin-6B) (CK 6B) (K6b | **54.79** | 81.70 | 7.82 | 621.20 | 11802664.5 | 50 | 29 | 8 | 564 | 22457 |
| 14 | 7 | >NP_001011598.1 alpha-amylase precursor [Apis mellifera] | **48.28** | 80.78 | 8.84 | 636.50 | 18376127.5 | 70 | 26 | 14 | 493 | 37 |
| 15 | 8 | >NP_001014429.1 major royal jelly protein 7 precursor [Apis mellifera] | **48.53** | 77.43 | 11.50 | 737.30 | 44956178.7 | 62 | 26 | 9 | 443 | 93 |
| 16 | 9 | >NP_001011574.1 glucose oxidase [Apis mellifera] | **30.89** | 73.34 | 9.35 | 653.20 | 26863308.7 | 59 | 23 | 12 | 615 | 13 |
| 17 | 10 | >NP_001011599.1 major royal jelly protein 5 precursor [Apis mellifera] | **36.12** | 59.62 | 7.19 | 587.50 | 24344734.6 | 61 | 25 | 16 | 598 | 38 |
| 18 | 11 | >gi\|386848\|gb\|AAB59562.1\| keratin | **35.59** | 58.50 | 8.33 | 628.50 | 8398284.1 | 36 | 18 | 5 | 472 | 22454 |
| 19 | 12 | >NP_001011622.1 major royal jelly protein 6 precursor [Apis mellifera] | **35.93** | 42.43 | 6.52 | 504.80 | 15239091.2 | 41 | 16 | 7 | 437 | 59 |
| 20 | 13 | >XP_016770320.1 PREDICTED: esterase B1-like [Apis mellifera] | 26.40 | 35.21 | 5.71 | 556.30 | 8924348.2 | 19 | 10 | 2 | 553 | 16162 |
| 21 | 14 | >XP_397512.1 PREDICTED: uncharacterized protein LOC408608 [Apis mellifera] | 38.12 | 32.27 | 11.77 | 765.80 | 7915582.7 | 16 | 9 | 1 | 181 | 21448 |
| 22 | 15 | >XP_016770797.1 PREDICTED: lipase member H-A-like [Apis mellifera] | 21.05 | 21.53 | 6.88 | 586.60 | 3632853.5 | 9 | 5 | 1 | 304 | 16639 |
| 23 | 16 | >XP_016770154.1 PREDICTED: hexamerin 110 isoform X1 [Apis mellifera] | 9.81 | 21.16 | 5.85 | 518.30 | 1226731.4 | 10 | 7 | 0 | 1009 | 15996 |
| 24 | 17 | >XP_003251148.1 PREDICTED: glucose dehydrogenase [FAD, quinone] [Apis mellifera] | 16.80 | 20.86 | 6.56 | 480.10 | 1565054.2 | 13 | 10 | 4 | 625 | 2239 |
| 25 | 17 | >XP_003251149.1 PREDICTED: glucose dehydrogenase [FAD, quinone] [Apis mellifera] | | | | | | | | | | |
| 26 | 17 | >XP_394209.2 PREDICTED: glucose dehydrogenase [FAD, quinone] [Apis mellifera] | | | | | | | | | | |
| 27 | 18 | >XP_016768806.1 PREDICTED: uncharacterized protein LOC413627 [Apis mellifera] | 9.94 | 19.30 | 4.91 | 447.30 | 1823466.9 | 8 | 5 | 0 | 654 | 14648 |
| 28 | 19 | >NP_001172074.1 actin related protein 1 [Apis mellifera] | 13.56 | 18.92 | 5.86 | 496.80 | 1136009.4 | 11 | 5 | 1 | 376 | 325 |

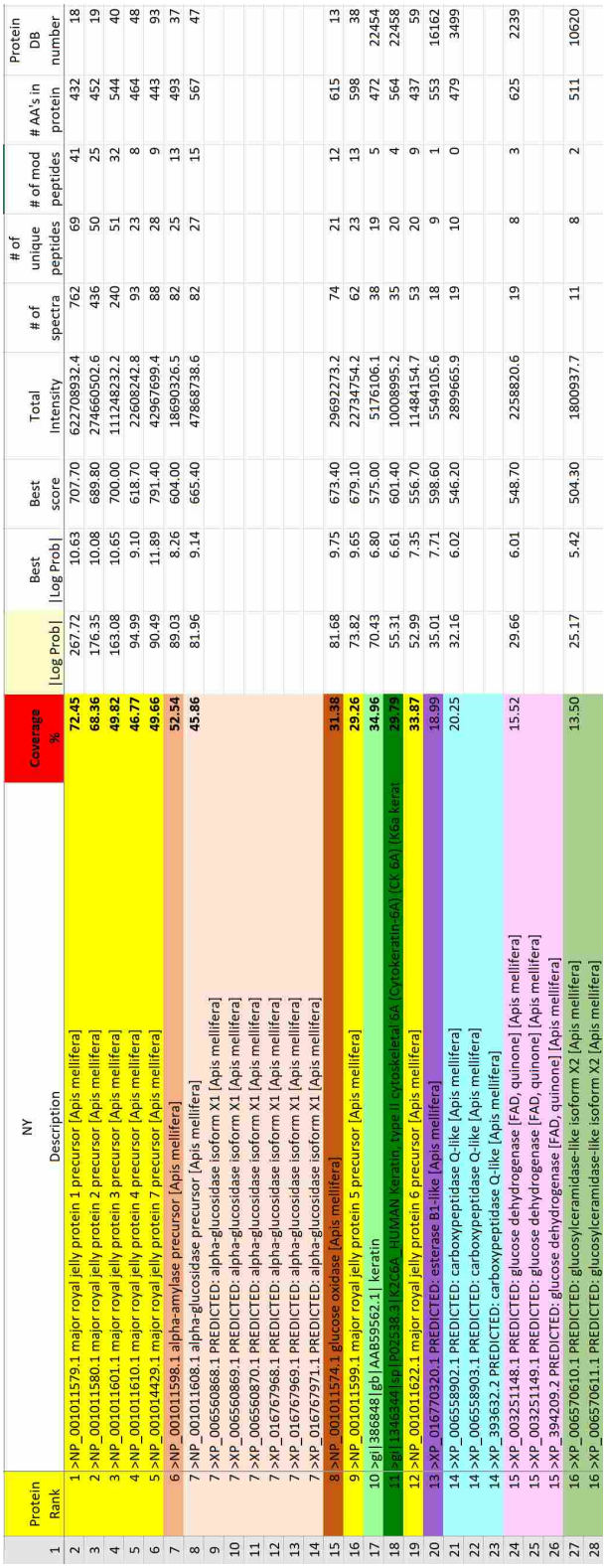
Figure 20. HPLC-MS-MS analysis of the total proteins present in honey sample 13. Major royal jelly protein 1 was found in greatest abundance.

| | Protein Rank | 14 Description | Coverage % | \|Log Prob\| | Best \|Log Prob\| | Best score | Total Intensity | # of spectra | # of unique peptides | # of mod peptides | # AA's in protein | Protein DB number |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 1 | >NP_001011579.1 major royal jelly protein 1 precursor [Apis mellifera] | **84.49** | 506.57 | 14.14 | 1080.40 | 703158456.7 | 935 | 188 | 41 | 432 | 18 |
| 2 | 2 | >NP_001011580.1 major royal jelly protein 2 precursor [Apis mellifera] | **79.87** | 290.55 | 9.28 | 841.20 | 308693481.5 | 437 | 117 | 37 | 452 | 19 |
| 3 | 3 | >NP_001011601.1 major royal jelly protein 3 precursor [Apis mellifera] | **61.03** | 224.83 | 10.23 | 879.00 | 176884709.9 | 260 | 80 | 23 | 544 | 40 |
| 4 | 4 | >NP_001011608.1 alpha-glucosidase precursor [Apis mellifera] | **72.31** | 187.31 | 7.02 | 729.20 | 183689601.0 | 261 | 95 | 29 | 567 | 47 |
| 5 | 4 | >XP_006560868.1 PREDICTED: alpha-glucosidase isoform X1 [Apis mellifera] | | | | | | | | | | |
| 6 | 4 | >XP_006560869.1 PREDICTED: alpha-glucosidase isoform X1 [Apis mellifera] | | | | | | | | | | |
| 7 | 4 | >XP_006560870.1 PREDICTED: alpha-glucosidase isoform X1 [Apis mellifera] | | | | | | | | | | |
| 8 | 4 | >XP_016767968.1 PREDICTED: alpha-glucosidase isoform X1 [Apis mellifera] | | | | | | | | | | |
| 9 | 4 | >XP_016767969.1 PREDICTED: alpha-glucosidase isoform X1 [Apis mellifera] | | | | | | | | | | |
| 10 | 4 | >XP_016767971.1 PREDICTED: alpha-glucosidase isoform X1 [Apis mellifera] | | | | | | | | | | |
| 11 | 5 | >NP_001011574.1 glucose oxidase [Apis mellifera] | **62.11** | 135.54 | 7.87 | 776.80 | 59253884.5 | 143 | 57 | 16 | 615 | 13 |
| 12 | 6 | >NP_001014429.1 major royal jelly protein 7 precursor [Apis mellifera] | **55.98** | 87.48 | 6.36 | 660.60 | 56306228.7 | 90 | 40 | 4 | 443 | 93 |
| 13 | 7 | >NP_001011599.1 major royal jelly protein 5 precursor [Apis mellifera] | **43.31** | 76.87 | 6.24 | 669.60 | 45885119.5 | 81 | 39 | 13 | 598 | 38 |
| 14 | 8 | >NP_001011610.1 major royal jelly protein 4 precursor [Apis mellifera] | **49.78** | 64.39 | 5.77 | 620.80 | 29662047.3 | 71 | 27 | 5 | 464 | 48 |
| 15 | 9 | >NP_001011598.1 alpha-amylase precursor [Apis mellifera] | **55.38** | 61.39 | 7.24 | 730.40 | 31812889.3 | 74 | 37 | 11 | 493 | 37 |
| 16 | 10 | >NP_001011622.1 major royal jelly protein 6 precursor [Apis mellifera] | **45.08** | 55.25 | 5.82 | 641.70 | 30649492.7 | 62 | 24 | 3 | 437 | 59 |
| 17 | 11 | >XP_003251148.1 PREDICTED: glucose dehydrogenase [FAD, quinone] [Apis mellifera] | **35.20** | 48.77 | 4.94 | 632.10 | 12542110.5 | 50 | 27 | 6 | 625 | 2239 |
| 18 | 11 | >XP_003251149.1 PREDICTED: glucose dehydrogenase [FAD, quinone] [Apis mellifera] | | | | | | | | | | |
| 19 | 11 | >XP_394209.2 PREDICTED: glucose dehydrogenase [FAD, quinone] [Apis mellifera] | | | | | | | | | | |
| 20 | 12 | >XP_016773212.1 PREDICTED: major royal jelly protein 3-like, partial [Apis mellifera] | **26.60** | 43.10 | 6.42 | 652.50 | 25432900.5 | 52 | 15 | 0 | 376 | 19054 |
| 21 | 13 | >XP_016770320.1 PREDICTED: esterase B1-like [Apis mellifera] | **31.28** | 36.45 | 4.40 | 617.80 | 15064443.7 | 27 | 13 | 2 | 553 | 16162 |
| 22 | 14 | >XP_397512.1 PREDICTED: uncharacterized protein LOC408608 [Apis mellifera] | **37.02** | 35.61 | 12.33 | 996.50 | 23810157.5 | 32 | 13 | 0 | 181 | 21448 |
| 23 | 15 | >XP_016770144.1 PREDICTED: major royal jelly protein 2 isoform X1 [Apis mellifera] | **4.42** | 24.75 | 6.95 | 682.30 | 10923133.6 | 24 | 7 | 0 | 452 | 15986 |
| 24 | 16 | >XP_016770797.1 PREDICTED: lipase member H-A-like [Apis mellifera] | **31.91** | 23.82 | 5.80 | 651.50 | 5734609.2 | 23 | 11 | 1 | 304 | 16639 |
| 25 | 17 | >XP_001120887.3 PREDICTED: chitinase-like protein Idgf4 isoform X2 [Apis mellifera] | **20.09** | 19.62 | 3.39 | 492.50 | 2187866.1 | 12 | 9 | 0 | 438 | 883 |
| 26 | 18 | >XP_006558902.1 PREDICTED: carboxypeptidase Q-like [Apis mellifera] | **21.50** | 17.64 | 3.65 | 523.70 | 5556139.6 | 19 | 10 | 1 | 479 | 3499 |
| 27 | 18 | >XP_006558903.1 PREDICTED: carboxypeptidase Q-like [Apis mellifera] | | | | | | | | | | |
| 28 | | | | | | | | | | | | |

Figure 21. HPLC-MS-MS analysis of the total proteins present in honey sample 14. Major royal jelly protein 1 was found in greatest abundance.

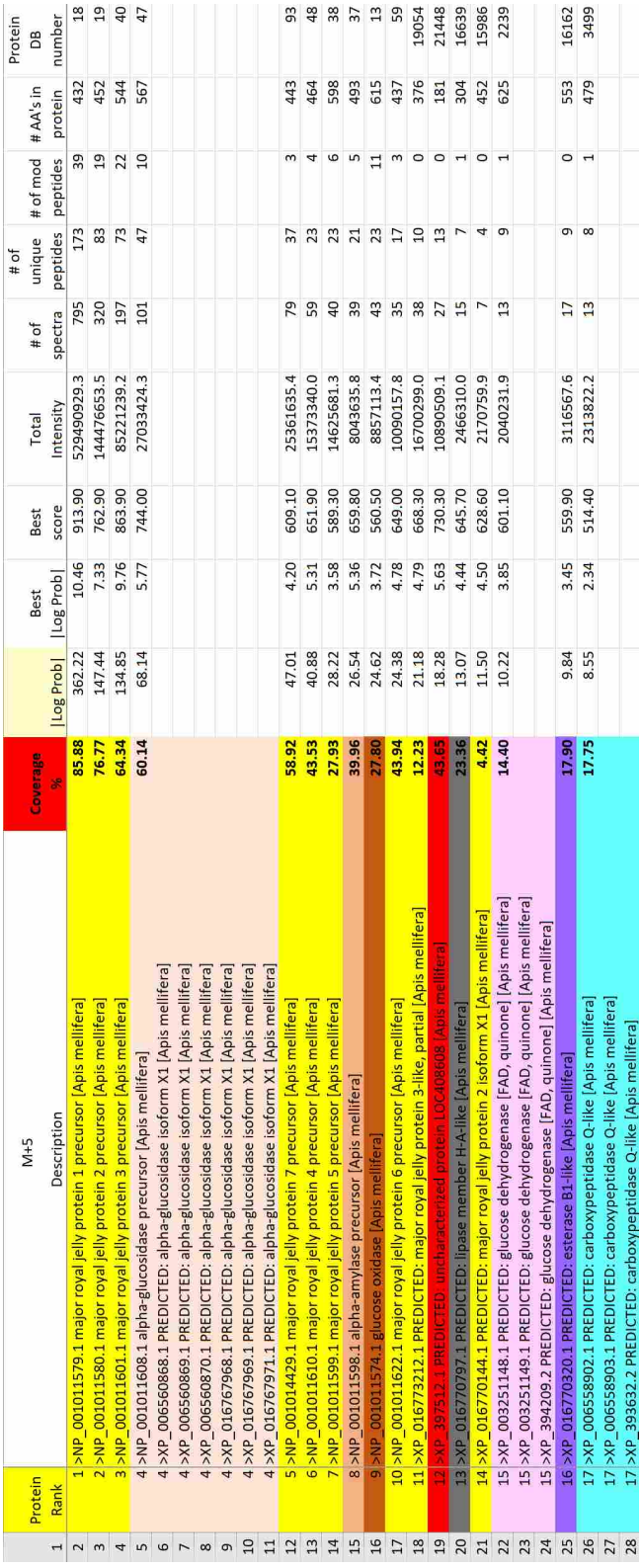
| | Protein Rank | 15 Description | Coverage % | \|Log Prob\| | Best \|Log Prob\| | Best score | Total Intensity | # of spectra | # of unique peptides | # of mod peptides | # AA's in protein | Protein DB number |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 2 | 1 | >NP_001011579.1 major royal jelly protein 1 precursor [Apis mellifera] | **81.94** | 355.20 | 9.24 | 872.70 | 676961339.3 | 813 | 156 | 36 | 432 | 18 |
| 3 | 2 | >NP_001011580.1 major royal jelly protein 2 precursor [Apis mellifera] | **79.42** | 249.64 | 8.27 | 823.90 | 359893236.1 | 471 | 112 | 27 | 452 | 19 |
| 4 | 3 | >NP_001011601.1 major royal jelly protein 3 precursor [Apis mellifera] | **69.49** | 178.15 | 8.19 | 809.20 | 202863324.8 | 269 | 88 | 26 | 544 | 40 |
| 5 | 4 | >NP_001011608.1 alpha-glucosidase precursor [Apis mellifera] | **61.55** | 101.28 | 7.70 | 770.30 | 85815365.1 | 155 | 59 | 13 | 567 | 47 |
| 6 | 4 | >XP_006560868.1 PREDICTED: alpha-glucosidase isoform X1 [Apis mellifera] | | | | | | | | | | |
| 7 | 4 | >XP_006560869.1 PREDICTED: alpha-glucosidase isoform X1 [Apis mellifera] | | | | | | | | | | |
| 8 | 4 | >XP_006560870.1 PREDICTED: alpha-glucosidase isoform X1 [Apis mellifera] | | | | | | | | | | |
| 9 | 5 | >NP_001014429.1 major royal jelly protein 7 precursor [Apis mellifera] | **56.43** | 73.82 | 4.87 | 623.70 | 39797238.4 | 96 | 46 | 4 | 443 | 93 |
| 10 | 6 | >NP_001011599.1 major royal jelly protein 5 precursor [Apis mellifera] | **40.30** | 63.45 | 6.68 | 710.00 | 51906783.2 | 89 | 39 | 7 | 598 | 38 |
| 11 | 7 | >NP_001011574.1 glucose oxidase [Apis mellifera] | **43.41** | 51.08 | 5.71 | 661.40 | 30585826.0 | 73 | 31 | 10 | 615 | 13 |
| 12 | 8 | >XP_016773212.1 PREDICTED: major royal jelly protein 3-like, partial [Apis mellifera] | **25.00** | 46.17 | 8.23 | 800.60 | 31136750.8 | 54 | 13 | 0 | 376 | 19054 |
| 13 | 9 | >NP_001011610.1 major royal jelly protein 4 precursor [Apis mellifera] | **40.09** | 45.39 | 4.88 | 640.00 | 26003641.9 | 61 | 21 | 4 | 464 | 48 |
| 14 | 10 | >NP_001011598.1 alpha-amylase precursor [Apis mellifera] | **50.10** | 42.45 | 5.59 | 658.60 | 27151376.7 | 66 | 31 | 8 | 493 | 37 |
| 15 | 11 | >NP_001011622.1 major royal jelly protein 6 precursor [Apis mellifera] | **41.19** | 42.21 | 5.23 | 641.80 | 28742601.2 | 56 | 24 | 4 | 437 | 59 |
| 16 | 12 | >XP_397512.1 PREDICTED: uncharacterized protein LOC408608 [Apis mellifera] | **42.54** | 29.74 | 7.59 | 687.60 | 18153249.5 | 38 | 14 | 0 | 181 | 21448 |
| 17 | 13 | >XP_016770320.1 PREDICTED: esterase B1-like [Apis mellifera] | **32.37** | 27.47 | 5.55 | 687.30 | 10483861.6 | 26 | 17 | 0 | 553 | 16162 |
| 18 | 14 | >XP_003251148.1 PREDICTED: glucose dehydrogenase [FAD, quinone] [Apis mellifera] | **30.40** | 24.71 | 4.19 | 585.20 | 7363081.2 | 31 | 20 | 4 | 625 | 2239 |
| 19 | 14 | >XP_003251149.1 PREDICTED: glucose dehydrogenase [FAD, quinone] [Apis mellifera] | | | | | | | | | | |
| 20 | 14 | >XP_394209.2 PREDICTED: glucose dehydrogenase [FAD, quinone] [Apis mellifera] | | | | | | | | | | |
| 21 | 15 | >XP_016770797.1 PREDICTED: lipase member H-A-like [Apis mellifera] | **39.47** | 23.24 | 7.04 | 746.20 | 10670775.6 | 31 | 14 | 1 | 304 | 16639 |
| 22 | 16 | >XP_016770144.1 PREDICTED: major royal jelly protein 2 isoform X1 [Apis mellifera] | **7.52** | 22.62 | 5.82 | 687.10 | 14625276.4 | 31 | 8 | 0 | 452 | 15986 |
| 23 | 17 | >XP_624728.5 PREDICTED: carboxypeptidase Q-like [Apis mellifera] | **13.57** | 11.65 | 3.24 | 532.60 | 3862950.2 | 10 | 7 | 0 | 479 | 22171 |
| 24 | 18 | >XP_016768806.1 PREDICTED: uncharacterized protein LOC413627 [Apis mellifera] | **12.39** | 11.31 | 3.36 | 573.90 | 2939891.9 | 13 | 10 | 0 | 654 | 14648 |
| 25 | 19 | >XP_001120887.3 PREDICTED: chitinase-like protein Idgf4 isoform X2 [Apis mellifera] | **11.87** | 10.98 | 3.14 | 520.10 | 1633119.6 | 8 | 5 | 0 | 438 | 883 |
| 26 | 19 | >XP_016769016.1 PREDICTED: chitinase-like protein Idgf4 isoform X2 [Apis mellifera] | | | | | | | | | | |
| 27 | 20 | >XP_392669.1 PREDICTED: venom serine protease 34 [Apis mellifera] | **21.98** | 10.91 | 5.13 | 601.80 | 924136.5 | 7 | 6 | 1 | 405 | 19813 |
| 28 | 21 | >XP_006570610.1 PREDICTED: glucosylceramidase-like isoform X2 [Apis mellifera] | **13.31** | 9.83 | 2.37 | 474.30 | 2553151.0 | 10 | 6 | 1 | 511 | 10620 |

Figure 22. HPLC-MS-MS analysis of the total proteins present in honey sample 15. Major royal jelly protein 1 was found in greatest abundance.



| | Protein Rank | NY Description | Coverage % | [Log Prob] | Best [Log Prob] | Best score | Total Intensity | # of spectra | # of unique peptides | # of mod peptides | # AA's in protein | Protein DB number |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 1 | >NP_001011579.1 major royal jelly protein 1 precursor [Apis mellifera] | **72.45** | 257.72 | 10.63 | 707.70 | 6227089324 | 762 | 69 | 41 | 432 | 18 |
| 2 | 2 | >NP_001011580.1 major royal jelly protein 2 precursor [Apis mellifera] | **68.36** | 176.35 | 10.08 | 689.80 | 2746605026 | 436 | 50 | 25 | 452 | 19 |
| 3 | 3 | >NP_001011601.1 major royal jelly protein 3 precursor [Apis mellifera] | **49.82** | 163.08 | 10.65 | 700.00 | 1112482322 | 240 | 51 | 32 | 544 | 40 |
| 4 | 4 | >NP_001011610.1 major royal jelly protein 4 precursor [Apis mellifera] | **46.77** | 94.99 | 9.10 | 618.70 | 226082428 | 93 | 23 | 8 | 464 | 48 |
| 5 | 5 | >NP_001014429.1 major royal jelly protein 7 precursor [Apis mellifera] | **49.66** | 90.49 | 11.89 | 791.40 | 429676994 | 88 | 28 | 9 | 443 | 93 |
| 6 | 6 | >NP_001011598.1 alpha-amylase precursor [Apis mellifera] | **52.54** | 89.03 | 8.26 | 604.00 | 186903265 | 82 | 25 | 13 | 493 | 37 |
| 7 | 7 | >NP_001011608.1 alpha-glucosidase precursor [Apis mellifera] | **45.86** | 81.96 | 9.14 | 665.40 | 478687386 | 82 | 27 | 15 | 567 | 47 |
| 8 | 7 | >XP_006560868.1 PREDICTED: alpha-glucosidase isoform X1 [Apis mellifera] | | | | | | | | | | |
| 9 | 7 | >XP_006560869.1 PREDICTED: alpha-glucosidase isoform X1 [Apis mellifera] | | | | | | | | | | |
| 10 | 7 | >XP_006560870.1 PREDICTED: alpha-glucosidase isoform X1 [Apis mellifera] | | | | | | | | | | |
| 11 | 7 | >XP_016767968.1 PREDICTED: alpha-glucosidase isoform X1 [Apis mellifera] | | | | | | | | | | |
| 12 | 7 | >XP_016767969.1 PREDICTED: alpha-glucosidase isoform X1 [Apis mellifera] | | | | | | | | | | |
| 13 | 7 | >XP_016767971.1 PREDICTED: alpha-glucosidase isoform X1 [Apis mellifera] | | | | | | | | | | |
| 14 | 8 | >NP_001011574.1 glucose oxidase [Apis mellifera] | **31.38** | 81.68 | 9.75 | 673.40 | 29692273.2 | 74 | 21 | 12 | 615 | 13 |
| 15 | 9 | >NP_001011599.1 major royal jelly protein 5 precursor [Apis mellifera] | **29.26** | 73.82 | 9.65 | 679.10 | 22734754.2 | 62 | 23 | 13 | 598 | 38 |
| 16 | 10 | >gi\|386848\|gb\|AAB59562.1\| keratin | **34.96** | 70.43 | 6.80 | 575.00 | 5176106.1 | 38 | 19 | 5 | 472 | 22454 |
| 17 | 11 | >gi\|136344\|sp\|P02538.3\|K2C6A_HUMAN Keratin, type II cytoskeletal 6A (Cytokeratin-6A) (CK 6A) (K6a kerat | **29.79** | 55.31 | 6.61 | 601.40 | 10008995.2 | 35 | 20 | 4 | 564 | 22458 |
| 18 | 12 | >NP_001011622.1 major royal jelly protein 6 precursor [Apis mellifera] | **33.87** | 52.99 | 7.35 | 556.70 | 11484154.7 | 53 | 20 | 9 | 437 | 59 |
| 19 | 13 | >XP_016770320.1 PREDICTED: esterase B1-like [Apis mellifera] | **18.99** | 35.01 | 7.71 | 598.60 | 5549105.6 | 18 | 9 | 1 | 553 | 16162 |
| 20 | 14 | >XP_006558902.1 PREDICTED: carboxypeptidase Q-like [Apis mellifera] | 20.25 | 32.16 | 6.02 | 546.20 | 2899665.9 | 19 | 10 | 0 | 479 | 3499 |
| 21 | 14 | >XP_006558903.1 PREDICTED: carboxypeptidase Q-like [Apis mellifera] | | | | | | | | | | |
| 22 | 14 | >XP_393632.2 PREDICTED: carboxypeptidase Q-like [Apis mellifera] | | | | | | | | | | |
| 23 | 15 | >XP_003251148.1 PREDICTED: glucose dehydrogenase [FAD, quinone] [Apis mellifera] | 15.52 | 29.66 | 6.01 | 548.70 | 2258820.6 | 19 | 8 | 3 | 625 | 2239 |
| 24 | 15 | >XP_003251149.1 PREDICTED: glucose dehydrogenase [FAD, quinone] [Apis mellifera] | | | | | | | | | | |
| 25 | 15 | >XP_394209.2 PREDICTED: glucose dehydrogenase [FAD, quinone] [Apis mellifera] | | | | | | | | | | |
| 26 | 16 | >XP_006570610.1 PREDICTED: glucosylceramidase-like isoform X2 [Apis mellifera] | 13.50 | 25.17 | 5.42 | 504.30 | 1800937.7 | 11 | 8 | 2 | 511 | 10620 |
| 27 | 16 | >XP_006570611.1 PREDICTED: glucosylceramidase-like isoform X2 [Apis mellifera] | | | | | | | | | | |
| 28 | | | | | | | | | | | | |

Figure 23. HPLC-MS-MS analysis of the total proteins present in honey sample NY. Major royal jelly protein 1 was found in greatest abundance.



| | Protein Rank | M+5 Description | Coverage % | \|Log Prob\| | Best \|Log Prob\| | Best score | Total Intensity | # of spectra | # of unique peptides | # of mod peptides | # AA's in protein | Protein DB number |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | | | | | | | | | | | | |
| 2 | 1 | >NP_001011579.1 major royal jelly protein 1 precursor [Apis mellifera] | **85.88** | 362.22 | 10.46 | 913.90 | 529490929.3 | 795 | 173 | 39 | 432 | 18 |
| 3 | 2 | >NP_001011580.1 major royal jelly protein 2 precursor [Apis mellifera] | **76.77** | 147.44 | 7.33 | 762.90 | 144476653.5 | 320 | 83 | 19 | 452 | 19 |
| 4 | 3 | >NP_001011601.1 major royal jelly protein 3 precursor [Apis mellifera] | **64.34** | 134.85 | 9.76 | 863.90 | 85221239.2 | 197 | 73 | 22 | 544 | 40 |
| 5 | 4 | >NP_001011608.1 alpha-glucosidase precursor [Apis mellifera] | **60.14** | 68.14 | 5.77 | 744.00 | 27033424.3 | 101 | 47 | 10 | 567 | 47 |
| 6 | 4 | >XP_006560868.1 PREDICTED: alpha-glucosidase isoform X1 [Apis mellifera] | | | | | | | | | | |
| 7 | 4 | >XP_006560869.1 PREDICTED: alpha-glucosidase isoform X1 [Apis mellifera] | | | | | | | | | | |
| 8 | 4 | >XP_006560870.1 PREDICTED: alpha-glucosidase isoform X1 [Apis mellifera] | | | | | | | | | | |
| 9 | 4 | >XP_016767968.1 PREDICTED: alpha-glucosidase isoform X1 [Apis mellifera] | | | | | | | | | | |
| 10 | 4 | >XP_016767969.1 PREDICTED: alpha-glucosidase isoform X1 [Apis mellifera] | | | | | | | | | | |
| 11 | 4 | >XP_016767971.1 PREDICTED: alpha-glucosidase isoform X1 [Apis mellifera] | | | | | | | | | | |
| 12 | 5 | >NP_001014429.1 major royal jelly protein 7 precursor [Apis mellifera] | **58.92** | 47.01 | 4.20 | 609.10 | 25361635.4 | 79 | 37 | 3 | 443 | 93 |
| 13 | 6 | >NP_001011610.1 major royal jelly protein 4 precursor [Apis mellifera] | **43.53** | 40.88 | 5.31 | 651.90 | 15373340.0 | 59 | 23 | 4 | 464 | 48 |
| 14 | 7 | >NP_001011599.1 major royal jelly protein 5 precursor [Apis mellifera] | **27.93** | 28.22 | 3.58 | 589.30 | 14625681.3 | 40 | 23 | 6 | 598 | 38 |
| 15 | 8 | >NP_001011598.1 alpha-amylase precursor [Apis mellifera] | **39.96** | 26.54 | 5.36 | 659.80 | 8043635.8 | 39 | 21 | 5 | 493 | 37 |
| 16 | 9 | >NP_001011574.1 glucose oxidase [Apis mellifera] | **27.80** | 24.62 | 3.72 | 560.50 | 8857113.4 | 43 | 23 | 11 | 615 | 13 |
| 17 | 10 | >NP_001011622.1 major royal jelly protein 6 precursor [Apis mellifera] | **43.94** | 24.38 | 4.78 | 649.00 | 10090157.8 | 35 | 17 | 3 | 437 | 59 |
| 18 | 11 | >XP_016773212.1 PREDICTED: major royal jelly protein 3-like, partial [Apis mellifera] | **12.23** | 21.18 | 4.79 | 668.30 | 16700299.0 | 38 | 10 | 0 | 376 | 19054 |
| 19 | 12 | >XP_397512.1 PREDICTED: uncharacterized protein LOC408608 [Apis mellifera] | **43.65** | 18.28 | 5.63 | 730.30 | 10890509.1 | 27 | 13 | 0 | 181 | 21448 |
| 20 | 13 | >XP_016770797.1 PREDICTED: lipase member H-A-like [Apis mellifera] | **23.36** | 13.07 | 4.44 | 645.70 | 2466310.0 | 15 | 7 | 1 | 304 | 16639 |
| 21 | 14 | >XP_016770144.1 PREDICTED: major royal jelly protein 2 isoform X1 [Apis mellifera] | **4.42** | 11.50 | 4.50 | 628.60 | 2170759.9 | 7 | 4 | 0 | 452 | 15986 |
| 22 | 15 | >XP_003251148.1 PREDICTED: glucose dehydrogenase [FAD, quinone] [Apis mellifera] | **14.40** | 10.22 | 3.85 | 601.10 | 2040231.9 | 13 | 9 | 1 | 625 | 2239 |
| 23 | 15 | >XP_003251149.1 PREDICTED: glucose dehydrogenase [FAD, quinone] [Apis mellifera] | | | | | | | | | | |
| 24 | 15 | >XP_394209.2 PREDICTED: glucose dehydrogenase [FAD, quinone] [Apis mellifera] | | | | | | | | | | |
| 25 | 16 | >XP_016770320.1 PREDICTED: esterase B1-like [Apis mellifera] | **17.90** | 9.84 | 3.45 | 559.90 | 3116567.6 | 17 | 9 | 0 | 553 | 16162 |
| 26 | 17 | >XP_006558902.1 PREDICTED: carboxypeptidase Q-like [Apis mellifera] | **17.75** | 8.55 | 2.34 | 514.40 | 2313822.2 | 13 | 8 | 1 | 479 | 3499 |
| 27 | 17 | >XP_006558903.1 PREDICTED: carboxypeptidase Q-like [Apis mellifera] | | | | | | | | | | |
| 28 | 17 | >XP_393632.2 PREDICTED: carboxypeptidase Q-like [Apis mellifera] | | | | | | | | | | |

Figure 24. HPLC-MS-MS analysis of the total proteins present in honey sample M+5. Major royal jelly protein 1 was found in greatest abundance.

| | Protein Rank | M+20 Description | Coverage % | \|Log Prob\| | Best \|Log Prob\| | Best score | Total Intensity | # of spectra | # of unique peptides | # of mod peptides | # AA's in protein | Protein DB number |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | | | | | | | | | | | | |
| 2 | 1 | >NP_001011579.1 major royal jelly protein 1 precursor [Apis mellifera] | **82.18** | 459.99 | 10.88 | 837.30 | 97765148.2 | 518 | 152 | 33 | 432 | 18 |
| 3 | 2 | >NP_001011580.1 major royal jelly protein 2 precursor [Apis mellifera] | **77.21** | 193.47 | 6.96 | 696.10 | 27218186.7 | 234 | 73 | 18 | 452 | 19 |
| 4 | 3 | >NP_001011601.1 major royal jelly protein 3 precursor [Apis mellifera] | **58.09** | 135.69 | 7.37 | 646.20 | 14611035.9 | 121 | 55 | 18 | 544 | 40 |
| 5 | 4 | >NP_001011608.1 alpha-glucosidase precursor [Apis mellifera] | **39.51** | 71.45 | 9.07 | 705.80 | 3766860.4 | 56 | 29 | 6 | 567 | 47 |
| 6 | 4 | >XP_006560868.1 PREDICTED: alpha-glucosidase isoform X1 [Apis mellifera] | | | | | | | | | | |
| 7 | 4 | >XP_006560869.1 PREDICTED: alpha-glucosidase isoform X1 [Apis mellifera] | | | | | | | | | | |
| 8 | 4 | >XP_006560870.1 PREDICTED: alpha-glucosidase isoform X1 [Apis mellifera] | | | | | | | | | | |
| 9 | 4 | >XP_016767968.1 PREDICTED: alpha-glucosidase isoform X1 [Apis mellifera] | | | | | | | | | | |
| 10 | 4 | >XP_016767969.1 PREDICTED: alpha-glucosidase isoform X1 [Apis mellifera] | | | | | | | | | | |
| 11 | 4 | >XP_016767971.1 PREDICTED: alpha-glucosidase isoform X1 [Apis mellifera] | | | | | | | | | | |
| 12 | 5 | >NP_001014429.1 major royal jelly protein 7 precursor [Apis mellifera] | **37.92** | 68.91 | 5.86 | 599.60 | 5233300.9 | 48 | 25 | 1 | 443 | 93 |
| 13 | 6 | >NP_001011599.1 major royal jelly protein 5 precursor [Apis mellifera] | **30.77** | 47.90 | 7.51 | 670.90 | 3177209.4 | 32 | 22 | 7 | 598 | 38 |
| 14 | 7 | >XP_016770147.1 PREDICTED: major royal jelly protein 4 isoform X1 [Apis mellifera] | **31.25** | 44.59 | 4.80 | 536.70 | 2051663.5 | 27 | 15 | 2 | 464 | 15989 |
| 15 | 8 | >XP_016773212.1 PREDICTED: major royal jelly protein 3-like, partial [Apis mellifera] | **18.35** | 34.57 | 5.97 | 620.60 | 3652698.3 | 36 | 11 | 0 | 376 | 19054 |
| 16 | 9 | >NP_001011574.1 glucose oxidase [Apis mellifera] | **23.41** | 33.13 | 7.82 | 688.70 | 1500654.8 | 28 | 14 | 6 | 615 | 13 |
| 17 | 10 | >NP_001011598.1 alpha-amylase precursor [Apis mellifera] | **30.63** | 29.93 | 7.30 | 608.20 | 1091962.0 | 24 | 15 | 6 | 493 | 37 |
| 18 | 11 | >XP_016770797.1 PREDICTED: lipase member H-A-like [Apis mellifera] | **39.14** | 27.02 | 6.20 | 614.30 | 1003358.2 | 20 | 11 | 1 | 304 | 16639 |
| 19 | 12 | >NP_001011622.1 major royal jelly protein 6 precursor [Apis mellifera] | **28.83** | 26.20 | 4.85 | 533.80 | 1896685.7 | 20 | 11 | 2 | 437 | 59 |
| 20 | 13 | >gi\|136429\|sp\|P00761.1\|TRYP_PIG RecName: Full=Trypsin; Flags: Precursor | **47.19** | 23.83 | 4.17 | 458.60 | 16009929.9 | 57 | 18 | 1 | 231 | 22466 |
| 21 | 14 | >gi\|386848\|gb\|AAB59562.1\| keratin | **16.31** | 16.55 | 4.79 | 529.90 | 1192742.8 | 13 | 7 | 0 | 472 | 22454 |
| 22 | 15 | >XP_016770320.1 PREDICTED: esterase B1-like [Apis mellifera] | **18.44** | 14.93 | 4.42 | 536.80 | 708852.7 | 13 | 8 | 0 | 553 | 16162 |
| 23 | 16 | >XP_016770144.1 PREDICTED: major royal jelly protein 2 isoform X1 [Apis mellifera] | **4.42** | 12.41 | 6.45 | 621.60 | 174168.5 | 3 | 3 | 0 | 452 | 15986 |
| 24 | 17 | >gi\|166900097\|sp\|P04259.4\|K2C6B_HUMAN Keratin, type II cytoskeletal 6B (Cytokeratin-6B) (CK 6B) (K6b keratin) | **18.79** | 12.34 | 3.69 | 553.60 | 473331.0 | 17 | 8 | 1 | 564 | 22457 |
| 25 | 17 | >gi\|13467344\|sp\|P02538.3\|K2C6A_HUMAN Keratin, type II cytoskeletal 6A (Cytokeratin-6A) (CK 6A) (K6a keratin) (Cytokeratin-6D) (CK 6 | | | | | | | | | | |
| 26 | 18 | >XP_003251464.1 PREDICTED: actin, clone 205-like [Apis mellifera] | **21.01** | 11.93 | 4.07 | 471.20 | 279183.0 | 8 | 6 | 1 | 376 | 2343 |
| 27 | 18 | >XP_016767697.1 PREDICTED: actin, clone 205-like [Apis mellifera] | | | | | | | | | | |
| 28 | 19 | >XP_397512.1 PREDICTED: uncharacterized protein LOC408608 [Apis mellifera] | **26.52** | 11.33 | 4.60 | 543.60 | 474310.8 | 6 | 5 | 0 | 181 | 21448 |

Figure 25. HPLC-MS-MS analysis of the total proteins present in honey sample M+20. Major royal jelly protein 1 was found in greatest abundance.

Appendix C

Measurement of hydrogen peroxide using chemical strips

One gram of honey was weighed into 15 ml conical tubes and 9 ml of DI $H_2O$ was added. The mixture was vortexed to get a homogenous solution. The mixture was allowed to sit for at least 1 hour at room temperature to allow $H_2O_2$formation. Quantofix peroxide 100 (1-100mg/L $H_2O_2$) strips were used from Sigma-Aldrich. The color produced was matched with the color bar given on the bottle.

Bradford assay Bovine serum albumin standard (BSA)

A bovine serum albumin (BSA) standard of 2 mg/ml (2,000 µg/ml) in original tubes (Thermofisher Cat # 23200) was used. Table 8 shows the different concentrations of BSA standards which were made.

Tube 1- 0.05 mg/ml → 1462.5 µl DI $H_2O$ + 37.5 µl BSA standard

Table 8. BSA standard setup and final concentration

| Tubes | DI $H_2O$ (µl) | 0.05 mg/ml BSA (tube 1) | Coomassie dye (µl) | Final Concentration (µg/µl) |
|---|---|---|---|---|
| Tube 2 | 500 (blank) | 0 | 500 | 0 |
| Tube 3 | 475 | 25 | 500 | 0.0025 |
| Tube 4 | 450 | 50 | 500 | 0.0050 |
| Tube 5 | 400 | 100 | 500 | 0.01 |
| Tube 6 | 350 | 150 | 500 | 0.015 |
| Tube 7 | 300 | 200 | 500 | 0.02 |

Table 8 shows the BSA standard setup scheme and the final concentrations that were used before doing the Bradford assays before each experiment. An initial stock of 0.05 mg/ml in tube 1 was used to make the final concentrations in the rest of the tubes (tubes 2-7).

Sodium Dodecyl Sulfate - Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Resolving and stacking gel solutions were prepared without APS or TEMED. Table 6 gives more detailed information on how these were made. Complete list of reagents for making 12.5% resolving gel and 4% stacking gel is given in Table 9.

Table 9. Resolving gel 12.5% and stacking gel 4%

| Reagents | Resolving gel 12.5% | Stacking gel 4% |
|---|---|---|
| 30% Acrylamide/bis | 6.25 ml | 1.98 ml |
| 0.5M Tris-HCl, pH 8.8 | - | 3.78 ml |
| 1.5M Tris-HCl, pH 8.8 | 3.75 ml | - |
| 10% SDS | 150 µl | 150 µl |
| DI $H_2O$ | 4.78 ml | 9 ml |
| TEMED | 7.5 µl | 15 µl |
| 10% APS | 75 µl | 75 µl |
| Total volume | 15 ml | 15 ml |

The prepared solutions of resolving and stacking gels were degassed under vacuum for at least 10-15 mins. While solutions were degassing, the glass cassette sandwich was assembled. A

comb was placed into the assembled sandwich. A mark 1 cm below the teeth of the comb on glass plate was placed to the level the resolving gel was be poured. The comb was removed.

APS and TEMED was added to the degassed resolving gel solution, and it was poured in the cassette to the mark. The gel was allowed to solidify/polymerize for at least 45 mins to 1 hour. Once an observable line was formed between stacking and resolving gel, the gel was polymerized. The overlay solution was poured the top of the gel was rinsed with DI $H_2O$.

The area above the separating gel was dried with Kim wipes. A comb was placed into the cassette and the stacking gel was poured. APS and TEMED was added to the resolving gel solution, and it was poured into the spacer nearest the upturned side of the comb. The solution was added until all the teeth of the combs were covered by the solution. The comb was re-aligned in the sandwich and then monomer was added to fill the cassette completely. The gel was allowed to solidify or polymerize for at least 35 to 45 minutes. The comb was removed by slowly pulling it up carefully without tearing the gel apart. The wells in the gel were rinsed completely 2 to 3 times with DI $H_2O$.

Hydrogen peroxide concentration assays using the Amplex® Red Hydrogen peroxide/peroxidase Assays kit

Amplex® Red Hydrogen Peroxide/Peroxidase Assay Kit (invitrogen, Catalog no. A22188) was used to make the different reagents. The stock solutions were made according to the kit protocol. The experimental protocol for preparing the $H_2O_2$ assay working solutions was followed way as shown in Table 10.

Table 10. Working solutions: preparation of $H_2O_2$ standards

| | |
|---|---|
| A. 20 mM $H_2O_2$ | Prepared in step 1.5 from protocol |
| B. 2 mM $H_2O_2$ | 20 µl from A + 180 µl 1X reaction buffer (RB) (prepared in step 1.3 from protocol) |
| C. 200 µM $H_2O_2$ | 20 µl from B + 180 µl 1X RB |
| D. 20 µM $H_2O_2$ | 20 µl from C + 180 µl 1X RB |
| E. 10 µM $H_2O_2$ | 75 µl from D + 75 µl 1X RB |
| F. 5 µM $H_2O_2$ | 30 µl from E + 30 µl 1X RB |
| G. 2 µM $H_2O_2$ | 15 µl from E + 60 µl 1X RB |
| H. 1 µM $H_2O_2$ | 10 µl from E + 90 µl 1X RB |

A working solution of 100 µM Amplex Red reagent and 0.2 U/mL Horseradish Peroxidase (HRP) was made by mixing following for a total of 5 ml:

- 50 µl of 10 mM Amplex® Red reagent stock solution (prepared in step 1.2 from protocol)
- 100 µl of 10 U/ml HRP stock solution (prepared in step 1.4 from protocol)
- 4.85 ml of 1X Reaction Buffer (prepared in step 1.3 from protocol)

This 5 ml volume is sufficient for ~100 assays.

For the reaction, 50 µl of Amplex Red reagent/HRP working solution was added to wells of a 96-well plate containing 50 µl of standards, controls, or samples. The 96 well plate was

allowed to incubate at room temperature for 30 minutes protected from light. Table 11 below shows the 96-well plate lay out and how different honey sample dilutions were added to each well. Column 1 shows the controls.

Table 11. 96 well plate showing different honey sample dilutions and controls.

| | | 12 | 12-w | 13 | 14 | 15 | NY | M+5 | M+20 |
|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
| A | 0 µM | (1:2) | (1:2) | (1:2) | (1:2) | (1:2) | (1:2) | (1:2) | (1:2) |
| B | 20 µM | (1:2) | (1:2) | (1:2) | (1:2) | (1:2) | (1:2) | (1:2) | (1:2) |
| C | 10 µM | (1:4) | (1:4) | (1:4) | (1:4) | (1:4) | (1:4) | (1:4) | (1:4) |
| D | 5 µM | (1:4) | (1:4) | (1:4) | (1:4) | (1:4) | (1:4) | (1:4) | (1:4) |
| E | 2 µM | (1:10) | (1:10) | (1:10) | (1:10) | (1:10) | (1:10) | (1:10) | (1:10) |
| F | 1 µM | (1:10) | (1:10) | (1:10) | (1:10) | (1:10) | (1:10) | (1:10) | (1:10) |
| G | -- | (1:20) | (1:20) | (1:20) | (1:20) | (1:20) | -- | -- | -- |
| H | -- | (1:20) | (1:20) | (1:20) | (1:20) | (1:20) | -- | -- | -- |

*(--) nothing was added to the well
**color shows different dilutions

Fluorescence was measured at an emission wavelength of 590 nm using an excitation wavelength of 530 nm employing the Synergy HT multi-detection microplate reader (Bio-Tek) in the RIC facility, at BYU. The OD reading are shown Table 12.

Table 12. Raw OD reading of the honey samples using Amplex Red assay.

| Raw OD | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
|---|---|---|---|---|---|---|---|---|---|
| A | 110 | 1100 | 907 | 1503 | 2313 | 1028 | 611 | 3075 | 1294 |
| B | 3252 | 920 | 873 | 1502 | 2409 | 933 | 674 | 2922 | 1288 |
| C | 1800 | 1333 | 879 | 1949 | 2512 | 1150 | 637 | 4623 | 1929 |
| D | 977 | 1269 | 906 | 1903 | 2682 | 1331 | 686 | 4677 | 2023 |
| E | 493 | 618 | 337 | 965 | 962 | 696 | 248 | 2035 | 1061 |
| F | 333 | 607 | 345 | 992 | 1012 | 700 | 255 | 2144 | 1237 |
| G | -- | 383 | 205 | 528 | 541 | 427 | -- | -- | -- |
| H | -- | 506 | 291 | 695 | 718 | 467 | -- | -- | -- |

*(--) nothing was added to the well
**color shows different dilutions

The background was corrected by subtracting the value derived from the no-$H_2O_2$ control in well A1. Corrected values are shown in Table 13.

Table 13. Corrected OD readings of the honey samples using the Amplex Red Assay.

| Corrected OD | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
|---|---|---|---|---|---|---|---|---|---|
| A | 0 | 990 | 797 | 1393 | 2203 | 918 | 501 | 2965 | 1184 |
| B | 3142 | 810 | 763 | 1392 | 2299 | 823 | 564 | 2812 | 1178 |
| C | 1690 | 1223 | 769 | 1839 | 2402 | 1040 | 527 | 4513 | 1819 |
| D | 867 | 1159 | 796 | 1793 | 2572 | 1221 | 576 | 4567 | 1913 |
| E | 383 | 508 | 227 | 855 | 852 | 586 | 138 | 1925 | 951 |
| F | 223 | 497 | 235 | 882 | 902 | 590 | 145 | 2034 | 1127 |
| G | - | 273 | 95 | 418 | 431 | 317 | - | - | - |
| H | - | 396 | 181 | 585 | 608 | 357 | - | - | - |

*(--) nothing was added to the well
**color shows different dilutions

Reactions containing 50µM Amplex® Red reagent, which had 0.1 U/mL HRP and the indicated amount of $H_2O_2$ in 50 mM sodium phosphate buffer, pH 7.4, and the indicated amount of $H_2O_2$ were incubated for 30 minutes at room temperature. A standard curve obtained for this assay is shown in Figure 26.

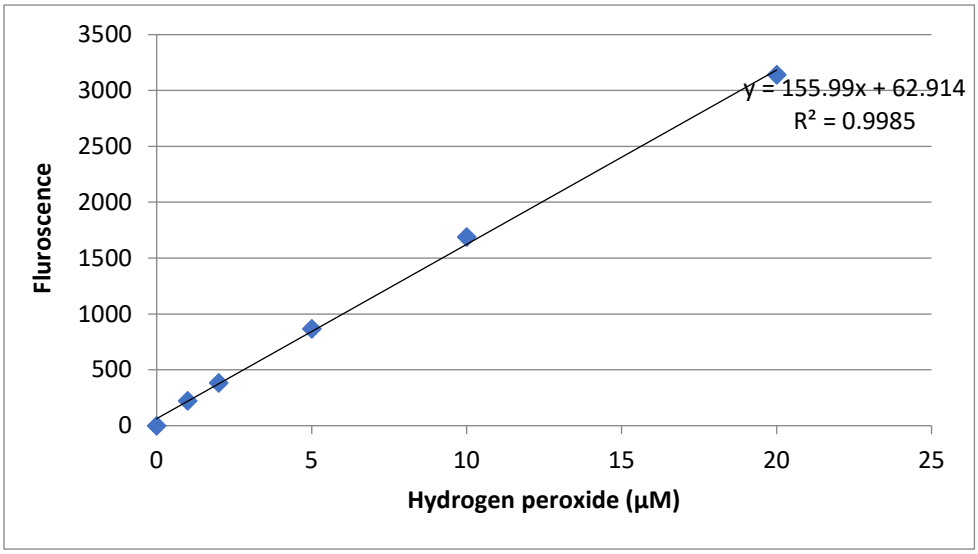



Figure 26. Standard curve for the detection of $H_2O_2$ using the Amplex® Red Hydrogen Peroxide/Peroxidase Assay Kit.

Fluorescence was then measured with a fluorescence microplate reader using excitation at 530 nm and fluorescence detection at 590 nm. Background fluorescence, determined for a no- $H_2O_2$ control reaction, was subtracted from each value.

The final $H_2O_2$ concentrations of honey samples based on the standard curve in Figure 26 is shown in Table 14.

Table 14. Final $H_2O_2$ concentrations in honey samples.

| Conc (μM) | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
|---|---|---|---|---|---|---|---|---|---|
| A | 0.0 | 11.9 | 9.4 | 17.1 | 27.4 | 11.0 | 5.6 | 37.2 | 14.4 |
| B | 19.7 | 9.6 | 9.0 | 17.0 | 28.7 | 9.7 | 6.4 | 35.2 | 14.3 |
| C | 10.4 | 29.7 | 18.1 | 45.5 | 60.0 | 25.1 | 11.9 | 114.1 | 45.0 |
| D | 5.2 | 28.1 | 18.8 | 44.4 | 64.3 | 29.7 | 13.2 | 115.5 | 47.4 |
| E | 2.1 | 28.5 | 10.5 | 50.8 | 50.6 | 33.5 | 4.8 | 119.4 | 56.9 |
| F | 1.0 | 27.8 | 11.0 | 52.5 | 53.8 | 33.8 | 5.3 | 126.4 | 68.2 |
| G | - | 26.9 | 4.1 | 45.5 | 47.2 | 32.6 | - | - | - |
| H | - | 42.7 | 15.1 | 66.9 | 69.9 | 37.7 | - | - | - |
| | | | | | | | | | |
| | Average: | 28.2 | 18.5 | 47.7 | 62.2 | 34.4 | 12.5 | 118.8 | 46.2 |