Analysis of essential oils and volatile organic compounds in various strains of *Humulus lupulus* and their effects on resistance to Downy and Powdery mildew

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Introduction

Humulus lupulus – commonly known as hops – is a popular crop in the brewing industry. The Brewers Association estimates the American beer market is worth 107.6 billion dollars and produces 420 thousand jobs. Due to its instrumental role in the production of beer, hops is an extremely valuable crop.

Hops produces flowers which, due to their shape, are referred to as cones. Within these cones there are granular structures known as trichomes (Booth et al. 2017). These trichomes are the production sites for terpenes and essential oils. Hops produces essential oils in the form of lupulin. The sappy lupulin is stored in the bracts of the hops cone.

Recently, scientists have begun to research medicinal applications for the essential oils produced by the hops plant (Wang et al. 2008). If hops can be used for medicinal purposes, the value of hops in the American economy will increase significantly. Additionally, the essential oils produced by other plants have been found to have antifungal properties (Zambonelli et al. 1996). With the growing popularity of environmentally friendly products, the oils produced by hops – if found to have anti-fungal properties – could be used to develop biodegradable fungicides. Anti-fungal applications of the essential oils produced by hops would further increase the importance of the hops crop to the American economy.

Throughout the 2017 -2018 academic year, research focused on developing techniques to facilitate the extraction of essential oils from the hops crop. Once extracted, the characterization would help narrow the focus of which variety of hops to continue to extract the oils from and test for anti-fungal properties.

The four selected varieties of hops were Cascade, Brewer’s Gold, Willamette, and Citra. The principle focus would be on Citra due to greater accessibility. All the extracted essential oils would be tested on *P. humuli*, downy mildew, and *P. macularis*, powdery mildew.

P. humuli and *P. macularis* are both fungi that are infamous for ravaging hops crops. These fungi cannot be detected until the growth season after the hop was infected. By that point, not much can be done to save the crop. Furthermore, the fungi can spread easily due to the proximity of the crops.

If lupulin proves to be an effective anti-fungal substance, farmers may be interested in developing a hop variety which produces greater quantities of lupulin. The greater amounts of lupulin produced may be a cheaper and safer alternative to the current anti-fungal sprays.

Principal Approaches

Isolation of Essential Oils.

The initial process of essential oil isolation was performed through several steps. Mature female hop cones were purchased from northern brewer for use. The hop cones are placed in an aluminum weigh tray which was placed in a dewar filled with liquid nitrogen. The hop cones were left in the dewar in order to thoroughly flash freeze them. The flash frozen cones are now very brittle and are pulverized into a fine powder using a mortar and pestle (Sanja *et al.,* 2000).

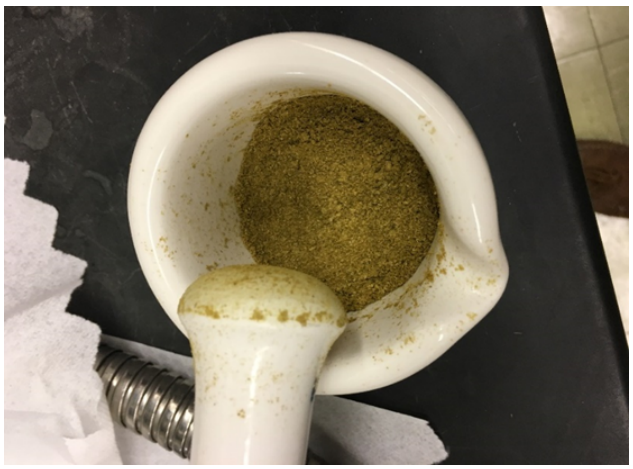

Figure 1: Mortar and Pestle with freshly ground hops cones.

Frozen stocks of *M. smegmatis* were propagated on Luria agar plates (incubating at 37°C), and grown in a Middlebrook 7H9 complete medium incubated at 37°C with shaking for 48h. This procedure was repeated weekly to maintain liquid bacterial cultures no older than 96h.

The pulverized hop cones are then weighed out using an analytical balance into ~0.500 gram samples and placed into a 10mL glass vial. This glass vial then has 5mL of hexane added to the sample (10:1 hexane: plant matter) (Wang 2008). The prepared vial was then lightly shaken to mix the solution and let to sit for 2-4 hours. After the sample has settled, roughly 3mL of the top layer was pipetted of and rotovaped down until all hexane was evaporated off.

A technique using steam distillation was also performed in an attempt to find an even more suitable method for essential oil extraction. A steam distillation apparatus was set up as shown in figure 2, and 10 grams of pulverized hops was added to the distillation flask. The apparatus was allowed to run for 8 hours.

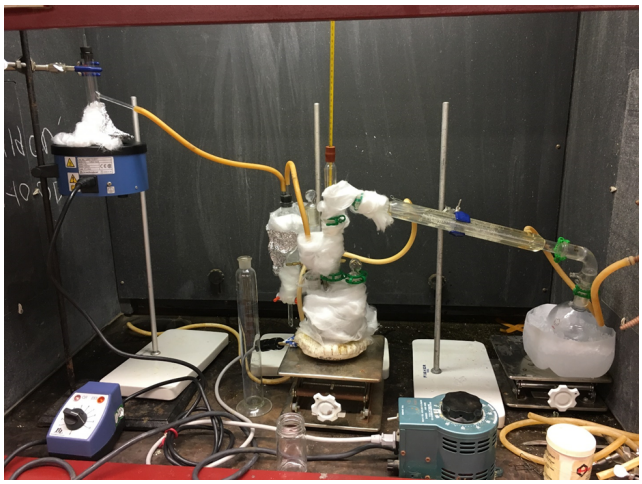

Figure 2: Steam distillation apparatus

Preparation for GC-MS.

The oil residue was then put back into solution using analytical hexane (provided by Sigma Aldrich), which acts as the solvent for Gas Chromatography Mass Spectroscopy. A GC-MS vial must be used and a solution of 1mL of oil solution and 0.5mL of analytical hexane must be mixed in this vial. The sample may then be run through the instrument using preset standards (lavender method file) by Dr. Kevin Dunn. This optimizes the coil's heating process in order to effectively pass oils through the capillary column. This process was repeated twice for every variety of hops in order to retrieve comparable chromatograms in order to confidently analyze the data.

Once the instrument concluded its run of the samples, the spectra was analyzed using the Post run program. For each chromatogram, 10 peaks were auto-integrated and any other relevant peaks were manually integrated and placed into a table for further analysis. A similarity search was then run on all of the acquired peaks. For the majority of the peaks, a similarity index above 80%, based on both retention index and mass spectra, was used to determine the molecule represented by each peak.

With qualitative profiles established for each variety of hops, an internal standard was to be established. The two chemicals (both provided by Sigma Aldrich) which were used to do this were cis-3-hexenol (established by Kishimoto 2005) and D-Toluene (established by Sanja 2000). Solutions containing these molecules and analytical hexane were made so that their addition to a GCMS sample vial would have a known quantity. 10mL of analytical hexane was added to a 50mL bottle then, 6 microliters of D-toluene was added and the solution was mixed. 10mL of analytical hexane was added to another 50mL glass bottle, then 7 microliters of cis-3-hexenol was added to this bottle and the solution was mixed. Both of these bottles were covered using Parafilm in order to create an airtight seal and keep the solution from evaporating. 0.5mL of these standards were added to GCMS sample vials containing 1mL of the plant matter/hexane solution. This created a solution which contained a standard which was 0.03% by weight. This known concentration allows for peaks to be compared to the standard, and for concentrations to be established for each molecule (by peak to standard peak ratio).

Results

The steam distillation apparatus proved to be ineffective. Multiple trials were run and adjustments made, but the device never managed to extract essential oils. Furthermore, the device was determined to be too burdensome to continue to modify. Steam distillation was not used to extract essential oils.

Two chromatograms were acquired for each variety of hops and qualitative profiles were established. These chromatograms provide both major peaks and minor peaks. The major peaks are essential oils which are present in each variety of hops and the minor peaks are the peaks which change and provide the different hops with their unique aromas.

After comparing all of the spectra, several peaks can be identified as standard, abundant, essential oils produced by the hops plant. These peaks are: Humulene, beta-myrcene, beta-caryophyllene, and lupulon. The other peaks present in the chromatograms vary greatly between different varieties. The table below shows the current established profiles of important minor peaks for each variety.

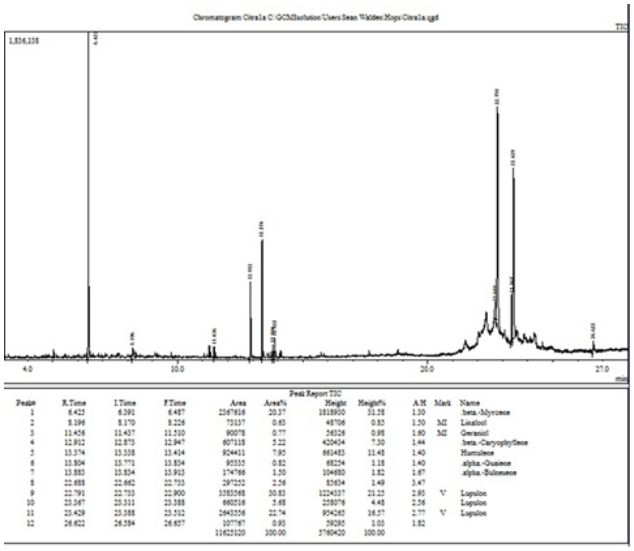

Figure 3: Sample of chromatogram

Once these qualitative profiles were established, Citra was run with the two internal standards and chromatograms were interpreted. Two Citra samples were prepared as stated previously; one was poisoned with D-toluene and the other was poisoned with cis-3-hexenol. These samples were analyzed using GCMS as all previous samples have been.

The cis-3-hexenol standard appeared clearly in the sample. The peak is of a similar size to the other peaks shown on the chromatogram. The peak was displayed around 4 minutes which is well within the range that was established in the batch method for GC. Using the peak table (Area %) values, quantities of the molecules present in Citra were calculated:

Molecule	Quantity in sample (µg)	Quantity in sample (% by weight of 0.496 gram sample)
Myrcene	1.794	0.004
Humulene	1.028	0.002
Beta-caryophyllene	0.583	0.001
Alpha-Guaiene	0.115	0.0002
Lupulon	8.004	0.016

Table 1: Quantity of individual molecules in Citra sample.

Initially, the Citra hops with the d-toluene standard did not seem to have any clear peak for d-toluene. The peak was very lightly seen at the beginning of the chromatogram, around 3 minutes. The peak was far enough off of the chromatogram that the frame of the chromatogram needed to be shifted. Thus, the batch acquisition method was altered slightly to accommodate the peak.

Once shifted, both a d-toluene and a toluene peak were present. The toluene peak was larger than the d-toluene peak. This lead to the d-toluene standard being run by itself to analyze its contents.

When run alone, the standard returned the above chromatogram. This clearly shows two peaks. The larger of the two is standard toluene. This shows that the d-toluene standard that was used is not pure and should not be used as an internal standard.

Discussion

Through research process, qualitative profiles have been established for Willamette, Brewer's Gold, Cascade, Chinook, and Citra hops varieties. These profiles have shown that beta-myrcene, humulene, beta-caryophyllene, and lupulon are essential oils which are present in each variety of hops and make up a quintessential piece of the aroma profile of hops. These oils may vary in quantity between different varieties, playing a role in the niche which each variety fills in the brewing industry, but their presence is universal. There are several other essential oils which are present in the individual varieties which also play an important role in establishing the aroma profile and niche for these hops. In order to further understand the profile of the hops, a quantitative profile needed to also be collected. Using Citra as a standard, both masses of individual molecules and percent of plant matter by weight were established. This method was done through the use of d-toluene and cis-3-hexenol as internal standards. D-toluene did not provide a single, clear, peak; rather, it provided two peaks. Further analysis of the content of the d-toluene standard showed that it contained more toluene (C7H8) than d-toluene (C7D8). This could be caused by contamination of the analytical hexane which was used for GCMS solution prep (this was proven to not be the case by running an analytical hexane background sample). It may also be that the product supplied from the company was improperly synthesized, or the standard was contaminated during its prep. Whatever the cause, being that this standard is not pure, it should not be used in any further profile establishment. The internal standard, cis-3-hexenol, was effective in providing a clear peak which could easily be compared to establish quantities for each other peak. This showed that beta-myrcene, humulene, beta-caryophyllene, and lupulon were the most prominent oils in the sample. Lupulon being the largest by percent weight. In light of this information, humulene, beta-caryophyllene, beta-myrcene, and lupulon should be used in further testing of the anti-fungal qualities of hops essential oils.

Future Research

As a continuation of this research, the anti-fungal qualities of the previously mentioned oils will be tested in the presence of Aspergillus fumigatus on potato dextrose agar (PDA) plates. After further

quantitative profiling of the other hop varieties, some of the minor essential oils will also be selected for testing. The contents of the d-toluene standards purchased from Sigma Aldrich will also be analyzed. The standard, from a new ampule, will be run through GCMS and compared to chromatograms of analytical toluene and analytical hexane to further confirm (or deny) beliefs that the standard may be contaminated.

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