# Can hops (cones of *Humulus lupulus* L.) cure what ails you? Relating hop chemistry to bioactivity

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# ABSTRACT

This thesis used biochemometrics, metabolomics, and bioassay-guided fractionation to quantify, group, and identify active chemical constituents in hop (*Humulus lupulus* L. Cannabaceae) extracts, a phytochemically unique plant of cultural and economic significance around the world. Hops produce infructescence (cones) from the female plant that contain < 30% (dry weight) secondary metabolites such as the polyketides humulones (α-acids), lupulones (ß-acids), and prenylated chalcones (e.g., xanthohumol), that were quantified via high-performance liquid chromatography with diode array detection (HPLC-DAD). Hops have anti-bacterial, anti-inflammatory, and sedative properties, though most are destined for the brewing industry. However, the traditional uses, unique metabolites, and reported bioactivity of hops have led to increasing interest in hops as a natural health product.

Hop breeders select hops for their unique chemical properties (aroma or bittering) that differ between varieties. Previously, it was found that the alpha acids were positively correlated to xanthohumol, though the relationship between alpha acids and beta acids remained unclear. Alpha acids (co- and ad-humulone) correlated with one another, beta acids (lupulone, colupulone, and adlupulone) correlated with one another, and the individual alpha acids correlated more strongly with xanthohumol than the individual beta acids did with xanthohumol. Indigenous North American hops usually have greater alpha acid content than European hops. Bittering hops and North American hop samples contained significantly more alpha acids (humulone, cohumulone, and adhumulone). They had a higher alpha-to-beta ratio than the aroma and European hop samples.

The biochemometric approach uses a chemically diverse set of plants and modelled bioactivity to chemistry from these HPLC data, along with testing isolated constituents of hops,

metabolomic analysis on the hop extracts using ultra-performance liquid chromatography with high resonance mass spectroscopy, and bioassay-guided fractionation to identify other compounds or confirm the identity of targeted compounds.

Free radicals are sometimes a natural byproduct of cellular respiration; enzymes typically scavenge these free radicals. However, oxidative stress occurs when free-radical production outpaces free-radical scavenging activity (RSA). Continued oxidative stress leads to an inflammatory response and activation of oxidase enzymes, such as cyclooxygenase-2 (COX-2). The endocannabinoid system consists of receptors, ligands, and enzymes that metabolize endocannabinoids (e.g., FAAH and MAGL). FAAH and MAGL inhibition increases endocannabinoids levels, reducing the quantity of arachidonic acid used by some inflammatory pathways (e.g., COX-2 and LOX).

Hop extract chemistry modelled RSA and FAAH inhibitory activity based on marker compounds. In contrast, extract chemistry did not model COX-2 and MAGL inhibitions as there was a narrow activity range among extracts. Isolated n-adlupulone demonstrated the strongest RSA, comparable to ascorbic acid. Xanthohumol potently inhibited COX-2, FAAH, and MAGL. Colupulone potently inhibited COX-2 and MAGL. Mass features were tentatively identified from the metabolomics dataset and modelled bioactivity, while bioassay-guided fractionation confirmed xanthohumol as a strong FAAH and MAGL inhibitor. This is the first study to use biochemometrics, metabolomics, and bioassay-guided fractionation to identify bioactive components in hops and that assessed isolated compounds in a concentration-dependent manner. These results will further support the development of hop-based natural health products.

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# CHAPTER 1: GENERAL INTRODUCTION

## ETHNOPHARMACOLOGY

Plant-based medicine is ubiquitous throughout the world and human history. Approximately 35,400 species of plants have reported medicinal properties, but the vast majority have yet to be extensively studied under laboratory conditions, and hundreds of thousands of species remain unstudied for potential therapeutic properties (Schultz & Garbe, 2023). Ethnopharmacology is a multidisciplinary holistic approach to studying natural organisms used traditionally and historically for medicinal purposes by combining qualitative social science data with quantitative physical science data (Schultz & Garbe, 2023). This approach identifies pharmacological mechanisms and possible compounds contributing to the healing properties anecdotally mentioned by traditional healers (Arnason et al., 2022; Schultz & Garbe, 2023).

Numerous plants have been found to have medicinal properties, supporting their traditional uses and development into natural health products (NHP). The following four plants serve as examples. Feverfew (*Tanacetum parthenium* L.) was traditionally used to treat headaches, migraines, psoriasis, toothaches, and nausea (Pareek et al., 2011). Some active constituents of feverfew were parthenolide lactone, and pinenes, which have been shown to reduce inflammation (Pareek et al., 2011). Valerian is another medicinal plant typically used as a sleep aid (Zanoli & Zavatti, 2008), with valerenic acid and valepotriates, among other compounds responsible for its sedative effects (Houghton, 1999). Copal (*Protium copal*) has been used for ceremonial purposes to heal community members and has also been shown to have anxiolytic properties due to the presence of α- and ß-amyrins (Merali et al., 2018). *Echinacea angustifolia* and *E. purpurea* have been used by the North American First Nations to treat sore throat and upper-respiratory infections and promote wound healing, and both species produce secondary

metabolites with anti-inflammatory, antibiotic, and cannabimimetic activity (Barrett, 2003; R. Liu, Caram-Salas, et al., 2021).

While ethnopharmacological research has supported and validated well-established medicinal herbs such as those described above. Ethnopharmacological research can also be applied to understudied botanicals, both to gain insight into historical uses and for their development as NHPs, which area viable commercial products that enhance health from natural sources (Szczurko & Boon, 2008). Furthermore, technological advances have improved the ease and accessibility of obtaining high-quality phytochemical and bioactivity data for medicinal plants, opening new avenues to identify potential bioactive compounds and mechanisms of action via metabolomic and biochemometric analyses (Arnason et al., 2022; Atanasov et al., 2021; R. Liu, Burkett, et al., 2021; Shang et al., 2015). Metabolomics uses high-resolution mass spectrometry to identify mass features that may differ between groups and identify those mass features through additional analyses (Witte & Overy, 2022). Meanwhile, biochemometrics attempts to link chemistry to bioactivity using multiple statistical approaches, resulting in greater confidence that the concentration of a given compound relates to the bioactivity (Kellogg et al., 2016). Focusing on hops (*Humulus lupulus* L. Cannabaceae), an ethnobotanically important plant with fascinating yet poorly studied medicinal potential, this dissertation investigated the phytochemistry and bioactivity of hops, aiming to characterize active constituents using metabolomics and biochemometric analyses.

## BIOLOGY OF HOPS

Common hop (*Humulus lupulus* L.) is a dioecious perennial bine (a term referring to twining stems particular of *H. lupulus*) that grows in temperate regions along rivers or open terrain and produces unique infructescence (cones) that varies in size, shape, and chemistry (Almaguer et al.,

2014; Koetter & Biendl, 2010; Neve, 1991; Zhang et al., 2021). Hops belong to the Cannabaceae family, and the genus *Humulus* is the closest extant relative of *Cannabis* (Small, 1978). Its aggressive wolf-like growth on other plants gives rise to its species name, *lupulus*, from *Canis lupus* (Zanoli & Zavatti, 2008). Hops originated in Asia, likely China, and spread eastward to North America from Asia and moved westward from Asia into Europe (Karabin et al., 2015; McCallum et al., 2019; Olšovská et al., 2016). *Humulus lupulus* can be subdivided into five varieties: *neomexicanus* (originating in Western North America), *pubescens* (originating in Midwest North America), *lupulus* (originating in Europe and spread to North America), *cordifolius* (found in Japan), and *lupuloides* (settling into Eastern North America) (Small, 1978). Each of these varieties is well-adapted to their respective environments.

## PHYTOCHEMISTRY

The vast majority of research on hop phytochemistry has focused on the infructescences (commonly referred to as "cones") because of their use in brewing. Lupulin glands, which are located in the seedless infructescence of female plants, contain over 40% w/w of the phytochemicals found in hops (Mishra et al., 2019). These phytochemicals include bitter acids (e.g., α-acids and ß-acids), prenylated chalcone (e.g., xanthohumol), flavonoids (e.g., 6- & 8-prenylnaringenin, kaempferol, quercetin) and terpenes (e.g., myrcene and ß-caryophyllene) (Eyres et al., 2007; Karabín et al., 2016; Zanoli & Zavatti, 2008) among other secondary metabolites.

Unique to hops, the bitter acids are synthesized using three malonyl-CoA with isovaleryl-CoA, isobutyryl-CoA, 2-methylbutyryl-CoA in the presence of valerophenone synthase to create deoxyhumulone, deoxycohumulone, deoxyadhumulone, respectively (Figure 1-1). This is later converted via oxidase or prenyltransferase enzyme to form humulone and lupulone, respectively

(G. Wang et al., 2008). The branch chain amino acid backbone leucine generates humulone and lupulone, whereas valine leads to cohumulone and colupulone, and isoleucine generates adhumulone and n-adlupulone (Clark et al., 2013; Tsurumaru et al., 2012). The most abundant prenylated chalcone, xanthohumol, is synthesized from 4-coumaroyl-CoA with three malonyl-CoA molecules in the presence of chalcone synthase, followed by modulation of a prenyltransferase enzyme and O-methyl-transferase (G. Wang et al., 2008).

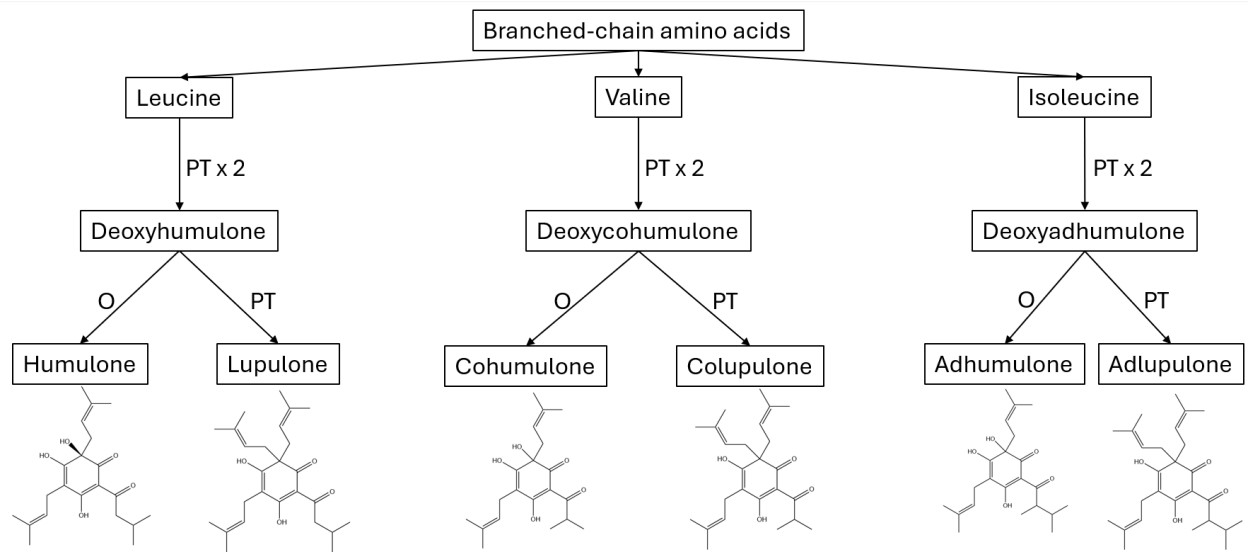


**Figure 1-1. The synthesis of hop bitter acids from the branched chain amino acids (BCAA) leucine, valine, and isoleucine, the substrates for deoxyhumulone, deoxycohumulone, and deoxyadhumulone, respectively**. Oxidase enzyme (O) forms the humulones, while prenyltransferase (PT) forms the lupulones (Clark et al., 2013; Tsurumaru et al., 2012; G. Wang et al., 2008).

Hop terpenes can be divided into three groups: hydrocarbons (e.g., mono and sesquiterpenes), oxygenated compounds (e.g., terpene alcohols), and sulphur compounds (e.g.,

thioesters, sulphides) (Karabín et al., 2016; S. Lafontaine et al., 2019). Hydrocarbons are generally not water-soluble and include monoterpenes such as α-pinene, myrcene, and limonene, along with sesquiterpenes such as α-humulene, farnesene, and caryophyllene (Olšovská et al., 2016). The oxygenated compounds have better aqueous solubility and are responsible for the taste and odour of beer, such as linalool and geraniol (Karabín et al., 2016). Lastly, sulphur compounds, such as dimethyl disulphide, make up less than 1% of aromatic compounds yet can profoundly influence the beer due to our sensitivity to these compounds (S. Lafontaine et al., 2019).

Flavonoids are found in the highest concentration within the infructescences and could reach concentrations up to 5 g/100g hop cones (Jelínek et al., 2010). Flavonoids commonly found in hops include xanthohumol, iso-xanthohumol, and 8-prenylnaringenin (8-PN) (Karabin et al., 2015). Polyphenols can be classified into flavonols, such as quercetin, kaempferol, and rutin, flavan-3-ols, such as catechin, epicatechin, oligomers, and tannins, phenolic carboxylic acids, and polyphenols such as resveratrol. The bulk weight of these polyphenols acts as anti-herbivory compounds and protects the plant from environmental impacts (Olšovská et al., 2016). Many flavonoids have a sugar attached that could impact their bioactivity (Heim et al., 2002; Karabin et al., 2015; Slika et al., 2022).

# ETHNOBOTANY

## Medicinal applications

Hops were grown around monasteries by the 8$^{th}$ Century CE (Krofta & Mikyška, 2014). However, the first written accounts for its use as a preservative and an anti-inflammatory herb date back to the 11$^{th}$ century (Karabin et al., 2015; Krofta & Mikyška, 2014). Around this time, Hildegard von Bingen (1098-1179) claimed that hops increase melancholy among men but have

preservative properties (Karabin et al., 2015; Koetter & Biendl, 2010). From the 1300s to 1500s AD, beer was used as a vehicle for various herbs. However, the German purity law of 1516 stated that only hops, barley, and water are permitted in beer (Koetter & Biendl, 2010). Around the 16$^{th}$ century, hops were used in Europe as a digestive aid and diuretic, encouraging bile production (Koetter & Biendl, 2010). Hops were also used as a mild sedative to treat sleeplessness and nervousness (Zanoli & Zavatti, 2008), and preparations made with valerian were used in Germany (Chadwick et al., 2006; Zanoli et al., 2005). Lastly, King George III (1905) recommended hop pillows and tea for sleep (Koetter & Biendl, 2010).

Beyond Europe, hops were used by Indigenous peoples of North America, Traditional Chinese Medicine, and Ayurvedic medicine to treat various conditions (Table 1-1). This includes using hops for the treatment of renal and gynecological issues; anxiety via its analgesic, sedative, and hypnotic properties; pain management in the treatment of headaches and tension, respiratory illness, digestive problems and appetite stimulant, wound healing, and antibiotic (Carbone & Gervasi, 2022; Karabin et al., 2015; Koetter & Biendl, 2010; Olšovská et al., 2016). In summary, around the world, for the last millennia, people have been using hop cones for their medicinal properties.

**Table 1-1. An overview of the medicinal uses of hops among traditional herbal uses.**

| Group | Condition | Reference |
|---|---|---|
| Cherokee Nation | Gynecological, kidney, urinary problems, analgesic, and sedative | (Hamel & Chiltoskey, 2002) |
| Delaware Nation | Earache and toothache | (Tantaquidgeon, 2001) |
| Navajo Nation | Coughs and colds | (Vestal, 1952) |
| Dakota Nation | Intestinal disorders and wound healing | (Gilmore, 1913) |
| Traditional healers | Leprosy and foot odour | (Karabin et al., 2015) |
| Traditional Chinese Medicine | Anxiety, insomnia, stimulate appetite | (Karabin et al., 2015) |
| Ayurvedic medicine | Tension, headaches, indigestion, sedative, hypnotic, and antibacterial | (Koetter & Biendl, 2010; Olšovská et al., 2016) |

## Brewing Application

Beer is one of the oldest beverages (S. Sun et al., 2022) and is made by steeping crushed malted barley and other grains in warm water and then straining this sugar-rich liquid called the wort. The wort is then brought to a boil, and hops are added at various stages during the boiling process based on the desired flavour profile and beer style (Zanoli & Zavatti, 2008). The alpha acids are isomerized during the boil to form iso-alpha acids that impart bitterness, as they are more soluble than the non-isomerized alpha acids and improve foam stability and preservation of the beer (Moir, 2000; Sanz et al., 2019; Zanoli & Zavatti, 2008). The early addition of hops also reduces the overall aromatic profile of the finished beer (González-Salitre et al., 2023; Sanz et al., 2019). Therefore, adding hops at the end of the boil or after the wort has cooled (known as dry-hopping) reduces isomerization but preserves many volatile aromatic compounds such as

terpenes and other flavour compounds that are maintained in the finished beer (González-Salitre et al., 2023; Kirkpatrick & Shellhammer, 2018; S. R. Lafontaine & Shellhammer, 2018; Sanz et al., 2019; Sharp, Qian, Y., et al., 2017). Therefore, the finished beer's style and flavour profile are impacted by when the hops are added to the boil.

Traditionally, the whole hop cones were added to the wort and then boiled. However, many commercial growers now create hop pellets by grinding and compressing the infructescence. This preserves the aroma and reduces oxidation. Pellets are categorized into two groups: T90 and T45, where the "T" stands for type, and the number corresponds to the percent quantity of whole plant material that was used to form the pellet, T90 having 90% original plant material and T45 having 45% dry plant material and supplemented with lupulin (Kowalczyk et al., 2013).

Hop cultivars are selected for particular traits, such as sensory profile of infructescence (e.g., the cultivar Cascade has fruity, citrus, and floral aromas) and disease-resistance (e.g., powdery mildew resistance) (Townsend & Henning, 2009). European hops tend to have subtle aromatic properties and are typically used as bittering hops at the beginning of the boil. Meanwhile, hops grown in Oceania and North America impart strong aromatic compounds, usually added at the end of the boil, to preserve these volatile flavour compounds (Sharp et al., 2016). The cultivars developed over 200 years ago in Europe likely originated from *Humulus lupulus var. lupulus*, while North American hops are European crossed with North American hop varieties (Neve, 1991; Small, 1978; Somalraju et al., 2024). Utilizing wild hop varieties in breeding programs could induce disease resistance and could add unique flavour profiles to new cultivars (McCallum et al., 2019; Zaidi et al., 2022).

## Applications

Today, over 90% of the hops harvested are used by the brewing industry (Kowalczyk et al., 2013; Krofta & Mikyška, 2014; Sanz et al., 2019), though the potential applications of hops beyond the brewing industry are bountiful. For pest control, hop essential oils have demonstrated toxicity for Varroa mites (*Varroa destructor*) that are detrimental to bee colonies around the globe. However, essential oils from specific cultivars such as Cascade, Spalt, and Mapuche had lower survival of bee larvae than the control, with the highest survival rate from the cultivar Victoria (Iglesias et al., 2020). Hop Guard® is a market-ready product implemented as a natural way to control Varroa mites (*Varroa destructor*) in bee colonies. Beta acids (i.e., lupulones) found in hops have been shown to elicit 93% mortality in mites with little impact on honeybee (*Apis mellifera*) mortality, making this a viable solution for controlling mite populations (Rademacher et al., 2015). The beta acids have also been proposed to be used as a preservative for meat without imparting its bitter flavour and by inhibiting *Listeria monocytogenes* and *Escherichia coli* growth in foods (Astray et al., 2020; Krofta & Mikyška, 2014; S. Sun et al., 2022; Zugravu et al., 2022). The lupulones also inhibit lactic acid-producing bacteria. Inhibition of these bacteria is helpful in sugar production and fermenting alcohol for distillation (Krofta & Mikyška, 2014; S. Sun et al., 2022). This may also explain why sour beers (fermented with a combination of lactobacillus and yeast) are less hoppy, as this would slow the fermentation process and result in a less sour beer. Hop extracts have even been added to bread to extend shelf life likely due to its polyphenol-rich content (Astray et al., 2020).

## BIOACTIVITY

The literature on the bioactivity of hops grew exponentially beginning in the 1990s and has since expanded across multiple mechanisms of action extending from its antioxidant properties

(Keskin et al., 2019; Kobus-Cisowska et al., 2019; Wu et al., 2020), anti-inflammatory (Hougee, 2008; Nicácio et al., 2022; Tripp et al., 2005; Yamamoto et al., 2000), to estrogenic effects (Aghamiri et al., 2016; Di Viesti et al., 2011; Krause et al., 2014; Milligan et al., 1999).

Hop extracts demonstrated anti-bacterial properties, specifically against gram-negative bacteria (Langezaal et al., 1992; S. Sun et al., 2022), such as the ulcer-causing bacteria *Heliobacter pylori* (Carbone & Gervasi, 2022).

The sedative and anxiolytic activity of hops could be linked to its interaction with the neurochemical gamma-aminobutyric-acid (GABA; inhibitory), whereby lupulone (i.e., beta acid) found in hops is a $GABA_A$ agonist (Zanoli et al., 2007). However, hop extracts inhibit Glutamic Acid Decarboxylase (GAD) activity that is responsible for the breakdown of glutamate into GABA, thus resulting in excess glutamate (excitatory) with less GABA being released (Awad et al., 2007). Hops may seem like a panacea, but it comes with some risks, as hops can negatively impact CYP enzymatic function, which may negatively interact with medications (Foster et al., 2009, 2011).

## DEVELOPMENT OF HOPS AS A NATURAL HEALTH PRODUCT

Hops is one of the closest relatives to cannabis (Olšovská et al., 2016) and humans have heavily selected both cannabis and hops throughout our history for diverse properties and applications. Biologically, a significant difference between cannabis and hops is that hops do not contain cannabinoids, which are the dominant class of bioactive chemicals found in cannabis and are responsible for the psychoactive effects of cannabis (Small, 2016). Instead, hops contain bitter acids and xanthohumol with promising medicinal applications (Stevens & Page, 2004; Zanoli & Zavatti, 2008). In contrast to cannabis, hops are easily accessible, affordable (25x less

expensive), and shipped around the world. Therefore, hops have fewer barriers than cannabis and could be useful as an NHP or dietary supplement.

Hop plants have been cultivated for years in the brewing industry (Kowalczyk et al., 2013; Krofta & Mikyška, 2014; Sanz et al., 2019). The industry has had annual hop harvests of over 100,000 tonnes since 2016 (BarthHaas, 2022; Macchioni et al., 2021). Hops are currently being used as a flavouring and preservative in beer (Sanz et al., 2019; Van Cleemput et al., 2009; Zanoli & Zavatti, 2008; Zhang et al., 2021), though a large proportion of the unique compounds found in beer are not water-soluble (e.g., beta-acids) and, therefore, do not end up in the finished beer (Foster et al., 2009). The necessary agricultural land and processing facilities are already present for developing hops as natural health products (NHPs). However, there is a lack of knowledge regarding the pharmacology of hops. There is also growing concern over the harms of alcohol (Nutt et al., 2010) that resulted in lower-risk use guidelines (CCSA, 2023). This could result in a surplus of hops as reduced beer consumption will result in reduced beer production. Knowledge transfer from the brewing industry to bioactivity could make for a profitable NHP. Research on the medicinal properties of hop constituents, namely the bitter acids and xanthohumol, has been gaining popularity (Carbone & Gervasi, 2022; Olšovská et al., 2016; S. Sun et al., 2022; Wu et al., 2020; Zugravu et al., 2022). Therefore, combining traditional knowledge, conventional uses, and bioactivity data positions hops to be an effective NHP.

The traditional uses of hops are supported to some degree by pre-clinical data and warrant further investigation as a candidate for an NHP (Koetter & Biendl, 2010). However, the fundamental research on hops as a medicinal plant has predominantly focused on isolated compounds found in hops instead of the whole infructescence (cone) extract (Hall et al., 2008; Minich et al., 2007; Tripp et al., 2009; Yamaguchi et al., 2009; Yamamoto et al., 2000). Whole

cone extracts were used traditionally and may be more (or less) bioactive than the individual constituents. The structural similarities of hop bioactive metabolites (Figure 1-1) could result in additive effects associated with hop extracts relative to isolated compounds as chemical combinations may interact synergistically or antagonistically with the various enzymatic receptors. The hop extracts would also contain minor metabolites that could also impact bioactivity. Furthermore, hop cultivars are easily accessible and vary greatly in their chemical diversity (Healey, 2016). Therefore, understanding hop chemistry and in relation to bioactivity is an essential first step toward selecting the hop material necessary for an NHP.

Free radicals have been known to cause oxidative stress, which can result in inflammation (Bouayed & Bohn, 2012; Slika et al., 2022; Yamaguchi et al., 2009; Zhou et al., 2018). Furthermore, endocannabinoids release arachidonic acid when they are broken down, which can be used as a substrate in an inflammatory pathway (Donvito et al., 2018; Sasso et al., 2015; Scarpelli et al., 2016; Schurman & Lichtman, 2017). Conducting tests to determine how hops interact with free radicals, inhibit inflammation, and affect the enzymes responsible for breaking down endocannabinoids can provide substantial evidence supporting hops as a natural health product.

## FREE-RADICALS AND RADICAL SCAVENGING ACTIVITY

Free radicals are molecules with unpaired electrons that are generated endogenously through metabolism and play roles in cell signalling and initiating an inflammatory cascade at the appropriate time (Bouayed & Bohn, 2012; Slika et al., 2022). However, left unchecked, free radicals can cause oxidative stress and damage cells that could result in a whole host of diseases, from DNA damage causing cancer to oxidized low-density lipoproteins contributing to atherosclerosis to neurotoxicity associated with neurodegenerative diseases (Beal, 2003;

Bouayed & Bohn, 2012; Galasko & Montine, 2010; Heim et al., 2002; Kleinrichert & Alappat, 2019; Lela et al., 2022; Slika et al., 2022; Wu et al., 2020). Two common types of free radicals are reactive oxygen species (ROS) and reactive nitrogen species (RNS). Both occur naturally in the body and are the by-product of normal cellular functioning (Bouayed & Bohn, 2012). Other free radicals could come from environmental toxins (e.g., polyaromatic hydrocarbons) or ultraviolet radiation (that radicalize compounds) and could overwhelm the body's natural systems of scavenging free radicals (Eom et al., 2013; Heim et al., 2002). Oxidative stress occurs when the endogenous free-radical scavenging system cannot process incoming free radicals, and insufficient radical scavengers are consumed in the diet (Bouayed & Bohn, 2012).

The endogenous systems to scavenge free radicals work via enzymatic and non-enzymatic processes. Enzymes involved in scavenging free radicals include superoxide dismutase, glutathione reductase, catalase, and peroxidase (Bouayed & Bohn, 2012; Zugravu et al., 2022). Non-enzymatic endogenous scavengers include glutathione and coenzyme Q10 (Bouayed & Bohn, 2012). Alternatively, diets rich in fruits and vegetables enhance the quantity of free-radical scavengers. Notable antioxidants found in plants include ascorbic acid (vitamin C), tocopherols (vitamin E), and polyphenols (Bouayed & Bohn, 2012; Heim et al., 2002; Shahidi & Ambigaipalan, 2015; Slika et al., 2022; Torres De Pinedo et al., 2007).

## Plant phenolics and their radical scavenging properties

Plants contain various antioxidants, such as phenolics, which are particularly interesting for free-radical scavenging research. One class of phenolic compounds is flavonoids which are characterized by two aromatic rings connected by three carbon (propyl) bridge, itself typically forming a third heterocyclic ring (Bouayed & Bohn, 2012; Heim et al., 2002; Karabin et al., 2015). A hydroxyl group on the phenolic could accept the free-radical electron or could be

scavenged by the double bond on the phenolic (Bors et al., 2001; Heim et al., 2002; Shahidi & Ambigaipalan, 2015; Slika et al., 2022). The phenolic hydrogen becomes radical but is replenished with ascorbic acid or glutathione (Shahidi & Ambigaipalan, 2015). More hydroxy groups on phenolics result in a higher correlation with free-radical scavenging activity (Bors et al., 2001). The alkyl length has a minor impact on antioxidant activity (Torres De Pinedo et al., 2007). Major subgroups of flavonoids include anthocyanidins, chalcones, flavones, isoflavones, flavanones, flavonols, flavanols, and non-flavonoid phenolic acids and stilbenes (Bouayed & Bohn, 2012). Plants produce these compounds for growth, reproduction, predation defence, disease resistance, and protection against ultraviolet light (Bouayed & Bohn, 2012; Heim et al., 2002; Karabin et al., 2015; Slika et al., 2022).

When consumed by animals, flavonoids act by multiple mechanisms, including free-radical scavenging, chelating metals, activating antioxidant enzymes, reducing α-tocopherol radicals, and inhibiting oxidases (Heim et al., 2002; Shahidi & Ambigaipalan, 2015). However, most flavonoids are consumed in the glycosylated form, which is generally less bioactive than aglycones (Bouayed & Bohn, 2012; Heim et al., 2002; Shahidi & Ambigaipalan, 2015; Slika et al., 2022). In the gut, enzymes and microbes (e.g., caecal bacteria) can transform these aglycones into a more bioactive form (Heim et al., 2002). Dietary antioxidants scavenge free radicals in healthy cells but may act as prooxidants in high doses or in malignant or cancerous cells (Slika et al., 2022). Excessive consumption of polyphenols can have a prooxidant effect whereby they initiate the opposite effect of antioxidants (Bors et al., 2001; Bouayed & Bohn, 2012; Carbone & Gervasi, 2022; Heim et al., 2002; Shahidi & Ambigaipalan, 2015; Slika et al., 2022). A balanced diet rich in fruits and vegetables is likely the best route for consuming polyphenols and could

lower the risk and mortality from cardiovascular disease and cancer (Bouayed & Bohn, 2012; Heim et al., 2002).

## Radical scavenging activity of hops metabolites and extracts

Flavonoids are the largest group of polyphenols and are well-known for their antioxidant activity in hops (Bartmanska et al., 2013; Karabin et al., 2015; Stevens & Page, 2004; Zhou et al., 2018). Though not polyphenols, the bitter acids are unique to hops and contain a variety of hydroxy groups and alkyl chains, with the alpha acids (i.e., humulones) containing more hydroxy groups than lupulone (Figure 1-1). The structural differences suggest that alpha acids are more potent antioxidants than beta acids. However, lupulones contain an extra double bond that could scavenge free radicals (Figure 1-1).

Researchers initially discovered that alpha and beta acids have comparable free-radical scavenging activity (RSA) to vitamins E and C (Tagasgira et al., 1995). However, some reports suggest that lupulone is stronger than humulone ($EC_{50}$ 25 vs. 35μM, respectively), while others found humulone stronger than lupulone ($EC_{50}$ 579 vs. 2315μM, respectively) (Y. Liu et al., 2007; Van Cleemput et al., 2009). Xanthohumol, a prenylated chalcone, has reported RSA stronger than humulone, lupulone, vitamin C and vitamin E (Karabin et al., 2015; Yamaguchi et al., 2009).

Beyond the bitter acids and xanthohumol, the essential oil of hops has also demonstrated antioxidant activity (Kowalska et al., 2022), highlighting the impact of extraction methods on hop chemistry and, thus, bioactivity. Medicinal plant extracts contain a plethora of phytochemicals that could act as additive, synergistic, or antagonistic effects, and quantifying how plant chemistry relates to bioactivity could optimize the efficacy of plant medicine (Arnason et al., 2022; Caesar & Cech, 2019). Comparing the hop cone's and hop leaf's antioxidant activity,

researchers discovered that the hop cone RSA outperformed hop leaves using the DPPH assay ($EC_{50}$ 0.78 and 0.99 mg/mL, respectively) (Keskin et al., 2019). Previous research demonstrated that various hop varieties and extraction protocols impact the RSA of hops (Kobus-Cisowska et al., 2019; Wu et al., 2020). For example, three varieties of hops (Magnum, Marynka and Lubelski) were made into either 40% ethanol or aqueous extracts and were tested on both the DPPH and ABTS assays (Kobus-Cisowska et al., 2019). The aqueous extracts ($IC_{50}$ = 0.31 - 0.44 mg/mL) outperformed ethanolic extracts ($IC_{50}$ = 0.56 - 0.98 mg/mL) for the DPPH assay, while the ethanol extracts ($IC_{50}$ = 0.92 - 0.99 mg/mL) outperformed the aqueous extracts ($IC_{50}$ = 1.23-1.26 mg/mL) on the ABTS free-radical scavenging assay (Kobus-Cisowska et al., 2019). In the following year, researchers tested a variety of extraction protocols on numerous bioassays (e.g., antioxidant, anti-inflammatory) to optimize extraction protocols for bioactive components in hops (Wu et al., 2020). Ethanol concentration was positively correlated to flavonoids extracted from hops. In contrast, ethanol concentration was negatively related to the quantity of total phenols content. They found that hops extracted in 95% ethanol had the strongest $EC_{50}$ (half maximal effective concentration) on the DPPH assay at 93.12 µg/mL, and aqueous extraction had the weakest $IC_{50}$ at 456 µg/mL. The authors concluded that the flavonoid concentration (40.9 mg/g) drives the antioxidant activity (Wu et al., 2020). Although hop extracts typically contain over 40 mg/g of total bitter acids and xanthohumol, the study did not evaluate if or how activity related to these known antioxidants.

## INFLAMMATION AND ANTI-INFLAMMATORY COMPOUNDS

Acute and inducible inflammation is an energetically costly adaptive response imperative for the body to heal properly after injury or infection (Straub, 2012; Straub & Schradin, 2016). However, chronic inflammation is generally associated with many diseases, including multiple

sclerosis, cancer, Parkinson's and Alzheimer's diseases (Beal, 2003; Galasko & Montine, 2010; Mesko et al., 2010).

## Inflammatory pathways

There are multiple pathways associated with inflammation. After experiencing a stressor, the body initiates an inflammatory response with positive and negative feedback loops to achieve healing and homeostasis. The immune response to inflammation results in white blood cells upregulating specific enzymes and releasing various chemical mediators (Pahwa et al., 2020). The body responds to inflammation by releasing two main categories of chemical messengers: cytokines (non-structural protein, e.g., IL-1) (Dinarello, 2000) and eicosanoids (e.g., fatty acids) (Denisenko et al., 2015). After these signalling molecules have been released, downstream cytokines respond to the inflammation. These cytokines differ in their function, with some having pro-inflammatory (e.g., TNFα, IL-1) (Scheller et al., 2011) and others having anti-inflammatory effects (e.g., IL-10) (Opal & DePalo, 2000). The acute response to inflammatory signals helps to protect the body from pathogens, heal after injury, and maintain homeostasis (Hougee, 2008; Straub & Schradin, 2016). However, chronic exposure to inflammatory cytokines may result in inflammatory diseases such as Alzheimer's, Parkinson's, cancer, and cardiovascular, along with metabolic syndrome (Gerhauser et al., 2002; Lugrin et al., 2014; Minich et al., 2007; Pahwa et al., 2020; Quiñones et al., 2013; Slika et al., 2022; Straub & Schradin, 2016; G. Y. Sun et al., 2014; Y. Sun et al., 2022; Tung et al., 2021).

Essential to the inflammatory response, several major classes of inflammatory mediators are derived from arachidonic acid. The substrate for two distinct enzymes and inflammatory pathways: (1) cyclooxygenase (COX) and (2) lipoxygenase (LOX) (Figure 1-2). Subtypes of these enzymes are activated by a stress response, such as an increase in free radicals, to initiate

upregulation of oxygenase enzymes (Bouayed & Bohn, 2012). There are two COX isoforms in humans: COX-1 and COX-2. COX-1 is expressed under basal conditions and associated with maintaining homeostatic functions (e.g., gastrointestinal and renal functions), while COX-2 is inducible and related to inflammatory pathways (Luo et al., 2005; Martel-Pelletier, 2003; Van Cleemput et al., 2009). When COX-2 catabolizes arachidonic acid, the by-products are prostacyclin, prostaglandins, and thromboxane, which lead to further inflammatory signalling (Martel-Pelletier, 2003). Though not the focus of this dissertation, the 5-LOX pathways result in various leukotrienes and may initiate other inflammatory cascades (Martel-Pelletier, 2003). COX and LOX inhibition tends to reduce inflammation. The chronic administration of COX inhibitors could lead to adverse effects such as vascular and gastrointestinal issues, renal and heart failure, psychiatric events, slowed wound healing, and glutathione depletion (Bak et al., 2003; Barker, 1977; Bhala et al., 2013; Bleumink et al., 2003; Brinks et al., 2010; Caughey et al., 2011; Fored et al., 2001; Hengge et al., 2006; Kelkar et al., 2012; Lanas et al., 2011; Poetker & Reh, 2010; Warrington & Bostwick, 2006). Fortunately, selective COX-2 inhibitors (e.g., celecoxib) have been discovered, thus mitigating the adverse effects of inhibiting COX-1 (Hall et al., 2008). Nevertheless, high doses of celecoxib inhibit COX-1, and users may have adverse events associated with non-targeted NSAIDs (Bak et al., 2003; Bhala et al., 2013; Bleumink et al., 2003; Caughey et al., 2011; Lanas et al., 2011). Therefore, it is imperative to seek new anti-inflammatory drugs with potentially fewer adverse effects.

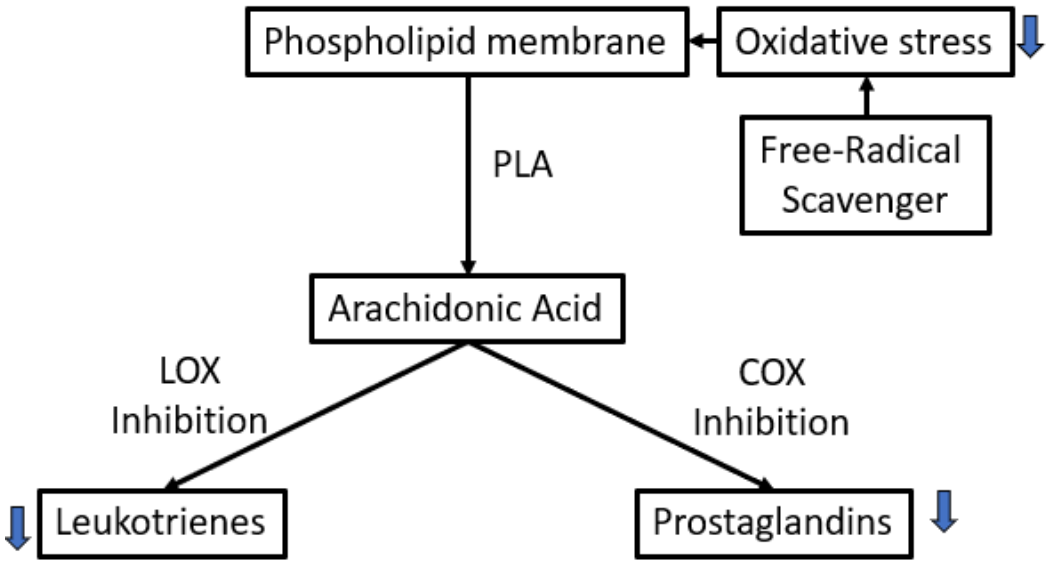


**Figure 1-2. A schematic diagram highlighting the production of pro-inflammatory mediators from the precursor. Arachidonic acid is released from the phospholipid membrane via phospholipase (PLA) after exposure to oxidative stress.** The arachidonic acid is a precursor for leukotrienes via the lipoxygenase (LOX) enzymes and prostaglandins via the cyclooxygenase (COX) enzymes. When radical scavengers are present, oxidative stress is lowered, and homeostasis is maintained. Meanwhile, when LOX or COX are inhibited, the production of leukotrienes and prostaglandins, respectively, are diminished (Herschman, 1996; M. Murakami & Kudo, 2002).

## Phytochemistry and its anti-inflammatory activity

Plants have been the source of anti-inflammatory agents, with phenolics being of particular interest (Surh et al., 2001). Notable antioxidant phytochemicals also interact with the COX-2 enzyme. Some of the notable phytochemicals with antioxidant activity also have anti-inflammatory activity in cell-based models, including curcumin, epigallocatechin gallate, and resveratrol, coming from turmeric, green tea, and grapes, respectively (Slika et al., 2022; Surh et

al., 2001; Van Cleemput et al., 2009). Furthermore, the compound Zerumbone, found in ginger, has also shown COX-2 inhibitory activity in a macrophage cell line (Kalantari et al., 2017; A. Murakami & Ohigashi, 2007). A Traditional Chinese Medicine concoction known as Huo-Lou-Xiao-Dan (HLXL) contains herbs from 11 different plants and was found to contain boswellic acid derivatives that are selective COX-1 inhibitors, while COX-2 was inhibited by senkyunolide *O* and cryptoanshione using an enzyme-based assay (Cao et al., 2010). The plant Agimantha brihat (*Premna integrifolia*), used in Ayurvedic medicine, was tested for its activity on COX-2, 5-LOX, TNF-α, IL-1ß and iNOS. Using bioassay-guided fractionation, the compound 6-hydroxy salvinolone was identified as having anti-inflammatory effects using a macrophage cell line (Azad et al., 2018). Alkaloids from *Cortex phellodendri* have also been of interest for their effects on 5-LOX and COX-2, whereby protoberbine and demethyleneberbine demonstrated strong COX-2 and 5-LOX inhibition using a benign prostatic hyperplasia rat model (S. Wang et al., 2021).

## Anti-inflammatory activity of hop metabolites and extracts

The anti-inflammatory properties of hops are supported by traditional practices (Carbone & Gervasi, 2022; Koetter & Biendl, 2010; Olšovská et al., 2016) as well as pre-clinical studies (Hall et al., 2008; Minich et al., 2007). Reviews of the ethnobotanical medicinal uses of hops (Table 1-1) highlight its ability to treat inflammation and improve immune function (Carbone & Gervasi, 2022; Neve, 1991; Zanoli & Zavatti, 2008). Moreover, hop extracts or metabolites have been reported to interfere with multiple proinflammatory pathways – including COX and LOX – and, thus, may help treat symptoms associated with inflammatory disease (Forino et al., 2016; Gerhauser et al., 2002; Hall et al., 2008; Hougee, 2008; Karabin et al., 2015; Minich et al., 2007;

Nicácio et al., 2022; Tripp et al., 2005, 2009; Trouillas et al., 2003; Wu et al., 2020; Yamaguchi et al., 2009; Yamamoto et al., 2000; Zhou et al., 2018).

Crude hop extracts also demonstrated anti-inflammatory potential. Prostaglandin E2 (PGE2) production was inhibited when peripheral blood mononuclear cells (PBMC) stimulated with lipopolysaccharide (LPS) were given a standard $CO_2$ extract of hops ($IC_{50}$ = 3.6 µg/mL) (Hougee, 2008). Using a gastric intestinal cell model, researchers determined that hop extracts inhibited COX-2 more potently than COX-1 (Tripp et al., 2005), though more is known about the role of isolated medicinal constituents from hops. They found that rho-iso-α-acid (RIAA) had an over 200-fold selectivity for COX-2 ($IC_{50}$ = 1.3 µg/mL) over COX-1 ($IC_{50}$ = 289 µg/mL). In a 14-day human trial, RIAA had no adverse effects, while naproxen increased calprotectin (a protein released by neutrophils found in fecal matter) by 200% (Hall et al., 2008).

Several medicinal constituents of hops elicit anti-inflammatory effects, including bitter acids, flavonoids, and terpenes. Xanthohumol (a flavonoid derivative) demonstrated concentration-dependent COX-2 and 5-LOX inhibition, as well as PGE2 synthase inhibition using an *in vitro* test (Forino et al., 2016; Gerhauser et al., 2002), and was uncovered as the active constituent downregulating IL-8 secretion in gastric epithelial cells (Sangiovanni et al., 2019). The bitter acids also possess anti-inflammatory activity. When RAW 264.7 mouse macrophages were stimulated with LPS and IFN-gamma (type II interferon), lupulone (ß-acids) treatment reduced levels of the inflammatory biomarker nitrous oxide compared to untreated stimulated cells (Lee et al., 2007). When LPS+IFN-stimulated RAW 264.7 cells were treated with α-acids, humulone inhibited COX-2 without affecting COX-1 (Tripp et al., 2005), and iso-α-acid inhibited COX-2 (Hall et al., 2008) and accumulation of PGE2, a byproduct of COX-2 activation (Nozawa et al., 2005). Furthermore, orally administered humulone inhibited 12-*O*-tetradecanoylphorbol-13-

acetate (TPA) induced epidermal COX-2 expression in rats (Lee et al., 2007) and inhibited COX-2 activity and gene expression in murine osteoblastic MC3t3-E1 cells (Yamamoto et al., 2000). Throughout this literature review, it has become apparent that previous research primarily focuses on single compounds found in hops without testing botanical extracts, providing little evidence of hops' anti-inflammatory potential.

## ENDOGENOUS CANNABINOID SYSTEM & PHYTOCANNABINOIDS

The endocannabinoid system (ECS) has been evolutionarily conserved across chordates and can influence central and peripheral nervous systems, metabolism, and immune function (Klein et al., 1998; Russo, 2016). The ECS consists of three main components: the cannabinoid receptors (e.g., CB-1 and CB-2), the endocannabinoids, and the enzymes responsible for endocannabinoid metabolism.

The cannabinoid receptors are G-coupled protein receptors known as cannabinoid receptor 1 (CB-1) and cannabinoid receptor 2 (CB-2) (Di Marzo, 2008). The central nervous system contains a high density of CB-1 receptors that, when activated, help with pain management, appetite, and mood (Katchan et al., 2016; Pertwee, 2006). CB-2, meanwhile, is primarily found on immune cells, modulating some of the inflammatory and immunomodulatory signalling (Katchan et al., 2016; Pertwee, 2006).

The two main endocannabinoids are N-arachidonoyl-ethanolamine (AEA) and 2-arachidonoyl-glycerol (2-AG), and both bind and activate the cannabinoid receptors to varying degrees (Di Marzo, 2008). AEA was the first endocannabinoid discovered in 1992 (Devane et al., 1992) and is more commonly known as anandamide, derived from the Sanskrit word for “bliss” and is a partial agonist to both cannabinoid receptors (Tsuboi et al., 2018). However, 2-AG levels in the brain, which act as a full agonist of CB-1 and CB-2, were an order of magnitude

greater than AEA (Schurman & Lichtman, 2017; Tsuboi et al., 2018). Numerous enzymes are known to break down endocannabinoids, but the two primary enzymes are fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL) that catabolize anandamide and 2-AG, respectively (Di Marzo, 2008; Izzo et al., 2009). Modulators of the endocannabinoid system have been the focus of much drug discovery in recent years; of particular interest to this thesis is the impact natural products may have on FAAH and MAGL activity (De Leo et al., 2018; Gertsch et al., 2010; R. Liu, Burkett, et al., 2021; R. Liu, Caram-Salas, et al., 2021).

There is growing interest in developing a drug that inhibits both FAAH and COX. For example, there were fewer gastrointestinal side effects after combining a nonsteroidal anti-inflammatory drug with an FAAH inhibitor (Scarpelli et al., 2016). Treatment with FAAH inhibitors has been shown to reduce negative symptoms associated with traumatic brain injury and lower COX-2 expression (Schurman & Lichtman, 2017). Like arachidonic acid, the endocannabinoids can be altered by COX-2-producing eicosanoids (Ueda et al., 2013). Alternatively, the endocannabinoids (2-AG and AEA) can also be catabolized (facilitated by MAGL and FAAH) into arachidonic acid (Figure 1-3) that later is converted into eicosanoid (Donvito et al., 2018; Sasso et al., 2015; Scarpelli et al., 2016; Schurman & Lichtman, 2017; Ueda & Tsuboi, 2012), though the majority of arachidonic acid in the body does not come from catabolized endocannabinoids but instead originates from the phospholipids (G. Y. Sun et al., 2014). This dissertation will focus on the role of FAAH and MAGL as modulators of inflammation without testing the synthesizing enzymes or cannabinoid receptor activity.

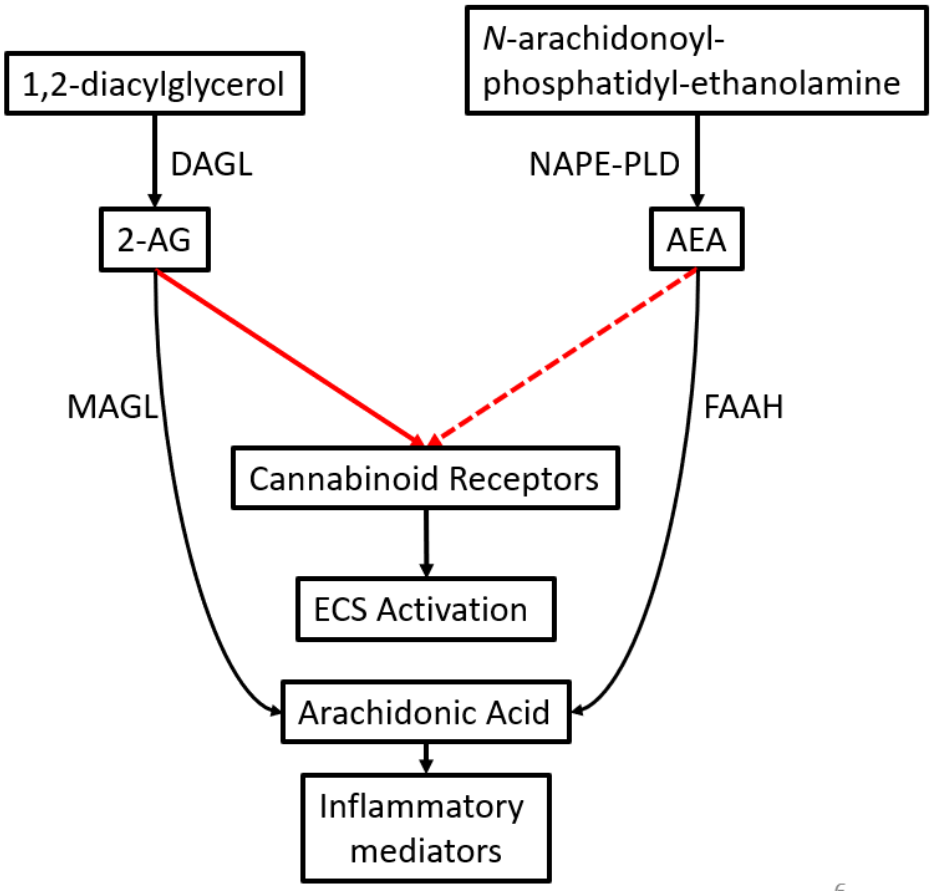


**Figure 1-3. A schematic diagram highlighting the synthesis, agonistic, and degradation of endocannabinoid 2-arachidonoylglycerol (2-AG) and arachidonoylethanolamide (anandamide (AEA)).** 1,2-diacylglycerol created 2-AG via diacylglycerol lipase (DAGL). *N*-arachidonoyl-phosphatidylethanolamine was converted into AEA via *N-acyl-phosphatidylethanolamine*. The solid red line represents full agonist, while the dashed red line represents partial agonist from AEA to the cannabinoid receptors. If AEA or 2-AG do not bind with the receptor, then they are broken down by fatty acid amide hydrolase (FAAH) or monoacylglycerol lipase (MAGL). Boxes represented compounds, black lines refer to enzymes. (Donvito et al., 2018; Sasso et al., 2015; Scarpelli et al., 2016).

## The ECS-modulating activity of plant metabolites

The search for phytochemicals beyond the cannabinoids is a growing area of research, with compounds produced by various plant taxa now known to modulate the endocannabinoid system (Appendino et al., 2022; Gertsch et al., 2010; Russo, 2016). Beta-caryophyllene, a sesquiterpene found in a multitude of plants including hops, has demonstrated affinity for the CB-2 receptors and acts as a selective CB-2 agonist (Gertsch et al., 2008). Echinacea extracts inhibit FAAH activity, partially explained by caffeic acid derivatives and alkyl amides, the latter bind to CB-2 and confer immunomodulatory effects (Chicca et al., 2009; R. Liu, Burkett, et al., 2021). The flavonoid kaempferol has also demonstrated FAAH inhibitory activity (Thors et al., 2008). Red clover contains an isoflavone, Biochanin A, a potent inhibitor of FAAH without affinity for CB1 or CB2 (Thors et al., 2010). Other isoflavone inhibitors include genistein, daidzein, and formononetin (Maccarrone, 2020). FAAH inhibitors from nutmeg, maca, and other plants have also been identified (Maccarrone, 2020).

As for MAGL inhibition, *Salvia pseudorosmarinus* contains Jewenol A, a diterpene that exhibits MAGL inhibition (De Leo et al., 2018). A desert plant, *Cistanche phelypaea*, contains a phenylethanoid glycoside that inhibits MAGL greater than other phenylethanoid glycosides discovered in the plant extracts (Beladjila et al., 2018). Previously, the triterpenoids pristimerin demonstrated MAGL inhibition (King et al., 2009). Furthermore, ß-amyrin showed potent MAGL inhibition without impacting FAAH (Chicca et al., 2012). Therefore, FAAH and MAGL inhibition is possible across multiple classes of plant compounds. This warrants further investigation into the FAAH and MAGL inhibitory activity of hops.

### The ECS-modulating activity of a hop metabolite

The use of bioassay-guided fractionation and metabolomics has helped identify specific phytochemicals that interact with bioactivity across a variety of plants, including hops (Awad et al., 2009; Nicácio et al., 2022; Sangiovanni et al., 2019; Shang et al., 2015). In addition to these approaches, *in silico* modelling and molecular docking studies screened thousands of compounds to identify potential structures of interest and test them using relevant bioassays (Kowalska et al., 2022; Tung et al., 2021). This was how the hop compound 8-prenylnaringenin (8-PN) was identified and demonstrated MAGL inhibition (Tung et al., 2021). However, no study has systematically tested hop extracts and other secondary metabolites from hops as potential sources of FAAH and MAGL inhibition. Therefore, it would be interesting to test if hops inhibit FAAH/MAGL and identify compounds in the extract that model activity using bioassay-guided fractionation and metabolomic analysis to identify other bioactive secondary metabolites.

## KNOWLEDGE GAPS

Whether targeting radical scavenging, anti-inflammatory, or ECS-modulating activity, previous research investigating the medicinal properties of hops primarily focused on isolated hop compounds rather than complex and variable plant extracts. For example, researchers tested isolated compounds such as humulone, lupulone, rho-iso-α-acid, xanthohumol, and 8-PN on COX-2 inhibition but seldom tested or compared hops extracts (Hall et al., 2008; Lee et al., 2007; Nozawa et al., 2005; Tripp et al., 2005, 2009; Tung et al., 2021; Yamamoto et al., 2000). Hence, the bioactivity of crude hop extracts remains unknown, as does the contribution of different hop metabolites to extract activity. Since people use and consume hop cones and extracts but not individual hop metabolites – whether in beer or NHPs – the bioactivity of

extracts warrants investigation to both gauge variability across different hop varieties and understand how phytochemical profiles impact (or predict) with activity of extracts.

## THESIS OBJECTIVES

Hop plants have been grown for the brewing industry, though the potential to develop hops as an NHP is ripe. The infrastructure, such as agricultural land, processing, and distribution, is already in place to establish hops as a Natural Health Product. Furthermore, over 100,000 tonnes of hops are harvested annually (BarthHaas, 2022). This would provide an alternative use of hops beyond the brewing industry, especially as alcohol harms are more widely accepted (CCSA, 2023).

With an overarching applied objective of developing a high-quality and evidence-based hop NHP and an overarching scientific objective of elucidating the active compounds contributing to hop bioactivity, my thesis combined approaches in phytochemical analysis *in vitro* pharmacology and biochemometrics to:

- Survey the phytochemical diversity of commercially available hop cultivars (Chapter 2)
- Evaluate the activity of hop extracts and marker compounds in assays of established activity (RSA, COX-2 inhibition) (Chapter 3)
- Evaluate the activity of hop extracts and marker compounds in previously unstudied assays (FAAH/MAGL inhibition) relevant to ethnobotanical uses (Chapter 4)

### Chapter 2: Chemodiversity of cultivated hops (*Humulus lupulus*)

Objective (1): Test the relationship among marker compounds (cohumulone, n-adhumulone, colupulone, n-adlupulone, xanthohumol).

Rationale: Previous reports suggest that alpha and beta acids are correlated to one another (Krofta, 2003; Likens et al., 1978; McAdam et al., 2014), and one report mentions a positive relationship between alpha acids and xanthohumol (Krofta et al., 2015; Patzak et al., 2015).

Objective (2): Statistically test if the differences in marker compounds differ between groups.

Rationale: Hops were cultivated for specific purposes, namely, for bittering or aromatic properties. This selection resulted in aromatic hops containing a lower alpha acid content than bittering hops. Similarly, North American varieties tended to have more bitter acids (Townsend & Henning, 2009), though each of the bitter acid constituents was not statistically tested. This study will statistically test the differences in marker compounds between bittering and aromatic hops along with North American and European hops to test if they significantly differed in secondary metabolites.

## Chapter 3: Identifying antioxidants and anti-inflammatory secondary metabolites in hops (*Humulus lupulus*) extracts: a biochemometric approach

Objective: Identify hop secondary metabolites that are responsible for RSA and COX-2 inhibition.

Rationale: Hop extracts have been studied for their RSA but not for COX-2 inhibitory activity. Hop constituents have been tested on both assays. In this chapter, a chemically diverse set of hop extracts was tested in a concentration-dependent manner using the free-radical scavenging and COX-2 inhibition assays. Metabolomic analysis was also conducted by grouping the three strongest (low $EC/IC_{50}$) and weakest (high $EC/IC_{50}$) hop extracts for each of the assays. Mass features that significantly differed between these two groups were identified, and modelled to bioactivity using all twenty-four hop extract data. Since xanthohumol demonstrated strong COX-2 inhibition, then it will likely be a strong inhibitor. Also, the alpha acids demonstrated

strong inhibitory activity relative to beta acids, and the COX-2 inhibition data will likely model this.

## Chapter 4: The inhibitory potential of hops (*Humulus lupulus*) on endocannabinoid degradation enzymes fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL): novel insights

Objective: Identify hop secondary metabolites and model them to FAAH and MAGL inhibition.

Rationale: Thus far, 8-PN is the only compound found in hops that has been shown to inhibit MAGL (Tung et al., 2021). Taking a similar approach to the previous chapter, hop extracts will be tested in a concentration-dependent manner, model chemistry to activity, test isolated hop compounds, and run similar metabolomic data analysis as mentioned above. The unique analyses will run an assay-guided fractionation using a hop sample with potent inhibition for FAAH. Since 8-PN has demonstrated MAGL inhibition activity and xanthohumol is structurally similar to 8-PN, it might similarly interact with MAGL inhibition. However, since the content of xanthohumol did not vary across extracts, there should be no association between xanthohumol content and FAAH/MAGL activity.

# CHAPTER 2: CHEMODIVERSITY OF CULTIVATED HOPS (*HUMULUS LUPULUS*)

## ABSTRACT

Hop (*Humulus lupulus*) is widely used in the brewing industry for its aromatic and bittering properties associated with the plant's unique phytochemistry. While the various properties and uses of different hop cultivars are believed to reflect the total and relative amounts of bitter acids and prenylated flavonoids, few scientific studies have investigated the relationship between these compounds among a variety of different brewing hop cultivars. A collection of hop samples (commercial pellets and field-grown infructescence) was extracted and analyzed using targeted and untargeted metabolomic techniques for phytochemical comparisons based on cultivar use (aroma vs. bittering) and origin (Europe vs. North America). Marker compounds were positively correlated with one another, most notably between co- and ad-humulone (alpha acids, $r = 0.90$) and co- and ad-lupulone (beta acids, $r = 0.72$), each with xanthohumol. Bittering hops had significantly more co- and ad-humulone than aroma cultivars, while North American hops had significantly more n-adhumulone and a higher alpha-to-beta acid ratio than cultivars originating from Europe. Elaborating upon previous reports, these results highlight statistically significant differences in chemistry between bittering and aroma hops and between North American and European varieties. Metabolomics analyses tentatively identified an adduct of adhumulone, 8-PN, and desmethylxanthohumol more abundant in cones than pelleted hops. These data will inform the decisions and practices of hop breeders, farmers, brewers, and consumers.

# INTRODUCTION

Hops, *Humulus lupulus* L. (Cannabaceae), is a dioecious perennial bine that produces infructescence containing unique chemicals such as the bitter acids (e.g., humulones and lupulones) and xanthohumol (a prenylated chalcone), as well as terpenes that generate the aroma of hops but are also found in numerous plant taxa (Killeen et al., 2017; Zanoli & Zavatti, 2008).

Most commonly used in the brewing industry, hop cultivars are categorized based on their use (aromatic or bittering properties). With hundreds of different cultivars bred, cultivated, and sold around the world, hops are also categorized based on origin (the region where it was bred) or the form of commercial product (e.g., whole cone, pellet, or lupulin-enriched product). Surprisingly, there is scant literature on phytochemical variation and relationships among hop varieties, with limited empirical evidence of chemical differences associated with their use, origin, or form.

Lupulin glands, which cover the inner infructescence, contain the highest density of bitter acids and xanthohumol (Carbone & Gervasi, 2022; Mishra et al., 2019; Sanz et al., 2019). The yellow resinous substance contains soft and hard resin that comprises up to 15-30% of the dry weight of the cones (Almaguer et al., 2014; Carbone & Gervasi, 2022). Soft resins include bitter acids such as humulones (alpha-acids) and lupulones (beta-acids) and are soluble in hexane. Meanwhile, hard resins include xanthohumol and hulupulones that are insoluble in hexane (Almaguer et al., 2014; Astray et al., 2020; Olšovská et al., 2016). While the biosynthesis of bitter acids uses branched-chain amino acids (leucine, valine, and isoleucine) as building blocks (Clark et al., 2013; Tsurumaru et al., 2012), xanthohumol is synthesized via chalcone synthase and prenyltransferase enzymes using 4-coumaroyl-CoA and malonyl-CoA substrate (G. Wang et al., 2008).

Hops are typically processed to preserve and accentuate their unique chemical properties (Srečec et al., 2010). The two leading commercial forms are cones or pellets (Rutnik et al., 2023). Cones are bulky, prone to oxidation (larger surface area relative to pellets), costly to transport, and challenging to measure accurately in beer production. In contrast, pelletizing hops improves freshness and mitigates oxidation by grinding and compressing the cones into pellets (Chadwick et al., 2006; Van Cleemput et al., 2009). The pellets are further preserved with a nitrogen flush, opaque packaging, and cold storage. The most common type of pellet, T90, contains 90% original plant material retained, and enriched with lupulin glands (Kowalczyk et al., 2013; Srečec et al., 2010). As a result, the pellets should contain higher levels of desirable secondary metabolites (i.e., the bitter acids) and less fibrous material relative to hop cones.

Since hop cultivars have been predominantly bred and utilized by the brewing industry, they are typically selected and developed for their unique sensory properties, yield, disease resistance, and antimicrobial activity (Chadwick et al., 2006; Neve, 1991). Accordingly, established hop cultivars are grouped based on their sensory attributes and organoleptic contribution to beer (bitterness and aroma), which are indirectly linked to the hopping time (i.e., when added to the unfinished beer). The bitterness component relates to α-acid content, while the aroma is related to both ß-acid and volatile oil content. Therefore, bitter hops tend to have more alpha acids and are added to the beginning of the boil (to enhance bitterness). In contrast, aroma hops tend to have fewer alpha-acids, with relatively more beta-acids and terpenes and are added at the end of the boil or during fermentation (González-Salitre et al., 2023; Killeen et al., 2014). Although widely accepted in the brewing industry, the chemical differences between aroma and bitter hops have rarely been tested scientifically (Krofta, 2003; McAdam et al., 2014).

Genetics and environment play an integral role in the development of phytochemical diversity for many crops, including hops. Thus, local conditions (e.g., weather) at the location (e.g., length of photoperiod) of cultivation impact their chemistry and sensory attributes (Féchir et al., 2023; Hagemann et al., 2024; Matsui et al., 2016; Ocvirk et al., 2019). Taxonomically, European varieties are classified as the *lupulus* variety (*Humulus lupulus* var. *lupulus*), while North American varieties include *lupuloides, pubescen*, and *neomexicanus* (Small, 1978). In addition to distinct genetic differences between European and North American varieties, the reported levels of bitter acids are, on average, greater among North American varieties than European varieties (Townsend & Henning, 2009). However, many European varieties have been crossbred with North American varieties – and *vice versa* – to prevent disease and achieve the desired flavour and aroma characteristics (Neve, 1991; Townsend & Henning, 2009). Cultivated varieties (i.e., cultivars) differ from the geographical taxonomic varieties mentioned above.

Beyond phytochemical variability rooted in genetic differences, the same hop cultivar may have different chemistry based on environment, especially growing location (Féchir et al., 2023) and harvest time (S. Lafontaine et al., 2019; Matsui et al., 2016). New technology using energy-dispersive x-ray fluorescence spectrometry, coupled with discriminant analysis, has been validated as a means to identify the geographic location of hops cultivation based on the sample's unique elemental fingerprint (Ocvirk et al., 2019). The Shellhammer Lab from Oregon State University found terroir differences between and within states (Washington and Oregon) for two popular cultivars (Cascade and Mosaic) grown in thirty-nine locations. Moreover, these chemical differences resulted in sensory differences in the finished beer (Féchir et al., 2023). The researchers found few differences in the total content of marker compounds between growing

locations. However, multifactorial analysis of 24 quantified terpenes uncovered clusters related to cultivars and locations (Féchir et al., 2023).

Metabolomics is a powerful tool that is starting to provide new insights into hop science. Metabolomics allows researchers to scan samples containing hundreds of compounds – known or unknown – and compare their results with those of other researchers worldwide. Furthermore, as instruments become more accessible, the vast amount of data from metabolomic datasets could enhance the identification of compounds that would have otherwise been overlooked. With advances in high-resolution mass spectrometry (HRMS) and metabolomics (Witte & Overy, 2022), phytochemical studies of hops have expanded from targeted profiling of major compounds to untargeted analyses that capture hundreds of mass features that can be compared to identify cultivars (Ikhalaynen et al., 2022), or metabolites (Prencipe et al., 2014; Salviati et al., 2021), including bioactive compounds (Brown et al., 2022; Chiancone et al., 2023; Maietti et al., 2017; Nicácio et al., 2022; Olšovská et al., 2016; Önder et al., 2013; Tanaka et al., 2014). To date, researchers have applied metabolomic approaches to study the chemical variability among hop samples regarding terpene profiles (Sharp et al. 2017), cultivar differences (Farag et al., 2012), and pest and disease resistance (Morcol et al., 2020).

The objective of this study was to investigate phytochemical correlations among marker compounds (cohumulone, n-adhumulone, colupulone, n-adlupulone, and xanthohumol) across a diverse collection of hop cultivars and to compare marker profiles based on (1) industry use (aroma vs. bittering), (2) origin (European vs. North American), and (3) form (cones vs. pellet). In addition to targeted profiling of marker compounds, metabolomic analyses were utilized to identify mass features that differ between cultivar groups (use, origin, form). Results from this

experiment provided a metabolic roadmap to the chemical profile of hops that brewers could use based on the content of marker compounds.

# METHODS

## Plant material

Hops samples (n = 32) were collected from various sources in 2019. Most hop pellets (n = 25) were obtained from www.ontariobeerkegs.com, which primarily sourced their hops from the Yakima Chief Hop Company (Yakima, Washington, USA) (n = 21) and LD Carlson Company (Kent, Ohio, USA) (n = 4). An additional pellet sample was provided by Kichesippi Brewery (Ottawa, Ontario, Canada) (n = 1). Cone samples were obtained from CWG Farm Inc. (St-Eugène, Ontario, Canada) (n = 1), Highland Farm (Meaford, Ontario, Canada) (n = 3), and Carleton University (Ottawa, Ontario, Canada) (n=2) (Table 2-1). All pellet samples were T90. All hop samples were kept in a freezer at -20 °C until extracted.

**Table 2-1 List of hop samples collected (n = 32)**, categorized as Cultivar, Source (CU: Carleton University, CWG: CWG hop farm, LD: LD Carlson Company, Highland: Highland hop farm, Kichesippi: provided by Kichesippi brewery, YCH: Yakima Chief Hops), Uses (Aroma or Bitter), Origin (EU: Europe, NA: North America), Form (cone or Pellet). Magnum (U): is grown in the United States of America, while Magnum (G): is grown in Germany. n/a: not available.

| Cultivar | Source | Uses | Origins | Form |
|---|---|---|---|---|
| Cashmere | Kichesippi | n/a | NA | Pellet |
| Citra | YCH | Aroma | NA | Pellet |
| Cluster | YCH | n/a | NA | Pellet |
| Columbus | YCH | Bitter | NA | Pellet |
| German Northern Brewer | YCH | n/a | EU | Pellet |
| German Spalt | YCH | Aroma | EU | Pellet |
| German Tettnanger | YCH | Aroma | EU | Pellet |
| Hallertau | YCH | Aroma | NA | Pellet |
| Hallertau Blanc | LD | Aroma | EU | Pellet |
| Hersbrucker | YCH | Aroma | EU | Pellet |
| Horizon | YCH | n/a | NA | Pellet |
| Huell Melon | LD | Aroma | EU | Pellet |
| Lemondrop | LD | Aroma | NA | Pellet |
| Magnum (G) | YCH | Bitter | EU | Pellet |
| Magnum (U) | YCH | Bitter | EU | Pellet |
| Mt Hood | YCH | Aroma | NA | Pellet |
| Nugget | YCH | n/a | NA | Pellet |
| Perle | YCH | Aroma | NA | Pellet |
| Saaz | YCH | Aroma | EU | Pellet |
| Simcoe | LD | n/a | NA | Pellet |
| Sorachi Ace | YCH | Aroma | NA | Pellet |
| Styrian Golding | YCH | Aroma | EU | Pellet |
| Summit | YCH | Bitter | NA | Pellet |
| UK East Kent Golding | YCH | Aroma | EU | Pellet |
| UK Fuggle | YCH | Aroma | EU | Pellet |
| Willamette | YCH | Aroma | NA | Pellet |
| Cascade | Highland | Aroma | NA | Cone |
| Centennial | Highland | Aroma | NA | Cone |
| Chinook | Highland | Bitter | NA | Cone |
| Hallertau Tradition | CU | Aroma | EU | Cone |
| Newport | CWG | Bitter | EU | Cone |
| Tettnanger | CU | Aroma | EU | Cone |

## Chemicals and reagents

The ICE-4 hop standard mixture containing the four major bitter acids, cohumulone, n-adhumulone, colupulone, and n-adlupulone (Saint Paul, Minnesota, USA) was purchased from the American Society of Brewing Chemists. Xanthohumol and iso-xanthohumol standards were purchased from Chromadex (Longmont, Colorado, USA). The HPLC columns were purchased from Phenomenex (Torrance, California, USA), while the 0.22µm filter tips and HPLC vials were purchased from Chromatographic Specialties Inc. (Brockville, Ontario, Canada). Mili-Q (MQ) water was purified in-house (Mobile Phase A). All other reagents were purchased from Fisher Scientific unless otherwise stated.

## Sample preparation

Hops pellets were crushed into a powder by hand, while hops cones were pulverized using a Magic Bullet Blender®. The hop extracts were made by adding 120 mL 99% ethanol to 6.00g crushed hop pellets or pulverized cones and sonicating samples in a water sonication bath for 10 minutes at 23°C. The ethanol was filtered using Whatman Grade A Filter paper under vacuum and transferred to a round-bottom flask. An additional 120 mL of 99% ethanol was added to the hop material, sonicated again for 10 minutes, and then filtered. The first and second extractions were combined and concentrated to ~20 mL by rotary evaporator (Yamato RE 500, Yamato Scientific, Japan) with a 40°C water bath and - 4°C condenser. The concentrated extract was placed into a weighed 50-mL Falcon Tube and was dried using a Centrivap Centrifugal Vacuum Concentrator (Labconco Centrivap, Model 7810014, Kansas City, Missouri, USA). Additional moisture from the extract was removed by lyophilization (SuperModulyo220 freeze dryer; Thermo Fisher Scientific, Nepean, ON, Canada).

Dried extracts were reconstituted to 10 mg/mL in 99% ethanol, filtered using a 0.22 µm polytetrafluoroethylene (PTFE) syringe filter, and stored in the -20°C freezer until targeted phytochemical analysis. HPLC-UV-DAD (High-Performance Liquid Chromatography with Diode Array Detection) analysis.

## Targeted profiling of marker compounds

### Bitter acids and Xanthohumol quantification

HPLC-UV-DAD was used to quantify the bitter acids and xanthohumol found in 2µL of hop extracts. A Kinetex C18 5U (100x4.6mm) column (Phenomenex, Torrance, California, USA) was used to quantify the compounds of interest. The flow rate was 1.5 mL/min, the stop time was after 17.1 minutes, and the post-run time was 3 minutes at a temperature of 40°C. Mobile phase A was MQ water, and mobile phase B was methanol. Both mobile phases have 0.1% trifluoroacetic acid (TFA). The gradient elution was as follows: the initial condition was 52.5% mobile phase B for one minute, changing to 90% at 13-min, by 13.1min 100% mobile phase B, and with a 4-minute wash with 100% mobile phase B and re-equilibrated for 3 minutes. The calibration curve ranged from 12.5-250 µg/mL for xanthohumol, 13.75-274.5 µg/mL for cohumulone, 39.5-790 µg/mL for n-adhumulone, 16.27-325.5 µg/mL for colupulone, 16.9-338 µg/mL for n-adlupulone. Quantification occurred at 300nm.

To obtain reference standards to build calibration curves, the ICE-4 standard was reconstituted to 100 mg/mL in dimethyl sulfoxide (DMSO), filtered through a 0.22µm PTFE syringe filter, and then repeatedly separated using a Phenomenex Luna Omega 5µM C18 100 (250 x 10 mm) column (Phenomenex, Torrance, California, USA) and HPLC-UV-DAD system (Agilent 1100). Peak-based fraction collection (Agilent 1200 G1364C Fraction Collector) isolated the four major peaks (cohumulone, n-ad-humulone, colupulone, n-adlupulone) using

isocratic elution with 90% mobile phase B (methanol + 0.1% TFA) for 10-min at 50°C with UV monitoring and fraction collection set at 330 nm. Once the run was completed, these aliquots were placed into a weighed 50-mL Falcon Tube. They were dried using the Centrivap Centrifugal Vacuum Concentrator (Labconco Centrivap, Model 7810014, Kansas City, Missouri, USA) followed by lyophilization (SuperModulyo220 freeze dryer, Thermo Fisher Scientific, Nepean, Ontario, Canada) to remove residual water. The ICE-4 standard and four isolated compounds were stored in a -20°C freezer until ready to use. To test for stability, each of these four compounds was tested on the HPLC using the hop extract method described above eight months after isolation and all were found to be stable.

## Untargeted metabolomics

Sample preparation for Ultra High-Resolution Mass Spectrometry

Dried hop extracts were reconstituted to 500 µg/mL using MS-grade methanol for metabolomic analysis. Analytical standards (ICE-4 and xanthohumol) were diluted to 100 µg/mL. Methanol blanks were injected every eight samples to monitor for sample carryover. Reserpine was injected with every set to evaluate mass accuracy and had an error of 12 ppm.

High-Resolution Mass Spectrometry Data Acquisition

Thermo Scientific Dionex Ultimate 3000 UPLC system coupled to a Thermo Scientific LTQ Orbitrap XL high-resolution mass spectrometer, with Phenomenex Kinetex C18 column (100Å, 2.1x50mm, 1.7µm) and C18 guard cartridge. Chromatography had an oven temperature set at 30°C and a flow rate of 0.35 mL/min. The Mobile phase (A) was LC-MS grade water (+0.1% formic acid), and the mobile phase (B) was acetonitrile (0.1% formic acid). The gradient began with 5% B and increased to 95% B in 4.5 minutes, held at 95% B for 3.5 minutes, returned to 5% B over 0.5 minutes and equilibrated for 6 minutes. The HRMS Orbitrap XL was operated in ESI+

mode monitoring *m/z* 50-2000, resolution = 30000, and an MS runtime of 10 minutes. Samples were injected at 5µL and run in triplicate with methanol as a blank and reserpine as an internal standard. These data files were received as .RAW files for analysis.

Metabolomic Analysis

ProteoWizard version 3 converted RAW files into mzML files. MzMINE version 2.53 was used for the analysis of the mzML files. The mass detection noise level was set at 1.0 E + 4. The automated data analysis pipeline (ADAP) set the following parameters: Minimum group size was 5, group intensity threshold was 1.0 E + 5, the minimum highest intensity was 1.0 E + 6, mass-to-charge (m/z) was 0.005 m/z or 5 ppm. Chromatogram deconvolution step parameters: algorithm was local minimum search, chromatographic threshold of 20, a search minimum in RT range (min at 0.05), minimum relative height was 15%, minimum absolute height was 1.0 E + 6, minimum ratio of peak top/edge was 1.2, peak duration range from 0.00-10.00 min, and the m/z center calculation was median. The isotopic grouper had m/z tolerance set at 5.0 ppm, retention time tolerance was 0.1 absolute (min), and maximum charge was set at 1.

The join aligner function joined peaks with m/z tolerance was 0.005m/z or 5ppm, m/z weight was twenty, retention time (RT) tolerance was 0.1 (absolute min), and weight for RT was ten. In the gap-filling process, the intensity threshold was 5.0%, m/z tolerance was 0.001m/z or 5.0ppm, and the RT tolerance was 0.05 (absolute min). These data were normalized to the total raw signal, and the peak measurement type was the peak area. The file was exported as a .CSV file with a field separator set to comma; export elements include export row ID, m/z, retention time, row identity (all IDs), and data file elements include peak area.

Data curation

The conditional formatting was set at 1.0 E + 5, and mass features abundant in methanol blanks were removed. There was a total of 642 mass features in the completed dataset. This master dataset was then used for the metabolomic analyses for each of the classifications (e.g., industry uses (aroma or bittering), origins (Europe or North America), or form (cone or pellet)).

The resulting metabolomics database was analyzed using MetaboAnalyst 5.0. Statistical Analysis [one factor] was selected, the CSV file was uploaded, and peak intensities were selected under the data upload tab. Interquartile range (IQR) removed outliers; data were normalized by sum, and Pareto scaled data. The volcano plot highlighted mass features that differ between groups, and these significant mass features will be presented. The false discovery rate adjusted p-value was set at 0.05 for the volcano plots.

## Statistical analysis

All statistics were conducted in R-studio (R Core Team, 2023) using version 4.3.1; graphs were created in ggplot2. The Pearson correlation table for hop marker compounds (i.e., cohumulone, n-adhumulone, colupulone, n-adlupulone, and xanthohumol) found in the extracts was presented first, followed by descriptive statistics for each group. Due to the differences in sample size among groups, nonparametric tests (Wilcoxon rank sum exact test or Kruskal Wallis rank sum test with pairwise Wilcoxon rank sum exact test) were tested for significant differences of marker compounds between groups. Finally, the groups were analyzed using the metabolomic datasets presenting how the mass features differ between groups using Partial Least Squares Discriminant Analysis (PLS-DA) and volcano plots, along with highlighting and identifying mass features that differ between groups using MetaboAnalyst (https://www.metaboanalyst.ca/). The error between the monoisotopic mass of a compound relative to the neutral mass feature was identified using an online tool

(https://warwick.ac.uk/fac/sci/chemistry/research/barrow/barrowgroup/calculators/mass_errors/). Reported mass features and monoisotopic masses were also compared to mass features identified from the volcano plot. These techniques permitted tentative identification of mass features.

# RESULTS

## Targeted profiling of marker compounds

Hop samples (n = 32) were homogenized, extracted in 99% ethanol, and then analyzed by HPLC-UV-DAD to quantify major marker compounds (cohumulone, n-adhumulone, colupulone, n-adlupulone, and xanthohumol). All markers were identified in all samples but varied in relative quantities. For all extracts, the marker compound accounted for >90% of the cumulative area under the curve (AUC) of all detected peaks, with a few minor peaks (>25 mAu·s) inconsistently observed among extracts (Figure 2-1). Across samples, cohumulone content varied 70-fold with an average of 5.77 ±0.64 mg/g, n-adhumulone averaged 16.16 ±1.80 mg/g and varied 30-fold, colupulone content averaged 7.32 ±0.54 mg/g and varied 5-fold, and n-adlupulone average of 7.46 (±0.52) and varied 7-fold. Xanthohumol content was lower than bitter acids, averaging 1.75 ±0.14 mg/g and varying 6-fold across samples. Finally, the alpha-to-beta ratio (ABR) ranged between 0.12-3.30, with an average of 1.56 (±0.18) varying 27-fold (Table 2-2).

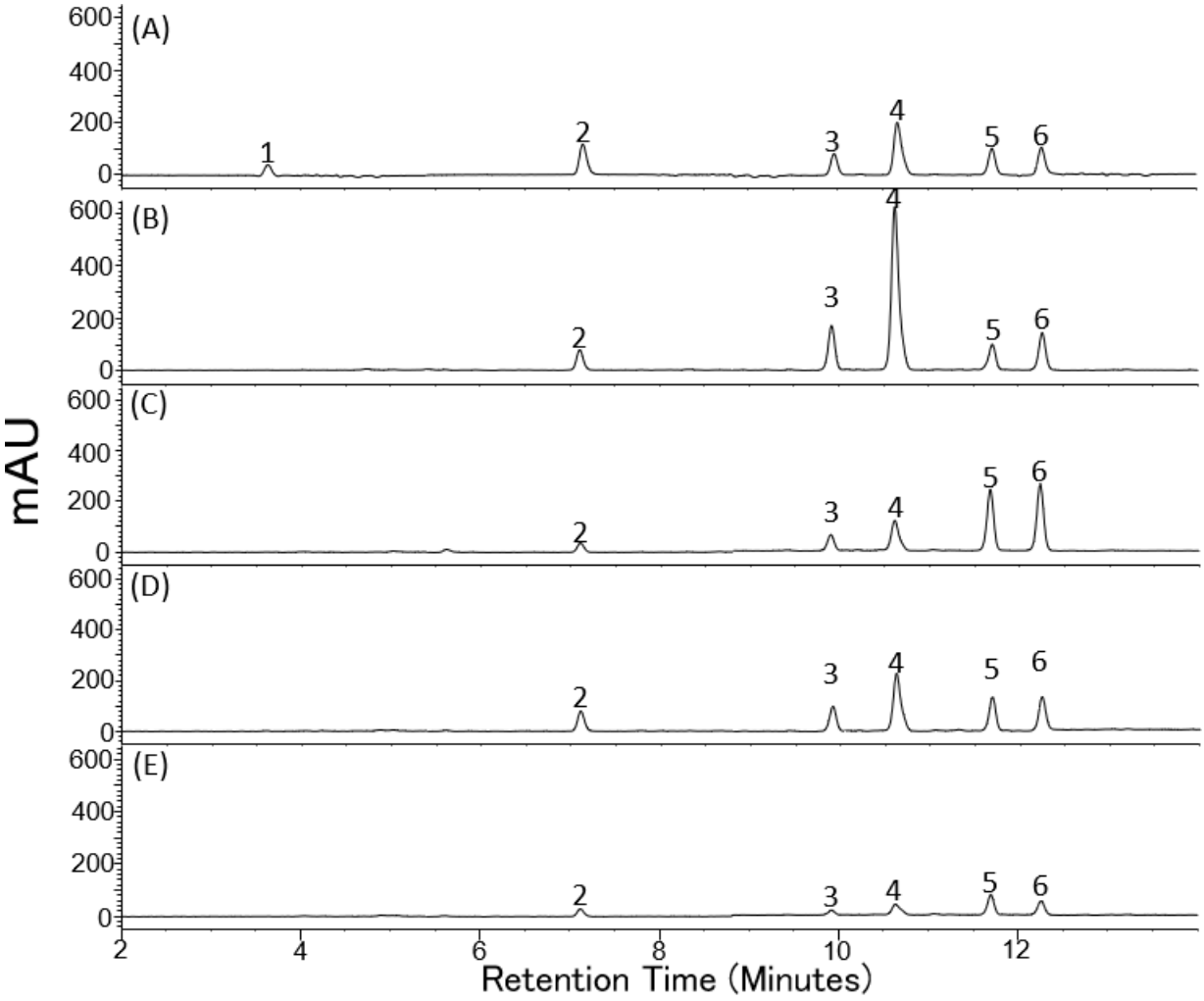


**Figure 2-1. HPLC-DAD chromatogram of hop standard (A) and representative *Humulus lupulus* L. extracts (B-E).** All compounds were monitored at the wavelength of 300nm between the retention time of 2 minutes to 14 minutes. 1: iso-xanthohumol (not detected in hop extracts), 2: xanthohumol, 3: cohumulone, 4: n-adhumulone, 5: colupulone, and 6: n-adlupulone.

**Table 2- 2. Quantified marker compounds (mg/g plant material) (cohumulone, n-adhumulone, colupulone, n-adlupulone, and xanthohumol) and the alpha-beta acid ratio (ABR) of all hop samples (n = 32).** Mean was the arithmetic mean, SEM was the standard error of the mean, Min (minimum) refers to the lowest observed concentration, and Max (maximum) refers to the highest observed concentration.

| | **Mean** | **SEM** | **Min** | **Max** |
|---|---|---|---|---|
| Cohumulone | 5.8 | 0.6 | 0.2 | 14.8 |
| N-adhumulone | 16.2 | 1.8 | 1.2 | 37.9 |
| Colupulone | 7.3 | 0.5 | 2.9 | 14.3 |
| N-adlupulone | 7.5 | 0.5 | 2.1 | 14.8 |
| Xanthohumol | 1.8 | 0.1 | 0.6 | 3.7 |
| ABR | 1.6 | 0.2 | 0.1 | 3.3 |

A strong positive correlation was observed between the alpha acids cohumulone and n-adhumulone ($R = 0.90$, $p < 0.001$, Table 2-3) and between the beta acids colupulone and n-adlupulone ($R = 0.67$, $p < 0.001$) (Table 2-3). Cohumulone and colupulone levels were positively correlated ($R = 0.45$. $p < 0.01$), but no other correlations were detected between bitter acids. Xanthohumol content, however, correlated positively with all of the bitter acids, most robustly with cohumulone ($R = 0.72$, $p < 0.001$) and n-adhumulone ($R = 0.67$, $p < 0.001$), and weakly with colupulone ($R = 0.54$, $p < 0.01$) and n-adlupulone ($R = 0.40$, $p < 0.05$) (Table 2-3).

**Table 2-3. Pearson R coefficient matrix among the five marker compounds (cohumulone, n-adhumulone, colupulone, n-adlupulone, and xanthohumol) quantified using HPLC-DAD of hop extracts *Humulus lupulus* L. (n = 32).** * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

| | Cohumulone | N-adhumulone | Colupulone | N-adlupulone | Xanthohumol |
|---|---|---|---|---|---|
| Cohumulone | 1.00 | | | | |
| N-adhumulone | 0.90*** | 1.00 | | | |
| Colupulone | 0.45** | 0.23 | 1.00 | | |
| N-adlupulone | 0.22 | 0.24 | 0.72*** | 1.00 | |
| Xanthohumol | 0.72*** | 0.67*** | 0.54** | 0.40* | 1.00 |

## Uses – aroma and bitter hop cultivars

Targeted profiling of marker compounds revealed a significant difference in the content of alpha acids (cohumulone and n-adhumulone) between aroma and bittering hop. The bittering hops, on average, had 2.6-fold more cohumulone, 2.3-fold more n-adhumulone, 2-fold more xanthohumol, and 2-fold greater alpha-to-beta ratio than the aroma hop group. Colupulone and n-adlupulone content were not significantly different between aroma and bittering hops (Table 2-4).

**Table 2-4. Quantified marker compounds (mg/g dry plant weight) and the alpha-beta acid ratio (ABR) in aroma (n=20) and bitter (n=6) hop samples.** Non-parametric pairwise Wilcoxon (*W*) test statistic was used to test for significant differences in compound content between the two groups. * $p < 0.05$, ** $p < 0.01$, * ***$p < 0.001$.

| | **Aroma** | **Bitter** | ***W*** | ***p*** |
|---|---|---|---|---|
| Cohumulone | 4.0 (0.6) | 10.6 (1.2) | 7 | <0.001*** |
| N-adhumulone | 11.4 (1.8) | 26.3 (3.4) | 11 | <0.01** |
| Colupulone | 6.9 (0.7) | 9.2 (1.1) | 29 | 0.06 |
| N-adlupulone | 7.3 (0.7) | 8.4 (1.1) | 52 | 0.66 |
| Xanthohumol | 1.5 (0.1) | 2.1 (0.3) | 23 | 0.02* |
| ABR | 1.1 (0.2) | 2.3 (0.4) | 21 | 0.02* |

To investigate whether the observed difference in cohumulone and n-adhumulone is accompanied by additional unknown chemical correlates, aroma and bittering hop samples were compared based on untargeted metabolomic data consisting of 642 mass features annotated by RT and [m/z]. The unsupervised PCA revealed no clustering of bitter and aroma groups (graph not shown). The PLS-DA resulted in more unique clusters but similarly explained 28.7% of the variance (Figure 2-2A). A volcano plot was used to identify mass features that significantly differed between groups (Figure 2-2B). This analysis identified 20 unique mass features that significantly differed between aroma and bittering hops, with only two mass features being significantly greater in aromatic hops (Figure 2-2B), although none were tentatively identified from the available literature.

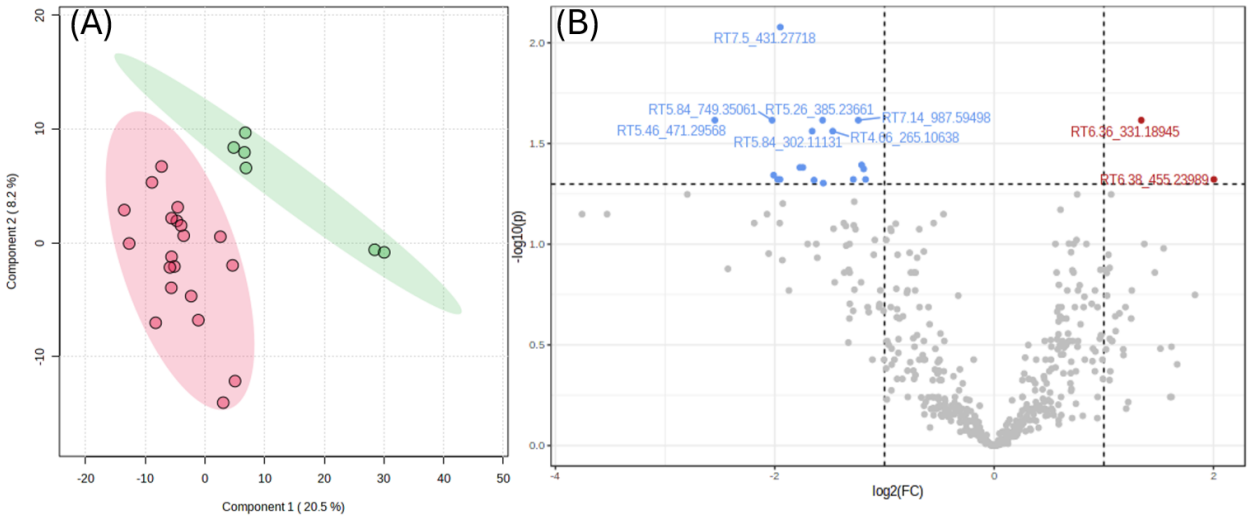


**Figure 2-2. Hop (*Humulus lupulus* L.) extracts (n = 26) comparing uses (aromatic or bittering) in a metabolomic dataset (containing 642 mass features).** Mass features are labelled as their retention time (RT) and mass-to-charge ratio [m/z]+. (A) Partial Least Squares Discriminant Analysis scores plot for hop extracts usage, the cumulative variance between PC1 and PC2 is 28.7%. Aroma (red, n = 20) and Bitter (green, n = 6). (B) The volcano plot depicts mass features that significantly differ from one another. Blue dots indicate those mass features that were significantly greater in the bittering hops, while red dots indicate mass features that were significantly greater in aroma hops.

## Origins – hop cultivars developed in Europe and North America

Quantitative profiling revealed two marker compounds that significantly differed between European and North American hop pellets (Table 2-5). North American hops had 1.5 times more cohumulone, 1.8 times more n-adhumulone, and twice the ABR as found in European hops (Table 2-5). Other marker compounds did not significantly differ between European and North American hop groups (Table 2-5).

**Table 2-5. Mean (SEM) quantified marker compounds (mg/g dry plant weight) and the alpha-beta acid ratio (ABR) in European (n = 15) and North American (n = 17) hop samples.** Non-parametric pairwise Wilcoxon (*W*) test statistic was used to test for significant differences in compounds between the two groups. * $p < 0.05$, ** $p < 0.01$, * ***$p < 0.001$.

| | **Europe** | **North America** | ***W*** | ***p*** |
|---|---|---|---|---|
| Cohumulone | 4.4 (0.8) | 7.0 (0.9) | 75 | 0.05* |
| N-adhumulone | 11.4 (2.1) | 20.4 (2.4) | 57 | <0.01** |
| Colupulone | 7.5 (0.9) | 7.2 (0.7) | 130 | 0.94 |
| N-adlupulone | 8.0 (0.9) | 7.0 (0.6) | 153 | 0.35 |
| Xanthohumol | 1.6 (0.2) | 1.9 (0.2) | 89 | 0.15 |
| ABR | 1.00 (0.1) | 2.1 (0.3) | 49 | <0.01** |

Metabolomic analysis was conducted to identify differences between European and North American hop samples (Figure 2-3). The PLS-DA demonstrated unique clusters; however, it only explained 28.8% of the variance (Figure 2-3A). A volcano plot was used to identify mass features that significantly differed between groups, whereby seven of the mass features were significantly greater in North America relative to the European hop samples (Figure 2-3B).

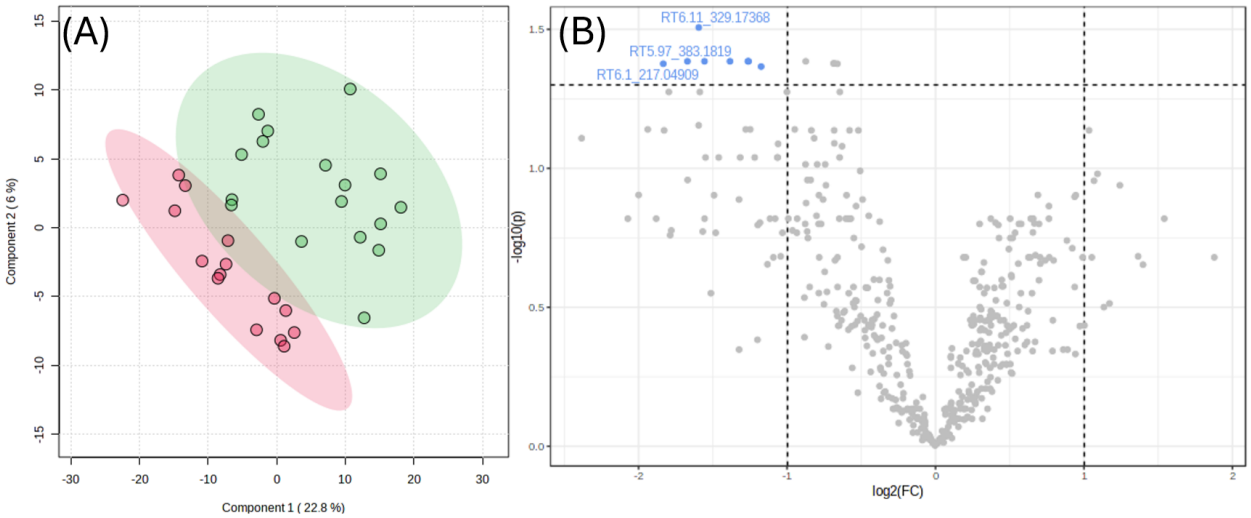


**Figure 2-3. Hop (*Humulus lupulus*) extracts (n = 32) comparing origins (European or North American) using a metabolomic dataset (containing 642 mass features).** Mass features are labelled as their retention time (RT) and mass-to-charge ratio [m/z]+. (A) In the partial least squares discriminant analysis, principal component 1 explains 22.8% of the variance, while principal component 2 explains 6% for a cumulative variance of 28.8%. Europe (red, n = 15) and North America (green, n = 17). (B) The volcano plot depicts mass features that were significantly greater in the North American hop samples (blue dots).

One of the mass features (neutral mass 216.05909) was significantly greater in the North American hop samples and, based on repoted metabolites of hops, was most closely related to 1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid (monoisotopic mass = 216.089874 g/mol, with an error of 142 ppm), which was tentatively identified from Tanaka and colleagues (2014), although further analysis is necessary to confirm their identity.

## Uses x Origin – Combining the uses variable and origin

Targeted profiling of marker compounds revealed a significant difference in the content of alpha acids (cohumulone and n-adhumulone) based on industry use and origin. Cohumulone was over 4-fold greater in the bitter North American group and almost 3-fold greater in the bitter European group than the aroma European group. Furthermore, the bitter North American group had 2.4 times more cohumulone than the aroma North American group. N-adhumulone content was four-fold greater in the bitter North American group relative to the aroma European hop groups. ABR was almost four-fold greater in the bitter North American group than the aroma European group (Table 2-6).

**Table 2-6. Quantified marker compounds (mg/g dry plant weight) and the alpha-beta acid ratio (ABR) in aroma European (A-E) (n = 11), Aroma North America (A-NA) (n = 9), Bitter European (B-E) (n = 3), and Bitter North American (B-NA) (n = 3) hop samples.** Non-parametric Kruskal-Wallis (KW) rank sum test (df = 3) was used to test for significance * $p < 0.05$, ** $p < 0.01$, ***$p < 0.001$. Marker compounds that significantly differed ($p < 0.05$) across groups were denoted by a letter, as determined by the Pairwise Wilcoxon Rank Sum Task test using Bonferroni correction.

| | **A-E** | **A-NA** | **B-E** | **B-NA** | ***KW*** | ***p*** |
|---|---|---|---|---|---|---|
| Cohumulone | 3.0 (0.6) $^{A}$ | 5.3 (1.0) $^{AB}$ | 8.4 (0.9) $^{B}$ | 12.8 (1.1) $^{B}$ | 13.9 | 0.03* |
| N-adhumulone | 7.87 (1.7) $^{A}$ | 15.7 (2.9) $^{AB}$ | 20.7 (4.4) $^{AB}$ | 31.9 (3.1) $^{B}$ | 14.0 | 0.02* |
| Colupulone | 6.4 (1.0) | 7.4 (1.0) | 11.0 (1.7) | 7.4 (0.5) | 5.4 | 0.14 |
| N-adlupulone | 7.3 (1.1) | 7.4 (1.0) | 10.0 (1.9) | 6.7 (0.5) | 1.8 | 0.62 |
| Xanthohumol | 1.3 (0.2) | 1.7 (0.2) | 1.9 (0.2) | 2.4 (0.6) | 6.8 | 0.08 |
| ABR | 0.8 (0.2) $^{A}$ | 1.5 (0.3) $^{AB}$ | 1.4 (0.2) $^{AB}$ | 3.1 (0.1) $^{B}$ | 11.3 | 0.01* |

Metabolomic analysis was performed for the Uses x Origin variable (Aroma European, Aroma North American, Bitter European, and Bitter North American). The PLS-DA resulted in more unique clusters and explained 30.2% of the variance (Figure 2-4A). The volcano plot only applies to metabolomics compared between the two groups. Since the Uses x Origin comparison has more than two categories, an ANOVA was used to identify mass features that significantly differ between groups (Figure 2-4B). Metabolomic analyses identified 12 mass features that significantly differ between use (aroma or bitter) and origin (European or North American) grouping. Ten mass features were significantly greater in the bitter North American group, while two were greater in bitter European varieties. One mass feature (neutral mass 414.20784) was tentatively identified as n-adlupulone (monoisotopic mass = 414.27700969 g/mol, with an error ppm of 167), and was significantly greater in the bitter North American group than the other three groups. Furthermore, this mass feature was cited in the literature as having a neutral reported mass of 414 g/mol (Chiancone et al., 2023; Prencipe et al., 2014).

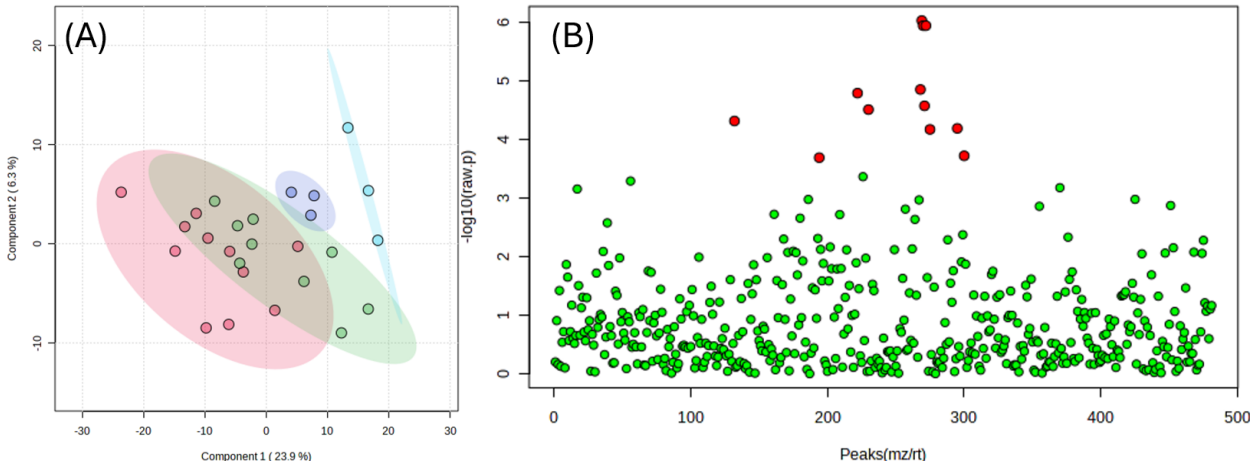


**Figure 2-4. Hop (*Humulus lupulus*) extracts (n = 26) comparing uses (aroma or bitter) x origins (European or North American) using a metabolomic dataset (containing 642 mass features).** Mass features are labelled as their retention time (RT) and mass-to-charge ratio (m/z). (A) Partial least squares discriminant analysis, principal component 1 explains 23.9% of the variance, while principal component 2 explains 6.3% for a cumulative variance of 30.2%. Aroma-European in Red (n = 11), aroma North American in green (n = 9), bitter EU in dark blue (n = 3), and bitter-NA in light blue (n = 3). (B) ANOVA identified 12 mass features (red dots) that differed between the four groups.

## Form – hop samples that were pelletized or dried unprocessed

Because T90 pellets are processed and have some plant matter removed in the processing, they have proportionally more lupulin relative to whole cones, I investigated chemical differences between forms (cone or pellet). No significant chemical differences were observed between the cone and pellet forms of hops ($p > 0.05$, Table 2-7). However, there was a trend toward a lower concentration of marker compounds in the cones, most notably for colupulone ($p = 0.055$).

**Table 2-7. Quantified marker compounds (mg/g dry plant weight) and the alpha-beta acid ratio (ABR) between cone (n = 6) and pellet (n = 26) hop samples.** Pairwise Wilcoxon test statistic was used to test for significant differences in compound between the two groups.

| | **Cone** | **Pellet** | ***W*** | ***p*** |
|---|---|---|---|---|
| Cohumulone | 7.6 (1.2) | 5.3 (0.7) | 108 | 0.16 |
| N-adhumulone | 16.6 (3.4) | 16.1 (2.1) | 84 | 0.80 |
| Colupulone | 9.8 (1.3) | 6.8 (0.5) | 118 | 0.05 |
| N-adlupulone | 7.9 (1.1) | 7.4 (0.6) | 80 | 0.94 |
| Xanthohumol | 1.8 (0.2) | 1.7 (0.2) | 89 | 0.62 |
| ABR | 1.5 (0.4) | 1.6 (0.2) | 79 | 0.98 |

Metabolomic analysis was performed for the form of hops. The PLS-DA resulted in unique clusters; however, it only explained 24.7% of the variance (Figure 2-5A). A volcano plot identified 11 mass features that were significantly greater in the cone than in pelletized hops (Figure 2-5B).

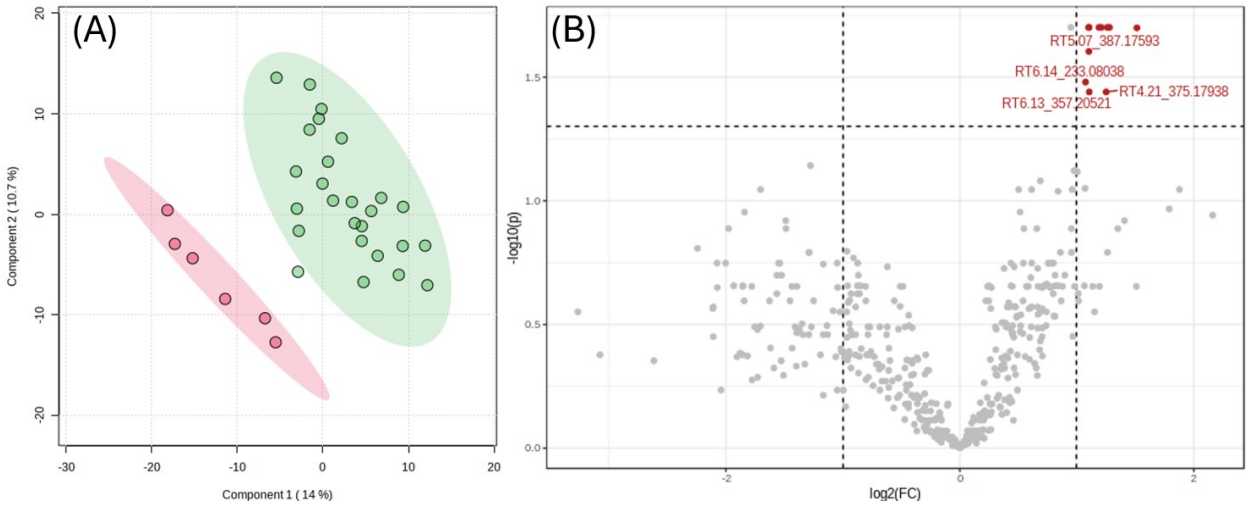


**Figure 2-5. Hop (*Humulus lupulus*) extracts (n = 32) comparing form (cone or pellet) using a metabolomic dataset (containing 642 mass features).** Mass features are labelled as their retention time (RT) and mass-to-charge ratio [m/z]+. (A) Partial Least Squares Discriminant Analysis: Principal component 1 explains 14% of the variance, while principal component 2 explains 10.7% for a cumulative variance of 24.7%. Hop cone samples were depicted in red (n = 6) and hop pellet samples were depicted in green (n = 26). (B) The volcano plot depicts mass features that were significantly greater in the cone hop samples (red dots).

The significant mass features were compared to those found in the literature, and two were tentatively identified. The first mass feature (neutral mass 340.1375) was tentatively identified as desmethylxanthohumol (monoisotopic mass = 340.13107373 g/mol) (Farag et al., 2012) and had an error of 19 ppm. However, the same mass feature was tentatively identified as adhumulone+2Na-H (monoisotopic mass = 340.1382 g/mol, error = 2 ppm). Once more, the same mass feature (340.1375) was also tentatively identified as 8-PN (monoisotopic mass = 340.13107373 g/mol, error = 18 ppm). The second mass feature (neutral mass 356.20521) was

tentatively identified as being dihydroxanthohumol (monoisotopic mass = 356.16237386 g/mol, error = 120 ppm) (Brown et al., 2022).

## DISCUSSION

The purpose of this study was to characterize the phytochemical variability of commercial hop varieties, first by identifying relationships among marker compounds (cohumulone, n-adhumulone, colupulone, n-adlupulone, xanthohumol) and second to investigate phytochemical correlates of different classifications of brewing hops.

The five marker compounds were detected in all samples and accounted for the majority (>90%) of the UV-detected peaks (Table 2-2, Figure 2-1). Correlations revealed that, across all samples (n = 32), the cohumulone and n-adhumulone (i.e., the alpha acids) content were highly correlated, as were colupulone and n-adlupulone (i.e., the beta acids), but only a weak positive correlation was observed between cohumulone and colupulone with no relationships between the other alpha and beta acids. While some studies have similarly detected weak positive correlations between alpha and beta acids (Likens et al., 1978; Patzak et al., 2015) others have not (Killeen et al., 2017). This discrepancy appears related to the tested hop material, with positive correlations observed among whole cone extracts and strong negative correlations when studying lupulin chemistry. Whereas the positive correlation in cones likely reflects infructescence structures (e.g., lupulin gland size and density) whereby more glands would result in proportionally more alpha and beta acids, the negative correlation in lupulin reflects a trade-off in production due to competition for the same substrate (Likens et al., 1978; Patzak et al., 2015). The positive correlation between cohumulone and colupulone may relate to the abundance of valine (BCAA precursor for both compounds) and the regulatory genes controlling the expression of valine bitter acid-specific biosynthetic enzymes (Clark et al., 2013). The lack of a correlation between

n-adhumulone and n-adlupulone, despite sharing isoleucine as a precursor, may be explained by differential regulation of n-adhumulone and n-adlupulone biosynthesis (Clark et al., 2013).

Previous studies reported positive correlations between alpha acids and xanthohumol (Killeen et al., 2014, 2017; Krofta et al., 2015), as supported by this current study, but significant (weaker) positive correlations were also observed relative to beta acids within our sample set. Xanthohumol is synthesized in the cytosol using a completely different pathway used for bitter acid synthesis in the plastid (Mishra et al., 2019). The quantity of specific BCAA (leucine, valine, isoleucine, Figure 1-1) along with enzymes responsible for creating the specific bitter acids are responsible for the final concentration of each specific bitter acid (Clark et al., 2013). Previously, no correlation was found between n-adhumulone and n-adlupulone; meanwhile, there were positive correlations between cohumulone and colupulone along with humulone and lupulone (Killeen et al., 2017). Both humulone (α-acid) and xanthohumol have demonstrated anti-herbivory properties towards insects, likely to protect the hop seeds that would develop in the fertilized infructescence (Stevens & Page, 2004). Perhaps both of these pathways are activated by the evolutionary threat of predation, which may explain the correlation between the alpha acids and xanthohumol.

This study demonstrates that bittering hop cultivars contained, on average, more cohumulone and n-adhumulone than aroma varieties with no difference in beta acid levels and significantly greater ABR values (Table 2-4). North American hops similarly had more alpha acids and a higher ABR than European hops (Table 2-5). Genetic differences between North American and European varieties have been reported in the literature (Townsend & Henning, 2009) with a potential impact on the expression and function of enzymes involved in secondary metabolism in hops (Clark et al., 2013; Killeen et al., 2017). Consistent with previous literature (Beatson et al.,

2003; Rutnik et al., 2023), North American hops had a higher ABR than European hops (Table 2-5), with European varieties averaging ratios around 1, while North American varieties are greater than 1. Our data suggest this difference is driven primarily by levels of n-adhumulone in North American hops, increasing the ABR with limited or variable contribution from the other bitter acids (Table 2-4, 2-6). Other studies also highlight the ABR as a way to categorize hops, with high values indicating high alpha (or bitter varieties) and low values indicating aroma varieties (Beatson et al., 2003; Jelínek et al., 2010; Rutnik et al., 2023; Stewart et al., 2017). However, ABR does not consider the total acid content. Consequently, some varieties might be very bitter, with high values of both humulone and lupulone (alpha and beta acid, respectively).

Previous research shows that the environment also impacts hop phytochemistry, with multiple studies systematically growing hop cultivars in different regions to test for terroir effects and reporting large effects on cone chemistry (Féchir et al., 2023; Hagemann et al., 2024; S. Lafontaine et al., 2019; Matsui et al., 2016). For example, minerals found in the soil have been linked to different characteristics of hops, likely due to the interaction of certain minerals with the biosynthetic enzymes involved in secondary metabolites (S. Lafontaine et al., 2022). The mineral profile of hop samples determined using X-ray technology is a useful tool to determine the growing location and could prevent food fraud by having mineral profiles linked to specific regions (Ocvirk et al., 2019). Beyond environmental differences, microsatellite tags are useful in determining the specific type of hops (Somalraju et al., 2024).

While terroir effects were not targeted as part of this study and no paired samples were included, *post-hoc* analyses comparing our samples grown in Europe and North America did not reveal a strong effect of growing location on chemistry (data not shown). To test for terroir effect, multiple hops cultivars (clones) could be grown in different regions while tracking and

then comparing the observed chemical differences relative to growing conditions (e.g., temperature, rainfall, photoperiod) and origin (Hagemann et al., 2024; Matsui et al., 2016). Additional studies could include metal and metalloid content (S. Lafontaine et al., 2022), proanthocyanin content (Mikyška et al., 2022), sensory attributes (Féchir et al., 2023), target compounds, and metabolomics could improve the chemotaxonomic approach to hops. To further tease apart the effect of terroir based on aroma and flavour, a sensory panel of judges could compare hops samples (Hagemann et al., 2024), and small batches of beer brewed from different hop terroirs (Féchir et al., 2023; S. Lafontaine et al., 2022; Matsui et al., 2016) warrants further investigation.

Notably, research on hop chemical diversity (including this study) typically investigates European and North American varieties. With hop breeding and cultivation expanding to other regions of the globe, particularly Oceania, future studies examining the impacts of origin and terroir on hop chemistry should include cultivars from outside the historic breeding centres of brewing hops. Future studies comparing hop chemistry should include varieties grown in Oceania, as this is a growing area of research into unique aromatic profiles of hops, and compare them to the relatives of these varieties grown in Europe or North America. Soil samples from hop fields in New Zealand and Great Britain have higher levels of P, K, and Cl, suggesting similar soil composition due to proximity to the ocean (Ocvirk et al., 2019). Knowing the lineage of these varieties grown in Oceania would also tease apart the environmental effect on chemistry. Transplanting hop cultivars from temperate environments (e.g., North America and Europe) to tropical environments (e.g., Brazil) has previously been tested, demonstrating that the cultivars Cascade and Chinook, had greater survival in tropical environments than other cultivars tested, though they had lower levels of bitter acids and volatile oils relative to their North American

grown counterparts (Contin et al., 2023). When hop varieties from North America and Europe were transplanted in Tasmania, there was a strong correlation between alpha and beta acid content, though, this disappeared by the second year of harvest (McAdam et al., 2014). The authors noted that there was a strong genetic component to alpha and beta acid content (McAdam et al., 2014) suggesting that the bitter acid could be predicted by the hop plant's genetics.

There was no significant difference in marker compound content between cones and pelleted hops. With the enrichment of lupulin glands to pellets, a minor yet consistent increase in marker concentration was expected. However, a similar chemical profile makes sense as the pellets are 90% hop cones compressed with lupulin glands (Kowalczyk et al., 2013). Previously, the chemical difference between cones and pellets was negligible when the hops were freshly harvested, but the alpha acids degrade more quickly in cones relative to pellets (Rutnik et al., 2023; Srečec et al., 2010). There is, however, an elemental impact of pelletizing such that pellets could contain more chromium, tin, and strontium (S. Lafontaine et al., 2022).

Whereas the presented comparison of marker compounds confirms and elaborates on well-studied aspects of hop chemodiversity, the untargeted metabolomics data add unique insight into the chemical similarity and variability among different hop varieties. For all classification-based comparisons (use, origin, use x origin, and form), multivariate analysis of metabolomic results among samples and between groups generated comparable results. As expected, PLSDA analyses yielded greater separation between groups with less variability <35%, explained by PC1 and PC2 (Figures 2-2 to 2-5). The use- and origin-based differences observed when comparing marker compounds were lost at the metabolomic level, reflective of human selection and experimental limitations. Because bitter acids are so abundant, closely tied to brewing-related

properties, and actively measured by industry, their production has long been an indirect or direct trait for breeding and cultivar development. Other metabolites, whether less abundant or unknown, would only contribute indirectly to trait selection, and their associated genotypes would more likely remain randomly distributed.

Metabolomics was nonetheless a powerful research tool that uncovered previously overlooked constituents in a plant extract. The untargeted metabolomic approach uncovered mass features that were differentially detected between groups that might represent markers of cultivar use or origin. The only tentatively identified compound associated with North American samples was ß-carboline, a class of indole alkaloids, with antimicrobial properties that also interact with the serotonergic 5-HT and GABA receptors, providing antidepressant and anxiolytic effects (Ferraz et al., 2019; Khan et al., 2013; Venault & Chapouthier, 2007). Note that the error ppm falls outside of the range set by the reserpine standard.

Metabolomic analysis also revealed that the cone samples contained considerably more xanthohumol-derivatives, such as desmethylxanthohumol and dihydroxanthohumol, that were tentatively identified based on reported HRMS data (Brown et al., 2022; Farag et al., 2012). These xanthohumol-derivatives are reduced xanthohumol and the quantity are controlled by gene-specific activity (Rutnik et al., 2023; Srečec et al., 2010). The processing time of hop material has a significant impact on the chemical and sensory characteristics of the hops. There is a significant loss of essential oils when the hop infructescence is not dried within a day post-harvest (Raut et al., 2021). The two-stage approach to pelletizing hops first involves compressing dried cones into bales, followed by pelletizing the plant material. Having a delay from harvest to pellets results in a greater loss of alpha acids likely caused by high-temperature kiln drying along with exposure to oxygen (Srečec et al., 2010; Taniguchi et al., 2013). The loss of secondary

metabolites is later supplemented with lupulin glands in the manufacturing of the T90 pellets (Kowalczyk et al., 2013; Srečec et al., 2010). However, this process would miss secondary metabolites found in the cone but not in the lupulin glands resulting in different metabolomic profiles between cones and pelletized hops.

To gain better insight into the relationship among marker compounds in hops, future studies should separate and quantify the major bitter acid isomers (humulone from adhumulone and lupulone from adlupulone), which co-eluted using our methods, since adhumulone production in different hop varieties is relatively stable whereas humulone production is more variable (Zhang et al., 2021). Alternative methods using a UPLC, published after the presented analyses are now available to differentiate n-lupulone from adlupulone (Schulz et al., 2019). Moreover, research on phytochemical biosynthesis and profiles should focus on lupulin while research on total phytochemical production should focus on cone chemistry (i.e. when floral structure like lupulin gland density is important.

## CONCLUSION

This was the first study to systematically test a diverse set of commercial cultivar samples to elucidate correlations among marker compounds and to test differences among various groupings used by brewers and the brewing based on both targeted and untargeted approaches. Across all of the samples, alpha acids correlated with one another, as did beta acids, and most bitter acids correlated with xanthohumol. Bittering hops and North American-bred hops contained more bitter acids and had higher ABR than aroma and European-bred hops. No significant differences in marker compounds were detected between cone and pellet; however, more xanthohumol-related compounds were observed at the metabolomic level. Overall, these results will help

breeders and brewers make informed decisions on the classification, development, and use of hop cultivars.

# CHAPTER 3: IDENTIFYING ANTIOXIDANTS AND ANTI-INFLAMMATORY SECONDARY METABOLITES IN HOPS (*HUMULUS LUPULUS*) EXTRACTS: A BIOCHEMOMETRIC APPROACH

## ABSTRACT

Aging is a natural process and is accelerated due to an excess of free-radicals leading to oxidative stress and inflammation. Free radicals are produced during natural processes (e.g., cellular respiration) and are typically scavenged via enzymatic processes. However, oxidative stress occurs when free-radical production outpaces free-radical scavenging activity (RSA). Continued oxidative stress leads to an inflammatory response and activation of oxidase enzymes, such as cyclooxygenase 2 (COX-2). Certain phytochemicals have demonstrated both RSA and anti-inflammatory effects. Hops (*Humulus lupulus* L., Cannabaceae) were traditionally used for their anti-bacterial, sedative, and anti-inflammatory properties, in addition to their widely known use as the bittering agent in beer. The objective of this study was to identify compounds responsible for RSA and COX-2 inhibition in a diverse set of hop extracts (n = 24) and test individual hop constituents. High-performance liquid chromatography quantified hops' unique secondary metabolites, such as cohumulone, n-adhumulone, colupulone, n-adlupulone, and xanthohumol. The extract's chemical components cohumulone and n-adhumulone modelled RSA, while n-adlupulone was the strongest compound tested for RSA. Meanwhile, hop extracts demonstrated COX-2 inhibition, and colupulone was the strongest compound tested followed by xanthohumol. Taken together, this is the first study to use these biochemometric approaches to identify bioactive components in hops along with testing individual compounds in a concentration-dependent manner. Knowledge of this bioactivity will further support the development of hop NHP targeted for antioxidant and anti-inflammatory effects.

# INTRODUCTION

Hops, *Humulus lupulus* L. (Cannabaceae), is a dioecious perennial bine that grows in temperate regions and produces unique infructescence with similarly unique phytochemistry (Teghtmeyer, 2018; Zhang et al., 2021). Global annual hop harvests have generally exceeded 100,000 tonnes since 2016 (BarthHaas, 2022), with the majority used by the brewing industry as a flavouring, foam stabilizer, and antimicrobial agent (Kowalczyk et al., 2013; Krofta & Mikyška, 2014; Sanz et al., 2019; Zanoli & Zavatti, 2008).

The unique chemistry and organoleptic properties of hops are concentrated within the infructescence (colloquially called cones or flowers) that house the lupulin glands. These lupulin glands contain the highest concentration of the bitter acids (α- and ß-acids, known as humulones and lupulones, respectively), terpenes (e.g., α-humulene, ß-caryophyllene), and xanthohumol – a prenylated chalcone unique to hops (Killeen et al., 2017; Mishra et al., 2019). The bitter acids are synthesized from branched chain amino acids leucine, valine, and isoleucine which make deoxyhumulone, deoxycohumulone, and deoxyadhumulone, respectively, after undergoing two prenylation steps via prenyltransferase enzymes (Figure 1-1) (Clark et al., 2013; Tsurumaru et al., 2012; G. Wang et al., 2008). These deoxy-molecules are enzymatically altered either by oxidases to form the humulones or by prenyltransferase to form the lupulones (Figure 1-1) (Clark et al., 2013; Tsurumaru et al., 2012; G. Wang et al., 2008).

Hop varieties differ in their concentration of secondary metabolites, resulting in different flavour profiles in beer and potentially possessing different bioactivity. Although over 339 varieties of hops are commercially available (Healey, 2016) the vast majority have not been studied beyond their use in brewing. Note that the term variety from the previous reference is not the same definition as the five botanically defined varietal groups of hops (Small, 1978). While

the therapeutic potential of hops has recently received increased scientific attention, particularly its anti-cancer and anti-inflammatory properties (Astray et al., 2020; Carbone & Gervasi, 2022; Zhang et al., 2021), the development of hops as a dietary supplement or Natural Health Product (NHP) has been overlooked by both science and industry (Sanz et al., 2019).

*Humulus lupulus* have been used medicinally for millennia in regions of Asia, Europe, and North America as an antibiotic, anti-inflammatory, and sedative, among other purposes (Korpelainen & Pietiläinen, 2021). Indigenous Peoples of North America used hops for a variety of conditions. The Delaware Nation used hops to treat ear and toothaches (Tantaquidgeon, 2001), while the Navajo used hops to treat coughs and colds (Vestal, 1952), the Cherokee used hops for sleep disorders (Hamel & Chiltoskey, 2002), and the Dakota used hops for intestinal disorders and wounds (Gilmore, 1913). In Traditional Chinese Medicine, hops were used as an anxiolytic, sedative and appetite stimulant (Karabin et al., 2015), while it was used for its anti-inflammatory and antibacterial properties in Ayurvedic medicine (Koetter & Biendl, 2010; Olšovská et al., 2016). Common to many of these conditions is the role of oxidative stress and inflammation in their pathophysiology and symptoms (Fernández-Sánchez et al., 2011; Galasko & Montine, 2010; Keskin et al., 2019). Indeed, hop cones and secondary metabolites elicit both radical scavenging and anti-inflammatory activity.

Hop cones are phytochemically rich and diverse, producing bitter acids most abundantly ($\leq$ 30% dry weight), with alpha acids (i.e., humulones) typically representing a greater proportion than beta acids (i.e., lupulones) (Almaguer et al., 2014), followed by polyphenols (3-5% dry weight (Kobus-Cisowska et al., 2019)), most notably xanthohumol and other flavonoids. Studies on the radical scavenging activity (RSA) of isolated hop metabolites show that, while humulone and lupulone exhibit strong RSA (Tagasgira et al., 1995), xanthohumol demonstrated stronger

RSA scavenger than the positive controls Vitamin C and E (Yamaguchi et al., 2009). Studies that compared the antioxidant and radical scavenging potential of crude hop extracts report that RSA associated with the level of either bitter acids (Keskin et al., 2019; Kobus-Cisowska et al., 2019; Lyu et al., 2022; Wu et al., 2020) or flavonoids (Kowalczyk et al., 2013; Lyu et al., 2022) and that ethanolic hop extracts tend to have stronger RSA than those made using other solvents (Kobus-Cisowska et al., 2019; Wu et al., 2020). Cultivars have also been shown to differ in RSA, suggesting that plants selected for greater quantities of bitter acids might confer greater RSA (Kobus-Cisowska et al., 2019). However, since these studies were limited by the number of included hop samples or phytochemical analytes, the relative contributions of bitter acids, xanthohumol, and other hop metabolites to RSA remains unknown.

Reactive oxygen species (ROS) naturally occur as a by-product of cellular respiration and are normally scavenged by superoxide dismutase or glutathione reductase, along with phytochemicals consumed in the diet such as phenolics (Bouayed & Bohn, 2012; Zugravu et al., 2022). The accumulation of ROS and other free radicals beyond the body's capacity to scavenge them results in oxidative stress linked to acute and chronic inflammatory conditions (Fernández-Sánchez et al., 2011; Galasko & Montine, 2010; Keskin et al., 2019; Lugrin et al., 2014) and could lead to cancer, metabolic syndrome, neurodegenerative and heart diseases (Bouayed & Bohn, 2012; Slika et al., 2022; Yamaguchi et al., 2009; Zhou et al., 2018; Zugravu et al., 2022). As such, the antioxidant activity of hops may also contribute to its traditional uses as an anti-inflammatory agent. Beyond scavenging free radicals to reduce downstream inflammatory signals and potential cellular damage, hop extracts and metabolites can impact inflammation through other mechanisms such as inhibiting cyclooxygenase (COX) activity.

In humans, COX enzymes exist in two forms (COX-1 and COX-2), both metabolizing arachidonic acid into lipid signalling molecules. COX-1 is constitutively expressed in most organs and produces metabolites important in maintaining homeostatic function (Hougee, 2008; Yamamoto et al., 2000). Meanwhile, COX-2 is induced in response to an inflammatory event, acting as an inflammatory signal via prostaglandin production. Both non-selective (e.g., ibuprofen) and selective (e.g., celecoxib) COX-2 inhibitors effectively reduce inflammation (Hall et al., 2008), although chronic use increases the risk of adverse events (Bhala et al., 2013; Luo et al., 2005). Limited *in vitro* studies suggest that certain hop extracts inhibit COX-2 activity (Hougee, 2008; Tripp et al., 2005), but researchers have primarily focused on isolated hop compounds rather than crude extracts or fractions (Hall et al., 2008; Tripp et al., 2005; Yamamoto et al., 2000). Xanthohumol (Gerhauser et al., 2002), humulone (Lee et al., 2007; Yamamoto et al., 2000), and bitter acid-derived compounds (e.g., iso-alpha-acid, rho-iso-alpha-acid) (Tripp et al., 2005, 2009) all exhibit COX-2 inhibition. In animal models, hop-derived compounds demonstrated greater COX-2 selectivity with fewer gastric side effects than the selective COX-2 inhibitor celecoxib (Hall et al., 2008). Using an untargeted approach to link anti-inflammatory activity to hop extracts, researchers identified humulones and their derivatives as responsible for reduced production of PGE2, a downstream product of COX-2 activity (Nicácio et al., 2022). As with RSA, the relative contribution of bitter acids, xanthohumol and other hop metabolites to COX-2 inhibiting activity of hop extracts has not been elucidated.

Aiming to identify contributing bioactive compounds, this study investigated the radical scavenging and COX-2 inhibitory activities of a chemically diverse collection of hop extracts and applied biochemometric approaches to compare activity relative to extract chemistry. Biochemometric analyses initially focused on the marker compounds since they are the most

abundant and well-studied, comparing activity between groups based on marker profile (high-alpha, high-beta, high-xanthohumol, and low bitter acids), to marker compound concentrations across samples using simple linear, and stepwise regression modelling, and testing isolated compounds. To help identify less abundant metabolites that may contribute to bioactivity, untargeted metabolomics data of the most and least active hop extracts were compared using multivariate methods. This approach was successfully applied by researchers who identified flavonoid glycosides as mediators of glucose uptake activity among medicinal plants using discriminant analysis (Shang et al., 2015).

With an established global infrastructure for hop cultivation and hundreds of cultivars available for study and commercial development, hops' medicinal properties and active metabolites warrant further investigation. Since all marker compounds exhibit RSA but alpha acids are consistently the most abundant metabolites in hop cone (Almaguer et al., 2014; Caballero et al., 2012; Carbone & Gervasi, 2022; Clark et al., 2013; Karabin et al., 2015; Killeen et al., 2014, 2017; Rutnik et al., 2023; Stevens & Page, 2004; Van Cleemput et al., 2009; Zanoli & Zavatti, 2008; Zhang et al., 2021), I hypothesized that alpha acids drive antioxidant activity among extracts, predicting that alpha acid content would associate positively with RSA activity. However, contributions from other bitter acids or flavonoids may contribute additively to RSA. According to several studies (Gerhauser et al., 2002; H. Liu et al., 2020; Tronina et al., 2017), xanthohumol is a potent COX-2 inhibitor and, while humulones and lupulones also inhibit COX-2 (Hougee, 2008; Tripp et al., 2005; Yamamoto et al., 2000), though their activities were less potent than crude extract (Tripp et al., 2005), suggesting other metabolites contribute to COX-2 inhibition. The objective of this study was to identify target compounds that model COX-2 inhibition and to test isolated compounds to identify the strongest inhibitors of COX-2 found in

hops. If xanthohumol is a potent inhibitor of COX-2, then the content within extracts would model inhibition, and the isolated compound would be a potent inhibitor. Meanwhile, if extracts inhibit COX-2 stronger than alpha acids and alpha acids stronger than beta acids, then isolated alpha acids should demonstrate greater activity than beta acids.

# METHODS

## Chemicals and reagents

The ICE-4 hop standard was purchased from the American Society of Brewing Chemists (ASBC) and was used to isolate the bitter acid constituents (cohumulone, n-adhumulone, colupulone, and n-adlupulone) as previously described (Chapter 2). In-house purification occurred for MQ water. Chromatographic Specialties Inc. (Brockville, ON, Canada) was the source for the 0.22µm filter tips and HPLC vials. Phenomenex (Torrance, CA, USA) was the source for HPLC columns. Sigma-Aldric (Oakville, ON, Canada) was the source of ascorbic acid. Unless otherwise stated, all other chemicals, reagents and labware were purchased from Fisher Scientific.

## Plant material

Most hops samples were purchased as hop pellets (n = 16) from www.ontariobeerkegs.com, which were primarily sourced from Yakima Chief Hop Company (Yakima, Washington, USA ) (n = 12) and LD Carlson (Kent, Ohio, USA) (n=1). Kichesippi Brewery (Ottawa, Ontario, Canada) generously donated three hop pellet samples (n = 3). All pellets were T90 except one that was a lupulin-enriched pellet. Cone samples were obtained from Carleton University (Ottawa, Ontario, Canada) (n = 2), CWG Farm Inc. (St-Eugène, Ontario, Canada) (n = 2), Highland Farm (Meaford, Ontario, Canada) (n = 3), and the author's private property (Ottawa, Ontario, Canada) (n = 1). All samples were kept in the freezer at -20 °C until extraction.

Briefly, hop pellets were crushed by hand, while hop cones were pulverized using a Magic Bullet Blender ®. Extracts were made by adding 120 mL of 99% ethanol to 6.00g of crushed or pulverized plant material and sonicating samples in a water bath for 10 minutes at room temperature. The ethanol was filtered and collected, and the plant material was extracted again (as above). The ethanol extracts were combined, and dried by a rotary evaporator (Yamato RE 500, Yamato Scientific, Japan), vacuum concentrator (Labconco Centrivap, Model 7810014, Kansas City, Missouri, USA), and freeze-dryer (SuperModulyo220 freeze dryer, Thermo fisher scientific, Nepean, Ontario, Canada) (see Chapter 2 for more details). The dried extracts were stored in a dark -20°C freezer until experimental use.

## Phytochemical analysis

Targeted phytochemical analysis

Extracts were reconstituted at 10 mg/mL 99% ethanol for high-performance liquid chromatography ultraviolet with photodiode array detection (HPLC-UV-DAD) analysis and were filtered using 0.22µm PTFE syringe filters. The HPLC was used to quantify cohumulone, n-adhumulone, colupulone, n-adlupulone, and xanthohumol using 2µL of injections. A kinetex C18 5U column (100 x 4.6mm, Phenomenex, Torrance, California, USA) was maintained at a temperature of 40°C, with a flow rate of 1.5 mL/min, time of 17.0 min followed by a post-run time of 3-min. Mobile phase A was water, and mobile phase B was methanol, with 0.1% trifluoroacetic acid (TFA) in each solution. The initial condition was 52.5% mobile phase B for one minute, then 90% B at 13 minutes, and 100% B by 13.1 minutes and held at 100% B until 17 minutes, monitored and quantified at 300nm. Calibration curve for xanthohumol ranged from 12.5-250 µg/mL, cohumulone 13.75-274.5 µg/mL, n-adhumulone 39.5-790 µg/mL, colupulone 16.3-325.5 µg/mL, and n-adlupulone 16.9-338 µg/mL.

Untargeted metabolomic profiling

The twenty-four dried ethanolic extracts were reconstituted to 500 µg/mL using LC-MS grade methanol. Extract and method control samples (e.g., solvent and reserpine) were each injected at 5µL onto a Thermo Scientific Dionex Ultimate 3000 UPLC system with Thermo Scientific LTQ Orbitrap XL high-resolution mass spectrometer (HRMS). The method and specifications used for the device have been outlined in previous literature (Witte & Overy, 2022) and the previous chapter. Briefly, the mobile phase (A) was LC-MS grade water with 0.1% formic acid and the mobile phase (B) was acetonitrile with 0.1% formic acid. The gradient commenced with 5% B before achieving 95% B in 4.5 minutes, held constantly at 95% B for 3.5 minutes returned to 5% B over 0.5 minutes, and equilibrated for 6 minutes. The HRMS Orbitrap XL was operated in $ESI_+$ mode monitoring *m/z* 50-2000, resolution = 30000, and an MS runtime of 10 minutes.

Metabolomic data processing and analysis

RAW files generated from each analysis were converted into mzML files using ProteoWizard, and further analysis was done using MzMine 2.53. Following optimization of processing parameters, the group intensity threshold was 1.0 E + 5, the minimum highest intensity was 1.0 E + 6, and the mass to charge (m/z) was set at 0.005 m/z or 5ppm. The reserpine drift accounted for 12 ppm when comparing tentative mass features to the literature. The analyses were conducted in MetaboAnalyst5.0 (MetaboAnalyst, 2023). The error between a compound's monoisotopic mass and the neutral mass feature was identified using an online tool: (https://warwick.ac.uk/fac/sci/chemistry/research/barrow/barrowgroup/calculators/mass_errors/). Reported mass features and monoisotopic masses were also compared to mass features identified

from the volcano plot. These techniques permitted a tentative identification of mass features, while the error from the reserpine standard established an anchor for relative error.

## DPPH protocol

Methanol was used to dissolve extracts and isolated compounds. Extracts were tested at concentrations of 6.25-200 µg/mL and isolated marker compounds (cohumulone, n-adhumulone, colupulone, n-adlupulone, xanthohumol) were tested at concentrations of 0.32-200 µg/mL. Positive controls were tested at compound-specific ranges (Vitamin C 0.625-20.0 µg/mL in-well; catechin 3-18 µg/mL and quercetin 0.6-9.6 µg/mL). Methanol was used as the vehicle control. The 1, 1-diphenyl-2-picrylhydrazyl (DPPH) powder was prepared into a 100 µM solution in methanol. The samples were loaded into a white 96-well clear-bottom plate followed by the DPPH solution. The plate was run in the Cytation 3 plate reader (BioTek, Winooski, Vermont, USA) with an absorbance reading after 10 minutes of incubation at room temperature in the dark.

DPPH scavenging activity was calculated based on the average absorbance from duplicate wells of the same sample concentration as follows (Yang et al., 2010):

$$\text{Percent radicals scavenged (\%RS)} = (1 - (Abs_{sample} / Abs_{vehicle\ control})) \times 100\%$$

All samples were tested in three or more independent experiments, with activity reported as the mean % RSA (± SEM, n = 3) at 12.5 µg/mL and the median effective concentration ($EC_{50}$). The $EC_{50}$ was calculated using the log-logistic four-parameter model found in R's dose-response curve package (Ritz et al., 2015), whereby the concentration was the predictor variable, and the percent RSA was the response variable. The three and four-parameter models were run for the isolated compounds, and the appropriate model based on these data was selected.

## COX protocol

Extracts and isolated compounds were diluted in dimethyl sulfoxide (DMSO) and tested at concentrations ranging from 1.6 – 25 µg/mL for extracts and 0.25-5.00 µg/mL for isolated compounds. The methodology for this assay has been adapted from Cayman chemical COX Fluorescent inhibitor screening assay kit (Item No. 700100), with arachidonic acid (100µM) as the substrate, ADHP (10-acetyl-3,7-dihydroxyphenoxazine) as the assay reagent, DuP-697 (60µM) as the positive control, DMSO (5%) as vehicle (negative) control, while using white 96-well clear-bottom plates. Half of the solution volume was used for this experiment relative to the assay protocol provided by Cayman. The fluorescence excitation was set between 535nm and the emission wavelength at 590nm. Readings were taken once at initiation and after 2 minutes after the reagent and arachidonic acid were added. The average inhibition was taken from the duplicate wells of the same concentration. Then, the percent inhibition was calculated as

$$\left[1 - \frac{(Sample\ Enzyme\ End - Sample\ Enzyme\ Start) - (Sample\ Blank\ End - Sample\ Blank\ Start)}{(Vehicle\ Control\ Enzyme\ End - Vehicle\ Control\ Enzyme\ Start) - (Vehicle\ Control\ Blank\ End - Vehcile\ Control\ Blank\ Start)}\right] * 100$$

All samples were tested in three or more independent experiments, with activity reported as the mean % inhibition (± SEM, n=3) at 6.25 µg/mL and the median inhibitory concentration ($IC_{50}$). The $IC_{50}$ was calculated using the log-logistic three-parameter model found in the dose-response curve package (Ritz et al., 2015), similar to what was mentioned above except that the response variable was percent inhibition, not percent RSA.

## Statistical Analysis

Chemical groups were created by selecting hop samples that were high in marker compounds alpha acids, beta acids, xanthohumol and ones that were low in alpha and beta acids. One-way ANOVAs for the group on each of the target compounds (cohumulone, n-adhumulone, colupulone, n-adlupulone, xanthohumol, total alpha + beta) with *post hoc* Tukey-HSD supported the groupings. The data summary (Min, Max, Mean, SEM) for each marker compound was

calculated across the whole sample set (n = 24) and further broken down into means and SEM for each of the four groups. Group differences were calculated using an ANOVA with Tukey-HSD *post hoc* test for each marker compound, percent activity, and $EC_{50}$ or $IC_{50}$. Simple linear regressions were used to model the concentration of marker compounds in the extract to RSA at 12.5 µg/mL and $EC_{50}$ for DPPH or percent inhibition at 6.25 µg/mL and $IC_{50}$ for COX-2. This was followed by stepwise regressions of extract RSA at 12.5 µg/mL and $EC_{50}$ values or for COX-2 at 6.25 µg/mL and $IC_{50}$, relative to each of the quantified compounds in the extracts.

Using untargeted analysis is another way to help identify metabolites that might have needed to be noticed from the abovementioned targeted approach. To do so, the three strongest and three weakest $EC_{50}$ or $IC_{50}$ samples were identified in each dataset (DPPH and COX-2 separately). Next, volcano plots were run, and mass features significantly differed between the strong and weak groups were identified. Simple linear regressions were run using the normalized-scaled and centred mass-feature value to all $EC_{50}$ or $IC_{50}$ values (n = 24). Those associated with activity were tentatively identified based on published literature, the molecular weight of these compounds, and open-sourced mass spectral libraries (e.g., Mass Bank). Statistical analyses were conducted using R-studio version 4.3.2. and $EC_{50}$/$IC_{50}$ values were calculated using the DRC package in RStudio (Ritz et al. 2015)

# RESULTS

## Targeted profiling of marker compounds

Hop samples (n = 24) were homogenized, extracted in 99% ethanol, dried and reconstituted at 10 mg/mL, and then analyzed by HPLC to quantify major marker compounds (cohumulone, n-adhumulone, colupulone, n-adlupulone, and xanthohumol) based on reference standards. All marker compounds were identified in all samples but varied in relative quantities (Figure 3-1;

Table 3-1; Supplemental Table 2). Alpha acid content ranged from 10.7-192.4 µg/mg (mean 94.6±11.6 µg/mg), with cohumulone and n-adhumulone between 1.61-54.0 µg/mg (mean 25.4±3.2 µg/mg), and 9.1-156.0 µg/mg mean (69.3±8.6 µg/mg), respectively. The beta acid content was lower (29.5-146.7 µg/mg, mean 74.6±6.4µg/mg), colupulone ranged from 14.0-71.21 µg/mg (mean 35.9 ±3.0 µg/mg), and n-adlupulone between 15.45-75.45 µg/mg (mean 38.7 ±3.7 µg/mg). Xanthohumol content varied (2.8-13.8 µg/mg) but was far less abundant (mean 9.0 ±0.7 µg/mg) than the bitter acids. This diversity in marker profile among extracts permitted groupings based on the relative quantity of specific marker compounds (representative HPLC chromatogram presented in Figure 3-1).

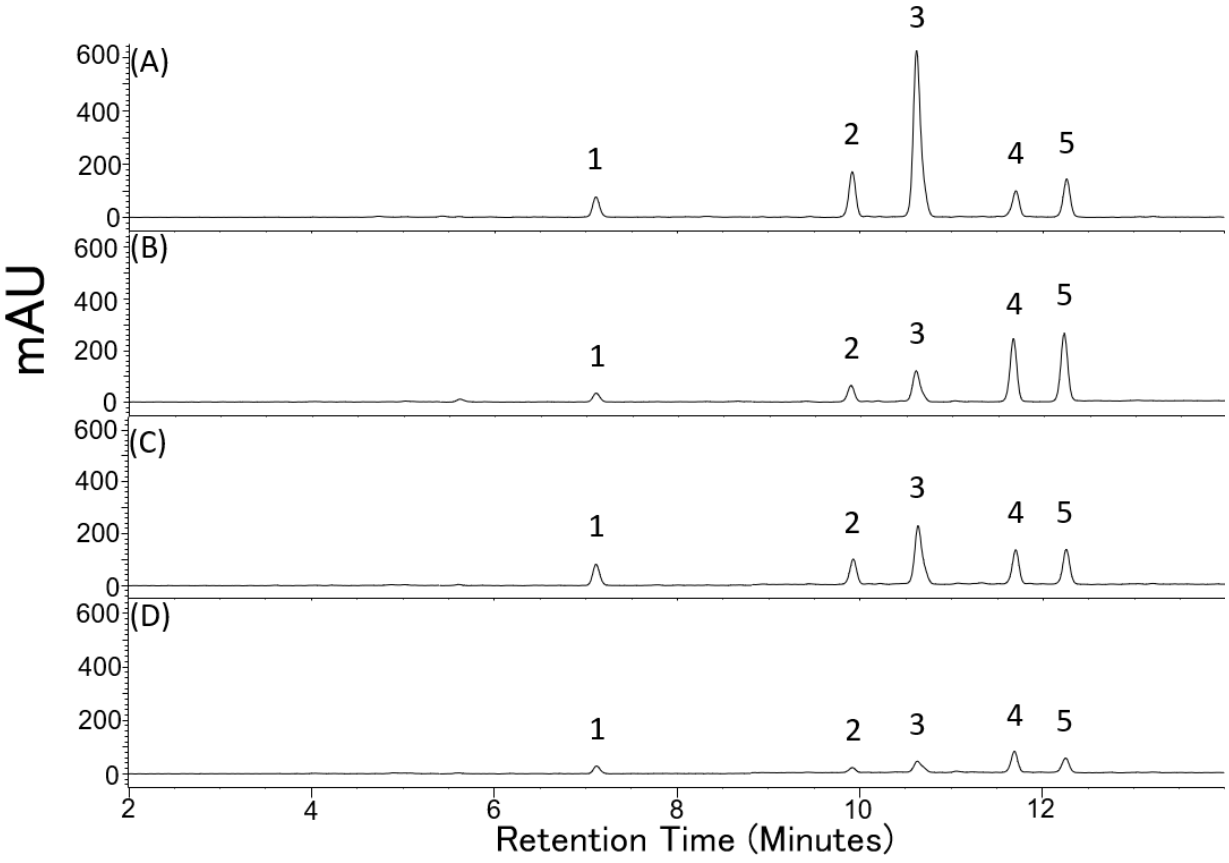


**Figure 3-1. HPLC-DAD chromatogram of representative *Humulus lupulus* L. extracts from the four assigned chemical groups (n = 24).** (A) High-Alpha acid group, (B) High-Beta acid group, (C) High-xanthohumol group, (D) Low-alpha-beta acid group. All compounds were monitored at 300nm, between 2-14 minutes. Target compounds are: (1) xanthohumol, (2) cohumulone, (3) n-adhumulone, (4) colupulone, and (5) n-adlupulone.

**Table 3-1. Quantified marker compounds (µg/mg plant extract) and the alpha-beta acid ratio (ABR) found in *Humulus lupulus* extracts (n = 24).** Alpha acid was the sum of cohumulone and n-adhumulone. Beta acid was calculated as the sum of colupulone and n-adlupulone. The mean was the arithmetic mean, SEM was the standard error of the mean, Min (minimum) refers to the lowest observed concentration, and Max (maximum) refers to the highest observed concentration.

| | Mean | SEM | Min | Max |
|---|---|---|---|---|
| Alpha Acid | 94.6 | 3.2 | 10.7 | 192.4 |
| Cohumulone | 25.4 | 3.2 | 1.61 | 54.0 |
| N-adhumulone | 69.3 | 8.6 | 9.10 | 156.0 |
| Beta Acid | 74.6 | 6.4 | 29.5 | 146.7 |
| Colupulone | 35.9 | 3.0 | 14.0 | 71.2 |
| N-adlupulone | 38.7 | 3.7 | 15.4 | 75.5 |
| Xanthohumol | 9.0 | 0.7 | 2.80 | 13.8 |
| ABR | 1.6 | 0.25 | 0.10 | 4.60 |

## Phytochemical correlation of hop samples

The quantitative data for the five marker compounds across 24 different hop samples revealed that concentrations of the alpha acids co- and ad-humulone were strongly correlated ($R = 0.92$, $p < 0.001$), as were levels of beta acids colupulone and n-adlupulone ($R = 0.83$, $p < 0.001$, Table 3-2). Both cohumulone ($R = 0.59$) and n-adhumulone ($R=0.54$) concentrations also correlated with those of xanthohumol ($p < 0.01$).

**Table 3-2. Pearson correlation associated with quantified marker compounds (xanthohumol, cohumulone, n-adhumulone, colupulone, n-adlupulone) (µg/mg extract weight) from hop extracts (n = 24) using high-performance liquid chromatography diode array detection.** Significance denoted with * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

| | Xanthohumol | Cohumulone | N-adhumulone | Colupulone | N-adlupulone |
|---|---|---|---|---|---|
| Xanthohumol | 1 | | | | |
| Cohumulone | 0.59** | 1 | | | |
| N-adhumulone | 0.54** | 0.92*** | 1 | | |
| Colupulone | 0.19 | -0.05 | -0.17 | 1 | |
| N-adlupulone | -0.02 | -0.36 | -0.35 | 0.83*** | 1 |

Individual extracts were then assigned to one of four groups: 1) High-alpha acid (HA), 2) high-beta acids (HB), 3) high-xanthohumol (HX), or 4) low alpha and beta acid (LAB) (n = 6 per group). The alpha acid content was significantly greater in HA than in HB, HX, and LAB (Table 3-3). Likewise, beta acid content was significantly greater in HB than in HA, HX, and LAB (Table 3-3). The HX group had 1.5x and 2.5x more xanthohumol than HB and LAB, respectively, but no significant difference was observed between the HX and HA groups. The LAB group had significantly less alpha and beta acids than the other three groups.

**Table 3-3. Quantified marker compounds (µg/mg extract weight) from hop extracts (n = 24) were categorized into distinct chemical groups (HA: High-Alpha, HB: High-Beta, HX: High-Xanthohumol, LAB: Low alpha-beta) using high-performance liquid chromatography-diode array detection.** Marker compounds were quantified using high-performance liquid chromatography with UV and diode array detector. Triplicate injection means ± SEM are presented. Significant differences are denoted with letters among groups for each marker compound using a one-way ANOVA (df = 3,20) with Tukey-HSD *post hoc* test to test for significance.

| | **HA** | **HB** | **HX** | **LAB** | | |
|---|---|---|---|---|---|---|
| | **Mean (SEM)** | **Mean (SEM)** | **Mean (SEM)** | **Mean (SEM)** | ***F*** | ***p*** |
| Alpha acids | 169.5 (7.5) $^{A}$ | 67.6 (11.6) $^{B}$ | 102.1 (18.1) $^{B}$ | 39.3 (8.2) $^{B, C}$ | 21.5 | <0.001 |
| Cohumulone | 43.2 (3.1) $^{A}$ | 18.3 (4.6) $^{B}$ | 29.5 (5.8) | 10.6 (2.6) $^{B}$ | 11.4 | <0.001 |
| N-adhumulone | 126.3 (6.9) $^{A}$ | 49.4 (7.2) $^{B}$ | 72.7 (12.4) $^{B}$ | 28.7 (5.7) $^{B,C}$ | 24.8 | <0.001 |
| Beta acids | 55.8 (4.9) $^{B}$ | 117.7 (7.1) $^{A}$ | 75.1 (7.0) $^{B}$ | 49.7 (8.7) $^{B}$ | 19.1 | <0.001 |
| Colupulone | 28.2 (3.2) $^{B}$ | 54.7 (5.3) $^{A}$ | 37.2 (2.5) $^{B}$ | 23.3 (3.0) $^{B}$ | 14.2 | <0.001 |
| N-adlupulone | 27.6 (1.9) $^{B}$ | 63.0 (3.7) $^{A}$ | 37.9 (5.0) $^{B}$ | 26.4 (6.1) $^{B}$ | 14.5 | <0.001 |
| Xanthohumol | 10.5 (0.4) $^{A,B}$ | 8.4 (1.1) $^{B}$ | 12.6 (0.4) $^{A}$ | 4.7 (0.5) $^{C}$ | 27.8 | <0.001 |

## DPPH scavenging activity

All tested hop samples exhibited concentration-dependent free-radical scavenging activity (RSA). At a standard extract concentration of 12.5 µg/mL, the percent RSA ranged from 13.3-61.3% with a mean of 33.2% ± 2.7. $EC_{50}$ values ranged from 5.5-35.0 µg/mL, with a mean of 14.0 ± 1.4 µg/mL (Supplemental Table 3).

There was a significant group effect on RSA ($F_{3,20} = 8.6$, $p < 0.001$) at a standard concentration of 12.5 µg/mL (Table 3-4). The HA group had nearly twice the RSA as HB and LAB, and 1.5 times greater than HX ($p < 0.05$) (Table 3-3). A significant main effect of the Group was similarly observed for $EC_{50}$ values ($F_{3,20} = 3.2$, $p = 0.046$), but with no significant difference among the groups (Table 3-4).

**Table 3-4. The mean (SEM) percent Radical Scavenging Activity (%RSA) at 12.5 µg/mL and $EC_{50}$ (µg/mL) among the four groups of hop extracts (n = 24).** Percent RSA was measured at an extract concentration of 12.5 µg/mL median effective concentration ($EC_{50}$ µg/mL) was calculated based on 6.25-200 µg/mL concentration-dependent curves (Ritz et al., 2015). One-way ANOVA (df = 3,20) and Tukey HSD post hoc test for significance, with significant differences among groups denoted by superscript letters.

| | % RSA (12.5 µg/mL) | $EC_{50}$ (µg/mL) |
|---|---|---|
| High-Alpha | 49.3 (2.8) $^{A}$ | 7.7 (0.5) |
| High-Beta | 25.6 (3.3) $^{B}$ | 14.8 (1.2) |
| High-Xanthohumol | 31.6 (5.6) $^{B}$ | 16.1 (4.0) |
| Low-Alpha-Beta | 26.2 (3.1) $^{B}$ | 17.3 (2.4) |
| *F* | 8.6 | 3.2 |
| *p* | <0.01 | <0.05 |

To explore the relationship between marker compound concentrations and radical scavenging activity, simple linear regressions were run for each quantified marker compound in each extract relative to the observed activity. Based on percent radical scavenging (at 12.5 µg/mL extract), the alpha acids strongly predicted activity (Table 3-5), with RSA increasing as the alpha-acids content of an extract increased. Specifically, n-adhumulone content was more robustly associated with RSA ($R^2 = 0.77$, $p < 0.001$) than cohumulone content ($R^2 = 0.60$, $p < 0.001$) (Table 3-5). No other marker compounds in hops were associated independently with RSA ($R^2 < 0.20$, $p > 0.05$

(Table 3-5). The step-forward regression analysis resulted in the predictive model (Adjusted $R^2$ = 0.79, $F_{2,21}$ = 43.8, $p < 0.001$):

$$\text{Percent RSA} = 19.8 + 25.0\ (\text{n-adhumulone}) - 73.2\ (\text{xanthohumol})$$

Similar trends were seen in $EC_{50}$ data (Table 3-5), with alpha-acids positively related to activity, although the slopes were negative as lower $EC_{50}$ indicates stronger activity. Both step-forward and step-backward regression analyses resulted in the predictive model (adjusted $R^2$ = 0.49, $F_{2,21}$ = 12.35, $p < 0.001$):

$$EC_{50} = 18.47 - 0.13\ (\text{n-adhumulone}) + 0.53\ (\text{xanthohumol})$$

**Table 3-5. Simple linear regression of percent free radical scavenging activity (RSA) or $EC_{50}$ (µg/mL) as a function of marker compounds quantified across twenty-four hops (*Humulus lupulus* L.) extracts.** Percent RSA was measured at an extract concentration of 12.5 µg/mL and the median effective concentration ($EC_{50}$ µg/mL) was calculated based on 6.25-200 µg/mL concentration-dependent curves (Ritz et al., 2015). Marker compounds were quantified using high-performance liquid chromatography with UV and diode array detector. Significant p-values ($p < 0.05$) are in boldface font.

| | Percent RSA | | | | | $EC_{50}$ | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Predictor** | **$R^2$** | **Slope** | ***SE*** | ***F*** | ***p*** | **$R^2$** | **Slope** | ***SE*** | **F** | ***p*** |
| Alpha acid | 0.74 | 15.9 | 2.0 | 63.6 | **<0.001** | 0.49 | -0.08 | 0.02 | 20.7 | **<0.01** |
| Cohumulone | 0.60 | 51.3 | 8.9 | 33.1 | **<0.001** | 0.42 | -0.27 | 0.07 | 15.7 | **<0.01** |
| N-adhumulone | 0.77 | 21.9 | 2.6 | 73.0 | **<0.001** | 0.49 | -0.11 | 0.02 | 21.3 | **<0.01** |
| Beta acid | 0.14 | -12.2 | 6.6 | 3.5 | 0.08 | 0.02 | 0.03 | 0.04 | 0.5 | 0.51 |
| Colupulone | 0.09 | -21.5 | 14.3 | 2.3 | 0.15 | 0.01 | 0.05 | 0.10 | 0.3 | 0.61 |
| N-adlupulone | 0.15 | -22.6 | 11.3 | 4.0 | 0.06 | 0.02 | 0.06 | 0.08 | 0.6 | 0.47 |
| Xanthohumol | 0.09 | 96.4 | 64.1 | 2.3 | 0.15 | 0.04 | -0.38 | 0.42 | 0.8 | 0.38 |

Among the isolated compounds, n-adlupulone had the strongest RSA of the bitter acids, followed by colupulone, n-adhumulone, and cohumulone, all of which were more active than positive controls vitamin C and catechin, and comparable to quercetin (Table 3-6). Xanthohumol exhibited the same radical scavenging as catechin but the weakest activity among hop marker compounds (Table 3-6). The $EC_{50}$ for isolated compounds (range: 2.6-7.0 µg/mL) outperformed most extracts (range: 5.5-35.0 µg/mL) for RSA (Table 3-6).

**Table 3-6. Median effective concentrations ($EC_{50}$) of hop marker compounds and positive controls.** The radical scavenging activity of each marker compound was measured using the DPPH assays and serial dilutions of each compound. Assays were performed thrice with $EC_{50}$ (±SEM) values were calculated using concentration dependent curves (Ritz et al., 2015).

| Isolated compound | $EC_{50}$ (µg/mL) | $EC_{50}$ (µM) |
|---|---|---|
| Cohumulone | 4.6 (0.04) | 13.3 (0.12) |
| N-adhumulone | 4.4 (0.08) | 11.7 (0.22) |
| Colupulone | 4.4 (0.09) | 11.0 (0.23) |
| N-adlupulone | 2.6 (0.07) | 6.3 (0.17) |
| Xanthohumol | 7.0 (1.26) | 19.6 (3.54) |
| Vitamin C | 2.9 (0.10) | 16.4 (0.58) |
| Quercetin | 2.7 (0.05) | 8.9 (0.17) |
| Catechin | 5.7 (0.20) | 19.6 (0.67) |

The metabolomic dataset was generated by analyzing the twenty-four hop extracts on an UPLC-Q-Orbitrap HRMS system with data subsequently curated to establish mass features in hop extracts. This approach resulted in 642 mass features. Comparing the metabolomes of the three strongest (mean $EC_{50}$ = 6.9 ± 0.6 µg/mL) and three weakest (mean $EC_{50}$ = 27.0 ± 3.8 µg/mL) samples by discriminant analysis identified seven mass features as significantly different between the two groups (Figure 3-2). The relative abundance (normalized ion count) of these mass features was then extracted from metabolomic data of all tested hop extracts and simple linear regressions were performed relative to the $EC_{50}$ values (n = 24). Of the six mass features modelled to $EC_{50}$, five were greater in the strong $EC_{50}$ group and the same four mass features significantly modelled $EC_{50}$ ($p < 0.01$) (Table 3-7). The only mass feature (210.0959, Table 3-7) that matched the available literature was phloroisovalerophenone (monoisotopic mass = 210.0892 g/mol, error = 141 ppm) (Nicácio et al., 2022). From the molecular adduct comparison, the mass feature 302.0483, did not model activity (Table 3-7), however, was tentatively identified as adhumulone+2ACN+H (monoisotopic mass = 302.049 g/mol, error = 2 ppm).

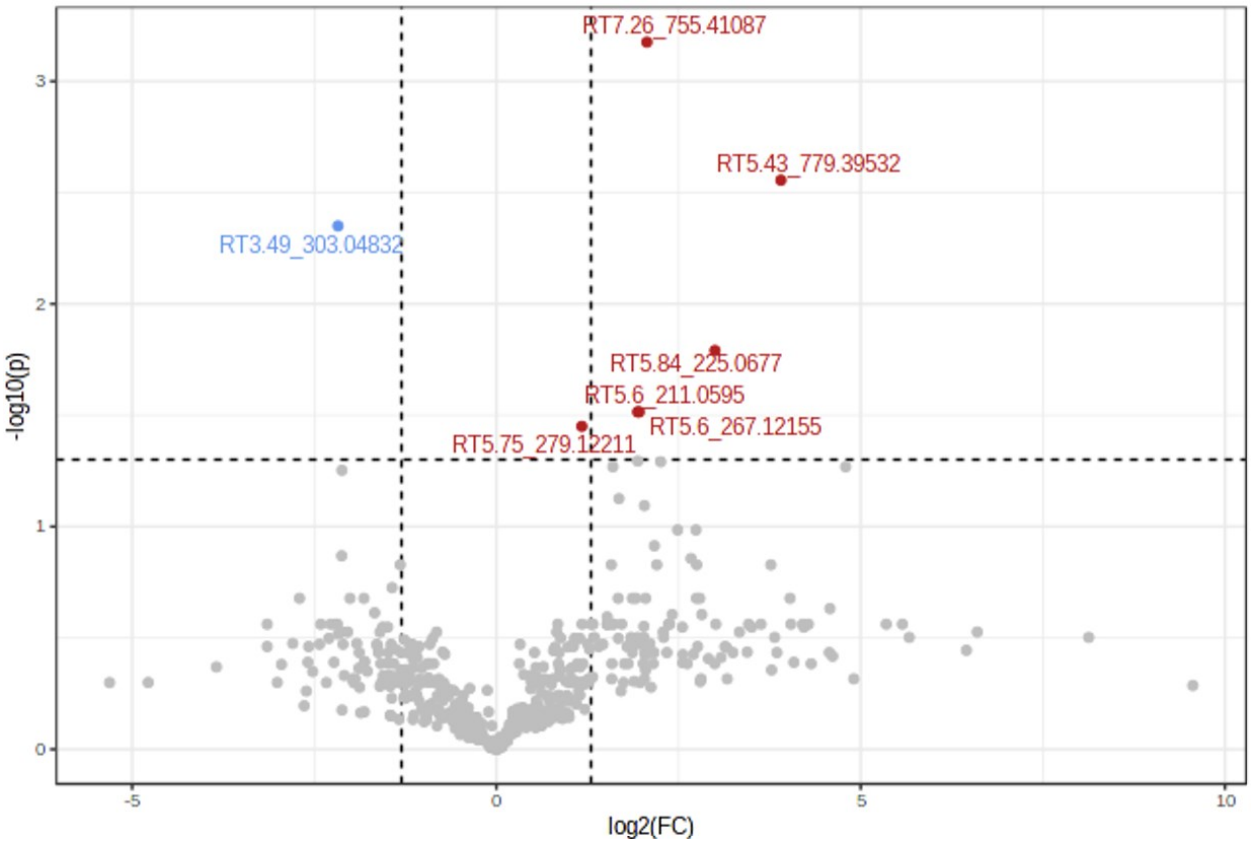


**Figure 3-2. Comparison of the three strongest to three weakest hop (*Humulus lupulus*) extracts on a free-radical scavenging assay using a metabolomic dataset (containing 642 mass features).** Mass features are labelled as their retention time (RT) and mass-to-charge ratio [m/z]+. Mass features that were significantly greater in the stronger hop extracts were depicted in red while those that were significantly greater in the weaker hop extract samples were depicted in blue.

**Table 3-7. Simple linear regressions between DPPH $EC_{50}$ (µg/mL) values and normalized-centred-scaled mass-feature data from the metabolomic dataset using *Humulus lupulus* extracts (n = 24).** *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

| Predictor ($M+H^+$) | $R^2$ | Slope | F | *p* |
|---|---|---|---|---|
| RT3.49_303.0483 | 0.11 | 2.16 | 2.61 | 0.12 |
| RT5.60_211.0595 | 0.41 | -2.82 | 15.49 | <0.01** |
| RT5.60_267.1216 | 0.38 | -4.13 | 13.74 | <0.01** |
| RT5.75_279.1221 | 0.49 | -6.67 | 20.97 | <0.001*** |
| RT7.26_755.4109 | 0.30 | -4.92 | 9.57 | <0.01** |

## COX-2 inhibition

Hops extracts demonstrated concentration-dependent COX-2 inhibition. At the standard extract concentration of 6.25 µg/mL, percent inhibition across extracts ranged from 40.8-67.6% with a mean of 53.2 % ± 1.6 (Supplemental Table 3). $IC_{50}$ values ranged from 0.9-21.8 µg/mL, with a mean of 6.6 µg/mL ± 0.7 (Supplemental Table 3). Groups (HA, HB, HX, and LAB) did not significantly differ in their COX-2 inhibition (6.25 µg/mL) ($F_{3,20} = 0.41$, $p = 0.70$) or $IC_{50}$ ($F_{3,20} = 0.16$, $p = 0.90$) (Table 3-8).

**Table 3-8. COX-2 mean (± SEM) percent inhibition (at 6.25 µg/mL) and $IC_{50}$ for hop (*Humulus lupulus* L.) extracts (n = 24).** $IC_{50}$ (µg/mL) was calculated based on 1.6-25 µg/mL concentration-dependent curves (Ritz et al., 2015). One-way ANOVA test was used to test for significance, followed by Tukey HSD *post hoc* test to compare among groups. There were no significant differences between groups.

| Group | Inhibition (%) | $IC_{50}$ (µg/mL) |
|---|---|---|
| High-Alpha | 54.2 (3.6) | 6.66 (2.3) |
| High-Beta | 53.3 (3.3) | 6.01 (1.2) |
| High-Xanthohumol | 50.3 (2.0) | 7.01 (0.5) |
| Low-Alpha-Beta | 55.0 (3.7) | 6.65 (0.9) |
| *F* | 0.41 | 0.16 |
| *p* | 0.70 | 0.90 |

Simple linear regressions were run to compare the relationship between marker compound concentrations and percent inhibition for COX-2 (Table 3-9) at 6.25 µg/mL. The hop extracts' alpha and beta acid content did not predict COX-2 inhibition (Table 3-9). Contrary to the hypothesis, the xanthohumol content of hop extracts was not predictive of COX-2 inhibition ($R^2$ = 0.005, $p$ = 0.75, Table 3-9). Correspondingly, there were no significant linear regressions between $IC_{50}$ and any of the marker compounds (Table 3-9). Furthermore, the stepwise regression did not result in a significant model for inhibition or $IC_{50}$ for the marker compounds.

**Table 3-9. Simple linear regression of COX-2 bioactivity, percent inhibition or $IC_{50}$ (µg/mL) as a function of marker compounds concentration found in hop extracts (n = 24).** Modelled using COX-2 percent inhibition (at 6.25 µg/mL) or $IC_{50}$ (µg/mL) was calculated based on 1.6-25 µg/mL concentration-dependent curves (Ritz et al., 2015). Marker compounds were quantified using high-performance liquid chromatography with UV and diode array detector.

| | **Percent Inhibition** | | | | | **$IC_{50}$ (µg/mL)** | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Predictor** | **$R^2$** | **Slope** | ***SE*** | **F** | ***p*** | **$R^2$** | **Slope** | ***SE*** | **F** | ***p*** |
| Alpha acid | <0.01 | 0.7 | 4.6 | 0.02 | 0.9 | <0.01 | 0.0 | 0.02 | 0.01 | 0.9 |
| Cohumulone | <0.01 | 4.4 | 6.2 | 0.07 | 0.8 | 0.01 | -0.03 | 0.06 | 0.21 | 0.6 |
| N-adhumulone | <0.01 | 0.7 | 6.2 | 0.01 | 0.9 | <0.01 | 0 | 0.02 | 0 | 0.9 |
| Beta acid | <0.01 | -3.0 | 8.2 | 0.13 | 0.7 | <0.01 | 0 | 0.03 | 0.01 | 0.9 |
| Colupulone | <0.01 | 2.8 | 17.6 | 0.03 | 0.9 | <0.01 | -0.02 | 0.07 | 0.05 | 0.8 |
| N-adlupulone | 0.03 | -10.8 | 14.2 | 0.58 | 0.5 | <0.01 | 0.02 | 0.06 | 0.13 | 0.7 |
| Xanthohumol | <0.01 | -25.8 | 78.6 | 0.11 | 0.8 | 0.01 | 0.17 | 0.30 | 0.31 | 0.6 |

When tested independently, colupulone, n-adlupulone, and xanthohumol all elicited concentration-dependent COX-2 inhibition curves with $IC_{50}$ values of 1.5-5.6µM (Table 3-10). Colupulone was the most potent inhibitor of COX-2, followed by xanthohumol and n-adlupulone (Table 3-10). Both alpha acids exhibited weak activity at the concentration tested. The $IC_{50}$ for active compounds (range: 0.6 - 2.3 µg/mL) outperformed most extracts (range: 0.9 - 21.8 µg/mL) for COX-2 inhibition (Table 3-10).

**Table 3-10. Mean COX-2 $IC_{50}$ (µg/mL) ± (SEM) values from isolated compounds found in hops.** Assays were performed thrice with $EC_{50}$ (±SEM) values were calculated using concentration-dependent curves (Ritz et al., 2015). Compounds not listed did not reach 50% inhibition at the highest concentration tested.

| Isolated compound | $IC_{50}$ µg/mL | µM |
|---|---|---|
| Colupulone | 0.6 (0.2) | 1.5 (0.5) |
| N-adlupulone | 2.3 (0.1) | 5.6 (0.2) |
| Xanthohumol | 1.2 (0.7) | 3.4 (2.1) |

As done with the DPPH results, metabolomic data of the three strongest (mean $IC_{50}$ = 1.4 µg/mL ± 0.4) and three weakest (mean $IC_{50}$ = 16.5 µg/mL ± 2.7) COX-2 inhibiting extracts were compared by discriminant analysis. Among the seven mass features that significantly differed between the strong and weak groups, only one was higher in the most active extracts, while six were associated with the weak extracts (Figure 3-3). Mass-feature content did not predict $IC_{50}$ values across extracts (n = 24, $p > 0.05$, Table 3-11).

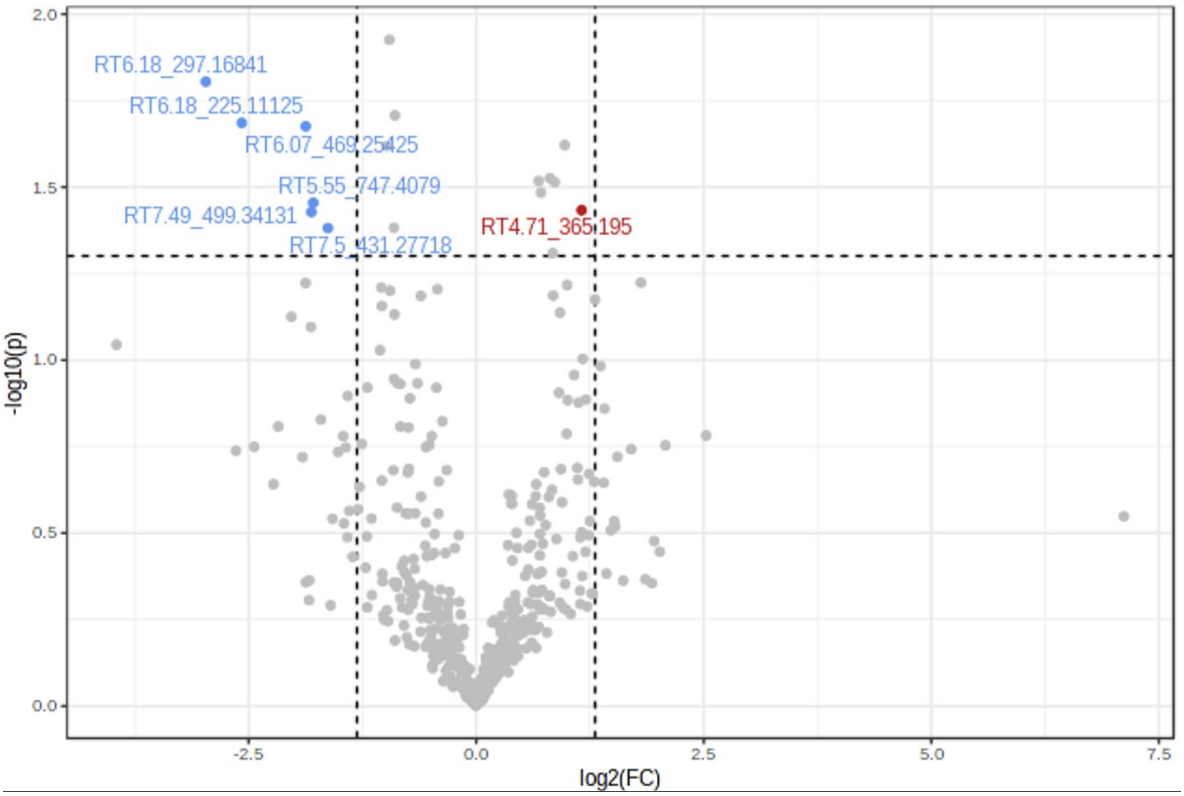


**Figure 3-3. Comparison of the three strongest to three weakest hop (*Humulus lupulus*) extracts on the COX-2 inhibition assay using a metabolomic dataset (containing 642 mass features).** Mass features are labelled as their retention time (RT) and mass-to-charge ratio [m/z]+. Mass features that were significantly greater in the stronger hop extracts were depicted in red while those that were significantly greater in the weaker hop extract samples were depicted in blue.

**Table 3-11. Simple linear regression for COX-2 $IC_{50}$ (µg/mL) as a function of normalized-centred-scaled mass features using *Humulus lupulus* extracts (n = 24)**. Chemical analysis occurred on a UPLC-Orbitrap. Mass-features were identified with the use of volcano plots followed by linear regressions presented below.

| **Predictor** | **$R^2$** | **Slope** | ***F*** | ***p*** |
|---|---|---|---|---|
| RT4.71_365.1950 | 0.085 | -1.02 | 2.03 | 0.17 |
| RT5.55_747.4079 | <0.01 | -0.004 | <0.001 | 0.99 |
| RT6.07_469.2543 | 0.072 | 0.64 | 1.70 | 0.21 |
| RT6.18_225.1113 | 0.004 | 0.15 | 0.08 | 0.79 |
| RT6.18_297.1684 | 0.005 | 0.14 | 0.13 | 0.73 |
| RT7.49_499.3413 | 0.007 | -0.27 | 0.15 | 0.70 |
| RT7.50_431.2772 | 0.078 | -0.16 | 0.08 | 0.78 |

There was no significant relationship between the $IC_{50}$ values for COX-2 inhibition and the $IC_{50}$ values for DPPH activity ($R^2 < 0.001$, $p = 0.9$).

## DISCUSSION

This study aimed to identify active metabolites underlying hops' radical scavenging and COX-2 inhibiting properties. Multiple biochemometric approaches were executed using targeted profiling and untargeted metabolomics data relative to single-point (% RSA or inhibition) and concentration-response ($EC_{50}$/$IC_{50}$) measures of activity across a collection of hops extracts.

The first approach, the comparison of activity between four groups (n = 6) with distinct marker profiles was facilitated by the variability of marker content across samples. These groups differed from one another based on significantly higher or lower levels of specific marker compounds relative to other groups. The one exception was for the HX group which contained similar xanthohumol levels as the HA group (Table 3-3). This could be partially attributed to the relatively low quantity of xanthohumol in the samples (Table 3-1) and the positive correlation between the alpha acids and xanthohumol among samples (Table 3-2), as previously noted in the literature (Killeen et al., 2014, 2017; Krofta et al., 2015). The second approach entailed regression analyses of marker compound concentrations relative to activity across all extracts (n = 24) followed by an assessment of marker compound activity. The third approach compared the untargeted metabolome of the three most and three least active extracts, with mass features associated with activity subsequently tested for associations to assay results across all samples.

For RSA, all three approaches yielded significant and converging results pointing to alpha acids as the primary radical scavenging metabolites. The HA group exhibited the greatest RSA response (Table 3-4), and both co- and n-ad-humulone concentrations were strongly associated with RSA and $EC_{50}$ (Table 3-5). High variability in n-adhumulone content among the extracts and lower variability across the other marker compounds (Table 3-3) could explain why other marker compounds did not significantly predict RSA (Table 3-5), despite their comparable

activity when tested as pure compounds. Notably, all marker compounds (based on mass) performed as well or better than standard dietary antioxidants vitamin C, quercetin, and catechin (Table 3-6). Lastly, untargeted metabolomics identified four mass features that were significantly associated with $EC_{50}$ (Table 3-7), one of which was tentatively identified as phloroisovalerophenone (Nicácio et al., 2022). The higher representation of phloroisovalerophenone, a leucine-derived precursor in the bitter acid biosynthetic pathway (Champagne & Boutry, 2017; Nicácio et al., 2022; Paniego et al., 1999; G. Wang et al., 2008), and bitter acid derivatives among more active samples suggests that elevated activity of bitter acid biosynthetic pathways rather than simply alpha acid concentration contributes to the RSA of a given hop sample.

Previous research has established the RSA of hop extracts and their constituents (Gorjanović et al., 2013; Keskin et al., 2019; Kobus-Cisowska et al., 2019; Kowalczyk et al., 2013, 2013; Lyu et al., 2022; Wu et al., 2020). Some of these studies similarly used the DPPH assay and cone extracts, associating RSA with total bitter acid content, total phenolic content and total flavonoid content (likely as an initial proof of concept) and subsequently observed a predictive relationship between RSA and phenolic and flavonoid content (Lyu et al., 2022; Wu et al., 2020), though they did not model RSA to specific bitter acid content. Other researchers found a strong positive correlation ($r = 0.99$) between total phenolic content and RSA (Gorjanović et al., 2013). They also tested a hop tannin mixture and found the strongest DPPH activity associated with quercetin, rutin, and catechin that demonstrated stronger RSA than xanthohumol, humulone, and lupulone (Gorjanović et al., 2013). However, based on the presented HPLC data (Figure 3-1), few flavonoids or phenolics were detected in our extracts beyond xanthohumol. While these more polar compounds were poorly extracted by our selected solvent (99% ethanol), they also

represent a smaller fraction of total metabolism (<5% of cones dry weight) relative to bitter acids (up to 30% dry weight) (Almaguer et al., 2014; Van Cleemput et al., 2009).

Indeed, previous studies highlight the impact of extraction solvent on the free-radical scavenging of hop extracts. For example, when testing hop pellet extracts in multiple RSA assays, Wu, and colleagues (2020) reported stronger activity in extracts made with a higher percentage of ethanol compared to more aqueous extracts. Meanwhile, Kobus-Cisowska and colleagues (2019) found that aqueous hop extracts were more active than ethanolic hop extracts using the DPPH assay but less active using the ABTS assay. The DPPH assay uses methanol as the primary solvent, while ABTS uses water (Wu et al., 2020), so the assay medium – in addition to the extraction solvent – contributes to inconsistencies in the literature. In particular, water-soluble phenolic compounds would be extracted more efficiently in more aqueous solvents and perform better in water-based assays while the bitter acids are better solubilized in ethanolic (organic) solvents and thus scavenge radicals more effectively in non-aqueous assays.

Biologically, the effect of a compound's RSA on different tissues can vary depending on the solubility in the medium (e.g., aqueous cytosol vs. lipid-rich membranes). The oxygen radical absorbance capacity (ORAC) was created that sums the RSA from both aqueous and lipophilic environments to achieve a total ORAC score (Yamaguchi et al., 2009). Xanthohumol had the highest total ORAC score, while lupulone had a high ORAC score in the lipid assay, and humulones performed moderately across both aqueous and lipophilic environments (Yamaguchi et al., 2009). Compared to the present results, each of the bitter acids outperformed isolated xanthohumol (Table 3-6) and the average $EC_{50}$ for the plant extracts (Table 3-4).

The inhibition of COX-2 by hop extracts has received less attention compared to the RSA of hop extracts (though see (Hougee, 2008; Tripp et al., 2005) for exceptions), as the primary focus has been on testing isolated hop-derived compounds (Gerhauser et al., 2002; Hall et al., 2008; Lee et al., 2007; Tripp et al., 2005, 2009; Yamamoto et al., 2000). The present experiment demonstrated the anti-inflammatory potential of hops via COX-2 inhibition in a concentration-dependent manner. In contrast to RSA, biochemometric analyses failed to identify metabolites driving COX-2 inhibition: there was no group effect (Table 3-8), no predictive models based on marker compounds (Table 3-9), and no HRMS mass features associated with inhibition (Table 3-11). Isolated xanthohumol inhibited COX-2 in a concentration-dependent manner with $IC_{50}$ < 10μM (Table 3-10), supporting the predictions and previous findings (H. Liu et al., 2020). In contrast to previous studies (Tripp et al., 2005; Yamamoto et al., 2000), the alpha acids failed to demonstrate a concentration-dependent relationship, though the beta acids demonstrated strong activity and should be explored further.

The lack of identified active compounds could be explained based on a variety of factors. First, in comparison to RSA, there was little variability of COX-2 inhibition and $IC_{50}$ values between hop groups (Table 3-8) or among samples ($IC_{50}$ range: 1-17 μg/mL), limiting the sensitivity for detecting changes in activity despite the extensive range of marker compound concentration. Second, active marker compounds, tested in isolation, elicited $IC_{50}$ values comparable to those of the extracts, which contained levels of these actives at levels far below their inhibitory concentration. Lastly, compounds that were not quantified for this study might have attributed to the inhibition.

Previously, selected hop compounds were screened for COX-2 inhibition and GI toxicity using RAW264.7 and AGS cell lines, respectively. The rho-iso-alpha-acids (RIAA) potently

inhibited COX-2 without impacting COX-1 and had low gastric toxicity (Hall et al., 2008; Tripp et al., 2005). RIAA was ranked as the strongest COX-2 inhibitor, followed by iso-alpha acids (IAA) and tetrahydroiso-alpha acids (THIAA), with alpha acids and beta acids acting less potently (individual alpha and beta acids cohumulone, n-adhumulone, colupulone, n-adlupulone were not tested) (Tripp et al., 2005). Trace amounts of IAA or derivatives of the bitter acids may have contributed to bioactivity and warrant further investigation as minor constituents that went undetected or co-eluted with major bitter acids.

In the present experiment, crude extracts and certain markers showed promising potency using a human recombinant COX-2 biochemical assay. More complex models suggest that hops' COX-2-mediated anti-inflammatory effects vary based on the model. Using Whole Blood Assay and LPS-stimulated PBMC model of inflammation, researchers reported that a standardized $CO_2$ hops extract inhibited COX-2 with an $IC_{50}$ of 20 µg/mL and 3.6 µg/mL, respectively (Hougee, 2008). Alpha acids constituted the majority of plant secondary metabolites in the standardized $CO_2$ extract (>70% humulone), and with only 2.3% beta acids (Hougee, 2008) but only one extract was evaluated.

Metabolomics is a new way of identifying many compounds that would typically be missed using conventional analytical methods (Arnason et al., 2022). Metabolomics can also identify clusters of different compounds that associate with one another or clusters based on different experimental protocols (Witte & Overy, 2022). The present study followed a published protocol whereby plant extracts that stimulate glucose uptake were compared to those that do not (Shang et al., 2015). In the present study, the most and least active RSA samples were analyzed. This resulted in six mass features being identified as significantly differing between the most and least

active groups, the majority significantly modelled the $EC_{50}$ values from the DPPH dataset, with phloroisovalerophenone was tentatively identified from the literature (Nicácio et al., 2022).

Bioassay-guided fractionation is a popular approach to identifying bioactive components found in natural health products (Awad et al., 2007; Kellogg et al., 2016; Shang et al., 2015), and this approach has been applied to hops that used machine learning to identify xanthohumol as an anti-inflammatory agent (Heussner, 2022) and 8-PN as a potent phytoestrogen (Milligan et al., 1999). A future experiment could take a single hop extract and create hundreds of fractions, followed by pooling similar fractions. Next, these pooled fractions can be screened at a single concentration on the DPPH and COX-2 inhibition assays, whereby active compounds with high yields could be further purified and identified using mass spectroscopy and NMR.

The use of *in silico* modelling would be another helpful approach to identifying bioactive metabolites in plants. For example, multiple phytochemicals, such as polyphenols, have been shown to inhibit COX-2 (Quiñones et al., 2013). An *in silico* study found that curcumin (a bioactive polyphenolic compound found in turmeric) to be a potent inhibitor of COX-2 along with kaempferol, apigenin, and genistein (Maldonado-Rojas & Olivero-Verbel, 2011). Furthermore, celecoxib and curcumin synergized because they have different binding sites on the COX-2 enzyme (Maldonado-Rojas & Olivero-Verbel, 2011). Testing the synergistic capabilities of hops constituents and celecoxib using a similar methodology would be fascinating. *In silico* modelling could screen for potentially potent phytochemicals, followed by metabolomics to identify clusters of compounds or samples that are associated with bioactivity. Lastly, assay-guided fractionation, though tedious at times, can screen dozens of fractions, followed by isolating, identifying, and testing these compounds in a concentration-dependent manner to demonstrate causality.

An attempt was made to run hop extracts in a concentration-dependent manner on 5-LOX inhibition, the complimentary arachidonic pathway to COX and is associated with immune signalling. There was no consistent concentration-dependent response using hop extracts, so the experiment was excluded from the thesis. However, testing hop extracts on a different LOX assay might yield more promising results and should be pursued.

Future experiments testing the anti-inflammatory properties of hops should test extracts and isolated compounds on ELISAs with an emphasis on IL-1ß, -6, -8, -10, and TNFα would be particularly useful to identify the interleukins and cytokines that hop targets. Furthermore, it is still unknown how hops would interact with COX-1. Future studies could include other hop-derived compounds that were tested previously, such as THIAA and RIAA (Tripp et al., 2005), along with hop tinctures that use ethanolic extraction with minimal drying to preserve volatile compounds (these volatile compounds could interact with bioactivity) and quantify the total phenolic content and total flavonoid content along with quantifying specific phenolics that could be driving activity. Another experiment would be to run an *in-silico* model to predict where the hop compounds interact with the COX-2 sites. Cytotoxicity was measured for cannabinoids and cannabis extracts using the WST-1 assay (Waldbillig, 2022), this same approach could be applied to hop target compounds and extracts. As for RSA, applying the biochemometric modelling approach with testing hop extracts in both hydrophilic and lipophilic environments would make for an interesting project. Future studies could also test the relationship between TPC, TFC, bitter acids, and free-radical scavenging activity. If additional free-radical scavenging assays were run, then a redundancy analysis could be used to identify compounds that correlate with each other and whether they impact bioactivity. Moreover, testing hop extracts and constituents on various chemical, cell-based, and animal-based experiments to identify RSA

could further support the medicinal properties of hops (Jainu & Devi, 2005; Yokozawa et al., 1998).

## CONCLUSION

Collectively, this was the first study to systematically test a chemically diverse set of hop samples on free-radical scavenging activity and COX-2 inhibition using both targeted and untargeted biochemometric approaches. The alpha acids (i.e., humulones) were significant models for RSA but not COX-2 inhibition. Meanwhile, isolated colupulone and n-adlupulone were potent in both assays. Future hop experiments could model hops' interaction on the COX-2 enzyme, test hop extracts on many other anti-inflammatory assays, and assay-guided fractionation on a specific hop extract. Overall, these results will help in the development of a natural health product or dietary supplement that targets free-radical scavenging activity and or anti-inflammatory properties.

# CHAPTER 4: THE INHIBITORY POTENTIAL OF HOPS (HUMULUS LUPULUS) ON ENDOCANNABINOID DEGRADATION ENZYMES FATTY ACID AMIDE HYDROLASE (FAAH) AND MONOACYLGLYCEROL LIPASE (MAGL)

## ABSTRACT

The human endocannabinoid system (ECS) consists of endogenously produced ligands that activate the cannabinoid receptors and are rapidly degraded, primarily by two enzymes: fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL). Inhibition of FAAH and MAGL can increase the concentration of the endocannabinoids and enhance ECS activation, can slow the production of pro-inflammatory arachidonic-acid-derived prostaglandins, and has been shown to reduce anxiety and neuroinflammation. Some plant metabolites have demonstrated ECS modulatory effects, most notably cannabis. Like cannabis, its closest relative hops (*Humulus lupulus*, Cannabaceae) were traditionally used to treat anxiety, spasms, and insomnia. The effects of hops, however, on ECS activity remain poorly studied despite their unique bitter acid and flavonoid secondary metabolites. In this study, ethanolic extracts of hops (n = 24) were tested for FAAH and MAGL inhibitions modelled to extract's chemistry based on quantitative profiling and untargeted metabolomic data. As colupulone concentrations increased so did FAAH inhibition, but negatively associated with MAGL inhibition. N-adhumulone concentrations were negatively associated with FAAH inhibition and showed no relationship to MAGL $IC_{50}$ values. The amount of xanthohumol in the plant extracts did not predict activity in either assay, although it demonstrated bioactivity when tested alone. Assay-guided fractionation identified cohulupone and hulupone as inhibitors of FAAH and reconfirmed xanthohumol as an inhibitor effect of both FAAH and MAGL. This was the first time that hop extracts and its constituents demonstrated FAAH and MAGL inhibition. These results could be applied in the development of Natural Health Products that modulate the ECS.

# INTRODUCTION

Hops (*Humulus lupulus* L., Cannabaceae) is a dioecious perennial bine typically grown in temperate regions (Koetter & Biendl, 2010; Olšovská et al., 2016; Zugravu et al., 2022). There are three species of "hops" or hop plants: *H. lupulus* (common hop), *H. japonicus* (Japanese hop), and *H. yunnanensis* (Yunnan hop) (Small, 1978). The term hop is employed conventionally to designate common hop, and the term hops is employed to designate the female flower clusters (strobili, cones) of the female plants, which are usually seedless. Female hop cones contain lupulin glands that house a yellow resin (lupulin) with unique secondary plant metabolites (Olšovská et al., 2016). These include xanthohumol and bitter acids (cohumulone, n-adhumulone, colupulone, and n-adlupulone). The bitter acids are derived from prenylated phloroglucinol and form the soft resin component of hops (Carbone & Gervasi, 2022). The majority (>95%) of cultivated hops (>100,000 metric tons annually) are harvested and used in the brewing industry (Kowalczyk et al., 2013; Krofta & Mikyška, 2014; Macchioni et al., 2021; Zhang et al., 2021), however, with a long history of medicinal use across its distribution (Carbone & Gervasi, 2022; Karabin et al., 2015; Karabín et al., 2016; Koetter & Biendl, 2010; Krofta & Mikyška, 2014; Moir, 2000; Olšovská et al., 2016; Teghtmeyer, 2018; Van Cleemput et al., 2009; Yamaguchi et al., 2009; Zanoli & Zavatti, 2008), a small proportion was used for human health as natural health products (NHPs) or dietary supplements (Sanz et al., 2019). Supporting the therapeutic potential of hops, extracts of infructescence from the female hop plant have demonstrated anti-inflammatory, antibacterial, anxiolytic, and sedative properties (Astray et al., 2020; Carbone & Gervasi, 2022; Zanoli & Zavatti, 2008).

Hops and cannabis belong to the Cannabaceae family (Olšovská et al., 2016). Secondary metabolites valuable to humans are found in the female reproductive plant parts in each species,

and both have been shown to treat similar conditions (Astray et al., 2020; Katchan et al., 2016). Chemically, cannabinoids are the dominant secondary metabolites in cannabis (Small, 2016) but are not present in hops. In contrast, bitter acids are the most dominant secondary metabolites in hops (Krofta & Mikyška, 2014) and are absent in cannabis. While cannabis and cannabinoids elicit their effects through modulation of the endocannabinoid system (ECS), among other mechanisms, hops and bitter acids have no reported cannabimimetic activity to date.

The endocannabinoid system is present in all Chordata (i.e., animals with a spine). It influences numerous biological functions, including but not limited to nociception, cognition, mood, metabolism, inflammation, growth, and development (Chanda et al., 2010; MacCallum & Russo, 2018). The ECS comprises the cannabinoid receptors, their endogenous ligands (endocannabinoids), and the enzymes responsible for endocannabinoid metabolism (Gasperi et al., 2014).

The cannabinoid receptors are G-coupled protein receptors with two known human subtypes, cannabinoid receptor 1 (CB-1) and cannabinoid receptor 2 (CB-2) (Di Marzo, 2008). CB-1 receptors are found primarily in the central nervous system, and activation of this receptor is responsible for some of the psychoactive properties of cannabis along with pain management, appetite, and mood (Katchan et al., 2016; Pertwee, 2006). Meanwhile, CB-2 is expressed primarily in immune cells and modulates inflammatory signalling and modulates the immune system (Katchan et al., 2016; Pertwee, 2006). Identifying CB-1 and CB-2 agonists could be useful in treating inflammatory and autoimmune conditions (Rom & Persidsky, 2013).

Ligands for the cannabinoid receptors can be broadly categorized into endocannabinoids, phytocannabinoids, and synthetic cannabinoids. Endocannabinoids, as the name implies, are produced within the body. The dominant endocannabinoids are anandamide (AEA) and 2-

arachinoylamide (2-AG). Anandamide acts as a partial agonist and has a more significant affinity to the CB-1 receptors, while 2-AG acts as a full agonist and binds with moderate affinity to CB-1 and CB-2 (Tsuboi et al., 2018).

Phytocannabinoids are derived primarily from the cannabis plant, most notably tetrahydrocannabinol (THC), which acts as a partial CB receptor agonist (MacCallum & Russo, 2018; Pertwee, 2008). Chemically, this term relates to the structure of cannabis-derived cannabinoids, but pharmacologically, the term has been applied to structurally unrelated natural products with CB agonist or antagonist activity. Synthetic cannabinoids such as WIN55-212 and HU-308, developed as pharmaceuticals and now used in research, typically have a high affinity for one or both CB receptors (Guindon & Hohmann, 2008; Klein, 2005).

Fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL) are the primary catabolic enzymes for AEA and 2-AG, respectively (Di Marzo, 2008). Inhibiting these enzymes increases the concentration of endocannabinoids that could further activate endocannabinoid receptors (Di Marzo & Maccarrone, 2008; Piomelli, 2014). Since arachidonic acid is a byproduct of AEA and 2-AG degradation, FAAH/MAGL inhibition has been proposed as a mechanism to reduce inflammation (Donvito et al., 2018; Maccarrone, 2020; Sasso et al., 2015; Scarpelli et al., 2016; Schurman & Lichtman, 2017; Tung et al., 2021). Moreover, MAGL inhibition is a potential target for reducing the neuroinflammation associated with Parkinson's and Alzheimer's disease, as half of the arachidonic acid in the brain (used as a substrate for inflammatory pathways) is produced by the breakdown of the endocannabinoid 2-AG by MAGL (Tung et al., 2021). In the brain, the 2-AG level was an order of magnitudes greater than AEA, and 2-AG acts as a full agonist to both CB-1 and CB-2 (Schurman & Lichtman, 2017; Tsuboi et al., 2018). Modulating the ECS has been proposed to help in the treatment of cancer and neurodegenerative

diseases via activation of the CB receptors (De Leo et al., 2018; Granchi et al., 2021; Maccarrone, 2020; Schurman & Lichtman, 2017).

Research into phytochemicals beyond cannabinoids that interact with the ECS is rapidly expanding as more plant-derived compounds are demonstrating their activity on the endocannabinoid system (Appendino et al., 2022; Gertsch et al., 2010; Russo, 2016). For example, beta-caryophyllene, a sesquiterpene found in many different types of plants, including hops and cannabis, is a selective CB-2 agonist (Gertsch et al., 2008, 2010). Furthermore, the activation of CB-2 via beta-caryophyllene has anti-inflammatory and sedative properties (Gertsch et al., 2010). Echinacea extracts have been shown to partially inhibit FAAH activity primarily due to its caffeic acid derivatives and alkylamides (R. Liu, Burkett, et al., 2021). The flavonoid kaempferol and the isoflavone biochanin A both displayed FAAH inhibitory activity without any affinity for CB-1 or CB-2 (Thors et al., 2008, 2010). Other inhibitors such as genistein, daidzein, and formononetin have also been identified as FAAH inhibitors along with compounds found in nutmeg and maca (Maccarrone, 2020; Thors et al., 2008, 2010). Jewenol A, a diterpenoid, found in *Salvia pseudorosmarinus*, has been shown to inhibit MAGL (De Leo et al., 2018). In addition, *Cistanche phelypaea*, a desert plant, contains a phenylethanoid glycoside that exhibits MAGL inhibition activity greater than other phytochemicals tested in the study (Beladjila et al., 2018). Studies have also demonstrated MAGL inhibition by triterpenoids such as pristimerin (King et al., 2009) and ß-amyrin (Chicca et al., 2012; Merali et al., 2018). Pertinent to this thesis, the flavonoid 8-prenylnaringenin (8-PN), found in hops, has been shown to inhibit MAGL, with other prenylated flavonoids predicted to inhibit MAGL as well (Tung et al., 2021).

Since the major hop metabolites, bitter acids and xanthohumol, are prenylated phloroglucinols and prenylated chalcones (Forino et al., 2016; Van Cleemput et al., 2009), respectively, share similar properties to 8-PN and other flavonoids, they may inhibit FAAH/MAGL through similar mechanisms. However, no studies have explored the potential of hop extracts and secondary metabolites as FAAH and MAGL inhibitors.

Accordingly, this study tested a collection of hops ethanolic extracts made from cultivated female hop infructescence for their ability to inhibit FAAH and MAGL. This was done using *in vitro* human recombinant enzyme assays to characterize inhibitory activity, phytochemical analyses to define hop samples, and multiple biochemometric approaches to identify contributing active metabolites.

# METHODS

## Chemicals and reagents

The ICE-4 hop standard was purchased from the American Society of Brewing Chemists (Saint Paul, Minnesota, USA). MQ water was purified in-house. Xanthohumol and iso-xanthohumol standards were purchased from Chromadex (Longmont, Colorado, USA). The HPLC columns were purchased from Phenomenex (Torrance, California, USA), while the 0.22µm filter tips and HPLC vials were purchased from Chromatographic Specialties Inc. (Brockville, Ontario, Canada). Bitter acid constituents (cohumulone, n-adhumulone, colupulone, n-adlupulone) with 95% purity were isolated using an HPLC, as described in Chapter 2. A hulupone standard mix was graciously donated by Kalsec Inc. (Kalamazoo, Michigan, USA). The following reagents were used for the bioassay-guided fractionation. Silica gel was SiliaFlash Irregular Silica Gels - F60,40, 63um, 60A (Silicycle Inc, Québec, Canada). Solvents were

sourced from ACS reagent MilliporeSigma Canada Ltd. (Oakville, ON, Canada): Hexane (≥98.5%), DCM (≥99.5%), methanol (≥99.8%). All other reagents were purchased from Fisher Scientific unless otherwise stated.

## Plant materials

Commercial hop samples (n = 24) were selected based on phytochemical profiles, which provided a broad range of marker compound profiles (bitter acid and xanthohumol) as well as four distinct chemical groups (n = 6). Sixteen hop samples were purchased as hop pellets from www.ontariobeerkegs.com. All pellets were T90 except for one that was a lupulin-enriched pellet. Yakima Chief Hop Company (Yakima, Washington, USA ) (n = 12) was the primary supplier. One sample was from LD Carlson (Kent, Ohio, USA) (n=1). Kichesippi Brewery (Ottawa, Ontario, Canada) generously donated three hop pellet samples. Cone samples were obtained from various sources, including Carleton University (Ottawa, Ontario, Canada) (n = 2), CWG Farm Inc (St-Eugène, Ontario, Canada) (n = 2), Highland Farm (Meaford, Ontario, Canada) (n = 3), and the author's private property. To ensure freshness and quality, all samples were stored in a dark freezer at a temperature of -20°C until extraction.

## Preparation of extracts

Ethanolic extracts of hops were made as described in Chapter 2. The hop pellets were manually crushed, whereas the cones were pulverized using a Magic Bullet Blender ®. The extracts were made using 6.00g dried plant material extracted twice with 20 mL/g 99% ethanol under sonication for 10 min. Supernatants were pooled, filtered using Whatman Grade A filter paper, then dried using a roto-evaporator (Yamato RE 500, Yamato Scientific, Japan) and Centrivap Centrifugal Vacuum Concentrator (Labconco Centrivap, Model 7810014, Kansas City, Missouri, USA), followed by freeze-drying (SuperModulyo220 freeze dryer, Thermo

Fisher Scientific, Nepean, Ontario, Canada). Dried ethanolic extracts were stored in a dark freezer (-20°C) until experimental use.

## Targeted phytochemical analysis by HPLC-UV-DAD

The methods for targeted phytochemical analysis were provided in greater detail in Chapter 2. Briefly, hop extracts were reconstituted in 99% ethanol at 10 mg/mL and were filtered through a 0.22 µM PTFE syringe filter. The High-Performance Liquid Chromatography Ultraviolet Spectroscopy Diode Array Detection (HPLC-UV-DAD) was used to quantify cohumulone, n-adhumulone, colupulone, n-adlupulone and xanthohumol. The HPLC was equipped with a Kinetex C18 5U (100 x 4.6mm) column, with a flow rate of 1.5 mL/min, a stop time of 17.1 min, and a post-run of 3 min at a temperature of 40°C. Mobile phase A was water, and mobile phase B was methanol, both with 0.1% trifluoracetic acid (TFA). The starting condition was 52.5% mobile phase B for 1 min, increasing to 90% B at 13 min, and 100% B by 13.1 min, monitored and quantified at 300nm. Cohumulone, adhumulone, colupulone, n-adlupulone, and xanthohumol were quantified relative to calibration curves generated using reference standards.

## Untargeted metabolomic analysis

Stock solutions of hop extracts (100 mg/mL DMSO) were diluted to 500 µg/mL aliquots using MS-grade methanol. The extracts were injected at 5µL onto the Thermo Scientific Dionex Ultimate 3000 UPLC system with Thermo Scientific LTQ Orbitrap XL high-resolution mass spectrometer. The run specifications can be found in a recent publication (Witte & Overy, 2022), and were outlined in Chapters 2 and 3, with reserpine as the internal standard and accounted for an error of 12 ppm. Metabolomic analyses using the curated dataset containing 642 mass features were conducted using the open-access online tool MetaboAnalyst5.0 (MetaboAnalyst, 2023). Volcano plots were produced to identify centred-scaled-normalized mass features that

significantly differ between the three most potent and three least potent extracts. Mass features were tentatively identified from the published literature on hop metabolomics. An online tool was used to determine the error between a compound's monoisotopic mass and the mass feature: (https://warwick.ac.uk/fac/sci/chemistry/research/barrow/barrowgroup/calculators/mass_errors/). Reported mass features and monoisotopic masses were also compared to mass features identified from the volcano plot. These two techniques permitted tentative identification of mass features, and the error from the reserpine standard established an anchor for relative error.

## Assay-guided fractionation and isolation of active compounds

Silica gel column fractionation

A glass column (38cm x 3.1cm) was packed with silica gel and equilibrated for 24h. Approximately 2.0g of crude ethanolic plant extract with potent FAAH activity was mixed with 2.0g celite and placed on the silica gel-packed column. The extract was separated using 500 mL of the following ratios of hexane-to-dichloromethane (DCM): 100:0, 90:10, 80:20, 70:30, 60:40, 50:50, 40:60, 30:70, 20:80, 10:90, and 0:100, followed by methanol to DCM mixtures of 0:100, 10:90, 20:80, 30:70, 40:60, 50:50, 60:40, 70:30, 80:20, 90:10, and 100:0. This was followed by 500 mL 50% methanol in MQ water. Fractions were collected every 20 mL, resulting in over five hundred separate fractions. Collected fractions were analyzed using thin-layer chromatography (TLC) and pooled based on shared chemistry for thirty-seven fractions. These fractions were subsequently dried and reconstituted for screening experiments.

Subfraction and phytochemical isolation

Once fractions were assessed on a bioassay, those with strong activity were run on the HPLC to observe complexity before sub-fractionation. The fractions were prepared at 5 mg/mL and filtered using a 0.22µm PTFE filter. A Kinetex C18 5U (100 x 4.6mm) column was used, with a

flow rate of 1.5 mL/min, a temperature of 40°C, a stop time of 20 min, a method wash of 5 min, and a 4-min post-run. The initial condition was 0% mobile phase B (i.e., methanol), moving to 100% B in 20 minutes.

To obtain sub-fractions and, if possible, pure compounds from the selected active fractions a fraction collector (Agilent G1364C, Mississauga, Ontario, Canada) was connected to the HPLC-DAD. A Phenomenex C18 (2) 100 Å (20 x 4.6mm) column was used with a flow rate of 2.0 mL/min, temperature of 40°C, mobile phase A is water and mobile phase B is acetonitrile (ACN), with 70% B until 8 min, then 100% B at 14.00, 100% B at 17.0 min, and 50% B at 17.10 min. Following peak-based fraction collection (300nm), sub-fractions were dried using the Centrivap Centrifugal Vacuum Concentrator, followed by lyophilization overnight in a dark room. This same protocol was used to fractionate the standardized hulupone mixture provided by Kalsec.

UHPLC-MS – of active subfractions and isolated compounds

A Shimadzu UHPLC-PDA-MS system consisting of LC30AD pumps, a CTO20A column oven, a SIL-30AC auto-sampler, an SPD-M20A Photodiode array detectors and an LCMS-2020 mass spectrometer was used to analyze the active subfractions and hulupones. A Phenomenex Kinetex PS C18 column 100 x 2.1 mm, 2.6 µm particle size with a Phenomenex C18 Guard column was used for separation. The mobile phase comprised $H_2O$ (A) and Acetonitrile (B) with 0.1% formic acid. The gradient elution method was initiated at 30% B, then increased to 100% B at 9 min and 70% B at 18 mins. The column was then washed with 100% B for 2 minutes and returned to the initial conditions for a 4-minute re-equilibration. The flow rate was set to 0.3 mL/min with the column temperature at 50˚C. The Photodiode array detectors were set to monitor from 190 to 600 nm. The mass spectrometer with electrospray ionization (ESI) interface

was operated in both positive/negative scan modes. The nebulizing gas flow was set at 1.5 L/min, and the drying gas flow was at 15 L/min. The desolvation line and heat block temperatures were set at 250°C and 400°C, respectively. The m/z range of positive and negative scans is from 100 to 1000 with 10000 μ/sec scan speed.

## Expression and purification of FAAH and MAGL protein

Expression plasmids for humanized rat FAAH and human MAGL protein were generously provided by Dr. B. Cravatt (Scripps Research, San Diego, USA). FAAH was expressed and purified by the postdoctoral fellow Dr. Graham Heberlig based on previously published methods (Mileni et al., 2008). Briefly, frozen transfected kanamycin-resistant *Escherichia coli* (*E. coli*) contained a humanized rat protein expression for FAAH. These cells were propagated in SuperBroth until the OD = 0.4-0.5 at 600 nm. The protein was then induced with 1M Isopropyl β- d-1-thiogalactopyranoside (IPTG) and grown overnight at room temperature. After sonicating, the supernatant ran through a nickel column containing 2 mL of nickel-resin solution (Profinite ™ IMAC Ni-Charged Resin CAT #156-0133), and the supernatant solution flowed through. The concentrated protein was combined with 50% glycerol, aliquoted to 50μL, and kept in the -80°C freezer until ready to use.

Human recombinant MAGL was expressed using an in-house protocol developed by the postdoctoral fellow Dr. Graham Heberlig and produced by Nick Coates. These methods were previously published (Zvonok et al., 2008). Briefly, transfected *E. coli* BL-21 with human-MAGL expression were propagated in LB broth until the OD=0.4-0.5 at 600 nm. The protein was then induced with 1M Isopropyl β- d-1-thiogalactopyranoside (IPTG) and grown overnight at room temperature. After centrifuging and sonicating the cells, the supernatant ran through a nickel-resin column containing 3 mL of nickel-resin solution (Profinite ™ IMAC Ni-Charged

Resin CAT #156-0133). The concentrated protein was then combined with 50% glycerol, aliquoted to 50 µL, and kept in the -80°C freezer until ready to use.

**Inhibition assays**

The FAAH inhibition protocol was adapted from the Cayman Chemical Fatty Acid Amide Hydrolase Inhibitor Screening Kit (Item No. 10005196). The enzyme concentration for fraction screening was adjusted to standardize enzyme activity across batches of enzymes made in-house. The Cytation plate reader incubator was set to 37°C. The vehicle control was 5% DMSO; the substrate was 20 µM AMC-AA. The FAAH protocol lasted 40 minutes, taking a fluorescence reading every minute with an excitation wavelength of 350 nm and an emission wavelength of 460 nm. Each hop extract (dissolved in DMSO) was tested at a range of concentrations (0.4-250 µg/mL), pooled fractions were screened at 250 µg/mL, the positive control was tested at a standard concentration of 5 µg/mL JZL-195, and the isolated compounds were tested from 0.02-50 µg/mL. The FAAH enzymes used to test the hop extracts and isolated compounds were made in-house from humanized rat FAAH. In contrast, the FAAH used for the assay-guided fractionation was a human-recombinant FAAH purchased by Cayman Chemicals.

The MAGL inhibition protocol is adapted from the Cayman Chemical Monoacylglycerol Inhibitor Screening Kit (Item No. 705192). JZL-184 was the positive control, DMSO was the vehicle control (5%) when testing hop extracts and 2.5% when screening the fractions, and the substrate was 236µM 4-nitrophenyl acetate. The MAGL protocol lasted 15 minutes with an absorbance reading every 5 minutes at 405nm; the third reading (at 10-min) was used for the analyses. Each hop extract (dissolved in DMSO) was tested at a range of concentrations (0.08-250 µg/mL), pooled fractions were screened at 125 µg/mL, the positive control was tested at a standard concentration of 5 µg/mL JZL-184, and the isolated compounds were tested from 0.02-

50 µg/mL. The MAGL enzymes used to test both the hop extracts, isolated compounds, and assay-guided fractionations were made in-house from human recombinant MAGL.

Percent inhibition was calculated by taking an average fluorescence (FAAH) or absorbance (MAGL) from three wells from the same sample. The inhibition was calculated as follows:

$$[1 - \frac{(Sample\ Enzyme\ End - Sample\ Enzyme\ Start) - (Sample\ Blank\ End - Sample\ Blank\ Start)}{(Vehicle\ Control\ Enzyme\ End - Vehicle\ Control\ Enzyme\ Start) - (Vehicle\ Control\ Blank\ End - Vehicle\ Control\ Blank\ Start)}] * 100$$

The $IC_{50}$ data utilized the triplicate inhibition data across all concentrations for each hop extract, fractions, and isolated marker compounds independently. These data were run using log-logistic 3 and log-logistic 4 models using the DRC package in RStudio (Ritz et al., 2015).

## Statistical analysis

All statistical analyses were run using RStudio 4.3.2. A one-way ANOVA accompanied by the Tukey HSD *post hoc* test was applied to the targeted HPLC results to establish chemical groupings (High-alpha acids HA; high-beta acids, HB; high-xanthohumol, HX; low alpha-beta LAB) as described in Chapter 3.

The statistical analyses for both FAAH and MAGL follow a similar analysis to the previous chapter (i.e., Chapter 3), whereby group differences were calculated using an ANOVA with a Tukey-HSD post hoc test for percent inhibition (at 10 µg/mL) and $IC_{50}$. This was followed by simple linear regressions and stepwise regressions with marker compound concentrations as predictors and either percent inhibition or $IC_{50}$ as the response variable.

For comparison of untargeted metabolomics data. Volcano plots were used to identify mass-features that significantly differed between the three strongest and three weakest samples from each dataset (FAAH and MAGL inhibition separately), followed by linear regressions with the relative abundance of mass-features and $IC_{50}$ from all 24 hop extracts as predictors and the

response, respectively. Mass-features that significantly modelled $IC_{50}$ were compared to mass-features in the published literature to tentatively identify these compounds.

# RESULTS

## Targeted profiling of marker compounds into chemical groups

Individual extracts were assigned to one of four groups based on marker compound profile: 1) High-alpha acid (HA), 2) high-beta acids (HB), 3) high-xanthohumol (HX), or 4) low-alpha and beta acid (LAB) (n = 6 per group). The high-alpha group (HA) had 2.5x more alpha acid than high-beta (HB), 1.67x more than high-xanthohumol (HX), and 4x more than the low alpha-beta (LAB) group (Figure 4-1). Similarly, the HB group had 2.1, 1.56, and 2.35x beta acid than HA, HX, and LAB, respectively (Figure 4-1). HX had 1.5 and 2.68x more xanthohumol than HB and LAB but no significant difference from HA (Figure 4-1). LAB had significantly less total bitter acids than the other three groups (Figure 4-1).

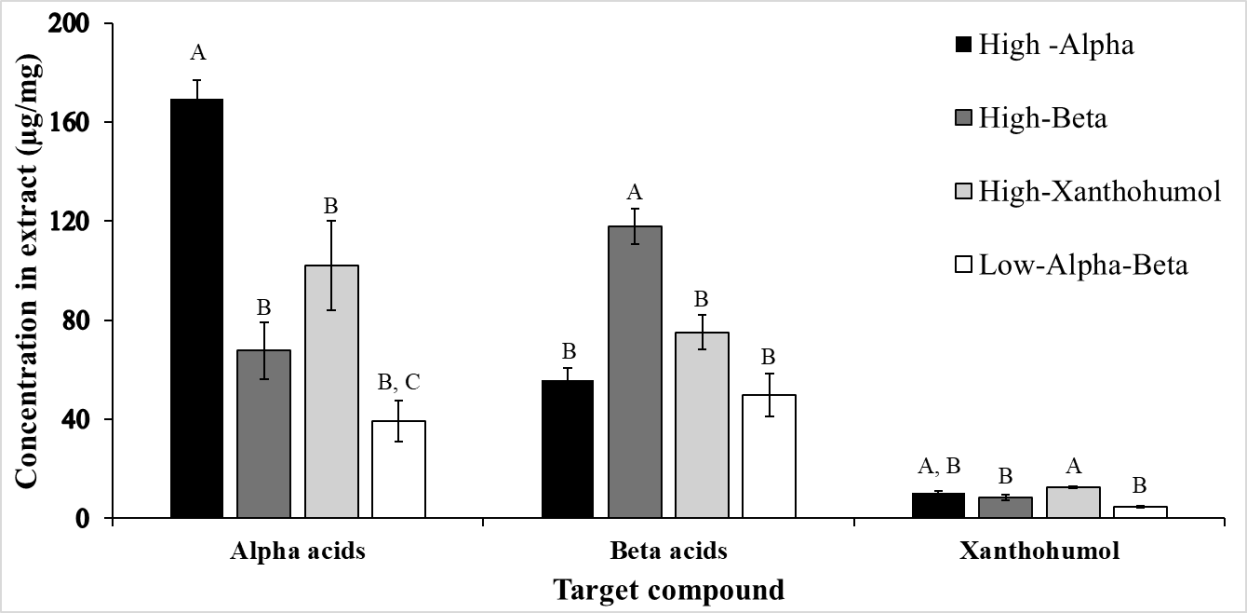


**Figure 4-1. Quantified marker compounds (µg/mg extract weight) from hop extracts (n = 24) using high-performance liquid chromatography diode array detection and categorized into chemical groups (High-Alpha, High-Beta, High-Xanthohumol, and Low Alpha-Beta).** Values expressed as µg/mg dried extract (mean ± SEM, n=3). Significant differences ($p < 0.05$) are denoted with letters among groups for each marker compound using one-way ANOVA (df = 3,20) with Tukey HSD *post hoc* test.

## Hop extracts inhibit FAAH and MAGL *in vitro*

All tested hop samples exhibited concentration-dependent inhibition of both FAAH and MAGL activity. For FAAH, at the standard extract concentration of 10.0 µg/mL, percent inhibition ranged from 39.1-68.9% with a mean of 60.1± 1.5%. $IC_{50}$ values ranged from 3.3-20.4 µg/mL, with a mean of 7.8± 1.1 µg/mL (Supplemental Table 3). For MAGL, at the standard

extract concentration of 10.0 µg/mL, percent inhibition ranged from 47.3-68.4% with a mean of 58.3± 0.9%. $IC_{50}$ ranged from 3.2-9.4 µg/mL, with a mean of 6.3± 0.3µg/mL (Supplemental Table 3).

Mean enzyme inhibition data for HA, HB, HX, and LAB groups are presented in Table 4-1. A significant main effect of chemical groups was observed for FAAH inhibition at 10 µg/mL of hop extracts ($F_{3,20} = 4.6$, $p = 0.01$, n = 24) as percent inhibition among HA samples was at least 9% lower than the other three groups (Table 4-1). The group effect was also significant for $IC_{50}$ values ($F_{3,20} = 3.3$, $p = 0.04$), with the only difference observed being between HB and HA, where HB was 3x more potent than HA (Table 4-1). Relative to FAAH, differences in MAGL inhibition varied less between groups, with no significant group effect detected for percent inhibition ($F_{3,20} = 1.7$, $p = 0.21$) nor $IC_{50}$ values ($F_{3,20} = 1.5$, $p = 0.24$).

**Table 4-1. The mean percent inhibition (±SEM, at 10 µg/mL) and $IC_{50}$ values (µg/mL) (±SEM) for FAAH and MAGL among the four groups of hop extracts (n = 24).** One-way ANOVA (df=3,20) and Tukey HSD post hoc tested for significance, with significant differences ($p < 0.05$) denoted by superscript letters.

| | FAAH | | MAGL | |
|---|---|---|---|---|
| **Group** | **Inhibition (%)** | **$IC_{50}$** | **Inhibition (%)** | **$IC_{50}$** |
| High-Alpha | 52.2 (3.7) $^{B}$ | 12.3 (2.1) $^{A}$ | 58.8 (1.8) | 6.1 (0.6) |
| High-Beta | 64.4 (1.3) $^{A}$ | 4.4 (0.6) $^{B}$ | 55.8 (1.9) | 7.4 (0.7) |
| High-Xanthohumol | 62.1 (1.7) $^{A}$ | 6.5 (1.4) $^{AB}$ | 61.2 (1.1) | 5.7 (0.4) |
| Low-Alpha-Beta | 61.6 (2.6) $^{A}$ | 8.0 (2.6) $^{AB}$ | 57.4 (2.3) | 5.9 (0.7) |
| *F* | 4.6 | 3.3 | 1.7 | 1.5 |
| *p* | 0.01 | 0.04 | 0.21 | 0.24 |

## Linear regression of marker compounds and enzyme inhibition across extracts

To investigate the potential contribution of hop marker compounds to the observed enzyme inhibition, percent inhibition and $IC_{50}$ values were analyzed relative to the concentration of markers in each corresponding extract using simple and multiple linear regression modelling. Note that a negative correlation with $IC_{50}$ values indicates greater potency as less extract is needed to achieve 50% inhibition of the enzyme.

For FAAH, the beta acids showed a positive relationship with percent inhibition ($R^2 = 0.18$, $p = 0.04$), primarily driven by the colupulone content ($R^2 = 0.23$, $p = 0.02$, Table 4-2). In contrast, the alpha acids were negatively associated with percent inhibition ($R^2 = 0.28$, $p = 0.01$, Table 4-2), driven through n-adhumulone ($R^2 = 0.31$, $p < 0.001$, Table 4-2). Xanthohumol content did not model FAAH inhibition. Backward stepwise regression analysis revealed a predictive model with n-adhumulone and colupulone (Adjusted $R^2 = 0.41$, $p = 0.001$):

FAAH percent inhibition = 59.0 – 8.8 (n-adhumulone) + 19.9 (colupulone).

The bitter acids content of hop extracts also modelled $IC_{50}$ data (Table 4-3). Most notably, a significant negative slope model existed between beta acids, primarily driven by colupulone and slightly by n-adlupulone (Table 4-2). Furthermore, extracts rich in n-adhumulone were less effective at inhibiting FAAH (Table 4-2). Backward stepwise regression analysis again resulted in the predictive model with n-adhumulone and colupulone (Adjusted $R^2 = 0.41$, $p = 0.003$):

$IC_{50} = 9.84 + 0.05$ (n-adhumulone) – 0.15 (colupulone).

**Table 4-2. Simple linear regression of percent inhibition or $IC_{50}$ for FAAH as a function of marker compounds quantified in the hop (*Humulus lupulus*) extracts (n = 24** Marker compounds were quantified using high-performance liquid chromatography with UV and diode array detector. Models used extract concentration set at 10 µg/mL or $IC_{50}$ (µg/mL) calculated using the concentration range of 0.4-250 µg/mL (Ritz et al., 2015), whereby significant p-values ($p < 0.05$) are in boldface font.

| | Percent Inhibition | | | | | $IC_{50}$ | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Predictor** | **$R^2$** | **Slope** | ***SE*** | **F** | ***p*** | **$R^2$** | **Slope** | ***SE*** | **F** | ***p*** |
| Alpha acid | 0.28 | -6.9 | 2.4 | 8.5 | **0.01** | 0.21 | 0.04 | 0.02 | 5.7 | **0.03** |
| Cohumulone | 0.17 | -19.3 | 9.1 | 4.5 | **<0.05** | 0.13 | 0.12 | 0.06 | 3.4 | 0.08 |
| N-adhumulone | 0.31 | -9.9 | 3.1 | 10.1 | **<0.001** | 0.23 | 0.06 | 0.02 | 6.5 | **0.02** |
| Beta acid | 0.18 | 10.1 | 4.5 | 4.9 | **0.04** | 0.24 | -0.08 | 0.03 | 7.1 | **0.01** |
| Colupulone | 0.23 | 24.0 | 9.4 | 6.6 | **0.02** | 0.26 | -0.18 | 0.06 | 7.7 | **0.01** |
| N-adlupulone | 0.13 | 14.4 | 8.1 | 3.2 | 0.09 | 0.20 | -0.12 | 0.05 | 5.4 | **0.03** |
| Xanthohumol | 0.02 | -33.2 | 47.2 | 0.5 | 0.49 | 0.01 | 0.13 | 0.32 | 0.2 | 0.69 |

For MAGL, marker compounds in the hop extracts did not model percent inhibition at 10 µg/mL (Table 4-3, $p > 0.05$). Backward stepwise regression analysis resulted in the predictive model that did not reach significance (Adjusted $R^2 = 0.15$, $p = 0.10$) due to the lack of variability in percent inhibition for hop extracts:

MAGL percent inhibition = 57.0 + 86.5 (xanthohumol) – 14.1 (cohumulone) – 7.6 (n-adlupulone).

Colupulone content predicted $IC_{50}$, whereby as colupulone content increased the $IC_{50}$ increased (Table 4-3). Backward stepwise regression analysis resulted in a significant predictive model that includes xanthohumol, cohumulone, n-adhumulone, and n-adlupulone (Adjusted $R^2 = 0.50$, $p = 0.001$):

MAGL $IC_{50}$ = 5.51- 0.32 (xanthohumol) + 0.06 (cohumulone) - 0.06 (n-adhumulone) + 0.05 (n-adlupulone)

**Table 4-3. Simple linear regression of percent inhibition or $IC_{50}$ for MAGL as a function of marker compound quantified in the hop (*Humulus lupulus*) extracts (n = 24) using high-performance liquid chromatography with a diode array detector.** Extract concentration for percent inhibition was shown at 10 µg/mL or $IC_{50}$ (µg/mL) calculated using the concentration range of 0.08-250 µg/mL (Ritz et al., 2015). Significant *p*-values ($p < 0.05$) are displayed in bold-faced font.

| | Percent Inhibition | | | | | $IC_{50}$ | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Predictor** | **$R^2$** | **Slope** | ***SE*** | **F** | ***p*** | **$R^2$** | **Slope** | ***SE*** | **F** | ***p*** |
| Alpha acid | 0.02 | 0.37 | 1.7 | 0.05 | 0.83 | 0.01 | 0.00 | 0.00 | 0.18 | 0.68 |
| Cohumulone | <0.01 | -0.24 | 6.2 | 0.00 | 0.97 | 0.04 | 0.02 | 0.02 | 0.82 | 0.37 |
| N-adhumulone | <0.01 | 0.72 | 2.32 | 0.10 | 0.76 | <0.01 | 0.00 | 0.01 | 0.06 | 0.82 |
| Beta acid | 0.02 | -2.19 | 3.1 | 0.52 | 0.48 | 0.14 | 0.02 | 0.00 | 3.64 | 0.07 |
| Colupulone | 0.02 | -4.76 | 6.5 | 0.54 | 0.47 | 0.17 | 0.04 | 0.02 | 4.55 | **0.04** |
| N-adlupulone | 0.02 | -3.46 | 5.3 | 0.42 | 0.52 | 0.10 | 0.03 | 0.02 | 2.46 | 0.13 |
| Xanthohumol | 0.12 | 47.53 | 27.6 | 2.96 | 0.10 | 0.05 | -0.10 | 0.10 | 1.09 | 0.31 |

## FAAH and MAGL inhibition by marker compounds

Most marker compounds exhibited concentration-dependent inhibition of both FAAH and MAGL. Xanthohumol ($IC_{50}$ = 13.3μM) was the most potent inhibitor of FAAH among the individual compounds tested (Table 4-4) as it was 7- and 8 times more potent than n-adlupulone and colupulone. Xanthohumol was also greater than 10x more potent than cohumulone and n-adhumulone (Table 4-4). Meanwhile, for MAGL, colupulone and xanthohumol demonstrated greater inhibition than other target compounds, though with less than 2x potency relative to cohumulone and n-adhumulone (Table 4-4), and comparable to those found in hop extracts (Table 4-3). All isolated compounds (except for n-adlupulone) had stronger activity on MAGL than FAAH.

**Table 4-4. Target compound FAAH and MAGL mean $IC_{50}$ values (SEM).** Compounds tested derived from hops (*Humulus lupulus*) were cohumulone, n-adhumulone, colupulone, n-adlupulone, and xanthohumol, each tested in triplicate at a concentration range of 0.02-50 µg/mL. JZL-195 (positive control) was tested at a concentration range of 0.0005-5 µg/mL, while JZL-184 (positive control) was tested at a concentration range of 0.00005-50 µg/mL.

| | FAAH | | MAGL | |
|---|---|---|---|---|
| **Compound** | **$IC_{50}$ (µg/mL)** | **$IC_{50}$ (µM)** | **$IC_{50}$ (µg/mL)** | **$IC_{50}$ (µM)** |
| Cohumulone | >50.0 | >143.0 | 8.6 (0.8) | 24.7 (2.4) |
| N-adhumulone | >50.0 | >132.8 | 12.4 (1.7) | 32.9 (4.5) |
| Colupulone | 46.1 (26.8) | 115 (67.0) | 6.13 (0.8) | 15.3 (2.0) |
| N-adlupulone | 38.3 (19.7) | 92.5 (47.3) | >50.0 | >121.0 |
| Xanthohumol | 4.7 (0.2) | 13.3 (0.67) | 6.9 (2.6) | 19.6 (7.4) |
| JZL-195 | 0.007 (0.002) | 0.017 (0.004) | - | - |
| JZL-184 | - | - | 0.049 (0.01) | 0.09 (0.02) |

## Untargeted Metabolomic – discriminant and linear regression analyses

The mass features that significantly differed between these two groups were identified by comparing the metabolomic profile of the three strongest and three weakest extracts based on $IC_{50}$ values using discriminant analysis. These mass-features were then extracted from the metabolomic data of all samples and using simple linear regression, their relative abundancies (centred-scaled-normalized mass-features) were compared to enzyme inhibition ($IC_{50}$ in µg/mL, n = 24).

Discriminant analysis of the most ($IC_{50}$ = 3.4 µg/mL ± 0.04) and least ($IC_{50}$ = 18.3 µg/mL ± 1.2) potent FAAH-inhibiting extracts revealed nine mass-features [RT, $(m/z)^+$], five were more abundant in the more active extracts (Figure 4-2). Based on linear regression of normalized relative abundance and activity across all 24 extracts, four mass features were significant predictors of FAAH $IC_{50.}$ (Table 4-5). The tentative identification of two mass features negatively associated with $IC_{50}$ values matched those of isocohumulone (monoisotopic mass = 348.193665 g/mol, error = 18 ppm) and n-adlupulone (monoisotopic mass = 414.277008 g/mol, error = 74 ppm) were previously reported in hops by HRMS (Brown et al., 2022; Chiancone et al., 2023; Farag et al., 2012; Nicácio et al., 2022; Prencipe et al., 2014; Salviati et al., 2021). The neutral form of cohumulone was also identified for the mass feature 348.19986 (monoisotopic mass = 348.19367399 g/mol, error = 17 ppm). Furthermore, the mass feature 360.19901 was tentatively identified as cohulupone+ACN+H (monoisotopic mass = 360.21693231 g/mol, error = 50 ppm). Similar to the literature search, the adduct search tentatively identified m/z 414.24646 as adlupulone (monoisotopic mass = 414.277008 g/mol, error = 73 ppm).

Discriminant analysis of the most ($IC_{50}$ = 4.1 µg/mL ± 0.4) and least ($IC_{50}$ = 9.1 µg/mL ± 0.1) potent MAGL-inhibiting extract revealed seven mass-features [RT, $(m/z)^+$], five more

abundant in the more active extracts (Figure 4-3). Based on linear regression mass-features abundance and activity of all 24 extracts, three features were significant predictors of MAGL $IC_{50}$ (Table 4-6). Unfortunately, after exploring molecular adduct ions of known compounds and comparing the mass features to the literature no tentative mass features could be identified.

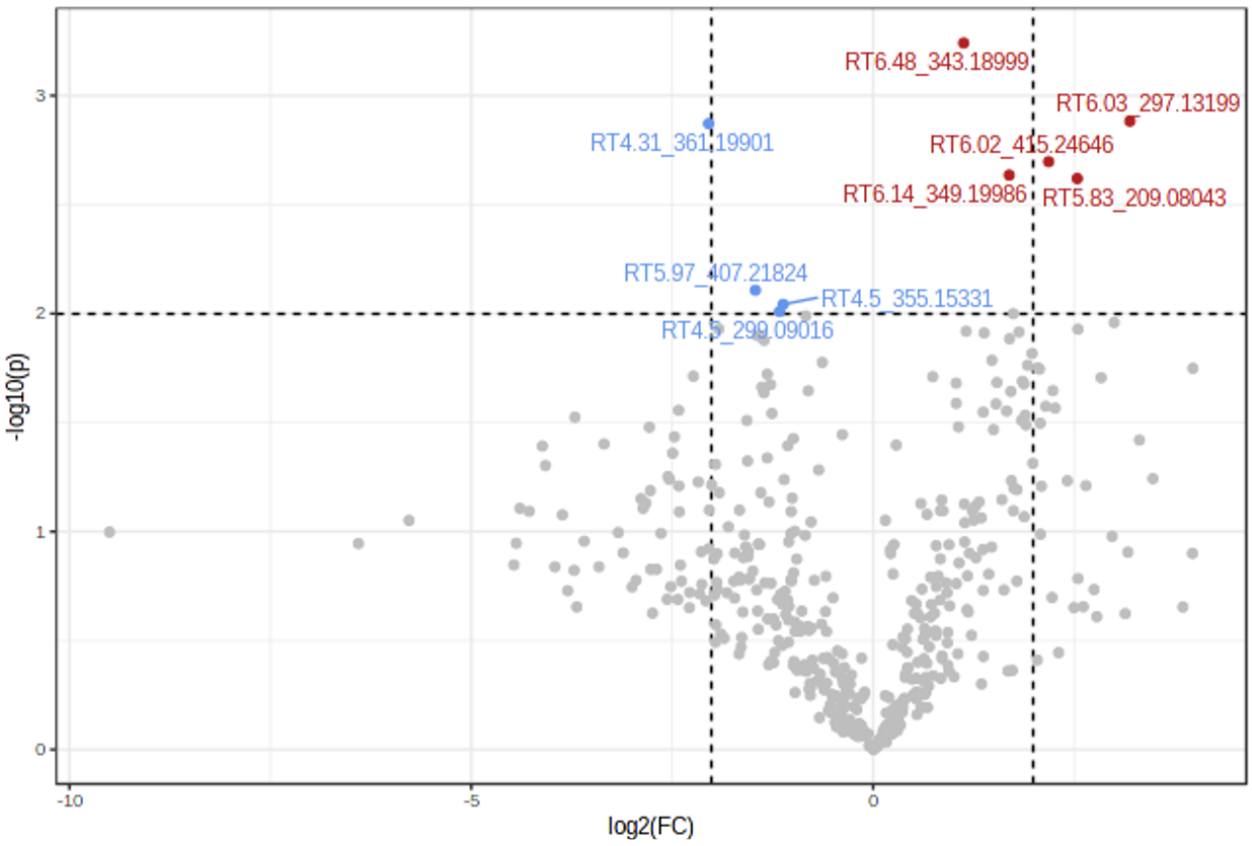


**Figure 4-2. Comparison of the three strongest to three weakest hop (*Humulus lupulus*) extracts on the FAAH inhibition assay using a metabolomic dataset (containing 642 mass features).** Mass features are labelled as their retention time (RT) and mass-to-charge ratio [m/z]+. Mass features that were significantly greater in the stronger hop extracts were depicted in red, while those that were significantly greater in the weaker hop extracts were depicted in blue.

**Table 4-5. Simple linear regression for FAAH $IC_{50}$ (µg/mL) as a function of mass features using *Humulus lupulus* extracts (n = 24).** Samples were run on UPLC-LTQ-Orbitrap HRMS, and these data were normalized-centred-scaled *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

| Mass-features | $R^2$ | Slope | F | *p* |
|---|---|---|---|---|
| RT5.83_209.08043 | 0.11 | -1.29 | 2.71 | 0.11 |
| RT6.03_297.13199 | 0.14 | -3.00 | 3.47 | 0.08 |
| RT6.48_343.18999 | 0.39 | -5.22 | 13.84 | 0.001*** |
| RT6.14_349.19986 | 0.36 | -2.43 | 12.27 | 0.002** |
| RT4.31_361.19901 | 0.43 | 5.04 | 16.92 | <0.001*** |
| RT6.02_415.24646 | 0.23 | -1.92 | 6.66 | 0.02* |

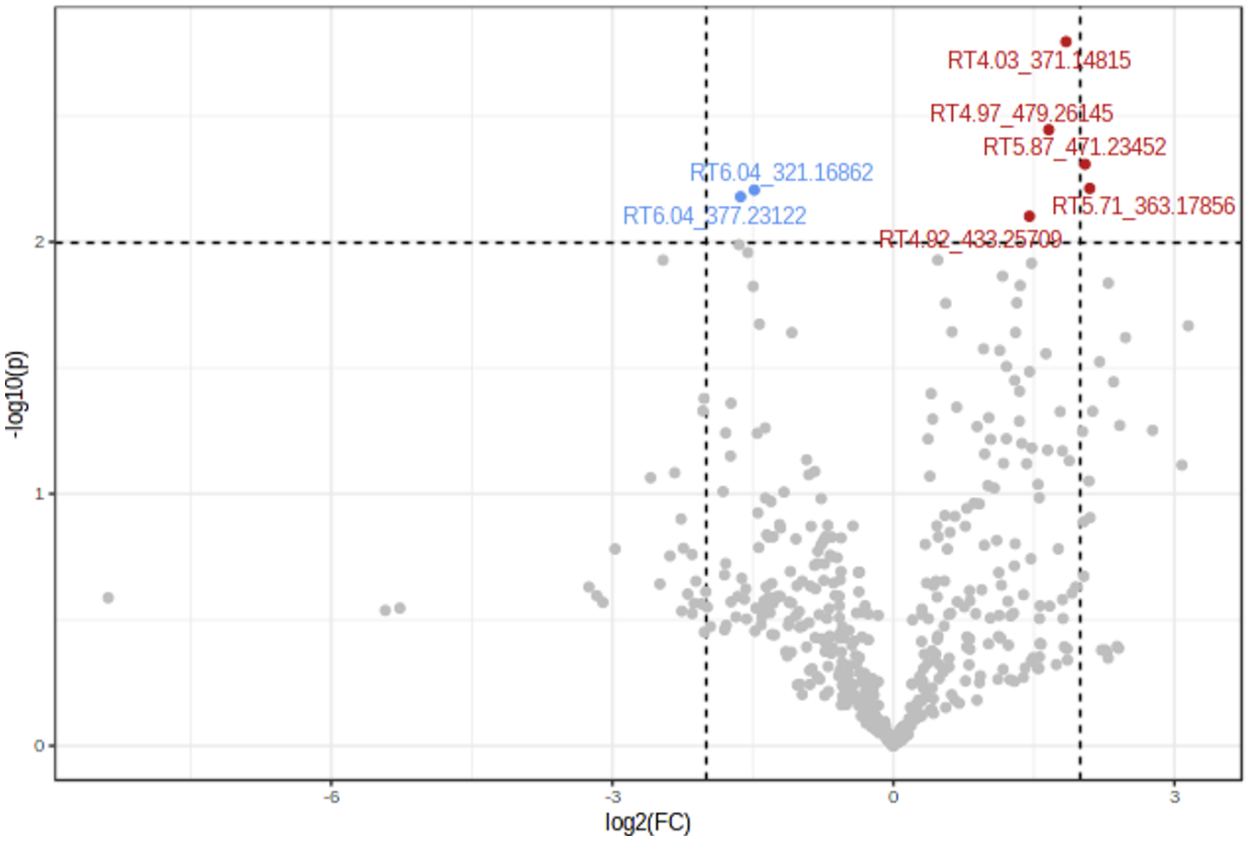


**Figure 4-3. Comparison of the three strongest to three weakest hop (*Humulus lupulus*) extracts on the MAGL inhibition assay using a metabolomic dataset (containing 642 mass features).** Mass features are labelled as their retention time (RT) and mass-to-charge ratio [m/z]+. Mass features that were significantly greater in the stronger hop extracts were depicted in red, while those that were significantly greater in the weaker hop extracts were depicted in blue.

**Table 4-6. Simple linear regression for MAGL $IC_{50}$ (µg/mL) as a function of mass features using *Humulus lupulus* extracts (n = 24).** Samples were run on UPLC-LTQ-Orbitrap HRMS, and these data were normalized-centred-scaled *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

| Predictor | $R^2$ | Slope | F | *p* |
|---|---|---|---|---|
| RT4.03_371.14815 | 0.27 | -1.16 | 8.06 | <0.001*** |
| RT6.04_321.16862 | 0.43 | 0.70 | 16.6 | <0.001*** |
| RT6.04_377.23122 | 0.43 | 1.10 | 16.78 | <0.001*** |

## Assay-guided fractionation

As a final approach to identifying active metabolites, assay-guided fractionation was completed based on both FAAH and MAGL inhibition. The hop extract selected for column fractionation had the lowest $IC_{50}$ for FAAH (3.4 µg/mL ± 0.8) and moderate $IC_{50}$ for MAGL (7.0 µg/mL ± 0.7). Low-pressure column chromatography using silica gel yielded 36 fractions screened in both assays at a standard concentration of 250 µg/mL for FAAH and 125 µg/mL for MAGL. For FAAH, the percent inhibition ranged from 15-94%, and for MAGL, the percent inhibition ranged from 24-93% inhibition (Figure 4-4). Fractions 20 and 21 were the only two fractions with strong activity for FAAH and MAGL (>75% inhibition) and had yields of 9% and 36% of the total quantity of fractions collected.

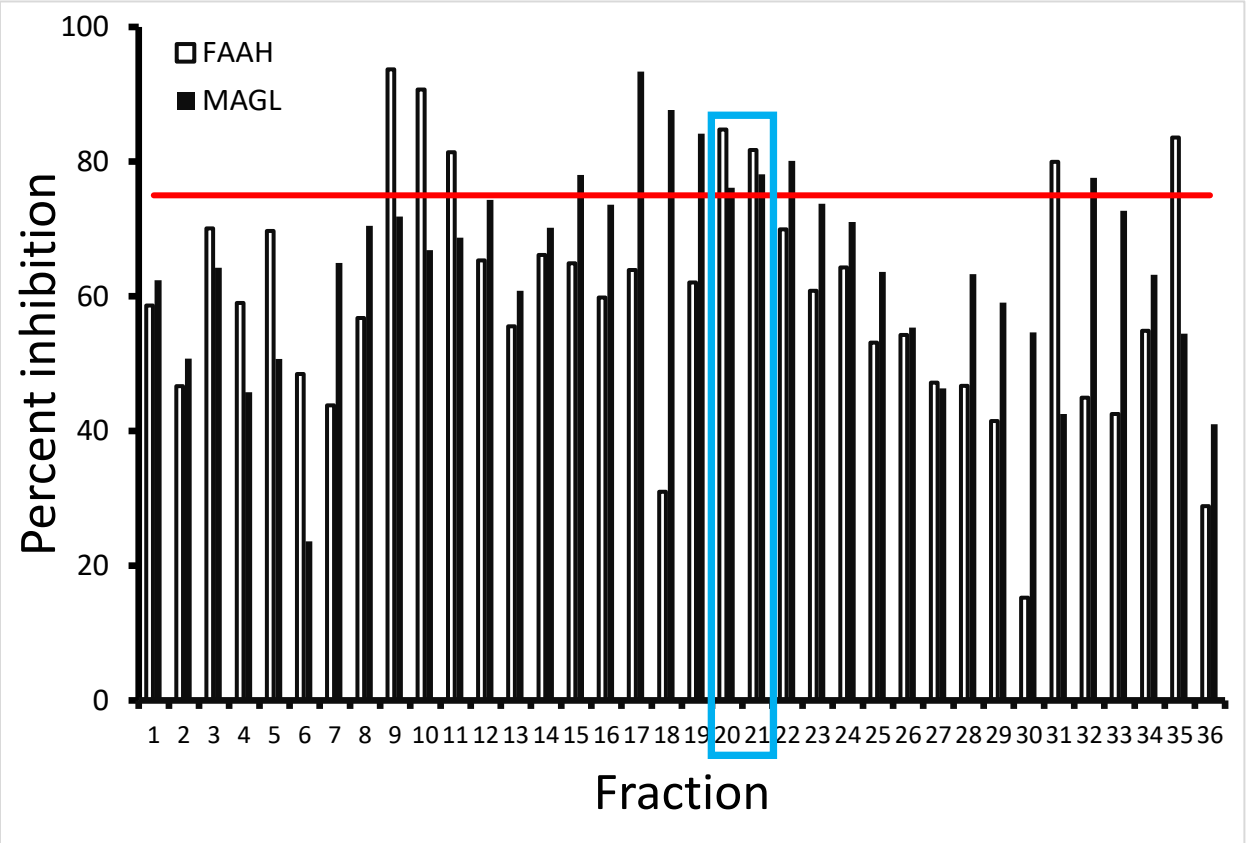


**Figure 4-4. FAAH (open bars) MAGL (closed bars) percent inhibition of hop (*Humulus lupulus*) fractions (n = 36)**. Fractions were collected and screened at 250 µg/mL and 125 µg/mL for FAAH and MAGL, respectively. The red horizontal bar represents the 75% threshold to be identified as a potent inhibitor during the screening phase. Fractions 20 and 21 are highlighted in a blue box and represent 9% and 36% of the total recovered fractions, respectively. All other fractions yielded ≤5%, except for fraction 22, which accounted for 11%.

Fractions 20 and 21 were combined and, using HPLC-DAD-based fraction collection, three major peaks were collected as sub-fractions. All three subfractions demonstrated a concentration-dependent FAAH inhibition, where sub-fraction two was almost twice as active as sub-fraction 3 (Table 4-7). The third sub-fraction inhibited MAGL in a concentration-dependent manner, while the other two subfractions did not (Table 4-7).

**Table 4-7. Mean FAAH and MAGL $IC_{50}$ (µg/mL ± SEM) ran in triplicate from the three subfractions isolated from fractions 20-21 along with 8-PN.** A concentration of 50 µg/mL was the highest value tested, therefore, samples with >50 were indicative that no concentration-dependent relationship was established. The enzyme used for the FAAH assays was human-recombinant enzymes.

| Subfraction | FAAH $IC_{50}$ µg/mL | MAGL $IC_{50}$ µg/mL |
|---|---|---|
| 1 | 13.2 (5.6) | >50 |
| 2 | 11.7 (1.3) | >50 |
| 3 | 20.4 (1.2) | 5.5 (0.1) |
| 8-PN | 37.4 (14.0) | 17.4 (2.6) |

After further examination, the principal component of subfraction three was identified as having a molecular weight of 354 g/mol and was identified as xanthohumol after running the sample on the UHPLC-MS (Figure 4-5). The primary compounds detected in the other subfractions were tentatively identified as cohulupone and hulupone, with [M+H]+ of 319 and 333, respectively. Upon obtaining a hulupone mixture and attempting to purify these two compounds, no clear concentration-dependent relationship emerged after running the hulupone mix or isolated hulupones on FAAH and MAGL.

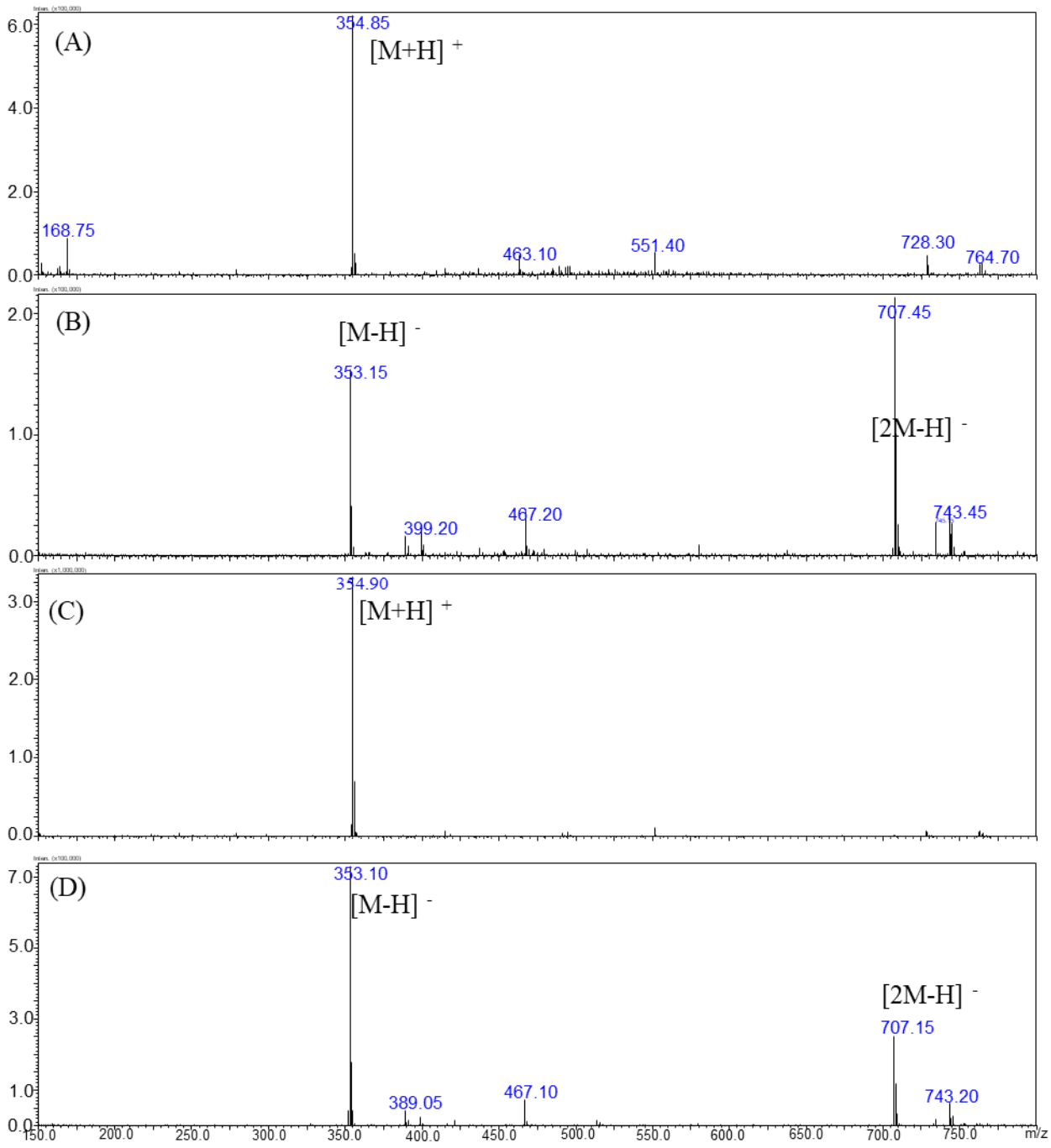


**Figure 4-5. UHPLC Mass Spectrum** for (A) subfraction three positive ion mode, central peak 354.85 g/mol, (B) subfraction three negative ion mode, major peak 353.15 g/mol, (C) Xanthohumol positive ion mode, central peak 354.90 g/mol and (D) Xanthohumol negative ion mode central peak 353.10 g/mol.

## DISCUSSION

This study tested whether hops and their constituents inhibit FAAH and MAGL to better understand their potential role in the ECS. To do so, I used *in vitro* enzyme assays alongside targeted and untargeted phytochemical analyses and various biochemometric approaches.

For FAAH inhibition, multiple approaches confirmed that beta acids and xanthohumol demonstrate bioactive properties. The HB group exhibited the greatest inhibitory response (Table 4-1), and colupulone (a beta acid) content modelled FAAH inhibition both at a standard concentration and in terms of $IC_{50}$ values (Table 4-2). The tentative identification of adlupulone from the metabolomic analyses comparing most and least active extracts provided additional support that the beta acids were driving FAAH inhibition. Meanwhile, HA had the lowest activity, and adhumulone was negatively associated with inhibition. The backward stepwise regression model reinforced this relationship, revealing that a lower level of n-adhumulone and a higher level of colupulone best-predicted inhibition. The negative relationship between n-adhumulone content and FAAH inhibition could be partially explained by the abundance of alpha acids resulting in a trade-off of beta acid production. Indeed, there was a negative, albeit not significant, correlation among the alpha and beta acid constituents (Table 3-2), and the individual beta acids were more active than individual alpha acids (Table 4-5). A similar dilution effect was proposed to explain negative correlations observed between FAAH inhibition and specific echinacea metabolites (R. Liu, Burkett, et al., 2021) but alternative mechanisms, such as allosteric modulation (Dainese et al., 2020) may also be involved.

Interestingly, isolated xanthohumol was the strongest inhibitor of the tested isolated compounds (Table 4-4), but xanthohumol concentration was unrelated to inhibition across

extracts. This lack of relationship can be attributed to xanthohumol levels in extracts (<1% of 10 µg/mL or 0.1 µg/mL) that were below active concentrations and did not vary among samples as much as other market compounds (Table 3-1; Figure 4-1). Indeed, most extracts inhibited FAAH more strongly than marker compounds (Table 4-2; Table 4-5), and the $IC_{50}$ for isolated compounds typically exceeded the in-well concentrations when extracts were assayed for activity. These observations suggest that additional hop metabolites contributed to activity.

The tentative identification of lupulones using the metabolomics dataset coupled with modelling mass features to bioactivity (Table 4-5) supports findings from the targeted approach (Table 4-2). Iso-cohumulone was tentatively identified from the literature with a low error value and cohumulone was tentatively identified while searching for adducts of cohumulone.

Adding to the growing list of NHPs that act as FAAH inhibitors, the isolation of xanthohumol through assay-guided fractionation was the first report of a prenylated chalcone with FAAH-inhibiting activity. Other phytochemicals with reported activity include Licarin A (benzofuran) from nutmeg, caffeoylquinic acids and alkylamides from echinacea, and macamides (benzylalkylamide) from maca (R. Liu, Caram-Salas, et al., 2021; Maccarrone, 2020). While direct comparison across studies is difficult, the potency of xanthohumol ($IC_{50}$ = 13.33µM) and the beta acids were less potent than had considerably greater potency than any of the isolated compounds from echinacea, where the most potent was cichoric acid ($IC_{50}$ = 45µM (R. Liu, Burkett, et al., 2021)). Most of these compounds, including xanthohumol, contain planar and polar moieties (e.g., amide, phenol) with flexible lipophilic alkyl or prenyl groups, similar to FAAH's primary substrate, AEA.

Whereas hop extracts potently inhibited MAGL activity, $IC_{50}$ values across extracts were less variable than observed for FAAH, affording reduced sensitivity for biochemometric analyses.

Neither group comparisons nor simple regression of modelling of MAGL inhibited that major marker compounds were driving activity, but stepwise multiple regression revealed an interactive effect; activity was most strongly associated with xanthohumol but modified both positively and negatively by bitter acids. While hop marker compounds inhibited MAGL more potently than FAAH, crude extracts again outperformed pure compounds (by weight), and the concentration of marker compounds at extract $IC_{50}$ values was well below their own when tested alone (Table 4-1, 4-4). Other contributing metabolites found in hops could be attributed to MAGL inhibition. For example, the results from Tung and colleagues (2021) identified 8-PN as a potent inhibitor of MAGL with an $IC_{50}$ = 9.5μM. Although it has demonstrated MAGL inhibition, 8-PN is also known for being the strongest phytoestrogen known and has been tested on various models of hormonal imbalance (Di Viesti et al., 2011; Erkkola et al., 2010; Krause et al., 2014). In contrast to 8-PN, xanthohumol is structurally similar with fewer endocrine disrupting effects, and xanthohumol was a stronger MAGL inhibitor at approximately double that of 8-PN reported in the literature (Tung et al., 2021) and thrice that of 8-PN tested in the present study (Table 4-5). The allosteric modulation from multiple phytochemicals could explain why the extract bioactivity outperformed isolated compounds. Surprisingly, isolated 8-PN (Table 4-7) was the second strongest FAAH but the second weakest isolated compound for MAGL (Table 4-4). Interestingly, the precursor to 8-PN is iso-xanthohumol (Dhooghe et al., 2010; Karabin et al., 2015; Nikolic et al., 2005), and the gut microbiome has been shown to complete this conversion (Carbone & Gervasi, 2022), though most xanthohumol does not get converted into 8-PN (Bartmanska et al., 2013). The structural similarities between 8-PN, iso-xanthohumol, and xanthohumol may partially explain why both compounds inhibit MAGL.

Jewenol A found in *Salvia pseudorosmarinus* aerial parts had a MAGL $IC_{50}$ of 46.8 μM (De Leo et al., 2018); therefore, the present study still has twice the $IC_{50}$ from xanthohumol (Table 4-4). Other phytochemicals with reported MAGL inhibiting activity include phenylethanoid glycoside and triterpenes beta-amyrin and pristimerin (Beladjila et al., 2018; Chicca et al., 2012; King et al., 2009; Merali et al., 2018).

This study demonstrated that xanthohumol is a phytochemical with promising applications for inhibiting both FAAH and MAGL. By targeting FAAH and MAGL activity, xanthohumol could potentially be useful in reducing oxidative stress associated with cancer (De Leo et al., 2018; Granchi et al., 2021; Tung et al., 2021) and neurodegenerative disease neurodegenerative disease (Di Marzo, 2008; Klein, 2005; Schurman & Lichtman, 2017; Tung et al., 2021).

The bioassay-guided fractionation resulted in two fractions showing considerable activity. The third subfraction had the strongest activity, later identified as xanthohumol. Meanwhile, the other two subfractions demonstrated FAAH inhibition but not MAGL. These were tentatively identified as hulupones, known for being byproducts of bitter acid oxidation (Salviati et al., 2021; Van Cleemput et al., 2009). When isolated hulupones (not from the bioassay-guided fractionation) were tested, they did not show a concentration-dependent relationship in either FAAH or MAGL. While these results support xanthohumol as a contributing active compound toward the FAAH and MAGL and inhibiting potential of hop extracts, they also highlight the risk of metabolite degradation through the assay-guided fractionation process as hulupones are not synthesized by hops and result from oxidation during storage and processing (Taniguchi et al., 2013).

The purpose of this study was to assess a chemically diverse set of cultivated hops for ECS modulatory potential and to identify contributing active compounds. Focusing on biochemical

enzyme assays, however, there is an added uncertainty when moving toward cell-based and *in-vivo* models. Future studies should validate FAAH and MAGL inhibition in models of inflammation, as the interaction of FAAH and COX-2 has been proposed (Di Marzo, 2008; Donvito et al., 2018; Schurman & Lichtman, 2017), and tested using pharmaceutical-based products (Sasso et al., 2015; Scarpelli et al., 2016) but not natural health products. The interaction of hop metabolites and FAAH and MAGL enzymes could be modelled using *in silico* docking software (Kowalska et al., 2022; Tung et al., 2021). Given the observed potential to modulate the ECS via endocannabinoid metabolism, hop extracts and isolated compounds should be assessed for CB-1 and CB-2 agonist, antagonist, or allosteric modulating activity. Lastly, (3) Beta-caryophyllene is a terpene found in a multitude of plants, including hops (Karabin et al., 2015; S. Lafontaine et al., 2019; Sharp, Vollmer, et al., 2017; Zanoli & Zavatti, 2008), lowers inflammation and reduces nociception via CB-2 agonist activity (Gertsch et al., 2008, 2010). Selecting hop extracts with varying levels of beta-caryophyllene content could be used as a model to predict CB-2 agonist activity.

## CONCLUSION

This was the first study to systematically test hop extracts on FAAH and MAGL inhibition. Colupulone modelled MAGL $IC_{50}$ with a positive slope and modelled FAAH $IC_{50}$ with a negative slope. Bioassay-guided fractionation reconfirmed xanthohumol as a strong inhibitor of FAAH and MAGL. The other two subfractions were cohulupone and hulupone, both of which were active for FAAH but not MAGL. Overall, colupulone content in the extract and xanthohumol as an isolated compound can modulate FAAH and MAGL. The application of these results could be useful in the development of a non-cannabinoid modulator of the ECS as an NHP or dietary supplement would be a worthwhile endeavour.

# CHAPTER 5: GENERAL DISCUSSION

## SYNTHESIS OF RESULTS

This dissertation presents novel methods for hop selection based on compounds that possess pharmacological effects. Such techniques will aid in the advancement of NHP and quality control practices. Alpha acids were associated with greater RSA, while beta acids inhibited FAAH, and isolated xanthohumol functioned as a panacea showing free-radical scavenging activity, and as a FAAH, MAGL, and COX-2 inhibitor.

Brewers use hops to provide bitterness to beer, though there is growing interest in exploring the medicinal applications of hops. In this thesis, hops were collected from various sources, and ethanolic extraction was used to optimize for bitter acid and xanthohumol yield. First, correlations among marker compounds across the sample collection, and statistical differences were observed between hop groupings (uses, origins, and form), particularly concerning alpha acid content and supporting both industry knowledge and limited scientific literature. In addition to confirming that bitter (relative to aroma) and North American (relative to European) hop samples, contained more alpha acids using a previously unmatched variety of hop cultivars (n = 32), my results refined these relationships by highlighting that a) beta-acid levels remained comparable across groups with increases in alpha-acids leading to higher ABR values (Table 2-4 & 2-5), b) use (bitter vs. aroma) was associated with greater differences in alpha acids than origins, and c) use-related differences remained consistent – even additive – with origin related differences and *vice versa* (Table 2-6), and d) metabolomic analyses identified desmethylxanthohumol in cone samples compared to pellets. These results also identified strong phytochemical correlations across all samples, regardless of groups, most significantly between the alpha acids (cohumulone and n-adhumulone) and between beta acids (colupulone and n-adlupulone), but also between xanthohumol and each bitter acid, with cohumulone and n-

adhumulone showing the strongest association (Table 2-3). This was the first experiment that empirically tested the chemical differences of the five most abundant secondary metabolites between bitter and aroma hops along with North American and European hops. Researchers previously combined genetic information about hop cultivars with metabolomics to achieve a hierarchical cluster analysis, and bitter acids were driving the cluster of hop samples (Ikhalaynen et al., 2022). This paper was published after my data collection but before this thesis was written. Breeding among varieties could be helpful in the development of novel cultivated varieties (i.e., cultivars) that could have higher yields, unique chemical profiles, and or disease resistance (McCallum et al., 2019; Zaidi et al., 2022).

Next, I demonstrated that hop extracts elicit a variety of concentration-dependent bioactivities: radical scavenging and the inhibition of three human enzymes, COX-2, FAAH, and MAGL. Extract chemistry predicted RSA with n-adhumulone content predicting activity, while FAAH inhibition was best modelled based on colupulone content. Biochemometric analyses failed to converge on any metabolites driving COX-2 or MAGL inhibition, although inter-extract variability in activity toward these enzymes was lower than those observed for RSA and FAAH. When modelled to $IC_{50}$ data, MAGL had a significant stepwise model that suggested extracts greater in xanthohumol and n-adhumulone while being lower in cohumulone and n-adlupulone would best predict $IC_{50}$. The addition of assay-guided fractionation to the workflow in Chapter 4 reconfirmed xanthohumol as a MAGL inhibitor.

In terms of marker compound activity, xanthohumol also exhibited moderate RSA and was a potent inhibitor of COX-2, similar to previous reports (Gerhauser et al., 2002; Yamaguchi et al., 2009). The previous reports of xanthohumol (Yamaguchi et al., 2009) having greater RSA than bitter acids were not supported due to different assays and scoring that were used. RSA among

bitter acids has also been inconsistently reported, such that humulone was reported as having stronger RSA than lupulone and *vice versa* (Van Cleemput et al., 2009). Meanwhile, it was a novel finding to discover that xanthohumol was also a potent inhibitor of both FAAH and MAGL, more potent than hop-derived 8-PN, which was previously identified as a MAGL inhibitor using *in silico* modelling (Tung et al., 2021). These results identified new compounds that inhibit FAAH and MAGL. The present results found cohumulone, n-adhumulone, and colupulone all having similar RSA ~ 4 µg/mL, though n-adlupulone was stronger at 2.6 µg/mL (Table 3-6). Previous reports of COX-2 inhibition of bitter acids had a standardized $CO_2$ extract as a stronger inhibitor than both beta and alpha acids (Tripp et al., 2005).

Combining conventional approaches such as bioassay-guided fractionation with untargeted metabolomics has broad applications across different scientific fields, its use in deciphering active metabolites in plants remains nascent (Arnason et al., 2022; Caesar & Cech, 2019; Y. Li et al., 2023; McCallum et al., 2020). Our approach, which screened a collection of qualitatively similar yet quantitively diverse extracts and then compared the metabolomes of the most active and less active samples, demonstrated promising results as it identified both a) major metabolites confirmed as active via targeted approaches and b) minor metabolites that, only tentatively identified, warrant further investigation in terms of chemical structure and activity. Metabolomic results tentatively identified phloroisovalerophenone – a bitter acid precursor – from the literature (Nicácio et al., 2022) along with an adhumulone adduct that both modelled RSA. Furthermore, n-adlupulone was tentatively identified from the literature (Chiancone et al., 2023; Prencipe et al., 2014; Salviati et al., 2021) and via molecular ion adduct analysis, confirmed its relationship to FAAH activity. The bioassay-guided fractionation revealed two subfractions as novel FAAH inhibitors, though, when identified and screened as cohulupone and hulupone did

not exhibit a concentration-dependent response. Meanwhile, it confirmed xanthohumol's bioactivity for FAAH and MAGL. Figure 5-1 illustrates the various mechanisms through which hop extracts and metabolites offer therapeutic potential. This could be applied to NHP, supplements, or pharmaceutical product development with free-radical, anti-inflammatory, and endocannabinoid modulatory properties.

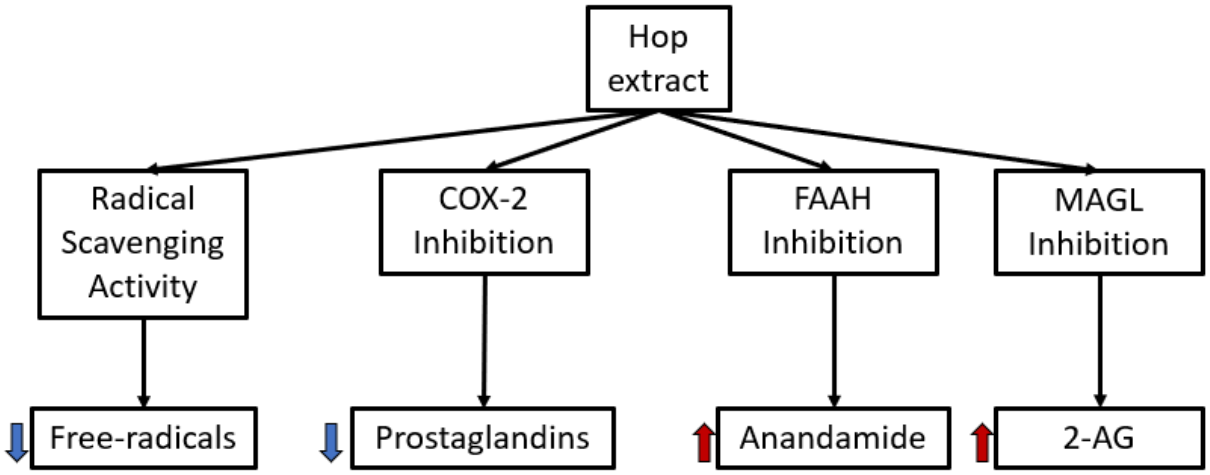


**Figure 5-1. Schematic diagram of the bioactivity of hops assessed in the current thesis.** Hop extracts were associated with greater RSA, COX-2, FAAH and MAGL inhibitions. The proposed *in vivo* downstream effects are represented by arrows. Blue arrows represent lower quantities of free radicals and prostaglandins, while red arrows represent an increase in anandamide and 2-AG. Increasing free-radical scavenging activity decreases oxidase activation. Furthermore, FAAH and MAGL inhibition results in less arachidonic acid available for COX-2 to produce inflammatory prostaglandins.

## COMPARISON OF EXTRACTS TO ISOLATED COMPOUNDS

The $IC_{50}$ of isolated compounds was much higher (at least an order of magnitude) than the concentration found in the extract at the median inhibitory concentration, suggesting that additional unidentified active metabolites contribute to bioactivity. Since the analytical methods relied on UV-absorbance, compounds lacking a chromophore would have escaped our detection and isolation including terpenes such as myrcene, ß-caryophyllene, and humulene (Formisano et al., 2019; Maillard et al., 1993). Similarly, the HRMS data was analyzed in ESI+ mode which has more noise than ESI- (Liigand et al., 2017), so some of the hop compound signals may have been missed in the noise. The bulk of hop secondary metabolites have been quantified as the bitter acids and xanthohumol (Figure 2-1). In the extracts, compounds that were not quantified via HPLC and HRMS could have impacted bioactivity via additive or synergistic effects associated with the plethora of phytochemicals in the hop extracts. Synergistic, additive, and inhibitory effects are common in food and plant-based medicine, with the concert of phytochemicals acting on a multitude of systems (Caesar & Cech, 2019; S. Wang et al., 2011). For example, the synergistic effects of *Echinacea purpurpea* extracts showed greater anti-inflammatory effects, via CB-2 activation, than isolated compounds tested on a cell-based assay (Chicca et al., 2009). The bioactivity of hop extracts over any of its purified metabolites supports the natural health product development approach to hops instead of a single compound pharmaceutical-based approach.

## DEVELOPING HOPS AS AN NHP

The vast majority of hops are currently being grown for use in the brewing industry (Kowalczyk et al., 2013; Krofta & Mikyška, 2014; Sanz et al., 2019), with over 100,000 tonnes harvested annually since 2016 (BarthHaas, 2022; Macchioni et al., 2021). Phytochemicals

unique to hops, such as bitter acids and xanthohumol, make up 10-30% of its weight and have demonstrated a wide spectrum of bioactive properties (Almaguer et al., 2014; Carbone & Gervasi, 2022; Elrod et al., 2019; Olšovská et al., 2016; Van Cleemput et al., 2009; Zhang et al., 2021). However, hop-based supplements continue to miss the top 40 herbal supplements threshold (Smith et al., 2019, 2022). The transfer of knowledge between brewing to NHP development could be one solution to this problem.

For example, hop supplements do not list the quantity of bitter acid components. However, hops purchased as pellets from a homebrew supply shop have the percentages for alpha and beta acids on their labels, as brewers select hops based on these values and sensory characteristics. Therefore, combining brewing industry knowledge of hop chemistry, coupled with the known bioactive properties of hops, along with consumer education could position hops in the top forty herbal supplements (Smith et al., 2024).

Developing new hop-based dietary supplements and NHPs should aim to identify optimal phytochemical profiles for various bioactivities (e.g., RSA, anti-inflammatory, and endocannabinoid modulatory properties). This will enable the identification of optimal source materials or formulations for new hop products with targeted therapeutic applications, such as high-alpha varieties selected for RSA (Chapter 3) or high-colupulone (and low-n-adhumulone) varieties developed as endocannabinoid system modulators (Chapter 4). The next steps would be *in vivo* animal models, followed by clinical trials, and then marketers could use this information to a target audience.

In contrast to the extract approach to product development, targeting specific isolated compounds for pharmacologically relevant applications could also be worthwhile. For example, colupulone demonstrated moderate RSA, strong COX-2 inhibition (Chapter 3), and the strongest

MAGL inhibitor (Chapter 4), suggesting that this could be a useful supplement for those with elevated oxidative stress and inflammation levels. Note that colupulone has also demonstrated cytotoxicity in brine shrimp (McCallum et al., 2020), and further *in vivo* trials should be completed before colupulone is administered to humans. Meanwhile, xanthohumol demonstrated moderate COX-2 inhibition though weaker RSA than other target compounds (Chapter 3) and was the strongest FAAH inhibitor (among the target compounds) and the second strongest inhibitor of MAGL (Chapter 4). Currently, there are 6 completed and 5 actively recruiting clinical trials testing xanthohumol's effects on multiple mechanisms, such as antibiotic properties to preventing DNA damage, whereas there are no clinical trials for any of the bitter acid constituents (https://clinicaltrials.gov).

Interestingly, beta acids and xanthohumol have received less attention from both the brewing and the NHP communities. It has been shown that xanthohumol and beta acids can be used effectively in both the food and pharmaceutical industries (Astray et al., 2020; Krofta & Mikyška, 2014; H. Liu et al., 2020; Zugravu et al., 2022). BetaBio 45, developed by Hopsteiner, contains 45% beta acids and could be used as a natural antimicrobial agent for various food products as beta-acids have established antimicrobial properties (Macchioni et al., 2021; Sanz et al., 2019; Zhang et al., 2021; Zhou et al., 2018; Zugravu et al., 2022).

Beta-acids (including colupulone) are hydrophobic (Krofta & Mikyška, 2014; Zhang et al., 2021), while alpha-acids are more water-soluble (Foster et al., 2009; Krofta & Mikyška, 2014). Therefore, the specific solvent used for extraction or product formulation should be selective for target compounds and appropriate for human consumption. Hence, high-percentage ethanol was an appropriate choice for extracting bitter acids and prenylated flavonoids, but it also translates well to NHP development, where food-grade ethanol is the organic solvent of choice to ensure

consumer safety and regulatory compliance. More recently, a nanoformulated (lipid-encapsulated compounds ranging from 1-100nm) hop extract was shown to have better antioxidant activity than conventional hop extraction techniques (Lela et al., 2022). It would be interesting to see if a nano-encapsulated formulation of hop extract has greater inhibitory potential on enzyme-based assays.

There was 1.89 billion hL of beer produced in 2022 (BarthHaas, 2022), this equates to more than 23L of beer per person. Imagine the quantity of hops added to beer that were not repurposed for other uses. Spent hops are the by-product of hops from the brewing industry and might be a viable way to repurpose a waste product into profit, considering that beta-acids have low solubility in aqueous environments (Foster et al., 2009). They would be commonly sourced from hops after being extracted (e.g., supercritical $CO_2$) or filtered before fermentation (Olivares-Galván et al., 2022). Spent hops are rich in polyphenols and have demonstrated anti-inflammatory properties (Caban et al., 2020; Hougee, 2008; Tripp et al., 2005), though sometimes they have considerably lower beta acid content relative to hop pellets (Sharp, Qian, Y., et al., 2017), though based on the quantity of beer produced annually, this might be a worthwhile endeavour to repurpose a waste product into an NHP.

## FUTURE STUDIES:

Results from this dissertation spawn a plethora of interesting experiments in hops research. From the chemistry chapter, the addition of different compound classes, terpene, total phenolic content, and total flavonoid content data would enhance the chemical separation. Having equal sample sizes for each of the groups could enhance the statistical power. Meanwhile, the hops growing location and harvest year have been shown to impact the number of secondary metabolites (Contin et al., 2023; McAdam et al., 2014), future work investigating the criteria to

enhance total yield or secondary metabolite yield across space and time could improve the efficiency of growing hops. The inclusion of hops grown in Oceania would be fascinating as their chemical profiles are sought after among craft brewers.

Moving to assay-based tests, the total ORAC score should be tested to explore the interaction between free-radical scavengers in aqueous and lipophilic environments, following the protocols that identified xanthohumol as the hop constituent with the greatest ORAC score (Yamaguchi et al., 2009). Applying how hop extracts interact with the natural enzymatic free-radical scavenging enzymes, such as glutathione reductase, would add external validity to the present results. Identifying the cytokines responsible for hops' anti-inflammatory properties would also expand the knowledge of how hops lowered inflammation. Testing hops on LOX inhibition would be another terrific addition to the DPPH and COX-2 data (Figure 5-2). The hulupones have been shown to enhance natural killer cells and could be useful alongside chemotherapy for cancer treatment (Salviati et al., 2019). Exploring the impact of hulupones on COX-2 inhibition might also be a worthwhile experiment.

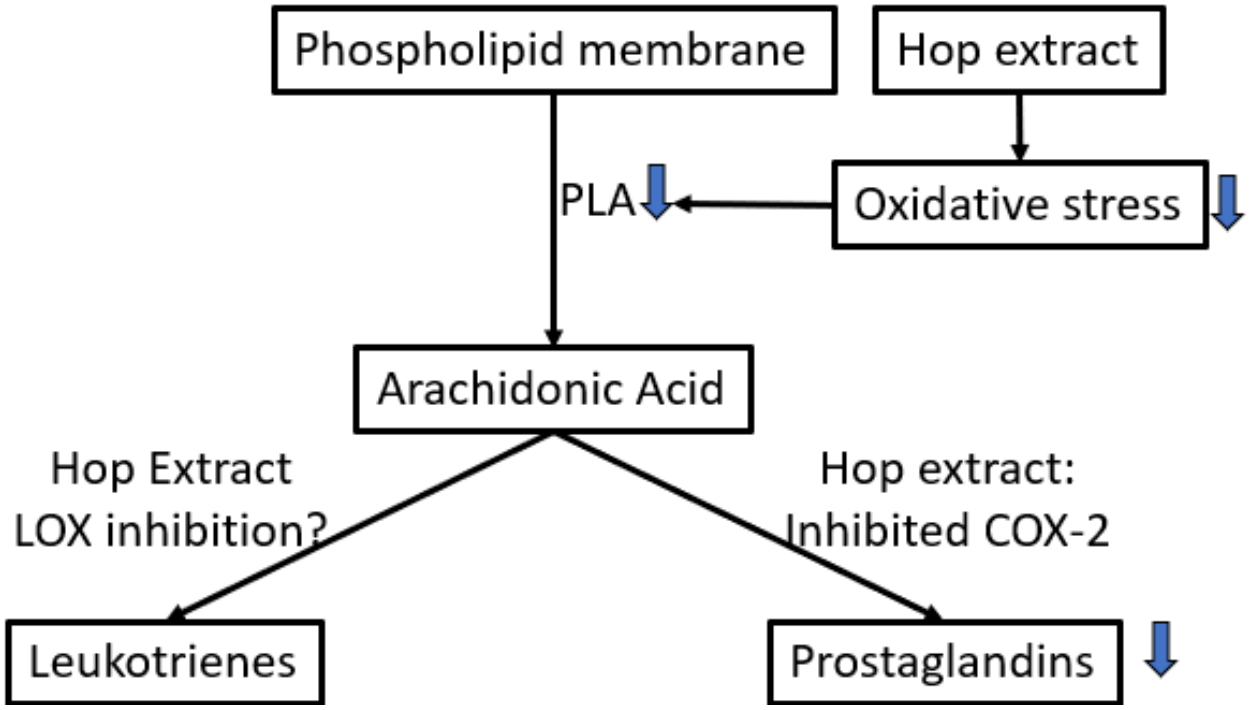


**Figure 5-2. A schematic diagram of when hop extracts interact with inflammatory pathways.** Hop extracts demonstrated free-radical scavenging activity. The RSA is associated with inhibiting oxidase enzymes (Heim et al., 2002; Shahidi & Ambigaipalan, 2015)**.** Hops demonstrated COX-2 inhibition, thereby reducing prostaglandin production. However, it remains unclear what role hops play in LOX activity inhibition, which is linked to lower leukotriene production. (Herschman, 1996; M. Murakami & Kudo, 2002).

Previously, colupulone has been shown to inhibit many of the CYP450 class of enzymes responsible for metabolizing various classes of drugs (Foster et al., 2009, 2011). Therefore, CYP inhibition assays should be completed to ensure a balance between safety and efficacy at an appropriate dose of specific plant compounds along with cone extracts. Moreover, colupulone was identified, as having cytotoxicity in a brine shrimp model (McCallum et al., 2020). Therefore, high-throughput toxicity screening of extracts and hop constituents should be

compared to the bioactivity to identify its therapeutic potential (D. Li et al., 2013). This could be followed up with a *in vivo* study to ensure the safety of consuming high concentrations of hop secondary metabolites.

*In silico* modelling and machine learning (Heussner, 2022; Tung et al., 2021) would be a useful next step for identifying the receptor binding location of hop compounds on COX-2, FAAH, and MAGL enzymes. Identifying the activity of hop constituents on CB-1 and CB-2 receptors would also be a useful addition to the FAAH and MAGL experiments.

An attempt was made to run hop extracts in a concentration-dependent manner on LOX-5 inhibition, the complimentary arachidonic pathway to COX and is associated with immune signalling, and these results would have been included in Chapter 3 (RSA and anti-inflammatory properties of hops). There was no consistent concentration-dependent response using hop extracts, so the experiment was excluded from the thesis.

## ADDITIONAL CONTRIBUTIONS

Other contributions made during my time in graduate school at the University of Ottawa include writing a peer-reviewed manuscript on the quality control and quality assurance of cannabis in Canada (Pusiak et al., 2021). This was followed up with a conference presentation and a book chapter exploring the quality control quality assurance of cannabis. Other cannabis-related presentations occurred at the quality assurance quality control of cannabis (QAQCC) annual symposium with a poster presentation outlining the dried cannabis equivalence and why they should be abolished, along with a 15-minute oral presentation outlining the variability of THC in dried flower cannabis. I have also been a guest lecturer three years in a row for a fourth-year drug policy class where I highlight cannabis science and nuances in testing protocols with an emphasis on dried flower variability and product recalls.

## OVERALL CONCLUSION:

This was the first study to systematically test a chemically diverse set of commercial hop samples to elucidate bioactivity across marker compounds using targeted and untargeted analytical techniques. Bitter and North American hops had more alpha acid than aroma and European hop samples, respectively. In hop extracts, alpha acid modelled RSA, beta acid modelled FAAH inhibition. Isolated colupulone showed moderate RSA, potent COX-2 inhibition, and the strongest MAGL inhibition across samples. Meanwhile, xanthohumol showed moderate COX-2 inhibition, the strongest FAAH inhibition, and the second strongest MAGL inhibition. Future work should test hops on cell-based models and use *in silico* models to predict where isolated compounds interact with COX-2, FAAH, and MAGL. The evidence for bioactivity presented in this research and that which has already been published warrants the development of hop-based nutraceuticals targeting free-radical scavenging activity and endocannabinoid system modulatory effects.

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# SUPPEMENTAL TABLES

**Table S1. Quantified chemistry in mg/g dried hops (n=32) using an HPLC-DAD:** cohumulone, n-adhumulone, colupulone, n-adlupulone, and xanthohumol. The alpha-to-beta acid ratio (ABR) is also included.

| Cultivar | Cohumulone | N-adhumulone | Colupulone | N-adlupulone | Xanthohumol | ABR |
|---|---|---|---|---|---|---|
| Cascade | 7.5 | 17.1 | 14.0 | 12.8 | 1.7 | 0.9 |
| Cashmere | 9.2 | 23.6 | 9.3 | 7.9 | 2.6 | 1.9 |
| Centennial | 9.0 | 25.0 | 7.7 | 7.0 | 2.3 | 2.3 |
| Chinook | 12.2 | 27.9 | 7.2 | 5.9 | 2.2 | 3.1 |
| Hallertau | 4.4 | 7.6 | 9.3 | 8.3 | 1.2 | 0.7 |
| Tradition | | | | | | |
| Tettnanger | 4.7 | 8.6 | 6.8 | 5.7 | 1.0 | 1.1 |
| Citra | 8.1 | 26.5 | 6.3 | 4.6 | 2.1 | 3.2 |
| Cluster | 4.4 | 7.1 | 9.5 | 4.8 | 1.4 | 0.8 |
| Columbus | 14.8 | 37.9 | 8.4 | 7.6 | 3.5 | 3.3 |
| German | 8.6 | 22.1 | 9.6 | 10.0 | 3.7 | 1.6 |
| Northern | | | | | | |
| Brewer | | | | | | |
| German Spalt | 1.6 | 5.5 | 7.0 | 9.4 | 1.4 | 0.4 |
| German | 1.8 | 5.5 | 6.3 | 8.3 | 1.2 | 0.5 |
| Tettnanger | | | | | | |
| Hallertau | 2.0 | 8.6 | 5.5 | 8.8 | 1.2 | 0.7 |
| Hallertau Blanc | 6.3 | 22.1 | 6.4 | 9.7 | 1.4 | 1.8 |
| Hersbrucker | 0.2 | 1.2 | 4.0 | 6.9 | 0.6 | 0.1 |
| Horizon | 4.9 | 30.2 | 4.7 | 8.6 | 1.6 | 2.7 |
| Huell Melon | 4.7 | 11.9 | 14.3 | 14.8 | 3.1 | 0.6 |
| Lemondrop | 4.2 | 8.8 | 8.1 | 5.7 | 1.1 | 1.0 |
| Magnum (G) | 10.1 | 28.3 | 11.3 | 13.8 | 1.9 | 1.5 |
| Magnum (U) | 6.9 | 20.4 | 7.8 | 8.7 | 1.5 | 1.7 |
| Mt Hood | 2.5 | 10.9 | 7.2 | 9.9 | 1.2 | 0.8 |
| Newport | 8.1 | 13.2 | 13.7 | 7.5 | 2.4 | 1.0 |
| Nugget | 9.0 | 28.7 | 5.8 | 6.2 | 2.9 | 3.2 |
| Perle | 2.7 | 7.8 | 3.0 | 3.3 | 0.9 | 1.7 |
| Saaz | 1.5 | 5.0 | 5.3 | 7.6 | 1.6 | 0.5 |
| Simcoe | 4.6 | 20.6 | 2.9 | 4.8 | 1.6 | 3.3 |
| Sorachi Ace | 8.9 | 28.4 | 9.7 | 8.8 | 2.6 | 2.0 |
| Styrian | 0.8 | 2.6 | 3.0 | 2.1 | 0.7 | 0.7 |
| Golding | | | | | | |
| Summit | 11.3 | 29.8 | 6.8 | 6.7 | 1.5 | 3.1 |
| UK East Kent | 4.5 | 10.8 | 4.9 | 4.4 | 1.3 | 1.6 |
| Golding | | | | | | |
| UK Fuggle | 2.0 | 5.7 | 3.1 | 3.1 | 1.0 | 1.3 |
| Willamette | 3.1 | 7.9 | 5.7 | 5.3 | 1.6 | 1.0 |

**Table S2. Extract chemistry (µg/mL) from ethanolic extracts of hops (n = 24) quantified using an HPLC-DAD.** Sorted by Group (HA: High-Alpha, HB: High-Beta, HX: High-Xanthohumol, LAB: Low alpha-beta).

| Group | Xanthohumol | Alpha Acid | Cohumulone | N-adhumulone | Beta Acid | Colupulone | N-adlupulone |
|---|---|---|---|---|---|---|---|
| HA | 11.2 | 170.1 | 47.8 | 122.3 | 51.6 | 27.1 | 24.5 |
| HA | 9.4 | 192.4 | 36.4 | 156.0 | 48.8 | 20.8 | 28.0 |
| HA | 9.7 | 140.0 | 33.4 | 106.6 | 69.2 | 36.2 | 33.0 |
| HA | 10.9 | 158.2 | 41.7 | 116.5 | 68.7 | 35.9 | 32.8 |
| HA | 9.7 | 177.7 | 54.0 | 123.6 | 58.1 | 31.8 | 26.3 |
| HA | 12.0 | 178.6 | 46.0 | 132.6 | 38.7 | 17.7 | 21.0 |
| HB | 7.1 | 69.9 | 21.4 | 48.5 | 146.7 | 71.2 | 75.4 |
| HB | 9.2 | 48.0 | 11.0 | 37.0 | 110.5 | 47.1 | 63.4 |
| HB | 12.0 | 64.0 | 18.2 | 45.8 | 111.6 | 54.7 | 56.9 |
| HB | 9.3 | 68.6 | 20.1 | 48.6 | 100.4 | 50.5 | 49.9 |
| HB | 4.3 | 36.3 | 3.0 | 33.3 | 107.0 | 36.8 | 70.2 |
| HB | 8.4 | 119.1 | 36.3 | 82.9 | 130.4 | 68.1 | 62.4 |
| HX | 13.8 | 114.5 | 32.0 | 82.5 | 73.1 | 35.8 | 37.3 |
| HX | 12.4 | 50.5 | 11.4 | 39.1 | 101.8 | 42.0 | 59.8 |
| HX | 12.3 | 162.9 | 50.8 | 112.2 | 53.2 | 29.3 | 23.9 |
| HX | 11.2 | 139.5 | 39.2 | 100.4 | 72.9 | 39.6 | 33.3 |
| HX | 13.5 | 83.2 | 23.9 | 59.3 | 63.6 | 31.4 | 32.2 |
| HX | 12.5 | 62.1 | 19.4 | 42.7 | 86.0 | 45.2 | 40.8 |
| LAB | 2.8 | 36.3 | 11.3 | 25.0 | 69.0 | 32.4 | 36.6 |
| LAB | 5.6 | 27.5 | 6.4 | 21.1 | 40.7 | 24.1 | 16.6 |
| LAB | 4.4 | 49.5 | 12.7 | 36.7 | 29.5 | 14.0 | 15.4 |
| LAB | 4.4 | 10.7 | 1.6 | 9.1 | 82.9 | 30.4 | 52.5 |
| LAB | 5.1 | 41.8 | 10.9 | 30.9 | 33.2 | 16.5 | 16.8 |
| LAB | 5.9 | 70.1 | 20.5 | 49.6 | 42.8 | 22.4 | 20.4 |

**Table S3: Bioactivity of dried ethanolic hop extracts (n=24) across the four assays: DPPH, COX-2, FAAH and MAGL.** Percent inhibition was calculated at 12.5 µg/mL for DPPH, 6.25 µg/mL for COX-2, 10 µg/mL for both FAAH and MAGL. $IC_{50}$ values are in µg/mL. Samples are sorted by Group (HA: High-Alpha, HB: High-Beta, HX: High-Xanthohumol, LAB: Low alpha-beta).

| Group | DPPH | | COX-2 | | FAAH | | MAGL | |
|---|---|---|---|---|---|---|---|---|
| | Inhibition | $IC_{50}$ | Inhibition | $IC_{50}$ | Inhibition | $IC_{50}$ | Inhibition | $IC_{50}$ |
| HA | 50.2 | 7.9 | 50.1 | 6.3 | 54.7 | 10.6 | 63.1 | 5.7 |
| HA | 61.3 | 5.5 | 44.8 | 17.2 | 47.9 | 15.5 | 58.4 | 5.2 |
| HA | 43.4 | 9.1 | 57.9 | 0.9 | 65.4 | 4.5 | 60.2 | 5.9 |
| HA | 49.4 | 7.3 | 59.3 | 1.1 | 49.0 | 13.8 | 60.2 | 4.9 |
| HA | 42.4 | 8.5 | 45.6 | 7.3 | 57.3 | 9.9 | 50.5 | 8.8 |
| HA | 49.2 | 7.8 | 67.6 | 3.2 | 39.1 | 19.0 | 60.3 | 6.1 |
| HB | 16.4 | 15.2 | 63.9 | 2.3 | 64.1 | 4.1 | 53.8 | 9.0 |
| HB | 23.4 | 15.1 | 50.7 | 6.6 | 65.1 | 4.0 | 57.5 | 5.2 |
| HB | 33.5 | 11.8 | 52.4 | 21.7 | 65.2 | 3.9 | 58.6 | 5.7 |
| HB | 22.3 | 17.0 | 51.7 | 2.6 | 67.0 | 3.4 | 47.3 | 9.3 |
| HB | 23.0 | 18.4 | 40.8 | 9.4 | 58.1 | 7.3 | 59.1 | 7.1 |
| HB | 35.3 | 11.1 | 60.5 | 4.3 | 66.7 | 3.4 | 58.2 | 7.8 |
| HX | 33.2 | 12.1 | 55.7 | 5.1 | 62.1 | 5.1 | 61.1 | 5.8 |
| HX | 22.9 | 16.4 | 50.1 | 6.7 | 63.1 | 5.4 | 63.7 | 4.6 |
| HX | 49.7 | 8.5 | 45.9 | 8.7 | 65.6 | 4.2 | 58.6 | 6.4 |
| HX | 44.8 | 9.3 | 54.6 | 5.1 | 61.9 | 7.2 | 58.3 | 6.9 |
| HX | 25.8 | 15.1 | 51.9 | 7.3 | 54.1 | 13.1 | 65.1 | 5.0 |
| HX | 13.3 | 35.0 | 43.3 | 8.2 | 65.8 | 4.0 | 60.5 | 5.4 |
| LAB | 37.6 | 10.5 | 54.6 | 3.7 | 60.9 | 7.4 | 57.1 | 7.7 |
| LAB | 17.2 | 26.9 | 61.6 | 4.4 | 68.9 | 3.3 | 68.4 | 3.2 |
| LAB | 31.0 | 12.6 | 64.8 | 4.8 | 56.8 | 8.0 | 54.7 | 6.6 |
| LAB | 25.2 | 19.0 | 46.1 | 8.2 | 62.8 | 5.2 | 55.3 | 6.2 |
| LAB | 19.7 | 19.1 | 61.0 | 6.2 | 67.6 | 3.8 | 53.9 | 4.4 |
| LAB | 26.2 | 15.8 | 42.1 | 10.5 | 52.4 | 20.4 | 54.9 | 7.5 |

# APPENDIX A: Growing pains: An overview of cannabis quality control and quality assurance in Canada – February 2021

## Growing pains: An overview of cannabis quality control and quality assurance in Canada

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**Abstract**

In the past decade, the predominant prohibition model for cannabis use has shifted towards a regulated legal model, most widely in the context of medical purposes. In 2018, Canada became the first G7 country to legalize cannabis for adult use, implementing a two-phase roll-out of cannabis regulations. A stated goal of the new legal framework is to minimize harms by providing a safe supply of cannabis to Canadian consumers. One way that this can be achieved is through appropriate Quality Control and Quality Assurance (QC/QA) measures. Canada has implemented stringent QC/QA measures for all classes of cannabis, which include requirements such as labelling THC and CBD content per product and limiting THC doses. This paper will provide an overview of the current QC/QA measures in Canada, highlighting differences based on class of cannabis and consider the strengths and weaknesses of the current standards. QC/QA standards represent a key safety feature that can enable informed purchasing and provide consumers with necessary information about various cannabis products. As Canada continues to progress its cannabis policies, QC/QA measures provide a key consideration for ensuring Canada meets its objective of providing a safe supply of cannabis to Canadian consumers.

**Keywords:** cannabis, legalization, Canada, product quality, regulations, production standards, variability, limits, testing, protocols, consumers

## Background

Canada legalized adult use cannabis in 2018 with the implementation of the *Cannabis Act* [1, 2], bringing with it an overhaul of the old, and an articulation of the new legal, socio-political, and economic frameworks related to this notorious plant. Despite criminal sanctions and social stigma, millions of individuals have continued to consume cannabis for centuries across the world, with Canadians representing one of the largest demographics of users in the world [3]. Canada's recent legalization can be held as an evolution from past prohibitionist cannabis policies in favour

of a strict regulatory framework that enables the legal manufacturing, sale, and consumption of cannabis [4]. Found in section 7 of the *Cannabis Act,* the main purposes of legalization are positioned as reducing access for youth, decreasing drug sale profits to criminals, and enabling access to quality-controlled and safe cannabis products for adult users [2]. There have been and continue to be wide-ranging considerations involved in the legalization of cannabis, from international trade to manufacturing processes to workplace safety. Setting up a distribution chain, creating a retail ecosystem, developing proper manufacturing and quality assurance protocols, and determining the general framework in which cannabis will be accessed in the country all offer examples of the various auxiliary tasks required in legalizing cannabis. Creating the legal cannabis industry in Canada has been a complex undertaking that remains in its infancy.

A fundamental element of legal cannabis production and sale in Canada and elsewhere is quality control and quality assurance (QC/QA), which requires the creation of new standards and processes [5, 6]. **QC** is a set of activities for ensuring **quality** in products. **QA** is a set of activities for ensuring **quality** in the processes by which products are developed. Illicit cannabis, prior to legalization, had limited (if any) standardized quality controls for the cannabis distributed and consumed in Canada – this is in contrast to the QC/QA of medical cannabis [7], which was first implemented with the *Marihuana Medical Access Regulations* in 2001 [8]. Illicit markets did not, and still do not, have any regulation or oversight pertaining to quality, no industry standardization frameworks that cannabis growers must follow, no surveys to capture or highlight consumers' concerns or protection from related harms [9]. This historical lack of enabling rather than prohibitory regulation resulted in potential hazards such as microbial and chemical contaminants (see Table 1, and [10]). Purchasers of cannabis from illicit markets across jurisdictions report concern about product safety and the methods of production [11]. In Canada, these concerns have

been addressed, in part, with the introduction of the *Cannabis Act,* and the subsequent implementation of stringent QC/QA protocols that allow safety assurances and seed-to-sale tracking mechanisms [12]. Theoretically, as a result of standardization through governmental oversight, Canadian consumers can expect safety, consistency and reliability when purchasing legal cannabis products. With ongoing calls for the safe supply of other drugs, the legalization of cannabis can be viewed as a mechanism for providing safe supply through a quality-controlled system as well as a potential example of safe supply in action [13, 14, 15].

This paper focuses on the QC/QA protocols and frameworks for cannabis in Canada, starting with a descriptive overview of the current regulatory regime, followed by an analysis of the strengths and weaknesses of the implemented framework, and finishing with considerations for the future. While cannabis for medical purposes has been legal in Canada since 2001 [8] the current focus is on adult-use cannabis, referring to previous Canadian regulatory regimes as needed. As other jurisdictions worldwide re-examine their approaches to cannabis, a critical review of Canada's pioneering QC/QA structure at a national scale can shed light on some of the benefits and tensions apparent since enactment of the *Cannabis Act*.

**Setting the Stage: Descriptive Overview of the QC/QA Requirements in Canada**

An underlying goal in legalizing adult-use cannabis is to protect consumers from the harms associated with illicit products. Creating a legal supply chain allows for the potency and safety of products to be tested and relayed to consumers so that they can make informed decisions about which products to buy; in turn, minimizing harms related to potency and contaminants commonly found in illicit cannabis (Table 1). In terms of accessible cannabis products in Canada, decision-makers opted for a phased roll-out by only allowing dried inflorescence, tinctures, and cannabis-

infused oils to be produced and sold as of October 2018 [16]. The second phase was implemented one year later in October 2019 and expanded the legislation to enable the production and sale of edibles (e.g., baked goods, drinks), topicals (e.g., skin cream or ointment), and concentrates (e.g., hash, shatters, and vape cartridges) [16]. The recent roll-out of these products has been dubbed by some as “Legalization 2.0” [17] and has introduced further complexity into existing QC/QA frameworks with food and beverage quality assurance guidelines also needing to be considered.

Health Canada is the federal government regulator charged with the oversight of cannabis production, manufacturing, and QC/QA. Licensed producers (LPs) of legal cannabis are required to have appropriate, specific, and validated protocols in place to ensure products are consistent and safe [18]. These measures include the mandatory testing of the levels of cannabinoids, microbial contaminants, pesticide residues, elemental impurities, and residual solvents in the products (Table 1), similar to the mandatory testing of medical cannabis in the Netherlands, and under previous Canadian regulations [19, 20]. Of these tests, cannabinoid content, specifically that of tetrahydrocannabinol (THC) and cannabidiol (CBD), represents a focus for consumers, industry, and public health, alike, and is therefore a focus of QC/QA regulations.

THC is the main cannabinoid responsible for the cannabis “high”, as well as certain therapeutic effects, and its levels define the potency of the product. Accordingly, legislation has defined limits on THC levels (taking into account the maximum amount of THC that would be obtained if delta-9-tetrahydrocannabinolic acid (THCA) was converted into THC with no further degradation of THC) to minimize potential harms associated with potent THC products as advocated for by public health experts [21]. CBD represents another common cannabinoid that has received recent attention from consumers, media, and industry for its potential therapeutic application and ability to mitigate certain harmful effects of a high-dose THC [22, 23]. Adult-use

cannabis products do not have a maximum CBD potency for any cannabis products [1]. However, since current consensus is that potential harms are associated mainly with THC, regulations (and our review) focus primarily on THC. Topicals and extracts are limited to 1000 mg THC per package and must have a mechanism to appropriately dose the amount of THC. Meanwhile, ingestion, nasal, vaginal, or rectal cannabis products are limited to 10 mg of THC per discrete unit, while the limits for tinctures are no more than 7.5 g of product per unit [16]. There is no legislative limit for the percent THC in flower cannabis, however, the biological upper limit for producing THC in cannabis flower is approximately 30%, and often these estimates are inaccurate [24].

The current *Cannabis Act* does require strict limits for the amount of THC in each respective class of product (e.g., 10 mg/ unit) and Canadian LPs are required by Health Canada to test and label the total content of THC and CBD in all commercial cannabis products [2]. This allows consumers, faced with a variety of products, to make informed decisions based on their preferences and past experiences. The mandatory testing of cannabis for total THC, total CBD, and potential contaminants by LPs in Canada are completed in-house or outsourced to an independent testing facility. For example, pesticides must be tested by a third-party laboratory [25]. Manufacturers have the option to test the final form (e.g., cannabis extract) or the final step of production (e.g., packaged cannabis edible). Each manufacturer requires a Quality Assurance Person (QAP) who is responsible to oversee in-house or external testing, as necessary, review complaints pertaining to quality of the cannabis, and investigate use of potentially harmful ingredients [16]. These experts ensure that the LP complies with the QC/QA regulations and oversees testing of potency, and microbial and chemical contaminants. Following legalization in 2018, numerous certificate programs and educational courses aiming to teach the skills required of a QAP have emerged, ranging from post-graduate diplomas to semester-long post-secondary

courses to week-long training events. However, there are currently no standardized industry requirements related to competencies for these key individuals.

Cannabis producers are also required to implement Good Production Practices (GPP) to ensure that a consistent, safe, and high-quality product is sold to consumers. GPP ensures that the production of cannabis products meets the QC/QA standards outlined in the *Cannabis Act*. This includes testing for and mitigating potential contaminants (Table 1), record keeping, and running recall scenarios. The GPP protocols and practices are conducted under the supervision of LPs; however, Health Canada audits the LP's facilities annually and reviews documentation as required to ensure that the LPs are following legislation and GPP [26, 27, 28]. LPs are also required to maintain documentation and records related to their production of cannabis for two years, alongside the annual practice of recall scenarios [2]. These documents must be retained for a minimum of two years and include the ingredients used, suppliers of the ingredients, dates the ingredients were obtained and produced, and a description. A list of adverse reactions are made annually, and a record of all serious adverse events must be retained for 25 years as per s.248 of the *Cannabis Regulations* [18].

In terms of packaging requirements, LPs must include the total THC and CBD content of the product, the standard cannabis symbol, THC warning label, health warning, child resistant packaging, ingredient list, packaging date, and common names [16]. Currently, cannabis products in Canada must not make health claims due to the recreational nature of their sale and the current scheduling of the drug. These packaging requirements are similar to those implemented for substances such as tobacco, and act to inform and warn consumers of potential risks associated with use. There have been recent calls for more effective labelling of cannabis products to allow consumers to have a better grasp of THC concentrations and to titrate their THC intake more

effectively [29]. Adapting labelling requirements from beer, which classifies products based on the percent alcohol-by-volume (ABV) categories [30], may be useful to classify levels of THC in cannabis products and enhance the consumer experience. Beer drinkers can tailor their purchases based on the categories of alcohol content (e.g., 2.6-4% ABV for light, 5.6-8.5% ABV for strong, etc.), which is the substance of concern. As an example of an industry solution, rather than policy solution, certain licensed cannabis stores (both online and brick-and-mortar) allow customers to select a range of both THC and CBD (among other variables) available in cannabis products. This tailors to the customers preference and reduces the need to create legal categories.

Regulating cannabis production, the quality and diversity of products, and accessibility for consumers remains a challenge to balance and implement due, in part, to the novelty of the *Cannabis Act* and the relative lack of available research to inform regulatory decisions. As more knowledge emerges about cannabis consumption patterns and health impacts, the QC/QA frameworks, and broader regulatory structures in general, will need to adapt in tune.

**Strengths of QC/QA requirements**

The introduction of QC/QA requirements for cannabis production and manufacturing for the first time with Canadian legalization is a large step forward, as it creates a standardized environment in which consumer safety and quality metrics are prioritized.. QC/QA testing and labelling *prima facie* acts as information production that provides producers and consumers with necessary data such as a THC and CBD potency ranges. The QC/QA requirements also form a system of record keeping that creates a trackable and auditable baseline of product information [12]. Such documentation ensures accountability and mandates transparency on the part of LPs and regulators. These measures also act as a consumer protection mechanism, by dissolving some

of the information asymmetry in the market [11]. This occurs when sellers have more information than consumers about product quality, while QC/QA frameworks provide a mitigation strategy for this asymmetry. Finally, in terms of producers, transparent quality assurance record-keeping allows companies to continually improve their processes and, in turn, their products [31]. The enforcement of industry-wide regulations keeps companies accountable while also providing internal information helpful in product development and research [28]. The most recent Summary of Inspection Data reported that, under the ACMPR (2017-2018), Health Canada conducted 240 regular inspections and 17 surprise inspections that collectively revealed 63 "major" and two "critical observations" leading to the issuance of five warning letters, three ratings of non-compliance, and one voluntary recall [28]. However, the number of licensed cultivators, processors and sellers has increased from >100 to <550 since that time [32] and, with no compliance data available under the Cannabis Act, the regulator's capacity to maintain oversight and enforcement of QA/QC regulations, although presumably strengthened, remains unknown.

*Informed Consumers*

For consumers, the QC/QA requirements enable a climate of knowledgeable purchasing for cannabis products, whereby consumers are assured that the cannabis has passed contaminant testing and relevant information is disseminated on the levels of THC and CBD [16]. Prior to legalization, recreational consumers had little to no information about the products they purchased, let alone whether they were safe to consume. The regulation of cannabis under the *Cannabis Act* and related QC/QA requirements for LPs minimizes risk and mandates standardized consumer-facing packaging. Required testing and labelling of THC and CBD percentages may also reduce risk of over-consumption and unintended effects, since novice and experienced consumers are able to select products with lower THC and/or higher CBD depending on their subjective reason for

use. Accordingly, (accurate) reporting of potency can reduce the chance of harms or a negative experience by allowing consumers to select products with cannabinoid levels that suit their preference. As new evidence is uncovered and knowledge on cannabis and its effects progresses, QC/QA requirements can help to inform and educate consumers on product types. The Canadian government has recently announced it is investing $100 million towards a cannabis education campaign to inform consumers on the impacts of consuming cannabis and education pertinent to safe use which can help support informed purchasing [33]. There are also online portals for some general information on cannabis use, along with a short document explaining the approximate onset and duration after consuming various cannabis products [34, 35, 36].

*Consistent (Formulated) Products*

For new classes of cannabis, including edibles and concentrates, regulations limit both the maximum content and the maximum variability of THC. Whereas limits on THC content may reduce risks associated with overconsumption and unintentional intoxication, limits on variability ensures that the amount of THC reported on the label and the actual amount of THC in the product is within an acceptable range of error [16]. The variability limits for cannabis are set out in Table 2. This is an added strength of the new consumer product regulations compared to earlier medical cannabis QA standards, which set no such limits on variability; although, there are still no limits for inflorescence flower products. For new classes of cannabis products such as edibles and concentrates, which are formulated and should therefore be more consistent across batches and lots than plant material, the *Cannabis Act* requires more accurate THC labelling. For example, if consumers purchase a 10 mg THC edible, then the product must contain 10 mg of THC with a 15% margin of error (8.5 - 11.5 mg, see Table 2). Placing legislative limits on variability increases the likelihood that people are indeed getting what is labelled, along with reducing adverse effects

of accidental or over-consumption. This is of particular importance for edible cannabis that, relative to smoked or vaporized inflorescence products, has a slower onset, therefore, poses a higher risk for over-consumption and related adverse events [37, 38].

The QC/QA requirements also bring a level of product consistency to the market. As highlighted by Thomas and Pollard (2016), consistency in chemical constituents is a basic requirement in the pharmaceutical industry for quality, safety, and efficacy of botanically derived drugs [39]. While non-medical cannabis and medical cannabis in Canada are not necessarily classified as pharmaceutical products with a Drug Identification Number (DIN), the incorporation of stringent QC/QA standards elevates consistency to the level of other botanically-derived health products. It is important to note however, that all purified phytocannabinoids (e.g., THC, CBD, etc.) from cannabis are classified as a pharmaceutical ingredient, thus requiring a prescription for sale, except derivatives of cannabis as defined by the *Cannabis Act* [2, 40]. Health Canada is currently considering an alternative framework for some cannabis health products (CHPs), with strict limits of 10 µg/g THC, that would allow some specific health claims to be made in alignment with current scientific evidence and subject to regulatory authorization [41, 42]. The QC/QA standards mandate producers to curate consistent product offering, in turn enabling consumers to know what to expect through labelling, past purchases, and other information sources.

*Minimizing Contamination of Products*

QC/QA standards that mitigate risk of microbial and chemical contamination of cannabis were introduced under previous regulations for the sale of cannabis for medical purposes [43]. While some fungi and bacteria can grow on living cannabis plants with low risk to the plants or to humans, others can pose a risk to the health of both the plant and consumer. Mold, for example,

can reduce the yield and quality of the inflorescence produced by living plants but can also persist and proliferate on cannabis inflorescence after harvest, during processing and storage, and during human consumption [9, 20, 44]. Some microbes may also produce toxins that contaminate and persist on plant material once the microbes are killed and the inflorescences have been dried and processed for recreational or therapeutic use. As levels of both microbes and microbial toxins must now be tested and determined to be absent or below specified thresholds for all legal cannabis products, risk of such contamination is far lower than in illicit products where no such testing or oversight occurs.

With the exception of hemp (defined as cannabis with <0.3% THC [20, 45]), legal cannabis must be cultivated in Canada indoors to provide security, to control growth conditions, and to limit variability. Cannabis is typically cultivated at high density to maximize yields and profits. This environment, however, is also favourable for many plant pests and pathogens. While pesticides can be an effective intervention to maintain plant health and avoid losses in production and sales, licensed cannabis producers are limited to a small number of approved natural pesticides with low risk of toxicity [46]. Interestingly, there are over 1000 approved pesticides for tobacco [47]. Regulated commercial cannabis products, unlike illicit ones, must also be tested by a third-party laboratory to confirm the absence of more than 90 different (unapproved) pesticides [48].

The remaining chemical contaminants covered under QA standard testing are introduced during processing and formulation of cannabis products. To make THC- and CBD-enriched products including edibles and beverages, vape pen cartridges or topicals, the cannabinoids must first be extracted from the plant for use as ingredients. The most common methods of extraction involve processing inflorescences in an organic solvent that captures the cannabinoids and is subsequently removed (e.g., evaporated off) to yield a concentrated extract. The extract, however,

may contain residual amounts of solvent that can lead to adverse effects if consumed above threshold levels. Whereas the majority of LPs in Canada use low risk solvents like food-grade ethanol, more toxic and lower-grade solvents (e.g., methanol, butane, hexane) may be used in un-regulated operations and persist in products at higher residual concentrations [49].

Although not a new element of cannabis QC/QA in Canada, mandatory testing of microbial and chemical contaminants became a more important harm reduction tool with the introduction of products that use extracts as an ingredient and that could deliver microbes or toxins to the lungs, gut, skin or other epithelia (without degradation related to combustion). Low-quality or otherwise unappealing crops are less marketable to consumers so often processed into extracts for use in other products. Without regulatory oversight, contaminated crops could be processed, and their contaminants transferred, potentially in a more concentrated form, to products and then to people. Since high levels of both residual solvents and pesticides have been reported among concentrates available in the California medical cannabis market [49], strict regulation of microbial and chemical contaminants promises to protect Canadian consumers from an avoidable risk.

**Potential Gaps and Weaknesses of the regulations**

Although there are many benefits to the current framework of QC/QA of cannabis in Canada, there are still knowledge gaps to fill, and weaknesses to strengthen, in order to further reduce risk of consumer harm. Weaknesses include defining cannabis categories; limits and variability of THC between LPs, batches, and individual packages relative to labelling; quality assurance on dispensing products; accurate vaping technologies; and consistent analytical testing.

*Inconsistency of strains and inflorescence products*

Defining subgroups of cannabis is a challenging endeavour. Small and Cronquist's (1976) is a widely but not universally accepted system which defines cannabis as one species (*C. sativa* L.), with two subspecies that, in turn, have two varieties [50]. Taxonomists Clarke and Merlin classify cannabis as a polytypic genus consisting of two extant species, *C. sativa* and *C. indica* Lam., each circumscribing several biotypes or subspecies [51]. Today, cannabis is colloquially categorized as lineages of sativa, indica, or hybrids of the two [52].

In the cannabis industry, the term "strain" is often used to refer to a type of cannabis yet there is no objective and widely accepted definition of a cannabis strain, and what differentiates one strain from another [53]. The word strain is not a botanical term and is often confused with cultivated varieties or cultivars of other economic crops developed over a long history of farmer selection and breeding of wild varieties and their hybrids. Historical cultivars are called "land races" in agriculture. Whereas hemp cultivars follow agricultural standards of stable and distinct traits, strains of drug-type cannabis are developed and named as no guiding rules or regulations. An initial database of strains and cultivars was formulated in 2002 listing over 700 varieties (strains of high-THC and cultivars of hemp) of cannabis [54]. More recently, over 1300 unique commercial strains of high-THC cannabis were documented in 2018 [22]. Strain names are commonplace, and consumers believe that strains have distinct characteristics (i.e., potency, aroma, appearance), though limited testing and almost no statistical analysis has been used to support classification of distinct strains of cannabis [24].

Perhaps the most rigorous way to classify plants is through internationally accredited cultivars for which phenotypic and chemical characteristics fall into a narrow-defined range as is common for agricultural crops. This would create an objective definition of cannabis varieties and clear characteristics that separate one variety from another (e.g., cannabinoid levels, aroma

(terpene profile), appearance, etc.) and that can be *consistently* reproduced (either sexually or asexually) with the same characteristics [55]. Some growers attempted to patent medical cannabis plants, though most of these patents were abandoned [56]. Producing and registering cultivars takes time and resources and has been successfully and widely accomplished with hemp (56 cultivars approved for cultivation in Canada for the 2020 growing season [57]). The same will likely be achieved for recreational and medical cannabis in the coming years and will greatly improve the reproducibility and characterization of commercial cannabis.

Under current use of strain names, variability of plants in a single strain may be much higher than registered cultivars. This can generate different chemical profiles from lot-to-lot [58]. In addition to genetic and environmental factors that contribute to variability, the processing of cannabis can have a major impact on the quality and consistency of the finished product. Growing conditions within a single facility can vary and certain plants grow more vigorously than others based on lighting, nutrients, temperature, pests/disease, etc. Different trimming and curing protocols could result in differences among batches of the same strain [59]. While GPP as outlined in the QC/QA guidelines can help mitigate some of this variability, consumers may purchase the same product twice and receive two chemically and pharmacologically different products. As demonstrated for medical cannabis in the Netherlands and is likely true with many Canadian LPs, optimized processes and standard operating procedures result in improved consistency between batches [59]. Therefore, the move towards developing standardized strains such as those in the Netherlands [60] and the United States [61] would improve consistency among batches of cannabis from the same strain. LPs and/or Health Canada should consider policy approaches in other jurisdictions and seek to develop their own consistent strains so as to mitigate risk of variability among lots.

The current regulations do not define any limit on the variability of cannabinoid (i.e., THC or CBD) content for cannabis inflorescence, in contrast to the defined limits for new classes of cannabis products (Table 2). This lack of regulatory control for THC variability reduces the QC/QA credibility of legally produced cannabis. Total THC in dried inflorescence (mg/g) of the same strain can vary widely between and within LPs. As described above, due to variable plant genetics and environmental conditions, two LPs could grow the same strain and produce inflorescences with different levels of THC [24]. Different batches of cannabis (produced by the same LP), moreover, will likely have different amounts of total THC as will different inflorescences from the same batch. Each batch is tested, and the chemical results, typically expressed as the average across all tested samples, are present on the product label. Even if the product label indicates the range of THC levels within a batch (e.g., 12-16% or 14±2%) and indicates that the range changed between batches, there is currently no requirement to include the range on the label or to limit either lot-to-lot or batch-to-batch variability. This creates a retail environment where consumers are left with opaque information on the actual THC levels in the inflorescence they purchased in relation to the labelled variability values.

A study of illicit cannabis grow-operations found that THC content of dried inflorescences can vary considerably within the same plant (e.g., from 20-30%) and between plants (4-25%) [62]. For other regulated substances, such as alcohol, there are strict laws in place to ensure that the ABV content that is labelled on a bottle of alcohol is accurately reported [63]. In contrast, cigarette packaging in Canada does not indicate the level of nicotine in their product. While it is unlikely to set such strict limits on THC for cannabis inflorescence, due to factors associated with the plant producing cannabinoids, a variability limit should be considered by policy makers. The variability limit for THC and CBD in the Netherlands for medical cannabis users is set at 15% [59] and the

Cannabis Expert Panel suggests a 20% variability limit [20]. Therefore, it is possible to apply these standards to Canadian cannabis. Products that are outside of the range can be repurposed for extraction and used in other products (e.g., concentrates, edibles, topicals, etc.).

An alternative way to group cannabis beyond strains or cultivars that considers the batch-batch variability would be to define the cannabis product based on its unique chemical profile. These chemical-varieties would be grouped based on their cannabinoid content (levels of THC and CBD) and terpenes profile (the compounds responsible for cannabis' distinct odour) and not necessarily be based on their lineage [59, 64]. Using a metabolomics approach, varieties can then be defined (e.g., high THC, caryophyllene dominant terpene) and compared to others (e.g., high CBD, high myrcene) to gauge degree of similarity based on chemistry [64]. This would bypass the resource-heavy international cultivar approval process for high-THC cannabis [59, 64]. Although currently beyond the capacity of most LPs, the gaining popularity and accessibility of metabolomics may pave the way toward defined varieties that would better inform consumers and reduce confusion about the properties of commercial cannabis products [64].

*New product classes reveal new challenges to QC/QA and harm reduction*

QC/QA protocols for limiting, testing, and labelling cannabinoid content of edibles, topicals, and concentrates have only recently been implemented in Canada. These new classes of products can be tested as the extract used as an ingredient or as the final product [16], with both options presenting drawbacks. For example, cannabinoids in baked goods may be lost (e.g., degraded or vaporized from the product), concentrated (e.g., due to water loss) or transformed (e.g., by decarboxylation of THCA, CBDA, etc.) during the cooking process or unevenly distributed across products within a batch. There is greater variability in levels of THC in the final

products because of the processing that occurs when producing the cannabis product. Testing the extract is less variable because it has not been chemically or mechanically altered in the finished product. If the company tests the extract/ingredient or pre-baked batch of batter, then labelled cannabinoid levels may differ from what is in the final product. To avoid this risk and more accurately measure both cannabinoid content and variability, LPs could simply test the finished product. However, with variability in production, testing finished products may yield greater variability in cannabinoids than testing extracts, and – if greater than 15% – would not meet QC standards and result in lost revenues. Moreover, analytical methods for testing cannabinoids in extracts are much less challenging and costly than for edibles, which require a new validation procedure for each product. Once QA-approved, an extract can also be used as an ingredient for multiple products which minimizes incentive for LPs to test the final product. This could result in less consistent products, whereas the risk would be higher for edibles and possibly topicals than for beverages and concentrates, for which batches are more homogenous.

Issues with consistency of edible products have been a concern where medical and recreational cannabis was available in the United States [37, 65, 66]. The upper limit for edible cannabis in Colorado is 100 mg, which can be divided into 10 portions of 10 mg each [5, 37]. After sampling edible cannabis from three American cities, Vandrey and colleagues (2015) found that most (60%) were over-labeled, meaning that the product had *less* THC than what was labelled, and 23% were under-labeled, indicating that the product had *more* THC than what was labelled [66]. The high degree of variability and under-reporting of THC in the low dose products (up to 70% more than labelled) is particularly concerning given the current 10 mg limit of THC per individual edible product serving in Canada, and could have serious consequences for consumers. This risk has been addressed, as limits on both edible dose (10 mg) and variability (15%) should

mitigate the harm of accidental- or over-consumption. However, for consumers seeking higher doses of THC in edible form, the price of individual products at 10 mg/unit may become cost prohibitive and push consumers to the more potent options available on the illicit market [67].

Dispensing methods and accurate measurements of THC in the new classes of cannabis products is another issue. Electronic cigarettes (for both nicotine and THC) are a growing concern [68, 69, 70] as the presence of substances toxic or harmful when inhaled (e.g., vitamin E acetate) are linked to health issues associated with vaping [71]. Accordingly, a list of prohibited substances for vaping products now exists, which includes vitamin E acetate [72]. Whereas the *Cannabis Act* regulates the use of flavours, additives and carriers, and limits individual doses to 10 mg of THC, it does not regulate the delivery technologies, such as vape pens and their metering mechanisms [1]. Although several patented technologies exist, no peer-reviewed literature is currently available to support the accurate metered dosing of THC using any hand-held vaporization device. Therefore, the consumer can only estimate how much has been consumed (perhaps like an actual cannabis cigarette). At a policy level, this may cause issues with attempting to limit the 10 mg per dose in these electronic devices as few producers claim accurate dosing of their product. However, 10mg dose of vaped THC is a large dose, particularly given the ease of repetitive dosing. Speculatively, a disposable vape pen may contain 0.5 g of a vape solution (concentrate plus carrier) with 700 mg THC/g solution, making the total THC 325 mg/pen. Hypothetically, some users might get 200 “pulls” (inhalation of the product) from a single pen. This results in THC levels around 1.625 mg/pull, which are well below maximum 10 mg/discrete unit [16] and probably safer as a result. Perhaps more of a concern is that, while the concentrate in the cartridge may have passed QC/QA standards, there are no regulations or mandated QA testing for the devices and their components or the vapours that consumers inhale into their lungs.

The devices used to vaporize cannabis may not only vary in their efficiency in decarboxylating THCA and dispensing THC but may also malfunction and result in user harm. Beyond delivering THC, the cannabis accessory cannot enhance the effects of cannabis, cause dependency, and must not be toxic to consumers [18]. The dependability, durability and reactivity of the device components that may malfunction or degrade, for example oxidized metals, overheating coils, or melting plastic [73, 74, 75], are given limited attention. Hence, vaping products should be examined more carefully, especially with the mounting concerns over vaping products [76] and the harm reduction goal of legalization in mind.

Labelling guidelines are also unique for each class of cannabis products [1] with different units of measurement for labelling THC as milligram (mg) per unit, mg per gram (g), or percent THC. This variability could lead to consumer confusion and mismanaged dosing of THC based on the range of labelling types used and lack of consistency across product categories [29]. For example, if a cannabis inflorescence contains 200 mg/g THC (20%), while an edible product contains 10 mg THC per discrete unit, then dosing the appropriate level of THC might be confusing (e.g., would 0.5 g of cannabis inflorescence be equivalent to 10 discrete units?). This is compounded with the fact that edible cannabis has delayed onset of effects and therefore may result in adverse effects if over consumed [38].

*Lab-to-lab variability in QC/QA testing*

Another weakness of the QC/QA standards for cannabis in Canada is the sampling protocols for potency and contaminant testing. While there is an established hemp testing protocol [77] the drug-type cannabis sampling protocols are defined in the standard operating procedures developed by the LP. Although the GPP guidelines mention that repeatedly sampling cannabis in

order to obtain the desired result is not allowed [7], there is a current lack of validated industry-wide sampling protocols for drug-type cannabis which means that some testing facilities may systematically measure higher or lower cannabinoid (or contaminant) levels than others (although see [78]). More inter-lab validation studies are clearly needed (although see [24].

The lack of nationally validated and implemented methods for testing cannabinoids [24]is a major weakness of the current QC/QA standards in Canada and abroad [20]. Inconsistent testing within and across different LPs and testing companies, as well as government laboratories, undermines the purpose and effectiveness of related QC/QA standards and harm reduction measures. If two companies have the same cannabis sample but their testing protocols differ, then the reported levels of cannabinoids may differ [24]. This inconsistency could be mitigated if Health Canada had the capacity to validate and compare existing industry methods but, more realistically, the uptake of emerging international standards for cannabis testing and QC should be encouraged [20, 78]. Such interventions would result in greater testing and reporting consistency across the industry and country. New classes of cannabis products will invariably require unique and validated methods to quantify the levels of cannabinoids and contaminants for each of the products (e.g., different candies, baked goods, and topicals would have different testing methods). To avoid replicating the “do-it-yourself” strategy that resulted in different testing protocols for cannabis inflorescence, the regulations could align more formally with international initiatives developing validated QC/QA standards for the new classes of cannabis [20, 78]. Adding mandatory third-party testing could further reduce variability but at the expense of cost and time for LPs.

The start-up cost to cultivate cannabis is expensive, so additional costs for purchasing equipment, validating methodology, and reviewing regulation and pharmacopeias could become

cost prohibitive. Depending on company size, third-party testing of cannabis can reduce or increase costs. If aligned through certified validated methods and expanded in capacity, third-party testing facilities in Canada could maintain consistent QC/QA for cannabis products nationwide. The use of third-party services is already commonplace within the supplement industry and credited with reducing the presence of contaminants in these products; completely independent third-party facilities, moreover, reduce risk of bias and conflict of interest [79].

**Future Considerations**

Addressing the weaknesses in QC/QA regulations should focus on reducing harm to consumers and protecting public health, which includes limiting costs and prices to attract and retain consumers. This can be accomplished, in part, by developing registered cultivars that reduce intrastrain variability, testing the final product, regulating dispensing products and devices, and consistent product sampling and testing protocols.

***Consumer safety***:

Harm reduction measures implemented through the *Cannabis Act* need to be communicated to the public. To date, most Public Service Announcements (PSAs) about cannabis have focused on the risks and harms of cannabis to children and adolescents, of impaired driving and, more recently, of edibles [34, 80, 81]. Given one of the stated objectives of the *Act* is to enable access to quality-controlled and safe cannabis products for adult users, it would be useful for Health Canada and LPs to educate consumers on why regulated cannabis products are safer than other sources of cannabis. Such messaging could be achieved through PSAs broadcast on conventional and social media. New PSAs could position the QC/QA measures for legally produced cannabis in contrast to illicit cannabis. Using targeted ads might persuade some

individuals to purchase from LPs instead of other avenues. The migration of consumers towards regulated rather than illicit cannabis would not only reduce risks associated with contaminated or unlabelled products but reduce both revenue for, and public funding to combat, the illicit cannabis trade. Legislation is in place to mitigate harms associated with contaminated or misrepresented alcohol products [63], and therefore, similar protocols could be implemented for cannabis products. PSAs such as these should not be taken as an endorsement for cannabis consumption, but as an educational tool that can be used to support informed decision-making (e.g., Ontario Cannabis Store blog [36]). Taken a step further, LPs and cannabis vendors can educate consumers as to why their product is a safer alternative to illicit cannabis.

***Market***:

The two-stage approach to legalizing cannabis, first with inflorescences and oils, followed by an array of products the following year, in theory was a wise decision for policy makers. This strategy allowed a pilot form of testing in terms of how the regulation, sale, and legal framework of cannabis will work in Canada. However, the first rollout of legalization saw an increase in sales of prohibited cannabis products, as their prices undercut the regulated cannabis market [82]. Moreover, the illicit market still accounts for more sales than the legal market [83] and offers lower prices [67]. Although administratively overwhelming, had phase one of legalization permitted the sale of more classes of cannabis products, the expansion of the illicit market may have been slowed. As a result, more consumers may have transitioned to legal products, bringing more revenue to LPs and less to the unregulated market. With calls for the legalization of other recreational and therapeutic-based drugs (e.g., psilocybin), cannabis could provide a litmus test for

how other illegal substances may be regulated [13, 14, 15] and demonstrate key inherent challenges when new legal markets compete with an already entrenched illicit market.

**Conclusion**

This paper provides a descriptive overview of the current QC/QA framework for regulated cannabis in Canada with an analysis of strengths and weaknesses through the lens of harm reduction and consumer safety. With the goal of providing adult Canadians access to a safe and reliable source of cannabis, Canada is the first G7 country to develop a non-medical cannabis market with manufacturing and regulatory systems that operate from seed-to-sale. The QC/QA requirements create the necessary oversight for production, testing, and distribution of cannabis that, in turn, enhance safety and transparency. As the illicit market in Canada continues to operate in parallel, the implementation and utilization of quality controls and quality assurances remain a critical priority. High quality processes, safe and consistent products, and market stakeholder accountability are needed in the new regulated market to retain consumers and create a dependable source of commercial cannabis.

The frameworks, however, are not without potential weaknesses. Consumer safety will need to be prioritized as the market matures, alongside attention dedicated to the balance of governmental control and private business practices. QC/QA frameworks can act as a control mechanism by creating benchmarks and industry standards within which private businesses can operate. Variability remains a current and pressing concern, with high variability in processes (sampling and testing) as well as products (THC and CBD levels), resulting in uncertainty at the consumer-level. While product consistency can be expected to improve over time with the development of stable cultivars (or chemovars); the optimization of operations and processes, and

the validation and implementation of national and/or international QC/QA standards specific to cannabis, can help guide regulators and accelerate progress. Accordingly, Health Canada has engaged with international initiatives attempting to formulate appropriate QC/QA protocols for cannabis testing [20, 78].

With Canada as the only G7 country to legalize cannabis for recreational use on a federal level, the *Cannabis Act* not only serves as an example, but positions Canada as a leader in cannabis quality standards [78, 84]. The Canadian QC/QA requirements, as mandated through legislation, can provide a starting point for broader regulatory frameworks focused on product safety and quality, including those in other countries proposing to legalize and regulate recreational cannabis use. The current QC/QA frameworks also provide a starting point for evidence-based consumer education and knowledgeable purchasing; where increased safety measures can be built upon and tweaked as the markets mature and knowledge of cannabis cultivation and manufacturing proceeds. Maintaining inherent flexibility will be necessary in Canada to reflect new knowledge, current gaps, and the ever-adapting international and domestic landscapes.

Table 1. Type of test, reference document, endpoints, labelling requirement, and product tested for potency, biological and chemical contaminants found in cannabis products. Reference document can be found in Schedule B [10].

| **Type of test** | **Endpoints** | **Labelling requirement?** | **Products tested** |
|---|---|---|---|
| Potency | % by weight: THC<br>CBD | Yes | All |
| Biological contaminants (microbes) | Thresholds (pass/fail) [1]:<br>● Total aerobic microbial count<br>● Total yeast and mold count<br>● Bile-tolerant gram-negative count<br>● Total coliform count<br>Specific microbes[2]:<br>● *Escherichia coli,*<br>● *Salmonella spp.*<br>● *Staphylococcus aureus* | No | All |
| Chemical Contaminants | | | |
| ● Mycotoxins | Aflatoxins | No | All |
| ● Pesticides | Absence of 96 banned pesticides | No | All |
| ● Metalloids & metals | Thresholds (pass/fail):<br>● Arsenic, cadmium, lead, mercury | No | All |
| ● Solvents | | No | Oils |
| ● Encapsulation degradants | | No | Capsules |

[1] Defined as Colony forming units (CFU) per gram or mL of material

[2] Different guidance documents may specify different microbes for testing, depending on expected route of administration

Table 2. Variability limits associated with the new classes of cannabis (edibles, extracts, and topicals)[1]. [16]]

| Dose of THC[2] or CBD[3] (mg) | Percent permitted variability (%) | Example of range in Percent (%) | Example of Range (mg) |
|---|---|---|---|
| <2 | 25 | 75-125 | 1mg: 0.75 - 1.25 mg |
| 2-5 | 20 | 80-120 | 4mg: 3.20 - 4.80 mg |
| >5 | 15 | 85-115 | 10mg[1]: 8.5- 11.5 mg |

[1] Limit per package for extracts and topicals is 1000mg THC, while edibles have a 10mg limit of THC per discreet unit

[2] Upper limits established at 10mg of THC per discreet unit

[3] No upper limits have been established, though variability limits still apply

## Abbreviations

| Abbreviation | Explanation |
|---|---|
| µg | Microgram |
| ABV | Alcohol-by-Volume |
| ASTM | American Society for testing and Materials |
| CBD | Cannabidiol |
| CBDA | Cannabidiolic Acid |
| CCSA | Canadian Centre on Substance Abuse and Addiction |
| DIN | Drug Identification Number |
| FDR | Food and Drugs Regulation |
| g | Gram |
| G7 | Group of 7 countries |
| GPP | Good Production Practices |
| LCBO | Liquor Control Board of Ontario |
| LP | Licensed Producers |
| mg | Milligram |
| NIDA | National Institute on Drug Abuse |
| PSA | Public Service Announcements |
| QAP | Quality Assurance Person |
| QC/QA | Quality Control Quality Assurance |
| THC | Tetrahydrocannabinol |
| THCA | Tetrahydrocannabinolic acid |
| TVPA | Tobacco and Vaping Products Act |

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[77] Health Canada, "Industrial hemp technical manual - standard operating procedures for sampling, testing, and processing methodology," Ottawa, 2004.

[78] ASTM, "D37 Committee," 2020. [Online]. Available: https://www.astm.org/COMMITTEE/D37.htm.

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[81] Drug Free Kids Canada, "Archives," [Online]. Available: https://www.drugfreekidscanada.org/archives/.

[82] The Daily, "StatsCannabis data availability: crowdsourced cannabis prices, fourth quarter 2019," Ottawa, 2019.

[83] Statistics Canada, "Number and percentage of current consumers who access cannabis from each source (exclusively or not), by gender, Canada, first half of 2019," Ottawa, 2019.

[84] S. Ash, "Setting quality assurance standards in the medical cannabis industry: QA workers and growers need to be part of the conversation," 11 April 2019. [Online]. Available: https://www.thegrowthop.com/cannabis-business/setting-quality-assurance-standards-in-the-cannabis-industry-qa-workers-and-growers-need-to-be-part-of-the-conversation.

# APPENDIX B: This is your country on cannabis: A review of legislation, testing, and pharmacology of legal cannabis in Canada

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Canada legalized the recreational sale of cannabis for adults on October 17th, 2018, in response to consumer demands for its legalization. This has not come without some issues along the cannabis supply chain from governmental policy, licensed production, and consumer confusion. This paper will provide an overview of the biology of cannabis, legalization, dosing measures, and variability of cannabis products.

**Cannabis:**

The following section will be a general overview of the cannabis plant and its interaction with humans. *Cannabis sativa* is one of the most notorious plants used for millennia by humans for its medicinal, recreational, spiritual, industrial, and nutritional properties[1] [2]. Cannabis shares some of the same terpenes (scent molecules) found in other plants such as, limonene dominant in lemons and linalool dominant in lavender[3]. The cannabinoids, however, are a unique class of

[1] Warf, Barney. "High points: an historical geography of cannabis." *Geographical Review* 104.4 (2014): 414-438.

[2] Small, Ernest. "Evolution and classification of Cannabis sativa (marijuana, hemp) in relation to human utilization." *The botanical review* 81.3 (2015): 189-294.

[3] Russo, Ethan B. "Taming THC: potential cannabis synergy and phytocannabinoid-terpenoid entourage effects." *British journal of pharmacology* 163.7 (2011): 1344-1364.

phytochemicals found almost exclusively in the species *Cannabis sativa* (though see [4]). The cannabinoids are responsible for the psychoactive and bioactive effects of cannabis. The highest density of cannabinoids are found within the trichomes on the female flowers (inflorescence) of the cannabis plant.

The most dominant cannabinoids found in the cannabis plant are tetrahydrocannabinol (THC) and cannabidiol (CBD) – along with over 60 minor cannabinoids[4] that may have therapeutic potential. THC is primarily responsible for the psychoeuphoric effects (i.e., feeling high), and adverse effects such as paranoia and delusions in susceptible individuals[5]. Meanwhile, CBD is known for its medicinal properties such as an anti-psychotic[6], anti-anxiety [7], anti-epileptic[8], and anti-inflammatory agent [3]. However, THC is also therapeutic by improving appetite for those undergoing chemotherapy or patients suffering from AIDS[9].

Only trace amounts of THC and CBD occur within the cannabis plant, instead they naturally occur in their acidic forms, THCA and CBDA. This acidic side-chain reduces the bioavailability

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[4] Degenhardt, F., F. Stehle, and O. Kayser. "The biosynthesis of cannabinoids." *Handbook of Cannabis and related pathologies*. Academic Press, 2017. 13-23.

[5] Freeman, Daniel, *et al.* "How cannabis causes paranoia: using the intravenous administration ofΔ 9-tetrahydrocannabinol (THC) to identify key cognitive mechanisms leading to paranoia." *Schizophrenia bulletin* 41.2 (2015): 391-399.

[6] Leweke, F. M., *et al.* "Cannabidiol enhances anandamide signaling and alleviates psychotic symptoms of schizophrenia." *Translational psychiatry* 2.3 (2012): e94-e94.

[7] Zuardi, Antonio W., *et al.* "Inverted U-shaped dose-response curve of the anxiolytic effect of cannabidiol during public speaking in real life." *Frontiers in pharmacology* 8 (2017): 259.

[8] Hanuš, Lumír Ondřej, *et al.* "Phytocannabinoids: a unified critical inventory." *Natural product reports* 33.12 (2016): 1357-1392.

[9] MacCallum, Caroline A., and Ethan B. Russo. "Practical considerations in medical cannabis administration and dosing." *European journal of internal medicine* 49 (2018): 12-19.

(e.g., psychoactive properties) of these cannabinoids relative to their neutral forms, THC and CBD[10]. Once heated, however, the acidic group detaches (i.e., decarboxylates) making the cannabinoids bioactive. This decarboxylation step is the reason why cannabis is typically smoked, vaporized, or cooked before being consumed. The effects of cannabis vary based on the concentration (e.g., mg/g) of THC and CBD along with the ratio of cannabinoids (i.e., THC: CBD). THC is responsible for the intoxicating effects of cannabis[11,12], whereas products dominant in CBD generally act as an anxiolytic[7] (although the full suite of psychoactive effects associated with both THC and CBD have yet to be established). A simple way to remember the effects of cannabinoids is through the mnemonic THC as The High Cannabinoid and CBD as Calm Back Down.

The endocannabinoid system could explain some of the benefits from cannabis as the cannabinoid receptors are the most abundant G-coupled protein receptor in the central nervous

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[10] Citti, Cinzia, *et al.* "Pharmaceutical and biomedical analysis of cannabinoids: A critical review." *Journal of pharmaceutical and biomedical analysis* 147 (2018): 565-579.

[11] Childs, Emma, Joseph A. Lutz, and Harriet de Wit. "Dose-related effects of delta-9-THC on emotional responses to acute psychosocial stress." *Drug and alcohol dependence* 177 (2017): 136-144.

[12] Green, B. O. B., David Kavanagh, and Ross Young. "Being stoned: a review of self-reported cannabis effects." *Drug and alcohol review* 22.4 (2003): 453-460.

system [13] and are ubiquitous throughout the human body[14, 15]. The endocannabinoid system works by producing endocannabinoids that bind to one of two cannabinoid receptors and then is later degraded. Exogenous cannabinoids (i.e., from cannabis) interact with the cannabinoid receptors within the body producing effects stronger than the endocannabinoids alone. Furthermore, unlike alcohol or opioids, a person cannot die from overconsuming cannabis [16] – instead, they may feel like they are dying. This is because the cannabinoids do not shutdown the part of the brain responsible for breathing[17].

The selection for different traits in cannabis resulted in two phenotypically distinct groups: high- and low-THC cannabis. High-THC cannabis (i.e., drug-type cannabis) was selected for high levels of THC and is traditionally used for its medicinal, recreational, and spiritual practices [18]. Meanwhile, low-THC cannabis (i.e., hemp), was selected by humans for fibre and food production and was quite a popular addition to bird feed in the early 20th century[19]. Hemp was

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[13] Russo, Ethan B. "Beyond cannabis: Plants and the endocannabinoid system." *Trends in Pharmacological Sciences* 37.7 (2016): 594-605.

[14] Pertwee, Roger G. "Cannabinoid pharmacology: the first 66 years." *British journal of pharmacology* 147.S1 (2006): S163-S171.

[15] Di Marzo, Vincenzo. "Targeting the endocannabinoid system: to enhance or reduce?." *Nature reviews Drug discovery* 7.5 (2008): 438-455.

[16] Small, Ernest. *Cannabis: a complete guide*. CRC Press, 2016

[17] Small, Ernest. *Cannabis: a complete guide*. CRC Press, 2016, P.314

[18] See Small (2015)

not selected for THC content (most cultivars <0.3% THC dry weight) and proportionally higher in CBD. A third lesser-known group of cannabis, ruderalis, is typically found along roadside ditches with low levels of either cannabinoids. Cannabis could be classified into sativa and indica. Sativa strains are said to grow tall and lanky with an uplifting high, while indica strains have a short stature with sedative effects[20]. Cannabis users report having different effects between indicas and sativas, however, there is little chemical difference between these two groups of cannabis[20].

A more precise way to categorize cannabis could be based on its THC-to-CBD ratio where: type I is THC dominant, type II is balanced between THC and CBD, and type III is CBD-dominant [21, 22]. While THC and CBD each have their own unique medicinal properties, the illicit market has seen a demand for greater amounts of THC in their product. For example, the average THC in the mid-1990s had approximately 40mg/g (i.e., 4%) THC, while the average THC in the mid-2010s had 120mg/g (i.e., 12%) THC [23].

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[19] Small, Ernest, H. D. Beckstead, and Allan Chan. "The evolution of cannabinoid phenotypes in Cannabis." *Economic Botany* (1975): 219-232.

[20] Elzinga, Sytze, *et al.* "Cannabinoids and terpenes as chemotaxonomic markers in cannabis." *Nat. Prod. Chem. Res* 3.81 (2015): 10-4172.

[21] See Small *et al.* (1975)

[22] Hillig, Karl W., and Paul G. Mahlberg. "A chemotaxonomic analysis of cannabinoid variation in Cannabis (Cannabaceae)." *American journal of botany* 91.6 (2004): 966-975.

> Note: Hillig uses the term chemotype (chemical type), while Small (1975) used the term phenotype. To avoid confusion between terms I will use the word *type* to describe these three categories

[23] ElSohly, Mahmoud A., *et al.* "Changes in cannabis potency over the last 2 decades (1995–2014): analysis of current data in the United States." *Biological psychiatry* 79.7 (2016): 613-619.

The cannabis from the 1970s was even less potent than the 1990s ranging from 0-3% THC[24]. In contrast, cannabis sampled in 2015, from licensed testing facilities report THC values over 25% in some cases[25]. Perhaps the shift from outdoor to indoor covert grow-operations and selective breeding regimen might possibly explain this dramatic increase in THC[26]. However, demand for higher THC content comes at a cost to greater THC variability within a single harvest of illicit cannabis[27].

Due to the lack of Quality Control Quality Assurance (QCQA) oversight, selection for more potent cannabis, and greater variability of THC between harvests could be most harmful to the consumer (especially medical cannabis users). When cannabis was prohibited, consumers were generally unaware of the amount of THC and the THC:CBD ratio[28], along with the potential contaminants on or within their cannabis products. Legalization and regulation of cannabis is one solution to provide a consistent cannabis product to consumers.

**QCQA and accessibility of cannabis: from legislation to the consumer**

Canada is the second country to legalize cannabis for recreational use. The Canadian government's three main goals for legalizing cannabis were to: (1) ensure safe-products for

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[24] Small *et al.* (1975)

[25] Jikomes, Nick, and Michael Zoorob. "The cannabinoid content of legal cannabis in Washington State varies systematically across testing facilities and popular consumer products." *Scientific reports* 8.1 (2018): 1-15.

[26] Knight, Glenys, *et al.* "The results of an experimental indoor hydroponic Cannabis growing study, using the 'Screen of Green'(ScrOG) method—Yield, tetrahydrocannabinol (THC) and DNA analysis." *Forensic Science International* 202.1-3 (2010): 36-44.

[27] See Knight *et al.* (2010)

[28] Hammond, David, and Samantha Goodman. "Knowledge of Tetrahydrocannabinol and Cannabidiol Levels Among Cannabis Consumers in the United States and Canada." *Cannabis and Cannabinoid Research* (2020).

Canadians, (2) reduce use among minors, and (3) reduce the proceeds to the illicit market[29]. In theory, the government oversight and regulation of legal cannabis provides rigorous control and product testing – something that was not apparent in the illicit market. However, the legal market is not without issue as recalls have been issued for improperly labeled cannabis products[30] and security breaches with the online cannabis transactions[31]. These scenarios could reduce consumer trust and strengthen the illicit cannabis market[32]. Legal cannabis can work if there is a cohesive partnership among government agencies, licensed producers (LP), and consumers as they each are integral to the cannabis supply chain.

*Legislation*

The Canadian Government opted for a two-phase approach to legalizing recreational use of cannabis products via the *Cannabis Act* [33] and *Cannabis Regulations*[34]. In October 2018, flower, oil, and tincture cannabis were permitted to be sold. There was no limit on the THC potency of flower cannabis, although it is generally less than 30% THC dry weight[35]. In some provinces, cannabis sales were limited to online transactions until physical stores (i.e., brick-and-mortar

[29] Cannabis Act, 2018 (Canada), S.c. 2018. c. 16

[30] Healthy Canadians Recall, search: Cannabis. https://healthycanadians.gc.ca/recall-alert-rappel-avis/search-recherche/result-resultat/en?search_text_1=cannabis

[31] Vuong, 2018. Canada Post data breach affected 4,500 customers, OCS says https://globalnews.ca/news/4639742/ocs-canada-post-hacked/

[32] Cox, Chelsea. "The Canadian Cannabis Act legalizes and regulates recreational cannabis use in 2018." *Health Policy* 122.3 (2018): 205-209.

[33] See Cannabis Act (2018)

[34] Cannabis Regulation, 2020 (Canada). SOR/2018-144

[35] See Jikomes and Zoorob (2018)

stores) were opened. In October 2019, the sale of concentrate, topicals, and edible products were available. The limit on edible products was set at 10mg THC per package[36], while there is no upper limit of CBD per package.

The slow release of approved products allows for adjustments in legislation to take place once flower cannabis was legal. This helped to tease apart the effects of having some or all products available[37]. The top-down approach permitted greater government control and QCQA oversight, and provide a safer supply to consumers compared with a prohibition or decriminalized approach to selling cannabis.

Having less government oversight might result in a higher risk for contamination, lot-to-lot inconsistency, and improperly labelled edible products [38,39]. Legal cannabis, however, is tested for the potency and contaminants[40, 41]. The legal limits on the potency of edible cannabis mitigates the harms of accidental or overconsumption, which might occur in jurisdictions with greater potency limits on edibles. The 10mg THC per package limit along with public education[42] about the effects of these products should reduce healthcare costs associated with

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[36] See Cannabis Regulation (2020)

[37] Pusiak, Ryan JP, Chelsea Cox, and Cory S. Harris. "Growing pains: An overview of cannabis quality control and quality assurance in Canada." *International Journal of Drug Policy* (2021): 103111.

[38] See Pusiak *et al.* (2021)

[39] Vandrey, Ryan, *et al.* "Cannabinoid dose and label accuracy in edible medical cannabis products." *Jama* 313.24 (2015): 2491-2493.

[40] See Cannabis Regulation

[41] See Pusiak *et al.* (2021)

[42] Hammond, David. "Communicating THC levels and 'dose' to consumers: implications for product labelling and packaging of cannabis products in regulated markets." *International Journal of Drug Policy* (2021): 102509.

accidental consumption by minors and reduce the risk of drug impaired driving[43, 44]. The *Cannabis Act* prohibits the sale containing a mixture of cannabis and alcohol (ethanol concentration <0.5w/w or limit of 7.5g ethanol tincture), tobacco, and or caffeine (maximum of 30mg natural caffeine permitted)[45]. Routes of consumption must not harm the user (e.g., abrasion to the skin, taken through the eye, or via injection), although smoking is arguably harmful. The goal of these regulations is to focus on consumer safety.

The roll out of legalizing cannabis had some issues. Provinces had their own regulations in place to how cannabis was sold. Some provinces such as Newfoundland and Labrador had physical stores, while other provinces such as Ontario began legal sales online. For online sales, customers had to input their credentials (e.g., name, address) and pay electronically. There have been rumours suggesting that the sales of legal cannabis are tracked and could result in issues when crossing international borders, however, there is insufficient evidence to suggest that this information is easily accessible to border security[46]. Furthermore, within weeks of legalization there was a security breach of 4500 customers from the Ontario Cannabis Store [47]. Another unforeseen issue with the two-stage framework was the consumer demand for the array of banned cannabis products that were not available until late 2019. This *hypothetically* provided an avenue for the illicit online sales of concentrates, topicals, and edibles.

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[43] See Cannabis Regulation

[44] See Pusiak *et al.* (2021)

[45] See Cannabis Act

[46] Savoie, Alexandra. "Cannabis purchases, privacy and the Canada – U.S. border background paper", Library of parliament (2019). https://lop.parl.ca/sites/PublicWebsite/default/en_CA/ResearchPublications/201913E

[47] See Vuong (2018)

Issues associated with illicit cannabis sale usually revolve around the QCQA of illicit vape cartridges [48] and concentrates [49, 50]. Legal vaping products must undergo rigorous product testing before being sold to consumers, whereas illicit products do not. Furthermore, the term vaping is used as a catchall statement for: (1) an e-cigarette containing nicotine, known as Electronic Nicotine Delivery System - ENDS[51]; (2) vape pens and cartridges with THC; and (3) vaping using a vaporizer (i.e., a device that decarboxylates cannabis using heat, thus making it bioavailable without combustion[52]), each of which could lead to consumer confusion and catchy media headlines. For example, researchers tested the effects of smoked vs. herbal vaporized cannabis flower[53]. The article was later cited by another researcher reporting on a young male who entered a psychosis after inhaling an illicit THC vape pen (i.e., cannabis concentrate)[54]. The former experimenter used herbal cannabis from the National Institute on Drug Abuse (NIDA) at known concentrations, while the latter researcher reported on illicit concentrate use. Having a clear definition of vaping, such as differentiating legal vs. illicit, nicotine vs. THC, and

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[48] Alchin, David R. "'Vaping psychosis': Ultra-acute onset of severe, persisting and unusual psychotic symptoms following first-time cannabis vaping in a previously well individual." *Australian & New Zealand Journal of Psychiatry* 54.8 (2020): 848-848.

[49] Bell, Cameron, *et al.* "Butane hash oil burns associated with marijuana liberalization in Colorado." *Journal of medical toxicology* 11.4 (2015): 422-425.

[50] Ottawa Police "Ongoing police and fire operation on Renaud Road", March 25th, 2021. https://www.ottawapolice.ca/Modules/News/index.aspx?page=2&newsId=7d00f83d-982b-4ff2-87ab-c3977be6a53e

[51] Glasser, Allison M., *et al.* "Overview of electronic nicotine delivery systems: a systematic review." *American journal of preventive medicine* 52.2 (2017): e33-e66.

[52] Gieringer, Dale, Joseph St. Laurent, and Scott Goodrich. "Cannabis vaporizer combines efficient delivery of THC with effective suppression of pyrolytic compounds." *Journal of Cannabis Therapeutics* 4.1 (2004): 7-27.

[53] Spindle, Tory R., *et al.* "Acute effects of smoked and vaporized cannabis in healthy adults who infrequently use cannabis: a crossover trial." *JAMA network open* 1.7 (2018): e184841-e184841.

[54] See Alchin (2020)

concentrate vs. flower could mitigate misinterpretations of data. However, please note that some herbal vaporizers are equipped to handle vape cartridges[55] – adding to the confusion. Finally, the difference in QCQA between legally regulated and illicit vape products should be clearly defined in any regulation pertaining to the harms of vaping.

*Product testing and creation*

Under the current cannabis regulations, individuals can grow up to four plants per adult for their own personal use. However, only licensed producers of cannabis are permitted to sell to licensed retailers. The initial start-up cost and approval process for the licensing, production, and product testing of cannabis is expensive. The LP must adhere to the government oversight for Good Production Practices, product testing, and hire a quality assurance person that has experience in cannabis product testing[56].

Product testing is one way to ensure a safe product is being sold to consumers. Each batch of product must be tested for fertilizer and pesticide residue, the presence of heavy metals (and metalloids), microbes, and the amount of THC and CBD. Additional product testing could include terpenes (i.e., odour profile) that would provide customers with more product information. In contrast, the illicit market has limited product testing (i.e., cannabinoid and contaminant testing) and claim to have high levels of THC in their products [57, 58]. This could

[55] PAX Canada. Dual use vaporizer. Accessed March 2022 https://ca.pax.com/products/pax-3?variant=32024009408625

[56] See Cannabis Regulation (2020)

[57] Mahamad, Syed, *et al.* "Availability, retail price and potency of legal and illegal cannabis in Canada after recreational cannabis legalisation." *Drug and alcohol review* 39.4 (2020): 337-346.

[58] See Vandrey *et al.* (2015)

result in improper dosing and adverse effects of cannabis. Legal packages must include proper documentation for cultivation and packaged on dates[59]. Moreover, LPs must practice recall scenarios and retain the records of recalls for 25 years[60]. There are also strict regulations pertaining to the production and sales of cannabis products along with proper disposal procedures[61].

During the first three years of legalization, however, it appeared that most cannabis consumed in Canada was not coming from the LPs but from alternative unregulated sources[62]. Prior to the second-wave of legalization in October 2019, the thriving illicit cannabis market was selling cannabis products (e.g., edibles and vape cartridges) that were not yet available from regulated sources and well above the proposed legal limits[63]. Furthermore, it is still unknown if these illicit products actually contained the level of THC mentioned on the label[64], however, a report from medical dispensaries in the United States demonstrated that the labelled amount of THC does not necessarily match its contents[65]. In Canada, there are also strict requirements for childproof and plain packaging making it unattractive to minors. Meanwhile, the illicit cannabis companies have attractive (usually non-childproof) packaging with various banned flavours or

[59] See Cannabis Regulation (2020)

[60] See Cannabis Regulation (2020)

[61] See Cannabis Regulation (2020)

[62] Rotermann, Michelle. "What has changed since cannabis was legalized?" Health Reports Statistics Canada. February 19, 2020. https://www150.statcan.gc.ca/n1/en/pub/82-003-x/2020002/article/00002-eng.pdf?st=2WULQWne

[63] See Mahamad *et al.* (2020)

[64] See Mahamad *et al.* (2020)

[65] See Vandrey *et al.* (2015)

forms of product[66]. The LPs were also not permitted to advertise their products. However, illicit cannabis ads were commonplace on various social media sites[67]. Not only did the illicit market have a wide-range of products but they are also undercutting the price of cannabis. While legal cannabis sold for $10.30/g in the fourth quarter 2019, illicit cannabis sold for on average $5.73/g[68]. This could nudge people to continue to purchase illicit cannabis based on the price alone. To adjust for this, the price for legal cannabis has since dropped, which may explain why half of the cannabis purchased in 2020 was from a legal source[69].

*Consumer protection: safe supply*

The legalization of cannabis came from consumer demand for a safer alternative to illicit cannabis, followed by a greater acceptance of its use[70]. The multitude of products available (e.g., flower, topical, edible, concentrates, etc.) allow customers to purchase a cannabis product that suits their needs. In addition, customers may through a legal online retailer (where applicable),

[66] Marchitelli, Rosa. "Copycat pot edibles that look like candy are poisoning kids, doctors say". CBC News British Columbia. January 25th, 2021. https://www.cbc.ca/news/canada/british-columbia/cannabis-gummies-poisonings-kids-illegal-sites-1.5879232

[67] Correa, Ginni. "Advertising illicit marijuana on social media" Addiction Centre. February 4th 2020. https://www.addictioncenter.com/community/advertising-marijuana-social-media/

[68] The Daily, "StatsCannabis data availability: Crowdsourced cannabis prices, fourth quarter 2019". https://www150.statcan.gc.ca/n1/en/daily-quotidien/200123/dq200123c-eng.pdf?st=i4Tz0SgZ

[69] See Rotermann (2020)

[70] Duff, Cameron, *et al.* "A Canadian perspective on cannabis normalization among adults." *Addiction Research & Theory* 20.4 (2012): 271-283.

offering anonymity from members of the community[71]. Alternatively, customers could go to a physical store where they can pay cash (less traceable) and can ask questions to the employees[72].

Although there are strengths associated with legal cannabis (both as a product and policy), there are still some weaknesses that should be addressed. Consumer trust could be one example, where cannabis consumers may initially trust their original, quasi-legal or illicit, source over novel legally regulated cannabis. Likewise, legal cannabis might not be available in the dosage they are looking for, believe that their current source is superior to legal sources, and or have not had problems with their original source. Furthermore, the customer might be cautious of legal cannabis as there has been growing list of recalls for cannabis products ranging from inaccurate measurements of THC to the presence of mold on edible products[73]. In contrast, there are no recalls in the unregulated cannabis market – where products could have more contaminants and inaccurate (if any) labels [74, 75]. There is also a legal purchasing limit of 30g of cannabis flower from licensed stores, which might not be enough for people who have difficulty accessing the cannabis store. This includes medical patients who may require more of a particular product and or those in communities with no nearby cannabis stores. However, note that in 2019, 45% of Canadians live within 10km of a legal cannabis store[76]. In contrast to the purchasing limits of legal cannabis, there is no advertised purchasing limit for at-home use of alcohol and tobacco

[71] No Author. "Online Cannabis Retailers in Canada" https://gotleaf.ca/online-dispensaries/

[72] See Pusiak *et al.* 2021

[73] See Healthy Canadians Recall

[74] See Vandrey *et al.* 2015

[75] See Mahamad *et al.* 2020

[76] Statistics Canada "The retail cannabis market in Canada: A portrait of the first year" December 11, 2019. https://www150.statcan.gc.ca/n1/pub/11-621-m/11-621-m2019005-eng.htm

products in Canada. Perhaps limiting the amount of alcohol and tobacco that could be purchased at a given time could be adopted as a public health measure, or to simply abolish the purchasing limit for cannabis.

Upon legalization in 2018, illicit sales were still on the rise because these illicit websites offered a wide-array of products at lower prices and with no purchasing limit. These illicit cannabis products had varying levels of QCQA (e.g., contaminant and potency testing) and no government approval or oversight[77]. The edible products were also well above the maximum 10mg/dose. Moreover, there were wholesale prices exceeding the 30g limit set by the government and the websites typically have loyalty reward programs. Even when the new classes of cannabis were legally available, the economic (price and dose) incentive to purchase from an illicit seller is alluring.

**Dosing and bio-equivalency**

With the array of cannabis products available, it would be useful to be able to compare the psychoactive effects among the various products available to customers. As of this writing, there is no defined dose of cannabis and no equivalency for what a dose means among products[78]. However, NIDA has set 5mg as a standard dose of cannabis for research[79]. One approach is to create a bio-equivalency table among products, first would be to describe the onset times and second, an equivalent dose (at a set concentration) of flower cannabis. Bio-equivalency is

[77] See Marchitelli (2021)

[78] See Hammond (2021)

[79] Freeman, Tom P., and Valentina Lorenzetti. "'Standard THC units': a proposal to standardize dose across all cannabis products and methods of administration." *Addiction* 115.7 (2020): 1207-1216.

defined as two or more substances having the same concentration in the body over time[80]. Any deviation between these products is a change in bio-equivalency. For example, the concentration of THC in the body is different whether the cannabis is an edible product or was inhaled[81, 82].

Cannabis is typically inhaled, reducing the onset time of cannabis' psychoactivity and providing users an opportunity to self-titrate an appropriate dose. Although flower is the most popular type of cannabis purchased[83], there are still harmful compounds present in cannabis smoke (e.g., pyrolytic (carcinogenic) compounds) that are absent in vaporized cannabis [84]. In contrast, other researchers demonstrate that there is no temporal link between cannabis smoke and cancer[85]. Similar to tobacco, the temporal link between smoking and lung cancer might take a few decades to hash out. Moreover, there have been anti-smoking campaigns aimed at reducing tobacco cigarette use and motions for a smoke-free Canada [86] – and it would likely transfer to smoked cannabis. Although, edible cannabis is not without consequence. The psychoactive effects after consuming edible cannabis (via the digestive tract) are delayed and may result in

[80] CADTH, "What are bioavailability and bioequivalence?" 2012. https://www.cadth.ca/sites/default/files/pdf/What_Are_Bioavailability_and_Bioequivalence_e.pdf

[81] See Small 2015

[82] Health Canada "Information for health care professionals: cannabis (marihuana, marijuana) and the cannabinoids. October 2018. https://www.canada.ca/content/dam/hc-sc/documents/services/drugs-medication/cannabis/information-medical-practitioners/information-health-care-professionals-cannabis-cannabinoids-eng.pdf

[83] Health Canada "Canadian cannabis survey 2020: Summary" December 21st, 2020 https://www.canada.ca/en/health-canada/services/drugs-medication/cannabis/research-data/canadian-cannabis-survey-2020-summary.html

[84] See Gieringer *et al.* 2004

[85] Melamede, Robert. "Cannabis and tobacco smoke are not equally carcinogenic." *Harm Reduction Journal* 2.1 (2005): 1-4.

[86] Physicians for a smoke-free Canada. 2021. https://smoke-free.ca/

adverse effects from overconsumption [87]. For example, the onset of inhaled cannabis is 5 minutes, peaking at 15-20 mins, and lasting 2-3hours, while the onset of edible cannabis is 30-90min, peaking at 2-3 hours, and lasting 4-12 hours[88] . Therefore, it is more challenging to dose edible cannabis due to its delayed effects, whereas, inhaling cannabis is easier to dose but comes at a potential risk of lung injury.

In order to reduce harm, setting a dose for cannabis could help consumers expect similar effects across products. For example, if someone occasionally smokes flower cannabis containing 200mg/g THC, then what would be an equivalent dose of edibles to achieve the same level of intoxication without adverse effects? The maximum level of THC in an edible is 10mg THC per package, so would the consumer aim for 7.5mg THC? How does flower compare with vape cartridges or other forms of concentrates? If a company claims their vape pen administers 2.5mg from the vape cartridge on every inhalation[89], then how does this compare to a 0.5g joint with 100mg THC? It is not surprising to know that there is very little published research on comparing the pharmacology of various cannabis products to one another (vaporized concentrates, smoked flower/concentrates, edibles, oils, topicals, toothpaste, suppositories, etc.). Yet, these products remain available to both recreational and medical consumers alike, with varying levels of guidance.

In contrast to cannabis, alcohol has a defined dose (in Canada) set at 17.05mL of pure alcohol (i.e., ethanol), the equivalent found in 341mL of beer at 5%, 142mL of wine at 12%, and

[87] See Health Canada 2018

[88] See Health Canada 2018 from Table 4: Comparison between cannabis and prescription cannabinoid medications

[89] Dosist 2021 https://dosist.com/dose-pens

43mL of spirits at 40%. This way consumers can assess an equivalent dose[90] and anticipate a similar effect among drinks. The standard drink also help set the social reference pricing for alcohol, where higher potency alcohol beverages are taxed at a higher rate[91]. Taxing higher potency cannabis flower and concentrates could reduce risk of adverse effects, although should be monitored to ensure consumers are not transitioning back to unregulated markets[92].

Defining a standard dose of cannabis could be useful as a public health approach and mitigate adverse reactions[93]. Yet, the main advice given by legal cannabis retailers along with some scholars and medical professionals is to "start low and go slow" [94]– without any defined standard dose. Do you consume 1/10 of a 10mg edible or one puff (2.5mg) from a vape pen at 85% THC, over 1 vs. 4 vs. 24 hours? What about oils, sprays, or topicals? In theory, there should have been evidence-based guidelines for the appropriate dosing for medical patients since its approval in 2001. Thus far, the general advice from Health Canada's guide for medical cannabis use for medical practitioners is to advise patients to use the lowest possible dose to achieve a therapeutic effect [95]. The report from Health Canada comes with a disclaimer indicating that there is no established standard dose for medical cannabis because every individual reacts differently to cannabis (although see Freeman & Lorenzetti, 2020). The complexity of cannabis (e.g., %THC/CBD, THC: CBD, batch variability), user experience with cannabis, and individual

[90] University of Victoria: Canadian institute for substance use research "Convert your drinks into standard drink sizes" http://aodtool.cfar.uvic.ca/index-stddt.html

[91] Canadian Centre on substance Use and Addiction "Social reference pricing for alcohol FAQs" 2017. https://www.ccsa.ca/social-reference-pricing-alcohol-faqs

[92] See Cox 2018

[93] See Freeman & Lorenzetti (2020)

[94] See MacCallum & Russo (2018)

[95] Health Canada (2018)

differences in their endocannabinoid system, makes it exceedingly difficult to provide an accurate population-wide dose of cannabis[96]. There have been attempts to accurately dose cannabis products in the pharmaceutical industry through products such as Sativex [97] and Epidiolex[98]. Setting a dose of cannabis across products is a critical knowledge gap that should be addressed in partnership from the government, industry, and consumers.

The labels of flower cannabis originally contained only the percent dry weight THC and CBD on the label[99]. Moreover, it must also contain the total amount of THC and CBD, that is, THCA+THC=total THC, CBDA+CBD=total CBD. Once the new products (e.g., edibles, topicals) were regulated in 2019, flower cannabis must include the milligrams per gram THC and CBD and could also contain the percent THC and CBD. At first glance this might be confusing to customers as a strain with 20% THC might be sold as 200mg/g [100]. Moreover, this has apparently led to the recall of various products [101] as the percent THC is incorrectly labeled as mg/g – thus underestimating the potency by a factor of 10 (e.g., labeled as 20mg/g but it is actually 200mg/g). When changes like this happen, it is important to inform consumers and explain the rationale for changing the labels. The labeling change for flower cannabis to mg/g

[96] Health Canada, (2018)

[97] Karschner, E. L., *et al.* "Subjective and physiological effects after controlled Sativex and oral THC administration." *Clinical Pharmacology & Therapeutics* 89.3 (2011): 400-407.

[98] Sekar, Krithiga, and Alison Pack. "Epidiolex as adjunct therapy for treatment of refractory epilepsy: a comprehensive review with a focus on adverse effects." *F1000Research* 8 (2019).

[99] See Cannabis Regulation

[100] See Hammond (2021)

[101] See Healthy Canadians Recall (2021)

may actually act as the first step towards bio-equivalency among products, although the pharmacology among products is still unknown.

Bio-equivalency starts in the product. Putting together a simple table comparing the average amount of THC per class of product is a good first step, along with the estimated onset time and duration of psychoactive effects. Researchers could then give a survey to customers when they purchase cannabis. With these data, researchers could formulate what experiments should be conducted to accurately assess the bio-equivalency of cannabis products. Next, an infographic can be created comparing the array of cannabis products to help better inform customers on the duration and intensity of a given cannabis product. Taken a step further, combining product information with user preferences [102] could enhance the experience to the cannabis consumer.

**Variability of THC in Cannabis Products**

Plants can greatly vary in the quantity of phytochemicals produced due to environmental, genetics, or epigenetic conditions[103]. Cannabis is no exception to this rule as cannabinoid levels can be altered at various levels of cultivation and processing[104].

*Strain credibility.*

[102] Jikomes, Nickolas, *et al.* "System for selection of regulated products." U.S. Patent Application No. 16/228,197.

[103] Boira, Herminio, and Antonio Blanquer. "Environmental factors affecting chemical variability of essential oils in Thymus piperella L." *Biochemical systematics and Ecology* 26.8 (1998): 811-822.

[104] See Knight *et al.* (2010)

The term strain is typically used to describe microorganisms, not plants[105]. Regardless, strains are the standard protocol when categorizing cannabis – without any consensus among growers on what defines a strain. Perhaps if there was a lineage for a particular strain, then there would be some credibility in the pedigree of such strains. However, written accounts of breeding lineages are exceedingly rare due to the risks of having these records confiscated by law enforcement or competing growers[106].

Traditionally, cannabis has been classified into sativa and indica types, though recent research suggests that there is no statistical difference in cannabinoid content between these categories of cannabis [107, 108]. Terpenes are chemicals responsible for the odour of cannabis and might be a better predictor on how to categorize cannabis. Elzinga and colleagues (2015) analyzed terpene profiles of various strains and found that OG tend to be different from Kush strains[109]. Although, the names of OG and Kush could mean something different based on where and how they were grown.

Even under a legal model of cannabis regulation, there is no penalty for selling an incorrectly labeled strain in Canada. In the Netherlands, THC content from the same strain varied greatly

[105] Russo, Ethan B. "The case for the entourage effect and conventional breeding of clinical cannabis: no “strain,” no gain." *Frontiers in plant science* 9 (2019): 1969.

[106] Hecht, Peter. *Weed Land: Inside America's Marijuana Epicenter and How Pot Went Legit*. Univ of California Press, 2014.

[107] See Elzinga *et al.* (2015)

[108] Hazekamp, Arno, Katerina Tejkalová, and Stelios Papadimitriou. "Cannabis: from cultivar to chemovar II—a metabolomics approach to Cannabis classification." *Cannabis and Cannabinoid Research* 1.1 (2016): 202-215.

[109] See Elzinga *et al.* (2015)

both between and within cannabis storefronts [110]. This could lead to consumer confusion as they may be expecting certain psychoactive effects from a particular strain, but if they received a different strain with the same strain name, then other effects might occur. In Canada, there has been one voluntary recall due to the improper labeling of strain names[111] – although, it is likely that this is not an isolated occurrence.

One solution for the improper labelling of strains could be to create cultivars. Cultivars are cultivated varieties of plants that have consistent traits among individuals and across generations[112]. This cultivar approach is not impossible for high-THC cannabis as there is a list of approved cultivars for hemp (i.e., low-THC cannabis)[113, 114]. However, there must be established definition for each of the high-THC cultivars in question and this could be challenging as there are differences among the definition (or lineage) for certain strains of cannabis[115].

*Cultivation and processing.*

[110] See Hazekamp *et al.* (2016)

[111] Healthy Canadians "Medical cannabis by Shoppers Drug Mart Inc. recalls one lot of WeedMD's Pedro's Sweet Sativa dried cannabis" November 20, 2020. https://healthycanadians.gc.ca/recall-alert-rappel-avis/hc-sc/2020/74363r-eng.php

[112] International Society for horticultural science "International code of nomenclature for cultivated plants, ninth edition", June 2016. https://www.ishs.org/scripta-horticulturae/international-code-nomenclature-cultivated-plants-ninth-edition

[113] See Pusiak *et al.* (2021)

[114] Health Canada, "List of approved cultivars for the 2021 growing season: industrial hemp varieties approved for commercial production", April 19th, 2021. https://www.canada.ca/en/health-canada/services/drugs-medication/cannabis/producing-selling-hemp/commercial-licence/list-approved-cultivars-cannabis-sativa.html

[115] See Pusiak *et al.* (2021)

Some of the variability within strains might be partially explained by the way cannabis flower has been cultivated and processed[116]. In attempts to recreate illicit cannabis grow operation Knight and colleagues (2010) found there to be inconsistency in the potency of cannabis grown under the same conditions from the same clonal mother[117]. Cloning cannabis is a process to ensure greater consistency among plants – a stem from an unmatured female cannabis plant is cut and propagated to maturity[118]. Even with clonal propagation, it is likely that the difference in production practices may explain why variability exists. Moreover, post-production processing (e.g., trimming, drying, and curing flower) could influence the levels of THC in the final product[119, 120].

The lack of a variability range results in the LPs announcing voluntary recalls on improperly analyzed cannabis flower and include the corrected values of THC[121]. This can be costly for LPs to issue the recall as it results in lost time, revenue, and credibility. Moreover, if there is no penalty for improperly labeled levels of THC in flower cannabis, then why would an LP issue a recall? Less than three years into legal cannabis there have been 15 recalls for inaccurate analysis

[116] See Pusiak *et al.* (2021)

[117] See Knight *et al.* (2010)

[118] Campbell, Lesley G., Steve GU Naraine, and Jaimie Dusfresne. "Phenotypic plasticity influences the success of clonal propagation in industrial pharmaceutical Cannabis sativa." *PloS one* 14.3 (2019): e0213434.

[119] See Knight *et al.* (2010)

[120] See Campbell *et al.* (2019)

[121] See Healthy Canadians

for THC[122]. Currently, there is no limit on THC variability within flower cannabis, however, it has been proposed that a 20% variability limit is reasonable[123].

Instead of focusing on the variability within the strain, another approach would be to focus on the chemistry and categorize the cannabis post-harvest and processing [124,125]. Categories must be created and named, along with a set variability range for each of the plant compounds. Using strain names may help the transition towards this chemical variety approach so as to avoid consumer confusion. If enough LPs accept the definition and thresholds for groups of cannabis, then the rates of variability would be reduced as the chemical profile will define the category and not the category defined by its alleged lineage.

Machine learning coupled with the chemical analysis could streamline the categories of cannabis, where the computer model can quickly categorize cannabis based on levels of cannabinoids (even beyond THC and CBD) and terpenes for cannabis flower[126]. Physical appearance (e.g., size, density, colour of the flower) could also be included in the computer model for these varieties. Once the chemical analysis for the particular plant is complete, then it could be binned into a particular chemical variety. This would make for an easier transition from strain to cultivar and would reduce variability within the strains as the cannabis industry moves

[122] See Healthy Canadians

[123] Sarma, Nandakumara D., *et al.* "Cannabis inflorescence for medical purposes: USP considerations for quality attributes." *Journal of natural products* 83.4 (2020): 1334-1351.

[124] Hazekamp, A., and J. T. Fischedick. "Cannabis-from cultivar to chemovar." *Drug testing and analysis* 4.7-8 (2012): 660-667.

[125] See Hazekamp *et al.* (2016)

[126] de la Fuente, Alethia, *et al.* "Relationship among subjective responses, flavor, and chemical composition across more than 800 commercial cannabis varieties." *Journal of Cannabis Research* 2.1 (2020): 1-18.

towards a more consistent product. Some of these ideas have already been patented and utilize user-data along with objective chemical analysis to arrange products on user-preference[127]. Creating a network combining user guided structured decision-making and chemical analysis could improve user-product outcomes and mitigate adverse effects with overconsumption, thus reducing harm.

*Consumer consideration*

The new classes of cannabis products typically use an extract or concentrate of cannabis and producers mix this extract or concentrate into their own product (e.g., edibles, topicals, oral sprays). Though the chemistry of these extracts and concentrates can be accurately measured, there are additional challenges to adding these extracts to another product, especially if they are produced at non-licensed facilities (such as at home).

If the home-cook is using homegrown cannabis flower to create their product, then they would likely be uncertain of the quantity of THC per gram of cannabis. The unknown potency of cannabis is then decarboxylated (making the cannabinoids more bioactive), adding more uncertainty with the level of decarboxylation (e.g., partially, or fully decarboxylated). If heated for too long the THC might be vaporized off of the cannabis and therefore, would not be present in the final product[128]. The bioactive cannabinoids are typically extracted using a carrier oil (e.g., MCT or olive oil) or high-potency alcohol, though the level of THC in the liquid would be unknown. The oil is added to a product and is sometimes baked. When making candy edibles,

[127] See Jikomes *et al.* (2020)

[128] Taschwer, Magdalena, and Martin G. Schmid. "Determination of the relative percentage distribution of THCA and Δ9-THC in herbal cannabis seized in Austria–Impact of different storage temperatures on stability." *Forensic science international* 254 (2015): 167-171.

the concentrate is dissolved in carrier liquid (e.g., oil or melted candy) and then is applied to candy. Uneven spraying or mixing of the cannabis concentrate could produce hotspots and lead to inaccurate dosing. Moreover, when calculating THC in confections, there is a matrix of carbohydrates, fats, and proteins that could interfere with pharmacology of the cannabinoids. Furthermore, replicating the exact process and achieving the same results every time is very unlikely due to the variability introduced in every step of manufacturing these types of products. All of this variability could result in the overconsumption of an edible product due to inaccurate dosing.

There are multiple areas where sources of error could arise in the production of homemade cannabis products. Therefore, the established protocols from LPs coupled with the upper limits of THC set at 10mg/discrete unit are safeguards against over/accidental-consumption of cannabis products[129]. Moreover, precise extraction protocols and testing the cannabis extract or concentrate will improve accuracy in the final product and ensure complete decarboxylation without vaporization (i.e., cannabinoids are not vaporized off). There is improved consistency between products and batches when produced at the industrial level used by most LPs (e.g., using industrial mixers to form a homogenous mixture between samples). Finally, there is less variability due to the well documented source and chemistry of the cannabis product.

*Chemical analysis:*

Legally produced cannabis products must be tested for THC potency, however, there is no validated method for quantifying THC. The LP could create and validate their own method for these tests in an analytical testing facility. Alternatively, the LPs could send their product to a

[129] See Cannabis Regulations (2020)

testing facility. This means that the variability in the amount of THC in a particular product (or flower) could vary among testing facilities if they have different analytical methodology [130,131]. This could lead to biases as an LP could use multiple analytical techniques (post-production) in order to achieve the "correct" level of THC to fit a given product. Alternatively, the LP could send their product to various third-party testing facilities and select the results that best suit their product, considering that the testing facilities use different analytical techniques to test for THC[132]. However, if there were validated testing methodologies from a reputable source that could be used industry-wide, then the variability of THC between labs would likely decrease.

Third-party testing facilities, with industry-wide, validated testing protocols, should reduce the cost and bias of testing their own product. The testing facility could also accept cannabis from home growers who want to test their product for levels of cannabinoids, terpenes, and contaminants. This would greatly reduce the harm toward the cannabis user as it provides an objective chemical overview of the product's contents. However, this approach would only work if there were approved and validated methods from a governing body that can ensure inter-lab consistency. Therefore, testing cannabis via independent third-party testing facilities that use validated methods that are approved by a governing body would reduce biases among LPs. This approach is already used in the supplement industry and is promising to reduce the number of tainted supplements[133].

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[130] See Jikomes & Zoorob (2018)

[131] See Pusiak *et al.* (2021)

[132] See Jikomes & Zoorob (2018)

[133] Eichner, Amy K., *et al.* "Essential features of third-party certification programs for dietary supplements: A consensus statement." *Current sports medicine reports* 18.5 (2019): 178-182.

Cannabis products, excluding flower, must fall within a range of THC, which changes with potency of the product, or else the product cannot be sold[134]. Cannabis flower, however, has no variability threshold. This means that flower cannabis labeled at 10% THC could be between 0-30% THC. A 20% variability threshold, as proposed by the United States Pharmacopeia [135] means that 10%THC cannabis flower could range from 8-12% THC. The governing body could also set a threshold on the THC variability in flower cannabis. The lack of a threshold has some LPs issuing recalls for products due to improper analysis and labeling of a product. For example, one product had the correct level of THC but mislabeled CBD at 0.4% when the corrected value is <0.07%[136]. It is unlikely that this minute difference in CBD will elicit a different psychoactive experience. In contrast, there was another recall where the labeled THC was 0.3%, while the actual amount was 20.66%[137]! This could be harmful for the naïve user who is seeking a low THC cannabis product.

*How does inaccurate analysis impact taxation?*

Some food and beverages have compositional standards outlined in the Food and Drugs Act[138]. This includes various types of alcohol (e.g., beer, wine, and vodka). Alcoholic beverages

[134] See Cannabis Regulation (2020)

[135] See Sarma *et al.* (2020)

[136]Healthy Canadians, Recalls and safety alerts. "Tweed inc. recalls one lot of LBS Sunset (Indica) dried cannabis. March 7, 2019. https://healthycanadians.gc.ca/recall-alert-rappel-avis/hc-sc/2019/69256r-eng.php

[137] Healthy Canadians Recalls and safety alerts "Aphria Inc. recalls one lot of Good Supply Royal Highness dried cannabis" October 29, 2019. https://www.healthycanadians.gc.ca/recall-alert-rappel-avis/hc-sc/2019/71407r-eng.php

> However, this strain is known to have between 170-240mg/g therefore, the purchaser might see the typical range prior to purchase

[138] Food and Drugs Regulation (Canada) C.R.C., c.870

that do not have a compositional standard are considered unstandardized and follow a different set of rules for labeling requirements. Adopting and applying the compositional standard approach to the categories of cannabis would permit a legal definition for cannabis products. Once these standardized products are established, then subsequent taxation for these products could be equivalent based on their level of THC (e.g., 2, 5, or 10mg THC per dose) and perhaps route of administration (smoked vs. edible). Alternatively, the government could tax all products the same or set-prices, which is similar to nicotine-based vaping liquid, whereby the price of a product is similar regardless of the amount of nicotine present. However, customers may select a higher potency product considering it costs the same as a lower potency product.

Currently, the taxation for all cannabis products (except flower) in Canada are based on the amount of THC (in mg) found in the product[139]. If the THC analysis overestimates the actual amount of THC in the product, then the LP will pay more taxes. However, if the THC analysis underestimates the level of THC, then the LP will pay fewer taxes. One solution is to set the tax based on the category and mass of product being sold. This is already the case for flower cannabis, where taxation is based on the mass of the flower instead of the quantity of THC[140]. Cannabis products (excluding flower) tend to contain other ingredients such as carrier oils, sugars, etc. Therefore, setting taxes based on the weight of a product would likely shift the

[139] Canada Revenue Agency "EDN60 calculation of cannabis duty and additional cannabis duty on cannabis oil, edible cannabis, cannabis extracts and cannabis topicals" April 2019. https://www.canada.ca/en/revenue-agency/services/forms-publications/publications/edn60/calculation-cannabis-duty-additional-cannabis-duty-cannabis-oil-cannabis-edibles-extracts-topicals.html

[140] Canada Revenue Agency "EDN55 Calculation of cannabis duty and additional cannabis duty on cannabis products" September 2018 https://www.canada.ca/en/revenue-agency/services/forms-publications/publications/edn55/calculation-cannabis-duty-additional-cannabis-duty-cannabis-products.html

market to manufacture products that weigh less and have proportionally more THC. Simply taxing based on the potency of the product could help favour less potent products that would (in theory) result in fewer adverse effects – similar to the social reference pricing for alcohol[141]. This would be a challenging task if there is no bio-equivalency or knowledge of the pharmacology of various cannabis products.

*Purchasing limits*

The purchasing limits for cannabis products is just as confusing as cannabis taxation. The government set the public possession and purchasing limit at 30g dried cannabis flower. However, since there are more products than just flower, the government also had to devise an equivalency based on the weight of the product – not based on the total THC of the product (see Table 1). It would be sensible to follow the logic used for taxing cannabis, whereby, taxation is based on the amount of THC in the product.

The current regulations for possession and purchasing limits need some rehashing. For example, if cannabis flower contains 200mg/g THC, then the 30g limit would contain 6000mg THC/ 30g flower. The greatest difference on purchasing limits for cannabis products is between bath bombs (solids containing cannabis) and oral sprays (non-solid containing cannabis). If a bath bomb contains 140mg THC and weighs 150g, then the legal purchasing limit is 3 as this would be the "equivalent" of 30g of dried cannabis (though it only contains 420mg THC/3 units – not 6000mg/30g THC). In theory, producers could reduce the final weight of their product, while keeping THC at a fixed amount, so as to facilitate a greater number of units sold per transaction. This strategy is apparent when it comes to oral sprays. For example, a 20mL bottle

[141] Canadian Centre on substance Use and Addiction

of oral spray contains 500mg THC/bottle and has a purchasing limit of over 110 bottles. Therefore, the purchase would contain over 55,000mg of THC, which is almost 10x the limit of THC from 6000mg/30g cannabis flower!

If the limits for possession and purchasing cannabis products were based on a set amount of THC (e.g., 6000mg THC), then this would eliminate the difference in the amount of THC being purchased at a single time between products. In contrast, there are no purchasing limits on tobacco or alcohol within Canada. If the cannabis purchasing limit does not change, then this would be an excellent opportunity to advertise and set purchasing and possession limits of tobacco and alcohol products in Canada.

*Table 1. Possession limits for products that contain cannabis*[142]

| Class of Cannabis | Equivalent to 1g dried cannabis | Possession limit |
|---|---|---|
| Dried cannabis | 1 g | 30 g |
| Fresh cannabis | 5 g | 150 g |
| Solids containing cannabis | 15 g | 450 g |
| Non-solids containing cannabis | 70 g | 2100 g |
| Cannabis concentrate | 0.25 g | 7.5 g |
| Cannabis plant seed | 1 | 30 seeds |

[142] See Cannabis Act (2018)

**Future directions and conclusions**

Moving forward, all parties involved in the legal cannabis industry should consider the following. First, there should be a defined standard dose of cannabis and clearly defined bio-equivalency among products. Dosing cannabis should have been established 20 years ago when cannabis was legalized for medical purposes. Second, categorizing cannabis flower based on its chemistry would reduce the level of variability within types of cannabis as the flower would be categorized based on its phytochemical profile instead of its lineage (where known lineages are unknown due to the prohibition of cannabis). The use of machine learning and user preference data can help consumers select a product that is best for them with a reduced likelihood of adverse effects. Homemade products could vary in its level of THC, even if the cannabis is legally purchased. Therefore, LPs should highlight their QCQA protocols as a less harmful approach to various cannabis products.

Perhaps most importantly, there is no government validated cannabis testing methodology. This results in various analytical methods being used by various labs in order to test for levels of THC – resulting in variability among testing facilities. Verified third-party testing facilities could reduce bias among LPs. Furthermore, all cannabis products, except for flower, have variability limits in order to reduce harm. Cannabis flower should follow with a variability limit at 20%. Currently there is no variability limit, so customers are uncertain of the THC (and or CBD) potency in their flower product with wide ranges of THC.

Finally, there should be a call for compositional standards for cannabis products, so consumers know that there is a legal definition of a given product. Either alter or abolish the purchasing limits of cannabis products as it is not based on the amount of THC, rather it is based

on the weight of the product. This could be adapted to limiting the amount of THC (in mg) purchased from each of the categories instead of the weight of the product (disregarding the amount of THC).

In conclusion, *Cannabis sativa* is a notorious plant which has a unique and rich history with humans. Canada legalized the recreational use of cannabis providing safer alternatives with goals to reduce proceeds to organized crime, reduce use among minors, and provide a safer supply of cannabis to Canadians. The two-stage approach to legalization had some benefits such as teasing apart the effects of permitting various cannabis products. Though it also had drawbacks such as ephemeral illicit cannabis retailers filling the void for novel products and lower prices. The illicit cannabis products likely do not follow the strict QCQA product testing protocols that LPs follow and could be harmful to the consumer. Legal cannabis, however, has had online security breaches, and recalls on products, which may nudge consumers to illicit market. The wide array of cannabis products available have some consequences and therefore, determining a standard dose for each of these products is challenging. Canada, being the first G7 country to legalize cannabis can be a leader in cannabis policy and cannabis science by addressing these and other concerns. This is Canada on cannabis.